CARBON-CARBON BOND FORMATION: REACTOR SELECTION FOR TRANSKETOLASE-CATALYSED BIOTRANSFORMATIONS

by

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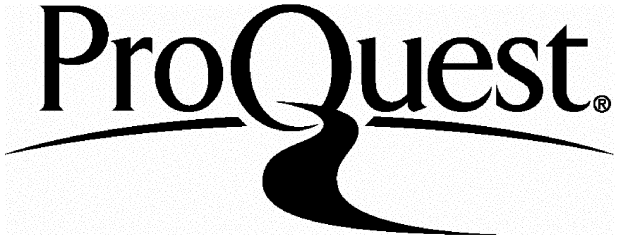

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To Anna, Lily and Louis.....

Acknowledgement

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Abstract

A rational approach to biotransformation process design can result in significantly reduced optimisation and scale-up times. This thesis describes the application of a structured approach to bioreactor selection for transketolase catalysed biotransformations. A decision making procedure based initially on characterisation of the reaction components, the biocatalyst, the reaction and the interactions between them was employed. These characteristics were used to set constraints and reactor options could then be evaluated in terms of relative performance within these constraints.

The identification of the key parameters and constraints on transketolase biocatalysis was conducted employing a model reaction as the research tool. Analytical methodologies were developed for the model reaction and utilised for biotransformation characterisation studies. These characteristics enabled tentative definition of the key constraints of the process and suitable reactor options for further evaluation. A number of the reactor options were investigated. Validation of the constraining parameters determined from these initial studies was conducted using a second reaction. Research involved the development of the analytical methods for this validation reaction, investigation of the key parameters perceived as constraints and a theoretical evaluation of the reactor options.

The constraints of transketolase-catalysed biotransformation processes utilising substrates exhibiting a range of industrially representative characteristics has therefore been defined. Based on this information the optimum reactor configurations for a range of transketolase catalysed biotransformations can be assigned based on the acquisition of a small but definitive set of data. The application of this type of approach to industry has been considered.

Contents

Index of Figures

Index of Tables

Nomenclature

A	absorbance (-)
c	concentration (μmol ml^{-1})
ε	extinction coefficient (ml $\mu mol^{-1}.mm^{-1}$)
E	total catalyst in reactor (moles)
Km	Michaelis constant (mol l^{-1})
Ki	Inhibition constant (mol l^{-1})
k	rate constant (sec^{-1})
l	light path (mm)
Q	volumetric reactant feed rate (l sec^{-1})
$R_{p/x}$	ratio of product per unit biocatalyst
So	initial or feed reactant concentration (mol l^{-1})
Sa(t)	specific activity as a function of time (g. g^{-1} d^{-1})
Tc	operational life of the catalyst (d)
t	batch reaction time (sec)
V	reaction volume (l)
Vo	initial reaction rate (mol min^{-1} l^{-1})
Vmax	maximum reaction rate (mol min^{-1} l^{-1})
X	fractional conversion of reactant to product

Units

Å	angstroms
°C	degrees centigrade
cm	centimetres
d	days
hrs	hours
kDa	kilodaltons
l	litre
m	metres
mg	milligrams
min	minutes
ml	millilitre
mM	millimolar
M	molar
mol	moles
nm	nanometres
rpm	revolutions per minute
sec	seconds
µl	microlitres
µm	micromolar
%	percentage
% v/v	percentage volume by volume
% w/v	percentage weight by volume

Abbreviations

BSA	bovine serum albumin
BSTR	batch stirred tank reactor
CISSR	continuous *in-situ* S-aldehyde removal
CST(R)	continuous stirred tank (reactor)
DSP	downstream processing
DHAA	dihydroxyacrylic acid
E. coli	*Escherichia coli*
FBSTR	fed-batch stirred tank reactor
HEPES	N-2-hydroxyethylpiperazine-N'-ethanesulphonic acid
H^1NMR	proton nuclear magnetic resonance
HPA	β-hydroxypyruvate
HPLC	high performance liquid chromatography
GC	gas chromatography
ISPR	*in-situ* product removal
KBS	knowledge based system
MW	molecular weight
NADH	nicotinamide adenine dinucleotide (reduced)
PFR	plug flow reactor
PIPES	piperazine-N-N'-bis(2-ethanesulphonic acid)
3-OBG	3-oxy benzylglyceraldehyde
5-OBX	5-oxy benzyl-D-xylulose
ODS	octodecyl silica
RCF	relative centrifugal force
TASA	tartronic acid semialdehyde
TLC	thin layer chromatography
TLPSTR	two liquid phase stirred tank reactor
TK	transketolase
Tris	tris(hydroxymethyl)aminoethane
UV	ultraviolet

1. INTRODUCTION

1.1. Biotransformations - a general overview

Enzymes are the biological catalysts necessary for the biochemical processes of life. Over the last fifty years industry has increasingly harnessed these biocatalysts for the synthesis of commercially important products. Due to the organic nature of enzymes, the reaction conditions for functional catalysis are constrained within narrow operational limits. However, unlike chemical catalyses, these conditions are frequently relatively mild being limited to ambient temperatures and pressures and near neutral pH. Enzymatic catalysis is inherently more selective than chemical synthesis. This results in reduced side reactions, easier separation and potentially higher economic efficiency. Enzymes exhibit a high degree of regio- and stereo-selectivity and specificity (Jones and Beck, 1976; Cannarsa, 1996). However, the exploitation of enzymes by industry arises from the ability to catalyse the conversion of a range of substrates in addition to the natural substrates encountered *in vivo.* Providing the substrate satisfies certain physical and chemical criteria dictated by the conformation of the 'active site' of the enzyme, a wide range of potentially useful substrates may be substituted. Where these reactions are limited to a few chemically defined steps the process is termed *biotransformation* .

The first biotransformation of industrial importance was the use of whole cells for steroid modifications in the early 1950's (Petersen *et al.*, 1952). However, since this time several technological advances have produced major impacts in the field. These are :

- the advent of large scale enzyme isolation techniques. This is due to developments in the design and manufacture of fermentation and downstream processing equipment and advances in control and monitoring technology

- the discovery and practical application of immobilisation methods for enzymes and cells. The main impact of this technology is the improved economics resulting from the reusability of immobilised biocatalysts.

- the use of biocatalysis in non-aqueous solvents. This technology extends the range of substrates amenable to biocatalysis to include aqueous immiscible substrates.

- the rapid developments in DNA technology. The major impact of DNA manipulation has been to decrease the cost of biocatalyst production by increasing the specific yield of biocatalyst. In addition, protein engineering by DNA manipulation is becoming increasingly useful for changing substrate range and improving enzyme stability (Wetzel, 1986).

Although chemical and biological synthesis can be viewed as competing technologies, there are many processes, particularly in the food and pharmaceutical industries, where biocatalysis has no chemical competition. Examples include the depolymerisation of starch, the synthesis and modification of biopolymers and the production of synthetic and semi-synthetic carbohydrates. However, it is the stereo-selective/-specific property of enzyme catalysed reactions which is attracting increasing interest particularly from the pharmaceutical industry not only for the synthesis of new drugs but for improving the formulation of existing drugs particularly drug racemates of which there are over 500 currently marketed (Cayen, 1991). Proposed changes in the regulatory climate suggest that racemic drugs will no longer be regarded as individual entities but as mixed entities. This will require the implementation of expensive validation studies to acquire pharmacological and toxicological data on each individual enantiomer. With advances in biotransformation technology, asymmetric chemical synthesis (Brown, 1989; Ward, 1990; Crosby, 1991), stereoselective crystallisation (Sheldon, 1990; Gottarelli and Spada, 1991; McBride and Carter, 1991) and chiral analytical techniques (Morrison, 1985; Trost *et al.*, 1986; Ferringa *et al.*, 1986; Krstulovic, 1989; Cannarsa, 1996), regulatory pressures are driving the pharmaceutical industry towards the

manufacture of single isomer drugs. In many cases biotransformation will be the only suitable methodology. It has been projected that 80% of all new synthetic drugs will be homochiral by the year 2000 (Margolin, 1993).

1.2. Biocatalysts for chemical syntheses

1.2.1. General

To date approximately 3000 enzymes have been partially or wholly characterised. For many, the structural and stereochemical specificities are well documented (Jones and Beck., 1976). Table 1.1 illustrates the main classes of enzymes based on the reactions catalysed *in vivo* and describes the properties of each class. Although over 300 different enzymes are commercially available (Hacking, 1986) with the total annual U.S. market volume of bulk industrial enzymes estimated at US$ 340 $x10^6$ (Hartmeier, 1988), the bulk of the industrial volume production consists of only a few different enzymes used mainly in the starch, washing powder and dairy industries. Enzymes can be classified according to the type of reaction catalysed. The proportion of commercially available enzymes in each class is shown in Table 1.2. (Roberts *et al.*, 1995).

Plant, animal and microbial cells are all sources of industrially useful biocatalysts. However, the microbial (bacterial, yeast, and fungal) enzymes typically account for 80 % of total industrial production (Godfrey and Reichelt, 1983). Microbial cells are relatively simple to grow, exhibit high growth rates and are extremely diverse in species. In addition, this group is receptive to genetic manipulation which may result in enzyme overexpression (Arbige and Pitcher, 1989; Tien and Tu, 1987) and may permit catalyst modification by protein engineering techniques (Wetzel, 1986). For industrial processes it is not always necessary to extract the enzyme in pure form since crude preparations, whole cells or even cell fragments may be sufficiently catalytically active.

Enzyme class	Reaction catalysed	Representative subclasses
Lyases	Cleaves C-C, C-N, C-O bonds by elimination to produce double bonds or add groups to double bonds	decarboxylases, aldolases, ketolases, hydrolases, hydratases, dehydratases
Oxidoreductases	Catalyses oxidation and reduction reactions	oxidases, dehydrogenases, oxygenase, peroxidase
Transferases	Transfers functional groups of one compound (donor) to a second molecule (acceptor)	transaldolases, transketolases, glycosyltransferases, methyltransferases, acyltransferases, transaminases, alkyltransferases
Hydrolases	Hydrolyse a variety of compounds such as esters, lipids, thiolesters, phosphates, pyrophosphates, glycosides, peptides and proteins	lipases, esterases, proteases, glycosidases, sulphatases, phosphatases, aminoacylases, nucleasess, halohydrolases
Isomerases	Catalyse intramolecular isomerisation	racemases, isomerases, epimerases
Ligases	Link together two or more molecules with the simultaneous hydrolysis of ATP. (C-O, C-N, C-S, C-C bonds)	synthetases, carboxylases

Table 1.1. Classes of enzymes and reactions catalysed *in vivo.*

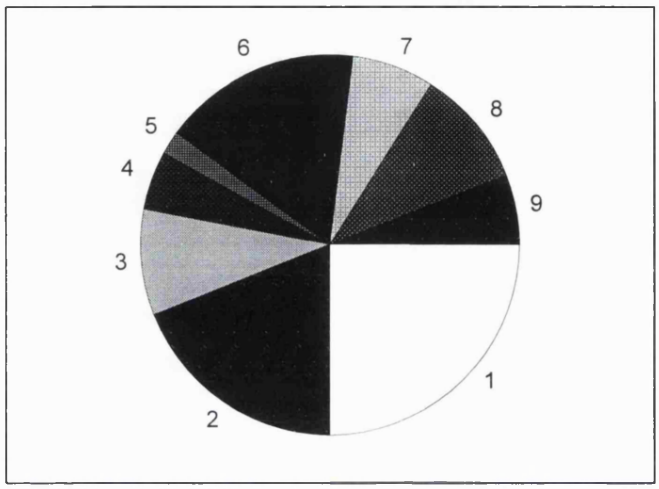
Enzyme classification	Number of enzymes identified	Number of enzymes commercially available
Lyases	255	35
Oxidoreductases	650	90
Transferases	720	90
Hydrolases	636	125
Isomerases	120	6
Ligases	80	5

Table 1.2. Number of enzymes identified and commercially available (Roberts *et al.*, 1995).

There are a number of actual and perceived problems associated with biocatalytic systems for chemical synthesis. It is widely believed that enzymes can only function in aqueous solutions thus creating problems of scale with poorly or none water soluble substrates. However, the use of enzymes in pure solvents or aqueous-solvent mixtures is receiving increasing attention (Lilly and Woodley, 1985; Klibanov, 1990; Woodley *et al.,* 1990; Halling and Valivety, 1992; Collins, 1995). High rates of conversion and good productivities have been obtained. Another misconception is that enzymes are too fragile for commercial chemistry. Although many enzymes denature above 65°C, heat stable enzymes from thermophilic micro-organisms are available (Fiala and Stetter, 1986, Huber *et al.*, 1987). In addition, rates of enzyme catalysis at ambient temperature are often of the same order of magnitude as chemical catalysis. Many surfactants, oxidants, reductants, and organic solvents deactivate biocatalysts but the majority of perceived problems with biotransformations can be overcome or at least significantly reduced by judicious selection of both the biocatalyst and the reactor configuration. The advantages and disadvantages of biotransformation over chemical synthesis must be compared on a case by case basis.

Although enzyme processes are now competing successfully against chemical methods in asymmetric syntheses, the frequency of use of a particular biocatalyst is not distributed evenly among the various types of biotransformations. Figure 1.1 illustrates the proportion of biotransformations performed using the different groups of enzymes (Faber and Franssen, 1993). However, it should be noted that many of these reported reactions were performed on analytical or preparative scale only and the number of commercially viable biotransformations is not illustrated.

Over 50% of all biotransformations are performed using the hydrolytic enzymes lipase, esterase and protease. This is mainly due to the availability, high stability in aqueous and organic solvents and no cofactor requirement. Many redox and oxidation reactions currently use whole cells instead of free enzyme due to the requirement for cofactors.



Key

1 lipases	6 whole cell oxidoreductases
2 esterases	7 (isolated enzymes)
3 proteases	8 oxygenases
4 nitrilases	9 lyases, transferases, isomerases
5 other hydrolases	

Figure 1.1. Frequency of use of a particular biocatalyst in biotransformations (Faber and Franssen, 1993).

The key to a successful industrial biotransformation process is reliability and predictability of the biocatalyst. Due to the complex nature of biological systems, batch to batch variability of the biocatalyst may be a problem particularly with crude biocatalyst preparations. For example, the selectivity of porcine liver esterase varies depending on the commercial source used (Polla and Frejd, 1991). Since only a small number of enzymes have been fully characterised with regard to reactivity and selectivity, trial and error methods are usually employed to determine the constraints on the range of substrates although substrate-selectivity models have been created for porcine liver esterase (Toone and Jones, 1991), Candida lipase and Pseudomonas lipase (Kaslauskas *et al.*, 1991).

In addition, although aqueous conditions were previously thought necessary for enzyme activity, it has recently been noted that some enzymes can be active in dry organic solvent (Klibanov, 1990). The activity is only observed in the more hydrophobic solvents (log P > 4) and the effects on substrate selectivity are still not fully understood.

The lack of appropriate literature databases severely limits the rate of progress in this area. The characterisation of the biocatalyst is generally regarded as of secondary importance to the organic chemistry when biotransformations are reported. Enzymes from unusual microbial sources, such as extremophiles, may exhibit novel and useful properties but data on these catalysts are rarely available.

1.2.2. Form of biocatalyst

The three main forms of biocatalyst currently used in biotransformations are :

1. soluble enzyme
2. immobilised enzyme
3. whole cells (proliferating and non-proliferating)

Enzymes are either retained intracellularly or secreted extracellularly by the microbial producer organism and therefore require varying degrees of processing prior to the biotransformation. While commercial preparations have usually undergone some purification procedure, intensive purification is expensive and usually limited to enzymes that can be immobilised and therefore reused.

The use of soluble enzyme for biotransformations has both advantages and disadvantages. The homogenous nature of these systems allows high levels of control and predictability. Also, there is negligible cost additional to producing the enzyme and presenting it to the substrate. However, the soluble enzyme is difficult to recover and therefore usually lost after each reaction. In addition, the loss of biocatalyst may increase product purification costs as the product stream will be contaminated with protein. A deactivation procedure may also be required before analytical measurement or downstream processing of the product can proceed. Many soluble enzymes display Michaelis Menten kinetics in aqueous solution where the rate of reaction is proportional to substrate concentration. Consider the case of single substrate to single product:

$$Vo = \frac{Vmax \,.\, [So]}{Km + [So]}$$

Vo = initial reaction rate
Vmax = maximum reaction rate
So = initial substrate concentration
Km = Michaelis Menten constant

If the initial substrate concentration [So] approaches the Michaelis Menten constant for the substrate, Km, the enzyme is used inefficiently. In industrial biocatalytic processes such as bioremediations and waste-treatment the substrate concentration may be low and the catalyst working sub-optimally. In synthetic biotransformation processes, higher substrate levels are preferred to maximise enzyme efficiency and reduce downstream costs. Substrate toxicity effects at these high substrate levels may affect the enzyme but the effects can be reduced by employing feeding strategies to control substrate concentration within the optimum range for enzyme activity and stability.

Immobilisation of the enzyme has many advantages. The ease of separation from the reactant mixture allows the catalyst to be re-used thereby producing a cleaner product stream. Also, the reaction can be terminated at a desired point just by

physical removal. In some instances, immobilisation of the enzyme has resulted in increased stability (Klibanov, 1979; Guisan, 1996; Lin *et al.*, 1996). However, immobilisation procedures may be expensive and may lead to partial or total inactivation of the enzyme. Diffusional mass transfer resistances are usually increased compared to soluble enzyme systems but this is dependent on the nature of the support and the mode of operation of the bioreactor. The three main methods of immobilisation are:

1. catalyst attached to a support
2. catalyst cross-linked to catalyst
3. entrapment within a support or membrane

Immobilisation, particularly on porous supports, may provide the enzyme with a microenvironment significantly different to the bulk phase of the solution. This may result from diffusional restrictions into and out of the bulk phase (Clarke and Bailey, 1985; Hu *et al.,* 1985) and also partition effects due to charge interactions with the support (Ruckenstein and Kalthod, 1982). These partition effects may then affect kinetic constants such as observed Vmax and Km. The nature of the immobilisation method to the support may affect the accessibility of the active site and strong interactions with the support may restrict the conformation of the protein to a more rigid structure. As many enzymes require a degree of flexibility to operate, such restrictions may affect the kinetics of the system.

Many enzymes are not secreted out of the cell but remain intracellular either bound to membranes or free in the intracellular matrix. If intact cells exhibit the desired biocatalytic activity, cell disruption is often unnecessary and biocatalyst purification costs therefore minimised. The cells may be permeabilised using surfactants to promote mass transfer of substrate and product through the cell wall (Gowda *et al.*, 1991; Sonmkuti and Steinberg, 1994). Although substrate diffusion may be perceived as an inherent problem of whole cells, many enzymes are inhibited by high substrate concentrations and the lower intracellular concentration may result in higher activities. Also, retaining the enzyme in the high protein, reductive conditions within the cell can result in improved biocatalyst stability. Intracellular

enzymes may be unstable once released by cell disruption and may require a stabilising procedure which is often expensive and may result in additional contamination of the product stream.

Intact cells are particularly useful catalysts for two major types of reaction. Firstly the catalysis of a desired sequence of reaction steps which may be difficult or uneconomic to reproduce with free enzymes. Unfortunately, the use of whole cell biocatalysts may therefore also promote undesirable side-reactions. Secondly, whole cell catalysis is useful when cofactors are required for the desired enzyme activity. By using metabolising cells, intracellular cofactor levels can be maintained sufficiently high to allow continuous product formation, although metabolic carbon co-substrates need to be supplied. For example, benzene cis-glycol production by the dioxygenase system in non-proliferating intact cells of *Pseudomonas putida* results in oxidation of NADH to NAD. By supplying ethanol or glucose as a co-substrate, the reduced cofactor is continuously regenerated and carbon dioxide released as a waste by-product (Brazier *et al.*, 1990; Collins *et al.*, 1995). However, in some instances it has been possible to perform biotransformations requiring reduced cofactors in cell-free systems by adding the appropriate cofactor regenerating enzyme and associated co-substrate (Bowan and Pugh, 1985; Hartmeier, 1985; Plant and Cowan, 1991). The regeneration system, however, must be retained within the reactor and be amenable to multiple re-use to prove economically viable (Chaplin and Bucke, 1990). However, few examples of this type have been identified as economic at industrial scale.

1.3. Biotransformation Process Design

1.3.1. Introduction

Prior to selecting biotransformation technology for performing a desired chemical reaction, competing technologies need to be evaluated thoroughly. Existing and potential chemical methodologies require detailed investigation as several different synthetic routes may exist to the desired product. The extraction of the desired product from natural sources may be economically viable as many enantiomerically pure compounds can be isolated from nature (termed the 'chiral pool'). If the competing technologies are inappropriate or considered uneconomic, then biotransformation methodologies may provide the solution.

The efficient development of a complete biotransformation process requires expertise in a range of scientific and engineering disciplines. Figure 1.2 summarises the areas of expertise involved in the different process steps. Input would be required at various stages from geneticists, biochemists, biochemical engineers and organic chemists to take the process from biocatalyst selection to final process.

1.3.2. Choice of Biocatalyst

Once the biotransformation route is selected, the initial search can be extended to investigate the reported data on known enzymatic reactions similar to the desired catalysis step. This should include the availability, cost and cofactor requirements of commercial enzyme preparations together with existing and expired patents on possible biocatalytic routes. This is usually followed by a screening program to evaluate both promising commercial enzymes and in-house and environmental microbial biocatalysts. Close interaction between microbiologists, biochemists and chemists is essential to develop rational screening methodologies. If no commercial enzyme is available but a potent microbial source is identified, optimisation and genetic manipulation can be used to increase the biocatalyst yield.

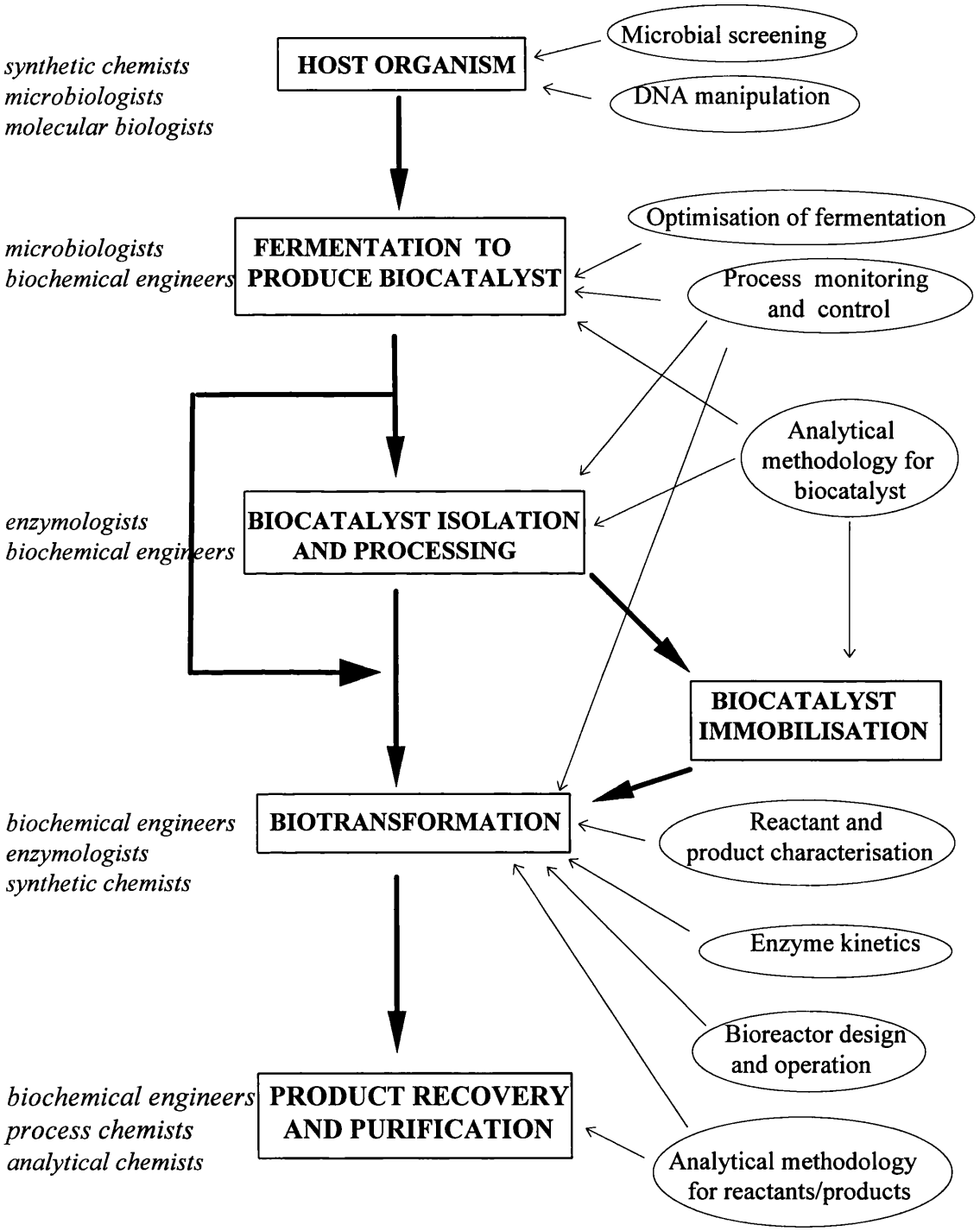


Figure 1.2. Scientific disciplines required for various aspects of biotransformation process design.

Simultaneous genetic manipulation studies can further improve yields and may also be instigated to improve enzyme characteristics. Wong and Whiteside (1994) produced a flow diagram (Figure 1.3.) illustrating the strategic approaches to enzyme catalyst development for a general biotransformation process.

The regulatory guidelines for production of biologicals also need to be addressed when identifying a potential source of enzyme. The safety aspect of large scale microbial biocatalyst production is important both in terms of microbial containment of recombinant strains and potential pathogenicity. Unknown microbial isolates should be identified, at least to generic level, before undergoing extensive investigation. Pathogenic micro-organisms should generally be avoided.

1.3.3. Reactor selection and operation

1.3.3.1. Introduction

The primary objective in the design of a bioreactor based process is to minimise the cost of producing a high quality product or service. The choice of reactor and method of operation are critical in the economics of the process as the reactor provides the link between the starting materials and the product. It is possible to separate processes that require bioreactors into those that are conversion cost-intensive (eg: biomass production, biotransformations) and those that are recovery cost-intensive (eg: antibiotic production, intracellular protein production). The volumetric productivity (space-time yield) is the crucial parameter in the economics of conversion cost-intensive processes whereas for recovery cost-intensive processes it is the product concentration that is the dominant criterion for minimising costs (Cooney, 1983). In addition, the processes can also be divided depending on the commercial price of the product, the size of the product market or the complexities of the relevant process technologies involved. Due to these differences in aims and

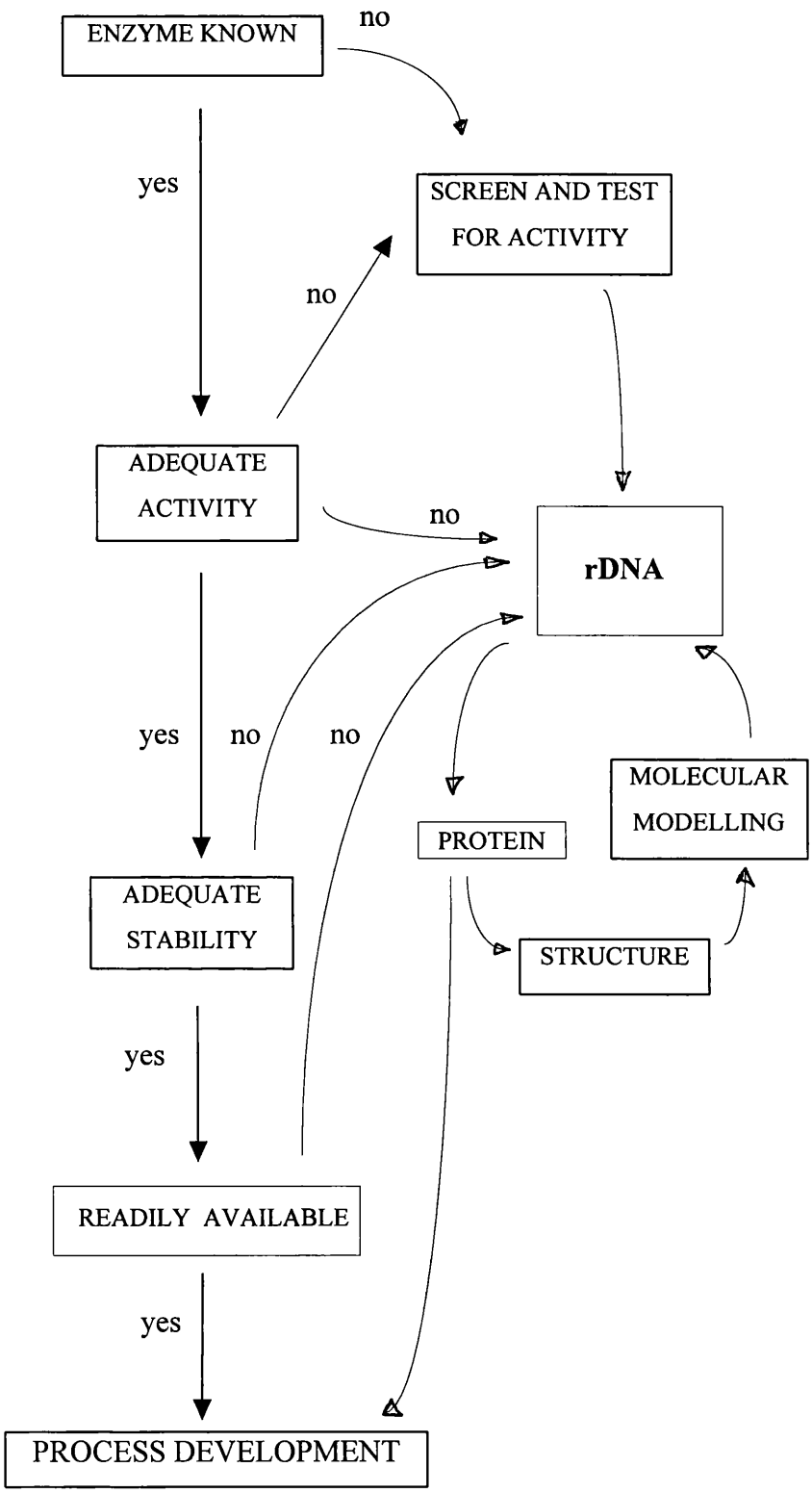


Figure 1.3. Strategic approach to enzyme catalyst development for biotransformations (Wong and Whiteside, 1994).

economics between bioprocesses, most studies on reactor selection and operation have been conducted on a case by case basis. In many cases this approach was encouraged by short term financial constraints as bioreactors were typically adopted for a particular process on the basis of available expertise and equipment rather than selected on the basis of defined process requirements. Although a variety of unit process bioreactors such as airlift (Katinger, 1973 Gasner, 1974), hollow fibre (Rony, 1971), packed bed (Lilly and Dunnill, 1976), fluidised bed (Andrews, 1982) and trickle bed reactors (Briffaud and Engasser, 1979) were reported in the 1970's, rational systematic approaches to bioreactor selection and operation were only considered relatively recently. Bliem (1989) described a rationale for selection and evaluation of animal cell bioreactors based on defining the operational constraints. In the field of biotransformation process design, Dervakos *et al.* (1995) proposed the use of a knowledge-based system (KBS) for design of biotransformation processes similar to that utilised in the chemical industry (Beltramini and Mortard, 1988). The use of KBS in this instance is, however, dependent on building a relevant and broad database based on a large number of diverse biotransformations and is therefore impractical in many industrial situations. Woodley and Lilly (1994; Lilly and Woodley, 1996) have proposed a detailed structured design philosophy for biotransformation reactor selection and operation. This philosophy is based on rational decision making procedures arising from determination of the characteristics of the various components of the bioprocess, both individually and on interaction. During this characterisation, it is necessary to collect only the data which required for the definition of constraints. Table 1.3 illustrates the constraints in several industrial biotransformation processes (Woodley and Lilly, 1996). The constraints can then be applied to screening different process options for reactor configurations which allow operation within these constraints. This screening is based on heuristics and fundamental design principles. Inappropriate reactor configurations are quickly rejected and suitable options selected for further evaluation. In cases where no economic process configuration is found, a viable process may be achieved if constraints are changed or removed by altering key biotransformation characteristics or through process techniques. Altering the biotransformation characteristics can be

Product	**Characteristics**	**Constraints**
6-APA	Acid degeneration pH dependent reaction	Control pH
Acrylamide	Reactant inhibition	Control reactant concentration
S-2 chloropropanoic acid	Acid generation pH dependent reaction	Control pH
6-hydroxynicotinic acid	Oxygen requirement	Supply oxygen
Toluene *cis* glycol	Poorly water soluble reactant Toxic reactant Oxygen requirement	Control reactant concentration

Table 1.3. Examples of constraints in industrial biotransformations (Woodley and Lilly, 1996)

achieved, for example, by altering properties of the biocatalyst using genetic and /or protein engineering. These techniques have been used successfully to increase enzyme thermostability (Perry and Wetzel, 1984; Villafranca *et. al.*, 1983), alter substrate specificity (Estell *et. al.*, 1986; Craik *et. al.*, 1985) and alter pH optima (Russell and Fersht, 1987). The utilisation of particular process techniques to overcome constraints can be illustrated by the examples of an *in-situ* product removal techniques to overcome the effects of product inhibition and product toxicity (Freeman *et al.*, 1993; Chauhan *et al.*, 1996) and by the introduction of an organic liquid phase to act as a reservoir for a biocatalyst-toxic reactant (Cremonesi, 1973; Woodley and Lilly, 1992; Collins, 1995). The ultimate aim of the structured approach is to identify the key constraints on choice of bioreactor for a particular biocatalyst. The most suitable reactor or process configuration can then be targeted with a high degree of confidence from the minimum amount of research.

This thesis challenges the structured approach to reactor selection for biotransformation process development proposed by Woodley and Lilly (1994) and summarised below (1.3.3.2.).

1.3.3.2. The structured design methodology

1.3.3.2.1. Introduction

The interrelationship between biocatalyst selection, reactor configuration, reactor operation and downstream processing is such that all these aspects of biotransformation process development should be considered concurrently. The choice of reactor configuration is dictated by constraints imposed by the characteristics of both the biocatalyst and the biotransformation. The evaluation of these constraints defines suitable reaction conditions within which a flexible and economic process can be conducted. The maintenance of these conditions defines the mode of operation of the reactor, the form of the biocatalyst and the requirement for *in-situ* operations such as continuous product removal. This 'structured' approach

to biotransformation process design is summarised in Figure 1.4. This approach allows the promising reactor designs to be targeted at an early stage in the process development. As a result, research and development studies can concentrate on evaluating and optimising these designs thus maximising the research effort. The net result of this should be an acceleration in the development from bench scale biotransformation to production scale and an early decision on the most suitable reactor configuration for the biotransformation. Fixing the biotransformation process early also has advantages regarding regulatory licensing. The FDA require validation of an entire process before granting a Process Licence. Any change introduced into the system may require extensive re-validation of all the steps downstream of the change. Changes introduced after the commencement of phase I or II clinical trials require extensive checks to demonstrate acceptable final product quality. The later the changes are introduced with regard to the progression of the clinical evaluation trials, the more exhaustive and expensive the re-validation requirement. Therefore, decisions for process changes are dominated by whether the process change might influence product identity or quality and by the economic value achieved by the change. It is therefore extremely important that the key unit operations such as the biotransformation are fixed early in the life of the project.

1.3.3.2.2. Characterisation of the biotransformation

1.3.3.2.2.1. Introduction

The properties of the substrates, products and the biocatalyst, the reaction characteristics and the interaction between these factors need to be evaluated before the reactor configuration can be selected. In order to do this rapidly and accurately, analytical methods need to be available.

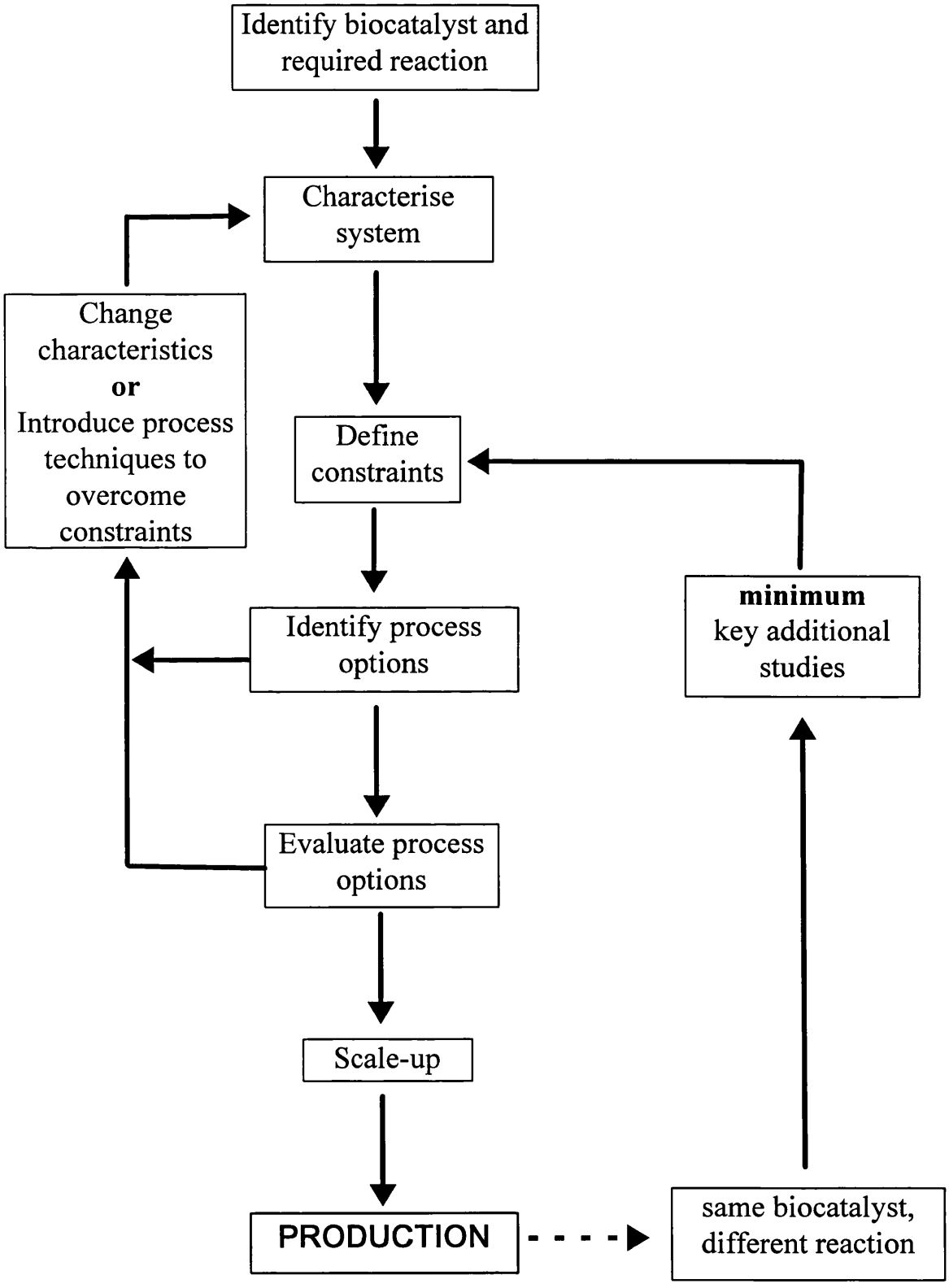


Figure 1.4. Structured approach to biotransformation process design utilising a defined biocatalyst

1.3.3.2.2.2. Properties of reactants and products

The properties of the substrates and products influences all aspects of the biotransformation process. As one of the major costs in biological syntheses is raw materials, it is important to maximise the substrate conversion yield. It is widely accepted that the cost of downstream processing is generally proportional to the concentration of the product stream. Since the limiting factor in the maximum concentration of the final product is often the maximum solubility of the substrates, a knowledge of the solubility characteristics of both reactants and product under a variety of conditions is important. These conditions should not be limited to the mild operation conditions of the biocatalyst as the substrates may be incorporated as a feedstream and would not necessarily dictate the environment around the biocatalyst.

The stability of the substrates and products needs to be evaluated under a range of pH, temperature, medium additives (buffers, antimicrobials and stabilising agents), and organic solvents. Physical and chemical differences between substrate and product can be exploited to determine the viability of *in-situ* product removal (ISPR). ISPR is useful if the product interferes with optimal activity of the catalyst or if the product is unstable under the reaction conditions. Possible ISPR techniques for different categories of biotransformation product (relative molecular weight < 1000) are shown in Table 1.4.

The properties and stability of any required cofactors may also be important. The cost of cofactors, particularly the organic cofactors such as ATP and NAD(P)H, may contribute a significant proportion of the raw material costs in a soluble enzyme system.

ISPR technique	Biotransformation product categories				
	1	**2**	**3**	**4**	**5**
Evaporation	+		+		
Extraction					
organic solvent	+	+	+	+	
aqueous biphasic			+	+	+
Size selective permeation					
dialysis			+	+	+
electrodialysis					+
perstraction	+	+	+	+	
Complexation					
chemical	+	+	+	+	+
biological	+	+	+	+	+
Immobilisation					
hydrophobic absorption	+	+	+	+	
ion exchange					+
biorecognition based	+	+	+	+	+

Key + = suitable

1. hydrophobic - volatile
2. hydrophobic - non-volatile
3. hydrophilic - neutral, volatile
4. hydrophilic - neutral, non-volatile
5. hydrophilic - charged

Table 1.4. Possible ISPR techniques for different biotransformation product characteristics (adapted from Freeman *et al.*, 1993)

1.3.3.2.2.3. Biocatalyst characteristics

The pH-activity profile and pH-stability profile for the enzyme provide important information concerning the pH window within which the biocatalyst can operate without associated loss of activity. The effect of temperature not only influences enzyme stability and activity but also the rate of growth of microbial contaminants. In addition to pH and temperature, the effect of ionic strength and the requirement for enzyme protectants and stabilising agents needs to be considered. Properties of the enzyme such as the isoelectric point and molecular weight may be utilised for purification procedures. The stability of catalysts to shear effects at different interfaces will directly determine the form of the active catalyst employed and the efficiency of mass transfer in the final process design. The use of organic solvents for poorly water soluble substrates can influence both the activity and stability of the biocatalyst. Over 2000 commonly used solvents/solvent mixtures have been utilised for biosynthetic purposes but high biocatalytic activity is generally favoured by relatively hydrophobic solvents (Laane *et al.*, 1987).

An important parameter when assessing the efficiency of a particular biocatalyst is the ratio of product per unit catalyst, $R_{p/x}$.

$$R_{p/x} = \int_0^{Tc} Sa(t)\,dt$$

Tc = catalyst half-life,
Sa(t) = specific activity as a function of time

Improvements in $R_{p/x}$ can be achieved by increasing the catalyst specific activity, the enzyme stability, and the yield of active enzyme per unit producer organism.
Loss of activity due to microbial growth during both operation and storage may also be a recurrent problem. Microbial activity can be restricted by storage at low temperatures, operation at high temperatures, operation at extremes of pH, or the addition of antimicrobials such as azide, toluene or chlorhexidine.

1.3.3.2.2.4. Reaction characteristics

The equilibrium of the reaction is governed by thermodynamics. However, in some instances, reversible reactions may be forced into the forward reaction by judicious selection of the substrates (Srere *et al.,* 1958).

The influence of reaction on pH and the requirement for control of pH is important. Buffers are commonly employed to reduce shifts during reaction but may prove difficult to remove downstream. Buffers are often used on small scale but are expensive to use on large scale. Acid and alkali titration to control pH may replace buffers but the less favourable mass transfer characteristics at large scale may prove problematic and require multiple titrant injection points. The increase in working volume by titrant addition and the associated dilution of product may also have an important influence on both the choice of reactor and on downstream processing costs.

Gas production during a reaction may also be an important parameter in consideration of reactor design. However, as the solubility of gases change with temperature, pressure and pH, the undesirable physical effects of gaseous products can be suppressed by manipulating one or more of these parameters (Takamatsu *et al.*, 1986; Palavra *et al.*, 1992). Conversely, the addition of a gaseous reactant may be a requirement of the process. For example, the supply of oxygen for oxidation reactions (Hudlicky *et al.*, 1988; Mountfort, 1990; Lynch, 1994) or to reduce the effects of reactant toxicity (Van den Tweel *et al.*, 1988). This would also exert a constraining influence on bioreactor selection and operation (Yagi *et al.*, 1969; Barzana *et al.*, 1989).

1.3.3.2.2.5. Interactions between substrates, products and biocatalyst

The substrate and product may affect the activity and stability of the enzyme and vice-versa. Anhydrides, acid halides, activated halides, activated aldehydes, alkyl halides and epoxide functional groups are all highly reactive and can cause severe inactivation of enzymes (Northeisz *et al.*, 1972). Crude biocatalyst preparations may result in side reactions and associated reduction in product yield. Increasing selectivity of the catalyst will therefore maximise conversion yield.

The kinetic constraints of the reactants and products on the biocatalyst affect the rate of reaction while the thermodynamics of the system govern the product yield. A kinetic model is useful to predict the effect of different substrate and product concentrations with V_{max}, K_m, K_a and K_i data providing the backbone of the model. This can be used to determine substrate feeding strategies and possible ISPR requirement.

The operational and storage stability of the catalyst are important criteria in the design of the process. The operational stability is the loss of enzyme during reaction but this parameter is difficult to measure for soluble enzyme systems. However, a method has been developed where the soluble enzyme is enclosed in a membrane and the reaction performed in a continuous stirred tank reactor (Tosa *et al.*, 1966). The operational stability is easier to determine for an immobilised system where the catalyst can be removed and placed in fresh reactant. Protein engineering has been employed to improve the operational stability of some enzymes. For example, by replacing oxidative unstable amino acids in the protease subtilisin with stable amino acids, enzyme stability was improved (Stauffer and Etson, 1969). Immobilisation has often been reported to have a stabilising effect on the enzyme (Alvaro *et al.*, 1989, Guisan, 1988). This probably results from stabilisation of the tertiary structure of the protein thereby reducing the possibility of conformational changes leading to denaturation of the enzyme.

1.3.3.2.3. Selection of reactor

The selection of an appropriate biocatalyst and identification of the operational windows will define constraints on the choice of reactor configuration. The bioreactor should be designed to meet the specific needs and constraints of the process and will affect both the cost and quality of the final product. However, the objective of this approach is to create a flexible biotransformation process thereby minimising the cost of switching between different substrates for a particular biocatalyst. For any particular biotransformation it is usually desirable to operate the reactor at the maximum limits of the constraints (Wang *et al.*, 1979).

The operational form of the biocatalyst has a major influence on the choice of reactor configuration. Table 1.5 illustrates the suitability of different reactor configurations for soluble and insoluble biocatalysts.

Reactors may operate in three different modes;

1. batch
2. semi-continuous
3. continuous

The choice of mode of operation is dependent on a range of factors such as substrate and product inhibition and toxicity, and reactant and product solubilities. There is extensive literature illustrating examples of novel bioreactor configurations (Atkinson, 1974). However, the main principles of reactor selection can be illustrated by comparing several basic reactor configurations - batch stirred tank, packed bed (plug flow) and continuous stirred tank reactors. Table 1.6 illustrates the general characteristics of these reactor modes (Lilly, 1992; Woodley and Lilly, 1994).

Reactor configuration	Biocatalyst form		
	Soluble	Insoluble	
		Fixed	Suspended
Stirred Tank	✓	✖	✓
Bubble Column	✓	✖	✓
Fluidised Bed	✖	✖	✓
Trickle Bed	✖	✓	✖
Packed Bed	✖	✓	✖
Membrane Reactor	✓	✓	✖

✓ suitable ✖ unsuitable

Table 1.5. Suitability of biocatalyst form to various reactor configurations (Woodley, 1992).

Characteristics	Batch stirred tank	Packed bed (plug flow)	Continuous stirred tank
Liquid flow pattern	well mixed	plug flow	well mixed
pH control	possible	difficult	possible
Temperature control	possible	difficult	possible
Gaseous reactants/ products	possible	unsuitable	possible
Presence of second liquid phase (solvent/reactant/product)	possible	unsuitable	possible
Control of substrate inhibition	poor	poor	good
Control of product inhibition	better	better	poor
Suspended solids in feed solution	possible	unsuitable	possible
Catalyst - mechanical damage	possible	unlikely	possible
- concentration limits	low	high	low

Table 1.6. General characteristics of three types of bioreactor (adapted from Lilly, 1992; Woodley and Lilly, 1994).

Assuming an irreversible reaction obeying the Michaelis Menten equation, a comparison of the kinetics of steady state can be made (Lilly and Dunnill, 1976).

Batch $$X.So + Km.\ln\left(\frac{1}{1-X}\right) = k.\frac{E.t}{V}$$

Packed bed $$X.So + Km.\ln\left(\frac{1}{1-X}\right) = k.\frac{E}{Q}$$

CST $$X.So + Km.\left(\frac{X}{1-X}\right) = k.\frac{E}{Q}$$

X = fractional conversion of reactant to product
So = initial or feed reactant concentration (mol l^{-1})
Km = Michaelis constant (mol l^{-1})
k = rate constant (sec^{-1})
E = total catalyst in reactor (moles)
t = batch reaction time (sec)
V = reaction volume (l)
Q = volumetric reactant feed rate (l sec^{-1})

When So is significantly greater than Km , the left side of all three reactions tends to X.So. When So is less than Km, the second terms on the left of each equation dominate. Under these conditions, the continuous reactor requires significantly more biocatalyst to achieve total conversion and is seldom used for enzyme mediated conversion reactions (Lilly and Dunnill, 1976). As mentioned previously, high final product concentration is desirable to reduce costs. Inhibition effects by substrate and product therefore need to be addressed. Constraints such as substrate inhibition and toxicity effects on the enzyme can be reduced by utilising process techniques such as feeding the reactant thereby maintaining the catalyst in favourable reaction conditions. In CST reactors the effect of substrate inhibition is reduced because the reactant is maintained at the relatively low level of the output concentration. For

product inhibition, however, the CST reactor is at a disadvantage to batch and plug flow as all the catalyst is maintained at the relatively high output product concentration. If product inhibition is a constraint of the biotransformation process, process techniques to overcome this constraint can be introduced such as ISPR or by introduction of an appropriate second liquid phase with favourable partitioning for the product. However, reactor design for bi-phasic catalysis is an area which will not be discussed here. The reader is referred to several good reviews available in the literature (Woodley and Lilly, 1992; Laane *et al.*, 1987). Examples of reactor configurations for some potential commercial biotransformations are shown in Table 1.7 (Woodley and Lilly, 1994). It should be noted that the assignment of these reactor configurations was performed on a kinetic basis with the assumption that the enzyme component cost contributed significantly to the overall process economics.

1.3.3.3. Scale-up and economic constraints

The considerations involved in designing biotransformation processes for large scale commercial reactions are wider than for laboratory scale research. In many cases the operating plant may be used for several different processes at different scales emphasising the requirement for flexibility of the reactor configuration. This is particularly important if the price of the product is prone to fluctuations on the world market. Ideally it should be possible to incorporate the reactor configuration into the existing downstream and purification plant. The scale-up of a process is often mistakenly considered to consist of a change in reactor volume. However, scale-up often requires the equipment to meet different design criteria. Re-validation costs for a single large reactor of new design may outweigh the costs of process scale-up using several smaller vessels of a known but less efficient design.

The safety of the process on large scale is paramount in obtaining regulatory licences. In order to satisfy regulations for biological products, particularly

Process	Substrate/ product aqueous solubility	Inhibited by:		Acid or base product[n]	Catalyst form	Catalyst stability	Reactor type
		subst	prod				
Amino acid ester hydrolysis	all high	no	no	acid	immob. enzyme	good	packed bed
Penicillin acylation	all high	yes	yes	acid	immob. enzyme	good	fed-batch
Benzene oxidation	substrate low	toxic	no	none	whole cell	poor	fed-batch
Fat interesterification	all low	no	yes	none	immob. enzyme	good	packed bed

Table 1.7. Reaction parameters for several biotransformations of commercial potential (Woodley and Lilly, 1994)

therapeutics, strict controls must be exercised over all the facilities utilised for manufacturing and testing the product. This also includes safe handling practices for both biologicals and solvents including waste disposal and pollution control. These may confer significant additional costs particularly at large scale.

1.4. Transketolase -A Model Biotransformation

1.4.1. Introduction

In order to challenge the applicability and feasibility of the structured approach to biotransformation design, a suitable biocatalytic reaction with significant potential for asymmetric synthesis was required as a model. Due to the development of an in-house source of recombinant transketolase at UCL (Hobbs *et al.*, 1995) as part of an intensive multidisciplinary program evaluating this biocatalyst, transketolase was used as a model system for this study.

1.4.2. Synthetic potential of transketolase

Enzymes whose function *in vivo* is the degradation of carbohydrates, are also versatile biocatalysts for stereoselective carbon-carbon bond formation. Non-enzymatic methods of this type of synthesis are usually performed in organic solvents and low temperatures. However, for carbohydrates this requires extensive strategies for protection of the numerous polyfunctional groups. The utilisation of enzyme mediated synthesis, specifically the use of lyases, aldolases and transketolases, allows the reaction to proceed in aqueous solution and at neutral pH without the need for protection. In general, aldolases catalyse the addition of a nucleophilic carbonyl group of up to three carbons to an electrophilic aldehyde

acceptor molecule via an aldol condensation. The nucleophile is usually dihydroxyacetone phosphate whereas the aldehyde species can be more diverse. Aldol reactions catalysed by DHAP-dependent aldolases are useful for elongation of aldehydes by two to three carbon units and are both diastereo and enantioselective. Different aldolases can be used to give both threo and erythro-diols.

The transketolase reaction is similar to the aldolase but is limited to two carbon transfer and the enzyme requires both metal ion and thiamine pyrophosphate (TPP) cofactors for activity. *In vivo*, transketolase forms part of the pentose phosphate pathway for the transfer of an hydroxyketo group from a ketose phosphate to an aldose phosphate. However, reactants need not be phosphorylated for transketolase catalysis. Transketolase recognises chirality in the aldehyde substrate to a greater extent than aldolases and generates products with D-threo stereochemistry. Figure 1.5 illustrates the general reaction for transketolase catalysis.

The use of hydroxypyruvate as ketol donor is useful for chemical synthesis as the reaction becomes irreversible resulting from the generation of carbon dioxide as one of the two products. The potential application of this to commercial syntheses has been demonstrated with the reported production of the beetle pheromone (+)-exo breviconin (Myles *et al.*, 1991), the valuable aromatic furaneol (Hequet *et al.*, 1994), and the azo sugar 1,4-imino-D-arabinitol (Ziegler *et al.*, 1988). Chiral resolution of racemic aldehyde using transketolase has also been used to remove the undesired R-enantiomer of several racemic 2, hydroxyaldehydes (Effenberger *et al.*, 1992) as optically active 2, hydroxyaldehydes are reported to be useful for various chemical syntheses (Mulzer, 1991). Transketolase has also been utilised in the synthesis of deoxysugars (Hequet *et al.*, 1994). These compounds are rare is nature but are important in cell adhesion processes and play a vital role in the metastasis of many human cancers.

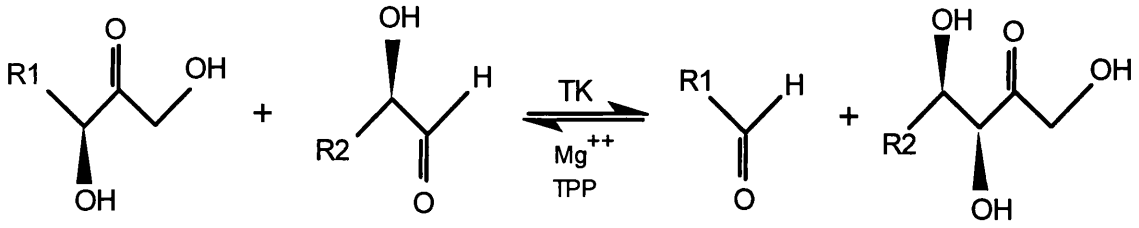


Figure 1.5. The general transketolase catalysed reaction.

1.4.3. General properties of transketolase

Although the cleavage of ribose-5-phosphate to triose phosphate by crude extracts of *Escherichia coli* (*E. coli*) bacterium was first observed in 1948 (Racker, 1948), transketolase was first identified and reported in 1952 (de la Haba and Racker, 1952; Horecker and Smyrniotes, 1952). The cofactor requirements and the reactant specificity were rapidly elucidated (Horecker *et al.*, 1953; Racker *et al.*, 1953). Transketolase has been isolated and purified from a wide variety of sources such as baker's yeast (de la Haba *et al.*, 1955), spinach leaves (Smyrniotes and Horecker, 1956), Torula yeast (Srere *et al.*, 1958) and *E. coli* (Sprenger, 1991; Iida *et al.*, 1993). However, the two main sources for study have been baker's yeast and spinach with almost all reported syntheses utilising transketolase from one of these origins. Significant quantities of pure crystalline transketolase were first obtained in 1961 (Datta and Racker, 1961) using 'new' cellulose chromatographic techniques (Sober *et al.*, 1956). Using this pure enzyme, kinetic and structural data was obtained. However, various researchers have reported a wide range of physical and kinetic properties for transketolase from the same sources as well as from different sources (Table 1.8). It is now generally accepted that transketolase from microbial sources consists of two identical sub-units each of approximately 70 kDa (Lindqvist *et al.*, 1992). Although Kochetov and Sovieva (1971) described each monomer as catalytically active, this has been refuted by several other groups (Egan and Sable, 1981; Lindqvist *et al.*, 1992). The recent elucidation of the crystallographic structure of the baker's yeast transketolase by the Lindqvist group (1992) has shown that the yeast transketolase consists of two identical subunits closely associated to give two identical active sites per dimer. Each subunit contributes residues involved in both active sites and therefore the subunit cannot be individually catalytically active. Based on structural data and supported by experimental evidence reported by other groups (Kremer *et al.*, 1979; Holzer *et al.*, 1962) a model has been suggested for the chemical steps of the reaction mechanism (Kluger, 1987, 1990; Schneider and Lindqvist, 1993). This model is shown in Figure 1.6.

Property	Source of transketolase			
	Baker's yeast	**Spinach**	***E. coli***	**Pig liver**
Molecular size in kDa [N° of subunits]	**158** [2] (Kochetov, 1982) **140** [1] (Datta *et al.*, 1961) **140** [2] Lindqvist *et al.*,1992)	**110** [1] (Horecker, 1953) **150** [4] (Murphey *et al.*, 1982) **100** [1] (Demuynck *et al.*, 1990)	**146** [2] (Sprenger, 1991)	**138** [4] (Phillipov, 1980)
Optimum pH	**7.6** (Datta *et al*, 1961)	**~8** (Villafranca *et al.*, 1971)	**8.0 - 8.5** (Sprenger, 1991)	**7.8 - 8.2** (Phillipov, 1980)
Km - HPA	**7** (Bolte *et al.*, 1987)	-	**18** (Sprenger *et al.*, 1995)	-
- TPP	**33** (Wood, 1973)	-	-	-
- Mg^{2+}	**0.032** (Kochetov, 1982) **0.4** (Heinrick *et al.*,1972)	-	-	-
Specific activity (U/ml) *[measured at 25-30°C at pH optimum using linked enzyme assay]* (Villafranca *et al.*, 1971)	**15-25** (Sigma,1995) **20** (Cavalieri *et al.*, 1975) **11** (Kotchetov *et al.*, 1978) **20** (Kotchetov,1982)	**7.8** (Villafranca *et al.*, 1971) **50** (Demuynck *et al.*, 1990)	**50** (Sprenger, 1995) **8** (Sprenger, 1991)	-

Table 1.8. Reported data on the structural and kinetic properties of transketolase enzyme from different sources.

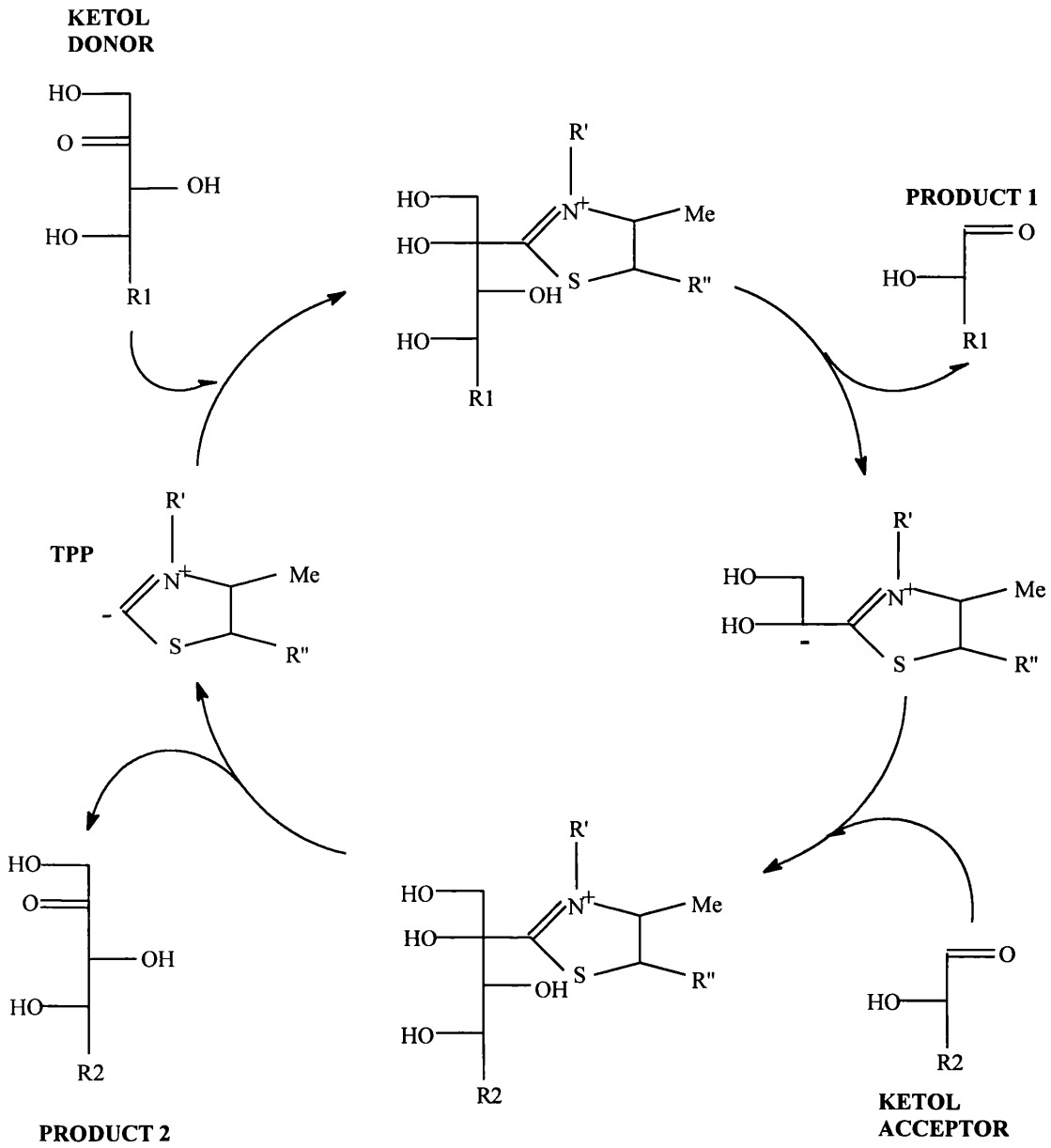


Figure 1.6. Proposed reaction mechanism for transketolase catalysis (Kluger, 1990).

The TPP is bound to the N-terminal domain of transketolase and interacts with the protein through hydrogen bonding and indirectly via the divalent metal cation. The metal ion acts as an additional anchor for the TPP. Leading to the bound cofactor is a channel or cleft, formed by loop regions of the proteins, which binds and orientates the substrates (Schneider and Lindqvist, 1993). No large conformational changes are associated with cofactor binding but it is postulated that there are two loops at the entrance to the active site cleft which are flexible enough to allow TPP and the metal cation to reach the active site. Once the cofactors are bound, the two loops interact with each other and the cofactors stabilise the conformation (Sundstrom *et al.*, 1992). Several divalent metal cations may act as a cofactor for yeast transketolase (Kotchetov and Meshalkina, 1979). In order of decreasing activity:

$$Ca^{2+} > Mn^{2+} > Mg^{2+} >> Zn^{2+} / Cu^{2+}$$

The presence of a phosphate binding site for TPP interaction may explain the observation that both sulphate and phosphate inhibit the trasketolase reaction at >5 mM concentration (Datta and Racker, 1961; Kotchetov, 1982).

A potentially unlimited source of transketolase has recently been reported by several workers using recombinant *E. coli* (Sprenger, 1991, Hobbs *et al.*, 1993). In both these cases the chromosomal transketolase gene from *E coli* was transformed back into *E. coli* at multiple copy number. Yield increases of thirty to forty fold over parent have been reported (Sprenger *et al.*, 1995). The crystalline structure of this *E. coli* transketolase has been elucidated by Littlechild and co-workers (1995). and found to show 43% sequence homology to the yeast enzyme. As the active site residues appears to be almost identical to the yeast enzyme, the reaction mechanism for the yeast and *E. coli* derived enzymes is probably also similar.

1.4.4. Measurement of transketolase activity

Several assays for transketolase have been reported in the literature. The most widely used assays use the natural phosphorylated sugar substrates and link the formation of product to a further dehydrogenase catalysed reaction. The transketolase is diluted such that this enzyme is rate limiting in the cascade reaction and the loss of NADH in the dehydrogenase step is monitored spectrophotometrically (Heinrick *et al.*, 1972; Villafranca *et al.*, 1971; Hecquet *et al.*, 1993). The main disadvantage with these assays are the high cost of both the substrates and additional enzymes.

The use of hydroxypyruvate (HPA) as the ketol donor in the transketolase reaction provides several theoretical routes for measurement of activity. The loss of HPA can be monitored directly by absorbance at 210-240 nm (Kotchetov and Phillipov, 1972) or enzymatically using glycerate dehydrogenase and alcohol dehydrogenase linked to NADH oxidation (Holldorf, 1966). The direct spetrophotometric method is not suitable for low levels of activity due to the high initial absorbance. Enzymatic determination of HPA concentration requires the transketolase to be denatured by acidification and the pH re-adjusted back to near neutral. This introduces significant errors into the system particularly with samples of low volume. Alternatively, the utilisation of HPA results in an increase in pH which can be monitored spectrophotometrically using colorimetric indicators such as p-nitrophenol (Hubner *et al.*, 1992). However, in practice, these absorbance changes are too small to be measured accurately (Hobbs, 1994). A coupled enzyme system using phospho-enol-pyruvate carboxylase (PEPC) linked to malate dehydrogenase (MDH) has also been reported (Burns and Aberhart, 1988). Carbon dioxide evolved during the transketolase reaction is one of the substrates for PEPC, the product of which is a substrate for MDH resulting in reduction of NAD to NADH. However, the difficulty with this system is maintaining the various substrate and enzyme solutions completely free of extraneous dissolved carbon dioxide. Transketolase may also be assayed by monitoring hexacyanoferrate reduction in the presence of a donor substrate (Usmanov and Kotchetov, 1991) although this is not a practical method as the sensitivity is reportedly very low (Hubner *et al.*, 1992).

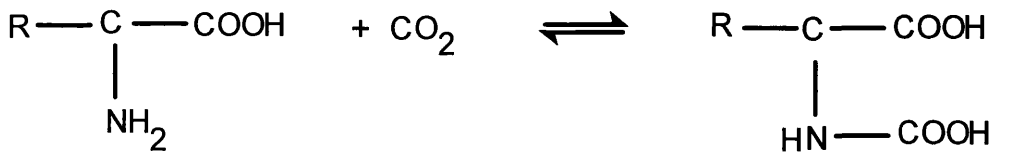
Therefore, although many different assays are to be found in the literature, none are without disadvantages. However, for research purposes, the measurement of transketolase using natural substrates via a multiple enzyme cascade appears to be the most accurate and the most suitable (Hobbs, 1994).

1.4.5. Choice of model transketolase reaction and process implications

In order to study the process leading to bioreactor design and operation for transketolase catalysed biotransformations, it is necessary to choose a model reaction. Commercial exploitation of transketolase for synthetic purposes is rarely concerned with reactions reaching equilibrium. The use of HPA as ketol donor results in irreversible reaction and stoichiometric production of carbon dioxide. Carbon dioxide production during reaction poses a number of potential process design problems. Gas production may result in foaming and denaturing of catalyst in addition to creating channelling and buoyancy effects in immobilised enzyme systems. However, reaction conditions can be manipulated to reduce these effects.

The stoichiometric production of carbon dioxide during production of the amino acid L-alanine was reportedly eliminated by pressurising the reaction system (Furui and Yamashita, 1983; Senuma *et al.*, 1989). These workers used immobilised Pseudomonas cells in a packed-bed reactor pressurised to 8 kg/cm^2 . Chemical reactions with carbon dioxide may also be a potential problem. The dissociation products of carbon dioxide dissolution may react with proteins to produce carbamates (Figure 1.7) (Mitz, 1979; Fox, 1991) and may also result in protein/bicarbonate colloid formation (Paxton, 1974). Also, as mentioned previously, the use of an HPA salt as ketol donor results in a significant pH shift to alkali during reaction. Although carbon dioxide dissociation partially offsets this shift, measures to control the change in pH, particularly on a large scale, need to be considered.

Substrate specificity studies on both yeast transketolase (Kobori *et al.*, 1992) and *E. coli* transketolase (Hobbs *et al.*, 1993) have shown that a wide range of non-phosphorylated α-hydroxyaldehydes can be substituted as ketol acceptors. Table 1.9



Protein | Carbon dioxide | Carbamate compound

Figure 1.7. Carbamate formation by reaction of proteins with dissolved carbon dioxide.

Aldehyde	**Reaction rate (relative to glycolaldehyde)**	
	E. coli T.K. (Hobbs *et al.*, 1993)	Yeast T.K. (Kobori *et al.*, 1992)
Glycolaldehyde	100	100
DL - Glyceraldehyde	41	56
D - Erythrose	87	56
L - Threose	64	-
D - Glucose	13	4
D - Mannose	4	-
Propanal	24	-
Pyruvaldehyde	21	-
Furfuraldehyde	2	-
Pyrrolaldehyde	2	-
But-2-enal	3	-

Table 1.9. Relative transketolase-catalysed reaction rates for different non-phosphorylated aldehydes with HPA as ketol donor.

illustrates the relative reaction rates for different α-aldehydes using transketolase from *E. coli* with HPA as the ketol donor (Hobbs *et al.*, 1993). In addition, α-unsubstituted aldehydes (Demuynck *et al.*, 1991) and nitrosoaromatics (Corbett and Corbett, 1986) have been shown to be acceptor substrates.

Glycolaldehyde is widely accepted as the most reactive non-phosphorylated ketol acceptor molecule for transketolase mediated catalysis (Kobori *et al.*, 1992; Hobbs *et al.*, 1993). This aldehyde is soluble in aqueous and generally stable under conditions suitable for enzymes. However, aldehydes are highly reactive compounds particularly with primary amine functional groups. Therefore reaction may occur with amino-rich proteins resulting in formation of Schiff base (Figure 1.8) and may result in loss of enzyme activity (Demuynck *et al.*, 1991). The use of HPA and glycolaldehyde as substrates for the model system therefore raises several potential problems for bioreactor design and is a pertinent reaction for study.

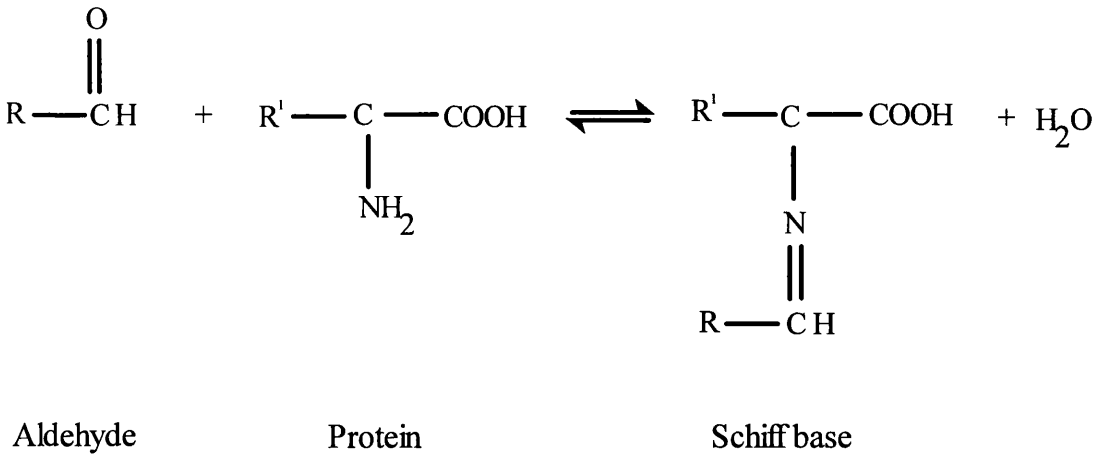


Figure 1.8. Schiff base formation resulting from interaction between protein and aldehyde.

1.5. Aims of the present work.

This study to apply the structured approach (Woodley and Lilly, 1996) to selection and operation of a bioreactor for general transketolase catalysed biotransformation processes is part of a larger multidisciplinary program to investigate transketolase from recombinant *E. coli.* The total program involves the production and development of the recombinant microbial source, evaluation of the synthetic potential of the enzyme, and development and characterisation of the biotransformation system. The large scale production of the *E. coli* transketolase has been performed to 1000 l scale (Hobbs *et al.,* 1995) at University College London. These cells are the source of transketolase for this study on biotransformation design.

The aims of the present study are as follows:

1. To design a suitable assay system for monitoring the model reaction and for measurement of other potential substrates.

2. To characterise the model biotransformation and define the constraints on the transketolase reaction based on these characteristics.

3. To design and evaluate suitable bioreactor configurations for the model biotransformation.

4. To define the key parameters for investigation in order to select, with only minimum additional studies but a high degree of confidence, the appropriate reactor options for transketolase-catalysed reaction of alternative reactant aldehydes.

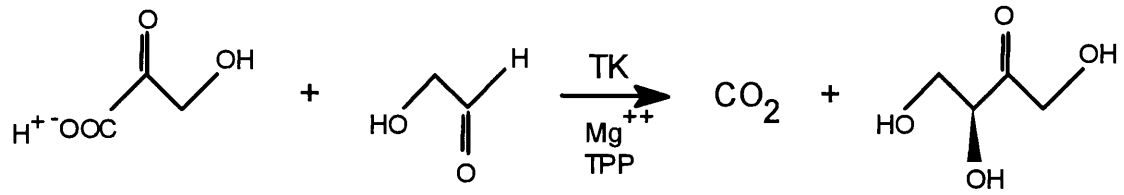
5. To validate the constraints and key parameters defined in (2) and (4) by application to a second transketolase reaction of inherently different characteristics to the model reaction (a validation reaction system).

2. MODEL SYSTEM

2.1. Introduction

As reported previously (Hobbs *et al.*, 1996), large scale fermentation of a recombinant *Escherichia coli* (*E. coli*) has provided a reliable and consistent source of transketolase A (hereafter referred to as transketolase). This thesis describes the use of this catalyst to demonstrate the feasibility of a structured design philosophy recently reported (Woodley and Lilly, 1994; Lilly and Woodley, 1996) for biotransformation reactor selection and evaluation. This procedure is based on the initial characterisation of the reaction components (substrate(s) and product(s)), reaction, biocatalyst and the interactions between them. Only data relevant to reactor design are collected during this characterisation stage and used to determine the constraints. Chapter 2 reports the results of characterisation studies on a model transketolase biotransformation and the general constraints imposed on choice and operation of a reactor for efficient transketolase catalysed processes.

It is probable that commercial biotransformations will utilise β-hydroxypyruvate (HPA) as the ketol donor owing to the consequent irreversibility of the reaction. The most rapid reaction of this type is reported to occur between HPA and water soluble glycolaldehyde (Bolte *et al.*, 1987; Dalmas and Demuynck, 1993) resulting in the production of L-erythrulose and carbon dioxide (Figure 2.1). This reaction was used as a model for investigation of the structured design philosophy although the product itself is of no commercial significance. In this instance the model reaction was simply used as a design tool to characterise the biotransformation and define the limits and constraints of the process.



β-hydroxypyruvate glycolaldehyde L-erythrulose

Figure 2.1. The model reaction adopted for study of transketolase-mediated biocatalysis.

2.2. Materials and Methods

2.2.1. Materials and equipment

2.2.1.1. Solvents and chemicals

All solvents were HPLC grade. The water used in these studies was produced by reverse osmosis followed by ion exchange and was of similar quality.
All chemicals were of analytical grade.

2.2.1.2. Chromatography

2.2.1.2.1. High performance liquid chromatography (HPLC)

The HPLC system consisted of ISS-100 (Perkin Elmer) autosampler fitted with a 20 µl fixed sample loop, a Series 200 (Perkin Elmer) isocratic pump, a dual column oven (Jones Chromatography) and a RI 71 (Shodex) refractive index detector. The data capture system was the Prime Chromatography P200-D computer software package (HPLC Technology Ltd).

2.2.1.2.2. Size exclusion chromatography

The equipment comprised a GradiFrac System (Pharmacia Biotech) fitted with a manual injector and a UV absorbance peak detector at 280 nm. The gel filtration medium for separation of cofactors and salts from transketolase consisted of a column (50 x 1 cm) of Sephadex G-25 (Pharmacia Biotech). The samples were collected

using an automated fraction collector. The sample loop volume for this study was fixed at 0.5 ml.

2.2.1.3. Model substrates and products

Lithium HPA, HPA (free acid), glycolaldehyde and L-erythrulose were obtained from Sigma Chemical Company. The purities of these compounds were determined independently by ^{1}H NMR. Although agreement with stated purity was found for the free acid of HPA (>99%) and glycolaldehyde (99%), lithium HPA and L-erythrulose were found to be 93% and 88% pure respectively (excluding water of hydration). Glycolaldehyde was supplied as a dimeric solid but monomerised in solution. The chloride salt of TPP was obtained from Sigma.

Multigram quantities of the potassium and lithium salts of HPA for larger scale biotransformations were synthesised from bromopyruvic acid by Dr Mark Smith of the University of Edinburgh (Morris *et al.*, 1996). Purities of these compounds were determined to be 99% and 97% respectively by ^{1}H NMR.

2.2.1.4. Transketolase from *E. coli.*

Transketolase was stored at -20°C stored in the form of a frozen wet cell paste of recombinant *E. coli* strain JM107/pQR701 (French and Ward, 1995). The recombinant strain possessed integral kanomycin resistance and was produced by fed-batch fermentation (Hobbs *et al.*, 1996). The cells were resuspended to 10% w/v (~2% dry w/v) in chilled 5 mM sodium phosphate buffer pH 7.0 and passed three times through an Lab 40 (APV Manton Gaulin) homogeniser at 1200 bar. The homogeniser was fitted with a heat exchanger to maintain the lysate in a chilled condition. The lysate was clarified by centrifugation (Biofuge 13, Heraeus Sepatech) at an RCF of 10500 g for 5 minutes and stored at -20°C for up to 4 weeks without loss of activity. Transketolase levels were typically 18-20 U/mg total soluble protein

(approximately 250 U/ml clarified lysate) as measured by the spectrophotometric assay. The lysate was used for all studies without further purification and contained approximately 35% w/w transketolase (protein basis) of which 80-90% was in the apo-form.

2.2.1.5. SDS PAGE buffers and stains

2.2.1.5.1. Sample buffer

The sample buffer was made up by addition of 1.0 ml 0.5 M Tris-HCl pH 6.8, 0.8 ml 10% (w/v) glycerol, 1.6 ml 20% (w/v) SDS, 0.4 ml 5% (w/v) 2-mercaptoethanol, 0.2 ml 0.05% (w/v) bromophenol blue and 4.0 ml water. The solution was stored at 4°C.

2.2.1.5.2. Running buffer

The running buffer was made up as a concentrate in 1 litre water consisting of 15 g/l Tris base, 72 g/l glycine and 5 g/l SDS. The solution was adjusted to pH 8.3 and stored at 4°C. For use, the buffer was diluted with four parts water.

2.2.1.5.3 Coomassie blue G-250 stain

The stain solution was made up to a total volume of 1 litre with water and contained 417 ml 45% (w/v) methanol, 167 ml 15% (w/v) acetic acid and 1 ml 0.1% (w/v) G250 Coomassie blue.

2.2.1.6. pH-stat equipment

A pH-stat was used for all investigations requiring pH control. The system utilised was a pH-stat unit (Radiometer Ltd) comprising an ABU Autotitrator, a PHM 82 standard pH meter, a TT 80 titrator and a TT80 titration assembly. The agitator comprised a belt-driven 3 sided shaft rotating at a fixed speed of 4800 rpm. The reactor was a 25 ml glass vessel fitted with a water jacket for temperature control. The glass vessel was manufactured for this study (Soham Glassworks Ltd) and the glass surface was pre-treated with silanising agents prior to use to reduce protein adsorption.

2.2.2. General methods

2.2.2.1. Spectrophotometric transketolase assay

Transketolase activity was determined by a modified version of the linked enzyme assay (Villafranca and Axelrod, 1971) as described below. The reaction mixture (1.5 ml final volume) consisted of 100 mM glycyl-glycine buffer (pH 7.6), 0.5 g/l BSA, 0.25 mM TPP, 9 mM $MgCl_2$, 0.154 mM NADH, 0.2 units(Sigma)/ml phosphoriboisomerase (PRI), 0.2 units(Sigma)/ml α-glycerophosphate dehydrogenase-triosephosphate isomerase (GDH-TPI), 0.2 units(Sigma)/ml D-ribulose-5-phosphate epimerase (RPE), 3 mM ribose-5-phosphate and transketolase. The assay was performed at pH 7.6, 35°C. The reaction was initiated by addition of the ribose component and the rate of NADH oxidation monitored at 340 nm using a Uvikon 922 spectrophotometer (Kontron Instruments). A small background loss of NADH was deducted from the reaction rate. All assay components were obtained from Sigma Chemical Company. 1 U of activity was defined as the amount of transketolase enzyme required to produce 1 μmol of glyceraldehyde-3-phosphate from xylulose-5-phosphate and ribose-5-phosphate per minute at 35°C, pH 7.6.

The activity was determined using the maximum rate of NADH utilisation attained over the duration of the assay as an initial lag period was always observed.
The relationship between rate of oxidation of NADH ($\Delta A/\Delta t$) and expressed transketolase activity is shown below:

$$c = A/\varepsilon.l$$

$$\Rightarrow \quad c/t = (\Delta A/\Delta t)/\varepsilon.l$$

$$= (\mu mol/ml)/min$$

$$= U/ml$$

$$= [transketolase] \text{ in assay}$$

Where: c = concentration (μmol/min)
A = absorbance
ε = extinction coefficient (ml/μmol.mm)
l = light path (mm)

Since $(\Delta A/\Delta t) = min^{-1}$ = rate of oxidation of NADH,

and $\varepsilon_{NADH} = 0.63$ ml/μmol.mm

then: $$[transketolase] \text{ in assay} = \frac{\text{rate of oxidation of NADH } (min^{-1})}{\varepsilon_{NADH} \quad (ml/\mu mol.mm)}$$

$$= \frac{\text{rate of oxidation of NADH}}{0.63}$$

Therefore, for a 1.5 ml final assay volume in a cuvette of 10 mm light path,

$$\text{Activity in assay (U/ml)} = \frac{\text{rate of oxidation of NADH}}{6.3} \times 1.5$$

The activity observed in the assay can be related back to the transketolase activity (U/ml) of the test sample using a multiplication factor of 1/sample volume (ml) added to the assay.

2.2.2.2. Substrates and product HPLC assay (see section 2.3.3.2)

The isocratic HPLC system consisted of 2 x Aminex® 87H (ion exclusion/ hydrogen bonding) columns (7.8 x 300 mm) (Biorad) in series with 0.006 M H_2SO_4 mobile phase running at 0.45 ml/minute. The columns were maintained at 65°C and the peaks were detected by change in refractive index. Samples were diluted in mobile phase to a final concentration of 1-2 mM and filtered to 0.45 μm prior to injection. All peak areas were determined by manual baseline designation to increase accuracy of integration.

2.2.2.3. Protein assay

Soluble protein levels were determined by the Biorad macro-protein assay (Biorad Laboratories Ltd.), a commercial version of the Bradford protein assay (Bradford, 1976). Bovine serum albumin (fraction V powder, Sigma Chemical Company) in the range 0 - 1.0 g/l was used to generate a standard curve (Appendix 1). Samples were diluted in water into the range 0.1 - 0.5 g/l for absorbance measurement at 595 nm, 25°C.

2.2.2.4. Trichloroacetic acid (TCA) precipitation of proteins

The protein sample volume was adjusted to 1.0 ml with water and 0.33 ml of 100% TCA, gently mixed by inversion and left for 2 hours at 4°C. A protein pellet was produced by centrifugation for 7 minutes at RCF 10500 g and the supernatant decanted to waste. The pellet was washed by addition of 1.0 ml of a solution of 5 mM HCl in acetone followed by severe vortex mixing for 1 minute to break up the pellet. The protein was spun down again to a pellet and the supernatant wash decanted to waste. The pellet was dried under gentle vacuum and stored dry at -20°C until required.

2.2.2.5. SDS-PAGE protein resolution

2.2.2.5.1. Resolving-gel

The protein electrophoresis system (Pharmacia Biotech) was cleaned with methanol and assembled. The medium for one 12 % resolving gel plate was produced using the following reagents:

Reagent	1:	Water	3.35 ml
	2:	1.5 M Tris-HCl, pH 8.8	2.50 ml
	3:	10% (w/v) SDS	0.10 ml
	4:	30% (w/v) acrylamide/bis acrylamide	4.00 ml
	5:	10% (w/v) ammonium persulphate	0.05 ml
	6:	TEMED	0.005 ml

Reagents 1-4 were mixed and degassed using a 0.2 μm syringe filter. Reagents 5 and 6 were then added, gently mixed and poured slowly into the plate cavity using a 1 ml pipette tip ensuring no air bubbles were entrapped. The gel cavity was filled to 70% and water added slowly onto the surface to ensure air exclusion and a level gel surface. The gel was left to set for 2 hours, the water removed and the surface blotted dry ready for the stacking gel.

2.2.2.5.2. Stacking gel

The reagents for the 4% stacking gel were as follows:

Reagent	1:	water	3.05 ml
	2:	0.5 M Tris-HCl, pH 8.8	1.25 ml
	3:	10% (w/v) SDS	0.05 ml
	4:	30% (w/v) acrylamide/bis acrylamide	0.65 ml
	5:	10% (w/v) ammonium persulphate	0.025 ml
	6:	TEMED	0.005 ml

Reagents 1-6 were added and treated as described for the resolving gel. The stacking gel was gently pipetted onto the surface of the resolving gel to the top of the glass plate. A 12 tooth comb was inserted into the surface and the gel allowed to set for 1 hour. The comb was removed and the wells rinsed with water. The gasket was removed and the plate inserted into running buffer (2.2.1.5.2) until submerged.

2.2.2.5.3. Protein reduction

The protein pellet from the TCA precipitation was dissolved in 100 µl sample buffer Any yellow colour indicated acidity and 1 µl 2.0 M Tris base was then added. The pellet was mixed thoroughly and any undissolved protein spun down and discarded. If required, the protein solution was further diluted in sample buffer into the range 1-10 mg/ml. 100 µl was incubated at 100°C in a pierced eppendorf for 3 minutes and then allowed to cool. The reduced protein samples were then loaded at 10 µl per well Non TCA-precipitated protein samples were diluted 1:1 in sample buffer, incubated at 100°C for 3 minutes and loaded at 16 µl per well. A standard marker solution of different protein molecular weights (12 kDa - 77 kDa) was reduced at a protein level of 1 mg/ml and loaded at 8 µl per well.

2.2.2.5.4. Running and staining the gel

A model 400C (Gibco) power supply was used at a voltage of 400 Volts. The current was set to 20 mA and the gel run until the blue protein band reached the resolving gel. The voltage was switched to 15 mA and the gel allowed to run until the blue band reached the base of the gel. The power was switched off and the gel removed from the glass support plates . The stacking gel was removed and placed in fixing stain (7% v/v acetic acid, 40% v/v methanol in water) overnight. The protein was then stained in Coomassie blue G-250 staining solution (2.2.1.5.3) for 3 hours. The gel

was transferred to destaining solution (10% v/v acetic acid, 25% v/v methanol in water) for 2 hours, rinsed in methanol and then rinsed in water.

2.2.2.6. Dialysis of protein solutions

Dialysis was utilised as a method of removing small compounds (MW < 12000) such as salts or cofactors from protein solutions. The dialysis membrane consisted of seamless cellulose tubing of diameter 16 mm. The membrane was prepared by washing in water for 3 - 4 hours to remove the glycerine humectant. Removal of residual sulphur compounds was not deemed necessary for this study. The dialysis buffer contained 10 mM 2-mercaptoethanol when the transketolase in the protein solution was present in the holo-form. For small volumes (<10 ml), the dialysis buffer to protein sample volume ratio was >500 : 1 and the dialysis was conducted under gentle stirring at 4°C overnight. For larger volumes, the dialysis was conducted with three changes of fresh dialysis buffer, each dialysis step performed at 4°C over 3 hours but with a reduced buffer to sample volumes ratio of ~ 50 : 1 . The conductivity of the dialysed sample was checked against the dialysis buffer to ensure dialysis was complete.

2.2.2.7. Concentration of protein solutions

In order to increase the concentration of transketolase solutions after diluting procedures such as dialysis, known volumes of the dilute protein solutions were encased in dialysis sacks (cellulose membrane) and buried in polyethylene glycol crystals of mean molecular weight of 20000 (Sigma Chemical Company). The concentration step was performed at 4°C, the duration depending on the concentration ratio required. 2-mercaptoethanol was included in the protein solution to 10 mM if transketolase was present in the holo-form.

2.2.2.8. Biotransformation using pH-stat apparatus

Biotransformations were conducted in the absence of buffers by utilising a pH-stat apparatus (2.2.1.6) controlled by automated addition of 1.0 M HCl. This apparatus provided mixing and temperature control at 20°C, 25°C, 30°C and 35°C. For 10 ml reaction volume the required quantities of lithium HPA, glycolaldehyde, TPP and $MgCl_2$ (based on 10 ml solution) were dissolved together in water to a volume of 8.0 ml in a pH-stat maintained at 25°C. The pH was adjusted to 7.0 with 0.25 M NaOH and the volume made up to 10.0 ml with water and transketolase (2.2.1.4) to give the required final activity. The reaction was controlled at pH 7.0 unless stated otherwise. Concentrations of the TPP and $MgCl_2$ cofactors at the start of reaction were 2.4 mM and 0.9 mM unless stated otherwise. The HPA and glycolaldehyde substrate concentrations were an equivalence, the concentration defined by the experimental studies (within the range 50 - 500 mM). The reaction was monitored by ion-moderated partition HPLC (2.3.3.2).
These biotransformation conditions were modified during the project based on the results of research studies performed.

2.2.2.9. Pre-activation of transketolase

The pre-incubation step involved addition of a pH 7.0 solution of TPP and Mg^{++} cofactors in water (adjusted to pH 7.0 with 0.1 M NaOH) to transketolase-rich lysate to give final cofactor concentrations of 2.4 mM and 0.9 mM respectively. 2-mercaptoethanol was then added to 10 mM concentration and the enzyme/cofactor solution incubated at 25°C for 20 minutes prior to addition to the biotransformation.

2.3. Analytical Method Development

2.3.1. Introduction

Previous work on transketolase utilised enzyme-linked spectrophotometer assays for quantitation of glycolaldehyde and HPA. However, measurement of the product L-erythrulose was not performed (Hobbs, 1994). Although thin layer chromatography could be used to detect all three compounds, this was usually qualitative only. Therefore, an assay was required to quantify the product. In addition, utilising enzyme-linked assays requires the transketolase reaction to be terminated, usually by acidification, and the conditions then re-adjusted to allow enzyme-mediated determinations. This introduces significant errors and delays into the assay system. Ideally, a single non-enzymic assay for all three compounds was required.

2.3.2. Colorimetric assay for L-erythrulose

Boratynski (1984) developed an assay for ketoses based on reaction with phenol-acetone-boric acid. Boratynski found that non-ketose carbohydrates did not interfere and different ketose sugars gave different absorbencies although L-erythrulose was not studied. This assay was therefore, investigated for L-erythrulose using D-fructose standards up to 0.5 mM. The reagents were made up as follows:

Reagent A: 2.5 g phenol was dissolved in 50 ml H_2O. 1.0 ml acetone was added dropwise over 10 minutes while stirring with a further 10 minutes stirring at room temperature. 2.0 g boric acid was dissolved into the solution and the reagent stored at 4°C for up to 2 weeks.

Reagent B: Concentrated sulphuric acid (>96%)

100 µl of the sample, standard and control was mixed with 0.5 ml of *Reagent A* and 1.4 ml *Reagent B* added directly to the surface. The solution was mixed for 5 minutes at room temperature and then incubated at 37°C. The absorbance was measured at 568 nm against a water sample/reagent control. The standard curve for D-fructose and for L-erythrulose shown in Appendix 2.

The D-fructose standard curve gave a good linear fit up to 1 mM concentration and an absorbance scan showed a maximum absorbance peak at 570 nm as expected. However, the L-erythrulose was less sensitive with no detection at concentrations below 1.0 mM. Also, a scan revealed a major peak at 480 nm and only minor absorption at 570 nm. The solutions were straw coloured instead of the pink colouration of the D-fructose samples. This phenomenon was not observed by Boratynski for any of the ketose sugars reported. This assay was therefore abandoned and other methods for quantitation of L-erythrulose were investigated.

2.3.3. HPLC

2.3.3.1. Introduction

The measurement of neutral monosaccharides by HPLC can be performed using two types of column:

1. Amino-bonded silica supports are the most common columns. However, these were not suitable for measurement of L-erythrulose due to the presence of glycolaldehyde in the biotransformation samples. Aldehydes would bind to the amines and cause loss of resolving power.
2. Ion-moderated partition columns can operate at up to 90°C (depending on ionic form) with weak acidified water as mobile phase. Carbohydrates (Kuo and Yeung, 1981), organic acids (Marsili *et al.*, 1981) and aldehydes (Pecina and Bonn, 1984) have all been measured using this type of separation.

2.3.3.2. Ion-moderated partition HPLC

2.3.3.2.1. Assay development

The column chosen for investigation of the model compounds was the Aminex® 87H organic acids column (Biorad Laboratories Ltd). A UV absorbance scan revealed that HPA and L-erythrulose absorb UV light at wavelengths up to 250 nm but glycolaldehyde does not absorb above 200 nm. Therefore, UV detection at 210 nm was used to detect the HPA and L-erythrulose while the glycolaldehyde remained undetected. The conditions for this investigation are shown below:

Column:	Aminex® 87H with Aminex® 87H pre-column
Mobile phase:	0.006 M H_2SO_4
Flow rate:	0.6 ml/minute
Sample size:	20 µl
Detection:	UV absorbance at 210 nm

Aminex® product literature specified peak resolution for the column as temperature dependent with better resolution at the higher temperature. Therefore the column temperature was set at 60°C. HPA and L-erythrulose were injected and the peaks identified as shown in Figure 2.2. This assay offered a rapid facile method of quantifying two of the three compounds of interest and included measurement of the product. However, further work was required to measure all three compounds in a single assay.

Measurement of the change in refractive index could also be used to detect elution peaks. Unlike UV absorbance, this method detects any compound with a different refractive index to the mobile phase but is therefore more prone to interference by contaminating compounds. In addition, the refractive index detection is less sensitive than UV absorbance methods and higher sample concentrations are required. However, change in refractive index was investigated for detection of all three compounds eluting from the Aminex® column. Identical conditions to the UV absorbance method were utilised except the column temperature was 35°C and a refractive index detector was used.

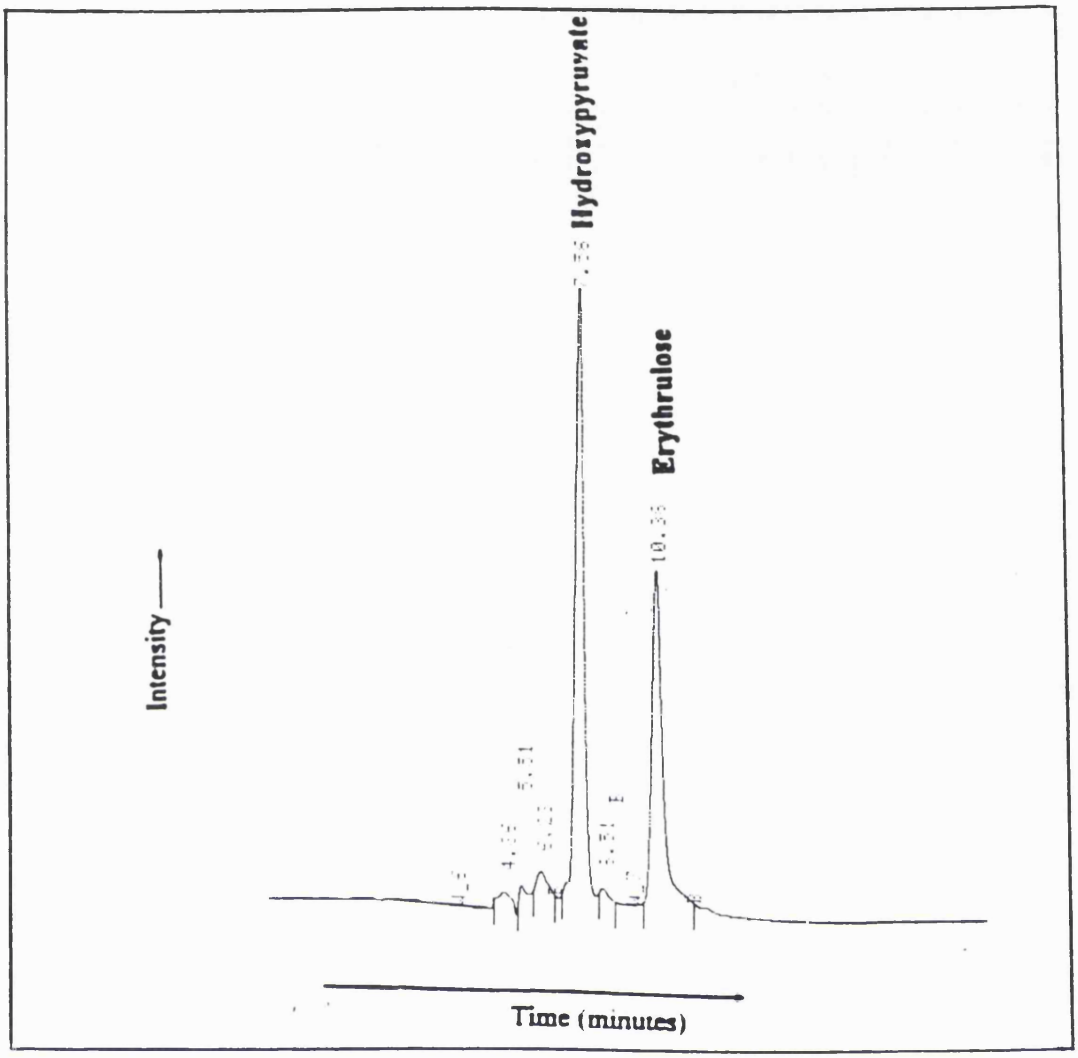


Figure 2.2. HPLC trace for rapid quantitative determination of HPA and L-erythrulose. The system consisted of an Aminex® 87H column at 60°C with 0.006 M H_2SO_4 mobile phase at a flow rate of 0.6 ml/minute. Peaks were detected by a change in UV absorbance at 210 nm.

At 35°C, the HPA eluted at 9.0 minutes but the L-erythrulose and glycolaldehyde co-eluted as a single peak at 12.6 minutes. Changing the concentration of sulphuric acid in the mobile phase between 0.001-0.02 M did not resolve the peaks. However, increasing the column temperature to 65°C, the design limit of the column, resulted in partial separation of the aldehyde and product (Figure 2.3). Unfortunately, no further separation was possible by manipulating the assay conditions. Therefore, a second Aminex® 87H column was introduced in series and the assay conducted under the following conditions:

Column:	2 x Aminex® 87H in series with Aminex® 87H pre-column
Column temperature:	65°C
Mobile phase:	0.006 M H_2SO_4
Flow rate:	0.45 ml/minute
Sample size:	20 µl fixed loop
Detector:	Refractive index

The separation of the three peaks is illustrated in Figure 2.4. The aldehyde and product were almost completely separated with only a slight overlap. The HPA remained completely resolved from the other peaks. However, the total assay time was approximately 40 minutes due to the introduction of the extra column and the lower flow rate required to remain within the maximum backpressure design limit of the columns.

Internal standards are an efficient method of reducing the errors introduced by slight differences in injection volumes. Different organic acids were investigated with the aim of identifying a suitable standard showing no interference with the three peaks of interest. Malic acid (retention time of 24.8 minutes) was found to be the most suitable internal standard and could be included in the dilution buffer at a fixed concentration if the HPLC system showed low reproducibility.

The double Aminex® column HPLC system illustrated in Figure 2.4 was adopted for quantitation of the (non-gaseous) reaction components of the transketolase model system.

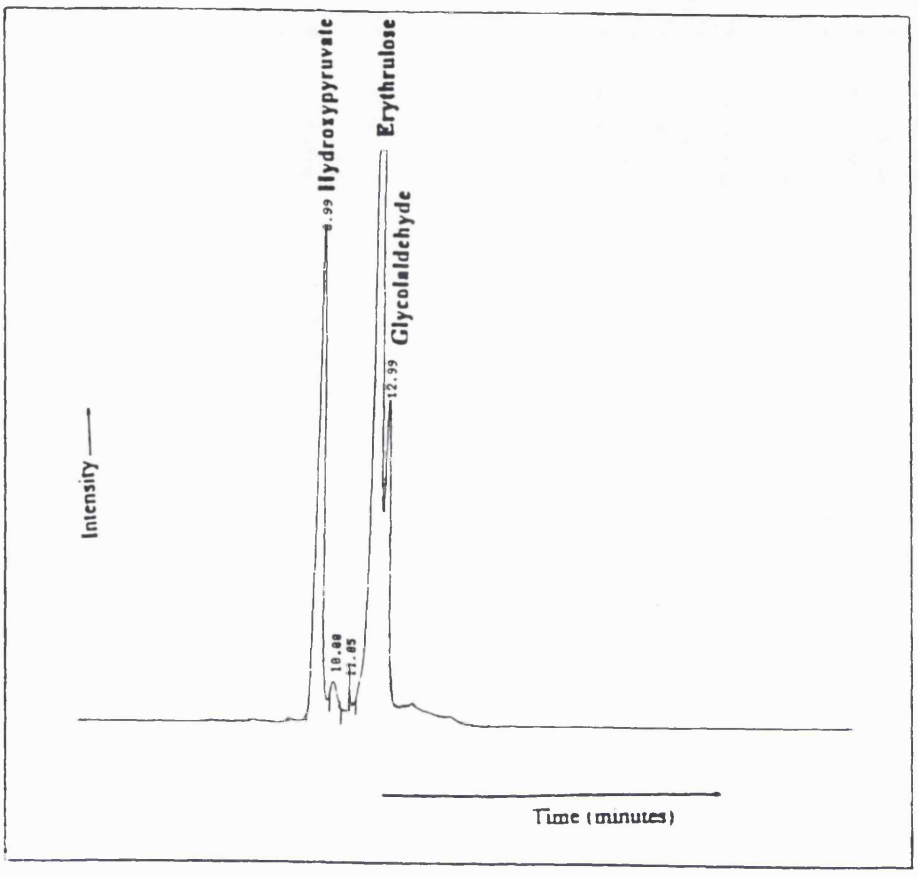


Figure 2.3. Partial separation of HPA, L-erythrulose and glycolaldehyde by HPLC using a single Aminex® 87H column at 65°C with refractive index detection.

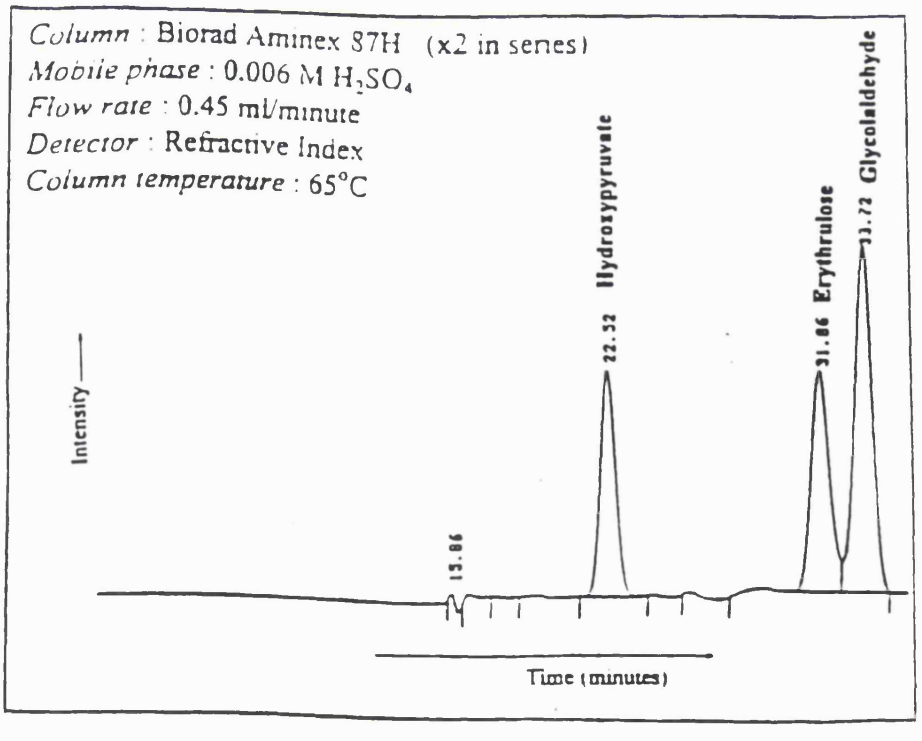


Figure 2.4. Typical HPLC trace for adopted assay of HPA, glycolaldehyde and L-erythrulose using an HPLC comprising of two Aminex® 87H columns in series.

2.3.3.2.2. Reproducibility of the adopted dual column HPLC system

The system was calibrated for HPA, glycolaldehyde and L-erythrulose within the range 0.1-5 mM. The system reproducibility was tested over five consecutive identical injections of 20 µl at the different concentrations over the calibration range. The standard error was calculated from the following equation:

$$\text{Standard error (+/- \%)} = \frac{\text{standard deviation}}{\text{mean}} \times 100\ \%$$

The standard error values (+/- one standard error) over the calibration range for the three model compounds are illustrated in Table 2.1.

It is evident from this data that the assay is reproducible for measurement of the individual reaction components at concentrations greater than 0.2 mM. However, concentrations greater than 5.0 mM resulted in column overloading and were generally avoided. Therefore, for measurement of HPA, glycolaldehyde and L-erythrulose in single component solutions, samples were diluted to within the range 0.2 - 5.0 mM.. Due to a partial overlap of peaks for solutions containing both glycolaldehyde and L-erythrulose, the useful concentration range for accurate analysis of mixtures of these two components was constricted to dilution to within the range 0.2 - 2.0 mM.

Component concentration (mM)	Standard error (+/- %)			
	HPA	Glycolaldehyde	L-erythrulose	Glycolaldehyde + L-erythrulose (equimolar)
0	0	0	0	0
0.1	4.7	5.1	6.4	10.1
0.2	3.0	2.8	3.2	3.9
0.5	2.1	3.3	2.2	2.5
1.0	2.0	2.5	1.6	2.8
2.0	2.3	2.7	2.0	3.1
5.0	2.0	2.7	2.3	7.8

Table 2.1. Standard error for the adopted dual column HPLC assay based on five consecutive 20 µl injections of the model reaction components

2.4. Characterisation of Substrates and Products

2.4.1. Substrate/product stability

2.4.1.1. Effect of buffers

The use of buffered systems for biotransformation pH control was considered an unnecessary burden on potential downstream processes. However, initial studies were undertaken to investigate the effect of buffers on substrate stabilities following observed mass balance deficiencies during glycyl-glycine buffered reactions (Hobbs *et al.*, 1993).

The stabilities of 100 mM HPA and 100 mM glycolaldehyde were monitored over 6 hours at pH 7.6, 35°C in the presence of several different buffers also at 100 mM concentration. The concentrations were monitored by HPLC (2.2.2.2). Table 2.2 illustrates the effect of the buffers on the model substrates :

Buffer Type (100 mM initial)	Residual concentration (% initial)	
	HPA	Glycolaldehyde
None	97	97
Glycyl-glycine	64	56
MOPS	8	68
Tris/HCl	85	93
Phosphate (sodium)	-	83

Table 2.2. Effect of buffers on stability of model reaction substrates

Although the effect of phosphate on HPA was not determinable (due to the interference of this compound with the HPA peak during the HPLC assay), all the buffers investigated were found to decrease the stability of the two substrates by

varying degrees. The losses observed in the presence of glycyl-glycine explained the incomplete mass balance previously observed for reactions conducted in the presence of this buffer. In the absence of buffers negligible losses were observed over the 6 hours incubation. These results confirm the undesirability of using buffers for pH control for this reaction. All further reactions were performed in the absence of buffers utilising acid/alkali titration *via* a pH-stat (2.2.1.6).

2.4.1.2. Effect of pH

10 ml solutions of HPA, glycolaldehyde and L-erythrulose were made up at 100, 250 and 500 mM concentrations and Mg^{++} and TPP cofactors added to 0.9 mM and 2.4 mM respectively. The pH was immediately adjusted to the required value (in the range pH 6.0-9.0) using 0.1 M HCl or 0.1 M NaOH as necessary. The pH was then maintained at the desired value over 6 hours using a pH-stat. Temperature was controlled at 25°C or 35°C as required. Samples were removed at hourly intervals and analysed by HPLC.

Figure 2.5 illustrates the rate of degradation of the substrates and products at different pH values at 25°C based on the mean loss over the entire 6 hours. The degradation profiles for 100 mM substrates and product at both 25°C and at 35°C are shown in Appendix 3. The rate of degradation was pH, temperature and concentration dependent. Near-neutral pH conditions resulted in only minor losses but alkali appeared to accelerate the degradation process. The mechanism behind alkali-mediated degradation of HPA is reported to occur via conversion to the tautomers dihydroxyacrylic acid (DHAA) and tartronic acid semialdehyde (TASA) (Hedrick and Sallach, 1961). These compounds are subject to auto-oxidation and spontaneous decarboxylation respectively. The presence of oxygen was reported to enhance the auto-oxidative degradation of DHAA while the presence of Mg^{++} was reported to enhance the conversion from DHAA to TASA. The pathway for the alkali-mediated autocatalytic degradation of glycolaldehyde and L-erythrulose is not known.

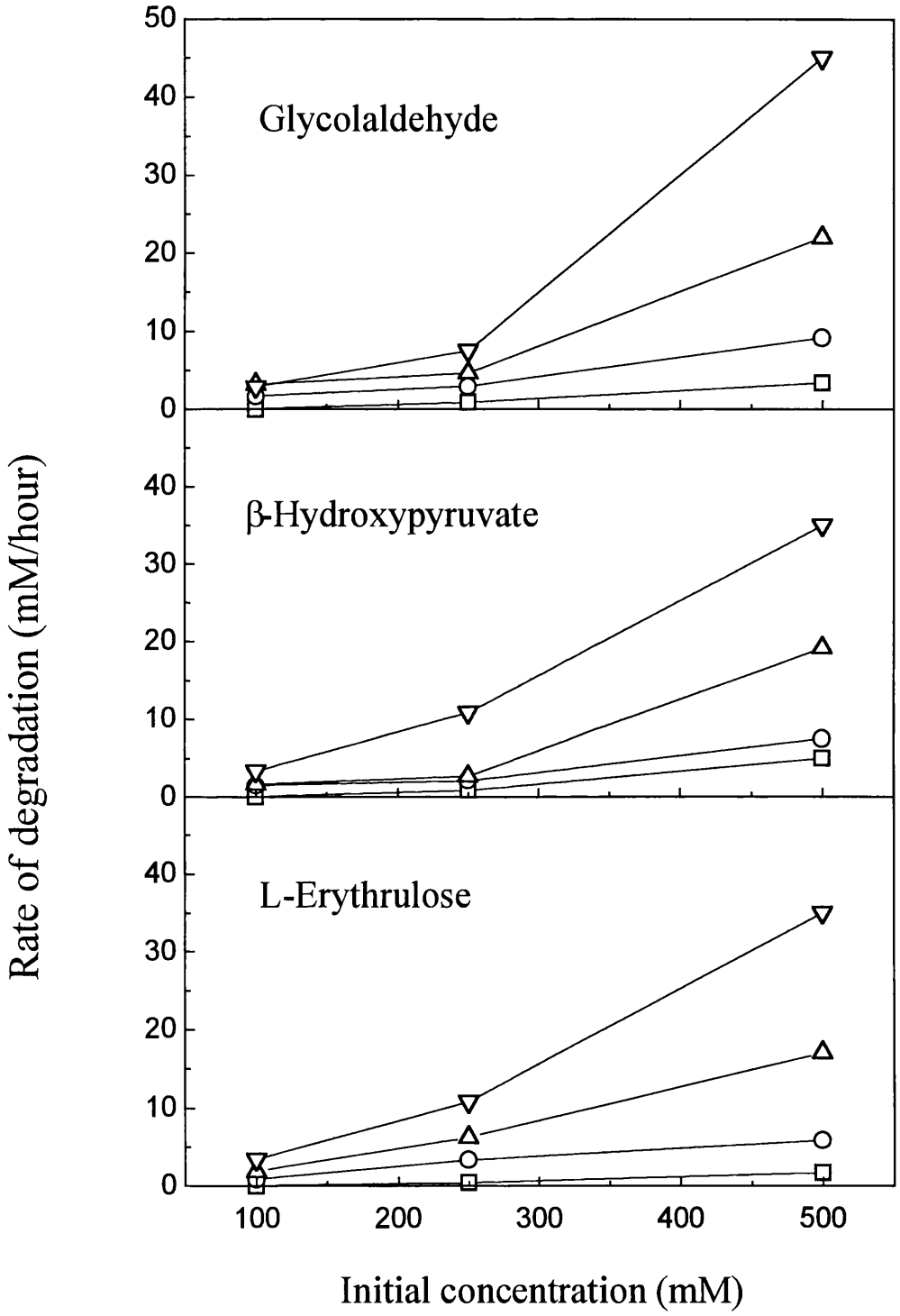


Figure 2.5. pH - stability profile for model reaction components at 25°C in water. The rates of degradation are indicated for pH 7.0 (□), pH 8.0 (○), pH 8.5 (Δ), and pH 9.0 (▽)

2.4.2. Solubility in aqueous systems

The maximum solubility of glycolaldehyde and lithium salt of HPA was investigated individually in the range pH 1.0-8.0 and temperature 25-35°C in a pH-stat. The solubilities were measured by HPLC analysis of 0.2 μm filtered solutions after 1 hour stirring under the chosen conditions. Cofactors were included at the concentrations used for the transketolase reaction studies (2.4 mM TPP, 0.9 mM $MgCl_2$). The solubility limit of glycolaldehyde was found to exceed 4.2 M at all pH values studied and the numerical limit was not determined. However, the HPA solution was loaded beyond the solubility limit at pH 8.0 for lithium HPA and the pH value adjusted stepwise to acidic pH values over a period of several hours while taking samples. Maintenance of stable pH was performed using 1 M HCl or 1 M NaOH as required. The solution was maintained saturated under all conditions by addition of the respective solid Li HPA as required. The solubility of the free-acid of HPA was also measured at pH 1.0, 25°C for comparison.

Figure 2.6 illustrates the solubility limit for (lithium) HPA at different pH and temperature. The solubility limit of the (lithium) HPA was constant as the pH was reduced from pH 8.0 to 3.0. As the pH was further reduced a rapid increase in solubility was observed. Interestingly the free acid exhibited slightly greater solubility over the (lithium) HPA at pH 1.0. This may have resulted from the lithium chloride by-product of acidification reducing the solubility limit of the HPA. Alternatively, the acidic conditions may have reduced the rate of solubilisation of the (lithium) HPA in suspension and samples may have been taken before equilibrium was reached. The solubility for the (lithium) HPA at 35°C was approximately 10% higher than at 25°C over the entire pH range investigated.

The lithium salt was still used for the majority of studies as this was the form of HPA manufactured in large quantities within the research program (and was therefore cheap and readily available).

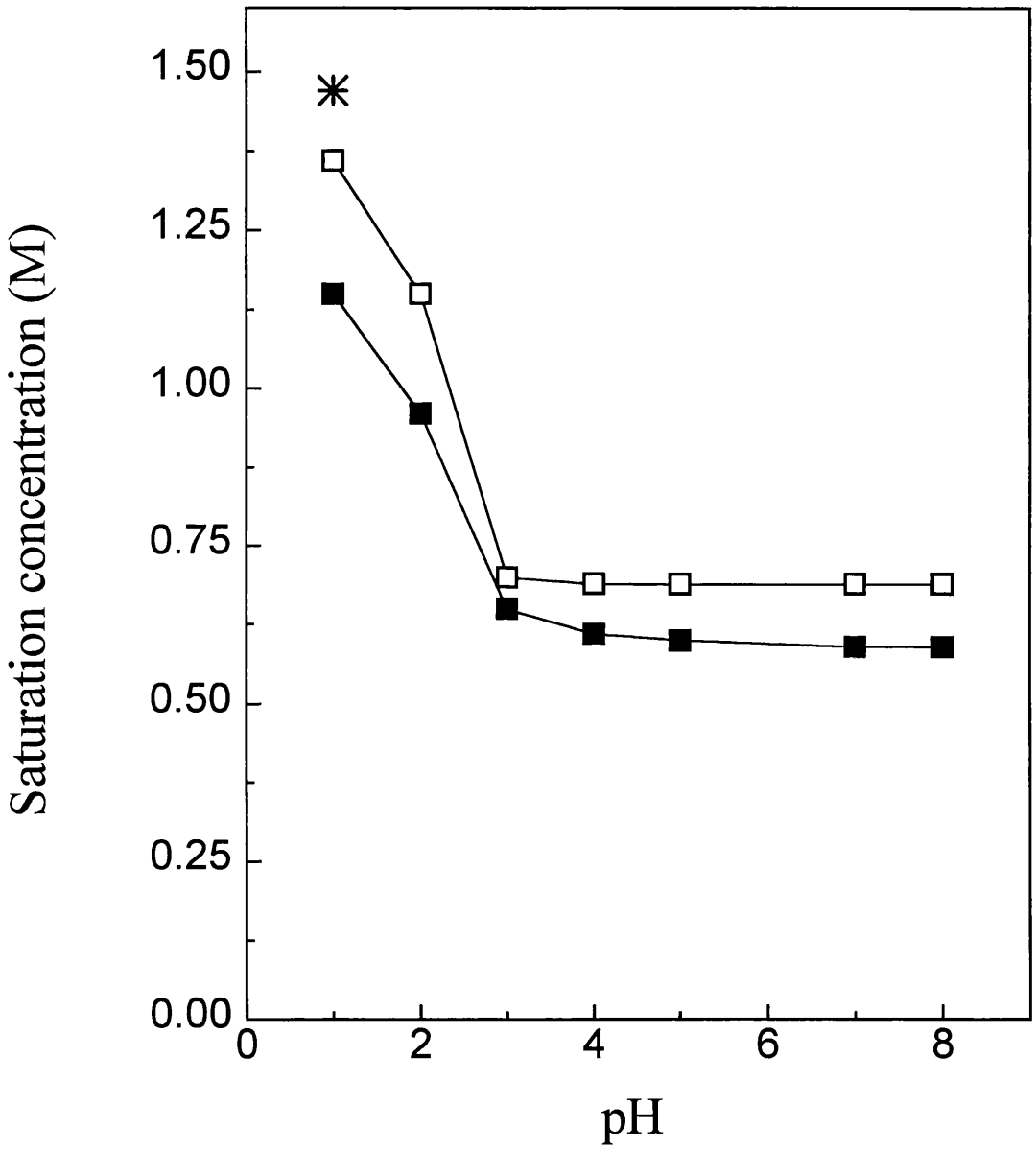


Figure 2.6. The solubility of lithium HPA (■, □) and the free-acid of HPA (✳) in water at a range of different pH. Solubilities are indicated after 1 hour incubation at 25°C (■, ✳) and 30°C (□).

The characterisation studies were not performed near the solubility limit of the lithium HPA and the use of the free-acid conferred no immediate advantage.

2.5. Characterisation of Transketolase

2.5.1. Effect of enzyme form on enzyme stability

Researchers have indicated that the holo-form of transketolase from bakers yeast may possess greater stability than the apo-form (Kochetov, 1986). Therefore, the effect of cofactors on *E. coli* transketolase stability was investigated. Filter sterilised lysate (25 U/ml final concentration) was incubated in the presence and absence of magnesium (0.9 mM) and TPP (2.4 mM) in 5 mM glycyl-glycine buffer at 25°C, pH 7.0 for 70 hours. Samples were removed aseptically at regular intervals and assayed for transketolase activity by the linked enzyme assay. The results are shown in Figure 2.7. Inclusion of TPP produced a significant increase in the rate of deactivation of the enzyme compared to the control (no added cofactors) while the presence of magnesium did not influence the deactivation. This was not the expected result. Further studies of this effect were therefore carried out with investigation of different TPP concentrations in the presence of 0.9 mM magnesium. The study was performed under similar conditions to that used previously but with a range of TPP concentrations and a fixed magnesium level. In case the instability of the holo-transketolase was specific to *E. coli*, the stability of transketolase from bakers yeast was also studied in the presence of 2.5 mM TPP. Table 2.3 illustrates the effect of TPP on the residual transketolase activity after 44 hours incubation at 25°C, pH 7.0. It is evident that TPP concentration influences the stability of the enzyme. However, this was almost certainly due to the direct influence of TPP level on the proportion of the holo-form of the enzyme as higher concentrations than 0.1 mM did not result in greater rates of deactivation. Thus, 0.1 mM was considered the TPP level at which

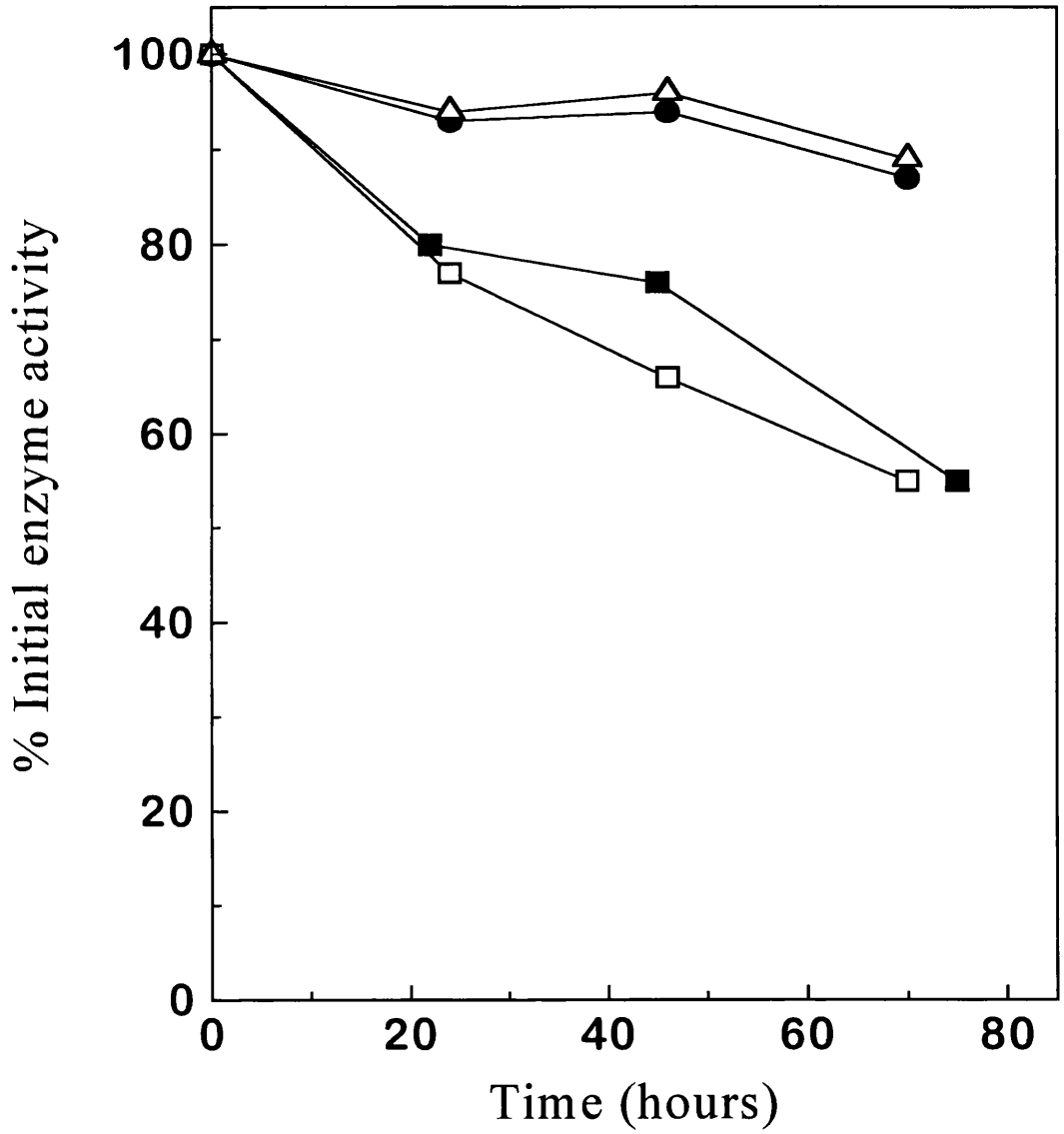


Figure 2.7. Effect of cofactors on transketolase stability (lysate) at 25°C, pH 7.0 Lysate was incubated in absence of added cofactors (●), in the presence of 2.4 mM TPP (□), 0.9 mM Mg^{++} (Δ), and both cofactors (■).

all the enzyme was in the unstable holo-form. The active site of the yeast enzyme was known to be highly homologous to the *E. coli* enzyme (Littlechild *et al.*, 1995) and was also found to be unstable in the presence of cofactors.

TPP concentration (mM)	Residual transketolase activity (% of initial)
0 (control)	82
0008	72
0.004	55
0.02	39
0.1	32
0.5	30
2.5	34
2.5 (Sigma baker's yeast transketolase)	43

Table 2.3. Effect of TPP concentration on stability of lysate transketolase over 44 hours incubation at 25°C, pH 7.0.

The instability of the active form of transketolase was a major problem when considering potential losses of biocatalytic activity during biotransformation reactions. Therefore effort was directed at stabilising the active enzyme.

2.5.2. Stabilisation of enzyme

2.5.2.1. Initial studies using 2-mercaptoethanol

The possibility of protease action on the enzyme was discounted as the apo-form was found to be stable but, as only minor conformational changes occurred on cofactor

binding (Sundstrom *et al.*, 1992), existed in a dimeric state of similar conformation to the holo-form.

Crystallisation studies (Littlechild *et al.*, 1995) had identified a cysteine residue in the active site close to key residues involved in TPP binding. Therefore, investigations concentrated on the effects of oxidation, a known deactivation mechanism of many enzymes containing cysteine and methionine residues in key functional positions (Wells and Estell, 1988; Bristow, 1990). The influence of added reducing agent was studied at a fixed cofactor concentration (2.4 mM TPP, 0.9 mM Mg^{++}) under conditions similar to those used in section 2.5.1. 2-mercaptoethanol was added to the holo-enzyme solution at a range of concentrations up to 20 mM. The presence of reducing agent dramatically improved the stability of the active enzyme (Table 2.4) but addition of higher concentrations (i.e. 20 mM) appeared to reduce the effectiveness.

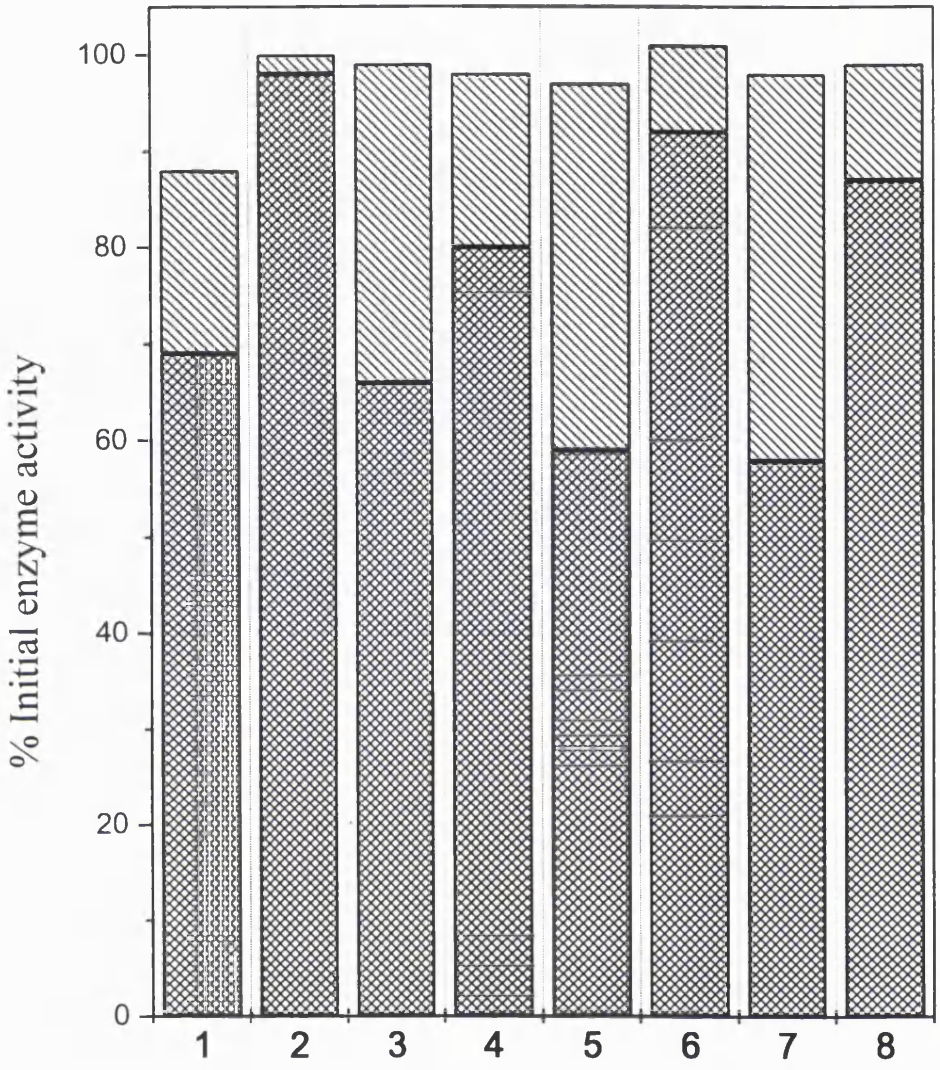
2-mercaptoethanol concentration (mM)	Residual transketolase activity (% of total initial)
0	34
2	72
10	67
20	59
0 (lysate. i.e. no added cofactors)	82

Table 2.4. Effect of 2-mercaptoethanol concentration on stability of holo-transketolase over 44 hours at 25°C, pH 7.0.

The effect of 10 mM 2-mercaptoethanol inclusion during a biotransformation was also studied (2.6.3). When 2-mercaptoethanol was included in model reaction studies, 10 mM was the concentration adopted unless stated otherwise.

2.5.2.2. Further studies

The use of mercaptoethanol was not practical on process scale due to the toxic nature of this compound. Two alternatives included the use of the less toxic but expensive reducing sugar dithiothreitol (DTT) and, secondly, the physical removal of oxygen by degassing and incubation under a nitrogen headspace. Both these options were compared to the use of 10 mM mercaptoethanol. Transketolase-rich lysate was diluted 1:9 in 20 mM Bis-Tris-Propane buffer pH 7.0 containing cofactors (2.4 mM TPP, 0.9 mM Mg^{++}) with no added reducing agents, 10 mM 2-mercaptoethanol, 1 mM DTT or 5 mM DTT. The solutions (5 ml) were filter sterilised into sterile sealable vials (10 ml total volume). The headspace of these vials was filled with nitrogen or with air and the vials incubated at 25°C. Samples were removed aseptically and assayed for activity by the linked enzyme assay. Figure 2.8 illustrates the activities measured after incubation for 4 and 72 hours. The presence of reducing agents and incubation under nitrogen both resulted in enzyme stabilisation over several hours but over several days incubation under nitrogen proved the most successful. Interestingly the presence of reducing agents resulted in a lower activities after several days under both air and nitrogen compared to incubation in the absence of the agents. Therefore, for studies in the short-term involving small volumes (<100 ml), reducing agents were included to stabilise transketolase. However, for long-term studies and larger reactor volumes degassing and incubation under nitrogen was the stabilising method of choice. For characterisation studies and small scale biotransformations 2-mercaptoethanol was used to prevent oxidation.



1. no reducing agent (air)
2. no reducing agent (nitrogen)
3. 10 mM mercaptoethanol (air)
4. 10 mM mercaptoethanol (nitrogen)
5. 1 mM DTT (air)
6. 1 mM DTT (nitrogen)
7. 5 mM DTT (air)
8. 5 mM DTT (nitrogen)

Figure 2.8. Effect of reducing agents on holo-transketolase stability in sealed vials in the presence of air and nitrogen headspace gases. Activities are illustrated as percentage of initial after 4 hours (▨)and 72 hours (▨) incubation at pH 7.0, 25°C.

2.5.3. pH/stability profile

2.5.3.1. Studies in presence of buffers

Initial studies of the stability of transketolase at different pH values involved the incubation of lysate (~ 85% apo-transketolase) at buffered pH for 68 hours at 20°C in the absence of both cofactors and reducing agent. The buffers utilised were 50 mM cacodylate/HCl for investigation of pH 5.5 to pH 7.4 and 50 mM Tris/HCl for pH 7.4 to pH 9.5. The lysate (250 U/ml) was diluted 1:4 with 62.5 mM of the appropriate buffer, filter sterilised and stored in sterile airtight vials. Samples were removed aseptically over 68 hours and assayed for activity by the linked enzyme assay. Figure 2.9 illustrates the change in transketolase activity observed over the incubation period. The apo-enzyme appeared stable in the range pH 7.0-9.5 although a 10-15% loss was observed initially in this range. (Subsequent studies on the effect of oxygen on the holo-enzyme explained this loss as oxidation of the small proportion of holo-enzyme present in the lysate.) However, the most interesting observation was the deleterious effect of pH <6.0 on the apo-transketolase together with formation of a proteinaceous precipitate. However, as the holo-enzyme was the active form of transketolase and buffers would not be present during the biotransformation, a more representative study was conducted (2.5.3.2).

2.5.3.2. Studies in absence of buffers

The irreversible effects of pH on holo-transketolase were therefore investigated in the pH range 5.5-10.5 in absence of buffers. Transketolase-rich lysate (200 U/ml) was diluted 1:9 in cofactor solution (2.4 mM TPP, 0.9 mM $MgCl_2$ final concentration) containing 2-mercaptoethanol (10 mM final concentration) at pH 7.0, 25°C for 20 minutes in a pH-stat. Transketolase activity and soluble protein were measured, the

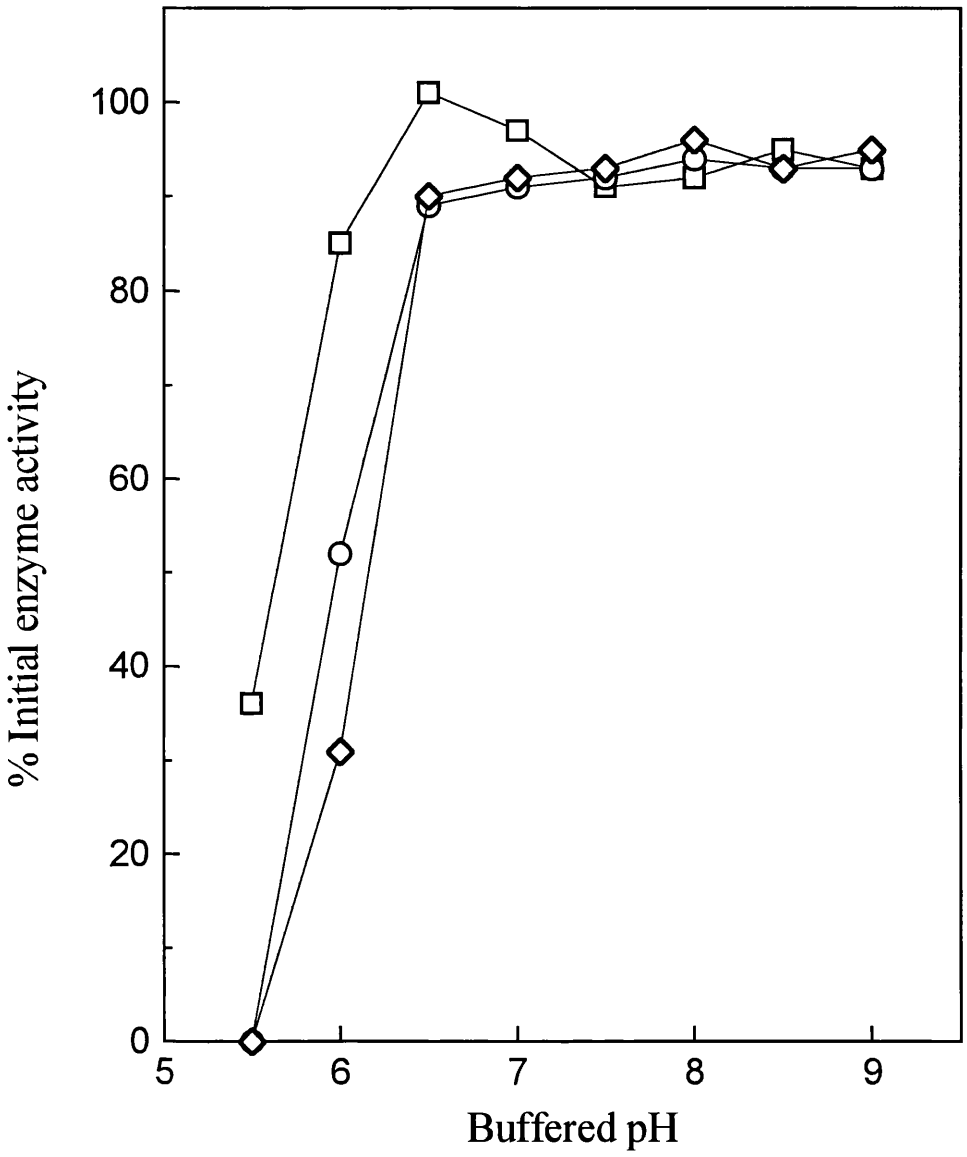


Figure 2.9. pH stability of crude transketolase enzyme during incubation at 20°C in the presence of 50 mM buffer but in the absence of both added cofactors and reducing agent. The initial transketolase level was 50 U/ml. Residual activity was measured at 1 hour (□), 44 hours (○), and 68 hours (◊) incubation.

pH then adjusted to the desired value with 100 mM HCl or 100 mM NaOH and subsequently incubated for 2 hours. Soluble protein levels were again determined, the pH then readjusted to 7.0 and transketolase activity remeasured by the linked enzyme assay. This provided information on irreversible loss of transketolase activity. Figure 2.10 shows the residual soluble protein and observed transketolase activity after 2 hours and 4 hours incubation at different pH expressed as percentage of the initial value (as measured after 20 minutes incubation at pH 7.0).

The upper pH limit on the transketolase appears to be approximately pH 10. The deleterious effects below pH 6.0 were slightly reduced compared to the study of the apo-rich enzyme but were still apparent A protein precipitate was observed to form immediately at pH 5.5 but more slowly at pH 6.0. The stable pH range for the active enzyme therefore appeared to be from pH 6.5 to pH 10.0.

The protein precipitate observed at pH 5.5 may offer a facile method of purifying transketolase to greater than 65% of the total soluble protein without using salting out methodologies. However it was not known whether transketolase may have precipitated out of solution at pH 5.5 and resolubilised on readjustment to pH 7.0. In order to investigated the nature of the precipitate and confirm the absence of transketolase at pH 5.5, PAGE techniques were utilised (2.2.2.5). The total initial protein at pH 7.0 was compared to the precipitate and supernatant isolated after 2 hours at pH 5.5 and after readjustment from pH 5.5 to 7.0. All samples were precipitated using TCA and acetone washed (2.2.2.4) prior to electrophoresis. Figure 2.11 illustrates the protein banding obtained. Transketolase monomer (MW ~ 70 kDa) was observed at high level in the supernatant at pH 5.5 but not in the precipitate. The precipitate at pH 5.5 was therefore free of transketolase. However, a protein band at 64 kDa was observed in the soluble fractions isolated after 2 hour incubation at pH 5.5 and after readjustment to pH 7.0. This band was not present in the initial lysate indicating that some protein, possibly transketolase, may have partially degraded to this smaller protein. For this reason, although acidification as a transketolase purification method could be used, incubation periods at pH 5.5 should be reduced, possibly to 1 hour.

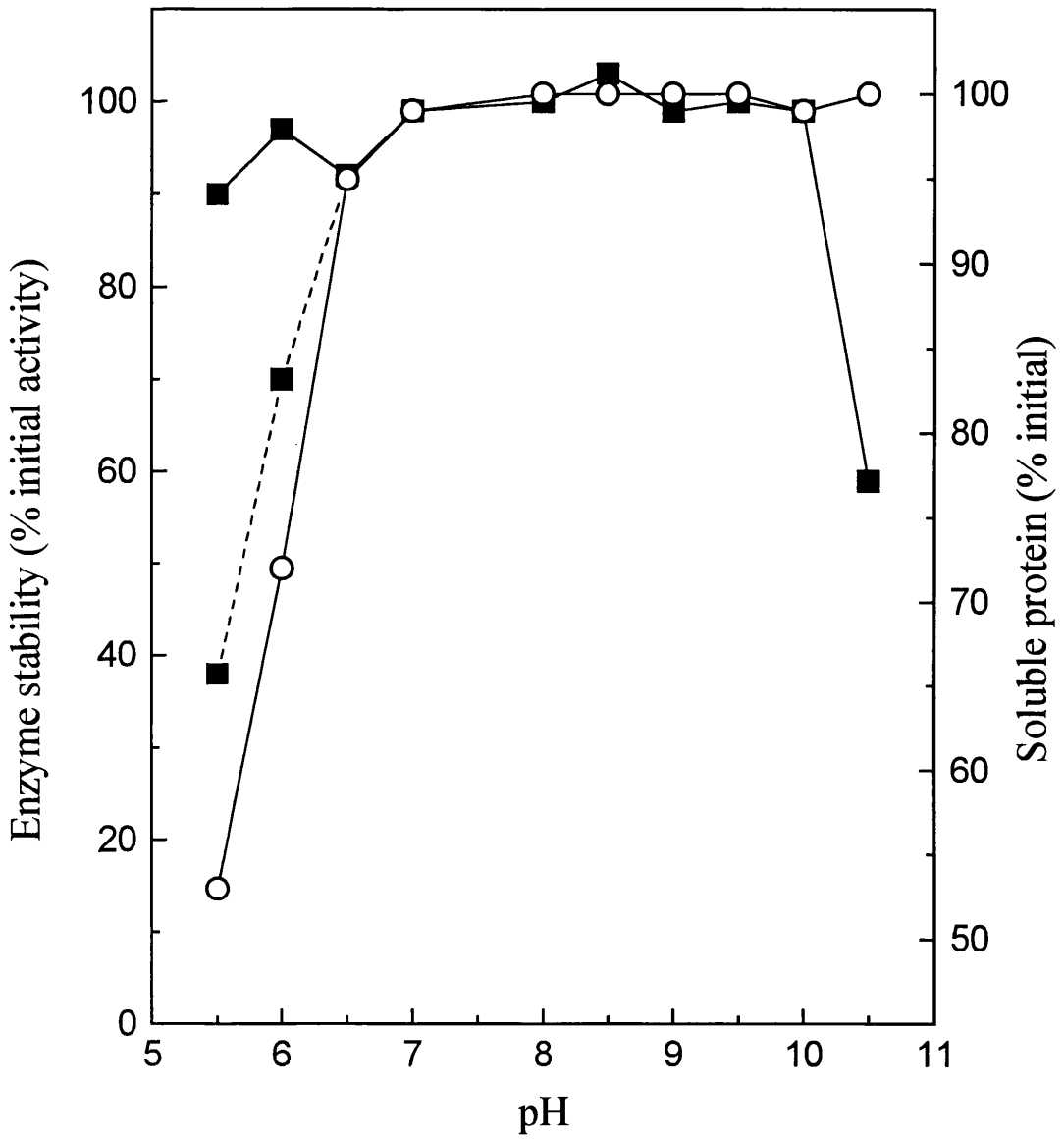
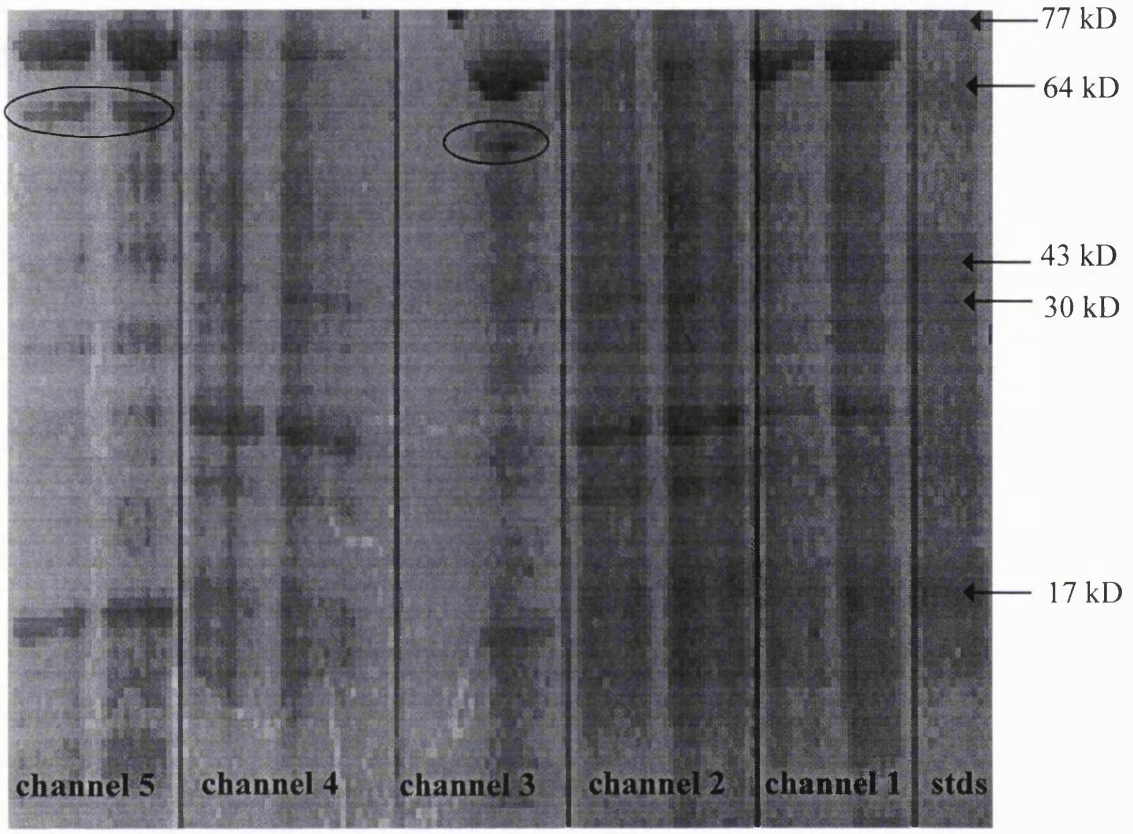


Figure 2.10. Effect of pH on transketolase stability (■).and soluble protein concentration (○) after 2 hours at 25°C in presence of cofactors. The dashed line indicates the measured transketolase activity after 4 hours. The initial transketolase level was 20 U/ml. 10 mM 2-mercaptoethanol was also included.



Channel 1: Total protein at pH 7.0
2: Protein precipitate after 2 hours at pH 5.5
3: Supernatant after 2 hours at pH 5.5
4: Protein precipitate after 2 hours at pH 5.5 and re-adjustment to pH 7.0
5: Supernatant after 2 hours at pH 5.5 and re-adjustment to pH 7.0

Figure 2.11. SDS-PAGE gel of components of the precipitation observed during incubation of transketolase lysate with cofactors at pH 5.5 for 2 hours. A possible breakdown product of transketolase observed after incubation at pH 5.5 is ringed.

2.5.4. pH/activity profile

The effect of pH on activity was determined by measurement of the initial rate of both product formation and substrate utilisation during a 100 mM biotransformation at various fixed pH over the range pH 6.5 - 9.0 using a pH stat (2.2.2.8). The enzyme was pre-activated in cofactor solution (2.2.2.9) and 10 mM 2-mercaptoethanol was included in the biotransformation to protect the enzyme from oxidation. The biotransformation contained 5 U/ml (final) transketolase and the reaction at 25°C was monitored over the initial 10 minutes by HPLC. Figure 2.12 shows the profiles for the initial rates of substrate utilisation and product formation expressed as a percentage of that observed at pH 7.0. The influence of pH-mediated substrate and product degradation on the activity/pH profile obtained was negligible as the measured rates of substrate utilisation and product formation were comparable. The pH profile for transketolase was found to be broad with an optimum at approximately pH 7.0. This was a useful characteristic as the operating pH was therefore not constrained by a narrow pH range of activity.

2.5.5. Cofactor resolution

2.5.5.1. Cofactor dissociation

The cofactor requirement for activation of transketolase is an important parameter to consider particularly if immobilisation is an option for the biocatalyst. It was important to define the conditions for cofactor dissociation as any requirement for cofactor inclusion in the reaction solvent may be an important parameter in both downstream processing operations and total process economics.

Cofactor dissociation studies were conducted in 50 mM Bis-Tris-Propane (BTP) buffer over the range pH 6.0-9.5. Enzyme lysate was incubated for 1 hour at 25°C in

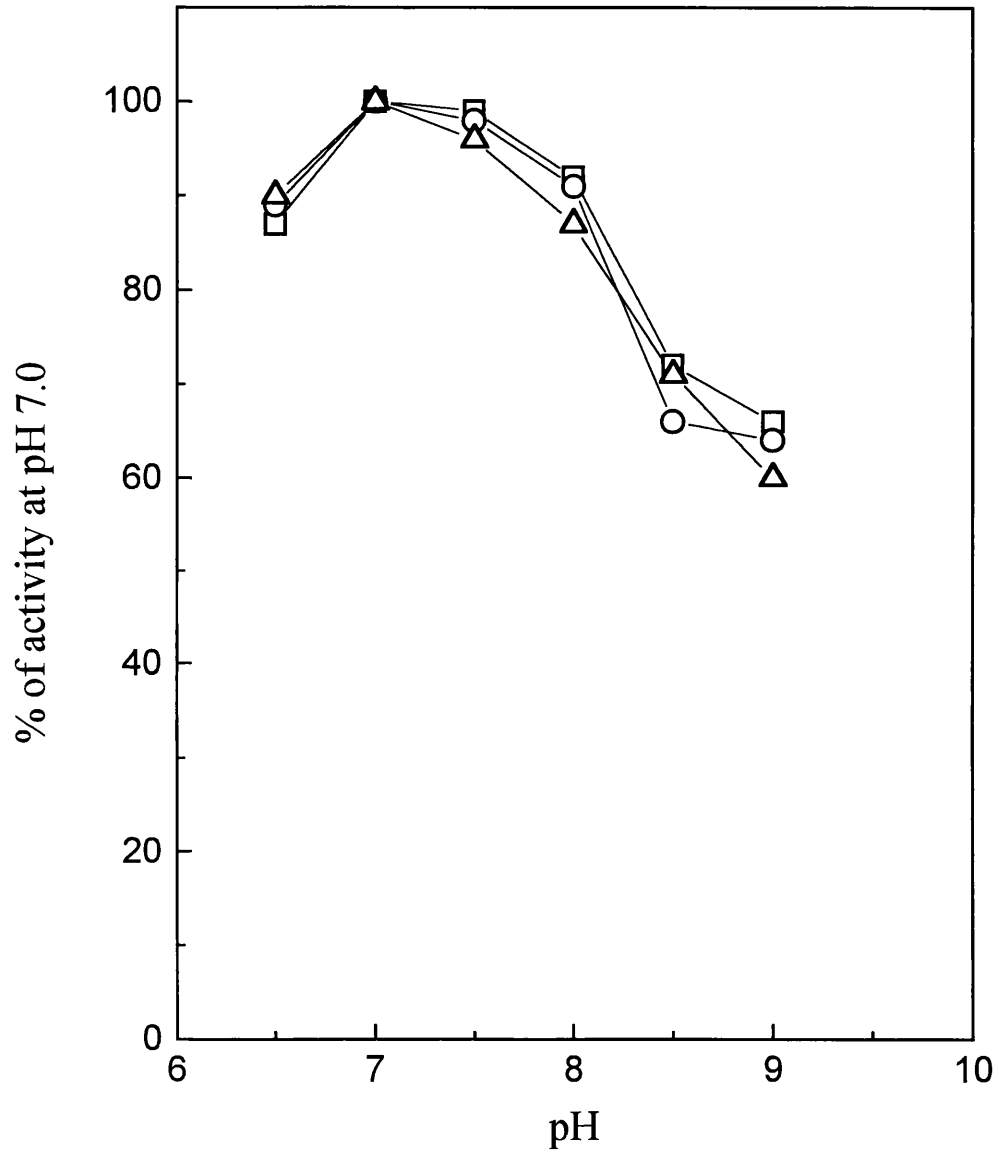


Figure 2.12. pH-activity profiles for transketolase (lysate) by monitoring initial changes in HPA (□), glycolaldehyde (○) and L-erythrulose (Δ) levels during 100 mM biotransformation at 25°C containing holo-transketolase at 5 U/ml (in presence of cofactors and 10 mM 2-mercaptoethanol).

the presence of 0.5 mM TPP and 5.0 mM $MgCl_2$ and 2 mM 2-mercaptoethanol at the appropriate pH under investigation. The TPP concentration was reduced as an excess was not required for this study. The reconstituted enzyme was passed down a pre-equilibrated gel filtration column (50 x 1 cm) containing Sephadex G-25 fitted to a GradiFrac chromatography system (2.2.1.2.2). The column was pre-equilibrated at the pH under investigation with 50 mM BTP containing 2 mM 2-mercaptoethanol in both the presence and absence of 5.0 mM $MgCl_2$. Appendix 4 shows the separation achieved between the protein peak and the free cofactors. Fractions (2.0 ml) containing the transketolase were pooled and assayed for activity at 35°C, pH 6.5 under the cofactor conditions of the resolution and also in the presence of both cofactors. The spectrophotometric transketolase assay buffer was 100 mM BTP for this study.

Figure 2.13 shows the effect of pH on cofactor dissociation by illustrating the residual activity of the enzyme after removal of unbound TPP only and after removal of unbound TPP and Mg^{++}. The activity was expressed as a fraction of that observed by assay in the presence of both cofactors. It was evident that cofactor dissociation occurred over the entire range of pH investigated but was significantly greater at alkaline pH. Previous researchers (Kochetov and Philippov, 1970) reported that transketolase containing bound TPP but no Mg^{++} showed partial activity but enzyme associated with Mg^{++} in the absence of TPP was inactive. The disparity between both the curves in Figure 2.13 and the maximum possible retention of activity (100%) shows that a proportion of previously bound TPP had dissociated and the reduced activity was not due solely to resolution of Mg^{++}. The difference between the two curves indicated that, for a proportion of the enzyme, Mg^{++} dissociated during gel filtration while TPP remained bound. The observed dissociation of TPP over the range of pH stability for the enzyme imposes a constraint on reactor operation. Cofactors must be included in the reaction solvent for maintenance of the active form of both free and immobilised transketolase.

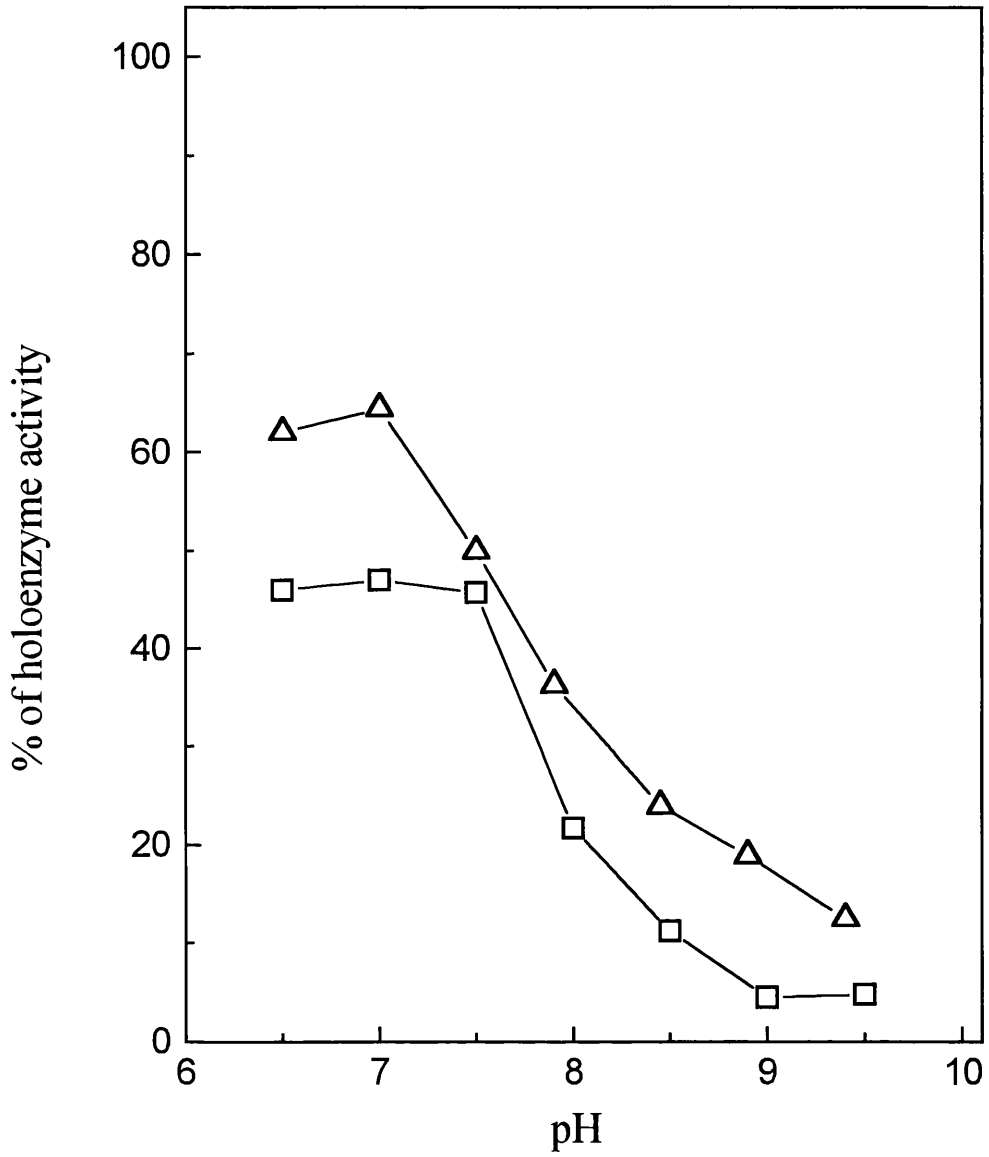


Figure 2.13. Effect of pH on resolution of cofactors from holo-transketolase. Transketolase (50 U/ml) in a solution of both cofactors was resolved by passage down gel a filtration column and assayed in presence and absence of cofactors. Profiles are illustrated for absence of TPP only during resolution and assay (Δ) and for absence of both TPP and Mg^{++} (□) during resolution and assay. Maintained cofactor/enzyme association is expressed as a percentage of the activity observed when measured in the presence of an excess of both cofactors. 2 mM 2-mercaptoethanol was included in all solutions.

TPP is reported to be labile in alkaline environments (Maier and Metzler, 1957). It was therefore possible that the TPP was degrading and not just dissociating from the enzyme. The stability of the TPP was investigated in the context of the dissociation effect.

2.5.5.2. TPP stability

Although several methods are reported for the quantitative determination of TPP (Bessey *et al.*, 1952; Penttinen, 1978; Ishii *et al.*, 1979), the most convenient method was utilising apo-transketolase. The enzyme lysate (~ 85% apo-transketolase) was gel filtered through a Sephadex G-25 column (previously described) and eluted with 50 mM BTP pH 9.5 equilibration buffer containing 2 mM 2-mercaptoethanol. The transketolase fractions were pooled, dialysed in 100 mM BTP buffer, pH 6.5, and concentrated using solid PEG 20000 (2.2.2.7). The resolved transketolase was found to contain 99.5 % apo-enzyme by assay in 100 mM BTP pH 6.5. The assay mixture containing apo-enzyme and excess Mg^{2+} could be used to measure up to 2.5 μM (final) added TPP in the assay . The calibration curve for the TPP assay is shown in Appendix 5. The loss of TPP from 0.5 mM initial concentration was determined after incubation for 2 hours in 50 mM BTP, 25°C, at a range of pH values. The conditions were chosen to reflect those encountered during the dissociation studies. All TPP samples were diluted in 50 mM BTP pH 6.0 to within the required concentration range for assay.

Figure 2.14 illustrates the residual TPP concentration measured after incubation and expressed as a percentage of the initial concentration. At pH 6.0, negligible loss of TPP was recorded but as the alkalinity increased, TPP stability decreased linearly with a recorded loss of 12% at pH 9.5. The pH lability might at first sight explain the apparent resolution of TPP from holo-transketolase. However, at pH 6.5 TPP degradation was only 2% but observed TPP 'dissociation' effects were high (2.5.5.1). Hence, the observed dissociation effects may be due to contributions by both TPP

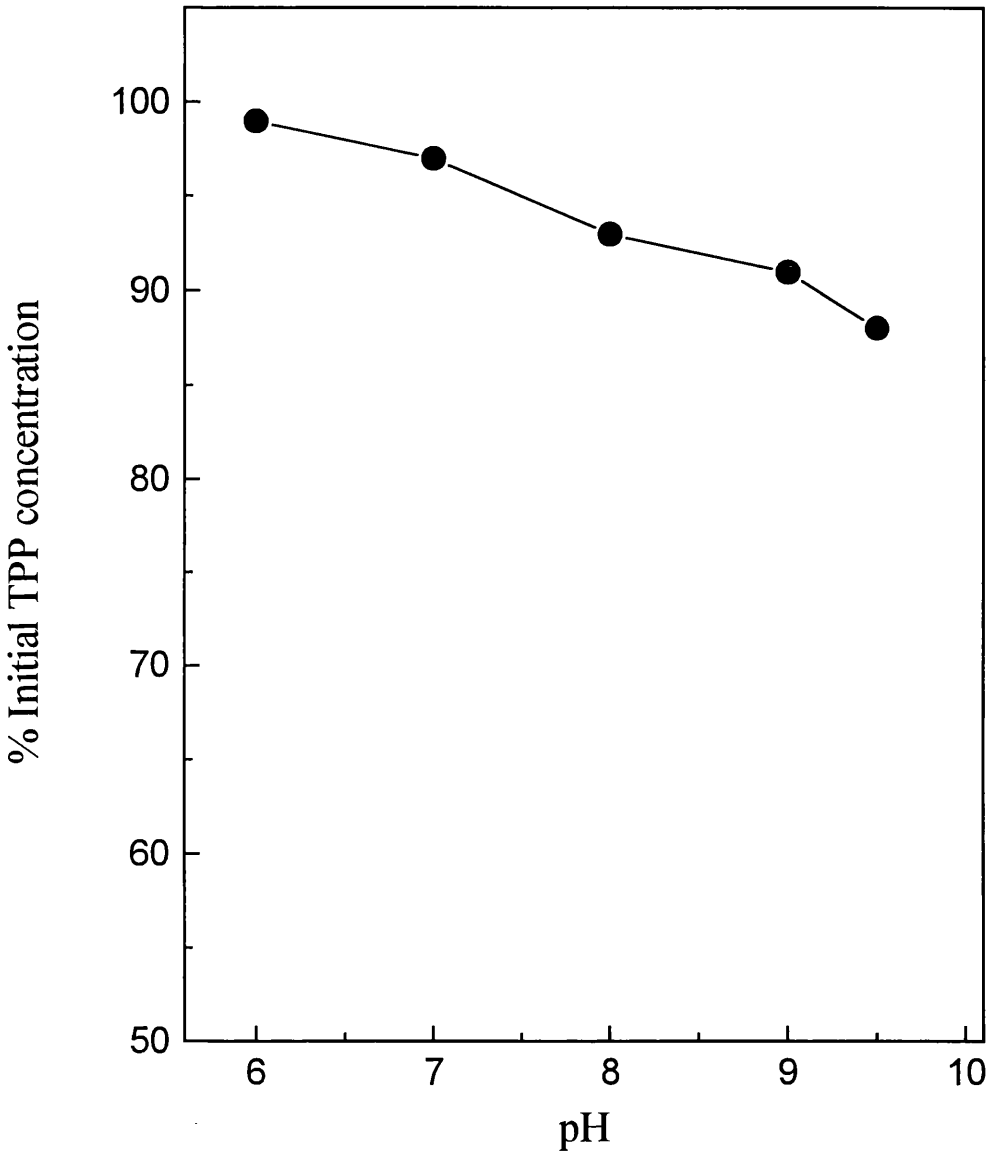


Figure 2.14. Stability of thiamine pyrophosphate (TPP) measured after 2 hours at 25°C in 50 mM Bis-Tris-Propane buffer at different pH values (assayed using *E. coli* apo-transketolase). The initial TPP level was 0.5 mM. 2 mM 2-mercaptoethanol was included in all solutions.

dissociation and by TPP degradation. The lability of TPP reinforced the necessity for inclusion of excess cofactors in substrate solutions in order to maintain active biocatalyst during biotransformation.

2.6. Reaction Characteristics and Interactive Effects

2.6.1. Effect of substrates and products on transketolase stability

2.6.1.1. Interference with analytical methods.

In order to determine the effects of various concentrations of biotransformation substrates and products on the stability of transketolase, the spectrophotometric multi-enzyme assay was used to monitor changes in the enzyme activity with time. It was therefore necessary to determine levels of any interference resulting from the presence of these biotransformation compounds in the assay. Fortunately, the spectrophotometric assay was a sensitive method requiring significant dilution of the enzyme, and therefore dilution of any interfering compounds, prior to assay. However, the effect of low concentrations of the model substrates in addition to the component substrates of the assay still required evaluation.

Interference effects were investigated by addition of various concentrations of HPA, glycolaldehyde and L-erythrulose to the assay (Table 2.5). Negligible interference was observed for both model substrates up to 1.0 mM concentration. For 500 mM biotransformations at 25 U/ml (a typical transketolase level) the dilution required for measurement of enzyme activity by the linked enzyme assay would be 1:100. Accounting for the dilution of the enzyme sample in the assay, this would result in a model substrate concentration in the assay of approximately 0.17 mM. At this level no interference of the assay would result from the presence of the model substrates. Minor interference (4% reduction in activity) was observed at 1.0 mM L-erythrulose which would equate to a 3 M L-erythrulose concentration in the biotransformation. This product level was unlikely to be attained in any characterisation studies.

Therefore, interference of the model substrates and products with the enzyme assay was acceptably low within the concentrations range of interest in the characterisation studies.

Concentration in assay (mM)	Measured transketolase activity (% of activity in absence of model compound)		
	HPA	glycolaldehyde	L-erythrulose
0	100	100	100
0.025	101	97	-
0.05	99	100	-
0.1	98	97	-
0.2	99	98	-
0.5	98	97	95
1.0	100	99	96
2.5	-	-	93
5.0	-	-	91

Table 2.5. Effect of concentration of model reaction components on the linked - enzyme transketolase assay.

2.6.1.2. Stability studies

Transketolase stability (25 U/ml final) in the presence of 500 mM substrates and product was investigated in the presence and absence of added cofactors (2.4 mM TPP, 0.9 mM $MgCl_2$) using a pH-stat (pH 7.0, 25°C). As preliminary studies had previously demonstrated (2.6.1.1), the substrates and products did not interfere with the spectrophotometric assay for transketolase at the dilutions required for enzyme measurement. Therefore, samples were simply removed at regular intervals over 8 hours from the reaction component/enzyme solution and assayed for transketolase activity (Figure 2.15). Differences in the stability of the holo and apo-transketolase

were observed in the presence of the substrates of the biotransformation. HPA at 500 mM exhibited a strong destabilising effect on the apo-enzyme while having little specific effect on the holo-enzyme. Glycolaldehyde deactivated both forms of the enzyme to the same extent at 500 mM concentration while the equivalent concentration of L-erythrulose product exerted no influence on the holo-transketolase.

Further studies investigated the effect of oxygen removal on holo-transketolase stability in the presence of aldehyde including the effect of different aldehyde concentration. For practical purposes over this short term study, oxygen removal was achieved by addition of the reducing agent 2-mercaptoethanol (10 mM). The effects of a range of concentrations of the aldehyde substrate on stability were investigated using the pH-stat over 6 hours in the presence of cofactors and 2-mercaptoethanol at the stated concentrations. The half-life of transketolase was calculated from the losses over the 6 hour incubation assuming a continued linear degradation with time (Figure 2.16). The level of deactivation increased with the aldehyde concentration and the presence of reducing agents did not increase the enzyme stability. The activity half-life at 500 mM glycolaldehyde was approximately 3.5 hours while the value at 50 mM was over 60 hours.

2.6.2. Mass balance on biotransformation

The biotransformations previously undertaken in glycyl-glycine buffer (Hobbs *et al.*, 1993) required an excess of the aldehyde component in order to reach completion. However, the mass balance for these reactions was not complete. The effect of the buffer on substrate stability has been shown to be the cause (2.4.1). A 500 mM biotransformation in absence of buffers, pH-stat controlled at pH 7.0 25°C, was studied (2.2.2.8) with respect to the total mass balance. The initial transketolase level in the biotransformation for this study was 26.0 U/ml.

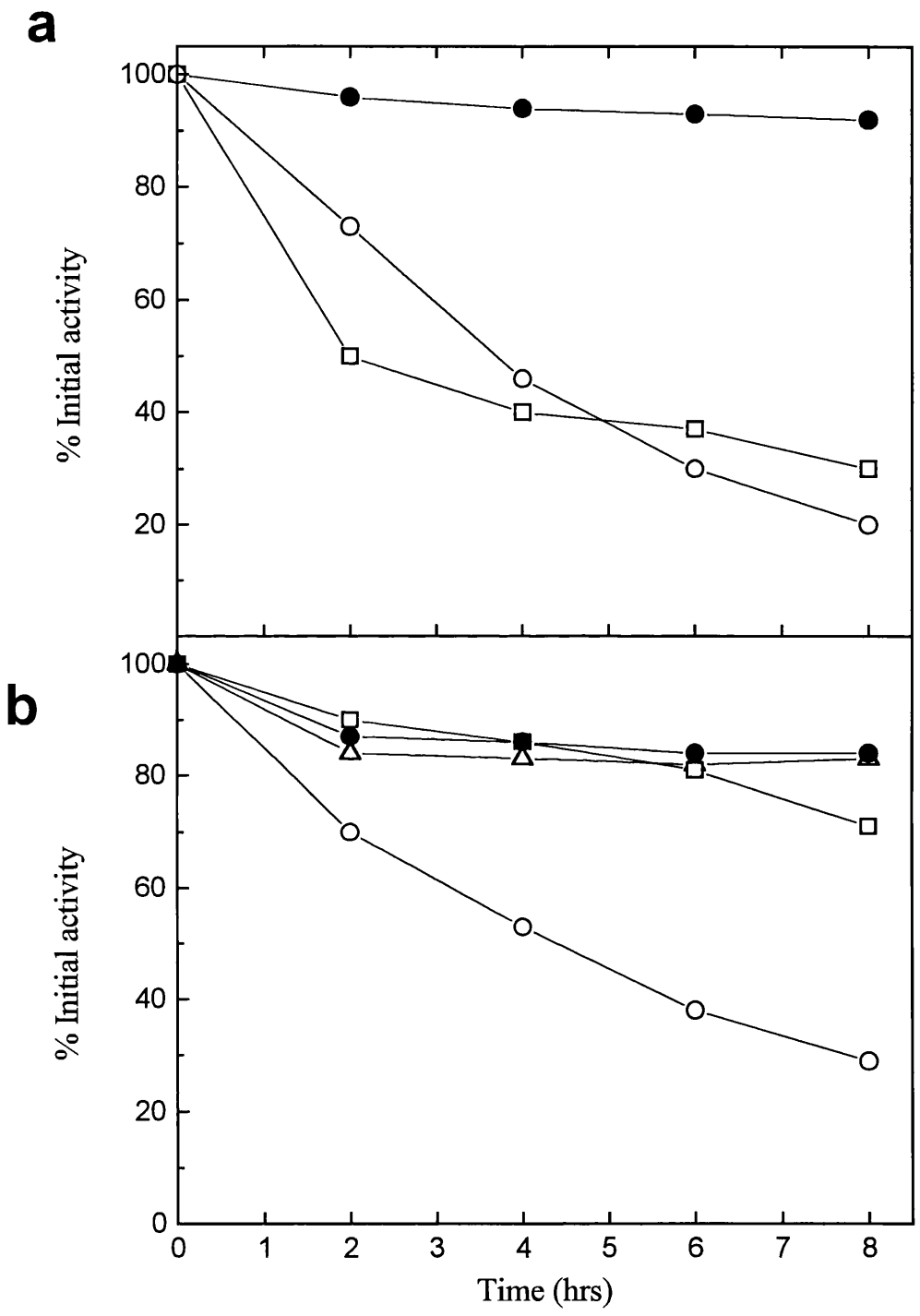


Figure 2.15. Effect of model substrates and product on lysate transketolase stability in absence (a) and presence (b) of added cofactors at 25°C, pH 7.0 . Cofactor levels were 2.4 mM TPP, 0.9 mM Mg^{++}. Substrates were HPA(□), glycolaldehyde (○) and L-erythrulose (Δ) at 500 mM concentrations. Incubation was also conducted in absence of model reaction compounds (●).

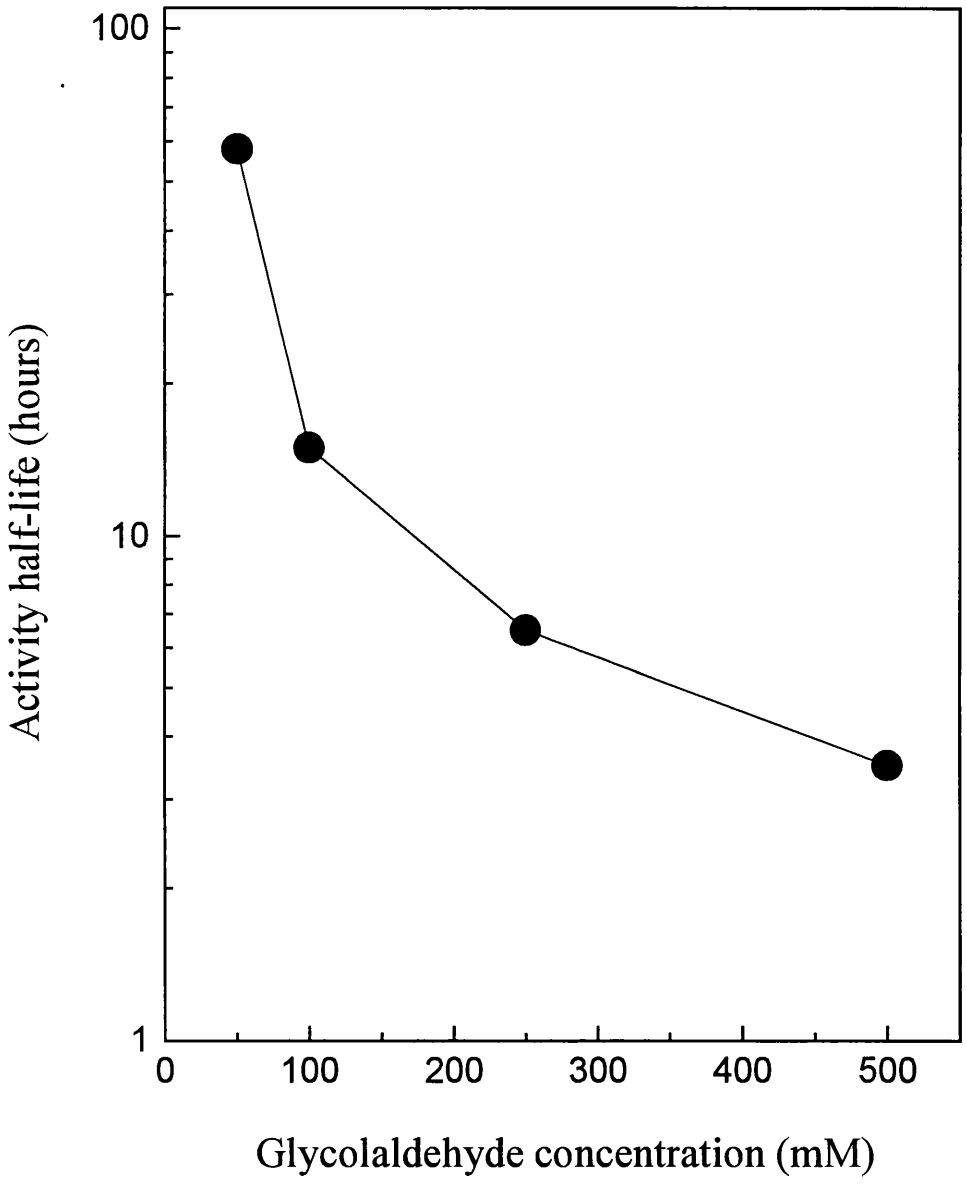
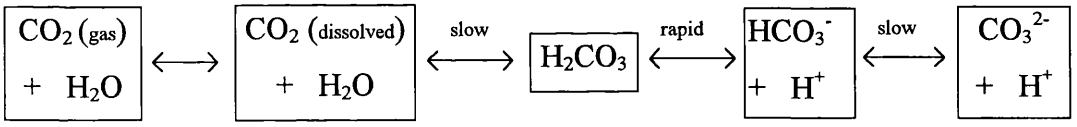


Figure 2.16. Effect of glycolaldehyde concentration on holo-transketolase stability at 25°C, pH 7.0 in absence of oxidation effects. Half-life values are based on loss of transketolase activity from an initial level of 25 U/ml over 6 hours incubation in the presence of 10 mM 2-mercaptoethanol.

Figure 2.17 shows the change in concentration of substrates and product with time while Figure 2.18 illustrates the mass balance for substrate, product and titrant during the reaction. Although total conversion to product was achieved with complete mass balance within approximately 2 hours, the dilution effect of the titrant addition was appreciable and resulted in a final product concentration of less than 350 mM from 500 mM initial substrates. However, although the substrate/product balance was complete, the titrant/product balance was incomplete with the total moles of titrant addition at only 85% of the total product formed. after 5 hours incubation. Comparing the molar rate of acid addition and product formation (Figure 2.19), it was evident that the initial rate of product formation was greater than the requirement for titrant. After approximately 40 minutes incubation, the rate of acid addition was greater than the rate of product formation. The explanation for these two phenomena is as follows. During the initial 30 minutes, the carbon dioxide by-product of the reaction dissolved in the reacting solution and dissociated as illustrated:



This generated protons which partially balanced the utilisation of the HPA and thereby offset the requirement for acidic titrant. Between 30 and 50 minutes of the reaction, the solution was saturated with carbon dioxide (and dissociation species) and the carbon dioxide bubbled out of the bulk solution at the same rate as the reaction was proceeding. At this point the titrant addition rate was similar to the reaction rate. However, as the biotransformation proceeded to completion, the rate of carbon dioxide generation decreased and the carbon dioxide in bulk solution equilibrated with the low carbon dioxide environment of the reactor headspace. This was a slow process hence the requirement for pH control despite the reaction having reached completion. It is possible that the titrant/product may have equated over an

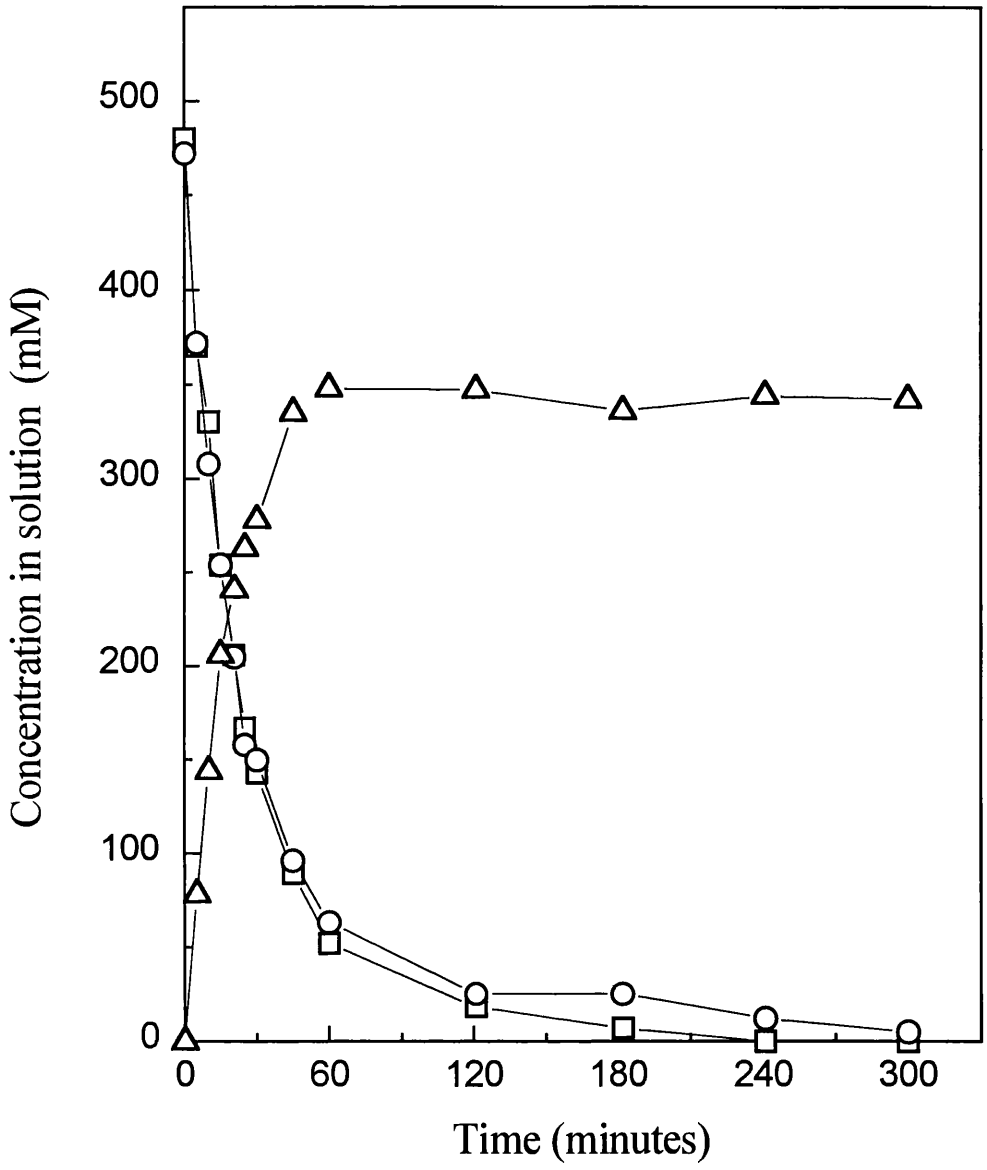


Figure 2.17. Reactor concentration profile for a 500 mM batch biotransformation illustrating HPA (□), glycolaldehyde (○),and L-erythrulose (Δ) concentrations in solution. The initial transketolase level was 26 U/ml.

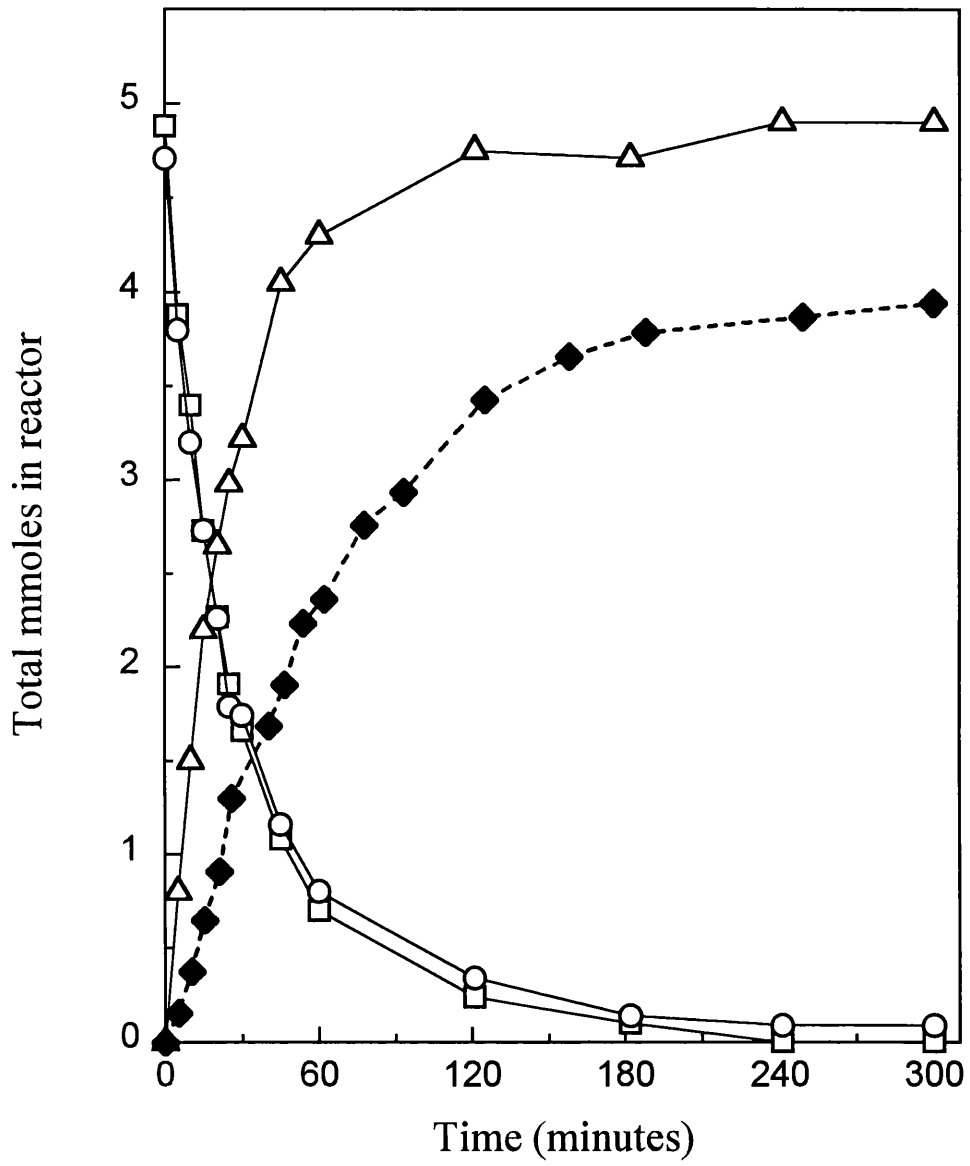


Figure 2.18. Mass balance for a 500 mM batch biotransformation controlled at pH 7.0, 25°C illustrating total μmoles of HPA (□), glycolaldehyde (○), L-erythrulose (Δ) and HCl titrant (◆) in the system. The initial transketolase level was 26 U/ml.

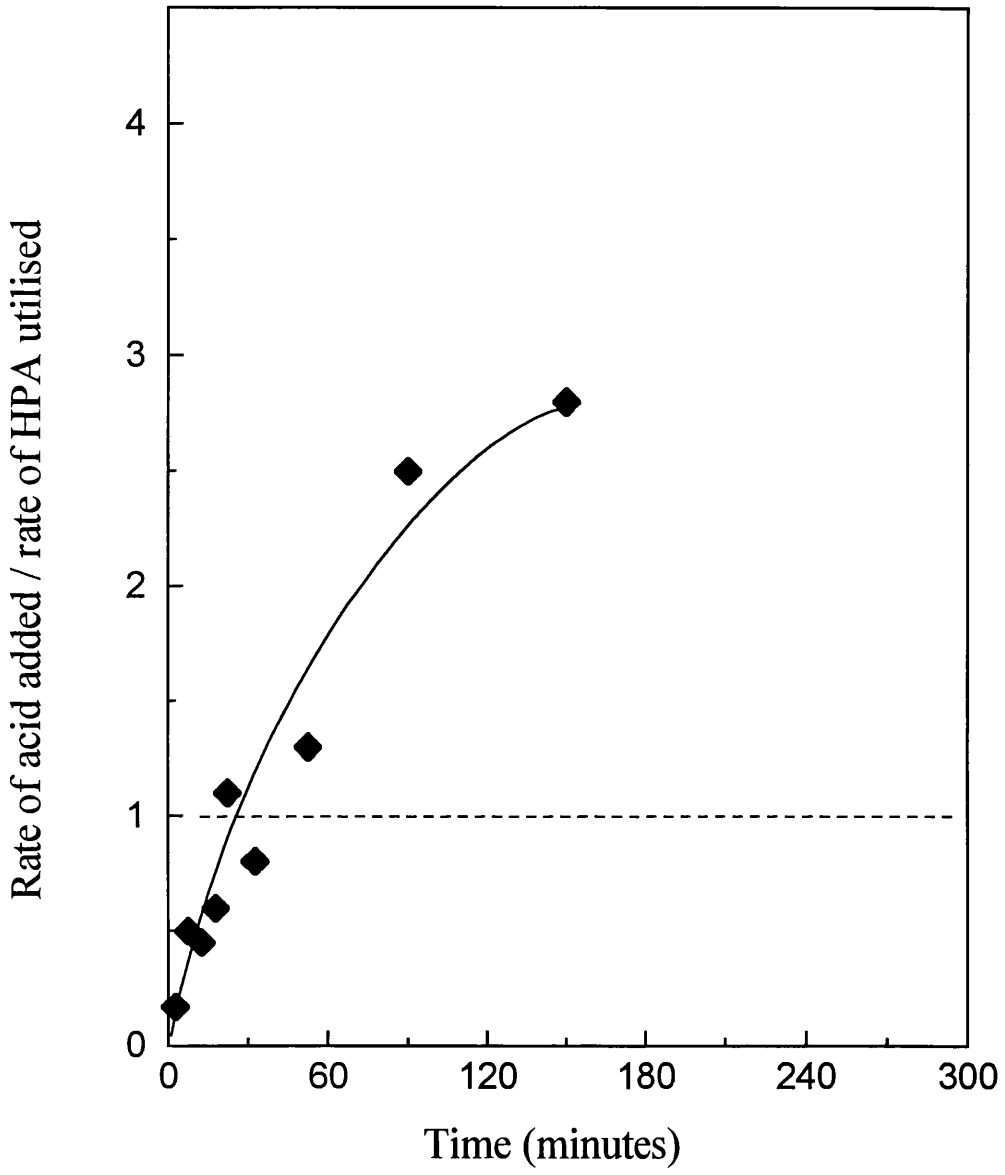


Figure 2.19. Illustration of the change in ratio of reaction rate to titrant addition rate during a 500 mM batch biotransformation controlled at 25°C, pH 7.0. The initial transketolase level was 26 U/ml. The ratio is not shown after 120 minutes reaction due to the large errors associated with the low rates involved.

extended incubation time but complete mass balance was unlikely due to the buffering effect of the protein in the bulk solution.

2.6.3. Effect of pH control on product yield

The 500 mM biotransformation procedure (2.2.2.8) was performed with and without pH control at pH 7.0. The reaction was monitored over 2 hours and both reactions reached completion as no substrates were detected after this time. Figure 2.20 compares the total L-erythrulose product and reaction pH for the two biotransformation conditions.

In absence of pH control the reaction pH rapidly increased to a final value of pH 8.7 by the end of the reaction. Comparison of the mass balance between the two reaction conditions clearly indicated a reduction in yield by up to 20% if the pH was not controlled. It was evident from stability studies on the model substrates and product at different pH values that all the model reaction components were alkali labile (2.4.1.2). Operation of the model biotransformation in alkaline conditions, as a consequence of uncontrolled reaction pH for example, would therefore be expected to result in lower final product yields. This was confirmed. However, although the pH increased during uncontrolled reaction, this increase was lower than expected from the theoretical equivalence of protons generated by the reaction. This observation was probably a direct result of the logarithmic increase in carbon dioxide solubility that is known to occur as pH is increased. The net effect of this would be a 'buffering' effect as the pH shifted towards the alkaline region. The raised solubility of carbon dioxide at the higher pH was confirmed by substantial gas evolution observed on acidification of the completed reaction.

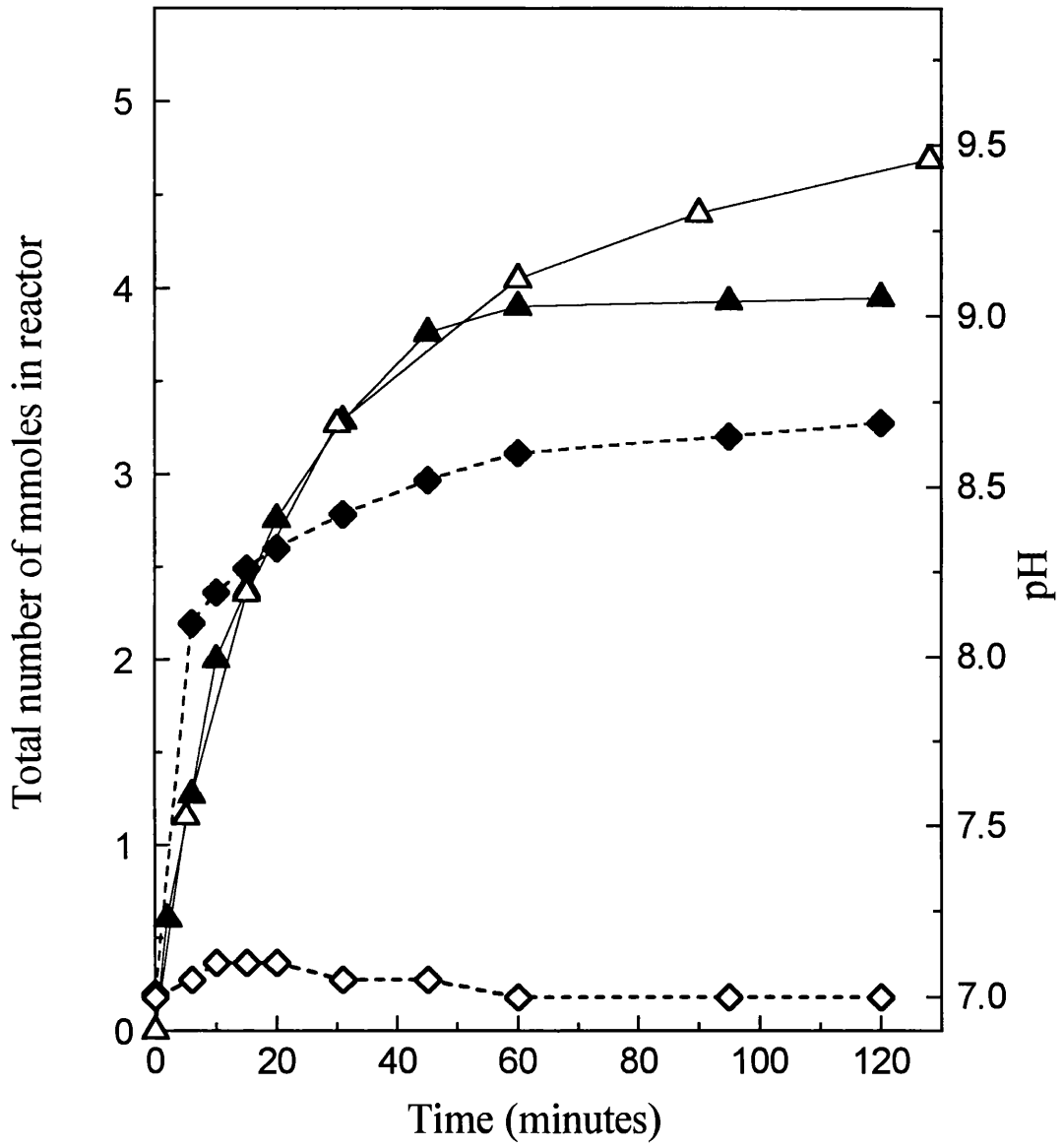


Figure 2.20. Effect of pH control on the product concentration (Δ, ▲) during a 500 mM biotransformation at 25°C. Total L-erythrulose formation is illustrated for reactions maintained at pH 7.0 (Δ) and pH uncontrolled reaction (▲) while the measured pH is described by (◊) and (◆) respectively. Initial transketolase levels were 25 U/ml.

2.6.4. Effect of reducing agent on biotransformation

Oxidative deactivation of holo-transketolase has been demonstrated during incubation in the absence of substrates and products (2.5.2). The effect of reducing agent on enzyme oxidation during a biotransformation required investigation.

A biotransformation at 500 mM initial substrate concentration (2.2.2.8) was conducted at pH 7.0, 25°C in the presence and absence of 10 mM 2-mercaptoethanol. The reaction was monitored by ion partition chromatography and the transketolase activity measured during the reaction by the linked enzyme assay (2.2.2.1).

The effect of reducing agent on enzyme stability during biotransformation and associated reaction rate is shown in Figure 2.21. The reducing agent stabilised the enzyme over the 4.5 hour study and resulted in a faster rate of reaction. Further incubation over 5 hours resulted in a gradual decrease in enzyme activity (results not shown) indicating a loss of reducing power. Glycolaldehyde-mediated deactivation of the enzyme was negligible in this instance as the duration at high aldehyde concentration was short due to the rapid reaction rate. However, heterocyclic and aromatic aldehydes with greater deactivating power and lower rates of reaction (Demuynck *et al.*, 1991) would be expected to exert a greater influence in similar batch biotransformations.

2.6.5. Effect of enzyme pre-activation on biotransformation

2.6.5.1. Effect of pre-activation on initial rate of reaction

500 mM biotransformations were set up (2.2.2.8) but the initial rate of reaction using transketolase-rich lysate was compared to reaction using lysate pre-activated with cofactor solution (2.2.2.9). The biotransformations were performed at pH 7.0 and 35°C in the presence of 10 mM 2-mercaptoethanol and the final enzyme level in both

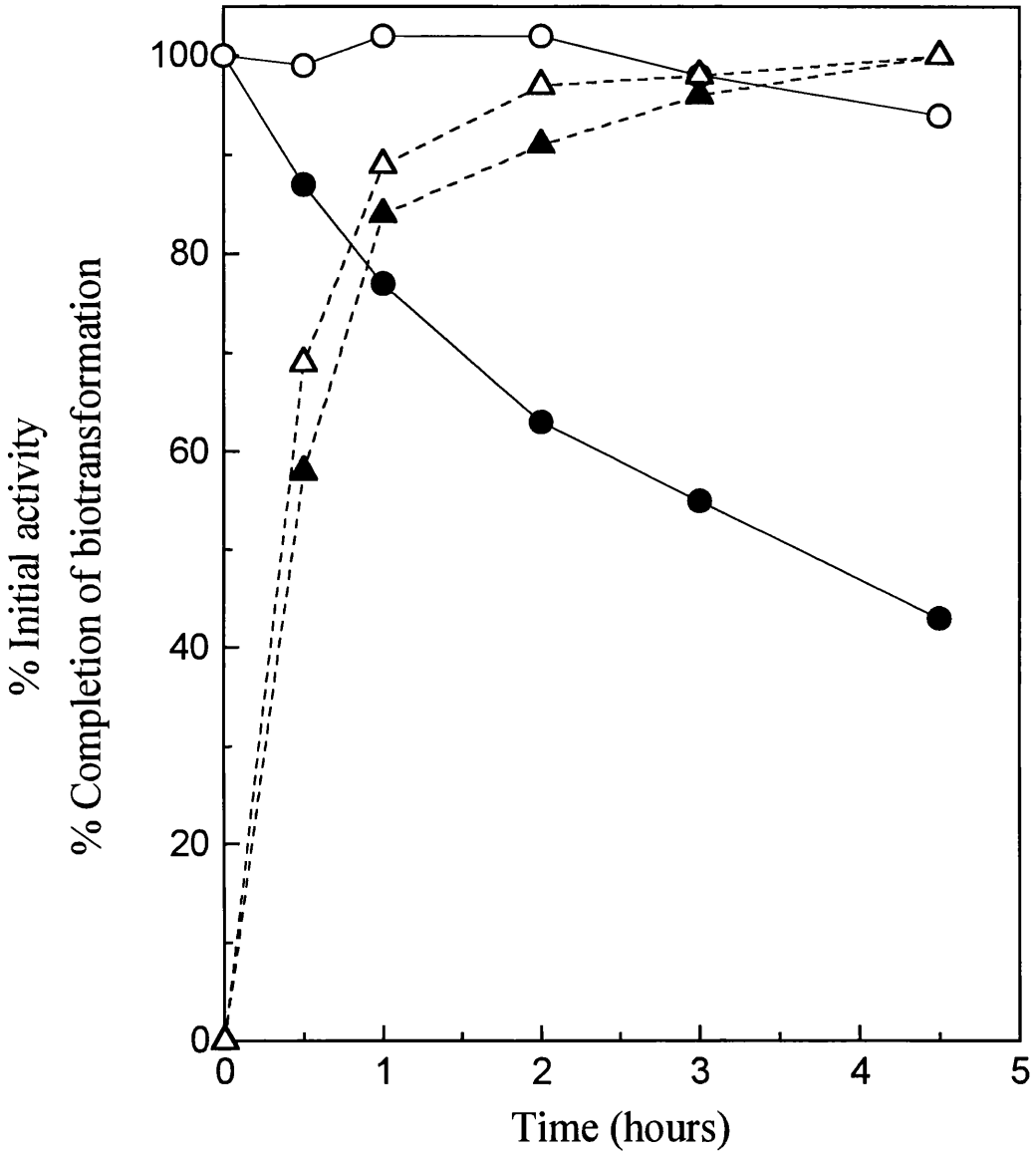


Figure 2.21. Effect of inclusion of reducing agent on total residual enzyme activity (O, ●) during a 500 mM biotransformation at 25°C, pH 7.0. The % completion of the biotransformation (--Δ-- , --▲--) is also illustrated. The reactions were conducted in the presence (O, Δ) and absence (●, ▲) of 10 mM 2-mercaptoethanol and the initial transketolase levels were 25 U/ml.

cases was 13 U/ml (equivalent to 25 U/ml if the biotransformation were performed at 25°C). HPA utilisation was monitored over the initial 20 minutes of reaction (Figure 2.22). Pre-activation of the enzyme resulted in reaction immediately on addition to the substrate whereas addition of the lysate without modification produced a lag phase of 4 minutes before full activity was observed. The utilisation of initial biotransformation rates, using activated enzyme, as an analytical tool for characterisation studies was investigated. The linearity of the initial rate of reaction with enzyme activity required evaluation.

2.6.5.2. Initial rate of biotransformation as a transketolase assay

In order to investigate the linearity of the initial reaction rate, 100 mM biotransformations were conducted (2.2.2.8) at a range of enzyme levels up to 15 U/ml. The reactions were performed with pre-activated enzyme (2.2.2.9) at 35°C, pH 7.0 for comparity to reaction conditions used in the spectrophotometric transketolase assay. The rates of HPA and glycolaldehyde utilisation and L-erythrulose production were monitored over the initial 5 minutes of incubation. A plot of measured rate of biotransformation against enzyme concentration (as determined by spectrophotometric assay) is shown in Figure 2.23. The profile is illustrated for HPA utilisation although profiles for each of the other components was comparable. Any minor changes in reaction volume by addition of titrant were accounted in the calculation. Under the described biotransformation conditions the reaction remained linear at transketolase activities up to 4.5 U/ml. At lower enzyme levels the initial reaction would remain linear for longer than 5 minutes.

Therefore, providing the enzyme was activated prior to addition to a biotransformation, monitoring initial biotransformation rates provided a simple method for investigation of kinetic parameters such as evaluating enzyme inhibitors and measurement of Km values. A modified version of this assay has been used for determination of several kinetic parameters of transketolase (Gyamerah and Willetts, 1996).

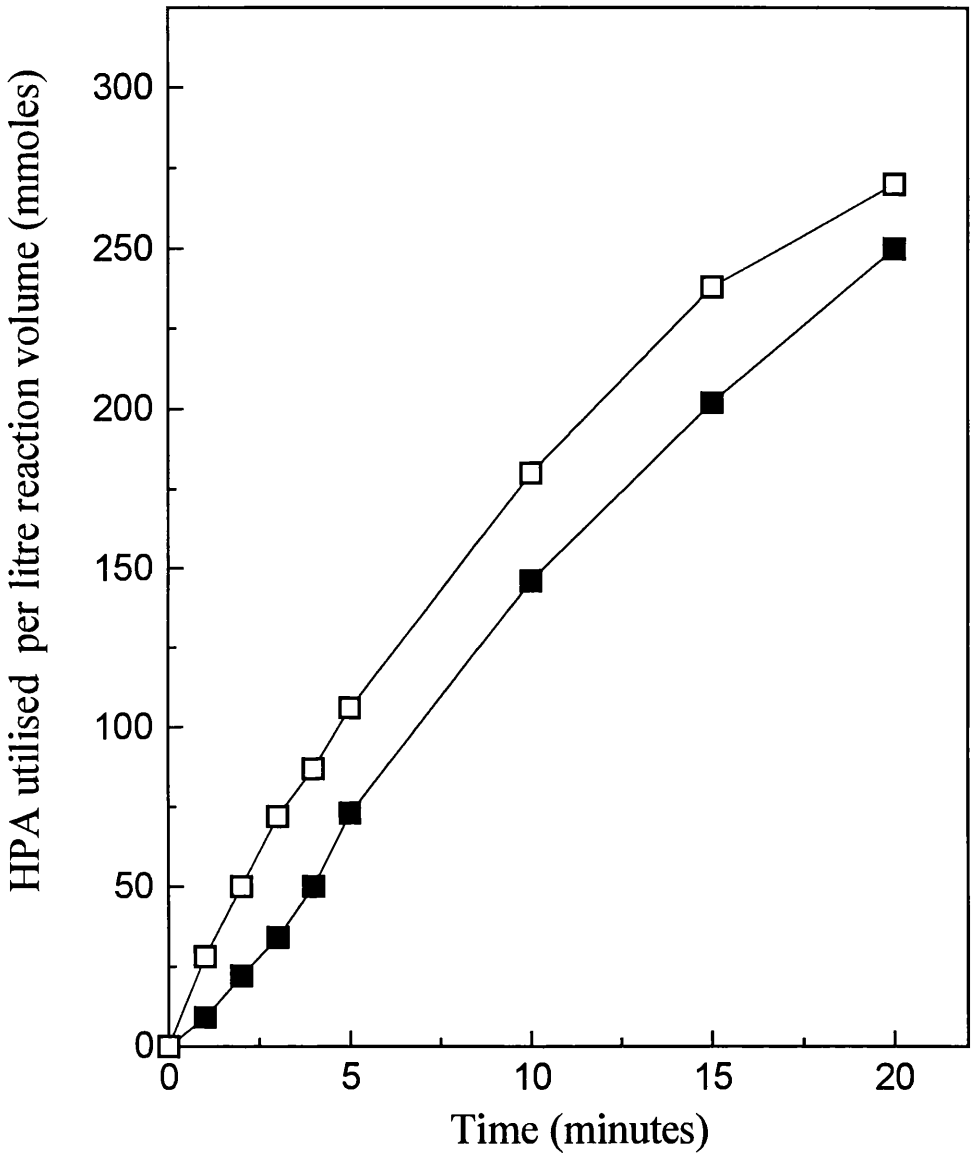


Figure 2.22. Effect of pre-activation on initial stages of a 500 mM biotransformation at pH 7.0, 35°C. The graphs illustrate the amount of HPA utilised for pre-activated (□) and non-pre-activated (■) transketolase-rich lysate. Initial transketolase levels were 13 U/ml. 10 mM 2-mercaptoethanol was included.

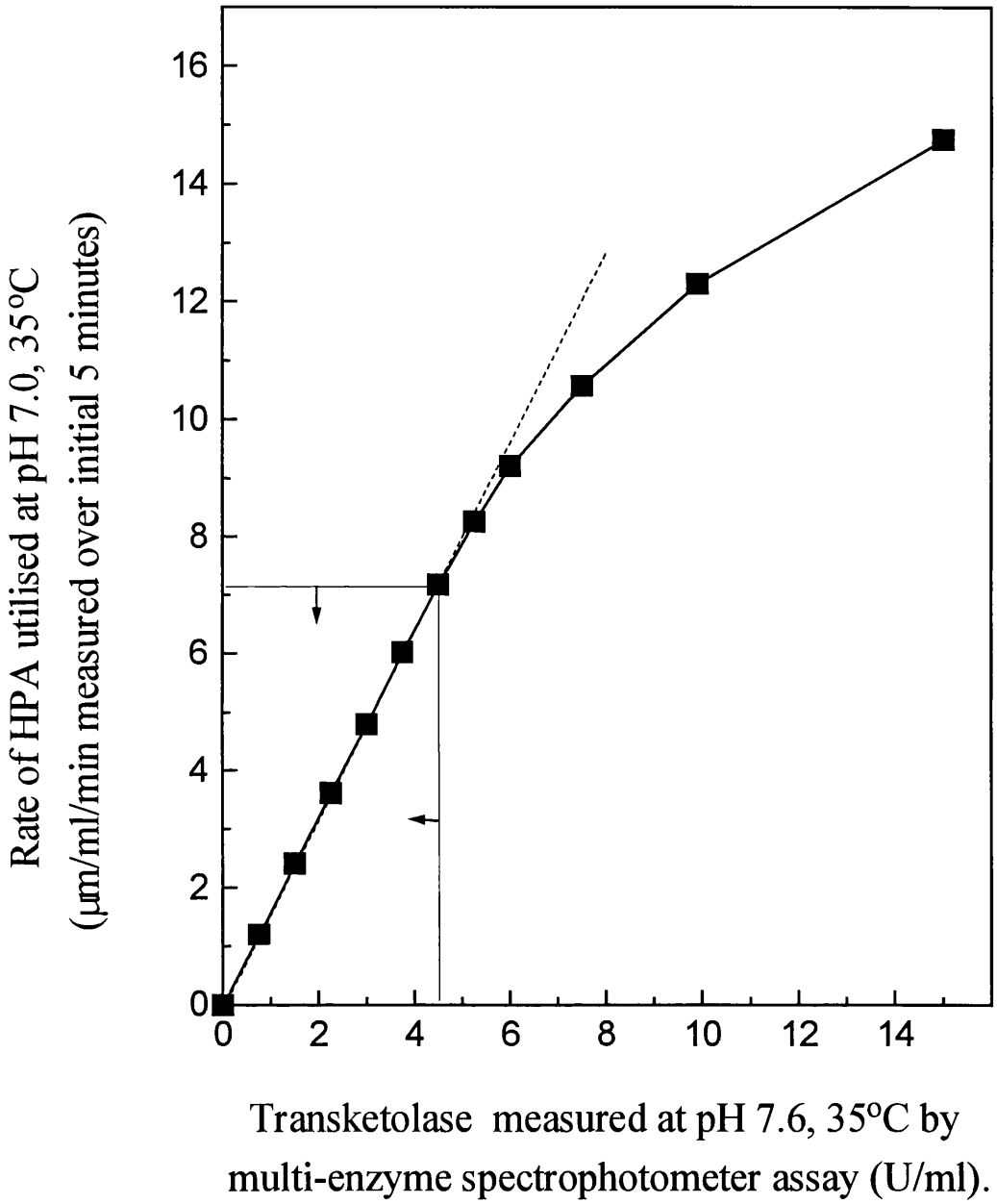


Figure 2.23. Comparison of initial rate of 100 mM model biotransformation at pH 7.0, 35°C (determined by HPLC measurement of residual HPA) for different transketolase concentrations (determined by the multi-enzyme spectrophotometer assay). The linear region is indicated by the arrows.

2.6.6. Effect of dissolved carbon dioxide on transketolase stability

Due to the difficulty involved in separating interfacial effects from biochemical effects with the use of carbon dioxide gas, bicarbonate addition was utilised to evaluate the biochemical interactions of carbon dioxide with the enzyme. An investigation was performed to test whether dissolved carbon dioxide (and related dissociation species) irreversibly denatured transketolase. The investigation was performed at pH 8.0 with addition of bicarbonate to 500 mM. pH 8.0 was chosen as all the carbon dioxide remained in solution at this pH value. Transketolase (23 U/ml final) was incubated in a pH stat at pH 8.0, 25°C in the presence of 2.4 mM TPP, 0.9 mM Mg^{++} and 10 mM 2-mercaptoethanol. 500 mM bicarbonate was added and the pH maintained at 8.0 over 4 hours incubation. Transketolase activity during incubation was compared to a control at the same ionic strength (using NaCl) and a control with activated enzyme only. In addition, the high ionic strength in the presence of 500 mM NaCl was also investigated. Table 2.6 illustrates the measured activities (as determined by the spectrophotometric assay) after incubation under the various conditions.

Additions	Transketolase activity (% of initial)			
	0 hours	1 hour	2 hours	4 hours
Negative control (no additions)	100	96	98	97
500 mM bicarbonate	100	93	101	97
Ionic strength control (NaCl)	100	91	100	97
500 mM NaCl	100	99	95	96

Table 2.6. Effect of dissolved carbon dioxide and ionic strength on holo-transketolase stability in the presence of reducing agent.

Neither bicarbonate nor NaCl at 500 mM exerted any permanent deactivation effect on the enzyme. However, it may be possible that during a biotransformation, reaction between carbon dioxide and the enzyme was resulting in carbamate formation (Mitz, 1979) and causing reversible inhibitory effects. This is difficult to measure as a reduction in levels of the dissolved gas would occur during sampling and assay for activity which may alleviate the inhibition effect. The only suitable method to determine such reversible effects would be to compare rates of biotransformation in the presence of different bicarbonate levels.

2.7. Biotransformations incorporating substrate feeding

2.7.1. Introduction

The synthetic potential of transketolase is due partly to the wide variety of aldehydes that can be used as acceptor compounds. However, as confirmed in this study, aldehydes can react with proteins such as transketolase, via imine formation, resulting in loss of catalytic ability (Demuynck *et al.*, 1991). Although glycolaldehyde exerts lower toxicity relative to other potentially useful aldehydes, substantial toxic effects were observed (2.6.1). There is clearly a need to investigate methodologies for maintaining low aldehyde concentration within the reactor in order to reduce or remove the influence of this constraint on the operating conditions. In addition, as the final product concentration in batch mode was limited by the aqueous saturation concentration of the hydroxypyruvate salt, this may constitute another constraint and, consequently, studies involving feeding of HPA were conducted.

2.7.2. Addition of a bi-substrate acidic feedstream at fixed rate

Reduction of the concentration of aldehyde in the vicinity of the biocatalyst to reduce enzyme deactivation may be achieved by feeding the aldehyde to the reactor at a fixed rate. Providing the feeding rate is lower than the total maximum reaction rate for the enzyme in the system, the residual concentration of aldehyde should remain low. The solubility of HPA has been shown to be significantly higher in free acid form at low pH (2.4.2). By combining the aldehyde with HPA in an acidic feed, high final concentrations of product should result with reduced requirement for external pH control and a minimum loss of catalytic activity. Therefore, the addition of an acidified bi-substrate feed at a fixed rate was investigated.

The initial reaction volume was 5 ml containing 110 U/ml activated transketolase in cofactor solution (2.4 mM TPP and 0.9 mM Mg^{++}) and 10 mM 2-mercaptoethanol. The solution was maintained at pH 7.0, 25°C in a pH-stat. Fine control of pH was conducted by automated addition of 2 M NaOH or 1.0 M HCl as required. The feed volume was 10 mls and contained 1000 mM HPA (free-acid), 1000 mM glycolaldehyde, 2.4 mM TPP, 0.9 mM Mg^{++} and 10 mM 2-mercaptoethanol. The pH of the feed was not adjusted but was approximately pH 2.7. The biotransformation was initiated by feeding at 0.57 vol./init.vol./hour. The substrates and product concentration, the enzyme activity and the total reaction volume were monitored over 6 hours (Figure 2.24). The feeding phase was complete in 3.5 hours but although the feeding rate was significantly below the total potential reaction rate, residual substrate concentrations reached ~50 mM during feeding. The final product level was > 600 mM with negligible levels of unreacted substrate in the product solutions enabling simple downstream processing. The total transketolase activity was maintained at 80% of the initial level, the 20% loss probably due pH/aldehyde concentration hot spots at the point of feed addition resulting from poor mixing.

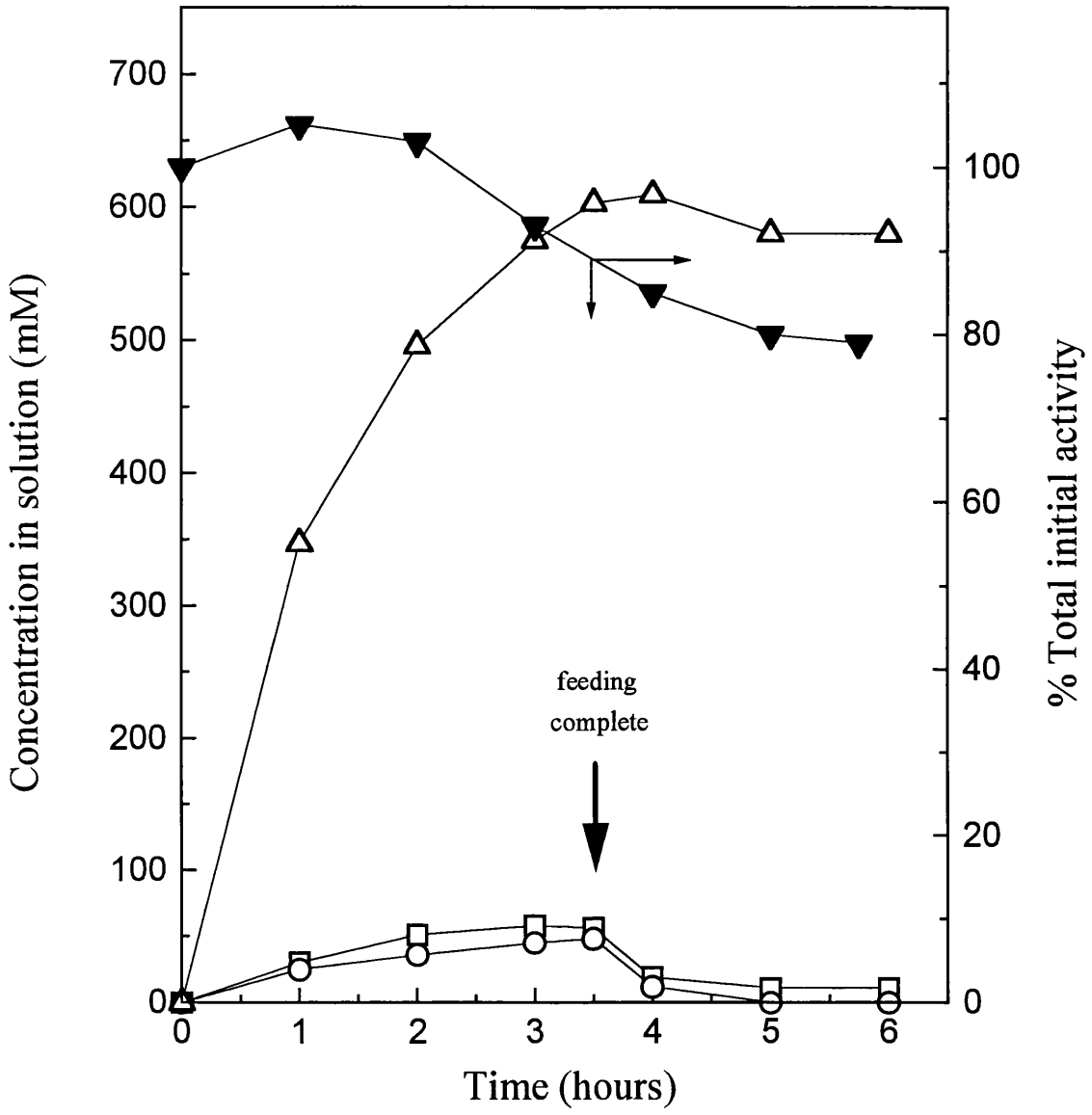


Figure 2.24. Fed-batch biotransformation at pH 7.0, 25°C with addition of a single bi-substrate acidic feed of 1000 mM HPA/1000 mM glycolaldehyde at a fixed rate of 0.57 vol./init. vol./hr. Total transketolase activity (▼) and reactor concentrations of HPA (□) glycolaldehyde (○), and L-erythrulose (Δ) are illustrated. The initial reaction volume (5.0 ml) contained 110 U/ml transketolase. 10 mM 2-mercaptoethanol and cofactors were included in both the initial reaction volume and the feed.

2.7.3. Use of bi-substrate acidic feedstream utilising a feed-on-demand strategy

The requirement for an acidic titrant to control the pH of a batch biotransformation could be utilised in a fed-batch process as a method of maintaining the feed addition rate at the maximum possible enzyme reaction rate and also maintaining the reaction pH near neutral. The acidic feed was therefore utilised in a feed-on-demand biotransformation.

A batch biotransformation at pH 7.0, 25°C was set up (2.2.2.8) with the following modifications. The glycolaldehyde and lithium HPA concentrations in the initial batch mode were 350 mM. The titrant addition vessel contained 10 ml of 1300 mM glycolaldehyde, 1300 mM HPA (free acid), 0.9 mM $MgCl_2^{++}$ and 2.4 mM TPP. The pH was not adjusted. 2-mercaptoethanol was included in both the initial batch reaction volume and the titrant/feed volume to 10 mM. The biotransformation was initiated by addition of pre-activated transketolase (2.2.2.9) to 25 U/ml. Minor adjustment of reaction pH during the study was performed manually by addition of 1.0 M HCl or 1.0 M NaOH as required. The substrates and product concentration and the change in reaction volume were monitored over 6 hours incubation as shown in Figure 2.25. Total transketolase was measured at the end of the study.

The initial drop in residual concentration of HPA was due to the solvation of carbon dioxide. However, once carbon dioxide equilibration was complete the rate of reaction and titration were equivalent and the system was operating on a feed on demand basis. The background level of substrates was maintained at approximately 100 - 150 mM during the duration of the feed-on-demand period. It was again evident that the final product concentration could be increased considerably by feeding acidic HPA at a high concentration. In this case, the final product level of >800 mM exceeded the maximum solubility limit of lithium HPA during operation in batch mode (~650 mM) and thus the constraint of limiting HPA solubility at pH 7.0 was overcome. On extending the study beyond the completion of feeding and manually controlling the pH, the remaining substrate was converted entirely to product.

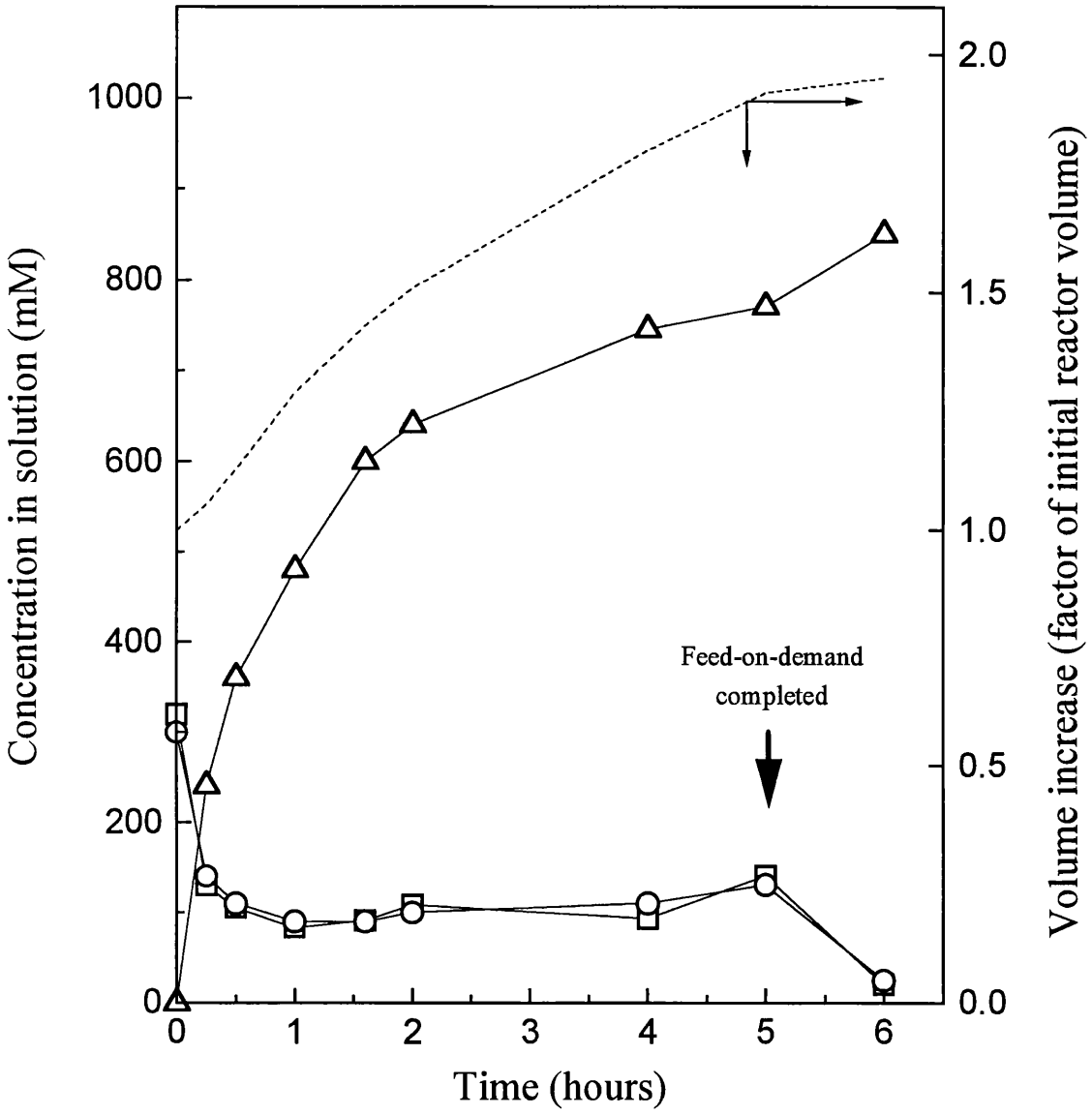


Figure 2.25. Fed-batch biotransformation at 25°C, pH 7.0 using an acidic bi-substrate feed-on-demand strategy. Total reaction volume (-----) and reactor concentrations of HPA (□), glycolaldehyde (○), and L-erythrulose (Δ) are illustrated. The initial transketolase level was 25 U/ml and 10 mM 2-mercaptoethanol was included in both the initial reaction volume (10.0 ml) and the feed (10 ml).

Although high final product level was achieved, an end-point determination of the residual transketolase activity showed a 39% loss in total activity over the 6 hour duration. This was attributed to the deactivation effect of the background aldehyde level coupled with pH/aldehyde 'hotspots' due to poor mass transfer at the point of titrant addition.

2.8. Discussion of Model Studies

2.8.1. Analytical tools

In this study, a systematic approach to reactor design has been adopted for transketolase catalysed reactions based on the characterisation of a model carbon-carbon bond synthetic reaction. In order to perform detailed investigations, the first priority was to develop the necessary analytical tools. The direct measurement of substrate and product offered the potential for complete characterisation of the catalytic process enabling evaluation of process options. Although the enzymatic assay of HPA and of glycolaldehyde was developed during the 1960s (Holldorf, 1966; Goedde *et al.*, 1966 respectively), the transketolase reaction first required termination and the conditions then re-adjusted to allow enzymatic quantification. This quenching procedure usually involved the addition of acid to denature the enzyme followed by the addition of an equal quantity of alkali to attain near-neutral pH. This introduced significant errors and delays into the assay system. As part of this study, an accurate and rapid method for the direct quantitative analysis of HPA, glycolaldehyde and L-erythrulose using HPLC was developed (Mitra and Woodley, 1996). Although discussed here with specific application to the model reaction system, the assay technique will theoretically detect most water-soluble carbohydrates. For transketolase catalysed biotransformation of HPA with water-immiscible aldehydes, the reaction may still be monitored quantitatively by measurement of the loss of HPA using the HPLC assay. For determination of

transketolase activity, a linked multi-enzyme spectrophotometric system was routinely used. However, although suitable for measurement of enzyme activity, this was not suitable for determination of the kinetic constants for the model reaction. By determination of initial reaction rates of the model biotransformation under different conditions using the HPLC assay, the kinetic parameters have been studied in detail (Mitra and Woodley, 1996; Gyamerah and Willetts, 1996).

2.8.2. Characteristics and constraints

Once the analytical tools were available, the biotransformation could be accurately characterised. The first consideration was the properties of the substrates and products. Both the substrates and product were shown to be sensitive to alkaline environments particularly at pH values above 8.0, the rate of degradation increasing with concentration of compound. This was an important observation with respect to HPA, an expensive and common component of many useful transketolase catalysed reactions, and thus imposes a constraint on the maximum pH of the model process. Clearly, for other reactions where the respective pH stabilities of the different aldehyde and product components are lower than HPA, these may define the highest acceptable limit of pH. Although the cofactor TPP was slowly degraded in alkaline conditions, the low concentration required and low commercial cost of this compound probably negates this as a limiting constraint. The limited increase in pH observed during the uncontrolled 500 mM batch reaction illustrated the buffering effect of the bicarbonate but the alkalinity was still sufficient to reduce final product yield by 20%. However, gas production was visibly decreased as the pH increased due to greater solubility of carbon dioxide in alkaline solutions. The reaction of proteins with the dissociation species of carbon dioxide has been reported to result in the formation of carbamates. Transketolase, however, showed no permanent damage after several hours of exposure to high levels of bicarbonate (and therefore to saturating levels of the associated ionic species). The method of investigation did not, however, rule out the possibility of a reversible but deleterious effect on activity only evident during reaction in the presence of the dissociation species. Although not addressed due to time constraints, this could be investigated by determination of initial reaction rates

during the model biotransformation in the presence of different levels of bicarbonate at different pH values. To predict the solubility of the carbon dioxide under a range of reaction conditions would be useful but the time constraints of this study ruled this unfeasible.

The lower pH limit for the reaction was defined by the enzyme stability and is another constraint on the system. The transketolase enzyme was irreversibly denatured at all pH values less than 6.5 . The rate of denaturation of transketolase at pH 5.5, however, was low, compared to the instantaneous precipitation of contaminating proteins in the lysate at this pH value. If required, this characteristic may be utilised as a simple purification step to increase transketolase purity.

The pH-activity profile for *E. coli* transketolase was based on the initial rate of L-erythrulose formation during a 100 mM batch reaction. The profile was broad maintaining at least 90% maximum activity over the range pH 6.5 - 8.0 . This broad pH profile is a useful characteristic for potential industrial syntheses. The pH optimum was observed between pH 7.0 and pH 7.5 measured in the absence of buffers. This is similar to a reported value of pH 7.6 for the baker's yeast enzyme (Datta and Racker, 1961) but lower than the pH range 8.0 - 8.5 reported for the *E. coli* transketolase (Sprenger *et al.*, 1995). However, these reported values for *E. coli* differed depending on the buffer system used suggesting an influence is exerted on transketolase kinetics by the use of buffers.

Some researchers (Dalmas and Demuynck, 1993) have incorporated the continuous removal of dissolved gases (by sparging with nitrogen) into transketolase catalysed syntheses without explaining the justification. The majority of research papers, however, do not report any effect of oxidation on the enzyme during transketolase catalysed processes (Effenberger *et al.*, 1992; Kobori *et al.*, 1992; Demuynck *et al.*, 1991). In this study, it was found that although the apo-enzyme was stable with time, the inclusion of the cofactor TPP increased the susceptibility of the enzyme to oxidative attack. The presence of HPA, however, appeared to cause rapid oxidation of the apo-form without influencing the holo-enzyme. The oxidation effect therefore appears to affect interacting groups in the active site region of the enzyme. One candidate for oxidation is the cys 157 residue reported to occur close to the diphosphate binding region for the cofactor (Littlechild *et al.*, 1995). Based on crystallographic investigation, this residue was reported to be present in an unusual

hydroxylated form. We suggest that this observation may be an artefact resulting from the presence of oxygen during or prior to the crystallisation process. The observation that the presence of the cofactor initiates oxidative deactivation is surprising since the cys 157 residue is shielded from the external environment on binding of the cofactor. It would therefore be expected that the apo-enzyme would be the oxygen sensitive form. However, the requirement for the exclusion of oxygen either by degassing or the addition of reducing agents is another constraint on the system. The physical degassing of the reacting solution could be achieved by sparging with nitrogen or by operation under vacuum. The inclusion of sulphydryl compounds such as dithiothreitol or mercaptoethanol is the less desirable option considering the cost, the toxic nature of these compounds and the possible additional downstream operations required to remove them.

The presence of aldehyde compounds was expected to exert some influence on the system. Reaction between aldehydes and the primary amines of proteins occurs through Schiff base (imine) formation and glycolaldehyde was observed to deactivate both the apo and holo-transketolase at similar rates. This effect was concentration dependent and not influenced by the exclusion of oxygen. It is important to note that the deactivating effect of cyclic and heterocyclic aldehydes has previously been reported to be greater than glycolaldehyde (Demuynck *et al.*, 1991). The severity of this constraint will therefore depend on the type and concentration of aldehyde in contact with the enzyme. These effects are likely to assume greater significance for resolution reactions where racemic aldehydes are used.

The detailed kinetics of the *E. coli* transketolase have been studied (Sprenger *et al.*, 1995; Gyamerah and Willetts, 1996) showing the enzyme is subject to low levels of product inhibition (Ki ~570 mM for L-erythrulose). Minor substrate inhibition effects during the reaction have also been reported (Gyamerah and Willetts, 1996). However, inhibition effects did not prevent the reaction running to completion. Although no information was found concerning the inhibitory effect of other transketolase reaction products, the potential for inhibition needs consideration during process design. *In situ* product removal (ISPR) is an option for limiting the concentration of product in the biocatalyst environment and is particularly relevant where hydroxypyruvate is not used as ketol donor and the reaction is reversible.

Cofactor dissociation studies indicated that both cofactors dissociate from the *E. coli* holo-enzyme at all values in the pH range 6.5-9.5 . However, the majority of the dissociation occurred as the pH increased from pH 7.5 . This differs from the yeast enzyme where pH 6.0 initiates the dissociation (Heinrich *et al* , 1972). However, it is possible that the bound TPP was not merely dissociating but degrading due to an observed alkaline instability. These effects of pH are important as enzyme immobilisation may result in greater pH shifts in the vicinity of the enzyme compared to the bulk solution. Based on the recent elucidation of the crystal structure of both yeast transketolase (Sundstrom *et al.*, 1992) and *E. coli* transketolase (Littlechild *et al.*, 1995), only a small defined conformation change is reported to occur within the active site cleft on cofactor binding. Therefore, it is unlikely that the cofactors can be permanently retained within the holo-transketolase using extensive external cross-linking immobilisation methods. An immobilised system would therefore require the inclusion of both cofactors in solution to maintain activity.

Based on the above information, it is possible to illustrate the general operational window for *E. coli* transketolase catalysed biotransformations (Figure 2.26). This is a useful visual tool for describing the effect of several constraints and parameters on the operation of transketolase catalysed processes. The upper limit of operational pH is determined by the acceptable rate of substrate or product degradation. The lower operational limit of pH is set by the deleterious effect of acidic conditions (less than pH 6.5) on the enzyme. Although the deactivation is relatively slow at pH 6.0, the rate increases as the pH is lowered. HPA does not affect holo-transketolase stability but there is an HPA solubility limit in the operational pH range of 650 mM and 1100 mM for the lithium and sodium salt respectively. The maximum operational concentration of the aldehyde component may be set by physical constraints such as solubility or, if exhibiting a high deactivation effect on

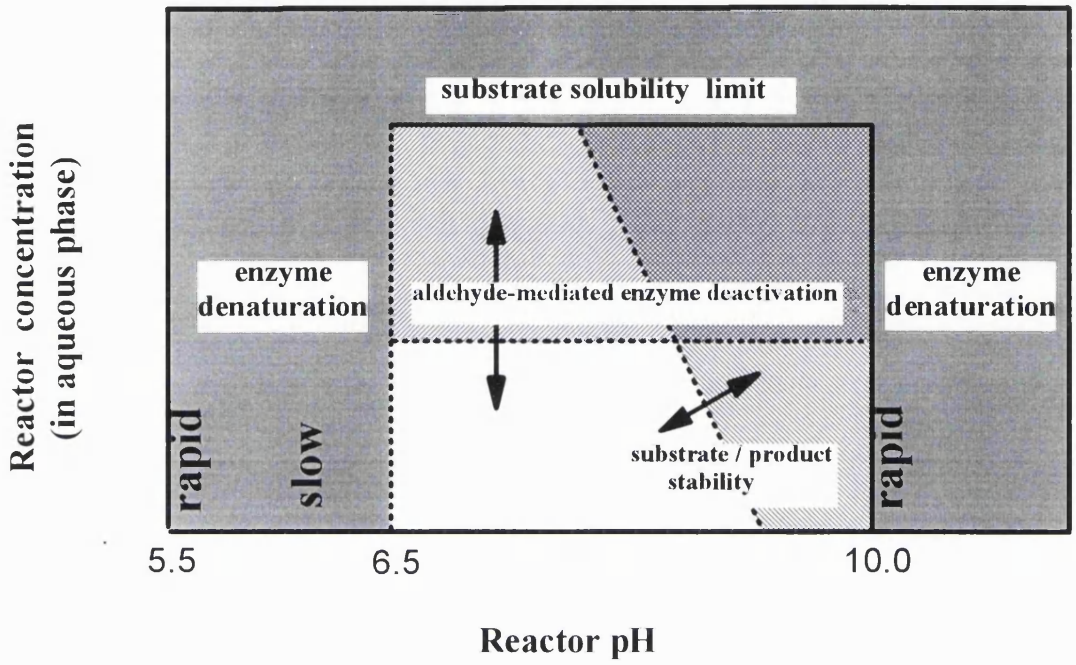


Figure 2.26. Schematic general operational window based on the characteristics of the model transketolase reaction. Oxidation effects on the holo-transketolase are not shown. The dotted boundaries indicate a degree of flexibility depending on acceptable rates of loss.

transketolase, may be controlled at lower values. However, during all these reaction processes the holo-enzyme is vulnerable to irreversible damage by oxidative attack. Therefore extended biotransformation times should be avoided or measures taken to remove and exclude oxygen from the vicinity of the active enzyme.

2.8.3. Reactor options

Table 2.7 summarises the constraints of the biotransformation characteristics on the selection of the most appropriate reactor configuration for the model reaction. For demonstrating the principle of the structured approach, equal operational importance has been allocated to all constraints and no economic factors have been considered. (The effects of various economic scenarios on reactor design are considered in section 4). As the substrates and products are labile in alkaline pH, the inherent problem of pH control in plug flow reactors eliminates this as an appropriate design. In addition, packed bed plug flow reactors using immobilised biocatalysts are ruled out for two additional reasons; the likelihood of channelling due to gas production and the toxic effect of the glycolaldehyde on the biocatalyst. The use of a pressurised reactor could reduce the physical release of carbon dioxide to a minimum and therefore prevent channelling but the toxicity problem would still be prevalent.

This toxicity of the aldehyde also rules out batch stirred tank reactor configurations where high initial substrate concentrations are required to generate the high product levels required for efficient downstream operations. Competitive inhibition by L-erythrulose, a potential ketol donor, has been observed at high product concentrations (Gyamerah and Willetts, 1996). Continuous stirred tank reactors expose the biocatalyst to constant high product concentrations and are therefore also inappropriate. Hence, by default, the fed-batch stirred tank reactor would be the design option of choice for this particular model system. To evaluate this option, feeding studies were performed employing a variety of different design configurations. Feeding both substrates at an acidic pH proved to be the most useful feeding strategy for the following reasons:

Characteristics		Constraints				
		Reactor selection				Reactor operation
		FBSTR	BSTR	CSTR	PFR	
Reactants / products	pH lability				●	pH 7
	Temp. lability					Operate at 25°C
Reaction	Acid consumption				●	pH stat
	Gas production				●	
Interactions	Substrate toxicity		●		●	low [glycolaldehyde]
	Product inhibition			●		low [L-erythrulose]
Selection		○	●	●	●	

● unsuitable
○ suitable

FBSTR = fed -batch stirred tank reactor
BSTR = bstch stirred tank reactor
CSTR = continuous stirred tank reactor
PFR = plug flow reactor

Table 2.7. Characterisation and constraints for reactor selection and operation for the model reaction

1. Possible substrate inhibition effects (i.e. reversible activity loss) of HPA and/or the aldehyde would be reduced.
2. Final product concentrations would be increased due to the greater solubility of HPA in an acidic feed and also due to the decreased extraneous titrant volume required to maintain pH.
3. Feeding the free-acid form of HPA would reduce the level of contaminating salts in the product stream.
4. The toxic effect of the aldehyde on transketolase (i.e. irreversible activity loss) would be reduced by feeding the aldehyde at a reaction limiting rate.
5. ISPR would be simplified by reducing interference from the substrates (Chauhan *et al.*, 1996).

In the case of fixed rate feeding of the substrates, a residual background level of both substrates built up to but did not exceed 50 mM. This occurred despite the feed addition rate being significantly lower than the transketolase Vmax for the reactor. On termination of feeding this residual level slowly disappeared indicating that a lower feed rate would reduce this residual substrate level. The rate of feed addition would, however, be an important parameter in the economics of a commercial fed-batch process as a low rate may greatly extend the total process time to final product. The effect of product inhibition coupled to the high Km values for the glycolaldehyde and HPA, 16.1 and 13.2 mM respectively (Gyamerah and Willetts, 1996) would explain this observed phenomenon. The Km describes the ability of the enzyme to recognise and bind low levels of a particular substrate and will therefore play an important role in any feeding strategy adopted.

The feed-on-demand reactor system utilised the requirement for acidic titrant addition during reaction at pH 7.0 to add both the aldehyde and HPA substrate in concentrated form. Unlike the fixed-feeding configuration, the feed rate was controlled automatically at the maximum kinetic reaction rate by the utilisation of HPA. Although significant enzyme was lost during the biotransformation due to a high background level of glycolaldehyde (100-150 mM), it may be possible to reduce this background aldehyde effect by reducing the substrate concentration in the initial batch

period of the biotransformation. However, based on the observed accumulation of substrates in the fixed-feed study, the background substrate level during the feed-on-demand strategy would need to exceed 50 mM.

The fixed-feed and the feed-on-demand strategies would be useful feeding regimes for biotransformations involving aldehydes of high water solubility (>1.0 M). Both systems would result in product concentrations at pH 7.0 greater than the maximum solubility of Li HPA at this pH.

One feature of the feeding regime that also needs to be considered is the effect of the mixing efficiency on the catalyst stability. The observation of protein precipitation at the site of feed addition during these small scale studies was a result of the inefficient mixing in the pH-stat reactors. The acidic and toxic nature of the feed necessitates a reactor of high mixing efficiency or the inclusion of a compartmentalised system where the site of feed addition is separate to the biocatalyst. Larger scale fed-batch reactors employing these types of feed may require multiple injection ports for feed/titrant addition.

The benefits of feeding both substrates in acidic form for the model biotransformation system have been clearly demonstrated for the model reaction.

2.8.4. Key parameters for investigation of new aldehyde substrates

Based on the reaction studies, the characteristics of *E. coli* transketolase catalysed biotransformations indicate the parameters and constraints which need to be considered regarding the general synthetic process. This is the first and most critical step in the rational choice of reaction conditions and reactor configuration for other transketolase catalysed syntheses, based on minimum additional data acquisition. In order to determine the more efficient reactor configurations for transketolase catalysis of HPA with useful aldehyde substrates, the number of additional investigations required can be reduced to four key areas as indicated by the constraints identified:

- Alkaline stability of aldehyde and product.
- Solubility of aldehyde and the effect of temperature and pH on solubility.
- Stability of the holo-transketolase in the presence of aldehyde substrate and the effect of aldehyde concentration.
- Kinetic constants. (The potential for product and substrate inhibition and the Km value for the aldehyde).

Since many of the most useful aldehydes are significantly less polar than glycolaldehyde, these conclusions from the structured approach require validation. A system more representative of synthetically useful reactions was therefore studied in relation to these four key areas .

3. VALIDATION SYSTEM

3.1. Introduction

As discussed in section 2.8, the structured approach applied to the transketolase system provided a useful tool for defining the enzyme characteristics. The model system is the simplest type of transketolase reaction but there are several possible levels of complexity depending on the properties of the ketol donor and the aldehyde. This is illustrated in Table 3.1. The achiral aldehyde substrate and the product of the model reaction (type 1) were not characteristic of the majority of aldehydes and products of commercial interest. The reactions of commercial interest are likely to be of type 2 or 4 utilising the enantiospecific properties of transketolase for reaction of racemic aldehydes with HPA (favourable equilibrium). Type 5 and 6 reactions are unlikely to be of industrial interest due to reversibility of reaction (as discussed in section 4.1). The key reactant and product specific parameters proposed in section 2.8 were therefore investigated for a type 4 transketolase catalysed reaction. This aldehyde was a poorly water soluble racemic aldehyde (i.e. significantly different chemical and physical properties to glycolaldehyde). This offered an extreme but ideal system to validate both the choice of the key reaction-specific parameters for transketolase-mediated catalysis and the structured approach in general.

The validation reaction consisted of the irreversible transfer of a two carbon group from HPA to 2RS, 3-O-benzylglyceraldehyde (3-OBG) to generate 5-O-benzyl-D-xylulose (5-OBX) and carbon dioxide. As discussed in section 1.4, the enzyme is specific for the R-enantiomer generating the product in the 3S, 4R configuration. However, the 3-OBG was supplied as a racemate of (2R) and (2S) enantiomers. The transketolase catalysed reaction utilising racemic 3-OBG is shown in Figure 3.1.

System characteristics	Type 1 e.g. model system	Type 2	Type 3	Type 4 e.g. validation system	Type 5	Type 6
Ketol donor:	HPA	HPA	HPA	HPA	alternative	alternative
Aldehyde solubility: (in water)	high	high	low	low	high	low
Aldehyde chirality:	homo/ achiral	racemic	homo/ achiral	racemic	racemic or homo/achiral	racemic or homo/achiral

Table 3.1. Transketolase reactions grouped according to the properties of the reaction substrates

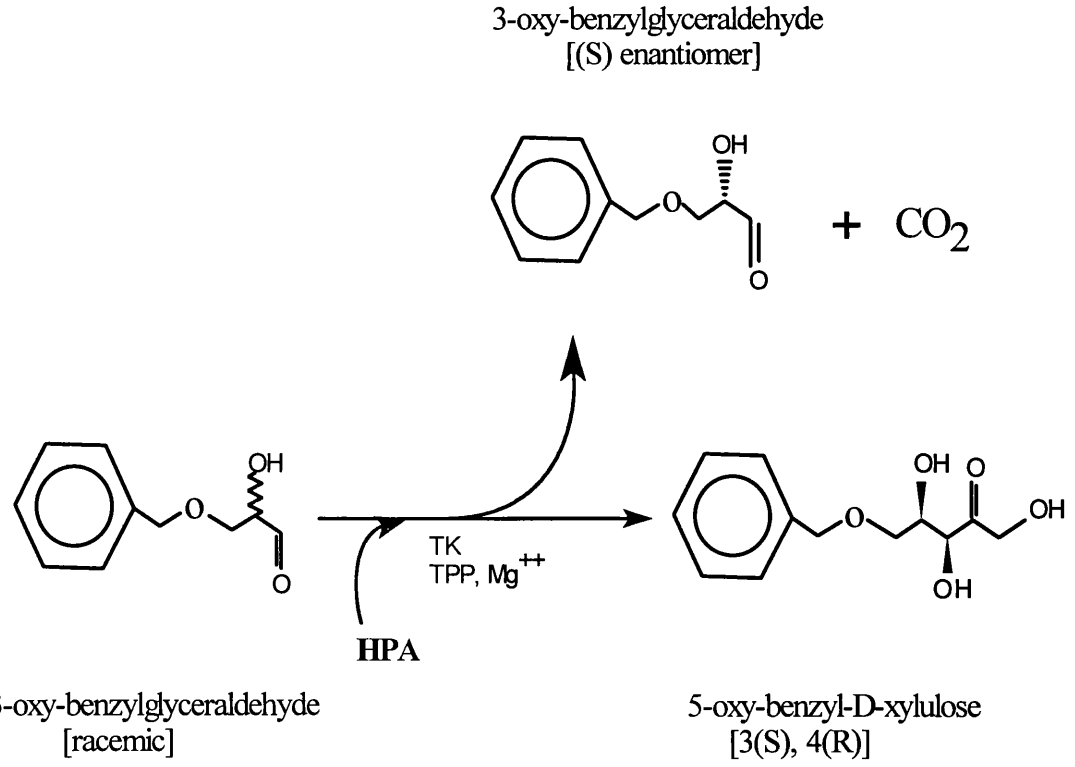


Figure 3.1. Validation reaction incorporating racemic 3-OBG as aldol acceptor.

The validation reaction product, 5-OBX, is one of two transketolase reaction products of potential as chiral synthons for the imino sugar nectrisine (Sawden and Turner, 1993). Indeed, the formal synthesis of nectrisine from 5-OBX has recently been reported (Humphrey, 1997). Nectrisine was first isolated in 1988 from the fungus *Nectria lucida* and shown to have potent activity as an immunoactivator and glycosidase inhibitor (Hashimoto *et al.*, 1988). Therefore, in addition to presenting a strenuous validation of the constraints defined from the model system, the validation reaction also provided useful starting material for investigation of the synthesis of a pharmaceutically active compound (Morris *et al.*, 1996; Humphrey, 1997).

3.2. Materials and Methods

3.2.1. Solvents

All organic solvents for analytical and product purification were of HPLC grade (Sigma Chemical Company) and were stored over 4Å molecular sieve as a standard storage precaution.

3.2.2. Substrates

3.2.2.1. Supply of substrates for validation reaction

3-OBG was kindly synthesised and supplied as the stable diethylacetal form by Dr Mark Smith (University of Edinburgh) (Smith *et al.*, 1996). The 3-OBG diethylacetal existed as a clear viscous liquid the purity of which was pre-determined to be >90 % by ^{1}H NMR. The compound was stored under anhydrous conditions at

4°C. The deprotection of the compound (3.2.3) was performed prior to biotransformation.

All other biotransformation components were obtained as described in 2.2.1

3.2.2.2. Purification of 3-OBG diethylacetal

Further purification of the 3-OBG diethylacetal was performed by the procedure utilised for separation of 5-OBX from residual aldehyde after biotransformation (3.2.9). Fractions containing the diethylacetal as monitored by thin layer chromatography (3.2.10.1), were pooled and residual solvent removed by evaporation under vacuum. This purified version of the 3-OBG diethylacetal was used to produce high purity 3-OBG (3.2.3) and high purity 5-OBX (by downstream purification (3.2.7) after biotransformation (3.2.6.1). These compounds were used to calibrate the HPLC assay (3.2.10.2). The characterisation studies did not utilise the purified forms of 3-OBG and 5-OBX as the supplied diethylacetal (and subsequently derived compounds) was considered sufficiently pure (>90% by ^{1}H NMR) for characterisation purposes.

3.2.3. Deprotection procedure for 3-OBG

The 3-OBG substrate was prepared *in-situ* in a pH-stat (2.2.1.6) by acid catalysed hydrolysis of the diethylacetal. The deprotection was performed in 0.1 M HCl (pH ~1.0) while vigorously stirring at 30°C and was complete in approximately 24 hours. The progress of the deprotection was followed qualitatively by TLC (3.2.10.1) or quantitatively by HPLC (3.2.10.2.1). The utilisation of lower pH values for the deprotection procedure proved problematic as this resulted in the formation of 3-OBG crystals which proved difficult to re-solubilise.

3.2.4. Conversion of 3-OBG to the diethylacetal form

3-OBG in organic solvent was recovered from the downstream purification step (3.2.9) following validation reaction. The solvent was removed under vacuum and the 3-OBG immediately dissolved in ethanol (50 volumes). Dry, beaded Amberlyst® 15 H^+ exchange resin (Sigma Chemical Company) was added to approximately 50% v/v and gently agitated at 25°C for 24 hours. Conversion to the diethylacetal form was confirmed by HPLC (3.2.10.2.1). The mixture was filtered and neutralised by addition of Amberlite® OH^-exchange resin (Sigma Chemical Company) to approximately 50% v/v (the resin having previously been rinsed with 1.0 M LiOH and then water). The mixture was gently agitated for 1 hour at 25°C, the resin removed by filtration, and the diethylacetal concentrated under vacuum to yield a clear oil.

3.2.5. Solubility studies

The solubility studies were conducted in a temperature controlled pH-stat apparatus (2.2.1.6). Solubility studies were conducted in water at 25°C and at 30°C (3-OBG only) at a range of different pH. The 3-OBG deprotection liquor (3.2.3) in 0.1 M HCl (pH 1.0), was the starting point for the study on the aldehyde. The pH was raised in steps of 1.0 pH unit using 5.0 M NaOH, held at the desired pH for 30 minutes and a single 0.5 ml sample removed. This was immediately filtered through an inorganic 0.45 μm syringe filter and assayed for 3-OBG (3.2.10.2.2). The product solubility study was initiated at pH 3.0 and was performed by adding product to excess (visible as a layer on the surface and as discreet droplets) and waiting 30 minutes prior to removing a sample, filtering and assaying for 5-OBX (3.2.10.2.2). This was repeated as the pH was increased in unit steps up to pH 8.0, additional product added as required. The solubility of the 5-OBX was then monitored as the pH was decreased stepwise back down to pH 3.0.

3.2.6. Effect of pH on substrates

The study was conducted in a pH-stat (2.2.1.6) at 25°C and 30°C with pH adjustment and maintenance using 1.0 M NaOH or 1.0 M HCl as appropriate. The studies were conducted at 40 mM for both 3-OBG and 5-OBX individually in the range pH 6.0 - 9.0. Rates of degradation of 100 mM 5-OBX at 25°C were also investigated. The bulk liquid volumes were loaded with 3-OBG and 5-OBX at the desired pH (concentrations checked by HPLC (3.2.10.2.2)). For 3-OBG, the deprotection liquor (3.2.3) was adjusted to the desired pH, filtered through a 0.45 μm inorganic syringe filter and suitably diluted. The solutions were incubated with agitation over 6 hours, samples removed at hourly intervals and the reaction components measured by HPLC (3.2.10.2.2).

3.2.7. Determination of 5-OBX (product) inhibition

The investigation was conducted in pH-stat apparatus (2.2.1.6) by initial rate measurements of the model biotransformation (2.6.5.2) in the presence of different added 5-OBX concentrations. The initial model substrates level was 100 mM and the reaction was maintained at 25°C, pH 7.0 in the presence of 2 mM 2-mercaptoethanol. Transketolase was added in pre-activated form (2.2.2.9) to 2.5 U/ml and the rate monitored at 5.0 minute intervals against a control containing no added 5-OBX. The samples were assayed using the Aminex® HPLC system (2.2.2.2) to give rates of HPA utilisation.

3.2.8. Batch Biotransformation

3.2.8.1. Analytical scale biotransformation run

The 3-OBG biotransformation studies were carried out in a pH-stat (2.2.1.6) but fitted with a larger volume reactor vessel allowing working volumes up to 50 ml. The deprotected aldehyde solution (3.2.3) was carefully adjusted to pH 7.0 using 5.0 M NaOH and the temperature adjusted to the desired value for the biotransformation. Any precipitate were removed by filtration through a 0.45 μm inorganic filter. This did not significantly affect the 3-OBG concentration in solution. For concentrations of 3-OBG lower than the limit of solubility, the saturated solution was diluted to the required concentration with water. $MgCl_2$ and TPP were then added to 0.9 and 2.4 mM concentration respectively followed by the required amount of solid HPA. After complete solubilisation, the solution was adjusted to pH 7.0 and 2-mercaptoethanol added to 2.0 mM. The reaction was started by addition of pre-activated transketolase solution (2.2.2.9). The pH was maintained during the biotransformation by automated addition of 1.0 M HCl.

3.2.8.2. Preparatory-scale batch biotransformation run

The deprotection was performed in a baffled glass fermentation vessel (LH Fermenters). 25g of the diethylacetal was added to 4.0 litres of 0.1 M HCl and the deprotection performed over 72 hours at 25°C with agitation at 200 r.p.m. The deprotection was monitored by HPLC (3.2.10.2.1) and the 3-OBG concentration was found to be sub-saturation negating the requirement for a filtration or further dilution step. The pH was adjusted to 7.0 by addition of 5.0 M NaOH and the cofactors added in solid form. The lithium salt of HPA was added in excess (~ 16 mM) ensuring the reaction was not limited by lack of the ketol donor. The pH was adjusted to 7.0 and the reaction initiated with pre-activated transketolase (2.2.2.9) to

a final level of 2.6 U/ml. The 2-mercaptoethanol was not added to this run as the removal of this compound was not assured during the downstream processing of the product. The pH was controlled at 7.0 by addition of 1.0 M NaOH as required. 1.0 ml samples were removed and diluted in the respective HPLC mobile phase. Samples were diluted 1:25 for HPA analysis (2.2.2.2) and 1:250 for analysis of 3-OBG and 5-OBX (3.2.10.2.2). After the reaction was complete, the solution was evaporated onto 50 g silica and the product purified (3.2.9).

3.2.9. Downstream separation and purification of product

Separation of the 5-OBX from the residual aldehyde on analytical scale was achieved using flash chromatography on Merck silica gel 60 (230-400 mesh ASTM) (Sigma Chemical Company). The wet silica packed bed dimension was approximately 15 x 2 cm in primary elution solvent. Dry silica was added to the biotransformation liquor at a loading ratio of approximately 1:3 and evaporated to dryness. The dried silica was then loaded onto the surface of the wet column to a height of approximately 3 cm and the solvent level gently raised ensuring no air was trapped. The surface of the silica sample was protected with sand (sulphuric acid washed) to a height of 0.5 cm. The solvents for the elution of the 3-OBG and the 5-OBX were selected from TLC studies using different solvent polarities as shown in Table 3.2. Solvents that resulted in Rf values of 0.3 - 0.4 were used for selective elution from the column. The 3-OBG was eluted using petroleum ether/ethyl acetate at 60:40 under a slight nitrogen head pressure (to give flow rate of 5 - 8 column centimetres per minute). 5.0 ml fractions were collected and aldehyde content monitored by TLC (3.2.10.1). Once the aldehyde was eluted and the eluent was clean, the product was eluted with ethyl acetate / methanol at 90:10 at the same flow rate. The samples were monitored for product using TLC (3.2.10.1). The fractions containing the compound of interest were pooled and the solvent removed by evaporation under vacuum at 40°C.

3-OBG (aldehyde)			5-OBX (product)		
solvent		Rf value	solvent		Rf value
Ethyl acetate	Petroleum ether		Ethyl acetate	Methanol	
100	0	0.58	100	0	0.28
90	10	0.57	90	10	0.38
80	20	0.54	80	20	0.53
70	30	0.52	70	30	0.64
60	40	0.50	60	40	0.67
50	50	0.44	50	50	0.74
40	60	0.39	40	60	-
30	70	0.29	30	70	-
20	80	0.17	20	80	-

Table 3.2. Evaluation of selective elution solvent during flash chromatography purification.

The purification of the product from the preparatory scale biotransformation was kindly performed by Dr Mark Smith (University of Edinburgh) utilising identical conditions to the analytical scale purification but using high volume chromatography columns.

3.2.10. Analytical

3.2.10.1. Thin layer chromatography (TLC)

The qualitative TLC assay initially used for monitoring the deprotection and biotransformation was performed on Merck Silica Gel 60 coated glass plates (Sigma Chemical Company). The mobile phase was ethyl acetate giving observed Rf values for the 3-OBG diethylacetal and 3-OBG of approximately 0.8 and 0.6 respectively. The Rf value for the 5-OBX product in ethyl acetate was approximately 0.3.
The compounds were visualised using potassium permanganate stain and stored at room temperature in the dark. The stain contained 1% w/v $KMnO_4$, 5% w/v K_2CO_3 and 1% w/v NaOH in water. The compounds appeared as bright yellow spots on gentle heating.

3.2.10.2. HPLC Assay (see also section 3.3.4)

3.2.10.2.1. Assay for the 3-OBG diethylacetal

Samples were diluted in mobile phase to within the range 0.01 - 0.5 mM and assayed by reverse-phase isocratic HPLC using an Ultracarb® ODS (20) (250 x 4.6 mm) reverse-phase column (Phenomenex) at 25°C. The mobile phase contained methanol and sodium phosphate buffer (50 mM, pH 7.0) at a ratio of 55:45. The flow rate was

0.6 ml/minute and the peak detection was by UV absorbtion at 220 nm. The injection volume was 50 µl via a fixed loop. The HPLC was a Perkin Elmer system consisting of an ISS 100 autosampler, an LC 250 gradient pump operated in isocratic mode and an LC 90J UV monitor. The data capture system was Nelson Systems PC Integrator version 5.1 (Nelson Systems Inc.).
The retention time of the diethylacetal was approximately 43 minutes and the deprotection was monitored primarily by loss of the diethylacetal.

3.2.10.2.2. Assay for 3-OBG and 5-OBX

This assay system was identical to the assay for the diethylacetal (3.3.5.3.1) with the exception that the mobile phase component ratio for MeOH/ phosphate buffer was changed to 45:55. The retention times for the 5-OBX and 3-OBG were 13 and 20 minutes respectively.

3.3. Analytical method development

3.3.1. Introduction

There was a requirement to develop the analytical tools necessary to characterise the 3-OBG reaction system and to provide a means of monitoring the progress of both the deprotection reaction and the biotransformation. Previously, the deprotection had been monitored semi-quantitatively by determination of the end-point using TLC (3.2.10.1) and the biotransformation had been monitored solely by the requirement for titrant addition (Smith, 1996). However, the use of titrant addition as an end-point determination of the biotransformation is inaccurate as titrant would still be

required several hours after the reaction was complete due to the slow equilibration of the carbon dioxide in solution with the atmosphere (2.6.2).

3.3.2. Normal phase HPLC

3-OBG diethylacetal, 3-OBG and 5-OBX are poorly water soluble compounds and were therefore ideal candidates for extraction from aqueous into an organic phase and analysis by normal phase HPLC. This extractant ideally being the HPLC mobile phase.

The column chosen for the investigation was a general purpose Techsphere® silica 5 μm (250 x 4.6 mm from HPLC Technology Ltd.) using the manufacturers recommended starting mobile phase comprising:

Hexane	49.75 % v/v
Ethyl acetate	49.75 % v/v
Acetonitrile	0.5 % v/v

Due to strong ultraviolet (UV) absorbance by ethyl acetate, a component of the mobile phase, the detection was by change in refractive index. The flow rate was 1.0 ml/minute and the sample size was 20 μl using a fixed volume loop for greater accuracy.

The HPLC traces for the 3-OBG, 3-OBG diethylacetal and 5-OBX are shown in Figures 3.2, 3.3, and 3.4 showing peak retention times of 2.1, 2.6 and 7.3 minutes respectively. It is evident that a contaminating peak was present with a retention time of 2.3 minutes. This was not observed in the 3-OBG trace as the compound used for this assay was partially purified by using crystals isolated from the deprotection liquor. In order to confirm the identities of the 3-OBG and diethylacetal peaks, the deprotection was monitored. 1.0 ml samples were removed from a deprotection at 0 and 4 hours and adjusted to pH 7.0 with 1.0 M NaOH. The compounds were extracted into 1.0 ml mobile phase by mixing the two phases for 1.0 hour at 25°C and isolating the organic phase. The HPLC traces for these samples

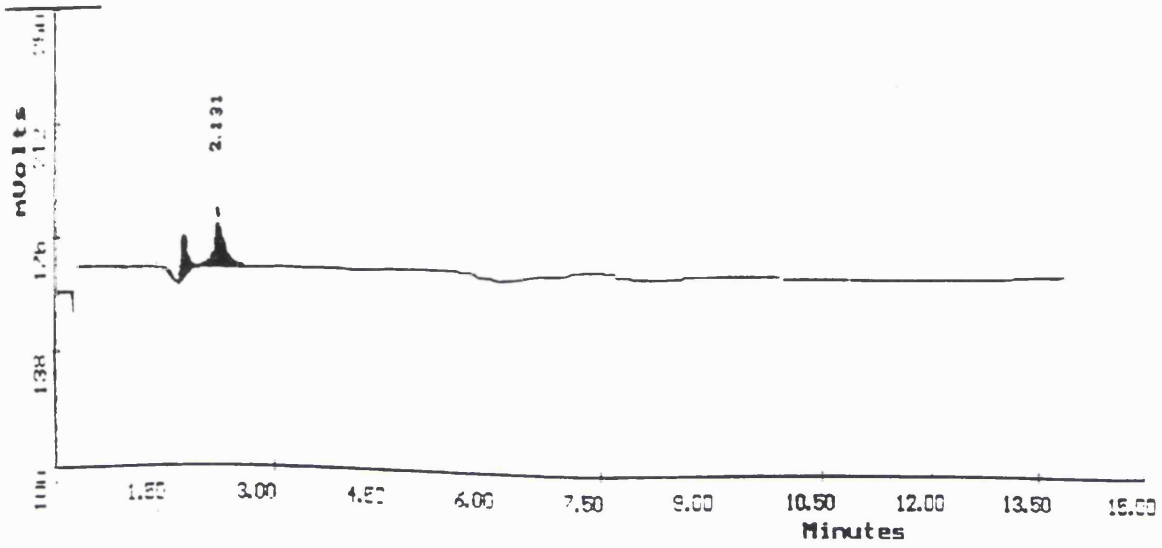


Figure 3.2. HPLC trace for 3-OBG using a Techsphere® silica 5 µm normal-phase column. Peaks were detected by U V absorbance

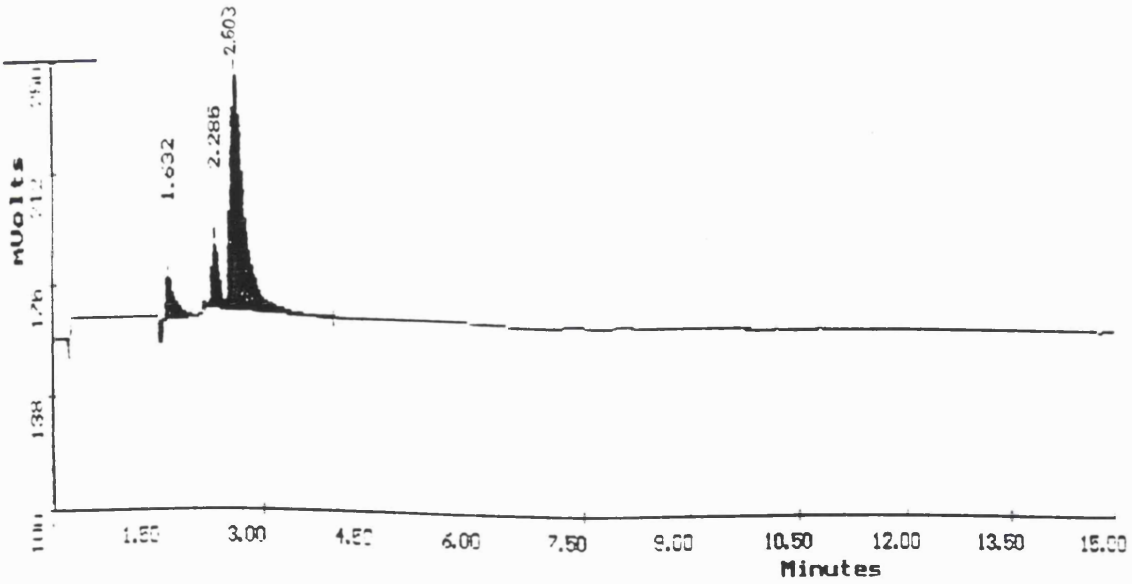


Figure 3.3. HPLC trace for 3-OBG diethylacetal using a Techsphere® silica 5 µm normal-phase column. Peaks were detected by U V absorbance

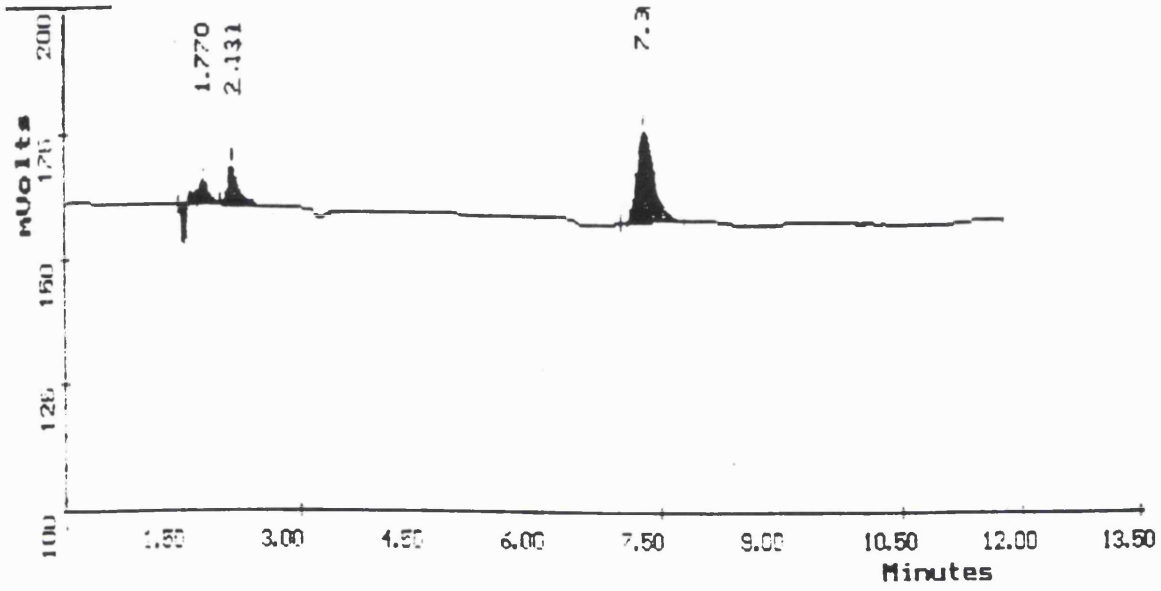


Figure 3.4. HPLC trace for 5-OBX using a Techsphere® silica 5 µm normal-phase column. Peaks were detected by U.V absorbance

are shown in Figures 3.5. The attribution of the peak identities was correct. However, the solvent extraction step also gave rise to partitioning of water into the extractant resulting in a broad trough at the point of product elution (Figure 3.6). Attempts were made to remove the water by the addition of excess anhydrous magnesium sulphate (to 10% w/v) to the extracted sample and mixing for 1.0 hour at room temperature. However, although there was some improvement, significant interference was still observed. Therefore, use of normal phase HPLC was suspended while other methodologies were investigated.

3.3.3. Gas chromatography

Due to the problems encountered with water in the samples for the normal-phase HPLC system, a suitable alternative was considered to be gas chromatography (GC). Water and organic solvents in the sample were not a consideration as these compounds are volatile.

The GC was a Shimazu system (Dyas Instruments Ltd.) comprising a GC 14A with an AOC 14 autoinjector and an AOC 1400 automated sampler. The initial column for these studies was a fused silica capillary column (30 m x 3.2 mm i.d.) with a CP-sil 5CB-MS coating (Chrompak). Initial conditions were set to determine the volatilities and relative retention times of the substrates without any method optimisation. The initial conditions were as shown in Table 3.3 for a broad temperature range of 40°C to 270°C at 5°C per minute. The 3-OBG diethylacetal peak was identified at 27 minutes and a contaminant peak at 8 minutes as shown in Figure 3.7. However, no identifiable peaks were obtained for either the 3-OBG or the 5-OBX other than a similar contaminant peak at 8 minutes. This indicated the requirement for a derivatisation procedure to increase the volatilities of the 3-OBG and 5-OBX. The most applicable derivatisation method for both these compounds was to make the trimethylsilyl derivative by reaction with N, O-bis trimethylsilyl acetamide (Pierce, 1968). This silyl compound reacts with hydroxyl groups. Pre-treatment of the aldehyde and ketone with hydroxylamine can also be performed to

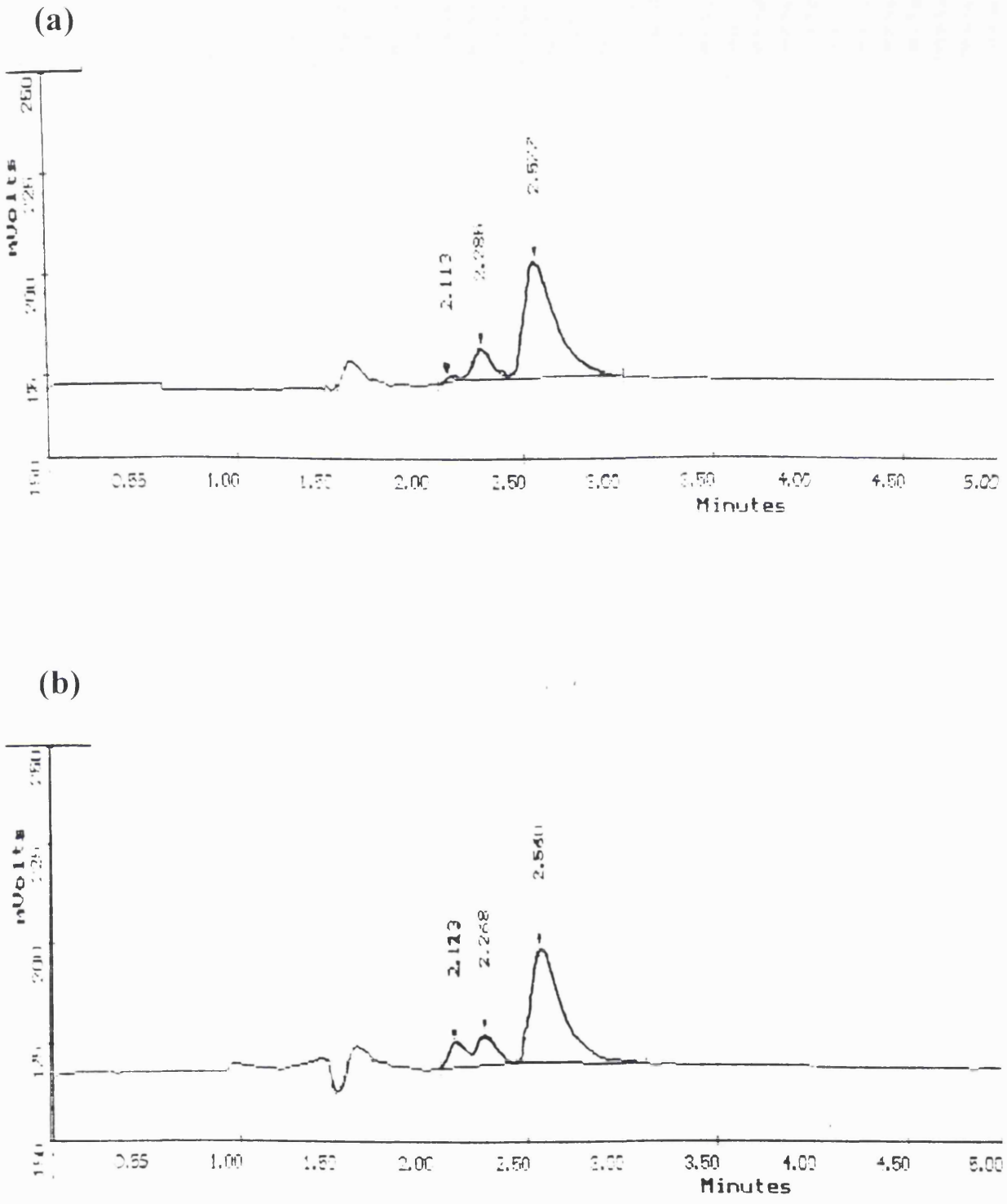


Figure 3.5. Normal-phase HPLC traces during the deprotection of 3-OBG. The traces are illustrated for deprotection at time 0 hours (a) and after time 4 hours (b).

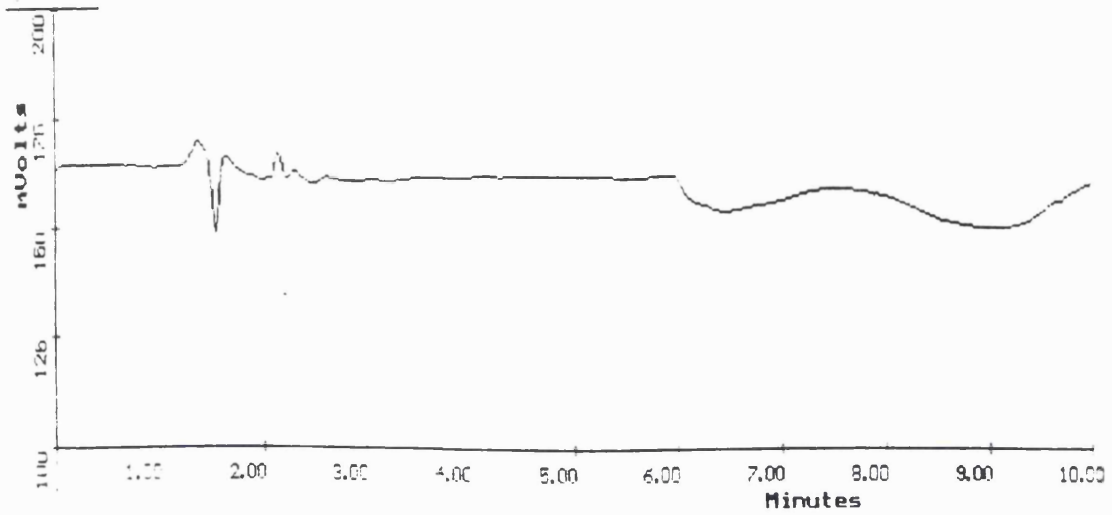


Figure 3.6. The effect of water contamination on normal-phase HPLC analysis after solvent extraction of a 3-OBG deprotection from aqueous into HPLC mobile phase.

Column (see text):	capillary (CP-sil 5CB)
Column length:	30 m
Inside diameter:	0.32 mm
Film thickness:	0.25 μm
Carrier gas (flow rate):	Helium (40 ml/min)
Detector:	FID
Injector temperature:	270°C
Detector temperature:	300°C
Temp. profile:	40 to 270°C at 5°C/min
Sample concentration:	~ 0.1 mM in MeOH
Sample size:	1 μl split 1:50

Table 3.3. Method parameters for gas chromatography analysis.

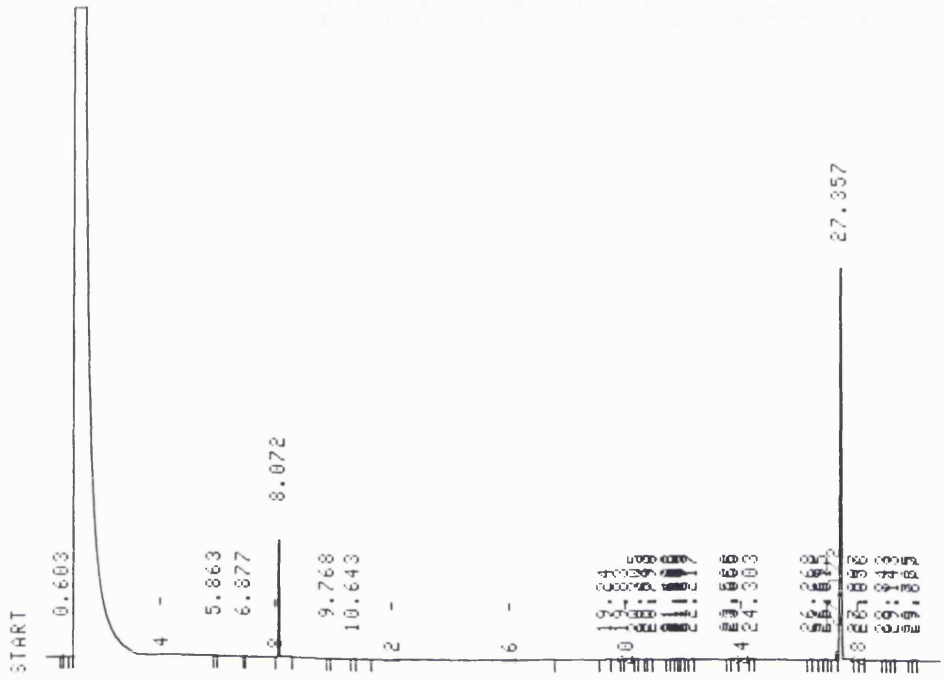


Figure 3.7. GC trace for 3-OBG diethylacetal using CP-sil 5CB capillary column.

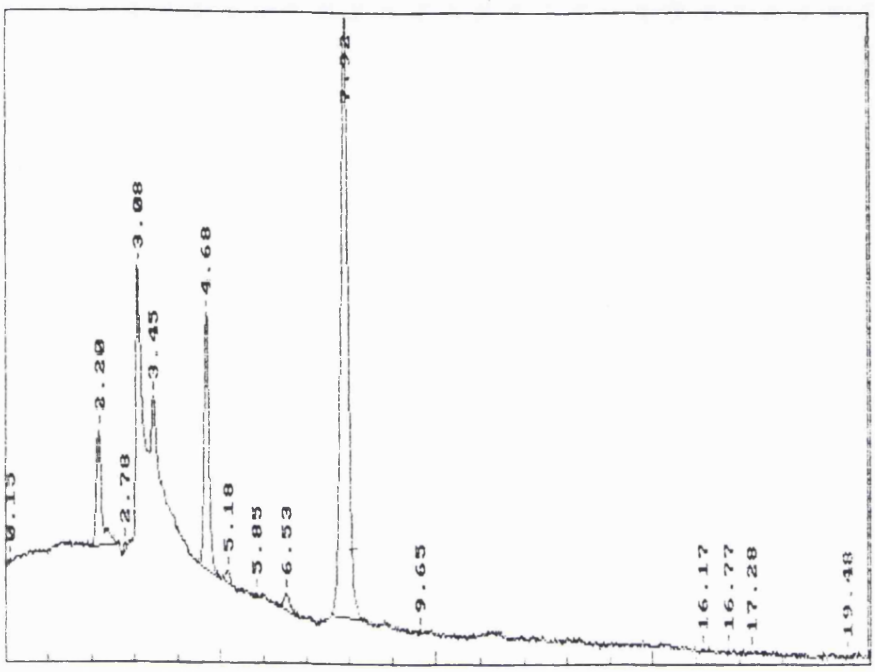
generate an oxime and therefore an extra available hydroxyl for derivatisation. Alternatively, the 3-OBG can be converted to the diethylacetal by incubation with ethanol and an appropriate H^+ exchange resin (3.2.4). This method could also be used to measure the enantiomeric excess (e.e.) of the remaining 3-OBG during the biotransformation by chiral GC (Humphrey, 1997). However, this is not necessarily a measure of the e.e. of the product (4.1.2). Unfortunately, due to time constraints, these derivatisation methods were not examined at this time and alternative methods were investigated.

3.3.4. Reverse phase HPLC

3.3.4.1. Development

Due to the presence of a UV absorbing benzyl group on all components of interest, the UV absorption spectra of 3-OBG and 5-OBX was measured. The wavelength generally utilised for detection of benzyl groups is 254 nm in order to minimise interference by other compounds. This was the wavelength of initial choice although, as a comparison, absorbtion at 220 nm was also investigated. The assay system utilised initially was isocratic HPLC using an Ultracarb® ODS (20) (250 x 4.6 mm) reverse-phase column (Phenomenex) at 25°C. The initial mobile phase for the study was based on the solvent utilised for analysis of benzyl compounds by reverse-phase HPLC (Wilkinson, 1996). This mobile phase consisted of 45% v/v acetonitrile, 0.1% v/v glacial acetic acid in water and the flow rate was 1.0 ml/minute. 3-OBG and 5-OBX were analysed by this method at the two wavelengths as shown in Figures 3.8 and 3.9 while the diethylacetal was assayed at 220 nm only (Figure 3.10). Comparing these traces it was evident that the peaks detected at 7.9 minutes (at 254 nm) and at 3.4 minutes (at both wavelengths) were contaminants. The peak at 4.7 minutes was also a possible contamination compound.

(a)



(b)

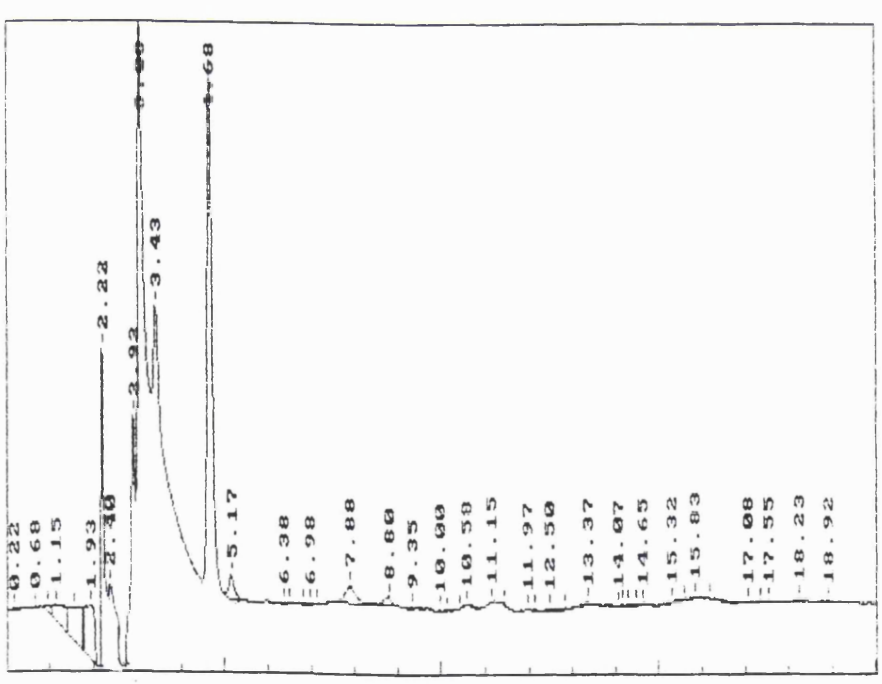
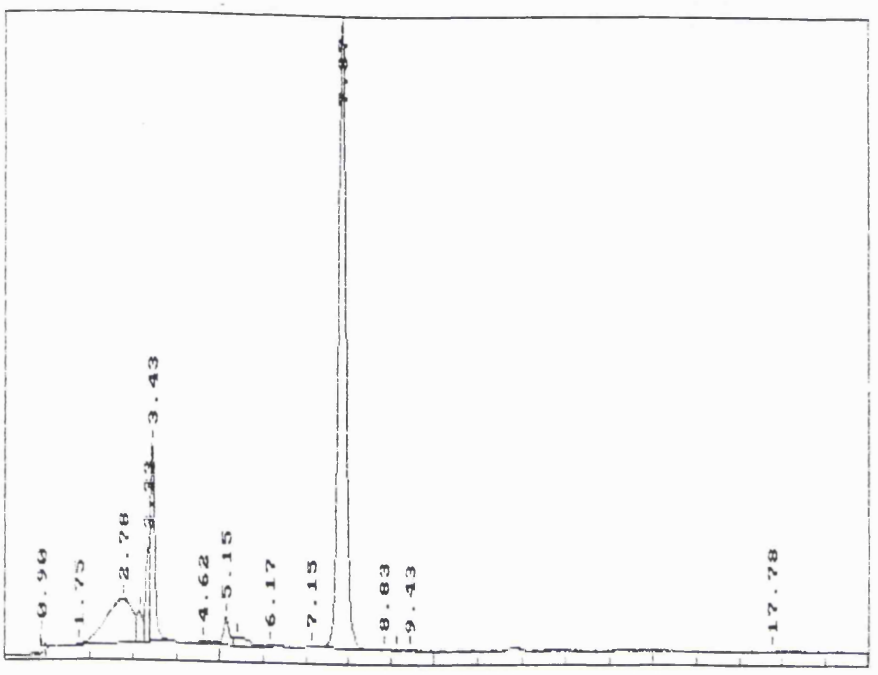


Figure 3.8. Reverse-phase HPLC traces for 3-OBG using the mobile phase utilised by Wilkinson, 1996. Peaks are illustrated for a detection wavelength of 254 nm (a) and 220 nm (b).

(a)



(b)

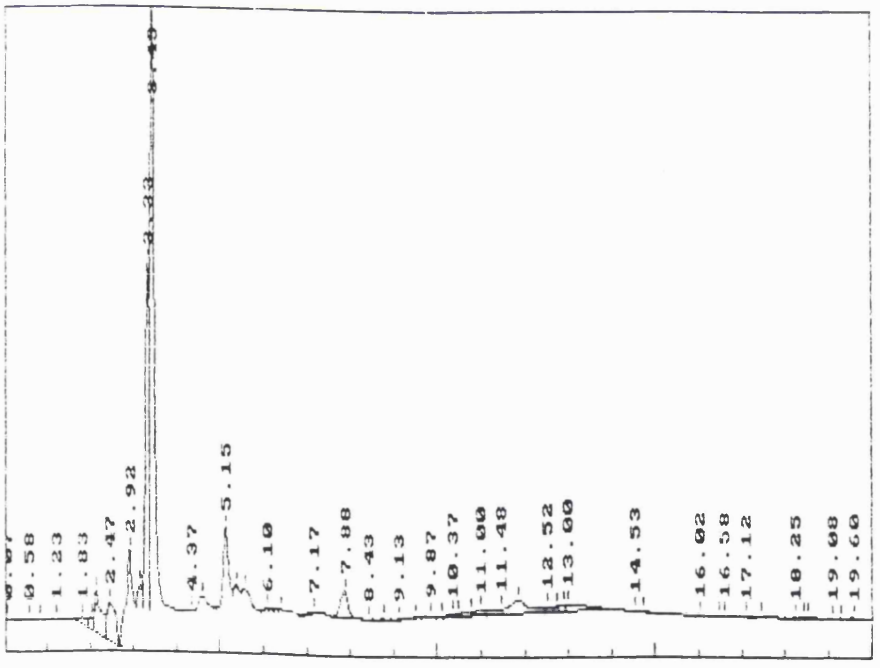


Figure 3.9. Reverse-phase HPLC traces for 5-OBX using the mobile phase utilised by Wilkinson, 1996. Peaks are illustrated for a detection wavelength of 254 nm (a) and 220 nm (b).

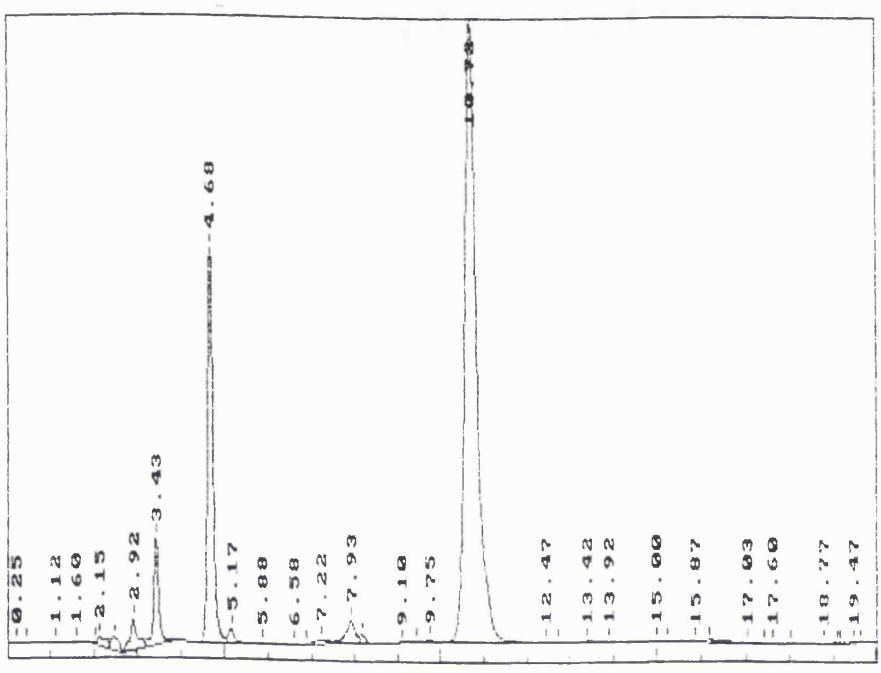


Figure 3.10. Reverse-phase HPLC traces for 3-OBG diethylacetal using the mobile phase utilised by Wilkinson (1996). Peaks are illustrated for a detection wavelength 220 nm.

It was difficult to assign compounds to peaks other than the diethylacetal (RT = 10.7 minutes) due to the number of contaminating and interfering peaks. However, due to the complete removal of the large contaminant peak at 7.9 minutes on reduction of the wavelength to 220 nm, it was decided to perform the next set of investigations at the lower wavelength. The initial peaks required separation by increasing the polarity of the mobile phase. However, even at 10% v/v acetonitrile the peaks were not separating fully. Therefore, a further increase in the polarity of the solvent was required necessitating a change in the components of the mobile phase.

Mobile phases for reverse phase HPLC often incorporate an alcohol component. Due to the reactive nature of aldehydes with alcohols at acidic pH, the use of alcohol based mobile phases was limited to near-neutral pH values. Therefore, incorporating an alcohol of low UV absorbance, such as methanol, into the mobile phase required the exclusion of the acetic acid component. In order to ensure the pH of the mobile phase remained near-neutral the aqueous component consisted of 50 mM phosphate buffer at pH 7.0. The 5-OBX, 3-OBG and 3-OBG diethylacetal were run isocratically in methanol/ phosphate buffer at a ratio of 1:1 and monitored at 220 nm. The traces are shown in Figure 3.11. The 5-OBX peak was identified at 7.6 minutes, the 3-OBG as the broad peak at 14 minutes and the diethylacetal at 72.3 minutes. Several contaminating peaks of various peak areas were observed at 4.3, 6.1, 7.6, 11.9, 16.1 and 64.9 minutes. Some slight interference was observed between the aldehyde and two of the contaminants and a further mobile phase modification was required.

The polarity of the mobile phase was increased by changing the ratio of the MeOH/ phosphate buffer to 45:55. The 3-OBG was assayed using this system and the peaks showed adequate separation. The system was suitable for monitoring the progress of a biotransformation (Figure 3.12). Single peaks for the 5-OBX (RT = 13.2 minutes) and the 3-OBG (RT = 20 minutes) were obtained.

Due to the isolated nature of the 3-OBG diethylacetal peak, adequate separation of this peak with minimal run time was attained using a less polar mobile phase of MeOH/ phosphate buffer ratio of 55:45. The retention time of the diethylacetal was 43 minutes and was a suitable assay for determination of the deprotection efficiency.

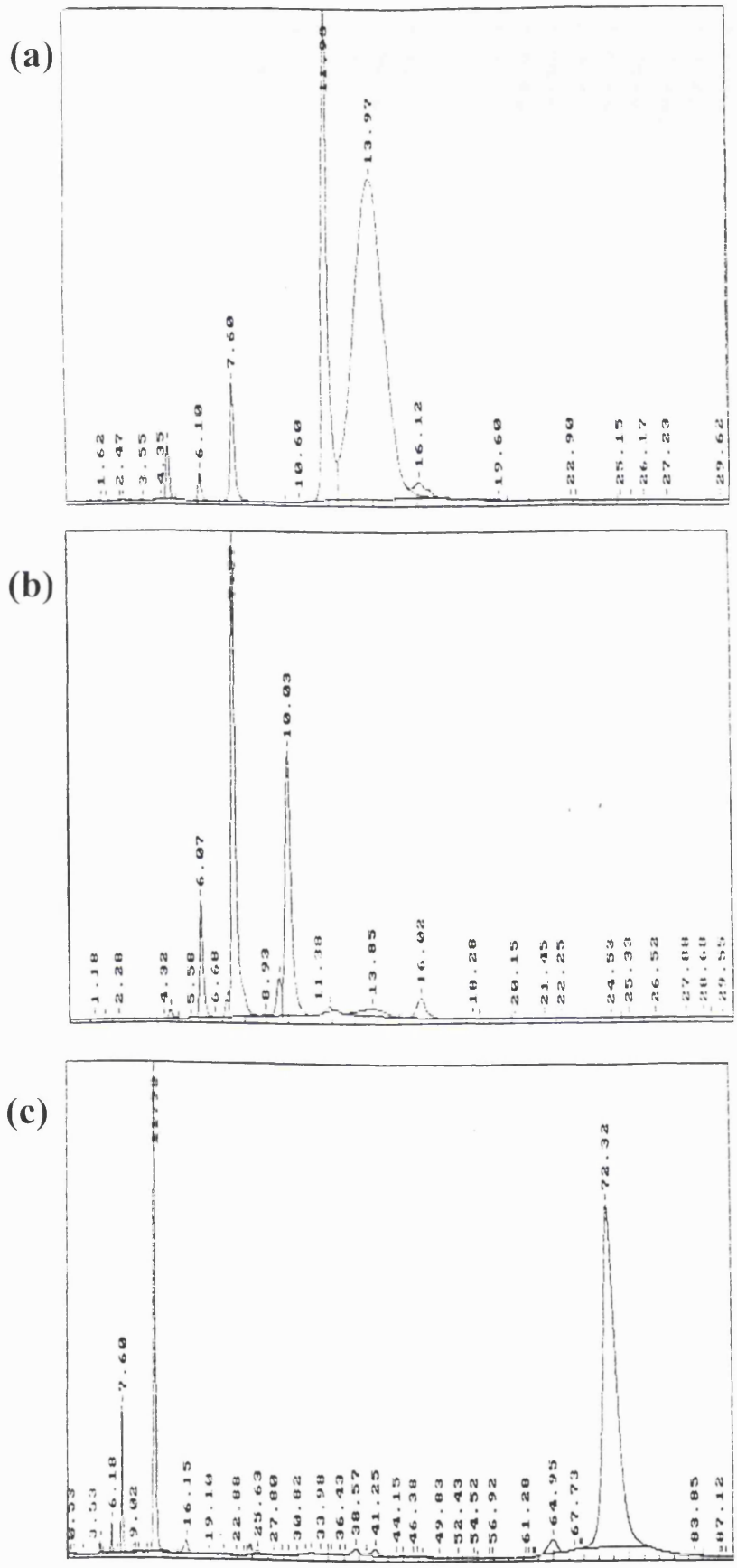
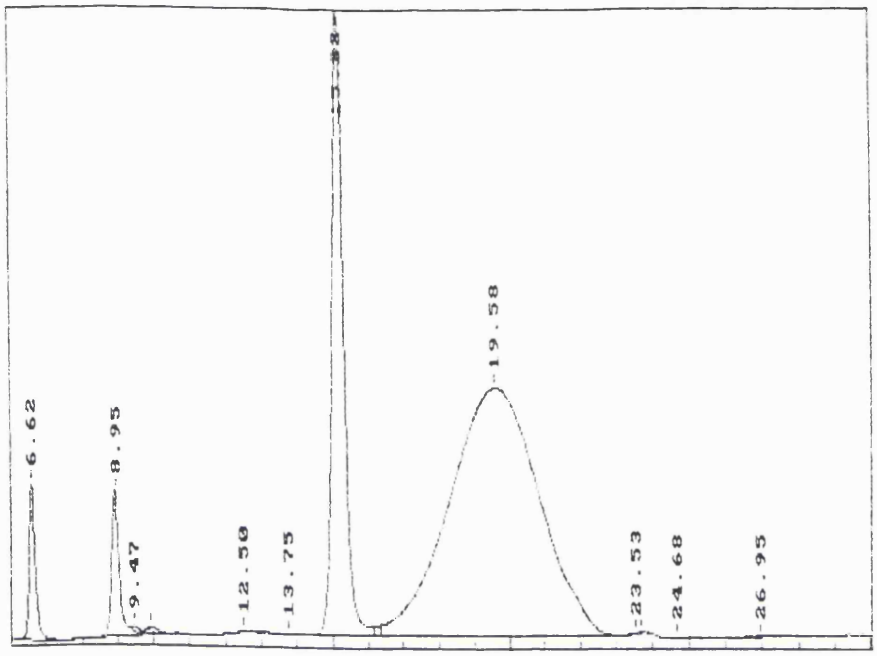


Figure 3.11. Reverse-phaseHPLC traces for (a) 3-OBG, (b) 5-OBX, and (c) 3-OBG diethylacetal utilising MeOH/phosphate buffer mobile phase at a volume ratio of 1:1. Peak detection was by absorbance at 220 nm.

(a)



(b)

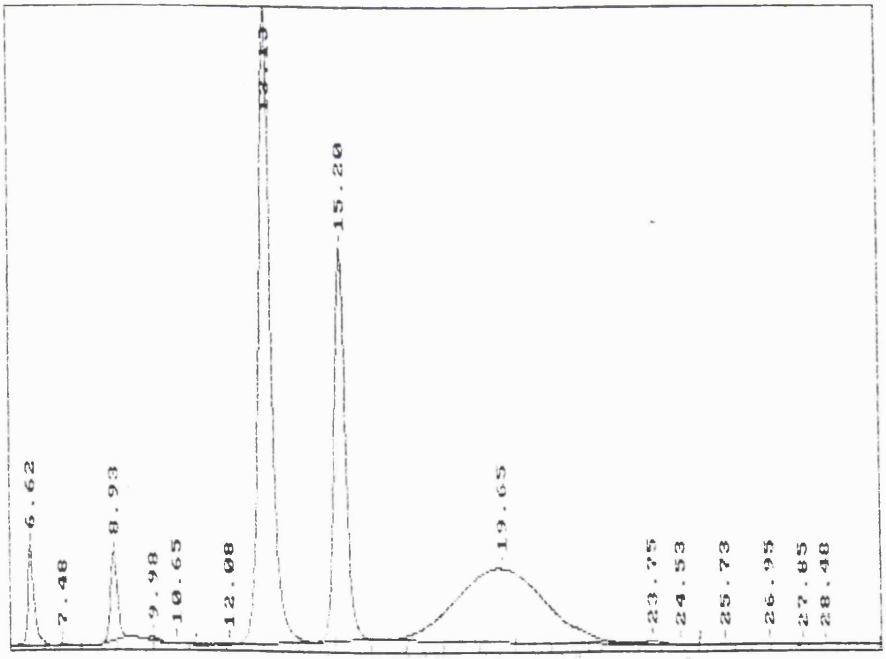


Figure 3.12. Reverse-phase HPLC of the validation biotransformation at (a) initiation and (b) completion of reaction. The HPLC mobile phase consisted of MeOH/phosphate buffer at a volume ratio of 45:55. Peak detection was by absorbance at 220 nm.

3.3.4.2. Reproducibility

The reverse-phase system was calibrated and curves generated for the 3-OBG and the 5-OBX shown in Appendix 5. The reproducibility of the system was also investigated and the expected error calculated over the range of the calibration. The errors differed between the two compounds due to the sharp defined nature of the 5-OBX peak and the broad nature of the 3-OBG peak. The integration of the broad aldehyde peak was prone to errors in baseline evaluation and therefore less accurate. The errors over the calibration range were calculated from five identical injections and are shown in Table 3.4. The errors are expressed as

$$\text{Standard error (+/- \%)} = \frac{\text{Standard deviation}}{\text{mean}} \times 100\%$$

Concentrations of 5-OBX of 0.05 mM or less were prone to significant errors due to an unstable baseline at this point but concentrations above this value exhibited high reproducibility. Therefore the dilutions of the 5-OBX for assay were calculated to give concentrations in the calibration range 0.1-2.0 mM. As expected for the 3-OBG the errors were generally higher due to broad peak integration errors. However, errors within the appropriate dilution range were considered acceptable for the purpose of characterising and monitoring the validation biotransformation.

	Concentration (mM)	Standard error (+/- %)
3-OBG	0	0
	0.16	6.2
	0.35	3.3
	0.76	6.8
	1.36	5.9
5-OBX	0	0
	0.05	12.2
	0.1	5.9
	0.2	4.8
	0.4	2.6
	0.6	1.2
	0.8	2.0
	1.0	2.7
	1.5	2.9
	2.0	3.9

Table 3.4. Errors calculated over the calibrated range of concentrations for the reverse-phase HPLC assay.

3.4. Results of validation studies

3.4.1. Component solubilities

While the solubility of the HPA had previously been evaluated (2.4.2) and found to be pH dependent, the maximum solubility of the model glycolaldehyde was not attained and any pH dependence therefore not evaluated. The solubility of the validation reaction components 3-OBG and 5-OBX was investigated in water over a range of pH values and temperatures (3.2.5) . The pH was adjusted stepwise from acidic to alkali conditions and, for 5-OBX only, the solubility was also monitored as the pH was returned to acidic values.

The profiles for the solubilities of the 3-OBG and 5-OBX are shown in Figure 3.13. At 25°C, the rapid increase in 5-OBX solubility observed above pH 5.0 was a solvation effect as confirmed by the maintenance of the solubility on subsequent reduction of the pH. The maximum concentration of 5-OBX at neutral pH was found to be approximately 114 mM at 25°C. As the pH was increased towards neutral, the solubility of the 3-OBG was slightly reduced reaching a soluble concentration of 43 mM at pH 7.0, 25°C. A 5°C increase in temperature resulted in a 22 % increase in 3-OBG solubility at pH 1.0 but was only a 10% increase within the biocatalytically useful pH range. However, at acidic pH and significantly higher temperatures than those investigated, the resulting increase in solubility of 3-OBG may be useful for a concentrated feed.

3.4.2. Effect of pH on substrates and products stability

The degradative effect of alkaline pH on HPA, glycolaldehyde and L-erythrulose stability was evident during the characterisation studies of the model reaction. Although the HPA is a common substrate to both the validation and model reactions, it was unknown whether the 3-OBG and 5-OBX lability was a characteristic that was

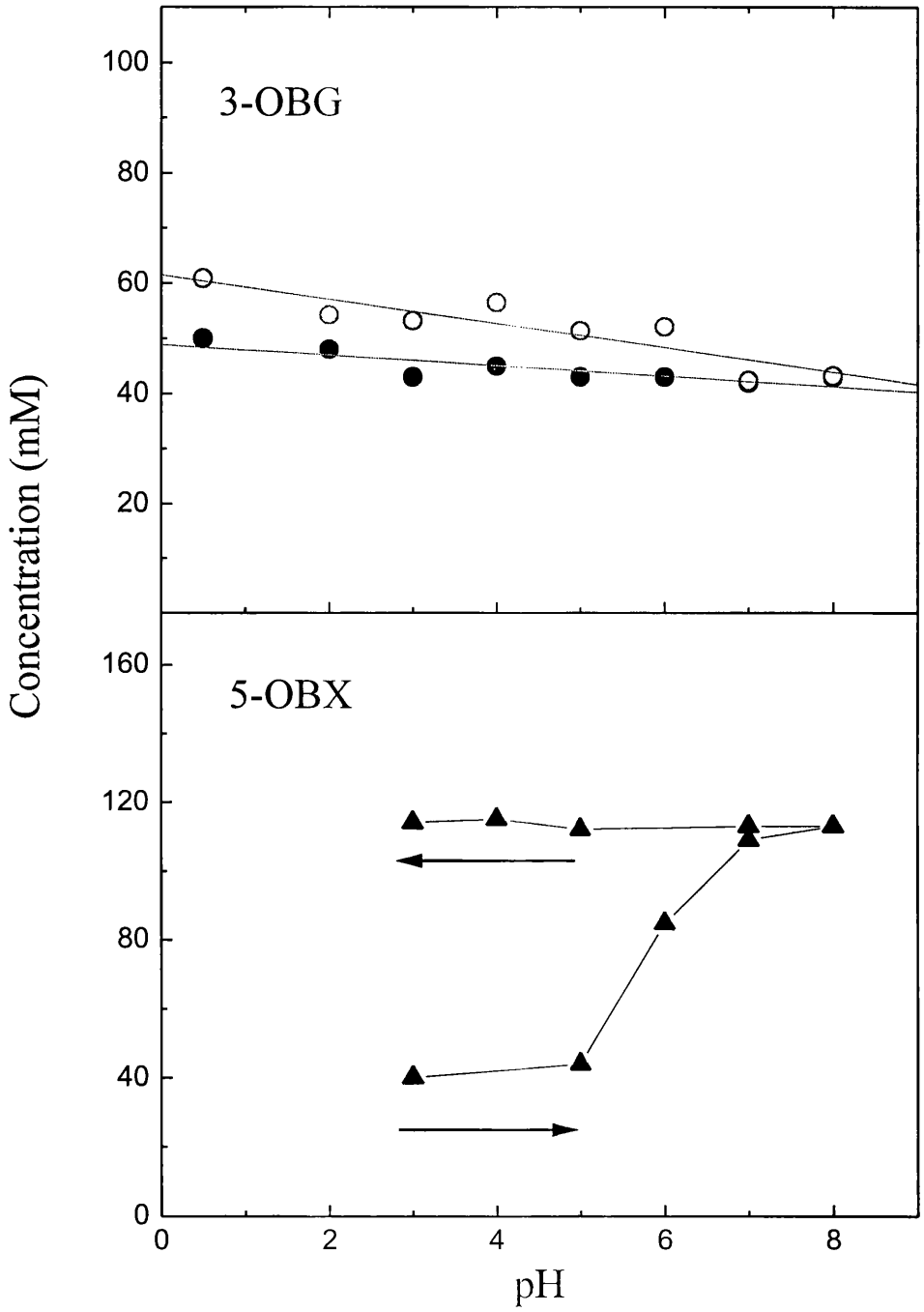


Figure 3.13. Aqueous solubility profiles for 3-OBG (●, ○) and 5-OBX (▲) at different pH. Profiles are illustrated for incubation at 25°C (●, ▲) and 30°C (○). The arrows on the 5-OBX profiles indicate the direction of pH adjustment during the study and illustrate a solvation or kinetic effect.

also maintained. Therefore the pH stability profile for 3-OBG and 5-OBX was studied at 40 mM over 6 hours over the range pH 6.0 - 9.0 (3.2.6). The degradation trends are summarised in Figures 3.14 for the 3-OBG and 5-OBX at two different temperatures.

The rate of degradation was a function of both pH and temperature. The rate of loss increased severalfold above pH 8.0 with no discernible loss at pH 6.0. The profiles follow similar trends to those observed for the model aldehyde and product suggesting a trait which may hold for all these types of transketolase substrates and products.

3.4.3. Effect of 3-OBG on transketolase

The toxicity of the glycolaldehyde model substrate on transketolase was previously observed to result in a 50% loss in activity over 3.5 and 60 hours at aldehyde concentrations of 500 and 50 mM respectively (2.6.1). Reaction of proteins with aldehyde groups is well known (Demuynck *et al.*, 1991) and this toxicity was not unexpected. However, the relative toxicity of 3-OBG required evaluation and was therefore investigated. The toxicity of 3-OBG to transketolase was investigated at a range of aldehyde concentrations up to 42 mM at pH 7.0 and 25°C in presence of cofactors and 2 mM 2-mercaptoethanol. The transketolase-rich lysate was added at a ratio of 1.0 ml lysate to 9.0 mls of (suitably diluted) aldehyde. The highest concentration of aldehyde was achieved by not removing the aldehyde haze post deprotection/neutralisation. The filtration step was only included after the addition of the enzyme thus allowing further solubilisation due to the dilution effect of the enzyme. The half-lives of the activity were calculated or extrapolated from the data obtained over 6 hours incubation and plotted against aldehyde concentration for the holo-enzyme (Figure 3.15).

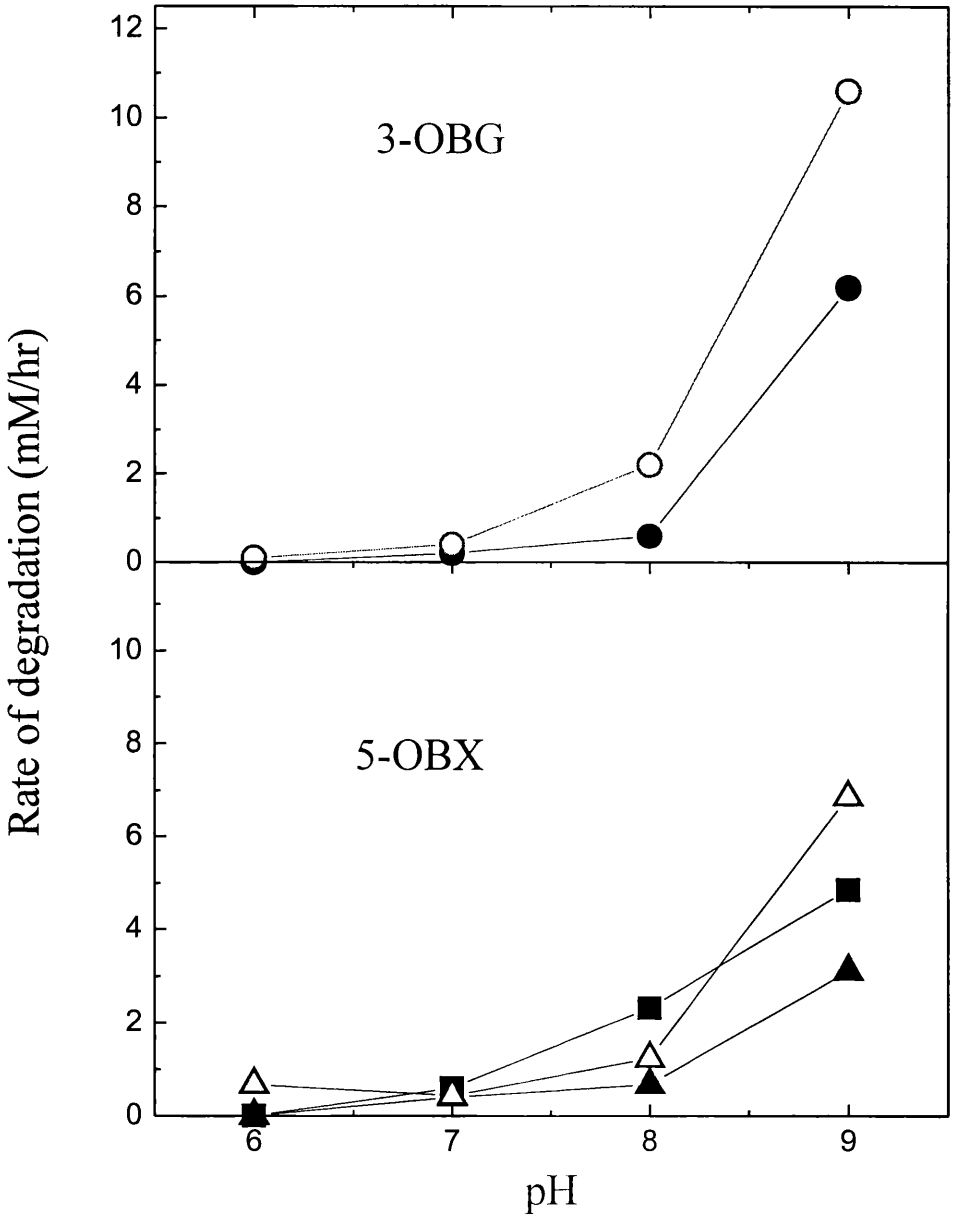


Figure 3.14. Effect of pH on the rates of degradation for 40 mM initial concentrations of 3-OBG (●, ○) and 5-OBX (▲, Δ) at 25°C (●, ▲) and 30°C (○, Δ). The profile for 100 mM 5-OBX at 25°C is also illustrated (■).

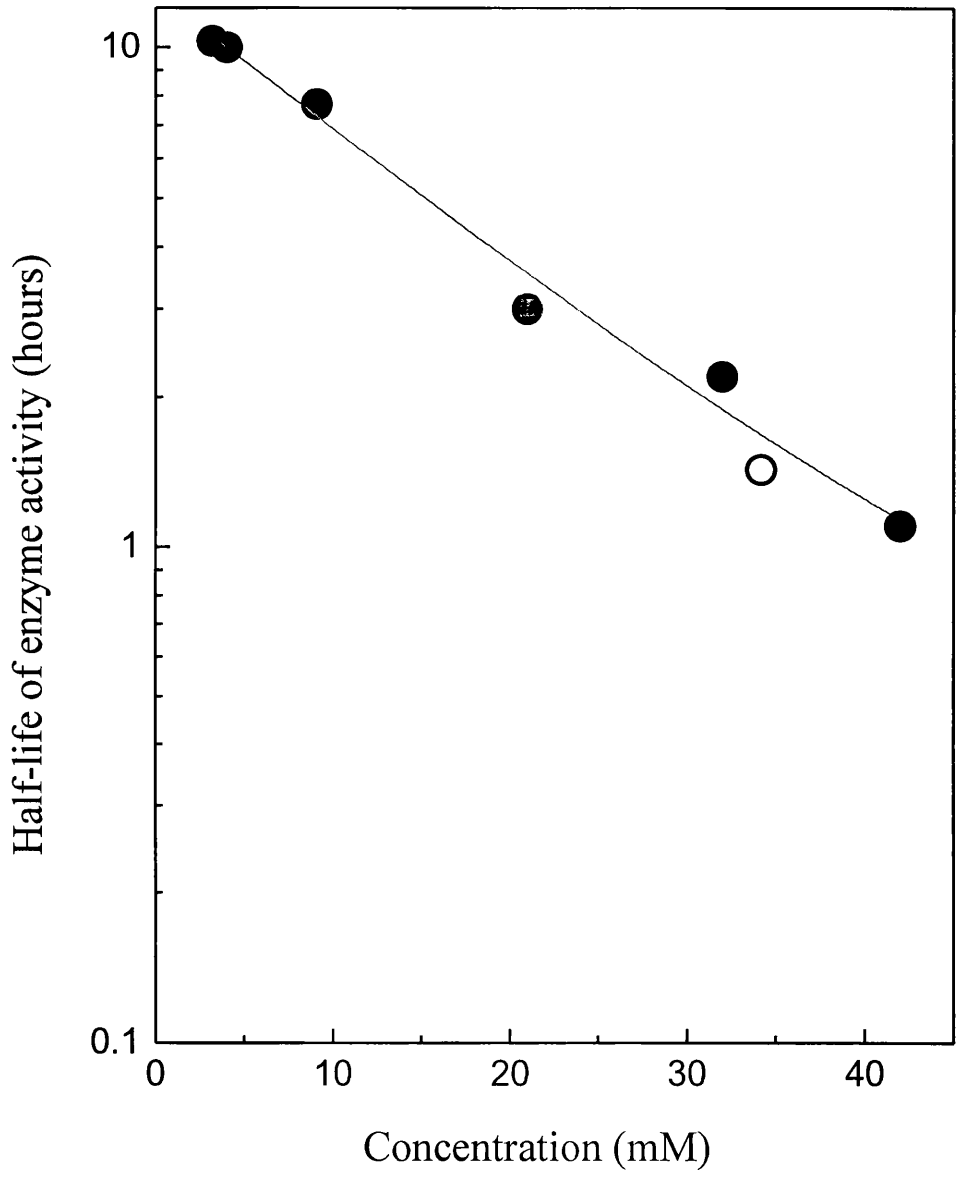


Figure 3.15. Effect of racemic 3-OBG concentration (●) on holo-transketolase stability at 25°C, pH 7.0 in absence of oxidation effects. Half-life values are based on loss of transketolase activity from an initial level of 23.7 U/ml during 6 hours incubation in the presence of 2 mM 2-mercaptoethanol. The effect of 34 mM (S)-rich 3-OBG (O) on holo-transketolase is also illustrated.

The toxic effect of 3-OBG was significantly greater than for the glycolaldehyde model substrate. Activity half-life values at 40 mM 3-OBG were approximately 1 hour compared to a projected 70 hours for the equivalent concentration of glycolaldehyde.

Increased enzyme toxicity of one enantiomer over the other was possible and required investigation. The remaining (S)-rich 3-OBG was recovered during downstream purification (3.2.9) of a completed validation biotransformation (3.2.8.1) and converted to the diethylacetal form (3.2.4) until required. Although not measured here, similarly recovered residual 3-OBG material was previously shown to have an e.e. of >72% in favour of the (S) enantiomer (Humphrey, 1997). The diethylacetal was deprotected using 0.1 M HCl and the neutralised 3-OBG used in the toxicity study. The effect on transketolase was investigated at a concentration of 34 mM and found to show similar toxicity to the same concentration of racemic 3-OBG (see Figure 3.15).

3.4.4. Kinetic studies

3.4.4.1. Evaluation of Km for (2R) 3-OBG

The Km values for HPA and glycolaldehyde were reported previously (Gyamerah and Willetts, 1996) using the HPLC assay (2.2.1.2.1) to measure initial rates of the model reaction (Mitra and Woodley, 1996). The Km values for HPA and glycolaldehyde were determined to be 13.2 mM and 16.1 mM respectively. The relatively high values were not a significant problem for reactor operation for the model system since the solubility of both these compounds is high, the toxicity relatively low and maximum reaction rates could therefore be achieved. The solubility of the 3-OBG, however, was found to be low but the toxicity was high and concentration related (3.4.3).

Therefore the Km value for (R) 3-OBG assumed greater importance and needed to be determined. Determination was based on the initial rates of the validation biotransformation (3.5.1), obtained at racemic 3-OBG concentrations up to 37 mM in the presence of 100 mM HPA (i.e. excess). The degree of conversion by pre-activated holo-transketolase (2.6.5) at 2.0 U/ml was measured over the initial 2.5 and 5.0 minutes by HPLC (3.2.10.2.2) and expressed as 5-OBX formed per minute. The comparative initial rates at different racemic 3-OBG concentrations are shown in Figure 3.16.

Superimposition of reaction rate data for 2.5 and 5.0 minutes confirmed a linear reaction at racemic 3-OBG concentrations up to 25 mM over the entire 5.0 minutes. However, reaction rates during 5.0 minutes incubation at the high 3-OBG concentrations were found to level off, a result of either achievement of Vmax or the effect of toxicity and/or substrate limitation. However, the reaction rate measured over the initial 2.5 minutes was still increasing linearly with increasing racemate concentrations up to 37 mM thus indicating that Vmax had not been achieved. Since racemic 3-OBG consists of 50% (R) enantiomer (Humphrey, 1997), then the concentration of the (R) component at 37 mM racemate concentration was 18.5 mM. The linearity of the activity/concentration profile observed in this study illustrates that the Km for (2R) 3-OBG was probably greater than 18.5 mM but the exact value was not determinable. The net result of this observation is that reduction of the toxic effect on the enzyme by restriction of the racemic 3-OBG concentrations will result in significantly reduced reaction rates. Higher enzyme levels would therefore be required to maintain productivity (per unit time).

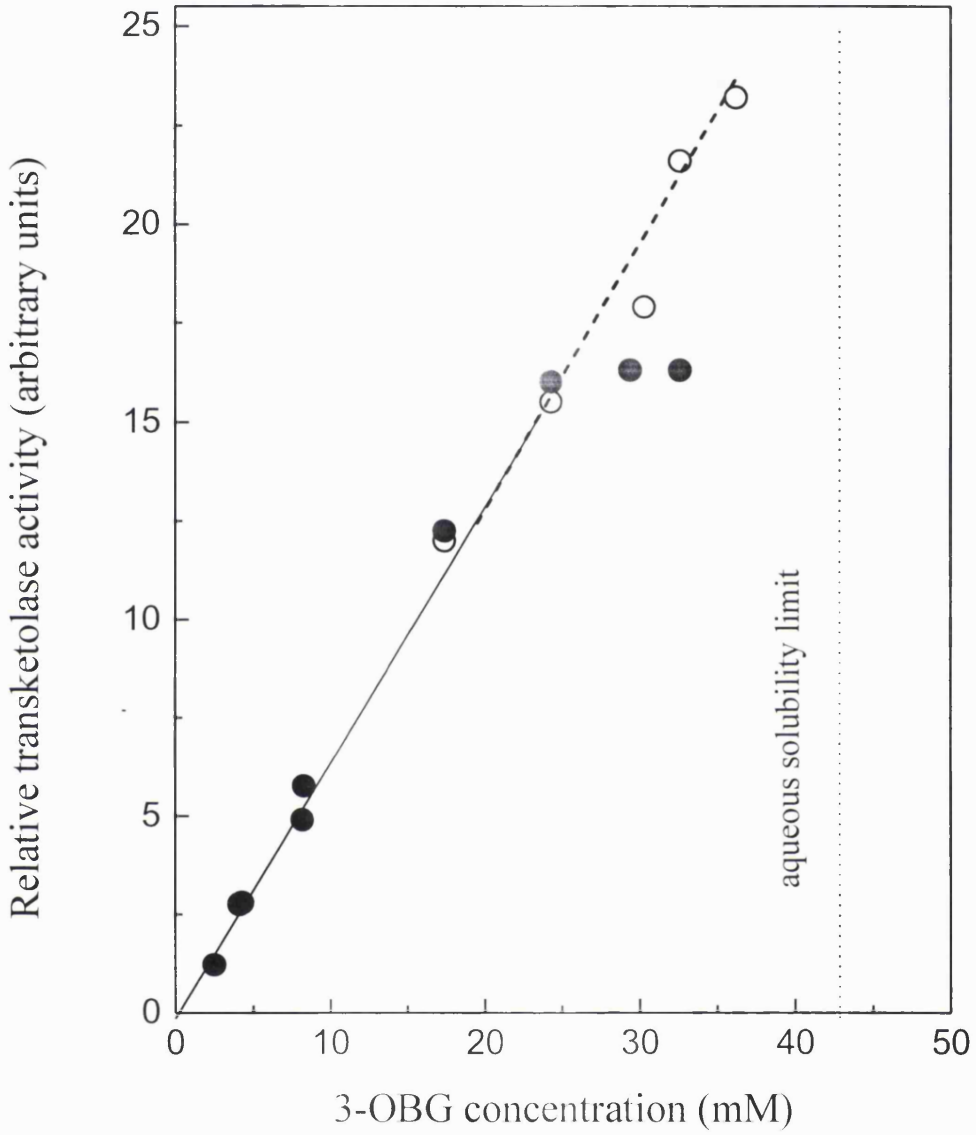


Figure 3.16. Effect of 3-OBG concentration on rate of transketolase reaction. Reaction rates were calculated in terms of HPA utilisation per minute based on measurement after the initial 2.5 minutes (○) and after the initial 5.0 minutes (●) of the validation biotransformation at pH 7.0, 25°C. The initial transketolase level was 2.0 U/ml (pre-activated).

3.4.4.2. Evaluation of product inhibition effects

The presence of inhibition by the model reaction product, L-erythrulose, on transketolase has been reported (Gyamerah and Willetts) although inhibition levels were low (Ki ~ 570 mM) and not a significant problem. However, the significance of product inhibition needs to be evaluated for the validation system. The effect of 5-OBX on the rate of reaction of the model biotransformation was measured at pH 7.0, 25°C for 2.5 U/ml holo-transketolase at 5-OBX concentrations up to 98 mM (3.2.7).

Table 3.5 illustrates the relative rates observed compared to control containing no 5-OBX. The results are based on comparison of the mean rate of HPA utilisation (mM per minute) over both the first 5.0 minutes and the first 15.0 minutes of reaction. Minor product inhibition was observed above 81 mM 5-OBX with rates reduced by nearly 20 % at concentrations approaching 100 mM.

5-OBX concentration (mM)	**Relative reaction rate (% of control)**	
	Initial 5 minutes	**Initial 15 minutes**
0 (control)	100	100
58	99.5	104
81	97.4	95.8
98	84.2	82.3

Table 3.5. Effect of 5-OBX concentration on initial rate of the model reaction.

It is possible that the 5-OBX may have competed with HPA as a ketol donor for reaction with glycolaldehyde thereby producing the observed effect. Measurement of loss of glycolaldehyde or production of L-erythrulose in addition to HPA utilisation would have resolved this but this data was not collected.

3.5. Batch Biotransformation

3.5.1. Analytical scale synthesis of 5-OBX

Batch biotransformations were performed in order to generate 5-OBX product for characterisation studies and to study the kinetics of the reaction. The biotransformations were performed at pH 7.0, 25°C with 1.0 M HCl titrant (3.2.8.1). The profile and mass balance for a 23.0 ml biotransformation containing 4.5 U/ml transketolase are shown in Figures 3.17 and 3.18 respectively. The reaction illustrated contained an excess of HPA to ensure the ketol donor was not a limiting substrate in the reaction. The reaction proceeded until 53 % of the total racemic 3-OBG was consumed in approximately 2 hours. The mass balance shows approximate agreement between the substrate utilisation and product formation profiles indicating negligible degradation of the reaction components under these conditions. The titrant requirement was lower than anticipated but can be attributed to an increased significance of the carbon dioxide level in solution as a proportion of the total carbon dioxide produced. Theoretically, if the initial racemate concentration were reduced to 20 mM, the titrant requirement would be minimal as a major proportion of the gas would stay in solution and offset the effect of HPA utilisation on the reaction pH.

The initial reaction rate for 3-OBG with HPA at pH 7.0, 25°C was approximately 0.3 µmol/min/ml for a transketolase level of 4.5 U/ml. This compares to approximately 4 µmol/min/ml expected for reaction of glycolaldehyde employing the same enzyme level, pH and temperature (Mitra and Woodley, 1996). However, this direct comparison is misleading as the Vmax for the 3-OBG reaction was not achieved due to the limiting solubility of 3-OBG in water. Several published reports have compared the reaction rate of transketolase on various aldol donors and arrived at numerous conclusions (Hobbs, 1995; Kobori *et al.*, 1992; Bolte *et al.*, 1987). These studies have not included any data on the respective solubilities and Km values of the acceptor substrates without which such comparisons are difficult to interpret.

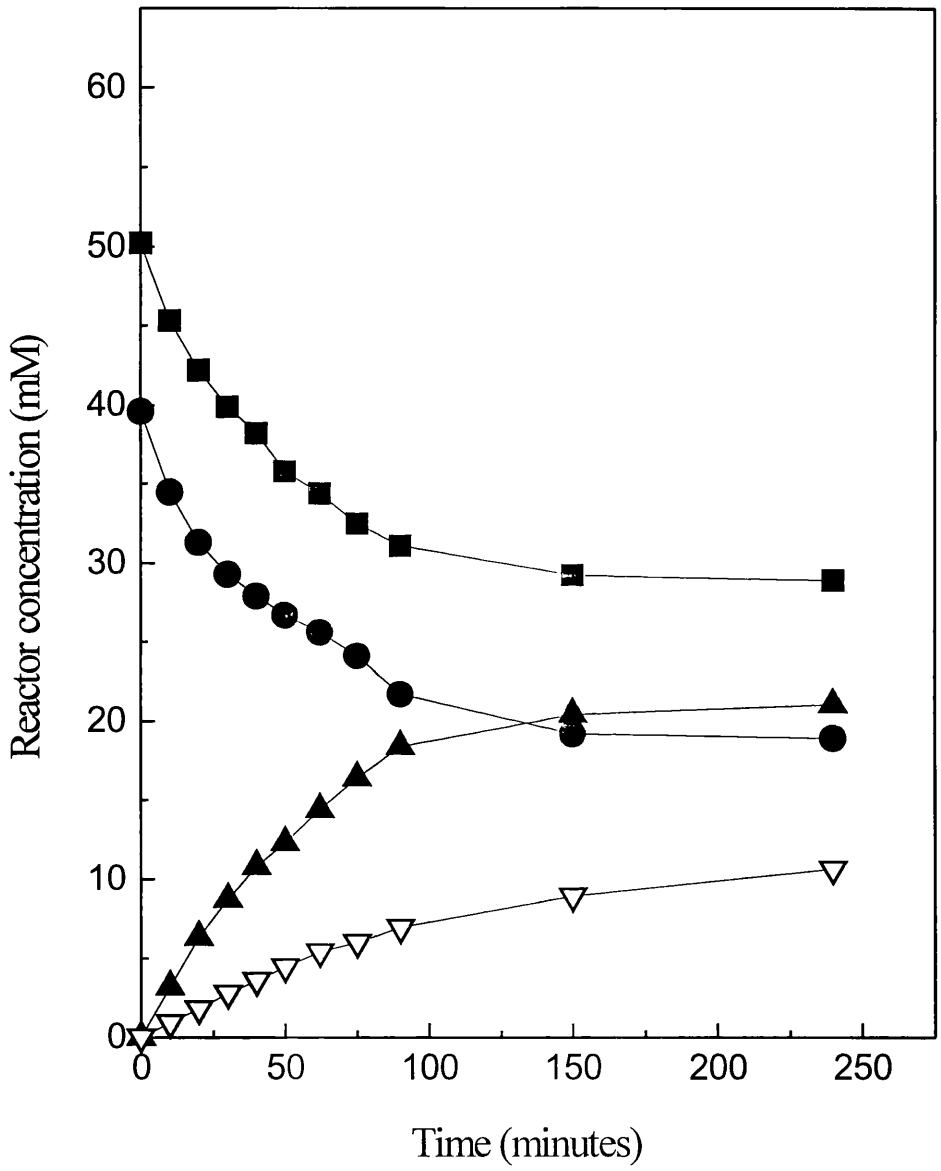


Figure 3.17. Concentration profile for a small scale validation biotransformation at pH 7.0, 25°C in the presence of excess HPA. The initial 3-OBG concentration was 40 mM and the initial transketolase level was 4.5 U/ml. The reaction pH was maintained at pH 7.0 by addition of 1.0 M HCl titrant. Profiles are illustrated for reactor concentrations of HPA (■), 3-OBG (●), 5-OBX (▲) and HCl titrant (▽).

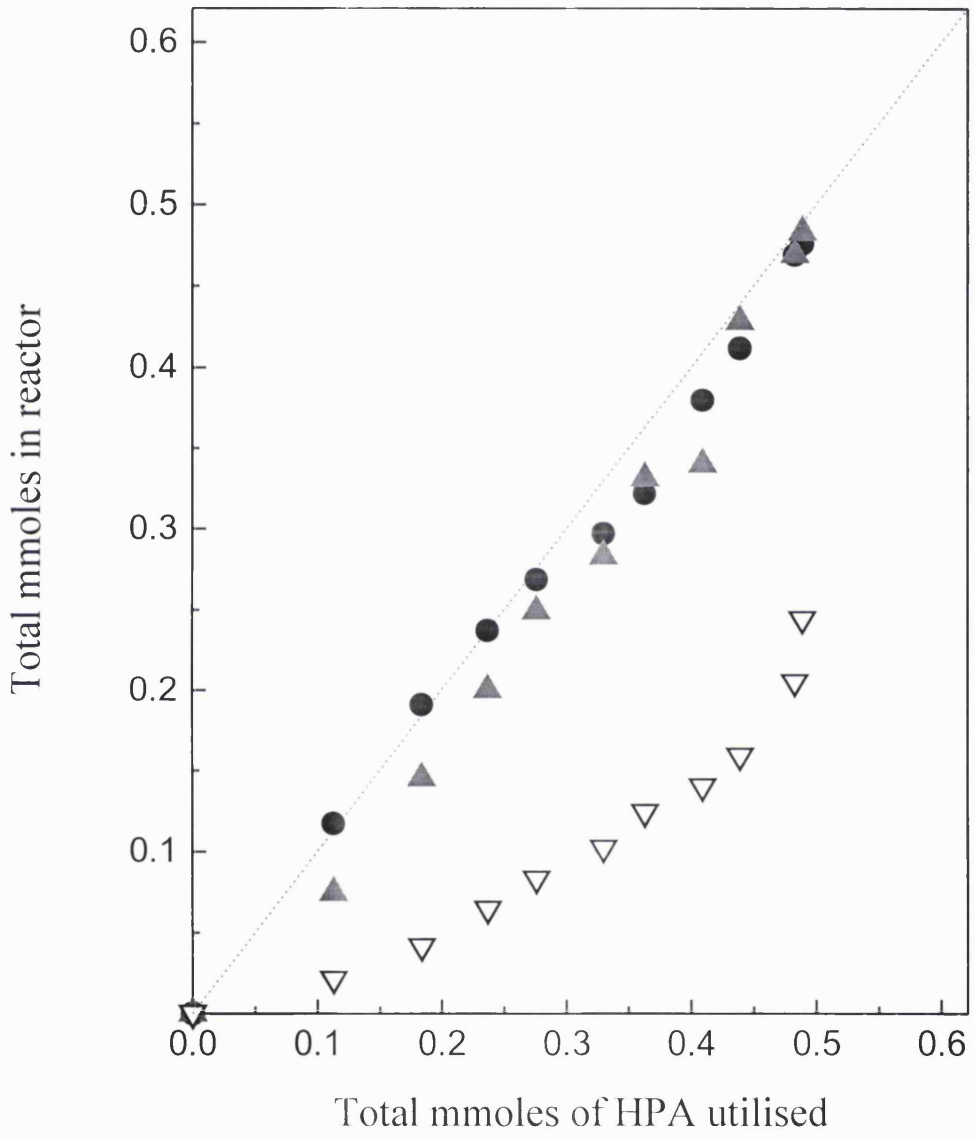


Figure 3.18. Mass balance for analytical scale validation biotransformation controlled at pH 7.0, 25°C. in the presence of excess HPA. The total moles of 3-OBG utilised (●), 5-OBX produced (▲) and HCl titrant added (∇) are plotted against total HPA utilised. The dotted line indicates the profile for molar equivalence of substrates to product.

3.5.2 Preparative scale synthesis of 5-OBX

In order to perform preparative scale syntheses of the interesting chiral synthons discussed in section 3.1, several grams of 5-OBX was required. A batch biotransformation was performed at 18 mM initial racemic 3-OBG concentration at a total initial volume of 4.0 litres. The bioreactor vessel was a fermentation reactor containing stainless steel baffles (3.2.8.2). The deprotection procedure was carried out *in situ* in 0.1 M HCl for 3 days. The deprotection was complete as monitored by HPLC (3.2.10.2.1). However, this deprotection procedure resulted in a pale green discoloration of the solution which changed to brown on neutralisation with NaOH. One potential explanation of the discoloration is the acid-mediated leaching of iron from the stainless steel baffles into the deprotection liquor. However, the biotransformation was not significantly affected except for a slight lag in the observed reaction profile as illustrated in Figure 3.19. No titrant was required as no pH change was observed during the reaction. This was not unexpected due to the low initial substrate concentration as discussed in section 3.5.1. This also illustrates the disadvantage of using titrant addition to monitor the rate of reaction.

Concentration and drying of the biotransformation liquor onto silica during downstream processing (3.2.9) resulted in a black powder but this did not confer any problems on subsequent processing or the appearance of the final product. The isolated yield of 5-OBX was 8.6 g.

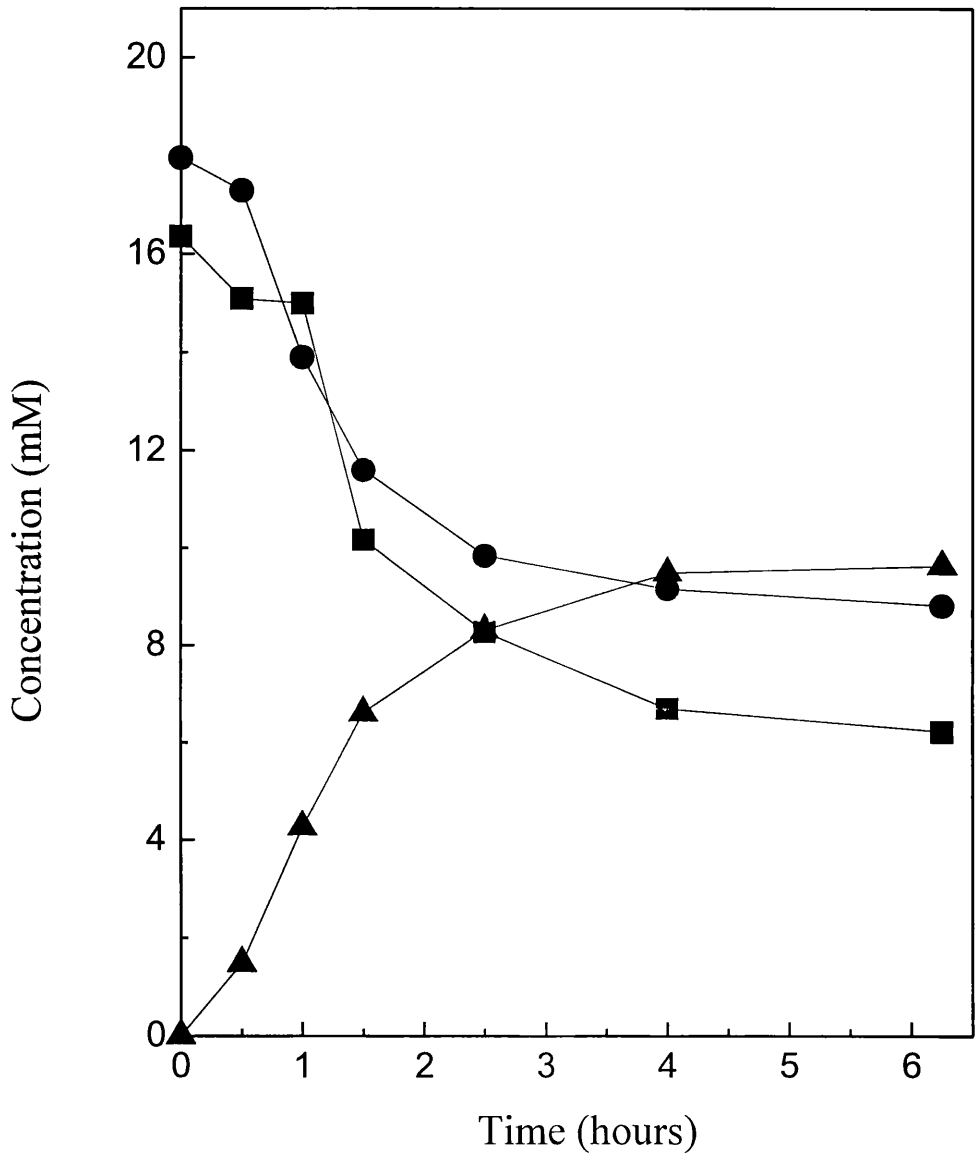


Figure 3.19. Concentration profile for a preparative scale validation biotransformation at pH 7.0, 25°C in the presence of excess HPA. The initial 3-OBG concentration was measured at 18 mM and the initial transketolase level was 2.6 U/ml. Profiles are illustrated for HPA (■), 3-OBG (●), and 5-OBX (▲).

3.6. Process Options

The reactor options for the model system were refined to fed-batch systems based on constraints imposed by the various components of the reaction and the interactions between them. A change in aldehyde substrate requires further investigation in several key areas before the process options can be redefined. These key areas were assigned in section 2 from the results of the model study.

The results of these studies, in conjunction with the characteristics of the model system, were used to define the constraints on the validation reaction. Based on these limitations, the suitability of different reactor configurations was evaluated as shown in Table 3.6. As with the choice of model reactor, all parameters have been allocated equal weight for the purpose of demonstrating the structured approach.

The results of these key studies indicated that the 3-OBG toxicity against transketolase was several fold greater than for the model aldehyde. Although this was not totally unexpected, the $T_{1/2}$ value of 1 hour was surprisingly high at 43 mM 3-OBG concentration, the solubility limit in water at 25^0C. Even at 5 mM concentration the enzyme was still severely affected. The use of a batch reactor would therefore result in significant loss of biocatalyst activity. This was observed at bench scale. This toxicity, coupled to the requirement for pH control due to the alkaline instability of the 3-OBG and 5-OBX, also precludes plug flow type reactors as an appropriate option. The pH change would only be negligible if initial concentrations of substrate were low thereby resulting in undesirable dilute product streams.

The observed toxicity effect would usually signify the requirement for feeding the aldehyde but the situation is not simple. The maximum racemate feed concentration, without incorporating miscible solvents in the feedstream, was only 43 mM. The addition of miscible organic solvent to the feed would result in only a limited increase in solubility. Therefore, a simple feeding regime would produce a dilute final product stream, an undesirable attribute for a commercial process. In addition, due to the method of manufacture, the 3-OBG is supplied as a racemic mixture.

Characteristics		Constraints					
		Reactor selection					Reactor operation
		BSTR	FBSTR	CSTR	PFR	TLPSTR	
Reactants/products:	pH lability				●		pH 7
	Low 3-OBG solubility in aqueous	●	●	●	●		
Reaction:	Acid consumption				●		pH-stat
	Gas production				●		
Interactions:	S/R 3-OBG toxicity	●	●	●	●		low [S/R 3-OBG]
Selection		●	●	●	●	○	

● Unsuitable
○ Suitable

BSTR = Batch stirred tank reactor
FBSTR = Fed-batch stirred tank reactor
TLPSTR = Two-liquid phase stirred tank reactor
CSTR = continuous stirred tank reactor
PFR = plug flow reactor

Table 3.6. The suitability of different reactor configurations to the validation biotransformation based on the defined constraints

Feeding this mixture would result in build up of the non-reacting (S) enantiomer in the bulk solution and lead to biocatalyst deactivation. (3.4.3). Despite the low final product concentration, fed-batch systems may still prove a useful option providing the residual concentration of the non-reacting (S) 3-OBG can be maintained low.

Competitive inhibition by 5-OBX was only found to be significant at concentrations approaching 100 mM where a 20 % reduction in observed activity was noted. Continuous stirred tank reactors could therefore also be a valid option but, as with the fed-batch system, the residual (S) 3-OBG concentration must be controlled. In addition, continuous reactors operate on the principle that the catalyst be retained in the reactor by immobilisation or compartmentalisation. However, there is a necessity to maintain the presence of both cofactors in free solution to maintain the active form of the free enzyme (2.5.5). This suggests that the cofactor would need to be included in the feedstream to maintain activity of the immobilised catalyst in a continuous reactor system. Covalent immobilisation of transketolase is currently under investigation within the transketolase program (Brocklebank *et al.*, 1996) while compartmentalisation of transketolase using membranes has been reported (Kragl *et al.*, 1996).

In order to maintain catalyst activity for extended production runs in both continuous and fed-batch systems, there is a requirement to maintain low residual (S) 3-OBG levels. One method may be to incorporate an *in-situ* removal operation such as a hydrophobic column recycle. This could be incorporated as an external loop to a bi-substrate fed-batch reactor (Figure 3.20). The reactive (R) 3-OBG from the feed should rapidly be converted to product in the bulk solution providing the Km for the (R) aldehyde is low. Rapid recycle through a packed bed of hydrophobic resin, for example, should maintain low (S) 3-OBG in the bulk solution and may also bind 5-OBX. In theory, the (S) 3-OBG and 5-OBX could then be selectively eluted using solvents of differing polarities. Unfortunately this reactor may not be suitable as kinetic studies showed that the Vmax for transketolase on 3-OBG substrate was not achieved at concentrations up to the solubility limit for the aldehyde in water (3.4.4.1). These studies suggest that the Km for (R) 3-OBG is high (probably greater than the solubility) and, as observed during the feeding studies for the model system, may result in the build up of a residual background level of the (R) in

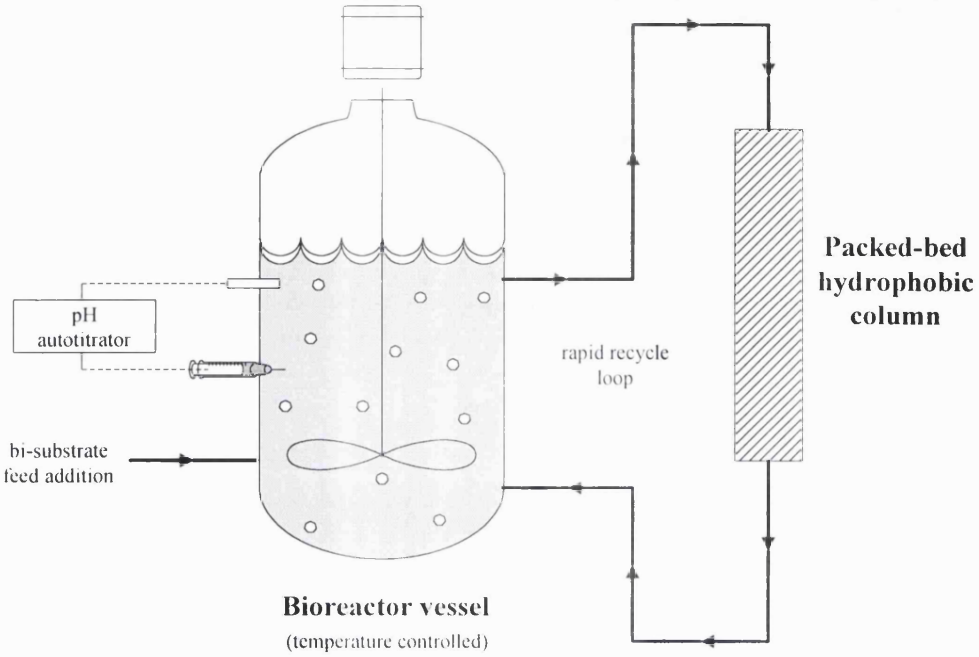


Figure 3.20. One possible configuration for an aldehyde/HPA bi-substrate fed-batch reactor incorporating a continuous *in-situ* (S) 3-OBG removal system. The system utilises a hydrophobic column to remove unreacted 3-OBG which may also remove 5-OBX product. Enzyme containment within the reactor may be required due to the presence of hydrophobic patches on the protein surface.

addition to the non-reactant (S) 3-OBG. If this occurs, (R) enantiomer would be bound to the hydrophobic column and the reaction yield would be reduced. In addition, if the enzyme is not retained in the bulk solution, it must not be prone to binding to the column resin. Although the active site does not contain hydrophobic groups, hydrophobic patches exist on the surface of the enzyme and may act as binding areas.

Another option is to use an 'inert' second liquid phase as a reservoir to hold the 3-OBG. By judicious selection of the polarity of this organic phase, the partitioning of the 3-OBG between the two phases can be controlled. This has been demonstrated over extended periods in batch operation for a single toxic reacting species (Cremonesi *et al*, 1973; Lilly, 1983; Woodley and Lilly, 1992). However, the 3-OBG system consists of the two enantiomers. The (S) 3-OBG would partition to equilibrium levels with the aqueous phase while the (R) 3-OBG would also partition but be constantly converted into 5-OBX in the aqueous phase thus creating a flux of (R) enantiomer across the interface. This is illustrated in Figure 3.21. Depending on the polarity, the organic phase may also act as an *in situ* product removal system providing a simple method of product concentration. As with the hydrophobic column, the unreacted 3-OBG and 5-OBX could easily be separated using organic solvents ready for further chemical processing. However, in order to maintain a low (S).3-OBG concentration in the aqueous phase, the polarity of the organic phase would need to be low thus reducing the rate of flux of the (R) 3-OBG to the enzyme. This flux may be the rate limiting step which, when coupled with the high observed Km for (R) 3-OBG, may rule out the two phase system for this biotransformation.

The suitability of two phase systems for this biotransformation requires a knowledge of the compatibility of the transketolase with a second organic phase particularly with regard to interfacial effects. This information is not available. However, reduction of the toxic effects of the (S) 3-OBG while maintaining rapid rate of reaction will be difficult to equate using multiphasic systems.

A third option to maintain the catalytic activity of the system is to resolve the racemate prior to the biotransformation in order to remove the (S) enantiomer.

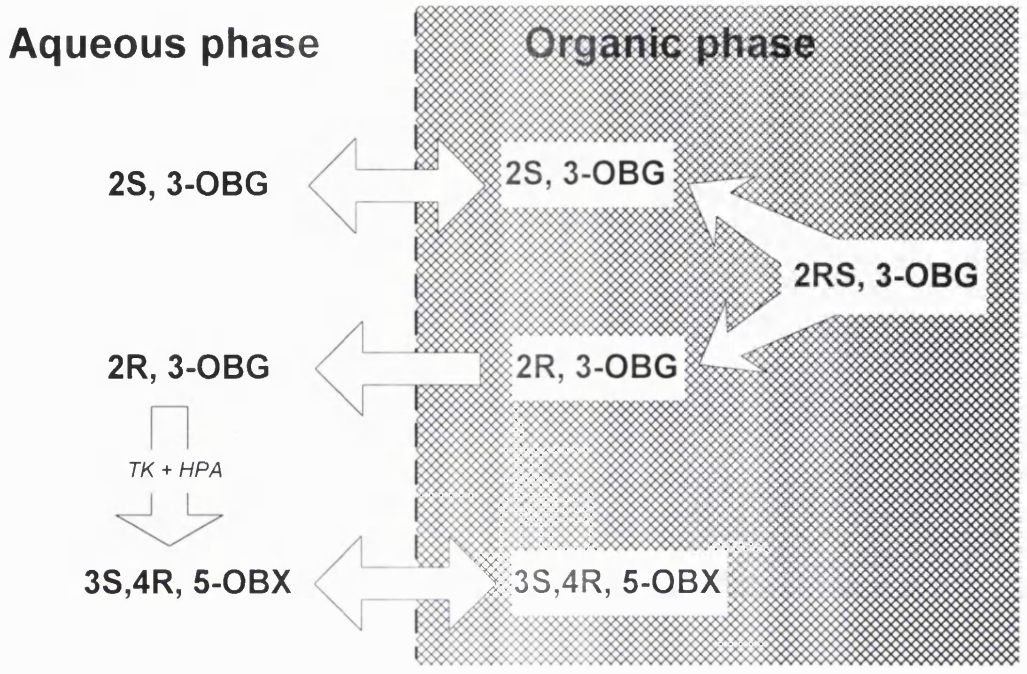


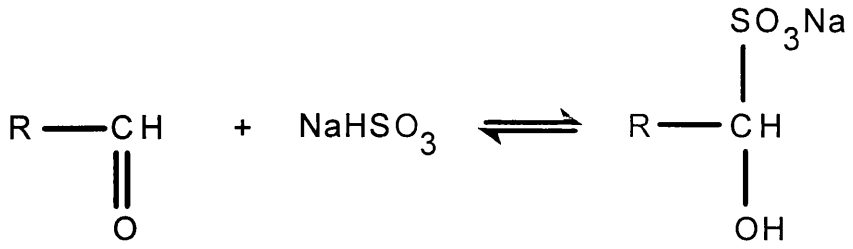
Figure 3.21. Schematic diagram of the expected mass transfer of racemic 3-OBG and 5-OBX between the organic and aqueous phase during a validation reaction in a bi-phasic batch stirred tank reactor.

Although this negates the inherent enantioselective advantage of using transketolase, it may be possible to perform a chiral resolution using either chiral chemistry techniques such as differential crystallisation or by biocatalytic resolution methods. The use of a lipase to stereoselectively esterify the hydroxyl on the α-carbon in the presence of a carboxylic acid could be one such biocatalytic method.

An *in-situ* racemisation step would be useful to reduce the concentration of the (S) enantiomer in solution thus reducing the toxic effect during biotransformation and also for improving the total process yield. However although racemisation of carbonyl compounds such as ketones via acid or base catalysed enolisation is straightforward (Finar, 1964; Black *et al.*, 1989), racemisation of α-hydroxyaldehydes is not well documented. This may be due to the low enolisation rates observed with aldehydes (Forsen and Nilsson, 1970) and the tendency of aldehydes to polymerise in strongly acidic conditions (Baron *et al.*, 1963).

In addition to selective elution from hydrophobic resins or silica columns (3.2.9), isolation of the (S) 3-OBG post-biotransformation may be possible directly from the reaction liquor by complex formation with sodium bisulphite as shown in Figure 3.22. The (S) 3-OBG may then be recovered by addition of acid (Youngs and Jencks, 1978) or using H^+ ion exchange resin (Khusid and Chizova, 1985) and then subjected to chiral inversion chemistry. Unfortunately, bisulphite complexation may also result in precipitation of ketones, such as 5-OBX, and an additional separation step may be required to separate 5-OBX from the (S) 3-OBG prior to aldehyde recycling.

It is evident that there are several options for bioreactor selection for the validation reaction depending on the relative importance of maintaining the stability of the different process components. Since the acceptable stability of the different reaction components is proportional to the component cost contribution to the total economics of the biotransformation, relative component cost is one economic constraint that can be used to rule out reactor options. This factor is discussed in chapter 4.2. The most important constraint which needs to be incorporated into the decision making procedure of the structured approach is the economic viability of the different process options available.



Aldehyde | Sodium bisulphite | Bisulphite addition product

Figure 3.22. The reaction of aldehyde with sodium bisulphite in aqueous conditions to form an insoluble precipitate.

4. GENERAL DISCUSSION

4.1. Characteristics of the transketolase system

4.1.1. Common and reaction-specific features

The model reaction proved a useful indicator of the key features of general transketolase-catalysed reactions and the important constraints on the reactions utilising HPA as the ketol donor. The model system utilised the highly water soluble substrates HPA and glycolaldehyde and transketolase catalysis resulted in complete conversion to the highly water soluble product L-erythrulose. In this system, the achiral glycolaldehyde substrate also exhibited a low level but concentration-dependent toxicity to the enzyme which could be further reduced by incorporating feeding strategies into the biotransformation. Although no new parameters were revealed during 3-OBG (validation) reaction studies, the dominating influence on reactor design of the 3-OBG substrate characteristics (i.e. solubility, toxicity and chiral purity) became apparent. The 3-OBG aldehyde proved to be a highly toxic racemic aldehyde of low water solubility. These are characteristics shared by many of the potentially useful transketolase reaction aldehydes (Kobori *et al.*, 1992, Turner, 1996) but not to any significant degree by the aldehyde component of the model system. The choice of model reaction, although providing a useful analytical tool, did not reflect the key constraining characteristics of potentially useful transketolase-catalysed biotransformations. An industrially representative system, such as the validation reaction, would have proved more beneficial as a model and the importance of a commercial awareness in choice of the model is apparent.

It is useful to divide the general characteristics of transketolase-catalysed biotransformations into those that appear to be common to all transketolase mediated reactions and those that appear to be reaction-specific. As the use of HPA results in a favourable equilibrium which is a commercially useful feature, it is probable that

the majority of commercial reactions will utilise this ketol donor. Therefore, the characteristics of this donor substrate can be grouped under common features. Although there may be instances when an alternative ketol donor is utilised, if the cost of HPA is a limiting factor in the process economics for example, this would result in less favourable equilibrium conversion. Due to the reported similarities in the chemico/physical characteristics of transketolase substrates and products (Chauhan *et al.*, 1997), this would increase downstream costs of product separation and reduce the economic benefit resulting from a change in donor substrate. Alternatively, it is possible that a different donor substrate may be required if the desired product is the reaction component arising from the removal of the 2-C unit. However, this is an unlikely scenario and there is no published precedent for a transketolase biotransformation where this has been the intention.

The common features for transketolase-mediated reactions using HPA as the ketol donor can be defined as;

- all substrate/product-independent transketolase characteristics (e.g. oxidation sensitivity of holo-transketolase, the pH/activity profile, the pH/stability profile, the pH-dependent dynamic equilibrium between free and enzyme-bound cofactor).
- the physico/chemical characteristics of HPA (e.g. maximum solubilities, the autodegradation of HPA in alkali).
- the HPA/transketolase interactive effects (e.g. the Km for HPA, the inhibition effects of HPA on transketolase, the toxicity of HPA to apo-transketolase).
- the reaction characteristics due to utilisation of HPA as the ketol donor (i.e. pH increase, carbon dioxide production, favourable reaction equilibrium).

In addition to HPA, the aldehydes and products for both the model and validation reactions were also found to degrade spontaneously in alkaline conditions. This may be a common feature for all transketolase substrates and products but this is not necessarily the case and has therefore not been included under common features. As a result of this project, the common features are now defined and need not be investigated further for other transketolase catalysed biotransformations.

The reaction-specific features are those characteristics that may alter the constraints on reactor selection whenever a new aldehyde substrate is employed (and therefore a new product generated). Before appropriate reactor designs can be assigned, these features need to be re-examined and therefore dictate the aims of key experiments to be performed. These specific features are:

- the solubility of the aldehyde and product in water and organic solvents.
- the chiral purity of the aldehyde (and the ratio of reactant to non-reactive aldehyde).
- the degree of toxicity of the aldehyde (both the reactant and non-reactive forms) to the enzyme.
- the value of kinetic constants (e.g. the Km for the aldehyde, the inhibition constants for the aldehyde and product).
- the enantiospecificity of transketolase for the aldehyde and enantioselectivity for the product (and the effect of different enantiomeric ratios of the aldehyde racemate).

In order to determine many of the reaction-specific features of transketolase catalysed biotransformations, the analytical procedures for measurement of the aldehyde and product need to be in place. The ion-partition and reverse-phase HPLC methods already developed for the model and validation reactions would probably be suitable for many of the useful transketolase reaction components but some modifications may be required. Although not measured directly in this project, methods for determination of enantiomeric excess (e.e.) of the product would also need to be developed.

4.1.2. Optical purity of the reaction product

The high enantiospecificity and stereoselectivity of transketolase are key features which illustrate the potential of this enzyme in asymmetric synthesis. It is useful to

define these attributes. Transketolase can react with achiral and chiral aldehyde substrates but, with chiral aldehydes, the enzyme is highly enantiospecific in that only aldehydes with (2R) configuration are substrates. The product of reaction of achiral aldehydes and of homochiral (2R)-hydroxyaldehydes is a ketose with a (3S) configuration and a (3S,4R) configuration respectively. The enzyme therefore also exhibits high stereoselectivity in the chirality of the product at the 3-carbon position. Evidence for these conclusions is illustrated below:

1. Racemic (2RS)-hydroxybutyraldehyde reacted to give the (3S,4R) chiral triol product with an e.e. >95 (by ^{1}H NMR of the Mosher's ester) and recovered residual aldehyde with an e.e. of 95% (chiral shift ^{1}H NMR analysis of the reduced aldehyde) (Kobori *et al.*, 1992). *(Illustrates high stereoselectivity and high enantiospecificity).*
2. Transketolase-mediated resolution of the α-hydroxyaldehydes lacaldehyde, 3,3-diethoxy-2-hydroxypropionaldehyde and 3-OBG yielded recovered residual aldehyde with e.e. values of >95% (Kobori *et al.*, 1992), 78% (Humphrey, 1997) and 72% (Humphrey, 1997) respectively. *(Infers enantiospecificity).*
3. Homochiral (2R)-hydroxyphenylacetaldehyde reacted to give a single diastereomer whereas homochiral (2S)-hydroxyphenylacetaldehyde was not a substrate (Humphrey, 1997). *(Infers stereoselectivity and enantiospecificity)*.
4. Individual biotransformations utilising (2R)-, (2S)-, and (2RS)-glyceraldehyde resulted in full utilisation, no utilisation and 50% utilisation of the total initial aldehyde respectively (Morris *et al.*, 1996). *(Infers enantiospecificity).*
5. Reaction of homochiral (2R)-hydroxy-3-phenylpropionaldehyde was terminated before completion and the remaining aldehyde isolated. The aldehyde was shown to be enantiomerically pure by chiral shift ^{1}H NMR confirming that racemisation was not occurring during the reaction.

The evidence for high enantiospecificity and stereoselectivity of transketolase for the model and validation reactions is inferred from the evidence below:

1. Measured optical rotations of L-erythrulose product from the model reaction (i.e. using the achiral glycolaldehyde substrate) correspond to literature values (Hobbs *et al.*, 1994). *(Illustrates stereoselectivity).*
2. NMR shows high diastereomeric purity of 5-OBX product formed from racemic (2RS) 3-OBG (Humphrey, 1997). *(Infers stereoselectivity and enantiospecificity).*
3. For racemic 3-OBG the reaction only proceeded to 50-55% of total initial aldehyde despite residual active enzyme. *(Infers enantiospecificity).*
4. For racemic 3-OBG the remaining aldehyde, after completion of reaction, was converted to the diethylacetal and found to have an e.e. of 72% by chiral GC (Humphrey, 1997). *(Illustrates enantiospecificity).*

It is important to note that the e.e. of the residual aldehyde after reaction using racemic aldehyde with HPA is not a definitive indicator of the e.e. of the product. The direct determination of e.e. of the products of the model and validation reactions was not performed in this study as this required the use of homochiral product standards of different stereochemistries. These were not available commercially and would require specific in-house synthesis involving multiple chemosynthetic steps. However, the accumulation of evidence described above is such that the products of the model and validation reactions are almost certainly of high e.e. For the validation reaction in particular, it is unfortunate that no direct product e.e. data was available as the chiral purity of the product may have changed as the biotransformation proceeded. This phenomenon is observed in many enzyme-mediated chiral resolution reactions where both enantiomers are substrates but react at different rates (Margolin, 1993; Wong and Whiteside, 1994). The requirement for high e.e. of the final product during transketolase catalysis from racemic aldehydes may provide an additional constraint on the reaction conditions. This may influence the choice of suitable bioreactor system and is, therefore, an important issue if the structured approach is to be applied to enzyme-catalysed asymmetric syntheses.

4.2. Economic considerations in the structured approach

For the purpose of demonstrating the principles behind the structured approach, the economics of the transketolase catalysis process have not been addressed in detail. This is obviously an artificial situation as the economics of the process are the driving force in the process development. The point in the decision making procedure at which the economics of the process are addressed is important. In industry the pressure to produce preparative quantities of a novel pharmaceutical for clinical trials, by biotransformation, often defers analysis of the economics of the adopted bioprocess. This is a potentially expensive approach as regulatory guidelines dictate that the compound under clinical trials, particularly in phases II and III, should be manufactured under conditions similar to those of production scale. By following the structured approach to reactor selection, the component costs should not influence the identification of the process constraints but will, however, influence the relative importance of the constraints and thus the identification of the most economic process options for further evaluation. Although the component costs should only be incorporated into the structured approach after identification of the process constraints, the economic information should be gathered early in the project timetable. This will identify the expensive components of the process and allow feasibility studies and component cost reduction programs to be completed before final bioreactor selection and evaluation. Before the introduction of genetic manipulation techniques, the cost of biocatalyst production in biotransformation processes was often the highest component cost. For transketolase the cost of the biocatalyst has been reduced by several orders of magnitude since 1990 by utilising recombinant technology to overexpress the enzyme in *E. coli.* In addition, the cost of the HPA component of the reaction has been dramatically reduced by development of an in-house synthesis methodology. The relative costs of the substrate and biocatalyst components utilised in the transketolase project are shown in Table 4.1.

Component	Approximate cost (1996)
Transketolase (Sigma)	£ 2000000 / 10^6 units
Transketolase (recombinant enzyme)	£ 10 / 10^6 units *
HPA lithium salt (Sigma)	£ 1800 - £2400 / mole
HPA free acid (Sigma)	£2400 - £3200 / mole
HPA potassium salt (in-house synthesis)	£ 220 / mole#
Glycolaldehyde (Sigma)	£ 280 - £370/ mole
3-OBG racemate (in-house synthesis)	£ 450 / mole# reactive (R) enantiomer
Cofactors: TPP (Sigma)	£ negligible (£ 0.6 /mmole)
$MgCl_2$ (Sigma)	£ negligible

* Based on ~ £2000 / 1000 l fermentation
\# Excludes any labour cost

Where a price range is shown, the lower price is based on a 25% discount quoted by Sigma for bulk (multikilogram) orders on catalogue price (higher price in the range)

Table 4.1. Model and validation reaction component costs based on commercially supplied (Sigma) purchase price and in-house manufacture cost.

For a monophasic aqueous biotransformation, the contribution of the TPP cofactor component to the overall economics of the biotransformation is linked to the solubility limit of the substrate(s) in the bulk solution. However, even for a poorly water-soluble substrate, such as 3-OBG, the TPP cost component is likely to be small. The minimum concentration of TPP for maximum holo-enzyme activity was found experimentally to be 0.1 mM. As the maximum solubility for 3-OBG in aqueous is 43 mM, then for a batch aqueous reaction, each mole of racemic 3-OBG will require approximately 26 litres of reactant solution containing a total of 2.6 mmoles TPP (at a total cost of less than £2). Even if the process were operated at significantly lower 3-OBG concentration, for increased stability of the biocatalyst for example, the TPP cost would still be comparatively low. A 1 mole 3-OBG (racemic) batch operation at 5 mM aldehyde concentration would require 200 litres of reactant solution with a total TPP cost contribution of approximately £13. Therefore, as the metal ion cofactor cost contribution is negligible, the cofactor requirement for transketolase catalysis on large scale is not an economic constraint.

Although the approximate component costs for the model and validation transketolase reactions are known, it would be a valuable exercise to consider the effect of different economic scenarios on the choice of reactor configuration. As the industrially useful transketolase reactions reported to date are likely to have similar characteristics to the 3-OBG validation reaction, it will be assumed that the aldehyde substrate for useful transketolase reactions is generally toxic to the enzyme. The influence of both highly water-soluble and poorly water-soluble racemic and homochiral aldehyde substrates on reactor choice will be considered. Table 4.2 summarises the reactor configurations most likely to provide an economic process based on the economic parameters presented. A low cost biocatalyst such as recombinant transketolase is unlikely to prove an economically viable candidate for immobilisation and can be regarded as a disposable commodity. The transketolase and contaminating proteins in free soluble form can easily be removed by acid-precipitation after completion of reaction with low risk of degrading the product. If the transketolase were isolated from naturally occurring sources such as spinach and baker's yeast, the commercial cost would be high and the biocatalyst would need to

Relative cost of reaction components		Aldehyde substrate characteristics		Relative[#] product recovery cost	Bioreactor options for further evaluation	
Biocatalyst	**Substrate(s)**	**Optical purity**	**Aqueous solubility**		**Recommended form of biocatalyst**	**Reactor configurations for further evaluation**
low	high	racemic	low	high	free soluble (disposable)[1]	BSTR, biphasic BSTR*
			high	high	free soluble (disposable)[1]	BSTR
		homo/achiral	low	low	free soluble (disposable)[1]	biphasic BSTR*
			high	low	free soluble (disposable)[1]	BSTR
high	low	racemic	low	high	immobilised (reusable)[2]	biphasic BSTR* with CISSR
			high	high	immobilised (reusable)[2]	FBSTR with CISSR
		homo/achiral	low	low	immobilised (reusable)[2]	CSTR, biphasic BSTR*
			high	low	immobilised (reusable)[2]	CSTR, FBSTR

1 = oxygen exclusion desirable
2 = oxygen exclusion vital, reaction conditions must minimise aldehyde-mediated enzyme deactivation
[#] Relative between process utilising aldehyde racemate and process using homo/achiral aldehyde substrate
* Inclusion of a hydrophobic organic phase to act as a substrate reservoir

BSTR = batch stirred tank reactor
CSTR = continuous stirred tank reactor
FBSTR = fed-batch stirred tank reactor
CISSR = continuous *in-situ* (S)-aldehyde removal

Table 4.2. The effect of different cost component economic scenarios on reactor selection for transketolase biotransformations

be reusable. This would likely be by physical immobilisation onto a support or by compartmentalisation within a selectively permeable membrane. Due to steric and diffusion limitations with immobilised biocatalysts, the expressed activities of such systems would be reduced compared to free enzyme thereby effectively reducing the specific activity of the enzyme. For transketolase the expressed activity of enzyme immobilised on Eupergit® was reported in the range 1-20% of the equivalent free enzyme activity (Hobbs, 1994; Brocklebank *et al.*, 1996). The economic necessity for multiple reuse or continual use of the immobilised or compartmentalised biocatalyst dictates that the catalyst possess high process stability. For transketolase-catalysed biotransformations the reaction conditions need to be controlled such that oxidative and aldehyde-mediated deactivation is minimised. The use of two phase systems for poorly water-soluble racemic aldehydes and reaction products was discussed previously (3.6). Although the selection and evaluation of suitable organic solvents and appropriate two phase reactor configurations is not a simple procedure, appropriate guidelines have been proposed and evaluated (Woodley *et al.*, 1990; Woodley and Lilly, 1992; Collins, 1995).

The use of homochiral or achiral aldehydes would eliminate the presence of a residual level of contaminating (S) aldehyde in the product stream. If the cost of the biocatalyst were high and the preservation of catalyst stability a high priority, the economics of methodologies to remove the (S) enantiomer from an aldehyde racemate, prior to the biotransformation, should be addressed. Possible methods have been discussed (3.6). For transketolase, however, the low cost of the biocatalyst is likely to negate this approach.

4.3. Application of the structured approach in industry

This structured approach to reactor design and operation is particularly useful for guiding researchers through biotransformation process development. Many scientists are aware of the synthetic potential of biocatalysts in organic synthesis but often fail to operate biocatalytic processes efficiently. From the evidence of published

research, in the many cases where enzymes are utilised, the reactions are performed on analytical scale at low concentrations with little consideration of biocatalyst limitations, process optimisation and scale-up parameters. For example; extended reaction times are often utilised with little regard for biocatalyst stability, non-quantitative reaction monitoring techniques such as TLC are often the only in-process analysis, and reaction yields are expressed as final recovered yield after downstream product purification. As a result, the source of problems such as low reaction yield cannot easily be ascertained. This approach is therefore not amenable to considerations of reaction optimisation and biotransformation process economics. It may also be the case that unstructured and disjointed approaches to potential commercial biotransformation processes, coupled with lack of knowledge of enzyme structure and function, have designated feasible processes as being unfeasible. The structured approach would provide guidelines for evaluation of all relevant parameters before determination of process feasibility with a high degree of confidence.

Industrial acceptance of the generic applicability of the structured approach to biotransformation reactor design and operation is a significant hurdle. One of the main benefits of the approach lies in the progressive reduction in the amount of research and development required for several different biotransformations performed using the same biocatalyst. This may be the case for biocatalysts involved in asymmetric syntheses which exhibit a chiral preference in substrate or product but a wide substrate specificity (e.g. *Candida cylindraceae* lipase), but is less useful for processes utilising commercial biocatalysts of narrow substrate range and limited applications. Although there is still an advantage in the structured approach when applied to a biocatalyst for a single type of reaction only, the efficiency is gained solely in rapidly identifying the inappropriate reactors for large scale production thereby concentrating the research effort on the viable process options. As the initial intensive characterisation studies on the first or model biotransformation are time consuming and, therefore, commercially expensive, the incentive to adopt a structured approach is reduced. For both generically useful and reaction-specific biocatalysts, the urgency for synthesis of a product sample for clinical trials coupled to the inherent limits on patent life may also dissuade

manufacturers from investing time and effort in the short-term for a gain in efficiency in the longer term.

A second possible hurdle to acceptance of the structured approach is whether the manufacturers would take the perceived risk in choosing an appropriate reactor configuration based solely on the results of the few key experimental studies defined from an initial/model biotransformation system. If the structured approach is to be adopted reliably, the intellectual and practical input during the initial characterisation and determination of constraints must be of high quality.

Although the structured approach to bioreactor design and operation has been discussed in detail for transketolase-mediated catalysis, the philosophy has also been applied to the chemoenzymatic synthesis of N-acetyl-D-neuraminic acid (Blayer *et al.*, 1996), to multi-step microbial conversions (Marshall and Woodley, 1995) and to evaluation of suitable methods for *in-situ* product recovery (Freeman *et al.*, 1993; Chauhan *et al.*, 1996). By application to a range of biotransformations the design philosophy will be constantly refined with the ultimate goal of developing an integrated decision flowsheet incorporating all the unit operations involved in a total biotransformation process.

5. CONCLUSIONS AND FUTURE WORK

5.1. Conclusions

5.1.1. The biocatalyst

- Transketolase from *E. coli* JM107 was obtained by fermentation at 1000 litre scale at an apo to holo-enzyme ratio of 85 : 15. Full activity was observed in the presence of 0.1 mM TPP and 0.9 mM Mg^{++}.
- The active holo-enzyme is deactivated by the presence of oxygen while the inactive apo-form is not affected. The inclusion of reducing agents and the removal of dissolved oxygen from a solution of the holo-enzyme significantly decreases the oxidation effect.
- The pH/activity profile is broad with an optimum activity at approximately pH 7.0.
- Holo-transketolase is pH stable in the range pH 6.5 - 10.0. Above pH 10.0 the enzyme is rapidly deactivated while below pH 6.5 the rate of deactivation increases with decreasing pH.
- Holo-transketolase is a complex of protein dimer and the cofactors TPP and Mg^{++}. In the range pH 6.5- 9.5 both 'bound' cofactors are in equilibration with free cofactors in the bulk solution. The dissociation of cofactors from the enzyme/cofactor complex increases with pH.

5.1.2. The model biotransformation system

- A single detection method for the model substrates HPA and glycolaldehyde and the triol product L-erythrulose has been developed.

- The model reaction components (excluding the CO_2 by-product) are alkali labile but stable at pH 7.0 and in acidic conditions. The rate of deactivation is both concentration and temperature dependent and increases with increasing alkalinity.

- All buffers investigated at pH 7.0 result in increased rates of degradation of both lithium HPA and glycolaldehyde at ambient temperature. No degradation occurs in absence of buffers under the same incubation conditions.

- Glycolaldehyde is soluble in water to at least 4.0 M over the range pH 2.0 - 8.0. HPA is significantly more soluble at pH 2.0 than at neutral pH, the lithium salt exhibiting a maximum solubility of 0.65 M at pH 7.0, 25°C.

- Glycolaldehyde is toxic to both apo and holo-transketolase. The activity half-life at pH 7.0, 25°C in the presence of 50 mM and 500 mM glycolaldehyde is 70 hours and 3 hours respectively.

- Lithium HPA at 500 mM rapidly deactivates apo-transketolase at neutral pH and ambient temperature but does not affect the holo-enzyme.

- The presence of dissolved CO_2 (500 mM sodium bicarbonate) does not have any permanent effect on holo-transketolase at pH 7.0, 25°C.

- Binding of TPP to the enzyme results in increased sensitivity to oxidative deactivation whereas the Mg^{++} cofactor has no effect on the enzyme stability.

- A 500 mM biotransformation at initial pH 7.0, 25°C without pH control results in an increase in pH and a reduced product yield despite complete disappearance of substrate. Control of the reaction pH at 7.0 results in complete conversion of substrate to product.

- The utilisation of acidic bi-substrate fed-batch reactor configurations can result in higher product concentrations than theoretically possible with batch systems. Transketolase activity can be maintained at a high level throughout. The process benefit of both fixed feed rates and feed-on-demand strategies has been demonstrated.

5.1.3. The validation biotransformation system

- A detection method for the aldehyde (2RS), 3-OBG and the triol product 5-OBX has been developed.
- (2RS), 3-OBG and 5-OBX are stable at acidic and neutral pH but labile in alkaline conditions. The rate of degradation is concentration and temperature dependent and increases with increasing alkalinity.
- (2RS), 3-OBG and 5-OBX are soluble in water to 43 mM and 110 mM at 25°C, pH 7.0.
- Racemic 3-OBG is toxic to the enzyme with activity half-lives at 5 mM and 40 mM of 1 hour and 10 hours respectively. The toxic effect is not enantiomer specific.
- At neutral pH and ambient temperature, the transketolase reaction is substrate limited by the (2RS), 3-OBG reaction component at all aqueous concentrations within the solubility range.
- Complete conversion of substrate to product has been demonstrated during batch biotransformation of the validation reaction at pH 7.0, 25°C.

5.2. Future work

Further work in the following areas would be of benefit:

1. The transketolase system:
    - development of an accurate and rapid method for determination of optical purity for transketolase products (e.g. 5-OBX) from racemic substrates. This would require the synthesis of different optical forms of the product in order to validate the assay.

- further evaluation of reactor configurations for transketolase reactions involving poorly water-soluble aldol acceptor substrates such as 3-OBG. For example, the use of a second organic phase as both a substrate reservoir and a possible method of *in-situ* product removal from the aqueous phase.

- development and validation of an accurate mathematical model for transketolase-catalysed reactions which could simulate the effects of various reactor configurations on important biotransformation parameters.

- detailed economic analysis of the various reactor options for a range of transketolase-catalysed biotransformation scenarios and the effect of integration of various process techniques.

- protein engineering to remove the oxygen sensitive amino-acid residues in the holo-enzyme and to increase catalyst efficiency.

- increasing the availability of the recombinant enzyme to interested parties on a commercial or non-commercial basis.

2. The industrial applicability of the structured approach to reactor selection:

   - application of the approach to several different biocatalysts of industrial interest using reaction components exhibiting characteristics typically observed in industry. This should provide data for the definition and eventual publication of generic guidelines for researchers to select with confidence the most applicable and economic reactor configurations based on acquisition of the minimum required research data.

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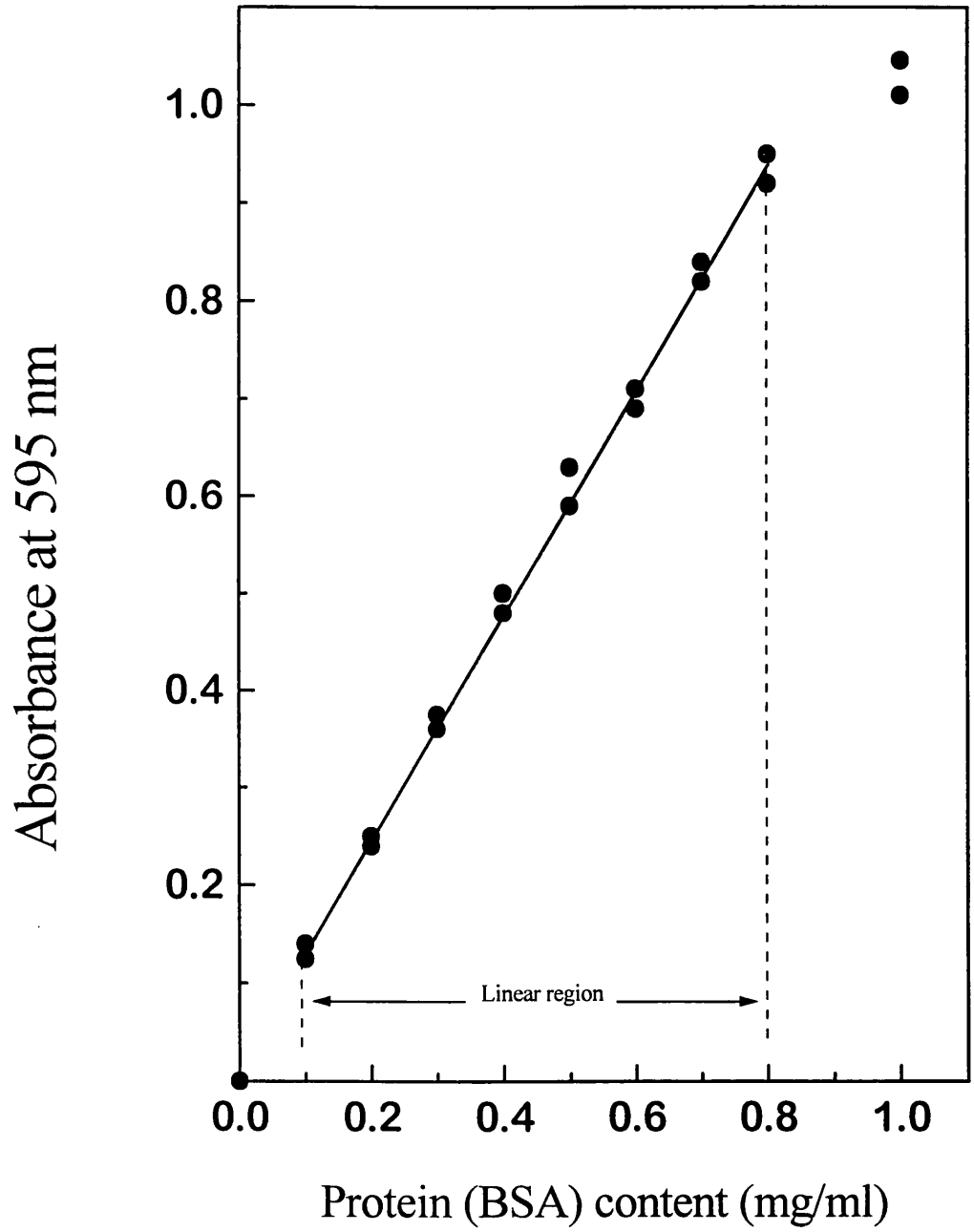
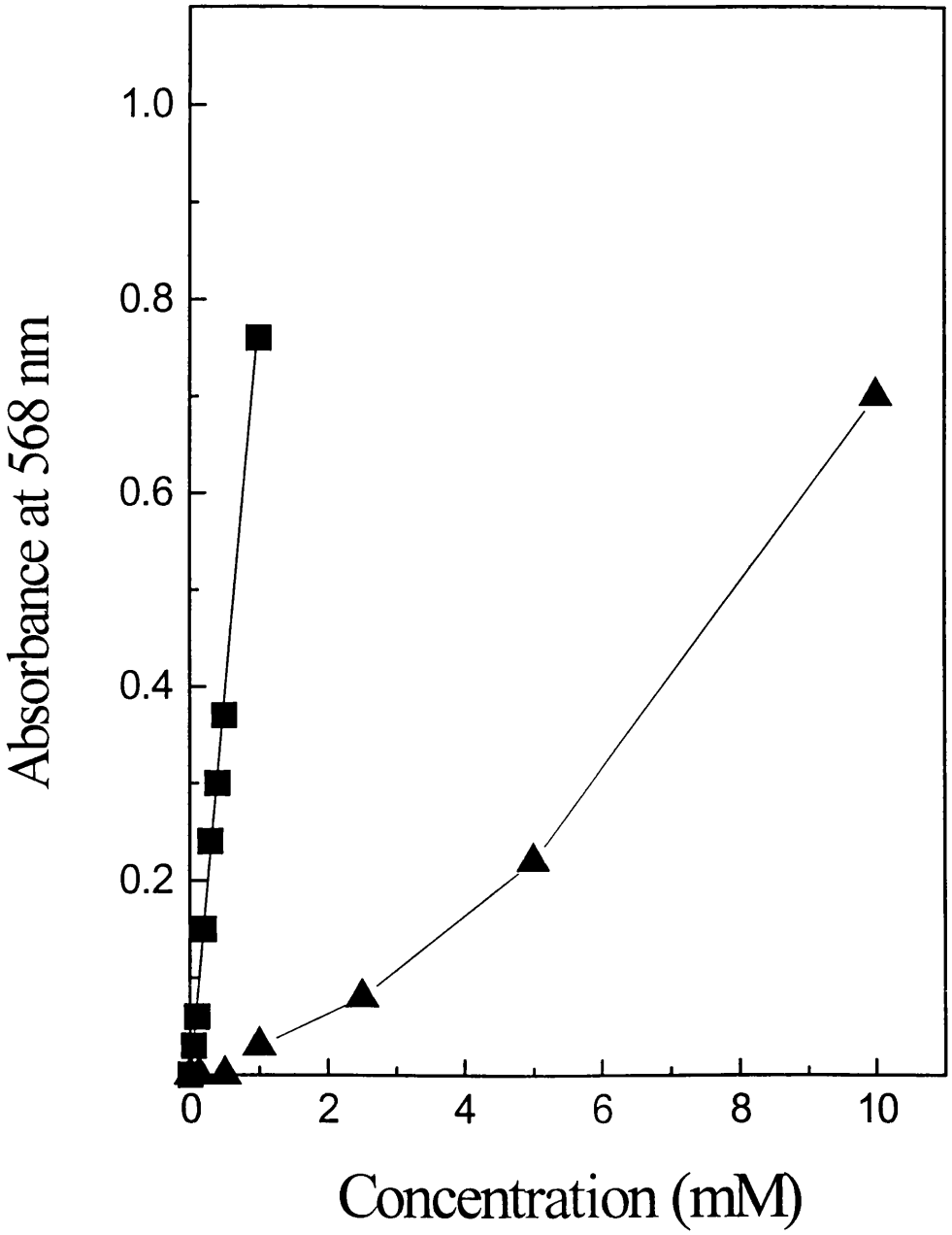
7. APPENDICES

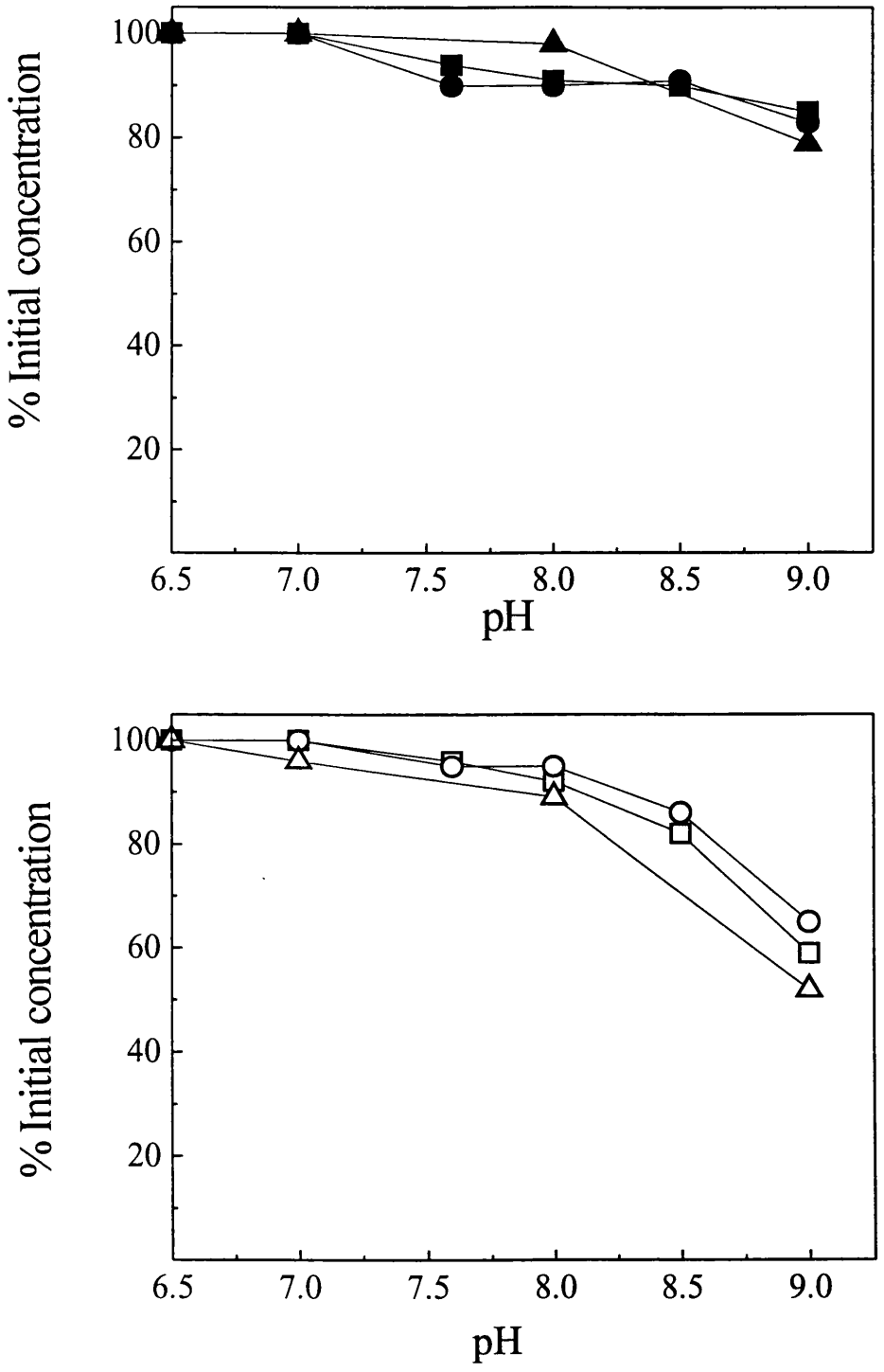
Appendix 1. Calibration curve for Bio-Rad macro protein assay utilising BSA as protein standard



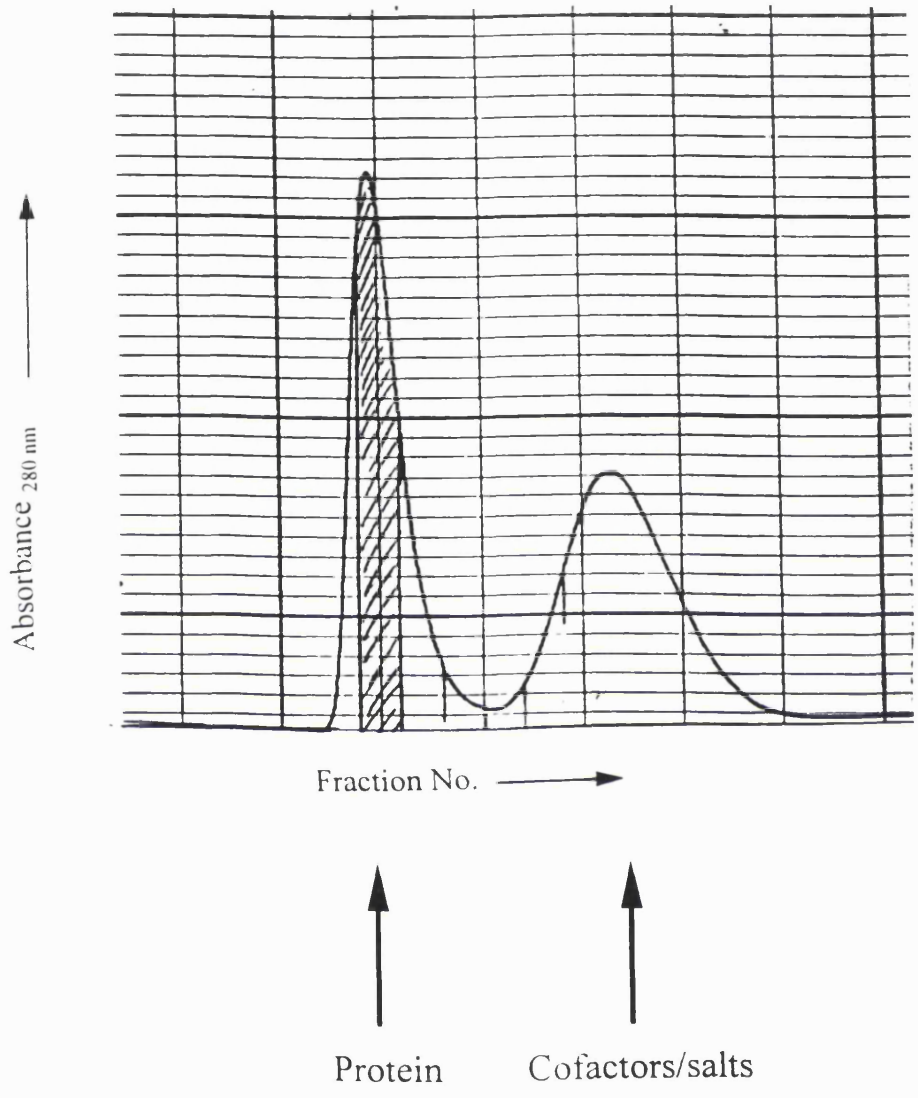
Appendix 2. Calibration curves for D-fructose (■) and L-erythrulose (▲)as determined by the colorimetric method of Boratynski (1984) for determination of ketoses.



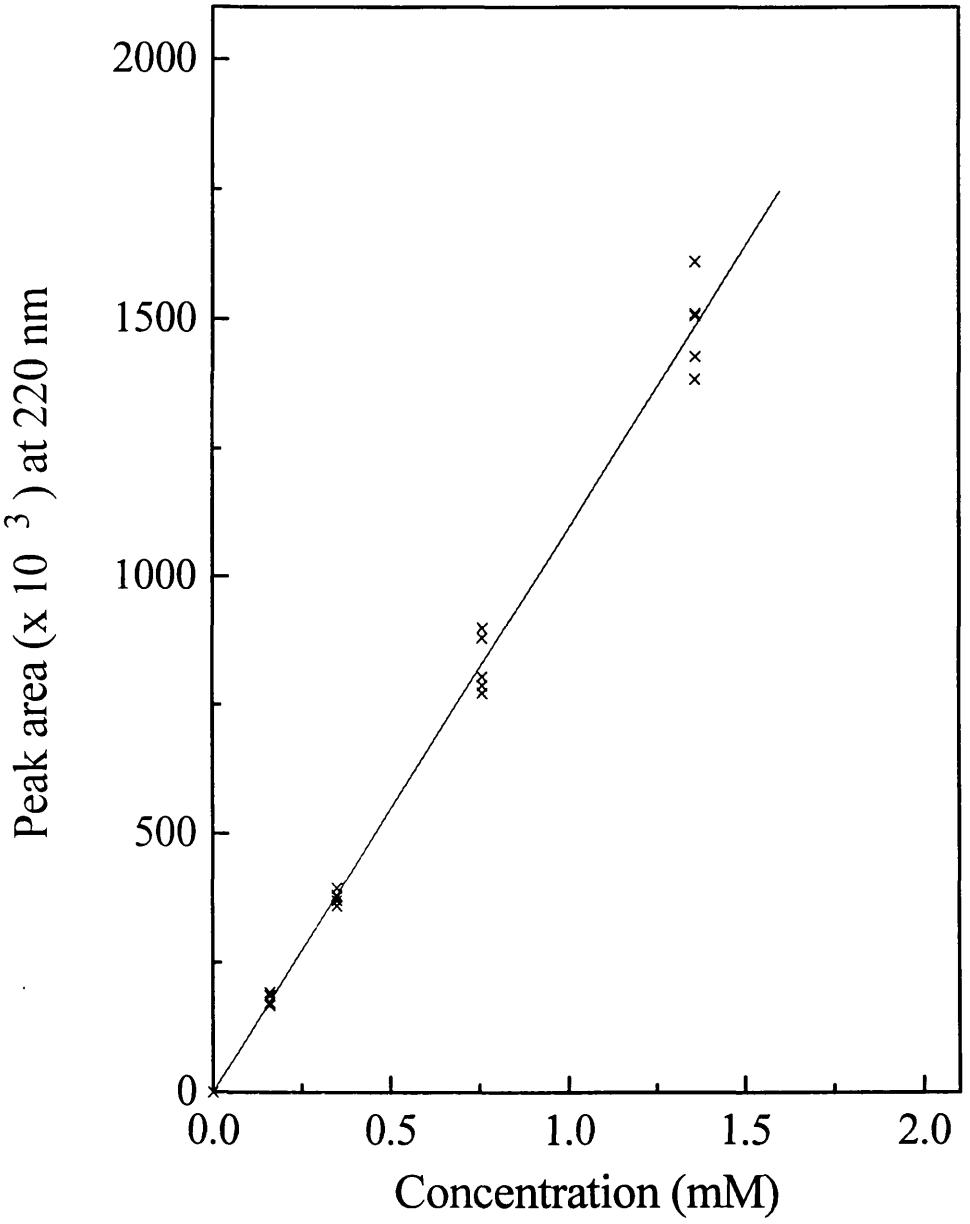
Appendix 3. Effect of pH on initial 100 mM level of HPA (■,❑), glycolaldehyde (●,○) and L-erythrulose (▲,Δ) at 25°C (■,●,▲) and 35°C (❑,○,Δ) in water.



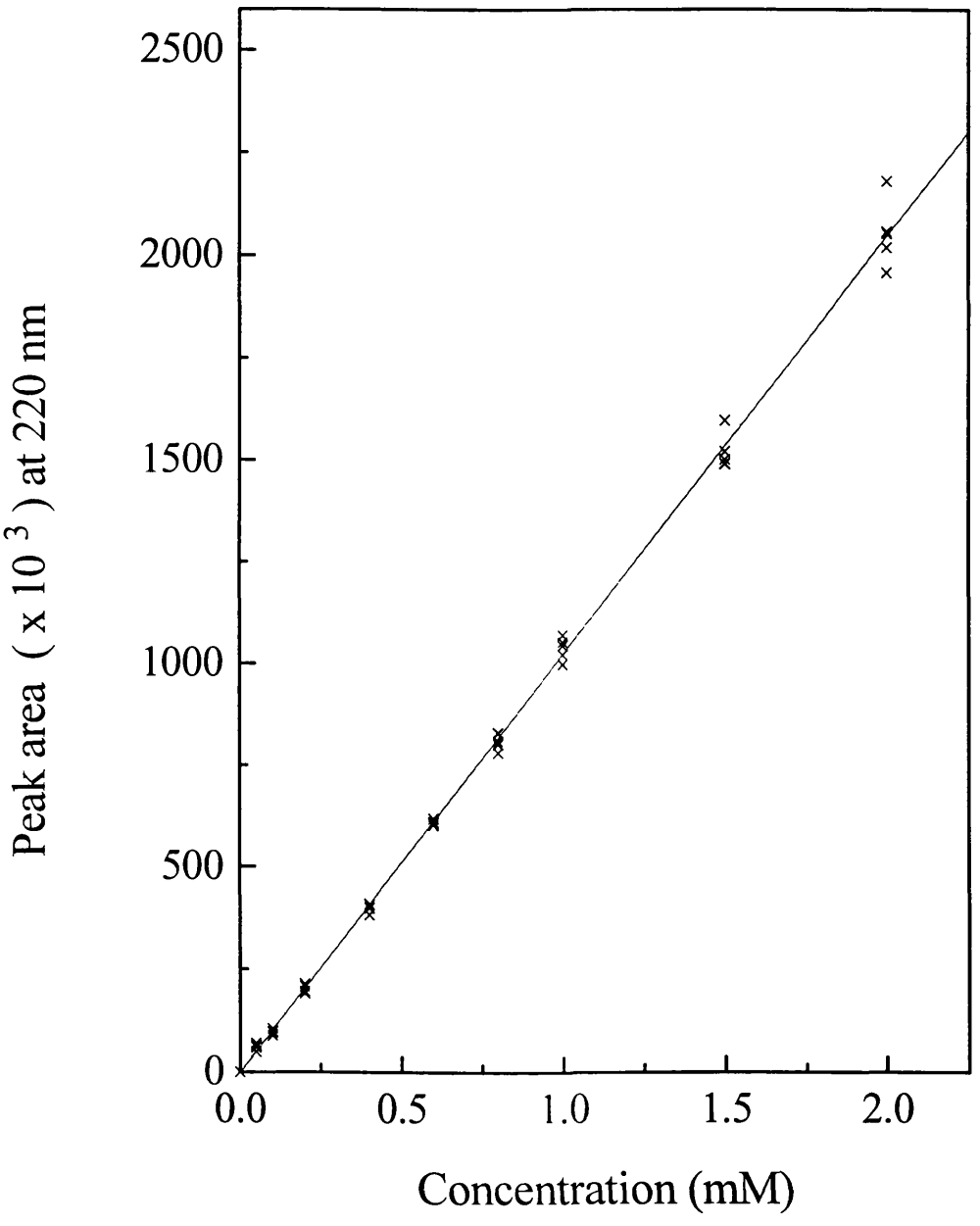
Appendix 4. Separation of transketolase from free cofactors achieved utilising a Sephadex G-25 size partition chromatography column. Shaded area indicates the protein fractions isolated for study.



Appendix 5a. Calibration curves for reverse-phase HPLC assay of 3-OBG. Assay details and assay reproducibility are described in section 3.3.4.



Appendix 5b. Calibration curves for reverse-phase HPLC assay of 5-OBX. Assay details and assay reproducibility are described in section 3.3.4.



Appendix 6. Addresses of suppliers

APV Manton -Gaulin GmbH:	Mecklenberger Strasse 223, Lubeck, Germany.
Bio-Rad Laboratories Ltd:	Bio-Rad House, Maylands Avenue, Hemel Hempstead, Herts. HP2 7TD, UK.
Chrompak Ltd:	Unit 4, Indescon Court, Millharbour, London, E14 9TN, UK.
Dyson Instruments Ltd:	Hetton Lyons Industrial Estate, Hetton, Tyne and Wear, DH5 ORH, UK.
Heraeus Sepatech GmbH:	Laboratory Division, Postbox 1563, D-63405 Hanau, Germany.
HPLC Technology Ltd:	Wellington House, Waterloo Street West, Maclesfield, Cheshire, SK11 6PJ, UK.
Jones Chromatography Ltd:	Hengoed Trading Estate, Hengoed, Wales, UK.
Kontron Instruments:	Blackmore Lane, Croxley Centre, Watford, Herts. WD1 8XQ, UK.

LH Fermenters Ltd;	Porton House, Maidenhead, Berks. SL6 4UB, UK.
Perkin Elmer Ltd:	Post Office Lane, Beaconsfield, Bucks. HP9 9PA, UK.
Pharmacia LKB Biotechnology AB:	Bjorkatan 30, S-751 82 Uppsala, Sweden.
Phenomenex:	Melville House, Hurdsfield Industrial Estate, Macclesfield, Cheshire. SK10 2BN, UK.
Radiometer Ltd:	Manor Court, Manor Royal, Crawley, Sussex. RH10 2PY, UK.
Sigma-Aldrich Company Ltd:	Fancy Road, Poole, Dorset. BH12 4QH, UK.

Appendix 7. Published work

1. Brocklebank, S. P., Mitra, R. K., and Lilly M. D., (1996) "Carbon-carbon bond synthesis: Preparation and use of immobilised transketolase", *Ann. N.Y. Acad. Sci.*, **799**, 729-738.

2. Hobbs, G. R., Mitra, R. K., Chauhan, R. P., Woodley, J. M. and Lilly, M. D., (1996), "Enzyme catalysed carbon-carbon bond formation: Large scale production of *Escherichia coli* transketolase", *J. Biotechnol.*, **45**, 173-179.

3. Lilly, M. D., Chauhan, R., French, C., Gyamerah, M., Hobbs G. R., Humphrey, A., Isupov, M., Littlechild, J. A., Mitra, R. K., Morris, K. G., Rupprecht, M., Turner, N. J., Ward, J. M., Willets, A. J., and Woodley, J. M., (1996), "The impact of rDNA technology on enzymic carbon-carbon bond synthesis", *Ann. N.Y. Acad. Sci.*, **782**, 513-521.

4. Mitra, R. K. and Woodley, J. M., (1996), "A useful assay for transketolase in asymmetric synthesis", *Biotechnol. Tech.* **10**, 167-172.

5. Morris, K. G., Smith, M. E. B., Turner, N. J., Lilly, M. D., Mitra, R. K., and Woodley, J. M., (1996), "A practical procedure for asymmetric carbon-carbon bond synthesis", *Tet. Asymm.*, 7, 2185-2190.

6. Woodley, J. M., Mitra, R. K., and Lilly M. D., (1996), "Carbon-carbon bond synthesis: Reactor design and operation for transketolase catalysed biotransformations", *Ann. N.Y. Acad. Sci.*, **799**, 434-450.