2) ISOLATION AND CHARACTERIZATION OF A CRUDE EXTRACELLULAR PHENOL OXIDASE FROM A RECIRCULATION BIOREACTOR

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ABSTRACT

The study of phenol biodegradation has been performed using calcium alginate entrapped activated sludge in a recirculation reactor. An effective recirculation reactor system may be used on the laboratory scale to produce phenol oxidase. Phenol oxidase was isolated and concentrated from the degradation solution of phenol by ultrafiltration and freeze-drying respectively.

The effects of various concentrations of phenol, pH, temperature, ionic strength, cations, anions and 2-chlorophenol were studied on the oxidation reaction of enzyme. The optimal conditions of activated sludge phenol oxidase are: [phenol] = 100 ppm; pH = 6.0; temperature = 37°C; ionic strength equivalent to 0.1 M Na_2HPO_4-NaH_2PO_4; The enzyme activity was increased by Cu^{2+}, inhibited by Ag^{+}, Cl^{-}, and unsignificantly changed by Zn^{2+}, Fe^{2+}, NO_3^{-}, and SO_4^{2-}; other substrates such as 2-chlorophenol, styrene, and methylene chloride, have also been shown to be oxidized by this enzyme; the enzyme activity was inhibited by beta-hydroxybutyric acid as a substract.

Preliminary studies indicate that phenol stimulates the activated sludge to release phenol oxidase at low concentrations of phenol and inhibit at high concentrations of phenol.

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CHAPTER I

INTRODUCTION

1.1 BIOREACTORS IN HAZARDOUS WASTE TREATMENT

It is well known that biodegradation of organic wastes is a good technology. For the present day, this technology also needs to be developed for treating highly toxic and much more complex industrial wastes. Biological treatment of several organic compounds has been realized in the last twenty years, but not many processes other than the conventional activated sludge treatment have been designed and implemented on a large scale for specific applications. Since the complexity of the waste stream, the design and development of high efficiency bioreactors to treat industrial waste is more difficult and challenging [1].

1.2 IMMOBILIZATION AND ITS ADVANTAGES

A major development in bioreactor designs has been the incorporation of immobilization techniques. Using immobilized microorganisms can be more advantageous than using free microorganisms for numerous reasons, especially for the treatment of aqueous wastes [2-4]. A free-cell system which is very sensitive to varying input

conditions and long residence times can be significantly improved using immobilized microorganisms. Immobilization facilitates separation and has very good operational flexibility, as continuous or semicontinuous processes become practical. In addition, immobilized cells are much more resistant to high concentrations of toxic chemicals [5] and the cell density can be much higher than that of the free cell, which results in higher rates of biodegradation per unit volume of the reactor. Immobilized cells can also be dried and stored as a convenient source of reusable biomass.

There are numerous methods for immobilized cell preparation [5]. The support material should be stable with the substrate, product and reaction conditions and suitable for continuous or repeated use in the desired scale.

1.3 REQUIREMENTS OF DISSOLVED OXYGEN AND SUBSTRATE

In a system utilizing aerobic microorganisms for the biodegradation of organic compounds, dissolved oxygen requirements are much more important. Aerobic microorganisms utilize oxygen primarily as the terminal electron acceptor for aerobic respiration. In general, bacterial respiration does not appear to be affected. A critical dissolved oxygen concentration at which the

respiration rate of cells is one half of the maximum rate. The respiration rate is generally lower for dispersed cultures than for flocculent cultures [6]. Relatively little is known about the influence of dissolved oxygen on the microbial degradation of toxic chemicals. In the case of microorganisms entrapped in alginate gel, diffusion can become a rate limiting factor for oxygen uptake and may further increase the half life of biodegradation. The degradative pathways involve a number of enzymes. If one of them is an oxygenase, then the degradative rate will also depend on oxygen concentration.

Although there is the disadvantage of increasing diffusional resistance of substrates and products through immobilization matrices, immobilization can be of advantage in case of system exposure to high concentrations of toxic chemicals. The lower solubility of oxygen in water and high local cell density, oxygen transfer is often the rate limiting factor [7]. Methods that have been used to by the N.J.I.T. Biotechnology Laboratory for increasing the availability of oxygen to immobilized cells include decreasing the particle size of the immobilization matrix and increasing the oxygen delivery capacity to the medium. Increasing the oxygen delivery to the medium has been attempted by the addition of H_2O_2.

1.4 ACTIVATED SLUDGE AND BIODEGRADATION

The activated sludge process is used widely for treatment of municipal and industrial wastes. The large variety of microorganisms in the activated sludge reactor and their interaction may hold the key to the effective treatment and removal of toxic compounds.

For microorganisms entrapped in alginate gel, the rate constants may not be the same as those for free microbes, and factors other than microbial activity may be rate limiting.

1.5 ISOLATION AND CHARACTERIZATION OF PHENOL OXIDASE

During biodegradation, microorganisms release certain enzymes according to the type of substrates which are used. There may be multi-enzyme systems, single enzyme or combinations. There are intracellular enzymes, extracellular enzymes and structural enzymes.

The crude phenol oxidase of this study is released by immobilized activated sludge during degradation of phenol in a recirculation bioreactor.

The mechanism of action of enzyme is in itself one of the most fascinating fields of scientific investigation being pursued at the present time.

The characteristic property of an enzymes is its power for catalyzing certain definite chemical reactions. The activity of enzyme depends on many different factors such as pH, temperature, buffer concentration, different substrates, and so on.

It is possible to fractionate the solution and obtain proteins of different molecular sizes from the mixed protein system. The target enzyme can then be crudely characterized.

CHAPTER II

LITERATURE SURVEY

Biochemists and chemical engieers have utilized their fields of expertise in developing high performance bioreactors. The literature survey for this work has been done to identify and site approaches taken in development of high efficiency bioreactors, phenol biodegradation and phenol oxidase characterization. Fundamental aspects of this technology have been probed, and better biocatalysts and have defined appropriate environments for the enzymes to perform specific functions have been defined.

In the present work the literature review is concentrated on the area of bioreactors which use immobilized cells or enzymes, phenol biodegradation, and phenol oxidase.

The entrapment of activated sludge in alginate is a good method of immobilization. When the biomass is entrapped inside a matrix, the selection criteria are primarily based on the pore size of matrix. With a proper pore size the biomass can be localized and the diffusion of nutrients and products across the matrix can be controlled as described by Tanaka et al. [8].

The parameters which are microbe-surface interaction, surface porosity, pore size opening, and

hydrophilic/hydrophobic characteristics of the surfaces are important for the immobilization process. For the transfer of oxygen across the biofilm, due to high local cell density, the requirements of dissolved oxygen are more than that required for a conventional free cell system. It has been hypothesized that DO limitations also occur in conventional activated sludge systems wherein an anoxic core forms inside the biological flock, void of sufficient DO [9]. Also bioparticle or pellet size can limit the availability of oxygen to the cell entrapped system. Chen and Humphrey [10] have determined that a simple relationship could be used for estimating the critical particle diameter. Under given conditions of biomass density, diffusivity, and specific respiration rate, the critical radius was given for zero and first order approximations of Michaelis Menten kinetics.

The supply and availability of oxygen at reaction sites within the bead or bioparticle is mainly dependent on diffusivity of oxygen in supports entrapping whole cells. Sato and Toda [11] found that diffusivity of oxygen in agar gel was 70% of that in water. Adlercreutz [12] concluded that diffusion of oxygen on calcium alginate gel was 25% of that in water.

Tomlinson and Snaddon [13] reported that oxygen diffuses in microbial slimes from 62% to 83% of that in water. The dependence of oxygen diffusivity on cell

concentration when entrapped in alginate gel was studied by Sun et al. [14]. They observed a 73% decrease in effective diffusivity by increasing the cell density four times.

Phenol degradation in a fluidized bed bioreactor was modeled by Livingston and Chase [15]. The model described simultaneous diffusion and reaction of both phenol and oxygen in the reactor packed with calcined diatomaceous earth particles to which the bacteria were attached. The model predicted a transition from phenol to oxygen limiting kinetics. this is the first time that such a model has been developed. They found that at a critical ratio in the range of 0.9 to 1.1 (phenol/oxygen) a transition from phenol to DO limiting kinetics occurred.

Also, the biodegradation on phenol in waste water was studied at the N.J.I.T. Biotechnology Laboratory [16]. In addition, a polymeric membrane reactor was used for phenol degradation. By using immobilized activated sludge, 2-chlorophenol was degraded in recirculation reactor [17]. Phenol degradation by Candida tropica by whole cells entrapped in polymeric ionic networks was studied by Klein [18]. Bettmann and Rehm [18] reported continuous degradation of phenol [5] by Pseudomonas putida P8 entrapped in polyacrylamide hydrazide. Similarly biodegradation methods of 4-chlorophenol and 2-chlorophenol by immobilized Acaligenes sp. A7-2 and

activited sludge have been developed respectively [17][20][21].

System response to variation in temperature and concentration of chlorophenol was studied in an air-sparged reactor. A recirculation reactor, run in a batch mode, were used to study kinetic parameters in this laboratory by Lakhwala et al [22]. Dwyer et al [5] used an immobilized methanogenic consortium to degradate phenol. The kinetic constants of phenol biodegradation were determined.

The rate of biodegradation of phenol may be affected by a variety of factors. These include induction or derepression of enzymes, genetic change, and growth of specific degrader organisms [23]. During the process of phenol degradation, the major enzyme is phenol oxidase. The isolation and characterization of phenol oxidase from other sources have been done previously [24-30]. The complex enzyme phenol oxidase also plays a major role of decolorization [31].

A specific phenol oxidase which is produced during conidiospore development in the fungus Aspergillus nidulans has been purified [32]. There are two active forms (A and B) and masses of 50 and 48 KDa respectively. This enzyme can be inhibited by 2,3-dihydroxynaphthalene, 8-hydroxyquinolene and diethyldithiocarbamate based on non-competitive and competitive forms. The peptide

mapping N-terminal sequence was completed in this metal ion containing enzyme. The presence of copper is typical of phenol oxidase [33]. This metal is required for activity. Two active forms (A and B) have been confirmed by using the N-terminal amino acid sequence to construct a DNA probe which hybridizes with an ivo B clone isolated by mutant complementation.

Ruckdeschel et al. [34] observed the effects of pentachlorophenol and 35 of its known and possible metabolites on 30 different species of bacteria. Significant results were obtained. Two chlorophenols, five dichlorophenols, four trichlorophenols, two tetrachlorophenols, and tetrachloro-1,2-benzenediol were more active than pentachlorophenol against some, but not all of the strains tested.

The quantitative studies of extracellular phenol oxidase production by eight species of wood-rotting fungi were performed by Daniel [35]. There are differences between two strains in phenol oxidase activity. The results suggest that fungi can lose their abilities for enzyme production when cultured for long periods of time. Phenol oxidase activity depends on contain strains representing different levels of phenol oxidase activity.

Mutations can affect phenol oxidase activity [36]. The structural gene for phenol oxidase has been established by Rizki [37]. Therefore, the large-scale

recombinant production of phenol oxidase may be possible in the future.

CHAPTER III

OBJECTIVES

The objective of this research is to use a recirculation flow reactor system employing immobilized activated sludge and degrading phenol for the production of phenol oxidase. Subsequents isolation and crude characterization thos enzyme is the primary goal of this work.

An assay for phenol oxidase was developed. An attempt was made to separate the phenol oxidase from the reaction solution after the biodegradation of phenol which at different starting concentrations, and with varying concentrations of oxygen.

In addition, the reaction conditions for and some characteristics of phenol oxidase were obtained.

CHAPTER IV

MATERIALS AND EXPERIMENTAL METHODS

4.1 MICROORGANISMS

Fifty liters of activated sludge were obtained from the Parsippany Water Pollution Control Plant (NJ) and sieved through a 297 um opening screen. The sludge was then acclimated with one 100 ppm phenol spike per day for three days, at room temperature ($27^{o}C \pm 2$). It was then centrifuged at 3000 rpm and $4^{o}C$ (IEC model PR-2 centrifuge), and the pellets so obtained were stored at $4^{o}C$, and were used for inoculating the bioreactor, and to make the biobeads.

4.2 NUTRIENT MEDIUM

The nutrient medium consisted of magnesium sulfate (10 mg), magnesium chloride (100 mg), ferric chloride (0.5 mg), potassium phosphate (10 mg), and tap water (100 ml for trace elements). The above solution was then diluted to 1000 ml by adding distilled water. The pH was adjusted to 7.2 using mineral acid or base.

4.3 MEASUREMENT OF BIOMASS

Dry biomass content of the sludge was determined by drying five samples of pellets in the oven at 120°C for 24 hours. The pellets used had a concentration of 50 mg dry biomass per gram of pellets.

4.4 IMMOBILIZATION

For a given batch of 50 g pellets a typical procedure for making beads was: Wet bacterial pellets and 0.5% NaCl solution were mixed in a ratio of 2:5 by weight in a blender. Sodium alginate (0.75% w/w) was then added to the mixture with stirring 2 to 3 minutes to obtain a homogeneous cell suspension. With the help of a syringe pump (Sage Instruments, model 351) the homogeneous cell suspension was then extruded as discrete droplets in a slowly stirred solution of 0.1 M $CaCl_2$. On contact with $CaCl_2$, the droplets hardened to form beads about 3 to 3.5 mm in diameter. The beads were then cured in $CaCl_2$ solution for 24 hours at 4°C before use.

4.5 DETERMINATION OF PHENOL OXIDASE ACTIVITY BY A MICROASSAY REACTOR

Phenol oxidase activity was determined by a microassay reactor with a Clark-type dissolved oxygen electrode as shown in Figure 1.

Before the start of each run the reactor was washed successively with ethanol and distilled water and rinsed several times with 0.1 M Na_2HPO_4-NaH_2PO_4 buffer solution (pH 6.0). This buffer solution (1.8 ml) was then added to the reactor and saturated with oxygen by bubbling air through it. The saturation concentration of oxygen from air in water at 35°C was estimated to be 228 nmol/ml. The reactor was then sealed from the top. After equilibrating with strong mixing for ten minutes, 10 ul 100 ppm phenol was injected into the reactor. Finally, 0.1 ml enzyme solution was added in the reactor and the concentration of dissolved oxygen was monitored on the strip chart recorder. Sufficient mixing was continued throughout the run to overcome any mass transfer resistance at the surface of the probe.

4.6 PHENOL BIODEGRADATION STUDIES USING A RECIRCULATION REACTOR

Experimental set-up of the recirculation reactor (batch mode) is shown in Figure 2. The reactor is 11.5 cm in diameter and 25.5 cm long. The reservoir is 6.5 cm in diameter and 20.5 cm long. The total reaction volume is 2 liters. The reaction medium is recirculated between reservoir and packed beads using a centrifugal pump. The linear velocities are maintained high enough to overcome

the solid/liquid film resistance to mass transfer by recirculating the stream at 1 l/min.

Temperature and pH were monitored inside the reactor. Typical experiments were done at room temperature (25°C to 27°C), and the DO level was maintained by sparging air/O_2. An impingement flow Clark-type dissolved oxygen probe monitored the concentration of dissolved oxygen.

In batch recirculation mode the parameter studied was concentration of phenol. The biomass loading in the reactor was selected to be 450 g biobeads.

A good recirculation rate is required to operate under a regime unlimited by solid/liquid mass transfer resistance of the rate limiting compound, and also to maintain physical integrity of the beads.

Phenol concentration was varied from 100 ppm to 1000 ppm, and the DO level (usually from 5 to 8 ppm) was monitored.

During phenol biodegradation, the concentration of phenol was detected by GC (Perkin-Elmer, model 8500) every 15 minutes. A typical removal rate for phenol in the recirculation reactor is shown in Figure 3.

4.7 ISOLATION OF PHENOL OXIDASE

After a 5 day run with beads in the recirculation

reactor, the entire solution was drained out, separated, concentrated and tested. This solution was first filtered through a 0.22 um filter to remove all the bacterial cells and other suspended solids from the protein solution. The procedure of separation was carried out in the presence of ice to decrease the temperature of solution and prevent denaturization of protein and loss of the enzyme activity.

The ultrafiltration (UF) is carried out in a stirred cell with selective barriers for the preparative isolation of differentsizes of molecules. In UF, the sample fluid passes through a porous membrane of well defined permeability. Molecules whose sizes are less than the membrane molecular weight cut-off (MWCO) will pass through the membrane (as permeate) while those whose sizes are greater than the MWCO become concentrated (as retenate).

The filtration was carried out in a stirred cell as shown in the Figure 4. The cell used was 21.6 cm in height and 11 cm in diameter and its capacity was 450 ml. The membrane diameter was 76 mm and the filtration area was 38.5 sq cm. The pressure of up to 70 psi N_2 was used during the operation.

The enzyme solution, after filtration through a 0.22 um filter, was poured in the stirred cell and the operation was carried out with 10 K, 50 K, and 100 K

ultrafiltration disc membranes. Four fractions were obtained:

1) protein cut more than 100 K,
2) protein cut more than 50 K, but less than 100 K,
3) protein cut more than 10 K, but less than 50 K, and
4) protein cut less than 10 K.

An attempt was made to obtain different protein fractions according to molecular sizes. During the operation the permeates were collected in a beaker and kept in ice.

The more than 10 K less than 50 K protein fraction, for which the phenol oxidase activity was greatest was further concentrated by using freeze-dryer (LABCONCO Freeze Dryer 5, model 75050, Labconco Corporation.) under -40°C temperature, 5 u Hg pressure.

4.8 DETERMINATION OF PROTEIN CONCENTRATION

The Lowry procedure has been found to be a reliable and satisfactory method for quantitation of soluble proteins. The procedure is based on Peterson's modification of the micro-Lowry method and utilizes sodium dodecylsulfate, included in the Lowry Reagent, to facilitate the dissolution of relatively insoluble lipoproteins.

This protein assay may be conveniently purchased

from Sigma chemical company, protein assay kit, No. 5656. A test tube was labeled blank and filled with 1.0 ml water. The samples were added to appropriately labeled test tubes, followed by diluting each to 1.0 ml with water, adding 1.0 ml Lowry Reagent Solution to each tube, mixing well and letting stand at room temperature for 20 minutes. With rapid and immediate mixing, 0.5 ml Folin & Ciocalteu's Phenol Reagent Working Solution was added into each tube and color was allowed to develop for 30 minutes. The solutions were then transfered to cuvets and the absorbance measured the samples vs. the blank at a wavelength 600 nm (Spectronic 20D, Milton Roy Company). The readings were completed within 30 minutes.

4.9 CONTROLS

4.9.1 Control Experiment in the Recirculation Reactor

Control runs were done under the same conditions of temperature and air flow (without biomass) to account for removal of phenol by physical processes, mainly stripping.

Alginate beads without any biomass were stirred in a solution of 100 ppm, 250 ppm, 500 ppm, 1000 ppm phenol to account for absorption by alginate gel. No absorption was seen over a period of 24 hours. The volatility rates

with these different concentrations of phenol in the recirculation reactor are shown in Table 1.

4.9.2 Control Experiment in the Microassay Reactor

Control analysis was done under the each condition of temperature, pH, ionic strength, and quality of enzyme (without substrate) to test the enzyme activity. No oxidation reaction took place in the microassay reactor in the absence of enzyme.

CHAPTER V

RESULTS AND DISCUSSION

5.1 EFFECTS OF ENZYME ACTIVITY

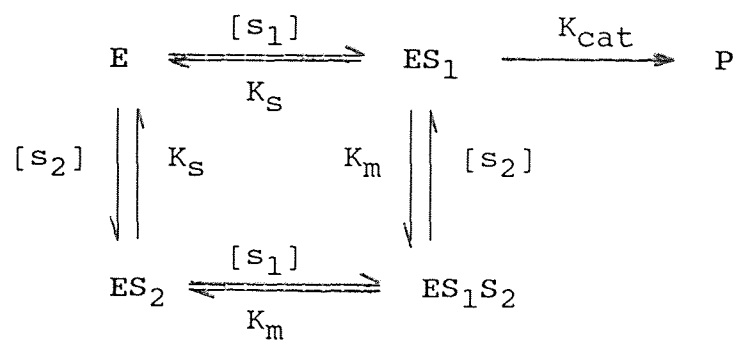
The enzyme solution was obtained from the recirculation reactor as described earlier. The initial rate of the oxygen consumption by different concentrations of protein was used as the measurement of the enzyme activity in the microassay reactor. This enzyme reaction is a bisubstrate enzyme reaction - oxygen and phenol.

The parameters studied were the concentration of phenol, pH, temperature, buffer concentration, different ions, different substrates, etc.

5.1.1 Variation of Phenol Concentration

Increasing the concentration of phenol as a substrate prolongs the induction period of phenol oxidase action [38]. The variation of phenol oxidase activity with phenol substrate is shown in Figure 5. The 100 ppm phenol concentration level is the optimal substrate concentration condition in this enzymatic reaction. Levels of 1000 ppm phenol inhibit phenol oxidase activity. In most normal enzymatic reactions the greater the concentration of the substrate the more rapid the

reaction. Although the phenol oxidase does not obey the ordinary Michaelis-Menten equation, the substrate inhibition reaction is in compliance with Michaelis-Menten kinetics. A second substrate molecule can bind to the enzyme-substrate complex, but the double substrate-enzyme complex reacts more slowly.



$$v = \frac{[E]_o[S]K_{cat}/(1+[S]/K_s)}{[S]+K_m}$$

This is termed noncompetitive inhibition: K_m is unaffected, V_{max} is changed [39].

5.1.2 Variation of pH

The initial rates of oxidation were investigated at several pH buffer solutions of 0.1 M Na_2HPO_4-NaH_2PO_4. The variation of phenol oxidase activity with pH is shown in Figure 6. The pH of about 6.0 is the optimal condition in this reaction.

The pH of the solution usually has a very great effect on the rate of an enzyme reaction. Effects of pH are irreversible if the acidity or basicity becomes too

high, since the tertiary structure of the protein is destroyed. Within a certain pH range the pH can be changed back and forth without any permanent effects ensuring. This behavior can be explained in terms of the ionizations of groups on the protein. There are at least several ionizations of groups which are $-NH_3^+$, -COOH and some function groups as playing an important role at the active center. The comformation of protein is dependent on the ionization of groups. The detailed treatment of pH effects is quite complicated, but the major effect of comformation of the enzyme active center is distributed by changes of ionizing groups, which changes affinity of enzyme with substrate. The ionization of substrate and intermediate effect the affinity, too [40].

Under assumption the enzyme molecule is not denatured within certain range of pH change, the observed rate dependence on the pH change usually reflects the requirement of some essential amino acid residues, in which one of their protonated or deprotonated forms is responsible for the enzyme catalysis.

5.1.3 Variation of Temperature

The variation of phenol oxidase activity with temperature is shown in Figure 7. The temperature at 37°C was the optimal temperature in this enzymatic reaction. Increasing the temperature up to certain limits

accelerated the phenol-phenol oxidase reaction.

In general, the rate of enzymatic oxidation of phenol increase with an increase in temperature. An increase in temperature also shortens the induction period in the oxidation of phenol. However, increasing the temperature reduces the oxygen solubility in the reactive solution.

The total oxygen concentration during a reaction with phenol-oxidase was directly related to the initial rate of the enzyme.

5.1.4 Variation of Buffer Concentration

Increasing the buffer concentration of the buffer solution (Na_2HPO_4-NaH_2PO_4) from 0.001 M to 0.5 M, reveals the variation of phenol oxidase activity as shown in Figure 8. 0.1 M Na_2HPO_4-NaH_2PO_4 buffer is the optimal ionic loading condition in this reaction. The higher concentration of the buffer solution, the stronger the ionic strengh in the solution. The ionic strength affects this enzyme activity probably because the enzyme conformation is changed due to the ionic strength. This causes affinity changes of the enzyme with substrate at the bonding site.

5.1.5 Variation of Ions

One of the characteristics of this phenol-oxidase

is that copper is associated with the activity of the enzyme. This enzymatic is reaction affected by several metal ions as shown in Table 2. Sufficient cupric ions resulted in almost complete activation of the enzyme. Other metal ions (iron, cobalt, nickel, magnesium, manganese, zinc, sodium, nitrate, sulfate, etc.) were ineffective in accelerating enzymatic activity. like silver, mercury and gold , these kinds of metal ions of the perodic table, which are in an adjacent group, compete with copper for sites on the enzyme. When these metals attacked the enzyme an inactive preparation was obtained.

5.1.6 Variation of Other Substrates

The phenol oxidase activity was also tested for other substrates such as 2-chlorophenol. The same kind of analysis method was repeated, the only change was the addition of different substrates. The variation of phenol oxidase activity with 2-chlorophenol, styrene, methylene chloride and beta-hydroxybutyric acid is shown in Table 3. The enzyme activity for 2-chlorophenol, styrene, and methylene chloride substrates were less than that for phenol substrate, and was completely inhibited by beta-hydroxybutyric acid.

5.2 EFFECT OF PROTEIN CONCENTRATION AND ENZYME ACTIVITY FOR DIFFERENT CONCENTRATIONS OF PHENOL IN THE RECIRCULATION REACTOR

The protein solutions from the recirculation reactor after a 5-day run at different phenol spiked concentrations were ultrafiltered and concentrated. The concentration of protein was analyzed with Lowry Reagent method and enzyme activity was determined by microassay reactor. The results are shown in Figure 9 and Figure 10 respectively.

From Figure 9 and Figure 10 it is observed that the suitable spiked concentration of phenol was 500 ppm to release the enzymes from microorganisms. Low concentration of phenol stimulated the microorganisms to release the enzyme whereas high concentration of phenol inhibited the release of enzymes.

5.3 THE DECAYING RATE CONSTANTS AND HALF-LIFE TIMES OF PHENOL OXIDASE ACTIVITY

The enzyme activity was assayed at different temperatures before and after 24 hours. According to the equations: $d(c)/d(t) = -kct$, $\ln(2)=kT_{1/2}$, (k is the rate constant, c is the concentration of the enzyme, t is the time.) the rate contstants and half-life times with different tempertures were obtained. These were shown in

table 4.

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

7.1 CONCLUSIONS

The following are some of the most important conclusions that can be drawn from this study.

1. An effective recirculation reactor system may be used on the laboratory scale to produce phenol oxidase.

2. Phenol oxidase is readily isolated from the reaction solution after the treatment with phenol. The isolation method is suitable for further studies of this enzyme.

3. The effects on enzyme activity for variations of phenol concentration, pH, temperature, buffer solution, different cations and anions, and other substrates were studied. Some optimal conditions were found: [phenol] = 100 ppm; pH = 6.0; temperature = 37°C; ionic strength equivalent to 0.1 M Na_2HPO_4-NaH_2PO_4. The enzyme activity was increased by Cu^{2+}, inhibited by Ag^{+} and Cl^{-}, and unsignificantly changed by Zn^{2+}, Fe^{2+}, NO_3^{-} and SO_4^{2-}; other substrates such as 2-chlorophenol, styrene, and methylene chloride, were also known to be oxidized by this enzyme; the enzyme activity was inhibited by beta-

hydroxybutyric acid as a substrate.

4. The experimental results indicate that high concentrations of phenol inhibit enzyme release from microorganisms. It is necessary to find optimal initial spiking concentration of phenol for effective release of enzyme in the recirculation bioreactor.

5. Phenol oxidase has potential use in studies of large-scale degradation of waste containing phenol and its homologues.

7.2 RECOMMENDATIONS

1. The further purification of phenol oxidase is necessary for studies of characterization.

2. Experiments should be repeated with the same kinds of mixed microbial species to observe any differences that would occur in oxidase properties.

3. Further investigations are needed to determine the kinetic parameters of degradation of phenol and its homologues by phenol oxidase.

4. The enzyme should be further purified and characterized. Any metals such as copper should be analyzed.

TABLE 1

Abiotic Removal of Phenol in the Recirculation Reactor
(Control runs without biomass)

Number	Initial Concentration of Phenol (ppm)	Volatile Rate (ppm/hour)
1.	100	2.2
2.	250	2.3
3.	500	2.5
4.	1000	2.8

TABLE 2

Effect for Phenol Oxidase Activity by some Ions

(at Temperature 35°C, 0.1 M Na_2HPO_4-NaH_2PO_4 Buffer, pH 6.0, 100 ppM Phenol Substrate).

Number	Typical Ions (1×10^{-4} M)	Enzyme Activity (10^{-2} nmol/minute-ug)
1.	Copper	4.563
2.	Zinc	3.221
3.	Iron	3.221
4.	Silver	1,836
5.	Chlorine	1.374
6.	Sulfate	3.098
7.	Nitrate	3.081
8.	Control	3.124

TABLE 3

Effect of Phenol Oxidase on Other Substrates

(at temperature 35°C, 0.1 M Na_2HPO_4-NaH_2PO_4 buffer, pH 6.0, 100 ppm substrate)

Number	Typical Substrates (100 ppm)	Enzyme Activity (10^{-2} nmol/minute-ug)
1.	Phenol	3.124
2.	2-Chlorophenol	2.808
3.	Styrene	2.282
4.	Methylene chloride	1.825
5.	beta-hydroxybutyric acid	0

TABLE 4
The Decaying Rate Constants and Half-life Times of Phenol Oxidase Acftivity

Temperature (^{o}C)	Rate Constant (day^{-1})	Half-life Time (day)
25.0	0.18	3.81
30.0	0.34	2.01
35.0	0.13	5.20
40.0	0.12	5.65
45.0	0.13	5.28

FIG. 1 EXPERIMENTAL SETUP OF MICROASSAY REACTOR

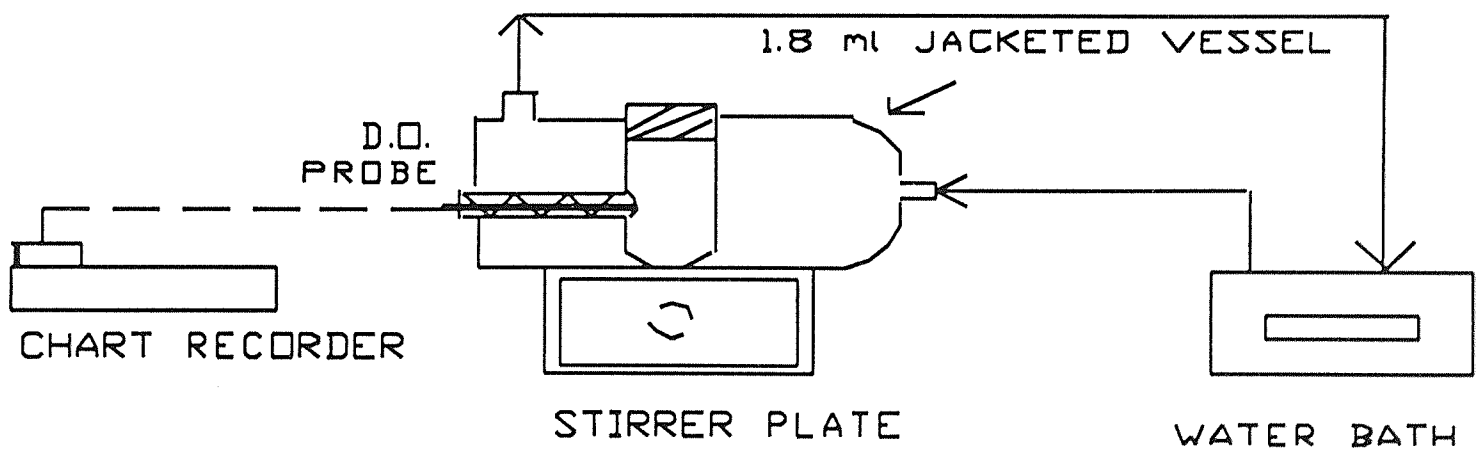


FIG. 2 EXPERIMENTAL SETUP OF CALCIUM ALGINATE BIOBEAD REACTOR (BATCH RECIRCULATION MODE)

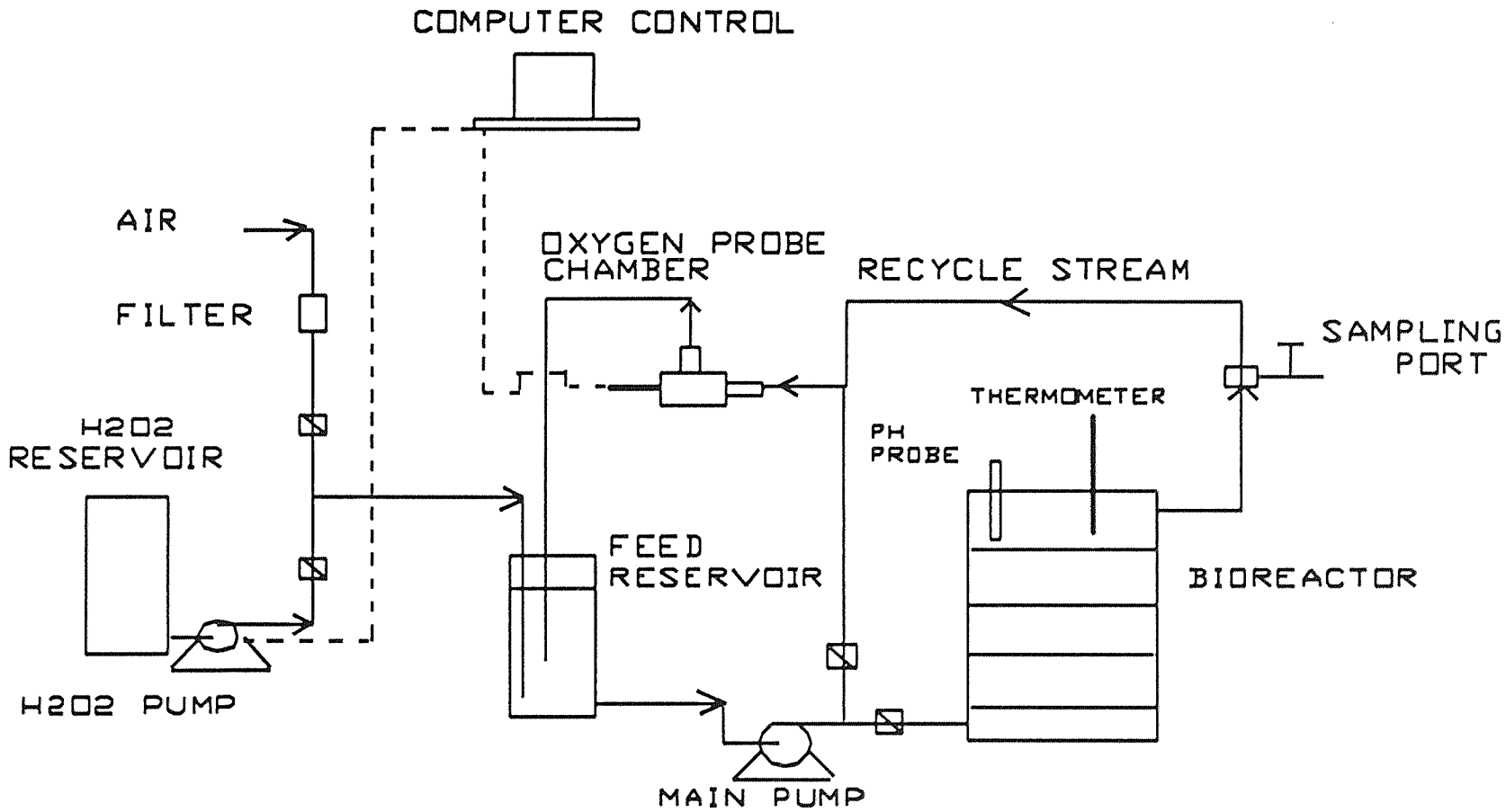


Fig 3 Biodegradation Rate of Phenol in the Recirculation Reactor

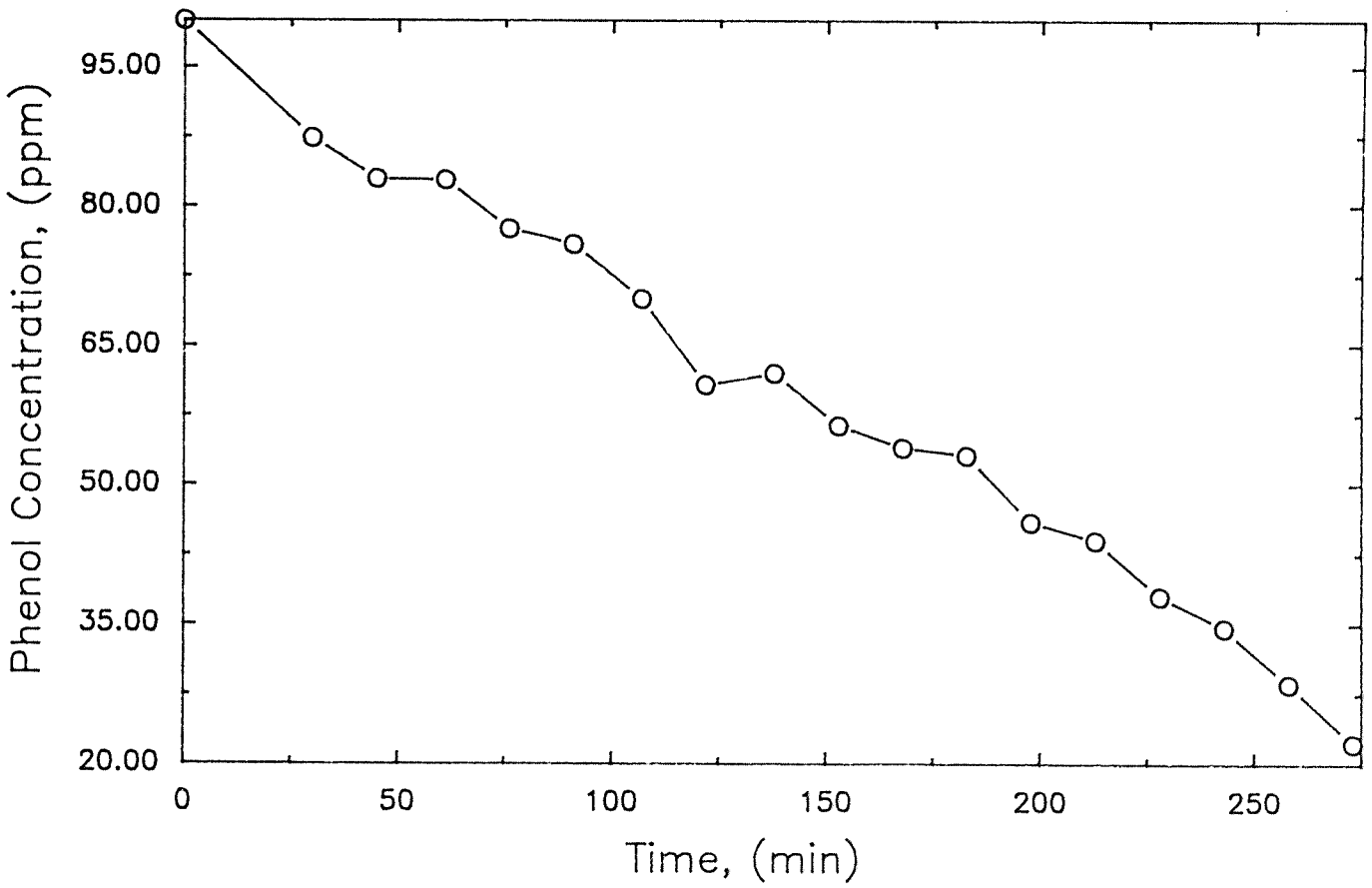


FIG. 4 ULTRAFILTRATION STIRRED CELL

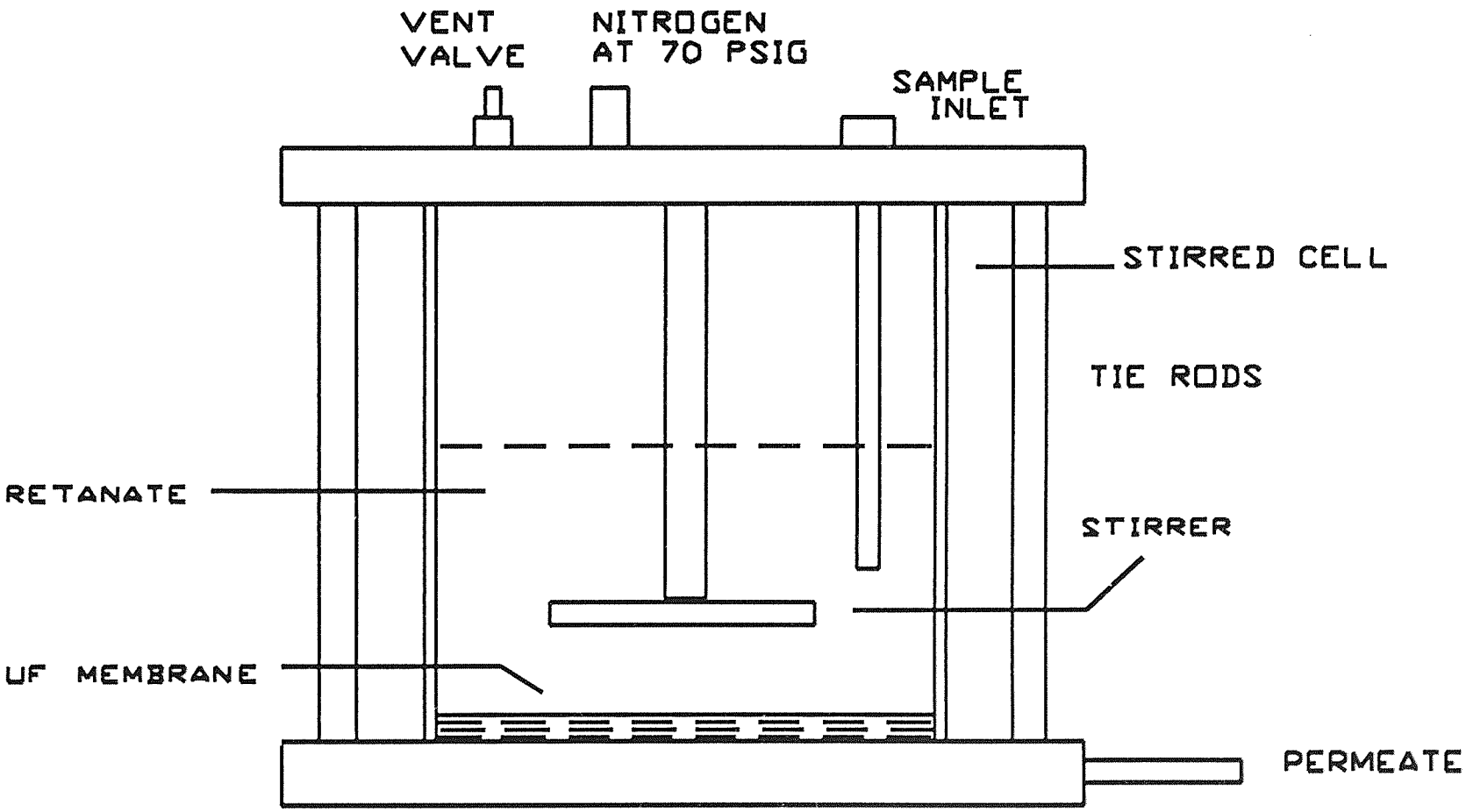


Fig 5 Variation of Phenol Oxidase Activity with Substrate

(at temperature 35 °C, 0.1 M Na_2HPO_4–NaH_2PO_4 buffer, pH 6.0)

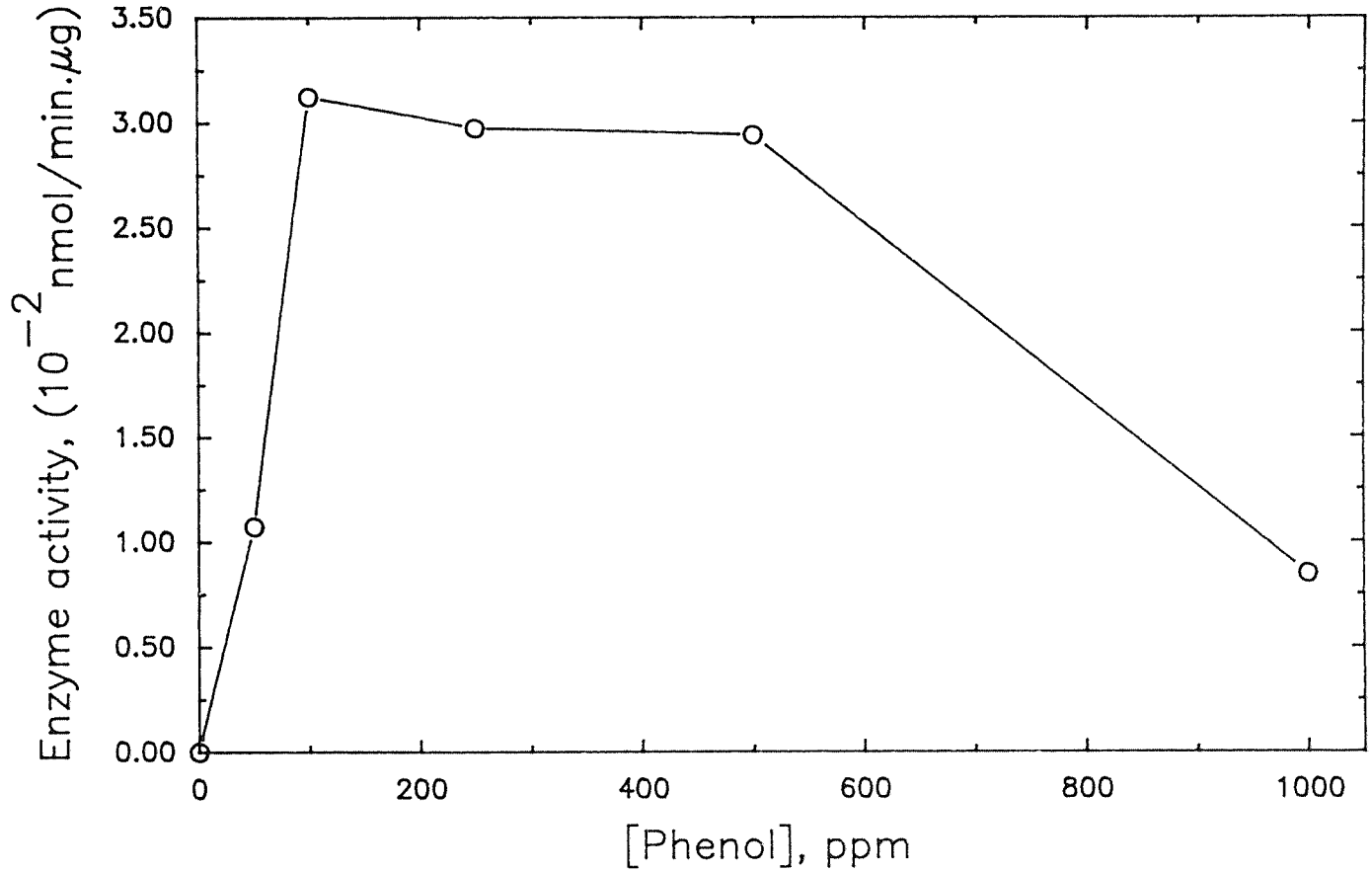


Fig 6 Variation of Phenol Oxidase Activity with pH

(at temperature 35°C, 0.1 M Na_2HPO_4–NaH_2PO_4 buffer, 100 ppm phenol substrate)

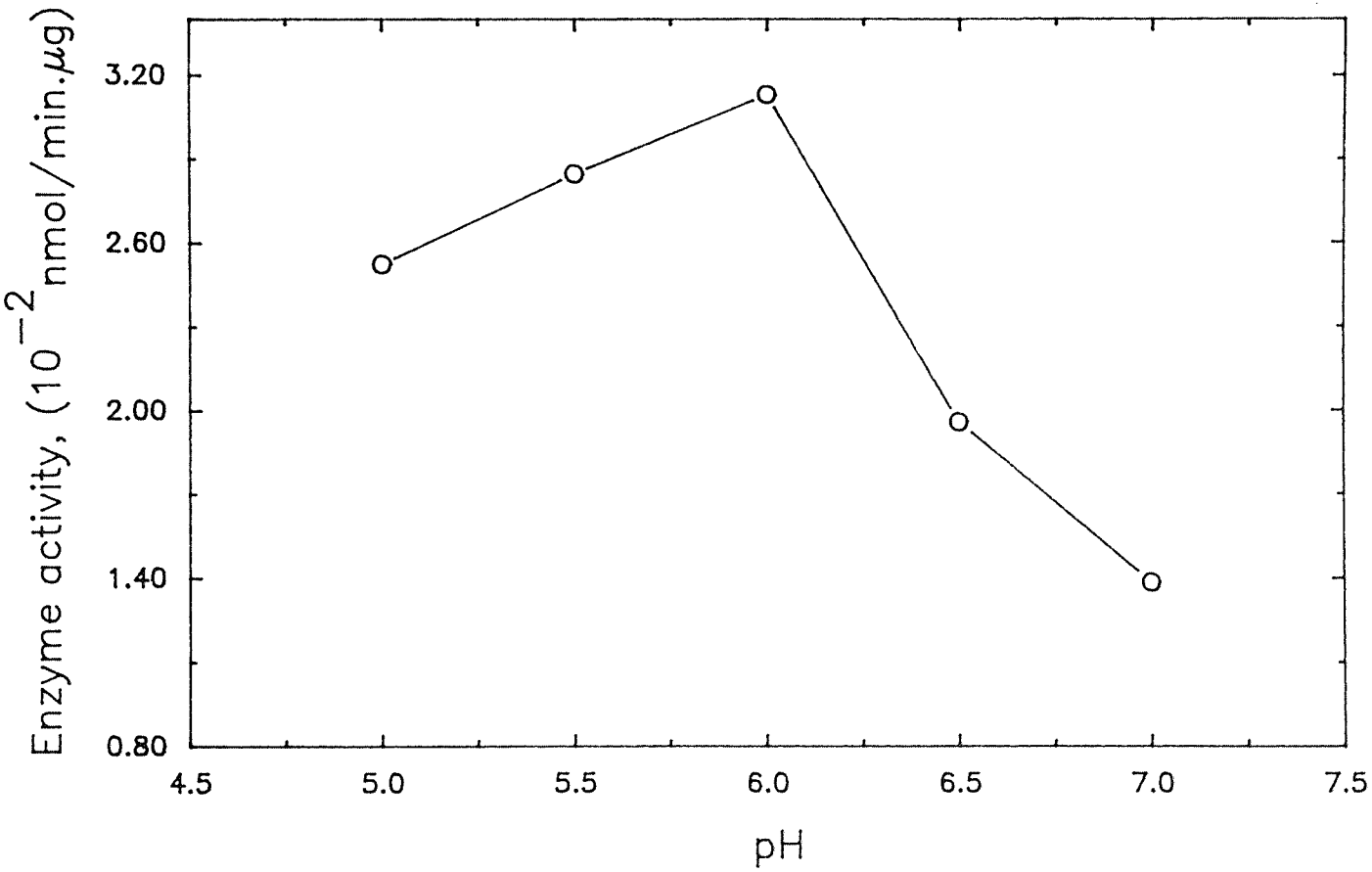


Fig 7 Variation of Phenol Oxidase Activity with Temperature

(at 0.1 M Na_2HPO_4–NaH_2PO_4 buffer, pH 6.0, 100 ppm phenol substrate)

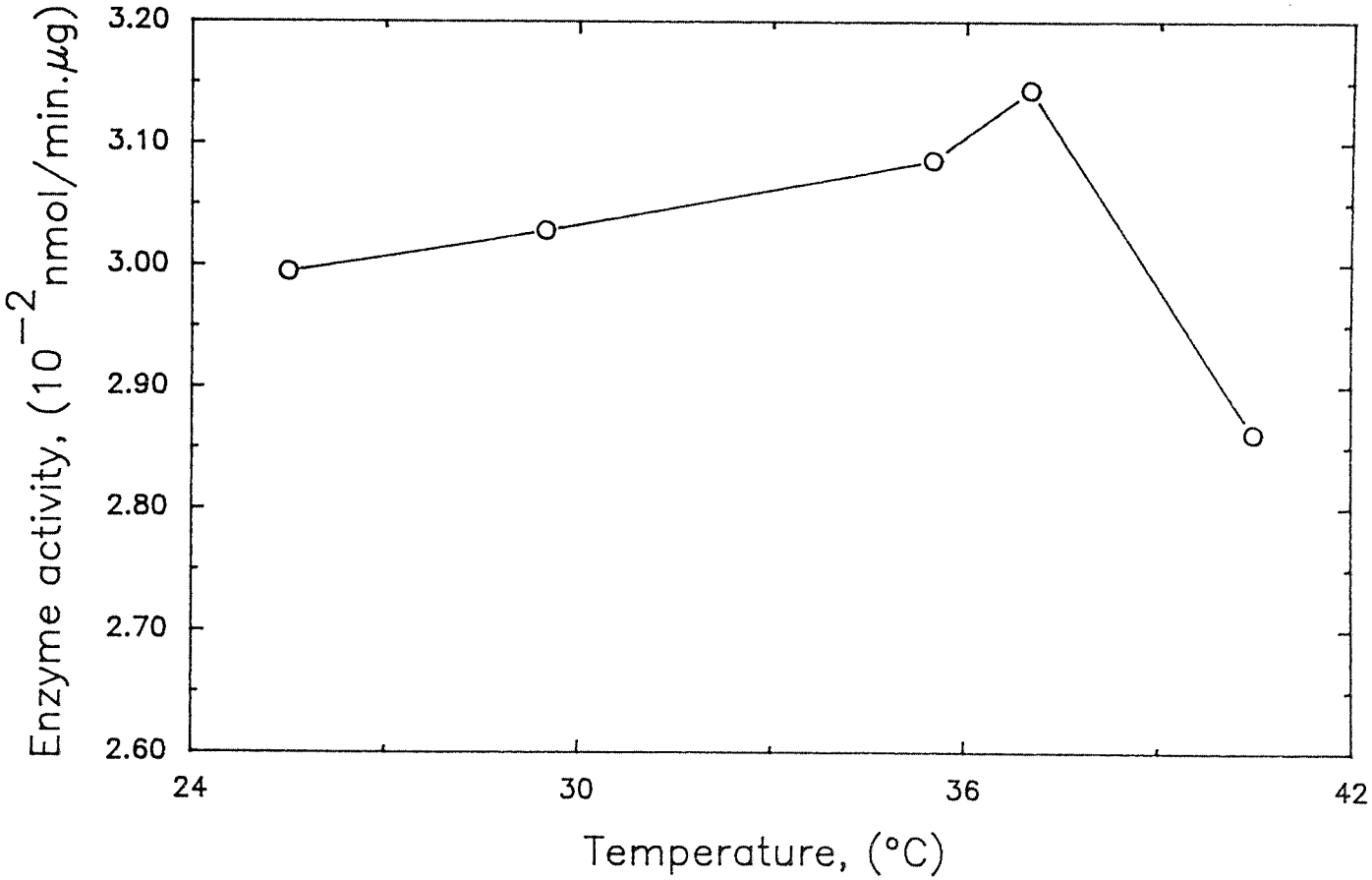


Fig 8 Variation of Phenol Oxidase Activity with Buffer Concentration

(at tremperature 35°C, pH 6.0, 100 ppm phenol substrate)

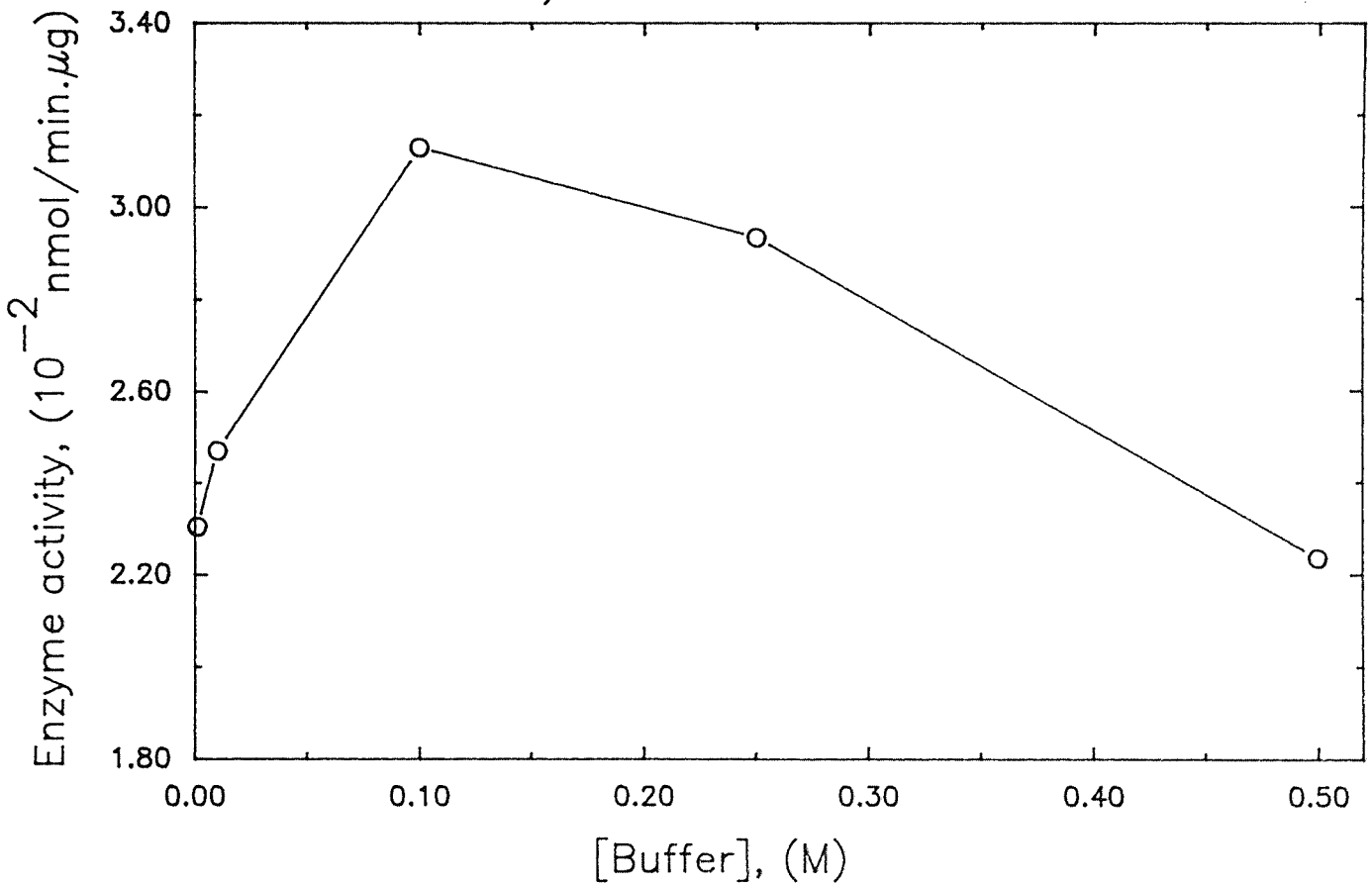


Fig 9 Variation of Protein Concentration with Phenol Concentration in the Recirculation Reactor

(at 600 nm, Lowry Reagent method)

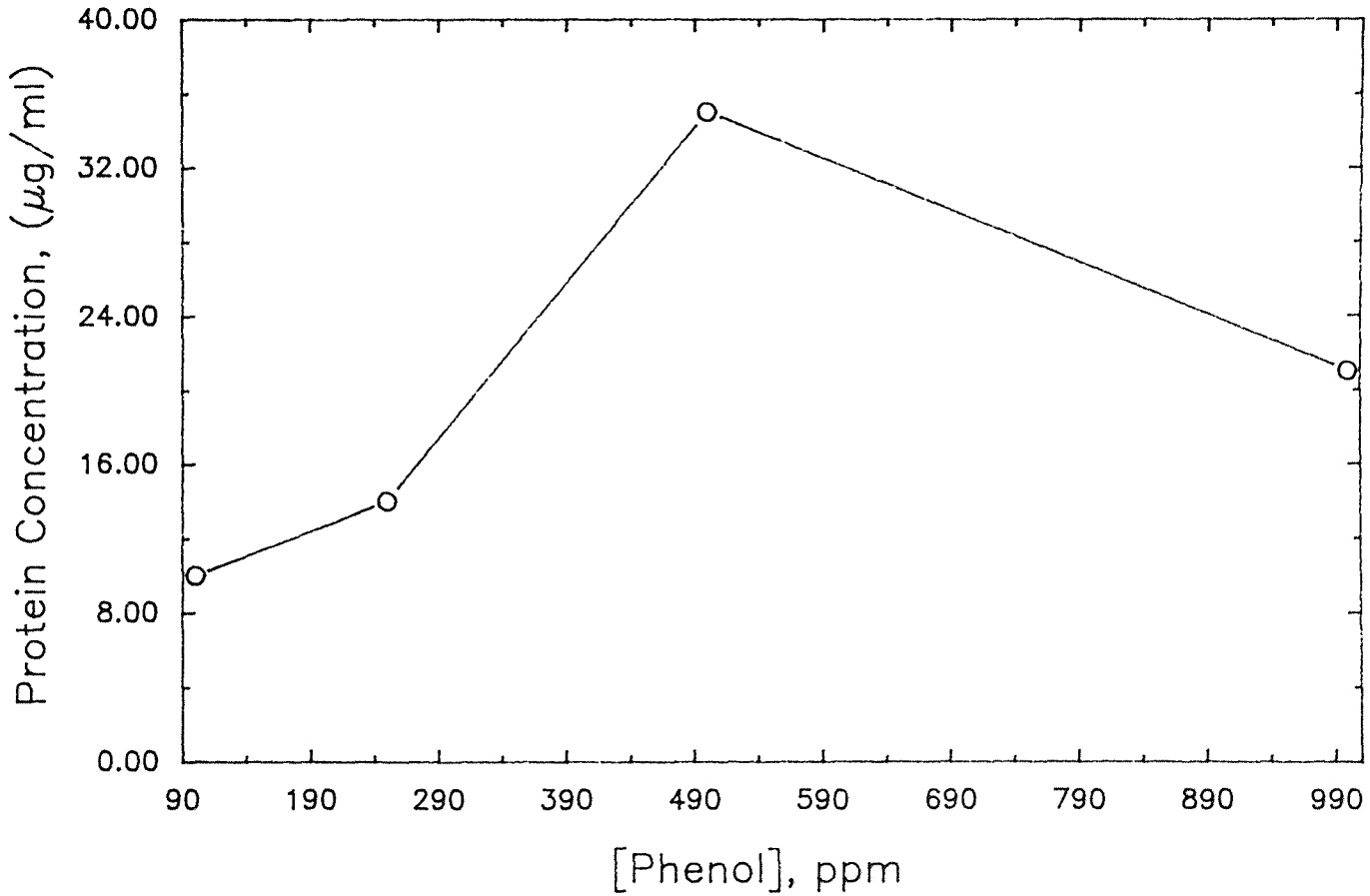
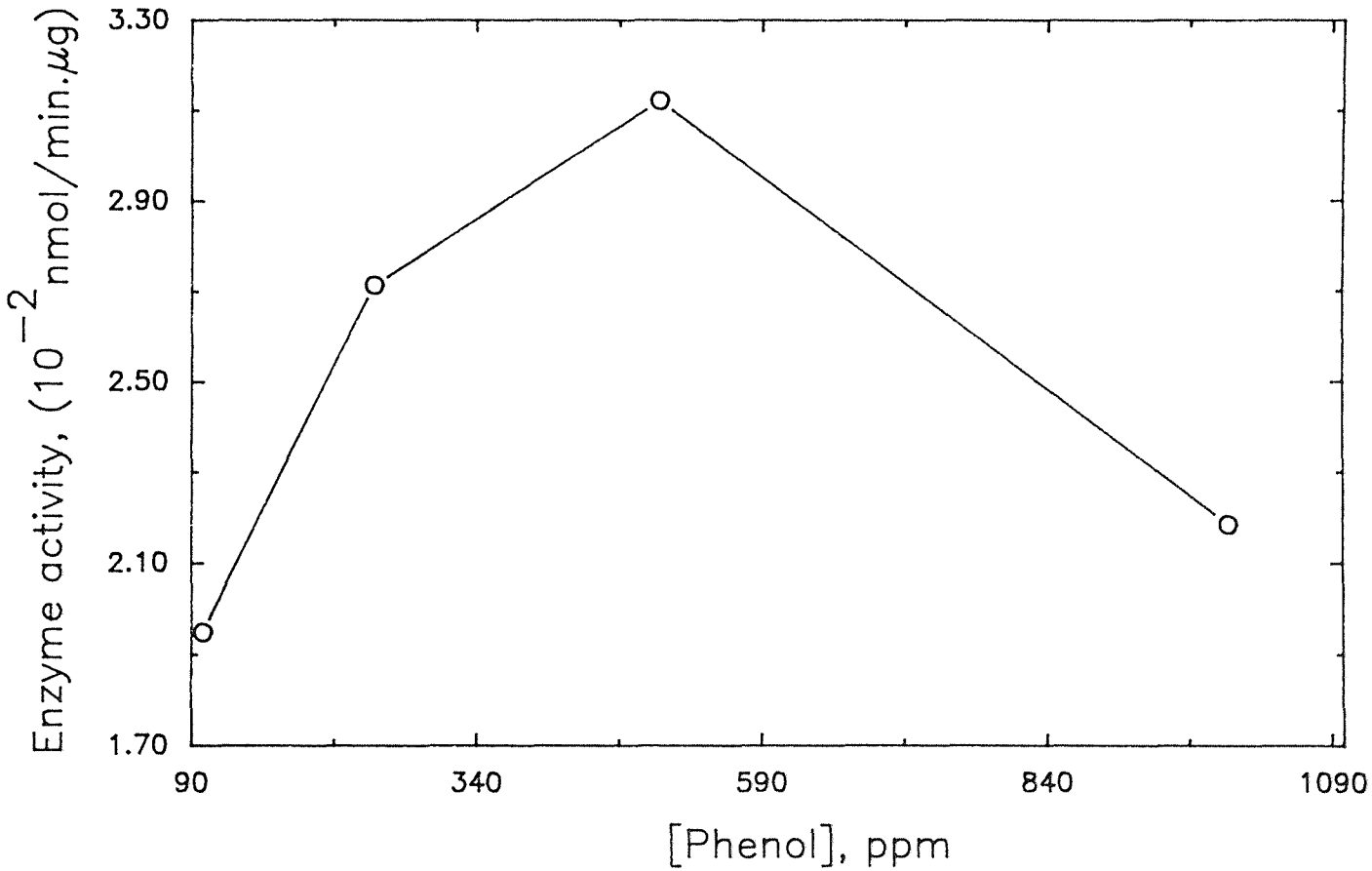


Fig 10 Variation of Phenol Oxidase Activity with Phenol Concentration in the Recirculation Reactor

(at temperature 35 °C, 0.1 M Na_2HPO_4–NaH_2PO_4 buffer, pH 6.0, 100 ppm phenol substrate)



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