Titre: Title:	Continuous penicillin V production by immobilized penicillium chrysogenum in an inverse fluidized bed reactor
Auteur: Author:	Daguang Wang
Date:	1990
Type:	Mémoire ou thèse / Dissertation or Thesis
Référence: Citation:	Wang, D. (1990). Continuous penicillin V production by immobilized penicillium chrysogenum in an inverse fluidized bed reactor [Master's thesis, École Polytechnique de Montréal]. PolyPublie. https://publications.polymtl.ca/56722/

URL de PolyPublie: PolyPublie URL:	https://publications.polymtl.ca/56722/
Directeurs de recherche: Advisors:	Claude Chavarie, & Denis Rouleau
Programme: Program:	Génie chimique

UNIVERSITE DE MONTREAL

CONTINUOUS PENICILLIN V PRODUCTION BY IMMOBILIZED PENICILLIUM CHRYSOGENUM IN AN INVERSE FLUIDIZED BED REACTOR

par

Daguang WANG

DEPARTEMENT DE GENIE CHIMIQUE

ECOLE POLYTECHNIQUE

MEMOIRE PRESENTE EN VUE DE L'OBTENTION

DU GRADE DE MAITRE ES SCIENCES APPLIQUEES (M.Sc.A.)

JANUARY 1990

ISBN 0-315-58127-1

UNIVERSITE DE MONTREAL

ECOLE POLYTECHNIQUE

Ce mémoire intitulé:

CONTINUOUS PENICILLIN V PRODUCTION

BY IMMOBILIZED PENICILLIUM CHRYSOGENUM

IN AN INVERSE FLUIDIZED BED REACTOR

présenté par: Daguang Wang

en vue de l'obtention du grade de: M.Sc.A.

a été dûment accepté par le jury d'examen constitué de:

M. Christophe Guy	Ph.D., président-rapporteur
M. Danilo Klvana	Ph.D.
M. Claude Chavarie	Ph.D., directeur de recherche
M. Denis Rouleau	Ph.D., Co-Directeur

To my parents

ABSTRACT

This research describes the continuous penicillin V production by immobilized Penicillium chrysogenum in an inverse fluidized bed bioreactor.

In this work, polystyrene beads were used as the immobilization matrix. Tyndallization and gamma radiation were found to be feasible for the sterilization of polystyrene beads.

The inverse fluidized bed bioreactor (IFBBR) which was previously used for the biochemical oxidation of ferrous iron by Thiobacillus ferroxidans was not designed for standard pure culture work. However, penicillin production requires that the reactor must be sterilized. The original reactor design had to be modified. The plexiglass reactor body was replaced with pyrex glass. An inoculation port was set up at the bottom of the reactor. An air diffuser was added on the aeration inlet. A steel wire eroder was placed in the inner draft tube to control the thickness of biofilm.

A biomass analysis method was developed by the use of sodium hypochlorite digestion. It is a quick and convenient method for biomass analysis.

The limiting phosphate concentration was determined in semicontinuous mode. Penicillin production was influenced by the change

of phosphate concentration. Higher specific productivity was obtained at lower phosphate concentration. As the phosphate concentration increased, the morphology of the biofilm changed from fluffy to compact. A phosphate concentration of 0.001% is recommended for penicillin production in IFBBR.

The maximum penicillin production and specific productivity in IFBBR are 0.178 (g/L) and $5.79*10^{-4}$ (g p-v/g cell /h) respectively. These results are comparable with that in other systems and furthermore the biofilm thickness can be controlled.

RESUME

Dans ce travail nous avons étudié la production de la penicilline V en continu par du Penicillium chrysogenum immobilisé dans un bioréacteur à lit fluidisé inversé.

Nous avons utilisé des particules de polystyrène comme matrice immobilisée. Ces particules sont stérilisées par tyndallization et irradiation gamma.

Le bioréacteur à lit fluidisé inversé (IFBBR) qui était déjà utilisé pour l'oxydation biochimique des ferreux en utilisant des Thiobacillus ferroxidans n'était pas conçu pour faire des cultures non contaminées. Cependant, pour la production de la penicilline il faut que le bioréacteur soit stérilisé. Donc le design original du réacteur se trouve être modifié pour satisfaire à cette condition. Le corps du réacteur en plexiglass est remplacé par du verre pyrex. Une porte d'inoculation est installée dans le bas du réacteur. Un diffuseur d'air est ajouté à l'aération à l'entrée. Pour contrôler l'épaisseur du biofilm nous avons placé un érodeur de fil de fer dans la partie intérieure du tube.

Une méthode d'analyse de biomasse a eté développée en utilisant la digestion de l'hypochlorite de sodium; c'est une méthode convenable et rapide pour l'analyse.

La concentration limitante de phosphate est déterminée en mode semicontinu. La production de pénicilline est influencée par la variation de la concentration en phosphate. Une productivité spécifique élevée est obtenue à basse concentration en phosphate. Quand la concentration en phosphate augmente, la morphologie du biofilm passe de la forme pelucheux à compact et dense. Une concentration en phosphate de 0.001% est recommendée pour une meilleure production de pénicilline dans un réacteur IFBBR. La production maximum de pénicilline et la productivité dans un réacteur IFBBR sont respectivement de 0.178 g/L et de $5.79*10^{-4}$ g p-V/g cell/h. Ces résultats sont comparables avec d'autres systèmes et, de plus, l'épaisseur du biofilm est contrôlée.

ACKNOWLEDGEMENTS

I would like to acknowledge Dr. Denis Rouleau, Dr. Claude Chavarie, Dr. Juliana Ramsay and Dr. Bruce Ramsay for their support and supervision of this thesis.

I wish to thank all of the students, secretaries and technicians who helped me throughout the course of this work.

Financial support for this work was provided by NSERC and Ecole Polytechnique de Montréal.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLES AND ABBREVIATIONS

C_D frictional coefficient

C_L oxygen concentration, mM

C_L^* solubility of oxygen in broth, mM

d_{BP} diameter of bioparticle, m

d_{fix} diameter of fix bed in das-siphon, m

d_p diameter of support particle, m

D_{int} diameter of central tube, m

D_{ext} diameter of external tube, m

f friction factor for monophasic flow

f_{TP} friction factor for diphasic

F feed rate of substrate, g/h

Fr_{TP} diphasic Froude number

g gravity, m/s^2

G volumic flow rate of gas, m^3/s

h_{fix} height of the fixed bed, m

H height of the fluidized bed, m

H_{init} height of initial fluidized bed, m

K_o Monod constant for growth dependence on oxygen, mM

K_{op} saturation coefficient for penicillin production dependence on oxygen, $(mM)^{2.74}$

K_{op}' Contois coefficient for penicillin production dependence on oxygen, $(mM)^{2.74}/g$

K_{ox} Contois coefficient for growth dependence on oxygen, mM/g

K_I substrate (glucose) inhibition constant for penicillin production, g/L

K_La volumetric mass transfer coefficient for oxygen, h^{-1}

K_p Monod constant for penicillin production dependence on glucose, g/L

K_s Contois constant for growth dependence on substrate, g/L

K_{sx} Contois coefficient for growth dependence on substrate, g/L

K_x Contois constant for growth dependence on glucose, g/g

L volumic flow rate of internal liquid recirculation, m^3

m_x maintenance requirement of substrate, g/g

m_o maintenance requirement of oxygen, mmol/g

N bioparticle concentration, g/l

P penicillin concentration in broth, g/mL

q_o specific rate of oxygen uptake, mmole/g-cell/h

q_p specific rate of penicillin production, g/g-cell/h

q_{pm} maximum specific rate of penicillin production, g/g-cell/h

q_s specific rate of substrate consumption, g/g-cell/h

Re Reynolds number

Re_{fix} fix bed Reynolds number

Re_{TP} diphasic Reynolds number

Re_t bioparticle terminal Reynolds number

S substrate concentration, g/L

S_F substrate concentration in feed stream, g/L

t time, h

U_t terminal speed of bioparticle, m/s

V volume of broth, L

We_{TP} diphasic Weber number

X free cell concentration, g/L

$Y_{p/o}$ yield coefficient for penicillin from oxygen, g/mmol

$Y_{p/s}$ yield coefficient for penicillin from substrate, g/g

$Y_{x/o}$ yield coefficient for biomass from oxygen, g/mmol

$Y_{x/s}$ yield coefficient for biomass from substrate, g/g

Z parameter defined in equation (1)

α volumetric fraction of the gas in the airlift section

ε fraction of liquid in the fluidized bed

ε_v friction loss factor

μ specific growth rate, h^{-1}

μ_m maximum specific growth rate, h^{-1}

μ_p maximum specific rate of product formation, g product/g cell/hρ_L density of the liquid, kg/m^3

ρ_p density of the support particles, kg/m^3

ρ_{BP} density of the bioparticles, kg/m^3

CHAPTER 1

INTRODUCTION

It was more than 60 years ago that Flemming first discovered that penicillin was produced by the mold Penicillium. Since then penicillin and its many semi-synthetic derivatives have been used to treat infectious diseases and much has been done to increase its productivity. Strain development (i.e. genetic mutation and manipulation) has led to high-yielding strains while process improvement (i.e. control of culture conditions and improvement of bioreactor design) has also played a major role. At the level of industrial scale production, the processing technology becomes particularly important.

Penicillin G and V are produced using submerged culture of Penicillium chrysogenum, a filamentous microorganism. Unfortunately, the growth of this mycelial organism results in high viscosity of the culture broth. Such a situation causes a decrease in the mass transfer capabilities of a conventional stirred tank reactor for oxygen and nutrients. The agitation and aeration rates can be increased to overcome these problems but severe shear stress may then be imposed on the cells causing a decline in cell productivity. The rheology of the broth can be changed by immobilizing the cells. There are a number of advantages in the use of immobilized cells, such as the increased lifetime of the catalyst, increased productivity, and from a chemical engineering point of view, a means of overcoming mass transfer

problems.

Many methods have been developed for the immobilization of spores or mycelium of P. chrysogenum. K-carrageenan beads [1], porous glass, the inner surface of nylon tubing [2], polyacrylamide gel, collagen membrane [3] and calcium alginate beads [4] have been used as matrices for the immobilization of P. chrysogenum. For surface immobilization, celite [5] and polycarbonate beads [6] have been used. The choice of matrix is primarily determined by its compatability with the organism and by its operational stability in a bioreactor. If an inverse fluidized bed bioreactor (IFBBR) is used, the density of the matrix must also be considered.

Conventional fluidized bed reactors have been studied for use in penicillin production using P. chrysogenum immobilized on celite beads [5] or in carrageenan beads [1]. However, the high cost of immobilization, the difficulty in controlling the size of the beads, growth within the beads and mass transfer limitation through the beads may make these methods unworkable on a commercial scale.

The IFBBR reactor has all the advantages of the airlift fermentor (efficient mass transfer and low shear) plus the advantages of surface immobilization (high catalyst concentration and good mass transfer through the film). There is also another advantage of being able to control the biofilm thickness by the use of a device in the draft tube to shear excess biomass from the beads.

The reactor has been evaluated for the oxidation of iron (for use in mineral leaching processes) [7] as well as wastewater treatment [8].

In order to work with a pure culture it was necessary to modify the design of the IFBBR and find a method for the sterilization of the polystyrene beads.

The objective of this research is to verify the use of polystyrene beads as an immobilization support matrix for the continuous production of penicillin V by P. chrysogenum in the inverse fluidized bed bioreactor.

To achieve the above overall objective the following specific objectives had to be met.

—— To develop a sterilization procedure for polystyrene beads

—— To immobilize P. chrysogenum on polystyrene beads

—— To develop a method for biomass analysis

—— To modify the reactor previously used in waste water treatment for pure culture and to control the biofilm thickness

—— To determine the limiting phosphate concentration for penicillin V production

—— To evaluate the potential of lactose as a substrate

—— To compare the specific productivity of IFBBR reactor with other systems

CHAPTER 2

GENERAL BACKGROUND AND THEORETICAL PRINCIPLES

2.1. Penicillin Fermentation

2.1.1. History of Penicillin Production

Penicillin, which was discovered by Alexander Flemming in 1929, is among the most effective antibiotics to combat infectious diseases. Considerable improvement in penicillin production from Fleming's culture has been achieved by the process of strain selection and culture method. High-yielding strains and new reactor designs made it possible to increase the productivity of penicillin.

Fleming's original strain of P. notatum produced only a few milligrams of penicillin per liter, but today, strains can produce up to 30 g L^{-1}. Figure 1 [9] shows that due to the improvement of medium, aeration, inoculum preparation, culture reisolation, programmed feeding and strain selection, the production of penicillin has increased significantly.

Surface culture and submerged culture were the original culture methods processed in batch. To obtain enough mold in surface cultivation for final production, the growth procedure must be repeated many times. Rigid aseptic technique is absolutely essential and considerable time is involved since each transfer of mold takes five to

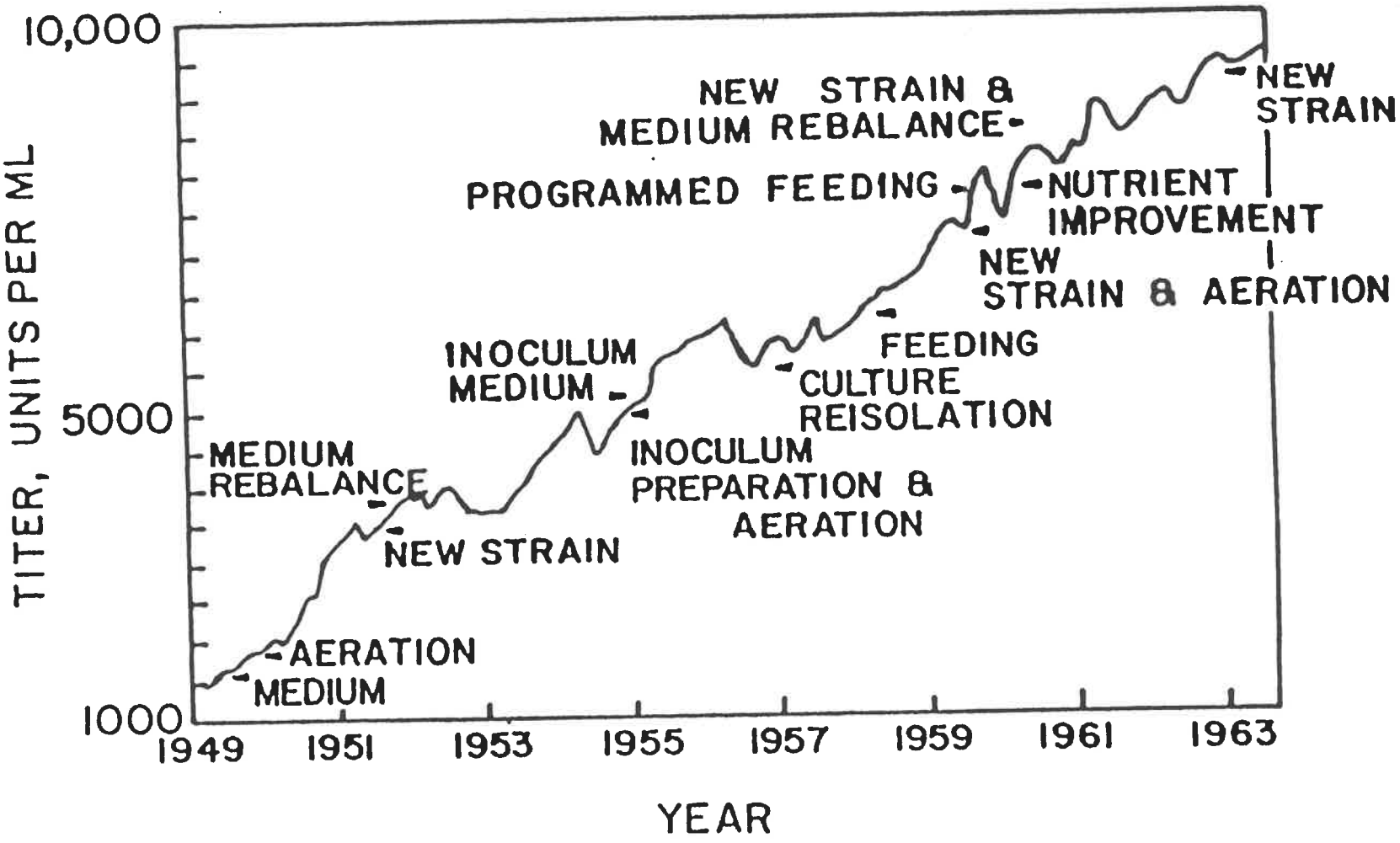


Figure 1 Influence of fermentation development studies on a typical antibiotic. (Courtesy of Bristol Laboratories.)

seven days to grow.

Submerged culture is used for the production of penicillin since automatic control is well developed and higher mycelium concentration can be maintained. A flowsheet of the penicillin manufacturing process used by Gist-Brocades is presented in Figure 2. In general, four stages can be distinguished: fermentation, product recovery, production of semisynthetic penicillin and medical production [10].

The advantage of the continuous culture is that it permits much closer control of the environment allowing a stable physiological state to be maintained. The stability of the penicillin production rate over a long period and its relatively high value are noteworthy. However, continuous culture washes out free mycelium and higher productivity is hard to achieve. The investigation of Kolachov & Schneider [11] did not show that the continuous process had any marked advantage over the batch process.

The immobilization of mycelium was introduced for penicillin production to overcome the problems such as high viscosity in submerged culture and washout of mycelium in continuous culture [1].

Air lift and fluidized bed reactors using immobilized biomass have been used in the research laboratory [1] for the penicillin production. Some problems exist in that the size of the bioparticles cannot be controlled.

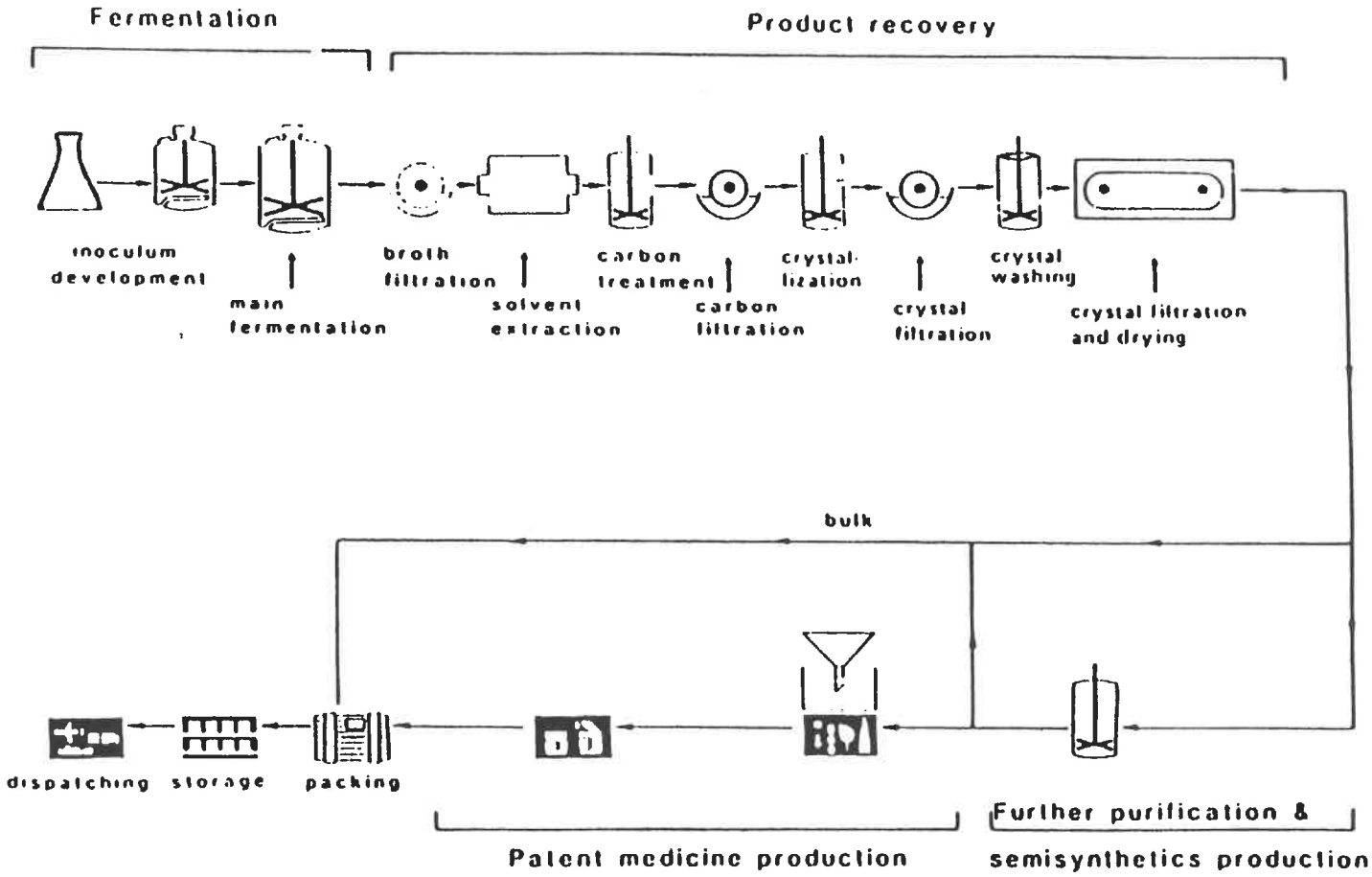


Figure 2 Penicillin manufacturing processes of Gist-Brocades.

2.1.2. Importance of Penicillin

Penicillin is a β-lactam antibiotic which is a specific inhibitor of bacterial cell wall synthesis. Since growing cells synthesize new cell wall, such antibiotics have the property of killing only growing cells and not resting cells.

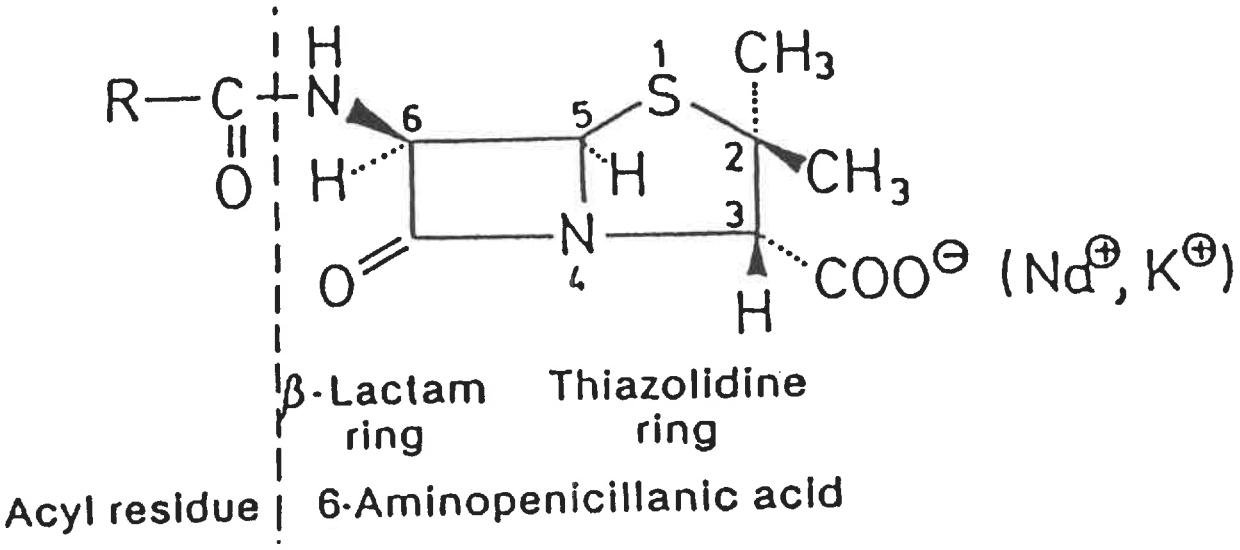
Penicillins are effective against numerous gram-positive bacteria. Because of its low toxicity, large doses of penicillin can be used. Only a small percentage (0.5-2%) of patients develops allergies.

In order to meet the great requirement of penicillin, worldwide production is increasing. The production of penicillin G increased from 12000 tons in 1982 to 20000 tons in 1987.

2.1.3. Biochemical Synthesis

Natural penicillins are produced during the fermentation if no side-chain precursors are added. Usually a side-chain precursor, like phenylacetic acid, is added so that only one desired penicillin is produced. Industrially, only penicillins G, V and O have been produced.

The basic structure of penicillin is 6-aminopenicillanic acid (6-APA), which consists of a thiazolidine ring with a condensed β-lactam ring as shown in Figure 3. At the acyl residue part, different



Penicillin G R = CH_2— Phenyl acetic acid

Penicillin V R = O-CH_2— Phenoxy acetic acid

Figure 3 : The basic structure of penicillin.

R group can form different kinds of penicillin.

The β-lactam thiazolidine ring of penicillin is constructed from L-cysteine, L-valine and L-α-aminoadipic acid. These two amino acids and L-α-aminoadipic acid are condensed to the tripeptide δ-(L-α-aminoadipyl)- cysteinyl-D-valine. Subsequently, the lactam and thiazolidine rings are closed, yielding isopenicillin N. Exchange of the α-aminoadipyl moiety for phenylacetic or phenoxyacetic acids yields penicillin G or V. Figure 4 illustrates the biosynthesis of penicillin G [12]. Penicillin V has a similar biosynthesis pathway except for the precursor side chain.

2.1.4. Fermentation Process Economics

An economic analysis of the penicillin G fermentation presented in Table 1 shows that about 80% of the penicillin production cost is associated with the fermentation step [13]. The glucose and precursor account for 30% of the fermentation cost. Raw material costs may constitute up to 50% of the total cost. Since only 10% of the consumed carbon [14] is used for penicillin production and about 65% for maintenance and 25% for mycelium growth, the efficiency of conversion of the raw materials into penicillin is therefore an important objective.

Mixing costs are considerable because of energy consumption. The

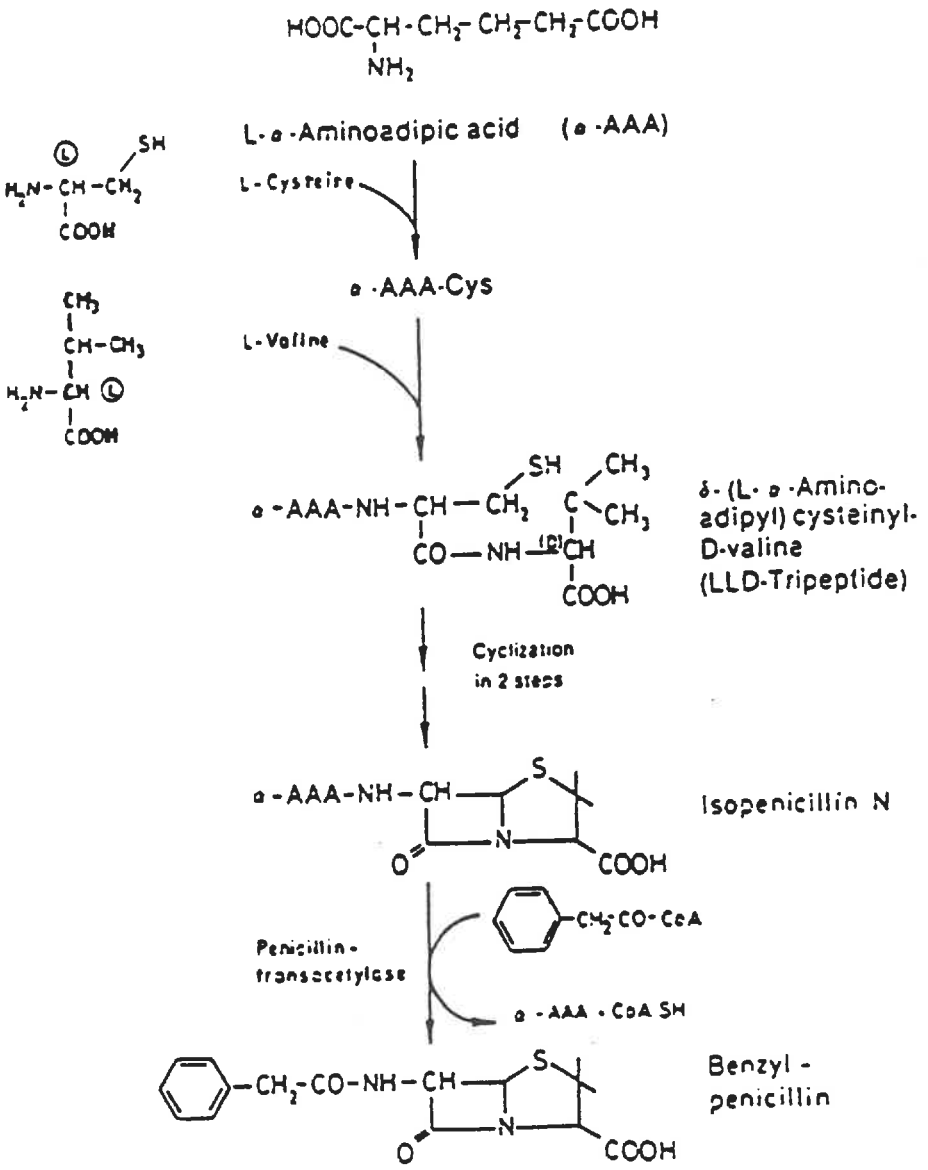


Figure 4 Biosynthesis of penicillin in *Penicillium chrysogenum*

Table 1: Penicillin manufacturing cost

Fermentation cost	%
Raw materials	
Glucose	12
Precursor	11
Others	5
Utility	12
Labor/Supervision	3
Maintenance/Labortory	9
Fixed charges	21
Plant overhead	6
	79
Purification cost	
Direct production cost	13
Fixed charges/Plant overhead	8
	100

separation and purification process constitutes only 20% of the penicillin manufacturing cost. The recovery yields reach 90% and Berdy [15] reported a penicillin production of 40-50 kg of sodium penicillin G per cubic meter of fermentation broth. The fermentation process economics of penicillin V have no great difference from penicillin G.

2.1.5. Regulation of Penicillin Production

2.1.5.1. Phosphate Regulation

High concentrations of inorganic phosphate inhibit the formation of many antibiotics while stimulating vegetative growth [16]. For example, when the phosphate concentration is above 10 mM, it has an inhibitor effect on the production of chlortetracycline, streptomycin oxytetracycline, aminoglycoside antibiotics, (such as neomycin, butirosin, and kanamycin) and peptide antibiotics (such as bacitracin, polymycin, gramicin, and actinomycin).

Several mechanisms have been proposed to explain the effect of phosphate on the production of secondary metabolites. Phosphate addition appears to disrupt the typical biphasic behavior of antibiotic fermentations [17]. In a normal phosphate-limited fermentation, the rates of protein and RNA synthesis increase rapidly during the first 15 to 24h and then decrease to minimum values at the time when the antibiotic is first detected. This decrease in the rate of RNA

synthesis is characteristic of the end of trophophase. When a high concentration of phosphate is added, this decrease does not occur and RNA synthesis continues at a very high rate. The transition from trophophase to idiophase in Penicillium urticae, as measured by appearance of 6-methyl salicyclic acid, is delayed by the addition of inorganic phosphate [18].

Phosphate shifts the carbohydrate catabolic pathways [19]. An increased phosphate concentration in the medium decreases the activity of the pentose phosphate pathway favoring glycolysis. The pentose phosphate route predominates in the rapid growth phase (trophophase), whereas glycolysis is more important in the production phase (idiophase) in P. urticae. Similarly, it was noted with P. chrysogenum that the pentose phosphate pathway is preferentially used during the growth phase of Penicillium prior to penicillin production.

Phosphate also limits the synthesis of the inducer of the antibiotic pathway [20]. This hypothesis has been receiving increased attention recently, owing to the discovery of inducers of antibiotic synthesis. Such effectors of antibiotic synthesis are recognized by their effect on product formation when added during the trophophase and their lack of effect when added during the idiophase.

Other possible mechanisms have also been suggested. It was reported that phosphate affects the level of precursors involved in antibiotic synthesis. Majumdar [21] postulated that phosphate inhibits or

represses phosphatases necessary for antibiotic biosynthesis. It was also suggested that inorganic phosphate suppresses antibiotic production by depriving the cell of an essential metal [22].

From the above, it would appear that phosphate acts by different mechanisms in inhibiting the antibiotic biosynthesis. However, it is possible that these different mechanisms are mediated by a common effector, such as the intracellular ATP concentration or the adenylate energy charge of the cell.

2.1.5.2. Catabolic Regulation

Glucose interferes with the biosynthesis of many antibiotics as shown on Table 2 [23]. The molecular mechanism of carbon catabolite regulation may be related to growth rate control of antibiotic biosynthesis. Thus, glucose interference with penicillin biosynthesis can be eliminated by slow addition of glucose to the fermentation to obtain a slow growth rate.

Recent studies on glucose regulation of penicillin biosynthesis in P. chrysogenum indicate that glucose represses the incorporation of [^{14}C]valine into penicillin [23]. It is also reported that too much glucose and consequently a high value of μ reduces penicillin production because, under the same aeration conditions, the glucose combustion results in a decrease of the oxygen concentration in the

TABLE 2 *Carbon catabolite regulation of antibiotic biosynthesis*

Antibiotic	Interfering carbon source	Noninterfering carbon source(s)
Penicillin	Glucose	Lactose
Actinomycin	Glucose	Galactose
Streptomycin[a]	Glucose	Mannan
		Slowly fed glucose
Siomycin	Glucose	Maltose
Indolmycin	Glucose	Fructose
Bacitracin	Glucose	Citrate
Cephalosporin C	Glucose	Sucrose
Chloramphenicol	Glucose	Glycerol
Violacein	Glucose	Maltose
Prodigiosin	Glucose	Galactose
Mitomycin	Glucose	Low glucose
Neomycin	Glucose	Maltose
Kanamycin	Glucose	Galactose
Enniatin	Glucose	Lactose
Puromycin	Glucose	Glycerol
Novobiocin	Citrate	Glucose
Candidin	Glucose	Slowly fed glucose
Candihexin	Glucose	Slowly fed glucose
Butirosin	Glucose	Glycerol
Cephamycin	Glycerol	Asparagine, starch

[a] Regulation refers to the enzyme mannosidostreptomycinase.

broth [24].

There are two solutions which can overcome glucose repression: to replace glucose with lactose which does not repress penicillin synthesis, or to add glucose slowly to the fermentation so that glucose concentration is always low.

2.1.5.3. Nitrogen Repression

Few studies have dealt specifically with nitrogen metabolite regulation of antibiotic biosynthesis, but several reports indicate that free cell cultures under NH_4^+ limitation lose their ability to produce penicillin G [25]. A critical NH_4^+ level is thus required.

In the continuous fermentation with Cellite R-630 as immobilization support matrix, the NH_4^+ level was maintained at 2.0mM, a level lower than the critical level required for free cells [5]. Ammonium sulphate, a common constituent of the media used in penicillin production, effects the morphology of the filamentous mould. The hyphal length is increased while the frequency of hyphal branching is decreased and the formation of swollen cells is prevented at pH 7.4. It is worth noting that the size control of bioparticles and prolonged production phase can be achieved by nitrogen limitation [26].

2.1.5.4. Lysine Regulation

The amino acid lysine is synthesized from L-α-aminoadipic acid and thus shares a common pathway for penicillin production. Lysine is a feedback inhibitor of homocitrate synthase which is an enzyme involved in L-α-AAA synthesis. Penicillin cannot be synthesized if L-α-AAA is deficient. However the inhibition by lysine of homocitrate synthase is less than the inhibition of penicillin formation, so that other regulatory processes are postulated on the branch from α-aminoadipate to penicillin. Figure 5 shows lysine regulation in penicillin biosynthesis [12].

2.1.5.5. Penicillin Feedback Inhibition

The cessation of penicillin accumulation during a fermention may be related to the end product itself. Exogenous penicillin added at any time throughout the fermentation has been shown to limit subsequent penicillin accumulation [27].

The synthesis-inhibiting level of antibiotics in a particular producing strain is usually similar to the production level of that strain. Table 3 shows that 15 mg of penicillin per mL completely inhibits production by the high-producing mutant E-15, whereas 2 mg/mL is sufficient to inhibit penicillin production by strain Q176 which produces 420 μg/mL and 200 μg/mL completely abolishes penicillin

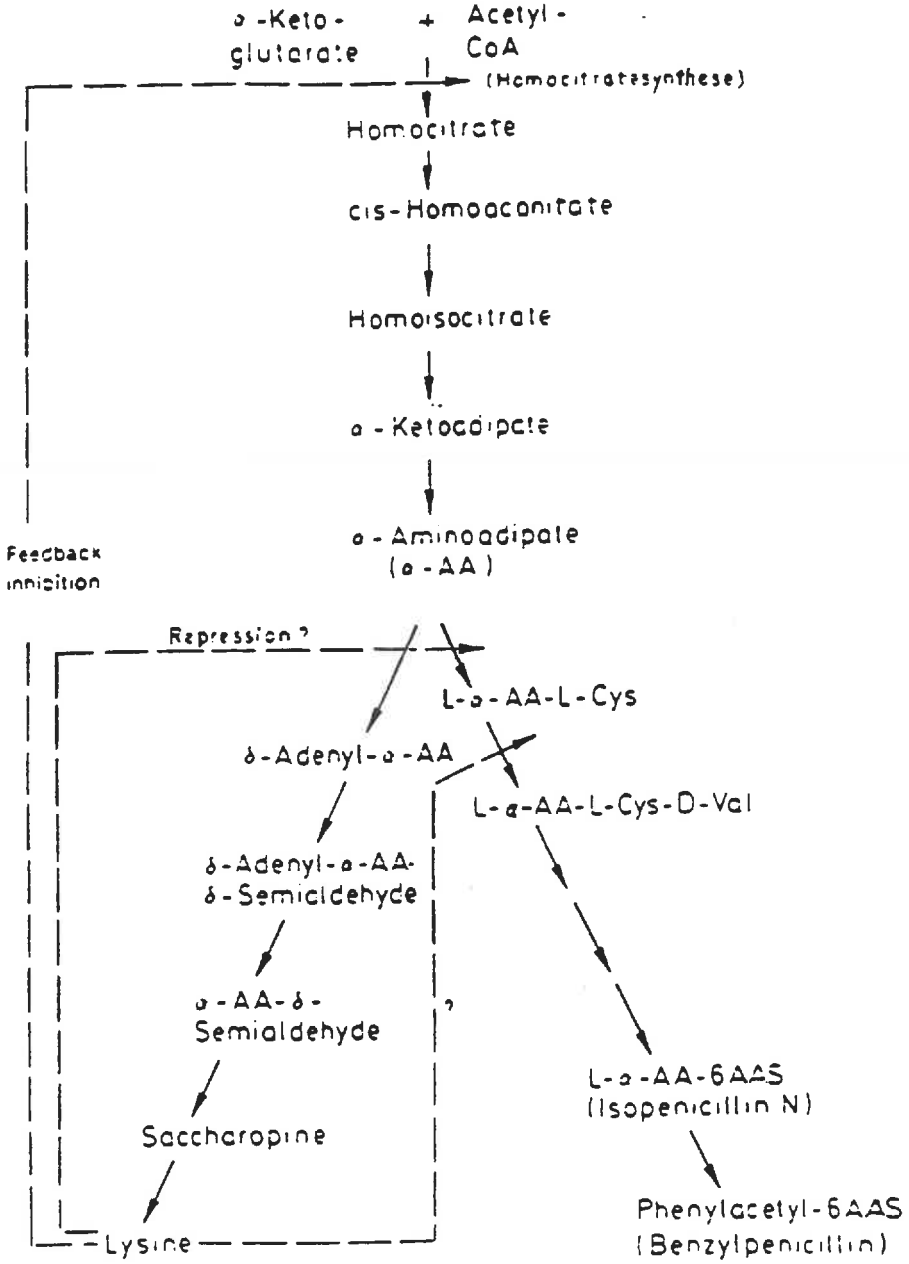


Figure 5 Regulation of penicillin biosynthesis by L-lysine in *Penicillium chrysogenum*

strains of *P. chrysogenum*	inhibitory penicillin concentration	maximum penicillin produced
E15	15mg/mL	very high
Q176	2 mg/mL	0.42 mg/mL
NRRL1951	0.2mg/mL	0.125mg/mL

Table 3: Inhibitory Effect Of Exogenous Penicillin On Defferent *Penicillium chrysogenum* Strains

production by strain NRRL1951 which produces 125 μg/mL [27].

2.1.5.6. The Effect of Dissolved Carbon Dioxide

The concentration of dissolved carbon dioxide is an important process variable in antibiotic fermentations. Carbon dioxide is a product of the oxidation of carbohydrates in the cell mitochondria. Both molecular species, CO_2 and HCO_3^-, are capable of inhibiting the growth rate as well as the cell yield. The inhibition appears to be a complex function of both dissolved carbon dioxide and aqueous HCO_3^-. It is probably based on the loss of balance between the increasing concentration of HCO_3^- and the protoplasm's protein buffer system.

Carbon dioxide was found to inhibit both cell growth and penicillin production for fermentations exposed to a steady-state injection of 12.6 and 20% CO_2 gases. For fermentations subjected to a steady-state injection of 12.6% CO_2 gas, penicillin production and cell growth rates were reduced approximately 23 and 40%, respectively. The fermentations exposed to 20% CO_2 gas resulted in a reduced cell growth rate and penicillin production to nearly nil [28].

While the specific growth rate and penicillin production rate were not affected by exposure to gases of 3 and 5% CO_2 partial pressures, scanning electron microscopy did reveal a significant increase in the hyphal branching frequency compared to the cells of the control

fermentation. For fermentations sparged with 12.6 and 20% CO_2, a significant quantity of swollen and deformed hyphae were observed.

Carbohydrate metabolism and penicillin biosynthesis have been reported [24] to be inhibited even when the concentration of dissolved oxygen is above the critical value of 0.022 mM/L. This decrease in respiration took place when the CO_2 concentration of the effluent gas rose above 4 per cent.

It appears that aeration is necessary for the biosynthesis process in two aspects. First, it should be assured that the oxygen supply is greater than the critical value where the antibiotic production is independent of the oxygen concentration. Secondly, an effect of ventilation should be maintained where the CO_2 concentration is less than its inhibitory critical value.

2.1.5.7. Oxygen

Oxygen is required for the penicillin biosynthesis by mycelium. The rate of oxygen uptake by the immobilized mycelium was found to be about 30% of that of the washed mycelium. Table 4 shows the necessity of air in the course of penicillin production by the immobilized mycelium. Under anaerobic conditions only a small amount of penicillin was produced [32].

TABLE 4

Effect of Air on Production of Penicillin by Immobilized Mycelium[a]

Conditions	Penicillin produced (units/ml hr)
Under air	0.65
Under nitrogen gas	0.04

[a] Ten g immobilized mycelium (containing the equivalent of 1 g of wet mycelium) were added to 25 ml of the medium in a Sakaguchi flask. Reactions were carried out under aerobic or anaerobic conditions.

It is assumed that the specific rate of penicillin production depends on the oxygen concentration in the broth, but high oxygen concentration involves a reduction of the growth rate. For fixed oxygen concentrations in the broth, the biomass reaches the maximum values, consequentially the fermentor cannot produce more than a fixed limit, whatever the nutrient feeding conditions. However, this limit varies providing more effective aeration or changing the agitation rate [24].

The specific oxygen uptake rate has been determined experimentally. Figures 6 and 7 show that the specific oxygen uptake rate which is necessary to maintain the maximum specific penicillin production rate is 1.6 mmol O_2/g cells/h. This is significantly greater than the specific oxygen uptake rate which corresponds to the specific growth rate of 0.015 h^{-1}. The value of the oxygen uptake rate at μ=0.015 h^{-1} is about 1.2 mmol O_2/g cells/h from Figure 7. Since the oxygen requirement for the maximum penicillin productivity is considerably greater than the oxygen requirement that corresponds to the specific growth rate of 0.015 h^{-1}, there is a strong indication that penicillin biosynthesis requires an amount of energy that is equivalent to 0.4 (=1.6-1.2)mmol O_2/g cells/h [33].

2.1.5.8. pH

It was reported that a pH above 7.5 leads to rapid destruction of penicillin, particularly in the presence of ammonium ions [29]. The

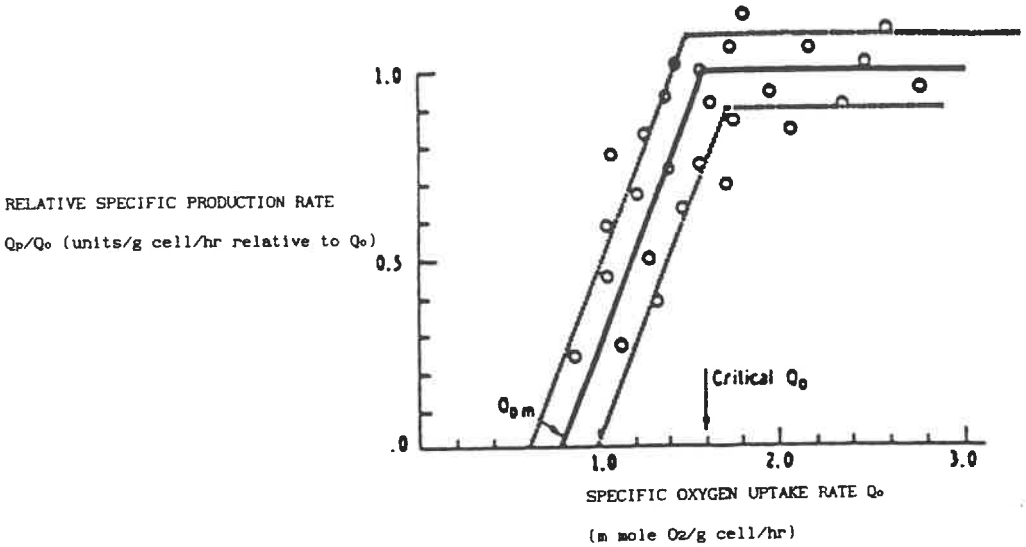


Fig. 6 Correlation between the specific penicillin production rate and specific oxygen uptake rate.

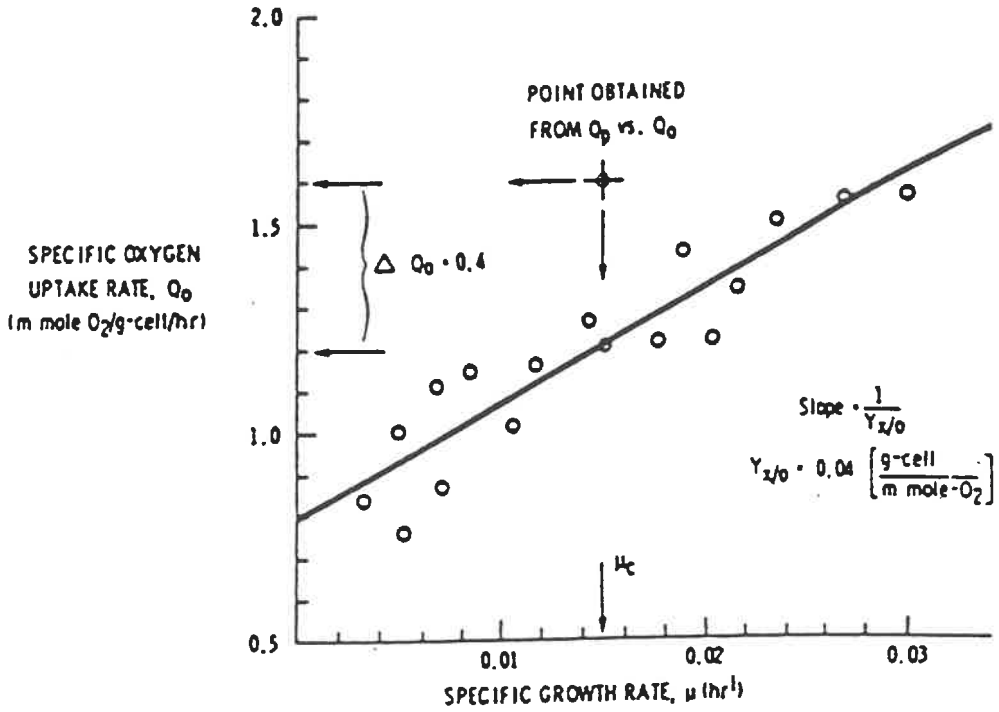


Fig. 7 Effect of specific growth rate on the specific oxygen uptake rate.

optimal pH plateau recommended for penicillin fermentation in early reports were high and ranged from pH 7.2 to 7.5 [30].

Later, Hockenhull [29] reported the optimal pH for maximal synthesis to be approximately 7.0. More recent reports indicate the best range to be pH 6.5 to 6.6 [31]. Thus, over the past two decades, there appears to have been a shift to a lower pH optimum. The change can probably be attributed to the introduction of improved mutant strains, less expensive crude fermentation nutrients, and improved engineering technology.

Deo and Gaucher [1] reported that a high concentration (6.8 g/L) of KH_2PO_4 prevented a rapid decrease in the medium pH, and the accompanying decrease in the penicillin production. In the absence of phosphate, the buffering capacity of the medium is markedly decreased and the medium responds to the pH changes more rapidly.

2.1.6. Penicillin Process Kinetics

In the following discussion of penicillin process kinetics, only the effects of sugar and oxygen are incorporated in the kinetic expressions as important regulating nutrients. All other nutrients are assumed to be present in non-regulating concentrations. Additional factors, such as the strain used, would influence growth and product formation rates. However, sugar and oxygen provide a realistic set of primary

environmental variables for the purpose of comparing the performance of free and immobilized cell reactors.

2.1.6.1. Growth Model

The most commonly used general growth equation is called the Monod equation since the relationship expressed was first described by Jacques Monod. The specific growth rate, μ, is generally found to be a function of three parameters: the concentration of limiting substrate S, the maximum growth rate μ_m and a substrate-specific constant K_s.

$$\mu = \mu_m \frac{S}{K_s + S} \qquad (1)$$

Considering the possibilities of obtaining very high microbial cell densities (up to 40 g/dm^3) the use of Contois kinetics which was developed by Bajpai and Reuss [27] is preferred:

$$\mu = \propto \frac{S}{K_x X + S} \qquad (2)$$

Similarly, the dependence of specific growth rate on dissolved oxygen concentration was assumed to be described by:

$$\mu = \propto \frac{C_L}{K_{ox} X + C_L} \qquad (3)$$

where S is the sugar concentration, C_L is the dissolved oxygen

concentration, and X, the cell density. Care must be taken in applying such an expression to an immobilized cell reactor where a biofilm may exist of approximately constant density but variable thickness.

The combined effect of equations (2) and (3) is

$$\mu = \mu_{max} \frac{S}{K_x X + S} \frac{C_L}{K_{ox} + C_L} \qquad (4)$$

2.1.6.2. Product Formation Model

It has been reported [35] that the amount of acyltransferase, the enzyme responsible for the introduction of a side chain on to 6-amino penicillinic acid, is greatly increased during penicillin production and is presumably subject to catabolite repression. It is also known that the presence of easily metabolised sugars in large quantities results in only very little production of antibiotic. This suggested substrate-inhibition kinetics of the following form:

$$q_p \propto \frac{S}{K_p + S(1 + S/K_I)} \qquad (5)$$

The effect of oxygen on the kinetics of penicillin production was quantified by Giona et al.[36]. The resulting model included the effect of oxygen diffusion limitation by making a saturation constant proportional to cell mass. This kind of functional dependence was determined from the experimental data under industrial conditions. It

was not based upon any enzyme kinetic principle. It is known that the rate of penicillin production is strongly dependent upon the dissolved oxygen concentration. Since oxygen is not directly involved in penicillin biosynthesis (it is needed for growth, for maintenance and for supply of the energy needed for biosynthesis), this dependence is expected to arise mainly due to the diffusional limitations. The following form was used for oxygen limitation of growth:

$$q_p \propto \frac{C_L}{K_{op} X + C_L} \qquad (6)$$

2.1.6.3. Substrate and Oxygen Consumption Model

The models for substrate and oxygen consumption were obtained by assuming constant yield coefficient and maintenance requirements by the growth and product formation kinetics [35], thus

$$q_s = - \frac{1}{Y_{x/s}} \mu - \frac{1}{Y_{p/s}} q_p - m_m X \qquad (7)$$

and

$$q_{o2} = - \frac{1}{Y_{x/o}} \mu - \frac{1}{Y_{p/o}} q_p - m_o X \qquad (8)$$

It should be noted that the definition of the maintenance term here excludes the requirement for penicillin formation.

2.1.7. Product Recovery

Figure 8 (Hersbach et al, 1984) [10] shows the purification process used by Gist-Brocades. The duration of the penicillin recovery cycle from one fermentor is about 15 hours.

In the first stage, mycelium is usually separated from the penicillin-containing broth on a rotary vacuum filter. The mycelium is washed on the filter. Whole broth extraction can be done as an alternative to filtration but mycelium components cannot be extracted.

Penicillin is extracted in the acid form into a solvent such as amyl acetate or butyl acetate in a continuous counter-current multistage centrifugal extractor at pH 2.5-3.0 and at 0-3°C. The penicillin-rich filtrate is mixed with dilute sulfuric acid. The mixture and the organic solvent are fed into the Podbielniak extractor (Podbielniak 1970) [41]. The flows are in the proportion of 4 or more to 1. Short residence times are necessary to prevent penicillin degradation under the acid conditions.

In the next stage, activated charcoal is used to remove pigments and impurities from the penicillin-containing solvent. The charcoal is separated from the extract on a precoated rotary vacuum filter and washed with the solvent.

Penicillin G or V can be crystallized as the potassium or sodium

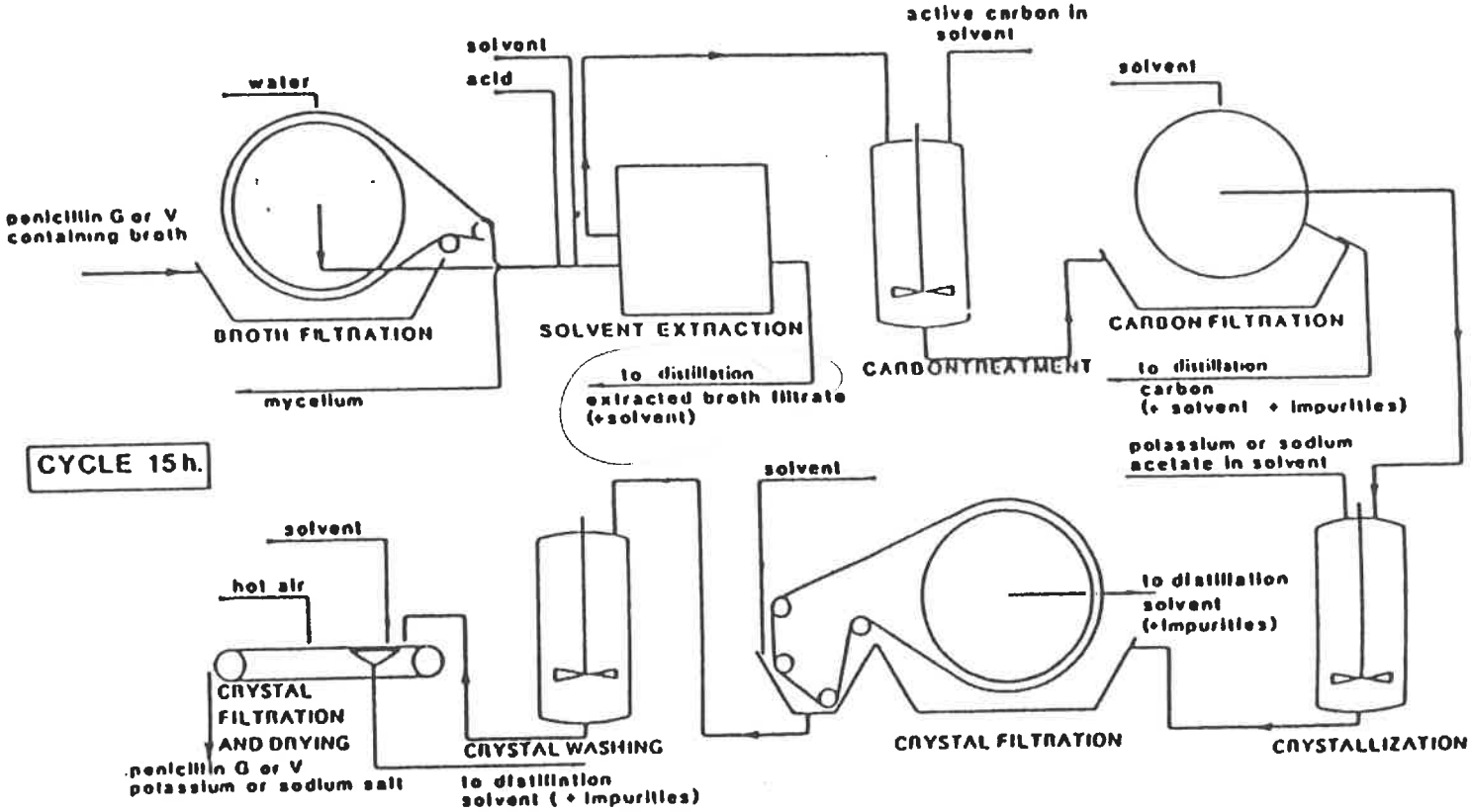


Figure 8 Penicillin purification process of Gist-Brocades.

salt from the solvent phase through the addition of potassium or sodium acetate. The penicillin potassium or sodium salt crystals are collected on a rotary vacuum filter.

The penicillin salt crystals are washed and predried with anhydrous isopropyl alcohol or butyl alcohol to remove residual impurities. The crystal slurry is then brought to a large horizontal vacuum belt filter, washed with fresh anhydrous solvent and dried with warm air. At the end of the belt the 99.5% pure penicillin salt is collected in a container. The streams containing spent solvents are processed for solvent purification by washing, distillation, and drying. The recovery solvents are reused in the purification process. Crystalline penicillin G or V potassium or sodium salt is further processed to pharmaceutical grade or used as an intermediate for the production of semi-synthetic penicillins.

2.2. Inverse Fluidized Bed Bioreactor

The inverse fluidized bed bioreactor (IFBBR) consisting of two concentric columns is a combination of an airlift loop reactor and a fluidized bed column. Oxygenation, recirculation of the liquid medium and control of the biofilm thickness take place in the inner draft tube. Buoyant beads covered by a biofilm are fluidized by the downflow of the liquid in the annulus forming an inverse fluidized bed (Figure 10).

This type of reactor has the merit of the airlift loop reactor and of surface immobilization. Also the biofilm thickness can be controlled by the use of an eroding device in the draft tube to shear excess biomass from the beads.

Chavarie et al [37] present a simple comprehensive model which link the bioparticle properties such as the biofilm thickness, the support particle diameter and density with the liquid and gas flow rate through the bed.

As shown in equation (9), the basis for this model is the mechanical force balance, between, on the one hand, the head difference between the inner tube and the annular region and on the other hand, the sum of the friction losses in the system.

The mechanical force balance is described by the equation:

$$Hg\,[\rho_{BP}(1-\varepsilon) + \rho_L\varepsilon] - Hg\,[\rho_L(1-\alpha) =$$

$$\rho_L[L/(\pi/4D_{int}^2)]/2g[1+2\alpha/(1-\alpha)+\varepsilon v+4f_{TP}H/D_{int}+f_{fix}] \quad (9)$$

where εv: in+out

and $\frac{2\alpha}{1-\alpha}$: acceleration through a gas section

Equations (10) to (15) report the various relations proposed by the authors to solve equation (9).

Friction factor for two-phase flow:

$$f_{TP}/f = 6.42\ Fr_{TP}^{-0.213}\ Re_{TP}^{-0.056}\ We_{TP}^{-0.157} \qquad (10)$$

where f = f(Re) for monophase tubular hydrodynamics

Gas retention:

$$(1 - \alpha)/\alpha = 4.6\ Z^{0.9}\ e^{1.2Dint} \qquad (11)$$

where $Z = (L/G)\ Fr_{TP}^{0.36}\ Re_{TP}^{-0.006}\ We_{TP}^{-0.57}$

Friction factor:

$$f_{fix}=18.74h_{fix}/d_{fix}[150/Re_{fix}+1.75] \qquad (12)$$

Bioparticle density:

$$\rho_{BP}=[\rho_p d_p^3+\rho_{BF}(d_{BP}^3-d_p^3)]/d_{BP}^3 \qquad (13)$$

Porosity of the fluidized bed under steady state conditions:

$$\varepsilon = 1 - 0.6\ H_{init}/H\ d_{BP}^3/d_p^3 \qquad (14)$$

$$L/[\pi/4(D_{ext}^2 - D_{int})] = U_t\varepsilon^n$$

where $n = n(Re_t)$ (model of Richardson & Zaki)

and $U_t = [4/3\ g\ d_{BP}(\rho_{BP} - \rho_L)/\rho_L\ 1/C_D]^{1/2}$

$C_D = C_D(Re_t)$

The number of bioparticles per unit volume:

$$N = 0.6\ H_{init}/H/(\pi/6\ d_p^3) \qquad (15)$$

2.3. Sterilization

2.3.1. Steam Sterilization By Autoclaving

Moist heat in the form of the pressurized steam such as produced in an autoclave is regarded as the most dependable method for the destruction of all forms of life, including bacterial spores.

The basic principle behind autoclaving is that the temperature of the gas increases proportionally as its pressure increases. Since steam is a gas, increasing its pressure in a closed system increases its temperature. As the water molecules in steam become more energized, their penetration increases substantially. This principle is used to reduce sterilizing time in the autoclave.

Basic conditions during the autoclaving process is to have 16 psig

steam to give 120°C. Therefore materials to be autoclaved must be able to withstand this temperature.

2.3.2. Dry Heat

Radiation dry heat is a type of energy which does not penetrate materials easily and thus, long periods of exposure to high temperature are necessary. The effect of dry heat on microorganisms is equivalent to that of baking. The heat changes microbial proteins by oxidation reactions and creates an arid internal environment, thereby burning microorganisms slowly. This method is useful for the sterilization of dry powder and water-free oily substances, as well as many types of glassware such as pipettes, flasks, and syringes.

2.3.3. Ethyl Alcohol Treatment

Many chemical sterilization procedures such as with ethyl alcohol are active against vegetative cells but have no effect on spores. These processes denature proteins and dissolve lipids, which leads to cell membrane disintegration.

2.3.4. Tyndallization

The method, tyndallization, was named after its developer, John Tyndall. It was also called fractional sterilization because a fraction was accomplished on each day. During the first day exposure, heat kills virtually all organisms except bacterial spores. When the material is incubated overnight, spores germinate into vegetative cells which are killed during the second day exposure to heat. Again, the material is cooled and the few remaining spores germinate, only to be killed on the third day.

Tyndallization has assumed importance in modern microbiology with the development of high-technology instrumentation and new chemical substances. Often, these materials cannot be sterilized at autoclave temperature, or by long periods of boiling or baking, or with chemicals. Polystyrene is such a material.

2.3.5. Ionizing Radiation

Gamma rays have wavelengths shorter than the wavelength of ultraviolet light. As gamma rays pass through microorganisms, they force electrons out of their shells in organic molecules, thereby creating ions. The ions attract one another and form unusual molecules that change the structure or chemistry of the microorganism.

Ionizing radiation is currently used to sterilize such heat-sensitive pharmaceuticals as vitamines, hormones, and antibiotics as well as certain plastics and suture materials.

CHAPTER 3

EXPERIMENTAL

3.1. Sterilization of Polystyrene Beads

In the initial stages of the research, various methods for the sterilization of the polystyrene beads were evaluated. Once sterilized, the degree of sterility was determined by tests in liquid medium or on solid medium.

3.1.1. Autoclave

Polystyrene beads were put into two beakers, one with water and another without water. Then the beakers were placed in an autoclave and sterilized at 120°C (16 psig) for 15 minutes.

3.1.2. Dry Heat

Polystyrene beads were placed in an oven at 85°C for 10, 24, and 48h, and at 96°C for one hour.

3.1.3. Chemical Treatment

Alcohol was used as a chemical sterilizing agent. For one period of treatment, the polysyrene beads were soaked in alcohol for three hours. Tests were done for one, two and three periods of treatment.

3.1.4. Tyndallization

Thirty milliliters of polystyrene beads, between 1.1 and 1.4mm in diameter, were distributed into 500 mL flasks containing 200 mL of water. These flasks were heated and shaken while the water was boiling. After one minute of boiling, the flasks were cooled to room temperature and put into an incubator at 28°C. Ten hours later, the same process was repeated until five successive ten hours. This experiment was done in duplicate.

3.1.5. Ionizing Radiation

Polystyrene beads were sealed in a ziplock plastic bag and sterilized with two and three million rads of gamma radiation.

3.2. Design of IFBBR To Be Used For Pure Culture Fermentation

Figure 9 shows a typical inverse fluidized bed bioreactor which was used for the biochemical oxidation of ferrous iron by Thiobacillus ferroxidans [38]. Owing to the nature of the Thiobacillus fermentation, this reactor was not designed to be sterilized. However, penicillin production requires that the reactor must be sterilized. Hence some changes had to be made to the original reactor design. Figure 10 shows an improved IFBBR for penicillin production.

3.2.1. Material of Reactor Body

The material of the original reactor was plexiglass. In order to meet the need of sterilization, the plexiglass was replaced with pyrex glass.

3.2.2. Inoculation Port

Under sterile conditions, inoculation at the top of the reactor with the biofilm-coated polystyrene beads was very difficult. A special method was developed to overcome this problem. Since the density of the bioparticles was less than water, it was possible to inoculate the reactor from the bottom as the bioparticles float through the reactor to the top of liquid surface. In this manner the reactor is kept

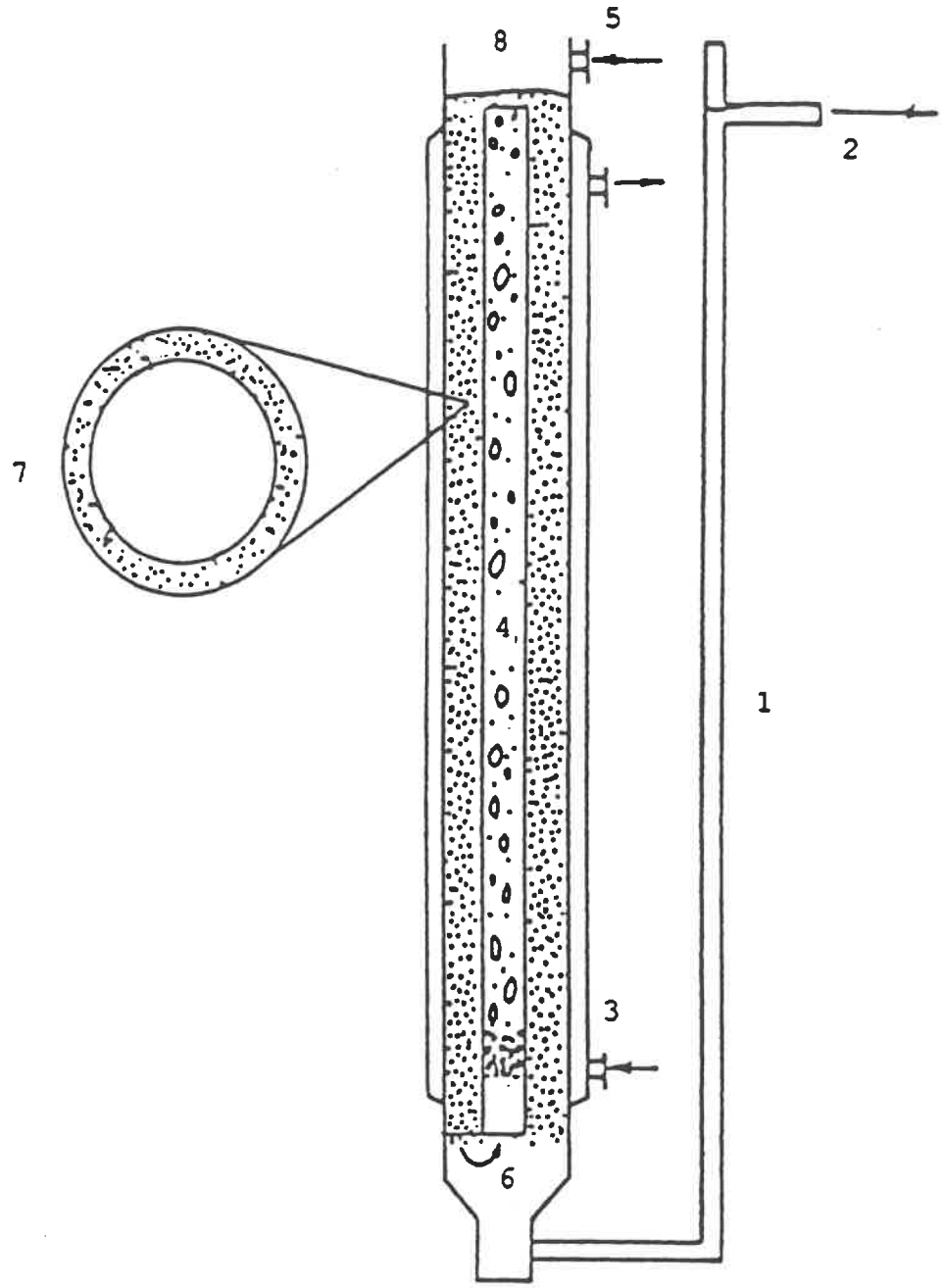


Figure 9: Typical IFBBR; (1) level control, (2) overflow device, (3) water jacket for temprature control, (4) inner tube, (5) medium in, (6) aeration inlet, (7) bioparticles, (8) top open to atmosphere.

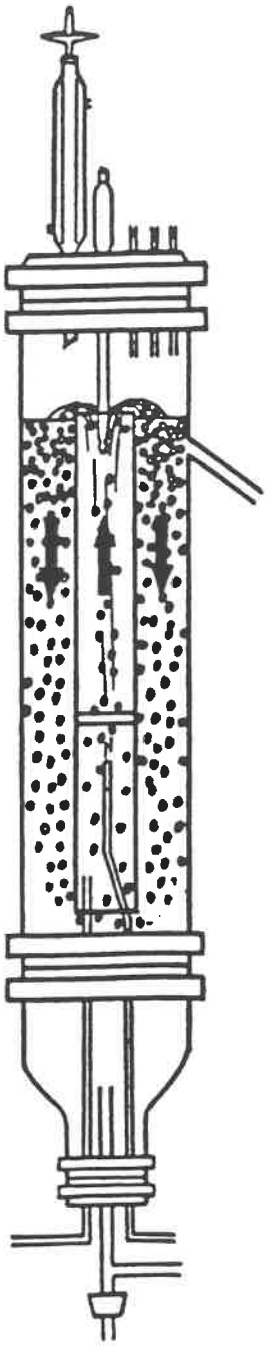


Figure 10: An improved IFBBR for penicillin production.

sterile. Figure 11 shows the modifications for the inoculation procedure.

3.2.3. Aeration

Where the aeration inlet opens into the reactor, an air diffuser has been added so that small air bubbles can be obtained. Figure 12 shows this configuration.

3.2.4. Eroder

Figure 13 shows that a plastic ring with steel grid was set up in the inner draft tube of the IFBBR reactor and located above the aeration inlet. The steel grid was used as an eroder to control the size of bioparticles. The ceramic eroder, stainless eroder and glass eroder were tested before steel grid was found to be the best.

3.2.5. Sampling

Immobilized biomass and fermentation broth were sampled by the method showed on Figure 14(b). When the clamp on the sampling outlet was opened, the immobilized biomass and fermentation broth went out at the same time. During the process of fermentation the sampling outlet

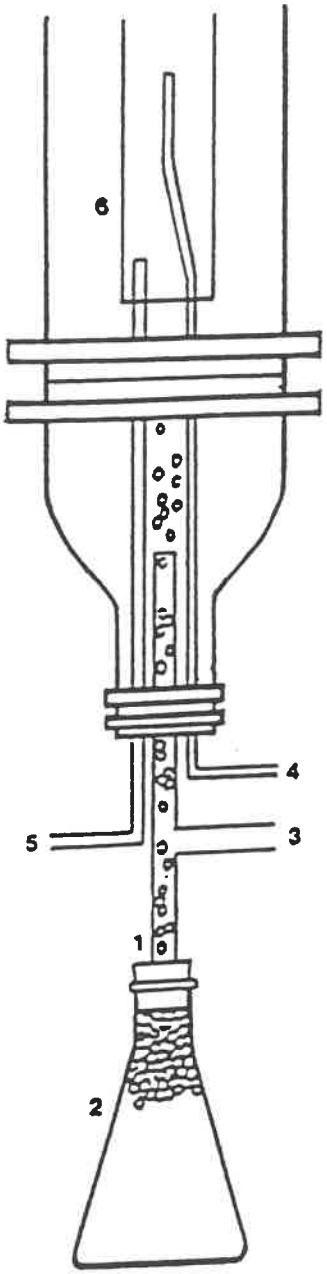


Figure 11: Inoculation of IFBBR; (1) inlet for inoculum, (2) immobilized beads, (3) outlet for mycelium, (4) aeration inlet, (5) medium out, (6) draft.

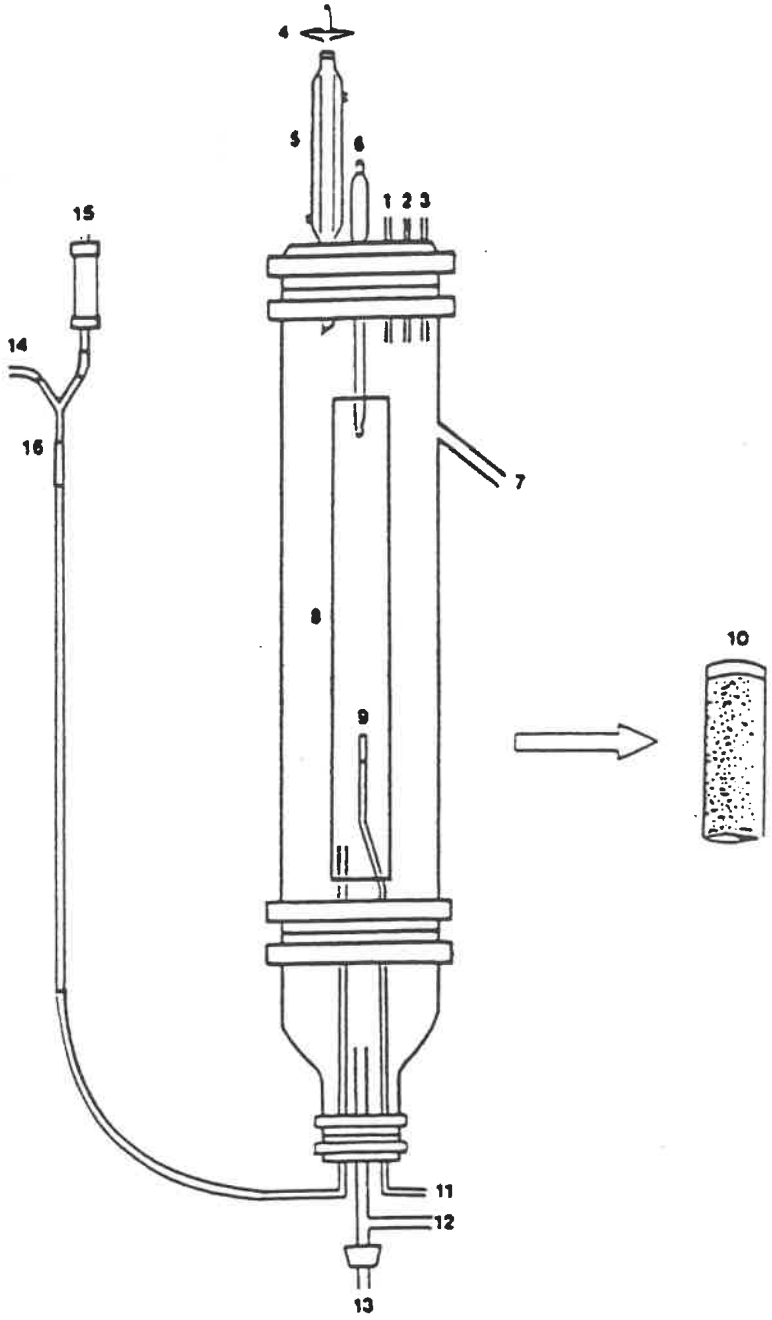


Figure 12 : The IFBBR reactor with sparger on aeration inlet;
(1) antifoam, (2) base, (3) medium in, (4) outlet air filter,
(5) condenser, (6) pH probe, (7) sampling line, (8) draft,
(9) sparger, (10) enlarged sparger drawing, (11) aeration inlet,
(12) mycelium outlet, (13) inlet for inoculum, (14) medium out,
(15) outlet air filter, (16) level control.

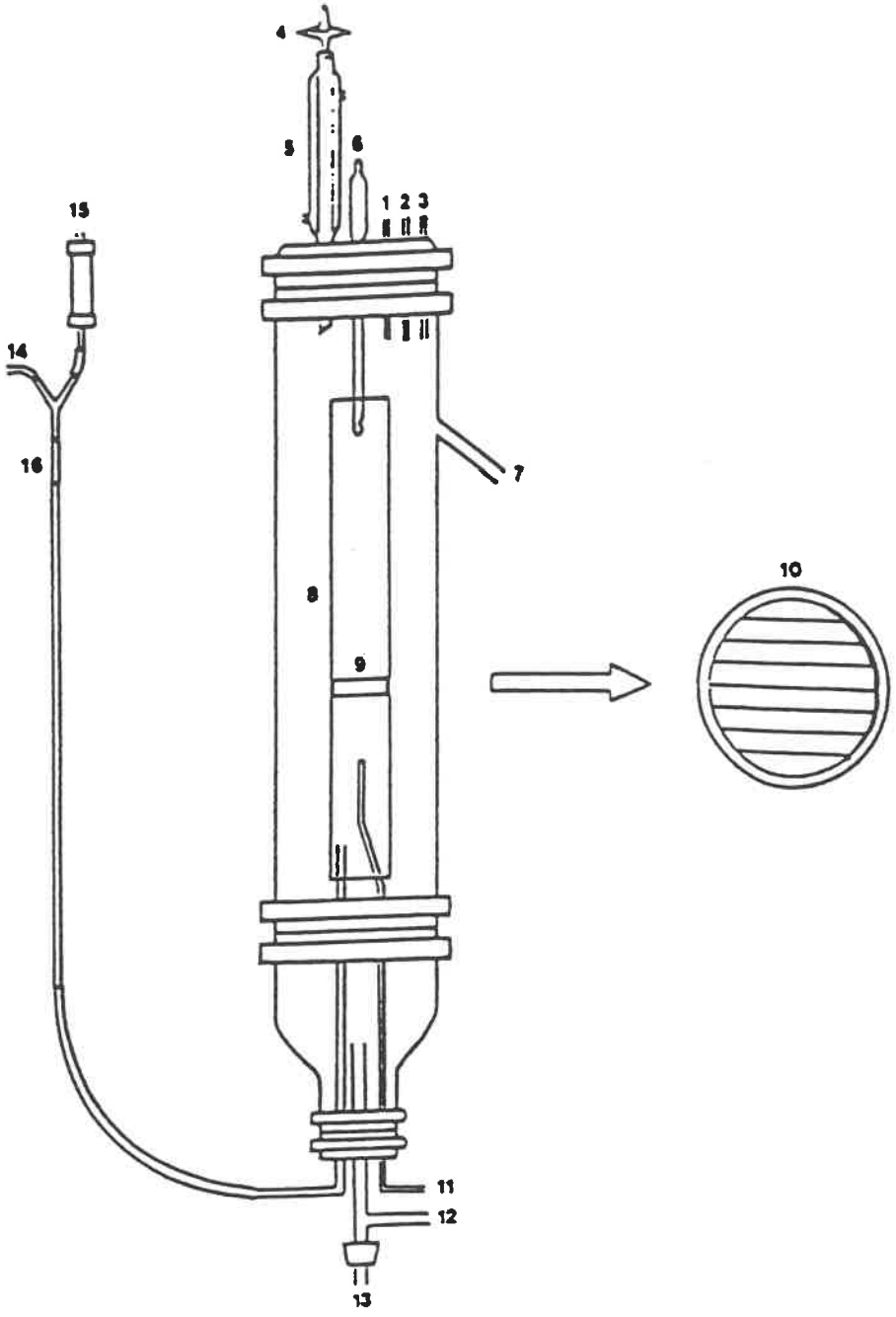


Figure 13 : The IFBER reactor with a grid in the draft;
(1) antifcam, (2) base, (3) medium in, (4) outlet air filter,
(5) condenser, (6) pH probe, (7) sampling line, (8) draft,
(9) grid, (10) enlarged grid drawing, (11) aeration inlet,
(12) mycelium outlet, (13) inlet for inoculum, (14) medium out,
(15) outlet air filter, (16) level control.

was immerged in formaline solution to keep the outlet sterile.

Figure 14(a) shows a proposed sampling method which has not been tested. A syringe could be added to the sampling outlet. When the sample is taken, a test tube will be connected to another opening. The plunger of the syringe is pulled, immobilized beads and fermentation broth will enter into the test tube.

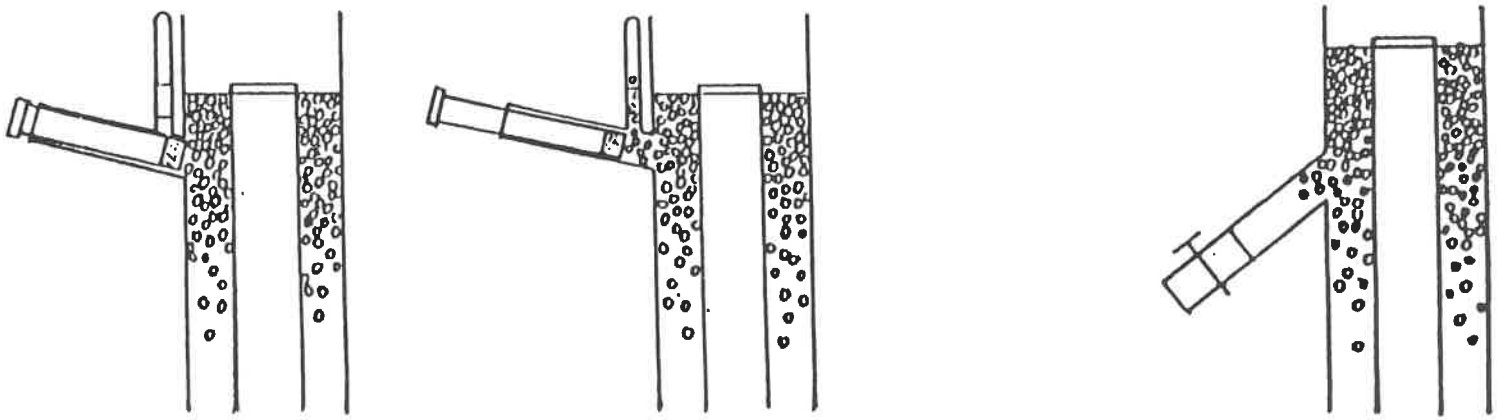
3.3. Composition of medium

The spore production medium consists of 50 g/L malt agar, 10 g/L glucose and 1 g/L peptone while the immobilization and germination medium contains 30 g/L malt extract broth, 10 g/L glucose and 1 g/L peptone.

Unlike the spore production medium, and immobilization and germination medium which are natural organic mixtures, the production medium is defined. It consists of 10g/L lactose, 3g/L $(NH_4)_2SO_4$, 0.6g/L phenoxyacetic acid, 0.1g/L KH_2PO_4 for IFBBR fermentation and 0.005, 0.01, 0.05, 0.1, 0.5, 1.0, 6.0g/L series for shake flask fermentation.

3.4. Spore production

P. chrysogenum wis. 54-1225 was used in this research. The culture



(a) (b)

Figure 14: The methods of sampling for IFBBR

(a) proposed sampling method

(b) actually used sampling method.

was inoculated on slants of the spore production medium which was incubated at room temperature until green velvet spores were produced. These slants were then kept in a refrigerator until later use. The spores which were used to colonize the polystyrene beads were cultivated on the spore production medium in 100mL Erlenmeyer flasks. The flasks were inoculated from the slants and were incubated at room temperature until spores were produced.

3.5. Immobilization on polystyrene beads

3.5.1. Pretreatment of polystyrene beads

P. chrysogenum cannot be immobilized on polystyrene beads without previous treatment. There are two ways of treatment which make immobilization easier.

The first method is the tyndallization of polystyrene beads in the liquid medium rather than in water. The process is the same as the tyndallization in water.

The second method is the treatment with celite. Polystyrene beads were mixed with 10% (V/V) celite powder in a blender for 15 minutes. Then the beads were separated from celite powder, sterilized by gamma radiation and washed with sterile water.

3.5.2. Immobilization

Spores were washed off the solid medium with 0.1% Tween 80 under sterile conditions. There were about 10^6-10^7 spores per mL.

Twenty milliliters of spore solution and 30mL of polystyrene beads were added into a 500mL baffled flask with 200mL immobilization and germination medium. The flask was placed in an incubator with a shaking speed of 150 RPM. After three days at 26-28°C , there was full growth on the beads. After these beads with biofilm were rinsed with sterile water, they were ready to inoculate the IFBBR and shake flasks.

3.6. Determination of limiting phosphate concentration

500 mL baffled flasks with 200 mL production medium were autoclaved and cooled to room temperature. They were inoculated with about 18,000 immobilized beads under sterile conditions.

Shake flasks experiments were performed in a Lab-line incubator at 27±1°C and 150 RPM. In each flask the production medium had a different phosphate concentration. Every 12 hours the same amount of beads and fermentation broth were sampled and an equal volume of fresh production medium was added to maintain a constant volume.

3.7. Evaluation of IFBBR

IFBBR reactor, production medium and other apparatus were steam sterilized in an autoclave for 30 minutes at 121°C, 15 psig and cooled to room temperature. The reactor was then inoculated with the immobilized beads through the inoculation port at the bottom of the reactor as shown on Figure 11.

After inoculation, fresh production medium was added to the reactor continuously at a flow rate 0.024 L/h. For the 1.5 L reactor which was used in this experiment, the residence time was 62.5 hours. The fermentation broth was removed to maintain a constant volume in the reactor.

The pH in the reactor was controlled between 6.5 and 6.8 by the addition of saturated sodium hydroxide. Foaming was controlled by the intermittent addition of 33% polypropylene glycol. Air was filter sterilized through a sterile filter with the air flow rate to the reactor being 0.9L/min. A condensor was added at the air outlet to minimize the loss of liquid. The free mycelium which was sheared off by a grid eroder and collected at the bottom of the reactor was pumped out of the reactor when there was an accumulation. Every 12 h, samples of beads and fermentation broth were taken.

3.8. Analytical Procedures

3.8.1. Analysis of Biomass

3.8.1.1. Dry Weight

Beads with biofilm were washed in distilled water, transfered into an aluminum dish and then dried in an oven to constant weight at 60°C.

The same number of beads without biofilm were used as controls and were dried under the same conditions.

3.8.1.2. Sodium Hydroxide Treatment

Beads with biofilm were dried to constant weight at 60°C. Then they were soaked in 20% (w/v) sodium hydroxide and heated at 100°C for 15 min. Normally, the biofilm on the beads should be digested at these conditions. The beads without biofilm could have been dried at 60°C to constant weight.

3.8.1.3. Sodium Hypochlorite Biomass Analysis

Concentrated sodium hypochlorite (10.4% w/w = 12% w/v) was used to digest the biofilm from the beads. Beads with biofilm were washed with

distilled water, transfered to an aluminum dish and dried to constant weight in an oven at 60°C. Then these beads were soaked in sodium hypochlorite for two hours until the biofilm on the beads disappeared. These beads were washed with distilled water and dried in the same aluminium dish under the same conditions until the beads reach constant weight.

3.8.2. Measurement of Diameter of Beads

The diameter of beads was determined from the average diameter of 20 beads mesured with a micrometer under a microscope.

3.8.3. Carbohydrate Analysis

Lactose, glucose and galactose were analyzed by HPLC using an Aminex HPX-87P column. Distilled deionized water which was filtered through a 0.45 μm filter was used as eluant with a flow rate of 0.7 mL/min. The column pressure was 800 psig and the column temperature was 80°C. A Waters differential refractometer (R401) was used as the detector. A typical chromatograph and standard curves are shown on Figure A1, A2, A3.

3.8.4. Penicillin V and Phenoxyacetic Acid

Penicillin V and phenoxyacetic acid were analyzed by HPLC using a Partisil 5 ODS-3 column at room temperature with a column pressure of 2000 psig. The eluant composition was CH_3OH : H_2O : (1%)$NaHCO_3$ (45 : 55 : 0.8) with a flow rate of 0.65 mL/min. The wavelength of the UV detector (100-40) was 254 nm. A typical chromatograph and standard curves are shown on Figure A4, A5.

CHAPTER 4

RESULTS

4.1. Sterilization

The results of the various methods of sterilization are summarized on Table 5.

The polystyrene beads lost their shape and contracted after sterilization in the autoclave at 120°C for 15 min, regardless of whether they were or were not in water during autoclaving.

After being in the oven at 85°C for 48 hours or at 96°C for one hour, the polystyrene beads also contracted. The bead shape did not change when they were baked at 85°C for 10 or 24 hours. The sterility test showed that these beads were not sterile.

The shape of the polystyrene beads was not effected by alcohol during the chemical sterilization. After one period of treatment, the beads were not sterile. The sterility tests showed that two or three periods of treatment were needed to sterilize the beads.

Five one-minute tyndallizations had no influence on the shape or size of the polystyrene beads. Sterility tests either in liquid or on solid medium showed that the beads were properly sterilized.

Table 5 : Different methods of sterilization of polystyrene beads

Sterilization Method	Bead Shape	sterility test in liquid (28 C)	sterility test on solid medium	Summary
Autoclaving in vapor 15 minutes	contract	ND*	ND*	
Autoclaving in water 15 minutes	contract	ND*	ND*	
Dry heat (oven) 96 C 1h	contract	ND*	ND*	
Dry heat (oven) 85 C 48h	contract	ND*	ND*	
Dry heat (oven) 85 C 24h	no change	not clear after 7 days	ND*	beads not sterile
Dry heat (oven) 85 C 10h	no change	not clear after 4 days	ND*	beads not sterile
Alcohol 1 time 3h	no change	not clear after 2 weeks	growth	beads not sterile
Alcohol 2 time 3h	no change	clear 20 days later	no growth	
Alcohol 3 time 3h	no change	clear 20 days later	no growth	
Tyndallization I 5 times (1 min per time)	no change	clear 20 days later	no growth	feasible
Tyndallization I 5 times (1 min per time)	no change	clear 20 days later	no growth	feasible
gamma radiation 2 million rads	no change	ND*	no growth	feasible
gamma radiation 3 million rads	no change	ND*	no growth	feasible

*ND refers to data not determined.

During the sterilization by gamma radiation, no heat which would affect the shape and size of polystyrene beads was used. The shape and size of beads remained unchanged while sterility tests showed that the beads were sterilized as there was no growth in liquid or on solid medium.

4.2. Eroder

Table 6 lists all types of eroders which were tested. These eroders were set on a steel wire grid. The ceramic eroder plugged up the grid while the stainless steel one was too heavy to move with the air flow. The glass eroder dropped through the grid. Only the steel wire grid by itself worked well. The distance between each steel wire was 4 mm. This steel wire eroder stands firmly when air flow passed through and controls bioparticle size.

4.3. Immobilization

4.3.1. Mycelium morphology

The mycelium morphology was influenced by both the size and the shape of the shake-flasks used in the experiment. The mycelium pellets were more fluffy in a baffled flasks than in a non-baffled flask and more fluffy in a 500 mL baffled flask than in a 250 mL baffled flask (Table 7).

ERODER	SIZE (mm)		RESULT
	Length	Diameter	
ceramic	9	9	plugged up grid
stainless steel	6	6	did not move with air flow
glass	7	6	dropped through grid
grid	5 (distance between wires)		worked well

Table 6: Eroders tested in IFBBR.

CONTAINER	regular flask		baffle flask	
	250mL	500mL	250mL	500mL
FREE MYCELIUM MORPHOLOGY	compact	compact	fluffy	more fluffy

Table 7: The effect of configuration and volume of flasks on mycelium morphology

4.3.2. Immobilization

Without pretreatment of the polystyrene beads, the beads were not immobilized by the spores of P. chrysogenum. The spores germinated into mycelium pellets instead of attaching on polystyrene beads. These mycelium pellets have a higher density and accumulated at the bottom of a shake flask.

After pretreatment by celite powder or nutrient medium, the polystyrene beads were immobilized by the spores. First there was growth on one spot of a bead. Then, little by little, the mycelium covered the whole bead.

4.4. Biomass analysis

The mycelium from P. chrysogenum was easily digested by sodium hypochlorite. Experiments showed that the higher the hypochlorite concentration the shorter the time needed to digest the mycelium. Figure 15 shows the effect of sodium hypochlorite concentration on digestion of biomass on polystyrene beads.

The diameter and weight of polystyrene beads did not change after treatment with sodium hypochlorite at different times (Figures 16 and 17). Also as shown on Figure 18, the weight of polystyrene beads did not change over time at 60°C.

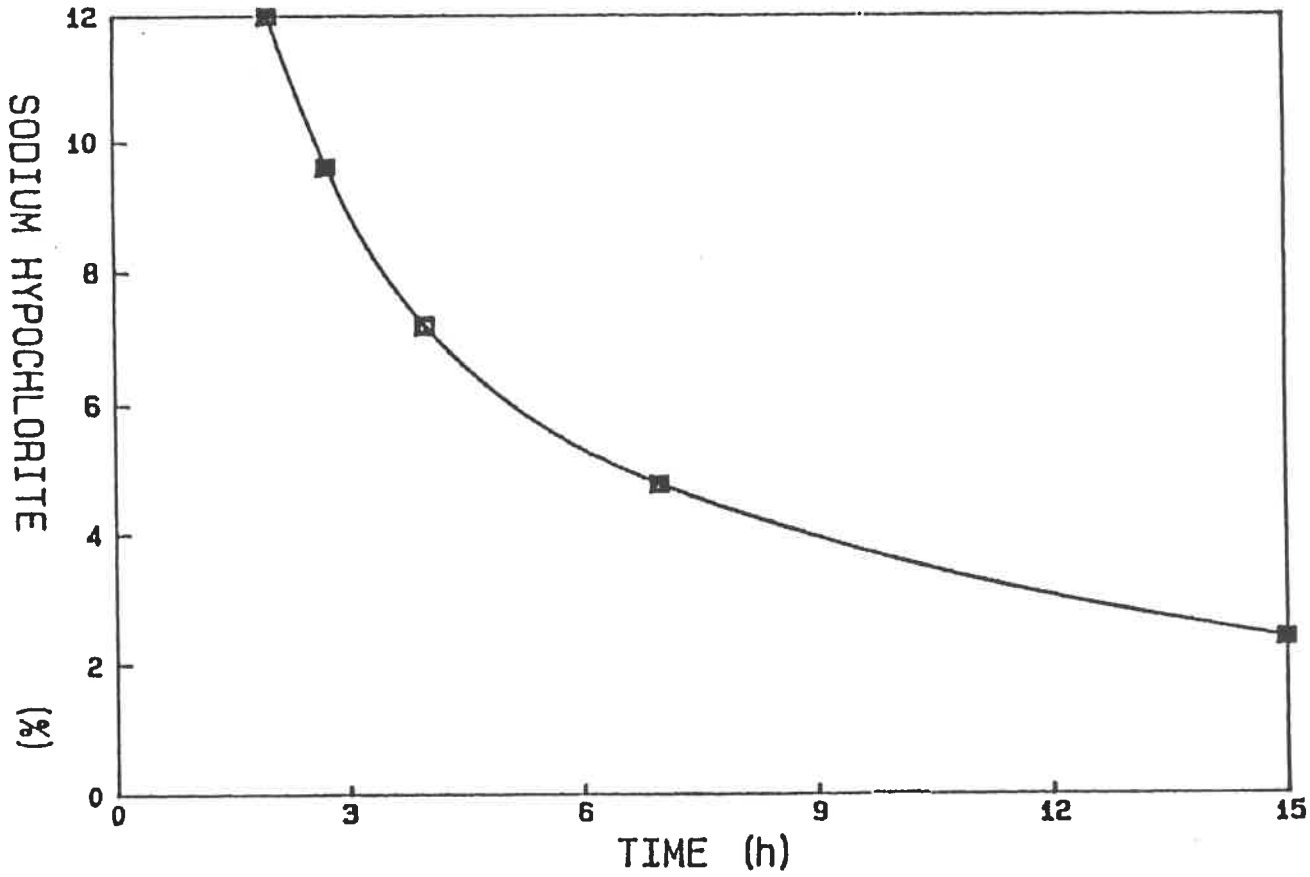


Figure 15: Change of sodium hypochlorite conc.

on resolving biomass on polystyrene beads.

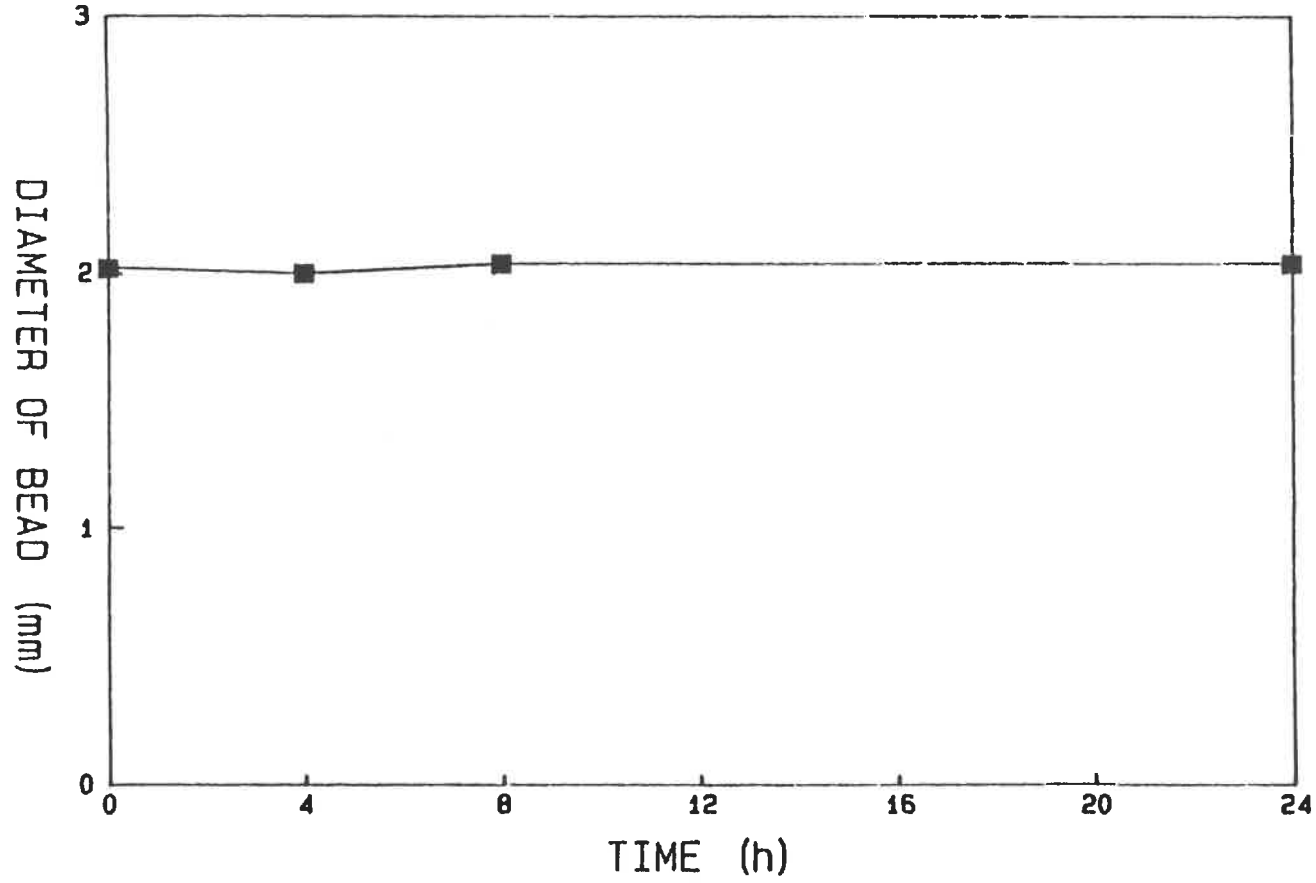


Figure 16 : Change of bead diameter with time at 12% sodium hypochlorite conc.

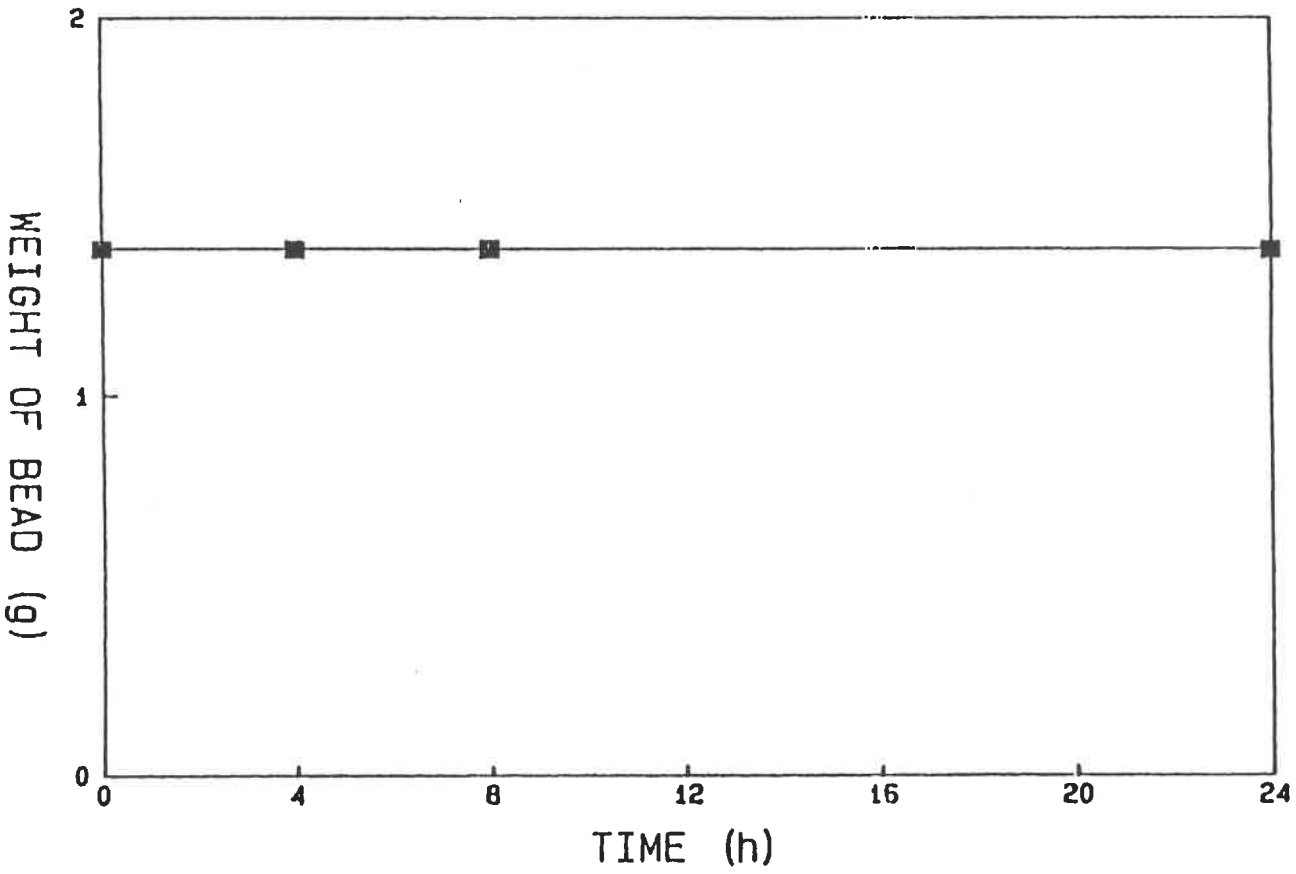


Figure 17 : Change of bead weight with time at 12% sodium hypochlorite conc.

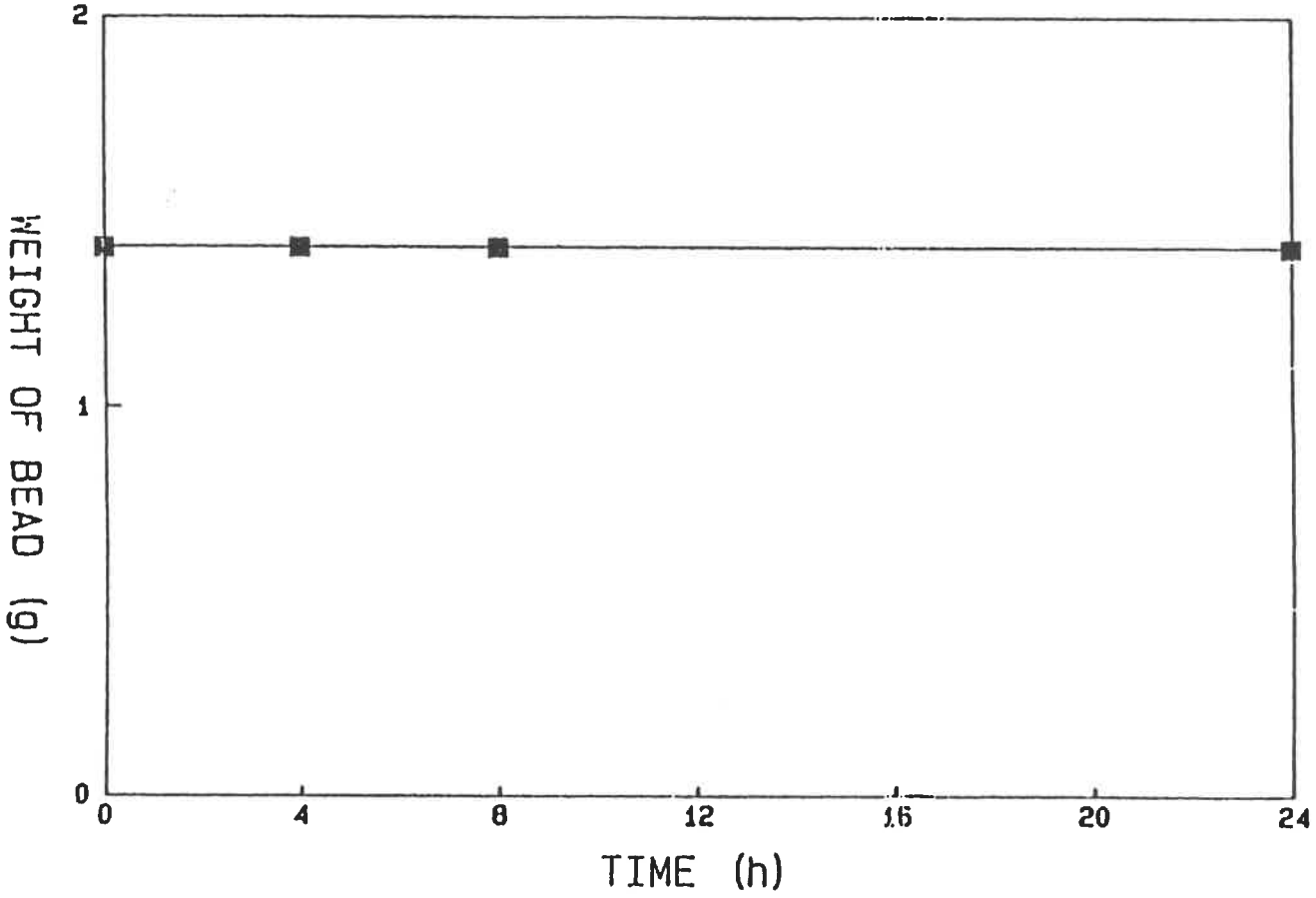


Figure 18 : Change of bead weight with time at 60 °C

4.5. Determination of Limiting Phosphate Concentration

To determine the limiting phosphate concentrating, a series of shake flasks with different phosphate concentration in semicontinuous mode were used. In this experiment, the zero time is the sampling time just before the first addition of nutrient medium.

Biomass morphology was observed in every shake flask at different phosphate concentrations during the fermentation. The results showed on Figure 19 indicated that as the phosphate concentration increased, biofilm morphology changed from fluffy to compact.

At all phosphate concentrations, the diameter of the immobilized beads had a tendency to increase while the immobilized biomass tended to decrease during the fermentation which is shown on Figures 20 and 21. This increase in diameter and decrease in the weight of biomass means the mycelium on the beads became more fluffy during the course of the fermentation, especially at lower phosphate concentration. The reason for this type of undesirable growth might be due to the lack of pH control and inadequate oxygen supply. There was much more free mycelium at higher phosphate concentration than that at lower phosphate concentration.

Figure 22 shows the lactose consumption at different phosphate concentrations. After inoculation, during the first three days in semicontinuous mode (fresh medium was added after each sampling), the

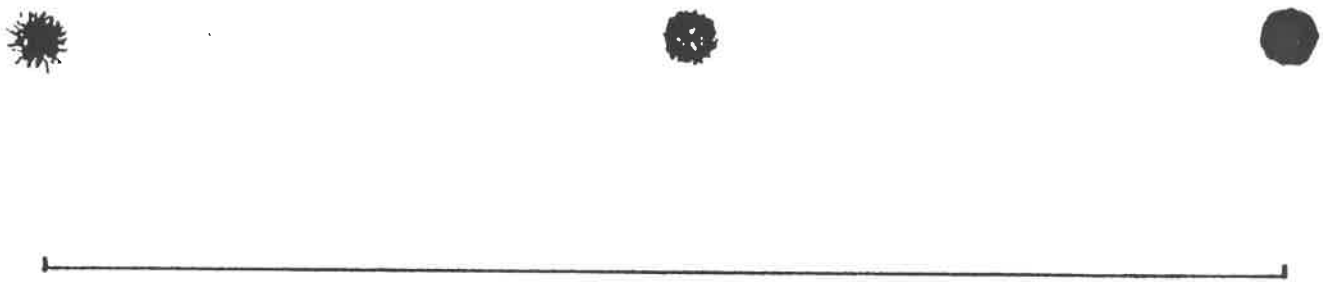


Figure 19: The morphology of biofilm at different phosphate conc.

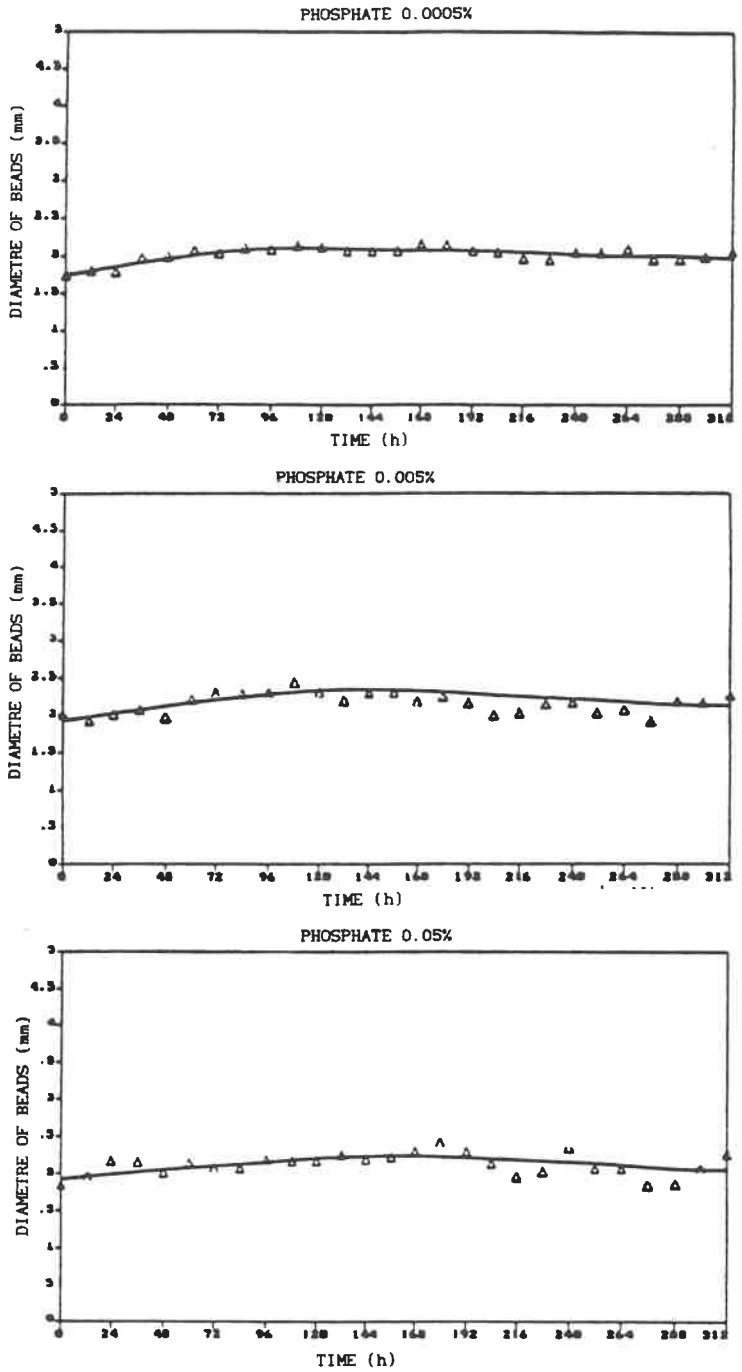


Figure 20: Diameter of bioparticles at different phosphate conc.

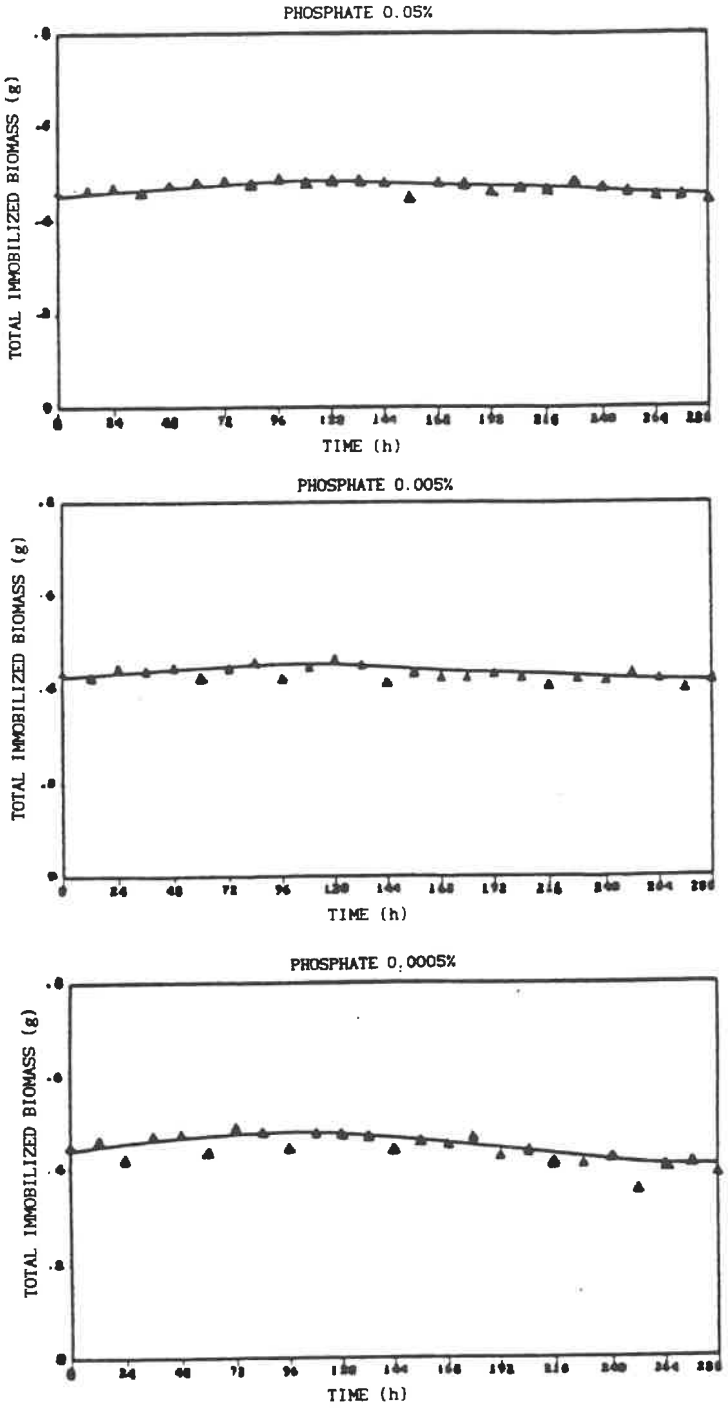


Figure 21: Total immobilized biomass at different phosphate conc.

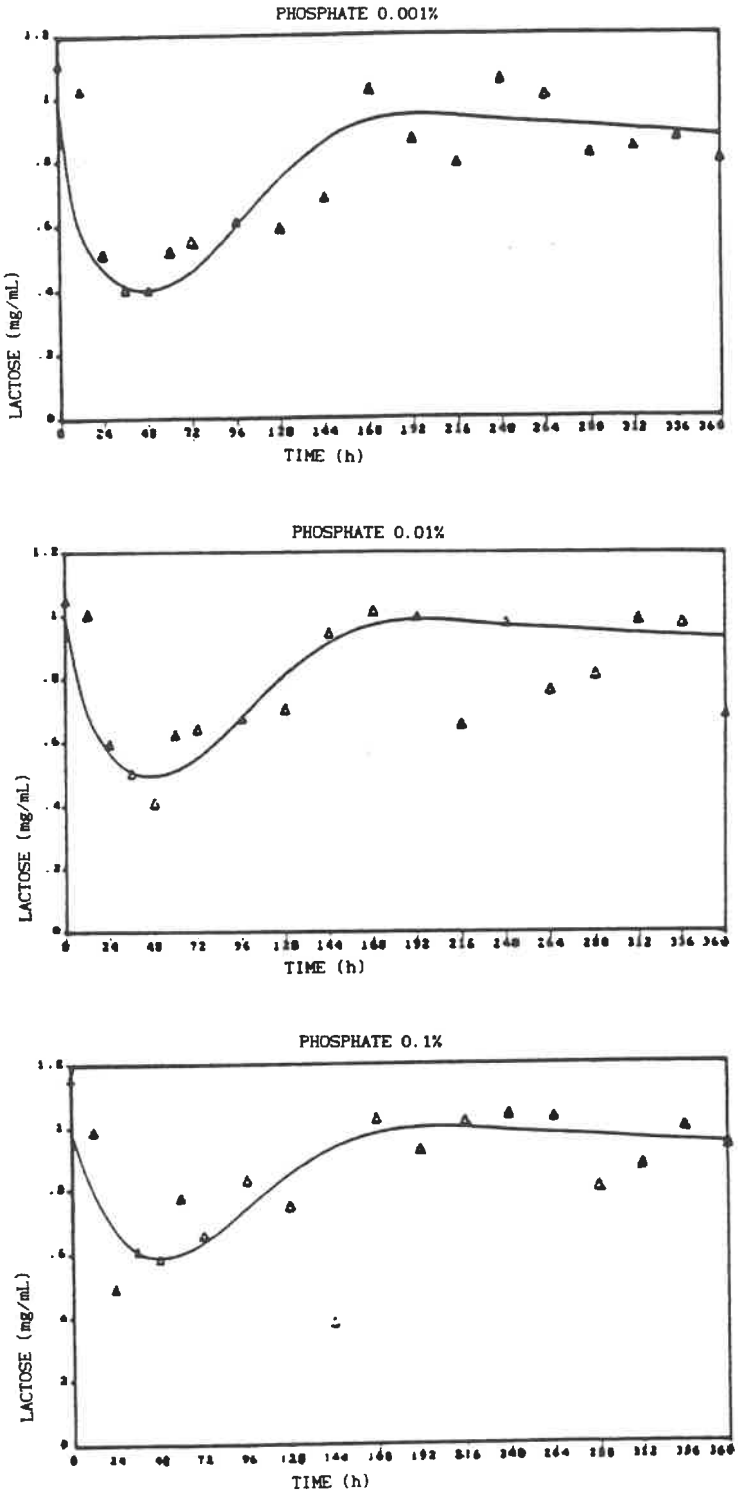


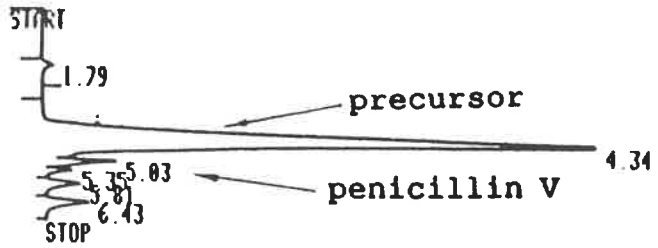
Figure 22: Lactose consumption at different phosphate conc.

lactose concentration decreased sharply and reached a minimum at the third day. Then the lactose concentration increased quickly and reached a maximum at the seventh day. Then the lactose concentration decreased slowly.

Lactose hydrolysis products, galactose and glucose, were analyzed by HPLC, but they were not found in the fermentation broth. The purpose of analysis of galactose and glucose is that they are the hydrolysis products of lactose. It was important to know if there were galactose and glucose in the culture broth. These results indicate that the mycelium of P chrysogenum consumes both the galactose and glucose.

Penicillin V in the fermentation broth was also analyzed by HPLC. On the chromatogram, there were several peaks which had a similar retention time. In order to find out which was penicillin V, two HPLC samples were prepared. Sample one (Figure 23a) is the fermentation broth and sample two (Figure 23b) is the same fermentation broth but mixed with the penicillin V standard. The peak at the retention time of 5.00 in Figure 23b had an area much larger than that on Figure 23a. This increase is due to the addition of the penicillin V standard. Thus the peak at about 5.00 is penicillin V.

Figure 24 shows the change in penicillin V production with time at different phosphate concentrations. During the first two days, the penicillin V concentration increased sharply and then decreased gradually.

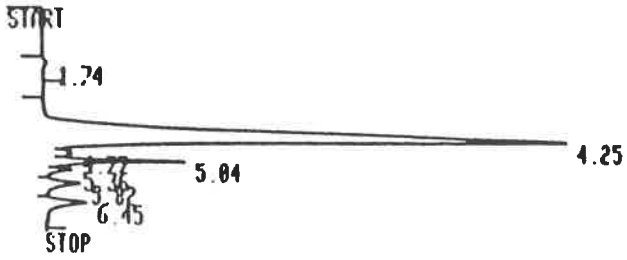


RUN # 77
WORKFILE ID: B
WORKFILE NAME:

AREA%

RT	AREA	TYPE	AR/HT	AREA%
1.79	8919	BB	0.231	0.791
4.34	982530	PV	0.430	87.108
5.03	47478	VV	0.185	4.209
5.35	18879	VV	0.181	1.674
5.81	30854	VV	0.239	2.735
6.43	39280	VV	0.247	3.482

(a)



RUN # 79
WORKFILE ID: B
WORKFILE NAME:

AREA%

RT	AREA	TYPE	AR/HT	AREA%
1.74	4785	BB	0.237	0.537
4.25	713810	PV	0.390	80.053
4.77	24012	VV	0.242	2.693
5.04	69324	D VV	0.141	7.775
5.36	16962	D VV	0.175	1.902
5.82	27070	VV	0.225	3.036
6.45	35708	VB	0.249	4.005

(b)

Figure 23 : Verification of penicillin v peak by addition of penicillin v standard in the fermentation sample. (a): fermentation sample. (b): fermentation sample plus penicillin v standard.

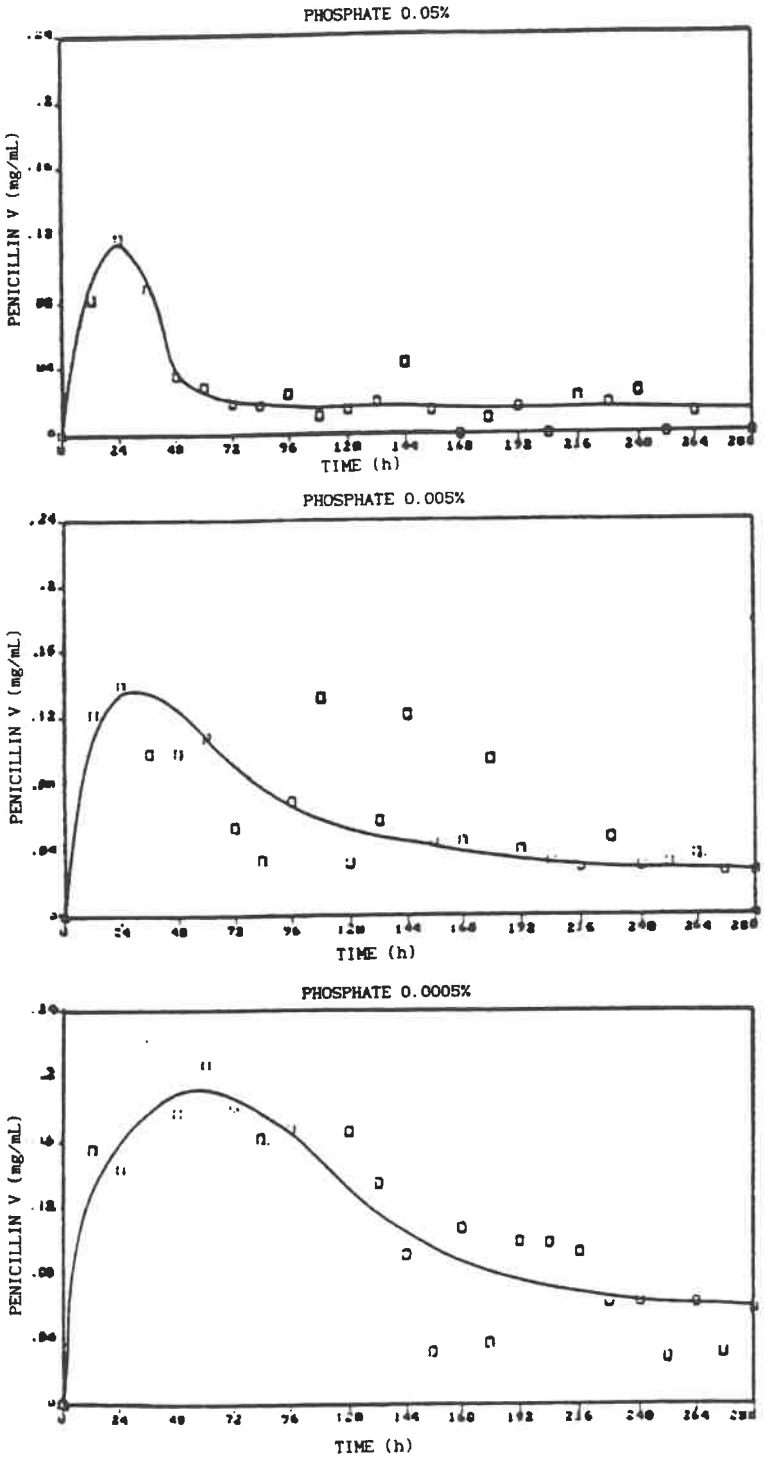


Figure 24: Penicillin V production at different phosphate conc.

Penicillin V specific productivity (q_P) was calculated according to the equation:

$$q_P = \frac{F*P}{X_T} \qquad (16)$$

where F is the feed rate (L/h), P is the penicillin V concentration (g/L), and X_T is the total biomass in the reactor (g cell). Penicillin V, biomass and respective q_P at different phosphate concentration are listed on Appendix B. Figures 25 and 26 show that higher penicillin V specific productivities (q_P) were obtained at lower phosphate concentrations.

4.6. Evaluation of IFBBR

The IFBBR reactor was evaluated in the continuous mode of fermentation. On all of the figures in this section, zero time is the start of the continuous feeding.

As illustrated on Figure 27, after inoculation, the bioparticles began to grow slowly. With growth, the density of the bioparticles increased. As the density increased, the bioparticles descended further down the annulus of the reactor. After four days the bioparticles half-filled the reactor. Ten days later they began to recirculate through the inner draft tube. In the inner draft tube, the eroder sheared off the excess biomass and controlled the diameter of the

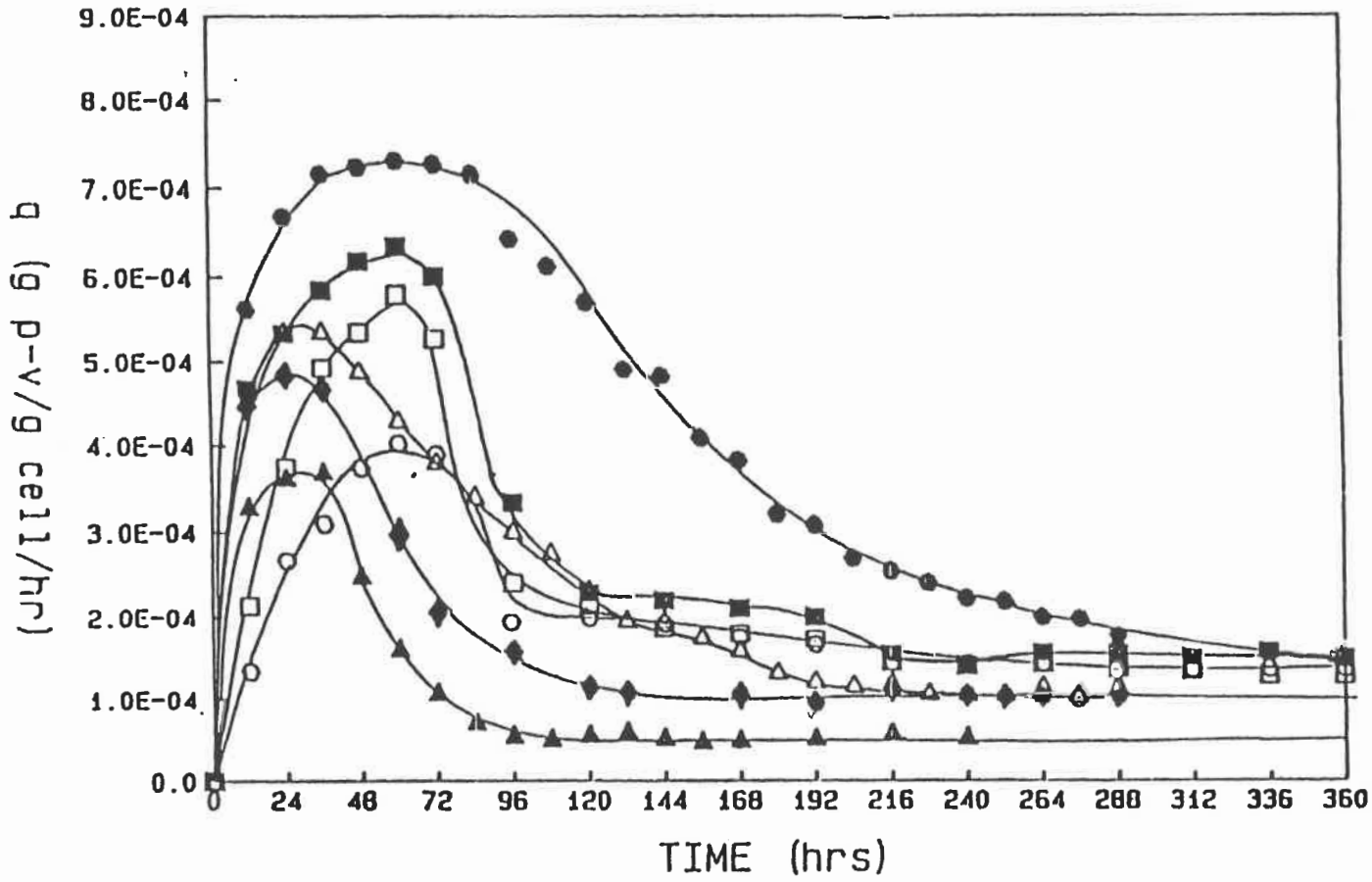


Figure 25 : q(p) in shake flasks with different phophate conc.

P (%): ◆ 0.6% ○ 0.1% ▲ 0.05% □ 0.01% △ 0.005% ■ 0.001% ● 0.0005%

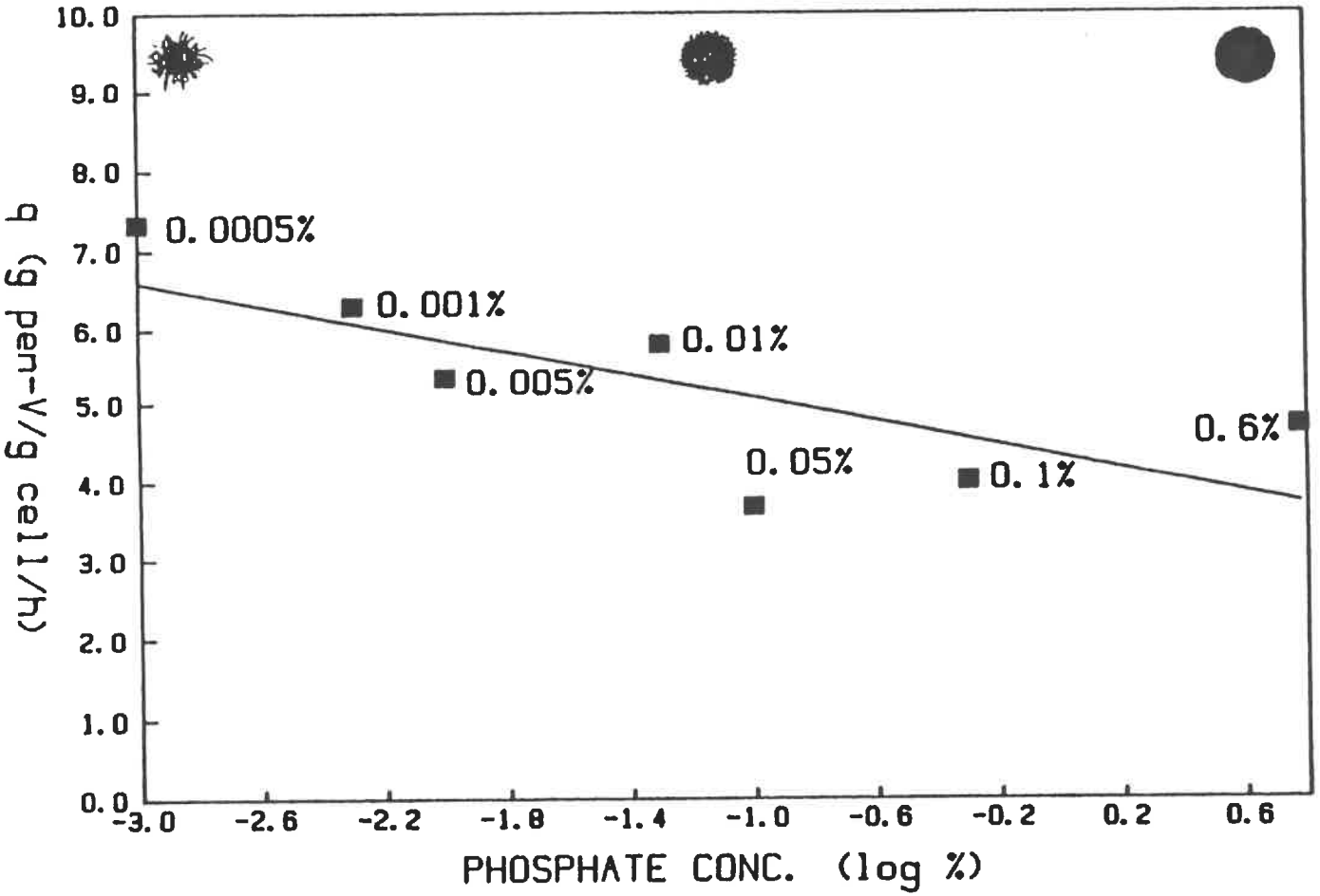


Figure 26 : The influence of phophate conc. on q(p)

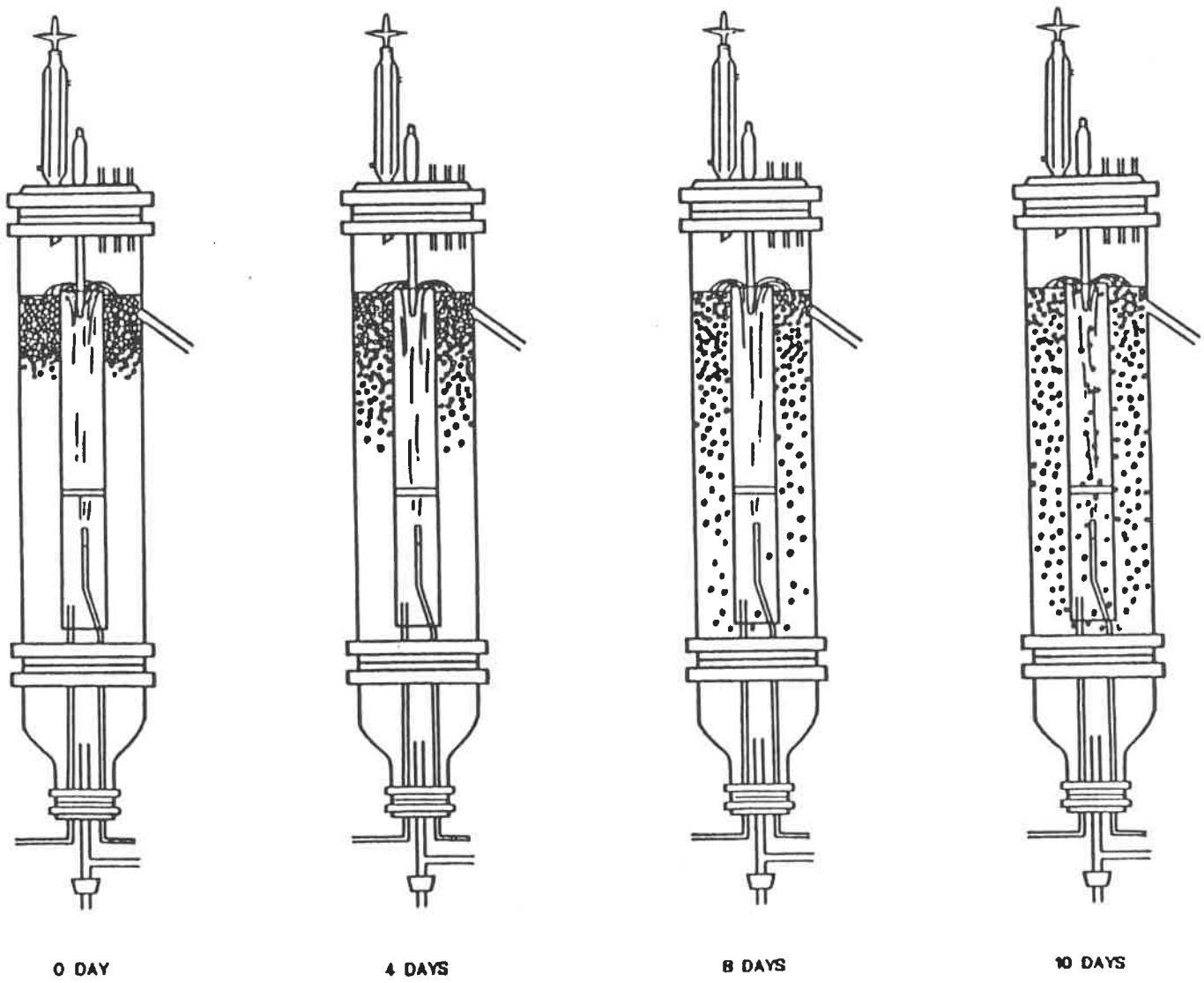


Figure 27 : Distribution of immobilized beads in IFBBR reactor.

bioparticles to about 4 mm with a biofilm thickness between 1.3 and 1.4 mm as seen on Figure 28 where the bioparticles achieved a maximum diameter of 4 mm after eight days.

The immobilized biomass was determined by the method of sodium hypochlorite biomass analysis. Figure 29 shows that the weight of immobilized biomass kept increasing during the first ten days after inoculation. Then the biomass attached to the beads was constant because the size of the bioparticles was controlled by the eroder.

On the same figure, it is shown that the initial lactose concentration was 1.0 % (w/v). During the first five days the lactose concentration decreased and then remained constant throughout the rest of the fermentation.

Phenoxyacetic acid was used as a precursor for penicillin V production. Figure 30 indicates that at the third day after continuous feeding, phenoxyacetic acid concentration reached a minimum while penicillin V was detected at that time. Then phenoxyacetic acid concentration increased until the tenth day, after which it remained constant.

Penicillin V in the fermentation broth was detected at the third day after inoculation. Ten days later the penicillin V concentration reached a maximum and then remained constant. Penicillin V production in an IFBBR is shown on Figure 31.

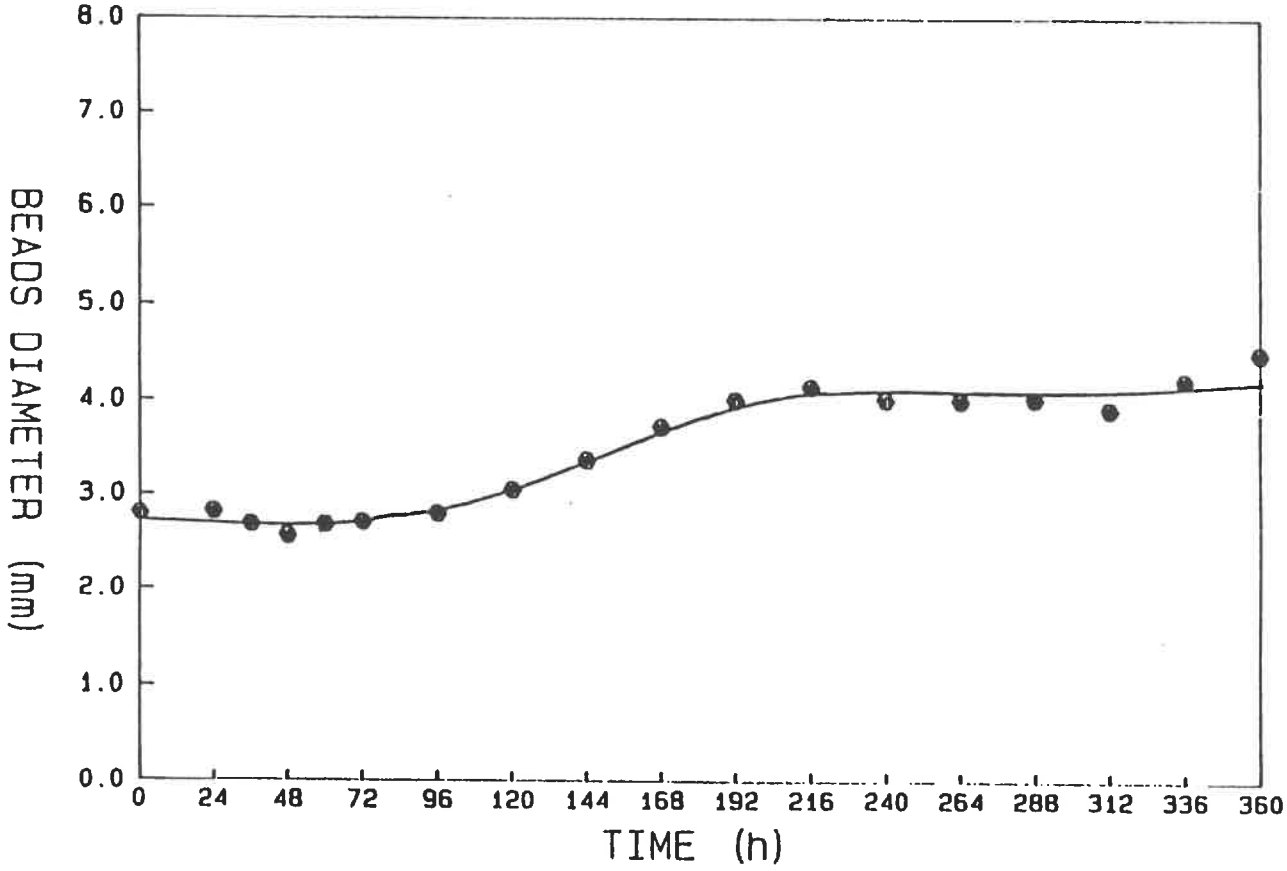


Figure 28: Diameter of beads in IFBBR reactor

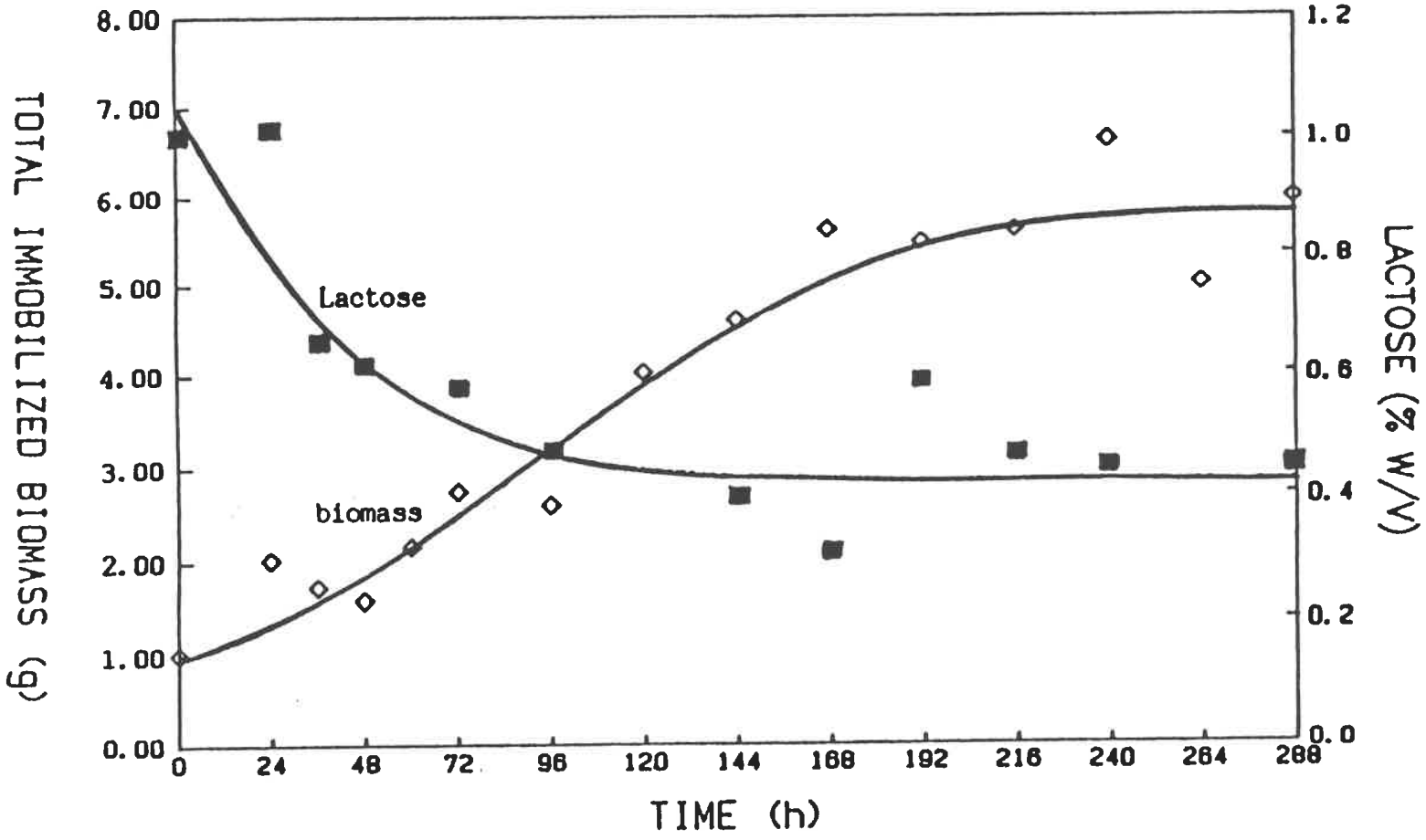


Figure 29 : Lactose consumption and biomass production in IFBBR reactor

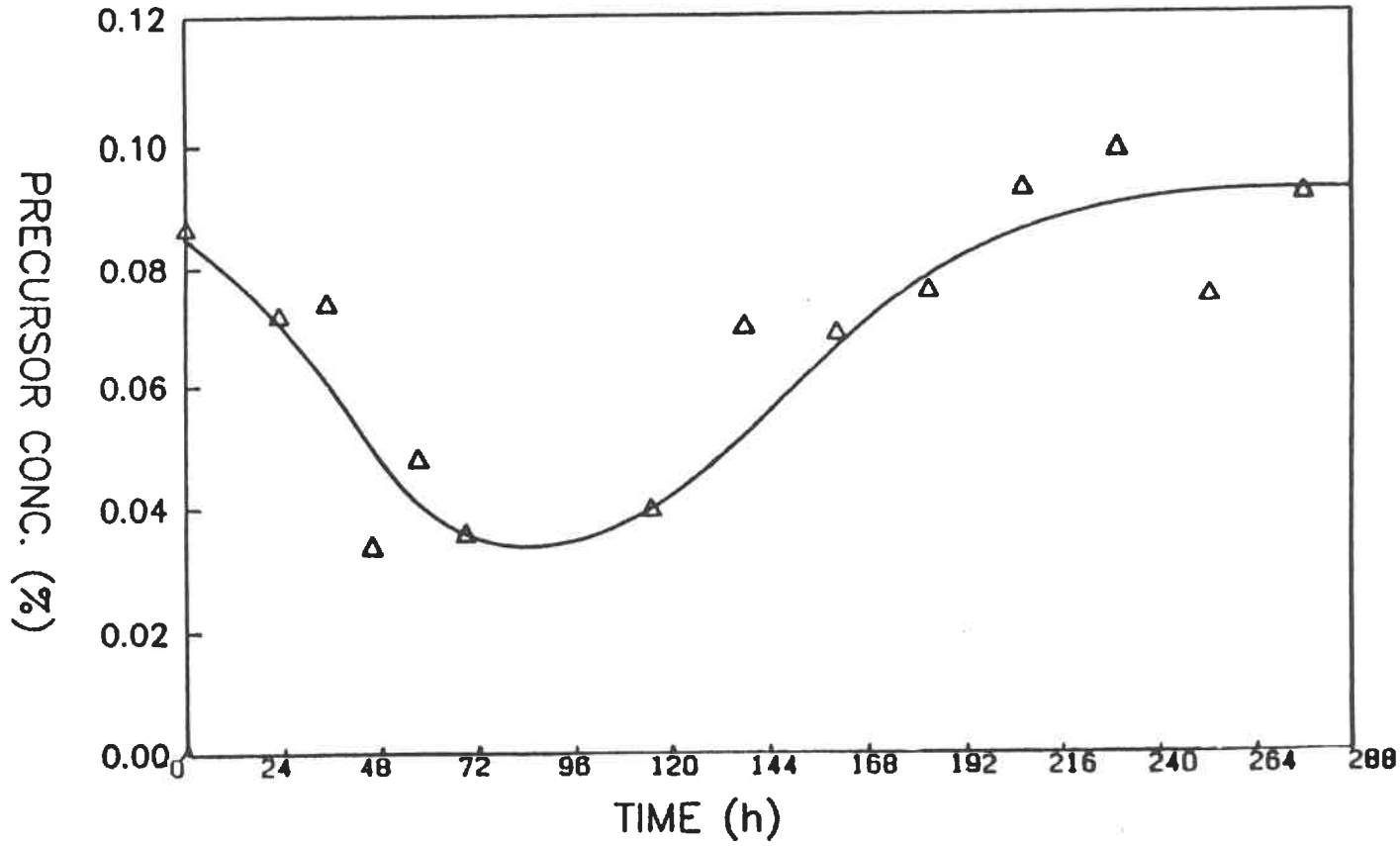


Figure 30: Phenoxyacetic acid consuption in IFBBR.

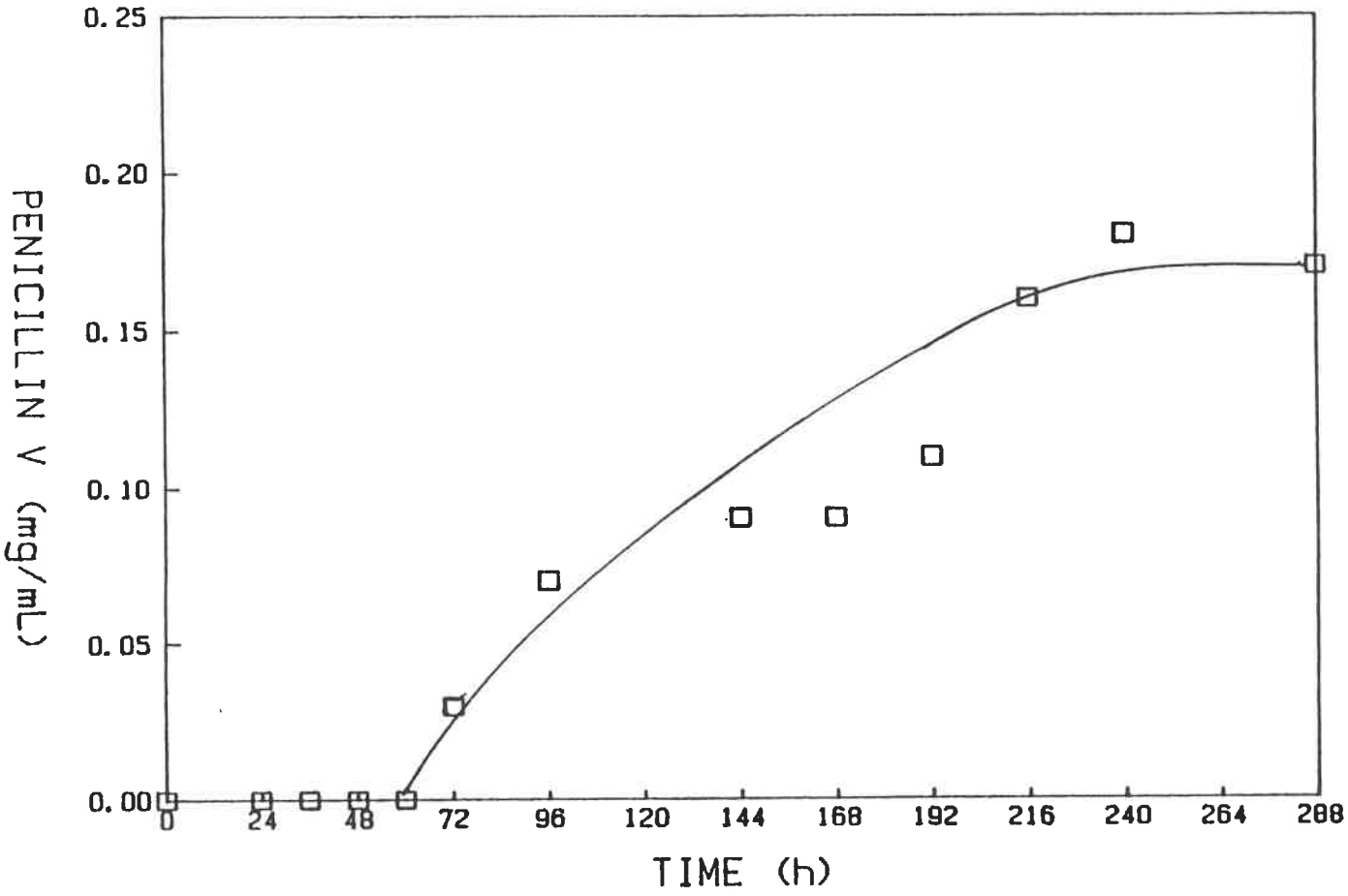


Figure 31 : Penicillin V production in IFBBR reactor

Penicillin V, biomass and respective q_P are listed on Appendix C. Figure 32 shows penicillin V specific productivity in IFBBR reactor during the course of the fermentation as calculated by means of equation (16) with X_T equal to the fixed biomass. The specific productivity increased from zero to a maximum after ten days.

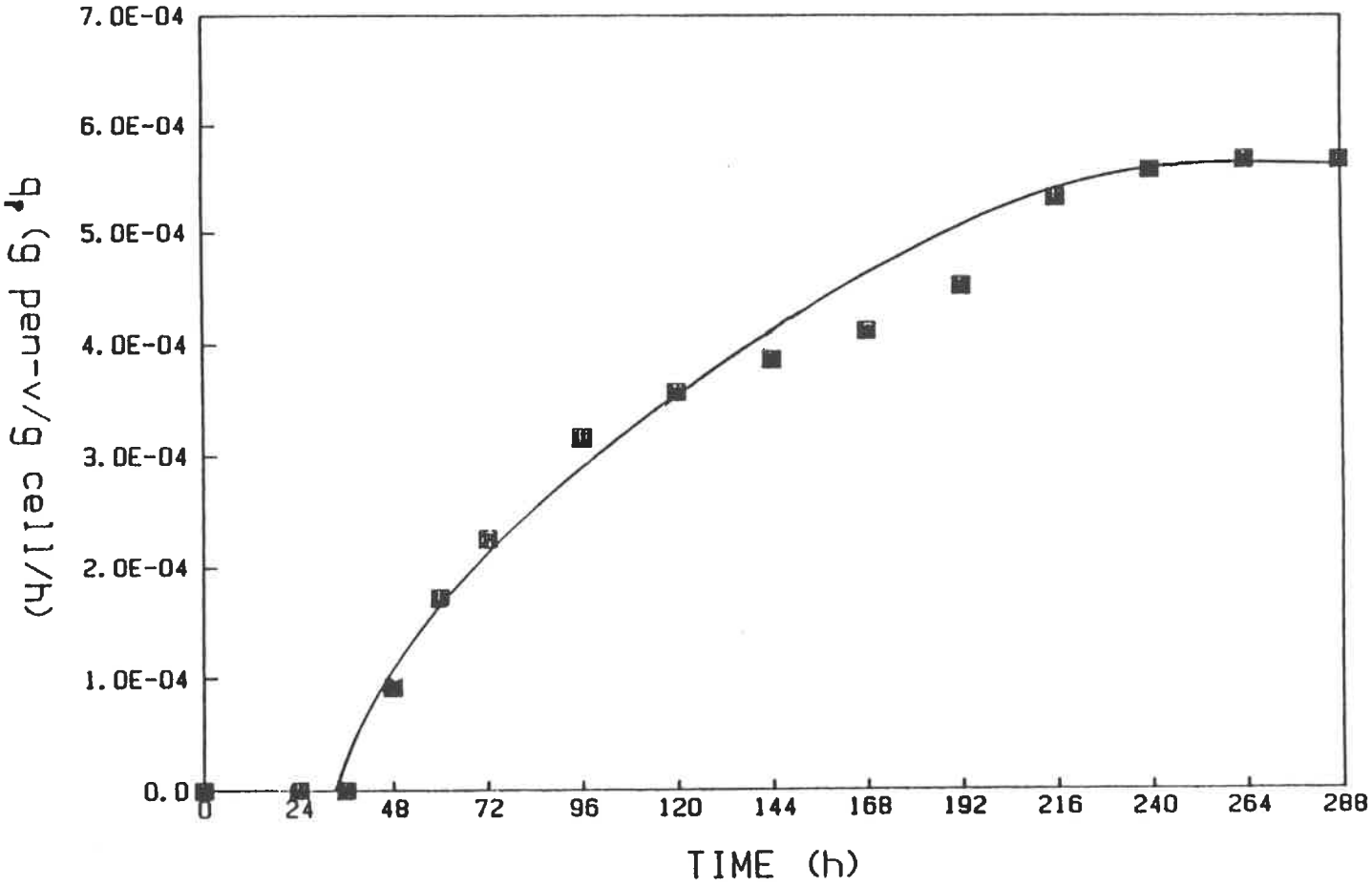


Figure 32 : q_p of penicillin v in IFBBR reactor

CHAPTER 5

DISCUSSION

5.1. Sterilization

Polystyrene beads were sensitive to higher temperature. Long exposure time to 85°C or short exposure time to 90°C made them contract. The contracted beads had a smaller size and a higher density. Such beads could not be used in the IFBBR. Short time exposure to 85°C did not sterilize the beads. So autoclaving and dry heat could not be used for the sterilization of the beads.

Ethyl alcohol treatment did sterilize the beads, but because it is toxic to microorganism, it may prevent the immobilization of fungi.

Tyndallization was effective for the sterilization of polystyrene beads. Repeated short exposure time to higher temperature had no influence on the shape and size of the beads but made the beads sterile. There was still one problem with this method in that a long time was needed for the whole process.

Gamma radiation was a quick and effective process for the sterilization of heat sensitive material such as polystyrene beads. The only problem was after gamma radiation, the beads must be washed by sterilized water so that there would be no free celite particles on the beads. Another solution is to wash the beads after treatment with

celite, dry them and then sterilize with gamma radiation.

In the industrial scale, gamma radiation will be a convenient procedure for the sterilization of polystyrene beads.

5.2. Immobilization

The immobilization was performed succesfully in the immobilization and germination medium with a spore suspension and polystyrene beads.

The spore suspension prepared with Tween 80 solution was used as soon as possible after preparation. Indeed, because of the surfactant activity of the Tween 80 solution on the spores, the spores are destroyed if left in contact with the solution for one hour.

Polystyrene beads were treated with celite powder before immobilization so that the surface of the polystyrene beads was suitable for the attachment and growth of spores. Then the beads were washed well until there were no more celite particles remaining on the beads. If there were any celite powder on the polystyrene beads before immobilization, these celite powder particles would enter into the immobilization and germination medium forming thousands of active cores for the growth of P. chrysogenum. Many mycelial pellets were produced in this manner. This growth competitively consumed nutrients in the medium and the polystyrene beads were not contacted by the spores. When

the growth of free mycelial pellets occurred, the polystyrene beads were transfered to a fresh immobilization and germination medium without adding spores. The spores left on the beads germinated and grew on the beads.

5.3. Biomass Analysis

The concept for this analytical method was taken from the biuret protein assay. Only the step of sodium hydroxide digestion was used. After the digestion, the weight of biomass should have been obtained from the difference between the weight of beads with biofilm and the weight of beads without biofilm. Unfortunately, the mycelium of P. chrysogenum was not digested by hot 20% (w/v) sodium hydroxide, so this method could not be used. Hence another method was developed.

Sodium hypochlorite treatment is a quick, convenient and accurate method for the analysis of biomass. Sodium hypochlorite digested biomass easily and did not affect the diameter or weight of the polystyrene beads (Figures 16 and 17). When the beads were dried in an oven at 60°C over several hours the weight was unchanged (Figure 18). The weight of biomass was obtained from the weight of beads with mycelium minus the weight of beads without mycelium.

This method may be used in the biomass analysis of immobilized microorganism as long as the immobilization matrix is stable to sodium

hypochlorite and temperature over 60°C.

5.4. Determination of Limiting Phosphate Concentration

As indicated in chapter 2, high concentrations of inorganic phosphate inhibit the formation of many antibiotics while stimulating vegetative growth [16]. The results from shake flasks fermentation showed that phosphate concentration really had a positive effect on biomass growth, the formation of penicillin V, and the specific productivity of penicillin V.

At higher phosphate concentrations, the mycelium on polystyrene beads was thicker and more compact than those at lower phosphate concentration. There was much more free mycelium in the fermentation broth. These free mycelium increased the viscosity of the fermentation and resulted in mass transfer problems. On the other hand, the higher biofilm thickness and the compact biomass probably inhibited internal mass transfer and the release of product through the bioparticle. Lower concentration of penicillin V (Figure 24) and a specific productivity (Figures 25 and 26) were obtained.

While at lower phosphate concentration, the mycelium on the beads were thinner and more fluffy. The mass transfer and the release of product could be easier. This is contrary to the result of Kim [39]. It was reported that bioparticles developed under phosphate-limiting

condition were smooth compact ones. In the fermentation broth, there was only a little free mycelium. The fermentation broth was less viscous. Higher penicillin V concentration (Figure 24) and specific productivity (Figure 25 and 26) were obtained.

Galactose and glucose in the fermentation broth were analysed based on the fact that these were the hydrolysis products of lactose. The object was to determine if galactose and glucose were present in the fermentation broth. Analysis results indicated that the mycelium of P. chrysogenum consumed both galactose and glucose. At different phosphate concentrations, the maximum lactose consumption was different. At higher phosphate concentration there was less lactose consumption while at lower phosphate concentration there was more lactose consumption (Figure 22).

As indicated above, at higher phosphate concentration less lactose was used and lower penicillin V production was obtained. This means that phosphate influenced the lactose metabolism and the penicillin synthesis.

In this experiment, the limiting phosphate concentration was found to be 0.0005%. At this phosphate concentration, the highest penicillin V concentration and specific productivity were obtained. In the industrial fermentation of penicillin, the natural organic component corn steep liquor, which contains 0.001% phosphate, is used. Hence a phosphate concentration of 0.001% is recommended to be used as the

appropriate limiting phosphate concentration.

It was reported that [40] bioparticles developed in rich media with other limiting nutrients such as carbon and nitrogen sources were all considered inadequate to limit biomass production since they resulted in a very fluffy loose morphology. In this research, the biomass attached to the beads was fluffier at lower phosphate concentration but at the chosen concentration, this degree of fluffiness was minor and did not hamper the functioning of the reactor.

5.5 Evaluation of the Performance of the IFBBR

The advantages of the IFBBR were observed during the continuous penicillin production. As mentioned before, the eroder which was set up in the inner draft tube of the IFBBR was an important component and enabled the control of the biofilm thickness between 1.3 and 1.4 mm. Because air was supplied upwards through the inner draft tube of the IFBBR, there was an upflow in the draft tube and a downflow in the annulus. The low density beads were fluidized by downflow of the liquid in the annulus and then recirculated through the eroder in the draft tube, the excess biomass was sheared off from the bioparticles and the biofilm thickness controlled (Figure 10).

After the excess biomass was sheared off from the bioparticles, these beads still appeared the same as before, having a smooth surface

and a round shape. The sheared off biomass was not uniform. It was difficult to determine the concentration of the free biomass. This free biomass settled to the bottom of the reactor and was pumped out of the reactor when there were visual accumulations.

One of the major problems in the fermentation of surface immobilization is the lack of control of biofilm thickness. As the biofilm thickness increases, mass transfer problems arise. The eroder installed in the IFBBR is an excellent device to help overcome this problem. The eroder sheared off excess biomass and controlled the bioparticle diameter within 4 mm.

Limiting the phosphate concentration not only limited the growth of immobilized mycelium on the polystyrene beads but also the growth of free mycelium which was sheared off by the eroder. This resulted in a less viscous fermentation broth. The control of biofilm thickness and fermentation broth viscosity lead to better mass transfer and higher penicillin V production.

From the data of the total immobilized biomass (Figure 29) and the bead diameter (Figure 28), it can be seen that after 8 days of fermentation the bioparticles began to pass through the eroder (Figure 27) and the total immobilized biomass and the bead diameter was kept constant from then on. This means that the eroder used in the IFBBR is really an effective device to control the diameter and hence the weight of the bioparticles.

In continuous culture, it was calculated by Pirt [31] that if a two-stage culture could be developed with a specific productivity (q_{pen}) of 3.6 mg/g/h and a cell weight of 40 g/L., it should be possible to reach a penicillin yield of 12.0 mg/mL. In practice, a cell weight of 30 g/L was used. For continuous culture of immobilized cells, this high cell concentration can easily be obtained. In this IFBBR experiment, the cell weight was only 5.0 g/L. Higher penicillin yield could have been obtained if more beads had been used. If a minimum liquid fraction of 50% in the fluidized bed is assumed, 5000 beads (about 30% more beads) can be added.

The initial phenoxyacetic acid concentration was 0.068% (Figure 30). After continuous feeding, phenoxyacetic acid was consumed for the penicillin V synthesis. From the third day, phenoxyacetic acid concentration increased. After the sixth day, the analyzed phenoxyacetic acid concentrations were higher than the initial concentration. This might be due to some by-products which had the same retention time as phenoxyacetic acid.

The change of the bioparticles, total immobilized biomass, lactose, precursor, penicillin V and specific productivity with dimentionless $t/t_{residence}$ is shown on Figure 33. For the 1.5 L reactor which was used in this experiment with a flow rate 0.024 L/h, the residence time was 62.5 hours.

It can be seen that when $t/t_{residence}$ is equal to 1, the diameter of

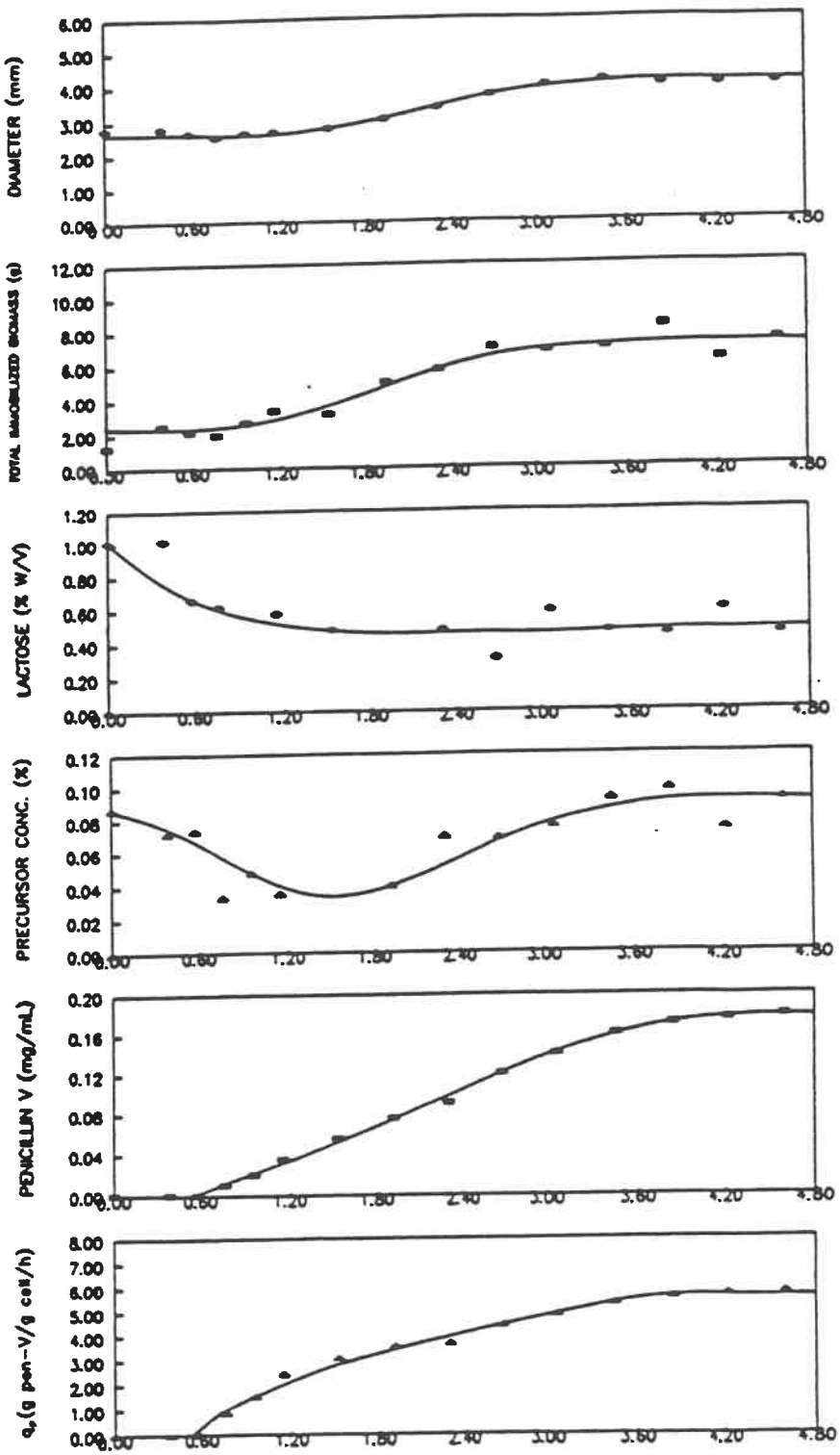


Figure 33: Bioparticle diameter, biomass production, lactose consumption, phenoxyacetic acid consumption, penicillin V production and specific productivity at different t/tresidence

bioparticles and total immobilized biomass did not change. During this time period, there was a change over from the growth to production medium and the mycelium of P. chrysogenum was adapting to the new environment, hence there was no evident growth. Lactose concentration decreased significantly because it was used to maintain the mycelium alive and meet the need to produce penicillin V which appeared at $t/t_{residence}$ of 0.6. Meanwhile, the precursor concentration decreased when it was used for penicillin V production.

When the $t/t_{residence}$ was 2, the diameter of bioparticles and total immobilized biomass were increasing. Lactose concentration decreased slowly and precursor concentration reached a minimum. Penicillin V kept increasing when there was biomass growth and lactose and precursor were consumed.

At $t/t_{residence}=3$, the diameter of bioparticles and total immobilized biomass achieved a constant value when the bioparticles began to circulate through the eroder. This occurs after 8 days or 192 hours (Figure 27). Lactose consumption was constant. Precursor concentration increased because of the reason mentioned before. Penicillin V production still increased slowly. It might be due to the free mycelium which was sheared off by the eroder.

When the $t/t_{residence}$ was 4, the diameter of bioparticles, total immobilized biomass, lactose, precursor, penicillin V and specific productivity achieved steady state.

Phosphate regulation was successfully used for the continuous penicillin V production in a IFBBR reactor, to increase the specific productivity as well as the operational stability of the bioreactor system.

The specific productivity of penicillin V in an IFBBR was comparable with those of other systems (Table 8). Higher specific productivity was obtained in the IFBBR than that in the systems such as column reactor with free mycelium [26] and fluidized bed reactor with immobilized biomass on k-carrageenan [1]. In the systems of El-sayed [4] and Behie [5], the higher specific productivities which were obtained may be attributed to the different strains which were used.

Specific penicillin productivities exhibited different patterns depending on the fermentation process. In the semicontinuous fermentation, the specific penicillin productivity reached a high level quickly and declined after a short time (Figure 25). While in the continuous fermentation in the IFBBR, q_p increased relatively slowly and achieved a constant rate after 4 reactor volumes and maintained this rate for at least three days (Figure 32). The decrease in q_p in semicontinuous fermentation might be due to oxygen limitation since during these fermentation, the shake flasks were mixed at 150 RPM. This resulted in a constant biomass (Figure 21) and a short stationary phase for penicillin production (Figure 24). However in the continuous fermentation, there was little chance of oxygen limitation as air was constantly sparged through the reactor at 0.9L/min. Other researchers

Table 8 : Penicillin Production in Different Systems

MODE OF FERMENTATION	TYPE OF REACTOR	IMMOBILIZATION MATRIX	q (mmol/g cell/h)	AUTHOR
semicontinuous	fluidized bed reactor	celite	0.0036	Kim (1986) [39]
	SHAKE FLASKS	POLYSTYRENE	0.0011-0.0021	Wang (1988)
continuous	column	free mycelium	0.0020	Pirt (1960) [26]
	fluidized bed reactor	k-carrogeenon	0.0036	Deo (1984) [1]
	IFBBR	POLYSTYRENE	0.0017	Wang (1988)
	bubble column	calcium alginate	0.0120	El-Sayed (1987) [4]
	fluidized bed reactor	celite	0.0500	Behie (1986) [5]

found that at this level of aeration there was no oxygen limitation in the IFBBR (unpublished results, M.Sc. thesis, S. Gavida 1990).

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

In this research, many factors were investigated in the evaluation of the inverse fluidized bed bioreactor. The following conclusions were obtained.

1. Tyndallization and γ-radiation were feasible for the sterilization of polystyrene beads. γ-radiation would be more convenient for large scale sterilization.

2. Sodium hypochlorite digestion was a good method for biomass analysis. The higher the sodium hypochlorite concentration used, the sooner the biomass was digested.

3. P. chrysogenum was easily immobilized on polystyrene beads if the beads were pretreated with celite powder.

4. A steel wire grid was found to be suitable as an eroder and sheared off excess biomass from the surface of the bead and hence controlled the biofilm thickness.

5. Growth and penicillin production were influenced by phosphate concentration. A phosphate concentration of 0.001% is recommended as the appropriate limiting phosphate concentration to maximize penicillin V production and to limit growth.

6. Specific penicillin productivity (q_p) in the IFBBR was comparable with that in other systems.

IFBBR is promising for penicillin production. Some work remains to be continued. The following further researchs are recommended:

1. An immobilization matrix which is stable at higher temperature should be found so that it can be sterilized with convēntional methods such as autoclaving.

2. Other cheap nutrients such as cheese whey can be tested in the penicillin production.

3. High yield strains are recommended to be examined in IFBBR.

4. The effect of feeding rate remains to be found.

REFERENCES

1. Deo, Y. M., and Gaucher, G. M., *"Semicontinuous and Continuous Production of Penicillin-G by Penicillium chrysogenum Cells Immobilized in k-Carrageenan Beads"*, Biotechnol. Bioeng., 26, 285(1984).

2. Mattiasson, B., Danielsson, B., Winquist, F., Nilsson, H., and Mosbach, K., *"Enzyme thermistor analysis of penicillin in standard solutions and in fermentation broth"*, Appl. Environ. Microbiol. 41(4), 903-908(1981).

3. Marikawa, Y., Karube, I., and Suzuki, S., *"Penicillin-G production by Immobilized whole cells of Penicillium chrysogenum"*, Biotechnol. and Bioeng. 21, 261-270(1979).

4. El-sayed, A-H. M. M., and Rehm, H. J., *"Continuous penicillin production by Penicillium chrysogenum immobilized in calcium alginate beads"*, Appl. Microbiol. Biotechnol. 26, 215-218(1987).

5. Jones, A., Wood, D. N., Razniewska, T., Gaucher, G. M., and Behie, L. A., *"Continuous production of penicillin-G by Penicillium chrysogenum cells immobilized on celite biocatalyst support particles"*, Can. J. Chem. Eng. 64(8), 547-552(1986).

6. Karhoot, J. M., Anderson, J. G., and Blain, J. A., *"Production of penicillin by immobilized films of Penicillium chrysogenum"*,

Biotechnol. Lett. 9(7), 471-474(1987).

7. Nikolov, L., and Karamanev, D., *"Experimental study of the inverse fluidized bed biofilm reactor"*, Can. J. Chem. Eng. 65(4), 214-217(1987).

8. Karamanev, D. G., Nikolov, L. N., and Elenkov, D. G., *"Light particles fluidized bed biofilm reactor"*,III International Summer School of Modelling of Chemical Reactors, Varna, Bulgaria. 109(1982).

9. Wang, D. I. C., Cooney, C. L., Demain, A. L., Dunnill, P., Humphrey, A. E., Lilly, M. D., *"Fermentation and enzyme technology"*, John Wiley & sons, New York. Chichester. Brisbane. Toronto. Singapore. p. 195.

10. Hersbach, G. J. M. & al. *"The penicillins: properties, biosynthesis and fermentation. Biotechnology of industrial antibiotics"*, E. Vandamme ed. Drugs and pharmaceutical science. 22, 45-140(1984).

11. Kolachov, P. J. & Schneider, W. C. (1952), Sept. 2. Continuous process for penicillin production. U.S. Pat. 2,609,327.

12. Crueger, W. and Crueger, A., *"BIOTECHNOLOGY: A Textbook of Industrial Microbiology"*, Science Tech, Inc. Madison, WI. pp. 203(1984).

13. Mou, D. G., *"Biochem. eng. & β-lactam antibiotic production in*

antibiotics containing the β-lactam structure", Part I: 255-284(1983).

14. Vandamme, J., "*Biotechnology of industrial antibiotics*", Drugs and the pharmaceutical sciences. 22, 86(1984).

15. Berdy, J., "*Recent advances and prospects of antibiotic research*", Proc. Biochem. Oct/Nov, 28-35(1980).

16. Martin, J. F., "*Control of Antibiotic Synthesis by Phosphate*", Adv. Biochem Eng. 6, 106-125(1977).

17. Mertz, F. P., Doolin, L. E., "*The effect of inorganic phosphate on the biosynthesis of vancocin*", Can. J. Microbiol. 19, 263(1973).

18. Bu'Lock, J. D., Hamilton, D., Hulme, M. A., Powell, A. J., Sepherd, D., Smalley, H. M., Smith, G. N., "*Metabolic development and secondary biosynthesis in Penicillium urticae*", Can. J. Microbiol. 11, 765(1965).

19. Herold, M., Hostalek, Z., In: Biogenesis of Antibiotic Substances. Z. Vanek, and Z. Hostalek (eds), Prague: Academia 1965, p.93.

20. Drew, S. W., Demain, A. L., "*Methionine control of cephalosporin C formation*", Biotechnol. Bioeng. 15, 743(1973).

21. Majumdar, M. K., Majumdar, S. K., "*Isolation and characterization of three phosphoamido-neomycins and their conversion into neomycin by*

Streptomyces fradiae", Biochem. J. 120, 271(1970).

22. Weinberg, E. D., "*Biosynthesis of secondary metabolites: roles of trace metals*", Adv. Microbial Physiol. 4, 1(1970).

23. Martin, J. F., and Demain, A. L., "*COntrol of Antibiotic Biosynthesis*", Microbiol. Review. 6, 230-251(1980).

24. Giona, A. R., De Santis, R., Marrelli, L., and Toro, L., "*The influence of oxygen concentration and of specific rate of growth on the kinetics of penicillin production*", Biotechnol. Bioeng. 18, 493-512(1876).

25. Court, R. K., and Pirt, S. J., "*Carbon- and nitrogen-limited growth of Penicillium chrysogenum in fed batch culture: the optimal ammonium ion concentration for penicillin production*", J. Chem. Technol. Biotechnol. 31, 235-239(1981).

26. Pirt, S. J., and Callow, D. S., "*Continuous-flow culture of the filamentous mould Penicillium chrysogenum and the control of its morphology*", Nature. 8, 309(1959).

27. Gordee, E. Z., and Day, L. E., "*Effect of exogenous penicillin on penicillin biosynthesis*", Antimicrob. Agents Chemother. 1, 315-322(1972).

28. Ho, C. S., and Smith, M. D., "*Effect of dissolved carbon dioxide on penicillin fermentations: mycelial growth and penicillin production*", Biotechnol. Bioeng. 28, 668-677(1986).

29. Hockenhull, D. J. D., Antibiotics, In: *Biochemistry of Industrial Microorganisms, C.* Rainbow and A. H. Rose (eds), Academic Press, Inc., New York. pp. 231-233(1963)

30. Hosler, L. M., and Johnson, M. J., "*Penicillin from chemically defined media*", Ind. Eng. Chem. 45, 871-874(1953).

31. Lurie, L. M., and Levitov, M. M., "*pH value in the biosynthesis of penicillin*", Antibiotiki. 12, 395-400(1967).

32. Morikawa, Y., Karube, I., and Suzuki, S., "*Penicillin G production by immobilized whole cells of Penicillium chrysogenum*", Biotechnol. Bioeng. 21, 268(1979).

33. Ryu, D. D. Y., "*Qantitative physiology of Penicillium chrysogenum in penicillin fermentation*", Biotechnol. Bioeng. 22, 289-298(1980).

34. Bajpai, R. K. and ReuB, M., "*A mechanistic model for penicillin production*", J. Chem. Tech. Biotechnol. 30, 332-344(1980).

35. Aiba, S., Humphrey, A. E., Millis, N. F., *Biochemical Engineering*. Academic Press Inc. New York, Lonton. pp. 85-86, 207(1973).

36. Giona, A. R., Marrelli, L. and Toro, L., "*Kinetic analysis of penicillin production by semicontinuous fermenters*", Biotechnol. Bioeng. 18, 473-492(1976).

37. Chavarie, C., Karamanev, D., Nikolov, L. N. and Champagne, J., "*Simulation of IFBBR*", (Simulation d'un réaction à biofilm à fluidisation inversée), 4th European Fluidization Conference, Toulouse, Sept., 1985. p.254.

38. Pirt, S. J., "*Application of continuous-flow methods to research and development in the penicillin fermentation*", Chem. & Ind. 601(1968).

39. Kim, J. H., Oh, D. K., Park, S. K., Park, Y. H., and Willis, D. A., "*Production of penicillin in a fluidized-bed bioreactor using a carrier-supported mycelial growth*", Biotechnol. Bioeng. 28, 1838(1986).

40. Oh, D. K., Hyun, C. K., Kim, J. H., and Park, Y. H., "*Production of penicillin in a fluidized-bed bioreactor: control of cell growth and penicillin production by phosphate limitation*", Biotechnol. Bioeng. 30, 569(1988).

41. Podbielniak, W. J. & al. Centrifugal solvant extraction. In: The history of penicillin production. Chem. Eng. Prog. Symp. Ser. 100. Vol. 66, pp. 45-50(1970).

APPENDIX A

STANDARD CURVES

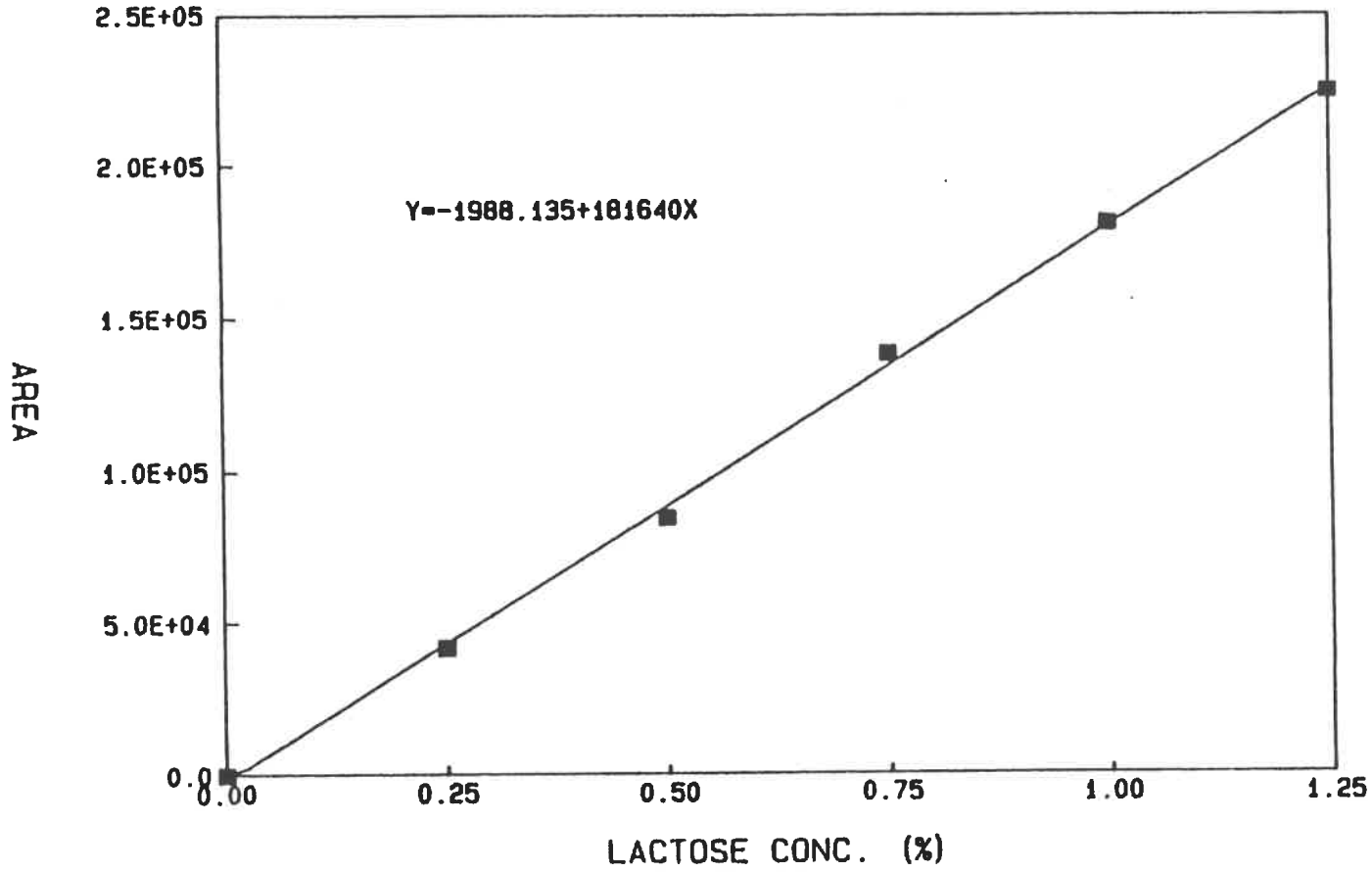


Figure A.1: Lactose standard curve

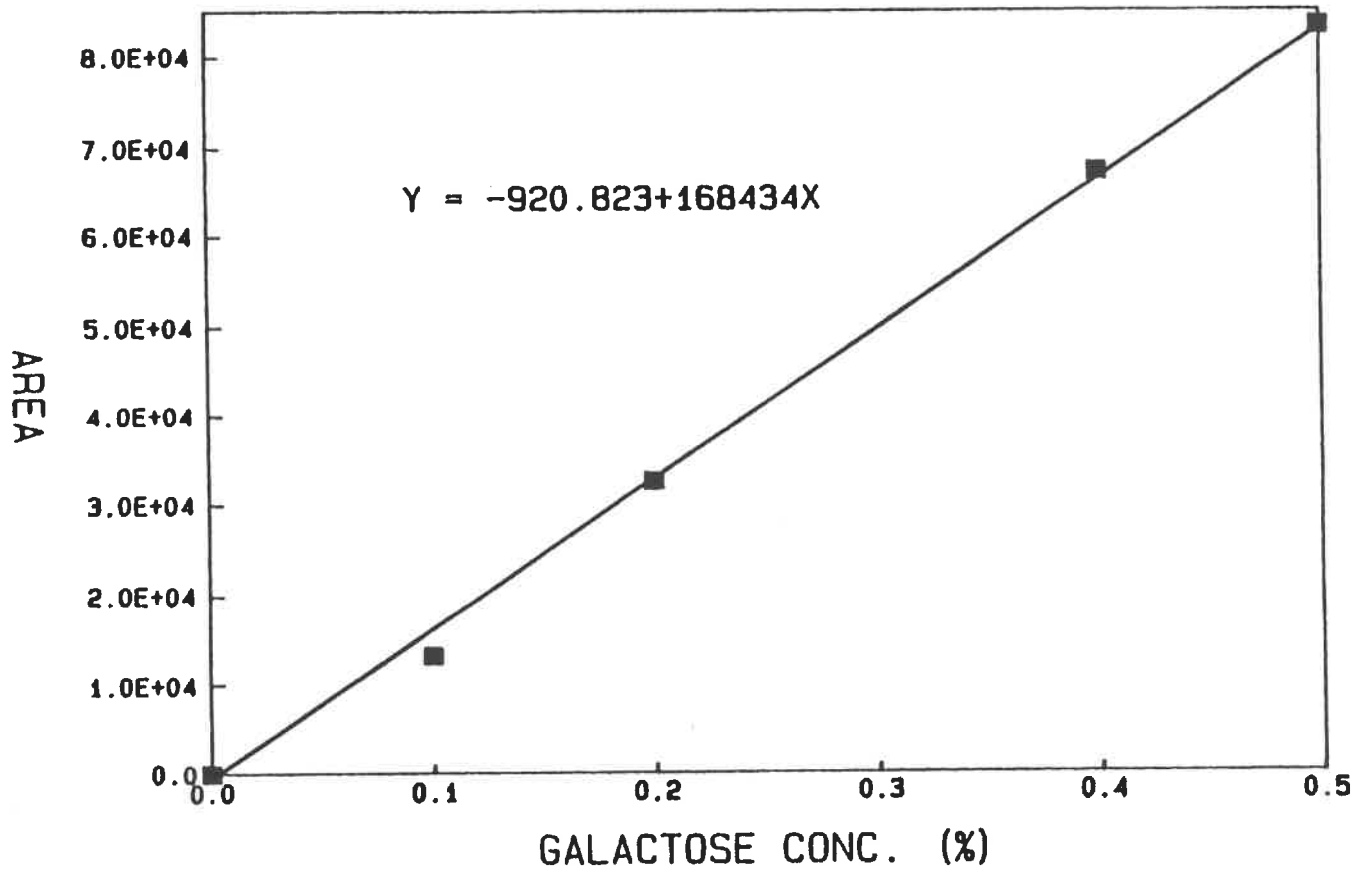


Figure A.2: Galactose standard curve

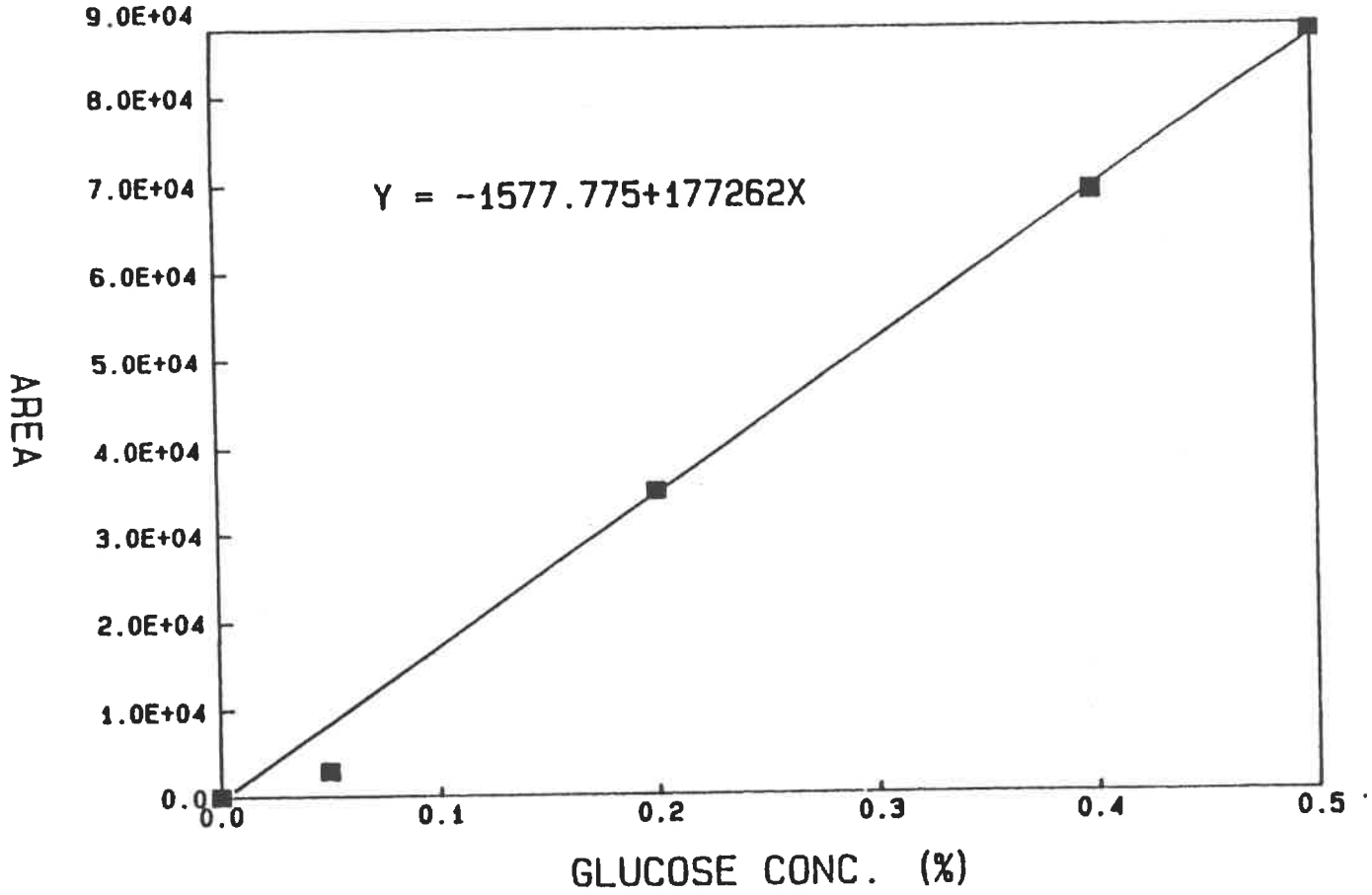


Figure A.3: Glucose standard curve

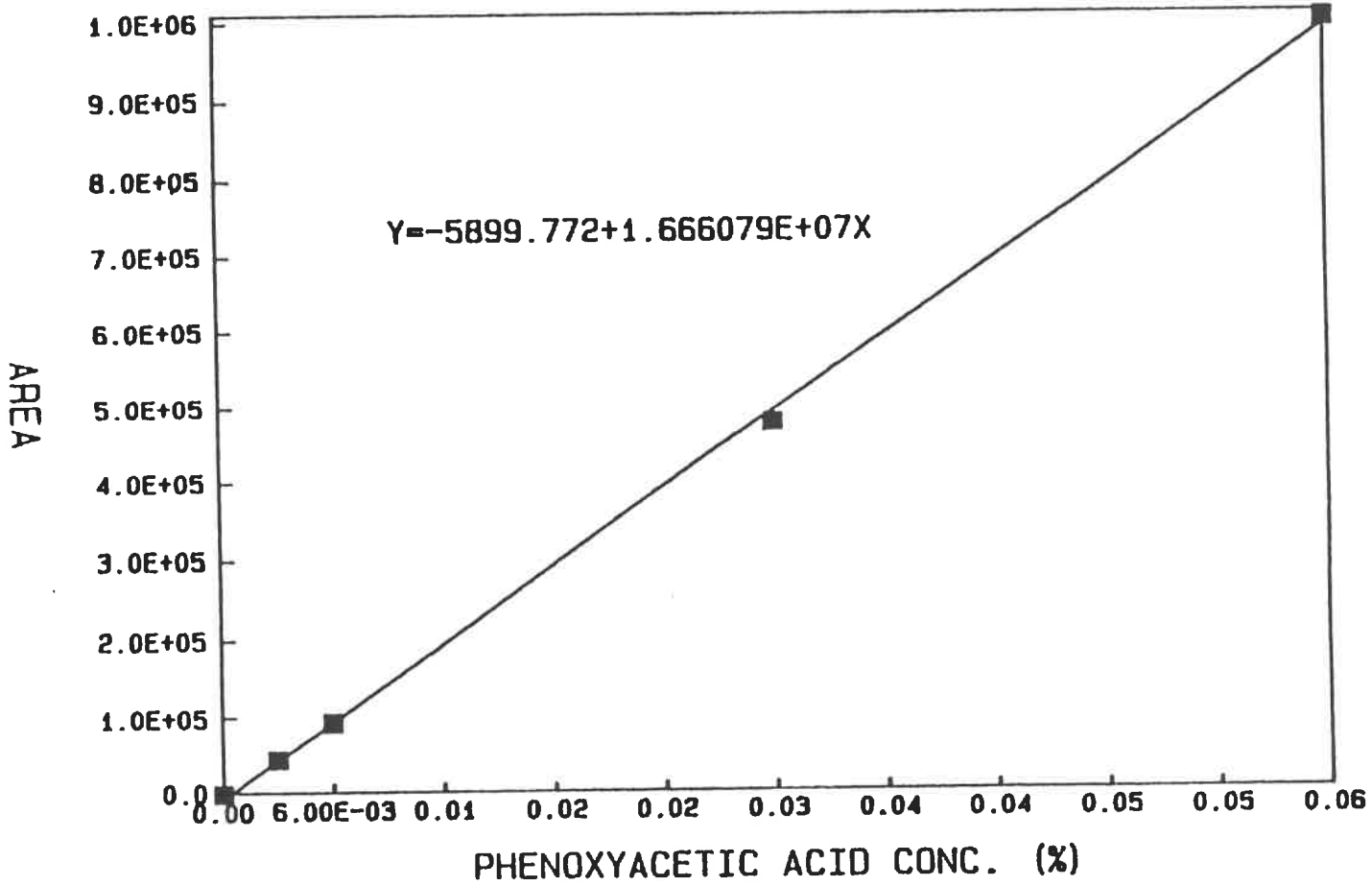


Figure A.4 : PHENOXYACETIC ACID STANDARD CURVE

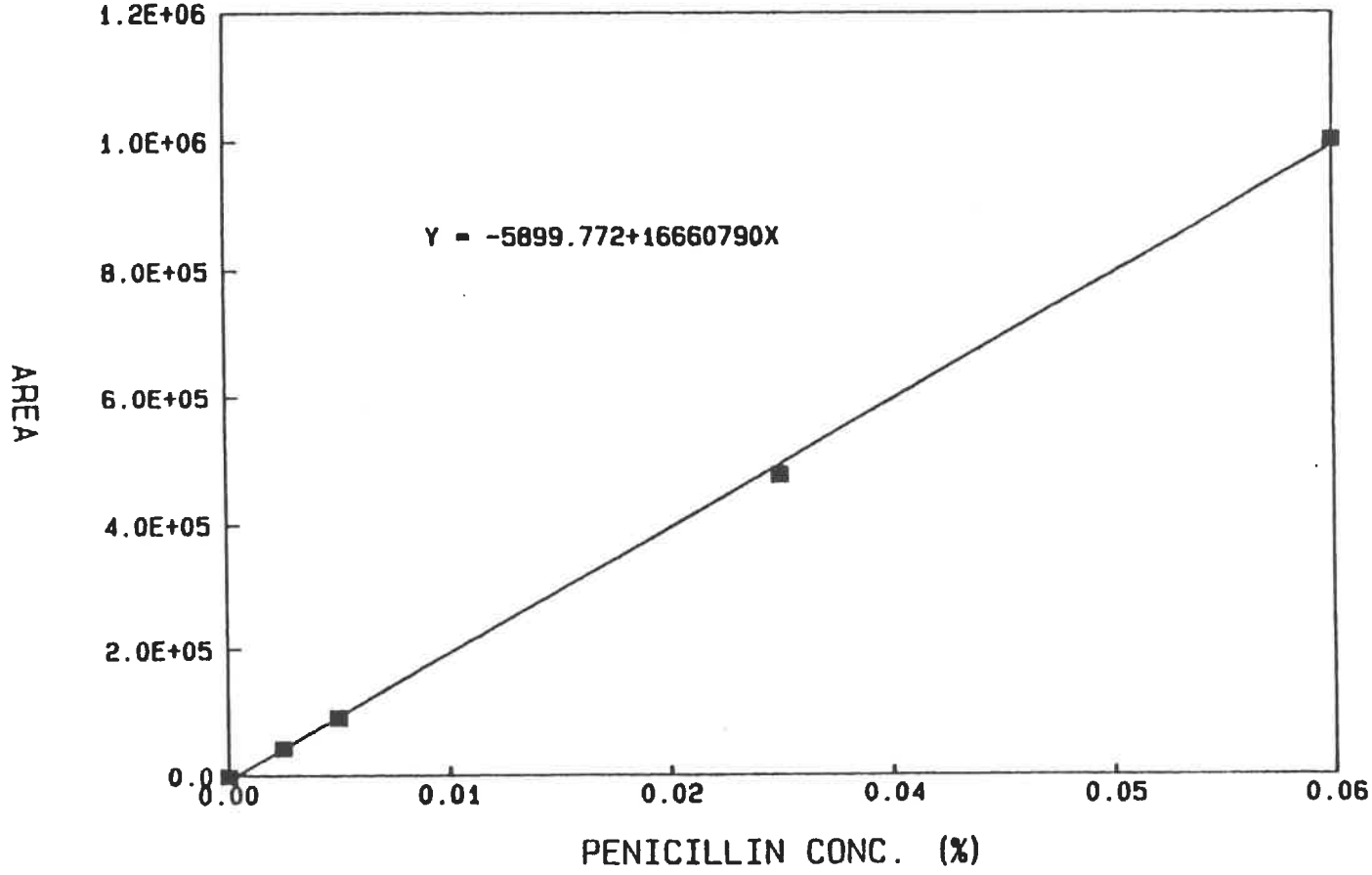


Figure A.5: Penicillin standard curve

APPENDIX B

PENICILLIN V, BIOMASS AND RELATIVE Q_P IN SEMICONTINUOUS FERMENTATION

Table 1: Penicillin V, biomass and rellative Q_P in semicontinuous fermentation at phosphate concentration 0.0005%.

Time (h)	Penicillin V (g/L)	Biomass (g)	Q_P (g p-V/g cell/h*10^{-4})
0	0.000	0.450	0.00
12	0.140	0.458	5.09
24	0.166	0.460	6.03
36	0.178	0.466	6.38
48	0.180	0.472	6.37
60	0.182	0.475	6.40
72	0.178	0.475	6.36
84	0.167	0.475	6.26
96	0.152	0.475	5.87
108	0.142	0.473	5.37
120	0.135	0.470	5.04
132	0.124	0.468	4.82
144	0.111	0.462	4.48
156	0.102	0.456	4.06
168	0.092	0.450	3.78
180	0.084	0.446	3.44
192	0.077	0.425	3.30
204	0.070	0.416	3.09
216	0.066	0.412	2.84
228	0.062	0.408	2.70
240	0.060	0.400	2.58
252	0.057	0.400	2.50

264	0.056	0.400	2.38
276	0.056	0.400	2.34
288	0.056	0.400	2.34

Table 2: Penicillin V, biomass and relative Q_P in semicontinuous fermentation at phosphate concentration 0.005%.

Time (h)	Penicillin V (g/L)	Biomass (g)	Q_P (g p-V/g cell/h*10^{-4})
0	0.000	0.430	0.00
12	0.116	0.432	4.48
24	0.134	0.435	5.14
36	0.134	0.438	5.11
48	0.122	0.439	4.64
60	0.108	0.438	4.12
72	0.095	0.437	3.63
84	0.085	0.435	3.26
96	0.075	0.432	2.90
108	0.069	0.432	2.67
120	0.061	0.431	2.36
132	0.055	0.430	2.14
144	0.049	0.429	1.91
156	0.044	0.426	1.72
168	0.040	0.424	1.58
180	0.036	0.423	1.42
192	0.034	0.422	1.34
204	0.033	0.421	1.31
216	0.031	0.419	1.24
228	0.030	0.418	1.20
240	0.030	0.415	1.19
252	0.029	0.414	1.17

264	0.029	0.412	1.18
276	0.029	0.410	1.18
288	0.029	0.409	1.18

Table 3: Penicillin V, biomass and relative Q_P in semicontinuous fermentation at phosphate concentration 0.05%.

Time (h)	Penicillin V (g/L)	Biomass (g)	Q_P (g p-V/g cell/h*10^{-4})
0	0.000	0.458	0.00
12	0.082	0.460	2.98
24	0.090	0.465	3.23
36	0.092	0.468	3.28
48	0.062	0.469	2.21
60	0.040	0.480	1.39
72	0.027	0.482	0.94
84	0.018	0.482	0.62
96	0.014	0.482	0.48
108	0.014	0.482	0.48
120	0.015	0.481	0.52
132	0.016	0.480	0.56
144	0.015	0.478	0.52
156	0.014	0.477	0.49
168	0.013	0.476	0.46
180	0.013	0.472	0.46
192	0.014	0.466	0.50
204	0.014	0.462	0.51
216	0.014	0.461	0.51
228	0.014	0.460	0.51
240	0.014	0.456	0.51
252	0.013	0.452	0.48

264	0.013	0.447	0.48
276	0.012	0.442	0.45
288	0.012	0.441	0.45

Table 4: Penicillin V, biomass and relative Q_P in semicontinuous fermentation at phosphate concentration 0.6%.

Time (h)	Penicillin V (g/L)	Biomass (g)	Q_P (g p-V/g cell/h*10^{-4})
0	0.000	0.390	0.00
12	0.111	0.400	4.63
24	0.120	0.425	4.72
36	0.116	0.446	4.34
48	0.098	0.492	3.33
60	0.074	0.500	2.47
72	0.051	0.519	1.64
84	0.045	0.534	1.41
96	0.038	0.555	1.14
108	0.034	0.563	1.01
120	0.031	0.595	0.87
132	0.031	0.596	0.87
144	0.300	0.600	0.84
156	0.029	0.600	0.81
168	0.028	0.598	0.78
180	0.028	0.596	0.78
192	0.029	0.579	0.84
204	0.029	0.562	0.86
216	0.029	0.535	0.90
228	0.029	0.526	0.92
240	0.029	0.502	0.96
252	0.029	0.485	1.00

264	0.029	0.466	1.04
276	0.028	0.460	1.02
288	0.028	0.460	1.02

Table 5: Penicillin V, biomass and relative Q_P in semicontinuous fermentation at phosphate concentration 0.001%

Time (h)	Penicillin V (g/L)	Biomass (g)	Q_P (g p-V/g cell/h*10^{-4})
0	0.000	0.602	0.00
12	0.140	0.602	3.38
24	0.160	0.600	4.45
36	0.175	0.575	5.08
48	0.185	0.562	5.50
60	0.190	0.540	5.88
72	0.180	0.538	5.59
96	0.150	0.538	4.66
120	0.130	0.538	4.04
144	0.125	0.550	3.80
168	0.120	0.562	3.56
192	0.120	0.575	3.48
216	0.120	0.600	3.34
240	0.110	0.600	3.06
264	0.110	0.602	3.05
288	0.110	0.600	3.06
312	0.100	0.575	2.90
336	0.100	0.538	3.10
360	0.095	0.530	3.00

Table 6: Penicillin V, biomass and relative Q_P in semicontinuous fermentation at phosphate concentration 0.01%.

Time (h)	Penicillin V (g/L)	Biomass (g)	Q_P (g p-V/g cell/h*10^{-4})
0	0.000	0.600	0.00
12	0.080	0.600	2.23
24	0.140	0.582	4.02
36	0.170	0.582	4.88
48	0.185	0.582	5.31
60	0.200	0.584	5.72
72	0.190	0.602	5.27
96	0.180	0.604	4.98
120	0.160	0.606	4.41
144	0.140	0.608	3.84
168	0.130	0.612	3.55
192	0.130	0.625	3.47
216	0.130	0.628	3.34
240	0.125	0.628	3.19
264	0.120	0.638	3.14
288	0.110	0.625	2.94
312	0.105	0.620	2.83
336	0.100	0.612	2.73
360	0.100	0.612	2.73

Table 7: Penicillin V, biomass and relative Q_P in semicontinuous fermentation at phosphate concentration 0.1%.

Time (h)	Penicillin V (g/L)	Biomass (g)	Q_P (g p-V/g cell/h*10^{-4})
0	0.000	0.775	0.00
12	0.070	0.750	1.56
24	0.120	0.725	2.76
36	0.130	0.700	3.10
48	0.140	0.625	3.74
60	0.145	0.600	4.04
72	0.140	0.600	3.90
96	0.135	0.575	3.92
120	0.120	0.525	3.82
144	0.115	0.500	3.84
168	0.105	0.500	3.51
192	0.100	0.500	3.34
216	0.095	0.525	3.02
240	0.090	0.550	2.73
264	0.090	0.550	2.73
288	0.090	0.550	2.73
312	0.090	0.550	2.73
336	0.090	0.550	2.73
360	0.090	0.550	2.73

APPENDIX C

PENICILLIN V, BIOMASS AND RELATIVE Q_P IN IFBBR

Table 8: Penicillin V, biomass and Q_P in IFBBR at limiting phosphate concentration 0.001%.

Time (h)	Penicillin V (g/L)	Biomass (g)	Q_P (g p-V/g cell/h*10^{-4})
0	0.000	1.650	0.00
24	0.000	2.100	0.00
36	0.000	2.400	0.00
48	0.010	2.750	0.87
60	0.020	3.150	1.52
72	0.035	3.420	2.46
96	0.055	4.300	3.07
120	0.075	5.050	3.56
144	0.090	5.800	3.72
168	0.120	6.500	4.43
192	0.140	6.900	4.87
216	0.160	7.200	5.33
240	0.170	7.300	5.59
264	0.175	7.34	5.72
288	0.178	7.38	5.79