Magnetically Stabilised Fluidised Bed Reactor For Biotransformations

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Abstract

A novel type of reactor, the magnetically stabilised fluidised bed reactor (MSFBR) aims to overcome the disadvantages of packed beds by increasing the bed voidage and linear velocity through the reactor. The fixation of enzymes to magnetic susceptible supports and placement in a magnetic field will introduce an extra degree of freedom in controlling the bioreactor. Applying a magnetic field increases the escape velocity of the particles allowing substantially higher flowrates through the reactor compared with a conventional fluidised bed.

A cost efficient method of producing magnetic particles has been developed to manufacture the particles to test the feasibility of the MSFBR as a biocatalytic reactor under various process conditions. Two enzymes, penicillin acylase and α-chymotrypsin have been successfully immobilised to the magnetic particles. The specific activity and operational stability for both enzymes were comparable to the respective commercially available enzymes.

A physical characterisation of both the magnetic particles and the MSFBR was undertaken. The horizontal magnetic field varied by 2% radially across the reactor. Bed expansion curves were completed to examine the effect of flowrate and magnetic field strength on the escape velocity of the particles and voidage of the bed.

The ability of the reactor to allow pH control has been tested by a first model system using the penicillin acylase catalysed conversion of Penicillin G to 6 aminopenicillanic acid. The performance of the MSFBR was comparable to that achieved in identical packed bed reactor experiments.

The ability to allow processing in the presence of solids has been tested by a second model system using a α-chymotrypsin catalysed reaction for the conversion of N-acetyl tyrosine ethyl ester to N-acetyl tyrosine. This model reaction also presented the problem of pH control. The MSFBR had shorter, 95% conversion times than identical runs in a conventional fluidised bed reactor. The reaction could not be operated in a packed bed reactor due to high pressure drops.
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Nomenclature

a  Radius of coils (m)
B  Magnetic Field Strength (Tesla, weber/m²)
D/UL  Dispersion coefficient (-)
H  Intensity of magnetic field (A/m)
I  Current (Amperes)
K_m  Michaelis Menton constant (mM)
K_IPAA  Inhibition constant for Phenylacetic acid (mM)
K_{6apa}  Inhibition constant for 6 amino penicillanic acid (mM)
K_{1h}, K_{IS}  Penicillin G substrate inhibition constant (mM)
n  Index in Richardson and Zaki equation (-)
N  Number of turns on helmholtz coil or solenoid
N_c  Number of Helmholtz coils
S  Substrate concentration (mM)
T  Temperature (°C)
u  Superficial linear velocity (m/s)
U_{mf}  Minimum fluidisation velocity (m/s)
\textit{u}_t  Terminal settling velocity (m/s)
V_{max}  Maximum enzyme reaction rate (μmol/min, U/g)
W_1...W_3  Mass (g)
Z  Bed Expansion (-)
\varepsilon  Bed voidage (-)
\theta_{95}  Time for 95% conversion (minutes)
\rho_r  Resistivity (ohm m)
\rho_{H2O}  Density of water (Kg/m³)
\rho_{MCP}  Density of magnetic carrier particle (Kg/m³)
\mu_0  Free permeability of space (Henry/metre)
\varphi  Magnetic flux (weber)
Abbreviations

6 APA  6 amino penicillanic acid
AT     N-acetyl-L-tyrosine
ATEE   N-acetyl-L-tyrosine ethyl ester
BSA    Bovine Serum Albumen
CSTR   Continuous stirred tank reactor
FB     Fluidised bed
GA     Glutaraldehyde
HMDA   1,6 – diaminohexane
IME    Immobilised enzyme
ISPR   In Situ Product Removal
MCP    Magnetic carrier particle
MSFBR  Magnetically stabilised fluidised bed reactor
PA – MCP Penicillin acylase immobilised to magnetic particles
PA     Penicillin acylase
PAA    Phenylacetic acid
PFR    Plug flow reactor
rpm    Revolutions per minute
RTD    Residence time distribution
STR    Stirred tank reactor
Chapter 1

Introduction
Chapter 1  Introduction

1.1 Biotransformations

The general definition of a biotransformation process is one in which a product is obtained from a substrate through one or more reactions catalysed by enzymes.

The majority of biotransformations involve the use of biocatalysts either as isolated enzymes or whole micro-organisms. Biological catalysts provide many advantages over non-biological conversions including substrate specificity, stereospecificity, regiospecificity and the mildness of the reaction conditions (Bailey and Ollis, 1986). Considering the high catalytic power and exceptional stereo, regio and chemoselectivity of enzymes, the small number of commercial applications in the pharmaceutical industry is quite surprising. The incorporation of enzymes to the pharmaceutical industry has been limited in the past by the high cost, relative instability in industrial conditions and competition with established chemical techniques (using fully paid back capital cost equipment) (Zaks and Dodds, 1997). Advances in enzyme purification and immobilisation have attempted to overcome these limitations. The use of automated screening and recombinant techniques have accelerated the selection and specificity of enzymes.

Conventionally most biochemical processes use a soluble enzyme or cell cultures to convert a substrate into product. The application of free enzymes as a viable process option has been limited by washout effects, problems with contamination of the product and further purification of the enzyme. In order to overcome these limitations, immobilised biocatalyst systems were introduced (Ballesteros et al, 1994), Chibata et al, 1986, Warburton et al, 1972, Zaborsky 1973).

Traditionally each individual industrial immobilised biocatalyst system has been developed separately. As each enzyme behaves in a unique way, a biocatalyst system was geared towards that specific enzyme. This lengthens the time required to
develop a biocatalyst system as the process is essentially starting from scratch each time. In an attempt to reduce biotransformation development time, Woodley and Lilly (1996) have outlined a generic approach to be applied to the biotransformation process. This includes analysis of various parameters from enzyme kinetics, reactor type, economics and indeed all relevant factors in the biotransformation development process. By selection of the appropriate parameters the time required for biocatalytic reactor selection can be reduced.

1.2 Immobilised Biocatalyst Technology

A biocatalyst is termed immobilised if its mobility has been restricted by chemical or physical means. This artificial limitation of mobility may be achieved by widely differing methods, such as binding the biocatalysts to one another or to carrier substances by entrapping in the network of a polymer matrix or by membrane confinement (Hartmeier, 1988).

In principle immobilisation of a biocatalyst separates it from a process stream. Immobilisation to a particular support enables easier recovery of the biocatalyst post treatment by separation methods of filtration or centrifugation. Similarly, the biocatalyst can be retained within the reactor using an appropriate outlet filter.

Most procedures for immobilisation modify the properties of the enzyme. In many cases this may be advantageous by increasing stability and possibly reducing substrate inhibition by the introduction of diffusional limitations. The enzyme can be protected from the high substrate concentrations in the bulk solution by the slow diffusion of substrate through the immobilised enzyme. However immobilisation can also have a detrimental effect on the enzyme process by reducing activity and destabilisation. It is only after careful deliberation on the suitable choice of immobilisation in terms of activity changes, stability effects and an economic evaluation that an appropriate methodology for immobilisation can be found.
Forcing the substrate solution to flow through the interparticle voidage of the immobilised beads or stirring the beads can reduce the external liquid mass transfer resistance.

1.3 Immobilisation Techniques

There are three techniques available for biocatalyst immobilisation. Firstly the biocatalyst can be adsorbed to a retaining support material. The second method involves entrapment of the biocatalyst in either a microcapsule, support medium or within a reactor. The third procedure and by far the most popular commercially, requires chemical linkage of the biocatalyst by covalent attachment to the support. Although these are the main types of immobilisation, they are by no means mutually exclusive and many newly developed hybrid systems have surfaced incorporating a mixture of these systems. Cross linked enzyme crystals (CLEC's) are also a useful development in enzyme immobilisation (Margolin, 1996, Vaghjiani et al, 2000).

Adsorption of an enzyme to a support is widely hailed as the easiest procedure with a wide choice of supports including glass, ion exchange resins, celluloses, agaroses and Sephadexes (Powell, 1990). In fact, the earliest immobilisation technique developed by Nelson and Griffin in 1916 used charcoal as a support for invertase. The most important feature of this mechanism is to ensure a large surface area for adsorption to maximise protein loading. Using a small particle size and choosing a porous support material can attain this. Adsorption of the enzyme to the support is by a range of forces including hydrophobic linkage to simple Van der Waal’s forces. All of the bonds can be broken by selective changes in environment from pH, temperature, ionic strength and solvent change. The ease of bond breakage is a major drawback of this type of immobilisation. However, in organic media where proteins are not usually soluble desorption does not occur as readily.

A range of methods can achieve immobilisation of enzymes by entrapment. The biocatalysts are embedded in natural or synthetic polymers, mostly of gel like structure.
Covalent attachment of the enzyme to a support often results in greater stabilisation of the protein. The atoms in covalent binding are linked by means of shared electron pairs. This can be manipulated to produce a tight association between the biocatalyst and carrier support. The amino, carboxyl, hydroxyl and phenolic groups have all been used for covalent binding. Some functional groups such as the sulphydryl group react directly with the relevant groups on the carrier material whilst others such as hydroxyl groups require ‘activation’ before attachment. Common activating reagents include cyanogen bromide (which activate hydroxyl groups on carrier) (Hartmeier, 1988).

The carrier support materials can be inorganic, natural polymers or synthetic polymers. Inorganic carriers and synthetic polymers are advantageous if harsh conditions are required for removal of protein when reuse of the support is essential (Powell, 1990). More popular are natural polymers such as celluloses and starch.

To date, little research has focused on trying to manipulate the mixing behaviour in the immobilised biocatalyst reactor. By immobilisation onto a magnetic support the position, strength and length of the magnetic field around a biocatalyst reactor could influence the mixing behaviour of the enzyme particles to potentially improve productivity.
1.4 Biocatalyst Reactor Systems

1.4.1 Biocatalyst Reactor Selection

There are a variety of parameters that must be taken into account when considering which bioreactor to perform a reaction in. Principally the enzyme kinetics and the reaction environment determine the type of reactor required although economies of scale play an important role. There are many hybrid reactors which have been developed for enzyme reactions such as membrane reactors although there remain two basic reactor types: the stirred tank reactor and the packed bed reactor.

1.4.2 Stirred Tank Reactors

The conventional stirred tank reactor has been a popular choice for both enzyme and whole cell biotransformations. This is mainly because of familiarity with this type of reactor system although there are many process advantages. The pH in the reactor is easily maintained by a pH control system which is absent or difficult to operate in a bed type reactor. The substrate concentration in the reactor can be manipulated by using a fed batch system if substrate inhibition is a determining factor in the biotransformation. Also if product inhibition is a significant factor, *In situ* product removal (ISPR) can be applied by recirculating the process stream through a column where the product is continuously removed.

1.4.3 Bed Reactors

Bed reactors are very common for performing reactions using particulate biocatalysts. Depending on the nature of the particles constituting the bed and the method in which the substrate flows through it the reactors are termed packed bed, fluidised bed or expanded bed reactors.
Introduction

The packed bed reactor is the most popular at present for immobilised biocatalysts since it allows, depending on the kinetics, the use of catalysts at the highest possible density. Consequently the volumetric productivity in a packed bed is greater than for other reactor configurations. However not all biocatalysts and reactions are suitable for use in a packed bed. The structural properties of the biocatalyst should be such that little pressure is required to allow substrate flow through the bed. Even after extensive use, the bed should not have a high pressure drop. In those reactions that create a gas, such as ethanol production with yeast cells, the gas produced may impede optimal contact between substrate and particles.

A gradient is established in the packed bed, with a higher concentration of substrate at the inlet and a lower concentration at the outlet. This distribution of residence times of the different parts of the fluid in the reactor can be held within very close limits, which makes possible an almost complete transformation of substrate into products (Hartmeier, 1988). For reactions involving a pH change, the packed bed can only be chosen if the pH range within the bed does not exceed the limits tolerated by the biocatalysts.

1.4.4 Fluidised Bed Reactors

1.4.4.1 Introduction to Fluidisation

Fluidisation is the operation by which solid particulates are placed in a fluid like state through suspension in either a gas or liquid environment. At low flowrates, when a fluid is passed upwards through a fine particle bed, the fluid merely filters and percolates through the small void spaces between stationary particles. This is the definition of a fixed bed. At higher flowrates, the particles move apart and begin to vibrate and expand in the direction of flow. This is the definition of an expanded bed.

In order to create stable fluidisation of particles within a liquid, the weight of the particles must equal the fluid - particle interactions. This is achieved when the flow
of upward fluidisation liquid exceeds a minimum value termed $U_{mf}$ the minimum fluidisation velocity. The substrate enters from below and is forced upward through the fluidised bed, keeping the biocatalysts in a state of loose suspension by the substrate flow, given the condition that the density of the particles is greater than that of the substrate.

1.4.4.2 Quality of Fluidisation

The ease with which particles fluidise and the range of operating conditions that sustain fluidisation vary widely among liquid systems and numerous factors can affect this. The size and distribution of particles has an affect on fluidisation. Generally the finer the particle the more likely the particles are to clump and form agglomerates. To alleviate this problem, the fluidised bed can be agitated either mechanically (Beveridge, 1997) or operated at higher velocities. Fine particles of a wide size distribution can be fluidised in a wide range of flowrates allowing flexible operation with deep beds. On the contrary, beds of large uniformly sized solids tend to fluidise poorly with bumping, spouting and slugging which can have serious structural implications on large scale beds (Kunni and Levenspeil, 1991).

1.4.4.3 Mixing regimes in Fluidised Beds

Under certain operational conditions, liquid flow in the fluidised bed bioreactor can approach plug flow, this is a kinetic advantage over completely mixed systems, such as a continuous stirred tank reactor, for reactions presenting product inhibition. Fluidised beds operated at high recirculation rates approach a well mixed system implying that reaction kinetics play an important role in the design and analysis of fluidised beds. Configurations favouring completely mixed regimes will be appropriate for substrate inhibited reactions which can be fed. Manipulation of the aspect ratio of a fluidised bed, producing a long thin column has resulted in a new breed of fluidised beds, commonly known as expanded beds, mimicking plug flow conditions well and are used industrially in affinity chromatography for protein
purification (Bailey and Ollis, 1986). It is these plug flow conditions, which may be advantageous in product inhibited enzyme reactions.

1.4.4.4 Design and Operation of Fluidised Beds

The physical construction of fluidised beds greatly influences the fluidised bed operation especially in terms of the aspect ratio. At high values of the aspect ratio the design will favour plug flow conditions. The factors, which primarily decide the bed expansion, are the particle diameter and flowrate through the bed. The effect of flowrate through the bed is directly proportional to the bed expansion.

From the mass transfer benefits of a fluidised bed bioreactor configuration, it is possible to gain higher levels of overall productivity than in packed bed reactors seemingly contradicting the lower volumetric fraction of immobilised biocatalyst particles in a fluidised bed (Klein and Kressdorf, 1983).

Fluidised beds are often used in a process to avoid excessive pressure drops prevalent in packed bed reactors. However, the range of operating velocity is severely restricted between the minimum fluidisation velocity and the maximum velocity where particles begin to leave the reactor (escape velocity). In some cases the enzymatic reaction rates are low requiring high liquid residence times. In order to obtain these large residence times the superficial linear velocity of the particles has to be high. Therefore to enable fluidisation external liquid recirculation is required. When compared with the more traditional packed bed reactor, fluidised bed bioreactors can be operated with smaller size particles without the drawbacks of clogging, high liquid pressure drop, creation of preferential flow paths (channelling) or compression of particles (Godia and Sola, 1995). The use of smaller particles then reduces internal diffusion limitations and the increased levels of mixing helps external mass and heat transfer from the liquid bulk to the solid particles.

For enzyme reactions with inhibition problems, an important criterion for effective transformation in the fluidised bed is the plug flow of the liquid in the bed. Ideally the residence time of all substrate molecules should be equal. Maintenance of plug
Introduction

flow behaviour is particularly difficult during scale up. In gas producing/consuming and solid containing reactions this is particularly difficult and often the flow reaches a well mixed bed or in fact does not flow at all. It is these problems of fluidised beds that drove the development of a novel type of reactor, a magnetically stabilised fluidised bed reactor.

1.5 Magnetically Stabilised Fluidised Bed Reactor

1.5.1 Magnetic Carrier Technology

Magnetic carrier technology is an area of increasing industrial interest especially in the field of biotechnology. It is possible to manipulate the chemical, physical and surface characteristics of magnetic carriers to allow selective or non selective attachment of molecules, colloidal particles and whole cells to the carrier (Pieters et al, 1991).

The basic concept of a magnetically stabilised bed was outlined in a ground breaking paper by Rosenweig (1979). In these novel beds, ferromagnetic particles were subjected to a spatially uniform magnetic field positioned axially to the fluidising gas flow. The main advantage of this type of reactor was to prevent bubbling behaviour that destabilises the gas fluidised bed at higher flowrates and which occurred in the conventional bubbling bed commonly used at the time.

1.5.2 Application of Magnetic Carriers

There are numerous types of magnetic particles that can be utilised in bioprocessing but most use natural or synthetic polymers often with further surface derivatisation to increase specificity. Within bioprocessing, magnetic carriers have been used in wastewater treatment (Cooper and Atkinson, 1981) and in fact this is the oldest application of this technology. Magnetic carrier particles have previously been adopted for use in immobilisation of enzymes by Robinson and coworkers (1973) and applications in drug delivery (Widder, 1978). Miyabashi and Mattiasson (1988)
Introduction

found improvements in biosensors using magnetic particles. Magnetic particles have been used to a greater extent in affinity separation of biomolecules (Dunnill and Lilly, 1974, Burns and Graves, 1985).

For immobilisation to biocatalysts, magnetic carrier materials consist of two components namely a polymeric carrier for enzyme immobilisation and a magnetic material. The magnetic material can be taken from different groups; ferrimagnets, ferromagnets, paramagnets and diamagnets. Both ferromagnets and ferrimagnets have been used in enzyme immobilisation previously (Defilippi, 1982 and Sada et al, 1981). Industrial companies such as Pharmacia (www.apbiotech.com) and Upfront (www.upfront-dk.com) offer magnetic particles for protein adsorption in expanded beds.

Magnetic particles are utilised in a wide range of industries using various attachment procedures. Table 1.1 shows the various types of magnetic particle (Law, 1994)

1.5.3 Application of magnetic carriers in a fluidised bed

At the minimum fluidisation velocity, the particles in the bed become fluidised. It has been shown that the minimum fluidisation velocity is independent of the applied field intensity (Hu and Wu, 1987). After applying a magnetic field, the bed will expand at the minimum fluidisation velocity but without relative movement of particles. Under these conditions, the bed is deemed to be stably fluidised. Any subsequent changes in flowrate will increase the bed height resulting in particle movement and the bed becomes unstably fluidised.

The development of a Magnetically Stabilised Fluidised Bed Reactor (MSFBR) by Webb and coworkers (1996) for use in immobilised enzyme systems showed increases in liquid mass transfer capabilities of the reactor. This increase in mass transfer is due to the increased flowrates that can be achieved through the reactor.
Introduction

This type of reactor has been used by workers before, Burns and Graves, 1985 in bioseparations, Hu and Wu, 1987 for immobilised cells, Bramble and coworkers (1990) for plant cell culture and Sada and coworkers (1981) for immobilised enzymes with many showing advantages which will be discussed later in section 1.5.8.

<table>
<thead>
<tr>
<th>Magnetic Particle</th>
<th>Immobilisation Technique</th>
<th>Manufacturing Method</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solid Particle</td>
<td>Adsorption</td>
<td>Crushed magnetite</td>
<td>Waste water treatment</td>
</tr>
<tr>
<td>Coated Particle</td>
<td>Adsorption, Ion exchange and Covalent binding</td>
<td>Silane derivatisation, Polymer adsorption</td>
<td>Enzyme Immobilisation</td>
</tr>
<tr>
<td>Encapsulated Particle (a) Non-porous polymer</td>
<td>Adsorption, Ion exchange and Covalent binding</td>
<td>Emulsion polymerization</td>
<td>Cell sorting Affinity separation</td>
</tr>
<tr>
<td></td>
<td>(b) Porous polymer</td>
<td>Entrapment</td>
<td>Ionic gelation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Enzyme immobilisation Drug delivery</td>
</tr>
<tr>
<td>(c) Microcapsule</td>
<td>Entrapment</td>
<td>Phase separation</td>
<td>Enzyme immobilisation Drug delivery</td>
</tr>
<tr>
<td>Non porous Polymer coated particle types (a) Polymer coating</td>
<td>Adsorption, Ion exchange and Covalent binding</td>
<td>Polymer adsorption and cross linking</td>
<td>Affinity separation Drug delivery</td>
</tr>
<tr>
<td></td>
<td>(b) Polymer grafting</td>
<td>Adsorption, Ion exchange and Covalent binding</td>
<td>Co-γ radiation</td>
</tr>
<tr>
<td>Magnetic cells and liposomes (a) Erthrocytes</td>
<td>Adsorption, Covalent binding</td>
<td>Blood cell fractionation</td>
<td>Cell sorting</td>
</tr>
<tr>
<td></td>
<td>(b) Magnetotactic bacteria / algae</td>
<td>Adsorption, Covalent binding</td>
<td>Fermentation</td>
</tr>
<tr>
<td></td>
<td>(c) Magnetoliposome</td>
<td>Adsorption, Entrapment</td>
<td>Phospholipids adsorption</td>
</tr>
</tbody>
</table>

Table 1.1 : Magnetic particle classification
1.5.4 MSFB Reactor Configuration

*Figure 1.1* illustrates the basic layout of the fluidised bed reactor with copper coils and a flow distributor plate.

A low magnetic field is applied to the copper coils to gain magnetism. The magnetic carrier particles are held within a finite region in the reactor. Prior to full stabilisation of the MSFBR, both the magnetic field strength and flowrate can be used to alter the bed expansion height. In a conventional fluidised bed the expanded bed height is predominantly controlled by the flowrate.

For ideal performance of a MSFBR, the bed expansion must be minimised whilst extending the stable fluidised regime to higher flowrates but also maintaining a low pressure drop across the bed. Once the bed is fully stabilised, increases in magnetic field have little effect on the bed expansion (Siegell, 1987).
Figure 1.1: Magnetically stabilised fluidised bed reactor (MSFBR)
1.5.5 Definitions of Magnetism

In order to understand the operating conditions of the MSFBR the following definitions commonly used in magnetics are outlined below:

**Magnetic field** : The region surrounding a magnetised body which is capable of inducing magnetism in other bodies. Either a magnetised body or an electric current can generate the magnetic field.

**Intensity of the Magnetic field (H)** : If a unit pole is placed at a specific point in a magnetic field it will be acted upon by a force that is taken as a measure of the intensity of a magnetic field (H). The intensity of the magnetic field is measured in Ampere/metre.

**Unit pole** : This is used to define the intensity of the magnetic field. It is specifically an entity that will repel an equal and similar pole placed 1 metre away (in a vacuum) with a force of 1 Newton.

**Pole strength** : This is defined as the strength (or power) of a magnet which is equal to the number of pole units.

**Lines of Magnetic Force** : These field lines show the path taken by a unit pole in a magnetic field due to the magnetic forces acting upon it. The number of lines of force per unit area is equal to the strength or the intensity of the field at that point.

**Magnetic Flux (\(\Phi_m\))** : The number of field lines of force emanating from the north pole face of a magnet per unit area. The unit is weber or tesla, T. The number of lines of force emanating from a pole of strength m is \(4\pi mT\).
1.5.6 Behaviour of magnetic carriers in a MSFBR

A very different behaviour is observed in the MSFBR compared to a fluidised bed due to the structural changes in the reactor bed. The arrangement of the particles is different due to the action of both the magnetic field and fluid flow. Casal and Arnaldos (1991), found that particles within a magnetically stabilised gas fluidised bed arranged themselves in chains following the magnetic field lines. According to Casal and Arnaldos (1991) this led to a significant increase in the bed voidage in gas fluidised beds. Figure 1.2 shows the field lines of the MSFBR operated in plug flow holding the particles in chains up the reactor.

Figure 1.3 demonstrates the effect of changes in magnetic field on fluidisation characteristics of a typical magnetically stabilised fluidised bed shown by Goto and coworkers (1995). Goto and coworkers (1995) found the distinction between an unstable and stable fluidised regime for a fluidisation velocity of $1.7 \times 10^3$ m/s corresponded to a bed expansion of 0.08 m (33% expansion above settled bed height) at a magnetic field strength of 10 kA/m.
Magnetic Field Line

Magnetic coil showing direction of current

Direction of Liquid Flow in MSFBR

Figure 1.2: Magnetic Field Lines in the MSFBR
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(a) Operating window for stable fluidisation of a MSFBR and (b) effect of fluidisation velocity and magnetic field on fluidisation 'regimes'

Figure 1.3: (a) Operating window for stable fluidisation of a MSFBR and (b) effect of fluidisation velocity and magnetic field on fluidisation 'regimes'
Introduction

Figure 1.3 shows behavioural observations of a magnetically stabilised fluidised bed and is shown here to demonstrate the effects of magnetic field strength and fluidisation velocity on mixing regimes in the bed. The definitions of roll cell and random motion are as follows. A roll cell mixing of the particles and fluid is circulatory movement of particles in axial layers along the bed. Random motion is gulf streaming and circulatory movement of the whole bed (Goto et al, 1995). An unstable fluidised bed is defined as a regime where substantial solids mixing occurs and a turbulent liquid flow is in place. From these observations it can be seen for reactions requiring plug flow mixing, a roll cell mixing of particles in axial layers is desirable. For reactions with well mixed requirements, a random motion of particles whereby the whole bed moves in a circulatory motion is desirable.

A scale up problem Siegell, 1987 found when investigating a liquid fluidised magnetically stabilised bed was the effect the distributor plate design has on the transition between the stable and roll cell mixing regimes. Ensuring a uniform distribution and size of pores in the distributor plate is paramount in the design of the plate and it seems alteration of the pore size will influence the mixing behaviour observed.
1.5.7 Aggregation of magnetic carrier particles

Aggregation is a particular problem for magnetic carriers and primarily results from residual magnetism of the particles. Magnetic carriers when released from a magnetic field clump together. A number of methods have been developed to prevent aggregation (Law, 1994) and these are shown in Table 1.2.

<table>
<thead>
<tr>
<th>Method</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Using superparamagnetic properties</td>
<td>Bioseparations</td>
</tr>
<tr>
<td>Polymer Coating</td>
<td>Bioseparations</td>
</tr>
<tr>
<td>Using curie temperature</td>
<td>Magnetic Labelling</td>
</tr>
<tr>
<td>Ultrasound treatment</td>
<td>Enzyme immobilisation</td>
</tr>
<tr>
<td>High flowrates between runs</td>
<td>Research only</td>
</tr>
<tr>
<td>Use small particles (2 μm)</td>
<td>Immunoassays</td>
</tr>
</tbody>
</table>

Table 1.2: Methods for preventing aggregation in magnetic carrier particles

Specifically in enzyme immobilisation Dekker (1989) found that the use of ultrasound before magnetic particles are placed in a magnetic field reduced the effect of agglomeration considerably. Cocker and coworkers (1997) recently found that the addition of sorbitan sesquioliate acting as a stabiliser to polyacrylamide / magnetite beads reduced bead agglomeration. The sorbitan placed a coating around the particles reducing the effect of residual magnetism. The addition of a stabiliser or polymer coating to particles that will be immobilised would have to be traded with the reduction in immobilisation efficiency that would result from addition of other compounds to the carrier material. The effect of agglomeration of the particles would have to be quite significant to justify the need for a coating.

Siegall (1987) developed a more easily applied and less intrusive method for prevention of aggregation. Between each experiment the bed was operated at a high liquid velocity without the magnetic field on. This reduced the effects of past experiments at high magnetic field. This method seems to separate the particles by
high flowrates forcing the particles to separate. As a short term solution this appears effective, although the effect of this method on the total process time would have to be considered before adopting.

1.5.8 Perceived advantages of the magnetically stabilised fluidised bed reactor

The main advantage of operating a fluidised bed by magnetic stabilisation is the ability to operate at higher velocities. The escape velocity, defined as the velocity of the substrate through the bottom of the column at which the particles begin to leave the reactor, is much higher in a magnetically stabilised fluidised bed. The magnetic field 'holds' the magnetic particles in place in the reactor. The obvious advantage that this phenomenon gives is increased productivity in terms of product transformation per unit time. However the kinetics of the reaction will determine the optimum contact time in the reactor and therefore define the maximum operating flowrate. Moffat and coworkers (1996) reported that under a 40A magnetic field the increase in operable flowrate in a MSFBR is as much as five fold. Webb and coworkers (1994) found that the escape velocity of the particles from the bed was proportional to the magnetic field intensity squared.

Although a fluidised bed can handle solids satisfactorily, the percentage solid content in the bed at any one time can be increased by using a MSFBR. Due to the fact that the Magnetic Carrier Particle's (MCP's) are held in place by the magnetic field, the solids cannot drag smaller biocatalyst particles out of the reactor as can happen at high solid concentrations in the conventional fluidised beds.

Since the bed is fluidised the pressure drop through the column remains the same regardless of the flowrate and is approximately equal to the bed weight divided by the column cross sectional area (Burns and Graves, 1988). Estevez and coworkers (1995) observed that operation of the fluidised bed under a magnetic field causes a slight decrease in the pressure drop across the bed as compared with a conventional fluidised bed, which builds on one of the main advantages of a fluidised bed over a fixed bed: a reduced pressure drop.
The operational conditions can be manipulated to create either a reactor mimicking plug flow conditions or a well mixed system. If plug flow conditions are required in a reactor for successful operation, but a packed bed reactor is not a viable solution due to operating difficulties (such as gas consumption/production or pH control limitations), a MSFBR can provide this condition. The magnetic field can reduce axial mixing of particles under the appropriate operating conditions. These conditions consist of alteration of the magnetic field intensity and positioning of the magnetic coils. A basic definition of axial mixing is the number of separate mixing stages between adjacent particles along the bed. In a plug flow mode the extent of axial mixing must be minimised to give a large number of separate mixing stages along the column. This low degree of dispersion for both phases allows many theoretical stages to be contained in a single column reactor as opposed to a normal single stage fluidised bed. This effect is very desirable in adsorption chromatography (Burns and Graves, 1985) and industrial expanded beds for these purposes are available, although trial and error methods are often incorporated to achieve low axial mixing conditions. The solid/liquid contacting characteristics of the MSFBR under these conditions are therefore more similar to that of a packed bed, than a fluidised bed. For immobilised enzyme catalysed reactions, inhibition problems with both substrate and product render this type of operation attractive. As an alternative to fixed bed operation, a magnetically stabilised fluidised bed was used by Sada and coworkers (1981) with immobilised urease and gave similar or higher conversions in comparison with the more traditional fixed bed reactor. The general advantages of a MSFBR over a packed bed are increased bed voidage (increasing substrate availability), decreased pressure drop and better plug flow conditions.

*Figure 1.4 and Table 1.3* show comparisons of conventional reactors with the magnetically stabilised fluidised bed. *Figure 1.4* illustrates the simplified type of mixing in each reactor and the type of mixing obtained in the MSFBR. *Table 1.3* illustrates the potential advantages of using the MSFBR technology.
Figure 1.4: Comparison of biocatalytic reactor types

- **Packed Bed Reactor**: Plug Flow
- **Batch Stirred Tank Reactor**: Well Mixed
- **Fluidised Bed Reactor**: Well Mixed
- **Magnetically stabilised fluidised bed reactor**: Well Mixed, Plug Flow

Magnetic coil showing direction of current
### Introduction

<table>
<thead>
<tr>
<th></th>
<th>Packed Bed</th>
<th>Stirred Tank</th>
<th>Fluidised Bed</th>
<th>MSFBR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ease of operation</td>
<td>●</td>
<td>●</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>Ease of Biocatalyst replacement</td>
<td>○</td>
<td>●</td>
<td>●</td>
<td>●</td>
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<tr>
<td>Pressure drop</td>
<td>●</td>
<td>○</td>
<td>○</td>
<td>○</td>
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<tr>
<td>Mixing characteristics</td>
<td>○</td>
<td>●</td>
<td>○</td>
<td>●</td>
</tr>
<tr>
<td>Plug flow characteristics</td>
<td>●</td>
<td>○</td>
<td>○</td>
<td>●</td>
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<tr>
<td>Oxygen Transfer</td>
<td>○</td>
<td>●</td>
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<td>●</td>
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<tr>
<td>Biocatalyst attrition</td>
<td>○</td>
<td>●</td>
<td>○</td>
<td>●</td>
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<tr>
<td>Ease of scale-up</td>
<td>●</td>
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<tr>
<td>Suitability for</td>
<td>●</td>
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<tr>
<td>1. Product inhibition kinetics</td>
<td>●</td>
<td>○</td>
<td>○</td>
<td>●</td>
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<tr>
<td>2. Substrate inhibited kinetics</td>
<td>○</td>
<td>●</td>
<td>●</td>
<td>●</td>
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<tr>
<td>3. Presence of solids</td>
<td>○</td>
<td>●</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>4. Presence of gas</td>
<td>○</td>
<td>●</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Ease of reactor control</td>
<td>○</td>
<td>●</td>
<td>●</td>
<td>●</td>
</tr>
</tbody>
</table>

- ● High
- ○ Adequate
- ○ Low

*Table 1.3: Comparison of conventional bioreactor configurations with the MSFBR*
If the reaction conditions require a well mixed system, the MSFBR conditions can be manipulated to gain this by altering the position of the coils as seen in Figure 1.4. As shown in Figure 1.3 in Section 1.5.6, when the coils are placed with the current running in the same direction, the coils join to produce parallel lines of field strength. When the MSFBR is operated for a well mixed system, the current in each coil is running in the opposite direction to the adjacent coil creating a random motion of magnetic carriers within the bed. Following the right hand rule of magnetics for each coil, the magnetic field lines run alternately up and down the column.

1.5.9 Flexibility of Operating Parameters

One of the main differences in operational procedure between a conventional fluidised bed and a magnetically stabilised fluidised bed is the extra degree of freedom in the control of fluidisation. The degree of fluidisation and indeed the stability of the fluidisation regime can be controlled by the strength of the magnetic field. Whilst conventional fluidised bed systems have had to rely on adjustment of the flowrate and aspect ratio, this new type of system adds another parameter to optimise performance.

In summary, the operational mode of the MSFBR is determined by the required application. When good solid/liquid contacting is desired, without solid mixing (such as sorption separations) the bed should be operated in the stable regime. If a degree of mixing is desired, operation may occur in either the roll cell or random motion regimes.
1.6 Project Aims

The project aims can be split into five major objectives.

1. The first aim was to physically characterise a magnetically stabilised fluidised bed. This would involve determining the optimum position of the coils to provide an uniform magnetic field, bed expansion profiles at various magnetic field strength and mixing studies in the reactor.

2. To immobilise two enzymes; penicillin acylase and α-chymotrypsin to carrageenan magnetic carrier particles, which involved determining the optimum glutaraldehyde concentration, immobilisation time and conditions. The pH and temperature stability of the immobilised enzymes would be found.

3. To test the MSFBR’s ability to perform biotransformations in the presence of a pH change. This was achieved by performing a series of 1 litre biotransformations of Penicillin G to 6 APA using immobilised penicillin acylase in the MSFBR. Effect of flowrate and substrate concentration on the reaction and comparison to conventional manufacturing was also investigated.

4. To test the MSFBR’s ability to perform biotransformations in the presence of a solid substrate in an ester hydrolysis reaction. This was achieved by carrying out a series of 1 litre biotransformations of N-acetyl-L-tyrosine ethyl ester to N-acetyl-L-tyrosine using immobilised α-chymotrypsin in the MSFBR. The effect of flowrate and substrate concentration on the reaction and comparison to conventional manufacturing was also investigated.

5. The final aim was to model the reactor to provide information on the scale up of the MSFBR technology and its potential use in other biocatalytic reactions.
Chapter 2

Experimental Systems
2.1 Magnetic Carrier Particles (MCP)

2.1.1 Proposed Carrier Support

The carrier support initially proposed for this project was magnetite embedded in a carrageenan gel that had been used by previous researchers (Pieters et al, 1991, Webb et al, 1996, Law, 1994). The particles were made by mixing magnetite and carrageenan then placed in hot vegetable oil to form beads, polymerised by addition of potassium chloride (KCl) and hardened by 1,6-diaminoehexane (HMDA). A detailed procedure for manufacture is outlined in Section 3.2.

These particles contain a previously immobilised matrix, κ-carrageenan first used by Tosa and coworkers (1979) and subsequently by many others (Lochmuller et al, 1987, Guiseley, 1989). In order for κ-carrageenan to be used as a carrier support it must be induced to a gel. This is initiated by contact with metal ions, amines, amino acid derivatives and water miscible organic solvents.

Carrageenans are naturally occurring hydrocolloids consisting of high molecular weight linear sulphated polysaccharides. They are widely used in the food and cosmetic industries as a gelling, thickening and stabilising agent (Chibata et al, 1987). There are various methods for gelation of κ-carrageenan that will influence the gel strength of the beads. In a survey conducted by Chibata and coworkers (1987) some of the procedures which resulted in gel strength of 1000 to 1500 g/cm² included contact with metal ions (such as potassium chloride), contact with amines (such as HMDA) and contact with amino acid derivatives. Carrageenan is a polysaccharide composed of unit structures of β-D-galactose sulphate and 3,6-anhydro-α-D-galactose with a molecular weight of between 100 and 800 kDa. κ-carrageenan beads appear to be less sensitive than other polysaccharides to disruption by the presence of salt and buffers used in immobilised systems (Guiseley, 1989).
To enhance the operational stability of the glutaraldehyde activated immobilised biocatalyst a hardening agent was used. There are a few diamines used for modification of carboxylates including 1,3 - diaminopropane, diaminodipropylamine, ethyl diamine and a short chain Jaffamine derivative (Hermanson, 1996). Ethylene diamine has been a common choice as its short chain ensures reduced steric effects and minimal hydrophobic interactions. Diaminodipropylamine has a longer spacer arm and has been widely used as a bridging molecule for coupling carboxylate containing ligands to insoluble supports (Hermanson, 1996). In some cases of immobilised whole cells for example, aspartase activity in immobilised E.coli cells, the addition of 1, 6 - diaminohexane resulted in a slight reduction in activity but a significant enhancement of stability (Tosa et al, 1979). The addition of a hardening agent is to improve the stability of the biocatalyst and increase its robustness. Any losses of activity by the presence of the hardening agent can be justified by the improved robustness of the carrier particle. The strength of the final immobilised biocatalyst will be of great relevance during operation of the MSFBR in terms of the pressure drop along the bed and potential attrition damage during processes requiring a well mixed reaction.

The advantages of using carrageenan as an immobilisation support are that it can be performed under very mild conditions and can theoretically be achieved without the use of chemicals that may alter the structure of enzyme proteins (Tosa et al, 1979). This method has also been used for immobilisation of whole cells and the number of living cells after treatment can easily be determined by dissolving the gel in sterilised physiological saline solution. Nagalakshi and Pai (1997) immobilised penicillin acylase producing E.coli cells in carrageenan achieving an immobilisation efficiency of 90%. To gain this high efficiency the beads were hardened by glutaraldehyde (85mM) and hexamethylenediamine (0.5mM).
2.1.2 Cross linking of magnetic carrier particles

The enzymes utilised in the project were immobilised to the carrier particles using glutaraldehyde and the optimum concentration of this reagent was determined in a trade off between the specific activity of the enzyme and the stability. It is uneconomical and impractical to manufacture a high activity immobilised enzyme that retains its activity for only a short period.

The exact nature of crosslinking glutaraldehyde to the magnetic carrier particles is indeterminable, although general information on glutaraldehyde has suggested that it is either through the formation of a Schiff base or through addition at other points of unsaturation (Hermanson, 1996). Figure 2.1 illustrates the method of attachment thought to occur between the magnetic particle, hardening agent, glutaradehyde and the enzyme. The exact chemistry of the linkage and stability of the magnetic particles in the absence of enzyme has been investigated elsewhere (Law, 1994) and is not a major part of this project. However, the main characteristics of the chemistry of glutaraldehyde attachment to enzymes are (a) the overall reaction is rapid and is usually complete within an hour; (b) the reaction can be considered a two step process, namely the formation of a soluble crosslinked complex followed by a rapid step resulting in insolubilisation and (c) enzyme modification is apparently irreversible (Zaborsky, 1973).
Figure 2.1: Components involved in the attachment of the enzyme to the magnetic carrier particle.
2.1.3 Previous Immobilisation to Magnetic Particles

There has been a wide range of enzymes immobilised to various types and sizes of magnetic particle. Cellulase has been successfully immobilised to 45 μm iron oxide particles using covalent bonding by Garcia and coworkers (1989), which utilises a high molecular weight spacer molecule to increase activity by improving the freedom of movement at the end of the particle. Davidson and Scott (1988) have reported using iron oxide in K-carrageenan immobilised cells to increase the specific density to aid their fluidisation capabilities in a conventional fluidised bed. The enzyme urease has been successfully immobilised to magnetite containing beads loading 4 mg/ml of protein (Sada et al., 1981). Shinkai and coworkers (Shinkai et al., 1991) successfully immobilised β-glucosidase to fine magnetic particles (4 - 70 nm) with 168 mg protein per gram magnetite. Most recently, Mehta and coworkers (1997) immobilised BSA directly to freshly precipitated magnetite (mean particle size 12 nm) retaining 90% of the protein.

Pieters (1989) first established the incorporation of commercially available magnetite into the cheap polysaccharide, carrageenan to create an inexpensive magnetic carrier particle as used in this work. Law (1994) optimised this process by examining various hardening agents and activating agents for attachment of enzymes.

2.1.4 Activity Changes to Immobilised Enzymes

The enzyme activity of the immobilised enzyme is likely to change, as this is a direct consequence of enzyme immobilisation. Generally the enzyme activity shows pH dependency due to the active sites on the enzyme possessing both acidic and basic functional groups. The sites become most reactive when a specific balance in ionised forms is found. The most common form is carboxyl and amino groups creating the required ionic atmosphere. The attachment of the enzyme to a support will alter the surface chemistry of the enzyme and more importantly the environment within the active sites. The immobilisation procedure for the first enzyme to be immobilised,
penicillin acylase, involves negatively charged carboxyl groups, which will create a net positive charge on the immobilised enzyme. This will affect the pH within the active sites increasing it as compared with the bulk solution. The glutaraldehyde also reacts with the NH$_2$ groups and reduced the pH of the proteins.

Generally, increasing the temperature will result in an increase in enzyme activity following an Arrhenius type relationship. However increases in temperature will promote the rate of protein denaturation, reducing the enzyme activity exponentially. On a temperature - enzyme activity plot, a temperature mid point can be seen where a trade off is achieved between these two contrasting events. To predict the temperature dependencies of immobilised enzymes is difficult. The thermal deactivation of free enzymes is known to be caused by disruption of the weak intermolecular forces and subsequent protein unfolding. Immobilisation of the enzyme will increase the thermal stability of the enzyme by stabilising the weak intermolecular forces increasing the temperature operating window.

When studying the effect of immobilisation on the enzyme itself, it is important to ensure that diffusional limitations do not affect the reaction rate and the kinetics of the immobilised enzyme. External and internal limitations can sometimes be overcome by rapid agitation and low enzyme loading respectively (Pencreac'h et al, 1997).
2.2 Reaction Types for biotransformations in the MSFBR

2.2.1 Biotransformation model enzyme selection

The key characteristics, which the model system must have, were identified from analysis of problem areas in biocatalytic reactions. Those reactions containing a change in pH and/or the presence of solids were chosen as the test systems.

2.2.2 Model system 1: Conversion of penicillin G to 6 APA using immobilised Penicillin acylase

The first system to be studied was the production of 6 amino penicillanic acid (6 APA) using immobilised penicillin acylase. This commercially successful system was chosen as the first model system to test the ability of the MSFBR to cope with pH control difficulties. The biocatalysis involves a significant pH change which necessitates a pH control system.

6 APA is the key intermediate in the production of semi synthetic penicillins. Although a mature technology the world market for penicillin acylase was between $8 and $10 million in 1988. (Cheetham, 1994). Figure 2.2 illustrates the biotransformation and its two products. In earlier processes the removal of the side chains of penicillin G and penicillin V was performed via protection of the \( \beta \) - lactam with trialkylsilyl chloride and subsequent treatment with phosphorus pentachloride. The enzyme system has now completely replaced the chemical method.

The penicillin acylases are widely available among the bacteria, yeast and filamentous fungi. However the acylases of bacterial / fungal origin display a preference for production of phenylacetic acid. Penicillin acylase is a heterodimer with a 20.5 kDa \( \alpha \) subunit and a 69 kDa \( \beta \) subunit (Bock et al, 1983). The crystalline structure of penicillin acylase from \( E. \) coli at 1.9 Å° resolution indicates the catalytically active centre to be the N terminal serine residue of the \( \beta \) subunit (Duggleby et al, 1995).
Penicillin G

\[ \xrightarrow{\text{Penicillin acylase}} \]

Phenylacetic acid (PAA) + 6-aminopenicillanic acid (6APA)

\[ \xrightarrow{} \]

Semi synthetic penicillins

*Figure 2.2: Biotransformation of penicillin G to 6 aminopenicillanic acid and phenylacetic acid catalysed by penicillin acylase*
However, as with other serine proteases, penicillin acylase does not appear to have a histidine residue in the vicinity of the active site that may act as the base in the catalytic process. The specific characteristic of this system is the pH control required to maintain optimum production. As well as the production of 6 APA, phenylacetic acid (PAA) is produced on a stoichiometric basis causing an decrease in pH that must be controlled by the addition of alkali solution. A higher starting pH is not desirable due to β lactam ring hydrolysis and penicillin inactivation (Vandamme, 1988). To quantify the importance of pH control in this biotransformation, Balasingham and coworkers (1972) investigated the pH dependence of the kinetic parameters in the pH range 7 - 8.5 and showed them to change by 30% over this relatively narrow pH range. The alkali solution often added is either sodium or ammonium hydroxide. The prerequisite of pH control may render operation in a packed bed impossible without recirculation through a stirred tank to maintain a constant pH.

The industrial production of 6 APA is predominantly performed in batch stirred tank reactors (Bailey and Ollis, 1986), which are limited in the concentration of enzyme permissible in the reactor. The abrasion of immobilised penicillin acylase particles by the impeller in a stirred tank may lead to the formation of fine particles, which will be lost from the reactor. The commercial value of 6 APA is only slightly higher than the cost of penicillin G and therefore a high conversion is necessary for the process to be economically efficient. There have been many papers proposing continuous production and various reactor configurations, which will be summarised in this section. Many innovations try to combine the advantages of a packed bed operation (whereby high enzyme concentrations are allowable) and stirred tank operation (ease of pH control) while ideally losing the disadvantages of both reactor types in the process. There have been many reviews published on production of 6 APA and the various reactor configurations used and kinetic studies undertaken such as Lowe and coworkers (1981). Continuous production of 6 APA has been achieved using a combination of a stirred tank and packed bed reactor configuration at a low substrate concentration (reducing the effects of substrate inhibition). By utilising a reverse osmosis system, Danzig and coworkers (1995) showed that the final product concentration could be concentrated to obtain the higher values for recovery of 6
APA by precipitation. Noworyta and Bryjak (1993) showed advantages of a CSTR system over a batch system of better reproducibility of product and process simplification although admitted that the CSTR systems required a larger volume of reactor per unit of product. 6 APA has been produced on a pilot plant scale utilising recirculating batch mode operation of a stirred tank and fixed enzyme bed with immobilised penicillin acylase. The immobilised enzyme used was a commercially available enzyme Semacylase™. Mollgaard and Karlson, 1988 found that the productivity levels were comparable with that of a conventional stirred tank reactor but the recirculating packed bed had advantages of less enzyme wear, shorter reaction time and less dilution of product due to alkali addition.

The use of a multicolumn recirculated packed bed batch reactor for 6 APA production substantially increases the overall production yield of immobilised penicillin acylase but reduces the 6 APA production rate (Lee, 1997). The production yield of immobilised penicillin acylase increases and 6 APA decreases with the number of packed columns installed in the system. This relatively new reactor configuration obviously requires an optimisation study to determine the overall advantages over a conventional system.

As a method for choosing an appropriate configuration for production of 6 APA, kinetic studies have been undertaken by many research workers (for example, Balasingham et al, 1972 , in immobilised form by Carleysmith et al, 1980 and most recently, Van der Weilen et al, 1997). The enzymatic deacylation of penicillin G is generally thought to follow a catalytic mechanism that has been investigated by Kasche and coworkers (1984). The catalytic site contains a catalytic Ser and various binding sites (Fink, 1989). The specificity of the side chain of penicillin G is thought to be due to specific binding by the subsites on the enzyme. The enzymatic action of penicillin acylase on penicillin G is described as a deacylation as it involves the reversal of the acylated enzyme by capturing a water molecule and subsequent release of phenylacetic acid. The two substrate two product deacylation reaction is described by ping pong bi-bi kinetics (Warburton et al, 1972).
Fed batch operation would in theory overcome substrate inhibition and is frequently utilised in industry for this reason. However, Ospina (1992) found no improvement by utilising a fed batch system over a batch reaction for 6 APA production and that the effects of product inhibition are stronger than substrate inhibition concluding that fed batch offers only marginal advantages.

A recent development in kinetic modelling of deacylation of penicillin G by Van der Weilen and coworkers (1997) involves taking into account the effects on thermodynamic non ideality in terms of electrostatic interactions. The majority of industrial scale 6APA production occurs at a high ionic strength and pH 8. A mechanistic model to be used as a basis for optimisation of these processes in terms of both ionic strength and pH is now available (Van der Weilen et al, 1997).

The system of 6 APA production is a well known and researched area allowing more time and effort in this project to concentrate on optimising the reactor choice rather than examining specific kinetics of production. Immobilisation of penicillin acylase to the magnetic carrier particles has not been attempted before and initial work concentrated on optimising activity levels and stability of penicillin acylase when immobilised to the magnetic carriers.
2.2.3 Model System 2: Conversion of N-acetyl tyrosine-L-ethyl ester to N-acetyl-L-tyrosine using α-chymotrypsin

α-chymotrypsin is mainly used as a purified enzyme for diagnostic and analytical applications.

α-chymotrypsin is specific for peptide bonds containing uncharged amino acid residues such as aromatic amino acids. α-chymotrypsin hydrolyses peptide bonds in which the carboxyl group is contributed by the aromatic amino acids phenylalanine, tyrosine or tryptophan or by one with a bulky non-polar functional group (Met) (Murray et al, 1993). α-chymotrypsin is a simple protein with a molecular weight of 21.6 kDa, 241 amino acid residues and 3 sub units. The enzymes consist of three polypeptides held together by two interchain disulphide bonds (Boyer, 1999).

The enzyme follows Michaelis Menton type kinetics although, as with most immobilisations, the kinetic parameters change when the enzyme is attached to a support.

Axen and coworkers (1971) immobilised α-chymotrypsin to an agarose polysaccharide using cyanogen halide. The polysaccharide was activated with cyanogen halide at 23°C under alkaline conditions. Axen and co-workers (1971) found that the higher the pH employed during cyanogen halide activation the greater the coupling capacity.

Unlike penicillin acylase, α-chymotrypsin has been immobilised previously to several magnetic supports. Robinson and coworkers (1973) embedded cellulose with precipitated magnetic iron oxide and successfully attached α-chymotrypsin using cyanogen bromide. The immobilised α-chymotrypsin was used in a stirred tank reactor to illustrate the effects of flocculation of magnetic particles. At high stirrer speeds (and therefore high shear rates) the particles will separate into individual particles and give high activity. At low shear rates, the particles aggregate and activity is reduced. The aim of the work was to show the benefits of using magnetic...
particles as a method of enzyme recovery after biocatalytic reactions. Continuing on the same process theme, Munro and coworkers (1977) immobilised chymotrypsin using glutaraldehyde as an activating agent to several magnetic supports with varying degrees of success. Enzyme activity was predictably high in small precipitated FeO\(_3\) particles (less than 1 \(\mu\)m in diameter) at 2600 Units / g. For larger more practical particle sizes, 65 U / g for Nylon – Fe\(_3\)O\(_4\) (60 – 130 \(\mu\)m) and 40 U/g for polyacrylamide – Fe\(_3\)O\(_4\). The majority of the particles used in the work of Munro and co-workers were non porous which tended to result in lower activities at higher particle sizes (with reduced surface area available for immobilisation).

The conversion of N-acetyl tyrosine ethyl ester to N-acetyl tyrosine is shown in Figure 2.3.
Figure 2.3: Biotransformation of N-acetyl-L-tyrosine ethyl ester to N-acetyl tyrosine catalysed by α-chymotrypsin. Ethanol is also produced (not shown on diagram).
2.3 Reactor Systems

2.3.1 Bed Type Reactors

All bed type reactions were performed in the same reactor column. The conditions of operation depended on whether a fluidised bed or magnetically stabilised fluidised bed was required. The reactor specifications are shown in Table 2.1.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Internal Diameter</td>
<td>5 cm</td>
</tr>
<tr>
<td>Reactor Column Height</td>
<td>50 cm</td>
</tr>
<tr>
<td>Column volume</td>
<td>1 litre</td>
</tr>
<tr>
<td>Material of construction</td>
<td>Perspex</td>
</tr>
<tr>
<td>Distributer Plate</td>
<td>100 μm pore size, Stainless Steel</td>
</tr>
<tr>
<td>Jacketed</td>
<td>1 cm surround</td>
</tr>
<tr>
<td>No of sampling ports</td>
<td>Two</td>
</tr>
</tbody>
</table>

*Table 2.1: Reactor specifications for operation in fluidised and MSFBR operation*

2.3.2 Packed Bed

Packed bed experimentation for comparison of the MSFBR to this conventional choice of reactor was an important part of the project as it would provide a benchmark for subsequent performance. The bed utilised was a Pharmacia XK 50 packed bed system. The column had an internal diameter of 5 cm identical to the fluidised and magnetically stabilised fluidised bed. End plungers were used to set the desired bed height.
2.3.3 Fluidised Bed

Fluidised bed operation required upward flow of the substrate to a designated expansion. The particle density and flowrate of substrate determined the bed expansion of the magnetic carrier particles. The bed was operated at various bed expansions, which were controlled by the flowrate. As the bed moves considerably when first fluidised, the bed was stabilised initially with fluidising buffer, before addition of substrate when plug flow conditions are required.

2.3.4 Magnetically Stabilised Fluidised Bed

Operation of this type of bed reactor was similar to the fluidised bed, but the degree of fluidisation and therefore the stability could be controlled by alteration of the magnetic field present around the reactor. Electromagnetic coils (RS Components Ltd, Corby, Northants) were placed around the fluidised bed in order to create the magnetic field required to stabilise the magnetic carrier particles as shown earlier in Figure 1.1. The reactor was designed in collaboration with EA Technology as part a DTI Link scheme. The reactor was built in the Chemical engineering workshop at University College London. Table 2.2 shows the additional specifications for the fluidised bed when operated as a magnetically stabilised fluidised bed. Figure 2.4 illustrates the magnetically stabilised fluidised bed containing magnetic particles.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Power</td>
<td>1W</td>
</tr>
<tr>
<td>Magnetic Field Strength range</td>
<td>0 – 20 mTesla</td>
</tr>
<tr>
<td>Number of coils</td>
<td>4</td>
</tr>
<tr>
<td>Electromagnetic coils</td>
<td>Copper, 1mm thick, 500 turns</td>
</tr>
</tbody>
</table>

Table 2.2 : Additional specifications for the magnetically stabilised fluidised bed reactor
Figure 2.4: Magnetically Stabilised Fluidised Bed Reactor
2.3.5 Fluid Mixing Characteristics

As an interesting basis for comparison, a graph illustrating the mixing types and operational ranges for each type of reactor has been produced. Figure 2.5 shows the approximate behaviour of each type of reactor. The fluidised bed and MSFBR reactor position in terms of their mixing behaviour can be manipulated by alteration of the bed set up (dispersion plate, aspect ratio, magnetic field strength and position).

![Diagram showing fluid mixing characteristics of each reactor type]

*Figure 2.5: Fluid mixing characteristics of each reactor type*

Changing the expansion of the bed can alter the extent of mixing seen in the fluidised bed reactor. The expanded height of the bed is determined by the flowrate through the bed.

2.3.6 Residence Time Distributions for each type of reactor

The wide scope and various mixing problems encountered in the different biocatalytic reactor configurations have resulted in several criteria being developed to assess the mixing quality in a particular reactor. There is no definitive guide to the
most accurate criteria (Coulson and Richardson, 1990). For analysis of non ideal flow in hybrid reactors such as a fluidised bed, the analysis must be completed at steady state flow with no reaction occurring. There exist many types of model which can be used to characterise non ideal flow within vessels. Many draw on the analogy between mixing in actual flow and a diffusional process and so are named dispersion models. For analysis of reactors such as a fluidised bed a dispersed plug flow model (dispersed model) is commonly used (Goetz and Graves, 1991).

The residence time distribution (RTD) of a reactor is a characteristic of the mixing that occurs and provides information on how long various fluid elements have been in the reactor. The RTD can be found by using stimulus response techniques such as tracer experiments. Providing the tracer material is completely miscible and has similar density and viscosity to the liquid in the reactor, the tracer concentration can be measured as a function of time at any point in the vessel by means of a suitable detector, such as a UV detector or conductivity meter. The tracer such as an inert dye, electrolyte or radioactive species can be introduced to the reactor in the form of a pulse input or step input. Figure 2.6 shows the ideal responses from a CSTR and plug flow type reactors using a pulse input of tracer. The responses are shown as an E curve, which is one method of representation of residence time distributions in reactors.
Figure 2.6: Residence time distributions for an ideal plug flow and stirred tank reactor
Chapter 3

Immobilisation of enzymes to magnetic carrier particles
Chapter 3 Immobilisation of enzymes to magnetic carrier particles

3.1 Chapter Aims

The main objective of the immobilisation experiments was to immobilise model enzymes to magnetic particles to test out the applicability of the MSFBR in biotransformations. The chosen model enzymes, penicillin acylase and α-chymotrypsin are well characterised biocatalytic systems as outlined in chapter 2. The aim of the work reported in this chapter was to obtain a stable immobilised enzyme comparable to that currently available commercially.

More specifically, the optimum conditions for immobilisation had to be determined in terms of the following:

- Optimum glutaraldehyde concentration and activation time for covalent attachment of the enzyme to the MCP’s
- Enzyme loading: The most efficient enzyme attachment conditions. This involves examination of the immobilisation time, temperature, pH and the amount of activity loaded per gram of magnetic particles
- pH, temperature and operational stability of the immobilised enzyme

Each of these conditions of immobilisation has been dealt with for each enzyme reaction in section 3.5 and 3.7 for immobilisation of the first enzyme penicillin acylase and in section 3.6 and 3.9 for immobilisation of the second enzyme α-chymotrypsin.

The first section of this chapter will deal with the manufacture of the magnetic particle for use as a matrix for attachment of enzymes.
3.2 Manufacture of Magnetic Carrier Particles

3.2.1 Precipitated magnetite

41.7 g of ferrous sulfate heptahydrate dissolved in 180 ml of deionised water was added to 80.7 g of ferric chloride hexahydrate dissolved in 400 ml of deionised water. The resultant solution was mixed using an overhead stirrer with a Rushton turbine (diameter = 5 cm) at 300 rpm for 45 minutes. Using dropwise addition, 20% w/v sodium hydroxide was added to the mixture to bring the pH to 11.5 using rapid stirring (1000 rpm). The mixture was left stirring at 300 rpm for 1 hour at 60°C. The magnetite was rapidly cooled, agitation stopped and the solution poured over 4 litres of ice cold deionised water. After repeated washes of the magnetite with water and removal of most of the water by décantation the magnetite was ready for use in the production of the MCP’s.

3.2.2 Magnetic Particles

7.68 g of Kappa - carrageenan (Sigma, Poole, Dorset) was added to 240 ml of distilled water and mixed at 200 rpm at room temperature into a gel. The mixture was heated to 75 °C whereby the mixture became less viscous. Using an overhead stirrer fitted with a Rushton turbine impeller (4 cm diameter), the speed was set to 800 rpm and checked using a tachometer. 40 - 60 g of precipitated magnetite was slowly dropped into the carrageenan - water mixture. The mass of precipitated magnetite added was varied depending on the required density of particle. Adding 40g of precipitated magnetite would result in a low density magnetic particle and adding 60g of precipitated magnetite would result in a higher density magnetic particle. Vegetable oil (Budgens, London) at 65 °C was stirred using the same size impeller at a rate of 1200 rpm at 65 °C. The carrageenan - magnetite mixture was poured on to the hot oil at a rate slow enough to produce evenly sized particles but quick enough to reduce the loss of mixture due to settling at the bottom of the beaker. 0.1 M Potassium chloride was added to the beds to initiate polymerisation. The particles were left to settle and the oil removed by repeated washing with distilled water and decanting the top oil layer.
To obtain the desired size range, the magnetic carrier particles were sieved using four sieve sizes, namely 65, 125, 250 and 355 μm in a Vibratory sieve shaker (Fritsch, Gateshead, Tyne and Wear) four times for 30 minutes and left overnight to evaporate the surface water on the beads.

3.2.3 Hardening of Magnetic Particles

To improve the robustness of the particles a surface agent was added. Using 2 % w/v solution of 1,6-diaminohexane (HMDA) (BDH, Lutterworth, Leics) (pH to 7.0 using concentrated HCl), 5ml was added per gram of beads and left stirring gently for 1 hour. The HMDA was decanted off and washed with distilled water. The beads were stored at 4°C in deionised water.

3.3 Magnetic Particle Drying

For analytical purposes, the magnetic particles could be dried. Particles were dried using a speed vacuum dryer at 60°C. The particles were put in 2 ml eppendorf vials and placed in the centrifugal bowl with open lids. Using a VP 100 two stage Savant vacuum pump, a refrigerated vapour trap RVT 1000 (using cryocool heat transfer fluid at -50°C) and a speed vacuum dryer CS 110 (Savent, Farmingdale, N.Y.) the particles were dried for 2.5 hours at 5000rpm.
3.4 Measurement of the protein content of the immobilised enzymes

3.4.1 Bradford Assay for protein determination

A common method for protein determination, Bradford (1976) will be adopted here of BSA equivalent protein spectrophotometry. The amount of protein loaded to the MCP was measured and also subsequent washes of the MCP and supernatant concentrations. Coomassie brilliant blue G-250 dye (Bio-Rad, Munchen, Germany) was used to determine the protein concentration of the samples using B.S.A (bovine serum albumen, Sigma, Poole, Dorset) as a basis. The typical standard curve for the protein assay is shown in Appendix 2, although a standard curve was produced for each batch of Coomassie used. Protein concentrations of 200 to 1,400 μg/ml could be measured using this technique by using the appropriate dilutions in distilled water to give readings within the spectrophotometry limits. 600 μL of the protein sample was added to 600 μL of diluted coomassie solution, mixed well and read between 5 and 30 minutes after mixing. The spectrophotometer measures the optical density at a wavelength of 595 nm and has limits of 0.2 to 0.6 absorbance units (Dunn, 1989).

3.4.2 Lowry Assay for protein determination

For analysis of protein concentrations in the α chymotrypsin immobilisation development, the Lowry method was used. Standard curves are shown in Appendix 3. All stock solutions were obtained from Sigma (Poole, Dorset, U.K.). The four stock solutions used were 5% K Na Tartrate, 0.5% copper sulphate hydrate, 0.1 M Sodium hydroxide in 2% Na₂CO₃ and Folin Ciocalteu solution. All working solutions were prepared fresh on the day of the assay. 2.5 ml of CUSO₄ solution was mixed with 2.5 ml tartrate solution and diluted to 100 ml with NaOH / Na₂CO₃ solution. The folin ciocalteu solution was diluted 1 part with 2 parts water to the desired assay volume required. The protein solution for assay was prepared in a volume of 125 μL (at a protein concentration of 5 to 50μg). 0.5 ml of the CuSO₄ alkaline solution was added and mixed and allowed to stand at room temperature for 10 – 30 minutes. 125 μL of diluted folin ciocalteu solution was added and mixed immediately and allowed to stand for 30 minutes. The mixture was placed in a quartz cuvette and read in a spectrophotometer at 660 nm.
3.5 Experimental Protocol for Immobilisation of Penicillin Acylase to magnetic carrier particles

3.5.1 Analysis Techniques

3.5.1.1 HPLC Analysis

A High Pressure Liquid Chromatography (Dionex (California, U.S.A) system with PeakNet software for data analysis) was used to determine the concentrations of penicillin G and 6 aminopenicillanic acid. The column used was a Lichrosorb Reverse Phase C18 Column (HiChrom, Reading, Berks, U.K.) at room temperature. Absorbance was measured using UV absorbance at 230 nm. Concentrations of 6 APA (Sigma. Poole, Dorset, U.K.) and Penicillin G (BDH, Lutterworth, Leics, U.K.) were measured using an isocratic method with a mobile phase of 0.3 M KH$_2$PO$_4$ - acetonitrile (8:2 v/v) at a flowrate of 0.8 ml/min. This method was adapted from previous research where similar methodologies were used such as Ishimura and Suga (1991) and Yang and coworkers (1996).

3.5.1.2 Preparation of mobile phase for HPLC

32.6 g of potassium phosphate (KH$_2$PO$_4$) (Sigma, Poole, Dorset, U.K.) was added to 800 ml of deionised water and mixed thoroughly. 200 ml of acetonitrile (HPLC grade, Sigma, Poole, Dorset, U.K.) was then added to complete the mobile phase. To prevent blockage of the HPLC column, both phases were firstly filtered using a glass Buchman filter with 0.2 μm filter paper (Whatman, Maidstone, Kent, U.K.) under vacuum and degassed for 15 minutes.

3.5.1.3 Preparation of samples for HPLC analysis

Repetitions were completed to give an indication of the error of both the HPLC machine and experimental/human error. Analysis of components during biotransformations was completed within 2 hours of the reaction to reduce effects of component degradation.
3.5.1.4 Enzyme titrimetric assay

The progress of the reaction was determined in an autoburette pH titrator (Radiometer, Crawley, West Sussex, U.K.) using a thermostated reactor. The progress of the reaction was monitored by noting the amount of 0.25 M sodium hydroxide added every minute. The characteristic of this reaction which is manipulated in this assay is the production of phenylacetic acid at the same rate as the production of the desired product, 6 aminopencillanic acid. More specifically the enzymatic deacylation of penicillin G at pH 7.8 produces 1 μmole of acid per mole of 6 APA. This enzyme assay was taken from original work by Carleysmith and coworkers (1980).

One unit of enzyme activity was defined as the amount to produce 1μmole of 6 APA per minute at 37 °C and pH 7.8. An initial penicillin G concentration of 30 mM was used and 0.25 M NaOH (Sigma, Poole, Dorset, U.K.) was used to maintain the pH at 7.8. The average initial reaction rate was calculated over ten minutes. In order to determine the relationship between the production of phenylacetic acid (PAA) and the corresponding addition of sodium hydroxide, standards of PAA were used in the pH stat under the assay conditions of 37 °C and pH 7.8 and the amount of sodium hydroxide added by the pH stat noted. The relationship is shown in Appendix 4.

A particular problem with using penicillin G as a substrate is the effect of background hydrolysis, which reduces the pH of the solution over time and is directly proportional to the concentration of the penicillin G. In order to establish the magnitude of this effect the addition of sodium hydroxide used in the assay was monitored over ten minutes and plots of background hydrolysis were established. The extent of background hydrolysis on different ages of penicillin G solutions was also investigated to determine any significant changes between fresh solutions and 1 to 2 day old solutions. The effect of background hydrolysis at various penicillin G concentrations is shown in Appendix 5. To reduce hydrolytic effects, penicillin G solutions were stored at 4 °C.
3.5.2 Effect of glutaraldehyde concentration on immobilisation efficiency of penicillin acylase to magnetic particles

Using set enzyme challenge conditions, the effect of glutaraldehyde concentration on the immobilisation of penicillin acylase to magnetic particles was examined. Glutaraldehyde (Sigma, Poole, Dorset) concentrations ranging between 1 and 10% (v/v) were investigated. Using a 2 ml eppendorf, 1.5 ml of the desired concentration of glutaraldehyde solution (in 50 mM potassium phosphate buffer pH 7.0) was added to approximately 500 μL of wet magnetic carrier particles. After 1 hour shaking on a Vibrax mixer (Staufan, Frankfurt, Germany) at room temperature, the particles were washed three times using phosphate buffer. 75 Units of penicillin acylase (Sigma, Poole, Dorset, U.K.) was added to the particles and immobilised by shaking at 400 rpm at room temperature for 3 days. After three washes of the particles, the activity was measured using the penicillin acylase assay. This would establish the relative merits of each concentration of glutaraldehyde on the immobilisation in terms of the specific activity and the retained activity.

A second set of experiments using 5 ml of magnetic carrier particles was repeated using the above procedure in a 20 ml Duran bottle to determine the stability of the immobilised magnetic particles. 5 ml of wet magnetic particles were added to 15 ml of the desired glutaraldehyde concentration in 50 mM phosphate buffer pH 7.0. After 1 hour shaking at 400 rpm on a Vibrax mixer at room temperature, the particles were washed three times using phosphate buffer. 750 Units of penicillin acylase was added to the particles and immobilised by shaking at 400 rpm at room temperature for 3 days. After three washes of the particles, the activity was measured using the penicillin acylase assay. The particles were stored at 4 °C in 50 mM potassium phosphate buffer pH 7.0 and the activity measured after 7 and 14 days.
3.5.3 Effect of glutaraldehyde attachment time on immobilisation efficiency of penicillin acylase to magnetic particles

5ml of wet magnetic particles were placed in 20 ml duran bottle with 15 ml solution of 5% v/v glutaraldehyde solution in 50 mM phosphate buffer pH 7.0. The particles were shaken at 400 rpm on a Vibrax shaker at room temperature. The particles were activated for 12, 21, 44, 70 and 95 hours. After the desired contact time with the glutaraldehyde solution, a sample was taken. The wash procedure used for all immobilisation work was as follows. The particles were washed three times with 50 mM phosphate buffer pH 7.0. The particles were spun at 3000 rpm between washes in a microcentrifuge and the spent wash removed by pipette. This stock of activated particles after washing was stored at 4 °C and used for the subsequent immobilisation work.

For each activation time experiment, 75 Units of penicillin acylase was added to the 0.5 ml of activated particles and the eppendorfs were shaken at 400 rpm at room temperature for 24 hours. After immobilisation, the particles were washed three times with phosphate buffer using the above washing procedure. The washed immobilised magnetic particles were assayed for activity using the penicillin acylase assay and the supernatent and spent wash from the immobilisation assayed for protein content by the Bradford assay.

3.5.4 Effect of penicillin acylase challenge on immobilisation efficiency

The most efficient loading to the MCP was calculated by examining the effects of increasing enzyme challenge on the specific activity (amount of activity per gram of MCP) and the retained activity (the activity present on the MCP's as a fraction of the total challenge).

Once glutaraldehyde activation was complete and the particles had been fully washed three times with phosphate buffer, 0.5 g wet weight MCP's were placed in 2 ml eppendorfs. The desired volume of free penicillin acylase and 1.5 ml of 50mM
phosphate buffer was added to each eppendorf. The challenge ranged from 2.5 μL to 100 μL which is equivalent to 2.5 and 75 units respectively per 0.5 g wet MCP. The particles were shaken at 400 rpm at room temperature for 24 hours. After immobilisation, the particles were washed as in section 3.5.3. The washed immobilised magnetic particles were assayed for activity using the penicillin acylase assay and the supernatent and spent wash from the immobilisation assayed for protein content by the Bradford assay.

3.5.5 Effect of buffer pH on the immobilisation efficiency of penicillin acylase to magnetic particles

0.5 g of wet glutaraldehyde activated magnetic particles were added to 75 Units of penicillin acylase in 50 mM phosphate buffer at various pH. The buffer pH investigated were 5.5, 7.0, 7.8 and 9.0. After 24 hours immobilising, the particles were washed three times in phosphate buffer and assayed for activity using the penicillin acylase assay.

3.5.6 Effect of immobilisation time on immobilisation efficiency of penicillin acylase to magnetic particles

5 g wet weight activated MCP were placed in 20 ml of 50 mM potassium phosphate at pH 7.0. 100 μL of penicillin acylase (equivalent to 7.6 mg protein, measured by the Bradford assay) was added and the mixture shaken at 400 rpm at room temperature. Samples of the supernatent were taken after 10, 20, 45, 70 and 100 hours and the protein concentration analysed using the Bradford assay outlined in section 3.4.

3.5.7 pH and temperature storage stability of immobilised penicillin acylase

The pH stability of immobilised penicillin acylase was investigated using a range of pH. Magnetic particles immobilised with penicillin acylase were stored in 50 mM phosphate buffer at pH 5.5, 7.0 and 9.0, at 4 °C for a period of two weeks. Samples were taken after 7 and 14 days and the activity measured using the penicillin acylase assay. The temperature stability of immobilised penicillin acylase was investigated by storing immobilised penicillin acylase in 50 mM phosphate buffer at pH 7.0 at 4, 10 and 22 °C for two weeks.
3.5.8 pH activity of free and immobilised penicillin acylase

The pH activity of both the free and immobilised penicillin acylase were investigated using a range of pH. The main characteristic of the penicillin acylase reaction is the production of an acidic by product, phenylacetic acid. The pH activity investigations were therefore more focused on the range acidic to pH 7.0 to determine the performance of the immobilised enzyme in acidic environments. The pH values used in this investigation were 5.2, 6, 6.5, 7.0, 7.8, 8, 8.8, 9.6 and 10.5. For pH values between 5.2 and 8.8, 50 mM potassium phosphate buffer was used. For pH values 9.6 and 10.5, 50 mM carbonate buffer was used. Carbonate buffer was made using 50 ml solution of 50 mM sodium bicarbonate (Sigma, Poole, Dorset, U.K.), 0.1 M sodium hydroxide was added to give the desired pH. The standard penicillin acylase assay, as described in 3.5.1.4, was used in the pH activity investigations. The pH maintained in the assay was changed from the standard pH 7.8 to the desired pH for analysis. 30 mM penicillin G was dissolved in each pH buffer and the assay run over ten minutes. The procedure was completed in triplicate for each pH.

3.5.9 Effect of substrate concentration on the activity of free penicillin acylase

The enzyme assay outlined in section 3.5.1.4 was used as a basis to analyse the effect of Penicillin G concentration on the activity of free penicillin acylase. 25, 100, 150 and 200 mM Penicillin G were dissolved in 100 ml of 50 mM potassium phosphate buffer pH 7.8. 2.5μL of free penicillin acylase was added to the assay and the initial reaction rate at each substrate concentration was noted.

3.5.10 Effect of substrate concentration on the activity of immobilised penicillin acylase

Using the penicillin assay outlined in section 3.5.1.4, the activity could be measured for various sizes of particle, namely 65 - 125, 125 - 250 and 250 - 350 microns. For each particle size, the penicillin acylase assay was run using various penicillin G concentrations. This ultimately meant a small scale (20 ml) biotransformation was...
run for 10 minutes using 2.5, 5, 10, 30, 50, 100 and 200 mM penicillin G. The production of 6 APA was measured by noting the consumption of alkaline sodium hydroxide.

The density of the immobilised magnetic particle and its resultant effect on enzyme activity within the particle were also examined by using two types of 125 - 250 micron particle, specifically particles with a density of a 1.04 and 1.2 g/ml.

Penicillin G was dissolved in 50mM phosphate buffer pH 7.8. 20 ml of the required penicillin G was placed in a thermostated reactor at 37 °C. 500 µL of a suspension of immobilised penicillin acylase to magnetic particle was added to the reactor and the pH taken to 7.8 by using dilute HCL or dilute NaOH, if required. Once the pH had reached 7.8, the reaction could be started and the volume of 0.25 M NaOH added recorded every minute. After 10 minutes, the biotransformation was stopped and the particles removed for analysis of dry weight.

**3.5.11 Product inhibition kinetics of immobilised penicillin acylase**

3 mM and 6 mM Penicillin G (in 50 mM potassium phosphate buffer pH 7.8) substrate solution spiked with set concentrations of 6 APA were prepared. 500 µL of wet magnetic particles were added. The reaction rate was measured by noting the volume of 0.1 M NaOH added in a small 20 ml reaction vessel over five minutes. By plotting the reciprocal of reaction rate over 6 APA concentration a value for the 6 APA product inhibition constant, $K_{IPA}$, could be found. This experiment was repeated with the second product from the penicillin acylase reaction, phenylacetic acid to gain a value for this product inhibition constant, $K_{IPAA}$. 
3.6 Experimental protocol for the immobilisation of chymotrypsin to magnetic particles

3.6.1 Analysis Techniques

3.6.1.1 HPLC Analysis

A High Pressure Liquid Chromatography system (Dionex, California, USA) with PeakNet software for data analysis was used to determine the concentrations of N-acetyl-L-tyrosine ethyl ester and N-acetyl-L-tyrosine. The column used was a phenyl-hexyl luna 5μ C8 150mm by 4.6 mm (Hichrom, Reading, U.K.) at room temperature. Absorbance was measured using UV detection at 210 nm. Concentrations of the components were measured using a gradient method with mobile phases, 25 mM Ammonium phosphate and 25 mM Ammonium phosphate – acetonitrile (50:50) at a flowrate of 1 ml/min.

3.6.1.2 Preparation of mobile phase for HPLC

4.3 g of ammonium dihydrogen phosphate (Sigma, Poole, Dorset, U.K.) was added to 1.5 litres of deionised water and mixed thoroughly to give a 25 mM ammonium phosphate solution. 500 ml of the solution was added to 500 ml of HPLC grade acetonitrile (Sigma, Poole, Dorset, U.K.), which was used as the gradient mobile phase. The remaining litre of 25 mM ammonium phosphate was used as the loading mobile phase. Both solutions were filtered separately using a glass Buchman filter with a 0.2 μm filter paper (Whatman, Maidstone, Kent, U.K.) under vacuum and degassed for 15 minutes.

3.6.1.3 Enzyme titrimetric assay

The enzyme activity was determined using a conventional chymotrypsin titrimetric method used by numerous researchers (Axen et al., 1970, Munro et al., 1977 and Pliura and Jones, 1980). α-chymotrypsin was sourced from Sigma, Poole, Dorset, U.K. The esterolytic activity was measured by monitoring the initial reaction rate in a pH stat (Radiometer (Crawley, West Sussex) autoburette pH titrator) using a 20 ml thermostat reactor. The progress of the reaction was monitored by noting the amount of 0.1 M sodium hydroxide added every minute. The enzyme activity is defined as the amount of enzyme which produces 1 μmole of N-acetyl Tyrosine per minute at 25
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°C and pH 8 using 25 mM N-acetyl Tyrosine Ethyl Ester in 50 mM potassium phosphate buffer as a substrate and the reaction rate was calculated over five minutes. The jacketed reactor volume was 20 ml and the stirrer speed 550 rpm.

In order to determine the relationship between the production of N-acetyl Tyrosine (AT) and the corresponding addition of 0.1 M sodium hydroxide, standards of AT in 50 mM potassium phosphate pH 8.0 were used in the pH stat under the assay conditions of 25 °C and the amount of sodium hydroxide added noted (see Appendix 6). The effects of non-specific hydrolysis of the substrate were accounted for in each assay. The non specific hydrolysis at various ATEE concentrations and at different pH and relationship between N-acetyl Tyrosine and 0.1M sodium hydroxide are shown in Appendix 7. After each enzyme assay the sample was transferred to a 20 ml plastic universal. The liquid was removed by pipette and the immobilised magnetic particles transferred to pre weighed dried 2 ml eppendorfs. The particles were dried to give accurate quantitative specific activities (Units/g dry magnetic particle).

3.6.2 Generic analysis

The protein concentration was measured using the Lowry method as outlined in section 3.4.2. After analysis using the assay for enzyme activity, the magnetic particles were dried using the procedure outlined in section 3.3.

3.6.3 Effect of glutaraldehyde concentration on the immobilisation efficiency of α-chymotrypsin to magnetic particles

0.5 ml settled volume of magnetic particles were placed in 2 ml eppendorfs with 1 ml solution of the desired glutaraldehyde solution. The glutaraldehyde solutions were made up with 50 mM phosphate buffer at pH 7.0 from a 50% glutaraldehyde solution (Sigma, Poole, Dorset, U.K.). The temperature of activation was 21 °C and the eppendorfs were shaken at 700 rpm on a thermomixer (Eppendorf, Cambridge, U.K.) for 25 hours. Glutaraldehyde concentrations of 1, 2.5, 5 and 10 % v/v were investigated.
After 25 hours contact with the glutaraldehyde solution, the particles were washed three times with 50 mM potassium phosphate buffer pH 7.0. The concentration of glutaraldehyde in the washes was periodically monitored using spectrophotometry at 280 nm. The washed particles were spun down in a micro centrifuge at 3000 rpm and the spent wash removed by pipette. This washing procedure was used repeatedly during the immobilisation work.

An arbitrary enzyme loading was used in these initial immobilisation studies of 10 mg/ml solution of α-chymotrypsin (3315 Units/ml). 1 ml of this stock enzyme solution was used in each eppendorf for this section of experiments. The eppendorfs were shaken at 700 rpm at 21 °C for 24 hours on the thermomixer. After immobilisation, the particles were washed as described above.

For each glutaraldehyde concentration, a 100 ul sample of washed MCP was assayed for enzyme activity using the method outlined in section 4.1.4. The protein content in the supernatant was calculated using the Lowry method for protein determination as outlined in section 3.4.2. The stability of immobilised penicillin acylase at various glutaraldehyde concentrations were examined after 4 days storage in 50 mM potassium phosphate buffer pH 7.0 at 4 °C.

3.6.4 Effect of glutaraldehyde attachment time on immobilisation efficiency of α-chymotrypsin to magnetic particles

0.5 ml settled volume of magnetic particles were placed in 2 ml eppendorfs with 1 ml solution of 2.5% v/v glutaraldehyde solution in 50 mM phosphate buffer pH 7.0. The temperature of activation was 21 °C and the eppendorfs were shaken at 700 rpm on a thermomixer for the desired activation time. Activation times of 2.5, 5, 25 and 48 hours were investigated. The particles were washed as described in section 3.6.3.

An arbitrary enzyme loading was used in these initial immobilisation studies of 10 mg/ml solution of α-chymotrypsin (3315 Units/ml, 5.6 mg B.S.A. equivalent protein/ml). 1 ml of this stock enzyme solution was used in each eppendorf. The
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Eppendorfs were shaken at 700 rpm at 21 °C for 24 hours on a thermomixer. After immobilisation, the particles were washed as described in section 3.6.3.

For each experimental condition, a 100 µL sample of washed MCP was assayed for enzyme activity using the method outlined in section 3.6.1.3.

3.6.5 Effect of temperature of glutaraldehyde activation on the immobilisation efficiency of α-chymotrypsin to magnetic particles

0.5 ml settled volume of magnetic particles were placed in 2 ml eppendorfs with 1 ml solution of 2.5 % v/v glutaraldehyde solution in 50 mM phosphate buffer pH 7.0. The eppendorfs were shaken at 700 rpm on a thermomixer for 25 hours at the desired temperature. The temperatures investigated were 4, 12, 21 and 30 °C. After 25 hours contact with the glutaraldehyde solution, the particles were washed as described in section 3.6.3.

1 ml of a 10 mg/ml α-chymotrypsin solution was added to each eppendorf. The eppendorfs were shaken at 700 rpm at room temperature (21 °C) for 24 hours. After immobilisation, the particles were washed as described in section 3.6.3.

For each experimental temperature, a 100 µL sample of washed MCP was assayed for enzyme activity using the method outlined in section 3.6.1.3. The protein content in the supernatant was calculated using the Lowry method for protein determination as outlined in section 3.4.2.
3.6.6 Effect of α-chymotrypsin challenge on the immobilisation efficiency

0.5 ml settled volume of magnetic particles were placed in 2 ml eppendorfs with 1 ml solution of 5 % v/v glutaraldehyde solution in 50 mM phosphate buffer pH 7.0. The eppendorfs were shaken at 700 rpm at 21 °C. After 25 hours contact with the glutaraldehyde solution, the particles were washed as described in section 3.6.3.

0.5 ml settled volume of magnetic particles were placed in 2 ml eppendorfs with a 1 ml solution of various enzyme concentrations. The enzyme concentration’s investigated were 1 – 25 mg enzyme/ml buffer, which is equivalent to 260 – 11,150 Units/ml (0.6 – 14 mg/ml BSA equivalent protein). The eppendorfs were shaken at 700 rpm at 21°C for 72 hours. After immobilisation, the particles were washed as described in section 3.6.3.

For each enzyme loading experiment, a 100 μL sample of washed MCP was assayed for enzyme activity using the method outlined in section 3.6.1.3. The protein content in the supernatent was calculated using the Lowry method for protein determination as outlined in section 3.4.2.

3.6.7 Effect of immobilisation temperature on the immobilisation efficiency of α-chymotrypsin on magnetic particles

200 μL from a stock solution of activated magnetic particles using the optimised glutaraldehyde conditions (5% v/v glutaraldehyde at 21 °C for 25 hours) was placed in a 1.5 ml eppendorf with 400 μL solution of a 10 mg/ml enzyme solution (3315U/ml) equivalent to a total activity loading of 1326 Units of α-chymotrypsin. The eppendorfs were shaken at 700 rpm at the desired temperature on a thermomixer. The immobilisation temperatures investigated were 4, 10, 21 and 30 °C. After immobilisation, the particles were washed as described in section 3.6.3.

For each immobilisation temperature, a 100 μL sample of washed MCP was assayed for enzyme activity using the method outlined in section 3.6.1.3.
3.6.8 Effect of immobilisation time on the immobilisation efficiency of α-chymotrypsin on magnetic particles

200 μL from a stock solution of activated magnetic particles using the optimised glutaraldehyde conditions was placed in a 1.5 ml eppendorf with 400 μL solution of a 10 mg/ml enzyme solution equivalent to 1326 Units of α-chymotrypsin. The eppendorfs were shaken at 700 rpm at room temperature for the desired time. Immobilisation times of 18, 25, 48 and 72 hours were investigated.

For each immobilisation time, a 100 μL sample of washed MCP was assayed for enzyme activity using the method outlined in section 3.6.1.3. The protein content in the supernatent was calculated using the Lowry method for protein determination as outlined in section 3.4.2.

3.6.9 pH activity of free α-chymotrypsin

For free α-chymotrypsin, the pH activity was measured at pH 5.5, 6.9, 7.6, 7.8, 8.4 and 9.5. For pH in the region 5.5 to 8.4, 50 mM phosphate buffer was used adding the appropriate volumes of monobasic (KH₂PO₄) and dibasic (K₂HPO₄) for the desired pH. For pH 9.5, sodium hydrogen carbonate buffer was used. To produce 100 ml of the pH 9.5 buffer, 50 ml of 50 mM sodium hydrogen carbonate (Sigma, Poole, Dorset, U.K.) was added to 6.2 ml of 0.1 M sodium hydroxide and diluted to 100 ml in deionised water.

Based on the enzyme assay described in section 3.6.1.3, 25 μL of a 1g/l solution of α-chymotrypsin was added to the assay with 20 mM N-acetyl tyrosine ethyl ester dissolved in the desired buffer. The activity of the enzyme was measured maintaining the pH at the desired buffer pH level by recording the volume of 0.1 M NaOH added over five minutes.
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3.6.10 pH activity of immobilised α-chymotrypsin

24 ml from a stock solution of activated magnetic particles using the optimised glutaraldehyde conditions were placed in a 100 ml duran bottle with 48 ml of a 10 mg/ml enzyme solution equivalent to a total enzyme loading of 159120 Units. The duran bottle was shaken at 400 rpm at room temperature for 90 hours.

For immobilised α-chymotrypsin, the pH activity was measured at pH 4.85, 5.73, 6.27, 6.6, 7.19, 7.82, 8.15, 8.3 and 9.9. For pH in the region 4.85 to 8.15, 50 mM phosphate buffer was used adding the appropriate volumes of monobasic (KH$_2$PO$_4$) and dibasic (K$_2$HPO$_4$) for the desired pH. For pH 9.9, sodium hydrogen carbonate buffer was used. To produce 100 ml of the pH 9.5 buffer, 50 ml of 0.05 M sodium hydrogen carbonate was added to 9.1 ml of 0.1 m sodium hydroxide and diluted to 100 ml in deionised water.

Using the enzyme assay described in section 3.6.1.3, a drop (using a plastic pasteur pipette) of immobilised MCP was added to the assay with 20 mM N-acetyl tyrosine ethyl ester dissolved in the desired buffer. The activity of the enzyme was measured maintaining the pH at the desired buffer pH level and recording the amount of 0.1 M NaOH added over 5 minutes. The specific activity was determined by drying the particles after the assay as described in section 3.3.

3.6.11 Effect of substrate concentration on the activity of free α-chymotrypsin

The enzyme assay outlined in section 3.6.1.3 was used as a basis to analyse the effect of N-acetyl tyrosine ethyl ester (ATEE) concentration on the activity of free α-chymotrypsin. 0.5, 1, 2.75, 5, 10, 15 and 20 mM ATEE were dissolved in 100 ml of 50 mM potassium phosphate buffer pH 8. For higher concentrations of ATEE, >5 mM, the samples were heated to 40 °C to aid dissolution. 25 μL of a 1mg/ml solution of α-chymotrypsin was added to 20ml of the desired ATEE concentration and the initial reaction rate at each substrate concentration was noted.
3.6.12 Effect of substrate concentration on the activity of immobilised α-chymotrypsin

0.5, 1, 2.75, 5, 10, 15 and 20 mM ATEE were dissolved in 100 ml of 50 mM potassium phosphate buffer pH 8. For higher concentrations of ATEE, greater than 5 mM, the samples were heated to 40 °C to aid dissolution. Based on the enzyme assay described in section 3.6.1.3, a drop of immobilised MCP was added to the assay with the desired concentration of ATEE dissolved in 50 mM potassium phosphate buffer pH 8.0. The activity of the enzyme was measured maintaining the pH at 8.0 using 0.1 M NaOH via the pH stat for each substrate concentration. The specific activity was determined by drying the particles after the assay as described in section 3.3.
3.7 Results and Discussion of the Immobilisation of Penicillin acylase to Magnetic Carrier Particles

3.7.1 Effect of glutaraldehyde concentration on the immobilisation of penicillin acylase to magnetic carrier particles

Using common immobilisation techniques, published literature on immobilisation of enzymes to these specific magnetic carrier particles (Law, 1994) and penicillin acylase immobilisation, specifically Burteau and coworkers (1989) and Fonseca and coworkers (1993), the initial range of conditions using glutaraldehyde could be established. Although glutaraldehyde is a common activating agent in immobilisation of enzymes it can be a problem if leaching occurs and separation is required in subsequent downstream processing. It was therefore important to select the optimum glutaraldehyde concentration.

This selection process consisted of firstly determining a trade off between the specific activity of the MCP (the amount of activity per gram of MCP) and the retained activity (the amount of activity on the MCP as a fraction of the original challenge to the MCP) and secondly by analysis of the stability of the immobilised penicillin acylase at various glutaraldehyde concentrations. This investigation aims to achieve optimisation conditions for both the immobilisation of penicillin acylase to magnetic carrier particles and the stability of these MCP’s over a period of two weeks. As outlined previously the aim of the immobilisation of penicillin acylase was to determine a reproducible literature comparable system not to find a substantially more efficient immobilisation. It was therefore decided that stability over a fortnight would be adequate for the experiments in the reactor.

The experiments were carried out in 50mM phosphate buffer at pH 7.8 and immobilisation occurred at room temperature at a scale of 2 ml. The activity of the MCP’s was measured using the penicillin acylase assay outlined in section 3.5.1.4. Figures 3.1, 3.2 and 3.3 illustrate the relationship between specific activity and
retained activity found for increasing enzyme challenge using three types of MCP activated with 2, 5 and 10% glutaraldehyde.

From Figures 3.1, 3.2 and 3.3, a general trend can be seen. As the penicillin acylase challenge increases, the specific activity increases allowing more enzyme attachment per gram of particle. However as the enzyme challenge increases the efficiency of immobilisation decreases giving rise to lower retained activity. From Figure 3.1, at a penicillin acylase challenge of 2750 Units/gram dry MCP, the retained activity is only 55% which may indicate that either the remaining activity is bound but in an inactive form or mass transfer of the substrates into the MCP was hiding the true activity of the MCP. This result may also mean that the MCP is completely saturated with enzyme and no active sites remain for attachment. Figures 3.2 and 3.3 show a similar trend.

There are a number of conclusions that can be drawn from these experiments. Up to a penicillin acylase challenge of 2000 units/gram dry MCP, a concentration of 2% glutaraldehyde is sufficient to bind the enzyme. At higher levels of penicillin acylase challenge, a 5% glutaraldehyde concentration binds the enzyme more efficient in terms of both specific activity and retained activity than using either 2 or 10% glutaraldehyde activation.

An enzyme challenge of 1750 Units/gram dry MCP using 5% glutaraldehyde solution would give a specific activity of approximately 1000 units/gram dry MCP and a retained activity of 60%.
Figure 3.1: Effect of increasing penicillin acylase challenge upon the specific activity and retained activity using 2% glutaraldehyde concentration during activation. Specific activity (■) and retained activity (▲).

Figure 3.2: Effect of increasing penicillin acylase challenge upon the specific activity and retained activity using 5% glutaraldehyde during activation. Specific activity (■) and retained activity (▲).
Figure 3.3: Effect of increasing penicillin acylase challenge upon the specific activity and retained activity using 10% glutaraldehyde concentration during activation. Specific activity (■) and retained activity (▲).

Figure 3.4: Stability of immobilised and free penicillin acylase
Free enzyme (■) and immobilised enzyme attached using 0.5% (★), 1% (●), 2% (▼) and 4% (▲) gluteraldehyde (v/v%).
Immobilisation of Enzymes to Magnetic Carrier Particles

In order to create a useable immobilised biocatalyst it must be stable over a period of time. This period of time is dependant on the final use of the biocatalyst. In this project a stability of at least 14 days was required. Figure 3.4 displays the results of immobilised MCP's using various concentrations of glutaraldehyde (0.5 – 4 % v/v) and their activity over time when stored in 50mM phosphate buffer pH 7.0 at 4 °C. It shows that a glutaraldehyde concentration of 4% yields a higher stability over 14 days compared with lower concentrations. At higher concentrations (> 4 % v/v, data not shown), the stability remained similar to that of 4% glutaraldehyde. The general trend in Figure 3.4 is that the stability of the immobilised penicillin acylase decreases with decreasing glutaraldehyde concentration below 4% v/v.

The optimum glutaraldehyde activation conditions were found to be of 5% v/v concentration in 50 mM potassium phosphate pH 7.0. This decision was taken based on the immediate immobilisation efficiency and the subsequent stability over 14 days. An enzyme challenge of 1750 U/g dry MCP using 5% v/v glutaraldehyde solution would give a specific activity of approximately 1000 U/g dry MCP and a retained activity of 60% at a 2mL scale.

3.7.2 Effect of glutaraldehyde attachment time on the immobilisation efficiency

Figure 3.5 illustrates the effect of glutaraldehyde activation time on the subsequent immobilisation of penicillin acylase to the activated magnetic particle. The amount of activity loaded to the magnetic particles increases with activation time up to 10 hours. At higher activation times, the activity loaded either remains constant (after 25 hours) or reduces (after 40 hours). For future activation of magnetic particles, 10 hours was chosen as the optimum activation time.
3.7.3 Effect of enzyme challenge on the specific and retained activity of immobilised penicillin acylase

As discussed earlier in section 3.7.1, the optimum glutaraldehyde concentration for attachment of penicillin acylase to magnetic carriers was 5% v/v. Figure 3.2 defines the optimum enzyme challenge for both retention of activity and a sufficient specific activity. Using a penicillin acylase initial challenge of 1750 Units per gram dry MCP resulted in a specific activity of 1000 Units / g dry MCP which is comparable to other immobilised penicillin acylase systems as described in section 3.7.11. The retained activity at this level of enzyme challenge was 60%.

The loss of activity could result from a chemical denaturation of the enzyme due to reaction conditions such as the pH or temperature. It also could be due to lower accessibility at the catalytic site and steric hinderance between substrate and support. However this hypothesis is unlikely due to the low molecular mass of the substrate. Complete attachment of the enzyme to a support is rarely achievable (Hartmeier, 1988).

3.7.4 Effect of buffer pH on immobilisation of penicillin acylase

One of the most important experiments in the initial binding studies was to determine the optimum pH for immobilisation. The immobilisation of protein to support matrices by covalent attachment is often carried out at high pH to deprotonate the target amino acid. Literature investigations revealed optima pH values for immobilisation of penicillin acylase ranged from the standard pH 7.0 (Carleysmith, 1979) to pH 8.0 (Nagalakshmi and Pai, 1997) to pH 8.7 (Self et al, 1969). pH optima determination involved carrying out identical immobilisation experiments at room temperature using 50 mM potassium phosphate buffer at pH 5.5, 5.5, 7.0 and 9.0. Figure 3.6 illustrates the results showing the optimum at pH 7.0. As a result all subsequent immobilisation experiments were carried out at pH 7.0.
3.7.5 Effect of immobilisation time on immobilisation efficiency

A series of experiments were performed to illustrate the protein binding curve of penicillin acylase to the MCP's. Figure 3.7 shows the protein binding curve for immobilisation of 100 µL of free enzyme (equivalent to 7.6 mg protein, measured by the Bradford assay) to 5.3 g of wet weight magnetic carrier particles. Almost 97% of the penicillin acylase in terms of protein is bound at 45 hours and only a 2.3% increase in protein is found after another 45 hours immobilisation. For subsequent experimentation, the immobilisation time was kept at 60 hours where a predicted 98.5% protein should be attached to the MCP.
**Immobilisation of Enzymes to Magnetic Carrier Particles**

*Figure 3.5:* Effect of glutaraldehyde activation time on the subsequent immobilisation of penicillin acylase

*Figure 3.6:* Effect of buffer pH on the immobilisation of penicillin acylase to MCP's
Figure 3.7: Protein binding profile of penicillin acylase to MCP
In section 3.7.1, an important parameter in determining the optimum glutaraldehyde concentration was the stability of the enzyme. It was therefore also important to establish the storage conditions in terms of pH and temperature. These parameters were especially important when the MCP’s would be utilised in the MSFBR as it would have been advantageous not to have to transfer the MCP’s to 4°C for overnight storage. Figure 3.8 (a) illustrates the effect of storage temperature on the relative activity of immobilised penicillin acylase. The activity was measured as a percentage of the activity seen after immobilisation of penicillin acylase to the magnetic particles. 25% of the activity is lost during storage at 22 °C over 14 days whilst at 4 °C only 5% of the activity is lost over 14 days storage. From the figure it is clear that storage at 4 °C reduces the activity lost over time by 20% as compared with 22 °C. Figure 3.8 (b) shows the effect of storage pH on the specific activity of immobilised penicillin acylase. The optimum pH for storage was found to be pH 7.0. At higher storage pH the reduction of activity over 14 days was 33%. Using an Arrhenius based model, the half life of immobilised penicillin acylase stored in 50 mM potassium phosphate buffer pH 7.0 and 4 °C was approximately 2807 hours.
Figure 3.8 : Storage stability of immobilised penicillin acylase. (a) Effect of storage temperature on the relative activity of biocatalyst in 50mM phosphate buffer pH 7.0 at storage temperature of 4 °C (■), 10°C (●) and 22°C(▲) and (b) Effect of storage buffer pH on specific activity of immobilised penicillin acylase in 50 mM potassium phosphate buffer at 4 °C at storage pH of 5.5 (■), 7 (▲) and 9 (●).
3.7.7 pH activity of free and immobilised penicillin acylase

The main characteristic of the penicillin acylase biotransformation is the pH change resulting from the by product formation of phenylacetic acid. It was therefore essential to ensure that the immobilised penicillin acylase retains sufficient activity in the range pH 6.0 to 7.8. Although pH control will be in place, it is likely that regions of the reactor will be in slightly acidic conditions.

The pH activity investigation involved running a 20 ml small scale biotransformation over 10 minutes in the specified pH solution. The free penicillin acylase and the different density MCP's of 125 - 250 microns were examined. Figure 3.9 illustrates the pH activity profiles of free penicillin acylase and magnetic carrier particles of densities 1.04 and 1.2 g/ml. For comparison purposes the activity of each preparation was normalised to the standard activity of the enzyme at conditions of 37 °C using 30 mM penicillin G concentration in 50 mM potassium phosphate pH 7.8. For free enzyme the optimum activity was 1015 Units at pH 8.0. For 125 – 250 micron particles of density 1.04 g/ml and 1.2 g/ml the optimum activity occurred at pH 8.5 and pH 6.7 respectively.

The pH activity profile of the free penicillin acylase is as expected when compared to published data (Ospini et al, 1992) showing a peak activity at a slightly higher than recommended standard conditions of pH 8.0. Data obtained from the supplier (Sigma, Poole, Dorset, U.K.) gave an optimum activity at pH 7.8. This anomaly is probably explained by assay experimental error (although is seems unlikely as experiments were completed in triplicate) or differences in reaction conditions (type and concentration of buffer solution).

The pH activity of the lower density particle (1.04 g/ml) displays a flat profile from pH 6.5 to 9.5 although drops sharply at pH values below 6.5. The immobilised MCP loses 65% activity at pH 5.5. Although the immobilised penicillin acylase lost activity on immobilisation, it shows significantly higher activity at pH 6.5. This is important is this type of bioreactor system as the pH in the MSFBR is likely to be
below 7.8 and very likely to be within the range 6.5 to 7.5 where this immobilised particle seems to show no significant loss in activity as compared to pH 7.8.

The pH activity profile for 1.2 g/ml MCP seen in Figure 3.9(b) is erratic although the profile shows a general flat nature with an unexpected peak in the acidic region of 6.7. No conclusive message can be derived from Figure 3.9(b).
Figure 3.9: pH activity profiles of free penicillin acylase and immobilised penicillin acylase.

(a) Free penicillin acylase (▲) and magnetic carrier particles of diameter 125 - 250 microns with a density of 1.04 g/ml (■) and (b) immobilised penicillin acylase of diameter 125 – 250 microns with a density of 1.04 g/ml (■) and 1.2 g/ml (●).
3.7.8 Kinetics of free and immobilised penicillin acylase to MCP’s

The evaluation of the kinetic parameters of immobilised penicillin acylase was needed to develop a rational design for future experimentation in a reactor. Not only is it important to compare kinetics of the immobilised system to the free enzyme but it is also vital to compare with other immobilisation attempts in literature and industry. This can be achieved by examining the effect the substrate concentration has on the initial enzymatic reaction rate and the values of the kinetic parameters, $K_m$, the substrate saturation constant and $V_{max}$, the maximum reaction rate.

As this first enzyme, penicillin acylase has not previously been immobilised to the magnetic carrier particles, a range of particle sizes and densities were investigated. The particle sizes used were 65 - 125, 125 - 250 and 250 - 350 microns and the densities were 1.04 and 1.2 g/ml. Figure 3.10 illustrates the relationship between penicillin G concentration and the relative activity for each particle size and compares with the free penicillin acylase. For comparison purposes the activity of each preparation was normalised to its activity at standard conditions of 37°C and pH 7.8 using 30 mM penicillin G concentration in 50 mM potassium phosphate buffer. The activities at these standard conditions were 778, 184 and 164 Units / g dry magnetic carrier particle for 65 - 125, 125 - 250 and 250 - 350 micron diameter particles. The activities seen are lower that those found during the immobilisation development work. The preparation of these particles was completed on a larger scale using 5 mL of magnetic particles rather than the typical 0.5 mL of magnetic particles used in the development work. The issue of scale up of the immobilisation is discussed in further detail in section 3.7.13. The free penicillin acylase kinetic curve is as expected from previous publications (Bryjak and Noworyta, 1993, Fonseca et al, 1993, Bianchi et al, 1996), displaying inhibition by penicillin G at high concentrations. For all sizes of particles, an increasing penicillin G concentration results in increased activity up to the standard conditions of 30 mM penicillin G at 37°C and pH 7.8. At higher concentrations, the activity decreases in all particle sizes. The decrease is significantly higher among larger diameter particles, with 250 - 350 micron particles showing 30% of its activity at standard conditions (30 mM penicillin G), at a penicillin G concentration of 200 mM. The 125 – 250 micron
penicillin acylase magnetic particles, which were used later in the MSFBR biotransformations, show only 40% of its activity at standard conditions, at a penicillin G concentration of 200 mM. At 100 mM penicillin G, the immobilised penicillin acylase shows 60% of its activity at standard conditions. The smallest particles used of 65 - 125 microns displayed a similar kinetic curve as for free penicillin acylase.

*Figure 3.11 (a)* illustrates the effect of substrate concentration on the activity of the 125 – 250 micron immobilised penicillin acylase. The values found for Km and Vmax were 5 (+/- 0.55) mM and 820 (+/- 164) U / g MCP respectively.
Figure 3.10: Relative activity versus penicillin G concentration for free penicillin acylase and immobilised penicillin acylase on magnetic carrier particles of various diameters.

Free penicillin acylase (▽) and magnetic particle diameters of 65 - 125 microns (■), 125 - 250 microns (●) and 250 - 350 microns (▲)
Figure 3.11: Michaelis-Menton kinetics for immobilised penicillin acylase at 37°C in 50 mM phosphate buffer pH 7.8 for initial rate experiments. (a) Lineweaver-Burk representation: (b) modification of the Lineweaver-Burk plot to determine the substrate inhibition constant
Figure 3.12 illustrates the specific activity of 125 - 250 micron magnetic carrier particles for increasing penicillin G concentration for two different particle densities, namely 1.04 g/ml and 1.2 g/ml. The method of standardisation of specific activity previously had been to use dry weight (effectively the weight of magnetite within the particle), however experiments involving differing densities invalidated this method. The lower density particles clearly contained a lower mass of magnetite leading to an inflated specific activity. Therefore each specific activity was defined per g of adjusted MCP. The adjusted MCP weight is equal to the dry weight of the particle multiplied by the magnetite composition of the magnetic carrier particle. Specifically the magnetite composition for each particle was 17% w/w for the 1.04 g/ml density particle and 27% w/w for the 1.2 g/ml density particle.

Figure 3.12 clearly shows that the magnetic carrier particle with a density of 1.04 g/ml displays higher specific activities at all concentrations of penicillin G. The specific activity of the MCP's at standard conditions are 138 and 25 Units / g adjusted MCP. The particles were loaded with the same enzyme challenge indicating that the immobilisation efficiency is lower for the higher density particles. The result indicates that it is the surface area of carrageenan that is important in the attachment of this enzyme.
Figure 3.12: Specific activity versus penicillin G concentration for 125 - 250 micron immobilised penicillin acylase on two different magnetic carrier particles. Densities for each magnetic carrier particle are 1.04 g/ml (•) and 1.2 g/ml (○)
3.7.9 Inhibition of penicillin acylase

3.7.9.1 Substrate Inhibition of free penicillin acylase

Penicillin acylase is inhibited by substrate as described by the model (Warburton et al, 1972):

\[
\text{rate} = \frac{V_{\text{max}}[S]}{\left( K_m \left( 1 + \frac{[PAA]}{K_{I_{PAA}}} \right) \left( 1 + \frac{[6APA]}{K_{I_{6APA}}} \right) + [S] \left( 1 + \frac{[6APA]}{K_{I_{6APA}}} + \frac{[S]}{K_{I_S}} \right) \right)}
\]  (3.1)

The effect of substrate inhibition on penicillin acylase is well documented in literature (Warburton et al, 1972). *Figure 3.13* illustrates the effect of substrate concentration on the inverse rate of reaction of free penicillin acylase at pH 7.8 and 37 °C. The substrate inhibition coefficient can be derived from this plot at the intersection of the substrate concentration axis. Using *Figure 3.13*, the substrate inhibition coefficient, \(K_s\), is 584 (+/- 24) mM. \(K_s\) is derived from the intercept of the x axis (substrate concentration) when the reaction rate is zero (at Y axis = 0) using a linear fit model. A table of comparison with other penicillin acylase systems is shown in *Table 3.1*. 
### Immobilisation of Enzymes to Magnetic Carrier Particles

<table>
<thead>
<tr>
<th>Researcher (s)</th>
<th>Year</th>
<th>Type</th>
<th>$K_m$ (mM)</th>
<th>$K_s$ (mM)</th>
<th>$K_{i(PAPA)}$ (mM)</th>
<th>$^*K_{i(PAA)}$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Self et al</td>
<td>1969</td>
<td>Free (source E. coli)</td>
<td>7.7</td>
<td>130-170</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cellulose derivative</td>
<td>3</td>
<td>200</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Balasingham et al</td>
<td>1972</td>
<td>Free (source E. coli)</td>
<td>0.67</td>
<td>270</td>
<td>7.1</td>
<td>4.8</td>
</tr>
<tr>
<td>Warburton et al</td>
<td>1973</td>
<td>Immobilised to DEAE cellulose (source E. coli)</td>
<td>0.63</td>
<td>250</td>
<td>9</td>
<td>4.6</td>
</tr>
<tr>
<td>Park et al</td>
<td>1982</td>
<td>Immobilised to gelatin/DEAE cellulose pellets (source E. coli)</td>
<td>3.78</td>
<td>NS</td>
<td>28.9</td>
<td>170</td>
</tr>
<tr>
<td>Haagensen et al</td>
<td>1983</td>
<td></td>
<td>1.56</td>
<td>NA</td>
<td>131</td>
<td>36.3</td>
</tr>
<tr>
<td>Suga et al</td>
<td>1991</td>
<td>Immobilised to porous polyacrylonitrile fibres (source Bacillus megaterium)</td>
<td>3.29</td>
<td>NA</td>
<td>104</td>
<td>22.6</td>
</tr>
<tr>
<td>Noworyta and Bryjak</td>
<td>1993</td>
<td>Free (source E. coli)</td>
<td>1.34</td>
<td>&gt;10$^{15}$</td>
<td>32.7</td>
<td>26.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Immobilised to acrylic carriers</td>
<td>4.29</td>
<td>652</td>
<td>10$^{15}$</td>
<td>20.9</td>
</tr>
<tr>
<td>SpieB et al</td>
<td>1999</td>
<td>Eupergit (source E. coli)</td>
<td>0.013</td>
<td>NA</td>
<td>0.56$^a$</td>
<td>60$^a$</td>
</tr>
<tr>
<td>This work</td>
<td></td>
<td>Free penicillin acylase (source E. coli)</td>
<td>2.7</td>
<td>584</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Immobilised carrageenan magnetic particles</td>
<td>5</td>
<td>704 (+/- 24)</td>
<td>105 (+/- 71)</td>
<td>109 (+/- 17)</td>
</tr>
</tbody>
</table>

Notes:

* Values quoted for $K_{i(PAA)}$ are at pH 8.0 except $^a$ Values at pH 7.0
NA : Not available

Table 3.1 : Comparison of Penicillin acylase Inhibition coefficients
3.7.9.2 Substrate Inhibition of immobilised penicillin acylase

The primary aim of determining the substrate inhibition coefficient for immobilised penicillin acylase was to determine the extent substrate inhibition occurred, which would justify the inclusion of a substrate term in any subsequent modelling of the penicillin acylase system. As is clear from Figure 3.11(a), there exists inhibition by the substrate, Penicillin G. Peter and coworkers (1981) have pointed out that the Lineweaver-Burk plot is inappropriate for immobilised enzymes since it gives particular emphasis to data obtained at low substrate concentrations. At low substrate concentration, the concentration gradient (the driving force) across the particle surface - bulk solution is too low and diffusional limitations tend to occur. Figure 3.11(b) shows a modified Lineweaver - Burk plot to determine the substrate inhibition constant, \( K_s \). The substrate inhibition constant, \( K_s \) was found to be 412 (+/- 68) mM. \( K_s \) is derived from the intercept of the x axis (substrate concentration) when the reaction rate is zero (at Y axis = 0) using a linear fit model.

3.7.9.3 Product (6 amino penicillanic acid) Inhibition of penicillin acylase

The deacylation of penicillin G by penicillin acylase is inhibited by the desired product 6 amino penicillanic acid (Balasingham et al, 1972). The inhibition is non competitive and is independent of pH. Figure 3.14 illustrates a Dixon plot (Friedenwald and Maengwn-Davies, 1954) used to calculate the product inhibition coefficient. This is calculated by examining the effect of 6 APA concentration on the reaction rate of immobilised penicillin acylase at two substrate concentrations. The low substrate concentration used (to reduce any effects from substrate inhibition) was 3 mM and 6 mM penicillin G. The slope of each curve equals the reciprocal of the inhibition coefficient. For each substrate concentration, the slope of the curve is identical yielding a 6 APA inhibition coefficient of 105 mM (+/- 71).

3.7.9.4 Product (Phenylacetic acid) Inhibition of penicillin acylase

The deacylation of penicillin G by penicillin acylase is also inhibited by the by product in the reaction, phenylacetic acid (Balasingham et al, 1972). The inhibition is competitive and is dependant on the pH of the reaction media. Figure 3.15
illustrates a Dixon plot used to calculate the product inhibition coefficient at pH 7.8. The effect of PAA concentration on the reaction rate of immobilised penicillin acylase was measured at a low substrate concentration of 3 mM. Low concentrations of penicillin G were used to reduce any effects of substrate inhibition. The inhibition constant for phenlyacetic acid was found to be 109 mM (+/- 17). This value is largely arbitrary as it is dependant on pH and will change during the biotransformation.
Immobilisation of Enzymes to Magnetic Carrier Particles

Figure 3.13: Substrate inhibition of free penicillin acylase
Figure 3.14 : Dixon plots to calculate the Inhibition coefficient for 6 APA on the deacylation of penicillin G by penicillin acylase at penicillin G concentrations of (a) 3 mM and (b) 6 mM

Figure 3.15 : Dixon plot to calculate the Inhibition coefficient for PAA on the deacylation of penicillin G by penicillin acylase
3.7.10 Summary of conditions for optimised immobilised penicillin acylase system

The optimised process for immobilisation of penicillin acylase to magnetic particles is as follows.

1 volume of wet magnetic particles was added to 3 volumes of a 5% glutaraldehyde solution in 50 mM potassium phosphate pH 7.0. The mixture was shaken for ten hours at room temperature. The activated particles were washed three times with 50 mM potassium phosphate pH 7.0 and the majority of the liquid removed from the particles. 30 Units of penicillin acylase was added per ml of settled volume magnetic particles. 3 volumes of 50 mM potassium phosphate buffer pH 7.0 was added per volume of magnetic particle and the mixture shaken for 60 hours at room temperature. The immobilised particles were washed three times with 50 mM potassium phosphate pH 7.0 at 4 °C. 1 g of wet magnetic particle was equivalent to approximately 0.06 g dry magnetic particle.

This process was used for all large scale production required in Chapter 5.

From the experience learnt during the immobilisation development of penicillin acylase, an immobilisation development chart was constructed shown in Figure 3.16. This pictorial chart shows the experiments required to develop an immobilisation process for an enzyme onto supports outlining the essential experiments that must be completed.
Figure 3.16: Generic chart for developing an immobilised biocatalyst
3.7.11 Comparison of the Immobilised PA-MCP to other industrial / Literature forms

As mentioned in 3.1, the main objective of the immobilisation experiments was to establish an immobilised penicillin acylase comparable to those either published previously in literature or industrially available to purchase. Table 3.2 illustrates some of the large quantity of literature published using an immobilised penicillin acylase system.

The groundbreaking work completed at University College London in the 1970's by Warburton, Carleysmith, Lilly and Dunnill and others laid the foundations for one of only a handful of success stories in the area of large scale immobilised enzyme technology. The relatively low activities found by Warburton and coworkers (1972) have increased over the next two decades using better techniques and novel designs such as pellets and fibres. Table 3.3 illustrates the better test for the immobilised PA-MCP, what industry is using as its immobilised penicillin acylase system.
### Table 3.2: A literature comparison of immobilised penicillin acylase systems

<table>
<thead>
<tr>
<th>Researcher</th>
<th>Year</th>
<th>Support Matrix</th>
<th>Method</th>
<th>Specific Activity (U/g)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Warburton et al</td>
<td>1972</td>
<td>DEAE cellulose</td>
<td>Tiazine activation</td>
<td>117</td>
<td></td>
</tr>
<tr>
<td>Marconi et al</td>
<td>1973</td>
<td>Cellulose triacetate</td>
<td>Entrapment</td>
<td>40</td>
<td>Whole cells</td>
</tr>
<tr>
<td>Lagerlof et al</td>
<td>1976</td>
<td>Sephadex G-200</td>
<td>CNBr activation</td>
<td>468</td>
<td></td>
</tr>
<tr>
<td>Carleysmith et al</td>
<td>1980</td>
<td>CM Cellulose</td>
<td>glutaraldehyde activation</td>
<td>272</td>
<td>Activity in wet weight</td>
</tr>
<tr>
<td>Park et al</td>
<td>1982</td>
<td>DEAE cellulose/gelatin</td>
<td>glutaraldehyde activation</td>
<td></td>
<td>cylindrica l pellets</td>
</tr>
<tr>
<td>Ishimura &amp; Suga</td>
<td>1990</td>
<td>HPF fibres</td>
<td>glutaraldehyde activation</td>
<td>1910</td>
<td>NA</td>
</tr>
<tr>
<td>Fonseca et al</td>
<td>1993</td>
<td>Silica gel, 0.5 – 1 mm</td>
<td>3-amino-propyltriethoxysilane activation</td>
<td>41</td>
<td>NA</td>
</tr>
</tbody>
</table>

### Table 3.3: Summary of industrial penicillin acylase systems

*(Shewale and Sivaraman, 1989)*

<table>
<thead>
<tr>
<th>Company</th>
<th>Support Matrix</th>
<th>Activity (U/g)</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astra</td>
<td>Sephadex G-200</td>
<td>225</td>
<td>1988</td>
</tr>
<tr>
<td>Boehringer Mannheim</td>
<td>Polyacrlamide</td>
<td>335</td>
<td>1989</td>
</tr>
<tr>
<td>Novo</td>
<td>NA</td>
<td>60</td>
<td>1989</td>
</tr>
<tr>
<td>SK Beecham</td>
<td>Dextran/Sephadex</td>
<td>NA</td>
<td>1990</td>
</tr>
</tbody>
</table>
3.7.12 Economic Comparison of immobilised penicillin acylase on magnetic carrier particles

The details of the cost of producing the magnetic carrier particles are shown in Appendix 10. In summary the magnetic carrier particles themselves can be manufactured inexpensively, it is the cost of purchasing the enzyme and activating agent (in this case glutaraldehyde) that escalates the raw materials costs. For a reactor capacity of 1.5 litres with 50% biocatalyst concentration, Table 3.4 illustrates the comparative costs of each stage of manufacture and the total cost for producing the magnetic particles. A total cost of under £200 for a 500 ml batch of particles (approximately 275 g) compared favourably with those immobilised penicillin acylases available commercially (seen in Table 3.5).

Table 3.4: Approximate manufacturing costs of producing immobilised penicillin acylase on magnetic carrier particles

<table>
<thead>
<tr>
<th>Source</th>
<th>Support</th>
<th>Specific Activity</th>
<th>Price</th>
<th>Price (per gram)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIGMA</td>
<td>Acrylic beads</td>
<td>60 - 120 U/g</td>
<td>£148 / 1000 Units</td>
<td>£8.80 / 17.76</td>
</tr>
<tr>
<td>FLUKA</td>
<td>Polyacrylamide copolymer</td>
<td>150 U/g wet</td>
<td>£57.30 per 5 grams</td>
<td>£11.46</td>
</tr>
<tr>
<td>FLUKA</td>
<td>Eupergit</td>
<td>100 U/g</td>
<td>£148.10 per 5 grams</td>
<td>£29.60</td>
</tr>
</tbody>
</table>

Table 3.5: Prices of commercially available penicillin acylase
3.7.13 Scale up of Immobilisation of penicillin acylase to magnetic carrier particles

Figure 3.17 illustrates the effect of scale up of immobilisation on the specific activity on the immobilised magnetic carrier particles. Initial scale up experiments yielded only 33 U/g dry MCP which is a 97% loss in activity when scaling up from 2 ml eppendorf to a 2 litre shake flask. Initial studies set the mixing rate at 250 rpm for immobilisation at 2 litre scale. This caused foaming in the flask indicating the protein had left solution. Subsequent studies used a lower speed of 100 rpm, which resulted in higher retained activities of 200 Units/g dry MCP. However this is still 20% of the activity achieved at a small scale of 2ml. A possible explanation for the loss of activity on scale up is insufficient contact of the enzyme with the activated magnetic particle although further work in this area is required to gain a full explanation. It was decided that these losses could be accepted in order for the project to move forward.

![Diagram](image)

Figure 3.17 : Effect of scale up of immobilisation on the specific activity of immobilised penicillin acylase
3.8 Summary of Immobilisation of penicillin acylase to magnetic carrier particles

The following conclusions can be drawn from the successful immobilisation of penicillin acylase to magnetic carrier particles.

- The optimum glutaraldehyde concentration for optimum activity and stability of penicillin acylase was 5%. An enzyme challenge of 1750 U/g dry MCP using 5% v/v glutaraldehyde solution would give a specific activity of approximately 1000 U/g dry MCP and a retained activity of 60% at a 2mL scale.
- The immobilisation time which bound 98.5% protein was 60 hours.
- The optimum pH for immobilisation of penicillin acylase was in 50 mM phosphate buffer pH 7.0.
- Storage of immobilised penicillin acylase in 50 mM phosphate buffer pH 7.0 at 4°C resulted in a loss of only 5% activity over two weeks.
- Immobilised penicillin acylase on a particle density of 1.04 g/ml and 1.2 g/ml displayed greater pH stability than free enzyme.
- The kinetics of immobilised penicillin acylase shows penicillin G, 6 APA and phenylacetic acid inhibition with \( K_m = 5 \pm 0.55 \) mM, \( K_s = 412 \pm 68 \) mM, \( K_{6APA} = 105 \pm 70 \) mM and \( K_{IPA} = 109 \pm 17 \) mM.
- 80% of enzyme activity is lost when scaling the immobilisation experiments from 2 ml to 2 litre scale. At 2 litre scale, an enzyme challenge of 1750 U/g dry MCP using 5% glutaraldehyde solution would give a specific activity of approximately 200 U/g dry MCP and a retained activity of 12%.
- The immobilised penicillin acylase is comparable to other commercially available enzymes regarding specific activity and is significantly cheaper to produce.
- An immobilisation development chart has been drawn up on the experiences learnt to enable reduced development time on subsequent immobilisation development projects.
3.9 Results and Discussion of the Immobilisation of α-chymotrypsin to magnetic particles

3.9.1 Effect of glutaraldehyde concentration on the immobilisation efficiency of α-chymotrypsin on magnetic particles

The hydrolytic activity of the immobilised α-chymotrypsin and the protein content were used as parameters to determine the optimum glutaraldehyde concentration during activation of the magnetic particle prior to immobilisation. Figure 3.18(a) illustrates the effect of glutaraldehyde concentration of the specific activity and retained activity of the immobilised particle. The specific activity is defined here as the amount of enzyme bound to the magnetic particles. The retained activity is the activity bound to the beads as a percentage of the activity challenge.

Figure 3.18(a) shows that the specific activity of immobilised α-chymotrypsin increases with glutaraldehyde concentration up to 5% v/v. At higher glutaraldehyde concentrations of 10% there is no further increases in activity. Using a glutaraldehyde concentration of 5% v/v gives a specific activity of 3000 U/g dry MCP.

Figure 3.18(b) illustrates the stability of immobilised α-chymotrypsin at 4 °C over 4 days. It can be clearly seen that higher glutaraldehyde concentrations prevent activity leakage during storage. The activity lost during storage of immobilised α-chymotrypsin was very small (less than 1.2% for all glutaraldehyde concentrations over 14 days storage at 4 °C). Bearing in mind higher concentrations are undesirable in terms of leakage of glutaraldehyde during processing, a glutaraldehyde concentration of 5% v/v was used for the remainder of the immobilisation work. This would give an immobilised magnetic particle with a specific activity of 3000 U/g dry MCP with a retained activity of 70%.

On a stoichiometric level using a simplistic basis of one mole of glutaraldehyde for one mole of enzyme. For a 1 ml (scale of all immobilisation studies) volume of 10
mg/ml enzyme solution, this is equivalent to $4 \times 10^{-4}$ mmoles of $\alpha$-chymotrypsin of 25 kDa. The smallest concentration of glutaraldehyde used of 1% v/v (in 1 ml volume) is equivalent to $1.2 \times 10^{-2}$ mmoles, which is a thirty fold excess of glutaraldehyde molecules to the enzyme.

Figure 3.19 illustrates a protein analysis of the effect of glutaraldehyde concentration on the attachment of $\alpha$-chymotrypsin. The percentage of protein attached to the magnetic particles is over 99% (as determined by the Lowry method of protein determination) for all glutaraldehyde concentrations. The protein concentration left in the supernatant is slightly higher at 32 $\mu$g/ml at a glutaraldehyde concentration of 1% v/v as compared to a supernatant protein concentration of 18 $\mu$g/ml at a glutaraldehyde concentration of 10% v/v.

There are a variety of problems with glutaraldehyde activation of particles prior to immobilisation. The reagent itself is homobifunctional in nature and the commercial product is rarely uniform in each sample leading to problems of indeterminable reaction products and losses in reproducibility (Hermanson, 1996). The activated magnetic particles may crosslink with other particles at large excesses of glutaraldehyde which would result in lower specific activities of subsequent immobilised $\alpha$-chymotrypsin. The modification of carboxylates with diamines such as the one used in the magnetic particle manufacture (1,6 diaminohexane) form terminal amino groups. The amide linkages on the magnetic particles can react with the aldehyde groups on the glutaraldehyde molecules. High concentrations of 25% glutaraldehyde have been used previously to attach $\alpha$-chymotrypsin to non porous magnetic nickel (Munro et al, 1977). Although from a processing point of view, high concentrations of glutaraldehyde used in immobilisation preparations are undesirable. High concentrations are more likely to result in greater leakage of glutaraldehyde during processing resulting in difficult downstream processing. Previous researchers have used glutaraldehyde as a pre immobilisation activating step. Lozano et al, 2000 attached $\alpha$-chymotrypsin via 2.5% glutaraldehyde activation to ultrafiltration membranes yielding 11 Units/g of enzyme activity.
Figure 3.18(a) : Effect of glutaraldehyde concentration on the immobilisation efficiency of alpha chymotrypsin on magnetic particles. Specific activity (▲) and Retained activity (■)

Figure 3.18(b) : Effect of glutaraldehyde concentration on the activity loss during 4 day storage of immobilised alpha chymotrypsin
Figure 3.19: Effect of glutaraldehyde concentration on the protein attachment of α-chymotrypsin

Protein concentration in the supernatant (■) and % protein attached to magnetic particles (▲).
3.9.2 Effect of temperature of glutaraldehyde activation on the immobilisation efficiency of α-chymotrypsin on magnetic particles

*Figure 3.20* illustrates the effect of temperature on the activation of magnetic particles by glutaraldehyde. This effect is measured by subsequent immobilisation of α-chymotrypsin and the resultant specific activity of the particles. The temperature, which gives the highest specific activity on later immobilisation, is at room temperature of around 21°C. There is a significant drop in activity of 30°C activated particles of almost half that seen at 21°C activated particles. At lower temperatures of 4 and 12°C, there is a slight drop in the activity of the immobilised magnetic particle. The drop in activity seen at 30 °C may be due to reduced crosslinking efficiency at higher temperatures. This temperature for activation of particles in preparation for immobilisation is typical. Axen *et al*, 1970 used 23 – 26 °C for cyanogen bromide activation prior to the attachment of α-chymotrypsin whilst Munro *et al*, 1977 used room temperature for glutaraldehyde activation prior to the attachment of α-chymotrypsin to magnetic particles.

3.9.3 Effect of glutaraldehyde activation time on the immobilisation efficiency of α-chymotrypsin to magnetic particles

*Figure 3.21* shows the effect of glutaraldehyde activation time on the subsequent specific activity of immobilised α-chymotrypsin. The resultant specific activity increases with glutaraldehyde activation time up to 25 hours. More specifically, by increasing the activation time from 2.5 hours to 25 hours, the specific activity on the immobilised enzyme increases from 2400 U/g dry MCP to 3500 U/g dry MCP. After 25 hours the specific activity decreases slightly. The effect seen in *Figure 3.21* may be due to over crosslinking of the particles to themselves reducing the free amino terminal groups available for covalent attachment of α-chymotrypsin.

Subsequent activation of magnetic particles with glutaraldehyde used an activation time of 25 hours.
Figure 3.20: Investigation into the effect of temperature of glutaraldehyde activation on the specific activity of immobilised α-chymotrypsin

Figure 3.21: Effect of time of glutaraldehyde activation on the specific activity of immobilised α-chymotrypsin
3.9.4 Effect of enzyme challenge on the immobilisation efficiency of α-chymotrypsin on magnetic particles

*Figure 3.22* illustrates the effect of enzyme challenge of the specific and retained activity. As the enzyme challenge to the magnetic particles was increased the specific activity of the enzyme increases with a corresponding decrease in retained activity. At the highest enzyme challenge of 11155 Units, the specific activity of the biocatalyst 7600 Units/g dry particle and the retained activity was 68%. Although at 11155 total enzyme challenge, the saturation point has not been reached, it is already clear that unacceptable low levels of retained activity are occurring and therefore higher enzyme activity loadings were not analysed as these would result in even lower retained activities. At the lowest enzyme challenge of 64 Units, the specific activity of the biocatalyst was 230 Units/g dry particle retaining 78% of the challenge activity. At the meeting point of the curves, at an enzyme challenge of 4100 Units, a trade off is reached between the need for a high specific activity and the resultant loss in retained activities at higher challenges. This enzyme challenge gave an approximate specific activity of 3000 U/g dry MCP.
Figure 3.22: Effect of enzyme challenge on the specific and retained activities of immobilised α-chymotrypsin. Specific activity (■) and Retained activity (▲)
3.9.5 Effect of immobilisation temperature on the immobilisation efficiency of α-chymotrypsin on magnetic particles

The immobilisation temperature for efficient attachment of α-chymotrypsin to supports varies extensively throughout literature. Immobilisation performed early on in the 1960's when immobilisation techniques started, used 0°C for dizotization of CM cellulose (Mitz and Summaria, 1961). Kay and Lilly (1970) attached chymotrypsin to various supports at room temperature whilst Pliura and Jones (1980) used 5 °C. The breadth of discrepancies in the literature led to this parameter being investigated.

Activated magnetic particles were immobilised at temperatures of 4, 10, 21 and 30 °C for 72 hours at pH 7.0. The specific activity was measured using the biotransformation assay for α-chymotrypsin. Figure 3.23 shows large scattering of the data obtained but illustrates a general trend of higher specific activities being achieved at 4 °C. The scattering of data points may be due in part for storage issues as the biocatalyst was stored for 7 days prior to analysis. The scattering of points may also partly explain the discrepancies in the standard temperature used for immobilisation of enzymes in the literature.

3.9.6 Effect of immobilisation time on the immobilisation efficiency of α-chymotrypsin on magnetic particles

Figure 3.24 illustrates the effect of immobilisation time on the efficiency of the attachment of α-chymotrypsin to magnetic particles. After 18 hours the specific activity is high at 3500 - 4000 U/g dry MCP. Incubating free enzyme with MCP's for longer periods of time, 25 - 72 hours result in lower specific activity. Further experiments could have been carried out to see the changes between 0 to 20 hours but at this stage a specific activity of 4000 U/g dry MCP was satisfactory and an immobilisation time of 25 hours acceptable.
Figure 3.23: Effect of immobilisation temperature on specific activity of immobilised α-chymotrypsin

Figure 3.24: Effect of immobilisation time on the specific activity of immobilised α-chymotrypsin
3.9.7 **pH activity of free and immobilised α-chymotrypsin**

The activity of free and immobilised α-chymotrypsin is shown in Figure 3.25. The optima pH is approximately 8.2 for free enzyme. The pH optima of immobilised α-chymotrypsin is around 8.4 which is slightly higher than for free α-chymotrypsin. The pH activity experiments were performed using the optimised process for α-chymotrypsin immobilisation to MCP’s.

Alkaline shifts in the pH activity profiles of immobilised α-chymotrypsin as compared with native α-chymotrypsin are due to pH gradients (created by the action of the enzyme itself) when the product diffusion is restricted (Goldstein, 1976).

3.9.8 **Effect of substrate concentration on the activity of free α-chymotrypsin**

The effect of substrate concentration on the activity of free α-chymotrypsin is shown in Figure 3.26. The figure shows the relationship between enzyme activity and substrate concentration rises sharply up to 5 mM N-acetyl-L-tyrosine ethyl ester and then drops off.

3.9.9 **Effect of substrate concentration on the activity of immobilised α-chymotrypsin**

The effect of substrate concentration on the activity of immobilised α-chymotrypsin is shown in Figure 3.27. The experimental data are quite scattered although a general trend line has been fitted (using a rectangular hyperbola model). The data has been used in the kinetic evaluation in section 3.9.12. The enzyme activity increases with substrate concentration up to around 25 mM ATEE. The aqueous solubility limit of ATEE is 4g/L (equivalent to 15 mM) and so further analysis at higher ATEE concentrations could not be carried out. Higher concentrations of up to 25 mM were achieved by dissolving ATEE at 40 °C.
Figure 3.25: pH activity of free (▲) and immobilised (■) α chymotrypsin
Figure 3.26: Effect of substrate concentration on the activity of free α chymotrypsin
Figure 3.27: Effect of substrate concentration on the specific activity of immobilised α-chymotrypsin
3.9.10 Evaluation of kinetic parameter of free and immobilised α-chymotrypsin

Figures 3.28 and 3.29 illustrate Eadie-Hofstee and Lineweaver-Burk plots of free and immobilised α-chymotrypsin respectively. The kinetic parameters calculated from these plots are shown in Table 3.6. Free enzyme has been measured as units of activity per mg of protein.

Using the LineWeaver –Burk method of parameter evaluation, a graph is made of the reciprocal of substrate against the reciprocal of reaction rate. The resultant linear fit reveals the following information. The intercept on the y axis (1/reaction rate) yields the 1/V_{max} value and the intercept on the x axis (1/substrate) yields the -1/K_m value.

Using the Eadie-Hofstee method of parameter evaluation, a graph was made of the enzyme reaction rate/substrate concentration versus the enzyme reaction rate. The points are scattered for the immobilised α-chymotrypsin although a linear fit was attempted yielding the K_m and V_{max} values.

<table>
<thead>
<tr>
<th>Form</th>
<th>LineWeaver Plot</th>
<th>Eadie Hofstee Plot</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free α-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chymotrypsin</td>
<td>K_m = 0.585 mM</td>
<td>K_m = 2.24 mM</td>
</tr>
<tr>
<td></td>
<td>V_{max} = 149 U/mg</td>
<td>V_{max} = 345 U/mg</td>
</tr>
<tr>
<td>Immobilised α-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chymotrypsin</td>
<td>K_m = 3.85 mM</td>
<td>K_m = 0.605 mM</td>
</tr>
<tr>
<td></td>
<td>V_{max} = 1470 U/g dry</td>
<td>V_{max} = 774 U/g dry</td>
</tr>
<tr>
<td></td>
<td>MCP</td>
<td>MCP</td>
</tr>
</tbody>
</table>

Table 3.6: Kinetic parameters of free and immobilised α-chymotrypsin
Immobilisation of Enzymes to Magnetic Carrier Particles

Figure 3.28: Eadie Hofstee plot for free (■) and immobilised α-chymotrypsin (∗)

Figure 3.29: Lineweaver-Burk plot for free (■) and immobilised α-chymotrypsin (∗)
3.9.11 Summary of conditions for optimised α-chymotrypsin immobilisation

The optimised process for immobilisation of α-chymotrypsin to magnetic particles is as follows.

1 volume of wet magnetic particles was added to 2 volumes of a 5% glutaraldehyde solution in 50 mM potassium phosphate buffer pH 7.0. The mixture was shaken for 25 hours at room temperature. The activated particles were washed three times with 50 mM potassium phosphate buffer pH 7.0 and the majority of the liquid removed from the particles.

120 Units of α-chymotrypsin was added per ml of settled volume magnetic particles and the mixture shaken for 25 hours at 4 °C. 2 volumes of 50 mM potassium phosphate pH 7.0 was added per volume of magnetic particle and the mixture shaken for 25 hours at 4 °C. The immobilised particles were washed three times with 50 mM potassium phosphate buffer pH 7.0. The immobilised magnetic particles were stored in 50 mM potassium phosphate pH 7.0 at 4 °C.

This procedure would give an immobilised α-chymotrypsin of approximately 3000 U/g dry MCP.

The process was used for all large scale production required in Chapter 6.
3.9.12 Analysis of α-chymotrypsin magnetic particle as a commercially viable support

α-chymotrypsin is a prevalent enzyme used commercially and therefore a large amount of literature is available on the various immobilisation techniques used. *Table 3.7* shows a selection of published immobilised α-chymotrypsin matrices including the specific activity obtained. There is a wide range of specific activities for these matrices from 15 U/g (Munro, 1977) to 5000 U/mg (Axen, 1970) with a variety of retained activity success form low figures of 16% (Axen, 1970) to high figures of 75% (Kay and Lilly, 1970).

The purchasing cost of an immobilised enzyme is a large proportion of the running cost of the biotransformation. Thus, the cost of the immobilised enzyme is a crucial factor in comparison studies. The current commercially available immobilised α-chymotrypsin is shown in *Table 3.8*. The approximate manufacturing costs of producing α-chymotrypsin immobilised on magnetic particles is £148 for a 500 ml batch. The breakdown of the costs is shown in *Table 3.9*. Details of the costs of the magnetic particles themselves are shown in the Appendix.
<table>
<thead>
<tr>
<th>Author</th>
<th>Enzyme Challenge for immobilisation (mg/ml)</th>
<th>Buffer for immobilisation</th>
<th>Enzyme Activity</th>
<th>Retained Activity (%)</th>
<th>Particle and Density (g/ml)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axen <em>et al, 1971</em></td>
<td>NA</td>
<td>0.1M sodium bicarbonate</td>
<td>5 U/mg</td>
<td>16</td>
<td>Agarose (4%)</td>
<td>activation pH 11</td>
</tr>
<tr>
<td></td>
<td>NA</td>
<td>0.1M sodium bicarbonate</td>
<td>3.2 U/mg</td>
<td>25</td>
<td>Sephadex G-200</td>
<td>activation pH 10</td>
</tr>
<tr>
<td>Barros <em>et al, 1998</em></td>
<td>NA</td>
<td>NA</td>
<td>13 – 17 U/g</td>
<td>NA</td>
<td>Polyacrylamide</td>
<td>Used -22°C acetone to precipitate enzyme on support</td>
</tr>
<tr>
<td></td>
<td>NA</td>
<td>Tris</td>
<td>15 U/g</td>
<td>N/A</td>
<td>polypropylene</td>
<td>BSA pre immobilisation</td>
</tr>
<tr>
<td>Kay and Lilly, 1970</td>
<td>20</td>
<td>0.5 M sodium bicarbonate</td>
<td>113 mg enzyme/g support</td>
<td>37.8</td>
<td>DE 52 - cellulose</td>
<td>Water regain is 4.2 wet wt/dry wt</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.5 M sodium bicarbonate</td>
<td>212 mg enzyme/g support</td>
<td>75</td>
<td>Sephadex G-200</td>
<td>Water regain is 18 wet wt/dry wt</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.5 M sodium bicarbonate</td>
<td>392 mg enzyme/g support</td>
<td>74</td>
<td>Sepharose 4 B</td>
<td>Water regain is 25 wet wt/dry wt</td>
</tr>
<tr>
<td>Munro <em>et al, 1977</em></td>
<td>2</td>
<td>Distilled water</td>
<td>15 U/g</td>
<td>43</td>
<td>8.9 g/ml</td>
<td>B.S.A. pre-immobilisation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6.4mg enzyme/g support</td>
<td></td>
<td>3 - 7 microns Nickel powder</td>
<td></td>
</tr>
<tr>
<td>Plura and Jones, 1980</td>
<td>0.3M potassium bicarbonate</td>
<td>57.3 mg protein/g particle</td>
<td>37</td>
<td>37</td>
<td>Sephadex G-200</td>
<td></td>
</tr>
<tr>
<td>Robinson <em>et al, 1972</em></td>
<td>5</td>
<td>50 mM phosphate</td>
<td>309 U/g dry st</td>
<td>2.5</td>
<td>Magnetite-cellulose 53 – 63 µm</td>
<td></td>
</tr>
</tbody>
</table>

*Table 3.7: A literature comparison of α-chymotrypsin immobilised systems*
Immobilisation of Enzymes to Magnetic Carrier Particles

<table>
<thead>
<tr>
<th>Support Material</th>
<th>Supplier</th>
<th>Activity</th>
<th>Price</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose</td>
<td>Sigma</td>
<td>2000 – 3500 U/g support 65 – 120 U/ml</td>
<td>£135 for 100 Units</td>
</tr>
<tr>
<td>Cellulose</td>
<td>Sigma</td>
<td>400 – 600 U/g support</td>
<td>£24 for 10 Units</td>
</tr>
<tr>
<td>Acrylic beads</td>
<td>Sigma</td>
<td>500 – 1500 U/g support</td>
<td>£92 for 100 Units</td>
</tr>
</tbody>
</table>

Table 3.8: Prices of commercially available α-chymotrypsin

---

MCP production  £ 3.81
α-chymotrypsin  £66.10
(purchased from Sigma)
Glutaraldehyde  £46.20
(purchased from Sigma)
Overheads (20%, Sinnott, 1991)  £32.80
Total cost for immobilised chy-MCP system £148.91 per 500 ml batch

Table 3.9: Approximate manufacturing costs of producing α-chymotrypsin immobilised on magnetic carrier particles
3.9.13 Scale up of Immobilisation of α-chymotrypsin to magnetic carrier particles

*Figure 3.30* illustrates the effect of scale up of immobilisation on the specific activity on the immobilised magnetic carrier particles. Initial scale up experiments yielded only 386 U/g dry MCP which is a 87% loss in activity when scaling up from 2 ml eppendorf to a 2 litre shake flask. It was decided that these losses could be accepted in order for the project to move forward.

![Diagram](image)

<table>
<thead>
<tr>
<th>Initial</th>
<th>Scale up</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 ml Eppendorf 0.5 g WET MCP's</td>
<td>200 ml Duran Bottle 40 ml WET MCP's</td>
<td>2 litre shake flask 0.2L WET MCP's</td>
</tr>
<tr>
<td>Specific Activity 3000 U/g DRY MCP</td>
<td>Specific Activity 2508 U/g DRY MCP</td>
<td>Specific Activity 386 U/g DRY MCP</td>
</tr>
</tbody>
</table>

*Figure 3.30 : Effect of scale up of immobilisation on the specific activity of immobilised α-chymotrypsin*
3.10 Summary of immobilisation of α-chymotrypsin to magnetic carrier particles

The following conclusions can be drawn from the successful immobilisation of α-chymotrypsin to magnetic particles.

- The optimum glutaraldehyde concentration for activity and stability of α-chymotrypsin magnetic particles was 5%. An enzyme challenge of 4100 U/g dry MCP gave a specific activity of 3100 U/g dry MCP with a retained activity of 77%
- The optimum immobilisation time was 25 hours
- The optimum immobilisation temperature was 4°C
- The optimum pH shifted from pH 8.2 for free α-chymotrypsin to pH 8.4 for immobilised α-chymotrypsin
- The pH activity profiles are very similar in shape
- Using a Lineweaver Burk analysis of the kinetic data, the Michaelis Menton constant, $K_m$, was found to be 0.58 mM for free α-chymotrypsin and 3.85 mM for immobilised α-chymotrypsin
- An 87% loss in activity was observed when scaling up the immobilisation from 0.5mL to 2 litre scale
- The immobilised α-chymotrypsin was comparable to other commercially available alternatives and is cheaper to produce
Chapter 4

Physical Characterisation of the MSFBR and MCP’s
Chapter 4  Physical Characterisation of the MSFBR and MCP's

4.1 Chapter Aims

This chapter will report the characterisation of both the magnetic carrier particles and the magnetically stabilised fluidised bed reactor. The magnetic carrier particles characterisation aims were to establish the relative merits of each particle size and differing densities. The aim was to characterise particles manufactured using an original method developed by Pieters (1989) and investigate improvements on these particles which culminates to the finished method, used for large scale biocatalysis, described in section 3.1. The range of particle sizes was obtained by using different impeller speeds during the production stage. Using Pieters (1989) method, Moffat (1990) recommended using agitation at 700 rpm on addition of the magnetite-gel mixture to the oil, which produced particles in the range 300 - 600 microns. This was envisaged as the largest size useable in this project and therefore particle size range studies started using 700 rpm (to give particles of sizes 300 - 600 microns) up to 1300 rpm (to give smaller particle sizes) during manufacture at 0.3 Litre scale.

One of the essential operating parameters of the MSFBR is the maintenance of a uniform magnetic field strength within the reactor. This is achieved by using theoretical equations to calculate the positions of the magnetic coils that will give uniform field strength throughout the reactor. The physical characterisation checked the theoretical predictions against those measured. To establish the relationship between flowrate of media, expanded bed height and magnetic field strength for the various sizes and densities of particles, bed expansion curve experiments were performed.

An important part of the characterisation studies was to establish physical operational limitations of the MSFBR and to compare this with those present in a conventional fluidised bed. This was achieved by analysis of the relationship between flowrate and bed height for a fluidised bed. For the MSFBR, the relationship was examined again with the extra parameter of magnetic field strength. This information defined
the increase in flowrate achieved by using the MSFBR for different particle sizes and densities.

The type of mixing required in the MSFBR is dependent on the type of biotransformation performed in the reactor. Both enzyme systems used in the reactor required to be run as a plug flow reactor for the reaction in combination with a stirred tank reactor for pH control. This resulted in the requirement of a set of mixing studies. The mixing studies primarily needed to establish that the reactor operated in plug flow mode. The most commonly used method for determining the mixing in plug flow reactors, by analysing the axial dispersion, was used.
4.2 Experimental protocol for characterisation of the magnetic carrier particles

4.2.1 Physical characterisation of the magnetic carrier particles

4.2.1.1 Particle size range

Particle sizes were analysed using four brass sieves (Endecotts, London, U.K.) of mesh size 65, 125, 250 and 350 microns using a Vibratory sieve shaker (Fritsch, Christianson Scientific Equipment, Gateshead, U.K.). The analysis was checked using a particle image analyser (Malvern mastersizer 2000, Malvern, U.K.).

4.2.1.2 Density

After hardening of the MCP’s the density of the beads was determined using volumetric flasks. Using a 25 ml flask, the vessel was filled to the calibration mark with distilled water and weighed (W1). A number of beads were added to the flask, at least 2 grams in wet weight, and weighed (W2). Using a pasteur pipette, the water was removed until the level in the flask equalled the calibration mark. Again the flask was weighed (W3). This procedure was repeated four times for each particle size range within each batch to minimise any errors.

The weight of the particles was the difference between W2 and W1. The volume of the particles was the difference between W3 and W2, divided by the density of distilled water (998 kg/m³). The density of the magnetic carrier particles (\( \rho_{MCP} \)) was subsequently calculated as follows.

\[
\rho_{MCP} = \frac{W_2 - W_1}{W_2 - W_3} \frac{1}{\rho_{H_2O}}
\]
4.3 Experimental protocol for characterisation of the magnetically stabilised fluidised bed reactor

4.3.1 Calculation of the magnetic field strength within MSFBR

The magnetic field strength in the reactor was measured using a Hall probe (Trilec portable gaussmeter 700, Magnetic Developments Ltd, Swindon, Wiltshire, U.K.). The readings were taken at various points both horizontally and vertically in the reactor, with the probe held horizontally for each reading.

The positions of the coils were adjusted by moving the screw supports on each of the four metal stands holding the coils. Each time the coils were moved, a spirit level was used to ensure each coil was axially level.

4.3.2 Bed Expansion curves experiments

The desired mass of particles was placed in the reactor, to give the required settled bed height. A 32-rpm peristaltic pump (Watson Marlow, Falmouth, Cornwall, U.K.) was used for flowrates 10 to 90 ml/min and a 250-rpm peristaltic pump (Watson Marlow, Falmouth, Cornwall, U.K.) was used for higher flowrates up to 1.25 litre/min. 50 mM potassium phosphate buffer pH 7.0 was used as the carrier fluid in all bed expansion experiments. Operating in recycle mode, for each flowrate and magnetic field strength the bed was left to stabilise for 30 minutes and the bed height recorded. This was completed at increasing flowrates until particle elution was observed.

The experiments were completed using two types of particle each of varying density. The first particle had a density of 1.04 g/ml with two particle size ranges of 125 - 250 microns and 250 - 350 microns. The second particle had a density of 1.2 g/ml with two particle size ranges of 125 - 250 microns and 250 - 350 microns.
The bed expansion curves ranged from using no magnetic field strength (a conventional fluidised bed) up to 22.5 mTesla.

4.3.3 Mixing studies in the MSFBR

The reactor was initially fluidised using 50 mM potassium phosphate buffer pH 7.0 at the desired flowrate and left to stabilise for a total of 30 minutes. When the magnetic field was required, it was switched on after 15 minutes. After 30 minutes, or until the bed appeared stable, the inlet feed switch was turned from buffer to a 1% acetone solution (Sigma, Poole, Dorset, U.K.). Samples were taken from the exit line every 2 - 5 minutes (depending on the flowrate) and measured using UV adsorption on a spectrophotometer at 280 nm.

Once the UV reading read 1% acetone the inlet feed switch was turned back to buffer and samples taken as before. A concentration - time curve was obtained for each experiment and an analysis of dispersion coefficient undertaken.
4.4 Results of the characterisation of the magnetic carrier particles

4.4.1 Particle size range

The primary aim of the particle size range experimentation was to establish the conditions of manufacture required for specific particle sizes. The range of particle sizes could be manipulated by alteration of the impeller speed during production. The manufacturing method as outlined in section 3.1 largely depends on the agitation rate for the range of particle sizes produced. Figure 4.1 illustrates the effect of impeller speed on the particle size range. The particles of most interest are the 125 - 250 and 250 - 350 microns as these would be used in the large scale biotransformations in the MSFBR experimentation (only 125 - 250 micron), the bed expansion profiles and mixing studies in the MSFBR. As can be seen from Figure 4.1, an impeller speed of 1250 rpm produces the desired particle size ranges.

*Figure 4.1* was produced by using wet sieving as the method of particle size analysis which is susceptible to errors from particle surface water affecting the size, adhesion of particles creating larger sizes and insufficient sieving time. The inherent errors of wet sieving were reduced by using a mechanical shaker (Vibratory sieve shaker) and a long sieving time of 2 hours. The wet sieving method provided a simple, low cost, quick method for separating the different particle sizes. However, in order to standardise this method a particle image analyser was used to give a detailed particle size distribution.

The particle image analysis was completed on one batch of magnetic carrier particles of 125 - 250 microns with a total volume of 300 ml. A Malvern Mastersizer was used which provides details of the volume and surface weighted mean, specific surface area and d50. These particles were originally taken from a batch separated using wet sieving. These particles were low density (1.04 g/ml) with a total sample weight of 312 g. *Figure 4.2* shows the size frequency curve obtained using 10
micron fractions. The mean equivalent diameter of the magnetic particles in this sample was between 165 and 175 microns.

As expected the size distribution analysis shows the existence of a significant mass of fine particles (25 - 125 microns). This function of the distribution represented 27% of the particles. The main drawback of the wet sieving method is the strong inclination of small particles to adhere to the larger particles preventing transit to the lower mesh size sieves. As mentioned earlier, increasing the sieve time reduces this effect. However, it was found that sieving times beyond 2 hours produced no significant weight increase on each sieve. 10% of the particle size distribution was above the 250 micron mark which illustrates the errors in the mesh size of the sieve or errors in the particle image analyser itself. Overall 63% of the particles analysed in the particle image analyser fell in the range 125 - 250 microns.
Physical Characterisation of the MSFBR and MCP's

Figure 4.1: Effect of impeller speed on the particle size range produced of magnetic carrier particles
Figure 4.2: Particle size range scan using Malvern Mastersizer, particle image analyser of particles labelled 125 - 250 microns by sieving method.
4.4.2 Density

The density of the magnetic carrier particles plays a significant role in determining the operating limits for the MSFBR as it defines the terminal settling velocity of the particles. The terminal settling velocity is one of the fundamental parts of the Richardson and Zaki equation for fluidisation of particles, shown below.

\[
\frac{u}{u_t} = \varepsilon^n
\]

(Richardson and Zaki, 1954)

where

- \(u\) : Linear velocity (m/s)
- \(u_t\) : Terminal settling velocity (m/s)
- \(\varepsilon\) : Voidage of the bed
- \(n\) : Function of Reynolds number, Re

The measurement of the particle density is an important step in the characterisation of the magnetic carrier particles. In order to alter the density of the particles, the mass of precipitated magnetite added during manufacture was altered. The standard procedure (section 3.1) resulted in the creation of a high density magnetic particle of 1.2 g/ml. Figure 4.3 illustrates the relationship found experimentally between weight of precipitated magnetite added and the density of particle produced. Although the density of particle produced could be theoretically calculated, process errors during manufacture, such as inability to remove the total mass of carrageenan magnetite composite from the vessel, resulted in the necessity to acquire this data.
4.4.3 Source of magnetite

The method for magnetic carrier particle manufacture differed fundamentally than that originally published by Pieters, 1989 in terms of the magnetite used to provide the magnetic part of the carrier particle. Original studies using magnetite purchased through Aldrich (Gillingham, Dorset, U.K.) (stated as having an average particle size of 5 microns) produced carrier particles with large pieces of magnetite in the particles as opposed to the required uniform dispersion of magnetite within the gel. Literature investigations into a possible solution to this problem resulted in the in house manufacture of magnetite by a precipitation method. Using a method originally published by Kobayashi and Matsunaga, 1991 whereby Fe$_2^+$ and Fe$_3^+$ ions are combined to produce uniform magnetite particles. This method was established to provide a rapid manufacturing technique for large quantities of magnetite (up to 1 kg). The mass of precipitated magnetite added during production was varied to produce particles of various densities.

Figure 4.4 shows a photograph of magnetic carrier particles manufactured using commercial coarse magnetite. Large masses of magnetite can clearly be seen. The non uniformity of magnetite within the particles will adversely affect the stabilisation of the MSFBR.

Figure 4.5 shows a photograph of the MCP’s generated using the precipitated magnetite. These pictures show particles of 125 - 250 micron size range with a density of 1.04 g/ml (the scale of each photograph is shown on the figure).
Figure 4.3: Effect of mass of precipitated magnetite during MCP manufacture of the density
Figure 4.4: Magnetic carrier particles manufactured using the coarse commercial magnetite
Figure 4.5: Magnetic carrier particles manufactured using precipitated magnetite
4.5 Characterisation of the MSFBR

4.5.1 Theoretical optimum position of coils for uniform magnetic field

Using the Biot-Savart Law, the field formed on the common axis of two coils can be related to the current applied, the diameter and the distance between the coils.

Two parallel, co-axial coils arranged as in Figure 4.6 are often used to provide a field that cancels out unwanted fields, like the Earth's field, or fields from iron building supports (Phillips and Grant, 1990). The coils, when arranged properly can provide a field which is almost uniform across an appreciable volume.
Physical Characterisation of the MSFBR and MCP's

\[ B = \frac{\mu_0 I}{4\pi} \oint \frac{dL' \cos \theta}{|r - r'|^2} \]  

(4.1)

where:

- \( B \): Magnetic field strength (Tesla)
- \( \mu_0 \): Permeability of free space
- \( I \): Applied current (Amps)
- \( dL' \): An element of the magnetic vector \( dB \)
- \( dB \): Magnetic vector in the plane OQP making an angle \( \theta \) with the axis which is also equal to the angle between lines OQ and QP as seen in Figure 4.6

\( \cos \theta \) and \( |r - r'|^2 \) are constant and equal to

\[ \cos \theta = \frac{a}{(a^2 + x^2)^{1/2}} \]  

(4.2)

\[ |r - r'|^2 = (a^2 + x^2) \]  

(4.3)

Substituting gives

\[ B = \frac{\mu_0 I}{4\pi} \frac{a}{(a^2 + x^2)^{3/2}} \oint dL' \]  

(4.4)

where \( dL' = 2 \pi \)

\[ B = \frac{\mu_0 I}{2} \frac{a^2}{(a^2 + x^2)^{3/2}} \text{ due to coil 1} \]  

(4.5)

The field at point P due to the second coil is in the same direction and its magnitude is obtained by replacing the variable \( x \) in the above equation with \( (L - x) \)
Combining 4.5 and 4.6 to give the total resultant field

\[ B = \frac{\mu_0 I}{2} \left( \frac{a^2}{(a^2 + (L-x)^2)^{3/2}} \right) \]

due to coil 2

Equation (4.7) can be applied to four coils by using the same method.

For a uniform magnetic field, the double differential must be equal to zero.

\[ \frac{d^2 B}{dx} = 0 \text{ which gives } L = a \text{ and } x = a/2 \]

This Biot–Savert based proof was taken from Dobbs, 1993. However for this purpose it needs to be taken one step further to gain an equation for the uniform field. Substituting \( x = a/2 \) into the total field gives an equation that can be used to calculate the uniform magnetic field strength (\( B \)) knowing the applied current (\( I \)) and radius of the coil (\( a \)).

\[ B = \frac{8\mu_0 I}{5\sqrt{5}a} \]

This equation can be used to calculate the parameters required to create an uniform field in the MSFBR.

4.5.2 Relating theoretical equations to the Helmholtz coils used in the MSFBR

The MSFBR contains four helmholtz coils each with 500 turns (\( N \)) and a diameter of 8.75 cm which adapts equation (4.8) to
Physical Characterisation of the MSFBR and MCP's

\[ B = \frac{16\mu_0 I}{5\sqrt{5}a} \]  

(4.9)

For an applied current of 5 amps the magnetic field predicted with the theoretical equations gives 5 mTesla, which matches that displayed on the Hall probe.

Table 4.1 illustrates the differences between the predicted magnetic field by the theoretical equations and that measured by the Hall probe on the gaussmeter. There is good agreement between the predicted and measured values with a maximum 2.8% error.

<table>
<thead>
<tr>
<th>Applied Current (Amps)</th>
<th>Magnetic Field Theoretical Equations (mT)</th>
<th>Measured Magnetic Field (mT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>2.6</td>
<td>2.5</td>
</tr>
<tr>
<td>5</td>
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</tr>
<tr>
<td>15</td>
<td>15.4</td>
<td>16</td>
</tr>
<tr>
<td>17.5</td>
<td>18.0</td>
<td>18</td>
</tr>
</tbody>
</table>

Table 4.1 : Comparison of the magnetic field predicted by the Biot - Savart Law and the measured magnetic field strength in the MSFBR
As the coils are manufactured by turning a long copper wire, manufacturing imperfections are frequent resulting in the coils producing often unpredictable fields. It was not surprising that the exact position of the coils to produce the desired uniform magnetic field was not as predicted by the theoretical equations. It is stated in the proof in section 4.5.1 that to create an uniform magnetic field, the coils should be placed apart a distance equal to the radius of the coils. Figure 4.7 shows the vertical variations in magnetic field strength measured using the portable gaussmeter at a distance of 8.75 cm apart (radius of the helmholtz coils). As can be seen, the vertical variation of magnetic field along the reactor is very large with 30% of the maximum field at the bottom of the reactor.
Figure 4.7: Variation of magnetic field strength when helmholtz coils are placed 8.75 cm apart as predicted by the theoretical equations to the optimum of an uniform field along MSFBR.
A series of experiments were subsequently performed to establish the optimum position of the coils to obtain the desired uniform magnetic field at different heights and distance between the coils.

Systematic alteration of the coil position vertically along the reactor from 1 cm to 20 cm determining an optimum position for a uniform magnetic field. Horizontal variations were determined at the following points:
(a) Centre of the reactor
(b) Outside edge of the reactor (2.5 cm from central axis)
(c) 5 cm from central axis
(d) Inside of helmholtz coil (8.75 cm from central axis)
Vertical variations were determined at arbitrary points along the reactor on the central axis.

Figure 4.8 shows the variations in magnetic field strength seen in the MSFBR when the helmholtz coils are placed 11 cm apart which was found to be the optimum. Figure 4.8 illustrates a three dimensional analysis of the horizontal variations across the helmholtz coil. Point 0, 0 on the x, y axis denotes the centre on the coil (and hence the centre of the MSFBR). The blue cross of lines on the top of the profile illustrates the uniformity of magnetic field strength within the MSFBR itself. The magnetic field strength was found to drop by only 2% towards the edge of the MSFBR but substantially drops between the outside of the MSFBR wall and the helmholtz coil as the diagram shows. Figure 4.9 shows the vertical variations in magnetic field strength. The magnetic field strength decreases by 5% towards the top of the reactor and by 40% at the bottom of the reactor. The helmholtz coils produce a uniform field horizontally when placed 11 cm apart. Clearly the four-coil rig can be moved vertically to decrease the magnetic field fluctuations at the bottom of the reactor but this would have a detrimental affect towards the top. As it is the escape of the magnetic particles, which the magnetic coils are attempting to prevent, it is more advantageous to have a lower drop in magnetic field strength at the top of the reactor.
Figure 4.8: Variations in magnetic field strength vertically horizontally at 10 mTesla (x and y axis are a cross section of the reactor measured in cm)
Figure 4.9: Vertical variation of magnetic field strength along the centre of the MSFBR at different applied currents of 2.5 (□), 5 (●), 7.5 (▲), 10 (○), 12.5 (★), 15 (■), and 17.5 (▼) Amperes.

Position 1 on the x-axis is the top of the reactor and position 5 is the bottom of the reactor.
The magnitude of the magnetic field strength (determined by the applied current) has no significant effect on the uniformity of magnetic field strength. This is clearly demonstrated in Figure 4.9.

4.5.3 Bed Expansion curves

The bed expansion curves largely define the physical operating limits of the MSFBR by identifying the escape velocity (the minimum flowrate at which particle elution occurs), voidage of particles within the reactor and biocatalyst concentration within the reactor at set conditions.

The variables that were identified as having the strongest influence on the expansion profiles were density of particle, particle size range, linear velocity and magnetic field strength.

As previously stated in section 1.5, the major advantage of the MSFBR is the ability to increase the linear flowrate through the reactor. Figure 4.10 shows the influence of magnetic field strength on the bed expansion profiles of a 19 cm settled bed height of 1.2 g/ml density magnetic particles. This is equivalent to a particle concentration in the reactor of 33%. The flowrate has been plotted against voidage, a commonly used parameter in expanded bed characterisation. To establish the link between the bed height, bed expansion (%) and voidage of the bed, the three parameters were plotted together against linear flowrate in Figure 4.11.

Figure 4.10 illustrates that for increasing flowrate the voidage increases as expected. Adding a further parameter of magnetic field strength shows that for up to 15 mTesla, the voidage decreases with increasing magnetic field strength for the same flowrate. This again is as expected as this is the main advantage of the MSFBR; the ability to operate at higher flowrates. By utilising a magnetic field strength of 15 mTesla, the reactor can double the throughput of fluid.

The comparison shown in Figure 4.11 shows expansions for two particle sizes, 125 - 250 and 250 - 350 microns from a settled bed height of 10 cm. But utilising a larger
particle size, the achievable linear velocity is increased by 50% from 200 cm/hr (125 - 250 microns) to 300 cm/hr (250 - 350 microns).

The bed expansion height (and therefore the % bed expansion) should be directly related to the linear velocity (Finette et al., 1996) although most expansions in Figure 4.11 (a) and (b) fail to show a linear relationship.

The density of the magnetic carrier particle will have a significant effect on the behaviour of the particles in the bed. Figure 4.12 shows the bed expansion profile of two different density particles of (a) 1.04 g/ml and (b) 1.2 g/ml for various magnetic field strengths. Increasing the density of the particle decreases the surface area available for immobilisation of enzyme and the effects of this are described in chapter 5. However decreases in density will reduce the operating flowrate of the reactor. A lower density particle has a lower escape velocity. The aim of this set of experiments was to quantify the reduced escape velocity resulting from a lower density particle, to enable an effective operating limit to be established. For both Figure 4.12 (a) and (b), the settled bed height was 19 cm. Under a conventional fluidised bed the low density particles begin to leave the reactor at flowrates above 30 ml/min (equivalent to 92 cm/hr) and the high density particles leave at 33 ml/min. Using a magnetic field strength of 12.5 mTesla, the low density particles begin to leave the reactor at flowrates above 37 ml/min whilst the high density particles have an escape velocity of over 60 ml/min.
Figure 4.10: Effect of magnetic field strength on the relationship between voidage and flowrate for magnetic particles of size range 125 - 250 microns and density 1.2 g/ml. Magnetic field strengths are 0 (■), 5 (●), 10 (▲), 12.5 (▼) and 15 (♦) mTesla.
Figure 4.11: The effect of linear flowrate on the expansion characteristics (a) bed height (cm), (b) bed expansion (%) and (c) voidage for MCP’s of 125 - 250 microns fluidised at 0 mTesla (■) and 10 mTesla (●) and 250 - 350 microns fluidised at 0 mTesla (○) and 10 mTesla (○)
Figure 4.12: Effect of magnetic field strength on the bed expansion profile of two different density magnetic carrier particle beds
Density of MCP is 1.04 g/ml (a) and 1.2 g/ml (b) fluidised at a magnetic field strength of 0 m Tesla (■), 5 mTesla (●), 7.5 mTesla (△), 10 mTesla (▲), 12.5 mTesla (▼) and 15 mTesla (○)
4.5.4 Mixing studies in the MSFBR

The primary aim of this section of the MSFBR characterisation was to establish the MSFBR as a plug flow type reactor. For the application of the first model system, penicillin acylase it was essential to operate in plug flow mode.

As described in section 4.3.3, a step input of 1% acetone was used as a tracer study investigation to characterise the type of mixing in the MSFBR. Using the two different particle sizes available, namely 125 - 250 micron and 250 - 350 micron, and varying the linear velocity, settled bed height and mode of operation a comparison could be made.

Table 4.2 illustrates the full set of results for the mixing study experiments.

Figure 4.13 illustrates the C curve concentration plots obtained from acetone tracer experiments using MCP’s of 125 - 250 microns. The settled bed height was 10 cm (equivalent to a particle concentration within the reactor of 20%) which was fluidised using 50 mM potassium phosphate buffer pH 7.0 using a flowrate of 35 ml/min to an expanded bed height of 34 cm. At time 0, the inlet switch was turned to an acetone feed. The switch was altered to buffer at time 76 minutes for operation in a fluidised bed mode (Figure 4.13 (a)) and at time 103 minutes for operation as a MSFBR mode (Figure 4.13 (b)). The dispersion coefficient (D / UL) for operation as a fluidised bed and as a MSFBR for the C curves are 0.08 and 0.025 respectively which according to Levenspeil, 1970 is equivalent to intermediate amounts of dispersion.

Previous attempts at characterising the mixing within a MSFBR have used the axial dispersion peclet number. Goetz and Graves (1991) found axial dispersion peclet numbers of 0.2 to 2.5 for 0.8 cm diameter by 11 cm. Siegall, 1987 using a 7.65 cm diameter by 50 cm found axial dispersion peclet number of between 0.2 and 0.9. Burns and Graves (1988) published a detailed paper on axial dispersion in a small scale 1 cm diameter by 9.8 cm and found peclet numbers of 0.3 to 4. For all of these axial dispersion investigations a pulse injection of tracer was used rather than the step...
input used in this work. The axial dispersion peclet number relies heavily on the shape of the response in particular the tail end of the peak. This effect has been highlighted in literature previously (Goetz and Graves, 1991). Using the variance matching procedure the whole peak is used to calculate the dispersion coefficient (D / UL). Therefore for the characterisation of real reactors this variance matching procedure is more reliable.

The dispersion model (or dispersed plug flow) is used in the analysis of packed beds and fluidised beds to analyse the amount of dispersion present in the reactor and the extent of plug flow behaviour. The model assumes that there exist no stagnant pockets and no gross bypassing or short circuiting of fluid in the vessel. It is based on Ficks Law for molecular diffusion and defines D / UL as the dispersion coefficient.

\[
\frac{D}{UL} \xrightarrow{\text{negligible dispersion}} \text{plug flow} \\
\frac{D}{UL} \xrightarrow{\text{large dispersion}} \text{mixed flow}
\]

It was anticipated that the MSFBR and certainly the fluidised bed would have intermediate degrees of dispersion. On a C curve (experimental C curve from this work seen in Figure 4.13), levels of D / UL above 0.01 skew the curve from a long needle shape to those seen in Figure 4.13. The mean and variance of these curves are given by equation 4.10.

\[
\sigma_0^2 = 2 \cdot \frac{D}{UL} - 2 \cdot \left( \frac{D}{UL} \right)^2 \cdot \left(1 - e^{-\frac{UL}{L}}\right)
\]  \hspace{1cm} (4.10)

The variance of each curve was found and through iteration, a value of D / UL for each tracer experiment was found. An example of the C curve obtained for one set of experiments is seen in Figure 4.13. The C Curve is a common method of normalising a RTD. The tracer response for the fluidised bed shows an interesting set of points towards the end of the curve after 160 minutes. This may be due to substantial mixing occurring at the top of the bed (the MSFBR stabilises the top)
resulting in a mixed regime typical response at the tail end of the C curve. The full results from the mixing analysis are seen in Table 4.2.

Levenspiel (1972) defined the following dispersion coefficients.

\[
\begin{align*}
D / UL &= 0.002 \quad \text{Small amounts of dispersion} \\
D / UL &= 0.025 \quad \text{Intermediate amounts of dispersion} \\
D / UL &= 0.2 \quad \text{Large amounts of dispersion}
\end{align*}
\]

Using a high flowrate of 525 cm/hour, a significant improvement was found by using a magnetic field. Experiments A and B show dispersion coefficients of 0.049 and 0.026 for fluidised and MSFBR mode reactors respectively. For 125 - 250 micron particles at a flowrate of 104 cm/hour and settled bed height of 10 cm the D/UL was smaller when using the MSFBR (indicating improved plug flow).

At 104 cm/hour, using a settled bed height of 10 cm (particles of 250 - 350 microns), the D/UL values in both reactor types are larger compared with a higher flowrate of a 4 cm settled bed height. No definitive conclusions can be drawn from this as both flowrate and settled bed height were different. However it can be seen that larger D/UL values are seen using a fluidised bed compared with the MSFBR. Expt E and F which are exactly the same experiment produced similar D/UL values of 0.238 and 0.185 respectively for the MSFBR operated at 104 cm/hour from a settled bed height of 10 cm of 250 - 350 micron particles. For the same set up without the magnetic field, the D/UL value was higher at 0.548.

The density of the particles will also have an effect on the axial dispersion in a fluidised bed. Tang and Fan (1990) showed that low density particles (1.05 - 1.3 g/ml) should significantly lower axial liquid dispersion in a fluidised bed than higher density particles. It has been shown be Asif (1991) that the distributor can cause severe distortion in the bed hydrodynamics when the particle density is 1.6 g/ml although at higher densities of 2.4 g/ml, no such distortion was observed.
### Physical Characterisation of the MSFBR and MCP’s

<table>
<thead>
<tr>
<th>Expt No</th>
<th>Particle Size Range (μm)</th>
<th>Flowrate (cm/hour)</th>
<th>Settled Bed Height (cm)</th>
<th>Mode of Operation</th>
<th>Voidage of bed</th>
<th>Dispersion Coefficient D / UL</th>
</tr>
</thead>
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<tr>
<td>A</td>
<td>250-350</td>
<td>525</td>
<td>4</td>
<td>FB</td>
<td>0.332</td>
<td>0.049</td>
</tr>
<tr>
<td>B</td>
<td>250-350</td>
<td>525</td>
<td>4</td>
<td>MSFB</td>
<td>0.332</td>
<td>0.026</td>
</tr>
<tr>
<td>C</td>
<td>125-250</td>
<td>104</td>
<td>10</td>
<td>FB</td>
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<td>0.08</td>
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</table>

*Table 4.2: Mixing study data*
Physical Characterisation of the MSFBR and MCP's

Figure 4.13: C curve concentration plots from tracer experimentation MSFBR conditions: Flowrate of 35 ml/min (equivalent to a linear velocity of 104 cm/hr) at an expanded bed height of 34 cm from settled bed height of 19 cm operated in (a) MSFBR mode at a magnetic field strength of 10 mTesla and (b) fluidised bed mode.
4.6 Summary of Physical Characterisation

The characterisation of the magnetic carrier particles has given specific manufacturing conditions for production of a specific particle size range. This has enabled bed expansion profiles of different particle sizes in the MSFBR. The method for separation of particle sizes, wet sieving has been checked using particle image analysis. The percentage of particles within the 125 - 250 micron range separated using wet sieving that were found to be within this range using the particle image analysis was 63%. A significant proportion of the particles (27%) that fell outside this range were 25 - 125 microns in size. During operation of the MSFBR the particle bed was conditioned for 1 hour to enable small particles to leave the reactor.

The most important aspect of the characterisation work has been the use of precipitated magnetite rather than coarse magnetite. Precipitated magnetite allows greater uniform distribution of the small magnetite particles through the carrageenan gel. Precipitated magnetite has a diameter of 1 -2 microns whereas the coarse commercial magnetite has a diameter of 5-10 microns. It is also possible for a desired particle density to be made by alteration of the mass of magnetite added during production.

The characterisation of the MSFBR was in three parts; establishing a uniform field within the reactor, bed expansion profiles and mixing studies in the reactor.

Theoretical equations were used to predict the position of the helmhoitz coils to give a uniform field. These were used as a basis for the investigation. It was found that the optimum position was 11 cm apart, slightly different from that predicted by the theoretical equations of 8.75 cm apart. This was probably due to different manufacture of each of the helmhoitz coils. At the optimum position of 11 cm apart, the horizontal and vertical variations of magnetic field strength across the reactor were 2 and 40% respectively.

The bed expansion profiles examined the effects of particle size, density, flowrate and magnetic field strength on the bed height. This gave an operating criterion for
each system type. The lower density particles (1.04 g/ml) had a lower escape flowrate of 30 ml/min compared with 34 ml/min for 1.2 g/ml particles in a fluidised mode (no magnetic field). The higher density particles produced a much higher increase in obtainable flowrate using a magnetic field. For 1.04 g/ml the escape velocity increased to 42 ml/min. Using 1.2 g/ml the escape velocity increased to 60 ml/min using a 12.5 mTesla field.

The mixing studies in the MSFBR involved using a step input of tracer to analyse the type of mixing occurring in the MSFBR and compare it with a fluidised bed. For a variety of flowrates the MSFBR displayed better plug flow characteristics (i.e. had a lower dispersion coefficient, D / UL) than the fluidised bed.

The combination of the characterisation of the MSFBR and MCP’s has provided a better understanding of the reactor itself and enabled production of different particle sizes and densities.
Chapter 5

Biocatalysis involving pH change in the
MSFBR
Chapter 5 Biocatalysis involving pH change in the MSFBR

5.1 Introduction

The biocatalytic conversion of penicillin G to 6 aminopenicillanic acid (6APA) was chosen as a model system for analysis of the performance of the MSFBR in biotransformations involving a pH change. The 6 APA synthesis was reviewed in detail in experimental systems in section 2.2.1 and this section will only briefly reiterate some of the important aspects relevant to operation in the MSFBR. The complex kinetics discussed earlier in section 3.7.9, involve substrate (penicillin G), non-competitive product (6 APA) and competitive product (PAA) inhibition resulting in a difficult choice of reactor.

The majority of industrial 6 APA is produced in fed batch stirred tank reactors with some application of packed beds and other novel types of reactor. The main aim of this chapter was to report on the characteristics of the MSFBR for this model reaction involving a pH change. Specifically the experimental aims were to:

- Investigate the effect of enzyme loading, substrate concentration and flowrate on the productivity of the reactor
- Compare the MSFBR with a conventional plug flow packed bed reactor
- Examine the operational stability of the immobilised biocatalyst in the MSFBR
5.2 Experimental protocol for the biocatalysis of Penicillin G to 6 APA in the MSFBR

5.2.1 Operation of the recycle MSFBR system

The process flowsheet for the production of 6 APA is shown in Figure 5.1 and the operational stages during each biotransformation experiment is shown in Figure 5.2. The process was operated using a buffered system of 75 mM potassium phosphate pH 7.8. Approximately 10 litres of the buffer was required for each biotransformation for use as an expansion media, in post reaction washing and subsequent storage media. The buffer was stored in a 12 litre sealed vessel and valve 1 (on Figure 5.1) was used to discharge buffer when required.

Penicillin G was dissolved in 75mM potassium phosphate pH 7.8 no more than 30 minutes prior to reaction start time to prevent non specific hydrolysis. A safety handling data sheet for penicillin G is in Appendix 8. The mixture was stirred using an overhead Rushton turbine impeller (diameter = 5 cm) at 200 rpm in the substrate vessel. The pH control vessel had a total capacity of 1 litre although the working volume was 700 ml. The sealed cylindrical glass vessel had a diameter of 10 cm and was temperature controlled at 37 °C using a water bath. The same water bath continually recirculated water through the jacket of the MSFBR maintaining a reactor temperature of 37 °C. An autoburette pH titrator (Radiometer, Crawley, West Sussex, U.K.) controlled the pH within the vessel. The reference electrode, pH electrode and the burette were placed through a custom-made rubber lid on the pH control vessel. 8 M sodium hydroxide was used to maintain the pH at the desired reaction pH of 7.8. The amount of 8 M NaOH added was recorded every 5 - 30 minutes, depending on the total reaction time.

The immobilised enzyme was added via a sampling port at the top of the reactor by pouring through a glass funnel. The top of the reactor was fitted with a 120 micron mesh to prevent magnetic particles leaving the reactor.
Biocatalysis involving pH change in the MSFBR

The main preparation step prior to the biotransformation reactions was the initial expansion of the MCP's using 50 mM potassium phosphate buffer pH 7.0 as seen in stage 1 on Figure 5.2. A peristaltic pump (101U/R, Watson Marlow, Cornwall, U.K.) was used to circulate the desired fluid through the MSFBR. All experiments were performed using a "flow-first' mode of operation (Siegell, 1987) whereby the bed is initially fluidised prior to application of a magnetic field. Once the bed was stabilised and the magnetic field of 10 mTesla was in place, valve 1 (V1) was switched to substrate feed. The residence time for the reaction was calculated prior to each biotransformation. This information would determine the time after the substrate feed, that recirculation of substrate could begin. It was important not to dilute the substrate feed tank with the buffer used to initially fluidise the MSFBR. Penicillin G was prepared in 1.5 litres of buffer and the total working volume of the recirculated MSFBR system was 1.8 litres and so some dilution was expected. The 300 ml safety zone ensured no loss of substrate or product to the waste stream. After the residence time (and allowing for a 300 ml buffering volume) the exit line was transferred from exit to waste, to recirculation through the pH control vessel.

Valve 2 (V2) was used for both monitoring of exit pH and sampling of components for analysis by HPLC. The HPLC method is detailed in 3.5.1. Samples were taken every 10 – 30 minutes depending on the flowrate. After the biotransformation was completed, valve 2 was switched to collect the product in the product collection vessel. Valve 1 was switched to 50 mM potassium phosphate pH 7.0 feed. The MCP’s were washed with 50 mM potassium phosphate buffer pH 7.0 for 30 minutes by fluidising the particles in the MSFBR with no magnetic field as seen in stage 3 of Figure 5.2. This post experiment break-up of particles prevented problematic long term aggregation of the particles. The immobilised magnetic particles were removed via a bottom sampling port and were stored at 4°C in 50 mM potassium phosphate buffer pH 7.0.
Figure 5.1: Process flowsheet for the operation of the recycle MSFBR
Biocatalysis involving pH change in the MSFBR

Figure 5.2: Operational stages for biocatalysis in the MSFBR

Stage 1: Expansion of MSFBR with fluidised buffer
Stage 2: Biocatalysis reaction
Stage 3: Washing/Realigning of dipoles in magnetic particles
Stage 4: Removal of magnetic particles through side exit port
5.2.2 Effect of substrate flowrate on the conversion of Penicillin G to 6 APA in the MSFBR.

Using the process outlined in section 5.2.1, the Penicillin G biotransformation was operated at flowrates of between 30 and 90 ml/min (equivalent to 90 and 275 cm/hour). All investigations into flowrate effects on 6 APA production were completed using an initial substrate concentration of 200 mM Penicillin G in 1.5 litres of 75 mM potassium phosphate buffer pH 7.8. The initial specific activity of the biocatalyst preparation was 33 U/g dry magnetic particle. The settled bed height in the reactor was 19 cm (equivalent to 11.1 g dry magnetic particles). This represented a total enzyme activity in the reactor, $E_q=365$ Units.

5.2.3 Effect of biocatalyst density on the conversion on Penicillin G to 6 APA in the MSFBR

Using the process outlined in section 5.2.1, the Penicillin G biotransformation was operated with two types of magnetic particle. The biocatalyst densities investigated were 1.04 and 1.2 g/ml. The substrate concentration used was 200 mM Penicillin G in 75 mM potassium phosphate pH 7.8.

5.2.4 Effect of enzyme loading on the conversion of Penicillin G to 6 APA in the MSFBR

Biocatalysts with relatively low specific activity of 33 Units/g dry MCP were compared to higher specific activity particles of 198 Units/g dry MCP. The experiments were run at a flowrate of 90 mL/min using a substrate concentration of 200 mM Penicillin G in 75 mM potassium phosphate pH 7.8.

5.2.5 Comparison on the MSFBR with a packed bed reactor

To determine the commercial applicability of the MSFBR as a realistic alternative to the traditional enzyme reactors, the MSFBR was compared directly with a packed bed reactor. The system used was identical to the flowsheet in Figure 5.1 except that
the MSFBR was replaced by a packed bed reactor. The packed bed reactor (as described in 2.3.2) was a 5 cm pyrex jacketed reactor (Pharmacia Amersham Biotech, Amersham, U.K.). An identical settled volume of immobilised magnetic particles was placed in the packed bed as had been used in the MSFBR. The bed was packed and operated based on recommendations from Pharmacia. This gave a settled bed height of 15 cm in the packed bed reactor.

5.2.6 Effect of substrate concentration on the conversion of Penicillin G to 6 APA

Penicillin G concentrations of between 100 and 200 mM were investigated in the MSFBR (equivalent to 37.2 g/l and 74.4 g/l) using a flowrate of 90 ml/min

5.2.7 Operational stability of immobilised penicillin acylase

Over the course of experimentation, the initial activity of the biocatalyst was repeatedly measured to determine the operational stability of the preparation. The initial activity was measured using triplicate assays using the penicillin acylase assay outlined in 3.5.1.4. The operational stability was measured over the time of operation and the mass of penicillin G converted.
5.3 **Effect of substrate flowrate on the conversion of Penicillin G to 6 APA.**

An important consideration in the operation of the MSFBR is the flowrate of substrate through the reactor. As part of the biocatalytic characterisation of the MSFBR, large scale biotransformations were performed at various flowrates. When using plug flow reactors the flowrates are often described as linear flowrates.

As illustrated in *Figure 5.1*, the MSFBR was operated in recirculation mode to counteract pH fluctuations resulting from phenylacetic acid by product formation. The MSFBR was operated at three different flowrates of 30, 60 and 90 ml/min representing 90, 180 and 275 cm/hour linear velocities in the MSFBR respectively.

*Figure 5.3* shows the biotransformation of 200 mM penicillin G to 6 APA using immobilised penicillin acylase on magnetic carrier particles in the MSFBR at a flowrate of 90 ml/min. *Figure 5.3 (a)* plots the 6 APA product concentration increase over the reaction time to a final product concentration of 30 g/litre after 1425 minutes. Also illustrated is the subsequent decrease in penicillin G concentration from an initial 56 g/L (200mM) to a final substrate concentration of 0.1 g/L. *Figure 5.3 (b)* plots the conversion rate achieved of mole product per mole substrate over the reaction time and the productivity levels seen over the course of the reaction.

As stated previously, the main advantage of using a plug flow type reactor is its ability to overcome product inhibition (or significantly decrease the effect as compared with a stirred tank reactor). One of the methods of establishing this is by examining the productivity levels over the reaction time. The productivity data seen in *Figure 5.3 (b)* is erratic in shape although shows a general slight decrease in activity over the run of the reaction. This shall be discussed later in the chapter in section 5.8. The conversion plots display a linear shape indicating no major effect of product inhibition either by the desired product, 6 APA or the by-product PAA.
Figure 5.4 and 5.5 illustrate the biotransformation of penicillin G to 6 APA using penicillin acylase immobilised to magnetic particles in the MSFBR at a recirculation flowrate of 30 ml/min and 60 ml/min respectively. These plots are similar in shape to Figure 5.3 for both 6 APA and Penicillin G concentration profiles. However there is an increase in 6 APA production towards the end of the 60 and 90 ml/min MSFBR run. This may be due to HPLC analysis error, pump fluctuations in flowrate or part of the reaction. Again for both Figure 5.4 and 5.5 the productivity curves are erratic and it is therefore impossible to comment on a general productivity trend. Figure 5.5 shows the final 6 APA product concentration of 30.6 g/l after 1600 minutes against a final substrate concentration of 2.5 g/l Penicillin G. At a higher flowrate of 60 ml/min the final 6 APA concentration after 1450 minutes was 31.7 g/l against a substrate final concentration of 1.1 g/l Penicillin G (seen in Figure 5.4).

The productivity levels for recirculation rates of 30, 60 and 90 ml/min are 244, 273 and 293 mmoles/min. Comparing these productivity levels with $E_0$, the total enzyme activity the reactor is showing only 67, 75 and 81 % activity. There are a number of possible explanations for this effect. The loss in activity could be due to experimental errors or different mixing regimes (the penicillin acylase assay was carried out in a 20 ml well mixed vessel and the MSFBR runs as a plug flow reactor) resulting in potential external mass transfer difficulties in the MSFBR. A likely explanation is external mass transfer problems restricting access of the substrate to the biocatalyst. As discussed in chapter 4, the magnetic particles when subjected to a magnetic field align themselves along magnetic field lines vertically along the reactor. In some cases the magnetic particles act like magnets themselves attracting those particles nearby. This will reduce the surface area available to the substrate reducing the apparent activity of the biocatalyst. At higher flowrates, the influence of external mass transfer difficulties is reduced. This can be seen in the productivity data. As the flowrate increases from 30 to 90 ml/min the % of the total activity used in the MSFBR increases. A further explanation for the drop in activity seen at lower flowrates is the pH level will be lower across the reactor at low flowrates. The pH control system was unable to cope with the drop in pH.
However, from section 3.7.8, at a substrate concentration of 200 mM, the activity drops to 60% of the activity measured during the penicillin acylase assay at standard conditions of 30 mM penicillin G concentration. Both product inhibition constants are around 100 mM and therefore an effect will be observed at concentrations above this value. The combination of these inhibition effects will overall reduce the activity seen in the reactor. This reduction in activity cannot be attributed to the MSFBR reactor itself.
Biocatalysis involving pH change in the MSFBR

(a)

Figure 5.3: Biotransformation of Penicillin G to 6 APA using immobilised penicillin acylase on magnetic carrier particles in the MSFBR at a recirculation flowrate of 90 ml/min. (a) The product (6 APA) concentration (■) and substrate (Penicillin G) concentration (▲) during reaction and (b) the conversion (mol/mol %) (■) and production rate (▲) during reaction.
Biocatalysis involving pH change in the MSFBR

Figure 5.4: Biotransformation of Penicillin G to 6 APA using immobilised penicillin acylase on magnetic carrier particles in the MSFBR at a recirculation flowrate of 60 ml/min.

(a) The product (6 APA) concentration (■) and substrate (Penicillin G) concentration (▲) during reaction and (b) the conversion (mol/mol %) (■) and production rate (▲) during reaction.
Biocatalysis involving pH change in the MSFBR

Figure 5.5: Biotransformation of Penicillin G to 6 APA using immobilised penicillin acylase on magnetic carrier particles in the MSFBR at a recirculation flowrate of 30 ml/min.

(a) The product (6 APA) concentration (■) and substrate concentration (▲) during reaction and (b) the conversion (mol/mol %) (■) and production rate (▲) during reaction.
5.4 Effect of biocatalyst density on the conversion on Penicillin G to 6 APA in the MSFBR

The initial strategy for the approach to performing large scale biocatalysis in the MSFBR was to manufacture a high density particle which would remain in the reactor at high throughput. The first batch for magnetic carrier particles immobilised with penicillin acylase utilised a high density particle of 1.2 g/ml. This had obvious process advantages with high escape velocity, increased susceptibility to magnetic fields and therefore potentially higher biocatalytic conversions. The relative merits of the different density particles are discussed in greater detail in chapter 3.

Figure 5.6 shows the biotransformation of penicillin G using the higher density magnetic carrier particles. It illustrates the product and substrate concentrations with final concentrations of 3.6 g/l of 6 APA and 49 g/l of penicillin G. Over the time the reaction was run of 300 minutes the conversion on a mole to mole basis only reached 10%. Figure 5.7 shows a comparison of the low and high density magnetic particles when used in the biotransformation of penicillin G. The reaction conditions were identical to standardise the comparison. The initial substrate concentration was 200 mM Penicillin G in 1.5 litres of 75 mM potassium phosphate pH 7.8. A 19 cm settled bed height of immobilised magnetic carrier particles was used. This is equivalent to a 33% biocatalyst concentration in the reactor. After 300 minutes of reaction time, the lower density particles had achieved 23% conversion. The specific activity of the immobilised magnetic particles were 33 U/g dry MCP for the 1.04 g/ml particles and 15 U/g dry MCP for the 1.2 g/ml particles.

As discussed in chapter 3, although the higher density particles improve the operating flowrates in the reactor, intrinsically this reduces the amount of enzyme that can be attached resulting in lower activity.
Biocatalysis involving pH change in the MSFBR

Figure 5.6: Biotransformation of Penicillin G to 6 APA using immobilised penicillin acylase on magnetic carrier particles in the MSFBR at a recirculation flowrate of 30 ml/min.

(a) The product (6 APA) concentration (■) and substrate concentration (▲) during reaction and (b) the conversion (mol/mol %) (■) and production rate (▲) during reaction.
Figure 5.7: Biotransformation of penicillin G to 6 APA using low and high density magnetic particles at a flowrate of 30 ml/min.

Initial conversion rates for immobilised magnetic carrier particles of density 1.04 g/ml (■) and 1.2 g/ml (▲).
5.5 Operational stability of immobilised penicillin acylase

An essential part of an immobilised biocatalyst is the operational stability during the biocatalytic process. Table 5.1 illustrates the operational stability in terms of the initial activity measured approximately every 24 hours during operation. The immobilised penicillin acylase on magnetic particles had a specific activity of 33 U/g dry MCP. The table also shows the operational stability against the mass of penicillin G converted by the immobilised biocatalyst. After 80 hours operation (equivalent to 320 grams of penicillin G) the biocatalyst has retained 93% of the initial activity. Fonseca and coworkers (1993) demonstrated retention of 86% of the initial activity of penicillin acylase immobilised to a silica carrier after 80 hours operation. Cardosa and Costa, 1985 retained 80% of the initial activity of an immobilised penicillin amidase over 1500 hours operation. Ospina and coworkers (1992) reported immobilisation of penicillin acylase on an epoxyacrylic carrier and retained 70% activity after 150 runs of a 2 hour reaction. There seems to be a wide range of reported literature on the operational stability of immobilised penicillin acylase. This biocatalyst seems to perform reasonably well with a loss of only 7% activity during 80 hours operation.

<table>
<thead>
<tr>
<th>Operation Time (hours)</th>
<th>Amount of Penicillin G converted (g)</th>
<th>Initial activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>26</td>
<td>95</td>
<td>97.4</td>
</tr>
<tr>
<td>50</td>
<td>213</td>
<td>94.2</td>
</tr>
<tr>
<td>75</td>
<td>324</td>
<td>93.6</td>
</tr>
</tbody>
</table>

Table 5.1 : Operational stability of immobilised penicillin acylase on magnetic carrier particles
5.6 Effect of enzyme loading on the conversion of Penicillin G to 6 APA

For the previous three sections, relatively low specific activities were used in the MSFBR. After initial immobilisation scale up difficulties (as discussed in 3.7), higher specific activities were achieved. This allowed higher enzyme loadings in the MSFBR. An improvement in specific activity was found increasing from 33 Units/g dry magnetic particle to 198 Units/g dry magnetic particle. Figure 5.8 illustrates biotransformations of penicillin G to 6 APA in the MSFBR with identical reaction conditions. The MSFBR was run using a magnetic field strength of 10 mTesla, a flowrate of 90ml/min and an initial substrate concentration of 200 mM. The total enzyme loading was 355 Units for the biocatalysis using magnetic particles with a specific activity of 33 U/g and 1740 Units for the improved specific activity biocatalyst of 198 U/g dry MCP. The figure illustrates the expected significant improvement in conversion times by increasing the specific activity of the biocatalyst. The higher activity particle, at 198 Units/d dry MCP was used in the remaining section of work in this chapter.

5.7 Comparison of the MSFBR with a Packed bed reactor

To determine the commercial applicability of the MSFBR as a realistic alternative to the traditional enzyme reactors, the MSFBR was compared directly with a packed bed reactor. The system used was identical to the flowsheet in Figure 5.1 except that a packed bed reactor replaced the MSFBR. The Pharmacia XK 50 packed bed reactor had an internal diameter of 5 cm and a total height of 50 cm. These were identical specifications as the fluidised bed/MSFBR reactor. Instead of an upward flow used in the fluidised bed/MSFBR reactor a downward flow was used in the packed bed system. The pH control system and product collection vessel were identical to the flowsheet in Figure 5.1. The time for 95% conversion was chosen with the reaction profiles as initial parameters of comparison of the MSFBR with a packed bed reactor at various substrate feed rates. The packed bed and MSFBR used identical 360 ml settled volume of 125 – 250 micron, 1.2 g/ml immobilised magnetic particles. Both reactors used an initial penicillin G concentration of 200 mM with a total activity loading of 1740 Units.
**Figure 5.9** shows the penicillin acylase biocatalysis at a low flowrate of 30 ml/min which is equivalent to 90 cm/hour. The time for 95% conversion was 220 and 219 minutes for operation in a packed bed and MSFBR respectively. The MSFBR matched the performance of the packed bed in terms of the reaction profile and the 95% conversion times. To investigate whether the performance matching remained at higher flowrates further biotransformations were carried out at 40 and 90 ml/minute. **Figure 5.10** shows the penicillin G biocatalysis at a flowrate of 40 ml/min equivalent to 122 cm/hour. The time for 95% conversion was 170 (+/- 21) and 220 (+/- 23) minutes for operation in a packed bed and MSFBR respectively. For the first 125 minutes of the biotransformation, the conversion rate in a packed bed is significantly higher than that in the MSFBR. However the 95% conversion times are not statistically dissimilar at 170 (+/- 21) and 210 (+/- 23) minutes. **Figure 5.11** illustrates the penicillin acylase biocatalysis at a high flowrate of 90 ml/min, the highest achievable flowrate in the MSFBR with this biocatalyst preparation. The reaction profiles are similar again with 95% conversion times of 160 (+/-19) and 195 (+/-25) minutes for operation in a packed bed and MSFBR respectively. At low flowrates of 30 ml/min, the MSFBR matches the packed bed 95% conversion times. At higher flowrates (>40ml/min), the packed bed reactor shows lower 95% conversion times than the MSFBR.

### 5.8 Effect of substrate concentration on the conversion of Penicillin G to 6 APA

**Figure 5.12** shows the relative conversion rates of biotransformations of Penicillin G to 6APA in the MSFBR at substrate concentrations of 100 and 200 mM. By using relative conversion rates, any substrate inhibition by the higher substrate concentration can be seen on the conversion graphs. Operating the Penicillin G biotransformation at a substrate concentration of 100 mM does not change the apparent activity in the reactor as compared to running the biotransformation with 200 mM Penicillin G. Although at the beginning of the synthesis the conversion rate is slower for a higher Penicillin G concentration of 200 mM. This may indicate the presence of substrate inhibition by Penicillin G.
Figure 5.8: Effect of enzyme loading on the conversion of Penicillin G to 6 APA in the MSFBR. Reaction conditions were a settled bed height of 15 cm, flowrate of 90 ml/min, magnetic field strength of 10 mTesla and an initial substrate concentration of 200 mM using a total enzyme loading of 355 Units (■) and 1740 Units (○).
Figure 5.9: Comparison of a packed bed and MSFBR biotransformation of penicillin G to 6APA under identical conditions at a flowrate of 30 ml/min. Reaction conditions were a settled bed height of 15 cm, flowrate of 30 ml/min, magnetic field strength of 10 mTesla and an Initial substrate concentration of 200 mM using a total enzyme loading of 1740 Units in a packed bed (●) and the MSFBR (■).
Biocatalysis involving pH change in the MSFBR

Figure 5.10: Comparison of a packed bed and MSFBR biotransformation of penicillin G to 6APA under identical conditions at a flowrate of 40 ml/min. Reaction conditions were a settled bed height of 15 cm, flowrate of 40 ml/min (122 cm/hour), magnetic field strength of 10 mTesla and an Initial substrate concentration of 200 mM using a total enzyme loading of 1740 Units in a packed bed (▲) and the MSFBR (■).
Biocatalysis involving pH change in the MSFBR

Figure 5.11: Comparison of a packed bed and MSFBR biotransformation of penicillin G to 6APA under identical conditions at the maximum operating flowrate of the MSFBR of 90 ml/min. Reaction conditions were a settled bed height of 15 cm, flowrate of 90 ml/min, magnetic field strength of 10 mTesla and an Initial substrate concentration of 200 mM using a total enzyme loading of 1740 Units in the MSFBR (●) and a packed bed (■).
Biocatalysis involving pH change in the MSFBR

Figure 5.12: Effect of substrate concentration on the biotransformation of penicillin G to 6 APA at 90ml/min in the MSFBR. Reaction conditions were a settled bed height of 15 cm, magnetic field strength of 10 mTesla. Substrate concentration was 100 mM (■) and 200 mM (●).
5.9 Discussion

In order for direct comparisons of these biotransformations at different flowrates, two illustrative figures have been plotted. Figure 5.13 shows the conversion of Penicillin G to 6 APA (mole of product/mole of substrate) at the three different linear velocities of 90, 180 and 275 cm/hour (67, 33 and 16 minutes residence time respectively). The plot clearly shows increased production at higher flowrates towards the beginning of the biotransformation although the distinction becomes less clear after 1200 minutes operation. This is probably explained by the increased degradation of 6 APA at higher concentrations rendering analysis by HPLC prone to larger errors.

Figure 5.14 shows the effect of linear flowrate on the time for 95% conversion, \( \theta_{95} \). The error bars are also shown (which were taken from the standard error of the original conversion versus time plot for each flowrate), as is a non linear curve fit to visibly illustrate the relationship. The curve seems to flatten at linear flowrates above 250 cm/hour, which indicates that mass transfer effects may occur at flowrates less than this value. Cardosa and Costa (1986) demonstrated that mass transfer effects were significant only below a linear velocity of 125 cm/hr in a packed bed recirculated with a STR for pH control. The last point on Figure 5.14 was obtained at a linear velocity of 275 cm/hour which is the operating limit of this system as particle elution occurs at higher flowrates. The particle elution velocity or more commonly the escape velocity is influenced by the magnetic field strength and the density of the particles. Increases in either or both of these parameters will result in higher escape velocities. However, as can be seen in Figure 5.14 it may be that further increases in flowrate result in only slight or no decreases in \( \theta_{95} \).

It is known that high concentrations of penicillin G will reduce the reaction rate as the substrate inhibits the reaction. Industrially concentrations of 8\% w/v (215 mM) (Shewale and Sivarman, 1989) are commonly used. Lee (1997) used a novel recirculated plate and frame filter reactor and found little increase in productivity at high substrate concentrations (>200 mM) by increasing recirculation rates.
Figure 5.13: Biotransformation of Penicillin G to 6 APA using immobilised penicillin acylase on magnetic particles in the MSFBR. The effect of linear flowrate on the conversion rate at 90 cm/hour (30 ml/min) (▲), 180 cm/hour (60 ml/min) (○) and 275 cm/hour (90 ml/min) (■).
Biocatalysis involving pH change in the MSFBR

Figure 5.14: Effect of linear flowrate on the time for 95% conversion, $\theta_{95}$, for the biotransformation of penicillin G to 6 APA using immobilised penicillin acylase on magnetic particles in the MSFBR.
5.10 Summary of Biotransformation of Penicillin G to 6 aminopenicillanic acid in the MSFBR

The following conclusions can be drawn from the successful biotransformation of penicillin G to 6 aminopenicillanic acid in the MSFBR

- Increases in substrate flowrate up to the maximum operating flowrate of 275 cm/hour result in lower time for 95% conversion times
- Examination of the effect of flowrate on conversion times suggest that further increases in flowrate will not result in lower time for 95% conversion due to external mass transfer limitations
- At substrate flowrates of 30, 60 and 90 ml/min in the MSFBR the apparent activity in the reactor was 67, 75 and 81% respectively of the total enzyme activity in the reactor as defined by the specific activity of the particles
- The use of lower density particles increases product rate of 6 APA but reduces the escape velocity of the particles (reducing maximum flowrate)
- The MSFBR compared favourably with the packed bed for the penicillin acylase biocatalysis at low flowrates
- The operational stability of immobilised penicillin acylase is comparable to industry with only a 7% loss in initial activity over 80 hours operation
- This reaction is not rapid enough to take advantage of the ability of the MSFBR to recirculate fast to avoid large pH drops
Chapter 6

Biocatalysis in the presence of a solid substrate in the MSFBR
Chapter 6  Biocatalysis in the presence of a solid substrate in the MSFBR

6.1  Introduction

The challenge of the second biotransformation model chosen was the ability of the MSFBR to perform a biocatalytic reaction in the presence of solids. The specific reaction studied was the α-chymotrypsin catalysed reaction of N-acetyl-L-tyrosine ethyl ester (ATEE) to N-acetyl tyrosine (AT). As with the first system, penicillin acylase, the reaction produces an acidic product resulting in the need for pH control. The α-chymotrypsin reaction was reviewed in detail in section 2.2.3.

The main aim of this chapter was to report the characteristics of the MSFBR for this model reaction involving a pH change in the presence of a solid substrate. Specifically the experimental aims were to:

- Investigate the highest substrate solids concentration achievable in the MSFBR
- Compare the MSFBR with the conventional reactors of a packed bed, fluidised bed and stirred tank reactor
- Examine the operational stability of the immobilised biocatalyst in the MSFBR
6.2 Experimental protocol for biocatalysis of N-acetyl tyrosine-L-ethyl ester to N-acetyl tyrosine in the MSFBR

6.2.1 Operation of the recycle MSFBR system for biocatalytic reactions

The process used for this biotransformation was identical to the schematic shown in Figure 5.1 in chapter 5 on the biocatalysis of penicillin acylase. A few operational changes were made to allow solid handling in the process.

At the start of the experiment, the required mass of N-acetyl-L-tyrosine ethyl ester was added to 50 mM potassium phosphate pH 7.0. The resultant slurry mixture was stirred using an overhead Rushton turbine impeller (diameter = 5 cm) at 700 rpm in the pH control vessel, which ensured the uniformity of solid substrate in the slurry feed to the reactor.

The pH control vessel was a 1 litre sealed glass vessel of diameter 10 cm which sat in a temperature controlled water bath. The system was operated at the optimum temperature for α-chymotrypsin of 25 °C. The water bath fed the MSFBR water jacket at the operating temperature of 25 °C. An autoburette pH titrator (Radiometer, Crawley, West Sussex, U.K.) controlled the pH within the vessel. The reference electrode, pH electrode and the burette were fixed within a custom made rubber lid on the pH control vessel. The alkali solution used in the pH control system was 4 M NaOH, which maintained the pH at 8.0. The amount of sodium hydroxide added over the reaction was used as the prime indicator for the end of the reaction as the amount of NaOH added for 100% conversion was known.

The immobilised α-chymotrypsin was added via a sampling port at the top of the reactor by pouring through a clean glass funnel. Immobilised magnetic particles of density 1.2 g/ml were used throughout this chapter. The top of the reactor was sealed with a 120 micron mesh to prevent magnetic particles leaving the reactor.
The MSFBR preparation involved initial fluidisation of the magnetic particles using a peristaltic pump (101 U/R, Watson Marlow, Falmouth, Cornwall, U.K.). The fluidisation buffer used was 50 mM potassium phosphate pH 7.0. All experiments were operated using a “flow-first” mode of operation whereby the magnetic particles are initially fluidised prior to application of the magnetic field as seen in stage 1 of Figure 5.2. After a pre determined length of time (which depended on the flowrate required), a 10 mTesla magnetic field was applied and the bed left to stabilise for 20 minutes until no visible movement was observed in the bed.

After bed stabilisation, the feed was changed using valve 1 (V1) from fluidisation buffer to substrate feed. The residence time for the reaction was calculated prior to each biotransformation. This information would determine at what time after the substrate feed, the recirculation of substrate could begin. It was important not to excessively dilute the substrate feed tank with the buffer used to initially fluidise the MSFBR. The N-acetyl-L-tyrosine ethyl ester was prepared in 1.5 litres of buffer and the total working volume of the recirculated MSFBR system was 1.8L and so 300 ml of dilution was permissible as a safety zone to ensure no loss of substrate/product. After the reactor residence time, the substrate recirculation began and the exit line was transferred from exit to waste, to recirculation through the pH control vessel.

Samples were taken from a sampling point at valve 2 (V2). Aliquots were taken and assayed for substrate and product by HPLC. The HPLC method is detailed in 3.6.2.1. The pH of the samples was taken to analyse the pH in the exit line over the course of the reaction.

After the biotransformation was complete, the magnetic particles were washed with fluidising buffer (50 mM potassium phosphate pH 7.0) for 30 minutes at 40 ml/min with no magnetic field applied. This procedure ensured adequate washing of the immobilised enzyme particles and prevented problematic long term aggregation of the particles. The immobilised enzyme particles were stored at 4 °C in fluidising buffer at pH 7.0.
6.2.2 Effect of solid substrate particle size on the biocatalysis of ATEE to AT in the MSFBR

100 g batches of N-acetyl-L-tyrosine ethyl ester were ground in a pestle and mortar based Vibratory shaker (Fritsch, Christianson Scientific Equipment Ltd, Gateshead, U.K.) for 30 minutes. The resultant ground substrate and unground particles were analysed in a Malvern Mastersizer 2000 Hydro SM (Malvern Instruments Ltd, Malvern, U.K.), which gave particle size distribution information and a mean particle size.

The dissolution rate was measured by analysis at 20 ml scale. 0.4 g of N-acetyl-L-tyrosine ethyl ester was added to 20 ml of 50 mM potassium phosphate pH 7.0 at room temperature and mixed at 500 rpm on a vibrax mixer (IKA vibrax, Janke and Kunkal, GmbH and Co., Germany) for 1 hour. 100 µL samples were taken at intervals and filtered using a 0.22 micron sterile filter and analysed for N-acetyl-L-tyrosine ethyl ester by HPLC as detailed in section 3.6.2.1.

Using the process outlined in section 6.2.1, ground and unground substrate particles were compared in the MSFBR by running experiments at a concentration of 100 mM N-acetyl-L-tyrosine ethyl ester (40.35 g of ATEE to 1.5 litres of 50 mM potassium phosphate pH 8.0) at flowrates of 33 – 85 mL/min and a magnetic field strength of 10 mTesla.

6.2.3 Effect of flowrate on the biocatalysis of ATEE to AT in the MSFBR

The flowrates investigated in this biotransformation were from 30 ml/min up to 90 ml/min. The substrate concentration used was 100 mM ATEE in 50 mM potassium phosphate pH 8.0.

6.2.4 Comparison of the biocatalysis of ATEE to AT in a fluidised bed and a MSFBR

Using the process outlined in section 6.2.1, identical biotransformations were completed using the MSFBR operated with a 10mTesla field and with no magnetic
field (effectively a fluidised bed). The biotransformation was performed using a substrate concentration of 100 mM ATEE and at a flowrate of 85 ml/min.

6.2.5 Limitations of a packed bed reactor for the biocatalysis of ATEE to AT

A 5 cm pyrex jacked reactor (Pharmacia Amersham Biotech, Amersham, U.K.) was packed using the packing protocol outlined in appendix 9 with immobilised α-chymotrypsin. Using the process outlined in section 6.2.1 and replacing the MSFBR with a packed bed reactor, biotransformations were completed at the highest soluble N-acetyl-L-tyrosine ethyl ester concentration, the solubility limit of N-acetyl-L-tyrosine ethyl ester in 50 mM potassium phosphate pH 8.0, which was 4 g/l. The biotransformation was completed at a flowrate of 33 and 65 ml/min.

6.2.6 Comparison of the biocatalysis of ATEE to AT in a stirred tank and a MSFBR

A small scale stirred tank biotransformation was completed in a 1 litre glass vessel of diameter 10 cm. An autoburette pH titrator (Radiometer, Crawley, West Sussex, U.K.) controlled the pH within vessel. The reference electrode, pH electrode and the burette were fixed within a custom made rubber lid on the pH control vessel. The sodium hydroxide feed used in the pH control system was 4 M NaOH, which maintained the pH at 8.0.

4.3 g of N-acetyl-L-tyrosine ethyl ester was added to 400 ml of 50 mM potassium phosphate pH 8. Using a 10% immobilised enzyme concentration basis in the stirred tank reactor, 40 ml settled volume of immobilised α-chymotrypsin on magnetic particles was added to the reactor. Samples were taken from the vessel using a syringe connected to tube with a sterile 0.22 micron filter at the end. The samples were analysed for N-acetyl-L-tyrosine ethyl ester and N-acetyl tyrosine by HPLC as detailed in section 3.6.2.1.

6.2.7 Investigation into the highest substrate concentration on the biocatalysis of ATEE to AT in the MSFBR

Using the experimental protocol outlined in section 6.2.1, biotransformations were run at increasing substrate concentrations. The substrate concentrations analysed
were 100 mM and 200 mM N-acetyl-l-tyrosine ethyl ester in 50mM potassium phosphate pH 8.0. In all experiments, ATEE was mixed vigorously at 700 rpm in the pH control vessel prior to the beginning of the experiment and the runs were performed at a flowrate of 90 mL/min.

6.2.8 Reproducibility studies

Three identical MSFBR biotransformations using the protocol outlined in section 6.2.1 with an initial N-acetyl-L-tyrosine ethyl ester concentration of 50 mM and a flowrate of 75 mL/min were carried out.

6.2.9 Operational stability of α-chymotrypsin

The enzyme activity of the immobilised α-chymotrypsin was monitored over the course of experimentation using the titrimetric enzyme assay outlined in section 3.6.2.3.
6.3 Effect of grinding solid substrate on the biocatalysis of ATEE to AT in the MSFBR

6.3.1 Dissolution rates of various substrate particle sizes

The overall process of enzymatic conversion of solid substrate can be described by two processes:

(a) Dissolution of solid substrate
(b) Enzymatic conversion of dissolved substrate

The kinetics of solid substrate dissolution can be described as

\[ \phi_A = K_L a (C^*_A - C^L_A) \]  \hspace{1cm} (6.1)

\( \phi \) : Dissolution rate of substrate
\( K_L \) : Mass transfer coefficient
\( a \) : Volume specific surface area of substrate
\( C^*_A \) : Substrate solubility
\( C^L_A \) : Concentration of dissolved substrate

Integrating equation 6.1 with the following boundary conditions

\[ \phi_A = \frac{dC^L_A}{dt} \] \hspace{1cm} (6.2)

gives

\[ C^L_A = C^*_A \left(1 - \exp\left(- \frac{1}{K_L a t}\right)\right) \] \hspace{1cm} (6.3)
Using non-linear regression $C^*_{A}$ and $K_{La}$ can be calculated and matched to experimental data (Wolff, 1999). This can be used as a dissolution rate model, which calculates approximate values for $K_{La}$.

The ATEE substrate was ground in a pestle and mortar based Vibratory shaker (Fritsch, Christianson Scientific Equipment Ltd, Gateshead, U.K.) to reduce the particle size, which would increase the value of $K_{Lu}$ and therefore the dissolution rate of substrate into solution. By increasing the dissolution rate of substrate, the rate-determining step in the reaction was shifted from dissolution rate to kinetic enzyme rate.

Figure 6.1 and 6.2 illustrate the particle size distribution of unground and ground N-acetyl-L-tyrosine ethyl ester gained from the particle size analysis performed using the Malvern Mastersizer. The mean particle size of unground ATEE is 125 μm and of ground ATEE is 95 μm. Figure 6.2 shows triplicate analysis of the ground particles to illustrate the accuracy of this method. All analyses gave an average particle size of 95 microns (+/- 5).

Figure 6.3 illustrates the measured dissolution rate versus that calculated using a dissolution model based on equation 6.3. The $K_{La}$ for unground ATEE (~125 μm) purchased off the shelf from Sigma was 0.02 (+/- 0.002) s⁻¹. Figure 6.4 illustrates the measured and modelled dissolution rate of ground ATEE (95 μm) which gives a ~70% higher value of $K_{Lu}$ of 0.07 s⁻¹ (+/- 0.01). From both figures, the dissolution rate model does not fit the experimental data well but demonstrates qualitatively the improvement in rate achieved by using smaller substrate particles.
Biocatalysis in the presence of a solid substrate in the MSFBR

Figure 6.1: Particle size distribution of unground and ground N-acetyl-l-tyrosine ethyl ester

Figure 6.2: Triplicate analysis of the particle size distribution of ground N-acetyl tyrosine ethyl ester (30 minutes at level 2 on Pestle and Mortar based Vibratory shaker (Fritsch, Christianson Scietific Equipment, Gateshead, U.K.))
Biocatalysis in the presence of a solid substrate in the MSFBR

Figure 6.3: Comparison of measured dissolution rate of 125 μm ATEE against a solid substrate dissolution model. Measured dissolution rate (■) and dissolution rate model (▲).

Figure 6.4: Comparison of measured dissolution rate of 95 μm ATEE against a solid substrate dissolution model. Measured dissolution rate (■) and dissolution rate model (▲).
6.3.2 Comparison of substrate particle size effects on the biocatalysis of ATEE to AT in the MSFBR

Figure 6.5 illustrates the effect of grinding the substrate, ATEE on the enzyme reaction rate in the MSFBR at a flowrate of 90 ml/min. The reaction rate for smaller particles clearly shows a different shape. Using unground ATEE with a particle size of 125 μm the reaction rate profile is linear. This linear shape is indicative of a dissolution rate determined reaction. The enzyme can convert only the substrate in solution and in this case the enzyme kinetic rate is higher than the dissolution rate. Using ground ATEE substrate particles of 95 μm the reaction rate profile is not linear although not the classical enzyme reaction shape. The time for 95% conversion is halved by using ground particles from 138 minutes for unground particles to 78 minutes for ground particles. For all the MSFBR experiments the ground particles with a particle size of 95 μm were used to ensure that the reaction rate seen was due to biocatalyst action and not limited by dissolution of substrate. The consumption of ATEE is 1826 μmole/min whilst the dissolution rate of ATEE is difficult to quantify using the simple model. The model requires the concentration of the substrate in solution, which is changing as it is used by the enzyme.

6.4 Effect of flowrate on the biocatalysis of ATEE to AT in the MSFBR

Figure 6.6 displays the effect of linear velocity on the time for 95% conversion, $\theta_{95}$. The error bars are also shown (which were taken from the standard error of the original conversion versus time plot for each flowrate) as is a non linear curve fit to visibly illustrate the relationship. Unlike with the penicillin acylase biocatalysis, the curve does not flatten out at higher flowrates. At 90 m/min, the MSFBR is operating at the maximum flowrate and so it is the reactor itself, which is limiting improved conversion times. The total activity in the MSFBR was 2282 Units. At the maximum flowrate, the total activity was 80% lower at 1826 Units. The 20% loss total activity in the reactor could be a consequence of mass transfer restrictions of substrate to the enzyme active sites.
Figure 6.5: Effect of grinding of the substrate, N-acetyl-L-tyrosine ethyl ester on the α-chymotrypsin catalysed reaction to acetyl tyrosine in the MSFBR at a flowrate of 90 ml/min and a magnetic field strength of 10 mTesla.

Substrate particle size 95 μm (*) and 124 μm (■).
Figure 6.6: Effect of substrate flowrate on the conversion of 100 mM N-acetyl-L-tyrosine ethyl ester to N-acetyl tyrosine in a MSFBR at a magnetic field strength of 10 mTesla and 10.5 cm settled bed height of immobilised α-chymotrypsin.
6.5 Comparison of the biocatalysis of ATEE to AT in a fluidised bed and a MSFBR

Figure 6.7 illustrates a direct comparison of the performance of a fluidised bed reactor and a magnetically stabilised fluidised bed for the conversion of N-acetyl-L-tyrosine ethyl ester to N-acetyl tyrosine by immobilised α-chymotrypsin. The figure also shows the exit line pH from the MSFBR reactor.

The biocatalysis performed in the fluidised bed is achieved with a constant reaction rate producing the smooth curve seen in Figure 6.7. The biocatalysis carried out in the MSFBR has a different shape with a low reaction rate initially followed by increased production after 60 minutes. The exit line pH from the MSFBR reaction is shown on the figure to explain the sluggish initial reaction rate. The exit line pH from the fluidised bed (data not shown) does not fall below a pH of 6.0. The pH, and therefore the enzyme activity in the MSFBR falls sharply from the optimum pH of 8.0 to 4.5 in 25 minutes. This is due to the inability of the pH control system to compensate for the high production rate of acidic product during the reaction. Examining the pH activity of immobilised α-chymotrypsin on magnetic particles in section 3 : Figure 3.9.8, the enzyme activity at pH 4.5 is less than 10% of the optimum activity at pH 8.0. This will significantly reduce the reaction rate and may explain the levelling off of the conversion rate at 50 – 60 minutes. During the fluidised bed reaction, the pH did not fall below pH 6.0 and examining the pH activity, at pH 6.0 the enzyme activity is still relatively high at 70% of the optimum activity at pH 8.0.

It is known that by applying a magnetic field, the particles align themselves in chains vertically in the reactor. This effect separates individual immobilised particles enabling 360 degree access of the substrate to active site in the immobilised enzyme. In the conventional fluidised bed experiment the same magnetic particles were used. However these particles will maintain some remnant magnetisation, which may result in them attracting each other. This will reduce the surface area available to the substrate reducing the reaction rate. The fluidised bed allows some movement of the particles, which will probably break up the particles reducing this effect.
Although this experiment was not operated at maximum efficiency, it does suggest that the MSFBR performs the biocatalysis at a higher rate than within a conventional fluidised bed.

6.6 Limitations of a packed bed reactor for the biocatalysis of ATEE to AT

Figure 6.8 illustrates the effect of two flowrates, 35 and 65 mL/min on the biocatalysis of ATEE to AT in a packed bed reactor. The biocatalysis was operated using an initial substrate concentration of 18 mM (4 g/l), which was the solubility limit of N-acetyl-L-tyrosine ethyl ester. It was not possible to increase the flowrate or substrate concentration further as the pressure drop across the reactor was too high (in excess of 1.5 bar) due to insoluble substrate. It is clear from this figure that the packed bed reactor provides an ideal environment for the reaction with near ideal plug flow conditions. It is the pressure drop, which severely restricts the use of this reactor in solid substrate reactions in large-scale industry.
Figure 6.7: Comparison of a fluidised bed and MSFBR biotransformation of ATEE to AT under identical conditions at an operating flowrate of 83 ml/min. Reaction conditions were a settled bed height of 10.5 cm, magnetic field strength of 10 mTesla and an initial substrate concentration of 100 mM using a total enzyme loading of 2.1 * 10^3 units of activity in the reactor. MSFBR (■) and fluidised bed (▲). The exit line pH during the MSFBR reaction is also shown for information (●).
Biocatalysis in the presence of a solid substrate in the MSFBR

Figure 6.8: Packed bed biotransformations of ATEE to AT at 33 ml/min (■) and 65 ml/min (●).
6.7 Comparison of the biocatalysis of ATEE to AT in a stirred tank and a MSFBR

*Figure 6.9* illustrates a comparison of the biocatalysis of ATEE to AT in the MSFBR and in a conventionally stirred tank reactor. The product concentration in the MSFBR increases steadily during the reaction achieving 95% conversions after 30 minutes. The product concentration in the STR increases at a greater rate achieving 95% conversion after 25 minutes. The figure clearly shows that the STR has a lower reaction time that the MSFBR for this specific reaction. However the STR used a much larger process volume. The biocatalyst is loaded to the STR at a 10% concentration. For example for a 1 litre immobilised enzyme batch, a 10 litre STR would be used whilst only a 1.5 – 2.5 litre MSFBR would be used for the same process.

6.8 Effect of substrate concentration on the biocatalysis

*Figure 6.10* illustrates the effect of substrate concentration on the conversion of ATEE to AT in the MSFBR. Using a higher substrate of 200 mM follows identical reaction curve as for a lower substrate concentration of 100 mM.

A concentration of 200 mM ATEE is the highest concentration that the MSFBR can handle. Concentrations higher than this block the flow distributor plate at the bottom of the MSFBR. Blockages in this plate significantly increase the pressure drop across the reactor.
Figure 6.9: Comparison of the biocatalysis of N-acetyl tyrosine ethyl ester to N-acetyl tyrosine in a STR and MSFBR at a flowrate of 150 ml/min. MSFBR (■) and stirred tank reactor (●).
Figure 6.10: Effect of substrate concentration on the conversion of N-acetyl-L-tyrosine ethyl ester to N-acetyl tyrosine using immobilised alpha chymotrypsin at a flowrate of 150 ml/min. ATEE initial substrate concentrations of 100 mM (●) and 200 mM (●).
6.9 Reproducibility studies

*Figure 6.11* shows three identical biocatalysis of N-acetyl-L-tyrosine ethyl ester to N-acetyl tyrosine in the MSFBR using an initial substrate concentration of 50 mM ATEE and a flowrate of 75 mL/min.

For the initial thirty minutes of the reaction, the first run is significantly different from the second and third runs. The first run produces N-acetyl tyrosine at a faster rate. After thirty minutes all the runs show similar product concentration profiles. The time for 95% conversion was 58, 60 and 62 minutes for run 1, 2 and 3 respectively.

6.10 Attrition of immobilised \( \alpha \)-chymotrypsin in the MSFBR

Immobilised \( \alpha \)-chymotrypsin magnetic particles were analysed before use in the MSFBR in a particle size analyser (Malvern Mastersizer 2000 Hydro SM, Malvern Instruments Ltd, Malvern, U.K.). The particles were re-examined after 24 hours use.

*Figure 6.12* shows the particle size distribution before use in the MSFBR. The mean particle size is 224 microns. After 24 hours use in the MSFBR, the average particle size has reduced to 217 microns. The particle size distribution is shown in *Figure 6.13*. Also shown on *Figure 6.13* is particle size distribution for immobilised penicillin acylase on 250 – 350 microns giving a mean particle size of 299 microns. Although some attrition damage has been done on the immobilised \( \alpha \)-chymotrypsin magnetic particles, the particle size has reduced by only 3% in 24 hours operation.
Figure 6.11: Batch to batch reproducibility studies of the conversion of 50 mM N-acetyl-L-tyrosine ethyl ester to N-acetyl tyrosine using immobilised \( \alpha \)-chymotrypsin in a MSFBR at a flowrate of 75 ml/min, a settled bed height of 10.5 cm and a magnetic field strength of 10 mTesla. Run 1 (■), 2(●) and 3(▲)
Figure 6.12: Particle size distribution of immobilised α-chymotrypsin on magnetic particles before use in the MSFBR (125 - 250 μm)

Figure 6.13: Particle size distribution of immobilised α-chymotrypsin on magnetic particles after 24 hours use in the MSFBR

Blue: Immobilised α-chymotrypsin on magnetic particles before use (125 - 250 μm)

Black: Immobilised α-chymotrypsin on magnetic particles after 24 hours use in the MSFBR (125 - 250 μm)

Red: Immobilised Penicillin acylase on magnetic particles (larger particles, 250 – 350 μm)
6.11 Summary and Conclusions

The following conclusions can be drawn from the successful biotransformation of N-acetyl-L-tyrosine ethyl ester to N-acetyl tyrosine in the MSFBR:

- Grinding the solid substrate from 125 to 95 microns increased the $K_{1,a}$ value in controlled small scale analysis.
- Running the ATEE biotransformations using smaller substrate particles improved the time for 95% conversion and switched reaction from a dissolution limiting to kinetic limiting reaction.
- The MSFBR performed well when compared with a conventional fluidised bed.
- Comparison with a stirred tank reactor showed that the reaction has lower conversion times than the MSFBR but requires larger process volumes.
- The reaction cannot be operated at higher flowrates than 200 cm/hour and/or higher substrate concentrations than 4 g/l (solubility limit of N-acetyl-L-tyrosine ethyl ester) in a packed bed due to high pressure drops.
- In the MSFBR, the time for 95% conversion decreases with increasing flowrates.
- A reduction in immobilised α-chymotrypsin magnetic particle size of 3% is seen after 24 hours operation in the MSFBR.
Chapter 7

General Discussion
Chapter 7  General Discussion

7.1 Introduction

There were three main aims of this final discussion chapter. The first general aim was to identify the window of operation for the two biotransformations, penicillin acylase and α-chymotrypsin in the MSFBR.

This will be achieved by identifying the following

- Limitations of substrate concentration
- Limitations of magnetic field strength
- Limitations of flowrate

The second aim of this chapter was to scale up the MSFBR. This was achieved by identifying the following

- The parameters which limit scale up
- Effect of reactor aspect ratio
- Recommend solutions to scale up difficulties

The final aim was to discuss the MSFBR and suggest suitable applications of this technology within the biotransformation area and other biotechnology applications.
7.2 Operating window for penicillin acylase and $\alpha$-chymotrypsin biotransformations in the MSFBR

7.2.1 Introduction

Operating windows are pictorial maps which show the process limitations of a system (Woodley and Titchener-Hooker, 1996). These limitations may be many parameters such as physical, kinetic or economic.

7.2.2 Limitations of substrate concentration

7.2.2.1 Conversion of Penicillin G to 6APA using immobilised penicillin acylase on MCP’s

The effects of substrate inhibition on the enzyme, limit the substrate concentration in this reaction. The maximum penicillin G concentration in this reaction was 200 mM. Shewale and Sivarman, 1989 found that industrial manufacture of 6 APA from penicillin G used between 7 and 10% (~ 187 – 264 mM). Effects of substrate inhibition can be reduced by feeding the substrate into the reactor at a set level.

7.2.2.2 Conversion of N-acetyl-L-Tyrosine ethyl ester to N-acetyl Tyrosine using immobilised $\alpha$-chymotrypsin on MCP’s

The effects of solid slurry concentration in the MSBFR limit the substrate concentration in this reaction. The current reactor design of a relatively narrow flow distribution area at the bottom of the MSFBR results in blockage at high substrate concentrations. The highest substrate concentration achieved in Chapter 6 was 200 mM.

7.2.3 Limitations of magnetic field strength

The maximum magnetic field strength in the MSFBR is generally influenced by the characteristics of the magnetic particles. It is therefore specific to each magnetic particle system. The magnetic particles used in both the immobilisation of penicillin
acylase and α-chymotrypsin were identical 125 – 250 μm in size with a density of 1.20 g/mL. In Chapter 4, the physical characterisation of the MCP’s and MSFBR aggregation of the these particles was identified at magnetic field strengths above 17.5 mTesla. Aggregation of particles occurs with inter – particle magnetic attractive forces from larger agglomerate particles. Although this has some advantages, as the escape velocity changes little with increases in flowrate through the MSFBR after particle agglomeration, it also has one severe disadvantage. The larger agglomerates have a reduced surface area, which significantly affects the enzyme activity in the reactor reducing the productivity of the biocatalysis.

The upper limit of magnetic field strength in this MSFBR system is 17.5 mTesla.

7.2.4 Limitations of flowrate

The effect of flowrate in each biotransformation was discussed in chapter 5 for penicillin acylase and chapter 6 for α-chymotrypsin. The main influences of the flowrate limits in the MSBFR are the escape velocity of the magnetic particles from the reactor and external mass transfer effects.

In Chapter 4, the escape velocity of various magnetic particles was investigated. The escape velocity at 10 mT identified for the magnetic particles used to immobilise the test enzymes, penicillin acylase and α-chymotrypsin (125 – 250 μm in size with a density of 1.20 g/mL) was 210 cm/hour (see Figure 4.11(b)).

Figure 7.1 (a) and (b) illustrate the operating window of a 19 cm bed height of unactivated magnetic particles (with no enzyme attached) of 125 – 250 μm and 250 - 350 μm, density 1.04 g/ml. The limitations of magnetic field strength, bed voidage and escape velocity are shown on the figure to show the area in which the MSFBR can be operated. To ensure adequate external mass transfer around the particles an arbitrary figure of 0.30 has been included as the minimum bed voidage at which to operate the MSFBR.
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Figure 7.1 (a) Operating window to inactivated magnetic particles of 125 – 250 μm, density 1.04 g/ml and (b) Operating window to inactivated magnetic particles of 250 - 350 μm, density 1.04 g/ml
By analysis of Figure 5.7 in Chapter 5, the time for 95% conversion in the penicillin acylase reaction begins to level off at flowrates of approximately 90 mL/min. This is equivalent of a linear velocity of 275 cm/hour. In a similar way, analysis of Figure 6.6 in Chapter 6 the time for 95% conversion in the α-chymotrypsin reaction does not have a levelling off suggesting that the conversion time can be decreased further. The difference in flowrate limitations between biotransformation types may be due to the presence of solid substrate in the α-chymotrypsin reaction as the solids may act to break up any aggregated MCP’s. This will increase the accessibility of the enzyme even at high flowrates. The escape velocity is higher than that identified in the physical characterisation work in chapter 4. Before attachment of enzyme, the escape velocity of 125 – 250 μm 1.20 g/mL particles was 210 cm/hour. After immobilisation of penicillin acylase, the achievable flowrate increases to 275 cm/hour. The differences between these two experiments were; (a) there was no enzyme attached during the physical characterisation work and (b) the fluid in which the particles were fluidised was 50 mM potassium phosphate pH 7.0 in the physical characterisation work and 200 mM Penicillin G, 50 mM potassium phosphate pH 7.8 for the penicillin acylase work and 200 mM N-acetyl-L-tyrosine ethyl ester, 50 mM potassium phosphate pH 8.0 in the α-chymotrypsin biotransformation work. The addition of glutaraldehyde and penicillin acylase to the magnetic particles would not increase the density of the particle significantly. The higher density and viscosity of the 30 mM Penicillin G solution would increase the drag forces on the immobilised magnetic particles, which would increase the escape velocity as observed here. There was substantial physical characterisation work completed on the magnetic particle prior to immobilisation of enzymes. During this work, the magnetic particles were fluidised for long periods of time. This results in smaller, less dense particles leaving the reactor. This is known as a conditioning effect, where smaller less dense particles are removed from the magnetic particle pool. This will however effect the overall escape velocity by increasing it over time as the magnetic particles are used in the reactor.

From the experimental work completed in Chapters 5 the flowrate limitation is defined by the insignificant increases in the time for 95% conversion above 275 cm/hour for the penicillin acylase reaction. From the experimental work in Chapter
the flowrate is not limited by the time for 95% conversion but by the escape velocity. The escape velocity for $\alpha$-chymotrypsin immobilised magnetic particles was 275 cm/hour.

*Figure 7.2 (a) and (b)* illustrate the operating windows for each biotransformation in this 5cm ID MSFBR with 125 – 250 micron particles. The operating window is the same for each biotransformations but for different reasons as discussed above. The limitations of substrate inhibition, kinetic limitations (no improvement in 95% conversion time at higher flowrates), large pH drop, excessive pressure drop, bed voidage and escape velocity are shown on the figure to show the area in which the MSFBR can be operated for each biotransformation.
Figure 7.2 (a) Operating window for Penicillin acylase biocatalysis in the MSBFR and (b) Operating window for α-chymotrypsin biocatalysis in the MSFBR
7.3 Scale up of MSFBR

7.3.1 Helmholtz coils

Initial studies on the scale up of this technology focused on the coils themselves and they were perceived as the main bottleneck. To maintain the constant magnetic field across the reactor both horizontally and vertically, the current needs to increase with square of the diameter. As the heat produced is directly related to the applied current, the heat produced by the field increases with the diameter squared also.

The resistivity, $\rho_r$ of copper increases with temperature, $T$ by the following relationship

$$\rho_r = 1.67 \times 10^{-8} + (0.0068 \times 10^{-8} [T - 20])$$  \hspace{1cm} (7.1)

Therefore the heat developed in a circuit by an electric current of $I$ amps flowing through a resistance of $R$ ohms for $t$ seconds is equal to $R \times I^2 \times t$. As previously mentioned above the current supplied to the coils must alter by the square of the diameter to maintain the desired field strength. This results in large increases in the heat production as the diameter increases. The precise nature of this relationship can be outlined by applying dimensionless analysis.

Using equation 7.2 for the relationship between the magnetic field strength and the applied current and diameter of the coil.

$$B = \frac{8N\mu_0 IN_c}{5\sqrt{5a}}$$  \hspace{1cm} (7.2)

where

$N$ : Number of turns on each helmholtz coil

$N_c$ : Number of helmholtz coils

$a$ : radius of the coils
The magnetic field strength is constant on scale up simplifying the relationship to

\[ a \propto I^2 N^2 \]  

(7.3)

### 7.3.2 Potential bottlenecks of MSFBR scale up

As with all novel types of reactor, the key question of success is the feasibility and economics of scale up. Initial examination of the magnetically stabilised fluidised bed reactor quickly establishes the method of providing magnetic stabilisation as the main bottleneck for scale up of this process.

*Figure 7.1* illustrates quite simply, the effect of scale up of the amount of current required in a four coil system as operated currently in the MSFBR. It also illustrates the effect of increasing the number of turns in each coil. Using equation 7.3, the amount of current required to create the uniform field of 40 Tesla for increasing scale was modelled. For coil diameters above 3 metres (which relates to a reactor diameter of 1.5 metres) the applied current required to maintain the desired magnetic field strength exceeds 400 Amps. The highest economically sound power pack produces 400 Amps and therefore current ratings above this value are inefficient. The number of turns in the magnetic coils are limited by physical size as a 4000 turn coil would have a height of approximately 10 cm and a bore size of 10 cm. The variation in wire placement within the coils reduces its capability to produce a uniform field. Helmholtz coils are only truly efficient with up to 2000 turns (Cook, 1975). Consequently the Helmholtz coils that can be feasibly used must require a 400 Amp current supply and have a maximum of 2000 turns.

The major effect of using a high current supply is the large amount of heat produced. Practically all of the power supplied to the coil will leave as heat and as the amount of heat produced increases to the square of the current supplied, the heat production levels escalate on scale up. *Figure 7.4* illustrates the heat production levels seen on scale up using equation 7.3.
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**Figure 7.3**: The applied current required to maintain a 40 mTesla magnetic field for increasing diameter of coil.

**Figure 7.4**: The heat produced and effect of temperature on the Helmholtz coils during scale up.
7.3.3 Solenoids

Depending on the aspect ratio of the solenoid, the design can be manipulated to give a constant magnetic field as given by (Purcell, 1985)

\[
B = \frac{N I \mu_0}{L_s} \left[ \frac{L_s}{\left( L_s^2 + D_s^2 \right)^\frac{1}{2}} \right]
\]

(7.4)

where

\[ L_s : \text{Length of solenoid (m)} \]
\[ D_s : \text{Diameter of solenoid (m)} \]

There are many variables in the equations for scale up of the MSFBR, although these can be reduced by the following assumptions.

(a) A standard power supply utilising 400 Amps is used to provide the current to the coils / solenoid. For industrial applications, capital expenditure on a power supply providing a single current supply is more economical than a variable supply.

(b) The magnetic field strength required for operation of the MSFBR is 15mTesla

The resistance of the solenoid is related to the diameter and number of turns in the solenoid by the following relationship.

\[
R = \frac{\pi ND}{\rho A}
\]

(7.5)

Power supplied to the coils is given by the simple relationship

\[
P = I^2 R
\]

(7.6)
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There are a variety of ways to scale up a process, ranging from aspect ratio to the linear flowrate through the reactor. It is these widely differing methods resulting in a long thin reactor and short fat reactor respectively which are used to illustrate the effects of heat production on scale up, which has been identified previously as the main concern.

A parameter that could be adjusted to increase the operating window of the reactor for both penicillin acylase and $\alpha$-chymotrypsin reactions is an increase in the aspect ratio of the reactor. Currently the aspect ratio is $1:10$: a 5cm internal diameter to a 50 cm reactor height.

Using aspect ratio, two different designs of reactor have been identified as possible large scale reactors. A long thin reactor with an aspect ratio of $1:10$, such as used in biocatalytic reactions and a short fat reactor such as used in chromatographic separations with an aspect ratio of $1:1$.

By using equation 7.4 to calculate the number of turns required for that aspect ratio, this result can then be fed into equation 7.5 to calculate the resistance. The power supplied and therefore the heat produced by the solenoid can be calculated from equation 7.6.

As can be seen from Figure 7.5, the amount of heat produced by the solenoid is much less than that produced by a set of helmholtz coils. The solenoid required for a reactor of aspect ratio $1:10$ requires 30kW of power at the 2 m diameter scale (equivalent to a reactor volume of 62 litres) as compared with 150 kW required by the helmholtz coils. The heat production at the 2 m diameter scale is reduced when the aspect ratio is small at $1:1$ (equivalent to a reactor volume of 6.2 litres), requiring only 5 kW of power.
Heat produced by a Solenoid of 1:10 aspect ratio, a Solenoid of 1:1 aspect ratio and four Helmhoitz coils on increasing diameter

Figure 7.5: Comparison of Helmholtz coils and a solenoid for scale up of the MSFBR
7.3.4 Further MSFBR design Improvements

There are further design improvements required before the reactor can be scaled up for industrial applications.

To reduce the heat production from the magnetic coils, a cooling system could be introduced. Instead of using solid copper wire in the solenoids, a small copper pipe with coolant inside could be used to dissipate the heat levels.

To improve the efficiency of magnetic field production, superconductor materials could be used instead of copper.

7.4 Potential Industrial Applications of MSFBR Technology

The future of the MSFBR as an economical industrial reactor lies in the areas of protein purification and some biotransformations.

The MSFBR could be used in the direct capture of proteins from cultures supernatent as the magnetic element can control the structure in the bed allowing good plug flow properties for efficient binding of proteins to a particular magnetic resin. The MSFBR could be used as a three phase in situ product removal reactor. A whole cell oxygen requiring biotransformation could be run in the reactor with the magnetic particles acting as a product binding resin to remove the product. This would be highly advantageous in a product-inhibited reaction. By combining production and recovery steps in one reactor the overall processing time is significantly shorter.

Although this chapter has established that it is possible to scale up the MSFBR, design improvements are required to make the MSFBR a realistic alternative to conventional reactors.
7.5 Discussion

The method for magnetic particle manufacture has been further developed to ensure homogeneity of magnetic particle size and shape within the magnetic particles. By using a precipitated magnetite rather than an off the shelf coarse magnetite, the magnetite within the magnetic particles was more uniformly spread through the particle volume. This ensured that magnetic particles remained stable when a magnetic field was applied. The magnetic particles could have applications in other areas of biotechnology such as biosensors or chromatography resins. Particle size analysis of the magnetic particles produced using the manufacturing method developed, showed a typical Guassian spread or sizes.

Two enzymes, penicillin acylase and α - chymotrypsin have been successfully immobilised to the magnetic particles using the bifunctional activating agent glutaraldehyde. The glutaraldehyde activation conditions were optimised by analysis of concentration, activation time and temperature. The immobilisation conditions were optimised by analysis of parameters such as enzyme challenge, time and buffer pH. Analysis of the kinetic properties of the immobilised enzymes confirms comparability with industrial immobilised forms of the enzymes. The storage and operational stability of the enzymes were optimised to ensure no significant losses occurred. This chapter of the project took a significant amount of time to complete and at the start of the project this part was not seen to be the main bottleneck to testing out the MSBFR. Prior to immobilisation of the second enzyme, α - chymotrypsin a decisional chart was produced which outlined the minimum experiments required to immobilise the enzyme to the magnetic particles. This significantly reduced the time required to optimise the immobilisation process. It is envisaged that this chart be used to immobilise any enzyme to these magnetic particles.

The characterisation of the MSFBR was approached from two angles. Firstly from a physical perspective, establishing the strength and uniformity of the magnetic field within the reactor. The horizontal magnetic field variation was up to 2% across the reactor. The vertical variation was much higher at up to 40 %. This large value is a
consequence of limiting the number of coils in the reactor to 4. Addition of a fifth magnetic coil would decrease this figure significantly.

The MSFBR at the pilot 1 litre scale was capable of producing up to 15 mTesla over 24 hours without significant temperature increases in the coils. At magnetic field strengths higher than this, the heat production levels began to effect the performance of the coils and the temperature regulation within the reactor. As discussed earlier in section 7.3.3, there are design improvements, which could be implemented to reduce the head production from the coils allowing higher magnetic field strengths and/or operation at a larger scale.

The bed expansion profiles of magnetic particles of size 125 – 250 and 250 – 350 micron and density 1.04 and 1.2 g/ml were established which gave valuable information on the bed voidage and escape velocity. Using a low density, small particle of 1.04 g/ml, 125 – 250 microns enabled up to 1.5 times the velocity through the reactor compared with no magnetic field. Using a high density, small particles of 1.2 g/ml, 125 - 250 microns the escape velocity is 1.8 times the velocity through the reactor compared with no magnetic field. Although the density of the particles could have been increased this would have reduced the amount of enzyme that could be attached to the magnetic particles.

The analysis of the mixing behaviour within the reactor using a dispersed plug flow model confirmed than the MSFBR yielded comparable axial dispersion numbers with a conventional fluidised bed.

The second part of the characterisation was the testing of the MSFBR with biotransformations. The reactions chosen were specifically targeted towards establishing the MSFBR as a reactor to be used with difficult biotransformations. The first key test was the potential ability of the reactor to handle pH changes. The reaction system chosen penicillin acylase presented an interesting challenge as both products and substrate inhibit the enzyme. Product inhibition, by far the strongest, suggests a packed bed reactor as a suitable reactor as in the first cycle the substrate reacts to product free enzyme. However the side product of the reaction is acidic (phenylacetic acid) which necessities the use of pH control which is difficult in a
General Discussion

packed bed. pH control can be achieved in packed beds using a recirculating system but high flowrates are required. High flowrates in packed beds are difficult to obtain without high pressure drops across the bed. In direct comparison experiments at various flowrates the MSFBR matched the packed bed in overall conversion times. At higher flowrates, the packed bed had shorter absolute 95% conversion times but the errors associated with calculating the conversion times resulted in no statistical difference between the packed bed and MSBFR conversion times.

The second test was the potential ability of the reactor to handle the presence of solids. This could either be solid substrate or product. The reaction system chosen of α-chymotrypsin used a partially soluble substrate, N-acetyl-L-tyrosine ethyl ester. As with the penicillin acylase system, the actual reaction chosen was arbitrary, as the way the reactor handles the presence of the solids is the real test. Industrially this reaction would be performed in a stirred tank reactor. The MSFBR was compared to a packed bed, fluidised bed and stirred tank. The ideal choice of reactor for this solid substrate biotransformation would be a stirred tank reactor. However this system was simply a model system to test the ability of the MSFBR to handle the presence of solids. When compared with the fluidised bed the MSFBR performs better with lower 95% conversion times. This may be attributed to the solid substrate mixing with the magnetic particle reducing external mass transfer limitations. The MSFBR is suited to this type of biotransformation and further work using different types of solid containing reactions should be tested out in the MSFBR.

However there are disadvantages of the MSFBR which must be detailed. The main disadvantage is the control of the reactor. A detailed study needs to be undertaken to understand the complex forces involved in the reactor. An attempt was made in this project but highly sophisticated mathematics is required and is certainly out of the scope of this thesis. Pre and post preparation are lengthy processes compared with conventional reactors. The power consumption on scale up is significantly higher than conventional reactors.

As mentioned Chapter 7 there are applications of the MSFBR in other areas of biotechnology. In the same way that the presence of solid N-acetyl-L-tyrosine ethyl
ester reduces external mass transfer around immobilized magnetic particles, protein could be captured from cultures supernatent in the presence of cells. The MSFBR could be used as a three phase in situ product removal reactor. A whole cell oxygen requiring biotransformation could be run in the reactor with the magnetic particles acting as a product binding resin to remove the product. This would be highly advantageous in a product-inhibited reaction and by combining production and recovery steps in one reactor the overall processing time is significantly shorter.

As with all novel types of reactor a lot to time, research and a leap of faith is required by industry to consider using these reactors.
7.6 Future Work

There are a number of recommendations from this thesis regarding any future work on the MSFBR.

1. The MSFBR may have applications in biotransformations containing either solid substrate or product. Other biotransformation reactions of this type should consider using the MSFBR.

2. The development of a mathematical model would enable more accurate scale up information on the MSFBR. This would include detailed force analysis on the magnetic particles.

3. The MSFBR has uses in intensifying biotransformation reactions.
Chapter 8

Conclusions
Chapter 8  Conclusions

The following conclusions can be drawn from this thesis.

1. A method has been established to produce cheap, uniform magnetic carrier particles for immobilisation of enzymes.

2. Two enzymes, penicillin acylase and α chymotrypsin have been successfully immobilised to magnetic carrier particles yielding 1000 U/g dry MCP and 3100 U/g dry MCP at a 2 ml scale.

3. Using Helmholtz coils, the horizontal variation of magnetic field strength across the MSBFBR is 2%.

4. The MSFBR matches the conversion times when compared with a packed bed reactor for the biotransformation of Penicillin G to 6 APA using immobilised penicillin acylase.

5. The experimental data suggests that the MSFBR has lower conversion times than a fluidised bed for the biotransformation of N acetyl tyrosine ethyl ester to N acetyl tyrosine using immobilised α-chymotrypsin at low flowrates.

6. Very high flowrates required to properly challenge the MSFBR were not possible using the present design of magnetic field generation.
References


Axen, R, Myrin, P-A, Janson, J-M (1970) Chemical fixation of chymotrypsin to water-insoluble crosslinked dextran (sephadex) and solubilisation of the enzyme derivatives by means of dextranase, Biopolymers, 9, 401 - 413.


References


References


Zaborsky, O (1973) Immobilized Enzymes, CRC Press, Ohio.

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Appendix 1

Glutaraldehyde Safety Data Sheet
Excerpts from GlaxoWellcome safety handling sheet

Chemical Name: Glutaraldehyde Molecular Formula: C₅H₁₀O₂

Hazards Identification

Health
Toxic if swallowed and by inhalation. Causes burns. May cause sensitisation by skin contact and inhalation.

Physio-Chemical
Corrosive to Metals

Environmental
Very toxic to aquatic animals

First Aid Measures

Symptoms and effects
A strong bronchial sensitiser that can cause asthma. Strongly irritant and can cause burns. Toxic if swallowed.

Treatment
Never attempt to induce vomiting. Do not attempt to give solid or liquid by mouth to an unconscious or semiconscious person.

Eyes
Irrigate thoroughly with plenty of water from an eye wash fountain or bottles and continue for at least 15 minutes.

Skin contact
Wash thoroughly with plenty of soap and water, preferably under a shower, removing contaminated clothing. Obtain medical attention. Thoroughly wash contaminated clothing before re-use.

Inhalation
Remove from exposure, keep warm and at rest. Obtain medical attention. If breathing is difficult or depressed ensure and maintain ventilation, giving oxygen as necessary.

Handling and Storage
Glutaraldehyde should be stored in clearly labelled, well filled, airtight and lightproof containers in a cool, well ventilated area. Containers should be opened cautiously – wear protective equipment. Laboratory use of glutaraldehyde should be restricted for fume cupboards or suitable work areas with forces ventilation.
## Appendix 2

Typical Bradford assay Protein calibration curve

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<tr>
<th>BSA Concentration (mg/ml)</th>
<th>Dilution Factor</th>
<th>Actual BSA Conc (mg/ml)</th>
<th>A595 Reading</th>
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<tr>
<td>0.1</td>
<td>10</td>
<td>0.01</td>
<td>0.298</td>
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<tr>
<td>0.15</td>
<td>10</td>
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<td>20</td>
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**Bradford Assay Standard Curve for B.S.A**

\[ y = 15.712x + 0.1437 \]

\[ R^2 = 0.9948 \]
Appendix 3

**Lowry method and calibration curve**

(relates to section 3.4.2)

All stock solutions were obtained from Sigma (Poole, Dorset, U.K.).

**Stock Solutions**

- 5% K Na Tartrate
- 0.5% copper sulphate hydrate
- 0.1 M Sodium hydroxide in 2% Na$_2$CO$_3$
- Folin Ciocalteu solution

**Working Solutions**

All working solutions were prepared fresh on the day of the assay. 2.5 ml of CuSO$_4$ solution was mixed with 2.5 ml tartrate solution and diluted to 100 ml with NaOH / Na$_2$CO$_3$ solution. The folin ciocalteu solution was diluted 1 part with 2 parts water to the desired assay volume required.

**Procedure**

The protein solution for assay was prepared in a volume of 125 µL (at a protein concentration of 5 to 50µg). 0.5 ml of the CuSO$_4$ alkaline solution was added and mixed and allowed to stand at room temperature for 10 – 30 minutes. 125 µL of diluted folin ciocalteu solution was added and mixed immediately and allowed to stand for 30 minutes. The mixture was placed in a quartz cuvette and read in a spectrophotometer at 660 nm.
Appendix

BSA Standard Curve for Lowry Assay

\[ y = 0.002x + 0.043 \]

Figure A1: BSA calibration curve for assay using Lowry method for determining protein concentration.

Figure A2: \( \alpha \)-chymotrypsin calibration curve for assay using Lowry method for determining protein concentration.
Appendix 4

Titration curve of Phenylacetic acid (PAA) against 0.25 M sodium hydroxide used to maintain pH during biotransformations
Appendix

Appendix 5

Non-specific hydrolysis of various concentrations of Penicillin G in 50 mM potassium phosphate pH 7.8 at 37 °C.
Appendix 6

Titration curve of N-acetyl-L-tyrosine against 0.1M sodium hydroxide used to maintain pH during biotransformation

\[ y = 0.4501x \]
\[ R^2 = 0.9983 \]

Appendix 7

Non-specific hydrolysis of N-acetyl tyrosine ethyl ester. For each analysis using ATEE, the non-specific hydrolysis was accounted for.
Appendix 8

Penicillin G Safety Data Sheet
Excerpts from Sigma, Poole, Dorset, U.K.
Chemical Name: Benzylpenicillin

Hazards Identification

Health
Toxic if swallowed and by inhalation. May cause sensitisation by skin contact and inhalation.

First Aid Measures

Symptoms and effects
Strongly irritant and can cause burns. Toxic if swallowed.

Treatment
Never attempt to induce vomiting. Do not attempt to give solid or liquid by mouth to an unconscious or semiconscious person

Eyes
Irrigate thoroughly with plenty of water from an eye wash fountain or bottles and continue for at least 15 minutes

Skin contact
Wash thoroughly with plenty of soap and water, removing contaminated clothing. Obtain medical attention. Thoroughly wash contaminated clothing before re-use.

Inhalation
Remove from exposure, keep warm and at rest. Obtain medical attention.

Handling and Storage
Benzylpenicillin should be stored in clearly labelled and airtight containers in a cool, well ventilated area. Laboratory use of benzylpenicillin should be restricted for fume cupboards.
Appendix

Appendix 9

Packing protocol for XR Pharmacia column

The magnetic particles were packed in a 5 cm ID XR Pharmacia column using a packing piece. The particles were added to the XR column and settled under gravity for 30 minutes. Any excess fines were removed by pipette. A peristaltic pump was attached to the column. The particles were packed under constant pressure at 100 mL/min (306 cm/hour) for 5 column volumes using 50 mM potassium phosphate at pH 7.0. If any air or uneven areas of packing were observed, the column was repacked. The column was stored at 4°C with top and bottom valves closed between uses.
Appendix 10

Economic Comparison of immobilised penicillin acylase on magnetic carrier particles

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<th>Quantity required per batch</th>
<th>Price</th>
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<td>ALDRICH</td>
<td>FeSO₄₇H₂O</td>
<td>41.7 g</td>
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<td>ALDRICH</td>
<td>FeCl₃H₂O</td>
<td>80.7 g</td>
<td>£20.30 per 2 Kg</td>
<td>82 p</td>
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<td>SIGMA</td>
<td>Sodium Hydroxide</td>
<td>60 g</td>
<td>£78.80 per 10 Kg</td>
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<td>Sinnott, 1991</td>
<td>Dimineralised water</td>
<td>20 litres</td>
<td>20 p /m³</td>
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Table A10.1: Summary of the costs for raw materials for the production of precipitated magnetite

Table A10.1 illustrates the cost for producing the precipitated magnetite for the magnetic particles. It can be seen from Table A10.1 that each batch of precipitated magnetite producing approximately of 350 ml costs £2.12 to manufacture. This can be used in Table A10.2 to illustrate the raw material costs in the overall production of the magnetic carrier particles.


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<thead>
<tr>
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<td>SIGMA</td>
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Table A10.2: Summary of the cost of raw materials used in the magnetic carrier particle manufacture

For each batch of magnetic carrier particles, the small scale raw material costs are £2.54 for approximately 500 ml.