

**Rational Immobilisation of Enzymes:
Immobilisation of Transketolase for Carbon-Carbon
bond synthesis**

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To my mother and father
and to
the memory of Professor Malcolm Lilly

“When you are a Bear of Very Little Brain, and you Think of Things, you find sometimes that a Thing which seemed very Thingish inside you is quite different when it gets out into the open and has other people looking at it”

Winnie-the-Pooh

from ‘The House at Pooh Corner’ by A.A.Milne (1928)

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Abstract

This thesis describes efforts in the development of an immobilised enzyme biocatalyst for a scaleable biotransformation. The enzyme transketolase, which catalyses the synthesis of carbon-carbon bonds, was chosen as a model. The enzyme has been cloned into *E.coli* and can be produced on a large scale and is consequently representative of an industrial enzyme.

Transketolase has been immobilised onto the commercially available materials Eupergit-C® and Amberlite XAD7. With a binding yield of ca90 % and a retention of activity between 20 % and 60 % depending upon enzyme load in the case of Eupergit-C® and a binding yield of 90 % and a retention of 20% activity in the case of Amberlite XAD7.

The cofactor thiamine pyrophosphate was successfully introduced to the Eupergit-C® immobilised enzyme, by co-immobilising the enzyme and cofactor, to yield a biocatalyst with a specific activity of 840 U.g⁻¹ and a retention of activity of 58 %. Although the cofactor was found to leach from the immobilised holo-enzyme as a consequence excess cofactor had to be maintained within the bioreactor environment.

Both immobilised enzymes were characterised and an improvement in the stability of both the Eupergit-C® and Amberlite XAD7 preparations against the toxic substrate glycolaldehyde was observed. The half life in the presence of 0.5 M glycolaldehyde was improved from 1 hour to 100 hours and 80 hours in the case of Eupergit-C® and Amberlite XAD7 respectively. An economic comparison of Eupergit-C® and Amberlite XAD7 was made and Eupergit-C® was found to be the more economic support material producing a biocatalyst with a space time yield of 674 kg.L⁻¹/batch and a calculated enzyme productivity of 100kU/kg product after 20 batches.

Nomenclature

D_{eff}	Effective diffusivity (cm^2s^{-1})
K_m	Michaelis constant (mmoles.L^{-1})
K_m'	Apparent Michaelis constant (mmoles.L^{-1})
Q	Volumetric flow rate (mL.min^{-1})
v	Reaction rate (mmoles.min^{-1})
V_m	Maximum reaction rate (mmoles.min^{-1})
V_m'	Apparent maximum reaction rate (mmoles.min^{-1})

Abbreviations

Ery	Erythrulose
E	Enzyme
GA	Glycolaldehyde
HPA	Hydroxypyruvic acid
NAD(P)(H)	Nicotinamide AdenosineDineucleotide (Phosphate) (Reduced form)
TPP	Thiamin pyrophosphate

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Introduction

1.1 Biocatalysis and Biotransformations

1.1.1 Background

Biological systems have been used in the manufacture of materials beneficial to mankind, such as bread, since the beginning of historical records. More recently biological activity has been applied to the production of compounds such as alcohol, amino acids, antibiotics, semisynthetic antibiotics, steroids, vitamins, proteins and other macromolecules. The use of biological activity can be divided into 2 discrete methods known as fermentation and biotransformation. During a fermentation useful compounds are produced from simple starting materials by whole metabolising cells through a variety of biochemical steps. A biotransformation on the other hand utilises the catalytic activity of a single (or in some instances a few) enzyme(s) to achieve the conversion of complex organic starting materials into similar organic products (Lilly M, D 1993).

Since the first synthetic use of biological activity, to introduce a hydroxyl group into progesterone in the 1950s, several biotransformations have been developed and used on an industrial scale (Lilly, M. D 1993). Along side this, biotransformations is becoming the focus of more research in chemistry, biochemistry and biochemical engineering. Enzymes, the biological catalysts, have been demonstrated as being able to provide a novel alternative to chemical catalysts, and in some cases their activity makes difficult or impossible novel reactions more feasible (Faber, K and Franssen, M. C. R 1993).

Reaction	Organism/Enzyme	Reference
11 α - hydroxylation of progesterone	<i>Rhizopus nigricans</i>	Peterson, D. H <i>et al.</i> , (1952)
Deacylation of Penicillin G	Penicillin acylase	Lily, M. D. (1994)
Production of D-amino acids	<i>Pseudomonas putida</i>	Elferink, V. H. M <i>et al.</i> , (1991)
Resolution of naproxen	Carboxylase NP	Faber, K and Franssen, M. C. R (1993)
Carbon carbon bond synthesis	Transketolase	Hobbs, G. H <i>et al.</i> , (1993)

Table 1.1 Table showing the variety of reactions to which biocatalytic activity can be applied.

The variety of biotransformation reactions and the variety of biocatalysts is demonstrated by table 1.1 above. Enzymatic activity has been put to use in reactions which are impossible or complex to carry out by conventional chemical routes, for example, the hydroxylation of progesterone requires a series of chemical steps, and the deacylation of penicillin G which is described in figure 1.1 Another point to note from the table is the variety of enzymes that have been used in biotransformations.

Enzymes are the basic biocatalytic materials upon which most of the reactions involved in life are based. They work similarly to chemical catalysts, by lowering the activation energy of the reaction allowing it to proceed faster. Like inorganic catalysts they neither shift the thermodynamic equilibrium of the reaction or are directly consumed as a part of the reaction. Unlike chemical catalysts, however, enzymes are proteinaceous macromolecules with the active centre being held in place by an amino acid backbone. They have some remarkable characteristics which give them advantages over chemical catalysts.

1. **Catalytic power.** Enzymes can increase the reaction rate by as much as 10^{14} -fold. Enzymes catalyse reactions under conditions remarkably less severe than conventional catalysis. For example, in the hydrolysis of urea catalysed by urease,

a rate constant of $5 \times 10^6 \text{ (mol dm}^{-3}\text{)}^{-1} \text{ s}^{-1}$ can be achieved at 21 °C, while a constant of only $7.4 \times 10^{-7} \text{ (mol dm}^{-3}\text{)}^{-1} \text{ s}^{-1}$ is achieved at 63 °C by acid catalysis of the same reaction (Price, N and Stevens, L, 1989). The remarkable catalytic power of enzymes is well illustrated by the fixation of N₂ gas to ammonia. This can be achieved by the industrial Haber process at high temperatures (700 K) and pressures (100 atms) in the presence of an iron catalyst. In contrast nitrogenase, an enzyme present in nitrogen fixing bacteria, catalyses this reaction at 300 K with a neutral pH.

2. **Specificity.** Enzymes are able to catalyse reactions with a high degree of substrate, stereo- and regio-specificity. The type and degree of this specificity varies between individual enzymes. Some are able to catalyse reactions with total selectivity for one enantiomer or one regioisomer. For example penicillin G amidase can achieve an enantiomeric excess (the ratio of one enantiomer to the other) of 100 % in the acylation of β-lactams to form an intermediate in the production of loracarbef, a cephalosporin antibiotic (Zmijewski, *Met al.*, 1991).

The milder conditions under which catalysis can be achieved using enzymes minimizes the problems of substrate or product decomposition and other side reactions. As a result the product isolation steps are often simpler than those involved in traditional chemical methods (Poulsen, P. B 1984). Furthermore the biological nature of proteins makes them a completely renewable resource and they are readily biodegradable. The current regulatory climate within the pharmaceutical industry dictates that, where a drug is a racemic mixture, pharmacological data must be presented for each enantiomer individually and for the mixture (Margolin, A, L, 1993). Many pharmaceutical companies have switched to the production of single enantiomers, while a small minority have begun to produce only achiral products (Chibata, *et al.*, 1992) . The ability of enzymes to distinguish between enantiomers is perhaps their most valuable attribute. Some of the most promising methods of producing chiral pharmaceutical compounds from prochiral substrates involves the use of enzymes (Kamphuis, *J et al.*, 1987).

One example of the use of an enzymes stereoselectivity is the production of L-amino acids. Here the stereo selectivity of amino peptidase from *Pseudomonas putida* is used to produce the L-amino acid to a high degree of optical purity. The kinetically resolved D-amino acid amide is then used to produce the D-amino acid (Elferink, V, H, M *et al.*, 1991).

It is easy to see therefore that the application of enzymes to industrial organic synthesis can result in greater product yields and the production of chiral intermediates. Nearly every type of enzyme catalysed reaction has been studied. The following sections will further outline the variety of biotransformations and the flexibility of the technology.

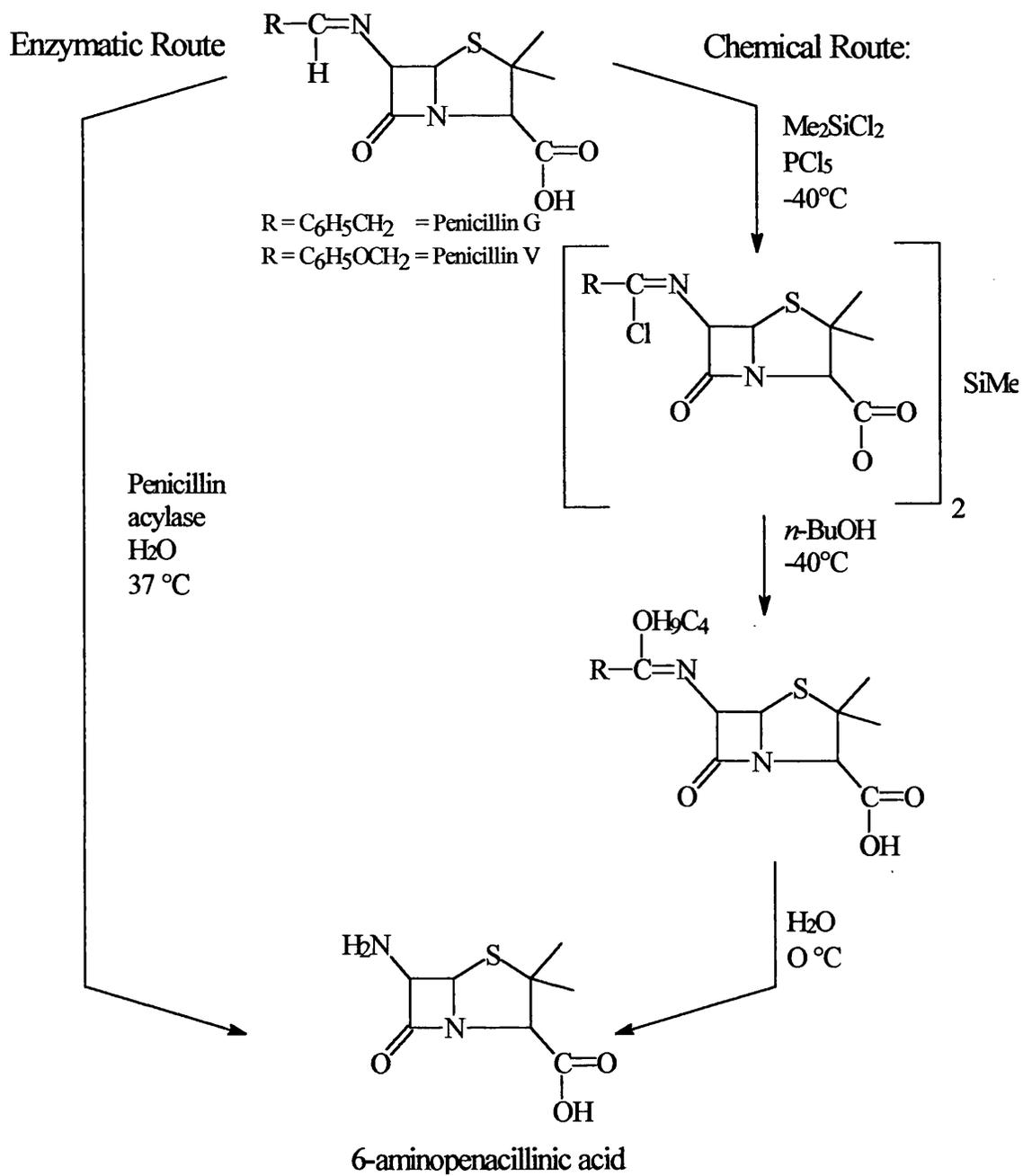


Figure 1.1. Comparison of the chemical and biological routes to 6-aminopenicillanic acid.

1.1.2 Enzyme catalysis

1.1.2.1 The mechanism of Enzyme catalysis

Any reaction must cross an energy barrier before it can occur. This barrier is known as the activation energy. Some reactions have an energy barrier so high that they cannot occur in the absence of a catalyst or without extreme environmental conditions. It is the function of any catalyst, whether biochemical or traditional, to provide an alternative reaction 'pathway' with a reduced energy barrier. This means that the catalyst cannot effect the thermodynamic equilibrium of the reaction, but will only affect the rate of the reaction. There are a variety of factors which will contribute to the reduction of the energy barrier.

- **Proximity and orientation.** The shape of an enzyme's active site allows substrate to be oriented correctly within the active centre. In the case of 2 substrate reactions this proximity can reduce the energy barrier.
- **Acid-Base catalysis.** Many of the reactions catalysed by enzymes are also known to be also catalysed by acids and/or bases as well. Since enzymes contain a large amount of amino acid side chains that can act as proton acceptors or donors it is reasonable to conclude that acid-base catalysis is important in the mechanism of enzyme catalysis
- **Covalent Catalysis.** Reactions can also be speeded up by the formation of intermediates, provided that these intermediates are readily formed or broken. Examples of intermediate formation can be found in the catalysis involving the serine side chain of serine proteases like chymotrypsin.
- **Strain and distortion.** It is possible that the binding of the substrate to the enzyme can cause a change in the conformation of the substrate. This change can lower the activation energy by making the substrate resemble the transition state more closely.

The substrate molecule(s) are able to fit within the active site where the interactions between the amino acid side chains and the substrate(s) give the enzyme its catalytic power and selectivity (Price, N and Stevens, L 1989). The nature and three dimensional conformation of an enzyme's amino acid backbone hold the shape of the

active site. The amino acids involved in substrate binding within in catalysis itself are held in place within the active site.

Some enzymes including dehydrogenases, lyases, isomerases, ligases and transferases require a non protein component for activity. This second 'helping' component is known as a cofactor or co-enzyme. There are 2 groups of cofactor; metal ions, and organic cofactors such as NADH or TPP. Some enzymes require both a metal ion and an organic cofactor, for example transketolase requires both TPP and a divalent metal ion (Lindqvist, Y, *et al.*, 1992). Cofactor requiring enzymes are inactive in the absence of their cofactors. The enzyme in the absence of its cofactor is known as the apo-enzyme. The cofactor attaches to the apo-enzyme by covalent attachment or non covalent linkage to form the enzyme cofactor complex known as the holo-enzyme.

Cofactor	Linkage to apo-enzyme	Type of reaction catalysed by holo-enzyme
Pyridoxal phosphate	Schiff's base formation to lysine residue	Transamination, decarboxylation and racemization
Biotin	Amide bond to lysine residue	Carboxylation reactions. e.g., pyruvate carboxylase
Lipoic acid	Amide bond to lysine residue	Acyl transfer
Thiamin diphosphate (TPP)	Non covalent binding	Decarboxylation
Flavin nucleotides	Non covalent and covalent binding, dependent upon individual enzyme	Redox reactions

Table 1.2 Some organic cofactors and the reactions they are involved in.

The above table demonstrates the variety of cofactors involved in enzymatic catalysis and the types of reactions catalysed by the holo-enzyme form. Some cofactors such as the nicotinamide adenine nucleotides are directly consumed in the reaction. For example, NAD⁺ is reduced to NADH during the oxidation of malate to oxaloacetate. Due to the cost of NAD(P)(H) stoichiometric quantities of the cofactor cannot always

be added to the reaction mixture. Therefore if the full variety of enzyme biotransformations are to be applied to the biotechnology industry the effective use of cofactor requiring enzymes in bioconversions must also be understood and developed.

1.1.2.2 Enzyme kinetics

In 1913 L. Michaelis and M. L. Menten developed theories on the mechanism and kinetics of enzyme action to propose a reaction scheme (1). The scheme divided the reaction into 2 steps. During the first step the enzyme (E) and its substrate (S) combine to form the enzyme substrate complex (ES). While in the second step the chemical processes occur to yield the product (P) and the unchanged enzyme. Although this is a simplification of enzyme kinetics the Michaelis Menten equation (1) is the basic model for enzyme kinetics. All other models for more complex kinetic processes such as 2 substrate reactions and equilibrium reactions are derived using the methods derived by Michaelis and Menten (Ferscht, A. 1984)

The Michaelis Menten reaction scheme:



This reaction scheme results in the following expression:

$$v = \frac{V_m \cdot [S]}{K_m + [S]} \quad (2)$$

Where:

v = the rate of change of substrate concentration or the rate of product formation,

V_m = the maximum reaction rate,

[S] = the substrate concentration, and

K_m = the Michaelis constant.

Analysis of the Michaelis Menten equation will show that the reaction rate describes a hyperbolic curve reaching a plateau at the maximum reaction rate. At low substrate

concentrations the rate of reaction is limited by the amount of substrate available to the enzyme and the binding of the substrate to the enzyme. Conversely at high substrate concentrations the rate of reaction is limited by the amount of free enzyme active sites available for the substrate to bind to. The maximum reaction rate (V_m) is a function of the rate constant of the slowest reaction step and the total concentration of enzyme, while the Michaelis constant (K_m) is treated as an apparent dissociation constant for the enzyme substrate complex. The Michaelis Menten equation can be applied to models containing diffusional resistances within solid phase biocatalyst particles and bioreactor dynamics.

1.1.3 Biocatalysis

1.1.3.1 Introduction

Biocatalysis can be defined as the application of biological activity to achieve a desired conversion under controlled conditions within a bioreactor (Tramper, J. 1996). Since enzymes display regio and stereo selection and they are catalytically powerful enough for more neutral conditions to be applied one would expect that biocatalysis would be the method of choice for much of the chemical industry. However, there is an enormous knowledge base for chemical catalysis compared to the more novel biochemical route. Consequently biocatalysis has been limited to those areas where the reaction shows stereo-chemistry or the reaction is impossible to achieve by chemical means (Elferink, V. H. M *et al.*, (1991) Katchalski-Kazir, E (1993)). In general this means that biocatalysis is applied to the production of high value low volume products in the:

- Carbohydrate chemistry
- Fine chemicals
- Pharmaceutical intermediates, and
- Semisynthetic antibiotics industries

In 1989 \$600M was spent on enzymes for use in the bioindustry, the majority of the enzymes produced were proteolytic enzymes and amylases for use in the food industry

(Arbrige, M and Pitcher, W 1989). Current use of enzymes in biotransformations are mainly limited to monomeric enzymes with simple mechanisms, namely the hydrolytic enzymes lipase and various proteases and esterases. Although more complex enzymes have been shown to produce useful products by biotransformation their industrial application has been limited. It is reasonable to deduce that this is due to the need for cofactor recycling and the cost of production and isolation of the more complex enzymes.

Developments in biotechnology, molecular biology and biochemical engineering have led to an increase in the use of enzymes as biocatalysts in chemical synthesis.

1. **Biochemical engineering.** Advances in fermentation technology have allowed high cell density fermentations and streamlined the isolation of intra- and extracellular enzyme products. This has made the production of enzyme biocatalysts cheaper and more efficient.
2. **Molecular Biology.** Advances in genetic engineering have allowed the cloning and overexpression of homologous and heterologous gene fragments in easily cultured organisms like *Escherichia coli* and *Saccharomyces cerevisiae*.
3. **Screening Technology.** Advances in the automation of screening technology have led to the isolation and characterisation of a wide variety of enzymes.
4. **Chemistry.** Advances in chemistry have seen biological catalysts being applied to novel chemical syntheses and novel biocatalysts being applied to conventional chemical reactions.
5. **Biochemical Reaction Engineering.** Advances in the development of biochemical reactors have led to a wide variety of operation strategies solving the unique engineering problems inherent with biocatalysis.

Advances in biocatalysis technology have also led to increased speculation that biocatalysis will be applied to a more broad spectrum of reactions. For example several techniques have been developed to alter the selectivity (stereo and substrate) of enzymes. The aim of this technology has been to enhance the reaction rate of the biocatalyst with non-natural substrates and/or with one enantiomer as opposed to the other. The three dimensional structure of the enzyme can be altered by chemical

modification or by genetic engineering, this has been demonstrated to allow the enzyme to react with non-natural substrates or one enantiomer (Faber, K and Franssen, M. C. R (1993)). An enzyme's three dimensional structure is also susceptible to changes brought about by non-covalent or ionic interactions. It has been demonstrated that the stereoselectivity of enzymes can be altered by altering the water content of enzymes in low water reactions (Stokes, T. M and Oehlshlager, A. C (1987)), the use of detergents (Wu S-H *et al.*, (1990)) and by immobilisation (Takahata, H *et al.*, (1992))

Other techniques used to alter the selectivity include the alteration of the substrate itself. This method includes the use of electronegative side chains and inert protecting groups. This method is often applied in cases where the chiral recognition process is dependent upon steric requirements or polarity. For example, the selectivity of *Pseudomonas* sp lipase can be altered by varying the acyl-donor involved in acyl transfer reactions. In the resolution of racemic methyl mandalate the selectivity could be increased by the alteration of the side chain length in the acyl donor (Faber, K and Franssen, M. C. R (1993)). It has also been demonstrated that 'media engineering' can also have an effect on stereo selectivity. This technique involves an alteration of the solvent system. The use of water miscible organic cosolvents can improve the selectivity of hydrolysis reactions catalysed by hydrolases (Guanti, G *et al.*, (1986)).

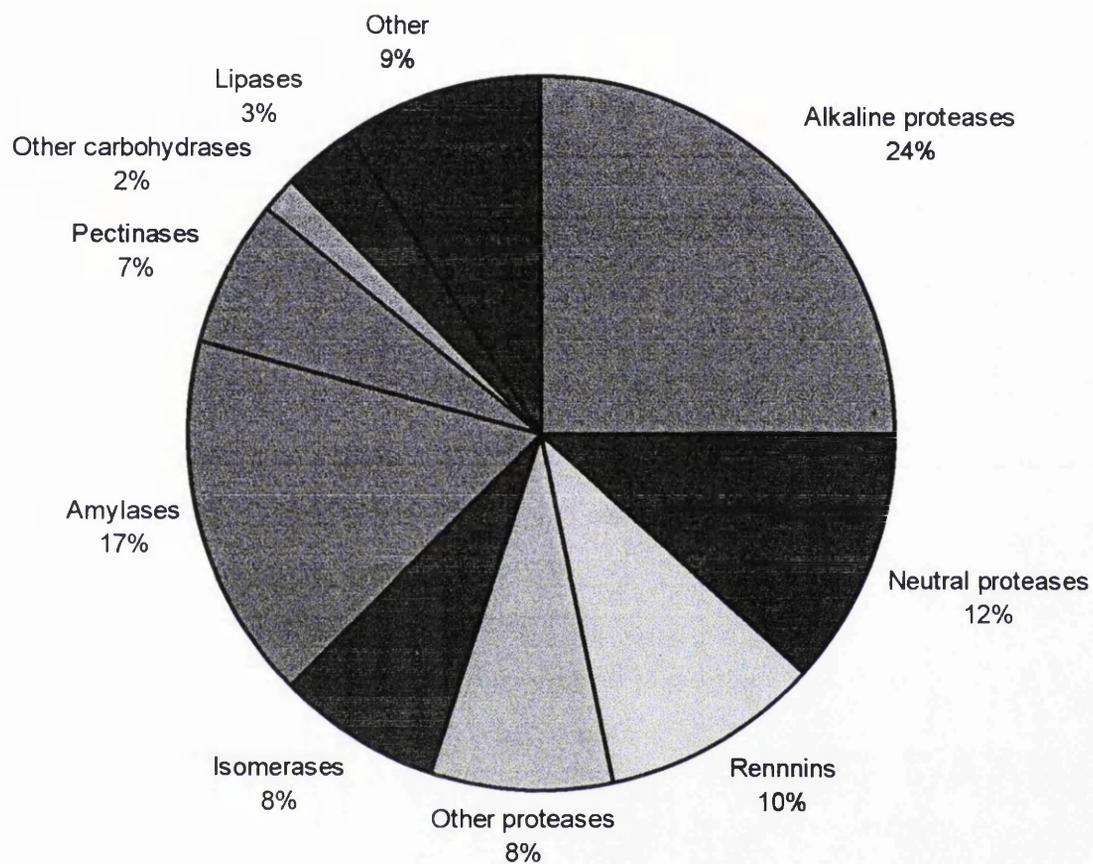


Figure 1.2 Approximation of the worldwide market for enzymes. The numbers are the approximate percentage of the worldwide market in 1989 totalling \$600M. (adapted from Arbrige, M and Pitcher, W 1989).

It is widely accepted that biocatalysis is a viable synthetic tool in the production of chiral synthons and in cases where the reaction would be impossible by traditional chemical means. It has also been demonstrated that there are a wide variety of enzymes and reactions available and that advances in supporting technologies have enabled more enzymes to be produced with higher yields. It has also been shown that the activity and selectivity of an enzyme can be altered by a variety of techniques. These advances make biochemical conversion a more attractive option in organic synthesis routes.

It is expected that these advances will have an increasing effect upon the properties of enzymes, their availability and their cost. Not only is it now possible to produce the simpler enzymes in larger quantities, but also more complex enzymes can be produced. Although new methods for the production of novel pharmaceutical compounds and novel methods for the production of old pharmaceutical compounds are available the development of this potential is dependent upon biochemical engineering as well as upon scientific developments (Mcauliffe, J and Hindsgaul, O. 1997).

Once it has been decided to use a biochemical conversion several questions need to be raised. Two of the most important questions involve the choice of the type of biocatalyst and the form that the biocatalyst should take within the bioreactor (Tramper, J. (1996)). The next section will discuss the questions involved in determining the type of biocatalyst to be used. The types of biocatalyst available:

- The advantages of each type of biocatalyst
- The disadvantages of each type of biocatalyst

1.1.3.2 Types of Biocatalyst

The selection of the type of biocatalyst is an important step in the development of a biotransformation process. The way that the biological activity is held within the bioreactor will effect the yield of the reaction, the productivity of the reaction and the economic viability of the biotransformation as a whole. There are several types of

biocatalyst available for application to specific biotransformations, as described in table 1.3.

A search through the biotransformations and biocatalysis literature will reveal that there are two major types of biocatalyst available. Namely the whole cell and the 'cell free' enzyme. The whole cell biocatalyst is easier to produce and consequently cheaper as enzyme isolation and purification is unnecessary. The cell free enzyme can be an ultrapure preparation or a crude extract. Whole cell biocatalysts are mainly used when the biological activity catalysing the biotransformation is unidentified, the cell's cofactor recycling system is required, or in a multistep biotransformation. Enzymes are normally only produced in catalytic quantities within cells and the substrate has to cross the cell membrane, as a result the whole cell biocatalyst normally has a low specific activity.

Whole cells contain the biochemical infrastructure for anabolism and catabolism and as a result many other biochemical steps can compete with or follow the desired reaction. Genetic engineering or other methods of deleting competing or ensuing activity, such as irradiation, have been employed to increase the yield of whole cell biotransformation reactions. However, due to the complexity of the cells metabolism and the antagonistic or toxic effects of some by-products cell free systems are often used. The use of a whole cell biocatalyst is also precluded when the substrate cannot penetrate the cell wall, side reactions inhibit the enzyme or are difficult to remove or when the activity is too low.

The selection of an appropriate biocatalyst must also take the characteristics of the reactants into account (Woodley, J.M and Lilly, M. D 1993). Many biotransformation reactions include one or more poorly water soluble substrates or products. In order to increase the concentration of reactant or product in the reactor, a second organic phase is often used. Enzymes have been shown to be tolerant of organic solvents and biphasic reaction media (Zaks, A and Klibanov, A. M 1985) while cell lysis can often occur at liquid liquid interfaces.. Consequently the cell free enzyme is often employed as a biocatalyst in reactions involving poorly water miscible reactants or products.

Biocatalyst	Form	Advantages	Disadvantages
Cell free enzyme.	Any.	Simple apparatus. High specific activity.	Cofactor recycling difficult. Costly to isolate and purify.
	Biphasic.	High concentration of organics possible.	Mass transfer limitations. Emulsion splitting.
	Very low water system.	concentrated organic reactants.	Low activities.
	Immobilised.	Reuse, Recovery easier. Stability can be improved.	Loss of activity. Mass transfer limitations.
Whole cell.	Any.	No cofactor recycling needed. Cheap to produce.	Low tolerance to organic solvents. Side reactions. Transfer of substrates across cell membrane.
	Resting.	Fewer by-products	Low specific activity.
	Immobilised.	Reuse, Recovery easier. Stability can be improved.	Mass transfer limitations. Low specific activity.

Table 1.3 The various types of biocatalyst available showing their advantages and disadvantages.

In order to select the appropriate type of biocatalyst some information about characteristics of the reaction and the biocatalyst is necessary.

- **Is the enzyme known?** In some cases the actual enzyme that catalyses the reaction is unidentified, in such cases the whole cell biocatalyst must be used.
- **Intra- or extracellular enzyme.** If the enzyme is extracellular the cell free enzyme must be used. While if the enzyme is intracellular the cost of isolation and purification must be balanced with the yield in activity.
- **Cell wall permeability.** Will the substrates or products penetrate the cell wall? Can the cell wall be made more permeable? If not then the cell free enzyme must be used in order for the reaction to take place.
- **Cofactor recycling.** Does the reaction require a cofactor, and does it need to be recycled? Although cell free cofactor recycling techniques are available, it is cheaper and easier to employ the cofactor recycling infrastructure of the whole cell.
- **Reactant and product solubility.** Is it necessary to use a 2 phase system? In which case it becomes important to know the stability of the cell free enzyme in organic solvents and in the presence of a liquid liquid interface.
- **Stability of the product in the presence of the whole cell.** Can a complete mass balance be achieved with the whole cell, or is the product involved in further metabolic steps? Is it possible to prevent this activity?

The answers to the above questions will enable the selection of either the whole cell or the cell free enzyme as the biocatalyst. It should be noted that due to advances in genetic and protein engineering it has become possible to alter the characteristics of the cell or the enzyme itself. These facilitating techniques broaden the scope or versatility of the various biocatalyst types. One such method of altering the enzyme is immobilisation. Although it is possible, and in some cases prudent to immobilise whole cells the next section will discuss the advantages of immobilisation of enzymes.

1.2 Immobilisation

1.2.1 Introduction

1.2.1.1 Background to immobilisation

Immobilisation has been defined by Gemenier, P (1992) as the localisation of biological activity inside a defined region of space within the bioreactor, with retention of activity so that the enzyme can be reused. In practice this has been achieved by linking the enzyme onto the surface of or within a solid matrix. Therefore the enzyme is no longer dispersed within the bulk phase, conversely the it results in a solid catalytic particle. This allows the biocatalyst to be recovered from the product stream, retained within the reactor and reused. The technique alters the characteristics of the biocatalyst and confers many advantageous properties.

- Reclamation of the enzyme from the product stream by physical means.
- Retention of the activity within the bioreactor, therefore allowing recycle or continuous reactors to be employed.
- Reuse of the biocatalyst, either in a continuous reactor, or in several discrete batches.

A glance through the literature will demonstrate that a wide variety of enzymes have been immobilised to a wide variety of support materials. The use of immobilised enzymes stretches from the food and pharmaceutical industries (Keller, R. *et al.*, (1987)) to biosensors (Fortier, G. *et al.*, 1992). The supports vary from controlled pore glass, natural polymers such as agarose (Guisan, J. (1988)) to synthetic polymeric materials (Raghyram Reddy, C. *et al.*, (1986)). Enzymes have been immobilised for a variety of reasons ranging from fundamental studies on enzyme kinetics or structure (Chan, W. *et al.*, 1973), to biocatalyst production (Keller, R. *et al.*, (1987)). This thesis will concentrate upon the immobilisation of enzymes for biotransformation processes, although it should be noted that enzymes can be immobilised for the reasons outlined above.

The expense of enzymes is one of the most important factors determining their use in technological processes (Poulsen, P. D 1984 and Rosevear, A 1984). Although DNA technology has provided a stable source for the hyperexpression of many enzymes the often costly problem of enzyme isolation still remains. The water soluble enzyme is often a contaminant of the product stream. This can result in increased cost of product isolation and/or a loss of some product during protein extraction. Secondly the presence of the enzyme within the product stream may be antagonistic to the product isolation procedure, this is especially important in cases where *in situ* product removal is employed. In cases where the enzyme is still active after the reaction it can be more cost effective to reuse the activity. The reuse of the enzyme is especially essential in cases where the enzyme is complex and difficult to produce and isolate.

Enzyme	Process
Glucose isomerase	Fructose enriched syrup.
Thermolysin	Ethylacetate production in aspartame synthesis.
Penicillin amidase	6-amino penicillanic acid (6-APA)
Aminoacylase	Resolution of racemic amino acids

Table 1.4 Examples of biotransformation processes using immobilised enzymes.

The variety of enzymes that have been immobilised and successfully to the food and pharmaceutical industries can be seen from the examples listed in table 1.3 above. Penicillin amidase is perhaps the most famous example of the application of an immobilised enzyme in the pharmaceutical industry. Initially cell suspensions were used to catalyse the hydrolysis of penicillin G on a single batch basis. The productivities achieved in this manner were very low, approximately 0.5 - 1 kg 6-APA / kg *E.coli* suspension. The productivities of the process has been enhanced by immobilisation of the enzyme. Productivities ranging from 500 to 2000 kg 6-APA / kg enzyme have been reported (Poulsen, P. D (1984)).

The impact of such an increase in productivity is well demonstrated by the resolution of racemic amino acids by immobilised aminoacylase. Aminoacylase catalyses the stereoselective hydrolysis of a chemically synthesised acylamino acid to yield the L-

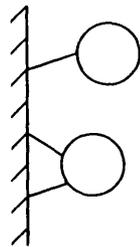
amino acid and leave the unhydrolysed D-acylamino acid. Immobilised onto DEAE Sephadex and the use of a continuous enzyme bioreactor allowed a 40% reduction in overall operating costs in comparison with the batch process using the soluble native enzyme (Chibata, I. Tosa, T and Shibata, T (1992))

Immobilisation of the biological activity can therefore often result in a more efficient reaction with much greater productivity and consequentially a reduction in the process cost. To date the technique has been used to augment a biotransformation process rather than enable one. The next section will describe and discuss the variety of techniques by which an enzyme can be immobilised.

1.2.1.2 Methods of Immobilisation

Enzymes can be attached to the surface of a support material or confined within a porous material. The complex must be stable enough to allow the repeated use of the enzyme and must allow the biocatalyst to be retained within the bioreactor by physical means.

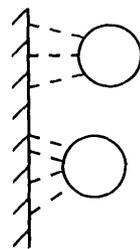
- **Attachment to the surface of the support.** This technique utilises covalent, ionic or physical forces to attach the enzyme to the surface of a support material. Since the activity is attached to the surface of the support the amount of diffusional resistances is reduced. However, the process of attachment to the surface of a solid surface can, in principle result in changes in the functioning of the enzyme. Surface attachment can be achieved by adsorption or binding the protein covalently to the support material.
- **Entrapment within a matrix.** The catalyst is enclosed behind a membrane or within a gel structure. Although this method is unlikely to cause direct changes in the structure of the enzyme interphase mass transfer often reduces the activity of this type of biocatalyst. Secondly conditions employed to entrap the enzyme are often stressful and result in some denaturation.



Covalent Bonding

Strong binding by single/few attachments.

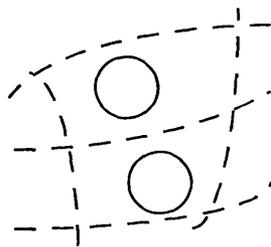
- + long term stability in aqueous solvents
- activity loss by interaction with protein structure or active centre



Non-covalent Bonding

Multiple binding through ionic, hydrogen, or hydrophobic interactions.

- + high activity retention
- + good performance in organic solvents
- binding strength is solvent dependent



Inclusion

Incorporation into polymeric network or membrane device.

- + No interaction with enzyme surface
- leaching
- polymerisation reaction can denature enzyme

Figure 1.3 Schematic diagram showing the variety of methods by which an enzyme can be immobilised.

During gel entrapment the biocatalyst is distributed within a porous three dimensional support matrix. Due to the aqueous solubility of enzymes hydrogels are normally used to entrap the enzyme. Hydrogels are easily wetted and have a high porosity to polar solutes. The actual entrapment of the enzyme can take place in 2 ways. The gel can be polymerized with the enzyme present, consequently producing a very stable gel, with the enzyme evenly distributed. However, the polymerization conditions will often severely deactivate the enzyme. The gels can also be formed from pre-existent polymers, however, the gels formed in this manner often have poor physical properties.

It should be noted that the term adsorption is used in the broad sense and includes all the interactions by which the protein is held at a solid liquid interface by non-covalent means. The term adsorption will include van de Waal's forces, hydrophobic interactions, ionic binding and metal chelation (Norde (1986)). The specificity of this adsorption can vary between general interactions to the interactions between biochemical surfaces. Adsorption can occur spontaneously therefore the enzyme need only be brought into contact with the solid surface. However, as described by Norde (1986) adsorption is highly susceptible to changes in the ionic environment and pH. Consequentially desorption of the enzyme can occur due to changes in either the micro- or macro-environment around the enzyme. As a result if the possibility of desorption it is inappropriate to immobilise by this method alone in cases where the pH or ionic environment will change during the course of the reaction.

Although adsorption is sufficient to bind a protein to a solid support the susceptibility of the method to desorption means that adsorption alone is insufficient to produce a stable immobilised enzyme. Crosslinking the adsorbed enzyme with a bifunctional agent such as glutaraldehyde will often prevent desorption and result in a stable source of activity.

A stable unassailable bond between the enzyme and support can be achieved through the formation of a covalent linkage between the two.. Covalent immobilisation has the advantage of being able to produce a permanent bond between the enzyme and support over the whole range of operating conditions. Since several pre-existing and pre-activated support materials are available commercially, covalent binding is the most

widely studied and applied method of immobilisation. Methods that use pre-activated supports are the simplest to use, however, techniques by which existing polymer matrixes are activated by chemical alteration of the polymer backbone (Guisan, J. 1988) or by coating with bifunctional agents have been reported (Carleymith, S. 1977).

Since the interaction between the enzyme and the support takes place at the surface of the polymer this method of immobilisation minimizes mass transfer limitations. In comparison entrapment methods suffer from mass transfer limitations. Table 1.5 compares the methods of immobilisation listing their advantages and disadvantages.

Method	Mass Transfer	Leaching	Inactivation
Adsorption	+	-	-
Covalent binding	+	+	-
Entrapment	--	+	-

Table 1.5 A comparative analysis of the methods of immobilisation.

Because covalent immobilisation produces the most stable interaction between the enzyme and its support its application is advantageous in the pharmaceutical industry where the leaching of protein into the product stream may be hazardous. Covalent immobilisation also has the advantage over entrapment of minimizing mass transfer limitation because the enzyme is not held within the matrix. As a result of these two major advantages of covalent immobilisation this thesis will concentrate upon this method.

1.2.2 Covalent Immobilisation

1.2.2.1 Description of Binding Chemistries

Several methods of covalently attaching proteins to the surface of solid supports have been developed and reported. Most methods have been derived from carbohydrate and protein chemistry. A glance through a biochemistry text book will yield a list of the variety of functional groups that will bind to various amino acid side chains on a proteins surface. Most methods involve nucleophilic attack of a functional group on

the support by amino, thiol, hydroxyl or phenolic side chains on surface amino acids. Like all nucleophilic reactions the speed of the reaction will be dependant upon the pKa of the attacking group and consequently the pH of the environment (Blanco, R. *et al.*, (1989)) .

Since this method requires the direct modification of the proteins primary structure, by chemical modification of amino acid side chains the selection of appropriate binding chemistries is important. Several methods of coupling have been reported in the literature and are described in table 1.6 below.

It is evident from table 1.6 that a wide variety of coupling chemistries are available. The selection of an appropriate binding method will be dependent upon several important criteria. Firstly, the functional group on the support must not be able to react with substrates or products. In order to minimize the amount of inactivation during the coupling process the reaction must take place under conditions that are not antagonistic to the protein. Finally the bond must be stable under all possible operating conditions.

Several preactivated support materials are commercially available for the immobilisation of protein. Suppliers such as Rohm, Sigma, Pharmacia and BioRad have support materials available for direct coupling. Ion exchange resins and controlled pore glass beaded materials are also commercially available for indirect coupling. Indirect coupling can be achieved by coating the bead with bifunctional agents such as gluteraldehyde or by the introduction of a functional group by derivitization of the material. Indirect coupling allows a greater flexibility of binding chemistries consequentially the material and the binding reaction can be separated. Also this method allows a greater degree of control over the degree of activation. Surface effects can also be reduced by the introduction of carbon chains imbetween the support before activation. However, these advantages must be balanced with the cost and complexity of the activation process.

Technique	Target group on protein	Reaction pH	Comments
Cyanogen bromide.	-OH -NH -SH	>8	<ul style="list-style-type: none"> • Some leaching of protein possible • Method produces cyanide as byproduct • Short spacer • Preactivated supports commercially available
Activated double bonds.	-SH -NH ₂ -OH	>8	<ul style="list-style-type: none"> • Rate highly dependant upon pH
Vinylketo groups	-SH -NH ₂ -OH	3 - 10	<ul style="list-style-type: none"> • Undesirable side reactions
Activated Aldehyde	-SH -NH ₂ -OH	>8	<ul style="list-style-type: none"> • Requires reduction step to 'fix' protien
Epoxides	-SH -NH ₂ -OH	3 - 12	<ul style="list-style-type: none"> • Rate and target group effected by pH • Commercially available
Gluteraldehyde	-NH ₂	>8	<ul style="list-style-type: none"> • Support requires activation

Table 1.6 Comparison of the characteristics of the variety of covalent coupling chemistries

1.2.2.2 Support Materials

There are a wide variety of support materials available that have been applied to the immobilisation of enzymes for biocatalytic purposes. The types of support available can vary between naturally occurring polymers such as agar, dextran or cellulose and inorganic materials such as glass and silicone. The classes of material available can be seen in table 1.7. Since the support material will create a second phase within the reactor and will be in direct contact with the enzyme and its reactants and products its physical form and physico-chemical properties are important. Finally the most important criteria in the selection of an appropriate support material is probably the cost and availability of the material.

Support	Diameter (μm)	Spacer length (Carbon atoms)	Coupling group	Supplier	Comment
Eupergit-C [®]	250	12	Epoxide	Röhm	• Slow
CNBr-Sepharose	45-165	1	CNBr	Pharmacia	• Release of a CN
ECH-Sepharose	45-165	9	COOH	Pharmacia	• Very stable a • Requires carbodiimide
XAD7- Gluteraldehyde	450	2	-CHO	Röhm and Haas Ltd	• Requires activation
Active CH- Sepharose	45-165	6		Pharmacia	• Requires a carbodiimide
Epoxy-Sepharose	45-165	12	Epoxide	Pharmacia	• Slow a

Table 1.7 Comparison of some commercially available support materials.

The support materials can be roughly divided into 2 classes namely, organic polymers and inorganic compounds such as glass or celite. The organic polymeric supports can be further subdivided into naturally occurring polymers and synthetic polymers (table 1.8). The biological polymers, such as cellulose, starch, agarose and dextran were originally the most popular support materials. In the early work on immobilisation, agarose and dextran based gels were most often used due to the high susceptibility of cellulose and starch to microbial attack (Porath, J and Axen, R (1976)). Polymer chemistry has led to the development and derivitisation of several synthetic polymers for the covalent immobilisation of enzymes. Materials such as polystyrene, polyacrylamide (Johansson, A-C, and Mosbach, K (1974)) and polyurethane (Wand, X and Ruckenstein, E (1993)) have been successfully used as supports.

Biological Polymers	Synthetic Polymers	Inorganic Materials
Dextran	Polyacrylamide	Glass
Agarose	Polystyrene	Celites
Cellulose	Polyurethane	
Starch		

Table 1.8 Materials used as supports for the immobilisation of enzymes.

In order to be viable immobilisation supports the materials need to have several characteristics.

- Inert in the bioreactor environment
- Chemically and biologically stable
- Physically durable
- Easily derivitisable

The shape, size, porosity and mechanical stability of the material will influence the properties of the final biocatalyst. Most polymers, whether natural or synthetic are available in a variety of shapes and sizes. For example cellulose is available as a fibre, spherical particles, microgranules, membranes and a variety of morphologies

(Gemenier et al., 1993). The shape of the support material can determine the kinetic parameters of the biocatalyst by affecting the mass transfer properties of the biocatalyst. The shape of the material can also influence the mixing and hydrodynamic properties of the material. Microgranular materials do not possess good flow properties, as a result the pressure drop along the length of the bed increases rapidly as the bed size is increased. As a result bead shaped materials are often preferred (Gemenier *et al.*, 1993).

Small materials exhibit lower transfer resistances than larger ones because the substrate doesn't have to travel as far and the surface area is greater (Carleysmith 1977). However, separation of the biocatalyst from the product stream can become more expensive for smaller materials. Larger materials can be segregated by coarse filtration while the cost of separation increases with the reduction in size. The smallest particles require ultrafiltration or centrifugation for isolation from the product stream. Small particles are easier to mix in stirred tank reactors, however, they often yield high pressure drops along packed bed bioreactors. Highly porous materials allow more enzyme to be bound, therefore the activity density is greater, however, the highly porous materials are subject to greater mass transport resistances.

1.2.3 Biocatalyst Development

The selection of an appropriate form for the biocatalyst is an important factor in the development of the biocatalyst. Once the enzyme has been isolated and characterised a variety of options are opened up. The enzyme can be present in the bioreactor in soluble form. In this case the enzyme can be removed from the product stream or maintained in the bioreactor by ultrafiltration. Although membrane technology is advanced and several laboratory scale bioreactions have been successfully performed in membrane bioreactors few commercial conversions use this technology. Ultrafiltration has also been used simply to remove the enzyme from the product stream. In this situation the enzyme cannot be reused, and as such the enzyme productivity is low.

As previously outlined the enzyme can be immobilised onto a solid support, this changes the properties of the enzyme to make it physically large and robust enough to

be removed from the product stream and reused. The cost of attaching the enzyme to the solid support and the development of the immobilised enzyme must be balanced with the economics of reusing the enzyme. In the situation where the enzyme is costly immobilisation will often make the process economically viable. However, in the current climate of genetic engineered protein and advances in fermentation technology may mean that immobilisation is no longer necessary on economic grounds alone (Poulsen 1984).

1.3 Transketolase

1.3.1 Introduction

Transketolase, D-seduheptulose-7-phosphate: D-glyceraldehyde-3-phosphate glycolaldehyde transferase, [2.2.1.1] is a dimeric protein with a molecular weight of around 130 - 170 kDa. In *vivo* the enzyme is involved in the pentose phosphate pathway. Transketolase has been successfully isolated from yeast, spinach, pig (Schneider *et al.*, 1992) and on a large scale from *E.coli* (Hobbs *et al.*, 1995). The enzyme catalyses the reversible stereospecific transfer of a two carbon ketol group to an aldol acceptor. The enzyme requires two cofactors; thiamine pyrophosphate and a divalent metal ion for full activity. The thiamine pyrophosphate binds to the inactive enzyme, the inactive enzyme is known as the apo-enzyme. The apo enzyme has two thiamine pyrophosphate binding sites (one for both subunits). The active enzyme containing thiamine pyrophosphate is known as the holo-enzyme.

The enzyme displays a very broad substrate specificity, for example, it has been shown to be able to use three carbon and seven carbon aldehydes (Schneider *et al.*, 1991), although cyclic aldehydes are very poor substrates, probably due to steric hinderances preventing their access to the active centre (Demuyck *et al.*, 1991). Although aldolase displays a similar activity to transketolase, both substrates involved in aldolase catalysis must be phosphorylated. Transketolase, on the other hand, does not require phosphorylated substrates (Villafranca and Axelrod 1971). Transketolase has been successfully applied to the synthesis of heptulose (Villafranca and Axelrod 1971), seduheptulose (Dalmas and Demuyck 1993), and several optically pure L-2-hydroxyaldehydes (Effenberger *et al.*, 1992). If hydroxypyruvic acid is used as the

donating ketol yields greater than 50 % can be routinely achieved (Kobori *et al.*, 1992).

1.3.2 Structure and Mechanism

Transketolase is a dimeric protein, both subunits are identical and contain a binding site for TPP and for the substrate. The binding site is made up of amino acids from both subunits, meaning that transketolase monomers cannot be active (Lindqvist *et al.*, 1992). In fact it has been observed that most of the intra-domain interactions in the yeast enzyme are present inside or close to the active site. TPP binds to the apo-enzyme at a tryptophan side chain in the active centre, this results in the ionisation of the C2 carbon on TPP making it a potent nucleophile. The electron is donated to the ketol donor making preparing it to transfer the hydroxyketo group to the accepting aldehyde (Schnieder *et al.*, 1991). The chirality is introduced at the accepting aldehyde moiety, which must contain a hydroxyl group in the S position. The thiamine pyrophosphate is consequently heavily involved in catalysis and is subsequently essential for the enzyme to display any activity.

The active centre of the enzyme is not large enough to accommodate both substrates simultaneously (Lindqvist *et al.*, 1992). As a result it is likely that the reaction mechanism follows a ping pong bi bi type mechanism. Kinetic data obtained by Gyemarrah *et al.*, 1996 also suggests that the reaction scheme follows that type of mechanism. The ping pong bi bi mechanism indicates that the first substrate enters the active site and complexes with the enzyme forming the first product. Only after this complex has been formed can the second substrate enter the active site to successfully form a second enzyme-substrate complex thereafter releasing the product and the enzyme. This would suggest that the reaction mechanism is as shown in figure 1.4.

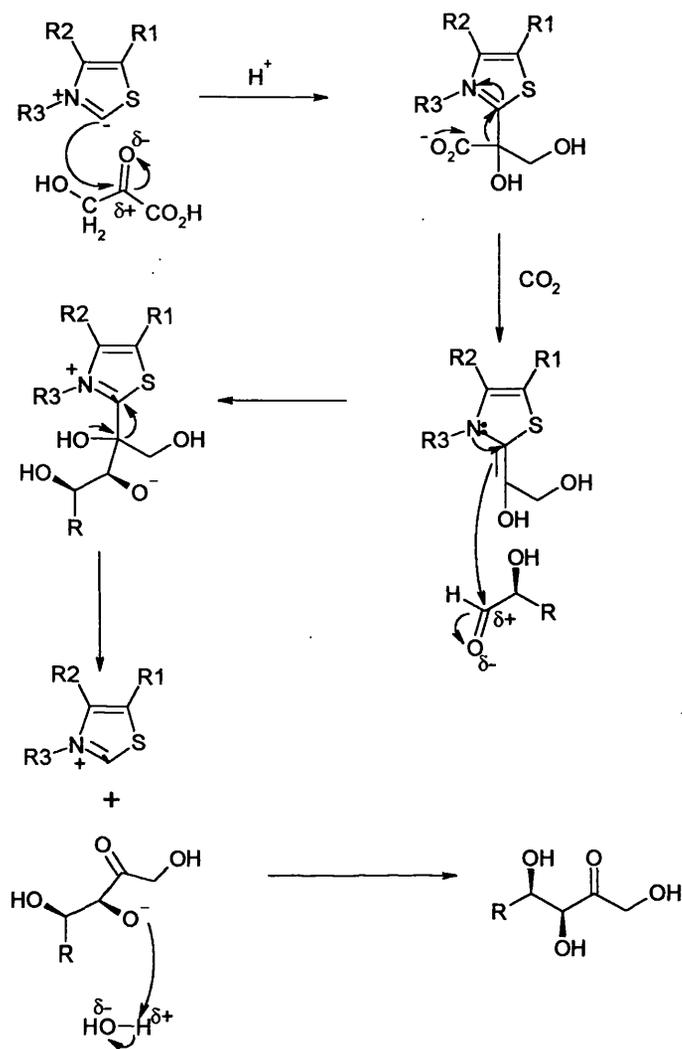


Figure 1.4 Transketolase reaction mechanism, showing the involvement of TPP in catalysis.

1.3.3 Model Reaction

The general transketolase reaction scheme is reversible. As a consequence complete conversion of substrate to product cannot be achieved. This would have several ramifications if the enzyme was to be used in the pharmaceutical industry as a biocatalyst. For example, the product stream will also contain substrate, as a result down stream processing will be more complex. The selection of hydroxypyruvic acid as a ketol donor will release carbon dioxide as the co-product of the reaction. The carbon dioxide can take no further part in the reaction and as a result the reaction becomes irreversible (Dalmas and Demuynck 1993). It is likely that most industrial reactions utilising transketolase will use hydroxypyruvic acid as the ketol donor to

overcome the problems associated with equilibrium, and most synthesis routes using transketolase described in the literature involve hydroxypyruvic acid as the ketol donor. The production of carbon dioxide will also mean that there is no soluble co-product which must be removed from the product stream, as a consequence the downstream processing costs of the bioreaction will be reduced.

Several aldehyde acceptor molecules have been tested with the transketolase and hydroxypyruvic acid system. The most reactive aldehyde has been shown to be glycolaldehyde (Dalmas and Demuyck 1993). The reaction between hydroxypyruvic acid and glycolaldehyde is shown in figure 1.5 below. The reaction, which yields L-erythrulose and carbon dioxide as products, proceeds with the net loss of one proton. This is not an unusual situation, several commercial bioreactions proceed with a pH change. Probably the most notable of these is the production of 6-amino penicillanic acid from penicillin by penicillin acylase. Since both reactants are unstable in most of the buffers tested the reaction is most often performed without buffers and the pH change can be overcome by the concomitant addition of acid.

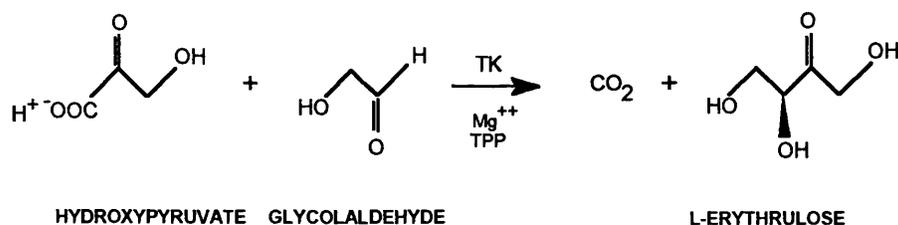


Figure 1.5 The molel reaction scheme between hydroxypyruvic acid and glycolaldehyde yielding L-erythrulose and carbon dioxide catalysed by transketolase.

1.5 Aim and Course of Study

The nature of immobilisation has been studied on a fundamental level and it has been demonstrated that a wide range of enzymes can be successfully immobilised. It has also been widely demonstrated that many enzymes can be stabilised by immobilisation, although this is by no means a general rule. This subsequently means enzymes may be applied to reactions and/or operated under conditions where the enzyme would not normally remain active. Consequentially some enzymes that would normally be inapplicable in a biotransformation process may be utilised in this way. Therefore immobilisation can offer specific advantages in the design of a biocatalyst. The aim of biocatalyst design is to produce the biocatalyst with the most effective properties for the biotransformation. If this biocatalyst is to an immobilised enzyme it will have to be stable and active under the biotransformation conditions.

The literature has demonstrated that immobilised enzymes can have a wide variety of advantageous and disadvantageous properties, and that these properties are largely dependant upon the selection of support material, the selection of active group and the procedure of immobilisation. Improvements in genetic engineering and fermentation technologies have lead to reduction in the prices of enzymes. Therefore immobilisation will cease to be necessary in order for many bioconversion processes to break even. However, it is possible that immobilisation can result in other advantages to the biotransformation process.

- Stabilisation of activity, therefore prolonging operation. This may not be necessary for the process to break even it may be advantageous on other grounds.
- Alteration of activity properties of the enzyme. For example to allow different internal and external pHs, temperatures or substrate concentrations and consequently shift equilibrium or prevent substrate inhibition. - characterisation of reaction necessary in order to determine the most important property of the immobilised biocatalyst.
- Integration with DSP operation, ie., removal of protein from product stream and consequential the reduction of DSP costs. - characterisation of down stream

processes to determine whether immobilisation is necessary and what is necessary ie, no cofactor in product stream, no enzyme, alteration of activity in order to change product stream.

Although, immobilisation is a 'black art' on a fundamental level and the 'science' of immobilisation has been reduced to a number of 'tricks' that improve the activity of the enzyme, there are some general rules that deserve to be highlighted. It is recognised that on a basic level the functional group, the enzyme challenge, the binding chemistry and the binding conditions are important in maintaining activity of the bound enzyme (by whatever means, eg., conformation change, enzyme distribution, amino acid involvement) and rigidifying the 3d structure of the enzyme. While the mechanical stability of the support is important for the operating conditions and reuse of the support. Finally, support chemistry is accepted as being instrumental in affecting the characteristics (such as pH profile, substrate penetration) of the final biocatalyst.

Subsequently if a rational biocatalyst design is to be applied to the question how the enzyme is to be immobilised and to understand whether an enzyme is to be immobilised in order to successfully integrate the biocatalyst with the whole process as well as the bioreactor itself it is important that:

- enzyme load
- functional group selection
- binding environment, and
- support stability

are investigated with respect to the biotransformation reaction itself and with the unit operations up and downstream of the bioreactor itself. It is the aim of this research therefore to investigate the development of an immobilised transketolase biocatalyst for carbon carbon bond synthesis.

The following areas will be investigated:

- The characterisation of the free enzyme, the support and the interaction between the enzyme and its support material in order to enable the selection of immobilisation conditions and binding reaction.
- The characterisation of the binding of cofactor to the enzyme and an investigation into the factors influencing the decision when to add the essential cofactor to the biocatalyst.
- The characteristics of the immobilised biocatalyst itself will be studied, in an attempt to investigate the advantages obtained by immobilising the enzyme.

These will be investigated using two commercially available support materials. Eupergit-C[®] and Amberlite XAD7. Both materials are available on a large scale and have a history of use in biocatalytic applications. An attempt to compare these support materials will also be made.

Materials and Methods

2.1 Analytical Methods

2.1.1 High Performance Liquid Chromatography

2.1.1.1 HPLC Equipment

The standard assay for the biotransformation reactants and products was a HPLC method. The HPLC system consisted of an ISS-100 autosampler as the master control unit (Perkin-Elmer Ltd., Beaconsfield, Buckinghamshire, England) with a Series 200-LC pump as the slave unit (Perkin-Elmer Ltd.). Detection of the separated components was carried out by refractive index using a Shodex RI-71 detector (Showa Denko K.K., Japan). Data collection and integration were performed on a PC driven Prime version 2.2.3 chromatography data station (HPLC Technology Ltd., Macclesfield, Cheshire, England).

2.1.1.2 HPLC Assay

The HPLC assay to quantify the concentrations of β -hydroxypyruvic acid, glycolaldehyde and L-erythrulose was performed using the methodology reported by Mitra and Woodley (1996). Separation of the various components was achieved on two Aminex HPX-87H columns in series (dimensions 300 x 7.8 mm, supplied by Biorad Ltd., Hemel Hempstead, Hertfordshire, England). The column temperature was maintained at 65°C in a Model 7970 Block Heater (Jones Chromatography Ltd., New Road, Hengoed, Mid Glamorgan). Separation was performed at a flow rate of 0.45 mLmin⁻¹ in 6 mM sulphuric acid (H₂SO₄) as the mobile phase. The retention times and sensitivities for the reaction components using the usual sample volume of 10 μ L are displayed in table 2.1. Samples from the biotransformation were diluted in 6 mM sulphuric acid to a suitable concentration, not exceeding 5 mM and passed through a 0.2 μ m filter prior to analysis. The peak areas are related to the concentration by the standard curves shown in figure 2.1. All points displayed and

used in calculating the enzyme rates were the mean of 2 duplicate, the standard error of the assay was found to be within 10 %.

	Retention time/ min	Minimum Detectable Concentration/ mM
L-Erythrulose	30.8	0.05
β -Hydroxypyruvic acid	21.5	0.05
Glycolaldehyde	32.4	0.1

Table 2.1 The retention times and minimum detectable concentrations of the reaction components in the HPLC assay.

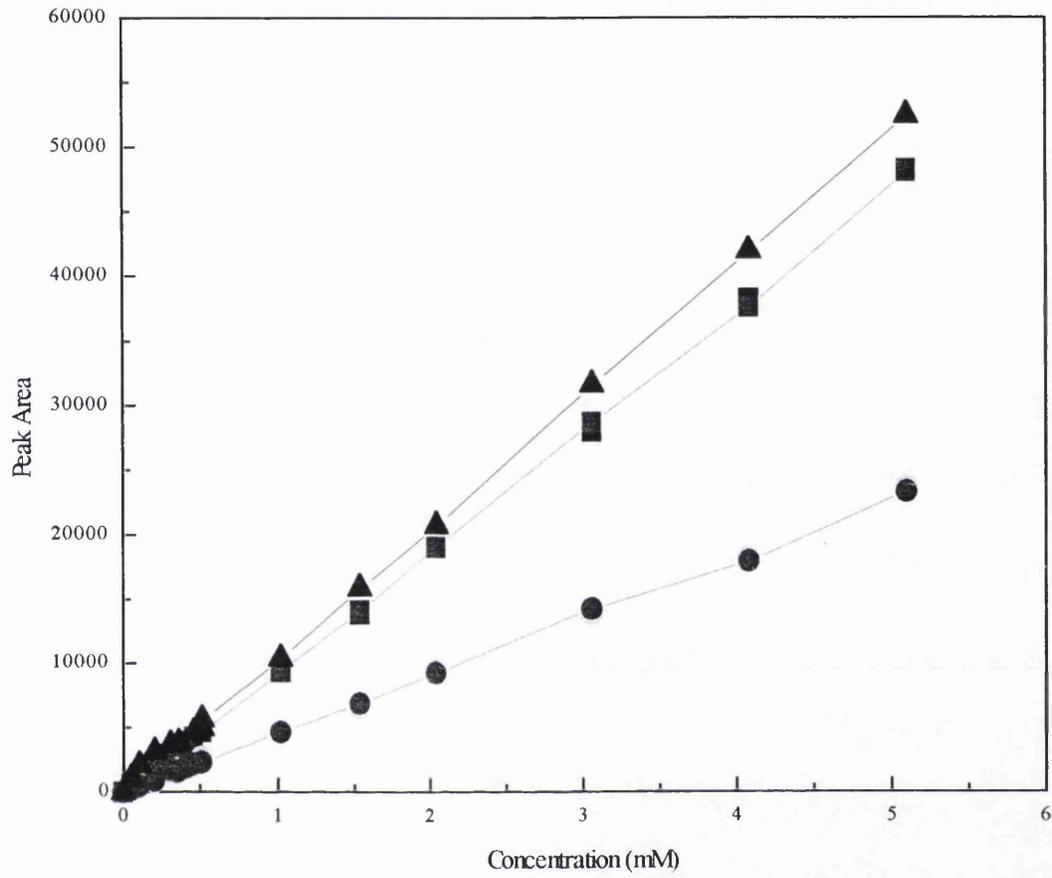


Figure 2.1 Calibration curve for the HPLC assay. Showing the correlation between peak area and hydroxypruvic acid (—■—) erythrose (—▲—) and glycolaldehyde (—●—) concentrations.

2.1.2 Spectrophotometric analyses

2.1.2.1 Spectrophotometer

All spectrophotometric assays were carried out on either a Kontron Uvikon 922 spectrophotometer (Kontron Instruments Ltd., Watford, Hertfordshire, England) or a Unicam UV2 spectrophotometer (Unicam Ltd., York Street, Cambridge, England).

2.1.2.2 Multi-Enzyme Linked assay for Transketolase

In order to quantify the amount of transketolase activity a variation of the linked enzyme spectrophotometric assay developed by Vilafranca and Axelrod (1971) was used as reported by French and Ward (1995) and Mitra and Woodley (1996). The assay links the activities of α -glycerophosphate dehydrogenase, triosephosphate isomerase, phosphoribose isomerase and D-ribulose-5-phosphate-3-epimerase with transketolase. The quantity of transketolase present was measured by the decrease in reduced nicotinamide adenine dinucleotide (NADH) concentration. The components of the assay were added in the following amounts to the final assay volume of 1.5 mL: 100 mM glycyl glycine buffer, pH 7.6 containing 0.5 mgmL⁻¹ bovine serum albumin (BSA), 0.25 mM thiamine pyrophosphate (TPP), 9 mM magnesium chloride hexahydrate (MgCl₂ · 6H₂O), 0.145 mM reduced nicotinamide adenine dinucleotide (NADH), 0.2 UmL⁻¹ α -glycerophosphate dehydrogenase-triosephosphate isomerase, 0.2 UmL⁻¹ phosphoribose isomerase and 0.2 UmL⁻¹ D-ribulose-5-phosphate-3-epimerase, a 50 mL of a suitably diluted sample of transketolase and 3 mM ribose-5-phosphate (added last). After the addition of the sample containing transketolase the enzyme was allowed to form its active complex by equilibration for 10 minutes, after which the reaction was initiated by the addition of the ribose-5-phosphate. The oxidation of NADH was monitored spectrophotometrically at 340 nm and equated to the amount of transketolase as outlined by Hobbs (1994). Assay conditions were 35°C and pH 7.6. All components of the assay were supplied by Sigma-Aldrich Company Ltd. (Poole, Dorset, England). All points were the mean of triplicate samples, the average standard deviation of the assay was found to be 15 %.

2.1.2.3 Protein Assay

Biorad protein assay reagent was diluted 1/5 with reverse osmosis water (RO water). The diluted dye was then filtered to 0.45 μm to remove particulate material. A 30 mL sample containing protein was added to 1.5 mL of the filtered Biorad reagent. This mixture was allowed to stand for at least 10 minutes after which the absorbance at 595 nm was measured. The value obtained was compared with the calibrated values obtained for BSA to quantify the amount of protein present in the sample.

2.2 Biotransformation Components

2.2.1 Transketolase and Cofactors

Transketolase was released from *E.coli* JM107 / pQR700, in which the enzyme is expressed at high levels (French and Ward, 1995; Hobbs et al., 1995). The centrifuged cells were resuspended at 12.5% w/v in 1M phosphate buffer at pH values ranging from 6 to 9. The cell extracts were prepared by homogenising the resuspended cells for 4 passes at 1200 bar in a Lab 40 Homogoniser (APV-Gaulin GmbH, Mecklenstrasse 223, Lübeck, Germany). The cell extract was then clarified by centrifugation at 13000 rpm for 5 minutes to yield clarified enzyme solutions containing approximately 250 U mL^{-1} transketolase and 20 mg mL^{-1} protein. Both thiamine pyrophosphate chloride (also known as cocarboxylase) and magnesium chloride hexahydrate were supplied by Sigma-Aldrich Company Ltd.

2.2.2 Reactants and Products

Glycolaldehyde, β -hydroxypyruvic acid both its lithium salt and its free acid form were purchased from Sigma-Aldrich while L-erythrulose hydrate was supplied by Fluka Chemicals Ltd. (Gillingham, Kent, England)

2.3 Immobilization Supports

2.3.1 Eupergit-C®

Eupergit-C® was described by Rohm GmbH (Kirschenallee, Darmstadt, Germany) as a polymer of methacrylamide, N,N'-methylene-bis(methacrylamide) and monomers containing the epoxide groups. The epoxide groups act as the functional groups allowing covalent attachment of proteins to the internal and external surfaces of the supports. The supports were white, opaque, spherical beads with a mean particle size distribution of 150 µm (Rohm GmbH). The epoxide content of the support had been determined by Rohm GmbH as exceeding 600 µmoles/g of dry beads. The morphological characteristics of Eupergit-C® as reported by the manufacturers are presented in table 2.2.

Characteristic	Data
Mean Particle Diameter	150 µm
Mean Pore Diameter	10-30 nm
Specific Surface Area	183 m ² /g (dry)
Interior Surface Area	48 m ² /g (dry)
Molecular Weight Excluded (Gel Permeation Chromatography)	5 x 10 ⁵ (globular proteins) 3 x 10 ⁵ (dextrans)

Table 2.2 Morphological data on Eupergit-C® as reported by the manufacturers (Rohm Pharma Polymers).

The literature cites several enzymes that have been successfully immobilized onto Eupergit-C®. These include phosphodiesterase, penicillin G amidase, lipase, mercuric amidase and alcohol dehydrogenase. A large selection of enzymes have been immobilized onto Eupergit-C® with a reasonable retention of activity and reasonable operational stability ranging from 5 batches to 500 days of continuous operation with 5-phosphodiesterase (Keller *et al.* 1987). Also a large variety of reactions catalyzed by an enzyme immobilized onto Eupergit-C® have been reported in the literature,

including NADH dependent oxoreduction reactions (Riva, S *et al.*, 1988 and Keinan, E *et al.*, 1986).

In the paper demonstrating the possibility of immobilization onto epoxy activated synthetic polymers (Drobnik, J *et al.*, 1979) it was demonstrated that immobilization onto Eupergit-C® was possible even at low pHs. They demonstrated that immobilization could be achieved at acidic pHs onto epoxide groups. This fact and supporting fact that several enzymes have been successfully immobilized onto Eupergit-C® gives the support a flexibility and a proven track record invaluable to this study.

2.3.2 Amberlite XAD7

Amberlite XAD7 (supplied by Rhöm and Haas (UK) Ltd., Croyden) is a spherical polymer of polymethylacrylate containing aliphatic reagents as cross linkers. The material is hydrophilic due to the presence of polar substituents, although there is no ion exchange capacity as these substituents are non-ionic. The beads are macroporous in nature with pores of between 4 and 20,000 nm, when wetted the liquid fills the continuous pore phase wetting the internal structure.

Characteristic	Value
Volumetric Porosity	0.55cm ³ .cm ⁻³
True wet density	1.05 g.cm ⁻³
Specific Surface Area	450 m ² g ⁻¹
Skeletal density	1.24 g.cm ⁻³
Average pore diameter	8 nm

Table 2.3 Some properties of Amberlite XAD7 as described in the manufacturers specifications.

2.3.3 Solids Handling Techniques

It was necessary that the beads were recovered after immobilization and since the beads were often reused after biotransformation. It is important to describe the techniques by which the solid supports were handled as it is possible that poor handling of solids can result in the generation of errors or a loss in reproducibility. After immobilization the solids were allowed to settle and the majority of the supernatant was removed by filtration through a filter gauze supplied by Rohm GmbH. After which the slurry was removed with a 5 mL pipette and filtered under vacuum onto 0.47 μm filter paper (Whatman International Ltd., Maidstone, Kent, England). The wet beads were weighed prior to charging the bioreactor.

After a biotransformation the majority of the supernatant was removed by filtration through a filter gauze, the final volume of liquid was decanted after the beads had settled. The beads were then washed *in situ* and reused as necessary. After reuse the settled bead slurry was removed into a 5 mL pipette and the wet weight measured. The beads were then dried at 45°C overnight and the dry weight recorded. It is important to note that in all cases the dry weight of the beads is used to normalize activity, capacity and binding yield.

2.4 Characterization of Free Transketolase

2.4.1 Resolution of Cofactors from Free Transketolase

The thiamine pyrophosphate (TPP) and MgCl_2 cofactors were resolved from transketolase by dialysis of the activated enzyme against buffers of a variety of pHs. The dialysis tubing was preprepared by boiling in EDTA and sodium bicarbonate for 20 minutes after which the dialysis tubing was washed thoroughly in RO water. The transketolase sample was preactivated by incubation with 2.4 mM TPP and 9 mM MgCl_2 for 30 minutes. After incubation 1 mL samples containing approximately 120 U mL^{-1} was added to the dialysis tubing and dialyzed against 10 mM phosphate buffer at pH 6.5, 7, 7.5, 8, 8.5 and 9 for 12 hours. Two 100 μL samples were taken from the dialyate, one sample was assayed for transketolase using the assay described in section 2.1.2.2. The second sample was assayed for transketolase using a modified

enzyme linked assay in the absence of cofactors. The total amount of transketolase was defined as the amount of enzyme activity measured in the presence of excess cofactors, while the amount of holo-enzyme was defined as the enzyme activity measured in the absence of added cofactors.

2.4.2 Stability of Free Transketolase

The stability of free transketolase was determined in the presence and absence of added cofactors to determine a difference between the stability of the holo- and apo-forms of the enzyme.

5 mL transketolase samples in 1M phosphate at a variety of pHs containing approximately 50 U/mL⁻¹ in the presence and absence of added cofactors were prepared aseptically. In cases where cofactors were required during incubation, a 1M phosphate solution containing 12 mM TPP and 45 mM MgCl₂ was passed through a sterile filter and added to the sample. 100 µL samples were taken at regular time intervals over a 72 hour period and assayed for transketolase activity.

2.4.3 Biotransformation with Free Transketolase

10 mL reaction mixture containing 100 mM glycolaldehyde, 100 mM HPA, 2.4 mM TPP, 9 mM MgCl₂, 0.5% (w/v) mercaptoethanol and transketolase at pH 7.5 and 25°C was charged into the pH stat reaction vessel. The pH was maintained at 7.5 by automatic titration with 1M HCl in the pH stat.

Transketolase was preincubated with cofactors prior to addition to the reaction mixture in order to reduce the lag time before the reaction reaches its maximum rate (Mitra 1997). The reaction was initiated by the addition of preincubated transketolase. 100 µL samples were taken from the biotransformation at regular time intervals and assayed by HPLC for reactants and products.

2.5 Characterization of Immobilization Supports

2.5.1 Determination of Bulk Expanded Volume

2 g of dry beads were allowed to swell in a variety of inorganic and organic solvents. The volume of the swollen beads was measured in a graduated measuring cylinder. The beads were then recovered and resuspended in 10 mL of the solvent in a graduated measuring cylinder and the volume that the beads displaced was measured. These data were used to record the bulk expanded volume and the displaced volume of the beads.

2.5.2 Protein Binding Capacity

The capacity of the beads for binding protein was measured at pH 6.5, 7.5 and 8.5. 6 mL of a solution of BSA (50 mgmL^{-1} and 100 mgmL^{-1}) was prepared in 1M phosphate buffer at the required pH, 1g of dry beads was added to the solution. At regular time intervals over a 72 hour period samples were taken and the total protein concentration was measured. After 72 hours the beads were recovered and washed 3 times in 50 mL 10 mM phosphate buffer. Samples from each wash were taken and the total protein concentration measured. The binding capacity of the beads was determined from a mass balance of the total amount of protein unbound and the amount of protein challenged to the support.

2.6 Immobilization of Transketolase

2.6.1 Immobilization onto Eupergit-C®

Clarified cell extract was prepared in 1M phosphate buffer at pH 6.5 for the holo-enzyme and pH 7.5 for the apo-form at a suitable transketolase concentration (between 50 and 500 UmL^{-1}) as described previously. After clarification 1 g of Eupergit-C beads were added per 8 mL of enzyme solution. When the holo-enzyme was immobilized the clarified extract was incubated with 2.4 mM TPP and 9 mM MgCl_2 to activate the enzyme prior to the addition of the Eupergit-C. After the addition of the beads a nitrogen head space was placed over the immobilization reaction mixture. The

mixture was shaken at 200 rpm for at least 24 hours to allow the enzyme to bind to the support. The amount of transketolase in the supernatant was measured after 24 hours.

After the binding reaction any unbound enzyme was removed by washing the support three times with 50 mL of 10 mM phosphate buffer at pH 6.5 containing 2.4 mM TPP and 9 mM MgCl₂. The amount of enzyme bound to the support was calculated from a mass balance of the starting amount of transketolase and the amount of unabsorbed and the amount of enzyme washed off the support.

After immobilization and washing, the beads were recovered as described previously. Any unreacted epoxide groups were capped with β -mercaptoethanol. The capping reaction was performed in 10 mL of 10 mM phosphate buffer at pH 6.5 containing 2.4 mM TPP, 9 mM MgCl₂ and 10 mM β -mercaptoethanol per 4 g (wet) beads. The capping reaction was allowed to proceed for 16 hours under a nitrogen head gas. After blocking the beads with mercaptoethanol the beads were recovered as described previously and washed in 500 mL reverse osmosis water.

2.6.2 Activation of Amberlite XAD7 with Gluteraldehyde

Amberlite XAD7 needs to be treated with gluteraldehyde in order to provide the ligands to which protein can be covalently bound to the support. The activation method employed in this work was developed by Carleysmith (1977). Amberlite resin (50 g) was mixed with 100 mL of a solution containing gluteraldehyde (10 % (w/v)) in deionised water at pH 10.5 for 16 hours. Changes in pH were controlled by the automatic addition of 5 M NaOH in the autotitrator. The activated resin was recovered by decanting away the supernatant. The activated resin was washed ten times with deionised water. The resin had a yellow colour after activation with gluteraldehyde.

2.6.3 Immobilisation of transketolase onto Amberlite XAD7

Clarified cell extract was prepared in 1M phosphate buffer at pH 6.5 for the holo-enzyme and pH 7.5 for the apo-form at a suitable transketolase concentration (between

50 and 500 U.mL⁻¹) as described previously. After clarification 1 g of Amberlite XAD7 beads were added per 8 mL of enzyme solution. In the case of the holo-enzyme, the clarified extract was incubated with 2.4 mM TPP and 9 mM MgCl₂ to activate the enzyme prior to the addition of the support material.

After the addition of the beads a nitrogen head space was placed over the immobilization reaction mixture. The mixture was shaken at 200 rpm for at least 24 hours to allow the enzyme to bind to the support. The amount of transketolase in the supernatant was measured after 24 hours.

After the binding reaction any unbound enzyme was removed by washing the support three times with 50 mL of 10 mM phosphate buffer at pH 6.5 containing 2.4 mM TPP and 9 mM MgCl₂ to ensure that the enzyme remained in the holo-form. The amount of enzyme bound to the support was calculated from a mass balance of the starting amount of transketolase and the amount of unabsorbed and the amount of enzyme washed off the support.

2.6.4 Assay for Immobilized Transketolase

The yield of immobilized enzyme activity was measured from the initial rate of erythrulose production during the first 20 minutes of a biotransformation with 100 mM reactants. The reaction mixture contained 100 mM glycolaldehyde, 100 mM hydroxypyruvic acid, 2.4 mM TPP and 9 mM MgCl₂ and 0.5% (w/v) mercaptoethanol, the pH of the reaction mixture was adjusted to pH 7.5. 10 mL of the reaction mixture was charged into the reaction vessel with 1 g (wet) biocatalyst beads. 100 µL samples were taken at regular time intervals over the initial 20 minutes. The biotransformation assay for transketolase was related to the enzyme linked assay for transketolase by the calibration curve shown in figure 2.2. The immobilised transketolase assay was found to have an average standard error of 20 %.

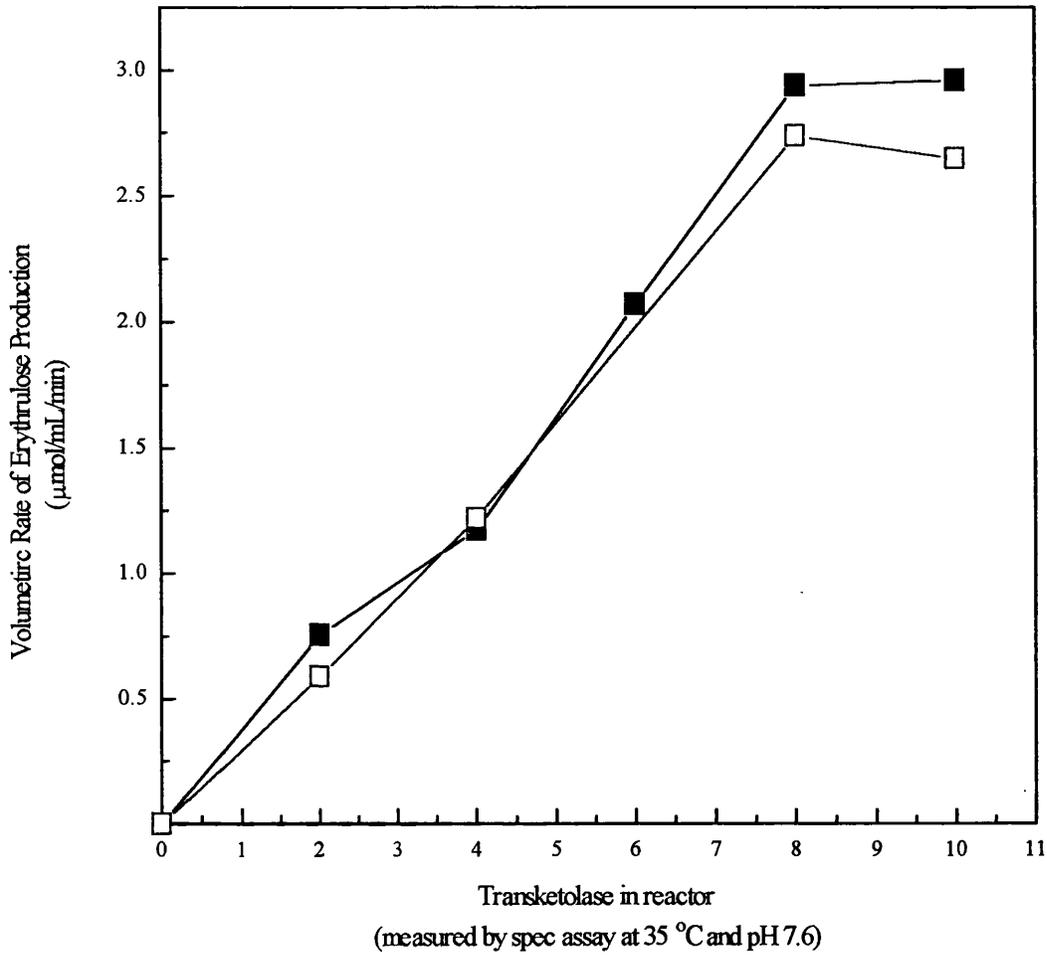


Figure 2.2 Calibration of the biotransformation assay for transketolase. Transketolase activity as measured by the enzyme linked assay was compared with the volumetric rate of erythrulose production as measured using the HPLC assay. The graph shows the values obtained with measurement times of 5 minutes (—■—) and 10 minutes (—□—).

2.7 Microscopic Study of Biocatalyst Beads

2.7.1 Staining of the Beads for Protein

The position of protein within the beads could be determined by staining with a protein stain Ponceau S ($M_w = 761 \text{ g.mole}^{-1}$). 300 mg Ponceau S was dissolved in a solution of glacial trichloroacetic acid (6 g) in 200 mL of deionised water. 5 mL of prepared dye was added to 1 g (wet) beads. After 30 minutes at room temperature the dye was pipetted off and any excess stain was washed off with 5 washes of 5 mL 2 % glacial trichloroacetic acid the acetic acid was removed by pipette and the wet beads were dried on a weighing boat at 45 °C for 30 minutes. The stained beads were mounted for microscopic analysis and photography as described below.

2.7.2 Microscopic Study of the Stained Beads

A bead was split in half to reveal the extent of staining. The bead was held on a glass surface within a piece of tissue paper, the bead was cut using a scalpel blade. A selection of beads were cracked using a pestle and mortar held in a liquid nitrogen bath. Prior to mounting the microscopic slide was prepared by smearing a thin layer of clear nail varnish. A small quantity of beads were sprinkled onto the varnish, excess beads were removed by inverting the microscope slide. The attached beads were allowed to dry for 5-10 minutes prior to their observation under microscope. An Olympus microscope (Olympus, Tokyo) was used to observe the beads under magnification by 10 times to give a view of several beads while a magnification factor of 40 allowed the observation of one individual bead. A tungsten-halogen light source was used to back light the microscopic slide to allow the slide to be photographed.

2.8 Statistical Testing of Data

Where described in the text the data were tested in order to determine whether they were significantly different. The test used was the Student T-test as described by Lennox and Chadwick (1977) and Rees (1985). Tests for significance are formulated by comparing the differences between the observed and the expected values of a

statistical value with the standard error. The distribution of most statistical values tends towards the Normal distribution as the number of samples increases. For small sample populations the distribution is far from Normal. The T-test uses the T-distribution which is valid for small and large sample populations.

Number of degrees of freedom	Significance level	
	5 %	1 %
1	12.71	63.66
3	3.18	5.84
5	2.57	4.03
10	2.23	3.17
15	2.13	2.95

Table 2.4 An extract from the Students T-distribution. (Rees (1985)).

Table 2.4 shows a small extract from the Students T-distribution. In order to test whether two sets of data are significantly different the data are deliberately paired. The null hypothesis is that there is no difference between the two sets of data. The test was performed at a confidence level of 5 %.

The observed T value was calculated using the following equation.

$$\text{The observed T value} = z/(s_z/\sqrt{n})$$

Where:

n = the number of samples,

z = the mean difference between the samples, and

$$s_z^2 = [\sum(z - \bar{z})^2]/(n - 1)$$

If $|\text{Obs T}| > \text{Calc T}$ then the difference between the data is statistically significant at the 5 % confidence level.

Results

3.1 Characteristics of Transketolase

When determining the mode of operation of a bioreactor the characteristics of the enzyme itself are important parameters. The characteristics of the enzyme will limit the conditions within the reactor. For example, the stability of an enzyme under alkaline conditions will help determine whether the bioreactor should be operated at pH values above 7. The characteristics of an enzyme which will affect operating conditions can be divided into those parameters which affect the stability of the enzyme and those parameters which will alter the activity of the enzyme.

It can be expected that some of the enzyme's characteristics would also affect how and whether the enzyme is immobilised. This section describes the investigation of the characteristics of the enzyme. The characterisation was limited to the following areas:

- Activation of the enzyme,
- Reconstitution of the enzyme with cofactors,
- Removal of the cofactors from the active site,
- Stability of the holo- and apo-forms.

These features were considered to be able to affect how much activity will be retained and in what form the enzyme can be immobilised.

3.1.1 Binding of TPP to Transketolase

Transketolase has been shown to be a dimeric cofactor requiring enzyme. The cofactors thiamin pyrophosphate (TPP) and a divalent metal ion are required for activity. In order to immobilise transketolase it is important to know how the cofactor molecule is bound to the enzyme. The shape of the TPP binding curve can be used to determine how much TPP is needed during immobilisation if the enzyme is to be immobilised in its holo-form.

In order to determine the characteristics of TPP binding to *E.coli* transketolase the standard transketolase assay (as described by Hobbs (1994)) was modified by the exclusion of added TPP cofactor. TPP was added to the assay cocktail separately to make known final concentration of cofactor and the activity of transketolase measured in the usual manner for a range of concentrations.

Figure 3.1 shows the effect of increasing concentrations of TPP upon the activity of transketolase. The assay was carried out at 36°C and pH 7.5 to simulate reaction conditions. As the TPP concentration increased the transketolase activity of the cell extract increased until it reached its maximum at a TPP concentration of 3.0 μM . The activity of the crude cell extract without any added cofactors demonstrated that approximately 10 % of the enzyme in the cell free extract contained TPP. An apparent K_m for TPP of 0.92 μM was calculated from these data and was compared with values obtained in the literature as shown in table 3.1.1 below.

Transketolase source	Apparent K_m (μM)	Reference
<i>E.coli</i> JM107 pQR700	0.92	This work
<i>S.cerevisiae</i>	1	Heinrich <i>et al.</i> , (1972)
Human erythrocyte	1.6	Booth and Nixon (1993)

Table 3.1 Comparison of the affinity of transketolases from different sources for TPP. All values were obtained in the presence of excess Mg^{2+} .

The value of the binding constant of TPP to this transketolase is comparable to the values observed in the literature for transketolases from other sources (table 3.1). The low apparent K_m demonstrates that transketolase has a strong affinity for its cofactor. There are, however, other factors of importance when determining whether the enzyme should be bound in the presence or absence of its cofactors. The effect of the ionic conditions during binding upon the dissociation of transketolase will constrain both whether the enzyme can be immobilised in its holo form, and the choice of binding method.

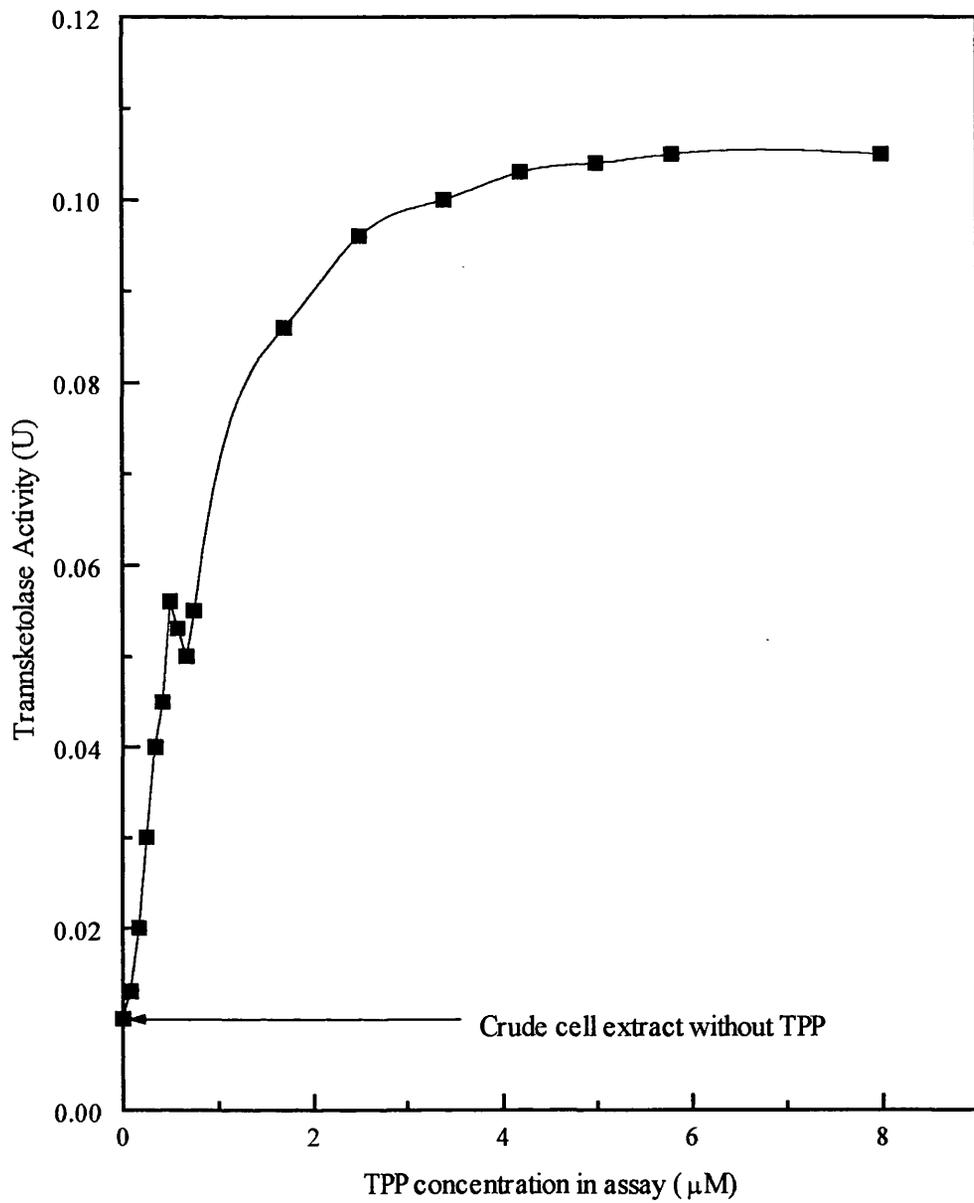


Figure 3.1 The effect of TPP concentration upon the transketolase activity of the *E.coli* crude cell extract. Transketolase activity was measured at pH 7.6 and 36 °C.

3.1.2 Effect of pH on the resolution of TPP from holo-transketolase

The covalent binding of protein to support materials is often carried out at high pH values in order to deprotonate the target amino acid side chains (Porath and Axen (1976)). However, the pH used for immobilisation can affect the formation of the holo-enzyme and the resolution of cofactor from the active site. Consequentially the effect of pH upon the resolution of TPP from the enzyme is an important factor in determining:

1. Whether the enzyme should be immobilised in its holo form or not, and
2. The pH conditions of immobilisation.

In order to determine the effect of pH upon the resolution of TPP from the holo-enzyme samples of the crude cell extract were reconstituted with 2.4 mM TPP for 30 minutes. After reconstitution the samples were dialysed for 24 hours against 10 mM phosphate buffer containing mercaptoethanol as a stabilising agent at a variety of pH values. It had been shown by Ikeda *et al.*, (1975) that in general the extent of cofactor binding to apo-enzyme can be measured by assaying the enzyme activity in the presence and absence of cofactor. To that end the amount of the enzyme remaining in the holo-form was measured using the transketolase assay prepared in the absence of TPP, while the total amount of transketolase was measured using the standard transketolase assay as described by Hobbs (1994).

Figure 3.2 shows how the amount of transketolase in the holo form varies with the external pH. As the pH was increased above 7.0 the amount of enzyme remaining in its holo-form after dialysis decreases rapidly to zero. It is important to note that even at low pH values the percentage of holo-enzyme is never 100% after dialysis. The results demonstrate firstly that the binding of TPP to *E.coli* transketolase is reversible, as shown by Heinrich *et al.* (1972) for *S.cerevisiae* transketolase and secondly that the pH is an important factor in the resolution of TPP.

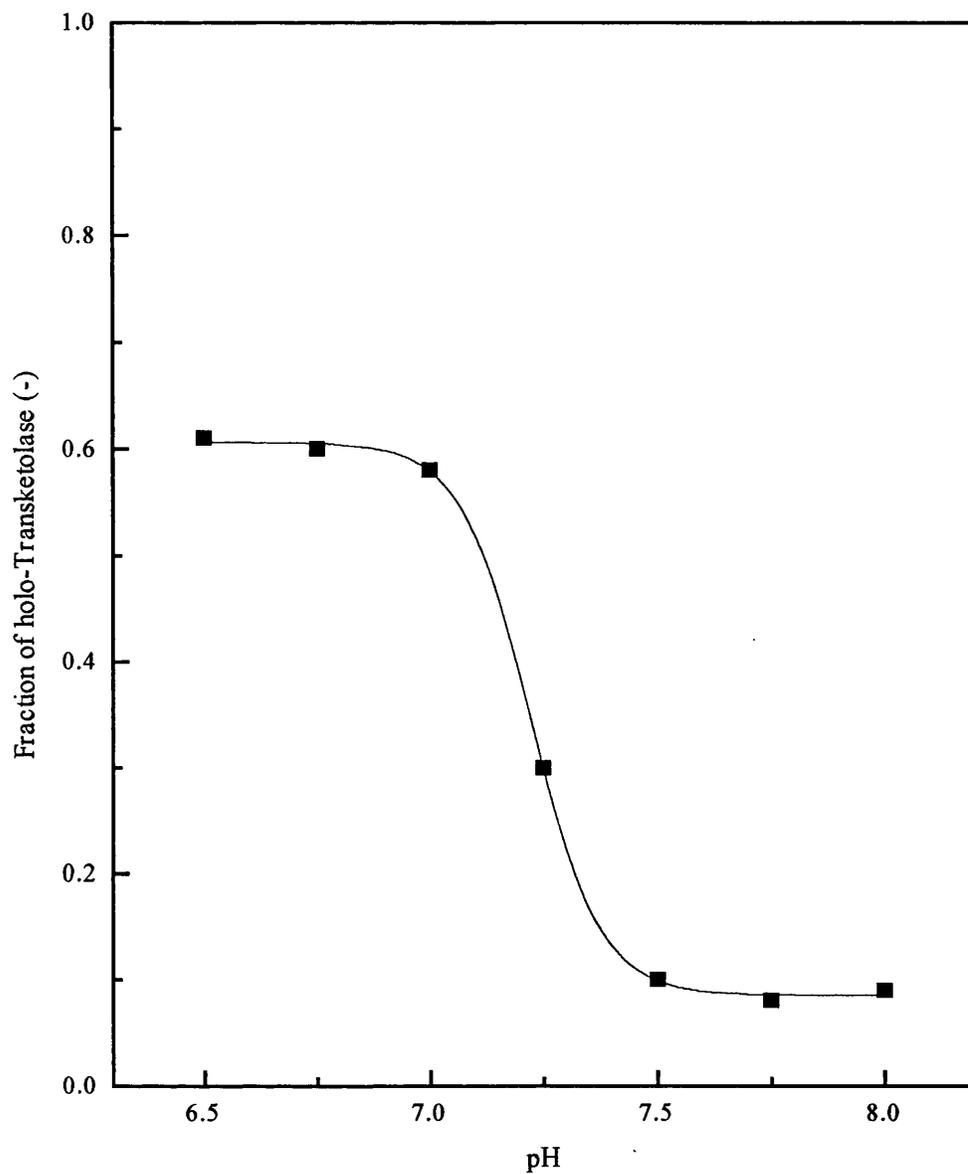


Figure 3.2 The effect of pH upon the dissociation of TPP from holo-transketolase. Activity was measured in the presence and absence of TPP.

3.1.3 Stability of holo- and apo-transketolase

The functional stability of the enzyme can be defined as its ability to maintain activity over a period of time irrespective to variations in its environment. It can be thought of as a 'bulk' parameter including the various effects that affect the activity of the enzyme, for example the ability of the enzyme's structure to maintain its integrity despite changes in its environment. The functional stability is determined by measurement of activity over a period of time whilst the enzyme is stored in a variety of conditions.

In order to demonstrate whether transketolase was stable enough to be immobilised with a reasonable retention of activity, the storage stability of soluble holo- and apo-transketolase was investigated. Enzyme was stored in phosphate buffer at pH 7.0 and activity was measured at regular time intervals.

It can be seen from figure 3.3 that the holo-enzyme is rapidly denatured under immobilisation conditions. All activity was lost within a 24 hour period. This means that if the holo-enzyme is to be immobilised the enzyme must be stabilised or the immobilisation must be complete within the 24 hour period. In order to demonstrate whether the enzyme could be stabilised the holo-enzyme was stored with 0.5% (w/v) mercaptoethanol and under a nitrogen head gas.

Figure 3.3 also shows that the activity of holo-transketolase could be maintained over a 72 hour period by incubation of the enzyme and cofactor in the presence of a nitrogen head gas or mercaptoethanol. These methods are known to stabilise proteins by preventing oxidation of essential cystine, methionine or tryptophan residues (Mazhaev (1993)). This indicates that oxidation of these residues is the main cause of inactivation of the holo-enzyme. Subsequent to these data unless otherwise stated in the text the immobilisation of holo-transketolase was carried out in the presence of a nitrogen head gas since mercaptoethanol would combine with the functional groups on the support material, while all reactions contained 0.5% (w/v) mercaptoethanol as a stabilising agent.

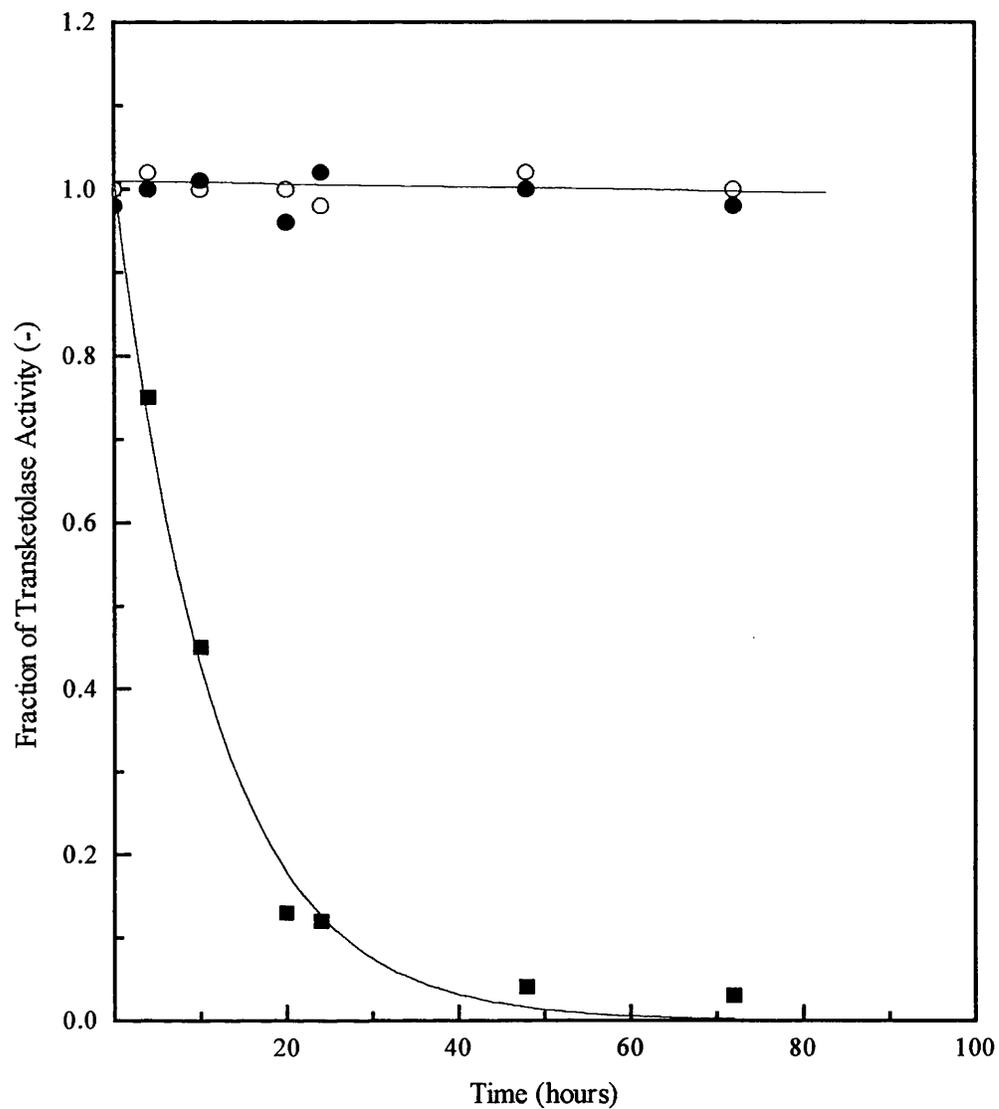


Figure 3.3 Stability of transketolase in immobilisation conditions. Transketolase was stored in 1M phosphate buffer at pH 7.5. Showing the stability of apo-transketolase (○), holo-transketolase (■) and holo-transketolase stabilised with 0.5% (w/v) mercaptoethanol (●).

3.2 Characteristics of Eupergit-C[®] and Amberlite XAD7

The supports will be in direct contact with the enzyme, its reactants and its products. It has been recognised that there are several characteristics of the support that can affect the activity and operational stability of the resulting biocatalyst and its operation in a bioreactor. It was the aim of this section to investigate the properties of the supports that will affect their ability to immobilise enzymes and its performance within a bioreactor.

3.2.1 Physico-chemical properties of Eupergit-C[®] and Amberlite XAD7

3.2.1.1 Bulk Expanded Volume

Naturally occurring biopolymers such as cellulose and synthetic polymers such as polyacrylamide are based upon flexible structures. As a result there can be significant levels of swelling when the polymer is in some solvents. The degree that a resin swells in a given solvent (also known as bulk expanded volume) is a multifaceted property that reflects the chemical structure of the polymer backbone, its compatibility with the solvent, the degree of cross-linking and the architecture of the polymer matrix (Arshady, R (1991)). The swelling properties of the support will enable the determination of the pore volume and consequentially allow the completion of a mass balance, taking into account the amount of reaction constituent that is drawn *into* the matrix.

Support	Measured variable	RO Water	1 M NaCl	1 M PO ₄ pH 3.5	1 M PO ₄ pH 7.5	1 M PO ₄ pH 10	AcN	Prop
Eupergit	BEV (mL.g ⁻¹)	4.75	4.75	4.75	4.75	4.75	4.5	4.5
	Displaced Volume (mL.g ⁻¹)	3.5	3.5	3.5	3.5	3.5	3.5	3.5
	External Volume (mL.g ⁻¹)	1.25	1.25	1.25	1.25	1.25	1	1
Amberlite	BEV (mL.g ⁻¹)	1.7	1.7	1.7	1.7	1.7	1.7	1.7
	Displaced Volume (mL.g ⁻¹)	1.5	1.5	1.5	1.5	1.5	1.5	1.5
	External Volume (mL.g ⁻¹)	0.2	0.2	0.2	0.2	0.2	0.2	0.2

Table 3.2 The change in bulk expanded volume (BEV) and other swelling characteristics of Eupergit-C[®] and Amberlite in a variety of solvents.

(Note: AcN = acetonitrile and prop = propanol)

In order to determine the swelling properties of the two support materials a known volume of dry material was allowed to swell in a variety of polar and non-polar organic and inorganic solvents. The selection of solvents was made with the eventual reaction and immobilisation conditions in mind. The change in volume was measured, with the change in the amount of solvent displaced by the swollen beads and these data along with the ratio of wet and dry weights were used to determine the pore volume and the external liquid volumes of the supports. The external liquid volume represents the void volume between the resin beads. The void liquid is loosely associated with the beads and readily exchangeable with the bulk liquid

Eupergit-C® and Amberlite both swell uniformly in the range of solvents used, despite changes in polarity, pH and ionic strength. Dry Eupergit and Amberlite both had a volume of 1.5 mL.g^{-1} . These data indicate that major reorganisation of Amberlite's tertiary structure is prevented, while Eupergit-C® swells to approximately 4 times its original volume. The stability of the swelling across the range of solvents is most likely due to cross-linking within the polymer matrix, the higher the degree of internal cross-linking the smaller change in the bulk expanded volume of the support (Arshady (1991)).

When these data are compared with that cited in the literature for other materials used for immobilisation it can be seen that neither Eupergit-C® or Amberlite swell to a high degree. Values of up to 20 mL.g^{-1} have been obtained for some polydimethylacrylamide preparations while values of approximately 5 mL.g^{-1} have been obtained for copolymers of polystyrene and polydimethylacrylamide (Arshady, R 1991).

3.2.1.2 Pore Volume and Specific Surface Area

In order to complete a reaction mass balance where a porous material is involved it is important to know how much of the external volume can be drawn *into* the resin. This can be determined from the bulk expanded volume and the change in the wet and dry weights of the support. The pore volume of a support material will determine the ease at which substrates can gain access to the enzyme during the biotransformation reaction. It will also influence the accessibility of the functional groups and the amount of the polymers surface area that is available to the protein for attachment.

Support	Wet weight (g)	Dry weight (g)	Pore Volume (mL.g ⁻¹)
Eupergit-C®	1.0	0.20	2.5
Amberlite	0.88	0.28	0.5

Table 3.3 Comparison of the wet and dry weights of Eupergit-C® and Amberlite and the pore volumes of these resins.

When wet Amberlite XAD7 and Eupergit-C® beads were transferred to a solution containing hydroxypyruvic acid and glycolaldehyde a change in concentration was observed. This change could be accounted for by the change of internal bead volume with the external liquid.

The specific surface areas of Eupergit-C® and Amberlite have been supplied by their manufacturers. Where the support is commercially produced this information is routinely measured by nitrogen adsorption as a Quality Assurance measurement. Table 3.4 compares the specific surface areas of Eupergit-C® and Amberlite with that of other supports sited in the literature. The specific surface area and porosity are easily controlled variables during the production of the resin, by alteration of the amount of monomer or cross-linking agent present.

Support	Specific Surface Area (m ² .g ⁻¹)	Reference
Eupergit-C®	180	Röhm
Amberlite	450	Röhm
Silica gel	20-498	Arshady (1991)
Polyurethane	ca. 486	Wang and Ruckenstein (1993)

Table 3.4 Comparison of the specific surface areas of Eupergit-C® and Amberlite with values obtained from the literature

These data show that the specific surface area of Eupergit-C® and Amberlite lie in the mid range of possible values. However, it should be noted that the pore volume and

specific surface area are strongly dependent upon the degree of swelling that the resin has undergone. Consequently, since Eupergit-C® swells to approximately 4 times its dry volume a change in surface area of approximately 2.5 times could be expected.

3.2.2 Binding of protein to Eupergit-C® and Amberlite XAD7

In order to determine how much protein can be bound to the resins and also to determine the pH conditions for immobilisation the capacities of the support materials were investigated for the immobilisation of bovine serum albumin (BSA) at pH 6.5, 7 and 10.

pH	Capacity Eupergit mg BSA.g ⁻¹ (dry beads)	Capacity Amberlite mg BSA.g ⁻¹ (dry beads)
6.5	155	-
7.5	155	240
10	155	-

Table 3.5 Capacity of Eupergit-C® and glutaraldehyde activated Amberlite for covalent binding of BSA at pH 6.5, 7.5 and 10.

These data indicated that with the average specific activity of 15 U.mg⁻¹ a capacity of 2325 U.g⁻¹ dry Eupergit and 3600 U.g⁻¹ dry Amberlite would be expected, if all the activity was retained after immobilisation.

3.3 Immobilisation of Transketolase

3.3.1 Binding of Transketolase

3.3.1.1 Effect of Salt Concentration

It has been previously shown that the presence of salts during immobilisation can enhance the rate of disappearance of protein from solution (Smalla, K *et al.*, (1988)). In order to investigate the effect of ionic strength upon the immobilisation of transketolase to Eupergit-C[®] and Amberlite immobilisation was performed in phosphate buffer at a variety of molarities.

The effect of phosphate concentration upon the immobilisation of transketolase to Eupergit-C[®] is shown in figure 3.4 while the influence of phosphate concentration upon the immobilisation onto Amberlite XAD7 is described in figure 3.5. In the case of Eupergit-C[®] the salt concentration had a strong influence upon the rate of disappearance of enzyme from solution. At phosphate concentrations of greater than 0.75M the rate of immobilisation onto Eupergit-C was unaltered. In the case of Amberlite XAD-7, salt concentration had little effect upon the rate of enzyme immobilisation.

This difference between the influence of phosphate concentration upon the rate of immobilisation was the first indication of a difference in the mechanism of immobilisation onto the two different supports. However, although the enzyme had disappeared from solution it may be held within the pore volume or be loosely associated to the support. In order to demonstrate how much enzyme could be washed off the support or merely adsorbed an immobilisation mass balance was carried out.

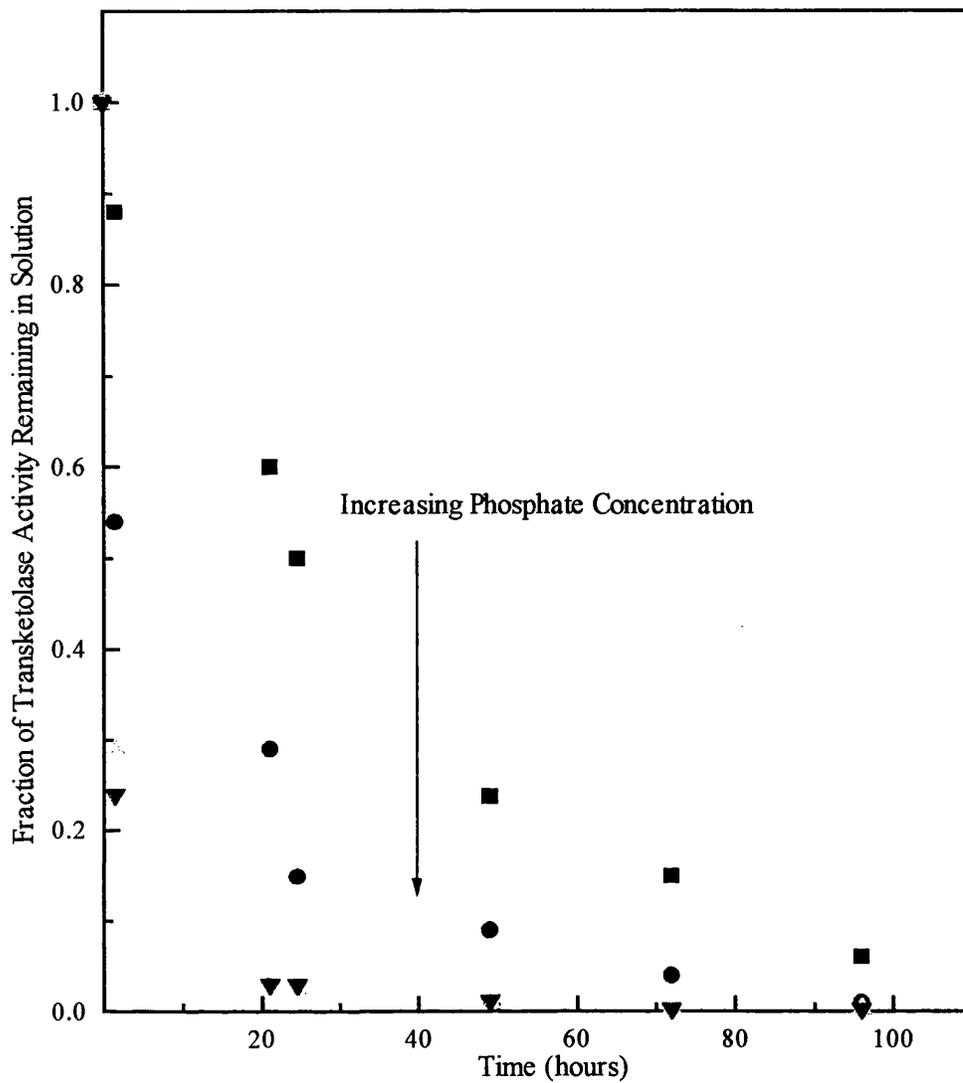


Figure 3.4 The effect of phosphate concentration upon the rate of immobilisation of transketolase to Eupergit-C. Showing the fraction of soluble transketolase remaining as immobilisation contact time increases. For (■) 0.1 M, (●) 0.5 M, (▲) 0.75 M and (▼) 1 M phosphate.

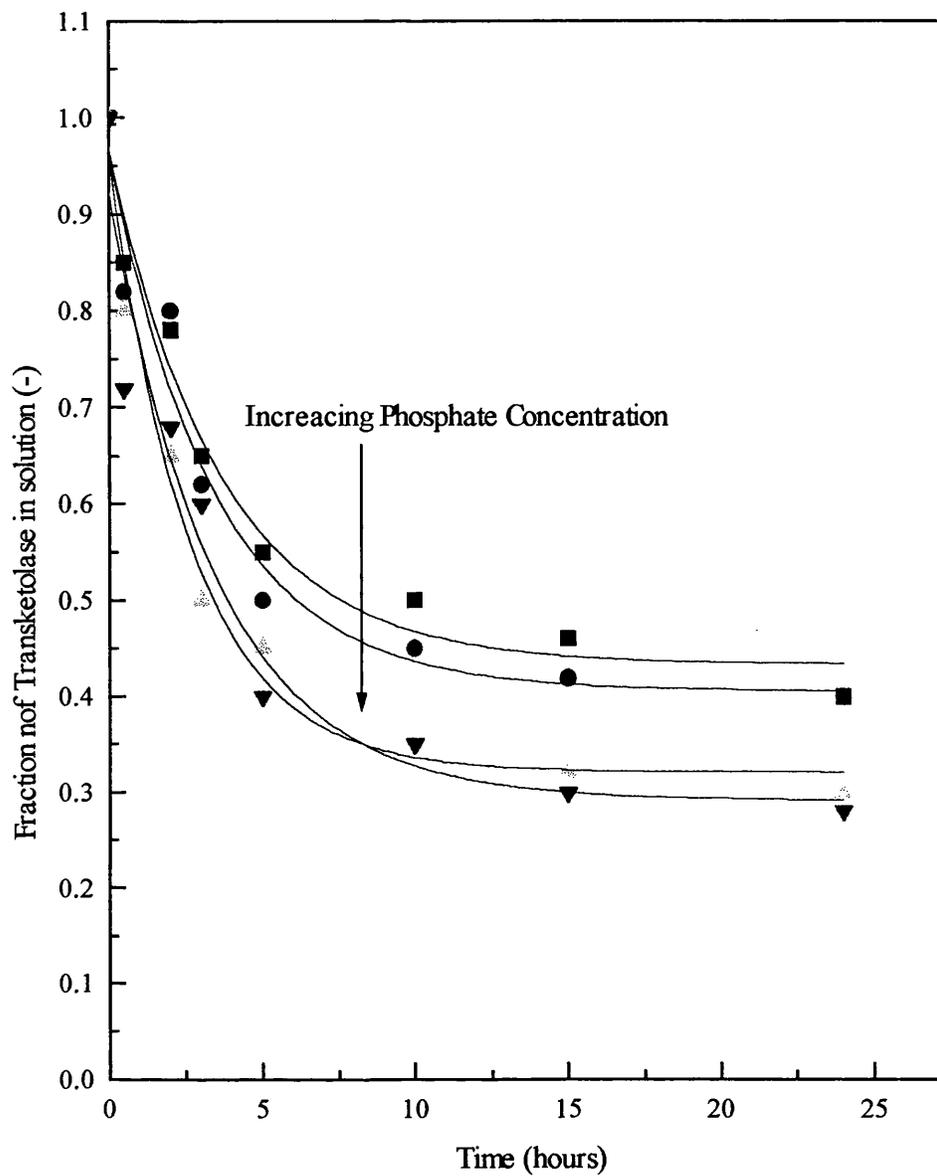


Figure 3.5 The effect of phosphate concentration upon the immobilisation of transketolase onto Amberlite XAD7. Showing the change in the fraction of transketolase activity in solution. Preparations were incubated at pH 7.5 in phosphate buffer at 10 mM (■), 100 mM (●), 0.5 M (▲) and 1 M (▼).

3.3.1.2 Immobilisation Mass Balance

In order to determine the true rate of binding the immobilisation was carried out in 1 M phosphate at pH 7.5. Samples were taken at regular time intervals and washed in buffer. The transketolase activity in the supernatant and the buffer were measured. This allowed a mass balance to be made. Table 3.6 shows the results of the mass balance giving the amount of enzyme measured in the supernatant and that could be washed off Eupergit and Amberlite.

In the case of Eupergit-C[®] the amount of enzyme washed off the support decreased as the contact time increased despite the fact that more enzyme activity has disappeared from solution. This allows the conclusion to be drawn that as the immobilisation time is increased the amount of enzyme merely trapped in the pores, or adsorbed to the support decreases as the slower covalent binding reaction takes place. Conversely in the case of Amberlite XAD7 the amount of activity in the wash increases as the contact time is increased. This would be expected if the association of the enzyme with the surface of the support is slower than the rate of covalent attachment.

Time h	Eupergit-C			Amberlite		
	Supernatant	Wash	Bound	Supernatant	Wash	Bound
	U	U	U	U	U	U
0	462	0	0	680	0	0
1	124	66.5	270	420	42	218
5	60	31.4	371	344	54	282
25	34.4	5.80	421	320	60	300
48	2	1.5	456	-	-	-
72	0.5	0.0	460	-	-	-

Table 3.6 The mass balance of transketolase during immobilisation onto 0.25 g dry Eupergit-C[®] and onto 0.25 g dry Amberlite XAD7. The table shows the amount of enzyme remaining in solution and the amount of enzyme washed off at each time point, the amount of bound enzyme was calculated from these two measured values.

In order to determine the overall efficiency of the immobilisation, in terms of the amount of actual biotransformation activity, immobilised samples of the biocatalyst were assayed in 100 mM biotransformation reactions. Table 3.7 shows the activity of each preparation in the biotransformation.

Time	Eupergit-C [®]		Amberlite	
	Specific activity	Retained activity	Specific activity	Retained activity
h	U.g ⁻¹ (dry)	%	U.g ⁻¹ (dry)	%
5	208	15	240	14
15	290	14	305	13
25	300	16	307	15
48	300	16	-	-
72	300	15	-	-

Table 3.7 The variation of the specific and retained activities of transketolase immobilised onto Eupergit-C[®] and Amberlite as contact time between the support and enzyme is increased. (Note: the enzyme was immobilised in the absence of TPP)

The retained activity of the Amberlite and Eupergit-C[®] was defined as the activity measured during the biotransformation reaction expressed as a fraction of the amount of activity bound. In both cases the amount of retained activity remained constant despite increases in the amount of bound enzyme. The specific activity of the biocatalyst (units of activity measured / dry weight of the biocatalyst) increased as the contact time was extended towards 24 hours. Increasing the contact time beyond 24 hours, however, did not affect the amount of transketolase bound or the amount of recovered activity, as a consequence, unless otherwise stated, all immobilisation procedures were carried out for 24 hours.

3.3.2 Effect of enzyme load

The effect of the amount of enzyme used to challenge Eupergit-C® was investigated. Differing quantities of enzyme were immobilised in 1 M phosphate buffer at pH 7.5 for 24 hours. The activities of the resulting biocatalysts were measured by the biotransformation assay. Figure 3.6 shows the effect that increasing the enzyme challenge has upon the specific activity and the retained activity of the final biocatalyst.

The maximum enzyme challenge was 2100 U.g⁻¹ (dry) which resulted in a 95 % yield of bound enzyme. This represented 91 % of the capacity of Eupergit-C®. When only 150 U.g⁻¹ of enzyme was bound a specific activity of 100 U.g⁻¹ was observed, this equates to 67 % of the bound enzyme expressing activity. However, as the amount of enzyme bound to the support was increased the retained activity of the biocatalyst decreased to less than 20 %. This indicated that the of the 1995 U.g⁻¹ of enzyme bound to the support, either 80 % was bound in an inactive state or mass transfer of the substrates into the support was masking the intrinsic activity of the biocatalyst. The investigation into the mass transfer properties of the support will be described in section 3.5.1.

3.3.3 Biotransformation with immobilised transketolase

3.3.3.1 Eupergit-C® immobilised transketolase

Figure 3.7 shows the reaction mass balance for a bioconversion with 100 mM substrate concentrations by transketolase immobilised onto Eupergit-C®. A mass balance was achieved by taking the pore and void volumes of the resin into account. Complete conversion was achieved with this biocatalyst. However, it is important to note that the amount of HCl titrant added to maintain pH conditions does not balance with the amount of HPA consumed or erythrulose produced, the ratio of titrant added to the amount of produced is shown in figure 3.8. This discrepancy was also observed by Mitra and Woodley (1996) for the free enzyme.

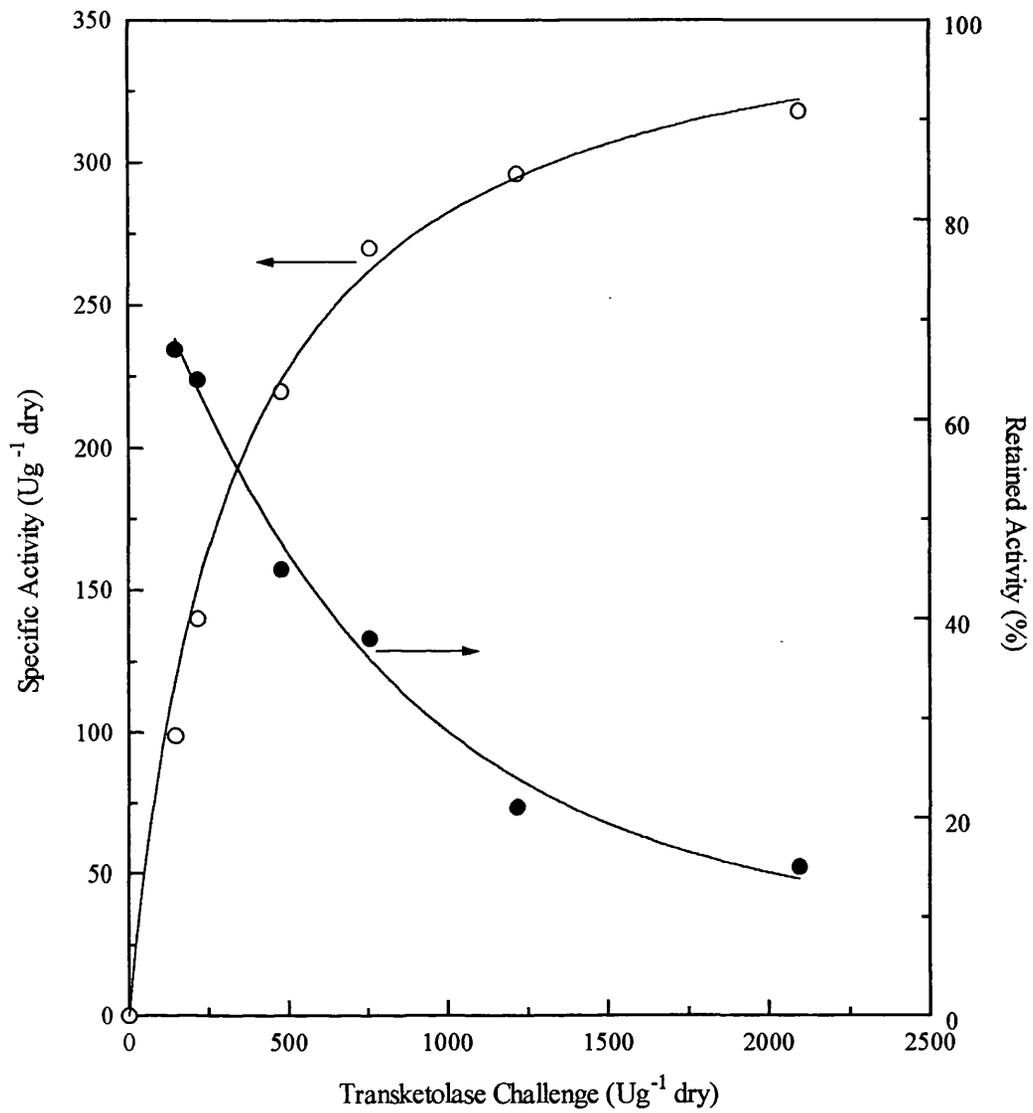


Figure 3.6 The effect of transketolase challenge upon the activity (○) and retained activity (●) of apo-transketolase immobilised onto Eupergit-C.

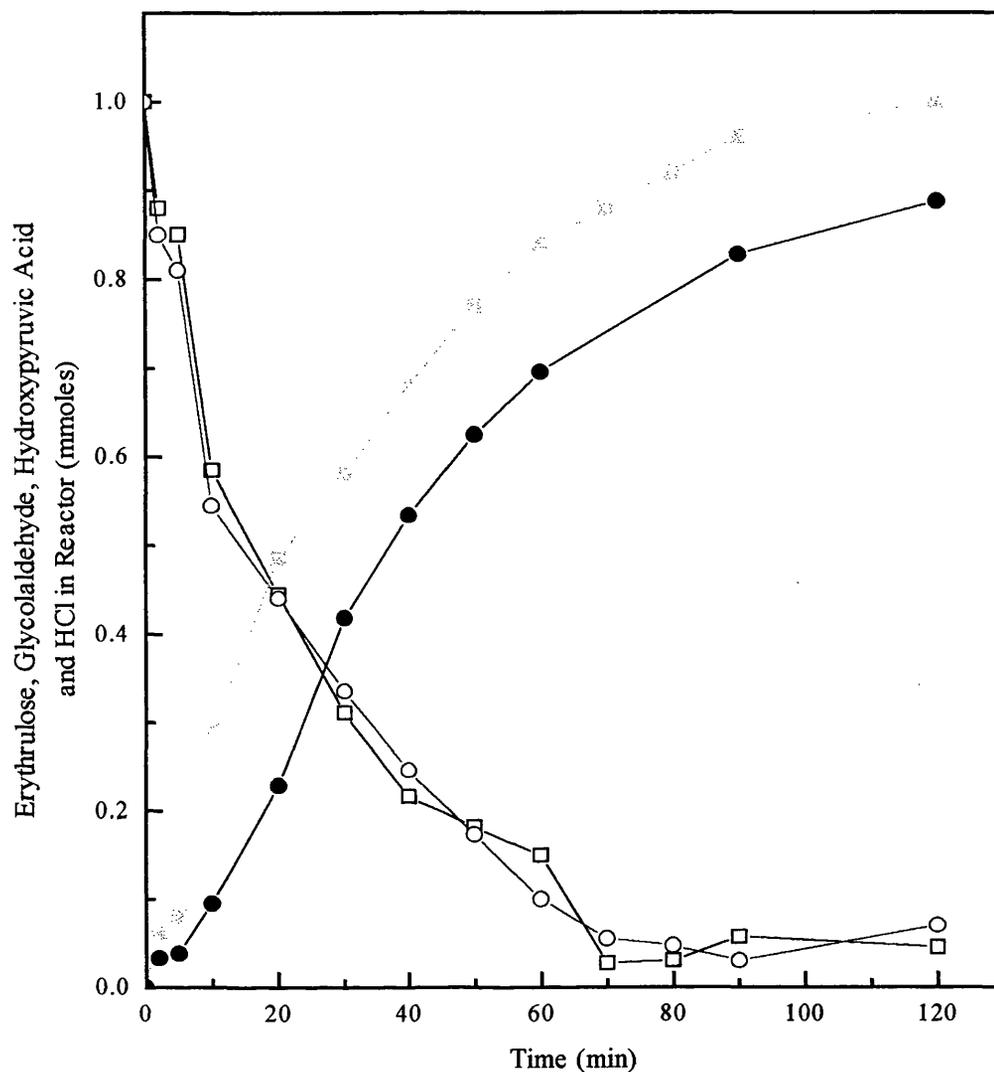


Figure 3.7 Biotransformation with apo-Transketolase immobilised onto Eupergit-C. Showing the number of mmoles of hydroxyppyruvic acid (—□—), glycolaldehyde (—○—), erythrulose (---*) and HCl titrant (—●—) in the reactor. Reaction was performed at 25°C and pH 7.5 in the presence of cofactors.

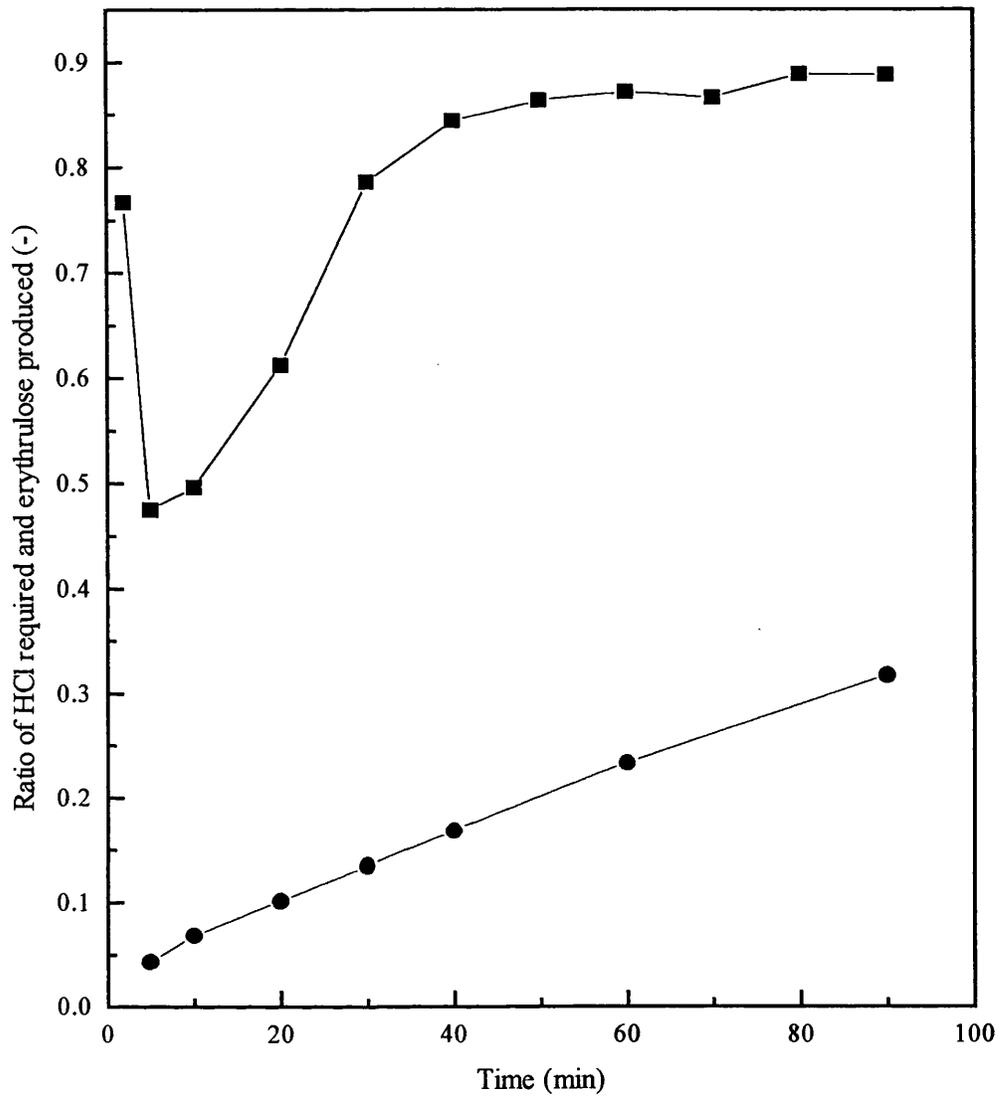


Figure 3.8 Comparison of the HCl titrant requirements of Eupergit-C and XAD7 immobilised transketolase. Showing the ratio of titrant required with erythrulose produced for Eupergit-C (—■—) and XAD7 (—●—).

3.3.3.2 Amberlite XAD7 immobilised transketolase

Figure 3.9 shows the reaction mass balance for a similar bioconversion by a transketolase preparation immobilised onto Amberlite XAD7. Again a mass balance was achieved by taking the pore volume into account. Complete conversion was achieved within 100 minutes. Again, the amount of titrant added does not balance with the amount of erythrulose produced or the amount of HPA consumed.

3.4 Activation of Immobilised Transketolase with TPP

Transketolase is a cofactor requiring dimeric enzyme. It has been demonstrated that the free enzyme requires TPP for activity and that the cofactor is exchangeable with the surrounding liquid at pH values greater than 7.25. However in the case of the immobilisation of the enzyme the absence of the cofactor from the active site may allow the enzyme to be denatured during the immobilisation process and/or the binding of the cofactor to the immobilised active site may be hindered by the support backbone. The question also remains whether the cofactors can be washed out of the active site of the immobilised enzyme. It was the aim of this section of the research to demonstrate whether transketolase can be immobilised in its holo-form, and secondly whether the cofactor can be washed out of the active site of the bound enzyme

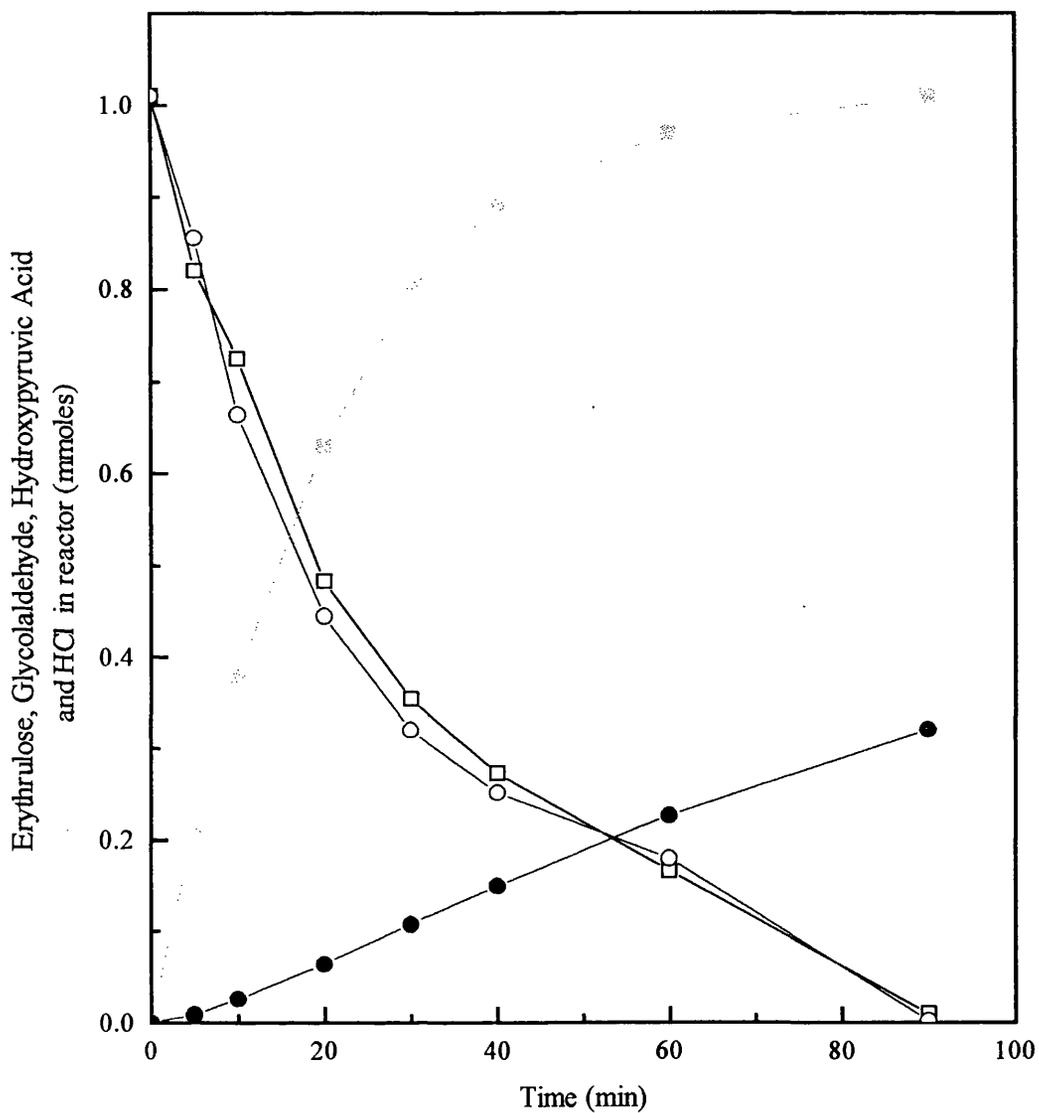


Figure 3.9 Biotransformation with apo-transketolase immobilised onto Amberlite XAD7. Showing the number of mmoles of hydroxypyruvic acid (—□—), glycolaldehyde (—○—), erythrulose (* * *) and HCl in the reactor (—●—).

3.4.1 Experimental Design

The aim of this series of experiments was to determine whether transketolase could be immobilised containing its cofactor TPP. Several preparations of immobilised transketolase were made. These were prepared in the absence and presence of the cofactor, in order to immobilise the apo- and holo-enzymes respectively. The preparations were assayed by biotransformation reaction in the presence and absence of cofactors. The experimental design is described in table 3.8.

Support	pH	Immobilisation	Biotransformation 1	Biotransformation 2
Eupergit-C Prep 1	7.5	+TPP	+TPP	-TPP To demonstrate whether pH effects immobilisation of holo -TK
Eupergit-C Prep 2	6.5	- TPP	+ TPP To demonstrate whether TPP is required during immobilisation to Eupergit	- TPP Eupergit-C apo-control.
Eupergit-C Prep 3	6.5	+ TPP	+ TPP Control - maximum activity	- TPP Holo-control - to demonstrate how much enzyme is bound to Eupergit in holo-form
XAD7 Prep 4	6.5	+ TPP	+ TPP Control - maximum activity	- TPP Holo-control. to demonstrate how much enzyme is present in its holo-form.
XAD7 Prep 5	6.5	- TPP	+ TPP To demonstrate whether TPP is required during immobilisation	- TPP Apo-control (-ve control). to demonstrate how much enzyme is in the apo form.

Table 3.8. Showing the experimental design for the analysis of the binding of holo-transketolase to Amberlite XAD7 and to Eupergit-C.

All biotransformations were carried out at 100 mM GA and HPA concentration containing 0.5 % (w/v) mercaptoethanol with and without 2.4 mM TPP at 25 °C, the pH was maintained at pH 7.5 by the addition of HCl as required.

3.4.2 Biotransformations with and without TPP

3.4.2.1 Activity of immobilised transketolase with and without TPP

Table 3.9 shows the results of the biotransformations reactions. In the case of both Amberlite and Eupergit-C[®], when transketolase was immobilised at pH 6.5 in the presence of TPP and the final biocatalyst assayed in the presence of TPP, the maximum activity was observed. When the enzyme was immobilised onto Eupergit-C[®] in the presence of TPP at the higher pH, the enzyme activity recovered after immobilisation was only 20 %. This indicated that the enzyme was not immobilised in its holo-form at high pHs. As previously demonstrated the fraction of enzyme in the holo-form is dependant upon pH.

The maximum possible retained activity achieved with Eupergit-C is approximately 60 %, whilst a value of 20% was obtained for Amberlite. The fraction of immobilised enzyme containing TPP was calculated from the ratio of the activity measured in the presence of TPP with that measured in the absence of the cofactor. When the enzyme is immobilised onto Eupergit-C[®] in the presence of TPP the fraction of TPP containing enzyme was 54 %. When the enzyme was immobilised in the absence of added TPP 33 % of the bound active enzyme was in the holo form. A further 200 Ug⁻¹ could be recovered by the addition of TPP during the reaction. However, the maximum activity obtained when immobilising in the absence of TPP is still significantly lower than that obtained when the enzyme was immobilised with its both of its cofactors.

In the case of XAD7 the maximum possible retained activity is 20 % at a specific activity of 260 U/g, while the amount of active bound enzyme in the holo form is 96 %. When the enzyme is immobilised to Amberlite in the absence of TPP the

specific activity achieved in the reaction in the presence of TPP was 193 U.g^{-1} , while the specific activity achieved in the reaction in the absence of TPP was 118 U.g^{-1} . This indicates that 61 % of the active bound enzyme is in the holo-form, the activity obtained in the absence of TPP is 74 % of the maximum activity obtained.

Support	Amount Bound Ug^{-1} (dry)	Biotrans +TPP		Biotrans -TPP		Comments
		U.g^{-1} (dry)	Retained Activity	U.g^{-1} (dry)	Retained Activity	
Eupergit 1 immob'd (pH 7.5) +TPP	1200	124	10 %	70	6 %	Holo-enzyme not bound at pH 7.5.
Eupergit 2 Immob'd +TPP	1434	840	58 %	460	32 %	Not all bound enzyme active sites are in holo-form.
Eupergit 3 Immob'd -TPP	1347	436	32 %	146	10 %	TPP can activate the immobilised enzyme.
XAD7 1 Immob'd +TPP	1330	260	20 %	250	19 %	All possible enzyme active sites in its holo-form.
XAD7 2 Immob'd -TPP	1300	193	13 %	118	9 %	TPP can activate the immobilised enzyme.

Table 3.9 Comparison of the activities of transketolase immobilised in its apo- and holo-forms to Amberlite XAD7 and to Eupergit-C[®] as measured by biotransformation in the presence and absence of TPP.

A comparison of the erythrose production profiles for the various Eupergit-C immobilised transketolase preparations described in table 3.9 above are shown in figure 3.10A.

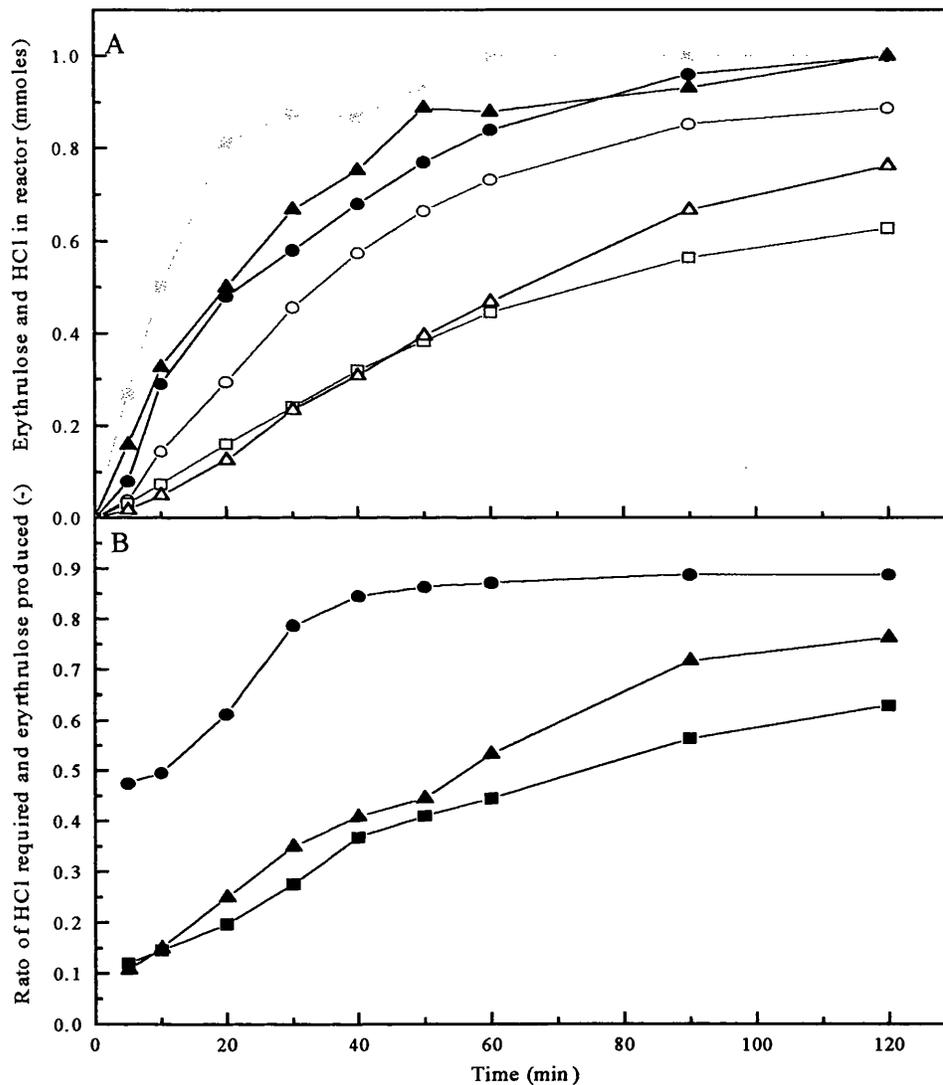


Figure 3.10 Comparison of Eupergit-C immobilised transketolase. A. Apo-transketolase (—●—), (—○—), holo-transketolase (—⊛—), (—□—) and holo-transketolase assayed in the absence of TPP (—▲—), (—△—). B. Comparison of the ratios of titrant addition to product production. Apo-transketolase (—●—), holo-transketolase (—■—) and holo-transketolase assayed without TPP (—▲—).

3.4.2.2 Titrant Profiles

The total requirement for acid is much lower for XAD7 than for the Eupergit-C preparation immobilised in the absence of TPP irrespective of the similarity in reaction rates (figure 3.10B, table 3.10). For the two Eupergit-C biotransformations more acid is required when cofactor is not present in the biotransformation batch despite the slower reaction rate and the fact that less material was present. The comparison of the ratios of titrant addition and conversion for Amberlite and Eupergit-C immobilised transketolase are shown in figure 3.8. In the case of Eupergit-C there is a clear difference in the amount of titrant required during operation in the presence and absence of TPP. While in the case of Amberlite the titrant requirements are similar for operation in the presence or absence of TPP.

Support	Reaction Rate $\mu\text{moles}\cdot\text{min}^{-1}$	Titrant Addition Rate $\mu\text{moles}\cdot\text{min}^{-1}$	Equilibrium Value mmoles	Comments
Eupergit C +TPP	67	7.8	0.628	0.16 g (dry) Eupergit
Eupergit-C -TPP	36	8.0	0.763	0.17 g (dry) Eupergit
XAD7 +TPP	38	3.5	0.310	0.30 g (dry) XAD7
XAD7 -TPP	37	3.5	0.300	0.29 g (dry) XAD7

Table 3.10. Comparison of the initial titrant addition rates for biotransformations with preparations of transketolase immobilised onto Eupergit and Amberlite with TPP.

3.4.3 Cofactor washout

Although the enzyme can be immobilised onto Eupergit-C® in its active form with an appreciable improvement in activity it is possible that the cofactor could be washed out of the enzyme's active site. The binding of TPP to the soluble enzyme has been shown in this work and by previous researchers (Heinrich, P. *et al.*, (1972)) to be reversible. In order to determine whether the cofactor could be removed from the Eupergit-C® enzyme, a packed bed bioreactor was operated in the presence and absence of TPP. The bed was operated at a flow rate of $0.5 \text{ mL}\cdot\text{min}^{-1}$ with a bed depth of 0.5 cm and a bed volume of 1.5 mL. The short bed height was chosen to minimise the effect of back-mixing and any product inhibition upon the reaction rate. Figure 3.11 shows the variation in the number of moles of erythrulose in the outlet of the reactor with throughput volume.

During the first stages of the reaction the bioreactor was allowed to equilibrate for 10 total column volumes in the presence of cofactors (stage 1 in figure 3.11). During this stage the maximum activity of $840 \text{ U}\cdot\text{g}^{-1}$ was observed. After the initial equilibration stage the cofactor was removed and the bioreactor was operated in the absence of cofactor (stage 2). During this period the amount of erythrulose in the outlet decreased from 0.38 mmoles to 0.25 mmoles within 26 total column volumes. In general the amount of enzyme in the holo-form can be determined by measuring the activity in the absence and presence of cofactors (Ikeda, S, I *et al.*, (1975)). The loss in conversion along the length of the column demonstrates that either the cofactor has been removed from the enzyme or the immobilised enzyme has been inactivated. However, in the case of the control, assayed continually in the presence of TPP, the degree of conversion at the column outlet remained constant. As a result, the reduction in activity of the immobilised holo-enzyme preparation in the absence of cofactors cannot be attributed to deactivation of the enzyme.

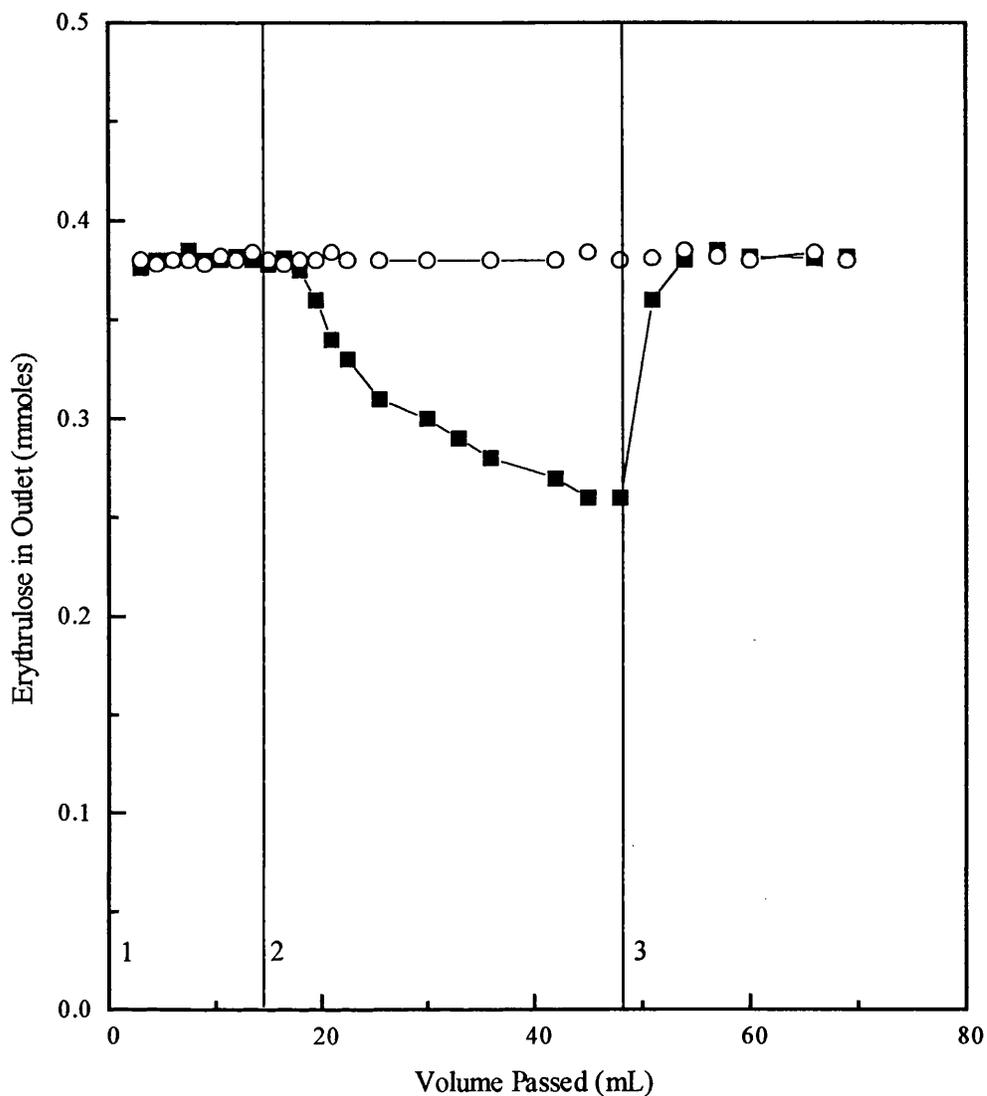


Figure 3.11 Dissociation of TPP from immobilised holo-transketolase. 100 mM reactants were passed through a 10 mm bed depth packed bed. Graph shows the change in erythrulose in the outlet during operation when TPP was not included in the reaction mixture (zone 2) and when it was returned (zone 3) (—■—). While operation solely in the presence of TPP (—○—) acted as a control.

In order to demonstrate whether the cofactor could be reintroduced into the immobilised enzyme the bioreactor was operated in the presence of TPP again (stage 3). As soon as the cofactor was returned into the system there was a rapid increase in the degree of conversion along the length of the bioreactor. The amount of erythrulose present in the outlet returned to the value obtained during stage 1 within 5 total column volumes. This demonstrates that the removal of TPP from the active site of immobilised transketolase is reversible.

Table 3.11 compares the specific and retained activities of holo-transketolase preparations immobilised to Amberlite XAD7. In order to demonstrate whether cofactor can be removed from Amberlite immobilised transketolase the preparations were used in successive biotransformations.. Successive biotransformations were performed in the presence of TPP as a control. While in order to demonstrate whether TPP could be removed, the first reaction with preparation 1 was carried out in the presence of TPP while the successive reaction was performed in the absence of TPP.

Prep ⁿ	Batch 1		Batch 2		Batch 2/Batch 1 %
	Ug ⁻¹	Retained Activity	Ug ⁻¹	Retained Activity	
1	464	36 %	406	31 %	87 %
Control	408	30%	361	28%	88%

Table 3.11. The stability of the activity of XAD7 immobilised holo-transketolase in the presence and absence of cofactors.

Both the control and preparation 1 lost activity between batches. These data would suggest that cofactor is not washed out of the XAD7 immobilised transketolase.

3.5 Characteristics of Immobilised Transketolase

The characteristics of the immobilised enzyme will determine how it is to be used within the bioreactor and consequently effect the productivity of the biocatalyst and the mode of operation of the bioreactor. The two predominant characteristics of an immobilised enzyme are its stability and its activity. This section describes the effect of mass transfer limitation upon the activity of the immobilised enzyme and the stability of the enzyme.

3.5.1 Mass transfer

3.5.1.1 The effect of Substrate concentration

The activity of the biocatalyst and productivity of the bioreactor will be influenced by the starting substrate concentration. Also, as the reaction proceeds the substrate concentration will be reduced appropriately. This in turn will influence the rate of reaction, and in many situations where immobilised biocatalysts are used the rate of reaction can become limited by the rate of transport of the substrate into the support material. Therefore in order to determine the effect of substrate concentration several biotransformations were carried out with various concentrations of HPA and GA, although the other conditions such as pH and cofactor concentrations were as usual. The reaction rates and percentage activities from these reactions are shown in table 3.12.

This demonstrated that the maximum activity for transketolase immobilised onto Eupergit-C® is achieved at around 50 mM substrate concentrations. Since there is little change in activity between the holo-enzyme preparations at 100 mM and 500 mM, it can be concluded that the apparent maximum activity of the immobilised holo-enzyme was $400 \mu\text{moles}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ ($800 \text{ U}\cdot\text{g}^{-1}$) while the apparent maximum activity of the apo-enzyme was $140 \mu\text{moles}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ ($280 \text{ U}\cdot\text{g}^{-1}$).

Preparation	Substrates concentration mM	Enzyme load U.g ⁻¹ (dry)	Activity $\mu\text{moles}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ (dry)	Percentage Activity %
Eupergit-C Apo-TK	15 HPA 15 GA	1660	31	4
Eupergit-C Apo-TK	50 HPA 50 GA	2200	160	15
Eupergit-C Apo-TK	100 HPA 100 GA	1520	160	21
Eupergit-C Holo-TK	100 HPA 100 GA	1430	418	60
Eupergit-C Holo-TK	500 HPA 500 GA	1051	400	70

Table 3.12 Comparison of the reaction rates and retained activities of a variety of immobilised enzyme preparations showing the effect of substrate concentration.

3.5.1.2 Effect of particle size

It was previously demonstrated that as the enzyme load was increased the efficiency of the binding was reduced, and the retained activity declined to 20 % at a loading of 2100 U.g⁻¹. This decrease in activity could be explained as being a function of the mass transport of the substrates into the centre of the immobilised enzyme particle. It has been shown that the amount of mass transfer will be a function of the radius of the support material (Carleysmith, S *et al.*, (1980)). Subsequently in order to demonstrate whether mass transport is the cause of the reduction of retained activity preparations of Eupergit-TK and Amberlite-TK at high enzyme load were ground to reduce the particle size. Figure 3.12. compares the erythrulose production profiles for Eupergit-TK ground and unground preparations, the figure also shows the erythrulose production profile achieved with soluble transketolase. While figure 3.13 compares the profiles for XAD7 ground and unground preparations.

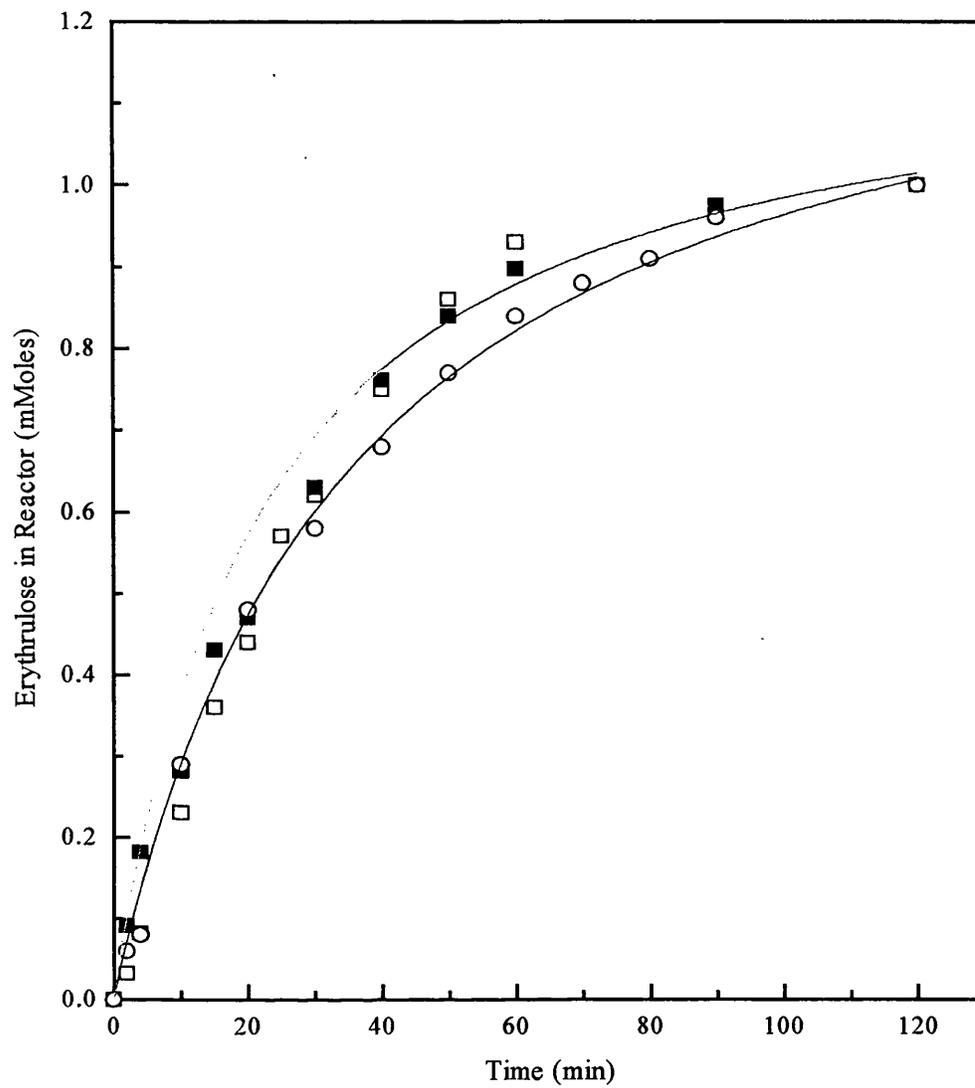


Figure 3.12. Comparison of the erythrulose production profiles for soluble (■) and ground (□) and unground (○) apo-transketolase immobilised onto Eupergit-C.

A statistical T-test as described in section 2.8 was performed on the erythrulose production profiles of the ground and unground preparations of Eupergit-C[®] immobilised transketolase. The difference in the amount of erythrulose produced at each time point was calculated for the three replicate pairs, this was then used to calculate the T-value for the data at each time point, the observed T-values were compared with the tabulated T-values (Rees (1985)).

Time	Difference (ground - unground)			Mean Difference	Obs. T-value	Table T- value
2	-0.026	-0.028	-0.025	-0.026	-30.6	4.303
4	0.035	-0.002	0.005	0.013	1.11	4.303
10	-0.096	-0.06	-0.012	-0.056	-2.3	4.303
20	-0.05	-0.04	-0.06	-0.05	-8.67	4.303
30	0.025	0.04	0.01	0.025	2.886	4.303
40	0.035	0.07	n/d	0.0525	3.67	63
50	0.035	0.09	-0.02	0.035	1.10	4.303
60	0.05	0.09	0.01	0.05	2.17	4.303
90	0.08	n/d	0.07	0.075	1.73	63
120	0.04	0.00	0.08	0.04	1.73	4.303

Table 3.14 Table showing the T-values for ground and unground preparations of Eupergit-C[®] transketolase. Tabulated T-values were obtained at a 5 % confidence interval.

The observed T-values were generally below the tabulated T-values. This signifies that at this confidence level, and with the low degree of freedom (2) the data all fall within the same T-distribution and are therefore not significantly different

There was no difference in the initial rates between the ground and unground immobilised enzyme preparations. Consequently the apparent maximum initial reaction rate was independent of particle size at this enzyme load. This demonstrated that the transport of substrate into the Eupergit-C beads was unlikely to limit the rate of reaction.

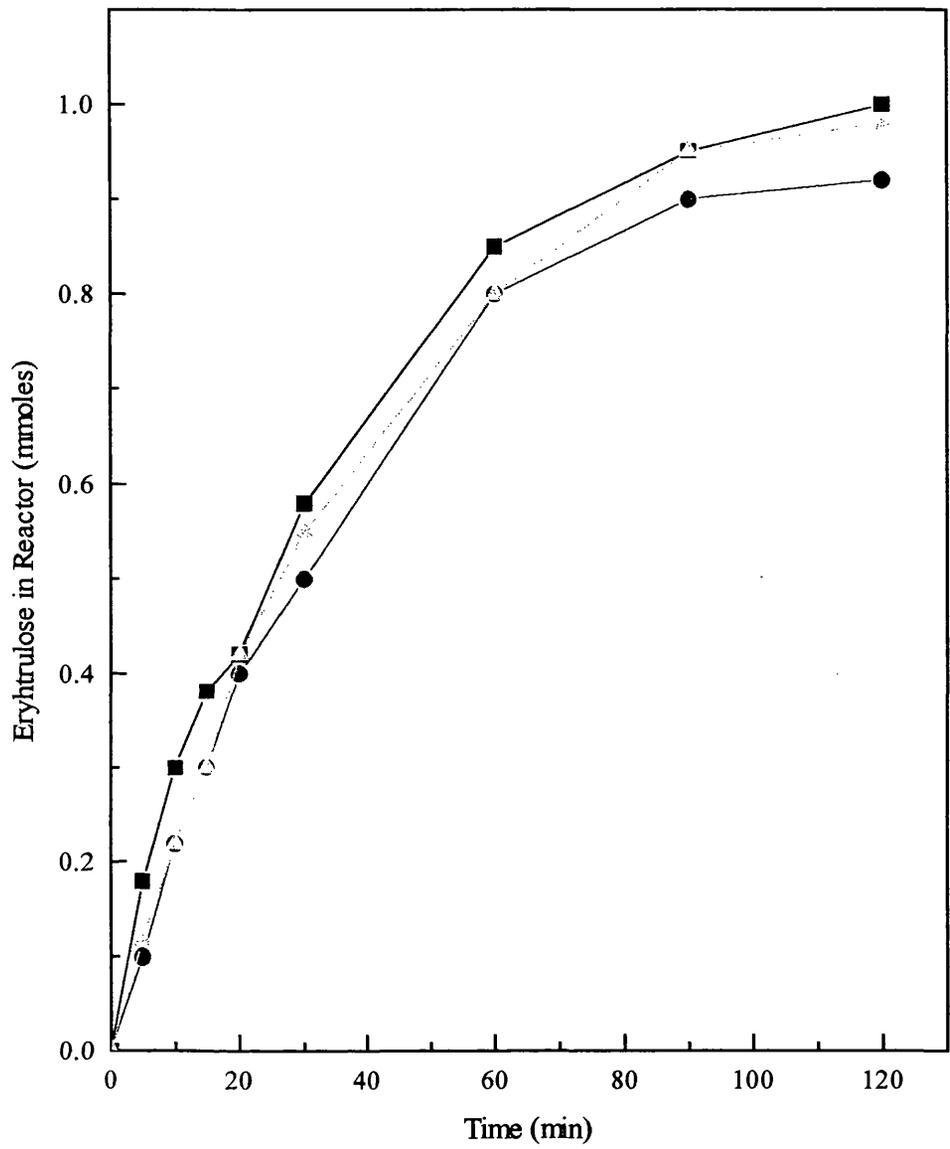


Figure 3.13 Erythrulose production profiles for XAD7 immobilised transketolase. Comparison of soluble transketolase 4.0 U mL^{-1} (—■—), ground immobilised transketolase 3.7 U mL^{-1} (· · · △ · · ·) and whole immobilised transketolase 3.8 U mL^{-1} (—●—).

Figure 3.13 compares the erythrulose production rates for ground and unground transketolase immobilised onto Amberlite XAD7 beads. The Amberlite beads are significantly larger than Eupergit-C[®], consequently the rate of transport of substrate into these beads can be expected to be slower than that achieved with the smaller Eupergit-C beads. However, again there was no difference between the rate of erythrulose production between ground and whole Amberlite beads. This demonstrated that even in the case of the significantly larger beads the rate of reaction is slower than the rate of transport of the substrate into the beads.

It would be expected that as the bulk substrate concentration is reduced the effect of mass transfer would increase since the concentration 'driving force' between the bulk environment and microenvironment is smaller. If mass transport of substrates into the support controls the observed reaction rate a greater difference between the reaction rates of ground and unground particles would be expected at lower substrate concentrations. Figure 3.14 compares the erythrulose production profiles for whole and ground Eupergit-C[®] beads showing the influence of substrate concentration upon the initial reaction rate and the shape of the profiles, and the initial reaction rates are given in table 3.14 below.

Substrate Concentration (mM)	Enzyme load (U)	Activity ($\mu\text{moles}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$)		Retained Activity (%)	
		Whole beads	Ground beads	Whole beads	Ground beads
15	1660	31	35	4	4
50	2200	160	170	15	16
100	1520	160	170	21	22

Table 3.14 comparison of activities of ground and unground Eupergit-TK beads at low substrate concentrations.

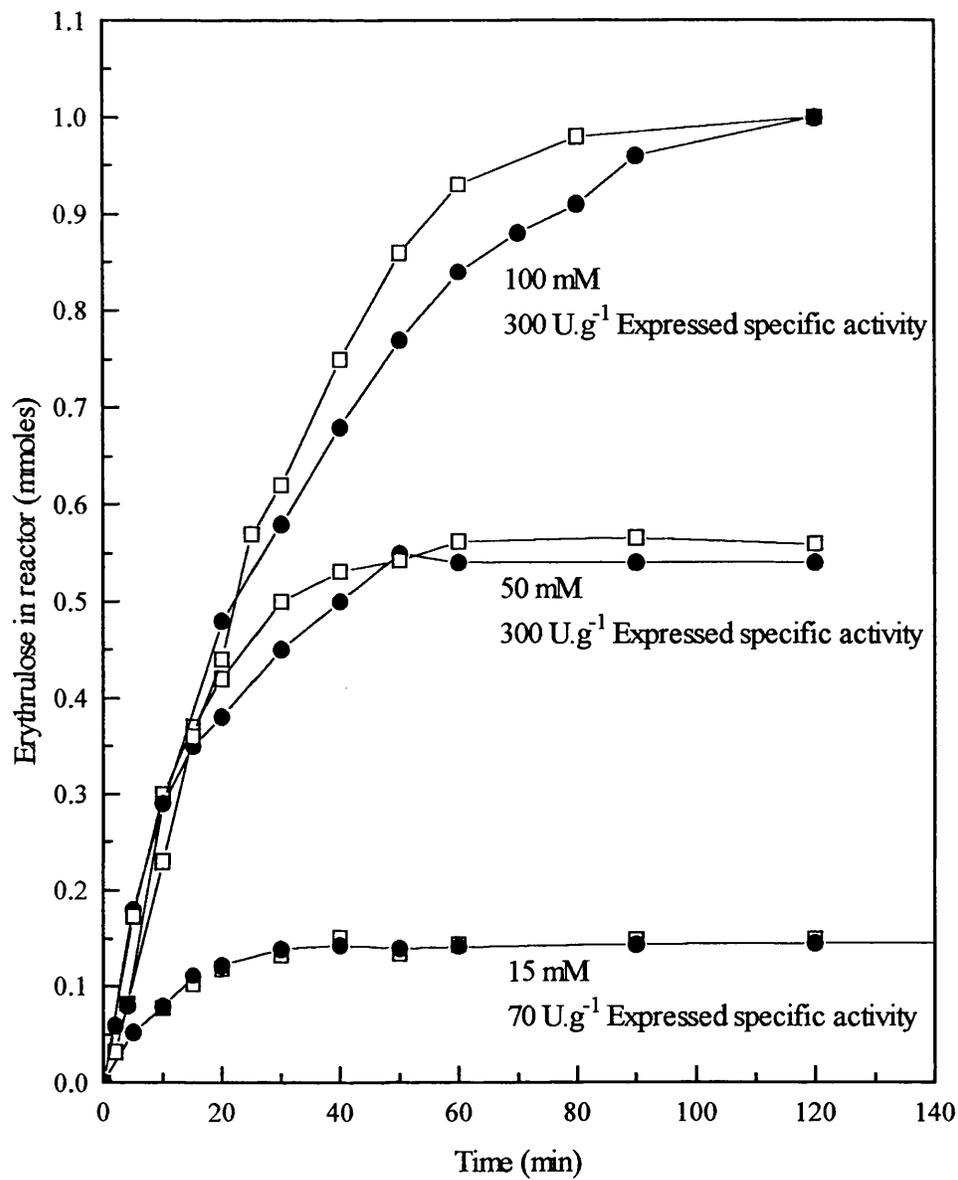


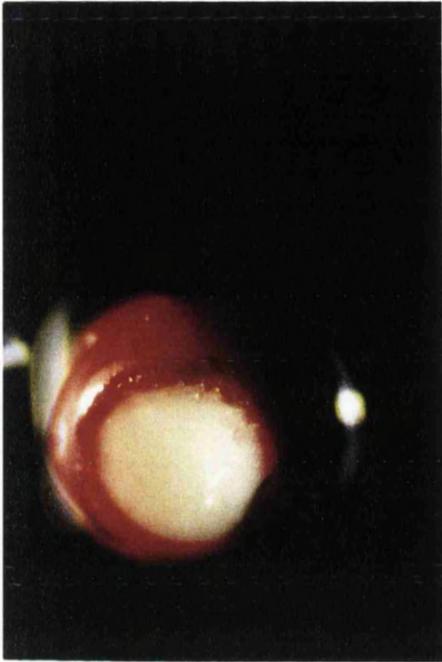
Figure 3.14 Effect of starting substrate concentration upon erythrulose production profiles. Showing a comparison of ground (□) and whole (●) Eupergit-C beads. Beads were loaded with 2000 U.g^{-1} .

The initial reaction is unchanged between whole and ground beads at the various substrate concentrations. However, at the higher substrate concentration (100 mM) the erythrulose profiles begin to diverge as the reaction proceeds towards complete conversion. This would be expected if erythrulose concentrations within the support are sufficient to cause product inhibition.

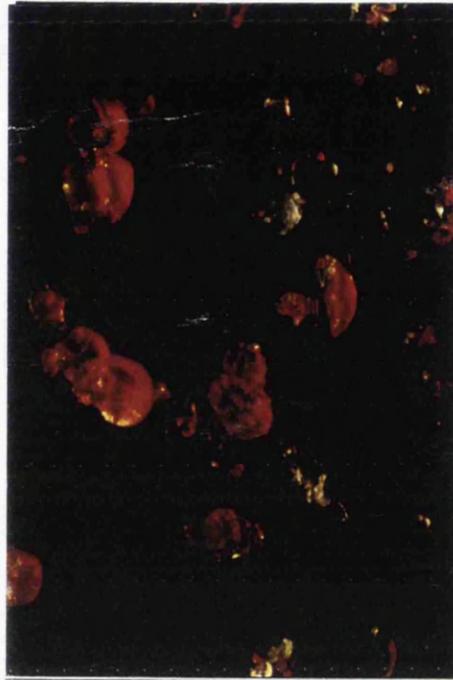
3.5.1.3 Penetration of enzyme into the supports

The depth of penetration of enzyme into the support material can effect the rate activity and retention of activity of a biocatalyst particle. If the protein is distributed heterogeneously, with the majority of the protein at the surface, the substrate will have to travel a smaller distance into the support material to reach an enzyme. Subsequently the affect of diffusion will be lower if the enzyme is predominantly distributed towards the surface. If the distribution is homogenous the affect of diffusion will be more pronounced, since in the extreme case the population of enzyme near the centre of the support will be not exposed to any substrate, rendering it unactive. In order to observe the distribution of protein within both Eupergit-C® and Amberlite XAD7 protein was visualised with Ponceau stain (Carleymith 1979). After staining the beads were smashed by pestle and mortar cooled with liquid nitrogen. This allowed cross sections of the beads to be microscopically visualised.

1



3



2



4



Figure 3.15 Shows the distribution of protein within the support materials Amberlite and Eupergit-C. Transketolase was immobilised onto Amberlite at low phosphate concentration (1) and high phosphate concentration (2), and onto Eupergit-C® with low (3) and high phosphate concentrations (4). Magnification X 10.

The protein stain was found to be distributed homogeneously through the Eupergit-C® whether the enzyme had been immobilised in the presence of high phosphate concentrations or not. While in the case of Amberlite XAD7, when the enzyme was immobilised in the presence of high phosphate concentrations the stain was distributed mainly at the surface of the material, leaving the centre of the particles unstained. When the enzyme was immobilised in the presence of low phosphate concentrations the stain was also distributed heterogeneously throughout the support material.

3.5.2 Stability

In order to obtain a stepwise increase the productivity of a biotransformation process it is often necessary to reuse the biocatalyst several times. Consequentially the stability of an immobilised biocatalyst is an important characteristic constraining both the decision whether to immobilise and the mode of operation of an immobilised enzyme bioreactor. Mitra (1997) demonstrated that transketolase is susceptible to irreversible denaturation by the aldehyde acceptor substrate (in this case glycolaldehyde) and that it could be stabilised by operating the bioreactor in fed batch mode. It has been widely demonstrated that immobilisation can result in the stabilisation of the enzyme, it is the aim of this section to determine whether transketolase can be stabilised by immobilisation onto Eupergit-C® or Amberlite XAD7. Although a substrate feed strategy can increase the operational stability of the biocatalyst operation in batch mode was selected for these experiments as it represents the worst case scenario.

3.5.2.1 Storage Stability

In order to investigate the storage stability of immobilised transketolase, preparations of the immobilised enzyme were stored in 10 mM phosphate buffer at pH 7.5 at 4 °C in the presence of a nitrogen headspace. Activity measurements were made at regular intervals under the usual conditions. After 2 months storage 50 % of the original activity had been retained by the immobilised enzyme. However when the enzyme was stored in the absence of mercaptoethanol or a nitrogen headspace 50 % of the activity was lost within 24 hours of storage.

3.5.2.2 Effect of mercaptoethanol

Holo-transketolase has been demonstrated previously to be susceptible to denaturation by oxidation. In order to demonstrate whether immobilisation was able to stabilise the enzyme against this denaturation mechanism successive biotransformations were performed in the presence and absence of mercaptoethanol. All biotransformations were carried out with 100 mM reactants and in the presence of cofactors.

Preparation	Batch1		Batch2		Batch 2 / Batch1 (%)
	Specific activity (U g ⁻¹)	Retained activity (%)	Specific activity (U g ⁻¹)	Retained activity (%)	
XAD7	250	20	145	10	58
Eupergit	250	20	140	10	57

Table 3.15. Batch to batch stability of immobilised transketolase in the absence of mercaptoethanol as a stabilising agent during the biotransformation. The table also gives a comparison between Eupergit-C[®] and XAD7 preparations. (the Eupergit-C biocatalyst was prepared in the absence of TPP)

In order to demonstrate whether the presence of a reducing environment is able to enhance the operational stability of the immobilised enzyme consecutive 100 mM biotransformation batches were performed in the presence of the reducing environment (figure 3.16). No activity was lost during 4 consecutive biotransformations with the Eupergit-C immobilised biocatalyst. This demonstrated that the main mechanism for the loss of activity by immobilised transketolase is the oxidation of the holo-form of the enzyme. Due to the cost of the reactants it was deemed too expensive to use the immobilised enzymes repeatedly (glycolaldehyde £ 74.mole⁻¹ and hydroxypyruvic acid £ 3,328.mole⁻¹). In order to simulate the operational stability of immobilised transketolase the biocatalyst preparations were stored in 500 mM glycolaldehyde at operational pH and temperature.

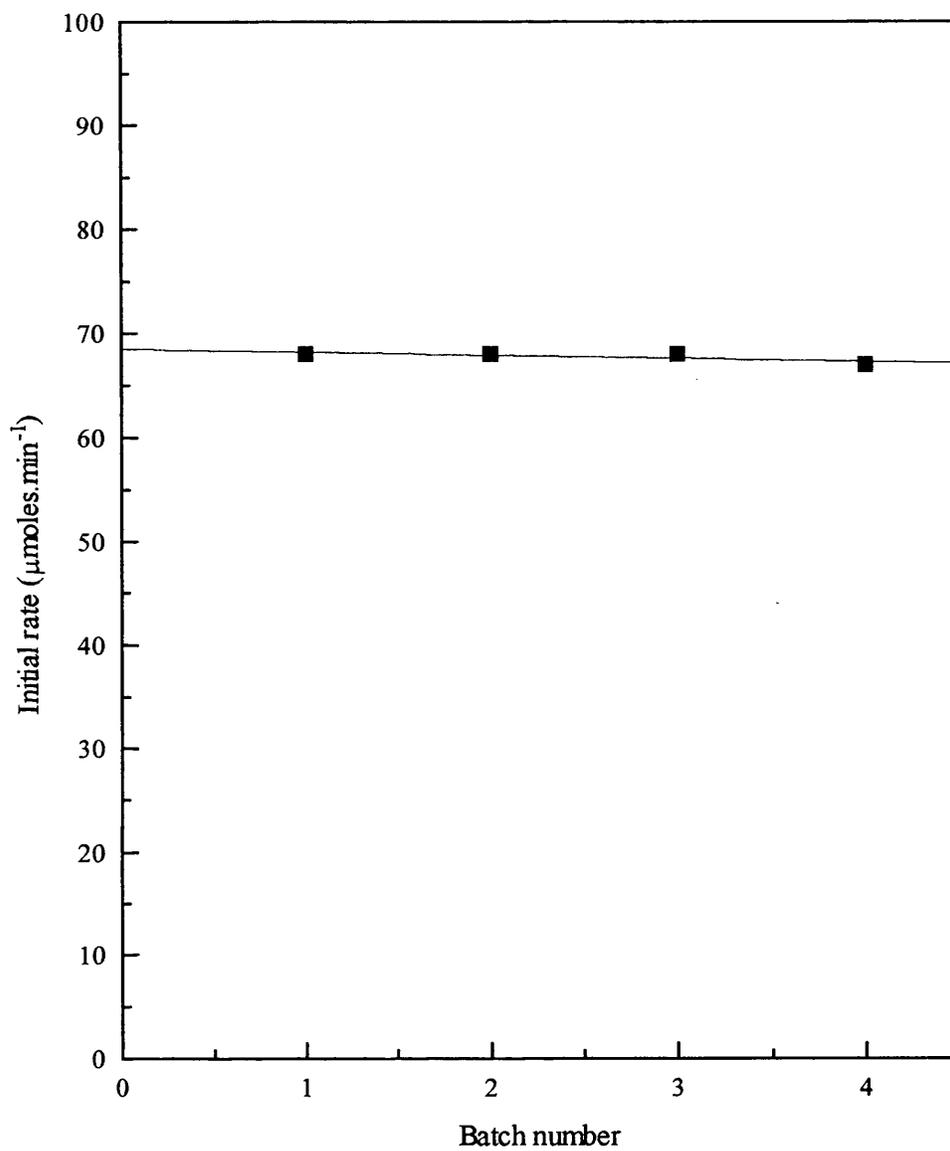


Figure 3.16 Erythrulose production rates of consecutive reactions with holo-transketolase immobilised onto Eupergit-C. All batches were performed in the presence of 0.5% (w/v) mercaptoethanol. $1300 \text{ U}\cdot\text{g}^{-1}$ (dry) transketolase bound with $20 \text{ U}\cdot\text{mL}^{-1}$ enzyme charged to reactor.

3.5.2.3 Stability of immobilised transketolase in Glycolaldehyde

The denaturing effect of glycolaldehyde has been demonstrated by Mitra, R, K (1997) to be influential in the determination of the mode of operation of the bioreactor. If the biocatalyst is to be reused economically its ability to withstand the denaturing effects of the toxic substrate is arguably one of its most important characteristics. To determine the effect of glycolaldehyde upon the stability of immobilised transketolase, beads were incubated in 500 mM glycolaldehyde under reaction conditions. Samples were taken and the remaining activity determined by biotransformation assay. Figure 3.17 shows that no activity is lost by Eupergit-C[®] immobilised transketolase over 6 hours of operation, while the free enzyme shows a significantly lower half life of less than 1 hour under the same conditions.

In order to simulate some of the effects of operation the immobilised enzyme was stored in glycolaldehyde under reaction conditions for an extended period of time. The results for the extended storage of immobilised transketolase are shown in table 3.15 below. The table also compares the stability of XAD7 and Eupergit-C[®] preparations of transketolase.

Time (hours)	Eupergit-C [®]		XAD7	
	Specific activity (U g ⁻¹)	Remaining activity (-)	Specific activity (U g ⁻¹)	Remaining activity (-)
0	1105	1	408	1
24	900	0.8	301	0.75
100	552	0.5	160	0.4
288	156	0.11	85	0.08

Table 3.16. The long term stability of immobilised transketolase in 500 mM glycolaldehyde. Comparing the remaining activities of XAD7 and Eupergit-C[®] preparations of the enzyme.

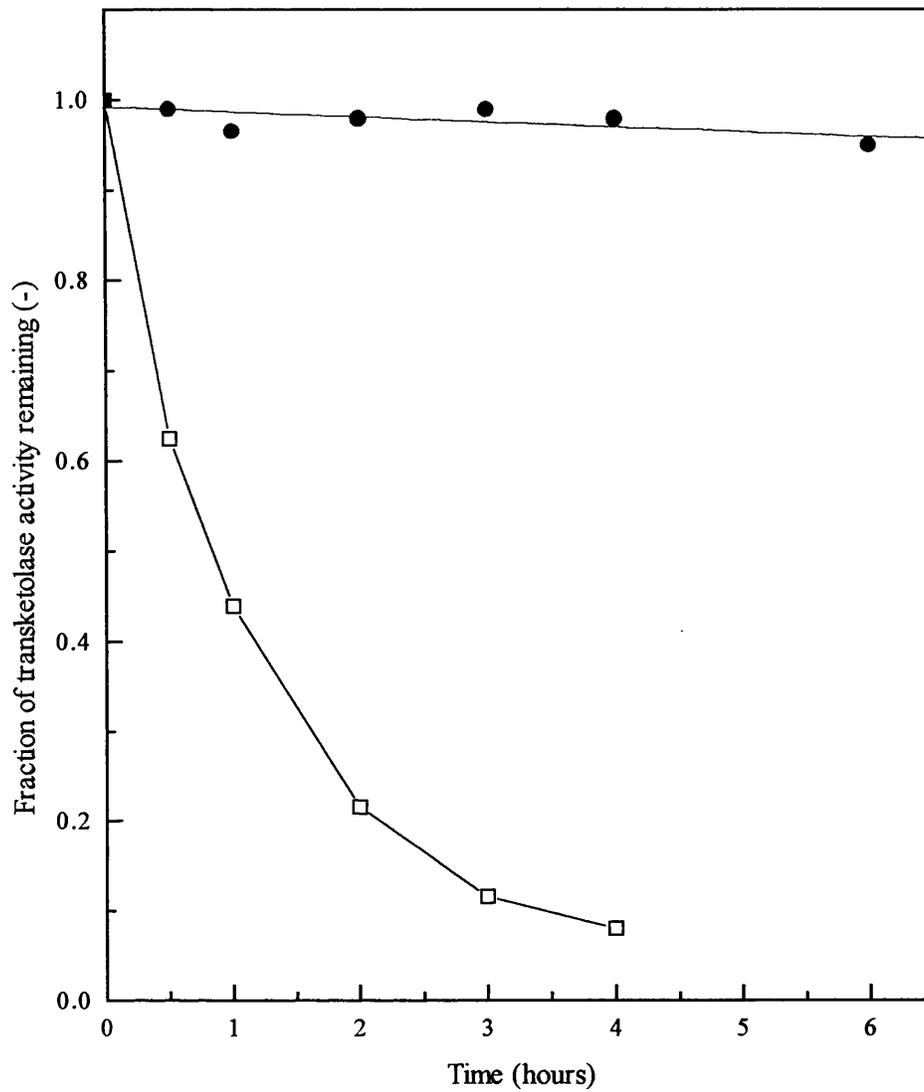


Figure 3.17 Comparison of the stability of free (\square) and immobilised (\bullet) transketolase in the presence of glycolaldehyde [500 mM] at pH 7.5 containing TPP [2.4 mM], $MgCl_2$ [9 mM] and 0.5 % (w/v) mercaptoethanol.

Discussion

4.1 Preparation and Use of Immobilised Transketolase

4.1.1 Immobilisation of Transketolase

4.1.1.1 Binding of Transketolase

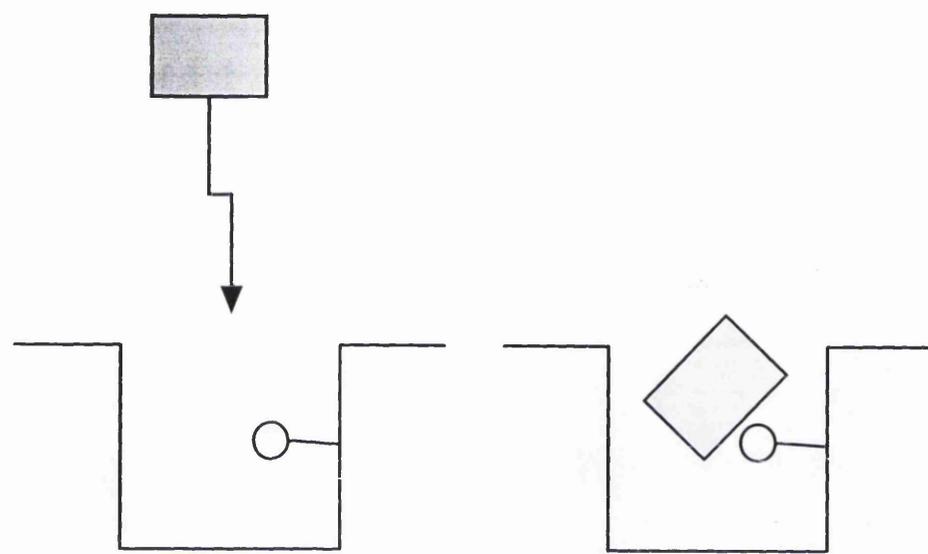
The swelling characteristics of the support materials demonstrated that Eupergit-C had a more open structure than XAD7 (Arshady, R. (1991)). Since Eupergit-C was able to swell to four times its dry volume the functional groups within the support material would be more accessible to the enzyme. This implies that of the two supports protein would be most likely to penetrate Eupergit-C more homogeneously than Amberlite XAD7. The staining of Eupergit-C confirmed that protein was distributed homogeneously throughout the structure, despite changes in the rate of binding of protein as the ionic environment was altered. Conversely the staining of Amberlite XAD7 showed that more protein concentrated near the surface of the support. Thus demonstrating that the nature of the support can influence the distribution of the material within its structure.

It is interesting to note the difference in the amount of enzyme that could be washed off Eupergit-C during the course of immobilisation, in comparison with that observed in the case of Amberlite XAD7. As the contact time between Eupergit-C and transketolase was increased the amount of protein in the wash declined. Conversely, as the contact between XAD7 and transketolase continued, the amount of transketolase in the wash increased. This implies that in the case of transketolase, the initial drop in soluble transketolase activity was due to enzyme becoming entrapped, or adsorbed into the more open pore volume of the material. This would indicate that the covalent attachment between the protein and the oxirane groups is slower than that between glutaraldehyde and the protein.

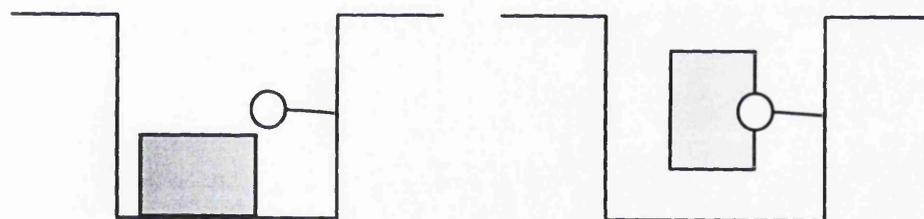
In their discussion of direct enzyme coupling methods Porath and Axen (1976) described oxirane coupling as being slow, but able to form stable bonds even at low

binding pH's. Since the ionic strength of the binding environment had a dramatic effect upon the rate of disappearance of enzyme from solution it would be expected that some form of hydrophobic or hydrophilic interaction between the support and the enzyme was promoted. Since protein penetrated through the whole bead despite changes in ionic environment, the hydrophobic\philic interaction is probably slower than the initial penetration. Consequently it is possible to propose a mechanism of immobilisation comprising of 3 stages. Firstly the rapid inclusion of protein within the pore volume of the support, followed by a slower hydrophobic interaction, where the protein is brought into contact with the oxirane groups, and finally a slower covalent attachment process (figure 4.1).

In the case of Amberlite XAD7, the ionic environment proved to have little effect upon the rate of immobilisation. Since the enzyme was distributed mainly at the surface of the support the rate of chemical attachment of the enzyme to the support by the glutaraldehyde and the interaction between the protein and the surface of the material are faster than the rate of diffusion of protein into the support material. This demonstrates that the mechanism of attachment to Amberlite XAD7 comprises of a hydrophobic step and a covalent attachment. The inclusion of the protein in the pore volume was much slower than in the case of Eupergit-C. This also indicates that the coupling between transketolase and glutaraldehyde is faster than the coupling of transketolase with oxirane groups.



Inclusion into pore volume



Adsorption followed by covalent attachment

Figure 4.1 Schematic representation of the proposed 3 stage mechanism of covalent attachment of transketolase to Eupergit-C[®].

4.1.1.2 Retention of Activity

The amount of activity retained upon the support after binding has been defined as the ratio of amount of activity measured in the 100 mM biotransformation and the amount of enzyme bound to the support, as measured by the mass balance of activity in solution. The retained activity is a factor which describes the efficiency of the immobilisation procedure. The retained activity will be expected to be a function of the following parameters:

- The amount of enzyme inactivated by the immobilisation process.
- The mass transport of substrate into the support.
- Electrostatic or other partitioning effects.

In addition to these factors, in the case of transketolase the cofactor is required for full activity. Therefore retention of activity will also be a function of the ability of the cofactor to bind to and subsequently activate the immobilised enzyme. Issues related to cofactor binding will be discussed in section 4.1.2.

The lowest retention of activity was recorded on both Eupergit-C and Amberlite XAD7 when the enzyme was immobilised in the absence of its cofactors. As the contact time between the supports and transketolase was increased the amount of specific activity increased, however, the retention of activity remained around 15 % in both cases. This would seem to indicate that prolonged contact between the enzyme and support has no effect upon the efficacy of the immobilisation procedure.

An appreciable change in the retention of activity was observed as the enzyme challenge was increased. The highest specific activity of 320 U.g^{-1} (dry Eupergit-C) was achieved with a low retention of activity (15 %). This low retention of activity can not be explained by transport of substrates into the support since the ground preparations did not give appreciably greater reaction rates. The open structure of Eupergit-C and the apparent ease by which the enzyme can permeate the material suggest that the smaller substrate molecules would be easily able to reach the enzyme bound towards the centre of the support.

Subsequently the retention of activity by transketolase immobilised covalently onto Eupergit-C[®] is apparently due to either inactivation of the enzyme during the binding processes or cofactor related issues. The former explanation does not seem to be as viable since preparations with a low enzyme loading showed a retention of activity of 70 %.

4.1.2 Implications of cofactor requirement

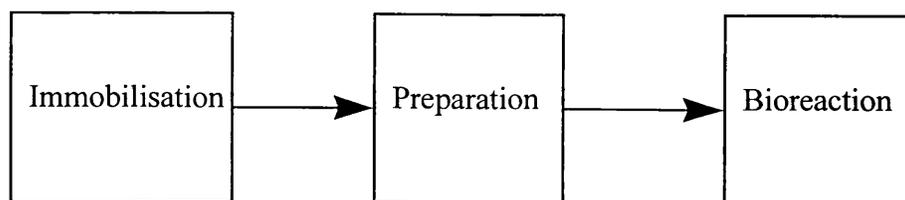
Transketolase is a homodimeric cofactor requiring enzyme. In the total absence of cofactor the enzyme is completely inactive. Consequently cofactor must be introduced into the active site at some point. This can be done before, during or after immobilisation. In other words, the cofactor can be co-immobilised with the enzyme, or it can be included during the reaction stages. Finally cofactor can be introduced at all stages leading up to and including the biotransformation reaction. The advantages and problems associated with these strategies are outlined in figure 4.2. The characteristics of the binding between the enzyme and its cofactors will determine when the cofactor must be introduced.

During the characterisation of the free enzyme it was demonstrated that the amount of enzyme in the holo-form was influenced by the pH of the surrounding solvent. It was also observed that the amount of enzyme in the holo-form was never 100 %, even in the more acidic pHs. Consequently it will be expected that even if the enzyme is immobilised in the presence of its cofactors, a further addition of cofactor will be necessary before full activity can be achieved.

It is striking to note the influence enzyme load had upon the retained activity of the enzyme immobilised onto Eupergit-C[®] in its apo-form. At the lowest enzyme load the amount of activity retained on the addition of TPP and Mg²⁺ was approximately 70 % of the maximum, while as the enzyme load was increased only 20 % activity could be recovered on the addition of the cofactors. While once the enzyme was immobilised in the presence of its cofactors the amount of activity recovered on the addition of TPP was 60 % at high enzyme loads. This surprising observation discounted mass transfer

of the substrates into the material as the cause of the lower retained activity of the enzyme immobilised without TPP.

The influence of pH upon the immobilisation of transketolase in its active state was also studied. The enzyme immobilised at pH's in the neutral region did not demonstrate an increased retention of activity. Whereas when the binding pH was in the acidic region a higher retention of activity was observed. This would seem to demonstrate that when the enzyme is immobilised at low pH's, the cofactor is retained in the active site. Whereas in more alkaline pH's the cofactor is more readily lost from the active site.



Disadvantages

- | | | |
|---|---|---|
| <ul style="list-style-type: none"> •pH conditions non ideal for binding reaction •Instability of holo-transketolase needs to be overcome •Subsequent wash out of TPP | <ul style="list-style-type: none"> •Access of TPP to active site •Instability of holo-transketolase •Subsequent washout of TPP | <ul style="list-style-type: none"> •Access of TPP to active site •Retained activity reduced |
|---|---|---|

Advantages

- | | | |
|--|---|---|
| <ul style="list-style-type: none"> •Possible protection of active centre •High retention of activity | <ul style="list-style-type: none"> •Negates problems of instability of holo-enzyme during immobilisation | <ul style="list-style-type: none"> •Negates problems of instability of holo-enzyme during immobilisation •Reduces amount of cofactor used overall |
|--|---|---|

Figure 4.2 The introduction of cofactor into the immobilised enzyme biocatalyst. Describing some advantages and disadvantages of each stage of introduction.

This difference in activation of the enzyme immobilised in the absence of cofactors and the preparation immobilised with the cofactors present could be a function of the parameters outlined below, acting individually or together:

- Bound monomers. The immobilisation of monomeric transketolase, which cannot be activated in the presence of TPP.
- Inactivation due to binding reaction. The incorporation of essential amino acids involved in TPP association during immobilisation. Several histidines and a tyrosine are involved in the binding of TPP to transketolase (Schneider and Lindqvist (1993)). These may be chemically modified during covalent attachment, rendering the bound enzyme unable to reassociate TPP.
- Steric hindrances. The orientation of the enzyme on the support material in a configuration that prevents the TPP from gaining access and subsequently activating the enzyme.
- Transport of TPP to the active site. The mass transfer of the cofactor molecule through the support material to the immobilised enzyme.

When the enzyme was immobilised in the presence of TPP the maximum specific activity was observed in the case of both support materials. However, Eupergit-C® demonstrated the highest activity. Since the apo-enzyme could be bound at low enzyme loading with a 70 % recovery of activity on the addition of cofactors, the presence of bound monomers is unlikely. When the Eupergit-C enzyme was operated by packed bed it was possible to remove cofactor from the enzyme's active centre, as was predicted previously. After removal of the cofactor, it was possible to completely reactivate the immobilised enzyme by the re-addition of cofactor to the reaction mixture. This indicated that the active sites of the enzyme had not been occluded, cofactor could penetrate the support material. Since the immobilised enzyme could be completely reactivated by the subsequent addition of cofactor to the feed stream it is also unlikely that the apo-enzyme dissociated to form monomers.

Steric hindrances due to the support material will be caused by the polymer backbone of the support material. In the case of an open material steric hindrances due to the

material are unlikely. However, it is possible that the enzyme could be packed so densely that enzyme molecules prevent the activation of their neighbours.

This observation was not repeated in the case of Amberlite XAD7. When the enzyme was operated in the absence of TPP there was no decline in activity and the inclusion of TPP did not increase the reaction rate. This would indicate that the closed structure of XAD7 is hindering the access of TPP to the enzyme's active site.

The data that has been discussed here demonstrates that the amount of activity recovered on the addition of cofactor is a function of the following:

- The enzyme loading.
- The presence of TPP in the enzyme.
- The support material.

Consequently some constraints upon the immobilisation of transketolase can be described. Firstly, whether the enzyme was immobilised onto Eupergit-C or Amberlite XAD7 the highest retention of activity was observed when the enzyme was immobilised with its cofactors. In the case of Eupergit-C it is most likely that the inclusion of TPP in the active site of transketolase prevents it from being damaged during the immobilisation process. As a result, the immobilisation conditions must be chosen to allow the cofactor to be included in the enzymes active site during coupling. In the case of transketolase the pH environment needs to be fairly acidic in order to ensure that the enzyme contains its cofactor. This will subsequently influence the selection of an appropriate binding method, since many coupling reactions require an alkaline environment.

4.1.3 Use of Immobilised Transketolase

4.1.3.1 Factors influencing the retention of activity

The use of an immobilised enzyme is affected by two major parameters. These are, the activity of the immobilised enzyme preparation, and the functional stability of the

preparation. The activity of the immobilised transketolase preparations described here is a function of the immobilisation support material, the amount of enzyme bound, the inclusion of cofactor during immobilisation and the transport of substrates into and products out of the support material. While the stability of the biocatalyst is often a function of the intrinsic stability of the enzyme, although sometimes tertiary structure of the enzyme can be rigidified by immobilisation (Guisard *et al.*, (1986)).

The rate of diffusion of substrate into the support is a function of the porosity of the matrix and the characteristic length (in the case of spherical particles, the radius) of the support. It has been demonstrated by Carleysmith (1977) that if the support material is ground to reduce the radius, an increase in activity will indicate the presence of internal diffusional limitation. However, in the case of immobilised transketolase no change in the initial rates of reaction were observed when the biocatalyst particles were ground. When the erythrulose production profiles of the ground and un-ground immobilised enzyme were compared with that produced by the free enzyme it was shown that the profile obtained with the ground biocatalyst was the same as that obtained with the free enzyme.

When substrate concentration is reduced the difference between the initial rate of reaction of Eupergit-C® immobilised transketolase was unaffected by grinding. While as the starting concentration of reactants was increased, the reaction rate of the ground preparation can be seen to divert from the profile observed with the un-ground beads as complete conversion is reached. This would seem to indicate that diffusion of product out of the support causes some product inhibition. When describing the kinetics of this enzyme Geyemarrah *et al.*, (1996) showed that transketolase was subject to product inhibition. One possible method for overcoming this reduction in reaction rate at higher product concentrations was described by Chauhan *et al.*, (1997), this involved the removal of product from the reactor during the reaction process.

The Thiele modulus is a dimensionless number which is often used to describe the internal mass transport conditions of an immobilised biocatalyst particle. The Thiele

modulus comprises of three major factors which determine the substrate concentration profile within the biocatalyst bead:

- The radius of the particle.
- The ability of the reactants to diffuse out of the support.
- The intrinsic activity of the biocatalyst.

The Thiele modulus (ϕ) can be described by the following equation:

$$\phi = R/3 (V_{\max} / K_m D_s)^{1/2} \quad (1)$$

Where:

R = the radius of the particle.

V_{\max} = the volumetric maximum reaction rate.

K_m = the apparent Michaelis-Menten constant.

D_s = the effective diffusivity of the substrate, taking account for the porosity of the support.

The Thiele modulus was estimated using the value of diffusivity for glucose in water obtained from Perry's Chemical Engineers Handbook ($6.7 \times 10^{-6} \text{ cm}^2 \cdot \text{s}^{-1}$), the radius of Amberlite (400 μm) and Eupergit-C (150 μm), the maximum reaction rate obtained (1.59 $\text{mmoles} \cdot \text{s}^{-1} \cdot \text{L}^{-1}$, Eupergit-C and , 0.92 $\text{mmoles} \cdot \text{s}^{-1} \cdot \text{L}^{-1}$ XAD7) and the value of K_m obtained for the free enzyme (16 $\text{mmoles} \cdot \text{L}^{-1}$). The effective diffusivity was estimated as being half of that for glucose in water, this estimation is equivalent the porosity factor used by Borchert and Buchholz in their analysis of the biocatalyst effectiveness (Borchert and Buchholz (1983)).

The Thiele modulus can be thought of as the ratio of the rate of reaction and the rate of diffusion. In the case of low biocatalyst effectiveness the reaction rate is much higher than the rate of diffusion the modulus will tend towards infinity. Conversely in the case of a high biocatalyst effectiveness the rate of reaction is slower than the rate of diffusion, as a result the modulus tends towards zero.

Thiele Modulus for Eupergit-C was calculated to be 0.87 while the value for XAD7 was determined to be 1.7. This would indicate that in no diffusional limitation would be expected in the case of Eupergit-C while the Amberlite XAD7 immobilised transketolase would be expected to be subject to some internal diffusional limitation, if the enzyme was uniformly distributed within the support. However, the staining of protein immobilised onto XAD7 demonstrated that protein was distributed mainly at the surface of the bead. Consequently diffusional limitation may not be expected, and when the erythrulose profiles of ground and un-ground XAD7 preparations were compared no difference in the initial rates were observed.

4.1.3.2 *The Stability of Immobilised Transketolase.*

The stability of an immobilised biocatalyst is the overriding factor which will determine the productivity of the biocatalyst. Since it is the stability of the preparation which determines how much enzyme must be added to the bioreactor to overcome destruction of the catalyst. In their paper on the characteristics of the transketolase reaction Mitra *et al.*, (1997) concluded that the stability of the enzyme in glycolaldehyde and other aldehyde substrates was one of the factors determining the mode of bioreactor operation. They demonstrated that feeding strategies were necessary to limit the concentration of aldehyde in the bioreactor and maintain enzyme activity.

The inactivation of enzymes can be attributed to several mechanisms which include (Fagain and O'Kennedy (1991)):

- Conformational scrambling. The unfolding of the 3 dimensional structure of the protein resulting in a reduction in the integrity of the active centre.
- Covalent alteration of the protein. (For example, deamidation of asparagine, peptide bond hydrolysis, destruction of disulphide bonds).
- Competition with structural water, altering inter- and intra- domain H-bonds (Gianfreda and Scarfi (1991)).
- Monomerisation of multimeric proteins.
- Loss of cofactor.

The stability of immobilised transketolase in the presence of glycolaldehyde is an important factor which may not only determine the productivity of a transketolase catalysed biotransformation but also the mode of operation of the bioreactor. Surprisingly however, when immobilised onto both Eupergit-C and Amberlite transketolase exhibited an improved stability in the presence of glycolaldehyde. This stabilisation effect may be a result of the rigidification of the tertiary structure of the enzyme. Rigidification has been observed in other immobilised enzymes, and accounts for the stabilisation of several enzymes reported in the literature (Blanco *et al.*, (1989), Guisan (1988), Gabel (1973)).

It has been observed that the denaturation of globular proteins occurs with conformational scrambling beginning at a destabilising 'nucleus' the effects of which radiating out to other parts of the protein resulting in inactivation (Wulthrich *et al* (1980)). A reduction in the mobility of this destabilising 'nucleus' is thought to result in the prevention of more global conformational changes resulting in a stabilised protein. The immobilisation of transketolase may prevent the initial unfolding of the destabilising 'nucleus' in the presence of glycolaldehyde

However, in the presence of oxygen, known to deactivate transketolase by oxidising essential cystine residues, immobilisation did not improve the stability of the enzyme. As a consequence immobilisation was unable to remove the constraint of operating in the presence of reducing agents (for example, mercaptoethanol) or under a nitrogen blanket. This would imply that the denaturation effect of oxygen is due to the chemical alteration of the enzyme as opposed to a change in its three dimensional conformation. The three dimensional structure and the amino acid sequence of *E.coli* transketolase resolved by Littlechild *et al.*, (1995) showed a cystine near to the TPP binding site. If this cystine was oxidised it may effect the ability of transketolase to bind TPP correctly. Since this would be a purely chemical effect it is unlikely that immobilisation would be able to prevent it.

The striking ability of immobilisation to stabilise transketolase against the denaturing effect of glycolaldehyde means that the model reaction with glycolaldehyde as a aldehyde acceptor can be operated with elevated glycolaldehyde concentrations.

Perhaps avoiding the use of the substrate feeding strategy to minimise reactor concentration as proposed for the soluble enzyme by Mitra and coworkers (1997).

4.1.3.3 pH control

The conversion of 1 mole hydroxypyruvic acid and glycolaldehyde to 1 mole L-erythrulose and carbon dioxide results in the net consumption of one proton. Consequentially one would expect a stoichiometric quantity of HCl to be added in order to control the reactor pH. The increasing pH of the bulk environment can alter the rate of reaction by moving the enzyme away from its activity pH optimum. Along side this, the pH can alter the productivity of the biotransformation by causing the destruction of both the substrates and the product. This is of particular importance for reactions using the relatively expensive hydroxypyruvic acid (Mitra *et al.*, (1997)). As a consequence it is important to control the reactor pH by the addition of an acid. One of the surprising observations made during the course of this work, however, was the difference in titrant addition profiles between the immobilised enzyme and the free enzyme. The difference in titrant addition was also seen to vary with the activity of the immobilised enzyme preparation, and with the type of support.

A stoichiometric quantity of titrant was not required for pH control by the free enzyme or by any preparation of the immobilised enzyme. The disparity between the molar production of erythrulose and the molar production of titrant required may be explained by the presence of the ionic species of carbon dioxide in solution. The pKa of H_2CO_3 is 6.4 while the pKa of HCO_3^- is 10.4. At pH values between 6 and 9 the most common species in solution will be the bicarbonate ion HCO_3^- . This will also result in the net generation of one proton. The ability of buffer salts to prevent changes in pH, or facilitate proton transport is restricted to whole pH units above or below its pKa. Therefore if the internal pH within the pores is between ca. pH 5.4 and 7.4 any change in pH will be strongly buffered by the presence of bicarbonate ions.

Subsequently as the reaction proceeds the formation of carbon dioxide and the subsequent production of bicarbonate will buffer changes in pH brought about by the reaction. As a consequence a stoichiometric quantity of titrant will not be required at pH's in this range. However, as the reaction proceeds the molar ratio of titrant

requirement and erythrulose production changes with more titrant being required. This can be explained in the following manner.

As the reaction proceeds towards completion, this apparent buffering capacity of the reaction mixture decreases. At the start of the reaction, there is little dissolved carbon dioxide or bicarbonate. As a consequence all most all of the carbon dioxide produced by catalysis will be present as bicarbonate ions. Subsequently at the earlier reaction coordinates, changes in pH will be buffered by the presence of bicarbonate ions. This situation will be improved at higher pH values where the bicarbonate solubility is improved, and where the amount of carbonate ions in solution is increased. As a consequence of the increasing bicarbonate concentrations, the reaction mixture becomes more saturated with bicarbonate. Subsequently more of the carbon dioxide produced by catalysis remains in solution, or as the un-disassociated acid. Which results in a reduction of the apparent ability of the reaction to buffer itself.

The data presented here suggest that in the case of Eupergit-C the saturation of solution within the pore volume with bicarbonate occurs sooner, or charged species exist on the surface of the support. However, before the biocatalyst is used in the biotransformation any unreacted epoxide groups are quenched with mercaptoethanol. If the unquenched support is introduced into the reaction mixture the pH increases causing an extra requirement of HCl. However, the quenched support had no observed effect upon the pH of the reaction mixture. Secondly, the grinding of the support would not have an effect upon the charge of the support. However, it was observed that the titrant profile of the ground support was similar to that for the free enzyme. Subsequently the presence of charged groups on Eupergit-C are unlikely to cause the remarkable difference in titrant requirement.

Another possible explanation for this discrepancy may be the transport of bicarbonate to the outside of the support. However, since it has been demonstrated that the transfer of glycolaldehyde and hydroxypyruvic acid were not limited by mass transport it is difficult to understand why bicarbonate transport would be limited. It can be seen therefore that there are a number of questions about the role of carbon dioxide in the

group of transketolase reactions involving hydroxypyruvic acid that need to be answered.

4.2 Implications of Immobilisation upon Transketolase Catalysed Biotransformations.

Until now the extended reuse of immobilised enzymes has justified the additional cost and effort of immobilising the biocatalyst. For transketolase, which can be produced in large quantities at a reasonable cost (Hobbs *et al.*, (1996)), reuse may not be a sufficient reason to justify immobilisation. It is important therefore to consider what other technical advantages are offered by immobilisation before this can be justified.

4.2.1 Cofactor Related Issues.

Transketolase can be immobilised in the presence of its cofactors, however, when operating the bioreactor in the absence of cofactors 35 % of the activity of Eupergit-C transketolase was lost within 40 minutes, in other words the activity is lost at a rate of 50 %·h⁻¹. Subsequently operation with immobilised transketolase will require permanent replenishment of TPP. The cofactor washout curve in figure 3.11 demonstrates that although the cofactor is removed, some interaction between the immobilised enzyme and cofactor is occurring. The almost instantaneous recovery of activity after the reintroduction of cofactor indicates that strong binding between the bound enzyme and its cofactor is still possible. However, since cofactor can be removed from the active each subsequent batch must also contain TPP in order to maintain the activity of the biocatalyst. While in continuous operation cofactor must be supplied in the feed stream, or recycled after separation from the product stream, or retained in the reactor by physical means.

Cofactor retention, by increasing the molecular weight of the cofactor and entrapping the cofactor behind an ultrafiltration membrane, has been demonstrated by Kragl and co-workers (1996) as being a viable method of operation with NADH requiring dehydrogenase enzymes. This may reduce activity because the larger NADH molecule

needs to get into the active site. When an expensive cofactor is being used the activity will be a secondary consideration after the efficient use of the cofactor. A glance at the respective costs of TPP and NADH informs us that TPP is much cheaper than its larger, rarer cousin NADH. Subsequently, the expense of retaining or recycling the cofactor is unnecessary purely on the basis of cofactor wash out.

Work carried out by Chauhan (1996) demonstrated that although ISPR (in *situ* product removal) was effective in removing the product from the bioreactor, it was also able to remove TPP from the bioreactor, resulting in a subsequent loss of enzyme activity. This was observed both in the case of the immobilised and the free enzyme. However the cost of TPP in comparison with the other reaction constituents means that the cofactor does not make up a major part of the cost and subsequently addition of cofactor during the process cannot be ruled out.

It follows therefore that although immobilisation will be effective in increasing the ratio of the amount of product that can be produced and the amount of enzyme that is required, in the case of transketolase the total turn over number (as defined by Kragl *et al.*, (1996)) will at best be unaffected. If the minimum amount of cofactor (in the case of Eupergit-C enough to replenish the 58 %h⁻¹ loss of activity) is continually added in the feed stream of a continuous biotransformation the molar ratio of product produced and cofactor required can be calculated on the basis of the 2.4 mM concentration charged to the reactor at start-up.

On this basis, the minimum cofactor addition rate for Eupergit-C is 1.39 mmoles cofactor L⁻¹.h⁻¹. The addition rate for XAD7 based upon the slower rate of loss of TPP from the active site is 0.31 mmoles.L⁻¹.h⁻¹. The total cofactor turnover of the XAD7 and Eupergit-C biocatalysts are given in table 4.1 below.

Support	Cofactor addition rate mmoles.L-1.h ⁻¹	Total cofactor turnover mmoles product. (mmoles cofactor) ⁻¹
Eupergit	1.39	168
XAD7	0.312	314

Table 4.1 Comparison of the total cofactor turnover numbers for Eupergit-C and XAD7 immobilised transketolase.

The major product of the model transketolase biotransformation, erythrulose, is of low commercial value. The prices quoted by Sigma-Aldrich for erythrulose and TPP are £3.20.g⁻¹ (or £384.mole⁻¹) and £4.9.g⁻¹ (or £2254.mole⁻¹) subsequently, the cost of the cofactor would be an appreciable fraction of the cost of this final product. However, the relative cost of TPP will be greatly reduced if other ketol donors or aldehyde acceptors are used. For that reason the requirement for extra cofactor due to the removal of cofactor from the active centre is not of economic importance, unless product inhibition is so severe that an ISPR technique is necessary to maintain enzyme activity.

For cofactor requiring enzymes therefore there are two major considerations to take into account:

- Productivity of the enzyme.
- Efficiency of use of the cofactor; the Total Turn Over Number.

The relative importance of these factors is dependent upon the cost of the cofactor relative to the final product. When the cofactor is expensive relative to the product the total turn over number is a critical factor. In this case it may be more important to reduce the amount of cofactor that is required. By either retaining the cofactor within the reactor system (with an enlarged cofactor for example), if possible by recycling the cofactor or by immobilising the biocatalyst in the absence of its cofactor.

4.2.2 Stabilisation of Transketolase

It is widely appreciated that transketolase has a reasonable potential as a biocatalyst for the production of chiral synthons. Although this work concentrated upon the transketolase catalysed reaction between hydroxypyruvic acid and glycolaldehyde, the enzyme shows a broad substrate specificity allowing its use in a variety of interesting bioconversions (Dalmás and Demuynck (1993)). Since the use of hydroxypyruvic acid as the ketol donor renders the reaction irreversible this substrate is probably the most important. Other ketol donors are possible, however, the reaction will be reversible and as such subject to the processing problems subject to equilibrium. The reaction in this study also used glycolaldehyde as the acceptor molecule. Glycolaldehyde is achiral, and subsequently the adduct of its reaction with hydroxypyruvic acid is of little commercial value. Other aromatic aldehydes or chiral aldehydes are also possible acceptor molecules.

Alternative aldehyde acceptors can be broken down into two categories, namely chiral and achiral. If the alternative aldehyde has a chiral centre, one of the enantiomers will be unreacted. As a result the unreacted enantiomer will remain in the bioreactor, or it will have to be removed by some recycling technique. The denaturing effect of several other aldehydes are reportedly more severe than that of glycolaldehyde (Dalmás and Demuynck (1993)B) subsequently the reactor concentration of aldehyde is an important consideration (Mitra, *et al.*, (1997)).

The strategy used to increase the productivity of reactions involving soluble transketolase and glycolaldehyde was the feeding of the aldehyde to minimise concentrations of the denaturant in the bioreactor. This strategy however, will not achieve the same result when the aldehyde acceptor has a chiral centre. Therefore an alternative strategy must be found. However, the immobilised enzyme has an appreciable improvement in stability against glycolaldehyde. Subsequently it can be expected that the immobilised enzyme will be more stable in the presence of any unreacted aldehyde. Therefore the immobilisation of transketolase is able to create a biocatalyst with a greater flexibility of reactions than the free enzyme.

The effect that stabilisation as a result of immobilisation will have upon the flexibility of transketolase catalysed reactions have been outlined in table 4.3 below. When HPA is used as a ketol donor the consequences to the process are beneficial because the reaction is irreversible. However, CO₂ is produced which has an effect upon the buffering capacity of the reaction environment, but no detrimental effect upon the retention of activity between multiple batches was observed. When an alternative ketol donor is used with glycolaldehyde the secondary product will no longer be CO₂, but will be an aldehyde, which can be expected to have a denaturing effect upon transketolase.

<u>Ketol Donor</u>	<u>Consequence</u>	<u>Free enzyme</u>	<u>Immobilised Enzyme</u>
HPA	CO ₂	+	+
Alternative	Aldehyde product toxicity	- requires feeding	+ stabilised
<u>Aldehyde Acceptor</u>			
GA	erythrose, aldehyde toxicity	- requires feeding	+ stabilised
achiral	Alternative product, aldehyde toxicity	-- denaturant	+ stabilised
chiral	Alternative product, Unreacted enantiomer	--- feeding ineffective	+ stabilised

Table 4.3 The implications of immobilisation on transketolase biotransformations with alternative substrates. (HPA, hydroxypyruvic acid, GA glycolaldehyde).

When glycolaldehyde is used as an aldehyde acceptor it has a toxic effect upon the enzyme. However, the stability of transketolase in glycolaldehyde is reportedly better than its stability in a variety of other aldehydes. If the alternative aldehyde has a chiral

centre the unreacted aldehyde enantiomer will be present within the reactor. As a result feeding this substrate into the reactor will not improve the operational stability of the free enzyme since the concentration of the unreacted enantiomer will steadily increase. However, since immobilisation with both Eupergit-C® and XAD7 improved the stability of the enzyme the operational life can be expected to be higher.

Another cause for the destabilisation of transketolase was the oxidation of essential cystine residues. The transketolase monomer contains two cystines and however no disulphide bridges were present (Isupov *et al.*, (1997)). The 1.9 Å data presented by Isupov *et al.*, (1997) showed that one of these cystines was present in its oxidized form. The stability of the holo-enzyme in solution was poor in the absence of either mercaptoethanol a potent reducing agent or a nitrogen head gas. The implication of this is that the cystines are somehow involved in the binding of the either the TPP cofactor or the metal ion. This argument may be further supported by the fact that one of the two cystines contained in each monomer is within the cofactor binding region of the protein.

As would be expected this chemical modification of transketolase was not prevented by immobilisation. The immobilised enzyme rapidly lost activity when used repeatedly in the absence of mercaptoethanol. This is perhaps a surprising contrast with the stabilisation against the inactivating effect of glycolaldehyde observed for the immobilised enzyme. As a consequence, although the glycolaldehyde concentration is no longer a constraint upon the bioreactor operation the presence of mercaptoethanol or a nitrogen gas head space is still necessary.

One of the major constraints upon the operation of the free enzyme bioreactor was the need to feed the substrate into the vessel in order to maintain a low aldehyde concentration. The constraint has been removed, or substantially reduced, by immobilisation. The immobilised enzyme was substantially stabilised against gluteraldehyde. In fact the immobilised enzyme showed a half life of approximately 100 hours. This would mean that the bioreactor would need to be recharged at a rate of approximately $0.5\%h^{-1}$. While the half life of the free enzyme with the same concentration of substrate [500 mM] was 1 hour. As a consequence the maintenance

of a low substrate concentration in order to reduce enzyme loss is no longer a major constraint.

Since the immobilised enzyme did not demonstrate a higher stability against oxidation of the essential cystine residues. As a result a reducing environment is still necessary. This will probably be maintained with an inert gas head space since mercaptoethanol is toxic and presents handling considerations. This is not an unusual situation and therefore will not be difficult to maintain.

The functional stabilisation of an enzyme can be achieved by a variety of strategies, the strategies can be subdivided into immobilisation (Guisan *et al.*, (1986) ; Klibanov (1979)), the inclusion of solutes into the reaction media, chemical modification of the enzyme (Fagian and O'Kennedy (1991)) and by genetic engineering. The results shown here demonstrate that a generic technique for the functional stabilisation of an enzyme is not available. It is necessary to understand the mechanism of inactivation prior to the selection of a technique or techniques for stabilisation.

The operational stability of transketolase is a function of the chemical inactivation by oxidation of essential amino acid residues and the inactivation due to the presence of glycolaldehyde. The mechanism of the latter is most likely due to changes in the three dimensional structure of the enzyme as a result of Schiff's base formation with glycolaldehyde. As a result a two pronged strategy for the stabilisation of the enzyme is necessary. Firstly 'medium engineering' to prevent oxidation, by the inclusion of a chemical reducing agent into the reaction media or by operation under nitrogen headspace. Followed by the prevention of structural changes in the protein as a result of Schiff's base formation with glycolaldehyde by immobilisation.

4.3 Comparison of Supports

Despite the number of publications describing the immobilisation of enzymes, hardly any make detailed comparisons of immobilisation of a particular enzyme by more than one support. One of the objectives of this work was to make such a comparison,

There are only a few immobilisation supports available commercially for covalent attachment of enzymes. One of the most commonly used is Eupergit-C[®] which is quite expensive and requires very prolonged use to justify its cost. This support material was compared to a technique involving relatively cheaper components. The system used was glutaraldehyde and XAD7 which had previously been successful with penicillin acylase (Carleymithet *al.*, (1980)).

As previously discussed the differences between Eupergit-C and XAD7 begin at a structural level. The structure of Eupergit-C was more open, which resulted in a uniform distribution of enzyme throughout the material despite changes in ionic environment. The structure of XAD7 was more closed and it was possible to achieve a degree of control of the distribution of protein within XAD7 by immobilising with high phosphate ion concentrations.

The capacity of the 2 supports to bind protein was also studied, a significantly higher capacity for BSA was observed for XAD7 than for Eupergit-C (240 mg.g⁻¹ and 155 mg.g⁻¹ respectively). Since BSA is a globular protein of approximately 70 kDa it could be expected that this will reflect the capacity of the supports for transketolase.

Both Eupergit-C[®] and XAD7 bound transketolase fairly quickly when high phosphate concentrations were used. However, the rate of immobilisation onto Eupergit-C[®] was considerably slower when the phosphate concentration was lower. This demonstrated that there was a difference in the coupling mechanism between Eupergit-C[®] and XAD7. In the case of the apo enzyme there is very little difference between the activity obtained with XAD7 and with Eupergit-C[®].

Since transketolase requires TPP for activity the addition of the cofactor to the immobilised enzyme is an important factor to be considered. In the case of Eupergit-C[®] the immobilised enzyme could be activated by the inclusion of the cofactor during immobilisation. However, this didn't lead to an appreciable increase in the activity of the Amberlite immobilised enzyme. The epoxide groups involved in attaching transketolase to Eupergit-C[®] are separated from the surface of the support by a 12 carbon atom long spacer arm. This long spacer arm may enable the epoxide groups to

penetrate into the active centre of the enzyme, perhaps coupling with amino acid side chains involved in the binding of TPP. As a consequence when transketolase is immobilised in its holo-form this inactivation will be prevented. Conversely the gluteraldehyde on the surface of Amberlite XAD7 doesn't have a long spacer arm. Subsequently it is not as likely to be able to penetrate into the core of the protein.

It has been reported that when epoxide groups bind to protein at acidic pH's the reaction is normally between tryptophan or tyrosine and the epoxide as opposed to histidines or lysines at more alkaline pH's (Drobnick et al., (1979), Zemanova et al., (1981)). The TPP binding site of yeast transketolase contains a hydrophobic pocket lined with several side chains, including Tyrosine448 (Schneider and Lindqvist (1993)). This pocket is involved in the binding of the pyrimidine ring and is essential for the binding of TPP (Schneider and Lindqvist (1993)). In the absence of TPP it would be expected that this side chain may be involved in binding the enzyme to Eupergit-C[®], which would result in a low retention of activity even on the introduction of excess TPP. It would be protected if the enzyme is immobilised in the presence of its cofactor. Also, Histidine481 is directly involved in catalysis by donating electrons to the TPP. Several other histidines are reported to hold the TPP in the correct configuration (Schneider and Lindqvist (1993)) while the diphosphate moiety of TPP is held in place by two other histidine residues (Lindqvist et al., (1992)).

In all there are 5 histidine groups which are essential to catalysis and TPP binding and one tyrosine group. Subsequently, if the immobilisation is performed at a low pH in the presence of TPP it is possible to prevent inactivation by chemical modification of these essential residues. Where as gluteraldehyde will bind protein by histidine and lysine side chains. Subsequently, it is possible that these residues may be involved in the attachment of transketolase to XAD7.

Possible methods of inactivation of the binding of TPP by Eupergit-C® include:

- Coupling by the histidine or tyrosine amino acid residues involved in binding TPP subsequently preventing the activation of the immobilised holo-enzyme.
- Interactions between the protein and the surface of the support which may cause steric hinderences preventing the activation the immobilised apo-enzyme.
- Alteration of the 3 dimensional structure of the enzyme altering the TPP binding site.

On the other hand, once further TPP was added in the reaction stage, the holo-enzyme immobilised onto Amberlite XAD7 didn't display more activity than the immobilised apo-enzyme. Therefore it is most likely that the inactivation of the enzyme by this support is not a result of changes in the TPP binding site. It is more plausible that the loss in activity on binding to Amberlite XAD7 is due to either structural changes in the active centre caused by the proximity of the enzyme to the support surface, or due to chemical inactivation of essential amino acid residues. It is possible that the inactivation by gluteraldehyde is similar in mechanism to the inactivation of transketolase by glycolaldehyde.

Eupergit-C® is able to bind protein at the relatively low pH of 6.5 where TPP can be maintained in the active centre of transketolase. This is a considerable advantage over other commercially available immobilisation techniques which require more alkaline pHs to bind protein. Amberlite XAD7 was also able to attach protein under mildly acidic conditions. However the retention of activity after immobilisation was not improved above that achieved when the apo-enzyme was immobilised.

At first glance therefore Eupergit-C appears to be the better of the two supports tested, the enzyme can be immobilised with a much greater retention of activity and as a consequence fewer batches will be required before economical use of the enzyme is achieved. The retention of activity obtained with Eupergit-C® means that it would only need to go through two cycles before the all of the enzyme has been used economically, whereas the 20 % retention achieved with XAD7 would mean that five operational cycles would have to be performed before the enzyme is efficiently used.

However, since the enzyme is recombinant and can easily be mass produced efficient use of the enzyme is no longer the major concern in determining which support material to employ. Several other parameters become more important in this case.

- Cost per unit activity, efficient use of the support material itself.
- Ease of handling, retention within the bioreactor and recovery from the product stream. This includes the size and density of the immobilised particles.
- Tightness of the binding. The importance of this factor will be increased with a pharmaceutical product.
- Mechanical stability of the material. This effects the stability of the particles within the bioreactor environment.
- Productivity of the immobilised enzyme.

Dealing with the first of these four factors, table 4.4 below shows a breakdown of the cost per unit of expressed activity for both holo- and apo-transketolase immobilised onto the two supports.

Support	Activation state	Binding yield (%)	Enzyme bound (kU.kg^{-1}) (dry)	Retained Activity (%)	Support Cost (£.kg^{-1}) (dry)	Cost/Unit Expressed Activity (£.kU^{-1})
Eupergit-C	apo	95	1995	18	235 ^a	0.65
	apo	95	150	70	“	2.35
	holo	95	1434	60	“	0.27
Amberlite XAD7	apo	90	1300	13	33	0.20
	holo	90	1330	20	“	0.12

Table 4.4 Comparison of the cost per unit activity of transketolase immobilised onto Eupergit-C[®] and Amberlite XAD7.^a based upon exchange rate of 2.969 DM/£ on 21-3-98.

Immediately a difference in the economics of the two biocatalyst particles can be seen. Amberlite XAD7 is approximately 10 times cheaper than Eupergit-C[®], as a result the cost per unit activity of transketolase upon Amberlite XAD7 is much lower than that achieved with any of the Eupergit-C[®] preparations. Amberlite XAD7 must be activated with gluteraldehyde before transketolase can be attached to the enzyme. This activation step will incur extra handling costs. Although this cannot be expected to account for the difference in cost, it will be sufficient to increase the cost of Amberlite-TK to a price similar to that of holo-transketolase immobilised onto Eupergit-C[®]. Since the specific activity of Eupergit-holoTK was higher than that obtained with XAD7, more material would be required to maintain an equal Space Time Yield.

Dealing with the second of the factors which will influence the choice of support, namely the ease of handling. Firstly it is important to note the difference in size between Amberlite XAD7 and Eupergit-C[®]. Amberlite is approximately 4 times larger than Eupergit-C[®], therefore it can be expected that this material will be much easier to remove from the product stream. Amberlite has a particle size of around 400µm which means that it can be easily removed by coarse filtration. Despite the fact that Eupergit-C[®] is much smaller, it too can be removed by coarse filtration. However, the settling velocity of Amberlite XAD7 could be seen to be much more rapid than that for Eupergit-C[®]. This would mean that the turn around time in between batches could be shorter for Amberlite than for XAD7.

Protein could not be found to desorb from niether Eupergit-C[®] nor Amberlite XAD7 even after 72 hours incubation. This would indicate that the binding of protein onto both supports was sufficient to prevent leaching into the product stream. This would allow the immobilisation of transketolase from a crude cell extract, subsequently reducing the cost of producing the enzyme since the costly enzyme purification stages could be avoided.

The productivity of the immobilised biocatalyst will be a function of the specific activity of the material and the number of cycles or batches that the biocatalyst can perform and the amount of product that it can produce. These two pieces of vital information can be calculated from the Space Time Yield (STY), that is the amount of

product produced per batch per litre of bioreactor (Kragl *et al.*, 1996) as shown in table 4.5. The amount of product produced per unit of enzyme, known as the enzyme productivity can be calculated from this and used to determine the break even point of the catalyst. This method of analysis is more dynamic than the previous, cost per unit activity analysis. It takes into account the amount of activity bound, the stability of the biocatalyst and the overall cost of the support.

The amount of enzyme required at start up was estimated from the Space Time Yield and an estimated annual production rate of 500 tonnes. It was financially impossible to perform repeat batches for the model reaction since hydroxypyruvic acid and glycolaldehyde were so expensive. However, it was possible to determine the stability of the immobilised catalysts for long term exposure to its denaturing substrate. This showed that unlike the free enzyme which will require replenishment after 3 hours of exposure, both preparations of the immobilised enzyme retained 50 % activity for an extended period of time. Eupergit-C[®] preparations would require replenishment at a rate of approximately 0.5%.h⁻¹ (3 % per batch) while XAD7 preparations would require replenishment at the slightly quicker rate of 0.6%.h⁻¹ (3.6 % per batch).

Support	Catalyst	STY (g.L ⁻¹ Erythrulose / Batch) ^a	Enzyme Required (kU) ^b
Eupergit-C [®]	holo	674	330.
Amberlite XAD	holo	282	920.

Table 4.5 A comparison of the Space Time Yields and the amount of enzyme required at the start of the reaction for Eupergit-holoTK and Amberlite-holoTK. ^a batch time approximated as 6 hours, ^b estimated from an annual production of 500 tonnes at 5 batches a week.

Table 4.6 shows a comparison of Eupergit-TK and Amberlite-TK preparations. The difference in the Space Time Yield of the two preparations of enzyme means that in practice more catalyst is required in a process employing the Amberlite preparation than the Eupergit-C[®] preparation. This means that the cost of the process with the

Amberlite support would be higher despite the fact that Amberlite XAD7 is a lot cheaper than Eupergit-C®.

	Eupergit-TK	Amberlite-TK
Enzyme at Start-Up (KU)	330	920
Bead load (kg)	0.4	3.4
Cost at Start-Up (£)	89.1	110
Recharge Rate per batch	3 %	3.6 %
Catalyst Cost/10 batches (£)	115	149

Table 4.6 Comparison of the biocatalyst economics of Eupergit-TK and XAD7-TK. Costs were estimated on a basis of an annual production of 500 tonnes with five 6 hour batches per week.

The comparison of the two materials can be made more complete with an assessment of how the productivity (kg product/kU enzyme) varies as the number of cycles is increased. This comparison can be made with the previous assumption of an annual production of 500 tonnes with five batches per week. As Amberlite-TK has such a low retention of activity in comparison with the Eupergit-C preparation more Amberlite is required at the start of the reaction. This would mean that the Amberlite preparation should be used in a packed bed reactor or a large stirred tank reactor to accommodate the larger quantity of biocatalyst beads.

Figure 4.3 shows how many cycles need to be performed before a Eupergit-C® transketolase biocatalyst would break even, while a similar break even graph for Amberlite is shown in figure 4.4. Immediately it can be seen that the Amberlite preparation needs to be used approximately 8 times as many cycles as the Eupergit-C® before the enzyme is used efficiently. The change in the enzyme productivity of the two materials with the number of operational cycles is shown in figure 4.5 (Kragl *et al.*, 1996). This is a tool for determining how efficiently the enzyme is being used. At the start of its use the enzyme will be used relatively inefficiently. However, as the

number of cycles performed is increased product specific catalyst decreases rapidly. The Amberlite preparation had a much lower enzyme productivity than the Eupergit-C preparation. After 100 batches the Amberlite XAD7 preparation was estimated to have a product specific enzyme value of 150 kU.kg^{-1} in comparison with 19 kU.kg^{-1} for the Eupergit-C preparation.

Consequently although Eupergit-C[®] is considerably more expensive than Amberlite, more biocatalyst would be required to be charged to the reactor to initiate the process. As a consequence although the cost of the material is a major consideration in support selection, a specific material cannot be ruled out on the grounds of cost alone. In the final analysis, the operational stability of the biocatalyst and the cost per unit activity are the most important factors involved in support selection.

The comparison of the two support materials has centred upon two factors; the retention of activity upon the support and the stability of the resulting enzyme. Although the cost of purpose developed commercial immobilisation support is much higher than the ion exchange resin the more expensive support had the considerable advantage of resulting in a biocatalyst with a much higher activity density. This was the major contributing factor in the difference between the two materials. As a guide to the selection of an appropriate material the relative costs of the support can be an effective decision making aid, however, it is unwise to ignore an expensive material on the basis of cost alone. The following parameters offer a much sounder basis for support selection:

- Cost per unit activity
- Retention of activity after immobilisation
- Operational stability

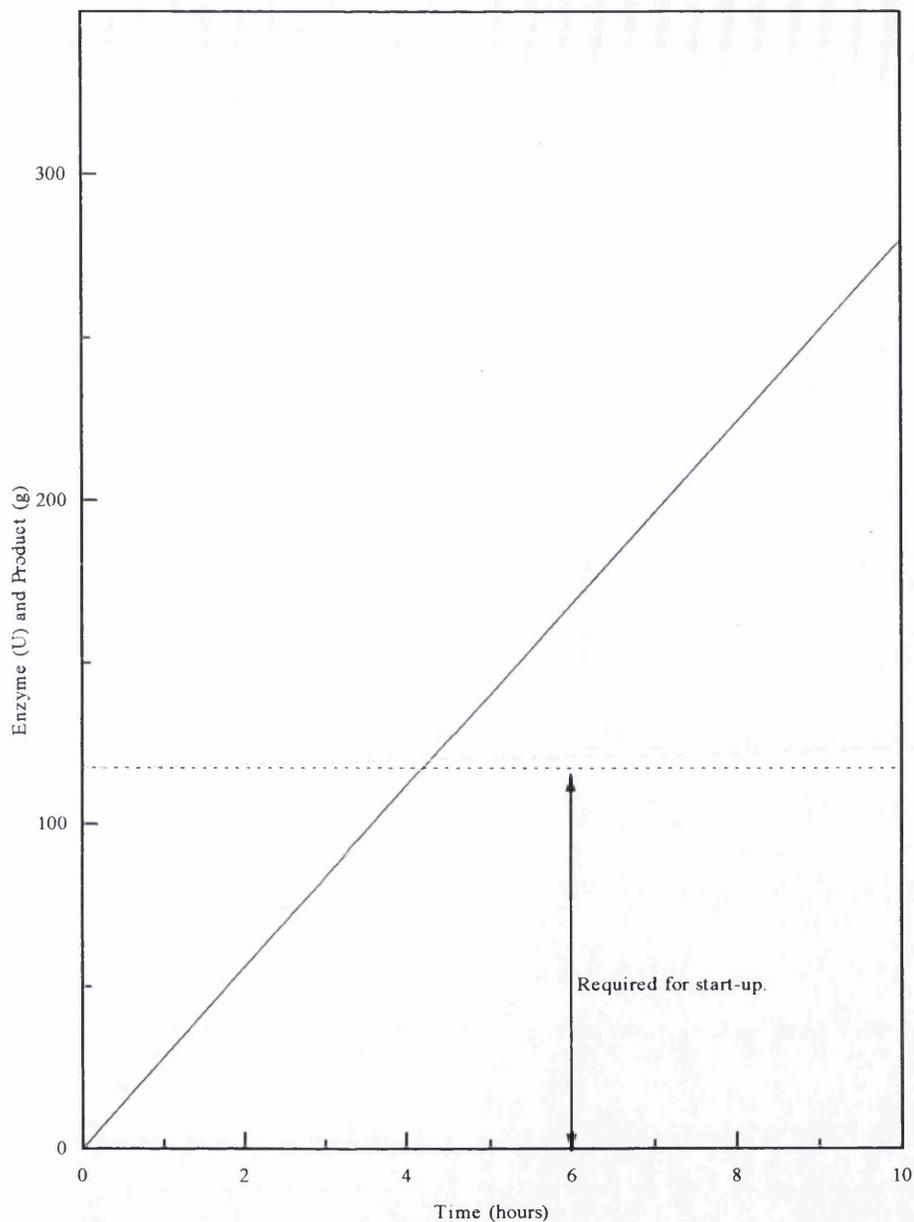


Figure 4.3 The amount of enzyme required and product produced by Eupergit-C immobilised transketolase. Values were calculated on the basis of Space Time Yield and the rate of enzyme destruction. (STY= 674.5 g/L/day and Enzyme destruction = 0.5% hr)
 (.....) Enzyme required at start-up, (---) enzyme loss through denaturation and (—) product formed.

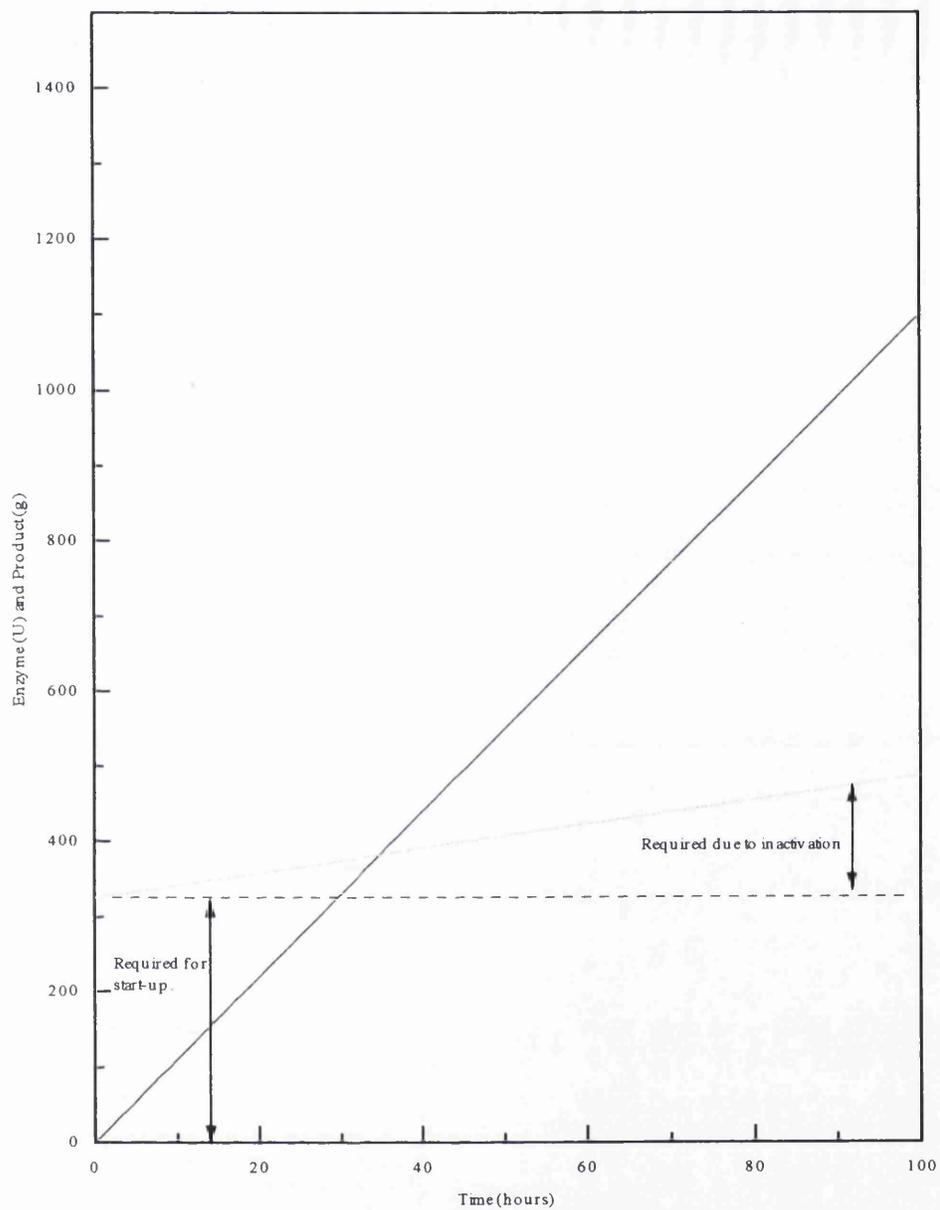


Figure 44 The amount of enzyme required and product produced by XAD7 immobilised transketolase. Values were calculated on the basis of Space Time Yield and the rate of enzyme destruction. (STY=282 g/L/day and Enzyme destruction = 0.5% hr)
 (-----) enzyme required at start-up, (——) enzyme lost through denaturation and (——) product formed.

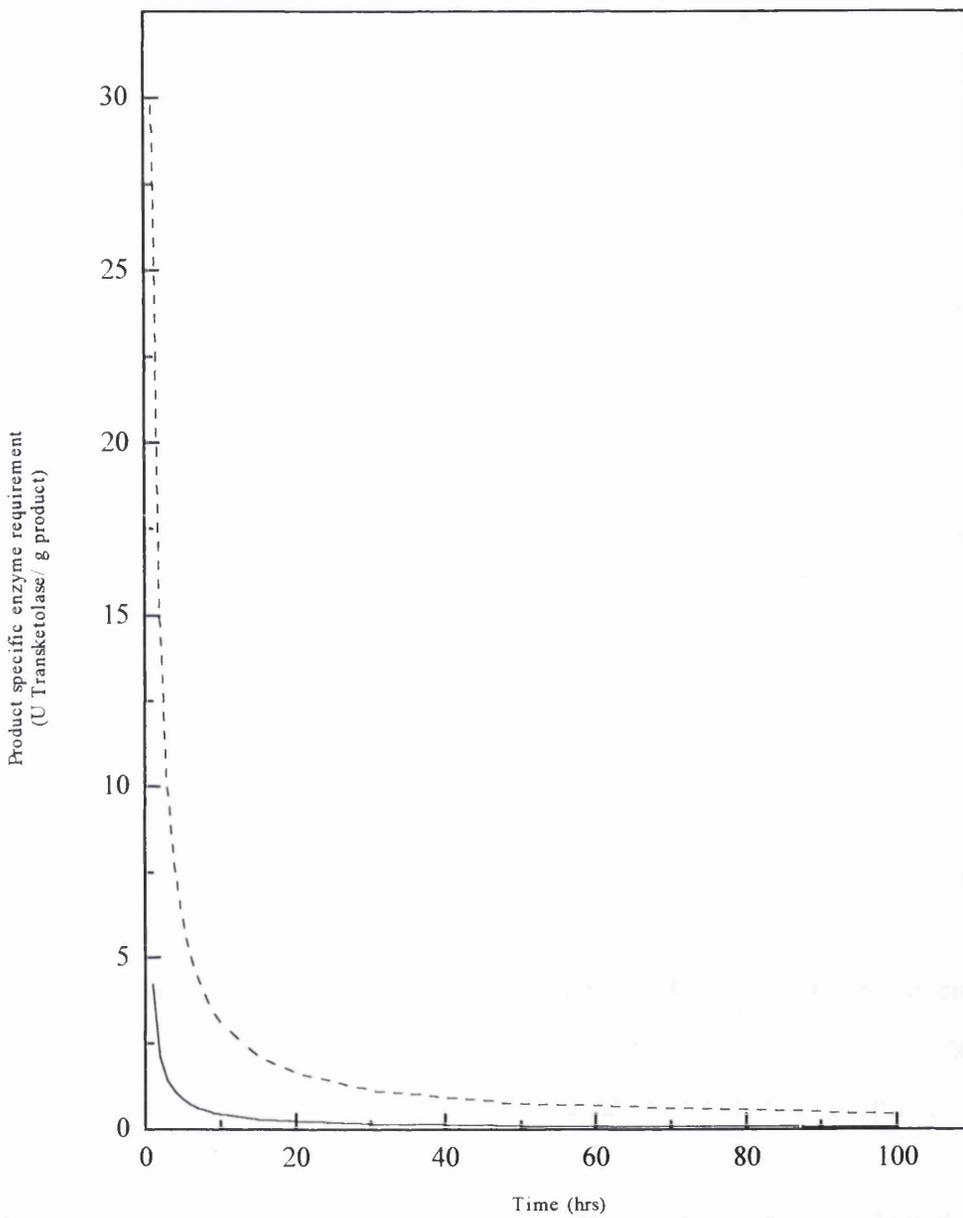


Figure 4.5. Comparison of the product specific enzyme requirements for transketolase immobilised onto XAD7 (----) and Eupergit-C(—).

4.4 Immobilising Cofactor Requiring Enzymes

When a cofactor requiring enzyme is to be immobilised and used as a biocatalyst a large amount of bound active or potentially active enzyme is required. In other words the enzyme must be immobilised in such a way that activity can be observed, or that the activity can be easily recovered by the subsequent addition of its cofactors. The use of the more complex enzymes pose problems associated with their requirement for cofactors. These problems are twofold, namely, the recycling of cofactors depleted during catalysis and the inclusion of cofactor into the active site of an immobilised enzyme.

The cofactor molecule can be introduced during two main stages of the process.

1. Immobilisation stage.
2. Reaction stage.

In the case of transketolase it was found that the introduction of the cofactor during the immobilisation stage could enhance the retained activity of the biocatalyst immobilised onto Eupergit-C®. However preactivation failed to improve the activity of the enzyme immobilised onto Amberlite XAD7. The inclusion of cofactor during immobilisation can have the effect of stabilizing the three dimensional structure of the enzyme, or preventing involvement of essential amino residues in coupling.

Preactivation, however, is not a guarantee that the enzyme will retain its cofactor during and subsequent to immobilisation. Consequentially it may be necessary to perform the immobilisation reaction in conditions that are not ideal for coupling but where the cofactor is retained. This was shown to be the case for transketolase. The cofactor was readily dissociated from the free enzyme under alkaline pH conditions. Coupling of the enzyme however, required deprotonated amine groups and was more efficient at higher pH's. As a consequence therefore it is possible that the selection of coupling method must take the necessity of preactivation into account.

The cofactor can also be introduced during the reaction stage. In this case the cofactor must travel through the support material to the active site of the enzyme and the active centre must be able to successfully incorporate the cofactor. As a consequence the morphology of the support material and the nature of the binding are of increasing importance. Most cofactor molecules are quite large in comparison with the substrate molecule. As a consequence it will be expected that the incorporation of the cofactor into the active centre will be subject to greater diffusional control than the substrate(s).

The data presented in this thesis demonstrated that the amount of potentially active enzyme, that is the amount of enzyme that had the potential for activity in the presence of cofactors, was a function of four parameters:

1. The amount of enzyme loaded on the material,
2. The presence of cofactor during immobilisation,
3. The conditions of immobilisation, and
4. The material and coupling method.

Since the cofactor must associate itself with the active centre of the enzyme the active site must be accessible. Subsequently the orientation of the enzyme on the support, the material morphology of the material and the site of the coupling reaction are important factors to consider. In the case of Eupergit-C[®], a porous, small support which swells readily, the enzyme only retained 20 % of its activity after immobilisation, while the inclusion of TPP increased the retention of activity to 60 %. This leads to the logical conclusion that the inclusion of the cofactor in the active site can somehow protect its integrity and allow a higher retention of activity. However, in order to achieve this, the conditions of immobilisation had to be altered. In the case of Amberlite XAD7 the inclusion of the cofactor during immobilisation did not result in a large gain of retained activity. This would indicate that perhaps the coupling of transketolase by gluteraldehyde involved essential amino acid side chains, most likely those involved in catalysis.

The alteration of immobilisation conditions to ensure that the cofactor is incorporated in the active centre can result in a large gain in retained activity. Subsequently when

immobilising a cofactor requiring enzyme this will be an important consideration. The selection of coupling technique, however, will affect the efficiency of this method. Firstly the functional group on the surface of the immobilisation support must be reactive under the altered pH and ionic conditions. Secondly the method must not incorporate amino acids in the active centre.

4.5 Immobilisation as a Process Option

Immobilisation is one alternative in a wide variety of options for the operation of a productive biotransformation process. Since advances in genetic engineering have resulted in the hyper-expression of a variety of enzymes, the use of immobilisation purely on the grounds of the cost of the enzyme can not be justified. Subsequently other reasons for the justification of immobilisation are necessary. It was part of the aim of this work to discuss the reasons for immobilisation in the light of current fermentation technology and genetic engineering advances. It is the aim of this section to discuss immobilisation as an option in the development and design of a biotransformation process. This will take the form of discussing the characteristics of immobilised enzymes and the constraints these have upon bioreactor operation. Finally a strategy for the development of immobilised enzyme biocatalysts will be discussed.

Immobilisation is carried out for one major reason, the reuse of the enzyme activity and subsequently the efficient use of the enzyme. However, immobilisation can also be used to improve the whole process. In this sense it becomes a factor in the integration of various process options. Each unit operation within a bioprocess flow sheet can be thought of as an individual process. When developing each option it is possible to do this in isolation of the whole process. This can be inefficient and result in a loss of yield. It is also possible to consider how each unit operation effects the subsequent operation. In this case each operation can be designed with the following operation in mind. In this case, individual yields may be compromised in order to increase the overall yield of the process.

Each unit operation has its own input and output stream which confers a particular 'shape' upon it. The subsequent operation needs to have the same input shape as its predecessor for the most efficient process. For example, the output stream of the bioreactor may contain protein, which subsequently adsorbs onto silica column used to remove the product. In this case the design engineer has the choice of altering the input stream of the subsequent product purification step or the output stream of the bioreactor. This can be achieved by the addition of an extra step for example ultrafiltration, altering the composition of the input stream to the product recovery operation to reduce or prevent adsorption of protein or by the alteration of the bioreactor output stream by using an immobilised enzyme to retain protein within the bioreactor.

Since every unit operation has its own 'step yield' some product will always be lost. The inclusion of the extra step between bioreactor and product recovery is likely to reduce the overall yield of the process. The inclusion of extra steps may also increase the overall cost of processing by increasing the equipment costs. The addition of steps should therefore be avoided if possible. The alteration of the input stream of the recovery operation will often result in the dilution of the product and result in the need for a concentration step further down stream it can be argued that the most efficient operation to change is the bioreactor itself. As a result immobilisation of the enzyme is one of the first options that should be considered during the development of the bioreaction, even if the enzyme can be produced in sufficient quantities that it is no longer a significant cost in the process.

Along side the benefit of removing the biocatalyst from the product stream, immobilisation also often improves the stability of the process (Guisan *et al.*, 1986 ; Klibanov 1979) as a result, in cases where the enzyme is denatured by a component in the reaction. For example the substrate, immobilisation can stabilise the proteins structure and subsequently the activity. In this way the productivity of the biocatalyst can be improved, and although it may not be critical due to the low cost of the catalyst, it will be an important contribution to the whole process.

When considering immobilisation of an enzyme several factors which have been highlighted during the course of this work need to be taken into account:

- The cost and nature of the enzyme. Is the enzyme hyper-expressed?
- Does the enzyme require a cofactor? How does the cofactor bind to the enzyme?
- Is the enzyme an oligomer? How easily do the subunits dissociate?
- How much activity can be retained after immobilisation?
- Cost of the support materials.
- The stability of the free enzyme in all of its states. How do immobilisation conditions affect the stability of the enzyme?

The cost of the enzyme will determine the reason why the enzyme is to be immobilised. If the enzyme is expensive immobilisation will often be necessary in order for the process to be economic. On the other hand if the enzyme is relatively cheap immobilisation will not be necessary on cost grounds alone. In the situation where the enzyme requires a cofactor it may be necessary to immobilise the enzyme in its active form, protecting the cofactor from involvement in the coupling reaction. As a result an understanding of cofactor binding will be necessary.

The quaternary structure of the enzyme can be an important factor in determining how an enzyme should be immobilised. In the case of transketolase, for example, the active site contained amino acid residues from both subunits. If the enzyme is immobilised in monomeric form it will be inactive even if cofactor is introduced after immobilisation. Knowledge of how the subunits dissociate can constrain the conditions of immobilisation and subsequently the support material and binding reaction.

As discussed previously the retention of activity after immobilisation is a measure of how efficiently the enzyme has been bound. This will effect the cost of the whole biocatalyst. When this is taken into account with the cost of the support material a clearer picture of the most effective support material can be built. In addition to the retention of activity, the stability of the biocatalyst will determine the effectiveness of the support material. In the case of a material which yields a biocatalyst with a low retention of activity after immobilisation, the final biocatalyst needs to demonstrate a

higher stability than the free enzyme and than the biocatalyst with a higher retention of activity after immobilisation.

An enzyme can be immobilised for two major reasons:

- Productivity (kg Product/kg Enzyme), where the enzyme is expensive and therefore needs to be used repeatedly.
- Yield (kg final product). In order to remove the enzyme from the product stream.

In the first case it is important that the enzyme is used effectively, as a consequence the resulting biocatalyst must express the maximum possible activity and operational stability. This will subsequently reduce the amount of enzyme required to produce the product. In the second case it is more critical, for whatever reason, that the enzyme does not contaminate the product stream. In this case the attachment between enzyme and support has to be secure enough that enzyme doesn't leach into the product stream, also, the support material must be sufficiently large to allow easy reclamation or retention. In the latter scenario the final activity of the biocatalyst can be sacrificed in favour of a support material that is easy to recover.

The development of a rationale for the immobilisation of an enzyme must include the selection of an appropriate support material, the definition of the immobilisation protocol and the determination of the operating conditions of the final biocatalyst. Woodley and Lilly (1996) developed a rationale for the selection and development of a bioreactor for a biotransformation process. This rationale was based upon the determination of constraints which limited the scope or range of operation of the process. The definition of these constraints subsequently allowed the variety of options available to be reduced and the most viable options to be determined and validated.

Constraints upon Immobilisation	Constraints upon Operation,
Compatibility of support with reactants	Stability of the biocatalyst
Support cost	Retention of activity after immobilisation
Support capacity	Specific activity
Stability of free enzyme	Particle size
Retention of activity	Support cost
Particle size	Mechanical Properties of material

Table 4.7 List of characteristics which will constrain the immobilisation protocol and the mode of operation of the bioreactor.

The constraints upon the immobilisation protocol are factors which influence the ability of the support to bind enzyme resulting in an biocatalyst with a reasonable cost per unit activity. The first question that should be posed, is what support materials are compatible with the reactants and products. The chemical inertness of the support material and the enzyme binding functional group is crucial.

As previously discussed the cost of the support material alone is not sufficient grounds to reject a material. The cost must be taken into account with the stability and retention of activity of the resulting biocatalyst. The support capacity and stability of the free enzyme are factors which will determine the conditions under which immobilisation should take place. These constraints include the stability of the structure of the enzyme, in other words, whether the enzyme is an oligomer or whether a cofactor is required.

Several questions need to be asked regarding the successful operation of a bioprocess with an immobilised biocatalyst. The stability of the biocatalyst will determine how many cycles of operation the biocatalyst can be used. Alongside the cost of the material and the retention of activity this factor can determine the final selection of support material.

Conclusions

In this section short conclusions to the work carried out are presented. These conclusions are intended to be specific to the findings for transketolase immobilised onto Eupergit-C[®] and Amberlite XAD7 and no attempt to analyze or expand these conclusions to the general case has been made.

- The fraction of soluble enzyme containing TPP (holo-enzyme) was found to be dependent upon the pH. At pH values above 7 all of the enzyme was found to be in the apo-form (i.e., without TPP).
- Holo-transketolase displayed a half life of 10 h in solution, this could be improved to the extent that no activity was lost over 72 hours, by the addition of mercaptoethanol, or by maintaining the enzyme under a nitrogen head space.
- The enzyme was immobilised onto Eupergit-C[®] and Amberlite XAD7 in the absence of cofactor with a retention of 20 % activity.
- The fraction of activity recovered when the enzyme was immobilised onto Eupergit-C[®] was dependent upon the amount of enzyme challenged to the support material, 20 % activity was recovered when the support was challenged with 2100 U.g⁻¹ (dry) while a retention of 67 % could be achieved with a challenge of 150U.g⁻¹ (dry).
- Co-immobilisation of transketolase with TPP could be achieved by immobilising in the presence of TPP at pH 6.5 and by maintaining a nitrogen head gas over the suspension. 60 % of the challenge activity could be retained by the enzyme immobilised onto Eupergit-C[®] in this manner even with an enzyme loading of 1434 U.g⁻¹ (dry).

- No improvement upon the low retention of activity obtained with Amberlite XAD7 could be achieved by immobilising in the presence of TPP, only 20 % activity could be recovered with the co-immobilised transketolase and TPP.
- Soluble transketolase was shown to be unstable in the presence of its aldehyde acceptor substrate, glycolaldehyde. A half life of 1 hour was achieved with the soluble enzyme. However the immobilised enzyme demonstrated a half life of 100 h and 80 h for Eupergit-C[®] and Amberlite XAD7 respectively.
- A space time yield of 674 kg.L⁻¹.batch⁻¹ was achieved with transketolase immobilised onto Eupergit-C[®] while a space time yield of 282 kg.L⁻¹.batch⁻¹ was achieved with the enzyme immobilised onto Amberlite XAD7.
- Based upon an annual production of 500 tonnes of erythrulose with 5 batches per week a start up charge of 330 kU or 920 kU of transketolase would be necessary in the case of Eupergit-C[®] and Amberlite XAD7 respectively.
- The cost per unit of immobilised activity based upon this work and the commercial costs of Eupergit-C[®] and Amberlite were 0.27 £kU⁻¹ and 0.12 £kU⁻¹ respectively.
- The amount of enzyme required to produce one kilogram of erythrulose was calculated to be 19 kU.kg⁻¹ in the case of the Eupergit-C[®] preparation and 150 kU.kg⁻¹ in the case of the Amberlite XAD7 preparation.
- The cost of producing erythrulose using Eupergit-C based catalyst would be 5.13 £.kg⁻¹ and 18 £.kg⁻¹ in the case of the Amberlite preparations.

Future Work

It is apparent from this work that several factors involved in the immobilisation and use of transketolase require further investigation. One of the primary future objectives involves determining the effect of carbon dioxide on the immobilised enzyme particle. Since there are several other biochemical conversions which result in the generation of carbon dioxide as a byproduct, for example the oxidation of formate to carbon dioxide which is used in NADH recycling, this effect is not specific to transketolase and would yield valuable information about the use of biocatalysts.

This work highlighted that, although there was no charge on the support material, the production of carbon dioxide resulted in a proton sink within the support material. This could be investigated in the future, perhaps with the aim of creating a mathematical model. Transketolase would perhaps present the ideal model system for the investigation of a carbon dioxide producing reaction because there is a pH change involved during catalysis and subsequently the effect of carbon dioxide is quite striking.

A second area that requires further investigation is the mechanism of denaturation of transketolase by its substrate glycolaldehyde. This would enable an investigation into the mechanism of stabilisation of the enzyme by immobilisation and subsequently give valuable insight into support material selection. The issue of enzyme stabilisation by immobilisation requires further investigation. Several questions about the stabilisation of proteins by immobilisation remain unanswered. Transketolase could perhaps be a model system for an investigation into stabilisation against denaturants like glycolaldehyde by a variety of different support materials, perhaps yielding some insight into the nature of protein stabilisation and yielding some general rules about the selection of an immobilisation support to stabilise a protein. This investigation could vary the binding chemistries and support materials to yield a picture about the nature of the binding chemistry, the target amino acids and the interaction between the support material and the protein itself.

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