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BIODEGRADATION OF POLYMERS AND ISOLATION AND CRUDE

CHARACTERIZATION OF PROTEINS FROM THESE STUDIES

by

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ABSTRACT

Title of Thesis: Biodegradation of polymers and isolation
and crude characterization of proteins
from these studies.

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The performance of immobilized and free microorganisms in the biodegradation of polymers was investigated using a recirculation flow reactor and an air-sparged reactor respectively. The bio-oxidation ability of these microbes towards polyamide emulsion was studied.

Extracellular protein cuts isolated were checked for oxidative activity in a microassay reactor. These fractions were compared from various biodegradation studies.

Microassay tests and monitoring of the oxygen consumption in recirculation flow confirmed substrate dependent oxygen consumption, polyamide being provided as the sole carbon source. Increases in extracellular protein buildup were a positive indication of biodegradation.

The protein cut greater than 500,000 daltons was found to be the most active (highest specific activity) and catalase and oxidase were found to be two of the enzymes in the extracellular protein mix.

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CHAPTER I

INTRODUCTION

The annual production of plastics in the United States has increased from 30 billion lbs to 60 billion lbs over a period of the last ten years [1]. Economic and safe disposal of plastics has been a major solid waste management problem.

The biological breakdown or degradation of both synthetic and natural polymers is becoming increasingly important for a broad spectrum of applications. Biodegradation is the assimilation, breakdown, or consumption of substrates by living organisms. For biomaterials, the biodegradation of solid polymers may be either wanted or unwanted depending on the application for which the polymer is designed.

Sofer et al. [2] and Lewandowski et al. [3] stated that the immobilized bacterial matrix forms an especially convenient research tool when used in recirculation configuration. In the NJIT Biotechnology Laboratory research involves use of enzymes, organelles, of whole cells for developing biochemical and biomedical technology. General research projects carried out have been in the area of hazardous waste decontamination using mixed bacterial cultures.

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Lodaya[4] studied the aerobic biodegradation of benzene using activated sludge immobilized in calcium alginate and also attached to a silica based catalyst support. Biological removal of a mixture of benzene, toluene and xylene (BTX) was also studied. Lakhwala[5] worked on the design of beads for biodegradation of 2-chlorophenol using microorganisms entrapped in alginate gel, while Yang[6] studied the biodegradation of 2-chlorophenol using a recirculation flow reactor. Sanji[7] showed that Aroclor 1242[R], a polychlorinated biphenyl could be biodegraded using a recirculation flow reactor.

A similar approach has been used for biodegradation of polyamide in the present study. This is a part of the recently initiated research co-op project with University of Massachusetts on qualitative and quantitative biodegradation analysis of both commercially and naturally available polymers. The program at NJIT has been divided into three tasks. Task I involves the evaluation and biodegradation studies of known and unknown polymers, intermediates and monomers using the bioreactor system developed at the NJIT Biotechnology Laboratory. Task II involves mass balance evaluation and CO_2 measurement to analyze the biodegradation efficiency and Task III involves the crude characterization and isolation of proteins secreted from polymer biodegradation studies.

For disposable plastics, especially packaging materials, biodegradation in the environment may well be required by law in the future. Unfortunately very little is known about the mode and mechanism of biodegradation of polymers and about the relationship between the chemical and solid state structure of polymers and their degradation by microorganisms.

Previous studies have indicated that many polymers can be biodegraded under aerobic conditions and microorganisms are capable of utilizing polymers as the sole source of carbon [8]. In the past, biological reactors for the treatment of aqueous wastes containing toxic compounds have typically utilized activated sludge in suspended form. With the severity in the problem increasing day by day, new and innovative technologies are now being investigated. Among these is the use of immobilized bioreactors.

In the present work, biodegradation studies were conducted using three different experimental configurations:

1) acclimation study using an air-sparged reactor,

2) shaker flask tests, and

3) oxygen consumption studies using a recirculation mode bioreactor.

Polyamide emulsion was the polymer which was tested and provided as the sole carbon source for experiments with an air-sparged reactor and a recirculation flow reactor. Dimer-based polyester was used as one of the substrates during the shaker flask tests. Phenol and glucose were used along with polyamide for the microassay reactor activity checks of the protein fractions.

1.1 IMMOBILIZATION AND ITS ADVANTAGES

The application of immobilized bacterial cells to biodegradation has been a subject of intensive study in recent years. The technique of immobilization has many advantages over the conventional free cell system in the treatment of aqueous wastes. Washout of biomass is one of the most common problems encountered in present day activated sludge processes. Moreover, the system is very sensitive to varying input conditions. Long residence times are required for the organisms to be acclimated and evolve a population that is compatible with the feed. All of these conditions can be significantly improved using immobilized microorganisms. This system facilitates separation and has a greater degree of operational flexibility, as continuous or semicontinuous processes become practical. Immobilized cells are much more resistant to high concentrations of

toxic chemicals [9]. In addition, the cell density of an immobilized system can be much higher than that of the free cell system, resulting 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.

Numerous methods have been developed for immobilized biocatalyst preparation [9]. There is no universal carrier or immobilization method for all living cells, and each application should be separately tested and optimized. The support material should withstand the substrate, product and reaction conditions and it should be suitable for continuous or repeated use in the desired scale. Moreover, the method should be sufficiently gentle for the living cells. For example, fungal mycelium may simply be dried and grown in a pellet form to be used as a biocatalyst [10]. Microbial cells may be flocculated or aggregated, they may be attached to a suitable carrier by adsorption or ionic bonding or they may be entrapped in a polymer matrix. Among the most used adsorption carriers are activated charcoal, porous ceramics and porous silica supports. Suitable polymers for entrapment of cells include alginate, K-carageenan, polyacrylamide and polyvinyl alcohol.

Attachment to the surface of a solid support offers an advantage because the support is stronger than the gel

matrix. Diffusion of oxygen and substrate is no longer a major problem, as growth is on the surface. The disadvantages are that the microorganisms cannot take shock loadings of toxic compounds because they are not protected, and the problem of washout still remains when working under severe operating conditions of flow.

In the present study, calcium alginate was used for the entrapment of cells.

1.2 REQUIREMENTS OF DISSOLVED OXYGEN

In a system utilizing aerobic microorganisms for the biodegradation of organic compounds and polymers, dissolved oxygen requirements are of great importance. Biodegradation of chlorinated organic compounds such as 2,4 dichlorophenoxyacetic acid requires molecular oxygen as a co-substrate for metabolism [11]. Aerobic microorganisms also utilize oxygen primarily as the terminal electron acceptor for aerobic respiration. In general, bacterial respiration does not appear to be affected above a critical dissolved oxygen concentration. The critical dissolved oxygen concentration has been defined as the concentration at which the respiration rate of cells is one half of the maximum rate. It is generally lower for dispersed cultures than for flocculant cultures [12]. Relatively little is

known about the influence of dissolved oxygen on the microbial degradation of toxic chemicals. In one of the studies, it was found that the half life of biodegradation of nitriloacetic acid in natural water samples increased from 1.3 to 5.8 days as the dissolved oxygen concentration decreased from a saturation level to about 0.3 mg/liter [13]. 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 oxygenase, then the degradative rate will also depend on oxygen concentration.

A disadvantage of immobilization is the increased diffusional resistance of substrates and products through immobilization matrices. But this can be of advantage in case of system exposure to high concentrations of toxic compound. Due to the solubility of oxygen in water and high local cell density, oxygen transfer is often the rate limiting factor in the performance of aerobic immobilized cell systems [14]. Methods that have been used to increase the availability of oxygen to immobilized cells include decreasing the particle size of the immobilization matrix and increasing the oxygen holding capacity of the medium have been developed in the NJIT Biotechnology Lab. Addition

of H_2O_2 was attempted with a view towards increasing the oxygen holding capacity of the medium [14,15].

1.3 ACTIVATED SLUDGE AND BIODEGRADATION

Biological treatment, and the activated sludge process specifically, are used widely for treatment of municipal and industrial wastes. The large variety of microorganisms present in the activated sludge reactor and their interaction may hold the key to the effective treatment and removal of chlorinated compounds. The diversity of microbial flora serves as a good environment for developing cultures that can biodegrade synthetic organics in general and chlorinated organics and plastics specifically.

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. Research in the past has indicated that acclimated cultures capable of utilizing chlorinated organic substrates can be derived from activated sludge, and are effective over a wide range of substrate concentrations [16].

In general, biological treatment systems involve two major competing removal mechanisms: physical removal and

biodegradation [17]. Thus it is necessary to investigate the physical removal mechanism, especially for plastics because the presence of suspended solids in the emulsion becomes critical.

1.4 Isolation and Crude Characterization of Proteins/Enzymes released during Biodegradation

It is believed that during biodegradation, microorganisms release certain enzymes according to the type of substrates which are used. There may be a mixed enzyme system which is prevalent or there may be a single enzyme which attacks the carbon source. The mechanism of action of enzymes is in itself one of the most fascinating fields of scientific investigation being pursued at the present time.

The characteristic property of enzymes is their power of catalyzing certain definite chemical reactions [18]. The activity of the mixture of enzymes depends on many different factors all of which are difficult to take into consideration, in an attempt to characterize the enzymes.

From the mixed enzyme system it is possible to fractionate the solution and obtain different molecular sizes of protein. The most active protein fraction can then

be determined and related essentially to the oxidative biodegradation process.

CHAPTER II

OBJECTIVES

The primary objective of this research was to determine the long-range feasibility of biodegradation of polymers.

The first step has been the development of a procedure for qualitative evaluation of the biodegradation of known polymers. Both free and immobilized microorganisms have been used for these studies.

The feasibility of biodegradation of polyamide was explored using a recirculation flow reactor system employing immobilized microorganisms. The analysis of polyamide was done using a UV-VIS spectrophotometer. The results of this study were compared to those obtained from an air-sparged reactor employing free microorganisms.

An attempt was made to separate the different protein cuts obtained from the enzyme solution after the biodegradation studies. An Ultrafiltration Stirred Cell was used for the above purpose. Protein cuts were checked for activity in a microassay reactor. These cuts were compared with the cuts obtained from other biodegradation studies.

CHAPTER III

LITERATURE SURVEY

Plastics are not inherently biodegradable and scientists have historically sought ways to make them more resistant to many kinds of degradation. Early environmental awareness in the 1960s fostered research to make plastics more degradable. Biodegradation of polymers still remains a relatively new and unexplored field.

Presently, municipal solid waste being placed in landfills consists of nearly 30 percent inorganics and plastics [19] which are not biodegradable and, therefore, will occupy some of the volume for a very long time.

Microorganisms secrete enzymes which can catalyze the biodegradation of plastics. Taylor[20] demonstrated in his experiments that in the presence of certain enzymes, plastics break down to products small enough to be assimilated by the microorganisms for digestion.

Efforts to make plastics biodegradable have pursued two courses; one to make the polymer itself susceptible to enzyme attack, and the other to incorporate biodegradable additives within the polymers. An extensive study and review by Potts[21] reveals that some synthetic polymers support microbial growth.

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PHBV is an acronym for polyhydroxybutyrate-valerate. This is an aliphatic polyester copolymer produced during fermentation of sugars. Holmes[22] stated that this polymer is biodegradable and resembles polypropylene in properties.

The use of starch as a biodegradable filler in plastics was reported by Griffin[23]. Griffin claimed that the addition of an autooxidant would degrade the synthetic polymer if buried. It was reported that following burial, a polyethylene film containing fifteen percent starch decomposes in six months and the one with six percent starch takes three to five years.

Delafield et al. [24] reported that poly-β-hydroxybutyrate (PHB), which is formed as a storage product by many microorganisms, is biodegradable. Winton[25] established the rate of biodegradability of PHB. Brandl et al. [26] and Lageween et al. [27] recently reported the synthesis of novel biodegradable poly-β-hydroxyalkanoates (PHAs) but their biodegradability has yet to be determined.

Macrae et al. [28] and Slepechy et al. [29] have established the physiological role of PHB. The regulation of PHB metabolism and its physiological significance among different species has been studied and well understood. Studies on the effects of intermediates formed during biodegradation have been performed by Nakata[30] and pH was also found to be a factor to be considered.

Several researchers[31-36] have performed studies on biodegradable polyesters and results have been obtained on polyesters as biomaterials. Comeau et al. [37] have reported a method for analysis of some selected polymers. This method was modified and used for the analysis of polyamide.

Westmeier et al. [38] studied the degradation of toxic chemicals such as 4-chlorophenol using immobilized alcaligenes. The technique of using immobilized microorganisms has been recognized as a promising method for treatment of hazardous and toxic wastes.

Ultrafiltration is a membrane separation process which has replaced or complemented some of the traditional processing methods, and has contributed to the making of some new products [39-44].

Lusty et al. [45] showed that some microorganisms like pseudomonas lemognei produce an extracellular depolymerase enzyme system which hydrolyzes PHB to hydroxybutyrate and the dimeric ester of the acid. Delafield et al. [46-47] further studied the degradation of the dimer by an intracellular hydrolase enzyme.

CHAPTER IV

MATERIALS AND EXPERIMENTAL METHODS

4.1 MICROORGANISMS

Activated sludge (mixed microbial population) from the Parsipanny Wastewater Treatment Plant (NJ) was used in this study. The microorganisms were acclimated to 500 ppm of polyamide over a period of 5 days at room temperature, with continuous aeration for the biodegradation run with immobilized beads. Unacclimated pellets were used for the air-sparged reactor studies. The culture was centrifuged at 3000 rpm and $5^{O}C$ to obtain concentrated pellets. These pellets were stored at $4^{O}C$ and used to prepare the beads.

4.2 NUTRIENT MEDIUM

The composition of the defined nutrient medium used in this study was as follows:

Magnesium chloride......................100 mg

Magnesium sulfate.........................10 mg

Ferric Chloride..........................0.5 mg

Potassium phosphate...................... 10 mg

Water..................................100 ml

The above solution was then diluted to 1000 ml by adding distilled water.

4.3 MEASUREMENT OF BIOMASS

A known weight of pellets obtained after centrifugation was dried at 105°C for 24 hours. The dry weight so obtained was expressed as dry biomass per unit weight of pellets. The pellets used had a concentration of 50 mg dry biomass per gram of pellets.

4.4 IMMOBILIZATION

The entrapment of microorganisms in calcium alginate gel was carried out as follows. Distilled water and concentrated pellets (50 mg dry biomass/g of pellet) were taken in a ratio of 5:2 by weight along with sodium chloride (0.5% w/w) in a blender. Sodium alginate (1.5% w/w) was then added slowly to the mixture, with continuous stirring to obtain a homogeneous cell suspension. With the help of a syringe pump, the homogeneous cell suspension was then extruded as discrete droplets in a slowly stirred solution of 0.1M calcium chloride. On contact with calcium chloride, the droplets hardened to form beads about 3-3.5 mm in diameter. Here, $CaCl_2$ acted as a cross-linking agent. The beads were then cured in calcium chloride for 24 hours at 4°C before use.

4.5 POLYMER BIODEGRADATION STUDIES USING AN AIR-SPARGED REACTOR

Acclimation studies on polymers were conducted using an air-sparged configuration reactor configuration as shown in Fig. 2. The objective of this experiment was to determine the viability response of free microorganisms to different concentrations of the polymer and monomer samples.

The reactor used was 5.08 cm in diameter by 20.32 cm long, with a working volume of 300 ml, and contained 30 g of unacclimated pellets. Water saturated air was sparged into the reactor to keep the pellets in suspension and also provide them with oxygen source. A known amount of polyamide emulsion was added periodically into the reactor as the sole carbon source. The emulsion was anionic polyamide resin with molecular weight of 8000 and had 45% solids by weight. The volume of the reactor was made up by adding the nutrient medium whenever necessary. Liquid samples were taken periodically to determine the activity or the rate of oxygen consumption and the protein concentration. Substrate dependent oxygen concentration was checked using a microassay reactor and it was also used for monitoring the consumption of dissolved oxygen. pH in the reactor was monitored throughout the run for 239 days. 11.85 Grams of polyamide were added during the entire run.

4.6 OXYGEN CONSUMPTION STUDIES IN A MICROASSAY REACTOR

The experimental setup of a microassay reactor is as shown in Fig. 1. A microassay reactor is a small jacketed vessel of 1.8 ml capacity, with provision for a Clark-type dissolved oxygen probe. This reactor is a valuable tool in enzyme kinetic studies due to the reproducibility and accuracy of the data required. It is also economical in the sense that it requires very small amounts of reagents. In this work, the reactor has proved suitable for easy yet explicit measurements of dissolved oxygen, the parameter to be monitored.

Before the start of each run the reactor was washed successively with methanol and sterile distilled water. It was then rinsed several times with sterile nutrient medium.

An aliquot (1.6 ml) was taken periodically from the air-sparged reactor during the span of the run and its activity was checked in the microassay reactor. After adding the sample in the microassay reactor it was saturated with oxygen by bubbling water saturated air through it. The saturation concentration of oxygen from air in water is about 220 nmoles/ml at room temperature. After saturating with oxygen, the concentration was monitored by the oxygen probe and recorded on a chart recorder. One

hundred ul of polyamide sample were added after obtaining the initial oxygen consumption to observe the change in activity and the substrate dependent oxygen consumption. Control runs were done by adding glucose and glycine as substrates.

4.7 BASKET EXPERIMENTS

A wire mesh basket containing 4.9 g of polyamide beads was dipped into the air-sparged reactor to observe the disappearance of the polymer in the reactor. The length of the basket was 5 cm and its diameter was 3 cm. The basket was taken out periodically to observe visually the physical disappearance and it was weighed on each occasion. During the span of the basket experiment no polyamide emulsion was added in the reactor, the polymer being the sole source of carbon.

A similar kind of basket experiment was also done in a preliminary run with the recirculation flow reactor. The basket was divided into two compartments, one was filled with alginate beads and the other contained polyamide beads. The objective of this particular study was to observe visually the attack of microbes on the beads.

4.8 SHAKER FLASK TESTS (PARTICLE COUNT)

Shaker flask tests were performed to check the protein buildup, count particle size, and determine the physical integrity and viability of using immobilized beads for polymer biodegradation studies. Two sets of experiments were conducted using 5 g of immobilized bacterial beads of NJIT (mixed culture) and U.Mass biomass (ps. oleovorans and r. rubrum). Calcium alginate was used for microbe immobilization. Control runs were performed in both cases using alginate beads. 50 Ppm of polyamide emulsion and dimer based polyester were added to the flasks containing beads and the experiment was conducted in an Environ Shaker at a temperature of 35^OC and a speed of 164 rpm. Initial and periodic samples were taken and analyzed for protein content and particle size of both supernatant and the beads. The particle size counts were done using a coulter multisizer.

4.9 BIODEGRADATION OF POLYAMIDE IN RECIRCULATION REACTOR

4.9.1 EXPERIMENTAL PROCEDURE AND SETUP

The scale-up from the microassay reactor to recirculation reactor was 1000 times (0.05 g wet beads in microassay reactor to 50 g wet beads in recirculation reactor). The experimental setup of the reactor was as

shown in Fig. 3. The reactor was 2.5" in diameter and 8" in length. The reservoir was 4.5" in diameter and 10" in length. The total reaction volume was 2 liters. The reaction medium was recirculated between substrate reservoir and packed reactor using a centrifugal pump. Typical recirculation flow-rate used was 300 ml/min.

An impingment flow Clark-type dissolved oxygen probe monitored the concentration of dissolved oxygen. Filtered air was sparged into the reservoir at the rate of 1.5 liter/min. The reactor contained a thermometer and a pH probe (Orion Cat no: 91-04). An on-line flow meter regulated the flow rate of the recycle stream. Samples were taken periodically from the reactor for analysis.

Two runs were performed using the recirculation mode. One was a control without using any beads. This was done to check the physical disappearance in the reactor system such as particles sticking to the tubes and to the sides of the reactor. The second run was done using immobilized bacterial beads. A starting concentration of 90 ppm of polyamide was used. Spikes of 200 and 100 ppm were given to the system on day 1 and day 8 in both the runs. The total duration of the runs was 25 days each.

4.9.2 METHOD OF ANALYSIS

Two ml of the sample from the reservoir were dissolved in equal parts by volume of isopropanol. The mixture was stirred well and an analysis was performed on a Perkin Elmer Lambda 38 UV-VIS spectrophotometer at a wavelength of 208 nm. A control was done on the isopropanol extract with no polymer which gave no peak at 208 nm.

4.10 SEPARATION OF DIFFERENT CUTS OF PROTEINS USING ULTRAFILTRATION STIRRED CELL

After the end of the run with the beads in the recirculation reactor, the entire solution was drained out and tested for enzymes. This solution was first filtered through a 0.45 um filter to remove all the bacterial cells and other suspended solids from the enzyme solution. The separation was carried out in the presence of ice to decrease the temperature of the solution and restore the activity of the enzymes. All of the protein cuts obtained were stored in a freezer.

The separations were carried out in a stirred cell using the ultrafiltration(UF) technique. Ultrafiltration is an exacting form of filtration. It uses selective barriers for the preparative isolation of molecular and macromolecular substances. In UF, the sample fluid is passed through a porous membrane of well defined permeability. Molecules whose sizes are less than the

membrane molecular weight cutoff (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 Fig. 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. A pressure of up to 70 psig was used during the operation. Stirred cells are used primarily to increase membrane life and throughput by efficient tangential fluid movement. Dewatering and concentrating proteins and enzymes are typical applications of ultrafiltration membranes.

Tangential flow is achieved with a teflon coated magnetic stirring bar. The stirring bar is mounted from the top cap and set for proper membrane clearance so there is no chance for the stirring bar to rip the membrane and also to minimize protein denaturation. Process fluids come in contact with non-metal surfaces only. A clear plastic reservoir allows for fluid level to be visually monitored. The cell is supplied with preset safety relief valves.

The enzyme solution, after filtration through 0.45 um filter, was poured in the stirred cell and operation was started beginning with 500 K ultrafiltration disc membranes. Predetermined volumes of the solution were used and two fractions were obtained:

1) Protein Cut > 500 K

2) Protein Cut < 500 K but > 50 K.

The whole procedure was repeated using 50 K, 20 K and 10 K cuts and an attempt was made to obtain different protein fractions according to molecular sizes. Filtration with 50 K filter was very slow and it was difficult to obtain further protein fractions. During the operation the permeates were collected in a beaker and kept in ice.

4.11 Activity Check of Proteins using a Microassay Reactor

These fractions obtained were then checked for activity and concentration of proteins. The protein concentration was determined using the standard Lowry procedure based on colorimetric analysis. 1.8 Ml of the protein sample was taken in the reactor and checked for rates of oxygen consumption as described earlier in section 4.6. The run was continued till the oxygen concentration almost reached zero; then it was resaturated using oxygen resaturated air. Resaturation was done twice and the run was continued for 5-8 hours to have rates of oxygen consumption at the same oxygen concentration over a period of time.

A similar kind of run was repeated; the only change was the addition of different substrates. The substrates

used in this study were phenol, glucose and hydrogen peroxide.

The protein separation procedure and the activity checks were also done for other studies such as biodegradation of phenol, benzene etc., and the cuts obtained from these studies were compared to those obtained from polymer biodegradation.

CHAPTER V

RESULTS AND DISCUSSION

5.1 AIR SPARGED REACTOR STUDIES

Free biomass is active in varying concentrations of polymer which is added during the entire run. The air-sparged reactor study lasted for 239 days. During this run, 11.85 g of polyamide emulsion was added as the sole carbon source. An analytical method based on suspended solids was attempted in order to quantify the biodegradation taking place. Samples were taken periodically from the reactor and dried in an oven to take the dry biomass weight. The results were not very consistent from this analytical set-up. Cell counts were also done and an increase in particle counts was observed along with a decrease in diameter, which may be due to the break down of the bigger size particles into smaller ones during the course of biodegradation.

5.1.1 VARIATION OF pH AND WEIGHT OF BASKET WITH TIME

Fig. 5 shows the variation of pH during the entire run. The operating regime for free cell experiments was found to be between 5 and 8; the average pH was around 6.

The polyamide basket was immersed into the reactor for 73 days. During this period, the weight of the basket

decreased from 4.94 g to 0.77 g which was indicative of oxidative attack (Fig. 6).

In the basket run with the recirculation reactor, the polyamide beads turned yellowish-brown and diminished in size while the alginate beads in the other compartments were unaffected, again indicative of oxidative attack.

5.1.2 VARIATION OF OXYGEN UPTAKE WITH TIME

Twenty spikes of varying concentrations were studied over the entire run (Fig. 8). Microassay activity checks confirmed substrate dependent oxygen consumption, as the biooxidation rates went up after the addition of polyamide into the reactor (Fig. 7). Controls were done using other substrates such as glucose and phenol to obtain the background rates of oxygen consumption. The rate increased from 0.286 nmoles/min to 0.392 nmoles/min with polyamide as the substrate while for glucose and phenol it increased to 0.532 and 0.570 nmoles/min respectively, after addition. The increase in oxygen consumption was not as fast for polyamide as it was when phenol or glucose was used as the substrate.

5.2 SHAKER FLASK TESTS (PARTICLE COUNT)

Shaker flask test results indicated that there were marked increases in protein values of three to five times

the initial protein value, Supernatant and bead particle counts indicated a decrease of 35% in particle count of substrate for both ps. oleovorans and NJIT biomass beads, with polyamide as substrate. In the case of dimer based polyester as the substrate, 70 and 38% decreases in particle counts was observed for NJIT biomass and ps. oleovorans respectively. r. rubrum did not react to both the substrates as the experiments were conducted under aerobic conditions and r. rubrum is known to perform the task better under anaerobic conditions.

Control runs were performed only with alginate beads along with different biomass runs and differences in particle counts in all cases were accounted for with respect to the values obtained from control runs, thereby accounting for physical removal of solids.

From the above results, the conclusion drawn was that immobilized bacterial beads could be employed for biodegradation experiments indicating that enzymes are possibly responsible for oxidative attack of the substrate.

5.3 IMMOBILIZED CELL EXPERIMENTS IN RECIRCULATION FLOW REACTOR

A control run without beads was performed on the physical removal of polyamide emulsion. 290 Parts per

million of polyamide emulsion was added before day 8 for stabilization of the system. Most of it was removed by means of adhesion to the surface of the system during the first and second spike. This includes the tubing, the reservoir and the reactor. Fig. 9 shows the decrease in concentration of polyamide with time after the third spike. The concentration decreases more rapidly during the run with the beads due to biodegradation as compared to the control without beads.

The variation of pH during the run with the beads is as shown in Fig. 10; the average pH was found to be 8.2. The protein concentration was low as compared to other biodegradation studies with phenol, benzene etc. However, it increased after every spike indicating that enzymes were released extracellularly and were responsible for initial oxidative attack.

5.4 CHARACTERIZATION AND ISOLATION OF PROTEINS FROM POLYAMIDE BIODEGRADATION STUDIES

At the end of the above mentioned run, the solution was drained and different cuts of proteins were obtained using the ultrafiltration stirred cell. The protein cut greater than 500 K was found to have the highest specific activity. All the studies to characterize the property of

the enzymes were done using this cut. The protein concentration for this cut was 30 ug/ml and the maximum rate of oxygen consumption was 2 nmoles/min-ml. Specific activity, which is the ratio of maximum rate of oxygen consumption to the protein concentration, was calculated to be 0.0666 nmoles/min-ug protein (Table 1).

Fig. 11 shows the variation of enzyme activity or rate of oxygen consumption of the cut > 500 K with time. The rates increased initially with time and then decreased after about 2 hours of reaction when low oxygen concentration became a factor. The rates increased over a large range of oxygen concentrations but at lower oxygen concentrations went down or levelled off (Fig. 12).

Fig. 13 shows the variation of relative rates of oxygen consumption with time at a fixed oxygen concentration. It can be seen that the rates decrease with time at the same oxygen concentration. The rates are lower for oxygen concentration of 147 nmoles as compared to the rates at 180 nmoles. The protein solution was resaturated twice in order to obtain the activity at the same oxygen concentration. Half lives were calculated for both the curves.

The oxidizing ability of the enzyme was checked by addition of substrates to the protein cut while checking

its activity. Fig. 14 shows that the rates increased after addition of glucose to the cut, giving a positive indication of the oxidizing ability of the enzyme. Fig. 15 shows the substrate dependent oxygen consumption with the addition of phenol. The rates were slower as compared to the glucose addition. Thus the enzyme showed the ability to oxidize two different substrates, and there may be a general oxidase enzyme system involved.

A H_2O_2 assay on this protein showed that the oxygen concentration rises after addition of H_2O_2 in the microassay reactor: this is due to the dissociation of hydrogen peroxide. Therefore, catalase may be one of the exoenzymes responsible for biodegradation of polyamide.

The other cut obtained from this run was the protein cut between 50 K and 500 K which was not as active as the cut > 500 K. Therefore, the enzymes responsible for biodegradation are those with molecular weight > 500 K. The cuts smaller than 50 K could not be obtained because of the extremely slow rate of filtration, indicating that most of the proteins are of size greater than 500 K. The observation here was in contrast with the protein cuts obtained from biodegradation of phenol, benzene etc., where cuts between 10 K and 50 K daltons had the highest specific activity in the range of 0.1 nmoles/ug-min.(Table 2).

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

These studies showed that microorganisms immobilized in calcium alginate gel can be used for biodegradation of certain polymers such as polyamide. Free microorganisms also showed stability and feasibility for polymer biodegradation.

Studies with the free biomass showed that the microorganisms were stable for 239 days and over 11.85 g of polyamide emulsion was added as the sole carbon source. The results obtained from the above experiment indicated that the microbial population was viable when subjected to varying concentrations of polyamide. A positive indication of biodegradation was achieved with increase in activity. The weight of the polyamide basket inserted decreased from 4.94 g to 0.77 g, indicative of an oxidative attack.

In the recirculation reactor studies employing immobilized microorganisms, the rate of polyamide consumption was found to be much faster when compared to the control without beads. However, due to the aggregated clusters of polyamide particles in the system after addition of the emulsion, physical removal was seen. The

two compartment basket experiment was indicative of oxidative attack on polyamide beads as compared to alginate beads. Free microorganisms may be better for solid polymer biodegradation. Polymer biodegradation is much slower than biodegradation of other compounds such as phenol and benzene. This may be attributed to its large molecular weight and stable cross-linking structure.

Out of the different fractions of proteins obtained from polymer biodegradation studies, the cut greater than 500,000 daltons was found to be the most active, and addition of substrates such as glucose and phenol showed substrate dependent oxygen consumption. This indicates that oxidase may be one of the enzymes responsible for biodegradation. An H_2O_2 assay showed that catalase is present in the system. The presence of a mixed enzyme system may be responsible for the biodegradation.

6.2 Recommendations

In the present work, preliminary experiments were conducted to assess the feasibility of the biodegradation of polymers using free and immobilized microorganisms.

Further studies should be conducted to find out the end products of polymer biodegradation. If biodegradation is complete, the end products are carbon dioxide and water.

It is known that CO_2 is released as one of the end products during the biodegradation process. A study can be conducted to achieve a stoichiometric material balance by measuring the amount of CO_2 released and performing periodic sample analysis for checking concentration of substrate.

The polymer particles should be ground in order to obtain the desired particle size. Sonication can be performed on samples to obtain a homogeneous emulsion which would facilitate studies with a recirculation flow reactor employing immobilized microorganisms.

Other parameters such as temperature, pH, concentration of dissolved oxygen and particle size of polymers, which may have significant influence on the activity of the microorganisms, can also be studied. A more detailed study on characteristics of enzymes is required in order to determine which enzymes are responsible for biodegradation.

Additives such as corn-starch can be added to the polymer matrix to facilitate the biodegradation process. Varying starch content will be added to the polymer emulsions and then tested for biodegradation in the future projects.

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Table 1: Maximum activity and protein concentration of
fractions from polyamide biodegradation.

Description of protein fractions	Activity nmoles/min/ml	Protein Concentration ug/ml	Specific Activity nmoles/min/ug
> 500 K	2.051	30.00	0.067
> 50 K and < 500 K	0.051	21.65	0.002
> 20 K and < 50 K	–	–	–
> 10 K and < 20 K	–	–	–

Table 2: Maximum activity and protein concentration
of fractions from phenol and benzene
biodegradation.

Description of protein fractions	Activity nmoles/min/ml	Protein Concentration ug/ml	Specific Activity nmoles/min/ug
500 K	0.185	15.26	0.012
> 50 K and < 500 K	0.543	39.24	0.014
> 20 K and < 50 K	2.036	19.32	0.105
> 10 K and < 20 K	1.400	19.64	0.071
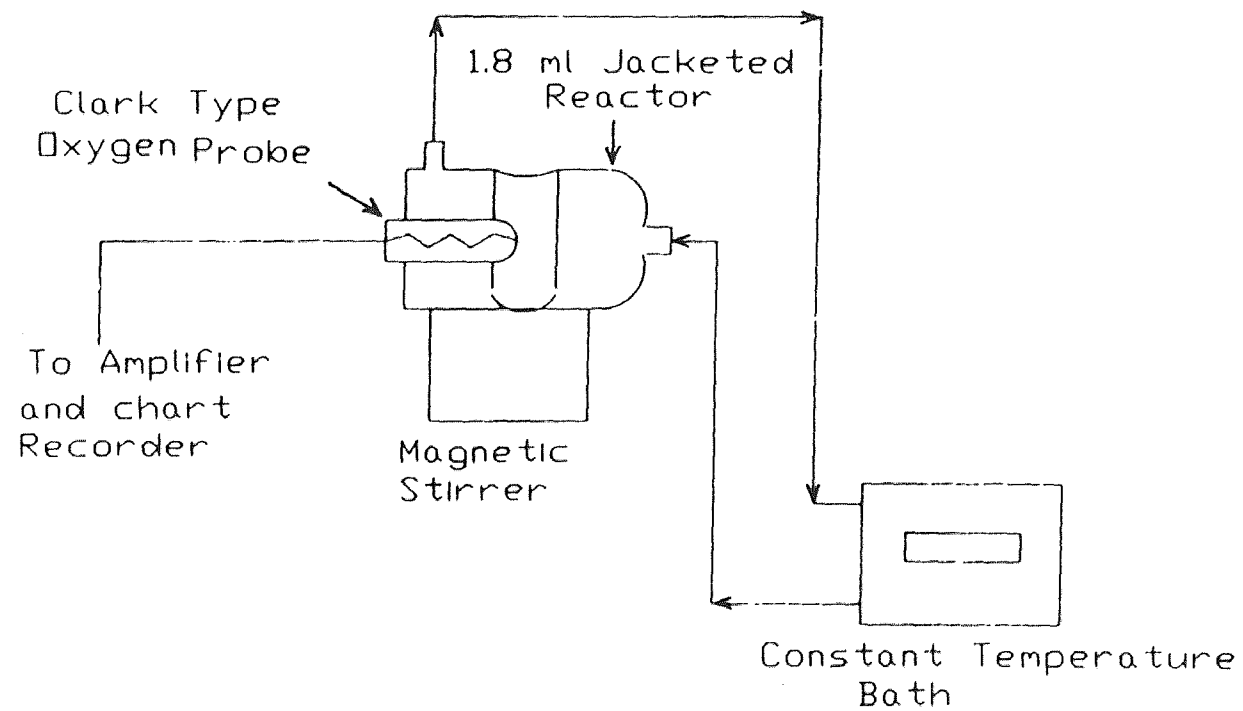
Fig. 1 Microassay Reactor

Fig.2 Air-sparged Reactor

Flow-meter

Filter

Water
Saturated
Air In

Reactor

Diffuser

Temperature
Controller

Constant
Temperature
Bath
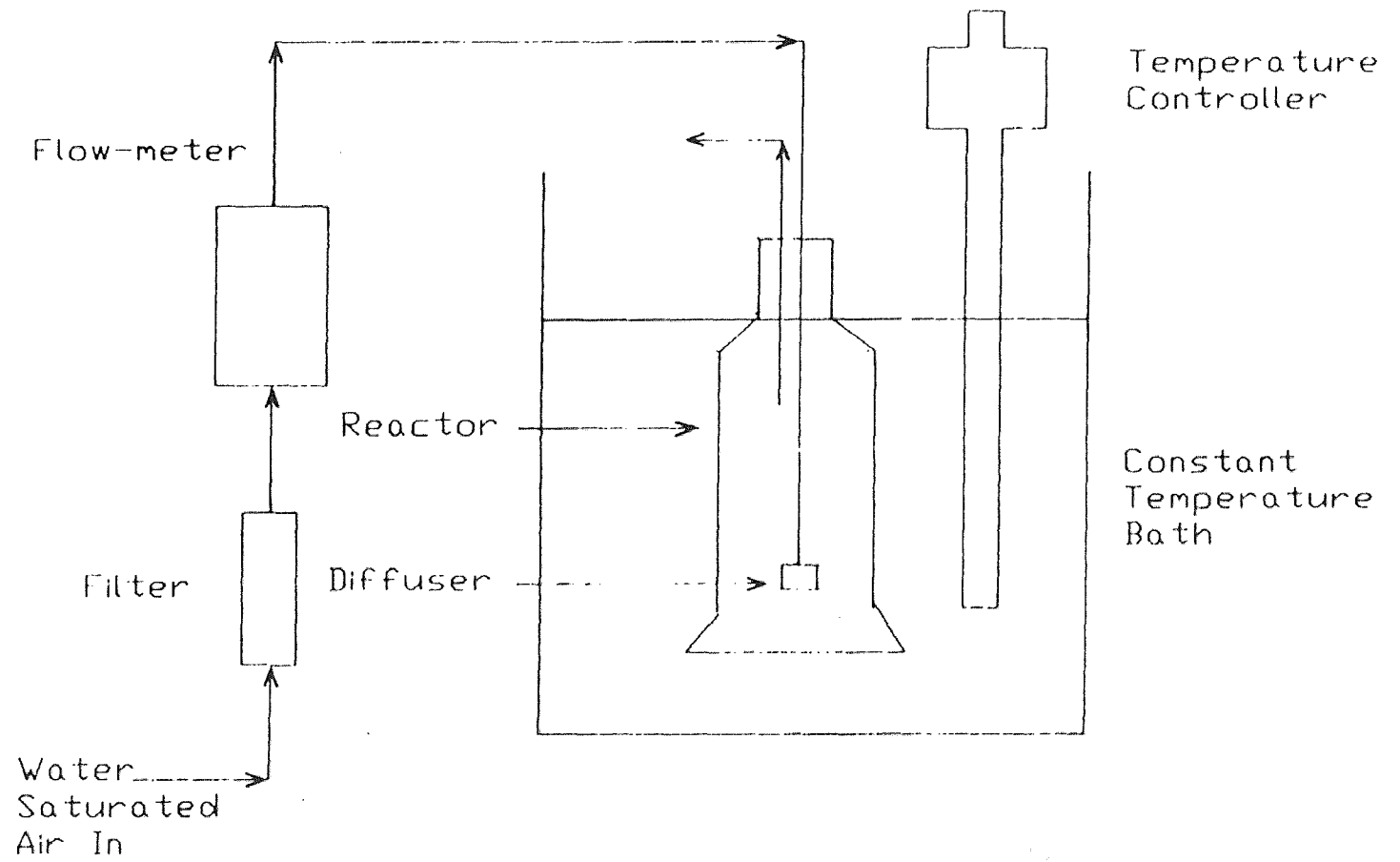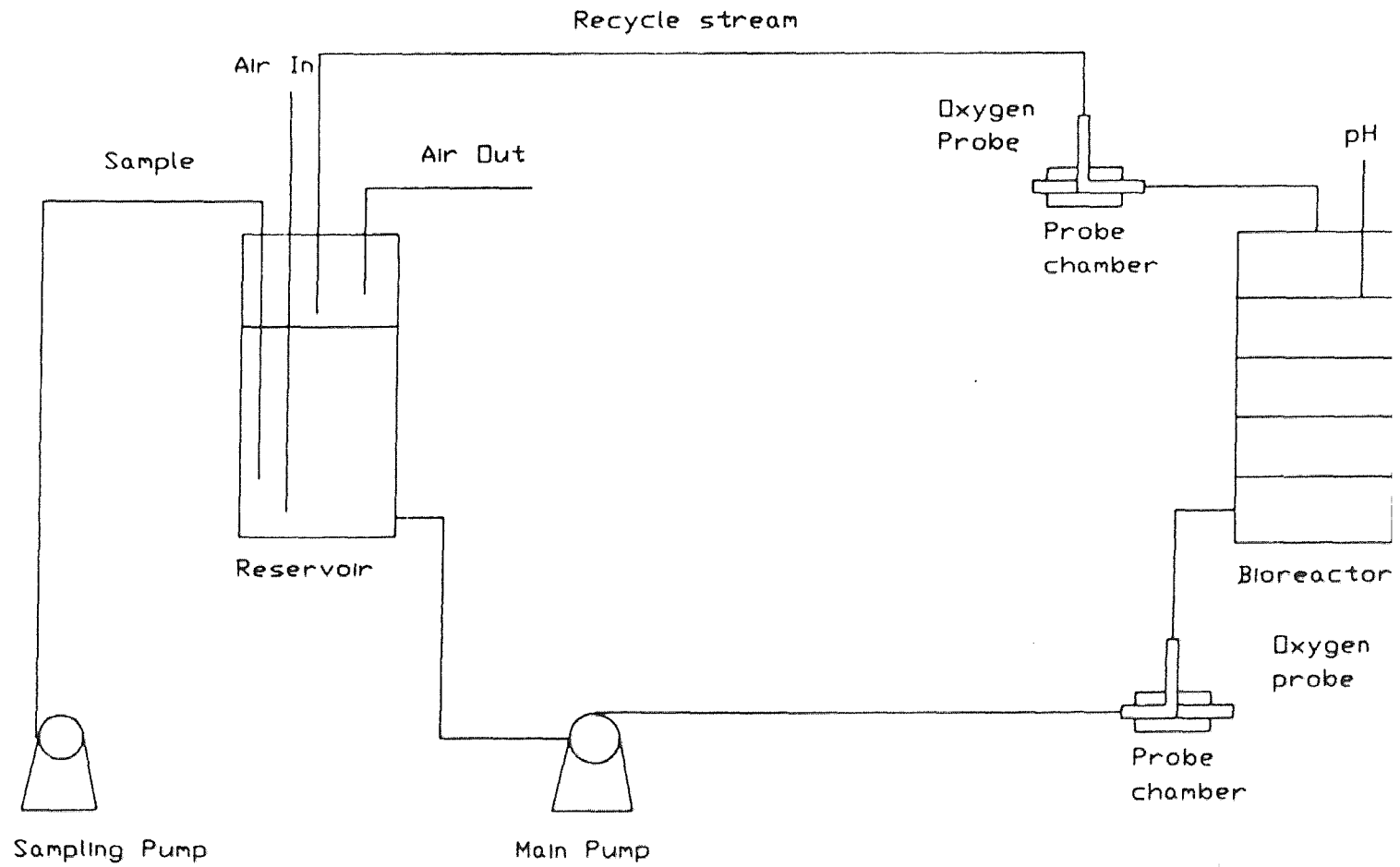
Fig. 3 Experimental Setup of Recirculation Reactor.
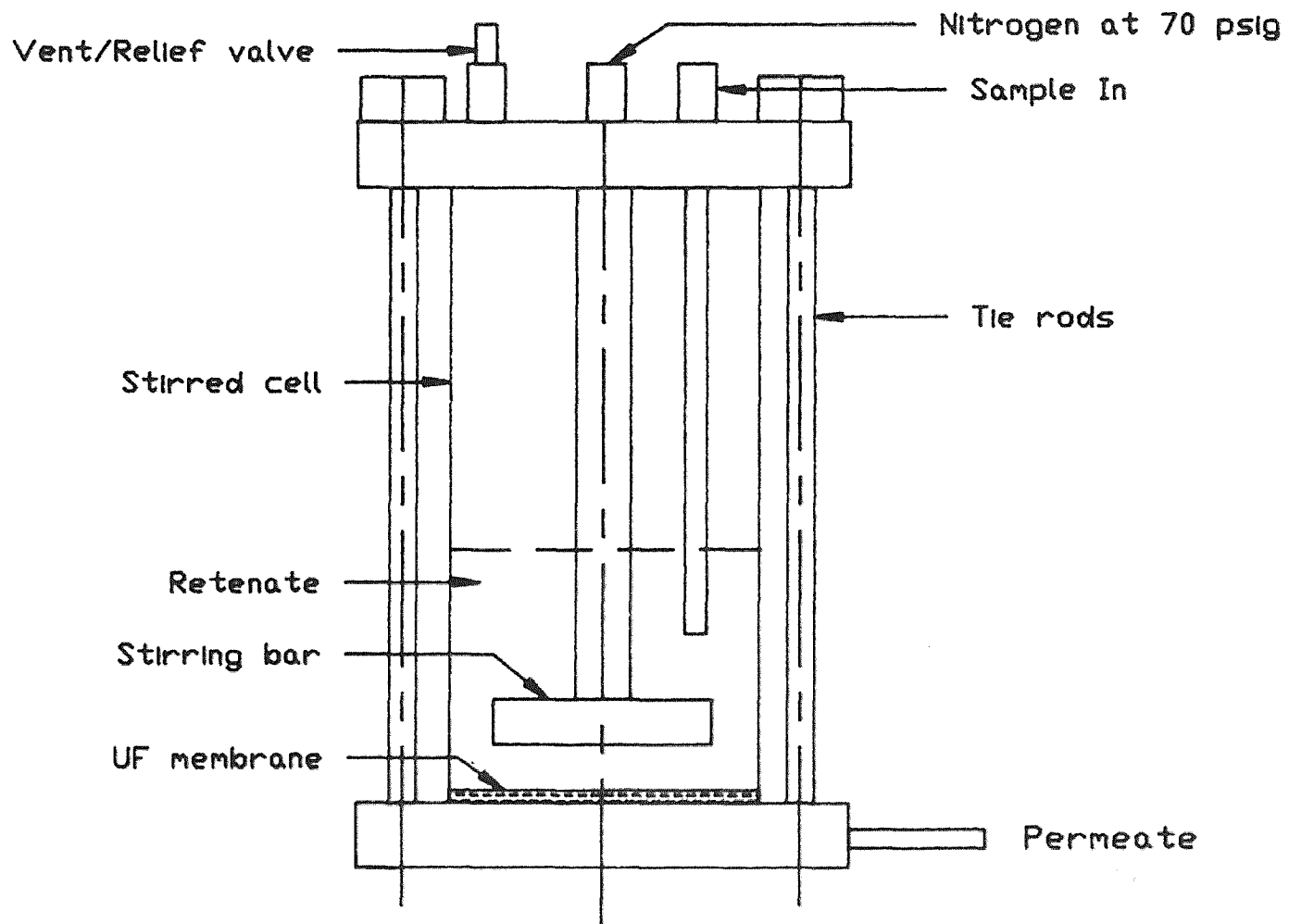
Fig. 4 Ultrafiltration Stirred Cell

Fig. 5 Air—Sparged Reactor:
pH vs Day.
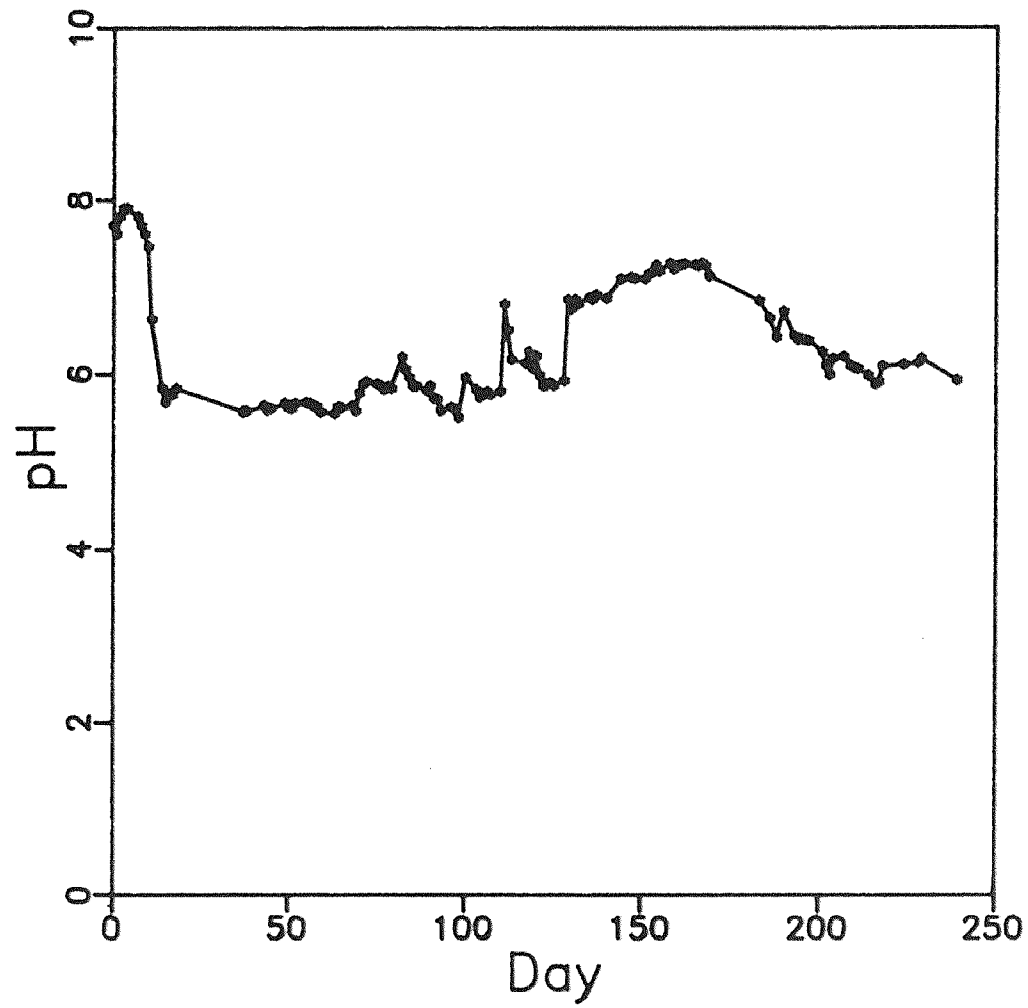
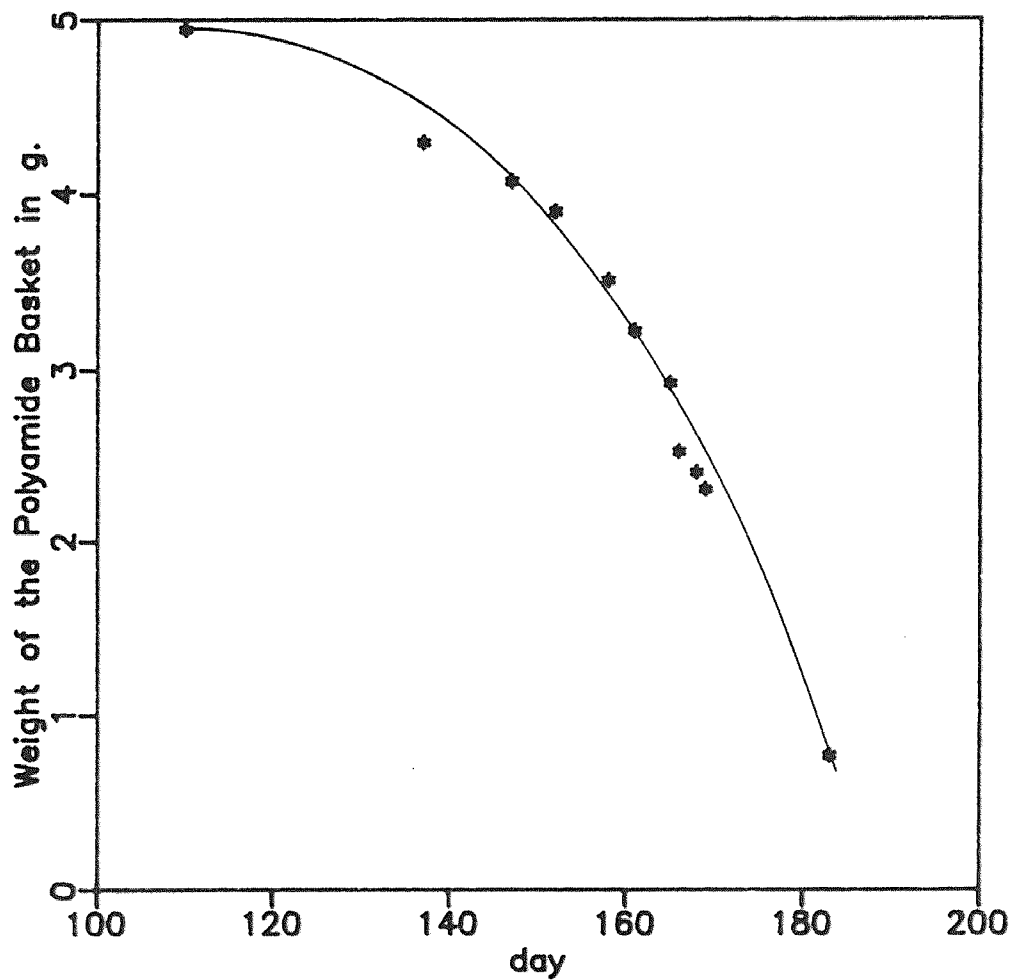Fig. 6 Air-Sparged Reactor: Basket Experiment
Weight vs Day.

Fig. 7 Air–Sparged Reactor:
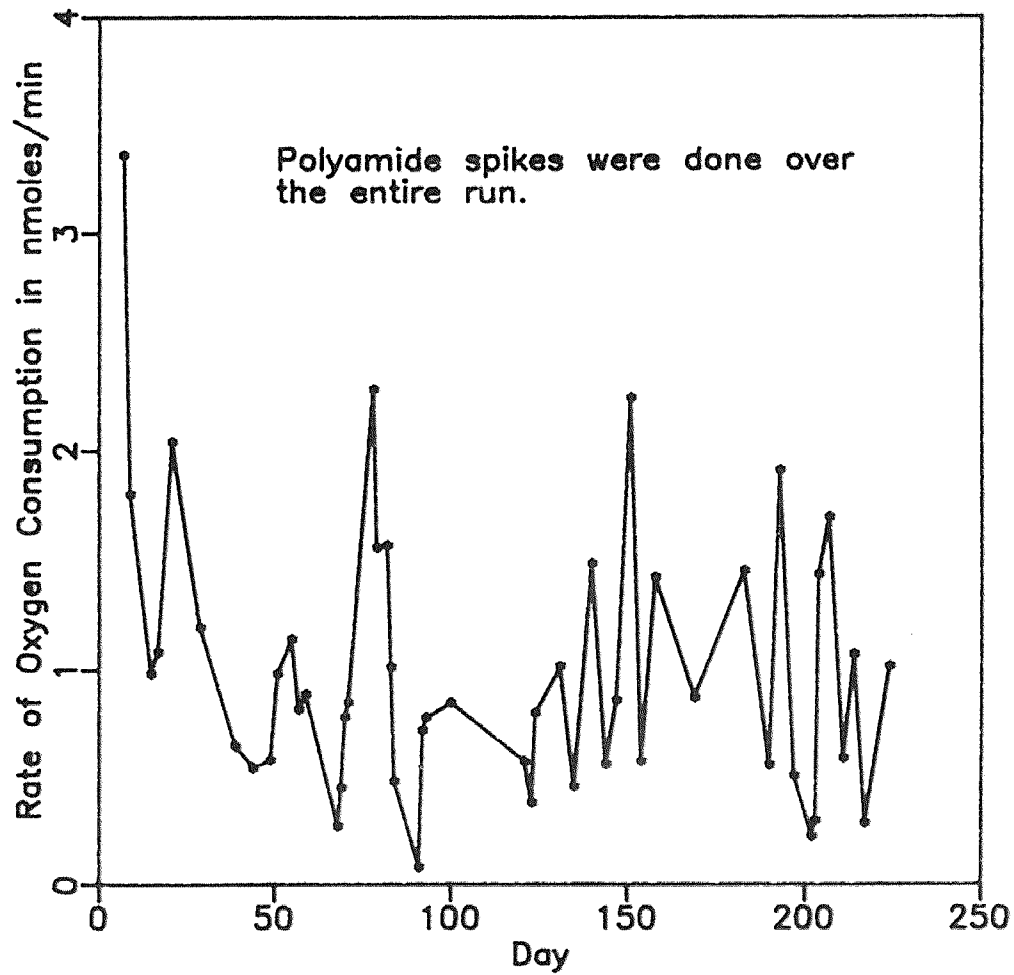## Rate of Oxygen Consumption vs. Day

Polyamide spikes were done over the entire run.

Fig. 8 Air—Sparged Reactor: Cumulative Polyamide Spikes vs Day
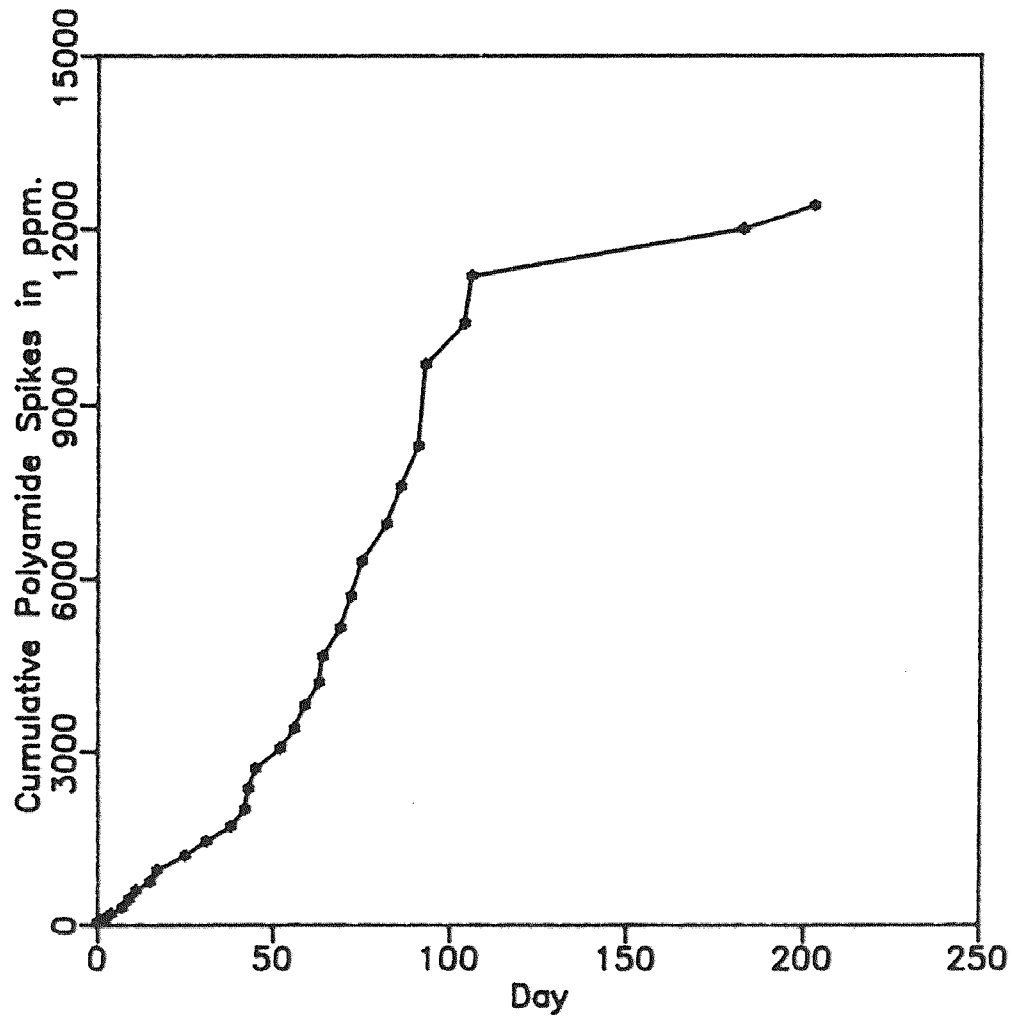
Fig. 9 Recirculation Reactor Runs
Concentration of Polyamide vs Day.

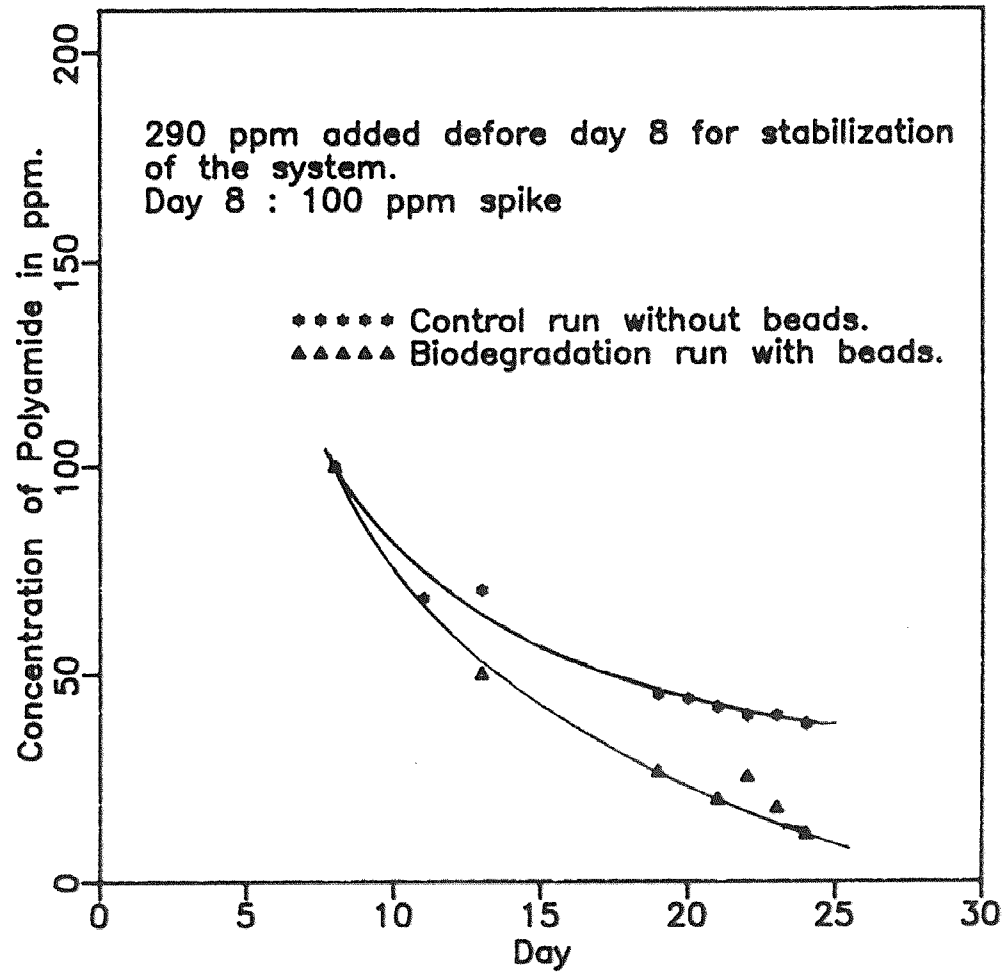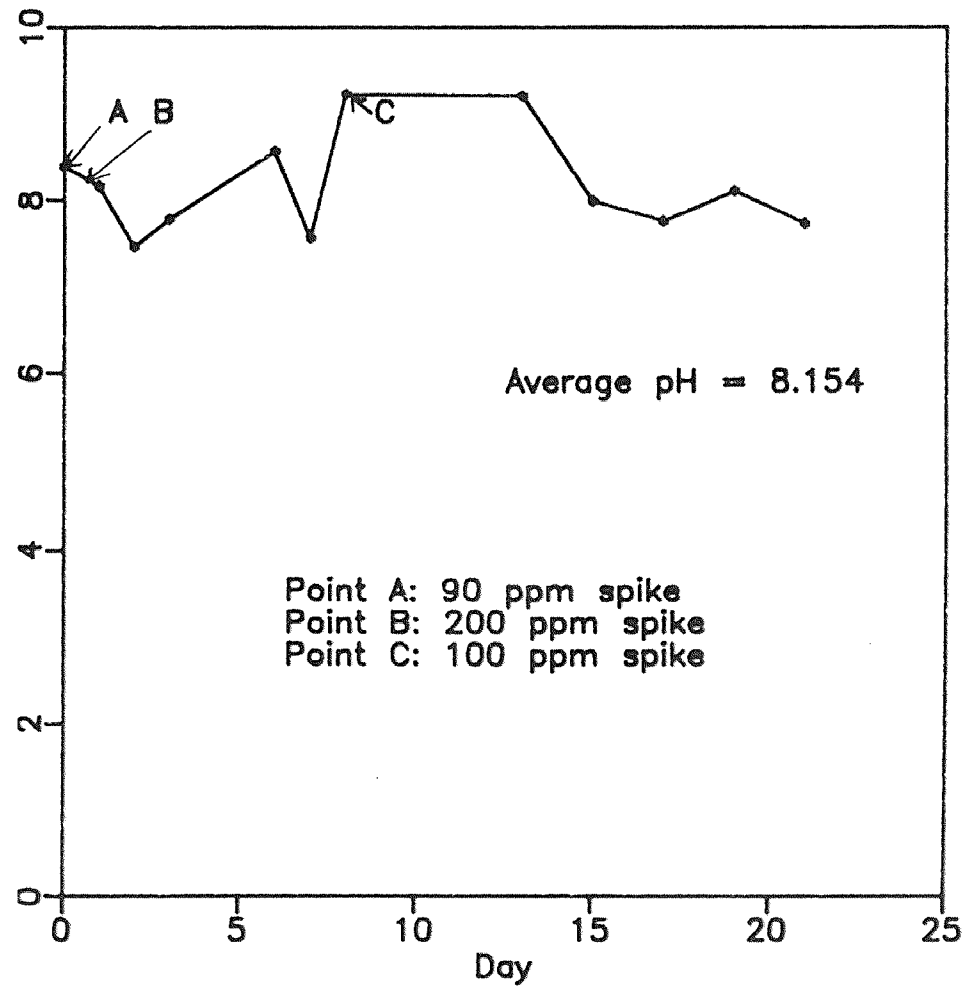Fig. 10 Polyamide reactor with Beads: pH vs Day.

Average pH = 8.154

Point A: 90 ppm spike
Point B: 200 ppm spike
Point C: 100 ppm spike

Fig.11 Variation of Enzyme Activity with time.

Rate of Oxygen Consumption in nmoles/min

Protein cut > 500 K was most active. Rate of oxygen consumption increases with time initially and then the rates level off at the end where oxygen conc. becomes a factor.

Time in Hours.
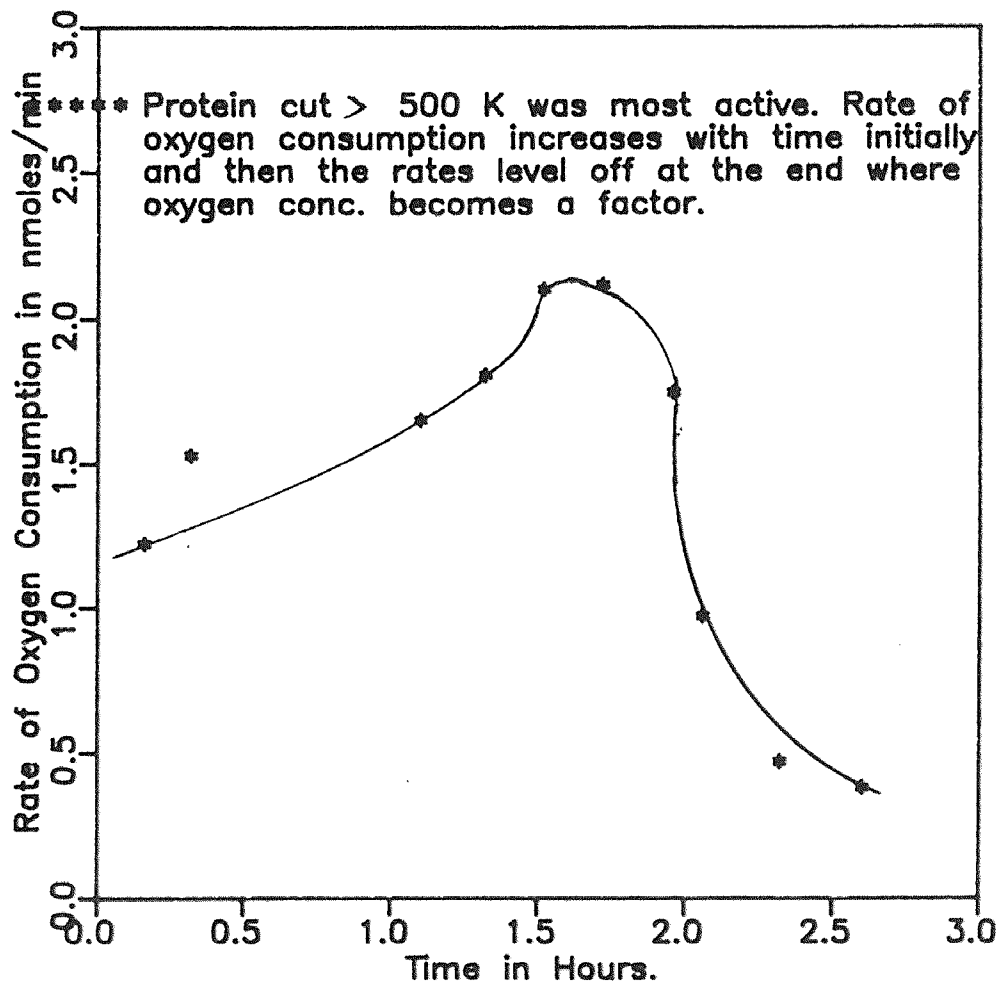
Fig.12 Variation of Enzyme Activity with Oxygen Concentration.

For Protein cut > 500 K, the rates increased starting from saturation (220 nmoles) upto 60 nmoles and then decreased at lower oxygen concentrations.

Rate of Oxygen Consumption in nmoles/min
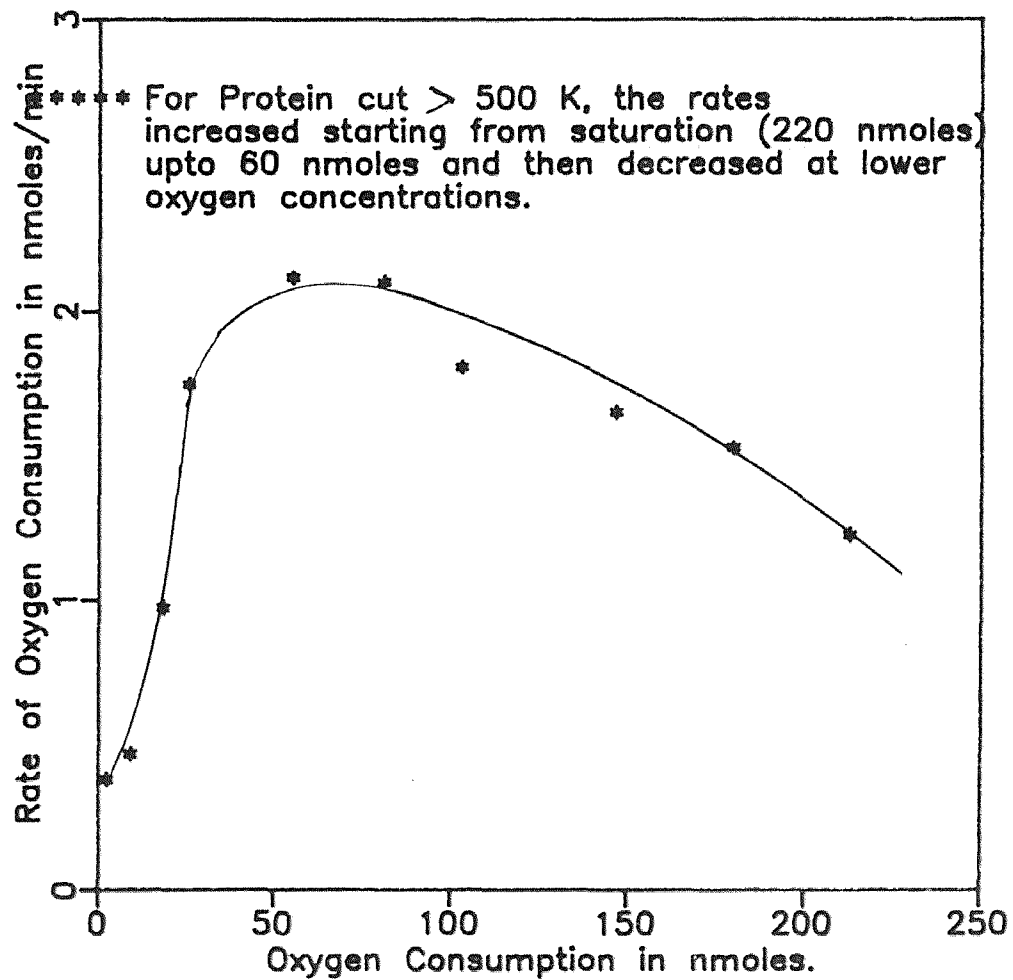
Oxygen Consumption in nmoles.

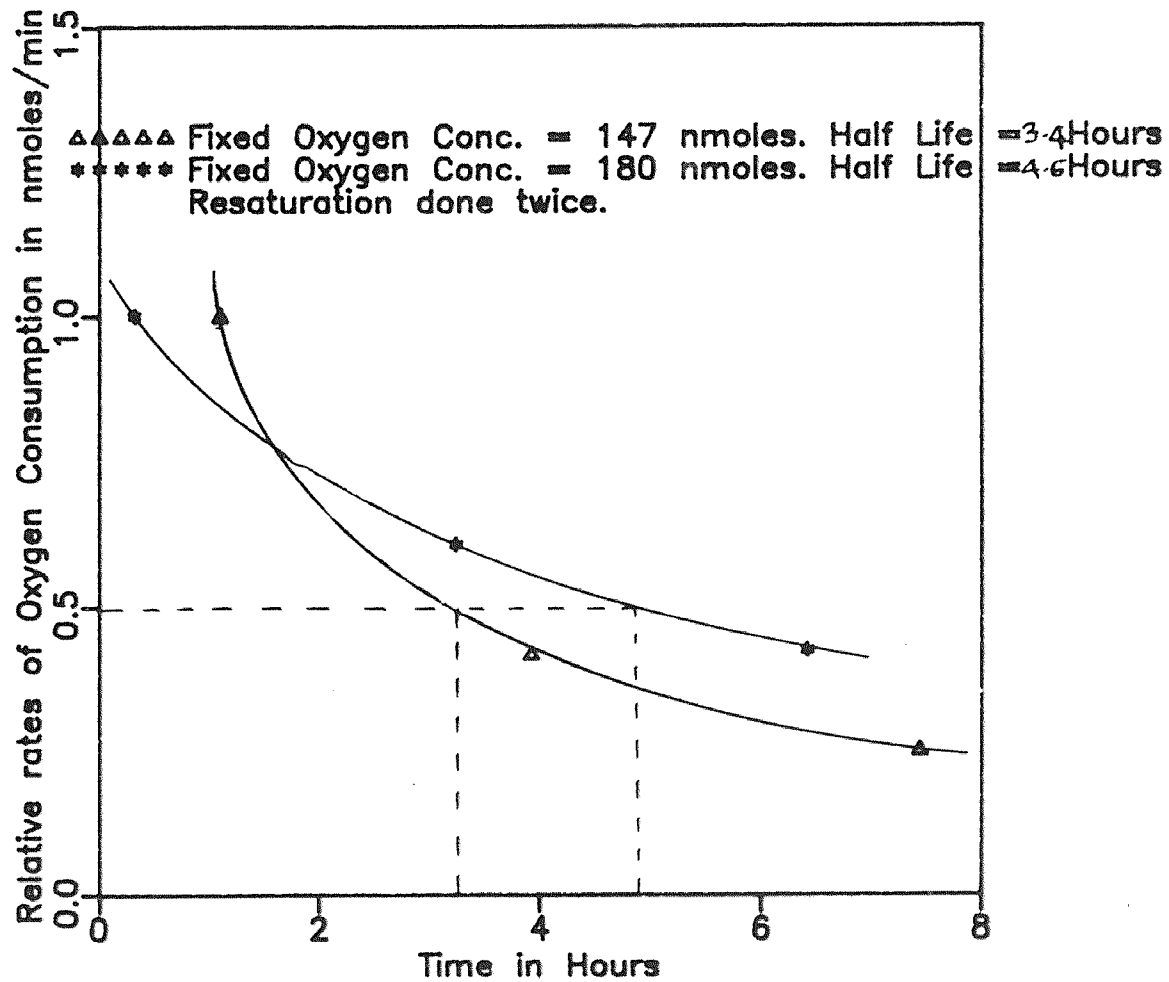Fig.13 Variation of Enzyme Activity with Time at fixed Oxygen Concentration.

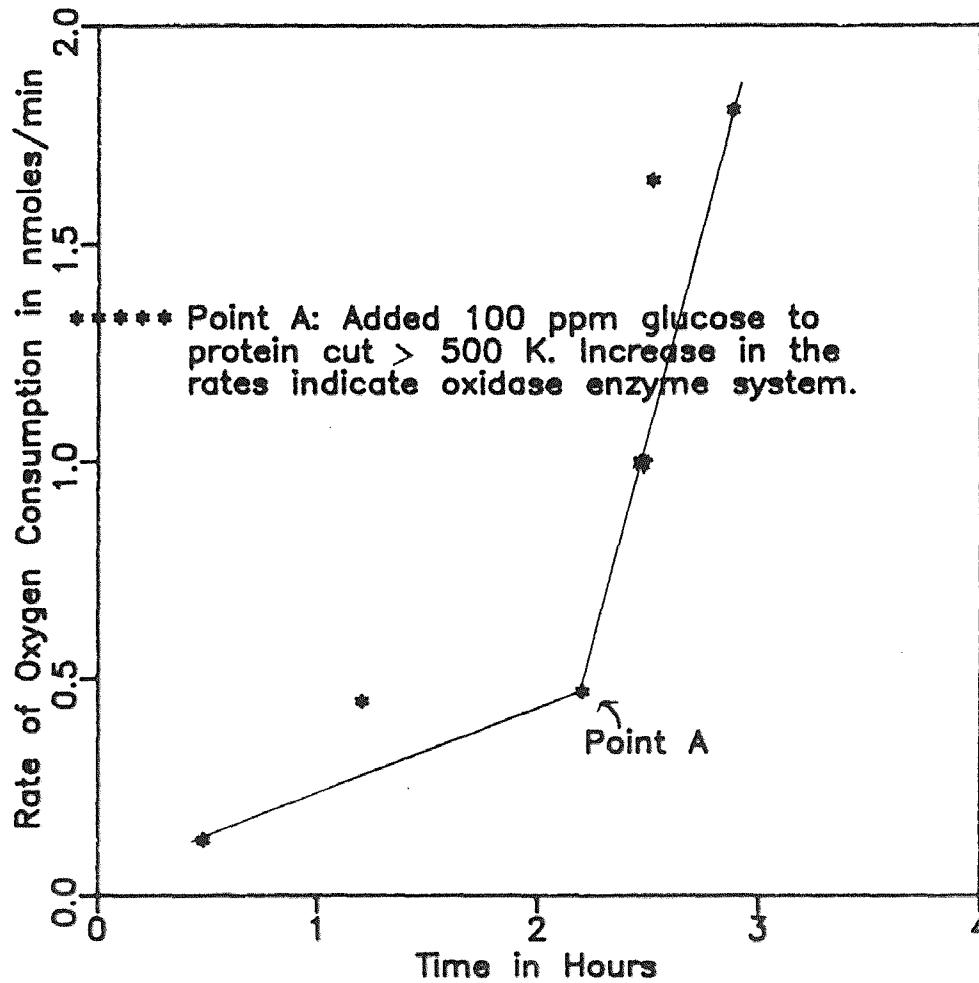Fig.14 Oxidizing ability of the enzyme with glucose as the substrate.

Fig.15 Oxidizing ability of the enzyme with phenol as the substrate.

Point B: Added 100 ppm phenol to protein cut > 500 K. Increase in the rates indicate oxidase enzyme system. Oxidase activity to two different substrates is observed.

Point B