THE WHOLE CELL CATALYSED HYDROLYSIS OF ACRYLAMIDE TO AMMONIUM ACRYLATE USING AN IMMOBILISED CELL BIOREACTOR

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ABSTRACT

Methods currently used for manufacturing the commodity chemical ammonium acrylate involve high temperatures that increases the risk of unwanted polymerisation and, in certain cases, leads to the generation of large amounts of unwanted by-product. The enzyme catalysed hydrolysis of acrylamide through to ammonium acrylate, however, may be carried out at ambient temperatures without by-product generation. Bioreactors operating with immobilised whole cell biocatalysts, have been examined as a means of producing ammonium acrylate.

Studies with the amidase active *C. nitrilophilus* showed that entrapment in cross-linked polyacrylamide gel was the best immobilisation method, resulting in a biocatalyst with good physical stability without a serious loss in amidase activity. Immobilisation scale-up was possible through the use of a suspension polymerisation technique to produce cells entrapped in cross-linked polyacrylamide beads. The beads exhibited amidase activity after drying and rehydration. The loss in amidase activity was reduced by decreasing the drying time while storage stability was increased when the beads were dried to a low water content.

Bioreactor studies were performed using *C. nitrilophilus* entrapped in cross-linked polyacrylamide gel cuboids. The changing conductance of reaction solutions, due to ammonium acrylate production, was used as an on-line method of monitoring amidase activity. Interfacing the conductance monitor to the acrylamide feed system, via a computer, allowed a 0.5 litre continuous stirred tank bioreactor to be operated at constant acrylamide and ammonium acrylate concentrations for several days at a time. It was shown that batch reactors were unsuitable for ammonium acrylate production as amidase activity was progressively and irreversibly deactivated in the presence of acrylamide and, to a lesser extent, ammonium acrylate. Amidase activity was decreased at lower reactor operating temperatures whilst amidase stability was increased. The automated bioreactor system was used to compare the stability of the amidase activity of *C. nitrilophilus* with that of two cell isolates: *R. rhodochrous* sp.632 and *Rhodococcus* sp.1068. The amidase activity of *R. rhodochrous* sp.632 was shown to be the most stable.

The amidase activity of *R. rhodochrous* sp.632 was found to be competitively inhibited by ammonium acrylate. Use of a fed-batch reactor for ammonium acrylate production was preferred over a continuous stirred tank reactor as the effects of product inhibition were reduced. Through monitoring of the conductance measurements, the fed-batch system was automated so that acrylamide concentrations were kept at a constant level. Operation of the system at different acrylamide concentrations showed that higher concentrations increased the rate of amidase activity loss.

Bioreactor scale-up was performed by designing, constructing and operating a stirred tank reactor system with a 6 litre working volume. The reactor was operated in fed-batch and continuous modes using computer control, and ammonium acrylate was produced on a kilogram scale. Performance of the 6 litre reactor operating with *R. rhodochrous* sp.632 immobilised in cross-linked polyacrylamide beads, was comparable to the performance of the 0.5 litre reactor.

Performance tests on polymers prepared from the bio-ammonium acrylate showed them to be indistinguishable from polymers of chemical origin.

CONTENTS

LIST OF SYMBOLS

ACM = Acrylamide
NH4ACR = Ammonium acrylate
[P] = Product Concentration (mol l^{-1})
[S] = Substrate concentration (mol l^{-1})
$[S]_0$ = Initial substrate concentration (mol l^{-1})
A = Amidase activity (μmoles NH4ACR g^{-1} dry cells min^{-1})
De = Diffusion coefficient (cm^2 s^{-1})
E_0 = Active enzyme concentration at time zero (mol l^{-1})
E_t = Active enzyme concentration at time t (mol l^{-1})
F = Reactor flow through rate (l s^{-1})
k_0 = Zero order rate constant (mol l^{-1} s^{-1})
k_1 = First order rate constant (s^{-1})
k_d = First order decay constant (min^{-1})
k_m = Michaelis constant (mol l^{-1})
k_p = Product inhibition constant (mol l^{-1})
k_s = Substrate inhibition constant (mol l^{-1})
P = Specific production rate (g NH4ACR/ g^{-1} dry cells hrs^{-1})
r = Distance from centre of particle (cm)
R = Reaction rate (mol min^{-1})
R_p = Sphere radius or half slab thickness (cm)
$S_{(B)}$ = Bulk substrate concentration (mol l^{-1})
$S_{(r)}$ = Substrate concentration at distance r (mol l^{-1})
t= time (s)
$t_{1/2}$ = Catalyst half-life (mins)
v = Rate of reaction (mol s^{-1})
V = Reaction volume (l)
V_{max} = Maximum rate of reaction (mol s^{-1})

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Dedicated to the memory of my grandfather, Frank Dobson.

INTRODUCTION

1. THE SYNTHETIC AND ENZYME CATALYSED PRODUCTION OF AMMONIUM ACRYLATE

1.1 The Uses and Manufacture of Acrylic Acid

The chemistry of acrylic acid and its associated salts gives rise to a large range of products serving many end-uses (Wood, 1993). Polymers of acrylic acid are utilised in the production of superabsorbent materials used in diaper manufacture and the formulation of reduced-phosphate detergents. Acrylic acid and acrylamide are copolymerised to produce flocculants used in the treatment of sewage. (Kirk-Othmer, 1991). Polymers and copolymers of acrylate esters give rise to resins and emulsions used in the manufacture of paints, coatings, adhesives, cement modifiers, textiles and vinyl mouldings. Water based acrylic emulsions used in place of traditional solvent-and-resin systems, combined with the demand for superabsorbents, led to a growth in global acrylates demand of 7 % per year, with 1993 requirements estimated at 1.7×10^9 kg per year (Wood, 1993).

The most common method of acrylic acid manufacture is based on the oxidation of propylene to first acrolein and then acrylic acid:

$$CH_2{=}CHCH_3 + O_2 \longrightarrow CH_2{=}CHCHO + H_2O$$

$$CH_2{=}CHCHO + \tfrac{1}{2}\, O_2 \longrightarrow CH_2{=}CHCOOH$$

These two steps are carried out using two reactors: the first packed with a bismuth molybdate catalyst and the second packed with a molybdenium vanadium oxide catalyst. The exothermic reaction leads to temperatures in the range of 330-430 °C and

hence a molten salt coolant is required. The acrylic acid is recovered by contacting with a suitable solvent such as butyl acetate which has a high selectivity for acrylic acid and low solubility for water and by-products. The extract is then vacuum distilled at low temperatures, to avoid polymer and dimer formation, before passing through a series of distillation columns under mild conditions and short residence times to again minimise dimer formation. Glacial acrylic acid is typically 95.5 % pure - with the predominant contaminants being water and acetic acid. The principal problem associated with acrylic acid manufacture is the risk of polymerisation. Inhibitors are added, such as the monomethyl ether of quinone, to prevent this. The chance of polymerisation occurring is increased at elevated temperatures as is the formation of dimers. For these reasons a storage temperature of 16-29 °C is preferred.

Other methods of manufacturing acrylic acid include the sulphuric acid catalysed hydrolysis of acrylonitrile. This method is economically unfavourable on a large scale when compared to the propylene based route. It is also unfavourable from an environmental view point due to the large amount of ammonium hydrogen sulphate waste generated.

1.2 Enzymes as Alternative Catalysts for Organic Synthesis

The industrial production of acrylic acid may be considered as a conventional chemical process involving a high temperature reactor containing a metal based catalyst. In contrast to this, the past twenty years has seen the development of several industrial processes utilising a quite different technology exploiting biological processes for the production of useful chemicals (Tanaka *et al.*, 1993). This will be referred to here as

biotechnology. In recent years the most significant development in the field of synthetic chemistry has been the application of biological systems to carry out chemical reactions (Yamada, 1993), and in particular the use of enzymes to catalyse these reactions. It is therefore poignant to outline the characteristics of enzymes that make them useful alternatives to traditional catalysts in chemical synthesis.

1.2.1 Advantages of Enzyme Catalysed Systems

Enzymes are the protein biocatalysts present in all living systems participating in the many chemical reactions necessary for metabolism. Because of the complexity of metabolism, there are thousands of different kinds of enzymes in just one typical cell (Mathews and Holde, 1990) capable of catalysing many thousands of reactions. Enzymes have distinctive properties offering several potential advantages over conventional catalysts and these are highlighted in Table 1.1 and discussed below.

Table 1.1 : Advantages of using Enzymes for Chemical Synthesis

Catalyse diverse range of reactions
Stereo specific, regio specific
Operate at moderate pH, temperature, pressure
High conversions
Environmentally acceptable

Not only is it possible to use enzymes to catalyse a diverse range of reactions, it is also possible to find enzymes capable of performing reactions that are difficult or impossible to emulate using conventional chemistry (Katchalski-Katzir, 1993). Enzymes are hugely important in the field of chiral synthesis (Darbourne, 1993) due to their stereo

and regio specificity, and many examples can be found in the literature of such reactions (Crosby et al., 1994, Chirality in Industry, 1992).

One of the main advantages of enzymes is their ability to catalyse reactions at moderate conditions of temperature, pressure and pH. These characteristics lead to a lower energy cost compared to comparable reactions where there is a need to maintain a chemical reactor at high temperatures to ensure adequate conversion rates. Less extreme operating conditions also mean that equipment can be manufactured from materials that are cheaper than those normally associated with conventional chemical processes. The ability to operate under moderate conditions is also useful when dealing with unstable substances where there is a need to reduce the formation of side-products. An example of this is the biotransformation of fats and oils where the scope for the application of lipases in the oleo chemical industry is enormous (Vulfson, 1993). The conditions for fat-splitting and the conventional glycerolysis of oils involve temperatures of 240-260 °C and high pressures, resulting in products that require distillation to remove impurities and products of degradation. The Miyushi Oil and Fat Co. have reported the commercial use of a lipase enzyme in the manufacture of fatty acids (Haq *et al.*, 1985) in a process that is cheaper to operate and yields a higher quality product.

The high efficiency of enzymes also gives them the benefit of being environmentally acceptable. Public concern with environmental issues has heightened the attractiveness of processes that operate with high selectivities and thus minimise the problems of waste and by-product disposal (Akiyama *et al.*, 1987). Being biological substances, even the enzymes themselves may be considered as biodegradable.

1.2.2 Choice of Catalyst Form

When designing an enzyme catalysed process there are several forms of biocatalyst that need to be considered (Lilly 1992). The term *biocatalyst* will be used here to refer to any of the enzyme catalyst forms given below:

Free Cells-Living

Free Cells-Resting

Immobilised Cells - Living

Immobilised Cells - Resting

Free Enzymes

Immobilised Enzymes

Living cells are often necessary when complex metabolic pathways are involved and coenzyme regeneration is required. Substrates must be provided for cell maintenance, and aeration and carbon dioxide release facilities may be required (Webb *et al*, 1986). For example, the production of ethanol from glucose involves five intermediates, the use and regeneration of the cosubstrate ATP and the coenzyme NAD^+ plus the release of carbon dioxide (Stanier *et al*, 1987).

For some simpler conversions involving one or a few enzyme steps the use of a respiring cell is no longer necessary, and the choice of a cell bound or extracted enzyme is available. Several factors need to be considered before making such a choice.

One of the enzymes used in the production of high fructose corn syrup, α-amylase, is an extracellular enzyme. Compared to an intracellular enzyme it is much easier and cheaper to extract, and therefore economical to use in its soluble form. The extraction of intracellular enzymes is expensive. Chaplin and Bucke (1990) estimate that generally, during purification, a 240 fold increase in enzyme concentration, results in a 6 fold increase in cost per unit volume of enzyme preparation. Obviously, the cost of extraction needs to be balanced against the increase in production rates available from the concentrated enzyme, and will vary from one process to the next.

Immobilisation is the process of physically confining a microorganism or enzyme to allow its reuse. Living cells may be immobilised as a means of increasing the cell density and allowing increased production rates while preventing washout from the reaction vessel. For example, sewage is often treated with bacteria adhering on to porous rock.

If the required process has a high profit margin and the biocatalyst has a short lifetime, then the biocatalyst may be used profitably on a once through basis. However, by immobilisation, a stable biocatalyst can be used more efficiently. The subject of immobilisation is dealt with in more detail in Chapter 2.

1.3 Large Scale Use of Enzymes

When considering any novel process with the aim of applying it on an industrial scale, it is necessary to consider the practicalities of such a process and determine how easy or difficult it will be to make the process commercially viable. The advantages of using an enzyme system have been outlined above but these alone do not justify their use. For example, if it is unlikely that the required enzyme can be produced on a large scale, it is difficult to justify further examination of the process in which it could be used. The key areas that need to be considered are listed below:

Availability of the required enzyme
Large scale production of the enzyme
Form in which the enzyme is to be used
Type of reactor in which the enzyme is to be used

This thesis is specifically concerned with the last two areas and these are discussed in detail in Chapter 2. However, it is important to consider the availability and large scale production of enzymes with regard to the possibility of their selection as a catalyst for an industrial scale process.

1.3.1 Commercial Availability of Enzymes

Biologically active enzymes can be extracted from the cells of any living organism such as animals, plants, yeasts and microorganisms (Chaplin and Bucke, 1990). Approximately 2500 types of enzyme can now be acquired from biochemical suppliers

and of these, around 50 are available on a multikilogram to ton scale (Gacesa and Hubble, 1987). However, these enzymes may not have the required activity for an envisaged bioconversion. In such a situation it is possible to screen for cells that have the required enzyme (Tweel, 1994).

Once an enzyme has been identified as having the required characteristics to perform a certain transformation, it is necessary to be able to produce the enzyme in quantity. This is achieved by culturing a cell that synthesises the enzyme at sufficient levels, typically this is around 1% of the total soluble protein but levels above 20% have been reported (Nagasawa and Yamada, 1990). It is also essential to grow the cells efficiently on a large scale. For example, being able to produce a high biomass concentration using a cheap culture medium (Kennedy, 1994). There are techniques available to increase the amount of enzyme that a cell produces. One such method is the use of continuous culture for the selection of over producing mutant strains (Leak, 1994). Over producing strains can also be produced by the use of molecular cloning which has made enzymes available at dramatically lowered costs (Akiyama *et al.*, 1987).

Microorganisms produce a tremendous range of useful enzymes, are quick and easy to grow and the technology of scale-up is well established (Gacesa and Hubble, 1987). Developments in recombinant DNA techniques have also made it possible to produce enzymes using microorganisms containing genetic information from animal and plant cells. In these ways it is often possible to increase the activity some hundred to thousand times that observed in the original isolate.

1.3.2 Disadvantages of Enzyme Systems

One of the key areas where enzymes show a disadvantage over traditional catalysts is their poor stability (Freeman, 1984) both in terms of storage (Kaul and Mattiasson, 1993) and operation.

Once an enzyme has been produced by a fermentation process it is beneficial to be able to store it without losing catalytic activity. Enzymes are susceptible to deactivation during storage due to unfolding of their three dimensional structure and spoilage caused by microbial growth. Industrial enzyme preparations are often < 10 % (w/w), the remainder being made up of stabilising agents, preservatives and diluents (Chaplin and Bucke, 1990). The action of many of these stabilising agents is to counteract the effects of water which promotes enzyme denaturation. Consequently, stabilisation of an enzyme preparation can often be achieved by drying (Tijesterman, 1993) in either a spray-drier or fluid bed drier. This method may also be applied to whole bacteria (Lievense and van't Riet, 1993) and even to immobilised bacteria (Kumakura and Kaetsu, 1983).

An important consideration in a bioconversion is the stability of the catalyst under reaction conditions. Enzyme stability is often referred to in terms of half-life: the time taken for the activity to be reduced by half. (Klein and Wagner, 1978). Biocatalysts with a longer half-life will produce more product per unit mass of catalyst within the catalysts operational life and this is particularly important when producing a commodity chemical (Moo-Young and Christi, 1994). Stability of the catalyst also effects the

operation of a reactor as a long half-life results in a decrease in the number of times the reactor is recharged with fresh catalyst.

Stability can vary greatly from one particular enzyme type to another, while stability of one particular enzyme can be affected by several factors such as the form in which it is used or the reaction conditions it experiences. Enzymes retained in a whole cell form have been found to be more stable than free enzymes (Ballesterios *et al.*, 1994), while immobilised cells can show much greater stability than freely suspended cells (Klein, 1988). By varying the immobilisation procedure it is also possible to greatly increase the apparent enzyme half-life (Takamatsu *et al*, 1981). These immobilisation effects are discussed in greater detail in Chapter 2.

1.4 The Industrial Production of Acrylamide

Acrylamide is a commodity chemical used in the production of flocculants and enhanced oil recovery products. Since 1985 it has been produced by the hydrolysis of acrylonitrile using an enzyme catalysed process operated by the Nitto Chemical Company.

The Nitto process is based on a nitrile hydratase active bacterium immobilised in a cross-linked polyacrylamide gel. This catalyst is used in packed-bed reactors at temperatures below 10 °C to hydrolyse an acrylonitrile solution to acrylamide (Ashina and Suto, 1993). Due to the toxicity and low solubility of acrylonitrile, it is added in

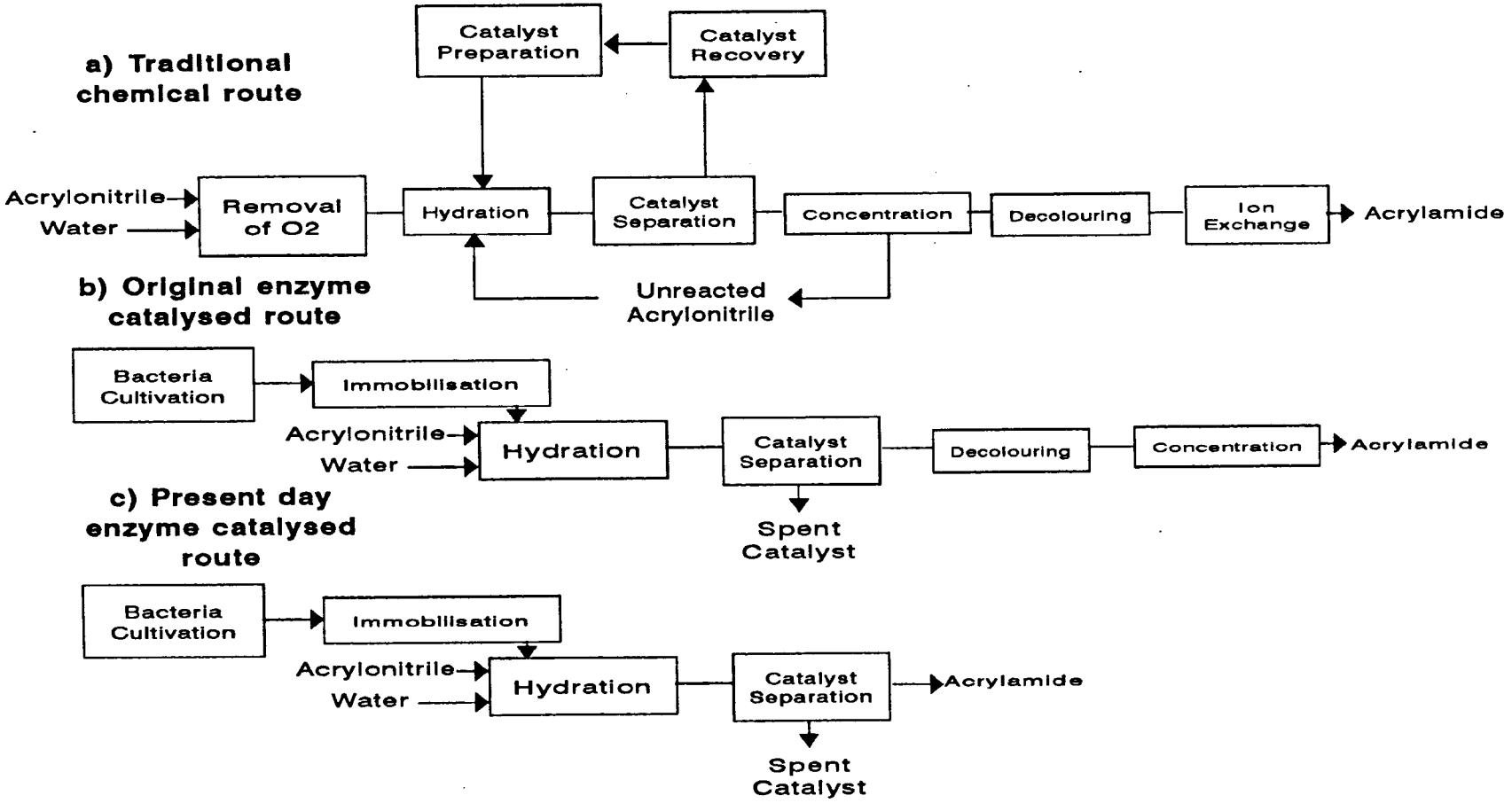
sequential batches to build the acrylamide concentration up to the required concentration.

The Nitto Chemical Company were already making use of an acrylonitrile degrading bacteria prior to 1985 in a process that eliminated acrylonitrile from the factory waste streams (Cheetham, 1994). Using a screening procedure, they were able to produce a nitrile hydratase active bacterium with sufficiently high enzyme activity and stability to warrant further study (Watanabe *et al.*, 1987a). They were able to develop this strain, *Rhodococcus* sp. N-774, by improvement of the growth media (Watanabe *et al.*, 1987a) until the level of cell growth and enzyme activity were high enough for industrialisation (Ashina and Suto, 1993).

1.4.1 Comparison of the Conventional Route for Acrylamide Production with the Enzyme Route

Figure 1.1 compares the conventional route for acrylamide production from acrylonitrile (Scheme a) against the enzyme catalysed route operated by the Nitto Chemical Company (Scheme b). The conventional route has several disadvantages. Compared to the Nitto Process, much higher temperatures are required (80-140 °C) which result in the production of toxic waste, including hydrogen cyanide. The copper catalyst is difficult to regenerate and traces of it in the product must be removed by ion exchange (Cheetham, 1994). As can be seen from Figure 1.1a and b, the enzyme catalysed process is much simpler. The lower operating temperatures mean that less energy is required and that construction of the plant equipment is simpler, though this is slightly offset by the need for refrigeration equipment.

Figure 1.1 Comparison of Traditional and Enzyme Catalysed Acrylamide Production Routes



1.4.2 Development of the Nitto Process

Since the enzyme catalysed industrial production of acrylamide started, the successful search for improved bacterial strains has continued, leading to a large increase in the efficiency of the process, as can be seen in Table 1.2 (Nagasawa *et al*, 1993). Improvements in the process mean that decolouring and concentration of the product are no longer required (Ashina and Suto, 1993) leading to an even simpler and more attractive process (Figure 1.1C). There has also been a reduction of impurities in the acrylamide produced. Because of this the maximum chain length of the polymers produced from the acrylamide has increased, (Cheetham, 1994), leading to a greater potential product range.

Table 1.2: Development of the Nitto Process since 1985

First year production	**1985**	**1988**	**1991**
Parameters	*Rhodococcus* sp. N-774	*P. chlorraphis* B23	*R. rhodochrous* J1
Acrylamide yield (g/g cells)	500	850	>7000
Total annual production (tons)	4000	6000	>30 000
Product concentration (% w/v)	20	27	40

The enzyme catalysed production of acrylamide is one of the first examples of biotechnology being used for the production of a commodity chemical, (Kabashi *et al.*, 1992), and serves as a useful illustration of several characteristics of an enzyme catalysed production process.

1.5 Nitrile and Amide Degrading Microorganisms

It has been known for several years that some microorganisms are capable of producing organic acids in the form of their ammonium salts via the enzyme catalysed hydrolysis of nitriles and amides. Examples of such organisms are given in Table 1.3.

Table 1.3: Nitrile and Amidase Degrading Organisms

Organism	Substrate	Enzyme	Reference
Pseuodomonas aeruginosa	acrylonitrile	nitrile hydratase	Clarke (1970)
Brevibacterium R312	acetonitrile acetamide	nitrile hydratase amidase	Miller and Knowles (1984)
Nocardia rhodochrous	acrylonitrile	nitrile hydratase amidase	Wyatt and Linton (1988)
Rhodococcus rhodochrous	acrylonitrile	nitrilase	Nagasawa *et al.* (1990)
Brevibacterium sp. R312	acrylamide	amidase	Bernet *et al.* (1987)

There are two possible routes by which nitriles and amides may be converted to acids. Firstly, nitriles may be hydrated to amides using a *nitrile hydratase* enzyme:

$$\text{ACRYLONITRILE} + H_2O \xrightarrow{\text{NITRILE HYDRATASE}} \text{ACRYLAMIDE}$$

$$RCN + H_2O \rightarrow RCONH_2$$

The amides may then be hydrolised to their corresponding acids and ammonia using an *amidase* enzyme:

$$\text{ACRYLAMIDE} + H_2O \xrightarrow{\text{AMIDASE}} \text{AMMONIUM ACRYLATE}$$

$$RCONH_2 + H_2O \rightarrow RCOOH + NH_3$$

Alternatively, nitriles may be converted directly to their corresponding acids

$$\text{ACRYLONITRILE} + 2H_2O \xrightarrow{\text{NITRILASE}} \text{AMMONIUM ACRYLATE}$$

$$RCN + 2H_2O \rightarrow RCOOH + NH_3$$

Several studies into nitrile and amide degrading organisms are reported in the literature. These studies are discussed below:

Miller and Knowles (1984) studied the nitrilase and amidase of *Brevibacterium* R312. They suspended non-growing cells in acetonitrile and observed the production of acetamide, acetic acid and ammonia. By lysing the cells they were able to show that nitrilase and amidase activity were located in the cytosolic fraction of the cell.

Wyatt and Linton, (1988), have studied the nitrile degrading bacterium *Nocardia rhodochrous*, and suggest that nitriles and amides enter the cell by diffusion alone. Indeed, Pryce (1988) observed that the very high enzyme activities of such microorganisms suggest that cell permeability can not be much of a constraint on the reaction rate.

1.5.1 Ammonium Acrylate Production using Nitrilase and Amidase Active Bacteria

Nagasawa *et al.*, (1990) reported the use of resting cells of *Rhodoccocus rhodochrous* J1, containing a nitrilase enzyme, for the hydrolysis of acrylonitrile to ammonium acrylate. By sequentially adding acrylonitrile to a buffered suspension of

whole cells, they claimed that an ammonium acrylate concentration of 5.45 M was reached.

Bernet *et al.*, (1987), have taken the enzyme catalysed production of ammonium acrylate a step further by operating an immobilised cell continuous reactor. By entrapping resting whole cells of the amidase active *Brevibacterium* sp. R312 in calcium alginate beads, they were able to hydrolyse acrylamide to ammonium acrylate in a continuous fluidised bed reactor without wash out of the cells occurring. Concentrations of up to 0.56 M ammonium acrylate were produced.

These findings demonstrate that enzymes are capable of acting as catalysts for the production of the ammonium salt of acrylic acid and that both acrylonitrile and acrylamide may be used as substrates. The Nitto Process proves that it is possible to use enzymes for the large scale production of a commodity chemical. It has been shown above that the Nitto Process also has several advantages over the conventional route to acrylamide, and it is possible that an enzyme catalysed process for ammonium acrylate production may benefit in a similar way.

The work reviewed above indicates that it may be possible to develop an enzyme catalysed process for the production of ammonium acrylate and that such a process may exhibit several advantages over a more traditional route. These reasons therefore justify further study into an enzyme catalysed process for the production of ammonium acrylate using acrylonitrile or acrylamide as a substrate.

2. PRINCIPLES OF IMMOBILISED CELL SYSTEMS

To be commercially viable, a biotransformation producing a commodity chemical must be operated at a high productivity (g l^{-1} hr^{-1}) without the need for frequent biocatalyst replacement. The product should be of high concentration and purity and be produced at an economic rate.

The performance of a biotransformation depends upon the reaction kinetics of the biocatalyst and on its enzymic stability; both of which depend partly on the environment of the enzyme. The adverse effects of high substrate and product concentrations and temperature can be minimised by correct operation of the bioreactor. Operating costs and product purification cost can be reduced by biocatalyst immobilisation. Each of these salient factors which have a bearing on the biotransformation performance will be discussed separately.

2.1 Enzyme Kinetics

Enzymes are large protein molecules containing an active site that will accept a substrate molecule and position it in such a way as to favour its subsequent transformation. Enzymes accelerate reactions by decreasing the activation energy requirement, but as is the case for all catalysts, do not alter the position of equilibrium. The making and breaking of chemical bonds by an enzyme is preceded by the formation of an enzyme-substrate complex:

$$E + S \Leftrightarrow ES \rightarrow E + P$$

The kinetic properties of many enzymes can be described using the Michaelis-Menten model:

$$v = \frac{Vmax \times [S]}{Km + [S]}$$

where v = Rate of reaction (mol s^{-1})
V_{max} = Maximum rate of Reaction (mol s^{-1})
[S] = Substrate concentration (mol l^{-1})
K_m = Michaelis constant (mol l^{-1})

A plot of this equation is shown in Figure 2.1. As the substrate concentration increases the reaction rate moves from being first order to zero order. The value of K_m is the substrate concentration at which the rate of reaction is half that of the maximum rate. The value of K_m indicates the extent of binding between the enzyme and its substrate, a lower K_m indicating a greater extent of binding (Chaplin and Bucke, 1990). Modifications to the Michaelis-Menten expression are required to describe the kinetics of enzymes which are affected by various types of inhibition. Enzymes are often reversibly inhibited by their substrates and products. Equations describing product and substrate inhibition are shown in Table 2.1. The product of a transformation will often resemble the substrate sufficiently to sit in the binding site of the enzyme molecule (Mathews and Holde, 1990) and reduce the turnover of the enzyme. This type of inhibition is referred to as *product inhibition*.

Enzyme catalysed reactions typically have a temperature and pH optimum at which maximum reaction rates are observed. Typical bell shaped activity curves are shown in Figure 2.2. Extremes of temperature and pH as well as of ionic strength may lead to a transformation of the complex molecular interactions present in the folded amino acid

Figure 2.1: Plot of Michaelis-Menten Equation

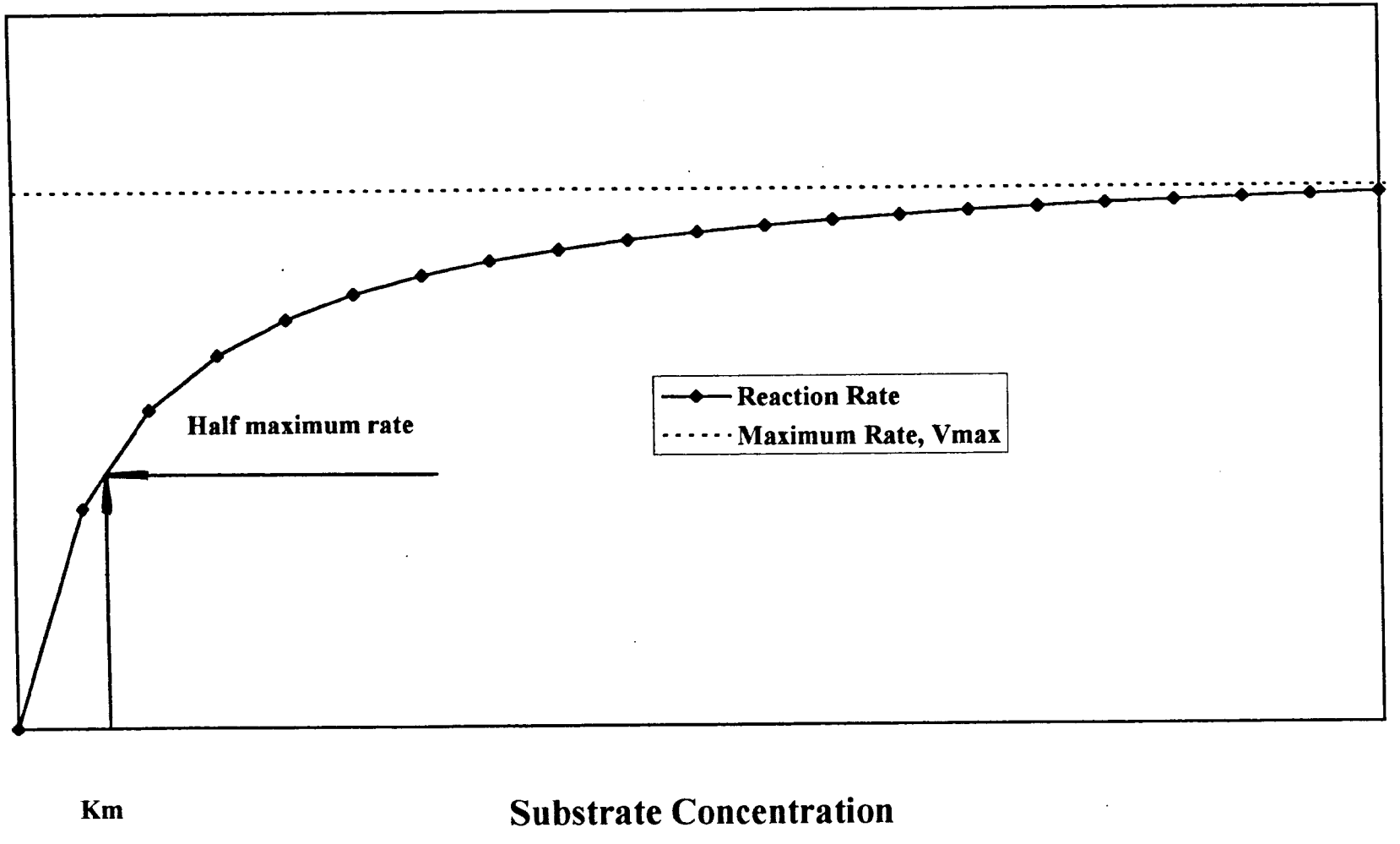
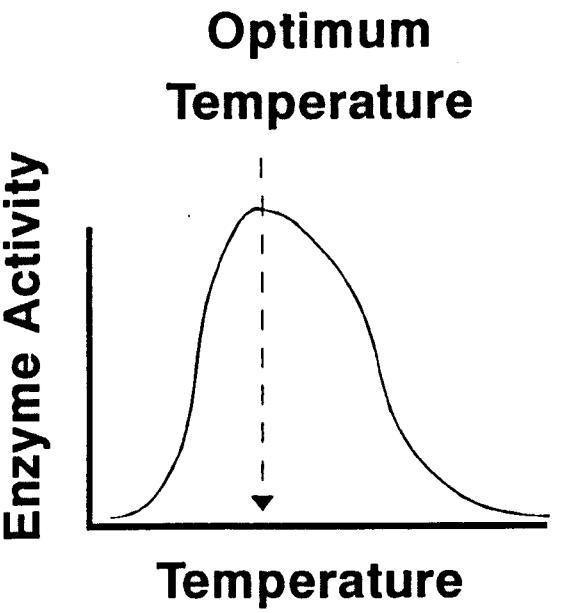
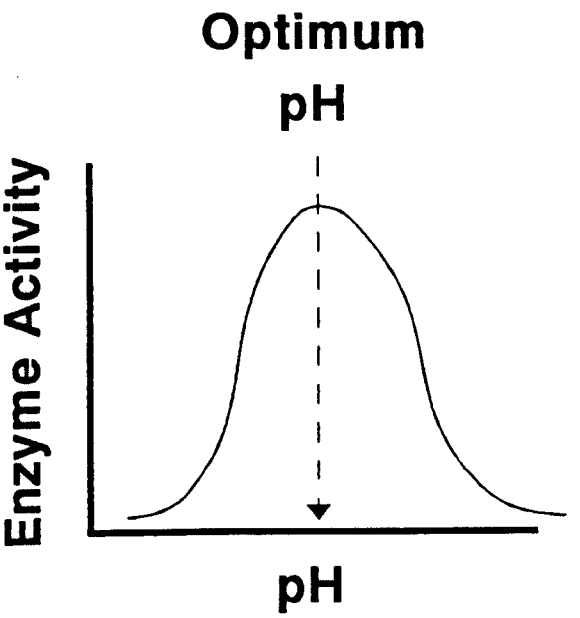


Figure 2.2: Effect of Temperature and pH on Enzyme Activity





sequence of an enzyme, leading to a possible irreversible denaturation of its structure and hence a loss in activity. Enzymes may also irreversibly bind to a substance causing permanent deactivation. The rate at which enzyme activity declines is of critical importance and this is especially true when considering its long-term use in continuous reactors (Lovitt and Jones, 1994). The characteristics of an enzyme play an important role in the choice of reactor when designing for optimum enzyme utilisation, and this is covered in greater detail in Section 2.3.

Table 2.1 : Equations Describing Various Types of Enzyme Kinetics

Kinetic Expression	Type of Enzyme Kinetics
$v = \frac{V\text{max} \times [S]}{Km + [S]}$	Simple Michaelis-Menten kinetics
$v = \frac{V\text{max} \times [S]}{Km(1 + [P]/Kp) + [S]}$	Competitive product inhibition
$v = \frac{V\text{max} \times [S]}{Km + [S](1 + [S]/Ks)}$	Substrate inhibition

Nomenclature

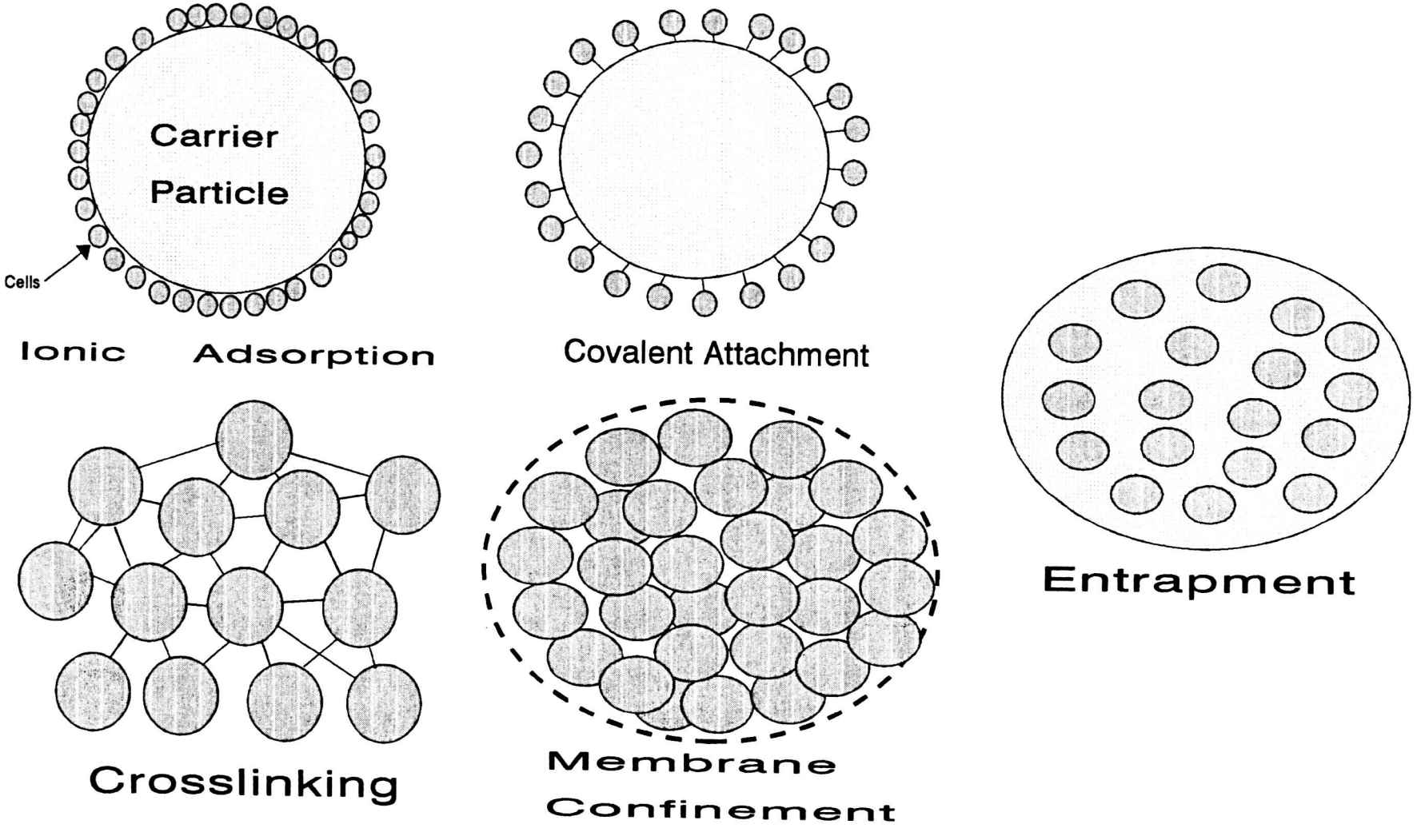
v = Rate of reaction (mol s^{-1})
V_{max} = Maximum rate of reaction (mol s^{-1})
[S] = Substrate concentration (mol l^{-1})
K_m = Michaelis constant (mol l^{-1})
[P] = Product concentration (mol l^{-1})
K_p,K_s = Inhibition constants (mol l^{-1})

2.2 Immobilisation of Cells

There are many techniques for the immobilisation of cells and these may be split into five groups (Birnbaum *et al.*, 1986). These are listed below and also shown in Figure 2.3:

Adsorption
Covalent Attachment
Cross-Linking
Entrapment
Membrane Confinement

Figure 2.3: Methods of Immobilisation



Entrapment is by far the most frequently used technique for cell immobilisation due to its simplicity and effectiveness (Philips and Poon, 1988). For this reason the discussion will be restricted to common methods of entrapment.

2.2.1 Entrapment in Calcium Alginate Gel

Entrapment of cells in calcium alginate is one of the most widely used methods of immobilisation, and is both simple and gentle (Smidsrod and Skjak-Braek, 1990). The biocatalyst is mixed with a solution of sodium alginate which is then dripped into a divalent metal salt, typically calcium chloride. The calcium ions form a bridge between the alginate polymer strands producing insoluble spherical particles with diameters of several mm. The use of compressed air (Klein *et al*, 1983) and a rotative atomiser (Begin *et al*, 1991) has led to control over the bead size.

2.2.2 Entrapment in Thermogels

Thermogels, like alginate, are polysaccharides and usually obtained from seaweed. Solutions of thermogels form a solid at temperatures below 40 ^{o}C, and this property can be exploited to immobilise cells. Agar, gellan and k-carrageenan are all thermogels.

Takata *et al*, (1980) investigated the immobilisation of bacteria containing fumarase activity. They were able to produce L-malic acid on an industrial scale by entrapment of *Brevibacterium ammoniagenes* in the thermogel k-carrageenan. They found that this method improved the enzyme activity retention by 1.6 times compared to entrapment in

polyacrylamide gel. The pH optimum of the entrapped enzyme was broadened in comparison to that of the free cells and enzyme.

Buitelaar *et al*, (1990), reported their investigations into immobilisation of yeast, bacterial and plant cells using agar, gellan and k-carrageenan. They produced spherical beads by dripping the gel/cell mixture through an organic solvent using a resonance nozzle and needle technique. Enzyme activity retention ranged from 20 to greater than 100 %, the latter result possibly being due to permeation of the cell wall during immobilisation.

2.2.3 Entrapment in Cross-Linked Polyacrylamide

Polyacrylamide is the synthetic polymer most often used for immobilisation of microbial cells (Phillips and Poon, 1988). Cross-linked polyacrylamide gel is formed by mixing acrylamide with methylene-bis-acrylamide (cross-linking agent) and then initiating the polymerisation. Cells or enzymes may be immobilised by mixing them with the monomers before gelation occurs. The resulting gel is then cut up or ground, to produce particles of a workable size.

Cottenceau *et al.*, (1990), used polyacrylamide gel to entrap *Streptococcus faecalis* for the conversion of arginine to citrulline. They found that the gel ensured an easy and physically stable immobilisation. Yamamoto *et al.*, (1976) immobilised *Brevibacterium ammoniagenes* in polyacrylamide for the fumarase-catalysed production of L-malic acid. Their investigations found that the stability of the fumarase enzyme activity was much increased by the immobilisation, compared to that of the free cells, and that the

apparent activation energy of the conversion was considerably lower than that of the native enzyme.

Koshcheyenko *et al.*, (1983), studied the characteristics of 3-ketosteroid-Δ'-dehydrogenase activity of *Arthrobacter globiformis* cells. They were immobilised by different methods; including cross-linked polyacrylamide, agar and membrane entrapment and adsorption onto ceramic carriers. Entrapment in cross-linked polyacrylamide gel gave an enzyme activity retention of 88% and the highest stability of the carriers tried. The immobilisation procedure produced a gel containing living cells which were reincubated in media, after 6 months of operation, to replace some of the lost activity.

One of the disadvantages of immobilising cells or enzymes in cross-linked polyacrylamide gel is the release of heat that accompanies the polymerisation. For example, during polymerisation of a 15 % w/v solution of acrylamide there is an approximate temperature rise of 40 °C, which may produce a reduction in enzyme activity. By suspending the monomer/cell mix in an immiscible phase, it is possible to produce uniform spherical beads. This method allows the heat of polymerisation to be dispersed into the oil phase and reduces the temperature rise experienced by the cells or enzyme.

Beck and Rase, (1973) used such a technique to produce immobilised glucose amylase. They added the monomer/enzyme/initiator system to a rapidly stirring bath of mineral oil which also contained sec-butyl alcohol as a dispersant. The immobilised biocatalyst formed retained 77 % of the free enzyme activity compared to only 22% using the

normal gel entrapment technique described previously. They reasoned that the improvement in activity retention was due to the greater control over the temperature rise that took place during polymerisation.

Klein and Schara, (1980) performed extensive investigations into immobilisation by suspension polymerisation using various monomers and solvent phases. They were able to retain up to 100% of the phenol degrading activity of free cells after immobilisation using polyacrylamide as the monomer and dibutylphthalate as the solvent. They also reasoned that the increase in activity retention over that of a normal gel immobilisation was due to the greater control over the temperature rise during polymerisation.

2.2.4 Advantages and Disadvantages of Immobilised Systems

The advantages and disadvantages of cell immobilisation are outlined in Table 2.2. Immobilisation allows the reuse of the cells for batch processes or their retention against washout in continuous process. Immobilisation also reduces the difficult problem of removing the cells or enzyme from the final product (Katchalski-Katzir, 1993). Immobilisation has been found to increase the stability of the cells by giving protection from fluid shear (Harrington *et al.*, 1991). The concentration of denaturing substrates may be limited in immobilisation matrices due to diffusional resistance, producing apparent increases in immobilised enzyme stability (Trevan, 1987). The effect of diffusion is an important factor and this is discussed below in more detail:

Table 2.2: Advantages and Disadvantages of Immobilisation

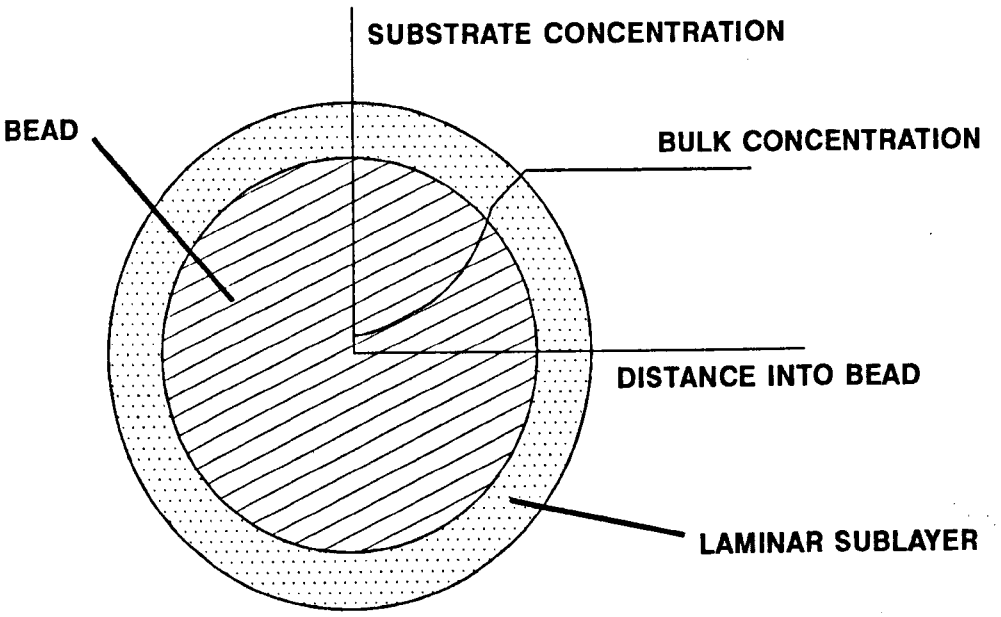
Advantages	Disadvantages
Ease of handling	Extra processing step
Biocatalyst reuse	Extra cost
Increased choice of reactor design	Loss in activity
Greater biocatalyst stability	Change in kinetics
Increased flow rates through continuous reactors	Waste disposal

2.2.5 Diffusion in Immobilised Cell Particles

Figure 2.4 shows a representation of an immobilised cell particle. Around a suspended particle there is typically a stagnant layer of fluid known as the laminar sublayer. However, if the reaction mixture around the particle is well stirred it can be assumed that there is no stagnant layer surrounding the particle surface. In this case substrate can pass directly into the immobilised cell particle from the surrounding solution.

In order for a reaction to occur, substrate must diffuse through the bead and enter through the cell wall before it encounters the active site of an enzyme. From the discussion given in Chapter 1, it can be assumed that the cell wall does not present a significant barrier to the passage of substrate and product in the case of acrylonitrile and acrylamide degrading bacteria. Therefore, the main rate limiting process will be the rate of substrate diffusion through the particle. As the substrate passes through the immobilisation matrix it is consumed by the enzyme. The substrate concentration therefore decreases towards the centre of the particle, as is shown in Figure 2.4. The equilibrium substrate profiles for immobilised biocatalyst particles with slab and sphere

Figure 2.4: Steady State Substrate Profile in an Immobilised Cell Particle



type geometries can be modelled using the equations given in Table 2.3, and taken from Geraats, (1992).

Table 2.3: Equations describing the Equilibrium Substrate Profile of an Immobilised Enzyme Particle under conditions of Zero and First Order Kinetics

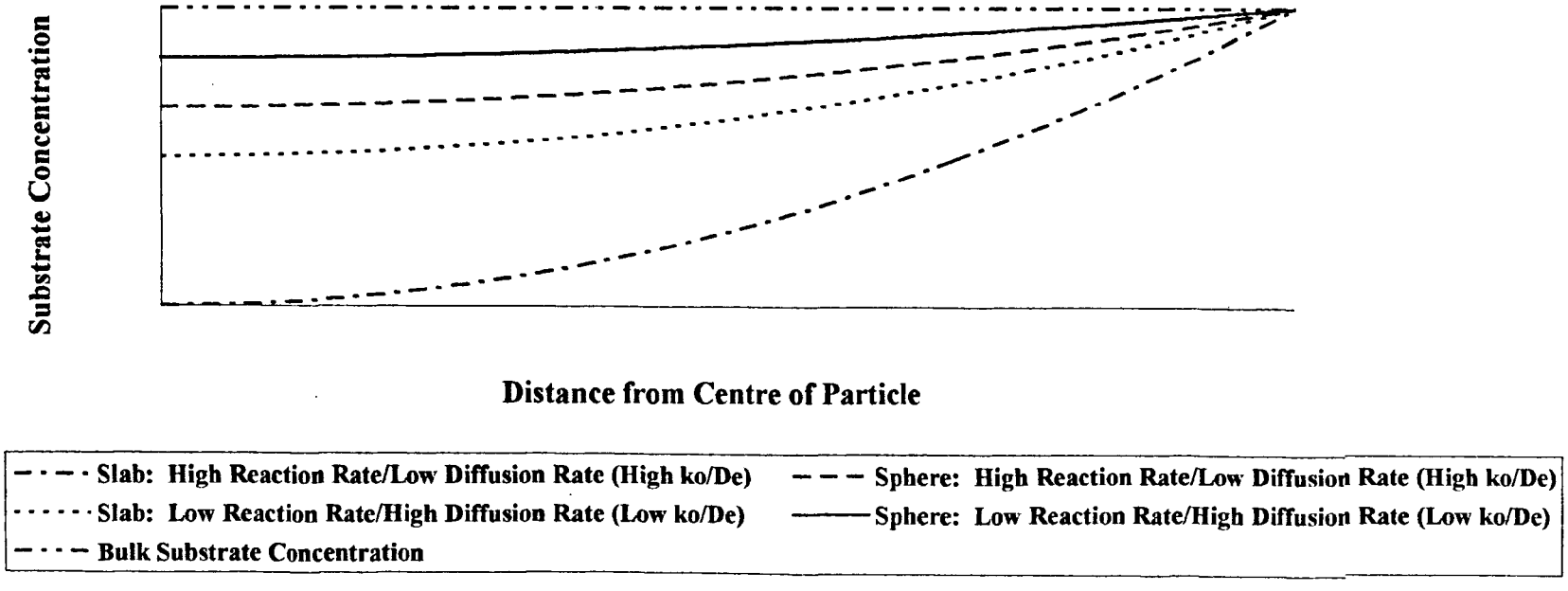
Geometry of Particle	Equation
Slab- Zero Order Kinetics	$S_{(r)} = S_b - 0.5\frac{k_0}{D_e}(Rp^2 - r^2)$
Sphere- Zero Order Kinetics	$S_{(r)} = S_b - \frac{1}{6}\frac{k_0}{D_e}(R_p^2 - r^2)$
Slab- First Order Kinetics	$S_{(r)} = S_b \frac{\cosh(\sqrt{(k_1 / D_e)}.\ r)}{\cosh(\sqrt{(k_1 / D_e)}.\ R_p)}$
Sphere- First Order Kinetics	$S_{(r)} = \frac{S_b . R_p}{r} \frac{\sinh(\sqrt{(k_1 / D_e)}.\ r)}{\sinh(\sqrt{(k_1 / D_e)} R_p)}$

Nomenclature

$S_{(r)}$ = Substrate concentration at distance r from centre of particle (mol l^{-1})
S_b = Bulk substrate concentration (mol l^{-1})
R_p = Sphere radius or half slab thickness (cm)
k_0 = Zero order rate constant (mol l^{-1} s^{-1})
k_1 = First order rate constant (s^{-1})
D_e = Diffusion Coefficient (cm^2 s^{-1})
r = Distance from centre of particle (cm)

Figure 2.5 shows a plot of the equations for the case of zero order kinetics i.e. the substrate concentration within the particles is high enough to allow all the immobilised enzyme to work at V_{max}. The value of (k_o/D_e) represents the ratio of the rate of substrate consumption to the rate that it can diffuse into the particle. As can be seen from Figure 2.5, the higher its value, the steeper is the substrate profile. In an immobilised cell particle, for example, increasing the cell concentration will increase the value of (k/D_e). This is because the rate of reaction per unit volume of particle increases, while increased cell concentration decreases the diffusion coefficient (Furui and Yamashita, 1985). Figure 2.5 also demonstrates the different substrate profiles of

Figure 2.5: Substrate Profile through an Immobilised Enzyme Particle Under Conditions of Zero Order Kinetics



catalyst particles with different geometries. It can be seen that the substrate profiles are steeper in the slab shaped particles compared to those of the spherical particles. This is due to the smaller surface area to volume ratio inherent in the slab geometry, leading to a reduction in the rate that substrate can diffuse into the particle.

It is shown in Figure 2.1 that the rate that an enzyme catalyses a reaction is dependant upon the substrate concentration when it falls below an enzyme saturating concentration. If the substrate concentration becomes low enough within the particle, the enzyme will no longer catalyse a reaction at its maximum possible rate. Such a situation can be modelled using the first order profile equations shown in Table 2.3. The observed enzyme activity of the particle will then be lower than would be expected if the entrapped cells were present in a free form. This phenomena is referred to as *substrate diffusion limitation*. The ratio of the free enzyme activity to that of the immobilised enzyme activity is known as the *effectiveness factor*.

Substrate diffusion limitation may also lead to apparent increases in enzyme stability. Some enzymes are irreversibly denatured by their substrates. This denaturation is dependant upon their concentration. In the case of the immobilised cell particle shown in Figure 2.4, the enzyme at the surface of the particle will become denatured more quickly than that at the centre. Such a phenomena may falsely indicate an increase in the immobilised enzyme stability compared to the free enzyme, (Klein and Vorlop, 1983). Such apparent increases in stability may be useful in enabling a reactor to be run for longer periods without recharging the reactor with catalyst (Trevan, 1987).

2.3 Bioreactor Types

Though there are many variations of bioreactor available, they are all based on the three basic reactor configurations:

Batch Stirred Tank
Continuous Stirred Tank
Plug Flow

These basic configurations are shown in Figure 2.6. The equations used to plot the reactor concentration profiles shown in this chapter are taken from Chaplin and Bucke (1990) and shown in Table 2.4.

2.3.1 Batch Stirred Tank Reactor

In an ideal batch reactor the substrate and product concentrations change with time as shown in Figure 2.6a. The use of an impeller to mix the reactor contents leads to good

Table 2.4: Reactor Design Equations for Simple Michaelis-Menten Kinetics

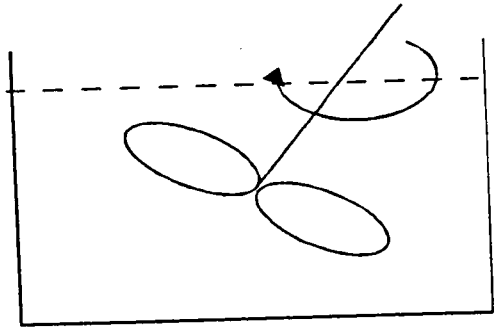
Design Equation	Reactor Type
$V_{max}\, t/V = ([S]_0 - [S]) - K_m \times \ln([S]/[S]_0)$	Batch
$V_{max}/F = ([S]_0 - [S]) - K_m \times \ln([S]/[S]_0)$	Plug-Flow
$V_{max}/F = K_m\,(([S]_0 - [S])/[S]) + ([S]_0 - [S])$	Continuous Stirred Tank

Nomenclature

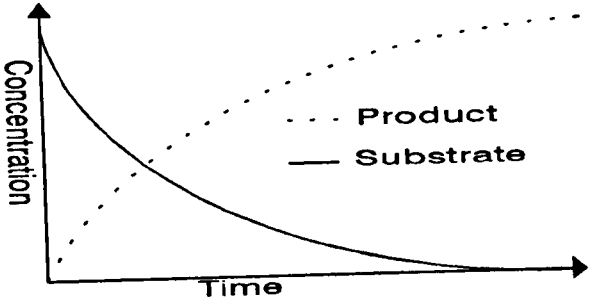
V_{max} = Maximum rate of reaction (mol l^{-1} s^{-1})
[S] = Substrate concentration (mol l^{-1})
$[S]_0$ = Initial substrate concentration (mol l^{-1})
t = Conversion time (s)
K_m = Michaelis constant (mol l^{-1})
F= Reactor flow through rate (l s^{-1})
V = Reaction volume (l)

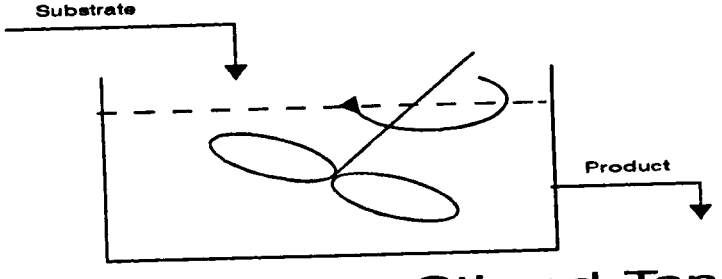
temperature and pH control. On a laboratory scale, they are useful for studying enzyme kinetics provided only initial rate data is used. On a commercial scale they are less

Figure 2.6: Types of Bioreactor

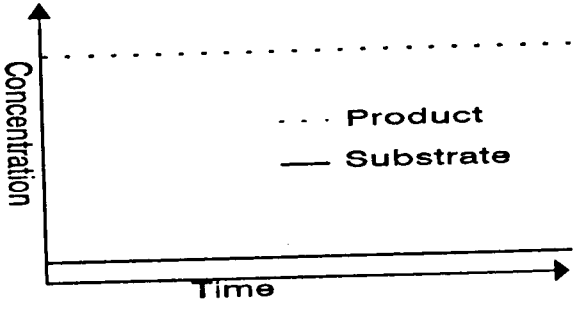


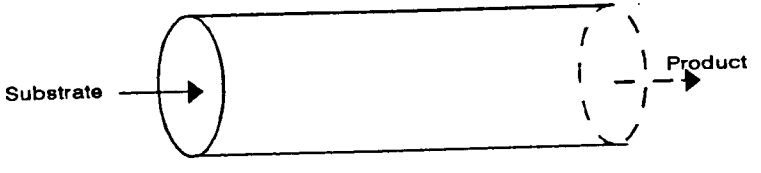
a) Batch Reactor



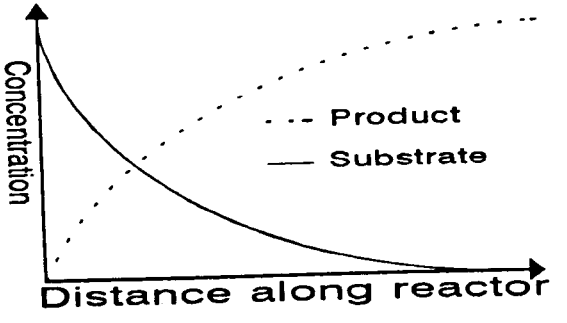


b) Continuous Stirred Tank Reactor (CSTR)





c) Plug Flow Reactor



suitable for producing large tonnages of product, compared to continuous reactors, due to lost production during down time between batches (Levenspiel, 1972).

2.3.2 Continuous Stirred Tank Reactor (CSTR)

In an ideal continuous reactor it is assumed that the contents are fully mixed so that the concentrations in the product stream are the same as in the reactor. Like the batch stirred tank, a CSTR allows good temperature and pH control. As is shown in Figure 2.6b, the concentrations in the reactor remain constant with time and for this reason they are useful for studying the effects of constant conditions on the stability of a biocatalyst. On an industrial scale they are more useful for producing large scale tonnages of product.

2.3.3 Packed Bed Reactor (PBR)

In an ideal packed bed reactor it is assumed that there is no axial mixing i.e. liquid travels along the length of the reactor as a plug of liquid. Packed bed reactors are often referred to as plug flow reactors. Conversion takes place along the length of the reactor as is shown in Figure 2.6c. In such a configuration, the control of pH and temperature is more difficult as alterations made to the fluid entering the reactor will not produce an immediate effect throughout the reactor.

2.3.4 Comparison and Choice of Reactor Type for Biotransformations

The choice of bioreactor for a certain process can be complicated due to the many factors involved: enzyme kinetics, form of biocatalyst and scale of production, mixing, pH and heat transfer.

The form of biocatalyst is an important factor in the choice of a bioreactor. If the reaction is catalysed by a soluble enzyme or free cell then a batch reactor will need to be used unless a membrane is incorporated to allow retention of the enzyme. Immobilisation increases the choice of bioreactor.

In a stirred tank configuration, the biocatalyst particles are subject to shear and must be able to withstand long periods of mixing without disintegrating. In a packed bed reactor, the contents are subjected to less shear, but problems may arise from the use of immobilisates that are compressible in nature, as this may lead to large pressure drops across the bed, and channelling of the fluid.

In a batch reactor, as can be seen from Figure 2.6a, the initial substrate concentration is high and therefore this reactor configuration is unsuitable for applications where the enzyme is inhibited or deactivated by its substrate. However, in the case of product inhibited reactions, batch reactors are favourable as the enzyme will only experience high product concentrations towards the end of the batch. A CSTR operating at a low substrate concentration is less suitable for product inhibited or deactivated reactions but more suitable for substrate inhibited reactions.

The equations listed in Table 2.4 give the production rates for reactors operating with enzymes that follow simple Michaelis-Menten kinetics. These equations can be modified using the inhibition equations given in Table 2.1, to describe reactors operating with enzymes that are substrate and product inhibited. These expressions may be found in most enzyme technology text books (Gemeiner, 1992). Figure 2.7 compares the effects of inhibition on the three reactor types described above in terms of quantity of enzyme required to produce a certain level of conversion. It can be seen from Table 2.4 that the design equations for plug and batch reactors are very similar and will produce the same fraction of substrate conversion after an element of reactant has spent time t in the reactor. The design equation for a CSTR, however, is quite different, and subsequently so is its performance under conditions of substrate and product inhibition, as can be seen from Figure 2.7.

Figure 2.7a shows that the CSTR reactor is less efficient, when the enzyme present suffers from product inhibition, because more enzyme is required to achieve the same production rate as a PFR or batch reactor . However, in the case of substrate inhibition (Figure 2.7b), the CSTR configuration is superior until the substrate concentration becomes very low.

2.3.5 Other Reactor Types and Configurations

Certain biocatalyst properties may demand the use of a variation on the standard reactor types or a combination of two or more different reactors. Three such examples are discussed below:

Figure 2.7a: Comparison of CSTR Bioreactor with Batch or Plug Flow Types in the case of Product Inhibited Enzyme

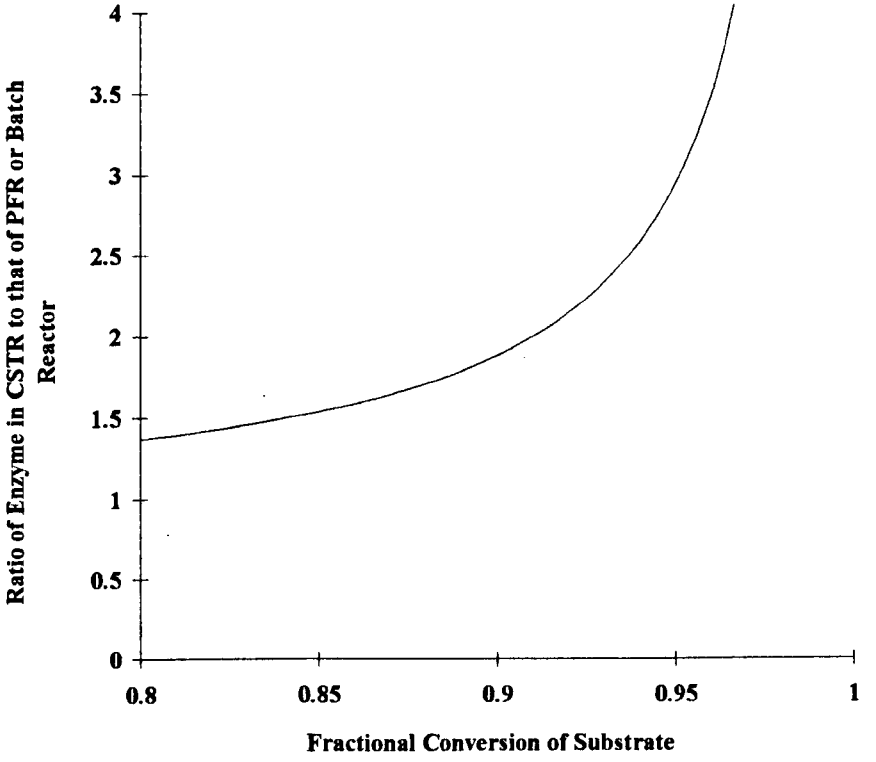
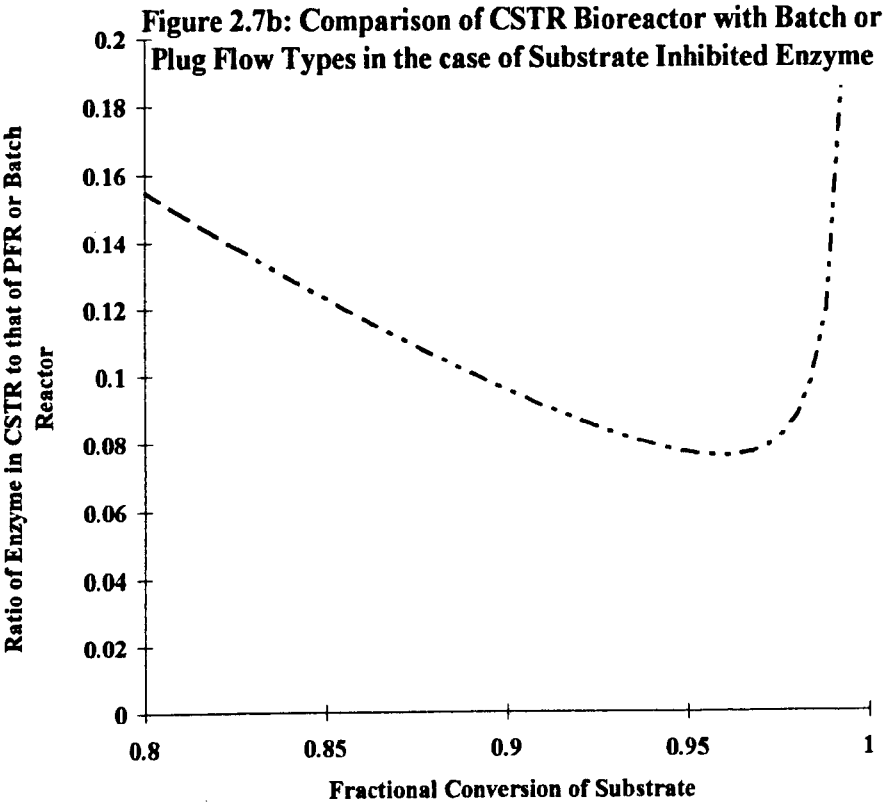


Figure 2.7b: Comparison of CSTR Bioreactor with Batch or Plug Flow Types in the case of Substrate Inhibited Enzyme



2.3.5.1 Fed-Batch Reactors

In certain cases it is possible that the enzyme is inhibited or deactivated by both the product and substrate. Each of the three reactor types shown in Figure 2.6 will have their drawbacks and it may be necessary to search for an alternative. One such solution is the use of a fed-batch reactor. This reduces the high substrate concentrations normally associated with a batch reactor and prevents the biocatalyst from experiencing high product concentrations until the end of each batch.

2.3.5.2 Multiple CSTR's

Karanth, (1979) made a theoretical comparison of a batch reactor with a single and 2-CSTR system for substrate and product inhibited kinetics. In the example discussed, it was theorised that control of pH was important and therefore a plug flow reactor was rejected. Even at 50 % conversion, the volume of a single CSTR needed to be twice that of a batch reactor to achieve an equivalent production rate. However, a 2-CSTR system was much more efficient because the lower product and higher substrate concentration in the first vessel reduced the inhibition effects present in a single vessel. At 98 % conversion , the volume of the 2-CSTR's needed to be 1.5 times that of the batch reactor to give an equivalent production rate. When the down time necessary with a batch reactor was considered, it was theorised that it may be more efficient to use the two CSTR system.

2.3.5.3 Fluidised Bed Reactors

In a fluidised bed reactor, catalyst particles are suspended and agitated by the upward flow of fluid or gas through the catalyst bed. Kinetically, fluidised beds can be modelled by a combination of the plug flow and stirred tank equations (Allen *et al.*, 1979) and the reactor performance can vary between both (Levenspiel, 1972). To their advantage, fluidised-beds do not suffer from the pressure drops of packed beds, or the high shear of stirred tanks. Mass and heat transfer characteristics are good, insoluble substrates can be processed and gas can be easily introduced or expelled. (Allen *et al.*, 1979). To their disadvantage, however, fluidised-beds are difficult to model and scale-up (Levenspiel, 1972).

2.4 On-Line measurement of Concentration

A knowledge of the composition of a process stream is often of major importance (Wardle, 1994). For example, when operating a reactor, it is important to know the concentration of reactants and products leaving the reactor. Such information may be required to determine whether the reactor is operating within specification, or may be used as the input to some form of controller making alterations to the reactor feed rate. For these reasons, before discussing bioreactor operation, it is poignant to briefly consider some general methods of on-line composition analysis.

2.4.1 Chromatographic Techniques

Examples of chromatographic techniques include Gas Chromatography (GC) and High Performance Liquid Chromatography (HPLC). In these techniques, a process stream is

separated into individual components by passing along a packed column such as silica. To its advantage, chromatography can be used for multicomponent analysis. To its disadvantage however, an on-line chromatograph must be used with a automatic sampler and diluter. Also, chromatographic analysis can suffer from a substantial time lapse between sampling and the results of the analysis (Wherry and Miller, 1974).

2.4.2 Electrochemical Analysis

2.4.2.1 Potentiometric Analysis

Measurement of chemical electromotive force (e.m.f.) is the basis for a number of analysis methods. An example of this type of detecting element is the pH probe which measures hydrogen ion concentration. The probe consists of two electrodes, one of which produces an electrical potential proportional to the electrolyte concentration of interest, in this case, the hydrogen ion concentration. The second electrode produces a constant electrical potential. The difference in potential between the two electrodes generates an e.m.f. which is related to the pH. Several types of ion selective probe are available including ammonium ion probes. Major difficulties with ion selective probes are non-linearity of signal and fouling caused by other components of the mixture being measured (Gostomski and Bungay, 1992).

2.4.2.2 Conductometric Analysis

Solutions of electrolytes in ionising solutions such as water will conduct a current, with the conductance being related to the ion concentration, ionic charge and ion mobility. Conductance measurement is ideally suited for the measurement of a single strong

electrolyte in dilute solution (Wherry and Miller, 1974). Conductance is measured using a conductivity cell consisting of two electrodes which are immersed into the solution so that the conductance of the solution between them is measured. A drawback of this method is interference caused by other ions i.e. conductance depends on total concentration of all ions. Also, at high concentrations, the conductance signal does not vary linearly with concentration.

2.5 Methods of Automatic Control

It has been shown how enzyme kinetics are dependant upon several parameters such as temperature, pH and reactant concentration. Therefore, when operating a reactor in which an enzyme catalysed reaction is taking place, it is important to be able to control such parameters. For example, substrate and product concentrations in a CSTR, can be controlled by adjusting the flowrate of substrate to the reactor. Such control can be achieved in several ways and these are discussed below:

2.5.1 Feed Forward and Feed Back Control

Consider the automatic control system shown in Figure 2.8a. It is required to keep the stirred tank at the set point temperature. Once the temperature of the tank drifts away from the required temperature the changing signal from the temperature sensor causes the controller to open or close the steam valve. This is an example of a feedback control system as the controlling action is only performed after the information showing the temperature deviation is fed back to the controller.

Figure 2.8a: Automatic Feedback Control of a Stirred Tank

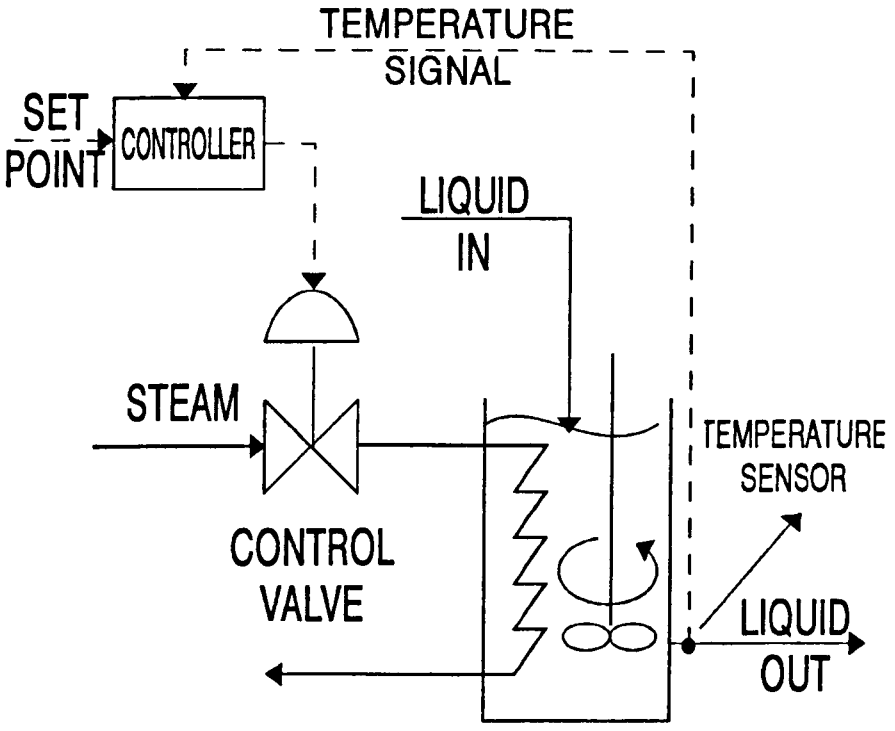
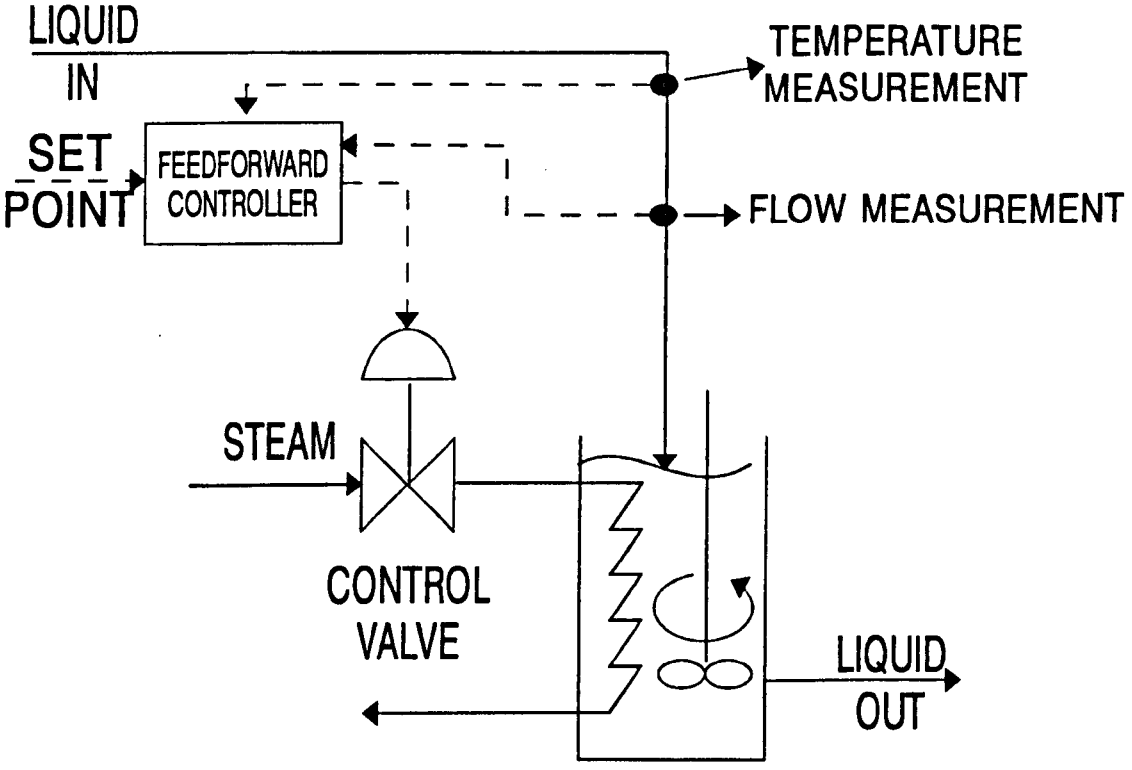


Figure 2.8b: Automatic Feedforward Control of a Stirred Tank



Now consider the automatic control system of the mixing tank shown in Figure 2.8b. This time the controller gathers information from the liquid input line i.e. it's temperature and flowrate. The controller then makes an appropriate change in the steam valve setting based on equations describing the heat and mass balance of the system. Such a set of equations is known as a *process model*. This is an example of a feedforward system as the controlling action is made before the temperature has deviated from the set point. The advantage of feedforward control is that disturbances to a system can be compensated for before the controlled variable deviates from the set-point. However, the process model required to produce such control is rarely seen (Wherry and Miller, 1974).

The feedback control equations shown in Table 2.5 and discussed below require certain constants to be calculated before they can be used as part of a control system. This can be achieved by either experimental investigation or a time-dependant mathematical analysis of the system being controlled (Wardle, 1994).

Table 2.5: Equations Describing Several Types of Feedback Controller

Type of Control	Control Equation
Proportional	$J = J_0 + K_P \cdot \varepsilon$
Proportional Integral	$J = J_0 + K_P \cdot \varepsilon + K_I \int_0^t \varepsilon \, dt$
Proportional Integral Derivative	$J = J_0 + K_P \cdot \varepsilon + K_I \int_0^t \varepsilon \, dt + K_D \frac{d\varepsilon}{dt}$
J = Controller Output J_0 = Controller Output when $\varepsilon = 0$ t = time	K_P, K_I, K_D = constants ε = Error

2.5.2 Types of Feedback Control

Four types of common control mechanism are discussed below and the equations describing their control action are shown in Table 2.5.

2.5.2.1 On-Off Control

In the case of the control system shown in 2.8a, an on-off controller would open the steam valve if the temperature of the tank was below the set point and close the valve once the temperature had risen above the set point. This will cause the tank temperature to oscillate around the set point.

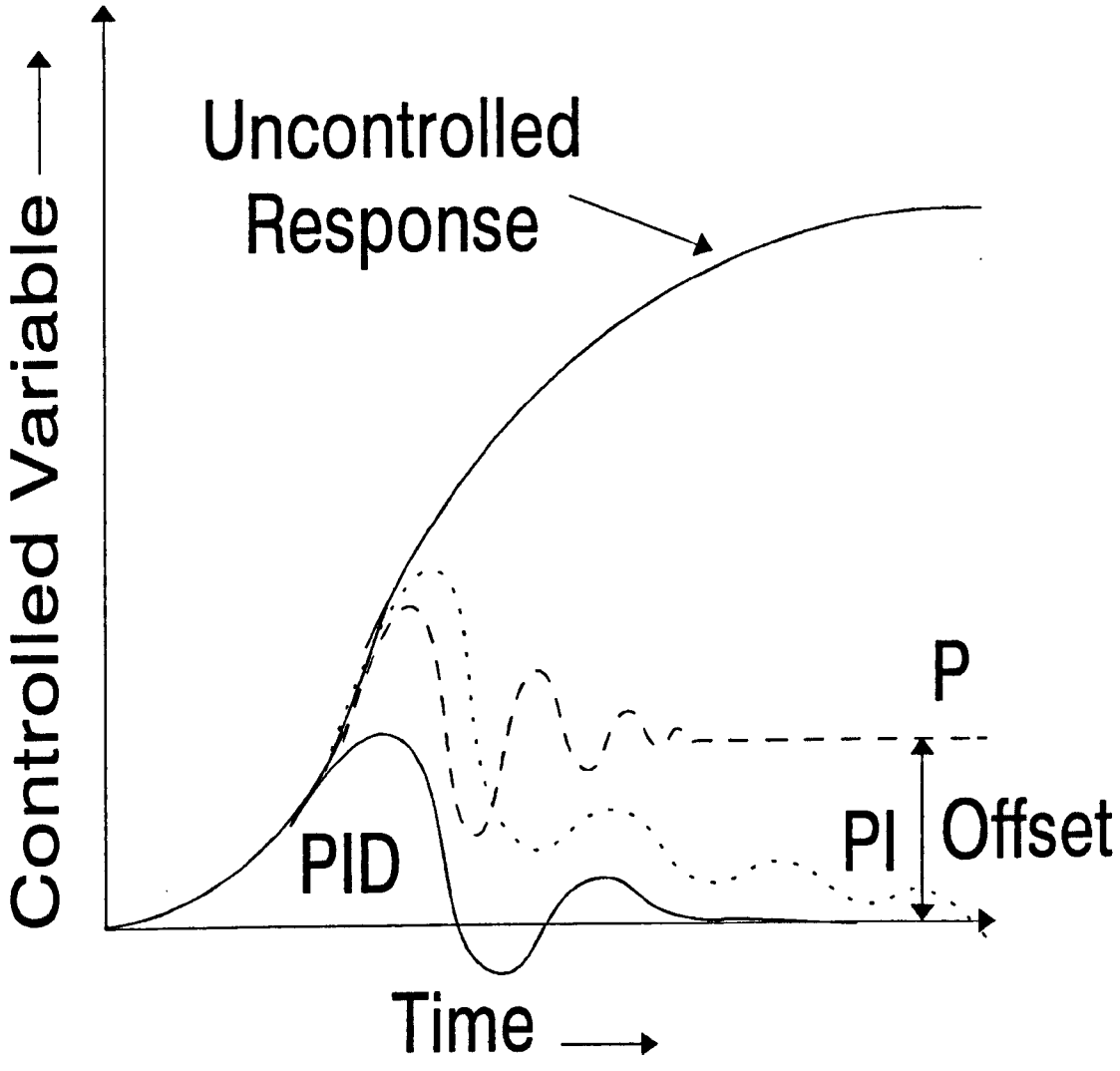
2.5.2.2 Proportional (P) Control

In the case of the control system shown in 2.8a, a proportional controller produces an output that is a fixed multiple of the measured error i.e. the difference between the set point and measured temperature. This type of controller will produce some oscillation and the controlled variable will be offset away from the set point, as shown in Figure 2.9.

2.5.2.3 Proportional Integral (PI) Control

This type of control produces a signal based on the size of the error and the time integral of the error. The integral action should eventually eliminate any off-set but does cause the controlled variable to oscillate for longer periods than with proportional control alone. This is shown in Figure 2.9.

Figure 2.9: Response of a Controlled Variable to a Disturbance in Load using Different Control Modes



2.5.2.4 Proportional Integral Derivative (PID) Control

The derivative part of this control action produces an output based on the rate that the error is changing. As is shown in Figure 2.9, this type of control produces a quicker response than P and PI control. The magnitude of oscillation is also reduced and the offset is eliminated.

AIM OF RESEARCH

3. AIM OF RESEARCH

The forms in which an enzyme may be used to carry out a biotransformation were discussed in the Introduction. It is clear that a choice of forms is available with which to perform the enzyme catalysed production of ammonium acrylate. However, it was also shown in the Introduction that whole, resting cells may be used for ammonium acrylate production. Attempts to purify the appropriate enzymes from *Nocardia rhodochrous* has led to their inactivation (Wyatt and Linton, 1988). Bernet *et al* (1987), viewed whole amidase active cells as 'bags of enzymes' which simplified operational conditions and were preferred to partially purified amidase. Ammonium acrylate is a low cost, bulk intermediate chemical. The necessity to keep production costs down dictates that the biocatalyst should be an immobilised resting whole cell. This system would eliminate any requirement for oxygen or nutrients to maintain cell viability and hence would simplify bioreactor design and downstream processing, as well as avoiding the expense of extracting the enzyme.

The work described in this thesis was performed as part of the Bio-Ammonium Acrylate Project supported by the SERC/DTI LINK Biochemical Engineering Programme. The initial intention of this research was to produce a bioreactor system for the enzyme catalysed hydrolysis of acrylonitrile to ammonium acrylate. It was also considered that the conversion of acrylamide to ammonium acrylate had commercial potential. Therefore, both these biotransformations were studied during the LINK Programme as two separate

projects. The work described here concentrates on the acrylamide to ammonium acrylate route.

The LINK Programme required study into several areas i.e. screening of suitable bacteria, cell immobilisation and bioreactor design and operation. The work described here concerns the latter two areas and was performed using bacteria from a culture collection as well as bacteria that have become available as a result of a screening programme that was simultaneously being carried out.

Initial studies into cell immobilisation and bioreactor design were carried out using an amidase active bacterium from a culture collection. This was to allow the development of a *model system* which could be used to compare bacteria that became available through the screening programme. This thesis, therefore, details studies pertaining to the design and operation of an immobilised, resting, whole cell reactor system for the production of ammonium acrylate from acrylamide.

EXPERIMENTAL

4. IMMOBILISATION STUDIES

4.1 Cell Culture and Use

The organisms used in the work described here are *Corynebacterium nitrilophilus* (11594), obtained from the National Collection of Industrial and Marine Bacteria, *Rhodococcus rhodochrous* sp.632 and *Rhodococcus* sp.1068. These latter two organisms were isolated from soil samples taken at the Allied Colloids site in Bradford. Media development, cell culture and separation are described below.

Corynebacterium nitrilophilus was grown routinely either on a minimal medium (pH 7.2), containing (in g/l): K_2HPO_4, 7; KH_2PO_4, 3; acetamide, 2; $MgSO_4.7H_2O$, 0.5; $CaCl_2.6H_2O$, 0.2; trace metals solution, 5ml; (Bauchop and Elsden, 1960); vitamins solution, 1ml; (Miller and Knowles, 1984); or a nutrient broth medium (Oxoid), supplemented with acetamide, 5 g/l. Fermentation was carried out at 30°C in a 20 l laboratory fermenter (Bioengineering). Bacteria were harvested during exponential growth by centrifuging at 10 000 × g for 10 min. at 4°C and then washing with physiological saline

Rhodococcus rhodochrous sp.632 and *Rhodoccocus* sp.1068 were grown routinely on a minimal medium (pH 7.2), containing (in g/l): K_2HPO_4, 7; KH_2PO_4, 3; acetamide, 2; sodium acetate, 10.0; $MgSO_4.7H_2O$, 0.5; $CaCl_2.6H_2O$, 0.1; trace metals solution, 5ml; (Bauchop and Elsden, 1960); vitamins solution, 1ml; (Miller and Knowles, 1984). Fermentation was carried out at 30 °C in a 280 l laboratory fermenter (New Brunswick Scientific). Bacteria were harvested at mid-late exponential phase (7 hrs) by

centrifugation 10 000 × g using a Westfalia Separator (TA 05-00-105) using a semi-continuous solid bowl and then washing with physiological saline.

Cell pellets were stored frozen and allowed to defrost before being used for experimental work.

4.1.1 Determination of Cell Suspension Concentration

A series of cell/saline suspensions of various absorbencies at 600 nm (A_{600}) were prepared. These suspensions (10.0 ml) were centrifuged at 10 000 × g for 8 minutes and 4 °C. The cell pellets produced were then resuspended in RO water to remove the saline and then recentrifuged. The resulting pellets were placed in pre-weighed test tubes and then dried at 110°C to give a dry weight. A plot of A_{600} against dry weight has a gradient equal to the value of grams dry weight of cells per litre per absorbance unit.

4.1.2 Immobilisation of *C. nitrilophilus* in Calcium Alginate Beads

Cells were immobilised in calcium alginate following the method of Martinsen *et al.*, (1989). Sodium alginate solution (8 ml at 4 % w/v) was mixed with *C. nitrilophilus* cells resuspended in 0.9 % (w/v) sodium chloride solution. The sodium alginate/cell mix was forced through a 0.6 x 25mm Microlance syringe needle, the end of which had been filed flat. The needle was positioned approximately 20cm above the surface of a stirred solution of 50 mM calcium chloride. Once sufficient beads had been formed they were allowed to mix for another 15 minutes before being transferred to a fresh

solution of 50 mM calcium chloride solution, in which they were kept over night at 5 °C.

4.1.3 Immobilisation of *C. nitrilophilus* in Cryptogrout Gel

Cryptogrout is a speciality polymer produced by Allied Colloids Ltd, and is a branched pre-polymer of cross-linked polyacrylamide with pendant double bonds. *C. nitrilophilus* cells resuspended in sodium phosphate buffer (50 mM, pH 7) were deaerated for several minutes using a vacuum pump before being mixed for several minutes with Cryptogrout (15g), triethanolamine (0.2 ml at 20 % w/v) and ammonium persulphate (0.2 ml at 20 % w/v). This mixture was then syringed into a plastic mould (60 × 60 × 1.5 mm). After setting for one hour, the resulting gel sheet was cut up into 3 x 3 x 1.5 mm cuboids which were washed with reverse osmosis water.

4.1.4 Immobilisation in Cross-Linked Polyacrylamide Gel

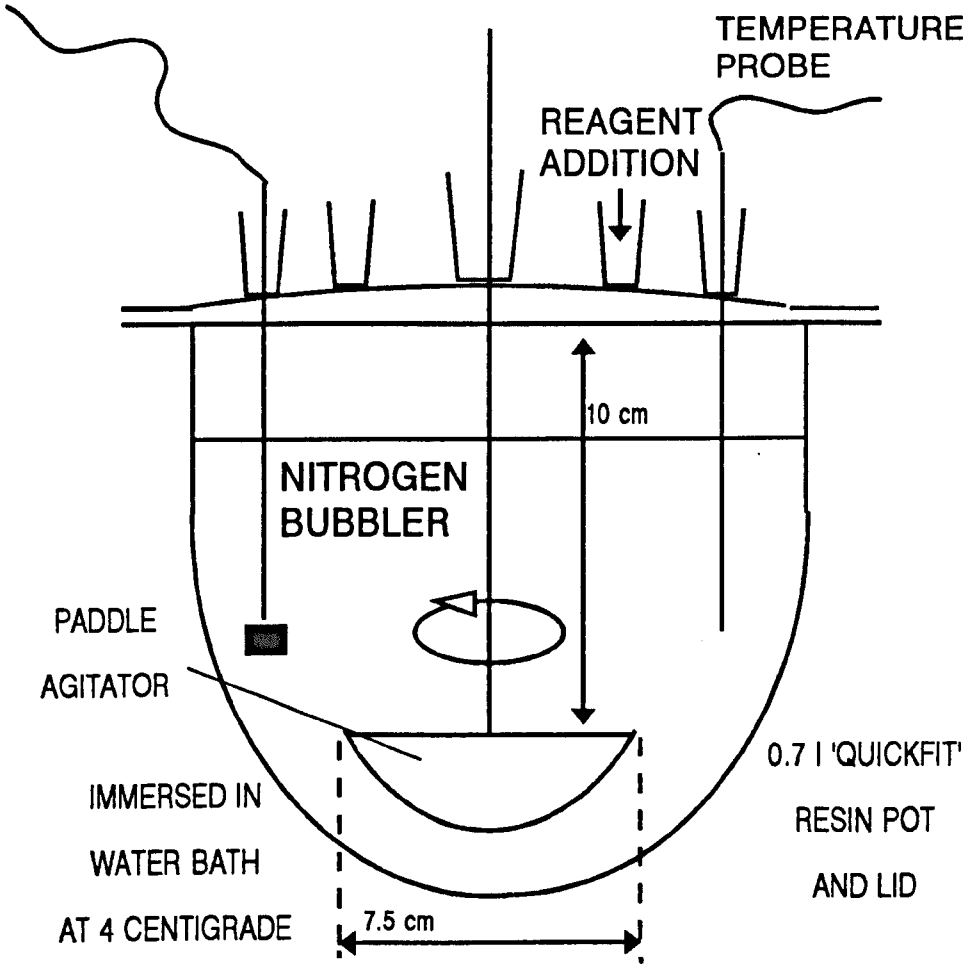
Immobilisation in cross-linked polyacrylamide gel was based on the method described by Chibata *et al.*, (1986). Acrylamide (3g) and methylene-bisacrylamide (0.15g) were dissolved in 6.85 ml of sodium phosphate buffer (50 mM at pH 7). This solution was cooled to 4 ^{o}C before being rapidly mixed with 8.3 ml of *C. nitrilophilus* cells resuspended in sodium phosphate buffer (50 mM, pH 7). Sodium sulphite (1ml at 1.6 % w/v) and tert-butyl hydroperoxide (0.7 ml at 1.6 % w/v) were then also added to this mixture and rapidly stirred before being poured into a petri dish lying on a layer of ice. The final acrylamide concentration was 15% (w/v). The gels were allowed to set for half an hour before being cut into 4x4x3 mm cuboids. These were then washed twice in 4l of reverse osmosis water at 4^{o}C for 3/4h.

4.1.5 Immobilisation in Cross-linked Polyacrylamide Beads by Suspension Polymerisation

This section is intended to outline the general procedures by giving an appropriate example of each type of suspension polymerisation. Variations in compositions and their consequences are given in the results section.

Mineral oil (300 g of Isopar G from Exxon Chemical) and methacrylic copolymer (0.5 g of MRM stabiliser from Allied Colloids Ltd.) were cooled to 4 °C by stirring in the 0.5 l reaction vessel shown in Figure 4.1. Oxygen was removed by bubbling nitrogen through the solvent phase for 30 minutes after which time the bubbler was raised above the solvent surface to maintain an inert atmosphere during bead formation. Acrylamide (6g) and methylene-bisacrylamide (0.3 g) were dissolved in 19 ml of sodium phosphate buffer (50 mM, pH 7.5) and this solution cooled to 4 °C before being rapidly mixed with 0.5 ml of sodium sulphite (0.16 % w/v) (or 0.09 g of ammonium persulphate) and *R. rhodochrous* sp.632 resuspended in sodium phosphate buffer (50 mM, pH 7.5) to a volume of 14.7 ml. This mixture was then poured into the reaction vessel and the stirrer speed adjusted until the cell/monomer phase was observed to be well suspended through out the oil phase. Tert-butyl hydroperoxide (60 µm at 70 % w/v) **or** 1 ml of N,N,N',N'-Tetramethyl-ethylenediamine were then added to the vessel and the temperature of the reactor contents monitored. Once the temperature had risen and then fallen again (~15 minutes), the stirrer and nitrogen were stopped and the reactor contents vacuum filtered to separate the beads from the solvent. The beads were then washed twice in 4l of reverse osmosis water at 4°C for 3/4h.

Figure 4.1: Suspension Polymerisation Equipment



Suspension polymerisation experiments were scaled up to 1l and 5l reaction vessels. The amounts given above were scaled up by the appropriate factor.

4.2 Preparation of Dried Cross-Linked Polyacrylamide Beads

R. rhodochrous sp.632 cells were entrapped in cross-linked polyacrylamide beads as described previously and then dried using the following methods:

4.2.1 Drying at 40 °C

Method 1: 4g aliquots of beads were placed in 20ml bijou bottles and left in an oven at 40 °C until the mass did not vary by more than 0.1 g and then stored at room temperature under airtight conditions.

Method 2: 4g aliquots were spread thinly over the bottom of a large beaker and dried overnight in an oven at 40 °C until the mass did not vary by more than 0.1 g and then stored at room temperature under airtight conditions.

4.2.2 Drying in a Fluidised-Bed Drier

Three batches of beads were dried at 30, 45 and 60 °C in a Sherwood Scientific Ltd Lab. Fluid Bed Drier operating at its maximum blower speed for 20-40 minutes or until they were fully fluidised within the drier and felt dry to the touch. They were then stored at room temperature under airtight conditions.

4.2.3 Drying under Vacuum

Two aliquots of beads were dried using Method 2 described in Section 4.2.1 and then further dried by subjecting them to a vacuum. The beads were maintained at a pressure of 0.1 mbar for 3 hours before being sealed at the said vacuum and stored at room temperature.

5. SMALL SCALE REACTOR STUDIES

5.1 Assay Methods

5.1.1 Determination of Ammonium Acrylate Concentration

Ammonium acrylate concentration was determined by either of two methods. Firstly, from the ammonium ion concentration which was itself determined using the colorimetric techniques of Muftic (1967) and Fawcet and Scott (1960). Secondly, from the change in conductance due to the production of ammonium acrylate, an electrolyte, from acrylamide, a non-electrolyte. Figure 5.1 shows an ammonium acrylate /conductivity calibration curve used to measure ammonium acrylate concentrations up to 500 mM.

5.1.2 Determination of Acrylamide Concentration

Acrylamide concentration was determined by H.P.L.C. using the conditions listed below:

Column: Lichrosorb, C18, Reversed Phase
Detection: 220 nm
Mobile phase: 95 % water, 5 % acetonitrile
Flow rate of mobile phase: 1ml/min
Injection volume: 20 µl

5.2 Determination of amidase activity.

The equipment used is that shown in Figure 5.2. Amidase activity was determined by stirring immobilised or free cells in a jacketed vessel at 30 °C in 50 mM sodium phosphate buffer (pH 7 for *C. nitrilophilus*, pH 7.5 for *R. rhodochrous*) and adding acrylamide to give a concentration of 500 mM unless otherwise stated. The rate of

Figure 5.1: Ammonium Acrylate Concentration/Conductivity Calibration Curve

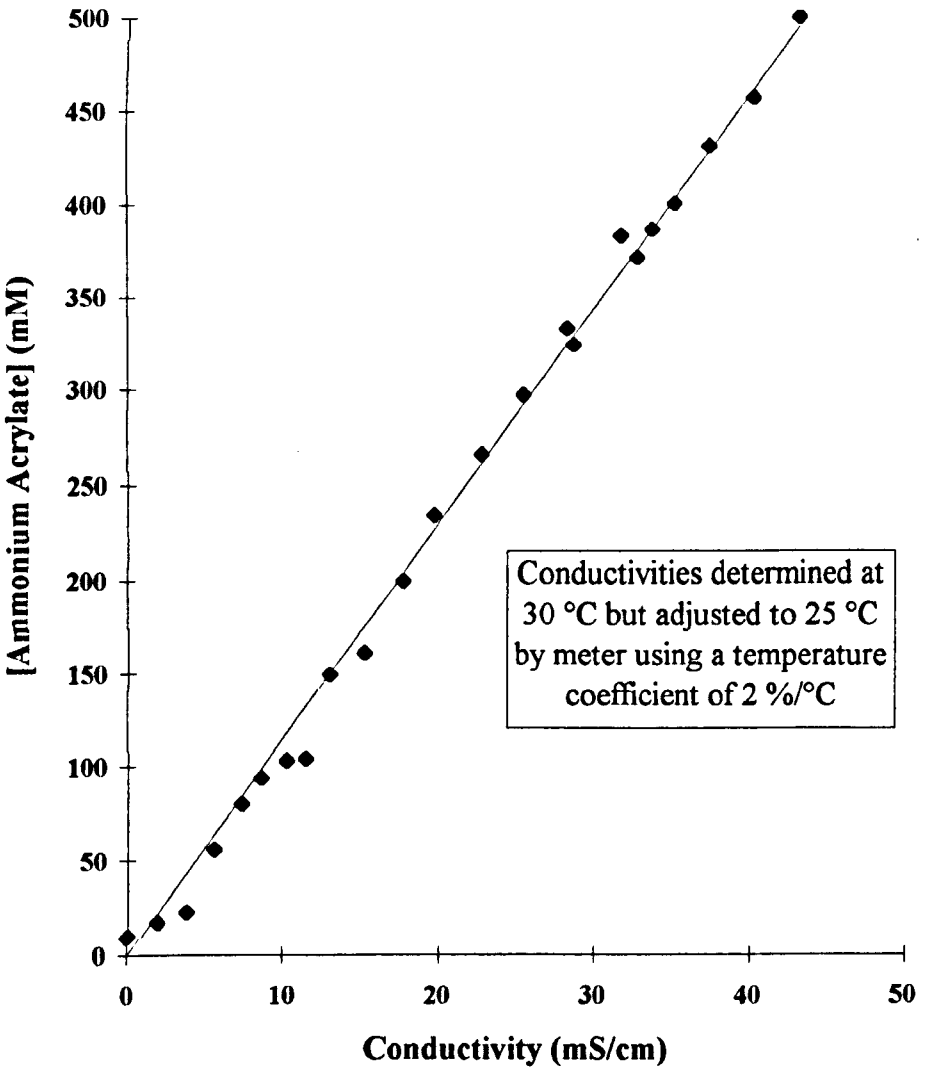
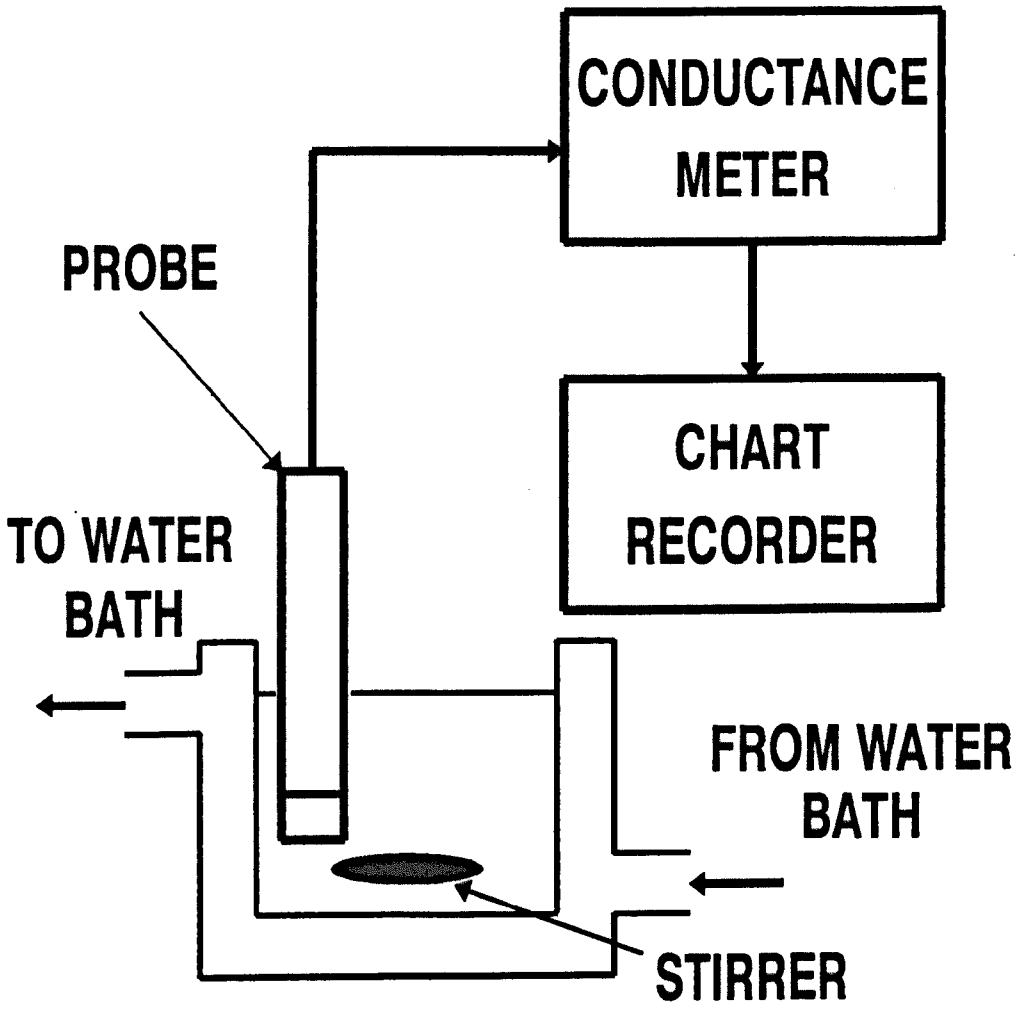


Figure 5.2: Experimental Set-Up for Amidase Assays of Free and Immobilised Cells



reaction was calculated from the maximum initial slope of the concentration/time profile. The specific amidase activity was defined as μmoles of ammonium acrylate formed per minute per gram of dry cells and is written in this report as U/g dry cells. The retention in amidase activity upon immobilisation was defined as:

$$100 \times \frac{\text{specific amidase activity of immobilized cells}}{\text{specific amidase activity of free cells}}$$

5.2.1 Effect of pH on Free and Immobilised Cell Amidase Activity

Free or immobilised cells of *C. nitrilophilus* and *R. rhodochrous* were assayed for amidase activity using the method described above but using the buffers shown in Table 5.1.

Table 5.1: Buffers used for Preparation of Amidase Activity/pH Profiles

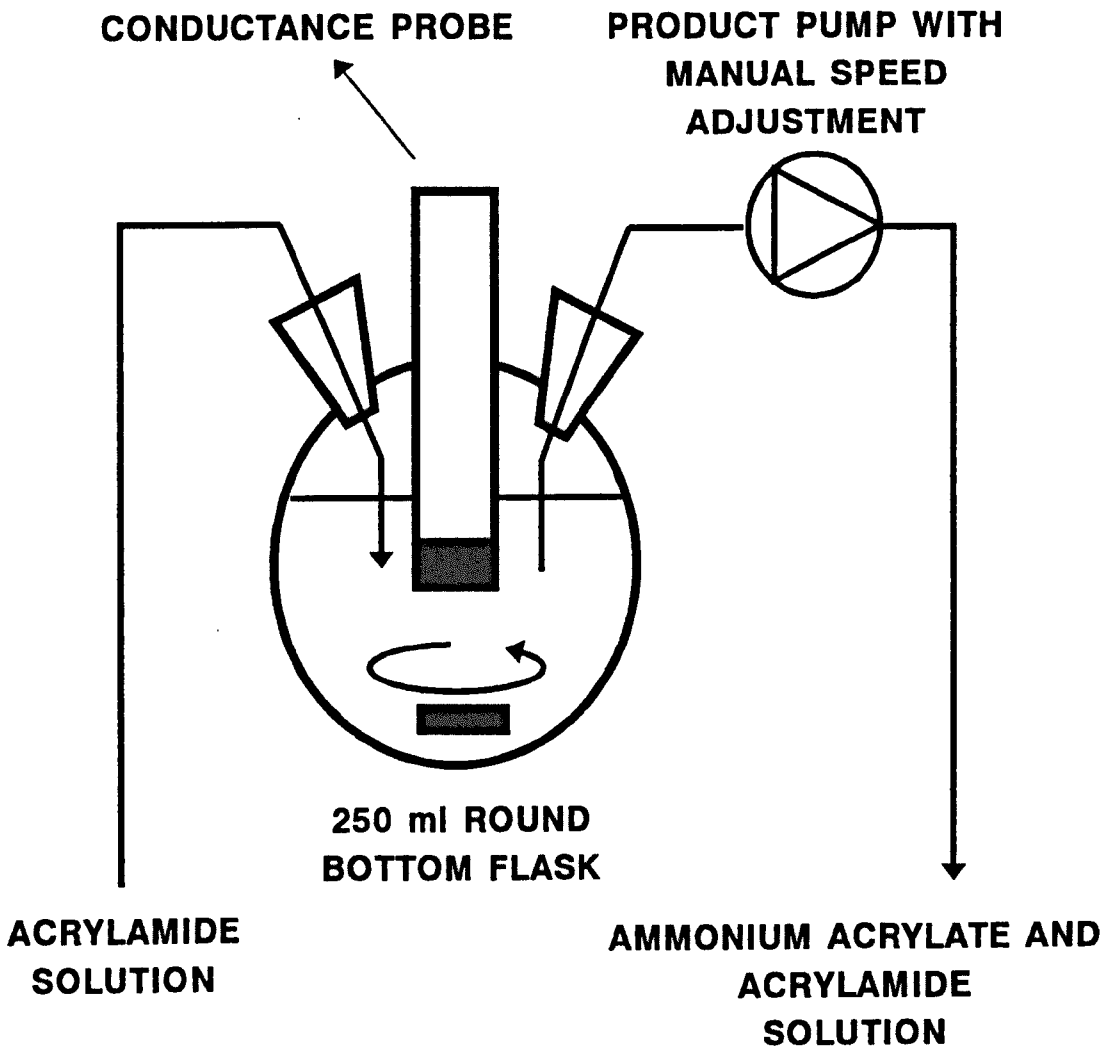
Buffer Type	pH
Citric acid/Disodium phosphate	4 to 6
Sodium phosphate	6.5 to 8
Potassium chloride/Boric acid	8.5 to 9

5.3 Continuous Stirred Tank Reactor (CSTR) Studies with *C. nitrilophilus* and *R. rhodochrous* sp. 632

5.3.1 Study of *C. nitrilophilus* Immobilised in Cross-Linked Polyacrylamide Gel Operating in a Manually Controlled Continuous Stirred Tank Reactor

The apparatus used is shown in Figure 5.3. Cells were immobilised in cross-linked polyacrylamide gel and suspended in water maintained at the required temperature by means of the cooling/heating finger. Acrylamide was added and the rise in ammonium acrylate concentration was followed by measurement of the conductance. Reaction

Figure 5.3: Equipment Set-Up for Manually Controlled CSTR Experiments



mixture was drawn continually from the reactor so that the resulting partial vacuum created in the system caused acrylamide solution to be continually drawn into the reactor thus maintaining a constant volume of liquid in the reaction vessel. The rate of liquid flow was periodically adjusted in order to keep the conductance constant

5.3.2 Study of *C. nitrilophilus* and *R. rhodochrous* sp.632 Immobilised in Cross-Linked Polyacrylamide Gel Operating in a Computer Controlled Continuous Stirred Tank Reactor (CSTR)

5.3.2.1 Equipment Set-up

The equipment used is shown in Figure 5.4. The reactor used is shown in Figure 5.5. It consisted of a 0.5 l vessel with a *Quick-fit* lid and an overflow to allow continuous operation. The signal from the conductivity meter was sent to a Unilab analogue/digital-digital/analogue converter. This sent a digital signal to a BBC Master computer which in turn sent a digital signal back to the converter. The resulting voltage was used to vary the substrate feed pump rate and so maintain the conductance at a constant level. The computer programmes used to control the reactor system were written in BBC basic and are described in the Results Section and in the Appendix Section.

Later CSTR studies were performed using a modified form of this equipment. The BBC computer and interface were replaced by a 486 personal computer containing a Keithley Metrabyte DAS1600 interface card, allowing a four fold increase in signal resolution. Programming on this system was performed using Easyest LX software purchased from Keithley Metrabyte.

Figure 5.4: Equipment Set-Up for Computer Controlled CSTR Studies

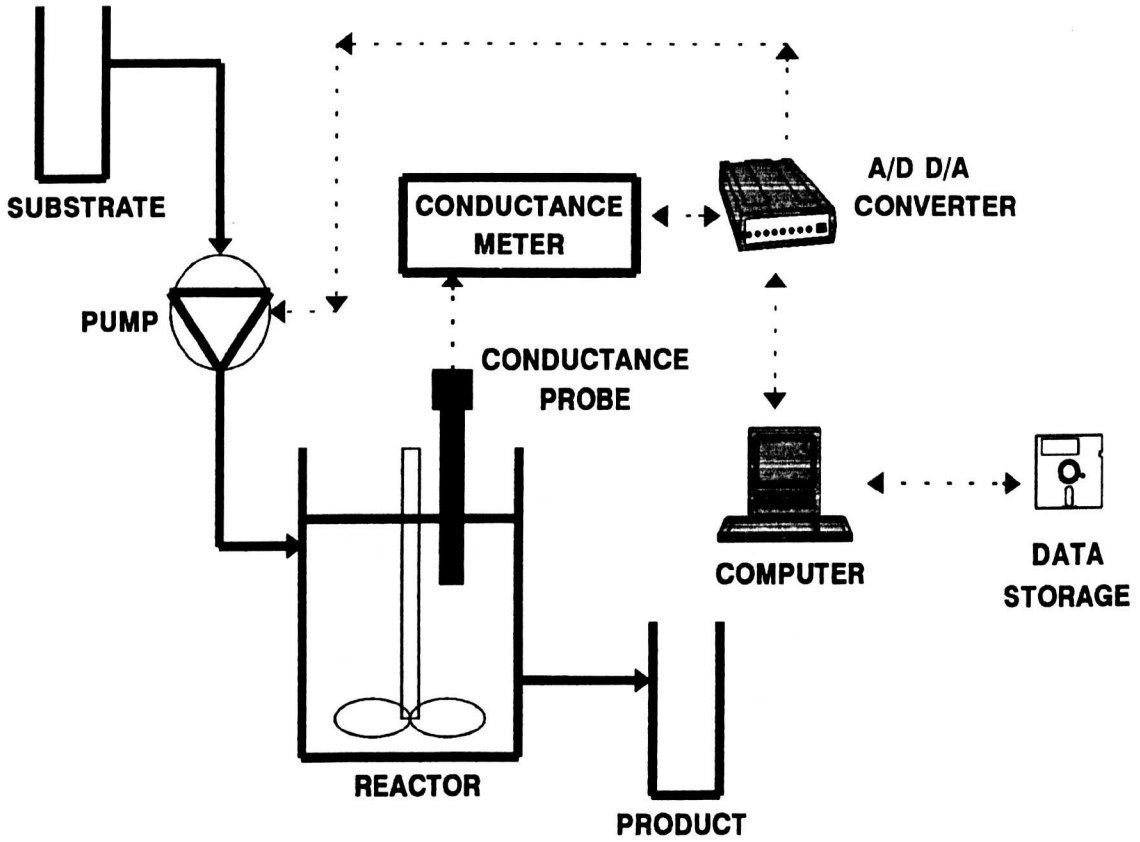
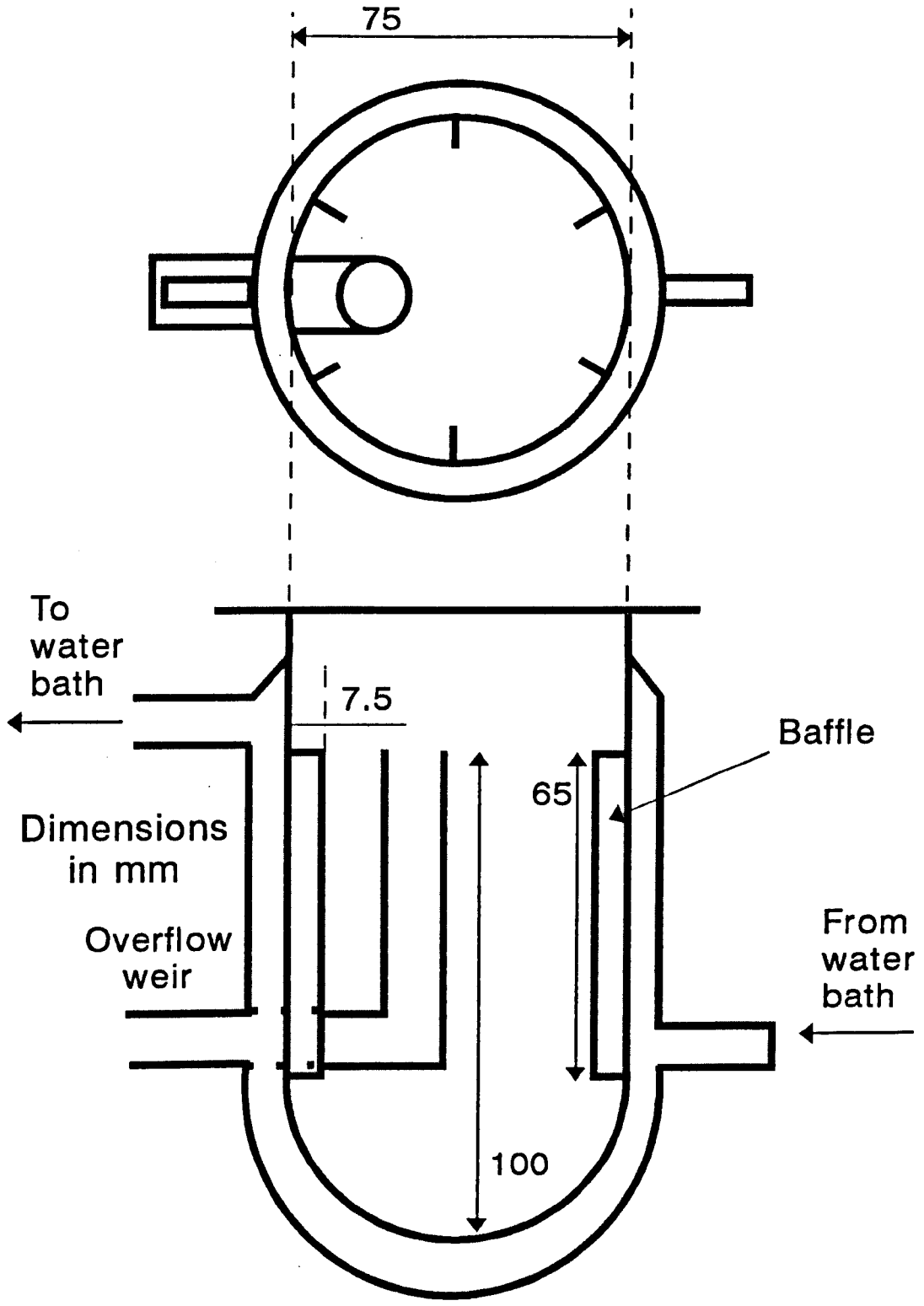


Figure 5.5: Vessel used for Continuous Reactor Studies



5.3.2.2 Calibration of the Pump and Conductivity Meter Signals

The signal from the conductance meter to the computer was calibrated by measuring the voltage output from the conductivity meter for known conductivity readings. The resulting linear relationship was determined using least squares analysis. To calibrate the substrate pumping rate, known voltages where sent from the computer to the pump and the flowrate was measured. A least squares analysis was again used to determine the relationship between the voltage sent and the resulting flowrate.

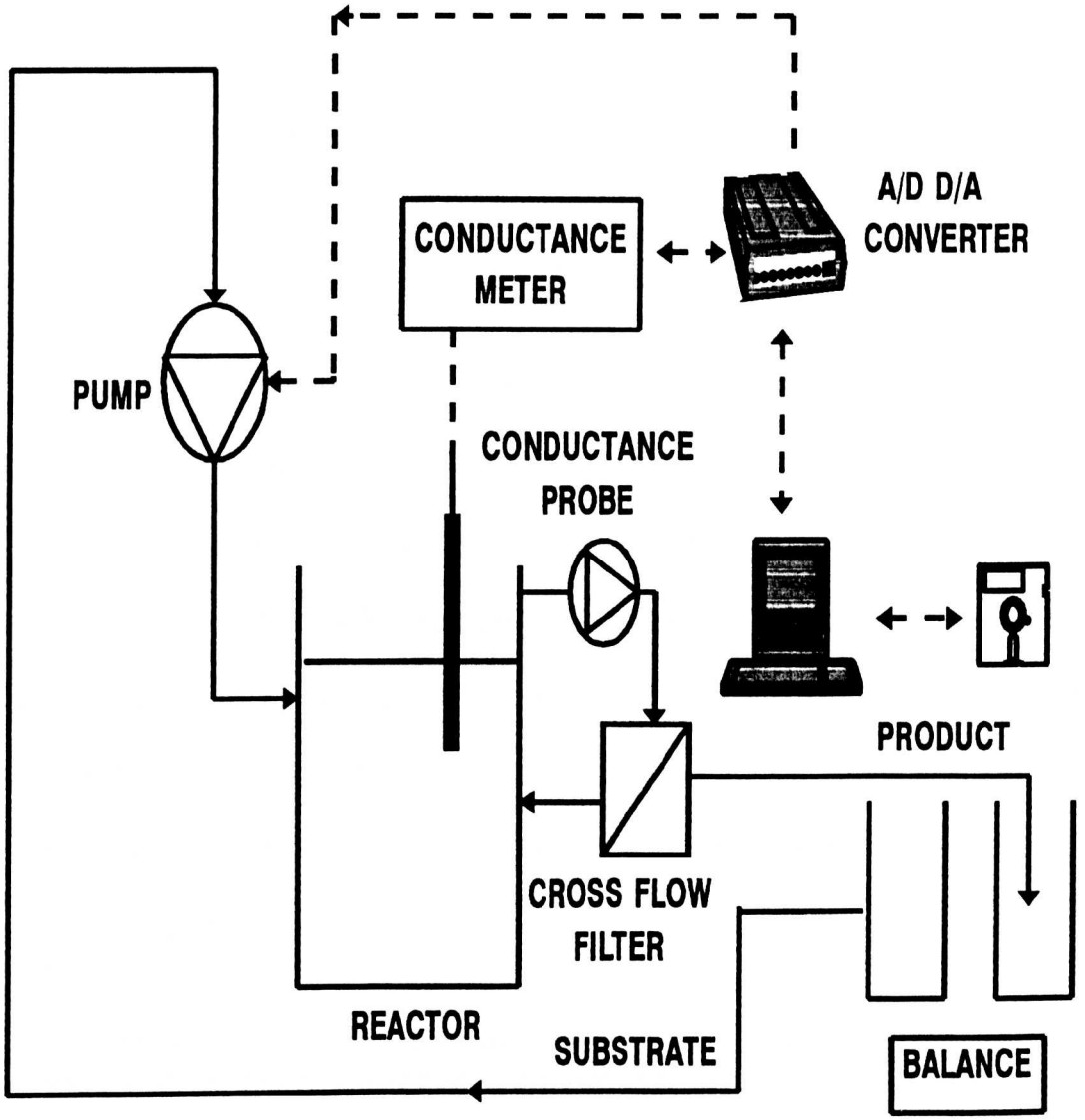
5.3.2.3 Preparation of the Reactor

Before starting up the CSTR, an ammonium acrylate solution was added to the reactor, to simulate the required operating conditions of the experiment. Because the biocatalyst is approximately 86% water, the solution needed to be more concentrated, to allow for the dilution that would occur when the biocatalyst was added to the vessel. After addition of the biocatalyst to the reactor the pH of the reaction solution was measured and adjusted using sodium hydroxide if required. Once the conductance became steady acrylamide was added to initiate the reaction and the main control programme was started. Several experiments were performed at various temperatures, concentrations and cell loadings, details of which are given in the Results Section.

5.3.3 Study of Free *C. nitrilophilus* Operating in a Computer Controlled CSTR using a Tubular Cross-Flow Ultrafiltration Unit

The equipment set up is shown in Figure 5.6. The start-up acrylamide/ammonium acrylate solution was added to the reactor and pumped around the system to ensure all the pipework and the filter was full of liquid, and all air had been removed. The filter was used to prevent the cells from being washed out of the reactor. A stirrer was not

Figure 5.6: Equipment Set-Up for Free Cell CSTR Experiments



required, as the pumping of liquid around the cross flow filtration unit was sufficient to agitate the reactor contents. When the temperature and conductance of the reaction solution had reached the desired values, a syringe was used to inject a suspension of *C. nitrilophilus* into the reactor. The main control programme was then run. When the computer turned on the substrate feed pump, the pump controlling the permeate flow rate was also turned on. Its speed was adjusted manually to ensure that the total mass of the feed and product beakers, shown on the balance, remained the same, keeping the volume in the reactor constant.

5.4 Fed-Batch Reactor Studies

5.4.1 Study of *R. rhodochrous* sp.632 Immobilised in Cross-Linked Polyacrylamide Beads Operating in a Fed-Batch Reactor using Manual Control

The equipment used was similar to that shown in Figure 5.2 except that the reaction vessel had a working volume of 150 ml. *R. rhodochrous* sp.632 immobilised in cross-linked polyacrylamide beads was stirred in water at 5 °C and acrylamide added to give a concentration of 350 mM. The pH was adjusted to 7.5 using sodium hydroxide. Further additions of acrylamide were made to bring the concentration to around 400 mM each time the rate of reaction had fallen to zero. When the ammonium acrylate concentration had risen above 1685 mM (15 % w/v) the liquid was decanted and the process repeated.

5.4.2 Study of *R. rhodochrous* sp.632 Immobilised in Cross-Linked Polyacrylamide Beads Operating in a Computer Controlled Fed-Batch Reactor

The equipment used was the same as that shown in Figure 5.4. The reactor vessel used was the same as that shown in Figure 5.5 except for the overflow weir which was

removed and the port blocked to allow batch operation. Cells of *R. rhodochrous* sp.632, immobilised in cross-linked polyacrylamide beads, were suspended in water and added to the reactor. The pH was adjusted to 7-7.5 using sodium hydroxide solution and the temperature maintained at 5 °C by the cooling jacket. The reaction was initiated by starting the computer control programme which controlled the addition of acrylamide to the reactor. (See Results Section and Appendix for a description of the control programme.) Once the ammonium acrylate concentration had exceeded 1685 mM (15% w/v) the control programme was stopped. The immobilised cells were removed from the vessel and separated from the reaction mixture by filtering under vacuum through filter paper. They were then returned to the reactor with fresh water cooled to 5 °C and the control programme restarted.

6. SCALE-UP REACTOR STUDIES

6.1 Operation of a Scaled-Up Reactor in CSTR and Fed-Batch Modes using Computer Control

The equipment used is shown in Figure 6.1 and the reactor vessel is shown in Figure 6.2. Design of the equipment is discussed in Section. 9. For fed-batch operation the overflow weir was removed and the port blanked off. The catalyst used for ammonium acrylate production was *R. rhodochrous* sp.632 immobilised in cross-linked polyacrylamide beads. The beads were formed by suspension polymerisation in the 5 litre resin pot as described in Chapter 4. Addition of beads to the reactor was achieved most easily by pumping a water/bead suspension into the reactor. Conductance was measured by constantly pumping reactant solution through the funnel shown in Figure 6.3 into the settling device before passing around the conductivity probe and back into the vessel. Operation of the reactor and the control programmes were the same as that described for the small scale CSTR and fed-batch experiments. At the end of a batch, during repeat fed batch operation, a drain port at the bottom of the vessel was opened and the liquid allowed to flow into the product barrel while the beads were retained by a filter. More water, that had been pre-cooled to 5 °C, was then added to the reactor and the computer control programme started again.

Figure 6.1: Equipment Set-Up used for Large Scale and Fed-Batch Reactor Studies

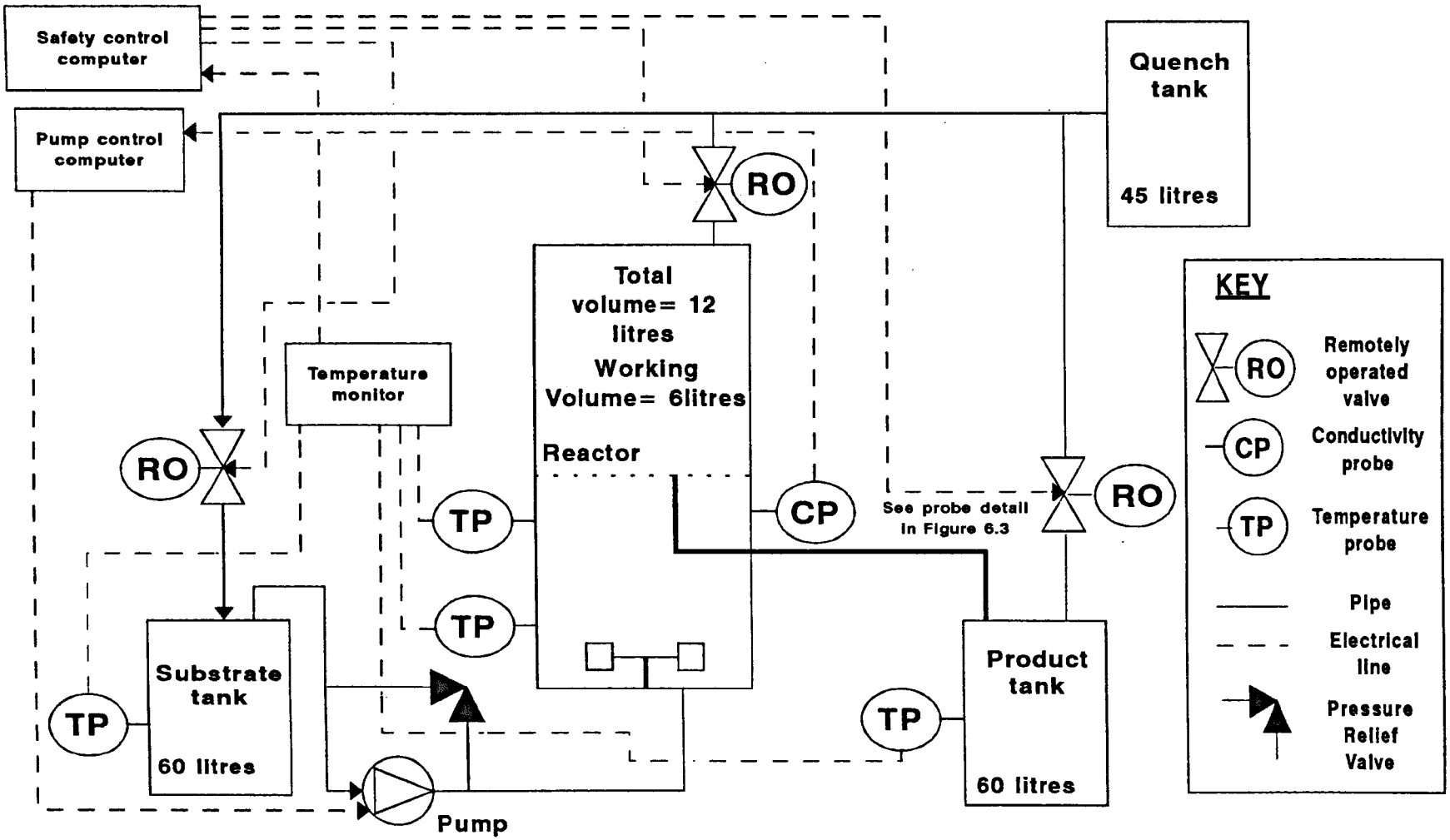


Figure 6.2: Vessel used for Large Scale CSTR and Fed-Batch Reactor Studies

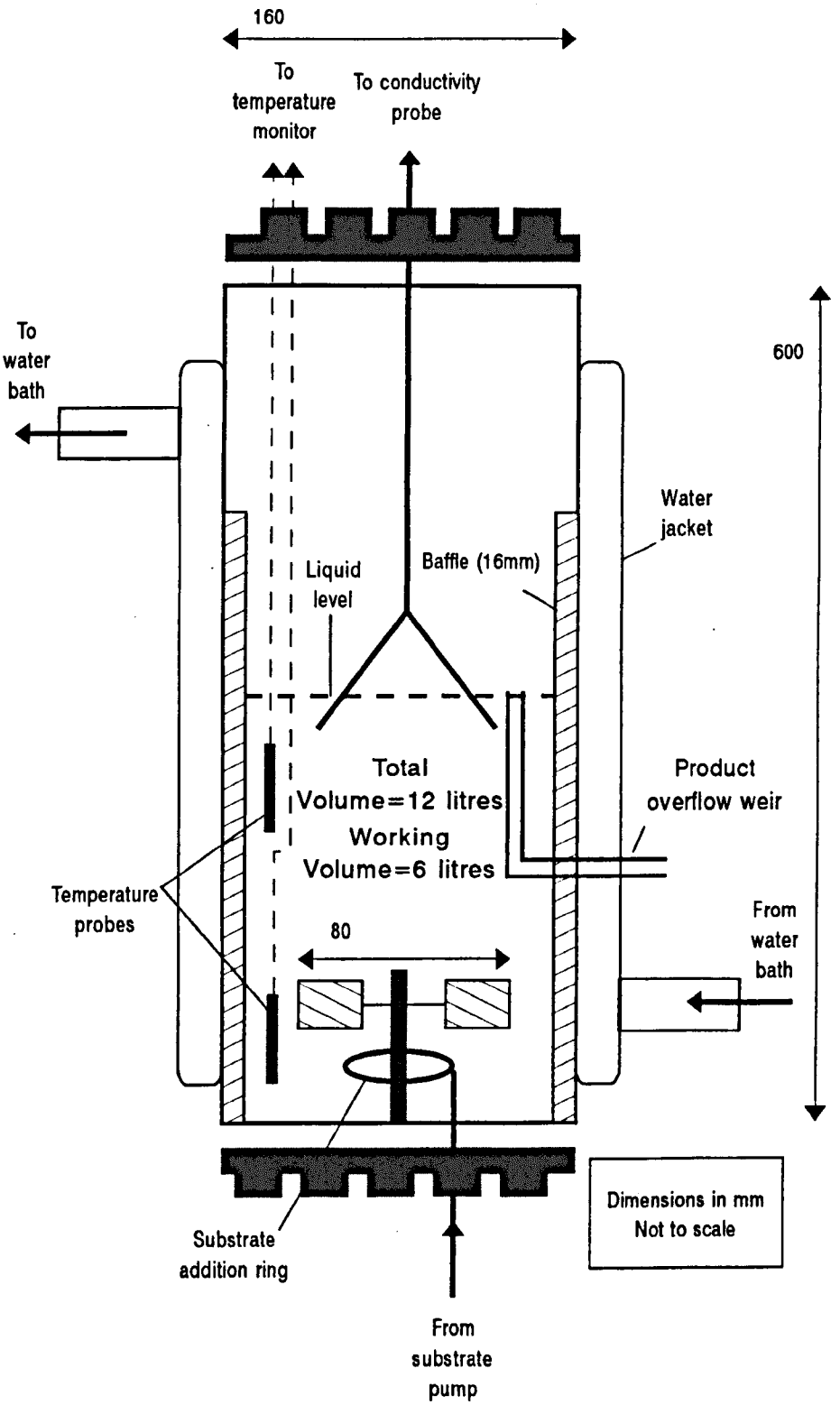
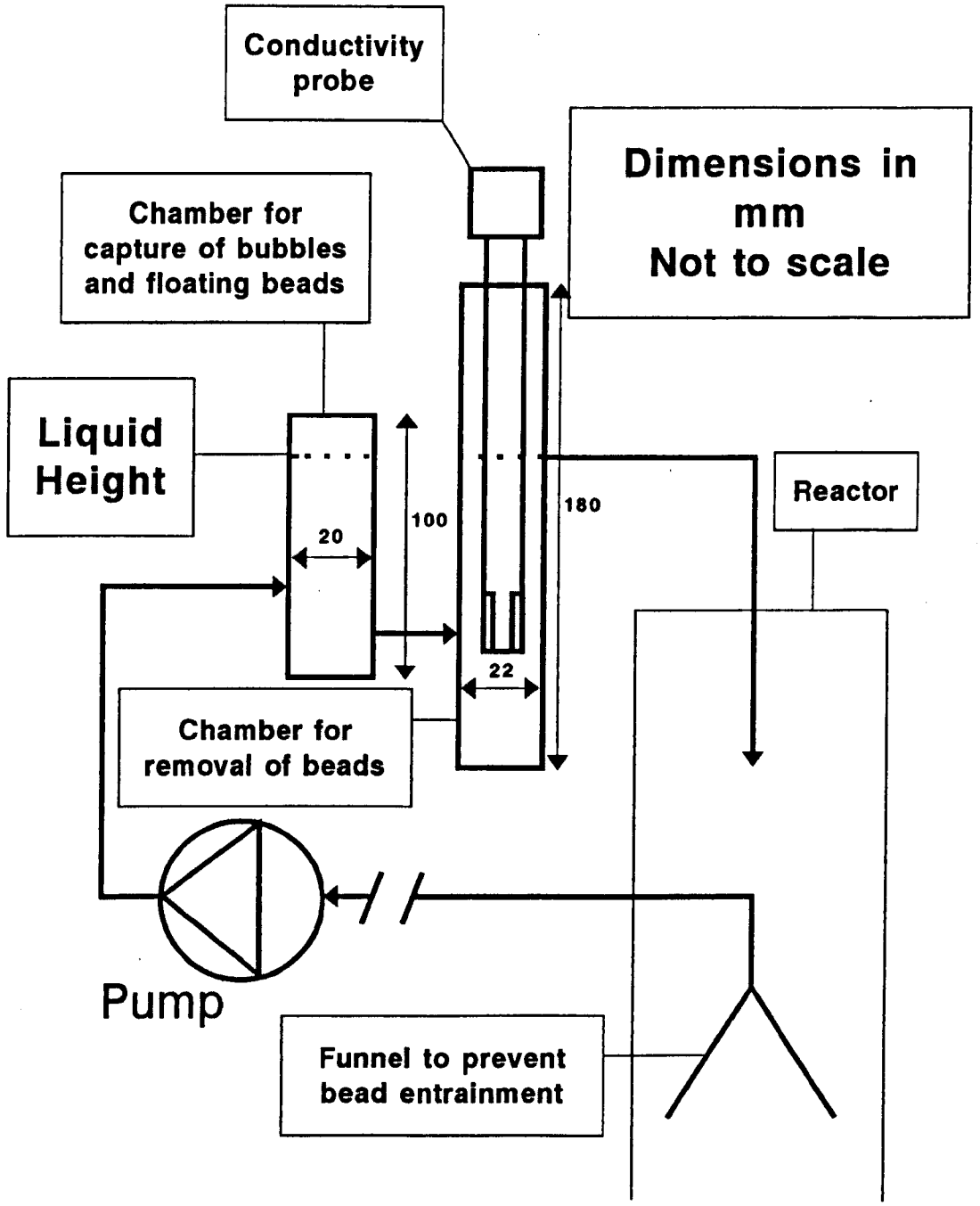


Figure 6.3: Equipment Set-Up used for Continuous Sampling of Large Scale Reactor



RESULTS AND DISCUSSION

7. IMMOBILISATION STUDIES

The initial aim of the immobilisation studies was to produce a biocatalyst with adequate physical stability and sufficient amidase activity to allow bioreactor studies to begin. By producing an adequate, rather than optimised biocatalyst, more effort could be directed towards the bioreactor studies. From the discussion on immobilisation in the Introduction, it is apparent that entrapment is the most widely used method of cell immobilisation, and several such methods were investigated using *C. nitrilophilus*, and later *R. rhodochrous sp.632*. These immobilised biocatalysts were assessed in terms of their resistance to attrition and retention in amidase activity.

7.1 Results of Immobilisation Studies

It can be seen from Table 7.1 that the apparent retention in amidase activity upon immobilisation in cross-linked polyacrylamide gel cuboids and Cryptogrout gel cuboids was very similar at 45 and 53 % respectively. The highest retention (80%) was exhibited by the cells immobilised in calcium alginate beads, as would be expected given the mild conditions of immobilisation that occur with this method (Bucke, 1986). However, the alginate beads were found to disintegrate when stirred in the presence of 200 mM ammonium acrylate. A similar observation was made by O'Brien *et al.*, (1990). A major limitation to the use of calcium alginate is its sensitivity to chelating agents, and in this case break-up is probably due to the sequestering of the calcium ions by acrylate and phosphate ions.

Table 7.1 Results of Immobilisation Studies

Method of Immobilisation	Cells in Immobilisation Matrix (% vol/vol)	Shape and Size of Matrix	L_b (g dry cells/100g wet gel)	Strength of Biocatalyst	Retention of Free Cell Activity (%)
Calcium alginate	28	Beads: 1.5-2 mm in diameter	6	Poor	81
Cryptogrout	9	Cuboids: 3×3 ×3 mm	2	Poor	53
Cross-linked Polyacrylamide Gel	23	Cuboids: 3×3 ×3 mm	5	Good	45

Cryptogrout is a speciality polymer produced by Allied Colloids, and is a branched pre polymer of cross linked polyacrylamide with pendant double bonds. As the polyacrylamide bonds have already been formed, less heat is produced during polymerisation and the temperature rise during gel formation is less. Reduction of the temperature rise during cell immobilisation is known to be an important factor when maximising enzyme activity, (Koshcheenko *et al*, 1981). Compared to cross-linked polyacrylamide gel, however, there was little difference in amidase activity retention.

On stirring under reaction conditions, it was noted that the cryptogrout gel broke-up after a few hours and released cell material into the reaction solution. Although some cell loss was observed when using cells entrapped in cross-linked polyacrylamide gel, this mainly occurred in the initial stages of stirring.

7.1.1 Choice of Immobilisation Method

As a consequence of the investigations described above, it was decided to use polyacrylamide gel as the model immobilisation method. There are several reasons to

justify this decision. The 45 % retention in amidase activity measured for the cross-linked polyacrylamide gel seemed adequate for future bioreactor studies. Information in the literature, discussed in the Introduction, suggests it is possible to improve the method to increase the amidase activity retention. An important factor in the choice of polyacrylamide gel over the other methods investigated was its superior physical stability during prolonged agitation. This method of cell entrapment has been used successfully by many other workers, (Philips and Poon, 1988), and has also been used as the immobilisation method for the industrial production of acrylamide (Ashen and Suttee, 1993). Also, acrylamide, the main component of cross-linked polyacrylamide, is widely available at a low cost which is important when considering an immobilisation method for a commercial process.

7.1.1.1 Activity Loss upon Immobilisation

It is shown in Table 7.1 that *C. nitrilophilus* cells immobilised in cross-linked polyacrylamide and assayed for amidase activity at 30 °C with 50 mM acrylamide retained an amidase activity of only 45 % of that of the free cells. This loss in activity may have been due to the rate of substrate diffusion into the gel being limiting, or due to deactivation of the enzyme during immobilisation. The amidase activity of gels at two different cell concentrations and two different substrate concentrations were measured. The conditions and results are shown in Table 7.2. A decrease in the observed specific amidase activity with an increase in cell concentration is indicative of the reaction rate being limited by the rate that substrate can diffuse into the gel matrix (Trevan, 1987). This can be countered by an increase in the substrate concentration as is observed in Table 7.2. The activity retention increased from 45 to 67 % when

500mM rather than 50 mM acrylamide was used as the initial substrate concentration. The remaining loss in amidase activity (23 %) is possibly due to further diffusion limitation or inactivation of the enzyme due to the toxic effect of the immobilisation reagents (Chibata *et al.*, 1986).

Table 7.2: Effect of Cell Concentration and Acrylamide Concentration on Amidase Activity of Cells Entrapped in Cross-Linked Polyacrylamide Gel Cuboids.

L_b	Initial Acrylamide Concentration (mM)	Retention in Activity (%)	Amidase Activity (U/ g dry cells)
1.9	50	89	630
7.5	50	45	180
7.5	500	67	470

7.1.2 Cell Immobilisation in Cross-Linked Polyacrylamide Beads

An essential part of the work described in this thesis was to build and operate a pilot scale immobilised cell bioreactor (circa 5l working volume) for ammonium acrylate production. The running of such a bioreactor would need a great deal more biocatalyst than could be reasonably produced using the method already described for immobilisation in cross-linked polyacrylamide.

It was suggested above that substrate diffusion limitation produces a reduction in the apparent amidase activity retention This could be partially overcome by increasing the acrylamide concentration. However, Brennan *et al.*, (1995), showed that acrylamide has a toxic effect on the amidase activity of *C. nitrilophilus* and that this effect increases with increasing acrylamide concentrations. An alternative method of reducing the effects of diffusion is to reduce the particle size. It may also be argued that small

beads are less likely to be eroded by the agitator in a reaction vessel than larger gel particles in the form of cuboids.

One way of producing smaller immobilised cell particles on a larger scale is through the use of suspension polymerisation. By dispersing the monomer/cell phase throughout an oil phase, prior to polymerisation, it has been shown that cells and enzymes can be immobilised in small, uniform beads (Beck and Rase, 1973, Klein and Schara, 1980). The reaction mixture is suspended in an inert medium, such as mineral oil, by the use of vigorous stirring and the presence of a dispersion stabiliser. Such stabilising agents are described by Anderson *et al.* (1977) and typically consist of a hydrophilic chain that resides in the dispersed aqueous phase, and a hydrophobic chain that extends from the particle surface into the oil phase. The oil phase also acts as a heat sink so that the problems of heat transfer associated with bulk polymerisation are reduced (Young and Lovell, 1991).

The arguments given above led to an investigation into cell immobilisation in cross-linked polyacrylamide beads using the suspension polymerisation technique. One of the difficulties encountered with suspension polymerisation is the method by which the polymerisation reaction is initiated. One way is to add the redox initiating agents to the monomer/cell phase before it is dispersed in the oil phase. However, there is a risk that polymerisation will begin before the monomer phase has been dispersed through the oil. To overcome this, methods were examined that would allow initiation of the monomer phase after it was dispersed through out the oil phase. The two initiation systems investigated made use of initiating agents that were soluble in both the oil and aqueous phases. This allowed them to be contacted with the monomer phase via the oil

once the monomer/cell mixture had been dispersed through the oil, and so avoid premature polymerisation.

7.1.2.1 Amidase Activity Retention of *R. rhodochrous* sp.632 Immobilised in Cross-Linked Polyacrylamide Beads

Table 7.3 shows the results of studies into the amidase activity retention of *R. rhodochrous* sp.632 cells immobilised in cross-linked polyacrylamide beads. The cross-linked polyacrylamide beads were typically spherical in shape with diameters ranging from 0.215 to 2.8 mm with the majority between 0.6 and 2 mm in diameter.

As is shown in Figure 7.1, during bead formation, the temperature of the oil phase rises and falls due to the exotherm of the polymerisation reaction and subsequent heat loss to the surroundings. The maximum temperature reached during each polymerisation is shown in Table 7.3 along with the amidase activity retention.

Initial studies into bead polymerisation were performed using the redox initiators ammonium persulphate (APS) and N,N,N',N'-Tetramethyl-ethylenediamine (TEMED) and the results are shown in rows 1-4. From row 1 it can be seen that an acrylamide concentration of 27 % w/v produces a higher than average temperature rise during polymerisation and the resulting retention in amidase activity is only 6%. By reducing the acrylamide concentration and consequently the temperature rise during polymerisation, a ten fold increase in amidase activity retention was obtained (Rows 2-4). For all further studies into suspension polymerisation an acrylamide concentration of 15 % w/v was used.

Table 7.3: Results of Suspension Polymerisation Immobilisation Studies

	Mass of Oil Phase (g)	Acrylamide Concentration (% w/v)	APS Conc . in aqueous phase (% w/v)	SS Conc. in Aqueous Phase (% w/v)	TEMED Conc. in Oil Phase (% w/v)	TBHP Conc. in Oil Phase (% w/v)	Maximum Temperature during Polymerisation (°C)	Retention in amidase activity (%)
1	300	27	0.770	0	0.2	0	19	6
2	300	20	0.26	0	0.2	0	6.4	60
3	300	20	0.260	0	0.2	0	5.4	60
4	300	15	0.2	0	0.2	0	3.3	65
5	300	15	0	0.02	0	0.015	3.6	81
6	300	15	0	0.02	0	0.015	3.9	74
7	300	15	0	0.02	0	0.015	7.2	89
8	300	15	0	0.02	0	0.015	7.3	76
9	300	15	0	0.02	0	0.015	17.5	58
10	600	15	0	0.02	0	0.015	4.2	61
11	600	15	0	0.02	0	0.015	4.3	84
12	600	15	0	0.02	0	0.015	5.7	62
13	3000	15	0	0.02	0	0.015	2.5	70
14	3000	15	0	0.02	0	0.015	3	72
15	3000	15	0	0.02	0	0.015	1.2	74
16	3000	15	0	0.02	0	0.015	3.8	63
17	3000	15	0	0.02	0	0.015	5.5	75
18	3000	15	0	0.02	0	0.015	5	78
19	3000	15	0	0.02	0	0.015	7	88

SS = sodium sulphite
APS = ammonium persulphate
TBHP = Tert-butyl hydroperoxide
TEMED = N,N,N',N'-Tetramethyl-ethylenediamine

Figure 7.1: Temperature profile of Oil Phase During A Suspension Polymerisation

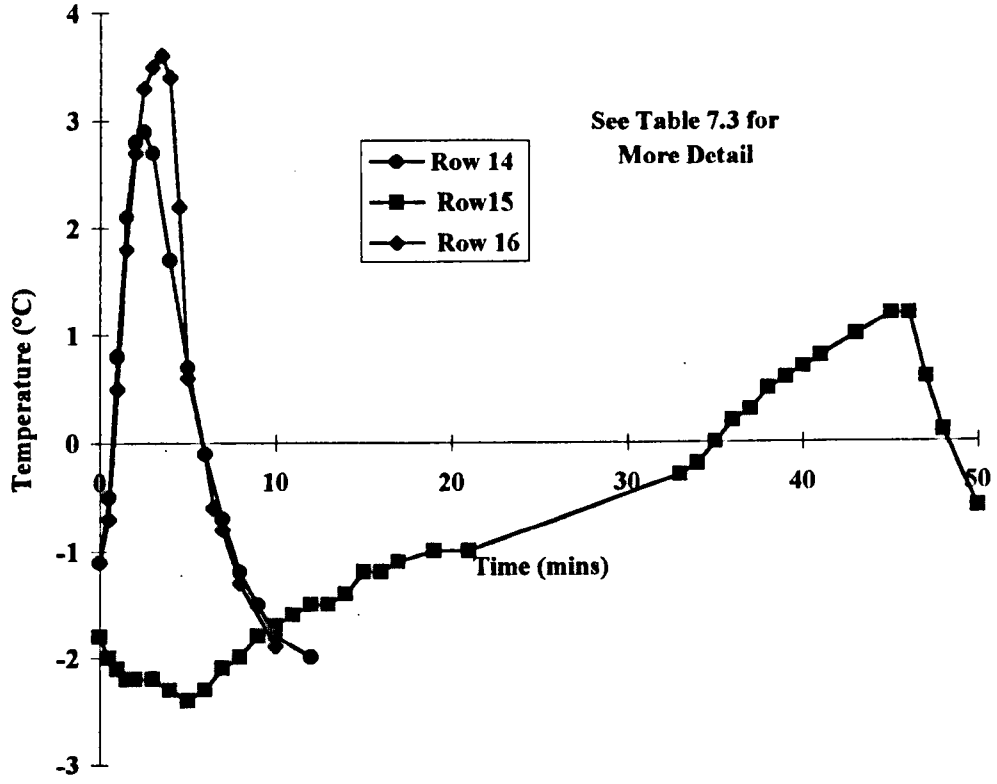
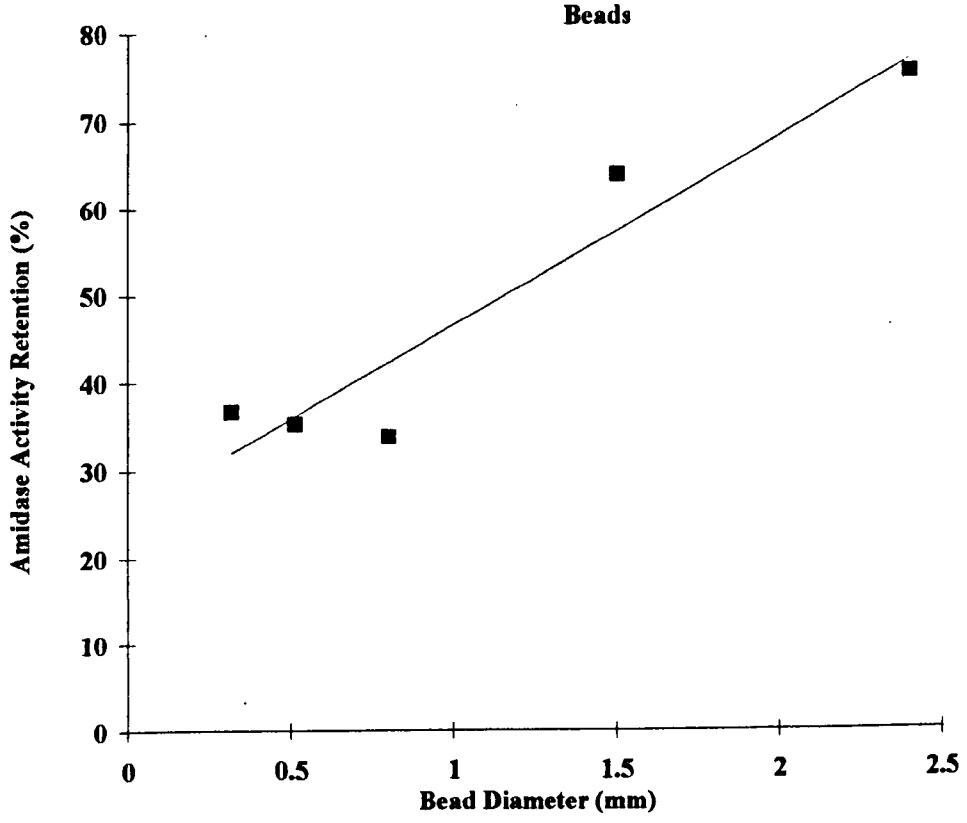


Figure 7.2: Effect of Bead diameter on Amidase Activity Retention of *R. rhodochrous* sp.632 Immobilised in Cross-Linked Polyacrylamide Beads



The majority of polymerisation studies were carried out using the redox initiators sodium sulphite (SS) and tert-butyl hydroperoxide (TBHP). This system was preferred as approximately only a tenth of the initiator concentration is required compared to the APS/TEMED system. Polymerisation initiators are known to reduce enzyme activity (Klein and Schara, 1980). However, the work performed using the APS/TEMED and SS/TBHP initiator systems are not directly comparable as higher acrylamide concentrations were used with the APS/TEMED studies. Further investigations would be required before it can be claimed with certainty that the APS/TEMED system causes more enzyme damage than the SS/TBHP system.

Watanabe, (1987b), studied the immobilisation of nitrile hydratase active whole cells in cross-linked polyacrylamide gel. He suggested that for maximal enzyme activity retention, the temperature during gelation should be kept below 30 °C. From Table 7.3 it can be seen that the temperature of the oil phase was typically kept below 8 °C for the suspension polymerisation experiments. However, the temperature inside the beads will be higher than this depending on the bead size and the rate of heat generation. The lowest retention in amidase activity (58 %) was observed when the oil temperature was allowed to rise from 11.6 to 17.5 °C.

7.1.2.2 Effect of Suspension Polymerisation Reagents on Amidase Activity

Figure 7.1 shows the temperature/time profiles of the suspension polymerisations detailed in rows 14-16 of Table 7.3. The polymerisation in row 15 was not initiated until after the monomer/cell phase had been in contact with the oil phase for 30 minutes. This is in contrast to the majority of bead polymerisations. Initiation typically occured just a few minutes after the monomer/cell phase had been added to the vessel.

However, as can be seen from Table 7.3, there is little difference in the amidase activity of the three polymerisations indicating that the increased contact time of the cells with the polymerisation reagents and mineral oil/stabilising reagents was not primarily responsible for the fall in amidase activity on immobilisation.

Once beads were formed they were removed from the oil phase by filtration and then washed in water before being assayed for amidase activity. Table 7.4 compares the activity of beads washed immediately after preparation with beads washed 20 hours after preparation.

Table 7.4: Effect of Washing Procedure on the Amidase Activity Retention of *C. nitrilophilus* Immobilised in Cross-Linked Polyacrylamide Beads

Washing Procedure	Relative Retention in Amidase Activity
Beads washed and assayed immediately after production	100
Beads washed immediately and assayed after 20 hours	93
Beads washed and assayed after 20 hours	46

It can be seen that the 20 hour delay before washing the beads causes the amidase activity to halve. This may be due to residual monomer and initiating reagents presence in the gel or mineral oil and suspension stabiliser remaining after filtration.

7.1.2.3 Effect of Bead Size on Amidase Activity Retention

Figure 7.2 shows the amidase activity of bead fractions from the same preparation after being separated into various sizes by sieving. It can be seen that amidase activity retention increases with increasing diameter. It has been mentioned previously that as catalyst particles decrease in size the effects of substrate diffusion limitation become

less significant. Therefore, it was expected that the smaller beads would have a greater or equal amidase activity retention when compared to the larger beads. The trend shown in Figure 7.2 is contrary to this but a possible explanation is given below.

It has been observed that washing of the beads after formation causes cell loss from the bead surface. The ratio of surface area to volume increases with decreasing bead size so that a greater fraction of the cells present in smaller beads will be lost from the bead surface during washing, thus leading to an apparent lower amidase activity retention.

7.1.2.4 Appearance and Physical Stability of Cross-Linked Polyacrylamide Beads

Figure 7.3 shows a scanning electron micrograph of *R. rhodochrous* sp.632 immobilised in cross-linked polyacrylamide beads formed by suspension polymerisation. Figure 7.4 shows the surface of the beads after washing in water but before having been used in a reactor for ammonium acrylate production. Cells can be seen entrapped under a layer of cross-linked polyacrylamide gel.

The physical stability of the beads was determined by stirring them in the bioreactor shown in Figure 5.4. The longest period of stirring was for 28 days in a solution of acrylamide (3.6 % w/v) and ammonium acrylate (15 % w/v) at a temperature of 5 °C. Cell leakage into the reaction solution was observed for the first two days, after which the suspending solution remained clear to the eye. Inspection of the beads using the scanning electron microscope, after stirring for 28 days, showed that a small number of the beads had broken-up (Figure 7.5). Figure 7.6 shows the surface of beads. Far fewer

Figure 7.3: Cross-linked Polyacrylamide Beads Formed by Suspension Polymerisation and Containing *R. rhodochrous* sp. 632

(Plate shows beads before being used for reactor studies).

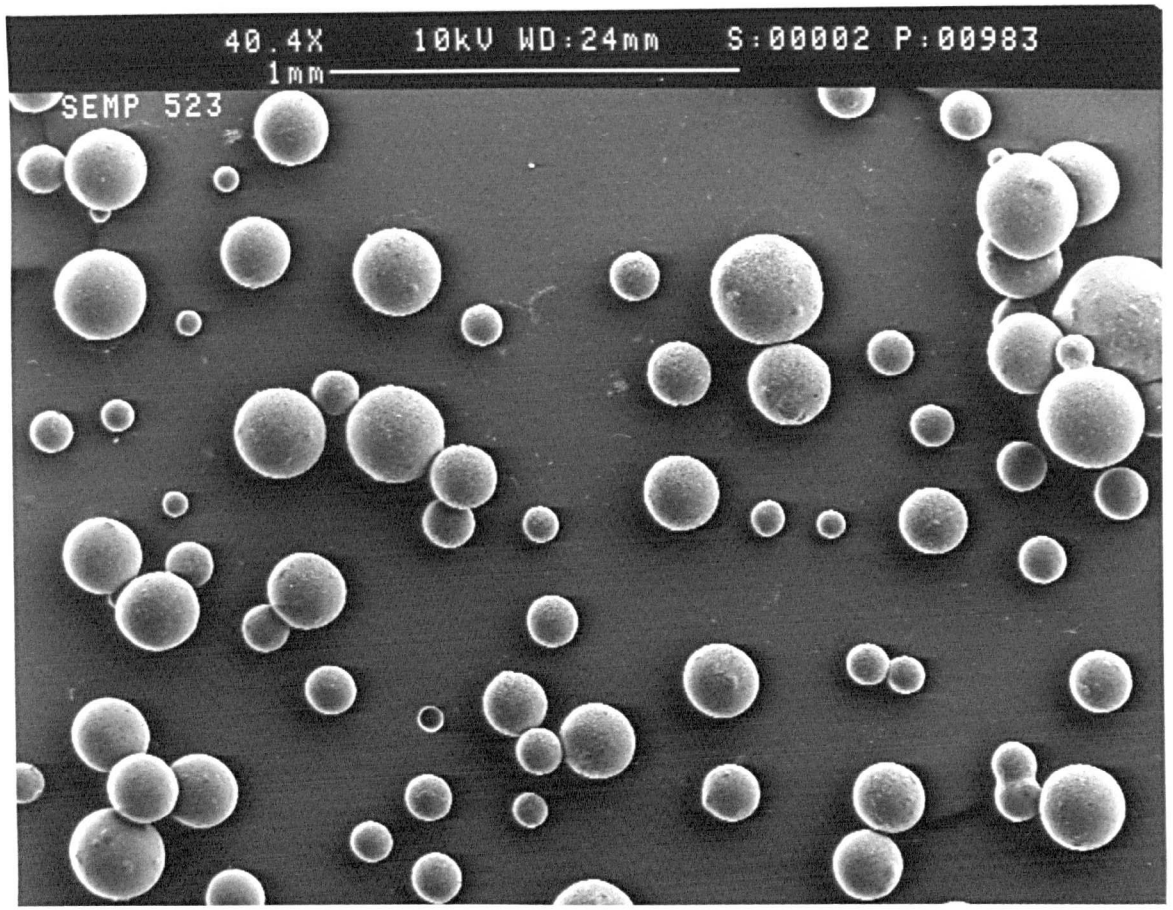


Figure 7.4: Cross-linked Polyacrylamide Beads Formed by Suspension Polymerisation and Containing *R. rhodochrous* sp. 632

(Plate shows bead surface before being used for reactor studies).

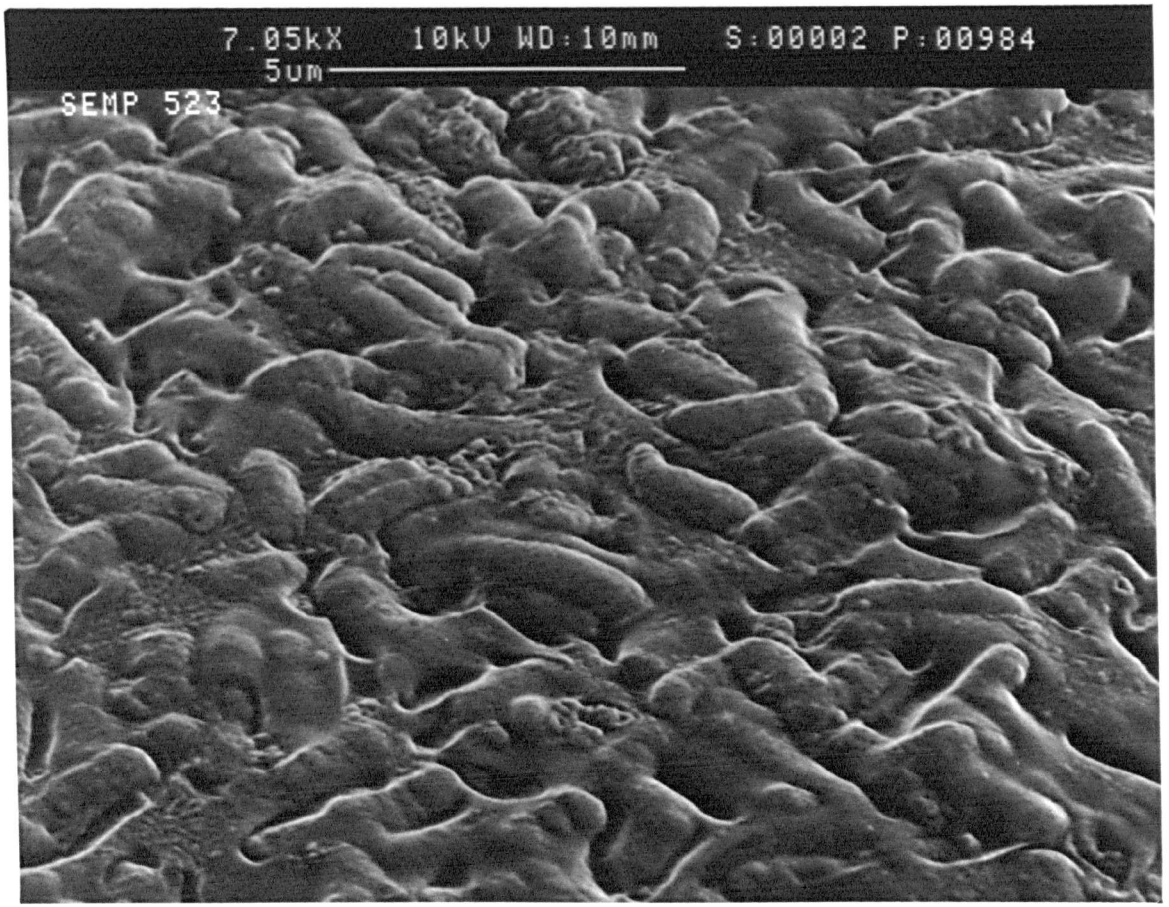


Figure 7.5: Cross-linked Polyacrylamide Beads Formed by Suspension Polymerisation and Containing *R. rhodochrous* sp. 632

(Plate shows beads after being agitated for 28 days in the reactor shown in Figure 5.4).

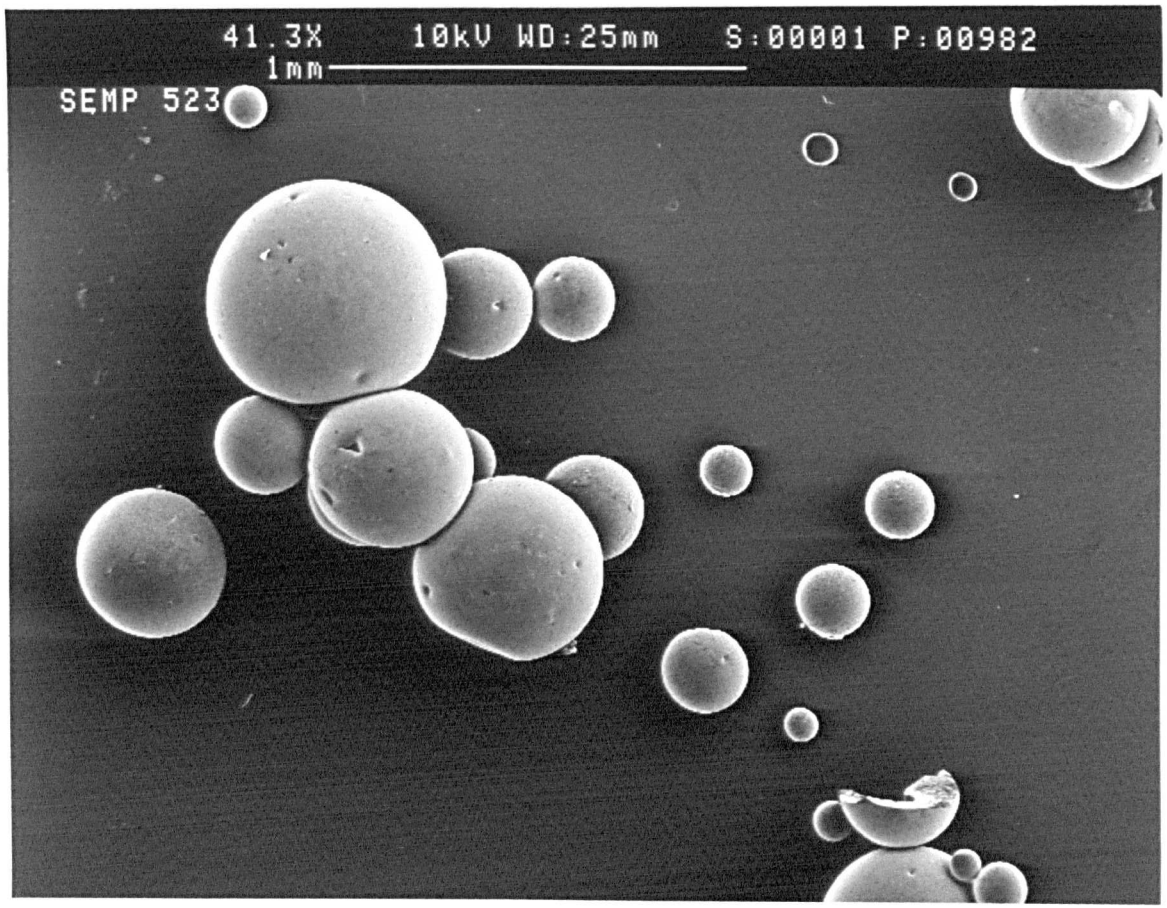
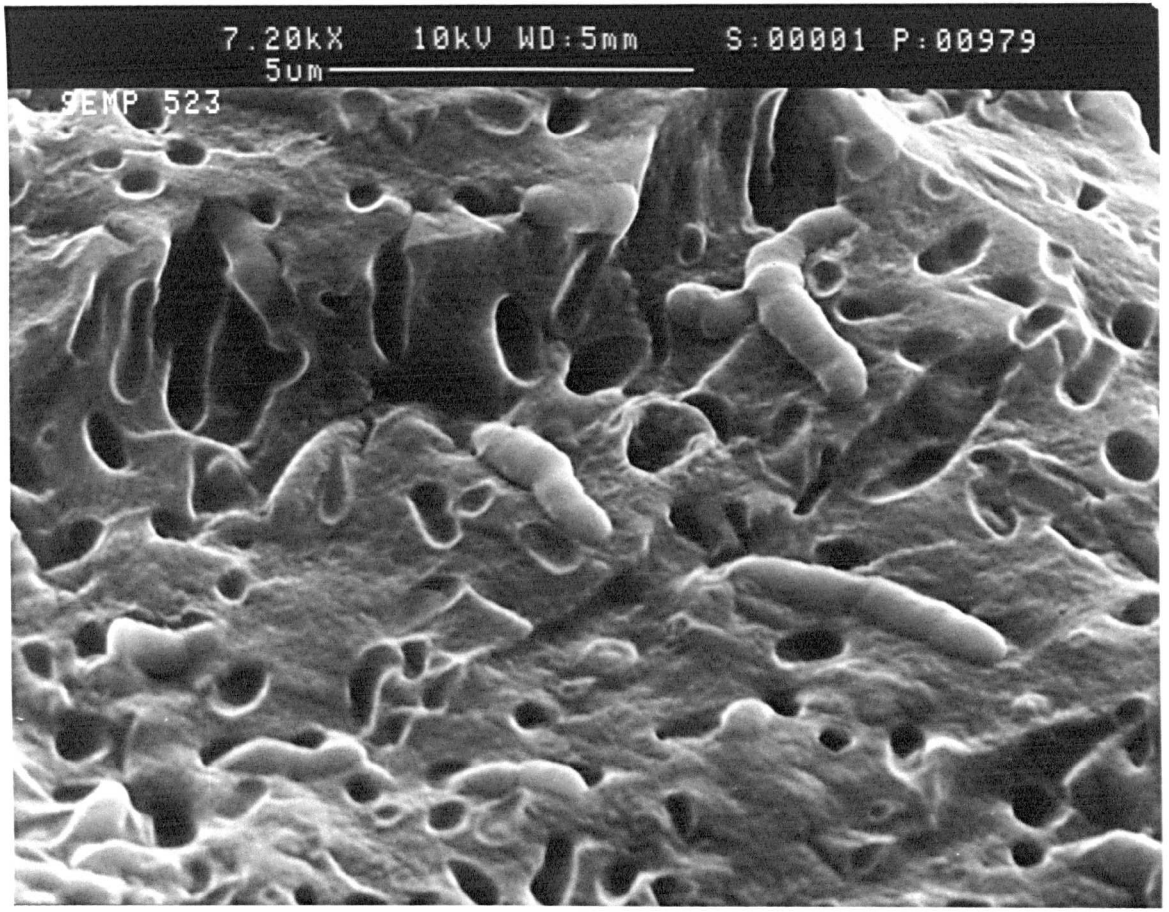


Figure 7.6: Cross-linked Polyacrylamide Beads Formed by Suspension Polymerisation and Containing *R. rhodochrous* sp. 632

(Plate shows bead surface after being agitated for 28 days in the reactor shown in Figure 5.4).



cells now remain on the bead surface indicating that some erosion has taken place. Further study is required to ascertain the degree of erosion taking place.

7.1.2.5 Scale-Up of Suspension Polymerisation

The suspension polymerisation technique was used to successfully scale-up cell immobilisation in cross-linked polyacrylamide gel. Beads were produced in 0.5, 1 and 5 litre vessels with the immobilised cells giving an average amidase activity retention of 76, 69 and 74 % respectively. The ratio of reagents used for the suspension polymerisation studies were identical for all three sizes of reaction vessel. The only equipment alteration required for polymerisations in the larger vessel was the addition of a cooling coil. This was required to compensate for the reduced surface area to volume ratio inherent in the larger vessel.

Further time could have been spent refining the suspension polymerisation technique in terms of the parameters that effect amidase activity retention. However, the main emphasis of the project was on bioreactor operation and the bead immobilisation process was regarded as satisfactory in that it could provide the required amounts of biocatalyst that was adequate in terms of amidase activity retention and physical stability.

7.2 Storage Stability of the Immobilised Cell Catalyst

Prior to operation of an immobilised cell reactor, a fermentation and immobilisation stage are required to provide the biocatalyst. Unless the biocatalyst can be stored, its

production would have to be coordinated with (or preceed) the immobilisation and bioreactor operation which would add to the complexity and cost of the process. This may also mean that the fermentation stage would need to be carried out on a smaller scale, with less favourable economics if the amount of cells required per bioreactor run was less than could be produced using the largest fermenter available. The operation of an enzyme catalysed process would therefore be simplified if it was possible to store the catalyst in a stable form that minimised enzyme activity loss and allowed the catalyst to be used when and where required (Tijsterman, 1993). However, a characteristic property of a large number of enzymes is their low storage stability (Kaul and Mattiasson, 1993).

On an industrial scale, bacteria are usually stored in a frozen or dried form (Lievense and van't Riet, 1993) with the latter being preferred in terms of cost. Therefore, in the case of an amidase active bacteria, it seems likely that the most practical way of increasing its storage stability would be to dry the bacteria. This may be possible for both free and immobilised cells. The investigations reported below, however, concentrate on the storage of cells in an immobilised form as this would negate the need to coordinate an immobilisation stage with the start-up of the bioreactor.

7.2.1 Effect of Drying and Rehydration on the Amidase Activity Retention of *R. rhodochrous* sp.632 Immobilised in Cross-Linked Polyacrylamide Beads

Table 7.5 shows the effect on amidase activity retention of immobilised *R. rhodochrous* sp.632 after drying and rehydration. It can be seen that amidase activity retention increases as the drying time decreases. This agrees with industrial practices where

methods of drying bacteria utilise short drying times in order to reduce thermal inactivation (Masters, 1985).

Table 7.5: Amidase Activity Retention of *R. rhodochrous* sp.632 Immobilised in Cross-Linked Polyacrylamide Beads after Drying and Rehydration

Drying Method	Drying temperature (°C)	Drying time	Water content kg/kg	Amidase Activity Retention (%)
Little surface area for drying	40	3 days	-	10
Beads spread thinly to increase area available for drying	30	12 hours	-	71
Fluidised-Bed Drier	30	40 mins	0.110	67
Fluidised-Bed Drier	45	30 mins	0.068	101
Fluidised-Bed Drier	60	20 mins	0.046	110

After drying at 30 °C in the fluidised bed, the beads had a higher residual water concentration compared to those dried at 45 and 60 °C using the same method, and this also seemed to reduce the amidase activity retention.

Apart from freeze-drying, fluidised-bed and spray drying are the most common methods used for drying bacteria (Lievense and van't Riet, 1993). Of these, the fluidised bed is the most applicable to cells entrapped in cross-linked polyacrylamide beads. This is because bacteria dried in such a way require immobilisation within or onto some form of support matrix such as starch, wheat bran or xanthanan gum, so as to prevent cell loss from the drier. Typically, drying temperatures ranging from 30 to 120 °C are used with residence times of 25 to 240 minutes. These figures compare well with those shown for the fluidised-bed results in Table 7.5. where the water content was reduced to 0.046 kg/kg at 60 °C in only 20 minutes. Because fluidised beds are

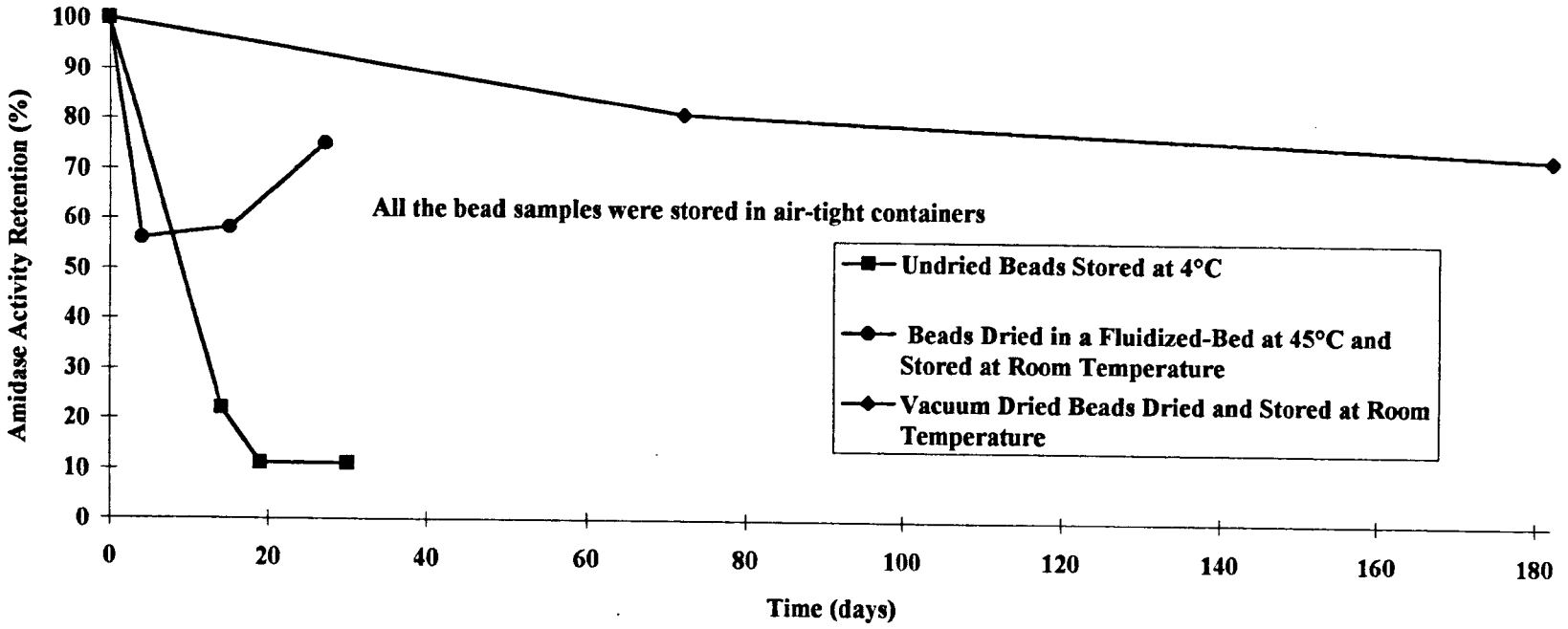
simple to model and control for drying processes (Lievense and van't Riet, 1993) it should be possible to produce the high amidase retentions seen above on a larger scale.

When particles dry in warm surroundings, i.e. an oven or fluidised bed, the temperature of the beads is much less than that of air surrounding them as heat is removed in the form of latent heat of vapourisation. Once the beads are dry, they rise in temperature to that of the surroundings. Therefore, they should be removed from the oven as soon as they are dry.

Figure 7.7 compares the amidase activity retention, after storage, of dried and undried *R. rhodochrous* sp.632 immobilised in cross-linked polyacrylamide beads. It can be seen that after 14 days the undried beads stored at 4°C had lost the majority of their amidase activity. After a total of 30 days storage, microbial contamination of the beads could be clearly seen in the form of a green 'mould' growing on the bead surface.

The amidase activity retention of the beads dried in the fluidised-bed was clearly greater than that of the undried beads but seemed to decrease and then increase with time. After drying in the fluidised-bed it was noted that some of the beads had coagulated together while some remained as separate species. It is possible that the coagulated beads were not dried to the same extent as the single beads and therefore did not retain their amidase activity to the same extent. The samples of beads assayed for amidase activity were not homogeneous in terms of the amount of coagulated and non-coagulated beads and this may explain the variations in amidase activity seen for the beads dried in this way.

Figure 7.7: Effect of Storage Time on Amidase Activity Retention of *R. rhodochrous* sp.632 Immobilised in Cross-Linked Polyacrylamide Dried Beads



The most stable of the bead samples were those which were further dried under vacuum. After 180 days the amidase activity retention had only fallen by 27 %. These results suggest that the storage stability of the amidase activity retention of the beads increases with decreasing water content.

In order to minimise the loss of amidase activity of the immobilised cells during storage, the results above suggest initial drying in a fluidised bed followed by further drying under vacuum. From an industrial view point, drying of the immobilised cells in this way would increase the total catalyst preparation costs. This would need to be balanced against the advantages gained by being able to store immobilised cells on a large scale for an extended period.

8. SMALL SCALE REACTOR STUDIES

8.1 On-Line Method of Analysis

A general discussion appears in the Introduction concerning various modes of reactor operation and the ways in which they can be monitored. For the operation of an enzyme reactor producing ammonium acrylate from acrylamide, there are several methods of analysis suitable for on-line monitoring of the bioconversion. Consideration of the advantages and disadvantages of these methods in relation to the concentration measurement of an acrylamide/ammonium acrylate solution is required in order to identify a suitable method of on-line analysis.

From consideration of the advantages and disadvantages outlined in Table 8.1 it can be seen that the measurement of conductivity is both a simple and cheap method of on-line analysis for the determination of the ammonium acrylate concentration. The main disadvantage of this method is that acrylamide, being a non-electrolyte, cannot be detected. However, knowing the total monomer concentration in the reaction solution, the acrylamide concentration can be determined indirectly from the ammonium acrylate concentration in the reaction mixture.

Figure 8.1a shows the increase in conductivity with time through the conversion of a 500 mM acrylamide solution to ammonium acrylate at 30 °C by *C. nitrilophilus* immobilised in cross-linked polyacrylamide gel. The equipment used is that shown in Figure 5.2. The initial conductivity of the solution is due to the presence of 50 mM sodium phosphate buffer (pH 7). Figure 5.1, in the Experimental Section, shows the conductivity/ammonium acrylate calibration curve used to convert the conductivity values shown in Figure 8.1a into the ammonium acrylate concentration shown in Figure 8.1b. It can be seen from Figure 4.1 that the conductivity increases linearly up to 500 mM ammonium acrylate and a conductivity of 44 mS/cm.

Figure 8.1a: Conductivity Change in a Batch Reactor Operating with *C. nitrilophilus* Immobilised In Cross-Linked Polyacrylamide Gel Cuboids Hydrolysing 500 mM ACM at 30 °C

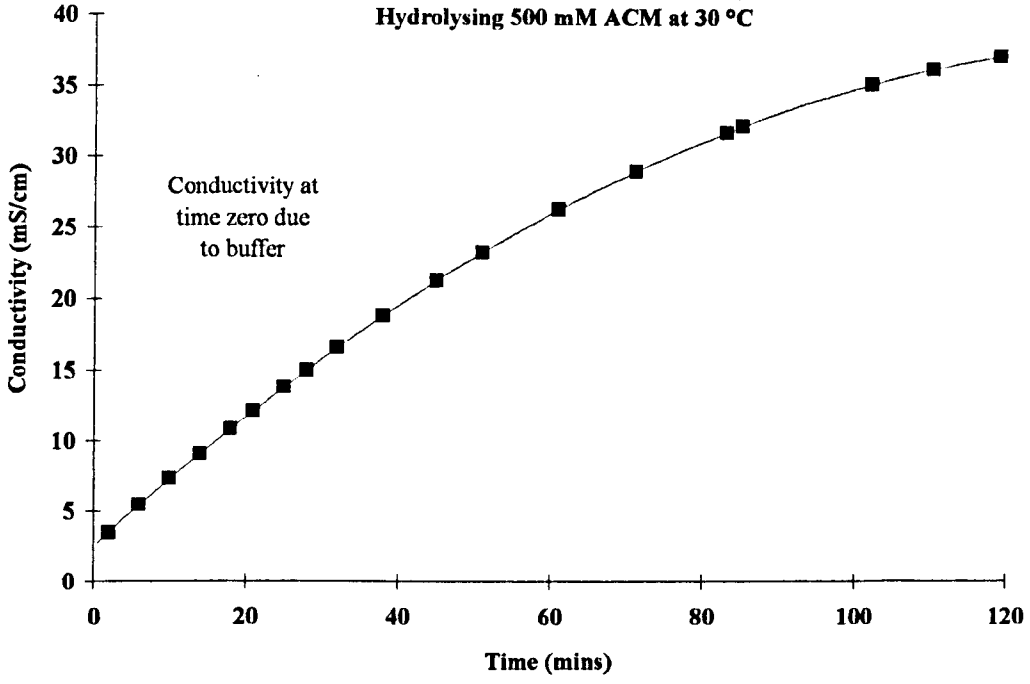


Figure 8.1b: Ammonium Acrylate Production in a Batch Reactor Operating with *C. nitrilophilus* Immobilised In Cross-Linked Polyacrylamide Gel Cuboids Hydrolysing 500 mM ACM at 30 °C

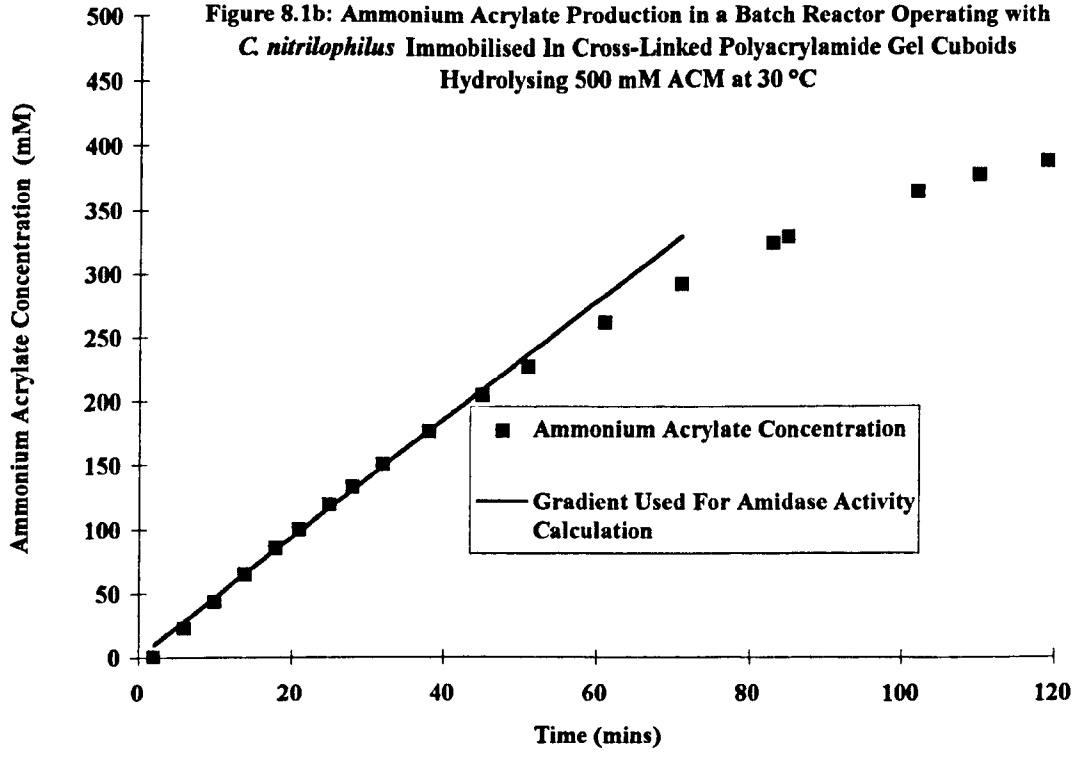


Table 8.1: Advantages and Disadvantages of Various On-Line Methods of Composition Analysis with Regard to an Acrylamide and Ammonium Acrylate Solution.

On-Line Analysis Technique	Advantages	Disadvantages
Chromatography	Can analyse both acrylamide and ammonium acrylate concentration	On-line sampler and diluter required Time delay between sampling and result Relatively Expensive
Ion Selective Probes	Relatively cheap	Can only determine ammonium concentration Sample dilution required for concentrations above 1 molar Fouling of probe Interference from other ions
Conductance	Can measure relatively high concentrations Quick response Relatively cheap Resistant to harsh conditions	Can only determine ammonium acrylate concentration Non-linear response Interference from other ions

The gradient of the line is 11.5 mM.cm/mS. It is possible to measure conductivity changes of 0.1 mS/cm in a solution of conductivity 44 mS/cm and so ammonium acrylate concentration changes can be determined to within 1.14 mM.

Figure 8.1b shows a plot of the ammonium acrylate concentration with time derived from Figures 8.1a and 5.1. This plot can be used to determine the rate of reaction. Despite the small contribution of the buffer to the conductivity, it is still possible to determine the ammonium acrylate concentration. It was decided, therefore, to perform all further ammonium acrylate production studies using conductivity measurement as

the on-line method of analysis. Additional off-line analysis was provided through the use of the colorimetric and HPLC methods described in the Experimental Section.

8.2 Choice of Reactor

The advantages and disadvantages of various types of reactor were discussed in the Introduction. The reactor studies reported below have made use of stirred tank vessels as opposed to packed or fluidised beds. This choice can be justified in several ways.

Temperature and pH are known to have marked effects on enzyme activity and their control is easier in a stirred tank than in a packed bed. Mixing in packed beds can be uncertain due to channelling of fluid. This is especially true when using compressible supports such as cross-linked polyacrylamide. An important aim of this work was to scale-up the reactor, and this is known to be difficult for fluidised bed reactors (Levenspiel, 1972). Although stirred tank reactors have been used for this work it is not necessarily true that they are the optimum choice for the production of ammonium acrylate from immobilised cells. However, their properties are easier to predict and therefore it can be more certain that the results of a particular experiment are due to the controlled conditions and not a problem with the reactor.

8.3 Batch Reactor Studies with *Corynebacterium nitrilophilus*

During the immobilised cell reactor studies described below, the degree of agitation in the vessels used was adjusted to ensure that all of the immobilised cell particles were fully suspended i.e. the stirrer speed was greater or equal to the *just suspended* speed (Nienow *et al.*, 1986). This was to ensure sufficient solid-liquid mass transfer between the catalyst particles and the liquid. Increased agitation above this point is known to have little effect upon the rate of mass transfer (Nienow *et al.*, 1986). However, the stirrer speed was occasionally adjusted to alter the degree of agitation in the vessel.

These adjustments had no subsequent effect upon the reaction rate. Therefore, it is likely that the rate of mass transfer of substrate and product at the particle surface was not limiting the rate of ammonium acrylate production in the reactor (Worstell and Ginestra, 1993).

8.3.1 Effect of pH and Temperature on the Amidase Activity of Free and Cross-Linked Polyacrylamide Gel Immobilised Cells

Figure 8.2a compares the specific amidase activities at 30°C of free cells using 50 mM acrylamide and immobilised cells using 500 mM acrylamide. The immobilised cells were assayed at 500 mM to reduce the effects of substrate diffusion limitation described in Section 7.1.1.1. It can be seen from Figure 8.2a that the amidase activity of the immobilised cells is less sensitive to pH but maximal activity was observed at pH 6.5 for both the free and immobilised cells. Hwang and Chang, (1989), found that the activity of nitrile hydratase active cells also became less sensitive to pH when immobilised in cross-linked polyacrylamide gel. Figure 8.2b compares the specific amidase activity of both free and immobilised cells over a range of temperatures at pH 7 using the concentrations stated above. Maximum activity was observed for both free and immobilised cells at 42°C and there was little appreciable difference between the two profiles. All subsequent investigations with *C. nitrilophilus* into amidase activity were carried out at a pH of 7, in the presence of buffer, unless otherwise stated.

Figure 8.2a: Effect of pH on the Amidase Activity of Free and Cross-Linked Polyacrylamide Entrapped *C. nitrilophilus* Cells

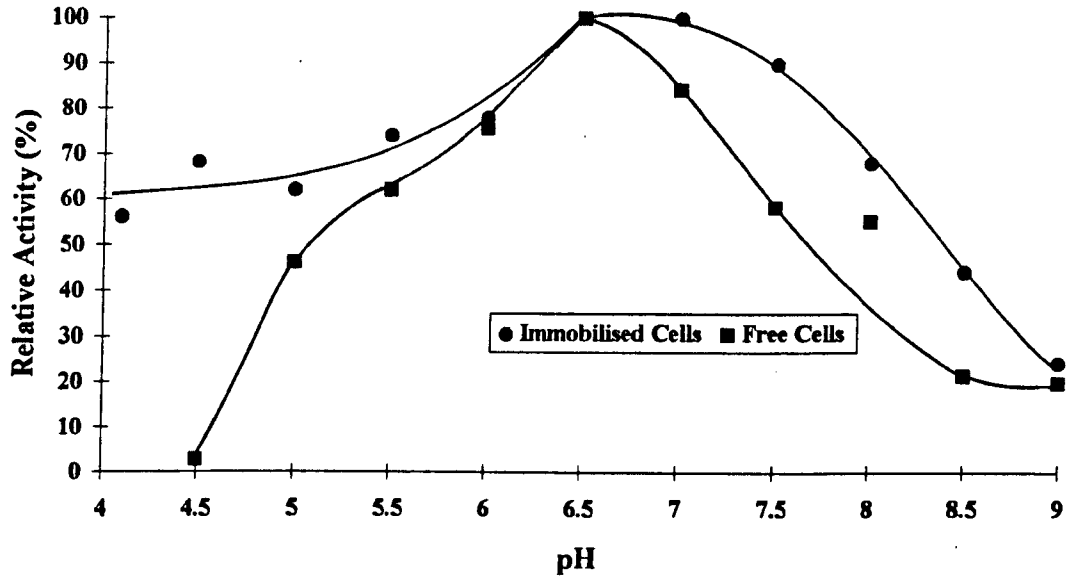
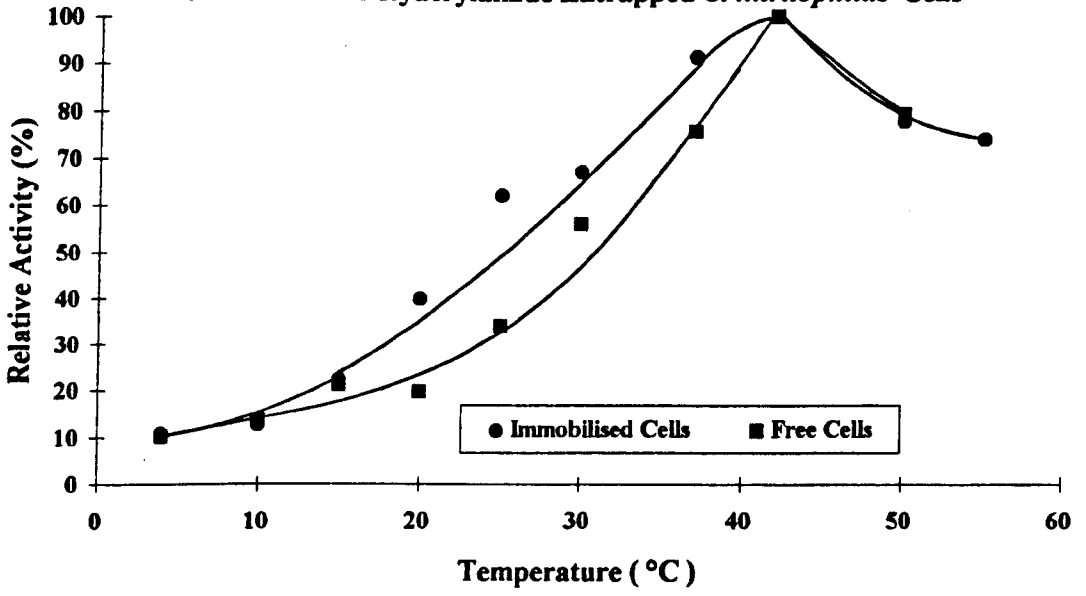


Figure 8.2b: Effect of Temperature on the Amidase Activity of Free and Cross-Linked Polyacrylamide Entrapped *C. nitrilophilus* Cells



8.3.2 Effect of Acrylamide Assay Concentration on the Amidase Activity of Free Cells of *C. nitrilophilus*

Table 8.2 shows the effect of acrylamide assay concentration on the amidase activity of *C. nitrilophilus* when assayed at 30 °C. The specific amidase activity decreased markedly at concentrations above (700 mM). It has been shown that the loss in activity is due to a progressive and permanent enzyme deactivation, that occurs in the presence of acrylamide and ammonium acrylate, and not due to the effects of inhibition (Brennan *et al.*, 1995). Bernet *et al.*, (1991), investigated the production of acrylamide from acrylonitrile using nitrile hydratase active cells. They found that acrylamide inhibited and denatured the enzyme activity. They limited the acrylamide concentration to 850 mM to avoid a rapid loss of activity.

Table 8.2: Effect of Acrylamide Assay Concentration on the Amidase Activity of Free Cells of *C. nitrilophilus*

Acrylamide Concentration (M)	Specific Amidase Activity (U/g dry cells)
0.05	2000
0.7	2300
1.4	950
2.8	400
4.2	0

Vinyl compounds such as acrylamide have been reported to alkylate the sulphydryl groups on proteins (Cavins and Friedman, 1968). It is also known that some amidases do have at least one sulphydryl group and that it is likely to play a part in the active site of the enzyme (Maestracci *et al.*, 1988). Modification of the sulphydryl group, therefore, is likely to affect the catalytic properties of the amidase enzyme. The extent of sulphydryl modifications by acrylamide varies with pH, temperature and vinyl compound concentration, (Cavins and Friedman, 1968). The loss of activity shown in Table 8.2 could be a result of this type of denaturation.

8.3.3 Operation of an Immobilised Cell Batch Reactor at Various Temperatures

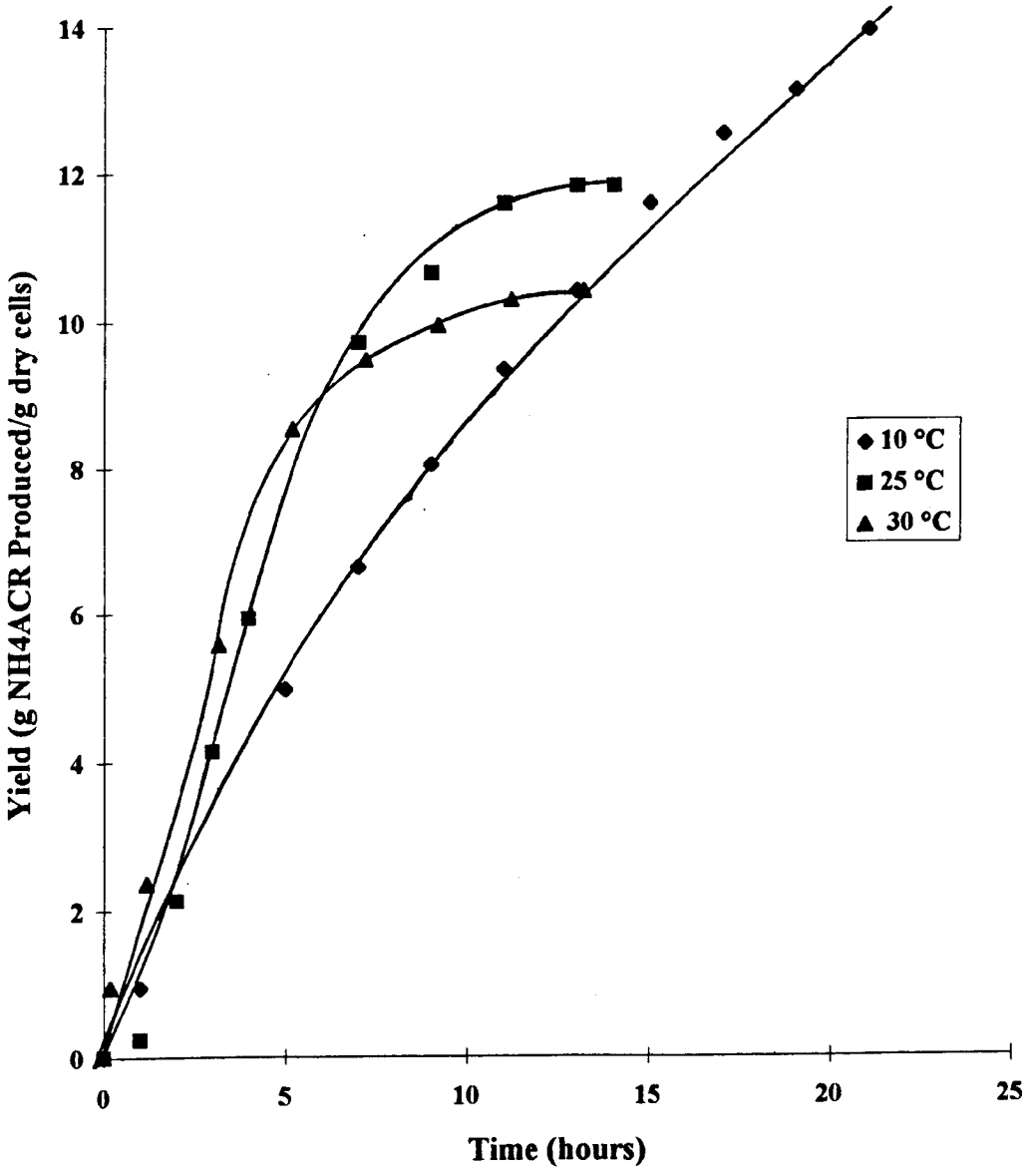
The batch production of ammonium acrylate from 500 mM acrylamide at three temperatures is shown in Figure 8.3. At high substrate concentrations the initial bioconversion rate would be expected to be zero order with the rate finally decreasing as a result of product inhibition or substrate limitation. The initial rate increases with increasing temperature as anticipated. However, in each case, the rate of conversion decreases progressively with time so that the reaction ceases well before complete conversion. This is considered to be due to a progressive deactivation of the enzyme under the conditions of the reaction.

It is clear from Figure 8.3 that the loss of activity decreases with decreasing temperature. Although the initial conversion rate is lowest at 10 °C, the slow loss of activity compensates for this, resulting in the highest overall yield of ammonium acrylate. Several workers studying the conversion of acrylonitrile to acrylamide (Hwang and Chang, 1989; Watanabe, 1987b), have similarly reported an increase in enzyme stability as the temperature is reduced. This fact is significant when considering the operating conditions of a bioreactor. The deactivation of the enzyme is due to the presence of acrylamide and acrylate, but it is impossible to distinguish the contribution of each since their concentrations are changing continuously throughout the batch conversion. It would be useful to be able to study the process of deactivation under conditions where the concentration of substrate and product remain constant. This is feasible in a properly controlled CSTR.

8.4 Manually Controlled Continuous Stirred Tank Reactor (CSTR) Studies With Immobilised *C. nitrilophilus* Cells

It is suggested in the discussion above that it would be useful to study immobilised enzyme kinetics under conditions of a constant substrate and product concentration.

Figure 8.3: Yield of Ammonium Acrylate from *C. nitrilophilus* Immobilised in Cross-Linked Polyacrylamide Gel Cuboids in a Batch Reactor



The characteristics of CSTR's were discussed in the Introduction. It was shown that at steady state the reactant and product concentrations in a CSTR remain constant with time. In a reactor in which the activity of the catalyst is slowly falling, a steady-state concentration of substrate and product can only be held constant by a reduction in the feed rate. Under such conditions, the catalysts activity is proportional to the feed rate. In this way the effect of temperature on activity over a range of concentrations can be studied.

Assuming the reactor is uniformly mixed, a CSTR can be described using the following equations:

Rate of substrate entering reactor	- Rate of substrate leaving reactor	- Rate of Reaction	= Rate of change of substrate in reactor.

$$FSo - FS - R = V(dS/dt) \qquad (1)$$

where R = Amidase activity at reaction conditions x mass of catalyst

At steady state, ds/dt = 0 and equation (1) becomes:

$$FSo - FS - R = 0$$

$$F(So - S) = R \qquad (2)$$

where
- F = Flow rate of substrate into reactor (l/min)
- S_0 = Input substrate concentration (moles/l)
- S = Substrate concentration in reactor (moles/l)
- V = Volume available for reaction (l)
- R = Reaction rate (moles/min)

Under conditions of constant concentration the reaction rate, R, is directly proportional to the flow rate of substrate into the reactor. Therefore, if the reactor conditions are maintained at steady-state the amidase activity of the biocatalyst is directly proportional to the substrate feed rate.

Figure 8.4a shows the decrease in feed rate with time required to maintain a 50% conversion in the reactor shown in Figure 5.3, when immobilised cells are used to convert a 500 mM acrylamide feed. Using equation (2), the flow rate/time profile can be converted to a reaction rate profile. This is shown in Figure 8.4b.

The decay rate of many enzyme systems can be described by a first order expression from which an enzyme stability half-life can be calculated (Gacesa and Hubbel, 1987):

$$\ln \frac{E_t}{E_0} = -k_d \times t \qquad (3)$$

where E_t = Active enzyme concentration at time t (mol/l)
E_0 = Initial active enzyme concentration (mol/l)
k_d = Decay constant (min^{-1}).
t = time elapsed (min).

It follows that:

$$\frac{R_t}{R_0} = \frac{E_t}{E_0}$$

and equation (3) can be written:

$$\ln \frac{R_t}{R_0} = -k_d \times t \qquad (4)$$

where R_0 = Reaction rate at time zero (moles/min)

R_t = Reaction rate at time t (moles/min)

Thus k_d can be determined from equation (4) by plotting ln (R_o/R_t) against time. Such a plot is shown in Figure 8.5 using the data from Figure 8.4b.

The fall in reaction rate for an immobilised cell system can not necessarily be described by a first order decay plot. Substrate diffusion limitation into the immobilisation matrix causes cells at the centre of an immobilised cell particle to experience different concentrations of product and substrate to those at the surface of the particle, leading to artificially high stability (Klein, 1988). For the experiments performed in the CSTR a

Figure 8.4a: Acrylamide Feedrate to a CSTR Operating at 30 °C with *C. nitrilophilus* Entrapped in Cross-Linked Polyacrylamide Gel Cuboids ACM=250 mM, NH4ACR=250 mM

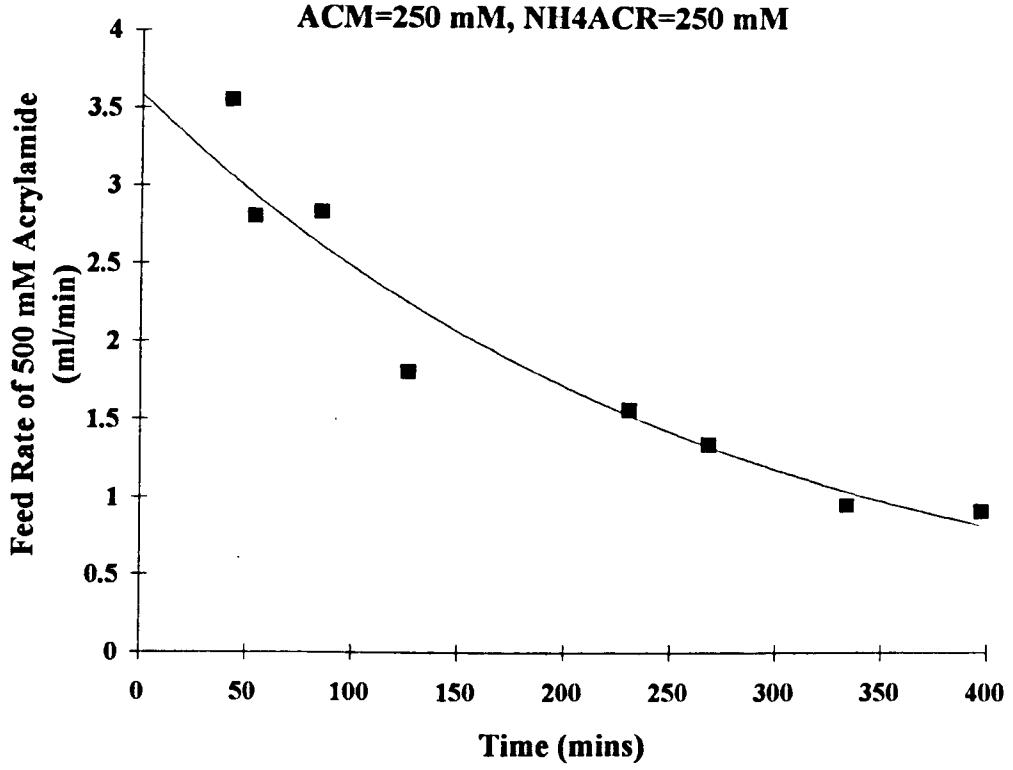


Figure 8.4b: Rate profile for a CSTR Operating at 30 °C with *C. nitrilophilus* Entrapped in Cross-Linked Polyacrylamide Gel Cuboids ACM=250 mM, NH4ACR=250 mM

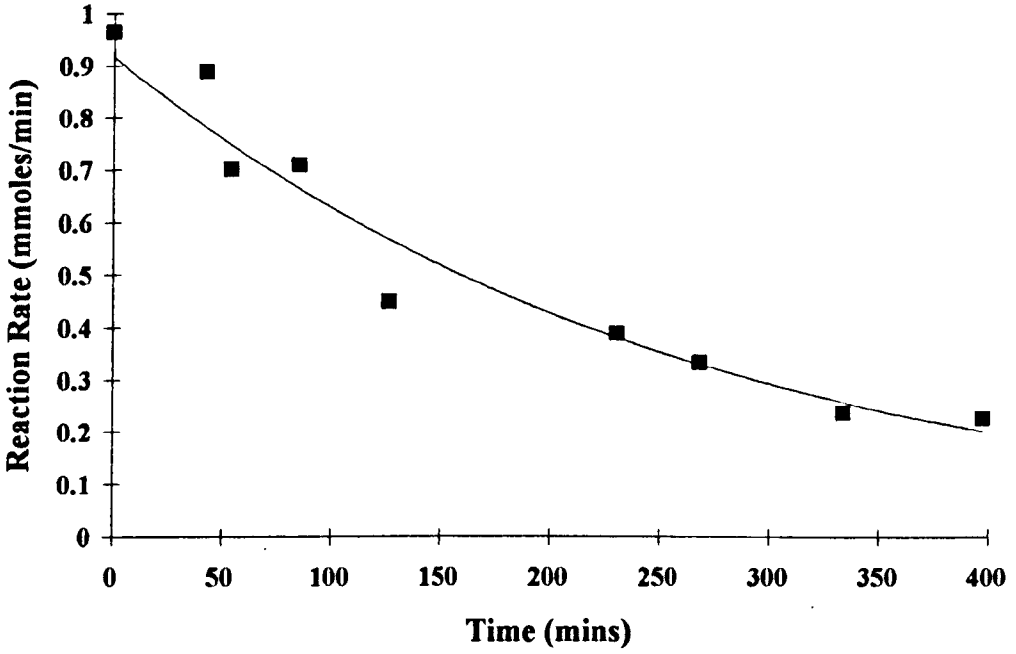
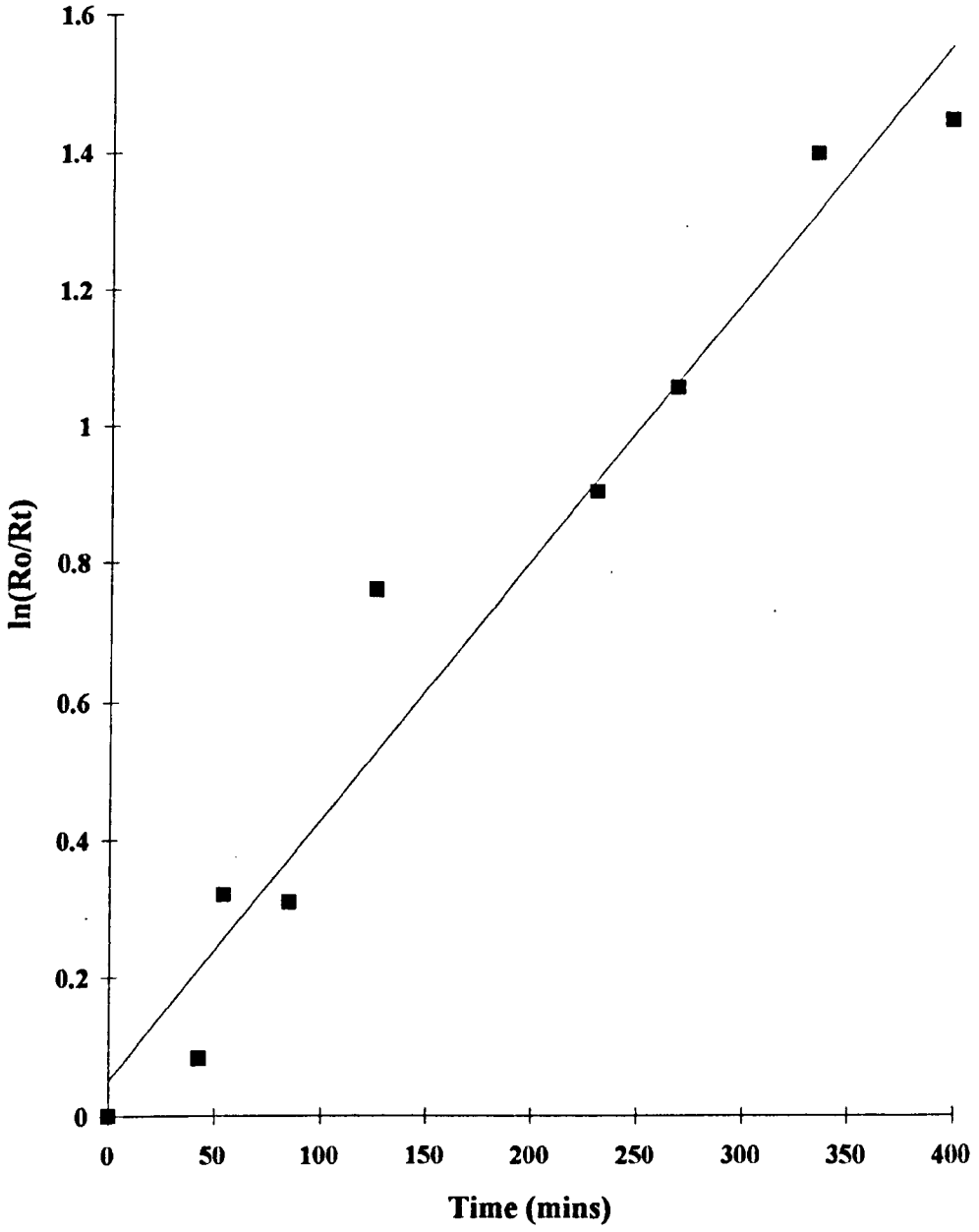


Figure 8.5: First Order Decay Plot of the Rate Data Shown in Figure 8.4b for Determination of Amidase Activity Decay Constant



low cell loading in the gel was used to minimise this effect. The first order decay plot, shown in Figure 8.5, describes the data well, and it is therefore likely that little diffusional resistance is occurring and the true biocatalyst decay constant can be derived. The time taken for the rate of reaction to become a half of the initial rate is known as the half-life. That is, the time when:

$$R_t = \frac{1}{2} R_o. \qquad (5)$$

Combining equations (4) and (5):

$$t_{1/2} = \frac{\ln 2}{k_d} \qquad (6)$$

Once k_d has been determined the half-life can be calculated using equation (6). Since the rate of reaction is directly proportional to the amidase activity of the immobilised cell catalyst, the half-life determined using equation (6) represents the half-life of the catalyst amidase activity.

Catalyst half-lives determined from runs at three different temperatures are shown in Table 8.3. It can be seen that as the temperature is reduced the amidase stability of the biocatalyst increases.

Table 8.3: Amidase Activity Half-Lives for *C. nitrilophilus* Cells Entrapped in Cross-Linked Polyacrylamide Gel Cuboids (L_b = 1.4 g dry cells/g wet gel) Operating in a Manually Controlled CSTR at 250 mM NH4ACR and 250 mM ACM

Temperature (°C)	Decay Constant (hrs^{-1})	Half-life (hrs)
30	0.23	3
25	0.15	4.6
15	0.06	12.4

8.5 Computer Controlled Continuous Stirred Tank Reactor (CSTR) Studies With Immobilised Cells of *C. nitrilophilus*

In order to determine the amidase activity half-life of immobilised *C. nitrilophilus* at 15 °C, when operating in the CSTR discussed above, it was necessary to operate the reactor for 24 hours. Clearly, operation of the reactor for periods of this length is impractical when the substrate and product concentrations are maintained at a constant level by manual adjustment of the feedrate. Some form of automatic control system is required to maintain the substrate and product concentrations when reactor studies are carried out for long periods of time. From an industrial perspective, the automation of a process leads to considerable reductions in operating costs (Katchalski-Katzir, 1993). Being able to automate the bioreactor would be an important step in showing its commercial viability. For these reasons, therefore, further studies into the amidase activity of *C. nitrilophilus* were performed in an automatically controlled CSTR, the design of which is discussed below.

8.5.1 Design of an Automatically Controlled CSTR

The equipment set-up used for the CSTR studies is shown in Figure 5.4 and the reactor vessel is shown in Figure 5.5. The substrate feed pump is controlled through the programming of the microcomputer. Methods of control were discussed in the Introduction and clearly several options are available through which to control the pump. Firstly, a choice needs to be made between a feedback and feedforward control system. An effective feedforward system requires a process model describing the system being controlled. In the case under question, a knowledge of the amidase activity and stability is needed. Clearly, such knowledge is not available and so a feedback system must be used.

The control system must be simple to use and provide reliable operation of the reactor. Initial control programmes used a proportional action, as discussed in Chapter 2.

However, determination of the proportional constant, K_p, proved difficult leading to large oscillations in the ammonium acrylate concentration. The control programme actually used is based on a derivative feedback controller and this is described below:

It was shown above that at steady state the flowrate in a CSTR can be related to the reaction rate using the following equation:

$$F(S_o - S) = R \qquad (2)$$

The value S_o - S is equal to the ammonium acrylate concentration, P, and so Equation (2) can be written:

$$F \times P = R \qquad (7)$$

It is desired to keep the ammonium acrylate concentration in the reactor constant with time, at the value P_{set}. If the flowrate is set equal to:

$$F = R/P_{set} \qquad (8)$$

then at steady state the product concentration will equal the desired value. Because the amidase activity, and therefore rate of reaction, is continually decaying, the ammonium acrylate concentration never actually reaches steady state. However, over a short period of time, a pseudo-steady state exists. For this period, the reaction rate can be determined from the rate of conductivity change in the vessel using a modified form of equation (1) shown in Section 8.4:

$$R = V \times dP/dt - FP \qquad (9)$$

The rate determined is then used in equation (8) to determine the new substrate feed rate. The equations above were incorporated into a computer programme written in

BBC Basic in order to control the system shown in Figure 5.4. A simplified flowsheet describing the programme is given in Appendix 1.

8.5.2 Operation of an Automatically Controlled CSTR using *C. nitrilophilus* Cells Entrapped in Cross-Linked Polyacrylamide Gel Cuboids

Table 8.4 summarises the results of studies into the amidase activity stability of *C. nitrilophilus* cells entrapped in cross-linked polyacrylamide gel in the automatically controlled CSTR. Control of the substrate feed using the automatic control system described above typically caused the ammonium acrylate concentration in the CSTR to remain within 5 % of the set-point which was deemed satisfactory. Samples from the reactor analysed by colorimetric analysis gave a value within ±1% of the concentration readings determined by the computer. Some problems were encountered with the control system and these are detailed more fully in Section 8.5.2.7.

Table 8.4: Amidase Activity Half-lives for *C. nitrilophilus* Immobilised in Cross-Linked Polyacrylamide Gel Cuboids Operating in a Computer Controlled CSTR

	Temp. (°C)	Lb (%)	[ACM] (mM)	[NH_4ACR] (mM)	Initial Amidase Activity (U/g dry cells)	$t_{1/2}$ (hrs)
1	30	11.1	72	428	83	3.8
2	30	7.8	157	343	126	4.5
3	30	7.7	200	500	278	2.2
4	30	10	257	243	381	1.9
5	30	7.8	368	132	206	1.7
6	30	9	384	116	379	2.5
7	30	7.4	400	600	320	1.6
8	30	5.7	500	200	278	1.6
9	15	8.2	195	1205	48	5.5
10	15	9.9	200	500	114	11.6
11	15	3	203	497	168	9.3
12	4	7.9	244	256	73	36
13	4	13.4	200	1200	7	23.8

8.5.2.1 First Order Decay Model

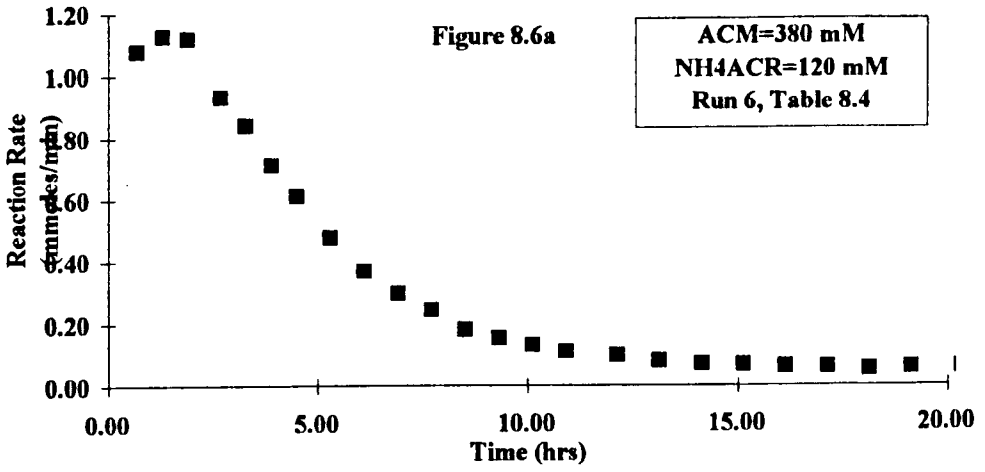
The results of stability tests carried out using the manually controlled CSTR showed that a first order decay model fitted the data well. However, further experiments with the automatically controlled system have shown that although most of the data obtained can be described by the model, it does not account for the whole process.

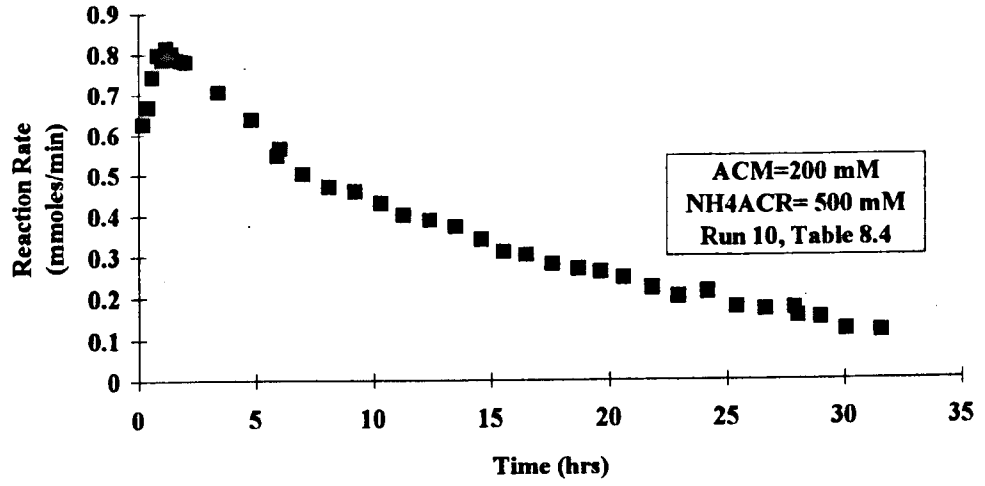
Figures 8.6a, b and c, show typical reaction rate/time profiles obtained from reactor experiments at three different temperatures. It can be seen that the maximum rate of reaction is not observed immediately after start-up of the reactor, a phenomena which becomes more apparent at lower operating temperatures.

Throughout the CSTR experiments a buffer was not used. The initial pH of the reaction solution was typically around 6, however, as the reaction continued this rose to, and remained at, 7.2- the normal pH of ammonium acrylate solution. On-start-up of the reactor, ammonium acrylate was added to simulate the required operating conditions. This ammonium acrylate was probably not fully neutralised leading to the low pH at the start of the reaction. Figure 8.2a shows the pH/amidase activity profile of cross-linked polyacrylamide entrapped *C. nitrilophilus*. It can be seen that 70 % of the maximum amidase activity is present at pH 6. The increase in amidase activity shown in Figures 8.6a, b and c may be explained by the pH rising during the initial reaction period.

Figure 8.7 shows an example of another phenomenon observed with some of the CSTR experiments. The first order decay plot shown, does give the expected straight line for the main portion of data analysed, but then gradually becomes curved. This indicates that the rate of decay in activity has decreased with time. There are several possible explanations for this trend:

Figure 8.6: Rate Profiles for a Computer Controlled CSTR Operating at 30 °C (a), 15 °C (b) and 4 °C (c) With *C. nitrilophilus* in Cross-Linked Polyacrylamide Gel





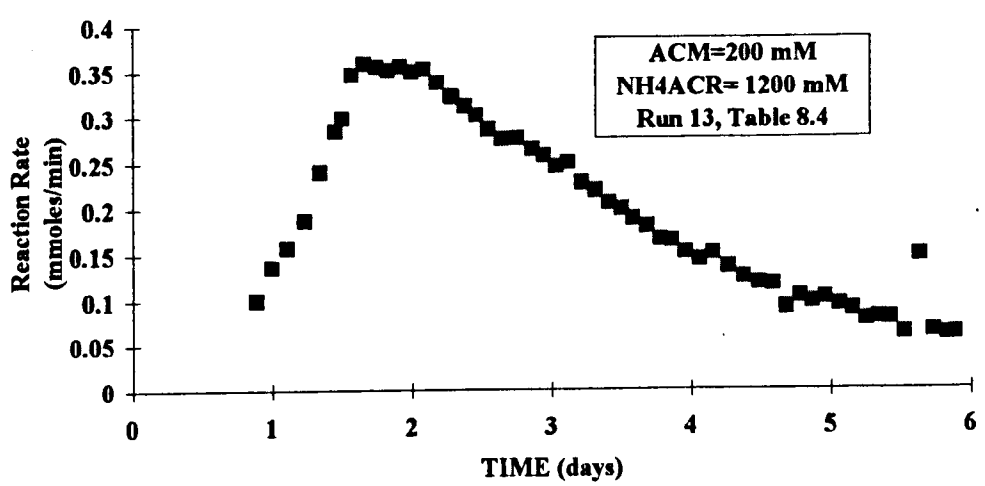
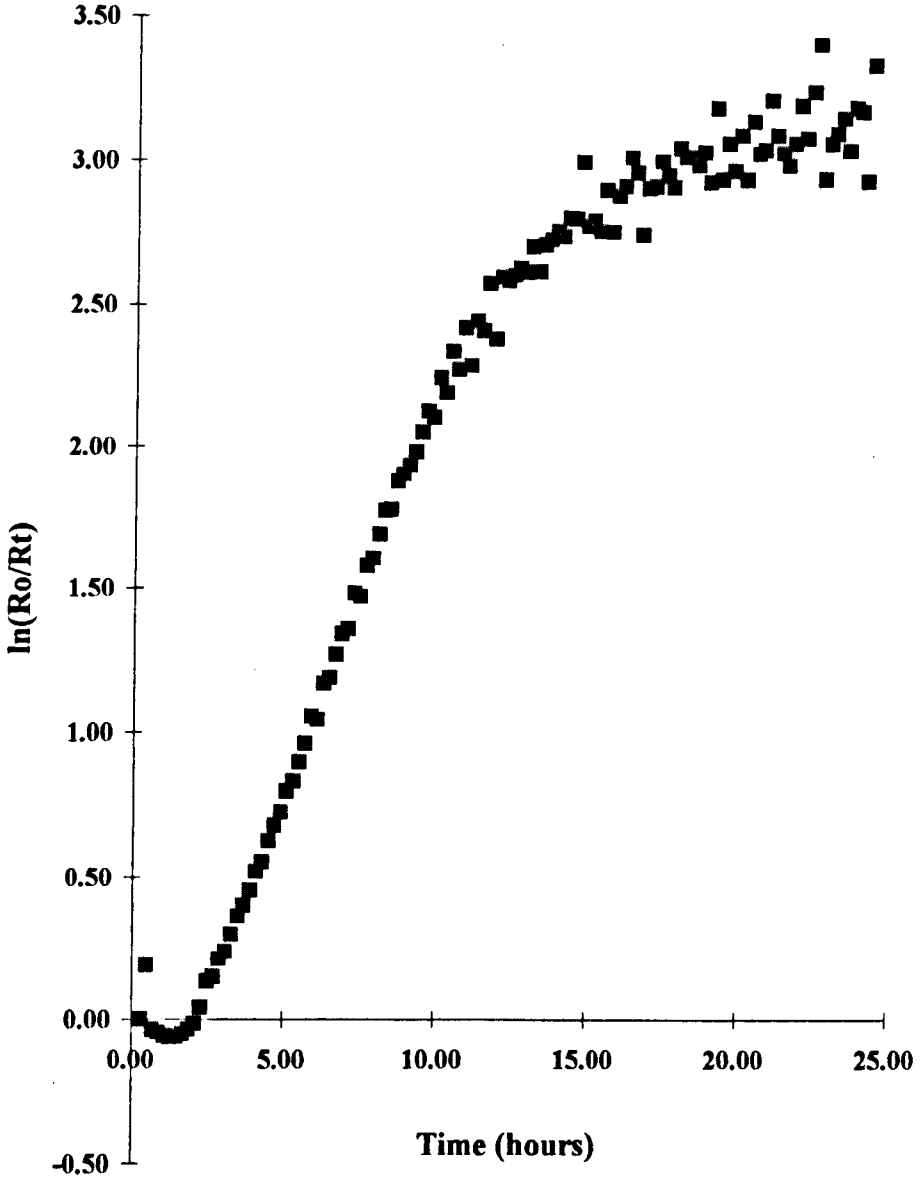


Figure 8.7: First Order Plot of the Rate Profile shown in Figure 8.6a



1) The bacteria immobilised to form the biocatalyst, *C. nitrilophilus*, like *Brevibacterium* sp. R312 may have two or more different amidase enzymes, (Mayaux *et al.*, 1990). One of these may hydrolyse acrylamide at a much higher rate than the other, but also decay at a faster rate. Therefore, it would only be when the activity of the more active enzyme has fallen off substantially, that the contribution of the second enzyme would become apparent.

2) Another possible explanation, is that the enzyme is most active when it is in the form of a complex. The complex, though more active, may break down readily into its less active, but more stable, subunits, (Lencki *et al.*, 1992).

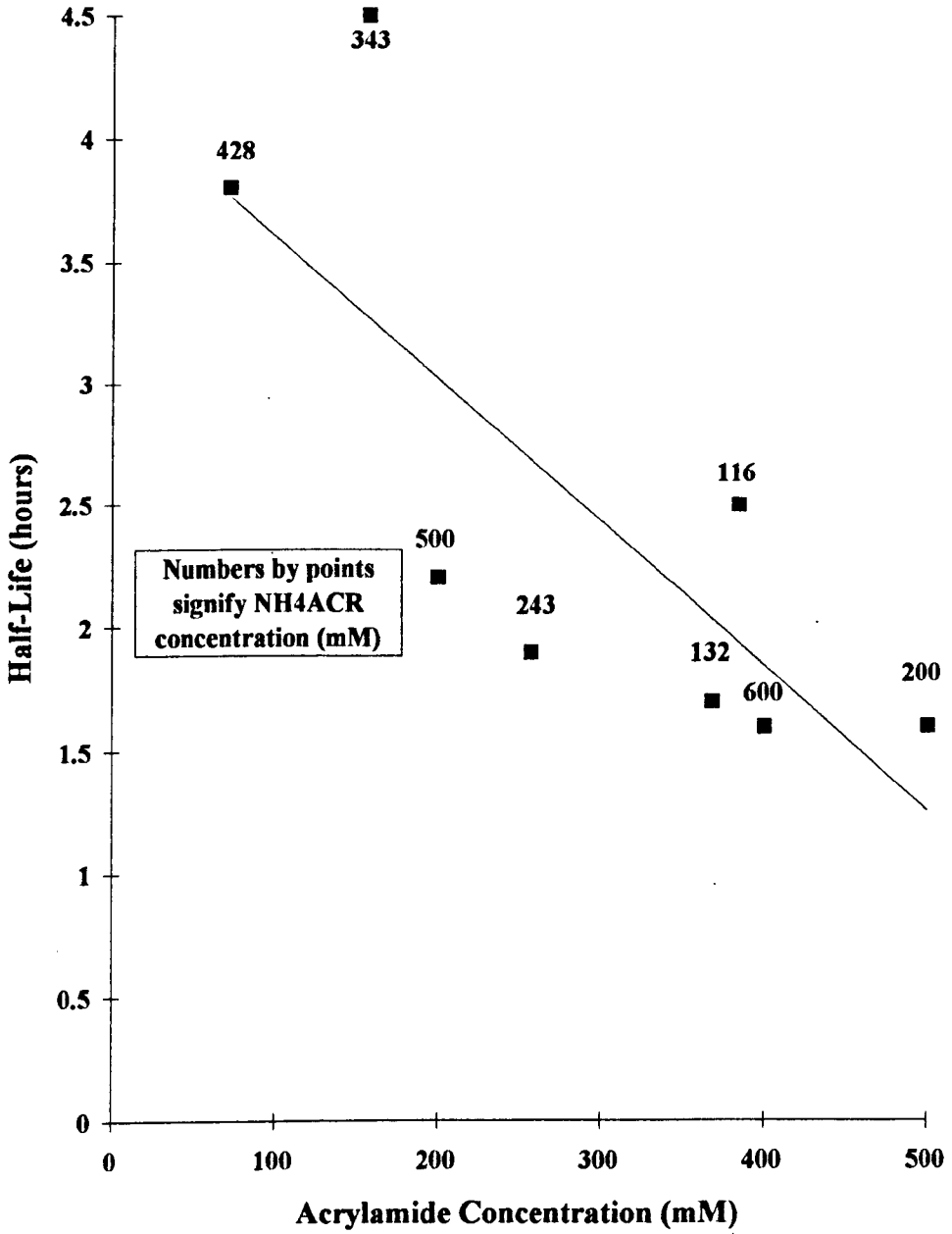
Though all the data from the CSTR experiments could not be described completely by the first order decay model, it was still used to calculate a value of the half-life, as it gave a good indication of the stability of the immobilised cell amidase activity under various conditions and in particular conditions likely to be encountered in a production situation.

8.5.2.2 Effect of Acrylamide and Ammonium Acrylate on the Amidase Activity Stability

Figure 8.8 is a plot of reactor acrylamide concentration against the half-life of the amidase activity determined when the reactor was operating at 30 °C. It is clear that the half-life is reduced by increasing the acrylamide concentration. Figure 8.8 also shows the ammonium acrylate concentrations present in the reactor for each experiment. It is apparent that the acrylamide concentration has a greater effect on the amidase stability than the ammonium acrylate concentration.

The effect of ammonium acrylate is easier to observe at high concentrations. Runs 9 and 10 in Table 8.4 are almost identical, except for the operating ammonium acrylate

Figure 8.8: Effect of Acrylamide and Ammonium Acrylate Concentration on Amidase Stability of *C. nitrilophilus* Immobilised in Cross-Linked Polyacrylamide Gel Cuboids when Operating at 30 °C in a CSTR



concentration. The half-life was measured to be 5.5 hours when the reactor was operated at 1200 mM ammonium acrylate and 11.6 hours when operated at 500 mM.

It was suggested that the loss in amidase activity is due to alkylation of the amidase sulphydryl groups by the vinyl groups of the acrylamide and/or ammonium acrylate. This idea can be extended to show that acrylamide is more likely to denature the amidase enzyme than ammonium acrylate. Figure 8.9a shows a possible reaction scheme for the modification of the thiol group, on the enzyme, by acrylamide. The vinyl group of acrylamide attacks the nucleophilic group, -SH, resulting in the structure shown. This type of reaction is known as Michael Addition (Sykes, 1986). Attack of the thiol group by the acrylate vinyl group would require formation of the double anion intermediate, shown in Figure 8.9b. Such an ion would be very unstable due to the double charge and therefore this reaction is much less likely to occur.

8.5.2.3 Effect of Temperature on Half-Life

Figure 8.10 is an Arrhenius plot of the half-lives for *C. nitrilophilus* amidase activity when the acrylamide concentration in the reactor was maintained between 200 and 250 mM. Reactor studies with a manually controlled CSTR are reported in Section 8.4 and these results are also shown. It is clear that the half-life can be greatly increased by lowering the operating temperature.

8.5.2.4 Effect of Cell Loading

The effects of cell loading can be seen by studying the results of Runs 10 and 11 in Table 8.4. The half-life is greater in the gel with the higher cell concentration, while the lower cell concentration produces a higher specific activity. The effects of diffusion were discussed in the Introduction. It is reasonable to assume that the difference in cell concentration of Runs 10 and 11 would produce a change in the substrate profile as demonstrated in Figure 2.5. It was shown above that high substrate concentrations

Figure 8.9a: Possible Mechanism of Reaction between the Thiol group on the Amidase Active Site and Acrylamide

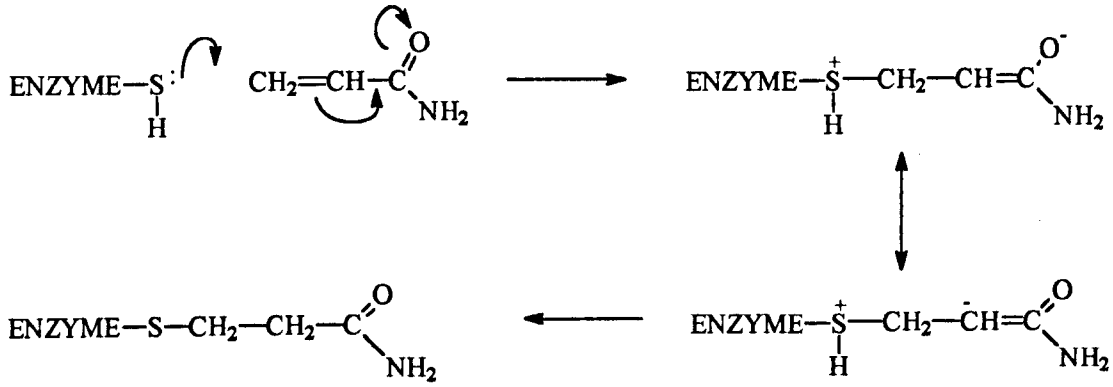


Figure 8.9b: Possible Mechanism of Reaction between the Thiol group on the Amidase Active Site and Ammonium Acrylate

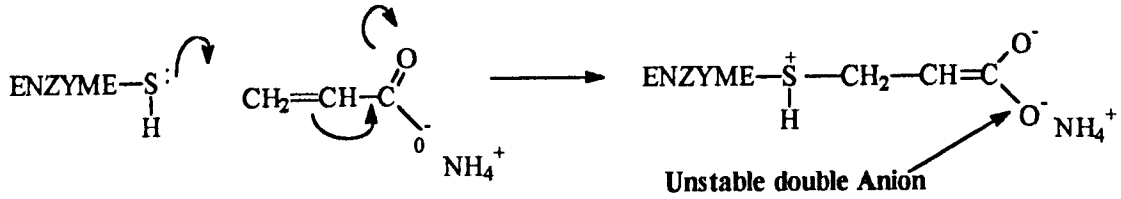
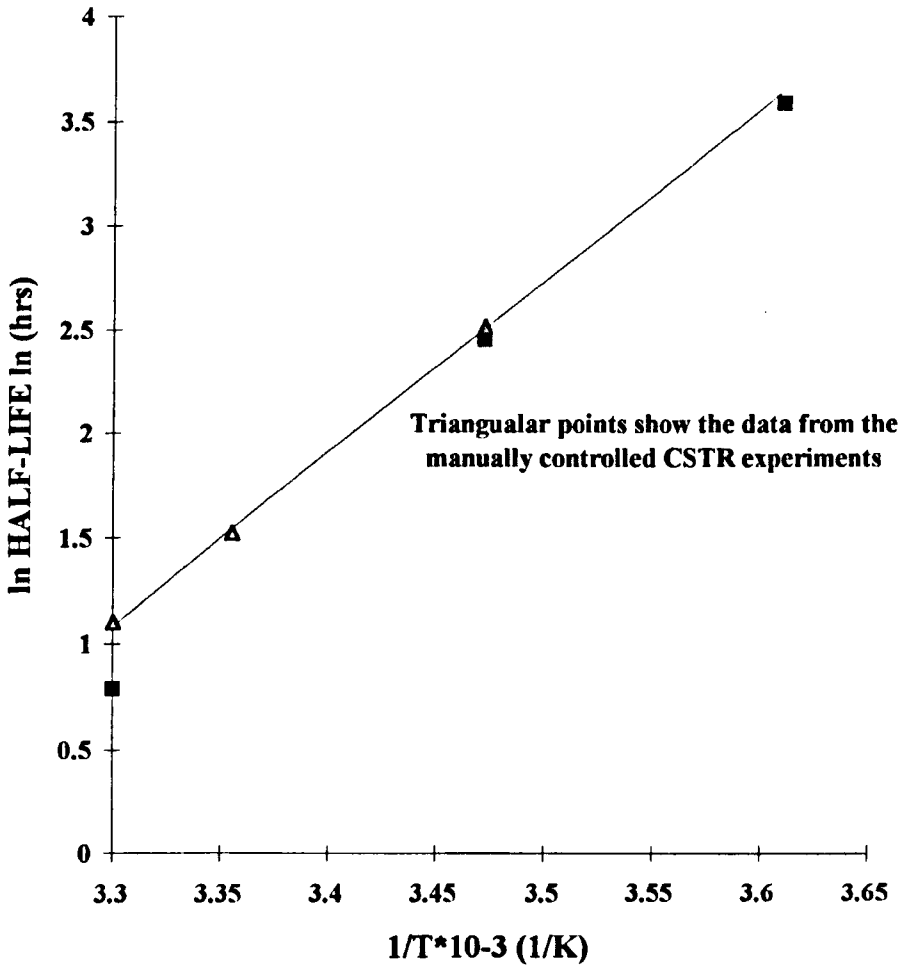


Figure 8.10: Arrhenius Plot of Amidase Activity Half-Lives of *C. nitrilophilus* Immobilised in Cross-Linked Polyacrylamide Gel Cuboids Operating in a Computer Controlled CSTR
ACM= 200-250 mM, NH4ACR= 250-500 mM



deactivate the amidase activity of the cell. The lower cell loading may have produced a higher average substrate concentration in the biocatalyst particle, and subsequent decrease in stability. Ultimately, the bead with the higher cell loading produced the greatest amount of ammonium acrylate per unit mass of cell material. Also, although the specific reaction rate per gram of cell material was lower with a high cell loading, the reaction rate per unit volume of reactor was higher, which may be more desirable from an economic view point.

8.5.2.5 Effect of Ammonium Acrylate Concentration on Amidase Activity

The effect of ammonium acrylate concentration on amidase activity can be seen from studying Runs 9 and 10 from Table 8.4. At 1200 mM ammonium acrylate concentration the initial amidase activity is less than half that observed when the reactor is operated at 500 mM. The cells used in each experiment had similar initial amidase activities. It is likely, therefore, that the difference in amidase activities observed for these two runs is due to inhibition of the amidase enzyme by the product.

8.5.2.6 Comparison of Free and Immobilised *C. nitrilophilus* Amidase Activity

Table 8.5 shows the results for the studies of free cells of *C. nitrilophilus* in the computer controlled CSTR.

The initial free cell experiment (Run 1, Table 8.5) used operating conditions similar to those used for Run 1 using immobilised cells (See Table 8.4.). A cross-flow filtration unit was used to prevent cell loss from the reactor. The amidase activity of the cells was very quickly lost, before the half-life could be measured. The catalytic properties of the cells were retained for longer when the pH was adjusted to 7 and the operating acrylamide and ammonium acrylate concentrations were both reduced to 50 mM (Run 2, Table 8.5). By doing this, a half-life of 26 minutes was measured. To ascertain

whether the use of the cross-flow filtration unit had a damaging effect on the cell amidase activity, a third experiment was performed, identical to the previous one, except that the cross-flow filtration unit was not used (Run3, Table 8.5) It can be seen that again the stability of the cells improved, with a half-life of 1.8 hours being observed. It is possible that the action of the filtration unit damaged the cells in some way. However, a sample of the cells used in Run 2, did not show any sign of lysis, when examined under a microscope at the end of the experiment. It is clear that immobilisation of the cells enables them to operate in much harsher conditions i.e. higher substrate concentrations and a lower pH.

Table 8.5: Free *C. nitrilophilus* Performance in a CSTR

	[ACM] (mM)	[NH4ACR] (mM)	TEMP. (°C)	pH	$t_{1/2}$ (hrs)	Comment
1	100	400	30	5.8	Very small	With Filtration
2	50	50	30	7	0.4	With Filtration
3	50	50	30	7	1.8	With out Filtration

8.5.2.7 Operational Problems With the Automatically Controlled CSTR

The main problem encountered, when operating the computer controlled CSTR, was the measurement of conductivity. There are two reasons for this. Firstly, the analogue signal sent by the conductivity meter is converted to a digital signal by an A/D interface before being sent to the computer. However, the accuracy of the interface is limited, making it more difficult for the computer to read small conductance changes at high ammonium acrylate concentrations.

The second problem encountered when measuring the conductance was caused by particles of biocatalyst entering the gap between the conductance probe electrodes and interfering with the readings. Covering the probe with a fine mesh failed to alleviate this problem, as the very small particles entered and gradually clogged the probe.

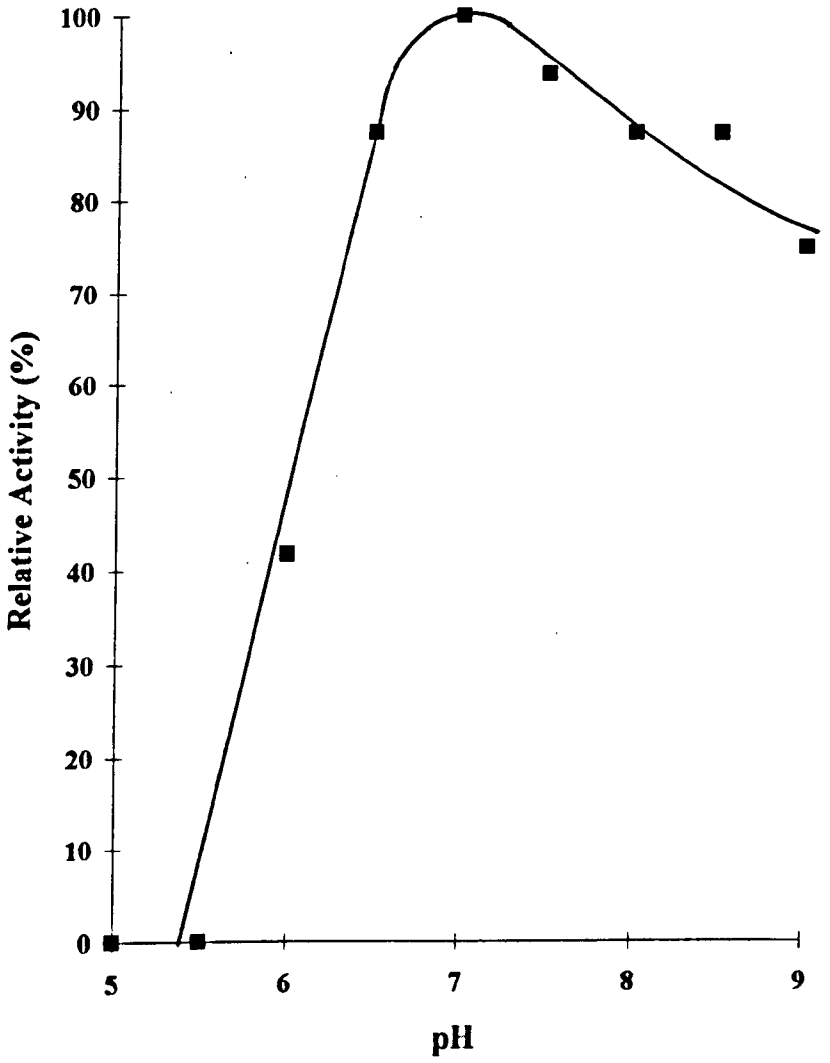
In order to improve the reliability of the automatic CSTR operation, several changes were made. Firstly, referring to Figure 5.4, the A/D converter was replaced, along with the BBC microcomputer, by a 486 personal computer containing an improved A/D converter capable of detecting smaller conductivity changes. Secondly, the reactor control programme described above was replaced by one utilising an *on-off* type control action. The control programme flow diagram is shown in Appendix 2. Using this method of control, acrylamide solution was no longer continuously pumped to the reactor. Instead, a set amount of solution was added to the reactor, once the ammonium acrylate concentration had risen above the set point, thereby causing the ammonium acrylate concentration to oscillate around the set point. This method of control was found to be much more reliable than the method previously used as it was less sensitive to erroneous conductance readings.

Two ways of preventing the immobilised cell particles interfering with the conductivity probe were tried. Firstly, the agitation rate was adjusted to produce full fluidisation of the beads without taking them into the top layer of fluid where the head of the conductivity probe was situated. However, this proved to be unreliable. Greater success was achieved by incorporating a command into the computer control programme to turn off the agitator for a short period prior to taking readings. This caused the particles to sink below the probe and greatly reduce the errors in the readings.

8.5.3 CSTR Operation Using *Rhodococcus. rhodochrous* sp.632 Entrapped in Cross-Linked Polyacrylamide Beads

Rhodococcus rhodochrous sp.632 is an amidase active bacterium isolated during the LINK Bio-Ammonium Acrylate Project. It was decided to compare its amidase stability against that of *C. nitrilophilus* using the computer controlled CSTR. Figure 8.11 shows the pH/amidase activity profile of *R. rhodochrous* sp.632 immobilised in cross-linked polyacrylamide beads. Compared to the pH profile of *C. nitrilophilus*,

Figure 8.11: Effect of pH on the Amidase Activity of *R. rhodochrous* sp.632 Immobilised in Cross-Linked Polyacrylamide Beads



R. rhodochrous sp.632 amidase activity drops much more sharply at a lower pH. For this reason the pH in the reactor at the start of the run was adjusted to 7 using sodium hydroxide solution. This had a negligible effect on the conductivity of the reaction solution and was only required at the start of the reaction.

Table 8.6: Comparison of Amidase Activities and Stabilities of various Immobilised Amidase Active Cells Operating in a Computer Controlled CSTR

Run	Organism	Temp. (^{0}C)	Lb (%)	[ACM] (mM)	[NH4ACR] (mM)	Initial Amidase Activity (U/g dry cells)	$t_{1/2}$ (hrs)
1	*R.rhodo-chrous sp.632*	40	5.6	200	500	200	1.2
2	*C.nitrilo-philus*	30	7.7	200	500	278	2.2
3	*R.rhodo-chrous sp.632*	30	2.1	200	500	-	58 (2.4 days)
4	*C.nitrilo-philus*	15	8.2	195	1205	48	5.5
5	*R.rhodo-chrous sp.632*	15	4.7	400	1200	40	588 (24.5 days)
6	*R.rhodo-chrous sp.632*	5	4.3	200	1200	44	1056 (44 days)
7	*Rhodococcus* sp.1068	5	2.0	200	1200	700	11.3

Table 8.6 compares amidase stability and activity data of *R. rhodochrous* sp.632 with *C. nitrilophilus*, and also with a second isolate *Rhodococcus* sp.1068. At 30°C *R. rhodochrous* sp. 632 is 26 times more stable than *C. nitrilophilus*, and at 15 °C, it 107 times more stable. The second cell isolate, *Rhodococcus* sp.1068, is also less stable than *R. rhodochrous* sp.632.

8.5.3.1 Specific Ammonium Acrylate Productivity of Amidase Active Isolates

Though the amidase activity of *R. rhodochrous* sp.632 is more stable than that of *Rhodococcus* sp.1068 it can be seen from Table 8.6 that *Rhodococcus* sp.1068 has the highest specific amidase activity. In order to decide which isolate is likely to be the

most efficient for ammonium acrylate production it is useful to consider the combined effects of both stability and activity on ammonium acrylate production.

Specific Production Rate, P, may be defined as:

$$P = \frac{A \times 89 \times 60}{1000000} \text{ (g NH4ACR/g dry cells. hr)} \qquad (10)$$

where A = Amidase Activity (U/g dry cells)

Assuming a first order decay rate, the amidase activity at any time, A_t, can be written:

$$A_t = A_0 \times e^{-kd\,t} \qquad (11)$$

where A_0 = amidase activity at zero time
k_d is the first order decay constant (hr^{-1})

Because the productivity is directly proportional to the amidase activity, productivity at time t can be written:

$$P_t = P_0 \times e^{-kd\,t} \qquad (12)$$

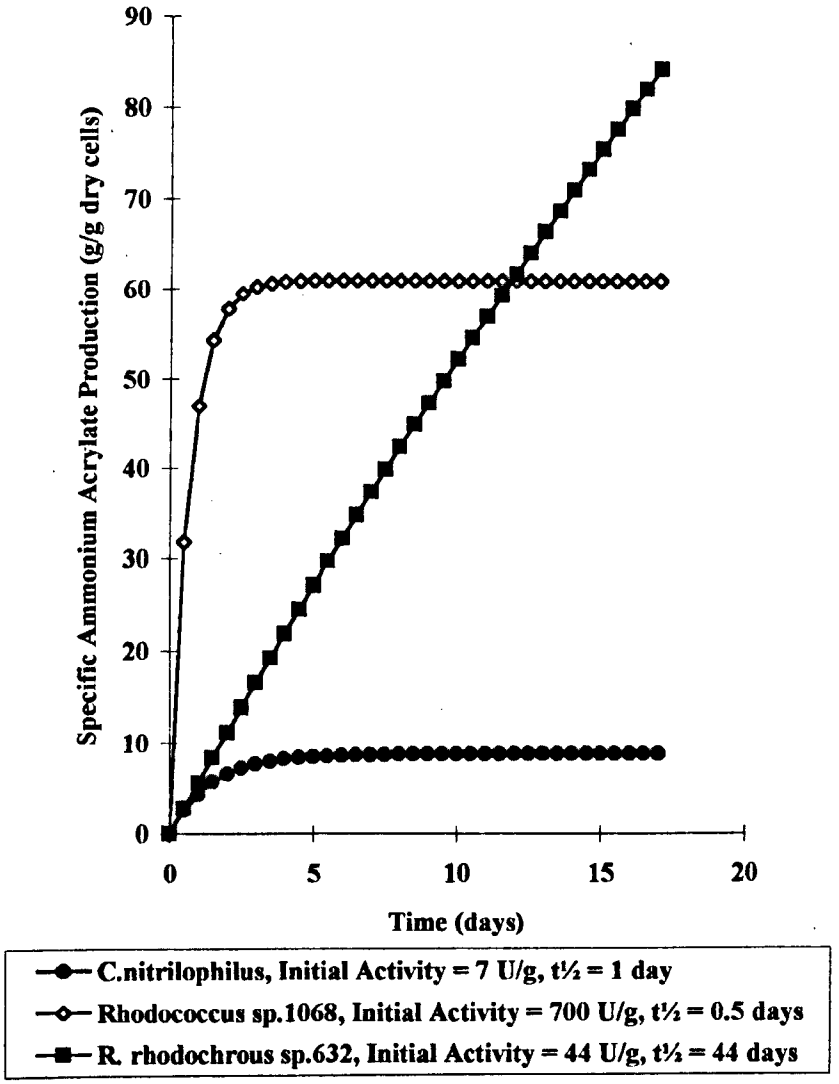
Integration of equation (12) with respect to time gives an expression describing the accumulation of product with time per mass of dry cells:

$$\text{Total product} = \frac{P0}{kd} \times (1 - e^{-kd\,t}) \text{ (g NH4ACR/g dry cells)} \qquad (13)$$

Figure 8.12 shows plots of equation (13) using half-life and activity data from Table 8.6 and 8.4. It can be seen that though *R. rhodochrous* sp.632 has a lower initial amidase activity than *Rhodococcus* sp.1068, its greater stability enables it to eventually produce more ammonium acrylate per mass of dry cells.

The culture of cells was discussed in Section 1.3.1. From the arguments presented there it is reasonable to assume that the specific amidase activity of *R. rhodochrous* sp.632 may be improved, using methods such as Chemostat culture or

Figure 8.12: Theoretical Specific Ammonium Acrylate Production of Three Immobilised Cell Types in a Computer Controlled CSTR Calculated using Equation (13) Reactor Operating at 5 °C, 200 mM ACM and 1200 mM NH4ACR



chemically induced mutation, to produce strains that over produce the amidase enzyme. In order to alter the stability or specificity of an enzyme, however, a change in the actual structure of the enzyme is required (Clark, 1986). For these reasons, therefore, it was decided to perform all further reactor studies using *R. rhodochrous* sp.632 as the biocatalyst.

8.5.4 Inhibition of the Amidase Activity of *R. rhodochrous* sp.632

Table 8.7 shows the results of repeated assays with *R. rhodochrous* sp.632 immobilised in cross-linked polyacrylamide beads. Assays 1 and 2 show that increasing the acrylamide concentration from 100 mM to 500 mM increases the amidase activity observed. This increase in rate shows that acrylamide is not inhibiting the initial amidase activity at these concentrations. Comparison of assays number 1 and 3 shows that ammonium acrylate does act as an inhibitor. However, by increasing the acrylamide concentration (assay 4), it is possible to overcome the inhibition, showing

Table 8.7: Effect of acrylamide and ammonium acrylate on *R. rhodochrous* sp.632 amidase activity.

Assay	[ACM] (mM)	[NH_4ACR] (mM)	Temp. (°C)	Note	Observed Amidase Activity (U/g dry cells)
1	100	0	30		1250
2	500	0	30		1400
3	100	300	30		1000
4	300	300	30		1100
5	300	0	30	Beads from 4 after washing to remove ACM + NH_4ACR	1400
6	500	0	5		100

that ammonium acrylate acts as a competitive inhibitor (Gacesa and Hubble, 1987). Assay 5 was performed using the beads from number 4 after they had been washed to remove any ammonium acrylate. It can be seen that the inhibitory effect is reversed by

its removal, again indicating that ammonium acrylate is a competitive inhibitor of *R. rhodochrous* sp. 632 amidase.

Assay 6 shows that amidase activity is greatly affected by temperature, the amidase activity at 5°C being only 7% of that at 30°C. From the results discussed above it can be seen that ammonium acrylate acts as a competitive product inhibitor which can thus be overcome by the addition of more substrate or by the removal of ammonium acrylate.

8.6 Ammonium Acrylate Production Studies in a Manually Controlled Fed-Batch Reactor using *R. rhodochrous* sp.632 Immobilised in Cross-Linked Polyacrylamide Beads

The effect of enzyme kinetics on the performance of reactor types was discussed in the Introduction. It was shown that CSTR's are an unsuitable choice when using biocatalysts that are inhibited by their product. In such a situation a batch or plug flow reactor is preferred. It has been shown that the amidase of *R. rhodochrous* sp.632 is competitively inhibited by ammonium acrylate. However, it has also been shown that acrylamide has a toxic effect on amidase activity. Since batch and plug flow reactors cause biocatalysts to be exposed to high substrate concentrations, this also makes them unsuitable for ammonium acrylate production when using *R. rhodochrous* sp.632. By sequential feeding of substrate to a batch reactor it is possible to avoid high substrate concentrations while only producing high product concentrations towards the end of the batch. It was decided, therefore, to operate a fed-batch reactor and compare its productivity with that of a CSTR when operating with *R. rhodochrous* sp.632 immobilised in cross-linked polyacrylamide beads and producing similar product concentrations.

Table 8.8 shows the performance of a reactor operating with *R. rhodochrous* sp.632 immobilised in cross-linked polyacrylamide beads. The reactor was operated in fed-

batch mode by repeated manual additions of 7M acrylamide until an ammonium acrylate concentration greater than 1685 mM was achieved.

Table 8.8: Comparison of a Manually Controlled Fed-Batch Reactor and a CSTR Operating with *R. rhodochrous* sp.632 Immobilised in Cross-Linked Polyacrylamide Beads

	First Batch of a Repeat Fed-Batch Reactor	CSTR
NH4ACR (mM)	1810 (Final)	1685
ACM (mM)	0 to 450	400
Working Volume in Reactor (ml)	Initial = 93.5 Final = 150	110
Operating Temperature (°C)	5	5
Mass of Beads (g)	30	30
Cell loading in Beads, L_b (g dry cells/100 g wet gel)	6	6
Amidase Activity (U/g dry cells) Before Reaction at Standard Assay Conditions (500 mM ACM, 30 °C)	2720	2720
Initial Activity (U/g dry cells) in Reactor	205	16
Final Activity (U/g dry cells)	31	-
Average Amidase Activity (U/g dry cells) over period of Reactor Operation	35	-
Initial Activity (U/g dry cells) of Second Batch	46	-

The product and substrate concentration profiles of the batch are shown in Figure 8.13a. It can be seen that the rate of product formation falls with time. (The periodic fall in ammonium acrylate concentration is due to the dilution of the reaction mixture caused by addition of acrylamide solution). The fall in rate is expected for several reasons. Firstly, the volume in the reactor is constantly increasing so that the catalyst concentration, and hence the amidase activity per unit volume of reactor, falls with time. The effect of volume can be eliminated if the specific amidase activity of the

Figure 8.13a: Batch 1 of a Manually Controlled Repeat Fed-Batch Reactor at 5°C using *R.rhodochrous* sp.632 Immobilised in Cross-Linked Polyacrylamide Beads

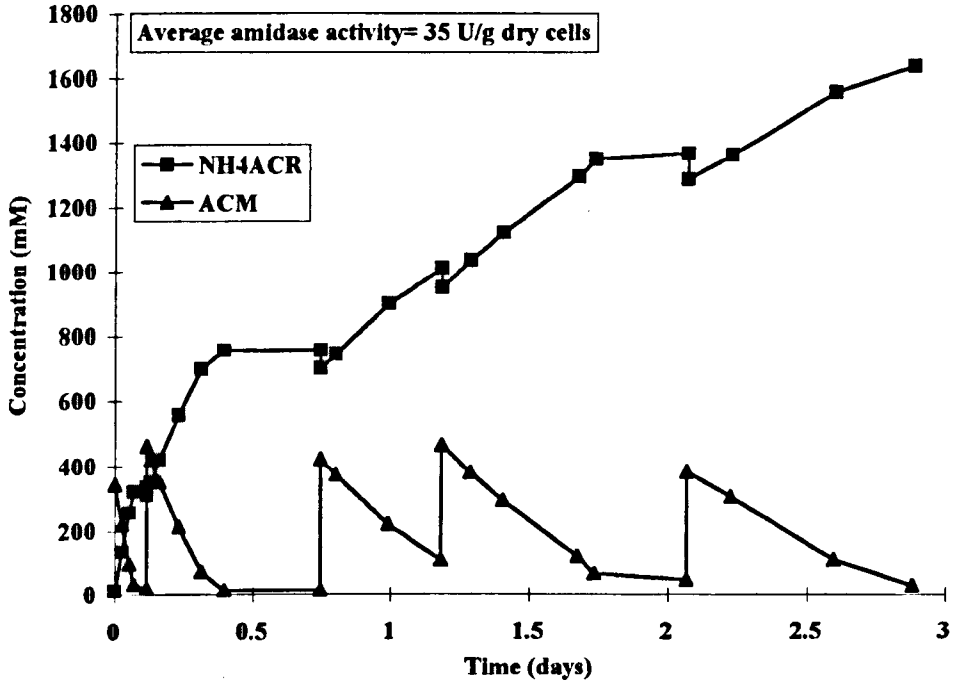
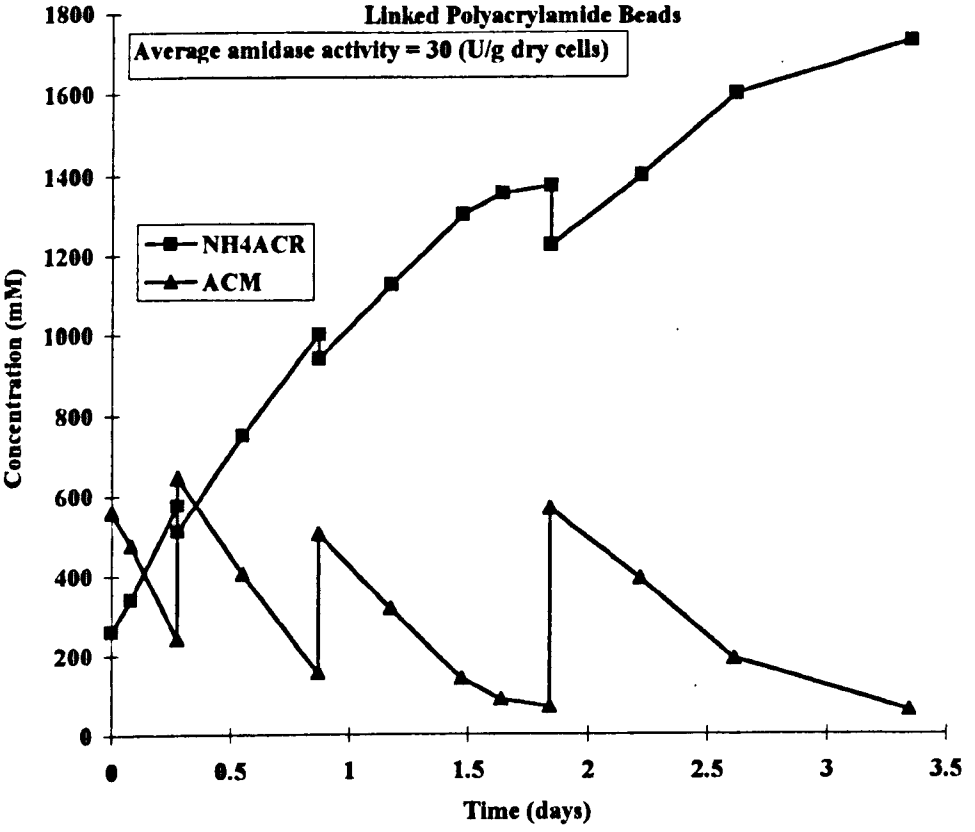


Figure 8.13b: Batch 2 of a Manually Controlled Repeat Fed-Batch Reactor at 5°C using *R.rhodochrous* sp.632 Immobilised in Cross-Linked Polyacrylamide Beads



catalyst is considered. Table 8.8 shows the amidase activity at the beginning and end of the first batch. The amidase activity fell throughout the batch due to the increasing ammonium acrylate concentration inhibiting the amidase enzyme. It is also likely that some permanent loss of biocatalyst activity occurred over the period of reactor operation.

Once the ammonium acrylate concentration in the reactor had risen above 1810 mM the liquid was removed and a second batch started by adding more water and then further additions of 7M acrylamide. Figure 8.13b shows the concentration profile of the second batch. Because ammonium acrylate was already present in the beads, the starting concentration in the reactor was around 270 mM. The presence of ammonium acrylate produced a pH of 7.2 in the reactor and no adjustment of the pH was necessary. This result is similar to that reported for the CSTR experiments discussed in Section 8.5.2.1. There being no need to buffer the pH of the reaction solution may be considered as an advantage of this particular bioconversion. The presence of a buffer in the product stream may well be an unwanted contaminant that would need to be removed from the final product (Watanabe, 1987b).

From Table 8.8 it can be seen that the amidase activity at the start of the second batch is greater than at the end of the first batch due to the lower ammonium acrylate concentration. The rate of reaction did not return to its original level, however, and this is probably due to a combination of several effects. Firstly, it is likely that there was an irreversible loss in amidase activity. Secondly, any amidase active cells that leaked from the immobilisation matrix during the run would have been lost when the beads were separated from the final reaction mixture. (Such cell loss was only observed during the first batch). Thirdly, ammonium acrylate present at the start of the second batch is likely to have caused some product inhibition which would not have occured at the start of the first batch.

The completion time of the second batch was 3.5 days compared to 2.9 days for the first batch. The beads used in the fed-batch reactor were used to complete a total of six fed-batch runs and Figure 8.14 shows that the average amidase activity for these batches decreased with each batch. The average amidase activity is calculated in the following way:

Average Amidase Activity =

$$\frac{\text{NH4ACR Produced in Reactor (g)over batch time t (mins)}}{\text{Mass Dry Cells in Reactor (g)} \times \text{t}} \quad \text{(Units/ g dry cells)}$$

Table 8.8 also shows the performance of *R. rhodochrous* sp.632 immobilised in cross-linked polyacrylamide beads operating in a CSTR at a high ammonium acrylate concentration. It can be seen that the initial specific amidase activity of the immobilised cells was only 16 U/g compared to an average activity of 35 U/g when used in the fed-batch reactor. Clearly, when producing ammonium acrylate using *R. rhodochrous* sp.632, the use of a fed-batch reactor is the preferred choice.

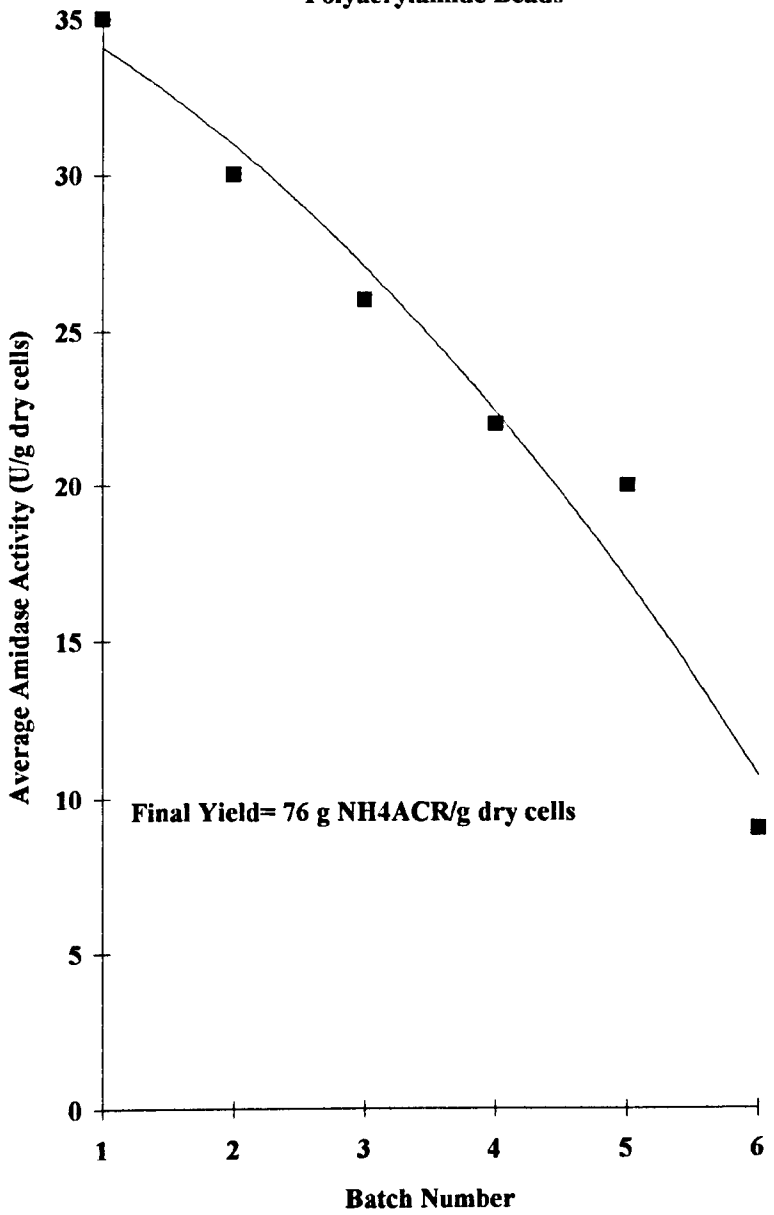
8.7 Automated Control of a Fed-Batch Reactor for Ammonium Acrylate Production

It can be seen from Figure 8.13a that the beads experienced concentrations of acrylamide as high as 450 mM followed by periods of very low concentration of acrylamide when no ammonium acrylate was produced. This was a result of the difficulty of arranging adequate manual control and therefore it was decided to automate the addition of acrylamide.

8.7.1 On-Line Measurement of Ammonium Acrylate Concentration

The automatic CSTR system used conductivity measurements to monitor the concentration of the electrolyte ammonium acrylate. The control system requires the

Figure 8.14: Fall in Average Amidase Activity of Repeat Batches Performed in a Fed-Batch Reactor Operating with *R. rhodochrous* sp.632 Immobilised in Cross-Linked Polyacrylamide Beads



determination of a calibration curve to convert the conductance readings to concentration. During a CSTR bioconversion the automatic control system only allows the substrate and product concentrations to vary over a small range and just a few calibration points are required, producing a linear calibration curve. In contrast to this situation, operation of a fed-batch reactor leads to acrylate concentrations ranging from zero up to the desired final product concentration. For this reason, a much more comprehensive calibration equation is required.

The points on Figure 8.15a represent the solutions of ammonium acrylate and acrylamide used to prepare the conductivity calibration curve for fed-batch operation. Figure 8.15b shows the corresponding conductivities of these solutions. It can be seen that at higher ammonium acrylate concentrations the conductivity/concentration plot becomes non-linear. It can also be seen that the acrylamide concentration has a significant effect upon the conductivity at high ammonium acrylate concentrations. Consider points A and B on Figure 8.15a and their corresponding conductivity points on Figure 8.15b. Though the solutions had a nearly identical ammonium acrylate concentration, the higher acrylamide concentration of point B significantly reduces the conductivity of the solution. Points C and D had a lower ammonium acrylate concentration. It can be seen that the difference in acrylamide concentration of these calibration points had a much smaller effect on the conductivity.

The data from Figures 8.15a and b was analysed using a statistical analysis computer package (Stats4Industry) in order to produce a calibration equation that would relate conductivity to acrylamide and ammonium acrylate concentration. The form of this equation is shown below:

$$\text{Conductivity} = z = Ax + By + Cxy + Dx^2 + E \qquad (14)$$

where x = ammonium acrylate concentration (mM)

y = acrylamide concentration (mM)

Figure 8.15a: Conductivity/ACM/NH4ACR Calibration data

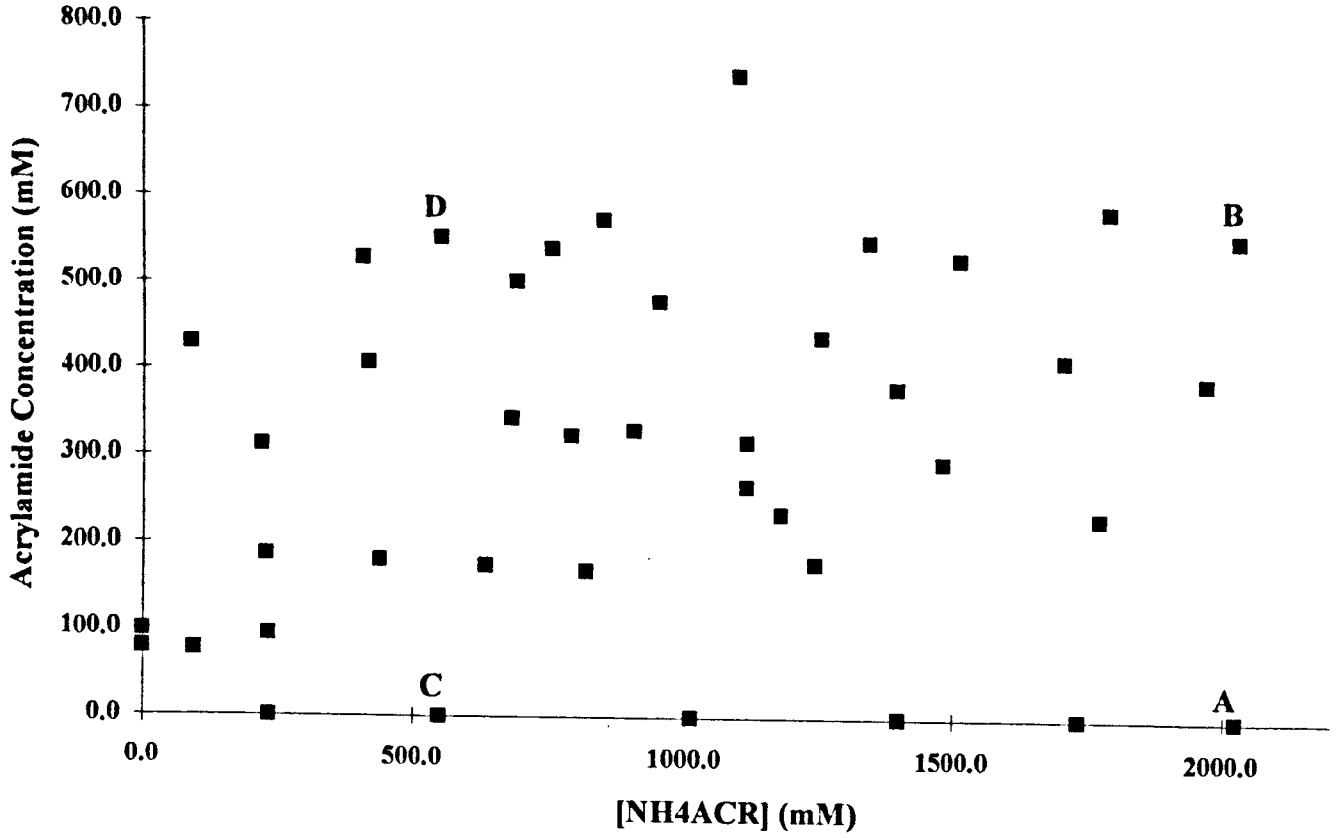
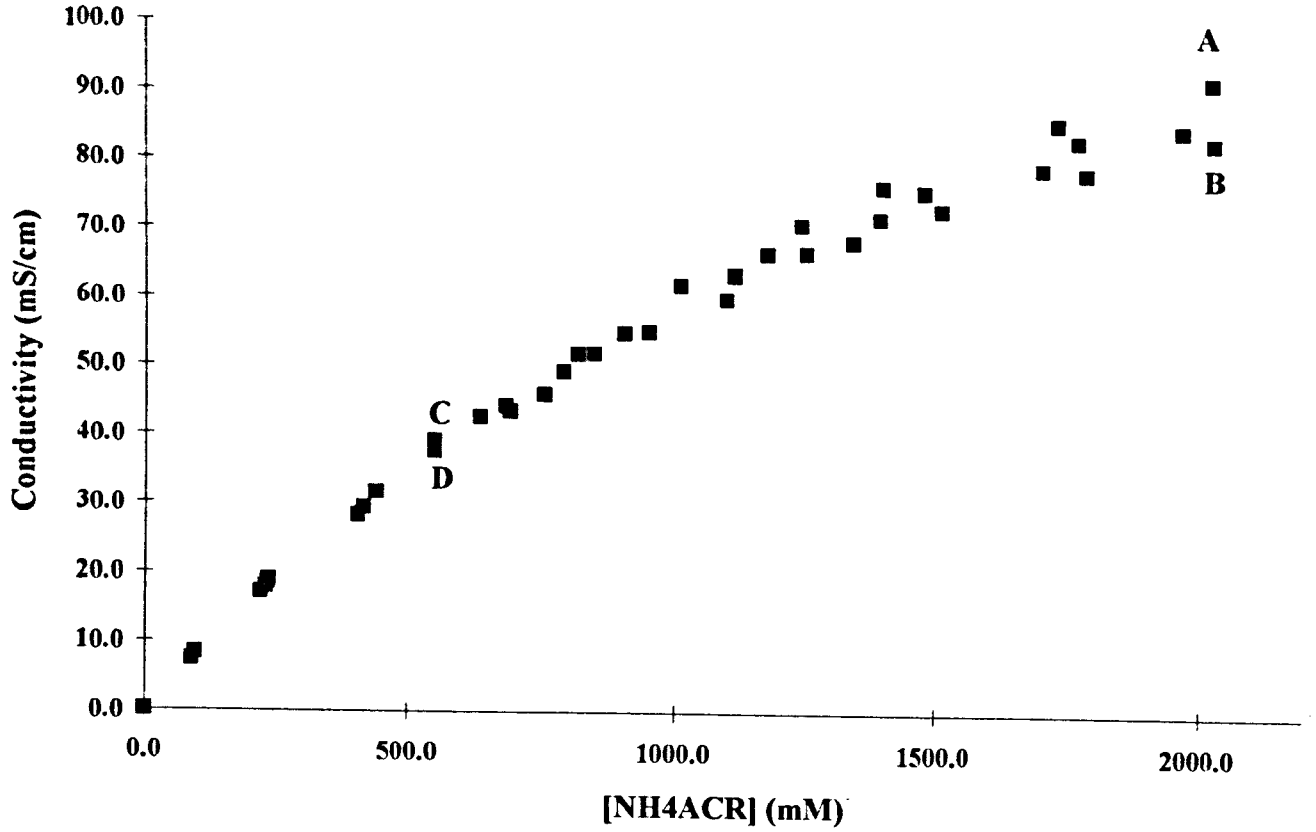


Figure 8.15b: Conductivity/ACM/NH4ACR Calibration data



A = 7.550E-2, B = 1.529E-3, C = -9.268E-6, D = -1.538E-5 and E = 1.091

A plot of the resulting calibration curve is shown in Figure 8.16. For the acrylamide and ammonium acrylate concentrations to be calculated from Equation (14), some further manipulation is required. Firstly when controlling a reactor, accurate addition of substrate will allow the total monomer concentration to be known at all times. Therefore:

If T = total monomer concentration (mM)

$$y = T - x \qquad (15)$$

Substituting equation (15) into (14) allows the term for acrylamide concentration to be removed. The resulting form can be re-arranged into a quadratic equation. In this case only the positive root is required:

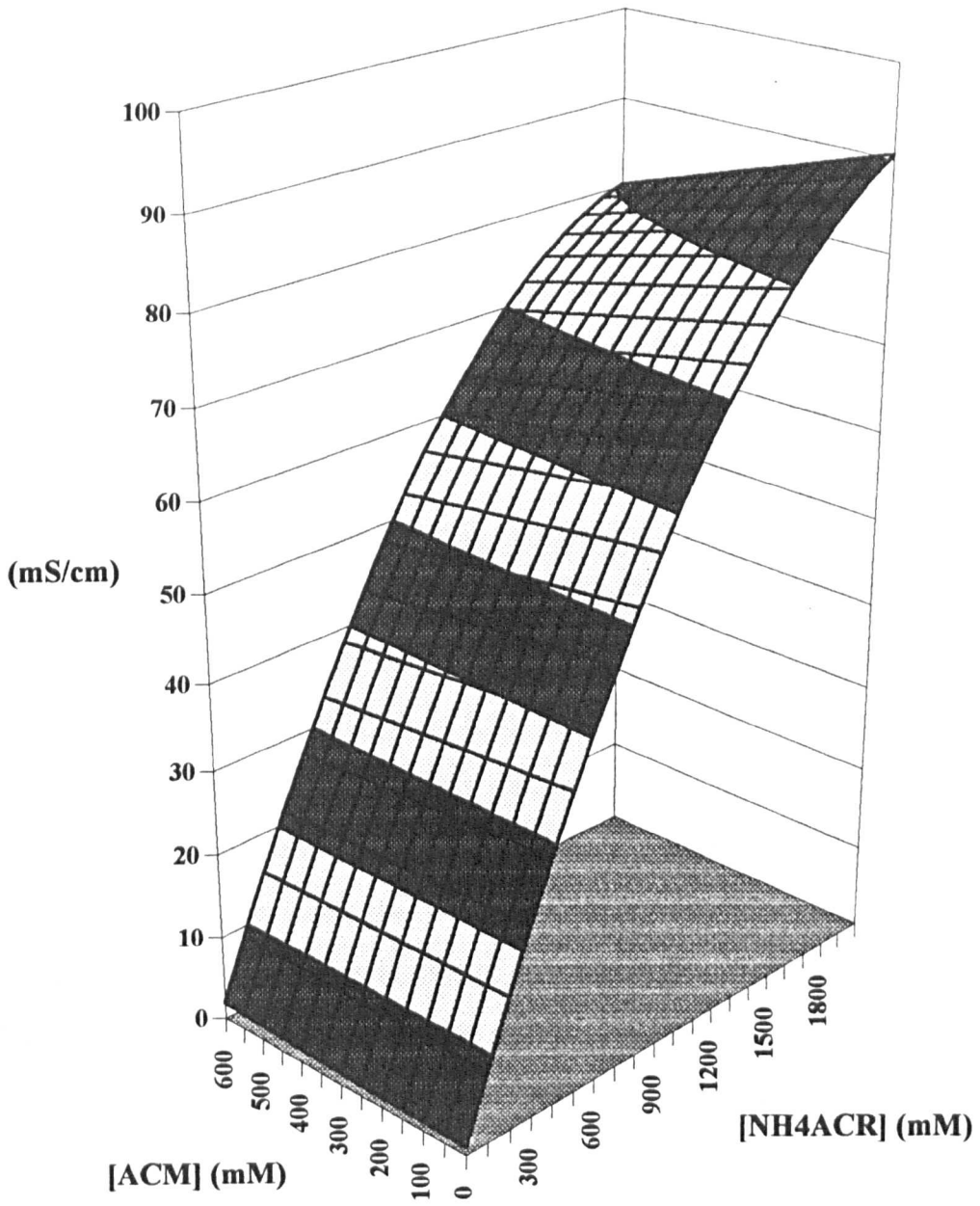
$$[\text{Ammonium Acrylate}] = \frac{-(A-B+CT)+\sqrt{(A-B+CT)^2-4(D-C)(BT+E-z)}}{2(D-C)} \qquad (16)$$

Therefore, conductance measurements and total feed data allow the ammonium acrylate and acrylamide concentrations to be determined during operation of a fed-batch reactor. Equation (16) was incorporated into the computer programme used to control the substrate feed rate to a fed-batch reactor so that the acrylamide concentration could be maintained at the desired level. The flowsheet for this programme is shown in Appendix 3.

8.7.2 Operation of a Fed-Batch Reactor using Computer Control

Figure 8.17 shows the ammonium acrylate and acrylamide concentration profiles for the 0.5 litre reactor (Figure 5.4 and 5.5) operating in fed-batch mode with computer control of the acrylamide feed rate. As with the manually controlled reactor, the rate of ammonium acrylate production falls with time due to dilution of the catalyst and

Figure 8.16: Plot of Calibration Equation Relating Conductivity to [NH4ACR] and [ACM]



product inhibition. The manually controlled fed-batch profile in Figure 8.13a, shows a large fluctuation in acrylamide concentration. At times (e.g. during the night) the substrate concentration fell to nearly zero, reducing the productivity of the reactor. In contrast to this, Figure 8.17 shows that it is possible, using computer control, to maintain the acrylamide concentration in the reactor at the desired level throughout the reaction. Thus, periods of high acrylamide concentration and very low acrylamide concentration are avoided. This is reflected in the average amidase activities determined for the manual and computer controlled reactor shown in Table 8.9.

8.7.2.1 Effect of Acrylamide Concentration on Reactor Performance

Figures 8.18a and 8.18b show the average amidase activity of each consecutive batch when the acrylamide concentration was maintained at 225 and 400 mM. The amidase activity of *R. rhodochrous* sp. 632 has been shown to be competitively inhibited by high levels of ammonium acrylate. It would therefore be expected that higher acrylamide concentrations would help to overcome this inhibition. However, the results suggest that there is no benefit gained in terms of increased production rates. The first batch performed at 225 mM acrylamide was complete after 27 hours while the batch at 400 mM took 35 hours. Figures 8.18a and 8.18b show that the average amidase activity of the immobilised cells falls with each consecutive batch. Comparing the two runs, it can be seen that the rate of activity loss is greater for the reactor operating at 400 mM acrylamide. Thus, the yield of ammonium acrylate per gram of dry cells is lower for this reactor. After 11 days of operation it can be seen from Figure 8.18b that the yield for the run operating at 400 mM acrylamide reaches 70 g ammonium acrylate per gram of dry cells, compared to 80 g/g for the 225 mM acrylamide run. As was the case for the *C. nitrilophilus* amidase, maintaining the acrylamide concentration at a higher level caused the amidase activity to fall more rapidly.

Figure 8.17: Concentration Profile of a Computer Controlled Fed-Batch Reactor using *R. rhodochrous* sp.632 Immobilised in Cross-Linked Polyacrylamide Beads

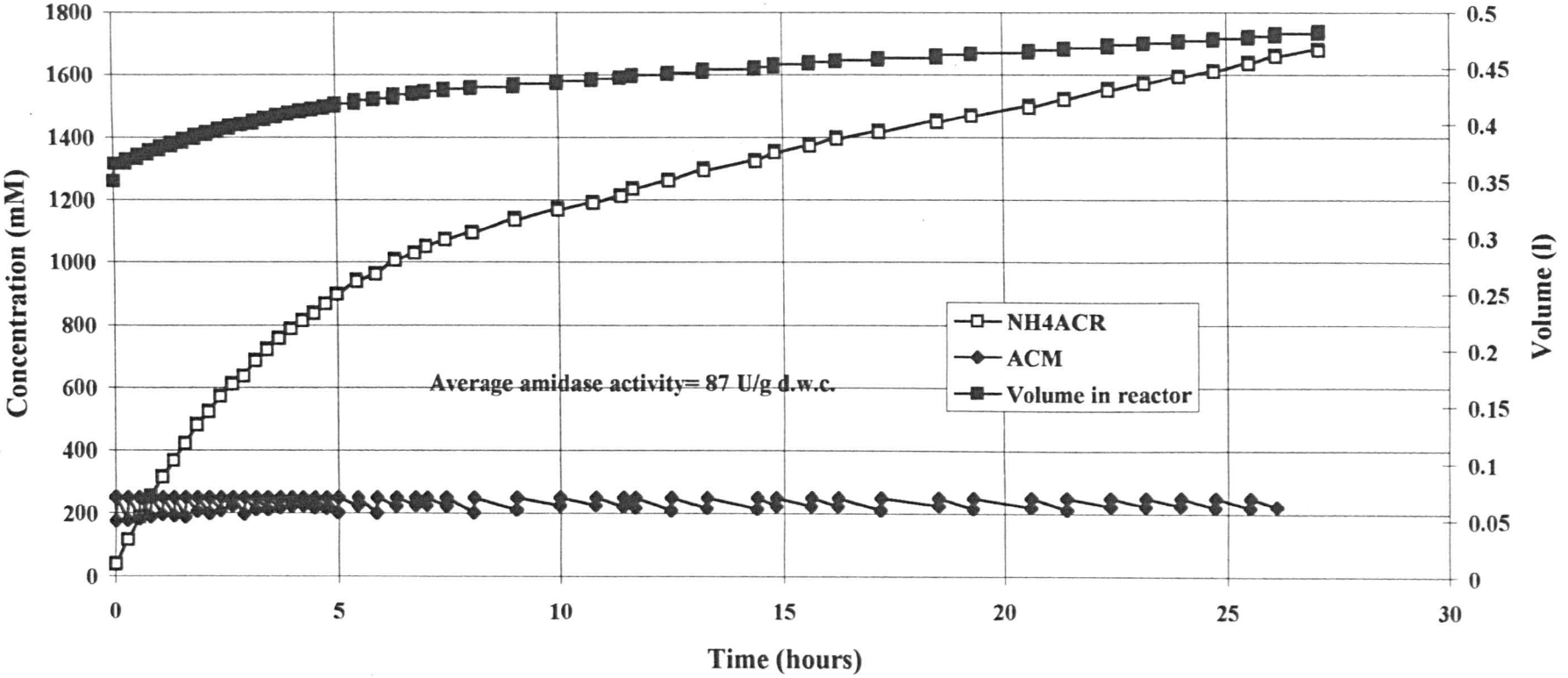


Figure 8.18a: Performance of *R. rhodochrous* sp.632 Immobilised in Cross-Linked Polyacrylamide Beads Operating in a Fed-Batch Reactor with [ACM]= 225 mM and final [NH4ACR]= 1685 mM

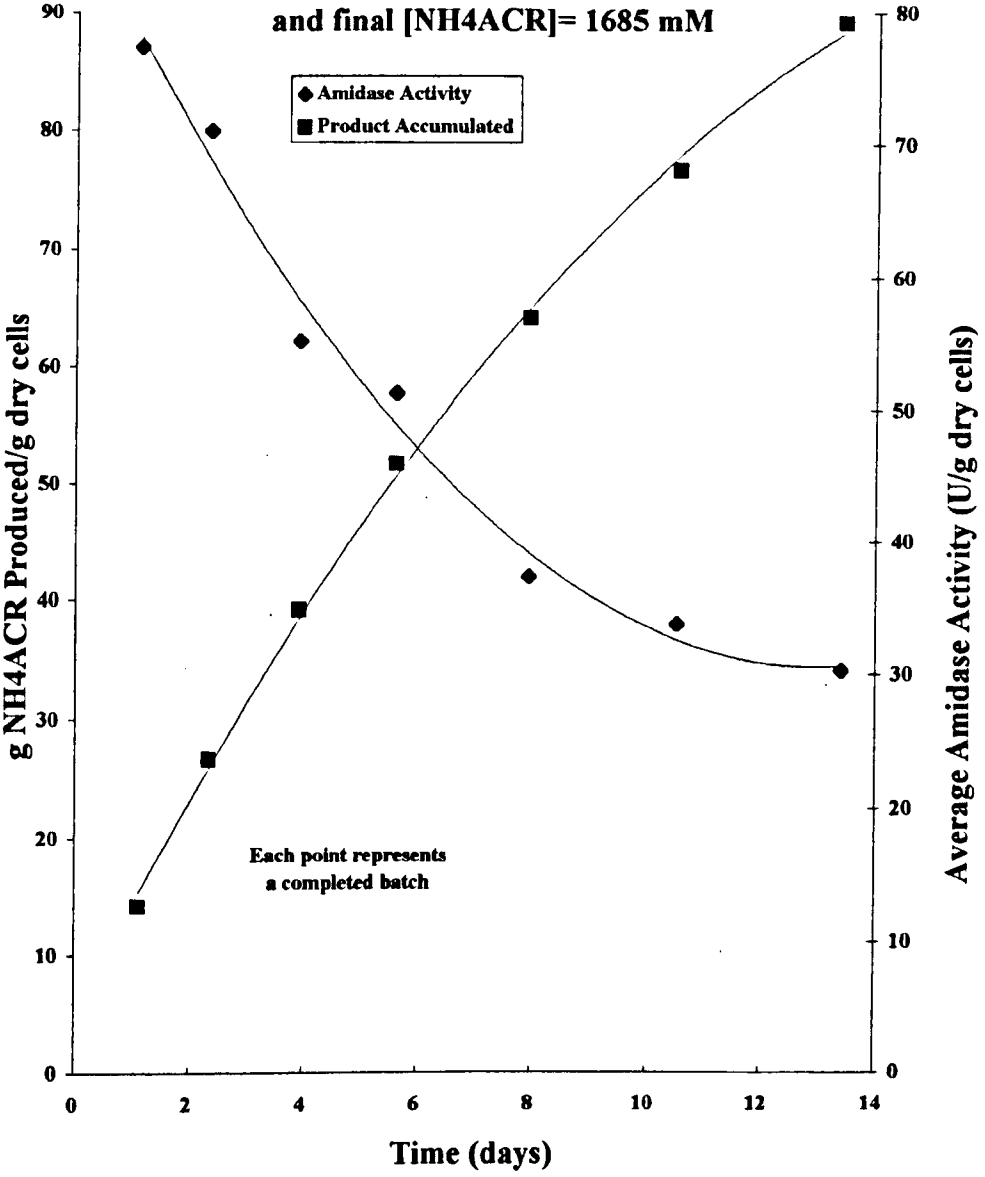
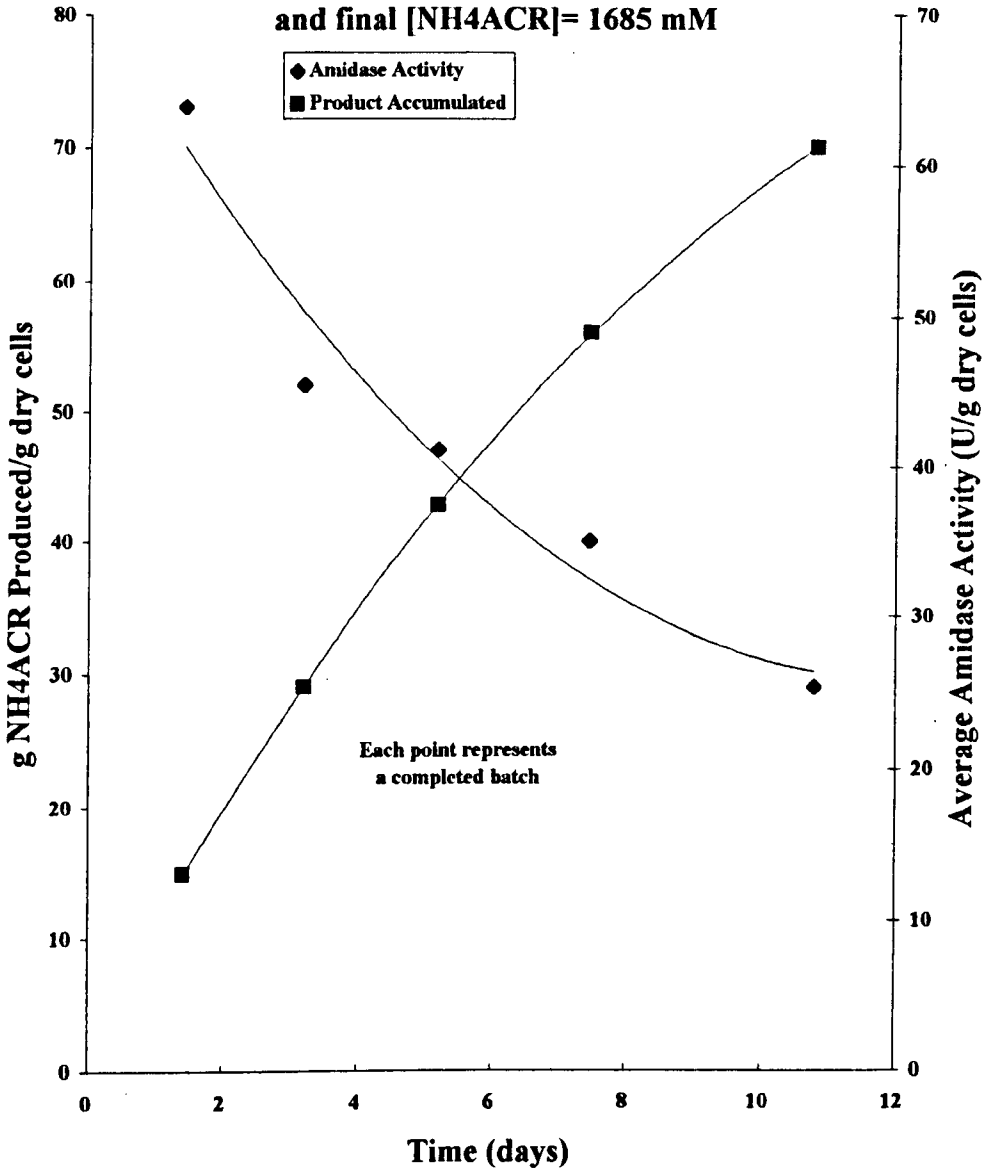


Figure 8.18b: Performance of *R. rhodochrous* sp.632 Immobilised in Cross-Linked Polyacrylamide Beads Operating in a Fed-Batch Reactor with [ACM]= 400 mM and final [NH4ACR]= 1685 mM



8.7.2.2 Rate of Amidase Activity Decay in the Reactor

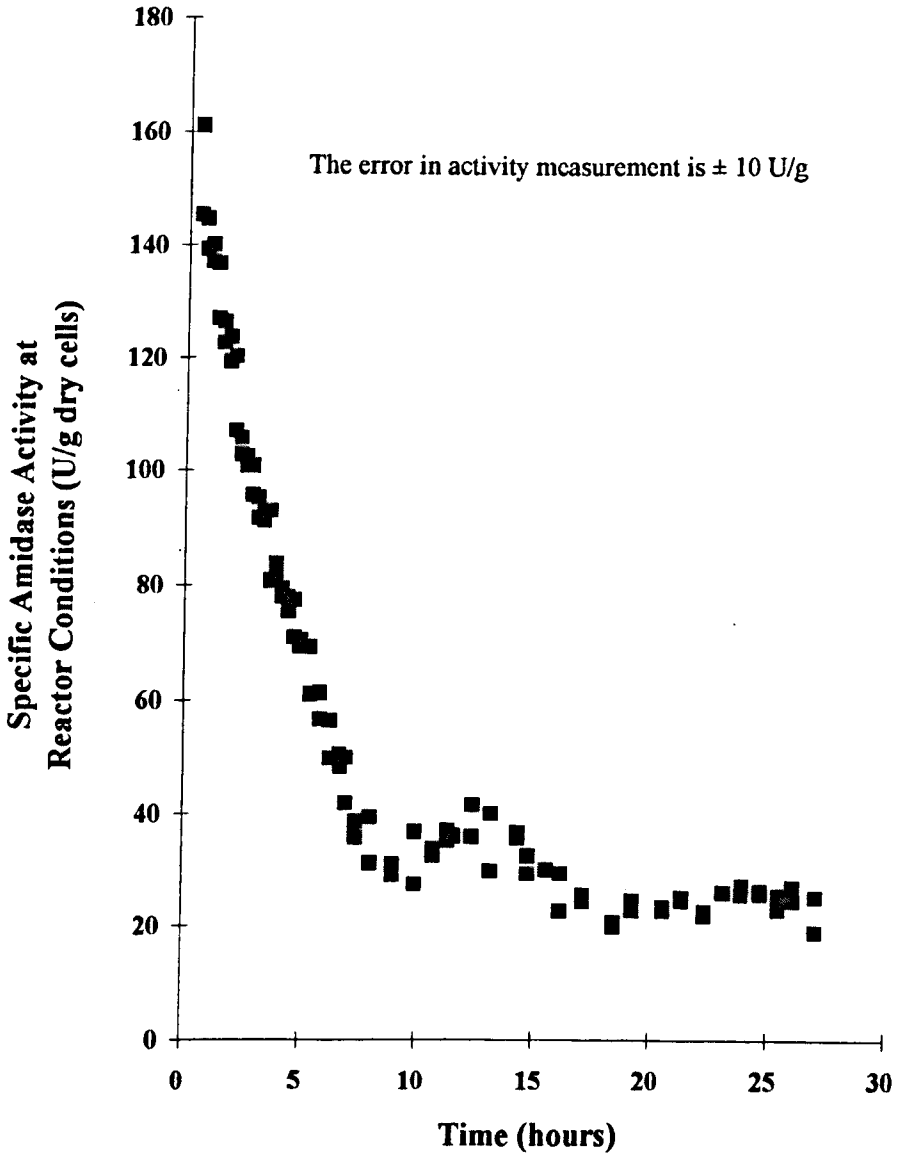
Figure 8.19 shows the specific amidase activity of the immobilised cells during the experiment detailed in Figure 8.17. The fall in activity is likely to be due to both product inhibition and an irreversible loss in amidase activity. It can be seen that the activity falls quickly at the start of the first batch and then seems to become more stable. The temporary increase in specific amidase activity after 10 hours, shown in Figure 19, may be due to an increase in the acrylamide concentration. This would overcome the competitive product inhibition and increase the reaction rate.

Table 8.9: Comparison of the First Batches of Manually Controlled and Computer Controlled Repeat Fed-Batch Reactors

	Manually Controlled Fed-Batch Reactor	Computer Controlled Fed-Batch Reactor	Computer Controlled Fed-Batch Reactor
NH4ACR (mM)	1810 (Final)	1685 (Final)	1685 (Final)
ACM (mM)	0 to 450	200-225	370-400
Operating Temperature (°C)	5	5	5
Cell loading in Beads, L_b (g dry cells/100 g wet gel)	6	5.7	5.2
Amidase Activity (U/g dry cells) Before Reaction at Standard Assay Conditions (500 mM ACM, 30 °C)	2720	2225	2010
Amidase Activity (U/g) Under Reactor Conditions of First Batch	35	90	70

This result is again similar to what was found for *C. nitrilophilus* and the arguments presented in Section 8.5.2.1 may apply here: The changing deactivation rate may be explained by the presence of two or more amidases in the immobilised cells with different levels of activity and stability. Alternatively, it may be that the amidase

Figure 8.19: Specific Amidase Activity of *R. rhodochrous* sp.632 Immobilised in Cross-Linked Polyacrylamide Beads in Batch 1 of a Repeat Fed-Batch Reactor Operating at 5°C



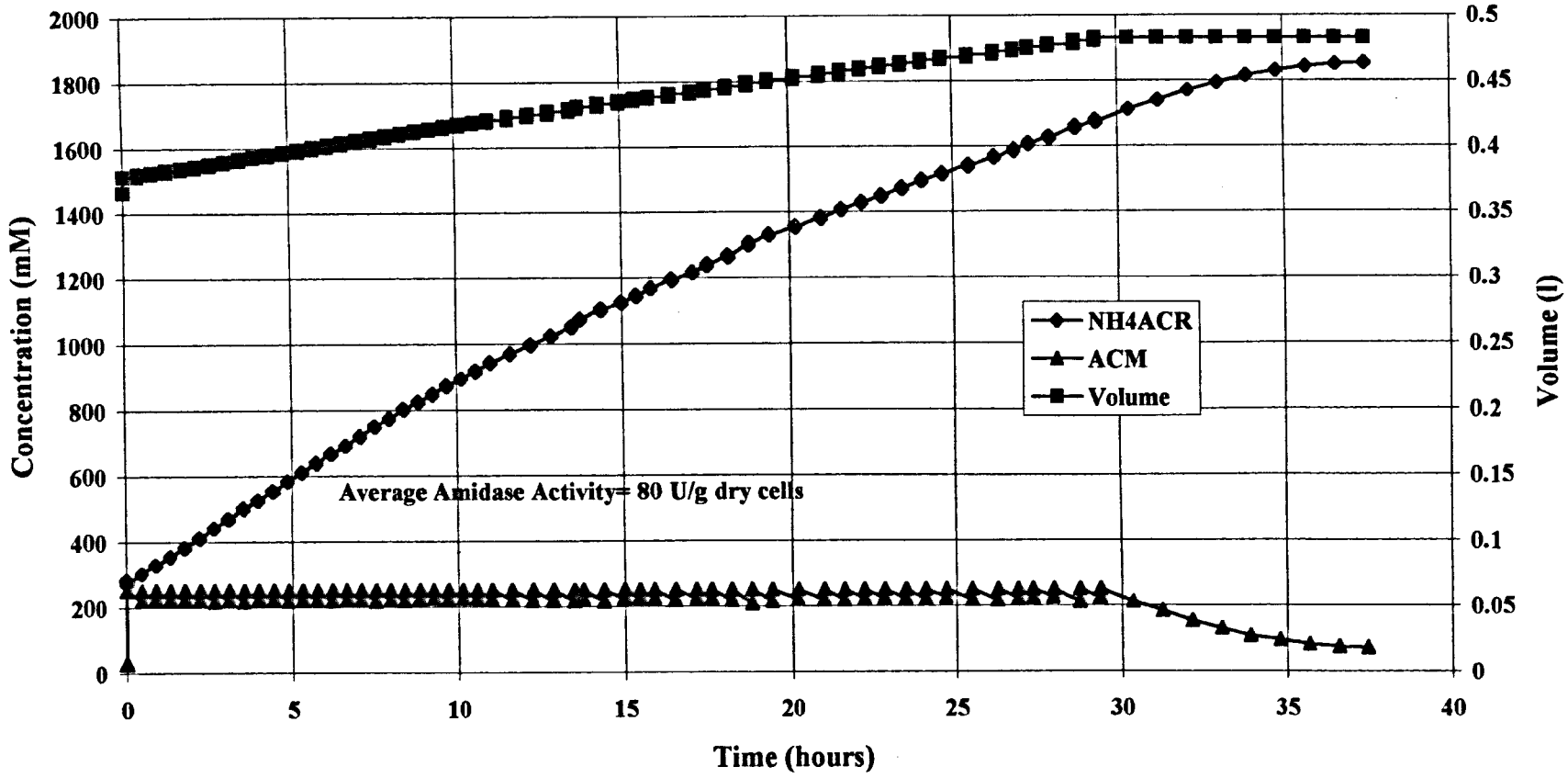
enzyme is most active when in the form of a complex. This complex may gradually break down into its less active, but more stable, subunits.

The reduction in deactivation rate described above highlights the difficulties of working with whole cell systems and care must be taken when attempting to measure constants such as the Michaelis-Menten constant, K_m, and the inhibition constant K_i. For example, the experiments demonstrating the product inhibition of *R. rhodochrous* sp. 632, described in Section 8.5.4, were performed under mild conditions which did not produce a loss in amidase activity. A value of K_i determined under such conditions may not be representative of the true level of inhibition occurring in the fed-batch reactor runs described above.

8.7.2.3 Extent of Acrylamide Conversion

Figure 8.20 shows the concentration profile of the third fed-batch of the reactor run operating with an acrylamide concentration of 225 mM. The computer control programme was instructed to stop acrylamide additions once an ammonium acrylate concentration of 1685 mM had been reached. Figure 8.20 shows that the acrylamide concentration falls to 70 mM, during which time the reaction rate also drops. An acrylamide concentration of 70 mM corresponds to 4 % of the total monomer in the reactor. The acrylamide concentration in the final product may not be important, depending on the final application of the ammonium acrylate. For example, many applications require the production of acrylamide/ammonium acrylate co-polymers. In such a case, the presence of acrylamide is not such a problem. However, some applications may demand a high product purity. A longer residence time in the reactor may increase the level of acrylamide conversion but will also reduce the production rate of the vessel due to slower reaction rates at higher ammonium acrylate concentrations.

Figure 8.20: Concentration Profile of the Third Batch of the Computer Controlled Fed-Batch Reactor



9. SCALE-UP REACTOR STUDIES

The reactor studies described in the last chapter made use of a reactor with a working volume of 0.5 litres or less. A commercial process for the production of a commodity chemical, such as ammonium acrylate, will require an increase in reactor size of several orders of magnitude. Such scaling-up can be performed in increments by studying various sizes of reactor up to a pilot plant size. This is to ensure that data produced from such studies can be relied upon when designing a full scale process. In order to make the first step in scale-up of the process, a bioreactor of 6 l working volume was designed, constructed and operated. By doing this it was hoped to achieve two main aims. Firstly, to demonstrate that ammonium acrylate can be produced on a kg scale using a biological route and secondly, to identify bioreactor operating and biocatalyst production problems.

9.1 Design of Reactor

The reactor vessel is shown in Figure 6.2. The vessel was constructed from glass to allow good visibility and samples ports were situated at the top and bottom of the vessel. Though investigations into mixing were not an intention of this work it was still felt to be important to use vessel and stirrer dimensions similar to those used by other workers studying particle suspension in stirred tanks (Chapman *et al.*, 1983). The agitator used was a downflow, 45°, 6 blade impellor. To avoid the need for a long drive shaft a bottom driven magnetically coupled agitator was used. Unfortunately, this

meant that the agitator was situated fairly high in the vessel. Low clearance, C, of the agitator is recommended in order to reduce the power required to suspend the beads (Chapman *et al.*, 1983). Clearance ratios down to one sixth of the tank diameter, T, are quoted in the literature. The minimum clearance possible with the bioreactor agitator was C=T/2.5. However, no problems with agitation were encountered at the biocatalyst loading employed. If mixing studies are to be made with the bioreactor the effects of the agitator height must be determined.

9.1.1 Safety Features

The main danger to be considered when working with the monomers acrylamide and ammonium acrylate is the possibility of an unwanted polymerisation. At high monomer concentrations the exotherm resulting from a polymerisation can lead to high temperatures and subsequent boiling of the liquid phase. For example a 30 % w/v solution could lead to a 90 °C rise in temperature. The chance of an unwanted polymerisation occurring is decreased at the low temperatures of the biotransformation, making the biological route inherently safer than the chemical route. However, it is still important to have a safety system in place that can cope with the results of an unwanted polymerisation.

The safety system used is shown in Figure 6.1. Temperature sensors were located in the reactor, substrate and product vessels. If a polymerisation did occur the subsequent temperature rise detected would have triggered an alarm signal to the separate computer operating the safety system. On detecting the alarm signal the computer was

programmed to switch off the substrate feed pump and open the remotely operated valves shown in Figure 6.1. This would have released water into the vessels and thus diluted the monomer solutions before a large temperature rise was produced. To this end, a sufficient head space was allowed for in the sizing of all the vessels to ensure flooding did not occur. The agitator was left stirring to aid the mixing of the monomer with the quench water.

9.2 Operation of the Reactor

9.2.1 Conductivity Readings

During the experiments described with the 0.5 litre reactor problems were encountered due to immobilised cell particles interfering with conductivity measurements. (See Section 8.5.2.7.). This problem was solved by turning off the stirrer for a short period of time while the particles settled away from the probe. Such a solution would be impractical with a large scale bioreactor due to the excessive wear on the agitation equipment. For the scaled-up reactor system the conductance probe was located outside of the vessel, and liquid from the reactor pumped through it, as shown in Figure 6.1. Initial attempts to remove the beads from the liquid stream were made by use of a filter mesh. However, this proved to be unsuccessful due to blockage of the filter. Greater success was achieved using the system detailed in Figure 6.3. Liquid was pumped from the reactor through a funnel. The large diameter of the funnel produced a low fluid velocity and prevented entrainment of the beads in the liquid pumped from the reactor. However, very small beads still become entrained in the stream being pumped to the conductivity probe. A very small percentage of the beads were also

observed to float and these too became entrained in the liquid stream. Using the bead capture chamber shown in Figure 6.3, however, these remaining beads were also removed before they interfered with the conductivity readings. There was a gradual build-up of beads in the chamber and these were periodically manually removed. Location of the conductivity probe exterior to the reactor also allowed easy access if cleaning or recalibration was required.

9.2.2 Addition and Removal of Catalyst from the Reactor

Another potentially difficult operation with regard to the reactor was the loading and removal of beads from the vessel. In practice this was not a problem due the ease in which the beads were fluidised. Loading of catalyst was easily achieved by suspending the beads in water and pumping the suspension into the reactor using a peristaltic pump. Damage to the beads caused by pumping was not observed.

9.3 Ammonium Acrylate Production Studies

Figures 9.1a and 9.1b show the ammonium acrylate production profiles for the 6l reactor when operated in CSTR and fed-batch modes respectively. The runs were continued for similar lengths of time (17-19 days) until the amidase activity was difficult to detect. Details of the runs are given in Table 9.1. It can be seen that, as with the small scale reactor studies, the performance of immobilised *R. rhodochrous* sp.632 was better when the reactor was operated in fed-batch mode. The final yield of the fed-batch reactor was 50 g ammonium acrylate per g dry cells after 19 days operation compared to a yield of 28 g in 17 days from the reactor operating in CSTR mode. The

Figure 9.1a: Product Accumulation from the 6l Reactor Operating in CSTR Mode with *R. rhodochrous* sp.632 Immobilised in Cross-Linked Polyacrylamide Beads

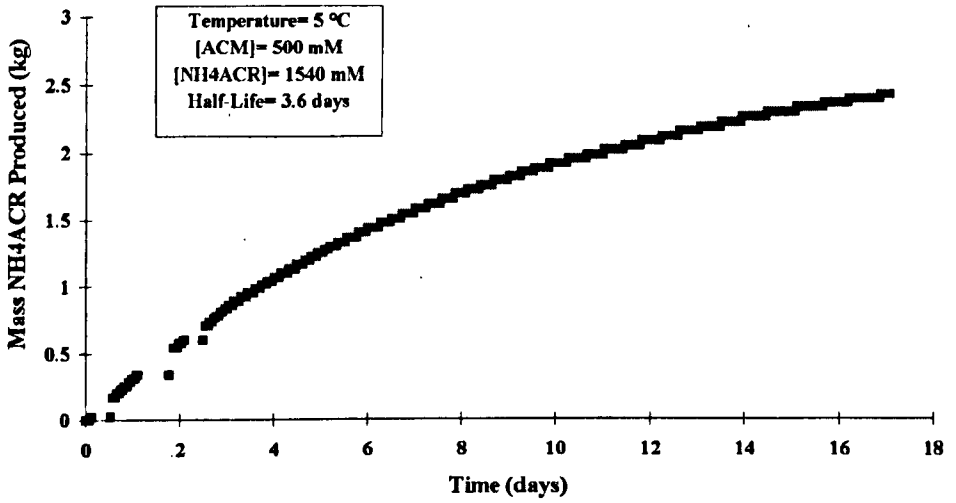
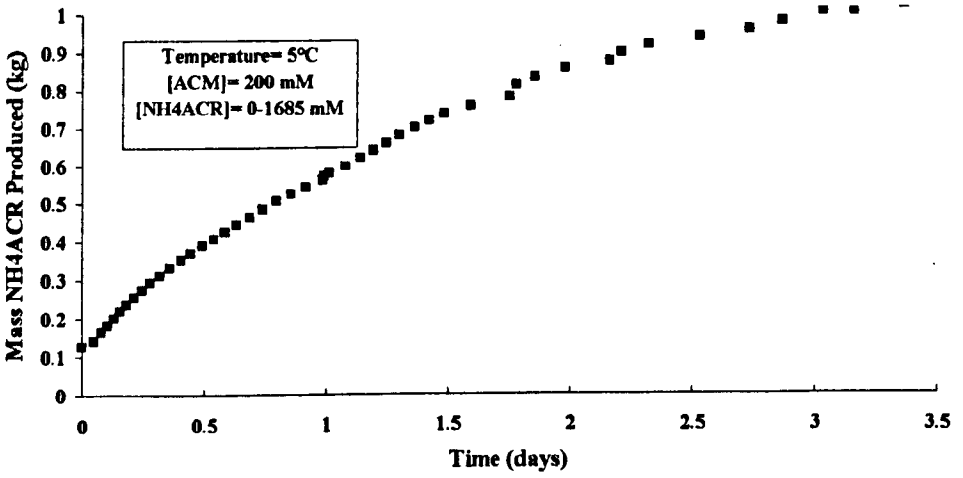


Figure 9.1b: Second Batch Concentration Profile for the 6l Reactor Operating in Repeat Fed-Batch Mode with *R. rhodochrous* sp.632 Immobilised in Cross-Linked Polyacrylamide Beads



acrylamide concentration during CSTR operation was 500 mM compared to 200 mM during fed-batch operation. The higher acrylamide concentration was used to try and overcome the effects of product inhibition that are constantly present when operating in CSTR mode. It is likely that the higher acrylamide concentration increased the deactivation rate of the amidase activity leading to the poorer specific yield in ammonium acrylate per gram of cells.

Table 9.1: Comparison of Fed-Batch and CSTR Operation of the Scaled-Up Reactor using *R. rhodochrous* sp.632 Immobilised in Cross-linked Polyacrylamide Beads

	Fed-Batch Operation	CSTR Operation
Operating temperature (°C)	5	5
[NH4ACR] (mM)	1685 (Final)	1535
[ACM] (mM)	180-200	460-500
Mass catalyst (kg)	2.15	1.38
Amidase activity at standard assay conditions (30 °C, 500 mM ACM)	1900	1750
Reaction volume (l)	5.86 to 7.57	4.37
Lb (g dry cells/100 g beads)	3.8	6.1
Mass dry Cells (g)	79	95
Total mass product (kg)	4.74	2.42
Operating time (days)	19	17
Number batches	4	-
Average productivity over total operating period (g NH4ACR/g dry cells/hr)	0.14	0.06
Yield (g NH4ACR/g dry cells)	50	28

When the small scale reactor was operated in fed-batch mode under similar conditions as the large reactor it gave a yield of 90 g ammonium acrylate per g dry cells. However, the initial amidase activity of the biocatalyst operating in the 0.5 l reactor was approximately double that operating in the 6 l reactor. This indicates that the increase in scale did not seriously affected the biocatalyst performance.

Through the operation of the 6l reactor in fed-batch or CSTR modes it was possible to produce ammonium acrylate on a kg scale. No serious problems were identified during operation of the 6 l reactor indicating that it may be possible to produce ammonium acrylate on an industrial scale. The availability, through continued research, of a more stable and active biocatalyst could lead to the bioconversion reaching commercial viability.

10. QUALITY TESTING OF AMMONIUM ACRYLATE PRODUCED DURING BIOREACTOR STUDIES

The commercial applications of ammonium acrylate were discussed in Chapter 1. It is intended to make use of ammonium acrylate produced through biocatalysis in such applications. Consequently, the quality of the Bio-Ammonium acrylate was compared with a commercially available chemically produced ammonium acrylate.

Samples of ammonium acrylate produced during the bioreactor studies were tested in the Monomer Research Laboratories of Allied Colloids Ltd, Bradford, UK.

10.1 Quality Testing of Ammonium Acrylate Produced using *C. nitrilophilus* Immobilised in Cross-Linked Polyacrylamide Gel Cuboids

A 1 molar solution of ammonium acrylate was prepared by hydrolysing a 1.2 molar solution of acrylamide at room temperature using *C. nitrilophilus* immobilised in cross-linked polyacrylamide gel. Due to the presence of cell and gel debris, it was necessary to centrifuge the sample for a few minutes at 10 000×g before decanting the monomer solution.

Using the Bio-ammonium acrylate it was possible to produce acrylamide:ammonium acrylate copolymers of 95:5 and 90:10 compositions (weight:weight). These were compared against copolymers produced using ammonium acrylate prepared by neutralising BASF acrylic acid with ammonia solution. No significant difference was

observed with regard to temperature rise during polymerisation. Measurement of the intrinsic viscosity's of the polymer solutions again showed no significant difference.

10.2 Quality Testing of Ammonium Acrylate Produced using *R. rhodochrous* sp.632 Immobilised in Cross-Linked Polyacrylamide Beads

Quality testing with ammonium acrylate prepared using *R. rhodochrous* sp.632 was performed with product taken from the 6 l fed-batch run described in Section 9.3. The sample was centrifuged for a few minutes at 10 000 × g before being used to prepare an 80:20 acrylamide: ammonium acrylate copolymer. This was tested against a copolymer prepared using standard ammonium acrylate as described above. Comparison of the polymerisation temperature profiles and intrinsic viscosity's showed little difference.

The Bio-ammonium acrylate was also tested by using the acrylamide:ammonium acrylate copolymer to prepare two potential end products: a flocculant and a print thickener. Comparison with standards again showed little difference in performance.

Polymerisations may be inhibited by low levels of impurities leading to no polymerisation occuring or low molecular weight polymers. The results obtained from the tests described above indicate that this problem does not occur with bio-ammonium acrylate and that there is no obvious reason why bio-ammonium acrylate may not be used in place of chemically produced ammonium acrylate.

CONCLUSIONS

11. CONCLUSIONS

11.1 Cell Immobilisation

A commercially viable biotransformation for the production of a commodity chemical, such as ammonium acrylate, will require the use of an immobilised biocatalyst. Several methods of entrapment were studied as a means of immobilising amidase active cells. The commonly used method of entrapment in calcium alginate beads was shown to be unsatisfactory due to the poor physical stability of the beads under reaction conditions. This was possibly due to chelation of the calcium ions in the presence of ammonium acrylate. Cross-linked polyacrylamide gel was found to be a satisfactory matrix for the immobilisation of cells giving good physical stability under reaction conditions without a serious loss in amidase activity.

An industrial scale biotransformation will necessitate an immobilised biocatalyst that can be produced on a large scale. Suspension polymerisations are carried out on an industrial scale. Laboratory scale investigations into such a technique showed it was possible to entrap cells within cross-linked polyacrylamide beads, suggesting that this method of immobilisation may be applicable on a large scale. The average loss in amidase activity was 32 % and the beads again showed satisfactory physical stability. Furthermore, the beads exhibited amidase activity when rehydrated after storage in a dried state. This is an important property allowing biocatalyst production at the most cost effective scale.

11.2 Bioreactor Studies

A bioreactor can be operated in several ways in order to optimise the biocatalyst performance. From an industrial perspective, batch reactors involve the least sophistication and therefore least capital outlay. It has been shown, however, that progressive and permanent deactivation of amidase activity occurs in the presence of acrylamide and ammonium acrylate. The high concentrations of these substances that are present in a batch reactor therefore make it unsuitable for ammonium acrylate production.

Continuous stirred tank bioreactors may be operated in such a way as to keep acrylamide and ammonium acrylate concentrations constant with time. Such studies with *C. nitrilophilus* immobilised in cross-linked polyacrylamide gel cuboïds showed that acrylamide has a greater deactivating effect on amidase activity than ammonium acrylate. Lower operating temperatures reduced the amidase activity but also increased the stability of the amidase activity. A first order decay equation was found to adequately describe the loss in amidase activity and the first order decay rate constant was a convenient way of comparing stability.

Under conditions of higher biocatalyst stability it was necessary to use an automatic acrylamide feed system as reactor operation could extend to several days. This was achieved fairly simply by interfacing the conductance monitor with the acrylamide feed pump via a computer control system. The possibility of automating the bioreactor in this way is an important step as the automation of a commercial production process leads to a considerable reduction in operating costs.

Through the use of a computer controlled 0.5 litre continuous stirred tank bioreactor it was possible to compare the immobilised amidase activity of *C. nitrilophilus* with two cell isolates- *R. rhodochrous* sp. 632 and *Rhodococcus* 1068. It was shown that *R. rhodochrous* sp.632 was the most stable biocatalyst while *Rhodoccocus* sp.1068 had the highest initial amidase activity. (Under reaction conditions of 200 mM acrylamide, 1200 mM ammonium acrylate and 5 °C, *Rhodococcus* 1068 was twice as stable as *C. nitrilophilus* while *R. rhodochrous* sp.632 was 93 times more stable.) The amidase activity of a biocatalyst may be increased through optimisation of cell growth conditions. Increasing the stability of a biocatalyst, however, requires an actual change in the enzyme structure and therefore is more difficult to achieve. On these grounds, *R. rhodochrous* sp.632 is the most favourable biocatalyst for ammonium acrylate production.

Studies with *R. rhodochrous* sp.632 immobilised in cross-linked polyacrylamide beads showed amidase activity to be competitively inhibited by ammonium acrylate. During operation of a continuous stirred tank bioreactor high levels of ammonium acrylate are continually present making this reactor type unsuitable for ammonium acrylate production. Comparison of a fed-batch bioreactor with a continuous stirred tank bioreactor showed the former to be optimum for ammonium acrylate production when operating with *R. rhodochrous* sp.632 due to the minimisation of product inhibition and biocatalyst inactivation.

Scale-up from a laboratory sized reactor to a commercial vessel is typically performed by studying various intermediate sizes of reactor. The first step in scaling-up the ammonium acrylate bioreactor was performed by designing, constructing and operating a 6l working volume reactor. It was shown possible to operate the bioreactor in repeat fed-batch and continuous stirred tank modes, using a computer to control the addition of acrylamide. The reactor was used to produce ammonium acrylate on a kilogramme scale (4.74 kg during fed-batch operation and 2.42 kg during CSTR operation). No obvious differences in biocatalyst performance were noted when the performance in a 0.5 l reactor was compared with that in the 6 l reactor.

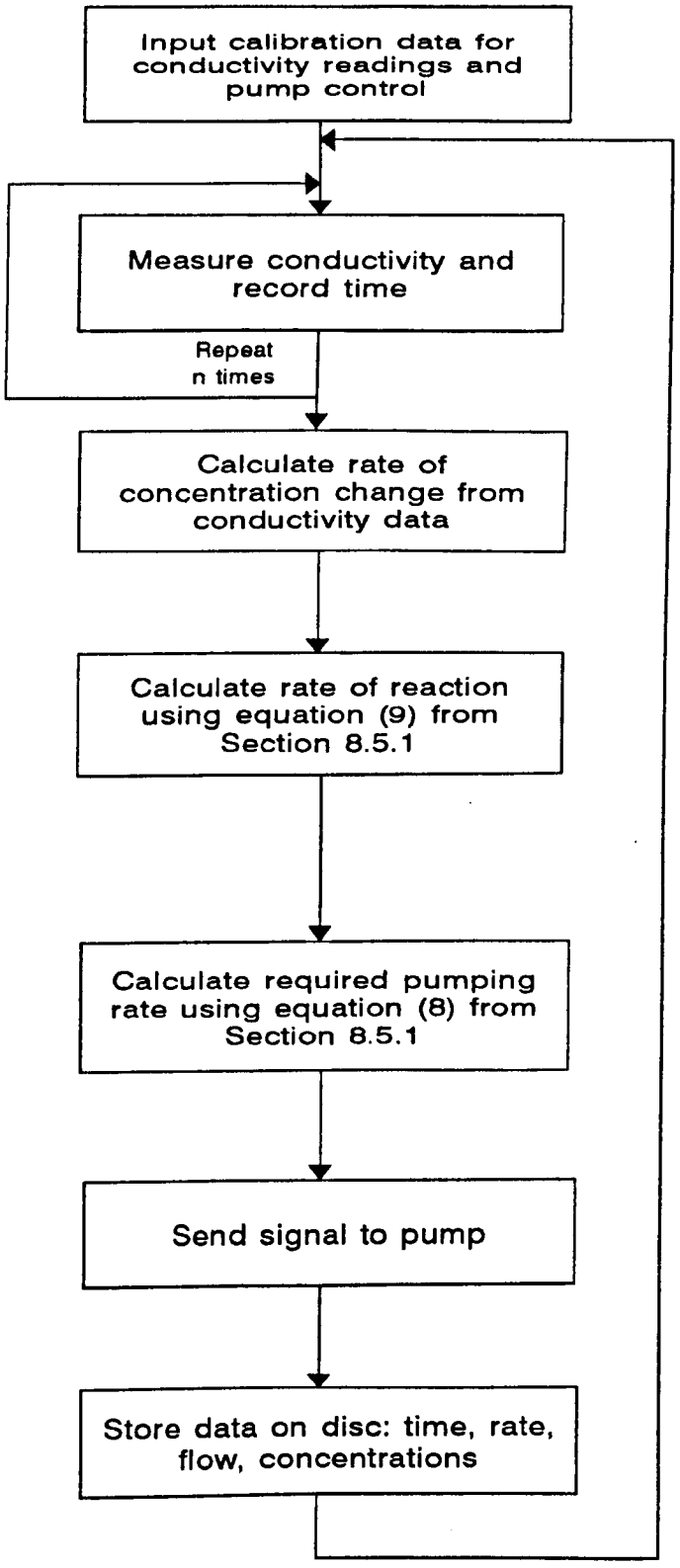
A comparison of ammonium acrylate produced from a bioreactor with a sample of commercial origin, synthesised chemically, is essential if it is to be a viable feed stock alternative. Ammonium acrylate from the 6l bioreactor, operating in fed-batch mode with *R. rhodochrous* sp.632 immobilised in cross-linked polyacrylamide beads, was used to produce acrylamide:ammonium acrylate copolymers. Physical tests on these polymers indicated that there is no obvious reason why biologically produced ammonium acrylate should not be used in place of chemically produced ammonium acrylate.

From an industrial viewpoint, the production of ammonium acrylate from acrylonitrile is more favourable than the acrylamide route. Acrylonitrile is difficult to handle, being highly volatile, flammable, toxic and poorly soluble in water. However, as a starting material it is typically several hundred pounds per tonne cheaper than acrylamide. The production of ammonium acrylate from both acrylonitrile and acrylamide has been

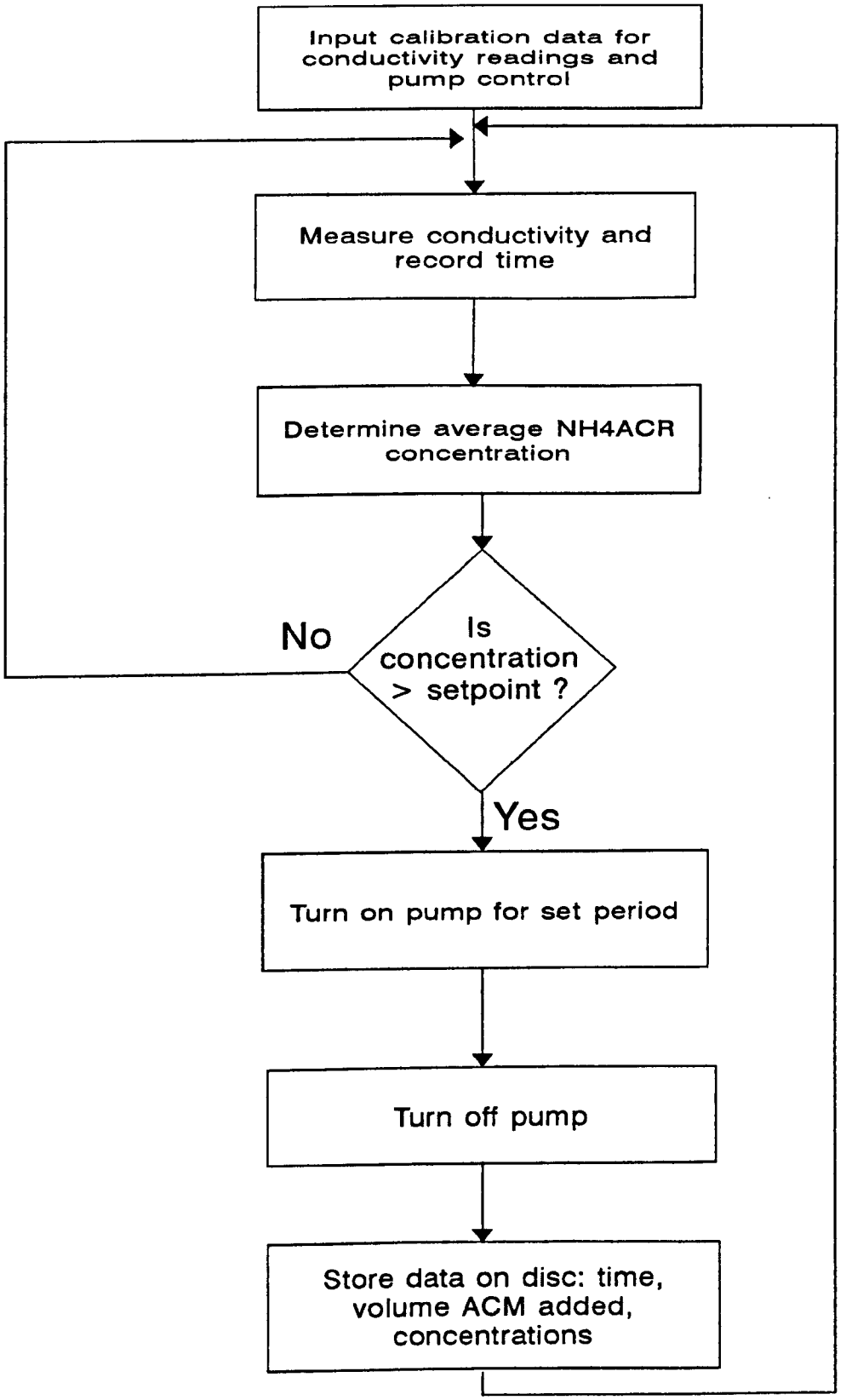
studied at Huddersfield University's Biotechnology Centre. The difference in value between acrylonitrile and acrylamide means that any further work into the acrylamide route will be very much dependant upon the success of the acrylonitrile conversion studies.

APPENDICES

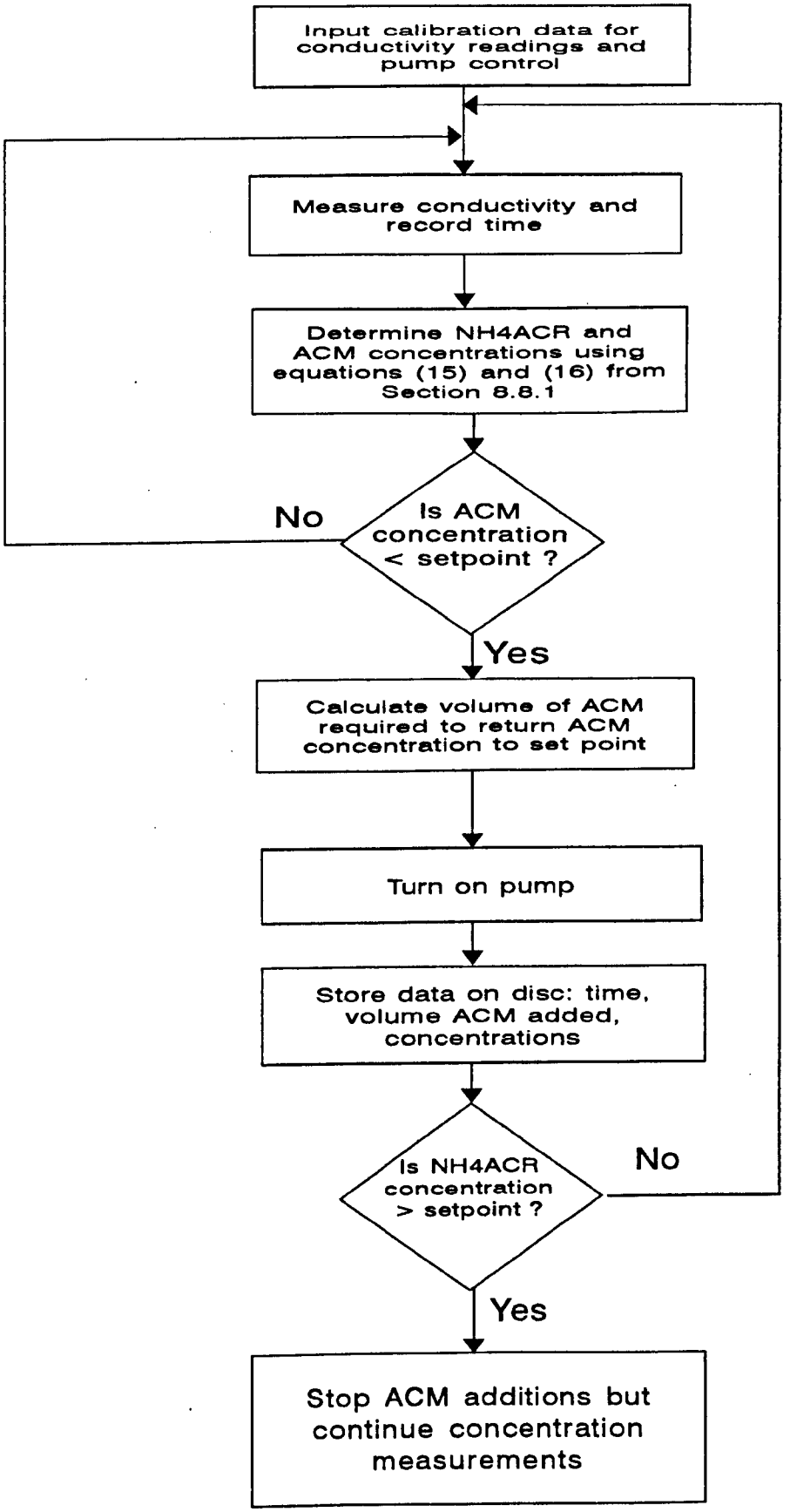
Appendix 1: Derivative Feedback Controller for Operation of the CSTR System shown in Figure 5.4



Appendix 2: On-Off Feedback Controller for Operation of the CSTR System shown in Figure 5.4



Appendix 3: On-Off Feedback Controller for Fed-Batch Operation of the System shown in Figure 5.4



REFERENCES

12. REFERENCES

Akiyama, A., Bednarski, M., Kim, M., Simon, E.S., Waldmann, H. and Whitesides, G.M. (1987). Enzymes in organic synthesis. *Chemistry in Britain*, **23**, 645-652.

Allen, B.R., Coughlin, R.W. and Charles, M. (1979). Fluidized bed enzyme reactors. *Annals of the New York Academy of Sciences*, **326**, 105-117.

Anderson, D.B., Forder, J. and Johnson, I.M. (1977). Allied Colloids Ltd. Improvements in and relating to polymers. U.K. Patent No.: 1482515.

Ashen, Y. and Suto, M. (1993). Development of an enzymatic process for manufacturing acrylamide and recent progress. In: Industrial application of immobilized biocatalysts. (Tanaka, A., Tosa, T. and Kobayashi, T., eds.). New York: Marcel Dekker.

Ballesterios, A., Boross, L., Buchholz, K., Cabral, J.M.S. and Kasche, V. (1994). Biocatalyst Performance. In: Applied Biocatalysis. (Cabral, J.M.S., Best, D., Boross, L. and Tramper, J., eds.). Reading: Harwood Academic Publishers.

Bauchop, T. and Elsden, S.R. (1960). The growth of microorganisms in relation to their energy supply. *Journal of General Microbiology*, **23**, 457-469.

Beck, S.R. and Rase, H.F. (1973). Encapsulated enzyme: a glucoamylase copolymer system. *Industrial and Engineering Chemistry Product Research and Design*, **2**, 260-267.

Begin, A., Caistaigne, F. and Goulet, J. (1991). Production of alginate beads by a rotative atomizer. *Biotechnology Techniques*, **5**, 459-464.

Bernet, N., Thiery, A., Maestracci, M., Arnaud, A., Rios, G.M. and Galzy, P. (1987). Continuous immobilized cell reactor for amide hydrolysis. *Journal of Industrial Microbiology*, **2**, 129-136.

Birnbaum, S., Larsson, P.O. and Mosbach, K. (1986) Immobilized biocatalysts, the choice between enymes and cells. In: Process engineering aspects of immobilized cell systems. (Webb, C., Black, G. and Atkinson, B., eds.). Oxford: Pergamon Press.

Bucke, G.M. (1986). Methods of immobilising cells. In: Process engineering aspects of immobilized cell systems. (Webb, C., Black, G.M. and Atkinson, B., eds.). Oxford: Pergamon Press.

Buitelaar, R.M., Hulst, A.C. and Tramper, J. (1990). Cell immobilization in thermogels: activity retention after gelling in various organic solvents. In: Physiology of immobilized cells. (de Bont, J.A.M., Visser, J., Mattiasson, B. and Tramper, J., eds.). Amsterdam: Elsivier Science Publishers.

Brennan, M.R., Armitage, Y.C., Mortimer, M.G., Hughes, J. and Ramsden, D.K. (1995). Amidase-active whole cells of *Corynebacterium nitrilophilus* for ammonium acrylate production. *Biotechnology Letters*, **17**, 513-518.

Cavins, J.F. and Friedman, M. (1968). Specific modification of protein sulfhydryl groups with α-β-unsaturated compounds. *Journal of Biological Chemistry*, **243**, 3357-3360.

Chaplin, M.F. and Bucke, C. (1990). Enzyme Technology. Cambridge: Cambridge University Press

Chapman, C.M., Nienow, A.W., Cooke, M. and Middleton, J.C. (1983). Particle-gas-liquid mixing in stirred vessels Part 1:Particle-liquid mixing. *Chemical Engineering Research and Design*, **61**, 71-81.

Cheetham, P.S.J. (1994). Case studies in Applied Biocatalysis-From ideas to products. In: Applied Biocatalysis. (Cabral, J.M.S., Best, D., Boross, L. and Tramper, J., eds.). Reading: Harwood Academic Publishers.

Chibata, I., Tosa, T. and Sato, T. (1986). Methods of cell immobilization. In: Manual of industrial microbiology and biotechnology. (Demain, A.L. and Solomon, N.A., eds.). Massachusetts: American Society for Microbiology.

Clarke, P. H. (1986). Experiments on the evolution of bacteria with novel enzyme activities. *Chemica Scripta*, **26B**, 337-342.

Clarke, P.H. (1970). The aliphatic amidases of *Pseudomonas aeruginosa*. *Advances in Microbiology and Physiology*, **4**, 179-222.

Collins, A.N., Sheldrake, G.N. and Crosby, J., eds. (1992). Chirality in Industry. The commercial manufacture and applications of optically active compounds. Chichester: John Wiley and Sons.

Cottenceau, G., Dherbomez, M, Lubochinsky, B., and Letellier, F. (1990). Immobilization and treatment of *Streptococcus faecalis* for the continuous conversion of arginine into citrulline. *Enzyme Microbial Technology*, **12**, 355-360.

Crosby, J., Moilliet, J., Parratt, J.S. and Turner, N.J. (1994). Regioselective hydrolysis of aromatic dinitriles using a whole cell catalyst. *Journal of the Chemical Society: Perkin Transactions 1*, **13**, 1679-1687.

Darbourne, A. (1993). Overcoming the problems of chiral synthesis. *Scrip Magazine*, 16-18.

Fawcett J.K. and Scott, J.E. (1960). A rapid and precise method for the determination of urea. *Journal of clinical pathlogy*, **13**, 156-159.

Freeman, A. (1984). Understanding enzyme stabilisation. *Trends in Biotechnology*, **2**, 147-148.

Furui, M. and Yamashita, K. (1985). Diffusion coefficients of solutes in immobilized cell catalysts. *Journal of Fermentation Technology*, **2**, 167-173.

Gacesa, P. and Hubbel, J. (1987). Enzyme technology. Stratford: Open University Press.

Gemeiner, P. (1992). Enzyme engineering, immobilized biosystems. London: Horwood.

Geraats, B. (1992). Modelling of immobilised systems. In: Operational modes of bioreactors. Oxford: Butterworth-Heinemann.

Gostomski, P. and Bungay, H. (1992). Reliable on-line monitoring of ammonium ion in a bioreactor. *Biotechnology Techniques,* **6**, 5-8.

Harrington, T.J., Gainer, J.L. and Kirwan D.J. (1991). Effects of fluid shear on immobilized enzyme kinetics. *Enzyme Microbial Technology*, **13**, 610-616.

Hoq, M.M., Yamane, T., Shimizu, S., Funada, T. and Ishida, S. (1985). *Journal of the American Oil and Chemical Society*. **62**, 1016-1021.

Hwang, J.S. and Chang, H.N. (1989). Biotransformation of acrylonitrile to acrylamide using whole cells of Brevibacterium CH1 in a recycle fed-batch reactor. *Biotechnology and Bioengineering*, **34**, 380-386.

Karanth, N.G. (1979). Multiple continuous stirred tank reactors vs. batch reactors-some design considerations. *Biotechnology Letters*, **1**, 139-144.

Katchalski-Katzir, E. (1993). Immobilized enzymes - learning from past successes and failures. *Trends in Biotechnology*, **11**, 471-478.

Kaul, R. and Mattiasson, B. (1993). Improving the shelf life of enzymes by storage under anhydrous apolar solvent. *Biotechnology Techniques*, **7**, 585-590.

Kennedy, M.J., Reader, S.L. and Davies, R.J. (1994). The kinetics of developing fermentation media. *Process Biochemistry*, **29**, 529-534.

Kirk-Othmer. (1991). Encyclopedia of Chemical Technology. 4th ed. USA: John Wiley and Sons.

Klein, J. (1988). Matrix design for microbial cell immobilization. In: Bioreactor immobilized enzymes and cells: fundementals and applications. (Moo-Young, M., ed.). Essex: Elsivier Applied Science.

Klein, J. and Vorlop, K.D. (1983). Immobilized Cells. In: Foundations of Biochemical Engineering. Kinetics and thermodynamics in biological systems. (Blanch, H.W., Papousakis, E.T. and Stephanopoulos, G., eds.). ACS Symposium series **207**. Washington DC: American Chemical Society.

Klein, J. and Schara, P. (1980). Entrapment of living microbial cells in covalent polymeric networks: 1. Preparation and properties of different networks. *Journal of Solid Phase Biochemistry*, **5**, 63-78.

Klein, J. and Wagner, F. (1978). Immobilized whole cells. In: Biotechnology Proceedings pt 3 of First European Congress in Biotechnology. New York: Verlag Chemie-Weinheir.

Klein, J.; Stock, J. and Vorlop, K-D. (1983). Pore size and properties of spherical calcium alginate biocatalysts. *European Journal of Applied Microbiology and Biotechnology*, **18**, 86-91.

Kobayashi, M., Komeda, H., Nagasawa, T., Yamada,H. and Shimizu, S. (1993). Occurrence of amidases in the industrial microbe *Rhodococcus rhodochrous* J1. *Bioscience, Biotechnology and Biochemistry.*, **57**, 1949-1950.

Koshcheenko, K.A., Sokhodol'skaya, G.V., Tyurin, V.S. and Skryabin, G.K. (1981). Physiological, biochemical and morphological changes in immobilized cells during repeated periodical hydrocortisone transformations. *European Journal of Applied Microbiology and Biotechnology*, **12**, 161-169.

Koshcheenko, K.A.; Turkina, M.V. and Skryabin, G.K. (1983). Immobilisation of living microbial cells and their application for steroid transformations. *Enzyme Microbial Technology*, **5**, 14-21.

Kumakura, M. and Kaetsu, I. (1983). Effect of drying on immobilised cell composites obtained by radiation polymerisation. *Process Biochemistry*, **18**, 3, 28-30.

Levenspiel, O. (1972). Chemical reaction engineering. 2nd. ed. Chichester: John Wiley and Sons.

Lievense, L.C. and van't Riet, K. (1993). Convective drying of bacteria 1. The drying processes. *Advances in Biochemical Engineering*, **50**, 46-63.

Lilly, M.D. (1992). The design and operation of biotransformation processes. In: Recent advances in Biotechnology. (Vardar-Sukan F. and Sukan, S.S., eds.). Netherlands: Kluwer Academic Publishers.

Martinsen, A., Skjak-Braek, G. and Smidsrod, O. (1989). Alginate as immobilization material: 1. Correlation between chemical and physical properties of alginate gel beads. *Biotechnology and Bioengineering*, **33**, 79-89.

Masters, K. (1985). Spray drying Handbook. 4th ed. New York: John Wiley and Sons.

Mathews, C. and van Holde K. (1990). Biochemistry. Redwood City: The Benjamin/Cummings Publishing Company.

Miller, J.M. and Knowles, C.J. (1984). The cellular location of nitrilase and amidase enzymes of *Brevibacterium* R312. *Microbiology Letters*, **21**, 147-151.

Moo-Young, M. and Chisti, Y. (1994). Biochemical Engineering in Biotechnology. *Pure and Applied Chemistry*, **66**, 117-136.

Muftic, M.K. (1964). A new phenol-hypochlorite reaction for ammonia. *Nature*, **201**, 622-623.

Nagasawa, T. and Yamada, H. (1990). Large-scale bioconversion of nitriles into useful amides and acids. In: Biocatalysis. (Abramowicz, D.A., ed.). New York: Van Nostrand Reinold.

Nagasawa, T., Nakamura, T., and Yamada, H. (1990). Production of acrylic acid and methacrylic acid using *Rhodococcus rhodochrous* J1 nitrilase. *Applied Microbiology and Biotechnology*, **34**, 323-324.

Nagasawa, T., Shimizu, H. and Yamada, H. (1993). The superiority of the third-generation catalysts, *Rhodococcus rhodochrous* J1 nitrile hydratase, for industrial production of acrylamide. *Applied Microbiology and Biotechnology*, **40**, 189-195.

Nienow, A.W., Konno, M. and Bujalski, W. (1986). Studies on three phase mixing: A review and recent results. *Chemical Engineering Research and Design*, **64**, 35-42.

O'Brien, D.J., Panzer, C.C. and Eisele, W.P. (1990). Biological Production of acrylic acid from cheeses whey by resting cells of *Clostridium propionicum*. *Biotechnology Progress*, **6**, 237-242.

Philips, C.R. and Poon, Y.C. (1988). Immobilization of cells. London: Springer-Verlag.

Pryce (Minutes of discussion group from: Wyatt, J.M. and Linton, E.A. (1988). The industrial potential of microbial nitrile biochemistry. *CIBA Foundation Symposium.*, **140**, 32-48.)

Scragg, A.H. (1991). Bioreactors in Biotechnology: a practical approach. England: Ellis Horwood Ltd.

Smidsrod, O. and Skjak-Braek, G. (1990). Alginates as immobilization matrix for cells. *Trends in Biotechnology*, **8**, 71-78.

Stanier, R.Y., Ingraham, J.L., Wheelis, M.L. and Painter, P.R. (1985). General Microbiology. 5th ed. Basingstoke: Prentice-hall, Macmillan Education.

Sykes, P. (1986). A guidebook to mechanisms in organic chemistry. 6th ed. New York: John Wiley and Sons.

Takamatsu, S., Yamamoto, K., Tosa, T. and Chibata, I. (1981). Stabilization of L-Aspartate ß-Decarboxylase activity of *Pseudomonas dacunhae* immobilized with carrageenan. *Journal of Fermentation Technology.*, **59**, 489-493.

Takata, I., Yamamoto, K., Tosa, T. and Chibata, I. (1980). Immobilization of *Brevibacterium flavum* with carrageenan and its application for continuous production of L-malic acid. *Enzyme Microbial Technology*, **2**, 330-36.

Tanaka, A., Tosa, T., Kobayashi, T. (1993). Industrial application of immobilized catalysts. (Tanaka, A., Tosa, T., Kobayashi, T., eds.). New York: Marcel Dekker.

Tijsterman, J.A. (1993). Product drying, conditioning and stabilisation. In: Recovery of bioproducts. (Schmidt-Kastner, G., Sell, D., Biedermann, K. Brocklebank, M.P., Jungbauer, A. and Tijsterman, J.A., eds.). London: SCI.

Trevan, M. (1987). Diffusion limitation- friend or foe ? *Trends in Biotechnology*, **5**, 7-8.

van den Tweel, W. (1994). Biocatalyst production. In: Applied Biocatalysis (Cabral, J.MS., best, D., Boross, L. and Tramper, J., eds.). Switzerland: Harwood Academic Publishers.

Vulfson, E.V. (1993). Enzymatic synthesis of food ingredients in low-water media. *Trends in food science and technology*, **4**, 209-215.

Wardle, A.P. (1994). 3rd. ed. Process Control. In: Chemical Engineering 3. (Richardson, J.F. and Peacock, D.G., eds.). Oxford: Pergamon Press.

Wardle, A.P. (1994). 3rd. ed. Sensors for Measurement and Control. In: Chemical Engineering 3. (Richardson, J.F. and Peacock, D.G., eds.). Oxford: Pergamon Press.

Watanabe, I.; Satoh, Y. and Enamoto, K. (1987a). Screening, isolation and taxonomical properties of microrganisms having acrylonitrile-hydrating activity. *Agriculturial and Biological Chemistry*, **51**, 3193-3199.

Watanabe, I. (1987b). Acrylamide production method using immobilized nitrilase-containing microbial cells. *Methods in enzymology*, **136**, 523-530.

Webb, C., Black, G.M. and Atkinson, B. (1986). Process engineering aspects of immobilized cell systems. Rugby: Pergammon Press.

Wherry, T.C. (1982). Process Control. In: Chemical Engineers' Handbook. 5th ed. (Perry, R.H. and Chilton, C.H., eds.). London: McGraw-Hill.

Wood, A. (1993). Acrylics: Versatile chemistry adapts to growth markets. *Chemical Week*, December, 22-24.

Worstell and Ginestra, J.M. (1993). Properly size fixed-bed catalytic reactors. *Chemical Engineering Progress*. June, **89**, 31-37.

Wyatt, J.M. and Linton, E.A. (1988). The industrial potential of microbial nitrile biochemistry. *CIBA Foundation Symposium*, **140**, 32-48.

Yamada, H. (1993). Microbial Reactions for the production of useful organic compounds. *Chimia*, **47**, 69-74.

Yamamoto, K., Tosa, T., Yamashita, K. and Chibata, I. (1976). Continuous production of L-malic acid by immobilized *Brevibacterium ammoniagenes* cells. *European Journal of Applied Microbiology*, **3**, 169-183.

Young, R.J. and Lovell, P.A. (1991). 2nd. ed. Introduction to Polymers. London: Chapman and Hall.

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AMIDASE ACTIVE WHOLE CELLS OF *Corynebacterium nitrilophilus* FOR AMMONIUM ACRYLATE PRODUCTION

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SUMMARY

Corynebacterium nitrilophilus amidase was studied with a view to it's use in ammonium acrylate production. Treatment of whole cells with 1 M acrylamide and 2 M ammonium acrylate solutions significantly reduced amidase activity. Immobilized *C. nitrilophilus* cells were used in batch and continuous bioreactors. Operation of the continuous reactor was found to be the most convenient way of producing steady state conditions for the study of enzyme stability.

INTRODUCTION

Acrylic acid and its salts are important commodity chemicals produced on a very large scale annually for the manufacture of a wide variety of polymers including flocculants, adhesives, thickeners and superabsorbents. The ammonium salt of acrylic acid of high purity has been produced under mild conditions by the hydration of acrylonitrile to the intermediate acrylamide by nitrile hydratase, followed by it's hydrolysis with bacterial amidase (DiGeronimo and Antoine, 1976; Bui *et al.*, 1984). Bacterial nitrile hydratases have been used commercially for over a decade for the conversion of acrylonitrile to acrylamide (Kobayashi *et al.*, 1992). Because acrylonitrile and acrylamide diffuse through the bacterial cell wall (Wyatt and Linton, 1988), immobilized whole cells with very high nitrile hydratase activity, stable to concentrated solutions of substrate and product are employed (Kobayashi *et al.*, 1992). The enzymic conversion of either acrylonitrile or acrylamide to ammonium acrylate has yet to be exploited commercially. The nitrile hydratase of *C. nitrilophilus* hydrates a wide range of aliphatic and aromatic nitriles, whilst the co-induced amidase is capable of hydrolysing a variety of aliphatic and aromatic amides (Amarant *et al.*, 1989).

This paper describes the growth of amidase active *Corynebacterium nitrilophilus*, the stability of the amidase within whole cells to high concentrations of acrylamide and ammonium acrylate and the subsequent use of immobilized cells in batch and continuous reactors for the conversion of acrylamide to ammonium acrylate.

EXPERIMENTAL

Organism and Culturing Conditions *Corynebacterium nitrilophilus* (NCIMB 11594) was grown either on a minimal medium (pH 7.2), containing (in g/l): K_2HPO_4, 7; KH_2PO_4, 3; acetamide, 2; $MgSO_4.7H_2O$, 0.5; $CaCl_2.6H_2O$, 0.2; trace metals solution, 5ml; (Bauchop and Elsden, 1960); vitamins solution, 1ml; (Miller and Knowles, 1984); or a nutrient broth medium (Oxoid), supplemented with acetamide, 5 g/l. Fermentation studies were carried out at 30°C in a 20 l laboratory fermenter (Bioengineering). Bacteria were harvested during exponential growth by centrifuging at 10000 x *g* for 10 min. at 4°C and then washed twice with physiological saline.

Enzyme Stability Tests Whole cells (250 mg dry weight) were suspended in 1 M acrylamide or 2 M ammonium acrylate in physiological saline solution (15 ml). The suspensions were incubated at 30°C for 0.5-2 h then centrifuged at 10000 x *g* for 10 min., washed twice with physiological saline, re-suspended in 50 mM sodium phosphate buffer at pH 7.0 and assayed as described below.

Immobilization Cells were immobilized in cross-linked polyacrylamide gel using a modification of the method described by Chibata *et al.* (1986). Cells suspended in 50 mM sodium phosphate buffer (pH 7.0) were cooled to 4°C and added to a mixture of acrylamide/N,N' methylenebisacrylamide (MBA), also cooled to 4°C, immediately before addition of the polymerisation initiating reagents; sodium sulphite and tert-butyl hydroperoxide (TBHP). The final concentration of reagents were: acrylamide, 15 % (w/v); MBA, 0.75 % (w/v); sodium sulphite, 0.08 % (w/v) and TBHP, 0.1 % (w/v). The cell/monomer mixture was poured into a petri dish which was cooled by standing in an ice bath as the polymerisation took place. The resulting gel was cut into cuboids of dimensions 4×4×2 mm^3 using a scalpel and washed in 50 mM sodium phosphate buffer (pH 7.0), prior to use.

Determination of Amidase Activity Amidase activity was determined by stirring washed free or immobilized cells in a jacketed vessel in 50 mM or 500 mM acrylamide respectively, at 30°C in 50 mM sodium phosphate buffer (pH 7.0). The reaction was followed by determining the concentration of ammonium ions produced as described by Fawcett and Scott (1960), or by measurement of the rate of conductivity change due to the production of ammonium acrylate. The amidase specific activity (U/g) was defined as µmoles of ammonium acrylate formed per minute per gram of dry cells.

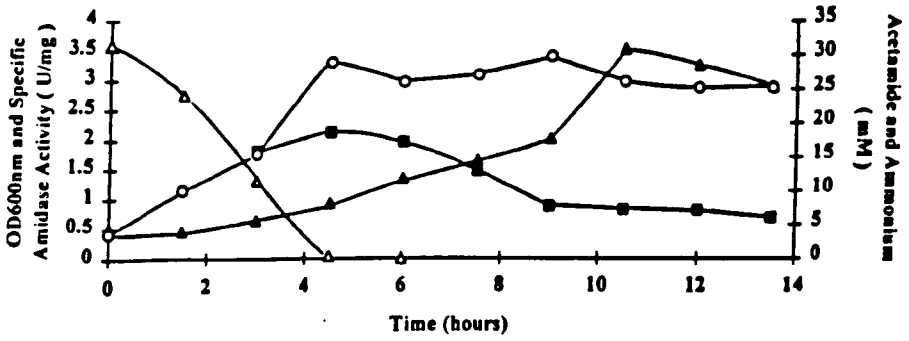
Operation of an Immobilized Cell Continuous Stirred Tank Bioreactor Immobilized cells were suspended in water in an air-tight 250 ml round bottom flask maintained at the required temperature by means of a cooling/heating finger and agitated with a 13mm magnetic stirrer bar. Upon acrylamide addition the rise in ammonium acrylate concentration was followed by measurement of the conductance change. When the desired ammonium acrylate concentration was reached, the reaction mixture was pumped continually from the reactor so that the resulting partial vacuum created in the system caused acrylamide solution to be continually drawn into the reactor, thus maintaining a constant volume of liquid in the reaction vessel. The pumping rate was periodically adjusted to maintain the conductance, and thus the ammonium acrylate concentration, at the desired level.

RESULTS AND DISCUSSION

Specific activity of the amidase was monitored during the growth of *C. nitrilophilus* in both minimal medium and nutrient broth supplemented with acetamide (5g/l). The amidase specific activity in the minimal medium (Figure 1) was highest in early exponential phase and coincided with the maximum ammonium ion concentration and the complete disappearance of

acetamide. The specific activity dropped to 50% of the maximum as the culture approached stationary phase. Similar observations have been made for *N. rhodochrous* LL100-21, (Linton and Knowles, 1986) and *Brevibacterium* sp. R312 (Maestracci *et al.*, 1984).

Figure 1: Growth and Amidase Synthesis: *C. nitrilophilus* in Acetamide Minimal Medium.



In acetamide-supplemented nutrient broth, amidase activity was also highest in the early exponential phase of growth. The specific activity decreased slightly thereafter but not as significantly as in minimal medium. Thus the mechanism repressing amidase synthesis in minimal medium is not so evident in the complex medium. It would seem unlikely that ammonium ions in the medium were the cause of the repression, since higher levels were observed in nutrient broth where repression of amidase synthesis was less pronounced. The amidase of *Pseudomonas aeruginosa* is reported to be subject to catabolite repression by acetate and other metabolites, (Brammar and Clarke, 1964).

Table 1 shows the relative activity of the amidase of washed cells, harvested during exponential growth, when assayed with different concentrations of acrylamide at 30°C.

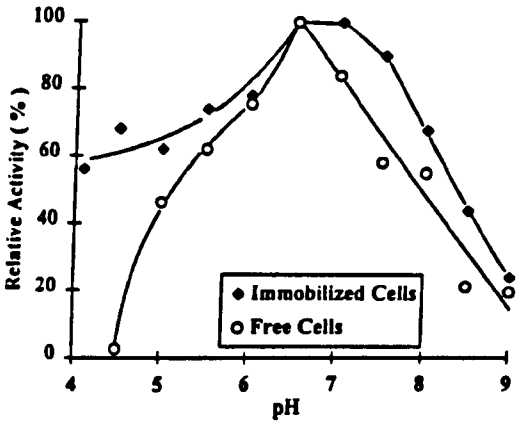
Table 1 : Amidase Activity of *C. nitrilophilus* At Different Acrylamide Concentrations.

Acrylamide Conc. (M)	Amidase Activity (U/g)	Relative Activity (%)
0.05	2000	87
0.7	2300	100
1.4	950	41
2.8	400	18
4.2	0	0

The amidase specific activity decreased markedly at higher acrylamide concentrations. To determine whether the observed reduction in amidase activity at high acrylamide concentration was due to deactivation, cells were assayed with 50 mM acrylamide following incubation in 1 M acrylamide and a cell washing process. The amidase activity was reduced by 44% after incubation in 1 M acrylamide for 0.5h, 50% after 1h and 71% after 2h. Cells were also incubated in 2 M ammonium acrylate for 0.5h, after which time 77% of the original activity was lost. This indicates that there is a permanent deactivation of the amidase.

The specific amidase activities at 30°C of free cells using 50 mM acrylamide and immobilized cells using 500 mM acrylamide were compared under different pH conditions (Figure 2). The amidase activity of the immobilized cells was found to be less sensitive to pH, though for both free and immobilized cells maximal activity was observed at pH 6.7. This is close to the pH of aqueous ammonium acrylate, removing the need for a buffer which would be an unwelcome contaminant. At pH 7, maximum activity was observed for both free and immobilized cells at 42°C and there was little appreciable difference between the two profiles.

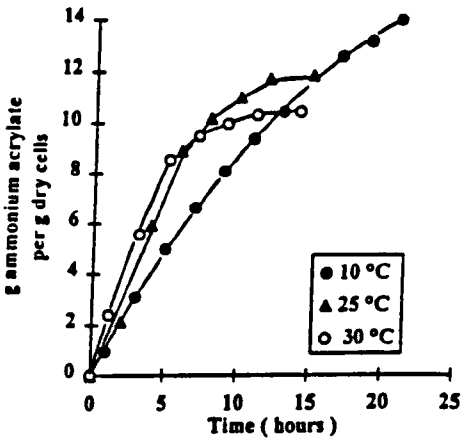
Figure 2: The effect of pH on the amidase activity of free and immobilized cells



The batch production of ammonium acrylate from 500 mM acrylamide by immobilized cells at three temperatures is shown in Figure 3. At high substrate concentrations the initial bioconversion rate would be expected to be zero order with the rate finally decreasing as a result of product inhibition or substrate limitation. The initial rate increases with increasing temperature as anticipated, however, in each case, the rate of conversion decreases progressively with time so that the reaction eventually ceases well before complete conversion. This is due to a progressive deactivation of the enzyme under the conditions of the reaction. It can be postulated that this is due to attack of the thiol groups of the enzyme by the vinyl group of acrylamide (Cavins and Friedman, 1968).

It is clear from Figure 3 that the loss of activity is much slower at the lowest temperature. Although the initial conversion rate is lowest at 10°C the slow loss of activity compensates for this, resulting in the highest overall yield of ammonium acrylate. Several workers studying the conversion of acrylonitrile to acrylamide (Hwang and Chang, 1989; Watanabe, 1987), have similarly reported an increase in enzyme stability as the temperature is reduced. This fact is significant when considering the operating conditions of a bioreactor. The deactivation of the enzyme is due to the presence of acrylamide and acrylate but it is impossible to distinguish the contribution of each since their concentrations are changing continuously throughout the batch conversion.

Figure 3: Yield of ammonium acrylate from cross-linked polyacrylamide gel entrapped cells operating in a batch reactor.



By controlling the acrylamide feed rate to an immobilized cell continuous stirred tank reactor, it was possible to hold the acrylamide and ammonium acrylate concentrations effectively constant and to study their effect on the activity of the amidase over a range of temperatures.

Figure 4 shows the decrease in reaction rate with time when the reactor was operated at a 50% conversion of a 500 mM acrylamide feed. The decay rate of many enzyme systems can be described by a first order expression from which an enzyme stability half-life can be calculated. The fall in reaction rate for an immobilized cell system can not necessarily be described by a first order decay plot. Substrate diffusion limitation into the immobilization matrix may cause cells at the centre of the particle to experience different concentrations of product and substrate to those at the surface of the particle leading to artificially high stability (Klein, 1988). For the experiments performed in the CSTR a low cell loading in the gel was used to minimise this effect. The first order decay plot, in Figure 4, describes the data well, and it is therefore likely that little diffusional resistance is occurring and the true biocatalyst half-life can be derived. Half-lives determined from runs at three different temperatures are shown in Table 2. It can be seen that as the temperature is reduced the amidase stability of the biocatalyst increases.

Figure 4: Rate profile for a CSTR operating at 30°C with cells entrapped in cross-linked polyacrylamide gel.

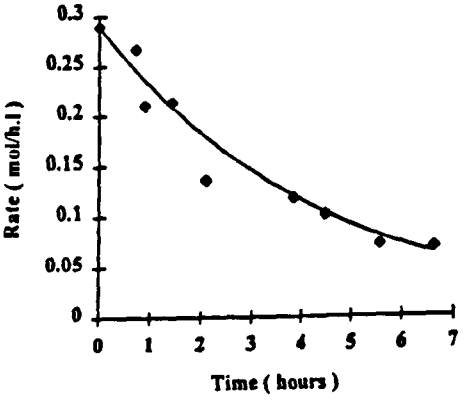


Table 2: Half-lives for a continuous stirred tank reactor operating with cells entrapped in cross-linked polyacrylamide gel.

Temperature (°C)	Half-life (hours)
15	12.4
25	4.6
30	3

CONCLUSIONS

Highest amidase specific activity in *C. nitrilophilus* was found in the early exponential phase of growth. The regulation of amidase synthesis in *C. nitrilophilus* has not been reported previously. Induction by an amide together with catabolite repression may be the controlling mechanisms.

The amidase specific activity of *C. nitrilophilus* is permanently reduced if the cells are pretreated by suspension in a solution of acrylamide or ammonium acrylate prior to assay. This toxic effect is manifest by a progressive decrease in activity reflected in a decreasing rate of bioconversion in both batch and continuous mode. The latter mode allows the contribution of each reagent and temperature to be quantified separately.

In a bioconversion designed for the commercial production of ammonium acrylate, deactivation of the type described would have to be minimised by a suitable choice of operating conditions. Optimum conditions could be deduced from a consideration of extensive stability data of the type presented here. Work is continuing to determine the effect of different acrylamide and ammonium acrylate concentrations on amidase activity, using an automated continuous stirred tank reactor and a comparative evaluation of other candidate amidases is also underway.

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REFERENCES

Amarant, T., Vered, Y. and Bohak, Z., (1989), Biotech. Appl. Biochem., 11, 49-59.

Bauchop, T. and Elsden, S. R., (1960), J. Gen. Microbiol., 23, 457-469.

Brammar, W. J. and Clarke, P. H., (1964), J. Gen. Microbiol., 37, 307-319.

Bui, K., Fradet, H., Arnaud, A. and Galzy, P., (1984), J. Gen. Microbiol., 130, 89-93.

Cavins, J. F. and Friedman, M., (1968), J. Biol. Chem., 12, 3357-3360.

Chibata, I., Tosa, T. and Sato, T. (1986), In: Manual of Industrial Microbiology and Biotechnology, Demain, A.L. and Solomon, N.A., eds. p. 218, Washington: American Society for Microbiology

DiGeronimo, M. J. and Antoine, A. D., (1976), Appl. Environ. Microbiol., 31, (6), 900-906.

Fawcett, J. K. and Scott, J. E., (1960), J. Clin. Path., 13, 156-159.

Hwang, J.S. and Chang, H.N. (1989), Biotech. Bioeng., 34, 380-386

Klein, J. (1988), In: Bioreactor immobilized enzymes and cells: fundamentals and applications, Moo-Young, M. ed. pp 1-8, Essex: Elsevier Applied Science.

Kobayashi, M., Nagasawa, T. and Yamada, H., (1992), Trends Biotechnol., 10, 402-408.

Linton, E. A. and Knowles, C. J., (1986), J. Gen. Microbiol., 132, 1493-1501.

Maestracci, M., Thiery, A., Bui, K., Arnaud, A. and Galzy, P., (1984), Arch. Microbiol., 138, 315-320.

Miller, J. M. and Knowles, C. J., (1984), FEMS Microbiol. Letts., 21, 147-151.

Watanabe, I. (1987), Methods Enzymol., 136, 523-530.

Wyatt, J.M. and Linton, E.A., (1988), In: Cyanide compounds in biology. (Ciba Foundation Symposium), 140 , 32-48, Chichester: Wiley.