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ABSTRACT

EFFECT OF VITAMIN SUPPLEMENTATION ON BIODEGRADATION OF ALIPHATIC HYDROCARBONS AND DIESEL FUEL

Susmita GuptaPal

Bioremediation of hazardous wastes is proven to be environmentally benign and most cost effective technology when proper conditions are provided. However, commercialization of this method still requires comprehensive research to enhance the degradation rate to make the technology more acceptable for implementation. In this research a unique approach to increase the rate of biodegradation by vitamins supplementation was studied.

Five vitamins, niacin, biotin, folic acid, thiamin and pantothenic acid were selected for the present study. Different short chain (C_8 and C_9), and long chain (C_{16} and C_{17}) aliphatic hydrocarbons and diesel fuel (a mixture of C_7 to C_{23}) were tested as the target compounds. The effects of the specific vitamins and mixture of vitamins on biodegradation of target compounds were investigated using a microbial consortium obtained from soil.

The degradation rate as well as the microbial growth were monitored. The optimal vitamin concentrations were experimentally determined. Then the kinetics of degradation of C_8, C_9, C_{16} and C_{17} were studied. For diesel, the degradation kinetics and also the fractionation pattern were investigated.

The results showed that the vitamin mixture was more effective than the individual vitamins. Individual vitamins were often inhibitory at certain concentrations. Therefore one needs to optimize individual vitamin concentrations to get the best effect. The effectiveness of arbitrary vitamin mixture (contained each of five vitamins at the same

concentration) was less than the optimized vitamin mixture which contained niacin, biotin, folic acid and pantothenic acid at their optimum concentrations.

The effectiveness of the optimized vitamin mixture was most prominent in reduction of lag phase, increasing overall growth rates and overall degradation rates. It has been found that the lag phase was reduced by 13 to 50% with optimized vitamin mixture as compared to that without vitamin. The lag phase reduction was very significant for nonane and heptadecane degradation. The overall growth rates and overall degradation rates increased significantly (increased by average 36% and 31% respectively) with optimized vitamin mixture supplementation

Increase of specific growth rates and degradation rates during exponential phase of microbial growth with the optimized vitamin mixture were not noticed in all cases. Although in some cases specific growth rates increased significantly.

Therefore it can be said that vitamins play important role to initiate growth of soil microbes and help them to degrade various hydrocarbons faster. Hence, vitamin supplementation can enhance the biodegradation of hydrocarbons on soil.

EFFECT OF VITAMIN SUPPLEMENTATION ON BIODEGRADATION OF ALIPHATIC HYDROCARBONS AND DIESEL FUEL

by
Susmita GuptaPal

A Dissertation
Submitted to the Faculty of
New Jersey Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy in Environmental Science

Department of Chemical Engineering
Chemistry, and Environmental Science

October 1996

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This Thesis is Dedicated to
my beloved late Grandma Suniti Gupta

ACKNOWLEDGMENT

The author would like to acknowledge Dr. David Kafkewitz and Dr. Piero M. Armenante who not only guided her for this study, but also offered her moral and financial support throughout the study. The author like to thank to Dr. R. Trattner, Dr. D. Burdick, and Dr. Gordon A. Lewandowski for their timely help and for serving as members of this committee.

Special thanks to Clint Brockway and Gwen Augustin of the analytical laboratory at the Center for Environmental Engineering & Science (NJIT) who were always available to help. The author is grateful to the technical reference librarian Bruce Slutsky of NJIT who assisted in the literature search.

The author also appreciates the timely help from fellow graduate students Monica Togna, Linda Thom, and Nelson. She is especially thankful to Ms. Jayati Mukhopadhyay, for her help during various experiments. She would also like to thank Takahiro Kemoda who translated the Japanese publications for her.

Finally she would like to express her gratitude to her beloved daughter Ria Pal and her husband Dr. N. Pal for their love and constant support, without which this study would never have been possible.

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CHAPTER 1

INTRODUCTION

Fuel contamination of soil is one of the major challenges environmentalists face today. Although contamination of soil by diesel oil is not usually considered a serious environmental threat, the contamination can spread to the ground water and enter drinking water systems, with consequent adverse health effects. Diesel is used in the trucking and railroad industries as fuel, and millions of gallons are stored and transported as part of daily operations. It is also used as a solvent and as a minor fuel for automobiles. The widespread use of diesel has resulted in numerous cases of soil contamination. Spillage, leakage from underground or aboveground storage tanks, or accidents during transportation are the common occurrence. In February 1975, a 59,000 gallons of diesel fuel no. 2 were accidentally spilled from a storage tank at Sandia National Laboratories, Livermore, CA, a Department of Energy facility [39, 46]. A ruptured pipe at a Burlington Northern Railroad fueling pump house resulted in over 60, 000 gallons of diesel spill onto the surrounding soil which contaminated soil 70 ft below ground surface [11]. During 1990 summer season, approximately 150 cubic yards of diesel contaminated gravel obtained from the Surfcote Pad in Prudhoe Bay [12]. It has been estimated that more than 10% of the 3.5 million petroleum product storage tanks in the U.S. are leaking and caused at least 300,000 environmental incidents [61].

Petroleum fuel contaminated soil is classified as hazardous waste. Cleanup by excavation and incineration or transport and burial of the hydrocarbon contaminated soil is expensive

contaminated soil has been used as an alternative treatment. It is a cost effective clean up processes compared to the excavation, incineration or disposal in landfill area etc.. The major disadvantage of biodegradation is its slowness. Numerous attempts have been made to promote degradation activity. The addition of fertilizers, largely nitrogen and phosphorous; control of various physical factors, such as temperature, pH, moisture; aeration to improve oxygen delivery; and emulsifiers to increase the oil-microbe contact are among the methods tested to accelerate the process. It has been found that even when all macronutrients are supplied and physical conditions are controlled, bioremediation is still slow, presumably due to limitations of the growth of microbes. One reason is the toxicity of the fuel components; another reason might be a limiting supply of micronutrients. It has been shown that the dominant microbial species in soil obtain their vitamin requirements either from the soil or they are able to synthesize them. Some microbes in soil need vitamins for growth.

The effect of vitamin supplementation on bioremediation does not appear to have been explored yet. Yet this seems to be a reasonable, and cost effective approach for enhancing the microbial growth and degradation of fuel in soil.

In the present study, we tried to improve biodegradation by supplementation with water soluble vitamins, namely biotin, niacin, folic acid, pantothenic acid, thiamine as well as mixtures of these vitamins. Diesel oil degradation was studied in detail to see if the vitamin supplementation changes the degradation pattern of the various subfractions of diesel oil. Though diesel fuel was the major target of our studies, we also investigated the effect of vitamins on the biodegradation of single hydrocarbons. We studied isooctane, octane and nonane (as models of short chain hydrocarbons), hexadecane and heptadecane

(as longer chain hydrocarbons). These compounds are components of diesel oil and are also widely used as solvents for the oil and paint industries and can cause disposal problems..

Our studies involved laboratory experiments using garden soil as inocula. Vitamins were tested for optimum dose for the soil inoculum. A soil population that was never exposed to diesel or other alkanes was used to simulate a real world case where an accidental spill may cause unexpected soil contamination. Therefore the present study is especially relevant for *in-situ* biodegradation of soil that is accidentally contaminated with diesel or alkanes.

CHAPTER 2

LITERATURE REVIEW

Microbial decontamination of chemicals in the environment is an emerging field. However the natural attenuation of the process is often slower than acceptable regulatory limits. Accidental fuel spills, leakage of gasoline and diesel from underground and above ground storage tanks, and widespread use of fuels for automobiles have resulted major environmental problems. Therefore a number of studies have been conducted to accelerate biodegradation, especially for various types of fuel. Many investigations have reported on the factors that might enhance bioremediation of diesel fuel and normal-alkanes and aromatics as the components of diesel fuel. There are also reports available for the properties and activities of individual vitamins and few reports on the study of biodegradation with vitamins. Based on that, we divided this chapter into three subsections.

2.1 Studies on Various Factors for Enhancing the Degradation of Hydrocarbons

McNabb et al. (1970), found that bacterial count of fuel contaminated soils was orders of magnitude higher than that in virgin soils [37]. This observation indicated that microbes can use the fuel oil fractions as their carbon source. This study also revealed that the concentration of macronutrients like oxygen, nitrogen and phosphorus are limiting

Biodegradation of aliphatic and aromatic hydrocarbons were of most important concerns for degradation of fuels. Since hydrocarbons are water-insoluble compounds, their uptake and enzymatic attack are difficult task for microbes. There are number of bacteria which can utilize hydrocarbons, though different population are able to use shorter chain (upto C_8) and longer chain C_{10} - C_{18}) alkanes. Microorganisms live in aqueous medium, can not live in oil, a large oil-water boundary layer is a prerequisite for a rapid degradation of oil. Attack of the hydrocarbon is possible only in the boundary layer.

Gotschalk [16] described possible mechanisms for bacterial growth on hydrocarbons. Some bacteria form trehalolipids, rhamnolipids, or some similar structures as constituent of their cell walls in which the hydrocarbons are dissolved and transported into the membrane. The first three reactions on the hydrocarbon proceed within the membrane, the membrane-bound enzymes monooxygenase catalyze the oxidation of alkanes to alcohols. An oxidizable cosubstrate is required for the alkane oxidation which is fulfilled by the reduced rubredoxin, a small protein contains Fe^{+2}, linked to cystein residues of polypeptide chains. In *Corynebacterium* species reduced cytochrome P_{450} is the cosubstrate. All these cosubstrates receive the reducing power from NADH. The primary alcohol that is formed by oxidation of alkanes is then further oxidized to corresponding aldehydes and the corresponding organic acids by NAD+ dependent dehydrogenases. Finally the fatty acids are degraded by β-oxidation.

A different mechanism of hydrocabon oxidation has been found in some organisms (*Nocardia* sp.). There a subterminal attack of the hydrocarbon chain is employed that leads to the formation of secondary alcohol which is further oxidized to ketone by monooxygenase reaction. The product is acetylester that on hydrolysis produces acetate

and alcohol. The alcohol oxidation follows the above mechanism. Therefore it is clear from this study that hydrocarbons, though water insoluble are degradable by bacteria and for degradation bacteria must need oxygen.

The effects of oxygen, nitrogen, and temperature on gasoline biodegradation on soil were studied by Zhou and Crawford [61]. They tested the biodegradation capacity of indigenous soil bacteria for gasoline remediation. Biodegradation kinetics and gasoline degradation rates were studied under different conditions, such as different soil cores, temperatures, oxygen concentrations, and nutrient concentrations. The V_{max}, K_s, and Q_{10} values were calculated for different conditions. Effect of temperature on microbial growth during biodegradation of gasoline are shown in Table 2.1.

Table 2.1 Effect of Temperature on Growth of Gasoline Degrading Soil Microbes

Temperature (°C)	V_{max} (ppm/day)	K_s (ppm)
11	62	281
25	114	120
37	135	117

The variation of the readings ranged within +/- 2 to +/- 6.

Under low nutrient conditions (C:N < 300:1) and very high nitrogen conditions (C: N > 1.8:1) biodegradation of total petroleum hydrocarbon was inhibited. Optimal C:N ratio was 50:1, though it varied for different soil types. Addition of macro-nutrients enhanced the biodegradation rates. The optimum oxygen concentration required for the soil population was unexpectedly high (about 10%). Not only oxygen, it requires optimum temperature and nutrient addition. It has generally been accepted that at normal temperatures short chain aliphatic hydrocarbons are more susceptible to microbial attack

and have faster degradation rates. However in this study the workers have seen an opposite in some of their experiments. Because soil population studied was acclimated in aromatic compounds, it was very likely that the selected microbial community had more aromatic degraders than the aliphatic degraders. Aliphatic hydrocarbons were degraded very slowly in some cases.

Biodegradation of diesel fuel by free and immobilized soil bacteria was studied by Grundmann et.al [17]. They looked at different parameters that influence the degradation pattern in sandy soil. They used several different soil bacteria for their experiments. The various parameters included the soil depth, carbon-nitrogen ratio, additional inoculation of the soil etc. The results showed that upto 70 cm depth of soil, oxygen was not limiting and 95% of the total hydrocarbon could be removed by biodegradation process. However, fertilizing soil with C:N ratio of 60:1 and active aeration of the soil reduced degradation time. With the additional soil inoculation, an immobilized culture bed showed a dramatic improvement in degradation efficiency (about 10 to 17% faster) of the microbes within the first week. The biodegradation pattern of the selected n-alkanes and iso-alkanes showed a characteristic pattern. Undecane >> Tetradecane >> Octadecane was the order of degradation of the investigated n-alkanes which took place recognizably faster with the additional inoculation. Also they noted that isoalkanes like pristane and phytane could be degraded only with the additional inoculation of soil with microrganisms. The degradation kinetics of diesel was found to be asymptotic, i.e. 50 to 85% of the hydrocarbons degraded within a few weeks, the upto maximum 95% were utilized within several months.

Aerobic biodegradation of jet fuel contaminated aquifer was studied by Aelion and Bradley [1]. To assess the aerobic biodegradation potential of the *in situ* microbial community, laboratory experiments were performed with contaminated sediments. Sediments were incubated with ^{14}C-labeled organic compounds and the evolution of $^{14}CO_2$ was measured over time. The results of this experiment showed the selected components of jet fuel like n-alkanes degraded whereas their branched isomers remained unattacked by the microbes. The results suggested that the *in situ* microbial community is active despite the JP-4 jet fuel contamination and that biodegradation is compound specific. Also it was found that nitrogen limitation is one of the important factors and that the addition of nitrogen enhanced the biodegradation significantly.

A study was conducted by Pritchard and Costa on an EPA bioremediation project for an oil spill in Alaska [43]. The experimental results showed a significant enrichment of oil degrading microorganisms in the beach of Prince William Sound, Alaska, but the efficiency of degrading oil was limited by nitrogen and phosphorous deficiency in the soil. The addition of fertilizers containing high amount of nitrogen and phosphorous enhanced the degradation of oil 2-3 fold. Pritchard and Costa also found that the most effective means of enhancing oil biodegradation was to increase the availability of nutrients to the microorganisms. An oleophilic fertilizer appeared to be very effective in this regard.

Study of Mills and Frankenberger showed that phosphorous is another important growth factor for bioremediation of diesel fuel [39]. Addition of phosphorous enhanced the degradation rates. However the source of the phosphorous was significant. In their experiments, the effectiveness of diethylphosphate, methylphosphonic acid, and potassium phosphate were compared as P fertilizers to stimulate the biodegradation of diesel fuel in

soil. The results showed that potassium phosphate of concentrations 100 to 500 ppm enhanced diesel biodegradation significantly. The potassium phosphate above 500 ppm was inhibitory for soil microbes. Diethylphosphate seemed to be the most- effective potassium source compared to potassium phosphate and methylphosphonic acid for the stimulation of diesel biodegradation by soil bacteria. On the other hand, methyl phosphoric acid was extremely toxic and inhibited diesel fuel biodegradation.

Toccalino and her co-workers investigated the nutrient limitations during propane and butane degradation [55]. They tested the biodegradation of hydrocarbons in an unsaturated sandy soil with the supplementation of macro-nutrients, and incubated aerobically at 20°C in controlled nitrogen environment. For first three months of incubation, propane and butane degradation occurred similarly utilizing the supplied nitrogen. After three months, the degradation pattern changed in two cases. The propane degradation became very slow due to nitrogen limitation and it remained slow till more N was added. In contrast, bacteria in butane degrading soil appeared to overcome their nitrogen limitation by nitrogen fixation. Analysis of total nitrogen and acetylene reduction assays supported the evidence of surplus nitrogen in butane amended soil. N_2 fixation was not noticed in propane amended soil.

Michaelsen et al. [38] studied the influence of dissolved oxygen tension (DOT) on microbial degradation of hexadecane in cultures with sediment seawater suspensions. These experiments were conducted in a fermentor system, where they were able to control the different oxygen concentrations (0.4 to 80% of oxygen saturation, and anoxic condition). When the DOT was reduced from 80 to 1%, no effect was noticed on hexadecane degradation rates or biomass production rates. The hexadecane degradation

rate was constant during this period and it was 0.15 mg of hexadecane / g of sediment / h. A significant influence on the degradation process was detected only with DOT below 1%. The amount of hexadecane was decreased with lower DOT but the degradation rates remained same. Under anoxic condition, no hexadecane degradation was found. The rate of hexadecane degradation remained constant down to 0.4% DOT showing that the actual oxygen concentration was not that important as long as the oxygen supply was high enough to ensure the oxygen-dependent degradation step.

Harder and Hopner, however, reported that oxygen is a very important factor for biodegradation of hexadecane [21]. They found a direct correlation between moisture content and oxygen concentration in soil with the rate of biodegradation of hexadecane. They found that both, moisture and oxygen content of soil were very important factors for *in situ* biodegradation but oxygen availability was negatively correlated with moisture. Hence they concluded that it was crucial to determine the optimum condition for both factors.

Song and Bartha studied the bioremediation potential of terrestrial fuel spills [53, 57] including diesel oil, gasoline, jet fuel, heating oil and bunker C fuel. They considered different parameters for kinetic studies of biodegradation of different fuels at a variety of temperatures, soil types, bioremediation treatments, and poisoned controls. The results showed that 27°C was the optimum temperature for biodegradation in all cases. Bunker C was structurally almost recalcitrant with 80% persisting after 1 year of incubation. Among the other fuels, diesel was the most persistent; however all of the fuels including the bunker C were biodegradable. Song *et. al* suggested, therefore, that hydrocarbons could be reduced to insignificant levels in contaminated soils within a year by biotreatment

process. In this study they also generated the depletion curves for all four fuels. Determination of kinetic constants were not possible because of the complex nature of fuels. However the kinetics for all four cases followed the intermediate of first order (exponential) and the zero order (linear) kinetics. Diffusion of C_6 to C_9 alkane components and increases in the degrading microbial populations made the kinetics more complicated. In addition, the kinetics became more complex as the fuels consist of numerous individual hydrocarbons and each degraded at different rates. The slowing tendency of utilization of hydrocarbons were caused by the depletion of substrates and also by the fact that the remaining hydrocarbons were structurally less degradable. For all these reasons, they did not try to convert the curves into constants. Instead they determined the half lives for all four fuel degradation which were simply the time required for 50% of fuel degradation. This gave a reasonable idea for biodegradability of all those fuels.

Although studies have shown that 27°C is the optimum temperature for diesel biodegradation by soil bacteria, Lidell et al. [34] reported that diesel degradation is also possible in colder temperatures (15° C or lower). They showed a case study for diesel bioremediation at Point Thomson in Alaska's arctic region where the temperature varies usually -21° C to -1° C. It took almost three years to degrade diesel to a insignificant level by biodegradation technology.

Systematic studies were performed by Wurdemann et al. [60] on the effects of physical and chemical conditions for biodegradation of n-hexadecane in sediments and soils. They performed a laboratory study of several ecological parameters that influenced the aerobic degradation of hexadecane. They tried to optimize physical factors such as

soil type, grain size, surface area etc. to enhance the biodegradation rate. Loess, BetDegan and Gilat soils were tested for degradation rate with hexadecane. It was found that if the sand or finer soil was replaced by loess soil, the lag phase was reduced. Similarly it was observed that with more the surface area, the degradation rates were higher.

Biodegradation of unresolved complex mixture (UCM) of hydrocarbons and aliphatics were studied by M.A. Gough and coworkers [15]. An UCM of hydrocarbons isolated from lubricating oil and a synthetic mixture of C_{25} hydrocarbons were subjected to biodegradation by *Pseudomonas fluorescens*, incubated for 25 days. It was found that molecular structure influences the degradability of a compound. Straight chain and monomethyl alkanes can be degraded rapidly in the order of n C_{25}>2-methyl-> 9-methyl tetracosane. The remaining alkanes degraded slowly and the UCM degraded as a whole, but no reduction in the proportion of resolved vs unresolved alkanes were observed.

Foght and her co workers investigated the effect of emulsifier on biodegradation of crude oil by mixed as well as pure bacterial culture [13]. They reported that the n-alkanes and the saturated hydrocarbons of crude oil were pretreated with purified emulsion, the heteropolysaccharide bioemulsifier produced by *Acinetobacter calcoaceticus* RAG-1. The emulsion treated crude oil were degraded by 50-90% in 14 days of incubation. Both pure culture and the mixed population of bacteria were effective. But the degradation of aromatic compounds by a mixed culture was inhibited by the emulsion. The aromatics degradation by the pure culture was either unaffected or slightly enhanced.

Another attempt of modifying methods for biodegradation enhancement was reported by Pritchard et al. [44]. They studied various fuels, including diesel for

biodegradation in multistage continuous culture system. For the multistage system, physical dispersion of oil layer was not necessary. Gas chromatographic analysis was performed on the alkane and aromatic fractions of diesel. Complete removal of diesel components were not possible for their process since many other transformation products were produced during degradation. A number of changes of diesel components were noticed, among which heptadecane-pristane ratio and octadecane-phytane ratios were decreased by maximum 10-25%. There was complete removal of C_{11} and C_{12}-n alkanes only, other peaks were decreased in chromatograms but not removed completely.

Mineralization of [^{14}C]hexadecane and [^{14}C]phenanthrene in crude oil were studied by Fought and others [13]. They isolated 138 bacteria from freshwater, marine, and estuarine samples and tested for the ability to produce $^{14}CO_2$ from n [1-^{14}C]hexadecane or [9-^{14}C]phenanthrene added to Prudhoe Bay crude oil. It was found that 39% of the bacteria mineralized the hexadecane and only 4% bacteria degraded phenanthrene. No single organism was able to mineralize both compounds. There was no correlation found between degradative ability, genus or source. An attempt to find bacterial strains able to degrade both the model compounds failed. Not all bacteria that degraded phenanthrene were able to mineralize other aromatics of the same group. Similar observations were noted for alkanes. Therefore a combined culture was used to mineralize the crude oil sample without any synergistic or antagonistic effect among the isolates.

It has been found from the above literature that the biodegradation of alkanes and diesel fuel by soil bacteria , though an efficient remediation technology, is often limited by several physical factors like oxygen deficiencies, moisture content of soil, soil types, temperature, pH etc.

Macro-nutrients such as nitrogen, phosphorous play a very important role in biodegradation rates by soil bacteria. However there is another important criterion which has not been explored yet. Even with all macronutrients and proper physical conditions, the microbial growth might still be inhibited unless micronutrients are present. Soil microbes need vitamins for their metabolism and growth. Most of the dominant species either get their required vitamins from soil or they are able to synthesize it. On the other hand, because of the severe competition among the soil population, some of the bacteria are likely to be deprived of the micronutrients. Vitamin supplementation can help them to grow.

Vitamin requirement for soil bacteria have been discussed in detail by S. A. Koser [31, 32]. The book reported many of the *Clostridium, and Bacillus,* required vitamins like nicotinic acid, biotin, pantothenic acid for their growth. The supplementation of those vitamins enhanced their growth in laboratory studies.

Lochheed, reported a study on vitamin requirements of soil bacteria [36], in which involved 499 organisms isolated from a field soil. It was found that 27.1% (corresponding to 14.1 million per g) of them required one or more vitamins for growth. The findings are summarized in the Table 1 where it shows Thiamin and biotin are very important growth factor either alone or with others.

Table 2. 2 Vitamin Requiring Bacteria Isolated from a Field /Soil.

Vitamin reqd. (either alone or in combination)	% of total soil isolates required vitamins	Approx. number of soil bacteria required vitamins (millions/g)
Thiamin	19.4	10.2
Biotin	16.4	8.6
Vitamin B12	7.2	3.8
Pantothenic acid	4.6	2.4
Folic acid	3.0	1.6
Nicotinic acid	2.0	1.1

Lochheed also discussed about the capability of predominant soil bacteria for vitamin synthesis in the same report. It was shown that 316 strains of bacteria from different types of soil were able to synthesize at least some part of vitamins. With all soils, riboflavin was produced by the highest number of isolates, followed by thiamin and B_{12}. A lower percentage of isolates were also able to synthesize biotin. Pantothenic acid synthesis by soil population was rare.

There is evidence of vitamin requirements of soil microbes reported by Korner and Laczko [30]. They tested different soils for diversified microorganisms and found that low-diversity soils responded positively to a small addition of a vitamin mixture of thiamin, biotin, folic acid, calpan and pyridoxine. The results indicated a connection between the microbial diversity and yet unspecified vitamin deficiency within the population. It was still under investigations whether the vitamin deficiency was the cause or the effect of the reduced diversity of microbial population

Most bacteria obtain their required vitamins from their own habitat. Hilde Lemmer and Lutz Nitschke were able to determine vitamins like thiamin, riboflavin and folic acid from dry solids, activated sludge and digested sludge respectively in municipal and

industrial wastewater treatment plants [33]. The concentration varied in different phase, and it was found that heterotrophic saprophytes obtained their required thiamin, and riboflavin from the sludge itself.

We can see from literature that vitamins or micronutrients are essential for microbial growth, consequently a discussion of the specific metabolic roles of certain vitamins would be useful. For our study we have already selected five major vitamins namely biotin, niacin, folic acid, pantothenic acid and thiamin. Hence, our discussion for the activity of specific vitamins concerned only these five vitamins. Structure and properties of these five vitamins are taken from the books of Koser [31, 32] and Wagner & Folkers [56].

2.2 Properties and Function of Specific Vitamins

In this subsection, five vitamins are described specifically. These five vitamins were chosen in our experiments based on their metabolic activities and chemical properties which are described briefly as follows

2.2.1 Biotin

Biotin is one of the most potent growth factors. It is widely distributed in cells and tissues but is present only in minute amounts.

Structure and Properties of Biotin : Biotin contains a bicyclic ring system, with a five membered urea ring and a five membered sulfur ring. Valeric acid is present as a side chain. There are four sterioisomers are possible. The structure of biotin is shown in the Figure 2.1. Only (+)-biotin is biologically active, and the (+/-)-biotin is 50% active.

Molecular weight of biotin is 244.31. It is sparingly soluble in water (0.02% at 25°C) and more soluble in 95% ethyl alcohol. It is practically insoluble in organic solvents. It is stable in autoclaving at 120°C for 1.5 h with sulfuric acid (2N) but loss of activity was found with 2N HCl.

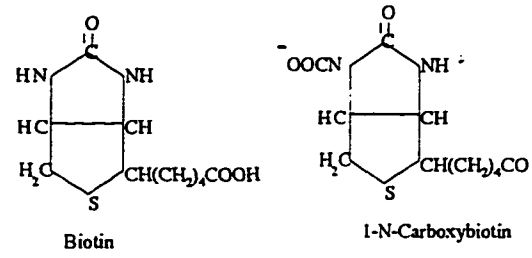


Figure 2.1. Structure of Biotin

Function of Biotin: Biotin is involved in different metabolic activities including the metabolism of carbohydrate, amino acid, protein, nucleic acids, or their components, and lipids.

Carboxylation: A thorough investigations for the activity of biotin has been done since long time. It has been established that the enzyme β-methylcrotonyl CoA carboxylase contains biotin as a prosthetic group. The carboxylation catalyzed by this enzyme converts a 5-carbon to a 6-carbon compound:

$$CH_3 - C(CH_3) = CH - CO - SCOA + CO_2 + ATP \xrightarrow{Mg^{++}} CH_3 - C(CH_2 - COOH) = CH - CO - SCoA + ADP + P_i$$

β-Methylcrotonyl -CoA biotin-enzyme β-Methylglutaconyl-CoA

The specific activity of the carboxylase was correlated with the biotin content of the enzyme. Avidin inhibited the reaction, the biotin addition protected against avidin.

The structure of biotin-CO_2 intermediate which transfers CO_2 was identified as 1'-N-carboxybiotin.

Several other biotin containing enzymes have been purified and implicated in other carboxylation reactions (eg. Propionyl-CoA carboxylase, methylmalonyl-CoA oxaloacetic transcarboxylase, and pyruvate carboxylase. Biotin also serves as a coenzyme in fatty acid biosynthesis [30]. Other investigations suggested that the biotin may have also role in purine biosynthesis, particularly in the change of aminoimidazole to carboxyaminoimidazole, a carboxylation process in which CO_2 is attached to the former compound. There have been several other suggestions about the involvement of biotin in synthesis of protein portion of enzymes [35], in the early stage of glucose catabolism, phosphorylation catalyzed by hexokinase [55] etc.

Therefore because biotin has very important physiological roles in microbial metabolism microbial growth may be stimulated by addition of biotin.

2.2.2 Folic Acid

Folic acid is a group of compounds of structurally and functionally similar or interrelated. The most important of them is pteroylglutamic acid. Some of other related compounds in the folic acid group are pteroyltriglutamic acid, Pteroylheptaglutamic acid, 5,6,7,8-tetrahydropteroylglutamic acid, folinic acid, 5-methyltetrahydrofolate etc.

Structure and Some Properties of Folic Acid Group Compounds: Molecular weight varies from 441.42 (pteroylglutamic acid) to 473.44 (folinic acid), sparingly soluble in room temperature. Solubility varies with pH. It is readily soluble in dilute solutions of alkali hydroxides but insoluble in alcohol, acetone, benzene, chloroform and ether. It is

more stable in alkaline than in acid solution. Loss of activity during autoclave varies with pH. The chemical structure of folic acid is given in Figure 2.

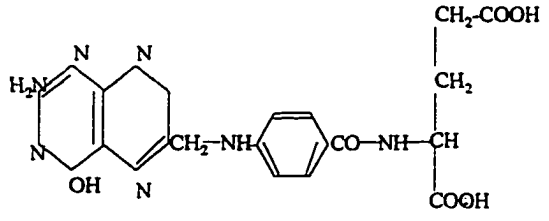


Figure 2.2. Chemical Structure of Folic Acid

The growth promoting activities of different folic acid group members were found to be varied with various genus and species of microbes as shown in the following Table[2.3].

Table 2.3. Comparison of Growth Promoting Activity of Different Folic Acid Members

Compounds	L. casei	S. faecalis	S. aginosus	P. cerevisiae	B. coagulans
Pteroglutamic acid (PGA)	+++	+++	++	-	+++
Pteroylheptaglu-	-	-	-	-	+
tamic acid	+++	+++	+++	+++	+++
Folinic acid					
N5-methyl tetrahydroPGA	+++	-	N.R	-	N.R

+++ = Very active; - = essentially inactive; N.R = not reported

Function of Folic Acid: Tetrahydrofolate compounds play the role of coenzyme in the transfer of one-carbon units. This occurs in several levels of oxidation-reduction: formyl (-CHO), hydroxymethyl (-CH_2OH), methylene (-CH_2), methyl (-CH_3) and forminino (-CH=NH) groups.

A generalized reaction mediated by folic acid coenzyme can be shown as follows:

D.X + C = C.X + D; C.X + A = A.X + C

where D is the donor molecule containing the potential one carbon unit X, C is the folic acid coenzyme, and A is an acceptor molecule.

Other metabolic changes mediated by folic acid coenzyme include the followings:

i) Introduction of C_2 and C_8 positions in purine biosynthesis;

ii) Formation of thymine by methylation of a pyrimidine intermediate,

iii) Conversion of glycine to serine,

iv) Formation of methionine by the methylation of homocysteine,

v) Introduction of #2 carbon in immidazole ring of histidine,

vi)methylation of nicotinamide to N-methyl-nicotinamide.

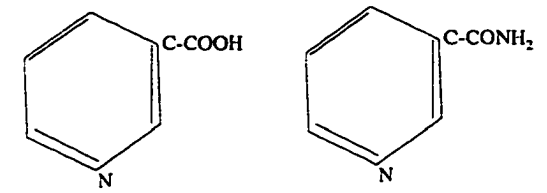
It is evident that folic acid derivatives are essential components of enzyme systems which by the transfer of one carbon units, synthesize a number of very important compounds, like precursor of nucleic acid, protein, fatty acids etc. Therefore it is essential for metabolism of microbes.

2.2.3 Nicotinic Acid

Nicotinamide is a constituent of both coenzymes NAD and NADP and plays an important role in biological oxidation. it has been recognized that nicotinic acid, nicotinamide or the coenzymes serve as growth factors for bacteria. The requirement for this vitamin for microorganisms can be satisfied with either nicotinic acid, nicotinamide or nicotinamide adenine dinucleotide. There are few exceptions like *Haemophilus influenzae* and *H. parainfluenzae* respond to NAD but not to nicotinamide or nicotinic acid. *Pasteurella*

multocida respond to nicotinamide and NAD but not to nicotinic acid. Similarly many species of *Leuconostoc* fail to utilize nicotinamide but respond to nicotinic acid and NAD.

Structure and Some Important Properties: Nicotinic acid, nicotinamide and nicotinamide adenine dinucleotide (NAD) are same compound, just in different substituted forms. These structures are given in Figure 2.3



Nicotinic Acid Nicotinamide

Figure 2.3. Chemical Structure of Nicotinic Acid and Nicotinamide

Properties of Nicotinic Acid: Molecular weight of nicotinic acid is 123.11, non hygroscopic, and sublimes without decomposition. It is soluble in water and ethanol, but insoluble in ether. It is comparatively weak acid, stable and the aqueous solution can withstand autoclaving at 15 pounds for 5 hours.

Nicotinamide has molecular weight 122.12, freely soluble in water and ethanol, slightly soluble in ether, on heating with strong acid or alkali it is hydrolyzed to nicotinic acid. NAD, NADP or the coenzyme form of nicotinic acid have much higher molecular weight (663.44 and 743.42 respectively). The stability of both oxidized and reduced NAD was determined in a wide range of pH over temperature 25-100°C.

Function of Nicotinic Acid: The coenzyme forms of nicotinamide occur in all living cells and play an essential role in hydrogen and electron transport in the process of biological

oxidation. Their important property is the ability to undergo reversible oxidation and reduction. It was early recognized that the center of coenzyme activity resides in a nicotinamide moiety. In the presence of appropriate substrate and apoenzyme, the process of reduction of the coenzyme involves the transfer of a pair of electrons and the hydrogen atom the pyridinium ring. The pentavalent nitrogen of the ring is reduced to the trivalent stage.

2.2.4 Pantothenic Acid

Pantothenic acid is an important growth factor occurs widely in natural products.

Structure and Properties Pantothenic Acid: Pantothenic acid has two components, pantoic acid and β-alanine. For some microorganisms a part of the pantothenic acid molecule suffices for growth; the intact molecule is not necessary. In certain cases the required moiety is the substituted butyryl residue, pantoic acid or its lactone form; in other cases β-alanine is the necessary part. It is usually assumed that the remaining portion of the molecule, other than the part supplied, is synthesized by the organism and pantothenic acid is formed. Other microorganisms are unable to synthesize either component and must be supplied the whole pantothenic acid molecule. Other forms of pantothenic acid are pantethine, pantetheine and coenzyme A. Pantethine and pantetheine represent the oxidized and reduced forms of a combination of pantothenic acid with β-mercaptoethylamine or cysteamine. Before the chemical structure was known, the growth promoting activity exerted by these compounds. Pantethine is a hygroscope non crystalline powder, colorless, heavy oil. It is reduced to form pantetheine. Coenzyme A is the functional form of pantothenic acid. The conversion of pantothenic acid into CoA by

microorganisms during metabolism was found by many investigators. Sulfur was recognized as a constituent of CoA. Adenine, ribose, and three phosphate groupings were also found as components of CoA [12, 18, 30]. The pure form of CoA is stable and can be stored as a powder form. Autoclaving of CoA solution causes some loss of activities [8]. The structure of the pantothenic acid is given in the Figure 2.4

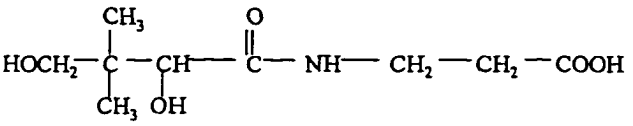


Figure 2. 4. Chemical Structure of Pantothenic Acid

Function of Pantothenic Acid: Pantothenic acid plays a metabolic role as a component of CoA and 4'phosphopantetheine. CoA acts as a carrier for acetyl and other acyl groups between donor and acceptor systems. In the process of acetyl transfer, acetyl CoA is formed and is the intermediary. There is a reversible attachment of acetyl radical to the coenzyme, with the binding occur at the -SH end of pantethiene to form thioester. The change can be pictured as:

CoA. SH + acetyl =CoA. S. CO-CH_3

The coenzyme shuttles back and forth between the free SH and thio-acetyl form. The acetyl transfer reaction may be considered in separate steps:

acetylation of CoA by various transferase enzymes (transacetylases) which derive the acetyl group from , acetyl phosphate, pyruvate, or acetoacetate; b) communications between acetyl donor and acceptor systems through acetyl CoA, c) the acetylation of the acceptor through specific enzymes (e.g. acetokinases).

As an acyl carrier, CoA plays a part in a variety of metabolic activities. It is active in the acetylation of amino acids, glucosamine, hydroxylamine, choline, sulfanilamide, and histamine in the formation of acetoacetate from two moles of acetate. It has been suggested that CoA plays a role in phosphorylation coupled with electron transport It is also found to be involved in fatty acid synthesis. In the breakdown of fatty acids, CoA along with appropriate enzymes plays a role in the removal of 2-carbon units from chain [1].

2.2.5 Thiamin (Vitamin B_1)

Thiamin requirement of soil bacteria were studied by Lochhead [36]. Though some soil bacteria showed better growth with thiamin supplementation, most of the soil microbes can synthesize it.

Some Properties of Thiamin: Thiamin is satiable in acidic pH, it can withstand autoclaving with 15 pounds pressure for 15 minutes in Phosphate -Noah buffer. Thiamin is split into its components pyrimidine and thiazole parts by sulfite in slightly acid solution at room temperature.

Among microorganisms which exhibit a thiamin requirement, there are marked differences in response toward the two parts of the vitamin molecule. Some organisms use the whole thiamin and unable to use the separate components, others are able to combine the two moieties to form thiamin. Some microbes even can synthesize some part of thiamin.

Although thiamin is required as nutritional supplement for some microorganisms, it has toxic or inhibitory effect in some other microorganisms. The pyrimidine component

has been reported to exert an anti-vitamin B6 effect and inhibit growth of several microorganisms including *Saccharomyces carlsbergensis* [42]. The pyrophosphate ester of thiazole part of thiamin was reported to inhibit yeast carboxylase by competing with coenzyme.

Thiamin pyrophosphate (TPP or Cocarboxylase) or thiamin monophosphate are the two functional forms of thiamin. Bacteria, yeasts and other fungi can produce TPP which act as the growth factor [7]. The structure of thiamin pyrophosphate is shown in Figure 2.5.

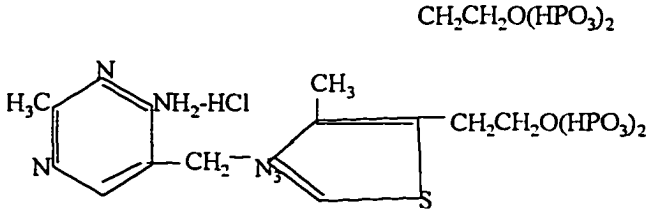


Figure 2. 5. Chemical Structure of Thiamin Pyrophosphate (TPP), The Functional Form of Thiamin

Function of Thiamin: Reactions catalyzed by enzymes containing TPP include decarboxylations of α-keto acids (pyruvic acid) with formation of acetaldehyde and CO_2.

$CH_3COCOOH \rightarrow CH_3CHO + CO_2$

The enzyme required for this reaction is pyruvate decarboxylase. It is nonoxidative decarboxylation and 'active aldehyde' is a likely intermediate in such reaction.

TPP is also involved in the oxidative decarboxylation of α-keto acids where keto acid undergoes decarboxylation to CO_2 and the next lower fatty acid. Dehydrogenation occurs along with decarboxylation , and a variety of electron transfer system is involved

here. This reaction sequence occurs in many bacteria and in animal tissues. TPP also plays a part in reactions catalyzed by transketolase in pentose phosphate shunt of carbohydrate oxidation. TPP serves as cofactor for classic enzymes found in many-cases of *Clostridium*.

Reviewing all these reports we have seen that vitamins are essential for microbial metabolism, thus required for their growth. Although many of the soil microbes are either able to synthesize or obtain required vitamins from soil. Due to tremendous competition among the soil population, it is likely that at least some organisms are always starved for micro-nutrients. Vitamin supplementation in the soil should help them to grow. Because soil microbes are used for bioremediation of diesel or other fuels, it might be advantageous to use vitamins to enhance fuel biodegradation. Only two reports relevant top this question were found after an extensive literature search. Both of the studies were restricted by single vitamin or single substrate.

2.3 Reports on the Effect of Vitamins on Biodegradation

A report from Bioprime ltd. Co. has suggested that folic acid enhanced the bioremediation of wastewater in a wastewater treatment plant [62]. The commercial product of folic acid is called dosfolat in Germany in 1986. This company tested dosfolat and found it very effective for toxic compounds like phenol, luryl sulfates degradation in wastewater. Also they reported that folic acid doubled number of colonies in agar plates for activated sludge bacteria. The study showed the addition of folic acid resulted advantageous shifts in the composition of the microbial population and increased metabolic rates slowed by the presence of toxic organics.

Another study reported by Masao Hirayama and his co workers on the efficiency of vitamins on biodegradation of oil. The report was originally written in Japanese language, and translated [23]. In the study, the researchers used marine bacteria to degrade crude oil with supplementation of vitamins B_1(thiamin), B_{12}(cyanocobalamine), H (biotin) and lipoic acid. They found that with vitamins B_1 and B_{12} the growth of bacteria was higher as well as the degradation rates were greater than the controls without vitamins. On the other hand the culture with vitamins H and lipoic acid showed inhibitory effect on bacterial growth as on degradation of oil. This was a shaker flask study in which they found that 50-80% of n-alkanes C_{11} to C_{27} were degraded by 233 hours. Degradation rates of C_{17} and C_{18} were less among the n-alkanes, probably because of the presence of pristane and phytane groups which were hardly affected by biodegradation. C_{28} and C_{30} were seemed to be extremely recalcitrant They used Gas Liquid Chromatography for quantitative analysis of carbon source in the culture. Although their results were not very consistent with the results shown for bacterial growth.

The first detail study of using vitamin supplementation for enhancing biodegradation has been started in our group. A study was done prior to the present work, which reported (unpublished) the use of vitamin mixture for biodegradation of monochlorophenols, 4-chlorobiphenyl and nonane [25]. It was found that vitamin mixture enhanced the degradation of both aromatic and aliphatic hydrocarbons by either pure culture of *Pseudomonas picketii*, strain LD1, CPE1 or by the mixed soil population. Further studies on this area can be obtained in the present dissertation.

The reports on preliminary studies have shown that the vitamin acted as a stimulator in the biodegradation process. This seems to be a unique approach. Therefore

it is worth to investigating the effect of vitamins on bioremediation of fuels in detail. If it is possible to stimulate biodegradation process by using vitamins, then it would not only be a benefit to our environment and economy, also it would open up a new research field for bioremediation of various other toxic compounds.

CHAPTER 3

OBJECTIVE

The objective of the present study was to determine the effect of five individual vitamins and vitamin mixtures on the biodegradation of short and long chain hydrocarbons and diesel fuel. Xenobiotic compounds such as isooctane, octane, nonane (representative short chain alkanes) , hexadecane, heptadecane (long chain alkanes) and diesel were tested for biodegradation by soil bacteria.

The specific objectives of this study were to investigate: (i) possible differences in the degradation pattern of odd and even numbered hydrocarbons, (ii) the effect of hydrocarbon chain length on degradation with and without vitamin supplementation, and (iii) the effect of five individual vitamins, namely biotin, thiamin, pantothenic acid, folic acid, and nicotinic acid, on the biodegradation of octane, nonane and heptadecane. To maximize the effect of vitamins, dose-response studies were conducted for each vitamin and their mixture. A vitamin mixture was made by using the individual vitamins at their optimum concentrations. This optimum vitamin mixture was used to compare the effect on biodegradation of isooctane, nonane, hexadecane, heptadecane and each fraction of alkanes in diesel fuel. Laboratory tests were performed to determine the effect of optimized vitamin mixture on total hydrocarbon and individual alkane fractions of diesel at two different concentrations.

CHAPTER 4

MATERIALS AND METHODS

4.1 Chemicals

Octane and nonane (98% purity for both) were obtained from EM Science (Cherry hill, NJ). Isooctane, hexadecane and heptadecane (all with 99% purity) were obtained from Fisher Scientific, Fairl Lawn, NJ. Culture medium constituents namely calcium chloride ($CaCl_2$, $2H_2O$), ammonium nitrate (NH_4NO_3), magnesium sulfate ($MgSO_4$. $7H_2O$), zinc sulfate ($ZnSO_4.7H_2O$) and phosphate buffers (K_2HPO_4 and KH_2PO_4), Glucose used as control in one of the experiments, and analytical grade hexane as solvents were also obtained from Fisher Scientific Company (Fair Lawn, NJ). Diesel fuels were obtained from a Hess gas station in East Newark (NJ). Sodium molybdate ($NaMo_4.2H_2O$) was from J.T. Baker Chemical Co., Phillipsberg, NJ. All vitamins were supplied by Hoffmann-La Roche, Inc., Nutley, NJ. All gases (hydrogen, nitrogen, helium) for GC analysis and oxygen for microbial growth were obtained from Matheson Co.(Rutherford, NJ).

4.2 Preparation of Various Stock Solutions

Prior to each experimental set up, various stock solutions for individual vitamins and a vitamin mixture, defined media, stock for fresh soil culture, standards were made.

4.2.1 Preparation of Vitamin Stock Solutions

The individual stock solutions of niacin, biotin, folic acid, pantothenic acid and thiamin were prepared by dissolving 50 mg of each vitamin in 1L of milli-Q water. Thus we had

each of 50 ppm. A mixture (250 ppm total) of all these five vitamins was also made by dissolving 50 mg of each vitamin in 1L milli-Q water. These stock solutions were used in different proportions in media for experiments. Another vitamin mixture (prepared after optimum concentrations were determined for individual vitamins) was prepared containing 20 mg of niacin, 20 mg of biotin, 40 mg of folic acid and 40 mg of pantothenic acid dissolved in 1L milli-Q water. This optimum vitamin mixture was used for the experiments during the last part of this study. All stock solutions were made ahead of the experiments and were stocked in refrigerator at 4°C.

4.2.2 Preparation of Inoculum

A mixed culture of soil microorganisms was used as inoculum in all experiments. The soil was collected from the grassy area in front of Boyden Hall on the Rutgers University Newark Campus. Since the soil population changes with change in seasons, a bucket (5 gallon size) of soil was preserved in the laboratory to avoid any of such effect due to seasonal variations. The soil was typical fertile garden soil, with no known exposure to any toxic chemicals. Before starting each experiment, a fresh soil culture was made by diluting 7 g of soil in 500 mL of milli-Q water in a flask. The mixture was shaken well and then heavy particles were allowed to settle to the bottom of the flask. Then the supernatant containing biomass, some water soluble soil ingredients and lighter soil particles were decanted and centrifuged at 3000 rpm for 10 minutes. The supernatant (free of any heavier soil particles) from the centrifuge was separated and again centrifuged at 10,000 rpm for 30 minutes. The biomass settled at the bottom of the centrifuge tubes were resuspended, washed with milli-Q water and centrifuged again at .10,000 rpm to

remove all the water soluble salts, vitamins and other soil components. This process was repeated for 3 to 4 times until a clear cell suspension appeared. This clean soil culture was used as inoculum for all of the experiments. The optical density of this clear soil suspension was measured by spectrophotometer (Hitachi, model U1100 with tube holder attachment).

4.2.3 Preparation of Culture Media

A modified mineral salt medium was used to grow the culture for all experiments. The composition of the medium was as follows(g/l): KH_2PO_4 (0.575); K_2HPO_4 (2.75); $MgSO_4$. $7H_2O$ (0.056); $ZnSO_4$. $7H_2O$ (0.005); $FeCl_2$. $4H_2O$ (0.001); $Na_2MoO_4.2H_2O$ (0.025); $CaSO_4.2H_2O$ (0.014); NH_4NO_3 (0.010). Final pH was maintained at 7.2. The phosphate buffer solutions (0.2M) were made beforehand and stored at 4° C. A day before starting the experiment, the buffer solution was Taken out from refrigerator and kept at room temperature and diluted to 0.02M with milli-Q water. Other salts were dissolved in the 0.02M buffer solution and autoclaved it at 120°C and 21 pounds pressure for 30 minutes and allowed to cool to room temperature.

4.2.4 Preparation of Alkane Standards

Different pure (99%) alkanes (such as nonane, hexadecane and heptadecane etc.) were taken and 100 ppm of solutions of respective alkanes were made by diluting the pure alkanes in hexane. After mixing well, the 100 ppm of alkane solutions were serially diluted to 50 ppm, 25 ppm, 12.5 ppm, 6.25 ppm and 3.125 ppm using hexane as solvent

(in a ratio of 1:1). The standards were made in larger volume, tightly sealed in glass bottles and preserved as stock solutions of standards.

4.2.5 Preparation of Diesel Standard

Since diesel composition varies with different sources, we bought a commercial standard of diesel (Tennessee/Mississippi mixture) from Restek Corporation (110 Benner Circle, Bellefonte, PA 8812). The standard was actually an artificial mixture of diesel range organics (alkanes) ranging from C_{10} to C_{25} (16 peaks). The standard contained 1000 ppm of each component dissolved in 1 ml methylene chloride. We diluted this 16000 ppm mixture into 1600 ppm of diesel using hexane solvent. Then the 1600 ppm mixture was again serially diluted to 800 ppm (i.e. each peak contained 50 ppm), 400 ppm (i.e. each peak contained 25 ppm), 200 ppm (i.e. each peak contained 12.5 ppm), 100 ppm (i.e. each peak contained 6.25 ppm) and 50 ppm (i.e. each peak contained 3.125 ppm), using hexane as solvent. GC runs were made with each concentrations. A calibration curve was made using the peak area in chromatograms and corresponding concentrations. This calibration curve (as shown in appendix I, Figure A1.2) was used to convert the sample peak area to their corresponding concentrations.

4.3 Measurement of Biomass Growth

Biomass growth was measured by optical density (OD) at a wavelength of 460 nm using a Hitachi spectrophotometer (model U 1100, with tube holder attachment). A calibration (Appendix 1) was built for OD vs biomass concentrations. During experiments the OD of previously standardized sample tubes was measured at regular time intervals till the culture

reached to stationary phase. The optical densities were converted into the corresponding biomass concentrations using the calibration curve.

4.4 Extraction and Quantitative Analysis of Alkanes and Diesel

Various alkanes and diesel were used as the substrate various experiments. Among alkanes, we used octane and nonane as representatives of short chain aliphatic hydrocarbons, hexadecane and heptadecane as longer chain hydrocarbons, and isooctane as a branched chain hydrocarbon. All the experiments were performed in 27 mL crimp sealed culture tube (glass) with teflon coated butyl rubber stopper. Since none of these alkanes or diesel were highly soluble in water, the alkanes and diesel were extracted with hexane before analysis. For this purpose, 10 mL of hexane (GC grade) were added to each of the sample tubes to be sacrificed at any given interval. After vigorous agitation on vortex shaker for five minutes, the hydrocarbons were sampled out from hexane layer of the tubes. The concentrations of alkanes and diesel were determined by gas chromatography (Hewlett-Packard 5890, series II). The GC was equipped with electronic pressure program (EPP) and autosampler. An Econocap 19660 (Alltech; Capillary column of 30m x 0.32 mm i.d.) was used. Nitrogen was used as the carrier gas with an FID detector. The data were integrated for all alkanes by a computer using minichrome® software. The GC conditions were different for short-chained hydrocarbons, longer chain hydrocarbons and diesel. The detail of GC conditions for quantification of various compounds are as follows:

4.4.1 GC conditions for Analysis of Octane, Nonane and Isooctane

Constant flow on, flow rate : 2.58mL/min

EPP on; with initial pressure : 11.8 psi

Injector temperature programmed with column; initial injector temperature : 43°C

Oven temperature : 40° -120°C with a rate of 7°C ramp

Detector temperature : 250°C

Autosampler Conditions:

Autosampler : HP GC system

Sample volume 1μL

1 injection / vial

solvent : hexane, 2 prewashes and 6 postwashes.

4.4.2 GC Conditions for Analysis of Hexadecane and Heptadecane

Since hexadecane and heptadecane are longer chain hydrocarbons, the temperature and pressure programs used for their analyses were different. Flow rate was also different as shown below. All other conditions for analysis of hexadecane and heptadecane were similar as for isooctane, octane and nonane.

Constant flow on, flow rate : 2.61mL/min

EPP on; with initial pressure : 13.5 psi

Oven tracked on-column injection with initial injector temperature : 90°C

Oven temperature : 90° -240°C with a rate of 10°C ramp

Detector temperature : 250°C

Autosampler conditions were same as mentioned for shorter hydrocarbons

4.4.3 GC Conditions for Analysis of Diesel:

Diesel oil contains aliphatic hydrocarbons ranging from C_{10} to C_{29}, and had to be analyzed using different instrument settings.

Constant flow on, flow rate : 3.35mL/min

EPP on; with initial pressure : 14.6 psi

Injector temperature programmed with column; initial injector temperature : 63°C

Oven temperature : 60°-290°C with a rate of 7°C ramp

Detector temperature : 300°C

All other conditions including column, carrier gas, autosampler were similar to those of alkanes. The data integration for diesel was not compatible with minichrome system. An integrator (HP system) was used for diesel chromatograms.

CHAPTER 5

EXPERIMENTAL PROCEDURE

This study included a large number of experiments, consequently the experiments are represented in four sections, each based on the specific objective of that set of experiments.

5.1 Part I: Initial Experiments to Determine Response of Soil Inocula to Vitamin Mixture

This experiment was performed to determine the effect of vitamin mixture (containing equal amount of all five vitamins) on microbial growth of soil inoculum with nonane as the sole carbon source. For this purpose, five different sets of test tubes were prepared and 10 mL of growth medium were transferred in each test tube. Each set of test tube was supplemented with one of the five (0.05, 0.10, 0.50, 1.0 and 5.0 ppm total) concentrations of vitamin mixture. Three different sets of controls were also made as follows:

i) V_o, i.e. control without vitamin

ii) C_1, i.e. control without nonane

iii) C_2, i.e. control without inoculum.

The proportions of media, inocula and vitamin mixture for different test concentrations are shown in Table 5.1. For all the experiments, 27 mL crimp seal culture tubes (Bellco, NJ) with teflon coated butyl rubber stoppers were used. Since the optical densities of each tubes varied, each tube was standardized by measuring optical densities

and marked the positions in each tube where all had the same (± 0.003 OD unit) initial OD. The design of the experiment was as follows:

No. of experimental concentrations : 5 (50, 100, 500, 1000 and 5000 ppb)

No. of controls : 3 (i.e., V_o, C_1, and C_2)

Experiment was performed in duplicate.

Therefore total tubes needed for each sampling : (5 + 3) x 2 = 16

Total sampling number considered for each experimental concentration and control= 10

So, total no. of tubes prepared : 16 x 10 = 160.

Since we had 20 samples for each experimental concentration and control and each sample contained 10 mL of experimental mixture of vitamin, inocula and media. Therefore 200 (20 x 10) mL of mixture for each vitamin concentration and control was made as given in the Table 5.1. Then each 200 mL mixture was apportioned into 20 tubes, each tube contained 10 mL

Table 5.1. Proportions of Inocula, Vitamins, and Media Used for Experiments of Part I and Controls

Experimental set (200 mL)	**Inoculum**	V_{mix}	**Media**
Vmix Conc.			
0.05 ppm	5 mL	0.04 mL	194.96 mL
0.10 ppm	5mL	0.08mL	194.92 mL
0.5 ppm	5 mL	0.4 mL	194.6 mL
1.0 ppm	5 mL	0.8 mL	194.2 mL
5.0 ppm	5 mL	4.0 mL	191 mL
Controls			
V_o	5 mL	0 mL	195 mL
C_2	0 mL	4.0 mL	196 mL
C_1	5 mL	4.0 mL	191 mL

Distributions of media, vitamin and inocula mixtures into the culture tubes were followed by crimp sealing each tube with teflon coated rubber stopper. 0.7 μL of nonane was carefully injected (by means of 1 μL glass syringe) through the self sealing stoppers. Each tube was then carefully swirled on vortex shaker to mix nonane with the media. The headspace contained enough oxygen (about twice the stoichiometric amount required to fully oxidize nonane) to ensure that oxygen was not limiting. All the tubes were then arranged in a covered box and put in a shaker (New Brunswick Scientific Co. Inc., Edison, NJ) for continuous shaking at 30°C at 155 rpm.

Sampling was performed at different time intervals. A test tube containing 10 mL media was used as the blank. For each sampling one of the tubes was randomly selected for measuring O.D at 460 nm and then the tube was sacrificed for extracting the nonane with hexane. To compare the growth, two standardized tubes of each experimental concentration and each control were measured for optical density during every sampling but not sacrificed till the experiment ended. After addition of nonane, the test tubes were agitated for 5 minutes on vortex mixture followed by 15 minutes on shaker. Then the phases (aqueous and hexane) were allowed to separate. Then 2 mL of the sample from hexane layer of each tube were drawn out for GC analysis.

5.2 Part II :Experiments for Kinetic Studies with Individual Vitamins and Their Mixture Using Nonane and Heptadecane as Carbon Sources

This part of the study included four different experiments as follows:

i) Experiment for degradation of nonane using 1 ppm of individual vitamins and 5 ppm of vitamin mixture and comparison with controls.

ii) Experiment for degradation of heptadecane using 1 ppm of individual vitamins and 5 ppm of vitamin mixture and comparison with controls

iii) Experiments for degradation of nonane using 20 ppb of individual vitamins and 100 ppb of vitamin mixture and comparison with controls.

iv) Experiments for degradation of heptadecane using 20 ppb of individual vitamins and 100 ppb of vitamin mixture and comparison with controls. .

For this part of the study three parameters were investigated. Those parameters were a) carbon source (nonane and heptadecane) b) individual vitamin concentrations(1 ppm and 20 ppb) and c) vitamin mixture concentrations (5 ppm and 100 ppb). Experimental design and procedures for all the experiments in this part were alike but different from that of part 1. Previously standardized tubes (27 mL crimp sealed culture tubes) were used. Each of the above mentioned five experiments followed the design as shown here:

No. of experimental sets with individual vitamins(5) and vitamin mixture : 5 + 1 = 6

No. of controls : 3 (V_o, C_1, C_2)

All experiments were performed in triplicate.

Therefore total tubes needed for each sampling : (6 + 3) x 3 = 27

Total sampling No. considered for each experimental concentration and control= 10

Total No. of tubes were standardized : 27 x 10 = 270.

Since there were 30 samples for each vitamin, vitamin mixture and controls and each sample contained 10 mL of experimental mixture of vitamin, inocula and media, Therefore 300 (30 x 10) mL of mixture for each vitamin concentration and controls was made as shown in the Table 5.2. Then each 300 mL mixture was apportioned into 30 tubes.

Table 5.2. The Proportions of Inocula, Vitamins and Media for Experiments of Part II and Controls

Conc. of Vitamins	Inoculum	Vitamin	Media	Experiment #	Alkanes used as C
Individual vitamins (1 ppm)	5 mL	6 mL	289 mL	1, 2, 3	Octane, nonane, heptadecane
Vmix (5 ppm)	5mL	6 mL	289 mL	1, 2, 3	Octane, nonane, heptadecane
Individual vitamins (20 ppb)	5 mL	120 µl	294.9mL	4, 5	Nonane and heptadecane
Vmix (100 ppb)	5 mL	120 µl	294.9mL	4, 5	Nonane and heptadecane
Controls					
Vo	5 mL	0 mL	295mL	all	Octane, nonane, heptadecane
C_2	0 mL	6 mL	294 mL	1, 2, 3	Octane, nonane, heptadecane
C_1	5 mL	6 mL	289 mL	1, 2, 3	No carbon
C_2	0	120 µl	300 mL	4, 5	Nonane and heptadecane
C_1	5 mL	120 µl	294.9mL	4, 5	No carbon

After the distribution of the mixtures in sampling tubes (as mentioned before), all of the tubes were crimp sealed with the stopper. Seven tenth µL of pure alkane (varied in different experiments, octane, nonane or heptadecane) was injected by means of a 1µL injection syringe into each tube through the self sealing stopper. The headspace contained more than enough oxygen for mineralization (Stoichiometrically 12.5 & 12.25 ml of air were required for complete oxidation of 100 ppm of nonane and heptadecane respectively, instead the headspace contained 17 ml of air in each tube and always we used less than 100 ppm of carbon source)

The controls were made accordingly as mentioned in Table 5.2. Then all the tubes were arranged in a closed box and put in a shaker (155 rpm) at 30°C. At regular time intervals triplicate samples were taken for each vitamin, vitamin mixture and the controls. Biomass growth in each tube was measured by optical density (at 460 nm), followed by the residual alkane extraction with 10 mL hexane. Three standardized tubes for each individual vitamin, vitamin mixture and controls were used with other sampling tubes for measuring microbial growth but not sacrificed until the end of the experiments. These tubes were measured to compare the biomass growth in the sacrificed tubes of the same group. GC analyses were performed to determine the alkane concentrations in the extracted samples.

5.3 Part III : Experiments for Optimization of Individual Vitamins with Heptadecane as Carbon Source

This part includes a series of experiments as follows:

i) Experiments to determine the optimum concentration of niacin for soil inoculum, using heptadecane as carbon source.

ii) Experiments to determine the optimum concentration of biotin for soil inoculum, using heptadecane as carbon source.

iii) Experiments to determine the optimum concentration of folic acid for soil inoculum, using heptadecane as carbon source.

iv) Experiments to determine the optimum concentration of pantothenic acid for soil inoculum, using heptadecane as carbon source.

v) Experiments to determine the optimum concentration of thiamin for soil inoculum, using heptadecane as carbon source.

All the above experiments were conducted simultaneously and in identical fashion. Experimental procedure for all these five experiments was identical except the vitamins were different. Each experiment included four different concentrations (50, 100, 500, 1000 ppbs) of a specific vitamin and three different controls V_o, C_1, and C_2. Each experiment in this part was designed exactly same as follows:

No. of experimental concentrations : 4 (i.e. 50, 100, 500, 1000 ppbs)

No. of controls : 3 (i.e., V_o = without vitamin, C_2 = without inoculum, and C_1 = without carbon)

These experiments were performed in triplicate.

Therefore total tubes needed for each sampling : (4 + 3) x 3 = 21

Total sampling No. considered for each experimental concentration and control= 10

So, total No. of tubes needed to be standardized : 21 x 10 = 210.

Since we had 30 samples for each experimental concentration and control and each sample contained 10 mL of experimental mixture of vitamin, inocula and media, Therefore 300 (30 x 10) mL of mixture for each vitamin concentration and control was made as given in the Table 5.3. Then each 300 mL mixture was apportioned into 30 tubes.

Table 5.3. Proportions of Inocula, Vitamins, and Media Used for Each Experiment of Part III and Controls

Experimental set (300 mL)	**Inoculum**	**Vitamin (50 ppm in store)**	**Media**
Specific vitamin			
0.05 ppm	5 mL	0.03 mL	294.97 mL
0.10 ppm	5mL	0.06mL	294.94 mL
0.5 ppm	5 mL	0.3 mL	294.7 mL

Table 5.4.(Continued)

Experimental set (300 mL)	**Inoculum**	**Vitamin (50 ppm in store)**	**Media**
1.0 ppm	5 mL	0.6 mL	294.4 mL
Controls			
V_o	5 mL	0 mL	295 mL
C_2	0 mL	0.6 mL	299.4 mL
C_1	5 mL	0.6 mL	294.4 mL

Distributions of media, vitamin and inocula mixtures into the culture tubes were followed by crimp sealing each tube with teflon coated rubber stopper. Seven tenth µl of heptadecane (pure) was carefully injected (by means of 1 µL glass syringe) through the self sealing stoppers. Each tube was then carefully shaken in vortex shaker to mix the hydrocarbon with the media. The headspace contained more than enough oxygen (about 200% the stoichiometric amount required to fully oxidize) to ensure that oxygen was not limiting. All the tubes were then arranged in a covered box and kept in a shaker (New Brunswick Scientific Co. Inc., Edison, NJ) for continuous shaking at 30°C and 155 rpm. Sampling was performed at different time intervals. For each sampling one of the tubes was used for measuring optical density at 460 nm and then the tube was sacrificed for extracting the residual alkane with hexane. To compare the growth, three standardized tubes of each experimental concentration and each control were measured for optical density during every sampling but not sacrificed till the experiment ended. The extraction procedure is identical as stated before.

5.4 Part IV : Experiments to Determine the Effect of Optimum Vitamin Mixture on Growth of Soil Micoorganisms with Isoctane, Nonane, Hexadecane, Heptadecane as Carbon Source

As we optimized each individual vitamin, we made a mixture of them containing optimum concentration of each vitamin. Thiamin was taken out from the mixture because it seemed to have no effect on biomass growth of soil inoculum. Therefore the mixture contained four vitamins at their optimum concentrations and we named it as optimum vitamin mix (V_{opt}). The recipe of this V_{opt} has already mentioned before. This mixture was used as vitamin supplement for various experiments in Part IV that includes 4 different experiments as:

i) Experiments to determine the effect of V_{opt} for isooctane degradation

ii) Experiments to determine the effect of V_{opt} for nonane degradation

iii) Experiments to determine the effect of V_{opt} for hexadecane degradation

iv) Experiments to determine the effect of V_{opt} for heptadecane degradation

All the above experiments were performed simultaneously. The procedure was same for all these four experiments. Only different alkanes were used as carbon source. The experimental design followed by all of these four experiments were exactly alike. The design for each experiment was:

No. of experimental concentrations : 1

No. of controls : 3 (i.e., V_o = without vitamin, C_2 = without inoculum, and C_1 = without carbon)

Experiment was performed in triplicate.

Therefore total tubes needed for each sampling : (1 + 3) x 3 = 12

Total sampling No. considered for each experimental concentration and control= 10

So, total No. of tubes needed to be standardized : 12 x 10 = 120.

We had 30 samples for V_{opt} mixture and each control and each sample contained 10 mL of experimental mixture of vitamin, inocula and media. Therefore 300 (30 x 10) mL of mixture for vitamin mixture and three different controls were made as shown in the table1. Then each 300 mL mixture was apportioned into 30 tubes, each tube contained 10 mL of it.

Table 5.5. Proportions of Inocula, Vitamins, and Media Used for Each Experiment of Part IV and Controls

Samples (300 mL))	**Inoculum**	V_{opt}	**Media**
Experimental sets	5 mL	0.75 mL	294.25 mL
Control sets			
V_o	5 mL	0 mL	295mL
C_2	0 mL	0.75 mL	294.25 mL
C_1	5 mL	0.75 mL	294.25mL

After the distribution of the mixtures in sampling tubes (10 mL in each tube), all of the tubes were crimp sealed with the stopper. A 0.7 μL of pure alkane (varied in different experiments, isooctane, nonane, hexadecane or heptadecane) was injected by means of a 1μL injection syringe into each tube through the self sealing stopper. Then all the tubes were arranged in a closed box and put in a shaker (155 rpm) at 30°C.

At each sampling, three sample tubes were used for measuring optical density at 460 nm and then sacrificed for extracting the residual alkane with hexane. To compare the growth, three additional standardized tubes of each experimental concentration and each control were measured for optical density during every sampling but not sacrificed till the

experiment ended.. 2 mL of the sample from hexane layer of each sacrificed tube were drawn out for GC analysis.

5.5 Part V : Experiments to Determine the Effect of Optimum Vitamin Mixture for Diesel Biodegradation by Soil Inoculum

Two experiments were performed in this part such as

i) Experiment to determine the effect of V_{opt} on biomass growth and degradation of diesel at lower initial concentration (approx. 70 ppm)

ii) Experiment to determine the effect of V_{opt} on biomass growth and degradation of diesel at higher initial concentration (approx. 150 ppm)

The procedure was same for both of these experiments. The pre-standardized 27 mL crimp seal culture tubes were used with teflon coated butyl rubber stoppers (same as other experiments before). The design for each experiment was:

No. of experimental concentrations : 1

No. of controls : 3 (i.e., V_o, C_1 C_2)

Experiment was performed in triplicate.

Therefore total tubes needed for each sampling : (1 + 3) x 3 = 12

Total sampling No. considered for each experimental concentration and control= 10

So, total No. of tubes needed to be standardized : 12 x 10 = 120.

We had 30 samples for V_{opt} mixture and each control and each sample contained 10 mL of experimental mixture of vitamin, inocula and media. Therefore 300 (30 x 10) mL of mixture for V_{opt} and three different controls were made as shown in the Table 5. Then

each 600 mL mixture was apportioned into 30 tubes, each tube contained 10 mL of it as mentioned for previous experiments.

Table 5.6 Proportions of Inocula, Vitamins, and Media Used for Each Experiment of Part V and Controls

Samples (300 mL))	**Inoculum**	**V_{opt}**	**Media**	**Diesel /10 mL of mixture (each tube)**
Experimental sets for exp. No. 1	5 mL	0.75 mL	294.25 mL	0.7 µL
Control sets for exp. # 1				
V_o	5 mL	0 mL	295mL	0.7 µL
C_2	0 mL	0.75 mL	294.25 mL	0.7 µL
C_1	5 mL	0.75 mL	294.25mL	0 µL
Experimental sets for exp. # 2	5 mL	0.75 mL	294.25 mL	1.5 µL
Control sets for exp. # 2				
Vo	5 mL	0 mL	295mL	1.5 µL
C_2	0 mL	0.75 mL	294.25 mL	1.5 µL
C_1	5 mL	0.75 mL	294.25mL	0 µL
Control for oxygen	5 mL	0 mL	295 mL	30 mg glucose

Distribution of media, vitamin and inocula mixtures into the culture tubes were followed by crimp sealing each tube with teflon coated rubber stopper. Diesel was carefully injected in each tube (by means of 5 µL glass syringe) through the self sealing stoppers. Each tube was then carefully shaken in vortex shaker to mix the hydrocarbon with the media. The oxygen in the headspace of the culture tubes was not enough (as calculated for the stoichiometric amount required to fully oxidize 500 ppm diesel was 12.33 ml oxygen or 58.81 ml of air and we had 17 ml headspace in each tube) for complete degradation of diesel. To avoid the problem of oxygen limitation, each tube

(after crimp sealing) was evacuated to 20 psi followed by introduction of .1.3 psig pure oxygen through the self sealing stoppers. The pure oxygen was also introduced into the control tubes with inoculum, media and glucose to ensure that the pure oxygen was not toxic for the biomass growth. All the tubes were then arranged in a covered box and put in a shaker (New Brunswick Scientific Co. Inc., Edison, NJ) for continuous shaking at 30°C at 155 rpm.

Sampling was performed at regular time intervals. For each sampling triplicate tubes of each set were used for measuring optical density at 460 nm and then the tube were sacrificed for extracting the residual diesel with hexane (as described for all other previous experiments). Two mL of the sample from hexane layer of each tube were drawn out for GC analysis.

CHAPTER 6

RESULTS AND DISCUSSION

In this section various experimental results are divided into five sections , as described in chapter 5 (Experimental Procedure section). In Each section the results are presented in tables along with corresponding figures. Discussion for each section is followed by the tables and figures. Results here are mostly interpreted from the original data (showed in Appendix B). The figures are representing the original data and used for determination of several parameters as shown below.

Interpretation of Experimental Results

Since mixed populations were used and samples were taken randomly from individual microcosms, the growth of biomass or the degradative activity started at various times. To analyze the data in a consistent fashion a number of variables were defined. The lag time (L_{av}) was defined here as the time corresponding up to 5% of degradation of the carbon source. The overall degradation rates and the overall growth rates were calculated as

$$O.D.R.(ppm/h) = \frac{R_0 - R_{95}}{t_{95}} \qquad (1)$$

[O.D.R. = Overall degradation rate (ppm/h);

R_0 = Initial carbon conc.(ppm);

R_{95} = Residual carbon conc. at 95% of carbon degradation;

$$O.G.R.(ppm/h) = \frac{B_0 - B_{95}}{t_{95}} \qquad (2)$$

[O.G.R. = Overall biomass growth rate (ppm/h);

B_0 = Initial biomass concentration.by dry weight (ppm);

B_{95} = Biomass concentration (ppm) when 95% of the initial target carbon source concentration had been degraded]

$$O.Y.C. = \frac{B_{95} - B_0}{R_0 - R_{95}} \qquad (3)$$

[O.Y.C. = Overall yield coefficient during the period between t = 0 and the time corresponding to 95% degradation of initial carbon concentration]

In order to describe the rate of carbon reduction during the logarithmic growth phase the average degradation rate (D.R.) was defined as

$$D.R. = \frac{R_{20} - R_{75}}{t_{75} - t_{20}} \qquad (4)$$

where:

[D.R. = Degradation rate during logarithmic growth phase (ppm/h)

R_{20} = Residual carbon concentration when 20% of the initial target carbon source concentration had been degraded (ppm);

R_{75} = Residual carbon concentration when 75% of the initial target carbon source concentration had been degraded (ppm)

t_{20} = Time required to achieve 20% degradation of the initial carbon source.

t_{75} = Time required to achieve 75% degradation of the initial carbon source]

Similarly, the specific growth rate (G.R.) during the logarithmic growth phase was defined as :

$$G.R. = \frac{\frac{B_{20} - B_{75}}{t_{75} - t_{20}}}{\frac{B_{75} + B_{20}}{2}} \qquad (5)$$

where:

[G.R. = Specific growth rate during the logarithmic growth phase (1/h)

B_{75} = Biomass concentration (ppm) when 75% of the initial target carbon source concentration had been degraded.

B_{20} = Biomass concentration (ppm) when 20% of the initial target carbon source concentration had been degraded].

Finally the yield coefficient (Y.C.) during the logarithmic growth phase was defined as:

$$Y.C. = \frac{B_{75} - B_{20}}{R_{20} - R_{75}} \qquad (6)$$

where:

[Y.C. = Yield coefficient during the period corresponding to the 20% and 75% degradation of target carbon source].

The above six equations were used to calculate the results shown in all of the tables in this chapter. Calculations of the percentage degradation and percentage growth were accomplished by using the figures presented with each corresponding tables.

6.1 Part I. Results of the Initial Experiments to Determine the Degradation Activity of Soil Inocula in the Presence of a Vitamin Mixture V_{mix}

This is the initial stage of the experiments developed to ascertain the response of soil inoculum to the V_{mix} (a mixture of 1 ppm of five individual vitamins), compared to the mixture without vitamin i.e. V_0. We selected five vitamins: niacin, biotin, folic acid, thiamin, and pantothenic acid, based on their roles in microbial metabolism (from literature review). The effect of this V_{mix} was studied at different concentrations ranging from 50 ppb to 1000 ppb against the control V_0 (without vitamin). Nonane was used as sole supplied carbon source. The results of this part are given in Table 6.1 and the corresponding parameter table. Figures 6.1a and 6.1b were used to calculate the percentage values as shown in Table 6.1.

Table 6.1. Results of Initial Experiments to Determine the Response of Soil Inocula to Different Concentrations of V_{mix} Using Nonane as Carbon Source

	Nonane as Carbon Source								
	V_0			V_{50}			V_{100}		
d (%)	R (ppm)	t (h)	B (ppm)	R (ppm)	t (h)	B (ppm)	R (ppm)	t (h)	B (ppm)
Initial	56	0	11.8	61	0	11.38	54.5	0	11.6
5	53.2	46	10.3	57.95	58	12	51.775	49	10.5
20	44.8	60	18	48.8	60	21	43.6	59	21
50	28	66	18.5	30.5	62	22.5	27.25	61	25
75	14	77	19	15.25	71	25	13.625	63	29
95	2.8	100	17.5	3.05	96	24.5	2.725	75	34
100	0	126	16.6	0	106	21.9	0	106	20.8

Table 6.2 (Continued)

	Nonane as Carbon Source					
	V_{500}			V_{1000}		
d (%)	R (ppm)	t (h)	B (ppm)	R (ppm)	t (h)	B (ppm)
Initial	53	0	12.23	53.5	0	9.59
5	50.35	48	12	50.825	56	10.5
20	42.4	60	20	42.8	61	17
50	26.5	61	22	26.75	70	17.5
75	13.25	65	29.5	13.375	80	31.5
95	2.65	95	31	2.675	103	30
100	0	106	28.6	0	124	21.08

Table 6.1a. Parameters Obtained from Results of Initial Experiments to Determine the Response of Soil Inocula to Different Concentrations of V_{mix} Using Nonane as Carbon Source

	V_o	V_{50}	V_{100}	V_{500}	V_{1000}
L_{av} (h)	46	32.5	49	48	56
O.D.R (ppm/h)	0.53	0.60	0.69	0.53	0.49
O.G.R (ppm/h)	0.06	0.14	0.30	0.20	0.20
O.Y.C	0.11	0.23	0.43	0.37	0.40
D.R (ppm/h)	1.81	3.05	7.49	5.83	1.55
G.R (ppm/h)	0.003	0.02	0.08	0.08	0.03
Y.C	0.032	0.12	0.27	0.33	0.49

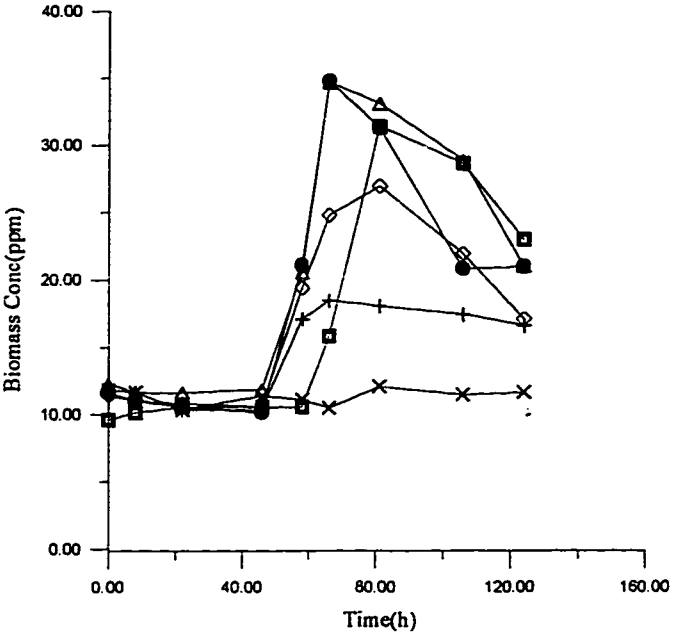


Figure 6.1a. Effect of Various Concentrations of V_{mix} on Biomass Growth Using Nonane as Carbon Source

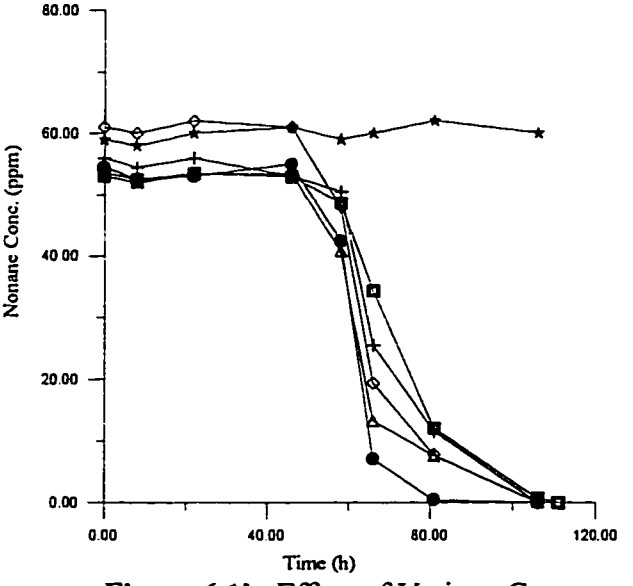


Figure 6.1b. Effect of Various Concentrations of V_{mix} on Nonane Degradation

+ Vo ● V100 ◘ V1000

◇ V50 △ V500 × C1 ★ C2

From the results of the initial experiments as shown in Table 6.1 we have seen that the lag phase was reduced with 50 ppb of V_{mix} by 14.5 hours as compared to that of V_0, but for other concentrations of V_{mix} (100 ppb, 500 ppb and 1000 ppb) lag phases were almost the same or longer than that of V_0. Overall degradation rate (O.D.R.) and overall growth rates (O.G.R.) were highest with 100 ppb V_{mix}. Degradation rate (D.R.) and growth rate (G.R.) at log phase were also highest with 100 ppb V_{mix}. At 1000 ppb V_{mix}, growth rates and degradation rates for both cases (overall and log phase) were less than those of V_0, which suggested that at 1000 ppb concentration, V_{mix} may inhibit the biomass growth and thus the degradative activity.

Figure 6.1a showed that the biomass growth with 1000 ppb V_{mix} started later than V_0 and other concentrations of V_{mix}. From this Figure we see that the maximum biomass concentration achieved with 1000 ppb V_{mix} was more than that with V_0 and V_{50} but less than that with 100 ppb and 500 ppb V_{mix}. In fact, biomass with 100 ppb V_{mix} reached the highest concentrations as compared to others. Five hundred ppb of V_{mix} also shows an almost identical growth pattern as the growth pattern with 100 ppb (Figure 6.1a). Figure 6.1b clearly showed that the times required for nonane degradation were minimum for V_{100}, closely followed by V_{500} ppb and maximum for V_{1000} (even greater than time required with V_0). C_1 (no carbon) and C_2 (no biomass) representing two controls (already mentioned in chapter 5) for alkane and for biomass respectively. C_1 shows no growth (Figure 6.1a) and C_2 showed no degradation (Figure 6.1b) which confirmed that the degradation of nonane occurred due to microbial growth.

Overall yield coefficient (O.Y.C) in the experiment also increased with addition of V_{mix}. The possible explanations for this might be that i) vitamins facilitated the growth of

some populations which could not grow on nonane without vitamin supplementation. ii) The microbes need vitamins for metabolism and most of the competent bacteria expend much ATP for the biosynthesis of some required vitamins. Exogenous vitamin supplementation may save bacterial energy from intracellular biosynthesis of vitamins and convert that energy into biomass. There were some exceptions observed in case of yield coefficient for log phase. For example, yield coefficient for logarithmic phase (Y.C) with 50 ppb V_{mix} was lower than that with V_o (Table 6.1a) . Because we used the mixed population, these kinds of discrepancies may be possible.

Since the O.D.R and O.G.R. and also D.R and G.R were highest at 100 ppb of V_{mix}, we therefore considered the 100 ppb of V_{mix} as the most appropriate micronutrient to enhance growth of the experimental inoculum. From this experiment, we have seen that 500 ppb was also almost equally effective as that of 100 ppb. However, the 1000 ppb of V_{mix} seems to be inhibitory. In order to get a clear-cut idea about how the inocula behave in regards to individual vitamins at lower and higher concentrations, experiments were conducted (as discussed in part II). One hundred ppb V_{mix} contained 20 ppb of each of the five individual vitamins (niacin, biotin, folic acid, thiamin, and pantothenic acid). Therefore, we selected 20 ppb of individual vitamins for studying the lower concentration and 1000 ppb of individual vitamins were selected for studying the higher concentration. Response of inocula to shorter- and longer-chain alkanes were also investigated, along with the individual vitamin studies.

6.2 Part II. Results of the Experiments with Individual Vitamins and Their Mixture Using Nonane and Heptadecane as Carbon Sources

This part of the study, four experiments were performed using individual vitamins (niacin, V_1; biotin, V_2; folic acid, V_3; thiamin, V_4; and pantothenic acid, V_5)and a vitamin mixture. Individual vitamins were tested at two different concentrations, 20 ppb and 1000 ppb. Vitamin mixture or V_{mix} was tested for 100 ppb and 5000 ppb concentrations in a ratio of $V_1:V_2:V_3:V_4:V_5$ = 1:1:1:1:1. Nonane or heptadecane were the supplied carbon source. All these experiments with a single vitamin or a vitamin mixture, with nonane or heptadecane were compared with controls without vitamin (V_0), without carbon (C_1) and without inocula (C_2). The setup of all four experiments are discussed in detail in chapter 5 (Experimental Procedure section)

The results are shown in the following Tables 6.2, 6.3, 6.4, 6.5 and their corresponding parameter tables 6.2a, 6.3a, 6.4a, 6.5a respectively. Figures 6.2a, 6.2b, 6.3a, 6.3b, 6.4a, 6.4b, 6.5a, and 6.5b are representing the graphical form of the same results.

Table 6. 3. Results of Experiments on the Degradation of Nonane Using 20 ppb of Individual Vitamins and 100 ppb of Vitamin Mixture

	Nonane as Carbon Source								
	V_0			**V_1 (20 ppb)**			**V_2 (20 ppb)**		
d (%)	R (ppm)	t (h)	B (ppm)	R (ppm)	t (h)	B (ppm)	R (ppm)	t (h)	B (ppm)
0	59.39	0	10.82	60.39	0	10.97	59.63	0	10.33
5	56.42	45	11	57.37	34	12	56.65	47	12
20	47.51	53	12	48.31	45	13	47.7	53	15
50	29.7	65	25.5	30.2	51	15	29.82	61	26
75	14.85	75	32	15.1	62	29	14.91	68	38
95	2.97	90	40	3.02	75	38	2.98	77	41
100	0	99	42.9	0	78	39.1	0	93	39.29

Table 6.4 (Continued)

	Nonane as Carbon Source					
	V_3 (20 ppb)			**V_4 (20 ppb)**		
d (%)	R (ppm)	t (h)	B (ppm)	R (ppm)	t (h)	B (ppm)
0	59.19	0	11.39	60.53	0	16.39
5	56.23	35.5	13	57.5	49	12
20	47.35	54	14	48.42	60	29
50	29.6	80	22	30.27	70	42
75	14.8	90	35	15.13	75	50
95	2.96	97	48	3.03	88	57
100	0	99	48.04	0	93	66

	Nonane as Carbon Source					
	V_5 (20 ppb)			**V_m (100 ppb)**		
d (%)	R (ppm)	t (h)	B (ppm)	R (ppm)	t (h)	B (ppm)
0	60.73	0	12.45	61	0	16.04
5	57.69	40	15	57.95	45	17
20	48.58	55.5	20	48.8	47	18
50	30.37	64	34	30.5	56	29
75	15.18	76	45	15.25	69	34
95	3.04	90	51	3.05	76	39
100	0	99	49.16	0	78	39.17

Table 6.2a. Parameters Obtained from Results of Experiments on the Degradation of Nonane Using 20 ppb of Individual Vitamins and 100 ppb of Vitamin Mixture

	Vo	**V_1**	**V_2**	**V_3**	**V_4**	**V_5**	**V_{mix}**
L_{av}(h)	52.5	52.5	52.5	68.5	52.5	52.5	44
O.D.R (ppm/h)	0.63	0.76	0.74	0.58	0.65	0.64	0.76
O.G.R (ppm/h)	0.32	0.36	0.34	0.38	0.46	0.43	0.30
O.Y.C	0.52	0.47	0.54	0.65	0.71	0.67	0.40
D.R (ppm/h)	1.48	1.95	2.19	0.90	2.22	1.63	1.53
G.R (ppm/h)	0.041	0.045	0.058	0.024	0.04	0.038	0.023
Y.C	0.61	0.48	0.70	0.65	0.63	0.75	0.48

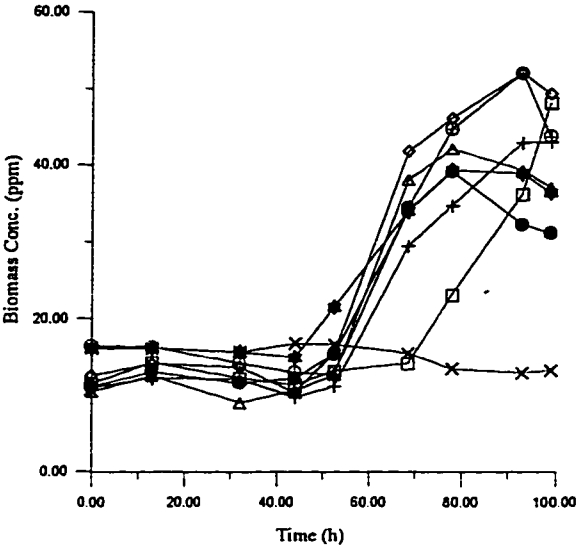


Figure 6.2a. Effect of 20 ppb of Individual Vitamins and 100 ppb of Vitamin Mixture on Biomass Growth, Using Nonane as Carbon source

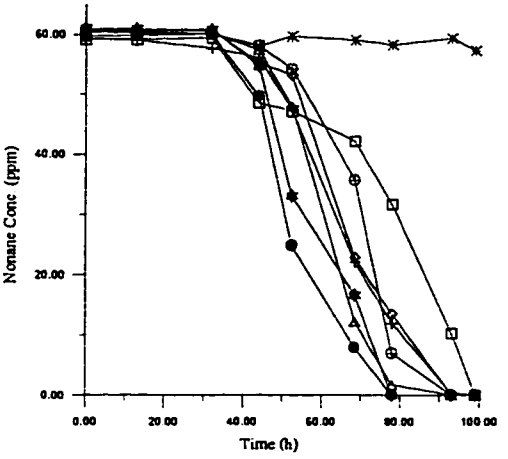


Figure 6.2b. Effect of 20 ppb of Individual Vitamins and 100 ppb of Vitamin Mixture (V_{mix})on Degradation of Nonane

Table 6. 5. Results of Experiments on the Degradation of Nonane Using 1000 ppb of Individual Vitamins and 5000 ppb of Vitamin Mixture

	Nonane as Carbon Source								
	V_0			**V_1 (1000 ppb)**			**V_2 (1000 ppb)**		
d (%)	R (ppm)	t (h)	B (ppm)	R (ppm)	t (h)	B (ppm)	R (ppm)	t (h)	B (ppm)
0	52.67	0	6.74	52.33	0	2.74	55	0	2.10
5	50.04	74	5	49.71	69	2.5	52.25	71	2
20	42.14	79	10	41.86	75	2	44	75	2.5
50	26.34	85	15	26.17	87	12	27.5	81	5
75	13.17	93	22	13.08	97	18.5	13.75	90	12
95	2.63	110	32	2.62	108	24	2.75	102	23
100	0	113	32.04	0	113	26.21	0	113	33.02

	Nonane as Carbon Source					
	V_3 (1000 ppb)			**V_4 (1000 ppb)**		
d (%)	R (ppm)	t (h)	B (ppm)	R (ppm)	t (h)	B (ppm)
0	56	0	3.16	53.67	0	7.24
5	53.2	67	3.5	50.99	61	4
20	44.8	73	4	42.94	75	6
50	28	80	11	26.84	81	11
75	14	85	14	13.42	88	17
95	2.8	100	25	2.68	100	20
100	0	105	29.44	0	105	21.78

	V_5 (1000 ppb)			**V_m (5000 ppb)**		
d (%)	R (ppm)	t (h)	B (ppm)	R (ppm)	t (h)	B (ppm)
0	59.33	0	3.65	46.67	0	3.65
5	56.36	73	4	44.34	52	3.5
20	47.46	88	17	37.34	62	5
50	29.67	99	20	23.34	69	7.5
75	14.83	110	23	11.67	72	8
95	2.97	114	24.5	2.33	82	26
100	0	120	14.9	0	86	35

Table 6.3a. Parameters Obtained from Results of Experiments on the Degradation of Nonane Using 1000 ppb of Individual Vitamins and 5000 ppb of Vitamin Mixture

	Vo	V_1	V_2	V_3	V_4	V_5	V_{mix}
L_{av}(h)	74	74	74	66.5	74	74	51.5
O.D.R (ppm/h)	0.45	0.46	0.51	0.53	0.51	0.49	0.54
O.G.R (ppm/h)	0.23	0.20	0.20	0.22	0.13	0.18	0.27
O.Y.C	0.50	0.43	0.40	0.41	0.25	0.37	0.50
D.R (ppm/h)	2.07	1.31	2.02	2.57	2.27	1.48	2.57
G.R (ppm/h)	0.054	0.073	0.087	0.093	0.074	0.014	0.046
Y.C	0.41	0.57	0.31	0.32	0.37	0.18	0.12

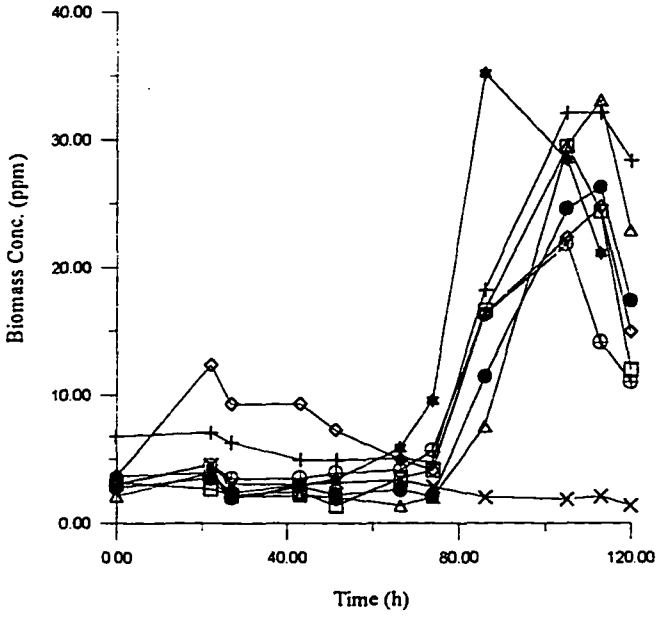


Figure 6.3a. Effect of 1000 ppb of Individual Vitamins and 5000 ppb of Vitamin Mixture on Biomass Growth Using Nonane as Carbon Source

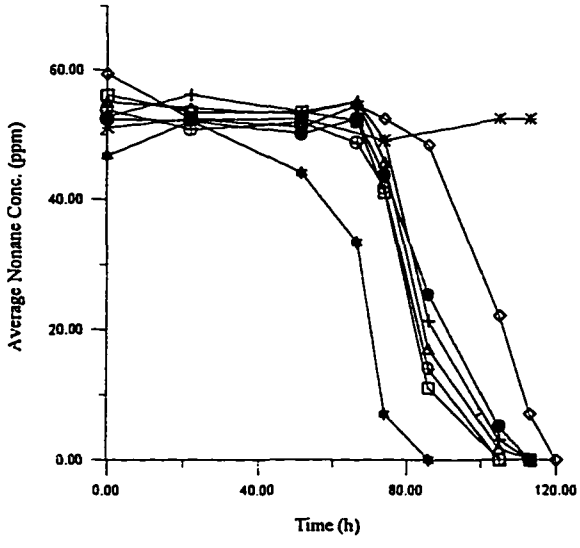


Figure 6.3b. Effect of 1000 ppb of Individual Vitamins and 5000 ppb of Vitamin Mixture on Degradation of Nonane.

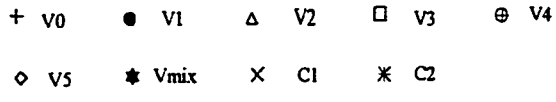


Table 6. 4. Results of Experiments on the Degradation of Heptadecane Using 20 ppb of Individual Vitamins and 100 ppb of Vitamin Mixture

	Heptadecane as Carbon Source								
	V_0			**V_1 (20 ppb)**			**V_2 (20 ppb)**		
d (%)	R (ppm)	t (h)	B (ppm)	R (ppm)	t (h)	B (ppm)	R (ppm)	t (h)	B (ppm)
0	60.05	0	8.92	59.39	0	8.85	62.29	0	9.91
5	57.05	19	9	56.42	23	12	59.18	24	10.5
20	48.04	32	10.5	47.51	31	15	49.83	34	18
50	30.03	40	22	29.7	37	28.5	31.15	39	29
75	15.01	49	35	14.85	42	44	15.57	42	38
95	3	56	39	2.97	55	42	3.11	55	44
100	0	65	42.58	0	58	41	0	58	41.17

Table 6.4 (Continued)

	Heptadecane as Carbon Source					
	V_3 (20 ppb)			**V_4 (20 ppb)**		
d (%)	R (ppm)	t (h)	B (ppm)	R (ppm)	t (h)	B (ppm)
0	62.09	0	10.19	61.86	0	13.91
5	58.99	30	10	58.77	30	11
20	49.67	37	11	49.49	32	12
50	31.05	44	18	30.93	42	24
75	15.52	54	28.5	15.47	50	32
95	3.1	64	44.5	3.09	56	42
100	0	65	44.48	0	58	42.65

	Heptadecane as Carbon Source					
	V_5 (20 ppb)			**V_m (100 ppb)**		
d (%)	R (ppm)	t (h)	B (ppm)	R (ppm)	t (h)	B (ppm)
0	59.74	0	5.76	60.72	0	10.19
5	56.75	30	4	57.68	22	14
20	47.79	35	16.5	48.58	27	18
50	29.87	40	20	30.36	33	21
75	14.94	47	31	15.18	39	35
95	2.99	56	41	3.04	47	45
100	0	65	42	0	58	41.32

Table 6.4a. Parameters Obtained from Results of Experiments on the Degradation of Heptadecane Using 20 ppb of Individual Vitamins and 100 ppb of Vitamin Mixture

	Vo	**V_1**	**V_2**	**V_3**	**V_4**	**V_5**	**V_{mix}**
L_{av}(h)	34	26	26	42	34	26	18
O.D.R (ppm/h)	1.02	1.03	1.08	0.92	1.05	1.01	1.23
O.G.R (ppm/h)	0.54	0.60	0.62	0.54	0.50	0.63	0.74
O.Y.C	0.53	0.59	0.58	0.58	0.48	0.62	0.60
D.R (ppm/h)	1.94	2.97	4.28	2.01	1.89	2.74	2.78
G.R (ppm/h)	0.063	0.071	0.089	0.052	0.05	0.051	0.053
Y.C	0.74	0.59	0.58	0.51	0.59	0.44	0.51

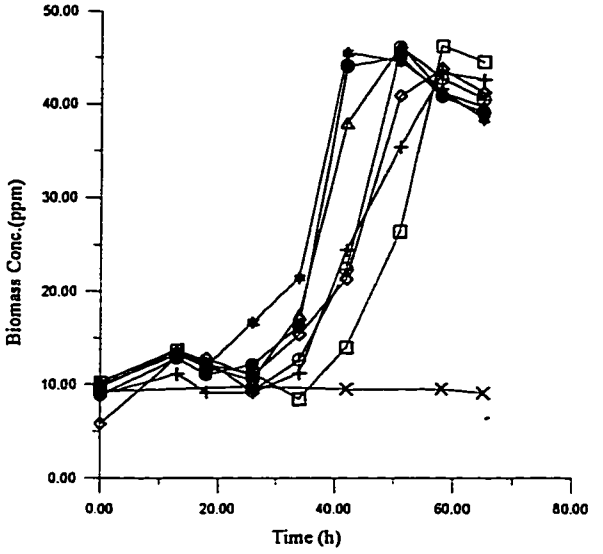


Figure 6.4a. Effect of 20 ppb of Individual Vitamins and 100 ppb of Vitamin Mixture on Biomass Growth Using Heptadecane as Carbon Source

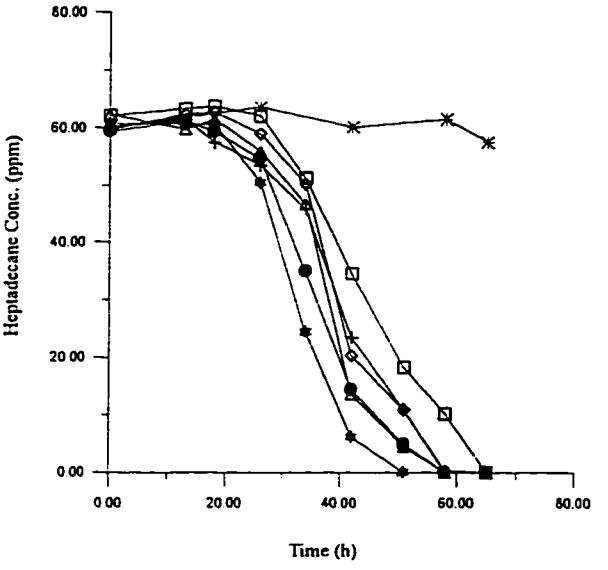


Figure 6.4b. Effect of 20 ppb of Individual Vitamins and 100 ppb of Vitamin Mixture on Degradation of Heptadecane.

+ V0 ● V1 △ V2 □ V3 ⊕ V4

◇ V5 ✱ Vmix × C1 ✳ C2

Table 6.5 Results of Experiments on the Degradation of Heptadecane using 1000 ppb of Individual Vitamins and 5000 ppb of Vitamin Mixture.

	Heptadecane as Carbon Source								
	V_0			**V_1 (1000 ppb)**			**V_2 (1000 ppb)**		
d (%)	R (ppm)	t (h)	B (ppm)	R (ppm)	t (h)	B (ppm)	R (ppm)	t (h)	B (ppm)
0	51.5	0	6.11	51	0	8.11	54.5	0	8.33
5	48.93	36	6	48.45	36	13.5	51.78	22	10
20	41.2	42	7	40.8	42	18	43.6	29	8
50	25.75	53	17	25.5	52	22.5	27.25	39	11
75	12.88	62	21	12.75	53	24	13.63	54	27.5
95	2.58	68	23	2.55	64	27	2.73	55	28
100	0	71	23.47	0	68	27.3	0	56	28.83

	Heptadecane as Carbon Source					
	V_3 (1000 ppb)			**V_4(1000 ppb)**		
d (%)	R (ppm)	t (h)	B (ppm)	R (ppm)	t (h)	B (ppm)
0	56	0	6.43	53	0	11.7
5	53.2	28	8.5	50.35	26	8
20	44.8	37	7	42.4	42	8
50	28	52	14	26.5	53	17
75	14	62	21	13.25	62	21
95	2.8	68	24.5	2.65	68	23
100	0	71	25.41	0	71	20.03

	Heptadecane as Carbon Source					
	V_5(1000 ppb)			**V_m(5000 ppb)**		
d (%)	R (ppm)	t (h)	B (ppm)	R (ppm)	t (h)	B (ppm)
0	53.5	0	8.22	55.5	0	6.85
5	50.83	35	18	52.72	12	8.5
20	42.8	45	12	44.4	18	8
50	26.75	55	23.5	27.75	25	13.5
75	13.38	66	23.5	13.88	31	20
95	2.68	70	22	2.78	39	28.5
100	0	71	21.82	0	40	30

Table 6.5a Parameters Obtained from. Results of Experiments on the Degradation of Heptadecane using 1000 ppb of Individual Vitamins and 5000 ppb of Vitamin Mixture

	V_o	V_1	V_2	V_3	V_4	V_5	V_{mix}
L_{av}(h)	40	28	28	40	40	40	21
O.D.R (ppm/h)	0.72	0.76	0.94	0.78	0.74	0.73	1.35
O.G.R (ppm/h)	0.25	0.30	0.36	0.27	0.17	0.20	0.56
O.Y.C	0.35	0.39	0.38	0.34	0.22	0.27	0.41
D.R (ppm/h)	1.42	2.55	1.20	1.23	1.46	1.40	2.35
G.R (ppm/h)	0.05	0.026	0.044	0.04	0.04	0.031	0.066
Y.C	0.49	0.21	0.65	0.45	0.45	0.39	0.39

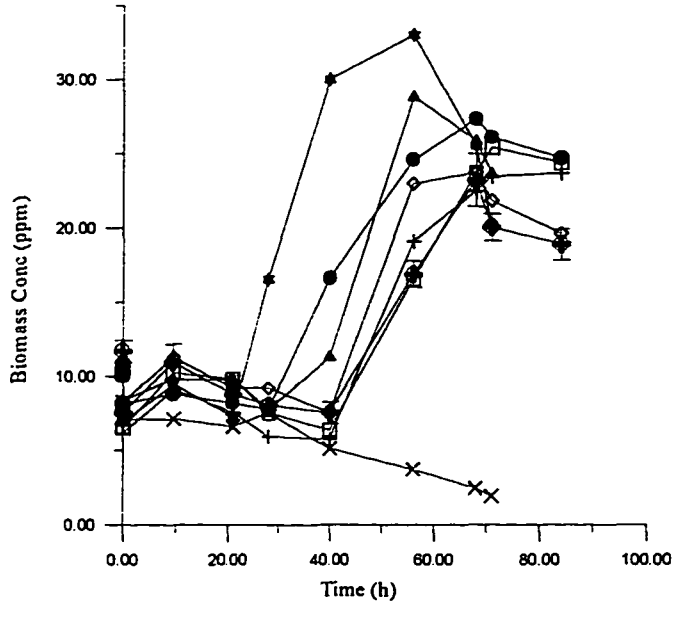


Figure 6.5a Effect of 1000 ppb of Individual Vitamins and 5000 ppb of Vitamin Mixture on Biomass Growth Using Heptadecane as Carbon Source

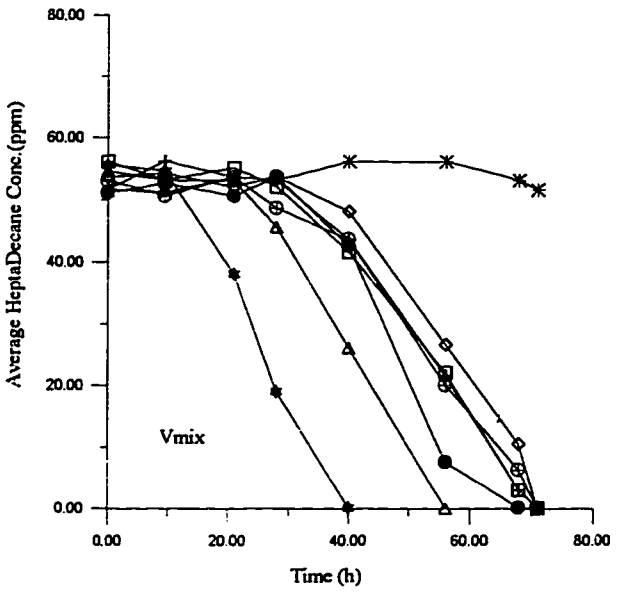


Figure 6.5b. Effect of 1000 ppb of Individual Vitamins and 5000 ppb of Vitamin Mixture on Degradation of Heptadecane.

+ V0 ● V1 △ V2 □ V3 ⊕ V4

◇ V5 ✱ Vmix × C1 ✳ C2

From the results as shown in Tables 6.2, 6.3, 6.4, 6.5 and Figures 6.2a, 6.2b, 6.3a, 6.3b, 6.4a, 6.4b and 6.5a, 6.5b respectively, one can draw a comparative picture among two different concentrations of vitamins. i) In the degradation of both nonane and heptadecane (Tables 6.2 and 6.4), the lag phases were longer when 20 ppb of V_3 (folic acid) were added as compared to the case with V_0. As the concentration of folic acid was increased to 1000 ppb, the lag phases were reduced drastically (Tables 6.3 and 6.5). Degradation rates and growth rates for both nonane and heptadecane, were smaller with 20 ppb of folic acid (Tables 6.2 and 6.4) and increased with addition of 1000 ppb of folic acid (Tables 6.3 and 6.5). The result suggested that 20 ppb of V_3 was not enough to enhance the microbial degradation of nonane or heptadecane.

Similarly with 20 ppb of niacin and pantothenic acid, growth rates and degradation rates were higher than those obtained with V_0 (Table 6.2). However, at a at higher concentration (1000 ppb) they became inhibitory for nonane degradation (Table 6.3). No inhibitory effect on heptadecane degradation was observed at higher concentrations of V_1 and V_5. Unlike the lower concentration, V_5 at 1000 ppb showed no effect. The degradation rate and growth rate with 1000 ppb of V_5 were same as of those with V_0. (Table 6.5).

V_2 (biotin) showed better response at the lower concentration (Table 6.4). At 1000 ppb it was inhibitory for heptadecane degradation (Table 6.5). V_4 (thiamin) did not produce any noticeable effect, though at 20 ppb it acted a little better for nonane degradation. The use of V_{mix} produced much better response for all cases.

A similar patterns of biomass growth and degradation of alkanes can be seen in the Figures 6.2a, 6.2b, 6.3a, 6.3b, 6.4a, 6.4b, 6.5a and 6.5b. Figure 6.2a shows that biomass growth with 20 ppb of V_3 was slower than biomass growth with V_0 or other vitamins. Biomass growth with V_1, V_2 and V_{mix} were almost the same or higher than that with V_0. Twenty ppb of V_5 seemed to be very effective for biomass growth (Figure 6.2a). However, in Figure 6.2b degradation of nonane did not show that effect with 20 ppb of V_5.

Almost identical observations can be made for the degradation of heptadecane, as shown in Figures 6.4a and 6.4b. In Figure 4a, biomass growth with heptadecane was slower with 20 ppb of V_3, same as found in Figure 6.2a. In Figures 6.4a and 6.4b V_3 seemed to be inhibitory same as observed in Figures 6.2a and 6.2b.

Therefore from the above discussion we can conclude that V_{mix} was most effective in all cases. V_1 and V_2 were effective at 20 ppb but showed no effect at 1000 ppb. V_5 was inhibitory at 20 ppb. However, at 1000 ppb of V_3 the inhibitory effect was not observed. This observation was true for both nonane and heptadecane degradation.

The results of the experiments reported in part I showed that 1000 ppb V_{mix} were inhibitory for nonane degradation. In the case of Part II experiments, irrespective of the concentrations and carbon sources, 1000 ppb of V_{mix} always reduced the lag phase substantially, and increased the degradation rates and growth rates (parameters of Tables 6.2a, 6.3a, 6.4a, 6.5a). One possible reason for this discrepancy might be the different batches of inoculum which were used for those two experimental parts. Although soil was collected from the same site at different times, seasonal changes probably caused the change of the soil population.

In part II experiments, the lag phase was longer for higher concentrations of vitamins in both cases of degradation in both nonane and heptadecane experiments. However, the lag phase for heptadecane degradation was much shorter than that of nonane. It took a longer period of time for complete degradation of nonane than the time required for complete removal of heptadecane, although heptadecane is a longer-chain hydrocarbon. Faster degradation of longer-chain aliphatic hydrocarbons were also reported by Zhou and Crawford [61]. Thus, there could be some specific populations in soil which attack heptadecane more easily than nonane.

6.3 Part III. Experiments for Optimization of Individual Vitamins with Heptadecane as Carbon Source

In Part II experiments, we saw that the individual vitamins produced different results at different concentrations. Therefore, it was reasonable to think that different vitamins had different optimum concentrations to degrade a specific carbon source. Based on this concept, Part III experiments were performed to optimize concentration of the individual vitamins using heptadecane as the carbon source. This part includes five experiments as follows:

Experiment to determine the optimum concentration of niacin

Experiment to determine the optimum concentration of biotin

Experiment to determine the optimum concentration of folic acid

Experiment to determine the optimum concentration of thiamin

Experiment to determine the optimum concentration of pantothenic acid

All the above experiments were conducted simultaneously and in identical fashion. The results are reported in Tables 6.6, 6.7, 6.8, 6.9, and 6.10 and Figures 6a, 6b, 7a, 7b, 8a, 8b, 9a, 9b, 10a, and 10b. Although control experiments with media, V_{mix}, and biomass, no alkane(C_1) or media, V_{mix}, alkane but no biomass (C_2) were conducted with each experiment, they are not shown in the Figures. The data for those two controls are presented in the data tables in Appendix II.

Table 6. 6. Results for Optimization of V_1 (Niacin) Using Heptadecane as Carbon Source

	Heptadecane as carbon source and Niacin as the vitamin supplements								
	V_0			V_{50}			V_{100}		
d (%)	R (ppm)	t (h)	B (ppm)	R (ppm)	t (h)	B (ppm)	R (ppm)	t (h)	B (ppm)
0	52.67	0	4.64	54.67	0	4.43	61.33	0	4.43
5	50.03	26	6	51.93	21	5	58.27	23	5
20	42.13	37	7	43.73	35	11	49.07	32	11
50	26.33	55	11	27.33	56	20	30.67	46	20
75	13.17	66	15	13.67	64	24.5	15.33	60	24.5
95	2.63	74	18	2.73	71	32	3.07	73	32
100	0	75.5	18.2	0	75.5	30.82	0	75.5	38

	Heptadecane as carbon source, niacin as the vitamin supplement					
	V_{500}			V_{1000}		
d (%)	R (ppm)	T (h)	B (ppm)	R (ppm)	T (h)	B (ppm)
0	51.93	0	5.06	53	0	4.78
5	49.34	23.5	5.5	50.35	10	5
20	41.55	31	8	42.4	33	8
50	25.97	44	12	26.5	56	12
75	12.98	51	15	13.25	65	18
95	2.6	67	20	2.65	73	25
100	0	69.5	22	0	75.5	27.83

Table 6.6a Parameters Obtained from the Results for Optimization of V_1 (Niacin) Using Heptadecane as Carbon Source

	V_0	V_{50}	V_{100}	V_{500}	V_{1000}
L_{av} (h)	26	21	23	23	20
O.D.R (ppm/h)	0.68	0.73	0.80	0.74	0.69
O.G.R (ppm/h)	0.18	0.39	0.38	0.22	0.28
O.Y.C	0.27	0.53	0.47	0.30	0.40
D.R (ppm/h)	1.00	1.04	1.21	1.43	0.91
G.R (ppm/h)	0.025	0.026	0.027	0.030	0.024
Y.C	0.28	0.45	0.40	0.25	0.34

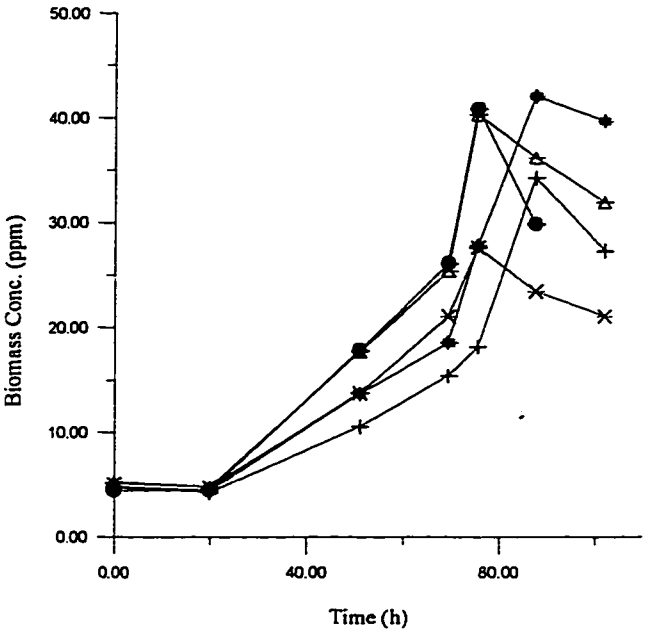


Figure 6.6a. Effect of Various Concentrations of Niacin on Biomass Growth, Using Heptadecane as Carbon Source.

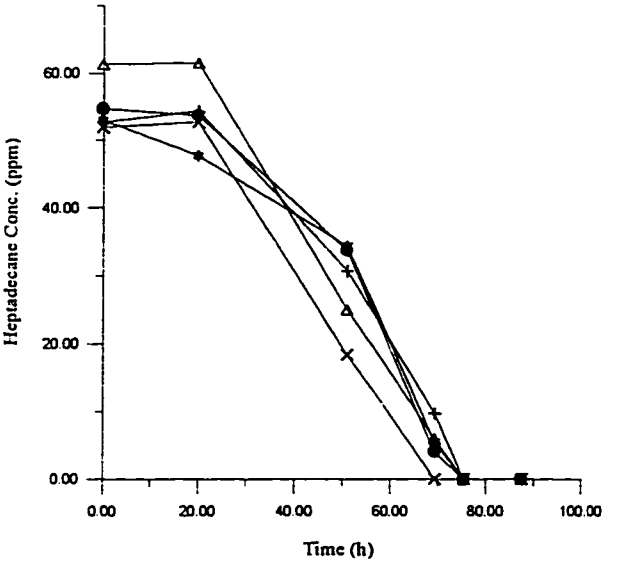


Figure 6.6b. Effect of Various Concentrations of Niacin on Degradation of Heptadecane.

+ Vo ● V50 Δ V100

× V500 ✱ V1000

Table 6.7. Results for Optimization of V_2 (Biotin) Using Heptadecane as Carbon Source

	Heptadecane as carbon source and biotin as vitamin supplement								
	V_0			V_{50}			V_{100}		
d (%)	R (ppm)	t (h)	B (ppm)	R (ppm)	t (h)	B (ppm)	R (ppm)	t (h)	B (ppm)
0	45.27	0	6.11	51.67	0	5.41	57	0	6.39
5	43.01	10	6.5	49.08	9	6.5	54.15	10	7.5
20	36.22	17	7.5	41.33	16	9.5	45.6	15	10
50	22.64	24.5	11	25.83	22	12.4	28.5	22	13
75	11.32	35	15	12.92	26	15	14.25	29	17
95	2.26	44	18.5	2.58	40	24	2.85	49	22
100	0	57	15.74	0	45	27.26	0	57	19.46

	Heptadecane as carbon source, biotin as vitamin supplement					
	V_{500}			V_{1000}		
d (%)	R (ppm)	t (h)	B (ppm)	R (ppm)	t (h)	B (ppm)
0	50.67	0	5.62	50.33	0	5.2
5	48.13	9	6	47.82	10	6.5
20	40.53	17	9	40.27	21	9
50	25.33	23	12.5	25.17	24	11.5
75	12.67	28	15	12.58	28	12.5
95	2.53	41	19	2.52	42	16.5
100	0	45	20.09	0	57	27.82

Table 6.7a. Parameters Obtained from the Results for Optimization of V_2 (Biotin) Using Heptadecane as Carbon Source

	V_o	V_{50}	V_{100}	V_{500}	V_{1000}
L_{av}(h)	9	8	10	8	8
O.D.R (ppm/h)	0.98	1.23	1.11	1.17	1.14
O.G.R (ppm/h)	0.28	0.46	0.32	0.33	0.27
O.Y.C	0.29	0.38	0.29	0.28	0.24
D.R (ppm/h)	1.38	2.84	2.24	2.53	3.96
G.R (ppm/h)	0.037	0.045	0.037	0.045	0.047
Y.C	0.30	0.19	0.22	0.22	0.13

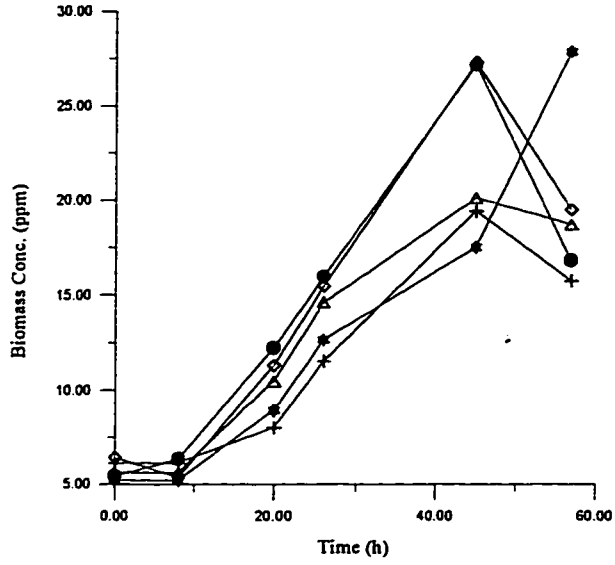


Figure 6.7a. Effect of Various Concentrations of Biotin on Biomass Growth, Using Heptadecane as Carbon Source

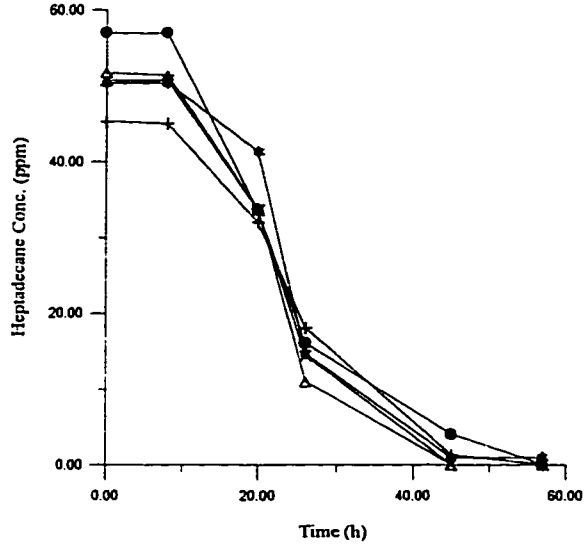


Figure 6.7b. Effect of Various Concentrations of Biotin on Degradation of Heptadecane.

+ Vo ● V50 Δ V100

× V500 ✱ V1000

Table 6. 8. Results for Optimization of V_3 (Folic Acid) Using Heptadecane as Carbon Source

	Heptadecane as carbon source, folic acid as the vitamin supplement								
	V_0			V_{50}			V_{100}		
d (%)	R (ppm)	T (h)	B (ppm)	R (ppm)	T (h)	B (ppm)	R (ppm)	T (h)	B (ppm)
0	40.07	0	14.91	38.29	0	13.36	39.02	0	15.4
5	38.07	26	20	36.38	20	14	37.07	30	26.5
20	32.06	34	31	30.63	24	18	31.22	35	32
50	20.04	38	35	19.15	29	22	19.51	38	35
75	9.76	42	39	9.57	45	28	9.76	42	42
95	1.95	63	29	1.91	52	27	1.95	51	43
100	0	76	21.94	0	68	27.79	0	68	38.77

	Heptadecane as carbon source, folic acid as the vitamin supplement					
	V_{500}			V_{1000}		
d (%)	R (ppm)	T (h)	B (ppm)	R (ppm)	T (h)	B (ppm)
0	40.73	0	14.42	40	0	14.63
5	38.69	15	8	38	10	14
20	32.58	24	13	32	23	18
50	20.37	31	21	20	30	22
75	10.18	40	27	10	45	28
95	2.04	52	30	2	51	20
100	0	68	26.3	0	76	19.79

Table 6.8a Parameters Obtained from the Results for Optimization of V_3 (Folic Acid) Using Heptadecane as Carbon Source

	V_o	V_{50}	V_{100}	V_{500}	V_{1000}
L_{av} (h)	40	20	33	26	31
O.D.R (ppm/h)	0.61	0.70	0.73	0.74	0.75
O.G.R (ppm/h)	0.22	0.26	0.54	0.30	0.11
O.Y.C	0.37	0.37	0.74	0.40	0.14
D.R (ppm/h)	2.79	1.00	3.07	1.40	1.00
G.R (ppm/h)	0.029	0.021	0.039	0.044	0.020
Y.C	0.36	0.47	0.47	0.63	0.45

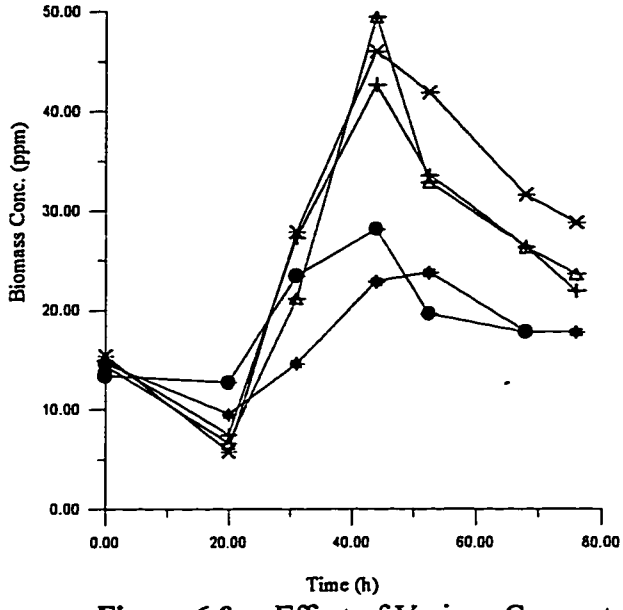


Figure 6.8a. Effect of Various Concentrations of Folic acid on Biomass Growth, Using Heptadecane as Carbon Source

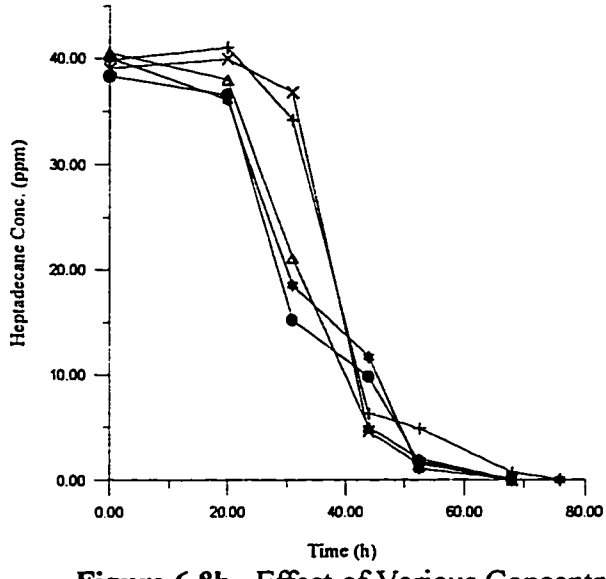


Figure 6.8b. Effect of Various Concentrations of Folic acid on Degradation of Heptadecane.

+ Vo ● V50 Δ V100

× V500 ✱ V1000

Table 6. 9. Results for Optimization of V_4 (Thiamin) Using Heptadecane as Carbon Source

	Heptadecane as carbon source, thiamin as the vitamin supplement								
	V_0			V_{50}			V_{100}		
d (%)	R (ppm)	T (h)	B (ppm)	R (ppm)	T (h)	B (ppm)	R (ppm)	T (h)	B (ppm)
0	52.44	0	7.03	50.67	0	7.46	51.67	0	6.4
5	49.82	23	7	48.14	25	7.5	49.08	23	7
20	41.95	27	9	40.54	29	11	41.33	26	7
50	26.22	33	11.5	25.34	40	15	25.83	32	11
75	13.11	42	16.5	12.67	50	18	12.92	40	13.5
95	2.62	52.5	17	2.53	59	17.5	2.58	51	18
100	0	56	15.47	0	69	16.46	0	56	18.36

	Heptadecane as carbon source, thiamin as the vitamin supplement					
	V_{500}			V_{1000}		
d (%)	R (ppm)	T (h)	B (ppm)	R (ppm)	T (h)	B (ppm)
0	49.93	0	6.89	50.33	0	7.46
5	47.44	25	7	47.82	24	7
20	39.95	28	7	40.27	27	8
50	24.97	33	12	25.17	32	12
75	12.48	44	17.5	12.58	42	18
95	2.5	53	18.5	2.52	53	21
100	0	56	18.08	0	56	19.2

Table 6.9a Parameters Obtained from the Results for Optimization of V_4 (Thiamin) Using Heptadecane as Carbon Source

	V_0	V_{50}	V_{100}	V_{500}	V_{1000}
L_{av}(h)	24	25	23	24	23
O.D.R (ppm/h)	0.95	0.82	0.96	0.89	0.90
O.G.R (ppm/h)	0.19	0.17	0.23	0.22	0.26
O.Y.C	0.20	0.21	0.24	0.24	0.28
D.R (ppm/h)	1.92	1.33	2.03	1.72	1.85
G.R (ppm/h)	0.039	0.023	0.045	0.054	0.051
Y.C	0.26	0.25	0.23	0.38	0.36

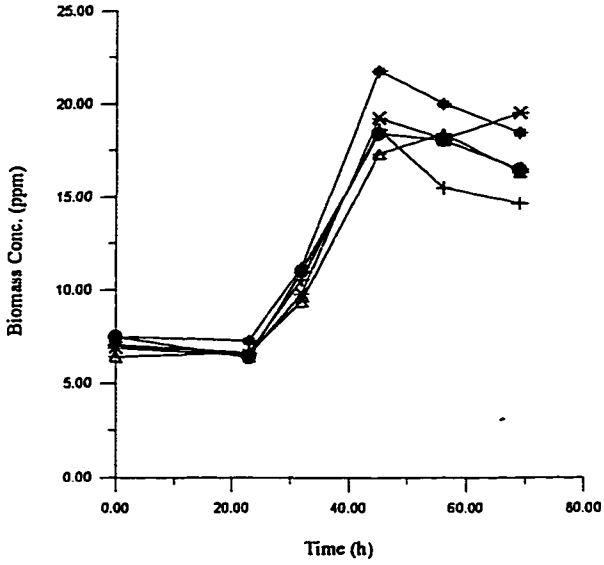


Figure 6.9a.. Effect of Various Concentrations of Folic acid on Biomass Growth, Using Heptadecane as Carbon Source

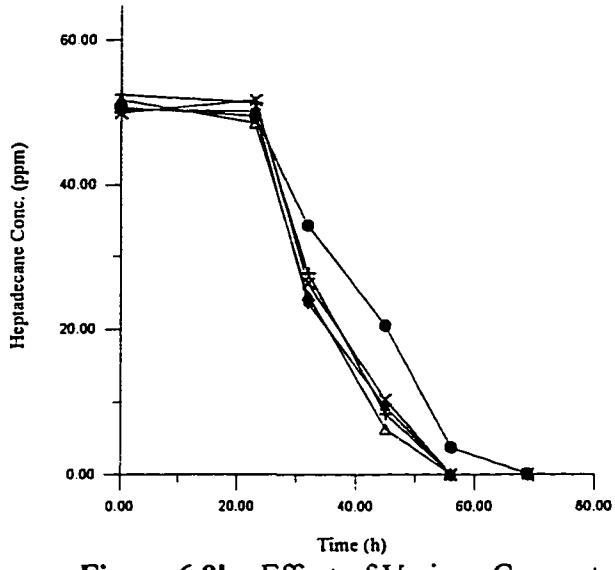


Figure 6.9b. Effect of Various Concentrations of Thiamin on Degradation of Heptadecane.

+ Vo ● V50 Δ V100

× V500 ✱ V1000

Table 6. 10. Results for Optimization of V_5 (Pantothenic Acid) Using Heptadecane as Carbon Source

	Heptadecane as carbon source, pantothenic acid as the vitamin supplement								
	V_0			V_{50}			V_{100}		
d (%)	R (ppm)	T (h)	B (ppm)	R (ppm)	T (h)	B (ppm)	R (ppm)	T (h)	B (ppm)
0	36	0	7.81	38.67	0	7.17	38.33	0	6.75
5	34.2	15	9	36.73	15	10	36.42	13	9
20	28.8	22	11	30.93	25	10	30.67	15	10
50	18	32	23	19.33	30	23	19.17	22	11
75	9	40	26	9.67	35	29	9.58	30	20
95	1.8	48	33	1.93	44	31	1.92	44	39
100	0	52	34.96	0	52	23.3	0	52	35.59

	Heptadecane as carbon source, pantothenic as the vitamin supplement					
	V_{500}			V_{1000}		
d (%)	R (ppm)	T (h)	B (ppm)	R (ppm)	T (h)	B (ppm)
0	36.67	0	6.96	37	0	6.68
5	34.83	18	10	35.15	17	10
20	29.33	26	12	29.6	27	12
50	18.33	35	21	18.5	39	25
75	9.17	46	26	9.25	48	29
95	1.83	64	38	1.85	65	32
100	0	69	31.58	0	69	32.71

Table 6.10a Parameters Obtained from Results for Optimization of V_5 (Pantothenic Acid) Using Heptadecane as Carbon Source

	V_0	V_{50}	V_{100}	V_{500}	V_{1000}
L_{av}(h)	13	15	13	15	15
O.D.R (ppm/h)	0.71	0.84	0.83	0.54	0.54
O.G.R (ppm/h)	0.52	0.54	0.73	0.45	0.39
O.Y.C	0.74	0.65	0.89	0.89	0.72
D.R (ppm/h)	1.10	2.13	1.41	1.01	0.97
G.R (ppm/h)	0.045	0.097	0.044	0.037	0.039
Y.C	0.76	0.89	0.47	0.69	0.84

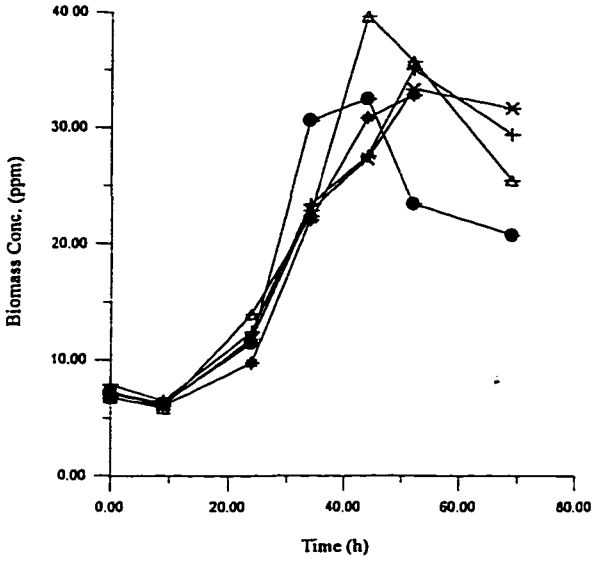


Figure 6.10a. Effect of Various Concentrations of Pantothenic Acid on Biomass Growth, Using Heptadecane as Carbon Source

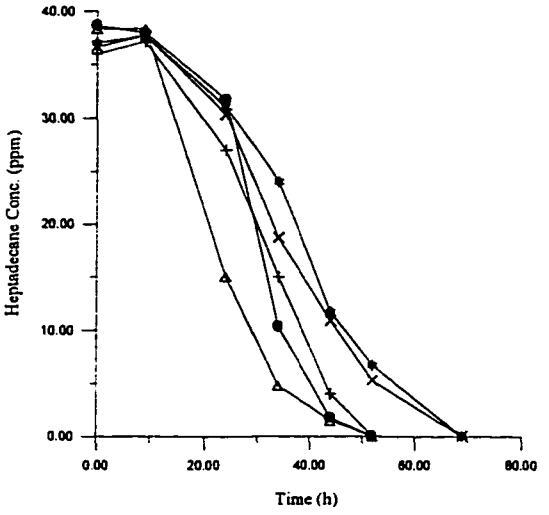


Figure 6.10b. Effect of Various Concentrations of Pantothenic Acid on Degradation of Heptadecane.

+ Vo ● V50 Δ V100

× V500 ✱ V1000

Among the five experiments for optimizing five individual vitamins, there was no significant lag phase reduction observed. Therefore for determining the optimum concentration of individual vitamins, O.D.R and O.G.R were taken into major consideration. Although Lag phase was noticed carefully during microbial growth in each case of optimization experiments.

In Table 6.6a, the overall degradation rate (O.D.R.) and overall growth rate (O.G.R.) were suddenly increased from 0.68 ppm/h and 0.18 ppm/h (with V_0) to 0.73 ppm/h and 0.39 ppm/h respectively with addition of 50 ppb of niacin. The O.D.R. and O.G.R. were 0.80 ppm/h and 0.38 ppm/h respectively with 100 ppb of niacin and 0.74 ppm/h and 0.22 ppm/h respectively with 500 ppb of niacin. O.D.R decreased to 0.69 ppm/h with 1000 ppb of niacin. Although the O.G.R. with 1000 ppb of niacin was 0.28 ppm/h (close to the O.G.R. value with 500 ppb niacin). We therefore considered 50 ppb niacin as the optimum concentration for the present study.

Degradation rate (D.R.) and growth rate (G.R.) during logarithmic phase did not change with 50 ppb niacin with respect to V_0. However, we still considered 50 ppb to be best because, in many cases with vitamin addition, growth rates and degradation rates at logarithmic phases were almost same or often even less than those without vitamin (V_0). We assumed that vitamins must have some role in growth initiation, thereby changing the overall growth or degradation pattern instead of accelerating D.R. and G.R. during logarithmic phase.

Similarly, biotin supplementation at concentrations of 50, 100 and 500 ppb enhanced the O.D.R and O.G.R for the (Table 6.7a). At 1000 ppb of biotin concentration, O.D.R was slightly higher than that with V_0 (1.14 compared to 0.98 ppm/h) but O.G.R

was same as that of V_0. Fifty ppb of biotin seemed to be most appropriate concentration for enhancing the overall growth or degradation in the present study. In this case D.R. and G.R. during the logarithmic phase were also higher with 50 ppb of biotin than those with V_0. Lag phase reduction for all concentrations of biotin was not clearly noticeable, (Table 6.7a). One thousand ppb of biotin enhanced degradation rates and growth rates substantially during the logarithmic phase, but the overall growth or degradation remained almost the same as that of V_0. Therefore, we considered 50 ppb biotin as the optimal concentration for our study.

Lag phase reduction was more prominent in the experiments involving folic acid. The lag phase was 40 hours without vitamin (V_0), whereas it was 20 to 32 hours with folic acid supplementation (Table 6.8a). Overall degradation rates (O.D.R.) and overall growth rates (O.G.R.) with vitamin addition at 50, 100, and 500 ppb were higher than those without vitamin (V_0). For 1000 ppb folic acid, O.D.R was higher than that with V_0, but the O.G.R was the same for V_0. Interestingly, degradation rates (D.R.) and growth rates (G.R.) during the logarithmic phase were higher than the control (V_0) only with 100 ppb vitamin. With 50, 500 and 1000 ppb of vitamins, D.R. and G.R., were less than that with V_0 (Table 6.8a). This result suggests clearly that folic acid only initiates microbial growth for heptadecane degradation, and not promoting growth rates during the logarithmic phase of microbial growth. Since it reduces the lag phase, it helps the microbes to start consuming a new carbon source earlier than the microbes without folic acid supplements. It thus completes consuming the carbon earlier, resulting in higher overall degradation rates and overall growth rates. We assumed 100 ppb folic acid as optimum concentration.

In this set of experiments, overall yield coefficient (O.Y.C) showed a big discrepancy between 100 ppb and 1000 ppb folic acid (0.74 and 0.14 respectively). This might indicate experimental error. The optical density of each tube often varied in a range from 20% to 50%. Also, different positioning of the same tube often gave different readings of absorbances in the spectrophotometer. This tube error might be possible, although we standardized each tube and marked a specific position for the same absorbances before we started each experiment.

No effect of thiamin was observed in the results of the present study of thiamin optimization. This observation was contradictory to the result to that reported by Hirayama et al [23]. In their study of diesel fuel degradation, they found that thiamin enhanced microbial growth as well as degradation. In our experiments, as we see in Table 6.9a, lag phases were almost identical for all different concentrations of thiamin and the control V_0. O.D.R and O.G.R were also the same as those of V_0. D.R. and G.R. during logarithmic phase were higher than those of V_0 only with 100 ppb of thiamin, all other concentrations showed no difference. Similar results were observed for the initial experiments of Part II. Therefore, we omitted thiamin from the optimum vitamin mixture (V_{opt}) that we used for the final part of our experiments. Yield coefficients were within a range of 0.2 to 0.4 for all cases in this set of experiments.

Pantothenic acid is another important vitamin which initiates the formation of α-keto- glutaric acid, a gateway enzyme for entering the Krebs cycle for aerobic carbon metabolism. The results (Table 6.10a) showed that the vitamin was not very effective in reducing the lag phase, but the O.D.R and O.G.R were increased with 50 ppb and 100 ppb as compared to the rates without vitamin (V_0). In particular, 100 ppb seemed to be the

most effective, and was considered as the optimum concentration for our further studies. Five hundred ppb and 1000 ppb of pantothenic acid showed lower O.D.R and O.G.R. D.R and G.R during log phase were also lower with 500 and 1000 ppb of pantothenic acid as well. We have seen similar effects in the experiments in Part II, where at 1000 ppb pantothenic acid showed growth inhibition (Table 6.3a). At 20 ppb it showed slightly higher growth but not very noticeable (Tables 6.2a and 6.4a). Therefore it seemed that pantothenic acid is effective at about 100 ppb. It might be a very sensitive vitamin, becoming inhibitory at higher concentrations (more than 500 ppb). The possible reason of its inhibitory effect could be that when the vitamin is present at high concentrations, it produces an excess of corresponding fatty acids which are cytotoxic. Yield coefficient in all cases were in a high range (0.5 to 0.89).

Based on the studies of optimization of individual vitamins we made an optimum vitamin mixture which contained 50 ppb of niacin, 50 ppb of biotin, 100 ppb of folic acid, and 100 ppb of pantothenic acid.

6.4 Part IV. Experiments to Determine the Effect of Optimum Vitamin Mixture on Growth of Soil Microbes with Isooctane, Nonane, Hexadecane, Heptadecane as Carbon Source

The optimum vitamin mixture was designated as V_{opt} and was tested against the control without vitamin (V_0) for degradation of various carbon sources such as isooctane, nonane, hexadecane, heptadecane, and two different concentrations of diesel fuel. Therefore, the object of our final study was to determine the effectiveness of V_{opt} on the degradation of

Four different experiments were included in this part as:

i) Experiments to determine the effect of V_{opt} for isooctane degradation

ii) Experiments to determine the effect of V_{opt} for nonane degradation

iii) Experiments to determine the effect of V_{opt} for hexadecane degradation

iv) Experiments to determine the effect of V_{opt} for heptadecane degradation

All the above experiments were performed simultaneously. The procedure was identical for all four of these experiments, except that different alkanes were used as carbon source.

Table 6. 11. Results for Degradation of Nonane With and Without V_{opt}

	Nonane as carbon source					
	V_0			V_{opt}		
d (%)	R (ppm)	T (h)	B (ppm)	R (ppm)	T (h)	B (ppm)
0	36.67	0	1.55	31.84	0	1.27
5	34.84	34	2.0	30.25	32.5	2.0
20	29.34	60	3.0	25.47	40	4.0
50	18.34	70	7.5	15.92	45	9.0
75	9.17	76	13.5	7.96	51	20
95	1.83	85	22	1.60	64	26
100	0	92	27	0	73	27

Table 6.11a. Parameters Obtained from Results for Degradation of Nonane With and Without V_{opt}

	V_0 (Nonane)	V_{opt} (Nonane)
L_{av} (h)	54	34
O.D.R (ppm/h)	0.41	0.47
O.G.R (ppm/h)	0.24	0.39
O.Y.C	0.59	0.82
D.R (ppm/h)	1.26	1.59
G.R (ppm/h)	0.08	0.12
Y.C	0.52	0.91

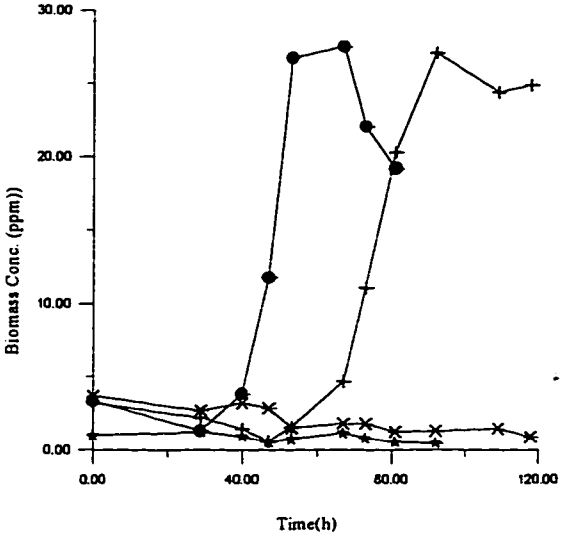


Figure 6.11a. Effect of V_{opt} on Biomass Growth Using Nonane as Carbon Source

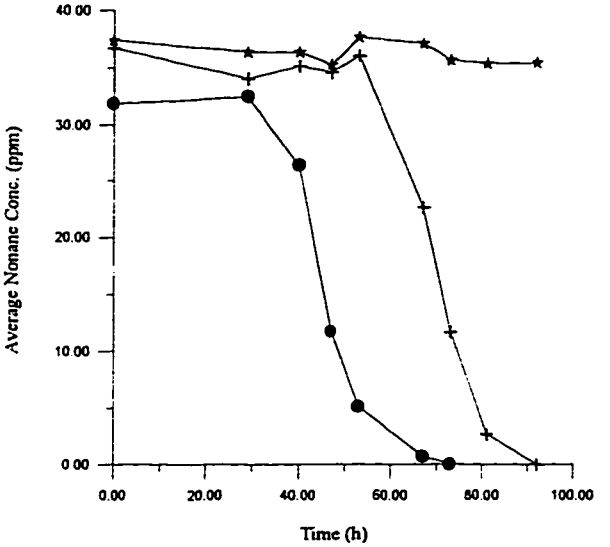


Figure 6.11b. Effect of V_{opt} on Degradation of Nonane.

+ Vo ● Vopt × C1 ✱ C2

Table 6. 12. Results of Degradation of Hexadecane With and Without V_{opt}

	Hexadecane as carbon source					
	V_0			V_{opt}		
d (%)	R (ppm)	T (h)	B (ppm)	R (ppm)	T (h)	B (ppm)
0	49.72	0	4.36	50.29	0	4.08
5	47.23	22	6.5	47.78	17	5.0
20	39.78	25	7.5	40.23	20	6.5
50	24.86	28	11.5	25.15	26	10.8
75	12.43	32	14.5	12.57	29	15.5
95	2.49	40	21.5	2.51	35	23
100	0	45	25.4	0	37	24.2

Table 6.12a. Parameters Obtained from Results of Degradation of Hexadecane With and Without V_{opt}

	V_0 (Hexadecane)	V_{opt} (Hexadecane)
L_{av} (h)	21	16
O.D.R (ppm/h)	1.18	1.37
O.G.R (ppm/h)	0.43	0.54
O.Y.C	0.36	0.40
D.R (ppm/h)	3.91	3.07
G.R (ppm/h)	0.091	0.091
Y.C	0.26	0.33

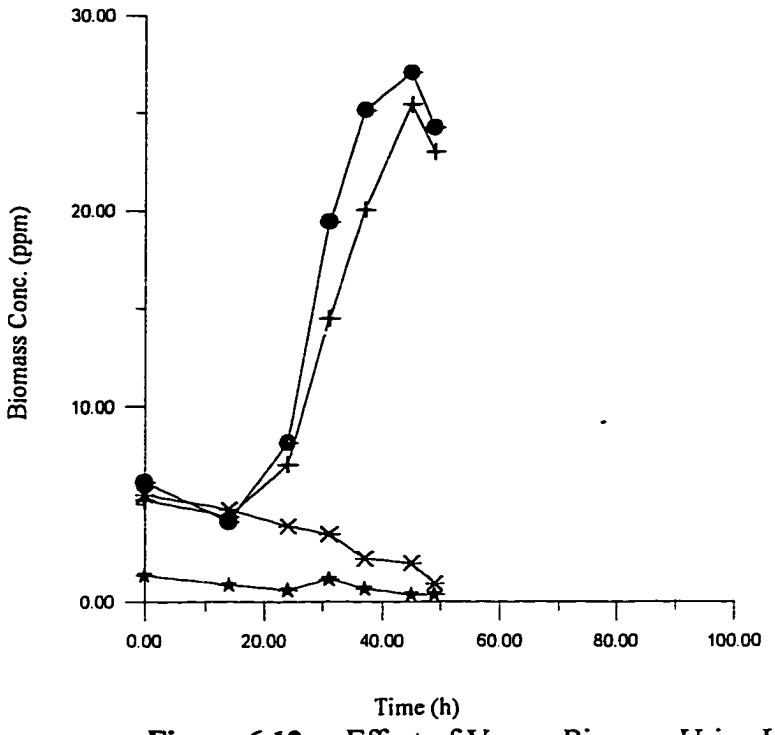


Figure 6.12a: Effect of V_{opt} on Biomass Using Hexadecane as Carbon Source

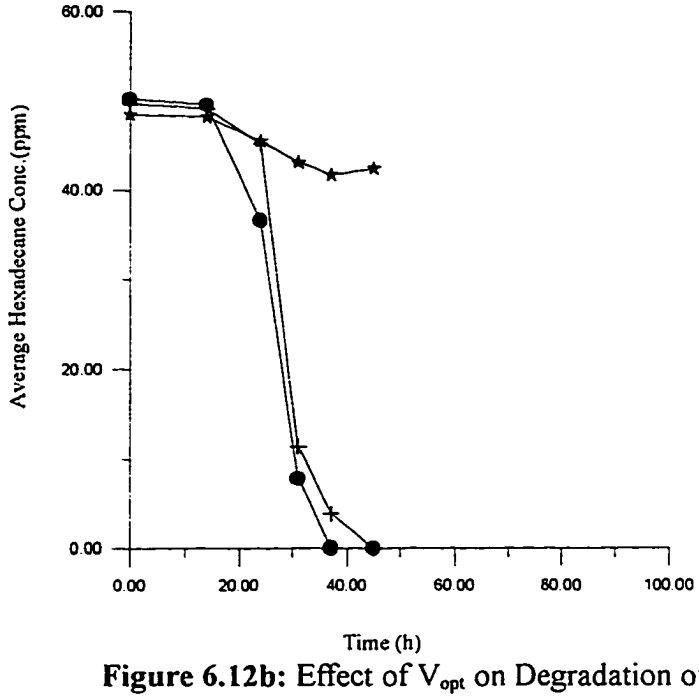


Figure 6.12b: Effect of V_{opt} on Degradation of Hexadecane

+ Vo ● Vopt × C1 ✱ C2

Table 6. 13. Results of Degradation of Heptadecane With and Without V_{opt}

	Heptadecane as carbon source					
	V_0			V_{opt}		
d (%)	R (ppm)	T (h)	B (ppm)	R (ppm)	T (h)	B (ppm)
0	34.12	0	3.80	35.38	0	5.06
5	32.41	32	4.20	33.61	15	4.8
20	27.30	38	5.0	28.30	27	5.0
50	17.06	47	8.5	17.69	33	7.5
75	8.53	53	12.5	8.85	37	12
95	1.71	57	14.5	1.77	42	18
100	0	70	16.31	0	46	23.5

Table 6.13a. Parameters Obtained from Results of Degradation of Heptadecane With and Without V_{opt}

	V_0 (Heptadecane)	**V_{opt} (Heptadecane)**
L_{av} (h)	33	21
O.D.R (ppm/h)	0.57	0.82
O.G.R (ppm/h)	0.19	0.32
O.Y.C	0.33	0.39
D.R (ppm/h)	1.25	1.95
G.R (ppm/h)	0.057	0.082
Y.C	0.40	0.36

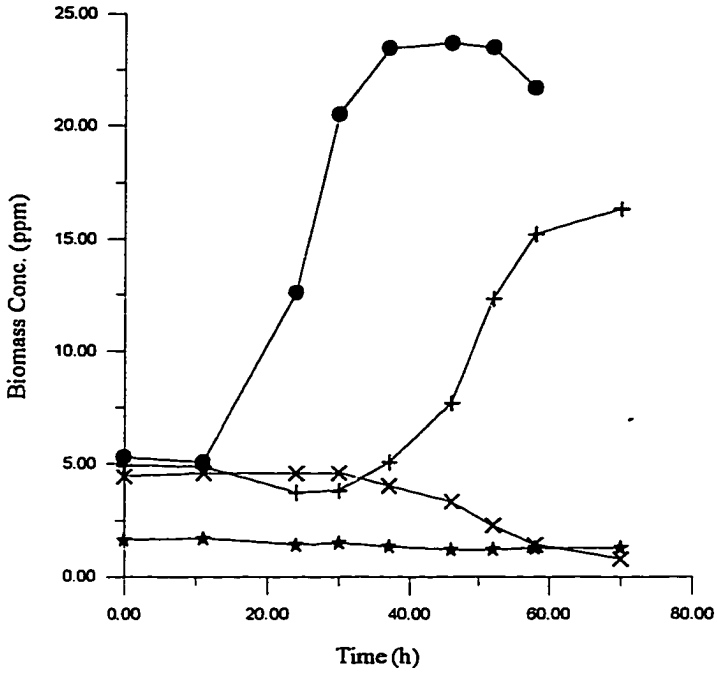


Figure 6.13a. Effect of V_{opt} on Biomass Growth Using Heptadecane as Carbon Source

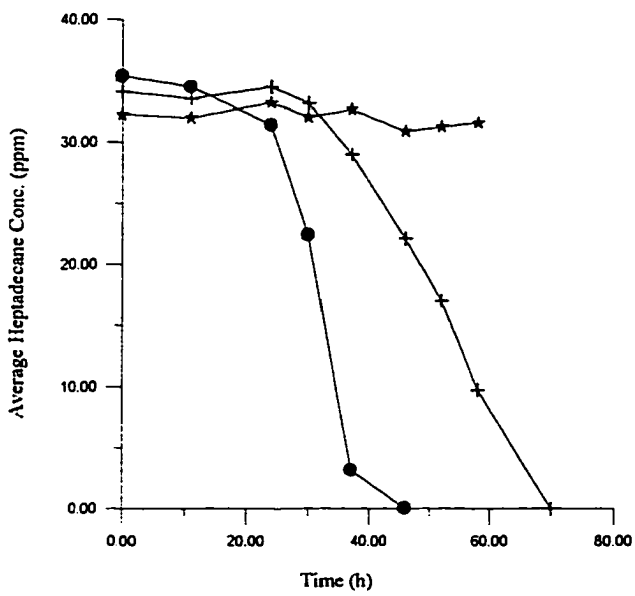


Figure 6.13b. Effect of V_{opt} on Degradation of Heptadecane

+ Vo ● Vopt × C1 ✱ C2

The results of these experiments are shown in Tables 6.11, 6.12, 6.13, and their corresponding parameter tables, and in Figures 6.11a, 6.11b, 6.12a, 6.12b, 6.13a, 6.13b. The results of the isooctane experiments will be discussed later. In the cases of nonane, hexadecane and heptadecane, we found a substantial reduction of lag phases with V_{opt} as compared to lag phases of V_0. In Tables 6.11a, 6.12a, and 6.13a the lag phases are calculated as 29 hours with V_{opt} vs. 53 hours without vitamin (V_0) for nonane, 17 hours with V_{opt} vs. 22 hours with V_0 for hexadecane, and 15 hours with V_{opt} vs. 32 hours with V_0 for heptadecane. Overall degradation rates (O.D.R) and overall growth rates (O.G.R) were increased with addition of V_{opt} for all three cases. Degradation rates (D.R) and growth rates (G.R) during logarithmic phase were higher than those with V_0 for nonane and heptadecane. For hexadecane degradation, D.R. was higher with V_0 than that with V_{opt}. G.R. with V_0 and V_{opt} were equal. Yield coefficient was also increased with the addition of V_{opt} for nonane degradation. For hexadecane and heptadecane the yield coefficient remained almost the same and within the range of 0.3 - 0.4. Therefore, it is clear that the optimum mixture of vitamin (V_{opt}) containing niacin, biotin, folic acid, and pantothenic acid (in a ratio of 1:1:2:2) is very effective for growth initiation. Since the growth started earlier, carbon degradation also started earlier and thus finished earlier than the control (V_0). V_{opt} was not particularly effective in enhancing the growth rates (G.R.) or degradation rates (D.R.) during logarithmic growth phase. However the V_{opt} was very effective to enhance O.D.R. and O.G.R. in the cases of degradation of nonane, hexadecane, and heptadecane.

The effect of V_{opt} is very prominent in Figures 6.11, 6.12, and 6.13. Figure 6.11a shows clearly that biomass growth with V_{opt} started earlier, and reached the maximum

concentration earlier than the biomass without V_{opt}. Microbial Growth with V_{opt} started at about 30 hours, whereas without V_{opt}, it started after 50 hours. There was a transition period noticed between the lag phase and exponential phase for both populations when growth rates were slower. Although it was also noticed in some previous experiments (especially for Part III experiments), it became increasingly prominent for the experiments in Part IV. V_{opt} might have some role in shortening the transitional period. In Figure 6.11a, we can see that the transitional period with V_{opt} was reduced to 10 hours instead of 20 hours with V_0. If we consider the time required from the initial point of exponential phase to reach the maximum biomass concentration, in both cases it took around 10 hours, with insignificant difference noticed between the two populations.

Figure 6.11b shows the degradation pattern of nonane with and without V_{opt}. It is very clear that degradation started earlier and finished earlier with V_{opt} With V_{opt} the consumption of nonane started at about 30 hours, whereas for V_0 the degradation of carbon started at about 50 hours. The transitional period for degradation with V_{opt} was noticeable but with V_0 was not prominent. It took about 35 to 40 hours from the starting point of degradation to consume nonane completely for both the population with and without V_{opt}. These observations are consistent with that of Figure 6.11a and are convincing in establishing that the role of V_{opt} was important only in the early stage of biodegradation.

Figure 6.12a and 6.12b are showing the biomass growth and degradation of hexadecane, a 16- carbon compound. This compound was selected as representative of even-numbered longer-chain hydrocarbons. The difference in transitional phase with V_{opt} is only noticeable in Figure 6.12b. In this case the difference between lag phase and

growth rates were not particularly evident from the figures. From Table 6.12a, we can say that lag phase was shortened by 5 hours with V_{opt} addition, O.D.R and O.G.R. were also higher by 13.9% and 21% respectively with V_{opt} than without V_{opt}. These differences in lag phase or O.D.R. and O.G.R. values are not very significant as compared to the results observed in case of nonane and hexadecane. Therefore it could be possible that the V_{opt} is only effective for degradation of odd numbered hydrocarbons with this particular inocula. It is difficult to draw any conclusion based on only this experiment, since we did not do any experiment with other even numbered hydrocarbons.

Figures 6.13a and 6.13b show the biomass growth and degradation of heptadecane respectively. Heptadecane was selected as a representative of odd-numbered longer-chain hydrocarbons. These two figures showed a substantial difference in growth and degradation pattern with V_{opt}. Although we did not have enough data points here to draw a demarcation line between the transitional phase and exponential phase, it is nonetheless very clear from the figures that the growth and degradation of heptadecane began earlier with V_{opt} than with V_0. Growth rates and degradation rates in exponential phase also seemed to be noticeably higher in this case.

In all these experiments we used two different controls (C_1 and C_2) in addition to V_0 . C_1 was the control for alkane contained inocula, V_{opt}, media but the hydrocarbon. C_2 contained Vopt, hydrocarbon, media but no inocula. The C_1 were used to measure if vitamins were used as carbon source for microbial growth and C_2 were used to see any interaction between the alkanes and vitamins in abiotic condition. In the above figures 6.11, 6.12 and 6.13, we have seen the gradual depletion of C_1 and the non depleting, steady curve of C_2, which suggests that the vitamins were not used just as carbon source

by the microbial population and there was no degradation of hydrocarbons in absence of inocula.

For isooctane, we could not see any microbial growth even after 52 days, which was unexpected. We repeated this experiment three times, but no growth was noticed at any time. Hence, we considered that this specific population was unable to utilize isooctane because of lacking the enzyme. Since we did not perform any experiment with other branched hydrocarbons (pure), we could not confirm whether the inoculum was lacking the enzymes to break just the specific isomer or all the branched chain hydrocarbons. Later in Part V experiments we found that diesel (containing many branched-chain alkane isomers) was degraded completely (below the detection limit) even at 150 ppm by this population. This observation suggests that the population might have needed some cometabolite or cosubstrate for degrading branched-chain hydrocarbons. In the case of diesel degradation the straight-chain hydrocarbons might have served as cosubstrates.

Difficulties in attacking isoalkanes by microbes were also reported by Grundmann et al [17]. Aelion and Bradley reported that for their study of degradation of some selected components of jet fuel, n-alkanes were degraded but the branched isomers remained unattacked [1]. There are also reports available [14] which mentioned that the microbial population degrading n-alkanes may not be able to degrade their isomers. Therefore this occurrence was not unusual.

6.5 Part V. Experiments to Determine the Effect of Optimum Vitamin Mixture for Diesel Biodegradation by Soil Inocula

Two experiments were performed in this part:

i) Experiment to determine the effect of V_{opt} on biomass growth and degradation of diesel fuel at lower initial concentration (approx. 70 ppm)

ii) Experiment to determine the effect of V_{opt} on biomass growth and degradation of diesel fuel at higher initial concentration (approx. 150 ppm)

Table 6. 6. Results to Determine the Effect of V_{opt} on Biomass Growth and Degradation of Diesel at Lower Initial Concentration (approx. 70 ppm)

	Diesel as carbon source					
	V_0			V_{opt}		
d (%)	R (ppm)	T (h)	B (ppm)	R (ppm)	T (h)	B (ppm)
0	68.17	0	9.7	62.9	0	8.43
5	64.6	3	12	59.85	1.5	10
20	54.4	8	14.5	50.4	7	14
50	34	36	31	31.5	16	21
75	17	63	39	15.75	44	39
95	3.4	96	57	3.15	75	60
100	0	110	49.5	0	80	60.5

Table 14a.Parameters Obtained from Results to Determine the Effect of V_{opt} on Biomass Growth and Degradation of Diesel at Lower Initial Concentration (approx. 70 ppm)

	V_0 (TDH of Diesel)	V_{opt} (TDH of Diesel)
L_{av} (h)	3	1.5
O.D.R (ppm/h)	0.67	0.80
O.G.R (ppm/h)	0.49	0.69
O.Y.C	0.73	0.86
D.R (ppm/h)	0.68	0.94
G.R (ppm/h)	0.02	0.03

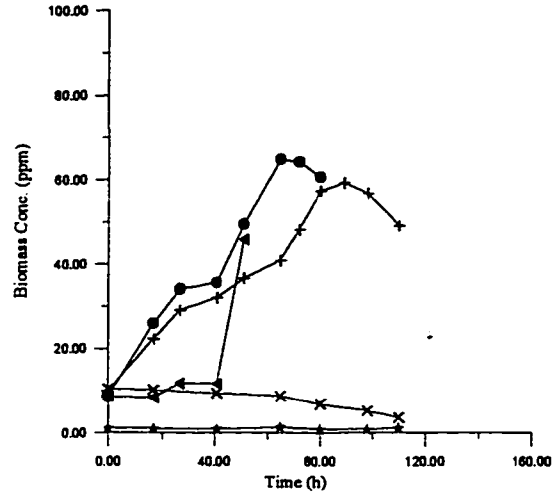


Figure 6.14a Effect of V_{opt} on Biomass Growth Using Diesel (low conc.) as Carbon Source

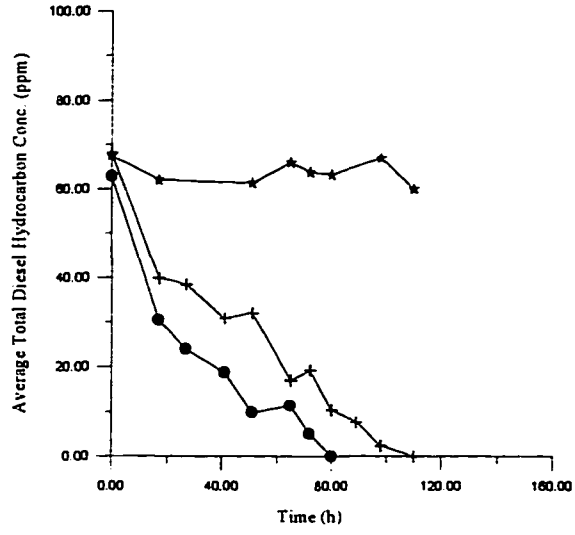


Figure 6.14b Effect of V_{opt} on Degradation of Total Diesel Hydrocarbon (at low conc.)

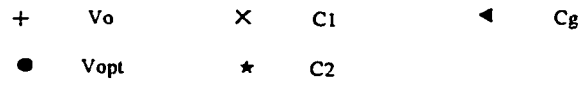


Table 6. 15. Results to Determine the Effect of V_{opt} on Biomass Growth and Degradation of Diesel at Higher Initial Concentration (approx. 150 ppm)

	Diesel as carbon source					
	V_0			V_{opt}		
d (%)	R (ppm)	T (h)	B (ppm)	R (ppm)	T (h)	B (ppm)
0	110.44	0	10.61	150.59	0	10.19
5	104.5	4	12	142.5	3	12
20	88	13	16	120	12	17
50	55	56	36	75	30	28
75	27.5	96	64	37.5	60	53
95	5.5	115	87	7.5	96	85
100	0	130	84.75	0	110	82.01

Table 6.15a Parameters Obtained from Results to Determine the Effect of V_{opt} on Biomass Growth and Degradation of Diesel at Higher Initial Concentration (approx. 150 ppm)

	Vo (TDH of Diesel)	Vopt (TDH of Diesel)
L_{av} (h)	4	3
O.D.R (ppm/h)	0.91	1.49
O.G.R (ppm/h)	0.66	0.78
O.Y.C	0.73	0.53
D.R (ppm/h)	0.73	1.72
G.R (ppm/h)	0.01	0.02
Y.C	0.79	0.45

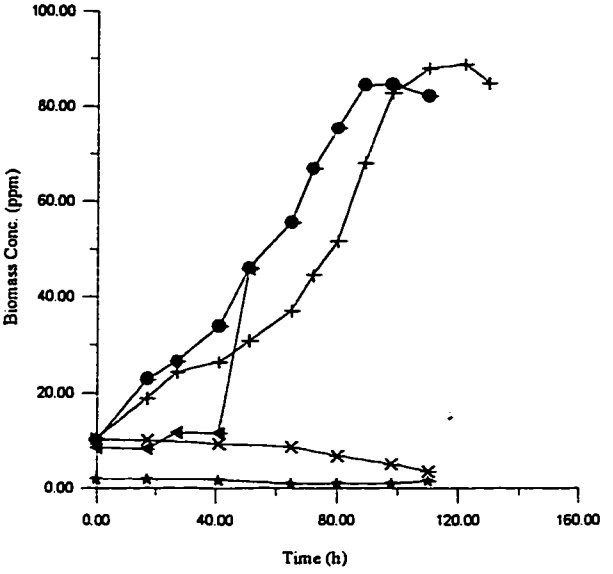


Figure 6.15a. Effect of V_{opt} on Biomass Growth Using Diesel (high conc.) as Carbon Source

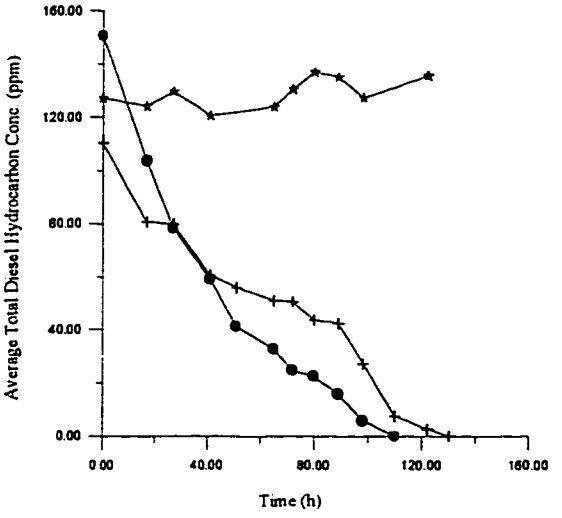


Figure 6.15b. Effect of Vopt on Degradation of Total Diesel Hydrocarbon

+ V'o × C1 ◂ Cg

● V'm ★ C'2

Table 6. 7. Effect of Vopt on Complete Degradation for n-Alkane Fractions of Diesel Fuel.

	Time taken for complete degradation of diesel fraction (h)			
Alkane components of diesel	V_0 (68 ppm of Diesel)	V_{opt} (63 ppm of Diesel)	V_0 (110 ppm of Diesel)	V_{opt} (150 ppm of Diesel)
C_{10}	27	17	72	41
C_{11}	51	27	89	65
C_{12}	65	65	89	72
C_{13}	72	65	89	72
C_{14}	72	65	89	89
C_{15}	72	65	89	89
C_{16}	72	65	89	72
C_{17} with Pristane	98	80	122	98
C_{18} with Phytane	65	65	89	72
C_{19}	65	51	89	89
C_{20}	65	51	89	72

Since the concentrations of individual alkane fractions were very low, and the biomass growth depended on total hydrocarbons of diesel, we therefore considered the total diesel hydrocarbon (TDH) degradation for comparison with and without optimum vitamin mixture (V_{opt}). From Table 6.14a and 6.15a we observed that in case of diesel degradation, the lag phase reduction is not significant with V_{opt} compared to V_0. In fact for diesel degradation, lag phase was very short with V_0 too. The possible reason is that the diesel fuel is a mixture of alkanes containing many short chain carbon compounds. The short chain alkanes are very easily attacked by soil microbes. Also there is a competition among microbes for carbon source. Therefore when diesel was added as carbon source the competent microbes immediately starts to consume the lower hydrocarbons. Thus the lag phase for diesel becomes very short. Similar observation was

reported by many other researchers. The O.D.R. and O.G.R. were noticeably increased with V_{opt} supplementation for both high and low concentrations of TDH. O.D.R. with V_{opt} supplement was increased by 16% to 39% depending on the initial concentration of diesel. The O.G.R. with V_{opt} increased by 15% to 30%. D.R. during logarithmic phase increased substantially (28% to 58%) for both concentrations of TDH with V_{opt}, G.R. increased by 33% to 50%. Two important things were noticed from the results of the last part of our experiments, i) the % increase of growth rates and degradation rates were higher at higher concentrations of diesel, which means at more stressed conditions, the effectiveness of V_{opt} might be more prominent, ii) the % increase of degradation rates (O.D.R. and D.R.) are higher than the % increase of growth rates (i.e. O.G.R. and G.R.) which means it will degrade much faster with V_{opt} producing little more biomass.

O.Y.C. and Y.C. readings are scattered in case of diesel experiments. We have seen this type of scattered yield coefficient for optimization experiments (Part III experiments) of folic acid (parameter Table 8) and pantothenic acid (parameter Table 10) as well. This variation of yield coefficient might be the result of using mixed populations, and also because fresh biomass was taken for each individual experiment. Another reason might be errors in reading optical densities for different microcosm tubes.

Figures 6.14a, 6.14b, 6.15a and, 6.15b were used to calculate the percentage values as shown in Tables 6.14 and 6.15 respectively. The figures do not show any lag phase in a true sense. We considered the biomass growth corresponding to 5% of the TDH degradation as lag phase.

The growth and degradation patterns seemed to be diauxic, which is a reasonable conjecture as diesel is a compound mixture of different hydrocarbons. V_{opt} seemed to

initiate growth and degradation of TDH faster than V_0. This enhancing effect of V_{cpt} was noticed for both concentrations of TDH (as shown in Figures 6.14a, 6.14b, 6.15a, and 6.15b).

Here we used three different controls other than V_0. C_1 (control for TDH) and C_2 (control for biomass) were the same as we used for prior experiments. C_g (the control for oxygen) was an additional control we used only for Part V experiments. Since diesel contains a mixture of carbon compounds ranging from C_{10} to C_{23}, we calculated that stoichiometrically the complete oxidation of 150 ppm diesel required 17.64 ml air or 3.53 ml pure oxygen. We had head space of 17 ml in each culture tube. To avoid oxygen limitation, we introduced pure oxygen at 1.3 psig into each tube (as we described in the Experimental Procedure section). To check the toxic effect of pure oxygen upon microbial growth, we used the control experiments with C_g that contained media, inocula, oxygen, and glucose. Glucose was used because it is the easiest carbon source for microbes to use for growth. Therefore, if any growth inhibition occurred in those tubes, it could be attributed to oxygen toxicity, and not due to TDH. Fortunately we did not find any growth inhibition due to oxygen toxicity. In fact, the microbial growth was so fast with glucose and the maximum biomass it reached was so high compared to that of others that it was off the scale. Hence we took only few initial data points of C_g as shown in Figures 6.14a and 6.15a just to show that biomass growth was not inhibited by pure oxygen.

Since our major concern was to study the degradation pattern of aliphatic hydrocarbons, we concentrated only on the n-alkane peaks in chromatograms. A few of the original chromatograms for each experiment with TDH are shown in Appendix III.

Although the n-alkanes ranging from C_{10} to C_{23} were present in the diesel sample, we considered up to C_{20} alkanes for measuring the total diesel hydrocarbon (TDH) concentrations. We ignored the three peaks of C_{21} to C_{23} as those were not calibratable. However, it was found in the chromatograms that those three peaks disappeared by 45-51 hours (for 70 ppm of diesel sample) and 72 hours (for 150 ppm of diesel sample) upon addition of V_{opt}. On the other hand, it took 65 and 89 hours, respectively, without vitamins.

Heptadecane (C_{17}) and octadecane (C_{18}) were always associated with two additional peaks which were identified as pristane and phytane. There are some reports saying those two compounds are hardly degradable by bacteria [42, 53]. In our study, the peak of phytane that is associated with octadecane degraded after 17 and 41 hours (for 60 and 150 ppm of diesel respectively) in both cases (V_{opt} and V_0). Heptadecane took a longer time to degrade, probably due to the presence of pristane (Hirayama, et al., 1978). Degradation of heptadecane was enhanced by V_{opt} supplementation (it took 80 and 98 hours respectively for TDH lo and TDH hi with V_{opt} instead of 98 and 122 hours without vitamin). Table 16 presents the results obtained for the degradation of individual peaks with and without V_{opt}. From two different concentrations of diesel, we observed that the degradation of total hydrocarbons was 15%-27% faster with V_{opt} compared to V_0.

Reviewing all the results of five different experimental parts, it seems V_{opt} had some advantage over the individual vitamins or the initial V_{mix} (that we used in Parts I and II experiments). The reduction of lag phase or transitional phase, and the increase of O.D.R and O.G.R were very clear and prominent with V_{opt} supplementation, whereas for V_{mix} or individual vitamins the results were not so clear. For example, at 1000 ppb V_{mix}

showed a prolonged lag phase and inhibition for the Part I experiment (parameter Table 6.1) but during Part II experiments even at 5000 ppb V_{mix} the lag phase was reduced, although it showed inhibition of O.D.R and O.G.R for nonane degradation (parameter Table 6.3). For heptadecane degradation in Part II experiment, lag phase was reduced, and O.D.R. and O.G.R were increased with 5000 ppb V_{mix}. (parameter Table 6.5). Individual vitamins responded differently at different concentrations. Some vitamins reduced lag phase at lower concentrations, while at higher concentrations they showed either no response or inhibition, or vice versa. In Part III experiments, except for folic acid, none of the individual vitamins showed any effect on lag phase whereas the results were very consistent and the effects were distinctly noticeable for V_{opt} irrespective of hydrocarbons.

To confirm our results, a statistical analysis (ANOVA test) was performed using raw data for each set with V_{opt} and V_0 for five different hydrocarbon sources we used in experimental Parts IV and V (i.e.; nonane, hexadecane, heptadecane, diesel at lower conc. and diesel at higher concentration). The relevant spread sheets are shown in Appendix IV. The output of the ANOVA test suggested the following:

Lag Phase: Lag phases for different hydrocarbons were significantly different (as p-value is 8.98E-09). Probability value or p-value for V_0 vs V_{opt} was 0.00014, which means that V_{opt} significantly reduced lag phases for all hydrocarbons. P-value for interaction between V_{opt} and different alkanes was 0.252, which means that there was no interaction. The reduction of lag phase with V_{opt} was true irrespective of all five hydrocarbons. Also, the average time of lag phase was 26.47 hours without vitamin (V_0) and 15.6 hours with V_{opt} for all hydrocarbons we used in our present study.

Overall Degradation Rates (O.D.R.): O.D.Rs. were significantly different among the different hydrocarbons (p-value is 1.61E-10). O.D.Rs were also very convincingly different with V_{opt} and V_0. The average O.D.R for all hydrocarbons was 0.745 ppm/h with V_0 and 0.975 ppm/h with V_{opt}. There was an interaction between the overall degradation rates with and without V_{opt} and various hydrocarbons. This means that if we change the hydrocarbons, the degradation rates with V_{opt} and V_0 will also change.

Overall Growth Rates (O.G.R.): The O.G.Rs were significantly different within various hydrocarbons (p-value is 1.8E-08). O.G.Rs were convincingly increased with V_{opt} as compared with V_0 (p-value was 0.000121). Average O.G.R with V_{opt} was 0.534 and that without V_{opt} was 0.394. Since p-value was not very small for interaction (0.0734), there is no convincing evidence that the effect of V_{opt} was affected by type of hydrocarbon.

Degradation Rates (D.R.) : Since the p-value is very low (3.32E-06), it is convinced that the D.Rs vary significantly with different hydrocarbons. There is no significant difference in degradation rates during logarithmic phase between V_0 and V_{opt} , as the p- value is high (0.377). Generally p-value below 0.05 is considered to be significant. The average D.R. were 1.98 and 2.26 with V_0 and V_{opt} respectively. The interaction between hydrocarbons and V_{opt} was also insignificant as the p-value is 0.449. Therefore, effect of V_{opt} did not significantly depend on hydrocarbons.

Growth Rates (G.R.) : Growth rates during logarithmic phase (G.R.) varied significantly with various hydrocarbons as the p-value is very low (1.27E-06). G.Rs with V_{opt} also were enhanced significantly as the p-value is 0.005. The average G.Rs with V_0 are 0.0486 and with V_{opt} 0.074, which shows that V_{opt} increased growth rates of inocula

during log phase too, especially for the present soil inocula. Interaction between V_{opt} and hydrocarbons was insignificant.

Yield Coefficient (Y.C.): Both the overall yield coefficient (O.Y.C.)-and yield coefficient during logarithmic phase (Y.C.) with V_{opt} and V_0 did not differ significantly. The p-values for both cases were high (0.144 and 0.198 for O.Y.C and Y.C respectively).

CHAPTER 7

CONCLUSION

From the above results, we can conclude that vitamins supplementation can enhance the biodegradation of various hydrocarbons including diesel fuel. Especially we have seen that the addition of optimized vitamin mixture (V_{opt}) reduced the total time of degradation significantly by changing some important factors. The lag phase was reduced by 35% in case of hydrocarbon degradation based on the average value of ANOVA data (Table D1.b). Overall growth rates (O.G.R.) and overall degradation rates (O.D.R.) also increased significantly (36% and 31% respectively, based on ANOVA Tables D2.b and D3.b) with the V_{opt} supplementation.

Lag phase reduction was very significant for individual hydrocarbons (eg. Nonane, and heptadecane). During lag phase, many metabolic changes occur (in particular, many enzymes required for growth in a new carbon source are synthesized), therefore vitamins possibly help microbes to cope metabolically with a new carbon source and to initiate their growth and reduce lag phase for individual alkanes. Reduction of lag phase was not very prominent for diesel degradation. There may be a number of reasons for that. i) Diesel fuel contains a number of short chain alkanes which are easily degradable by soil microbes. ii) Since diesel is a mixture of different hydrocarbons, some compounds may work as cometabolite and help the microbes to consume other hydrocarbons easily., iii) Also soil population contains many competing bacteria which would be capable of degrading short

depending on the initial concentration of diesel fuel (4.6 hours with V_0 and 2.3 hours with $V_{opt.}$ for lower concentration and 5.3 hours with V_0 and 4.6 hours with V_{opt} for higher initial concentration of diesel fuel).

The overall growth rates (O.G.R.) and overall degradation rates (O.D.R.) are noticeably higher with V_{opt} in all experiments (including the diesel fuel experiments). ANOVA analysis shows that O.D.R. was increased by 31% (Table D2.b) and O.G.R. increased by 36% (Table D3.b) with the addition of $V_{opt.}$

Growth rates (G.R.) and degradation rates (D.R.) during exponential phase were also higher in many cases with V_{opt} but not for all experiments. The ANOVA analysis shows that the increase of degradation rate during exponential phase (D.R.) with V_{opt} was not significant. However, the G.R. was increased significantly with V_{opt} supplementation.

Vitamins do not appear to be utilized just as additional carbon source. In fact, in most cases vitamins showed inhibitory effect at higher concentrations (1000 ppb). From this study we can assume that vitamins probably initiate growth of microbes, thus it helps in faster degradation of hydrocarbons. Once growth starts, the role of vitamins may not be that important because we have seen in many cases, the degradation and growth rates during logarithmic phases remained equal or even less than with those of V_0.

Since this is the first detailed study of the effect of vitamins on biodegradation, we had to develop methods with very few background references. The method which we followed was the best one among various methods we tried. There were several problems raised which could have introduced some errors in our results. i) It is advisable to work with fresh new culture tubes for each experiment to minimize the errors in reading absorbances. Also, each tube has to be standardized before starting the experiment. This

process was tedious and sometimes the errors were unavoidable. ii) Since we worked with culture tubes (27 ml), we were limited by the working volume of media and consequently confined our experiments within a small range of concentrations of hydrocarbons to avoid oxygen limitation. Nonetheless, for diesel we still encountered this problem, and we used pure oxygen in headspace to overcome the problem. Higher volume or higher concentrations of hydrocarbons may elicit a more prominent vitamin effect. iii) Although in real life fresh culture is more applicable, for initial testing one might need to work with the same biomass instead of using fresh culture for each experiment.

The present work is obviously significant not only because this was the first detailed study of vitamin effect on biodegradation, but also because the effectiveness of V_{opt} on biodegradation was found. Also in the present study, we found that the complete degradation of diesel was probably difficult previously. In some prior reports it was stated that it was almost impossible to degrade diesel completely, as some newer compounds were developed in course of degradation [45]. There are also reports mentioning that diesel was hard to degrade completely because pristane and phytane were almost unattackable by soil microbes [19, 25]. For the present study, we followed up the degradation of individual peaks of diesel (as shown in chromatograms in Appendix III). Although it took a slightly longer time, 150 ppm of diesel was completely degraded within 122 hours even without vitamins. The similar observation on the effect of individual vitamins as we found in the present study was also previously reported [32, 33, and 38].

The present work has already shown a significant effect of vitamin mixture for enhancing bioremediation of individual hydrocarbons and diesel fuel. Future research

might explore the effectiveness of other vitamins and other carbon sources including gasoline and polycyclic aromatic hydrocarbons (PAHs), which would open up a promising avenue for bioremediation.

APPENDIX A

CALIBRATION AND CONVERSION

A.1 Calibration of Optical Density vs. Biomass Concentration

Growth of the bacterial species was determined by measuring optical density of the experimental cultures in UV-VIS spectrophotometer (Hitachi, model U1100) at the wavelength of 460 nm. 10 ml modified salt medium was used as the reference sample in the spectrometer. A calibration of OD vs biomass concentration was made as follows: The clean soil inoculum was grown in an autoclave bottle (500 ml) in 100 ppm phenol medium until it reached to the maximum OD (the end of the logarithmic growth phase). Then the culture was serially diluted as 1/10, 2/10, 3/10, 4/10, 5/10 and so on. The turbidity of each dilution was measured by UV-VIS spectrophotometer in reference to the salt medium.

For dry weight determination of cell mass, three samples of 20 ml were taken from the original culture (undiluted) and cetrifuged them. After centrifuging the culture, the supernatant was decanted and the biomass were washed thoroughly with distilled water to remove the water soluble salts and cetrifuged again. The process was repeated several times and then placed the biomass in three pre-weighed aluminium dishes and dried them in oven at 55°C for 24 hours. After 24 hours the dishes are taken out from the oven and placed them in a dessicator until they come to the room temperature. Then the three dishes with dried biomass were reweighed. Thus the weight and the concentration of the 20 ml of biomass of the original culture was obtained. The concentrations of the serially

One pre-weighed aluminium dish was used as control. Calibration curve of OD vs biomass concentrations are shown in the Figure A.1

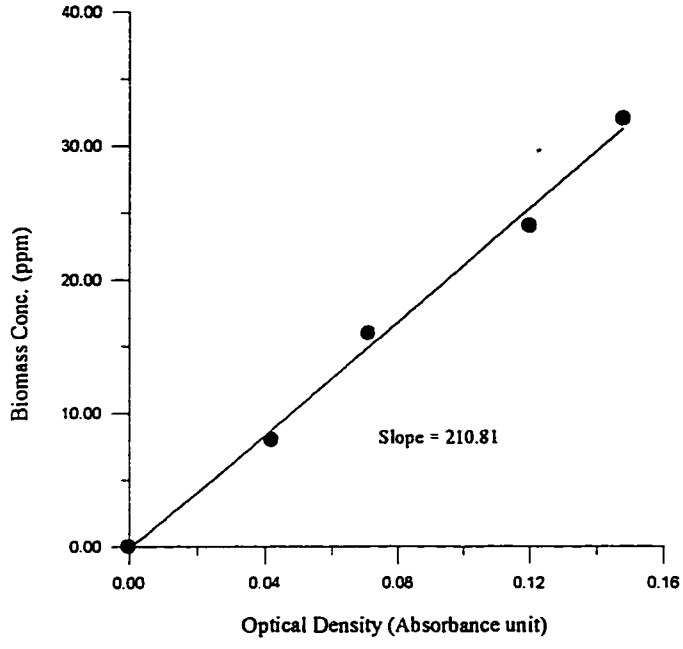


Figure A.1a. The Calibration Curve for Optical Density vs Biomass Concentration

A.2 Conversion of Peak Areas to Corresponding Concentrations (ppm) of TDH

Since the individual peaks were very small, and the biomass growth as we measured depended directly on the consumption of total hydrocarbons of diesel, therefore we considered the total diesel hydrocarbons (TDH) as the carbon source for biomass growth. We compared the growth rates and degradation rates of two different concentrations of TDH. Since diesel chromatograms showed the peak area of each of the hydrocarbons ranging from C_{10} to C_{23}, (as shown in the chromatograms in appendix III), we had to convert the total peak area into the corresponding concentrations by using a calibration.

The calibration was done by using commercial diesel standards (Tennesse std.) serially diluted at different known concentrations and their corresponding peak areas. The total peak area was calculated as:

TDH area = Total peak area in chromatograms - total peak area of the solvent (hexane)

Biomass growth was measured by optical density as mentioned before. The optical densities of biomass was converted to corresponding biomass concentrations by using the slope of calibration curve 1a (Figure 1a). The calibration curve for conversion of peak areas to the corresponding TDH concentrations is shown below in Figure A.1b.

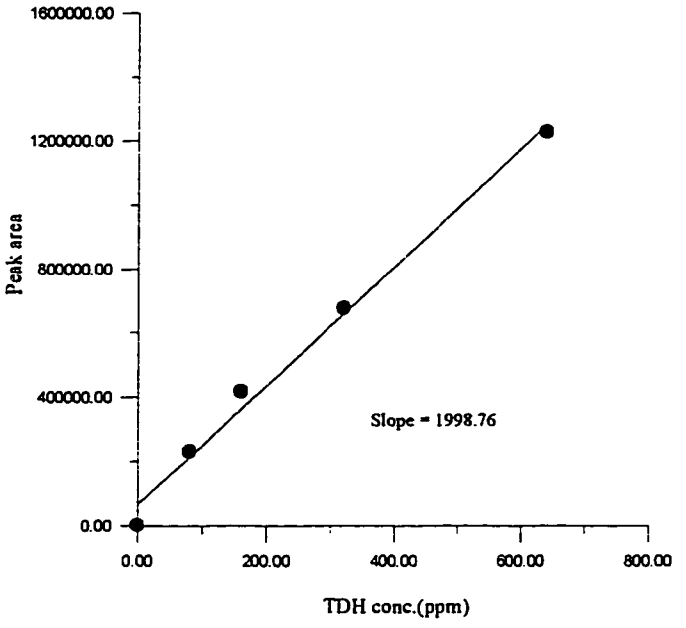


Figure A.1b. Calibration cureve for peak area vs TDH concentration

A.3 Conversion of Peak Areas of Individual Alkane Components of Diesel Into Corresponding Alkane Concentrations

In the experimental part V, as we described that along with the degradation studies of TDH, we also monitored the individual alkane components of each sample. Though the individual peak areas were often very small, we still converted all recognisable peaks into the corresponding concentrations. We used calibration curves for each individual alkanes ranging from C_{10} to C_{20}. C_{21} to C_{23} peaks were too small and were not consistent in all standards. Therefore we could not calibrate them. For the individual alkane peaks' calibrations, we used the same artificial diesel standard (Tenesse standard) a mixture of n-alkane components (ranging from C_{10} to C_{25}) each of 1000 ppm concentrations. The standard mixture was serially diluted in such a way that each alkane component was diluted to 40 ppm, 20 ppm, 10 ppm and 5 ppm. GC runs were made for these different diluted samples. Calibration curves were made by using those known concentrations vs the corresponding peak areas for each alkane component of diesel.

APPENDIX B

EXPERIMENTAL DATA

This section of appendix is showing all data used for figures in results and discussion part. The data are shown in tables in the same sequence as we discussed in the results and discussion part as follows:

Table B.1a. Effect of Various Concentrations of V_{mix} on Biomass Growth Using Nonane as Carbon Source

	Biomass Conc. with different V_{mix} conc (ppm)				
Time (h)	V_0	V_{50}	V_{100}	V_{500}	V_{1000}
0	11.81	11.38	11.60	12.23	9.59
8	11.60	10.96	10.96	11.60	10.12
22	10.33	10.75	10.54	11.60	10.54
46	10.33	10.54	10.12	11.81	10.54
58	17.08	19.40	21.08	20.56	10.54
66	18.45	24.77	34.68	34.58	15.81
81	18.03	26.88	31.20	32.99	31.31
106	17.39	21.93	20.77	28.78	28.57
124	16.59	17.08	20.98	20.98	22.98

Table B.1b. Effect of Various Concentrations of V_{mix} on Nonane Degradation

	Nonane Conc. with different V_{mix} conc (ppm)					
Time (h)	V_0	V_{50}	V_{100}	V_{500}	V_{1000}	C_2
0	56	61	54.5	53	53.53	59
8	54.5	60	52.5	52	52.5	58
22	56	62	53	53.5	53.5	60
46	53	61	55	53.5	53	61
58	50.5	48	42.33	40.67	48.63	59
66	25.5	19.33	7	13.22	34.33	60
81	11.5	7.67	0.33	7.5	12	62
106	0	0	0	0	0.67	60

Table B.2a. Effect of 20 ppb of Individual Vitamins and 100 ppb of V_{mix} on Biomass Growth Using Nonane as Carbon Source

	Biomass Conc. with different 20 ppb individual Vit. & 100 ppb V_{mix} (ppm)							
Time(h)	V_0	V_1	V_2	V_3	V_4	V_5	V_m	C_1
0	10.82	10.97	10.34	11.39	16.39	12.45	16.04	16.11
13	12.10	12.87	12.38	14.14	16.18	13.94	16.04	16.04
32	11.96	11.46	8.86	12.10	14.00	13.50	15.54	15.54
44	9.57	12.03	10.41	11.18	12.8	10.20	14.77	16.67
52.5	11.04	15.19	12.52	13.00	12.59	15.33	21.45	16.53
68.5	29.40	33.97	37.98	14.00	34.32	41.71	33.97	15.33
78	34.60	39.04	41.99	23.00	44.59	46.00	39.25	13.29
93	42.83	32.14	39.25	36.08	51.91	51.91	38.82	12.73
99	42.90	31.09	36.93	48.04	43.61	49.16	36.29	13.08

Table B.2b. Effect of 20 ppb of Individual Vitamins and 100 ppb of V_{mix} on Degradation of Nonane

	Nonane conc. with different 20 ppb individual Vit. & 100 ppb V_{mix} (average of 3 data sets, ppm)							
Time(h)	V_0	V_1	V_2	V_3	V_4	V_5	V_m	C_2
0	59.39	60.39	59.63	59.19	60.53	60.73	61.00	60.53
13	59.05	60.21	59.77	59.18	60.41	60.59	60.89	60.41
32	57.76	59.97	60.07	59.42	60.14	60.62	60.82	60.14
44	56.14	49.75	57.40	48.54	58.09	55.33	54.71	58.09
52.5	47.48	24.76	48.06	47.20	54.32	53.25	33.10	59.65
68.5	22.08	7.86	12.17	42.12	35.65	22.78	16.59	58.98
78	11.94	0	1.70	31.68	6.95	13.52	0	58.28
93	0.35	0	0	10.30	0	0.16	0	59.33
99	0	0	0	0	0	0	0	57.33

Table B.3a. Effect of 1000 ppb of Individual Vitamins and 5000 ppb of V_{mix} on Biomass Growth Using Nonane as Carbon Source

	Biomass conc. with different 1000 ppb individual vitamins. & 5000 ppb V_{mix} (average of 3 data sets, ppm)							
Time	V_0	V_1	V_2	V_3	V_4	V_5	V_{mix}	C_1
0	6.75	2.74	2.1	3.16	3.02	3.65	3.65	3.02
22	7.03	3.44	3.86	2.60	4.50	12.30	3.86	4.50
27	6.25	1.90	2.04	2.18	3.44	9.28	2.32	3.02
43	4.92	2.81	2.11	2.39	3.44	9.28	2.88	2.95
51.5	4.92	2.18	1.97	1.34	3.86	7.24	3.37	3.16
66.5	4.99	2.53	1.34	3.44	4.08	4.92	5.83	3.30
74	4.71	2.04	1.97	4.08	5.62	4.08	9.56	2.81
86	18.20	11.38	7.52	16.58	16.23	16.30	35.13	1.97
105	32.04	24.52	29.44	29.44	21.78	22.28	28.39	1.83
113	32.04	26.21	33.03	24.31	14.05	24.66	21.08	2.04
120	28.32	17.29	22.84	11.95	10.96	14.90		1.34

Table B.3b. Effect of 1000 ppb of Individual Vitamins and 5000 ppb of V_{mix} on Degradation of Nonane

	Nonane conc. with different 1000 ppb individual Vitamins. &5000 ppb V_{mix} (average of 3 data sets, ppm)							
Time(hrs)	V_0	V_1	V_2	V_3	V_4	V_5	V_m	C_2
0	52.67	52.33	55	56	53.67	59.33	46.67	51
22	56	52	54	53.33	50.67	52.33	52	52.33
51.5	53.33	50	53	53.33	51.67	51	44	52.33
66.5	55	52.33	55	52	48.67	54.33	33.33	
74	49	43.67	45.67	41	41.67	52.33	7	49
86	21.33	25.33	17	11	14	48.33	0	
105	3	5	1.67	0	0	22		52.33
113	0	0	0	0	0	7		52.33
120						0		

Table B.4a. Effect of 20 ppb of Individual Vitamins and 100 ppb of V_{mix} on Biomass Growth Using Heptadecane as Carbon Source

	Biomass conc. with different 20 ppb individual vitamins. & 100 ppb V_{mix} (average of 3 data sets, ppm)							
Time(h)	V_0	V_1	V_2	V_3	V_4	V_5	V_m	C_1
0.00	8.92	8.85	9.91	10.19	9.70	5.76	10.19	9.28
13.00	11.10	12.79	13.21	13.70	13.63	12.93	13.56	
18.00	9.14	11.03	11.88	12.09	12.51	12.79	11.95	
26.00	9.14	12.09	10.40	11.17	9.28	10.89	16.65	9.77
34.00	11.17	16.23	17.43	8.43	12.58	15.32	21.43	
42.00	24.38	43.99	37.95	13.98	22.35	21.29	45.39	9.49
51.00	35.35	44.97	46.10	26.35	45.96	40.83	44.48	
58.00	43.29	40.76	41.18	46.17	42.65	43.71	41.32	9.49
65.00	42.58	39.05	39.63	44.48	40.48	41.18	38.37	9.06

Table B.4b. Effect of 20 ppb of Individual Vitamins and 100 ppb of V_{mix} on Degradation of Heptadecane

	Heptadecane conc. with different 20 ppb individual vitamins. & 100 ppb V_{mix} (average of 3 data sets, ppm)							
Time(h)	V_0	V_1	V_2	V_3	V_4	V_5	V_m	C_2
0.00	60.05	59.39	62.29	62.09	61.86	59.74	60.72	60.05
13.00	61.39	61.15	59.77	63.24	61.39	62.27	60.59	
18.00	57.43	59.29	61.40	63.67	61.35	62.65	60.50	
26.00	53.47	54.61	55.94	62.06	63.98	58.89	50.52	63.47
34.00	45.82	34.84	46.75	51.24	44.62	50.21	24.43	
42.00	23.42	14.30	13.50	34.49	29.24	20.26	6.12	60.08
51.00	10.94	4.83	4.36	18.18	11.44	10.78	0.00	
58.00	0.16	0.00	0.00	10.11	0.00	0.00	0.00	61.39
65.00	0.00			0.00	0.00	0.00	0.00	57.43

Table B.5a. Effect of 1000 ppb of Individual Vitamins and 5000 ppb of V_{mix} on Biomass Growth Using Heptadecane as Carbon Source

	Biomass conc. with different 1000 ppb individual vitamins. & 5000 ppb V_{mix} (average of 3 data sets, ppm)							
Time(h)	V_0	V_1	V_2	V_3	V_4	V_5	V_m	C_1
0.00	11.07	10.02	10.54	10.44	11.70	11.07	10.54	8.22
0.00	6.11	8.11	8.33	6.47	7.49	8.22	6.85	7.06
9.50	9.07	8.75	9.81	10.23	10.97	11.28	9.38	7.06
21.00	7.49	8.12	9.81	9.81	8.71	9.17	7.17	6.54
28.00	5.90	7.70	7.70	7.49	8.01	9.17	16.55	7.49
40.00	5.69	16.60	11.28	6.33	7.49	7.49	30.00	5.06
56.00	19.08	24.60	28.83	16.55	16.87	22.98	33.00	3.69
68.00	22.47	27.30	25.83	23.68	23.20	23.72	25.52	2.43
71.00	23.47	26.10	23.62	25.41	20.03	21.83	20.14	1.90
84.00	23.68	24.68		24.36	18.87	19.61		

Table B.5b. Effect of 1000 ppb of Individual Vitamins and 5000 ppb of V_{mix} on Degradation of Heptadecane

	Heptadecane conc. with different 1000 ppb individual vitamins. & 5000 ppb V_{mix} (average of 3 data sets, ppm)							
Time(h)	V_0	V_1	V_2	V_3	V_4	V_5	V_m	C_2
0.00	51.50	51.00	54.50	56.00	53.00	53.50	55.50	51.50
9.50	56.00	52.50	53.00	53.00	50.50	54.00	54.50	51.00
21.00	53.50	50.50	53.00	55.00	53.50	52.00	38.00	53.50
28.00	53.00	53.50	45.50	52.00	48.50	53.50	19.00	53.00
40.00	43.50	42.50	26.00	41.50	43.50	48.00	0.00	56.00
56.00	21.50	7.50	0.00	22.00	20.00	26.50		56.00
68.00	3.00	0.00		3.00	6.25	10.50		53.00
71.00	0.00	0.00		0.00	0.00	0.00		51.50

Table B.6a. Effect of Various Concentrations of Niacin on Biomass Growth Using Heptadecane as Carbon Source

	Biomass conc. with different conc. of niacin (average of 3 data sets, ppm)				
Time (h)	V_0	V_{50}	V_{100}	V_{500}	V_{1000}
0.00	4.64	4.43	5.20	5.06	4.78
20.00	4.22	4.43	4.71	4.71	4.43
51.00	10.54	17.85	17.71	13.77	13.70
69.50	15.39	26.07	25.37	21.08	18.57
75.50	18.20	40.83	40.26	27.62	27.83
87.50	34.22	29.79	36.12	23.40	42.02
102.00	27.26		31.90	21.08	39.63

Table B.6b. Effect of Various Concentrations of Niacin on Degradation of Heptadecane

	Heptadecane conc. with different conc. of niacin (average of 3 data sets, ppm)				
Time (h)	V_0	V_{50}	V_{100}	V_{500}	V_{1000}
0.00	52.67	54.67	61.33	51.93	53.00
20.00	54.27	53.63	61.53	52.67	47.67
51.00	30.67	33.67	25.00	18.33	34.33
69.50	9.67	4.00	6.00	0.00	5.53
75.50	0.00	0.00	0.00	0.00	0.00
87.50	0.00	0.00	0.00	0.00	0.00

Table B.7a. Effect of Various Concentrations of Biotin on Biomass Growth Using Heptadecane as Carbon Source

	Biomass Conc. with different conc. of biotin (average of 3 data sets, ppm)				
Time (h)	V_0	V_{50}	V_{100}	V_{500}	V_{1000}
0.00	6.11	5.41	6.39	5.62	5.20
8.00	6.11	6.32	5.34	5.62	5.20
20.00	8.01	12.23	11.31	10.47	8.92
26.00	11.52	15.95	15.46	14.62	12.65
45.00	19.39	27.12	27.26	20.10	17.50
57.00	15.74	16.78	19.46	18.69	27.83

Table B.7b. Effect of Various Concentrations of Biotin on Degradation of Heptadecane

	Heptadecane conc. with different conc. of biotin (average of 3 data sets, ppm)				
Time (h)	V_0	V_{50}	V_{100}	V_{500}	V_{1000}
0.00	45.27	57.00	51.67	50.67	50.33
8.00	45.00	57.00	51.33	50.67	50.33
20.00	32.00	33.67	33.90	33.67	41.29
26.00	18.00	16.00	10.93	14.33	14.67
45.00	1.33	4.00	0.00	0.00	1.00
57.00	0.00	0.00	0.00	0.00	1.00

Table B.8a. Effect of Various Concentrations of Folic Acid on Biomass Growth Using Heptadecane as Carbon Source

	Biomass Conc. with different conc. of folic acid (average of 3 data sets, ppm)				
Time (h)	V_0	V_{50}	V_{100}	V_{500}	V_{1000}
0.00	14.91	13.36	14.42	15.40	14.63
20.00	7.46	12.73	6.61	5.77	9.49
31.00	27.29	23.46	21.17	27.85	14.63
44.00	42.62	28.13	49.37	45.93	22.86
52.50	33.55	19.62	32.85	41.85	23.77
68.00	26.38	17.79	26.30	31.58	17.86
76.00	21.94		23.63	28.77	17.79

Table B.8b. Effect of Various Concentrations of Folic Acid on Degradation of Heptadecane

	Heptadecane conc. with different conc. of folic acid (average of 3 data sets, ppm)				
Time (h)	V_0	V_{50}	V_{100}	V_{500}	V_{1000}
0.00	39.86	38.29	40.49	39.02	40.00
20.00	41.02	36.52	37.95	39.91	36.06
31.00	34.17	15.15	21.06	36.75	18.48
44.00	6.29	9.76	4.95	4.54	11.68
52.50	4.86	1.73	1.98	1.48	1.06
68.00	0.68	0.00	0.00	0.00	0.02
76.00	0.00				0.00

Table B.9a. Effect of Various Concentrations of Thiamin on Biomass Growth Using Heptadecane as Carbon Source

	Biomass Conc. with different conc. of thiamin (average of 3 data sets, ppm)				
Time (h)	V_0	V_{50}	V_{100}	V_{500}	V_{1000}
0.00	7.03	7.46	6.40	6.89	7.46
23.00	6.61	6.33	6.61	6.47	7.24
32.00	10.55	10.97	9.35	9.78	11.18
45.00	18.57	18.36	17.30	19.20	21.73
56.00	15.47	18.01	18.36	18:08	19.97
69.00	14.63	16.46	16.32	19.48	18.43

Table B.9b. Effect of Various Concentrations of Thiamin on Degradation of Heptadecane

	Heptadecane conc. with different conc. of thiamin (average of 3 data sets, ppm)				
Time (h)	V_0	V_{50}	V_{100}	V_{500}	V_{1000}
0.00	52.44	50.67	51.67	49.93	50.33
23.00	51.35	49.43	48.50	51.67	50.10
32.00	27.72	34.24	24.67	26.33	23.67
45.00	8.40	20.46	6.22	10.33	9.33
56.00	0.00	3.67	0.00	0.00	0.00
69.00	0.00	0.00	0.00	0.00	0.00

Table B.10a. Effect of Various Concentrations of Pantothenic Acid on Biomass Growth Using Heptadecane as Carbon Source

	Biomass Conc. with different conc. of pantothenic acid (average of 3 data sets, ppm)				
Time (h)	V_0	V_{50}	V_{100}	V_{500}	V_{1000}
0.00	7.81	7.17	6.75	6.96	6.68
9.00	6.40	6.19	5.77	6.12	5.98
24.00	12.38	11.39	13.93	11.75	9.71
34.00	23.28	30.52	22.30	22.79	22.01
44.00	27.43	32.35	39.46	27.22	30.74
52.00	34.96	23.35	35.59	33.20	32.71
69.00	29.33	20.68	25.39	31.58	

Table B.10b. Effect of Various Concentrations of Pantothenic Acid on Degradation of Heptadecane

	Heptadecane conc. with different conc. of pantothenic acid (average of 3 data sets, ppm)				
Time (h)	V_0	V_{50}	V_{100}	V_{500}	V_{1000}
0.00	36.00	38.67	38.33	36.67	37.00
9.00	37.13	37.93	38.33	37.67	37.67
24.00	27.00	31.67	15.00	30.33	31.00
34.00	15.00	10.33	4.73	18.67	24.00
44.00	3.98	1.73	1.46	10.87	11.67
52.00	0.00	0.00	0.00	5.23	6.67
69.00				0.00	0.00

Table B.11a. Effect of V_{opt} on Average Biomass Growth Using Nonane as Carbon Source

	Average biomass concentration (ppm)			
Time (h)	V_0	V_{opt}	C_1	C_2
0.00	3.17	3.31	3.65	0.98
29.00	2.16	1.27	2.65	1.19
40.00	1.41	3.80	3.16	0.91
47.00	0.56	11.75	2.81	0.49
53.00	1.55	26.66	1.48	0.70
67.00	4.64	27.43	1.76	1.12
73.00	11.04	22.01	1.76	0.77
81.00	20.26	19.13	1.19	0.49
92.00	27.01		1.26	0.42
109.00	24.34		1.41	
118.00	24.83		0.84	

Table B.11b. Effect of V_{opt} on Degradation of Nonane (Average Data)

	Average nonane concentration (ppm)		
Time(h)	V_0	V_m	C_2
0.00	36.67	31.84	37.33
29.00	34.03	32.44	36.33
40.00	35.12	26.34	36.33
47.00	34.56	11.78	35.23
53.00	36.00	5.11	37.64
67.00	22.63	0.69	37.06
73.00	11.67	0.00	35.60
81.00	2.67		35.33
92.00	0.00		35.40

Table B.12a. Effect of V_{opt} on Average Biomass Growth Using Hexadecane as Carbon Source

	Average biomass concentration (ppm)			
Time (h)	V_0	V_{opt}	C_1	C_2
0.00	5.20	6.12	5.49	1.34
14.00	4.36	4.08	4.71	0.84
24.00	7.03	8.16	3.87	0.56
31.00	14.49	19.41	3.45	1.13
37.00	20.05	25.11	2.18	0.63
45.00	25.39	27.01	1.97	0.35
49.00	23.00	24.19	0.91	0.35

Table B.12b. Effect of V_{opt} on Degradation of Hexadecane (Average Data)

	Average hexadecane concentration (ppm)		
Time(h)	V_0	V_m	C_2
0.00	49.72	50.29	48.50
14.00	49.14	49.53	48.18
24.00	45.20	36.57	45.47
31.00	11.37	7.77	43.07
37.00	3.84	0.00	41.64
45.00	0.00	0.00	42.37

Table B.13a. Effect of V_{opt} on Average Biomass Growth Using Heptadecane as Carbon Source

	Average biomass concentration (ppm)			
Time (h)	V_0	V_{opt}	C_1	C_2
0.00	4.92	5.28	4.43	1.62
11.00	4.85	5.06	4.57	1.69
24.00	3.73	12.59	4.57	1.41
30.00	3.80	20.49	4.57	1.48
37.00	5.06	23.42	4.01	1.34
46.00	7.67	23.63	3.30	1.19
52.00	12.31	23.42	-2.25	1.19
58.00	15.19	21.63	1.41	1.26
70.00	16.32		0.77	1.26

Table B.13b. Effect of V_{opt} on Degradation of Heptadecane (Average Data)

	Average heptadecane concentration (ppm)		
Time(h)	V_0	V_m	C_2
0.00	34.12	35.38	32.26
11.00	33.56	34.49	31.99
24.00	34.51	31.42	33.22
30.00	33.17	22.34	32.04
37.00	29.04	3.18	32.68
46.00	22.06	0.00	30.89
52.00	16.99		31.26
58.00	9.76		31.60
70.00	0.00		

Table B.14a. Effect of V_{opt} on Average Biomass Growth Using Total Diesel Hydrocarbon (TDH) as Carbon Source

	Average biomass concentration (ppm)				
Time (h)	V_0	V_{opt}	C_1	C_2	C_g
0.00	9.70	8.43	10.33	0.98	8.50
17.00	22.21	25.93	10.05	0.84	8.22
27.00	29.09	34.01			11.66
41.00	32.04	35.56	9.35	0.77	11.59
51.00	36.61	49.33			45.75
65.00	40.90	64.72	8.64	1.19	
72.00	48.21	64.09			
80.00	57.27	60.50	6.75	0.70	
89.00	59.17				
98.00	56.57		5.06	0.56	
110.00	49.05		3.51	0.98	

Table B.14b. Effect of V_{opt} on Degradation of Total Diesel Hydrocarbon at Low Concentration (Average Data)

	Average TDH (low) concentration (ppm)		
Time(h)	V_0	V_m	C_2
0.00	68.17	62.90	67.38
17.00	39.79	30.38	62.03
27.00	38.39	23.85	
41.00	30.80	18.70	
51.00	31.97	9.74	61.35
65.00	17.05	11.39	65.99
72.00	19.26	4.95	63.81
80.00	10.37	0.00	63.27
89.00	7.59		
98.00	2.32		66.91
110.00	0.00		59.99

Table B.15a. Effect of V_{opt} on Average Biomass Growth Using Total Diesel Hydrocarbon (TDH hi) as Carbon Source

	Average biomass concentration (ppm)				
Time (h)	V_0	V_{opt}	C_1	C_2	C_g
0.00	10.61	10.61	10.33	1.90	- 8.50
17.00	18.83	18.83	10.05	1.76	8.22
27.00	24.24	24.24			11.66
41.00	26.42	26.42	9.35	1.62	11.59
51.00	30.78	30.78			45.75
65.00	37.03	37.03	8.64	0.77	190.71
72.00	44.55	44.55			259.15
80.00	51.65	51.65	6.75	0.84	240.18
89.00	68.02	68.02			
98.00	82.71	82.71	5.06	0.84	
110.00	87.84	87.84	3.51	1.41	
122.00	88.68	88.68			
130.00	84.75	84.75			

Table B.15b. Effect of V_{opt} on Degradation of Total Diesel Hydrocarbon at High Concentration (Average Data)

	Average TDH (high) concentration (ppm)		
Time(h)	V_0	V_m	C_2
0.00	110.44	150.59	127.10
17.00	80.77	103.76	124.10
27.00	79.84	78.16	129.51
41.00	60.44	58.96	120.44
51.00	55.94	41.18	
65.00	50.83	32.61	123.83
72.00	50.45	24.67	130.45
80.00	43.56	22.49	136.89
89.00	42.39	15.82	135.05
98.00	27.17	5.73	127.17
110.00	7.61	0.00	
122.00	2.68		135.49
130.00	0.00		

APPENDIX C

CHROMATOGRAMS OF DIESEL

The chromatograms of diesel at various times are shown here. From the Figure 2 and Figure 3 in this chapter, are representing the chromatograms of high concentration of diesel with V_{opt} and without V_{opt} respectively. Since it is impossible to show all the chromatograms, only three chromatograms of each V_0 and V_{opt} at various time intervals were chosen to give a clear idea of the effectiveness of V_{opt} on diesel degradation. In Figures 2 and 3 the chromatograms at time intervals of 0, 65 and 110 hours are shown. Figure 1 is representing the chromatogram of standard of diesel contained 10 ppm of each alkane ranging from C_{10} to C_{25} (as shown in the figure). Figure 4 and 5 are showing the chromatograms of low concentration of diesel with V_{opt} and V_0 respectively. To give a clear view of comparison, chromatograms with V_{opt} were chosen at 0, 72 and 80 hours whereas chromatograms with V_0 were taken at 0, 72 and 98 hours. These later figures tell us that with V_{opt}, low concentration of diesel were completely used up by 80 hours whereas it was still there at 98 hours for the case of V_0. Pristane and phytane peaks were labelled in only one chromatogram in Figure 2 though, these two peaks were present in other initial chromatograms too.

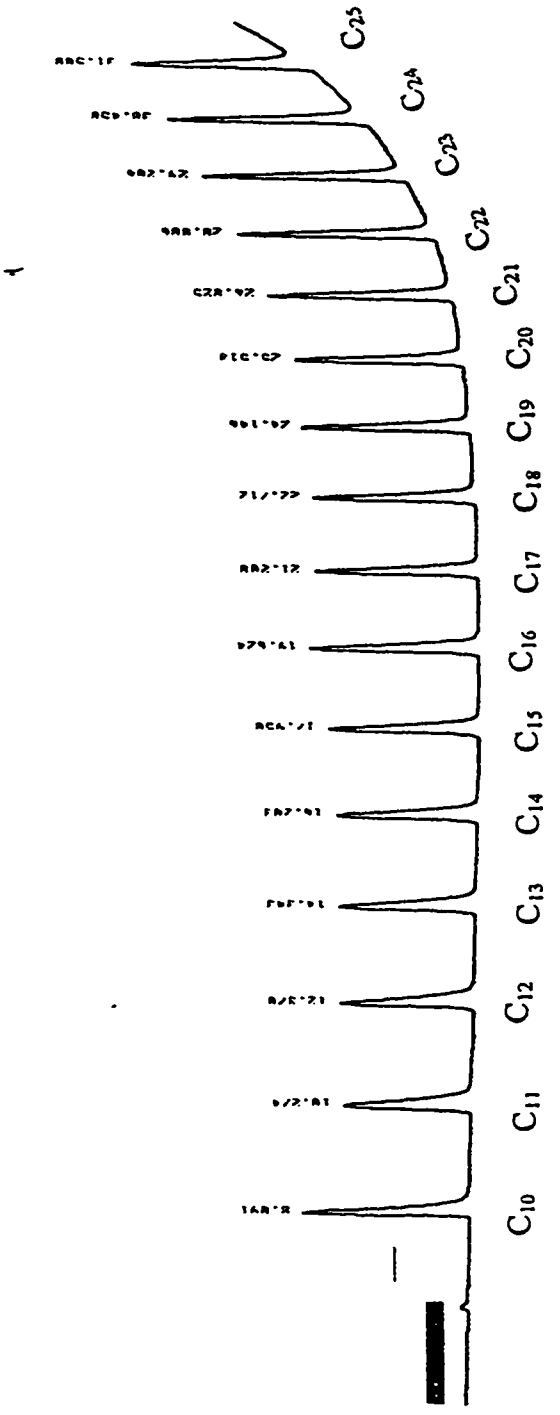


Figure 1. Standard of diesel containing n-alkanes of C_{10} to C_{25}, each of 10 ppm.

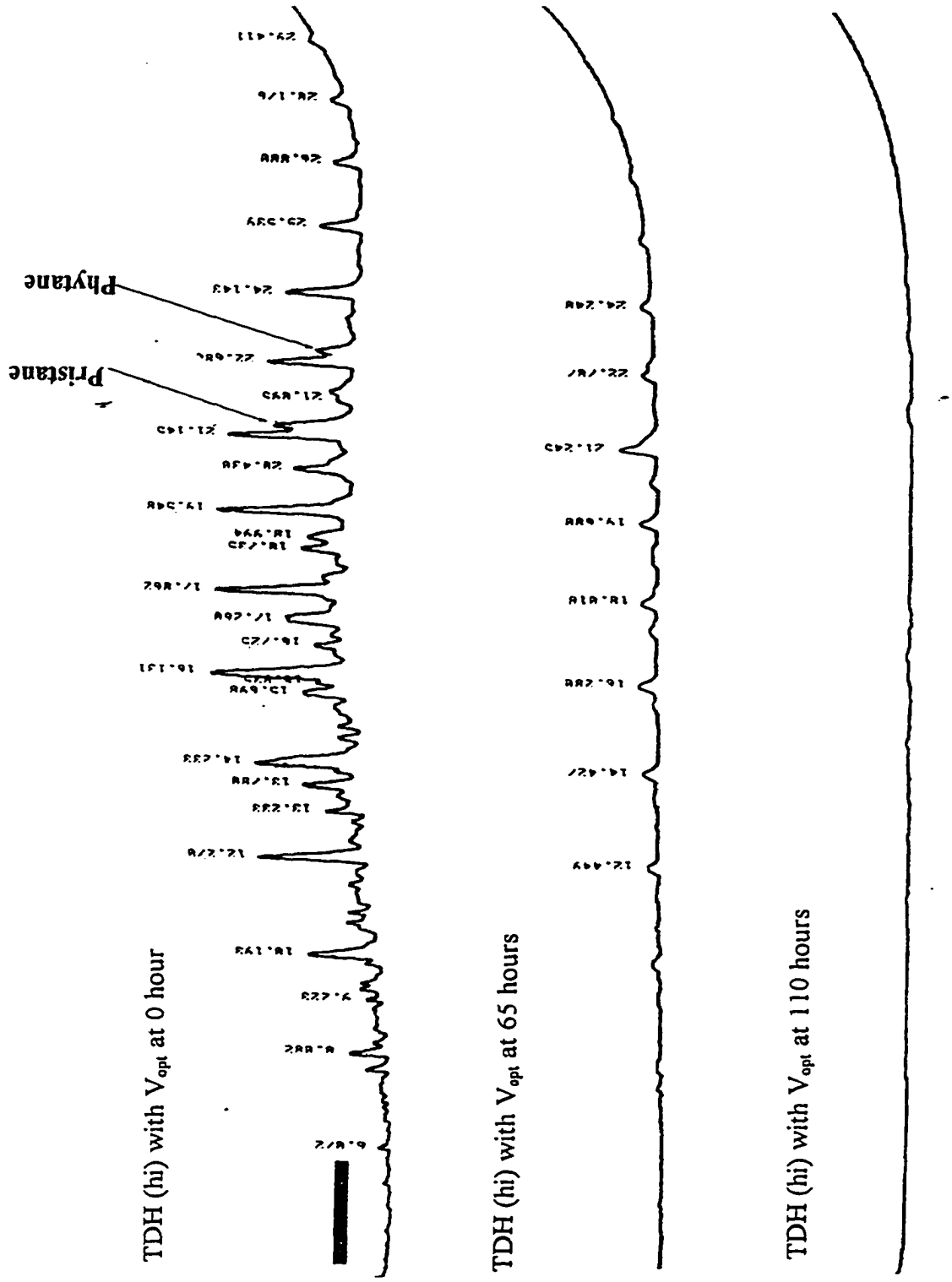


Figure 2. Chromatograms of diesel (initial concentration 150 ppm) at various time intervals with V_{opt}

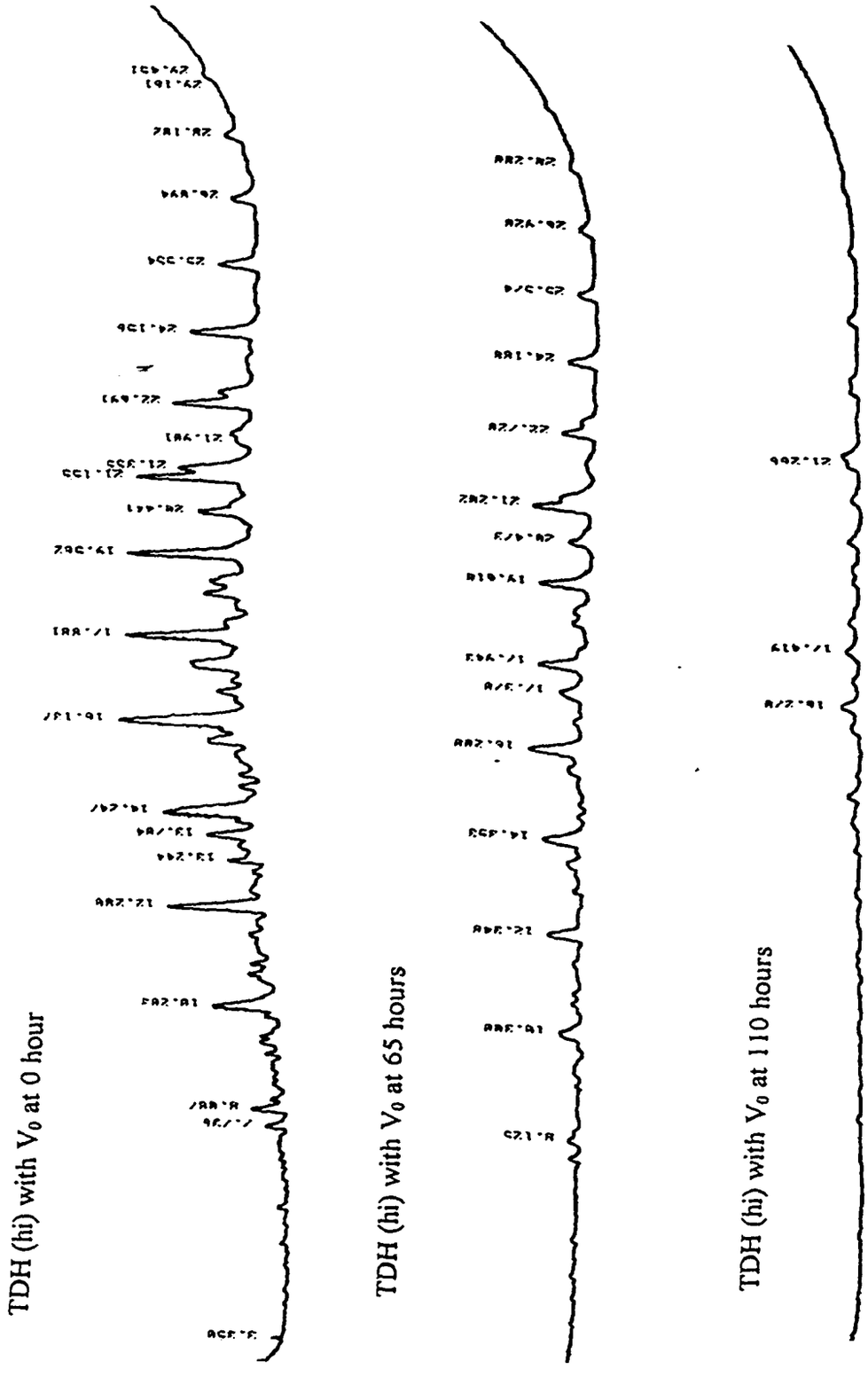


Figure 3. Chromatograms of diesel (initial concentration 110 ppm) at various time intervals with V_{opt}

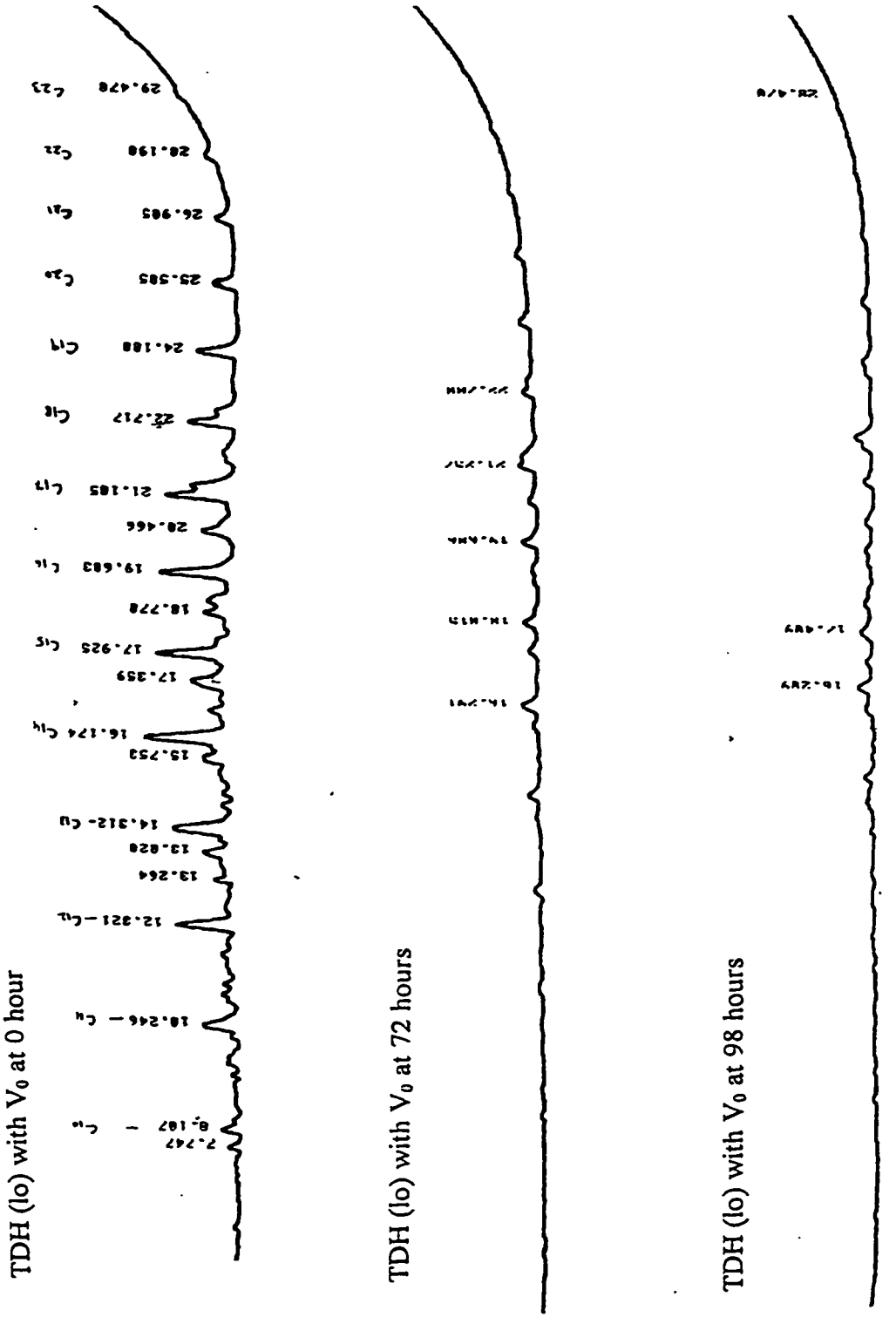


Figure 4. Chromatograms of diesel (initial concentration 63 ppm) at various time intervals with V_{opt}

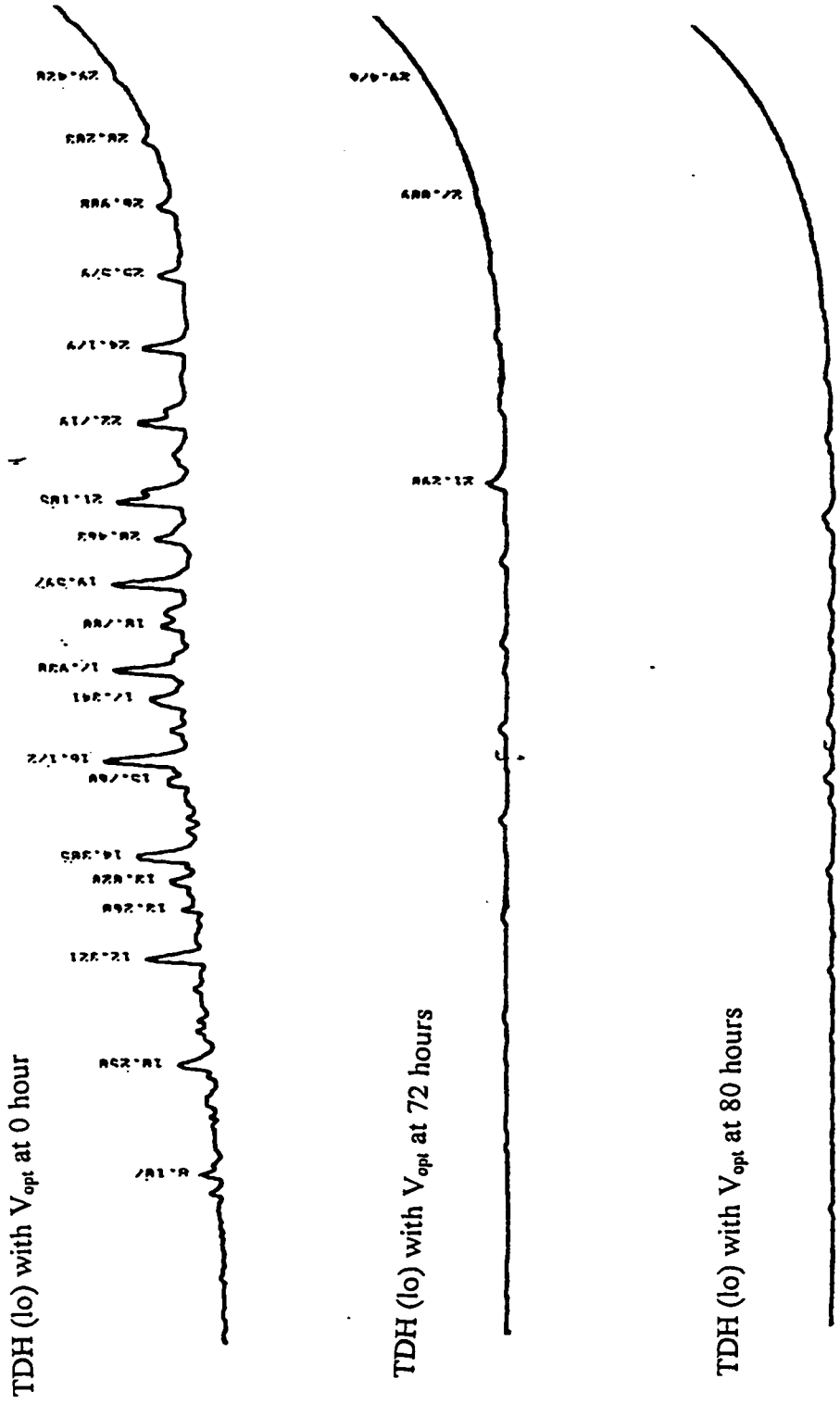


Figure 5. Chromatograms of diesel (initial concentration 68 ppm) at various time intervals with V_{opt}

APPENDIX D

TABLES FOR ANOVA ANALYSIS

Table D1a. Data for Lag Phase

	Lag phase of individual data set for various carbon source (h)	
Carbon Source	V_0	V_{opt}
C_9 set # 1	56	37
set # 2	50	33
set # 3	57	32
C_{16} set # 1	19	16
set # 2	20	16
set # 3	24	15
C_{17} set # 1	32	20
set # 2	36	19
set # 3	32	25
TDH (lo) set # 1	7	2
set # 2	7	2
set # 3	2	3
TDH (hi) set # 1	4	4
set # 2	9	5
set # 3	5	5

Table D1.b. Results for ANOVA Analysis for Lag Phase

Source of Variation	SS	df	MS	F	P-value	F crit
Sample	6701.13	4	1675.283	277.67	3.25E-17	2.87
Columns	529.2	1	529.2	87.71	9.42E-09	4.35
Interaction	365.8	4	91.45	15.16	7.51E-06	2.87
Within	120.67	20	6.03			
Average	**24**	**15.6**				

Table D2.a. Data for Overall Degradation Rates

	O.D.R of individual data set for various carbon source (ppm/h)	
Carbon Source	$\bar{V}_0$	V_{opt}
C_9 set # 1	0.39	0.46
set # 2	0.45	0.44
set # 3	0.37	0.43
C_{16} set # 1	1.28	1.36
set # 2	1.32	1.34
set # 3	1.11	1.39
C_{17} set # 1	0.57	0.8
set # 2	0.56	0.78
set # 3	0.53	0.77
TDH (lo) set # 1	0.69	0.54
set # 2	0.48	0.88
set # 3	0.81	1.04
TDH (hi) set # 1	0.88	1.37
set # 2	0.91	1.35
set # 3	0.83	1.68

Table D2.b. Results for ANOVA Analysis for Overall Growth Rates

Source of Variation	SS	df	MS	F	P-value	F crit
Sample	3.19	4	0.80	55.11	1.61E-10	2.87
Columns	0.40	1	0.40	27.43	4E-05	4.35
Interaction	0.28	4	0.07	4.76	0.007	2.87
Within	0.29	20	0.01			
Average	**0.75**	**0.98**				

Table D3a. Data for Overall Growth Rates

OGR		
	Group1	Group2
Trial 1	0.28	0.38
	0.26	0.36
	0.26	0.28
Trial 2	0.35	0.48
	0.39	0.52
	0.44	0.56
Trial 3	0.2	0.31
	0.23	0.26
	0.2	0.39
Trial 4	0.4	0.77
	0.35	0.72
	0.56	0.78
Trial 5	0.68	0.59
	0.6	0.66
	0.72	0.95

Table D3.b. Results for ANOVA Analysis for Overall Degradation Rates

ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Sample	0.83	4	0.21	32.32	1.8E-08	2.87
Columns	0.15	1	0.15	22.61	0.0001	4.35
Interaction	0.06	4	0.016	2.52	0.073	2.87
Within	0.13	20	0.006			
Average	**0.39**	**0.53**				

Table D4.a. Data for Degradation Rates

DR		
	Group1	Group2
Trial 1	1.36	2.21
	1.39	1.54
	1.75	1.38
Trial 2	3.82	2.5
	5.85	3.89
	5.55	6.27
Trial 3	1.68	1.94
	1.84	2.04
	0.97	2.8
Trial 4	0.88	0.4
	1.51	2.83
	0.92	1.36
Trial 5	0.75	1.54
	0.72	1.13
	0.67	2.07

Table D4.b. Results for ANOVA Analysis for Degradation Rates

ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Sample	49.80	4	12.45	16.92	3.32E-06	2.87
Columns	0.60	1	0.60	0.81	0.378	4.35
Interaction	2.83	4	0.71	0.96	0.450	2.87
Within	14.72	20	0.74			
Average	**1.98**	**2.26**				

Table D5.a. Data for Growth Rates

GR		
	Group1	Group2
Trial 1	0.074	0.147
	0.08	0.111
	0.069	0.1
Trial 2	0.095	0.066
	0.093	0.118
	0.073	0.127
Trial 3	0.061	0.067
	0.044	0.079
	0.044	0.157
Trial 4	0.008	0.022
	0.018	0.048
	0.026	0.017
Trial 5	0.016	0.019
	0.013	0.015
	0.015	0.023

Table D5.b. Results for ANOVA Analysis for Growth Rates

ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Sample	0.036	4	0.009	19.19	1.27E-06	2.87
Columns	0.005	1	0.005	10.56	0.004	4.35
Interaction	0.003	4	0.0007	1.400	0.27	2.87
Within	0.009	20	0.0005			
Average	**0.049**	**0.074**				

Table D6.a. Data for Overall Yield Coefficient

0.Y.C		
	Group1	Group2
Trial 1	0.071	0.82
	0.58	0.81
	0.7	0.66
Trial 2	0.27	0.35
	0.29	0.39
	0.39	0.4
Trial 3	0.34	0.39
	0.41	0.33
	0.37	0.5
Trial 4	0.58	1.43
	0.73	0.82
	0.69	0.75
Trial 5	0.77	0.43
	0.66	0.49
	0.86	0.57

Table D6.b. Results for ANOVA Analysis for Overall Yield Coefficient

ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Sample	0.93	4	0.23	7.93	0.0005	2.87
Columns	0.07	1	0.068	2.32	0.144	4.35
Interaction	0.36	4	0.090	3.06	0.040	2.87
Within	0.59	20	0.029			
Average	**0.51**	**0.61**				

Table D7.a. Data for Yield Coefficient

Y.C		
	Group1	Group2
Trial 1	0.073	0.65
	0.29	0.91
	0.29	0.92
Trial 2	0.26	0.29
	0.18	0.39
	0.16	0.28
Trial 3	0.27	0.28
	0.24	0.31
	0.41	0.87
Trial 4	0.24	1.97
	0.59	0.38
	0.79	0.4
Trial 5	0.98	0.48
	0.87	0.59
	1.07	0.41

Table D7.b. Results for ANOVA Analysis for Yield Coefficient

ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Sample	1.03	4	0.26	2.35	0.09	2.87
Columns	0.19	1	0.19	1.78	0.197	4.35
Interaction	0.99	4	0.25	2.27	0.098	2.87
Within	2.18	20	0.11			
Average	**0.45**	**0.61**				

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