Chemoenzymatic synthesis of novel, structurally diverse compounds

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I, Lydia Grace Coward confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

For my fantastic family.

Abstract

The use of Diels Alder cycloaddition chemistries to access a diverse range of useful cyclic structures is well established throughout literature. However, the value of the product may be enhanced further still by linking this reaction with subsequent (biocatalytic) steps to create novel, structurally demanding, optically pure compounds. This project investigates the linking of Diels Alder (DA) chemistry to the enzyme, transketolase (TK) as a model integration pathway of a chemical syntheses and a biological transformation. The two-step process aims to provide a framework to synthesise small structurally diverse compounds with high enantiomeric excess. The demand for optically pure compounds is becoming a necessity due to the adverse affects frequently introduced by racemic compounds and the cost implications of the material possessing often only 50% active compound.

Recombinant wild type *Eschericha coli* transketolase (EC 2.2.1.1) (WT-TK) was overexpressed in *E. coli* for the biocatalytic step of this two step synthesis. A substrate walking approach whereby a range of sequentially linked cyclic aldehydes, were applied to wild type transketolase and potential activity detected. Transketolase mutants, previously constructed based on information derived from the structural position within the active site of the dimeric enzyme were subsequently screened for activity with the cycloadduct of the Diels Alder reaction as aldehyde acceptor substrate for TK. Following identification, selection, culturing and sequencing of variants indicating enhanced activity towards the novel, bulky, hydrophobic, cyclic aldehyde the enantioselectivity and absolute stereochemistry of the product were determined. Activity displayed by wild type transketolase indicated an 18,000 fold activity improvement. Michaelis-Menten kinetic parameters were subsequently determined to have an apparent K_m of 69.9 mM, k_{cat} of 17.5 s^{-1} and a v_{max} of 0.07 $mM.min^{-1}$ and preliminary process compatibility including product and substrate inhibition issues were highlighted.

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Abbreviations

ABT	2-amino-1,3,4-butanetriol
ACN	Acetonitirile
AmpR	Ampicillin resistant
AP	Acetophenone
BSA	Bovine serum albumin
CO_2	Carbon dioxide
DA	Diels Alder reaction
DCHCA	3,4-dimethyl-3-cyclohexene-1-carboxaldehyde
DCDHP	1-(3',4'-dimethylcyclohex-3'-enyl)-1,3-dihydroxypropan-2-one
DHP	1,3-dihydroxypentan-2-one
DNA	Deoxynucleic acid
DOT	Dissolved oxygen tension (%)
E.C.	Enzyme commission
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
L-ery	L-erythrulose
EtOAc	Ethyl acetate
GA	Glycolaldehyde
GC	gas chromatography
His-tag	Histidine-tag
HPA	Hydroxypyruvic acid
HPLC	High Performance Liquid Chromatography
IPTG	Isopropyl-β-D-thiogalactosidase
ISPR	*in situ* product removal
kDa	kilo dalton
K_m	Michaelis constant
k_{cat}	Enzyme turnover rate
LB	Luria -Bertani
Li-HPA	Lithium hydroxypyruvate
MBA	Methylbenzylamine
MeOH	Methanol
mg	milligrams
mL	millilitre
MgCl	Magnesium chloride

MPTA	2-methoxy-2-(trifluoromethyl) phenylacetic acid
NaOH	Sodium Hydroxide
Ni-NTA	Nickel – Nitriloacetic acid
OD	Optical Density
PA	Propanal
PCR	Polymerase chain reaction
PLP	Pyridoxal phosphate
PMP	Pyridoxamine phosphate
PMA	Phosphomolybdic Acid
RO	Reverse osmosis
rpm	revolutions per minute
[S]	Substrate concentration
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SFPR	substrate feed product removal
SRW	Standard round well
TAm	Transaminase
TEMED	Tetramethylethylenediamine
TFA	Trifluoroacetic Acid
TLC	Thin Layer Chromatography
THF	Tetrahydrofuran
TK	Transketolase
TPP	Thiamine Pyrophosphate
TPTC	2,3,5-tripheyltetrazolium chloride
Tris-HCl	Tris(hydroxymethyl)aminomethane-hydrochloric acid
UCL	University College of London
UV	Ultraviolet
v/v	volume by volume
v_{max}	Maximal velocity
WT	Wild type
X5P	xylulose-5-phosphate
μ_{max}	Maximal specific growth rate
µL	microlitre
µg	microgram

Contents

List of figures

List of Tables

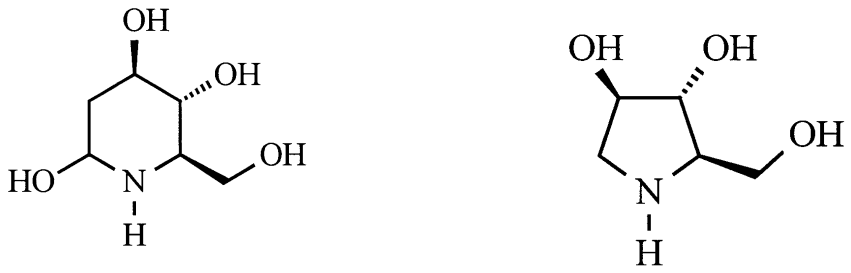
List of schemes

1 Introduction

1.1 Chemoenzymatic synthesis: Integrating chemistry and biology

Chemoenzymatic synthesis is a flexible approach to synthesising complex intermediates where the yield and knowledge of powerful conventional chemistry is combined with the potentially high enantio- regio- and chemo- selectivity of biocatalysts. Integrating the 'best' of two disciplines is a powerful tool for synthesising novel, structurally complex, chiral synthons, where demand for optically pure pharmaceuticals alongside greener more efficient processes is ever increasing (Dalby *et al.,* 2005). Whilst biotransformations are particularly elegant at selective conversions and atom efficiency, chemistry often affords high productivities. In order to truly impact drug intermediate synthesis at industrial scale in terms of productivity, cost effectiveness and environmental burden, biocatalysis and chemical synthesis must be integrated (Tao *et al.,* 2007).

An example of a process utilising a transferase enzyme, transketolase (TK) within a chemoenzymatic system is the production of the glycoside inhibitors, fagomine and natural compound 1,4-dideoxy-1,4-imino-D-arabinitol shown in Figure 1.1 (Hecquet *et al.,* 1994).



(a) (b)

Figure 1.1: Products from chemoenzymatic synthesis involving transketolase. Glycoside inhibitors (a) synthetic fagomine and (b) natural 1,4-dideoxy-1,4-imino-D-arabinitol.

The demand for more complex, optically pure compounds calls for alternative routes and novel pathways, such as chemical synthesis coupled with biocatalytic steps. The integration of the two steps requires process design consideration and the investigation of compatibility, such as the toxicity of components likely to interact and optimal reaction conditions. The presence of activating or toxic reactive groups on intermediates within the chemical synthesis will affect the kinetics of the biocatalyst, potentially damaging productivity (Kim *et al.,* 2007). Recent reviews demonstrate the application of biology and chemistry interfacing in highly significant fields, for instance the development of cancer therapeutics via a drug discovery process involving chemical biology and structural biology (Collins and Workman, 2006). Targeting novel compounds for new drugs often demands an efficient synthetic route in order to provide the quantities required for clinical trials, however, this can prove difficult and sometimes impossible for more complex small molecules. Utilising the knowledge from chemistry, chemical biology and structural biology, novel predictive and innovative technologies for pharmaceuticals may be developed with greater ease (Sawyer, 2006). A chemoenzymatic approach to the synthesis of non-ribosomal peptides and polyketides were previously reported where the combination of synthetic chemical tools and recombinant metabolic enzymes aimed to generate the structurally demanding compounds (Kopp and Maraheil, 2007). This illustrates not only the advantages of integration being to produce optically pure compounds more efficiently and to develop more direct, environmentally friendly routes but also to improve accessibility to more structurally demanding compounds.

A number of issues arise with the true integration of chemistry and biology. The impact that each process may have on the other, requires consideration and characterisation, such as the toxic effect of chemical reagents on enzymes or the presence of proteins inhibiting chemical reactions (Hailes *et al.,* 2007). Additionally, compatibility issues, such as reaction rates, reactant concentrations, optimal pH and optimal temperatures require consideration. Approaches to overcome the problems, may involve compromisation, whereby lower optimal reaction conditions are utilised to enable both reactions to occur. This however, is not ideal with regard to yield and productivity and therefore, approaches to alter

the steps to improve compatibility are considered. For instance, the modification of an enzyme at the genetic level, to withstand the presence of organic solvents or to accept substrates synthesised directly from a chemical reaction, without the need for purification processing (Bruggink *et al.,* 2003). The concept of a 'one-pot synthesis' is defined by the lack of intermediate purification processes required within a cascade reaction system, needless to say within a single reactor vessel (Koeller and Wong, 2000). Ultimately this further addresses the advantages associated with reducing the number of steps within a process, potentially reducing cost and environmental burden. Further more the linking of additional reactions such as a second biotransformation enhances the product potential tremendously.

The development of a process for the synthesis of chiral compounds with high enantiomeric excess is crucial with current regulatory demands within industry including pharmaceutical industry. It is rational to develop a pathway from components that are well established throughout the literature such as a well characterised enzyme with available structural data and activity profiles (Schorken and Sprenger, 1998). Transketolase has been extensively studied, providing excellent access to TK enzymes and established detection methods (Littlechild *et al.,* 1995; Brocklebank *et al*., 1996; Hobbs *et al*., 1996; Mitra and Woodley 1996; Mitra *et al.,* 1998; Morris *et al*., 1996; Hibbert *et al.,* 2007; Hibbert *et al.,* 2008). Also, the synthetic potential of TK has been demonstrated by coupling with a further biotransformation involving aminotransferase enzyme, Transaminase (Tam) (Ingram *et al.,* 2007). Similarly, the chemistry component of the pathway should be well examined and known to be commercially useful, such as the valuable Diels Alder (DA) cycloaddition, which is well reported in a variety of reaction systems with a huge array of potential cycloadducts (Kagan and Riant, 1992; Griffiths and Previdoli, 1993; Nicolaou *et al.,* 2002). The reaction should possess considerable future potential and be particularly applicable for exploitation with the ability to generate intermediates with extensively differing architectures.

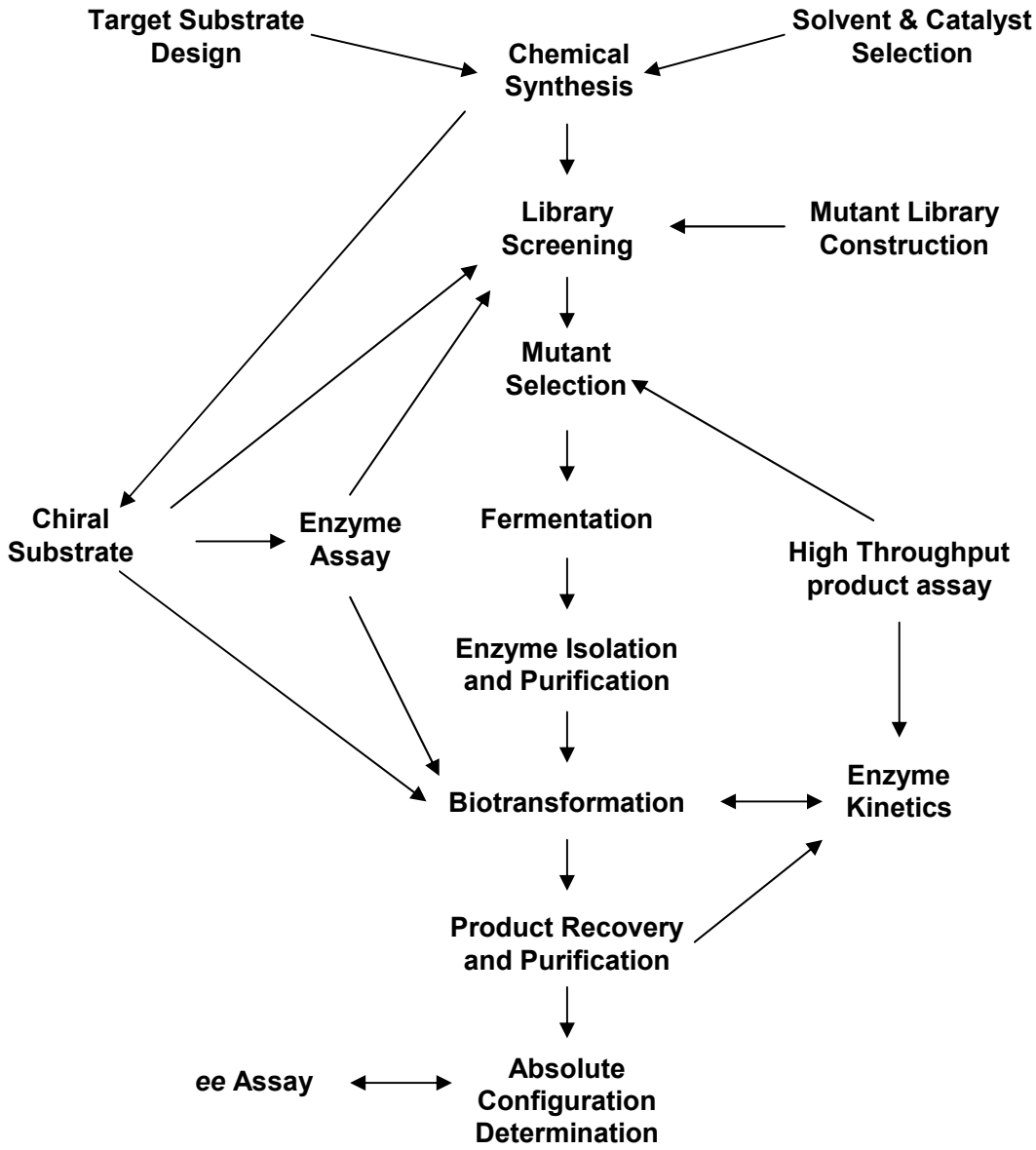
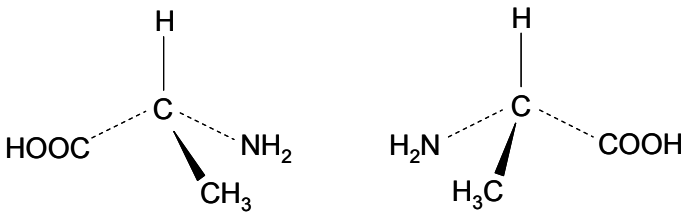


Figure 1.2. Process development for integration.

1.2 Chirality

Chirality is a significant feature of many biologically active molecules requiring much consideration during process design. As early as 1815, Jean-Baptiste Biot, a French physicist, whilst studying the polarisation of light, discovered optical purity. Using liquids such as turpentine and essential oils, he determined that light could be rotated clockwise or counter clockwise, dependent on the optical axis of the organic substance being penetrated. Later on in 1948, Louis Pasteur, rather crudely with a microscope and pair of tweezers isolated two enantiomer crystal forms, recognised by their mirror image of each other. The next era, saw Jacobus van't Hoff and Joseph Achille Le Bel, in 1874 proposing a theory of molecular asymmetry explaining the isomerization phenomenon by the configuration of groups on the tetravalent atom being tetrahedral (Meijer, 2001). This is demonstrated by the enantiomers of an amino acid in Figure 1.3.

The 'handedness' of a molecule can have a dramatic effect on the activity of a drug, where one enantiomer is active towards its target protein, and the other may be inadvertently active elsewhere causing potentially devastating side reactions. Although in many cases the second enantiomer is inactive, the demand for optically pure pharmaceuticals is greater then ever. It is reported that 80% of the compounds currently in development by the pharmaceutical industry are single isomer compounds (Breuer *et al.*, 2004).



D-amino acid L-amino acid

Figure 1.3. Enantiomers of an amino acid. The two molecules have the same components but may not be superimposed onto each other, they are mirror images.

In addition to pharmaceuticals, chiral technology is important to biochemicals, pesticides, aroma and flavourings, dyes and pigments, nonlinear optical materials, and polymers shown in Table 1.1.

Table 1.1: **Enantiomeric compounds with differing biological activity between enantiomers.**

Industry	Enantiomeric compound	Biological Activity
Pharmaceuticals	amphetamine	D-isomer acts as a potent central nervous system stimulant, L-isomer has no effect
	epinephrine	L-isomer is up to 10 times more potent as a vasoconstrictor than D-isomer
	propranolol	S-isomer acts by blocking beta-adrenergic receptors
Aroma/Flavourings	limonene	S-limonene smells like lemons, while R-limonene smells like oranges
	asparagine	D-asparagine tastes sweet, while L-asparagine tastes bitter
	carvone	S-carvone smells like caraway, while R-carvone smells like spearmint
Vitamins	ascorbic Acid	L-isomer is good ascorbic acid (vitamin C), while D-isomer has no such properties
Insecticides	bermethrine	D-isomer is much more toxic than L-isomer

1.2.1 Optically pure pharmaceuticals

Chirality becomes highly significant when the two optical isomers exhibit different chemical behaviours. In the early 1900s, Cushny, saw importance of chirality in pharmaceuticals come to light, where one of the Atropine enantiomers, L-hyoscyamine, possessed significantly greater potency than its D-isomer

(Cushny, 1904). Then, during the 1950s the drug thalidomide, a sedative antimuscurinic agent, was distributed to pregnant women as a drug to alleviate morning sickness. However it was soon realised that the two enantiomers of the compound behaved differently once administered. One remedied morning sickness whilst the other was teratogenic, causing malformation to the foetus, a somewhat devastating example of the administration of a racemic drug without the knowledge of the action of enantiomers.

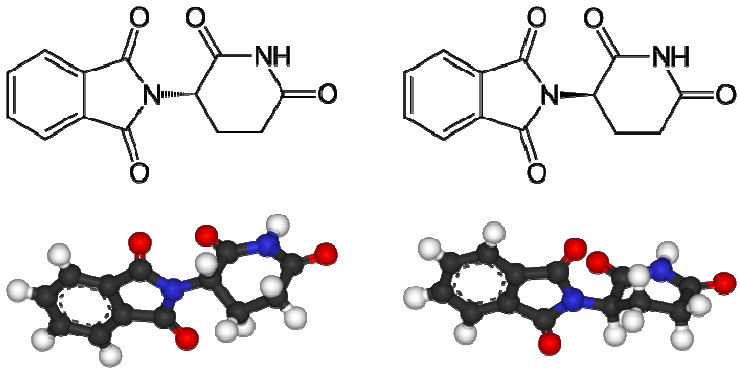


Figure 1.4: Thalidomide enantiomers (Rouhi, 2005).

This problem cannot be resolved by the administration of the single, effective, positively acting enantiomer however, due to the action whereby thalidomide isomers are reversibly converted into each other *in vivo*. This characterisation is therefore critical in the production of pharmaceuticals. Ethambutol, a bacteriostatic antimycobacterial drug prescribed for the respiratory disease, tuberculosis is also known to possess adverse side effects. One enantiomer of the drug successfully inhibits bacterial growth, whilst the other acts on the optic nerve causing, optic neuritis and potentially partial or complete blindness (Kanther, 1970; Tugwell and James, 1972). A further example of extreme differences in the activity of two enantiomers of a drug is amphetamine and methamphetamine. The

D-enantiomer acts on the central nervous system with stimulant effects, whilst the L-enantiomer acts on the peripheral nervous system having drastically differing effects (Tocco *et al.,* 1985).

The administration of drugs is highly dependant on the knowledge of correct doses and upper and lower limitations associated with the biological effects of the drugs. With racemic mixtures, where both enantiomers act identically but exhibit varying potencies, especially in cases where one enantiomer is inactive, theoretically larger doses than required are administered (Cannarsa, 1996), to compensate for the lesser acting enantiomer. The pain killing drug, Ibuprofen (iso-butyl-propanoic-phenolic acid), is an example of such a chiral pharmaceutical, where it is not economically feasible with the current process to isolate the active isomer but to simply double the recommended dose (Stinson, 1994). It becomes of particular consideration where side effects are observed, where increasing the dose may increase the possibility of these side effects and their intensities. Optically pure drugs would see an improvement in the control of pharmacokinetics (Caner *et al.,* 2004).

Apart from increased risk of adverse effects, and possibility that the other enantiomer is toxic, racemic mixtures also possess other undesirable properties. Such properties include the possible interaction with other commonly administered drugs, the lack of specificity of the other enantiomer, and the possible competitive nature between the two enantiomers which could reduce the effectiveness of the drug. The lack of control over the two different enantiomers is commercially unattractive and consequently being more and more closely regulated by agencies (i.e. FDA), which further creates the great demand for enantiomerically pure drugs and other chemical intermediates (Stinson, 1998). The Food and Drug Administration (FDA) of the USA have reported recommendations to provide patients with enantiopure pharmaceuticals and not racemic forms where available (FDA, 1992).

The production of biologically active molecules for use in pharmaceuticals, where restraints have been placed with regard to their optical activity and specificity has therefore instigated the demand and manufacture of more structurally complex

intermediates (Lye and Woodley, 1999). Many drugs that are now in production and use, are chiral and many possess multiple chiral centres. Chirality is increasingly important for pure, valuable compounds such as pharmaceuticals (Caner *et al.,* 2004).

Table 1.2: **Examples of racemic mixtures and the corresponding single-enantiomer products that have been marketed.** (The marketed brand is in brackets, data collected from numerous internet sources).

Racemic drug	Single enantiomer drug
amphetamine (Benzedrine)	dextroamphetamine (Dexedrine)
salbutamol (Ventolin)	levabuterol (Xopenex)
bupivacaine (Marcain)	levobupivacaine (Chicaine)
omeprazole (Prilosec)	esomeprazole (Nexium)
zopiclone (Imovane)	eszopiclone (Lunesta)
cetirizine (Zyrtec / Reactine)	levocetirizine (Xyzal)
ofloxacin (Floxin)	levofloxacin (Levaquin)

In simple terms, two general options are available for consideration, namely, the 'chiral approach' and the 'racemic approach'. The chosen approach is dependent largely on the target intermediates and the planned development time frame, costs and scale up feasibility of specific processes (Francotte, 2001; Francotte and Linder, 2006). The chiral approach involves the synthesis of the single enantiomer through specifically designed methodologies such as stereoselective catalysis using synthetic chiral transition metal complexes, organocatalysts, enzymes and chiral pool synthesis.

1.2.2 Enantioselectivity

On synthesis of a chiral compound it is necessary to determine the optical purity. The enantiomers of a compound will each rotate plane, polarized light in opposite directions and when a compound contains an equal mixture of enantiomers, termed 'racemic' no optical activity is exhibited, (the net rotation of plane-polarized light is zero). When the balance of enantiomers in a compound is unequal, light is rotated in relation to the proportions and is known as the enantiomeric excess (ee).

Enzymes which are chiral, often distinguish between the two enantiomers of a chiral molecule. There has been an increasing awareness of the potential of both microorganisms and enzymes for the transformation of synthetic compounds with high chemo, regio- and enantio- selectivity (Roberts *et al.,* 1995). Cyanohydrins, acyloins (α-hydroxy ketones), α- hydroxy acids and aldols (β-hydroxy ketones) were very efficiently synthesised enantioselectivity using carbon-carbon bond forming enzymes (Sukumaram and Hanefield, 2005).

1.3 Carbon-carbon bond forming

Carbon-carbon bond formation is a significant process in the synthesis of intermediates for the production of pharmaceuticals, and lies at the heart of organic synthesis. Synthetic carbon-carbon bond forming reactions include a well utilised Diels Alder reaction, which has been well studied and numerous processes are well established within industry (Nicolaou *et al.,* 2002).

Several enzymes are capable of carbon-carbon bond forming reactions, and many are involved in important industrial processes. N-acetylneuraminic acid aldolase (NcuA: EC 4.1.3.3) catalyses the carbon-carbon bond forming synthesis of sialic acid through the reversible addition of pyruvate to N-acetyl-D-mannosamine (Fessner, 1998). The importance of utilising such enzymes is recognised by the large range of processes involving compounds like sialic acids, such as cell-cell recognition during development and differentiation and tumour metastasis. The

ability of NcuA to accept a variety of substrates enables the synthesis of a whole host of derivatives. It demonstrates the significance of the development of enzymes for the synthesis of complex, multifunctional compounds that are otherwise difficult to achieve.

An enzymatic carbon-carbon bond synthesis is also demonstrated by the transferase class enzyme, transketolase, which has become a key enzyme within rational engineering and directed molecular evolution studies (Morris *et al.,* 1996; Woodley *et al.,* 1996; Hibbert *et al.,* 2007). The high stereoselectivity of transketolase reported, and the relatively low substrate specificity describe the characteristics of a potentially viable biocatalyst for asymmetric carbon-carbon bond synthesis of structurally diverse compounds (Littlechild *et al.,* 1995; Morris *et al*., 1996; Koeller and Wong, 2001).

1.4 Biocatalysis

Biochemical catalysts providing the appropriate kinetic environment for a chemical reaction to be initiated and proceed are suitably named biocatalysts. Thousands of biocatalytic processes go on within living organisms, enabling the formation of essential compounds from the substrates that are consumed, for example, as food. Biocatalysis having been used for some of the oldest chemical transformations known to man, including fermentation for production of beer, are now being realised for their huge potential in a numerous variety of areas. More recent uses for biocatalysts are being studied for structurally complex pharmaceuticals where they have been either impossible or extremely difficult to develop via conventional chemical synthesis. 'Biocatalysis can be defined as the application of a biocatalyst to achieve a desired conversion under controlled conditions in a bioreactor' (Krieger *et al.,* 2004).

Application in industry of biocatalysts has become widespread with the demand for complex products including pharmaceuticals, agrochemicals and plastic materials (Schmid *et al.,* 2001; Schmid *et al.,* 2002; Pollard and Woodley, 2007). For example, the synthesis of an optically pure carbonic anhydrase inhibitor

TRUSOPT, by Merck for the treatment of glaucoma, employed two biocatalytic steps instead of a complex chemical synthesis (Kim *et al.,* 2007). The further demand for enzymes with characteristics able to act on alternative substrates, and the demand for more complex molecules such as chiral products provides a greater need for biocatalysts due particularly, to their exquisite enantioselective ability (Adam *et al.,* 1999; Caner *et al.,* 2004). The development of processes integrating biocatalysis is therefore, essential for the next generation of greener, more efficient, industrial processes and requires improvement of product yield to become feasible. The development of biocatalysts with respect to the environments that reactions may be performed, i.e. organic solvents, pH, temperature, that were previously detrimental to the catalyst, have increasingly benefitted biocatalytic processes and therefore facilitated the progression of more sophisticated synthetic systems (Adams *et al.,* 1995; Schmid *et al.,* 2001).

Biocatalysts can take the form of isolated, purified enzymes, enzyme complexes, or whole cells (lysate or intact whole cells) (Krieger *et al.,* 2004). Within industry, isolated biocatalysts are mainly hydrolytic or isomerisation enzymes, whereas whole cells are utilised for synthetic reactions which necessitate the need for cofactors that may be regenerated in a whole cell system, eradicating the need for continual feeding of cofactors, thereby reducing costs (Schmid *et al.,* 2001).

1.4.1 Advantages and disadvantages of biocatalysis

A particularly useful characteristic of a biocatalyst is the ability to take part in a reaction and remain unchanged and therefore be available for further substrate conversion. Regenerated cofactors create an advantageous system reducing both costs and process time by eliminating purification and washing steps and the need for new cofactors for each batch.

Biocatalysts provide a process that may be a single or two step scheme, whereas the alternative chemical synthesis may require a multitude of stages with complex, costly separation, cleaning and purification protocols. The need for protection and

deprotection steps required in chemical synthesis is often eliminated with the use of biocatalysts (Lye *et al.,* 2002; Sheldon and Rantwijk, 2004). As a consequence of fewer steps in biocatalytic processes compared to conventional chemical syntheses there may be less waste and lower emissions, potentially reducing environmental burden.

Biocatalysts also boast an environmentally friendly nature, compared to their conventional chemical counterparts (Pereira *et al.,* 2005). This is due to the mild conditions the enzymes are able to perform in, relating to the biological origin of the catalyst (Sheldon and Rantwijk, 2004) resulting in a reduction in the use of organic solvents and an increased use of renewable resources (Schmid *et al.,* 2001). For example, a particular reaction within the body where fatty acids are oxidised to form carbon dioxide and water, the reaction conditions are mild i.e. body temperature and pH, and the reaction runs efficiently and rapidly. However, when the reaction is performed synthetically, high temperatures, extremes of pH and hazardous chemicals are required. There also remains the advantage that the enzyme is less likely to produce undesirable and/or toxic by-products that may be seen regularly in the chemical synthesis (Schmid and Verger, 1998).

The mild conditions associated with biocatalysts, also provide a means of eliminating problems that often afflict conventional methods. These problems include undesirable side reactions such as isomerisation, racemisation, epimerisation and rearrangement, which will affect the product yield and therefore the efficiency of the reaction (Burton *et al.,* 2002). Improved environmental impact may also be attributed to the fact that the enzyme, through conformational change in the protein structure, lowers the activation energy required for a reaction to occur, whereas chemical synthesis requires an external source of energy, such as heat.

A significant advantage associated with the use of biocatalysts is the ability to produce optically pure, enantioselective products. The enzyme preferentially discriminates between enantiomers during a reaction, related to the shape of the molecule and specific interactions within the enzyme active site. This is another

factor that afflicts the chemical synthesis, which would require further racemisation and/or kinetic resolution steps.

An important feature which many enzymes inherit from their natural environment is the sensitivity to both substrates and products, in particular at critical concentrations. Often, high product concentrations within a reactor will become toxic or inhibitory to the enzyme. This may be due to a feedback mechanism of the cell of origin to switch off the enzyme when the product is at optimal concentrations within the cell. This feature inevitably causes limitations within a process and therefore often requires solutions to realise optimal productivity.

Drawbacks of the use of biocatalysts are associated largely with the production of the enzyme and their stability. The production of the enzyme becomes difficult with particularly labile enzymes. The reputation remains that the immobilization to stabilise enzymes is effective yet costly, and the stability of the enzymes is 'inherent to their complex structure' (Llanes, 2001).

1.4.2 Ideal biocatalysts

Designer biocatalysts are becoming increasingly commonplace throughout industry, enabled by the use of recombinant DNA technology. Further to the advantages associated with the use of enzymes in synthesis, is the potential held by enzymes to be adapted and manipulated genetically (Burton *et al.,* 2002). The development of several techniques such as directed mutagenesis, has provided, and will continue to do so, the numerous industries with an increasing supply of specific, process-compatible biocatalysts. The observation that the range of reactants is not limited by the original metabolism of the biocatalyst signify that they are able to transform both natural and non-natural compounds is amplified by such processes (Held *et al. ,*2000). The alteration of specific amino acid residues within an enzyme gene, based on a particular approach, such as phylogenetic or structural, followed by screening and characterisation is a useful tool for the development of the biocatalyst pool. An expanding repertoire of enzymes with a large range of capabilities and characteristics is hugely valuable.

'Ideal' biocatalysts for industry would possess high specific activity, high storage and process stability, and would also be unaffected to issues such as substrate and product inhibition (Hibbert *et al.,* 2005). Enzyme specific activity may be quantified by k_{cat} and specificity k_{cat}/K_m and are significant parameters requiring characterisation for the determination of the applicability of a reaction. The degree of substrate specificity is a significant parameter requiring characterisation to realise its potential for synthetic transformations (Woodley, 2006). Together with these parameters, the cost of effectiveness of the biocatalyst largely determines its synthetic utility at industrial scale, determined by the kinetic parameters and its production.

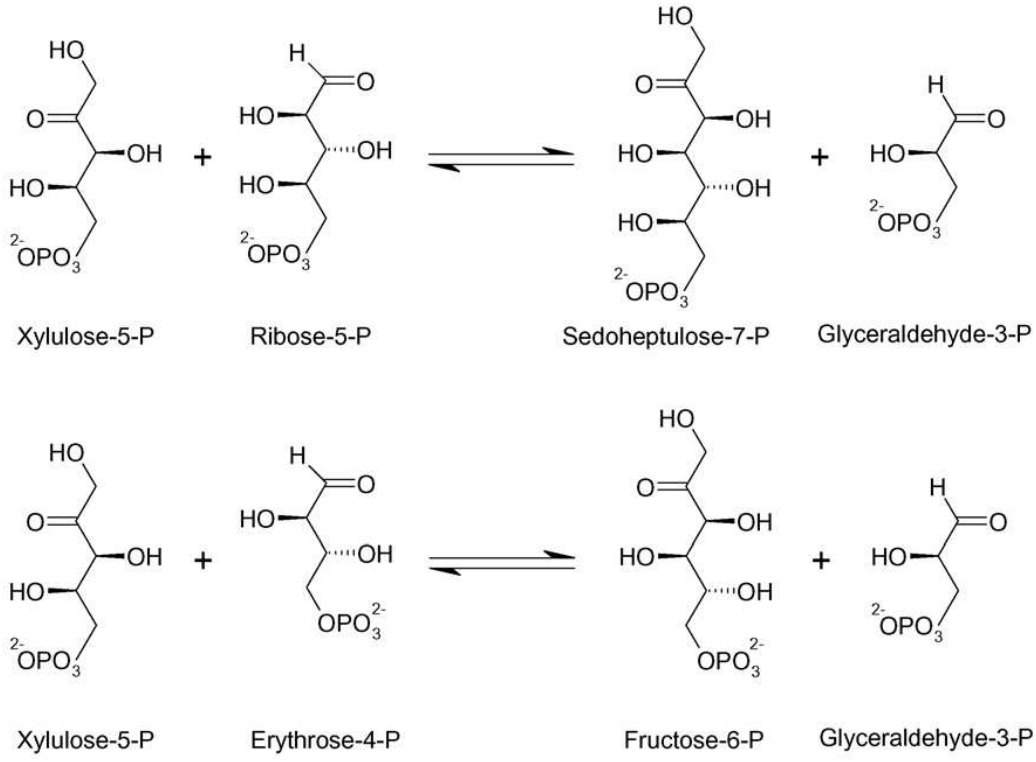
1.4.3 Biocatalysts in Industry

The transfer of techniques established in the laboratory to industrial scales involves a number of considerations. The effectiveness of a biocatalyst in terms of selectively and activity, must remain as high as possible with the scale up of substrate and subsequent product concentrations. Many processes to date have been engineered successfully and are currently in use for production of a wide range of fine chemicals (Shulze and Wubbolts, 1999; Panke *et al.,* 2004), including those used in the food industry (Hecquet *et al.,* 1996), pharmaceuticals (Hecquet *et al.,* 1994), agriculture and paper and dye production among many more. The wide range of enzymes available, i.e. oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases has enabled this flexibility, providing a means for many syntheses. The synthesis of aspartame, an artificial sweetener, is an example of a bioprocess within the food industry employing biocatalysts, where the precursor, L-phenylalanine, an aromatic amino acid, is synthesised by a thermolysin catalysed reaction (Oyama, 1985). The pharmaceutical industry, of particular relevance here, utilises biocatalysis for numerous drug synthesis processes, such as anticancer, antiviral, antihypertensive, anti-Alzheimer's drugs and for example the synthesis of semi-synthetic β-lactam antibodies with the use of acylases (Schoemaker *et al.,* 2003). A further example of the production of chiral products for the pharmaceutical industry includes the generation of S-ibuprofen by Sepracor, using a lipase biocatalyst from *Candida cylindracea*

(Krieger *et al.,* 2004). The increasing use of biocatalysts within the food industry is instigated by the demand for natural flavourings, i.e. not compounds made synthetically (Schrader *et al.,* 2004; Cheetham, 1997). Vanillyl-alcohol oxidase (VAO) is a versatile biocatalyst used in the enantioselective production of the flavouring vanillin (van den Heuvel *et al.,* 2001; van den Heuvel *et al.,* 2004).

1.4.4 Transketolase (TK)

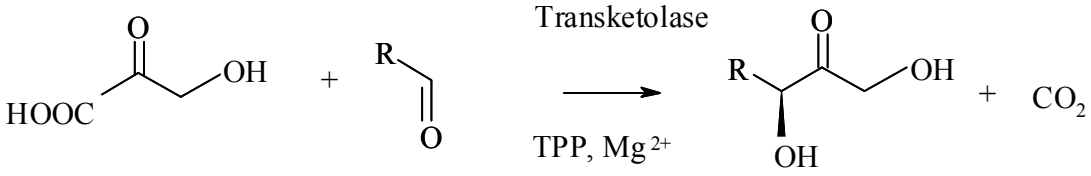
Transketolase, (TK; E.C. 2.2.1.1) a thiamine dependant enzyme, catalyses the formation of carbon-carbon bonds within the non-oxidative branch of the pentose phosphate pathway in animals and the Calvin cycle in plants. In addition to the cofactor thiamine diphosphate, transketolase also requires divalent metal ions such as Ca^{2+}, Co^{2+} and Mg^{2+} (Racker *et al.,* 1953).



Scheme 1: Natural TK Reactions. TK catalysed reactions of the oxidative pentose phosphate pathway.

A broad range of substrates have been reported for TK's from yeasts, plants (Kobori *et al.,* 1992) and bacteria (Sprenger *et al.,* 1995) and, since the products of these enzymes have been seen to serve as precursors for a number of synthetic compounds, the enzyme is being exploited for industrial application (Wohlgemuth *et al.,* 2009). TK catalysed reactions have been seen as dominant tools in the toolbox of routes to sugars, sugar analogs, glycoprocessing enzyme inhibitors and carbohydrate probes (Takayama *et al.,* 1997). The compounds produced using TK are also useful building blocks for conversion to other chiral synthons such as aminodiols (Schmid *et al.,* 2001; Ingram *et al.,* 2007).

E. coli TK was successfully overexpressed (Lilly *et al.,* 1996) and an irreversible reaction was made possible by the use of ketone donor, hydroxypyruvate, due to the release of CO_2 driving the equilibrium to completion (Srere *et al*., 1958).



Scheme 2: **The transketolase catalysed reaction using hydroxypyruvate (HPA) in the presence of cofactors, thiamine pyrophosphate (TPP) and Mg^{2+}.**

1.4.4.1 Synthetic potential of transketolase

The synthesis of carbohydrates and sugars and important intermediates for the investigation of metabolic diseases are a significant example of the synthetic potential of transketolase (Shaeri *et al.,* 2006). The conventional chemical synthesis of the particular metabolite, D-xylulose-5-phosphate (X5P) requires numerous steps including protection and deprotection processing causing low product yields (Breuer and Hauer, 2003), instigating alternative routes of production (Sprenger *et al.,* 1995; Sprenger and Pohl, 1999; Zimmerman *et al.,* 1999; Faber and Patel, 2000;). A two step enzymatic pathway utilising transketolase and triosephosphate isomerase has been characterised and operational guidelines determined (Shaeri *et al.,* 2008).

The synthesis of aromatic amino acids L-tryptophan, L-tyrosine and L-phenylalanine from D-glucose by TK-catalysed reactions (Frost and Draths, 1995), are examples of its use within a range of industries L-tryptophan is useful in the medical, food and animal feed industries, and also used as a precursor in the synthesis of the dye, indigo (Draths and Frost, 1991). L-tyrosine is used as a precursor for a UV-absorbing substance, eumelanin and L-phenylalanine is further transformed within the food industry for the production of aspartame, an artificial sweetener, and is also used within the pharmaceutical industry (Shenck *et al.,* 1998).

TK has also been employed within the flavour and aroma industries, due particularly to the demand for 'natural' flavourings and aromas. The use of biocatalysts for the synthesis of these compounds is stoked by this demand and the premium price allocated to such food additives. A compound with a caramel-like flavour, furaneol, is made though a chemoenzymatic synthesis involving a TK-catalysed reaction forming the furaneol precursor, 6-deoxy-L-sorbose (Schenk *et al.,* 1998; Turner, 2000).

Chiral amino alcohols are potentially synthesised by a multi-enzyme pathway of transferase enzymes, transketolase and transaminase. The products of the

transketolase catalysed reaction have been seen to be accepted by transaminase for optically active chiral amines with multiple chiral centres (Ingram *et al.,* 2007). The demand for both amino acids and optically pure non-proteinogenic amines from the nutrition and pharmaceutical industry is ever increasing in line with the demand for optically pure compounds in general. The large scale synthesis of single enantiomer peptidomimetic drugs calls for the synthesis of chiral amines as precursors, for example, L-tert-leucine in an HIV protease inhibitor as part of AIDS therapy (Kempf *et al.,* 1991) and D-phenylalanine in a remedy for thrombosis (Bajusz *et al.,* 1990).

1.4.4.2 TK function *In vivo*

In vivo, TK catalyses two reactions of the metabolic pathway which utilises free energy of metabolite oxidation in order to generate NADPH for reductive biosynthesis. The pathway also serves to synthesise ribose-5-phosphate for the synthesis of both nucleotides and nucleic acids.

In the first reaction a thiamine diphosphate-mediated reversible transfer of a 2-carbon unit from D-xylulose-5-phosphate to D-ribose-5-phosphate affords sedoheptulose 7-phosphate. In the second reaction, also thiamine diphosphate mediated, the transfer of a 2-carbon unit from D-xyulose-5-phosphate to the aldose erythrose-4-phosphate, affording fructose-6-phosphate and glyceraldehydes-3-phosphate.

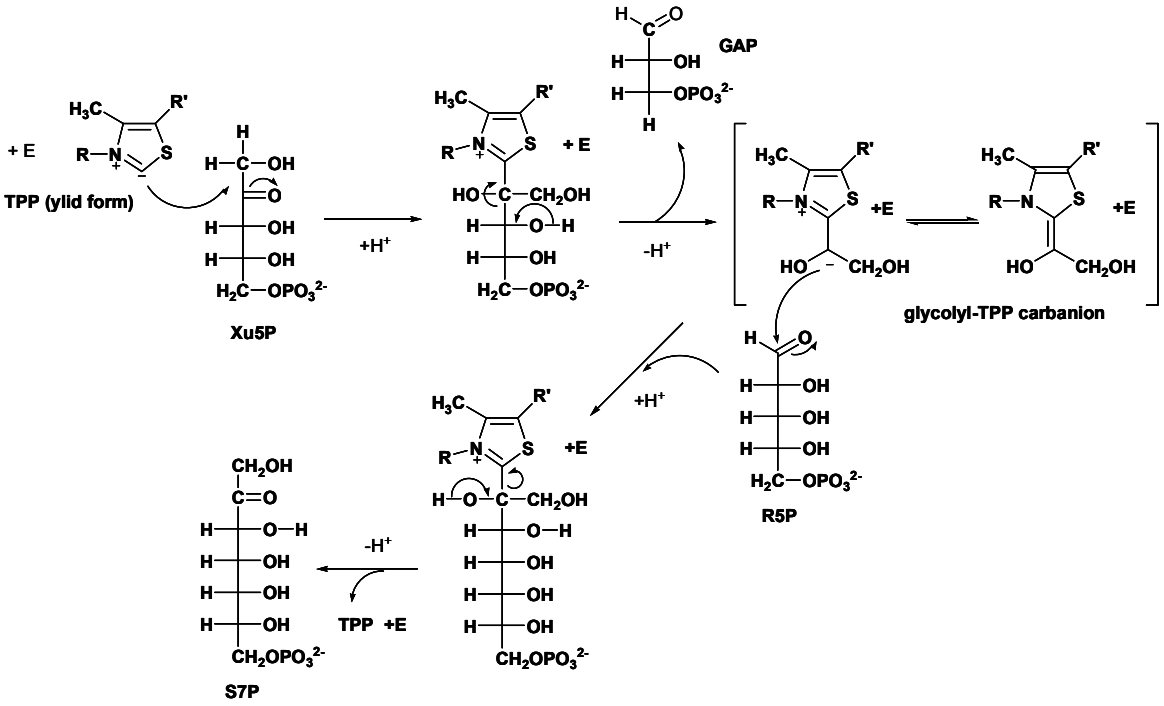
1.4.4.3 Transketolase mechanism

The catalytic mechanism of transketolase, particularly TK from yeast, has been well studied including subunit interactions and cofactor binding (Datta and Racker, 1961, Nilsson *et al.,* 1997, Fiedler *et al.,* 2001). The apo-enzyme of TK requires two cofactors, TPP and divalent metal ions to become the holo-enzyme which exists as a dimer. The active sites of the enzyme are formed at the interface

of the monomers and TK is therefore only active as a dimer. Each monomer or subunit has a molecular weight of 73 kDa (Brocklebank *et al.,* 1999).

X-ray studies of the transketolase structure indicated each subunit consisting of three domains, the N-terminus or PP-domain (residues 3-332), the middle or Pyr-domain (residues 323-538), and the C-domain (residues 539-680). Whilst reports suggest the N-terminus and Pyr-domain are involved in cofactor binding, it is unclear of the function of the C-domain (Kochetov, 2001).

Transketolase binds TPP in a 'V' conformation, which is energetically less favourable and is activated by the deprotonation of the C2 atom of the thiazolium ring of TPP which has been indicated as necessary for catalysis (Schenk *et al.,* 1998). This C2 deprotonation yields a carbanion which then attacks the carbonyl group of the ketol donor (nucleophilic attack). The C-C bond cleavage yields the aldose molecule and an enzyme bound TPP with a two carbon fragment attached (a resonance stabilised C2 carbanion). A new C-C bond is formed by the subsequent attack of the C2 carbanion on the terminal carbon of the acceptor aldehyde substrate. The TPP is subsequently available for the next reaction (See scheme 3).



Scheme 3. Transketolase mechanism. (E) transketolase enzyme (Voet & Voet, 2004).

1.4.4.3.1 Thiamine pyrophosphate (TPP)

Transketolase is dependent upon the presence of the cofactor thiamine pyrophosphate (TPP) also known as thiamine diphosphate (ThDP) shown in Figure 1.5. Thiamine diphosphate consists of an aromatic methylaminopyrimidine ring which is connected to a methylthiazolium ring via a methylene bridge. The thiazole ring contains nitrogen and sulphur atoms with a diphosphate functional group bound to the hydroxyethyl side chain. The nitrogen and sulphur within the thiazole ring portion of TPP, act by donating a proton and forming a carbanion.

Previously, studies involving the catalytic activity of thiamine pyrophosphate demonstrated that the thiazolium ring alone can in fact catalyse reactions similar to TPP dependent enzymes, however at much slower rates (Krampitz, 1969; Schenk *et al.,* 1998).

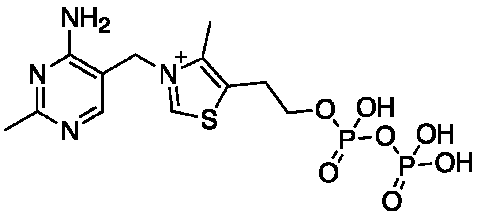


Figure 1.5: Thiamine pyrophosphate (TPP).

1.4.4.4 Sources of TK

Transketolase has been located in varying quantities within all organisms to date. Sources include yeast (Kotchetov, 1982), rat and pig liver (Paoletti and Aldinucci, 1986; Kotchetov, 1982), *E .coli* (Sprenger, 1991) and spinach (Demuynck *et al.,* 1990). Previously, for scientific purposes TK was sourced mainly from spinach

and *Saccharomyces cerevisiae* until more recently when the TK gene was successfully overexpressed in recombinant *E. coli* (Draths and Frost, 1990; French and Ward, 1995). This allowed for the production of TK in large quantities and at a relatively low cost in fermentations up to 1000 L scale (Hobbs *et al.,* 1996).

1.4.4.5 TK production

The large scale production of TK though fermentation of *E. coli* has been successfully implemented and bench scale fermentations are possible to yield sufficient quantities of biocatalyst. DNA technology has enabled high protein productivity utilising fermentation where the micro-organism is manipulated to over produce (over-express) a target enzyme. A recombinant micro-organism often consists of a host organism, such as *E. coli* and a vector, such as a plasmid. The plasmid, a DNA molecule, is modified by the insertion of a specific gene encoding the target protein, for instance an enzyme such as transketolase. Analogous to naturally occurring processes, the micro-organism replicates in the correct conditions along with the plasmid vector, of which the organism is hosting. Over-expression of the inserted gene is amplified by the ability of the plasmid to replicate up to 1000 times more than the host. The cost and simplicity of producing the enzyme is therefore attractive for industrial processes.

Release of the enzyme from the intracellular environment of the bacterium has been demonstrated by sonication (Bonsignore *et al.,* 1962) for smaller processes and homogenisation (Gray *et al.,* 1972) for larger volumes for improved consistency.

1.4.4.5.1 TK purification

In order to purify the enzyme from contaminants and other native enzymes potentially acting in similar ways to the target enzyme, a specific procedure is required. A number of methods exist, such as, ammonium sulphate fractionation, protein specific antibodies, SDS-PAGE and size exclusion chromatography. A

more specific and somewhat simpler method however, involves affinity chromatography. A method whereby a polyhistidine-tag (his-tag or his_6-tag) which is an amino acid sequence in proteins consisting of up to 6 histidine residues is attached to the N- or C-terminus of the protein. The procedure is often used for affinity purification of recombinant proteins that are expressed in *E. coli* or other prokaryotic expression systems. The process works by the specific binding of the his-tag to nickel groups of Ni-NTA (nickel-nitrilotriacetic acid) resins within a column, followed first by washing and then elution from the column providing a purified enzyme solution.

The application of the his-tag / Ni-NTA technology also lends itself well to enzyme immobilisation, a process used within biochemical engineering to overcome an array of process limitations (Krenkova and Foret, 2004). Such as, increased biocatalyst stability, increased durability, potential re-use of the biocatalyst, optimal control of the biocatalyst within solution, where it may be removed rapidly from contaminants and simplified removal from product solutions (Chibata, 1978). The stability of enzymes within industrial processes is a problem being faced more widely, as the advantages of biocatalysts become recognized. During industrial processes where high substrate concentrations are required and high ionic environments are concerned, proteins become denatured and processes become unfeasible. Immobilisation has been investigated and in many cases found to increase operational stability of the biocatalyst. The attachment of the protein to solid interfaces of films or coatings can result in a more stable protein structure and therefore more active and efficient biocatalysts. Several studies have reported reduced thermal denaturation of immobilised enzymes (Brocklebank, 1999) and is often thought to be due to the rigidification of the proteins tertiary structure.

1.4.5 Enzyme modification

The application of enzymes within industrial processes for synthesis of specific intermediates relies upon the ability of the enzyme to act on desired substrates and within certain reaction conditions. To enable the continued growth of biocatalysis application within further disciplines and a wider range of industries, the pool of available enzymes must be augmented. An alternative to searching for or creating enzymes with specific properties for target compound synthesis is the enhancement of existing enzymes. Modifying enzyme characteristics such as activity, substrate specificity or selectivity, pH optimum, stability in organic solvents and thermostability are ways in which the advantages of biocatalysis can be better suited to reactions conditions and requirements that are commercially useful. A number of approaches currently exist to take advantage of the capacity of enzymes to embrace modification for improvement.

1.4.5.1 Enzyme evolution

In biology, enzymes successfully evolve, and adapt at the molecular level. Evolution is a natural process within an organism's population, where inherited traits change from one generation to the next on the basis of improvement with regard to survival and reproduction. The evolution of enzymes has created a huge array of diverse proteins capable of performing within a diverse range of environments. This diversity provides a reservoir of enzymes to choose from for industrial applications that may require, for example high temperatures or acidic process conditions. Some enzymes will possess these characteristics naturally and may be known as extremozymes (Kirk *et al.,* 2002; Straathof *et al.,* 2002; Robertson and Steer, 2004). These enzymes have successfully adapted to extreme environments such as temperatures (hyperthermophiles), high salt (halphiles), pH (acidophiles and alkaliphiles) and pressure (barophiles).

The natural evolution of enzymes may be exploited and performed within the laboratory, providing enzymes with often specific properties. Powerful recombinant DNA technology and enzyme-screening technology enable the

development of ‘designer biocatalysts’ providing ‘ideal biocatalysts’ (Cowan *et al.,* 2004; Kourist *et al.,* 2008).

1.4.5.2 Directed evolution

Directed evolution is a powerful algorithm aiming to modify proteins, introducing mutations at the molecular level potentially introducing desirable properties, not otherwise found naturally (Schmidt-Dannert and Arnold, 1999; Schmid *et al.,* 2001; Zhao *et al.,* 2002; Dalby, 2003). This powerful tool may be implemented within the laboratory, where recombinant organisms are utilised and their amino acid sequence modified though alteration of the DNA sequence. Within a short period of time compared to the lengthy process of natural evolution, just a matter of days, a generation of mutants can be made, screened and selected.

It is true, however, that the complexity of a protein amino acid sequence holds back the process of simply changing a sequence for a target property. The stability of enzymes, for example relies upon the well orchestrated folding and specific arrangement of amino acids. The successful improvement of one characteristic such as enhanced activity, may be detrimental to this protein stability. Mutagenesis studies enable the identification of protein structure and function and the involvement of particular amino acid residues. It subsequently enables DNA, RNA and protein design with specifically altered stabilities, activities, enantioselectivities and tolerance to organic solvents to name but a few (Miyazaki and Arnold, 1999).

Directed evolution is based upon the generation of diversity proceeded by the screening of target characteristics, such as substrate acceptance and the selection of these mutants. The technology has been exploited as a more general approach to the alteration and improvement of enzyme characteristics, through random mutagenesis and recombination (Arnold *et al.,* 2001; Sylvestre *et al.,* 2006). Indeed saturation mutagenesis of selected sites has also proven to be a successful strategy for improving enzyme properties (Miyazaki and Arnold, 1999; Shinkai *et al.,* 2001; Goud *et al.,* 2001; Hayes *et al.,* 2002; Santoro and Schultz, 2002;

Peimbert and Segovia, 2003; Mullegger *et al.,* 2005; Ashworth *et al.,* 2006; Hibbert *et al.,* 2007).

The directed evolution process begins with the selection of an enzyme with the ability to catalyse a required reaction system for synthesis of target compounds. Appropriate reaction conditions may then be optimized, and the enzyme is then subjected to an *in vitro* directed evolution cycle. Through screening of the generated mutants for improved or new activity, mutants are selected and further directed evolution cycles may be applied to create a more stable and robust biocatalyst (Schmidt-Dannert, 2001; Walsh, 2001; Dalby, 2003).

The enantioselectivity of an enzyme is a significant target characteristic for enhancement using such processes as directed evolution. For instance, a directed evolution process applied to a lipase-catalysed hydrolytic kinetic resolution of a chiral ester improves the enantioselectivity of 2% ee to >90% ee (Reetz and Jaeger, 2000). The use of enzymes such as TK for studies involving mutagenesis is beneficial due to the considerable knowledge already reported. Several transketolase protein sequences in conjunction with a number of crystal structures are available including transketolase from *S. cerevisiae* both with and without the substrate bound and also from *E. coli* (Littlechild *et al.,* 1995).

'Site specific' mutagenesis (rational design) is used to describe the method where a single base is targeted within a very small known sequence of DNA (Reetz and Carballeira, 2007) as opposed to random base mutations throughout the coding gene (Kazlauskas, 2005; Paramesvaran, 2009). This method is used for the determination of protein structure and function as well as altering or improving enzyme function. The basic procedure relies upon the design of a sequence specific oligonucleotide or primer containing the desired base change. This primer is hybridized to a single stranded DNA containing the target protein gene, and the primer extended enzymatically with DNA polymerase. The subsequent double stranded gene containing the base change is inserted into a chosen host cell and cloned. A significant advantage associated with site directed mutagenesis is the relatively small number of mutant libraries required for the identification of improved properties in well known protein structures (O'Fagain, 2003).

Polymerase chain reactions, known as PCR, methods are now used increasingly for the amplification of DNA strands containing specific mutations.

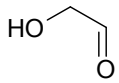
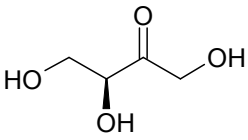
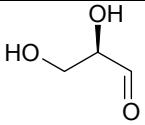
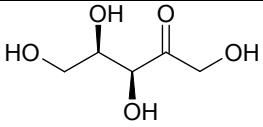
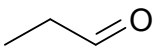
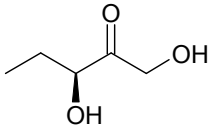
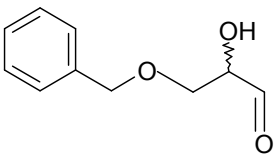
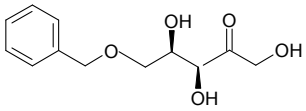
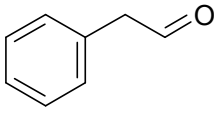
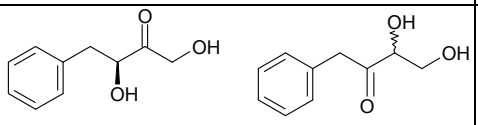
1.4.6 TK biotransformation

1.4.6.1 Reactants and products

In vivo, ribose-5-phosphate is the readily accepted α-hydroxyaldehyde donor molecule by TK within the pentose phosphate pathway. The synthesis of both phosphorylated carbohydrates (structurally homologous to its natural substrates) (Srere *et al.,* 1958; Mocali *et al.,* 1985) and non-phosphorylated carbohydrates (Villafranca and Axelrod, 1971; Demuynck *et al.,* 1990) have been reported. Many acceptor aldehydes were investigated for activity with transketolase, predominantly α-hydroxyaldehydes (Effenberger *et al.,* 1992; Kobori *et al.,* 1992) until a broader range of aldehydes were investigated (Demuynck *et al*., 1991). The subsequent investigation into substrate tolerance using *E. coli* Transketolase, highlighted the 'promiscuity' of the enzyme by its notable lack of specificity for aldehyde acceptor substrates compared to Yeast TK.

Preliminary studies using yeast and spinach TK with a variety of aldehydes enabled some conclusions to be drawn with regard to the acceptor aldehyde. It was seen that the presence of a hydroxyl group or an oxygen atom at the α-carbon or/and β-carbon enhanced relative rates. Also, steric hindrance near the aldehyde group causes a reduction in relative activity. In addition, α,β-unsaturated aldehyde are accepted as TK substrates, benzaldehyde is a poor substrate but the presence of hydroxyl-groups on C2 leads to higher activities. Finally, heterocyclic aldehydes are accepted better than benzaldehyde but low initial velocities were still observed (Demuynck *et al.,* 1991).

Table 1.3: Aldehyde substrates and conversion levels reported in literature.

Acceptor aldehyde substrate	Reaction product (Ketol donor is HPA)	Conversion %	Reference
 Glycolaldehyde	 L-erythrulose	74	Morris *et al.,* 1996 and Hobbs *et al.,* 1993.
 Glyceraldehyde	 Xylulose	37 (racemic substrate)	Hobbs *et al.,* 1993
 Propanal	 dihydroxypentan-2-one	29	Hobbs *et al.,* 1996 and Morris *et al.,* 1996
 3-O-benzglyceraldehyde	 5-o-benzyl-D-xylulose	80	Humphrey *et al.,* 2000
 benzaldehyde	 5-benz-1,3-dihydroxypentan-2-one	33:18	Morris *et al.,* 1996

The transketolase catalysed conversion of carbohydrates is a reversible reaction shown in Scheme 2. It was previously demonstrated (Srere *et al.,* 1958), that β-hydroxypyruvic acid (HPA) was accepted as a ketol donor substrate by TK, driving the equilibrium to completion. The decarboxylation of β-hydroxypyruvate and the subsequent release of CO_2 from the reaction render it an irreversible condensation reaction. This creates a commercially viable reaction and therefore HPA has since been used as a substrate in many transketolase reactions as an alternative to ketose sugars. As one of the TK substrates, measuring HPA depletion is a method of analysing activity, HPLC is a useful technique for this.

Where HPA is utilized for its irreversible quality, a disadvantage stands that a pH change occurs due to the consumption of the substrate, although this is offset by the by-product CO_2 reacting with water to produce H^+ ions, therefore the monitoring and subsequent addition of acid is required (Brocklebank *et al.,* 1996; Lilly *et al.,* 1996).

1.4.7 TK stereoselectivity

Previous studies examined the enantioselectivity of TK-catalysed reactions and found the condensation of α-hydroxyaldehydes with HPA to be specific to aldehyde acceptor molecules with the α-hydroxyl group in the D (i.e. 2*R*)-configuration (Hobbs *et al.,* 1993; Humphrey *et al.,* 1995). Preceding experiments indicated the preference of TK for the D-enantiomer of glyceraldehydes by demonstrating similar activity with this enantiomer to achiral glycolaldehyde, whilst no activity was seen at all with the L-enantiomer. Furthermore, racemic glyceraldehyde (D/L-glyceraldehyde) indicated half the activity exhibited by the stereospecific D-enantiomer substrate shown in Figure 1.6 (Morris *et al.,* 1996). In summary, 1,2-diols in the D-threo configuration are synthesised by TK in the presence of TPP by transfer of a hydroxyketo group from a donor substrate to an acceptor aldehyde.

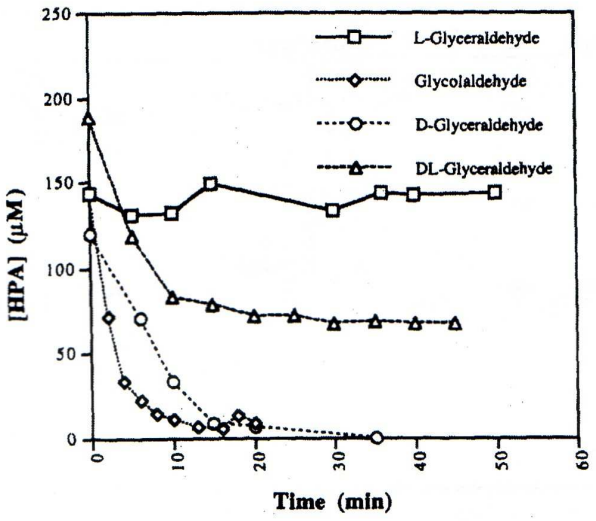


Figure 1.6: Enantioselectivity of *E. coli* towards D-, L- and DL-glyceraldehyde. Activity indicated by the consumption of HPA over time (Taken from, Morris *et al.,* 1996).

Table 1.4: TK Activities towards various substrates relative to glycolaldehyde. (Adapted from, Bolte *et al.,* 1987).

Substrates	$V_{substrate}/V_{glycolaldehyde}$	Products
D-glyceraldehyde 3-phosphate	44	D-xylulose-5-p
D-erythrose 4-phosphate	33	D-fructose-6-p
D-arabinose 5-phosphate	24	D-octulose-8-p
D-glucose 6-phosphate	9	L-erythrulose
D-glyceraldehyde	78	D-xylulose
L-glyceraldehyde	<0.01	-
DL-glyceraldehyde	56	D-xylulose
D-erythrose	56	D-fructose
D-glucose	4	D-octulose

1.5 Diels Alder reaction

The 'Diels Alder reaction' (DA) was discovered by Kurt Alder and Otto Paul Hermann Diels, and earnt them the Chemistry Nobel prize in 1950 (Diels and Alder, 1928). The discovery and subsequent development of this cycloaddition reaction has become an invaluable tool for organic chemists and has since been adopted as the standard method of six-membered ring formation. The reaction is widely used in industry for the construction of cyclic and heterocyclic compounds from simple materials.

The organic chemical reaction, involves the addition of a conjugated diene and a dienophile, forming a cycloadduct more commonly known as a substituted cyclohexene system, giving six new stereocentres in a single step. The most basic of the Diels Alder reactions occurs between the simplest conjugated diene, 1,3-butadiene and the simplest alkene (dienophile), ethene to form cyclohexene shown in Figure 1.7. The variety of starting materials that can be used, leads to an array of useful cycloadducts.

Figure 1.7: Diels Alder cycloaddition reaction proposed mechanism.

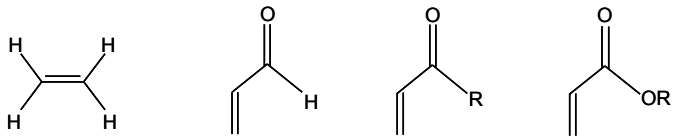
1.5.1 Diels Alder reaction substrates

The driving force behind the reaction involves the formation of two new δ-bonds replacing two π-bonds. The mechanism of the reaction involves a single step with no intermediate. The reaction is concerted and almost synchronous, and is largely

favoured by the presence of electron withdrawing groups on the dienophile and the corresponding presence of an electron-donating group on the diene.

The diene component of the reaction is an intermediate between an alkene (with a single bond) and polyenes (with multiple double bonds). Dienes suitable for the Diels Alder reaction are conjugated (two double bonds separated by a single bond). The diene is reactive in the *s-cis* conformation for the efficient addition to a conjugated dienophile. The corresponding *s-trans* diene is relatively unreactive.

Ethene is the simplest dienophile that has been utilised for the Diels Alder reaction. Acrolein is a commonly used dienophile and is a substrate for the production of cyclohexenecarboxaldehyde. Simply, dienophile means 'diene loving' and therefore shows reactive tendencies in the presence of appropriate conjugated dienes.



Ethene Conjugated carbonyls (aldehydes, ketones and esters)

Figure 1.8: Examples of good dienophiles as substrates for DA reactions.

1.5.2 DA cycloadducts

Cyclohexenecarboxaldehyde is a possible product of the Diels Alder reaction, and is also a potential substrate for the transketolase reaction. Although it has been reported that cyclic-aldehydes (Hobbs *et al*., 1993) were not good substrates for transketolase, advances in enzyme engineering can now aim to broaden the substrate range including these bulky aldehydes thought to cause steric hindrance. Some Diels Alder reactions may be performed enantioselectively providing a

means of supplying stereoselective biocatalysts with chiral starting materials for the potential of 100% conversion with no waste (i.e. unreactive enantiomer) (Thadani *et al.,* 2004).

1.5.3 Solvents and catalysts for Diels Alder reaction

Several studies have looked at the effects of solvents on reaction rate of the DA reaction, in order to determine the actual mechanisms behind the observed kinetic changes. Many solvents have been used to improve reaction rates, however it has been concluded that many of these unusual media were not the cause of the enhanced reaction rate, it was due to the involvement of a Lewis acid catalyst.

It has been well observed over the years that DA reactions performed in aqueous media can vastly increase the reaction rate (Breslow and Guo, 1988; Pindur *et al.,* 1993; Li, 1993). Although this discovery occurred early on in the DA reaction life time, it was not fully exploited and did not receive much attention. However, more recent studies have examined the effect of the media on the reaction rates and much has been considered, including the substituents of the substrates, such as the hydrophobicity of the dienophiles resulting in increased reaction rates (Pindur *et al.,* 1993). The use of water as a solvent for the DA reaction has been largely reported and accelerations of up to 13,000 times compared to organic solvents have been seen (Otto *et al.,* 1996). Proposed mechanisms for this outstanding reaction rate enhancement consist of 1) enforced hydrophobic interaction and 2) hydrogen bonding to the activating group of the dienophile. The evidence that water acts as a powerful catalytic media even without the presence of a catalyst, for the DA reaction prompted experiments using water as the solvent. It has also been observed that selectivity was enhanced when reactions were performed in aqueous media compared to similar reactions in organic solvents (Lubineau and Queneau, 1987; Lubineau *et al.,* 1994).

There are a number of systems used widely to catalyse the DA reaction, in particular, the well known acid catalysts. The acid catalysts act on the dienophile component of the reaction. Protonation or complexation of the dienophile by the

acid catalysts result in an energy decrease and is thought to cause the acceleration and improved selectivity of the DA reaction. Other, more recent systems such as TADDOL (α,α,α',α'-tetraaryl-1,3-dioxolane-4,5-dimethanol), a 'simple, commercially available chiral alcohol', has been developed for specific dienes and without the use of metal substituents, enhances the Diels Alder reaction similarly to the metal-based Lewis acid catalysts (Thadani *et al.,* 2004).

In 1942, the Brønsted acid catalyzed Diels Alder reaction was reported by Wasserman (Wasserman, 1942) however has received less attention than the well established Lewis-acid catalysts (Nakashima and Yamamoto, 2005). A Brønsted acid is a molecular entity that, in the presence of a base or corresponding molecule, is able to donate a proton. This protonating ability causes an activation of the dienophile and has a subsequent catalytic affect. Brønsted catalysts include acetic acid, bromoacetic acid, dichloroacetic acid and trichloroacetic acid.

Utilising chiral Lewis acids for the asymmetric catalysis of Diels Alder reactions is well established in literature (Aggarwal *et al.,* 1995). Lewis acids such as zinc chloride ($ZnCl_2$) and aluminium chloride ($AlCl_3$) act by coordinating to the dienophile substrate, resulting in a more electrophilic complex more reactive towards the diene substrate. The rate of the reaction is often therefore increased. The majority of studies associated with the catalysis of the Diels Alder reaction with Lewis acids, are limited to the use of organic solvents. This is surprising, when in the past it has been observed that the reaction is superior in aqueous media with Lewis acids when compared to organic solvents (Otto *et al.,* 1996). An example of a Lewis acid is nobium pentachloride (Figure 1.9), where previously unreactive dienes and dienophiles are activated by the compound and undergo the Diels Alder mechanism.

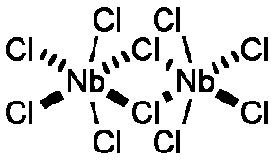


Figure 1.9: Lewis acid: nobium pentachloride structure.

Ionic liquids are also reported as a useful component for the catalysis and solvents simultaneously of DA reactions (Chen *et al.,* 2004). In this project I have used a moisture stable, Lewis acidic, room temperature ionic liquid containing a quaternary ammonium salts and functionalised side chain, $[Me_3NC_2H_4OH]Cl$ (Abbott *et al.,* 2001). The development of this ionic liquid attempted to both improve moisture stability and reduce costs, where aluminium containing imidazolium-based liquids were particularly costly and difficult to make due to the water insensitivity of the aluminium anions. Also, the alteration of the ionic liquid freezing point was investigated so as to reduce it and therefore create a simple and easy to make ionic liquid. The variation of metal anions and use of functionalised side chains successfully produced ionic liquids at 23-25 °C.

1.6 Enzyme kinetics

The investigation of reactions catalysed by enzymes involves the determination of the enzyme kinetics and the effect of reaction conditions such as pH, temperature and ionic strength on reaction rate. Enzyme kinetic mechanisms are generally described by the Michaelis-Menten theory, named after Leonor Michaelis and Maude Menten (Michaelis and Menten, 1913). In simple terms the theory proposes that the reaction proceeds in two steps, the first, a reversible binding of the substrate and enzyme followed by the irreversible reaction (whereby the product does not bind to the enzyme) of the enzyme-substrate complex to form the product and release the enzyme. An additional assumption that the rate of binding is more rapid than the rate of reaction describing that the enzyme-

substrate (ES) complex is in equilibrium with the concentration of free enzyme (E) and substrate (S) at all times.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_2} E + P$$

Where, k_1 represents the rate of the forward reaction of E and S, k_{-1} represents the rate of dissociation of ES to E and S and k_2 represents the rate of formation of product (P) and subsequent release of E.

The first assumption of the mechanism describing the more rapid change in concentration of P and S than ES can be written as:

$$\frac{d[ES]}{dp} = k_1\,[E][S] - [ES]\,(k_{-1} + k_2) = 0$$

The second assumption of the theory that the total enzyme concentration ($[E]_0$) does not change over time and can therefore be written as:

$$[E]_0 = [E] + [ES] = \text{const.}$$

Due to the concentration of substrate changing as the reaction proceeds, the initial reaction rate (v_0) is analysed for simplicity (as it is approximately linear) and can be defined as:

$$v_o = k_2\,[ES]$$

And therefore maximum velocity (V_{max}) can be defined as:

$$V_{max} = k_2 [E]_0$$

The rate of reaction may then be expressed as:

$$v_0 = \frac{V_{max}[S]}{K_m + [S]}$$

Where the Michealis-Menten constant, K_m be written as:

$$K_m = \frac{k_{-1} + k_2}{k_1}$$

The determination of enzyme kinetics largely rely on the determination of the Michaelis-Menten constants K_m and v_{max} (or k_{cat}). Enzyme assays are often performed with small quantities of enzyme, a range of substrate concentrations and detection of product formation over time and characterised using calibration curves with substrate concentrations that exceed the K_m.

Enzyme inhibition issues may also be characterised by Michaelis Menten theory, such as competitive and non-competitive, product and substrate inhibition. The mechanism behind such inhibitory effects is an inherent ability of enzymes for complex control of reaction within natural environment. Reflecting their involvement in complex biochemical pathways *in vivo* for control of homeostasis, enzymes are often inhibited by their own substrates or products, either of which may severely limit the productivity of a biocatalytic process.

1.6.1 Detection methods

Enzyme activity may be detected using many methods of analysis such as chromatography methods including HPLC, GC and TLC. Other methods include NMR, where sequential spectra of the metabolites provide a reaction profile, potentially of both reaction substrates and products and calorimetry, based on the proportionality between the rate of a reaction and the thermal power (heat/time) generated (Todd and Gomez, 2001).

Some enzymatic reactions are detected colorimetrically or fluorometrically where absorbance/fluorescence or optical density is measured by a spectrophotometer. For example, the detection of carbonic anhydrase by carbon dioxide hydration (Livesey, 1977) and an assay for amino acids using ninhydrin (Schwert, 1948). Many reactions involve a colorimetric component attached to the substrate or one of the substrates of the reaction being measured. Such as, a functionality that once reacted emits a specific colour that may then be measured. The amount of colour is then proportional to the amount of product directly.

A reaction may also be detected microbiologically, where the reaction product is used as a subsequent substrate for the growth of a microorganism, and organism growth is measured by optical density. The amount of growth is proportional to the amount of reaction product present.

Traditionally, TK activity was measured using a spectrophotometric assay, involving an NADH dependent enzyme, a method based on a system used by Heinrich and co-workers in 1972 (Figure 1.10). Additionally a pH indication assay using para-nitrophenol was used, and also importantly an HPLC assay based on the detection of residual substrates and formation of product (Mitra *et al.,* 1996; Miller *et al.,* 2007).

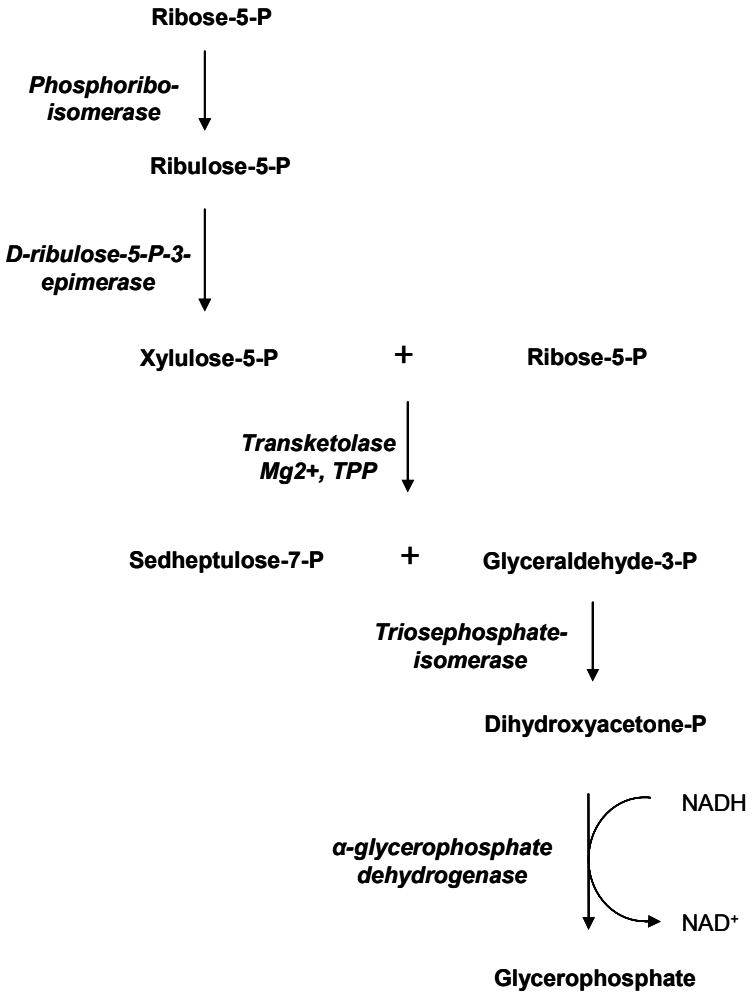


Figure 1.10: Linked enzyme assay for transketolase catalysed reaction product detection.

1.6.1.1 Colorimetric assay

A tetrazolium-red colour assay, shown in Figure 1.11, was developed for the detection of ketodiol products of the transketolase catalysed reaction (Smith *et al.,* 2006). Studies regarding the screening of a large number of novel mutant libraries made though directed evolution techniques, required a simple, sensitive and high-throughput assay (Hibbert *et al.,* 2007). Efforts were targeted to generate TK mutants with enhanced rate of reaction with a variety of acceptor aldehyde substrates including propanal (Hibbert *et al.,* 2008). The assay was developed to quantitatively distinguish between mutants that had improved activity compared to WT-TK.

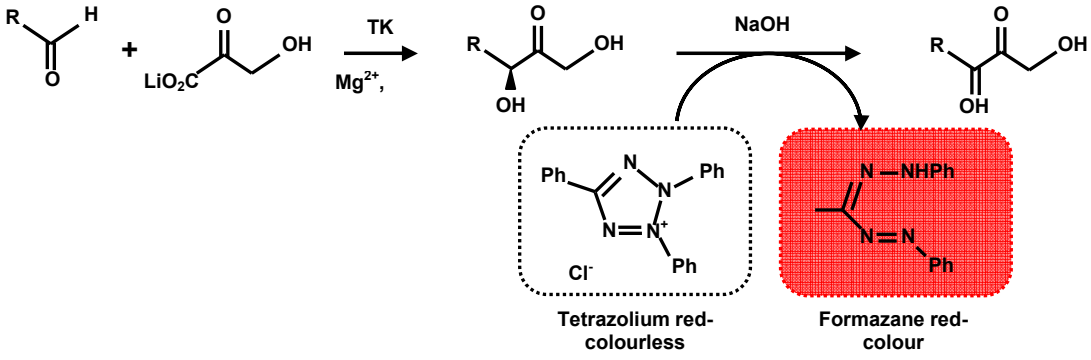


Figure 1.11: Colourimetric assay for TK. The assay ustilises the reduction of colourless tetrazolium red in the presence of non-α-hydroxyaldehydes forming red coloured compound formazane.

1.7 Summary, aims and objectives

The main objective of this project was to establish and present a model chemoenzymatic pathway for the synthesis of structurally diverse compounds with the potential for optical purity. Whilst aiming to explore the compatibility of a compound synthesized chemically and applied directly to a biotransformation, but also providing preliminary indications of a potential 'one-pot synthesis'. The use of a valuable Diels Alder reaction and well studied transketolase reaction provided good model components for integration and subsequent characterization. Determining the kinetic parameters of a substrate relatively far removed from the natural substrates of TK with possible TK mutants is significant for understanding such a system. In addition, due to the demand of optically pure pharmaceuticals the determination of enantiomeric purity is a significant aim of the project. The challenges highlighted, include increasing substrate complexity and therefore synthesizing more interesting and complex products. Also, identifying issues such as low substrate solubility in aqueous media and enzyme inhibition, for instance product toxicity at critical concentrations.

Chapter two provides general materials and methods paramount to this investigation including *E. coli* fermentation and TK isolation. Chapter three aims to investigate the target acceptor aldehyde substrate for the transketolase reaction and address the synthesis using a potentially compatible chemical method. The screening and subsequent identification of a TK mutant with the ability to accept the novel substrate is presented. Chapter four continues with the highlighted TK-mutants and determination of reaction rates and yields at preparative scales, where optimal conditions are analysed including the study of cosolvent addition where substrate solubility in aqueous media is limited. Chapter five presents the determination of the Michaelis-Menten kinetic parameters, K_m, k_{cat} and v_{max} for the mutants of TK with the cyclic aldehyde and compared to WT-TK kinetics utilising a modified colorimetric detection assay validated by HPLC. Chapter six, provides preliminary investigation into product and substrate inhibition associated with the pathway together with initial compatibility limitations. Prospective solutions are discussed. Chapter seven summarises the findings throughout the

investigation and offers a final discussion of the topics highlighted. Chapter eight continues by describing potential extensions and the future implications to this work.

The overall aims of the project:

- To establish a novel model pathway demonstrating chemoenzymatic synthesis of novel compounds.
- To identify new TK mutant enzymes with desired substrate specificity and preserved high selectivity.
- To demonstrate substrate walking which will allow the substrate specificity of an enzyme to be evolved beyond currently perceived limitations.
- To extend the synthetic repertoire of TK bioconversions by introducing a novel, cyclic aldehyde substrate.
- To determine the kinetic parameters and enantioselectivity of the novel transketolase reaction.

2 General Materials and Methods

2.1 General

Unless otherwise noted, solvents and reagents were reagent grade from commercial suppliers (Sigma-Aldrich) and used without further purification. Dry CH_2Cl_2 was obtained using anhydrous alumina columns. All moisture-sensitive reactions were performed under a nitrogen or argon atmosphere using oven-dried glassware. ^{1}H NMR and ^{13}C NMR spectra were recorded at the field indicated using Bruker AMX300 MHz, Avance-500 MHz and Avance-600 MHz machines. Coupling constants are measured in Hertz (Hz) and unless otherwise specified, NMR spectra were recorded at 298 K. Mass spectra were recorded on a Thermo Finnegan MAT 900XP and Micro Mass Quattro LC electrospray mass spectrometers VG ZAB 2SE. Infrared spectra were recorded on a Shimadzu FTIR-8700 and Perkin Elmer Spectrum 100 FTIR spectrometer. Optical rotations were recorded on a Polarimeter Perkin Elmer model 343 polarimeter at 589 nm, quoted in deg $cm^2.g^{-1}$ and conc (c) in g.100 mL^{-1}.

2.2 Reagents

Proteins were made in house by fermentation of glycerol stocks of *E. coli* pQR412:XL10-gold AmpR made for previous study. The D469T-TK mutant and WT-TK was expressed with a His_6 tag from pQR412:XL10-gold (AmpR) as described previously (Hibbert *et al.,* 2008) (previously cloned from expression construct pQR706 obtained by Dr John Ward, Department of Biochemistry and Molecular Biology, UCL and transformed by Dr Christine Ingram). RO water was used in all experiments. All other reagents including cyclic aldehydes were of analytical grade and were obtained from Sigma-Aldrich (Gillingham, UK), apart from the following: acetophenone (Acros Organics, Geel, Belgium), β-hydroxypyruvic acid lithium salt (Fluka, UK) and pyridoxal-5-phosphate (Calbiochem/Merch Chemicals, Nottingham, UK). (5*S*)-2,2,3-Trimethyl-5-phenylmethyl-4-imidazolidinone monohydrate chloride was purchased (Sigma-Aldrich).

2.3 Biocatalyst preparation

2.3.1 LB-agar plates and streaking

LB-agar was prepared by adding 15 $g.L^{-1}$ select agar to LB-media. The agar solution was then autoclaved at 121 °C. Once cooled to approximately 40 °C, ampicillin (150 $\mu g.mL^{-1}$) was added and dissolved by gentle mixing and 20 mL of the media was poured into standard-sized petri dish, being careful not to introduce bubbles. LB-agar plates were streaked with cells (XL10 pQr412) from glycerol stocks (50% v/v glycerol stored –80 °C) and incubated for 16 hours at 37 °C.

2.3.2 Luria Bertani (LB) media

All media components shown in Table 2.1, are weighed, added to RO water, stirred and autoclaved at 121 °C in fermentation shake flasks (ampicillin is added after autoclaving). Fresh media was made for each fermentation batch.

Table 2.1: Luria Burtani (LB) media components.

Component	Concentration ($g.L^{-1}$)
Yeast extract	25.0
Tryptone	10.0
Glycerol	10.0
NaCl	10.0
Ampicillin	0.15

2.3.3 Ampicillin

Fresh solutions of ampicillin for bacterial selection were prepared for each fermentation at a concentration of 150 $\mu g.mL^{-1}$ in RO water. The antibiotic solution is added to the autoclaved media through a 0.2 μm filter (Pall, MI, USA).

2.3.4 Overnight culture

A single colony was picked from streaked and cultured agar plates (described above) and used to inoculate 250 mL shake flasks containing 25 mL LB-glycerol media (containing ampicillin 150 $\mu g.mL^{-1}$) and incubated overnight at 37 °C and 200 rpm using an SI 50 orbital shaker (Stuart Scientific, Redhill, UK).

2.3.5 Shake flask fermentation

The overnight culture was used to inoculate a 1 L flask containing 100 mL LB-glycerol Amp media (10% v/v) , and incubated at 37 °C and 200 rpm. The fermentation was monitored by OD_{600} in triplicate using Unicam UV2 UV/vis spectrometer (Cambridge, UK) and related to dry cell weight of previous calibration until stationary phase was reached. The samples were diluted with fresh LB-media to keep concentrations within visible range (0.1-0.3 A.U). No induction was necessary due to the constitutive nature of the recombinant strain.

2.3.6 20 L fermentation of D469T-TK

A 20 L fermentation of *E. coli* pQR412:XL10-gold (AmpR) expressing TK mutant D469T was performed in a LH20L01/02 fermenter (LH Engineering Ltd 2000 Series) with 3000 Series instrumentation and Adaptive Biosystems PC software. Temperature was controlled via cold water circulation in the external jacket of the glass vessel bioreactor and maintained at 37 °C using a temperature probe and heating element (Bioprocess Engineering Services Ltd, Kent, UK). pH was measured by a sterilisable Ingold pH probe (Ingold Messtechnik, Urdorf, Switzerland) and controlled at a constant value of pH 7.0 (±0.05) by the metered addition of 5 M H_3PO_4. DOT was measured by a polarographic oxygen electrode (Ingold Messtechnik, Urdorf, Switzerland) and maintained above 40 %. PPG (polypropylene glycol) was added automatically to suppress formation of foam to a final concentration in the bioreactor of approximately 0.2 $mL.L^{-1}$. The in-going air flow was set at 0.5 vvm and dispersed through a membrane filter and sparger at the base of the bioreactor. Out going air was filtered with a Domnick Hunter tetpor air filter cartridge in housing on the exhaust air line. Online data logged, e.g. impeller speed, pH, DOT, temperature and exhaust gas measurements were recorded with the RT−DAS program (real-time data acquisition system) (Acquisition Systems, Guildford, Surrey, UK).

Overnight cultures (250 mL shake flasks) 2 x 20 mL broth were used to inoculate larger 2 L shake flasks (100 mL culture) and incubated at 37 °C for 8 hours. The 4

x 200 mL cultures were then used to inoculate the bioreactor grown from single colonies of AmpR agar plates incubated for 16 hours. The bioreactor contained two Rushton turbines for stirring and was sterilised *in situ* for 30 minutes at 121 °C and ampicillin added (150 $\mu g.mL^{-1}$) once cooled to 37 °C. The fermentation was performed until the end of the exponential phase, approximately 10 hours. The cells were harvested and centrifuged using CARR P-6 centrifuge and homogenised (APV CD60 homogeniser) in cold phosphate buffer (5 mM, pH7.0) and clarified by centrifugation (4000 rpm for 10 minutes at 4 °C) in 500 mL centrifuge bottles and stored at -20 °C.

2.3.7 Glycerol stocks

Overnight growth culture with shaking at 200 rpm was added to 50% v/v of sterile filtered glycerol and mixed in aseptically. Aliquots of 1 mL eppendorfs were frozen at -80 °C and used for plate streaking.

2.3.8 Sonication for clarified lysate

For clarified lysate preparations used in biotransformations, the culture broth was thawed, centrifuged for 10 minutes at 4000 rpm at 4 °C on a Hettich Universal 320R mid-bench centrifuge (DJB Labcare, Newport Pagnell, UK) and the supernatant discarded. The cell pellet was resuspended in cold phosphate buffer (5 mM) and sonicated (on ice) in a Soniprep 150 (MSE, Sanyo, Japan) with 6 cycles of 10 second 8 μ pulses with 15 second pauses. The sonicate was clarified by centrifugation as above and the supernatant decanted and filtered (0.45 μm GD/X PVDF syringe filters; Whatman, UK) to remove excess cell debris, and used immediately or frozen at -20 °C.

2.3.9 Sonication for purified enzyme

For lysis of cells in preparation for purification the culture broth was thawed and the broth (in 50 mL Falcon tubes) was centrifuged for 10 minutes at 4000 rpm on a Hettich Universal 320R mid-bench centrifuge (DJB Labcare, Newport Pagnell, UK) and the supernatant discarded. The cell pellet was resuspended in binding buffer (500 mM NaCl, 20 mM Tris-HCl, 5 mM imidazole; pH 7.0), with a v/v ratio of 1:10 buffer to broth. The resupended cell pellet sonicated with an ice jacket, using 6 cycles of 10 seconds 8 μ pulses with 15 seconds intervals for each 50 mL of culture broth using a Soniprep 150 sonicator (MSE, Sanyo, Japan). The lysate was clarified by centrifugation at 4000 rpm at 4˚C as above. The supernatant was decanted and filtered (0.45 μm GD/X PVDF syringe filters; Whatman, UK) to remove excess cell debris, and purified immediately or frozen at -20 °C.

2.4 Biocatalysis quantification

2.4.1 SDS-PAGE and densitometry

Protein analysis was determined by SDS gel electrophoresis using a Mini-Protean II system (Bio-Rad Laboratories Inc., Hemel Hempstead, UK) with 7.5% w/v acrylamide gels (prepared with Protogel® stacking and resolving buffers (National Diagnostics, Atlanta, GA). with 10% ammonium persulphate and TEMED and a 0.05% w/v Coomassie brilliant blue stain. After destaining, the protein was visualised and quantified on a GEL-Doc-It bioimaging system with labworks 4.5 software (Bioimaging systems, Cambridge) with a marker lane of known concentration.

2.4.2 Bradford assay

Quantification of total protein concentration was measured additionally using a Bradford assay (Bradford, 1977). A protein assay kit was used (Bio-Rad Labs

Inc., UK) and protein concentration calibrated with a BSA (bovine serum albumin) standard curve. Absorbance was measured at 595 nm using a UV/Vis spectrophotometer (Spectronic, Leeds, UK). The Beer-Lambert law was used to determine protein concentrations from absorbance measurements.

$$A = \varepsilon\lambda c$$

Where, A=absorbance at 280 nm, ε = extinction coefficient (93,905 M. cm^{-1} for transketolase) λ = path length (1 cm) and c = protein concentration ($mol.L^{-1}$).

2.4.3 Spectrophotometry for pure TK

Pure transketolase preparations were quantified by UV absorbance at 280 nm using a UV/VIS spectrophotometer (Spectronic Leeds, UK) and also with a Nanodrop™ 1000 UV/Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA,USA) and blanked with Tris-HCl buffer (50 mM, pH 7.0) and cofactors (TPP, 2.4 mM and $MgCl_2$, 9 mM). The Beer-Lambert law was also used here to determine protein concentration from absorbance measurements.

2.5 Transketolase activity test

2.5.1 Tris-HCl buffer

50 mM Tris-HCl (hydroxymethylaminomethane) buffer with a pH of 7.0 was prepared by dissolving 6.35 mg mL^{-1} Tris hydrochloride and 1.18 mg mL^{-1} Tris base in RO water and adjusted to pH 7.0 using HCl and NaOH if necessary.

2.5.2 Cofactor solution

Cofactor stock solution for GA and HPA activity tests were as follows: TPP (2.4 mM, 5.854 mg) and $MgCl_2$ (9 mM, 4.536 mg) were dissolved in 3 mL Tris-HCl buffer and pH adjusted to pH 7.0 whilst stirring (Reaction 300 µL total volume, 10 % enzyme (30 µL), 170 µL cofactor solution, 100 µL substrate solution).

Cofactor stock solution for PA and HPA reaction were as follows: TPP (2.4 mM) and $MgCl_2$ (9 mM) were dissolved in 1 mL Tris-HCl buffer and pH adjusted to pH 7.0 whilst stirring (Reaction: 600 µL total volume, 30% enzyme (200 µL), 200 µL cofactor stock solution, 200 µL substrate solution).

Cofactor stock solutions for DCHCA and HPA were made as follows: TPP (2.4 mM, 6.635mg) and $MgCl_2$ (9 mM, 5.141mg) were dissolved in 1 mL Tris-HCl buffer and adjusted to pH 7.0 whilst stirring. (Reaction: 600 µL total volume, 50% enzyme (300 µL), 100 µL c factor stock solution and 200 µL substrate solution).

2.5.3 Substrate solutions

All substrate stocks were prepared as fresh solutions for each reaction as a mixture of lithium hydroxypyruvate (HPA) and aldehyde. All activity assays performed maintained equimolar substrate concentrations of 50 mM, unless otherwise stated. Lithium hydroxypyruvate was synthesised as described and used for all substrate solutions.

2.5.4 Activity assay with GA and HPA

The enzyme (30 µL pure or lysate) was pre-incubated with cofactor solution (170 µL, TPP, 2.4 mM, and $MgCl_2$, 9 mM) to reconstitute the holoenzyme (Sprenger *et al.,* 1995). Substrate solution, glycolaldehyde and HPA (100 µL, 50 mM each) were added and incubated at 20 °C in SRW 96 microwell plates. Samples were

taken at regular intervals, quenched and diluted with 0.2% v/v TFA and analysed by HPLC for HPA depletion and L-erythrulose production.

2.6 Synthetic chemistry

2.6.1 Lithium hydroxypyruvate

Bromopyruvic acid (0.06 mol) was dissolved in water (100 mL) and carefully added 1 M LiOH solution at such a rate that the pH does not exceed 9.5. Glacial acetic acid was added dropwise in solution to pH 5. The mixture was concentrated *in vacuo* to approximately 20 mL final volume. The concentration was left to crystallise overnight. The crude product was washed with ethanol and filtered off. The crude product was suspended in ethanol (50 mL) at 40 °C for 30 minutes. White solid was filtered and washed with more ethanol affording a white powder (46%). V_{max} (KBr/ cm $^{-1}$) 3462-2619 (broad,O-H from hydrogen bond dimers), 1608 (strong, C=O, 1244 (strong, C-O). ^{1}H NMR (300 MHz; D_2O) δ_H = 3.60 (2H, s, methylene protons), 4.64 (2H, s, O*H*) 13C NMR (75 MHz; MeOD) δC = 65.8 (CH_2), 66.5 (CH_2), 94.8 (*gem*-diol), 167.9 (C=O), 177.0 (C=O, 203.0 (C=O). MS: *m/z* (ES+) (%): 220 (100) $[2M]^+$, 152 (20) $[M+Na]^+$ (See appendix).

2.6.2 1,3-dihydroxypentan-2-one (DHP)

In a 500 mL round bottomed flask, dissolve lithium hydroxypyruvate (HPA) (1.10 g, 10 mmol) and propanal (720 µL, 10 mmol) in RO water (200 mL). To this solution, 4-methylmorpholine (1.10 mL, 10 mmol) was added and stirred at room temperature for 17-24 hours. Silica (1 g) is added to the mixture and concentrated to dryness under vacuum on a rotary evaporator (avoiding temperatures of more than 50 ^{o}C). Flash chromatography was performed as described to give the desired product as an oil in approximately 350 mg (28%) yield (an impurity was eluted just prior to the desired product and this was thought to be a related triol). The product was stored at 4 °C and overtime appears to solidify.

2.7 Detection and quantification

2.7.1 Flash chromatography

Silica gel (BDH silica beads 40-63 μm) columns were prepared with a layer of sand for neat separation and resolution of components. The columns were equilibrated with mobile phase EtOAc:hexanes (2:1) prior to dry loading of the silica bound compounds (dried previously on a rotavap). After dry loading the product (absorbed onto silica) onto a flash silica column, pure DHP was obtained by chromatography eluting with EtOAc:hexane (2:1), fractionation of the eluent is analysed by TLC (visualised by PMA and heat) and dried on a rotavap (avoiding temperatures of more than 50 °C).

2.7.2 Thin layer chromatography

Reactions were monitored by pre-coated aluminium backed silica (TLC) plates (Kieselgel 60 F_{254}) with detection by UV (254 nm and 297 nm), potassium permanganate and phosphomolybdic acid (PMA), PMA hydrate (12 g) dissolved in ethanol (250 mL) stains. Mobile phase solvents system consisted of a 1:1 ratio of ethyl acetate and hexanes. Stained plates were visualised by heating with a heat gun.

2.7.3 HPLC

All HPLC analysis were performed on a Dionex (Camberley, Surrey, UK) microbore HPLC system and controlled by Peaknet 5.1 software. A multiwell plate autosampler (Spark, Emmen, The Netherlands) was integrated into the Dionex system with a GP40 gradient pump, and LC30 column oven, a PC10 pneumatic controller NaOH addition unit and an AD20 UV detector or ED40 electrochemical detector (ECD). Chiral HPLC analysis was performed on a Varian Prostar instrument equipped with a Chiralcel AD chiral column (Diacel) 25 cm x 0.46 cm.

2.7.3.1 HPLC Standards

Calibration curves were used to quantify absorbance responses for the reaction substrates and products. Standards of L−erythrulose, HPA and DHP were prepared to a range of concentrations in triplicate and detected by HPLC as described (Section 2.7.3.3). Standards of acetophenone and MBA were prepared separately and analysed by HPLC in order to obtain standard curves for calibration of the TAm reaction also (See appendix).

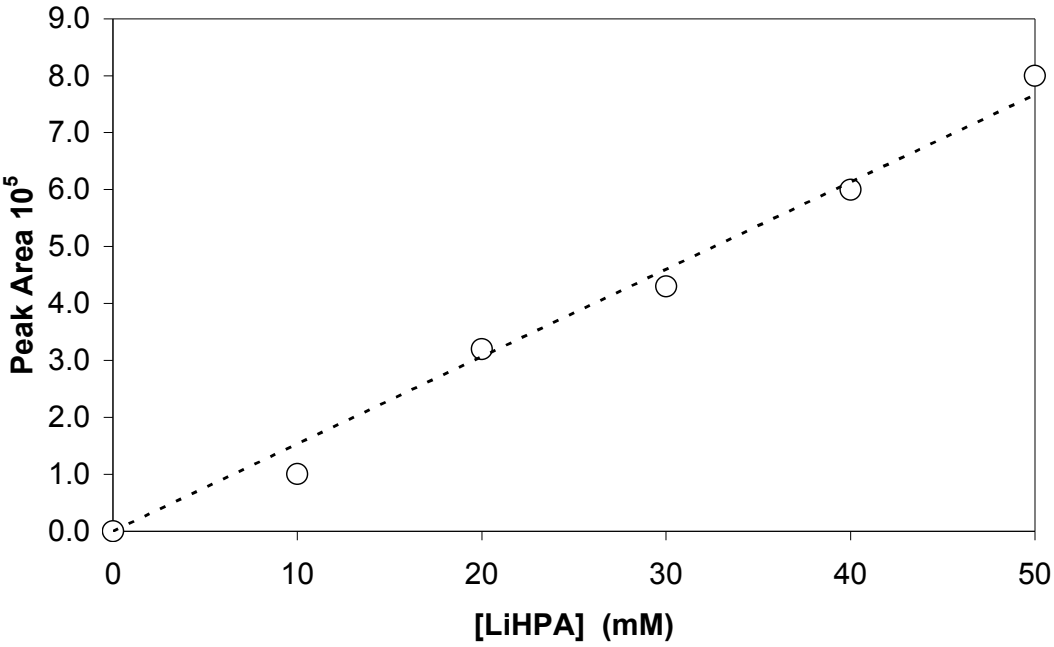


Figure 2.1: Li-HPA calibration curve. Stock solutions of HPA synthesised as described, were made ranging 0-50 mM in pH7.0 Tris-HCl buffer (50 mM, pH7.0).

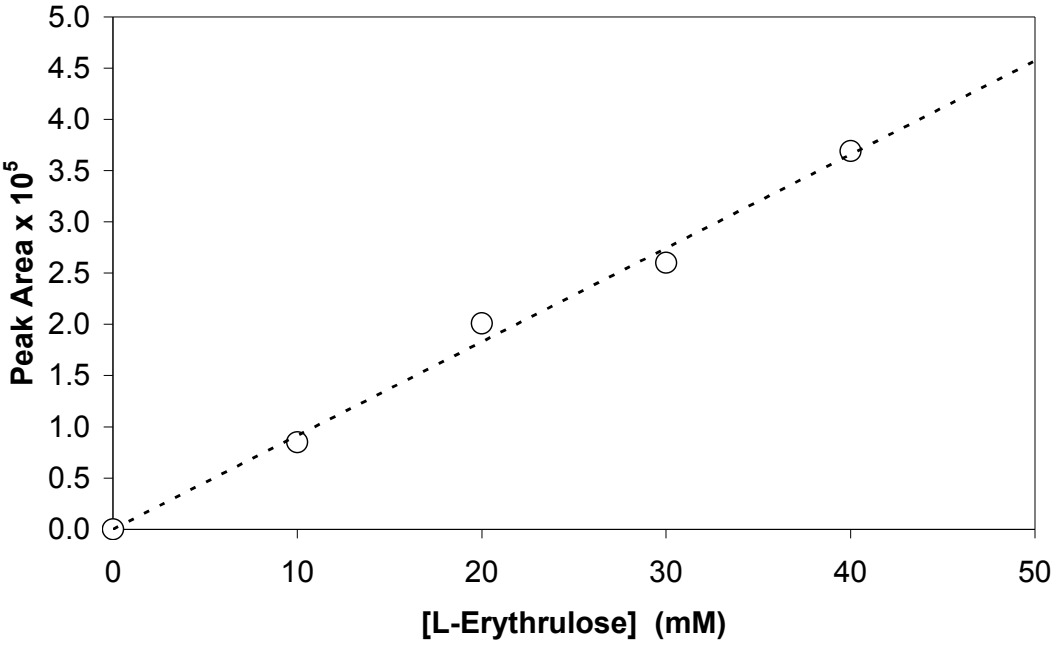


Figure 2.2: L-erythrulose calibration curve. Stock solutions of L-erythrulose (Sigma-Aldrich) ranging 0-40 mM were made in Tris-HCl buffer (50 mM, pH7.0).

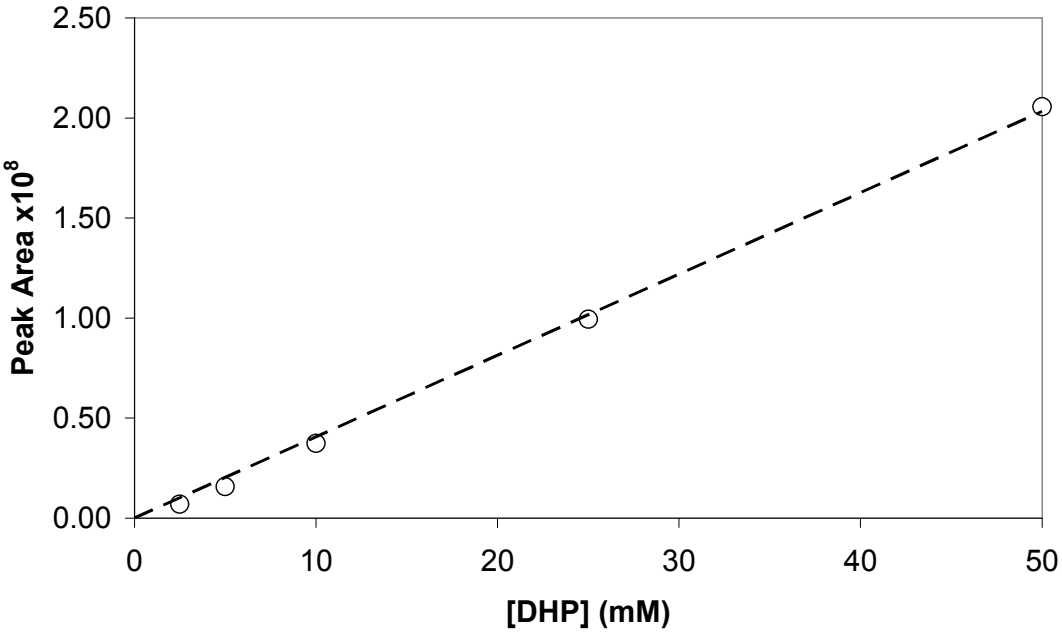


Figure 2.3: 1,3-dihydroxypentan-2-one (DHP) calibration curve. Stock solutions of DHP (synthesized as described in Section 2.6.2) ranging 0-50 mM were made in Tris-HCl buffer (50 mM, pH7.0).

2.7.3.2 HPLC assay for GA and L-erythrulose

A 15 minute isocratic assay was adapted from the existing HPLC assay for the TK reaction components glycolaldehyde, HPA and L-erythrulose (Mitra and Woodley, 1996). An Aminex 87H column (Bio-Rad, Hemel Hempstead, Herts., UK) at 60 °C using a mobile phase of 0.1% (v/v) trifluoroacetic acid (TFA) in water at 0.6 $mL.min^{-1}$. Detection was via a UV detector at 210 nm. Retention times were 8.40 min HPA, 11.40 min erythrulose, 12.28 min glycolaldehyde.

2.7.3.3 HPLC assay for DHP and HPA

An 8 minute isocratic assay was adapted from the existing HPLC assay for the TK reaction components HPA and DHP (Mitra and Woodley, 1996). An Aminex 87H column (Bio-Rad, Hemel Hempstead, Herts., UK) at 60 °C using a mobile phase of 0.1% (v/v) trifluoroacetic acid (TFA) in water at 0.4 $mL.min^{-1}$. Detection was via an electrochemical detector (ECD40), set to a standard triple potential, following adjustment of pH to above pH 12.0 by post column addition of NaOH.

3 Aldehyde design, library screening and mutant selection

This chapter investigates the initial design and synthesis of a new target aldehyde substrate for transketolase that is also the product of a Diels Alder reaction, and can therefore be used to demonstrate a chemoenzymatic process. The design of an appropriate acceptor aldehyde had to balance both the selection from 'the best of' conventional chemical synthesis options with the requirement of the chemical product to have activity with at least one mutant in a TK biocatalyst library. This would then provide a basis for a more in-depth investigation of the full chemoenzymatic synthesis in Chapter 4. This chapter also describes the process of TK-mutant library screening for enhanced activity with the target acceptor aldehyde, sequencing the top performing mutants and then producing pure enzyme preparations.

3.1 Introduction

3.1.1 Natural substrates for transketolase

TK catalyses a reaction that is stereospecific, whereby the (*S*)-configuration at the C3 of the natural ketol donor is maintained within the new chiral centre of the product. The enzyme is also stereoselective, whereby TK selects α–hydroxyaldehydes with the (*R*) - configuration at C2 over other configurations. Characteristics such as these are particularly attractive for the synthesis of commercial compounds, such as pharmaceuticals.

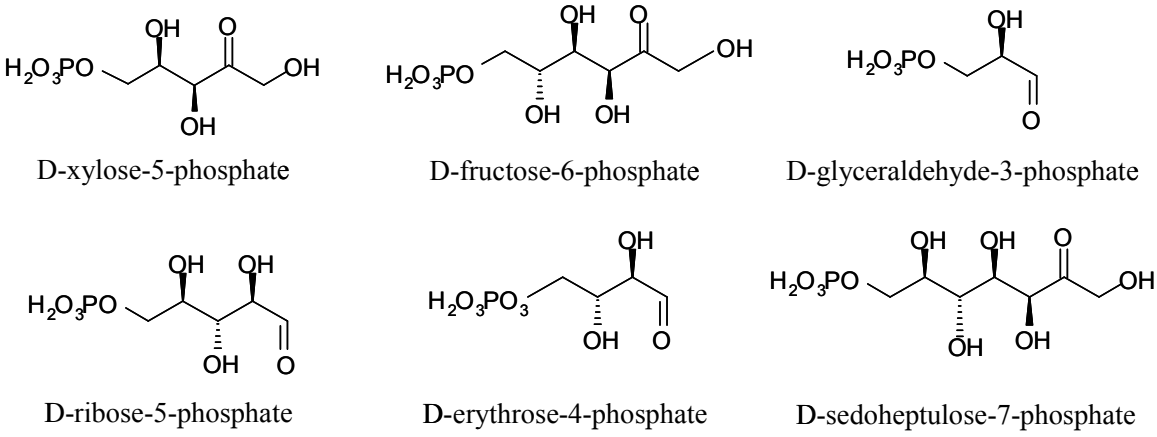


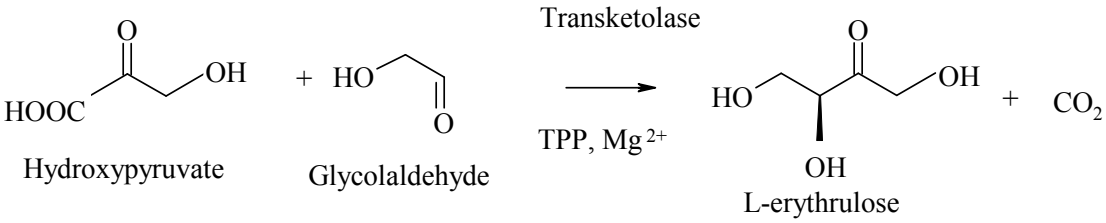
Figure 3.1: Natural transketolase substrates.

Natural substrates of *in vivo* TK catalysed reactions, shown in Figure 3.1, include phosphorylated sugars, including D-xylose-5-Phosphate, D-erythrose-4-phosphate, D-sedoheptulose-7-phosphate and D-fructose-6-phosphate. The phosphate group provides a platform for stabilising the substrates through hydrogen bond interactions between the phosphate group and residues within the active site (Nilsson *et al.,* 1997). Reports have demonstrated that residues 359, 528 and 469 were highly conserved in TK sequences and lay close to the phosphate group of the substrate. These studies concluded that the replacement of these residues with alanine caused considerable residual activity but with large increases in K_m for phosphorylated substrates (Schneider and Lindqvist, 1998). However, recent investigations regarding the use of non-phosphorylated substrates have provided evidence that reaction can occur without the presence of a phosphate group (Bolte *et al.,* 1987; Turner *et al.,* 2000; Hobbs *et al.,* 1993; Hibbert *et al.*, 2007). For industrial syntheses it is favourable to eliminate phosphate groups from substrates due to the complication they cause in product isolation and also to the subsequent removal from reaction products (Schenk *et al.,* 1998; Turner, 2000). The application to further non-phosphorylated substrates provides a greater repertoire of industrially useful compounds.

3.1.2 Non-natural substrates for transketolase

Initially, non-phosphorylated substrates were demonstrated as having limited activity and suggested to be due to the reduced substrate binding stability within the active site (Villafranca and Axelrod, 1969; Nilsson *et al* 1997). More recent studies have concentrated on providing directed evolution strategies to improve this limitation, in order to gain the benefits of increased activity towards phosphate free substrates (Hibbert *et al.,* 2007). The same strategies may be used to gain the further benefits of introducing more activity towards non-natural substrates into TK, particularly for the synthesis of target compounds that are otherwise difficult or impossible to synthesise with conventional chemistry.

Hydroxypyruvate has been well demonstrated throughout literature as a particularly useful donor substrate, due to the evolution of CO_2 creating an irreversible reaction (Scheme 4). This reaction system makes the industrial use of TK as a biocatalyst very favourable (Bolte *et al.,* 1987).



Scheme 4: **Transketolase catalysed reaction.** Transketolase, in the presence of cofactors TPP and Mg^{2+}, catalyses the transfer of a 2-carbon ketol group from ketol donor, HPA to acceptor substrate, glycolaldehyde, forming an asymmetric carbon-carbon bond in end product L-erythrulose. Carbon dioxide is consequently produced, rendering the reaction irreversible.

Glycolaldehyde (R=CH_2OH) (GA), alongside glyceraldehyde has been well reported as non-phosphorylated, acceptor aldehyde substrate for analysis of the model TK catalysed reaction (Datta and Racker, 1961; Mitra *et al.,* 1996; Hibbert *et al.,* 2007). Furthermore, the ketose product, L-erythrulose, of the TK-catalysed reaction with GA and HPA is synthesised as a bulk intermediate within several industries (Shaeri *et al.*, 2008). The cosmetic industry utilises L-erythrulose as a self tanning agent, and the pharmaceutical industry as an intermediate for the production of Nelfinavir™, an antiretroviral drug acting on HIV-1 and HIV-2 proteases (Kaldor *et al.,* 1997).

Approximately 60 aldehydes have previously been identified as acceptor substrates for TK indicating its 'promiscuous' nature, although certain characteristics of the aldehydes have been seen to be crucial for TK activity. In particular, the (R)-configuration of a hydroxyl group at position C2, despite the acceptance of substrates without a hydroxyl group at position C2.

3.1.3 Substrate walking

Applying increasingly diverse substrate acceptance into TK in a stepwise fashion described as 'substrate walking' gives a greater potential understanding of the versatility of both native and mutant TK enzymes. Catalysis by WT-TK with a previously reported substrate such as glycolaldehyde at well established reaction conditions (Gyamerah and Willetts, 1997), indicates the potential activity that might be achieved by directed evolution towards new substrates for which the current activity is poor or undetectable. For example, exploring the scope of TK by directed (or forced) evolution has led to the application of a slightly more complex and hydrophobic aliphatic substrate, propanal, which is no longer hydroxylated at the C2 position (Hibbert *et al.,* 2008; Smith *et al.,* 2008). This in turn may lead to an increased likelihood for access to substrates with a further increment of chemical diversity. Repeating this in a step-wise manner leads to a potential substrate series such as cyclopropane-carboxaldehyde, cyclopentane-carboxaldehyde, cyclohexane-carboxaldehyde and cyclohexene-carboxaldehyde. Each modification links the natural substrate to the desired, substantially altered, industrially useful substrate shown in Figure 3.2, where the desired substrate is out of scope at the first or second round of directed evolution.

Sequential evolution of the enzymes, through forced evolution strategies, may be required to allow further complex substrates to be applied to the TK-catalysed reaction. Where enzyme activity may no longer be detected, further cycles of directed evolution alongside the application of increasingly modified substrates may prove valuable.

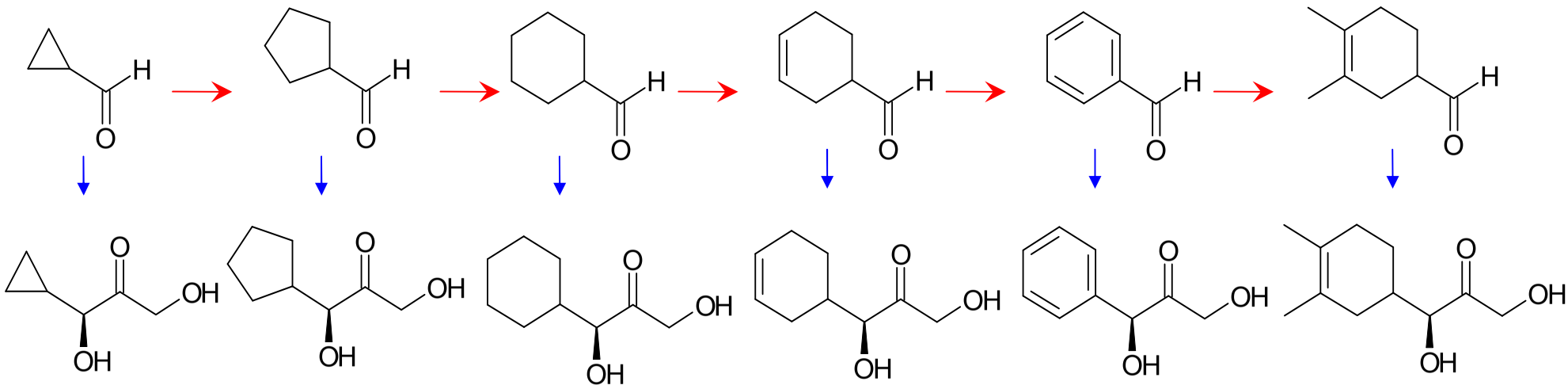


Figure 3.2: Substrate walking. Sequentially linked cyclic aldehydes and the corresponding ketodiol product.

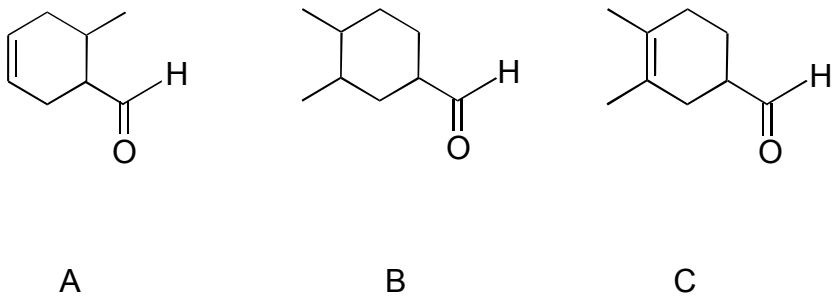
Here we apply the substantially altered substrates directly to both WT-TK and a range of first generation TK variants, based on the residue associated with stereospecificity and better acceptance of a non-alpha hydroxylated aldehyde. The aim is to provide a sound principle for the chemoenzymatic synthesis of structurally complex intermediates through non-phosphorylated, cyclic, and hydrophobic precursors. This analysis also begins to define the boundaries of detectable WT and first generation mutant activities towards substrates with increased chemical diversities.

3.1.4 Cyclic aldehyde series

The sequentially linked cyclic aldehydes shown in Figure 3.2 are particularly interesting due to the link back to substrates structurally homologous to natural substrates. Screening of these cyclic aldehydes not only provides a link to more highly complex aldehydes but highlights mutants with varied activities towards each aldehyde and also mutants with enhanced activity towards all screened aldehydes. Such mutants may therefore provide knowledge of the effect of particular mutations on selectivity and perhaps structural effects on the active site.

Aldehydes containing cyclic structures greatly increase the potential for the synthesis of complex intermediate precursors, through a single biotransformation step. Alternative methods of synthesis of such cyclic structures would involve numerous multi-step chemical transformations using harsh conditions and many protection and de-protection steps. The additional introduction of a double bond within a cyclic structure not only pushes the boundaries of currently accepted substrates, but further broadens the potential for modification of functionality. A greater number of intermediates may be synthesised from this precursor owing to the greater number of approaches available, including the reactivity of the double bond and the dimethyl groups attached to the cyclic ring.

Potential candidates meeting these criteria included 6-methyl-3-cyclohexene-1-carboxaldehyde, 3,4-dimethylcyclohexane-1-carboxaldehyde, and 3,4-dimethyl-3-cyclohexene-1-carboxaldehyde (Figure 3.3).



A B C

Figure 3.3: Potential target aldehydes. A) 6-methyl-3-cyclohexene-1-carboxaldehyde B) 3,4-dimethylcyclohexane-1-carboxaldehyde, and C) 3, 4-dimethyl-3-cyclohexene-1-carboxaldehyde.

3.1.5 Aldehyde design for a chemoenzymatic reaction cascade

The cyclic aldehydes **A** and **C** in Figure 3.3, introduce the potential for their synthesis using a Diels Alder route in the first step of a chemoenzymatic synthesis using TK in the second step. It was decided to study 3,4-dimethyl-3-cyclohexene-1-carboxaldehyde (**C**) due to the greater complexity of the aldehyde, therefore providing a greater insight to the potential of substrate acceptance by WT-TK, TK mutants or both.

Demonstrating TK activity towards **C** was the first step to defining such a chemoenzymatic cascade reaction. Probing libraries of existing enzyme mutants with novel substrates that are bulky, contain functionality for further chemical modification, or that are more hydrophobic and relatively far removed from currently accepted substrates, would enhance the potential of TK for the synthesis of more complex intermediates.

Compound 3,4-dimethyl-3-cyclohexene-1-carboxaldehyde (DCHCA) was also targeted as an acceptor aldehyde for TK mutant screening studies due to the potential modifications that may be applied subsequent to the TK reaction. Employing a substrate with a bulky structure was considered for pushing the boundaries of currently perceived limits, whilst maintaining a structural relationship with cyclic aldehydes used in preliminary screens. Introducing an alkene group provides further potential for synthetic manipulation. In addition, the use of a chiral substrate allows for the assessment of TK selectivity, a significant feature of ideal biocatalysts.

A symmetrical diene, 2,3-dimethyl-1,3-butadiene was selected, as opposed to asymmetrical isoprene, to avoid in this first system problems with DA regioisomers being formed. The essential *cis*-configuration for the Diels Alder reaction is however a limiting factor on reaction rate of the cycloaddition. If the diene constituent is part of a cyclic compound such as cyclopentadiene, the *cis*-configuration is fixed and therefore the reaction (in appropriate conditions) may proceed rapidly. The reaction is limited however, when the diene does not have the fixed *cis*-configuration where the single bond connecting the double bonds is continually rotating. The cycloaddition may only proceed at a rate allowed by the speed of rotation of the diene to the *cis*-position. The synthesis of further complex, bi-cyclic aldehyde substrates may therefore benefit from enhanced DA reaction rates.

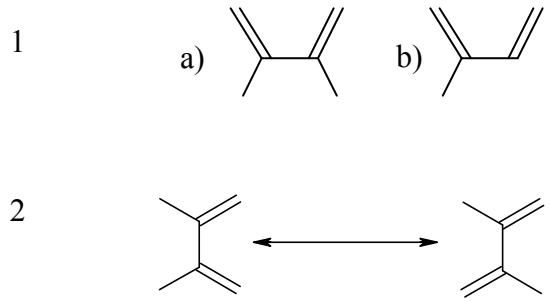


Figure 3.4: Dienes. 1) Dienes considered for application to Diels Alder reaction a) 2,3-dimethyl-1,3-butadiene and b) isoprene **2)** cis-trans rotation about the single bond.

Our aim was to use a racemate generated in the DA reaction as a substrate for TK initially for simplicity. The use of a well studied reaction such as the DA reaction system is beneficial when integration into a novel multistep reaction requires characterisation of individual aspects of each step, as well as complications and compatibility between them.

3.1.6 Aldehyde synthesis

The catalysis of such reactions has received widespread attention due to the valuable nature of cyclic compounds and therefore the methods of 6-membered ring formation. Countless DA reactions have been investigated with regard to catalysis, media and reaction conditions, including microwave irradiation, sonication, high pressure and extreme temperatures (Coda *et al.,* 1984; Blokzijl *et al.,* 1991; Otto *et al.,* 1994). Noteworthy efforts have seen great success in the effective rate acceleration and also selectivity enhancement of DA reactions, particularly in water since the 1980s (Rideout and Breslow, 1980; Sauer and Sustmann, 1980).

A recyclable organotungsten Lewis acid $[O=P(2\text{-}py)_3W(CO)(NO)_2](BF_4)_2$ was reported to effectively catalyse synthesis of DCHCA in ionic liquid media ($bmimPF_6$) with a yield of 92% at room temperature within 4 hours (Chen *et al.,* 2004). The same catalyst was also reported to catalyse the same reaction with an 80% yield within 30 seconds, when microwave irradiation was applied. The preparation of the catalyst involved several process steps including stirring, filtration, cooling, washing and drying.

An alternative method for the synthesis of the same cycloadduct was reported, where a moisture stable, Lewis acid containing room temperature ionic liquid produced yields of 91% within 2 hours. The process included a simple stirring step with no complicated or lengthy extraction methods, and also no purification steps were necessary. The preparation of the catalyst, $[Me_3NC_2H_4OH]Cl\text{-}ZnCl_2$ (1:2), required only two, relatively low cost components, choline chloride and zinc chloride, to be stirred whilst heated and cooled to room temperature in air. The ionic liquid was also reported to be recyclable (Abbott *et al.,* 2001).

3.1.7 Diels Alder (DA) reaction

The substrates of the DA reaction are simple to depict from the chosen target product as the reaction is a simple cyclic addition reaction. In detail, the three π (pi) bonds of the substrates transfer to give two σ (sigma) bonds and one π (pi) bond within the cycloadduct or cyclohexene ring of the product. A functional group may be attached to the substrates and will not play a role in the DA reaction, but remain within the cycloadduct. The substrates elucidated from the target cyclic aldehyde, DCHCA, are diene, 2,3-dimethyl-1,3-butadiene and dienophile, acrolein shown in Figure 3.5.

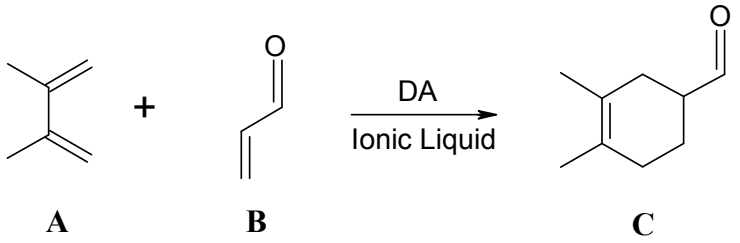


Figure 3.5: Target Diels Alder reaction. A) 2,3-dimethyl-1,3-butadiene, **B)** acrolein, **C)** DCHCA (3,4-dimethyl-3-cyclohexene-1-carboxaldehyde).

3.1.8 TK structural mutant library D469

Active site targeted directed evolution by saturation mutagenesis was previously used on *E. coli* transketolase in order to improve activity towards non-phosphorylated substrates. Two separate approaches to residue selection, phylogenetic and structural were used (Hibbert *et al.,* 2007). The phylogenetic approach realises the evolutionary variation of transketolases throughout an array of species. A number of residues were identified as lying within 10 Å of the TPP binding site, and 10 of these, K23, A29, N64, M159, S188, D259, A383, P384, V409 and L466 were found to vary since the common TK ancestor. The structurally defined sites were selected due to their vicinity to the erythrose-4-phosphate substrate binding position within the active site of yeast transketolase (Nilsson *et al.,* 1997). Residues H26, H100, I189, H261, G262, R358, S385, H461, D469 and R520, were all located within 4 Å of the defined active site. These previously generated phylogenetic and structural TK mutant's libraries for enhanced activity towards propanal were considered for screening with the cyclic aldehydes including DCHCA (Hibbert *et al.,* 2007).

The tetrazolium red assay was developed specifically for the screening of these 20 mutant libraries targeting enhanced activity towards non-α-hydroxylated aldehydes. Establishing a rapid and sensitive assay for the identification of new mutants with activity towards novel substrates is fundamental considering the enormous number of potential mutants required for screening (Smith *et al.,* 2006) (Refer to figures 3.6 and 3.7).

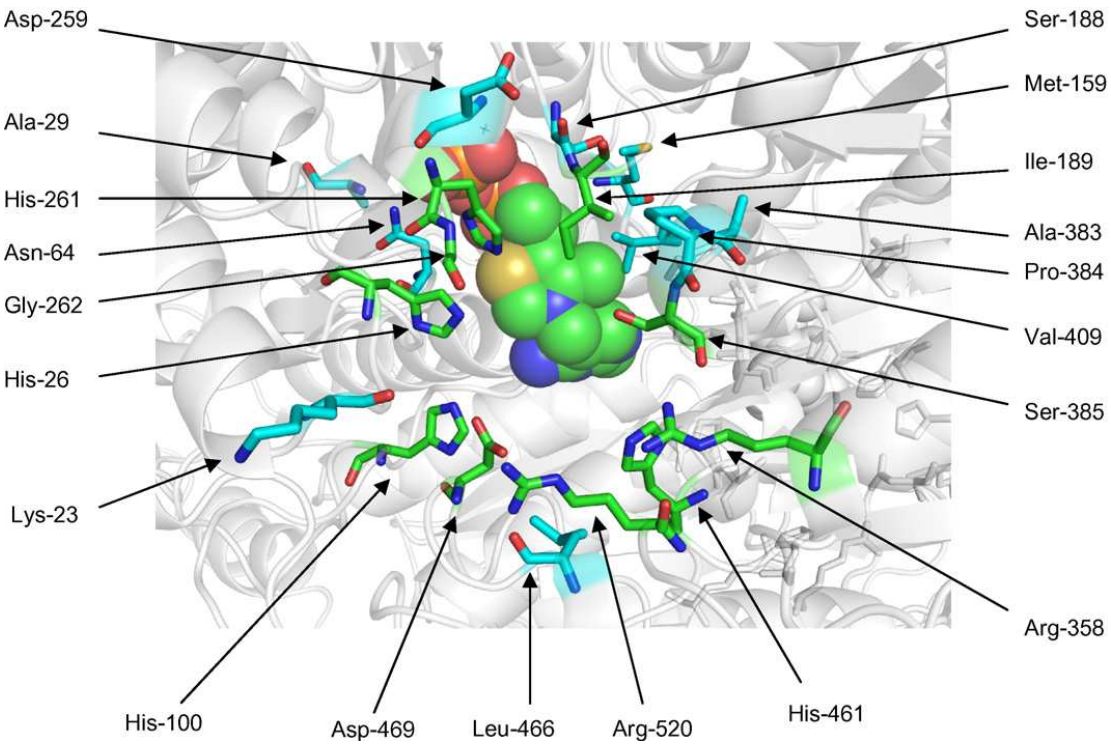


Figure 3.6: Transketolase active site. Highlighted residues selected for directed evolution through two separate approaches, structural and phylogenetic, and subsequently considered for screening with cyclic aldehydes including DCHCA. (Taken from Hibbert *et al.,* 2007).

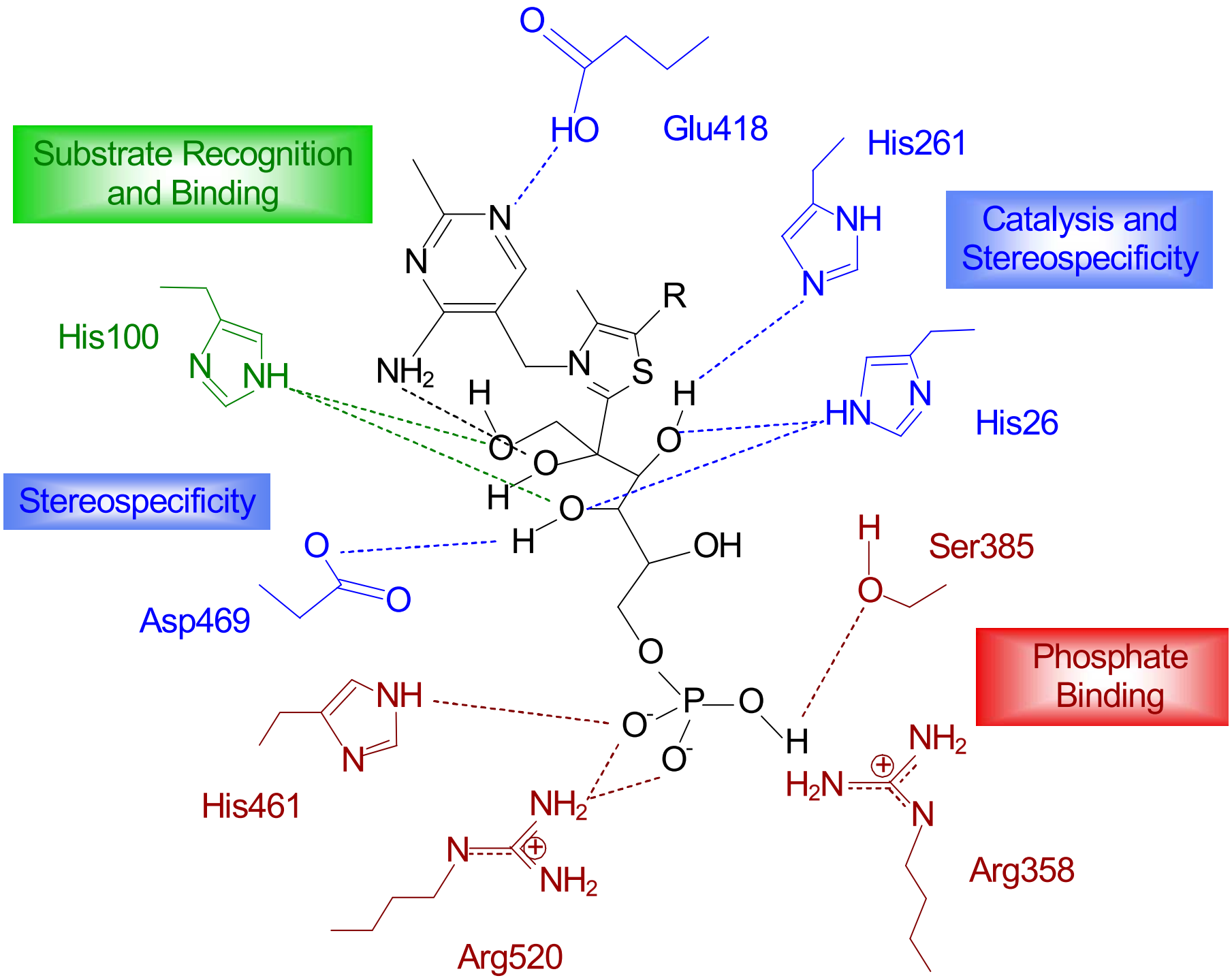


Figure 3.7: Structural library residues. Ketol donor and cofactor TPP within active site of transketolase.

3.1.9 Mutant library selection

Directed evolution strategies have proven to be extremely successful in generating enzymes with improved catalytic properties (Hibbert *et al.,* 2007). Previously, twenty saturation mutagenesis libraries with regard to specific residues were found to enhance activity towards both glycolaldehyde and propanal and also enhanced specificity towards propanal (Hibbert *et al.,* 2008). Several factors indicating involvement with substrate acceptance led us to explore the structural library D469 further. In particular, a number of TK-mutants were identified from library D469X as having enhanced activity towards propanal, despite the reported decrease in activity of D469E towards formaldehyde ($R=CH_2$) and glycolaldehyde. Interestingly, a yeast TK structure with erythrulose-4-phosphate adjunct, showed the hydrogen bonding of residue D469 (highlighted in orange on figure 3.8) with the C-2 hydroxy group of α-hydroxylated aldehyde substrates in the active site.

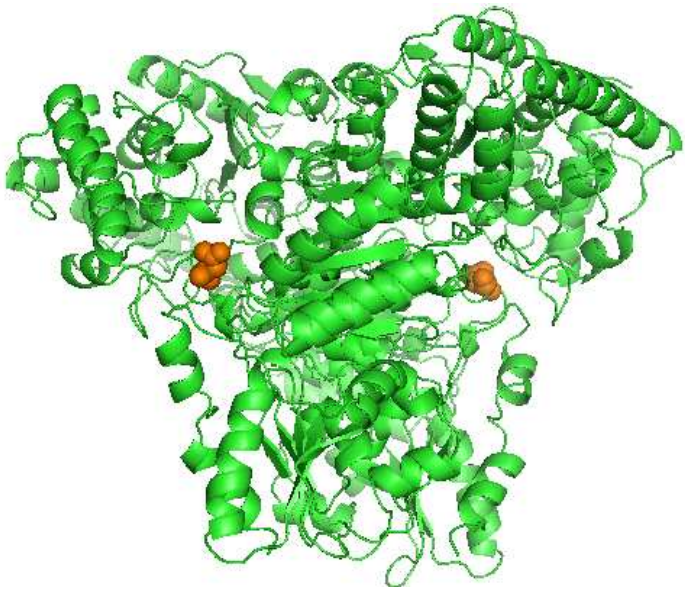


Figure 3.8: Transketolase dimer. Residue D469 highlighted in orange previously identified as having significant participation in stereospecificity. Generated using PyMOL with data from PDB (Littlechild *et al.,* 1995).

3.2 Materials and methods

3.2.1 TK reaction with cyclic aldehydes

Substrate solutions were prepared immediately prior the enzyme reaction to maintain homogeneity due to the relatively low solubility in water. Stock solutions of each cyclic aldehyde were prepared in water and sonicated on ice, for 3 cycles of 10 second pulses with 20 second intervals. The assay for cyclic aldehydes was performed as described in section 2.5.4 and analysed at 24 hours.

3.2.2 Ionic liquid $[Me_3NC_2H_4OH]Cl\text{-}ZnCl_2$ (1:2) preparation

The room temperature ionic liquid synthesised here, $[Me_3NC_2H_4OH]Cl\text{-}ZnCl_2$ (1:2), is available commercially, however, is simple, relatively quick and cheap to make. To prepare the ionic liquid (IL) a mixture of zinc chloride (5.45 g, 40 mmol) and choline chloride (2.79 g, 20 mmol) mixture was heated to 150 °C and stirred magnetically or mechanically (120 rpm) for 10 minutes in a paraffin bath until no solid remained. It was then cooled to approximately 28 °C and water added (0.72 mL, 40 mmol) with continued stirring. The liquid was cooled further to ambient temperature or to 26 °C. Portions of this material were then used in the cycloaddition reaction.

3.2.3 Ionic liquid recycling

Following use in the Diels Alder cycloaddition reaction the zinc chloride-choline chloride-RTIL ($[Me_3NC_2H_4OH]Cl\text{-}ZnCl_2$ (1:2)) was washed thoroughly with hexanes and dried for at least an hour on a rota-vap. The catalyst was stored in glass screw top vials under argon. The catalyst was heated to a viscosity enabling magnetic stirring and cooled to ambient temperature. The RTIL was subsequently used as described in section 3.2.4.

3.2.4 3,4-Dimethyl-3-cyclohexene-1-carboxaldehyde (DCHCA) synthesis using Diels Alder reaction in RTIL

2,3-dimethyl-1,3-butadiene (1.36 mL, 12 mmol) and acrolein (0.80 mL, 12 mmol) were added to the zinc chloride-choline chloride RTIL (0.5 mL) and the reaction mixture was stirred for 2 hours at ambient temperature. The mixture was allowed to separate by ceasing mixing. The upper layer containing the aldehyde was decanted or pippetted from the ionic liquid to give DCHCA as a colourless oil (1.53 g, 91%). Smaller scale reactions using 0.5 mL IL and 12 mM of 2,3-Dimethyl-1,3-butadiene and acrolein required a reaction time of 2 hours, however for larger scales (> 5 mL IL) longer reaction times were required (up to 24 hours) and the yield was reduced (500 mM 2,3-Dimethyl-1,3-butadiene and acrolein; 5 mL IL; 65%) while maintaining the high purity of the product. ^{1}H NMR (300 MHz; $CDCl_3$) δ 1.54 (3H, s, CH_3), 1.61 (3H, s, CH_3), 1.87–2.44 (7H, m), 9.67 (1H, s); ^{13}C NMR (75 MHz; $CDCl_3$) δ 18.9 (*C*H_3), 19.1 (*C*H_3), 22.8, 30.1, 30.5, 46.9, 123.4, 125.8, 204.7 (C=O) (see appendix).

3.2.5 (1*R*)-3,4-Dimethyl-3-cyclohexen-1-carboxaldehyde (DCHCA 1*R* isomer) synthesis

2,3-Dimethyl-1,3-butadiene (1.13 mL, 10 mmol) and acrolein (2.0 mL, 30 mmol) were added to a solution of (5*S*)-2,2,3-trimethyl-5-phenylmethyl-4-imidazolidinone monohydrate chloride as catalyst (255 mg, 1 mmol) in acetonitrile/water (95:5, 40 mL). The reaction was stirred at 25 °C for 48 hours. The reaction was partitioned between diethyl ether (100 mL) and 0.3 M $KHSO_4$ (100 mL) and the aqueous phase extracted with diethyl ether (2 × 100 mL). The combined organics were dried ($MgSO_4$) and the solvent removed in vacuo to give (1*R*)-DCHCA (1.10 g, 80%). $[\alpha]^{20}_{D}$ = +24.0 (*c* 1.0, CH_2Cl_2).

3.2.6 Diels Alder cyclo-adduct distillation purification

If necessary the DA cycloadduct (DCHCA) was purified though distillation. The aldehyde was heated with anti-bumping granules until boiling point was reached, and the more highly volatile, DA substrate contaminants evaporated and condensed. The remaining liquid was cooled and analysed for purity by TLC and $^{+}$H NMR. However, purification of the aldehyde was rarely required and all aldehyde stocks used for kinetic analysis were not purified.

3.2.7 D469X library screening assay

TK mutant libaries were previously produced using saturation site-directed mutagenesis using Quikchange kit (Stratagene, Netherlands) and plasmid transformed into electrocompetent XL10 (Stratagene) *E. coli* cells. The D469 mutant library reaction plate was thawed on ice in order to cause freeze-thaw lysis of the 100 µl culture in each SRW 96 microwell plate. The lysed cells were then incubated with 12× cofactor stock solution (25 µl of 28.8 mM TPP, 108 mM $MgCl_2$ in 50 mM Tris-HCl buffer, pH 7.0) and HPA stock (70 µl of 200mM LiHPA in 50mM Tris–HCl, pH 7.0) at room temperature for 20 minutes to reconstitute the holo-enzyme. Aldehyde substrate (100 µL, 100 mM DCHCA in 50 mM Tris-HCl buffer, pH 7.0) was sonicated and then added to initiate the reaction, and the microwell plate was sealed using a uniseal polypropylene mat (Whatman, Brentford UK) and incubated at room temperature for 24 hours. The colour assay was performed as described below.

3.2.8 Colorimetric assay for screening

Fifty µL of each microwell was transferred to a fresh SRW 96 microwell plate and 10 mg MP-carbonate resin added and left to incubate for 3 hours. Fifty µL of Tris-HCl buffer was added to each microwell and pipette mixed. This solution (50 µL) was then transferred to a fresh microwell plate. Automated injection of 20 µL tetrazolium red solution (0.2% 2,3,5-tripheyltetrazolium chloride in methanol) and

10 µL, 3 M NaOH (aq) with shaking by FLUOstar Optima plate reader (BMG Labtechnologies GmbH), was followed by immediate measurement at $OD_{485nm.}$

0.2% solution of 2,3,5-triphenyltetrazolium chloride in methanol (200 mg of 2,3,5-TPTC in 100 mL of MeOH). 3 M NaOH (aqueous) (12 g of NaOH dissolved in water to give 100 mL final volume of solution).

3.2.9 Sequencing

Small scale (10 mL culture in 100 mL flasks) overnight cultures were grown from single colonies as described in section 2.3. The culture was centrifuged to isolate the cell pellet and a QIAprep Spin Mini Prep (QIAGEN, UK) protocol performed to isolate DNA samples. The samples were diluted to within specific range (16-18 fmol) and sent (12 µL in 1.5 mL eppendorfs) to The Wolfson Institute for Biomedical Research, UCL for DNA sequencing. Primers used were:

TKmidSeq 5'- GTATGAAAGGCGAAATGCCGTCTGACT-3'.

Primers were prepared at concentration of 5 $pmol.\mu L^{-1}$. Sequences were subsequently analysed by Bioedit software available on the internet: www.mbio.ncsu.edu/BioEdit/bioedit.html.

3.2.10 TK Mutant production

The TK-mutants and WT-TK had been previously expressed in *E.coli* strain BL21 (DE3), pQR412. Allowing for the effective purification of the enzyme, the transketolase gene was His_6-tagged in previous studies (Ingram *et al.,* 2007). The selected mutants from microwells F12, B6, B7, F1, D5 and E2 were streaked from glycerol stocks of the same libraries which had been stored at -80 °C, onto LB agar plates and incubated for 16 hours. Small overnight cultures were inoculated with single colonies, which were subsequently used to inoculate larger scale shake flasks. Glycerol was used as a carbon source additive to the culture media to

support the constitutive expression of the TK gene. Cell growth was monitored by measuring OD_{600} and the fermentation was ceased once log-phase (exponential growth) had been observed and stationary phase was evident.

3.2.11 TK purification (His-tagged pQR412)

Fresh fermentation broth was centrifuged at 4000 rpm for 10 minutes, to obtain a compact cell pellet and clear media supernatant. Cell pellets were immediately re-suspended in cold binding buffer (500 mM NaCl, 20 mM Tris-HCl, 5 mM EDTA, pH 7.95) with gentle pipette mixing and sonicated with a Soniprep 150 on ice to counteract any heating generated. Equilibration of His bind Quick 900 cartridges with a cellulose matrix an immobilised Ni-NTA was performed prior to sample loading, followed by a series of washes using specific wash buffer 1 M EDTA, 500 mM NaCl and 20 mM Tris-HCl. Loading of each sample was passed through the same column twice to maximise protein binding to the Ni^{2+} cations within the column at a rate of 2 drops s^{-1}. The pure TK was then eluted with an EDTA elution buffer then dialysed overnight against 50 mM Tris-HCl buffer, pH 7.5, and then stored at 4 °C.

In order to improve enzyme stability further, cofactor stock solution was added to the dialysis buffer (TPP, 2.4 mM and $MgCl_2$, 9 mM) and two 8 hour dialysis steps replaced a single overnight dialysis in fresh buffer for each step to ensure the removal of potentially detrimental buffer (EDTA).

3.3 Results and discussion

3.3.1 TK wt on GA, PA and cyclic aldehydes

The product, L-erythrulose (L-Ery) and donor substrate, HPA concentrations were detected by HPLC and compared to previous measurements. The GA reaction was used because of its rapid reaction rate, well reported activity with WT-TK (Hobbs *et al.,* 1994; Mitra, 1997; Mitra *et al.,* 1998), readily available substrates and products and crucially its well established analytical HPLC method (Mitra and Woodley, 1996).

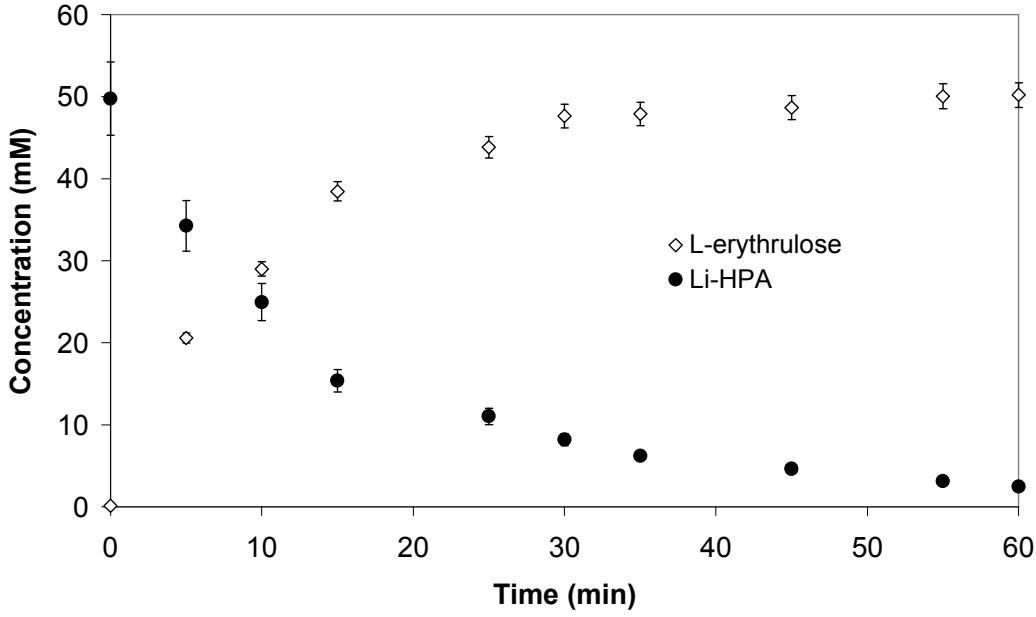


Figure 3.9: GA and HPA reaction profile. Representative TK catalysed reaction using 10% clarified TK-lysate, 50 mM (equimolar) substrate concentration at 22 °C (see section 2.5.4 for details).

The TK catalysed reaction of HPA and glycolaldehyde, synthesising L-erythrulose, is able to run to completion within 60 minutes. The measurement of the depletion of substrate HPA and the formation of product L-erythrulose is shown in Figure 3.9 and indicates a good stoichiometric correlation between the substrate consumption and product synthesis by the enzyme. The pure WT-TK catalysed reaction toward glycolaldehyde and HPA has a specific activity of 0.61 μmol.mg.min^{-1}.

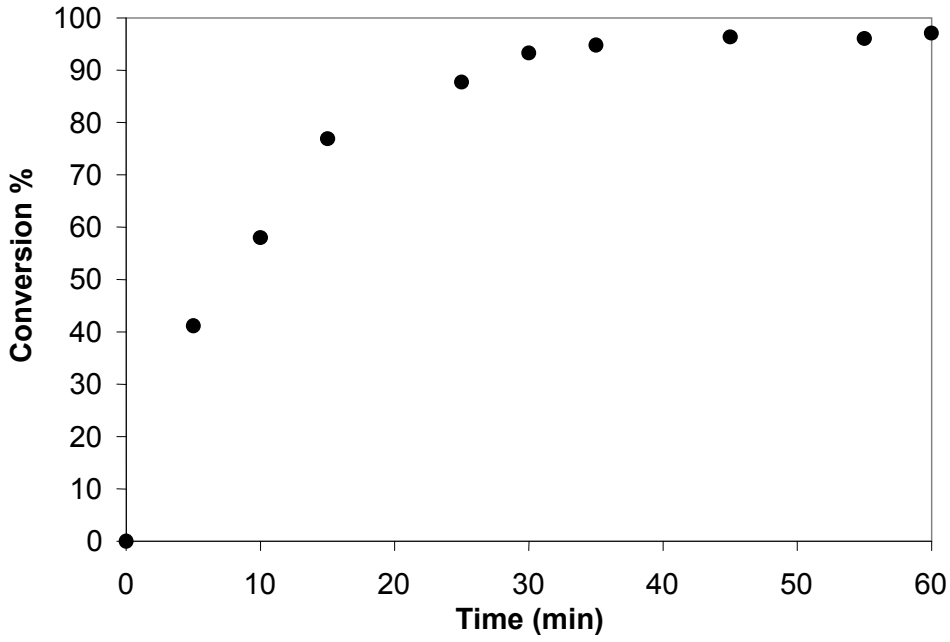
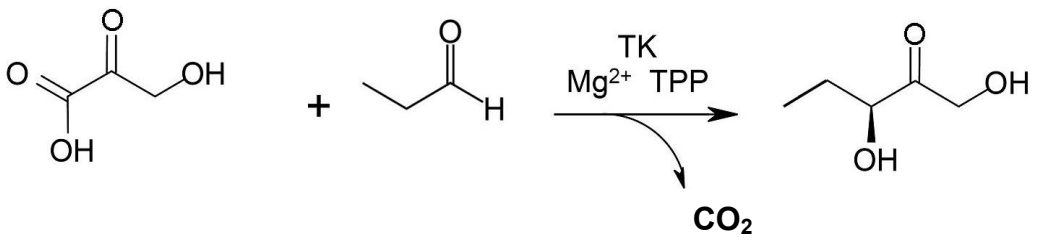


Figure 3.10: GA and HPA activity assay. A pure TK catalysed glycolaldehyde and HPA conversion to L-erythrulose and CO_2 based on results shown in Fig. 3.9.

Propanal (R=CH_2CH_3) is the next stepping stone of the substrate walking pathway and the reaction has been analysed for optimal TK catalysed reaction conditions. The use of this non-α-hydroxylated, aliphatic aldehyde with mutants of TK was previously described as having improved specific activity of 0.14 μmol.mg.min^{-1}, with a 4.9 fold activity improvement relative to WT-TK (Hibbert *et al,* 2008).



Hydroxypyruvate **Propanal** **DHP**

Scheme 5: Transketolase (TK) catalysed reaction. Propanal (PA) and HPA form 1,3-dihydroxypentan-2-one (DHP) via the transketolase catalysed reaction.

For identification and subsequent calibration of the propanal and HPA reaction product, 1,3-dihydroxypentan-2-one (DHP) was synthesised biomimetically in water (discussed further in section Chapter 4), and reaction progression analysed by TLC. The propanal (PA) reaction profile indicates lower specific activity than glycolaldehyde, however establishes the ability of WT-TK to accept non-phosphorylated, aliphatic substrates. WT-TK activity towards propanal and HPA was determined to be 0.056 $\mu mol.min^{-1}.mg^{-1}$.

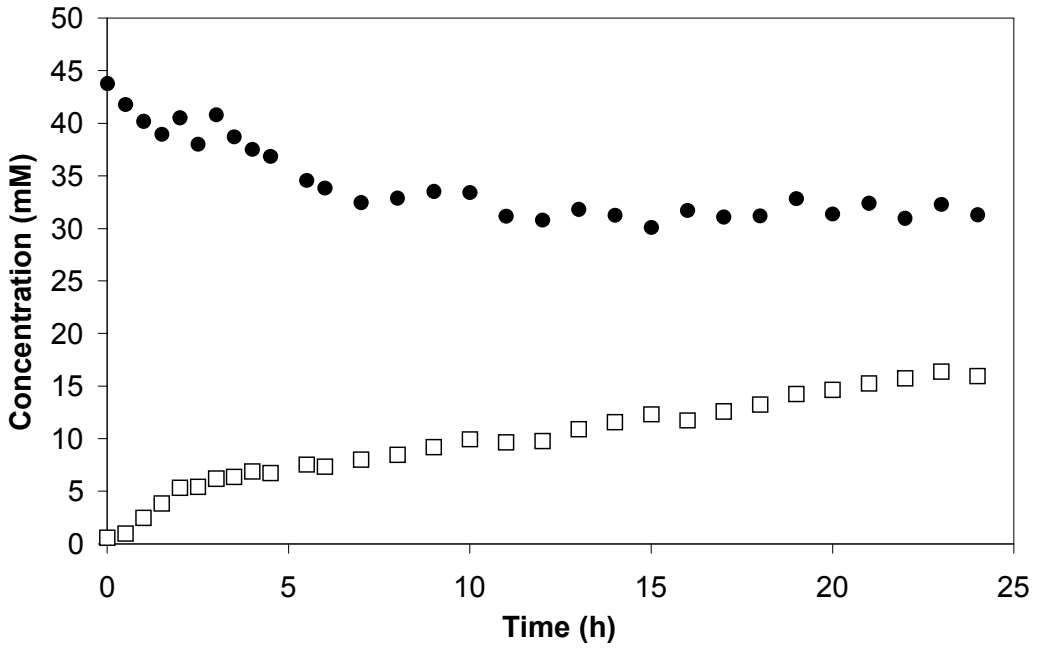


Figure 3.11: Transketolase reaction profile. Propanal (50 mM) and HPA (50 mM) WT- transketolase catalysed reaction in Tris-HCl buffer (50 mM, pH7.0) and cofactors TPP (2.4 mM) and $MgCl_2$ (9 mM) at 20 °C. Substrate HPA (●) and product DHP (□) detected by HPLC.

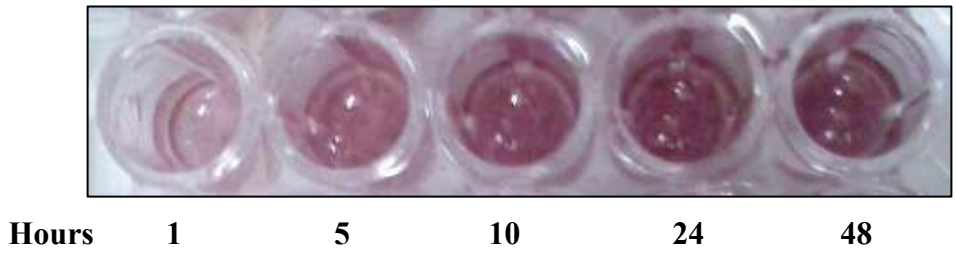


Figure 3.12: Colorimetric assay photograph. Propanal (50 mM) and HPA (50 mM) transketolase catalysed reaction in Tris-HCl buffer (50 mM, pH7.0) and cofactors TPP (2.4 mM) and $MgCl_2$ (9 mM) at 20 °C for 48 hours.

In order to establish the potential for activity with an aldehyde substrate greatly removed from currently reported substrates with respect to its, size, hydrophobicity and functionality, initial studies using lesser complex aldehydes were done. Screening reactions using WT-TK were subjected to a large range of increasingly complex cyclic aldehydes i.e, cyclopropancarboxaldehyde, cyclopentanecarboxaldehyde, cyclohexanecarboxaldehyde and 3-cyclohexene-1-carboxaldehyde. A 50 mM substrate reaction incubated at room temperature for 24 hours, and visualised on TLC using PMA and heat, indicated some product formation from all of the tested aldehydes.

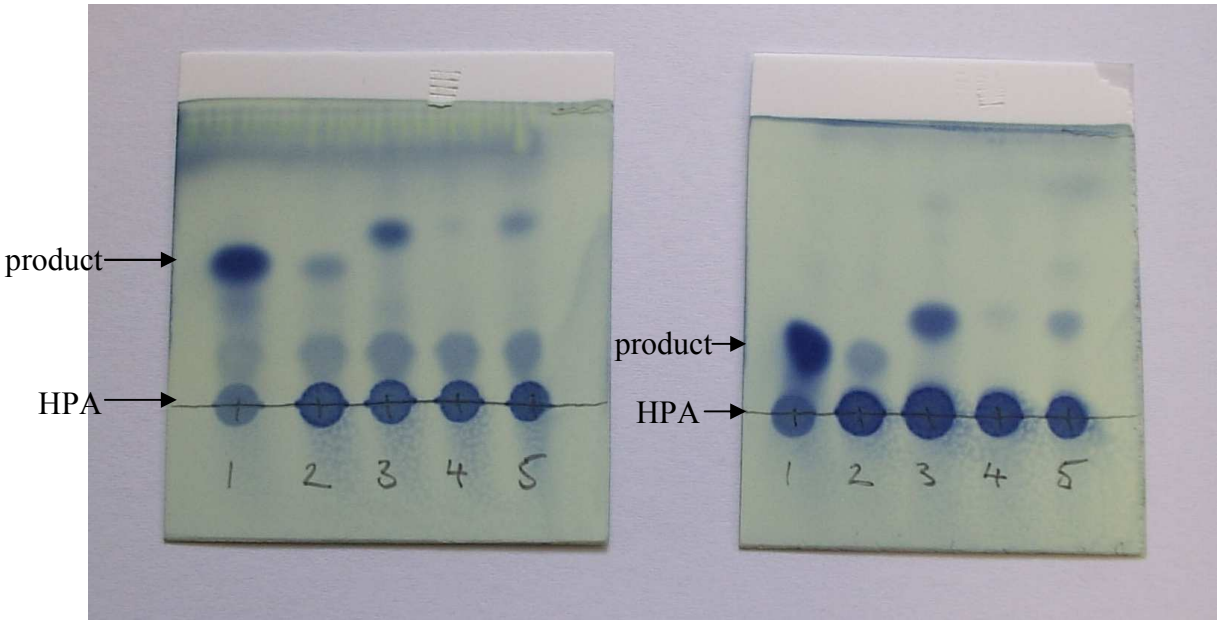
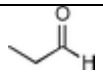
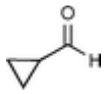
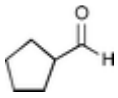
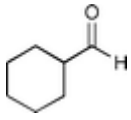


Figure 3.13: Substrate walking TLC. Plate A, mobile solvent 2:1 Ethyl acetate/hexane and Plate B Mobile solvents 1:1 Ethyl acetate/hexane, 24 hours, 50 mM 1. propanal, 2. cyclopropancarboxaldehyde, 3. cyclopentanecarboxaldehyde, 4. cyclohexanecarboxaldehyde and 5. cyclohexencarboxaldehyde (see section 2.7.2 for details).

Furthermore, the cyclic aldehydes were analysed for activity with the mutant D469T comparing the initial rates of the cyclic aldehydes with propanal shown in Table 3.1. Each of the specific activity and initial rates indicate a decrease with increasing ring size of the aldehyde. Steric hindrance of the aldehyde within the active site of the enzyme may be responsible for the decreasing activity, or decreasing conformational flexibility of the ring potentially reducing the probability of correct orientation.

Table 3.1: Specific activity of D469T against cyclic aldehydes compared to propanal (TK 0.3 $mg.mL^{-1}$).

Aldehyde	Initial Rate $mM.h^{-1}$	Specific activity $\mu mol.mg^{-1}.min^{-1}$	Initial relative rates
 Propanal	12.5	0.69	1
 Cyclopropane-carboxaldehyde	7.45	0.41	0.6
 Cyclopentane-carboxaldehyde	4.47	0.25	0.4
 Cyclohexane-carboxaldehyde	3.46	0.19	0.4

3.3.2 New aldehyde synthesis

3.3.2.1 Ionic Liquid

The production and use of a quarternary ammonium, lewis acidic, moisture stable, ionic liquid was reported for the synthesis of cyclic intermediates including this aldehyde, 3,4-dimethyl-3-cyclohexene-1-carboxaldehyde (DCHCA) (Abbott *et al.* 2001; Abbott *et al.* 2002). Several volume scales and substrate concentrations with and without the addition of water were investigated, stirring both magnetically and mechanically, with an overhead anchor type impeller. Using previously reported reaction conditions of 12 mmol substrates in 0.5 mL of a 1:2 ratio of zinc chloride-choline chloride and stirring for two hours at ambient temperature, the cycloadduct yield was 91%.

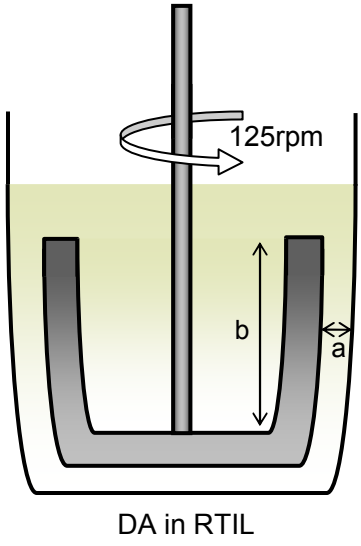


Figure 3.14: Mechanical stirring of large volume RTIL and DA reactants. a) (10mm used here) This width is significant for the effective stirring of viscous liquids, avoiding stagnant areas on the surface of the vessel. b) (60% of the height of the reactor used here) The height of the anchor-type impeller is also significant for allowing rotational flow throughout the viscous liquid.

Table 3.2: Effect of ionic liquid volume on yield. All reactions except those mechanically stirred had 1 molar equiv. water added. * Mechanically stirred as shown in Figure 3.14. Reaction times given are the times maximum yield was reached. (Ambient temperature ~20 °C).

Substrate Concentration	Volume mL (Ionic Liquid)	Temperature °C	Reaction Time (h)	Yield %
12 mM	0.5	Ambient	2	91
12 mM	5	25	2	90
24 mM	0.5	Ambient	4	85
24 mM	0.5	28	4	85
50 mM	0.5	28	24	84
0.1M	5	Ambient	24	90
0.1M	0.5	28	24	55
0.2M	0.5	Ambient	24	40
0.5M	5	Ambient	24	65
0.5M*	100	25	-	-
1 M*	100	Ambient	-	-

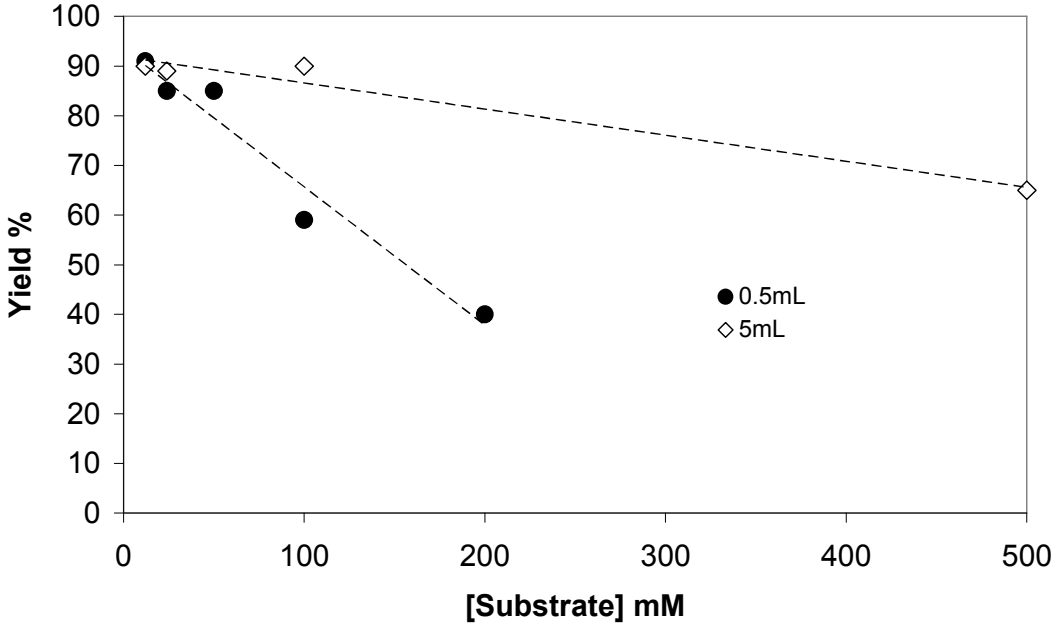


Figure 3.15: Effect of ionic liquid volume on DA reaction yield. Ionic liquid ($[Me_3NC_2H_4OH]Cl$-$ZnCl_2$ ratio 1:2) (●) 0.5 mL and (◊) 5 mL, volumes affecting aldehyde (DCHCA) yields of the Diels Alder reaction.

The addition of water to the ionic liquid was reported to ease stirring due to the high viscosity of the solvent and catalyst mixture. It was seen that the addition of water to the ionic liquid during its cooling stage was critical for stirring magnetically. The viscosity of the ionic liquid as it neared its freezing point of 23-25 °C was far too high for the magnetic stirrer bar to consistently stir the mixture prior to the addition of the substrates. It was observed that the absence of water during the late stages of cooling caused the ionic liquid to solidify, disabling the substrates from mixing with the solvent and catalyst, therefore no reaction occurred.

Maintaining the temperature at just above the reported freezing point of 23-25 °C for the ionic liquid proved advantageous for the ease of stirring magnetically and mechanically. Mechanical stirring was used at larger scales >5 mL and although the addition of water eased stirring it was not necessary at a maintained temperature of 28 °C in an oil bath. However, for both magnetic and mechanical stirring without the addition of water at ambient temperature (22 °C) at a scale of 100 mL ionic liquid, stirring became very difficult and the formation of emulsions between the ionic liquid and substrates solution were evident. The emulsion failed to separate into the ionic liquid and product phases. It was also observed that the mixture turned a vivid orange colour, possibly due to hotspots within the viscous emulsion causing adverse reactions.

3.3.2.2 DA reaction optimisation

Acrolein and 2,3-dimethyl-1,3-butadiene were easily depicted through simple evaluation of the target compound, due to the reaction being an atom efficient cyclo-addition. The uncatalysed reaction between 2,3-dimethyl-1,3-butadiene and acrolein was reported to take over 2000 hours (Odenkirk *et al.,* 1992). No reaction product was seen, however, after 4 weeks of stirring at room temperature as a neat mixture of the two substrates, or as a 0.5 M reaction in water.

To a stirred ionic liquid the substrates were added and continued stirring until reaction reached completion. The product (cycloadduct) was then allowed to separate from the ionic liquid and subsequently removed by decanting. The aldehyde product was analysed for purity and found to be >90% pure.

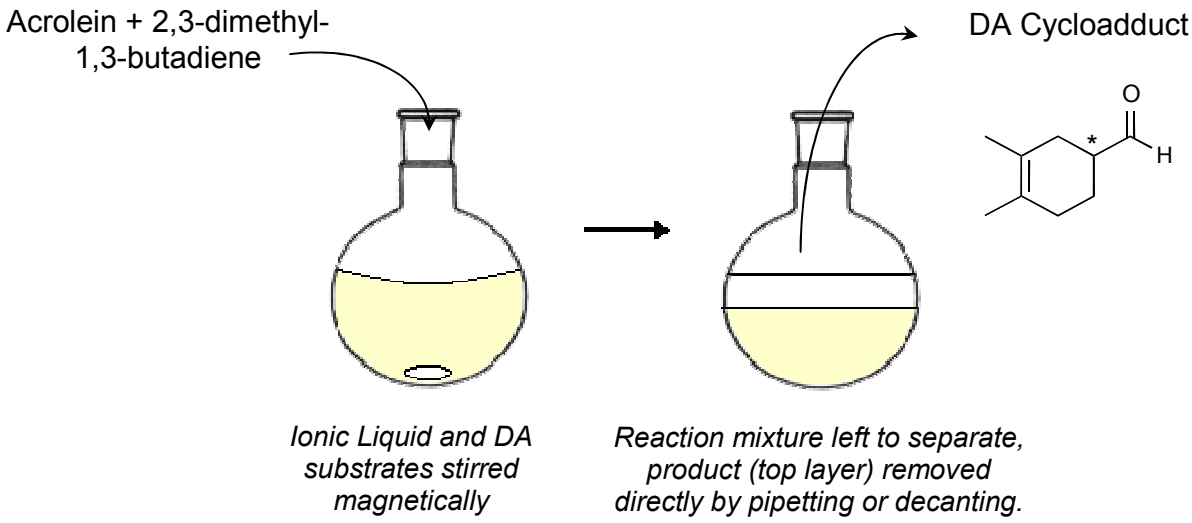


Figure 3.16: Diels Alder reaction in room temperature ionic liquid (RTIL) (See section 3.2.4).

Yield dropped as scale increased, possibly due to the increased reaction time allowing for side reactions to occur such as acrolein polymerisation. The longer reaction periods may have also seen an increased level of substrate loss through evaporation. Acrolein possesses a relatively low boiling point of 53 °C and similarly for 2,3-dimethylbutadiene at 68 °C. Improved sealing of the reaction

vessel may reduce this loss along with more effective temperature control and stirring at scale may aid reaction productivity.

It was found that the ratio of ionic liquid to substrate volume was critical with regard to overall product yield and reaction time. An increased ratio of substrate volume to ionic liquid volume was shown to reduce yield greatly from 91% to 40% (Table 3.2). Larger scale reactions, where >5 mL of ionic liquid and equal volumes of substrates were used required lengthy reaction times, of up to 24 hours, compared to the 2-4 hours required for completion with smaller scales <5 mL ionic liquid.

Control reactions were performed to establish any interaction between the substrates without the ionic liquid solvent and catalyst. The two DA reaction substrates were stirred neat, for 24 hours at room temperature and no product formation was observed. The ionic liquid was replaced with water and the reaction stirred for up to 24 hours, again no product formation was observed at room temperature. It was previously reported that the reaction time without the presence of the ionic liquid, or any catalyst was as long as seven weeks (Odenkirk *et al.,* 1992).

Removal of the product from the ionic liquid mixture was a particularly simple procedure, as it neatly separated from the ionic liquid/water solution as a layer on top (Figure 3.15). It was found that reducing the temperature to below the freezing point of the RTIL enabled the product to be simply poured from the reaction vessel. Subsequent purification of the product was not required, however distillation and flash chromatography were performed if necessary.

3.3.2.2.1 Ionic liquid recycling

It was reported that the ionic liquid was reusable and no drop in yield was seen here after 3 uses, however, the ionic liquid appeared to take on a yellow colouration. This may be due to an accumulation and subsequent polymerisation of the residual acrolein and also the presence of any contaminants post reaction,

bearing in mind the reaction takes place in air. No yellow colour was seen in ionic liquid stored under argon at 4 °C, which had not been used for a reaction over a period of 6 months. A small drop in activity was seen however after 4 cycles of use from 90% (±1%) to 88% (±2%) and then further to 79% (±2%) conversion after 5 cycles. This may be due to the increased level and therefore contamination of acrolein polymers within the ionic liquid despite a thorough washing procedure. A report suggests that mass spectrometric analysis of the ionic liquid after recycling shows that the alcohol side chain of some ammonium cations may have reacted with the carbonyl starting material (Abbott *et al.,* 2002).

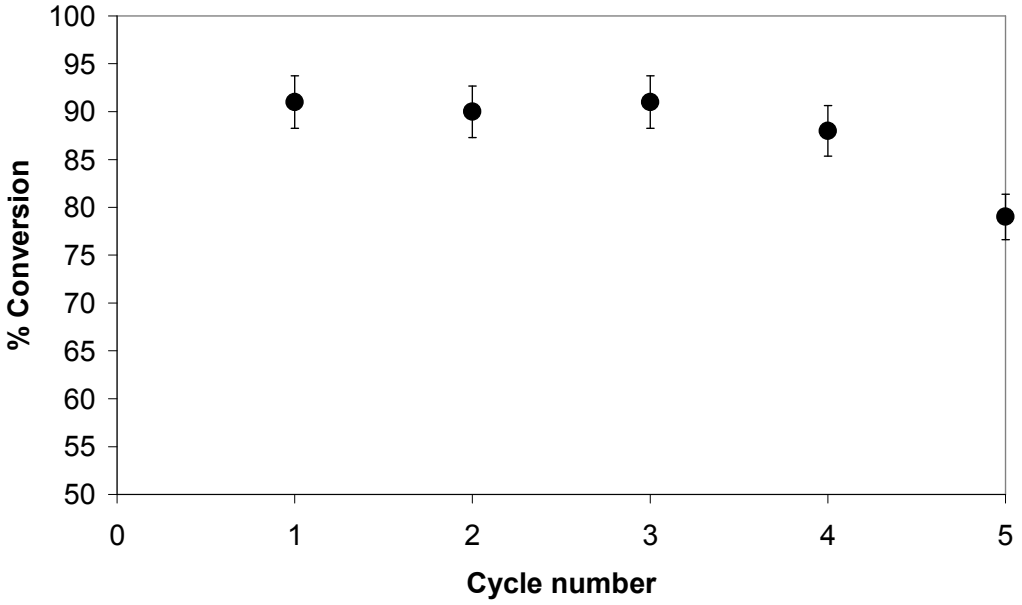


Figure 3.17: Room temperature ionic liquid recycling. Reaction conditions 0.5 mL ionic liquid (RTIL) ([$Me_3NC_2H_4OH$]Cl-$ZnCl_2$ (1:2)), 12 mM substrates, ambient temperature with water added. At the end of each cycle the ionic liquid was washed in hexanes and stirred at 30 °C for 2 hours to dry the ionic liquid. The ionic liquid was stored under argon at 4 °C between uses.

3.3.3 WT-TK on DA product

Initial activity studies of WT-TK with a range of cyclic aldehydes, although relatively low, indicated some acceptance and product synthesis shown in Figure 3.13. However, in contrast to the previous substrates, WT-TK enzyme showed negligible activity towards DCHCA. Of particular interest was the activity recorded with 3-cyclohexene-1-carboxaldehyde. As this was the most closely related aldehyde structurally to our target substrate, DCHCA, it was interesting to see WT-TK accepting an aldehyde with a bulky cyclic ring and also with an alkene moiety.

The capability of TK to physically allow the acceptance of larger, bulkier, substrates, although with limited activity, may be described by the shape of the active site. The cleft formed between the two enzyme subunits is funnel-shaped (French and Ward, 1996), potentially allowing for the substrate structure to be larger at the opposing end of the compound to the reactive aldehyde moiety. Limitations arise with orientation and proximity of the substrate with cofactors within the active site. Modelling experiments have been performed with WT-TK and a range of aldehydes, including linear aliphatic acceptor aldehydes (C_4-C_8) and the same cyclic aldehydes presented here (Càzares *et al.,* 2010). The cyclic aldehydes were seen to preferentially dock at the lip of a hydrophobic region at the opening of the active site of WT-TK, where no product formation was possible, providing a possible cause of limited activity with WT-TK.

3.3.4 TK library on propanal and cyclic aldehydes

All previously tested cyclic aldehydes (Figure 3.13) with WT-TK were screened with library D469 for enhanced activity and detected using the specifically designed tetrazolium red colour assay.

The cyclic aldehyde substrates that indicated some activity with WT-TK were applied to the library D469 for the possible identification of improved activity and production of cyclic ketodiol compounds. The cyclic aldehydes are all non-α-hydroxylated aldehydes and therefore applicable for the colorimetric assay. It was noticed that all 5 aldehydes maintained a common pattern within the microwell plate with regard to improved activity compared to WT-TK (Figures 3.18, 3.19, 3.20). Other cyclic aldehyde screens are shown in the appendix.

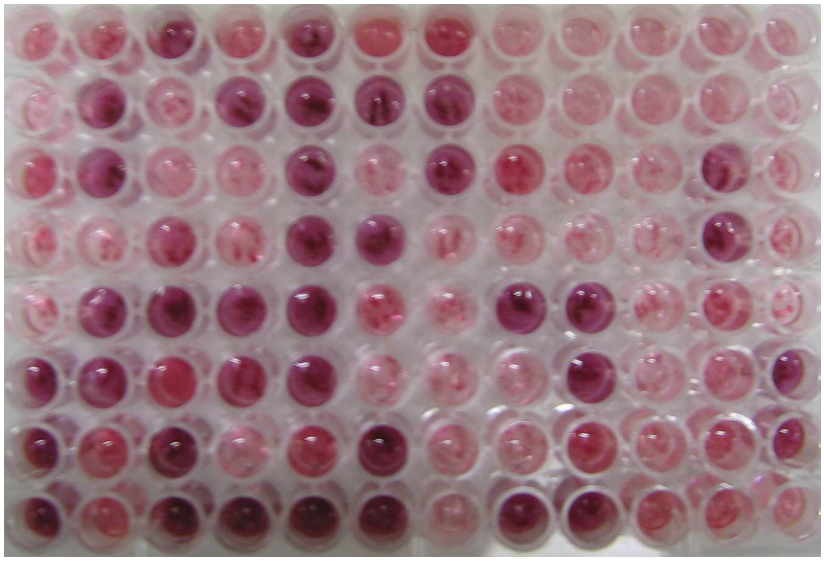


Figure 3.18: Propanal screen of library D469X. Photograph of microwell plate containing structural library D469 TK-mutants with enhanced activity towards propanal and HPA. (See section 3.2.7 for details)

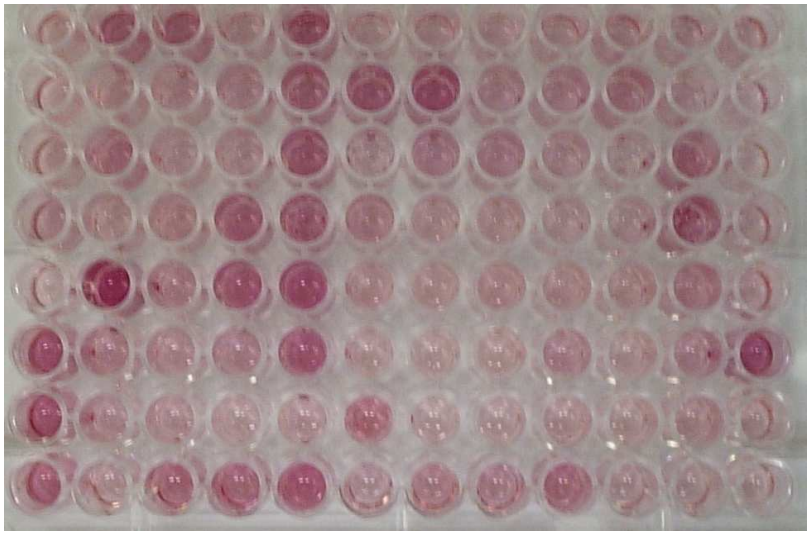


Figure 3.19: Cyclohexanecarboxaldehyde screen of library D469X. Photograph of microwell plate containing structural library D469 TK-mutants with enhanced activity towards cyclohexanecarboxaldehyde and HPA.

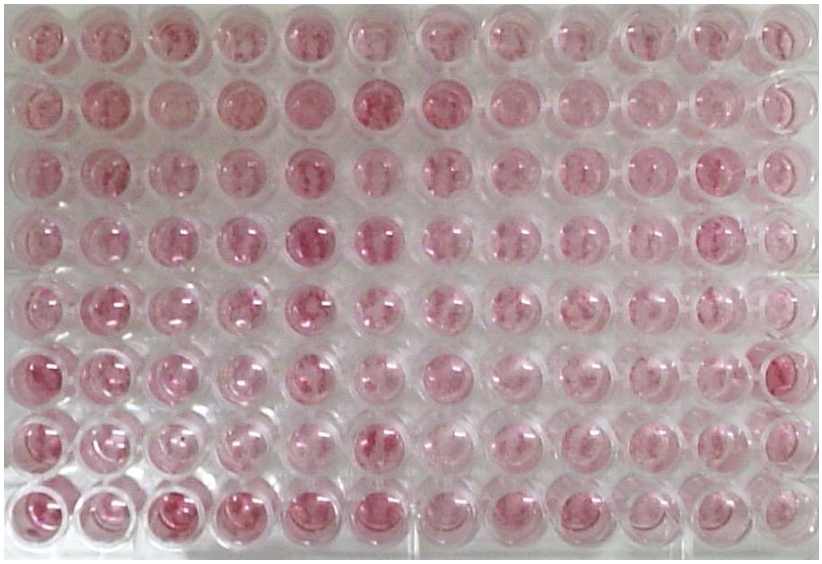


Figure 3.20: 3-cyclohexene-1-carboxaldehyde screen of library D469X. Photograph of microwell plate containing structural library D469 TK-mutants with enhanced activity towards 3-cyclohexene-1-carboxaldehyde and HPA.

3.3.5 TK D469X library on DA cycloadduct

Following screening with the commercially available cyclic aldehydes, the DA cycloadduct, 3,4-dimethyl-3-cyclohexene-1-carboxaldehyde (DCHCA) was applied to the same structural D469 library. Figure 3.21 shows the colorimetric detection assay indicating several mutants with activity towards the bulky, hydrophobic, cyclic acceptor aldehyde substrate.

Despite the lack of product formation from the WT-TK catalysed reaction with DCHCA, several mutants from library D469 indicted some activity. Figure 3.22 shows the correlation between active mutants of this library with all the cyclic aldehydes screened. All cyclic aldehydes appear to have a relationship with regard to mutant hits, with Pearsons correlation ranging from 0.62-0.74 for all cyclic aldehydes with respect to propanal.

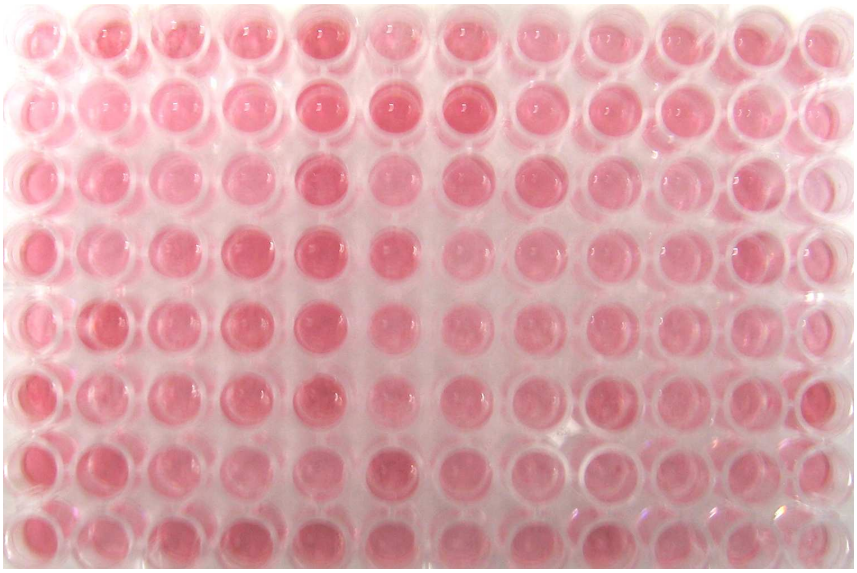


Figure 3.21: 3,4-dimethyl-3-cyclohexene-1-carboxaldehyde screen of library D469. Photograph of microwell plate containing structural library D469 TK-mutants with enhanced activity towards 3,4-dimethyl-3-cyclohexene-1-carboxaldehyde and HPA.

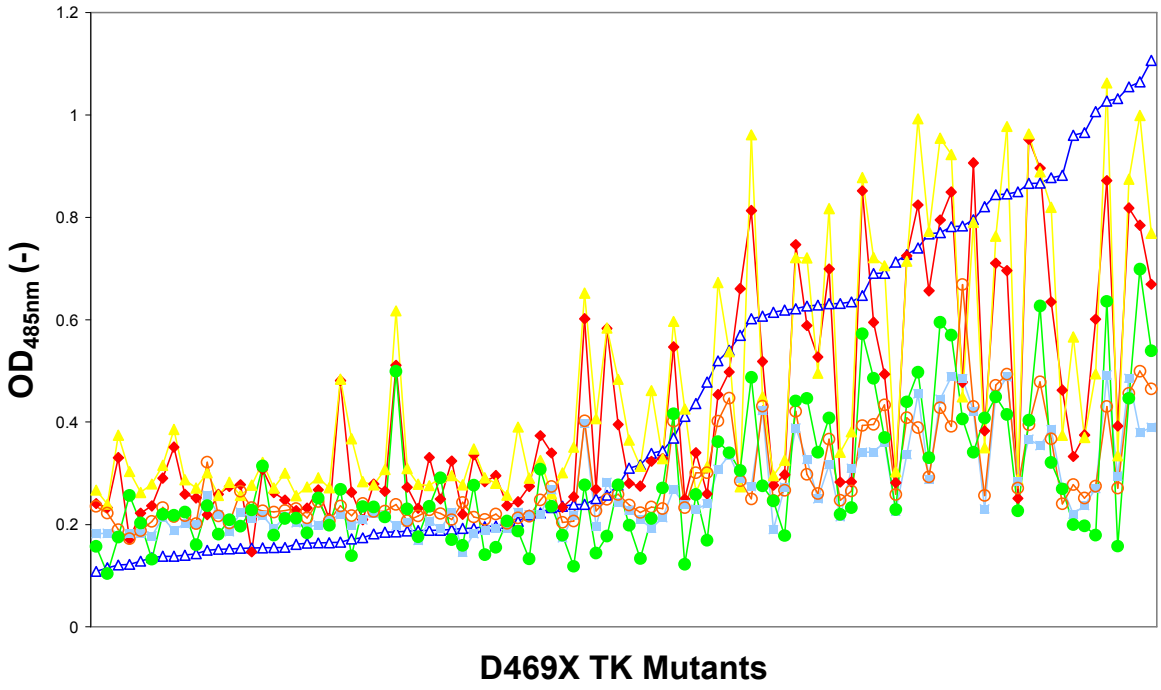


Figure 3.22: Correlation of cyclic aldehyde D469 screens in relation to propanal. Propanal (Δ) cyclopropane-carboxaldehyde (♦) cyclopentane-carboxaldehyde (▲) cyclohexane-carboxaldehyde (■) cyclohexene-carboxaldehyde (○) 3,4-dimethyl-3-cyclohexene-1-carboxaldehyde (●). OD is proportional to formazane production and is relative to ketodiol synthesis.

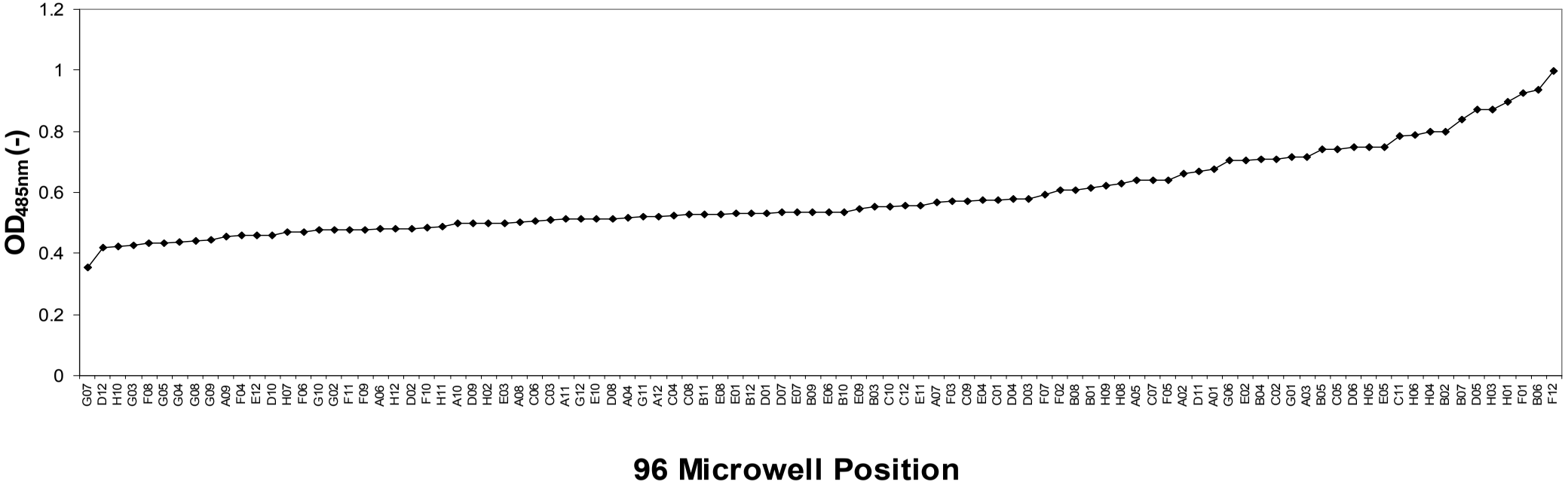


Figure 3.23: D469 Library screen, using 3,4-dimethyl-3-cyclohexene-1-carboxaldehyde (DCHCA). OD is proportional to formazane production and is relative to ketodiol synthesis.

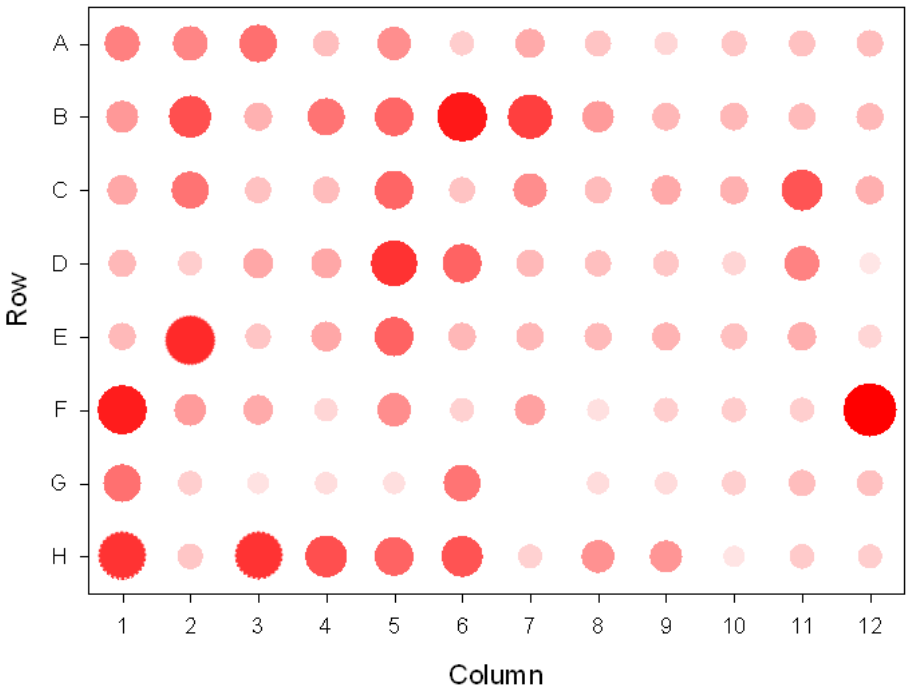


Figure 3.24: Intensity spots. Graph representing the absorbance intensity of 96 microwell plate containing library D469 active mutants measured towards DCHCA and HPA.

3.3.6 TK sequencing and mutant identification

The top six performing D469X TK-mutants with activity towards DCHCA on the basis of reduction to formazane, relative to ketodiol synthesis were those residing microwells F12, B6, B7, F1, D5 and E2 represented in Figure 3.24 and 3.23. Figure 3.25 shows the activity development of TK, where WT-TK shows no activity at all through the sensitive detection method of TLC and visualisation by PMA and heat alongside 2 novel active mutants, F12 and D5 towards a novel acceptor substrate.

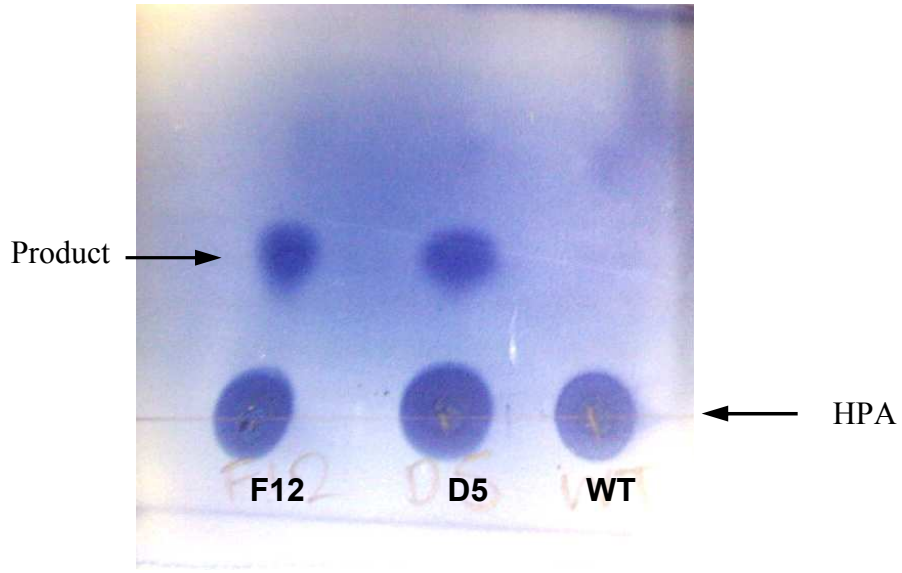


Figure 3.25: Comparison of top performing TK-mutants and WT-TK. Thin layer chromatography D469T (F12) D469L (D5) and (WT) over 48 hours. Product visualised with phosphomolybdic acid (PMA) and heat. Residual HPA does not migrate and resides at the baseline.

3.3.7 Fermentation scale up and synthesis at scale

The typical lag phase of approximately one hour was observed on all mutant fermentations in line with WT-TK *E. coli* BL21 (DE3), indicating the exponential growth of the inoculum. All mutant growth profiles followed WT growth except that of mutant residing in microwell F1. Fermentation was ceased on determination of the end of the linear exponential phase Ln [OD_{600}], allowing for the calculation of growth rate for the mutants based on the exponential growth rate and linear regression. All fermentations of the same *E. coli* strain, including both WT-TK and all mutants reached the end of exponential growth within 8 hours (± 1.0 hours).

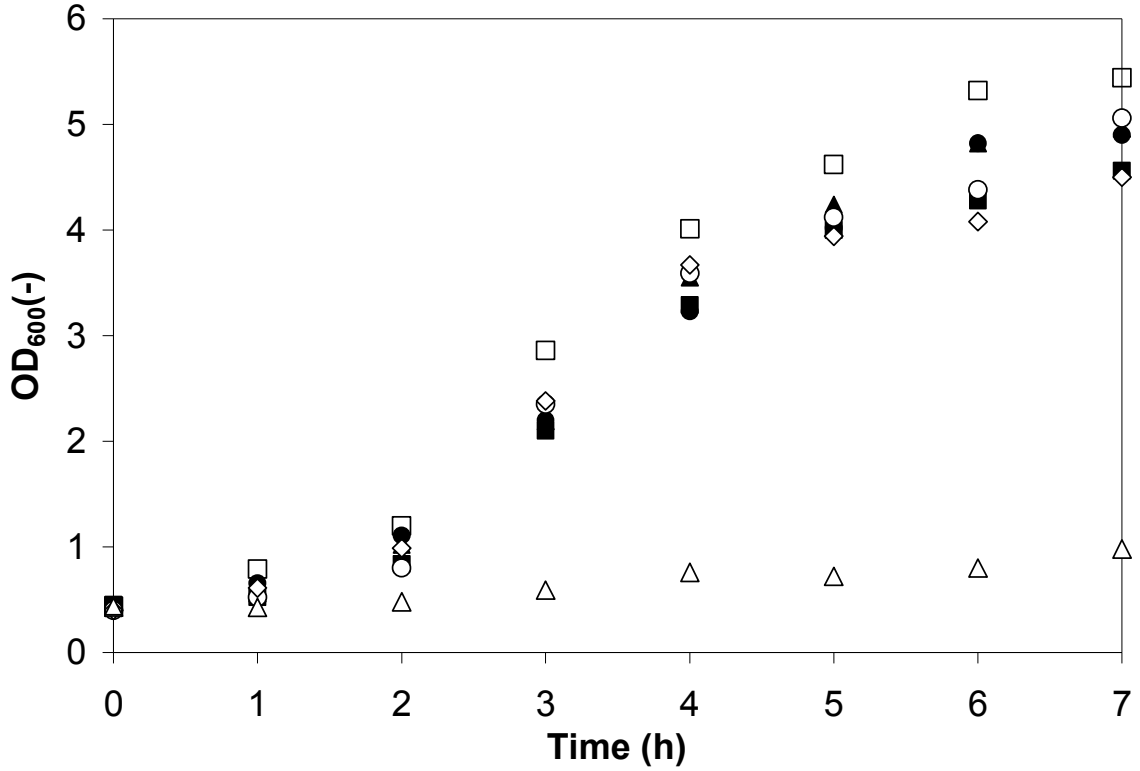


Figure 3.26: Typical fermentation growth curve of WT-TK and TK-Mutants from library D469. The growth of *E. coli* expressing WT-TK monitored by OD_{600}, (●) was compared to the growth of *E. coli* expressing selected top performing TK-mutants, B6 (■), F12 (□), E2 (○), B7 (▲), F1 (Δ) from library D469X, *E. coli* strain BL21(DE3) pQR412. All fermentations were carried out in parallel under exactly the same conditions with 10% v/v inoculum, 37 °C, 200 rpm in 1 Litre baffled shake flasks with 100 mL LB media and 150 $\mu g.mL^{-1}$ ampicillin. Data shown represents an average of 10 fermentations (see section 2.3 for details).

Slight variations in growth curves between the selected 6 TK mutants compared to WT-TK are not significant, except microwell F1. The average fermentation of *E. coli* expressing WT-TK and those expressing the top performing mutants maintained a specific growth rate of 0.8 h^{-1}. Growth for TK residing in F1, was inconsistent and often significantly lower than the other 5 TK mutants and WT-TK. An average growth rate of 0.12 h^{-1} (± 0.08) indicated a growth retardation possibly caused by the directed evolution processing. The TK levels obtained from microwell F1 were also significantly lower than WT-TK and the other TK-mutants. Potential issues with the cell host may have caused growth inhibition.

A 20 L fermentation of *E. coli* expressing D469T was performed in order to establish the stability of the mutant biocatalyst at scale and also to provide significant quantities of enzyme for future experiments. The growth of the bacteria was excellent reaching OD levels of 18 A.U. compared to the 5-6 A.U. of the smaller 1 L bench scale fermentations (Figure 3.27). The activity of the biocatalyst however once purified was significantly lower than that measured of the smaller scale fermentation preparations. It was considered that the larger scale homogenisation was detrimental to the enzyme activity. Centrifugation at large scale did not appear to be responsible for the reduced activity due to comparable activities from small scale centrifugation of the same 20 L fermentation culture broth. Due to the reduced activity, the enzyme preparation was not used for kinetic analysis and 1 L shake flask fermentations were used throughout. Optimisation of the purification process at scale would inevitably improve enzyme activity levels.

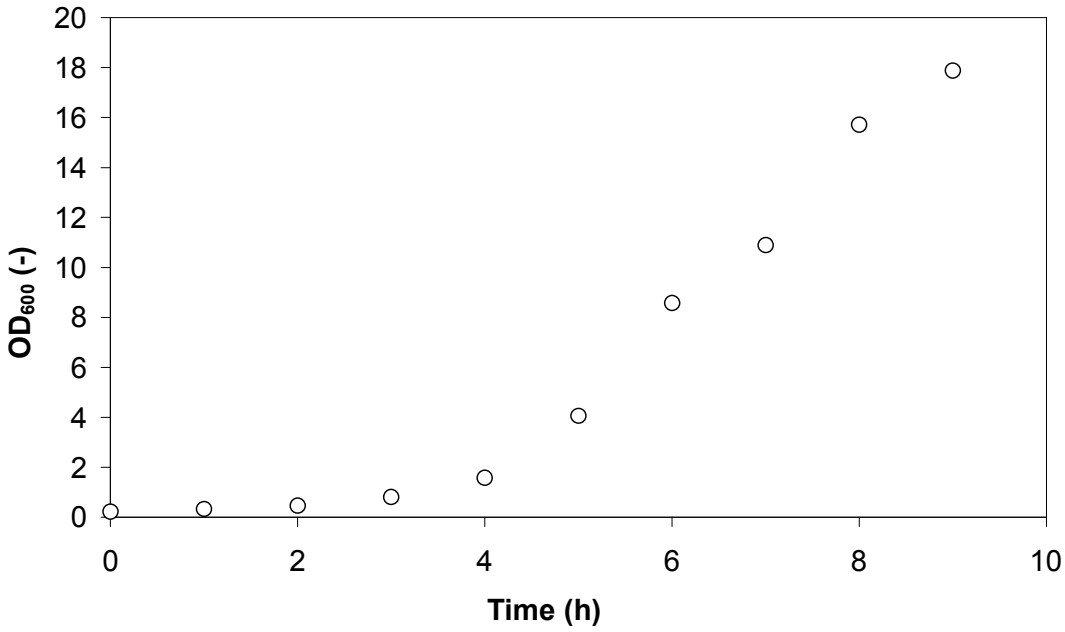


Figure 3.27: 20 L fermentation growth curve of D469T (Single fermentation performed and conditions described in section 2.3.6)

The use of pure enzymes for kinetic data is a controversial topic owing to the end process utilising a reaction system closer to that of enzyme lysate or whole cells. However in order to obtain data that is free of any potential interference from other enzymes that may be present in the protein lysate, pure TK has been used here. Also the potential presence of WT-TK, despite the lack of activity noted with DCHCA, may give false positive data.

The purification process was based upon a small scale nickel chelate affinity chromatography method for the binding of histidine-tagged proteins (Zeng *et al.,* 1999) using His·Bind® Quick 900 affinity columns. A detailed scheme of the purification process is shown in Figure 3.28. Samples were taken throughout the process for analysis of any TK protein loss by SDS-PAGE, and final determination of enzyme concentration measured by spectrophotometric wavelength scanning, and confirmation at wavelength 280 nm. Controlling temperature during the sonication procedure was found to be significant, alongside shorter pulses and longer interval periods, as protein losses reached

60% without the use of ice. The sonication procedure was seen to be detrimental to the activity of the enzyme if the sample was not cooled sufficiently. Where possible fresh buffer was cooled prior to use and at each stage samples were kept on ice. Centrifugation was also performed in a pre-cooled centrifuge at 4 °C.

SDS-PAGE was performed to determine enzyme purity and concentration with densitometry alongside the Bradford assay for total protein concentration. Initially, however, spectrophotometric wavelength scanning at 280 nm was performed. The fast, non-consuming, absorbance assay is based upon the knowledge that Tyr and Typ in particular, absorb UV absorbance near 280 nm and that the relationship between absorbance and concentration is linear. A known extinction coefficient of the pure TK, 93905 $M.cm^{-1}$ is required (Wetlaufer, 1963; Martinez-Torres *et al*., 2007). The value measured, however, must be considered for error, in that different proteins maintain greatly varying absorption characteristics. Also the presence of any non-protein contaminant may absorb UV at 280 nm giving false positive measurements. Pure TK preparations of both WT and mutants were repeatedly measured with concentrations ranging from 0.3-0.9 $mg.mL^{-1}$.

SDS-PAGE was subsequently used to measure purity of the enzymes. Figure 3.29 shows a typical 7.5% gel with a marker lane verifying the position of TK band, at just below 75 kDA, as TK monomers have a molecular weight of 72260 Da (72.3 KDa). Employing densitometry analysis of the gel, also allows for the quantification of the proteins separated by the 80-150 V run. Pure TK preparations including mutants often had a purity of >94%. The lysate preparations indicate the proportion of TK with regard to total protein concentration, which varied depending upon TK mutant and initial cell growth. Total protein concentration was determined by the Bradford assay where the absorbance shift between starting Coomassie solution and protein bound Coomassie is measured (Bradford, 1976).

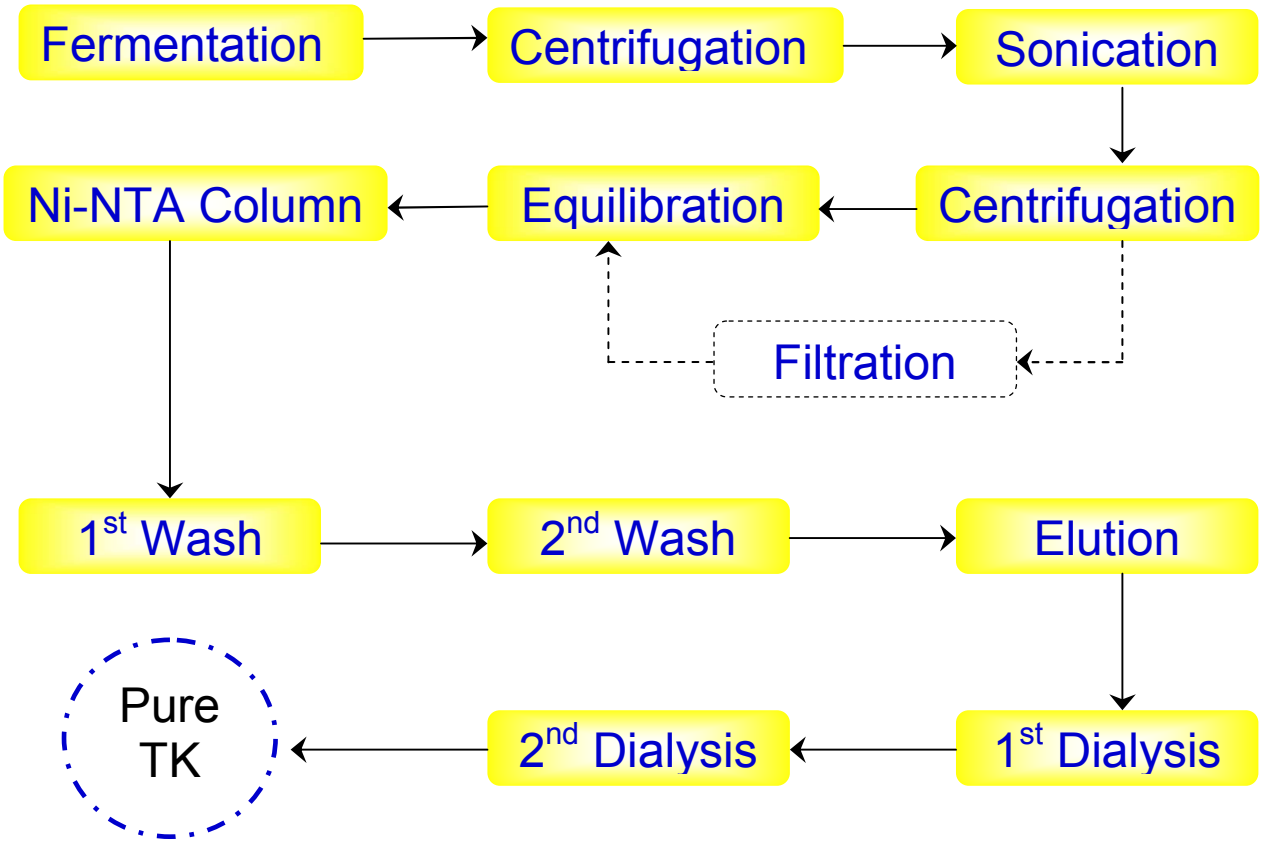


Figure 3.28: TK purification process. His x 6-tagged protein production and purification from fermentation and isolation using Ni-NTA columns, based on Nickel Chelate metal affinity chromatography.

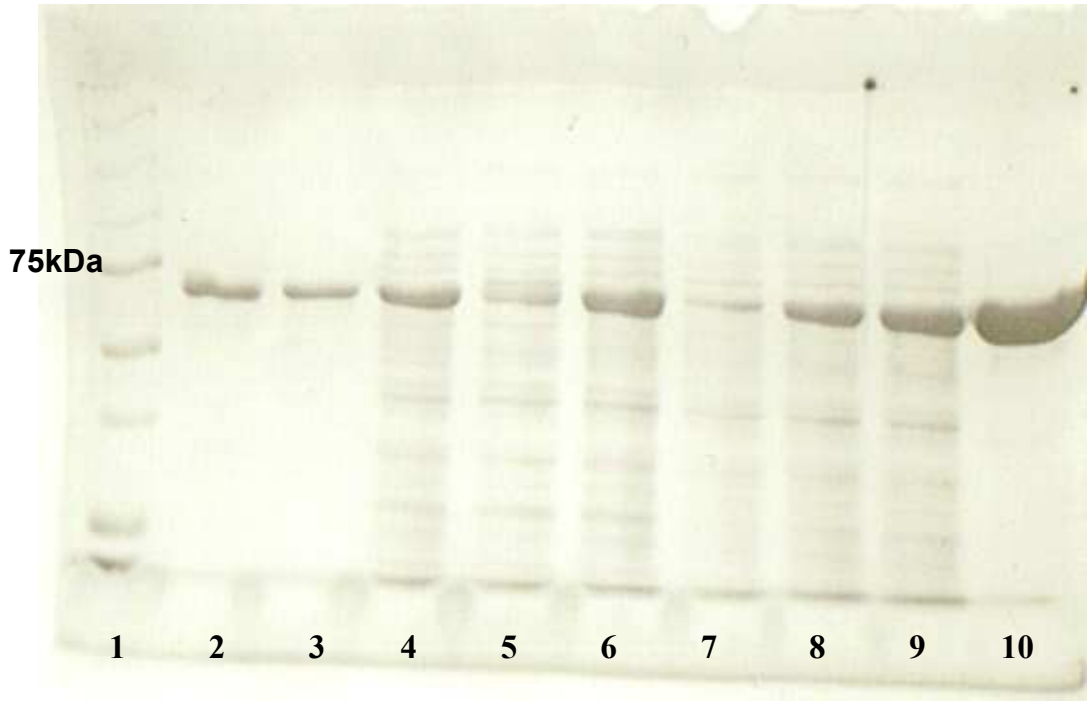


Figure 3.29: SDS-PAGE gel photograph. Lanes 1. Marker, 2. Pure WT-TK, 3. Pure D469T TK, 4. D469T lysate 33%, 5. D469L lysate 25%, 6. D469K (B6) lysate 35%, 7. F1 Lysate 19%, 8. WT 31%, 9. D469K (E2) Lysate 35% 10. Pure TK D469T (x5) (See section 2.4.1 for details).

WT-TK expressed TK as 30% of its total protein, microwell F12 expressed 33% TK, B6 35% TK, B7 32%, D5 25% and F1 an average of 19% TK of total expressed protein. Further indication of an issue with the host cell or plasmid vector of microwell F1.

Each batch of pure-TK and clarified lysate-TK preparations were analysed for activity with glycolaldehyde and HPA (Figure 3.8). The reaction products are L-erythrulose and carbon dioxide rendering an irreversible reaction (Bolte *et al.*, 1987). The standard glycolaldehyde activity assay entails a 20 minute pre-incubation of enzyme with a cofactor solution at room temperature to reconstitute the holoenzyme. An equimolar substrate concentration was performed, samples taken at regular intervals and a specific activity of 0.63 $\mu mol.mg^{-1}.min^{-1}$ was measured.

Initial activity tests using substrate glycolaldehyde and protein measurements of purified WT-TK and D469T-TK showed significantly lower amounts of enzyme, with lower activities than expected. The purification was analysed at each step to determine any protein losses. It was found the filtration step using 0.2 μm cellulose acetate syringe filters prior to column equilibration caused a 70% loss in TK levels. The filtration step was not performed in subsequent purifications, and appeared to have no detrimental effects on the loading of the sample onto the Ni-NTA column or on purity of the pure TK samples.

Two shorter dialysis stages replaced one lengthy stage, after initial activity assays indicated potential activity losses at the dialysis stage of 16 hours. A larger volume of 50 mM Tris-HCl dialysis buffer also containing cofactors, TPP and $Mg2^{+}$ was also used to increase removal of free EDTA and EDTA nickel complexes and maximise TK stability in solution (Matosevic, 2009).

3.3.7.1 Bioedit

Bioedit is a biological sequencing alignment tool, allowing for a relatively simple visualisation of gene sequences. The tool permits both the analysis of sequences and useful manipulation functions, whereby simple single point mutations are easily determined. The sequence mutations were determined and are presented in table 3.3.

Table 3.3: TK-Mutant Characterisation. Top performing mutants from D469 library screen with HPA (50 mM) and DCHCA (50 mM) in Tris-HCl buffer (50 mM pH 7.0). [a] Total activity measured using the colorimetric assay screen.

Well Number	Mutation	Residue	Relative Activity[a] (D469T = 1)	TK expressed (% total protein)
WT	D469	Aspartic acid	0	31 ± 5.0
F12	D469T	Threonine	1	33 ± 2.9
B6	D469K	Lysine	0.82	35 ± 4.5
B7	D469S	Serine	0.81	32 ± 4.3
F1	D469T	Threonine	0.79	19 ± 7.6
D5	D469L	Leucine	0.76	25 ± 3.1
E2	D469K	Lysine	0.82	35 ± 4.3

The top performing mutant F12 was sequenced as D469T (threonine), the same as the mutant sequenced in microwell F1. The inconsistent growth ranging from OD_{600} levels of 1-5 A.U., significantly lower expressed TK levels, up to 50% less than WT, and inconsistent activity levels with acceptor aldehyde substrates DCHCA and glycolaldehyde, indicated the possibility of an additional mutation within the gene not present in F12 or problems with the host.

Microwells E2 and B6, were both sequenced and confirmed to have the same mutation, D469K. Both microwells correlated well with regard to their TK expression levels and activity towards DCHCA, indicating that both mutants maintain the same mutation without any evidence of further mutations affecting these characteristics.

Initial growth and activity studies indicated that microwell E2 was contaminated with multi variants, as activity was inconsistent with both substrates propanal and DCHCA however consistent with glycolaldehyde reactions. Sequencing of the mutants from microwell E2 confirmed the presence of at least two different mutants, namely, D469K (leucine) and D469N (asparagine). The mutants were separated with repeated streaking and growing up of individual *E. coli* colonies as described in Chapter 2. D469K showed enhanced activity with DCHCA whilst D469N was relatively inactive, with no improvement on WT-TK activity.

3.3.8 Summary

In this chapter, a potential novel acceptor aldehyde for application to the transketolase catalysed reaction was investigated. A chiral, bulky, hydrophobic aldehyde was considered for probing the effects of TK activity and selectivity. The far removed substrate, yet sequentially linked to natural substrates provided the basis of broadening the range of TK acceptor aldehydes beyond currently perceived limitations.

The aldehyde, 3,4-dimethyl-3-cyclohexene-1-carboxaldehyde (DCHCA), was successfully synthesised using Diels Alder chemistry in a room temperature ionic liquid (RTIL) and the process assessed for optimal reaction conditions. The addition of water to the system was critical for the effective stirring of the viscous liquid magnetically. The water insensitive, catalyst/solvent system of zinc-chloride/choline-chloride melt, gave excellent aldehyde yields of up to 91% and high purity of 98% without lengthy or complicated purification steps.

Library D469X was selected for screening due to its relationship associated with substrate binding and substrate stereoselectivity. Initial screening with a range of cyclic aldehydes showed low but promising activity with WT-TK, however a number of D469X mutants showed considerable activity enhancement. A specific colorimetric assay was employed for its high sensitivity, rapid and relatively easy detection of a large number of mutants. Despite applying highly sensitive detection methods including thin layer chromatography, WT-TK indicated no activity towards the novel substrate. However, screening of TK-Mutants previously made through saturation mutagenesis highlighted several 'hits'.

The top six performing mutants towards the novel substrate were subsequently grown and their specific activity determined. Activity towards the novel substrate was demonstrated as infinitely enhanced with all mutants, due to the total lack of activity observed with WT-TK. Small scale fermentations were performed for the isolation of DNA, and the TK gene sequenced for identification of the single point mutation at position 469. A range of mutations were observed, and Mutant D469T (threonine) was identified as the most improved active mutant towards the novel substrate.

The identified D469T mutant was then subjected to a Histidine-tag purification process and analysed for optimal process conditions and storage stability. The purification process saw that eliminating a filtration step prior to his-tag binding improved protein yield and also identified the importance of maintaining consistently cool temperature (>4 °C where possible), particularly during sonication and centrifugation steps.

4 Synthesis of 1,3-Ketodiol and enantiomeric excess

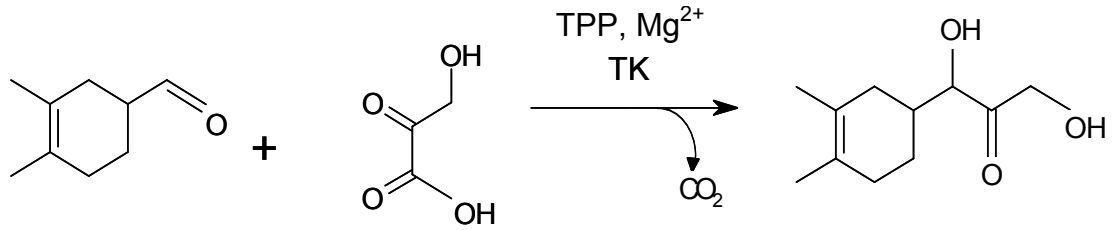
This chapter describes investigations into the enzymatic and biomimetic synthesis of the novel compound 1-(3',4'-dimethylcyclohex-3'-enyl)-1,3-dihydroxypropan-2-one (DCDHP). TK variants identified as having enhanced activity towards the cyclic, acceptor aldehyde 1,3-dimethyl-3-cyclohexene-1-carboxaldehyde (DCHCA), were applied to preparative scale reactions where products were then isolated, purified and subsequently characterised. This chapter also describes the determination of the optical purity of the product from TK catalysed reactions and compares the enantioselectivities of enhanced mutants to that of the WT-TK. The absolute stereo-configuration of the product from TK variant catalysed reaction of HPA and 1,3-dimethyl-3-cyclohexene-1-carboxaldehyde was also determined from a recently modified Mosher's ester methodology.

4.1 Introduction

4.1.1 TK reaction with complex substrates

A wide range of α-hydroxy and α-unsubstituted aldehydes have been previously reported as substrates accepted by transketolase from yeast (De La Haba *et al.*, 1955), spinach (Horecker *et al.,* 1956), and *E. coli* (Racker, 1948; Hibbert *et al.,* 2008). The ability of TK from yeasts, plants and bacteria to achieve enantioselective carbon-carbon bond formation, and tolerance for a wide range of aldehyde acceptors to give (*S*)-α,α'-dihydroxyketones has made it an attractive enzyme for industrial applications (Bongs *et al.,* 1997; Turner, 2000; Shaeri *et al.,* 2006). *E. coli* TK has been successfully over expressed (Lilly *et al.,* 1996) and shown to give up to six times greater specific activity than yeast TK, and as much as thirty times greater specific activity than spinach TK, with donor substrate HPA (Sprenger and Pohl, 1999). *E. coli* TK has also been successfully applied to a number of potentially commercial biocatalytic processes eg. the production of xylulose-5-phosphate (Shaeri *et al.,* 2008; Hecquet *et al.,* 1994).

A number of TK reports have described the use of α-hydroxyaldehyde acceptors and HPA where it was stereospecific for the α-(*R*)-hydroxyaldehyde (Bolte *et al.,* 1987; Effenberger *et al.,* 1992; Kobori *et al.,* 1992; Hecquet *et al.,* 1994; Mitra *et al.,* 1998) and stereoselectively forms a ketodiol product with the *S*-configuration. Further TK investigations have also reported that it can accept several non-α-hydroxylated aldehydes, although lower relative rates of reaction (typically 5-35% of hydroxylated substrates) were observed (Demuynck *et al.,* 1991; Hobbs *et al.,* 1993; Morris *et al.,* 1996). The activity of *E. coli* TK towards propanal as a substrate has been recently increased by directed evolution (Hibbert *et al.*, 2008; Smith *et al.*, 2008), paving the way for acceptance of other non-α-hydroxylated aldehydes. For example, 1-(3',4'-dimethylcyclohex-3'-enyl)-1,3-dihydroxypropan-2-one (DCDHP) was synthesised in Chapter 3 via a TK variant (Scheme 6).



DCHCA HPA DCDHP

Scheme 6: Transketolase catalysed reaction. HPA and 3,4-dimethyl-3-cyclohexene-1-carboxaldehyde (DCHCA) for the synthesis of 1-(3',4'-dimethylcyclohex-3'-enyl)-1,3-dihydroxypropan-2-one (DCDHP).

4.1.2 TK catalysed reaction conditions

Optimal TK reaction conditions are well established due to the continued investigation of activity (Cazares *et al.,* 2010) and the more recently studied structure and stability (Martinez-Torres *et al.*, 2007). However, with altering process conditions, such as scale, reaction vessels, titre concentration, enzyme concentration and enzyme preparation used, optimal conditions may require further modification tailored to a specific substrate. Also, the use of novel reaction components, may lead to required changes to the process conditions such as temperature increases, stirring rate and the use of alternative solvents to improve substrate solubility. Temperature and pH are important reaction parameters requiring consideration with TK, as with the majority of biological catalysts. *E. coli* TK has been seen to possess very low activity in acidic and basic conditions, with a typical bell-shaped activity profile with optimal activity at close to neutral pH (Mitra *et al.,* 1998).

The stability of the enzyme under storage and biocatalytic reaction conditions is also an important consideration when investigating kinetic activity with novel

substrates. It is crucial that the biocatalyst maintains native activity over a period of time and that no destabilisation or degradation of the enzyme occurs. This is fundamental to ensure that measurements between separate reactions are accurate and in particular between separate batches of TK preparations. For example, it is known that wild-type *E. coli* TK is susceptible to deactivation by dissociation of the TPP cofactor (Brocklebank *et al.,* 1999; Martinez-Torres *et al.,* 2007), and irreversible inactivation by oxidation (Brocklebank *et al.,* 1999).

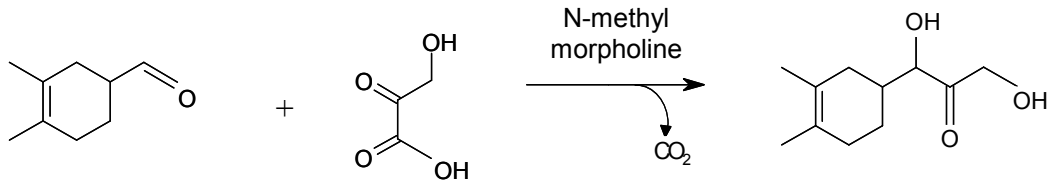
4.1.3 Biomimetic TK reaction

The monitoring and characterisation of new enzyme-catalysed reactions requires that a product standard can be synthesised to enable assay calibration and also identification of the correct product peaks if HPLC assays are used. Previously reported syntheses of α,α'-dihydroxyketones include methods such as a ruthenium-catalysed oxidation of allenes (Laux and Krause, 1997) and the double hydroxylation of silyl enol ethers with *m*-chloroperbenzoic acid (Horiguchi *et al.,* 1989). A particularly useful and somewhat relevant approach however, describes a mimetic of the transketolase catalysed reaction (Smith *et al.,* 2006).

The biomimetic reaction successfully synthesises a racemate of the 1-(3',4'-dimethylcyclohex-3'-enyl)-1,3-dihydroxypropan-2-one (DCDHP), useful for analysing the enantiomeric excess (*ee*) of the TK reaction product, and deciphering the corresponding enantiomer and therefore absolute configuration. The reaction is also advantageous in synthesising a standard, prior to the identification of TK accepting mutants of this aldehyde, enabling a comparison of detection methods including TLC, HPLC and calibration of the colorimetric assay.

The mimetic of the transketolase catalysed reaction was discovered during TK control experiments, where the formation of product was evident without the presence of the enzyme or cofactors. The formation of a quaternary ammonium enolate is postulated as the mechanism in the atom efficient one pot synthesis. The substrates and products are analogous with the TK reaction however, the cofactor,

TPP relied upon by TK for conversion, is not required here. The mechanism is not fully understood, however, is proposed to follow a different pathway to TK and involves tertiary amine catalysis and quaternary amine enolate formation. This mimetic reaction was used as described in Section 4.2.5 to synthesise the racemic 1-(3',4'-dimethylcyclohex-3'-enyl)-1,3-dihydroxypropan-2-one (DCDHP) using N-methylmorpholine shown in Scheme 7.



Scheme 7: The mimetic of the TK reaction. In the presence of tertiary amine, N-methylmorpholine the reaction is stirred in water at pH7.0 and monitored by TLC.

4.1.4 Substrate solubility in biocatalysis

For hydrophobic substrates, solubility is a challenge that potentially needs to be overcome amongst other process limitations to achieve both maximum productivity and accurate kinetic data. Reduced accessibility of the substrate to the biocatalyst may severely inhibit activity and many approaches therefore exist to improve such situations. A number of measures are employed for altering substrate solubility in process chemistry and biocatalysis including co-solvent addition (Carrea, 1984; Seng Wong *et al*., 2004; Miyako *et al.,* 2010), stirring, (Woodley *et al.,* 1991; Michelletti *et al.,* 2006) and increased temperature. All of these approaches subsequently generate considerations for process design and engineering, such as reaction vessel design, substrate feeding strategies and product isolation. All of these methods however, possess the potential to significantly affect the activity and / or stability of the biocatalyst. Some organic solvents as well as elevated temperatures may irreversibly cause protein

degradation. This may occur through the penetration of the cosolvent to the aqueous microenvironment surrounding the protein, potentially altering its three dimensional structure or stripping tightly bound water from the enzyme crucial for catalytic activity (Deetz and Rozzell, 1988; Klibanov, 1997). However, on the other hand, some enzymes have exhibited increased stability in the presence of organic solvents (Kilbanov, 1989; Moore and Arnold, 1996; Hao and Berry, 2004), such as porcine pancreatic lipase (Zaks and Klibanov, 1985) ribonuclease (Volkin *et al.,* 1991) and α-chymotrypsin (Zaks and Klibanov, 1988) at 100 °C exhibits a half life of several hours in anhydrous solvents, compared to complete deactivation within seconds in water.

The transketolase reaction has previously been performed in aqueous media and therefore all reaction components were characterised in water. The use of alternative or additional solvents may also have adverse effects on reaction components other than the biocatalyst, potentially causing decomposition over periods of time. Selectivity of the enzyme may also be affected by organic solvents altering enantio- regio- and chemo- selectivity characteristics. Many reports over recent years have presented data that not only shows an improvement in the enantioselectivity of the enzyme but complete inversion of the enantioselectivity (Klibanov, 2001). For instance, the transesterification of methyl-3-hydroxy-2-phenylpropionate with propanol saw a 20-fold rate change with differing organic solvents. Additionally, the enantioselectivity was seen to be completely reversed, in that with cyclohexene as a solvent the *S*-enantiomer was preferentially transesterified compared to the *R*-anitpode in acetone (Wescott *et al.,* 1996).

4.1.5 Enantioselectivity of wild-type-TK and its variants

The enantioselectivity of an enzymatic reaction if generating chiral compounds is one of the most significant factors determining the usability of a commercial biocatalyst. The direct synthesis of single enantiomer products is not only preferential but becoming more and more fundamental in most disciplines. Enantiomeric excess (*ee*) is a measure of the degree to which one or another

enantiomer is present in a greater quantity. WT-TK stereoselectivity towards glycolaldehyde and HPA was determined by chiral HPLC to be (*S*) 95% (Smith *et al.,* 2008).

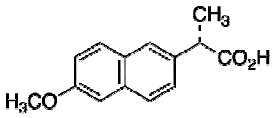
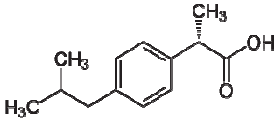
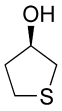
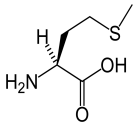
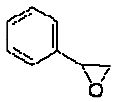
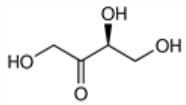
C_4-C_8 linear aldehydes (non-α-hydroxyaldehydes) were recently investigated as aldehyde acceptors with a range of single-point mutant TK enzymes (Cazares *et al.,* 2010). Several improvements in stereoselectivity were observed, ranging from 0% to >99%. It is particularly interesting that the degree of stereoselectivity of the transformation varied with the length of the aliphatic chain of the aldehyde. High stereoselectivities were noted with mutant TK-D469E against butanal, pentanal and hexanal giving 97-98% *ee* and the highest *ee* was observed with aldehydes of a similar size to *in vivo* TK substrates. WT- TK stereoselectivity with propanal (PA) was noted as only 58%; however D469E-TK improved this stereoselectivity to 90% of (3*S*)-DHP. The overall product yield was also enhanced from 36% to 70% (Smith *et al.,* 2008).

Mutant D469T- TK also exhibited both stereoselectivity and yield enhancement with PA, with the *ee* rising from 58% to 64% and yield improving from 36% to 68% (Smith *et al.,* 2010). TK residue D469 has previously also been noted as being involved in the enantioselectivity of the reaction when using α-hydroxyaldehydes (Nillson *et al.,* 1997). Remarkably, active-site single-point mutants Asp469Glu (D469E) and His26Tyr (H26Y) were identified and were able to both enhance and reverse the stereoselectivity from 58% (3*S*)-DHP to 88% (3*R*)-DHP (Smith *et al.,* 2008). Stereoselectivity reversals were also noted throughout a range of linear aldehyde substrates C_3-C_8. Mutant D469E-TK was also noted to possess high enantioselectivities with the cyclic aldehydes of the substrate walking approach, whereby cyclopropanecarboxaldehyde and cyclopentanecarboxaldehyde adducts were formed in 99% ee and cyclohexanecarboxaldehyde formed in 97% *ee.* Similarly D469T exhibited high enantioselectivities with these cyclic aldehydes forming the (1*S*)-isomer.

The equivalent residue to *E. coli* TK D469 in yeast transketolase is D477 which has also been reported as a determinant of enantioselectivity. This residue is

located in the active site channel and forms a hydrogen bond with the C2-hydroxyl group of the acceptor aldehyde. It was seen that substrates with an L-configuration at the C2-atom had k_{cat}/K_m values 3-4 orders of magnitude lower for the D477A mutant than with the wild-type enzyme (Nilsson *et al.,* 1998).

Table 4.1: Highly enantioselective biocatalysts reported in literature.

Enzyme	Source of Enzyme	Product	*ee* (%)	Ref.
(S)-specific Nitrilase	*Rhodococcus C311 &MP50*	*(S)*-naproxen 	>99	Layh *et al.,* 1994.
(S)-specific Nitrilase	*Acinetobacter sp. AK226*	*(S)*-Ibuprofen 	90.8	Goodhue and Schaeffer, 1971
Ketoreductase	*Lactobacillus kefir*	*(R)*-tetrahydrothiophene-3-ol 	99.3	Huisman *et al.,* 2010
Hydantoinase	*Escherichia coli*	L-methionine 	90	May *et al.,* 2000
Epoxide Hydrolase	*Agrobacterium radiobacter*	Styrene Oxide 	>99	Spelberg *et al.,* 1998
Transketolase	*Escherichia coli*	L-Erythrulose 	95	Smith *et al.,* 2008

4.1.5.1 Methods of enantiomeric excess (*ee*) determination

The products of reactions such as the TK catalysed reaction are often analysed for their enantiomeric ratio through optical rotation, derivatisation and chromatography. X-ray crystallography and NMR spectroscopy by the use of lanthanide shift reagents (Wenzel *et al.,* 1992) or chiral derivatising agents (Dale and Mosher, 1973; Pehk *et al.,* 1993) are also widely used methods for the determination of *ee* and absolute configuration.

A recent investigation has described the determination of absolute chemistry of 1,3-dihydroxy ketones through acetate derivatisation and chiral HPLC and GC alongside Ender's SAMP methodology to synthesise the products in high optical purity (Smith *et al.,* 2008). Enders methodology involves the utilisation of chiral auxiliaries, temporary chiral compounds employed to introduce chirality into racemic compounds allowing for the asymmetric alpha-alkylation of aldehydes and ketones. However, the use of this lengthy, multistep procedure and the addition of chiral auxiliaries is not ideal in terms of time scale and is also not suitable for aromatic α,α'-dihydroxyketones (Enders *et al.,* 1986).

The method developed by Mosher, using 2-methoxy-2-(trifluoromethyl) phenylacetic acid (MPTA) has been utilised widely for the determination of absolute stereochemistry of both secondary alcohols and primary amines (Ohtani *et al.,* 1991). MPTA is a chiral molecule, available commercially as both the R and S enantiomers shown in Figure 4.1. The approach works by forming an ester or an amide with the compound of unknown stereochemistry, which may be then determined by ^{1}H NMR spectroscopy of the ester or amide.

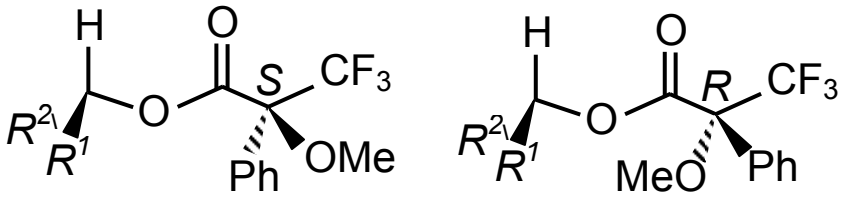


Figure 4.1: Mosher Conformers. (S)-MPTA and (R)-MPTA derivatives.

The use of Mosher's acid and ^{1}H chemical shift to aid configurational assignment of chiral secondary alcohols has been demonstrated by two separate, analogous experiments using each Mosher's acid enantiomer (Figure 4.1). The ester protons at R^1 and R^2 were assigned through the determination of the difference between their chemical shift, such that $\Delta\delta=\delta_{S\text{-}MPTA} - \delta_{R\text{-}MPTA.}$ The protons at R^1 of the (*R*)-MPTA diastereoisomer were observed upfield in relation to the (*S*)-MPTA ester due to current effects of the phenyl ring (Pehk *et al.,* 1993).

It was also recently demonstrated that application of a modified Mosher's method for the determination of enantiomeric excess (*ee*) and also the absolute configuration of chiral 1,3-dihydroxyketones at C3 was possible (Galman and Hailes, 2010). The *ee* of a range of aliphatic α,α'-dihydroxyketones with lipophilic and polar R groups were demonstrated to be accessible within 5% accuracy of GC or HPLC methods. In this chapter this method was used to determine the absolute stereochemistry of the 1-(3',4'-dimethylcyclohex-3'-enyl)-1,3-dihydroxypropan-2-one (DCDHP) product from a mutant TK-catalysed reaction.

4.2 Materials and Methods

4.2.1 TK Catalysed synthesis of 1-(3',4'-dimethylcyclohex-3'-enyl)-1,3-dihydroxypropan-2-one (DCDHP)

TPP (22 mg, 48 μmol; 2.4 mM) and $MgCl_2$ (39 mg, 180 μmol; 9 mM) were dissolved in H_2O (10 mL) and the pH adjusted to 7 with 0.1 M NaOH. To this stirred solution, at 20 ºC, was added D469T-TK (clarified lysate or pure) (2 mL) and the mixture stirred for 20 minutes. In another flask, HPA (330 mg, 3 mmol; 50 mM) and DCHCA (414 mg, 3 mmol; 50 mM) were dissolved in H_2O (8 mL), sonicated and the pH adjusted to 7 with 1 M NaOH. Following the 20 minutes enzyme/cofactor pre-incubation, the substrate solution was added to the enzyme solution and the mixture stirred at 25 ºC for 24 hours. During this time, the pH was maintained at 7.0 by addition of 1 M HCl using a pH stat (Stat Titrino, Metrohm). Silica was added and the reaction mixture concentrated to dryness before dry loading onto a flash silica gel column (ethyl acetate) to give DCDHP (0.090 g, 15% ±2%). $[\alpha]^{20}_{D} = +140$ (*c* 0.5, $CHCl_3$); ^{1}H NMR (300 MHz; $CDCl_3$) δ 1.38 (2H, m), 1.59 (3H, s, C*H*$_3$), 1.61 (3H, s, C*H*$_3$), 1.96 (4H, m), 2.12 (1H, m), 2.98 (1H, m) 4.22 (1H, *J* 2.6 Hz, C*H*OH), 4.30 (1H, d, *J* 18.8 Hz, C*H*HOH) 4.48 (1H, d, *J* 18.8 Hz, CH*H*OH); ^{13}C NMR (125 MHz; $CDCl_3$) δ 18.8 (*C*H$_3$), 19.1 (*C*H$_3$), 23.3, 31.5, 34.3, 38.9 (*C*H), 66.1 (*C*H$_2$OH), 78.2 (*C*HOH) 125.4, 130.4, 211.6 (C=O) (see figure 4.2).

The same protocol was followed with the addition of 5% EtOAc with the substrate solution for increased yields of the same product (19% (±2%).

4.2.2 D469T-TK reaction pH dependence

The reaction was performed exactly as described in section 2.5.4 using pH stat (Stat Titrino, Metrohm) at pH 3, 5, 7, 9 and 11. The pH was maintained at the corresponding pH by addition of 1M HCl or 1M NaOH. Samples were taken at regular intervals and analysed by HPLC.

4.2.3 D469T-TK reaction temperature dependence

Reactions were performed exactly as described in section 4.2.1 however at both 20 °C and 40 °C in a water bath and stirred magnetically. Samples were taken at regular intervals and analysed by colorimetric assay.

4.2.4 Biomimetic reaction with propanal to give product 1,3-dihydroxypentan-2-one (DHP)

To a stirred solution of HPA (330 mg, 3.00 mmol) in water and THF (1:1, 60 mL) at room temperature, was added propanal (PA) (720 μL 3.00 mmol) and N-methylmorpholine (0.33 mL). The reaction mixture was stirred at 20 °C for 48 hours. The solvent was removed *in vacuo* and the crude product dry loaded onto flash silica chromatography column (EtOAc/hexene, 1:1) to afford (DHP) as a white solid (22% ± 3%).

4.2.5 Biomimetic synthesis of 4-isomer mixture of (1*RS*,1'*RS*)-1-(3',4'-dimethylcyclohex-3'-enyl) -3-hydroxy-2-oxo-propyl ester

To a stirred solution of HPA (330 mg, 3.00 mmol) in water (60 mL) at room temperature, was added 3,4-dimethyl-3-cyclohexen-1-carboxaldehyde (DCHCA) (414 mg, 3.00 mmol) and *N*-methylmorpholine (0.33 mL). The reaction mixture was stirred at room temperature for 48 hours. The solvent was removed *in vacuo* and the crude product dry loaded onto a flash silica chromatography column (EtOAc/hexene, 1:1) to afford (1*RS*,1'*RS*) -1- (3',4'-dimethylcyclohex-3'-enyl) -3-hydroxy-2 -oxo-propyl ester (racemic DCDHP) as a colourless oil (0.020 g, 3%). R_f = 0.49 (EtOAc/hexene, 1:1); ν_{max} (KBr)/cm^{-1} 3456br, 2893m, 1721s; ^{1}H NMR (300 MHz; $CDCl_3$) δ 1.40–2.08 (13H, m), 2.93 (1H, m), 4.26 (1H, m, C*H*OH), 4.36 (1H, br d, *J* 19.3 Hz, C*H*HOH) 4.49 (1H, dd, *J* 19.3 and 4.2 Hz, C*H*HOH); ^{13}C NMR (75 MHz; $CDCl_3$) δ 18.8, 19.1, 22.7, 23.5, 31.5, 34.3, 38.9, 66.1, 78.2, 125.4, 130.4, 211.6 (C=O); *m/z* (+FAB) 221 (MNa^+, 35%), 199 (MH^+, 100); Found (+HRES) MH^+ 199.13381. $C_{11}H_{18}O_3$ requires 199.12895 (Figure 4.2a).

The 2-isomer mixture of (1*RS*,1'*R*)-1-(3',4'-dimethylcyclohex-3'-enyl)-3-hydroxy-2-oxo-propyl ester was prepared using an identical method and (1*R*)-3,4-Dimethyl-3-cyclohexen-1-carboxaldehyde (3-1*R* isomer) synthesis described in section 4.2.6.1.

4.2.6 Enantioselectivity of D469T – TK

4.2.6.1 Synthesis of (1*R*)-3,4-Dimethyl-3-cyclohexen-1-carboxaldehyde ((1*R*-3)-DCHCA)

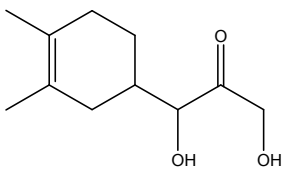
2,3-Dimethyl-1,3-butadiene (1.13 mL, 10 mmol) and acrolein (2.0 mL, 30 mmol) were added to a solution of (5*S*)-2,2,3-trimethyl-5-phenylmethyl-4-imidazolidinone monohydrate chloride as catalyst (255 mg, 1 mmol) in acetonitrile/water (95:5, 40 mL). The reaction was stirred at 25 °C for 48 hours. The reaction was partitioned between diethyl ether (100 mL) and 0.3 M $KHSO_4$ (100 mL) and the aqueous phase extracted with diethyl ether (2 × 100 mL). The combined organics were dried ($MgSO_4$) and the solvent removed *in vacuo* to give (1*R*)-DCHCA (1.10 g, 80%). $[\alpha]^{20}_D$ = +24.0 (*c* 1.0, CH_2Cl_2) (Benaglia *et al.,* 2002).

4.2.6.2 Benzoylation of DCDHP for chiral HPLC (Benzoic acid (3'RS,1''RS)-3'-(3'',4''-dimethylcyclohex-3''-enyl)-3'-hydroxy-2-oxo-propyl ester)

The reaction was carried out under anhydrous conditions. To a stirred solution of DCDHP (0.030 g, 0.10 mmol) in dichloromethane (20 mL) was added triethylamine (20 µL, 0.20 mmol) and benzoyl chloride (28 µL, 0.20 mmol). The reaction was then heated under reflux for 6 hours, cooled and added to dichloromethane (30 mL) and washed with 0.3 M $KHSO_4$ (2 × 20 mL), sat. $NaHCO_3$ (2 × 20 mL) and brine (2 × 20 mL). The organic layer was dried ($MgSO_4$) and evaporated to obtain the crude product. The residue was purified

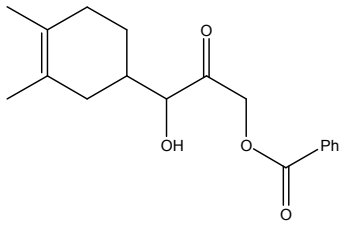
using silica gel column chromatography (EtOAc/hexane, 1:4) to afford the *benzoate* product as a colourless oil (0.040 g, 70%) (figure 4.2b) . R_f = 0.34 (EtOAc/hexane, 1:4); ν_{max} (KBr)/cm^{-1} 3429br, 2925s, 1785s, 1724s, 1452m; ^1H NMR (300 MHz; $CDCl_3$) δ 1.23–2.04 (13H, m), 3.00 (1H, br s, OH), 4.26 (1H, d, *J* 2.7 Hz, C*H*OH), 5.01 (1H, d, *J* 17.1 Hz, C*H*HOH), 5.15 (1H, d, *J* 17.1 Hz, CH*H*OH), 7.60 (3H, m, Ph), 8.13 (2H, m. Ph); ^{13}C NMR (75 MHz; $CDCl_3$) δ 18.8 (signals superimposed), 19.2, 22.6, 31.6, 31.9, 34.5, 38.4, 38.5, 66.6, 78.3, 78.7, 124.6, 128.5, 129.9, 133.6, 165.8 (*C*=O ester), 205.5 (*C*=O, ketone); Found (+HRFAB) M^+ 303.15995. $C_{18}H_{22}O_4$ requires 303.15963. Chiral HPLC: Chiracel-AD column (Daicel); mobile phase, isopropanol/hexane (3/97); flow rate, 0.8 mL/min, detection, UV 214 nm. Retention times; 28.6 min, 30.8 min, 34.0 min, 43.1 min.

a)



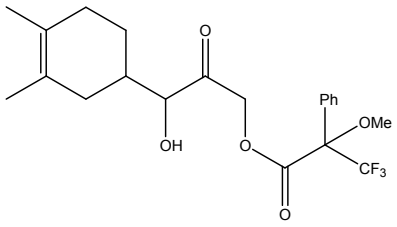
$C_{11}H_{18}O_3$
Exact Mass: 198.12560
Mol. Wt.: 198.25882
m/e: 198.12559 (100.0%), 199.12895 (12.2%)
C, 66.64; H, 9.15; O, 24.21

b)



$C_{18}H_{22}O_4$
Exact Mass: 302.15181
Mol. Wt.: 302.36488
m/e: 302.15181 (100.0%), 303.15516 (20.0%), 304.15852 (1.9%)
C, 71.50; H, 7.33; O, 21.17

c)



$C_{21}H_{25}F_3O_5$
Exact Mass: 414.16541
Mol. Wt.: 414.41541
m/e: 414.16541 (100.0%), 415.16876 (23.4%),
416.17212 (2.6%), 416.16965 (1.0%)
C, 60.86; H, 6.08; F, 13.75; O, 19.30

Figure 4.2: MS data for monobenzoylated DCDHP and Moshers acid.

The 2-isomer mixture of benzoic acid (3'*RS*,1"*R*)-3'-(3",4"-dimethylcyclohex-3"-enyl)-3'-hydroxy-2-oxo-propyl ester (DCDHP-1'*R* isomer) was prepared using an identical method. Chiral HPLC as above, retention times 28.6 min, 34.0 min.

The D469T-TK product was also converted to the monobenzoate as described above. $[\alpha]^{20}_{D}$= +7.6 (*c* 0.5, $CHCl_3$). Chiral HPLC as above, retention times 28.6 min (1%), 30.8 min (1%), 34.0 min (96.5%), 43.1 min (1.5%) to give a *de* for (1*S*,1'*R*)-DCDHP of 93%.

4.2.6.3 Mosher's Acid

(2*S*,3'*S*,1''*R*)-3,3,3-Trifluoro-2-methoxy-2-phenylpropionic acid 3'-(3'',4''-dimethylcyclohex-3''-enyl)-3'-hydroxy-2-oxo-propyl ester. The reaction was carried out under anhydrous conditions. To a stirred solution of the biotransformation product (DCDHP, 0.010 g, 0.05 mmol) in dichloromethane (5 mL) was added triethylamine (20 µL, 0.25 mmol) and (*R*)-MTPA chloride (15 µL, 0.08 mmol) in CH_2Cl_2 (2 mL) and the reaction was stirred for 12 hours at room temperature. The product was dry loaded onto silica gel and purified using flash chromatography (EtOAc/hexane, 1:4) to afford product as a colourless oil (0.015 g, 75%), (2*S*,3'*S*) >98% *ee*. R_f = 0.45 (EtOAc/hexane; 1:4) $[\alpha]^{20}_{D}$= +7.6 (*c* 0.5, $CHCl_3$); ν_{max} (KBr)/cm^{-1} 3429br, 2925s, 1758s,1734s, 1170s; ^{1}H NMR (500 MHz; $CDCl_3$) δ 1.38 (2H, m), 1.60 (3H, s, C*H*$_3$), 1.62 (3H, s, C*H*$_3$), 1.98 (4H, m), 2.12 (1H, m), 3.65 (3H, s, OMe), 4.23 (1H, m), 4.91 (1H, d, *J* 17.0, C*H*HO (2*S*,3'*S*)), 5.18 (1H, d, *J* 17.0, CH*H*O (2*S*,3'*S*)), 7.42 (3H, m, Ph), 7.63 (2H, m, Ph), (no 2*S*,3'*R* isomer detected); ^{13}C NMR (125 MHz; $CDCl_3$) δ 18.8, 19.1, 22.7, 31.6, 34.3, 38.6, 55.8 (O*C*H$_3$), 67.7, 78.6, 124.5, 125.5, 127.5, 128.5, 129.9, 131.9, 166.2 (*C*=O ester), 204.2 (*C*=O, ketone); ^{19}F NMR (282 MHz; $CDCl_3$) δ -71.0; *m/z* (+FAB) 437 (MNa^+-H, 43%), 415 (M^+, 56), 307 (65), 189 (100); Found (+HRFAB) M^+ 415.17372; $C_{21}H_{26}O_5F_3$ requires 415.17322 (Figure 4.2c).

4.3 Results and Discussion

4.3.1 D469T-TK catalysed reaction with DCHCA

The indication in Chapter 3 that transketolase variants were able to accept a hydrophobic, bulky, cyclic substrate provided evidence of the versatility introduced through genetic modification of the enzyme. Initial activity tests followed by TK mutant screening highlighted several mutants capable of accepting DCHCA. Subsequent cell culture growth and bench-scale production of the highest performing mutant TK enzyme enabled the preparative scale D469T-TK catalysed reaction for the synthesis and isolation of the reaction product 1-(3',4'-dimethylcyclohex-3'-enyl)-1,3-dihydroxypropan-2-one (DCDHP).

4.3.1.1 Determination of optimum reaction conditions

Reactions using both, clarified lysate and pure, D469T-TK preparations, were optimised using reaction substrates, HPA with GA and PA to synthesise L-erythrulose and 1, 3-dihydroxypentan-2-one (DHP) respectively as described below.

4.3.1.1.1 D469T-TK pH optimum

The natural progression of the TK-catalysed reaction has a net increase on the pH of the reaction mixture due to the consumption of hydroxypyruvate. The synthesis of carbonates is offset to a small degree by the evolution of carbon dioxide, however to maintain a constant, optimal pH, some level of control is required. Larger volume reaction systems are performed in a pH stat which continually monitors and fine tunes the pH by controlled continuous titration of concentrated acid and base solutions. Smaller scale reactions systems for kinetics analysis, enantioselectivity and high throughput reactions, utilising small volumes of reagents, reactions are performed in the presence of buffers.

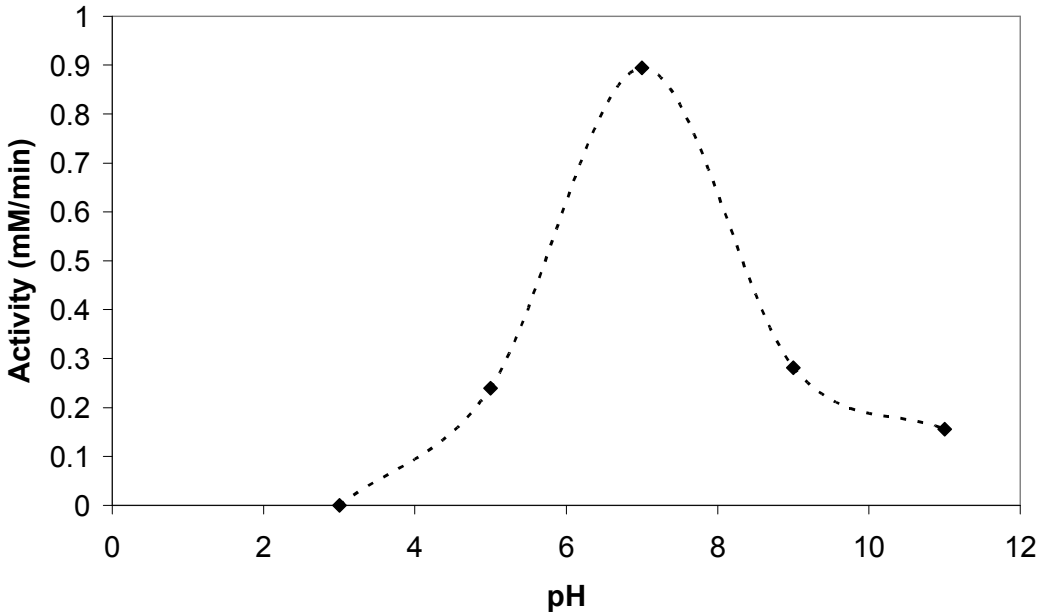


Figure 4.3: D469T-TK pH optimum investigation. D469T-TK with HPA (50 mM) and glycolaldehyde (50 mM) in Tris-HCl buffer (50 mM, pH 7.0), 20° C, to afford L-erythrulose analysed by HPLC.

The optimal pH of the D469T-TK reaction was analysed and found to peak at pH 7.0 as observed previously for WT-TK reactions (Mitra *et al.,* 1998). No activity was seen at all at pH 3, little activity at pH 5, and very little activity at pH 11 dropping from 0.9 mM/min to less than 0.175 mM/min. The optimal pH of D469T-TK corresponds very well with WT-TK behaving in the same manner at low pH and high pH alike. Previously reported data also indicated that exposure to pH below 6.5 results in a decrease in activity of WT-TK, and was assumed to be the result of protein denaturation. Maintaining a pH within the range 6.5-8.0 lead to >90% of TK enzyme activity (Mitra *et al.,* 1998). All biotransformations performed throughout the investigation were controlled at pH7.0 by automated titration of acid or by Tris-HCl buffer (50 mM, pH7.0).

4.3.1.1.2 Investigation of temperature optimum

Higher temperatures are seen not to be detrimental to the stability of the enzyme, in fact it is reported that exposing it to temperatures reaching 55 °C actually shows an improvement in residual activity (activity determined at ambient temperature after the enzyme is exposed to increased temperatures and cooled). However, exposure to temperatures above 55 °C resulted in a significant drop in activity. The report suggested a potential denaturation and refold mechanism enabling a structural re-annealing at around 55 °C leading to a more highly active protein (Martinez-Torres, 2009).

In this work a range of temperatures were investigated for its effect on D469T-TK activity, whilst attempting to improve the low solubility of the novel cyclic aldehyde substrate. In conjunction with pre-ultrasonication of the aldehyde stock solutions, reactions performed at 40 °C resulted in a slight initial reaction rate enhancement, however the overall yield remained the same (Figure 4.4). This indicated an intrinsic 'supply and demand' process occurring whereby, as substrate was being consumed more substrate dissolved in solution, thus introducing a substrate solubility partitioning pre-equilibrium. The initial rate of the enzyme was greater at 40 °C compared to 20 °C by a phenomenon seen with the majority of both biological catalysts and chemical reactions, whereby the number of energetic collisions increases and therefore reaction rate is more rapid according to the Arrhenius equation (below). The transmission coefficient- ratio (*k*, rate constant) represents the probability that the activated complex (enzyme / substrate complex) will go on to form products rather than return to reactants.

$$k = Ae^{-Ea/RT}$$

Where *k* is the rate constant, *T* is temperature, *R* is the gas constant, *A* is the pre-exponential factor (Arthenius constant) and E_a is the activation energy.

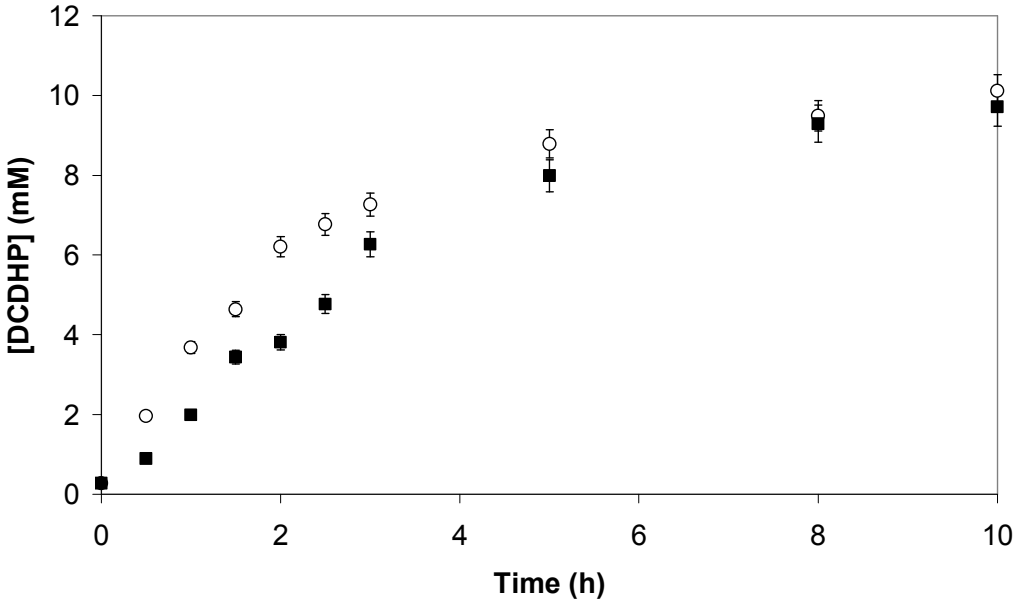


Figure 4.4: Effect of temperature on D469T-TK activity. Comparison of D469T activity against DCHCA and HPA at (○) 40 °C and (■) 20 °C over 10 hours (HPA and DCHCA 50 mM and cofactors TPP 2.4 mM and $MgCl_2$).

The lack of effect on overall yield of the reaction at 40 °C compared to ambient temperature indicates temperature independent reaction issues. Potential substrate and / or product inhibition may cause this trend when a particular concentration is reached and is investigated further in Chapter 6. Additionally, the stability of all reaction components may be compromised at the elevated temperature. A more rapid degradation of substrates may be occurring shown by a drop in reaction activity.

4.3.1.1.3 Reaction component stabilisation

4.3.1.1.3.1 Biocatalyst stability

Pure enzymes D469T and WT-TK storage stability was investigated, to evaluate potential structural affects of the mutation within the active site. Samples of both biocatalysts were stored at room temperature (20 °C), in the fridge at 4 °C, and at -20 °C. No difference in activity between the mutant and WT-TK in the various storage situations was seen at all, indicating no immediate structural effect of the mutation, shown in Figure 4.5. It was found that storage at 4 °C was sufficient for up to two months with no significant drop in activity. Storage at ambient temperature saw a rapid drop in activity over 2 weeks, with negligible activity after 1 month. Samples stored at -20 °C also showed no significant drop in activity at all over 2 months.

Each batch of protein purified for use in kinetic studies was therefore stored in the fridge at 4 °C. Batches were discarded after two weeks and new preparations made. GA and HPA activity tests established comparable activity of each batch prior to application to kinetic experiments.

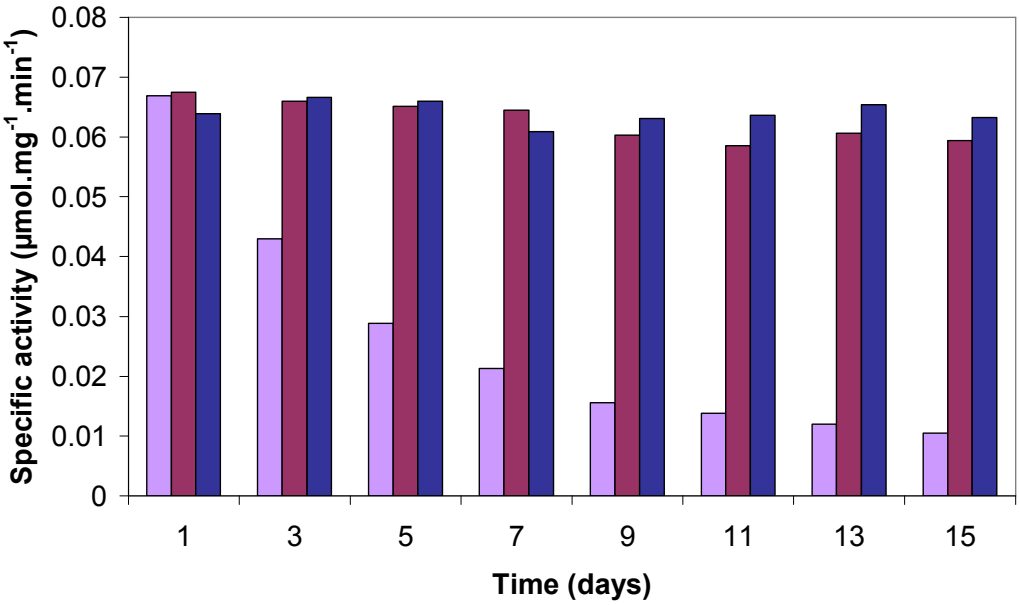


Figure 4.5: Storage stability of D469T-TK His-tagged pQ412. L-erythrulose formation using enzyme stored at (■) Room Temperature (■) 4 °C (■)-20 °C. Reaction conditions as described in section 2.5.4.

4.3.1.1.3.2 HPA

HPA appears to maintain sufficient stability throughout a 2 hour reaction time scale, whereby no significant drop in HPA is detected in control experiments at 20 °C and pH 7.0 (Figure 4.6). However with lengthier reaction time scales of up to 72 hours, the stability of HPA appears to become compromised (Figure 4.7). A starting concentration of 50 mM, equal to the starting concentration in a standard activity assay with glycolaldehyde, propanal and DCHCA, is seen to drop to approximately 35 mM over 72 hours.

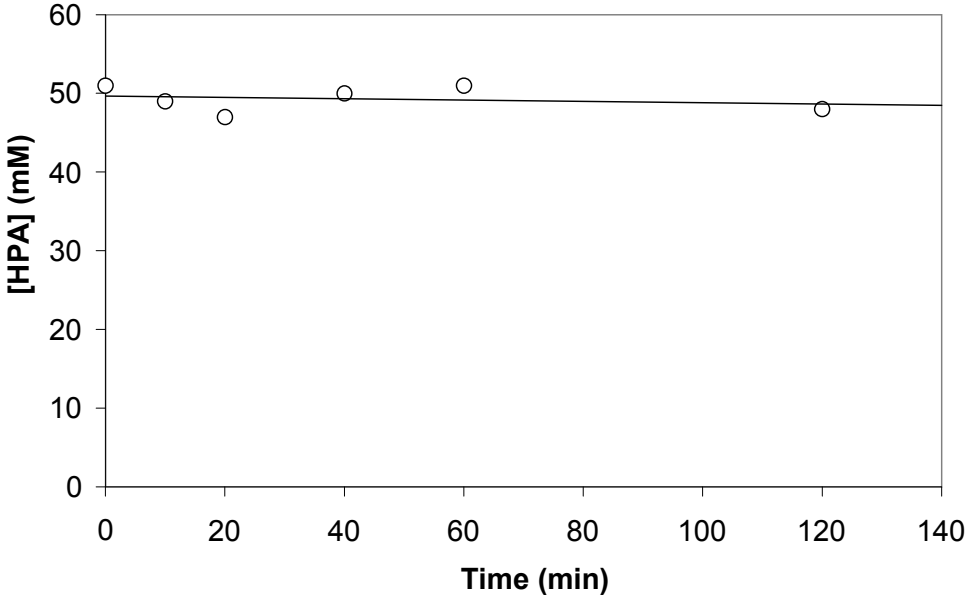


Figure 4.6: Stability of HPA during short time scale reactions. Experiment conditions, HPA (50 mM) Tris-HCl buffer (50 mM pH 7.0), 20 °C, in the presence of cofactors (TPP, 2.4 mM and $MgCl_2$, 9 mM).

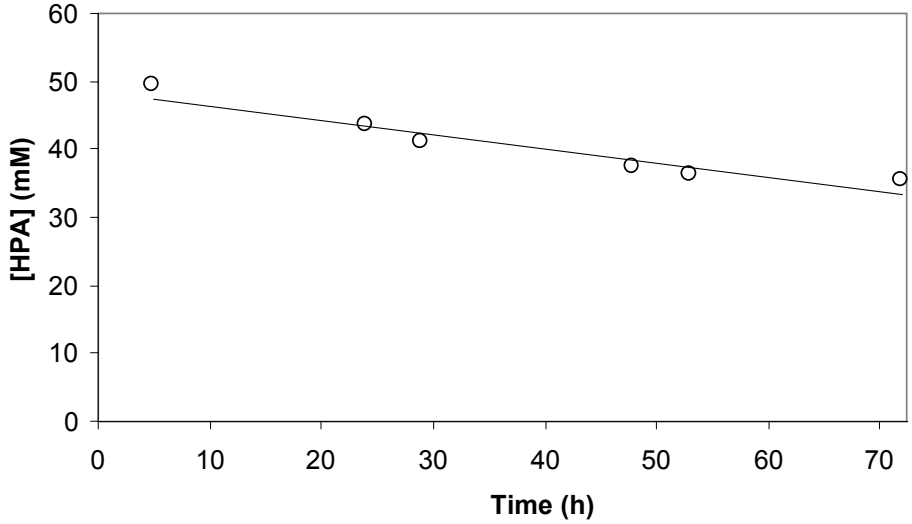


Figure 4.7: HPA stabilization during long time scale reactions. Experiment conditions, 50 mM Tris-HCl buffer, pH 7.0, 20 °C, in the presence of cofactors, (TPP, 2.4 mM and $MgCl_2$, 9 mM).

4.3.1.1.3.3 DCHCA and R-isomer-DCHCA

The storage stability of the racemic aldehyde and *R*-isomer aldehyde was investigated. ^{1}H NMR analysis and mass spectrometry (figure 4.8 shows a typical spectra used for comparison) was performed fortnightly on the compounds which were stored under argon at 4 °C in glass screw top vials. No structural changes or oxidation of the aldehyde were evident indicating a stable compound throughout a period of 6 months.

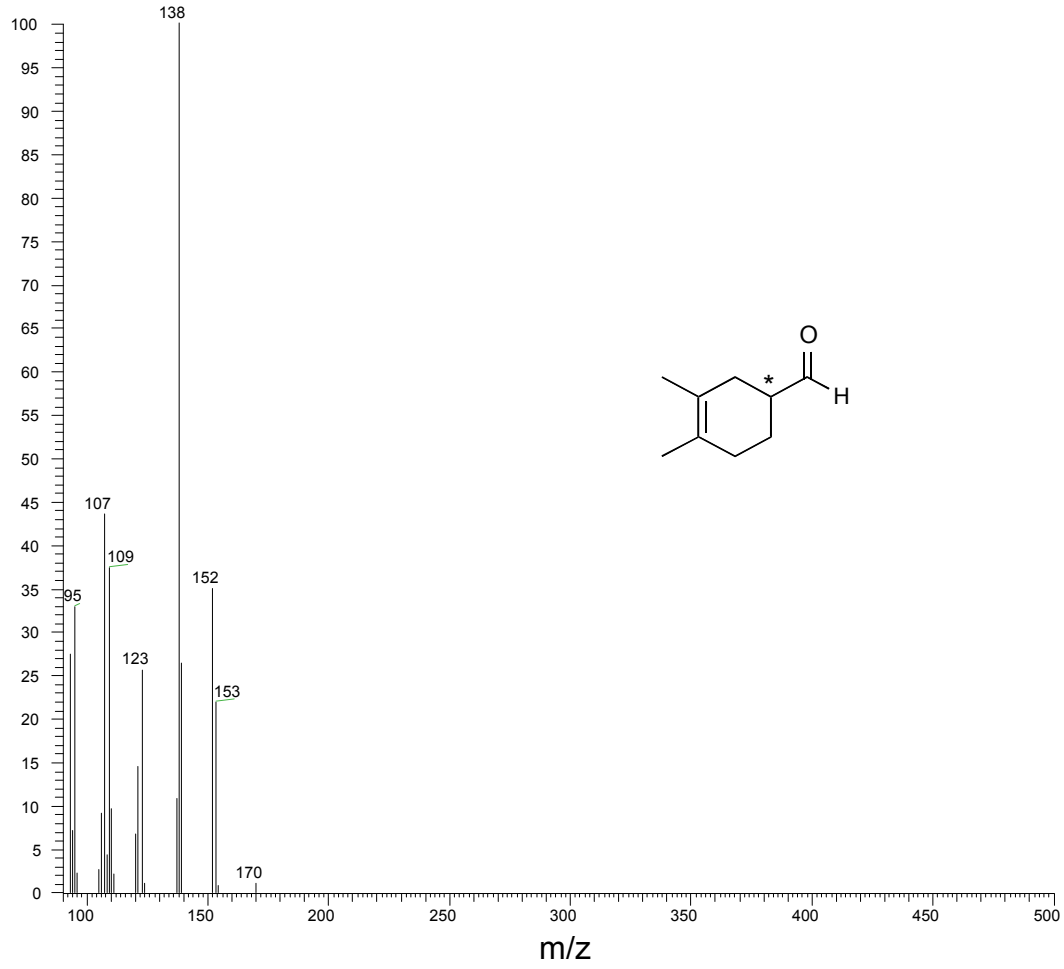


Figure 4.8: Mass spectrometry of DCHCA. MW 138 (m/z =mass to charge ratio).

4.3.1.1.4 Reaction vessels, plastic eppendorf or glass vials?

The reaction vessel was examined for optimal reaction conditions and to establish any effect on the different material of the vessel surface when using the novel aldehyde substrate. Figure 4.9, shows a fairly significant effect of two different reaction vessels, a closed top silanized glass vial and a closed top plastic eppendorf at 40 °C. Initially activity appears to follow a similar rate within the two different vessels, however at approximately 20-30 minutes reaction time, the rate of the reaction in the plastic eppendorf vial slowed significantly and after 50-60 minutes of reaction time activity appeared to cease. A subsequent study indicated that the same effect was evident when using plastic microwell plates, and is thought to be caused by an adverse adhering of the aldehyde to the vessel walls. Glass vials were subsequently used throughout for all reactions.

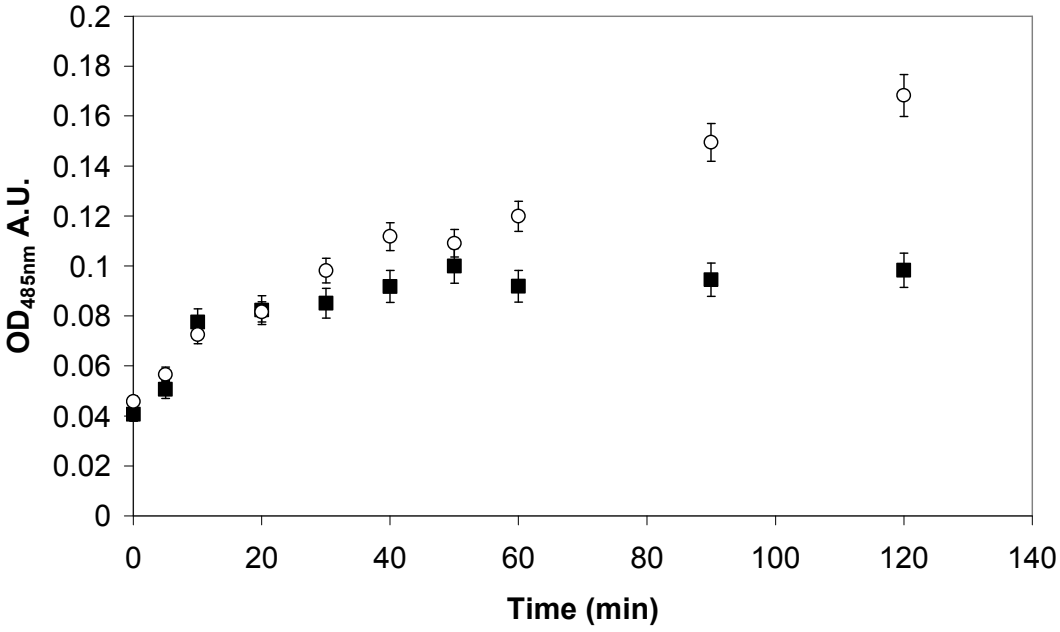


Figure 4.9: Comparison of reaction vessel effect on DCDHP synthesis. (○) 40 °C silianized glass vial (■) 40 °C plastic eppendorf vial. Reaction conditions, 40 °C maintained in a water bath, 50 mM DCHCA and HPA and cofactors (TPP 2.4 mM and $MgCl_2$ 9 mM).

Sealed reaction vials were also deemed necessary due to the volatile nature of propanal and potentially other substrates used with TK. Figure 4.10 indicates PA depletion within a closed vial compared to microwell plates without secure seals. An average of 75% loss was seen within the microwell plates with loose lid compared to 12% loss seen from the screw top vials, a significant improvement. The length of the reaction times make it crucial to ensure the reaction substrates remain within solution.

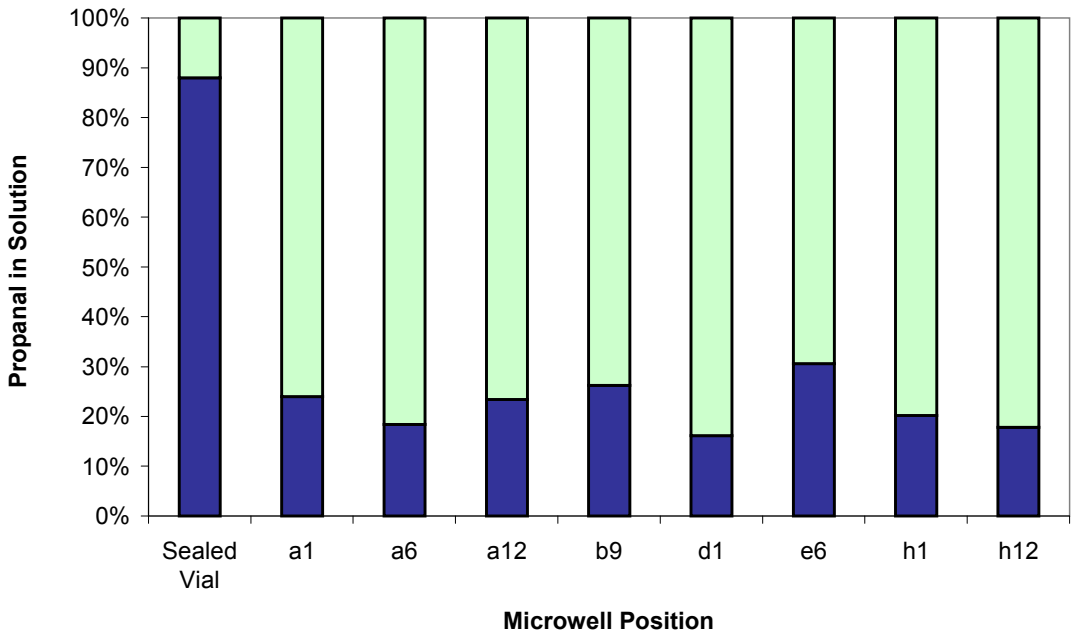


Figure 4.10: Comparison of propanal depletion within screw top glass vials and microwells with plate lid. 50 mM propanal (PA) in Tris-HCL buffer (50 mM, pH 7.0) measured by HPLC after 48 hours.

4.3.1.1.5 Impact of reaction scale for D469T-TK and WT-TK activities

4.3.1.1.5.1 Kinetic assays at low reaction volumes (300 µl -1.2 mL)

Small scale reactions were performed for glycolaldehyde activity assays, propanal activity assays and DCHCA kinetic analysis reactions. Small scales of 300 µL-1.2 mL were sufficient to provide a solution of enzyme and substrate to allow effective diffusion through the mixture with reaction sampling. No significant difference in reaction rate was seen between the three different scales of 300 µL, 600 µL and 1.2 mL.

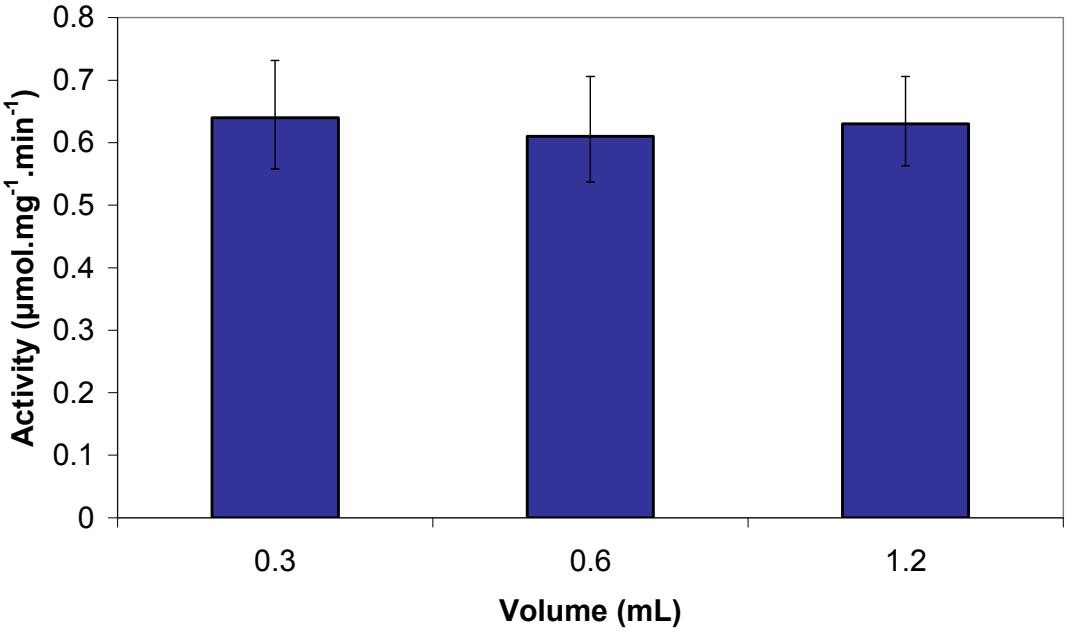


Figure 4.11: Comparison of activity of D469T-TK at small assay scales. Substrates glycolaldehyde and HPA (50 mM, Tris-HCl buffer 50 mM pH7.0) at 20 °C in 3 different small scale volumes, 300 µL, 600 µL and 1.2 mL and analysed by HPLC in triplicate.

A range of volumes (10% - 70% of reaction volume) of enzyme were investigated for the optimal level of reaction rate and balance of reaction components and solvent, whilst keeping substrate (50 mM) and cofactor (TPP 2.4 mM, Mg^{2+} 9 mM) concentrations constant and allowing for sampling at 300 µL scale. For the PA reactions 30% v/v clarified lysate was found to be optimal and for the DCHCA 50% v/v enzyme (pure and clarified lysate). A reaction with 70 % enzyme caused an issue with aldehyde solubility where substrate stock solutions required higher starting concentrations and so this was not considered for further investigation. A 10% reaction of DCHCA saw very little product formation over 24 hours and 30% relatively low product formation. (Figure 4.12). All GA activity tests maintained 10% v/v enzyme which was found to be effective for the rapid reaction rate and determination of activity within 60 minutes.

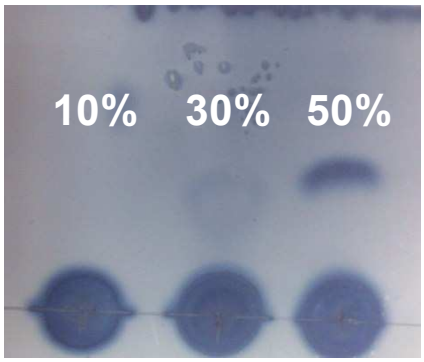


Figure 4.12: Comparison of enzyme concentration on product formation. D469T-TK mediated synthesis of DCDHP. DCHCA and HPA (50 mM) and cofactors TPP (2.4 mM) and $MgCl_2$ (9 mM) in Tris-HCl buffer (50 mM, pH7.0) at 20 °C for 24 hours.

4.3.1.1.5.2 TK kinetics at preparative reaction scales (90 mL-500 mL)

A 90 mL scale D469T-TK lysate reaction using a pH stat, at pH 7.0, 50 mM substrate concentration, in 100% water yielded 18% of DCDHP (± 3%). A Tris-HCl buffered (50 mM Tris-HCl, pH 7.0) reaction with the same reaction conditions yielded very similar conversions of 15% DCDHP (±2%), in a magnetically stirred closed reaction vessel over 72 hours.

Large scale volumes ranging from 90 mL up to 500 mL were performed for the preparation and isolation of 1-(3',4'-dimethylcyclohex-3'-enyl)-1,3-dihydroxypropan-2-one (DCDHP), the analysis of enantioselectivity and determination of absolute configuration. All reactions scales corresponded well with regard to product yield and reaction rate.

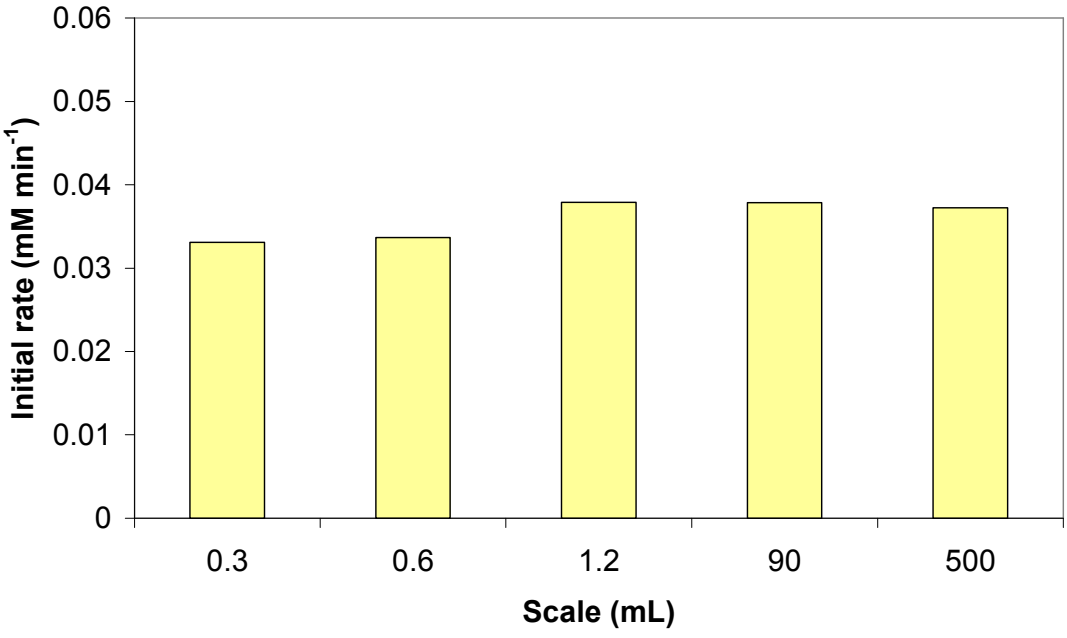


Figure 4.13: Comparing effect of scale on initial rate of D469T-TK. Substrates DCHCA and HPA (50 mM, Tris-HCl buffer 50 mM pH7.0) at 20 °C at different scale volumes, 300 µL, 600 µL, 1.2 mL, 90 mL, 500 mL and analysed by colorimetric assay.

4.3.1.2 Investigation into improving substrate solubility

4.3.1.2.1 Co-solvent addition to water

A small yield improvement was seen with the addition of a co-solvent ethyl acetate (EtOAc) to the TK-catalysed reaction with DCHCA and HPA in water. The Tris-HCl buffered D469T-TK lysate reaction yield was improved from 15% (±2%) to 19% (± 2%) with the addition of 5% v/v EtOAc, due possibly to an improvement in substrate solubility. This is also an indication of the tolerance of enzyme stability in organic solvents, as when using a 1:1 ratio of water and EtOAc there was no product formation seen at all. A 2:1 ratio of water and EtOAc also yielded no product. Reported data suggests an effect of the solvent on the activity of an enzyme catalysed reactions is due to differential Gibbs free energy of desolvation for the transition states of the reactants (Wescott and Klibanov, 1993; Ke *et al.,* 1996).

The cosolvent was therefore added at a concentration of 5% v/v to subsequent preparative scale reactions. However, for activity and kinetic analysis the solvent remained 100% aqueous, to establish the native activity and provide a direct comparison to previous substrates.

4.3.1.2.2 Comparison of shaking and non-shaking D469T reaction

The effect of shaking on the TK reaction with the hydrophobic substrate was briefly investigated. To establish potential significant rate enhancement and to evaluate an improvement on solubility, shaken vessels were compared to non-shaken reactions. No significant effects of shaking were seen at scales of 300 µL in glass screw top vials compared to non-shaken vials. Investigation of platform shaking at 200 rpm, at ambient temperature indicated no effect on the solubility of the substrates or on the mass transfer of solutes. The introduction of a magnetic stirrer bar into the kinetic assay reaction at such small scales was problematic. The aldehyde, as with the plastic vessels and microwells appeared to adhere to the plastic stirrer bar and cause slightly inconsistent reaction rates.

Stirring large scale reactions however, did have a significant effect and all preparative scale TK reactions and also biomimetic reaction were stirred magnetically. Unstirred preparative scale reactions produced very low yields of product <3%. The effect of the stirrer bar material did not appear to have an effect on reaction yields at larger scales, as the aldehyde loss (adhered to stirrer bar) was relatively less significant than the smaller scales.

4.3.2 Biomimetic reaction for racemic ketodiol synthesis

Biomimetic reactions using both PA and DCHCA were performed to synthesise standard racemic compounds of each. Initial chiral HPLC data, after conversion to the monobenzoate, indicated equals amounts of each of the four enantiomers, as shown in 4.14. Four peaks relating to each of the diastereoisomers was observed with a ratio of 1:1:1:1. Varying the reaction scales and use of co-solvents within the biomimetic reaction mixture did not affect the enantiomeric excess or distribution of diastereomers which in all cases were found in equal quantities.

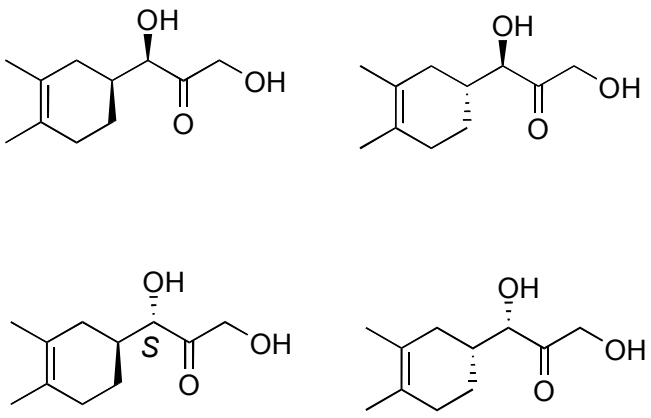


Figure 4.14: Four possible ketodiol product diastereomers.

4.3.2.1 Co-solvent addition to the biomimetic reaction

Initial preparative reactions in 100% water yielded very low conversions of racemic DCDHP, 3%. Co-solvents were assessed for their effect on reaction yields, in order to improve substrate solubility. The use of a co-solvent to improve precursor solubility in water was investigated using tetrahydrofuran (THF) and ethyl acetate (EtOAc). The use of EtOAc as a cosolvent did not lead to a significant improvement in productivity. The lower solubility of EtOAc in water may mean it is not suitable as a cosolvent possibly causing separation of reagents through a biphasic system. Ethyl acetate may also not be suitable due to potential hydrolysis. THF, a five membered ring containing an oxygen atom is soluble in water at the ratios used and due to the lone pair of electrons can hydrogen bond to reactants present in solution.

Figure 4.15: Tetrahydrofuran, THF. Cosolvent used in the transketolase mimetic reaction.

A 10% v/v addition of THF to the biomimetic reaction solution saw a small improvement from yields of <3% DCDHP, to 8 (±2%). Increasing cosolvent concentration to 50% v/v, saw a significant yield enhancement from <3% to 22 (±2%). A ratio of 1:1 water and THF was subsequently used in all biomimetic preparative reactions.

4.3.2.2 Scale

Increasing the scale of the reaction from 100 mL solvent volume in a magnetically stirred RB flask at ambient temperature to 500 mL did not have any effect on the overall product yield. Larger volume reactions were more useful with regard to product yield and were performed regularly for synthesis of racemic DCDHP.

4.3.3 Enantioselectivity of TK-D469T with DCHCA

4.3.3.1 Cyclic aldehyde enantioselectivity and absolute configuration

The methods described for determining ee vary depending on the compounds involved. The stereoselectivity and enantioselectivity were explored with this novel chiral substrate against mutant TK, D469T. Previously D469T-TK stereoselectivity against propanal was determined to be 90% for (*S*)-isomer compared to 58% for WT-TK (Hibbert *et al.,* 2008).

The product of the D469T-TK reaction alongside the product of the biomimetic reaction were subjected to the same benzoylation procedure in order to generate the D469T product and a four isomer mixture (racemate) of the same benzoylated compound (fig 4.16).

The HPLC spectra of the benzoylated products, Figure 4.17 revealed that the D469T-TK product consisted of a single major diastereoisomer with traces of the remaining three isomers. Analysis of another variant, D469L-TK, was performed for comparison of the unusally high enantio- and stereoselectivity exhibted by D469T-TK. Both TK variants demonstrated the formation of the same major diastereoisomer, indicated by peak **3** in Figure 4.17.

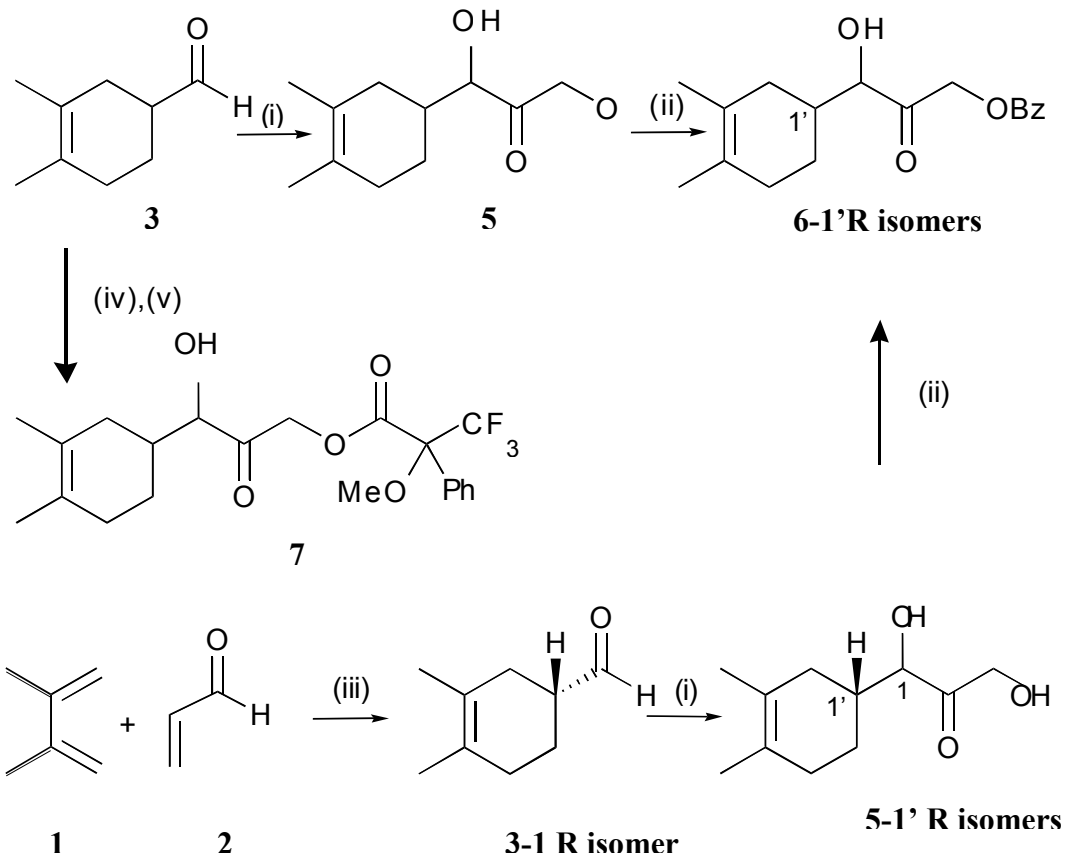


Figure 4.16: Reaction sequence for chiral HPLC and *ee* assay. Reaction conditions: (i) H_2O, N-methylmorpholine, 10%; (ii) NEt3, PhCOCl, 70%; (iii) (5S)-2,2,3-trimethyl-5-phenylmethyl-4-imidazolidinone, CH3CN/H2O, 83%; (iv) D469T-TK, 4, TPP, $MgCl_2$, 15% (rel. to 1RS-3), 30% (relative to 1R-3); (v) (R)-MTPACl, NEt3, 75%.

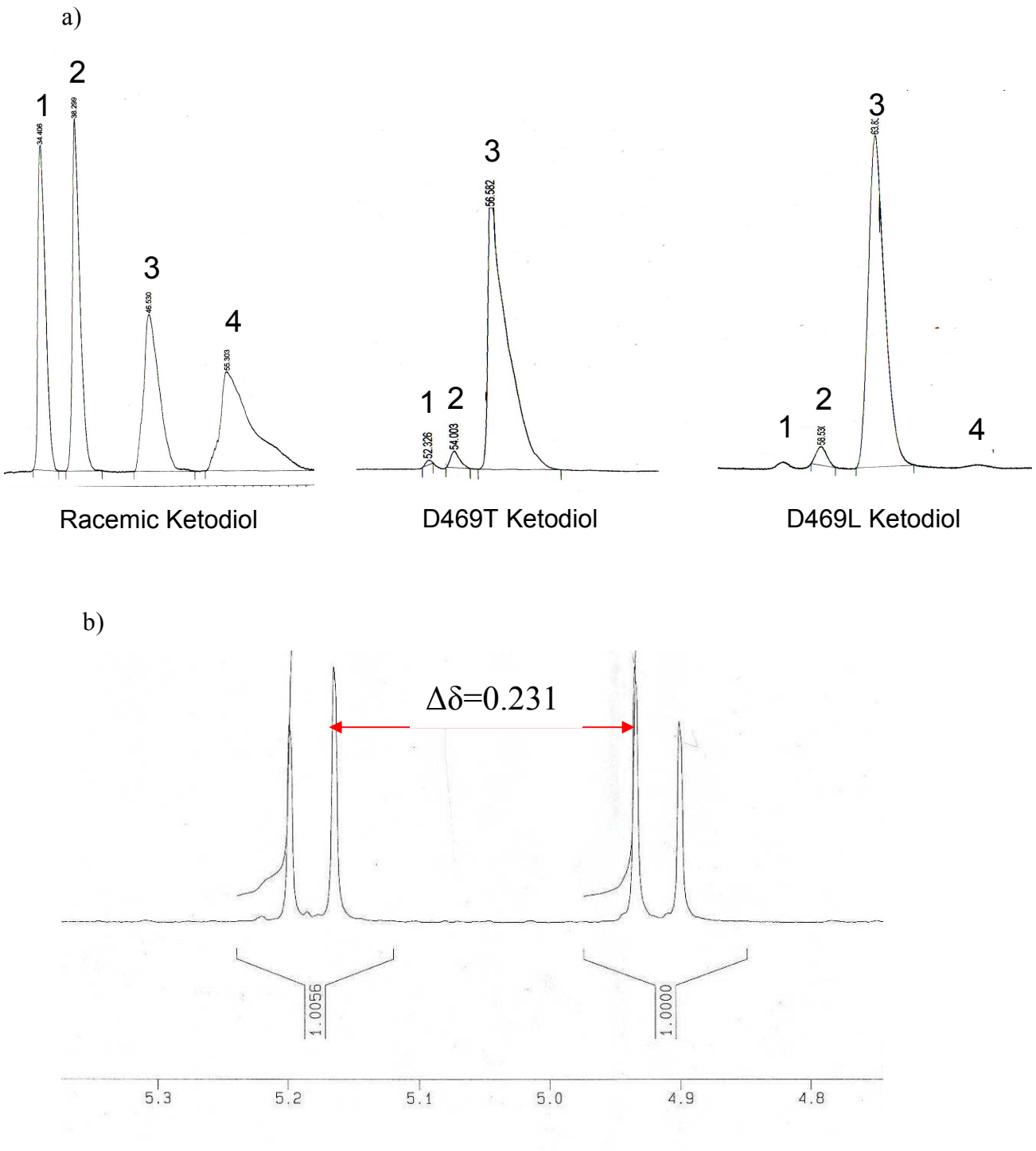


Figure 4.17: Chiral HPLC spectra. a) Comparison of the monobenzoylated reaction products from D469T and D469L reactions against DCHCA with racemic mixture formed biomimetically. (Four possible enantiomers indicated by peaks 1-4) **b)** S-MPTA DCDHP representative spectra, indicating a Δ_L-Δ_H = > 0.2 ppm.

The enantioselectivity of D469T-TK was determined and the absolute configuration of the product established through the preparation of the *R*-aldehyde and the recently modified Mosher's acid method (Benaglia *et al.,* 2002; Galman and Hailes, 2010). To begin with, the Macmillan's catalyst (5*S*)-2,2,3-trimethyl-5-phenylmethyl-4-imidazolidinone was reacted with the Diels Alder substrates to form the R-isomer of DCHCA (Figure 4.16, (iii)) with a yield of 83%). The biomimetic reaction was performed with this aldehyde, to form DCDHP which was then benzoylated. HPLC peaks corresponding to the 1'R-isomers were assigned, indicating that the major product formed by D469T-TK possessed a 1'R-centre in DCHCA (R-isomer). Also, the product (DCDHP) of the D469T-TK reaction was reacted with (*R*)-MTPACl to form the Mosher's ester at C-1. A recent protocol to assign the absolute stereochemistry at C-3 of α,α'-dihydroyketones, determined that the separation of the oxymethylene protons at C-2, derivatised as Mosher's esters, differed with the trend that $\Delta\delta_H^{2R,3'R \text{ and } 2S,3'S} > \Delta\delta_H^{2R,3'S \text{ and } 2S,3'R}$. Analysis of known stereocentres allows for the prediction that the Δ_L-Δ_H = > 0.2 ppm shown in Figure 4.17b. Therefore, the stereochemistry at C-3 of the product formed by D469T-TK, was assigned as (1*S*). Considering the data from both the HPLC experiments and the Mosher's ester derivatisations, the absolute stereochemistry of the major isomer of the product formed from D469T-TK reaction was established as (1*S*,1'*R*). HPLC analysis of the benzoylated ketodiol product indicated that the D469T-TK product (1*S*,1'*R*) was formed in 93% ee.

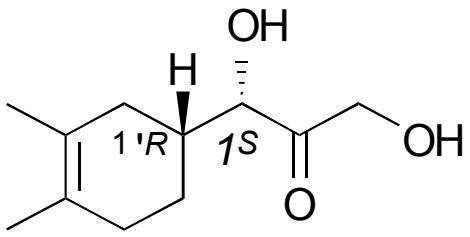


Figure 4.18: Stereochemistry of the major diastereoisomer formed by D469T-TK. (1'*R*,1*S*)-(3,4-dimethylcyclohex-3-enyl)-1,3-dihydroxypentan-2-one (DCDHP).

4.4 Summary

In this chapter the preparative scale transketolase mediated synthesis of 1-(3',4'-dimethylcyclohex-3'-enyl)-1,3-dihydroxypropan-2-one (DCDHP) using TK variant D469T was performed and analysed. The optimal reaction conditions were investigated for the novel enzyme mutant and compared to previously reported data for WT-TK. The pH optimum of around neutral, corresponded well with current data for TK, despite altering the TK gene by single point mutation and addition of a His tag at the N-terminus.

Alongside the TK catalysed reaction against DCHCA for the synthesis of the cyclic ketodiol product a very useful mimetic of the TK reaction was performed and optimised. The use of THF as cosolvent to improve solubility of the hydrophobic aldehyde, DCHCA, worked well and improved reaction yield almost 10 fold. HPLC spectra of the biomimetic product which had been monobenzoylated highlighted an equal mix of all four possible diastereo-isomers. The racemic compound provides a useful standard for both the calibration of kinetic assays and the determination of enantiomeric excess and absolute configuration of the D469T-TK reaction product.

A benzoylation procedure was applied to both the racemic cyclic ketodiol and the D469T-TK reaction product and analysed by chiral HPLC. Analysis of the HPLC spectra revealed the presence of a single major enantiomer with trace amounts of the other three enantiomers. Analysis of a further TK variant D469L also revealed the formation of the same single major enantiomer. An *ee* was calculated for the variant D469T and found to be in excess of 93%.

A recently modified Mosher's ester methodology was employed for the determination of the absolute chemistry of the single major enantiomer and was calculated by assigning protons of the compound. The absolute configuration was subsequently calculated and elucidated as the (1*S*,1'*R*) enantiomer.

5 D469T-TK and Wild-Type-TK kinetics

This chapter describes investigations into the characterisation of the highest performing D469 library mutant D469T identified for activity towards the novel, bulky, hydrophobic aldehyde acceptor 3,4-dimethyl-3-cyclohexene-1-carboxaldehyde (DCHCA). The enzyme kinetics of D469T–TK were analysed using a modified colorimetric assay and compared to WT-TK kinetics. The colorimetric assay, involving tetrazolium red, was further developed from a recently established assay for high throughput screening of 1,3-ketodiols, by modifying it for use as a quantitative kinetic assay. Michaelis-Menten kinetic parameters, K_m, V_{max} and k_{cat} were determined for the mutant D469T transketolase variant against the novel chiral acceptor aldehyde.

5.1 Introduction

5.1.1 Wild-type TK kinetics

Transketolase follows a mechanism known as the 'ping pong bi-bi' mechanism, whereby a two substrate reaction forms two products. The enzyme reacts with a single substrate to release a single product and the subsequently modified enzyme then reacts with the second substrate to form the second, final product and renders a regenerated enzyme. For instance with TK, the ketol donor (HPA) is first bound to the enzyme, followed by the release of the first product (CO_2) followed by the binding of the acceptor aldehyde (glycolaldehyde, propanal or 3,4-dimethyl-3-cyclohexene-1-carboxaldehyde) and subsequently followed by the release of the second product (L-Erythrulose, 1,3-dihydroxypentan-2-one, or 1-(3',4'-dimethylcyclohex-3'-enyl)-1,3-dihydroxypropan-2-one, respectively) and the regenerated enzyme.

Kinetic parameters for WT-TK overexpressed in *E. coli* host JM107 and plasmid pQR700 with substrates HPA and glycolaldehyde and product L-erythrulose have been reported. The enzyme was shown to be free from excess substrate inhibition up to 40 mmol 1^{-1} for HPA and 100 mmol 1^{-1} for glycolaldehyde. Inhibition by L-erythrulose was found to be competitive, as in the predicted mechanism (Gyamerah and Willets, 1997).

5.1.2 TK variant kinetics

Transketolase has been subjected to a number of engineering procedures such as mutagenesis techniques and directed evolution (Hibbert *et al.,* 2007; Hibbert *et al.,* 2008). Transketolase variants have been identified to have enhanced activity towards non-phosphorylated substrates such as glycolaldehyde and the non-natural aliphatic aldehyde propanal. Several mutants from a phylogenetically and structurally guided library were found to possess enhanced specific activity of up

to 3-fold higher than WT-TK under biocatalytically relevant conditions. These mutants were found to have substituted residues that differed from those found in nature. However, other mutants with conserved residues which are seen to be involved in phosphate binding also yielded mutants with almost 5-fold improved specific activity against non-phosphorylated substrates. The report suggests that the phylogenetically variant active-site residues are useful for modulating activity on natural or similar substrates and that conserved residues which no longer interact with modified target substrates are significant for application of saturation mutagenesis in order to improve activity (Hibbert *et al.,* 2007).

5.1.3 Colorimetric assay

Finding alternative methods of detecting substrates and products of a reaction creates a method of validating new raw data. A new specific technique for measuring reaction components is valuable for accurate data accumulation and may be simpler and quicker to perform than traditionally used assays. The method may also be modified for generic use.

Using a colorimetric assay to determine kinetic data is a relatively novel and interesting method compared to standard detection methods such as HPLC and gas chromatography. It allows for compounds with low responsive chromophores to be detected with ease and relative simplicity. It provides a means of validating standard data from sources such as chromatography to gain more accurate and precise data. The HPLC methods used for transketolase-catalysed reactions with glycolaldehyde and propanal accurately detect one of the two substrates, HPA. The other substrates are not detected with the same method due to low absorbance extinction coefficients. The product is detectable but with a relatively low and less well resolved response. The colour assay allows a faster and potentially more accurate measurement of the reaction product, while the HPLC method is complementary to that of substrate depletion.

TK has also been measured by monitoring the residual HPA via an NADH-dependent enzyme assay after the preparative scale isolation of the reaction product. However this method can prove costly due to large scales required and may also be prone to product losses through isolation and purification processes. Fluorescence has also been reported as a technique for *in vitro* TK activity producing D-threo aldoses and L-erythro ketoses, but is limited to particular substrates. Those containing an umbelliferone moiety were used, which could potentially affect the TK biotransformation (Sevestre *et al.,* 2003; Simon *et al.,* 2009).

Chromogenic compounds have been used as substrates for many enzymatic reactions in order to produce a coloured product for ease of detection (Erlanger *et al.,* 1961; Wahler *et al.,* 2001). Common chromogenic substrates include 5-bromo-4-chloro-3-indolyl derivatives and p-nitrophenyl. The use of dyes or stains and colour indicators are integral for the detection of such compounds. The use of tetrazolium salts is often demonstrated, due to the sensitive nature of its signal compared to reduction equivalents. The detection of aromatic acyloins, such as (R)-phenylacetylcarbinol ((R)-PAC) a chiral intermediate of industrial ephedrine synthesis, was developed utilising tetrazolium red (Brèuer *et al.,* 2002). The bioconversion of benzaldehyde by pyruvate decarboxylase is detected by the reduction of tetrazolium red by the product and measured spectrophotometrically.

Development of the colorimetric assay initially for screening of 2-non-hydroxylated aldehydes was based upon the reduction of 2,3,5-triphenyltetrazolium (tetrazolium-red) (Smith *et al.,* 2006). Whereby a colourless compound (tetrazolium-red) is reduced in the presence of diol products and becomes a red coloured formazane compound, providing a linear relationship between the formazane detection and transketolase cataysed reaction ketodiol product. In this chapter the screening assay is developed for use as a kinetic measurement detection assay, providing an alternative method besides HPLC.

5.2 Materials and methods

5.2.1 TK activity at various substrate concentrations

Pure TK (50% of reaction volume) (prepared as described in Section 3.2.11) was pre-incubated with cofactor solution (TPP 2.4 mM and $MgCl_2$ 9 mM as described in Section 2.5.2) for 20 minutes at 20 ºC. A range of substrate stock solutions were made with aldehyde concentrations of 10 - 50 mM and 50 mM HPA. Substrate solutions were sonicated to assist dissolution for 3 cycles of 10 second 8 µ pulses with 10 second intervals using a Soniprep 150 sonicator (MSE, Sanyo, Japan). Substrate solutions are added to the enzyme preparation with pipette mixing and samples taken at regular intervals. Samples were quenched with methanol to stop the reaction and stored at -80 °C. The colorimetric assay is performed as described in Section 5.2.3.2

5.2.2 TK Kinetic assay reaction quenching methods

Kinetic assay reactions were performed as described in Section 5.2.4 and 20 µL samples taken at regular intervals, however quenched with either 200 µL ethanol, 200 µL 0.2% TFA, or the samples were placed immediately in plastic eppendorf vials and immersed into an acetone and dry ice bath for rapid freezing and all samples stored at -20 °C. The flash frozen samples were diluted with 200 µL Tris-HCl buffer (50 mM, pH 7.0). The colour assay performed as in section 5.2.3.2.

5.2.3 Colorimetric assay for kinetic analysis

5.2.3.1 Carbonate resin quenching

100 µl of a 2.5 mM stock solution of HPA was added to 10 wells of a 96 microwell plate. Twenty µg MP-carbonate resin beads were added to the microwell at t_0, and samples taken from wells in order 1-10 (so as not to alter the

level of HPA within solution-due to sample taking) at regular intervals over 3 hours. The automated colour assay was performed as described in Section 5.2.3.2 to all samples and the OD measured at 485 mM.

5.2.3.2 Colorimetric assay

Samples were taken (50 μL) and transferred onto a microwell plate containing 10 mg MP-Carbonate resin (Biotage AB) and 50 μL Tris-HCl buffer (50 mM, pH 7.0) and incubated at 20 °C for 2 hours. A 50 μL sample of the quenched reaction was transferred without resin into a microwell plate. Automated injection of 20 μL tetrazolium red solution (0.2% 2,3,5-tripheyltetrazolium chloride in methanol) and 10 μL 3 M NaOH (aq) with shaking by FLUOstar Optima plate reader (BMG Labtechnologies GmbH), was followed by immediate measurement at $OD_{485nm.}$

5.2.3.3 Calibration curve

Stock solutions of DCDHP were made at concentrations of 50, 40, 30, 20, 10, 5, 2.5 mM in Tris-HCl buffer (50 mM, pH 7.0). For each concentration 50 μL was diluted with 50 μL of Tris-HCl buffer (50 mM, pH 7.0). The colorimetric assay was performed as in section 5.2.3.2. The calibration curve is shown in figure 5.1.

5.2.4 TK Kinetics Assay

Purified D469T-TK, 300 μL was incubated with 50 μL cofactor solution, TPP (2.4 mM), and $MgCl_2$ (9 mM) for 20 minutes at 20 °C. HPA (0–50 mM, Tris-HCl buffer, 50 mM, pH 7.0) and DCHCA (0–50 mM Tris-HCl buffer 50 mM, pH 7.0) were added to the reaction mixture. HPA was kept constant at a concentration of 50 mM, while DCHCA ranged, 0, 5, 20 30, 40, 50, mM and then DCHCA was kept constant at 50 mM while HPA ranged from 0 – 50 mM. Samples of the reaction mixtures were taken at regular intervals (20 μL) and the reaction quenched in 200 μL methanol. The samples were then taken (50 μL) and

transferred onto a microwell plate containing 10 mg MP-Carbonate resin (Biotage AB) and 50 µL Tris-HCl buffer (50 mM, pH 7.0) and incubated at 20 °C for 2 hours. Fifty µL of the quenched reaction sample was transferred without resin into a microwell plate. The automated colour assay was performed as described in section 5.2.3.2.

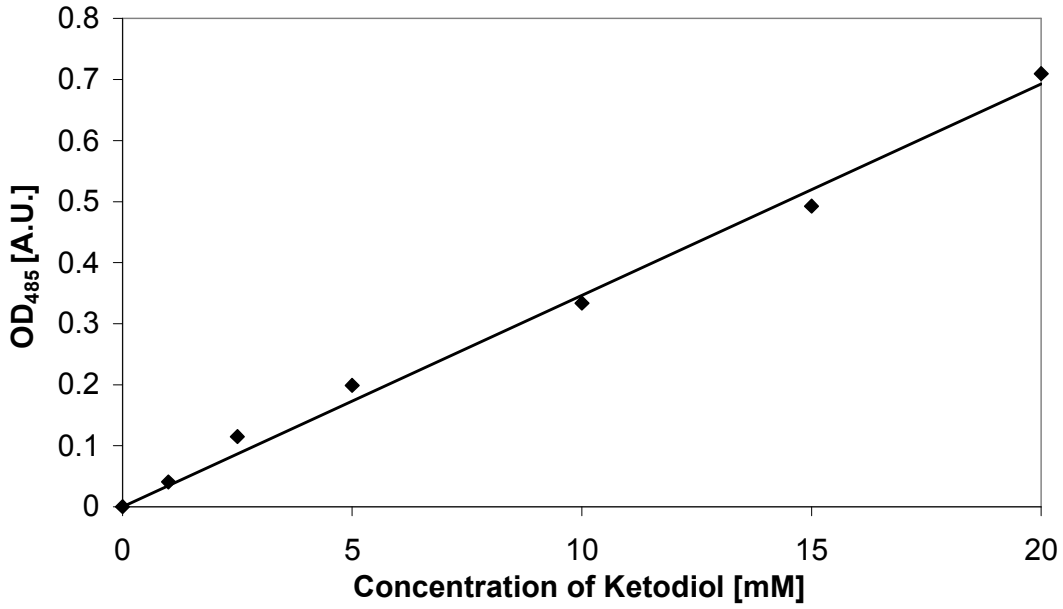


Figure 5.1: Calibration curve for formazane after addition of 1-(3',4'dimethylcyclohex-3'enyl)-1,3-dihydroxypentan-2-one, DCDHP (r^2= 99.3%).

5.3 Results and discussion

5.3.1 Kinetic assay development

The development of the kinetic assay was required for the application of a novel substrate with greatly differing properties than previous aldehydes such as glycolaldehyde and propanal. The reaction profile shown in Figure 5.10 indicates a reaction time of up to 48 hours, compared to the 60 minutes for glycolaldehyde and 24 hours for propanal.

5.3.1.1 Kinetic assay optimisation

The differing characteristics of the novel substrate compared to the well established substrates used previously in this TK assay, including in particular the much lower solubility in water, demands modifications. In order to improve this solubility limitation for the kinetic assay without the use of previously discussed co-solvents in preparative scale reactions (Chapter 4), substrate solutions were subjected to ultra sonication. The solubility of HPA is not limiting at the concentrations used here for kinetic analysis, however the industrial-scale economic requirements for high titres may find the solubility in water of 0.65 M to be problematic.

Cyclic aldehyde substrate stock solutions were sonicated using an ultra-sound probe (Soniprep 150 sonicator) in order to maximise solubility of the aldehyde within the buffer solution. The solution was placed within an ice jacket during sonication to counteract any heating affect of the procedure. In conjunction with the use of an ice jacket, ‘pause’ intervals were introduced between 10 second pulses to allow intermittent cooling of the solution. The ultra sonication of the aldehyde appeared to temporarily improve solubility in water turning the hydrophobic globules of the aldehyde into a homogenous ‘milky white’ solution.

The ultrasonic waves will cause a periodical compression and rarefaction of the buffer and aldehyde solution, potentially and significantly causing an increase in mass transfer (Mason, 1997). The increase in mass transfer may be attributed to a sonochemical effect, whereby the reactants are transformed into more water-soluble reaction products by the formation and rapid collapse of gas bubbles within solution. In addition, the microcurrents induced by the ultrasound procedure reduce diffusion layer differences between phases, promoting faster mass transfer in solution (Mason, 1999). .

The sonication method appeared to offer a more accurate transfer of substrate from stock solution into reaction solution, and was therefore performed before each reaction. ^{1}H NMR analysis of the sonicated aldehyde indicated no immediate effect on chemical structure, however stock solutions were discarded after one use and fresh solutions prepared for each separate reaction. HPA was added to the substrate solution following sonication to avoid any destabilization of the ketol donor compound. The solution was applied to the pre-incubated TK cofactor mixture immediately.

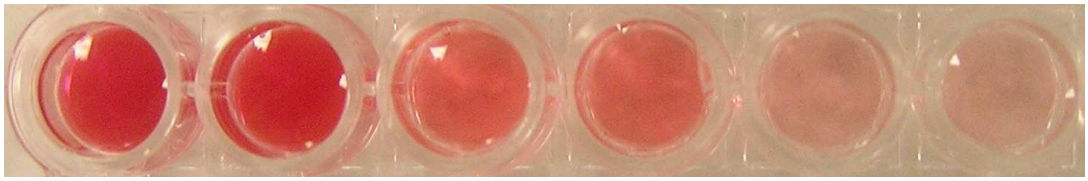


Figure 5.2: Optimisation of dilutions in the colorimetric assay. A range of dilutions of the solubilised 25 mM DCDHP product stock solution were investigated for optimal sensitivity, linearity and the limit of detection in kinetic experiments. Actual concentrations after assay dilution: the six wells containing from left to right 2.5 mM, 2.5 mM, 0.25 mM, 0.25 mM, 0.025 mM and 0.025 mM DCDHP.

A number of dilutions were analysed for optimisation and evaluation of the colorimetric assay for use in quantitative kinetic measurements. Concentrations that lay within the detection limits of the assay were investigated. Dilutions were required to provide product levels above the lower limits of the colour assay where traces of HPA are detected as shown in control experiments (Figure 5.7). Also, dilutions were required to remain within the lower maximum capacity range of the MP-carbonate resin beads, 2.5-3.5 $mmol.g^{-1}$ (based on nitrogen elemental analysis, Biotage, AB), otherwise the HPA substrate would saturate the resin and not be entirely removed from the reaction, thus causing inaccurate measurement. Dilutions were investigated to avoid signal saturation of the colour assay and also to optimise the sensitivity and ability to measure very low level concentrations and thus low reaction conversion levels. The assay was previously reported as capable of detecting as little as 2.5 mM (actually 12.5 nmol after assay protocol dilutions), however slight improvements in sample handling and dilution control provided a limit of detection (LOD) of just 0.5 mM. Comparable detection levels of DCDHP were detected here (Figures 5.2). It was found that it was absolutely crucial that samples were pipette-mixed at each transfer and particular attention to pipetting accuracy was fundamental when measuring such low product concentrations.

5.3.1.2 Reaction quenching methods

The standard method for sampling and quenching TK-catalysed reactions against GA and PA for detection by HPLC is by the addition of 0.2% trifluoroacetic acid (TFA) causing denaturation. The same method however, proved problematic (Figure 5.3) for samples of the reaction against DCHCA despite neutralisation, due to the new colorimetric detection method, as data was inconsistent and did not correspond well with HPLC data of the same reaction. Several alternative methods were investigated for reaction quenching including methanol addition and flash freezing in an acetone and dry ice bath (Figure 5.3).

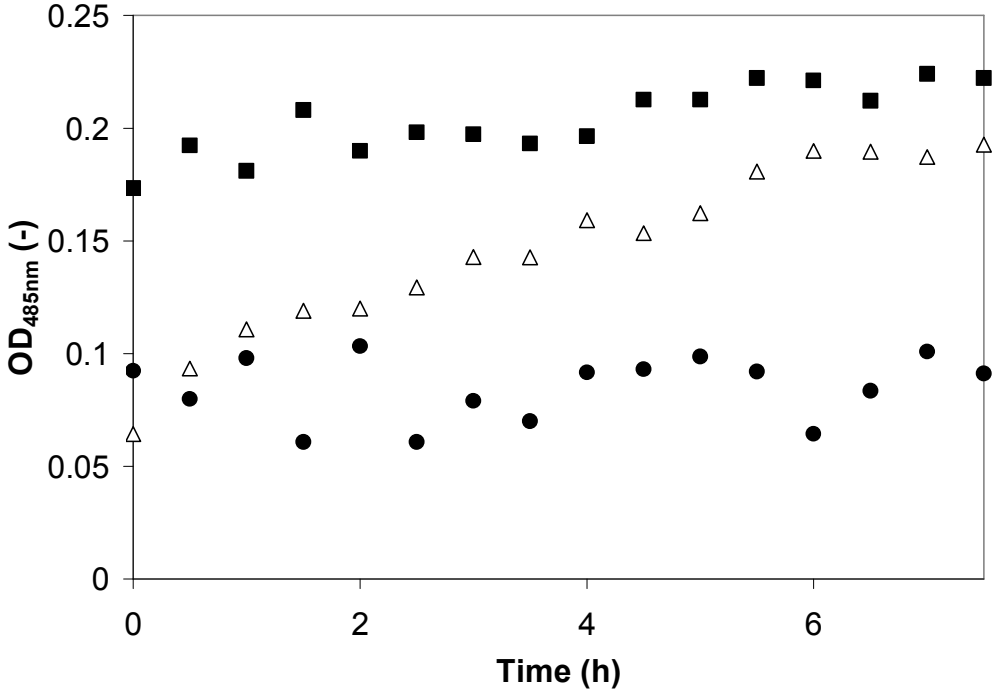


Figure 5.3: Enzyme reaction quenching methods. Comparison of quenching by: (Δ) Methanol (50% v/v); (■) Flash freeze (acetone and dry ice); and (●) TFA 0.2%. Activity assay performed as described in section 5.2.4.

Flash freeze quenching was not a successful method for initial rates experiments of this particular reaction. Results indicated it may have been possible that on thaw of the frozen samples the enzyme continued to transform the substrates within the sample during the 3 hour MP-carbonate resin adsorption for removal of the HPA. Therefore a method that instantly and irreversibly inhibited the enzyme (deprotonated or denatured) and therefore prevented the reaction from proceeding was required, due to the length of the HPA quench time relative to the short sample times of a matter of minutes.

The use of methanol as a quenching agent was based upon the high concentration of an organic solvent causing irreversible degradation to the enzyme within the sample. At a concentration of 50% methanol, Figure 5.3 indicates an effective quench (reaction inhibition) of the reaction and no adverse effect on the colorimetric detection assay. Using methanol within the system was simplified by its presence already within the colorimetric assay (solvent for tetrazolium red) reducing further potential complications of the presence of numerous organic solvents.

5.3.1.3 Resin quenching

The removal of HPA from the reaction system prior to tetrazolium red addition was fundamental. The presence of the hydroxyketone functionality within HPA reduces tetrazolium red in the same way as the dihydroxyketone products of TK to form the red coloured formazane, therefore, masking the effect of the TK reaction product on the formazane levels within the sample. Removal by an ion-exchange resin was developed (Smith *et al.,* 2006) and a quaternary amine functionalised MP-carbonate resin (Biotage) was found to be effective (Figure 5.4). The resin 'quench' required optimisation to ensure complete HPA removal from the system.

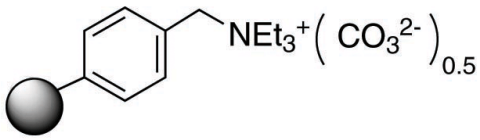


Figure 5.4: MP-Carbonate resin (Biotage). Used for scavenging acids, such as lithium hydroxypyruvate (HPA).

The length of time that the HPA is reacted (quenched and removed from the sample) with the MP-carbonate resin was investigated. It was found that two hours was adequate for removal of HPA, however three hours was optimal for complete confidence of removal of all traces of HPA. It is critical that the HPA is properly removed so as not to interfere with the relatively low ketodiol concentrations within the samples.

Shaking of the system was also investigated to assess potential increases in the adsorption rate of HPA to the resin beads, whereby the microwell plates were subjected to a 200 rpm platform shaker. However, shaking did not significantly reduce quenching time and it was considered unnecessary also due to the potential contamination caused between microwells. It was found, however, that volumes of sample solution greater than 200 μL for 10 mg resin did not effectively remove all of the HPA. The larger ratio of sample volume to resin bead mass was found to insufficiently quench the HPA possibly due to insufficient mass transfer rather than due to reaching the limit in chemical capacity of the resin. Mixing of the samples could potentially improve mass transfer throughout the solution of larger volumes (>200 μL), however as previously mentioned using a SWR-96 microwell format, contamination between wells was an issue.

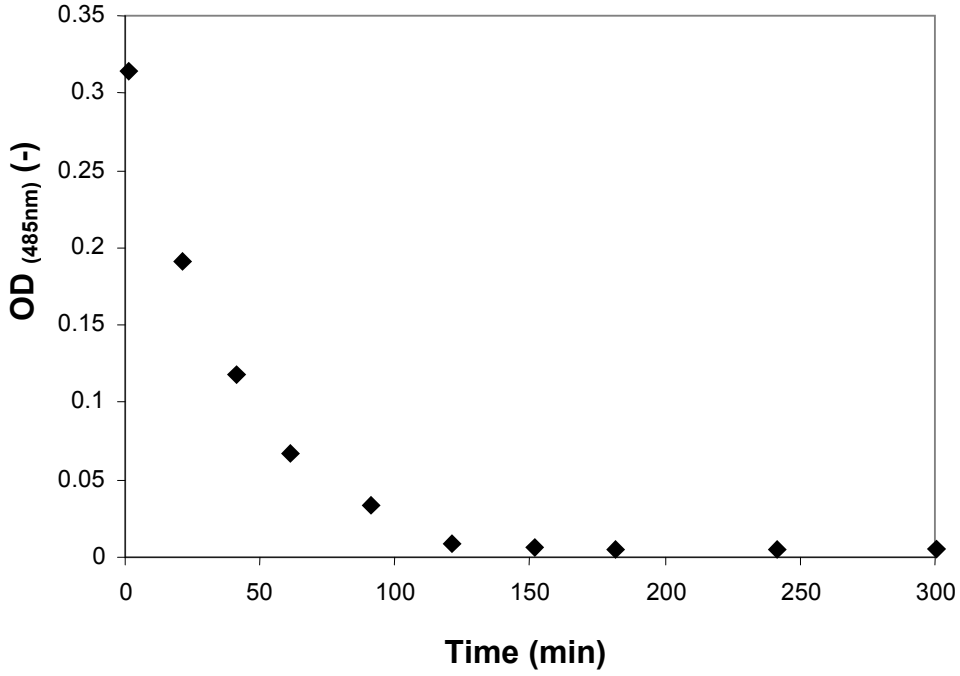
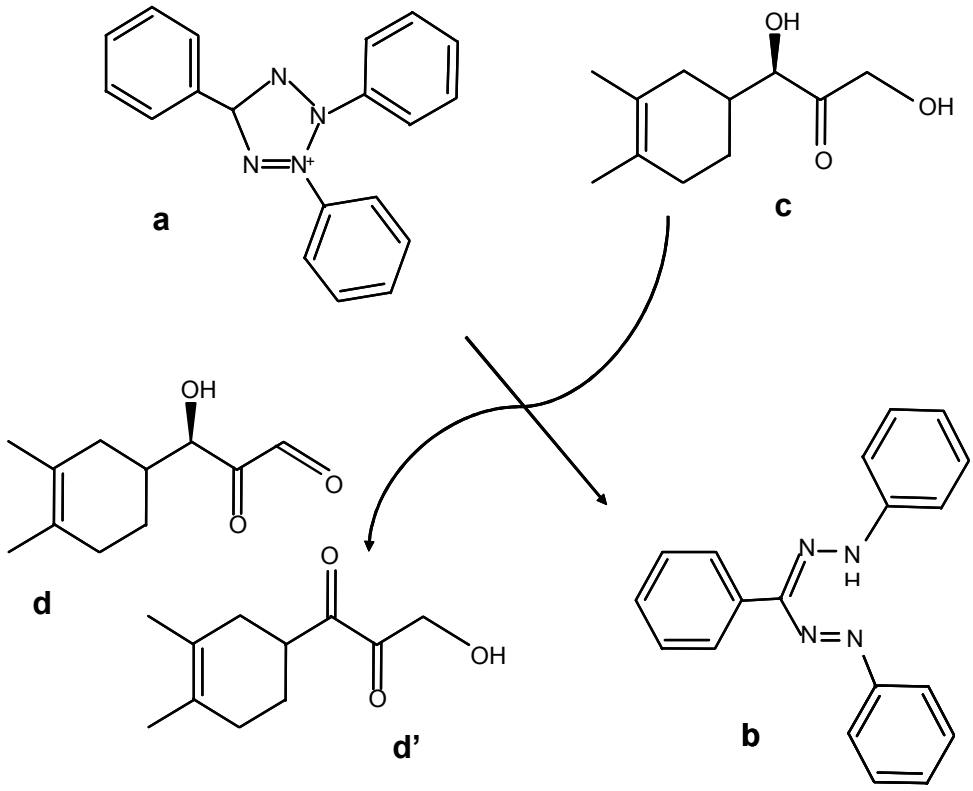


Figure 5.5: Time-dependence of Li-HPA quench using quaternary amine MP carbonate resin. 50 mM HPA solution (actual concentration 2.5 mM) to remain within resin maximum capacity and in line with actual kinetic reaction conditions, 20 °C, 10 mg MP-carbonate resin, assay performed as in section 3.2.8.

5.3.1.4 Colorimetric assay development for kinetic analysis

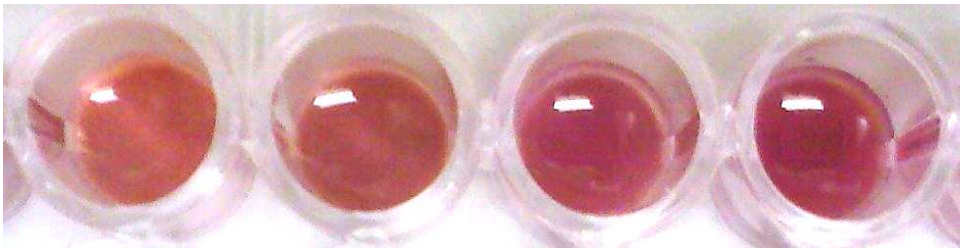
The tetrazolium-red colorimetric assay was initially developed for use as a high throughput screening assay for TK mutant libraries (Smith *et al.,* 2006). Here the assay has been modified for use as a kinetic assay for detection of the products of the transketolase reaction, including DCDHP. Modifications developed here includes the TK reaction times, sampling over time, reaction sample dilutions, reaction quenching method and assay component concentration. The assay provides an alternative, simpler and more rapid detection method of numerous samples at one time, and is also applicable to several TK products from non- α-hydroxylated aldehydes. Here, the 2-hydroxyketone TK reaction product reduces colourless 2,3,5-triphenyltetrazolium to red coloured formazane and the corresponding oxidised product, diketone.



Scheme 8: Colorimetric assay mechanism. Reduction of colourless a) tetrazolium red to b) red coloured formazane by oxidation of D469T-TK product c) 1-(3',4'-dimethylcyclohex-3'-enyl)-1,3-dihydroxypropan-2-one (DCDHP) to d) two possible oxidised products.

5.3.1.4.1 Colorimetric assay calibration curve

The ketodiol product (DCDHP from biotransformation and racemic biomimetic reactions) proved to be very difficult to re-dissolve in water after isolation and purification. Although kinetic experiments indicated solubility in water of up to ~12 mM, it seems that once the compound had been isolated it was very difficult to re-dissolve into water even using ultra-sonication. Therefore, a cosolvent was considered possible candidates included methanol (CH_3OH), ethanol (C_2H_5OH), ethyl acetate (EtOAc), and tetrahydrofuran (THF). The selected solvents were assessed for both their improvement on solubility and the possible effects on the colorimetric assay chemistry. Due to the presence already within the colorimetric assay, methanol was selected as cosolvent for the standard DCDHP solution for calibration curves. Methanol had no effect on the fundamental chemistry of the assay and also proved particularly effective as a cosolvent for the hydrophobic, cyclic, 1,3-ketodiol. The additional methanol within the system did, however, cause a very slight alteration in colour, from the distinctive pink/red seen during screening experiments, to a red/orange (λ_{max} = 485 nm) shown in Figure 5.6.



1 **2** **3** **4**

Figure 5.6: Effect of addition of methanol into colorimetric assay samples. Wells **1** and **2** show the additional methonal compared to original assay components in wells **3** and **4**. Use of cosolvent to improve solubility and therefore accuracy of calibration curve of TK product DCDHP.

5.3.1.4.2 Limit of detection and control assay

Control TK-reaction experiments analysed with the colorimetric assay indicated a small interaction between HPA and TK might occur, where despite quenching of the HPA from reaction solution, traces of HPA were still measured (Smith *et al.,* 2006). Where HPA was present (prior to resin quench) not in the presence of TK, no traces of HPA were measured. The detection limit is the mean of the blank and the lower detection limit is the lowest quantity of a substance that can be distinguished from the absence of that substance within a confidence limit. The limit of detection here was found to be 0.5 mM comparable to the signal of a WT-TK against DCHCA and HPA over 48 hours.

1 **2** **3**

Figure 5.7: Evaluation of the colorimetric assay on TK reaction control experiments. 1: 24 hour reaction with TK, Cofactors, HPA. 2: 24 hour reaction with cofactors, DCHCA and HPA 3: 24 hour reaction with TK, cofactors and DCHCA. (Cofactors: TPP and $MgCl_2$) and 3 hours quenching on MP-Carbonate resin at 20 °C (Biotage, AB).

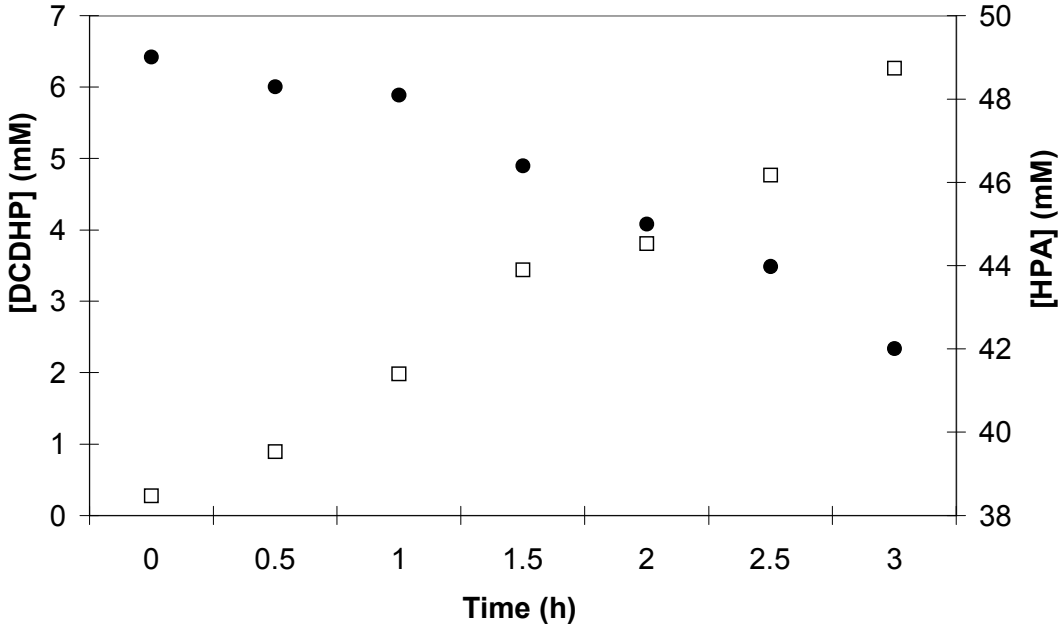
5.3.2 D469T kinetic parameters

The colorimetric assay for product formation was used to obtain kinetic data of mutant D469T-TK against DCHCA, in addition to mutant screening, as it was found to quantitatively compare with HPLC. The analysis of enzyme kinetics is vital to the development of new biotechnologies, such as screening for drug candidates and the identification of useful biocatalysts. The knowledge of kinetic parameters allows for the effective integration of individual enzymes or biocatalysts into current chemical syntheses or the development of new pathways with optimal productivity.

The depletion of HPA was monitored using the same HPLC-based method developed for assaying the glycolaldehyde reaction, and compared to the formation of DCDHP as monitored by the colorimetric assay. The Pearson correlation was calculated to be 0.98 indicating comparable data from the two different detection methods. To further initially validate the colorimetric assay the formation of DHP from the bioconversion of propanal (PA) and HPA was monitored by both HPLC and the colorimetric assay (fig 5.8). The Pearson correlation value calculated between the two sets of data was 0.9808.

His-tagged D469T-TK was purified and characterised using DCHCA at a range of concentrations (0–50 mM) and 50 mM HPA. Complete two substrate anaylsis was not performed due to the inhibition seen by HPA of WT-TK with a K*i* of 42 mM and also to concentrate on the aldehyde pathway only. The enzyme was first incubated with cofactors, Mg^{2+} and TPP to ensure formation of the holoenzyme, found to be crucial for the transketolase reaction to proceed (Sprenger *et al.,* 1995). The lack of pre-incubation was previously seen to slow intial reaction rates, due possibly to competitve binding between substrates and cofactors when prepared and applied to the enzyme simultaneously. Reaction volumes of 600 uL were performed in order to provide sufficient sample for the larger volumes required for accurate measurment by the colorimetric assay.

A)



B)

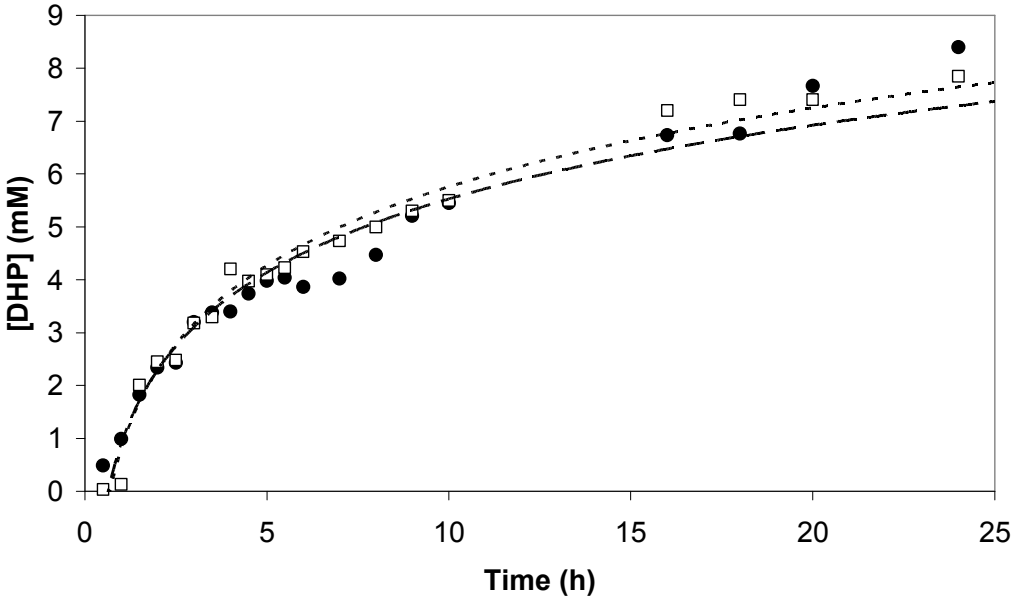


Figure 5.8: Colorimetric assay validation. A) HPA (●) depletion measured by HPLC and DCDHP (□) formation detected by colorimetric assay. 50 mM DCHCA and 50 mM HPA in Tris-HCl buffer (50 mM pH 7.0) 20 °C. B) DHP formation detected by HPLC (□) 15 min isocratic assay as described in section 2.6.2.3 and colorimetric assay (●) performed as described in section 3.2.8. Reaction, 50 mM propanal and 50 mM HPA, in Tris-HCl buffer (50 mM, pH 7.0) at 20 °C.

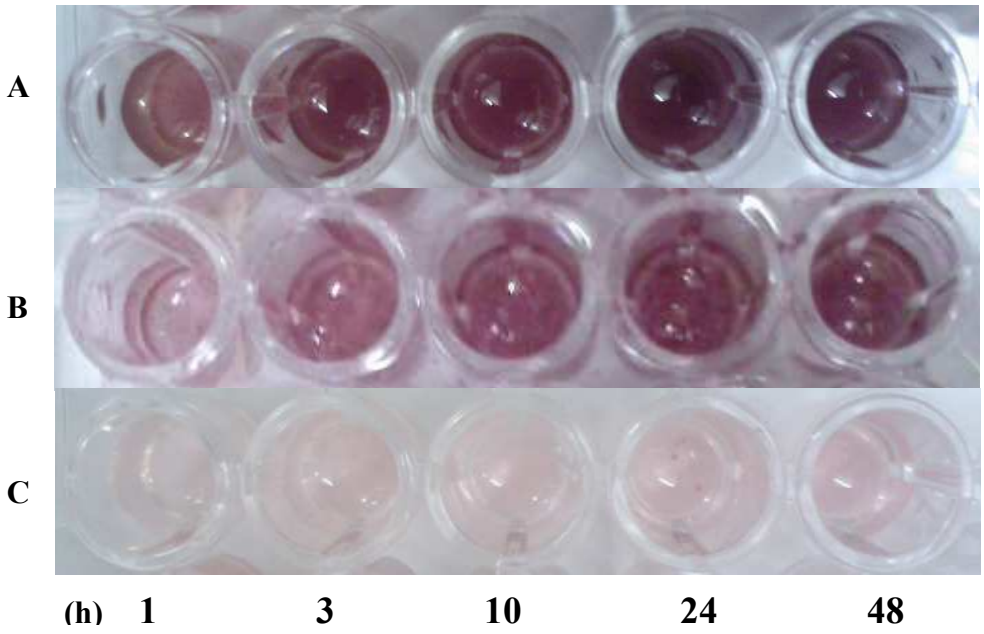


Figure 5.9: Comparison of colorimetric assay wells from different TK catalysed reactions. Comparison of reactions over 48 hours for A) D469T with propanal (PA); B) D469T-TK with DCHCA; and C) WT-TK with DCHCA. Reactions contained 50 mM substrates, 2.4 mM TPP, 9 mM MgCl2, 1.0 $mg.mL^{-1}$ TK, in Tris-HCl buffer (50 mM, pH 7.0) at 20 °C.

The reaction profile over 48 hours for activity of both WT-TK and D469T against the novel acceptor aldehyde DCHCA is shown in both figure 5.9 and 5.10. It may be seen that the activity of WT-TK against DCHCA is negligible which is also demonstrated by the sensitive TLC plate photograph, Figure 5.11.

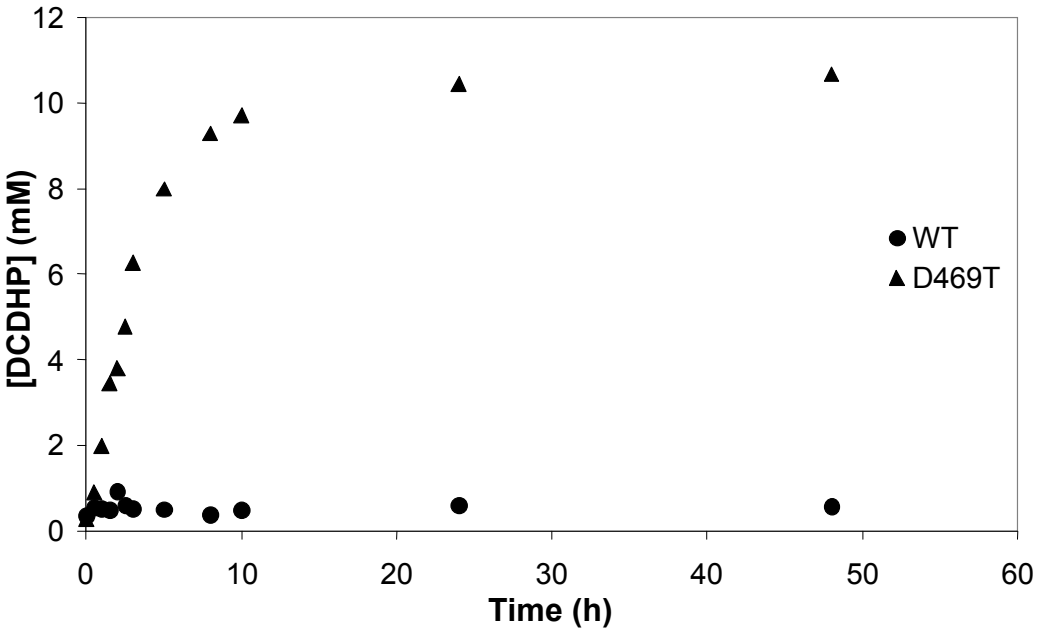


Figure 5.10: Comparison of TK reaction profiles for WT-TK and D469T-TK towards DCHCA. Reactions were analysed over 48 hours in triplicate, 10 mL reaction scale, 0.6 $mg.mL^{-1}$ in the presence of cofactors (TPP 2.4 mM and $MgCl_2$ 9 mM), stirred magnetically and controlled in a pH stat at pH 7.0. (●) WT-TK (▲) D469T-TK 50 mM DCHCA and 50 mM HPA for the synthesis of DCDHP.

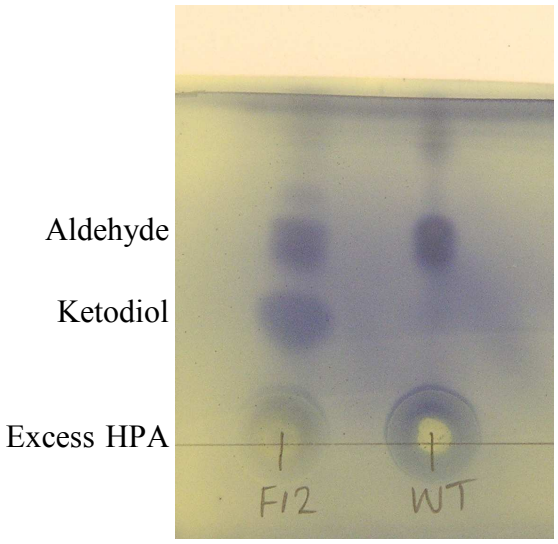


Figure 5.11: Typical TLC plate for the conversion of DCHCA to DCDHP by TK. TK reaction product (F12=D469T) by resolution with 1:1 EtoAc and hexane mobile phase on silica TLC plates and visualisation by phosphomolybdic acid (PMA) and heat.

The reaction profile indicates the level of product formation attainable, and it is obvious no product formation at all was possible from WT-TK, while D469T- TK reached levels of approximately 12 mM. Preparative scale WT-TK reactions yielded no DCDHP at all and pH stat preparative scale reactions of WT-TK indicated negligible pH changes within reaction solution by the lack of HCl consumption, normally seen with TK reactions to maintain constant pH 7.0. For kinetic analysis, the initial rate of the reaction is used to accurately measure and provide comparable data for previous TK substrates and other TK variants. Initial rate WT-TK is represented by Figure 5.12, further indicating the lack of activity of WT-TK with this cyclic, hydrophobic substrate.

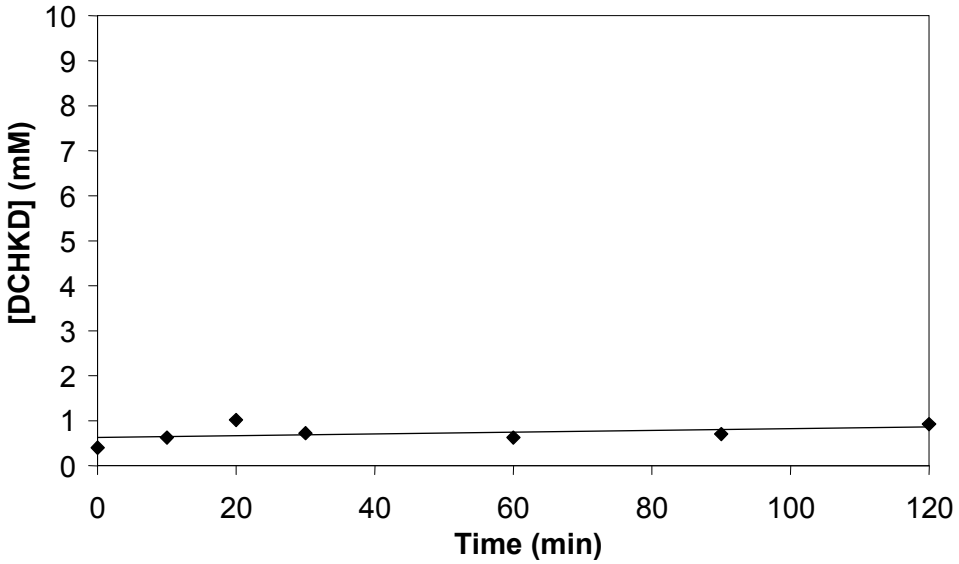


Figure 5.12: Initial rate of WT-TK against DCHCA. Reaction scale 600 µL pH 7.0 in 50 mM Tris-HCl buffer with cofactors TPP (2.4 mM) and $MgCl_2$ (9 mM) (TK 0.4 $mg.mL^{-1}$) at 50 mM equimolar substrate concentration and measured colorimetrically.

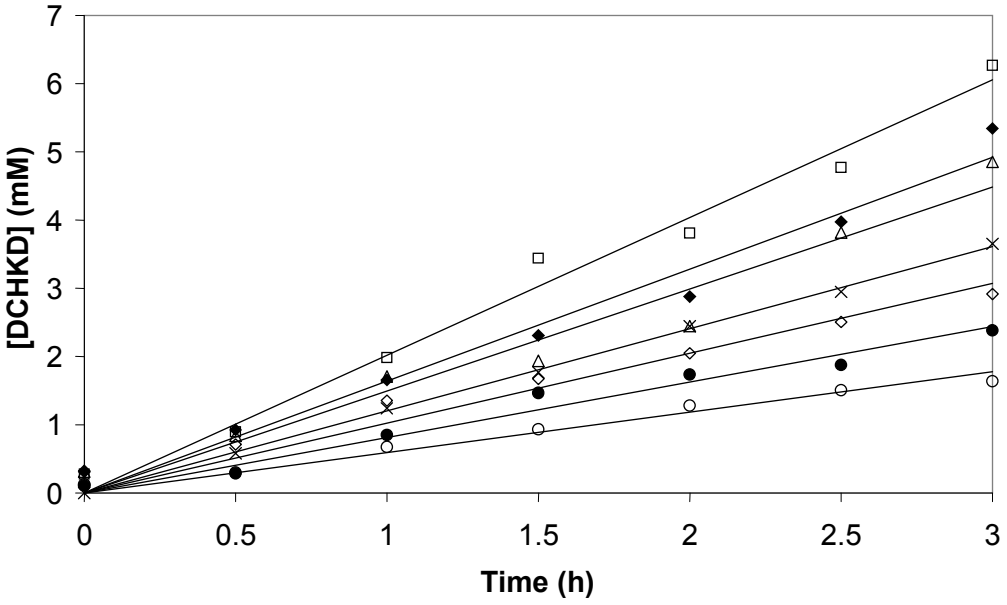


Figure 5.13: Initial reaction progress curves for D469T-TK catalysed reactions of DCHCA. HPA (50 mM) and DCHCA at (○) 10 mM (●) 15 mM (◊) 20 mM (X) 25 mM (Δ) 30 mM (♦) 40 mM (□) 50 mM were reacted with the

enzyme using conditions as described in section 5.2.4. The data were fitted by linear regression.

The data obtained colorimetrically (Figure 5.10) from the D469T-TK reaction with HPA and DCHCA, was fitted to the standard Michaelis-Menten model (below) utilising the Lineweaver-Burk plot to determine the kinetic parameters, V_{max} , k_{cat} and K_m. The following equation describes the basic model where, V_{max} is the maximum initial rate of synthesis of the product, v is the initial rate of product formation, K_m is the Michaelis–Menten constant, and [S] is the substrate concentration.

$$v = \frac{V_{max}[S]}{K_m + [S]}$$

Although transketolase has two substrates, it was assumed that the reaction would be limited by only one (HPA) and that the substrate concentrations were in excess of previously measured activity profiles.

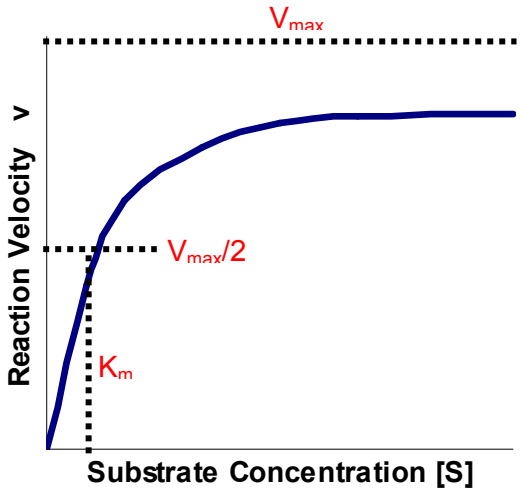
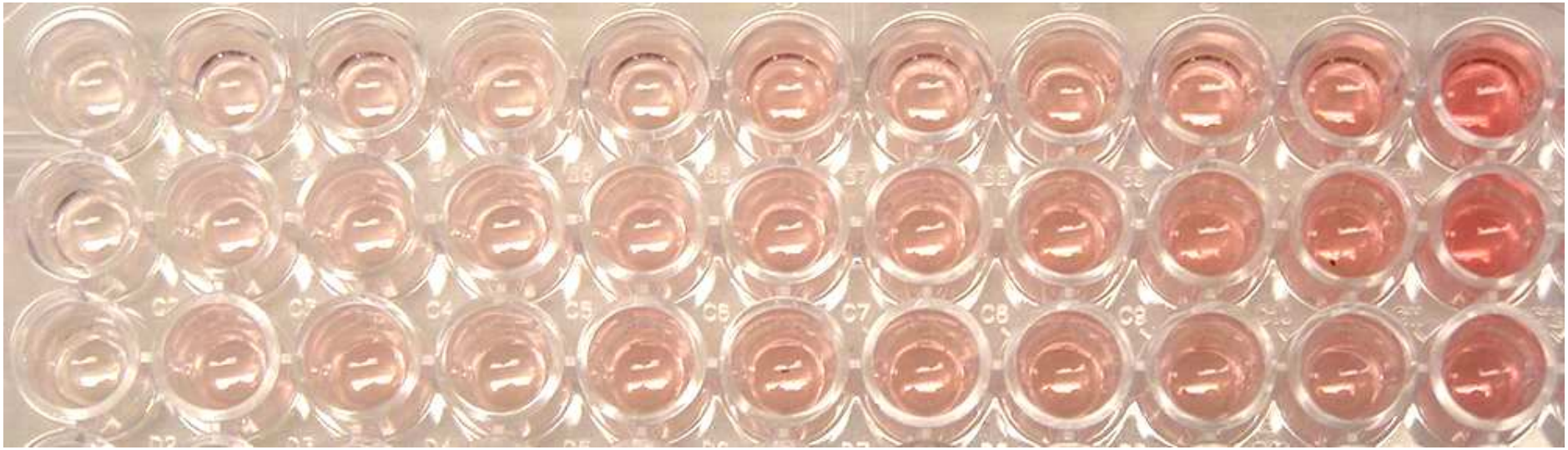


Figure 5.14: Michaelis Menten standard.

The basic Michaelis-Menten model shown in Figure 5.14 illustrates that the Michaelis –Menten constant, K_m is equivalent to the substrate concentration when the rate of conversion is half of $V_{max.}$ V_{max} is equivalent to the product of the catalyst rate constant (k_{cat}) and the concentration of the enzyme. As described by Michaelis and Menten in 1913, the K_m of a particular enzyme allows for the prediction of how the formation of product will be affected by the availability of the substrate, if at all. A substrate with a small K_m indicates high affinity which will saturate the enzyme more and thus advance closer to V_{max} at lower substrate concentrations. In order for a reaction to actually even approach V_{max}, a significant excess of [S] is required, where the enzyme becomes completely saturated.



(h) **0** **0.5** **1** **1.5** **2** **2.5** **3** **3.5** **4** **4.5** **5**

Figure 5.15: Colorimetric assay for pure D469T-catalysed conversion of different concentrations of DCHCA. Reactions were carried out over 0-5 hrs with pure D469T (0.5 $mg.mL^{-1}$), 50 mM HPA, 50 mM Tris-HCl buffer, pH 7.0, 2.4 mM TPP, 9 mM $MgCl_2$ and: top 30 mM; middle 40 mM; bottom 50 mM DCHCA.

The kinetic parameters can only be read approximately from a direct plot of the kinetic substrate concentration and initial velocity data. For accuracy the data can be fit to the Michaelis-Menten equation either by a linear or non-linear regression analysis. Such linear regression analysis methods including, Eadie-Hofstee, Hanes-Woolf and Lineweaver-Burk possess varying degrees of error distortion or exclusion within the data. The insensitivity to errors inherent in all inverse plots is taken into consideration and the Lineweaver-Burk plot is complemented by the Hanes-Woolf plot here. The Lineweaver-Burk (Figure 5.16) is particularly useful for representing the kinetic data graphically as a double reciprocal plot first described by Hans Lineweaver and Dean Burk in 1934 (Lineweaver and Burk, 1934).

$$\frac{1}{v} = \frac{K_m}{v_{max}} \cdot \frac{1}{[S]} + \frac{1}{v_{max}}$$

It is useful for analysis of the Michaelis Menten equation and although it adversely distorts error-prone data, particularly by placing greater weighting upon the less accurately measured lower substrate concentrations, it is also useful for graphically determining the type of competitive inhibition that may be present. The Hanes-Woolf plot (Figure 5.17) further represents Michealis-Menten data and an equation can be written:

$$\frac{[S]}{v} = \frac{[S]}{v_{max}} + \frac{K_m}{v_{max}}$$

A basic drawback of the use of Hanes-Woolf analysis is that the ordinate and abscissa are both dependent on substrate concentration and therefore do not represent independent variables resulting in error on both axes, as the correlation coefficient R becomes redundant.

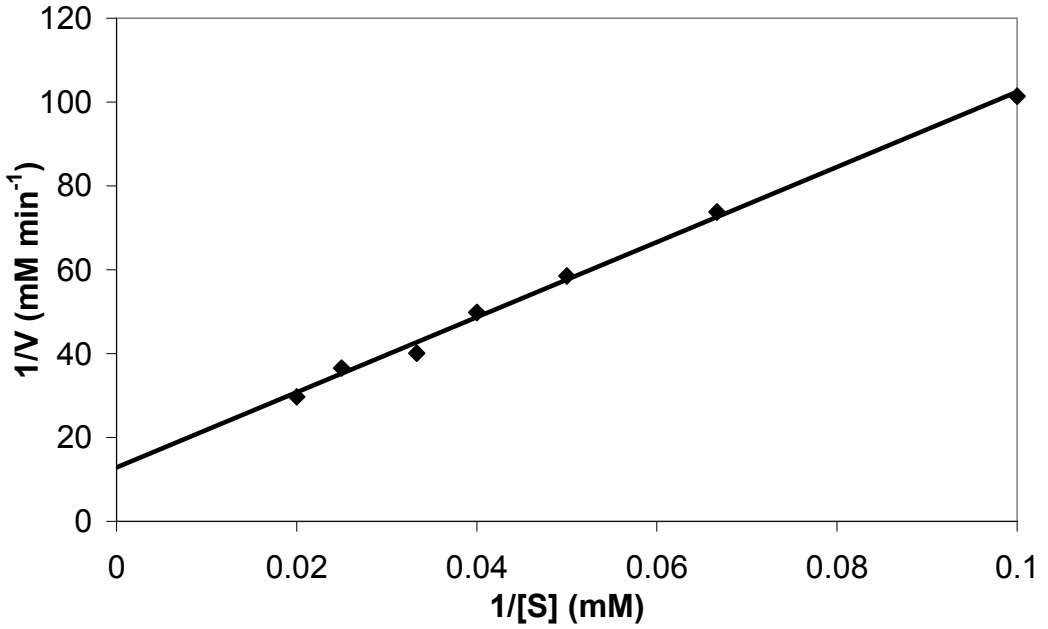


Figure 5.16: Lineweaver-Burk Plot for the D469T catalysed reaction of HPA and DCHCA. Reaction conditions: 20 °C, HPA (50 mM), 0–50 mM 3,4-dimethyl-3-cyclohexene-1-carboxaldehyde and 50 mM Tris-HCl buffer, pH 7.0, 2.4 mM ThDP, 9 mM $MgCl_2$, pH 7.0 (TK 0.5 mg.mL^{-1}) (R^2 = 0.9962).

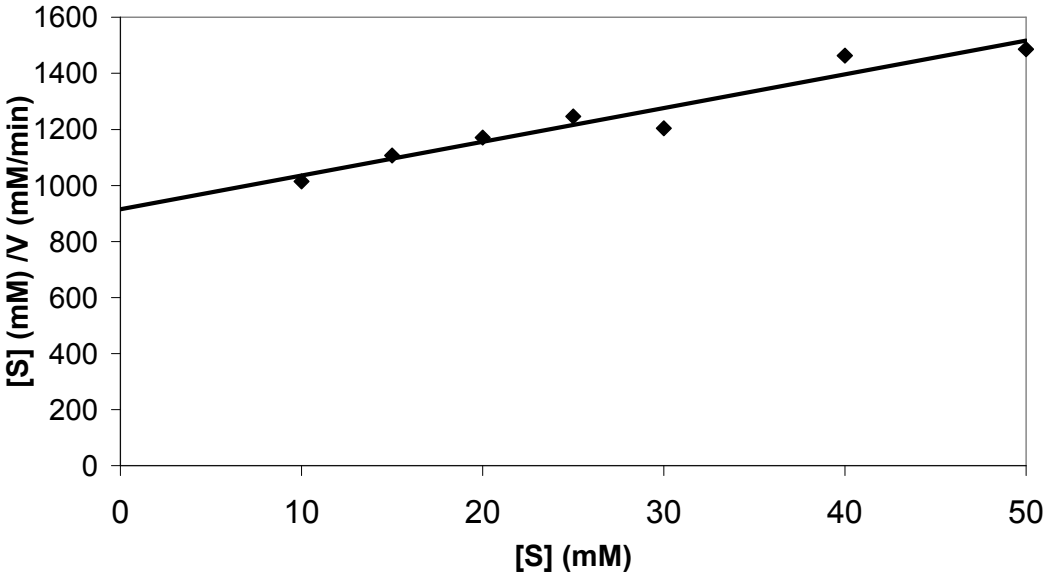


Figure 5.17: Hanes-Woolf Plot. (The same data from figure 5.16 is used here). (R^2=0.9332).

For D469T-TK with DCHCA, we observed a V_{max} of 0.07 mM min^{-1}, an apparent K_m of 69.9 mM, and a k_{cat} of 17.5 s^{-1}. Taking WT-TK activity to be at the limit of detection (0.5 mM at 48 hours), this represents a minimum of 18,000-fold improvement in substrate specificity (k_{cat}/K_m) for D469T-TK compared to WT-TK. The K_m value calculated is relatively high compared to previous measurements of WT-TK with a range of other aldehyde substrates, such as 0.16 mM for xylulose-5-phosphate and 1.1 mM for glyceraldehyde (Sprenger *et al.,* 1995). However it corresponds well with the increase in K_m seen for non-phosphorylated substrates, where D-ribose gave a K_m of 45 mM, L-xylose 55 mM, L-arabinose 250 mM and D-xylose 230 mM (Villafranca and Axelrod, 1971). The D469T mutant has previously been shown to have 8.5-fold improved specificity towards propanal relative to glycolaldehyde when compared to WT-TK, highlighting that the conversion of an acidic residue to threonine (Thr,) may favour conversions with more lipophilic substrates (Hibbert *et al.,* 2007; Hibbert *et al.,* 2008).

5.4 Summary

Product detection of kinetic assays for the determination of kinetic parameters are measured using a number of different methods such as chromatography, colorimetry and fluorescence. The transketolase catalysed reaction has been well reported to be measured, by a convenient and accurate HPLC assay, however is not time effective for the screening of numerous samples. A SRW-96 microwell format can take up to 24 hours for analysis. Previously a time-consuming and somewhat less sensitive method involving the coupling of an additional enzyme was used. The development of more specific and highly sensitive assays is therefore preferential, particularly for high-throughput procedures measuring a large number of samples, in the case of numerous enzyme variants.

Here, the recently developed tetrazolium red colorimetric assay for the high throughput screening of mutant TK libraries was modified for use as a kinetic

assay detection system. Alternative reaction quenching methods were analysed to improve compatibility with the colorimetric assay. As a replacement for the standard acid quench (0.2% TFA for deprotonation) for the transketolase catalysed reaction, flash freezing and methanol were analysed, and methanol was found to be the most effective and had the least impact on the colorimetric assay.

Michaelis-Menten kinetic parameters were determined for the novel TK mutant D469T against novel substrate 3,4-dimethyl-3-cyclohexene-1-carboxaldehyde (DCHCA). Solubility proved to be an issue with the novel substrate with very low solubility in water at room temperature. Attempts were made in order to overcome this for the kinetic analysis, however as for large scale preparative reactions, additional solutions will be required such as mixing and the use of a co-solvent, as discussed in Chapter 4 (Matosevic *et al.,* 2008). The kinetic parameters were characterised as having a significant 18,000-fold improvement in substrate specificity (k_{cat}/K_m) for D469T-TK compared to WT-TK. The activity enhancement further encourages the suggestion that the conversion of the acidic aspartic acid residue to the less polar and uncharged residue threonine, may prefer more lipophilic substrates such as propanal and now DCHCA (Hibbert *et al.,* 2008).

6 Product inhibition, substrate inhibition and product extraction

This chapter describes initial investigations into the transketolase reaction with DCHCA, including both substrate and product inhibition. The use of a novel substrate with additional functionalities, introduces problems that affect the enzyme in ways that require further characterisation. The chapter also looks initially at the compatibility between the Diels Alder reaction and transketolase for a chemo-enzymatic reaction. Initial identification of product inhibition and potential methods of product removal including the use of another enzyme transaminase (TAm) and phenylboronic acid are presented. Transaminase has been previously identified as an appropriate candidate for a multi-enzyme pathway following transketolase (Ingram *et al.,* 2007), as it has been seen to effectively transform ketodiol products of the transketolase reaction forming chiral amines (Matosevic *et al.,* 2010).

6.1 Introduction

The use of biocatalysis in place of conventional chemical syntheses maintains a number of significant advantages such as selectivity and operation under ambient conditions (described in section 1.4.1). However, due particularly to inherent mechanisms of biological enzymes, drawbacks include both substrate and product inhibition or even toxicity towards the enzyme (Dalby *et al.,* 2007) and limited substrate solubility in aqueous media.

Previously reported data regarding the stability of TK in the presence of the other reaction components, highlighted the requirement to perform a pre-incubation of the enzyme with its essential cofactors, TPP and Mg^{2+} (Mitra *et al.,* 1998). It was seen that in the absence of the reaction cofactors, the donor substrate HPA caused irreversible enzyme inhibition, however in their presence no inhibition was exhibited. TK is also susceptible to oxidation (Brocklebank *et al.,* 1999), irreversible inactivation at pH <6.5 (Mitra *et al.,* 1998), and dissociation of TPP upon removal of excess of TPP (Mitra *et al.,* 1998). It is significant therefore to establish the effect of novel substrates and other components on enzyme activity such as components of potentially integrated reactions.

6.1.1 Enzyme substrate inhibition

The phenomenon of substrate inhibition is described by the reduced activity of an enzyme-catalysed reaction in the presence of excess substrate. The relationship between substrate concentration and enzyme reaction rate can be quantitatively modelled by the Michaelis-Menten theory. Glycolaldehyde has been reported to show substrate inhibition towards transketolase (Mitra *et al.,* 1996; Woodley *et al.,* 1996; Bongs *et al.,* 1997; Vasic-Racki *et al.,* 2003; Chen *et al.,* 2007) and HPA indicated some inhibition without the pre-incubation of cofactors. The inherent effect of aldehydes on proteins has been widely identified as the aldehyde potentially forming Schiff bases with the primary amine groups on the surface of the enzyme (Snell and Di Mari, 1970).

Several methods have been employed to overcome substrate inhibition issues throughout industrial biocatalysis. For example, keeping substrate concentrations at around the K_m for maximum potential activity whilst maintaining a concentration low enough to eliminate substrate inhibition and yield high productivities overall is often the objective. Substrate feeding strategies provide solutions for such situations, where industrially relevant titre concentrations are not feasible due to substrate inhibition, substrate solubility or both.

6.1.2 Enzyme product inhibition

Inhibition often refers to a reversible loss of activity while toxicity further represents an irreversible and time-dependent loss of activity. The products of enzyme reactions have been seen in many biological reactions to be inhibitors of their corresponding enzyme (Walter and Frieden, 1963). Although commercially undesirable, this is an inherent feature of enzymes relating back to their natural environment in which it often provides a negative feedback loop for metabolic pathway regulation. In other words, an enzyme may synthesise product until a critical concentration is reached, at which point the product becomes inhibitory (at this critical concentration) to stop the enzyme from synthesising more product, as in the regulation of *in vivo* metabolic pathways (Hutson *et al.,* 1979). The negative feedback inhibits flux through the metabolic pathway and is significant in homeostasis mechanisms of biological organisms.

Forms of reversible and irreversible inhibition may be present within an enzymatic reaction, whereby the enzyme may become temporarily inactivated or where the enzyme may become truly incapacitated by the covalent bonding of an inhibitor. Competitive inhibition occurs, in simple terms, when the product of the reaction is 'mistaken' for the substrate, making the active-site unavailable for substrate conversion. Non-competitive inhibition occurs when the product adversely interacts with the enzyme by binding to it and causing an alteration in its three dimensional structure therefore preventing the substrates from interacting with the enzyme. Irreversible degradation may also occur by the structural

alteration of the enzyme caused by the product. Inhibition in any form is detrimental to the productivity of an enzymatic reaction and must first be characterised to establish the appropriate approach for subsequent solutions.

In addition to the direct toxic or inhibitory effects that reaction products may have on biocatalysts, is the effect the products have on the composition of the reaction mixture. The physio-chemical changes to the solution such as pH fluctuations, changes in ionic strength and solution viscosity necessitate the development of processes accordingly. For instance, the formation of CO_2 in the transketolase reaction, dissociating into carbonate and bicarbonate ions as the CO_2 dissolves into the aqueous media, causing a net increase in pH.

6.1.3 Approaches to overcome inhibition issues

6.1.3.1 Substrate feeding strategies

In addition to controlling substrate concentrations because of inhibitory effects at critical concentrations and limited solubility of target substrates, a relationship between high substrate concentrations and enantiomeric excess (D'Arrigo *et al.,* 1998; Houng and Liau, 2003; Kim *et al.,* 2007) has been reported indicating low substrate concentrations giving high selectivity. Fermentations have employed substrate feeding strategies to closely control the culture environment and yield higher product concentrations (Qureshi *et al.,* 1992) such as, ethanol production by *s. cerevisiae* (Hong, 1986) and the avoidance of oxidoreductive metabolism in bakers yeast production (Wang *et al.,* 1977). The supply of substrates to enzymatic bioconversions has also been described (Carragher *et al.,* 2001; Rojanarata *et al.,* 2004) although not as comprehensively as the removal of products from biocatalytic reactions. Commonly, fed-batch stirred tank reactors (STRs) are utilised to maintain low substrate concentrations. The biotransformation of toxic substrate, toluene to toluene *cis*-glycol (*cis*-1,2-dihydroxy-3-mthylcyclohexa-3,5-diene,TCG) by *Pseudomonas putida* UV4 was shown to follow Michaelis-Menten kinetics until toluene concentrations reached

2.4 mM, above which irreversible denaturation of the biocatalyst was observed. In order to overcome the low aqueous solubility of the substrate and highly toxic nature, an STR with a feed-back control algorithm based on a mass balance system was used (Hack *et al.,* 2001). Fed-batch modes are commonplace in substrate feeding strategies and have shown to be useful in bioconversions that require low aqueous substrate concentration for higher yields and/or higher *ee* (Phumathon and Stephens, 1999). Syringe pumps or peristaltic pumps simplify the fed-batch methodology for bench scale investigations.

The use of auxiliaries, such as water immiscible solid phase resins (Simpson *et al.,* 2001) and multiphases such as gas (Letisse *et al.,* 2003), or water-miscible organic solvents (Collins and Daugulis, 1997) have been reported as a means of substrate supply to eliminate substrate inhibition issues. Amberlite XAD 2 resin was reported to improve a *Saccharomyces cerevisiae* asymmetric reduction of ethyl-4- chloroacetoacetate (ECA) to *S*-4-chloro-3-hydroxybutyric acid ethyl ester. A yield improvement from 88% to 93%, alongside the optical purity increasing from 75 % to 84 % was observed when the substrate (ECA) was pre-absorbed onto resin and slowly released into solution. Mass transfer of the substrate from the solid-phase resin into the liquid phase is the driving force for release of the substrate into solution. The use of the resin within the reaction solution reduced substrate inhibition affects as well as preventing the spontaneous chemical hydrolysis of ECA, demonstrating stabilising effects of such an approach (Houng and Liau, 2003). Amberlite (IRA 400), another adsorbing resin, was utilised for the controlled release of benzoylformate in an aminotransferase bioconversion to synthesise D-phenylglycine. In order to improve the otherwise unfavourable equilibrium constant and the presence of substrate inhibition the resin was adsorbed with benzoylformate substrate and slowly released throughout the reaction on a demand-based supply, resulting in a four fold improvement in product yield (Rojanarata *et al.,* 2004).

6.1.3.2 Product removal

Product removal strategies traditionally occur as a separate batch operation once the reaction is complete by filtration, centrifugation or precipitation. This however, does not address product inhibition issues and therefore the product requires removal during the reaction. Multi-phasic strategies have been employed for such a process including, solid-phase resins and two-liquid phase reactors (Woodley and Lilly, 1990). As well as substrate feeding, resins have been largely used as tools for product removal in an approach named ISPR (*in situ* product removal) (Freeman *et al.,* 1993; Roddick and Britz, 1997; Lye and Woodley, 1999; Stark and von Stockar, 2003; Woodley *et al.,* 2008). Continuous removal of the reaction product is advantageous for removing inhibitory and toxic products but it also acts to drive the reaction equilibrium forward aiding completion of the reaction. A biocatalytic synthesis of 3-tert-butylcatechol by an *E. coli* variant 2-hydroxybiphenyl-3-monoxygenase was reported by the hydroxylation of toxic substrate, 2-tert-butylphenol which was supplied via a limited feed to the reaction mixture (Meyer *et al.,* 2003). In addition to the substrate control the product was removed from culture by a continuous *in situ* product removal with the hydrophobic resin Amberlite XAD-4, which was seen to stabilize the otherwise labile product. Boronates have also been investigated as a means of removal of *cis*-diols, such as the products of the transketolase reaction (Chauhan *et al.,* 1996; Chauhan and Woodley, 1997).

Simultaneous substrate feeding and product removal techniques (figure 6.1) represents a step further in the complete control of reaction component concentration for optimal reaction process conditions affording subsequent maximal yields and *ee* when toxic substrates form toxic products (Straathof, 2003). An example of such a concept is demonstrated by a preparative scale asymmetric Bayer-Villiger oxidation, where an *in situ* SFPR (substrate feed product removal) resin is used (Hilker *et al.,* 2004).

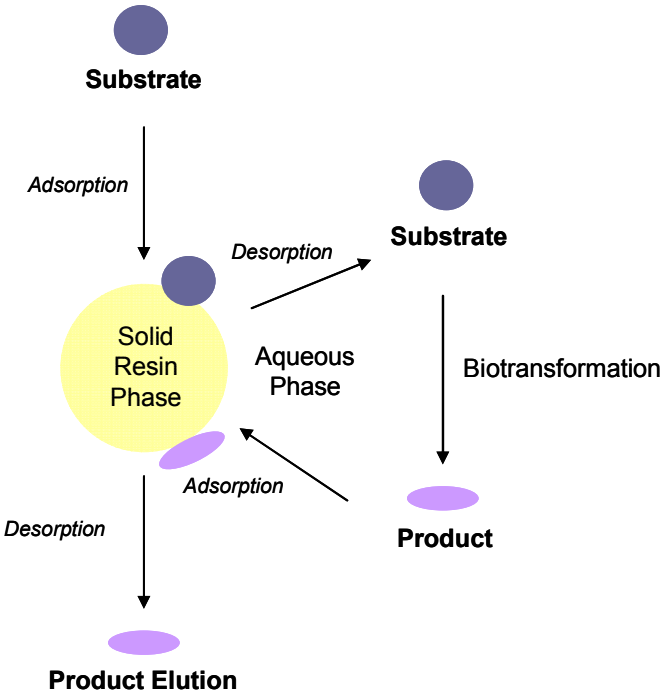


Figure 6.1: SFPR concept (substrate feeding product removal).

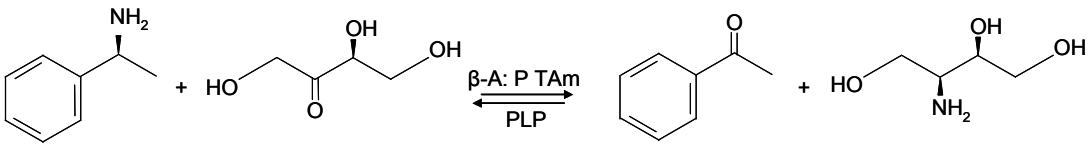
The use of a second enzyme is similar to the concept of ISPR. However, the removal process is not only designed to prevent product inhibition or to isolate the product, but also feeds a subsequent step in a useful pathway without the need for purification or isolation steps. A two-step transketolase and transaminase pathway has been previously investigated and is described below.

6.1.3.2.1 Transaminase

The compounds produced from transketolase catalysed reactions are potentially useful building blocks for conversion to other chiral synthons such as aminodiols. Another transferase enzyme, transaminase (TAm) is able to accept products of the transketolase reaction to form these chiral aminodiols. Previous investigation into dual strain recombinant *E. coli* for the 'one-pot synthesis' of chiral amino diols

has been performed (Ingram *et al.* 2007) by coupling of native transketolase and a β-alanine: pyruvate aminotransferase, transaminase. It demonstrated the integration of separate reactions establishing a symbiotic relationship whereby, the potentially toxic product of the first reaction (TK) feeds the second reaction (TAm). It provides a means of product removal, equilibrium driving force and synthesis of optically pure commercially interesting compounds in significantly reduced process steps.

In vivo transaminase has a molecular weight of 51.2 kDa and is involved in the synthesis of non-essential amino acids and also in the tricarboxylic acid and urea cycles. Like transketolase, the action of transaminase may be described by the 'ping-pong' mechanism. In the first half of a model TAm reaction (Scheme 9), binding of the amino-donor to the enzyme is followed by amination of the coenzyme pyridoxal 5'-phosphate (PLP) to pyridoxamine 5' phosphate (PMP) causing a release of the keto product. The second half of the mechanism transfers the amino group from the enzyme-bound PMP to the acceptor substrate, releasing a recycled cofactor PLP (Kaulmann *et al*, 2007).



L-MBA L-erythrulose Acetophenone ABT

Scheme 9: Model TAm reaction. Synthesis of optically active L-2-amino 1,3,4-butanetriol catalysed by β-alanine: pyruvate transaminase enzyme and cofactor pyridoxal 5'-phosphate (PLP) from L-erythrulose (synthesised by TK) and the amine donor L-methylbenzylamine.

6.1.3.2.2 Boronates

Boronic acids have been widely recognised as useful compounds for the derivatisation and protection of sugars, amino acids and hydroxamic acids with 1,2- or 1,3-diols within carbohydrate chemistry (Springsteen and Wang, 2002). The interaction of boronic acids and diols has been described as unique in supramolecular chemistry in that a reversible pair of covalent bonds is formed (Bromba *et al.,* 2009). The simple and straightforward reaction of boronic acids with diols has led to wide spread application in many processes including product removal in biotransformations. The removal of transketolase reaction products with boronic acid derivatives has been demonstrated by specifically extracting the ketodiol product from the reaction mixture (Chauhan *et al.,* 1997).

Boronic acids are alkyl or aryl substituted boric acids containing a carbon-boron bond such as phenylboronic acid (Figure 6.2). The compounds are able to rapidly and reversibly bond by a covalent pair-wise interaction to 1,2- and 1,3-diols in aqueous media in ambient conditions, making them excellent candidates for the removal of transketolase reaction products.

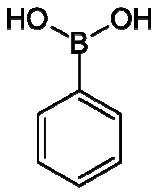


Figure 6.2: Phenylboronic acid

An investigation for the optimal removal of ketoses from aldose-containing reaction mixtures, illustrated that 3-amino phenylboronic acid (PBA) should be coupled to a polymeric carrier via alkylamino chemistry instead of an amido derivative. The effect of immobilisation chemistry on the preferential binding of

ketoses on PBA derivatives was shown previously (Dukler and Freeman, 2001), and indicated an increase in selectivity of xylulose and fructose binding throughout isomerisation at pH7.0 - 8.0.

6.1.4 Compatibility between biological and chemical processes

Biological processes within industry face many complications, particularly the stability of the enzyme. The integrity of the enzyme may be compromised by the effects of stirring such as shearing, lengthy reaction times and high ionic environments. High substrate titres required for industrial scale processes often cause proteins to become denatured. Solutions to overcome these limitations include strategies to increase enzyme stability, such as immobilisation to solid interfaces, films or coatings. The attachment of the protein to solid phases can result in a more stable protein structure and therefore more active and efficient biocatalysts. Several studies have reported reduced thermal denaturation of immobilised enzymes (Brocklebank *et al.,* 1999), and this is often thought to be due to the rigidification of the protein tertiary structure when bound to the resin support, or upon chemical cross-linking of the enzymes.

Similar to the effects of the industrial processing, the combining of processes with differing optimal conditions or the use of toxic components implores the development of compatible processes. For example, the optimal pH or temperature for the enzymatic reaction may not be the favourable optimum for the preceding synthesis step or the proceeding isolation and purification steps. In addition, the components of separate reactions may be detrimental to components of another such as aldehydes forming Schiff bases with proteins, or the competitive inhibition of one reaction substrate on the biocatalyst.

6.2 Materials and Methods

6.2.1 Substrate inhibition studies

6.2.1.1 Addition of neat aldehyde

Purified D469T-TK, 300 µL was incubated with 50 µL cofactor solution, TPP (2.4 mM), and $MgCl_2$ (9 mM) for 20 minutes at 20 °C. HPA (50 mM final) in 50 mM Tris-HCl buffer, pH 7.0) was added to the reaction mixture. Simultaneously 2.07 mg of neat, undiluted, DCHCA was added to the reaction mixture and samples of the reaction mixtures were taken at regular intervals (20 µL) and the reaction quenched in 200 µL methanol. The colour assay was performed as described in section 5.2.3.2**.**

The reaction was also performed without a pre-incubation of the enzyme and cofactor solution. Pure D469T-TK, cofactor solution, HPA stock and neat aldehyde were combined simultaneously and samples taken at regular intervals and colour assay performed.

A further substrate feeding simulation experiment was performed. A preparative scale assay was carried out as described however, the neat volume of aldehyde was added drop wise at regular intervals throughout the reaction. However, samples were not taken and the end point of the reaction analysed by TLC and colorimetric assay.

6.2.1.2 Increased substrate concentrations of 200 mM DCHCA

Purified D469T-TK, 300 µL was incubated with 50 µL cofactor solution, TPP (2.4 mM), and $MgCl_2$ (9 mM) for 20 minutes at 20 °C. HPA (50 mM in Tris-HCl buffer, 50 mM, pH 7.0) and DCHCA (200 mM in Tris-HCl buffer 50 mM, pH 7.0) were added to the reaction mixture with 5 % v/v EtOAc. Samples of the reaction mixtures were taken at regular intervals (20 µL) and the reaction

quenched in 200 µL methanol. The automated colour assay was performed as described in section 5.2.3.2.

The same reaction was performed with 50 mM DCHCA with 5% EtOAc for comparison.

6.2.2 Product inhibition study

6.2.2.1 D469T-TK reaction spiked with product at t_0

Purified D469T-TK, 300 µL was incubated with 50 µL cofactor solution, TPP (2.4 mM), and $MgCl_2$ (9 mM) for 20 minutes at 20 °C. HPA (50 mM in Tris-HCl buffer, 50 mM, pH 7.0) and DCHCA (50 mM in Tris-HCl buffer 50 mM, pH 7.0) and DCDHP (10 mM, Tris-HCl buffer 50 mM, pH 7.0) were added to the reaction mixture. Samples of the reaction mixtures were taken at regular intervals (20 µL) and the reaction quenched in 200 µL methanol. The automated colour assay was performed as described in section 5.2.3. The addition of 10 mM product was taken into consideration on analysis of data.

6.2.2.2 D469T-TK reaction spiked with product at t_5

Purified D469T-TK, 300 µL was incubated with 50 µL cofactor solution, TPP (2.4 mM), and $MgCl_2$ (9 mM) for 20 minutes at 20 °C. HPA (50 mM in Tris-HCl buffer, 50 mM, pH 7.0) and DCHCA (50 mM in Tris-HCl buffer 50 mM, pH 7.0) were added to the reaction mixture. Samples of the reaction mixtures were taken at regular intervals (20 µL) and the reaction quenched in 200 µL methanol. At reaction t_5, DCDHP (10 mM, Tris-HCl 50 mM, pH 7.0) was added to the reaction with pipette mixing. The automated colour assay was performed as described in section 5.2.3.2**.** The addition of 10 mM product was taken into consideration on analysis of data.

6.2.2.3 Enzyme product complex experiment

Purified D469T-TK, 300 µL was incubated with 50 µL cofactor solution, TPP (2.4 mM), and $MgCl_2$ (9 mM) for 20 minutes at 20 °C. HPA (50 mM in Tris-HCl buffer, 50 mM, pH 7.0) and DCHCA (50 mM in Tris-HCl buffer 50 mM, pH 7.0) were added to the reaction mixture and left to react for 48 hours. The reaction mixture was micro-dialysed (due to the small sample volume) in Tris-HCl buffer (50 mM, pH 7.0) and cofactors, TPP (2.4 mM), and $MgCl_2$ (9 mM) for 8 hours, then transferred into fresh buffer containing cofactors and dialysed for a further 8 hours (see Figure 6.3 for microdialysis). The dialysed solution was analysed by spectophotometry at OD_{280nm} for protein concentration. The solution was then applied to a TK catalysed reaction with glycolaldehyde and DCHCA as described and analysed by HPLC and colorimetric assay.

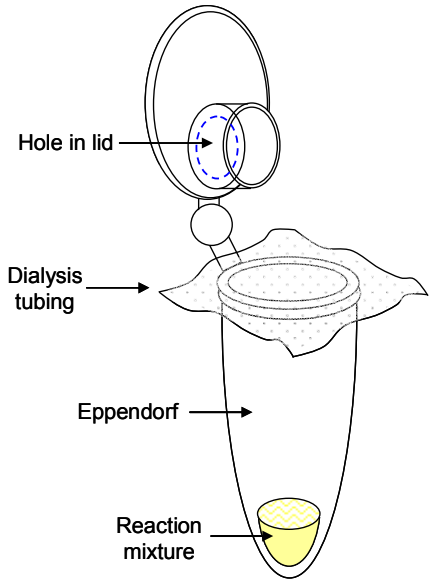


Figure 6.3: Micro-dialysis. Dialysis for small sample volumes. A hole is made in the lid of the eppendorf or microfuge tube by melting. The reaction mixture is placed in the bottom of the tube and a section of dialysis tubing trapped on closing of the tube, creating a tight seal. The sealed tube is inverted and attached (making sure the reaction mixture is in contact with the dialysis tubing) to the side of a vessel containing dialysis buffer and stirred magnetically at 4 °C for 16 hours.

6.2.3 Transaminase (TAm) preparation and purification

Purification of his-tagged β-A:P transaminase TAm (pQR801) was performed using a HisBind Quick 900 cartridge (EMD Biosciences, Darmstadt, Germany), packed with a pre-charged large-diameter cellulose matrix with tethered Ni^{2+} complex immobilised on NTA. Following fermentation, the broth (in LB-glycerol) was centrifuged for 10 minutes at 4000 rpm and the supernatant decanted. The pellet was resuspended in binding buffer (500 mM NaCl, 20 mM Tris-HCl buffer, 5 mM imidazole; pH 7.95; 0.1 mM PLP) and then sonicated to disrupt the cells. Following sonication, the lysate was centrifuged at 4000 rpm at 4 °C. The supernatant was recovered, filtered through a 0.45 and 0.1 μm Whatman syringe filter. Purification was carried out by equilibrating the cartridge with binding buffer, after which the cell extract was loaded and the cartridge washed with the same buffer. Elution was carried out with buffer containing 1 M imidazole, 500 mM NaCl, 50 mM HEPES and 0.1 mM of PLP.

6.2.4 Transaminase reaction

β-A:P TAm activity (with plasmid pQR801) was measured using an assay with (S)-α-methylbenzylalamine (MBA) and DCDHP as substrates. Substrates MBA (10 mM) and DCDHP (50 mM) were added to the reaction and incubated at 22 °C for 48 hours. Reactions were performed in 1.5 mL clear glass vials. Heating was provided by an Eppendorf Thermomixer Comfort shaker (Cambridge, UK). Reactions were monitored for MBA depletion and acetophenone (AP) production by sampling at t_0 and end point and analysed by HPLC. Samples were quenched by 0.2% TFA and centrifuged briefly at 2000 rpm to remove any precipitate prior to HPLC.

6.2.5 Transaminase HPLC analysis

AP and MBA were analysed using a CE 5 C18 reverse phase column (150 mm x 4.6 mm, 5 mm particle size; Advance Chromatography Technologies, Aberdeen,

UK). A gradient was run from 15% acetonitrile/85% 0.1% (v/v) trifluoroacetic acid (TFA) to 72% acetonitrile/28% TFA over 8 minutes, followed by a re-equilibration step for 2 minutes (oven temperature 30 ºC, flow rate 1 $mL.min^{-1}$). UV detection was carried out at 210 nm and 250 nm. The retention times (in min) under these conditions were: MBA 3.59 and AP 7.39.

6.2.6 Phenylboronic acid reaction with ketodiol products

6.2.6.1 L-erythrulose and phenylboronic acid

Phenylboronic acid (0.5 g) was added to separate, 5 mL L-erythrulose, 500 mM and 100 mM and stirred magnetically at room temperature at pH7.0 for 48 hours on pH stat. The solution was filtered and analysed by HPLC.

6.2.6.2 DCDHP and phenylboronic acid

Phenylboronic acid (0.5 g) was added to 5 mL DCDHP (10 mM) and stirred magnetically at room temperature at pH 7.0 for 48 hours at pH 7.0 controlled on a pH stat . The solution was filtered and analysed by colorimetric assay

In addition, 33 mg DCDHP was added to 1.7 mL water sonicated by 6 cycles 8 second pulses and 8 second pauses, stirred and heated to 40 °C until dissolved. 0.17 g phenylboronic acid was added to the solution and stirred at 40 °C for 48 hours at pH 7.0 controlled on a pH stat. The solution was filtered and analysed by colorimetric assay.

6.2.7 Compatibility studies

Purified D469T-TK, 300 µL was incubated with 50 µL cofactor solution, TPP (2.4 mM), and $MgCl_2$ (9 mM) for 20 minutes at 20 °C. HPA (50 mM in Tris-HCl buffer, 50 mM, pH 7.0) and DCHCA (50 mM in Tris-HCl buffer 50 mM, pH 7.0)

and one reaction with acrolein (50 mM in Tris-HCl buffer 50 mM, pH7.0) one with 2,3-dimethyl-1,3-butadiene (50 mM) and one with acrolein and 2,3-dimethyl-1,3-butadiene (50 mM each in Tris-HCl buffer 50 mM, pH7.0) added to the enzyme mixture. The automated colour assay was performed as described in section 5.2.3.2.

A separate reaction was performed in the same way however, with acrolein (50 mM in Tris-HCl buffer, 50 mM, pH7.0) in place of DCHCA. Control reactions were performed as set out in Table 6.1.

Table 6.1: Control reactions for compatibility studies. Reaction conditions: 20 °C in Tris-HCl buffer (50 mM substrates, pH 7.0) 0.5 mg/ml TK, cofactors TPP (2.4 mM) and $MgCl_2$ (9 mM) over 48 hours measured colorimetrically. [1]Additional buffer was added to reactions to maintain consistent reaction volumes and component concentration between reactions. [2] The OD measurements were below detection limit.

Control	TK	Buffer[1]	Co-factors (TPP and $MgCl_2$)	DCHCA 50 mM	Acrolein 50 mM	2,3-dimethyl-1,3-butadiene 50 mM	Li-HPA	Activity detected[2]
1		■	■	■			■	
2	■	■	■	■				
3	■	■	■			■		
4	■	■	■		■			
5	■	■	■				■	
6	■		■		■		■	

6.3 Results and discussion

6.3.1 Investigation into substrate inhibition

Substrate inhibition was previously reported for transketolase, particularly in the absence of the pre-incubation step with the cofactors to reconstitute the holo-enzyme (Mitra *et al.,* 1996). Schiff base formation is widely known as a complication of the interaction between aldehydes and proteins, whereby the amino acid residues form imines in the presence of aldehyde functionality, potentially altering the protein structure and active site composition. Initial substrate inhibition experiments were performed and found that greater than 50 mM substrate saw a decline in enzyme activity. Figure 6.4, represents the effect of higher substrate concentration on TK. The use of 5% ethyl acetate enabled the dissolution of the higher aldehyde concentration and was compared to the same conditions as the standard 50 mM substrate reaction. The yield improvement in the presence of cosolvent ethyl acetate, is indicated in Figure 6.4, and further represented by the yield improvement in preparative scale reactions (Chapter 3).

There was no significant difference in reaction rate when 50 mM DCHCA was added prior to the incubation of the enzyme with cofactors. This was considered to be due to the already relatively slow reaction rate, and the expected lag time was lost, compared to the reaction of glycolaldehyde occurring within just minutes. The affect of adding neat aldehyde to the reaction also indicated no difference in reaction rate, in experiments where total concentration was added at the start and also as a substrate feed simulation experiment. Small scale substrate feeding experiments yielded product levels no greater than previous preparations (Chapter 3) thought to be due to the low solubility in the aqueous media. A combination of increased temperature, use of cosolvent and feeding strategy may be beneficial here.

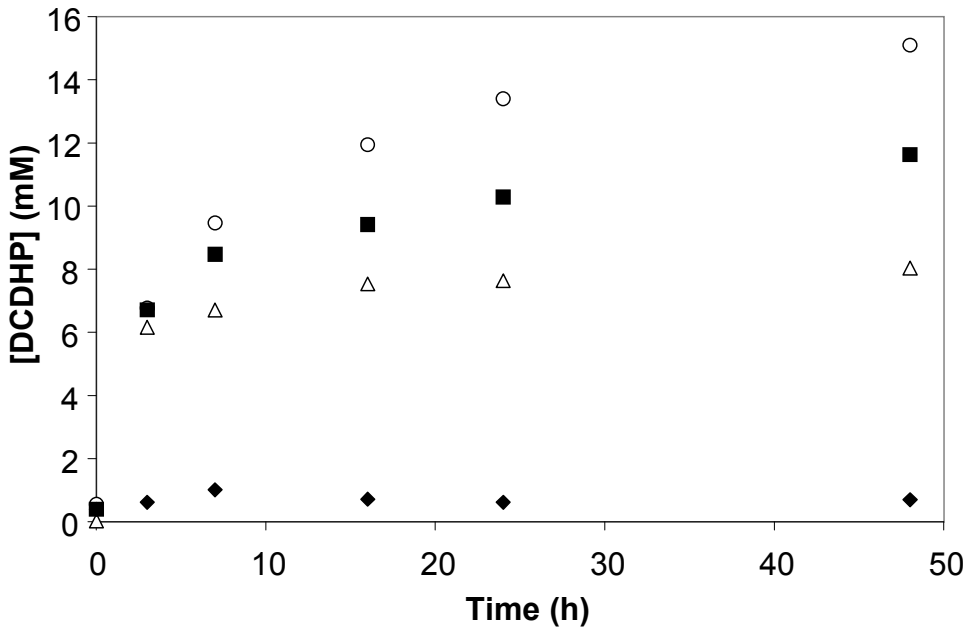


Figure 6.4: Varying reaction conditions of TK catalysed DCHCA and HPA reaction. ○ 50 mM substrates with 5% v/v EtOAc ■ 50 mM substrates Δ 200 mM DCHCA with 5% v/v EtOAc ♦ WT-TK 50 mM substrates. All in the presence of cofactors (TPP 2.4 mM and $MgCl_2$ 9 mM and TK 0.5 $mg.mL^{-1}$).

6.3.2 Investigation into product inhibition

The addition of fresh reconstituted transketolase to the reaction after 7 hours appeared to have a dramatic effect on the normal progression of the reaction (Figure 6.5). The reaction seems to continue at a similar rate to the first 7 hours of reaction, unlike the normal reaction which begins to plateau at around 7 hours. The slowing of the normal reaction in comparison to the spiked reaction indicates some inhibition whether it be competitive inhibition by the reaction product or a non-competitive toxicity towards the enzyme for instance.

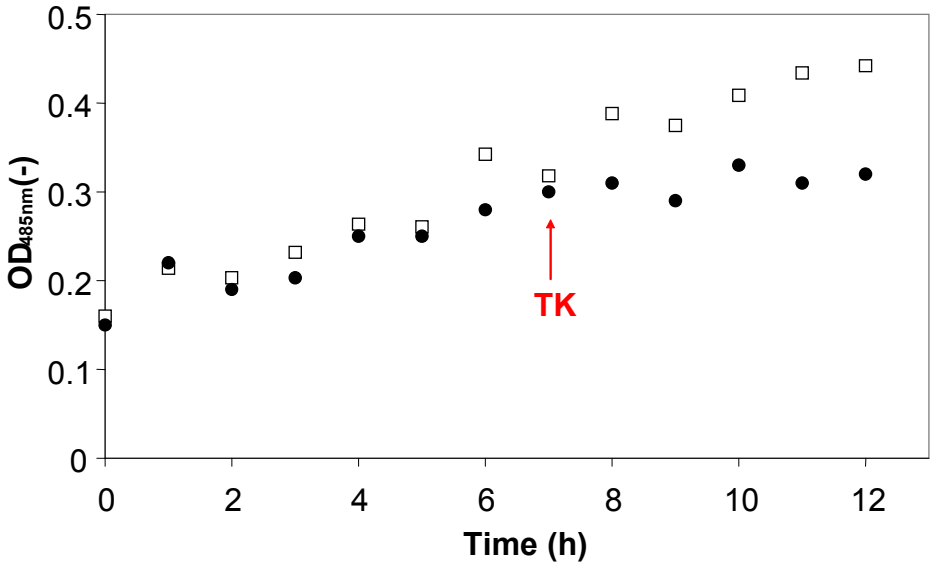


Figure 6.5: Enzyme spike reaction. The effect of spiking a 50 mM substrate reaction with fresh, pre-incubated enzyme at the point the reaction usually begins to plateau (□) spiked at t_7 with TK (indicated by arrow) (●) normal progression of 50 mM D469T-TK reaction. Reaction conditions: 20 °C, 50 mM HPA and DCHCA (Tris-HCl buffer 50 mM, pH7.0 and TK 0.5 mg.mL^{-1}).

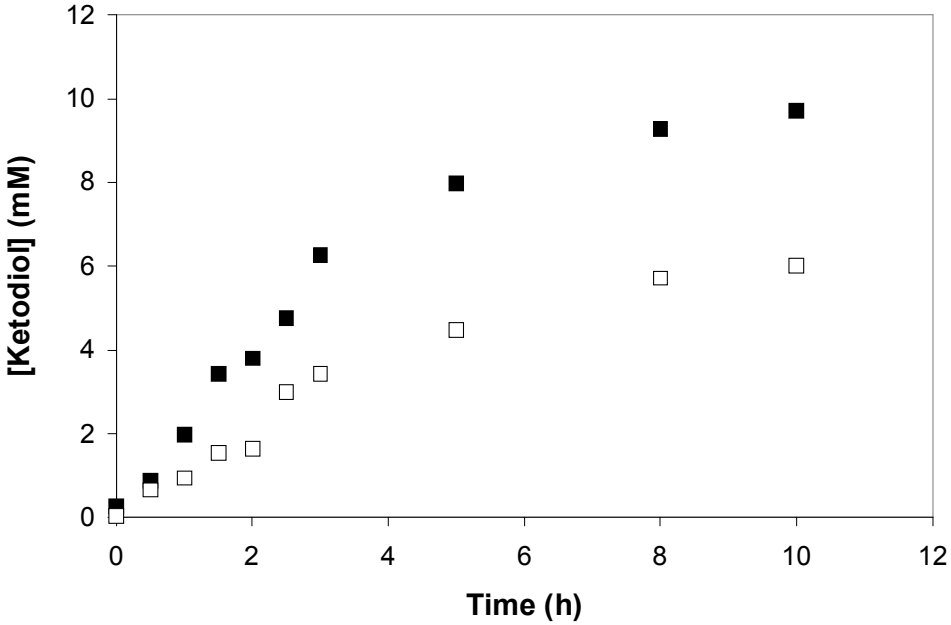


Figure 6.6: Effect of reaction product on reaction. □ DCDHP solution (10 mM, Tris-HCl buffer, 50 mM, pH7.0) added with reaction substrates ■ no product added. Reaction conditions 20 °C, 50 mM HPA and DCHCA in Tris-HCl buffer (50 mM pH7.0), cofactors (TPP 2.4 mM and $MgCl_2$) and TK 0.5 $mg.mL^{-1}$.

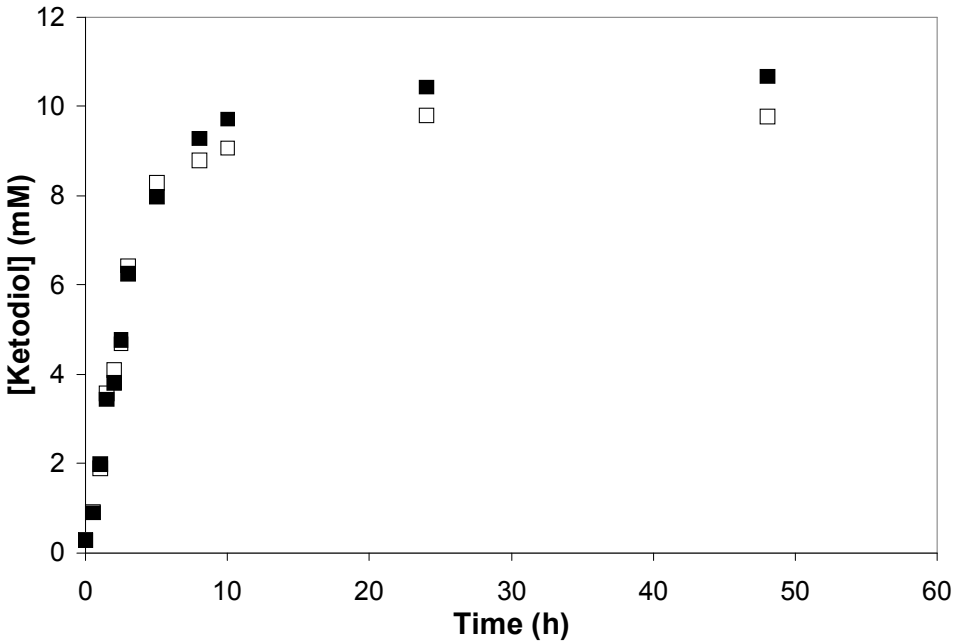


Figure 6.7: Effect of spiking TK reaction with product at 5 hours. (□) spiked at 5 hours with 10 mM product solution (■) no product added. Reaction conditions, 20 °C, 50 mM HPA and DCHCA in Tris-HCl buffer (50 mM pH7.0), cofactors (TPP 2.4 mM and $MgCl_2$) and TK 0.5 $mg.mL^{-1}$.

The transketolase catalysed reaction of DCHCA was subjected to product addition at both the beginning of the reaction and at 5 hours reaction time to determine any product inhibition issues. Both Figures 6.6 and 6.7 indicate some level of product inhibition. The rate reduction in the presence of DCDHP from the beginning of the reaction is significant confirmed by the spiking of the reaction later in reaction time.

The effect of the reaction on transketolase was investigated by recycling of the enzyme from a previous reaction. A DCHCA and HPA reaction was left to proceed for 48 hours, and analysed by TLC, to ensure product formation. The solution was subjected to a microdialysis (Figure 6.3) in order to remove the reaction components from the protein portion of the mixture. The mixture was dialysed against Tris-HCl buffer alongside cofactor solution (TPP and $MgCl_2$) at reaction concentrations to maintain TK stability through dialysis. The enzyme was then applied to a glycolaldehyde reaction and a DCHCA reaction. Activity was severely retarded as no product formation was seen at all for the DCHCA reaction, with detection by TLC and colorimetric assay and very low levels of L-erythrulose were detected by HPLC, <10% conversion. The dialysis may have caused protein destabilisation, the product may have been irreversibly bound to the enzyme or Schiff base formation between the aldehyde and protein may have caused considerable denaturation over 48hr reaction time. Further characterisation is required to provide a clearer picture of the possible causes of inhibition.

6.3.3 Product Removal

6.3.3.1 Phenylboronic acid

Free phenylboronic acid was reacted with L-erythrulose, the product of a transketolase reaction with glycolaldehyde, to form a boronic ester-ketodiol complex. The reactive tetrahedral phenylboronate anion is converted to a trigonal phenylboronic acid structure. HPLC analysis of the reaction saw that 76% (± 3%) of a 0.5 M solution of L-erythrulose was removed by the boronic acid. Lower concentrations, 0.1 M of the product solution saw lower levels of complexation

with the boronic acid, around 11 ± 2% corresponding well with previous investigations (Chauhan, 1996). Similar reactions with phenylboronic acid and the product of the DCHCA D469T-TK reaction, DCDHP, proved less effective due to the very low solubility of the product in aqueous solution at ambient temperature. Product solutions of 10 mM were made and stirred with the phenylboronic acid for 48 hours, the solution was filtered initially through filter paper followed by syringe filtration to remove the boronic ester-diol solid complex. No reduction in ketodiol concentration was evident after detection by colorimetric assay indicating no interaction between the boronic acid and ketodiol. It was considered that the low product concentration was preventing an effective interaction, and so the reaction was repeated at an elevated temperature in order to improve aqueous solubility. At 40 °C, DCDHP was dissolved to 100 mM and reacted with phenylboronic acid and stirred for 48 hours. After elimination of the binding component the colorimetric assay indicated a 9% ± 2% reduction in DCDHP in solution, indicating a positive reaction between the boronate and the novel TK product. The removal of the ketodiol product for future investigation is a simple alteration in pH, effectively stripping the covalent bonds. Lowering the pH causes the reactive tetrahedral phenylborate anion to be reverted back to the original structure. The seemingly generic reactivity of boronic acids with such diol products and the simplicity of removal firstly from reaction mixture by filtration and then separation of the complex by simple change in pH, lend themselves well to an *in situ* product removal tool for TK reactions.

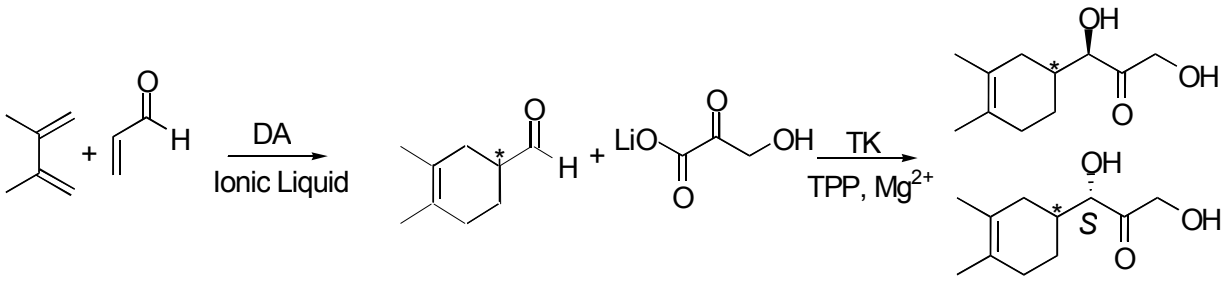
6.3.3.2 Transaminase (TAm)

A transaminase (TAm) reaction was performed with the ketodiol product (DCDHP) of the transketolase reaction product towards DCHCA and HPA. The reaction was performed to establish the potential of product removal technique utilising a second enzyme and also to determine potential future work involving a 'one pot synthesis' integration of a chemical synthesis step (DA) and 2 enzyme *de novo* pathway (TK and TAm). Previously, work has been reported regarding the use of an *E. coli* hosted dual plasmid strain expressing both TK and TAm (Ingram *et al.,* 2007). Initial detection of product by HPLC indicated some low level

acceptance of less than 5% (data not shown), and so this investigation was discontinued.

6.3.4 Compatibility studies between DA and TK reactions

The lack of a purification step for the cycloadduct of the Diels Alder reaction (DCHCA), despite 98% purity, called for an investigation into the effect of the other Diels Alder reaction components on the subsequent transketolase step. The potential for traces of acrolein, 2,3-dimethyl-1,3-butadiene, water and ionic liquid was high due to the simple decanting of the aldehyde from the reaction mixture. The effect the reaction components of each step have on other steps is also an indication of the potential 'one- pot synthesis' approach.



Scheme 10. Two step reaction sequence. Diels Alder reaction with substrates 2,3-dimethyl-1,3-butadiene and acrolein to afford 3,4-dimethyl-3-cyclohexene-1-carboxaldehyde (DCHCA). Followed by the second step, transketolase catalysed reaction of DA reaction cycloadduct (DCHCA) and lithium hydroxypyruvic acid (HPA) to afford 1-(3',4'-dimethylcyclohex-3'-enyl)-1,3-dihydroxypropan-2-one.

The dienophile substrate was analysed for activity with both WT-TK and D469T-TK, due to the compound possessing aldehyde functionality. The aldehyde group of the dienophile substrate of the cycloaddition reaction is present to afford a cycloadduct with the corresponding aldehyde group, essential for activity with TK. The colorimetric assay is applicable to the reaction of acrolein and HPA as it is a non-α-hydroxylated aldehyde unlike that of glycolaldehyde shown in the control reaction 6 of Table 6.1 This shows that the aldehyde is not accepted as an acceptor substrate and optical density measurements were in line with the limit of detection. WT-TK indicated the same negligible activity with acrolein. The effect of the diene component of the DA reaction on TK was also investigated. Control reactions confirmed the applicability of the colorimetric assay in the presence of the diene and dienophile shown in Table 6.1. 2,3-Dimethyl-1,3-butadiene appeared to have no adverse effects on the transketolase reaction when added at a concentration of 50 mM. The reaction proceeded as normal in the same way the reaction was unaffected in the presence of acrolein and also a mixture of the two DA substrates.

The presence of even traces of ionic liquid in the transketolase reaction saw completely toxic consequences, whereby the reaction did not proceed at all. The detrimental effect of ionic liquid on the enzyme indicates that further development of this system is required to be used as a model for 'one- pot syntheses'. The catalysis of the DA reaction has been widely reported by numerous methods including aqueous systems (Diego-Castro and Hailes, 1998; Otto *et al.,* 1998; Marchàn *et al.,* 2006; Windmon and Dragojlovic, 2008) and organic solvents (Laszlo and Lucchetti, 1984) or in the presence of a catalyst. An aqueous medium would be ideal in terms of compatibility with the biotransformation and also because water is an abundant and often cost effective solvent.

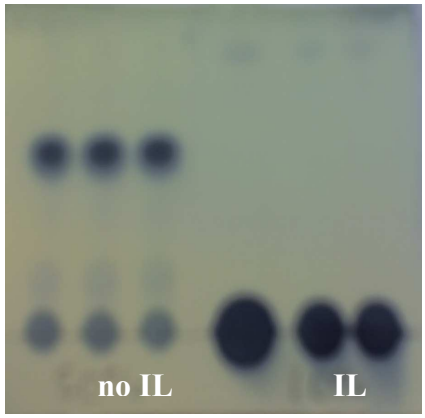


Figure 6.8: Effect of ionic liquid on D469T-TK reaction. Traces of the ionic liquid present appeared to completely prevent the TK reaction from proceeding.

6.4 Summary

Preliminary experiments were performed to investigate potential inhibition issues associated with the use of the novel, cyclic hydrophobic substrate for transketolase. The presence of product inhibition was indicated by spiking reactions with the product at various points of the reaction progression curve. Previously L-erythrulose was observed to similarly inhibit TK-catalysed reactions, and was presumed to be a competitive inhibitor of TK. It was also suggested that a futile reaction can exist where L-erythrulose and glycolaldehyde react to form the same compounds, indicating that the ketodiol can mimic the ketol donor substrate (Hobbs *et al.,* 1993). This could potentially occur with other aldehyde substrates including DCHCA.

The presence of substrate inhibition was also briefly investigated and the reaction found to be inhibited in concentrations of 200 mM DCHCA compared to 50 mM. Substrate feeding experiments were not significantly different and considered to be due to the low substrate solubility. Where standard, unfed, unstirred, batch reactions were possibly simulating the substrate 'supply and demand' effect of substrate feeding, through a hydrophobic/aqueous phase interface.

The compatibility between the two steps of the novel chemoenzymatic reaction, DA and TK, were also briefly analysed. The presence of even small traces of ionic liquid completely stopped the TK reaction preventing any product formation shown in figure 6.8. The presence of the other DA reaction substrates on the TK reaction however indicated no detrimental effects and the aldehyde did not appear to be accepted as a substrate by TK. These findings indicate good compatibility between the DA substrates and the biotransformation steps, however alternative solvent and catalyst for a single pot approach will be required.

The transketolase catalysed product of DCHCA and HPA reaction, with phenylboronic acid was investigated and found to positively interact. Although relatively low removal levels from solution were measured in comparison to L-erythrulose. The low solubility of the compound in water reduced the potential for higher levels as reactions with similar concentrations yielded similar levels of interaction. The possible conversion of the DCDHP compound by further biocatalyst, transaminase was also briefly investigated as potential synthesis of commercially valuable chiral amine intermediates. Unfortunately, conversion levels were low and insignificant, however, alongside reaction optimisation, processes such as the substrate walking approach and directed evolution methods used for transketolase may improve such activity.

7 Summary and final discussion

The integration of two separate fields is not only an attractive means of synthesis but it is becoming more of a necessity. The demand for optically pure pharmaceuticals is a hard task for a solely chemical synthesis to remain cost effective and to be developed within a feasible time scale. The integration of two different processes such as a chemical reaction and a biocatalytic step requires numerous considerations including process design, reactor design, and compatibility studies. The engineering of enzymes provides a closer link, whereby the biocatalysts are engineered to possess more desirable characteristics. For instance, to improve the biocatalyst activity over a wider range of pH or temperature to be compatible with chemical reaction conditions or with a broader range of more complex substrates to afford commercially valuable, structurally demanding, compounds.

The model reaction pathway designed for chemoenzymatic synthesis of optically pure compounds involved a DA cycloaddition which is a well established synthetic chemistry step, and an extensively investigated biotransformation with

the transferase enzyme, transketolase. The utilisation of a well established reaction is crucial to enable characterisation of the issues arising upon integration, as it is advantageous to already have established detection methods, knowledge of optimal reaction conditions, and the potential to achieve high yields.

Target compounds for the transketolase reaction were examined via a substrate walking strategy, whereby sequentially linked cyclic aldehydes with increasing complexity were investigated for activity with both WT-TK and TK variants. Positive results were obtained from screening experiments where several 'hits' were highlighted. The use of a further complex cyclic aldehyde with additional functionalities, including a double bond and two methyl groups was useful for probing TK activity and selectivity. The differing characteristics of previously examined substrates, particularly the low aqueous solubility, introduced factors for consideration, including the use of co-solvents and alternative reaction quench methods. The synthesis of the cyclic aldehyde acceptor substrate via a DA cycloaddition provided a point of integration for analysis and characterisation of a chemoenzymatic reaction sequence, such as enzyme kinetics and enantioselectivity.

The synthesis of a novel acceptor aldehyde by a well studied chemical synthesis was optimised here for application to a biocatalytic step to demonstrate the integration of the two processes. The acceptor aldehyde was synthesised by a Diels Alder cycloaddition reaction using a room temperature ionic liquid in ambient conditions (Abbott *et al.,* 2002). Ultimately with various scales and reaction optimisation experiments, very high purity of 98% and particularly high yields of around 91% were established without the need for separate purification techniques. The lack of additional purification steps however, did have potential knock-on effects for the following biological enzymatic step.

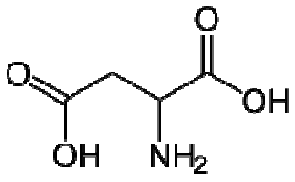
Previously produced, phylogenetic and structurally defined transketolase libraries (Hibbert *et al.,* 2007) were screened for activity with the sequentially linked cyclic aldehydes providing evidence for TK ability to accept such compounds. The DA cycloadduct was subsequently applied to the library with potential for activity, namely the D469X structural library, previously indicated for involvement with

stereoselectivity. Utilising a sensitive colorimetric assay that was specifically optimised for transketolase catalysed reaction products, a number of D469X mutants showed considerable activity enhancement. Subsequent selection and culture of the highest performing mutants enabled gene sequencing and identification of the sequence mutation. Corresponding well with a previous observation (Hibbert *et al.,* 2008), D469T was highlighted as having the highest level of activity enhancement towards the DA cycloadduct. A common pattern was seen throughout all the cyclic aldehyde screens including that for the simpler compound propanal, indicating some variants inability to accept such compounds in contrast to the more 'promiscuous' variants, for example D469T-TK.

Analysis of the initial rates of the cyclic aldehydes compared to propanal indicated a slight drop in reaction rate as ring size increased from cyclopropane 7.45 $mM.h^{-1}$ to cyclohexane 3.46 $mM.h^{-1}$.The potentially lower conformational flexibility of the cyclic aldehyde structures within the active site of the enzyme compared to less complex substrates including propanal and linear chain aldehydes, may explain the lower initial rates. Also the increasing ring size may have increasing steric hindrance within the active site or increasing Van der Waals interactions with residues reducing the flexibility of the substrate further for correct orientation.

Large scale preparations of D469T-TK followed by purification using an N-terminal His-tag and a Ni-NTA resin, enabled preparative scale biotransformations with DCHCA and HPA. The use of co-solvents for improving aldehyde solubility saw an increase in product yield and novel compound 3',4'-dimethylcyclohex-3'-enyl-1,3-dihydroxypropan-2-one was synthesised, isolated and analysed. Chiral HPLC techniques and modified Moshers' ester method permitted the determination of the enantioselectivity of the novel TK catalysed reaction. It was calculated that an *ee* in excess of 93%, for the single point mutation transketolase towards a cyclic, bulky, hydrophobic aldehyde was produced. The absolute configuration was subsequently calculated and the single major enantiomer elucidated as the (1*S*,1'*R*) enantiomer, corresponding well with previous observations that TK is stereospecific for (*R*)-hydroxyaldehydes and stereoselectively synthesises ketodiol products with the S-configuration.

The colorimetric assay for screening was successfully adapted for use as a kinetic measurement assay to complement HPLC analysis and Michaelis-Menten kinetic parameters were determined. For D469T-TK with DCHCA, we observed a V_{max} of 0.07 mM min^{-1}, an apparent K_m of 69.9 mM, and a k_{cat} of 17.5 s^{-1}, representing at least an 18,000-fold improvement in substrate specificity (k_{cat}/K_m) for D469T-TK compared to WT-TK. The mutation from a polar, charged, aspartic acid residue (Figure 6.9 **1**) previously highlighted as potentially being involved with enantioselectivity (Nilsson *et al.,* 1998), to a less polar and uncharged residue threonine (Figure 6.9 **2**) appears to be preferential for activity with more lipophilic substrates. Corresponding well with previously reported data (Hibbert *et al.,* 2008) D469T-TK exhibited enhanced activity with the lipophilic, aliphatic aldehyde propanal and here the lipophilic DA cycloadduct. Further emphasising this potential relationship, TK variant D469L, a conversion from aspartic acid to the non-polar, hydrophobic leucine (Figure 6.9 **3**), indicated enhanced activity towards the cyclic aldehyde. In addition, the top five performing mutants highlighted in library screening experiments, all possessed residues uncharged at neutral pH unlike the wild type TK residue aspartic acid, which indicted negligible activity towards the novel substrate. The well known phenomenon that the chemical property of an amino acid determines the substrate specificity of the enzyme is demonstrated here.



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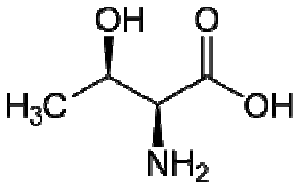

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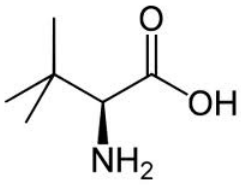

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Fig.6.9. Amino acid side chain comparison. 1) aspartic acid **2)** threonine and **3)** leucine.

The physical integration of the two-step reaction was briefly investigated alongside inhibition issues towards the enzyme, transketolase with aldehyde 3,4-dimethyl-3-cyclohexene-1-carboxaldehyde. Substrate inhibition became evident at concentrations above kinetic analysis concentrations and solutions to resolve this issue were briefly considered. Reported data regarding transketolase inhibition and also general enzyme inhibition, indicated the tendency for aldehyde substrates to induce Schiff base formation with the side chains of lysine residues lying on the surface of the protein. This may adversely alter the enzyme structure including the active site and therefore potentially affect enzyme activity, particularly at higher concentrations.

Product inhibition was also investigated for the D469T–TK catalysed reaction affording 3',4'-dimethylcyclohex-3'-enyl-1,3-dihydroxypropan-2-one. A futile reaction was seen previously with wild type transketolase between glycolaldehyde and the reaction product L-erythrulose synthesising the same compounds, suggesting the reaction of the product was acting as a competitive inhibitor (Hobbs *et al.,* 1993). The product of the reaction here may also be mimicking the cyclic aldehyde substrate causing product inhibition. In addition, at critical concentrations the product may be exhibiting toxicity towards the enzyme possibly causing an irreversible degradation of the protein structure. Further, more in depth analysis will be required to determine the nature of the product inhibition.

Compatibility between two processes upon integration is a significant issue requiring characterisation prior to establishing process design requirements. Initial experiments indicated the detrimental effect of the DA solvent/catalyst room temperature ionic liquid on the progression of the transketolase reaction. No contamination of ionic liquid in aldehyde preparations was evident at any point through decanting, however in the event that even traces of the ionic liquid was transferred from one step to the next the transketolase reaction was completely inhibited. This indicated the need for development of DA catalysis which is a topic widely discussed throughout literature, including aqueous catalysed cycloadditions (Diego-Castro and Hailes, 1998) for further development of a ‘one-pot’ strategy. The effect of the DA reaction substrates however, appeared to be insignificant with no activity differences shown in their presence at

concentrations of 50 mM. The dienophile substrate was also shown to have no enzymatic activity with transketolase given the aldehyde functionality possibly acting as acceptor substrate.

Many solutions exist to overcome process limitations such as substrate and product inhibition, low substrate solubility, pH fluctuations, etc. The feeding of substrates though pump fed or resin fed strategies, enable the continuous supply of substrate below critical inhibitory concentrations and also to aid solubility limitations. Product removal was approached using similar techniques including resin adsorption via methods known as *in situ* product removal (ISPR). A well known interaction of compounds synthesised by transketolase catalysed reactions, 1,2- and 1,3-diols and boronic acids was demonstrated here as a potential method of product extraction. Although relatively low yields were recovered compared to other *cis*-diol products, increased product concentrations and pH optimisation may positively influence interaction.

The ultimate objectives of the project were achieved whereby, a novel chemoenzymatic reaction, integrating a chemical reaction and biotransformation was performed to potentially synthesise novel, optically pure compounds. A transketolase variant able to accept a markedly different acceptor aldehyde substrate, synthesised by a DA cycloaddition reaction was identified and subsequently analysed. The enantioselectivity and absolute configuration were determined alongside Michaelis-Menten kinetic parameters, utilising a colorimetric assay formerly developed for screening high-throughput transketolase variant libraries.

8 Future work

The successful integration of a chemical synthesis and biotransformation has been demonstrated in this project. However, despite an excellent enantiomeric excess the enzyme reaction rate remains relatively low. The stability of the enzyme was demonstrated to be compromised at room temperature after even three days (Figure 4.5). It was evident, however, that the reaction equilibrium had not been reached, as indicated by further product synthesis upon addition of fresh holo-enzyme to the reaction at a point where activity began to diminish (Figure 6.5). The reaction rate and yield could therefore be optimised by improving the stability of the enzyme by immobilisation, possibly by a His-tag, Ni-NTA interaction, also utilised for the purification of the enzyme.

Further investigation for improving TK product yield could involve overcoming the issues associated with both substrate and product inhibition. The immobilisation of the enzyme would potentially improve the issue of substrate inhibition by rigidifying the enzyme so it becomes less susceptible to the formation of Schiff bases, also due to the fact that less surface residues of the enzyme are accessible. In addition, improvements could be made through substrate feeding strategies with pump-fed control systems, or by the use of resin pre-adsorbed with the substrate which then gets released through mass transfer kinetics. Simultaneous substrate delivery and product removal techniques are valuable tools for overcoming the additional effects of product inhibition, evident with the reaction demonstrated here. The implementation of boronates or other specific resins for product removal of *cis*-diols may be further characterised and improved by increasing the reaction and hence product concentration, and by examining the optimal pH for interaction as well as trials of different boronate resins.

Removal of product could also be achieved using a second enzyme step. The further integration of an additional enzyme step to the pathway for removal of TK product from reaction and synthesis of commercially valuable chiral amines could involve the transaminase (TAm) enzymes. Previously transaminases have been demonstrated to accept TK products and have also been investigated in a dual strain enzyme system (Ingram *et al.,* 2007). TAm activity was examined briefly with the product of the sequence described in this project and low activity was detected (> 5%). Development of a range of mutant libraries like the phylogenetic and structural libraries constructed for TK would potentially provide mutant TAm enzymes with enhanced activity. However, unlike transketolase, a high resolution crystal structure is not available for all transaminases so random mutagenesis approaches would be required as opposed to rational mutagenesis design approaches.

Building of the ‘one-pot synthesis’ concept for the ultimate integration of a chemoenzymatic synthesis would require an alternative DA reaction solvent or catalyst. The cycloadduct synthesis here used a room temperature ionic liquid consisting of a zinc chloride and choline chloride complex which was detrimental

to the progression of the TK reaction. The performance of DA cycloaddition reactions in aqueous media has been well investigated throughout the literature and would provide a sound basis for identifying a compatible solvent system for the two-step pathway.

Automation of the process at microwell scale could also enhance the process whereby, alternative process options could be evaluated within a reduced time frame ustilising less, often costly reagents such as HPA. A direct link between the process performance in varying conditions at small scale and modelling of the catalyst would potentially aid in achieving higher productivities.

In summary, possible extensions to this project to further optimise the chemoenzymatic reaction:

- Immobilisation to improve stability of TK throughout lengthy reaction times.
- Further investigation into substrate inhibition and substrate feeding strategies at scale.
- Characterisation of product inhibition and determination of suitable solutions such as *in situ* product removal using boronates or an additional enzyme such as, transaminase (TAm).
- Performance of Diels Alder reactions in aqueous media.
- Automation of the process for rapid analysis of compatible reaction and process options enabling prediction of the parameters at larger scales.

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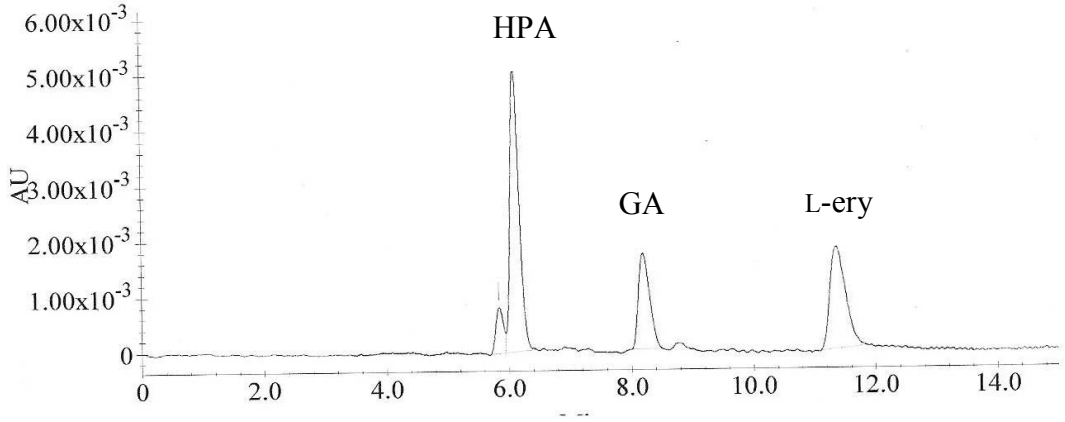
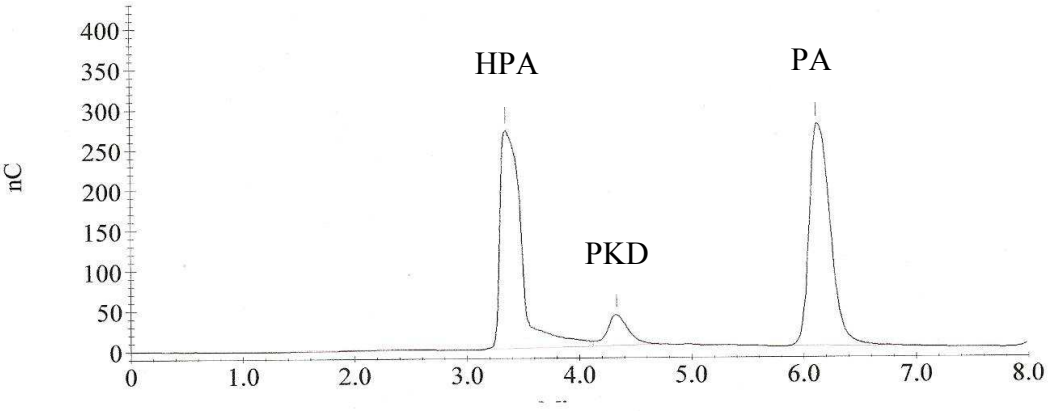
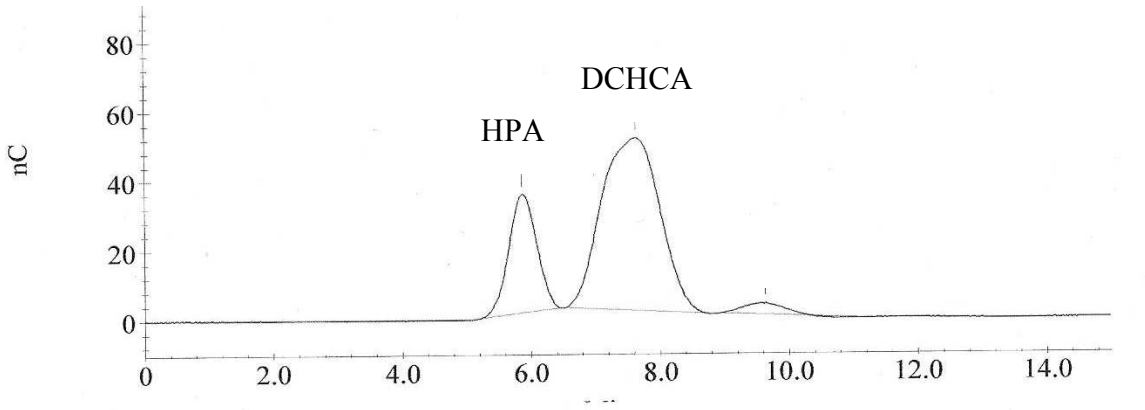
Appendix



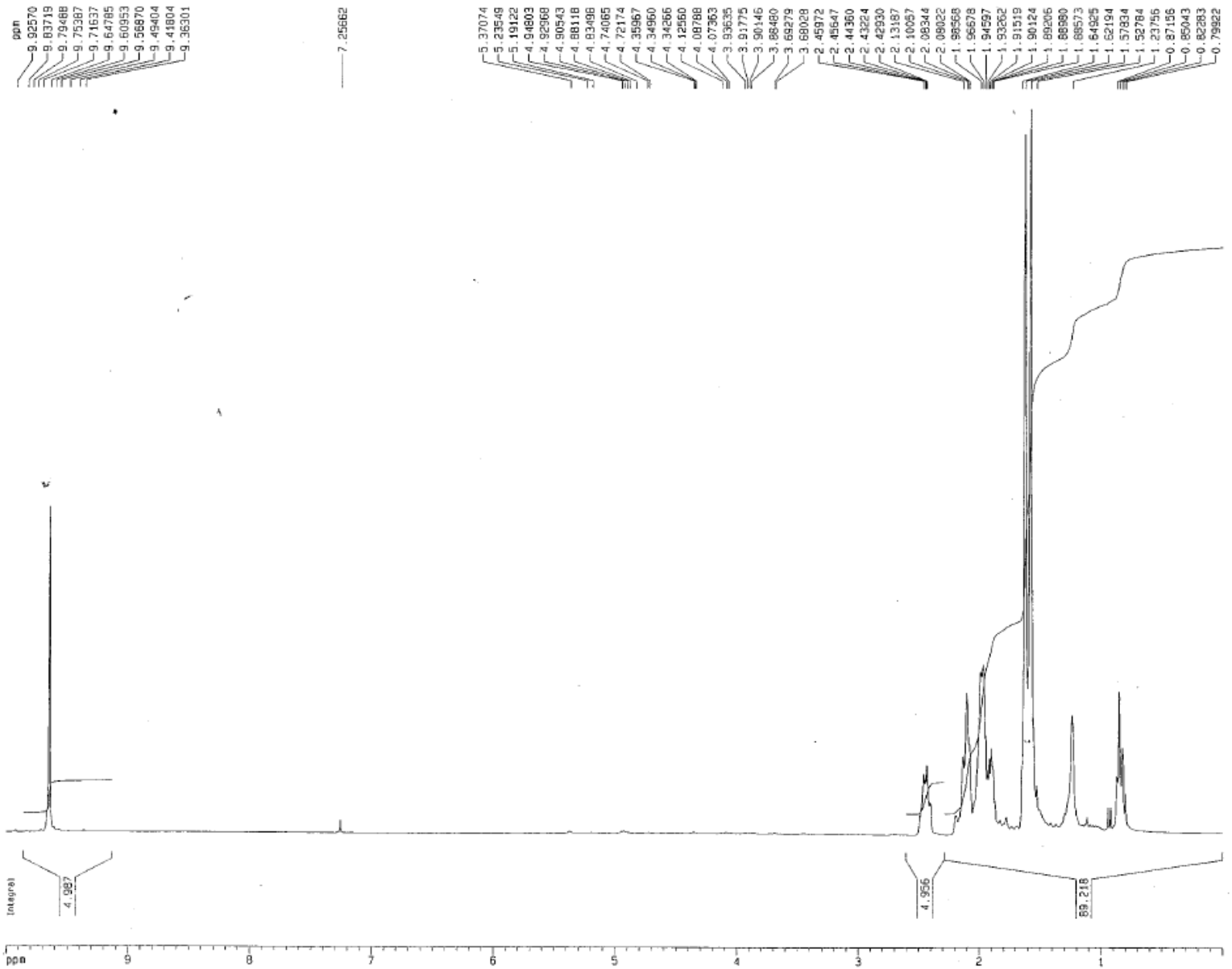
Appendix 1: Standard chromatogram for transketolase reaction detecting HPA, GA and L-erythrulose (UV).



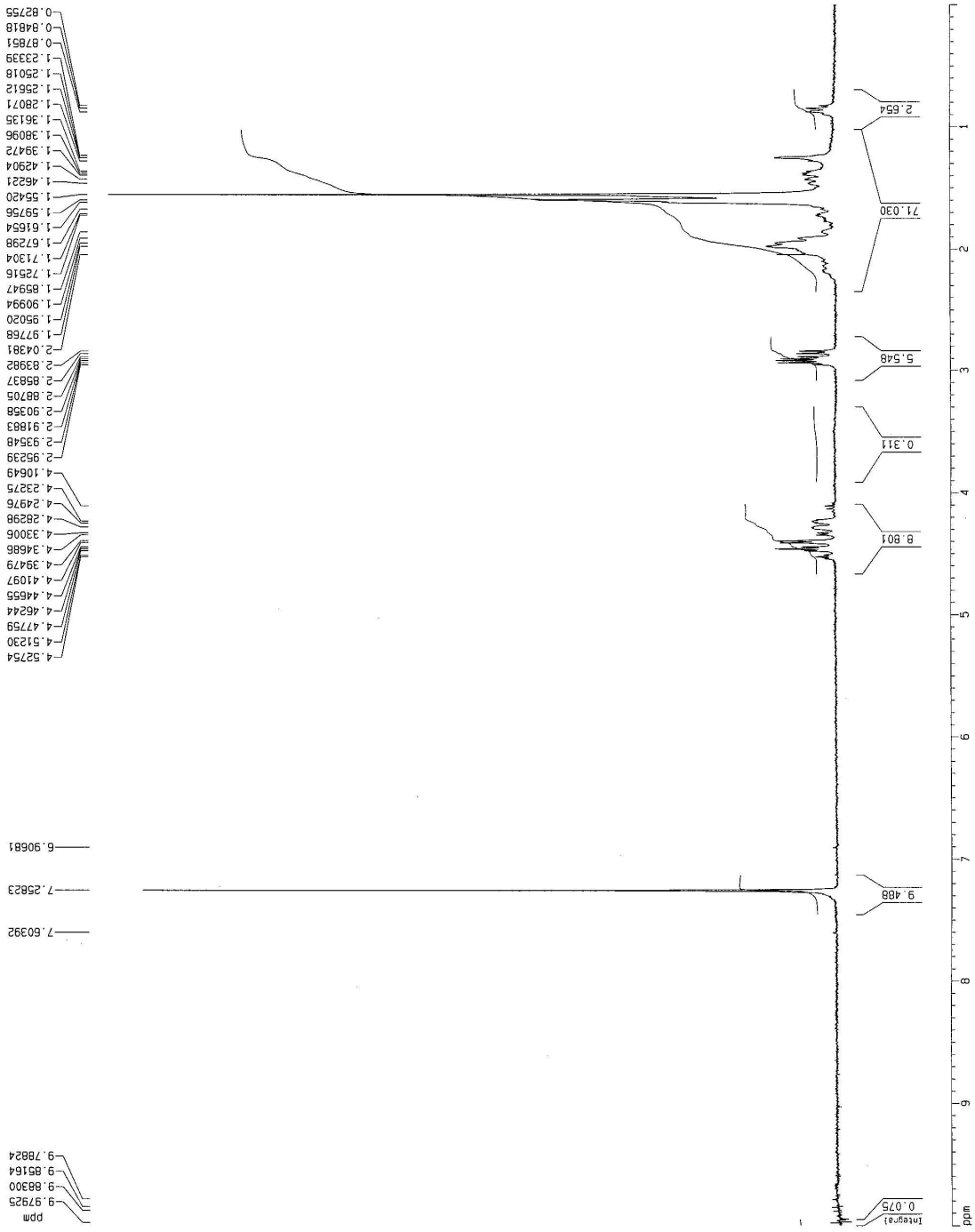
Appendix 2: Standard chromatogram for transketolase reaction. HPA and DHP and PA (ECD).



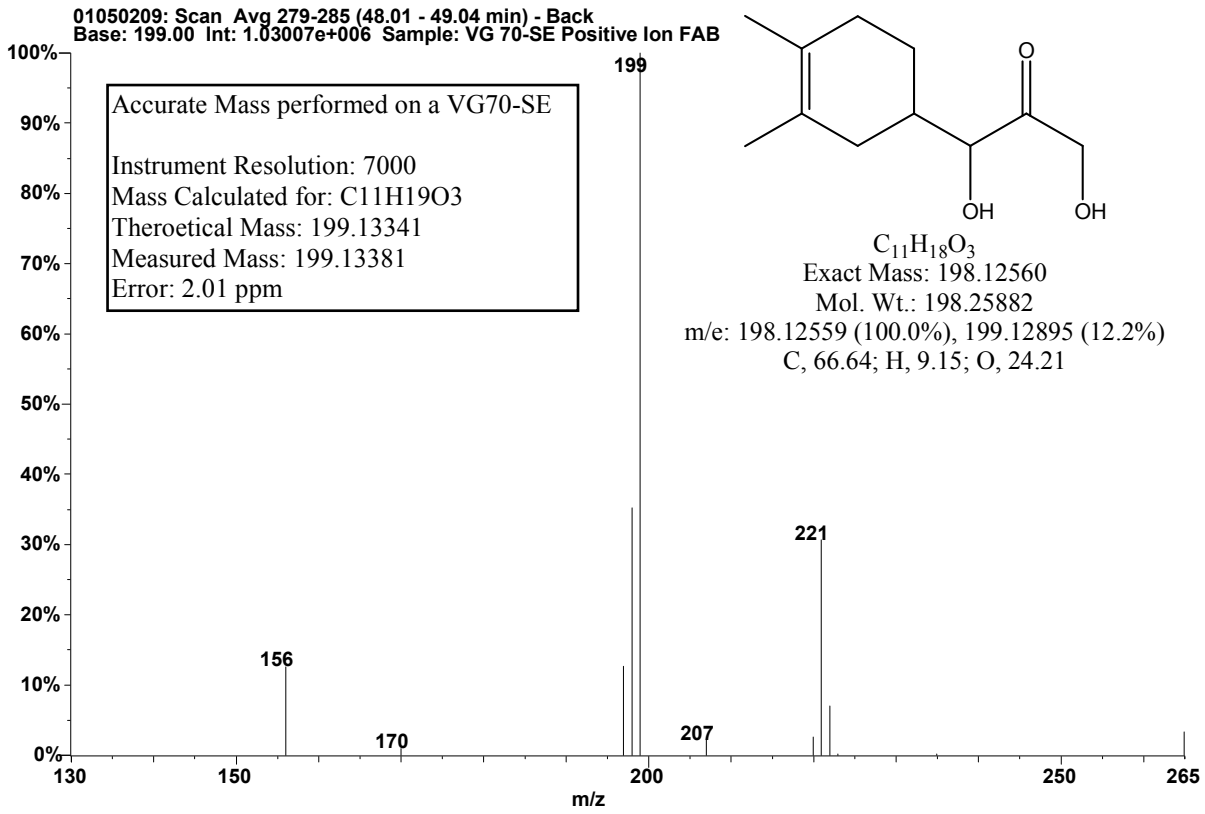
Appendix 3: Chromatogram for DCHCA (ECD).



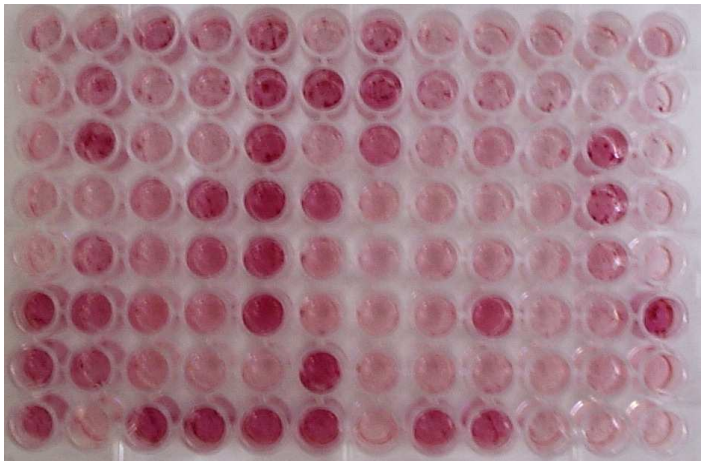
Appendix 4.. ^{1}H NMR Spectra for DCHCA. $CDC1_3$



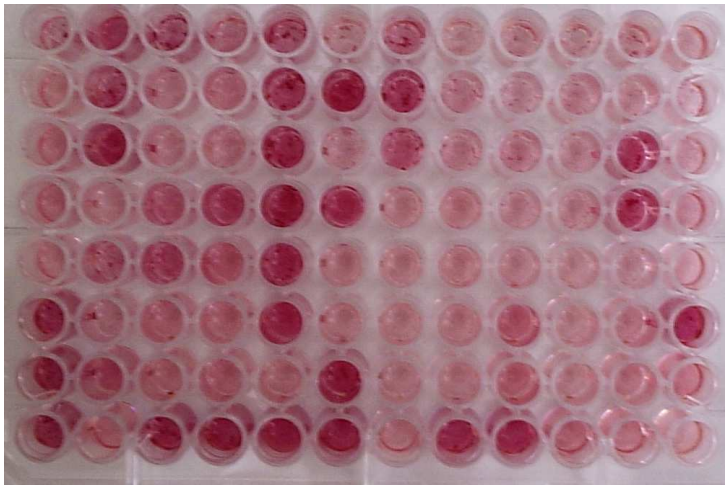
Appendix 5: ^{1}H NMR Spectra for DCDHP. $CDCl_3$



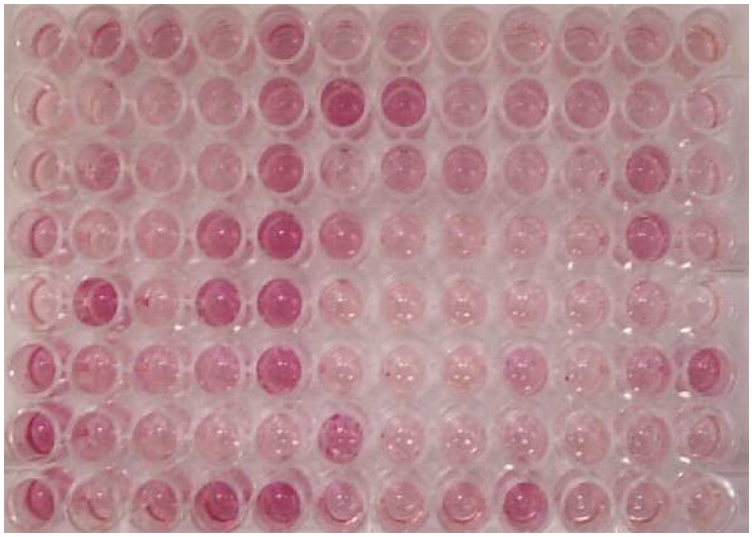
Appendix 6. Mass spec DCDHP



Appendix 7. Cyclopropanecarboxaldehyde screen of TK library D469.



Appendix 8: Cyclopentanecaboxaldehyde screen of TK library D469.



Appendix 9: Cyclohexanecarboxaldehyde screen of TK library D469.