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PRODUCTION OF THE ANTIBIOTIC PATULIN IN A CONTINUOUS THREE PHASE FLUIDIZED BED REACTOR USING IMMOBILIZED CELLS OF PENICILLIUM URTICAE

by

ARUN KUMAR NAYAR

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled,

"PRODUCTION OF THE ANTIBIOTIC PATULIN IN A CONTINUOUS THREE PHASE FLUIDIZED BED REACTOR USING IMMOBILIZED CELLS OF PENICILLIUM URTICAE"

submitted by Arun Kumar Nayar in partial fulfillment of the requirements for the degree of Master of Science in Engineering.

Dr. L. A. Behie, Chairman
Department of Chemical and Petroleum Engineering

Dr. A. A. Jeje
Department of Chemical and Petroleum Engineering

Dr. W. Y. Svrcek
Department of Chemical and Petroleum Engineering

Dr. G. M. Gaucher
Department of Chemistry

December 11, 1984.
Date

ABSTRACT

Antibiotics are the valuable products of the fermentation industry. Most antibiotics are still produced by batch or fed batch submerged free cell methods. In view of increasing demand for higher productivities, there is a continual effort to develop new more efficient processes for the production of antibiotics.

Keeping this in mind, the purpose of this study was to produce the antibiotic patulin in a continuous reactor for as long as possible while at the same time maintaining a sufficient biomass in the reactor. Spores of Penicillium urticae were immobilized in κ-carrageenen beads of sizes 3.2 and 1.75 mm. They were then incubated in growth medium for about 48 hours before being transferred to a reactor containing production medium. Three phase fluidized bed reactors were run successfully for a production period of about 325 hours to produce the antibiotic patulin. Smaller bead size resulted in a higher specific rate of reaction for patulin production but did not have any effect on the specific rate of reaction for glucose utilization. The maximum yield coefficient for patulin was found to be 0.065 as compared to the theoretical maximum of 0.43. About 29 and 22% increase in the protein content of the reactor was found in the case of large and small beads respectively during the continuous reactor runs. About 42% of the glucose was utilized in maintaining the cell mass during the production phase.

Microscopic examination of the beads showed the presence of a thick layer of mycelium near the surface of the bead at the end of the exponential growth phase. In order to study the effect of this physical barrier offered by the cells themselves, diffusion experiments were conducted. Diffusivity studies were performed on carrageenan beads with and without dead cells (48 hours old). The results showed no change in the diffusion coefficient of glucose but the diffusion coefficients of patulin and 6-MSA decreased by 20 and 33% respectively for the beads containing dead cells indicating the presence of an additional diffusional resistance.

Finally, to enable the operation of the reactor at higher flow rates without causing entrainment of the beads, denser carrageenan beads were formed by introducing fine inert stainless steel particles inside the beads. A 15 L fluidized bed was constructed to study the hydrodynamic characteristics of the bed. Gas hold-up was found to increase from 0.08 to 0.176 with increasing gas superficial velocity ranging from 1.4 to 6.4 cm/s. On the other hand solid and liquid hold-ups decreased from 0.33 to 0.26 and 0.59 to 0.56 respectively over the same range of gas velocity.

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dedicated to .....

my parents.

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NOMENCLATURE

A = crossectional area of the fluidized bed

a = radius of the bead

ATP = adenosine triphosphate

C = concentration of patulin in the reactor

C_B = concentration of solute in the bead

C_{BE} = concentration of solute in the bead at equilibrium

$C_{g_{in}}$ = concentration of glucose in the feed

$C_{g_{out}}$ = concentration of glucose in the outlet stream

C_L = concentration of solute in the solution

C_{LE} = concentration of solute in the solution at equilibrium

C_m = concentration of 6-MSA in the reactor

C_p = concentration of patulin in the reactor

$C_{p_{in}}$ = concentration of patulin in the feed

$C_{p_{out}}$ = concentration of patulin in the outlet stream

CMC = critical micelle concentration

D = diffusion coefficient

d = diameter of the column

dd = double deionized

FAD = Flavin Adenine Dinucleotide

g = acceleration due to gravity

H = height of the bed

K = partition coefficient

k_m = external mass transfer coefficient

M_s = maintenance coefficient

n = number of beads

NAD = Nicotinamide Adenine Dinucleotide

NADP = Nicotinamide Adenine Dinucleotide Phosphate

Q = feed flow rate

q_p = specific productivity of the cells

q_n = nonzero positive root of equation (9)

Q_{O_2} = rate of oxygen utilization

r = global rate of reaction for patulin production

$\bar{r}$ = specific rate of reaction for patulin production

t = time

t_d = doubling time for cells

V = volume of the reactor

V_s = volume of the solids

X = biomass concentration

X_o = initial biomass concentration

$Y_{x,s}$ = yield coefficient for biomass

$Y_{p,s}$ = yield coefficient for patulin

Greek Symbols

α = parameter defined in Section 5.7

ε_g = gas hold-up

ε_l = liquid hold-up

ε_s = solid hold-up

ρ_g = gas density

ρ_l = liquid density

ρ_s = density of the solids

η = pathway efficiency

μ = specific growth rate of the cells

μ_m = maximum specific growth rate of the cells

CHAPTER 1

INTRODUCTION AND THEORETICAL CONSIDERATIONS

1 GENERAL

"Small living creatures called microorganisms interact in numerous ways with human activities. On the large scale of the biosphere ,which consists of all regions of the earth containing life, microorganisms play a primary role in the capture of energy from the sun. Their biological activities also complete critical segments of the cycles of carbon, oxygen, nitrogen and other elements essential for life "(Bailey and Ollis,1977).

More purposeful utilization of microbes, however, is in converting various compounds present in nature to more useful forms. This can also be achieved by physical or chemical processes or a combination of both, but there are situations where these processes are not feasible and economical. At this point, these transformations are carried out by biological processes. Although these processes are very slow as compared to most physical or chemical processes in terms of converting reactant (substrate) into desirable product, microbes carry out these reactions under very mild conditions (i.e. temperature and pH). A wide spectrum of examples of biological processes include, food processing, manufacture of alcoholic beverages and the production of such complex organic comp-

pounds as vitamins and hormones. Fermentation processes are the main examples of biological processes and the antibiotics are some of the most important products of the pharmaceutical industry produced by fermentation processes.

Enzymes can be viewed as catalysts which are used for carrying out desired reactions in two ways. Firstly,insitu and secondly after being removed from the cell (i.e.cell free). The ability of an enzyme to catalyze any reaction is usually much more in the case of an intracellular environment. Outside the cells, enzymes are active only in aqueous solutions and under strict conditions of temperature, substrate (reactant) concentration and pH. Redox reactions require complex reducing and oxidation agents like Nicotinamide Adenine Dinucleotide,its phosphorylated form and Flavin Adenine Dinucleotide (NAD,NADP,FAD) which are present in and can be regenerated most effectively by living cells. Each cell contains thousands of enzymes and these are used as catalysts in various biochemical reactions. During these reactions the growth and multiplication of these cells take place. When a reactant enters a cell, it is converted into a variety of products. Some of these products are retained inside the cell while others diffuse outside. Since most of these reactions are quite slow as compared to the reactions in the chemical processes, therefore, usually a very small fraction of the substrate ends up as product. Since active enzymes (the ones producing the desired product) usually have a short life span, the reactions catalyzed by these enzymes last for a short period of time. Because of all these

shortcomings, fermentation processes have to be carried out in such a way, so as to achieve maximum efficiency.

The following points have to be considered in order to achieve maximum efficiency of the fermentation process.

i) better stability of the cells.
ii) manipulation of the cell's metabolic function to achieve maximum conversion of the desired product.
iii) better permeability of the cells for entry and exit of substrate and product respectively.

Complete knowledge of a cell's physiology is required in order to achieve the above mentioned goals. In most of the cases, however, we don't have the desired control over the cells' metablic functions. Hence, empirical methods are used to optimize fermentation processes in the industry.

1.1 REQUIREMENTS FOR GROWTH

It is important to appreciate that the cultural conditions that achieve maximum cell mass may not necessarily be those that give maximum yield of some product of metabolism. For example, the microbe called Aspergillus niger gives an optimum yield of citric acid when growth is restricted by semi-starvation concentrations of nitrogen, phosphorus, and trace metals, but a high concentration of sugar.

The best temperature for cultivation varies with the species, but organisms naturally occuring in soil, air or water usually grow best at 25°C to 30°C while those isolated from animals grow best at 37°C . Some organisms are thermophilic; some of industrial importance, like Lactobacilli, cellulose digesters and methane producer grow best at 40° to 45°C. Organisms with high temperature optima offer technical advantage in that contaminants usually grow better at lower temperatures, and thus growth of contaminants is likely to be inhibited at the temperature of fermentation (Shuichi et al., 1973).

The products of microbial metabolism often cause major changes in pH of reaction medium; hence, to maintain rapid growth , the pH must be close to the optimum for the particular organism. For most organisms this is close to pH 7. The pH for optimum product formation may be different from that for optimum growth, as is the case with Clostridium acetobutylicum. When acid by-products accumulate in the medium, causing an undesirable decrease in pH, it is at times possible to feed ammonia slowly to the culture, so supplying nitrogen for growth while controlling pH.

Microorganisms vary in their need for oxygen. Fungi, algae and a few bacteria are obligate aerobes, a few bacteria are strict anaerobes, while many bacteria can grow in both situations (i.e.facultative aerobes).

1.2 FORMULATION OF MEDIA

Detailed investigation is required to establish the most economic medium for any particular fermentation, but certain basic requirements must be met for any growth medium. These are discussed in Chapter 4.

1.2.1 Source of Energy

Growth proceses are endergonic; so that energy for growth must come from the oxidation of medium components or from light. ATP(adenosine triphosphate) is the most important compound in energy transformations in cells. The coupling of ATP to thermodynamically unfavorable reactions enables them to proceed at a useful rate.

In industrial fermentations, the commonest source of energy used to be starch or molasss, but the growing world population and the increasing cost of plant products has forced a change in the pattern. Autotrophic microorganisms and those which can oxidise hydrocarbon substrates like alkanes for energy are therefore of present industrial interest.

1.2.2 Source of Carbon

Frequently, the carbon needs of a cell are supplied with the energy source, but the autotrophic and photosynthetic bacteria use carbon dioxide. The pathway by which heterophic metabolize substrate

carbon is important in determining the amounts of carbon converted to cell material. It is found that facultative organisms incorporate about 10% of substrate carbon in cell material when metabolizing anaerobically.

1.2.3 Source of Nitrogen

Nitrogen can be supplied for most industrially important organisms by ammonia or ammonium salt, although growth may be faster when using organic nitrogen. Certain organisms, however, have absolute requirements for organic nitrogen. When products with a high nitrogen content are required, the greater the amount of nitrogen that can be assimilated by the organisms , the greater the chance of high yield of product, provided the organism has the genetic potential to produce the product.

Organic nitrogen compounds are relatively expensive. The cheap forms are soybean, peanut, fish or meat meals, malt combings, yeast extract and various enzymes digests of protein rich materials. Plant by-products like molasses and corn steep liquor can also provide small amounts of growth factors. Nitrogen constitutes 10% of the dry weight of most organisms, so that the minimum nitrogen content of the medium can be calculated for a desired cell yield.

1.2.4 Source of Minerals

Table 1.1 shows the range of concentrations of some elements

Table 1.1 Inorganic Constituents of Different Microorganisms (From Shuichi et al., 1973).

	% dry weight (g/100 g)		
Element	Bacteria	Fungi	Yeast
Phosphorous	2.0 - 3.0	0.4 - 4.5	0.8 - 2.6
Sulphur	0.2 - 1.0	0.1 - 0.5	0.01 - 0.24
Potassium	1.0 - 4.5	0.2 - 2.5	1.0 - 4.0
Magnesium	0.1 - 0.5	0.1 - 0.3	0.1 - 0.5
Sodium	0.5 - 1.0	0.02 - 0.5	0.01 - 0.1
Calcium	0.01 - 1.1	0.1 - 1.4	0.1 - 0.3
Iron	0.02 - 0.2	0.1 - 0.2	0.01 - 0.5
Copper	0.01 - 0.02	-	0.002 - 0.01
Manganese	0.001 -0.01	-	0.0005 - 0.007
Molybdenum	-	-	0.0001 - 0.0002
Total ash	7 - 12	2 - 8	5 - 10

found in bacteria, fungi and yeast. Clearly, phosphorus,potassium, sulfur and magnesium are major compounds and these and other elements present in significant amounts must be supplied in the medium. Trace metals like iron, copper, cobalt, manganese, zinc and molybdenum are essential but are usually present as impurities in other ingredients of the medium. Organic constituents of microorganisms are listed in Table 1.2.

1.3 PRIMARY AND SECONDARY METABOLISM

Microbial metabolites are generally divided into two categories, namely, growth-associated and non-growth associated metabolites. The growth -associated metabolites are called primary metabolites where as non-growth associated are referred to as 'secondary metabolites'. Alcohol, vitamins and amino acids are some of the examples of the primary metabolites. Examples of secondary metabolites include antibiotics. These substances (secondary metabolites) are of no apparent importance to the growth and maintenance of the producing organism itself, but their true function within the cellular metabolism is still unknown although there have been reports on this in the literature (Mothes, 1980, Demain, 1981).

When supplied with necessary nutrients, microbial cells break down complex carbon, energy and nitrogen sources to form low molecular weight intermediary metabolites. These metabolites are then used to form all of the other molecules necessary to cell construction and

Table 1.2 Organic Costituents of Microorganisms (From Shuichi et al., 1973).

	% dry weight					
	Bacteria		Yeasts		Molds	
Component	Av *	Range	Av *	Range	Av *	Range
Carbon	48	46-52	48	46-52	48	45-55
Nitrogen	12.5	10-14	7.5	6-8.5	6	4-7
Protein	55	50-60	40	35-45	32	25-40
Carbohydrate	9	6-15	38	30-45	49	40-55
Lipid	7	5-10	8	5-10	8	5-10
Nucleic acid	23	15-25	8	5-10	5	2-8
Ash	6	4-10	6	4-10	6	4-10

* Average

survival. These microorganisms respond quickly to any changes in the surroundings (Leegwater et al., 1982).

Sometimes these metabolic reactions produce low molecular weight products, which are not required for growth. These are called secondary metabolites and generally the synthesis of secondary metabolites starts after the maximal growth rate of cells (exponential growth phase) has subsided (Berry, 1975). When the concentration of one or more of the growth supporting nutrients becomes limiting, key intermediates of primary metabolism are often diverted into multistep metabolic pathways which are non-growth associated ; thus the principal precursors for secondary metabolism are also key intermediates of primary metabolism (Bu'lock, 1975). Antibiotic biosynthesis stops mainly because of the following reasons ,

(i) depletion of primary metabolic precursor

(ii) loss of pathway enzymes

(iii) feed back inhibition of secondary metabolism enzymes by end product (Neway et al., 1981).

Therefore, the duration of the active production phase of an antibiotic (secondary metabolite) could be increased if the following occured -

(i) there are no changes (the one causing enzyme degradation) in the environment,

(ii) the stability of the enzymes is improved,

(iii) the continuous synthesis of these enzymes is promoted.

1.4 ANTIBIOTICS

Antibiotics are the products of secondary metabolism which inhibit growth processes of other organisms even when used at low concentrations. During World War II, the demand for chemotherapeutic agents to treat wound infections led to the development of a production process for penicillin and the beginning of the era of antibiotic research. This continues to be the most important area of the industrial microbiology today. An estimated 100 - 200 new compounds are discovered annually.

Antibiotics are produced by bacteria, actinomycetes and fungi. Only 10 of the known fungal antibiotics are produced commercially and only the penicillins, cephalosporin C, griseofulvin and fusidic acid are clinically important.

Penicillins, cephalosporins and cephamycins are peptide antibiotics with unique structures (β-lactam). These are among the most effective antibiotics to combat infectious diseases.β-Lactam antibiotics are specific inhibitors of bacterial cell wall synthesis. These antibiotics have the interesting property of killing growing cells but not killing resting cells. The basic structure of the penicillin is 6-aminopenicillanic acid (6-APA). If the penicillin fermentation is carried out without addition of side chain precursors, the natural

penicillins are produced. From this mixture only benzylpenicillin is therapeutically useful. The fermentation of penicillin can be better controlled by adding a side chain precursor, so that only one desired penicillin is produced. Over 100 biosynthetic penicillins have been produced in this way. In commercial processes, however, only penicillin G, penicillin V and very limited amounts of penicillin O have been produced.

World wide production of antibiotics is over 100,000 tonnes per year and estimated gross sales for 1980 were 4.2 billion dollars. The annual gross sales in the United States alone were 1 billion dollars (Crueger,1982). Therefore, there is greater interest than ever before to improve the efficiency of the fermentation processes existing in the industry.

1.4.1 Methods Of Production Of Secondary Metabolites By Batch Fermentations

A biochemical reactor is a device in which materials are treated to promote biochemical transformations of matter by the action of living cells or cell free enzyme systems. It could be before or during the production of a desired product. Most of the antibiotics in industries are produced in stirred tank batch reactors. In these reactors, the cells are subjected to lot of stress and changing surroundings. Therefore, even if the fermentation conditions are controlled very strictly, the efficiency of the cells to produce the secondary metabolite starts declining just after 20 -25 hours

after the initiation of the production phase (Calam and Ismail, 1980). The production of a secondary metabolite can be prolonged by adding essential nutrients intermittently (Martin and Demain, 1980). In batch reactors, the products get accumulated after some time and cause inhibition of their own synthesis. Therefore, the best process for the production of antibiotics would be the one in which reactants(i.e. substrates or nutrients) are added continuously and at the same time products are removed while maintaining sufficient amount of biomass in the reactor for continued production of the desired product.

At present, most of the the antibiotics are produced by fed batch free cell process. In these processes, nutrients are added intermittently to the reactor. In the continuous production of secondary metabolites by submerged free cell process, there always comes a point when the growth rate of cells is less than their removal rate and they tend to be washed out leaving the reactor without any catalyst. Figure 1.1 shows the basic flow chart of the penicillin production (Swartz,1979). Most of the antibiotics go through more or less the same unit operations. Figure 1.2 illustrates the purification operation which inturn consists of various operations (Swartz, 1979). An indication of the duration of the production phase of an antibiotic in an industrial fermenter can be obtained from Figure 1.3 (Swartz, 1979). To maintain the high concentration of the antibiotic penicillin in the reactor, the nutrients are added intermittently as shown in the Figure 1.3.

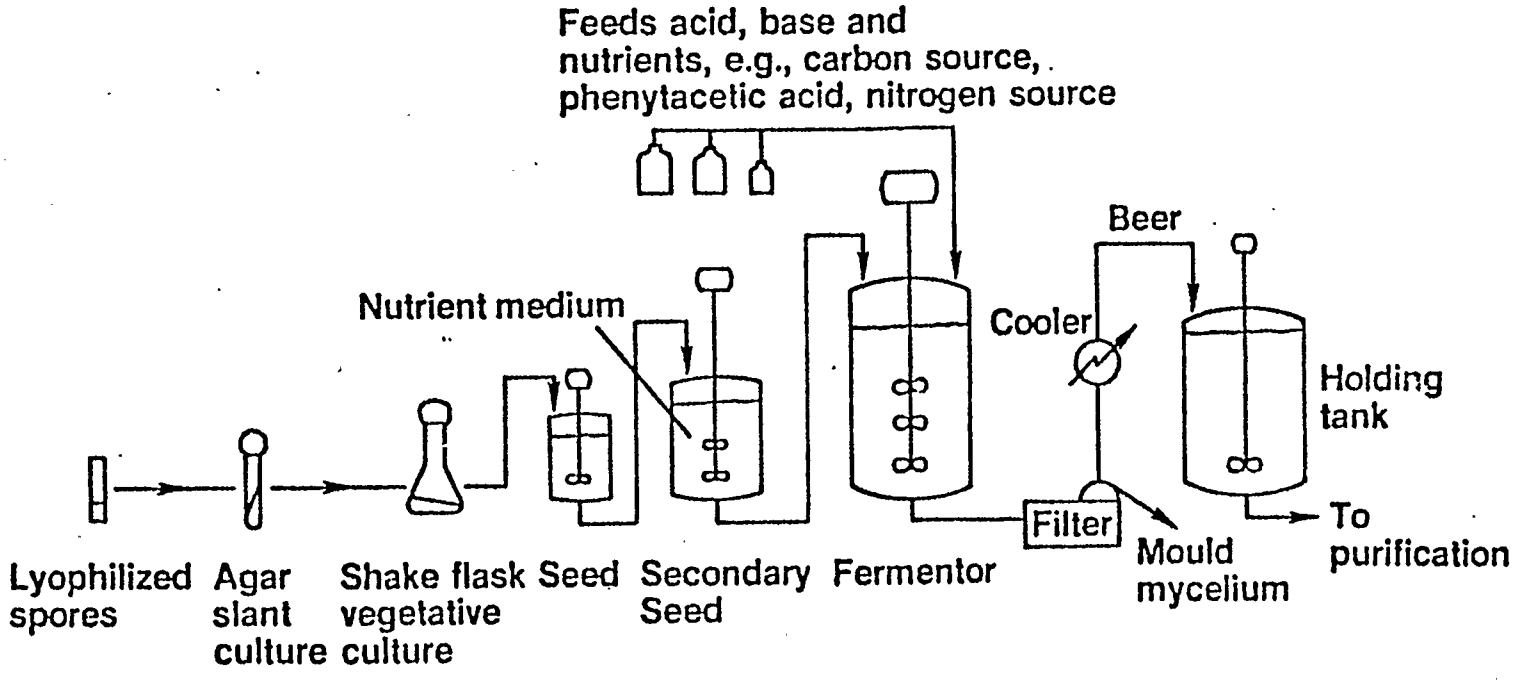


Figure 1.1 Flow Chart for Penicillin -G Production.
(From Swartz,1979)

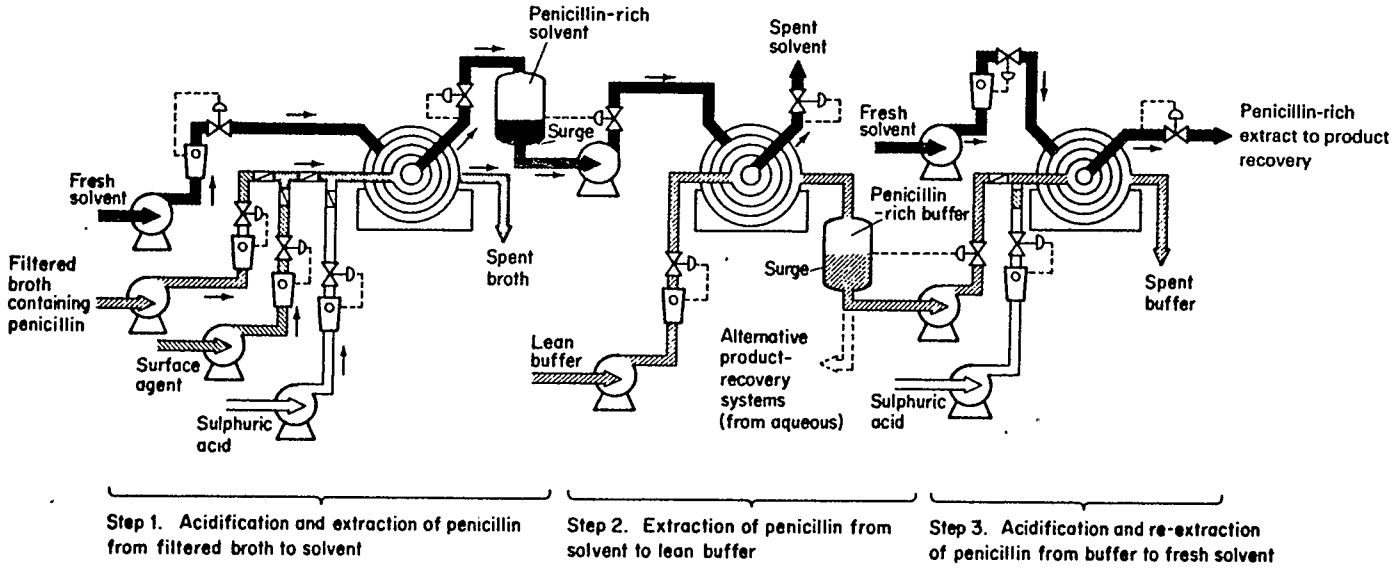


Figure 1.2 Purification Operation for Penicillin -G.
(From Swartz, 1979)

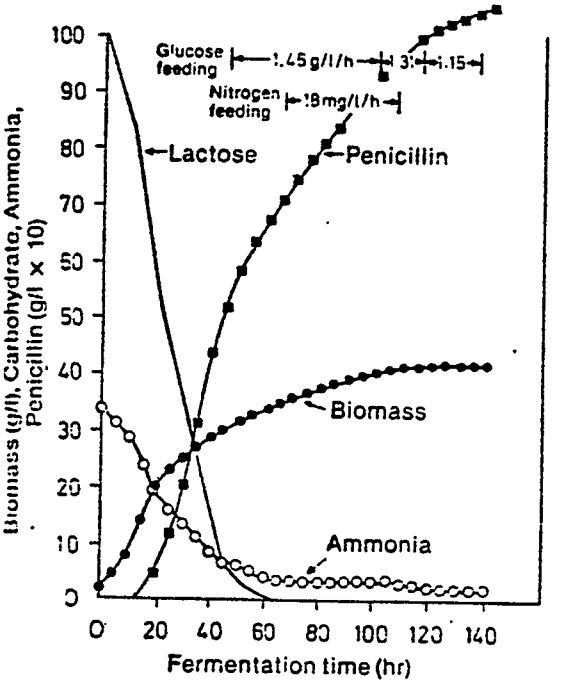


Figure 1.3 Fermentation Cycle for Penicillin G. (From Swartz,1979).

1.5 IMMOBILIZATION OF MICROBIAL CELLS

In order to prevent cell-wash-out during continuous production of an antibiotic, the cells are immobilized with the help of some sort of support matrix.

Immobilized microbial cells have been defined by Chibata (1978) as "microbial cells physically confined in a region of space retaining their ability to catalyze the reaction and which can be used repeatedly."

Immobilization of microbial cells offers the following advantages over free cells.

i) Multiple or repetitive use of a single batch of catalyst(s).

ii) Ability to stop the reaction rapidly by removing the catalyst from the reaction solution.

iii) Better stability of cells by bonding with the support matrix.

iv) The processed solution is not contaminated with the catalyst.

v) Longer half life (i.e. the time required for the catalytic ability of the cells to reduce to half of its original value).

Immobilization of cells can economically extend the life time of these biologically active molecules (Chibata, 1978) and this explains all the attention being given to this area of study.

1.5.1 Immobilization Techniques And Proliferation Of Carriers

A variety of methods for immobilizing microbial cells have been

developed. These may be classified as four different approaches to immobilization. They are:

i) Crosslinking without the benefit of carrier,

ii) crosslinking within a carrier or on the surface of carriers,

iii) encapsulation,

iv) entrapment,

Of all the above techniques cell entrapment has been found to be the most suitable for different types of single or multiple microbial reactions carried out in a continuous biochemical reactor. As compared to other methods, immobilization by cell entrapment offers longer half life, higher reaction rates and better protection of cells by the supporting matrix.

The carriers utilized for the immobilization of enzymes may be classified in to two groups : 1) organic ; 2) inorganic. Table 1.3 gives the list of various supports available. Morikawa et al. (1979) found out that cells entrapped in polyacrylamide and carrageenan remain active for longer periods of time and can also grow inside the matrix. There is very little information published in the literature about continuous production of secondary metabolites using immobilized cells. Morikawa et al. (1979) reported the production of penicillin using Penicillium chrysogenum cells immobilized in poly-

Table 1.3 List of Supports available for immobilization of cells (Modified from Messing, 1975 and Kolot, 1981).

SUPPORTS	
Inorganic carriers	Organic carriers
Celite	Carrageenan
Kaobinite	Cellulose
Colloidal silica	Agar
Glass particles	Starch
Alumina	Dextran
Titania	Agarose
Nickle oxide	Pectate
Zirconia	Phenolic resins
Carbon (Charcoal)	Nylon
Hydroxyapatite	Polyacrylamides
Iron oxide	Organic Copolymers
Magnesia	Collagen (protein)

acrylamide gels. Deo et al. (1980) reported the production of patulin by immobilized cells of Penicillium urticae using carrageenan as a support matrix and found the half life of the catalyst to be 16 days as compared to 6 days in case of free cells.

1.6 CARRAGEENAN MATRIX FOR CELL ENTRAPMENT

1.6.1 Carrageenan And Its Source

Carrageenan is a food additive which can be added directly to food for human consumption(F.M.C.Corporation, 1981). It is essentially a gelling material. The two water gelling carrageenans are kappa and iota. Kappa- carrageenan is most frequently obtained from sea- weeds such as Chondrus crispus and Eucheuma cottonii while iota carrageenan is most frequently obtained from Eucheuma spiosum. Figure 1.4 shows the structures of two gelling carrageenans i.e kappa and iota, on the right, while their non - gelling precursors, mu and nu, respectively, are shown on the left. Note that B units are the same in the non-gelling and in the gelling carrageenans. The A units are different in that the non- gelling carrageenans containing 6 SO_4 where as the gelling carrageenans contain a 3,6-anhydro ring structure. The precursors are non - gelling because the conformation of the 6 - sulfated residues introduces kinks which inhibit the formation of double helices, the mechanism by which aqueous gelation takes place.

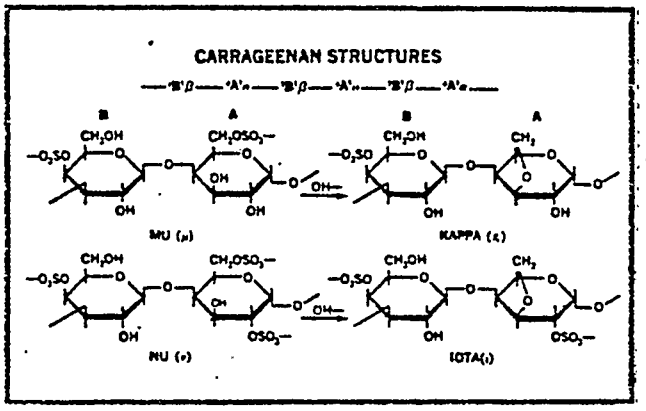


Figure 1.4 Structure of kappa and iota Carrageenans.
(From FMC Corporation,1981)

1.6.2 Gelation Mechanism & Commercial Production Of Carrageenan

Aqueous gelling carrageenan forms gels through the formation of double helices as shown in Figure 1.5. As the solution is cooled, double helices begin to form (Gel I) and aggregate to form junction zones (Gel II). All of the carrageenan gels are thermally reversibile, - they can be molded into their original form on melting without causing any degradation due to heat.

Dependent upon the requirements of the system, carrageenan may be produced in a variety of ways. Three typical processes are shown in Figure 1.6. Common to all three is the alkaline cook of seaweeds. The degree of alkalinity and time of cook determines the amount of conversion to the gelling form. Method 1 is applicable to those sea weeds that can be cooked with alkali without the carrageenan. Products of this type are generally the most economical to produce. Since they contain cellulosic matter, however, they are most suited for applications where clarity and smoothness are not required. Typical examples are pet foods and air freshner gels.

Methods 2 and 3 involve extraction of carrageenan followed by filteration to remove insoluble matter. Method 2 involves recovery of the carrageenan by roll drying where as Method 3 utilizes alcohol precipitation. The latter method produces carrageenan with the highest degree of purity and is more widely used than the roll drying process. Table 1.4 lists the properties of Kappa-carrageenan.

The choice of a matrix for immobilization of cells is determined by the following factors.

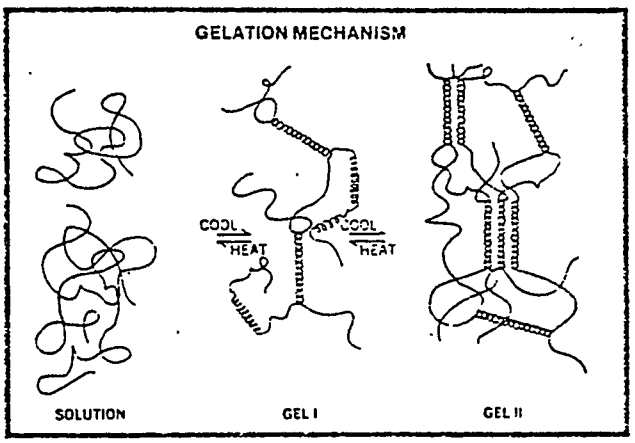


Figure 1.5 Gelation Mechanism for Carrageenans (From FMC Corporation, 1981).

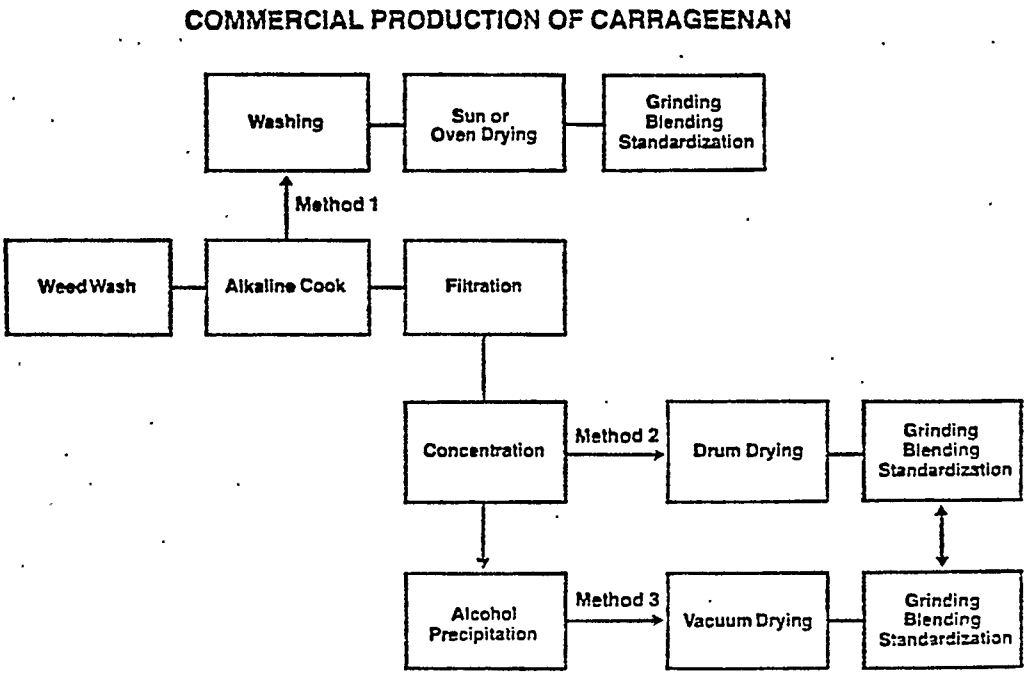


Figure 1.6 Methods of Commercial Production of Carrageenan (From FMC Corporation,1981).

Table 1.1 Properties of Kappa and Iota Carrageenan
(From FMC Corporation 1981).

PROPERTIES OF KAPPA AND IOTA CARRAGEENAN		
	Kappa	iota
Solubility Temperature	Depends on cations present.Normally above $60^{o}c(140^{o}F)$	Depends on cations present.Normally above $60^{o}c(140^{o}F)$
Viscosity of 1.5% soln. at $75^{o}c$	10 to 50 cps	15 to 50 cps
Gelling Effects	Na^{+} salt gives no gelation. Gels strongest with K^{+}, less with $(NH_4)^{+}$, Ca^{++}	Na^{+} salt gives no gelation. Gels strongest with Ca^{++}, less with K^{+}, $(NH_4)^{+}$
Type of Gel	Brittle	Elastic
Synersis of Gel	Moderate --- High	None
Freeze/Thaw of Gel	Poor	Good
Reforming Tendency of Broken Gel	Poor	Good
Gelling Temperature	Depends on K^{+} ion concentration	Depends on Ca^{++} ion concentration
Melting Temperature	Ususlly $10^{o}c$ to $20^{o}c$ above gelling temperature, depending on type of Kappa	Usually $5^{o}c$ above gelling temperature
Solution Clarity	Slight haze at neutral and alkaline pH.Clear at low pH	Slight haze at neutral and alkaline pH.Clear at low pH
pH Stability Neutral and Alkaline Acid(pH=3.5)	Both sol and gel are stable.Sol hydrolysis accelrated by heat, Gelled state is stable.	Both sol and gel are stable.Sol hydrolysis accelrated by heat, Gelled state is stable.
Effect of Locust Bean Gum on: Gel Strength Synersis Type of Gel	 Synergistic Enhancement Marked reduction Becomes elastic	 Negligible change No change Heavier body

(i) It's toxicity towards the organism.

(ii) It's operational stability in the bioreactor(temperature,pH).

(iii) The applicability of the matrix to a wide variety of cells.

(iv) It's feasibility to produce different sizes and shapes of gel particles in the reactor.

(v) It's cost of production.

(vi) It's ability to retain the activity of microbial cells producing various products.

Most of the reactions involving microbial cells take place in the temperature range of 25 - 38^{o}C . Carrageenan gels are stable in this temperature range and they also satisfy all the above mentioned requirements and are relatively cheap and available readily. All these qualities make it an ideal matrix for the production of patulin (Deo *et al.*, 1982). Takata *et al.* (1977) found carrageenan suitable for immobilization purposes.

CHAPTER 2

PATULIN — A MODEL SYSTEM FOR THE PRESENT STUDY

Patulin is one of the most potent known antibiotics and mycotoxins. It is produced by filamentous fungi such as Aspergilli and the Penicillia. Like other antibiotics it is also a secondary metabolite. The reason for choosing it as a model system is that unlike most antibiotics its biosynthetic pathway (Figure 2.1) as well as the enzymology and regulation of the pathway has been well understood (Gaucher et al.,1980). The prefered route for patulin biosynthesis is via m- hydroxybenzaldehyde. Glucose is decomposed into Acetyl-CoA and Malonyl-CoA in Tricarboxylic acid (TCA) cycle. These two compounds react to give 6-MSA which is present all along the pathway suggesting that decomposition of 6-MSA to m - HO - benzyl alcohol reaction is the slowest step as all the other intermediates decompose at a relatively faster rate. The biosynthesis of patulin can be considered as a consecutive series of atleast ten reactions with a corresponding number of intermediates and enzymes. It has been shown by GrootWassink and Gaucher (1980) that in free cell batch cultures of P.urticae the appearance of pathway enzymes follows the cessation of growth caused by the depletion of nitrogen nutrients. Pathway enzymes gradually loose their activity causing a decline in patulin production (Neway and Gaucher,1981). Also the organism P.urticae has been studied throughly by Sekiguchi et al.(1977) and the same authors (1977) reported immobilization of P.urticae in a

polyacrylamide gel. All this information in the literature makes it an ideal choice for the present study.

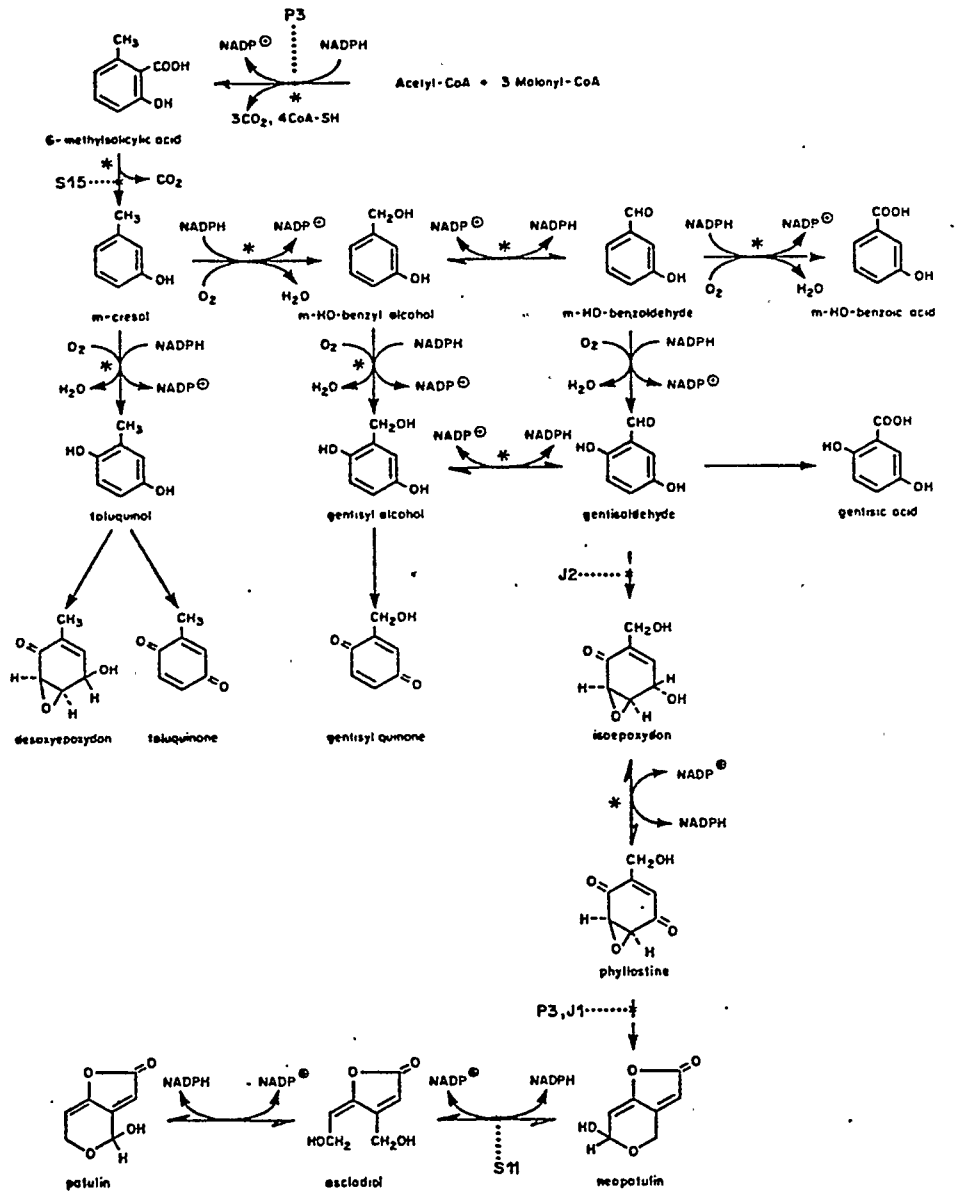


Figure 2.1 Biosynthetic Pathway of Patulin in Penicillium urticae.(From GrootWassink et al.,.1980).

CHAPTER 3

RESEARCH OBJECTIVES

In view of the increasing demand for higher productivity of antibiotics in the fermentation industry, there is a continual effort to develop new processes for the production of antibiotics. In the modern day industry most of the antibiotics are still produced by fed batch submerged free cell processes. About 80% of the total production cost of antibiotic is associated with the fermentation process and 20% with purification operations (Swartz, 1979). Therefore, there is a great incentive to improve the performance of existing fermentations. Keeping this in mind, the objectives of the present study were:

(1) To produce the antibiotic patulin in a continuous three phase fluidized bed reactor by immobilized cells of Penicillium urticae in order to investigate in detail, the problem of internal diffusion caused by the support matrix and its attendant cell population.

(2) To carryout diffusivity studies to determine the diffusion coefficients of substrate and products into the carrageenan cell support matrix.

(3) To study the hydrodynamic characteristics of the carrageenan beads in a three phase fluidized bed reactor to achieve the proper disengagement of the carrageenan beads at higher air flow rates.

CHAPTER 4

EXPERMENTAL METHODS AND MATERIALS

4.1 Strain Preservation, Agar Slant Cultures & Preparation Of Inocula

Cultures of P.urticae (NRRL 2159 A) were grown on agar slants from spores on a lyophilized filter paper strip. The filter paper strips were made from isolated single colonies of P.urticae and these were stored in an evacuated ampule at -70^{o}C . Spores from five such agar slants were suspended in 10 mL of sodium chloride solution containing 450 µL/L of aerosol OT.(Fisher) This was mixed with 5.0 g of silica gel (70 -230 mesh ASTM, Merck type 60) and lyophilized in an 30 mL vial (Fisher,3 - 338 J). This freeze dried spore stock was stored at 4^{o}C in a tightly capped vial and all subsequent inocula were grown from this (Deo, 1982).

Spore inocula of P.urticae for the cultivation of immobilized cells were prepared using agar slant cultures. Czapek Dox agar slants were used. Its composition is given in Table 4.1.

The agar media were heated till it melted and 10 mL portions were autoclaved for 15 minutes at 120^{o}C in 30 mL vials. Agar slants were prepared by keeping these vials on a bench top at an angle by keeping some cotton below so as to yield approximately 15cm^{2} of surface area. The agar was allowed to solidify in this position and then these slants were stored at 4^{o}C.

Table 4.1 : Composition of Media used for Inoculation of Spores of *P.urticae*.

INGREDIENTS	AMOUNT (g/L)
Czapek - Dox Agar solution (Difco)	49.0
Difco Bacto Agar	5.0
double deionized water	1.0

Spores of Penicillium urticae for inoculation of Czapek-Dox agar slant surfaces were obtained from silica - gel spore stocks using a sterile wire loop. These slants were incubated at 28°C until a thick layer of mycelium covered the entire agar surface. These slants were stored at 4°C to be used within 5 - 6 days.The spores from one slant were suspended in 5 mL of 50.0 mg/L aerosol OT solution by stirring on a vortex mixer (Fisher Scientific, Model K - 550 G). Spore count of P.urticae spore suspensions was done under the microscope. Typical values were approximately 4×10^6 spores/cm^3.

4.2 IMMOBILIZATION METHODS

Cells were immobilized in Kappa - carrageenan (FMC Corp.) for the reasons mentioned in Section 1.6. Kappa - carrageenan (4% wt/vol) was heated to boiling in double deionized (dd) water in a 2.0 L flat bottom flask. It was then covered with cotton and sealed with aluminium foil. The flask was autoclaved for 20 minutes and was later transferred to a water bath maintained at 37°C so that the carrageenan solution remained as a liquified gel. A spore suspension of P.urticae was added to the solution of carrageenan and stirred thoroughly to achieve a homogenous mixture.

This mixture of P.urticae spore suspension and carrageenan (at 37°C) was transferred to a cylindrical multineedle device called a bead machine made of steel (Needle size - 21 gauge and 1 inch long for large beads and 26 gauge and 1 cm long for small beads). The bead machine was pressurized with an inert gas (nitrogen) up to 275

kPa, thus, forcing gel to come out of the needles in the form of droplets (3.2 ± 0.13 and 1.75 ± 0.16 mm in diameter) and falling into a beaker containing a salt solution of aqueous 0.3 mol/L KCl and 50 mmol/L $CaCl_2$ kept at 4°C. The beads that were formed in the salt solution were stirred constantly for about 2 - 3 hours to allow the beads to harden. The reason for choosing the salt solution instead of water was that the K^+ and Ca^{++} ions help in gelation of carrageenan solution by acting as crosslinking agents.

4.3 SURFACE TENSION STUDIES

For making beads smaller than 3.2 mm, various techniques were tried. Surface tension studies were performed on Kappa - carrageenan solution at 37°C because the surface tension determines the gravity force necessary to break the liquid film from the tip of the needle. Silicon antifoaming agent (SAG 471,Union Carbide) was chosen for this purpose, the reason being that it was already being used in the reactor to overcome the problem of foaming and was also non-toxic. A Fisher Scientific Tensiomat (Model 20) was used for measuring the surface tension of the carrageenan solution. Increasing amounts of surfactant was added gradually. This was repeated until there was no further change in surface tension of the solution .This value of surfactant concentration is called the Critical Micelle Concentration(CMC). Figure 4.1 shows the plot for determination of Critical Micelle Concentration(CMC), which was found to be 525.0 mg/L.

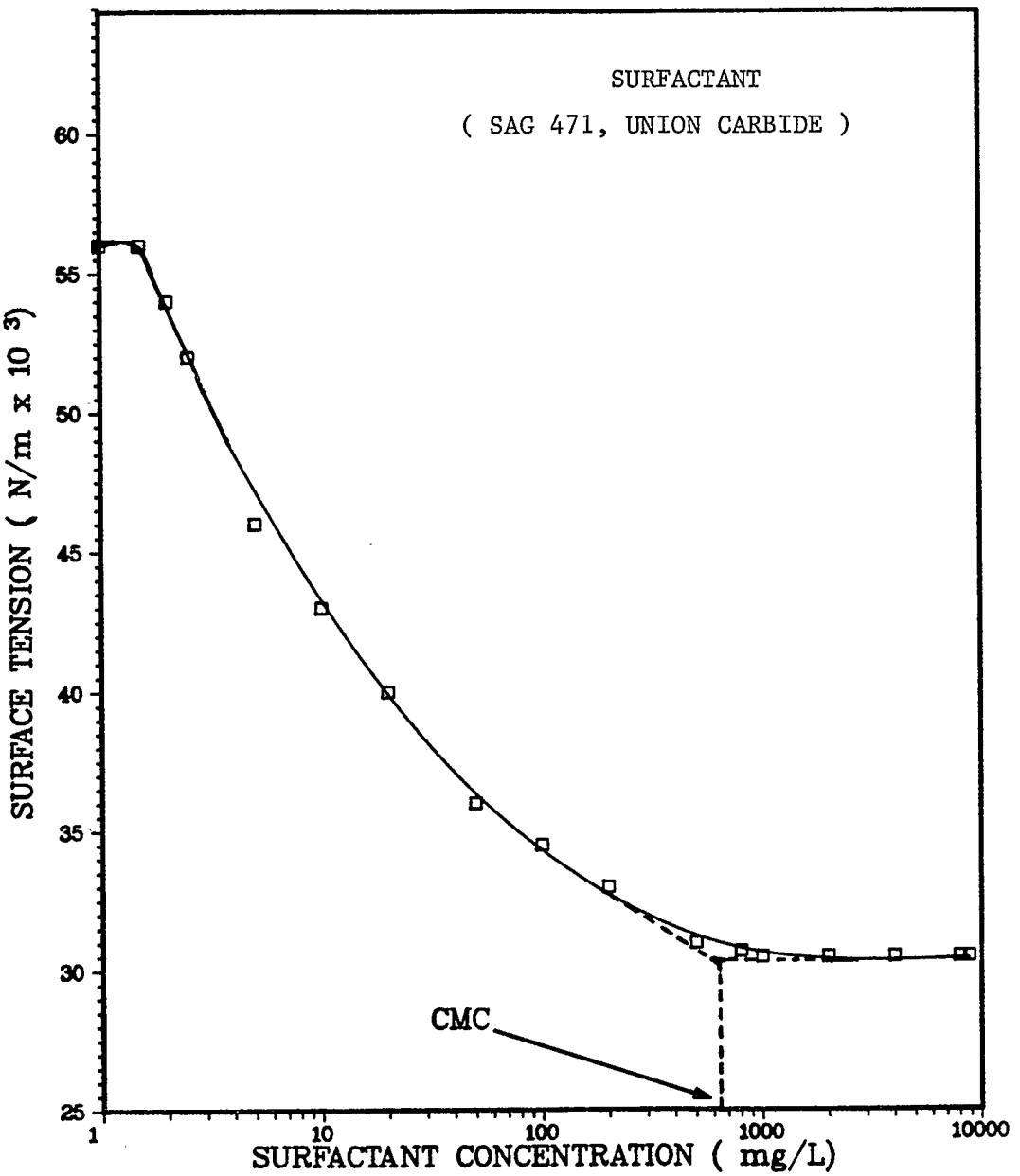


Figure 4.1 Variation of the Surface Tension of Carrageenan Solution with Surfactant Concentration.

For all the subsequents experiments involving smaller carrageenan beads 525.0 mg/L of the above mentioned surfactant was added to the carrageenan solution before sterilization. The Bead machine was modified to produce smaller beads (Needle size - 26 gauge). These needles were cut straight across to give a flat opening and then the outside wall was tapered inwardly at the tip so as to have a smaller surface area available for the drop to form. This also resulted in the early fall of the drop from the tip of the needle and hence smaller beads (1.75±0.16 mm). Beads of even smaller sizes than just described were produced by vibrating the entire bead machine. This was achieved by fixing a small pneumatic piston (7 cm long, inside a steel casing) to the bead machine. Air was introduced at 276 kPa, this resulted in the movement of the piston, thus, vibrating the needles while carrageenan beads were being formed. Beads of sizes ranging from 1.0 ~ 1.8 mm were obtained by this method. Due to this large distribution in sizes, it was decided not to make the beads by this method. Instead the beads of diameter 1.75 mm were used in the reactor run.

4.4 LIQUID MEDIA

The liquid media used for the growth of immobilized P.urticae cells was developed by GrootWassink and Gaucher (1980). The composition of the media is given in Table 4.2. The trace metal solution used in the growth medium was adopted from Yamamoto and Segel (1967). Table 4.3 lists the composition of the trace metal solution. This

Table 4.2 Composition of Growth Medium.

INGREDIENTS	AMOUNT (g/L)
Yeast Extract	5.0
Glucose	40.0
KH_2PO_4 (anhydrous)	13.6
Citric Acid (anhydrous)	9.8
Na_2SO_4 (anhydrous)	1.0
Trace Metal Solution	10.0 *

* (mL/L)

Table 4.3 Composition of Trace Metal Solution.

INGREDIENTS	AMOUNT (g/L)
$Na_3Citrate.2H_2O$	5.0
$MnCl_2.4H_2O$	3.0
$ZnCl_2$	2.0
$FeCl_3.6H_2O$	2.0
$MgCl_2.6H_2O$	50.0
$CuCl_2.2H_2O$	0.2
$CaCl_2.2H_2O$	0.75
$CoCl_2.6H_2O$	0.2
$MoCl_5$	0.1
$Na_2B_4O_7.10H_2O$	0.1
d d water	1 L

solution was stored at $4^{o}C$ for later use.

4.4.1 Growth Medium For P.urticae

All the ingredients of growth medium except glucose were dissolved in 2 litres of double deionized (dd) water. The pH of this solution (solution A) was adjusted to 6.5 with solid NaOH pellets. This solution was transferred to a 6 litres flask. Glucose was dissolved in 1 litre of dd water and kept in 4 litre flask (solution B). Both the flasks were plugged with cotton plugs and autoclaved for about 25 miinutes. Both these solutions (A and B) were mixed together aseptically after sterlization and the solution was ready to be used as a growth medium.

4.4.2 Production Medium For P.urticae

The composition of the production medium used for the continuous bioreactor runs is listed in Table 4.4. Berk *et al.* (1984) tried two runs for the production of patulin by changing the concentration of yeast extract in the first run to 0.25 g/L, while the second run was carried without the trace metal solution , Na_2SO_4 and citric acid. But both these changes in the production medium resulted in less production of patulin as compared to the one achieved by using the composition of production medium listed in Table 4.4 (Berk *et al.*, 1984). Therefore, all subsequent experiments were carried out using this production medium.

Table 4.4 Composition of Production Medium Used for the Continuous Bioreactor Runs (From Berk et al., 1983).

INGREDIENTS	AMOUNT (g/L)
Yeast Extract	0.05
KH_2PO_4	13.6
Glucose	25.0
Citric Acid	0.49
Na_2SO_4	0.05
Trace Metal Solution	0.5 *

* mL/L

All the ingredients of the production medium except glucose were dissolved in 4 L of dd water and the pH was adjusted to 6.5. This mixture was transferred to a large container and 10 litres of dd water was added. Glucose was dissolved separately in 6 L flask. Both the flasks were plugged with cotton and aluminium foil and autoclaved for 45 and 25 minutes respectively. Glucose was added aspetically to the solution containing all other ingredients before using the medium in the reactor.

4.5 REACTOR SYSTEM AND PROCESS DESCRIPTION

A schematic diagram of the fluidized bed reactor used for the production of patulin is shown in Figure 4.2 (modified from Berk et al., 1982). Filtered air was introduced from the bottom of the reactor. A porous fritted glass distributor was used to get good air distribution in the reactor. A peristaltic pump was used to pump the liquid feed into the reactor. The overflow outlet was plugged with a piece of coarse mesh crumpled wire that allowed the exit of the liquid but prevented the loss of beads. A 10 mL syringe was used to inject the antifoaming agent from the top the reactor. As shown in the Figure 4.2 the inlet at the top was covered with a rubber stopper, thus minimizing the risk of contamination. The temperature inside the reactor was maintained at 28^{o}C by continuously circulating water in the jacket from the constant temperature water bath.

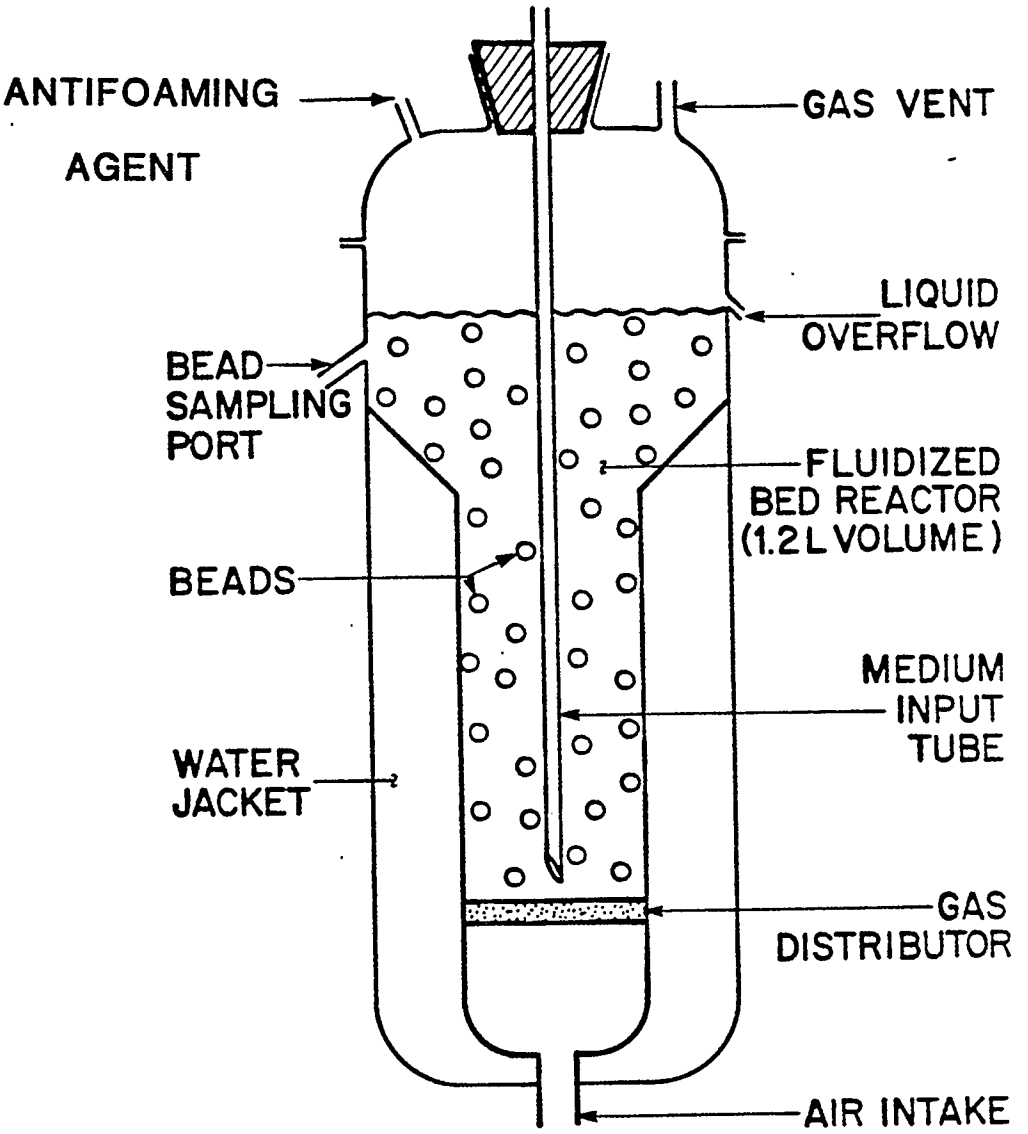


Figure 4.2 Schematic diagram of the three phase fluidized bed reactor used for Patulin production (Modified from Berk et al., 1982).

Total working volume of the reactor was 1.2 litres. Diameter of the reactor was 7.5 cm, the height 17.0 cm and the diameter of the disengaging section was 10.5 cm. Under typical operating conditions the superficial air velocity was 1.4 cm/s. The gas was uniformly distributed throughout the reactor in the form of tiny bubbles. The initial volume of the beads was approximately 400 mL (about 20,000 large beads, 3.2 mm diameter) resulting in initial solid hold up of approximately 33%. Liquid and gas holdups typically were 60% and 7% respectively.

Figure 4.3 illustrates the sequence of the various operations involved in the process (Gaucher and Behie, 1984).Beads of very narrow size distribution (3.2±0.13 and 1.75±0.16 mm) from the 4% (wt/vol.) carrageenan solution containing spores of P.urticae (mixture kept at 37°C) were made using the multiple syringe device (bead machine). Beads were stirred constantly for 3 - 4 hours in the salt solution maintained at 4°C to allow the beads to harden. After washing the beads with dd water for 3-4 times, they were transferred into a 2 L Fernbach flask containing growth medium. This flask was kept on a rotary shaker (300 rpm, 28°C) for a 48 hour incubation period. Then these beads containing grown cells were washed with phosphate buffer (KH_2PO_4 concentration of 13.6 g/L,pH= 6.5) and transferred into the fluidized bed reactor for the production of the antibiotic patulin. Humidified air was introduced from the bottom and at the same time, the pump for feeding liquid feed was switched on. Samples of reaction medium weretaken regularly (after every 24 hours) and were

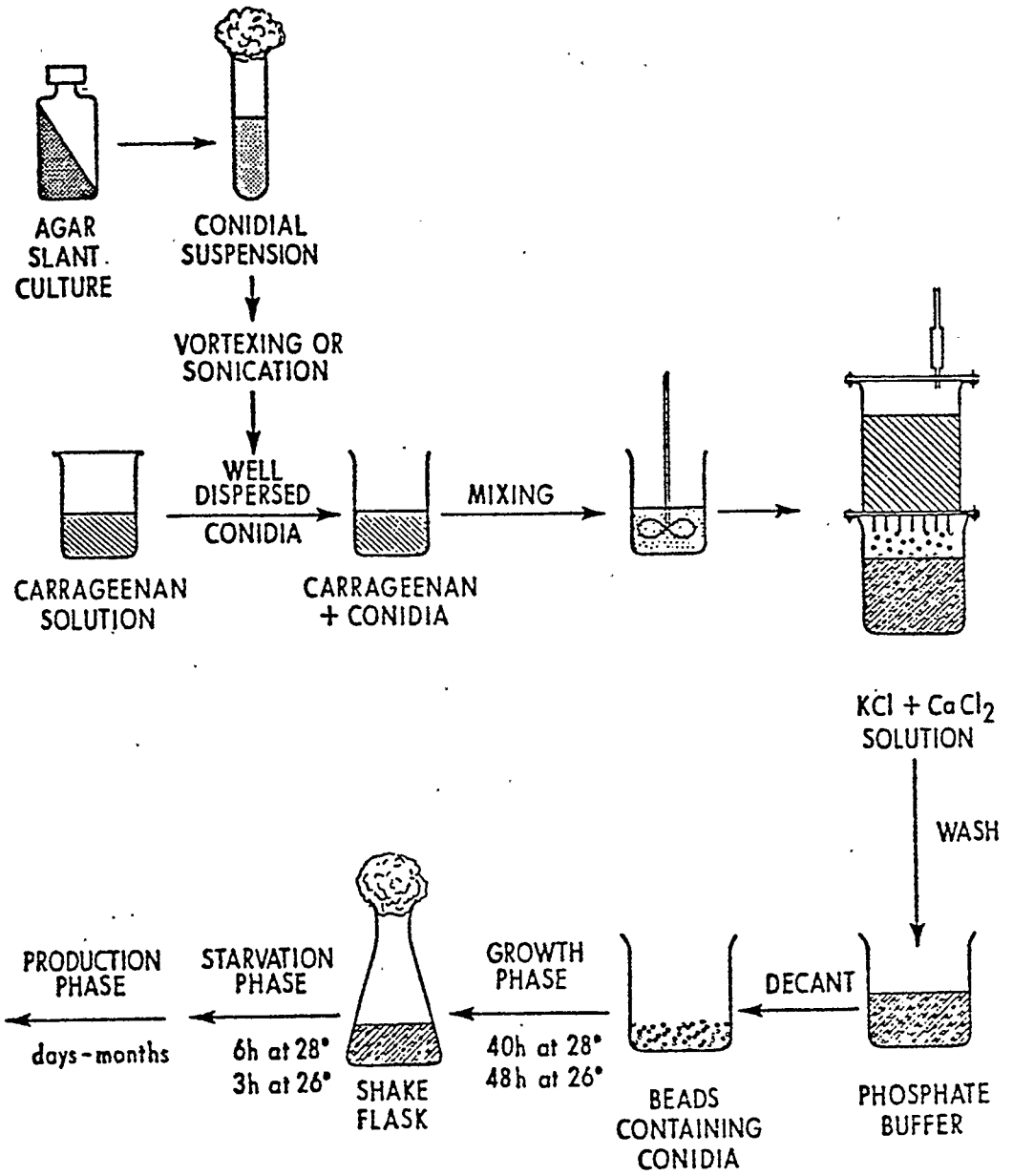


Figure 4.3 Process Sequence for Patulin Production.
(From Gaucher and Behie,1984)

frozen immediately in order to minimize any chances of changes in the concentration of product and other intermediates due to evaporation.

4.6 SAMPLING METHODS

Various sampling procedures employed during the course of a reaction run are described below.

4.6.1 Sampling For Protein

Samples of the beads were collected from the reactor at various time intervals during the production phase. These beads were washed 2-3 times with double deionized water and then transferred into a 100 mL beaker. Liquid nitrogen was poured into the beaker. This beaker was covered with parafilm and aluminium foil and kept in a round bottomed freeze drying flask. The beads were lyophilized for about 72 hours and were used for subsequent protein assays (Jones et al.,1984). At the end of the reaction run, all the beads were removed from the reactor, washed, freeze dried as described above and weighed for determining the total number of beads and amount of protein in the reactor.

4.6.2 Sampling For Patulin And 6-MSA

After about every 15 - 24 hours, about 2 - 3 mL of liquid sample was withdrawn from the reactor into a 22 mL vial. It was directly used for measuring pH using a pH meter (Fisher Acumet, Model 320). The sample was then frozen in a tightly capped vial for subse-

quent High Pressure Liquid Chromatographic (HPLC) analysis of secondary metabolites (discussed in Section 4.7) and also for glucose assay (Section 4.9).

4.6.3 Sampling For Microscopic Analysis

A sample of beads(10 - 12) containing immobilized cells of P.urticae were removed from the shaker flask before and after the growth phase. Beads for scanning electron microscopy (SEM) were washed 7 - 8 times with phosphate buffer (KH_2PO_4, 0.1M and pH = 7.0) and then fixed in a 5% glutaraldehyde solution in phosphate buffer for about 4 to 5 hours. This helps in driving water out of the carrageenan beads. Again these beads were washed with phosphate buffer 6 - 7 times so that there was no glutaraldehyde solution left along with the beads. These fixed beads were dehydrated in an ethanol - water mixture in series starting from 10% (v/v) ethanol in double deionized (dd) water upto 70% (v/v) ethanol in dd water with a increment of 20%. At every step the beads were kept in the ethanol-water mixture for about 20 minutes. Beads were further dehydrated in ethanol concentrations of, 90%, 95%, 98% and 100% .

Beads for phase contrast microscopy were dehydrated as per Deo, 1982. Beads for scanning electron microscopy were further dehydrated in an ethanol - amyl acetate mixture in series starting from 10% (v/v) amyl acetate in ethanol up to 90% amyl acetate in ethanol with a increment of 20%. Then the beads were kept in 98% and 100% (twice) amyl acetate for about 20 minutes before wrapping them in

parafilm and freezing under liquid nitrogen. After freezing the wrapped beads were kept on a glass petri dish and cracked in to halves and both the halves were transferred to 100% amyl acetate. After peeling off the parafilm the beads were critical point dried in liquid carbon dioxide in a critical point drier.

The dry beads were placed on SEM specimen holder with the help of 5 minute epoxy. The specimens were coated with a very thin layer of metallic gold by using an Edwards S 150A Sputter Coater. The beads were then ready for examination under SEM (Cambridge S 150).

4.7 CHROMATOGRAPHIC ANALYSIS OF PATULIN AND 6-MSA

Patulin and 6 methyl-salicylic acid in culture filtrates were quantitated by high pressure liquid chromatography (HPLC). Prior to injection, the samples which were stored at -15^{o}C in 22 mL vials were kept in warm water for about 10 minutes and then filtered through 0.45 µm regenerated cellulose filters (RC 55). This was performed to ensure the complete removal of cells and particles which would clog filters on the liquid chromatograph or be deposited on the surface of the column. This procedure thus lengthens the life time of the column. The chromatographic system involved a RP 8 guard column(200 X 4.6 mm ID; 10 µm particle size , Hewlett Packard, Avondale Division, PA, USA). The guard column provides extra protection for the main column but effects only minimal separation of metabolites. The guard column and separation column were attached to an HP 1084 B (Hewlett Packard) liquid chromatograph equipped with an automatic sampling device,

variable volume injector and a variable wave length detector. The former allows upto 60 samples to be loaded and run automatically. The variable volume injector can inject from 10 to 200µL of sample. This volume depends upon the titre of the culture medium being analysed and in these assays, routinely 20 µL aliquots were injected. The variable wave- length detector offers the capability of changing the detection wavelength at any time during a run so that a compound can be quantitated at its absorption maximum, (i.e. 276 nm for patulin and 308 nm for 6-MSA). The liquid chromatograph is programmed with a 79850 B LC terminal(Hewlett Packard) which also integerates peaks on the chromatogram as compounds are eluted. Injection of a known amounts of standards (eg, patulin and 6-MSA) is used to calibrate the machine by the external standard methods and any peaks eluting at the retention time of the standards is quantitated by the terminal using the amounts to area ratio. By virtue of functions such as minimum area rejection, adjustable slope senstivity, automatic correction for an extra area due to drifting of base line etc., this integerator's reproducibility of area counts was approximately 95%. The minimum detectable quantity of patulin was approximately 2 mg/L, far below the concentration range of the analysed samples.

To separate patulin, 6-MSA and other pathway metabolites, the program listed in Table 4.5 was used. At time t = 0, the detector was set to measure at 254 nm (with reference beam at 430 nm). During the run at time 5.5 minutes, the wavelength was changed to 276 nm to maximize the quantitation of patulin which eluted at 7.8 $\pm$ 0.10

Table 4.5 Gradient Elution Program for the Separation of Patulin and 6-MSA.

Oven Temperature	35°C
Solvent Temperature	35°C
Solvent 'A' Acetic Acid.	Water containing 0.5%
Solvent 'B' (v/v)	50% acetonitrile/water containg 0.5% acetic acid.
Gradient Elution	Time(min) % B
	0.0 10.0
	30.0 70.0
	33.0 10.0
	36.0 STOP

minutes. After elution at time 10.50 minutes, the wavelength was returned to 254 nm for quantitation of other metabolites. The compound 6-MSA elutes at 24.05 ± 0.13 minutes. A typical chromatogram is shown in Figure 4.4. Retention times for patulin and 6-MSA are 9.46 minutes and 24.17 minutes respectively.

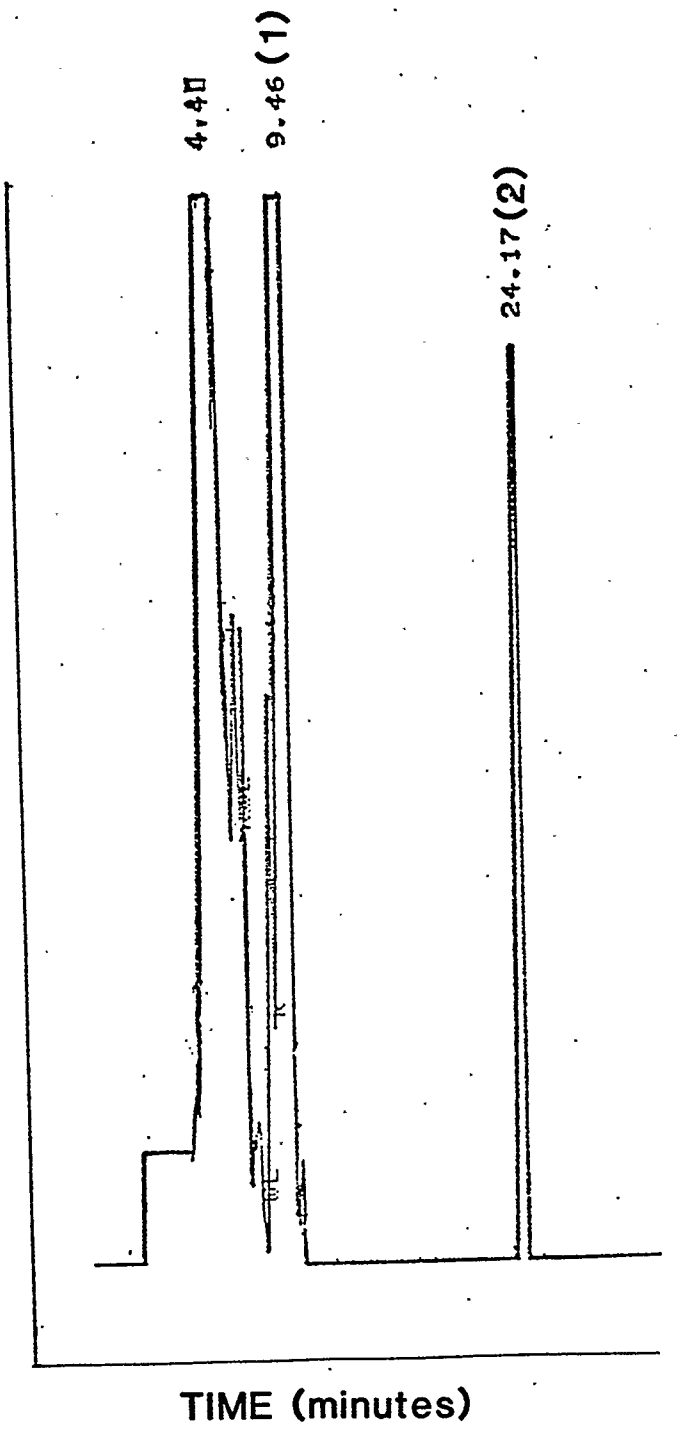


Figure 4.4 High Pressure Liquid Chromatogram.
(1) Patulin, (2) 6-MSA.

4.8 PROTEIN ASSAY

Immobilized spores of Penicillium urticae initially germinate and form hyphal strands throughout the bead. By 16 hours in growth medium, a thick mycellial mass developes around the periphery of the bead (Deo, 1982). A true estimate of dry weight can not be made because it has proven difficult to dissolve away the cell encapsulated carrageenan. Therefore, it was decided to use cell protein as a measure of the growth and productivity of immobilized cells. In addition, protein is also a better measure of fungal growth, since these organisms are known to increase their dry weight by accumulation of carbohydrates during the production phase of secondary metabolites.

The protein assay developed in our laboratories by Jones et al.(1983) was used for estimating the growth and productivity of immobilized cells of Penicillium urticae. The details of the assay are as follow.

Various samples of beads at different times during the bioreactor run were taken. After washing these beads with dd water(2 - 3 times) they were frozen in liquid nitrogen and freeze dried for about 3 days. After freeze drying, the beads were kept in open air for about an hour and groups of 50, 100, 150 beads were weighed to find the average weight per bead. The protein varied from 0.38 - 0.85 mg/bead. These beads were then crushed to a fine powder using a mortar and pestle. To this dry powder (about 20 mg) was added 10 mL of HCl - NaCl solution (0.9 g NaCl in 100 mL 1N HCl). After mixing on a

vortex mixer, this slurry was heated for about 15 - 20 minutes at 90°C. This partially hydrolysed the carrageenan so that it did not gel upon cooling. Once cool, the slurry was centrifuged 12,800 X g for 5 minutes in an Eppendorf centrifuge (Brinkman). The clear supernatant was removed with a Pasteur pippet and discarded. The precipitate was again washed with 1.0 mL of HCl - NaCl (0.9 g NaCl in 100 mL 1N HCl). After centrifugation and removal of this supernatant with a Pasteur pippet, 0.75 mL acetone was added to the residue. This removed excess HCl and phenolics. After a third centrifugation, the supernatant was removed by decantation and solids were heated at 90°C for 2 - 3 minutes to remove excess solvent. To this residue was added 1.0 mL of 1N NaOH and each residue was heated to 90°C for about 15 minutes. Upon cooling, each sample was centrifuged for the last time for about 5 minutes and 50 - 100 µL samples of the clear supernatant were analysed for protein by Folin and Lowry method (Lowry et al., 1951).

The Folin Lowry assay was carried out as follows. The following reagents were used.

(1) 1% $CuSO_4.5H_2O$

(2) 2% Na_2CO_3 in 0.1N NaOH

(3) 2% Sodium Potassium Tartarate

(4) Folin Lowry reagent diluted appropriately in double deionized water

(1), (2) and (3) were added in the ratio of 1:1:100. A sample containing 5 mL of this mixture was added to 0.5 mL of appropriately diluted supernatant. It was allowed to equilibrate for 20 - 25 minutes and then 0.5 mL of Folin phenol reagent was added. Colour was allowed to develop for about 10 minutes and then the optical density was measured at 660 nm using a spectrophotometer. Bovine serum albumin (BSA) was used as a standard and it gave a linear plot as shown in Figure 4.5 Hence, the protein content of immobilized cells was determined by using a standard as described above. About 300 mg of BSA was added to 45 mL of 1.25% carrageenan containing 0.05% LBG. This mixture was solidified by cooling and then freeze dried. Freeze dried block was then powdered and stored at $4^{o}C$ for subsequent preparations of standard plots for estimating the protein concentration in carrageenan beads.

4.9 ASSAY OF GLUCOSE IN CULTURE FILTRATES

In order to find the specific productivity of the secondary metabolite, the concentration of the substrate (glucose) in the reactor has to be known. Samples of culture filtrates collected from the bioreactor during the run and frozen in 22 mL vials were kept in warm

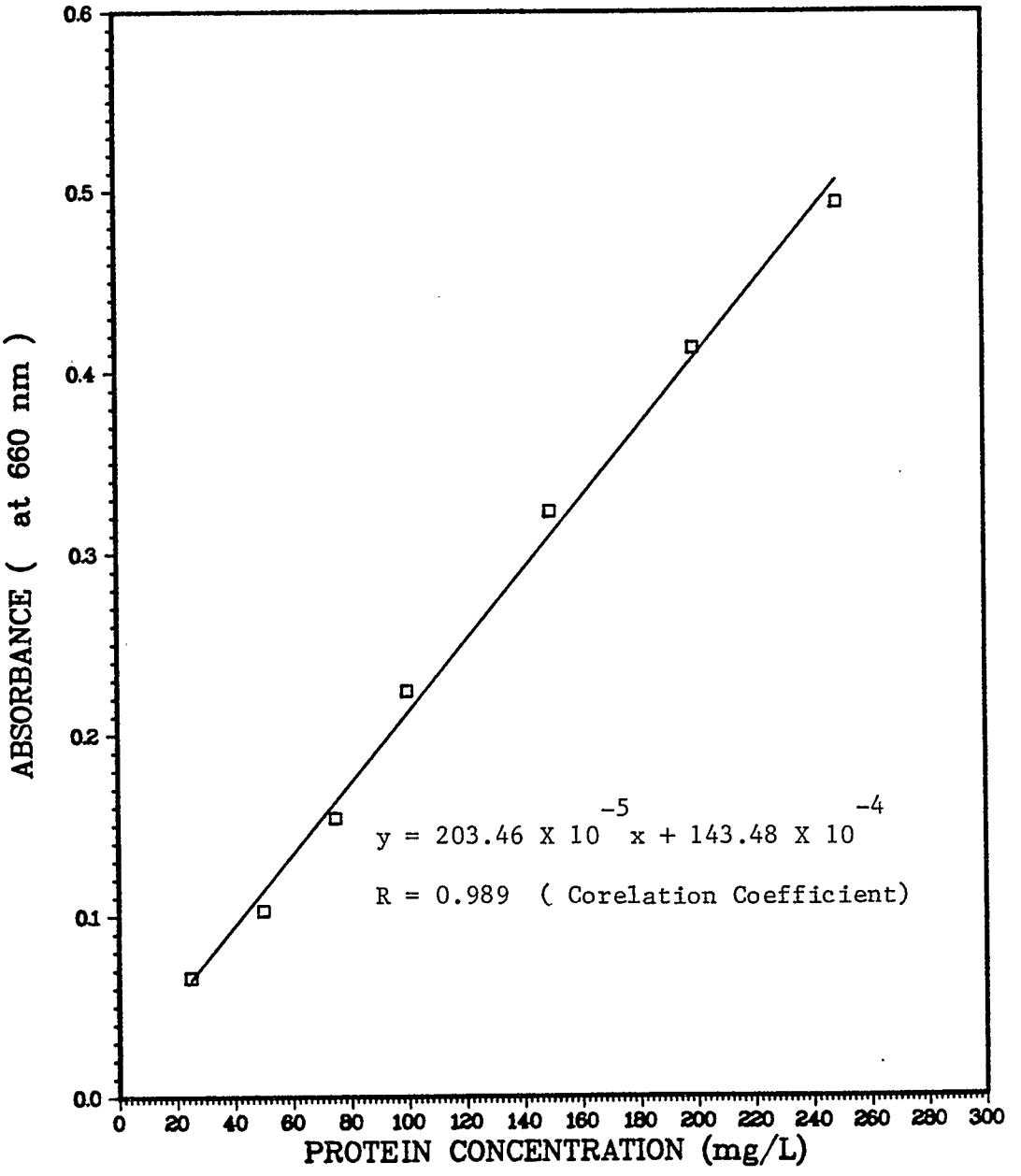


Figure 4.5 Standard Calibration Plot for the Protein Assay Using Bovine Serum.

water for about 10 minutes. before using them for estimation of glucose content in the reactor. Glucose assay was performed with the Worthington Statzyme kit. The procedure depends on the following series of reactions:

$$\beta\text{-D-glucose} + O_2 + H_2O \longrightarrow \text{D-glucono-}\delta\text{-lactone} + H_2O_2$$

$$H_2O_2 + \text{4-amino-antipyrene} + \text{p-hydroxybenzoate} \longrightarrow \text{quinoamine dye} + H_2O$$

In the initial reaction, β-D-glucose is oxidised by glucose oxidase to produce D-gluconolactone and hydrogen peroxide. In the second reaction the peroxide is oxidatively coupled with the chromogen, 4-amino-antipyrene in the presence of p-hydroxybenzoate to yield quinoamine chromophore which absorbs maximally at 500 nm. The change in absorbance is directly proportional to the glucose concentrations in the samples.

4.9.1 Materials and Methods

Test tubes : 14 X 100 mm

Eppendorf pipettes

100 mL volumetric flask.

Water bath at 37°C.

uV/vis Spectrophotometer(Varian 200) and 1 cm cuvettes.

Worthington Statzyme glucose reagent was reconstituted with 50.0 mL distilled water. In dissolving the mixture care should be taken not to denature the enzyme by excess shaking. The reagent is

stable for 12 hours at room temperature or 5 days at 2^{o}-6^{o}C.

A glucose standard was prepared by dissolving 100 mg glucose in 100 mL distilled water in a volumetric flask. The assay was performed by adding 1.0 mL of the glucostat reagent to the appropriately diluted sample of the culture filtrate (10 µL) and incubating at 37^{o}C in water bath for exactly 10 minutes. After cooling to room temperature, the absorbance was read at 500 nm by using a Spectrophotometer(Varian 200).

A stock solution of glucose was prepared to contain 1.012 g/ L. From this stock solution 50 µL aliquots were removed and diluted with water as shown in Table 4.6. These dilutions were used in construction of standard curve(Figure 4.6).

From each standard solution a 20 µL aliquots was added to a test tube. To these samples was then added 1.0 mL Glucostat reagent, mixed on a vortex mixer and tubes incubated at 37^{o}C. After 10 minutes, the tubes were removed and after cooling to room temperature the absorbance was read at 500 nm. The concentration of glucose, expressed as mg/L is then plotted against the E_{500} (absorbance at 500 nm) and a typical result is shown in Figure 4.6. For analysis of culture samples, these were diluted to contain 450 - 700 mg glucose/L. Aliquots (20 µL) of these diluted samples were then assayed for glucose by the method described. Usually two different dilutions of each sample were prepared. The standard curve was constructed by entering the data in HP 41-CV calculator and performing a linear regression.

Table 4.6. Glucose Dilutions for the construction of Standard Curve for Glucose Assay.

TUBE #	VOL.STOCK SOL.(mg/L)	VOLUME H_2O (µL)	GLUCOSE (mg/L)	E_{500}
1	50	25	674.67	0.437
2	50	50	506.0	0.346
3	50	150	253.0	0.179
4	50	200	202.4	0.147
5	50	350	126.5	0.093
6	50	0	1012.0	0.651

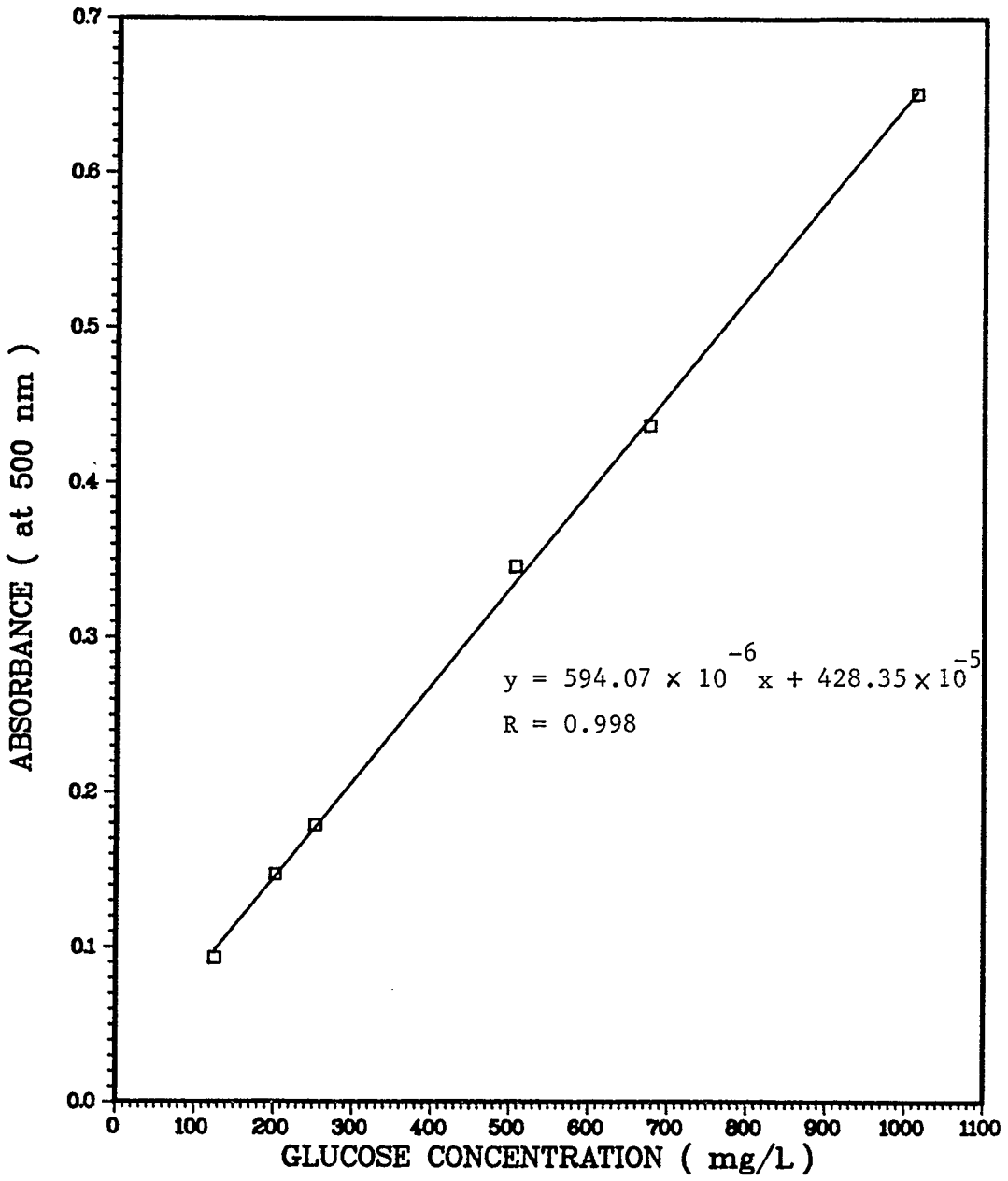


Figure 4.6 Standard Calibration Plot for Glucose Assay.

4.10 DIFFUSION CHARACTERISTICS OF SUBSRATES IN CARRAGEENAN BEADS

Natural or synthetic polymers are used as the matrix in some of the immobilized techniques to entrap protein, enzymes, whole microbial, plant and animal cells. The physiochemical characteristics of such a matrix in gel form can have an effect on the reactions of the biologically active material entrapped in the gel. The pore size of the gel, reflected by the viscosity of the carrier, due to the size of the molecule, and/or its concentration can effect the diffusion of the substrates or products and limit the reaction rates of the entrapped cells or enzymes.

The diffusion characteristics of the synthetic polyacrylamide gels have been studied by White et al.(1960,1961). Proteins and enzymes as well as whole cells, can be immobilized in polyacrylamide gels. The leakage of proteins and cells is minimal because of the small pore size of the carrier. On the other hand, the diffusion of low molecular weight substrates like KCl and urea is affected by the pore size. Tanaka et al.(1984) studied the diffusion characteristics of substrates in Ca-Alginate gel beads. They studied the diffusion of glucose, albumin, δ- lactoalbimin etc. into and out of gel beads.

There is no information in the literature on any studies done on diffusion characteristics of various substrates in polysaccharide gel beads. As carrageenan was the support matrix for immobil-

izing the whole cells of Penicillium urticae for the reasons described in Section 1.6, it was decided to study the diffusion of substrates like glucose, 6-MSA and patulin into these beads so that their effective diffusivities could be determined. Effective diffusivities of the reactants and products have to be known in order to model the reactor.

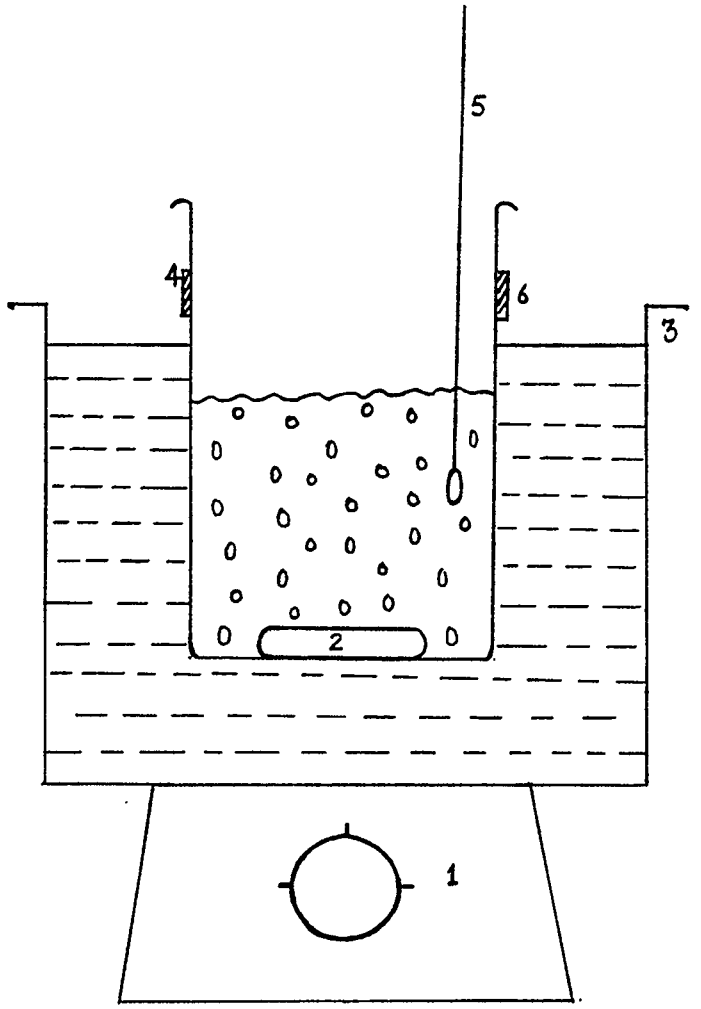
4.10.1 Materials And Methods

The experiment was divided into two parts. In first part diffusivity studies were performed using plain carrageenan beads and in the second part carrageenan beads containing dead cells were used. The reason for using carrageenan beads containing dead cells for diffusion experiments was, to study the effect of the physical barrier offered by these cells near the surface of the bead (discussed in Section 5.7) on the diffusion of substrates into the support matrix.

Beads of size 3.2 mm were prepared as described in Section 4.2. The experimental set-up is shown in Figure 4.7. About 200 mL of a solution of 4.3 g/L of glucose was made. The water bath was maintained at 28^{o}C so as to conduct the experiment at the actual temperature of the reactor. A sample containing 40 mL of carrageenan beads (including the buffer) barely covered by phosphate buffer, was tran-

Figure 4.7 Schematic diagram of the reactor system used for the diffusion experiments.

1. Magnetic Stirrer and Heater
2. Teflon Bar
3. Water Bath
4. Glass Beaker Containing Carrageenan Beads and Solute Solution.
5. Thermometer
6. Clamps


5
4
6
3
2
1

sferred to a 250 mL beaker kept in the water bath and the magnetic stirrer was switched on. Then 80 mL of glucose solution was poured into the beaker containing the beads. The stop watch was started and the first sample (100 µL) of the solution was immediately taken using an Eppendorf pipette. This was the initial concentration used in calculating the diffusion coefficients. Out of the 100 µL of the sample taken 20 µL was transferred into each of two test tubes to duplicate the results. Samples were taken from the beaker at various times during the experiment. These samples were assayed for glucose by the method described in Section 4.9 The same procedure was repeated for the solute (glucose) concentration of 9.2 g/L and 22.5 g/L. For a glucose concentration of 22.5 g/L the experiment was repeated with two different stirrer speeds of 650 rpm and 173 rpm. A digital tachometer was used to measure the rpm. Diffusion characteristics of patulin and 6-MSA were also studied. Samples of Patulin and 6-MSA were obtained from our laboratory. A sample containing 200 mg/L of patulin and 6-MSA solution (in phosphate buffer) was made for this study.

In the second part of the experiment, a spore suspension of P.urticae was added to the solution of 4% (wt) carrageenan as described in Section 5. Beads were made in the salt solution and after washing 4 - 5 times with dd water (sterile), they were transferred into a flask containing growth medium (composition given in Table 4.2) and incubated for about 48 hours. These beads, on being removed from the shaker flask were washed thoroughly (6 - 7)

times with KH_2PO_4 buffer (pH = 6.5) and the cells were killed by keeping the beads in a 250 mL flask containing 0.02% solution of highly toxic sodium azide. The flask was covered with parafilm and aluminium foil. These beads were used for all subsequent experiments.

Beads were taken out of the azide solution and washed for about 9 - 10 times with phosphate buffer so that there were absolutely no traces of azide solution (slightly yellow in colour) are left. The experiment was performed with glucose concentration of 22.5 g/L using the beads containing cells and the samples were assayed for glucose. The next two runs were performed with patulin and 6-MSA as the solutes. Initial concentration of both patulin and 6-MSA was 200 mg/L (in phosphate buffer). In these two runs, 100 µL of sample was taken from the beaker at a given time and at least 20 µL was transferred to each of the two 22 mL vials. Samples were taken after about 1 minute until 30 minutes. The samples were kept in tightly capped vials and frozen for subsequent High Pressure Liquid Chromatographic assay of patulin and 6-MSA.

Before starting the experiment, 40 mL of beads and buffer were measured in to a graduated cylinder. The volume of buffer (clear supernatant) was measured and the volume of the liquid covering the bead surface was measured by drying all the beads on the paper towel and weighing the paper before and after drying the beads. The total volume of the supernatant varied from 12.8 mL to 13.2 mL. Beads were counted after the experiment. The average size of the bead was found

by subtracting the volume of the supernatant from 40 mL and then dividing this volume by the total number of beads to get volume/bead. The measurement of the bead size with vernier caliper was in close agreement with the one found by the volume method.

4.11 HYDRODYNAMIC STUDY OF A THREE PHASE FLUIDIZED BED REACTOR

The effectiveness of a three phase fluidized bed as a chemical reactor may be determined largely by its hydrodynamic properties. The term three phase fluidization is used to denote a variety of rather different operations. The classification of Figure 4.8 (Epstein, 1981) provides some clarification. The three phase referred to are gas, liquid and solids, though recently investigations have been performed using two immiscible liquids and particulate solids (Dakshinamurty et al., 1979, Roszak et al.,1979).

The reactor system used in this study comes under bubble supported solids (Figure 4.8). In this fluidization category the liquid is below its minimum fluidization velocity or even stationary (Roy et al., 1964) and serves mainly to transmit to the solids the momentum and potential energy of the bubbles (Naryanan et al., 1969) thus suspending the solids. This mode of operation is also referred to as three phase bubble fluidization. According to Volpicelli and Massimilla (1970) much greater gas to liquid flow ratios are possible with this mode of fluidization making it a more practical mode for certain processes. It is different from bubble column slurry opera-

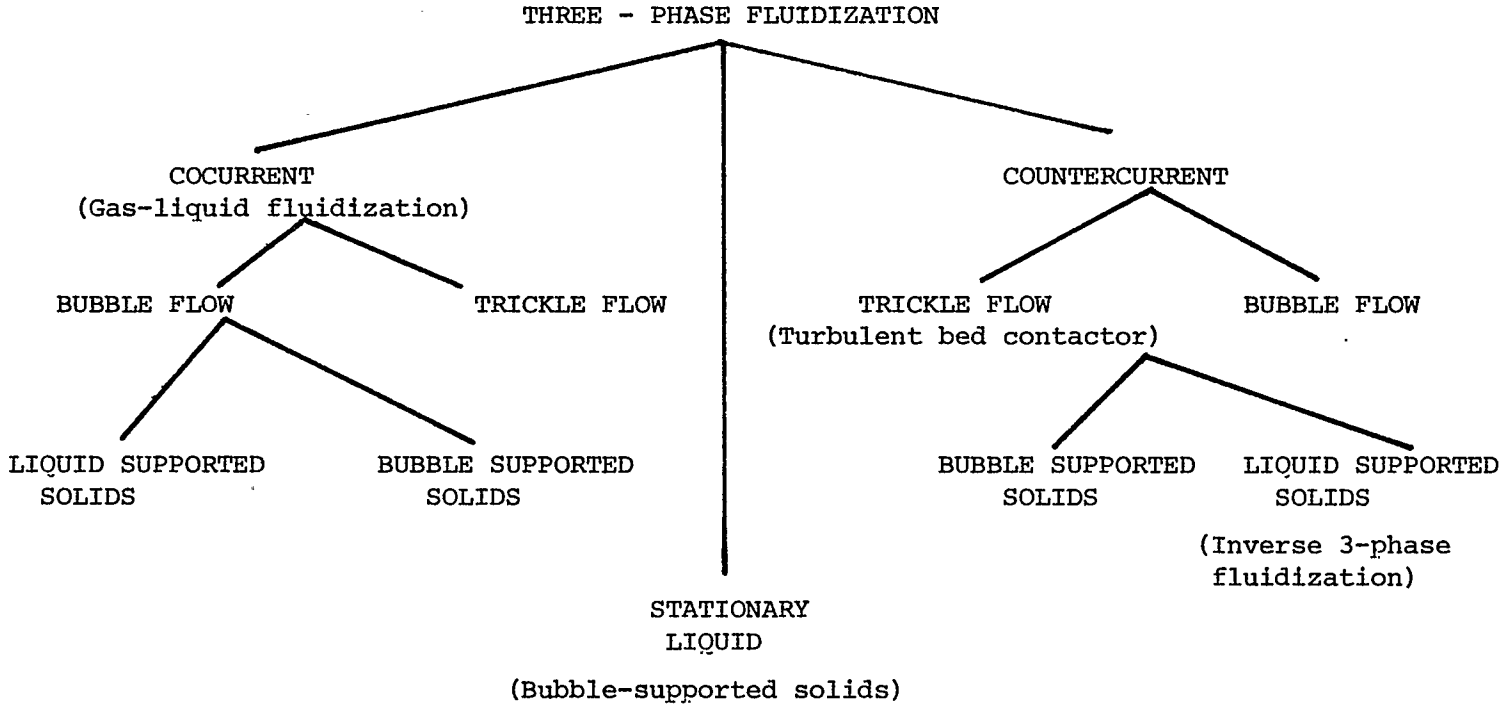


Figure 4.8 Classification of Three Phase Fluidisation.
(From Epstein,1981)

tion in its use of larger and/or heavier particles which are not subject to the hydraulic transport, characteristic of slurry operation.

For the present study, denser carrageenen beads (3.2 ± 0.17 mm) were made by mixing 140 -325 mesh stainless steel powder (4 - 5% wt/vol) with 4% (wt/vol) solution of carrageenan to ensure disengagement at the top of the reactor. The reason for mixing 4-5% of stainless steel was purely because of the area available for the flow of this mixture without blocking the needles. Higher concentration of stainless steel caused clogging of the needles. The mixture was delivered through an 18 gauge needle with the help of 30 mL syringe. The needle was 1 cm long, cut straight across and the wall tapered inwardly at the tip.

A fluidized bed reactor was constructed from 0.3 inch thick plexiglass. Total height of the reactor was 417 mm. Height of the main section of the column was 373.5 mm. Internal diameter of the column and the disengaging section was approximately 16.5 cm and 20.3 cm respectively. A plexiglass distributor (140 holes) was designed for thorough circulation of fine air bubbles. Six pressure taps were installed along the main column at a distance of approximately 6.2 cm from each other. Reference tap was about 2 cm above the distributor. Fine wire screen was used to prevent the loss of beads from the reactor and thereby, causing the blockage of the tubing leading to manometer. The ends of the pressure taps (A & B as shown in Figure 4.9) were connected to a differential manometer for measuring the static pressure along the column. Meriam fluid of specific gravity 1.75 was

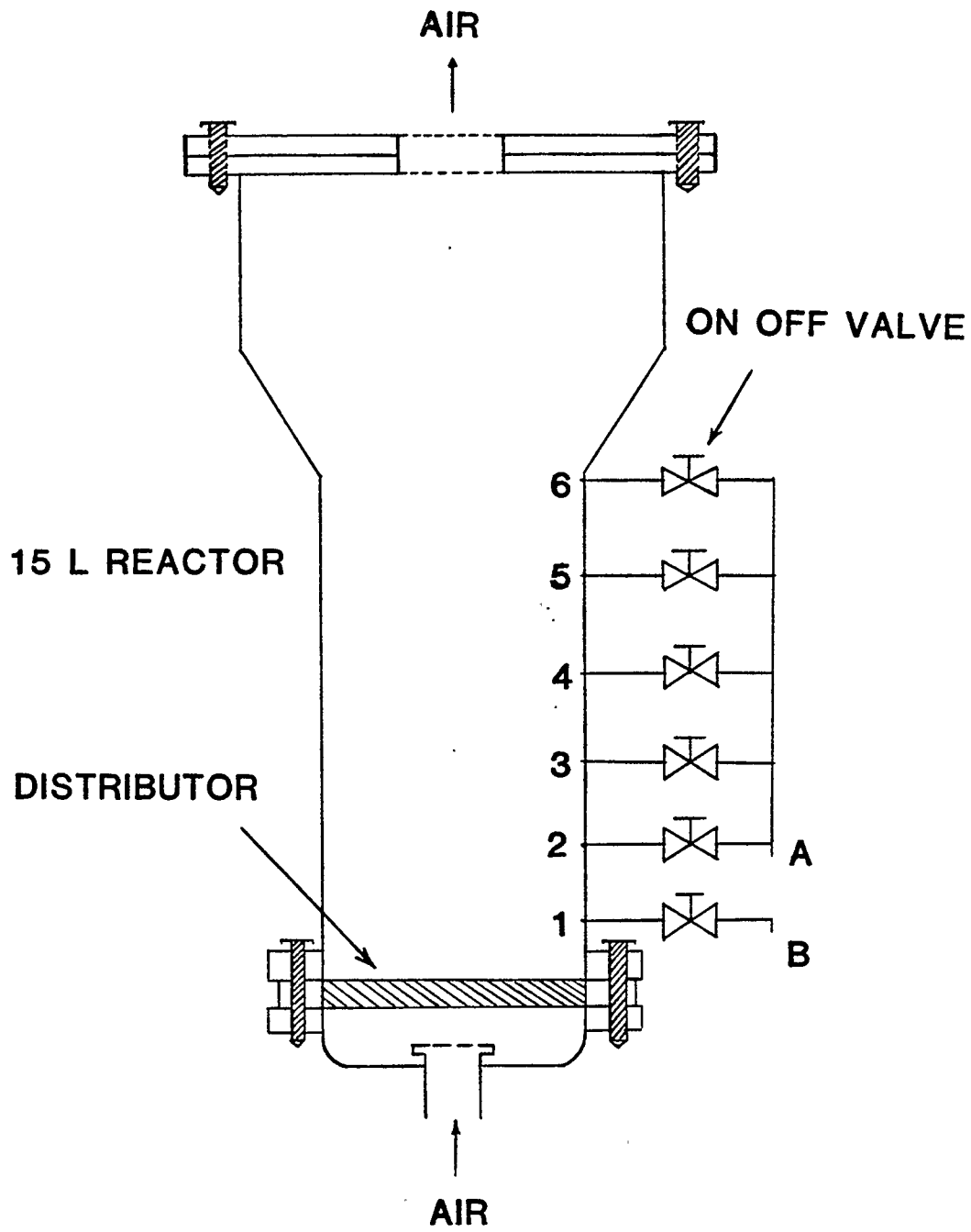


Figure 4.9 Schematic diagram of the fluidized bed used for the Hydrodynamic Study.
(Column ID=16.5 cm, Height = 37.35 cm).

used as a manometer fluid. Top of the disengaging section had an outlet for the air.

A weighed amount of solids was loaded into the reactor to give an initial bed height. Water was used as the liquid phase, air as the gas phase and 3.2 mm carrageenan beads (containing stainless steel powder, ρ=1.41 gm/cm^3) as the solid phase. Water was stationary throughout the experiment because in our experiment for the production of patulin, the liquid has a residence time of approximately 64 hours. The superficial velocity of the gas ranged from 1.4 cm/sec to 6.4 cm/sec. After transferring the solids and the liquid into the reactor, air was introduced at the desired superficial velocity. When steady state was reached, the pressure profile up the entire height of the column was measured using the manometer. Gas, liquid and solid hold-ups are calculated for the whole range of gas superficial velocity.

CHAPTER 5.

RESULTS AND DISCUSSION

5.1 CARRAGEENAN BEADS OF TWO SIZES

Spores of Penicillium urticae were immobilized in κ-carrageenan as described in Section 4.1. Beads formed by delivering the mixture of 4% wt/vol carrageenan and spore suspension of P.urticae were quite stable and did not have a large size distribution. As found by Deo (1982), the κ - carrageenan matrix did not have any deleterious effects on the ability of the cells to produce the antibiotic.

Beads of sizes 3.2 and 1.7 mm were cast successfully. Various methods were tried to produce smaller bead sizes (< 3.2 mm). These involved the use of nozzles, smaller needle size with pneumatic vibrations. But all these methods resulted either in no reduction in sizes or smaller size with very large size distribution and were therefore, rejected. It was decided to do detailed surface tension studies using SAG-471 (Union Carbide) surfactant because surface tension determines the force necessary to break the drop from the tip of the needle. The Critical Micelle Concentration (CMC) was found to be 525 mg/L. The needles were cut at the tip and the walls were tapered inwardly so that there was a smaller surface area available for the drop to form. This decrease in surface tension of the carrageenan mixture combined with the modified shape of the needles

resulted in an early fall of the drop from the needle and thereby producing beads of approximately 1.7 mm diameter.

5.2 GROWTH PHASE STUDY

Batch growth of P.urticae cells immobilized in carrageenan beads follows typical growth kinetics with lag, exponential and stationary growth phases. Figure 5.1 (Berk et al., 1983) shows the growth phase concentration of total biomass, glucose, patulin and 6-MSA(during stationary phase) for free cells of P.urticae.

The growth of the immobilized Penicillium urticae spores in carrageenan beads depends upon the specific rates of utilization of the nutrients and the rates at which they are transported. As shown in Figure 5.1 the specific rate of oxygen utilization, Q_{O_2}, of free P.urticae cells is observed during the exponential growth phase. After declining rapidly, the oxygen utilization remains constant during the stationary phase. Figure 5.1 also supports the established fact that the production of secondary metabolites begins only after the cessation of the active growth of the microbial cells has subsided (i.e. during the stationary phase). In case of patulin production by P.urticae the biosynthesis of the antibiotic is initiated by a limitation of nitrogen (GrootWassink and Gaucher, 1980). Glucose concentration declines slowly and reaches negligible values at about 75 hours which clearly indicates that the limitation of glucose will not have any effect on the producton of patulin. Maximum biomass concentration of 6.5 g/L is observed at about 80 hours. In case of

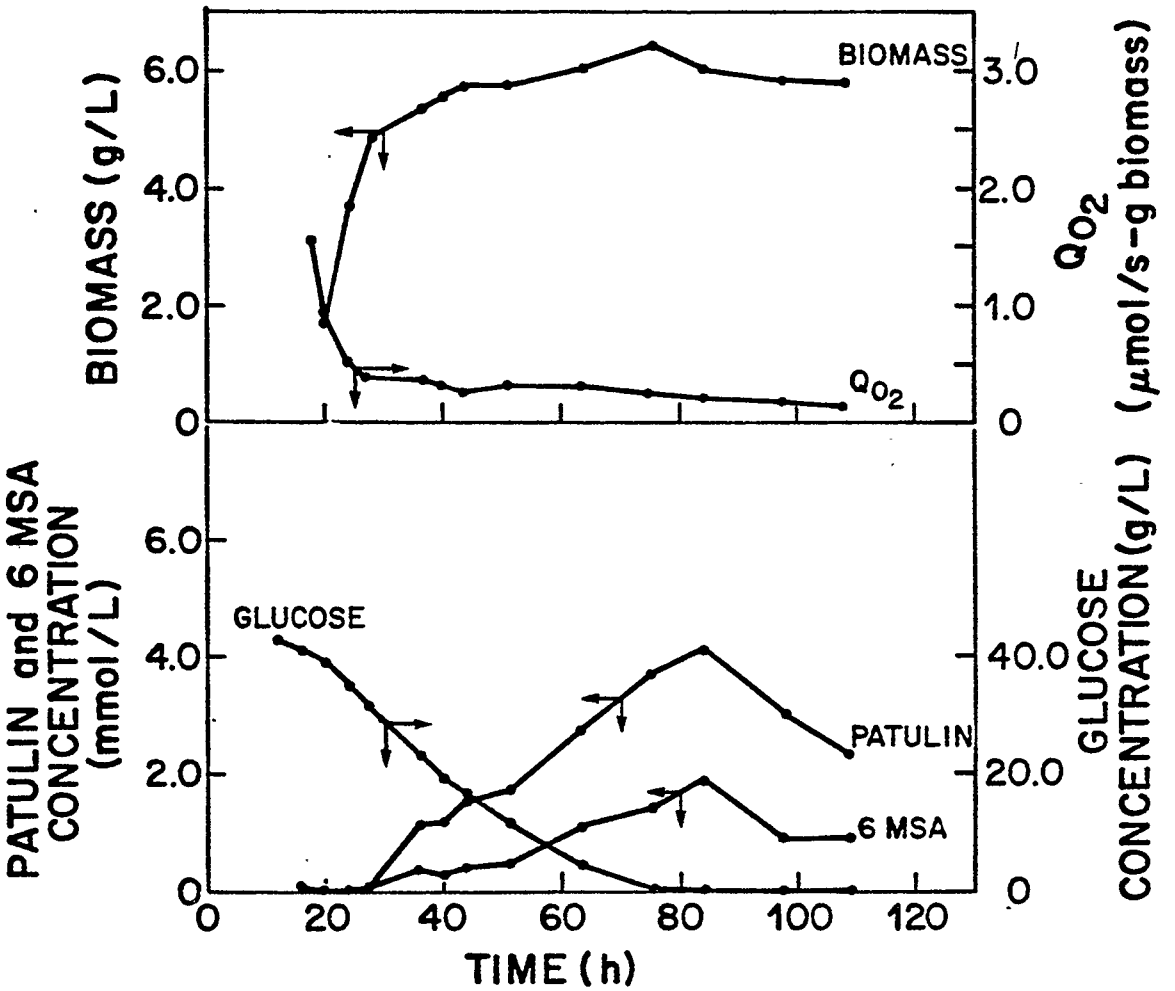


Figure 5.1 Batch growth of free P.urticae cells. The maximum oxygen utilization rate is observed during the rapid growth phase. Production of secondary metabolites starts during the stationary phase (Berk et al.,1983).

immobilized P.urticae cells it has been proven to be very difficult to find the dry weight of the cells. Therefore, all the reaction rates are on a per gram of protein basis which is representative of cell growth. Usually, the cell protein content is about 20% of the total biomass on a dry weight basis.

5.2.1 Specific Growth Rate And Yield Coefficients

The yield coefficient for cell growth, $Y_{x,s}$ was calculated for the growth phase (Figure 5.2). It is defined as the ratio of biomass produced per mass glucose utilized. As shown in Figure 5.2, it is 0.9 at about 20 hours and declines rapidly to about 0.15 at 50 hours and then remains almost constant. $Y_{x,s}$ is calculated from the following equation;

$$-\frac{ds}{dt} = M_s X + \frac{1}{Y_{X,s}} \frac{dX}{dt} \qquad (1)$$

where M_s is called the maintenance coefficient and depends upon the amount of substrate required just for the survival of the cells. $\frac{dx}{dt}$ represents the growth of the cells. M_s equals 0.027 g biomass/g glucose -h for Penicillium chrysogenum (Cooney, 1979) and is assumed the same for P.urticae because both P.chrysogenum and P.urticae come under molds and also there is no information about P.urticae in the literature. M_s has a negligible effect during growth phase and therefore, the first term on right side equals zero and equation simplifies to the following form:

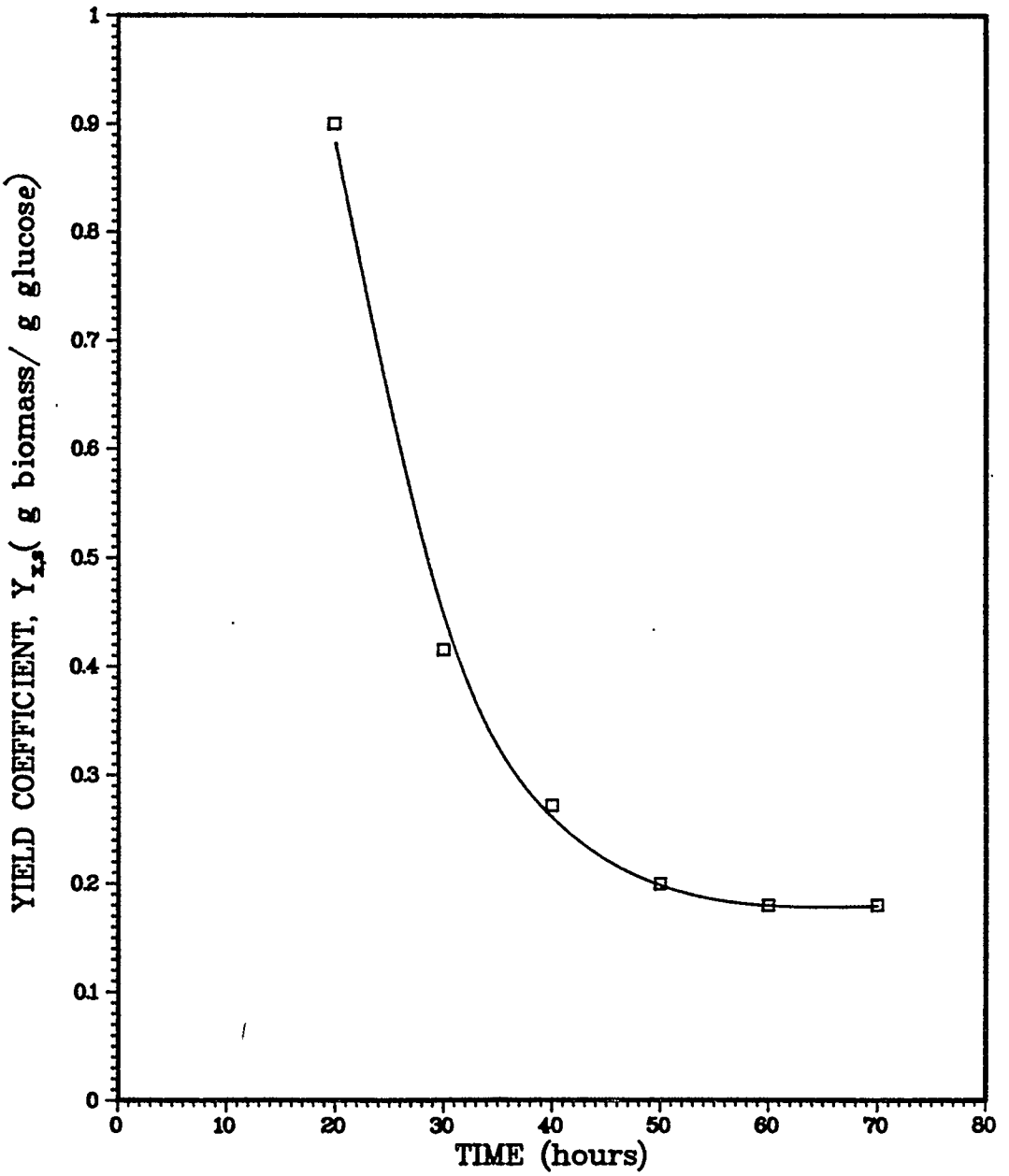


Figure 5.2 Variation of Yield Coefficient of biomass during growth phase of P.urticae cells.

$$-\frac{ds}{dt} = \frac{1}{Y_{x,s}} \frac{dx}{dt} \quad (2)$$

For exponential growth phase $Y_{x,s}$ is calculated to be 0.315 g biomass/g glucose for free cells of P.urticae.

During the exponential growth phase, only a single parameter μ is required to characterize the population. It is called the specific growth rate, and its magnitude is widely used to describe the influence of the cell's environment on its performance. μ is defined as follows:

$$\frac{1}{X} \frac{dX}{dt} = \mu \quad (3)$$

$$\text{or} \quad \ln \frac{X}{X_o} = \mu t \quad (4)$$

$$\text{or} \quad X = X_o \exp(\mu t) \quad (5)$$

where X_o is the biomass at the beginning of the exponential phase and X at any time 't'. From the equations above. we can readily deduce the time required to double the population (t_d).

$$t_d = \frac{\ln 2}{\mu} \quad (6)$$

Maximum specific growth rate of the free cells during the exponential growth phase (μ_m) was calculated to be 0.11 h^{-1} giving doubling time (t_d) of approximately 6.30 hours.

5.3 MICROSCOPIC ASSESSMENT OF GROWTH

Samples of the beads were taken at the end of the exponential growth phase. These beads were sliced into very thin sections (10 μ thick) using a rotary microtome with the assistance of Dr. Yeung of the department of Biology of University of Calgary. The complete procedure of making thin sections of the beads for the microscopic assessment of growth involved the following steps.

1. Parafilm Procedure.
2. TBA Series
3. Plastic Procedures
4. Staining.

Steps 1 - 3 were as per Jensen, (1962). The step 4 (staining) was modified from Fisher, (1968). Paraffin was removed from the sections by placing the slides in xylene for 5 minutes. These sections were then partially hydrated by passing through a series of alcohols of decreasing concentration: absolute, 95%, 70% and 50% (5 minutes in each). Aniline blue-black (C.I. 20470, 100 mL of 7% acetic acid and 1 g Aniline blue-black) was used for staining (approx. 1 minute). Excess stain was removed in 0.5% acetic acid. The slides were washed in distilled water before placing briefly in 95% ethanol and then dehydrating the sections rapidly in 100% ethanol. These sections were passed through a mixture of 100% xylene and ethanol (1:1) and then placed in xylene for 5 minutes (2 changes) before mounting in Harleco. This procedure stained proteins a deep blue so that they could be easily seen under the phase contrast microscope.

Photo 5.1 and 5.2 clearly show that the maximum growth of the cells takes place near the surface of the bead. Higher density of the protein is observed in case of small beads (Photo 5.1). Photo 5.3 shows the scanning electron microscopic picture of the hyphae at the surface of the bead. The presence of this thick layer of mycelium near the surface of the bead may cause resistance to the transport of nutrients from the bulk liquid to the cells present at the center of the bead.

5.4 PRODUCTION OF PATULIN IN A CONTINUOUS BIOREACTOR

Patulin was produced in the 1.2 L continuous three phase fluidized bed reactor described in Section 4.5. The amount of glucose available to the cells in a continuous bioreactor depends upon the concentration of glucose in the feed and its flow rate. In patulin biosynthesis, the flow rate of the liquid medium was 12 mL/h giving a mean residence time of about 64 hours. The reactors were run successfully for more than 300 hours.

Diffusional effects during the producton of patulin were studied by conducting two simultaneous reactor runs using beads of diameter 3.2 and 1.75 mm. Figure 5.3 shows the variation in concentration of patulin with time in the reactor as quantitated by HPLC. In this Figure time zero refers to the beginning of production phase. The concentration of patulin is higher in the case of smaller beads which suggests that 1.75 mm beads have a higher rate of production of patulin than 3.2 mm beads. Antifoaming agent (SAG 471) was added to the

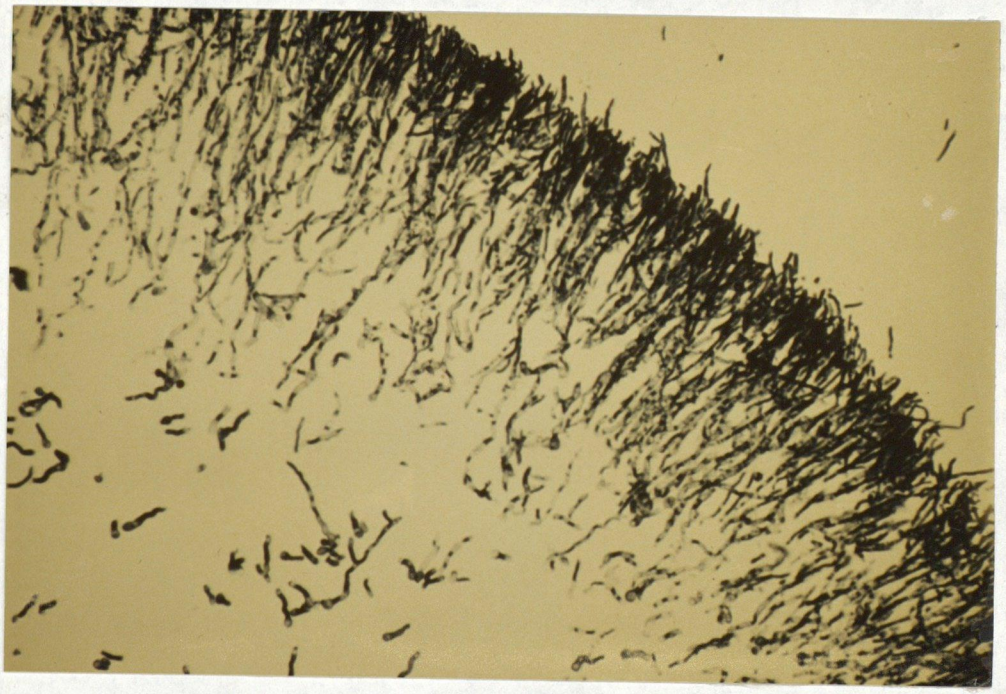


Photo 5.1 Phase contrast microscopic examination of a 1.75 mm carrageenan bead at the end of growth phase. Note the presence of relatively large amount of protein at the periphery (Magnification 200 X).

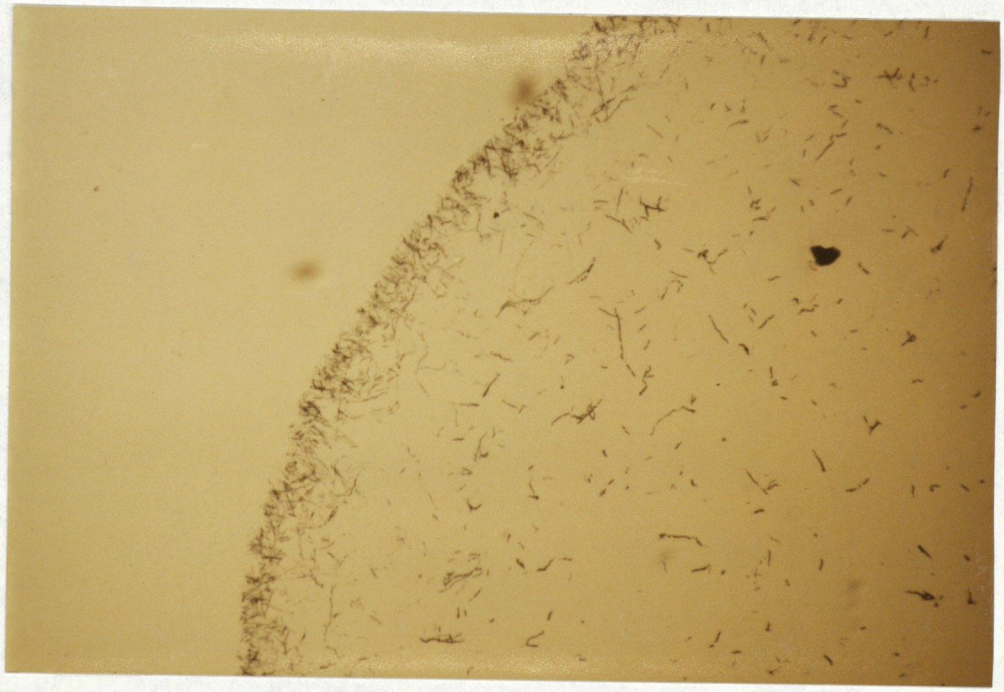


Photo 5.2 Microscopic examination of a 3.2 mm bead at the end of growth phase. There is less amount of protein towards the center as compared to the 1.75 mm bead (Magnification 100 X).

Photo 5.3 Scanning electron microscopic examination of the bead surface at the end of the growth phase.

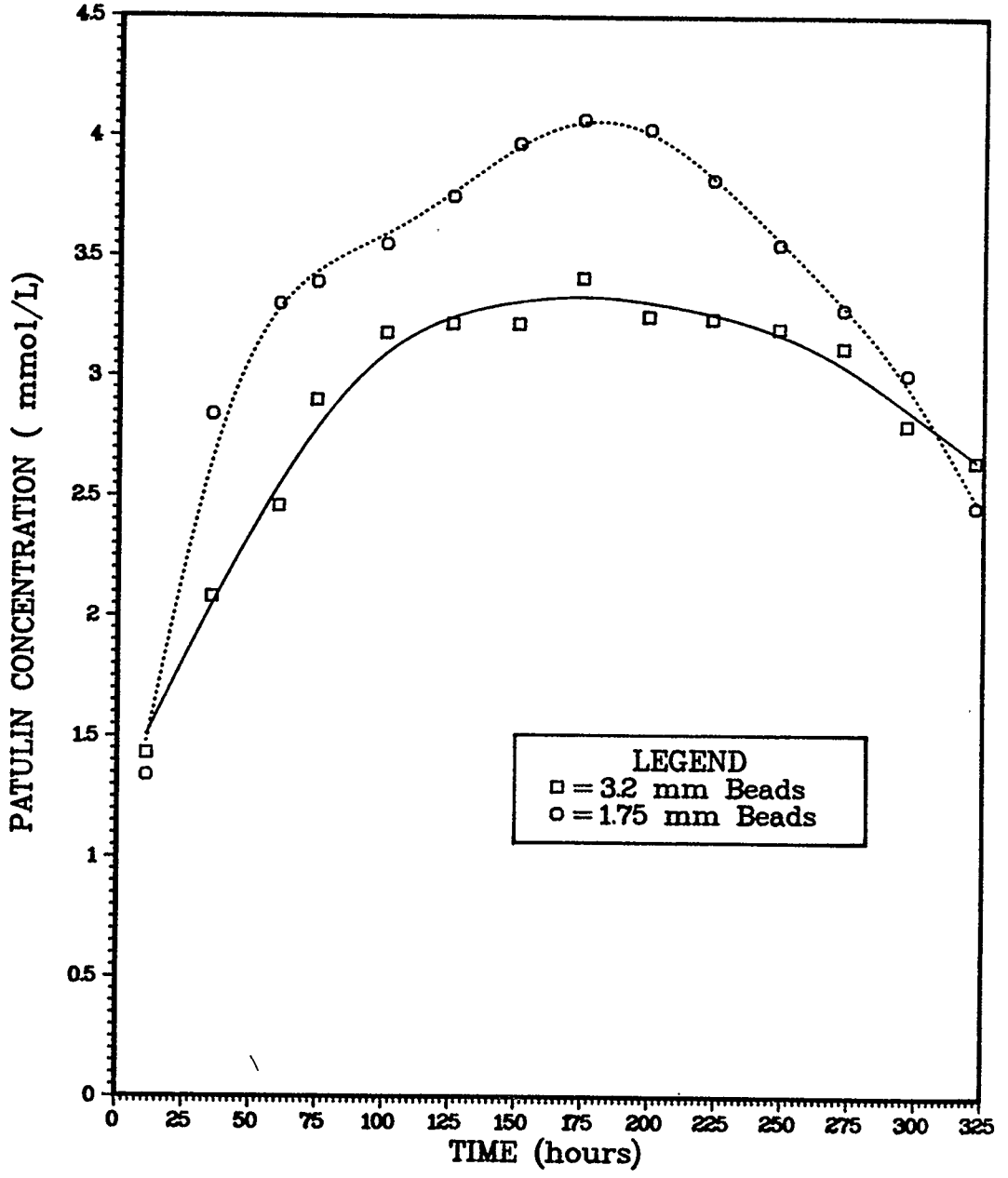


Figure 5.3 Variation in Patulin concentration during the production phase.

reactor medium to overcome the problem of extensive foaming during first 24 - 30 hours of the run. From 100 hours to 225 hours the concentration of patulin did not change much. Variation of 6-MSA concentration during the reactor run is shown in Figure 5.4. Concentration of 6-MSA in case of smaller beads is appreciably lower than that for larger beads. As discussed in Chapter 2, the first intermediate formed during the production of patulin is 6-MSA and this is the only intermediate which is present all along the reaction path suggesting that the decarboxylation to m-cresol is the slowest reaction step while other reactions proceed at much faster rates. Some of the 6-MSA formed is converted into patulin and the rest remains in the culture filterate. This agrees with the fact that the enzyme which catalyzes this reaction step is relatively labile (Gaucher et al., 1981). An indication of the catalytic ability of the cells to convert 6-MSA to patulin can be obtained from Figure 5.5. 'η' is called pathway efficiency (Berk et al.,1984 a) and is defined as the ratio of patulin concentration in the reactor to the total available 6-MSA, (i.e.the fraction of 6-MSA converted to patulin). Since 1 mole of 6-MSA produces 1 mole of patulin, therefore, the sum of the two concentrations ($C_p + C_m$) gives the total 6-MSA available in the reactor for biosynthesis.

$$\eta = \frac{C_p}{C_p + C_m}$$

The pathway efficiency for the large beads starts at around 40% at 75 hours and goes up to 53% and stays there till 250

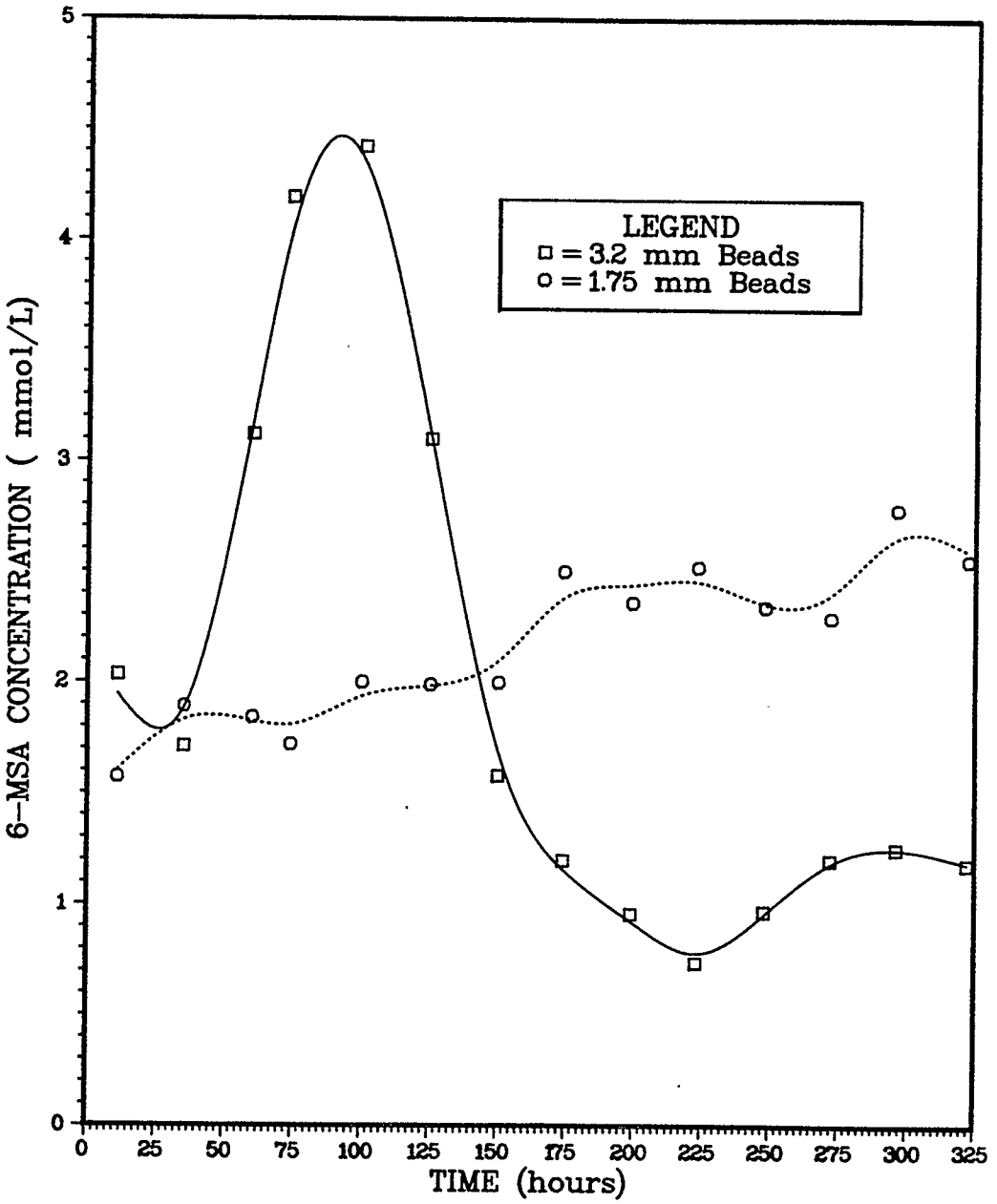


Figure 5.4 Variation in 6-MSA concentration during the continuous reactor run.

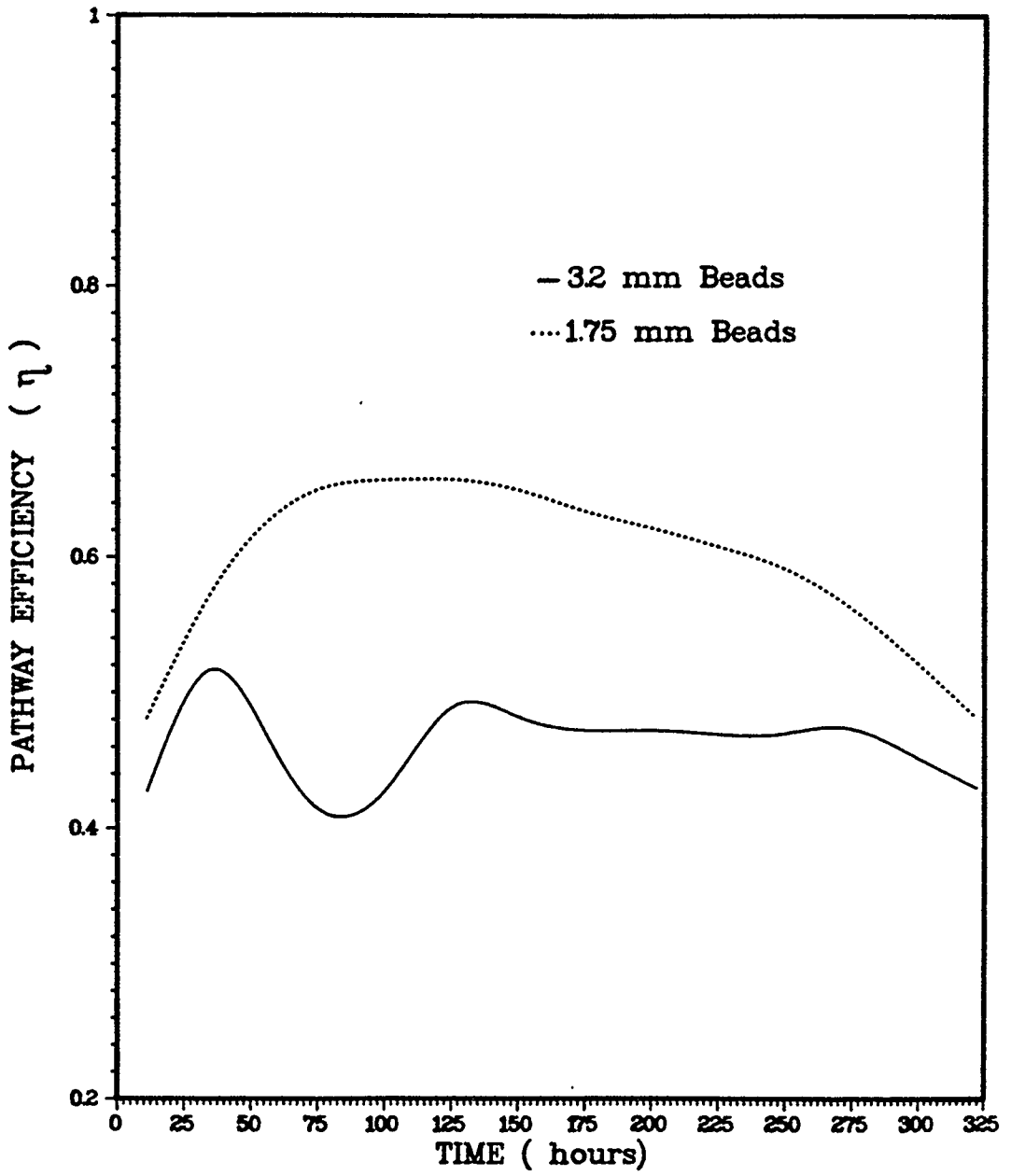


Figure 5.5 Variation of Pathway Efficiency with time during the continuous reactor run.

hours before declining again. For smaller beads, η is much higher. Initially it is 45% but goes upto 68% and is fairly constant till 210 hours before declining to 43%. All through the reactor run it is higher in the case of smaller beads which implies that the cells are more active (in terms of converting 6-MSA to patulin).

Growth of the cells is reflected by the total protein present in the reactor. Samples of beads were taken at various time intervals and were assayed for protein (Table 5.1). Total amount of protein in the reactor was calculated by multiplying the protein/bead and total number of beads in the reactor. Figure 5.6 shows the variation in the total amount of protein in the reactor during the patulin production phase. All the reaction rates (calculated later in the chapter) would be based on the unit mass of the protein present in the reactor at that time. There was approximately 29 and 22% increase in the amount of protein during production phase for the large and small beads respectively indicating that more substrate is utilized for the growth of cells in large beads as compared to the small ones.

5.5 GLOBAL RATES OF REACTION FOR PATULIN AND GLUCOSE

Material balance was performed on patulin to calculate the global rate of reaction for patulin production as follows,

Rate of production of patulin = Output - Input + Accumulation

The Accumulation term consists of accumulation in the liquid and

Table 5.1 Protein Content per Bead as a function of Time During the Production Phase.

Time (days)	mg protein /bead (Small Beads)	mg protein/bead (Large Bead)
0	12.7	38.83
6.25	13.80	45.41
13.42	15.80	49.71

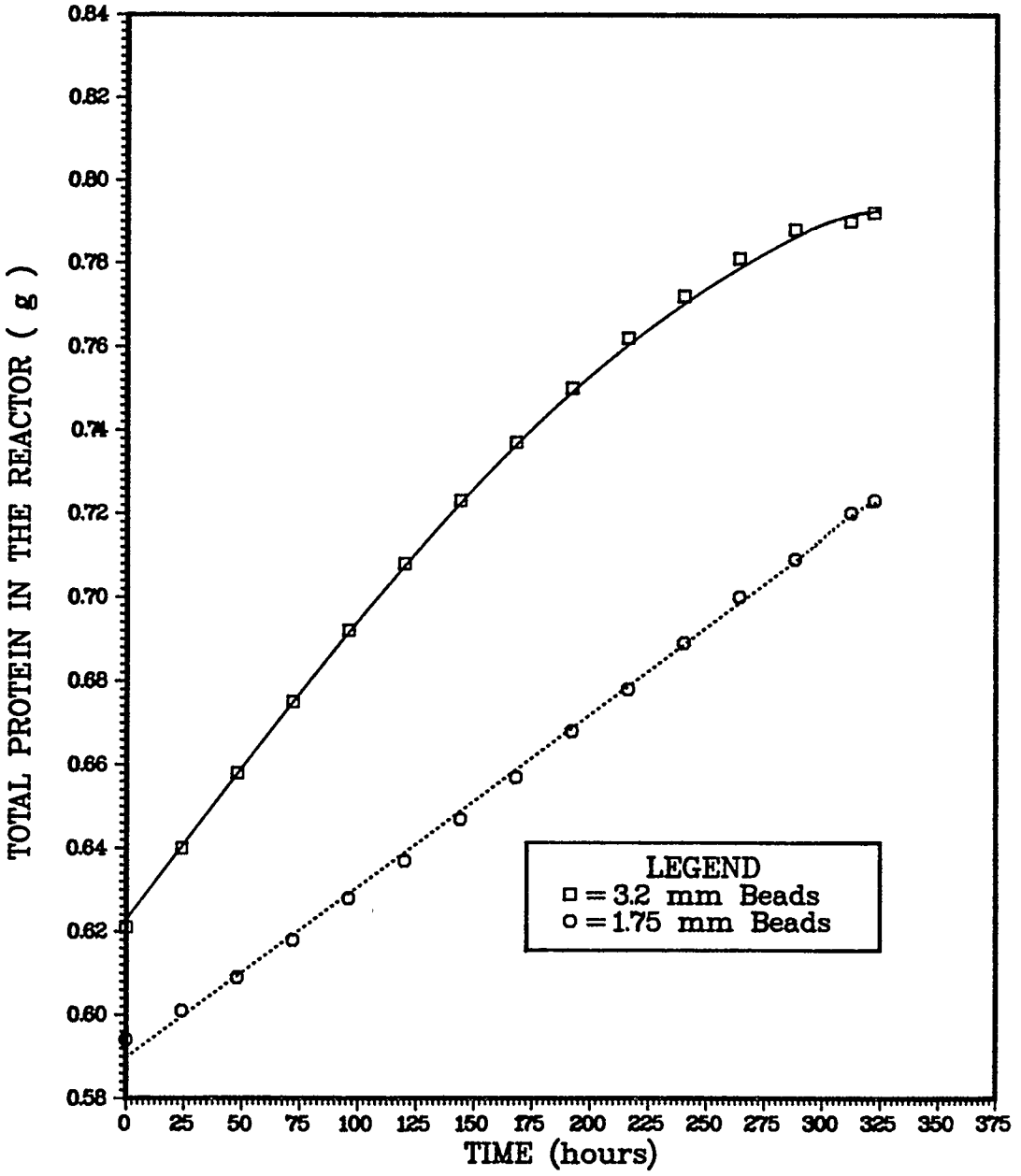


Figure 5.6 Variation of protein content of the reactor during the continuous reactor run.

solid phase.

$$r = Q\,C_{pout} - C_{pin} + V\frac{dC}{dt} + V_s\frac{dC_s}{dt} \qquad (7)$$

Where

Q = volumeteric flow rate of liquid (L/h)

C_p = concentration of patulin in the culture filtrate, (mmol/L)

C = concentration of patulin in the reactor, (mmol/L)

V = volume of the liquid in the reactor, (L)

V_s = volume of the solids in the reactor, (L)

C_s = concentration of patulin in the solid phase, (L)

r = global rate of production of patulin, (mmol/h)

$C_{p_{in}}$ = 0 as there is no patulin in the liquid feed. $(C_p)_{out}$ = C_p, concentration in mmol/L of the patulin in the reactor because the liquid phase is perfectly mixed. Accumulation of patulin in the solid phase is relatively very small and therefore, term $V_s\frac{dC_s}{dt}$ can be neglected (Berk et al., 1984 b). Equation (7) reduces to;

$$r = Q\,C + V\frac{dC}{dt} \qquad (7a)$$

In equation 7a, V and Q are known independent variables and C is found experimentally by HPLC. Concentration of patulin vs. time data

is fitted (Figure 5.3) with a best fit polynomial (Steffen,1982). This program calculates the polynomials of degree 1 to 12 and fits the statistically best polynomial. This polynomial is differentiated to obtain dC/dt at any time 't' in the reactor (see Appendix I). To compare the results of the two reactors containing different bead sizes, a common base needs to be established. Therefore, the global rate at any time t in the reactor is divided by the amount of protein present in the reactor at that time to calculate the specific global rate ($\bar{r}$) of the reaction for patulin production. Results for the two bead sizes are shown in Figure 5.7. As can be seen, the specific rates for the smaller beads are higher than that for the larger beads throughout the production phase indicating a higher diffusional mass transfer resistance for the larger beads. The protein content of the two reactors containing large and small beads changes only by 29 and 22% respectively during the 322 hours of antibiotic production. We assume this much change in the cell mass of the bead would not change the mass diffusivities of the substrates and products and therefore, they are assumed constant. Since the transport properties of the system remain almost the same through out the reactor run, the decline in the specific global rates is probably due to decline in their intrinsic rates. As discussed in section 5.2, at the end of the exponential growth phase, there is a thick layer of biomass at the surface of the beads. Specific rates of reaction for patulin production on a per unit surface area basis are plotted in Figure 5.8. In this plot also the smaller beads have higher production rates during the production run, suggesting that the difference in the specific

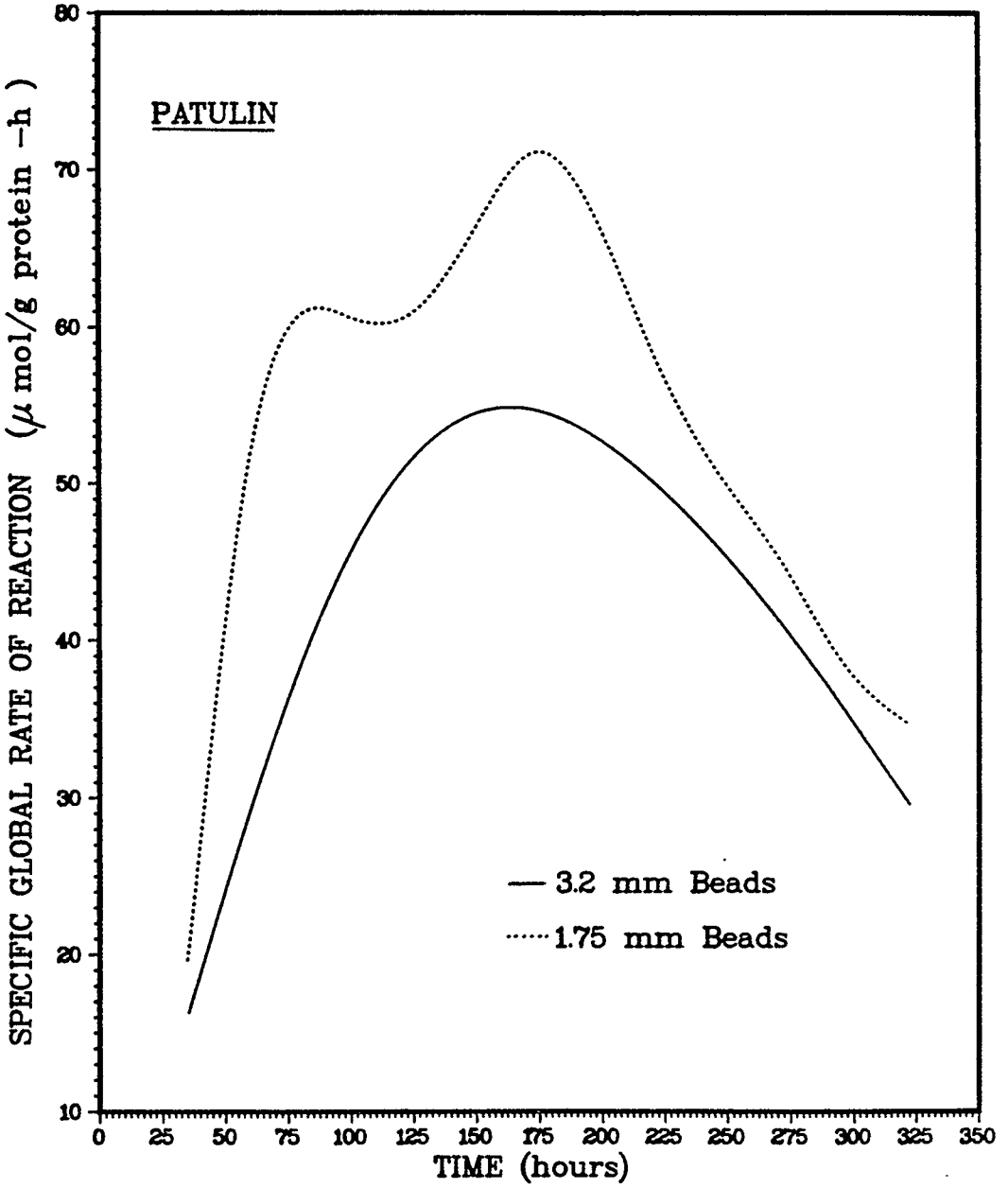


Figure 5.7 Specific global rate of reaction for Patulin production during the continuous reactor run.

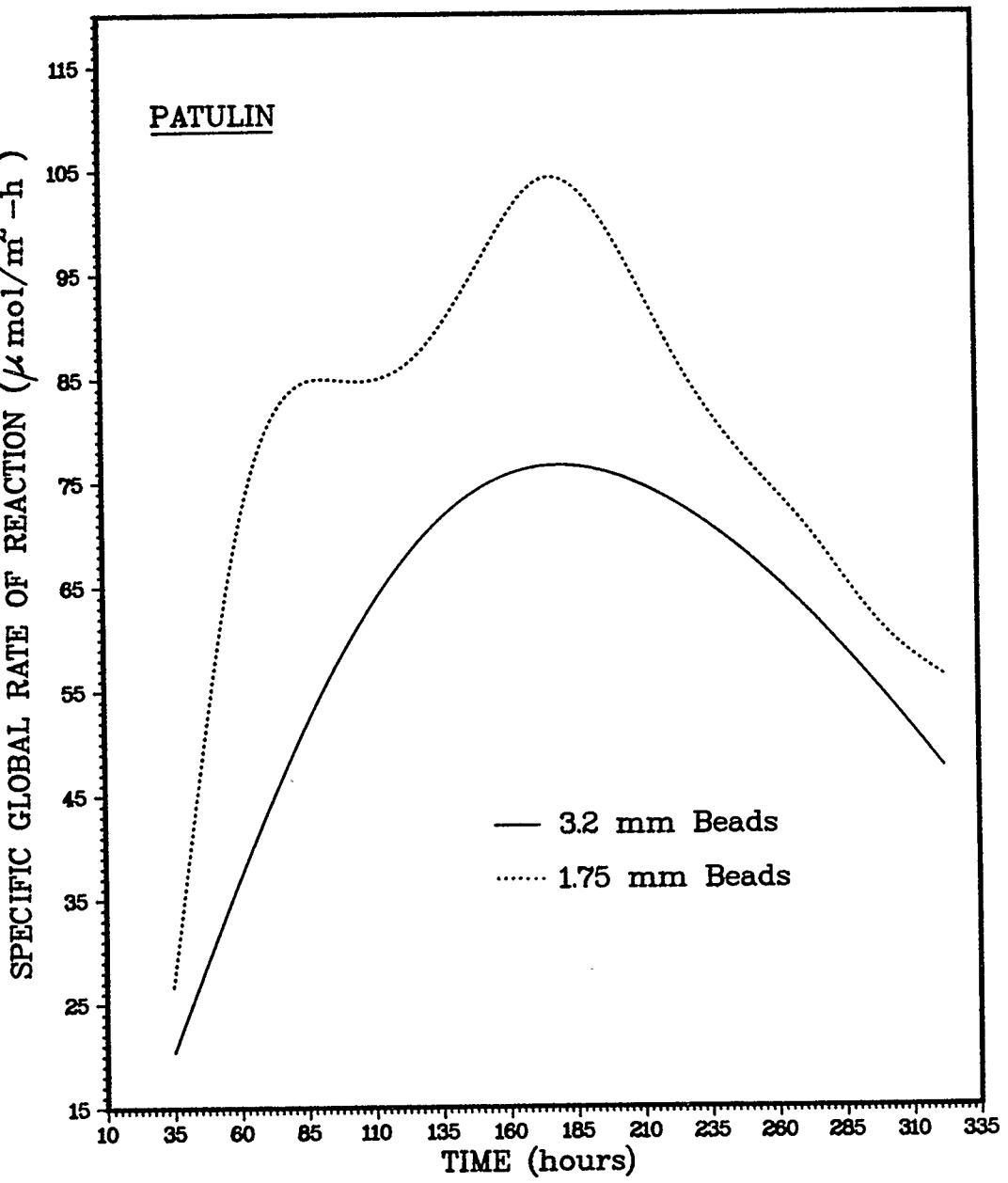


Figure 5.8 Specific rate of reaction for Patulin production (area basis) during the continuous reactor run.

rates for the two bead sizes is not associated with their difference in surface area.

Specific global rate of glucose utilization is also calculated in the same manner as the rate of patulin production (Appendix I). Inlet concentration of glucose is known and outlet concentration is found experimentally. Results are plotted in Figure 5.9. It is clear that smaller bead diameter does not have any effect on the specific global rate of glucose utilization. The specific rates in both the cases decline quite rapidly from 2.8 mmol/h.g protein at 75 hours to about 0.4 mmol/h.g protein at 322 hours. The reason for the same utilization rate could be that glucose is supplied in excess during the production phase and therefore, all the cells present even in the interior of the bead have access to the glucose. These cells may not be producing patulin (inactive cells) but they can still consume the nutrient (glucose in this case). This inability to distinguish between these two types of cells is the main problem in quantifying the diffusional effects.

Figure 5.10 shows the variation in the pH of the medium in the reactor with time. As the cells produce more secondary metabolites during the run, the pH starts declining from the initial value of 6.5 and is minimum at around 130 hours for both the beads. This minimum in the pH profile corresponds to the maximum value for the specific global rate of patulin production (Figure 5.7). After about 140 hours the pH starts increasing beacause of the depletion of the acid metabolites in the reactor and at the end of the reactor run its

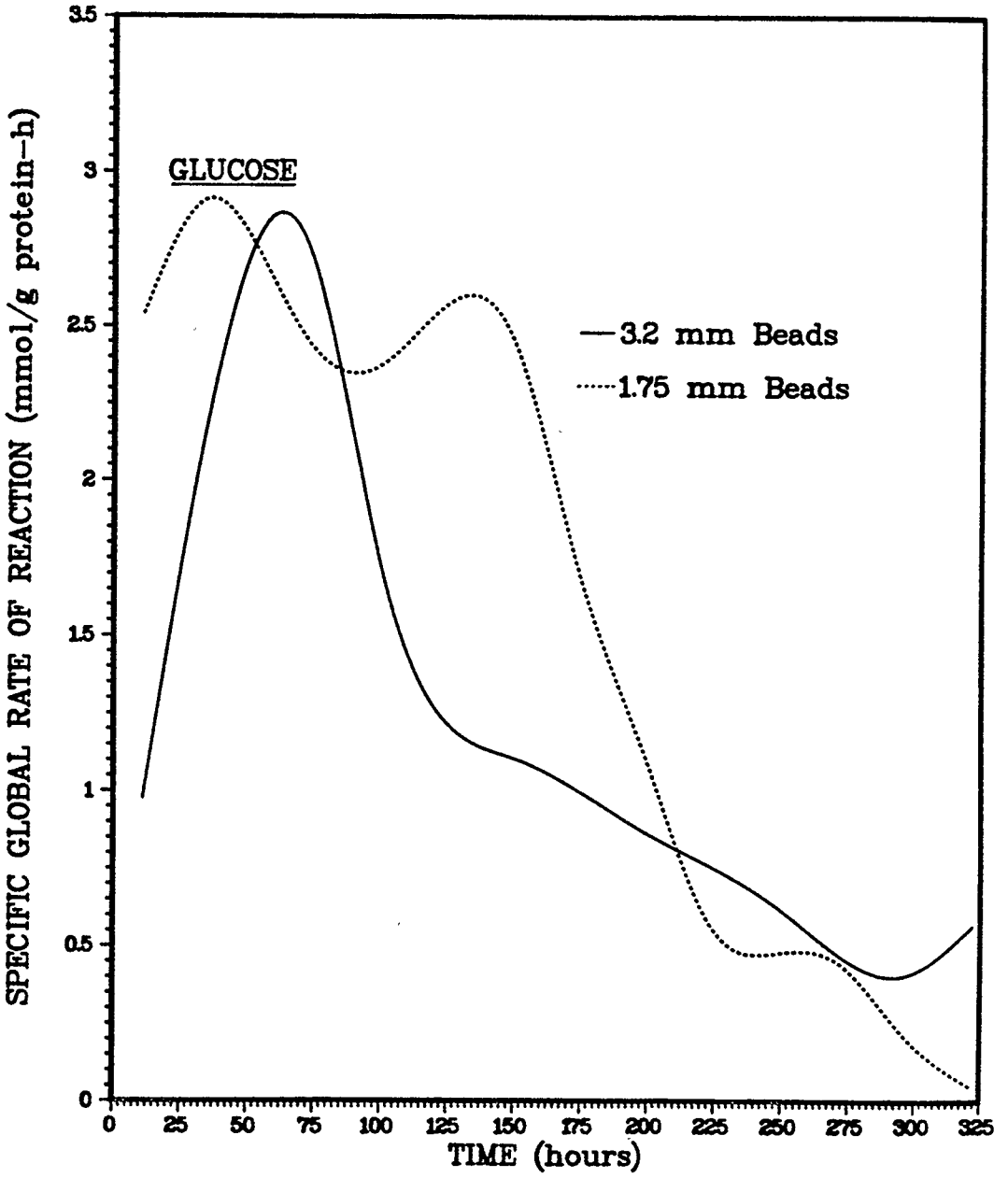


Figure 5.9 Specific global rate of reaction for Glucose utilization during the continuous reactor run.

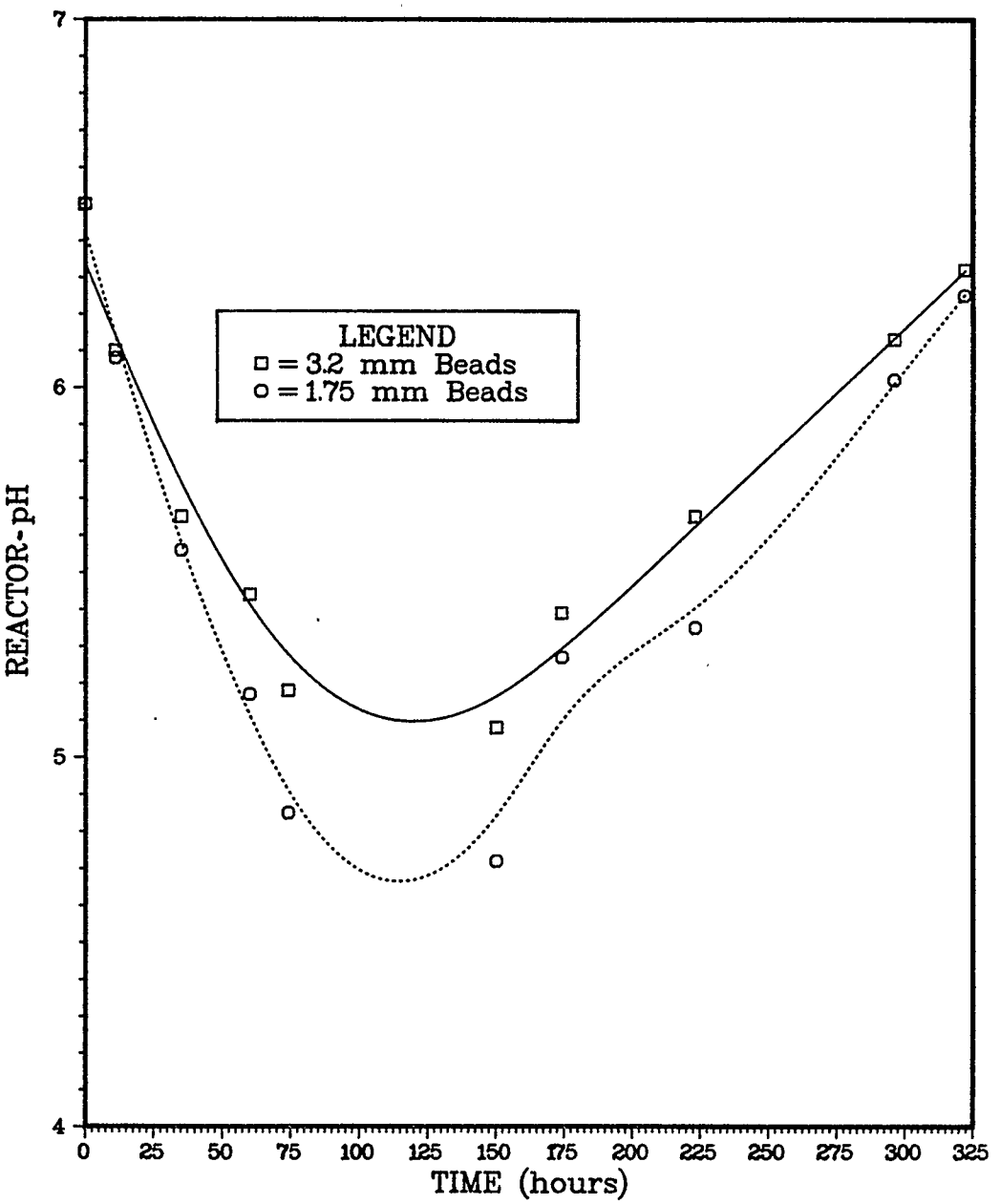


Figure 5.10 Variation in reactor pH during the production phase.

value is quite close to the initial value. As can be seen in the, Figure the pH of the medium containing the smaller beads is consistently lower than the one containing large beads.

Deo, (1982) performed various shake flask experiments with the controlled pH of the reaction medium. The production of patulin was not affected much between the pH range of 4.6 to 5.7 and therefore, the pH of the reaction medium was not controlled. Maximum production of patulin was observed in this pH range. It is clear from Figure 5.10 that during most of the production phase (about 250 hours) pH of the system remained in the above mentioned range.

5.6 CONVERSION YIELD IN PATULIN PRODUCTION

The carbon energy source is a major cost in fermentation processes. For this reason, considerable effort has been expanded in research and development to maximize the conversion of substrate to product. In evaluating a fermentation process, however one must know how well the observed conversion efficiency compares with the theoretical maximum. Determining the theoretical yield of relatively complex fermentation products, such as antibiotics, is difficult because the biosynthesis pathways are often unknown. Cooney (1979) has developed an alternative approach which can be used in the absence of information on the biosythetic pathway. It is based on the reaction stoichiometry and involves general equations for synthesis of antibiotic, cell mass and maintenance metabolism. All these equations are used to describe the utilization of glucose and other

nutrients for the synthesis of secondary metabolite, maintenance and cell mass. For obtaining the stoichiometric coefficients of the equation describing cell mass synthesis, one has to know the composition (Molecular formula) of the cell along with the conversion yield of glucose. Then the equation can be balanced for C,H,O,S & N.

The composition of P.urticae cells is unknown. Also, the exact composition of yeast extract, which is the source of nitrogen and carbon is not known, therefore,this method can't be used to its fullest accuracy. Since, biosynthesis of patulin has been well studied, therefore, theoretical conversion yield of patulin can easily computed. Overall stoichiometry of patulin formation is,

$$2\ \text{Glucose} + 5.5O_2 + 12\ \text{ADP} + 12\ \text{Pi} \longrightarrow \text{Patulin} + 12\ \text{ATP} + 5\ CO_2 + 21H_2O$$

Maximum possible conversion yield , $(Y_{p,s})_{theor.}$ is 0.43 g patulin/g glucose from the above equation. The actual yield coefficient obtained was much lower than the theoretical maximum of 0.43. Figure 5.11 shows the variation in $(Y_{p,s})_{act.}$ (g patulin/g glucose) with time during the production phase. Bead size did not seem to have any effect on the yield coefficient.

For subsequent calculations, $Y_{p,s}$ was taken to be 0.065. Table 5.2 lists the specific growth rates and the doubling times for the two sizes of beads during the production phase.

For calculating the glucose demand for cell mass, maintenance and patulin synthesis, the following assumptions were made;

i) All the carbon requirement is fulfilled by glucose

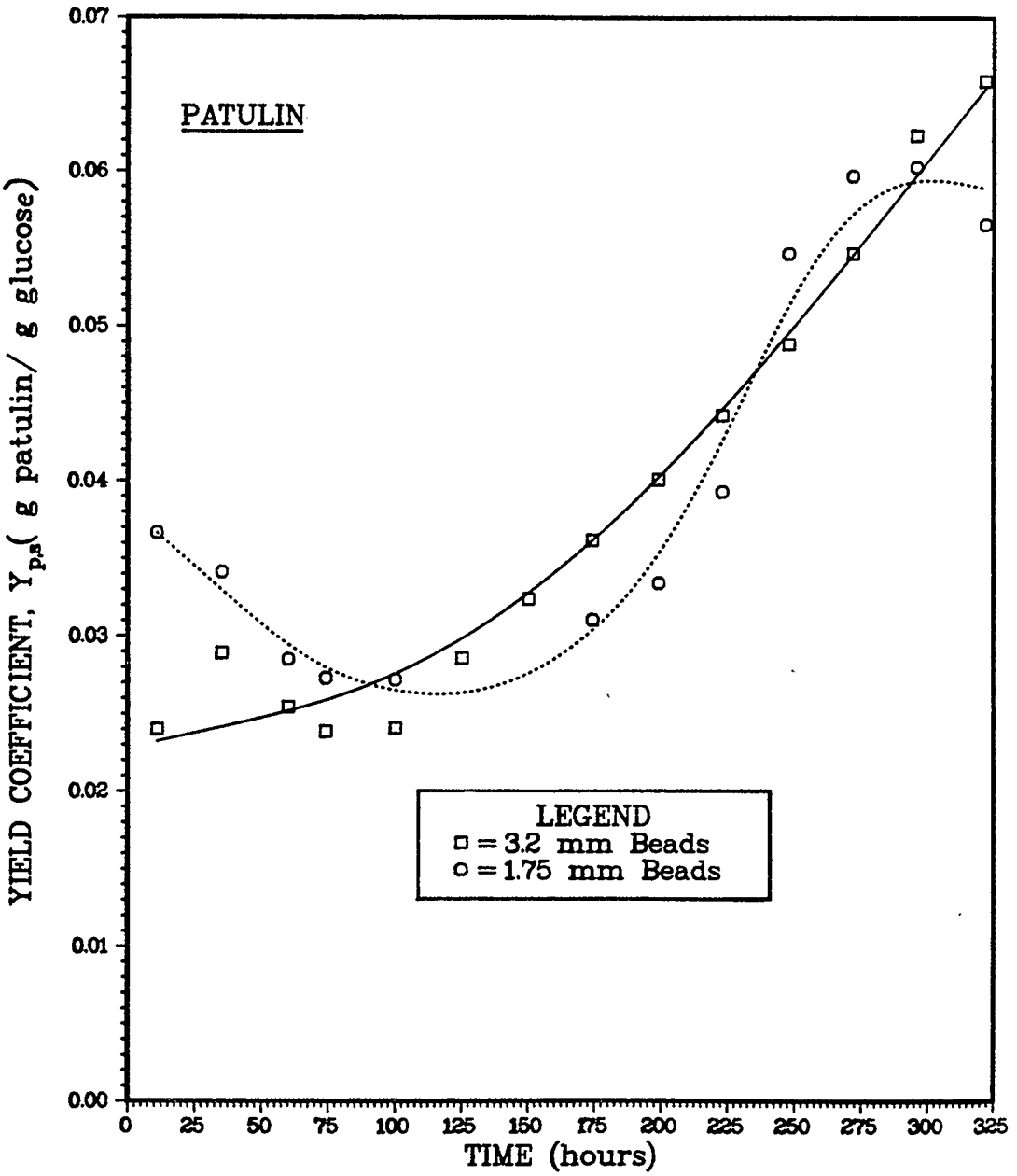


Figure 5.11 Yield Coefficient curve of Patulin.

Table 5.2 Specific Growth Rates and Doubling Times During the Production Phase.

Bead Size	Specific Growth Rate μ ($days^{-1}$)	Doubling Time t_d (days)
3.2 mm	0.0184	37.67
1.75 mm	0.0163	42.52

μ_m (Maximum Specific Growth Rate during Exponential Growth Phase) = 0.11 hr^{-1}.

Doubling Time = 6.30 hours.

ii) Maintenance coefficient for P.urticae is assumed to be the same as for P.chrysogenum, i.e 0.027 g biomass/g glucose.

It has been proved that it is always easier to fulfill energy requirements by carbon from glucose because it is more readily available than from amino acids. Besides this, the concentration of yeast extract in the production medium is so low that for all practical purposes the contribution of carbon by yeast-extract is almost negligible.There is no information about maintenance coefficient of P.urticae in the literature and since P.urticae and P.chrysogenum are both Penicillia, one would not expect much difference in the value of maintenance coefficients. Table 5.3 lists the glucose demand for cell mass, maintenance and patulin synthesis using the yield coefficients calculated earlier. Percentage of glucose utilized for cell mass,maintenance and patulin was found to be 33,42 and 25% respectively.

5.7 DIFFUSION COEFFICIENTS OF SUBSTRATES IN CARRAGEENAN BEADS

Diffusivity studies were carried out as discussed in Section 4.10.1. Diffusivities of the substrates and the products into carrageenan beads have to be known to assess the effect of the problem of internal diffusion. The molecular diffusion coefficients (D) of the substrates and products in carrageenan gel beads were obtained by observing the change in the concentration of the substrates and the products in the well stirred solution when the carrageenan beads

Table 5.3 Glucose Demand in Production of Patulin by *P.urticae*

Requirement	Glucose demand in the reactor (g)
Cell mass synthesis $\frac{0.75 \text{ g protein}}{0.14 \text{ g/g glucose}}$	5.35
Maintenance demand $\frac{0.027 \text{ g}}{\text{gcell-h}}$ (322h) (0.8 g protein)	6.96
Patulin synthesis $\frac{2.0 \times 0.154 \text{ g patulin}}{0.065 \frac{\text{g patulin}}{\text{g glucose}}}$	4.73
Total	17.04

were suspended in the solution. When the beads are spherical and free of the solute whose diffusion coefficient is sought and are suspended in a solution with an initial concentration of C_{LO}, the solute in the solution is diffused into the beads and the solute concentration in the beads, C_B, is given by the following equation (Crank, 1975),

$$C_B = \frac{\alpha C_{LO}}{(1+\alpha)} \left| 1 + \sum_{n=1}^{\infty} \frac{6(1+\alpha)e^{-Dq_n^2 t/a^2}}{9 + 9\alpha + q_n^2\alpha^2} \cdot \frac{a}{r} \frac{\sin(q_n r/a)}{\sin q_n} \right| \qquad (8)$$

where r is the distance from the center of a bead, t is the time, V is the volume of the solution excluding the space occupied by beads, α is defined by $\frac{V/n}{4\pi a^3 K/3}$, where n is the total number of beads, 2a is the diameter of the bead, K is the partition coefficient (defined as the ratio of solute concentration in the bead to the solute concentration in the solution at equilibrium), and the q_n terms are the non zero roots of

$$\tan q_n = \frac{3\, q_n}{3 + \alpha\, q_n^2} \qquad (9)$$

The liquid film resistance around the bead is zero (i.e. $k_m \longrightarrow \infty$) because of mixing. Therefore, the concentration of solute just with in the surface of a bead, $(C_B)_{r=a}$, is same as that in the solution (C_L), therefore, from equation 8, the solute concentration in the solution is expressed as follows:

$$C_L = \frac{\alpha\, C_{LO}}{(1+\alpha)} \left| 1 + \sum_{n=1}^{\infty} \frac{6(1+\alpha)e^{-Dq_n^2 t/a^2}}{9 + 9\alpha + q_n^2\alpha^2} \right| \qquad (10)$$

It was proven that at equilibrium, the concentration of solute in the solution and inside the bead were the same, i.e $C_{BE} = C_{LE}$, which implied that K = 1 (for the present system only). Program listings and the solution procedure of the above equations is given in Appendix II. Figure 5.12 and 5.13 show the variation in glucose concentration in the solution for 3 different initial glucose concentrations and fraction of initial glucose concentration present in the solution. Concentration of glucose did not have any effect on the diffusion coefficient. Stirrer speeds of 173 rpm and 650 rpm were used in two different runs. Both the runs yielded same glucose diffusion coefficient (Figure 5.14) suggesting that there is no external mass transfer resistance or in other words the bead external mass transfer coefficient is infinite. As illustrated in section 5.3 using phase contrast microscopy, thick layer of mycelium was observed near the surface of the bead at the end of exponential phase. In order to study the effect of this layer on the diffusion of substrate into the bead, experiments were conducted with beads which contained 48 hours old dead cells (in cubated in growth medium). In case of glucose, the same diffusion coefficient of $4.01\times10^{-4}cm^2/min$ was obtained (Figure 5.15). This could be because of the fact glucose was always present in large excess. In all the experiments the predicted values are quite close to the ones found experimentally.

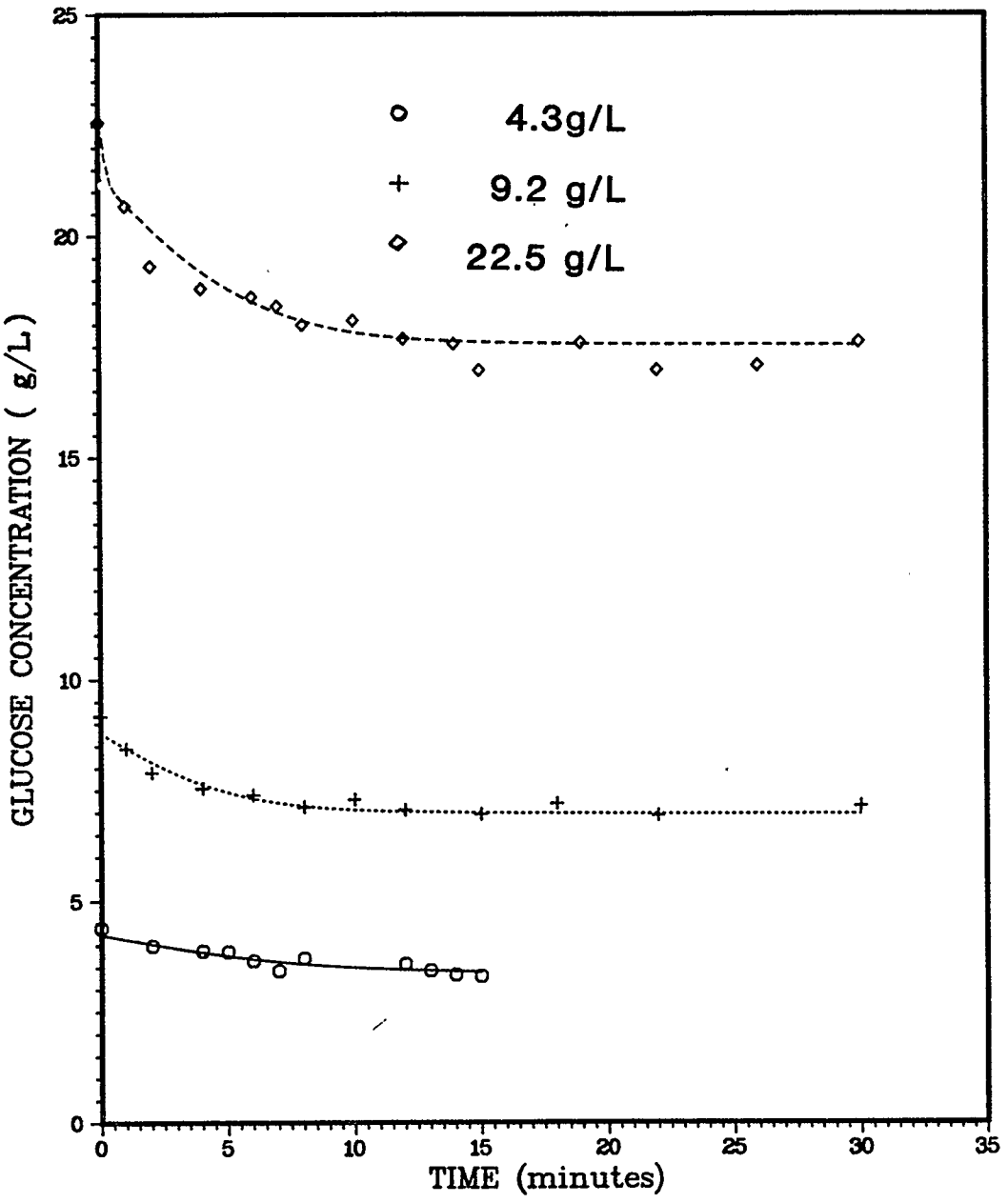


Figure 5.12 Variation in Glucose concentration of the solution during the diffusion experiment.

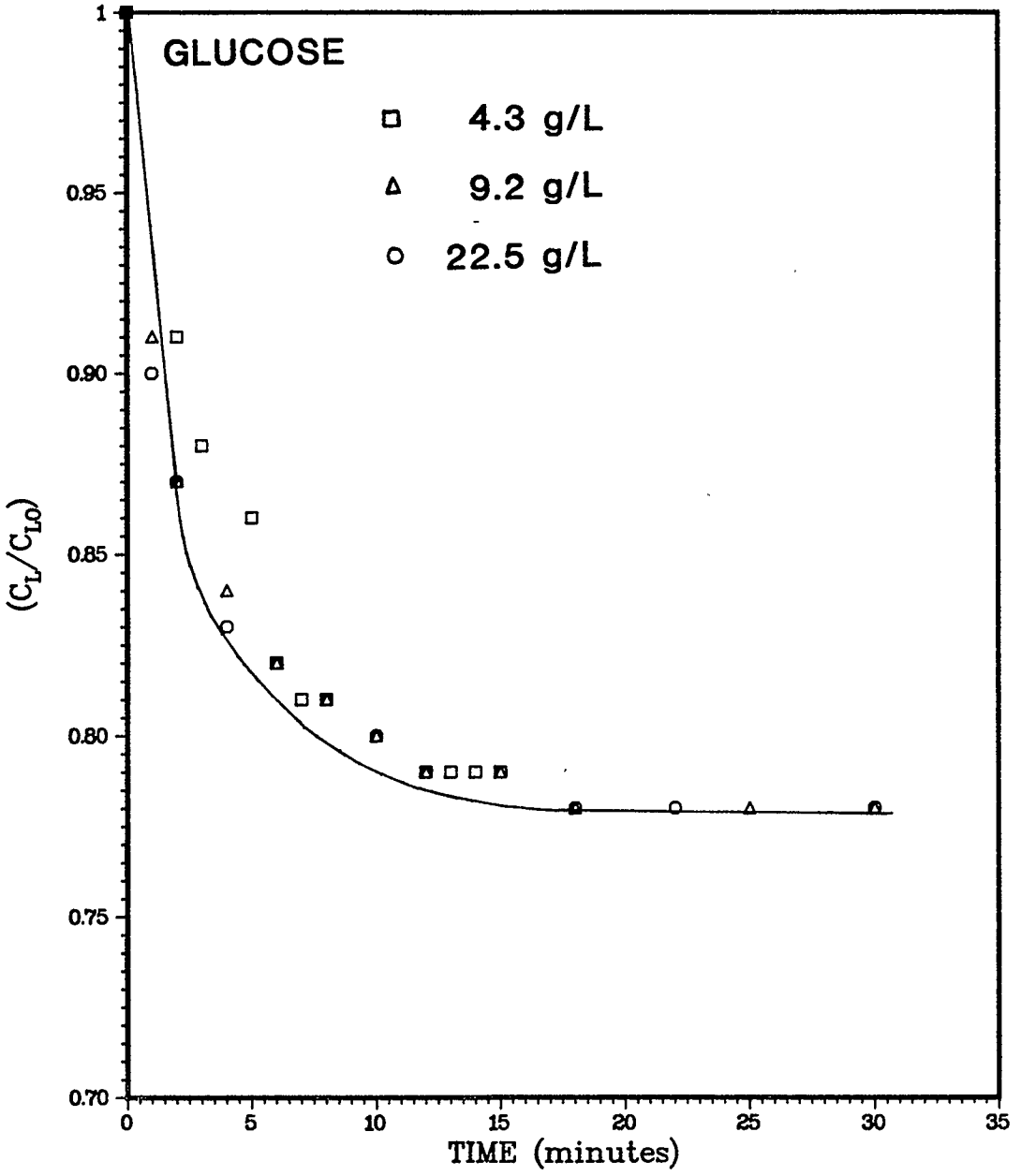


Figure 5.13 Fraction of Glucose concentration present in the solution.

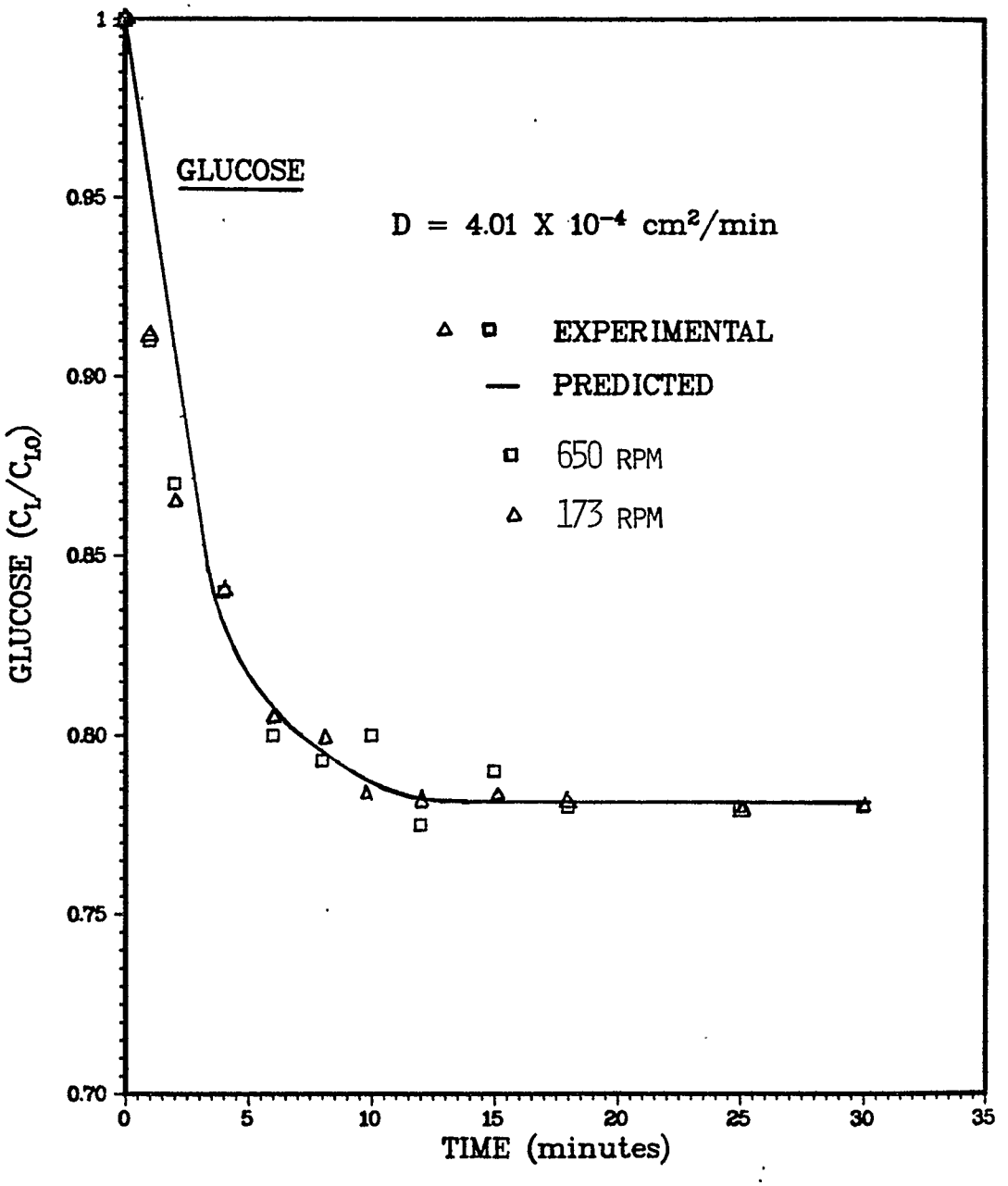


Figure 5.14 Plot for deterimination of diffusion coefficient of Glucose in Carrageenan Gel Beads.

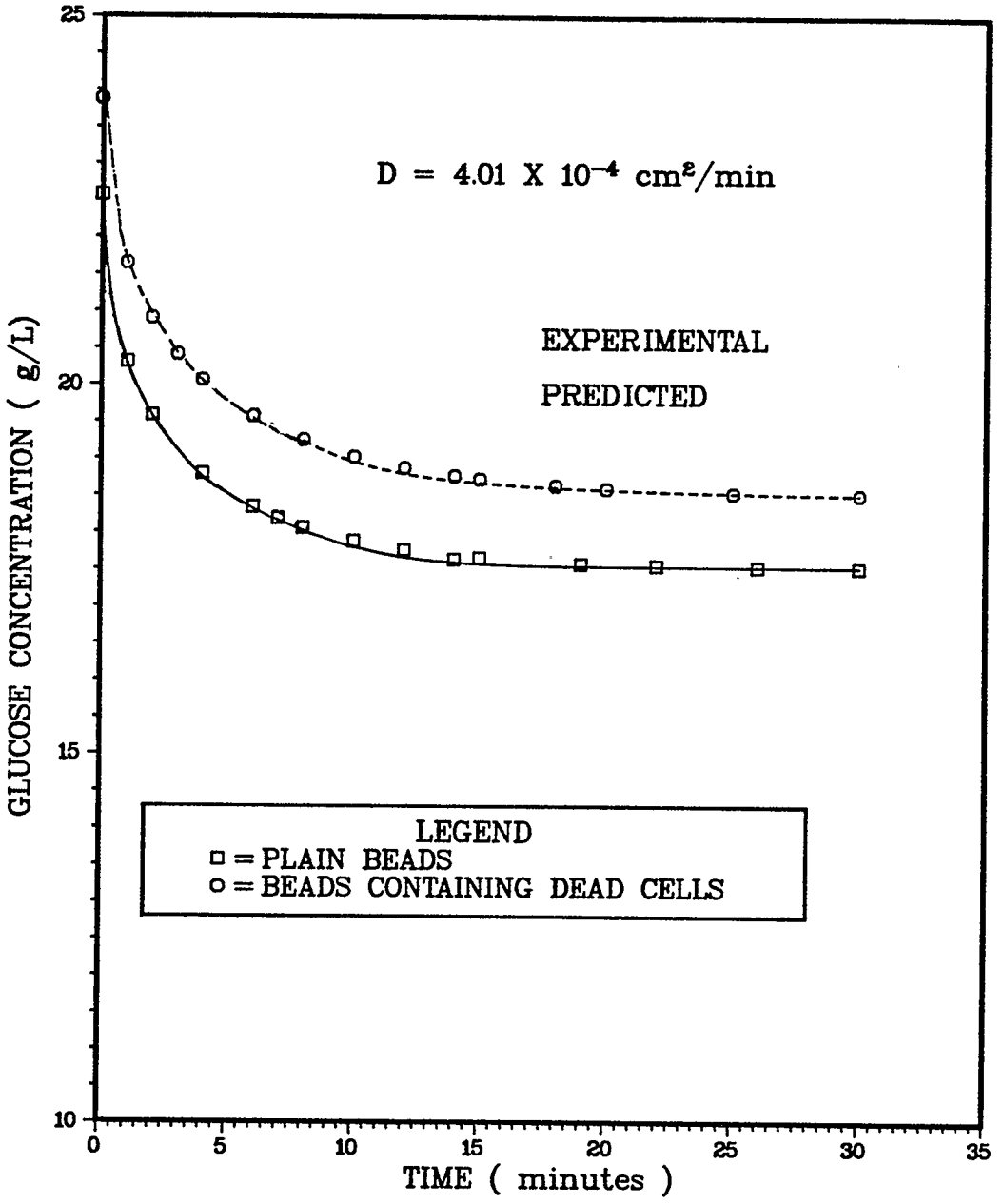


Figure 5.15 Effect of cell growth near surface of the bead on the diffusion coefficient of glucose.

Diffusion of 6-MSA and patulin into carrageenan gel beads (with and without dead cells) was also studied. Figure 5.16 and 5.17 illustrate the diffusion of patulin and 6-MSA into the beads without cells. Variation in concentration of patulin and 6-MSA in the solution in the case of beads containing dead cells is shown in Figure 5.18 and 5.19 respectively. Table 5.4 summarizes the results of all the diffusion experiments. It can be seen from this Table that diffusion coefficients of patulin and 6-MSA were affected by the increase in cell population during the growth phase. This means that cell constituents create an additional resistance to diffusional transfer.

The smaller value of the diffusion coefficients may be because of the fact that both 6-MSA and the carrageenan matrix of the beads are negatively charged species,thereby, offering resistance to diffusion. The relatively large diffusion coefficient of patulin may be attributed to the fact that it is a molecule with only one OH group and is relatively hydrophobic in nature.

5.8 HYDRODYNAMIC STUDY OF THE CARRAGEENAN BEADS

5.8.1 Static Pressure Profiles Along The Column

To enable the operation of the fluidized bed reactor at higher flow rates without causing any entrainment problems, the basic hydrodynamic characteristics of the beads were studied in a non reacting system and for this purpose the carrageenan beads containing fine stainless steel particles were made. The composite density of the

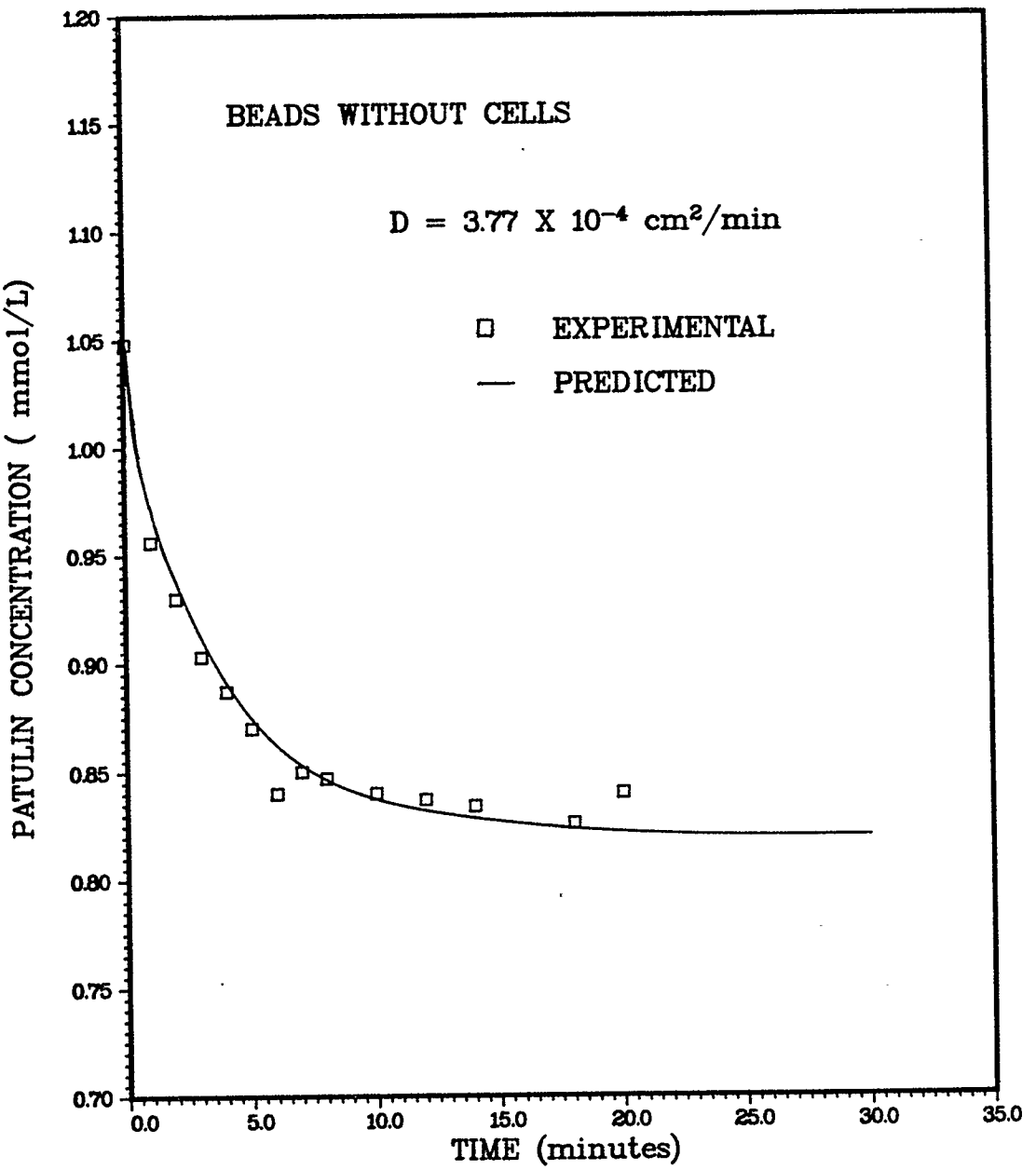


Figure 5.16 Plot for determination of diffusion coefficient of Patulin in Carrageenan Gel Beads without any cells.

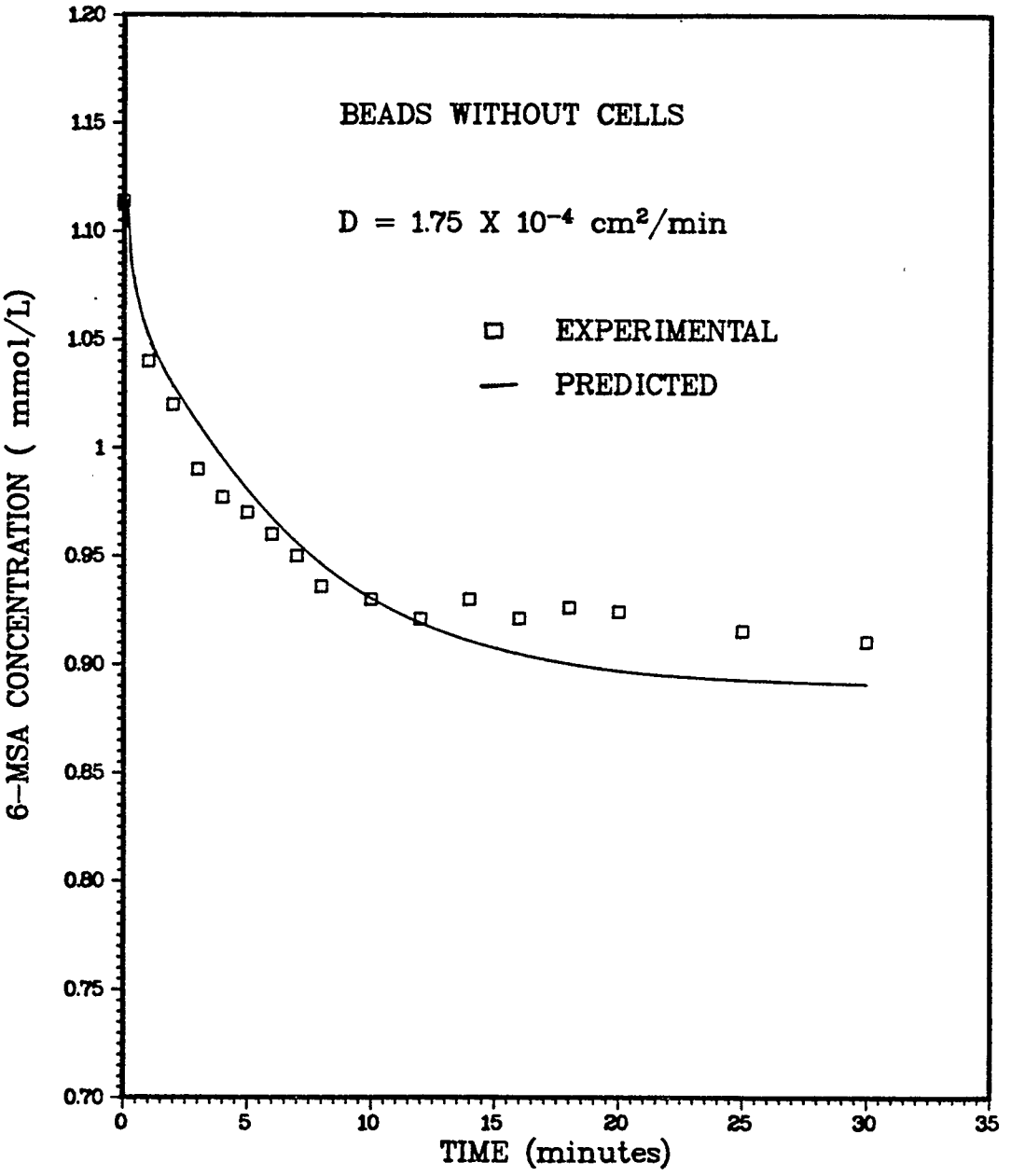


Figure 5.17 Plot for determination of diffusion coefficient of 6-MSA in Carrageenan Gel beads without any cells.

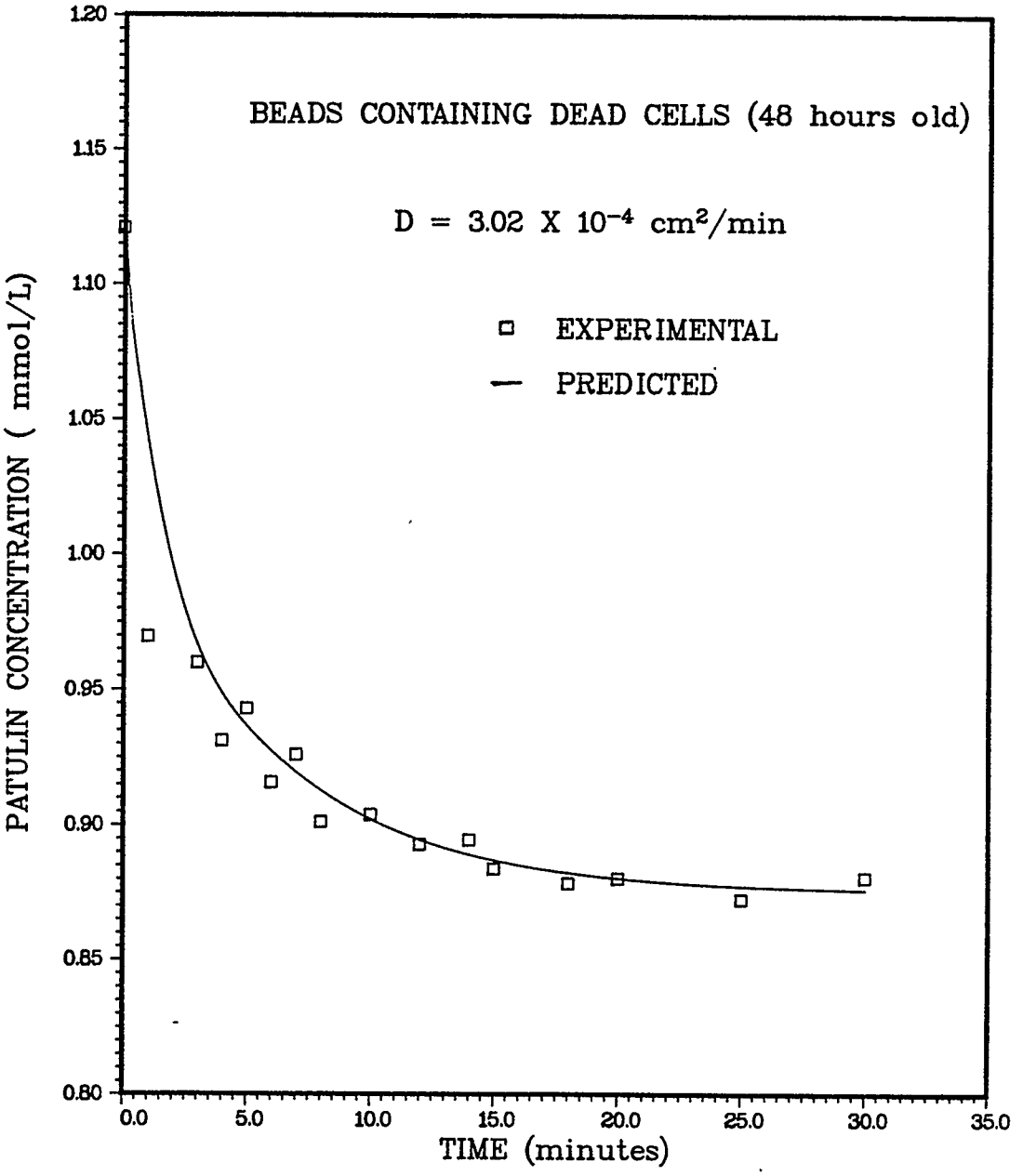


Figure 5.18 Plot for determination of diffusion coefficient of patulin in beads containing dead cells.

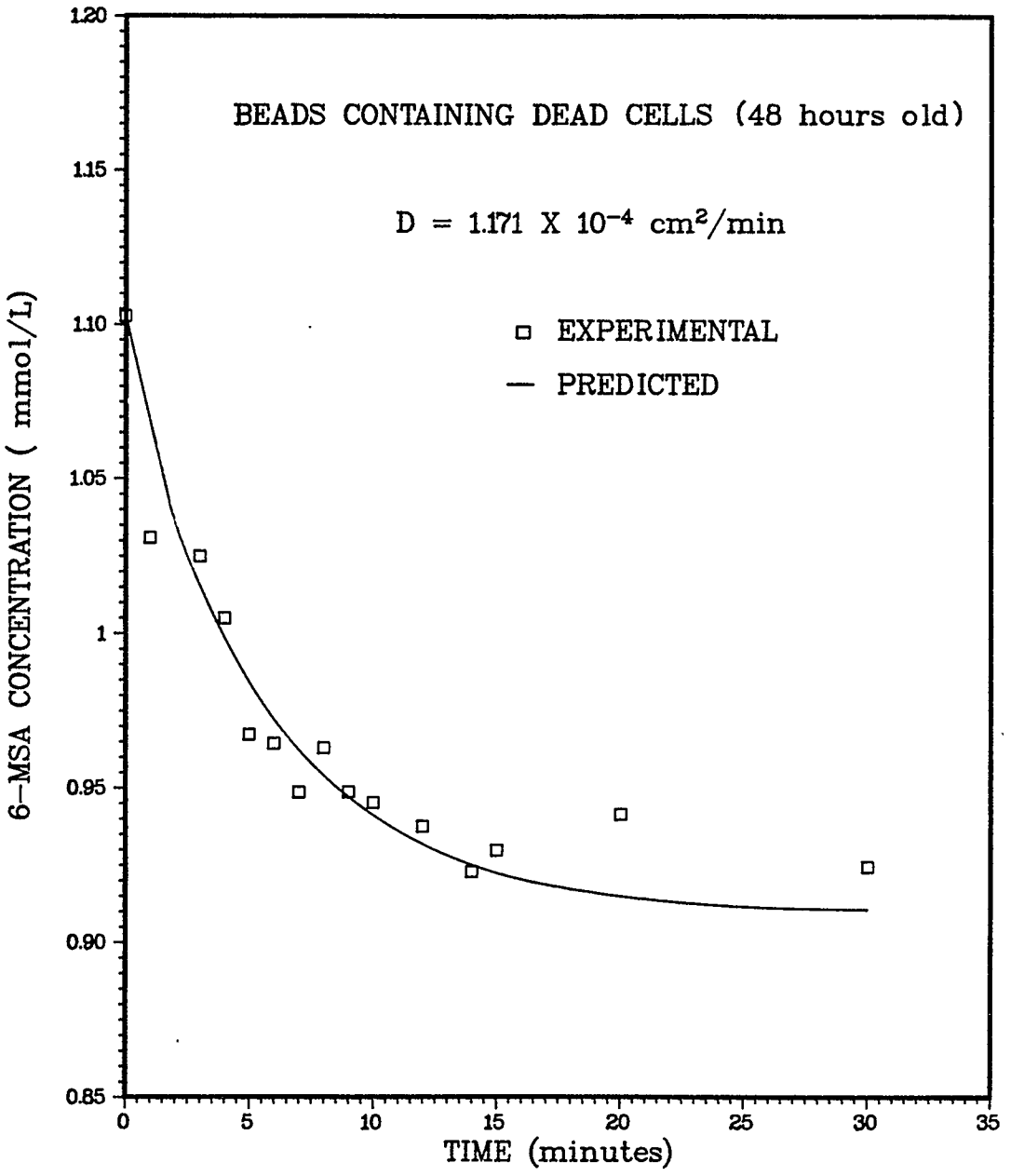


Figure 5.19 Plot for determination of diffusion coefficient of 6-MSA in beads containing cells.

Table 5.4 Diffusion Coefficients of Substrates in Carrageenan Beads.

DIFFUSION COEFFICIENTS (x 10^4), cm^2/min			
Substrate	in water	in carrageenen beads without cells	in carrageenen beads with cells
Glucose	4.04 *	4.01	4.01
Patulin	unknown	3.77	3.02
6-MSA	unknown	1.75	1.171

* from CRC Handbook of Phys./Chem., p f 37.

bead was 1.41 g/cm^3. The higher density of the beads ensured their proper disengaging at the top of the reactor. Static pressure was measured along the main section of the reactor using a differential manometer. The results are plotted in Figure 5.20. Most of the pressure drop was observed across the distributor. The slope of the plot yields the effective bed density. It was found to be approximately 1.16 g/ cm^3.

5.8.2 Solid, Liquid and Gas Hold-ups

The individual phase hold ups were calculated using the following equations,

$$\varepsilon_l + \varepsilon_g + \varepsilon_s = 1 \qquad (11)$$

$$\Delta P = g\,H\,(\varepsilon_l\rho_l + \varepsilon_g\rho_g + \varepsilon_s\rho_s) \qquad (12)$$

$$\varepsilon_s = M_s/A\,H\,\rho_s \qquad (13)$$

Equation 12 assumes there are no wall frictional losses. ε_l, ε_g and ε_s are the hold-ups in the liquid, gas and solid phase respectively, M_s the mass of the solids, ρ_i is the density in phase i (i = l,g,s,), A is the crossectional area of the column and H is the height of the bed. The exact calculation procedure is given in Appendix III. The effect of gas superficial velocity on the gas, liquid and solid hold up is shown in Figure 5.21 to 5.23. Gas hold up was found to increase with the gas superficial velocity where as the liquid and solid hold up decreased slightly.

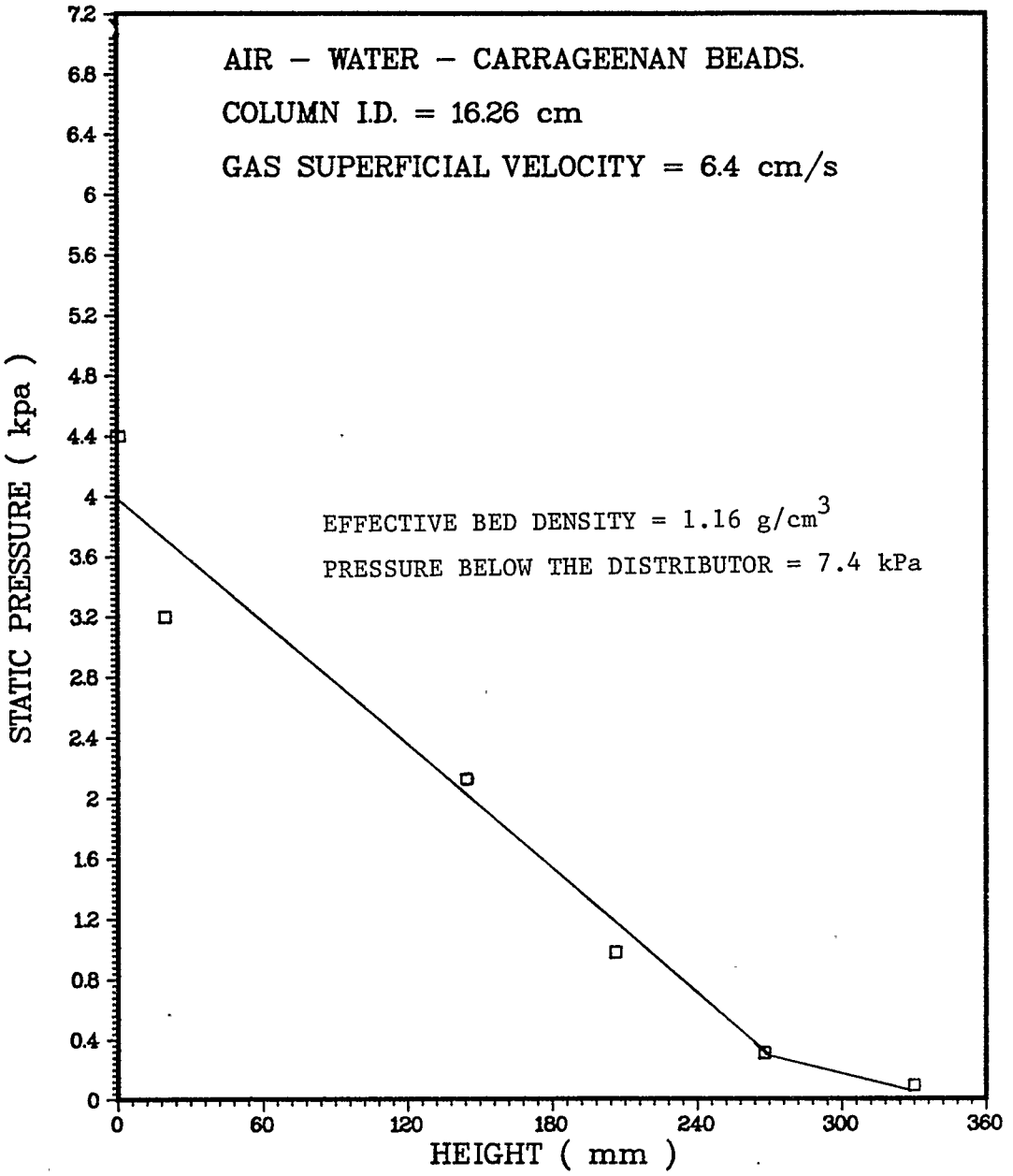


Figure 5.20 Plot of Static Pressure profile along the column.

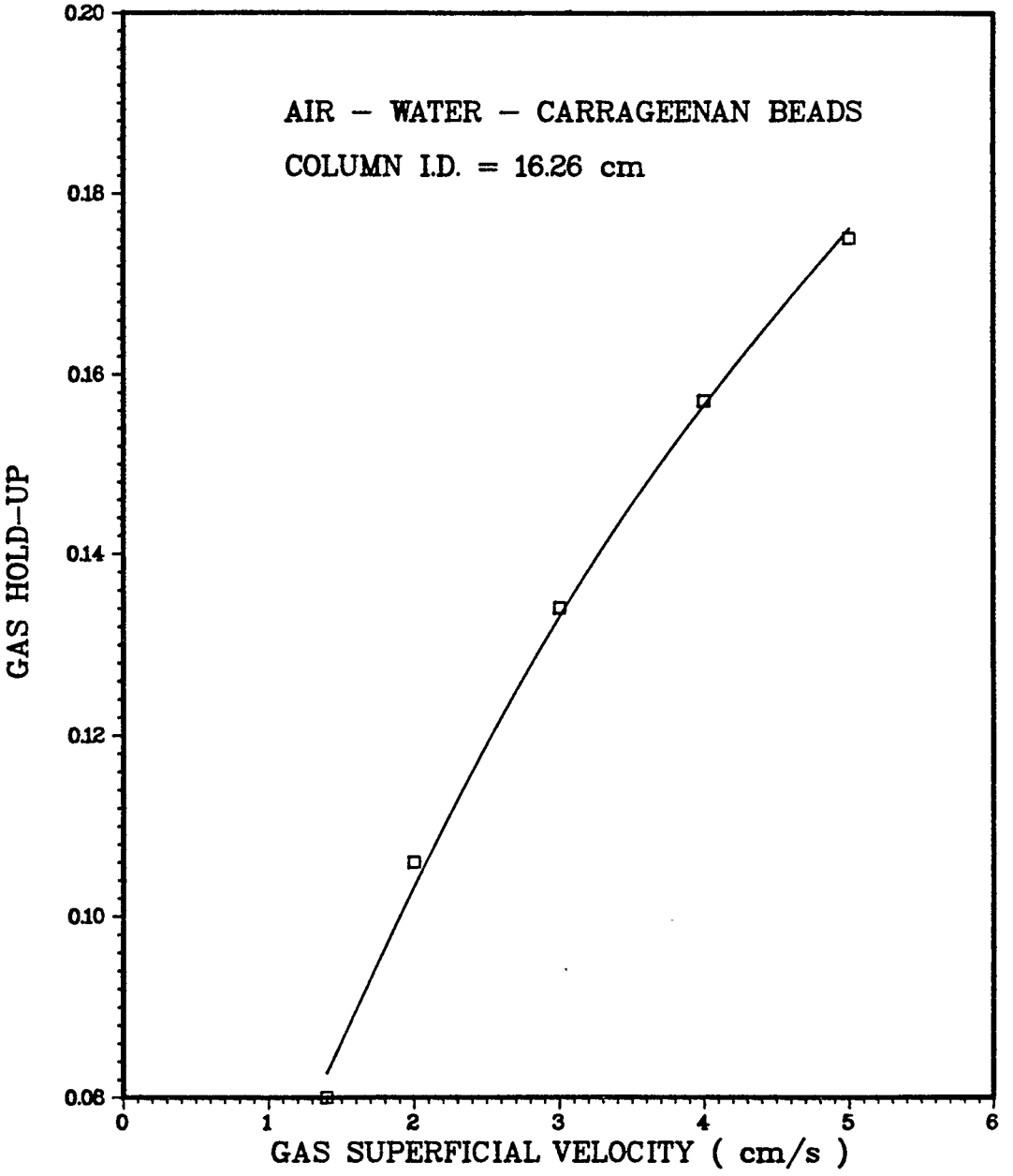


Figure 5.21 Effect of Gas superficial velocity on Gas hold-up.

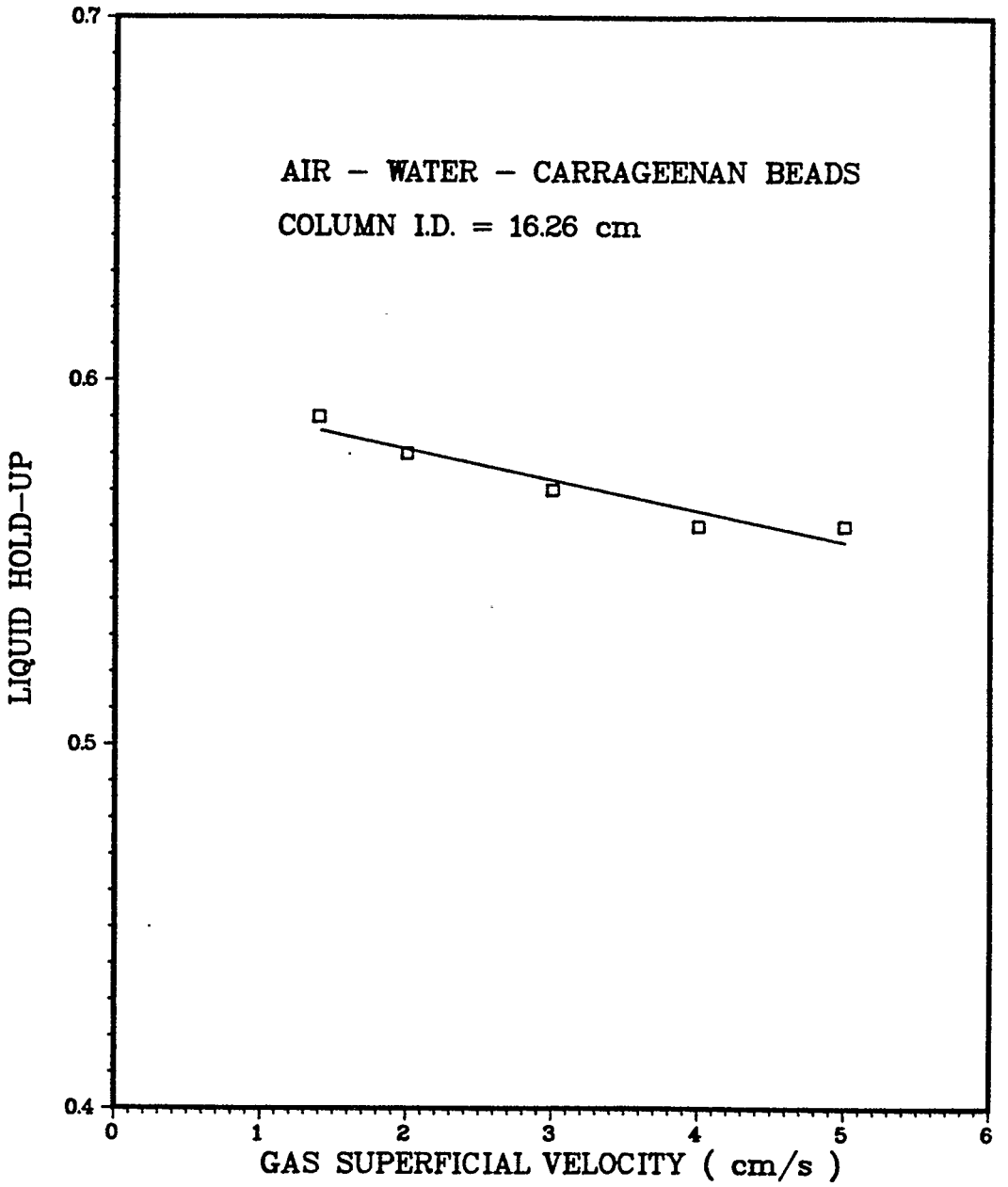


Figure 5.22 Effect of Gas superficial velocity on Liquid Hold-up.

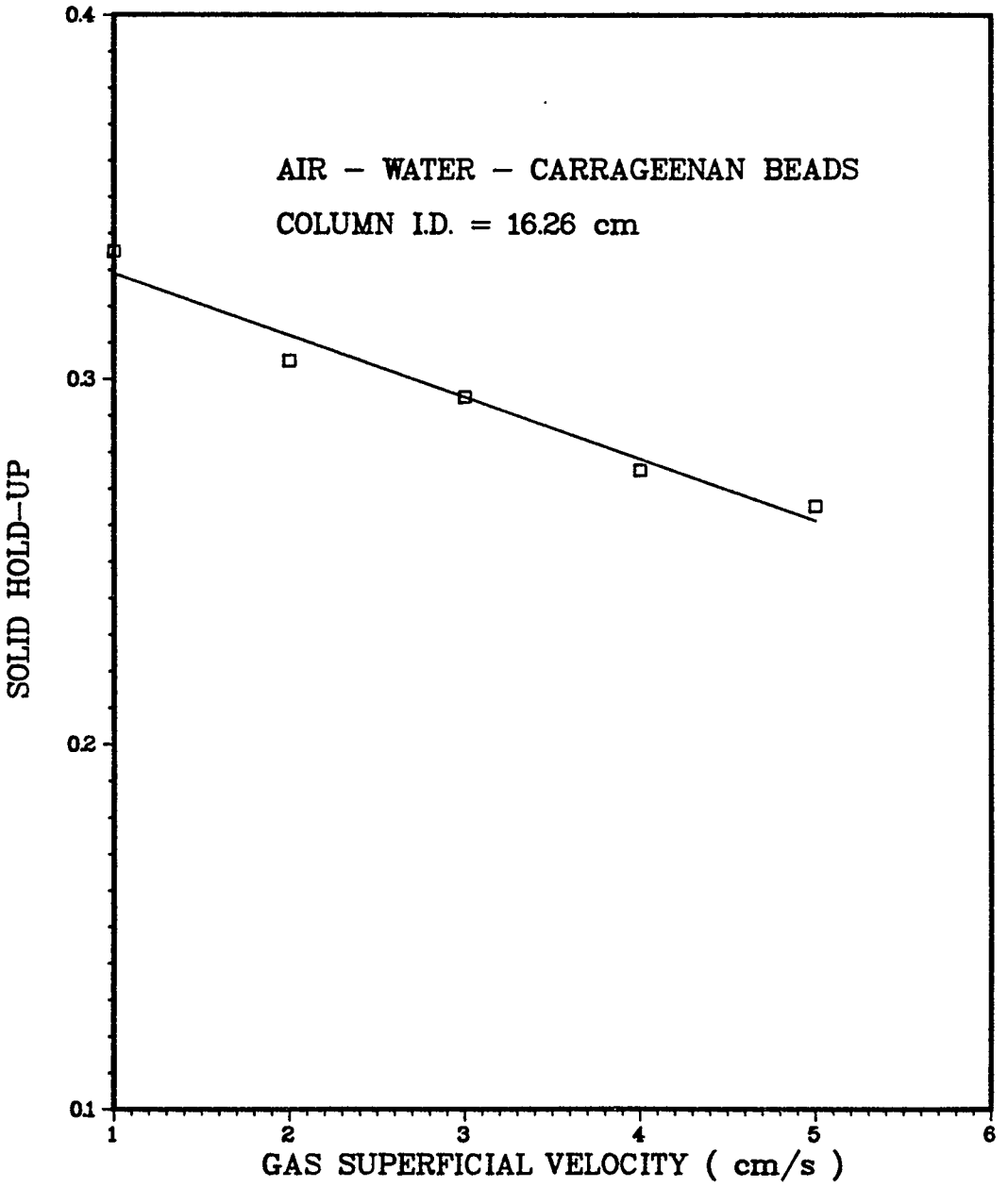


Figure 5.23 Variation of Solid Hold-up with Gas Superficial velocity.

CHAPTER 6

COMMERCIAL USE OF THE PROCESS USING IMMOBILIZED CELLS

Although the potential advantages of continuous fermentation are widely recognized and the theoretical basis is established, industrial use of the operation has been restricted almost entirely to yeast and yeast production, vinegar and sewage treatment processes, all of which yield bulk products and depend for their advantageous operation on a highly restrictive growth situation. Application of continuous culture as judged by current trend in research and development, will extend to the fermentations, where culture filtrate purity is much more precarious. One such application of considerable potential importance is in antibiotic production. An idea of the future potential of the antibiotic industry can be obtained from the fact the total antibiotic industry in United States itself was worth about 2.7 billion dollars in 1981 (World Predicast, 1984).

The following factors determine the commercial success of any fermentation process.

i) Conversion yield of the product, (g product/ g substrate)

ii) Specific rate of production, (g product/ g catalyst -h)

iii) Half life of the catalyst, ($t_{1/2}$)

iv) Solid (catalyst) hold up, (g catalyst/ vol of reactor)

v) Net productivity,(g product/g catalyst -useful life of cat.)

The efficiency of conversion of the carbon energy source to product is of primary importance in many fermentations processes because the carbon energy is a major cost in many fermentation processes. Table 6.1 gives a clear idea of the percentage of total cost for glucose in the case of penicillin production.

Most of the antibiotics in the industry are still manufactured by batch or fed batch free cell process. Fluidized beds utilizing the immobilized cells for production of secondary metabolites have the following advantages over the conventional reactors used in the industries:

a) sufficient biomass in the reactor is maintained at all times,

b) better temperature control,

c) less physical abrasion of the cells in the reactor,

d) increased duration of the production phase,

e) operation of the reactor at low viscosity because the cells can not mix with the liquid, thereby, reducing the cost of separation and purification of the product.

In case of fluidized bed reactors some hydraulic factors also have to be considered for the commercial use of the process. They include, catalyst hold up, pressure drop and channeling of liquid and gas phases.

Economic viability of the fermentation process depends upon

Table 6.1 Manufacturing Costs for the Production of Penicillin G expressed as percent of total Fermentation and Purification cost (From Cooney, 1979).

Item Direct Cost	% Total Cost
Glucose	12
PAA	11
Other Chemicals	5
Labour and Maintenanace	12
Other Charges	6
Utilities	10
Purification	23
Fixed Charges	23
Plant Overhead	8

the integeral $\int_{t_o}^{t_1} q_p(t) \cdot X(t)\, dt$ (Swartz, 1979), where $q_p(t)$ is the specific productivity of the cells, $(t_1 - t_o)$ is the total fermentation time and X is the total amount of biomass present at time 't'. To optimize this integeral we must have maximum X and maximum q_p for maximum period of time. In the industrial reactors X and q_p change appreciably with the fermentation time. Usually X increases till it reaches maximum and then stays there whereas q_p increases rapidly to a maximum and then declines sharply. In contrast to this, the fluidized bed reactors used in the present study were successfully run for more than 300 hours and at the same time maintaining a steady state for about 160 hours.

It will take some time before these fluidized bed reactors using immobilized cells are used in the well established fermentation industry because of the large fixed cost associated with them. But, it is definitely a more promising alternative for a new facility.

CHAPTER 7.

CONCLUSIONS AND RECOMMENDATIONS

7.1 CONCLUSIONS

1. Insitu grown immobilized cells of P.urticae in carrageenan gel beads were made successfully in two bead sizes of 3.2 and 1.75 mm.

2. Continuous bioreactors were run for more than 300 hours at 28^{o}C to produce the antibiotic patulin. The concentration of patulin did not change appreciably for about 150 hours which is much more than for the batch and fed batch reactors presently used for the production of antibiotics.

3. The decrease in bead size led to a higher specific global rate of production of patulin indicating there is a significant increase in diffusional resistance for the larger beads.

4. Smaller bead size however, didn't show any effect on the specific global rate of glucose utilization. This could be because of the fact that glucose is always present in large access.

5. Microscopic examination of the bead showed the presence of thick layer of biomass at the surface of bead suggesting that there is

not uniform growth inside the bead and this could influence the diffusion of substrates and the products into and out of the bead.

6. About 29 and 22% increase in cell mass was found in case of large and small beads respectively during the patulin production phase indicating a greater resistance to diffusional mass transfer for the larger beads.

7. It was found that about 42% of the glucose utilized during the production phase was for maintenance where as for cell mass and patulin synthesis the percentage was 33 and 25% respectively.

8 Maximum conversion yield of patulin ($Y_{p,s}$), was calculated to be 0.065 (g patulin/g glucose) as compared to the theoretical maximum of 0.43.

9. There is no resistance to the diffusion of glucose into the carrageenan beads indicated by no change in the diffusion coefficient of glucose for the beads with and without the dead cells.

10. There is no resistance to transport of nutrients from the bulk liquid to the surface of the beads.

11. The values of diffusional coefficients for patulin and 6-MSA were lower in case of beads containing the cells, suggesting the

added resistance to diffusion of these metabolites into/out of the support matrix.

12. Proper disengaging of the beads was ensured by making denser beads(using stainless steel powder) to enable the operation of the reactor at higher flow rates. Gas hold-up increased with increasing gas superficial velocity, whereas solid and liquid hold-ups decreased.

7.2 RECOMMENDATIONS FOR FUTURE WORK

1. Experiments should be conducted with celite micro beads as the support matrix. These beads are as small as 200 μ and have a very large internal area. This would give an insight into the problem of internal diffusion. These beads are also easier to handle as compared to carrageenan beads.

2. Ammonia instead of yeast extract should be used as a source of nitrogen so as to get an exact idea about nitrogen and carbon utilization.

3. Computer controlled experiments should be done to achieve better control over various experimental variables, which would help in determining certain parameters essential for the modelling of the system.

4. Optimization of feed flow rate and conversion yields should be investigated.

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APPENDIX I

Calculation of Specific Global Rate of Reaction for Patulin Production

Material balance on patulin yielded the following equation(Section 13.4),

$$r = V \frac{dC}{dt} + Q\ C \qquad (I)$$

Concentration vs. time data for the large beads yielded the following polynomial,

$$C(t) = 10161.13\times10^{-4} + 379.77\times10^{-4}t + 2.28\times10^{-4}t^2 + 10^{-6}t^3 + 718745.8\times10^{-15}t^4$$

On differentiation the above equation becomes,

$$\frac{dC(t)}{dt} = 379.77\times10^{-4} - 456676.26\times10^{-5}t + 186788.23\times10^{-5}t^2 - 28749.83\times10^{-4}t^3$$

At t = 199 hours,

$dC(t)/dt = -11.12 \times 10^{-4}$

$Q = 1.2\times10^{-2}$ L/h

C = 3.33 mmol/L

V = 0.75 L

All these valuse are substituted in equation (I) to give

$r = 3.86 \times 10^{-2}$ mmol/h

Total amount of protein in the reactor at 199 hours(Figure 5.6) = 0.755 g

Dividing 'r' by the total amount of protein gives the specific global rate of reaction for production of patulin ($\bar{r}$).

$\bar{r} = 51.1$ μ mol/g protein -h

The same procedure was repeated for all the data points for calculating the specific rates for large and small beads. Specific rate of reaction for utilization of glucose was also calculated in a similar way. In this case the material balance gives the following equation,

$$r = V \frac{dC}{dt} - Q(C_{g_{in}} - C_{g_{out}})$$

where $C_{g_{in}}$ and $C_{g_{out}}$ is concentration of glucose in the inlet and outlet stream respectively. Concentration of glucose in the inlet stream is known and concentration in the outlet stream is same as the concentration in the reactor because the liquid phase is perfectly mixed.

APPENDIX II

Calculation of Diffusion Coefficients

The diffusion coefficients of the substrates in carrageenan beads are calculated by solving the following equations.

$$\tan q_n = \frac{3q_n}{3 + \alpha q_n^2}$$

where

$$\alpha = \frac{(V/n)}{(4/3)\pi a^3 K}.$$

V = volume of the solution excluding the space occupied by beads.

n = number of beads.

a = radius of the bead,

K = partition coefficient, defined as the ratio of the solute concentration in the bead to the solute concentration in the solution at equilibrium.

q_n terms are the non zero positive roots of the above equation.

All the non zero positive roots of this equation are found by using Newton-Raphson method. The value of α is known as all the parameters are measured during the experiment. It was proved that at equilibrium the concentration of the solute in the bead and in the solution was the same or in other words K = 1.

The concentration of solute (substrate) in the solution at any time is given by the following equation.

$$C_L = \frac{\alpha\, C_{LO}}{(1+\alpha)} \left| 1 + \sum_{n=1}^{\infty} \frac{6(1+\alpha)e^{-Dq_n^2 t/a^2}}{9 + 9\alpha + q_n^2 \alpha^2} \right|$$

In this equation all the parameters except 'D' are known. A computer program was written to solve this equation by guessing the value of 'D' . The calculated value of C_L was compared to the measured one and least square technique was used to find the value of 'D' which gave the best fit. Program listings from an IBM XT microcomputer are attached at the end of this Appendix.

```
10 ' NEWTON -- A PROGRAM TO CALCULATE ROOTS OF A SPECIAL EQUATION BY
20 '           THE NEWTON-RAPHSON METHOD
30 INPUT "Do you want to find the roots?  y/n?  " , ANS$
40 IF ANS$ = "y" GOTO 50
50 DIM X(50)
60 DIM ROOT(50)
70 RAD = 1.54
80 LQDVOL = 47200!
90 BEADNM = 865
100 BEADVOL = 4! * 3.1415927# * RAD*RAD*RAD / 3!
110 ALPHA = ( LQDVOL / BEADNM )/ BEADVOL
120 PRINT ALPHA
130 DEF FNMAIN(X) = TAN(X) -  ( (3 * X)/ (3 + ALPHA * X^2) )
140 DEF FNDERIV(X)= 1 / ((COS(X))^2) - ( (9- 3*ALPHA*X^2) / ((3+
150 M = 1
160 FOR NZ = 0 TO 50
170     X(NZ) = 0!
180 NEXT NZ
190 NZ = 0
200 X(0) = 3.1415927# / 2 - .01 + M *  3.1415927#
210 M = M + 1
220 IF M = 20 GOTO 320
230    X(NZ + 1) = X(NZ) - FNMAIN( X(NZ) ) / FNDERIV( X(NZ) )
240 IF ABS(X(NZ + 1 )-X(NZ )) < .00001 GOTO 270
250 NZ = NZ + 1
260 GOTO 230
270 ROOT(M - 1) = X(NZ + 1)
280 OPEN "roots" FOR APPEND AS #3
290 PRINT #3  , ROOT(M - 1)
300 CLOSE #3
310 GOTO 160
320 END
```

```
10 ' TESTGNRT --  A PROGRAM TO GENERATE THE CONCENTRATIONS IN THE BULK
20 '              SOLUTION FOR GIVEN TIMES, T. PARAMETERS MAY VARIED AS
30 '              DESIRED. SEE "DIFFUSION  CHARACTERISTICS OF SUBSTRATES
40 '              IN Ca-ALGINATE GEL BEADS" BY TANAKA AND COHORTS. THIS
50 '              PROGRAM RUNS IN COLLUSION WITH THE PROGRAM "NEWTON"
60 '              WHICH GENERATES THE NECESSARY ROOTS (STORED IN FILE "ROOTS")
70 '              RESULTS ARE STORED IN FILE "EQUATION" AND CAN BE USED
80 '              BY THE PROGRAM "VARIANCE" TO COMPUTE THE 'BEST' DIFFUSION
90 '              CONSTANT.
100 'V -- volume of liquid
110 'n -- number of beads
120 't -- run time in minutes
130 ' A -- the radius of a bead
140 'qn -- the nonzero roots of tan(qn) = 3(qn)/(3 + alpha * qn)
150 ' CL0 -- the initial concentration of the solution
160 'ALPHA -- (V/N)/(4*PI*(A^3)/3)
170 V = 47.5
180 N=809
190 A = .154
200 PI = 3.1515927#
210 D = .000408
220 CL0 = 8.38
230 ALPHA = (V/N)/(4*PI*(A^3)/3)
240 FOR T = 0 TO 10
250 CL = (ALPHA * CL0)/(1 + ALPHA)
260 PRINT CL
270 PRINT ALPHA
280 OPEN "roots" FOR INPUT AS #1
290 WHILE NOT EOF(1)
300 INPUT #1, QN
310 PRINT QN
320 IF  EXP( -D * QN^2 * T / A^2) > .00001 GOTO 330 ELSE GOTO 350
330 CL =  CL + (ALPHA * CL0 )/(1 + ALPHA) * (  (6 * (1 + ALPHA) * EXP( -D * QN^2
340 WEND
350 OPEN "equation" FOR APPEND AS #2
360 PRINT T, CL
370 PRINT #2, T, CL
380 CLOSE #2
390 CLOSE #1
400 NEXT T
410 CLOSE #1
```

```
10 ' GRID -- A PROGRAM TO THAT DRAWS A GRAPH WITH AN ORIGIN AT ANY POINT ON
20 '        THE SCREEN . IT CAN PLOT THE EQUATION STORED AS COORDINATES
30 '        IN THE FILE "EQUATION" (IN THIS CASE GENERATED BY THE PROGRAM
40 '        "TESTGNRT") AND ANY SPECIAL DATA POINTS INSERTED ON REQUEST.
50 '        UNFORTUNATELY THIS GRAPHICS PROGRAM CANNOT BE PRINTED ON THE
60 '        PRINTER AT PRESENT. A COMMAND TO DO THIS MAY BE BURIED IN THE
70 '        EPSON MANUAL.
80 CLS
90 SCREEN 2
100 ' YVALUE -- location of Y = 0 in pixels (0 - 199)
110 'XVALUE -- location of X = 0 in pixels ( 0 - 615)
120 ' xpixnumber -- number of pixels from origin in + X direction
130 ' ypixnumber - number of pixels from origin in + Y direction
140 ' xpixdiv -- the number of pixels per x division
150 ' ypixdiv -- the number of pixels per y division
160 'xp -- number of pixels per unit -- x-axis
170 'yp -- number of pixels per unit -- y-axis
180 '
190 INPUT "Do you want diffusion equation plotted? y/n  " ,CHOICE$
200 IF CHOICE$ = "y" GOTO 210 ELSE GOTO 340
210 OPEN "equation" FOR INPUT AS #1
220 INPUT #1, X,Y
230 MAX.X =X : MIN.X = X : MAX.Y = Y : MIN.Y = Y
240 WHILE NOT EOF(1)
250 INPUT #1, X, Y
260 IF X>MAX.X THEN MAX.X = X
270 IF X<MIN.X THEN MIN.X = X
280 IF Y>MAX.Y THEN MAX.Y = Y
290 IF Y<MIN.Y THEN MIN.Y = Y
300 WEND
310 PRINT "Range of values stored in datafile."
320 PRINT "max.x =  " MAX.X "min.x =  " MIN.X  "max.y =  " MAX.Y  "min.y =  "            MIN.Y
330 CLOSE #1
340 '
350 INPUT "Do you have special data points to plot?  y/n  ",  PTPLOT$
360 IF PTPLOT$ = "y" GOTO 370 ELSE GOTO 440
370 OPEN "ptfile" FOR APPEND AS #2
380 INPUT "Give values of individual points. (0,0) to end.  ", XPT,YPT
390 WHILE XPT <> 0 OR YPT <> 0
400 PRINT #2, XPT,YPT
410 GOTO 380
420 WEND
430 CLOSE #2
440 '
450 INPUT "Choose location of x-axis (0 - 180 )  ", YVALUE
460 INPUT "Choose location of y-axis ( 0 - 600 )  ", XVALUE
470 INPUT "What is the value of the y offset?  ", YOFFSET
480 '
490 XPIXNUMBER = 639 - XVALUE
500 YPIXNUMBER = YVALUE
510 '
520 INPUT "What are the number of units for the x-axis?  ", XSIZE
530 INPUT "What are the number of units for the y-axis?  ", YSIZE
540 '
550 XP = XPIXNUMBER / XSIZE
560 YP = YPIXNUMBER / YSIZE
570 '
580 INPUT "Number of units per division on x-axis?   ", XDIVSIZE
590 INPUT "Number of units per division on y-axis?   ", YDIVSIZE
600 '
```

```
610 '
620  XPIXDIV = XP * XDIVSIZE
630 YPIXDIV = YP * YDIVSIZE
640 CLS
650 LOCATE 1 ,18 : PRINT " yoffset =  ", YOFFSET
660 LOCATE 2,18  : PRINT " units per x division =  ", XDIVSIZE
670 LOCATE 3, 18 : PRINT " units per y division =  ", YDIVSIZE
680 LINE (0, YVALUE) - (639, YVALUE)
690 '
700 FOR N = 1 TO INT( XVALUE / XPIXDIV)
710 LINE (XVALUE - N * XPIXDIV, YVALUE) - (XVALUE - N * XPIXDIV, YVALUE - 3)
720 NEXT N
730 '
740 FOR N = 1 TO INT (XSIZE / XDIVSIZE )
750 LINE (XVALUE + N * XPIXDIV, YVALUE) - (XVALUE + N * XPIXDIV, YVALUE - 3)
760 NEXT N
770 '
780 LINE (XVALUE, 0) - (XVALUE, 199)
790 '
800 FOR N = 1 TO INT (YSIZE / YDIVSIZE)
810 LINE (XVALUE, YVALUE - N * YPIXDIV) - (XVALUE - 6 , YVALUE - N * YPIXDIV)
820 NEXT N
830 FOR N = 1 TO INT ( (199-YVALUE)/ YPIXDIV)
840 LINE (XVALUE, YVALUE + N * YPIXDIV) - (XVALUE - 6, YVALUE + N * YPIXDIV)
850 NEXT N
860 '
870 IF CHOICE$ = "y" GOTO 880 ELSE GOTO 940
880 OPEN "equation" FOR INPUT AS #1
890 WHILE NOT EOF(1)
900 INPUT #1, X , Y
910 PSET (XVALUE + XP * X, YVALUE - YP * Y + YP *YOFFSET)
920 WEND
730 CLOSE #1
940 '
950 IF PTPLOT$ = "y" GOTO 960 ELSE GOTO 1010
960 OPEN "ptfile" FOR INPUT AS #2
970 WHILE NOT EOF(2)
980 INPUT #2, X,Y
990 PSET (XVALUE + XP * X, YVALUE - YP * Y + YP * YOFFSET)
1000 WEND
1010 'the end
```

```
10 ' VARIANCE -- A PROGRAM TO CALCULATE THE VARIANCE OF THE EQUATION
20 '            STORED AS COORDINATES IN THE FILE "EQUATION" AND THE
30 '            EXPERIMENTAL DATA POINTS, FROM THE BEAD DIFFUSION EXPERIMENTS,
40 '            STORED IN THE FILE "PTFILE". THE PROGRAM IS DESIGNED FOR THE
50 '            OPERATOR TO PICK THE SUCCESSIVE VALUES OF D, THE DIFFUSION
60 '            CONSTANT, FOR WHICH THE VARIANCE WILL BE CALCULATED. THIS
70 '            PROGRAM OPERATES IN CONJUNCTION WITH "GRID" (OR A SMALL PROGRAM
80 '            TO MAKE UP "PTFILE" OF EXPERIMENTAL POINTS) AND "TESTGNRT".
90 'V -- volume of liquid
100 'n -- number of beads
110 't -- run time in minutes
120 ' A -- the radius of a bead
130 'qn -- the nonzero roots of tan(qn) = 3(qn)/(3 + alpha * qn)
140 ' CL0 -- the initial concentration of the solution
150 'ALPHA -- (V/N)/(4*PI*(A^3)/3)
160 DIM T1(62), Y1(62), T2(61), Y2(61)
170 D = .000408
180 '
190 V = 47.2
200 NUM=865
210 A = .152
220 PI = 3.1515927#
230 CL0 = 7.151
240 ALPHA = (V/NUM)/(4*PI*(A^3)/3)
250 FOR T = 0 TO 20
260 CL = (ALPHA * CL0 )/(1 + ALPHA)
270 PRINT CL
280 PRINT ALPHA
290 OPEN "roots" FOR INPUT AS #1
300 WHILE NOT EOF(1)
310 INPUT #1, QN
320 PRINT QN
330 IF  EXP( -D * QN^2 * T / A^2) > .00001 GOTO 340 ELSE GOTO 360
340 CL =  CL + (ALPHA * CL0 )/(1 + ALPHA) * (  (6 * (1 + ALPHA) * EXP( -D * QN^2
350 WEND
360 OPEN "equation" FOR APPEND AS #2
370 PRINT T, CL
380 PRINT #2, T, CL
390 CLOSE #2
400 CLOSE #1
410 NEXT T
420 CLOSE #1
430 OPEN "equation" FOR INPUT AS #1
440 FOR N = 0 TO 20
450 INPUT #1, T1(N), Y1(N)
460 NEXT N
470 CLOSE #1
480 '
490 OPEN "ptfile" FOR INPUT AS #2
500 M = 1
510 WHILE NOT EOF(2)
520 INPUT #2, T2(M), Y2(M)
530 PRINT " t2 and y2 are ??????", T2(M), Y2(M)
540 M = M + 1
550 WEND
560 CLOSE #2
570 M = M - 1
580 PRINT M
590 '
600 N = 0
```

```
610 SUM = 0
620 FOR COUNT = 1 TO M
630 IF T1(N)<>T2(COUNT) GOTO 640 ELSE GOTO 660
640 N = N + 1
650 GOTO 630
660 PRINT "t1(n) and t2(m) =  ", T1(N), T2(COUNT)
670 PRINT "y1(n) and y2(count)  ", Y1(N) , Y2(COUNT)
680 SUM = SUM + (Y2(COUNT) - Y1(N))^2
690 PRINT "sum =  " , SUM
700 NEXT COUNT
710 LPRINT "  "
720 LPRINT "lactose diffusion, 0.1%, column 2, theoretical normalized to Co"
730 LPRINT "  "
740 LPRINT "D = ", D
750 LPRINT "sum =  ", SUM
760 KILL "equation"
770 INPUT "Choose a new value for diffusion constant.  ", D
780 GOTO 180
```

APPENDIX III

Calculation Procedure For Individual Phase Hold-ups

For gas liquid fluidization the total pressure gradient at any bed level is simply the bed weight per unit volume at that level (i.e. no wall frictional losses),

$$-\frac{dP}{dz} = (\varepsilon_l \rho_l + \varepsilon_g \rho_g + \varepsilon_s \rho_s)\, g \qquad \text{(III.1)}$$

where the individual phase hold-ups are inter-related as

$$\varepsilon_l + \varepsilon_g + \varepsilon_s = 1 \qquad \text{(III.2)}$$

The total pressure drop across a bed of height H is given by

$$-\Delta P = g \int_0^H (\varepsilon_l \rho_l + \varepsilon_g \rho_g + \varepsilon_s \rho_s) \qquad \text{(III.1a)}$$

in which $\varepsilon_g \rho_g$ can usually be neglected relative to the other terms to which it is added. When liquid is the continuous phase, the dynamic pressure gradient as measured by differential manometery is the total pressure gradient corrected for the hydrostatic head of the liquid:

$$-\frac{dp}{dz} = (-\frac{dP}{dz}) - \rho_l g \qquad \text{(III.3)}$$

Substituting Equations (III.1) and (III.2) in to Equation (III.3),

$$-\frac{dp}{dz} = [\varepsilon_s(\rho_s - \rho_l) - \varepsilon_g(\rho_l - \rho_g)]g \qquad \text{(III.4)}$$

When $\varepsilon_g = 0$, $\varepsilon_s = 1-\varepsilon_l$ and Equation (III.4) reduces to the following form:

$$-\frac{dp}{dz} = \varepsilon_s(\rho_s - \rho_l)g = (1-\varepsilon_l)(\rho_s - \rho_l)g \qquad \text{(III.5)}$$

The gas phase hold-up is calculated by subtracting equation (III.4) from equation (III.5). This was done by measuring the pressure gradient for the three phase bed and for the corresponding two phase solid-liquid bed at the same value of H (and therefore of ε_s) using differential manometery. Solid hold-up (ε_s) is given by

$$\varepsilon_s = M_s/AH\rho_s \qquad \text{(III.6)}$$

where M_s is mass of the solids, A is the area of cross section of the bed and ρ_s is the density of the solids. One sample calculation of the phase hold-ups is presented below.

$M_s = 2200.0$ g

$d = 16.26$ cm

$A = \pi d^2/4 = 207.55$ cm^2

$H = 22.86$ cm

$\varepsilon_s = 2200/(207.55 \times 22.86 \times 1.41) = 0.33$

On subtracting equation (III.4) from equation (III.5) we get,

$(\text{pressure drop})_{3-2} = \varepsilon_g(\varepsilon_l - \varepsilon_g)g$

$\rho_g = 129.28\times10^{-5}$ g/cm^3

$\rho_l = 1.0\ g/cm^3$

Substituting these bvalues in equation (III.7) gives,

$\varepsilon_g = 0.08$

From equation (III.2)

$\varepsilon_l = 1 - 0.33 - 0.08 = 0.59$