THE USE OF COMPLEX TOXIC INDUSTRIAL WASTE AS A FERMENTATION SUBSTRATE

by

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ABSTRACT

Two complex wastes were considered for biological conversion into a marketable product. One waste, peat run-off water (the waste-water that remains after the mining of peat), was found to be unsuitable for biological conversion to any product since it contained an insufficient quantity of carbon. The other waste, NVR (non-volatile residue, the major waste from the manufacture of nylon 6'6'), was found to be a suitable carbon and energy source for the production of PHB (poly-β-hydroxybutyric acid) by Pseudomonas cepacia ATCC 17697. A general approach to the development of complex toxic wastes as fermentation substrates was formulated.

NVR was found to be toxic to microorganisms. None grew in enrichment culture containing 2.0 % NVR. P. cepacia was the most resistant microorganism found. It could grow well in up to 1.3 % NVR. It also grew on butanoic, pentanoic, and hexanoic acid as well as 6-hexanolactone. These were found to be the major toxic components of NVR. P. cepacia was grown in a NVR-limited chemostat with a NVR feed concentration well in excess of the toxic NVR concentration. In nitrogen-limited, batch fermentation on fructose, P. cepacia accumulated PHB in excess of 50 % of its dry weight. A 2-stage chemostat process for the production of PHB from NVR by P. cepacia was investigated with encouraging results.

RESUMÉ

Deux genres d'effluents polluants furent étudiés pour leur conversion en un produit de mise en marché. Le premier, l'eau de la récolte de la tourbe, est insatisfaisant pour toute conversion biologique intéressante, ne contenant pas suffisamment de matière carbonnée. Le second genre, soit les résidus non-volatiles (NVR) de la production du nylon 6'6', est le polluant principal de ce procédé. Le NVR est une bonne source du carbone et d'énergie pour la production de l'acide poly-β-hydroxybutyrique (PHB) par la bactérie Pseudomonas cepacia ATCC 17697. Une approche générale fut élaborée pour le développement de procédés de transformation de résidus industriels toxiques en produits utilisables en fermentation.

Le NVR est toxique pour les microorganismes. Aucun n'a pu croître en milieu contenant 2 % de NVR. P. cepacia fut l'organisme le plus résistant trouvé. Il peut croître jusqu'à une concentration de 1.3 % de NVR. Il peut également croître, avec comme source de carbone, les acides butyrique, pentanoïque et hexanoïque ainsi que 6-hexanolactone. Ceux-ci sont les composants toxiques principaux du NVR. P. cepacia fut cultivé dans un chemostat limité en NVR dont la concentration en NVR dépasse largement celle de sa toxicité. Lors d'une fermentation discontinue,

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limitée en azote, cette bactérie a accumulé le PHB jusqu'à 50 % son poids sec. La production de PHB par P. cepacia, à partir du NVR avec un procédé à deux chémostats en série, a produit des résultats encourageants.

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Experiments investigating the effects of NVR and NVR components on growth and production of biosurfactants by Corynebacterium lepus, Corynebacterium insidiosum, Corynebacterium fascians, Torulopsis bombicola and Torulopsis petrophilum were conducted with great skill by Mr. G.M. Znoj.

PURPOSE OF STUDY

The purpose of this work was to find a use for the major waste of the nylon manufacturing industry by using it as a fermentation substrate to produce a product of economic value. A suitable product, microorganism and process were to be chosen. The possibility of using another waste (peat run-off water) as a fermentation substrate for the production of xanthan gum was also to be examined. The methodology developed should be of value in the future development of other toxic complex wastes as fermentation substrates.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

ATCC	American Type Culture Collection
BOD	biochemical oxygen demand
COD	chemical oxygen demand
CMC	critical micelle concentration
D	dilution rate
D_A	dilution rate of the first stage
DNA	deoxyribonucleic acid
GM	glucose mineral salts medium
K_s	the substrate concentration that supports 1/2 μ_{max} (analogous to the Michaelis-Menten coeficient for enzyme kinetics)
K_s	K_s for microorganism A
I_1	lowest NVR concentration at which less biomass is produced than when NVR is absent
I_2	lowest NVR concentration at which no growth occurs (ie the toxic concentration)
ICPB	Internation Collection of Phytopathogenic Microorganisms
M	molar (moles liter^{-1})
mM	millimolar
MSC	mineral salts citrate medium
MSM	mineral salts medium
NB	Nutrient broth medium
NVR	non-volatile residue (from the manufacture of nylon 6'6')
OUR	oxygen uptake rate
P	probability that the Y-axis values are not linearly related to the X-axis values
P_A	quantity of high protein biomass produced annually

PHA	poly-β-hydroxyalkanoic acid
PHB	poly-β-hydroxybutyric acid
PPM	parts per million
RPM	revolutions per minute
q_p	specific rate of production
Q_g	volumetric air flow rate
Q_m	molar air flow rate
Qo_2	specific oxygen uptake rate
r	rate of production of biomass or product ($g\ l^{-1}\ h^{-1}$)
RQ	respiratory quotient (carbon dioxide production rate divided by oxygen uptake rate (moles $mole^{-1}$))
S	concentration of the growth-limiting substrate
T	temperature
v/v	volume per volume
V_A	total fermentation capacity needed for production of high protein biomass
VVM	void volumes per minute (based on the liquid volume of the fermentor
w/w	weight per weight
X	biomass concentration
X_A	biomass concentration of the first stage
$Y_{P/S}$	yield of product from substrate
$Y_{P/X}$	yield of product from biomass
$Y_{X/S}$	yield of biomass from substrate
μ	specific growth rate (r/X)
μ_{max}	maximum specific growth rate
μ_a	specific growth rate (r/X) of microorganism A
μ_{max_a}	maximum specific growth rate of organism A

CHAPTER 1

INTRODUCTION

The treatment of complex and/or toxic wastes from chemical processes is a large rapidly expanding industry. There are many methods for dealing with waste materials. Concentrated chemical wastes may be burnt for their heating value. They may be buried at substantial cost and ecological risk. Often they are treated as sewage and biologically converted into harmless substances. Although it would be preferable to convert wastes into products of value, this is rarely done.

Sometimes the methane gas produced during anaerobic treatment is collected. However, many toxic wastes cannot be metabolized anaerobically and methane is a low value product. Aerobic fermentation processes are able to utilize a wider variety of toxic substrates but aerobic sewage treatment processes utilize complex mixed cultures which produce no products of value.

Mixed cultures are used in waste treatment for two major reasons. The first is that the cost of sterilization is eliminated. The other reason is that the waste composition is often unknown and even if it is, a mixed culture is usually necessary for the complete utilization of complex wastes. If the waste stream composition is known and is relatively constant, it may be possible to make a

profit from the treatment process by using the appropriate microorganism(s) and culture conditions.

Some complex waste materials, such as molasses and distillers dried solubles, have become substrates in the fermentation industry. There are undoubtedly many other wastes which would have an increased value if a suitable use could be found.

One such waste is produced by the nylon manufacturing industry. Cyclohexane is the only feedstock used in the production of nylon 6'6'. During the initial stage of the production process cyclohexane is oxidized to adipic acid. but undesireable side products are also formed Adipic acid is separated from these by a distillation process. The resulting waste is known as the nonvolatile residue or simply NVR. It accounts for in excess of 15 % of the original cyclohexane feedstock.

World-wide, hundreds of millions of liters of NVR are produced each year. At present there is no better use for NVR than burning it for its heating value. However, its composition suggests that NVR could be used as a feedstock in the fermentation industry.

NVR is a dark brown liquid. It is slightly denser than and only partially soluble in water at 25 °C. It is a variable mixture of monobasic acids, dibasic acids, aldehydes, esters and other organic compounds. Since it is

an industrial waste, NVR could not be used to produce food or health related products. However, its use in the production of biopolymers, biosurfactants and enzymes for industrial purposes appears to be more feasible. The composition of NVR used in this study is given in Table 1.1

The possibility of using peat run-off water or peat hydrolyzate as a source of carbon and energy for the production of xanthan was also investigated since previous work had shown this to be a possibility (104). Peat is decomposed plant material. It is formed in wetlands under specific conditions. Peat contains up to 85 % water and must be substantially dewatered before use as a fuel. The waste-water produced during mechanical pressing (pressate) and during the drainage of peat bogs (peat run-off water) should be purified before its return to the natural environment. Biochemical oxygen demand (BOD) should be reduced and phenolic materials should be eliminated.

One process used to dewater peat involves a pretreatment step before mechanical pressing. The pretreatment step, called wet carbonization involves the use high temperature and pressure to break down the colloidal structure of peat allowing water to be released more easily. The subsequent pressate is very high in organics. Waste-water from wet carbonized peat may have a BOD of 7000 mg l^{-1}, a chemical oxygen demand (COD) of 17,000 mg l^{-1}, a pH of 4 to 5 and may contain 20 mg l^{-1} of suspended

Table 1.1 Composition of NVR used in this Study[1]

Component	Concentration (% w/w)
Butanoic acid	3.9
Pentanoic acid	13.3
Hexanoic acid	3.4
5-Hydroxypentanoic acid[2]	2.3
6-Hydroxyhexanoic acid[2]	13.9
Succinic acid	0.4
Glutaric acid	1.7
Adipic acid	8.4
Water	21.7
Others[3]	31.0
Trace metals	125 PPM

[1] Analysis supplied by Du Pont Canada Inc.

[2] Analyzed by gas chromotography as hydroxymonocarboxylic acids but probably occur as lactones in NVR.

[3] Including cyclohexanediols which account for approximately 4 to 5 % of NVR.

solids. The aerobic/anaerobic treatment process of Sorigama AB of Sweden reduces BOD by 95 %, and COD by 75 % (48). Sixty per cent of the COD is converted anaerobically to a gas composed of 70 % methane and 30 % carbon dioxide by volume.

In another de-watering process, peat is subjected to wet-carbonization at 200 °C and 50 atmospheres pressure. This results in a waste-water with a COD of 21,000 mg l^{-1} (99) and 8000 ppm of soluble organics (140). Of the soluble organics, 80 % are methanol, acetone and acetic acid (162). All of these can be easily metabolized by a wide variety of microorganisms. However, 218 mg l^{-1} of phenolics are also produced. Phenol is very toxic and even at sublethal concentrations it is not easily broken down by microorganisms.

Successful attempts have been made to grow microorganisms on peat hydrolyzates. Peat hydrolyzate is similar to waste-water from the wet carbonization process except that acid is added to aid in the hydrolysis of the hemicellulose found in peat fibers. Thus the sugar content of peat hydrolyzate is much higher. Peat hydrolyzates contain mainly glucose and xylose with arabinose and galactose in lower concentrations (89). The glucose concentration may be as high as 7.5 % w/v (90). The commercially valuable polysaccharide, pullulan can be produced by Aurobasidium pullulans when grown on peat

hydrolyzates (91). The addition of nitrogen and phosphorous is not necessary for pullulan production, although sodium chloride and magnesium sulphate must be added to the medium (13). A. pullulans has the advantage of the ability to grow on pentoses (such as xylose) as well as hexoses.

In this thesis the possibility of using peat run-off water to produce xanthan gum was considered. However, the major objective of this work was to develop a process for the biological conversion of NVR into a product of commercial value.

CHAPTER 2

THEORY

2.1 Growth on Multiple Substrates

NVR, like most wastes, is a complex growth substrate. The use of complex substrates in industrial fermentations often causes difficulties. In conventional batch culture diauxy may result if two sources of the same chemical element are supplied. Diauxy can be defined as two growth phases separated by a lag phase. It results from the sequential utilization of two different elemental sources separated by a period of adaptation. On complex carbon sources this phenomenon becomes even more of a problem for the fermentation engineer (ie. multiauxy). For example E. coli exhibits triauxic growth when grown on a mixture of sorbitol, glucose and glycerol (100).

Diauxic growth is a problem for two reasons. The additional lag phase results in a longer fermentation which is more expensive and more susceptible to contamination. Also, if the substrates are very different, their matabolism will result in different products or at least different production kinetics.

Most bacteria utilize glucose first out of a mixture of carbon sources but this is not always the case. Pseudomonas aeruginosa uses citrate before glucose (59), and

Arthrobacter crystallopoietes uses succinate in preference to glucose (87).

In chemostat culture multiple sources of the same element are usually used simultaneously at low dilution rates but often not at higher dilution rates. Saccharomyces fragilis utilizes glucose and fructose simultaneously until a dilution rate of approximately 75 % μ_{max}. At higher dilution rates fructose uptake is inhibited (141). However, a member of the same genus, Saccharomyces cerevesiae, cannot use fructose at any dilution rate if glucose is in the medium (96). Often the growth of microorganisms in chemostat culture on two substrates containing the same elements selects for constitutive mutants which do not grow diauxically in batch culture. Such is the case with E. coli B6 grown on glucose and lactose where mutants constitutive for β-galactosidase predominate in chemostat culture (139).

One factor that complicates the use of multiple substrates at high dilution rates is the variation in μ_{max} with substrate. For example Nocardia salmonicolor has a μ_{max} of .14 h^{-1} when grown on acetate, .06 h^{-1} when grown on glucose and only .03 h^{-1} when grown on d,l-isocitrate (126). Unless the dilution rate was kept below 0.3 h^{-1}, d,l-isocitrate would not be fully utilized and could rise to an inhibitory concentration in the fermentor.

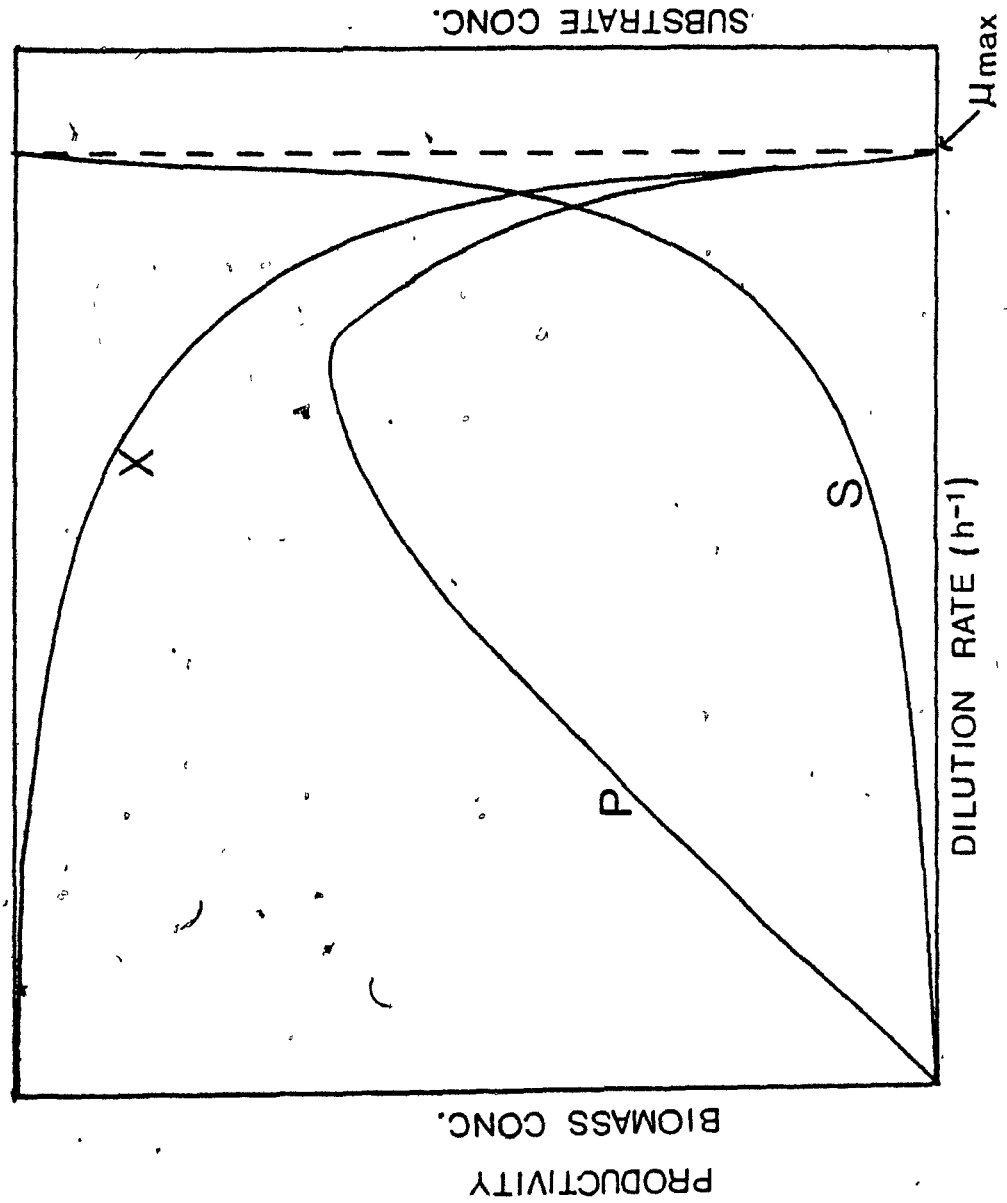
The most important mechanism limiting the simultaneous use of multiple sources of a growth-limiting element can be

described as follows. The production of enzymes is often controlled by the concentration of certain chemicals in the cell. Often the catabolites of one substrate inhibit the production of enzymes necessary for utilization of another substrate. This is known as catabolite repression. Figure 2.1 shows that a growth-limiting substrate will be in a concentration close to zero at low dilution rates. As a result the catabolites are also in low concentration. However, at high dilution rates, the concentration of a growth-limiting nutrient and its catabolites rise. When they reach a certain level they may inhibit the production of enzymes necessary for the utilization of another nutrient containing the same chemical element (ie. carbon). The result may be oscillatory behavior. This occurs when the first substrate or its catabolites inhibits use of the second substrate causing an increased demand for the first substrate thus reducing its concentration. The subsequent decrease in the intracellular concentration of the first substrate and its metabolites causes derepression of production of the enzymes necessary for catabolism of the second substrate and the cycle continues. Oscillations of this nature have been observed in beer wort (16) and Bakers' yeast (109) fermentations

Biological feedback mechanisms are usually so tightly controlled that the oscillations are not measurable. However, since oscillations of this nature occur in all living organisms, no biological system can be said to reach

Figure 2.1. A typical product (ie. biomass), substrate and dilution rate relationship in a well-mixed chemostat.

(X) Biomass (mass volume-1).
(S) Limiting substrate concentration (mass volume-1).
(P) Productivity (mass volume-1 time-1).


SUBSTRATE CONC.
μmax
X
S
P
DILUTION RATE (h⁻¹)
BIOMASS CONC.
PRODUCTIVITY

a true steady state. Steady state, as reported in the literature and in this thesis, is a relative term.

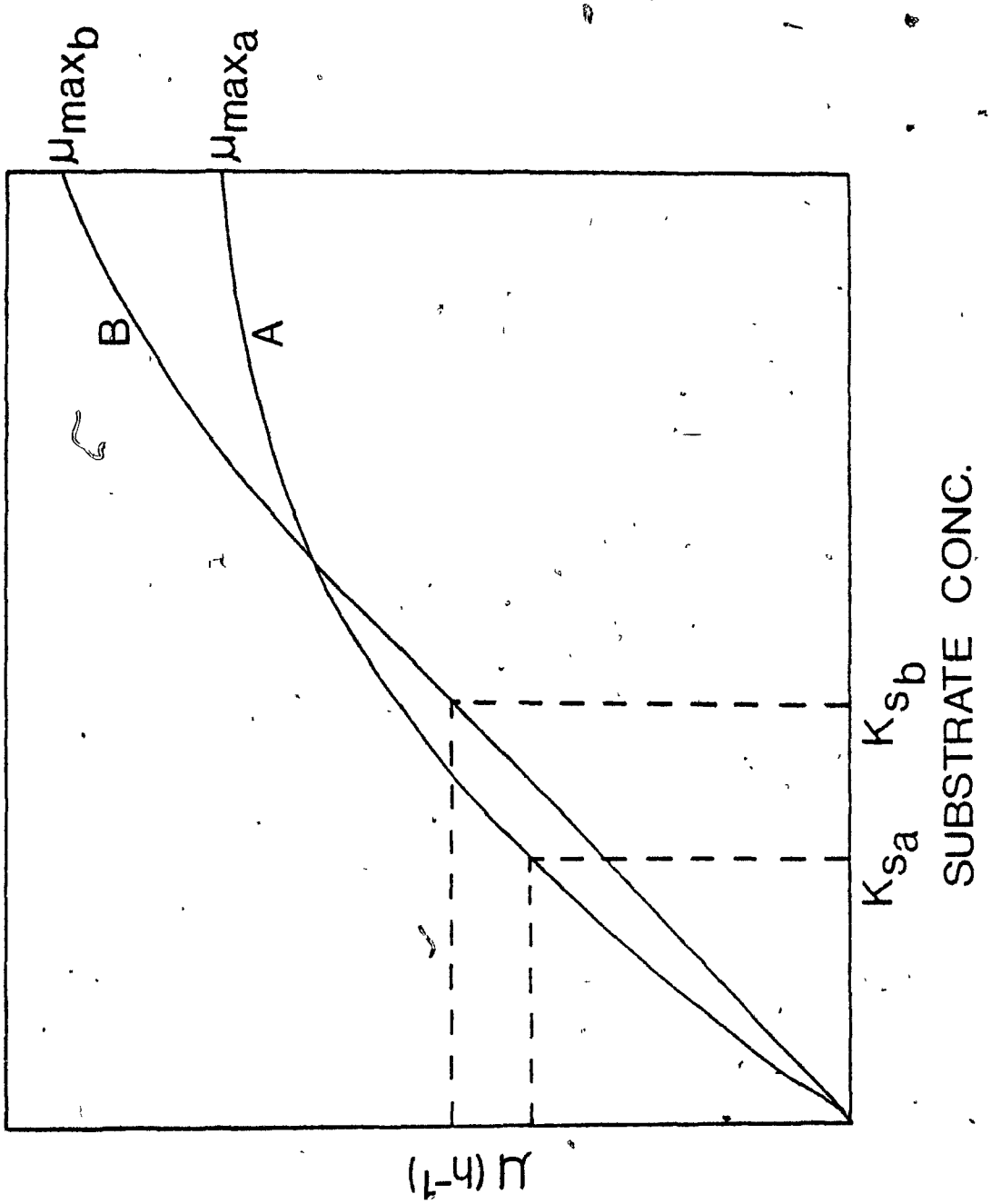
2.2 Microbial Competition and Selection

When grown in batch mixed culture, microorganisms with the highest μ_{max} (corresponding to the substrates and fermentation conditions), proliferate most quickly and dominate the culture. If one wishes to select for organisms posessing specific properties, conditions can be set whereby these organisms will be the most successful. This is called enrichment culture. For example if one wishes to obtain Clostridium pastorianum, it is only necessary to add some fertile soil to a flask containing glucose, calcium carbonate, water and a nitrogen atmosphere (ie. no oxygen). After several days at 25 °C, large numbers of C. pastorianum will be present. However, there will also be considerable contamination from other organisms which may not have grown very much but which were present in the initial inoculum.

An even more powerful tool is continuous enrichment culture. This technique can be used to isolate cultures relatively free of contamination. Since a steady-state culture can be easily achieved, continuous enrichment culture can also be used to select for microorganisms which have very special characteristics At low dilution rates, organisms with low K_s values for that substrate will proliferate. Thus organism A in Figure 2.2 would dominate the culture while organism B would wash out unless low K_s

Figure 2.2. A hypothetical relationship between specific growth rate and substrate concentration for two microorganisms.

(A) Microorganism A.
(B) Microorganism B.


μmaxb
μmaxa
B
A
μ (h⁻¹)
Ksa
Ksb
SUBSTRATE CONC.

mutants of B developed. However, organism B has a higher μ_{max} on the limiting substrate and at high dilution rates it would dominate and A would wash out. Only where the lines of Figure 2.2 intersect would a stable mixed culture result. Therefore, not only is it possible to select for microorganisms that use certain substrates, one can also select for organisms with a high μ_{max} or a low K_s for a specific substrate. The use of automatically controlled continuous culture systems such as the turbidostat (105), pH-stat (107,121), and the viscostat (138) provide even more power to the selection system.

The use of selective pressure, especially in the presence of mutagenic agents, is often a much more powerful tool than any genetic engineering technique. Gene splicing may be used to increase the copy number of a gene coding for an enzyme necessary for the production of a specific biochemical However, these creations are often very unstable and loss of culture potency may occur even in batch production systems. Often, the desired goal may be achieved much more easily through the use of selective pressure. Many methods have been developed for the application of selective pressure (36,128). One of the simplest methods is to limit the growth of an organism in a chemostat by limiting the supply of a growth factor that the organism cannot make for itself. For example, a thiamine-requiring organism increased its production of thiamine phosphate

phosphorylase by 1500-fold when it was grown in a thiamine-limited chemostat (81).

A similar selective pressure can be applied to microorganisms that require inducers for enzyme production. Growth of *E. coli* in a chemostat with a low lactose concentration resulted in the production of mutants that did not require lactose for β-galactosidase synthesis. The β-galactosidase produced accounted for 25 % w/w of the microorganism's protein content (69).

High production levels can also be achieved by the selection of mutants resistant to inhibitors or toxic materials. By growing *Rhodopseudomonas spheroides* in the presence of 0.1 M H_2O_2, a constitutive mutant was obtained whose total protein content was 25 % w/w catalase (22).

An unusual use of a continuous selection process was the isolation of a yeast-lyzing species of *Arthrobacter*. This organism was obtained from a compost inoculum in a chemostat where the limiting nutrient (carbon source) was *Saccharomyces fragilis* (123).

2.3 Microbial Growth on Water-insoluble Substrates

NVR is only sparingly soluble in water. Considerable research has been reported concerning the growth of microorganisms on n-alkanes which are also water-insoluble liquids (8,49). Apart from the generation of large amounts of heat and the consumption of large quantities of oxygen (98) due to their reduced nature, insolubility is the most important problem involved in using hydrocarbons as fermentation substrates.

Batch growth of microorganisms on n-alkanes is characterized by a short exponential growth phase which is followed by a much longer linear growth phase. Most explanations for this phenomenom relate to hydrocarbon transport kinetics. There are two major groups of hydrocarbon transport models (95).

One group of models attributes the linear growth phase to the saturation of available hydrocarbon droplets with cells. These models cite hydrocarbon droplets larger than cells as being the most important uptake form and thus responsible for growth kinetics. Evidence (135) that growth rate is directly related to droplet coalescence frequency (which increases with increased agitation) supports this model type.

The other group of models cites submicron droplets as controlling the growth kinetics (2). They describe the linear growth phase as a constant linear rate of diffusion of hydrocarbon droplets through the aqueous phase to the cell surface. The finding that the growth rate of Candida lipolytica increases with decreased droplet size (also due to increased agitation) supports this model type (103).

2.4 Biosurfactants

The earliest report of a microbially-produced surfactant (biosurfactant) was in 1947 (171). Desulfovibrio was shown to cause the release of bitumen from Alberta tar sand. Biosurfactants have certain properties that are superior to many synthetic surfactants. These include biodegradability (94) and great variety in chemical structure and thus in their surfactant properties (27).

Biosurfactants have been the subject of several reviews (27,169,170). They have been categorized as amino acid-containing biosurfactants, phospholipids, fatty acids and neutral lipids, and glycolipids.

Amino acid-containing biosurfactants include the lipopeptide produced by Corynebacterium lepus (30), the ornithine lipids of Pseudomonas rubescens (164) and Thiobacillus thiooxidans (84), and subtilysin (surfactin) which is one of several lipopeptides produced by B. subtilis (27).

All microorganisms contain phospholipids (7) but few excrete them in significant quantities (170). Exceptions include Thiobacillus thiooxidans (12), and Corynebacterium alkanolyticum (83,106). C. alkanolyticum must first be treated with cephalosporins and penicillins. C. lepus also secretes several surface-active phospholipids (30).

In addition to lipopeptide and phospholipid biosurfactants, C. lepus also produces surface-active corynemycolic acids (30). These and other hydroxy fatty acids are more effective surfactants than ordinary fatty acids (29). The fatty alcohols produced by Arthrobacter paraffineus (154) are examples of neutral lipid biosurfactants.

Glycolipid biosurfactants containing trehalose (ie. mycolic acids) are excreted by many actinomycetes when grown on hydrocarbons (116). Rhamnolipids are produced by Pseudomonas aeruginosa (71) and sophorose lipids by Torulopsis species (26,28).

Patents have been issued for the recovery of oil using the trehalose biosurfactant of Rhodococcus erythropolis (161) and for the use of an emulsifier produced by an Arthrobacter species (57,58). This emulsifier is a polysaccharide-lipid complex marketed under the name "emulsan". It is presently used to clean the hulls of oil

tankers (122). Biosurfactants can also be used for the cold water extraction of bitumen from Athabaska tar sands (168)

Many microorganisms that produce biosurfactants, do so only in the presence of hydrocarbons (27). It may be that hydrocarbons simply are convenient building materials for the lipid component which invariably serves as the hydrophobic component of the biosurfactant. There are other theories which attempt to explain this phenomenon. One suggests that since hydrocarbons are water-insoluble, the microorganism must produce surfactants in order to solubilize its carbon source (88,117). Another theory claims that the hydrocarbon substrate serves as a solvent for the extraction of biosurfactant from the microbial cell surface (40). If this theory is correct, then a substrate (NVR for example) need not be metabolized by an organism in order to stimulate biosurfactant production.

2.5 Extracellular Microbial Polysaccharides

In the past almost all economically significant polysaccharides were extracted from higher plants and algae. The most important polysaccharides are still plant-derived starches and cellulose. Recently, microbially-produced polysaccharides have begun to replace plant-derived polysaccharides in certain specialized areas. Xanthan, for example, has captured much of the market previously held by the tree exudate known as gum tragacanth (163). It has also been replacing the synthetic polymer, polyacrylamide, for

use as a viscosifier in tertiary oil recovery (125). Deacylated PS-60 polymer, which is produced by a Pseudomonas species (102), is beginning to replace agar as an inert solid medium on which microorganisms can be grown.

Microbial polysaccharides can be classified as homopolymers (containing a single structural unit) or as heteropolysaccharides (containing two or more different monomers). Dextrans and levans are homopolysaccharides. They are synthesized from sucrose by microbially-produced extracellular enzymes. Dextran is a homopolymer of glucose (101) while levan is composed exclusively of fructose (10). Dextran is used as a blood expander (ie. a plasma substitute) and can be cross-linked for use as a chromatographic gel (ie. Sephadex, Pharmacia AB, Sweden). Dextran may also be of value as a plugging agent, particularily in tertiary oil recovery where "viscous fingering" has occurred during polymer flooding (72). An estimated 2000 tons of dextran are produced annually world-wide (115,151). Levans may be used in photographic emulsions to improve the quality of silver granularity (10).

Few homopolymers synthesized by cell-bound enzymes have received attention for commercial applications (152). Curdlan is a non-branched linear homopolymer of d-glucopyranosyl units joined by β-d-(1-3)-glycosidic linkages (60,80). It is produced by Alcaligenes faecalis var myxogenes (5,111). Aqueous curdlan solutions form resilient

gels upon heating to 54 °C. Gel strength increases with temperature and is constant in a pH range of 2.5 to 12 0 (22). Scleroglucan, produced by Sclerotium and related genera (124), and pullulan, produced by Aurobasidium pullulans (18,19,134), are other potentially important homopolysaccharides. Pullulan is marketed by the Hayashibara Corporation of Japan (152).

Many microbial heteropolysaccharides are of commercial interest. Alginic acids contain d-mannuronic acid and l-glucuronic acid in various ratios. They are generally extracted from sea weed (Phaeophyceae) but can also be synthesized by Pseudomonas aeruginosa (93) and other microorganisms (32,68). Calcium and other divalent cations precipitate alginic acids fom aqueous solution. Calcium alginates are used as sizes in the textile and paper industry. Fifty per cent of all alginic acids produced are used in the food industry to stabilize ice cream, instant desserts, custards, creams and other emulsions (152).

Xanthan gum is produced by Kelco (U.S.), General Mills (U.S.), Rhone-Poulenc (France) and Tate and Lyle (U.K.). Xanthan has unique physical properties. It dissolves in hot or cold water producing high viscosity at low concentrations. It is highly pseudoplastic and is temperature, salt and pH insensitive (31). Although not a gelling agent by itself, xanthan can be used in combination with locust bean gum to produce a gel (110). It has

applications in textile printing, drilling muds, rust removal agents, liquid animal feeds and tertiary oil recovery (163). Other uses are in gelled explosives (ie. water-proof dynamite), as a paint stabilizer and as a flocculating agent for water clarification (80) Derivatives of xanthan can also be of value. Hydroxyalkylethers can be used in cosmetic formulations and dialkylaminoalkoxyethers as sizes (80). Graft copolymers with acrylamide, acrylic acid and other monomers can be synthesized.

Viscous heteropolysaccharides are produced by the gliding bacterium Lysobacter gummosus (21), Erwinia tahitica (Zanflo) (80), and Arthrobacter viscosus (17,50). Other microbial exopolysaccharides are currently being marketed or are of potential economic importance (80,151,152).

There are many application for polysaccharides outside of the food and health industries These include use in drilling muds, flocculating agents, rust removers, ceramics (both as a binder and a glaze), inks, paints, dyes, textiles, enhanced oil recovery, films, slurry explosives, pesticide sprays, adhesives, welding rod fluxes, silver recovery and many other processes (31,163).

2.6 Catalase

Catalase is an enzyme composed of four tetrahedrally arranged 60,000 dalton subunits Each subunit is a single

polypeptide chain that associates with a single prosthetic group, ferric protoporphyrin IX (131). Catalase can be used as an antioxidant because it catalyzes the reaction shown below

$$2\ H_2O_2 \rightleftharpoons 2\ H_2O + O_2$$

Catalase is used in conjunction with glucose oxidase to stabilize food products (ie juices, beer, wine, powdered milk, mayonaisse) against loss of flavour and colour by oxidation (52). It is also used in a process called "cold pasteurization". Conventional pasteurization is used to destroy pathogenic microorganisms in milk but the heat inactivates lipases, proteases, and phosphatases desirable for cheese-making. Hydrogen peroxide may be used to destroy microorganisms but later must be removed to allow growth of the starter culture. Catalase is employed to destroy the hydrogen peroxide (64). Other processes using catalase include the bleaching of textiles and hair so that dyes can be applied and the generation of oxygen from hydrogen peroxide for the production of foam rubber, plastics, porous cement and baked goods (166).

Commercial catalase is extracted from beef liver or produced in microbial fermentations (usually Aspergillus niger or Micrococcus lysodeikticus). The catalase of A niger has a much greater pH range and temperature stability than beer liver catalase It retains 65 % of its activity in a pH range of 3 0 to 9.0 and can withstand heating at 65

°C for several minutes (52). The commercial unit for catalase activity is the Baker unit (132).

2.7 POLY-β-HYDROXYBUTYRIC ACID (PHB)

As a survival mechanism, most living organisms produce an energy storage material when carbon and energy are freely available The chief energy storage materials are lipids, polysaccharides and polyphosphates. Generally these materials are in polymeric form, contain large amounts of energy per unit volume and contribute very little to intracellular osmotic pressure.

Poly-β-hydroxybutyric acid is produced only by microorganisms. Unlike other energy storage compounds there is no direct utilization of ATP involved in PHB generation. Procaryotic genera with members capable of PHB accumulation include Actinomyces, Bacillus, Beijerinckia, Chromatium, Derxia, Hyphomicrobium, Lampropaedia, Micrococcus, Moroxella, Nocardia, Photomicrobium, Pseudomonas, Rhizobium, Rhodopseudomonas, Rhodospirillum, Sphaerotilus, Spirillum, Streptomyces, Vibrio, and Zooglea (34,145) The polyester structure of PHB is given in Figure 2.3 while its chemical and physical properties are listed in Table 2.1.

This short review will concentrate on PHB accumulation by Alcaligenes eutrophus and other bacteria that neither fix nitrogen nor have photosynthetic capabilities. It should be noted that until recently A. eutrophus was known as

Figure 2.3. The structure of poly-β-hydroxybutyric acid.

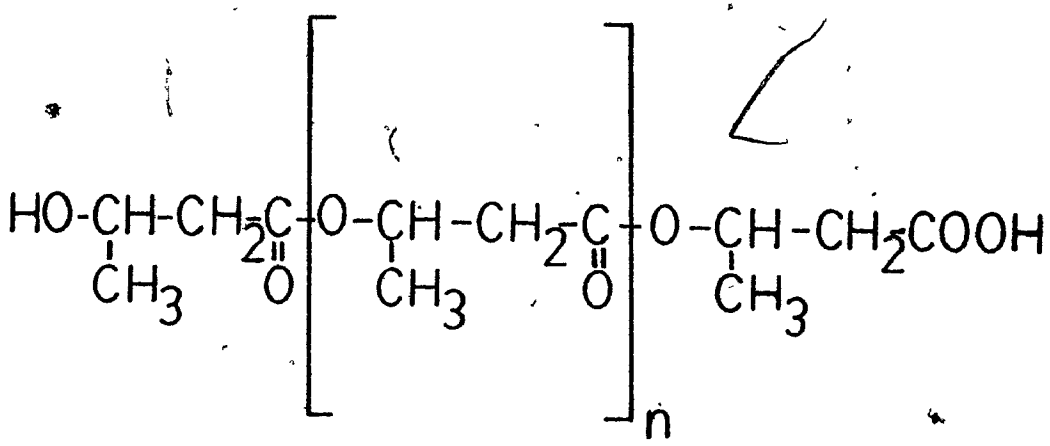


Table 2.1 Chemical and Physical Properties of PHB (34)

EMPIRICAL FORMULA	$(C_4H_6O_2)_n$
MELTING POINT	Range 160-172 °C
SPECIFIC GRAVITY	1.23-1.25
INTRINSIC VISCOSITY	0.2 to 1.5 % (w/v) chloroform solutions measured in an Ubbelhode viscometer. Hypochlorite isolation 0.04-1.05 dl g-1 Neutral solvent extraction 2.60-11.45 dl g-1
MOLECULAR WEIGHT	Calculated from intrinsic viscosity or by the sedimentation method of Archibald. Hypochlorite isolation 1-22 kg mol-1 Neutral solvent extraction 59-256 kg mol-1 Thioglycollate-chloroform 140-400 kg mol-1
SOLUBLE IN	chloroform, trichloroethylene, dichloroacetate, triflouroethanol, dimethylformamide, triolein, ethlyl acetoacetate, glacial acetic acid, NaOH (1 M), hyamine hydroxide (1 M), phenol (1 M aqueous).
PARTIALLY SOLUBLE IN	dioxane, octanol, toluene, pyridine.
INSOLUBLE IN	methanol, ethanol, acetone, ether, hexane, water, dilute mineral acids, alkaline hypochlorite, ethyl acetate, carbon tetrachloride.

Hydrogenomonas eutropha. It is capable of both hydrogen and carbon dioxide fixation but grows well heterotrophically.

Since it grows well autotrophically on a mixture of hydrogen, carbon dioxide and oxygen in an inorganic medium, A. eutrophus has been considered as an inexpensive source of protein. Its potential for use as a food in space travel has been investigated (73). One of its major liablities as a food is its propensity for the production of PHB which causes indigestion in mammals (130). While the nitrogen-fixing bacterium Azotobacter beijerinckia has been shown to accumulate up to 74 % of its dry weight as PHB (149), A. eutrophus has been reported to accumulate up to 86 % PHB growing autotrophically (127). Attempts have been made to select for strains of A. eutrophus deficient in the ability to produce PHB in order to use them for single cell protein production (129).

It was noticed that PHB has many properties in common with polypropylene (Table 2.2). It thus has the distinction of being the only biologically produced thermoplastic. As a plastic, PHB takes glass fiber filling very well (82). Since it is biologically produced, it has the advantage of being optically pure. Partially for this reason it has piezoelectric properties as do a small number of other polymers such as polyvinylidene fluoride (PVDF) (70). PHB is also biodegradable and it is possible to make biodegradable copolymerlizates with other substances which

TABLE 2.2 A Comparison of the Physical Properties of PHB with Those of Polypropylene (82)

PROPERTY	PHB	POLYPROPYLENE
CRYSTALLINE MELTING POINT (°C)	175	176
CRYSTALLINITY (%)	80	70
MOLECULAR WEIGHT ($g\ mole^{-1}$)	500,000	200,000
GLASS TRANSITION TEMPERATURE (°C)	15	- 10
DENSITY ($g\ ml^{-1}$)	1.250	0.905
FLEXURAL MODULUS (GPa)	4.0	1.7
TENSILE STRENGTH (MPa)	40	38
EXTENSION TO BREAK (%)	6	400
UV RESISTANCE	GOOD	POOR
SOLVENT RESISTANCE	POOR	GOOD

are not in themselves biodegradable (137). PHB is also immunologically compatible with human tissue and its film has gas barrier properties similar to the best coated films (133). As of April, 1981 Imperial Chemical Industries (ICI) was producing PHB on a pilot plant scale of 10 kg of per week. Although glucose was being used as the carbon and energy source, ICI was investigating the possibility of using CO_2 and H_2 from coal (82).

One of the organisms most studied with regards to PHB accumulation is Bacillus megaterium. Recently it was discovered that the B. megaterium storage polymer is actually a mixed polymer of β-hydroxy acids (46). Using capillary column chromatography, ten poly-β-hydroxyalkanoates (PHA's) other than PHB where found in B. megaterium and in samples of marine sediments. One sediment examined had PHA containing only 30 % β-hydroxybutyric acid while B. megaterium PHA was 95 % β-hydroxybutyrate (46). Candida rugosa and mutants thereof have been used to produce β-hydroxypropionic acid from propionic acid and β-hydroxyisobutyric acid from isobutyric acid whether or not they could use these substrates as carbon and energy source for growth (62,63). In the latter case an alternate carbon and energy source was supplied. Thus depending on the organism and the substrate PHB may not be the only PHA produced.

Microbial PHB is mostly found in granular form. The PHB granules of B. megaterium are spherical and average 0.5 μm in diameter (41). PHB can also be found in the inner membranes of bacteria where an increase in its level has been correlated with an increase in susceptibility to DNA transformation (.ie competance) (120). PHB can be produced synthetically. Triethylaluminum with water (1:1) as a co-catalyst polymerizes d,l-β-butyrolactone into a crystalline polymer with properties virtually identical to microbial PHB with the exception of optical purity (1,136).

Due to their refractivity, PHB granules can be seen with either phase contrast or dark microscopy. They stain with Sudan Black when in vivo but not when isolated. Sudan Black probably stains only the lipid membrane that surrounds the granules in vivo. This membrane is probably removed during isolation procedures. Intracellular PHB cannot be attacked by PHB depolymerases until they are subjected to treatment such as alkali extraction which removes a proteinaceous depolymerase inhibitor as well as PHB synthetase (34). It is believed that when the physiological conditions are correct for PHB degradation to be initiated, an activator is produced that neutralizes the depolymerase inhibitor. The activator is probably a Ca^{++} requiring proteolytic enzyme. Its action is mimicked by trypsin (145).

Many procaryotes can produce extracellular PHB depolymerases. These may not be specific for PHB and often release dimers and trimers as well as monomers of D(-)-3-hydroxybutyric acid. All pseudomonads able to use PHB secrete PHB depolymerases constitutively. Since PHB is not water soluble it is assumed that the soluble products released by constitutive enzymes induce additional extracellular PHB depolymerase production (35).

There are many methods for the extraction and purification of PHB. Centrifugal methods following lysis by sonication have been described (156). Generally, however, cells are digested in an alkaline hypochlorite solution leaving PHB granules (153). Imperial Chemical Industries have developed an elaborate process involving proteolytic enzyme or surfactant solubilization of cells, heat denaturing of nucleic acids, boiling under reflux for 10 minutes, incubation at 55 °C for 1 h, cooling, and centrifugation (67). A gas chromatographic method for the determination of intracellular PHB concentration has been developed. It is highly accurate and sensitive to 10^{-5} g l^{-1} (14). This supercedes the more cumbersome and less accurate crotonic acid colourimetric method.

A. eutrophus has been grown autotrophically to a dry biomass concentration of 25 g l^{-1} in 25 h with a doubling time of 1.5 to 2.0 hours (118). A 1.5 to 2.0 hour doubling time is the maximum autotrophic growth rate determined by

both batch and continuous techniques (11). Using radioactive tracers it was determined that 2 % of the total carbon assimilated by A. eutrophus during autotrophic growth appeared in the supernatant during exponential phase and 5 % in stationary phase. The carbon was mostly in the form of amino acids (15). The trace elements required for autotrophic growth include cobalt, chromium, and copper. There may also be a requirement for zinc. Foaming (suppressable by the addition of castor oil) occurs in iron deficient media. It is associated with the production of a yellow-green pigment (119) which is probably an iron chelator (61). Iron deficient cultures do not produce PHB but PHB synthesis in autotrophically grown cells can be initiated by nitrogen, phosphate, magnesium, or sulphur limitation (119).

The asporogenous mutant B. megaterium KM in nitrogen, sulphur, potassium and carbon-limited chemostat culture accumulates a maximum amount of PHB at a dilution rate of 0.4 h^{-1}. Interestingly, this microorganism stores up to 12 % PHB under carbon and energy limited conditions and accumulates glycogen as an alternate storage polymer (165).

PHB production by autotrophically growing A. eutrophus is fully growth associated even when there is no growth-limiting nutrient but under these conditions the PHB content is less than 1 % (127). Shortly after ammonium-limitation begins, in autotrophic batch culture, the specific PHB

production rate (q_p) reaches its maximum and then slowly decreases to zero during the remainder of the fermentation. The transition period is less than one generation time (143). After the onset of nitrogen limitation the protein content and number of cells of A. eutrophus H16 remain constant (144). The highest maximum PHB content of autotrophically grown A. eutrophus H16 was found to be 78 % (144).

Since PHB is a storage polymer, factors influencing endogenous metabolism (suspending medium, pH, temperature etc.) probably also influence PHB accumulation (33). Carbon sources that are metabolically related to acetyl-Co A favour PHB production (146). The Alcaligenes and Pseudomonas genera contain many members which can both accumulate large amounts of PHB and have the ability to grow on a very wide variety of carbon sources. Some A. eutrophus (75) strains have been shown to use such recalcitrant carbon sources as phenol and p-cresol as sole sources of carbon and energy if supplied in low concentrations (ie 2.5 mM). Alcaligenes paradoxus (47) and some Pseudomonas species (112) posess plasmids enabling them to degrade 2,4-dichlorophenoxyacetic acid (2,4-D). A continuous enrichment culture using linear alkyl benzenesulphonates as the limiting carbon source resulted in a stable mixed culture of many Pseudomonas and Alcaligenes species. The Alcaligenes species predominated at low dilution rates while the Pseudomonas species flourished at higher dilution rates. None of the

microorganisms could grow on linear alkyl benzenesulphonates in axenic culture (74).

Differential petri plate methods for the selection of PHB-producing strains of bacteria, as well as enrichment methods using the ^{32}P phosphate inactivation technique and sucrose density gradient centrifugation are available (129). A flow cytometry cell-sorting method has also been used to enrich for wild type PHB-producing cells from a predominantly PHB-negative population (144).

As early as the late 1950's, W.R. Grace and Co. of the United States was producing small quantities of PHB for the evaluation of its economic potential as a themoplastic. Inadequate production and purification processes led to its abandonment (66). Imperial Chemical Industries (ICI) began working on the development of PHB manufacturing processes for the bulk and specialty plastics markets in 1976. As of 1984, ICI was interested in PHB production by Azotobacter sp grown continuously on glucose under oxygen limitation but were experiencing difficulties with unwanted extracellular carbohydrate synthesis and strain instability. Researchers at ICI found that when grown on glucose, nitrogen-limited A. eutrophus contained less PHB per cell as the dilution rate was increased but that without a continuous process, production costs would be too high (133). Therefore a two stage process was being considered in which the first stage would be for high protein biomass

production and the second stage would be for PHB accumulation. ICI also has a methylotroph which produces PHB. Its PHB has a molecular weight of 50,000 and has different properties from the PHB of either Alcaligenes or Azotobacter.

CHAPTER 3

MATERIALS AND METHODS

3.1 Nonvolatile Residue (NVR)

NVR was supplied by the Du Pont Canada Inc Research Center, Kingston, Ontario The same batch was used for all work in this thesis. Its composition is given in Table 1.1.

3.2 Peat and Peat Run-off Water

Peat and peat run-off water were obtained from a bog in Barrington, Nova Scotia. The same batch was used for all work in this thesis.

3.3 Microorganisms

The organisms used in this thesis are listed in Table 3.1. Information about A. eutrophus , P. cepacia ATCC 17697, and X. campestris, the most important organisms in this research are listed in Tables 3.2, 3.3, and 3.4 respectively.

3.4 Media

The mineral salts medium (MSM) consisted of 0.4 % w/v $(NH_4)_2SO_4$, 0.2 % KH_2PO_4, 0.1 % $Na_2HPO_4 \cdot 7H_2O$, 0.02 % $MgSO_4 \cdot 7H_2O$, 20 mg l^{-1} $CaCl_2$, 1.6 mg l^{-1} $(CH_3COO)_2Zn \cdot 2H_2O$, 0.3 mg l^{-1} $FeSO_4 \cdot 7H_2O$, 0.6 mg l^{-1} $(NH_4)_8Mo_7O_{24} \cdot 4H_2O$, 0.6 mg l^{-1} H_3BO_3, and 0.01 % yeast extract. Glucose medium (GM) contained 2 % glucose in MSM. Mineral salts citrate medium (MSC) contained 2 % sucrose and 0 2 % citric acid in

Table 3.1 Microorganisms Used in this Study

Alcaligenes eutrophus ATCC 17697[1]
Alcaligenes faecalis subsp. myxogenes ATCC 14434[1]
Arthrobacter viscosus ATCC 19584[1]
Aurobasidium pullulans Pp KM 149[5]
Azotobacter indicus subsp. myxogenes ATCC 21423[1]
Bacillus subtilis[5]
Corynebacterium equi subsp. mucilaginosus ATCC 21521[1]
Corynebacterium fascians ICPB CF15[2]
Corynebacterium insidiosum ICPB CIBA[2]
Corynebacterium lepus[5]
Escherichia coli[4]
Isolate 1, and Isolates P_2, P_4, and B[7]
Isolates SS_1 and SS_2 (both identified as P. aeruginosa)[8]
Isolate C_{12}[8]
Lactobacillus brevis ATCC 14434[1]
Leuconostoc mesenteroides ATCC 10830[1]
Lysobacter gummosus ATCC 29489[1]
Mycobacterium rhodochrous ATCC 19067[1]
Pseudomonas acidovorans ATCC 17476[1]
Pseudomonas aeruginosa NRC 2786[3]
Pseudomonas cepacia ATCC 17759[1]
Rhodococcus rhodochrous NRC 43002[3]
Saccharomyces cerevesiae[4]
Sclerotium rolfsii ATCC 15202[1]
Staphylococcus epidermidis[6]
Torulopsis bombicola ATCC 22214[1]
Torulopsis petrophilum ATCC 20225[1]
Xanthomonas campestris ATCC 13951[1]
Xanthomonas campestris NRC 2146[1]

Sources

1 American Type Culture Collection(ATCC).
2 International Collection of Phytopathogenic Bacteria(ICPB).
3 Division of Biological Sciences, (Mr. R. Latta), National Research Council of Canada.
4 Department of Microbiology, McGill University.
5 University of Western Ontario's Biochemical Engineering Culture Collection.
6 Microbiology Dept., Royal Victoria Hospital, Montreal.
7 Isolated from continuous enrichment culture using NVR as the sole source of carbon.
8 Isolated from continuous enrichment culture using caproic acid as the sole source of carbon.

TABLE 3.2 Characteristics of Alcaligenes eutrophus (86).

Morphology	straight Gram negative rods 0.7 μm by 1.2-1.6μm.
Motility	motile by 1 to 4 pritrichous flagella.
Pigmentation	none.
Pathogenicity	none known.
Growth temperature	optimum is about 30 °C.
Metabolism	facultatively chemolithotrophic in an atmosphere containing H_2, O_2 and CO_2; strictly aerobic.
Growth	on glucose (mutant strains; freshly isolated strains cannot use glucose), fructose, d-gluconate, acetate, adipate, pimelate, sebacate, suberate, meso-tartrate, itconate, 2-ketogluconate, mucate, propionate, butyrate, isobutyate, caproate, succinate, fumarate, d,l-lactate, d,l-β-hydroxybutyrate, glutarate azelate, glycolate and most strains use d-malate, l-malate, pyruvate, levulinate, α-ketoglutarate, aconitate, citraconate, mesaconate, citrate, m-hydroxybenzoate, p-hydroxybenzoate and phenylacetate
No growth	on all sugars other than fructose and mutant growth on glucose, glycerol, valerate, isovalerate, heptanoate, caprylate, pelargonate, caprate, malonate, oxalate, maleate, d,l-glycerate, d-tartrate, l-tartrate, o-hydroxybenzoate, d-mandelate, l-mandelate, pthalate, quinate, glycine, l-serine, threonine, l-lysine, gamma-aminovalerate, kynurenate, anthranilate, ethanolamine, benzylamine, putrecine, spermine, histamine, tryptamine, butylamine, betaine, pentylamine, sarcosine, creatine, acetamide, m-aminobenzoate and p-aminobenzoate.

Table 3.3 Characteristics of Pseudomonas cepacia ATCC 17759 (86,147).

Morphology	Gram-negative rod 0.8-1.0 μm by 1.6-3.2 μm.
Motility	motile by multiple polar flagella.
Pigmentation	produces a yellow diffusible pigment.
Pathogenicity	opportunistic human pathogen, occassionally found associated with various infections of nosocomial origin.
Growth temperature	optimum is 30 to 35 °C; can grow at 41 °C.
Metabolism	strictly a heterotrophic aerobe.
Growth	on d-ribose, d-arabinose, l-arabinose, d-fucose, d-glucose, d-mannose, d-galactose, d-fructose, sucrose, trehalose, d-xylose, l-rhamnose, cellobiose, salicin, gluconate, 2-ketogluconate, saccharate, mucate, acetate, butyrate, isobutyrate, valerate, isovalerate, caproate, heptanoate, caprylate, pelargonate, caprate, malonate, succinate, fumarate, glutarate, adipate, pimelate, suberate, azelate, sebacate, d-malate, l-malate, meso-tartrate, d,l-β-hydroxybutyrate, d,l-lactate, d,l-glcyerate, hydroxymethylgluterate, citrate, α-ketoglutarate, pyruvate, aconitate, laevulinate, citraconate, mannitol, sorbitol, meso-inositol, adonitol, glycerol, 2,3-butylene glycol, benzoate, phenylacetate, o-hydroxybenzoate, m-hydroxybenzoate, p-hydroxybenzoate, quinate, delta-aminovalerate, gamma-aminobutyrate, many aliphatic and ring structure containing amino acids, benzylamine, putrescine, spermine, sarcosine, hippurate, acetamide, tryptamine, butylamine, alpha-amylamine, betaine, sarcosine, ethanol, n-propanol, n-butanol, l-mandelate, benzoylformate, and testosterone.
No growth	on maltose, isobutanol, phthalate, phenol, l-valine, l-leucine, nicotinate, trigonelline, n-dodecane, n-hexadecane, d-arabinose, d-(-)-tartrate, erythritol, itconate, geraniol, glycine propyleneglycol, glycine, creatine and pantothenate.

TABLE 3.4 Characteristics of Xanthomonas campestris (86).

Morphology	Gram-negative rods 0.4 by 0.7 μm, usually found singly.
Motility	motile by polar flagella.
Pigmentation	produce a cell-associated yellow xanthomonadin pigment (a brominated polyene).
Pathogenicity	cause a disease in many plant species especially Brassica sp.
Growth temperature	25 to 30 °C.
Metabolism	strictly a heterotrophic aerobe.
Acid	from glucose, sucrose, arabinose, mannose, galactose, trehalose, cellobiose, fructose, and most strains from lactose, maltose, xylose, ribose, melobiose, raffinose, melozitose, dextrin, glycogen and glycerol.
No acid	from adonitol, mannitol, sorbitol, dulcitol, rhamnose, salicin, meso-inositol, inulin and alpha-methylglucoside.
Growth	on acetate, citrate, malate, propionate, succinate and lactate.

MSM Nutrient broth medium (NB) was 0.8 % nutrient broth and yeast malt medium (YM) was 2.1 % yeast malt broth.

3.5 Shake-flask Experiments

All shake-flask experiments used unbaffled Erlenmyer flasks, shaken at approximately 180 RPM at a specified temperature. The pH was 6.0 unless otherwise stated. For shake-flask experiments incorporating different concentrations of NVR or NVR components, the NVR or appropriate component was added on a weight basis. The pH was adjusted with 20 % NaOH since NVR and its components are either acidic or do not effect pH. Flasks were sterilized by autoclaving at 121 °C and 15 psi for 20 minutes. Gas chromatographic analysis showed no detectable change in the concentration of volatile carboxylic acids after autoclaving.

In peat run-off water experiments, peat run-off water was added by volume. The appropriate media were made up in both peat run-off water and distilled water. The two media were then mixed to give the correct peat run-off water concentrations The pH was adjusted and the flasks were autoclaved as above.

3 6 Determination of the Growth Effect, I_1 and I_2 Values

The growth effect, I_1 and I_2 values were determined in order to summarize the effeets of NVR on the growth of microorganisms in shake-flasks Different amounts of NVR were added to 500 ml shake-flasks containing 50 ml of an

appropriate medium. A 5 % v/v inoculum was added and the flasks were shaken at a specified temperature (usually 26 or 37 °C). Flasks were sampled after a period of time sufficient to allow measurable growth but before the maximum biomass concentration was attained. The I_1 value was the lowest NVR concentration at which less biomass was produced than the control. The I_2 value was the lowest NVR concentration at which no growth occurred. NVR was said to have a positive growth effect if some flasks containing NVR had greater than 5 % more biomass than the control lacking NVR. A negative growth effect was assumed if at 50 % of the I_2 concentration there was greater than 5 % less biomass than the control.

3.7 Batch Culture of Microorganisms

All batch fermentations utilized Multigen F-2000 2 l fermentors (New Brunswick Scientific, Edison, New Jersey). The initial working volume was approximately 1.7 l. The medium was sterilized in the fermentor vessel in an autoclave at 121 °C and 15 psi for 30 minutes. Caramelization of sugars and precipitation of salts was prevented by the addition of 0.7 % concentrated HCl before sterilization. Agitation was kept at approximately 660 RPM. Sterile air was introduced below the impellors at approximately 1.7 l min^{-1}. In non-viscous fermentation broth the maximum oxygen transfer rate was 22 mM l^{-1} min^{-1} at 660 RPM, an air flow rate of 1.1 l min^{-1}, a temperature of 30 °C and a liquid volume of 1.3 l. This was measured

biologically in chemostat culture as shown in Appendix A (Figure A.1). Foaming was prevented by the addition of 500 ppm polypropylene glycol 2000 before autoclaving. Forty-five ml samples were taken from the bulk medium after the first 5 ml had been discarded (ie. what remained in the tubing after the previous sample).

3.8 Batch Enrichment Culture

Batch enrichment culture was performed in 500 ml shake-flasks containing unsterilized nutrient broth and a specified amount of NVR. Various inocula (as reported in the results section) were added in order to supply the culture with a diverse selection of seed microorganisms. After several days growth at 26 °C, 5 % v/v was transferred to a new flask containing the same medium. Several transfers were performed. Growth was monitored by measuring pH and by plating on YM agar.

3.9 Continuous Culture

The same fermentors used in batch culture were set up as chemostats. The same sterilization and foam prevention procedures were used as in batch culture except that the medium was sterilized for 45 minutes. Volume in the single-stage chemostat was kept at approximately 1.3 l by means of a Y-shaped draw-off tube (85) attatched to silicone tubing under vacuum supplied by a peristaltic pump. The Y-shaped draw-off allowed bulk medium rather than foam to be withdrawn from the fermentor. The same method was used to

transfer fermentation broth to the second stage of the 2-stage chemostat and to maintain the liquid level in each stage. Mineral salts medium was added via a peristaltic pump from a 10 l polypropylene resevoir. It was mixed with a heavy duty magnetic stirrer (Bellco Glass, Inc., Vineland, New Jersey). The flow rate of medium was determined by volumetric measurement of the resevoir contents after it was exchanged for another. Some carbon sources such as NVR and many of its components were not water soluble. Since these were liquids, they were added to the fermentor via a highly accurate peristaltic pump (UltraMasterflex, Cole-Parmer Ltd., Chicago, Ill.) with solvent resistant small bore tubing (Viton fluoroelastamer, 1/16 inch inside diameter). NVR was kept mixed with a magnetic stirrer and a teflon coated stir bar. The flow rate was measured by frequent weighing of the resevoir with a triple beam balance.

3.10 Continuous Enrichment Culture

As an inoculum, an aqueous soil extract, some refinery waste and sludge from the activated sludge tank of a nylon manufacturing plant (Du Pont Canada, Maitland, Ontario) was diluted in 0.05 M K_2PO_4 buffer adjusted to pH 7.0 with KOH. Several milliliters of this were added as an inoculum to 1.35 l of nutrient broth. After 24 h, dilute carbon source and mineral salts medium as well as more inoculum were added slowly but continuously to the reactor. Over a period of days the dilution rate and the carbon source concentration were gradually increased and eventually no more inoculum was

added. The fermentation conditions were then kept constant for several days. This resulted in a stable continuous culture.

3.11 Screening Microorganisms for their Ability to Use Major NVR Component as their Sole Source of Carbon and Energy

The cultures were maintained on agar (Difco, Detroit, Michigan) containing 0.4 % nutrient broth (Difco) and 0.55 % yeast malt extract. They were transferred to agar plates containing mineral salts medium (but lacking yeast extract) and 0.05 % w/v of the carbon source to be tested. After 72 h at 24 °C the cultures were again transferred to agar plates containing mineral salts medium (lacking yeast extract) and with 0.05 % of the desired carbon source. The plates were observed every 24 h for 4 days and were evaluated as follows: no detectable difference from the control (-); noticeable growth (+); good growth (++).

3.12 Accuracy of Results

Batch fermentations were repeated at least twice except for the X. campestris fermentations at 0.5 % and 1.0 % glucose concentrations which formed part of a series. As many samples as feasible were analyzed in all experiments in order to obtain the greatest possible precision and accuracy. Usually at least 10 flasks were used for each shake-flask experiment where the concentration of NVR or one of its components was varied. Approximately 15 samples were taken during each batch fermentation. Biomass, total

protein and PHB were always analyzed at least in duplicate. Internal standards were used in all quantitative gas chromatographic analyses.

3.13 Biomass Dry Weight Determination

Ten ml of culture broth was centrifuged for 10 minutes at 24,000 g (15 minutes at X 36,000 g for samples containing xanthan) at 4 °C. The resulting pellet was resuspended in 10 ml of distilled water and recentrifuged. The pellet was then washed out of the centrifuge tube into a preweighed aluminum pan and dried to a constant mass at 105 °C.

3.14 Cellular Protein Determination

Ten ml of culture broth was centrifuged, washed and recentrifuged as above. The resulting pellet was analyzed for protein content by the Biuret reaction (150). The standard curve used to determine the region of linear response is given in Appendix A (Figure A.2). Two 1.0 % w/v bovine serum albumin (Sigma Chemical Co., St.Louis, Mo.) external standards were used for each batch of samples analyzed.

3.15 Gas Analysis

Fermentor exhaust gas was passed through a water-cooled condenser and two drying columns. The dried gas was passed through the oxygen analyzer at a controlled rate of 100 ml min^{-1}. The exhaust from the oxygen analyzer was then passed through the carbon dioxide analyzer at the same flow rate. Oxygen was measured paramagnetically with a Taylor Servomex

oxygen analyzer (Crowborough, Sussex, England). Carbon dioxide was measured using a Lira infrared analyzer model 303 (Mine Safety Appliances, Pittsburgh, USA, 15208). Ambient air was used to calibrate the oxygen analyzer after passage through the drying columns and flow rate regulation to 100 ml min^{-1}. This was done before and after every fermentor exhaust gas sampling. The following equations were used to determine the oxygen uptake rates.

$$Q_m = Q_g \times [(1 \text{ mole}/22.4 \text{ l}) \times (273 \text{ °K}/T \text{ °K})] \quad (1)$$

where Q_g = air flow rate ($l\ l^{-1}\ h^{-1}$)

and T °K = fermentor temperature (°K).

$$\text{Oxygen Uptake Rate (OUR)} = O_2 \times Q_m \times 10 \quad (2)$$

where O_2 = Ambient oxygen concentration (% v/v) - Exhaust gas oxygen concentration (% v/v).

$$Q_{O_2} = OUR/X = \text{Specific oxygen uptake rate} \quad (3)$$

where X = Biomass or total protein concentration ($g\ l^{-1}$) as specified.

The rate of CO_2 production was not determined. The relative CO_2 uptake rate was monitored in order to determine

whether the respiratory quotient (RQ) was constant at a constant oxygen uptake rate. Both O_2 and CO_2 were continuously monitored on a chart recorder.

3.16 Analysis of NVR Components

NVR components were quantified using a Hewlett-Packard 5890 gas chromatograph with a Hewlett-Packard 3392 integrator. The helium flow rate through the DB-wax-60N 0.25 μm capillary column was 5 ml min^{-1}. The flame ionization detector was supplied with 400 ml min^{-1} air, 50 ml min^{-1} hydrogen and 30 ml min^{-1} of auxillary helium. An injection split ratio of approximately 170:1 was used. The internal standard and the temperature-time profile was varied according to the component to be analyzed. 6-Hexanolactone and hexanoic acid were esterified by dilution with the appropriate internal standard in 24 % tetramethyl ammonium hydroxide to yield volatile methyl esters. The injection port was maintained at 350 °C to insure complete conversion of tetramethyl ammonium salts to methyl esters. Pentanoic acid was used as the internal standard for hexanoic acid and butanoic acid determinations. Hexanoic acid was the internal standard for pentanoic acid and 6-hexanolactone determinations.

3.17 Glucose Analysis

Glucose concentration was measured by the glucose oxidase method (23). The standard curve used to determine the region of linear response is given in Appendix A (Figure A.3). A glucose solution was used as an external standard for each batch of samples analyzed.

3.18 Fructose Analysis

Fructose concentration was determined by high pressure liquid chromatography using a refractive index detector. An Animex HPX-87C column was used (Bio-Rad Laboratories Ltd., 3140 Universal Drive, Mississauga, Ontario). The column temperature was isothermal at 80 °C. The carrier liquid was HPLC water (Waters Scientific, 3688 Nashua Drive, Mississauga, Ontario) at a flow rate of 0.5 ml min^{-1}. The standard curve shown in Appendix A (Figure A.4) demonstrates linearity up to at least 40 g l^{-1} fructose. External standards (aqueous fructose solutions) were run after every fourth sample or more frequently if the baseline signal was varying.

3.19 Total Carbohydrate Analysis

Samples were diluted to contain between 10 and 80 µg ml^{-1} of carbohydrates. One milliliter of sample was added to 1 ml of a 5 % aqueous phenol solution and five ml of concentrated H_2SO_4. The standard curve used to determine the linear response zone is given in Appendix A (Figure A.5).

External standards (aqueous glucose) were run with each batch of samples analyzed.

3.20 Polysaccharide Analysis

Polysaccharides were analyzed gravimetrically after precipitation with methanol or ethanol and washing in distilled water. For example, during xanthan analysis, 5 g of supernatant obtained from the biomass determination was vortexed vigorously with 15 ml of methanol and centrifuged for 15 minutes at 36,000 g. The pellet was washed in 5 ml of distilled water and centrifuged again after precipitation with 15 ml of methanol. The resulting pellet was washed with distilled water into a preweighed aluminum pan which was dried to a constant weight at 105 °C.

For shake-flask cultures where the vicosity was relatively low, a 40 ml sample was placed into a 50 ml beaker and analyzed using a Brookfield RVT viscometer (spindle 4 at 100 RPM). For samples obtained from a fermentor a Brookfield LVT viscometer (spindle 3 at 30 RPM) was used. The small spindle allowed for more accurate viscosity measurements (ie. no wall effects).

3.21 Catalase

Catalase activity was measured by the titanium dioxide colourimetric method (159). Washed cells from a 10 ml sample were lyzed by sonication at 4 °C for 20 minutes. Lysis was monitored by following optical density at 410 nm

versus time. The change in optical density versus time of sonication is depicted in Appendix A (Figure A.6).

3.22 Biosurfactants

The presence of biosurfactants was monitored by measurement of whole broth surface tension with a Fisher Autotensiomat. The autotensiomat is a modified duNouy surface tensiometer equipped with a motorized sample stage with a sensitive strain gauge attatched to a platinum ring (51).

3.23 Poly-β-hydroxybutyric acid

Intracellular poly-β-hydroxybutyric acid was measured by the gas chromatographic method (14) using benzoic acid as the internal standard. Samples were prepared as follows. Ten ml of whole broth was centrifuged, washed with 10 ml of distilled water and recentrifuged. The cells were washed into screw-capped tubes with 2 ml of methanol containing 0.1 % benzoic acid as an internal standard and 3 % v/v concentrated H_2SO_4. The calibration standard was 2 ml of 0.1 % β-hydroxybutyric acid and 0.1 % benzoic acid in acidified (3 % v/v concentrated H_2SO_4) methanol. A calibration table was determined at the start of each batch of analyses This information was entered into the integrator for the automatic calculation of PHB concentrations. One microliter samples were injected. The standard curve used to determine the area of linear response is given in Appendix A (Figure A.7) along with a figure

showing the variation of benzoic acid and β-hydroxybutyric acid with time of heating in the sample preparation process (Figure A.8). The gas chromatograph, integrator, column type, gas flow rates and the split ratio were the same as for measurement of NVR and its components. The temperature profile was 90 °C for one minute, increasing at a rate of 8 °C per minute until 150 °C where the temperature was held constant for 5 minutes.

CHAPTER 4

RESULTS

4.1 Peat Run-off Water as a Fermentation Substrate for *X. campestris*

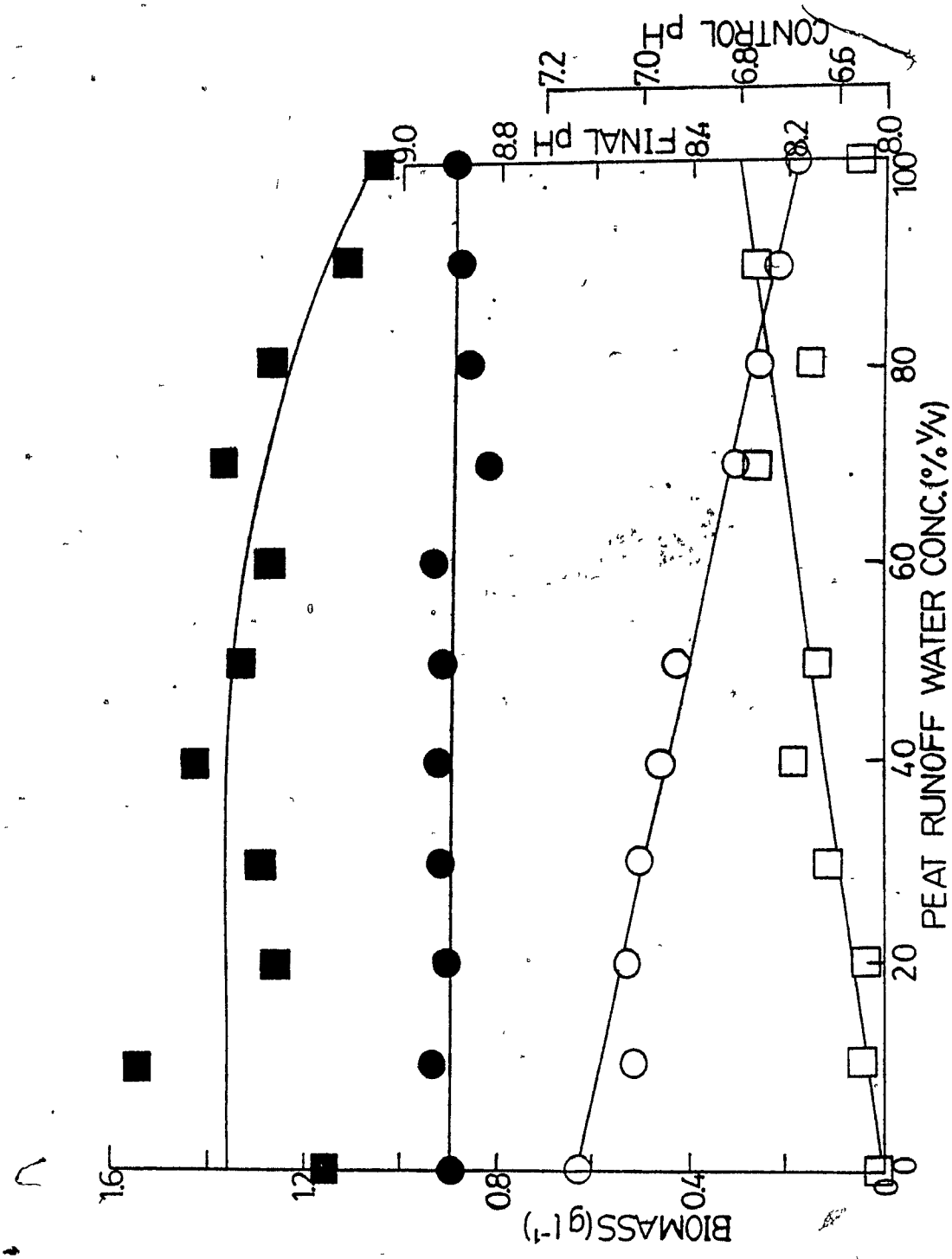
4.1.1 The Effect of Peat Run-off Water on Xanthan Gum Production and Growth of *X. campestris*

In order to determine the effect of peat run-off water on xanthan production, *X. campestris* NRC 2146 (ATCC 2146) was grown on 0.8 % nutrient broth made up with different concentrations of unfiltered peat run-off water. As shown in Figure 4.1, the control flask biomass data indicated that the solids content of unfiltered peat run-off water was approximately 0.35 g l^{-1}. Since the pH had been adjusted to 7.0 before autoclaving, the control flasks also showed that the autoclaving of peat run-off water resulted in a pH decrease.

After growth at 24 °C for 3 days, the pH in all flasks was approximately 8.9. This was probably an inhibitory level. Since flasks containing peat run-off water had a lower initial pH, one would expect these flasks to have produced more biomass but such was not the case. Even without subtracting the peat solids, the biomass concentration tended to decrease as the concentration of peat run-off water increased. No xanthan was produced and the final viscosity was identical to that of water.

Figure 4.1. *X. campestris* ATCC 2146 grown on nutrient broth medium with different concentrations of peat run-off water. The culture was grown at 26 °C for 72 h.

(■) Final biomass dry weight.
(□) Control biomass dry weight.
(●) Final pH.
(○) Control pH.


BIOMASS (g l⁻¹)
1.6
1.2
0.8
0.4
0
FINAL pH
9.0
8.8
8.4
8.2
8.0
CONTROL pH
7.2
7.0
6.8
6.6
0
20
40
60
80
100
PEAT RUNOFF WATER CONC.(% V/v)

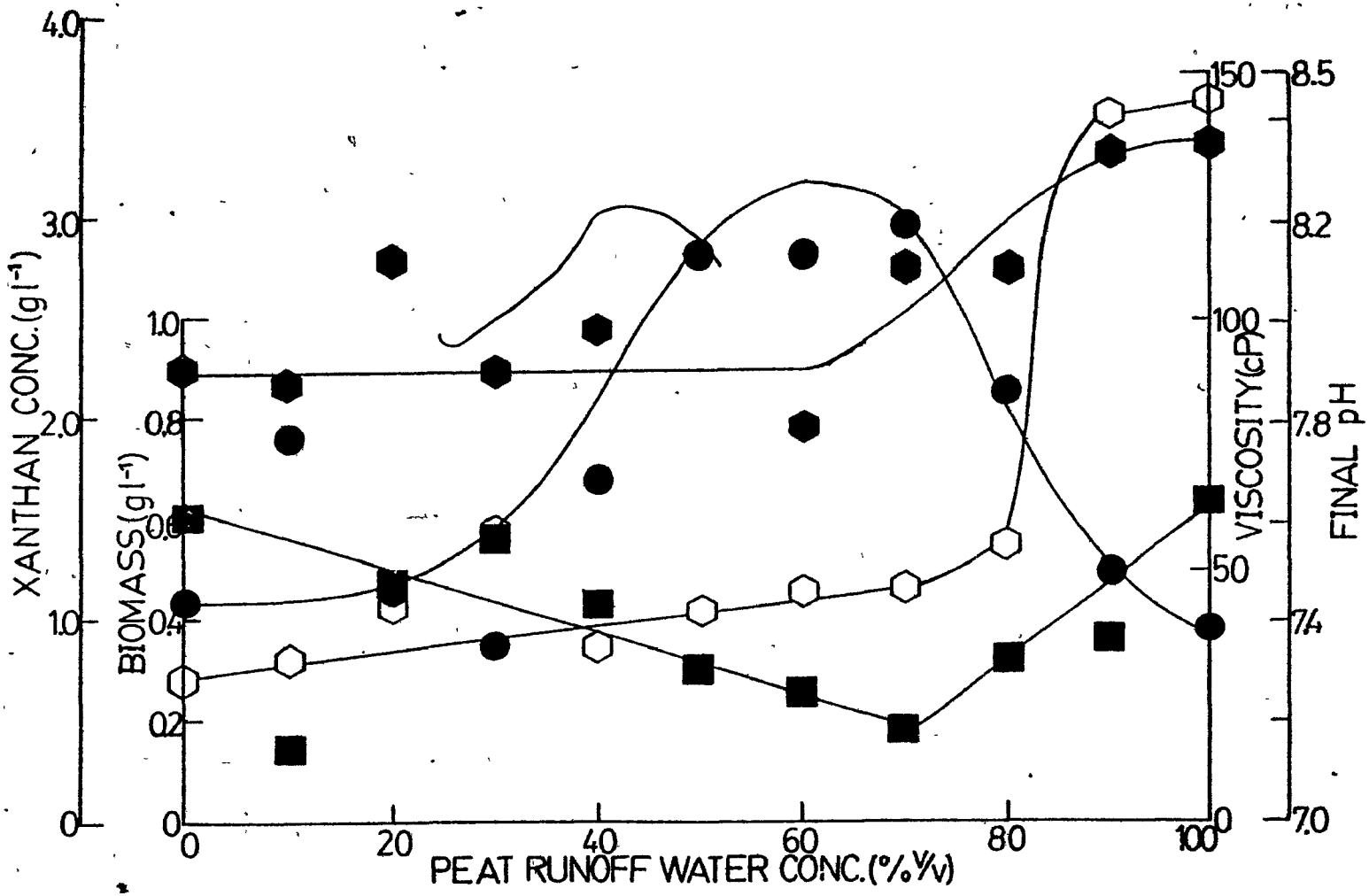
To facilitate more accurate biomass determinations the peat run-off water was filtered before use in all other experiments. Since no xanthan had been produced even in the control flask, it was decided to change both the medium and the strain of microorganism. Thus X. campestris ATCC 13951 (NRRL B-1459) was grown in shake-flasks of yeast malt medium made up with various concentrations of peat run-off water and distilled water. X.campestris ATCC 13951 is a production strain developed by the Northern Regional Research Laboratory, Peoria, Illinois. Yeast malt medium is excellent for high yield xanthan production.

In this experiment, a large increase in xanthan concentration and medium viscosity occurred when the peat run-off water concentration exceeded 60 % (Fig. 4.2). In the absence of peat run-off water, the biomass was 0.62 g l^{-1}. As the concentration of peat run-off water was increased to 70 %, this level of biomass decreased but increased again at higher concentrations of peat run-off water. The final pH reached a maximum at a 60 % peat run-off water concentration. These data indicated that either the xanthan yield or the rate of production was greater when peat run-off water was present.

In order to ascertain whether the presence of peat run-off water resulted in increased xanthan production by X. campestris ATCC 13951 and to aid in the determination of the cause of this effect, pH-controlled batch fermentations were

Figure 4.2. X. campestris ATCC 13951 grown on yeast malt medium with different concentrations of peat run-off water. The culture was grown at 26 °C for 60 h with an initial pH (before autoclaving) of 7.0.

(■) Biomass dry weight.
(●) Final pH.
(●) Xanthan concentration.
(○) Viscosity.


XANTHAN CONC.(g l⁻¹)
BIOMASS(g l⁻¹)
VISCOSITY(cP)
FINAL pH
PEAT RUNOFF WATER CONC.(%V/V)
0
20
40
60
80
100
4.0
3.0
2.0
1.0
0
1.0
0.8
0.6
0.4
0.2
0
150
100
50
0
8.5
8.2
7.8
7.4
7.0

performed using either distilled water or peat run-off water to make the medium. Mineral salts medium was used and glucose was the sole source of carbon and energy (except for that contained in peat run-off water). Table 4.1 shows that when peat run-off water was used in place of distilled water, more biomass but less xanthan was produced. The maximum viscosity/maximum xanthan value indicated that the gum that was produced was of similar viscosity. The maximum oxygen uptake rate was higher when peat run-off water was present. In all fermentations, glucose had been totally consumed well before monitoring of the fermentation had ceased. Therefore all yields were based on total glucose consumption. The fermentations were run until oxygen uptake had almost ceased, indicating exhaustion of the carbon source. Thus any usable carbon contained in the peat run-off water should have contributed to the yield values.

As shown in Figure 4.3 and Table 4.1, fermentations were run in which the initial glucose concentration was varied in mineral salts media made up with peat run-off water. In Figure 4.3 the data have been interpolated towards the zero point on the x-axis. This was done to assess the effects of using no glucose. If an actual fermentation had not incorporated glucose, the data values would have been too small to have been accurately determined. Figure 4.3 indicates that if the medium had not been supplemented with glucose, no biomass would have been produced, that the final medium viscosity would be close to

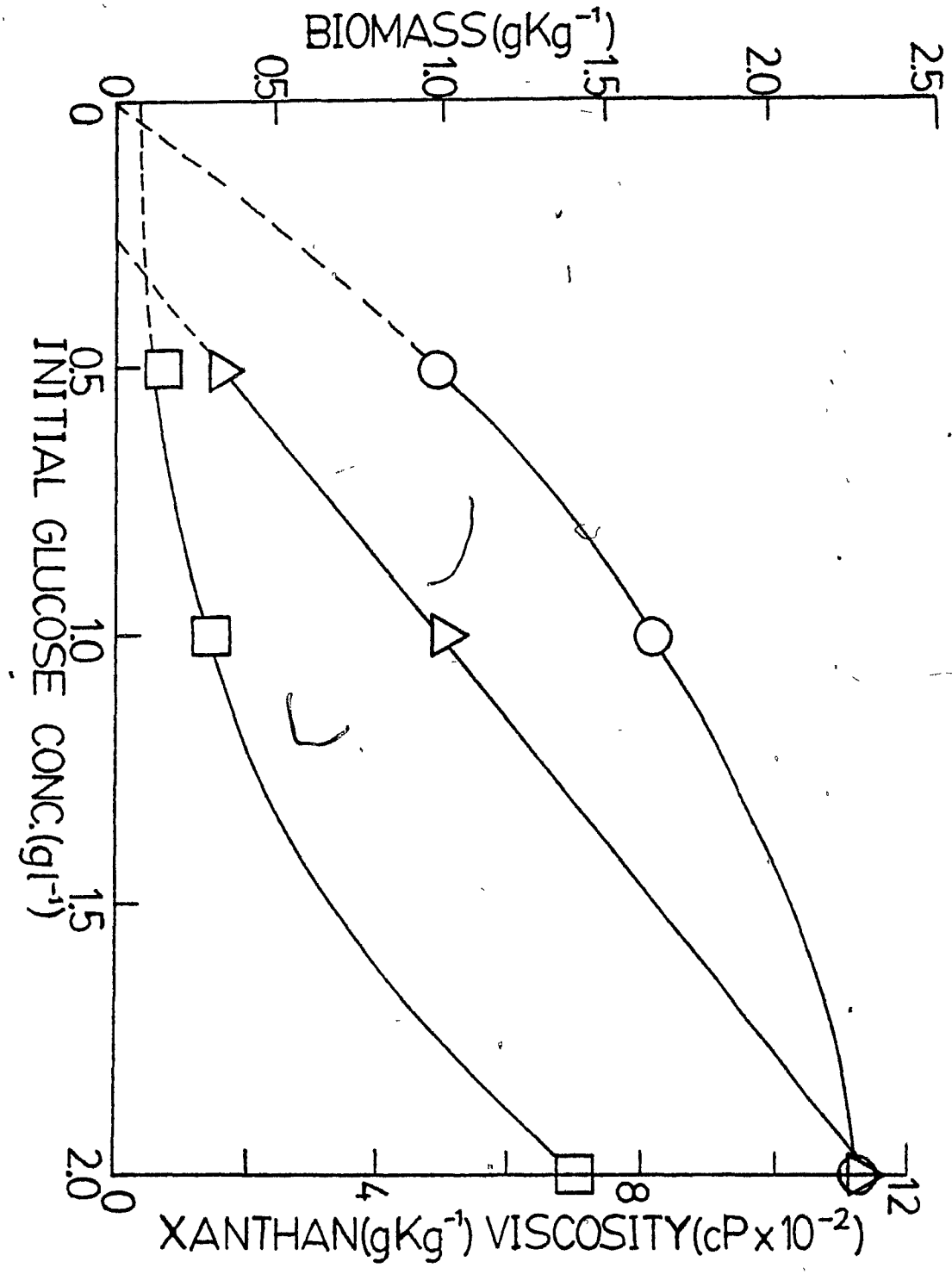
Table 4.1 The Effect of Peat Water on Xanthan Production in Fermentations using Mineral Salts Medium

Peat Water Present	Glucose Initial Concentration g kg^{-1}	Maximum Biomass g kg^{-1}	Maximum Xanthan g kg^{-1}	Y X/S W/W	Y P/S W/W	Y P/X W/W	Max. Visc. cP RV	Max. Visc./Max. Xanthan $cP/g^{-1}kg^{-1}$	Max. Oxy. Uptake Rate $mML^{-1}h^{-1}$
yes	5	0.98	1.6	0.20	0.318	0.162	76	48	5.4
yes	10	1.63	5.0	0.163	0.503	3.09	14	29	13.6
yes	20	2.24	11.3	0.112	0.565	5.04	75	67	11.1
yes	20	1.79	10.9	0.09	0.552	5.03	66	63	17.05
no	20	1.08	13.3	0.054	0.663	12.3	114	86	5.8
no	20	1.15	13.5	0.057	0.665	11.5	78	58	6.1

Figure 4.3. The effect of the increasing initial glucose concentration on xanthan production in MSM medium made up with peat run-off water.

(○) Maximum biomass dry weight.
(□) Maximum viscosity.
(△) Maximum xanthan concentration.

pH = 7.0 T = 30 °C Agitation = 660 RPM
Air flow rate = approximately 1.2 l min^{-1}.
Medium: MSM (glucose with 0.4 $(NH_4)_2SO_4$ in peat run-off water).


BIOMASS(gKg⁻¹)
0
0.5
1.0
1.5
2.0
2.5
INITIAL GLUCOSE CONC.(gl⁻¹)
0
0.5
1.0
1.5
2.0
XANTHAN(gKg⁻¹) VISCOSITY(cP x 10⁻²)
4
8
12

that of uninoculated medium and that the addition of 0.25 % glucose would be required to produce any xanthan.

These results conflicted with the results of the shake-flask experiment in which the concentration of peat run-off water was varied in yeast malt medium. Therefore, this shake-flask experiment was repeated with one change. The initial pH (before autoclaving) was lowered from 7.0 to 6.25 to allow for more growth. The results (after 3 days of growth) are shown in Figures 4.4 and 4.5. They showed that up to a concentration of 70 % peat run-off water, the biomass concentration increased from 0.55 g l^{-1} to 1.55 g l^{-1}. The final xanthan concentration was directly dependant on the peat run-off water concentration and the final viscosity was significantly higher in flasks containing peat run-off water. Probably because of the difference in initial pH, there were differences between the results of this experiment and the first shake-flask experiment that used both this medium and strain of microorganism. However, in both experiments the final xanthan concentration and viscosity were much greater when peat run-off water was used in place of distilled water.

The yeast malt broth experiments had been conducted in shake-flasks while the mineral salts medium experiments utilised well-mixed and aerated fermentors. In order to remove this variable the shake-flask experiment was repeated with mineral salts medium rather than yeast malt broth. The

Figure 4.4. The effect of peat run-off water on xanthan production in yeast malt medium in shake-flasks. The culture was grown at 26 °C for 65 h with an initial pH of 6.25.

(○) Biomass dry weight.
(△) Xanthan concentration.

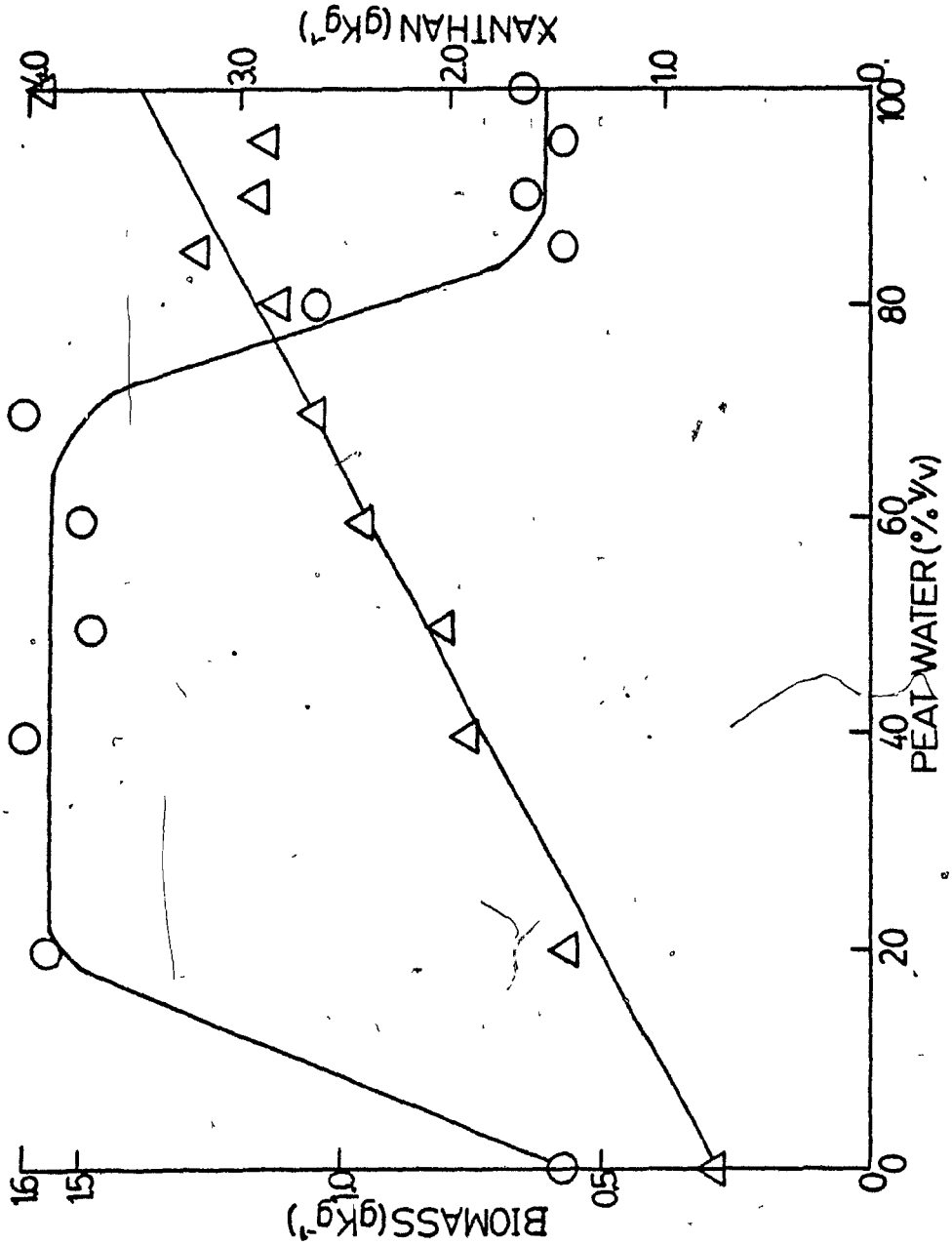
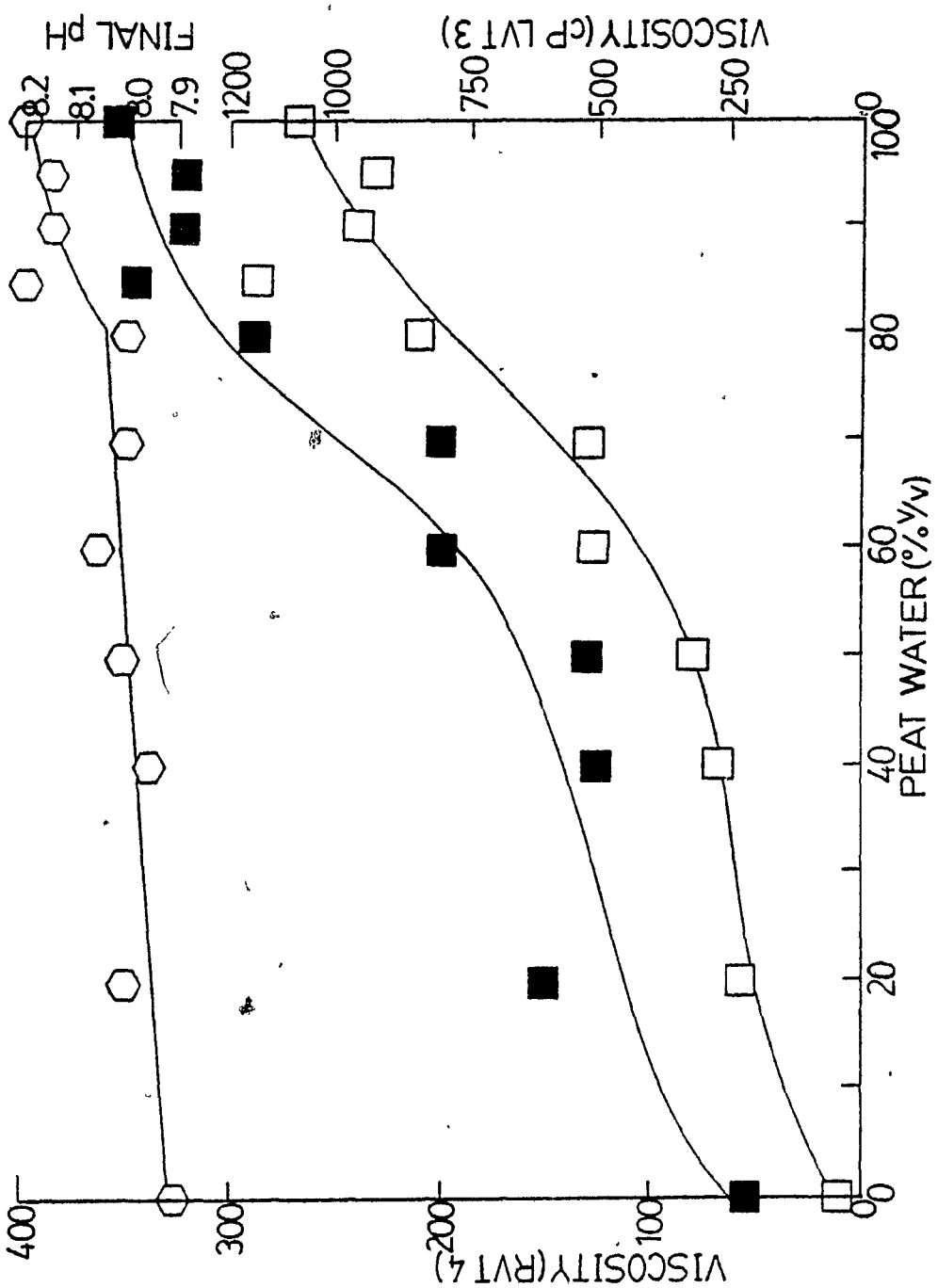

XANTHAN (gkg⁻¹)
40
30
20
10
0
BIOMASS (gkg⁻¹)
1.6
1.5
1.0
0.5
0.0
PEAT-WATER (% v/v)
0
20
40
60
80
100

Figure 4.5. The effect of peat run-off water on xanthan production in yeast malt medium in shake-flasks. The data are from the same experiment as described in Figure 4.4..

(⬡) pH.
(□) Viscosity (Brookfield RVT 4, 100 RPM for 1 min).
(■) Viscosity (Brookfield LVT 3, 30 RPM for 1 min).


FINAL pH
8.2
8.1
8.0
7.9
VISCOSITY (cP LVT 3)
1200
1000
750
500
250
0
100
80
60
40
20
0
PEAT WATER (%v/v)
VISCOSITY (RVT 4)
400
300
200
100
0

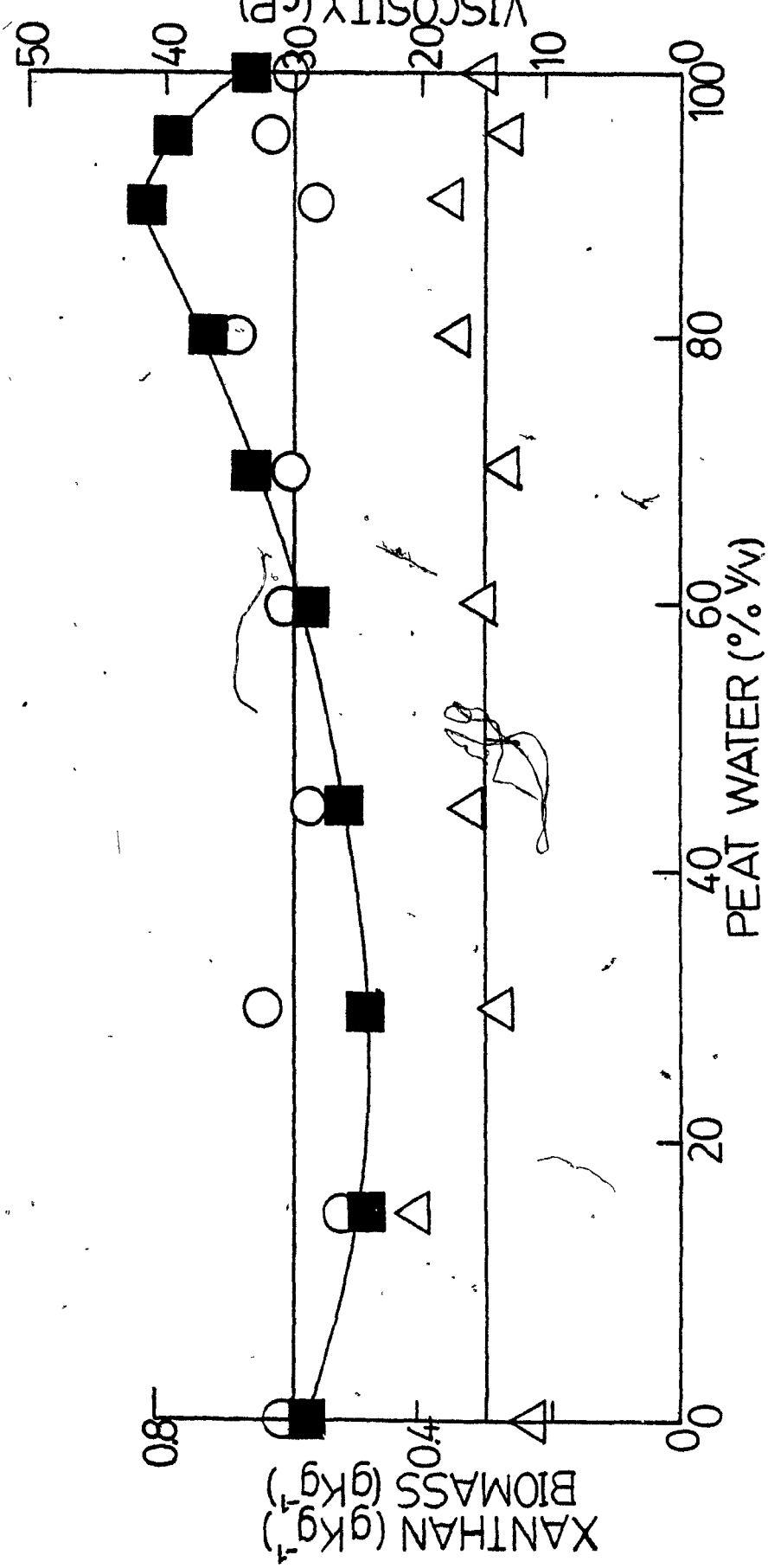
results (Fig. 4.6) showed that peat run-off water had little effect on biomass or xanthan production in this medium although the final viscosity was slightly improved. The results were taken after 3 days growth at 24 °C Glucose analysis showed that less than 25 % of the glucose had been used although all other medium components were present in excess. In order to achieve greater glucose conversion the experiment was repeated and was not sampled until 11 days after inoculation. The results were essentially the same as when sampled only 3 days after inoculation.

4.1.2 Inhibition of X. campestris Growth by Peat Hydrolyzate

Peat hydrolyzate has a much higher carbon content than peat run-off water. After finding that peat run-off water had no detrimental effect on the growth of X. campestris, an attempt was made to grow the organism on peat hydrolyzate. The hydrolyzate was made by adding Barrington peat to the peat run-off water to make a 10 g l^{-1} concentration The pH was adjusted to 2.9 with concentrated hydrochloric acid. This was autoclaved for 15 minutes at 15 psi and 121 °C. Suspended solids were then removed by filtration. Glucose (2.0 %), $(NH_4)_2SO_4$ (0 4 %) and mineral salts medium were added. After sterilization by autoclaving, a pH-controlled fermentation was attempted (using this medium). No growth occurred. This indicated that this concentration of peat hydrolyzate was toxic to X. campestris.

Figure 4.6. The effect of peat run-off water on xanthan production in MSM medium containing 2.0 % glucose and 0.4 % $(NH_4)_2SO_4$. The culture was grown for 72 h at 24 °C.

(○) Biomass dry weight.
(■) Viscosity.
(△) Xanthan concentration.


VISCOSITY (cP)
50
40
30
20
10
0
100
80
60
40
20
0
PEAT WATER (% v/v)
0.8
0.4
0.0
XANTHAN (gkg⁻¹)
BIOMASS (gkg⁻¹)

4.1.3 The Effect of Peat Run-off Water on P. cepacia

Figure 4.7 shows the results of a shake-flask experiment in which P. cepacia was grown in yeast malt medium made up with different concentrations of peat run-off water and distilled water. The presence of peat run-off water resulted in increased total protein production but there was no significant effect on PHB production. There was a substantial difference in final pH between flasks containing peat run-off water and the control, which indicated that peat run-off water had a significant effect on metabolism.

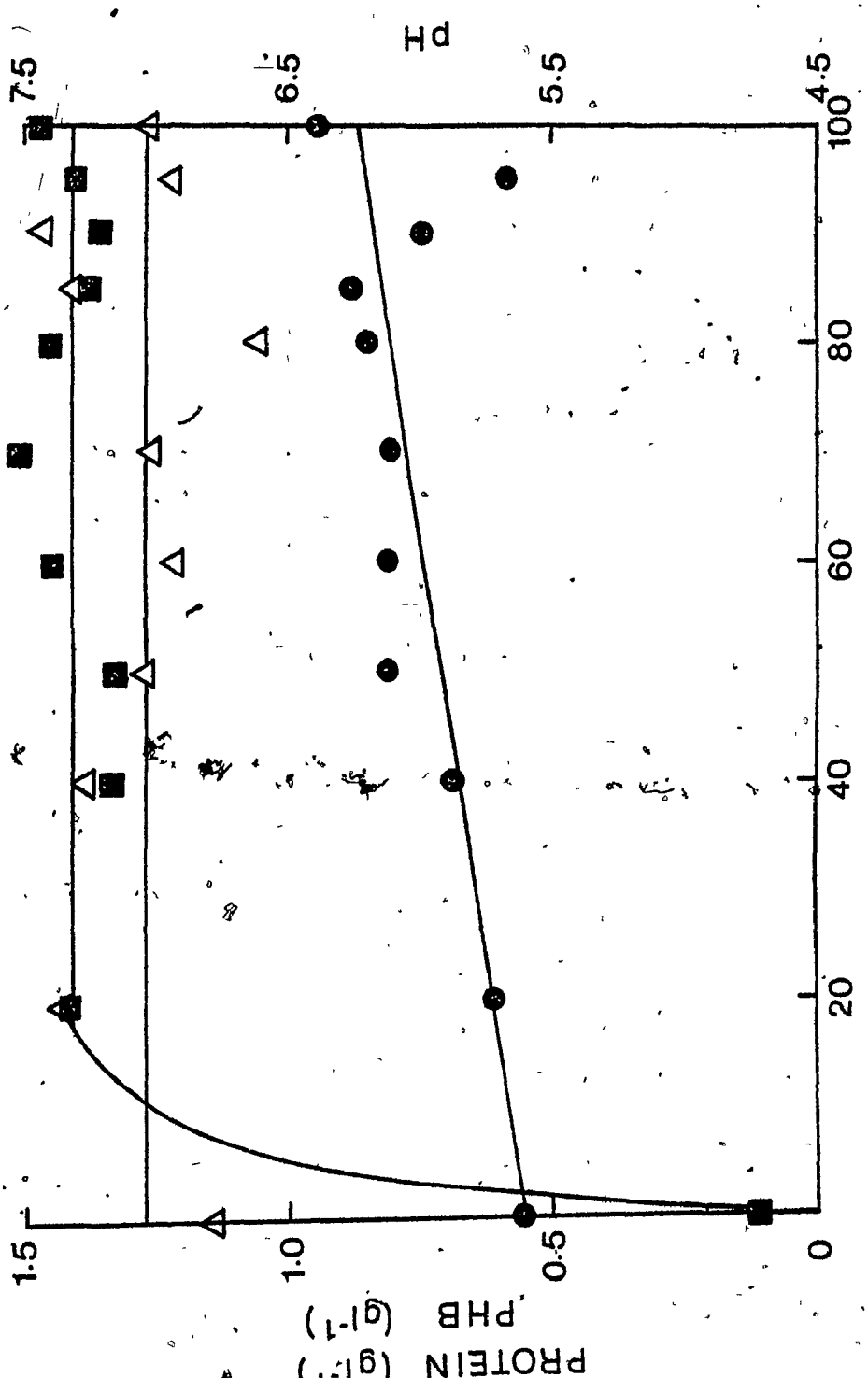
When 25 ml of sterile (autoclaved) peat run-off water was added to a 1.4 liter, fructose-limited, continuous fermentation of P. cepacia (dilution rate = 0.14 h^{-1}), there was no change in the oxygen uptake rate of the culture.

4.2 Use of NVR Components as Carbon and Energy Sources by Microorganisms

A mixed continuous aerobic fermentation was established using 1 % w/v NVR in mineral salts at 37 °C and pH 6.0. The inoculum was a mixture of samples from petroleum refinery sewage treatment facilities and activated sludge from Du Pont's Maitland, Ontario nylon manufacturing plant. It was found that the dilution rate could not exceed 0.21 h^{-1} as washout would occur. Strains isolated included P_2, P_4 and B.

Figure 4.7. The effect of peat run-off water on PHB accumulation by P. cepacia in yeast malt medium. The culture was grown at 26 °C for 66 h.

(●) Total cellular protein.
(△) PHB concentration.
(■) pH after growth.


pH
7.5
6.5
5.5
4.5
PEAT WATER. (% v/v)
0
20
40
60
80
100
PROTEIN (gl-1)
PHB (gl-1)
0
0.5
1.0
1.5

Another continuous fermentation was begun but at a temperature of 30 °C. The complex inoculum was added several times during the first days of the fermentation. The culture was left unperturbed at a dilution rate of 0.21 h^{-1} until a steady-state was achieved as monitored by differential viable cell counts on nutrient agar. This fermentation was dominated by a single strain which was named Isolate 1. Ninety-six % of the viable cells in the culture were Isolate 1 . Isolate 1 formed smooth, opaque, circular and somewhat volcano-shaped colonies when grown on nutrient agar. It was a Gram negative, strictly aerobic, catalase-positive, motile rod.

As will be discussed later, the monocarboxylic acids were found to be the most toxic components of NVR. With this in mind, another continuous enrichment culture was begun using caproic acid as the sole carbon source. At 30 °C and a pH of 6.0, the caproic acid concentration was gradually increased from 0.05 % to 1.0 %. The direct addition of NVR to the 1.0 % caproic acid culture resulted in a large increase in oxygen uptake by the culture. Only two colony types were detected. These were designated as strains SS_1 and SS_2. They were strictly aerobic Gram negative rods producing β-hemolysis on blood agar. In liquid culture they both produced a fluorescent, water-soluble pigment which was red under acid conditions and blue under neutral conditions. No slime was produced on 10 %

sucrose agar. Both isolates grew on glucose, sucrose and maltose. They grew slowly on galactose and trehalose, and and there was no growth on meso-inositol. Both were identified as isolates of Pseudomonas aeruginosa. This identification was confirmed by the microbiology department of Montreal's Royal Victoria Hospital.

The ability of various microorganisms to use individual NVR components as sole sources of carbon and energy is shown in Table 4.2. Pseudomonads and corynebacteria were the procaryote genera most capable of using NVR components. No microorganism could use 1,2 or 1,4-cyclohexanediol but bacteria such as P. cepacia, P. aeruginosa and isolate C12 could use all of the other major NVR components as sole carbon and energy sources. A. eutrophus, C. lepus and C. equi could use most of the major NVR components as sole carbon and energy sources. A. eutrophus is not known to use pentanoic acid (86) and did not when it was supplied in solid agar. However, when grown using the same mineral salts medium in a carbon (NVR or fructose)-limited chemostat, a pulse of pentanoic acid elicited a sharp increase in oxygen uptake by this strain of A. eutrophus. This indicates the ability to use that component as a carbon source especially since the shape of the oxygen uptake response curve was essentially identical to those elicited by butanoic and hexanoic acids which this organism can use as carbon and energy sources.

TABLE 4.2 THE USE OF MAJOR NVR CONSTITUENTS AS SOLE SOURCES OF CARBON AND ENERGY BY VARIOUS MICROORGANISMS[1]

Organism	NVR	6-Hexanolactone	Monocarboxylic acids			Dicarboxylic Acids		
			C_4	C_5	C_6	C_4	C_5	C_6
A. eutrophus	+	+	+	-	+	+	+	+
A. indicus	-	-	-	-	-	-	-	-
A. viscosus	-	-	-	-	-	++	+	+
B. subtilis	-	-	-	-	-	+	-	-
C. equi	+	+	+	+	+	+	-	+
C. fascians	-	-	-	-	-	+	-	-
C. lepus	+	+	+	+	-	+	+	+
E. coli	-	-	-	-	-	+	-	-
Isolate B	-	-	-	-	-	-	-	-
Isolate CAP 12	+	++	+	+	+	+	+	+
Isolate I1	+	-	+	+	+	+	+	+
Isolate P2	-	-	-	-	-	+	+	+
Isolate P4	-	-	-	-	-	+	-	-
L. brevis	-	-	-	-	-	-	-	-
L. gummosus	-	-	-	-	-	-	-	-
L. mesenteroides	-	-	-	-	-	-	-	-
M. rhodochrous	+	-	+	+	-	+	+	+
P. acidovorans	+	-	+	+	+	+	+	+
P. aeruginosa	+	+	+	+	+	+	+	+
P. aeruginosa (SS1)	+	+	+	+	+	+	+	+
P. aeruginosa (SS2)	+	+	+	+	+	+	-	+
P. cepacia	+	+	++	++	++	+	+	+
R. rhodochrous	+	-	-	-	-	+	+	+
S. cerevesiae	-	-	-	-	-	-	-	-
S. epidermidis	-	-	-	-	-	+	-	-
S. rolfsii	-	-	-	-	-	+	-	-
T. bombicola	-	-	-	-	-	-	-	-
T. petrophilium	+	-	+	+	+	-	-	-
X. campestris	-	-	-	-	-	+	-	-

[1] no detectable difference from the control (-); noticeable growth (+); good growth (++).

4.3 The Effects of NVR on Biosurfactant Production

4.3.1 Microorganisms that do not Require Hydrocarbons for Biosurfactant Production

In order to determine the effects of NVR on biosurfactant production, microorganisms known to produce biosurfactants or to utilize NVR components were grown in shake-flasks containing different concentrations of NVR. After a period of growth, the flasks were analyzed.

B. subtilis is an organism that produces a peptide biosurfactant (called surfactin) when grown on carbohydrates such as glucose (76,77). It was grown in a medium which contained sucrose and citric acid as alternate carbon sources to NVR. When surfactin is in excess of its critical micelle concentration (CMC), the surface tension should be approximately 24 mN m^{-1} (25). The results, as shown in Figure 4.8, indicate, that in the absence of NVR, surfactin was produced in a concentration somewhat lower than its CMC. The addition of small quantities of NVR stimulated growth of *B. subtilis* but had no significant effect on biosurfactant production. NVR concentrations of 0.12 % w/v or greater inhibited both growth and production of biosurfactant. The surface tension measurements at these inhibitory concentrations reflected the biosurfactant activity of the inoculum and of NVR.

Figure 4.8. The effect of NVR on growth and biosurfactant production by B. subtilis. The culture was grown on MSC medium at 26 °C.

(■) Biomass dry weight.
(●) Surface tension of the whole broth.

SURFACE TENSION(mNm⁻¹)
50
40
30
0.20
0.15
0.10
0.05
0
NVR CONC.(%W/V)
BIOMASS CONC(gl⁻¹)
0.6
0.4
0.2
0.0

4.3.2 Microorganisms that Require Hydrocarbons for Biosurfactant Production

C. fascians is known to produce large quantities of biosurfactant in the presence of hydrocarbons such as n-alkanes (24). As demonstrated by Figure 4.9, the presence of sublethal quantities of NVR did not stimulate biosurfactant production by C. fascians. Similar results were found wih C. insidiosum (4), C. lepus (30), M. rhodochrous (65) and P. aeruginosa (79), which also normally produce biosurfactants when n-alkanes are present.

4.3.3 Microorganisms that Require Both Hydrocarbons and Carbohydrates for High Yield Biosurfactant Production

T. bombicola is unusual in requiring both hydrocarbon and carbohydrate in its medium for high yield biosurfactant production, although it grows on either (26). When grown on mineral salts citrate medium, T. bombicola lowered the surface tension of the medium to 32 mN m^{-1} (Figure 4.10). Addition of NVR had no effect on biosurfactant production.

When grown on a mixture of n-alkanes and carbohydrates, T. petrophilum produces the same glycolipid as T. bombicola but in lower yield (28). When grown on a carbohydrate such as glucose in a medium lacking hydrocarbon, T. petrophilum produces an extracellular emulsifier but very little biosurfactant. The addition of NVR to MSC medium stimulated neither biosurfactant nor bioemulsifier production by this microorganism.

Figure 4.9. The effect of NVR on growth and biosurfactant production from C. fascians. The culture was grown on MSC medium at 26 °C.

(●) Biomass dry weight.
(■) Surface tension of the whole broth.

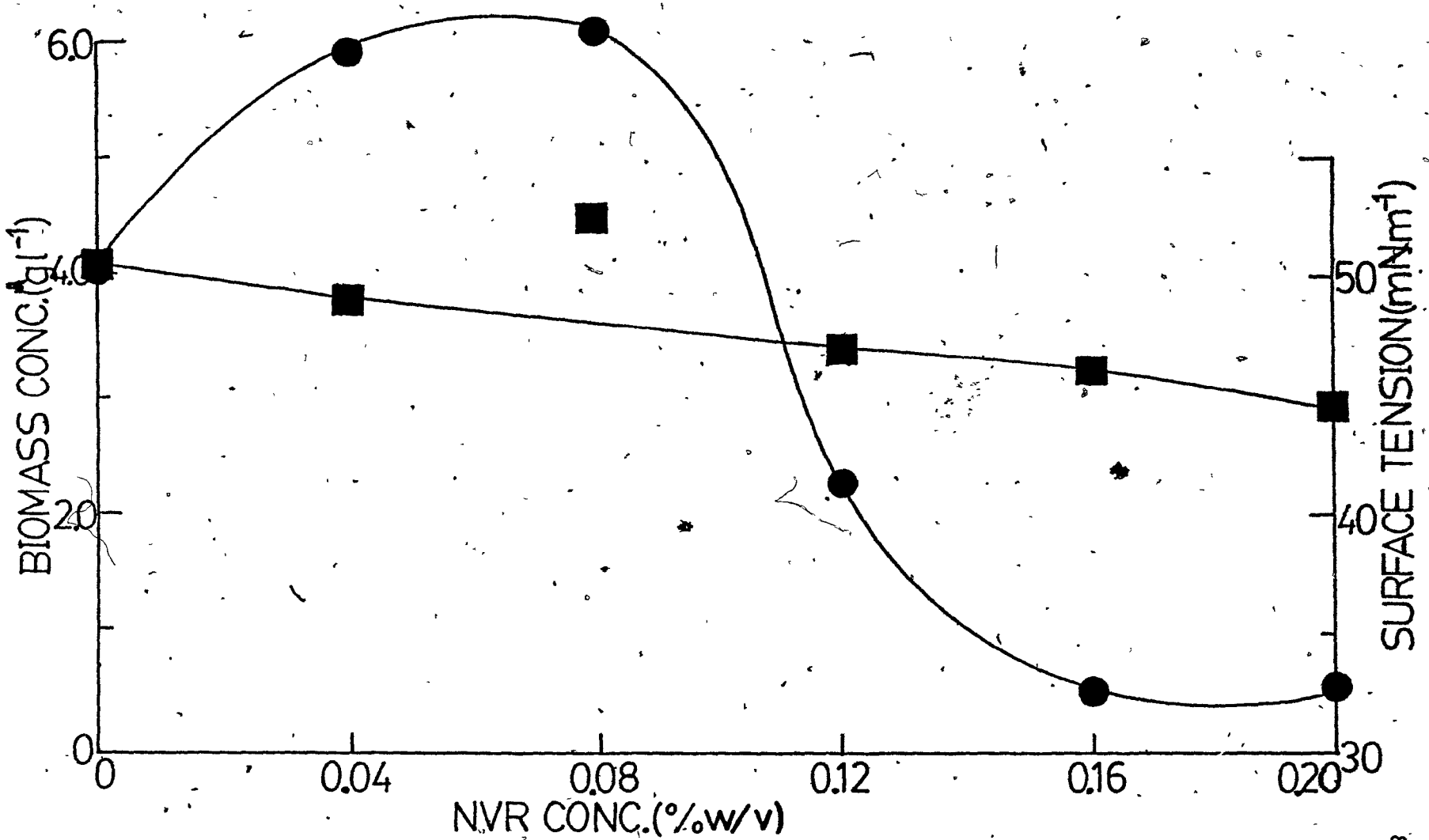
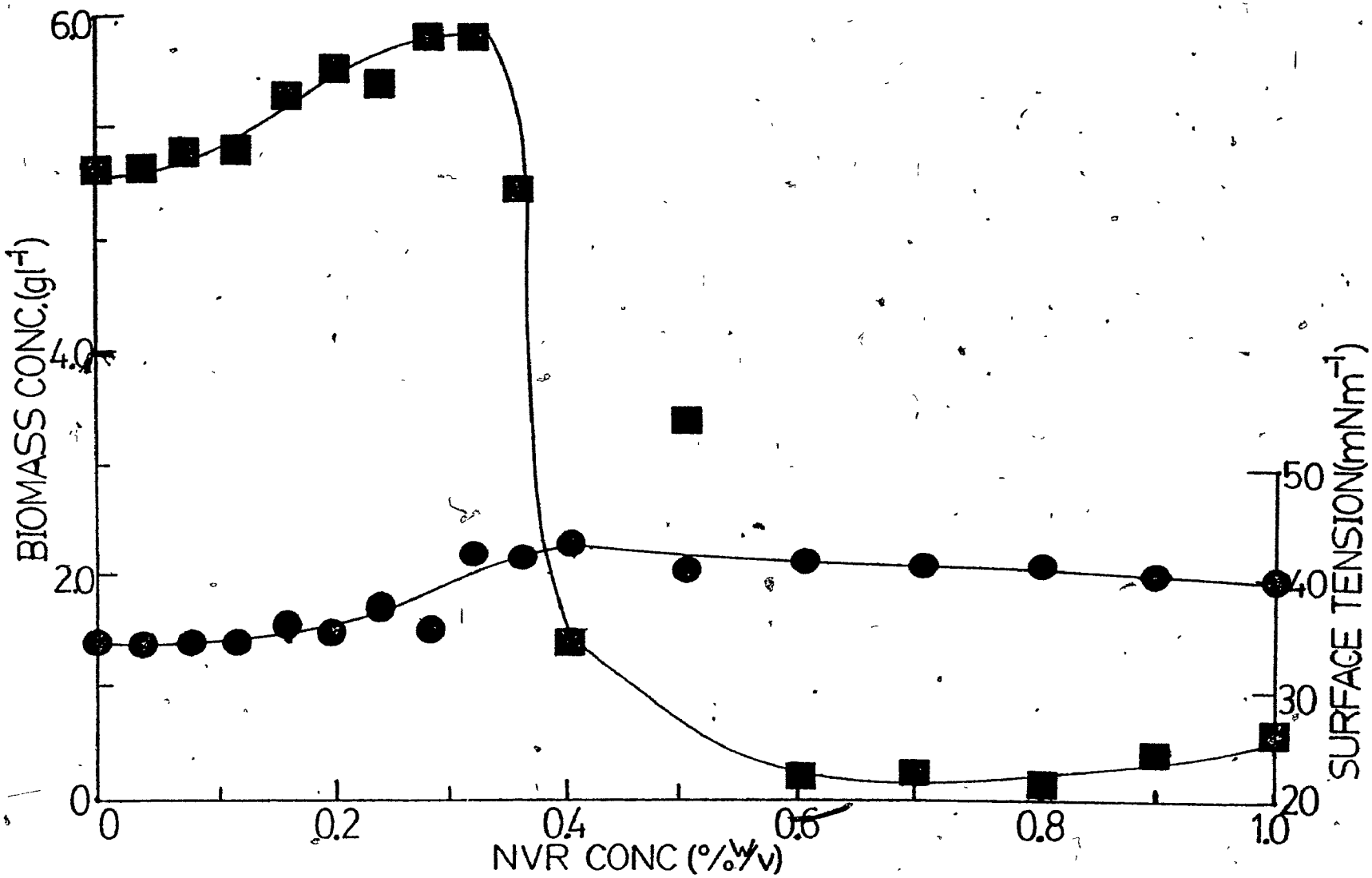

6.0
4.0
2.0
0
BIOMASS CONC.(gl⁻¹)
0
0.04
0.08
0.12
0.16
0.20
NVR CONC.(%w/v)
50
40
30
SURFACE TENSION(mNm⁻¹)

Figure 4.10. The effect of NVR on growth and biosurfactant production by T. bombicola. The culture was grown on MSC medium at 26 °C.

(■) Biomass dry weight.
(●) Surface tension of the whole broth.


6.0
4.0
2.0
0
BIOMASS CONC.(gl⁻¹)
0
0.2
0.4
0.6
0.8
1.0
NVR CONC (%W/V)
50
40
30
20
SURFACE TENSION(mNm⁻¹)

4.3.4 Organisms that were isolated from Continuous Enrichment Culture on either NVR or Caproic Acid

A lowering of the surface tension in the nutrient broth medium of isolate SS_2 on addition of NVR was due to the surface activity of NVR itself (Fig. 4.11). The surface activity of NVR can be observed at NVR concentrations where no growth occurred. Since no growth occurred the lowering of the surface tension was solely due to NVR and any biosurfactant that may have been present in the inoculum. Although growth of SS_2 was greatly stimulated at 0.2 and 0.3 % NVR, the medium surface tension was still 46 mN m^{-1} which is the same as at higher NVR concentrations where there was no growth.

Isolate G_5, a Gram negative rod, appeared to raise the surface tension of the broth (Fig. 4.12). This can be seen by comparing the surface tension at 0.6 % NVR, where there was growth, with that at 0.7 % NVR where there was no growth. The increase in surface tension could be due to metabolism of the surface active components of NVR by Isolate G_5.

Addition of NVR to the media of Isolate 1, or isolates B and G_3 did not stimulate biosurfactant production by these microorganisms.

The addition of up to 0.5 % NVR to the mineral salts citrate medium of isolate P_2 resulted in a decrease of the surface tension after growth (Figure 4.13). This indicated

Figure 4.11. The effect of NVR on growth and biosurfactant production by isolate SS_2 (*P. aeruginosa*). The culture was grown on glucose medium at 37 °C for 46 h.

(□) Biomass dry weight.
(△) Surface tension of the whole broth.
(○) pH after growth.

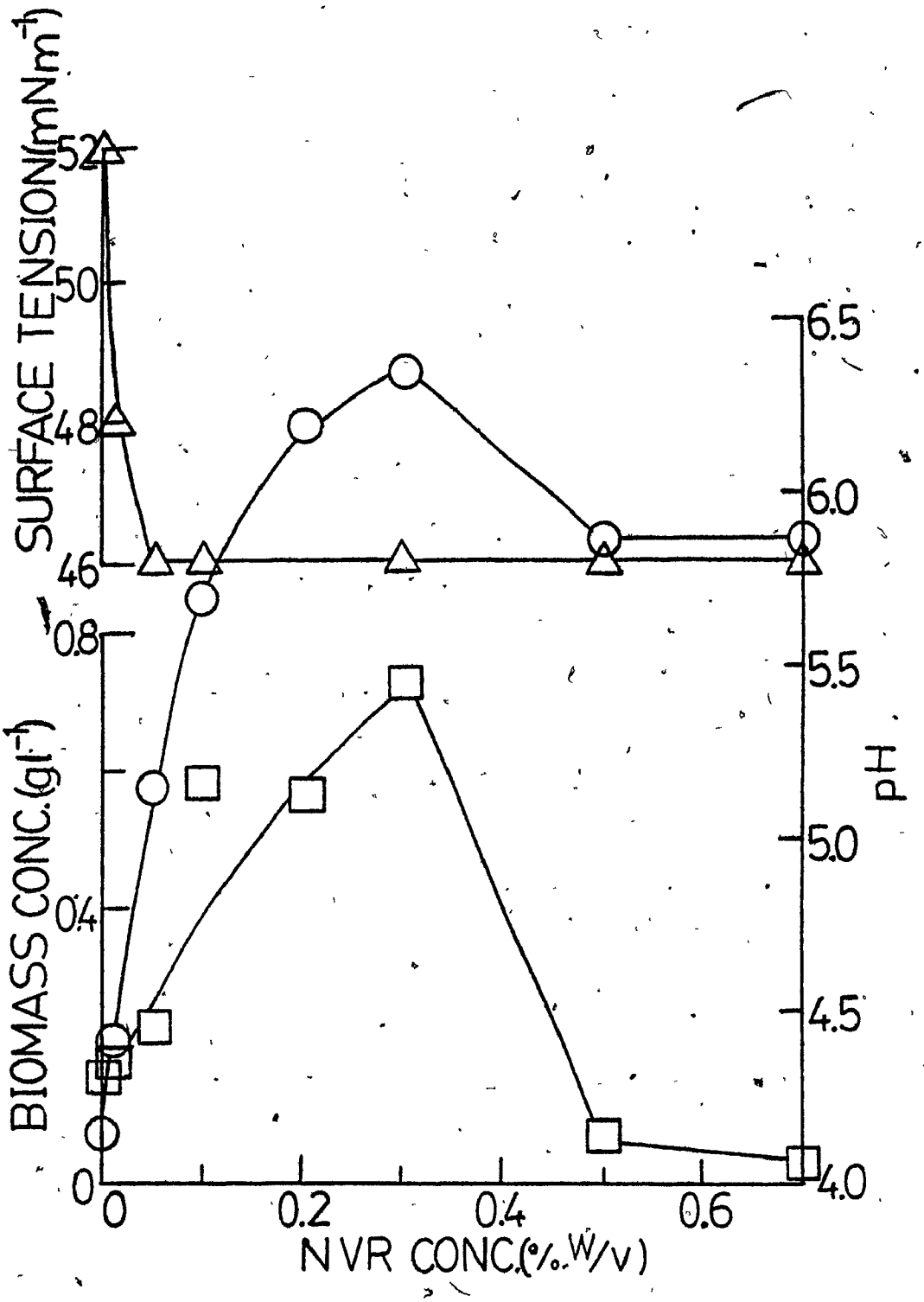

SURFACE TENSION(mNm⁻¹)
52
50
48
46
BIOMASS CONC.(gl⁻¹)
0.8
0.4
0
0
0.2
0.4
0.6
NVR CONC.(%.W/V)
6.5
6.0
5.5
5.0
4.5
4.0
pH

Figure 4.12. The effect of NVR on growth and biosurfactant production by isolate Gs. The culture was grown on MSC medium at 37 °C for 60 h.

(□) Total cellular protein.
(△) Surface tension of the whole broth.
(○) pH after growth.

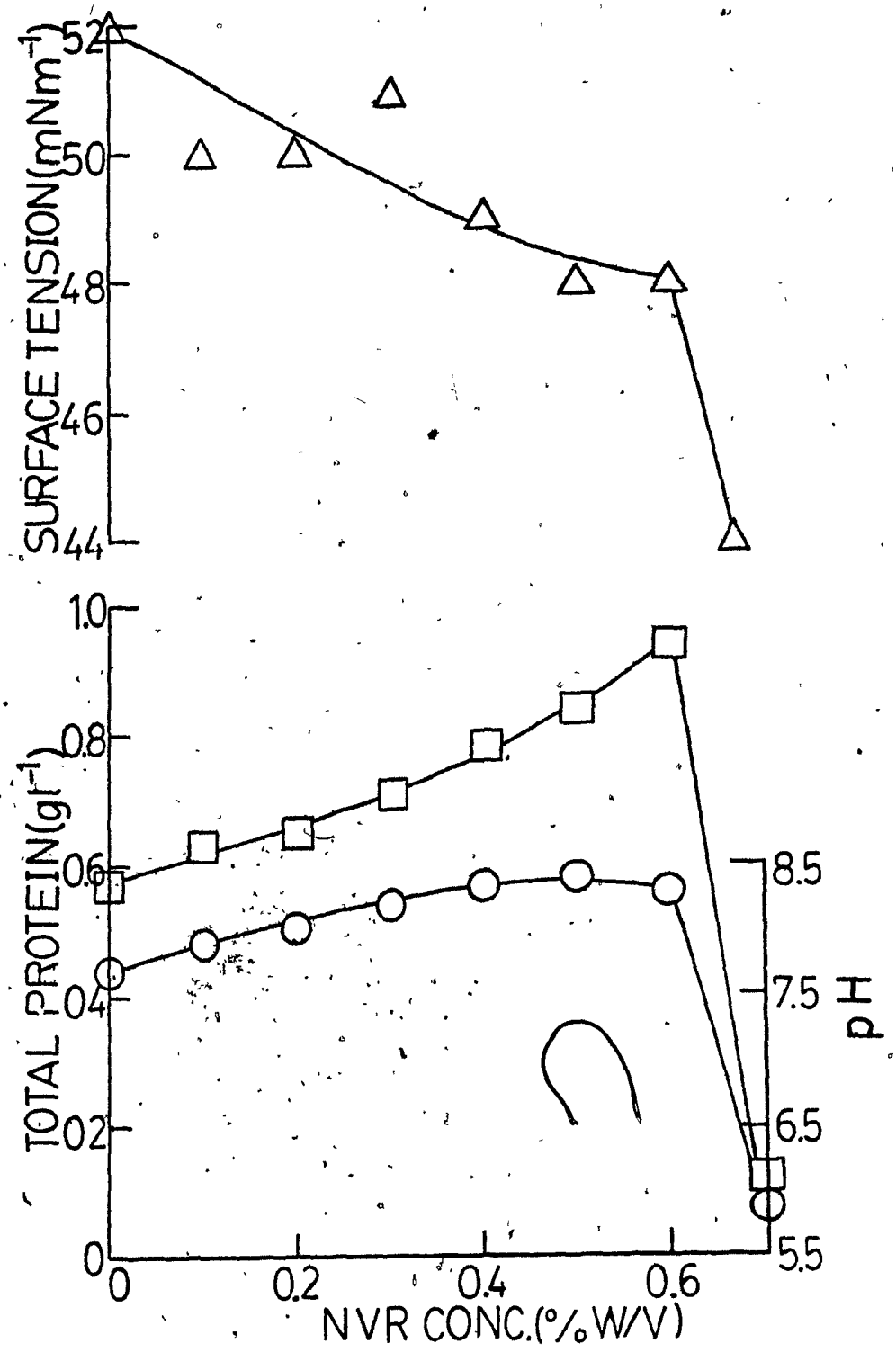
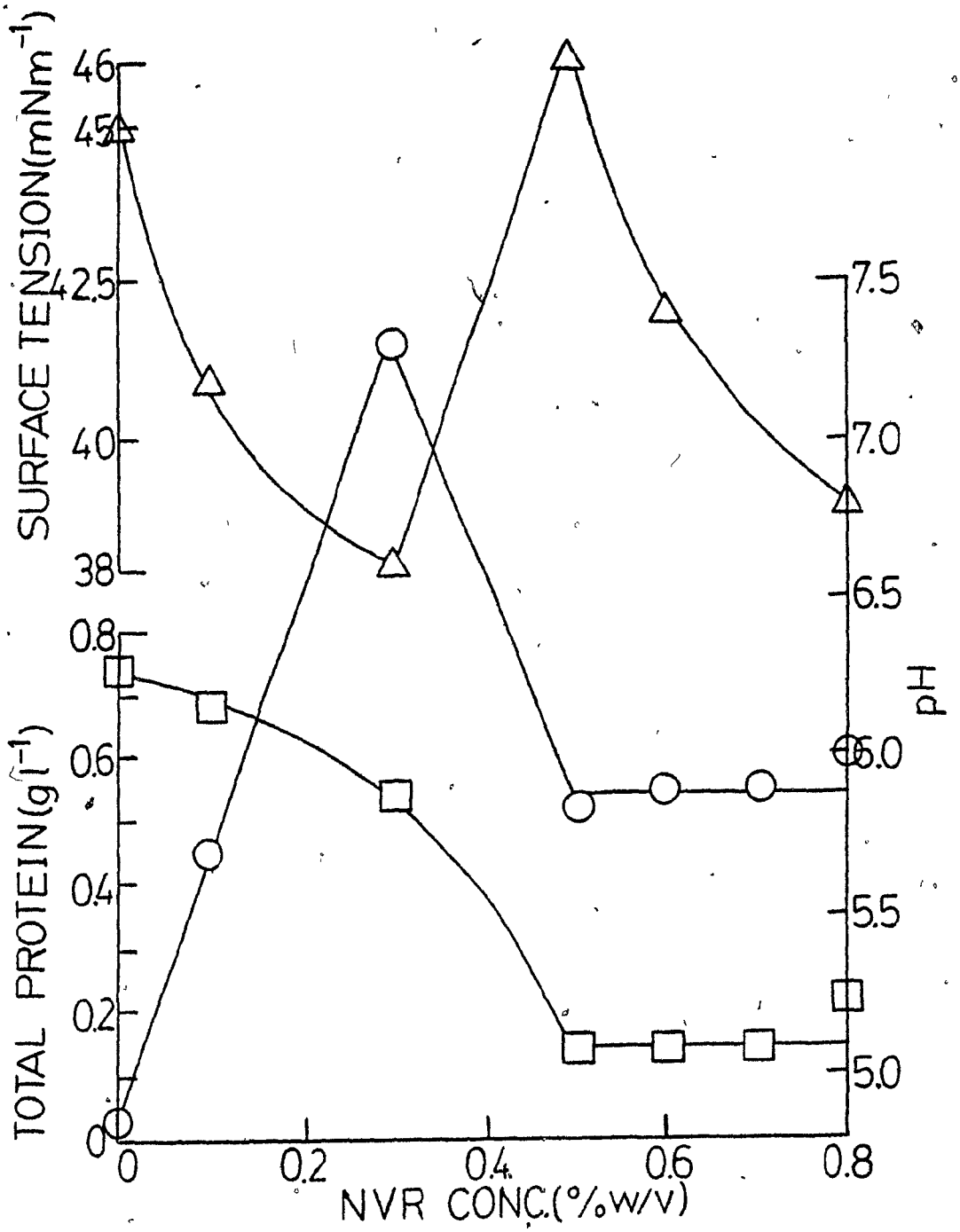

SURFACE TENSION(mNm⁻¹)
52
50
48
46
44
TOTAL PROTEIN(gl⁻¹)
1.0
0.8
0.6
0.4
0.2
0
pH
8.5
7.5
6.5
5.5
0
0.2
0.4
0.6
NVR CONC.(%W/V)

Figure 4.13. The effect of NVR on growth and biosurfactant production by isolate P2. The culture was grown on MSC medium at 26 °C for 60 h.

(□) Total cellular protein.
(△) Surface tension of the whole broth.
(○) pH after growth.


SURFACE TENSION(mNm⁻¹)
46
45
42.5
40
38
TOTAL PROTEIN(gl⁻¹)
0.8
0.6
0.4
0.2
0
pH
7.5
7.0
6.5
6.0
5.5
5.0
0
0.2
0.4
0.6
0.8
NVR CONC.(%w/v)

that NVR stimulated the production of a small amount (in terms of effectiveness) of biosufactant, which could lower the surface tension of water to 38 mN m^{-1} or lower.

4.4 The Effect of NVR on Production of Extracellular Polysaccharides by Microorganisms

The effect of NVR on polysaccharide production by X. campestris ATCC 13951, L. gummosus, A. viscosus, L. mesenteroides, P. aeruginosa NRC 2786 and the isolates was tested. Generally NVR was inhibitory to exopolysaccharide production. Growth and production of xanthan by X campestris was greatly inhibited by just 0.05 % NVR (Fig. 4.14). Metabolism, as indicated by the production of acid or base, was not completely inhibited until a concentration of 0.30 % NVR. However, at sublethal concentrations, NVR caused the organism to lower rather than raise the pH during growth indicating a significant effect on metabolism

NVR had a similar effect on L. gummosus. Polysaccharide production was almost totally prevented by the presence of just 0.01 % NVR. While the whole broth viscosity was 70 cP in the control flask, it was just 4 cP in the flask containing 0 01 % NVR. L. gummosus raised the medium pH in all flasks in which it grew

NVR did not stimulate increased production of exopolysaccharide by any microorganism tested except one. Isolate P2 produced small quantities of a non-viscous

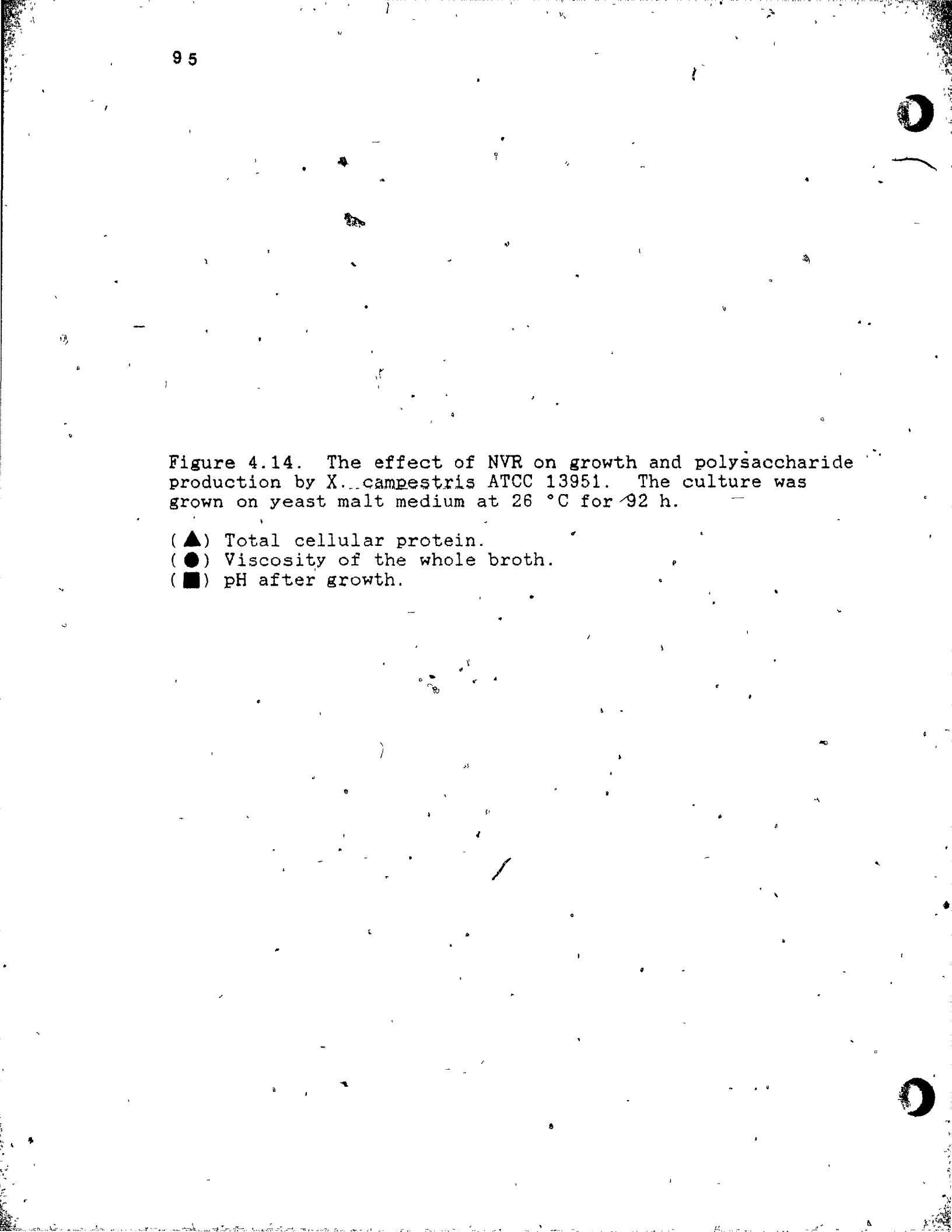
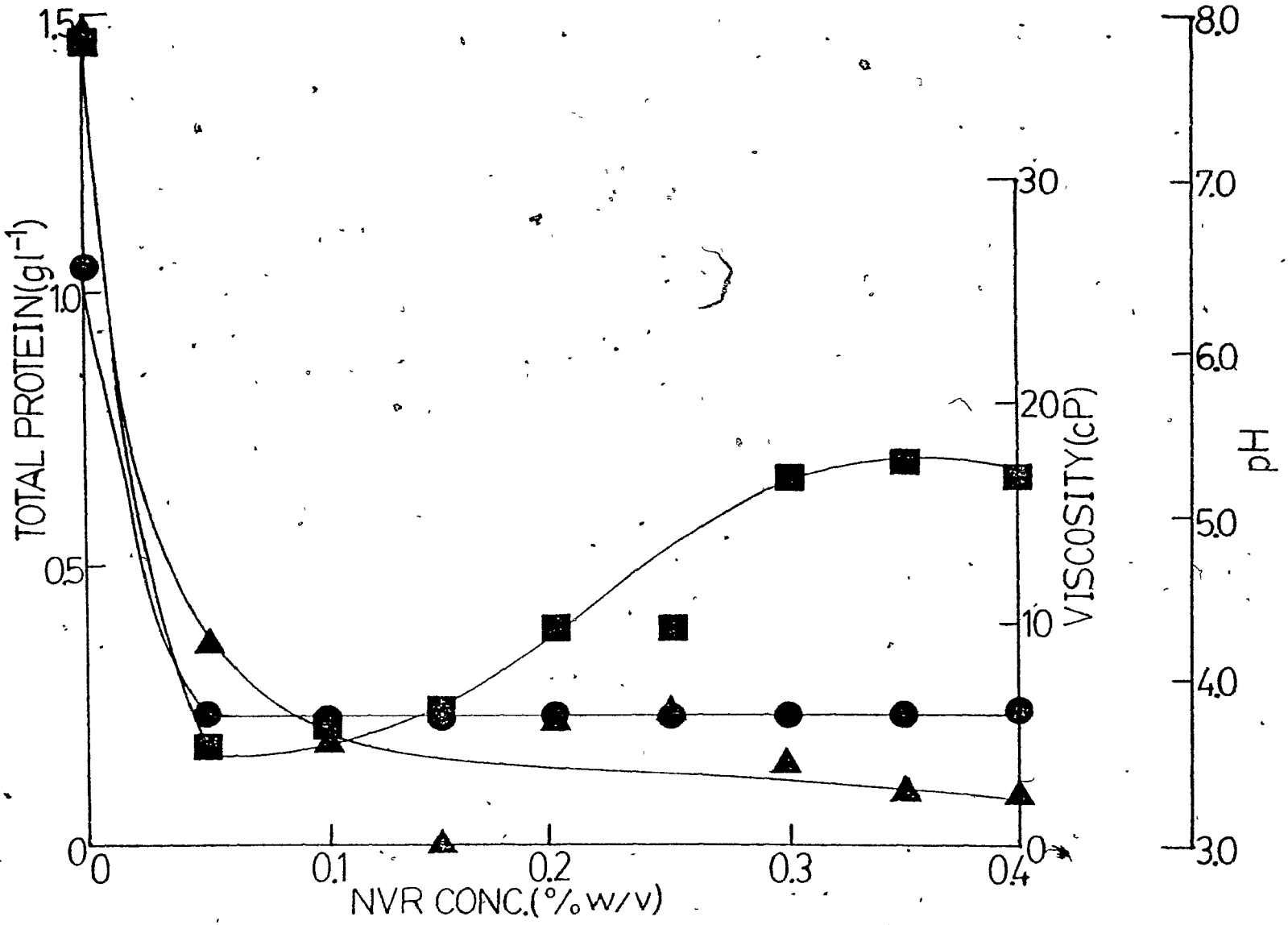


Figure 4.14. The effect of NVR on growth and polysaccharide production by X. campestris ATCC 13951. The culture was grown on yeast malt medium at 26 °C for 92 h.

(▲) Total cellular protein.
(●) Viscosity of the whole broth.
(■) pH after growth.


TOTAL PROTEIN(gl⁻¹)
1.5
1.0
0.5
0
VISCOSITY(cP)
30
20
10
0
pH
8.0
7.0
6.0
5.0
4.0
3.0
0
0.1
0.2
0.3
0.4
NVR CONC.(% w/v)

extracellular polysaccharide when sublethal quantities of NVR were added to the medium.

4.5 The Effect of NVR on the Production of Catalase by P. aeruginosa

NVR was found to greatly stimulate the growth of isolate SS2 (identified as P. aeruginosa) as well as its production of the enzyme catalase on glucose mineral salts medium (Fig. 4.15). The biomass concentration, as reflected by the total protein concentration, was greatly increased as the concentration of NVR was raised. Since the culture was sampled 46 hours after inoculation and the medium originally contained 20 $g \cdot l^{-1}$ glucose, this increase was related to the microorganism's rate of growth rather than its yield. As indicated by the specific catalase activity shown in Figure 4.15, the catalase activity was increased to an even greater extent than was the biomass concentration. Additional data from this experiment are given in Figure 4.11.

4.6 The Effect of NVR on the Production of PHB by Microorganisms

When grown for 28 hours at 26 °C on nutrient broth, A. eutrophus produced much more PHB as the concentration of NVR was increased (Fig. 4.16). The same effect was observed when pentanoic acid was added to the NB medium (Fig. 4.17). In this case A. eutrophus had been grown for 43 hours at 26 °C before sampling. As shown in Figure 4.18, the addition of 2-methylbutanoic acid to its NB medium stimulated PHB accumulation by this P. cepacia (24 h growth at 26 °C)

Figure 4.15. The effect of NVR on catalase production by isolate SS2 (*P. aeruginosa*). The culture was grown on yeast malt medium at 37 °C for 46 h.

(●) Total cellular protein.
(■) Specific catalase activity.

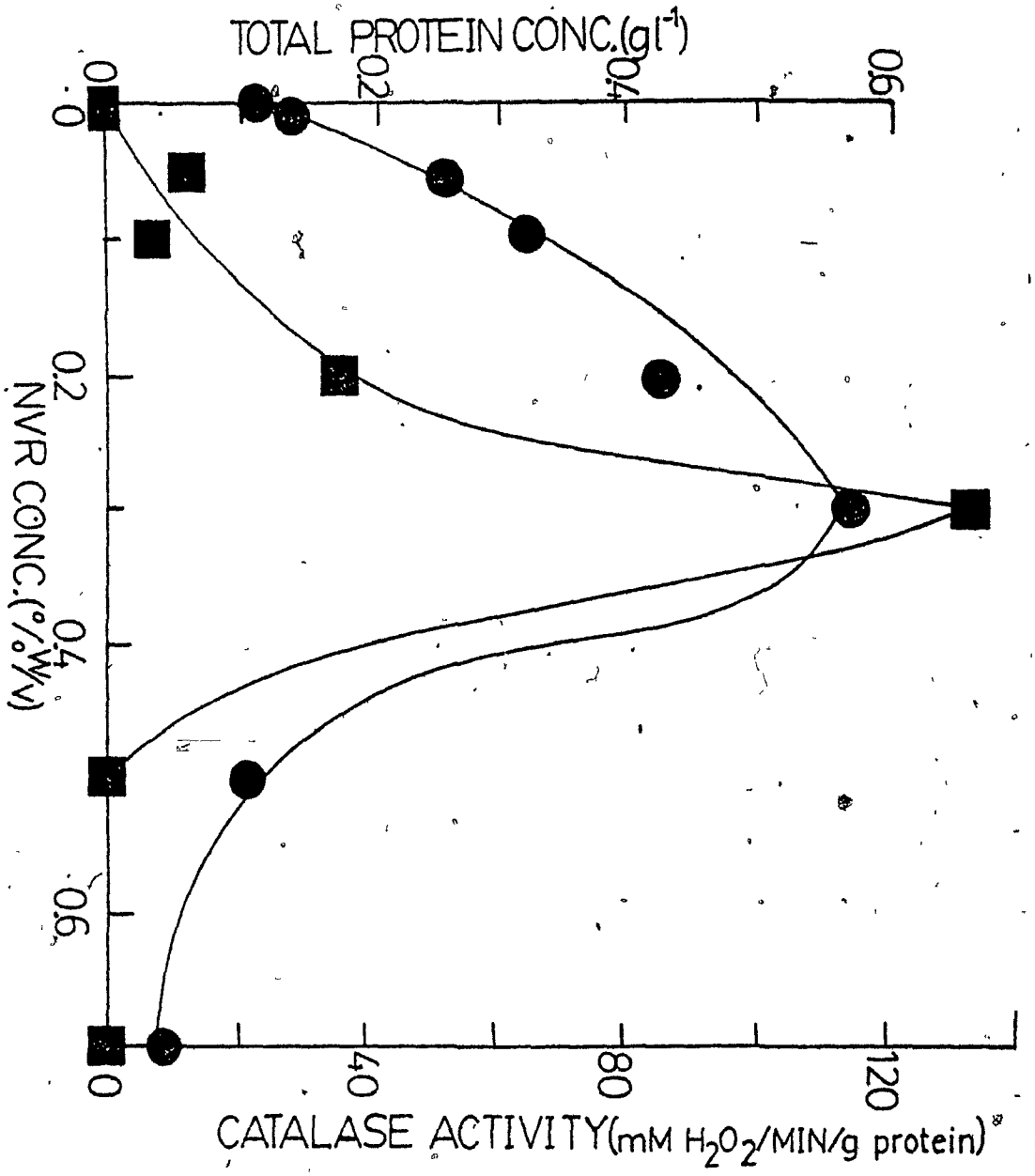

TOTAL PROTEIN CONC.(gl⁻¹)
0
0.2
0.4
0.6
0.0
0.2
0.4
0.6
NVR CONC.(%W/v)
0
40
80
120
CATALASE ACTIVITY(mM H2O2/MIN/g protein)

Figure 4.16. The effect of NVR on growth and PHB production by A. eutrophus. The culture was grown on nutrient broth medium at 26 °C for 28 h.

(■) Biomass dry weight.
(△) PHB concentration.
(○) pH after growth.

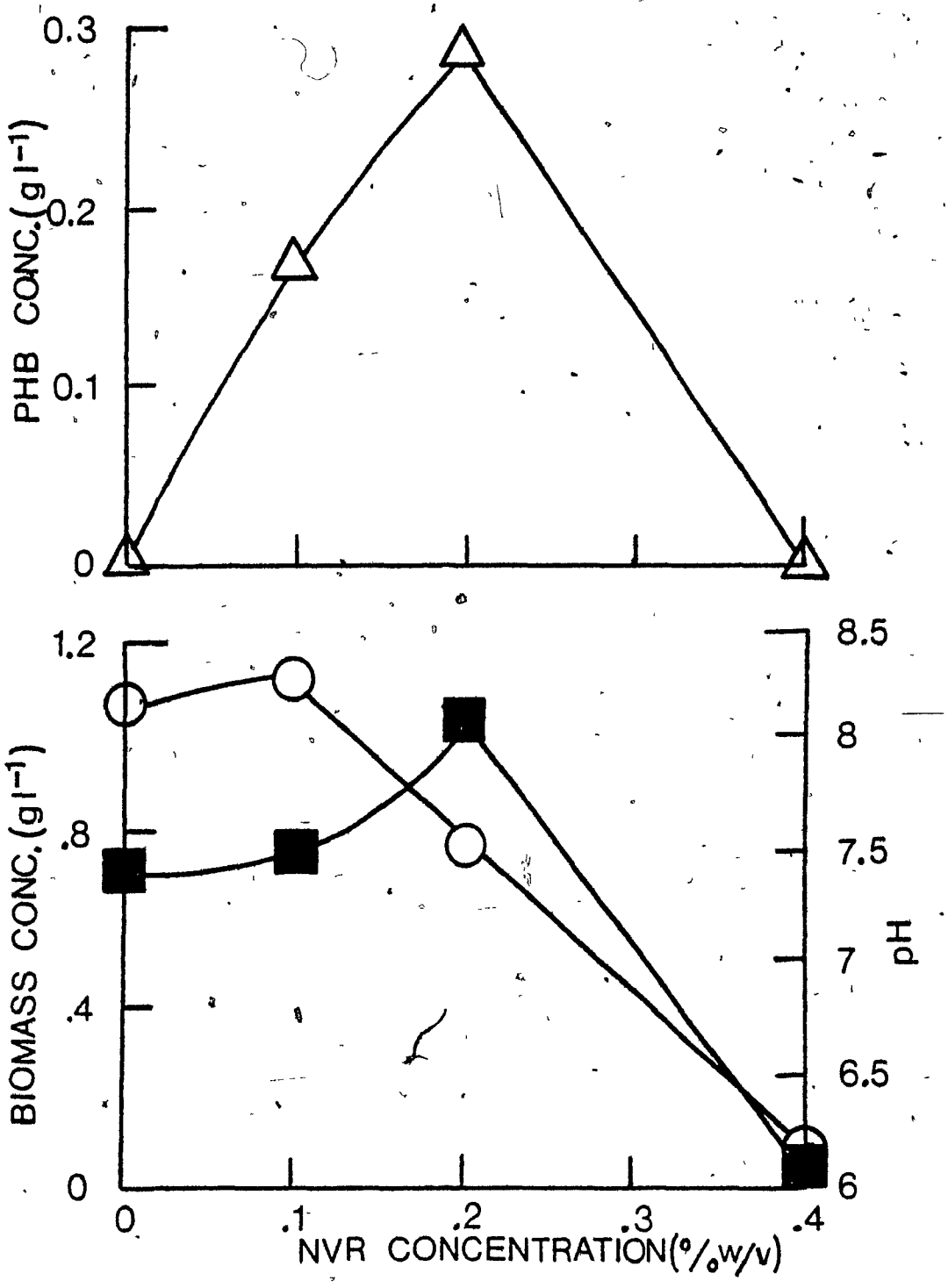

0.3
0.2
0.1
0
PHB CONC.(g l⁻¹)
1.2
.8
.4
0
BIOMASS CONC.(g l⁻¹)
8.5
8
7.5
7
6.5
6
pH
0
.1
.2
.3
.4
NVR CONCENTRATION(%w/v)

Figure 4.17. The effect of pentanoic acid on growth and PHB production of A. eutrophus grown on nutrient broth medium at 26 °C for 43 h.

(■) Biomass dry weight.
(Δ) PHB concentration.
(O) pH after growth.

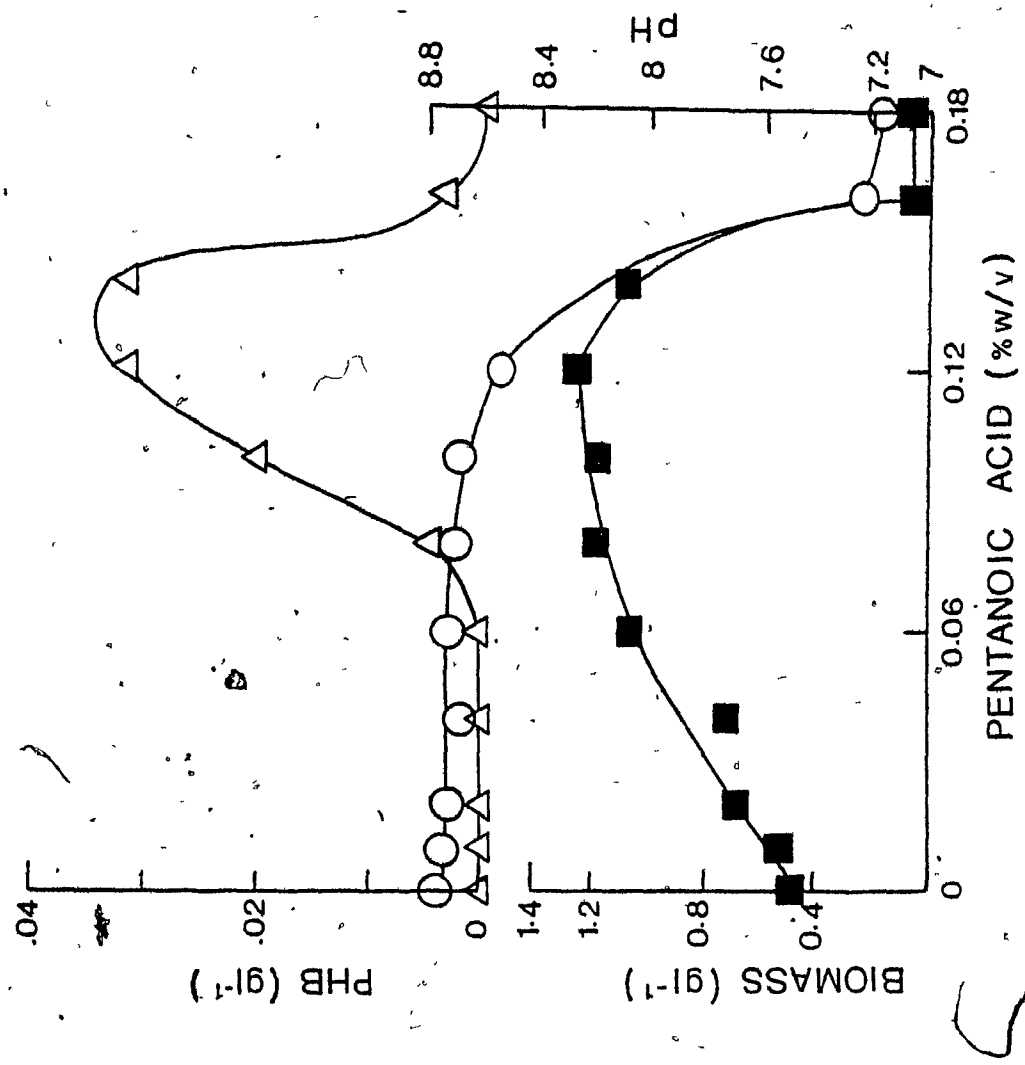
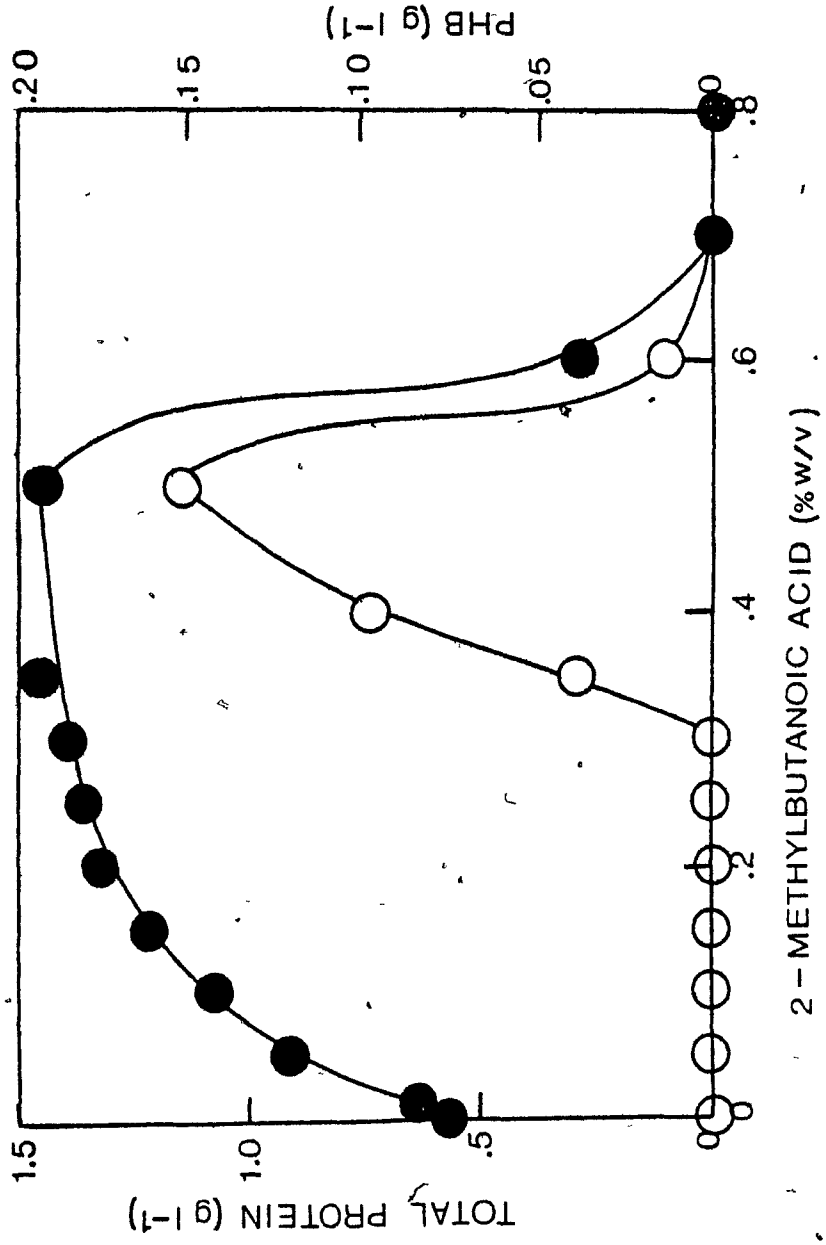

pH
8.8
8.4
8
7.6
7.2
7
0.18
0.12
0.06
0
PENTANOIC ACID (%w/v)
BIOMASS (gl⁻¹)
1.4
1.2
0.8
0.4
PHB (gl⁻¹)
.04
.02
0

Figure 4.18. The effect of 2-methylbutanoic acid on growth and PHB production by P. cepacia. The organism was grown on nutrient broth medium at 26 °C for 24 h.

(●) Total cellular protein.
(○) PHB concentration.


PHB (g l⁻¹)
.20
.15
.10
.05
0
.8
.6
.4
.2
0
2 – METHYLBUTANOIC ACID (%w/v)
1.5
1.0
.5
TOTAL PROTEIN (g l⁻¹)

4.7 The Effects of NVR on Growth of Microorganisms

4.7.1 Batch Enrichment Culture with NVR as the Carbon Source

The batch enrichment culture medium consisted of 4.0 % v/v NVR, 0.4 % w/v yeast extract and 0.4 % w/v nutrient broth. The inoculum to the 500 ml shake-flask containing 50 ml of medium was 1 ml activated sludge from a petroleum refinery sewage treatment plant and 1 g fertile soil (no Du Pont activated sludge was available at this time). After two weeks at 37 °C no growth had occurred. The experiment was repeated with 8 shake-flasks in which the initial pH was varied from 2 to 9 by the addition of KOH. There was no growth at any pH.

The first experiment (ie. initial pH = 7.0) was repeated again but with only 2.0 % w/v NVR and at 26 °C. Again there was no growth.

4.7.2 The Effects of NVR on Microbial Growth in Shake-flasks

The results of a growth inhibition study are listed in Table 4.3 where each line refers to a separate shake-flask experiment. The organisms are listed in decreasing order of their I_1 value. There was a wide variation in the effect of NVR on growth. Just 0.16 % w/v NVR prevented any growth of *B. subtilis*, *C. fascians* and *C. lepus* while an NVR concentration of 1.3 % w/v was necessary to totally prevent growth of *P. cepacia*. Growth of *L. gummosus* was inhibited by only 0.01 % NVR while growth of *P. cepacia* was not inhibited until a concentration of 1.2 %. *P. cepacia* could

4.3 The Effect of NVR on Growth of various Microorganisms

ORGANISMS	MEDIUM[1]	I_1[2] (% w/v)	I_2[3] (% w/v)	GROWTH EFFECT
P. cepacia	NB	1.2	1.3	++
P. acidovorans	NB	0.9	0.9	++
T. petrophilum	MSC	0.8	0.8	±
E. coli	NB	0.7	1.5	±
Isolate G_5	MSC	0.7	0.7	++
Isolate 1	NB	0.6	0.7	++
P. aeruginosa (SS_2)	GM	0.5	0.5	++
A. eutrophus	NB	0.4	0.4	++
T. bombicola	MSC	0.36	0.6	+
Isolate B	MSC	0.3	0.3	+
S. epidermidis	NB	0.3	0.3	±
A. viscosus	YM	0.3	0.3	±
Isolate G_3	MSC	0.2	0.5	-
C. lepus	MSC	0.12	0.16	±
C. fascians	MSC	0.12	0.16	±
B. subtilis	MSC	0.12	0.14	+
Isolate P_2	MSC	0.1	0.5	-
C. insidiosum	MSC	0.10	0.18	-
L. mesenteroides	YM	0.05	0.5	-
X. campestris	YM	0.05	0.3	-
L. gummosus	NB	0.01	0.5	-

[1] NB Nutrient broth
MSC Mineral salts citrate broth
GM Glucose mineral salts broth
YM Yeast malt broth

[2] Lowest concentration of NVR that was inhibitory to growth.

[3] Lowest concentration of NVR that totally prevented growth.

[4] ++ Great stimulation of growth below the I_2 concentration.
+ Some stimulation of growth below the I_2 concentration.
± No effect on growth below the I_2 concentration.
- Inhibition of growth at 50 % of the I_2 concentration.

grow at a higher concentration of NVR than any other organism tested.

There were three distinct ways in which microorganisms reacted to increasing concentrations of NVR (Fig. 4.19). Some, such as T. bombicola, were stimulated in growth by low NVR concentrations (ie. positive growth effect). Some, as in the case of T. petrophilum, were not affected by NVR until the I_2 concentration was approached (ie. no growth effect). Others, such as Isolate G_3, were inhibited at NVR concentrations much lower than I_2 (ie. negative growth effect). The growth effects of NVR on various microorganisms are summarized in Table 4.3. Generally, organisms that could grow at relatively high NVR concentrations were stimulated in growth by low concentrations of NVR.

4.8 The Effects of NVR Components on Microbial Growth

4.8.1 Identification of the Major Toxic NVR Components

Of the major NVR components (listed in Table 1.1), only the monocarboxylic acids and 6-hexanolactone were found to inhibit the growth of microorganisms. This was determined by exposing the microorganisms to relatively large concentrations of individual NVR components. The results of one such experiment are listed in Table 4.4. In this experiment, dicarboxylic acids stimulated growth of Isolate 1 while 1,2-cyclohexanediol and the trace metals found in NVR had no effect. Monocarboxylic acids and 6 -

Figure 4.19. The effects of NVR on the growth of microorganisms.

(▲) Stimulatory effect (T. bombicola).
(●) No effect (T. petrophilum).
(■) Inhibitory effect (Isolate G_3).

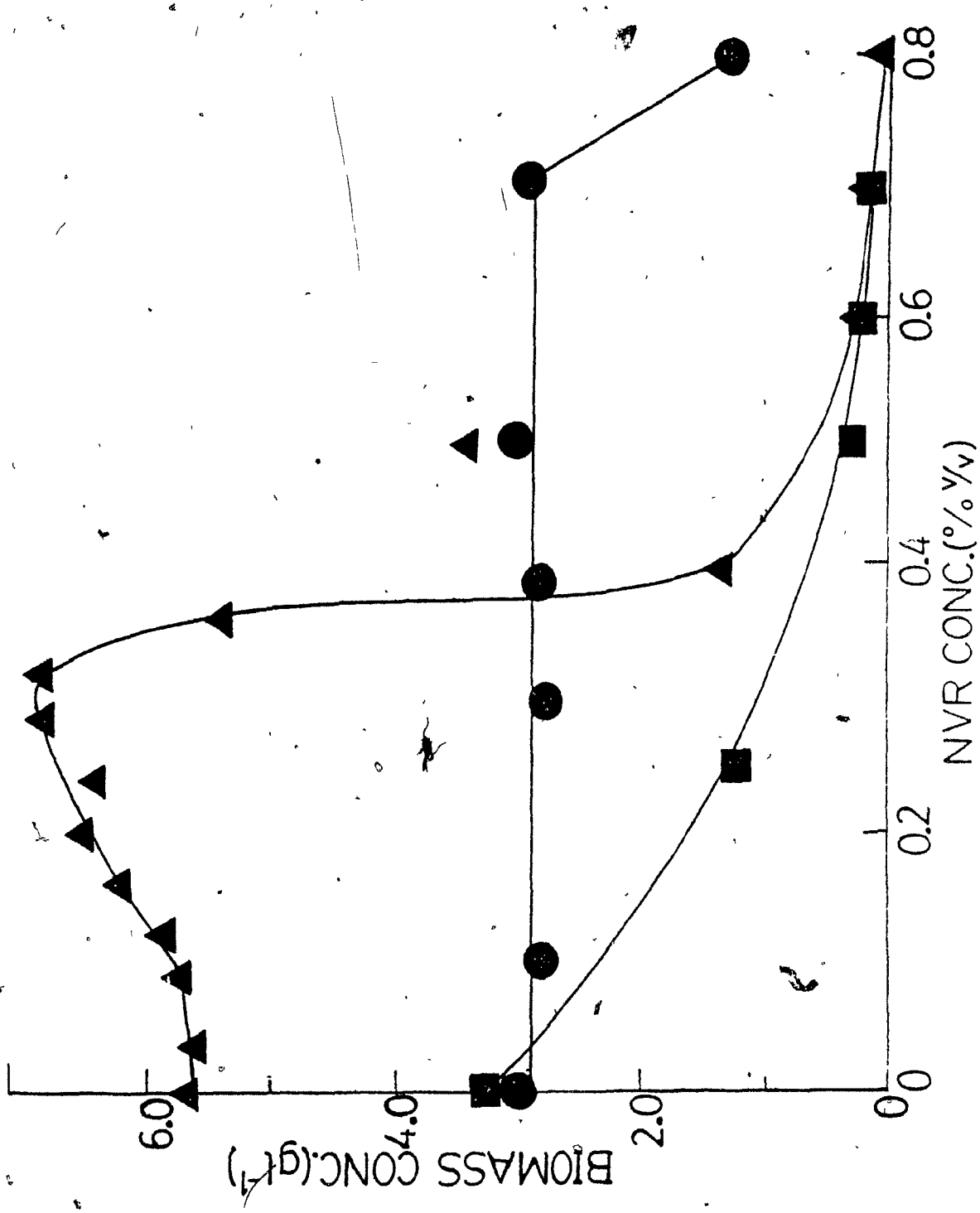

BIOMASS CONC.(gl⁻¹)
6.0
4.0
2.0
0
0
0.2
0.4
0.6
0.8
NVR CONC.(% v/v)

TABLE 4.4 THE EFFECT OF THE MAJOR NVR COMPONENTS ON THE GROWTH OF ISOLATE 1

NVR COMPONENTS	CONCENTRATION (% w/v)	BIOMASS DRY WT. (gl^{-1})	FINAL pH	GROWTH EFFECT
CONTROL	0	1.96	7.6	-
1,2 CYCLOHEXANEDIOL (CIS + TRANS)	0.6	1.95	7.9	NONE
TRACE METAL 1	-	1.68	7.5	NONE
TRACE METAL 2	-	1.85	7.6	NONE
6-HEXANOLACTONE	0.6	0.11	6.5	INHIBITORY
BUTANOIC ACID	0.6	0.10	6.4	INHIBITORY
PENTANOIC ACID	0.6	0.02	6.4	INHIBITORY
HEXANOIC ACID	0.6	0.15	6.4	INHIBITORY
SUCCINIC ACID	0.6	3.56	9.5	STIMULATORY
GLUTARIC ACID	0.6	3.81	9.5	STIMULATORY
ADIPIC ACID	0.6	5.48	9.2	STIMULATORY

hexanolactone greatly inhibited growth of Isolate 1. These results were also found with T. petrophilum (Table 4.5), B. subtilis (Table 4.6), and X. campestris ATCC 13951 (Table 4.7) with several exceptions. The growth of T. petrophilum and B. subtilis was not totally prevented by 0.6 % w/v 6-hexanolactone. Growth of T. petrophilum was only slightly stimulated by dicarboxylic acids while B. subtilis and X. campestris were not stimulated at all by dicarboxylic acids in a 0.6 % w/v concentration.

All NVR components appeared to inhibit emulsifier production by T. petrophillum. Trace metal #1 and 1,2-cyclohexanediol were the least inhibitory to emulsifier production.

Where growth occurred, the only NVR components that had an effect on biosurfactant production by B. subtilis were the trace metals. Trace metal #1 appeared to inhibit biosurfactant production while trace metal #2 was stimulatory.

4.8.2 The Effects of the Major Toxic NVR Components on the Growth of Microorganisms

Figure 4.20 shows the effects of NVR and pentanoic acid on the growth of 3 microorganisms. Pentanoic acid was toxic at a lower concentration than was NVR. For each organism, the pentanoic acid I_2 concentration was at least 50 % less than the corresponding NVR I_2 concentration. The I_1 concentration for Isolate 1 was much less than that of P.

TABLE 4.5 THE EFFECT OF MAJOR NVR COMPONENTS ON GROWTH AND BIOEMULSIFIER PRODUCTION BY *I. PETROPHILIUM*

NVR COMPONENT	CONCENTRATION (% w/v)	BIOMASS ($g l^{-1}$)	TOTAL PROTEIN ($g l^{-1}$)	FINAL pH	GROWTH EFFECT	% EMULSION
CONTROL 1◆	-	0.39	0.15	5.7	-	0
CONTROL 2◇	-	1.37	0.49	5.0	-	41
1,2 CYCLOHEXANEDIOL (CIS +TRANS)	0.6	1.01	0.30	5.2	NONE	28
TRACE METAL 1	-	1.41	0.59	5.0	NONE	5
TRACE METAL 2	-	1.57	0.60	5.1	NONE	33
6-HEXANOLACTONE	0.6	0.98	0.39	4.5	INHIBITORY	3
BUTANOIC ACID	0.6	0.35	0.12	5.9	INHIBITORY	0
GLUTARIC ACID	0.6	1.91	0.67	6.0	STIMULATORY	8
ADIPIC ACID	0.6	1.55	0.52	5.7	NONE	4

◆ : ANALYSED AFTER INOCULATION BUT BEFORE GROWTH TOOK PLACE
◇ : ANALYSED AFTER GROWTH TOOK PLACE

TABLE 4.6 THE EFFECT OF MAJOR NVR COMPONENTS ON GROWTH AND BIOSURFACTANT PRODUCTION BY *B. SUBTILIS*

NVR COMPONENT	CONCENTRATION (% v/v)	BIOMASS (gl^{-1})	TOTAL PROTEIN (gl^{-1})	FINAL pH	GROWTH EFFECT	SURFACE TENSION (mNm^{-1})
CONTROL 1 ◆	-	0.09	0.03	6.0	-	43
CONTROL 2 ◇	-	0.73	0.29	6.8	-	37
1,2 CYCLOHEXANEDIOL (CIS +TRANS)	0.6	0.52	0.34	7.1	NONE	40
TRACE METAL 1	-	0.73	0.36	6.8	NONE	42
TRACE METAL 2	-	0.62	0.41	7.0	NONE	33
6-HEXANOLACTONE	0.6	0.23	0.17	5.4	INHIBITORY	46
BUTANOIC ACID	0.6	0.08	0.05	6.0	INHIBITORY	43
ADIPIC ACID	0.6	0.53	0.34	6.9	NONE	39

◆ :ANALYSED AFTER INOCULATION BUT BEFORE GROWTH TOOK PLACE
◇ :ANALYSED AFTER GROWTH TOOK PLACE

TABLE 4.7 THE EFFECT OF MAJOR NVR COMPONENTS ON GROWTH OF *X. CAMPESTRIS* ATCC 13951

NVR COMPONENT	CONCENTRATION (% w/v)	TOTAL PROTEIN (gl^{-1})	FINAL pH	GROWTH EFFECT
CONTROL 1 ◆	-	0.04	5.5	-
CONTROL 2 ◇	-	0.56	4.1	-
1,2 CYCLOHEXANEDIOL (CIS + TRANS)	0.6	0.58	4.9	NONE
TRACE METAL 1	-	0.55	4.1	NONE
TRACE METAL 2	-	1.05	3.0	STIMULATORY
6-HEXANOLACTONE	0.6	0.08	4.9	NEGATIVE
BUTANOIC ACID	0.6	0.07	5.9	NEGATIVE
PENTANOIC ACID	0.6	0.10	6.0	NEGATIVE
HEXANOIC ACID	0.6	0.07	5.9	NEGATIVE
ADIPIC ACID	0.6	0.49	4.8	NEGATIVE

◆ : ANALYSED AFTER INOCULATION BUT BEFORE GROWTH TOOK PLACE
◇ : ANALYSED AFTER GROWTH TOOK PLACE

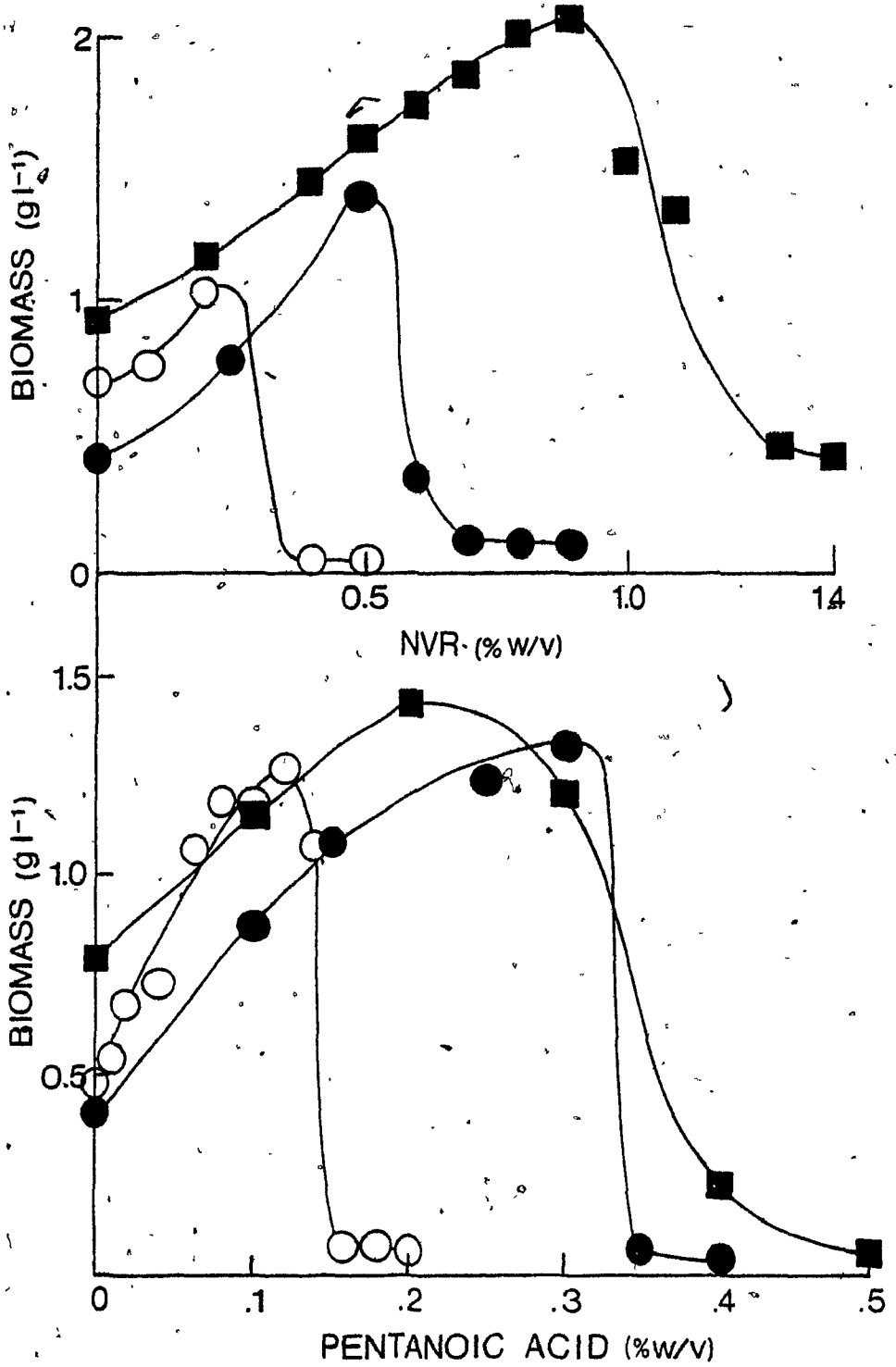
Figure 4.20. A comparison of the effect of NVR with that of pentanoic acid on the growth of microorganisms.

(○) A. eutrophus.
(●) Isolate 1.
(■) P. cepacia.

A. eutrophus was grown on nutrient broth medium at 26 °C for 28 h when grown with NVR and 43 h when grown with pentanoic acid.

Isolate 1 was grown on nutrient broth medium at 37 °C for 60 h when grown with NVR and 48 h when grown with pentanoic acid.

P. cepacia was grown on nutrient broth medium at 26 °C for 14 h when grown with NVR and 12 h when grown with pentanoic acid.


BIOMASS (g l⁻¹)
2
1
0
0.5
1.0
1.4
NVR (% w/v)
BIOMASS (g l⁻¹)
1.5
1.0
0.5
0
.1
.2
.3
.4
.5
PENTANOIC ACID (%w/v)

cepacia for NVR but almost equal for pentanoic acid. These results indicated that a component other than the monocarboxylic acids was responsible for inhibition of growth (if it occured) well below the toxic NVR concentration. Figures 4.21 and 4.22 indicate that this component was 6-hexanolactone. This component had a negative effect on the growth of both Isolate 1 and P. cepacia well below its lethal (I_2) concentration. This effect was more pronounced with P. cepacia.

Figure 4.21 also shows that a higher concentration (w/v) of hexanoic acid than butanoic acid was needed to prevent the growth of Isolate 1. Even less pentanoic acid was required. Similar amounts (in terms of both weight and molar concentrations) of all three monocarboxylic acids were required to prevent the growth of P. cepacia.

4.8.3 Use of the Major Toxic NVR Components by P. cepacia and their Effects on its Growth

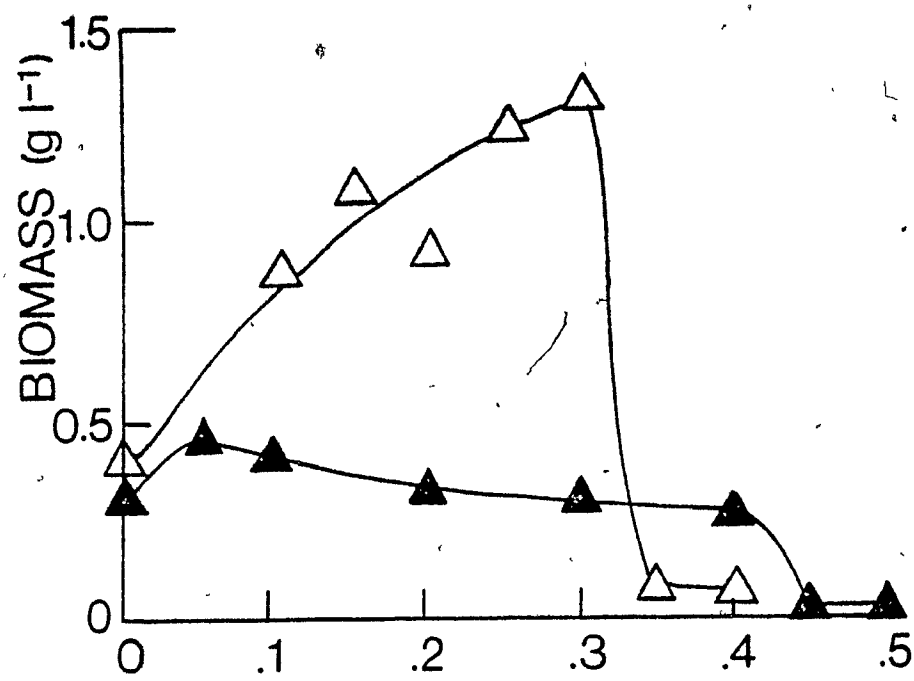
As shown in Figure 4.23, NVR stimulated the growth of P. cepacia until close to the lethal concentration. Although many NVR components are acidic and there was increased growth, the medium pH was raised to a lesser extent when NVR had been added to the medium.

Sublethal concentrations of 6-hexanolactone, above 0.2 %, inhibited the growth of P.cepacia (Fig. 4.24). The pH did not rise to as great an extent as the control when 6-hexanolactone was in the medium. Although Table 4.2 shows

Figure 4.21. Effects of the major toxic NVR components on the growth of isolate 1.

(□) Growth with NVR for 60 h.
(△) Growth with butanoic acid for 24 h.
(●) Growth with pentanoic acid for 48 h.
(○) Growth with hexanoic acid for 18 h.
(▲) Growth with 6-hexanolactone for 24 h.

All experiments were conducted with nutrient broth medium at 37 °C.


BIOMASS (g l⁻¹)
1.5
1.0
0.5
0
0
.1
.2
.3
.4
.5

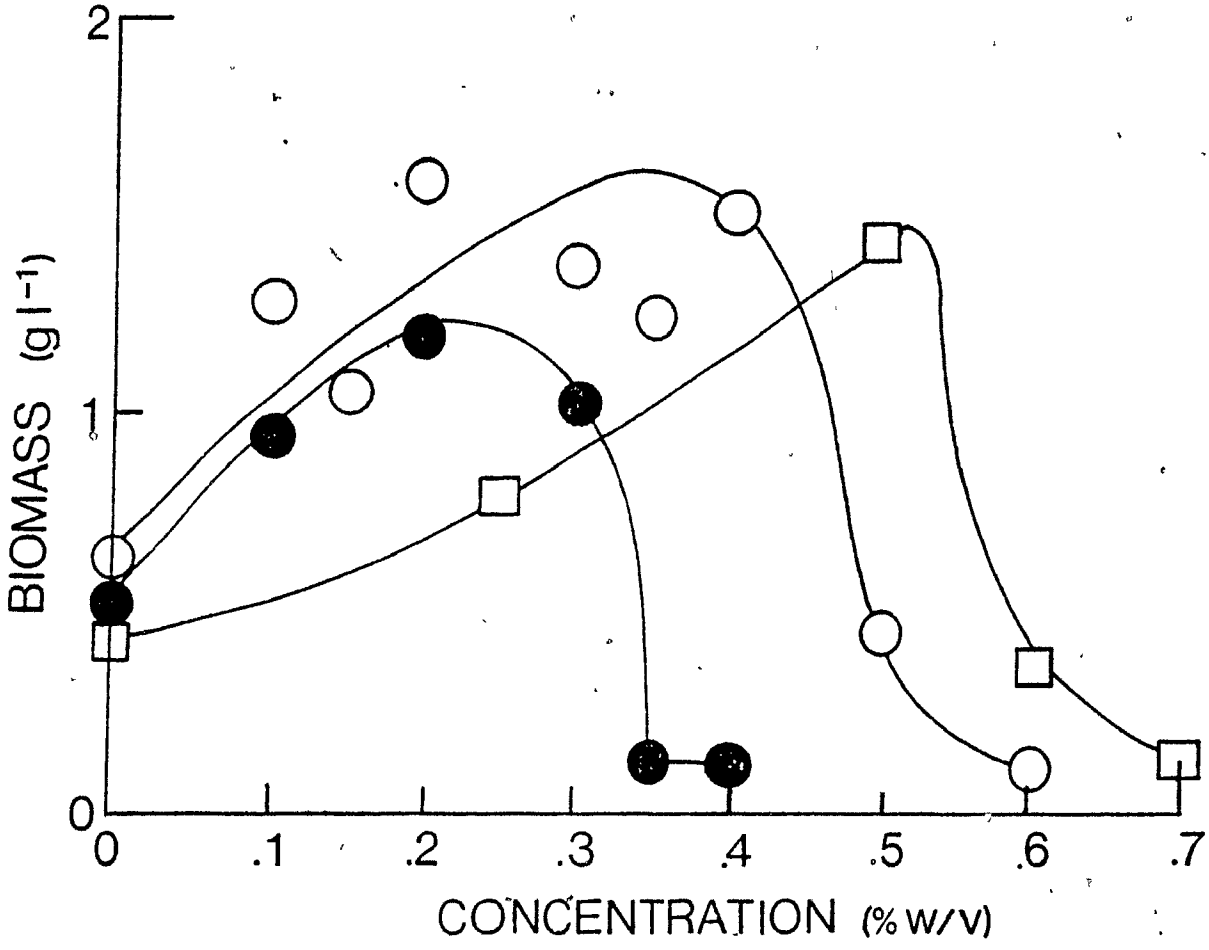

BIOMASS (g l⁻¹)
2
1
0
0
.1
.2
.3
.4
.5
.6
.7
CONCENTRATION (% W/V)

Figure 4.22. Effects of the major toxic NVR components on the growth of P. cepacia.

(●) NVR.
(○) Butanoic acid.
(▲) Pentanoic acid.
(□) Hexanoic acid.
(■) 6-Hexanolactone

All experiments were conducted with nutrient broth medium at 26 °C.

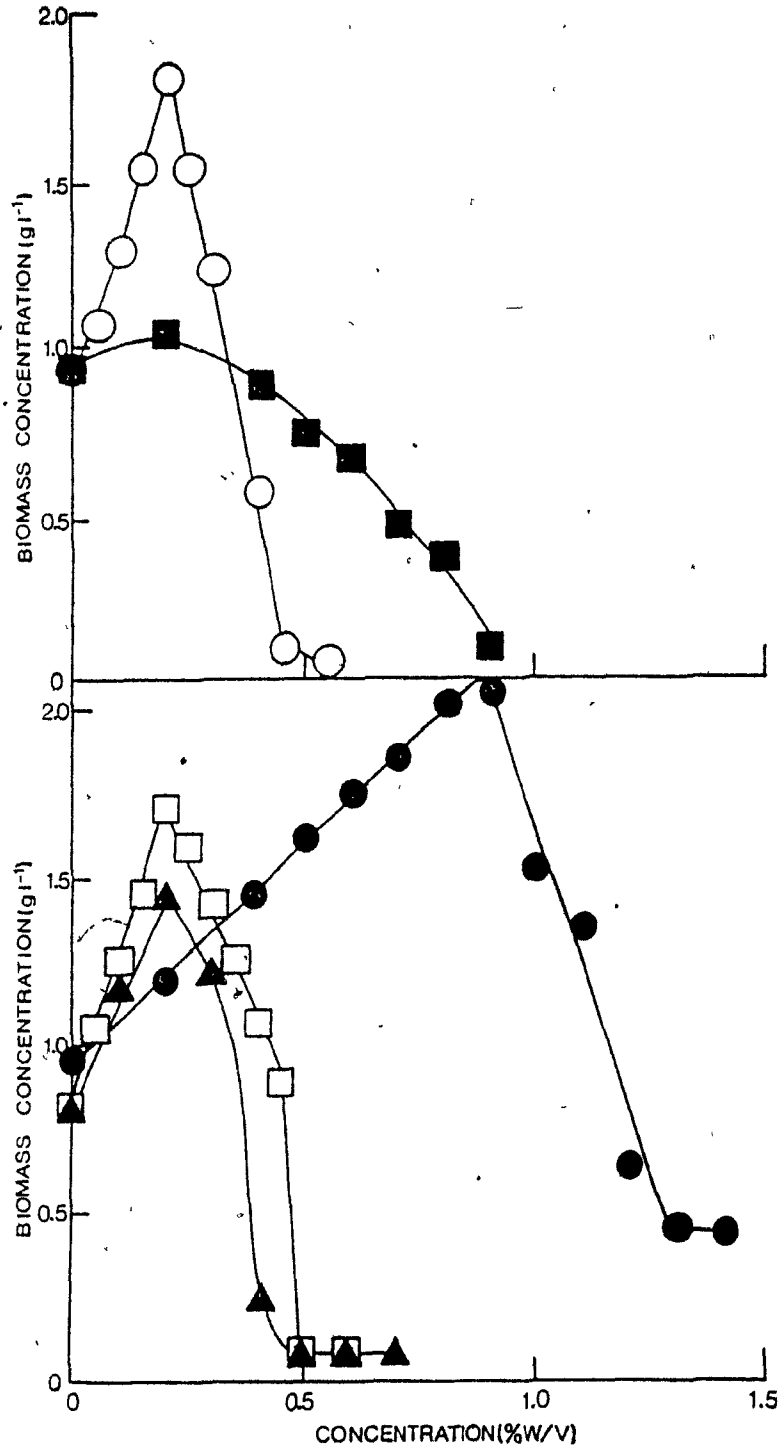

2.0
1.5
1.0
0.5
0
BIOMASS CONCENTRATION (g l⁻¹)
2.0
1.5
1.0
0.5
0
BIOMASS CONCENTRATION (g l⁻¹)
0
0.5
1.0
1.5
CONCENTRATION (%W/V)

Figure 4.23. Effects of NVR on growth of P. cepacia. The culture was grown on nutrient broth medium at 26 °C for 14 h.

(■) Biomass dry weight.
(○) pH.

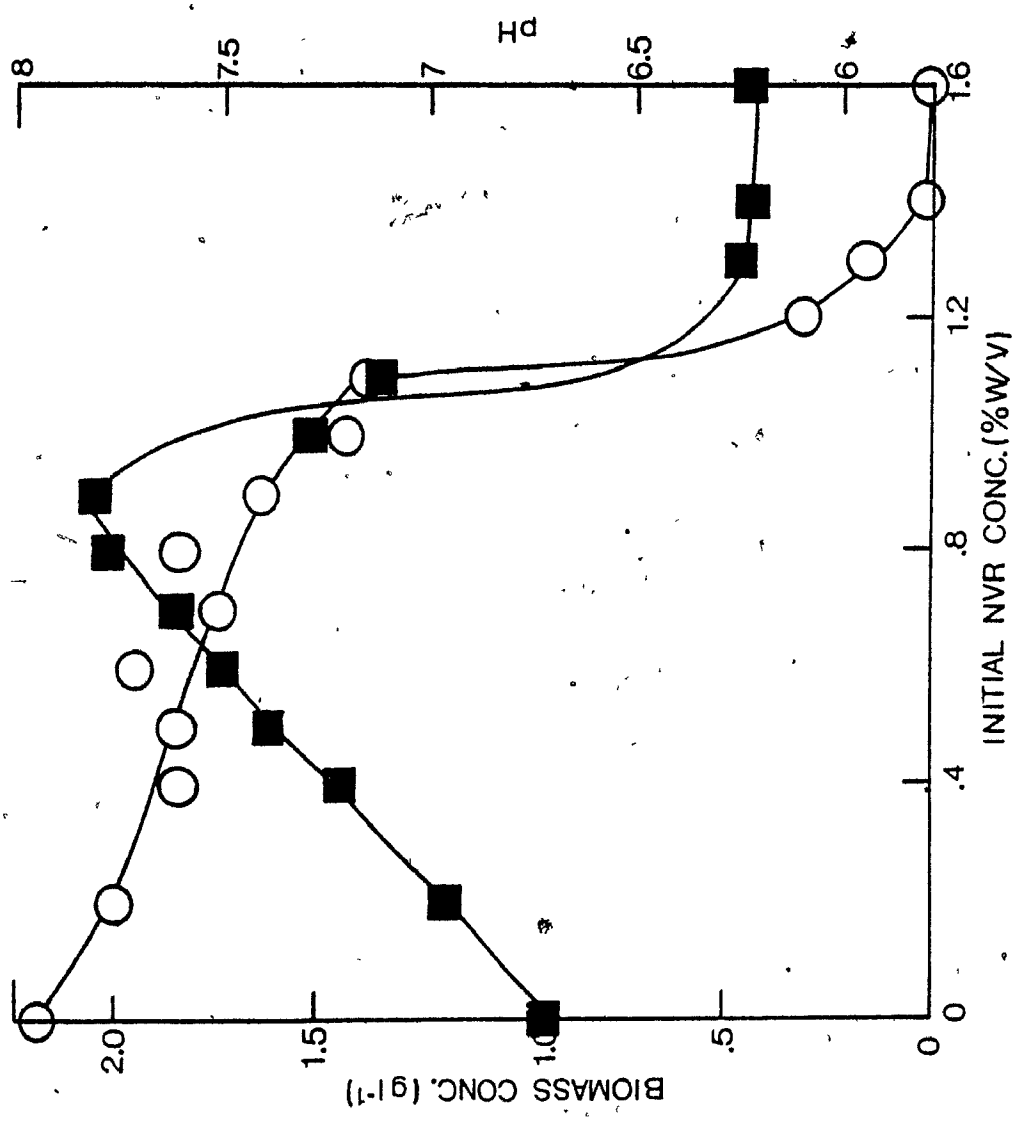
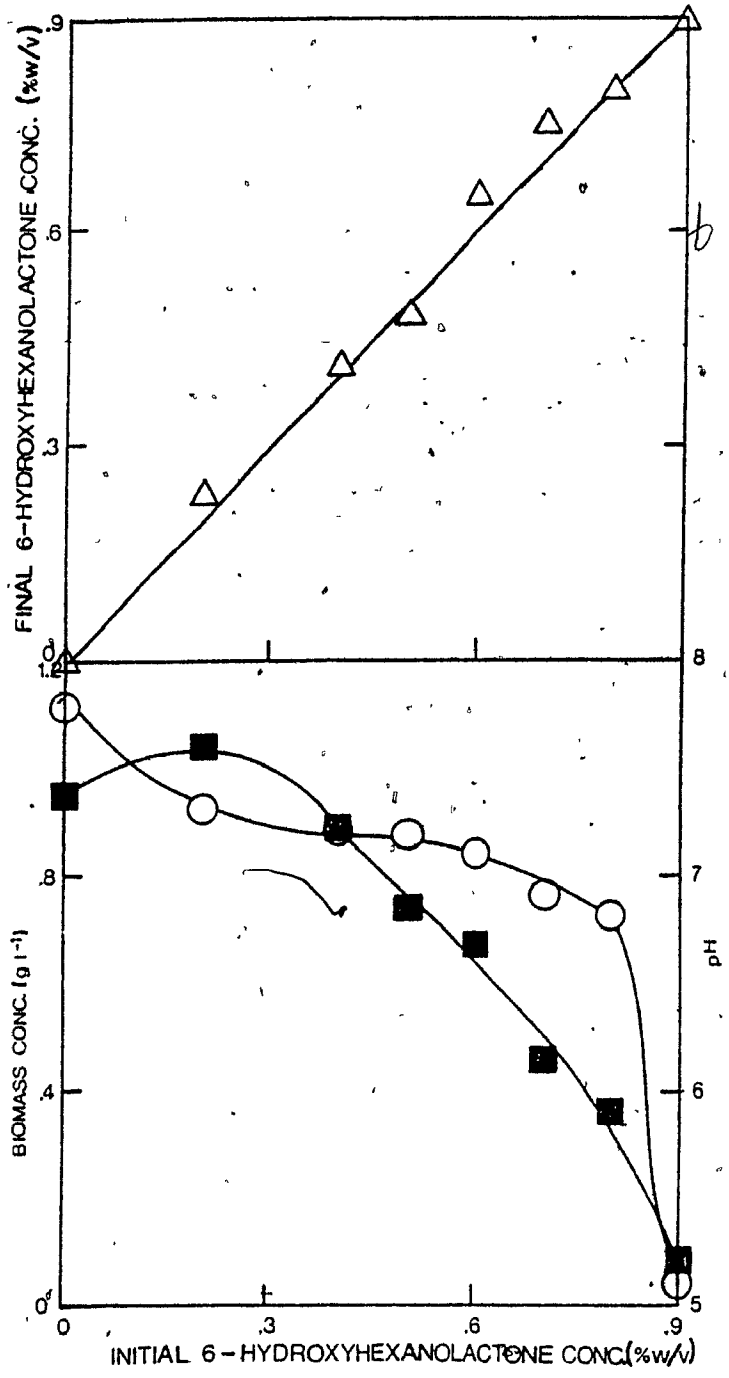

pH
8
7.5
7
6.5
6
BIOMASS CONC. (g l⁻¹)
2.0
1.5
1.0
.5
0
INITIAL NVR CONC.(%W/V)
1.6
1.2
.8
.4
0

Figure 4.24. Effects of 6-hexanolactone on growth of P. cepacia. The culture was grown on nutrient broth medium at 26 °C.

(■) Biomass dry weight.
(○) pH after growth.
(△) Concentration of 6-hexanolactone after growth.


FINAL 6-HYDROXYHEXANOLACTONE CONC. (%w/v)
.9
.6
.3
0
1.2
.8
.4
0
BIOMASS CONC. (g l⁻¹)
8
7
6
5
pH
0
.3
.6
.9
INITIAL 6-HYDROXYHEXANOLACTONE CONC.(%w/v)

that P. cepacia could grow using 6-hexanolactone as its sole source of carbon, the microorganism did not metabolize any in this experiment.

As shown in Figures 4.25, 4.26 and 4.27, butanoic, pentanoic and hexanoic acids, all stimulated the growth of P. cepacia on nutrient broth. Their presence (after the initial medium pH adjustment) had little effect on the final pH. All 3 monocarboxylic acids were utilized by P. cepacia in these experiments.

An unidentified microorganism (Gram positive coccus) was grown in axenic culture on nutrient broth in the presence of varying concentrations of pentanoic acid. As indicated by Figure 4.28, growth of this organism was greatly stimulated by pentanoic acid, yet it was not metabolized. This raised the question of whether monocarboxylic acids could stimulate the growth of P. cepacia even when not used as a carbon or energy source. The butanoic acid analogue, 2-methylbutanoic was employed in the hope that it would not be used by P. cepacia. However, as seen in Figure 4.29, P. cepacia metabolized 2-methylbutanoic acid. Its growth was stimulated by 2-methylbutanoic acid which was not as toxic (in terms of I_1 or I_2 values) as butanoic acid even when expressed as a molar concentration.

Figure 4.25. Effects of butanoic acid on growth of P. cepacia. The cuture was grown on nutrient broth medium at 26 °C.

(■) Biomass dry weight.
(○) pH after growth.
(△) Butanoic acid concentration after growth. The broken line represents the initial concentration.

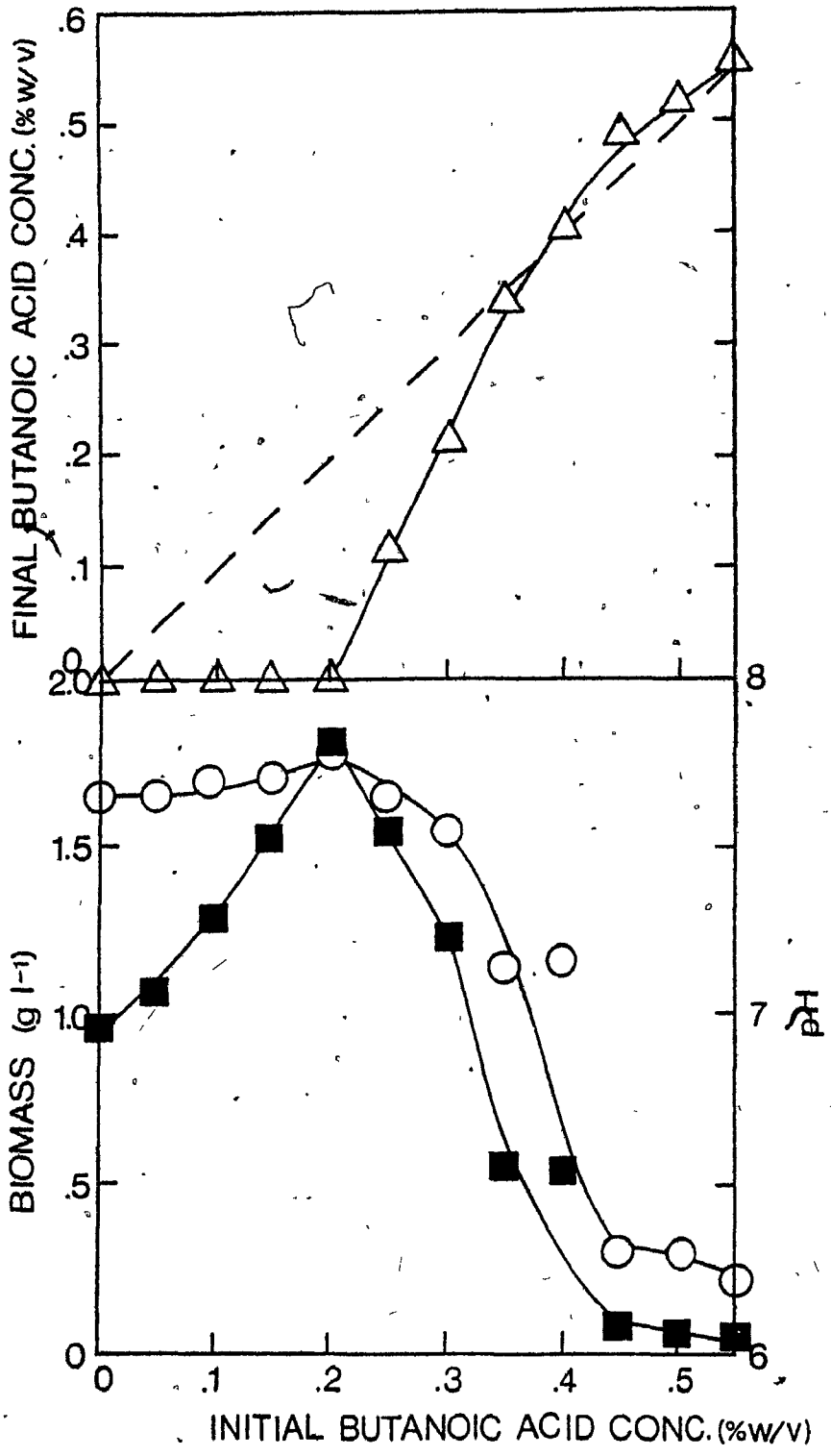

FINAL BUTANOIC ACID CONC. (%w/v)
.6
.5
.4
.3
.2
.1
0
2.0
8
BIOMASS (g l⁻¹)
1.5
1.0
.5
0
7
pH
6
0
.1
.2
.3
.4
.5
INITIAL BUTANOIC ACID CONC. (%W/V)

Figure 4.26. Effects of pentanoic acid on growth of P. cepacia. The culture was grown on nutrient broth medium at 26 °C for 12 h.

(■) Biomass dry weight.
(○) pH after growth.
(△) Pentanoic acid concentration after growth. The broken line represents the initial pentanoic acid concentration.

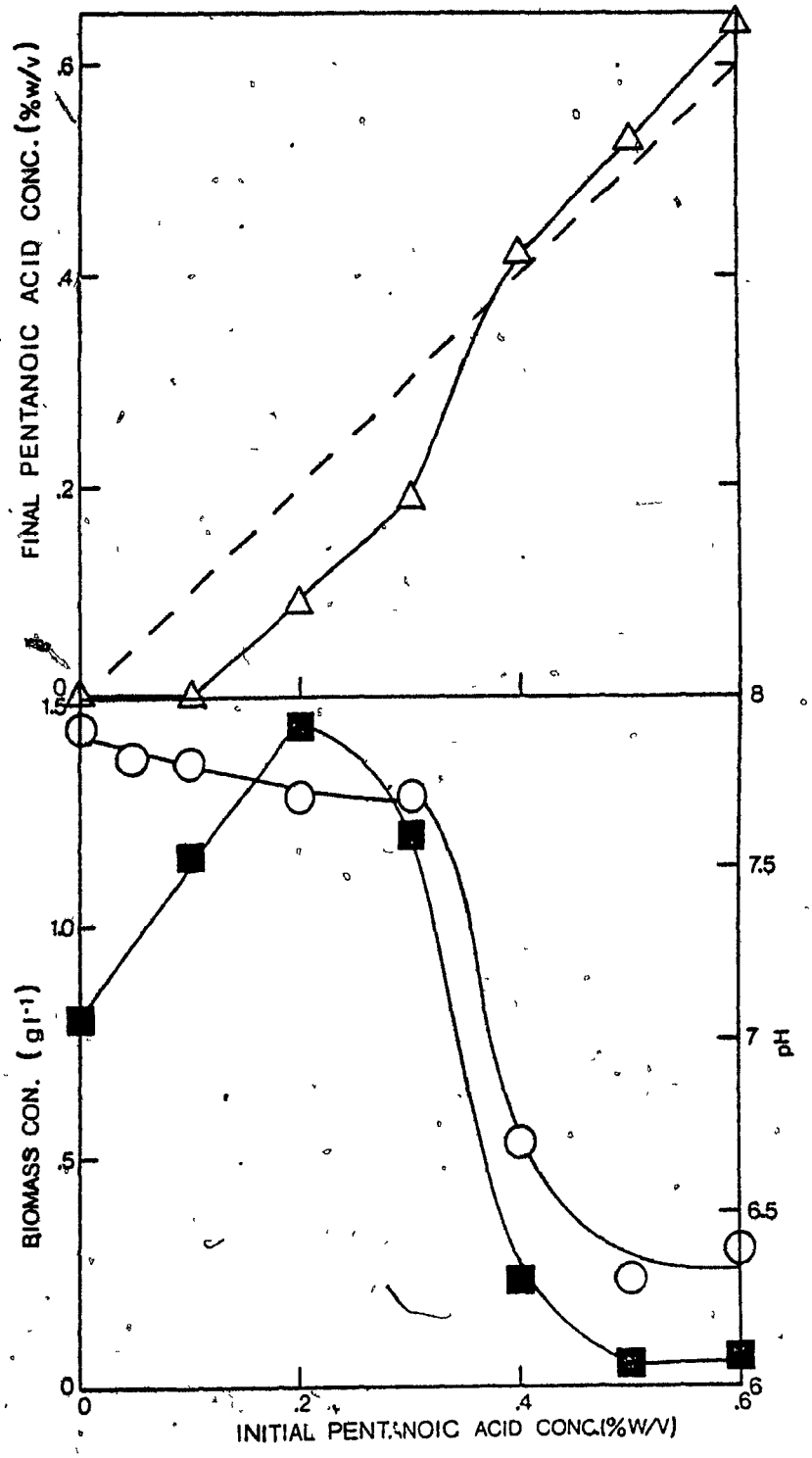

FINAL PENTANOIC ACID CONC. (%w/v)
.6
.4
.2
0
1.5
1.0
.5
0
BIOMASS CON. (g l⁻¹)
8
7.5
7
6.5
6
pH
0
.2
.4
.6
INITIAL PENTANOIC ACID CONC.(%W/V)

Figure 4.27. Effects of hexanoic acid on growth of P. cepacia. The culture was grown on nutrient broth medium at 26 °C for 48 h.
(■) Biomass dry weight.
(○) pH after growth.
(△) Hexanoic acid concentration after growth. The broken line represents the initial hexanoic acid concentration.

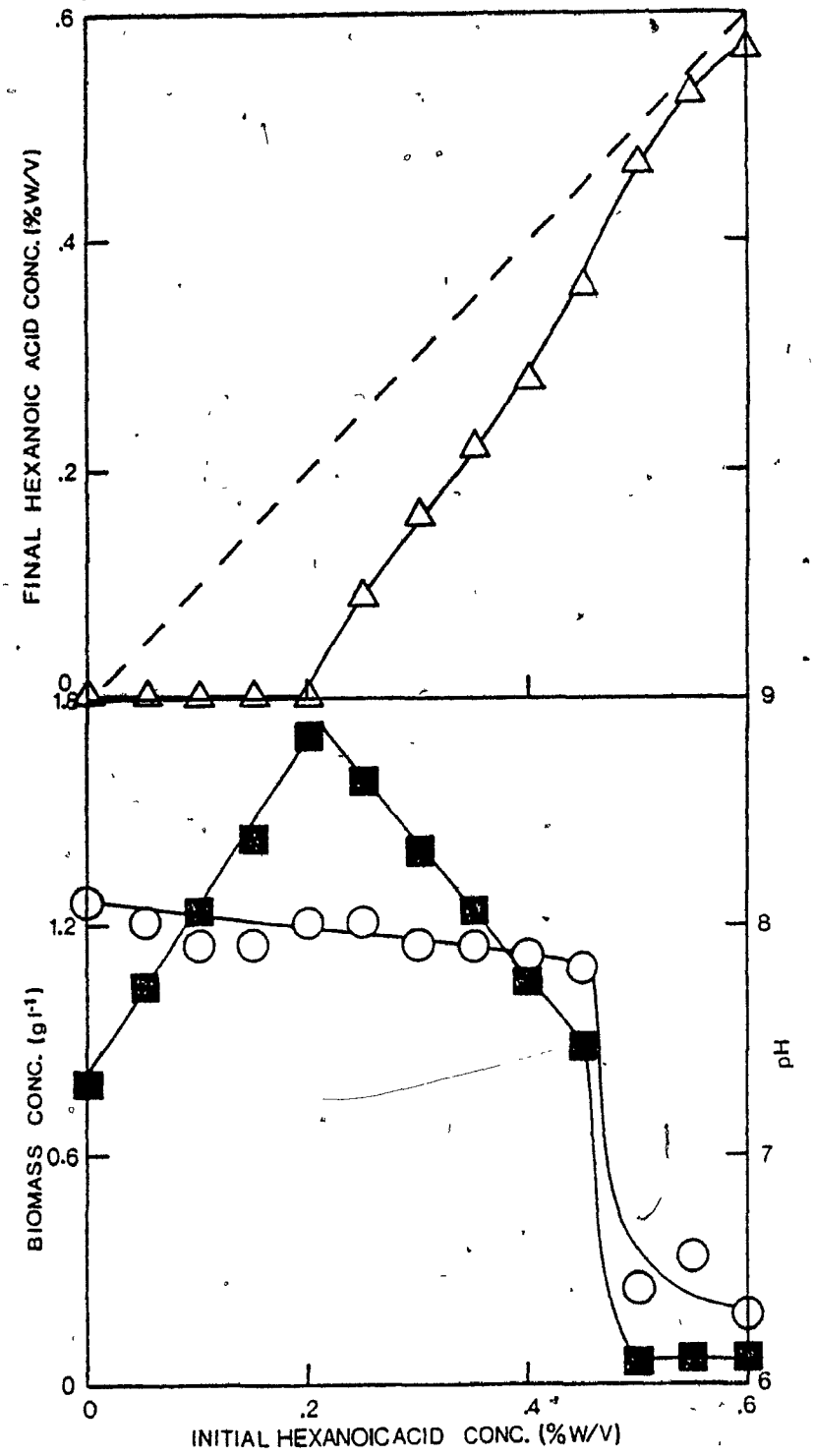

FINAL HEXANOIC ACID CONC. (%W/V)
.6
.4
.2
0
1.8
1.2
0.6
0
9
8
7
6
pH
BIOMASS CONC. (g l⁻¹)
0
.2
.4
.6
INITIAL HEXANOIC ACID CONC. (%W/V)

Figure 4.28. Effects of pentanoic acid on growth of an unidentified coccus. The culture was grown on nutrient broth medium at 26 °C for 36 h.

(■) Biomass dry weight.
(○) pH after growth.
(△) Pentanoic acid concentration after growth.

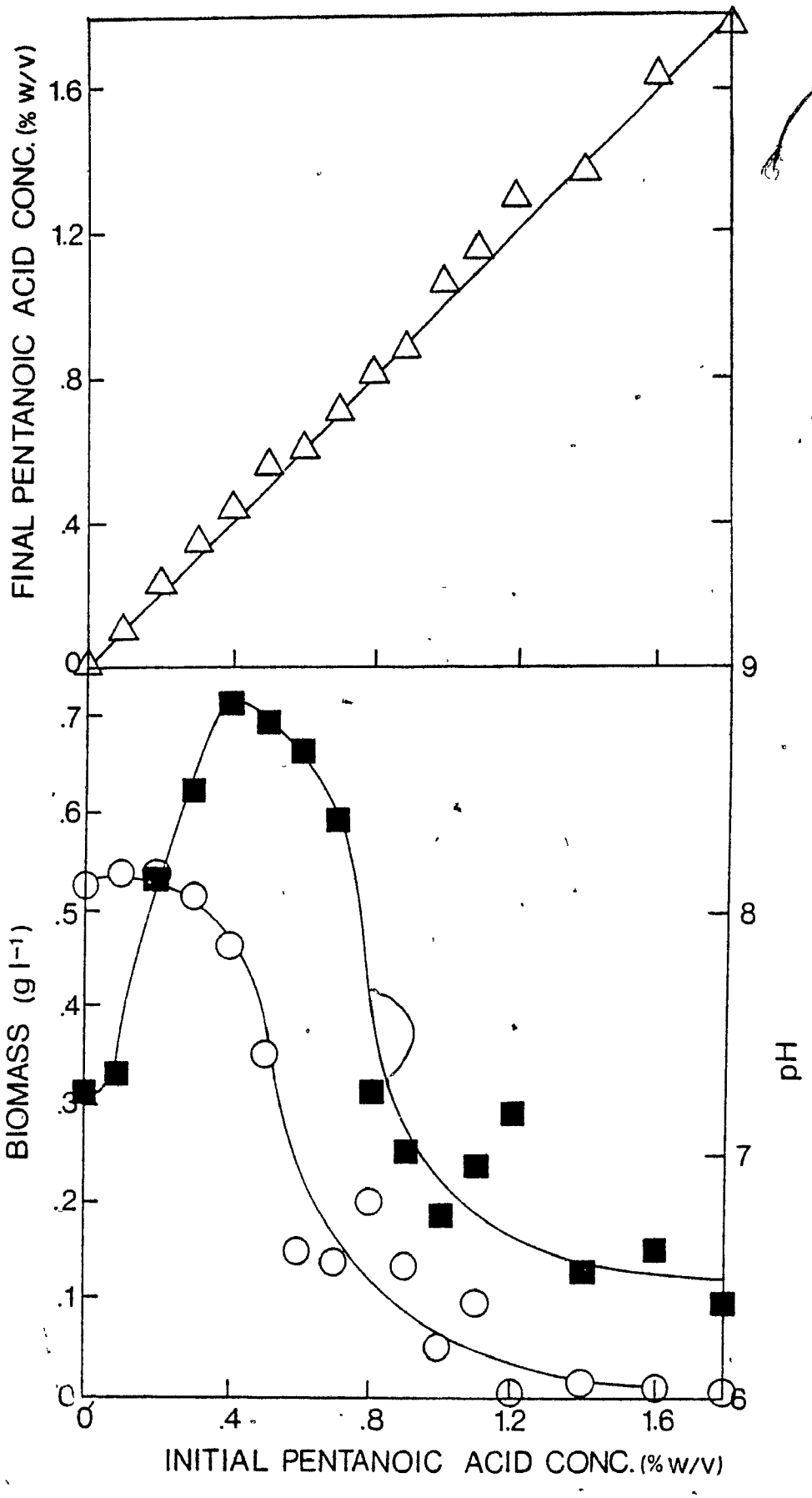
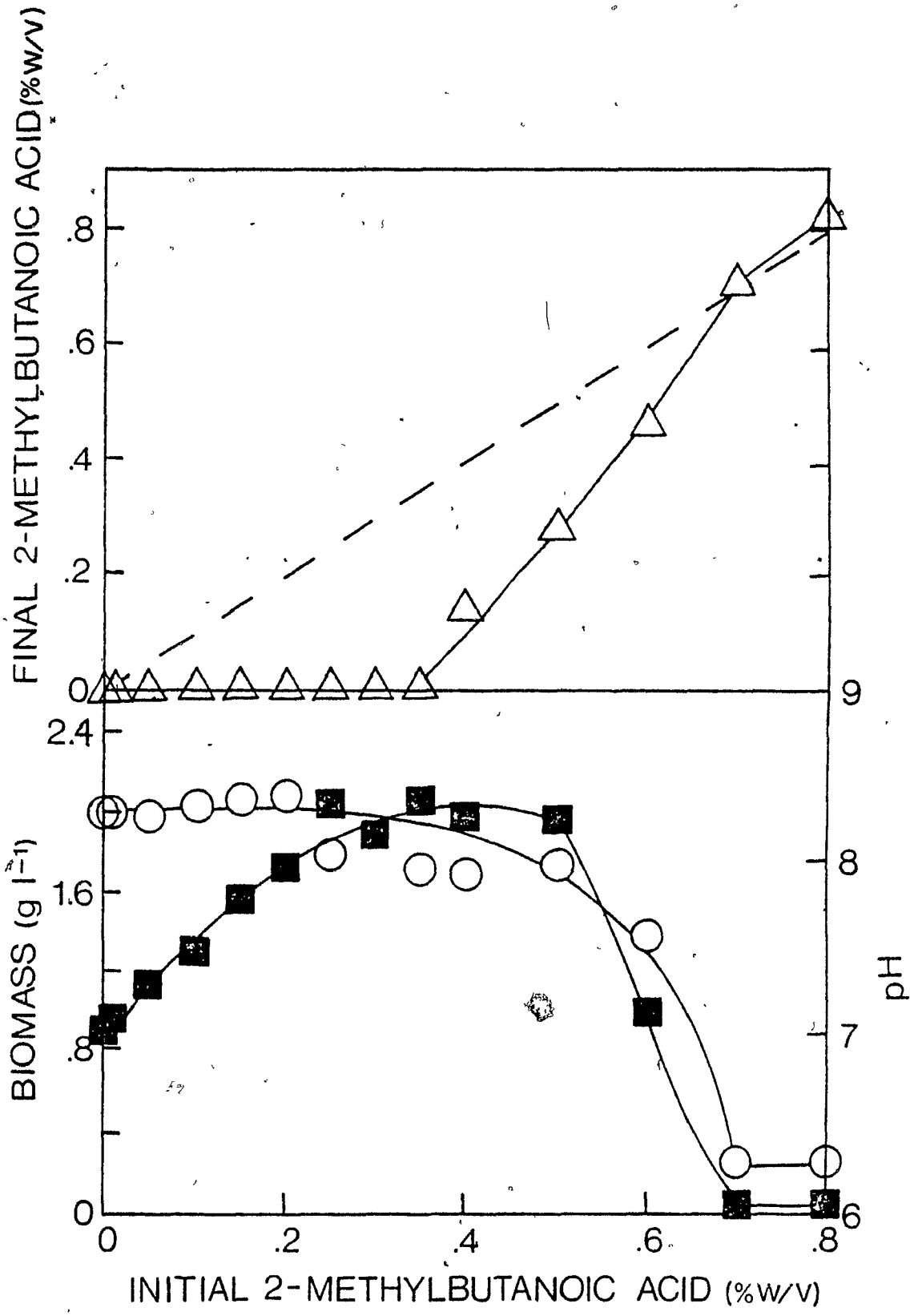

FINAL PENTANOIC ACID CONC. (% w/v)
1.6
1.2
.8
.4
0
9
.7
.6
.5
.4
.3
.2
.1
0
BIOMASS (g l⁻¹)
8
7
6
pH
0
.4
.8
1.2
1.6
INITIAL PENTANOIC ACID CONC. (% w/v)

Figure 4.29. Effects of 2-methylbutyric acid on growth of P. cepacia. The culture was grown on nutrient broth at 26 °C for 24 h.

(■) Biomass dry weight.
(○) pH after growth.
(△) 2-Methylbutyric acid concentration after growth. The broken line represents the initial concentration.


FINAL 2-METHYLBUTANOIC ACID (%W/V)
.8
.6
.4
.2
0
9
2.4
BIOMASS (g l⁻¹)
1.6
.8
0
8
7
6
pH
0
.2
.4
.6
.8
INITIAL 2-METHYLBUTANOIC ACID (%W/V)

4.9 Growth of P. cepacia on NVR in a Single-stage Chemostat

In order to prove conclusively that microorganisms can grow on NVR, P. cepacia was grown in a carbon-limited chemostat with NVR as its sole source of carbon and energy. The dilution rate was constant at 0.14 h^{-1}. Steady-state growth was achieved at a feed concentration of up to 17.8 gl^{-1} NVR. Above this level oxygen limitation occurred due to the mass transfer limitations of the fermentor.

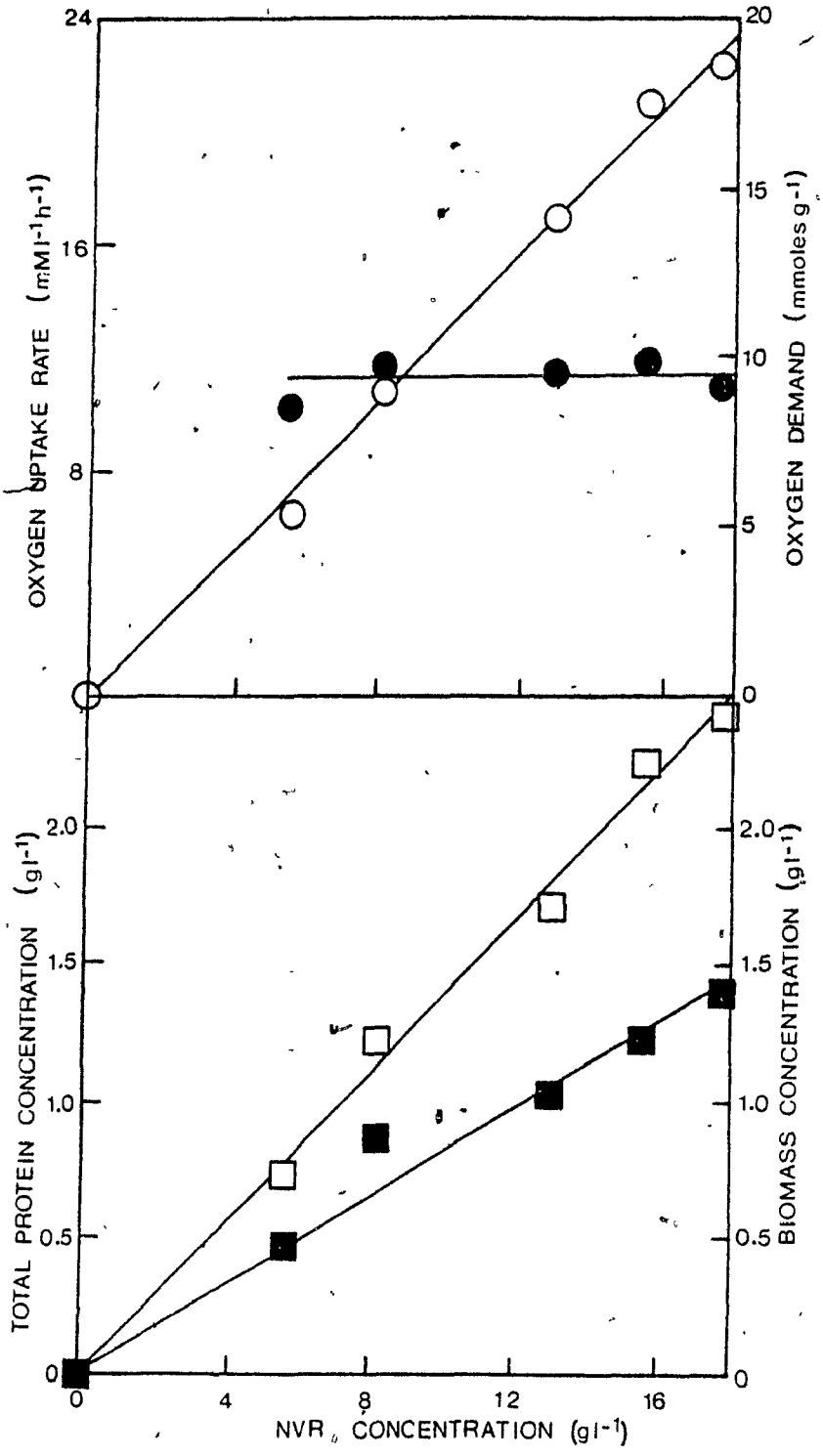
A statistical analysis was done to determine whether the oxygen uptake rate, the biomass concentration and the protein concentration were linearly related to the NVR concentration. All three relationships were highly significant ($P < 0.0005$) using Students t-distribution test with 4 degrees of freedom. It could therefore be concluded that as the NVR concentration was increased (to 17.8 g l^{-1}), the microbe was using it as its source of carbon and energy.

As seen in Figure 4.30, the yield ($Y_{X/S}$) was 13.6 grams of biomass for every 100 grams of NVR supplied. When the water content of NVR was subtracted, the calculated yield ($Y_{X/S}$) was 18 %. Under carbon-limited conditions, the protein content of P. cepacia was 60 % when grown on NVR using $(NH_4)_2SO_4$ as the nitrogen source. The Q_{O_2} was 9.2 mM $g^{-1} \cdot h^{-1}$ and 9.5 millimoles of O_2 were consumed for every gram of NVR supplied under carbon-limited conditions at this dilution rate.

Figure 4.30. Growth of P. cepacia on NVR in a single-stage chemostat.

(■) Total cellular protein.
(□) Biomass dry weight.
(○) Oxygen uptake rate.
(●) Oxygen demand (amount of O_2 required to use a given quantity of NVR).

D = 0.14 h^{-1} pH = 7.0
T = 30 °C Agitation = 660 RPM
Air flow rate = 0.83 VVM
Medium: MSM (0.40 % $(NH_4)_2SO_4$)


OXYGEN UPTAKE RATE (mMl⁻¹h⁻¹)
OXYGEN DEMAND (mmoles g⁻¹)
TOTAL PROTEIN CONCENTRATION (gl⁻¹)
BIOMASS CONCENTRATION (gl⁻¹)
NVR CONCENTRATION (gl⁻¹)
0
4
8
12
16
24
20
15
10
5
2.0
1.5
1.0
0.5

At a lower dilution rate (.09 h^{-1}) steady-state was achieved at an NVR feed concentration of 20.5 g l^{-1}. This could be attained because the oxygen requirements were less at the lower dilution rate. The biomass yield was the same as at the 0.14 h^{-1} dilution rate.

4.10 Balancing the Carbon to Nitrogen Ratio in the Mineral Salts Medium of P. cepacia

The amount of ammonium needed for the complete utilization by P. cepacia of a given amount of fructose was determined in a single-stage chemostat (D = 0.14 h^{-1}). As seen in Figure 4.31. above a C/N ratio of 15 moles $mole^{-1}$, there was no increase in protein production. The rate of oxygen utilization also leveled off at this point.

4.11 Batch Production of PHB by P. cepacia

Using the C/N ratio study as the basis for the design of an ammonium-limited medium, P. cepacia was grown in batch culture with fructose as the sole carbon source. As seen in Figure 4 32, when grown under these conditions, P. cepacia exhibited two linear growth phases in addition to the usual lag and exponential phases.

The rate of exponential growth could not be determined accurately since it was relatively short. However, the yield of biomass ($Y_{X/S}$) was 12.3 %. During the first linear growth phase the rate of biomass production was 0 12 g l^{-1} h^{-1} while the rate of substrate utilization was 0.69 g l^{-1} h^{-1}. The yield of biomass was 16 5 %. During the second

Figure 4.31. Effect of carbon/nitrogen ratio on growth of P. cepacia in a single-stage chemostat.

(■) Fructose.
(○) Total cellular protein.
(●) Oxygen uptake rate.

D = 0.14 h^{-1} pH = 7.0
T = 30 °C Agitation = 660 RPM
Air flow rate = 0.85 VVM
Medium: MSM (0.50 % fructose)

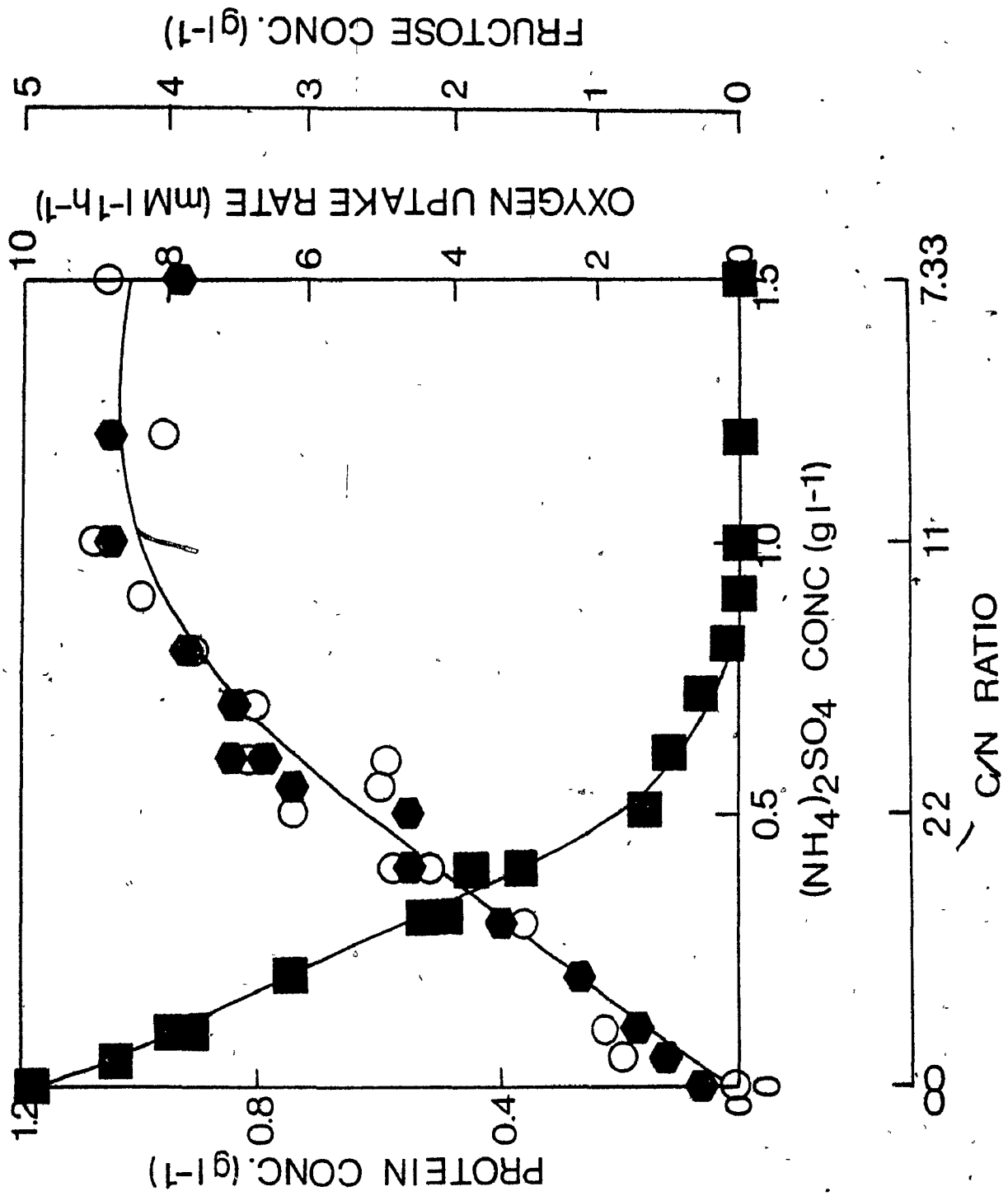
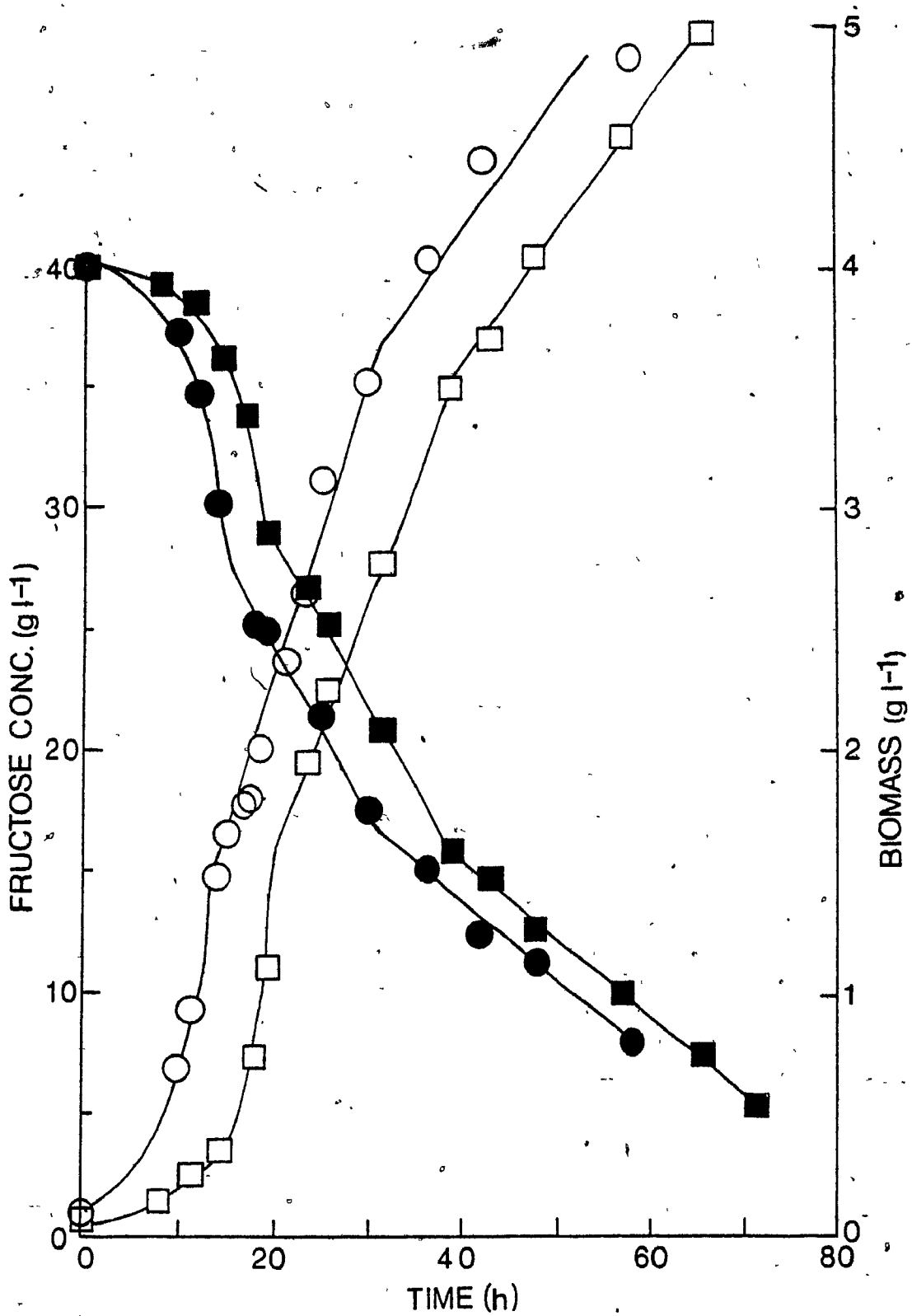

FRUCTOSE CONC. (g l-1)
0
1
2
3
4
5
OXYGEN UPTAKE RATE (mM l-1h-1)
0
2
4
6
8
10
PROTEIN CONC. (g l-1)
0.0
0.4
0.8
1.2
(NH4)2SO4 CONC (g l-1)
0.0
0.5
1.0
1.5
C/N RATIO
7.33
11
22

Figure 4.32. Growth and carbon source utilizaton by P. cepacia throughout ammonium-limited batch fermentation.

(○) Biomass dry weight of fermentation #1.
(●) Fructose concentration of fermentation #1.
(□) Biomass dry weight of fermentation #2.
(■) Fructose concentration of fermentation #2.

pH = 7.0
T = 30 °C Agitation = 660 RPM
Air flow rate = 1.7 l min^{-1}.
Medium: MSM (4.0 % fructose and 0.2 % $(NH_4)_2SO_4$)


FRUCTOSE CONC. (g l-1)
BIOMASS (g l-1)
TIME (h)
0
10
20
30
40
0
1
2
3
4
5
0
20
40
60
80

linear growth phase the rate of growth was 0.06 g l^{-1} h^{-1} while the rate of fructose utilization was 0.32 g l^{-1} h^{-1}. The yield of biomass was 16.9 %.

Figure 4.33 gives a breakdown of the biomass composition of P. cepacia throughout the fermentation that was represented by the square symbols in Figure 4.32. During the first 20 hours mostly high-protein biomass was produced. This corresponded to the period of exponential growth. For the next 20 hours mostly PHB was produced. This time period matched that of the first period of linear growth. After this, although some protein and PHB were produced, production of some other biomass component(s) predominated. This corresponded to the second period of linear growth.

Figure 4.34 shows the oxygen uptake rates of the two batch fermentations. In each case, after a period of exponential increase, the oxygen uptake rate decreased sharply. These peaks corresponded exactly with the start of the first linear growth phase, in which PHB was produced

4.12 Growth and PHB Production in a 2-Stage Chemostat

Since PHB production had been found to be separate from the exponential production of high-protein biomass, the possibility of using a 2-stage production process was investigated. The results are shown in Figures 4.35A and 4.35B and are summarized in Table 4.8 The working volume of the first stage was 0.78 l while the second stage was

Figure 4.33. The composition of P. cepacia biomass throughout ammonium-limited batch fermentation on fructose (fermentation #2 of Figure 4.32).

(▲) Total cellular protein.
(●) Total cellular PHB.
(■) Other biomass (ie. biomass other than protein and PHB).

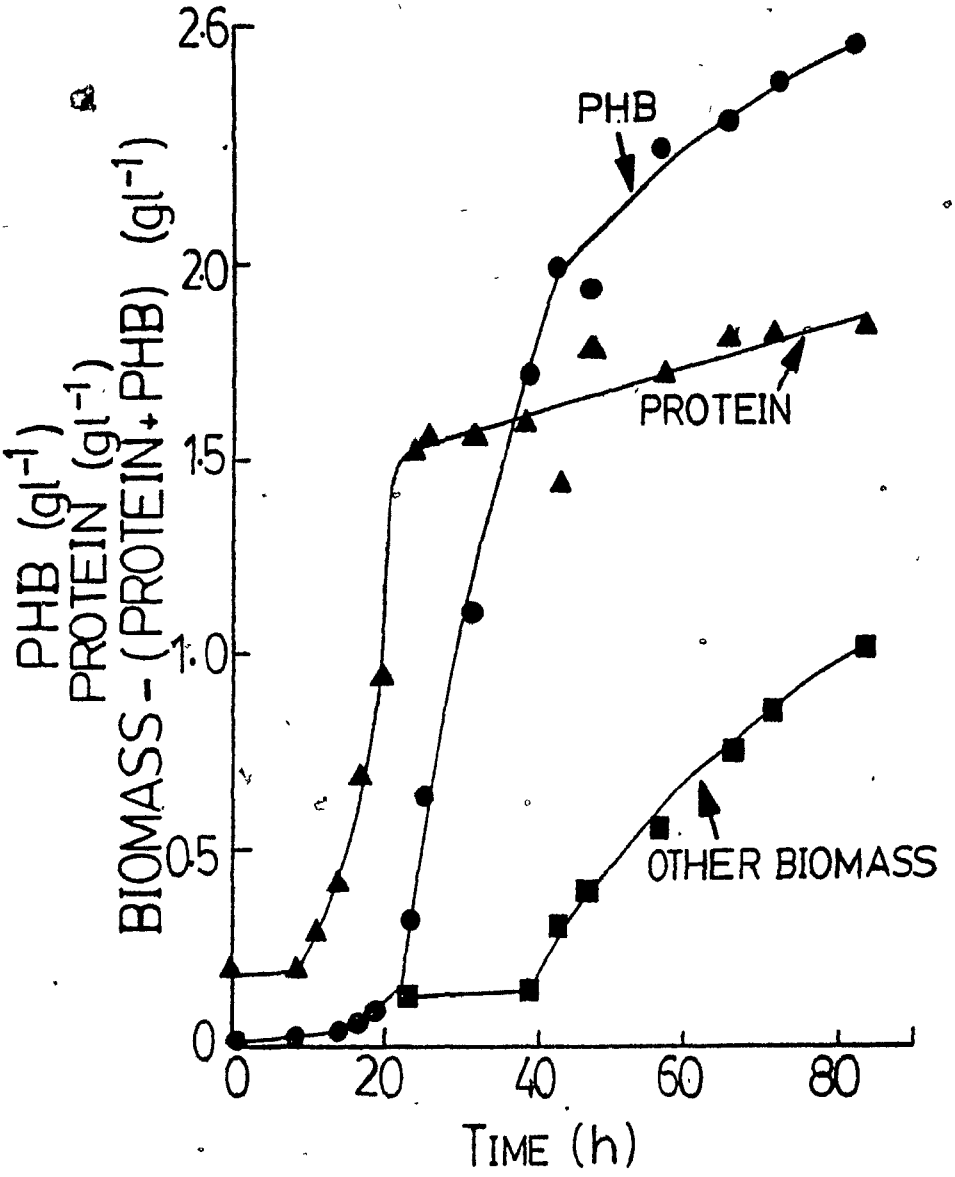

PHB
PROTEIN
OTHER BIOMASS
PHB (gl⁻¹)
PROTEIN (gl⁻¹)
BIOMASS-(PROTEIN+PHB) (gl⁻¹)
TIME (h)
0
0.5
1.0
1.5
2.0
2.6
0
20
40
60
80

Figure 4.34. The change in oxygen uptake rate of P. cepacia throughout ammonium-limited batch fermentation on fructose.

(○) Oxygen uptake rate (fermentation #1 of figure 4.32).
(■) Oxygen uptake rate (fermentation #2 of figure 4.32).

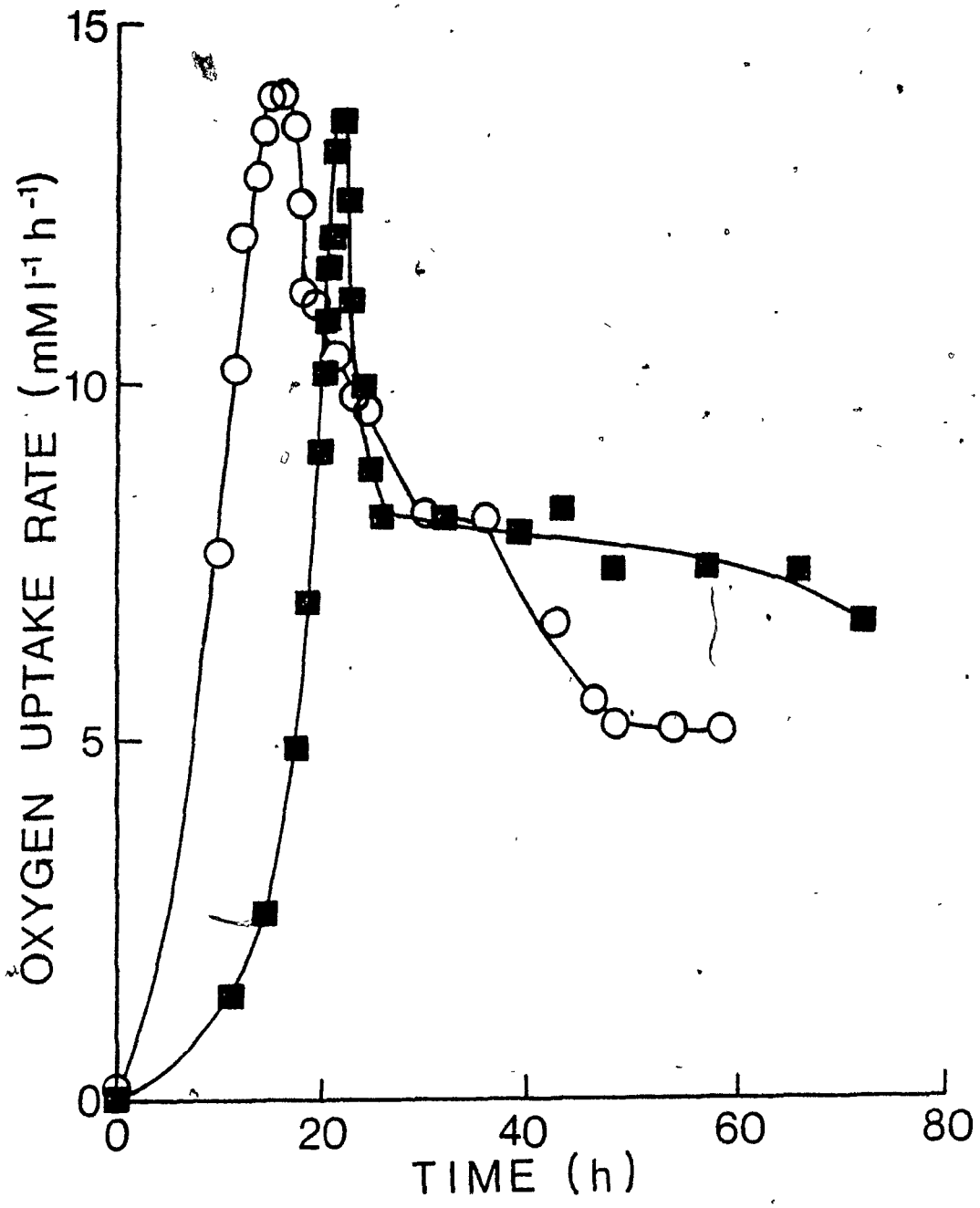

15
10
5
0
OXYGEN UPTAKE RATE (mM l-1 h-1)
0
20
40
60
80
TIME (h)

Figure 4.35A. Growth and production of PHB by P. cepacia in a 2-stage chemostat.

(○) First stage data.
(●) Second stage data.

pH = 7.0
T = 30 °C Agitation = 660 RPM
Air flow rates = 1.0 VVM (first stage) and 0.6 VVM (second stage).
Medium: Mineral salts medium.

Changes in the Medium Composition

Af : First stage feed contained 1.0 % fructose and 0.2 % $(NH_4)_2SO_4$. Fructose was fed into the second stage.

Anvr : First stage feed contained 1.0 % fructose and 0.2 % $(NH_4)_2SO_4$. NVR was fed into the second stage.

Bnvr : First stage feed contained 0.5 % fructose and 0.05 % $(NH_4)_2SO_4$. NVR was fed into the second stage.

Bf : First stage feed contained 0.5 % fructose and 0.05 % $(NH_4)_2SO_4$. Fructose was fed into the second stage.

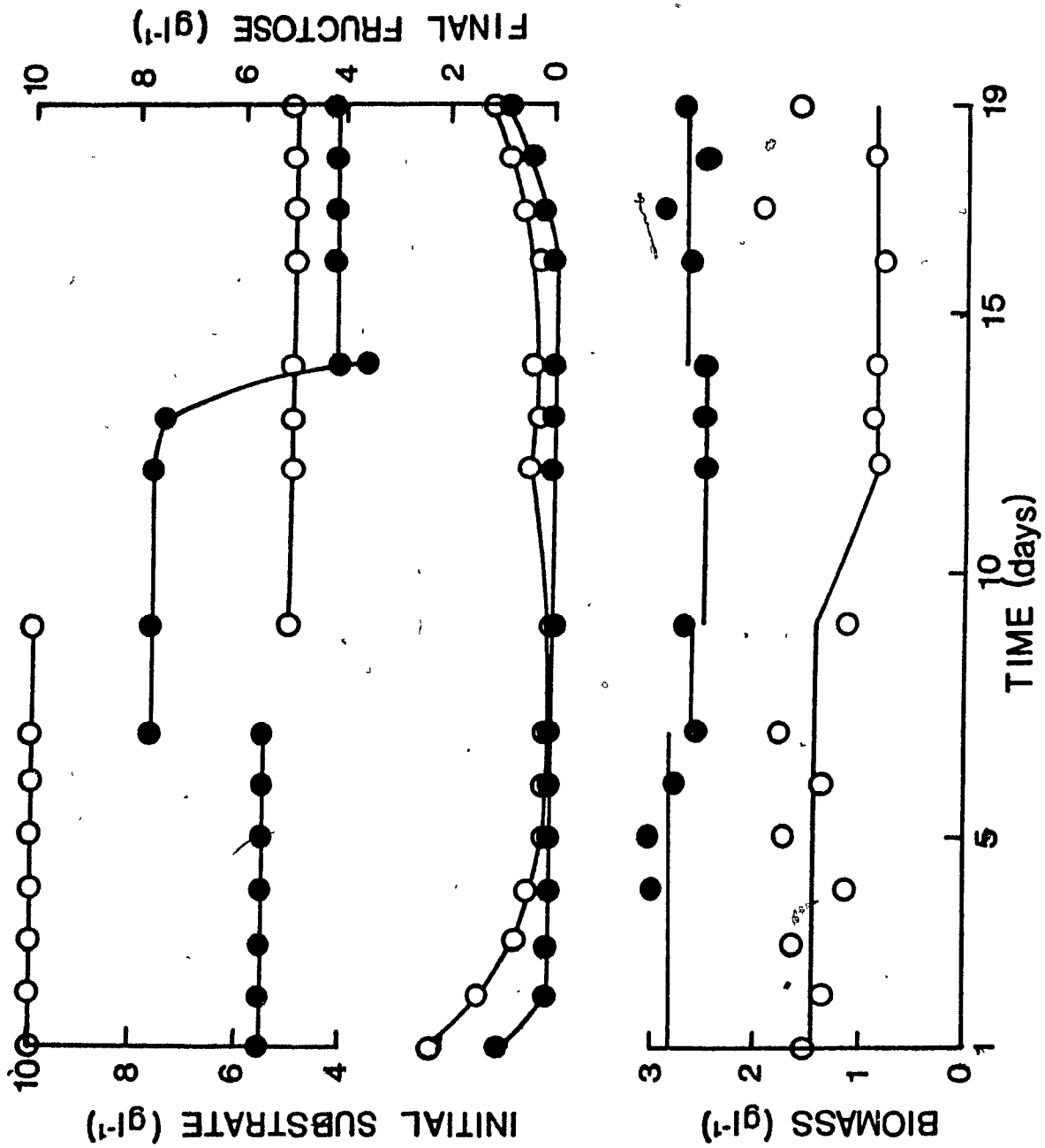

FINAL FRUCTOSE (gl⁻¹)
0
2
4
6
8
10
INITIAL SUBSTRATE (gl⁻¹)
10
8
6
4
BIOMASS (gl⁻¹)
0
1
2
3
TIME (days)
1
5
10
15
19

Figure 4.35B. Growth and production of PHB by P. cepacia in a 2-stage chemostat.

(○) First stage data.
(●) Second stage data.

pH = 7.0
T = 30 °C Agitation = 660 RPM
Air flow rates = 1.0 VVM (first stage) and 0.6 VVM (second stage).
Medium: Mineral salts medium.

Changes in the Medium Composition

A_f: First stage feed contained 1.0 % fructose and 0.2 % $(NH_4)_2SO_4$. Fructose was fed into the second stage.

A_{nvr}: First stage feed contained 1.0 % fructose and 0.2 % $(NH_4)_2SO_4$. NVR was fed into the second stage.

B_{nvr}: First stage feed contained 0.5 % fructose and 0.05 % $(NH_4)_2SO_4$. NVR was fed into the second stage.

B_f: First stage feed contained 0.5 % fructose and 0.05 % $(NH_4)_2SO_4$. Fructose was fed into the second stage.

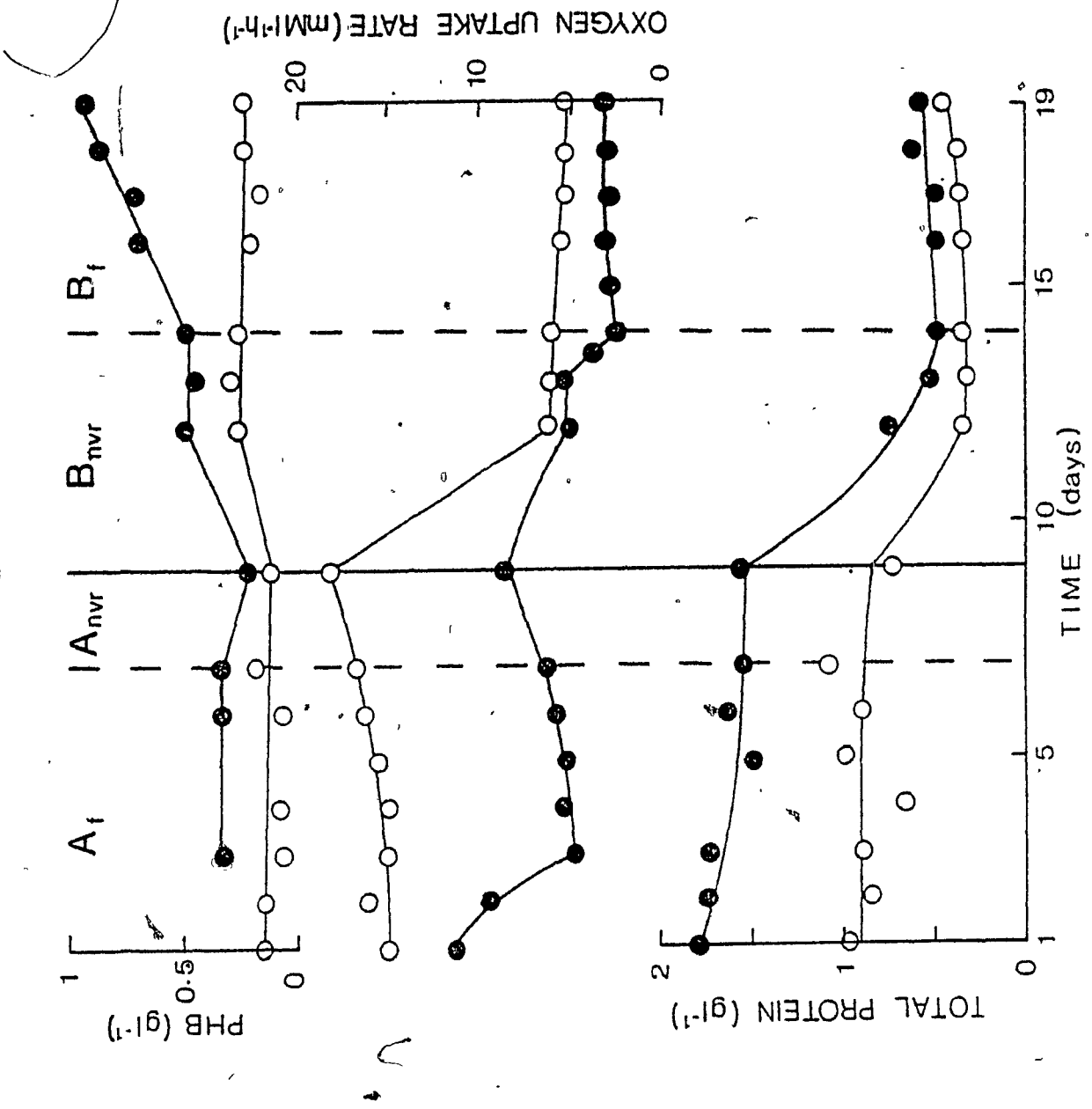

OXYGEN UPTAKE RATE (mMl⁻¹h⁻¹)
0
10
20
PHB (gl⁻¹)
0
0·5
1
TOTAL PROTEIN (gl⁻¹)
0
1
2
A_f
A_{nvr}
B_{nvr}
B_f
1
5
10
15
19
TIME (days)

TABLE 4.8 A SUMMARY OF THE 2-STAGE CHEMOSTAT RESULTS

AMMONIUM SULPHATE (gl^{-1})	2		2		0.5		0.5	
FIRST STAGE CARBON SUPPLY (gl^{-1})	10 (FRUCTOSE)		10 (FRUCTOSE)		5 (FRUCTOSE)		5 (FRUCTOSE)	
SECOND STAGE CARBON SUPPLY (gl^{-1})	5.2 (FRUCTOSE)		7.6 (NVR)		7.6 (NVR)		4.2 (FRUCTOSE)	
STAGE	1	2	1	2	1	2	1	2
BIOMASS (gl^{-1})◇	1.45	2.85 (1.40)	1.45	2.65 (1.20)	0.90	2.50 (1.60)	0.90	2.70 (1.80)
PROTEIN (gl^{-1})◇	0.90	1.55 (0.65)	0.90	1.55 (0.65)	0.40	0.50 (0.10)	0.40	0.50 (0.10)
PHB (gl^{-1})◇	0.15	0.35 (0.20)	0.15	0.22 (0.07)	0.25	0.50 (0.25)	0.25	0.80 (0.55)
OXYGEN UPTAKE RATE ($mMl^{-1}h^{-1}$)	16	6	17	8.5	6	5	6	3
Q_{O_2} ($mMg^{-1}h^{-1}$)◆	11.0	2.1	11.7	3.2	6.7	2.0	6.7	1.1
Q_{O_2} ($mMg^{-1}h^{-1}$)◁	17.8	3.9	18.9	5.5	15.0	10.0	15.0	6.0
$Y_{X/S}$ (%)	14.5	26.9	14.5	16.0	18.0	21.1	18.0	42.9
$Y_{P/S}$ (%)	1.5	3.8	1.5	0.9	5.0	3.3	5.0	13.1
$Y_{P/X}$ (%)	10.3	14.3	10.3	5.8	27.8	15.6	27.8	30.6
$Y_{PROTEIN/S}$ (%)	9.0	12.5	9.0	8.6	8.0	1.3	8.0	2.4

◇:VALUES IN BRACKETS INDICATE THE AMOUNT PRODUCED IN THE SECOND STAGE (i.e. STAGE2 - STAGE1)
◆:Q_{O_2} BASED ON BIOMASS CONCENTRATION
◁:Q_{O_2} BASED ON PROTEIN CONCENTRATION

maintained at 1.55 l. The flow rate of the mineral salts medium was 96 ml h^{-1} resulting in a first stage dilution rate of 0.123 h^{-1} and a second stage dilution rate of 0.062 h^{-1}. Fructose was in the mineral salts medium to supply carbon and energy to the first stage. Additional carbon in the form of either NVR or 50 % w/v fructose was added directly to the second stage.

When the mineral salts medium contained 10 g l^{-1} fructose and 2 g l^{-1} $(NH_4)_2SO_4$, only 0.15 g l^{-1} PHB was produced in the first stage. Under these conditions, when fructose was added to the second stage, the yield of PHB was only 3.8 g per 100 g fructose. The second stage yield from NVR (assuming total utilization and not accounting for the water content) was less than 1 %.

When the first stage carbon to nitrogen ratio was increased from 11 to 22, the first stage PHB yield ($Y_{P/S}$) increased from 1.5 % to 5.0 %. The second stage yield consequently increased to 3.3 % for NVR and 13.1 % for fructose. The second stage protein yield was considerably decreased when the first stage carbon to nitrogen ratio was increased.

CHAPTER 5

DISCUSSION

5.1 The Possibility of Using Peat Run-off Water as a Fermentation Substrate

It had been reported (104) that X. campestris NRC 2146 (ATCC 9924) could produce xanthan gum using peat pressate (water removed from peat by a pressing process) as its carbon source. Similar results were expected using peat run-off water since it is essentially the same material (ie. water that has been closely associated with peat). Since nutrient broth is deficient in carbon, the addition of peat run-off water was expected to result in increased biomass and/or xanthan production. There was no increase in either xanthan or biomass concentration (sect. 4.1.1), although the same strain of X. campestris was used as in the previously reported study. This indicated (contrary to the previously reported work (104)) that peat water contained no carbon source utilizable by X. campestris but additional experiments were needed to verify this.

Yeast malt broth contains glucose and sucrose in addition to nutrient broth. It thus has a surfeit of carbon and nitrogen but is lacking in mineral salts and trace elements. The yeast malt broth shake-flask experiments showed that the addition of peat run-off water resulted in an increased xanthan concentration. Since the shake-flasks

concentrations were not measured, conclusions could not be drawn as to whether the increased xanthan concentration was due to increased yield or to an increased rate of production.

In order to answer this question a series of batch fermentations were conducted. The mineral salts medium used was essentially the same as one developed specifically for X. campestris ATCC 13951 (37). It contained all the necessary mineral salts and trace elements but only a limited amount of glucose as a carbon source.. The results of these fermentations showed very little difference in biomass and xanthan yields or production rates when peat run-off water was added. The shake-flask study which used the same mineral salts medium as employed in the batch fermentations also showed no effect of peat run-off water addition. These results conflicted with the yeast malt broth shake-flask experiments.

Organic acids such as succinic acid have been shown to stimulate xanthan production (38). Peat is rich in humic acids. However, since there was no stimulation of xanthan production in the mineral salts medium, humic acids could not be responsible for the positive effect seen with yeast malt medium. Peat run-off water may contain some mineral salt, trace element or growth factor beneficial to xanthan production (and lacking in yeast malt broth), but it has no significant carbon content utilizable by X. campestris

P. cepacia may use more carbon sources than any other microorganism. It is known to grow on all four of the major sugars found in peat hydrolyzate and grows very well on organic acids (Table 3.3). The shake-flask experiment (Fig. 4 7) indicated that peat run-off water did not contain a significant amount of carbon utilizable by P. cepacia. The lack of response by a carbon-limited chemostat culture of P. cepacia to a pulse of peat run-off water verified this conclusion.

The COD of Barrington peat pressate is approximately 0.7 g l^{-1} (104). Peat run-off water from the same bog would be expected to have a similar COD. The COD value is roughly equivalent to the total organic carbon content (20). Therefore, even if all of the COD was contributed by sugars utilizable by X. campestris, peat run-off water would still not be a significant source of carbon.

Other waste products of the peat processing industry may be of some value as fermentation substrates. As described in the introduction, organisms have been grown on peat hydrolyzates and the processing of peat can produce effluent with a substantial COD. For example, one such process produces an effluent with a COD of 21 g l^{-1} (99). It may be possible to produce xanthan, PHB or some other biological product using such a waste.

It was shown that peat hydrolyzate was toxic to X. campestris (sect. 4.1.2). Peat processing wastes, which contain phenolic materials, are undoubtedly also toxic to microorganisms However, if the wastes were added at a suitably slow rate to a fed-batch or continuous culture, and if the chosen organism could metabolize the toxic components, the toxicity problem (as well as the potential for multiple growth phases) would be eliminated.

5.2 The Formation of a Strategy for the Development of NVR as a Fermentation Substrate

Experience with peat run-off water influenced the formation of a strategy for the development of toxic complex wastes as fermentation substrates. This strategy is outlined in Appendix B. It was used in the development of NVR as a fermentation substrate.

The mistake in attempting to develop peat run-off water into a fermentation substrate for xanthan production was in assuming (based on published data) that peat run-off water had a significant carbon content. In contrast the composition of NVR was known Apart from a 23 % water content, NVR is rich in carbon and chemical energy

The problem was to find one or two microorganisms that could use the majority of NVR components and produce a suitable product in high yield. In order to achieve this goal, the toxicity of NVR was investigated and the major

toxic components were identified. Microorganisms were selected based on their ability to metabolize these and other major NVR components. A study was made of the effects of NVR on the production of certain products. Using this knowledge, a product was chosen and a production process was developed.

5.3 Consideration of NVR Toxicity during Process Development

By definition, shake-flasks containing I_1 and I_2 concentrations of NVR contained less biomass at the time of sampling than did the control. Since most flasks were sampled relatively soon after inoculation, the I_1 and I_2 values reflected the effects of NVR on growth rate rather than on yield (ie. the flasks were sampled before growth had ceased). Most of the organisms tested were inhibited in growth when NVR was in excess of 0.35 % (Table 4.3) Such low I_1 values make these organisms unsuitable for industrial fermentations lacking rigid control. Growth would be inhibited during chemostat fermentation if the concentration of NVR reached the I_1 value. If the specific growth rate decreased to less than the dilution rate, washout would occur.

Since the highest I_2 value found was only 1.3 % w/v, conventional batch culture technique must be discounted as an economical method of using NVR as a fermentation substrate. Carbon-limited fed-batch and carbon-limited chemostat culture are the likeliest alternatives. These

fermentation methods would be successful, provided the microorganism used in the process was capable of utilizing the major growth-inhibiting components of NVR. Under carbon-limited conditions, this would keep the steady-state concentration of these components well below their inhibitory concentrations. This is verified by the fact that P. cepacia can be grown in a chemostat with a feed concentration of NVR much higher than the I_2 value for that organism (sect 4.9)

The use of carbon-limited fed-batch or chemostat culture also eliminates the problem of multiple growth phases (ie. diauxy) associated with batch growth on mixed substrates (sect. 2.1).

Since 6-hexanolactone and the monocarboxylic acids are the major toxic components found in NVR, only microorganisms able to degrade these components were considered for an industrial fermentation process using NVR.

Although the toxic effects of monocarboxylic acids on bacteria has been studied, the cause of their toxicity is unknown (43). It should be noted that temperature, pH and the degree of mixing greatly influences the apparent I_1 and I_2 values. Any factor affecting the solubility of carboxylic acids in the medium effects the apparent I_1 and I_2 values for NVR since monocarboxylic acids are the most toxic components of NVR (sect 4.8.1)

Those microorganisms with the highest I_1 and I_2 values (ie with the greatest ability to utilize toxic NVR components) and those which were stimulated in growth by low NVR concentrations, belonged to the genus Pseudomonas and related genera such as Alcaligenes (Table 4.3, sect. 4.7.2). NVR could most easily be used as a fermentation substrate in processes involving these organisms.

5.4 The Effects of NVR on the Growth of Microorganisms

As previously discussed (sect. 5.3), the results from shake-flask experiments reflect the effect of NVR on growth rate more than on the yield of biomass. The stimulatory effect of NVR on the growth of some microorganisms was particularily noticeable on nutrient broth medium which is very limited in carbon content. According to the Monod description of growth kinetics for microorganisms that divide by binary fission (eq. 5.1), the specific growth

$$\mu = (\mu_{max}\ S) / (S + K_s) \qquad \text{(eq. 5.1)}$$

rate (μ) is dependant on the concentration of the limiting substrate(s). Since the limiting substrate was carbon, the addition of NVR should have resulted in an increased growth rate. This leads to the corollary that NVR was used as a carbon source by microorganisms that had growth stimulated by NVR. The fact that one microorganism (Fig 4.28) was found to be stimulated in growth by pentanoic acid but did

not use pentanoic acid, shows that this conclusion cannot generally be drawn. The basis for the growth stimulation phenomenon was not investigated but since carboxylic acids are amphipathic, this effect may involve the cell membrane.

5.5 The Use of NVR Components by Microorganisms and its Influence on Process Development

As shown above, the growth stimulation effect was not a conclusive indication of whether, or how well, microorganisms could use NVR as a carbon source. The petri plate NVR component utilization test was a much better way of determining which microorganisms could use NVR as a carbon and energy source. Although this test only included a fraction of all NVR components, it did include all of the major components and classes of components.

The major classes of NVR components include monocarboxylic acids, dicarboxylic acids, lactones and cyclohexanediols. None of the organisms tested grew on cyclohexanediols. Fortunately the cyclohexanediols were found to be non-toxic. Thus, although inability to use cyclchexanediols would result in a reduced yield, their presence would not limit the maximum substrate concentration attainable in chemostat or fed-batch culture. Since some microorganisms are known to use cyclohexane and/or cyclohexanecarboxylic acids (6,44,142,143,160), microorganisms capable of using cyclohexanediols should exist. As corynebacteria, pseudomonads and related bacteria

were able to use the most NVR components (especially the toxic ones), these organisms would most likely be of value in a commercial process for the utilization of NVR as a fermentation substrate (Table 4.2, sect. 4.2). It is probable that mutant strains of some of these organisms capable of cyclohexanediol utilization could be isolated.

There should be little problem with strain stability in terms of substrate utilization since any commercial process using NVR would naturally select for strains which could utilize all NVR components. There would be some chance of strain specialization in substrate usage. Some strains of the production organism might only use certain NVR components while other strains would use other components. The result would effectively be a mixed culture. This is not necessarily undesireable since if the yield ($Y_{p/s}$) was maintained for all strains, then productivity could increase due to greater utilization of substrate.

5.6 Selection of a Product

5.6.1 Possible Products that could be Produced Biologically from NVR

Although it may be possible to enzymatically convert NVR into some product(s) of value, the search for a product was directed towards microbially rather than enzymatically produced substances. Since NVR is an industrial waste, medical or food-related products were not considered. Even if such a product was purified

economically, it may be difficult to obtain government approval for its sale.

Most NVR components are in a more chemically reduced state than carbohydrates Thus the search for a product was centered on those that could be produced or were preferentially produced from hydrocarbons. Until the early 1970's, when the price of crude oil rose substantially, alkanes were thought of as cheap fermentation substrates. Because of this, a considerable quantity of research was devoted to the development of products that could be made from them (49). Ones that fulfill the requirement of having uses outside of the food and health industries include biosurfactants, polysaccharides, catalase and PHB.

5 6.2 Biosurfactants

A surfactant would be an excellent product to make from NVR. Indeed attempts (unpublished) have been made to chemically convert NVR into a surfactant. Unfortunately, with the possible exception of isolate P2, none of the microorganisms tested were stimulated in biosurfactant production by NVR (sect. 4 3).

5.6.3 Extracellular Polysaccharides

NVR was not found to have a beneficial effect on polysaccharide production by any of the microorganisms tested (sect 4.4) Most of these organisms could not use many of the major NVR components. The exceptions were C.

equi and P. aeruginosa. C. equi produces a viscous polysaccharide when grown on n-alkanes (79). Many strains of P. aeruginosa are known to produce an alginic acid exopolysaccharide (113). Some strains of Pseudomonas are known to produce a viscous polysaccharide when grown on lower alcohols or glycols (157,167). Polysaccharide-producing strains of Pseudomonas can be isolated using a selective medium containing carbenicillin (53). Further research, preferably using a chemostat with NVR or monocarboxylic acids as the carbon source, would probably lead to the development of a process for the production of a polysaccharide from NVR.

5 6.4 Catalase

Hydrocarbon stimulate catalase production in microorganisms (108,158,159). NVR greatly stimulates the production of catalase by isolate SS_2 (a strain of P aeruginosa) (sect. 4.5). SS_2 grew well on most major NVR components (Table 4.2). However, the present market for catalase is small (9).

5.6.5 PHP

Hydrocarbons and lipids (such as carboxylic acids are oxidised by organisms to yield acetyl coenzyme A (39,92). PHB is preferentially produced from substrates related to acetyl Co-A (146). Thus NVR should be an appropriate substrate for the production of PHB. Indeed NVR and NVR

components were found to stimulate PHB accumulation in both A. eutrophus and P. cepacia.

5.6.6 Choice of Product

The ideal product to make from NVR would probably be a plastic. Plastics have many uses outside of the food and health industries. They could most easily be purified and marketed by a company that produces other polymers (such as nylon). PHB is the only microbially-produced thermoplastic. The greatest producers of PHB are also some of the organisms which grew best on NVR components and were most insensitive to NVR toxicity.

Strains of A. eutrophus are known to accumulate more PHB than any other microorganism (127). P. cepacia also accumulates PHB but the extent of accumulation had not previously been studied. P. cepacia was the microorganism found to be most insensitive to NVR toxicity (Table 4.3) and it could use as many or more major NVR components than any other organism studied (Table 4.2). If it was found that P. cepacia could accumulate large quantities of PHB, then it would be the organism of choice for PHB production from NVR.

5.7 Batch Growth and Accumulation of PHB by P. cepacia

As is the case with most storage polymers, PHB is generally accumulated in large quantities only after growth (as monitored by protein production) has ceased (143). Therefore some medium component which is not necessary for

PHB production must be limited (130). Since it was not possible to grow microorganisms in batch culture on useful quantities of NVR (ie. NVR is toxic even in relatively low concentration), P. cepacia was grown on fructose under nitrogen-limited conditions. Under these conditions, P. cepacia was found to accumulate PHB in excess of 50 % of its dry weight. This led to its choice as the organism to be used for further study of the production of PHB from NVR.

The C/N ratio study had shown that 1 mole of nitrogen was required for every 15 moles of carbon consumed (sect. 4.10). Since 2 g l^{-1} of $(NH_4)_2SO_4$ was supplied, 13.6 grams of fructose should be used before the nitrogen would be exhausted. At this point in the batch fermentations the oxygen uptake rate reached its peak, the fructose uptake rate and biomass production rate became linear, and PHB production began. Therefore, it can be assumed that nitrogen had become limiting especially since the mineral salts which had been previously balanced for another bacterium (X. campestris) using twice as much nitrogen (37).

The third growth phase (second linear phase) was probably caused by the exhaustion of a nutrient needed for production of PHB. It was probably not P, Mg, or S because these are not needed for PHB production (119). It could be identified by monitoring chemostat pulse experiments using a nitrogen limited medium at a low dilution rate. The appropriate nitrogen concentration and the dilution rate for

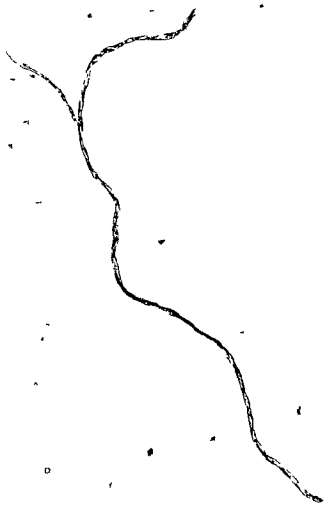
this experiment could easily be determined from the batch data. The third growth phase is undesirable because little PHB is produced. Therefore identification of this key nutrient would be of great value in medium formulation.

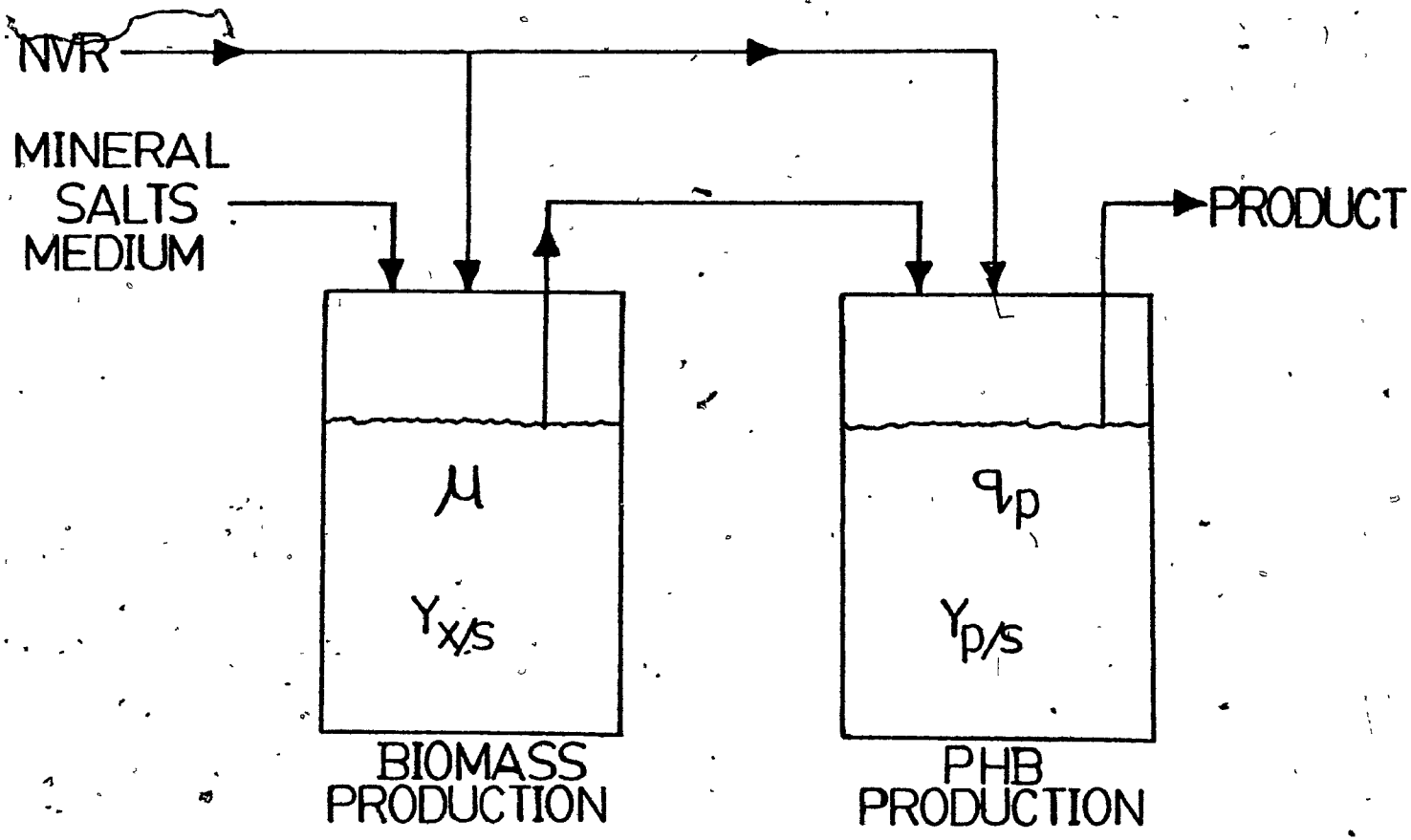
5.8 PHB Production in a 2-Stage Chemostat

The NVR toxicity study had shown that a fed-batch or chemostat production system would be necessary to avoid inhibition of growth by toxic NVR components (sect. 5.3). Because it involves less "down-time" and can be operated continuously at close to the maximum production rate, continuous culture is much more efficient than fed-batch culture (provided toxic products are not produced and the yield can be maintained). Batch fermentations had demonstrated that growth and production of PHB occur in separate stages. Therefore a 2-stage chemostat, as depicted in Figure 5.1, should be the most efficient method for the production of PHB from NVR. Each stage could be operated at its optimal conditions of pH, temperature and medium composition.

Two-stage chemostats are often used to provide a non-growing stage where secondary metabolite production can occur (114). An example of this is seen in the production of α-amylase. The rate of α-amylase synthesis by B. licheniformis is inversely proportional to the growth rate (97). It has been found with B. subtilis that 2-stage production results in higher levels of α-amylase than

Figure 5.1. Schematic drawing of a 2-stage chemostat.




NVR
MINERAL
SALTS
MEDIUM
PRODUCT
μ
$Y_{X/S}$
q_p
$Y_{P/S}$
BIOMASS
PRODUCTION
PHB
PRODUCTION

single-stage production (45). The best results are obtained when the growth rate in the first stage is much higher than that of the second stage.

P. cepacia grew well on NVR in a single-stage chemostat (sect. 4.9). PHB was produced by P. cepacia from NVR in the second stage of a 2-stage chemostat with a nitrogen-limited first stage (sect. 4.12). When R. cepacia was grown on fructose in a 2-stage chemostat with a nitrogen-limited first stage, PHB accumulation approached that of the PHB production phase of batch growth (sect. 4.12). Thus the 2-stage chemostat appears to be a workable method of production.

The optimization of a 2-stage chemostat process for the production of PHB from NVR would require a considerable amount of experimentation. The appropriate fermentation conditions for high yield PHB production have not yet been determined.

There are several key factors which determine the overall productivity of the 2-stage production system. The most important parameters governing first stage operation are μ and $Y_{x/s}$. The dilution rate of the first stage should be set at close to μ_{max}, provided that the yield ($Y_{x/s}$) of high-protein biomass from NVR could be maintained. The most important parameters governing operation of the second stage are the specific production rate (q_p) and the product yield ($Y_{p/s}$). The dilution rate in the second stage should be set

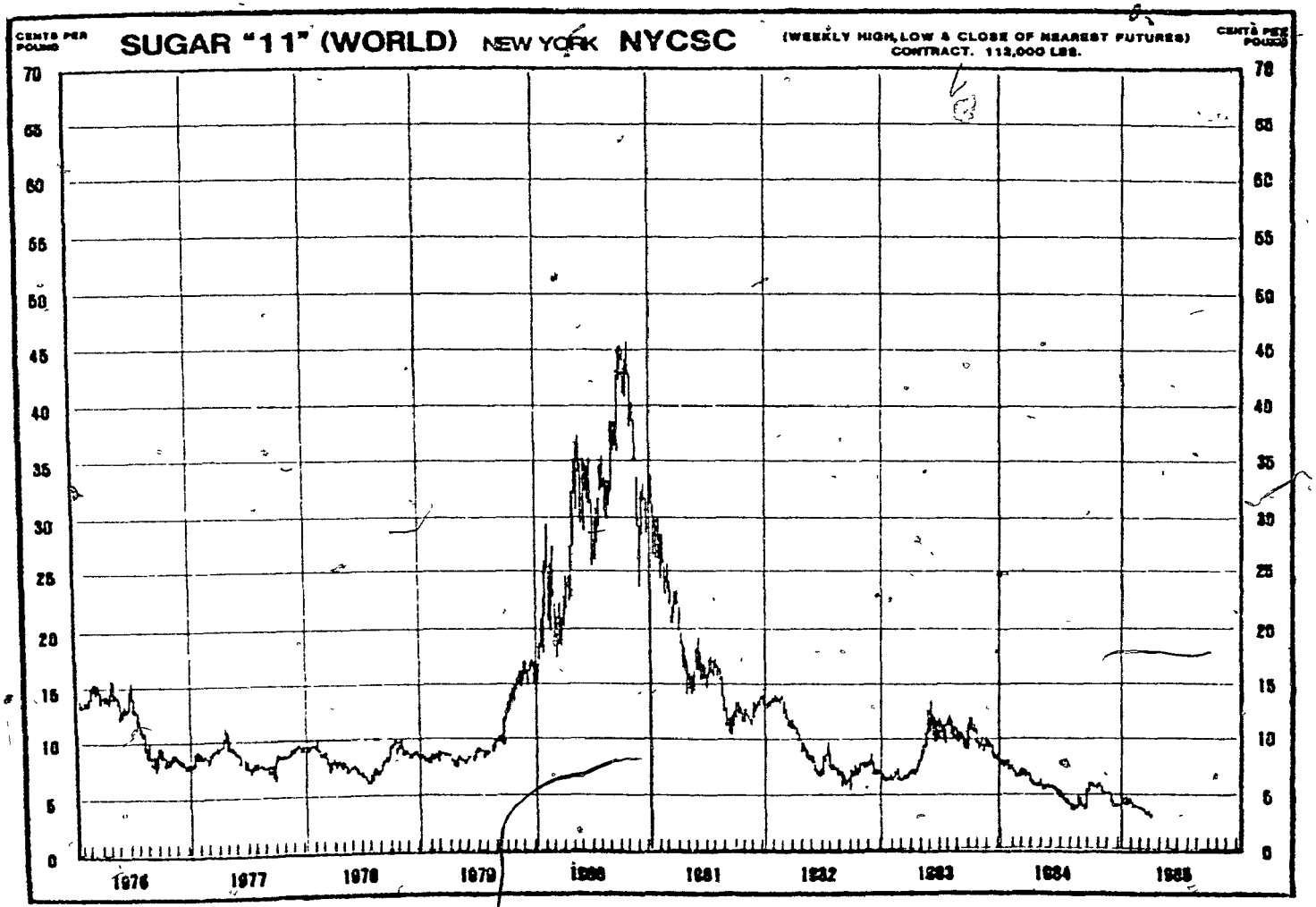
at near the maximum q_p, provided that the yield of PHB could be maintained.

5.9 The Economic Feasibility of Using NVR to Produce PHB

Without an extensive study of the relationships between $Y_{x/s}$ and μ (ie the dilution rate) in the first stage and $Y_{p/s}$ and q_p in the second stage, it is impossible to make an accurate prediction of the cost of PHB production. Other unknown economic factors include the cost of PHB extraction and the market value of PHB. However, it is possible to make an estimate of the substrate (carbon) cost. The carbon source is always a major expense in any fermentation process (78). Thus the determination of the substrate cost gives a good indication of whether the overall process is economically feasible.

As calculated in Appendix A, it would require 10.7 kg of sucrose or 13.7 kg of NVR to produce each kilogram of PHB. Thus NVR must be worth less than 78 % of the cost of sucrose in order for NVR to compete with sucrose as a carbon source. Although it fluctuates greatly (Fig. 5.2), the price of bulk sucrose is currently (June, 1986) 8 cents lb^{-1} or 17 1/2 cents kg^{-1} (all values are in U.S. currency) (38). The value of NVR (as a fuel) to Du Pont Inc. is classified information. However, NVR is much cheaper than bulk sucrose and at the present value of NVR, its use in place of sucrose would result in a substantial cost reduction.

Figure 5.2.. The price of sugar (42). The high low and closing prices of March world sugar futures at the Coffee, Sugar and Cocoa Exchange, Inc., New York.

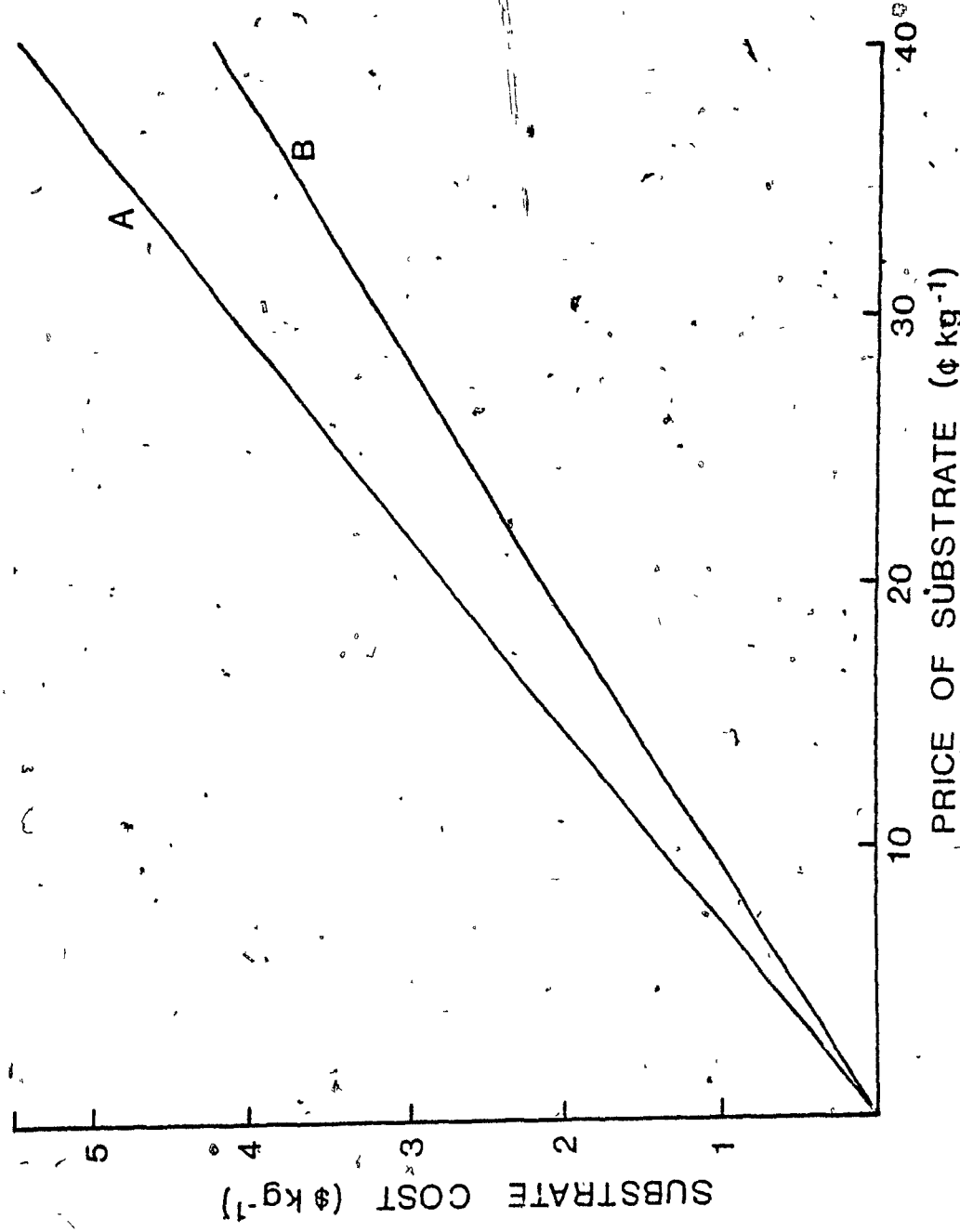

CENTS PER POUND
SUGAR "11" (WORLD) NEW YORK NYCSC
(WEEKLY HIGH, LOW & CLOSE OF NEAREST FUTURES)
CONTRACT. 112,000 LBS.
CENTS PER POUND
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
1976
1977
1978
1979
1980
1981
1982
1983
1984
1985

A greater yield of PHB from fructose might be obtained by using A. eutrophus H16 instead of P cepacia. This organism accumulates more PHB than any other microorganism yet tested (127). However, fructose is much more expensive than sucrose. Sucrose is a dimer of glucose and fructose and only mutant strains of A. eutrophus are capable of using glucose. Even if glucose and fructose could be used simultaneously, sucrose would probably have to be hydrolyzed before use by A. eutrophus. Nevertheless, it may be possible to obtain some microorganism that produces PHB in high yield and at a high production rate from sucrose. It is certain that a process using NVR could also be improved by the development of strains of P. cepacia which could use more of the minor NVR components and/or produce PHB at a higher yield from the components that it already uses. Thus a process using NVR would likely maintain its competitive edge.

The economic feasibility of using NVR rather than sugar to produce PHB depends on the relationship between the heating value of NVR (i.e. its value as a replacement for heating oil or other fuels) and the price of bulk sugar. Figure 5.3 shows the relationship between substrate cost and substrate price. The cost of substrate for the production of a given amount of PHB increases linearly with the market price of the substrate. If the fuel value of NVR increases to the price of bulk sucrose, it would not be economical to

Figure 5.3. The relationship between the price of substrate and its contribution to the cost of producing a specific amount of PHB. The slope of the line is equal to the inverse of $Y_{P/S}$ for a given substrate.

(A) NVR as a substrate.
(B) Sucrose as a substrate.


A
B
10
20
30
40
PRICE OF SUBSTRATE (¢ kg-1)
1
2
3
4
5
SUBSTRATE COST ($ kg-1)

use NVR. Since P. cepacia can use either sucrose or NVR as a carbon source, it may be possible to alternate or to use a blend of the two, depending on their relative value. The price of bulk sugar is presently considered to be unrealistally low (38). In the past, it has sold for as much as 45 cents lb^{-1} (99 cents kg^{-1} in 1980) (Fig. 5.2).

PHB has many properties in common with polypropylene (Table 2.2). Polypropylene is a cheap bulk polymer. It is made by the polymerization of polypropylene (sometimes with small quantities of comonomers) catalyzed by metallic halides and oxides and aluminum alkyls (54). It is used to make injection moulded items (especially for the transportation industry), fibers and films. As of June, 1985, the list price for large volume grades of polypropylene homopolymer varied between 88 cents and $1 05 kg^{-1} (55) as opposed to 77 to 92 cents kg^{-1} six years earlier (54). As can be seen from Figure 5 3, the price of sucrose or NVR would have to be less than 5 cents kg^{-1} for PHB to be competitive with bulk polypropylene if the substrate cost amounted to 50 % of the total production cost. Recently (June, 1986), bulk sucrose (contract #11) was selling for 17 1/2 cents kg^{-1}. Even using NVR as a substrate, it is unlikely that PHB could occupy much of the market presently held by polypropylene.

In 1984 nylon production in the United States was about 1.1 billion kilograms. About 65 % of this was nylon 6'6

(56). Assuming that NVR production amounted to 30 % (an unrealistically high value) of nylon 6'6' production, and the yield of PHB from NVR was 8 % (as calculated in Appendix C), then only 16.8 million kilograms of PHB could have been produced from all of the NVR in the United States. Also in 1984, about 2.2 billion kilograms of polypropylene was produced in the United States (55). Thus if PHB was produced from NVR, it would require a market of less than 1 % that of polypropylene. Considering the special properties of PHB, it should find a niche in the polymer market even at several times the price of polypropylene.

Nylon is a polymer posessing some properties superior to those of polypropylene. Because of this, in 1984 its price ranged from \$2.40 kg^{-1} to \$2.75 kg^{-1} for carpet staple and up to \$6.60 kg^{-1} for apparel grade (56). PHB could certainly be produced at these prices from NVR and it would be targeted at a much smaller market than nylon.

5.10 The Technical Feasibility of Producing PHB from NVR

5 10 1 Introduction

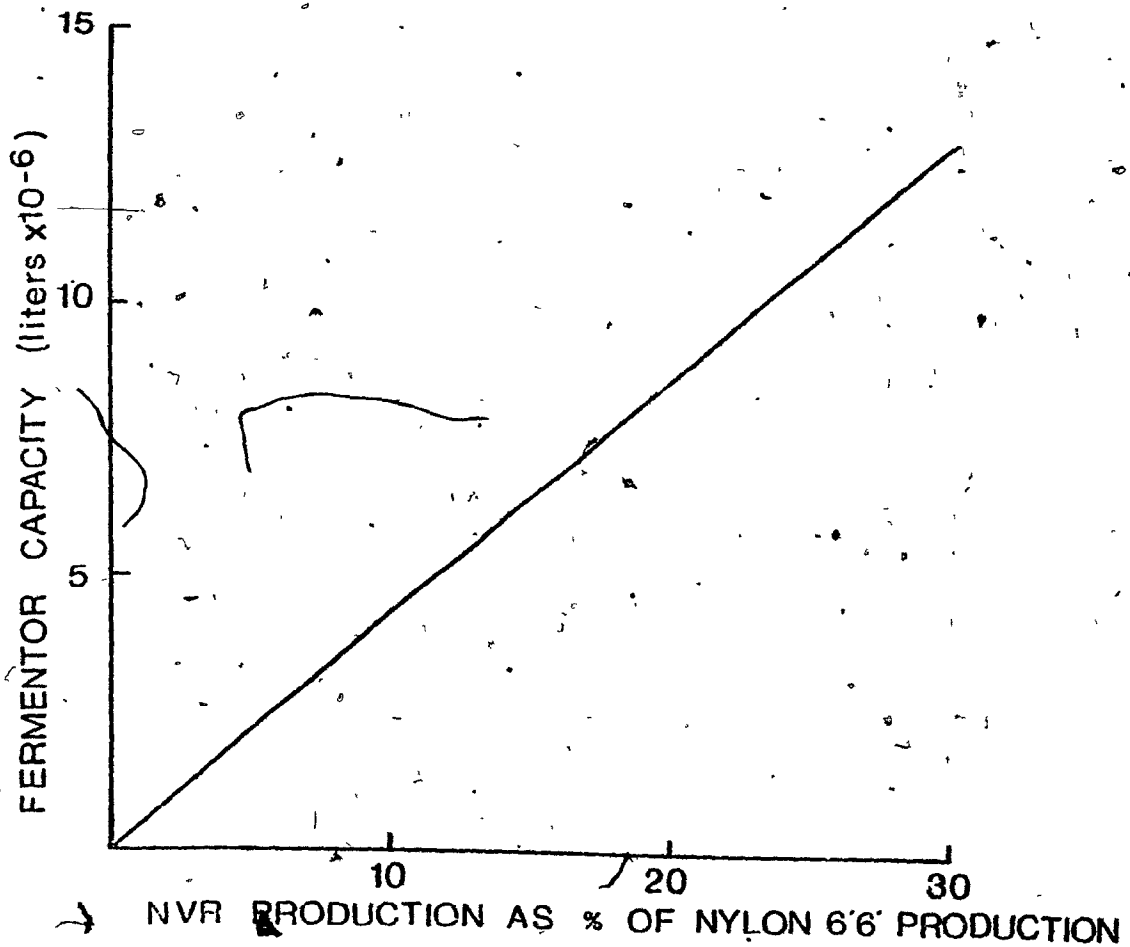
There would be several major stages involved in the process of converting NVR into a product incorporating PHB. These are production, extraction, processing and application. One must look at the feasability of each of these stages in order to determine if a process involving the conversion of NVR to PHB is practical.

5.10.2 Production

Much of this thesis has dealt with the subject of production. There are, however, two issues which have not yet been discussed. The organism chosen for this process is known to be an opportunistic pathogen (86). It is not particularily virulent but its use would pose a hazard to workers. Although the strain used in this study was found to be sensitive to chloramphenicol, it is resistant to most other antibiotics. Thus infections would be difficult to treat. It would be desireable and should easily be possible to develop an avirulent strain. P. cepacia is closely related to A. eutrophus (86). It is probable that strains incorporating the metabolic versatility of P. cepacia and the high PHB yield and avirulence of A. eutrophus could be isolated or engineered.

It is necessary to have some idea of the fermentor capacity needed to produce PHB from NVR. These calculations are given in Appendix C. This number was based on a biomass concentration of 15 g l^{-1} in the second stage. Recently fed-batch fermentations for the production of PHB have reached biomass concentrations of up to 206 g l^{-1} with a concommitant PHB concentration of 136 g l^{-1} (155). Figure 5 4 shows the fermentor capacity needed to use all of the NVR production in the United States in 1984 as a function of total U.S. nylon 6'6' production. For example, if the

Figure 5.4. Fermentor capacity required for the conversion to PHB of all of the NVR produced in the United States in 1984. The amount is given as a % of the total nylon 6'6' production.


15
10
5
FERMENTOR CAPACITY (liters x10-6)
10
20
30
NVR PRODUCTION AS % OF NYLON 6'6' PRODUCTION

production of NVR amounted to 30 % of total nylon 6'6' production (an unrealistically high value), then a fermentor capacity of 8.5 million liters would be needed. Thus, the equivalent of 85 one hundred thousand liter fermentors would be required. This number is practically achievable and the "economy of scale" would aid in reducing their cost.

5.10.3 Extraction of PHB

As previously discussed, there are many methods for extracting PHB from bacterial cells. As was shown in Table 2.1 PHB is soluble in some solvents but not in others. Thus a process involving the separation of cells from the whole broth, disruption of cells, and solvent extraction would probably be used. All of these processes are unit operations currently used in the chemical and/or biochemical industries although some adaptations would have to be made. Since much of the process could be automated and the solvents recovered, the major costs would be the initial capital cost and the energy cost for plant operation.

5.10.4 Processing of the Extracted PHB

PHB would undergo processing after extraction. Depending on the ultimate use, this may be as simple as the preparation of standard sized beads for use in injection moulding or more complex processes such as copolymerization with petrochemically-derived monomers in order to achieve

certain desired characteristics. Copolymerization processes have already been developed for PHB (137).

5.10.5 Summary

The production of PHB from NVR is, technically feasible although design of an efficient plant would require considerable research effort. The economic feasibility of PHB production depends on the development of suitable applications.

CONCLUSIONS

1. Peat run-off water does not contain a sufficient amount of carbon to be of value as a fermentation substrate.
2. Non-volatile residue is toxic to microorganisms.
3. The most toxic of the major NVR components are the monocarboxylic acids and 6-hexanolactone.
4. P. cepacia and some other microorganisms can use the major toxic components of non-volatile residue as their sole source of carbon.
5. P. cepacia can grow continuously with non-volatile residue as its sole source of carbon.
6. P. cepacia can grow in a carbon-limited chemostat with the feed concentration of non-volatile residue in excess of the concentration which is toxic in batch culture.
7. Catalase, poly-β-hydroxybutyric acid, and probably polysaccharides can be produced biologically from .
8. Poly-β-hydroxybutyric acid is the most economically suitable product to produce from non-volatile residue.
9. When grown on fructose in batch culture, P. cepacia can accumulate poly-β-hydroxybutyric acid in excess of 50 % of its dry weight.
10. Poly-β-hydroxybutyric acid can be produced by P. cepacia in the second stage of a 2-stage chemostat using either fructose or non-volatile residue as the carbon source.
11. It is cheaper to produce PHB from non-volatile residue than from conventional carbon sources such as sucrose.

RECOMMENDATIONS

1. The kinetics of PHB production using a 2-stage chemostat should be modeled.
2. The effect of substrate and physiological conditions on PHB quality should be investigated.
3. The effect of extraction procedures on PHB quality should be studied.
4. The minor components of NVR not utilized by P. cepacia should be identified.
5. The possibility of using mixed cultures of a small number of specific microorganisms to produce PHB from NVR should be studied.
6. The fed-batch production of PHB should be studied and compared with the 2-stage chemostat method in terms of economic feasibility.
7. The pathogenicity of each strain of microorganism used in these investigations should be considered before use.

REFERENCES

1. Agostini, D.E., J.B. Lando, and J.R. Shelton. 1971. Synthesis and characterization of poly-β-hydroxybutyrate. I. Synthesis of crystalline d,l-poly-β-hydroxybutyrate from d,l-butyrolactone. J Polymer Sci. A-1 9:2775-2787

2. Aiba, S., K.L. Huang, V. Moritz, and J. Someya. 1969. Cultivation of yeast cells by using n-alkanes as the sole carbon source. II. Mechanism of microbial uptake of hydrocarbons. J. Ferment. Technol. 47:211-219.

3. Aiba, S., A.E. Humphrey, and, N.F. Millis, 1973. Biochemical Engineering. Academic Press, New York.

4. Akit, J., D.G. Cooper, K.I. Manninen, and J.E. Zajic. 1981. Investigation of potential biosurfactant production among phytopathogenic bacteria and related soil microbes. Curr. Microbiol. 6:145-150.

5. Amemura, A., M. Hisamatsu, and T. Harada. 1977. Spontaneous mutation of polysaccharide production in Alcaligenes faecalis var myxogenes 10C3. Appl. Environ. Microbiol. 34:617-620.

6. Anderson, M.S., R.A. Hall, and M. Griffin, 1980. Microbial metabolism of acyclic hydrocarbons. cyclohexane catabolism of a pure strain of Pseudomonas sp. J. Gen. Microbiol. 120:89-94.

7 Asselineau, J., 1966. The Bacterial Lipids. Hermann, Paris.

8. Atlas, R.M. 1981. Microbial degradation of hydrocarbons: an environmental prospective. Microbial Rev. 45:180-209.

9. Aunstrup, K., 1979. Production and isolation of intracellular enzymes, p. 28-70. In Wingard, L.B. Jr. (ed.) Applied Biochemistry and Bioengineering II. Enzyme Technology. Academic Press, New York

10 Avigard, G., 1968. Levans, p. 711-718. In Mark, H.F., and N.G. Gaylord (ed.) Encylopedia of Polymer Science and Technology Vol.8.

11. Bangers, L., 1970. Some aspects of continuous culture of hydrogen bacteria. Dev. Ind Microbiol. 12:241-255.

12. Beebe, J., and W. Vinbreit. 1971. Extracellular lipid of Thiobacillus thiooxidans. J. Bacteriol. 108:612-614.

13. Boa, J.M., and A. LeDuy, 1984. Peat hydrolyzate medium optimization for pullulan production. Appl. Environ. Microbiol 48:26-30.

14. Braunegg, G., B. Sonnleitner, and R.M. Lafferty, 1978 A rapid method for the determination of poly-β-hydroxybutyric acid in microbial biomass. Europ. J Appl. Microbiol. Biotechnol. 6:29-37.

15. Brown, L.R., D.W. Cook, and R.G. Tischer, 1964. Preliminary studies on the extracellular products of Hydrogenomonas eutropha. Dev. Ind. Microbiol. 6:223-228.

16. Bull, A.T., and C.M. Brown, 1979. Continuous culture applications to microbial biochemistry. p 177-226. In J.R. Quayle (ed.) International Review of Biochemistry. Vol. 21. Microbial Biochemistry.

17. Cadmus, M.C., H. Gasdorf, A.A. Lagoda, R.F. Anderson, and R.W. Jackson, 1963. New bacterial polysaccharide from Arthrobacter Appl. Microbiol. 11:493-497.

18. Catley, B.J., 1971. Utilication of carbon sources by Pullularia pullulans for the elaboration of extracellular polysaccharide Appl. Microbiol 22:641-640.

19. Catley, B.J., 1971. Role of pH and nitrogen limitation in the elaboration of the extracellular polysaccharide pullulan by Pullularia pullulans. Appl. Microbiol. 22:650-654.

20. Cheremisinoff, P.N., and R.A. Young (ed.), 1975. Pollution Engineering Practice Handbook. Ann Arbour Science Publishers, Inc., Ann Arbour, Michigan.

21. Christenson, P., and F.D. Cook, 1978. Lysobacter, a new genus of nonfruiting, gliding bacteria with a high base ratio. Int. J. Syst. Bacteriol. 28:367-393.

22. Clayton, R.K., and C. Smith, 1960. Rhodopseudomonas spheroides: High catalase and blue-green double mutants. Biochem. Biophys. Res. Comm. 3:143-145.

23. Colowick, J., and N. Kaplan (ed.), 1966. Methods in Enzymology, Vol.8, Complex Carbohydrates. Academic Press, New York

24. Cooper, D.G., J. Akit, and N. Kosaric, 1982. Surface activity of the cells and extracellular lipids of Corynebacterium fascians CF 15. J. Ferment. Technol. 60:19-24.

25. Cooper, D.G., C.R. MacDonald, S.J.B. Duff, and N. Kosaric, 1981. Enhanced production of surfactin from Bacillus subtilis by continuous product removal and metal cation addition. Appl. Environ. Microbiol. **42**:408-412.

26. Cooper, D.G , and D.A. Paddock, 1984. Production of biosurfactant from Torulopsis bombicola. Appl. Environ. Microbiol. **47**:173-176.

27 Cooper, D.G., and J.E. Zajic, 1980. Surface-active compounds from microorganisms. Adv. Appl. Microbiol. **26**:229-253.

28. Cooper, D.G., and D.A. Paddock, 1983. Torulopsis petrophilum and surface activity. Appl. Environ. Microbiol. **46**:1426-1429.

29. Cooper, D.G., J.E. Zajic, and C. Denis, 1981. Surface active properties of a biosurfactant from Corynebacterium lepus. J. Am. Oil Chem. Soc. **58**:77-80.

30. Cooper, D.G., J.E. Zajic, and D.F. Gerson, 1979. Production of surface-active lipids by Corynebacterium lepus. Appl. Environ. Microbiol. **37**:4-10.

31. Cottrell, I.W., 1979. Industrial potential of fungal and bacterial polysaccharides. Presented at ACS Symposium, Hawaii.

32. Davidson, I.W., I.W. Sutherland, and C.J. Lawson, 1977. Localization of o-acetyl groups of bacterial alginate. J. Gen. Microbiol. 98:603-606.

33. Dawes, E.A., and D.W. Ribbons, 1962. The endogenous metabolism of microorganisms. Ann. Rev. Microbiol. 16:241-264.

34. Dawes, E.A., and P.J. Senior, 1973. The role and regulation of energy reserve polymers in microorganisms. Adv. Microbiol. Physiol. 10:135-266.

35. Delafield, F.P., M. Doudoroff, N.J. Palleroni, N.J. Lusty, and R. Contopoulos, 1965. Decomposition of poly-β-hydroxybutyrate by pseudomonads. J. Bact. 90: 1455-1466.

36. Demain, A.L., 1971. Overproduction of microbial metabolites and enzymes due to the alteration of regulation. Adv. Biochem. Eng. 1:113-142.

37. Demain, A.L., and P. Souw, 1981. Fermentative production of xanthan gum with organic acids. U.S. patent 4,245.046. 5 pp.

38. Donnelly, R.A., May, 1986. Barron's National Business and Financial Weekly.

39. Dunn, W.D., 1986. A molecular view of fatty acid metabolism in Escherichia coli. Microbiol. Rev. 50:179-192.

40. Duvjnak, Z., D.G. Cooper, and N. Kosaric, 1982. Production of biosurfactant by Arthrobacter paraffineus ATCC 19554. Biotechnol. Bioeng. 24:165- 176.

41. Ellar, D., D.G. Lundgren, K. Okamura, and R.H. Marchessault, 1968. Morphology of poly-β-hydroxybutyrate granules. J. Molecular Biol. 35:489-502.

42. Emery, W.L., S. Gaylinn, J. Allen, S.W. Cox, and W.L. Jiler, 1985. Sugar, p. 254-260. In CRB Commodity Year Book. Commodity Research Bureau, Jersey City, N.J.

43. Fay, J.P., and R.N. Farias, 1975. The inhibitory action of fatty acids on the growth of Escherichia coli. J. Gen. Microbiol. 91:233-240.

44. Feinberg, E.L., P.I.N. Ramage, and P.W. Trudgill, 1980. The degradation of n-alkylcyclohexanes by a mixed bacterial culture. J. Gen. Microbiol. 121:507-511.

45. Fencl, Z., J. Ricicia, and J. Kodesova, 1972. The use of the multistage chemostat for microbial product formation. J. Appl. Chem. Biotechnol. 22:405-416.

46. Findlay, R.H., and D.C. White. 1983. Polymeric beta-hydroxyalkanoates from environmental samples of Bacillus megaterium. Appl. Environ. Microbiol. 45:71-78.

47. Fisher, P.R., J. Appleton, and J.M. Pemberton, 1978. Isolation and characterization of the pesticide-degrading plasmid pJP1 from Alcaligenes paradoxus. J. Bacteriol. 135:798-804.

48. Frankel, I., and M. Drabkin, 1984. Wastewater treatment systems for synthetic fuels plants based on peat and oil shale. Presented at A.I.Ch.E. National Meeting, Aug. 19-22.

49. Fukui, S., and A. Tanaka, 1980. Production of useful compounds from alkane media in Japan. Adv. Biochem. Eng. 17:1-35.

50. Gasdorf, H.J., R.G. Benedict, M.C. Cadmus, R.F. Anderson, and R.W. Jackson, 1965. Polymer-producing species of Arthrobacter. J. Bacteriol. **90**:147-150.

51. Gerson, D.F., and J.E. Zajic 1978. Surfactant production from hydrocarbons by Corynebacterium lepus, sp. nov. and Pseudomonas asphaltenicus, sp. nov. Dev. Ind. Microbiol. **19**:577-599.

52. Gould, B.J., 1975. Enzyme data, p. 128-162. In Wiseman, A. (ed.) Handbook of Enzyme Technology. John Wiley and Sons, Toronto.

53. Govan, J.R.W., J.A.M. Fyfe, and T.R. Jarman, 1981. Isolation of alginate-producing mutants of Pseudomonas putida and Pseudomonas mendocina. J. Gen. Microbiol. 125:217-220.

54. Greek, B.F., Sept. 3, 1979. Thermoplastics boom may be easing. Chem. Eng. News. **57(No.36)**:10-16.

55. Greek, B.F., June 25, 1984. Upswing continues in demand for thermoplastics. Chem. Eng. News. **62(No.26)**:13-18.

56. Greek, B.F., Sept 3,1984. Slumping synthetic fibers may get help from import limits. Chem. Eng. News. 62(No.36):11-14.

57. Gutnick, D.,and E. Rosenberg, 1976. Cleaning of Cargo Compartments. U.S. Patent 3,941,692. 10 pp.

58. Gutnick, D.L., and E. Rosenberg, 1977. Oil tankers and pollution: A microbiological approach. Ann. Rev. Microbiol. 31:379-396.

59. Hamilton, W.A., and E.A. Dawes, 1959. A diauxic effect with Pseudomonas aeruginosa. Biochem. J. 71: 25p.

60. Harada, T., 1977. Production, properties and application of curdlan, p. 265-282. In Sandford, P.A., and A. Laskin (ed.) Extracellular microbial polysaccharides. American Chemical Society, Washington.

61. Harder, W., and L. Dijkhuizen, 1983. Physiological responses to nutrient limitation. Ann. Rev. Microbiol. 37:1-23.

62. Hasegawa, J., M., Ogura, H., Kanema, H., Kawaharada, and K. Watanabe. 1982. Production of d-β-hydroxyisobutyric acid from isobutyric acid by Candida rugosa and its mutant. J. Ferment. Technol. 60:501-508.

63. Hasegawa, J., M. Ogura, H. Kanema, H. Kawaharada, and K. Watanabe, 1982. Production of poly-β-hydroxypropionic acid by a Candida rugosa mutant unable to assimilate propionic acid. J. Ferment. Technol. 60:591-594.

64. Hasselberger, F.X., 1978. Uses of enzymes and immobilized enzymes. Nelson-Hall, Chicago.

65. Holdom, R.S., and A.G. Turner, 1969. Growth of Mycobacterium rhodochrous on n-decane: a new growth factor and emulsifying agent. J. Appl. Bact. 34:448-456.

66. Holmes, P.A., 1985. Applications of PHB - a microbially produced biodegradable thermoplastic. Phys. Technol. 16:32-36.

67. Holmes, P.A., and G.B. Lum (Imperial Chemical Industries PLC), Eur. Pat. Appl. EP 145,233. June 19,1985. GB Appl. 83/31,199. Nov.28,1983. 33 pp.

68. Horan, N.J., T.R. Jarman, and E.A. Dawes, 1981. Effects of carbon source and inorganic phosphorus concentration on the production of alginic acid by a mutant of Azotobacter vinelandii and on the enzymes involved in biosynthesis. J. Gen. Microbiol. 127:185-191.

69. Horiuchi, T., J. Tomizawa, and A. Novick, 1962. Isolation and properties of bacteria capable of high rates of β-galactosidase synthesis. Biochem. Biophys. Acta. **55**:152-163.

70. Howells, E.R., 1982. Single-cell protein and related technology. Chem. Ind. 15:508-511.

71. Itoh, S., H. Honda, F, Tomita, and T. Suzuki, 1971. Rhamnolipids produced by Pseudomonas aeruginosa grown on n-paraffin (mixture of C_{12}, C_{13} and C_{14} fractions). J. Antibiot. 24:855-859.

72. Jack, T.R., and E. Diblasio, 1985. Selective plugging for heavy oil recovery, p. 205-212. In Zajic, J.E., and E.C. Donaldson (ed.) Microbes and Oil Recovery. Vol. 1. Bioresource Publications, El Paso, Texas.

73. Jenkins, D.W., 1966. Bioregenerative life-support systems. NASA SP-165. U.S. Government Printing Office. Washington, D.C.

74. Johanides, V., and D. Hsrak, 1976. Changes in mixed bacterial cultures during linear alkylbenzenesulphonate (LAS) biodegradation. p. 426. In H. Dellweg (ed.) Proc.Fifth Int., Ferm. Symp. Westkreuz, Berlin.

75. Johnson, B.F., and R.Y. Stanier, 1971. Dissimilation of aromatic compounds by Alcaligenes eutrophus. J. Bacteriol. 107:468-475.

76. Kakinuma, A., M. Hori, M. Isono, G. Tamura, and K. Arima, 1969. Determination of amino acid sequence in surfactin, a crystalline peptolipid surfactant produced by Bacillus subtilis. Agric. Biol. Chem. 33:971-972.

77. Kakinuma, A., H. Sugino, M. Isono, G. Tamura, and K. Arima, 1969. Determination of fatty acid in surfactin and elucidation of the total structure of surfactin. Agric. Biol. Chem. 33:973-976.

78. Kalk, J.P., and A.F. Langlykke, 1986. Cost Estimation for biotechnology projects, p. 363-385. In Demain, A.L., and N.A. Solomon (ed.) Manual for Industrial Microbiology and Biotechnology. American Society for Microbiology, Washington.

79. Kanamaru, T., and S. Yamatodani. 1969. Production of acid heteropolysaccharides from n-paraffins. Agr. Biol. Chem. **33**:1521-1522.

80. Kang, K.S., and I.W. Cottrell, 1979. Polysaccharides, p.418-481. In Peppler, J., and D. Perlman (ed.) Microbial Technology, Vol. 1, Academic Press, New York.

81. Kawasaki, T., A. Iwashima, and Y. Nose. 1969. Regulation of thiamine biosynthesis in Escherichia coli J. Biochem. **65**:407-425.

82. King. P.P., 1982. Biotechnology. An industrial view. J. Chem. Technol. Biotechnol. **32**:2-8.

83. Kikuchi, M., T. Kanamaru, and Y. Nakao, 1973. Action of penicillin barrier to l-glutamic acid II. Relation between extracellular accumulation of l-glutamic acid and the excretion of phospholipids by penicillin-treated Corynebacterium alkanolyticum. Agr. Biol. Chem 37:2495-2408.

84. Knoche, H.W., and J.M. Shively, 1972. Structure of an ornithine-containing lipid from Thiobacillus thiooxidans. J. Biol. Chem. 247:170-178.

85. Koopman, B.L , A.O. Lau, P.F. Strom, and D. Jenkins (Assigned to the University of California), 1984. A liquid level subsurface draw off. U S. patent 4370418.

86. Krieg, N.R., and J.G. Holt (ed.), 1984. Bergey's Manual of Determinative Bacteriology. Williams and Wilkins, Baltimore.

87. Krulwich, T.A., and J.C. Ensign, 1969. Alteration of glucose metabolism of Arthrobacter crystallopoietes by compounds which induce sphere to rod morphogenesis J. Bacteriol 97:526-534

88. Kulkarni, K., and S.M. Barnett, 1979. Microbial growth on hydrocarbons: role of flocs. Biotechnol. Bioeng. 21:337-339.

89. Kuster, E., J. Rogers, and A. Mclaughlin, 1968. Stimulating effect of peat extracts on microbial metabolic reactions. In: Proc. Third Ann. Peat Congress, Quebec. pp.23-27.

90. Le Duy, A., 1979. Single cell protein from peat hydrolyzates. Process Biochem. 14:5-7

91. Le Duy, A., 1982. Pullulan production from peat hydrolyzate. Can. J. Microbiol. 29:143-146.

92. Lehninger, A.L., 1975. Biochemistry. Worth Publishers, Inc., New York.

93. Linker, A., and R.S. Jones, 1966. A new polysaccharide resembling alginic acid isolated from pseudomonads. J. Biol. Chem. 241: 3845-3851.

94. Margaritis, A., and E. Creese, 1980. Toxicity of surfactants in the aquatic environment p. 445-462. In Moo-Young, M. and G. Farquar (ed.) Waste Treatment and Utilization. Pergamon Press, Oxford.

95. Mallee, F.M., and H.W. Blanch, 1977. Mechanistic model for microbial growth on hydrocarbons. Biotechnol. Bioeng. **29**: 1793-1816.

96. Mateles, R.I., S.K. Chian, and R. Silver, 1967. p. 232. In Powell, E.O., C.G.T Evans, R.E. Strange, and D W. Tempest (ed.) Microbial Physiology and Continuous Culture, H.M.S.O., London.

97. Meers, J.L., 1972. The regulation of α-amylase production in Bacillus licheniformis. Antonie van Leewenhoek; J. Microbiol. Serol. **38**:565-570.

98. Mimura, A., M. Sugenò, T. Ooka, and I Takeda, 1971. Biochmeical engineering analysis of hydrocarbon fermentation II. Oxygen demand of hydrocarbon-assimilating yeasts. J. Ferment. Technol. **49**:238-254.

99. Monenco Ontario Ltd., 1983 Process evaluation and conceptual design of a plant based on a technical assessment of dewatering and peat fuel processes. Phase 1 Report (up-M-319-1) National Research Council Contract #DSS. ISX82-00030.

100. Monod, J., 1947. The phenomenon of enzymatic adaption. Growth. 11:223-289.

101. Monsan, P., and A. Lopez, 1981. On the production of dextran by free and immobilized dextransucrase. Biotechnol. Bioeng. 23:2027-2037.

102. Moorehouse. R., G.T. Colegrove, P.A. Sandford, P.A. Baird, and K.S. Kang, 1981 p.111-124. **In** D.R. Brant (ed.) Solution Properties of Polysaccharides. American Chemical Society, Washington.

103. Moo-Young, M., and T. Shimizu. 1971. Hydrocarbon fermentations using Candida lipolytica II. A model for cell growth kinetics. Biotechnol. Bioeng. 13:761-778.

104. Mulligan, C N , and D.G. Cooper, 1985 Pressate from peat dewatering as a substrate for bacterial growth. Appl Environ. Microbiol. 50:160-162.

105. Munson, R.J., 1970. Turbidostats. **In:** Norris, J.R., and D.W. Ribbons (ed) Methods of Microbiology Vol. 2. New York: Academic Press. pp. 349-376.

106. Nakao, Y., T. Kanamura, M. Kikuchi, and S Yamatodani. 1973. Action of penicillin on membrane permeabilty barrier to l-glutamic acid I. Extracellular accumulation of phospholipids by penicillin-treated Corynebacterium alkanolyticum. Agr. Biol Chem. 37:2399-2404.

107. Oltmann, L.F., G.S. Schoenmaker, W.N. Reijnders, and A.H. Stouthamer. 1978. Modification of the pH-auxostat method for the mass cultivation of bacteria. Biotechnol. Bioeng. 20:921-925.

108. Osumi, M., N. Miura, Y. Teranishi, A. Tanaka, and S Fukui, 1974. Ultrastructure of Candida yeasts growing on n-alkanes. Arch. Microbiol. 99:181-201.

109. Parulekar, S.J., G.B. Semones, M.J. Rolf, J.C. Lievense, and H.C. Lim, 1986 Induction and elimination of oscillations in continuous cultures of Saccharomyces cerevesiae. Biotechnol. Bioeng. 28:700-710.

110. Pederson, J.K., 1980 Carrageenan, pectin, and xanthan/locust bean gum gels. Trends in their food use: Food Chem. 6:77-88.

111. Philips, K.R., and H.G. Lawford. 1983. Theoretical maximum and observed product yields associated with curdlan production by Alcaligenes faecalis. Can. J. Microbiol. 29:1270-1276.

112. Pierce. G.E., T.J. Facklam. and J.M. Rice. 1981. Isolation and characterization of plasmids from environmental strains of bacteria capable of degrading the herbicide 2,4-D. Dev. Ind. Microbiol. 22:401-408.

113 Piggott. N.H., I.W. Sutherland. and T.R. Jarman. 1981. Enzymes involved in the biosynthesis of alginate by Pseudomonas aeruginosa. Europ. J. Appl. Microbiol. Biotechnol. 13:179-183.

114 Pirt, S.J., 1975. Principles of microbe and cell cultivation. Halstead Press, New York.

115. Prave, P., U Faust, W. Sittig, and D.A. Sukatsch, 1982. Handbuch der Biotechnologie. Akademische Verlagsgesellschaft, Wiesbaden.

116. Ramsay, B.A., A. Margaritis, J.E. Zajic, and D.G. Cooper, 1983. Rhodochrous bacteria: biosurfactant production and demulsifying ability. p. 61-65. In Zajic, J.E., D.G Cooper, T.R Jack, and N. Kosaric (ed.), Microbial enhanced oil recovery. Pennwell Books, Tulsa, Oklahoma.

117. Rapp, P and F. Wagner, 1976. Formation of trehalose lipid by Nocardia sp. grown on n-alkanes. Proc. Fifth Int. Ferm. Symp, Westkreutz, Berlin.

118. Repaske, R., and R. Mayer, 1976. Dense autotrophic cultures of Alcaligenes eutrophus. Appl. Environ. Microbiol. **32**:592-597.

119. Repaske, R., and A.C. Repaske, 1976. Quantitative requirements for exponential growth of Alcaligenes eutrophus. Appl. Environ. Microbiol. **32**:585-591.

120. Reusch, R.N., and H.L. Sadoff, 1983. D-(-)-Poly-β-hydroxybutyrate in genetically competent bacteria. J. Bacteriol. **156**:778-788.

121. Rice, C.W., and W.P. Hempfling, 1984. Nutrient-limited continuous culture in phauxostat. Biotechnol. Bioeng. **27**:187-191.

122. Rosenberg, E., 1986. Microbial Biosurfactants. Crit. Rev. Biotechnol. **3**:109-132.

123. Rowley, B.I., and A.T. Bull, 1977. Isolation of a yeast-lysing Arthrobacter species and the production of the lytic enzyme complex batch and continuous-flow fermentors. Biotechnol. Bioeng. **19**:879-899.

124. Sandford, P.A., 1979. Extracellular microbial polysaccharides. Adv. Carbohydr. Chem. Biochem. **36**:265-313.

125. Sandvick, K.I., and J.M. Maerker, 1977. Application of xanthan gum for enhanced oil recovery. p. 242-263. In Sandford, P.A. and A. Lasskin (ed.) Extracellular Microbial Polysaccharides. ACS Symp. #45, ACS Symp. Series, American Chemical Industry, Washington.

126. Sariaslani, F.S., A.W. Westwood, and I.J. Higgins, 1975. Control of isocitrate lyase in Nocardia salmonicolor (NCIB 9701). J. Gen. Microbiol. 91:315-424.

127. Schlegel, H.G., G. Gottschalk, and R. von Bartha, 1961. Formation and utilization of poly-β-hydroxybutyric acid by knallgas bacteria (Hydrogenomonas). Nature 191:463-465.

128. Schegel, H.G., and H.W. Jannasch, 1967. Enrichment cultures. Ann. Rev. Microbiol. 21:49-70.

129 Schlegel, H.G., and I. Kraus, 1970. The isolation of mutants not accumulating poly-β-hydroxybutyric acid. Arch. Mikrobiol. 71:283-294.

130. Schlegel, H.G., and R.M. Lafferty, 1971. Novel energy and carbon sources. A.The production of biomass from hydrogen and carbon dioxide. Adv. Biochem. Eng. 1:143-168.

131. Schonbaum, G.R., and B. Chance, 1976. Catalase. p. 363-408. In Boyer, P.D. (ed.) The Enzymes. Vol. 13. Academic Press, New York.

132. Scott, D., and F. Hammer, 1960. Properties of Aspergillus catalase. Enzomologia 22:229-237.

133. Senior, P.J., 1984. Polyhydroxybutyrate, a specialty polymer of microbial origin. p. 266-271. In Dean, A.C.R., D.C. Ellwood, and C.G.T. Evans (ed.) Continuous Culture. Vol.8. Biotechnology, Medicine, and the Environment. Ellis Horwood Ltd., Chechester.

134. Seviour, R.J., and B. Kristiansen, 1983. Effect of ammonium ion concentration on polysaccharide production by Aurobasidium pullulans in batch culture. Europ. J. Appl. Microbiol. Biotechnol. 17:178-181.

135. Shah, P.S., L.E. Erickson, and L.T. Fan, 1972. Growth models of cultures with two liquid phases. VI. Parameter estimation and statistical analysis. Biotechnol. Bioeng. 14:533-570.

136. Shelton, J.R., D.E. Agostini, and J.B. Lando, 1971. Synthesis and characterization of poly-β-hydroxybutyrate. II. Synthesis of d-poly-β-hydroxybutyrate and the mechanism of ring opening polymerization of β-butyrolactone. J. Polymer Sci. A-1. 9:2789-2799.

137. Siapkas, S., 1976. Diplomarbeit. Technical University Graz.

138. Silman, R.W., and E.B. Bagley, 1979. The viscostat: productstat method of feed-rate control in continuous fermentations. Biotechnol. Bioeng. 21:173-179.

139. Silver, R.S., and R.I. Mateles, 1969. Control of mixed substrate utilization in continuous cultures of Escheriohia coli. J. Bact. 97:535-543.

140. Smith, R.T., 1981. Environmental aspects of alternative wet technologies for producing energy/fuel from peat. Report 1981 DOE/FC/10169-T1.

141. Smith, D., and A.T. Bull, 1976. Studies on the utilization of coconut water waste for the production of the food yeast Saccharomyces fragilis. J. Appl. Bacteriol. 41:81-95.

142. Smith, D.I., and A.G. Callely, 1975. The microbial degradation of cyclohexane carboxylic acid. J. Gen. Microbiol. 91:210-212.

143. Sonnleitner, B., E. Heinzle, G. Braunegg, and R.M. Lafferty, 1979. Formal kinetics of poly-β-hydroxybutyric acid (PHB) production in Alcaligenes eutrophus H16 and Mycoplana rubra R14 with respect to the dissolved oxygen tension in ammonium-limited batch cultures. Eur. J. Appl. Microbiol. Biotechnol. 7:1-10.

144. Srienc, F., B. Arnold, and J.E. Baily, 1984. Characterization of intracellular accumulation of poly-β-hydroxybutyrate (PHB) in individual cells of Alcaligenes eutrophus H16 by flow cytometry. Biotechnol. Bioeng. 26:982-987.

145. Stanier, R.Y., E.A. Adelberg, and J. Ingraham (ed.), 1976. The Microbial World. Toronto: Prentice Hall.

146. Stanier, R.Y., M. Doudoroff, R. Kunisawa, and R. Contopoulou, 1959. The role of organic substrates in bacterial photosynthesis. Proc. Natl. Acad. Sci. U.S. 45:1246-1260.

147. Stanier, R.Y., N.J. Palleroni, and M. Doudoroff, 1966. The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. **43**:159-271.

148. Stirling, L.A., R.J. Watkinson, and I.J. Higgins, 1977. Microbial metabolism of acyclic hydrocarbons: isolation and properties of a cyclohexane-degrading bacterium. J. Gen. Microbiol. **99**:119-125.

149. Stockdale, H., D.W. Ribbons, and E.A. Dawes, 1968. Occurence of poly-β-hydroxybutyate in the Azotobacteriaceae. J. Bact. **95**:1798-1803.

150. Strickland, L.H., 1951. The determination of small quantities of bacteria by means of the biuret reaction. J. Gen. Microbiol. **5**:689-703.

151. Sutherland, I.W., 1983. Extracellular polysaccharides. p. 531-574. In Biotechnology: A comprehensive treatise in 8 volumes. Vol. 3. Verlag Chemie, Weinheim.

152. Sutherland, I.W., and D.C. Ellwood, 1979. Microbial exopolysaccharides - industrial polymers of current and future potential. p. 107-150. In Bull, A.T., D.C. Ellwood, and C. Ratledge (ed.) Microbial Technology. Current State. Future Prospects. Cambridge University Press, New York.

153. Sutherland, I.W., and J.F. Wilkinson, 1971. Chemical extraction methods of microbial cells. p. 345-383 In Norris, J.R., and D.W. Ribbons (ed.) Methods in Microbiology Vol. 5B Academic Press, New York.

154. Suzuki, T., and K. Ogawa. 1972. Transient accumulation of fatty alcohols by n-paraffin-grown microorganism. Agric. Biol. Chem. **36**:457-463.

155. Suzuki, T., T. Yamane and S. Shimizu. 1986. Mass Production of poly-β-hydroxybutyric acid by fully automated fed-batch culture of a methylotroph. Europ. J. Appl. Microbiol. Biotechnol. **23**:322-329.

156. Sykes, J., 1971. Centrifugal methods for the isolation and characterization of sub-cellular components from bacteria. p. 55-207. In Norris, J.R., and D.W. Ribbons (ed.) Methods in Microbiology Vol. 5B. Academic Press, New York.

157. Tanaka, A., Y. Cho, Y. Teranishi, S. Nabeshima, and S. Fukui, 1974. Production of polysaccharides from lower alcohols and glycols by nitrogen-fixing Pseudomonas sp. J. Ferment. Technol. **52**:739-746.

158. Teranishi, Y., S. Kawamoto, A. Tanaka, M. Osumi, and S. Fukui, 1974. Induction of catalase activity by hydrocarbons in Candida tropicalis pK 233. Agr. Biol Chem. 38:1221-1225.

159. Teranishi, Y., A. Tanaka, M. Osumi, and S. Fukui, 1974. Catalase activities of hydrocarbon-utilyzing Candida yeasts. Agr. Biol. Chem. 38:1213-1220.

160. Trower, M.K., R.M. Buckland, R. Higgins, and M. Griffin, 1985. Isolation and characterization of a cyclohexane-metabolizing Xanthobacter sp. Appl. Environ. Microbiol. 49:1282-1289.

161. Wagner, F., P. Rapp, H. Bock, W. Lindorfer, W. Schultz, and W. Gebetsberger, 1978. German Patent PS 28 05 823.

162. Washburn and Gillis Associates Ltd. Identification of the potential of harvesting and preutilization of processing of peat as an energy commodity. Final report submitted to Environment Canada, March, 1983.

163. Wells, J., 1977. Extracellular microbial polysaccharides - a critical overview. p. 299-313. In Sandford, P.A., A. Laskin (ed.) ACS Symp. Series #45, American Chemical Society, Washington.

164. Wilkinson, S.G., 1972. The structure of ornithine-containing lipid from Pseudomonas rubescens Biochim. Biophys. Acta 270:1-17.

165. Wilkinson, J.F., and A.L.S. Munro, 1967. p. 173-185. In Microbial Physiology and Continuous Culture. Powell, E.O., Evans, Strange, and Tempest (ed.) H.M.S.O., London.

166. Wiseman, A., 1975. Industrial practice with enzymes. p.243-272. In Wiseman, A. (ed.) Handbook of Enzyme Technology, John Wiley and Sons, Toronto

167. Yamatodani, S., and T. Kanamura, 1972. Novel microbial acid heteropolysaccharide and production thereof. Jap. Pat. 3,674,642

168. Zajic, J.E., and D.F. Gerson, 1978. Microbial extraction of bitumen from Athabaska tar sands. American Chemical Society Preprints, Washington. 22(3):195-203.

169. Zajic, J.E., and C. Panchal, 1976. Bioemulsifiers. Crit. Rev. Microbiol. 5:39-66.

170. Zajic, J.E., and W. Seffens, 1984. Biosurfactants. Crit. Rev. Biotechnol. 1:87-107.

171. Zobell, C.E., 1947. Bacterial release of oil-bearing materials. World Oil 126:36-40.

APPENDIX A

STANDARD CURVES

Figure A.1. The biological determination of the maximum oxygen transfer rate in a New Brunswick Multigen fermentor.

Medium: mineral salts medium.
Temperature. 30 °C.
Agitation. 660 RPM.
Aeration. 1.1 VVM.

(■) Dilution Rate = 0.24 h^{-1}.
(○) Dilution Rate = 0.14 h^{-1}.
(●) Dilution Rate = 0.09 h^{-1}.

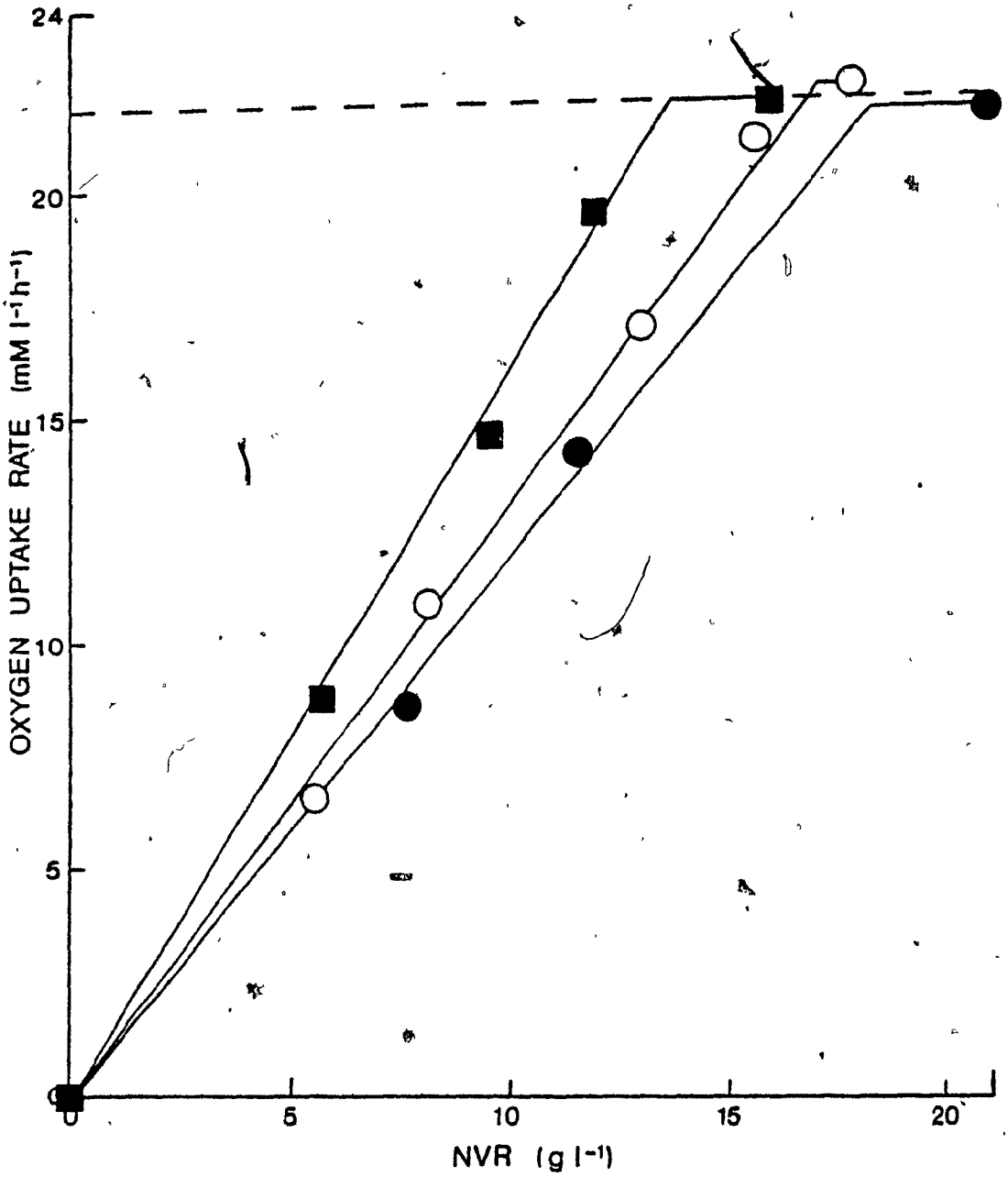

24
20
15
10
5
0
OXYGEN UPTAKE RATE (mM l⁻¹h⁻¹)
0
5
10
15
20
NVR (g l⁻¹)

Figure A.2. Optical density at 560 nanometers versus protein concentration for the biuret test.

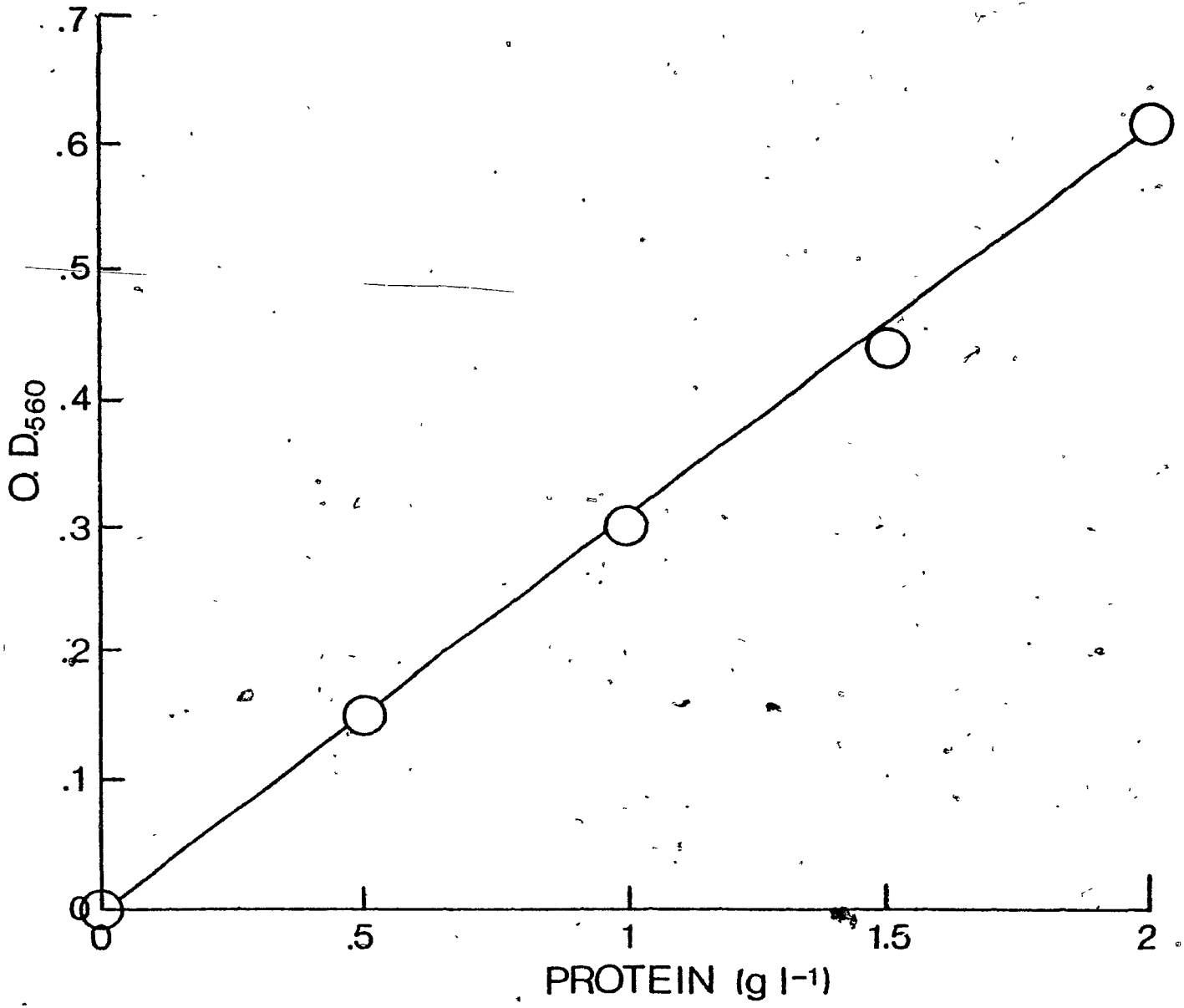

.7
.6
.5
.4
.3
.2
.1
0
O. D560
0
.5
1
1.5
2
PROTEIN (g l-1)

Figure A.3. Optical density at 450 nanometers versus glucose concentration for the glucose oxidase test.

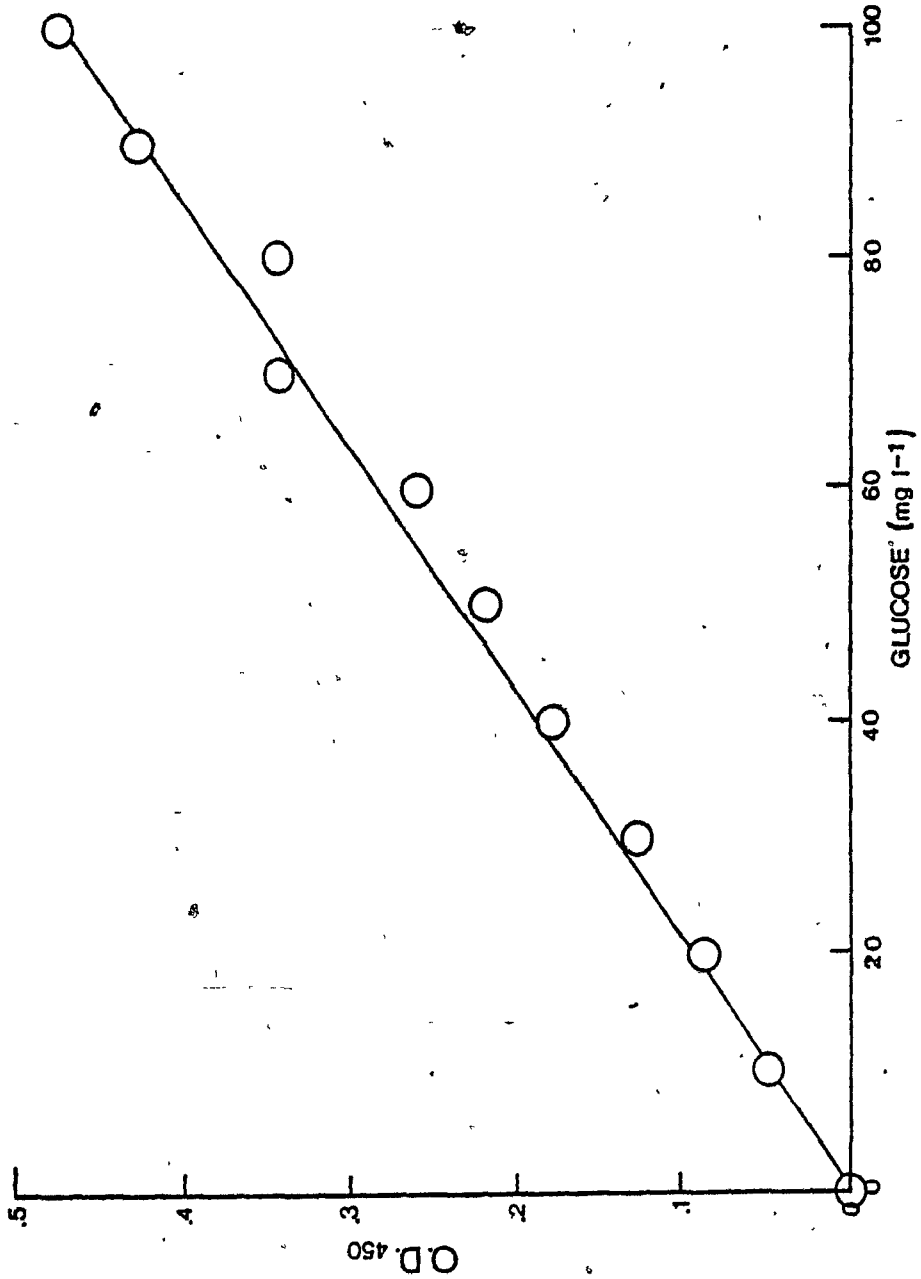

O.D. 450
.5
.4
.3
.2
.1
0
GLUCOSE (mg l⁻¹)
20
40
60
80
100

Figure A.4. Detector response (area) versus fructose concentration (50 µl injections).

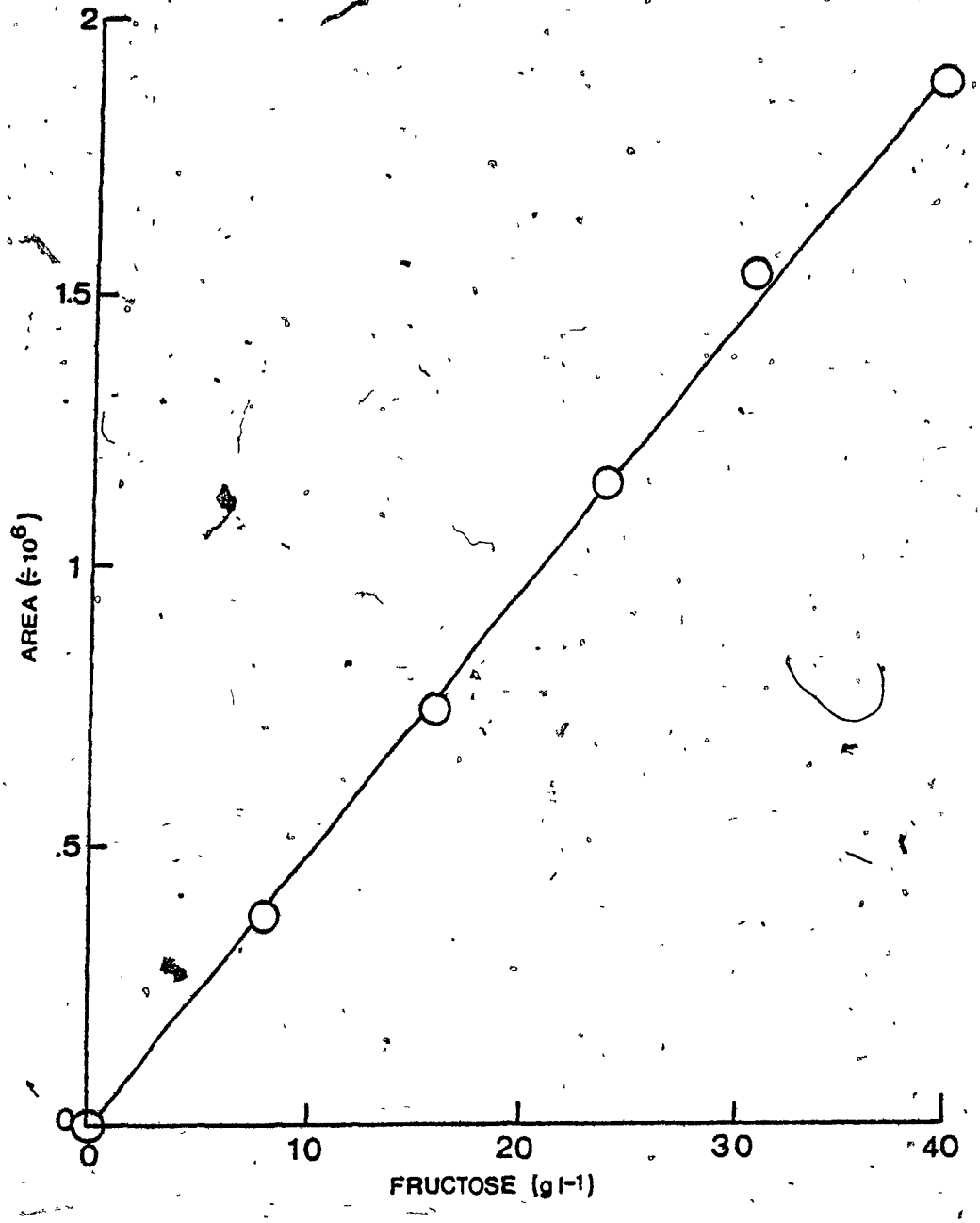

2
1.5
1
.5
0
AREA (÷10⁶)
0
10
20
30
40
FRUCTOSE (g l⁻¹)

Figure A.5. Optical density at 490 nanometers versus carbohydrate (glucose) concentration for the phenol test for total carbohydrates.

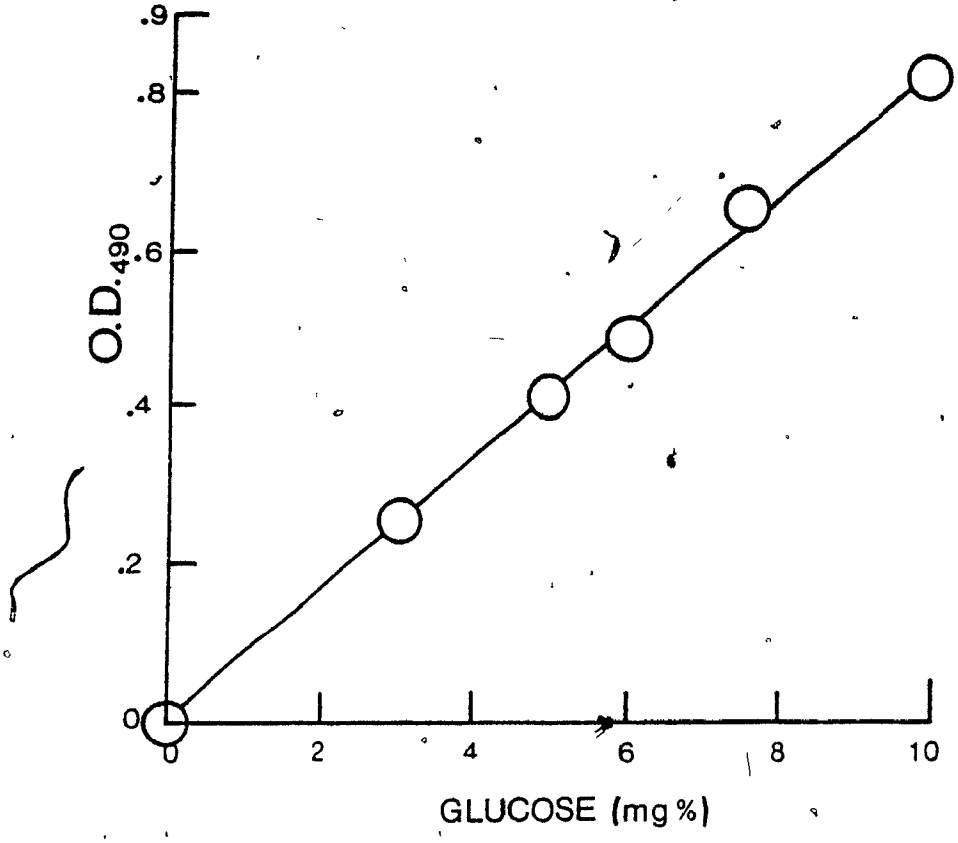

.9
.8
.6
.4
.2
0
O.D.490
0
2
4
6
8
10
GLUCOSE (mg %)

Figure A.6. Optical density at 410 nanometers versus time of sonication of Isolate SS_2.

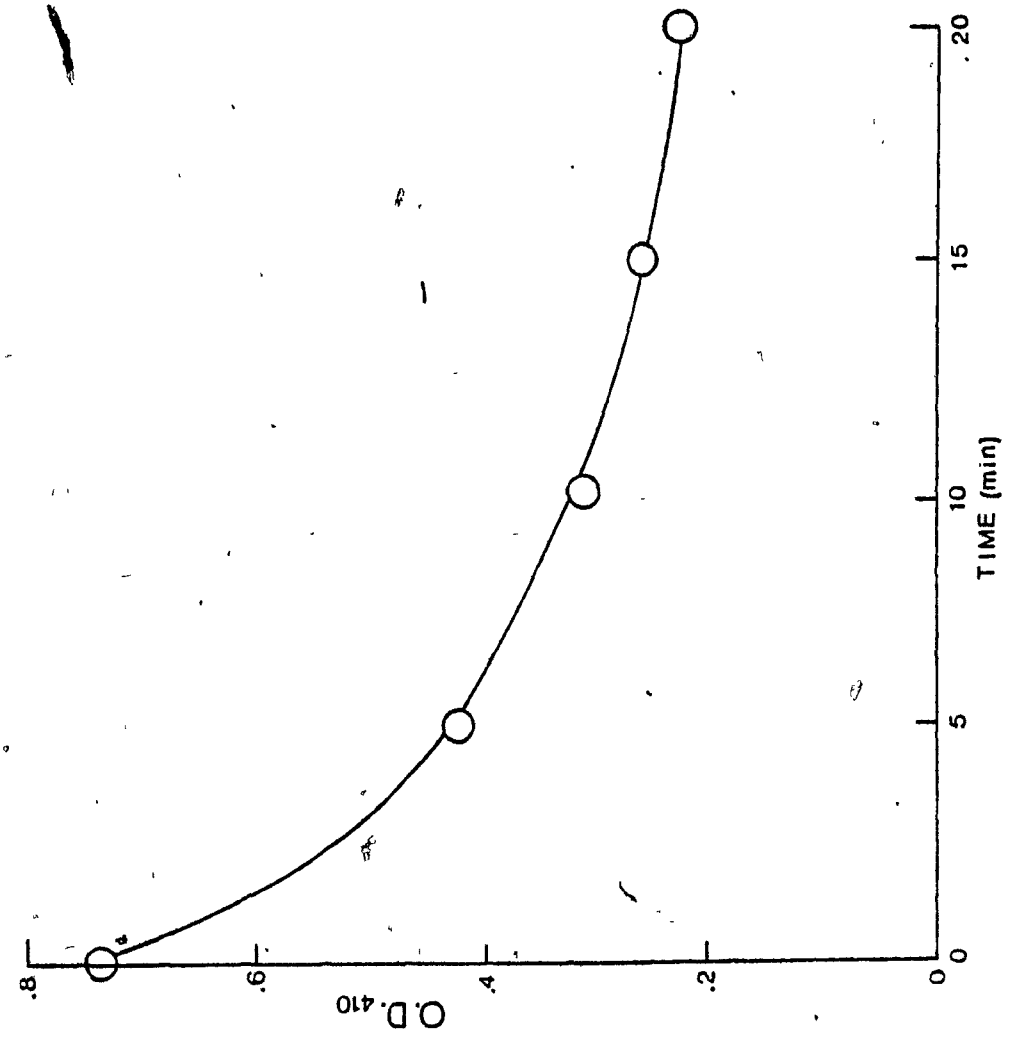

O.D.410
.8
.6
.4
.2
0
0
5
10
15
20
TIME (min)

Figure A.7. Detector response (area) versus quantity of internal or external standard injected.

(●) Internal standard (benzoic acid).
(▲) External standard (β-hydroxybutyric acid)

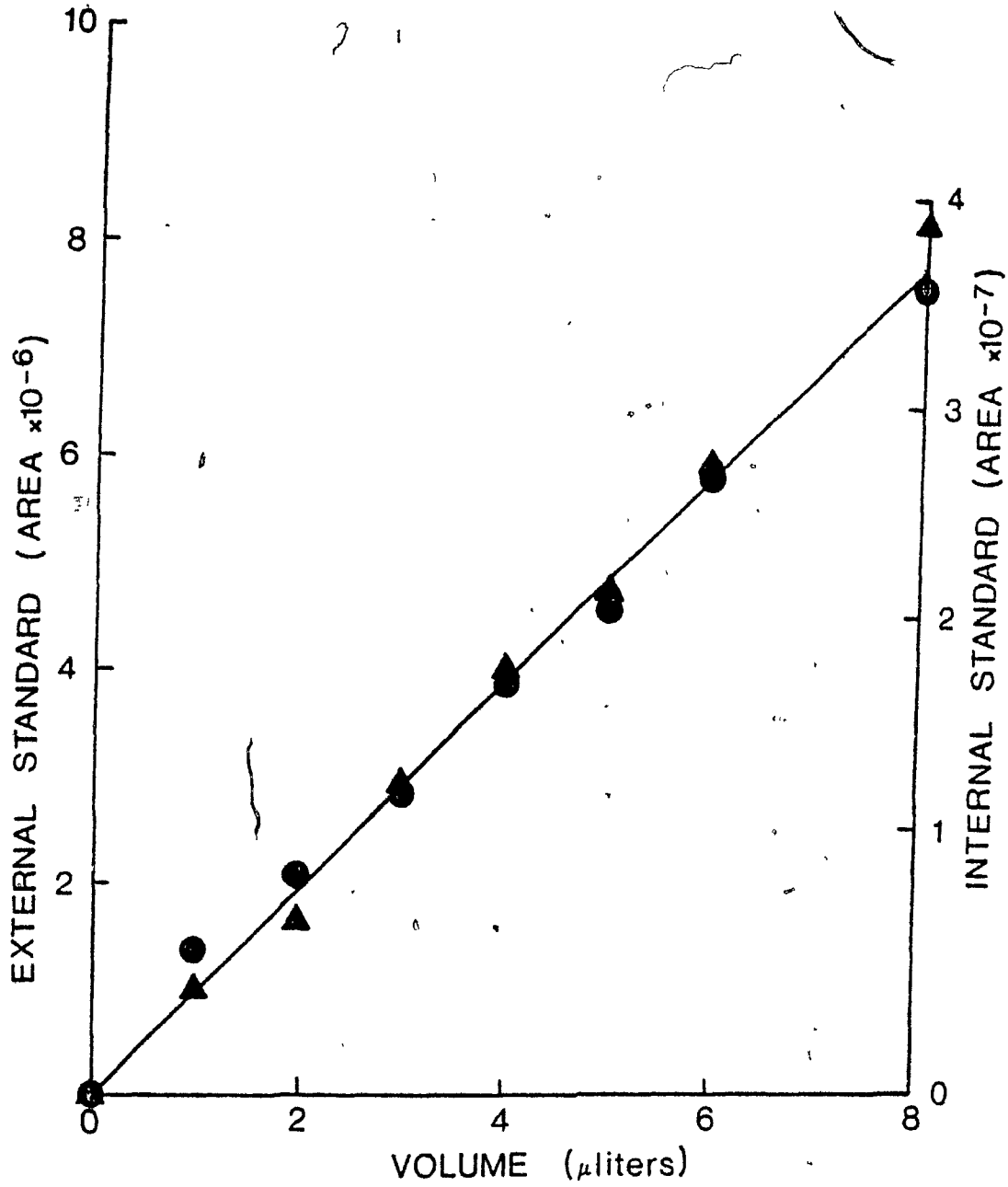
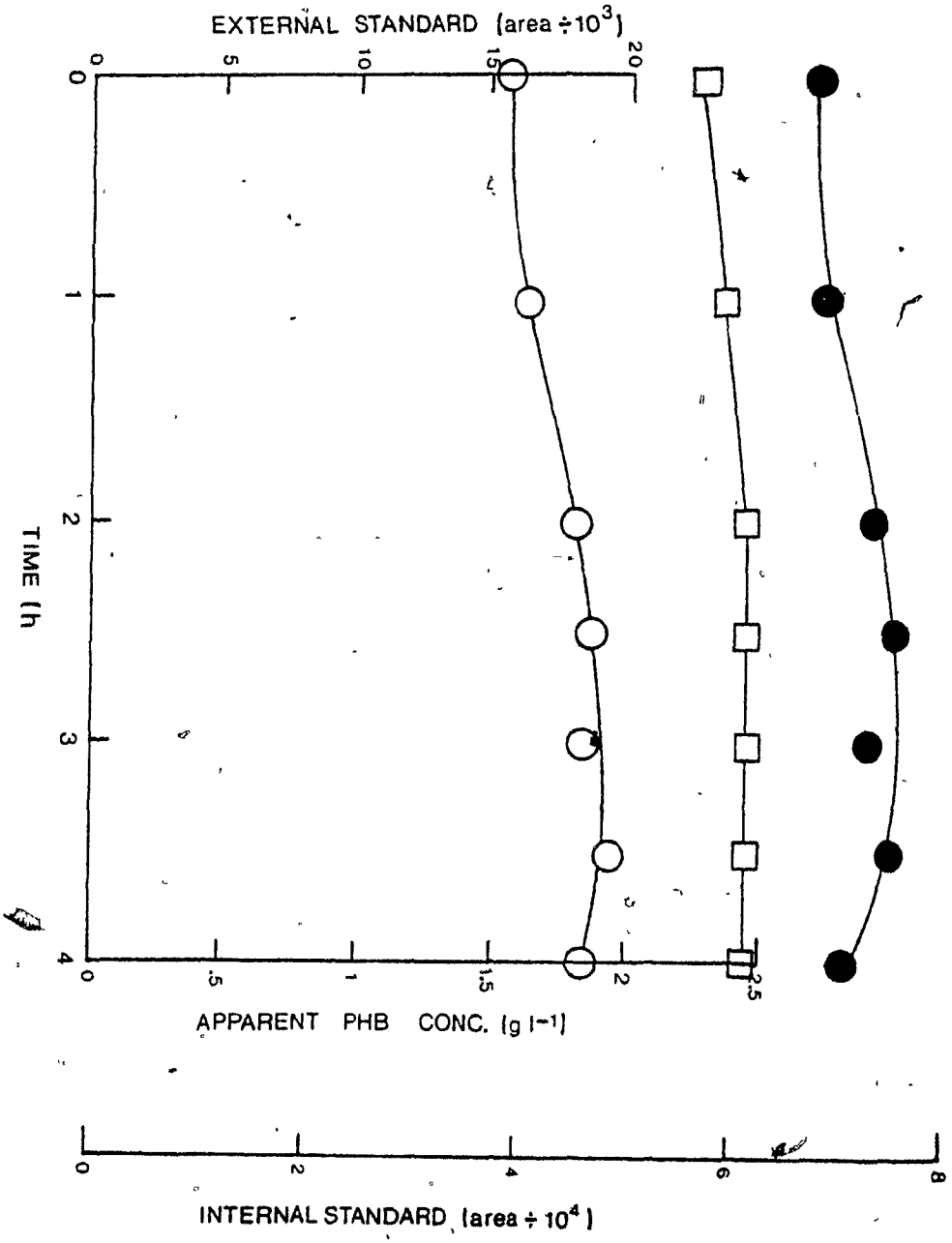

EXTERNAL STANDARD (AREA ×10-6)
INTERNAL STANDARD (AREA ×10-7)
VOLUME (μliters)
0
2
4
6
8
10
0
1
2
3
4

Figure A.8. The effect of heating time at 105 °C on the measurement of the internal and external standards used in PHB analysis.

(●) Internal standard (benzoic acid).
(□) Apparent PHB concentration reported by the integrator.
(○) External standard (β-hydroxybutyric) acid.


EXTERNAL STANDARD (area ÷10³)
0
5
10
15
20
TIME (h
0
1
2
3
4
APPARENT PHB CONC. (g l⁻¹)
0
.5
1
1.5
2
2.5
INTERNAL STANDARD (area ÷ 10⁴)
0
2
4
6
8

Appendix B

A GENERAL PROCEDURE FOR THE DEVELOPMENT OF PROCESSES THAT PRODUCE BIOLOGICAL PRODUCTS FROM COMPLEX TOXIC WASTES

B.1 Introduction

Many biochemical engineering research projects have involved attempts to produce a specific product from a specific waste (104,141). Often no set procedure is used to develop such a project. This may result in an attempt at the impossible, such as using a production strain that is known to be unable to use most components of the waste or attempting to produce single cell protein with a strain known to produce carcinogenic metabolites. The following is a simple generalized procedure that should prevent most of these common errors.

B.2 The Development Process

B.2.1 The Choice of a Suitable Waste

Not all wastes are suitable for use in biological processes. If the waste contains toxic components that are not known to be metabolized by any microorganisms or require a complex biological ecosystem for their degradation, then, without some form of chemical or physical pretreatment, it should be classified as being unsuitable. However, if a small number of microorganisms are capable of utilizing all of the major toxic components and most of the other components, then this waste can be considered for biological conversion into a product of value.

B.2.2 The Choice of a Suitable Product

It must first be determined which microorganisms are capable of using most of the waste components (especially the toxic components). This can be determined from published data (ie. theoretically) or, if this is insufficient, experimentally by identifying the microorganisms that proliferate in enrichment culture using the waste as the sole source of the particular element(s) in which the researcher is interested (ie. usually carbon and/or nitrogen). The most valuable products that can theoretically be produced by these microorganisms from these components can then be determined. The potential value of the product must outweigh the cost of production and recovery. Thus yield is of great importance and the theoretical yield should be considered.

B.2.3 Process Development

The choice of a fermentation process is the most important consideration at this stage. The economic considerations in choosing between a batch and a continuous process have been well documented (3). However, if the substrate is toxic, only fed-batch and chemostat methods are suitable. These methods allow for the use of toxic substrates as long as they are kept near a growth limiting supply. If both substrate and product are toxic, then fed-batch or multistage chemostat process may be employed.

B.3 Conclusions

Due to the complexity of the problem, the above approach is not specific. However, it does give a simple framework for theoretical process development without expensive or time-consuming laboratory experimentation. Laboratory time can be used instead to confirm or deny predictions, obtain accurate yield and kinetic values and to prepare for the commercialization of the process.

Appendix C

DETERMINATION OF THE REQUIREMENTS FOR CARBON SOURCE AND FERMENTATION CAPACITY

C.1 Determination of the Carbon Source Requirements

Data: $Y_{x/s}$ = 18 % (for high-protein biomass from fructose)

$Y_{P/s}$ = 14 % (for fructose)*

$Y_{x/s}$ = 14 % (for high-protein biomass from NVR)

Since the yield of PHB from NVR was never determined, the following estimation was made: $Y_{x/s}$ for NVR =

($Y_{x/s}$ for NVR / $Y_{x/s}$ for fructose)($Y_{P/s}$ for fructose)

= (14/18) (14) = 11 %

Assumption: PHB accounts for 60 % of the biomass in the second stage.

Therefore $Y_{x/s}$ (for biomass with a high PHB content) for fructose = (0.4) (18) + (0.6) (14) = 15.6 %

and $Y_{x/s}$ (for biomass with a high PHB content) for NVR = (0.4) (14) + (0.6) (11) = 12.2 %

Therefore to produce 1 kg of PHB requires

100 / ((15.6) (0.6)) = 10.7 kg of fructose and

100 / ((12.2) (0.6)) = 13.7 kg of NVR.

* $Y_{x/s}$ was 16.5 % in batch culture and 13 % in the second stage of the 2-stage chemostat.

C.2 Determination of the Fermentor Capacity Requirements

Assumptions: NVR production is 10 % of the total nylon 6'6' production (the actual value is higher but is a trade secret).

Only high-protein biomass is produced in the first stage and only PHB is produced in the second stage.

Since U.S. production of nylon 6'6' was about 7.15 X 10^8 kg of PHB in 1984 (56), 7.15 X 10^7 kg of NVR would have been produced.

Therefore (7.15 X 10^7 kg) (12.2 %) = 8.72 X 10^6 kg of high PHB content biomass could have been produced.

The annual first stage production would have been (8.72 X 10^6 kg) (40 %) = 3.49 X 10^6 kg of high-protein biomass.

The annual second stage production would have been (8.72 X 10^6 kg) (60 %) = 5.23 X 10^6 kg of PHB.

$$V_A = P_A / ((t)\ (D_A)\ (X_A))$$

where: V_A = Total fermentation capacity needed for production of high protein-biomass.

P_A = Quantity of high-protein biomass to be produced annually.

t = Total annual fermentor operation time.

D_A = Dilution rate of the first stage.

X_A = Biomass concentration of the first stage.

Assumptions: t = 300 days.

X_B = 15 g l^{-1}.

D_A = 0.4 h^{-1} (ie. μ_{max}).

No PHB is produced in the first stage.

X_A = 15 g l^{-1} X 40 % = 6 g l^{-1}.

t = (300 days) (24 h) = 7,200 h.

V_A = (3.61 X 10^9 g) / ((7,200 h) (0.4 h^{-1}) (6.0 g l^{-1})

= 2.09 X 10^5 liters.

$V_B = P_B / ((t) (D_B) (X_A))$

where P_B = amount of PHB produced in the second stage.

t = Total annual fermentor operation time.

D_B = Dilution rate of the second stage.

X_A = the protein concentation in the second stage.

Assumption: D_B = 0.08 h^{-1} (ie. the maximum q_p).

X_B = 60 % of the second stage biomass.

V_B = 5.33 X 10^9 g / ((7,200 h) (0.08 h^{-1}) (6.0 g l^{-1}))

= 1.54 X 10^6 liters.

Total fermentor capacity = $V_A + V_B$

= 2.09 X 10^5 + 1.54 X 10^6

= 1.75 X 10^6 liters.