REFERENCE ONLY

UNIVERSITY OF LONDON THESIS

Degree PhD Year 2006 Name of Author INGRAM, G.U.

Biocatalytic Synthesis of Amino-Alcohols Using a *de novo* Designed Transketolase-β-Alanine: Pyruvate Transaminase Pathway in *Escherichia coli*

A Thesis Submitted for the Degree of
Doctor of Philosophy
to the
University of London

Christine U. Ingram

Department of Biochemical Engineering
University College London

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

For my Grandpa, Maurice Ingram,
who departed before I arrived.

Abstract

Biocatalysis continues to emerge as a powerful technique for the efficient synthesis of optically pure pharmaceuticals that are difficult to access via conventional chemistry. The power of a biocatalytic stage can be enhanced if two or more reactions can be achieved by a single whole cell biocatalyst containing a pathway designed *de novo* to facilitate a required synthetic sequence. This will allow higher space-time yields and reduce the number of intermediate process steps required. The enzymes transketolase (TK) and transaminase (TAm) respectively catalyse asymmetric carbon-carbon bond formation and amine group addition to suitable substrate molecules. The ability of a transaminase to accept the product of the transketolase reaction can allow the two catalysts to be employed in series to create chiral amino alcohols from achiral substrates. These compounds are synthetically very useful in the production of a range of compounds with pharmaceutical application.

The β-alanine: pyruvate aminotransferase (β-A: P TAm) from *Pseudomonas aeruginosa* has been cloned, to create plasmid pQR426, for over-expression in *E.coli* strain BL21gold(DE3). The aromatic transaminase (ArTAm) from *E.coli* was also cloned, to create plasmid pQR416. The substrate specificity of the transaminases has been explored in order to assess the feasibility of functioning downstream of TK and the potential to access a wider range of TK-TAm pathway products. Both directed evolution and site directed mutagenesis have also been applied in order to facilitate acceptance of transketolase products. Over-expression of the β-A: P TAm alongside the native transketolase (from plasmid pQR411), in a single *E.coli* host, has created a novel bacterial biocatalyst capable of the synthesis of chiral amino alcohols via a synthetic two-step pathway. The feasibility of using the biocatalyst has been demonstrated by the formation of 2-amino-1,3,4-butanetriol (ABT) product, in up to 21 % yield, by the β-alanine: pyruvate transaminase, via transamination of *L*-erythrulose synthesised by transketolase, from glycolaldehyde (GA) and β-hydroxypyruvate (β-HPA) substrates. The degradation of the amino alcohol product and the low activity of the transaminase, initial rate 0.1 $mM.hr^{-1}$, relative to TK, initial rate 2.1 $mM.min^{-1}$, are found to be limiting the process requiring further investigation.

Acknowledgements

To all those people who have assisted me in the course of my PhD, thank you. In particular I would like to thank my supervisor Professor Gary Lye and advisor Dr Paul Dalby. Additionally Dr John Ward and Dr John Woodley both of whom gave me extensive advice and support.

Thank you to Dr Mark Smith to whom I am extremely grateful for not only helping me with my chemistry quandaries but who brought with him enthusiasm and inspired motivation as well as lending a sympathetic ear in the closing months of my project. Additionally to Martin Bommer and Kirsty Smithies for lending their molecular biology and analytical skills respectively. Thank you to Sarah Horton with whom I travelled through this journey sharing experiences and frustrations throughout and to Brenda Parker and Ryan McCoy for their support.

A special thank you to my parents, Jan and Clarice, and my sister Pauline for their love, encouragement and patience and continuing to show interest in my work when I either gave endless intricate details of exciting results or refused to tell them anything.

Abbreviations

ABT	2-amino-1,3,4-butanetriol
ACN	Acetonitrile
AmpR	Ampicillin resistance
AQC	6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate
β-A: P TAm	β-alanine: pyruvate transaminase
ArTAm	Aromatic transaminase
AspTAm	Aspartic acid transaminase
A_{xnm}	Absorbance at x nm
DNA	Deoxynucleic acid
dNTP	Deoxynucleotidetriphosphate
DoxP synthase	1-deoxy-D-xylulose-5- phosphate synthase
EBA	Ethylbenzylamine
E.C.	Enzyme commission
E.coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
E-PLP	Transaminase –PLP complex
E-PMP	Transaminase-PMP complex
GA	Glycolaldehyde
β-HPA	β-hydroxypyruvate
HPLC	High performance liquid chromotography
IPTG	Isopropyl-β-D-thiogalactosidase
KanR	Kanamycin resistance
α-KG	α-ketoglutarate
K_m	Michaelis constant
MBA	Methylbenzylamine
NADH	Nicotinamide adenine dinucleotide
OD	Optical density
P.aeruginosa	*Pseudomonas aeruginosa*
PCR	Polymerase chain reaction
PHA	Polyhydroxlalkanoates
PLP	Pyridoxal phosphate

PMP	Pyridoxamine phosphate
P.putida	*Pseudomonas putida*
RBS	Ribosome binding site
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TAm	Transaminase
TFA	Trifluoroacetic acid
TK	Transketolase
TK-TAm	Co-expressed transketolase and transaminase pathway
TLC	Thin layer chromatography
TPP	Thiamine pyrophosphate
Tris	Tris(hydroxymethyl)aminomethane
UV	Ultraviolet
V_{max}	Maximal velocity
μ_{max}	Maximal specific growth rate
wpepPCR	Whole plasmid error prone PCR

Contents

List of Figures

List of Tables

Chapter 1 – Introduction and Project Aims

1.1 Development of chiral pharmaceuticals

With the cost of drug discovery estimated at 0.8 to 1.3 billion $US per new compound (Schmidt, E. and Blaser, H.-U., 2003) the need for a reduction in overall costs of development and production are obvious. Additionally the competition from the drug candidates of other companies requires the timely development of a drug to minimise the time to market. The technology harnessed, to both develop and manufacture a drug, therefore needs to be cost effective, rapid and sustainable. The emphasis of either time or cost depends on the phase of development of a candidate drug (Yazbeck, D.R. *et al,* 2004). Initially, during the discovery phase of a candidate compound, when small amounts of material, i.e. 10mg – 100g are required, it is the time taken to produce material for further investigation and characterisation of pharmacological properties that is vital. Later on, when process development is being carried out and production at the kilogram and potentially the ton-scale, is required, process cost and sustainability become the defining parameters. A cost effective process requires conversion at the highest possible yield with the minimum of side products utilising materials that are affordable and available from reliable sources. The cost of goods is impacted by the value of process reagents. This includes both the starting materials and reagents used in each processing step, thus the number of processing steps can significantly increase overall costs. Productivity includes the output for time spent in the factory, which defines how many processing cycles can be completed in a given time, as well as the overall process product yield. Overall product yield is also impacted on by the number of processing steps since yield losses will be incurred at each stage. The type of process developed is further restricted by the sustainability of supply of materials, the availability of suitable facilities for operating the process, and in an age where the environmental issues are an important factor, the production and disposal of waste streams must be accounted for. Additionally, intellectual property issues may prevent the use of a certain technologies in a commercial process.

The increase in demand for the production of optically pure compounds has been largely driven by the pharmaceutical industry with 80% of compounds in the development pipeline being single-isomer compounds (Breuer, M. *et al* 2004). It is

now required by the regulatory bodies that characterisation of the single optical isomers of a chiral drug compound is carried out before licensing a drug for the marketplace (FDA, 1992). This has been forced by the recognition that two enantiomers of the same compound can have dramatically different pharmacological activities. For example, Verapamil is a drug where the *(S)*-enantiomer is used to alleviate high blood pressure, whilst the *(R)*-enantiomer is used to inhibit the resistance of cancer cells to chemotherapy agents (Wainer, I.W., 1993). Secondly, the single isomers may have different selectivity, for example Ibuprofen where the *(S)*-enantiomer of the drug is more selectively effective than the *(R)*-enantiomer (Kelley, M.T. *et al.*, 1992). Therefore, a single enantiomer formulation would have higher potency and thus require lower dosages, subsequently reducing the potential side effects of the drug (Stinson, S.C., 1994). Similarly, in the agro-chemical industry, single enantiomer compounds may reduce application volumes thus reducing cost and lessening the environmental impact.

The synthesis of optically pure products is often multi-step, employing complex chemistry and producing low product yields. This increase in complexity and subsequent cost of the production of homochiral compounds, versus racemates, has led to the development of several 'toolboxes' for asymmetric synthesis (Yazbeck, D.R. *et al,* 2004). These approaches include asymmetric chemocatalysis, use of a precursor from a chiral pool, crystallisation of diastereomeric salts, biocatalysis, and chromatographic separation. These will be assessed during development of a process to find the most efficient process. The use of asymmetric chemocatalysis is an attractive option, particularly for hydrogenation reactions, although the scope of this technique may be limited by the commercial availability of catalyst, patent restrictions and the rarity of broad spectrum catalysts. Resolutions of racemic mixtures may be carried out by direct crystallisation, diastereomeric crystallisation, chromatography or enzyme kinetic resolution. However these all result in at least a 50% yield loss so that, integration, especially later on in the processing scheme, is not ideal. Higher yields may be possible by the introduction of a re-racemisation step, of the unwanted isomer, into the process. For example, in the production of D-amino acids from racemic hydantoins using D-hydantoinase (Park, J.-H., Kim, G.-J., and Kim, H.-S., 2002). Crystallisation faces limitations due to the lack of development of efficient high throughput screening methods. However, the

generation of homochiral salts does result in ease of downstream processing. Chromatography faces high costs due to large waste streams and high cost of resins and scalability. Another approach to the synthesis of complex chiral compounds is the use of chiral synthons at the beginning of the synthesis. These are relatively simple enantiomerically pure starting compounds which provide the desired chirality in the reactions that follow. The selection of a chiral precursor from an existing chiral pool of compounds may be limited by the type of chemicals available, with the majority of commercial chiral molecules (being both cost effective as a precursor molecule and available at suitable quantities) being amino acids and simple carbohydrates.

Nature itself has demonstrated biological catalysts to have a role in the production of chiral molecules. For example, simple carbohydrates can serve as chiral starting materials for the synthesis of more complex molecules (Draths, K.M., *et al.* 1992). This is carried out by plants and microbes where the simple carbohydrate *D*-glucose is used as a starting material for the synthesis of amino acids and related metabolites. Biocatalysts may perform a synthetic or resolution role depending upon the particular reaction in question. The mild conditions under which most biocatalytic processes are operated reduce both running costs and the generation of toxic waste stream. Processes may also be applied to the synthesis of compounds other than that for which it is originally developed. This is due to the ability of many enzymes to accept a broad range of substrates. Biocatalysis faces limitations due to laborious screening, sensitivity to substrate and product concentrations, the need for specialist factory facilities and the problems of recovering products from aqueous process streams (Yazbeck, D.R. *et al,* 2004) . However, the intrinsic regio, chemo and stereo selectivity of biocatalysts along with advancements in both protein engineering and biochemical process engineering make biocatalysis a toolbox which now demands consideration for inclusion in a synthetic process (Rozzell, J.D., 1999).

An example of the assessment of different toolboxes has been described for the synthesis of a key chiral building block for the production of Ruprintrivir^{TM}, (Figure 1.1) a remedy for the common cold (Tao, J., McGee, K., 2002; Yazbeck, D.R. *et al* , 2004). An initial process used a chiral non-natural amino acid **a** as a starting material in the discovery and development phase.

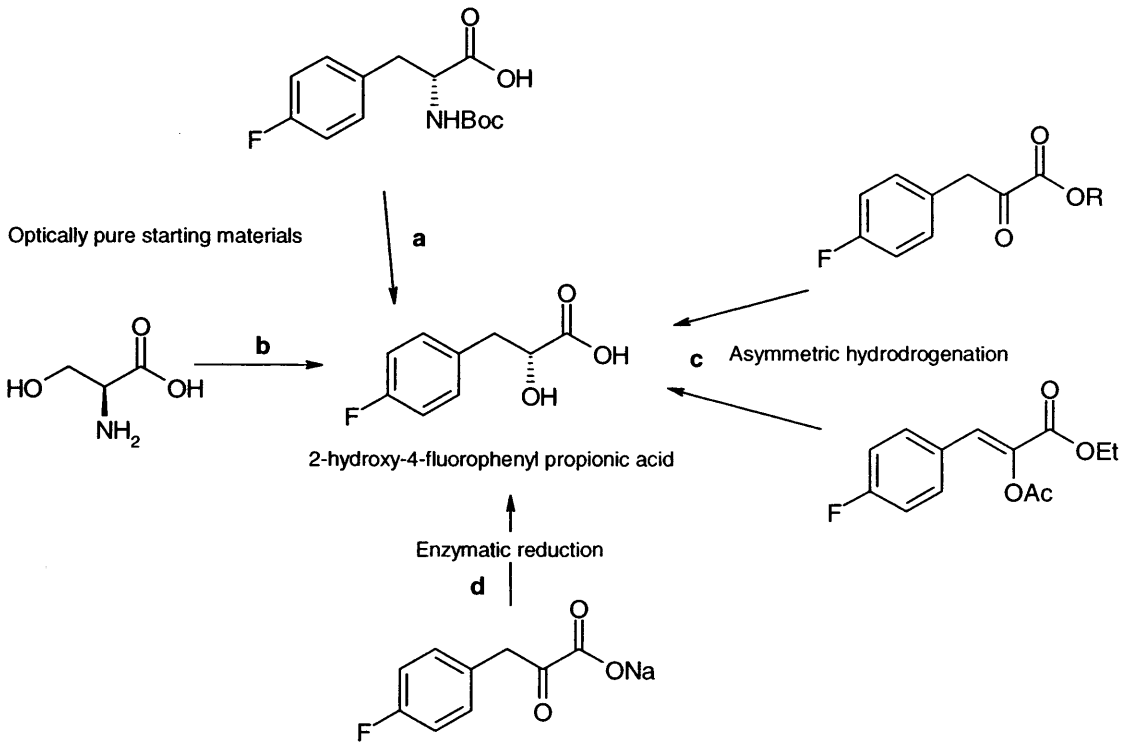


Figure 1.1: Synthetic strategies for the production of Ruprintrivir™ chiral building block 2-hydroxy-4-fluorophenyl propionic acid.

(Adapted from (Yazbeck, D.R. *et al.*, 2004)

However, yields were below 50% and the starting material was very expensive therefore a better process was required for production scale. The use of an *L*-serine chiral synthon **b** was also investigated but the synthesis was long and resulted in yields below 42%. An asymmetric chemocatalysis approach for the hydrogenation of achiral ketone substrates **c** was assessed but low enantioselectivity or poor yield (for high enantioselective process) was prohibitive. Eventually a process employing lactate dehydrogenase to reduce an achiral ketoacid salt **d** to the 2-hydroxy-4-fluorophenyl propionic acid intermediate was developed. This process was useful not only in this instance but has broad selectivity giving the potential for the conversion of a range of ketoacid substrates to chiral hydroxy acids (Kim, M.-J. and Whitesides, G.M., 1998; Schmidt, E. *et al.*, 1992; Simon, E. S., Plante, R. and Whitesides, G. M., 1989).

1.2 Industrial Biocatalysis

Biocatalysis has huge potential and despite much recent research, remains a relatively unexplored technology, with many organisms yet to be discovered and within those organisms many enzymes yet to be investigated (Cowan, D. *et al.*, 2005). Even with the already known organisms many proteins have unknown or only putative functions and even many of the identified enzymes have substrate ranges that are uninvestigated. Nevertheless the repertoire of biocatalysis tools is increasing steadily and it is becoming a toolbox that is considered as a viable option for many industrial chemical syntheses. This is reflected in a poll where it was reported 22 out of 38 large scale asymmetric syntheses involve enzyme, or whole cell bioconversions (Schmidt, E. and Blaser, H.-U., 2003). Processes including biocatalytic steps are extensively reviewed (Breuer, M. *et al*, 2004; Panke, S., Held, M. and Wubbolts, M., 2004; Patel, R.N., 2001; Schmid, A. *et al.*, 2001; Schoemaker, H.E., Mink, D. and Wubbolts, M.G., 2003; Schultze, B.M.W., Wubbolts, M.G., 1999; Straathof, A., Panke, S. and Schmid, A., 2002). Some examples of the successful application of biocatalysis in industry are the use of acylases in the production of β-lactam antibiotics (De Vroom, E., 1999), the production of acrylamide from acrylonitrile employing nitrile hyratase (Nagasawa, T and Yamada, H. 1990) and the industrial scale synthesis of chiral cyanohydrin *(S)-meta*-phenoxybenaldehyde, an intermediate in the synthesis of pyrethroid insecticide using recombinant *(S)*-hydroxynitrilase lyase (Griengl, H. *et al*, 1998).

Alongside the increasing number of large-scale processes is the development of process technology to overcome some of the limitations of processes involving biological systems thus enhancing the viability of a biocatalytic step. This technology includes the use of continuous reaction and separation bioreactors (DeRoode, B.M. *et al*, 2001) and '*in situ*', product removal techniques (Hilker, I. *et al.*, 2004; Lye, G.J.and Woodley, J.M., 1999), for continuous product removal and the use of packed bed reactors (Jeong, S. *et al*, 2000) which can improve volumetric productivity as well as alleviate product inhibition.

1.3 Multi-step Biocatalysis and Pathway Engineering

The literal meaning of 'biocatalysis' is the catalysis of a reaction using a biological entity. This includes fermentation methods where the metabolism of a living organism is used to produce desired compounds from simple starting materials. Biocatalysis also encompasses the use of enzymes discrete from the metabolism of the host strain and the use of isolated enzymes.

Advances in cloning and over-expression techniques have contributed to the construction of industrially useful recombinant biocatalysts. Cloning techniques enable the introduction of genes into a host organism where they may be more easily utilised than in the native host. Additionally, extension of existing pathways to reach a commercially desirable product may be possible by the addition of non-native enzymes. Over-expression of cloned genes can enable the production of large amounts of protein catalyst. This gives access to a huge number of gene products from organisms that are extremely challenging to cultivate on a scale that enables the generation of useful amounts of protein. The use of genetic manipulation to create a suitable recombinant biological catalyst to carry out desired reactions can be difficult and is a time consuming task. However, once completed a catalytic entity to carry out the conversion is gained. The recombinant strain itself may be used as the catalyst or the over-expressed enzyme more easily isolated for use in a purified enzyme process. Whilst this has been extensively investigated in the form of pathway engineering (Chotani, G. *et al*, 2000) the construction of non-natural pathways discrete from the host organisms metabolism is less well addressed.

Pathway engineering is extensively used to exploit the native synthetic functions within the organism during fermentation to produce a higher titre of useful end product. An increase in product yield may be achieved by modifying metabolism to divert carbon towards the desired product. An example of this is the modification of the central metabolism in *E.coli* to divert carbon towards aromatic biosynthesis (alongside interruption of primary aromatic synthetic pathway regulation) to produce a strain overproducing tryptophan (Ikeda, M. and Katsumata, R., 1999). Similarly, the process developed for the manufacture of cystine (Maier, T. and Winterhalter, C, 2000) utilises a metabolically engineered strain of *E.coli* which produces elevated levels of the amino acid. The excess cysteine, which is not utilised in metabolism, leaves the cells and is oxidised to cystine in the fermentation media. The addition of further enzymes to utilise naturally occurring compounds from an existing pathway, to form useful products, is also extensively exploited. There are many possible examples but one is the cloning of a dioxygenase from *Pseudomonas* into *E.coli* which enabled the production of indigo from indole, an intermediate of tryptophan biosynthesis (Ensley, B.D. *et al*, 1983).

Another example of pathway engineering is the production of polyhydroxlalkanoates (PHA), reviewed by Steinbuchel (Steinbuchel, A. and Lutke-Eversloh, T., 2003). PHAs are biopolymers synthesised by prokaryotic microorganisms (e.g. *Ralstoni eutropha, Pseudomonas aeruginosa*, and *Bacillus megaterium*) with industrially interesting properties. An example of an application is in the manufacture of biodegradable plastics. These polyoxoesters accumulate in the cytoplasm as inclusion bodies for the storage of energy and carbon. A range of PHAs can be produced by the use of different precursor substrates as the carbon source during cultivation of the microorganism. Metabolic engineering of various strains has been carried out to enable synthesis of various PHAs from metabolic pathways using different carbon sources.

The advancements in protein engineering and mutagenesis techniques have led to the possibility of the creation of a 'designer' biocatalyst that can carry out multi-step biocatalysis. Such an engineered biocatalyst will produce large quantities of the multiple enzymes involved in the desired synthesis. In this instance multi-step biocatalysis will refer to the use of multiple synthetic enzymatic reactions, not

naturally carried out in series, independent from the metabolism of the host organism for the synthesis of a desired product within a single operation. The harnessing of multiple enzyme reactions has produced multiple examples of efficient biocatalytic pathways.

One example is the construction of a novel biochemical pathway for the production of *L*-2-aminobutyric acid (Fotheringham, G. *et al.*, 1999; Fotheringham, G.I., 2001). The over-expression, of not only the key enzyme, *E.coli* aromatic transaminase, but of two further enzymes, to facilitate the reaction, was shown to significantly improve the yield and purity of the product (Figure 1.2). The auxiliary enzymes were *E.coli* K12 threonine deaminase, catalysing the *in situ* formation of transaminase substrate, 2-ketobutyrate, from commercially available substrate *L*-threonine, and *Bacillus subtilis* acetolactate synthase, which eliminates the pyruvate by-product which enables conversion of over the 50% equilibrium limitation that the transaminase experiences.

The production of amino acids has been the subject of several coupled enzyme approaches. Using dehydrogenases (deaminating amino acid oxidoreductases) for amino acid production has been hampered by the high costs of the nicotinamide cofactors. This has been overcome by the simultaneous over-expression of heterologous genes to combine the synthetic enzyme and a cofactor recycling system employing formate dehydrogenase into the process (Galkin, A. *et al.*, 1997). Subsequently, multi-enzyme schemes for the synthesis of both natural and non–natural amino acids using dehydrogenases to carry out the reductive amination of keto acids to amino acids have been developed (Hanson, R.L. *et al.*, 1999). Examples of this approach are two alternative syntheses for the antihypertensive Omapatrilat developed by Bristol Myers Squibb (Patel, R.N., 2001). The production of *L*-hydroxynorleucine, a useful chiral synthon used in Omapatrilat synthesis (Hanson, R.L. *et al*, 1999) was carried out using beef liver glutamate dehydrogenase (isolated enzyme).

This was coupled with a second dehydrogenase to carry out NADH cofactor regeneration. Initially the generation of the ketoacid substrate was via a multi-step chemical synthesis. This was overcome by the generation of the ketoacid substrate

from the *D*-enantiomer of the more easily preparable racemic 6-hydroxynorleucine (by hydrolysis of the corresponding hydantoin) using *D*-amino acid oxidase (and catalase to remove H_2O_2 by product). Near complete conversion of the racemate to the desirable *L*-isomer was achieved using this isolated enzyme scheme (Figure 1.3). Similarly, an alternative approach to the drug synthesis (Figure 1.4) utilised the production of amino acid acetal building block from keto-acid acetal by phenylalanine dehydrogenase along with the second dehydrogenase for cofactor recycling (Hanson, R.L. *et al*, 2000). Another multi-enzyme scheme for the production of *L*-amino acids involves the over-expression of enzymes *L*-hydantoinase, *L-N*-carbamoylase and hydantoin racemase from *Arthrobacter aurescens* in *E.coli.* The resulting biocatalyst is capable of the fast and complete conversion of racemic hydantoins to the corresponding *L*-amino acids (Wilms, B. *et al.*, 2001).

A final example is, again, the synthesis of polyhydroalkanoic acids (polyesters). Whilst much work has been done to metabolically engineer strains to produce these useful materials via metabolic pathways from simple carbon sources, reviewed in Steinbuchel, A. and Lutke-Eversloh, T., 2003, a novel bacterial biocatalyst to process unnatural substrates independent of metabolism has also been engineered (Liu, S.-J. and Steinbuchel, A., 2000). This involved the construction of a novel pathway over-expressing two *Clostridium acetobutylicum* genes, butyrate kinase and phosphotransbutyrylase alongside the polyhydroalkanoic acid synthase from *Thiocapsa pfenniggi* (or *Allochromatium Vinosum)* in *E.coli.* The non-native pathway had low substrate specificity with the ability to accept un-natural substrates thus creating a versatile catalyst. The pathway has been demonstrated to synthesise various homo and co-polyesters as well as polythioesters (Steinbuchel, A. and Lutke-Eversloh, T., 2003). A recombinant *E.coli* strain with a similar pathway has been constructed for the production of *(R)*-3-hydroxybutyrate (Gao, H.-J., Qiong, W.U. and Chen, G.-Q., 2002). This involves the two *Clostridium acetobutylicum* genes in the previous pathway, functioning in the reverse, along with β-ketothiolase and acetoacetyl-CoA reductase from *Ralstonia eutrophia.*

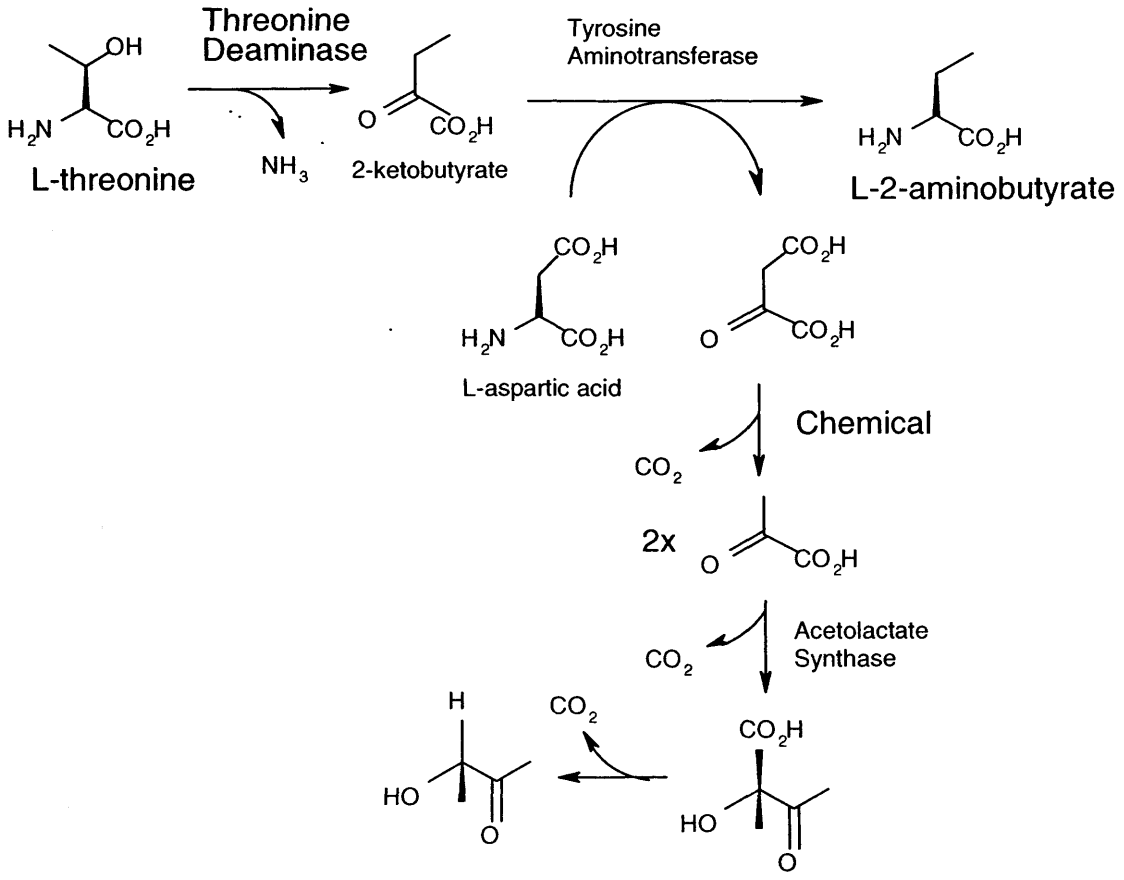


Figure 1.2: The use of an engineered multi-enzyme pathway for the synthesis of *L*-aminobutyric acid

(Reproduced from Fotheringham, G. et al., 1999)

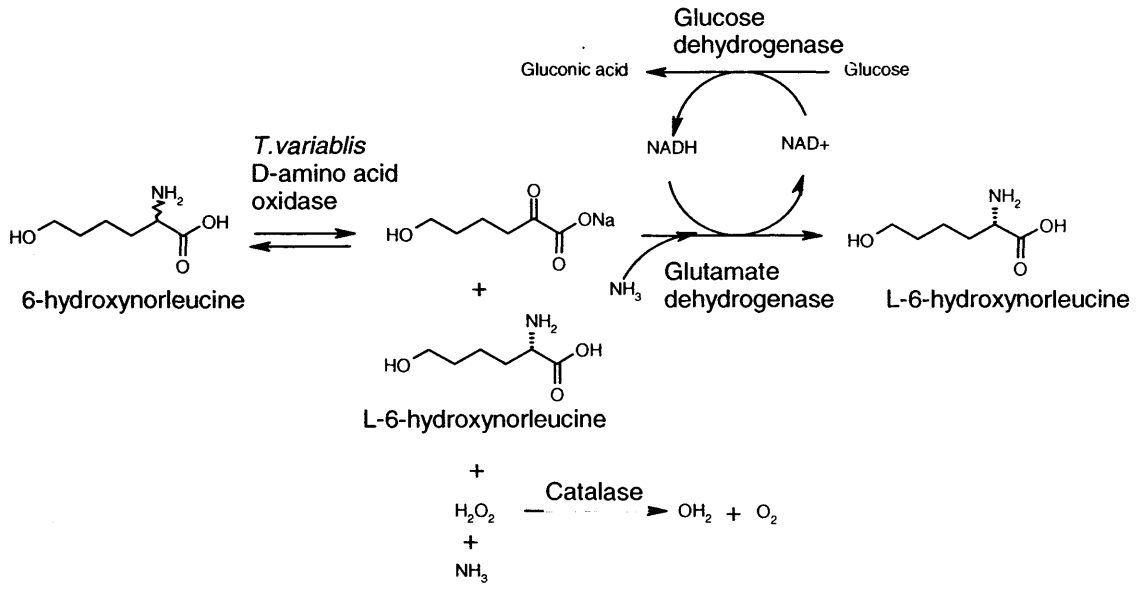


Figure 1.3: Enzymatic scheme for the conversion of racemic hydroxynorleucine to *L*-hydroxynorleucine for the synthesis of Omapatrilat.

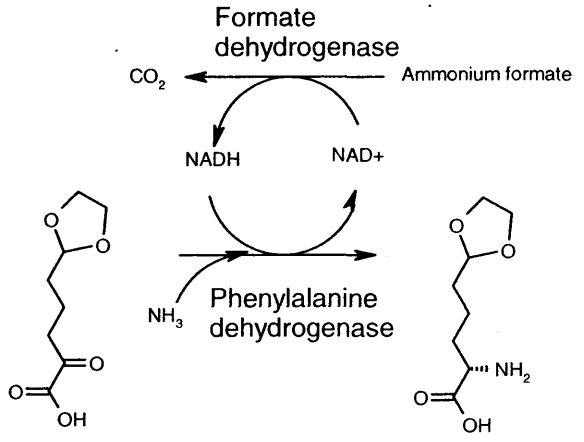


Figure 1.4: Enzymatic preparation of acetal intermediate for the synthesis of Omapatrilat.

1.4 Chiral amines

Whilst amino acids are in huge demand, predominantly by the nutrition industry, an increase in demand for optically pure non-proteinogenic amino acids and amines is coming from the pharmaceutical sector. The large scale synthesis of chiral amine products has been increasingly investigated due to the demand for single enantiomer peptidomimetic drugs. The development of rational target structure based drug design has led to an increased number of these drugs in development.

Optically pure non-proteinogenic amino acids and amines serve as precursors and building blocks for these complex molecules. Some examples of chiral amines in commercial molecules include *L-tert*-leucine (Kempf, D.J. *et al.*, 1991) in an HIV protease inhibitor used in AIDS therapy (Figure 1.5 (A)), *D*-phenylalanine (Bajusz, S., *et al.*, 1990) in a structure which targets thrombin to provide treatment for thrombosis (Figure 1.5 (B)) and *L*-6-hydroxynorleucine (Patel, R.N., 2001) an intermediate for antihypertensive Omapatrilat (Figure 1.5 (C)). The non-proteinogenic amino acids can not be manufactured by the fermentation strategies used in the production of *L*-amino acids. Thus significant effort has been focused on identifying strategies for the synthesis of both optically pure un-natural amino acids, amines and hydroxy amines (Breuer, M., *et al.*, 2004).

1.4.1 Chemical synthesis routes to chiral amines

Chemical routes to chiral amines are both via resolutions of racemic amines and synthetic strategies. Amines can be synthesised in the racemic form then a resolution carried out (therefore 50% max yield) to obtain the optically pure amine. A resolution technique that could be employed for chiral amines is a diastereomeric crystallisation with chiral carboxylic acids (Jacques, J., Collett, A. and Wilen, S., 1980). This is the formation of a diastereomeric ammonium salt followed by fractional crystallisation separating out the enantiomers. The scope of this is limited by the chemical characteristics of the amine and availability of a carboxylic acid resolving agent of the required chirality. A variation on the classical resolution approach is the 'Dutch resolution' (Vries, T. *et al.*, 1998). This method combines a mixture of optically active acids to precipitate the salt. It is a more versatile approach than the use of a single carboxylic acid.

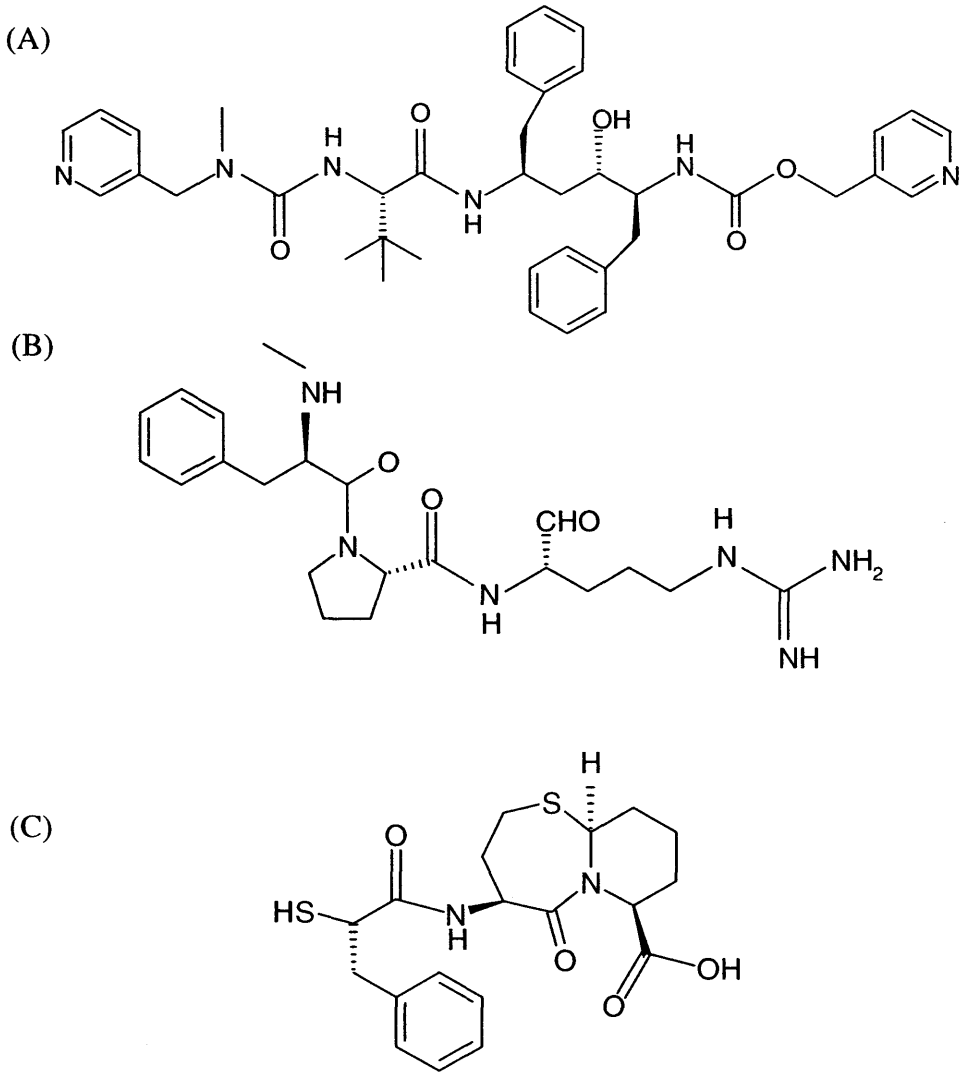


Figure 1.5: Examples of peptidomimetic drugs

(A) Peptidomimetic HIV protease inhibitor containing *L-tert*-leucine

(B) Compound containing *D*-phenylalanine used in thrombosis therapy

(C) Omapatrilat an antihypertensive synthesised from the chiral amine intermediate *L*-6-hydroxynorleucine

However recovery of the chiral acids for reuse is difficult as they become a complex mixture in the mother liquor of the precipitated salt. The 50% sacrifice in the yield by the use of a resolution can be lessened by re-racemisation of the unwanted enantiomer and further resolution carried out.

More favourable are synthetic routes enabling yields above the 50% limit of a resolution of a racemate. Synthetic methods include the chemical reduction of amino acids to form the corresponding amino alcohols as has been developed by chemists at Degussa. Starting with a chiral amino acid reduction of the carbonyl of the acid group is carried out with reducing agent $NaBH_4$ in the presence of an activator (either I_2 or H_2SO_4) (Bommerius, A. *et al*, 1996; Meyers, A.I. *et al*, 1994). Alongside numerous methodologies for the production of both unnatural and natural amino acids this methodology has the scope to access a broad range of amino alcohols. The asymmetric production of amines can also be carried out by the hydrogenation of C=N bonds (Johansson, A., 1995).

1.4.2 Enzymatic approaches to chiral amines

Enantiopure amino acids can be accessed via several enzymatic syntheses and resolutions. Resolution of racemates includes the resolution of racemic hydantoins by hydantoinase with carbomylase (May, O., Nguyen, P.T. and Arnold, F.H., 2000; Park, J.-H., Kim, G.-J. and Kim, H.-S., 2000; Park, J.H. *et al.*, 2002; Wilms, B. *et al.*, 2001), resolution of D,*L*-acyl amino acids using acylases (Machado, G.D.C. *et al.*, 2005), resolution of racemic amino acid amides with amidases (Eichhorn, E. *et al.*, 1997; Wang, M.-X. *et al.*, 2005) and the production of chiral amines via lipase catalysed resolution of racemic amines (Goswami, A. *et al.*, 2005). The resolution approach to a product not only requires a racemic amine substrate but, as stated previously, is limited to a 50% maximum yield. Systems to overcome this yield limitation have been developed for example in the synthesis of *L*-hydroxynorleucine (Patel, R.N., 2001), previously discussed in Section 1.3, where the unwanted isomer is converted into a ketone by an oxidase, followed by stereospecific reductive amination to the desired chiral amine. This is not always a feasible approach requiring two enzymes with the correct substrate range to synthesise the amine. Thus it is the synthetic approach from achiral ketones that attention has been focused on.

Common synthesis reactions include the use of lyases (van der Werf, M.J. *et al*, 1995), dehydrogenases and transaminases (Stewart, J.D., 2001). The number of papers and US patents filed, by biotechnology companies such as Diversa Corporation, (Warren, P.V. and Swanson, R.V., 1999), NSC Technologies (Fotheringham, G.I., 2001) and Celgene corporation (Stirling, D.I., 1992; Stirling, D.I. *et al.*, 1992) involving transaminase reactions perhaps illustrates their potential as commercial synthetic tools. Transaminases can be utilised in either a resolution or synthetic capacity and require no external cofactor recycling as the pyridoxal cofactor is recycled during the reaction cycle. The resolution of a racemic amine is achieved by the transfer of the amine group from the unwanted enantiomer to a ketone substrate leaving the desired isomer intact. An asymmetric synthesis reaction catalysed by a transaminase is carried out by transamination of the corresponding ketone precursor. Additionally ω-transaminases enable synthesis of various non acidic amines (Stirling, D.I., 1992) .

1.5 Asymmetric carbon-carbon bond formation

If asymmetric amine synthesis can be carried out using a chiral substrate the complexity of the product is increased. The synthesis of a ketone substrate containing a chiral centre from achiral starting materials requires asymmetric carbon-carbon bond formation. As with the production of optically pure amines asymmetric carbon-carbon bond formation presents significant challenges to synthetic chemistry. The production of side products and the requirement for protection (and subsequently de-protection) of other functional groups on the reactants make chemical routes cumbersome (Schoemaker, H.E. Mink, D. and Wubbolts, M.G., 2003)

1.5.1 Enzymatic synthesis of C-C bonds

Several enzymes are capable of the synthesis of ketones containing asymmetric carbon-carbon bonds. These include pyruvate decarboxylase (Shin, H.S.and Rogers, P.L., 1996), Dox P synthase (Kuzuyama, T. *et al.*, 2000), Fructose-1,6-diphospahte aldolase (Fessner, W.-D. and Helaine, V., 2001) and transketolase (TK) (Turner, N.J., 2000). TK (E.C.2.2.1.2) catalyses the formation of asymmetric keto diols via the transfer of a two carbon ketol group between several acceptor and donor substrates in a reaction dependant upon the presence of thiamine pyrophosphate and a divalent metal ion cofactor. The formation of the new carbon-carbon bond is

highly stereospecific and the reaction can be effectively irreversible if a β-hydroxypyruvate ketol donor substrate is employed yielding CO_2 as a product (Hobbs, G.R. *et al.*, 1993). A wide range, of both phosphorylated and un phosphorylated compounds, have been found to be accepted as substrates for transketolases (Andre, C. *et al*, 1998; Draths, K.M. *et al.*, 1992; Effenberger, F. Null, V. and Zeigler, T., 1992; Hobbs, G.R. *et al.*, 1993; Humphrey, A.J. *et al.*, 2000; Morris, K.G. *et al.*, 1996; Sprenger, G.A. *et al.*, 1995). This has led to the enzyme being extensively investigated for its synthetic potential. The development of a stable strain over expressing TK (French, C. and Ward, J.M., 1995) and a large scale production process (Hobbs, G.R. *et al*, 1996) has enabled the enzyme to be produced in significant quantities for use as a biocatalyst.

1.6 Enzymatic synthesis of amino diols

The production of chiral amino diols via biocatalysis has the potential to yield amino alcohols with multiple chiral centres from achiral starting materials. This can be achieved if transketolase catalysing chiral ketone formation (Section 1.5.1), from either achiral or chiral substrates, is coupled with the biocatalytic addition of an amine to the TK product using a transaminase (Section 1.4.2). Using transketolase a variety of reaction products can be accessed. In order to couple the two enzymes together assessment of possible TK reactions and possible transaminase candidates to react with the product of the TK catalysis is required. These are considered in Section 1.8 and Section 1.10 respectively.

1.7 Properties of Transketolase

Bacteria are dependent on transketolase for the catabolism of pentose sugars. These provide *D*-ribose 5-phosphate, the precursor for nucleotide and histidine biosynthesis and erythrose-4-phosphate required for the biosynthesis of aromatics. *E.coli* mutants deficient in TK are auxotrophic for pyridoxine and a combination of aromatic acids and are unable to grow on pentoses, *D*-arabinose, *D*-ribose and *D*-xylose, alone (Zhao, G. and Winkler, M.E., 1994). TK has been identified as a candidate enzyme for manipulation to enable improved percent conversion of *D*-glucose into aromatic biosynthesis in *E.coli.* Increasing TK activity has been found to increase the level of *D*-erythrose-4-phosphate (Draths, K.M. *et al.*, 1992) which is a substrate for 3-deoxy-*D*-arabino-heptulosonic acid 7-phosphate (DHAP) synthase, the first enzyme

unique to the pathway for aromatic amino acid synthesis. The increase was proposed as beneficial for the production of fermentation products such as aromatic amino acids as well as other commercially useful products. This was demonstrated by the synthesis of higher concentrations of the commercially valuable fine chemical 3-dehydroxyshikimic acid from glucose with an *E.coli* strain expressing elevated TK and DHAP synthase (Li, K. and Frost, J.W., 1999).

The activity of bacterial transketolase was first discovered in *E.coli* in 1948 (Racker, E., 1948) when cleavage of ribose-5-phosphate by *E.coli* crude extract was first observed. In 1952 transketolase was identified as the enzyme catalysing this process (de la Haba, G. and Racker, E., 1952). Transketolase is involved in metabolic regulation playing a key role in the non-oxidative pentose phosphate cycle. It catalyses the reversible transfer of a two carbon ketol group between several acceptor and donor substrates in a reaction dependant upon the presence of thiamine pyrophosphate and a divalent metal ion cofactor (Figure 1.6). The catalytic mechanism, proposed by Schneider and Lindqvist (Schneider, G. and Lindqvist, Y., 1993), involves the formation of an addition complex with the carbonyl of the ketose substrate and the ionised TPP cofactor. Substrate cleavage occurs yielding activated glycolaldehyde and an aldose is liberated. Nucleophilic attack upon the aldehyde acceptor substrate then yields the new ketose product. *In vivo,* TK provides the link between the glycolytic and pentose phosphate pathways catalysing the reversible transfer of a two carbon ketol group between ribose-5-phosphate and *D*-erythrose and donor phosphorylated sugar substrates xylulose-5-phosphate, fructose 6-phosphate and sedoheptulose-7-phosphate (Datta, A.G. and Racker, E., 1961; Draths, K.M. *et al.*, 1992).

The transketolase enzyme exhibits a high degree of stereoselectivity recognising the chirality of a hydroxyaldehyde substrate with a preference for the *(R)* isomer. This has been demonstrated with *E.coli* TK by a comparison of catalysis with *D* and *L*-glyceraldehyde. In reactions with β-HPA the reaction with *D*-glyceraldehyde was rapid whilst reactivity with *L*-glyceraldehyde was negligible in the reaction time monitored (Morris, K.G. *et al.*, 1996).

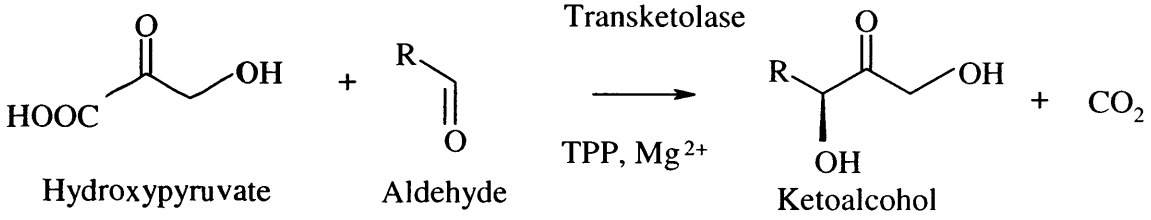


Figure 1.6: The transketolase catalysed reaction scheme

Transketolase catalyses the transfer of a 2-carbon ketol group from β-HPA to an aldehyde acceptor. This catalysis requires a divalent cation Mg^{2+} and TPP cofactor. The liberation of CO_2 from the reaction renders the reaction irreversible.

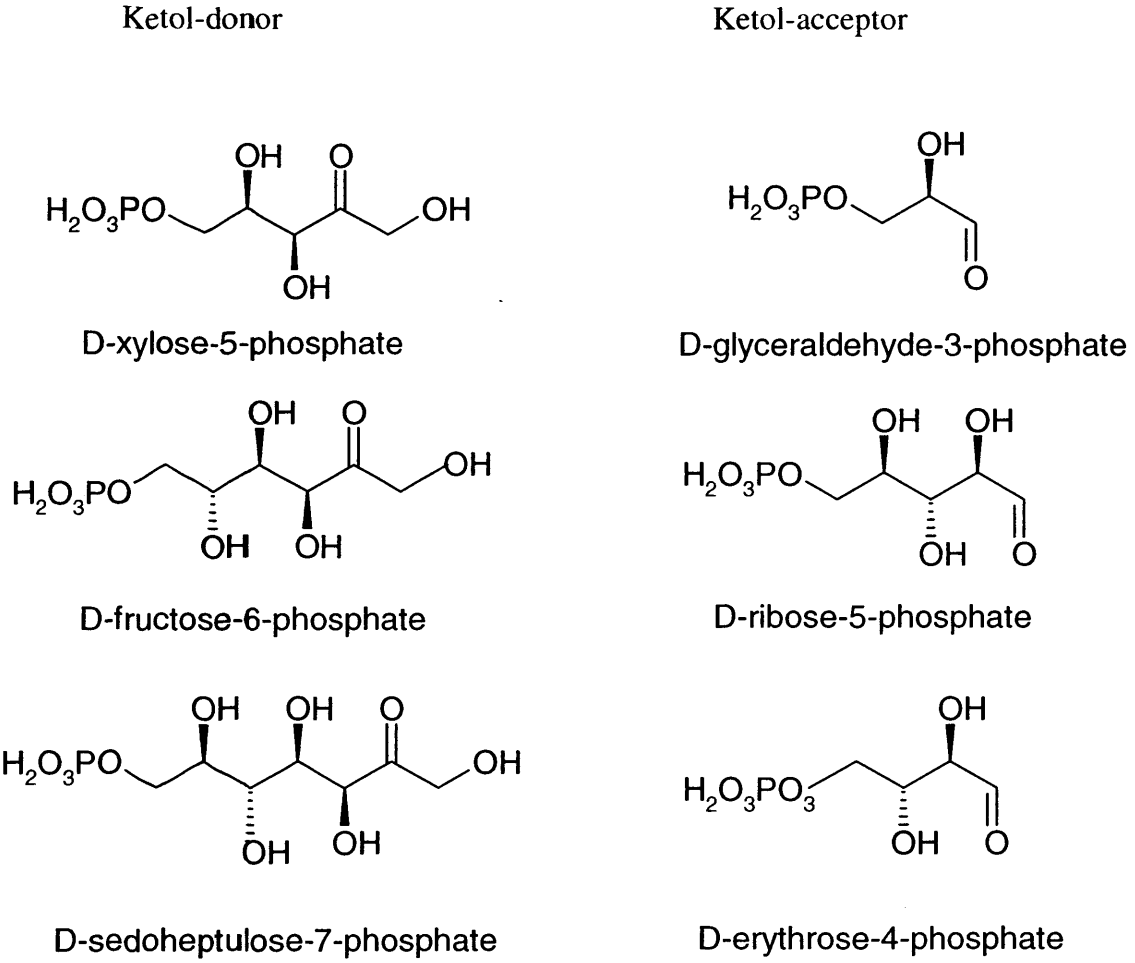


Figure 1.7: The natural substrates for bacterial transketolase

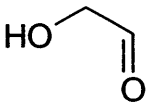
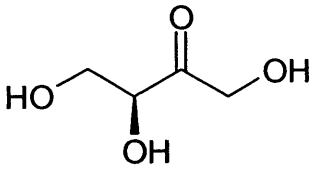
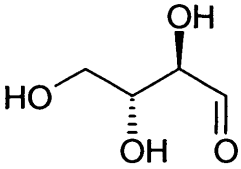
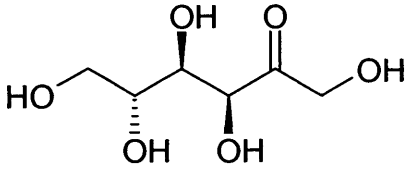
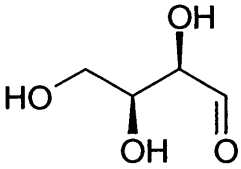
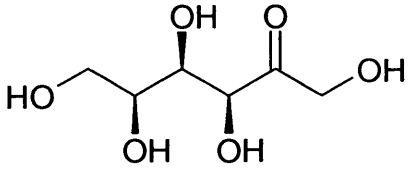
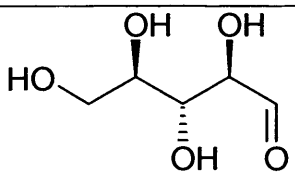
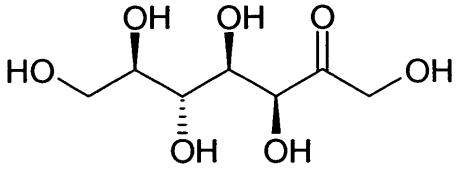
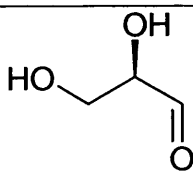
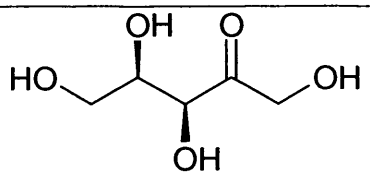
The product of a TK synthesised reaction contains an *(S)* stereo centre yielding products with *D*-threo (3*S*,4*R*) stereochemistry. The ability of TK to accept a range of aldehyde substrates gives the potential to access a large number of chiral products. Aldehyde specificity has been found to vary between transketolases from different organisms (Sprenger, G.A. *et al.*, 1995). The TK enzyme from *E.coli* has been found to catalyse a range of reactions with both phosphorylated (Figure 1.7) and non-phosphorylated substrates (Table1.1). Due to this and the availability of a strain already established for production of the enzyme it was chosen as the transketolase for this work.

1.8 Selection of target reaction

It is sensible to select a target reaction for the linked enzymes for which one reaction is already well established. Since TK has been extensively studied 'in house' (Brocklebank, S. *et al.*, 1996; Hobbs, G.R. *et al.*, 1996, 1996; Mitra, R.K., 1997; Mitra, R.K. and Woodley, J.M., 1996; Mitra, R.K., Woodley, J.M. and Lilly, M.D., 1998; Morris, K.G. *et al.*, 1996) it is logical to pick an already established TK reaction and investigate the transaminases to accept the product. The chosen reaction should yield products resembling known TAm products as far as possible. The selection of the transketolase catalysed reaction for the pathway target reaction requires the consideration of several factors. These factors are the availability of substrates, the percentage conversion achievable, the rate of reaction and the ability to monitor the reaction components including the availability of product standards. The published data on substrate specificity is presented as either relative velocity of reaction or the percentage conversion achieved depending on the individual study, comparing data is, therefore, a little difficult. The reactions that are most successfully demonstrated in the literature are illustrated in Table 1.1. Additionally the availability of transketolases that can accept heteroaromatic substrates further widens the potential choice of reaction (Bacon, S.L., 2001). However these enzymes require further characterisation so were not considered as candidates for the development of the initial demonstration of the TK-TAm pathway.

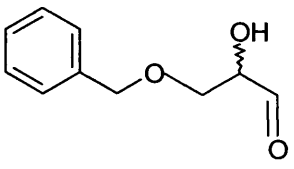
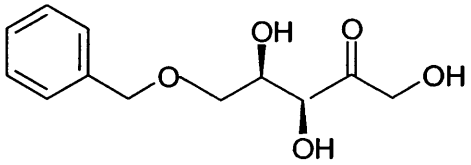
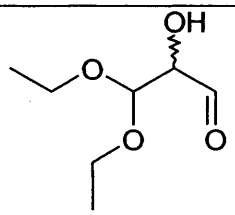
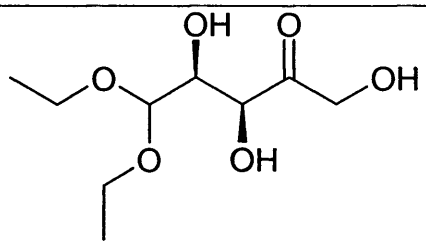
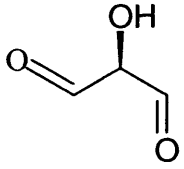
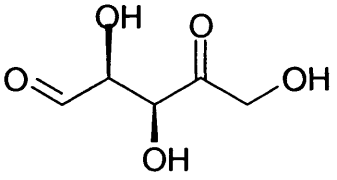
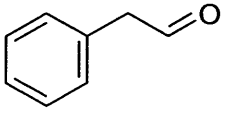
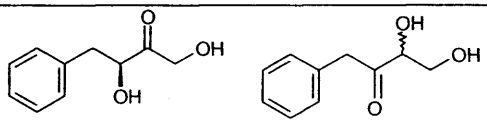
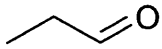
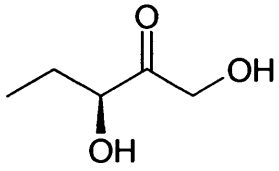
Table 1.1: Transketolase catalysed reactions

The most successful TK aldehyde substrates and products from reaction with β-HPA found in the published literature are shown. Activity is measured as percentage conversion or V_{rel} where $V_{rel} = 100$ for glycolaldehyde.

Aldehyde substrate	Product from reaction with HPA	V_{rel} or % yield	Reference
 Glycolaldehyde	 *L*-erythrulose	% yield = 74 V_{rel} = 100	(Morris, K.G. *et al.*, 1996)[a] (Hobbs, G.R. *et al.*, 1993)[b]
 *D*-erythrose	 *D*-fructose	V_{rel}= 89	(Hobbs, G.R. *et al.*, 1993)
 *L*-threose		V_{rel} = 56	(Hobbs, G.R. *et al.*, 1993)
 *D*-ribose	 Sedoheptulose	V_{max} = 25 relative to V_{max} = 60 for GA	(Sprenger, G.A. *et al.*, 1995)[c]
 glyceraldehyde	 xylulose	V_{rel} = 37 (using racemic substrate)	(Hobbs, G.R. *et al.*, 1993)

Continued

Continued from previous page

Aldehyde substrate	Product from reaction with HPA	V_{rel} or % yield	Reference
 3-O-benzylglyceraldehyde	 5-O-benzyl-*D*-xylulose	% yield = 80%	(Humphrey, A.J., *et al.*, 2000)[d]
		% yield = 56%	(Humphrey, A.J., *et al.*, 2000)
		% yield = 23%	(Morris, K.G. *et al.*, 1996)
		% yield = 33:18	(Morris, K.G. *et al.*, 1996)
 Propionaldehyde		V_{rel} = 20 % yield = 29%	(Hobbs, G.R. *et al.* 1996) (Morris, K.G. *et al.*, 1996)

a = reactions run at 6.9 mM β-HPA 13.8 mM aldehyde, with TK 600U, autotitrated to pH7.0 at room temperature, % yield calculated with respect to β-HPA concentration. **b**= reactions run at 7.5 Mm HPA 100 mM aldehyde, with TK 50U at pH 7.6, in glygly buffer at 37°C. V_{rel} was determined by analysis of HPA and is presented relative to the rate of the reaction with glycolaldehyde = 100. **c** = V_{max} determined using enzyme linked assays see reference for details, **d** = conditions as in **a** except racemic aldehyde used with yield calculated with respect to the reactive enentiomer concentration.

The TK reaction with glycolaldehyde is both a swift reaction, with the V_{rel} being used as 100 to compare other substrates to, and giving high yields of 74% under the conditions tested. Other promising reactions considered are with 3-*O*-benzylglyceraldehyde (80% conversion) and *D*-erythrose. Both of these reactions yield multiple stereocentres. This may present significant challenge to a TAm in the multi-enzyme pathway requiring accommodation of multiple branching from the main carbon chain (Section 1.7). Additionally the accommodation of the ester and benzyl group of 3-*O*-benzylglyceraldehyde may be troublesome. Whilst the product from the erythrose reaction is commercially available that from the 3-O-benzylglyceraldehyde reaction would require synthesis to provide TAm substrate to investigate the candidate transaminases and for product standards. The reaction with glycolaldehyde has previously been studied 'in house' (Hobbs, G.R., 1994; Hobbs, G.R. *et al.*, 1993; Mitra, R.K., 1997; Mitra, R.K., Woodley, J.M. and Lilly, M.D., 1998) therefore the reaction protocols, substrates and product standards and analytical assays (Mitra, R.K. and Woodley, J.M., 1996) are readily available. The reaction with glycolaldehyde and β-HPA was therefore selected as the first reaction in the target two-step synthesis.

The second enzyme in the pathway is a transaminase catalysing the transfer of an amine onto a ketone substrate. The model reaction for the TK-TAm pathway (Figure 1.8) with the first reaction forming *L*-erythrulose will result in the synthesis of 2-amino-1,3,4-butanetriol (ABT). Note that the final stereochemistry of the product will depend upon the stereospecificity of the enzyme used to catalyse the transamination step. However, to date, no transaminase that catalyses the transamination of *L*-erythrulose has been reported. It is therefore necessary to investigate which transaminases are likely candidates for use in the pathway.

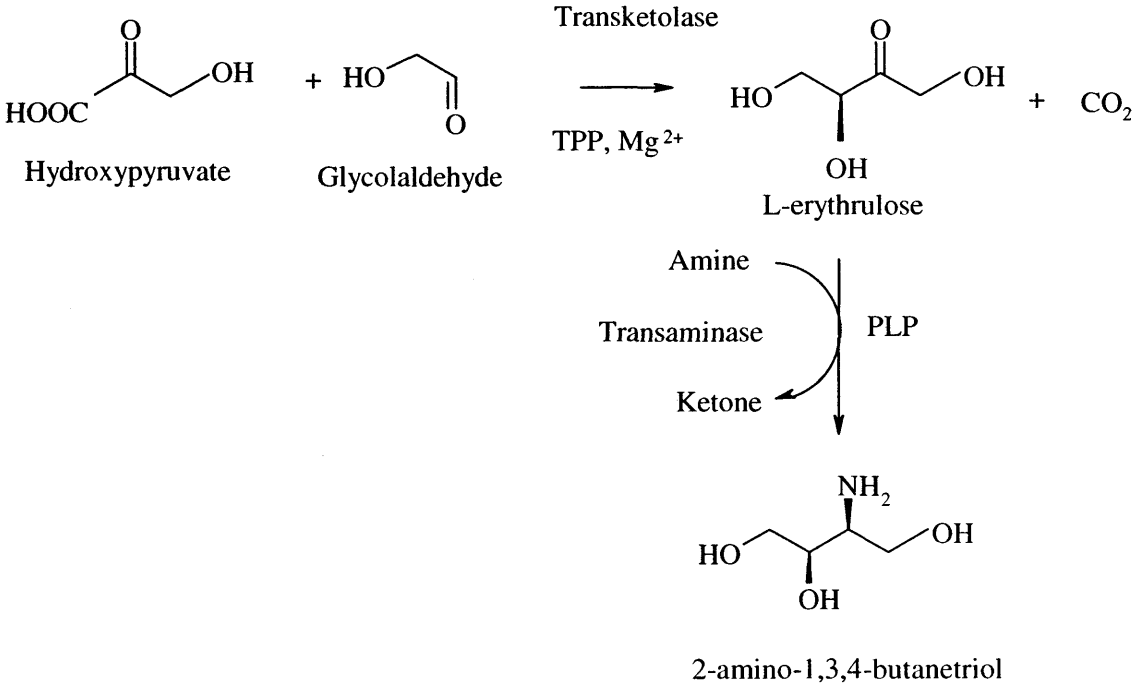


Figure 1.8: Target reaction scheme

The synthesis of chiral amino alcohol 2-amino1,3,4-butanetriol from achiral substrates glycolaldehyde and hydroxypyruvate using a TK-TAm pathway.

1.9 Chemical synthesis of pathway product

Although the target reaction is primarily selected to demonstrate the feasibility of the engineering of a novel biocatalyst to carry out the TK and TAm reactions in series, the pathway product 2-amino-1,3,4-butanetriol (ABT) is coincidently a useful product itself. The number of recent papers addressing the chemical synthesis of chirally pure ABT illustrates the importance of these compounds as chiral synthons (Dequeker, E. *et al.*, 1995; Fadnavis, N.W., Sharfuddin, M. and Kumara Vadivel, S., 2001; Kwon, S.J. and Ko, S.Y., 2001; Kwon, S.J. and Ko, S.Y., 2002; Merino, P. *et al.*, 2002). These compounds have been used as building blocks for statins used in the synthesis of protease inhibitors (Kwon, S.J. and Ko, S.Y., 2002), in particular in the synthesis of Nelfinavir™ (Figure 1.9), an HIV-protease inhibitor (Kaldor, W.K., *et al.*, 1997). ABT is also (in its fully protected form) an intermediate in the synthesis of Detoxinine a detoxifying agent used for reducing the toxicity of the antibiotic treatment for rice blast disease (Monache, G.D., Misiti, D. and Zappia, G., 1999). The broad substrate ranges of the TK and TAm enzymes to be investigated, and the scope for enlarging the substrate repertoire by mutagenesis, suggest the novel biocatalytic TK-TAm pathway will be able to produce other synthetically useful amino diol compounds.

1.10 Transaminases for investigation

Transaminases, or aminotransferases, belong to the E.C.2.6.1 class of transferases and are widely distributed throughout nature owing to their important role in metabolic pathways. They are involved *in vivo* not only in the synthesis of non-essential amino acids but also in the tricarboxylic acid and urea cycles. They catalyse the transfer of an amine group from an amine donor substrate to the carbonyl of an acceptor substrate (Figure 1.10). The mechanism of catalysis involves the cofactor, pyridoxal phosphate (PLP), bound to the enzyme (E-PLP) accepting an amine group from the amine donor substrate and release of the resulting ketone. This forms an intermediate enzyme pyridoxamine-complex (E-PMP). The amine group then donated to a second substrate, a ketone, to produce the corresponding amine and regenerate the E-PLP (Arnone, A. *et al*, 1985).

The transaminases selected for investigation into the acceptance of the TK products will have the potential to accept a range of substrates. For example although branching off the main carbon chain is found in the *L*-erythrulose substrate the branched chain transaminase (E.C.2.6.1.42) has not been found to have a wide substrate specificity (Inoue, K., *et al.*, 1988), therefore, would be an unwise choice for employment in a pathway that will potentially be manipulated to create a broad specificity biocatalyst. Additionally, the amount of information already known about enzyme structure as well as activity can be important. For example if a TAm candidate exhibits low activity towards a desired substrate it may be possible to rationally mutate the protein to enable more favourable catalysis of that substrate. This sort of rational mutagenesis requires knowledge of the protein structure, substrate-enzyme binding contacts and catalytic interactions. However the use of enzymes for which little is known, of which there are many, is very important to explore the potential of less studied enzymes. For this reason two transaminases were picked for investigation as the second enzyme in the TK-TAm pathway.

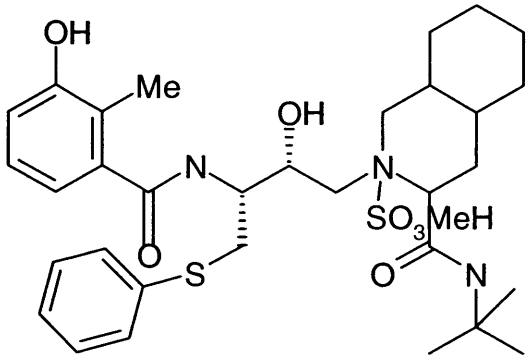


Figure 1.9: Structure of the protease inhibitor drug Nelfinavir™

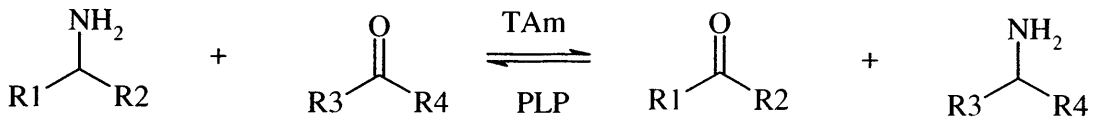


Figure 1.10: Transaminase catalysed reaction

Transaminase catalyses the transfer of an amine functionality between an amine and a ketone substrate in a reaction requiring a pyridoxal phosphate cofactor.

1.10.1 Aromatic transaminase

First the ArTAm (E.C.2.6.1.5) from *E.coli,* which *in vivo* catalyses the last step of tyrosine and phenylalanine synthesis was selected for investigation. This enzyme is well studied and is closely related to the extremely well studied AspTAm. The *E.coli* isoforms of the two enzymes were firstly identified by the activity of 'transaminase A' when Rudman and Meister observed transamination reactions involving aspartate, tryptophan, tyrosine and phenylalanine (Rudman, D. and Meister, A., 1953). Later the activity described as transaminase A was found to be two enzymes, AspTAm and ArTAm (Gefland, D.H. and Rudo, N., 1977a; Gefland, D.H. and Steinberg, R.A., 1977b). These two enzymes were cloned from *E.coli* and their primary structure compared (Fotheringham, G. *et al.*, 1986). Identity of the primary structure between these two transaminases is 43%. Additionally, they share similarity between the twenty eight residues that form the active site differing at only six residues. The characterisation of the ArTAm and comparison to AspTAm (Hayashi, H. *et al.*, 1993) revealed similarities between both the structural and catalytic properties of the two enzymes. The ArTAm protein was found to be a dimer made up of two 43 KDa subunits associated with one molecule of PLP per subunit as had been previously determined for AspTAm (Kondo, K., Wakabayashi, S. and Kagamiyama, 1987). The absorption spectra of the two enzymes were comparable, with two peaks at 358nm and 430nm, as were the circular dichroism (CD) spectra suggesting that the two enzymes are structurally similar. The catalytic activity of the two enzymes towards dicarboxylic substrates was found to be comparable whilst with aromatic substrates the activity of ArTAm was found to be three orders of magnitudes higher. The similarity between the two transaminases is such that substituting the six active site residues of the AspTAm, that differ between the enzymes, with those found at the corresponding positions in ArTAm increase the activity of the AspTAm towards phenylalanine to within seven fold of that of ArTAm (Onuffer, J.J. and Kirsch, J.F., 1995). Crystallographic analysis of the ArTAm has shown that the structure was similar to that of the AspTAm (Ko, T.-P. *et al.*, 1999). The close similarity between the two enzymes allows speculation about the properties of ArTAm from studies carried out on AspTAm.

ArTAm has relaxed substrate specificity towards the side chain of the amino acid substrate with the ability to catalyse reactions with both aromatic and dicarboxylic acid substrates. Therefore neither the hydrophobic R group nor the acidic R group is essential for catalysis. This flexible specificity may also enable the accommodation of the β-hydroxyl group resulting from TK catalysis (Figure 1.6). Additionally this enzyme has recently been demonstrated to accept heteroaromatic acid substrates (Cho, B.-K. *et al.*, 2004). This transaminase may therefore be useful in the longer term when TK mutants accepting aromatic or heteroaromatic substrates (Bacon, S.L., 2001) are substituted into the TK-TAm synthetic pathway.

The acceptance of serine as the amine donor substrate may enable the regeneration of TK substrate β-HPA during catalysis. It is not only advantageous to regenerate the costly TK substrate but also removal of one of the TAm products may enable the 50% equilibrium limit of α-TAm catalysed reactions to be overcome allowing higher conversions to be achieved. This has been previously demonstrated by the removal of oxaloacetate product during the production of *L*-2-aminobutyrate by α-TAm enabling yields of above 90% to be achieved (Fotheringham, G. *et al.*, 1999). However, the acceptance of serine by ArTAm has been shown to be extremely low (Hayashi, H. *et al.*, 1993). There is an enzyme class that is specific for serine, namely the serine:glyoxylate transaminases (E.C.2.6.1.45). These enzymes are found in plants and mammals with reportedly wide substrate specificity (Hagishita, T. *et al.*, 1996). The spinach isoform of this enzyme has successfully been demonstrated to catalyse a reaction preceeding TK to generate the β-HPA *in situ* as the substrate for TK (Hecquet, L., Bolte, J. and Demuynck, C., 1996). However, the complexities of cloning genes from higher organisms into the chosen *E.coli* host ruled this enzyme out of the pathway. These enzymes are also found in certain methylotrophs where they play a role in the assimilation of 1 carbon compounds (Antony, C., 1982). The enzyme from *Hyphomicrobium methylovorum* was successfully cloned and expressed in *E.coli* (Hagishita, T. *et al.*, 1996). Investigation into catalysis found the enzyme exhibited high substrate specificity (Izumi, Y., Yoshida, T. and Yamada, H., 1990) therefore ruling it out as a candidate for the pathway at this stage.

1.10.2 β-alanine: pyruvate transaminase

The family of transaminase enzymes is comprised of both the α-TAms and the ω-TAms. Subgrouping of the enzymes is by mutual similarities of primary structure, this corresponds with structural classification of the substrates utilised by the enzymes within subgroups. Groups I, III and IV are comprised of α-transaminases whilst the ω-TAms make up group II (Mehta, P., Hale, T.I. and Christen, P., 1993). Whilst the α-transaminases are well studied, an example of the acceptance of a substrate which is a non-keto acid, such as the TK reaction yields, has not been reported. With this in mind the less studied class II ω-transaminases were considered as potential TAm candidates. These enzymes can catalyse reactions with substrates where the amine is not adjacent to a carboxylate group. The ω-class is comprised of several enzymes catalysing reactions with particular substrates. The most frequently investigated for their application in biocatalysis are the so called ω-amino acid: pyruvate transaminases.

Within this group the amine: pyruvate transaminase from *Vibrio fluvialis* has been extensively studied by the Kim group (Park, J.H. *et al.*, 2002; Shin, J.-S., Kim, B.-G., 2001; Shin, J.-S. *et al.*, 2003). This enzyme has been successfully used to catalyse reactions with a range of compounds containing both aromatic and non aromatics and acidic and non acidic substrates (Shin, J.-S. and Kim, B.-G., 2002b). The use of this enzyme has been demonstrated in a multi enzyme scheme with ArTAm. The enzyme is used to simultaneously resolve racemic methyl benzylamine using pyruvate resulting from the spontaneous decarboxylation of oxaloacetate produced from transamination by ArTAm using an aspartic acid amine donor (Cho, B.-K. *et al.*, 2003a; Cho, B.-K. *et al.*, 2003b). Researchers at Celgene have also recognised the synthetic potential of the ω-amine: pyruvate TAms. An investigation into the synthetic potential of ω-TAms from *Bacillus megaterium*, *Pseudomonas aeruginosa* and *Pseudomonas putida* has been reported (Stirling, D.I., 1992). The use of the ω-TAms for the production of several chiral amines by either resolution or synthesis is demonstrated. The ω-TAms are loosely classified by those that accept β-alanine and pyruvate (E.C.2.6.1.18) named the β-alanine: pyruvate TAms (β-A: P TAm) and those that do not (E.C.2.6.1.19).

One of the early identifications of the activity of a β-A: P TAm was during the investigation of propionic acid metabolism in *Clostridium propionicum* (Goldfine, H. and Stadtman, E.R., 1960). Partially purified enzyme extracts were found to consume β-alanine at rates several times greater than that measured for propionate formation, which would result from deamination of β-alanine. Therefore a simple deamination reaction could not account for the alanine consumption. Furthermore the process required catalytic amounts of pyruvate and α-ketoglutarate and led to the formation of α-alanine or glutamate respectively. These findings led to the conclusion that transamination of the β-alanine was being carried out as an intermediate step during propionoate formation.

The isolation of β-A: P TAm was reported by Hayaishi *et al* in 1960 (Hayaishi, O. *et al.*, 1960). The enzyme was purified from *Pseudomonas fluorescens,* cultivated with β-alanine as the major source of carbon and nitrogen. An investigation of the reaction with β-alanine and pyruvate showed consumption of these substrates matched the production of α-alanine and malonic semi-aldehyde (Figure 3.11). The reaction was demonstrated to be both reversible and dependent on the presence of a pyridoxal phosphate cofactor. The optimum pH was found to be 9.2 and the K_m values determined as 62 mM and 14 mM for β-alanine and pyruvate respectively. A study of a selection of amine compounds to explore the substrate specificity of the enzyme, using pyruvate as the amino acceptor, found that monocarboxylic amino acids with the amine at either the β, γ and ε position were good amino donors. However α-amino acids proved poor substrates and dicarboxylic acids, aromatic acids and hydroxyacids, methionine and dipeptides were reported not to act as substrates.

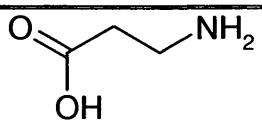
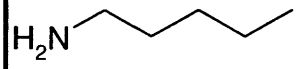
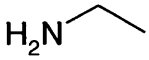
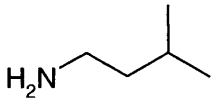
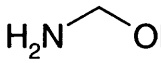
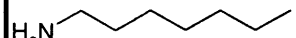
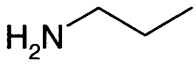
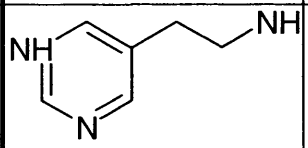
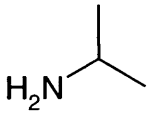
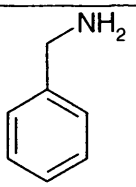
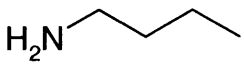
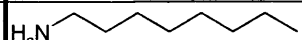
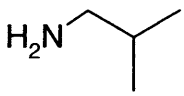
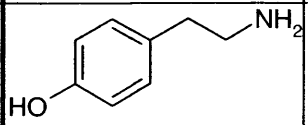
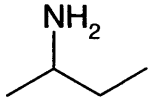
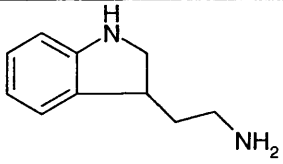
A related enzyme was isolated from *Pseudomonas sp. F-126* (Yonaha, K. *et al.*, 1976). Several studies have been conducted with this enzyme (Yonaha, K., Nishie, M. and Aibara, S., 1992; Yonaha, K., Toyama, M. and Kagamiyama, H., 1983; Yonaha, K. *et al.*, 1976; Yonaha, K., *et al.*, 1997) including the analysis of a low resolution crystal structure (Watanabe, N., *et al.*, 1989) and investigations into the catalysis with a range of amines. The enzyme is a tetramer composed of four identical 43 KDa subunits (Yonaha, K. *et al.*, 1976; Morita, Y. *et al*, 1979). Low

resolution crystal structure analysis (Watanabe, N. *et al.*, 1989) found the monomers, composed of a large and small domain, strongly associate as dimers which then loosely associate to form tetramers. The globular shape of the ω-TAm dimer is likened to that of a rugby ball shape whilst that of the α-TAm is an S-shape. However the secondary structure topology has been found to have similarities, providing a similar environment in the large domain for the PLP cofactor, although little homology in the amino acid sequences has been found (Yonaha, K., Nishie, M., and Aibara, S., 1992). These structural similarities are indicators that the two types of transaminases, α and ω, have evolved from a common ancestor.

The selectivity of the *Pseudomonas sp. F-126* ω-transaminase toward the amine donor substrate has been investigated (Yonaha, K. and Toyama, S., 1978) (Table 1.2). Although no β-branched hydroxylated substrates were tested the substrates explored show a favourable range. Enzyme activity was affected by the carbon chain length of primary alkyamines. Catalysis was most favourable with the 5 carbon n-amylalanine V_{rel}=80 (V_{rel}=100 for β-alanine) as opposed to equivalent substrates of varied chain length with the relative reactivity of the 4 carbon equivalent being V_{rel} = 60. This implies that the 4-carbon erythrulose is an acceptable chain length for this enzyme. Branched molecules were accepted with β-aminoisobutyric acid and isobutylamine giving V_{rel} of ~50. Hydroxylated molecules, whilst showing lower activities than their un-hydroxylated counterparts, for example ethylamine V_{rel} = 30 and ethanolamine V_{rel} = 5, still were substrates for the enzyme suggesting that the hydroxy-ketone substrates from TK may be accepted as substrates. Additionally the investigation into more bulky substrates such as benzylamine and histamine showed these substrates were also accepted. Therefore this enzyme, or one closely related may have the scope to catalyse the transamination of a range of TK products. This enzyme is not identified in *E.coli* therefore an alternative source was sought. Since *Pseudomonas aeruginosa* and *Pseudomonas putida* were both available 'in house' and have previously been demonstrated to have useful ω-TAms (Stirling, D.I., 1992) the β-A: P TAm from both organisms were initially investigated as transaminase candidates for the linked pathway with TK.

Table 1.2: Amine substrate reactivity for ω-TAm from *Pseudomonas putida*

Reproduced from (Yonaha, K. and Toyama, S., 1978). Activity is calculated as the amount of alanine produced relative to the reaction with β-alanine.

Substrate	Relative activity	Substrate	Relative activity
 β-alanine	100	 n-amylamine	80
 Ethylamine	30	 Isoamylamine	30
 Ethanolamine	5	 n-heptylamine	60
 n-propylamine	35	 Histamine	10
 Isopropylamine	40	 Benzylamine	45
 n-butylamine	60	 n-octylamine	45
 Isobutylamine	50	 Tyramine	5
 sec-butylamine	30	 Tyrptamine	20

1.11 Project aims and objectives

The use of enzyme pathways to yield useful products is extensively exploited throughout the chemical, pharmaceutical, food and agrochemical industries. However the construction of novel biocatalysts with *de novo* non-native pathways of synthetic enzymes, discrete from the host organism's metabolism, to carryout specific chemical transformations, is poorly explored. The ability to co-express multiple enzymes in a single host to carry out a sequence of desired chemical reactions could enable several synthetic processing steps to be carried out in one biocatalytic step. The aim of this project is therefore to demonstrate the construction of a transketolase – transaminase pathway, two enzymes not naturally functioning in series, and demonstrate the pathway for a model reaction system (Figure 1.8).

In order to achieve this aim the following objectives were set:-

- To identify and clone candidate transaminases and examine their activity with the TK reaction product *L*-erythrulose. This in itself requires the establishment of a cloning strategy to enable the co-expression of the successful TAm candidate together with TK in an *E.coli* host. This is detailed in Chapter 3.
- To establish methodology to assay the activity of the candidate transaminases with the TK product *L*-erythrulose and related compounds. This also requires the chemical synthesis of the model reaction product 2-amino-1,3,4-butanetriol. This is described in Chapter 4.
- To assess the transaminase candidates for activity with *L*-erythrulose or potential activity with compounds related to TK products, discussed in Chapter 4.
- To evaluate the selected transaminase with both natural and non-natural substrates. This is described in Chapter 5.
- To demonstrate synthesis of the 2-amino-1,3,4-butanetriol using the TK-TAm pathway in a one-pot process, this is detailed in Chapter 6.

Chapter 2 - Materials and Methods

2.1 Materials

Molecular biology enzymes were obtained from New England Bio-laboratories (NEB, Hitchin, UK). Nutrient broth and nutrient agar were obtained from Oxoid (Basingstoke, UK). Commercially competent *E.coli* cells (XL1-red genotype endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lacmutD5 mutS mutt Tn10 (Tet^r), XL1 blue genotype recA1 endA1 gyrA96 thi-1 hsdR17 spE44 relA1 lac [F' poAB $lacI^qZ\Delta M15Tn10$ (Tet^r)] and BL21gold (DE3) genotype E.coli B F^- ompT hsdS (r_B^- m_B^-) dcm^+ Tet^r galλ(DE3) endA) were obtained from Stratagene (Amsterdam, NL). Vectors pCR 2.1 TOPO (pUC ori f1 ori Kan^r Amp^r T7 promoter Plac lacZα) and pET24(a) (pBR322 ori Kan^r T7 promoter lacI) were obtained from Invitrogen (Paisley, UK). Primers and oligonucleotides were purchased from Operon (Cologne, DE). All other reagents were obtained from Sigma-Aldrich (Gillingham, UK) unless otherwise stated. Water was purified to a 15MΩ.cm resistance.

2.2 Culturing methodology

Overnight cultures of *E.coli* strains were grown from inoculation of 5 mL nutrient broth with a single colony obtained by streaking out cells from glycerol stocks (25% v/v glycerol stored at –80°C) on nutrient agar plates. Growth was for 16 hours at 37°C, with orbital shaking at 200 rpm using an SI 50 orbital shaker (Stuart Scientific, Redhill, UK). Growth selection was achieved by addition of the appropriate selection antibiotic (150 $\mu g.mL^{-1}$ ampicillin or 30$\mu g.mL^{-1}$ kanamycin) to the agar or broth. The optical density (OD) of liquid cultures was measured in triplicate by absorbance at 600nm using a Unicam (Cambridge, UK) UV2 UV/vis spectrometer. This measurement was related to cell concentration, $g.L^{-1}$, by the conversion factor of 0.47. This factor was obtained by the calibration of cell density relative to OD prepared by measurement of the dry weight of a series of culture aliquots of washed resuspended cells of known OD.

When induction of a cloned enzyme was required cells were prepared by a 1/20 inoculation of nutrient broth, (plus selection antibiotic), from a liquid overnight culture of the appropriate *E.coli* strain. Culture scale was either 100 mL culture in a 250 mL baffled shake flask or 500 mL in a 2 L flask. Growth was carried out at

37°C and 200rpm. Induction of enzyme expression was achieved by the addition of isopropyl-β-D-thiogalactosidase (IPTG) when an OD_{600nm} of ~ 0.6 was reached, followed by a further 4 hours growth.

Cell lysates were prepared from cultures (OD_{600nm} of 3.5) by removal of the supernatant following centrifugation and re-suspension of the cell pellet in 1/10 of the original volume of 25 mM phosphate buffer, pH 7.0. These were stored at –20°C. Upon thawing sonication to lyse cells was carried out using 5 cycles of 10 second 8 μ pulses with 10 second intervals using a Soniprep 150 sonicator (MSE, Sanyo, Jp), prior to use in reactions.

The maximum specific growth rate for liquid cultures, μ_{max} was determined by preparation of a semi log plot of growth data (Doran, P.M., 2000). According to the equation $\ln x = \ln x_0 + \mu t$ the linear portion of the graph, where the maximal growth rate is constant, has a slope μ_{max}. This is used to calculate doubling time, t_D from the equation $t_D = \ln 2/\mu$.

2.3 Standard molecular biology techniques

Recovery of plasmid DNA, from host *E.coli* cells, was carried out employing a QIAprep spin miniprep kit (Qiagen, Crawley, UK) unless otherwise specified. Gel extraction or DNA purification was carried out using a QIAquick PCR purification kit (Qiagen, Crawley, UK). The concentration of DNA was determined by measuring absorbance at 260nm (A_{260}) where 1 absorbance unit corresponds to 50 $ng.\mu L^{-1}$ of DNA. Restriction digests were carried out for three hours at 37°C in the recommended NEB restriction buffer, according to the enzyme used, at an appropriate scale (10μL for analytical digests and 40μL for preparative digest) according to the manufacturer's protocol. All ligations were carried out with T4 DNA ligase with T4 ligase buffer at 15°C for a minimum of 12 hours.

Agarose DNA gels were run on 1% w/v agarose gel with 0.5X tris-borate-EDTA buffer, markers were either lambda DNA cleaved with *pst*1 or a 1KB ladder (NEB, Hitchin, UK) for quantification, staining was with ethidium bromide. Smaller fragments of DNA were separated and quantified by 7.5% v/v acrylamide gel

electrophoresis against a 100bp ladder (NEB, Hitchin, UK). Competent cells were made from cultures grown in nutrient broth containing 20 mM $MgCl_2$ at 37°C, 200 rpm to an OD of 0.6. Cells were harvested by centrifugation and chilled on ice for 30 minutes. Resuspension was carried out in 75 mM $CaCl_2$ and then the cells were re-pelleted by centrifugation. This was carried out three times. Following the final centrifugation cells were resuspended in 75 mM $CaCl_2$ with 15 % v/v glycerol. Cells were aliquoted and either placed on ice for immediate transformation or stored at -80°C. Transformations were carried out using heat shock at 42°C for 45 seconds of either chemically competent cells made 'in house' or commercially available competent cells (Stratagene, Amsterdam, NL) of the appropriate strain.

All constructs were commercially sequenced by the service available at The Wolfson Institute for Biomedical Research, UCL. SDS-PAGE gel electrophoresis for protein analysis was carried out using a Mini-Protean II system (Bio-Rad Laboratories Inc., Hemel Hempstead, UK) with 12% w/v acrylamide gels and stained with 0.05 % w/v coomassie brilliant blue. Solubility of protein was assessed by clarification of the supernatant of lysate and SDS-PAGE analysis of the supernatant and the resuspended pellet alongside crude lysate. Quantification of protein was carried out according to the Bradford method (Bradford, M., 1976) using a protein assay kit (Bio-Rad Laboratories Inc., Hemel Hempstead, UK). The protein concentration was calibrated against a bovine serum albumin standard curve. All gels were visualised and quantified (where appropriate) on a Gel-Doc-it bioimaging system with labworks 4.5 software (Bioimaging systems, Cambridge).

2.4 Cloning of synthetic enzymes

2.4.1 Cloning and Expression of TK

2.4.1.1 Construction of plasmid pQR411

Plasmid pMMB67HE was purified by caesium chloride-ethidium bromide gradient large scale plasmid preparation (Sambrook, J., Fritsch, E.F. and Maniatis, T., 1989). This was followed by an ethanol precipitation step carried out by the addition of 1/10 of the volume of NaCl and 2 volumes of ethanol followed by incubation at 4°C for 1 hour. The precipitated DNA was recovered by centrifugation. The *E.coli* transketolase gene (with native promoter) was cleaved out of an existing expression

construct, pQR706 (obtained from Dr J.Ward, Department of Biochemistry and Molecular Biology, UCL), using *BamH1* and *Sac1*. Plasmid vector pMMB67HE (RSF1010 origin of replication) was digested with the same enzymes. The digested components were separated by agarose gel electrophoresis and the excised TK gene fragment was gel extracted (Qiagen kit). This was then ligated into the pMMB67HE vector and transformed into XL1 blue. Successful insertion of the gene into the vector was verified by restriction digest and commercial sequencing. The resulting ampicillin resistant plasmid, pQR411, was then purified by miniprep. and used to transform JM107 (chemically competent cells) and BL21gold(DE3) *E.coli* expression strains.

2.4.1.2 Verification of the expression of active TK

The over production of TK from the plasmid pQR411 in an *E.coli* host was assessed by SDS-PAGE electrophoresis (Section 2.3) relative to the production of TK from the original pQR706 construct at comparable cell densities. The TK produced from the new construct pQR411 in the BL21gold(DE3) host was determined to make up 24% of the total cellular protein. The production of active TK in a JM107 host was also assessed relative to the original construct. Activity of the transketolase from both strains and the native host strain (un-transformed JM107) was tested in 1 mL reactions in deep square well microtitre plates incubated at ambient temperature (~ 24°C). Lysate was incubated for 30 minutes with cofactors thiamine pyrophosphate and $MgCl_2$ prior to the addition of substrate to initiate the reactions. Each reaction contained 100mM β-hydroxypyruvate (lithium salt) and glycolaldehyde in 50 mM tris-HCL, pH 7.0 with 2.4 mM thiamine pyrophosphate and 9 mM $MgCl_2$. Lysate comprised 50% v/v of the reaction. Samples were quenched by dilution in 0.1% TFA for analysis using the RP-HPLC method for transketolase activity (Section 2.5.2).

2.4.1.3 Growth and activity profile of BL21gold(DE3) pQR411

Cultures, 100 mL, of BL21gold(DE3) pQR411 and control strain BL21gold(DE3) were grown from a 1/20 inoculum under the conditions previously described (Section 2.2). Aliquots were removed at intervals and the OD and activity measured. Activity was measured as initial rate of reaction carried out as describe in Section 2.4.1.2 except for the scale of reaction, 300 µL, and concentration of substrate, 50

mM. The growth and activity were performed in duplicate on separate occasions yielding comparable trends in data.

2.4.2 Cloning and Expression of ArTAm

2.4.2.1 Construction of plasmid pQR416

The *Tyr B* gene was obtained by PCR from the genomic DNA of *E.coli* strain MG1665 using Taq polymerase. This was inserted into a PCR 2.1 TOPO capture vector (Invitrogen) and transformed into *E.coli* TOPO 10 cells to create pQR400 and pQR401. This initial work was carried out by Dr J.Ward, The Department of Biochmistry and Molecular Biology, UCL. The presence of the correct gene sequence was confirmed by sequencing and the orientation of the gene in the vector verified by restriction digestion. Construct pQR400 was found to have the gene in the same direction as the T7 promoter of the vector whilst pQR401 has the gene in the opposing orientation.

In order to insert the T7 ribosome binding site, complimentary oligonucleotides encoding the T7 ribosome binding site with terminal *Nde1* and *Xba1* restriction sites and an internal *HindIII* site were obtained (Operon);
5'CTAGAAAAGCTTATAATTTTGTTTAACTTTAAGAAGGAGATATACA3'
and 5'TATGTATATCTCCTTCTTAAAGTTAAACAAAATTATAAGCTTTT3'.
These were diluted to 10 μM and annealed by addition of an equal volume of each oligonucleotide, heating in boiling water for 5 minutes and cooling to room temperature in a water bath. Plasmid pQR400 was digested with *Xba1* and *Nde1* and purified on a PCR purification column (Qiagen, Crawley, UK) to remove the excised fragment. Ligation of the annealed ribosome binding site oligonucleotides into the cleaved pQR400 preceded transformation into XL1 blue. Successful insertion of the oligonucleotides into the pQR400 vector was verified by restriction digest using *HindIII* and commercial sequencing. A digest of the construct with *Acl1* and re-circularisation enabled the removal of a portion of the ampicillin resistance gene to yield kanamycin resistant pQR416.

2.4.2.2 Verification of the expression of active ArTAm

The production and solubility of the ArTAm from plasmid pQR416 in *E.coli* BL21gold(DE3) was assessed by SDS-PAGE electrophoresis (Section 2.3). Activity was assayed using an enzyme linked spectrophotometric assay (Section 2.7.1). The activity of the ArTAm lysate with various native substrates was assayed by obtaining reaction profiles by HPLC using the method for native ArTAm assay (Section 2.5.3). Reactions contained 50% reaction volume lysate, 25mM potassium phosphate buffer, pH 7.0 with 10 mM ketoacid and various amino acid substrates with 0.2mM pyridoxal phosphate cofactor. Reactions were carried out in duplicate at 25°C with agitation at 200 rpm.

2.4.2.3 Growth and activity profile of BL21gold(DE3) pQR416

Cultures, 100 mL, of BL21gold(DE3) pQR416 and control strain BL21gold(DE3) were grown from a 1/20 inoculum under the conditions previously described (Section 2.2). Induction was carried out by addition of IPTG to 1mM after 4.5 hours of growth. Aliquots were removed at intervals, the OD measured and activity tested by enzyme-linked assay (Section 2.7.1). This was carried out in duplicate on separate occasions yielding comparable data.

2.4.3 Expression of β-A: P TAm

2.4.3.1 Construction of plasmids pQR426 and pQR427

Constructs pQR426 and pQR427 were obtained from Mr M. Bommer (Department of Biochemistry and Molecular Biology, UCL). These encode the β-A: P TAm genes amplified from the genomes of *Pseudomonas aeruginosa* (*PA0132*) and *Pseudomonas putida* (PPO596) respectively, ligated into the *Xho1* and *Nde1* sites of pET24(a)+. These constructs were transformed into BL21gold(DE3) for expression and DNA was prepared for sequencing.

2.4.3.2 Construction of pQR428

Plasmid pQR426 was further modified by insertion of oligonucleotide 5'CTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGGGCCAT CATCATCATCATCA3' into the *Xba1* and *Nde1* sites as described for the addition of the oligonucleotide into pQR400 (Section 2.4.2.1). The resulting plasmid,

pQR428, encodes the β-A: P TAm with an N terminal 6-histidine tag. This construct was then transformed into the *E.coli* BL21gold(DE3) host strain.

2.4.3.3 Nickel column purification of histidine tagged β-A: P TAm

Purified β-A:β TAm was obtained by purification of the histidine tagged product from pQR428 on a nickel column (Hochuli, E., Dobeli, H. and Schacher, A., 1987). At a flow rate of 1 $mL.min^{-1}$, the chelating Sepharose fast flow resin (Amersham Biosciences, Little Chalfont, UK) was charged with 1 column volume of 0.2 M $NiSO_4$ followed by washing with 10 column volumes of dH_2O and equibrilation with 2 column volumes of 25mM Tris buffer, pH 8.5. Lysate was prepared from induced cultures of BL21gold(DE3) pQR428. This was clarified by centrifugation at 20 000g for 15 minutes at 4°C and loaded onto the column. A wash of 10 column volumes of 25 mM Tris buffer, pH 8.5 was carried out to remove non-specifically bound material. The bound protein was then eluted with 25mM Tris buffer, pH 8.5, containing 250 mM imidazole, pH 8.5. Fractions of 5 mL were collected. These were assayed for protein concentration using a standard Bradford assay (Section 2.3). Fractions containing eluted protein were combined and dialysed (10,000 molecular weight cut off) against 50 mM potassium phosphate buffer, pH 7.2 overnight at 4°C. The enzyme was then concentrated in a Vivaspin (Sartorious), molecular weight cut off 10,000, at 1800 rcf for 15 minutes. An equivalent of 10% v/v of glycerol was added and the protein aliquoted for storage at –20°C.

2.4.3.4 Verification of the expression of active β-A: P TAm

The expression of soluble β-A: P TAm by *E.coli* BL21gold(DE3) was assessed by SDS-PAGE analysis (Section 2.3). Activity was verified by reaction, 0.3 mL, with substrates β-alanine and pyruvate, 15mM, with 2mM pyridoxal phosphate cofactor in 25mM potassium phosphate, pH 7.0. The reactions were carried out with 50% v/v lysate at 25°C with agitation at 200 rpm. Samples were taken at regular intervals and prepared for analysis by the HPLC method for native β-A: P TAm activity (Section 2.5.4) by derivatisation (Section 2.6.2). A 95% confidence interval was calculated from experiments performed in triplicate. Following storage of both whole cells and lysate overnight at –20°C the above reactions were repeated. A second assay was employed to confirm the activity of the β-A: P TAm using a spectrophotometric assay measuring activity with β-aminobutyrate and pyruvate (Section 2.7.2).

2.4.3.5 Growth and activity profile of BL21gold(DE3) pQR426

Cultures, 100 mL, of BL21gold(DE3) pQR416 and control strain BL21gold(DE3) were grown from a 1/20 inoculum under the conditions previously described (Section 2.2). Induction was carried out by addition of IPTG to 1mM after a 1 hour growth period. Aliquots were removed at intervals and the OD measured and activity tested by reaction with native substrates (Section 2.4.3.4). This experiment was carried out in duplicate on separate occasions yielding comparable data.

2.5 HPLC

2.5.1 HPLC system

A Dionex (Camberley, UK) microbore HPLC system controlled by Peaknet 5.1 software was employed for all RP-HPLC analysis. The system comprised of a GP40 gradient pump, an LC30 column oven, a PC10 pneumatic controller post column NaOH addition unit and either a AD20 UV detector or ED40 electrochemical detector module. An Endurance multiwell plate autosampler (Spark, Emmen, NL) was integrated into the Dionex system.

2.5.2 Assay for TK native activity

A 15 minute isocratic assay was adapted from the existing HPLC assay for the TK reaction components (Mitra, R.K. and Woodley, J.M., 1996). HPLC analysis of glycolaldehyde, β-HPA and *L*-erythrulose was carried out using an Aminex 87H column (Bio-Rad, Hemel Hempstead, UK) at 60°C, mobile phase 0.1% v/v trifluoroacetic acid (TFA), flow rate 0.6 $mL.min^{-1}$. Detection was via an electrochemical detector (ECD40), set to a standard triple potential, following adjustment of pH to above pH 12 by post column addition of NaOH. Samples were quenched and diluted with 0.2% v/v TFA. Retention times were HPA 8.40 min, erythrulose 11.40 min, glycolaldehyde 12.28 min (Appendix (A)). The linear range for all components was determined to 10 mM.

A shorter assay, duration 10 minutes, for β-HPA and *L*-erythrulose with a flow rate of 0.8 $mL.min^{-1}$ and UV detection at 210 nm was also used. Retention times were HPA, 6.38 min, and erythrulose, 8.82 min (Appendix (B)), and a linear range determined to 20 mM.

2.5.3 Assay for native ArTAm activity

To analyse aspartic acid, glutamate and serine a 14 minute gradient HPLC method was developed on an ACE 5 C18 column with UV detection at 254nm. The solvent program employed two phases A = 140mM sodium acetate, pH 5.05 and B = 100% ACN. Separation was achieved by a gradient, curve 7, from 100% A to 40% A at 1 mL.min over 10 minutes. A wash phase and re-equilibrium step is then incorporated into the method with a step to 80% B for 2 minutes before the final 2 minutes at 100% A. Derivatisation of the amines with AQC was required for separation and detection using this method (Section 2.6.3). Retention times were aspartate 6.17 min, glutamate 6.45 min (Appendix (C)) and serine 6.65 min. Linearity was confirmed to 1 mM (post derivatisation).

2.5.4 Assay for native β-A: P TAm activity

To analyse α- alanine, β-alanine and ABT a gradient HPLC method was established using an ACE 5 C18 column (Advance Chromatography Technologies, Aberdeen, UK) with UV detection at 254 nm. The solvent program employed mobile phases A = 140mM sodium acetate, pH 5.05 and B = 100% ACN. A gradient, curve 8, was run from 85% A to 100% A over 10 minutes, flow rate 0.5 $mL.min^{-1}$, followed by a column wash phase and re-equilibrium step at 1 $mL.min^{-1}$. Derivatisation of the amines with AQC was required for separation and detection using this method (Section 2.6.3). Retention times for the standard mixture of ABT diastereomers were 8.11 min and 8.20 min with the diastereomer generated by the β-A: P TAm eluting second (Appendix (D)i) α-alanine elutes at 8.32 min and β-alanine at 8.50 (Appendix (D)ii). Components below 1 mM post derivatisation were determined to lie within linear range.

2.5.5 Assay for transamination of aromatics

An 8 minute assay for the analysis of aromatic compounds was established using an ACE 5 C18 column with mobile phase A=100% ACN and B= 0.1% v/v TFA. The method employed a linear gradient of mobile phase A from 20% to 40% over 6 minutes at a flow rate of 1 $mL.min^{-1}$, followed by 2 minutes of re-equilibration at 20 % A. Detection was by UV adsorption at 216nm. Samples were quenched with 0.2% v/v TFA. Retention times were phenylalanine 2.1 min, MBA 2.40 min, EBA 3 min and acetophenone 6.1 min with linearity to 5 mM.

2.6 Synthetic Chemistry

2.6.1 Synthesis of ABT product standard

L-erythrulose (4 g, 33 mmol), **1**, was suspended in acetone:dimethoxypropane 9:1 (30 mL) and p-toluenesulfonic acid (catalyst) was added (Figure 4.1). The mixture was stirred at room temperature under N_2 for 3 h during which time the reaction became homogeneous. The reaction was monitored by TLC (2:1, ethyl acetate:hexane). Sodium acetate (0.5 g) was added and the reaction stirred for a further hour before the mixture was filtered and concentrated to dryness. The residue was purified by flash chromatography on silica gel (ethyl acetate/hexane 1:2) to yield the desired 3,4-*O*-isopropylidene acetal **2** (1.9 g, 38%). To an aliquot of acetal **2** (487 mg, 3.02 mmol) in methanol (10 mL) was added benzylamine (660 μL, 6.04 mmol) and then sodium cyanoborohydride (570 mg, 9.06 mmol). The pH was adjusted to pH 6.0 (indicated by bromocresol green) using acetic acid and the reaction was stirred at room temperature for 6 hours. The reaction was monitored by TLC (1:1, ethyl acetate:hexane). The mixture was concentrated under vacuum and the residue partitioned between dichloromethane (100 mL) and sodium hydrogen carbonate (aq, sat) (100 mL) with vigorous stirring. Solid sodium carbonate was added to the suspension in small portions to ensure that the final pH of the aqueous layer was ~9. The layers were then separated, the aqueous phase was extracted with further dichloromethane (2 x 100 mL) and the organic phases combined and concentrated to dryness. The residue was purified by flash chromatography on silica gel (ethyl acetate/hexane 1:1 then neat ethyl acetate) to yield the desired amine **3** as a 1:1 mixture of diastereomers (320 mg, 43%). The diastereomers could have been resolved at this stage but this was considered unnecessary.

To a solution of amine **3** (200 mg, 0.80 mmol) in MeOH (5 mL) was added drop wise 1 M HCl (aq) to give a mixture that gave a dark red colour to moist litmus paper. The reaction was stirred at room temperature and was monitored by TLC (1:1, ethyl acetate:hexane). After 2 h the reaction was complete and the mixture concentrated to dryness to yield crude amine **4** as the hydrochloride salt. This residue was re-dissolved in MeOH (5 mL) and hydrogenated (balloon pressure) in the presence of palladium on carbon (10% wt/wt, 100 mg) at room temperature overnight. The catalyst was removed by filtration and the solvent removed under

vacuum to yield 2-amino-1,3,4-butanetriol **5** as a 1:1 mixture of *threo* and *erythro* diastereomers (75 mg, 60 %). The NMR spectral data for compounds **2**, **3** and **4** was in accordance with those reported in the literature (Dequeker, E. *et al.*, 1995). A further NMR spectrum was obtained to confirm the structures of the final mixture of diastereomers of 2-amino-1,3,4-butanetriol, **5** and was compared with the literature valuesfor the single diastereomer (Appendix J).

2.6.2 Synthesis of derivatising agent

Derivatising agent 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) was synthesised 'in house' according to the protocol detailed by Cohen, S.A. and Michaud, D.P. (Cohen, S.A. and Michaud, D.P., 1993). Di(*N*-succinimidyl)carbamate, 1.5g, 6 mmol, was refluxed in 25 mL of dry acetonitrile. Dropwise addition of a 6-aminoquinoline, 0.75g, 5 mmol, solution in dry acetonitrile was carried out over 45 minutes. Following a further 30 minutes reflux, concentration was carried out to half the original volume. The solution was stored at –20°C overnight. The crystals were then filtered off and stored *in vacuo*. An 8 $mg.ml^{-1}$ AQC solution was prepared for use by dissolving crystals in dry acetonitrile by heating at 50°C for ten minutes.

2.6.3 Derivatisation of amines for HPLC analysis

Samples for amine HPLC analysis were diluted in 0.02M borate buffer, pH 8.8, to below 1 mM and derivatised by addition of an excess of 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate.

2.7 Spectrophotometric assays for TAm activity

Enzyme linked spectrophotometric assays were used to test the activity of the various transaminases. The rate of TAm substrate turnover was linked to an NADH dependent dehydrogenase, which accepts the product of the TAm reaction. The millimolar extinction coefficient of NADH, 6.22, at 340 nm, was used to convert absorbance readings to concentration. Therefore, the rate of TAm activity could be quantified by analysis of the change in NADH concentration.

2.7.1 ArTAm enzyme linked assay

Reactions, 1 mL, containing 19.7 mM of *L*-aspartate and α-ketoglutarate substrates, 0.2 mM pyridoxal phosphate cofactor, 0.1 mM β-nicotinamide adenine dinucleotide

(NADH), 20 units of malic dehydrogenase (E.C.1.1.1.38), 38 mM potassium phosphate buffer, pH 7.0 were started by the addition of 50 µL of cell lysate. Reactions, performed in triplicate, were monitored spectroscopically by measuring NADH depletion at A_{340nm}.

2.7.2 ω-TAm enzyme linked activity assay

Reactions, 1 mL, containing 50 mM β-aminobutyrate, 2 mM pyruvate, 0.001 mM PLP, 0.1 mM NADH and 0.5 U β-hydroxybutyrate dehydrogenase were started by the addition of TAm, (quantity varied). Reactions were monitored spectroscopically by measuring NADH depletion at 340nm. All reactions were performed in triplicate.

2.8 Investigation of ArTAm substrate specificity

Reactions contained 50% v/v lysate, 25mM potassium phosphate buffer, pH 7.0 with 10 mM of the keto acid and amino acid substrates and 0.2 mM pyridoxal phosphate cofactor. Reactions were carried out in duplicate at 25°C with agitation at 200 rpm. Reactions were analysed by the appropriate HPLC assay according to the substrates employed.

2.9 Mutagenesis of ArTAm

2.9.1 Random mutagenesis

2.9.1.1 Random mutagenesis of ArTAm using XL1-red

Plasmid pQR416 was transformed into commercial XL1-red (Stratagene, Amsterdam, NL) and grown on nutrient agar plates, containing 20 $\mu g.mL^{-1}$ kanamycin, overnight. All colonies (~150) were harvested directly from the surface of the plate and used to inoculate 100 mL nutrient broth, 20 $\mu g.mL^{-1}$ kanamycin in a baffled 250 mL shake flask. This was incubated for 12 hours at 37°C. The resulting culture was used to inoculate a second 100 mL nutrient broth, containing 20 $\mu g.mL^{-1}$ kanamycin, which was incubated for 12 hours. This cycle was repeated a further 7 times. The OD_{600nm} was measured at each inoculation and it was estimated that the cells had been propagated for a total of approximately 50 generations.

The pQR416 plasmid was then recovered by miniprep and transformed into the BL21gold(DE3) expression host. Individual colonies were then picked into standard

96 well microtitre plates containing 100 µL nutrient broth, kanamycin 15 $\mu g.mL^{-1}$ using the colony picking mode of a Qpix robot (Genetix, New Milton, UK). The plates were covered with an identical (empty) inverted plate and sealed with plastic tape to prevent evaporation. The sealed plates were then incubated at 37°C, on a Variomag Teleshake unit (Camlab Ltd., Cambridge, UK) at 1000 rpm, for 16 hours. The resulting optical densities of the individual cultures of the plate were measured using a FLUOstar Optima plate reader (BMG Labtechnologies GmbH., Offenberg, DE) at 600 nm. Following centrifugation the supernatant was aspirated from the plate and the cell pellets resuspended in 100 µL 50 mM potassium phosphate buffer, pH 7.0. The resuspended cells were then distributed into plates as 20 µL aliquots, for use in screening reactions, and frozen at –80°C. The remaining culture was stored at –80°C as 25% v/v glycerol stocks. A plate of wild-type ArTAm cells BL21gold(DE3) pQR416 was used to prepare wild-type ArTAm aliquots for use as a control for the substrate screens.

2.9.1.2 XL1-red library screening

Library plates containing 20 µL of cells in 50mM potassium phosphate buffer, pH 7.0 were thawed at room temperature. To ensure lysis of cells 20µL bugbuster™ (Novagen) was added and incubated for 45 minutes. The substrates for the screen, 10mM, in 50mM potassium phosphate buffer, pH 7.0 were then added with 0.2 mM pyridoxal phosphate cofactor. The plate was incubated at 25°C for four hours. The reactions were quenched by addition of 100 µL 0.1% TFA and subject to HPLC analysis for substrate consumption and product formation using the appropriate method (Section 2.5).

2.9.2 Whole plasmid error prone PCR

The initial reactions were all carried out, according to protocol from the Matsumura laboratory at Emery University, Atlanta, USA (Rowe, L.A. *et al.*, 2003), with 0.2 µM primers, M13 (-20) primer 5' GTAAAACGACGGCCAGT 3' and the reverse complement, template 34 ng, 0.2 mM dNTPs and 1.5 mM $MgCL_2$. Polymerases, Pfu turbo and Taq polymerase, were added to a concentration of 2.5 U in a 50 µL reaction. The Tth/ Vent mix was made up with a ratio of 13 U Tth to1 U vent and added to a concentration of 2.5 U of Tth in the reaction. The commercial reaction buffer for each enzyme was used (except for the reaction with Tth/Vent where the

buffer composed of 25 mM tricine, 5% v/v glycerol, 5% v/v DMSO, 85 mM potassium acetate). The thermocycle used with the pUC18 template was denaturation 30s at 95°C, annealing 1 minute at 60°C and extension for 5 minutes at 72°C, for 24 cycles. Cycling was completed by a final 7-minute extension. Reactions with Taq polymerase were carried out with the addition of more polymerase after 12 cycles. The PCR reactions were then treated with Dpn1 to remove methylated template DNA and run on an agarose gel. The PCR product was purified from the gel, treated with DNA T4 polymerase and ligase and transformed into BL21gold(DE3).

2.9.3 Site directed mutagenesis

2.9.3.1 Generation of ArTAm Arg386 mutants

Quickchange PCR was used to introduce mutations at amino acid position 386.
Primers used were
5'ATCTCATCGCCAGCGGGTNNSATGTGTGTGTCGCCGGGTT 3'.
PCR reactions , in a volume of 50μL, were 0.2 mM primers, 0.2 mM dNTPs, 37.5 ng pQR416 template, 1x Pfu reaction buffer and 1.25 units of Pfu turbo. Ten cycles of denaturation at 94°C for 30 seconds, annealing at 64°C for 1 minute and extension at 68°C for 11 minutes was carried out. The resulting 5.1 Kb product was transformed into *E.coli* strain BL21gold(DE3) individual colonies were selected and the DNA isolated for sequence analysis.

2.9.3.2 Activity of ArTAm Arg386 mutants

The activity of the variant transaminases was investigated by reaction with various substrates and analysis by HPLC as described in Section 2.8.

2.10 Investigation of β-A: P TAm activity

2.10.1 Investigation of β-A: P TAm activity with various substrates

Reactions, 1 mL, were carried out with substrates, 5 mM, in 25mM potassium phosphate buffer, pH 7.0 with 0.2 mM pyridoxal phosphate cofactor at ambient temperature (~25°C) and agitation at 200 rpm. Catalyst was added to 0.61 $mg.mL^{-1}$ for purified transaminase or lysate to give a concentration of the aminotransferase of 0.573 $mg.mL^{-1}$.

2.10.2 Transamination reactions catalysed by β-A: P TAm

Reactions, were carried out, unless otherwise stated, at a 1 mL scale in sealed glass vials with *L*-erythrulose and *L*-MBA substrates,10 mM, (or 10 mM pyruvate and β-alanine where stated) in 100 mM potassium phosphate buffer, pH 7.0 with 2mM pyridoxal phosphate cofactor at ambient temperature (~25°C) and agitation at 200 rpm. Catalyst was added as purified transaminase (Section 2.4.3.3) to a concentration of 0.61 mg.mL^{-1}. Where stated catalyst was added as lysate from BL21gold(DE3) pQR426 comprising 50 % v/v of the reaction to give a concentration of the aminotransferase of 0.573 mg.mL^{-1}. Analysis was carried out using the β-A: P TAm HPLC assay (Section 2.5.4) unless otherwise specified.

2.10.3 Investigation of substrate concentration on the native β-A: P TAm reaction

Reactions were carried out as described in Section 2.10.2 at an enzyme concentration of either 0.573 mg.mL^{-1} or 1.15 mg.mL^{-1} added in lysate from BL21gold(DE3) pQR426 for each substrate concentration, 5 mM, 10 mM, 20 mM and 40 mM. Reactions with enzyme were repeated at the lower enzyme concentration with lysate from different cultures of the strain to ensure the enzyme activity is consistent.

2.10.4 Investigation of substrate concentration on the target β-A: P TAm reaction

Reactions were carried out as described in Section 2.10.2 with *L*-MBA and *L*-erythrulose substrate concentrations of 5mM, 10mM, 20 mM and 40mM. The reaction at 10 mM was carried out in duplicate.

2.10.5 Investigation of individual substrate concentration on target β-A: P TAm reaction

Reactions were carried out as described in Section 2.10.2 except that the concentration of either L-MBA or *L*-erythrulose was varied, 2.5mM, 5mM, 10 mM and 20mM, whilst the other substrate was held constant at 10 mM. Analysis was carried out in triplicate using the β-A: P TAm HPLC assay (Section 2.5.4).

Duplicate reactions were carried out at 10 mM and compared to data from previous experiment to ensure reproducibility.

2.10.6 Investigation into product inhibition of target reaction with β-A: P TAm

Reactions were carried out as described in Section 2.10.2 with the addition of acetophenone and ABT simultaneously at 2.5 mM, 5 mM or 10 mM. This experiment was also repeated with individual addition of each product over the same concentration range.

2.10.7 Investigation into reaction equilibrium

Three reactions were run to steady state under the conditions described in Section 2.10.2. After 72 hours additions to the reactions were made. Enzyme was added to the first reaction to give a fresh enzyme concentration of 0.61 $mg.mL^{-1}$ with cofactor to a final concentration of 2 mM fresh cofactor. To a second reaction, substrates, in100 mM phosphate buffer, pH 7.0, with cofactor, were added, to raise the substrate concentration by 5 mM. Phosphate buffer with cofactor was added to the third reaction as a control. Analysis of ABT concentration was carried out using β-A: P TAm HPLC assay (Section 2.5.4).

2.11 Dual strain construction and activity

2.11.1 Dual strain construction and activity testing

Cells from BL21gold(DE3) pQR411 were made chemically competent (Section 2.3) and transformed with plasmid pQR426. The resulting transformed cells were cultured in the presence of both kanamycin and ampicillin to maintain the two plasmids. The dual plasmid strain was assayed for activity of both TK (Section 2.4.1.2) and β-A: P TAm (Section 2.4.3.4).

2.11.2 Coupling of TK and TAm reactions

Reactions, 1 mL, were carried out in sealed glass vials with β-HPA, GA and *L*-MBA substrates, 10 mM or 20 mM, in 100 mM potassium phosphate buffer, pH 7.0 with cofactors thiamine pyrophosphate, 2.4 mM, $MgCl_2$, 9 mM, and pyridoxal phosphate, 2mM, at ambient temperature (~28°C) with agitation at 200 rpm. The enzyme catalysts were added in lysate from the dual plasmid strain BL21gold(DE3) pQR411+ pQR426 comprising 50 % v/v of the reaction to give a concentration of

transketolase of 0.437 $mg.mL^{-1}$ and β-A: P TAm of 0.609 $mg.mL^{-1}$. In the case of whole cell reactions freshly cultured induced un-lysed cells were added to obtain the same enzyme concentrations as the lysate. Analysis was carried out using HPLC assays to analyse each component, β-HPA, GA and erythrulose (Section 2.5.2), MBA and acetophenone (Section 2.5.5) and β-A: P TAm HPLC assay (Section 2.5.4).

Chapter 3 – Cloning and expression of individual synthetic enzymes

The aim of this project is to demonstrate the potential of creating non-natural (*de novo*) pathways of synthetic enzymes to access desired chiral synthons as outlined in Section 1.11. To achieve this the synthesis of chiral amino alcohols is considered using an engineered transketolase and transaminase pathway (Section 1.6). Selection of the target reaction and the rationale for the candidate transaminase enzymes choices is detailed in Section 1.8 and Section 1.10 respectively. In this chapter the cloning strategy and the production of active transketolase and transaminase catalyst in an *E.coli* host strain is described.

3.1 Introduction

The molecular cloning of a gene enables control of the gene product expression in a specific host organism. Over-expression of genes encoding useful biocatalytic enzymes can enable production of a desired catalyst in much higher titres leading to improved activity per unit biomass. Other advantages of using cloned genes are the use of a more suitable host organism than the native host, control of expression using an already characterised promoter, the potential to co-express genes from different organisms and the ease of further genetic manipulations of a sub-chromosomal plasmid, such as mutagenesis, to change the gene product characteristics. Over-expression of a desired protein is achieved by inserting several copies of the gene into a cell and/or using a strong promoter to control the expression of the gene. The increase in enzyme produced in a whole cell biocatalyst minimises the impact of any competing side reactions catalysed by other enzymes in the host organism. Subsequently catalytic yields may be improved due to minimised loss of substrate or product and downstream purification may be simplified by lower levels of structurally similar by-products.

A host organism should be safe and easily handled, such as a non-pathogenic strain of *E.coli,* but must also share common biochemistry with the native host, such as compatible codon usage, to enable production of the functional gene product. Cloning is particularly valuable in the transfer of a gene from a pathogenic organism, such as the opportunist pathogen *Pseudomonas aeruginosa* (Sadikot, R.T. *et al.*, 2005) to a non-pathogenic host strain. This opens the opportunity to use a gene

product without the need for high containment rated facilities. The use of an already characterised host can also greatly simplify the development of fermentation strategies. Genes from organisms that are not easily cultured may be cloned into hosts for which growth strategies have already been developed. The existing growth protocols can then be further refined to enable optimal production of the gene product. Use of a rapidly growing host not only increases the cell biomass achievable but also reduces the risk of fermentation contamination. A well-characterised host such as *E.coli* is easily manipulated by standard techniques. The use of subchromosomal plasmid constructs, which are easily introduced, propagated and isolated, enables discrete manipulations of the gene sequence as required. In this project the cloning of candidate genes from *E.coli* and *Pseudomonas* strains will enable the use of an *E.coli* host. The *E.coli* strain BL21gold(DE3) will be employed as the host strain. This strain has been modified to encode the T7 polymerase under the inducible control of the LacUV promoter enabling the use of a T7 promoter to control gene expression (Studier, W.F. and Moffatt, B.A., 1986). The strain is naturally deficient in the La protease (Chin, D.T. *et al.*, 1988) and has been further engineered to remove the OmpT protease (Grodberg, J. and Dunn, J.J., 1988) thus degradation of recombinant protein is reduced.

Native promoters may be tightly controlled by cellular processes and expression of the gene product only instigated under certain conditions. For example the native promoter of *E.coli* ArTAm has been found to be regulated by the tyrosine repressor (Collier, R.H. and Kohlhaw, G., 1972; Silbert, D.F. Jorgensen, S.E. and Lin, E.C., 1963). The use of an already characterised promoter enables more predictable expression. Additionally the ability to switch on gene expression, using a characterised inducible promoter, can enable higher expression yields where the product is detrimental to cell growth and survival. A reasonable cell density can be reached before the added metabolic burden of protein over production and any toxicity to the cells is experienced, thus enabling higher protein titres to be reached than if a protein is expressed earlier in a culture.

The *a priori* design and construction of a biosynthetic pathway in *E.coli* requires the co-expression of the pathway enzymes. In the case of amino-alcohol synthesis, the over-expression of transketolase to make asymmetric carbon-carbon bonds, and a

transaminase to transfer an amine group on to the product of the transketolase reaction product, is required. In order to over-express both a transketolase and a transaminase within the same host cell, alternative cloning strategies can be used. The first is having the two genes encoded on the same plasmid. The second gene can be placed directly after the first gene and be read through from the promoter of the first gene. This allows little scope for control of expression and the efficiency of the 'read-through' to the second gene may be unpredictable. Alternatively the second gene can be inserted into the same plasmid under the control of a second promoter. This may enable more flexibility allowing the two genes to be controlled separately therefore the expression levels or time of expression of the two genes can be different (Jensen, P.R. and Hammer, K., 1998; Ruhdal, P. and Hammer, K., 1998). Another approach employs the two genes carried on separate plasmids and most usefully under the control of separate promoters in the same host cell. For the stable existence of two plasmids within the strain, differences in two of the plasmids components are important. Firstly, in order for multiple plasmids to coexist in the cell without compromising the copy number of either plasmid, different origins of replication must be used. If two different plasmids with the same origin are used the numbers of both plasmids may be reduced (but not necessarily by the same amount) to meet the origin dictated copy number. Secondly there must be a different selectable marker on each plasmid to enable selection for cells harbouring each plasmids.

The dual plasmid system has the advantage of allowing the two genes, carried on separate compatible plasmids, to be individually manipulated (e.g. mutated) and then introduced into a shared host. This is advantageous for the construction of an initial system to demonstrate the model reaction allowing the construction of a plasmid carrying the transketolase gene, which can be introduced with the plasmid carrying the selected transaminase gene without any further cloning. Furthermore manipulation of the transaminase gene can be carried out independently to accept the product of the established transketolase step as required. Additionally, other enzyme candidates can be cloned into compatible vectors to construct alternative pathways. For example a non pBR322 vector encoding DoxP synthase (Sprenger, G.A. *et al.*, 1997) can be used to replace the TK construct to be investigated in a pathway with the transaminases cloned. The dual plasmid approach was thus employed in this

work and all cloning steps carried out with this in mind. This chapter describes the construction of expression plasmids for the TK and candidate transaminases and verification of active enzyme production. The co-transformation of the vectors encoding TK and the candidate transaminase to make the dual plasmid expression strain is described later (Section 6.2.) following the investigation of the candidate transaminases.

3.2 Subcloning and expression of TK

3.2.1 Subcloning of TK

An *E.coli* strain JM107, pQR706, over-expressing the endogenous transketolase gene (tkt) was obtained from Dr J. Ward, Department of Biochemistry and Molecular Biology, UCL. The pQR706 construct is composed of PCR-Script^{TM}, a pBR322 derivative, carrying the transketolase gene under the control of the native *E.coli* promoter. This strain was developed to improve production of transketolase for biotransformation applications (French, C., Ward, J.M., 1995). Expression of active TK in JM107, pQR706 was found to make up 24% of the total cell protein in a 100 mL overnight shake flask cultures. A closely related parent plasmid pQR700 (yielding 17% of total cellular protein at 100 mL scale) was used to study large-scale transketolase production. Significant quantities of active transketolase enzyme were obtained with the enzyme constituting ~ 43% of the total cell protein in a fed batch 1000 L fermentation (Hobbs, G.R. at al., 1996).

Many commercial plasmids are descended from pBR322. For the successful construction of a dual plasmid biocatalyst, it is required that one gene of interest is carried on a plasmid with a non-pBR322 derived origin of replication. Since pQR706, a pBR322 derivative, has already been demonstrated as a suitable expression vector for production of the TK catalyst it would have been desirable to insert the transaminase gene into a non-pBR322 descendant. This would enable the use of the existing TK expression construct. It was apparent, however, that using expression vector pMMB67HE, an ampicillin resistant (AmpR), non pBR322 derivative (Furste, J.P., *et al.*, 1986), provided convenient restriction sites (*BamH1* and *Sac1)* to receive the 2.4 kb transketolase gene, with promoter, cleaved from pQR706 with the same restriction enzymes in a direct translocation . Consequently

this subcloning was carried out and confirmed successful by sequencing (Section 2.4.1.1). The new construct was named pQR411 (Figure 3.1). This expression plasmid is 11.2 Kb in size. The large size is due to the long coding sequence in the vector required for broad host compatibility. This includes the IncQ replicon RSF1010, which can be mobilised by IncP helper plasmids to transform several Gram negative bacteria (Scherzinger, *et al.*, 1984). For use in the chosen *E.coli* host for this work these components are not required. However since the vector was stable no attempt to further modify it was deemed necessary at this stage.

3.2.2 Expression of active TK

The activity of transketolase is assessed by the analysis of the model reaction where TK catalyses the formation of *L*-erythrulose and carbon dioxide from β-HPA + glycolaldehyde substrates (Figure 3.2). An HPLC method to monitor TK activity, via the electrochemical detection of β-HPA and glycolaldehyde substrates and *L*-erythrulose product, was developed by refinement of an existing method (Mitra, R.K. and Woodley, J.M., 1996). The method duration was shortened from 45 minutes to 15 minutes thus improving the throughput of analysis (Section 2.5.2). This was achieved by the use of a single Aminex 87H column, where previously two had been employed, reducing the retention times of all three components. This was feasible due to the substitution of the refractive index detector, employed in the original method, for an electrochemical detector. Using this detector resolution of each component was achievable following separation on a single column and the TK catalysed reaction progress could be monitored. The measured decline in HPA and glycolaldehyde during the catalysis corresponds to the formation of *L*-erythrulose (Figure 3.3). The reaction repeatedly yields above 95% conversion of HPA to erythrulose (time scale depends upon substrate and enzyme concentration). This is as expected by the 1:1 substrate to product stoichiometry of the reaction (Figure 3.2). The reaction is able proceed to completion due to the formation of CO_2, which leaves the aqueous reaction in gaseous form under the experimental conditions, rendering the reaction irreversible.

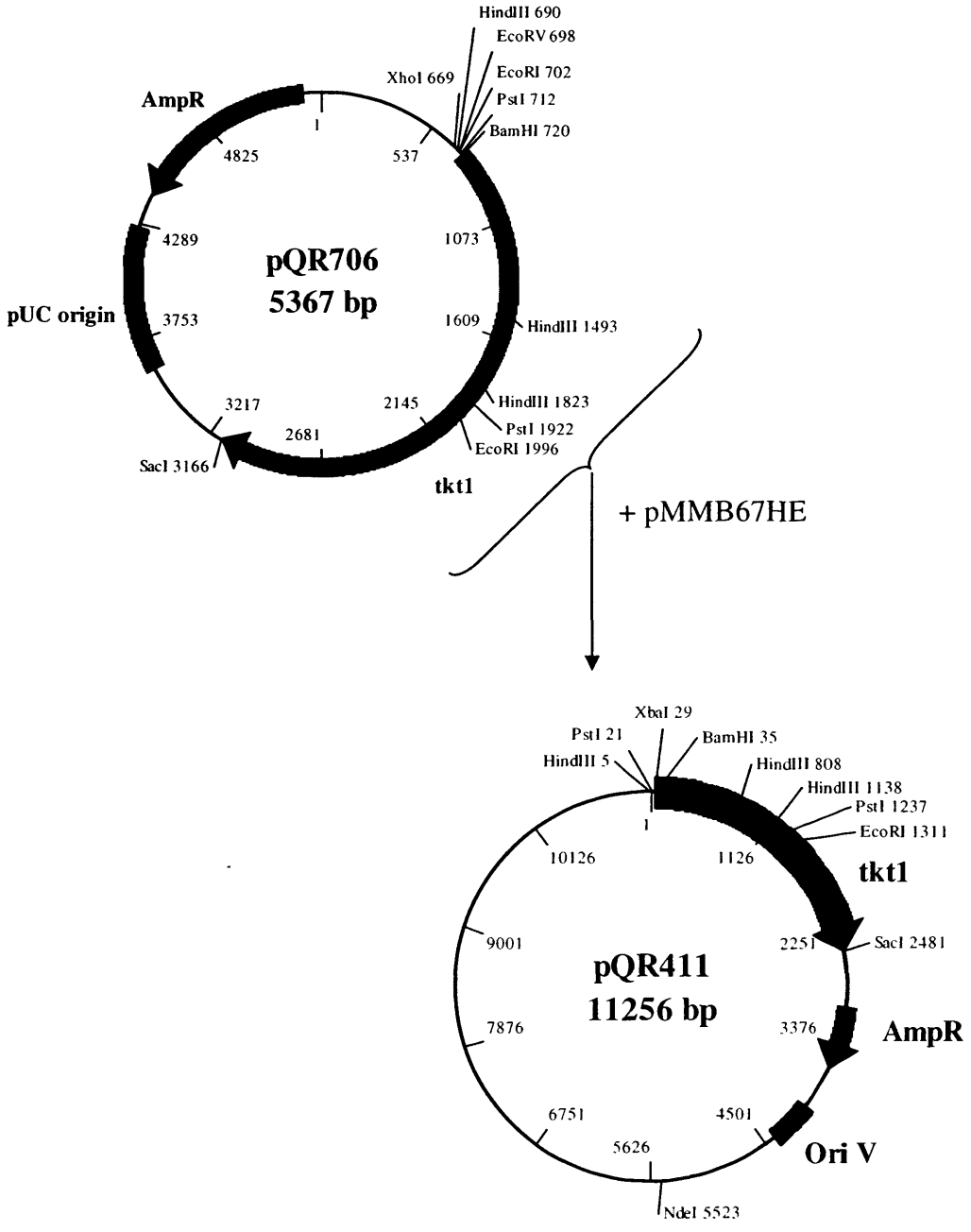


Figure 3.1: Construction of TK expression plasmid pQR411

The excision of the TK gene (including native promoter element) from pQR706, using restriction enzymes *SacI* and *BamHI,* was followed by ligation into the corresponding sites in plasmid vector pMMB67HE. This formed TK expression plasmid pQR411(AmpR). The subcloning is described in Section 2.4.1.1.

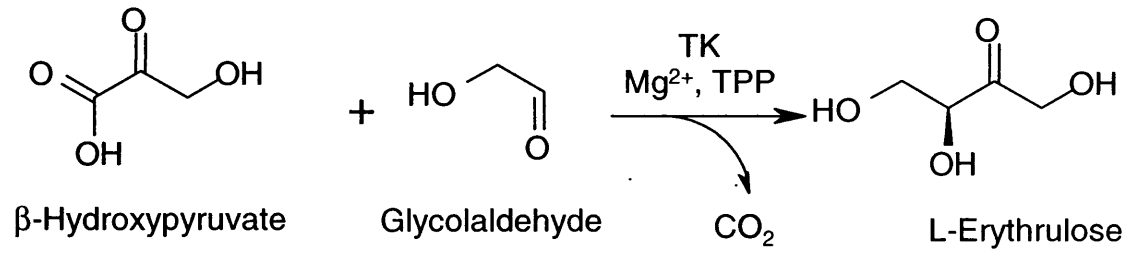


Figure 3.2: Model transketolase catalysed reaction

Transketolase catalyses the formation of an asymmetric carbon –carbon bond by the transfer of a 2-carbon ketol group to an aldehyde acceptor substrate. In the model reaction β-HPA is the ketol donor substrate resulting in the elimination of CO_2 rendering the reaction irreversible. The TK catalysed reaction, requiring TPP and Mg^{2+} cofactors, of β–HPA with glycolaldehyde results in the formation of *L*-erythrulose product.

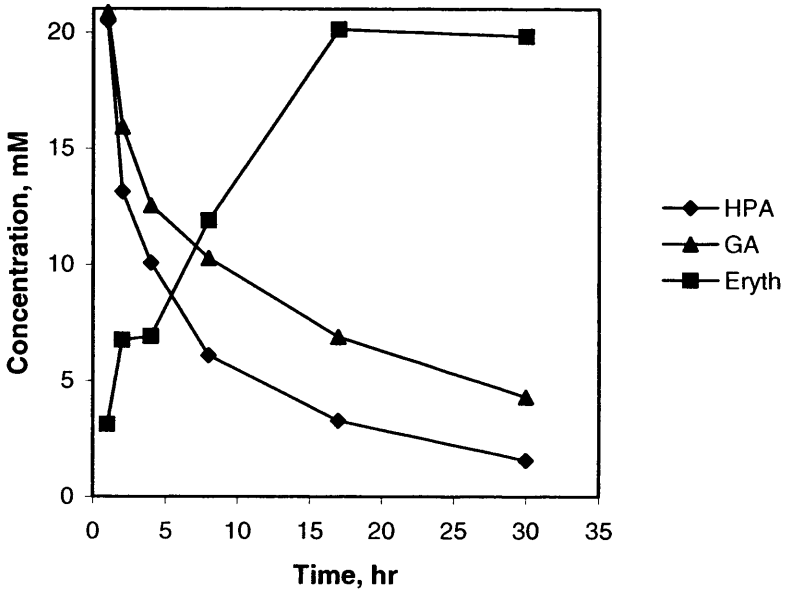


Figure 3.3: Profile of the TK model reaction

The reaction profile seen for the model TK reaction with 20mM β-HPA and glycolaldehyde substrates in 100 mM potassium phosphate buffer, pH 7.0 with cofactors thiamine pyrophosphate, 2.4 mM, $MgCl_2$, 9 mM, and pyridoxal phosphate, 2mM, at ambient temperature (~28°C) with agitation at 200 rpm (Section 2.11.2). Transketolase was added in lysate, giving a transketolase concentration of 440 μg/mL. Both substrates and products are monitored by HPLC with electrochemical detection (Section 2.5.2).

The production of active transketolase from the newly constructed plasmid pQR411 was assessed by activity assay with the model reaction relative to transketolase produced from the original strain. Cultures of the original TK strain JM107, pQR706, newly constructed JM107, pQR411 and the wild-type host strain control were grown overnight and diluted to comparable cell densities prior to lysis by sonication.

Pre-incubation of the lysates with cofactors TPP, 2.4 mM, and $MgCl_2$, 9 mM, was carried out to reconstitute the holoenzyme (Sprenger, G.A. *et al.*, 1995). Substrates β-HPA and glycolaldehyde, both 100 mM, were added and reactions incubated at 25°C. Reactions were monitored for β-HPA and glycolaldehyde depletion and *L*-erythrulose production by the refined HPLC assay (Section 2.5.2) over a four hour time period. Upon comparison of the erythrulose production of the strains (Figure 3.4) that harboured the new construct, pQR411, was found to produce transketolase activity significantly above that of the host strain not over-producing transketolase. Some production of *L*-erythrulose was evident from the wild-type host strain over the monitoring period. This is due to the endogenous TK of the bacterial host (Sprenger, G.A. *et al.*, 1995). The activity of the transgenic strain harbouring the pQR411 construct gave product formation at approximately half the rate of the original pQR706 construct. The new pQR411 strain reaching 60% conversion in the four hour monitoring period whilst the original pQR706 strain reached completion. The lower activity of the newly constructed strain can be attributed to the lower copy number of pMMB67HE, the parent vector of pQR411, relative to PCRScript™ parent vector of pQR706, resulting in less protein production. This difference in protein synthesis is observed by SDS-PAGE analysis of the cultures (Figure 3.5). Both strains carrying TK encoding plasmids show a strong band at 72 KDa as expected for the monomer that makes up the 145 KDa TK homodimer (Sprenger, G.A. *et al.*, 1995). This intense band was not evident in the JM107 wild-type lane. The band intensity (by eye) is lower for the strain harbouring the pQR411 construct, than for that carrying pQR706 at a comparable cell concentration. This lower TK concentration from JM107, pQR411 corresponds to the lower measured activity of this strain than that measured for JM107, pQR706 (Figure 3.3).

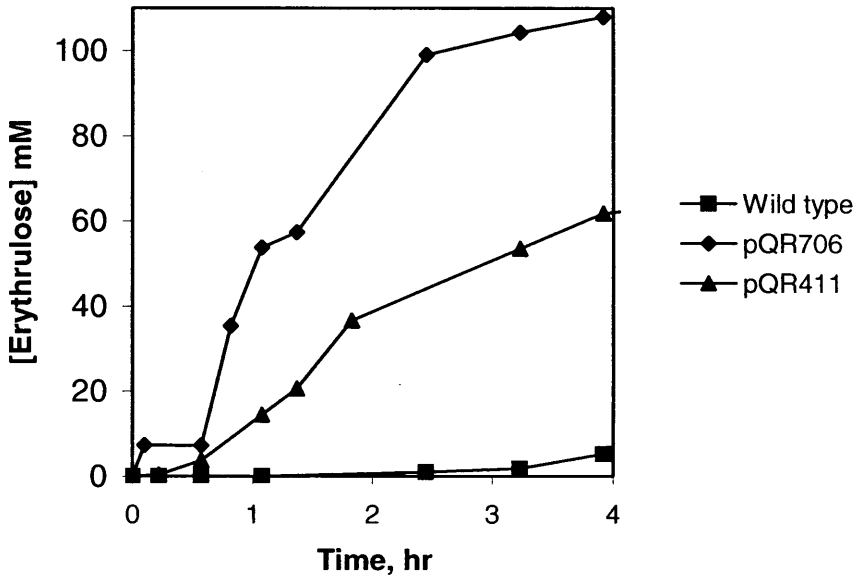


Figure 3.4: Comparison of the activity of TK produced from different plasmid constructs

Lysates of *E.coli* strains JM107 wild-type, JM107, pQR706 and JM107, pQR411 were monitored by HPLC for TK activity forming *L*-erythrulose from β-HPA and glycolaldehyde. Reactions were carried out with lysates from overnight cultures of comparable cell densities for each strain. Reaction contained β-HPA and glycolaldehyde, 100mM of each, cofactors TPP, 2.4 mM and $MgCl_2$, 9mM, in 50mM Tris HCL, pH 7.0 (Section 2.4.1.2).

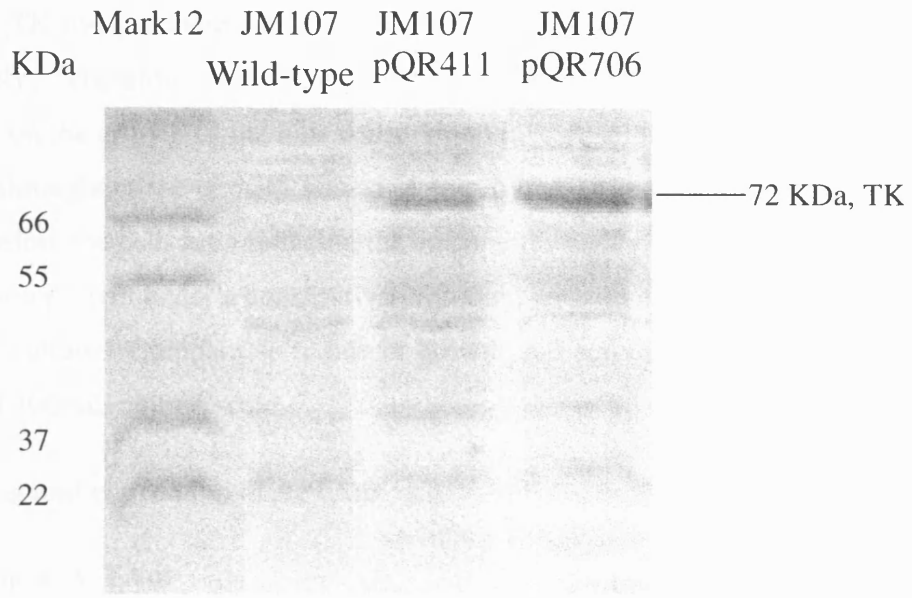


Figure 3.5: SDS-PAGE analysis of TK produced from *E.coli* JM107, pQR706 and JM107, pQR411

Samples of *E.coli* strains JM107 wild-type, JM107, pQR706 and JM107, pQR411 were diluted to comparable cell density and run on a 12% v/v acrylamide SDS-PAGE gel and stained with commassie blue. The intense band at 72 KDa corresponds to the overexpressed TK and was not seen in the wild-type JM107 control.

The SDS-PAGE analysis of BL21gold(DE3) pQR411 enabled quantification of the TK at 33 % of the cellular protein.

3.2.3 Growth-activity relationship of *E.coli* BL21gold(DE3) pQR411

The pQR411 construct was transformed into BL21gold(DE3) the chosen host *E.coli* strain for this project (Section 3.1). The growth of the recombinant strain constitutively over producing the TK catalyst reproducibly had the same growth characteristics as the wild-type host strain (Figure 3.6). The doubling time (t_D) during the exponential growth phase was calculated as 0.70 and 0.71 hours for the wild-type and TK overproducing strain respectively (or a μ_{Max} of 0.98 hr^{-1} and 0.97 hr^{-1} respectively). Therefore, production of the TK catalyst does not have significant adverse effect on the growth of the host cells. Upon investigation of the TK activity of the strain throughout the growth period it was found to increase with the cell density. Therefore the cells are producing the enzyme throughout the growth period, as expected for a protein under a constitutive promoter, without adverse effect on the growth of the culture. Comparable trends in growth and activity were observed at the 50 mL and 100 mL culture scale.

3.3 Cloning and expression of ArTAm

3.3.1 Cloning of ArTAm

The first transaminase candidate to be investigated for activity with the *L*-erythrulose product of the TK reaction was ArTAm from *E.coli* (Section 1.10) A strain overexpressing aromatic transaminase was not available 'in house' when this work commenced. Consequently cloning of the *E.coli Tyr B* gene encoding the aromatic transaminase (E.C.2.6.1.57) was undertaken (Figure 3.7). The *Tyr B* gene was amplified by PCR, from the genomic DNA of *E.coli* strain MG1665, without its native promoter. This was to enable expression of the gene to be under the control of an already characterised promoter commonly used for over-expression in *E.coli*. The gene product was captured with capture vector PCR TOPO 2.1 (Invitrogen), to form pQR400/pQR401. The presence of the *Tyr B* gene was confirmed by sequencing. These initial cloning steps were carried out by Dr J.Ward.

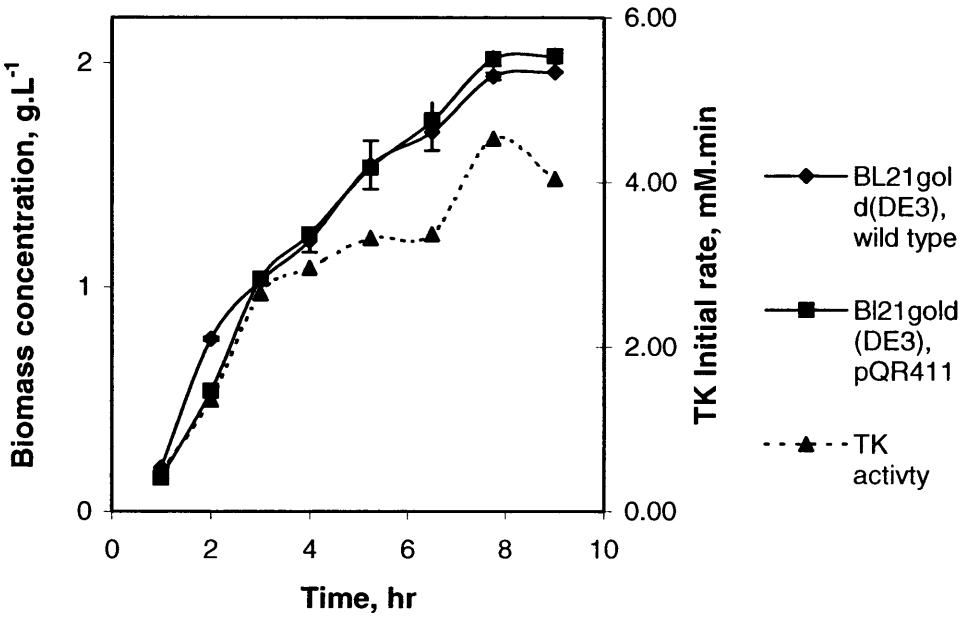


Figure 3.6: Growth and activity of TK expressing strain BL21gold(DE3) pQR411.

Cells were cultured, in 100mL nutrient broth with ampicillin, in a baffled shake flask at 37°C, 200rpm. Growth was monitored by $OD_{600\ nm}$ measurements and activity measured by analysis of the initial rate of the model reaction product, *L*-erythrulose, formation at each time point (Section 2.4.1.3).

The TOPO vector is designed to capture a PCR product generated with Taq polymerase, but can not function as an expression vector due to the absence of a ribosome binding sequence. In order to obtain expression of the *Tyr B* gene product insertion into an expression vector is required. Since transketolase was inserted into a non pBR322 derivative the transaminase gene could be cloned into an expression plasmid with a pBR322 derived origin (Section 3.1). Initially insertion of the *Tyr B* gene into kanamycin resistant pET24a(+), *Xho1* and *Nde1* sites, was attempted. Following many attempts no success was gained and an alternative approach was sought.

The PCR TOPO 2.1 capture vector has a pUC origin (pBR322 derivative) and carries a T7 promoter sequence close to the site of PCR product capture. However, it is deficient in the ribosome binding site thus the promoter is inactive. Consequently a second approach was carried out to convert this plasmid into an expression vector by the insertion of a ribosome binding sequence in front of the PCR product gene, inserted in the correct orientation with respect to the promoter. Plasmid pQR400 has the *tyrB* gene in the same direction as the plasmid T7 promoter (whilst the PCR fragment in pQR401 lies in the opposing direction). Insertion of the ribosome binding site sequence enabling the T7 promoter to be used to express the gene product was therefore possible by modification of plasmid pQR400. This was undertaken by the insertion of a 44 base pair oligonucleotide encoding the ribosome binding sequence for a T7 promoter sequence. To enable quick screening of transformants a *HindIII* restriction site was included into the oligonucleotide sequence. The presence of another single *HindIII* cleavage site elsewhere in the construct enabled detection of oligonucleotide insertion by *HindIII* digest of the plasmid yielding two fragments of 1296 bp and 3826 bp. Plasmid pQR400 without the insert would be linearised. This insertion of the ribosome binding site was successful and the resulting expression vector was named pQR415.

The pQR415 plasmid has both the β-lactamase gene conferring AmpR and the aminoglycoside phosphotransferase gene conferring resistance to kanamycin (KanR). In order to remove one of the antibiotic resistance genes further modification was required.

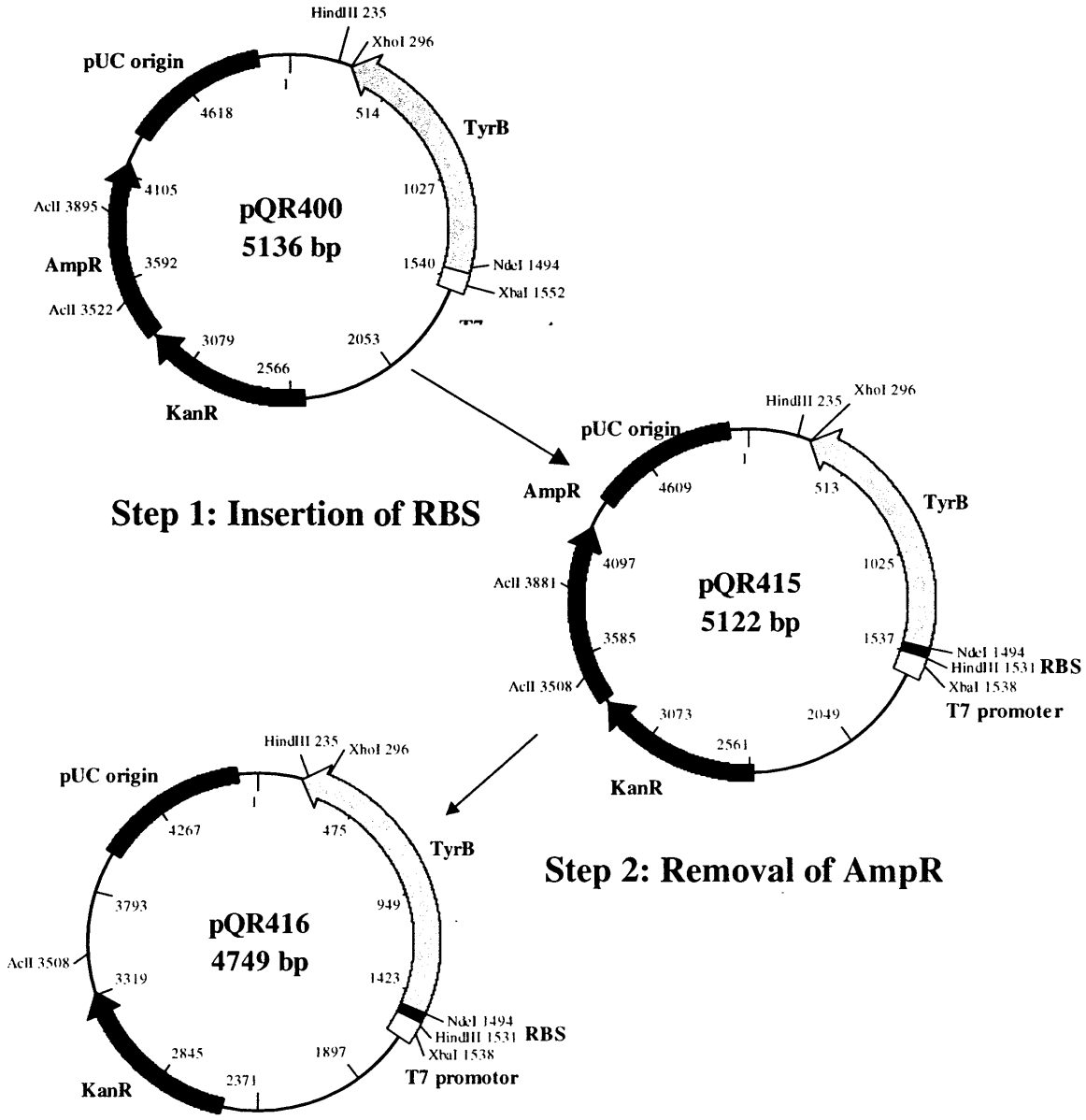


Figure 3.7: Construction of vector pQR416 for ArTAm expression

Plasmid pQR400 is constructed of the *tyrB* gene (PCR product) encoding ArTAm from *E.coli* captured by pCRTOPO 2.1 (Section 2.4.2.1). An oligonucleotide encoding a ribosome binding sequence was inserted, between the *Nde*1 and *Xba*1 restriction sites, to create pQR415. This is a functional expression vector. Further modification by digestion with *Acl*1 and re-circularisation was carried out to remove a Section of the β-lactamase gene conferring AmpR. This formed pQR416 a KanR plasmid encoding the ArTAm under the control of an inducible T7 promoter.

Since this vector was being made to potentially coexist with ampicillin resistant, TK encoding, pQR411 removal of the β-lactamase expression was required. Plasmid pQR415 was thus further modified to inactivate the ampicillin resistance gene. This was carried out by cleavage with *AclI* to remove a fragment from the β-lactamase gene. The resulting plasmid, pQR416 confers resistance to kanamycin and not ampicillin. The use of a T7 promoter required the use of a DE3 positive host strain carrying the T7 polymerase. Subsequently the plasmid was transformed into *E.coli* BL21gold(DE3) for the investigation of ArTAm expression.

3.3.2 Expression of active ArTAm

A convenient spectrophotometric enzyme linked assay for the activity of the ArTAm was initially used to assess TAm activity (Section 2.7.1). The aromatic transaminase can catalyse the transfer of the amine group from aspartic acid to an acceptor ketone substrate α-KG yielding glutamate and an oxaloacetate ketone product. This reaction can be coupled to the reduction of the carbonyl of the ketoacid group of the oxaloacetate, by lactate dehydrogenase, which requires an NADH cofactor. This consumption of oxaloacetate linked to NADH can therefore be measured at 340nm.

Using this assay, somewhat surprisingly, lysate from un-induced cultures of BL21gold(DE3) pQR416 was found to have activity thirty fold higher than that measured for the wild-type host strain at an equivalent cell density. Some activity was detected for the untransformed host due to the presence of the *TyrB* gene in the host genome. It was expected that prior to induction the measurable activity would be close to that of the wild-type activity. The T7 promoter requires the T7 polymerase encoded in the host (DE3) strain whose expression is induced from the lacUV5 promoter using IPTG (Studier, W.F. and Moffatt, B.A., 1986). Therefore without addition of the IPTG inducer the level of T7 polymerase should be minimal and therefore protein expression driven by the T7 promoter would be very low. It is probable that the production of ArTAm seen without induction is due to the leaky expression of the T7 polymerase. Induction of cultures with IPTG was expected to yield higher activity due to a large increase in protein production. However, induced cultures yielded half the activity seen without induction.

Upon investigation of ArTAm protein production by the strain by SDS-PAGE analysis it was confirmed that both un-induced and induced BL21gold(DE3) pQR416 cells were producing significant amounts of the ArTAm. It was therefore apparent that the transaminase is constitutively expressed from the construct due to the leaky expression of the T7 polymerase. Furthermore when assessing the soluble and insoluble fraction it was found that post induction much of the transaminase was in the insoluble fraction, whilst in un-induced cells the majority of the ArTAm remained in the soluble fraction. The increase in protein expression by induction is therefore leading to increased aggregation of the catalyst, which is detrimental to both the ArTAm activity and the growth of the host cells. In subsequent work the strain was therefore used to produce ArTAm without IPTG induction.

3.3.3 Growth activity relationship of BL21gold(DE3) pQR416.

Upon investigation of the growth of the ArTAm expressing strain BL21gold(DE3) pQR416 it was found that whilst the un-induced BL21gold(DE3) pQR416 strain exhibited slower growth than the wild-type BL21gold(DE3) culture it reached a comparable cell density over an 8 hour incubation period. When induced with IPTG the growth of the BL21gold(DE3) pQR416 was significantly retarded (Figure 3.8(a)). Growth ceased approximately 1-hour post induction thus reaching only half the cell density observed for the other cultures. Since it appears that production of ArTAm retards the growth of the cells, even in the un-induced strain, induction, further increasing the protein production, may well be expected to further inhibit growth. However the increase in protein production retarding the growth would be expected to yield higher activity. The trend of activity with respect to cell density may be expected to increase sharply as the catalyst concentration increases independently of the cell density. However, the culture at one hour after the time of induction, when the induced and un-induced cultures were at comparable cell densities, yielded ~70% of the activity of the un-induced culture (Figure 3.8(b)). This corroborates the data from the initial exploratory experiments (Section 3.3.2) to demonstrate active ArTAm production from the newly constructed strain.

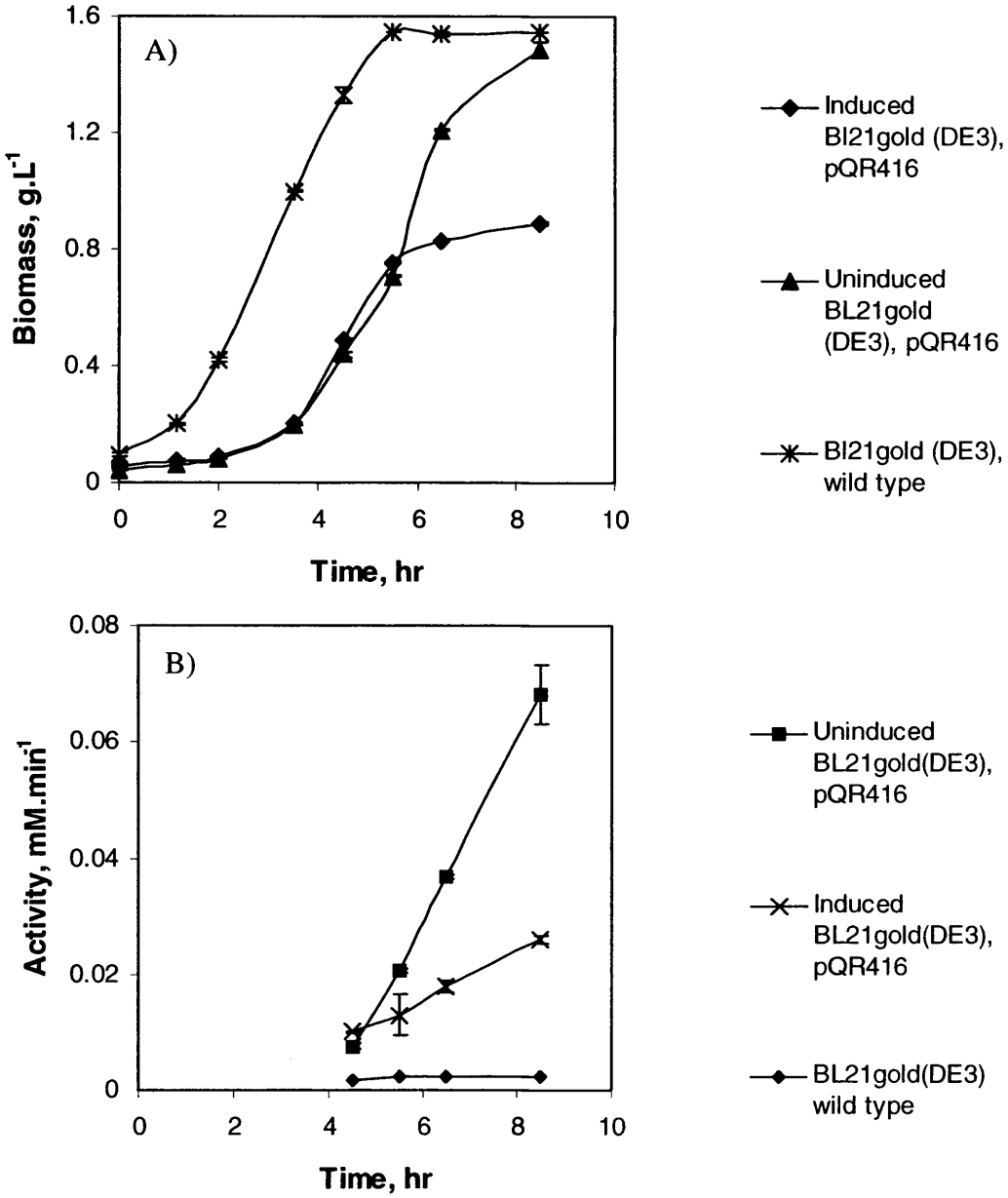


Figure 3.8: Investigation of growth of ArTAm producing strain BL21gold(DE3) pQR416.

The growth of BL21gold(DE3) pQR416 was monitored for cultures, 100 mL, grown from a 1/20 inoculum both induced, with 1mM IPTG at 4.5 hours, and un-induced alongside the wild-type host strain (Section 2.4.2.3). The growth (A) of the three cultures was monitored by measurement of cell density by OD 600nm. Activity (B) following induction was monitored by enzyme linked assay (Section 2.7.1).

3.3.4 Native activity of ArTAm

The native activity of ArTAm catalyses the transfer of the amine group from either aspartic acid or phenylalanine (Hayashi, H. *et al.*, 1993). With the activity of the cloned enzyme already confirmed with aspartic acid using the enzyme linked assay (Section 3.3.2) it was desirable that the activity with phenylalanine, a reportedly more favourable substrate (Hayashi, H. *et al.*, 1993), was demonstrated. The reactions were assayed by HPLC to monitor both the amine substrate and the glutamate produced by transamination of the α-ketoglutarate ketone substrate. The method developed for this (Section 2.5.3) was 14 minutes long and required amine derivatisation of the samples (Section 2.6.3) prior to injection to enable both separation on a C18 column and detection by UV. The activity of the ArTAm was high with the reactions going to equilibrium, with the production of 5 mM glutamate from 10 mM substrates, within one minute.

3.4 Cloning and expression of β-A: P TAm

3.4.1 Cloning of β-A: P TAm

The second transaminase candidate was β-alanine: pyruvate transaminase (Section 1.10.2) The β-alanine-pyruvate transaminase was cloned from the genomes of *Pseudomonas.* Following amplification of the PAO132 gene it was ligated into the *NdeI* and *XhoI* sites of expression vector pET24a(+). This places the gene under the control of the T7 promoter of the vector. This initial work was carried out by M.Bommer. The resulting construct was sequenced and named pQR426 (Figure 3.9). In parallel the β-A: P transaminase from *Pseudomonas putida* (PPO596) was also cloned into pET24a(+) to form expression construct pQR427. These plasmids were constructed to be compatible for coexistence with pQR411 carrying the transketolase gene (Section 3.1).

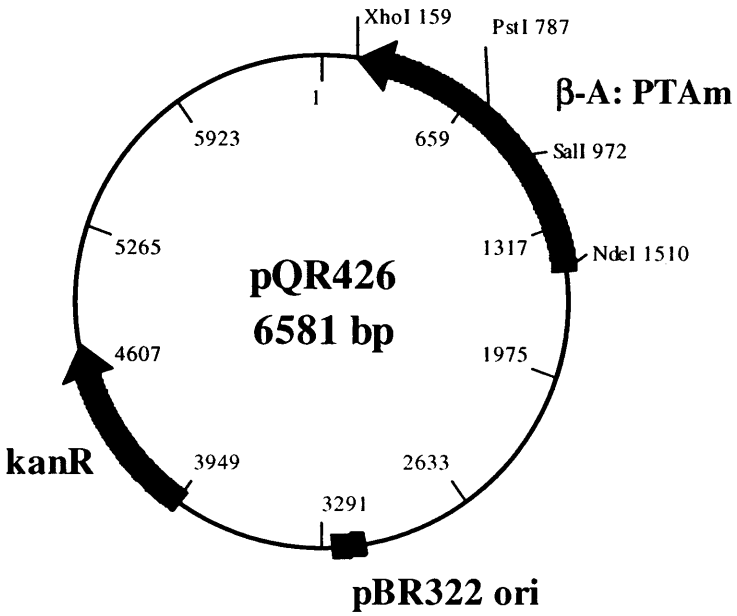


Figure 3.9: Expression vector for β-alanine: pyruvate transaminase

Expression construct pQR426 was constructed by the insertion of the β-alanine: pyruvate transaminase gene from *Pseudomonas aeruginosa* (PCR product from genome template) into the *Xho1* and *Nde1* sites of pET24a (+) (Section 2.4.3.1).

3.4.2 Expression of β-A: P TAm

The initial investigation into the expression of the two β-A: P transaminases cloned was carried out by SDS-PAGE analysis (Figure 3.10). Here the presence of a strong 50 KDa band, in induced cultures, comprising ~37 % of cellular protein, not present in the host strain, indicated the over-expression of the β-A: P transaminase protein from both *Pseudomonas putida* (lane 6) and *Pseudomonas aeruginosa* (lane 4). This band is the correct approximate size compared with the 48.4 KDa weight calculated from the amino acid composition. Additionally it corresponds to the ~50 KDa band seen for the single subunit that makes up dimeric amine: pyruvate transaminase from *Vibrio fluvialis* (Shin, J.-S. *et al.*, 2003). The β-A: P transaminase protein was found to be mainly in the soluble fraction by analysis of clarified supernatant (lane 3) compared to the pellet fraction (lane 2) for lysate from β-A: P TAm from *Pseudomonas aeruginosa*. This was similarly found for *Pseudomonas putida* (data not shown).

3.4.3 Native activity of β-A: P TAm

The activity of the β-A: P TAms from *Pseudomonas aeruginosa* and *Pseudomonas putida* was verified by reaction with the native substrates, pyruvate and β-alanine (Figure 3.11). Activity was confirmed by monitoring the depletion of β-alanine substrate and formation of α-alanine product (Figure 3.12) by HPLC analysis (Section 2.5.4). The use of a curved acetonitrile gradient on a C18 column enabled the separation of ACQ derivatised (Section 2.6.3) β- and α-alanine. Although baseline resolution was not achieved (Appendix D) the ability to see both substrate depletion and product formation made this assay accurate enough to quantitatively assess enzyme activity. The reaction initially proceeds quickly but levels off after an hour and a half at ~ 30% conversion. Both transaminases were found to have comparable activity with this substrate pair. The wild-type host control strain showed negligible α-alanine product formation (or loss of β-alanine substrate) over the reaction period. Comparable activity was obtained for the β-A: P TAm following storage at –20°C overnight as both lysate or whole cells.

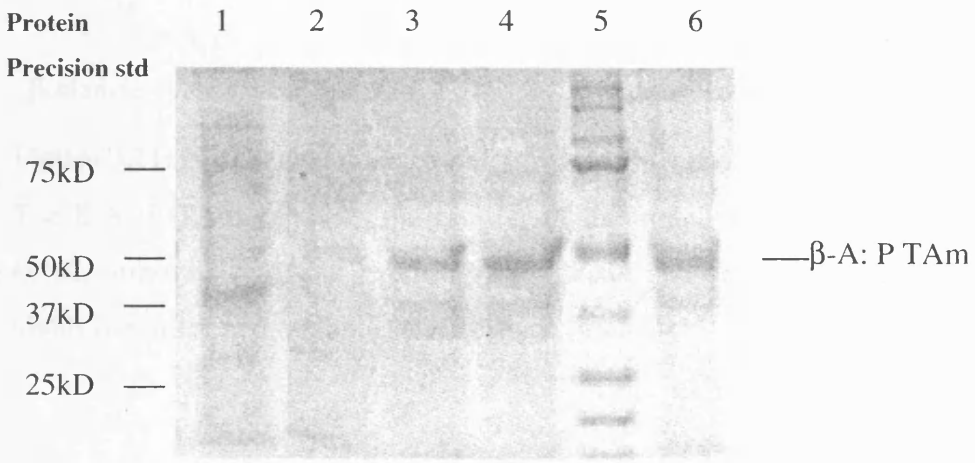
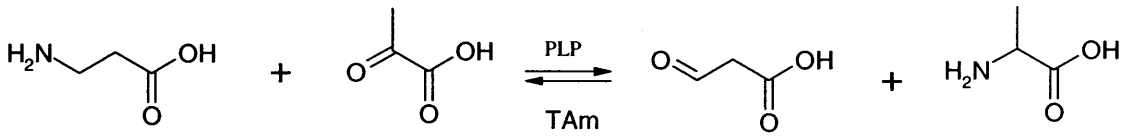


Figure 3.10: Analysis of β-A: P TAm expression

The expression of *Pseudomonas aeruginosa* β-A: P TAm from the constructed *E.coli* strain BL21gold(DE3) pQR426 was analysed on a 12% v.v acrylamide SDS-PAGE gel and stained with commassie blue (Section 2.3). Both the soluble and insoluble fractions were examined for the transaminase. Sample lanes: 1= wild-type BL21gold(DE3) 2= BL21gold(DE3) pQR426 insoluble fraction, 3= BL21gold(DE3) pQR426 soluble fraction, 4= BL21gold(DE3) pQR426 lysate, 5=MW marker, 6= BL21gold(DE3) pQR427 (β-A: P TAm *P. putida*). Strains over expressing β-A: P TAm show a prominent band at 50 KDa not present in wild-type host strain (lane 1).



β-alanine pyruvate malonic semialdehyde α-alanine

Figure 3.11: Native reaction scheme for β-A: P transaminase

The β-A: P TAm catalyses the transfer of the amine group from β-alanine to the ketone substrate pyruvate requiring a pyridoxal phosphate cofactor. This reaction yields α-alanine and malonic semialdehyde products.

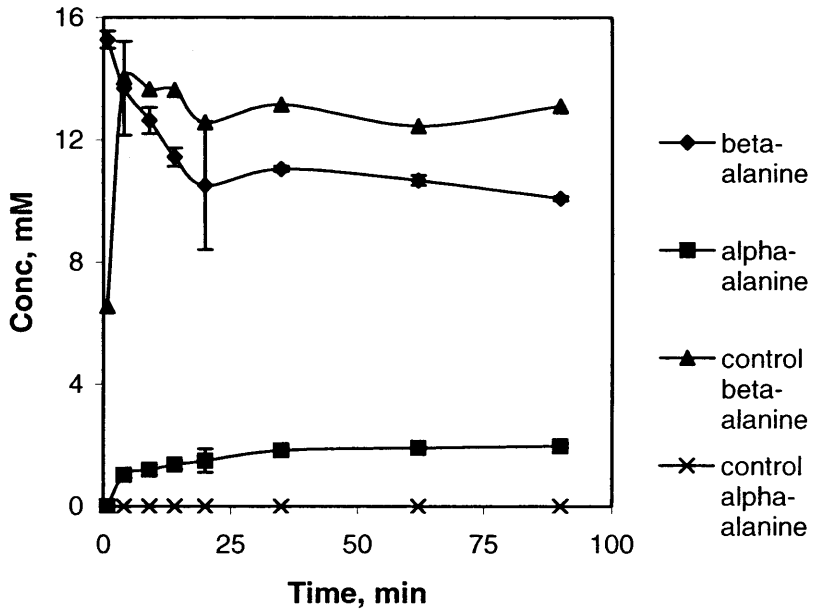


Figure 3.12: Activity of β-A: P TAm from *P. putida* with β-alanine and pyruvate

The lysate of BL21gold(DE3) *E.coli* strains over producing β-A: P TAm were assayed for transamination of pyruvate forming α-alanine using a β-alanine amine donor substrate. Reactions contained 15mM β-alanine, 15mM pyruvate, 2mM PLP in 25mM potassium phosphate buffer, pH7.0 and were incubated at 25°C (Section 2.4.3.4). Error bars represent a 95% confidence interval.

A second test for activity was carried out using a spectroscopic enzyme-linked assay (Burnett, G. *et al.*, 1980). This uses β-aminobutyric acid as the amine donor substrate and pyruvate as the ketol-donor substrate. The generation of β-hydroxybutyrate allows coupling with NADH-dependant β-hydroxybutyrate dehydrogenase (E.C..1.1.30) allowing the reaction to be monitored spectroscopically at 340nm (Section 2.7.2). This assay would provide a rapid method to assess the acceptance of different ketone substrates by the candidate transaminases. This would enable high-throughput analysis and may also be applicable to the screening of mutant libraries. The activity with the β-aminobutyrate substrate was confirmed for both of the cloned β-A: P TAms. However, the background signal of this assay was found to be very high with the wild-type host strain exhibiting half the rate detected for the transgenic strains.

3.4.4 Growth activity relationship of BL21gold(DE3) pQR426

Investigation into the growth characteristics of the *P. aeruginosa* β-A: P TAm producing strain BL21gold(DE3) pQR426 were carried out relative to the wild-type host strain (Figure 3.13). The induction of β-A: P TAm expression in the recombinant strain retarded host cell growth reaching just 55% of the cell density achieved by the wild-type host strain. Activity of the β-A: P TAm, not detectable in the host strain, followed a similar trend to the growth profile reaching near maximal activity 4 hours following induction and rising little after that.

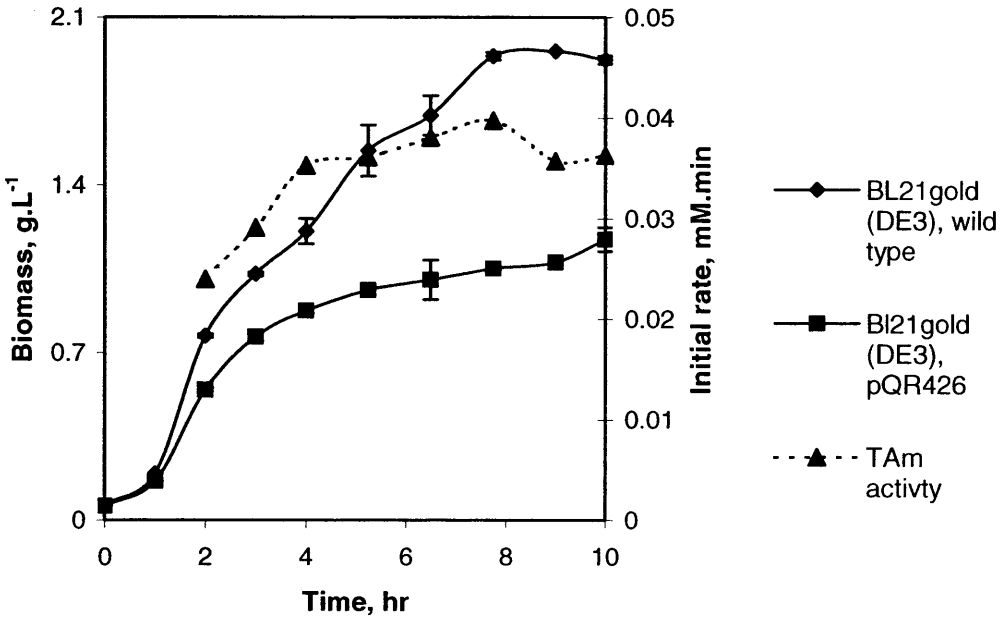


Figure 3.13: Growth activity relationship of β-A: P TAm expressing strain BL21gold(DE3) pQR426

The growth of BL21gold(DE3) pQR426 over expressing β-A: P TAm from *P.aeruginosa* was monitored alongside the host strain. Incubation was at 37°C with agitation at 200 rpm. Induction was carried out by addition of 1mM IPTG after one hour of growth. Growth was monitored by measurement of OD_{600nm} and activity was assayed by reaction with the β-alanine and pyruvate (Section 2.4.3.5).

3.5 Summary

This chapter describes the construction of plasmid vectors for the expression of the candidate enzymes. TK was subcloned from the existing vector pQR706 into an alternative expression vector, pMMB67HE, to form pQR411 (Section 3.2.1). Active enzyme production (Section 3.2.2) was confirmed using the model reaction with substrates β-HPA and glycolaldehyde. The growth of the strain carrying the pQR411 was found to be comparable to that of the host strain and TK was produced constitutively throughout the growth period of the culture (Section 3.2.3).

The ArTAm from *E.coli* was cloned in to pCRTOPO2.1 which was further modified to form expression vector pQR416 (Section 3.3.1). Once transformed into the BL21gold(DE3) host strain production of active over expressed protein was initially demonstrated, without the need for induction, using both an enzyme linked assay (Section 3.3.2) and an HPLC assay (Section 3.3.4), enabling different substrates to be investigated. The production of the transaminase in un-induced cultures was seen to slightly inhibit the rate of cell growth, due to inclusion body formation, but the culture reached a comparable cell density as the non-recombinant host strain over an eight hour growth period. Induction of transaminase expression led to the culture reaching only 55% of the cell density of the un-induced culture. Additionally the ArTAm activity of the induced culture was less than 50% of that observed with out induction therefore induction was not advantageous to the production of active enzyme.

The β-A: P TAm from *Pseudomonas aeruginosa*, in parallel with *P.putida* β-A: P TAm, was cloned directly into a pET vector forming pQR426 and pQR427. Expression was under the control of the T7 promoter therefore requiring induction of the T7 polymerase by IPTG. Activity of the expressed β-A: P transaminases was confirmed (Section 3.4.3) by monitoring the reaction with native substrates β-alanine and pyruvate by HPLC (Section 2.5.4). The growth-activity relationship showed that the activity of the transaminase increased sharply following induction and levelled off after 3 hours. This was following the same trend as the growth which was severely retarded upon induction reaching only 55% of that achieved by the native host strain.

The cloning of the two candidate transaminases and subcloning of transketolase has produced the vectors with which a dual TK-TAm expression system can be created (Section 6.2). Prior to construction of the dual plasmid strain further investigation of the substrates ranges of the transaminases will be carried out as described in the next chapter.

Chapter 4 - Substrate specificity and mutagenesis of candidate transaminases

The previous Chapter detailed cloning and expression of various candidate transaminases. The ArTAm, from *E.coli*, and the β-A: P TAm, from both *Pseudomonas aeruginosa* and *Pseudomonas putida,* were each cloned into a pBR322 derived expression vector compatible for co-existence with the constructed TK plasmid. The enzymes were over-expressed separately in the *E.coli* strain BL21gold(DE3) and demonstrated to be active. In this Chapter the acceptance of the transketolase product *L*-erythrulose by the candidate transaminases, for use in the TK-TAm biosynthetic pathway, is described.

4.1 Introduction

Enzymes are selective towards the substrates that they accept for catalysis. This selectivity involves chemo, regio, and stereoselectivity. Catalysis of a particular compound is dependent on both the chemical properties and the physical structure of the compound being compatible with insertion into the active site in such a way that catalysis can occur. Chemically unfavourable interactions of the substrate and the active site residues may disrupt substrate binding. The binding of a substrate may involve very few contacts with the active site residues. Any repulsion between the substrate molecule and the amino acids lining the active site may be sufficient to disrupt these interactions or cause a change in enzyme conformation making catalysis unfavourable. Furthermore, many enzymes exist in a distinct open and closed structure with the closure being induced by the substrate binding, an example of such an enzyme is aspartate transaminase (Hayaishi, H.,*et al.*, 2003; Jager, J. *et al.*, 1994) a close relative of ArTAm. The open conformation allows the substrate molecule to pass into the active site before the structural change into the closed confirmation required for catalysis. This conformational change may be reliant on the contacts between the substrate molecule and certain residues of the enzyme. Once in the active site, the alignment of the group at which catalysis is to occur must be in sufficient proximity of the catalytic residues of the enzyme to allow the necessary chemical interactions to occur. A molecule can have more than one group of a certain chemistry but catalysis is regiospecific to that which correctly aligns with

the catalytic residues. A preference for either the use of a chiral substrate or the production of a chiral product is also dictated by the positioning of the molecule within the active site. An enantiomer that is not accepted as a substrate may either not be able to bind in the active site due to steric hindrance, or may unfavourably position the target site of catalysis relative to the active site residues.

Enzymes with a known function are often discovered as part of a metabolic pathway and the so-called 'native activity' is described by this function. It is often the case that the substrate range of a particular catalyst is only explored as far as the interests of a particular investigator. The lack of reports on the activity of an enzyme with a particular substrate is often not a good indicator that it does not react. The search for a suitable transaminase involved the investigation of two transaminases (Section 1.10). Both were evaluated on their ability to accept *L*-erythrulose and other molecules that share structural similarity to transketolase products. This provides not only information about the suitability of the catalyst for the desired reaction but also possible information about the enzyme:substrate interactions eluding to other potential substrate candidates. Additionally the development of a mutagenesis strategy was investigated. This was in order to enhance the activity of the enzyme with the desired substrate. This Chapter is therefore divided into four sections. The first (Section 4.2) details the synthesis of the pathway product and assay development, the second (Section 4.3) probes the substrate range of ArTAm, the third (Section 4.4) investigates the mutagenesis, both random and site directed, of ArTAm and the fourth (Section 4.5) looks at the substrate range of β-A: P TAm.

4.2 Product synthesis and analytical assays

4.2.1 Synthesis of 2-amino-1,3,4-butanetriol product standard

In order to develop analytical assays to quantitatively monitor the bioconversions, standard compounds for both substrates and products are required. The target pathway product 2-amino-1,3,4-butanetriol (ABT) was found not to be commercially available, thus it was achieved by chemical synthesis (Section 2.6.1).

Initially a reductive amination of *L*-erythrulose was carried out using ammonia and a sodium cyanoborohydride catalyst in methanol at pH 6.0. The formation of ABT was monitored by TLC and a sample isolated from a preparative plate was confirmed by mass spectrometry. However, due to the product being soluble in aqueous solution, isolation proved difficult. Protection of the hydroxyl groups using tert-butyl-dimethyl-silyl chloride (TBDMS) was carried out to enable extraction into an organic solvent and separation on a silica column. However the isolation of any significant quantity of the product was not achieved.

A second approach was therefore investigated (Figure 4.1) which was adapted from published methodology (Dequeker, E. *et al.*, 1995). Following the conversion of *L*-erythrulose, **1** to the 3, 4 *–O* – isopropylidene acetal **2**, reductive amination was carried out using benzylamine and sodium cyanoborohydride to yield **3** as a mixture of diastereomers. De-protection of **3** was carried out with methanolic HCl to afford the aminotriol hydrochloride **4,** which was subsequently subjected to hydrogenolysis to yield the desired 2-amino-1,3,4-butanetriol product **5** as a 1:1 mixture of *threo* and *erythro* diastereomers. It should be noted that this synthesis allows for the resolution of diastereomers at intermediate **3** should this be considered appropriate. This was not carried out at this stage due to the lack of *L*-erythrulose starting material.

4.2.2 Analysis of 2-amino-1,3,4-butanetriol

The development of methods to enable quantitative analysis of the pathway product ABT was required. The previously developed method for β-A: P TAm activity (Section 2.5.4), following derivatisation, resolved the products from the native substrates of both the transaminases. This assay was found to also resolve the ABT. Baseline resolution of the diastereomers was not achieved but the resolution was considered sufficient at this stage.

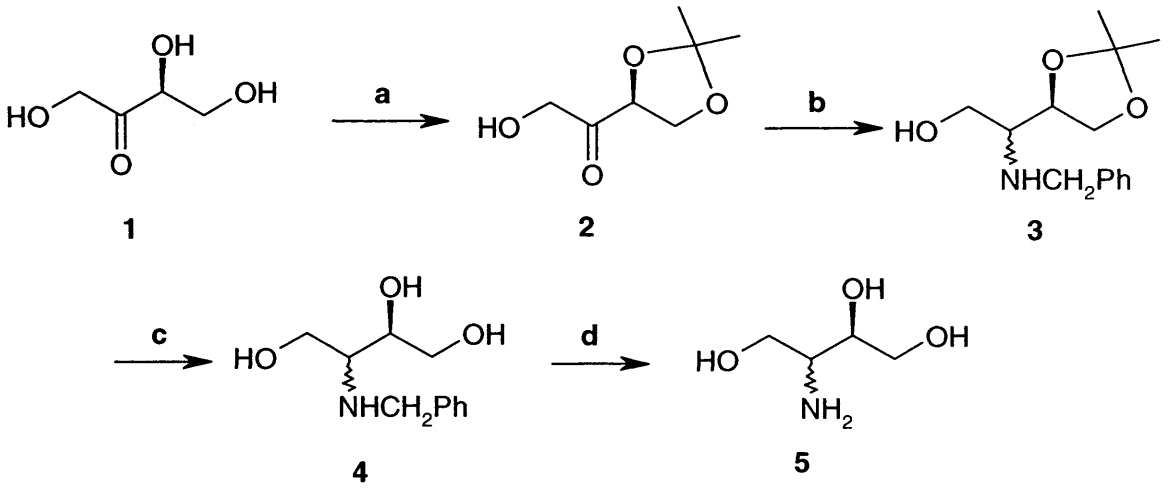


Figure 4.1: Synthetic pathway for 2-amino-1,3,4-butanetriol product standard.

a) Me_2CO/2,2-DMP (9:1), TsOH (cat.); b) $BnNH_2$, $NaCNBH_3$, MeOH, pH 6; c) 1 M HCl/MeOH; d) H_2, Pd/C, MeOH. Method described in section 2.6.1.

4.3 Substrate specificity of ArTAm

4.3.1 Activity with *L*-erythrulose

The activity of the cloned ArTAm from *E.coli* was previously demonstrated with the native substrates *L*-aspartic acid, or *L*-phenylalanine, and α-ketoglutarate (Section 3.3.4). The investigation into the acceptance of other substrate compounds was carried out under conditions (Section 2.8) where the native conversion, with *L*-phenylalanine and α-ketoglutarate, with lysate went to equilibrium (50% conversion) within one hour (Section 3.3.4).

The activity of the aromatic transaminase with target substrate *L*-erythrulose was initially investigated. A reaction containing *L*-erythrulose, and *L*-phenylalanine, 10 mM each, was carried out. The reactions were monitored for ABT formation over a 24 hour time period, but no activity was observed. A final sample taken at 72 hours again showed no detectable product formation. A second experiment, in which acceptance of *L*-erythrulose with aspartic acid was tested, corroborated the initial results with no detectable product formation.

4.3.2 Activity with serine

Serine has previously been reported to be a poor substrate for the ArTAm (Hayashi, H. *et al.*, 1993). However, it is a desirable amine source for the transaminase in the linked TK-TAm pathway, as transamination using a serine amine donor would yield β-hydroxypyruvate as the ketone product. This could feed back into the beginning of the pathway as a transketolase substrate. This would not only regenerate the TK substrate but would also enable the ArTAm reaction to be pulled past the 50% equilibrium by removal of one transamination product. The activity of ArTAm with serine as the amino donor was tested initially with phenylpyruvate. The reaction was monitored by analysis of the phenylalanine product. No appearance of the product was detected. This was in keeping with the results of another reaction of ArTAm with serine and α-ketoglutarate where the formation of the glutamate transamination product of α-ketoglutarate was not detected. However, in this reaction it was possible to monitor both the formation of glutamate and the consumption of serine substrate. It was noted that, whilst no glutamate product was detected, the serine

substrate was being consumed. The appearance of a peak co-eluting with ammonia suggested that the serine substrate was being deaminated but was not further investigated.

4.3.3 Activity with aromatic amine donors

The key differences between compounds known to be accepted as aromatic transaminase substrates (Hayashi, H. *et al.*, 1993) and TK generated substrates, such as *L*-erythrulose, are the presence of a hydroxyl group (from the β-HPA) instead of an acid group adjacent to the keto group (i.e. a non keto acid) and the hydroxyl branching off the main carbon chain at the-position adjacent to the carbonyl to be transaminated (Figure 4.2) generated by the TK catalysed carbon-carbon bond formation.

A number of aromatic amine substrates (commercially available) with the structural and chemical characteristics of the TK products (Figure 4.3), were tested as substrates with the ArTAm. Aromatic substrates were chosen since the reported activity of the enzyme with *L*-phenylalanine is many fold greater than with aspartic acid (Hayashi, H. *et al.*, 1993). Therefore the use of an aromatic substrate may improve the chances of compounds, lacking the keto acid group or with the addition of a β-hydroxyl, binding and reacting with the enzyme. The presence of the aromatic ring also makes direct analysis possible, allowing shorter assay development and both substrate loss and product formation to be monitored in a single assay, without the need for derivatisation.

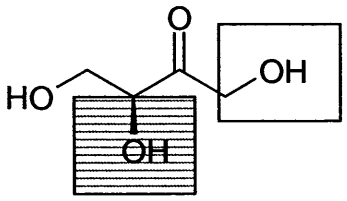
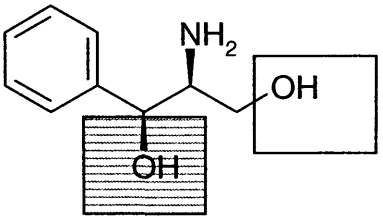


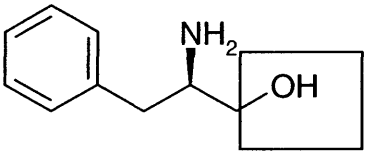
Figure 4.2: Illustration of key differences between *L*-erythrulose and known ArTAm substrates

The boxes illustrate the differences between known ArTAm substrates and the target substrate. The striped box highlights the hydroxyl branching off the main chain adjacent to the carbonyl group. The clear box highlights the presence of a hydroxyl instead of an acid group which would form the keto acid functionality.

Phenylaminopropandiol, **1**



Phenylaminopropanol, **2**



Phenylserine, **3**

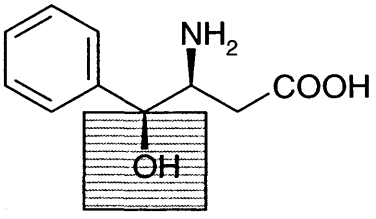


Figure 4.3: Alternative aromatic substrates for investigation with TAm

Boxes as described in legend to Figure 4.2

Phenylaminopropandiol,**1**, is the aromatic equivalent of *L*-erythrulose. However it may be a more favourable substrate for ArTAm than *L*-erythrulose due to the presence of the benzyl ring. Phenyl aminopropanol, **2**, is a step towards the known phenylalanine substrate with no branching hydroxyl, however the favoured keto acid moiety is still lacking. Conversely, phenyl serine, **3**, is a ketoacid but differs from phenylalanine by the presence of the hydroxyl at the carbon adjacent to the carbonyl. Although in the case of the target pathway the ketone would be the substrate, only the amine forms of the compounds were commercially available. Since the equilibrium constant of the enzyme is typically found to be 1, the reversible nature of this transaminase suggests that activity with an amine could be used as an indicator towards the acceptance of the corresponding ketone as a substrate for this enzyme.

4.3.3.1 Investigation into acceptance of phenylserine as a substrate

The reaction of ArTAm with *D-/L-threo*-phenylserine and phenylpyruvate yielded formation of phenylalanine product (Figure 4.4). The recombinant strain showed formation of 2 mM phenylalanine product within one hour. This then levelled off and no increase in product was seen in a four and a half hour period. The wild-type host strain also showed phenylalanine production but at a much lower rate, this is probably due to catalysis of the reaction with phenylserine by the chromosomal ArTAm in the *E.coli* host.

The yield of the product did not reach the 50% equilibrium normally observed for catalysis by ArTAm. This can be attributed to the *D-/L* nature of the substrate amine; half of the amine is not a substrate to the transaminase, which exclusively accepts L amino acids. The theoretical maximum yield for this substrate is therefore 25%. The observed yield of 18% is a little lower than the expected maximum yield. An investigation into whether it was inhibition that halted the reaction short of the theoretical maximum conversion for this particular form of the substrate was carried out. Identical reactions were set up (in triplicate) with 10 mM and 5 mM substrate.

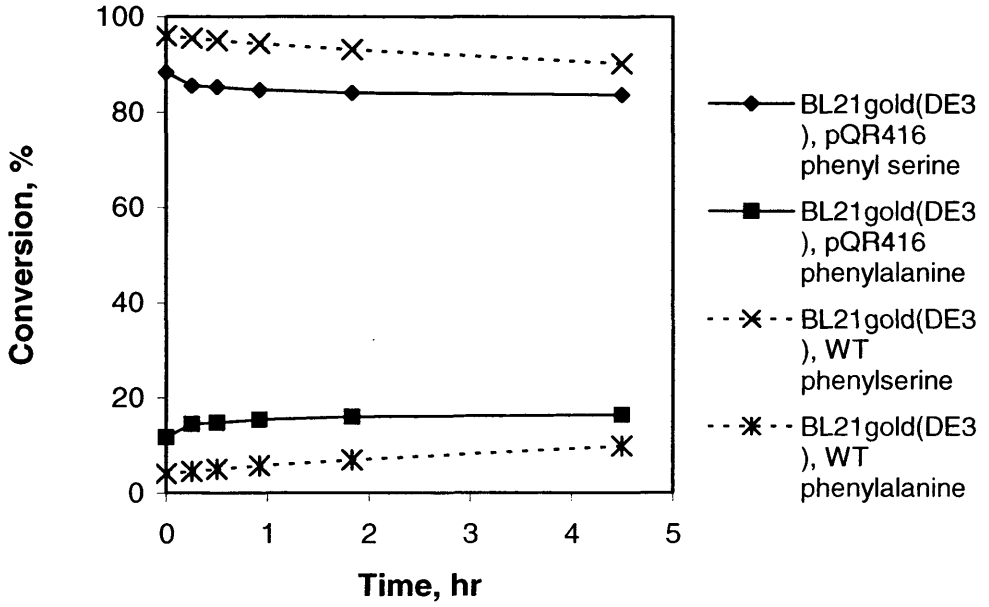


Figure 4.4: The activity of ArTAm with *D-/L*-threo β-phenyl serine

The activity of the ArTAm with *D-/L*-threo-β-phenyl serine was tested with phenyl pyruvate, both at 10mM each in 25 mM potassium phosphate buffer, pH 7.0 with 10 mM of the keto acid and amino acid substrates, 0.2 mM pyridoxal phosphate cofactor at 25°C with agitation at 200 rpm. (Section 2.8). The reaction was monitored by HPLC for both phenylserine depletion and phenyl alanine formation (Section 2.5.5). WT = *E.coli* host strain without a plasmid.

The reactions were monitored until steady state was reached after 4 hours. The steady state point for the reaction with 5 mM substrate was approximately half that of the 10 mM reaction (Figure 4.5a). Normalising the results showed (Figure4.5.b) that increasing the original substrate charge from 5 mM to 10 mM reduces the maximum percent conversion reached by 4 %. This could imply that one of the components of the transamination is inhibitory.

It has previously been noted that the *erythro* form of a *L*- phenylserine binds and gives a distinctly different absorption spectra to that for the *threo* form of *L*-phenylserine or other ArTAm substrates (Hayashi, H. *et al*, 1996). During the same study, when reactions were carried out in a stopflow reactor, it was observed that little of the PMP-ArTAm was produced with the *erythro*-phenylserine. This was postulated to be due to the high affinity of the oxo-acid product for the enzyme. An intense quinoid absorption around 500 nm was seen for the *erythro* form not seen in the absorbance spectrum for the *threo* isomer. This stabilisation of the quinoid intermediate by the interaction of the branching-hydroxyl, in the *erythro* configuration, may be extremely detrimental to catalysis. An investigation into the removal of the hydroxy functionality at tyrosine 70 (Hayashi, H. *et al.*, 1996) found that this destabilised the quinoid intermediate formation with *erythro* phenylserine, yielding a decrease in the quinoid absorption band at 500 nm. Accordingly the measured free energy levels of the quinoid intermediates showed a large decrease upon the Y70F mutation for the phenylserine substrate whilst for a phenylalanine substrate this mutation showed little change. The interaction of the branching hydroxyl with tyrosine residue 70 accounts partly for the stabilisation of the quinoid intermediate and the remaining stabilisation energy is due to interactions with other active site residues. Therefore, it is possible that the non-reacting diastereomer of the *D*-/L *threo* phenylserine, the *D*-amine which will have the hydroxyl in the equivalent position to the *L*- *erythro*-phenylserine, is entering the active site and binding, therefore acting as an inhibitor.

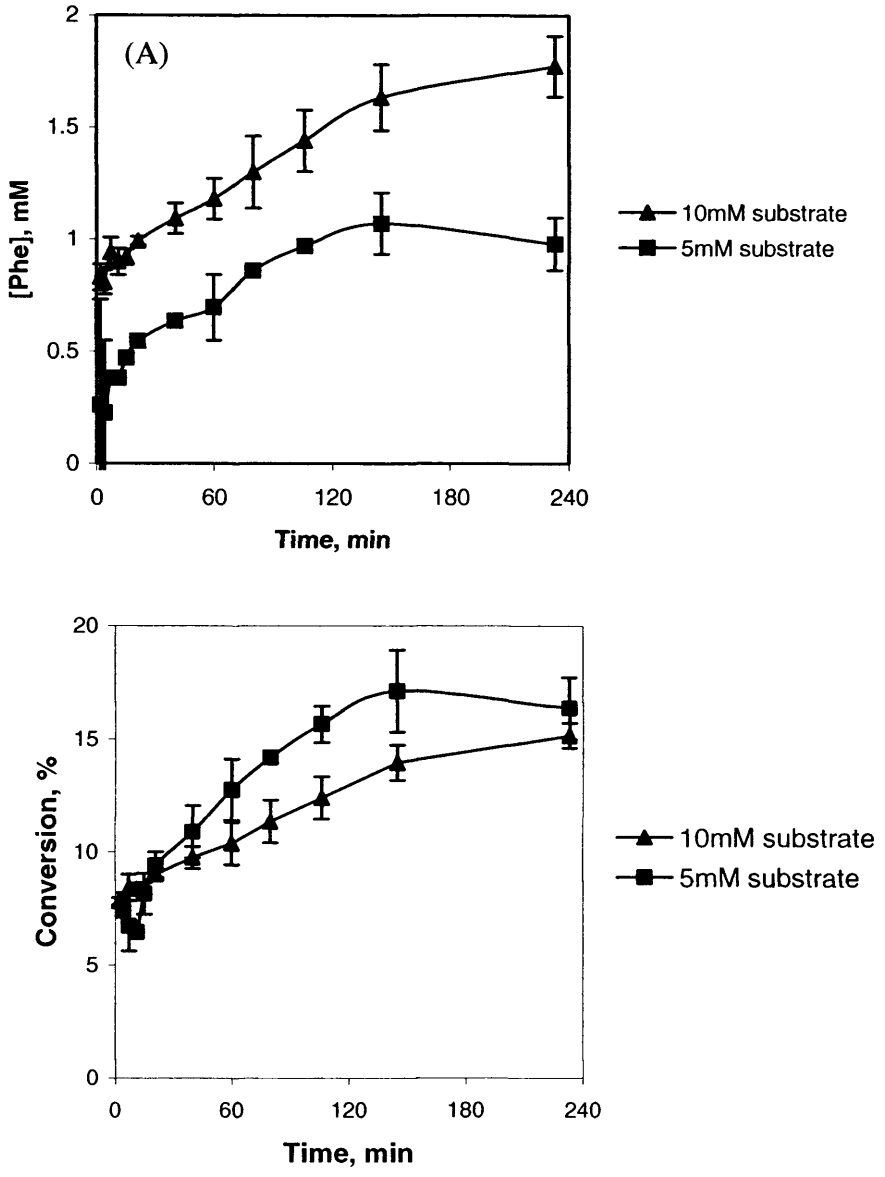


Figure 4.5: Investigation of the effect of substrate concentration for ArTAm using β-phenylserine as an amino donor.

(a) The formation of phenylalanine was measured (Section 2.5.5) for the transamination between phenylserine and phenyl pyruvate catalysed by ArTAm. Two substrate concentrations, 5 mM and 10 mM with reaction conditions 25 mM potassium phosphate buffer, pH 7.0 with 10 mM of the keto acid and amino acid substrates, 0.2 mM pyridoxal phosphate cofactor at 25°C with agitation at 200 rpm. The data in (b) is normalised to the total substrate plus product to enable comparison of the equilibrium position of the reactions at the two different substrate concentrations. Error bars represent 95 % confidence interval calculated from triplicate reactions.

Further work is required to investigate the catalysis of aromatic hydroxy-ketone substrates with ArTAm to investigate the stereochemistry of the amine resulting from a transamination. The enantiomer produced will depend upon the orientation of the binding of the substrate in the active site. The orientation of binding of the amino-β-hydroxyketone substrate may cause the *(S)* hydroxyl to experience the unfavourable interactions with the active site as the *erythro* phenylserine experiences. Using mutagenesis to reduce the interactions of the hydroxyl branch with the active site may be possible. The ability to change the activity of a transaminase towards branched substrates has previously been carried out on AspAT to enable a five-fold increase in the acceptance of branched amino acid valine (Yano, T., Oue, S., and Kagamiyama, H., 1998). However it is conceivable that if the substrate is not a keto-acid the binding of the substituted alcohol will not hold the substrate in such a rigid position as an acid at the equivalent position therefore allowing substrate rotation and the possibility of the hydroxyl branching being accommodated.

4.3.3.2 Activity of ArTAm with aminoalcohols

In order to test the acceptance of an aromatic substrate without the acid group phenylaminopropandiol, **2**, the diol form of phenylserine (Figure 4.3, was investigated. This substrate was tested in the 1*(S)*, 2*(S)*-2-amino-1-phenyl-1,3-propandiol form (equivalent to the reactive *L-threo* phenylserine). This contains the *(S)* stereochemistry of the β-hydroxyl like a TK product. Also tested was 1*(R)*,2*(R)*-2-amino-1-phenyl-1,3-propandiol. Additionally the transamination of the *(R)* and *(S)* stereoisomers of 2-amino-3-phenylpropanol was investigated. Since the use of amino alcohol substrates was investigating the use of a totally new class of compounds for ArTAm it is hard to predict the stereochemistry that would result from the transamination of the ketone precursor. Therefore both the *(R)* and *(S)* stereoisomers were tested.

Neither of the phenylaminopropanol isomers with α-ketoglutarate, both at 10 mM, yielded any detectable glutamate product formation with ArTAm over a 24-hour reaction period. Similarly the reactions with the phenylpropandiol diastereomers as the amine donor yielded no glutamate product.

The branching hydroxyl in *L*-erythrulose (Figure 4.2) is formed during the TK catalysed formation of the asymmetric carbon-carbon bond and is a characteristic of all TK products. The terminal hydroxyl is a product of the reaction of TK employing β-HPA and is not an intrinsic property of a TK product. The accommodation of the hydroxyl branch of phenylserine implies that this enzyme may be able to accommodate a molecule with a TK synthesised asymmetric carbon-carbon bond but the challenge is the acceptance of a non-keto acid substrate with no examples of acceptance of such a substrate for this enzyme. The acceptance of ketomalonic acid, in place of β-HPA, by TK was considered as a potential solution. This would result in a ketoacid product. This substrate was tested with TK, but again no activity was detected. Subsequently finding a TAm to accept a non-ketoacid was focused upon.

4.4 Investigation into the mutagenesis of ArTAm

4.4.1 Consideration of mutagenesis protocols

It was desirable to have a random mutagenesis strategy established both for development of the initial pathway (Figure 1.8) and the more long-term goal of evolving the constructed pathway to accept alternative substrates. A mutagenesis strategy includes the protocols for generating mutants of an enzyme as well as the ability to screen the mutant library for the desired activity. Since wild-type *E.coli* ArTAm did not have the desired activity towards any of the substrates resembling a TK product (Section 4.3) it could be a candidate for random mutagenesis techniques. Therefore, protocols to investigate both the random and site directed mutagenesis of ArTAm substrate specificity were investigated.

4.4.2 Mutant library generation by XL1-red mutator strain

XL1-Red allows the generation of large numbers of random mutants (Greener, A., Callahan, M. and Jerspeth, B., 1997). XL1-Red is an *E.coli* strain engineered to be deficient in three of the primary DNA repair pathways. Such a strain, often referred to as a 'mutator strain', can be used to propagate DNA and insert random mutations at a rate much higher than would naturally occur in a wild-type *E.coli* strain. This technique may be more convenient than other techniques such as error prone PCR as it bypasses the need for many tedious DNA manipulations. In addition, the problem of optimising the mutation bias is not present (Cline, J. and Hogrefe, H.H., 2000). In

order to achieve the increased mutation rate exhibited by XL1-red, the mutS, mutD and mutT mutations were introduced into a single bacteria (Greener, A., Callahan, M and Jerspeth, B., 1997). These mutations result in disruption of error-prone mismatch repair, loss of DNA polymerase III 3'-5'exonuclease activity and loss of hydrolysis of 8-oxodGTP lesions respectively. The engineered XL1-Red strain has a reported mutation rate approximately 5000 times higher than the parent strain assuming the wild-type mutation rate is 10^{-10} per nucleotide per generation. This strain has been successfully used in several instances. Using XL1-red generated mutants the enhancement of the activity of alkaline phosphatase towards chromogeneic substrate BCIP was achieved (Greener, A., Callahan, M. and Jerspeth, B., 1997), a variant of an esterase from *Pseudomonas fluorescens* able to carry out the resolution of a sterically hindered 3-hydroxy ester was obtained (Bornscheuer, U.T., Altenbuchner, J. and Meyer, H.H., 1998) and the activity of cycloartenol synthase was altered to function as a lanosterol cyclase (Wu, T.-K.a.G., J.H., 2002).

The pQR416 plasmid (Figure 3.7), encoding ArTAm, was propagated through 50 generations in the XL1-red mutator strain (Section 2.9.1.1). This required several re-inoculations as the strain achieved an average of 5 generations in a 12-hour growth period. The DNA was then isolated and transformed back into the BL21gold (DE3) expression host. Sequencing of DNA was then carried out for 10 colonies and the screening for activity of a small library (386 mutants) carried out. (Section 4.4.2.2).

4.4.2.1 Quantification of rate of mutagenesis

From sequencing a total of 10 x 1.2 kb genes, only three were found to have point mutations and one further sequence had an insertion. These results are summarised in Table 4.1. In an attempt to increase the observed mutation rate the mutant plasmid preparation used to make this library was reintroduced into XL1-red. A further fifty generations were carried out. However, upon sequencing a similar selection of random colonies a high frequency of stop codons was detected. This DNA was subsequently not used to make a library for screening. The introduction of two different stop codons into the sequences, both appearing in more than one sequence, implies there may be selection pressure on the cells expressing the protein.

Table 4.1: XL1-red derived mutations

Sequence mutations in ArTAm gene plasmid from pQR416 after passage through 50 generations in XL1-red mutator strain (Section 2.9.1.1).

DNA Mutation	Effect on amino acid sequence
491 T→ G	Leu → Trp
542 G → A	Cys → tyr
212 G → A	Gly → Glu
Ins G 581	Frame shift

It has already been shown that over-expression of ArTAm retards growth of the host strain (Section 3.3.3.), however XL1-red is not supposed to function as an expression strain for a gene under the T7 promoter. To check that expression was not occurring SDS-PAGE analysis was carried out. No over-expression of the ArTAm in XL1-red, pQR416 was detectable (data not illustrated). Thus the introduction of the stop codon is apparently incidental rather than selected for. It is possible that the stop codon was introduced early in the mutation cycle and is therefore propagated to subsequent daughter generations. This highlights a problem when using the mutator strain strategy. A further attempt at generating ArTAm mutant populations using XL1-red was unsuccessful with the growth of the cells and plasmid retention proving a problem. A different commercial batch of the cells then yielded no mutations in 6 Kb sequenced following propagation through 100 generations. The XL1-red mutation strategy was subsequently abandoned for this work.

4.4.2.2 Screening of mutant library with alternative substrates

The screening of the 386 mutants from the 50 generation library (Section 2.9.1.2) was initially for increased activity towards serine with α-KG. The analysis was initially carried out by monitoring the serine depletion (Section 2.5.3). This proved unreliable and the analytical methodology was reassessed. Subsequently the library was re-screened with phenylpyruvate as the amine acceptor enabling the formation of phenylalanine to be monitored. The 50-generation library was screened (against a wild-type ArTAm control) for increased activity with serine, phenylaminopropanol and phenylaminopropandiol. Acceptance of *L*-erythrulose was also screened although this was less reliable as it required the monitoring of phenylalanine amino donor substrate depletion. No activity was found to fall outside the variance seen for the wild-type for any substrate. This lack of 'hits' can be attributed to the low rate of mutation and the small library size investigated. The generation of larger mutant libraries with higher mutation rates is required. Additionally a more rapid way of assaying the samples for potential hits is desirable. This may be achieved by the use of auxotrophic strains, colormetric assays or the development of higher throughput automated analytical techniques (HPLC or LC-MS).

4.4.2.3 Native activity screen of library

One 96-well plate from the library was screened for the native activity with aspartic acid and phenylpyruvate allowing the formation of the phenylalanine product to be monitored (Section 2.5.5). This study enabled the estimation of the number of inactivated mutants created by the mutator strain. There were six mutants on the plate that yielded no phenylalanine formation. This may be due to the introduction of stop codons, or deletions or insertions resulting in a frame shift.

4.4.3 Whole Plasmid error prone PCR

Error prone PCR is an established method for random mutagenesis with many examples of successful evolution of desired characteristics including the evolution of cytochrome p450 to improve the activity towards non-natural substrates (Gleider, A., Farinas, E.T., Arnold, F.H., 2002), the evolution of a restriction enzyme to increase substrate specificity (Samuelson, J.C. and Xu, S.Y., 2002) and the modulation of the substrate specificity of an acylase (Otten, L.G. *et al.*, 2002) The use of manganese to reduce the fidelity of the polymerase used for amplification is used to achieve the desired rate of mutagenesis. However, it can be a laborious technique requiring optimisation of manganese concentration to obtain the desired mutation rate and ligation of the mutant PCR products into a vector prior to screening. Additionally mutation rates can be unpredictable and a bias in the observed nucleotide changes occurs with Taq polymerase being 4 times more likely to mutate A and T bases than C or G.(Matsumara, I. and Ellington, A.D., 2001).

The methodology to carryout successful PCR amplification of large segments of DNA from the β-globin gene cluster from the human genome has been successfully demonstrated (Cheng, S. *et al.*, 1994). The thermostable DNA polymerase rTth from *Thermus thermophilus* was shown to reliably amplify 21Kb PCR products. rTaq from *Thermus aquaticus*, was also demonstrated to successfully carryout such long amplifications. Using these enzymes it is therefore feasible that error prone PCR can be carried out on a whole plasmid circumventing the, often troublesome, ligation of the PCR product into a vector by simply re-circularising the linear PCR products (and digesting the circular template DNA) to form the complete plasmid for transformation into the *E.coli* host. This technique has been found to yield a high frequency of transition mutations (Matsumara, I. Unpublished result). This is a slight

variation of the methodology published for the production of a β-glucoronidase mutant library by cassette mutagenesis (Rowe, L.A. *et al.*, 2003).

4.4.3.1 Establishment of wpepPCR methodology

To investigate a protocol for a whole-plasmid error prone PCR (wpepPCR) method the pUC18 plasmid vector was used for a control experiment. This plasmid provided a convenient way to monitor the rate of mutagenesis as mutations inactivating the β-galactosidase gene in the plasmid could be detected by blue and white screening. It should be remembered that this is a large underestimate of the actual rate of mutation since only changes in the amino acid sequence inactivating the enzyme are detected whilst during the generation of a library it is the changes altering, but not inactivating the enzyme, that are important. However it was considered a suitable system for preliminary investigation into the mutagenesis strategy.

Selection of a suitable enzyme for amplification required not only a polymerase that could complete the PCR of the whole plasmid but also the ability to manipulate the mutation rate by addition of manganese. Investigation into the suitability of 3 different DNA polymerases, Pfu turbo, Tth/vent (vent was not able to yield a product of the right size alone) and Taq, was carried out by amplification of the plasmid in the presence of varying concentration of manganese. The enzymes were chosen for their proven ability to amplify long Sections of DNA (Cheng, S. *et al.*, 1994) and their different proof reading activity. Pfu turbo is a high fidelity polymerase (Lundberg, K.S. *et al.*, 1991) producing an amplified plasmid, which can be directly transformed in to an *E.coli* host. The Tth/vent combination should have minor proofreading capability, from the vent, whilst taq has no 3'-5' exonuclease activity so is not capable of proof reading at all. The use of a T4 DNA polymerase step to fill in any gaps in the plasmid product and a ligation step should enable transformation of the PCR products from all of the enzymes.

Table 4.2: Results of whole plasmid error prone PCR of pUC18

The fidelity of different DNA polymerises was tested by PCR amplification of the pUC18 plasmid (Section 2.9.2), at different manganese concentrations, transformation of the resulting products and analysis of the rate of inactivation of the β-galactosidase gene by blue-white screening.

Enzyme	$[Mn^{2+}]$ mM	Blue colonies	White colonies	% Inactivated
Vent/Tth	0	165	1	0.6
	0.01	603	17	2.8
	0.1	785	26	3.3
Pfu turbo	0	889	7	0.8
	0.01	763	4	0.5
	0.04	421	7	1.7
	0.1	699	12	1.7
Taq	0	164	13	7.9
	0.01	105	10	9.5
	0.04	184	21	11.4
	0.1	28	6	21.4

The wpep PCR protocol was found to be successful for the pUC18 control plasmid (Table 4.2). Each polymerase enzyme yielded detectable PCR product of the correct size although smearing was apparent for all but Pfu (Gels not shown). Transformation was successfully carried out for all the reactions and the resulting colonies analysed by blue and white screening by plating on X-gal. Pfu turbo yielded the highest number of colonies with the lowest mutation rate. This was as expected since this enzyme has been specifically engineered to carry out high fidelity amplifications and manganese has little effect on the efficiency of the proof reading activity. The Tth/vent polymerase combination also showed the ability to yield a high number of colonies, again this enzyme blend has previously been found to be very reliable for long amplifications (Cheng, S. *et al.*, 1994). The rate of mutation was seen to increase with manganese concentration with a 3.3% inactivation rate at the highest concentration of Mn^{2+} tested. Taq polymerase yielded the least colonies under the PCR conditions but also the highest rate of mutation, even without Mn^{2+} again as expected since this enzyme lacks proof reading activity.

Since identical conditions were used for each polymerase, whilst information about the rate of mutagenesis is gained, the number of colonies is not an accurate measure of the ability of the enzyme to carryout the wpepPCR. For example it may be possible to improve the number of colonies yielded from the Taq reactions by refining the PCR conditions to yield a more defined product band. However, even under sub-optimal conditions it may be possible to obtain enough colonies by plating more culture to enable a mutant library to be generated.

4.4.3.2 Error prone PCR of ArTAm

When the wpepPCR methodology was transferred to mutate the ArTAm encoding plasmid, pQR426, it was less successful. Refinement of the PCR conditions for both Taq and Tth/vent produced bands with a little smearing similar to the result with pUC18. However, the transformation yielded no colonies. This may have been due to the plasmid not being intact, therefore, not able to transform or the quality of the plasmid being low due to the presence of other products from the messy PCR reactions. Both of these issues were addressed. Firstly, the product band was excised from the gel and transformed, again this yielded little success with few colonies. Several modifications to the protocol were carried out. These included

further refinement of the PCR reactions to obtain a more defined product band and the treatment of the purified product with a kinase to phosphorylate the 5'terminal, DNA T4 polymerase to polish the plasmid DNA by filling in any gaps and T4 ligase to ensure re-circularisation, finally the use of primers with an internal restriction site were used to enable cleavage and re-ligation at this site. Again this was unsuccessful. The reasons for the failure of this technique to work for the ArTAm plasmid were not identified and investigation was ceased due to other more promising leads.

4.4.4 Site directed mutagenesis of Arg386 of ArTAm

4.4.4.1 Interactions of substrates and Arg386

The binding of a dicarboxylic substrate into the active site of *E.coli* AspTAm involves the interaction of the substrate carboxylate groups with Arg386 and Arg292 (of the other subunit of the dimer). This has been elucidated from crystallographic studies (Jager, J. *et al*, 1994; Kirsch, J. *et al.*, 1984). Each carboxylic group of the substrate forms a coplanar bifurcated bond with the guanidium side chains of the corresponding arginine residue. This interaction has also been observed with the aspartate transaminase from other species for example pig AspTAm, complexed with 2-methyl aspartate (Arnone, A. *et al*, 1985). These active site residues are similarly conserved in the *E.coli* ArTAm. Though it is noted that the interaction with Arg292 of ArTAm is non-essential for catalysis due to the ability of this enzyme to catalyse the reaction with aromatic substrates. It is widely accepted that the enzymes substrate and active site interactions and catalytic mechanisms are very similar (Section 1.10.1) including the interaction of the substrates carboxy group with Arg386.

A study of the substrate recognition of ArTAm from *Paracoccus denitrificans* (PdArTAm) gives an insight into the role of Arg386 of *E.coli* ArTAm in substrate binding (Okamoto, A. *et al.*, 1998). The PdArTAm shares only 30% amino acid sequence homology with the *E.coli* enzyme. However, all but three active site residues are identified as being conserved or homologous (four residues) between the two organisms. These conserved residues include all those with direct contact with the PLP cofactor or inhibitor studied. Therefore it is probable that the two ArTAms

share a common substrate recognition mechanism. Crystallisation of the *Paracoccus denitrificans* enzyme with substrate analogs, maleate and phenylpropionate, enabled the interactions of the compounds with the active site to be studied. The interaction with Arg386 was observed for both substrate analogs binding to subunit B of ArTAm which was in the closed conformation. Conversely it was observed that the binding of maleate to subunit A (3-phenyl propionate does not bind to subunit A) did not involve the formation of a salt bridge with Arg386 or Arg292. This subunit bound maleate weakly but remained in the open conformation.

The desire to use the transketolase non-keto acid products (Figure 4.2), with a hydroxyl group in place of the acid, as substrates for the transaminase led to investigation into the effect of mutagenesis of the arginine at position 386 on catalysis. With the keto acid substrate interaction of the α-carboxylic acid and Arg 386 occurs during catalysis by ArTAm. It has been shown that this interaction, in both ArTAm (Islam, M.M. *et al.*, 2000) and AspTAm (Mizuguchi, H. *et al.*, 2001), (Hayaishi, H., *et al.*, 2003a and b), provides the strain in the enzyme for domain closure and catalysis to occur. It is possible that the absence of the carboxylic acid group contacting with the arginine this residue can be substituted by an amino acid that can interact with the hydroxyl which replaces the carboxy acid in the TK product.

4.4.4.2 Generation of mutants

Mutants were generated by Quickchange PCR using a wobble primer with the sequence encoding amino acid position 386 defined as NNS (Section 2.9.3.1). This coding sequence allows the introduction of any of the 4 bases in the first two positions but only either cytosine or guanine in the third position. The introduction of any amino acid is possible, however, the use of S in the third position, enabling insertion either C or G, of reduces the number of degenerate codon sequences therefore reducing the number of mutants with different codons but the same amino acid at position 386. Access to all 20 amino acids can be achieved in this way. Initially 18 mutants were sequenced to investigate the efficiency of the mutagenesis. Out of 18 randomly picked colonies sequenced amino acid changes were achieved in 14 sequences accessing 10 different amino acids (Table 4.3). Additionally 3 were wild-type with Arg at position 386 and one was truncated by a stop codon at 386.

Table 4.3 Plasmids encoding ArTAm 386 variants generated by site directed mutagenesis.

The Arg 386 site directed mutants were made via Quickchange PCR using wobble primers (Section 2.9.3.1).

Plasmid	Codon encoding position 386	Amino acid 386
pQR419	GTG	Valine
pQR420	CCC	Proline
pQR421	TAG	Tyrosine
pQR422	GGG	Glycine
pQR427	CTC	Leucine
pQR428	TTC	Phenylalanine
pQR429	ACC	Threonine
pQR430	CAG	Glutamine
pQR431	AAG	Lysine
pQR432	TCC	Serine

4.4.4.3 Expression and activity of ArTAm Arg386 mutants

The alteration of a single amino acid residue in a protein can lead to a change in the protein structure and can lead to insolubility. Therefore it was first confirmed that the transaminase protein was found in the soluble fraction of the host cell for each mutant by SDS-PAGE. For each mutant, protein was confirmed in the soluble fraction (but was not quantified).

The activity of the ArTAm site directed mutants was then tested with various substrates (Section 2.9.3.2). Each mutant strain was tested alongside the wild-type BL21gold(DE3) host strain and BL21gold(DE3) pQR416 overproducing native ArTAm. The substrate combinations tested were: (1) aspartic acid and phenyl pyruvate; monitored for phenyl alanine production, (2) phenylalanine and α-ketoglutarate; monitored for phenylalanine formation, (3) phenylaminopropanol and phenylpyruvate; monitored for both phenylaminopropanol substrate disappearance and phenylalanine formation, (4) phenylalanine and *L*-erythrulose; monitored for phenylalanine disappearance and ABT formation and (5) phenylaminopropanol and *L*-erythrulose; monitored for both phenylaminopropanol disappearance and ABT formation. The reactions were sampled over a 6-hour period. It was considered that any activity detected under these conditions and within this time frame may be useful for further investigation.

The activity of the strain carrying pQR431, encoding the mutant with lysine substituting arginine at the 386 position, exhibited activity in reactions (1) and (2), each containing known ArTAm substrates comparable to that seen with the ArTAm wild-type strain. The other mutants exhibited the baseline activity for these native reactions of that measured for the wild-type host strain contributed by the endogenous transaminases. For the other substrate combinations no activity was detectable for any of the strains tested. In a previously reported study the direct substitution of Arg386 with a similarly positively charged lysine in AspTAm from *E.coli* was investigated (Inoue, Y. *et al.*, 1989). Activity was evident as seen here. The mutation was reported to yield a decrease in substrate interaction and catalysis with aspartic acid, glutamate, oxoglutarate and oxaloacetate. The decrease in activity was demonstrated by a decrease in V_{max} and an increase in the K_m value upon Arg

386 substitution. The increase of K_m ranged from 3 to 30 fold depending on the substrate measured. This was considered due to the inability of the lysine to form the strong bifurcated bond with the substrate α-carboxylate and subsequent alignment of the substrate in the active site required for catalysis to occur. The measurements made in this work were not accurate enough to detect the difference in rate of catalysis between the mutant Arg386Lys and the wild-type enzyme. Interestingly, whilst the Arg386Tyr mutant showed no measurable product formation a study by Danishefsky *et al* (Danishefsky, A.T. *et al.*, 1991) investigating Arg386Phe and Arg386Lys in AspTAm from *E.coli* reported activity. It is possible that the amount of soluble enzyme in our constructed Arg386Tyr strain was low and subsequently catalysis was not detectable under the conditions tested.

In the Danishefsky study, the rationale behind the mutation Arg386Tyr was to remove the positive charge but to maintain a group capable of interacting with the substrate α-carboxylate. The phenylalanine substitution has steric similarity with the tyrosine substitution but without the ability to form a hydrogen bond with the substrate. A reduction in activity relative to the wild-type AspTAm was observed for both mutants. Activity (presented as second order rate constant K_{cat}/K_m measured under single turnover conditions rather than steady state) was measured relative to previously determined values for the wild-type enzyme (Cronin, C.N. and Kirsch, J.F., 1988). The mutation Arg386Tyr gave a reduction of over 5 orders of magnitude. However, the activity was 3 fold greater than measured for Arg386Phe. This difference in activity was postulated to be due to the ability of tyrosine hydrogen bond with the substrate or small changes in local packing of the two different residues.

Any residue that could form an electrostatic contact with the substrate would need to be not only chemically compatible but also be orientated in such a way that interaction of the side chain with the substrate can occur and induce movement required for domain closure. This was investigated in the Danishefsky (Danishefsky, A.T. *et al.*, 1991) study where crystallisation of the Arg386Phe was carried out (but crystallisation of Arg386Tyr was not achieved). The structure of Arg386Phe implied that the orientation of the aromatic group points away from the substrate carboxylate.

Therefore if the orientation of the tyrosine at this position took a similar position it would also point away from the carboxylate thus being unfavourable for the hydroxyl to hydrogen bond with the substrate. It is further suggested that the orientation of the residue at 386 may affect the positioning of neighbouring residues and their interaction with the substrate. Also noted was the length of extension, from the α-carbon, of the side chain involved in hydrogen bonding would differ between arginine and tyrosine. The nitrogen atoms of the guanidium group of the arginine residues extend 6.4 and 6.8 angstroms whilst the tyrosine's oxygen reaches only 6.4 angstroms. This may further weaken the hydrogen bonding of the tyrosine with the substrate and may account for the large reduction in activity observed relative to wild-type. Additionally during this study they also investigated two non-native substrates with the mutant enzymes. These were β-glutamate whose carboxylate could extend further towards position 386 to enable better interaction, and isoglutamine, which has a neutral amide group in place of the carboxylate which may make a more favourable interaction with the hydroxyl of Tyr386. However, it was observed that the second order rate constant for both substrates was at least 10 fold lower than that obtained for glutamate. It was thus postulated that interactions other than that with Arg386 stabilise the negative charge of the carboxylate. Therefore the catalysis of substrates lacking the acid group may require not only substitution of the Arg386 interacting residue but also a change in neighbouring residues in the 3D structure to enable the exact positioning apparently required for interaction and domain movement. Therefore substituting a single active site residue may not be adequate to enable catalysis.

This investigation was not exhaustive of all possible amino acid substitutions at position 386. It is possible that one of the amino acids not yet tested could yield the desired activity. Additionally, the solubility of some of the mutants was lower than others therefore the amount of active protein available for catalysis would have been lower. This may have led to activity not being detectable under the conditions tested despite lysates from cultures with comparable cell densities being used. However this investigation was put aside without further characterisation of the mutants generated. This was due to encouraging results from the β-A: P TAm.

4.5 Substrate specificity of β-A: P TAm

4.5.1 β-A: P TAm activity with *L*-erythrulose

The activity of the cloned β-A: P TAms was initially determined with the native transamination (Section 3.4.3). An initial experiment into the acceptance of *L*-erythrulose was carried out with both the β-A: P TAm from *Pseudomonas putida* and *Pseudomonas aeruginosa*. In reactions, carried out with 5 mM substrates (Section 2.10.1), both of these enzymes were found to be inactive with *L*-erythrulose with a β-alanine amine donor. *L*-methylbenzylamine (*L*-MBA), a known substrate for the ω-TAm from *Vibrio fluvialis* (Shin, J.-S. and Kim, B.-G., 2001), was considered as an amine donor substrate for the β-A: P TAms with the native ketone substrate pyruvate. *L*-MBA is easier to assay than β-alanine as it does not require derivatisation for separation on a C18 column or detection by UV. The reactions with *L*-MBA as the amine donor can be monitored for both amine donor depletion and formation of the resulting acetophenone product in one assay (Section 2.5.5).

The activity in lysates from both the β-A: P TAm strains was tested with *L*-MBA and pyruvate as the amine acceptor substrate (Figure 4.6). The conversion of *L*-MBA and pyruvate, with both of the β-A: P TAm, was found to be very slow with the reaction occurring over a matter of hours. The native reaction of β-alanine and pyruvate was previously found to rapidly reach steady state at ~30% conversion after a thirty minute reaction period (Section 3.4.3). The reaction of *L*-MBA and pyruvate, catalysed by β-A: P TAm from *Pseudomonas aeruginosa,* reached 30% conversion of *L*-MBA to acetophenone after 40 hours. The reaction of the enzyme cloned from *Pseudomonas putida* was slightly less favourable showing lower rates of reaction and achieving just 15% percent conversion over the same time period. Reactions to test the acceptance of *L*-MBA and *L*-erythrulose by the β-A: P TAms were carried out simultaneously (Figure 4.7). This reaction proceeded to 20% conversion of *L*-MBA to acetophenone in 40 hours, a third lower than the conversion observed with pyruvate as the amine acceptor.

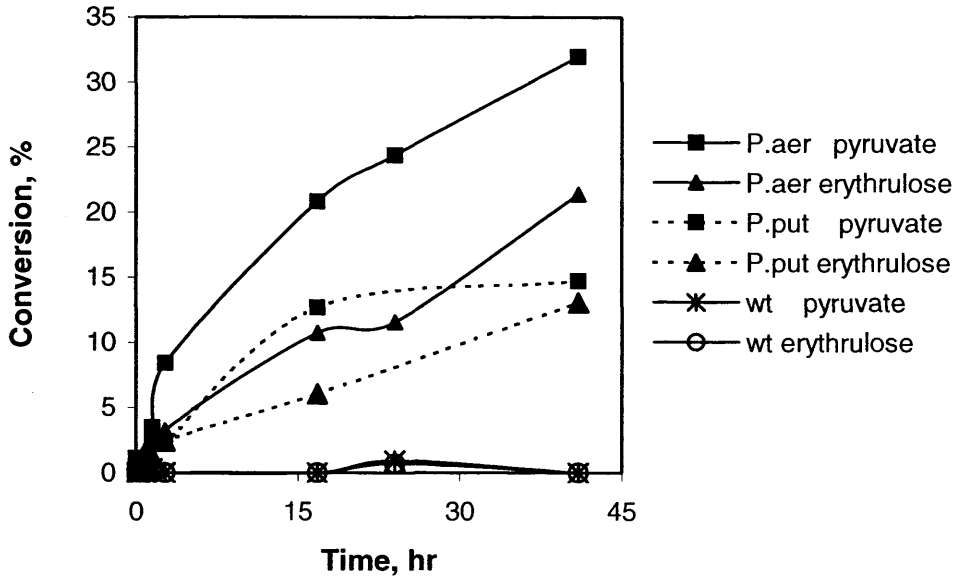


Figure 4.6: The activity of β-A: P TAm with *L*-MBA as the amine donor

Reactions containing *L*-MBA and either pyruvate or *L*-erythrulose substrates, each at 5 mM, were set up with lysate from the BL21gold(DE3) *E.coli* strains overexpressing the β-A: P Transaminase from either *Pseudomonas aeruginosa* (P.aer) or *Pseudomonas putida* (P.put.) alongside a wild-type control. Conditions were 25 mM potassium phosphate buffer, pH 7.0 with 0.2 mM pyridoxal phosphate cofactor at ambient temperature (~25°C) and agitation at 200 rpm (Section 2.10.1). Reactions were sampled and assayed for *L*-MBA consumption and acetophenone formation.

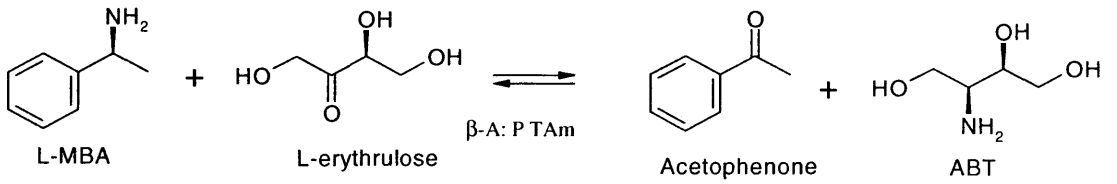


Figure 4.7: The reaction scheme for the transamination of *L*-erythrulose by β-A: P TAm from *Pseudomonas aeruginosa*

Transamination of *L*-erythrulose by β-A: P TAm using *L*-MBA as an amine donor.

A reaction with the β-A: P TAm from *Pseudomonas putida,* with *L*-erythrulose, once again gave less favourable conversion than the corresponding reaction with the *Pseudomonas aeruginosa* enzyme. Subsequently the β-A: P TAm from *Pseudomonas aeruginosa* was chosen as the candidate enzyme for further substrate specificity investigation. The wild-type host strain was tested alongside and yielded little acetophenone product formation with either ketone substrate.

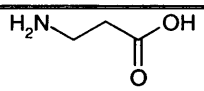
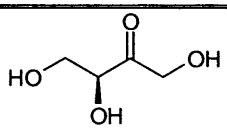
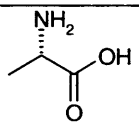
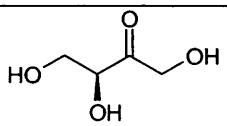
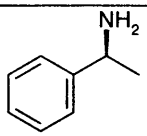
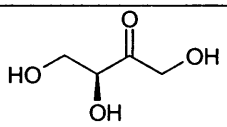
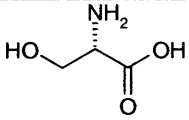
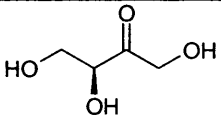
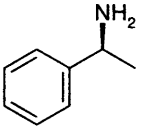
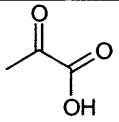
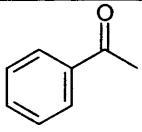
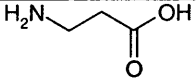
It is interesting that *L*-erythrulose is accepted as a substrate with *L*-MBA but not with the β-alanine amine donor favoured in the reaction with pyruvate. The mechanism elucidated for the transaminases involves the binding of the amine substrate, dissociation and then binding of the second substrate, no physical interaction of the two substrates occurs during catalysis. However it is essential that the ketone resulting from the amine substrate should not bind more favourably in the active site than the second substrate, This is because the ketone product from the first half of the reaction will compete with the ketone substrate entering the active site for transamination.

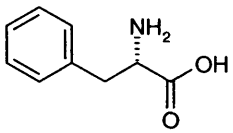
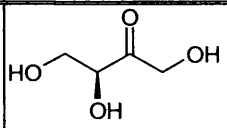
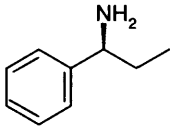
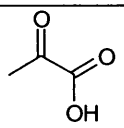
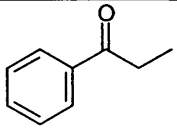
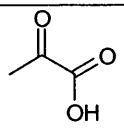
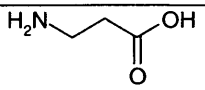
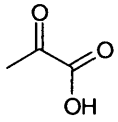
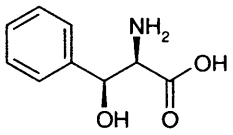
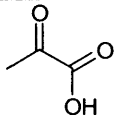
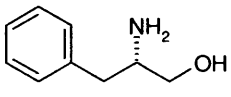
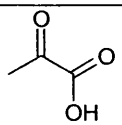
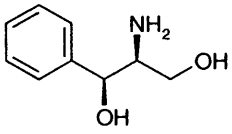
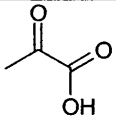
4.5.2 Further investigation of the substrate range of β-A: P TAm from *Pseudomonas aeruginosa*

The acceptance of other related substrates was investigated to try and explore the substrate repertoire of the *Pseudomonas aeruginosa* β-A: P TAm enzyme (Table 4.4). Interestingly it was found that whilst the reaction of β-alanine and *L*-erythrulose produced no detectable product, the reaction with α-alanine and *L*-erythrulose yielded a small amount of ABT (Data not shown). The rate was, however, a fraction of that observed with *L*-MBA and *L*-erythrulose. All other substrate combinations tested yielded no product, under the conditions investigated. Notable is the inactivity of the enzyme with acetophenone and β-alanine. In retrospect a further interesting reaction would have been acetophenone with ABT. It should also be noted that at this stage catalysis was assessed using the β-A: P TAm in crude lysate preparations. Therefore the rate of any product accumulation must been above the rate of any degradation of the compound by the lysate that may occur in order to be detected.

Table 4.4: Substrate combinations tested with β-A: P TAm

The compound pairs illustrated were tested as substrates, 5 mM, for reactions with β-A: P TAm from *Pseudomonas aeruginosa* (Section 2.10.1). Reactions in 25mM potassium phosphate buffer, pH 7.0 with 0.2 mM pyridoxal phosphate cofactor at ambient temperature (~25°C) and agitation at 200 rpm, were monitored periodically using the HPLC method appropriate to the substrates used, over a 40-hour period.

Amine donor substrate	Ketone substrate	Analysis method	Activity detectable
 β-alanine	 L-erythrulose	ABT formation	No
 α-alanine	 L-erythrulose	ABT formation	Yes
 L-Methylbenzylamine	 L-erythrulose	ABT formation	Yes
 L-serine	 L-erythrulose	ABT formation	No
 L-Methylbenzylamine	 Pyruvate	*L*-MBA depletion Acetophenone formation	Yes
 Acetophenone	 beta-alanine	*L*-MBA formation Acetophenone depletion	No

Amine donor substrate	Ketone substrate	Analysis	Activity detected
 L-Phenylalanine	 L-erythrulose	ABT formation	No
 L-Ethylbenzylamine	 Pyruvate	Ethylbenzylamine depletion	No
 propiophenone	 Pyruvate	Ethylbenzylamine formation	No
 beta-alanine	 Pyruvate	α-alanine formation and β-alanine depletion	Yes
 D,L-*threo-3*-phenylserine	 Pyruvate	α-alanine formation	No
 (S)-2-amino-3-phenylpropanol R isomer also tested	 Pyruvate	α-alanine formation	No
 (1S)-(2S)-2-amino-1-phenyl -1-3-propandiol (1R)-(2R) isomer also tested	 Pyruvate	α-alanine formation	No

4.6 Summary

This Chapter describes an investigation into the acceptance of *L*-erythrulose for transamination by the cloned candidate transaminases (Chapter 3). Initially the better characterised ArTAm (Section 1.10.1) was investigated. The acceptance of *L*-erythrulose as a substrate was not evident (Section 4.3.1). This was attributed to the two key differences between *L*-erythrulose and the native substrates of the enzyme (Section 4.3.3). These are the replacement of the acid functionality with a hydroxyl group and the branching of a hydroxyl from the main carbon chain adjacent to the carbonyl to be transaminated. Investigation into the acceptance of aromatic amines with one or both of these differences was carried out (Section 4.3.3), again no acceptance was detected.

The investigation of random mutagenesis strategy for the ArTAm was desirable. This was potentially useful for both the enhancement of any modest ArTAm activity towards a TK product discovered at this stage and also to provide the methodology to generate mutant libraries to screen against other substrates in the future to change the specificity of the target pathway. The use of mutator strain XL1-Red proved unpredictable and the achieved rate of mutagenesis of the ArTAm was not considered sufficiently high (Section 4.4.2). This strategy was subsequently abandoned. A second strategy examined was whole plasmid error prone PCR (Section 4.4.3). This showed promising results for the control experiments with pUC18 but the generation of mutants with the ArTAm encoding plasmid was less successful. In the meantime, site directed mutagenesis of the arginine residue interacting with the acid moiety of the ketoacid substrate was examined (Section 4.4.4). The mutagenesis of this residue has been previously examined but the assessment of the activity of the mutants with a non-keto acid substrate has not been reported. Of the twelve mutants assessed one with the substitution of arginine 386 to lysine, retained the ability to catalyse transamination with the native substrates. Activity was not detectable with *L*-erythrulose or the other non-keto acid aromatic amine substrates with any of the mutants created. Whilst this study was not exhaustive of all the possible substitutions at arginine 386 it was ceased due to more promising results for the β-A: P TAms.

The activity of the β-A: P transaminases of *P.aeruginosa* and *P.putida* with native substrate β-alanine and desired substrate *L*-erythrulose was not detected (Section 4.5.1). Since little is known about the substrate repertoire or structure of these enzymes the acceptance of a range of substrates was examined (Section 4.5.2). The reaction with *L*-erythrulose with *L*-MBA as the amine substrate resulted in product formation (Section 4.5.1). The enzyme from *P.aeruginosa* yielded the highest activity under the conditions investigated therefore this was selected as the candidate transaminase for further investigation into the target reaction.

Chapter 5 - Characterisation of the synthesis of 2-amino 1,2,4-butanetriol from *L*-erythrulose by β-Alanine: pyruvate Transaminase

The previous chapter described an investigation into the acceptance of *L*-erythrulose as a substrate for transamination. The β-alanine: pyruvate transaminase from *Pseudomonas aeruginosa* was identified as a promising candidate to function in a multi- enzyme conversion with transketolase. This enzyme is little studied and the reaction of interest (Figure 1.4) has not been reported to date. In this chapter the characterisation of the *L*-erythrulose transamination with the β- A: P transaminase to produce 2-amino-1,3,4-butanetriol (ABT) is described.

5.1 Introduction

The purification of the β-A: P TAm was carried out in order to further characterise the enzyme for use in series with TK for the bioconversion of TK product *L*-erythrulose to ABT, a useful chiral synthon, containing two stereo centres. The purification of an enzyme from the host strain offers the benefit of the removal of any competing side reactions that may occur in lysate (or transport problems where whole cells are concerned). Once purification has been carried out investigation of the reaction of the enzyme with substrates and the effect of products on catalysis can be assessed. The operational stability of the enzyme is also studied. The information gathered from such studies may be applied to the selection of the conditions for the initial demonstration of the synthesis of ABT, via a bioconversion carried out with lysate from an *E.coli* strain constructed to over-express both the TK and β-A: P TAm (Chapter 6).

5.2 Characterisation of the transamination of *L*-erythrulose by β-A: P TAm

5.2.1 Purification of β-alanine: pyruvate transaminase

In order to further study the transamination of *L*-erythrulose by β-A: P TAm, purification of the enzyme was desirable to remove the complications of other components in the lysate. The addition of a histidine tag (His_6 tag) to an enzyme can enable ease of purification from other cellular components by affinity

chromatography (Porath, J. *et al.*, 1975). The addition of a His_6 tag was carried out by the insertion of an oligonucleotide, encoding 6 histidine residues, in between the *Xba1* and *Nde1* sites of the β-A: P TAm encoding plasmid pQR426 (Section 2.4.3.2). The new plasmid, encoding the β-A: P transaminase from *Pseudomonas aeruginosa* with an N terminal His_6 tag, was named pQR428.

The addition of the histidine residues may cause a change in enzyme conformation which can lead to loss of activity and aggregation. Therefore testing of the activity of an enzyme after the addition of the histidine tag is important. Lysate from cultures of BL21gold(DE3) pQR426 and BL21gold(DE3) pQR428 were compared for activity with β-alanine and pyruvate (Section 2.4.3.4). The two strains over producing β-A: P TAm, with and without a histidine tag respectively, were found to have comparable levels of soluble enzyme and activity profiles for this reaction (data not shown). Therefore the addition of the His_6 tag is not significantly retarding the activity of the β-A: P transaminase.

The β-A: P transaminase was purified to homogeneity by the capture of the His_6-tag using immobilised nickel-ion affinity chromatography (Section 2.4.3.3). The protein concentration of the final preparation was 5.25 $mg.mL^{-1}$ based on the Bradford assay (Section 2.3). The activity of the purified enzyme was verified by enzyme-linked assay (Section 2.7.2). This purified enzyme was subsequently used to further investigate the characteristics of the β-A: P TAm.

5.2.2 Verification of the transamination of *L*-erythrulose

The transamination of *L*-erythrulose was earlier demonstrated by *P. aeruginosa* β-A: P TAm using *L*-MBA as an amine donor (Section 4.5.1). Further confirmation of the transamination of *L*-erythrulose was obtained by the demonstration that the apparent rate of conversion (measured by acetophenone formation), in the absence of *L*-erythrulose, is significantly below that observed when *L*-erythrulose is present (Figure 5.1). In the absence of *L*-erythrulose a low level of acetophenone production by the β-A: P TAm strain was observed. This was ~12% of the observed rate of acetophenone production in the presence of the *L*-erythrulose substrate.

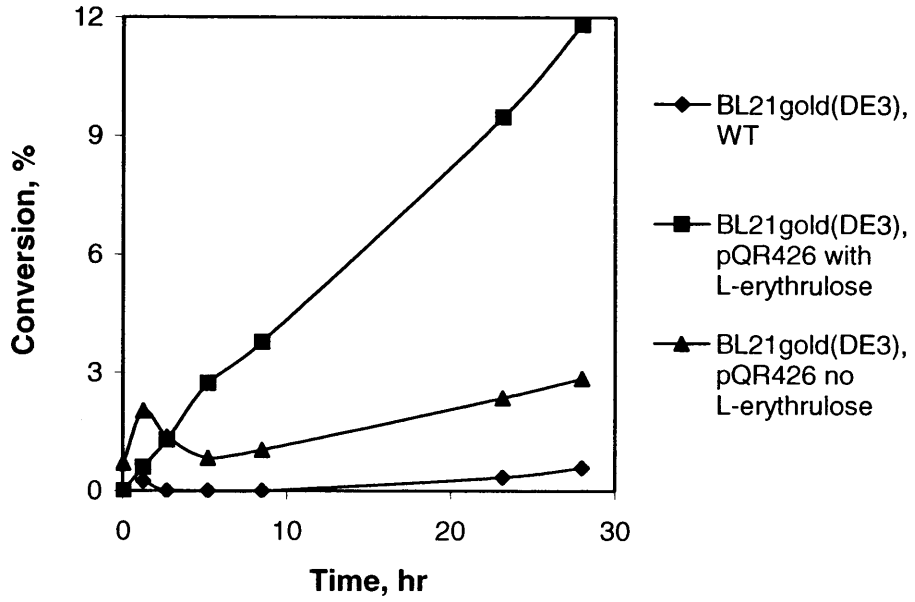


Figure 5.1: Acetophenone formation from the β-A: P TAm catalysed transamination between *L*-MBA and *L*-erythrulose.

Two reactions were carried out with lysate from BL21gold(DE3) pQR426. Reactions contained *L*-MBA, 10 mM, in 100 mM potassium phosphate buffer, pH 7.0 with 2 mM pyridoxal phosphate cofactor at ambient temperature (~25°C) and agitation at 200 rpm (Section 2.10.2). *L*-erythrulose, 10 mM was added to one of the reactions. A control reaction containing both substrates was carried out with BL21gold(DE3) Wild-type host strain. The progress of each reaction was monitored by the decline of *L*-MBA substrate and the formation of acetophenone product (Section 2.5.5).

The apparent turn-over of *L*-MBA to acetophenone in the absence of *L*-erythrulose may be due to transamination of other molecules with ketone functionality, present in the lysate, by the β-A: P TAm. The rate of acetophenone formation by the wild-type host strain was 3% of that with the strain BL231gold(DE3), pQR426, over producing β-A: P TAm. This implies the majority of acetophenone formation is due to the activity of the β-A: P TAm in the engineered strain.

End-point analysis of a similar experiment, using purified enzyme (Section 5.2.1) with *L*-MBA, in the absence of a ketone substrate, yielded no detectable ABT product. In the reaction with the *L*-erythrulose ketone substrate, 1.25 mM of ABT product was yielded over a 65-hour reaction period.

5.2.3 Effect of substrate concentration on native reaction

Transfer of an amine group from β-alanine to pyruvate by β-A: P TAm, under the conditions tested, is extremely limited with a maximum achievable conversion of ~30% (Section 3.4.3). Transaminases belonging to the ω subclass have previously been reported to be subject to both substrate and product inhibition (Shin, J.-S. and Kim, B.-G., 1999; Shin, J.-S. and Kim, B.-G., 2001). The initial rate and final conversion was monitored at substrate concentrations from 5 mM to 40 mM in reactions, with both β-alanine and pyruvate added at the same concentration, using the purified β-A: P TAm catalyst. Normalising the data enabled the direct comparison of the reaction progress at the different initial substrate concentrations (Figure 5.2). The initial rate of catalysis increased with increasing substrate concentration, and comparable reaction profiles reaching a final ~30% conversion of substrate to product, were obtained for each of the reactions. These findings imply that it is the reaction equilibrium for this enzyme and not inhibition by the substrate or product that is limiting the reactions to 30% conversion under the conditions tested.

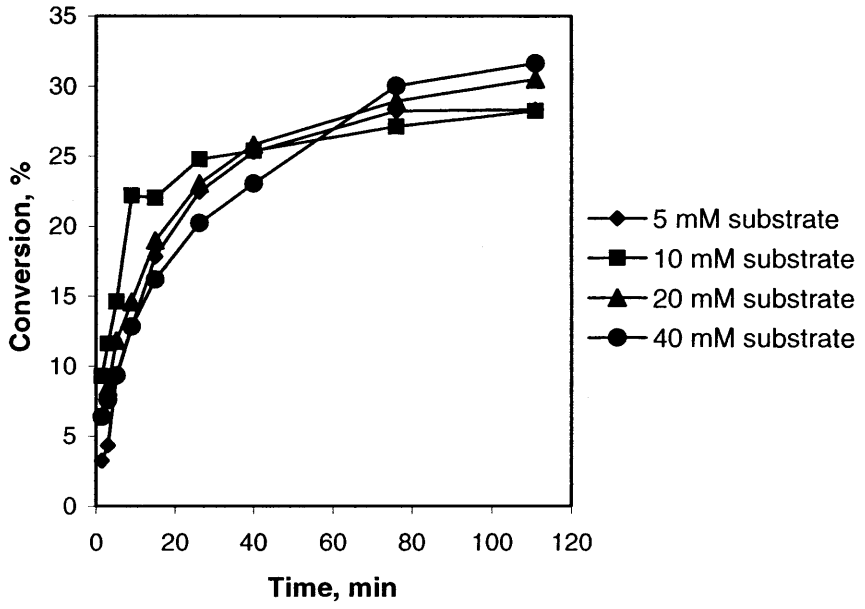


Figure 5.2: The transamination of β-alanine and pyruvate at varied initial substrate concentrations

Reactions to investigate the transamination of β-alanine and pyruvate by purified β-A: P TAm at initial substrate concentrations between 5 mM and 40 mM (Section 2.10.4) were carried out in 100 mM potassium phosphate buffer, pH 7.0 with 2 mM pyridoxal phosphate cofactor at ambient temperature (~25°C) and agitation at 200 rpm. Catalyst was added as purified transaminase (Section 2.4.3.3) to a concentration of 0.61 $mg.mL^{-1}$.The data was normalised against initial substrate concentration to enable comparison of the final equilibrium at each substrate charge.

5.2.4 Effect of substrate concentration on 2-amino-1,3,4-butanetriol formation

The effect of the substrate concentration on the transamination between *L*-MBA and *L*-erythrulose by purified β-A: P TAm was examined. Substrate concentrations from 5 mM to 20 mM were investigated. Both substrates were added at the same concentration and the progress of each reaction was determined by monitoring the formation of ABT after 100 hours. The substrate concentration was found to effect the product yield (Figure 5.3) presented as a percentage conversion of the initial substrate concentration. As the initial concentration of the two substrates was increased from 5 mM to 20 mM the observed conversion of substrate to product decreased from 18% of the initial substrate concentration, at 5 mM, to 10%, at 20 mM substrate. When the production of ABT was compared at increasing substrate concentrations it was found to increase whilst the percentage conversion of substrate to product was reduced. This implies that it is the equilibrium limiting the reaction at the lower substrate concentrations rather than the product reaching inhibitory levels. At higher substrate concentrations where higher levels of product are reached it is possible that the product reaches inhibitory levels and therefore product inhibition should be assessed (Section 5.2.5).

The ω-TAm from *Vibrio fluvialis* has been found to suffer substrate inhibition from both amine and ketone substrates (Shin, J.-S. and Kim, B.-G., 2001). In order to investigate the effect of the individual substrates of the erythrulose transamination independently, one substrate was held at 10 mM whilst the other was varied over a 5 to 40 mM range. The effects on the initial rate of conversion and the ABT product formation (Figure 5.4) were examined. Increasing the concentration of either *L*-erythrulose or *L*-MBA from 5 mM to 10 mM showed an increase in both the initial rate (Figure 5.4 (A)) and the concentration of ABT produced (Figure 5.4 (B)). Therefore, it is likely that the reaction is being limited by subsaturating substrate supply when the concentration of either substrate is 5 mM or below. When either substrate concentration is further increased a decrease in both the initial rate (Figure 5.4 (A)) and the yield of ABT (Figure 5.4 (B)) is observed.

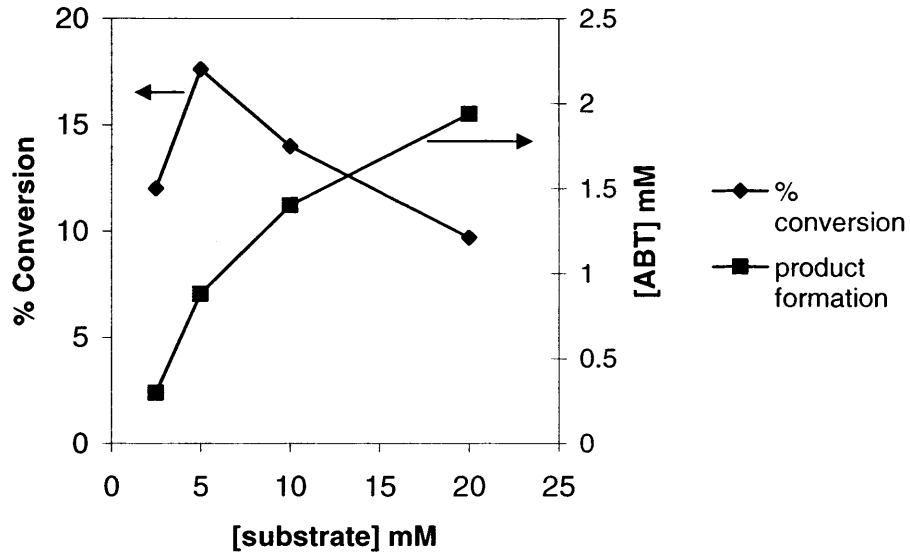


Figure 5.3: Substrate inhibition of β-A: P TAm catalysed transamination of *L*-erythrulose and *L*-MBA

Inhibition by both substrates was assessed by reactions with varied concentration of substrate from 2.5 to 20 mM (Section 2.10.4). Reaction conditions were 100 mM potassium phosphate buffer, pH 7.0 with 2 mM pyridoxal phosphate cofactor at ambient temperature (~25°C) and agitation at 200 rpm. Catalyst was added as purified transaminase (Section 2.4.3.3) to a concentration of 0.61 $mg.mL^{-1}$. Analysis of each reaction was by assaying ABT product formation after a 100 hour reaction period.

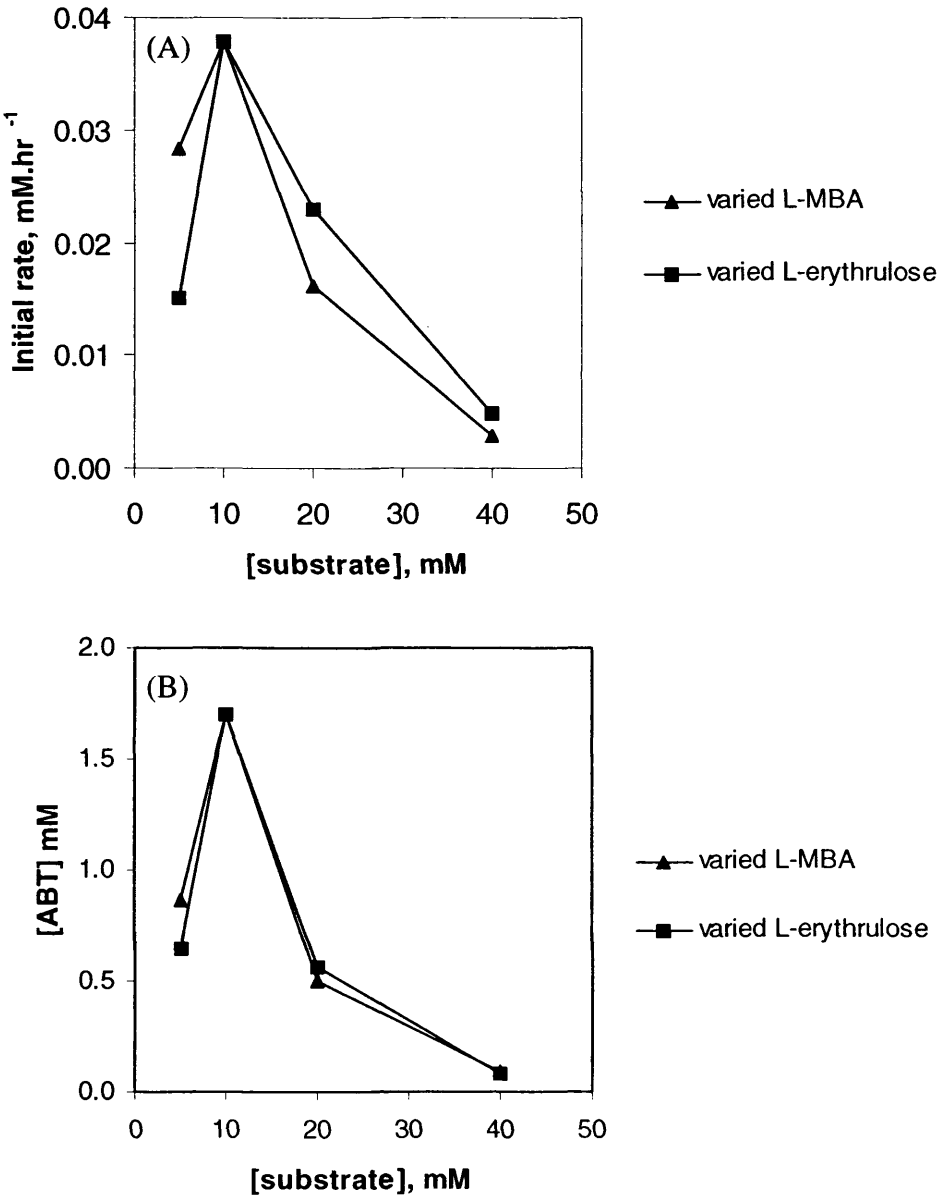


Figure 5.4: Individual substrate inhibition of β-A: P TAm by *L*-erythrulose and *L*-MBA

Inhibition by each substrate was assessed by varying one substrate from 5 to 40 mM whilst keeping the other substrate fixed at 10mM (Section 2.10.5). Reaction conditions were 100 mM potassium phosphate buffer, pH 7.0 with 2 mM pyridoxal phosphate cofactor at ambient temperature (~25°C) and agitation at 200 rpm. Catalyst was added as purified transaminase (Section 2.4.3.3) to a concentration of 0.61 $mg.mL^{-1}$. Analysis of each reaction was by assaying ABT product formation (Section 2.5.4) over a 100 hour reaction period. A) initial rate of ABT formation, B) final ABT concentration achieved.

The decrease in initial rate, from 10 mM to 20 mM substrate, is slightly higher for *L*-MBA than for *L*-erythrulose. A 51 % decrease in initial rate at 20 mM *L*-MBA relative to 10 mM is observed whereas at 20 mM *L*-erythrulose the decrease was 39%. This effect on initial rate implies that the substrates are inhibitory to the reaction. Furthermore a 70 % decrease in the final ABT yield was seen for an increase in either substrate concentration from 10 mM to 20 mM. Increasing the concentration of either substrate to 40 mM gives a further reduction in initial rate and ABT yield.

From the data obtained it is difficult to predict the mode of inhibition of the reaction. The substrates may be physically destabilising the enzyme. Inactivation has been observed for purified ω-amino acid: pyruvate TAm from *Vibrio fluvialis* which suffered complete inactivation by 5 mM *(S)*- MBA over a 12 hour incubation period (Shin, J.S. *et al.*, 2003). Addition of PLP with the MBA counteracts the destabilising effect. In the same study the addition of pyruvate enhanced the half-life of the enzyme. It is postulated by the authors that the inactivation of the enzyme is caused by the formation of E-PMP which is more un-stable than E-PLP. Alternatively the substrate could be binding with the enzyme in the incorrect form creating abortive complexes, for example the amine bound with E-PMP or the ketone bound to E-PLP. Experimental data for the substrate inhibition observed for an ω-transaminase from *Bacillus thuringiensis* was consistent with the kinetic model of this mode of inhibition (Shin, J.S. and Kim, B.G., 1998).

5.2.5 Investigation into product inhibition of *L*-erythrulose transamination

Product inhibition can occur when the enzyme has higher affinity for the product than the substrate. Therefore the product can inhibit the reaction by competitively binding at the active site. Additionally, the product may be toxic to the enzyme, destabilising it and therefore reducing the activity over time. Product inhibition of the transamination of *L*-erythrulose was investigated by the addition of both products simultaneously at the start of the reaction.

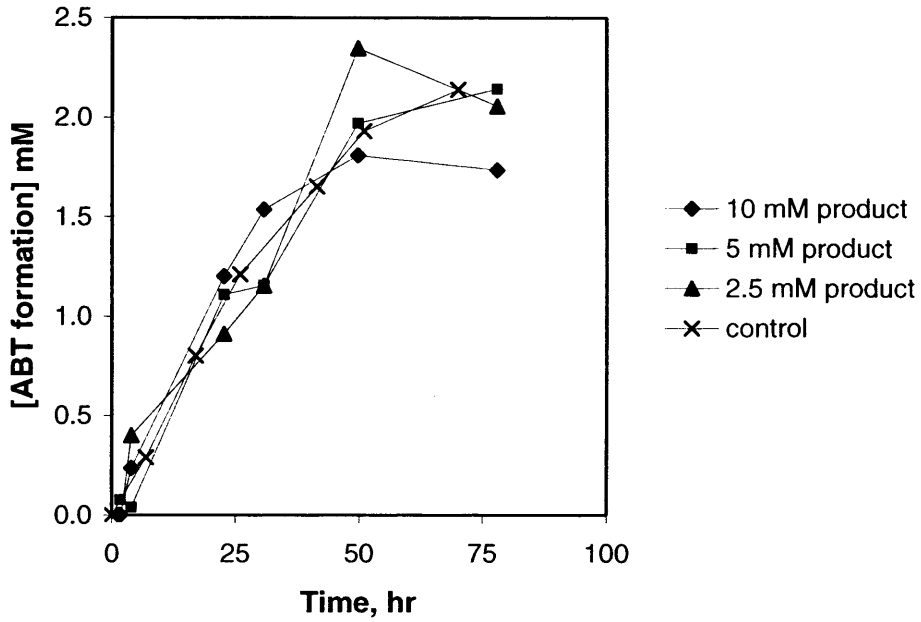


Figure 5.5: Investigation of the product inhibition of *L*-erythrulose transamination by β-A: P TAm

Reactions were spiked with both acetophenone and ABT at the start of the reactions. Reactions contained substrates, 10 mM, in 100 mM potassium phosphate buffer, pH 7.0with 2 mM pyridoxal phosphate cofactor at ambient temperature (~28°C) and agitation at 200 rpm (Section 2.10.6). Catalyst was added as purified transaminase (Section 2.4.3.3) to a concentration of 0.61 mg.mL^{-1}. The progress of the reactions was monitored by ABT formation (Section 2.5.4).

The reactions to investigate the effect of the product on the enzyme initially contained 10 mM substrates and were monitored for further ABT formation. The profiles of reactions spiked with both acetophenone and ABT (Figure 5.5) show little deviation from the control reaction, containing no added product. Thus the products at concentrations up to 10 mM are not detrimental to the reaction progress. This result was confirmed by reactions with the addition of individual products over the same concentration range (data not shown). Therefore, at the product levels achieved in the previous reactions with β-A: P TAm and substrates, *L*-erythrulose and *L*-MBA, product inhibition is not limiting the reaction.

5.2.6 Stability of purified β-A: P TAm

The progress of the *L*-erythrulose transamination reactions have been found to level off after approximately 50 hours of incubation at 28°C (Figure 5.5). This has been shown not to be attributable to product inhibition (Section 5.2.5), however, there may be destabilisation of the catalyst over time. In order to investigate the operational stability of the purified enzyme it was incubated under reaction conditions (Section 2.10.2) in the absence of substrate. Aliquots were removed over a 30 hour period and activity tested using the native β-alanine and pyruvate reaction (Section 2.4.3.4).

The activity of the enzyme declined over the incubation period with a loss of 19% of the initial activity after 30 hours (Figure 5.6). Therefore, over the 75 hour reaction period for the *L*-erythrulose transamination (Figure 5.5) approximately 50% of the enzyme activity could be assumed to be lost. This may be exacerbated by increasing substrate concentrations, suggested by the inactivation of the enzyme at elevated substrate concentrations (Section 5.2.4), but further experiments to investigate the effects of substrates and products on enzyme stability are required.

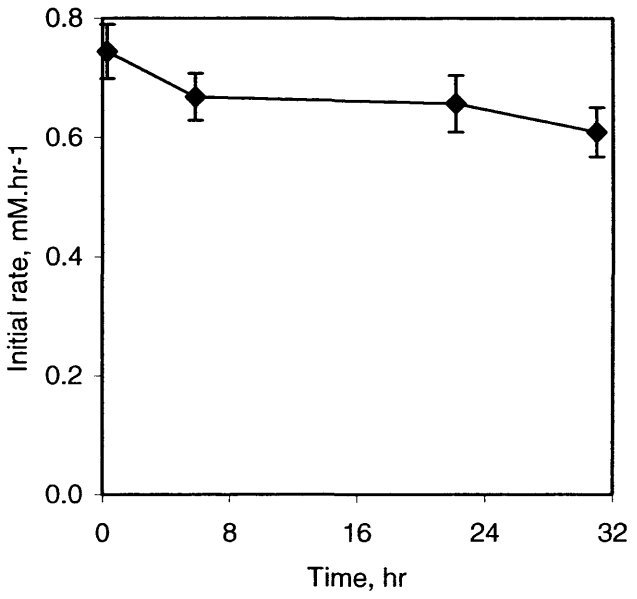


Figure 5.6: Operational Stability of β-A: P TAm

The purified enzyme at a concentration of 0.61 $mg.mL^{-1}$was incubated under the reaction conditions of 100 mM potassium phosphate buffer, pH 7.0 with 2 mM pyridoxal phosphate cofactor at ambient temperature (~28°C) and agitation at 200 rpm (Section 2.10.2) in the absence of substrate. Activity was assessed by removal of an aliquot and testing for the reaction with native substrates (2.10.2). Results are presented as an average of 3 replicates with a 95 % confidence interval.

5.2.7 Investigation into the *L*-erythrulose transamination equilibrium position

If the bioconversion is limited by enzyme inactivation before equilibrium is reached, then addition of further catalyst should enable a higher yield to be achieved. Similarly if the equilibrium position is the limiting factor, then addition of further substrate should promote further product accumulation back to the equilibrium position. This was investigated by allowing reactions to proceed to the apparent equilibrium at 72 hours before making an addition of either more substrate or catalyst (Figure 5.7).

In the reaction with the addition of more substrate (with cofactor) no further product formation was seen. The addition of more catalyst to the reaction resulted in the accumulation of a further 1.2 mM product over a further 70 hours at a slightly lower rate. A control reaction was also run with the addition of phosphate buffer. This reaction yielded no further product accumulation after 72 hours as expected. These results imply that the reaction is being limited by the degradation of the catalyst over the long reaction period. Addition of further catalyst resulted in an extra 12 % conversion reaching a total of 39 % conversion. Additional experiments are needed to further investigate the equilibrium position of the erythrulose transamination by the β-A: P TAm.

5.2.8 Determination of the pH optimum of the native β-A: P TAm reaction

Since the duration of the bioconversion is long (>70 hrs) the instability of the enzyme has a large impact on the achievable reaction yield. If the reaction rate could be increased, by a change in temperature or pH, it may be possible not only to reduce the reaction time but also increase the final product yield. This could only be achieved if the increase in reaction rate was significantly higher than any increase in enzyme instability due to the change in reaction conditions, as well as any elevated degradation of the reaction components.

The ω-pyruvate transaminase from *Pseudomonas sp.*F-126 has a reported optimum activity at a reaction temperature of 60°C without any apparent compromise of enzyme stability over a 10 minute incubation (Yonaha, K. *et al.*, 1977). The ω-TAm from *Vibrio fluvialis* JS17 was found to have highest activity at 37 °C, but was

rapidly inactivated at temperatures exceeding this over a 30 minute incubation period (Shin, J.S. *et al.*, 2003). Aside from the potential impact upon enzyme and reaction component stability, temperature increases at industrial scale are often unfavourable due to the high energy input required. The temperature optimum of the β-A: P TAm from *P. aeruginosa* was not investigated at this time although it remains a parameter that could potentially be manipulated to enhance the activity of the enzyme.

An investigation of pH was carried out, though it should be noted that the use of a whole cell biocatalyst expressing an engineered enzyme pathway is desirable. If a whole cell catalyst were to be employed the manipulation of pH may have different effects on the process to those seen with purified enzyme.

Previously reported investigations have found that several ω-transaminases have pH optimums of pH 9.0 (Shin, J.-S., *et al.*, 2003) or above (Kim, K.-H., 1963; Yonaha, K. and Toyama, S., 1978). TK has a reported pH optimum of 7.5 but retains 90% of the maximal activity from pH 6.5 – 8.0 (Mitra, R.K. *et al.*, 1998). Therefore, an increase in pH within this range for the multiple-enzyme scheme can be predicted to have little detrimental effect on the activity of TK. However, an increase in pH has an impact on the stability of the TK reaction components, particularly the substrates (Mitra, R.K., 1997), although this could be circumnavigated by feeding the substrates at a rate similar to the rate of TK activity to reduce their residence time at the unfavourable pH. The pH optimum of the native transamination between β-alanine and pyruvate was found to be above pH 8.5 (Figure 5.8) with a near doubling of initial rate from pH 7.0 to pH 8.5. A conversion of 30 % was achieved for reactions at each pH. Values above pH 9.5 were not investigated due to the likely instability of reaction components at higher pH.

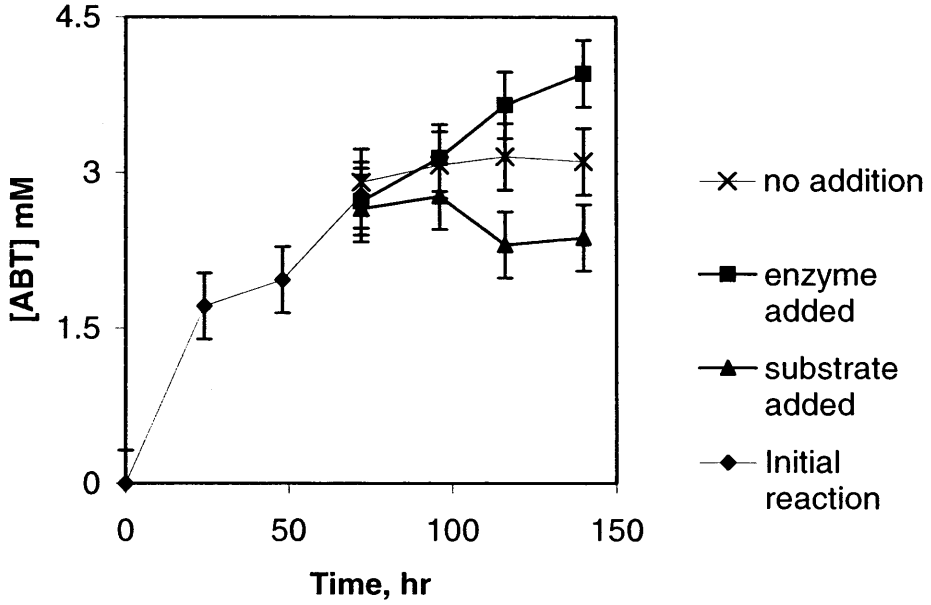


Figure 5.7: Investigation into reaction equilibrium

A reaction at 10 mM initial substrate concentration with purified enzyme at a concentration of 0.61 $mg.mL^{-1}$was monitored for ABT formation (Section 2.5.4) over 72 hours. Reaction conditions were 100 mM potassium phosphate buffer, pH 7.0 with 2 mM pyridoxal phosphate cofactor at ambient temperature (~28°C) and agitation at 200 rpm (Section 2.10.2). Additions (enzyme added = enzyme + 2 mM cofactor, no addition = phosphate buffer only, substrate added = substrates to increase reaction concentration by 5 mM + 2 mM cofactor) were then made to three reaction samples and the further accumulation of ABT monitored (Section 2.10.7). Data shown accounts for volume adjustment, error bars are 95 % confidence calculated from triplicate analysis of the control reaction.

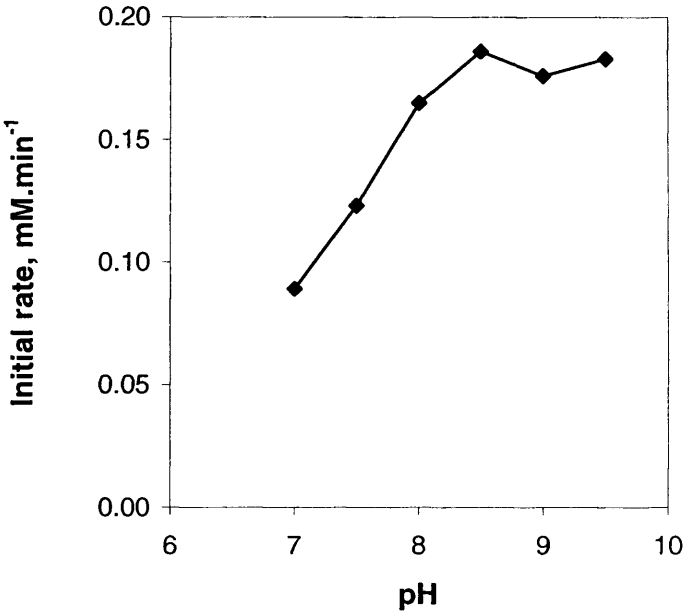


Figure 5.8: The effect of pH on the native reaction of β-A: P TAm

The effect of varying the pH from pH 7.0 - 9.5 was investigated by comparison of the initial rate of reactions investigated with 10 mM substrates β-alanine and pyruvate, in 0.1 M potassium phosphate buffer at the appropriate pH, 2 mM pyridoxal phosphate (Section 2.10.2).

5.2.9 Determination of pH optimum of *L*-erythrulose transamination

A difference in pH optima has previously been reported for ω-TAm from *Vibrio fluvialis* JS17 with different reaction substrates (Shin, J.-S. and Kim, B.-G., 1999). In this case the reaction between *(S)*-MBA and pyruvate had an optimum at pH 9.0. However the reverse transamination between *L*-alanine and acetophenone was found to have an optimum of pH 7.0 (comparison of relative activity). The authors postulated that the difference in optima between the reactions was due to the difference in pK_a of the substrates. Therefore, the pH required to enable the formation of the internal aldimine during catalysis would be higher for MBA (pK_a=10.65) than for *L*-alanine (pK_a=9.69). Additionally there may be an influence of pH on substrate binding. At different pH, the amino acid side chains of the active site may exist in different protonation states. This could affect the interactions both with one another and with the substrate or cofactor molecules.

The results for the effect of pH on native β-A: P TAm activity (Section 5.2.8) show that the enzyme activity is higher under more alkaline reaction conditions than pH 7.0, although no information has been obtained about stability of the enzyme at different pH. Therefore the effect of pH between pH 6.0 and pH 8.5, upon the transamination of *L*-erythrulose by the β-A: P TAm, was investigated. Higher pH was not considered due to the probability of reaction components being unstable.

Upon examination of the reaction profiles (Figure 5.9 (A)), it is evident that a comparison of initial rates could be misleading. Whilst an increase in pH has a little effect on the initial rates, the conversion has a distinct pH optimum with the maximum accumulation of product being achieved over 70 hours at pH 7.0 (Figure 5.9 (B)). At higher pH values, whilst the initial rate is comparable to that at pH 7.0, the rate of reaction quickly slows reaching just 32 % of the product yield obtained at pH 7.0 over a 50-hour reaction period. Further experimentation is required to determine whether it is the instability of either substrates or products, or inactivation of the enzyme leading to the reduced conversion at higher pH. This experiment shows that increasing the pH of the bioconversion above pH 7.0 to enhance the product yield is not a viable option.

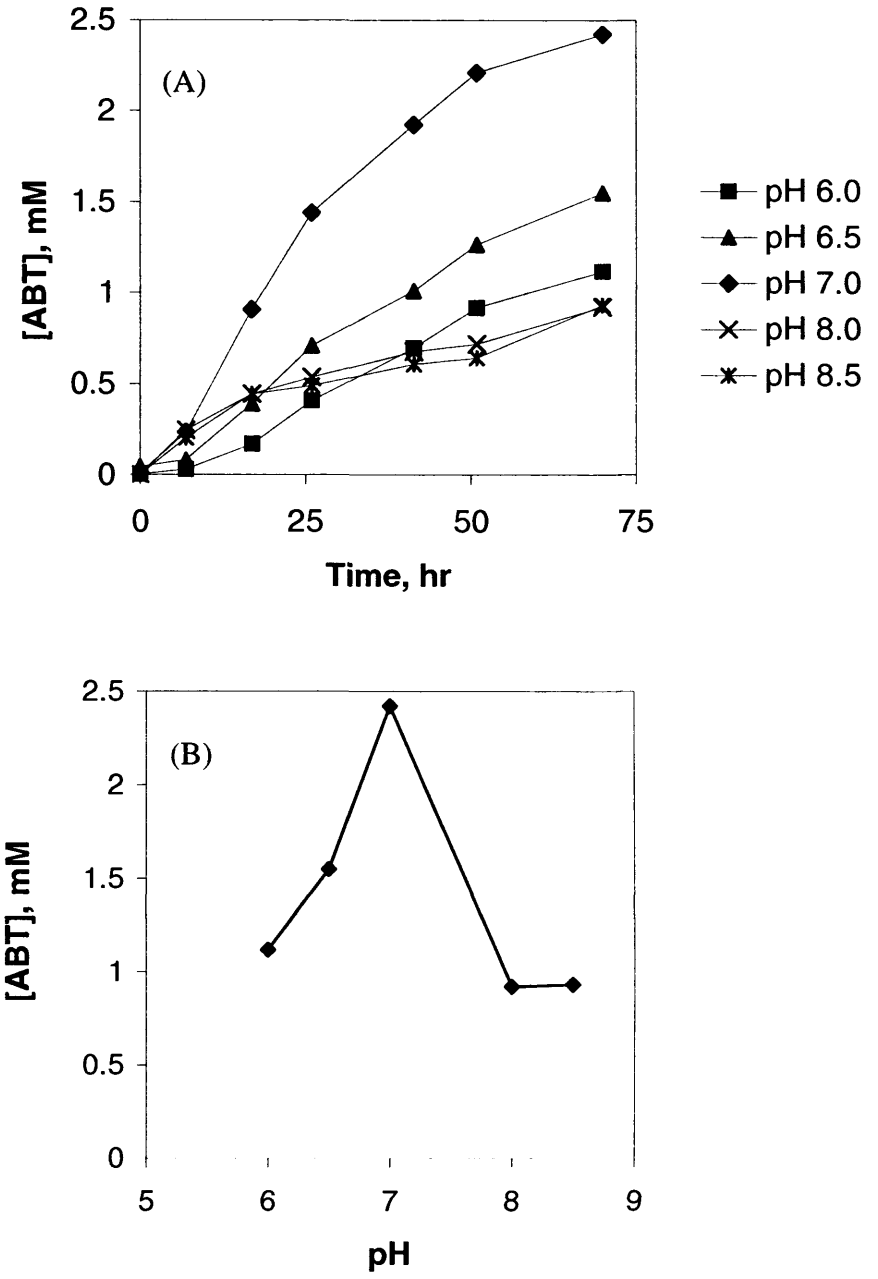


Figure 5.9: The effect of pH on the transamination of *L*-erythrulose by β-A: P TAm

The effect of varying the pH from pH 7.0 - 9.5 was investigated with 10 mM *L*-erythrulose and *L*-MBA substrates, 2 mM pyridoxal phosphate with purified enzyme at a concentration of 0.61 $mg.mL^{-1}$ in 0.1 M potassium phosphate buffer at the appropriate pH (Section 2.10.2). (A) reaction profiles (B) ABT production over 70 hour reaction period.

5.2.10 Demonstration of β-A: P TAm stereoselectivity

At this point in time an assay to determine the chirality of the ABT synthesised by the β-A: P TAm is not available. A simple way to demonstrate whether the enzyme is enantioselective is to investigate the acceptance of the commercially available L and D enantiomers of MBA. This can be used as an indicator of possible chiral product synthesis. However, it will not give any indication of the chirality of the amine product formed, as the stereoselectivity will be dependent on the ketone substrate. The enantioselective substrate dependence of ω-transaminases has been previously demonstrated for the ω-amino acid transaminase from *Bacillus megaterium* with a range of substrates (Stirling, D.I., 1992). Most compounds tested were accepted as amine donors in the *(S)* configuration, however for related hydroxylated compounds the *(R)* enantiomer was preferred.

Reactions using the cloned β-A: P TAm with either one of the single isomers of MBA or the racemic D/*L*-MBA mixture were set up with pyruvate as the amine acceptor. The formation of acetophenone was monitored to compare the transamination of the different enantiomers (Figure 5.10). It is apparent from the data obtained that the *L*-enantiomer of MBA is accepted as a substrate whereas the D-enantiomer is not a desirable substrate. The substrate in the racemic form also functioned as a substrate reaching 50% of the conversion reached with the *L*-stereoisomer this is as expected as the racemic mixture contains 50% of the favoured *L*-MBA substrate.

Studies on the *(S)*-specific ω-TAm isolated from *Vibrio fluvialis* JS17 have shown that the *(R)*-enantiomer of an amine substrate is not a substrate for the enzyme and actively inhibits the rate of reaction of the desired enantiomer (Shin, J.-S. and Kim, B.G., 2002a; Shin, J.-S., Kim, B.-G., 2002b). The data collected for the β-A: P TAm does not allow the comparison of the initial rates of catalysis of the single enantiomer and racemic form of MBA. In order to investigate whether this enzyme is subject to inhibition by the 'wrong' enantiomer investigation of the initial rates is required.

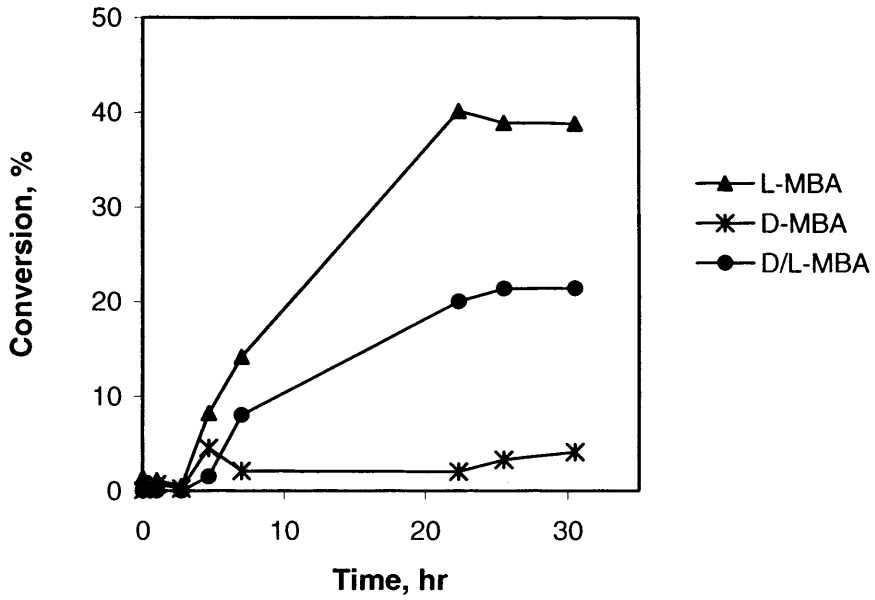


Figure 5.10: Investigation into the acceptance of the MBA stereoisomers by β-A: P TAm

Reactions were set up with 10 mM pyruvate and 10 mM of the specified MBA amino donor with 100 mM potassium phosphate buffer, pH 7 and 2 mM pyridoxal phosphate cofactor at ambient temperature (~28°C) and agitation at 200 rpm (Section 2.10.2). Enzyme was added as lysate from BL21gold(DE3) pQR426. Reactions were analysed for the formation of acetophenone product (Section 2.5.4).

5.3 Summary

In the previous chapter the transamination of *L*-erythrulose was detected by the β-A: P TAm from *Pseudomonas aeruginosa*. This chapter has further investigated the transfer of the amine group between *L*-erythrulose and *L*-MBA, using β-A: P TAm. In order to simplify the study of the reaction the β-A: P TAm was purified (Section 5.2.1). This was carried out by the addition of a histidine tag and purification by affinity chromatography on a Ni^{2+} column.

To confirm that the ABT was the product of the enzyme catalysis, the erythrulose ketone substrate was not added. This retarded the formation of both the ABT and acetophenone products (Section 5.2.2) implying that the generation of these products are dependent on the addition of *L*-erythrulose.

A summary of the characterisation of the desired transamination of *L*-erythrulose with *L*-MBA is presented in Table 5.1 alongside the results for the native transamination, between β-alanine and pyruvate. Whilst the product accumulation in the native reaction proceeds to 30 % over 100 minutes, before ceasing, the desired reaction is significantly slower reaching a comparable point over 75 hours. The substrate concentration had little effect on the native transaminase reaction up to 40 mM (Section 5.2.3) whilst increasing the substrate concentration in the transamination between *L*-erythrulose and *L*-MBA, with purified enzyme, from 10 mM to 20 mM was detrimental to the reaction progress (Section 5.2.4). The reaction products of this reaction had no apparent negative effect on the initial rate nor the yield of ABT over the concentration range investigated (Section 5.2.5).

The catalytic activity of the β-A: P TAm was seen to degrade over the long reaction period (Section 5.2.6). Addition of fresh enzyme when the product accumulation ceased led to further product accumulation (Section 5.2.7). Therefore the reaction is being limited by enzyme degradation. It is possible that the negative effect of increasing substrate concentration is due to the compounds further destabilising the enzyme.

In order to increase the rate of the reaction to try and improve the rate of product accumulation the pH optimum was investigated. Whilst the optimum for the native reaction was above pH 8.5 (Section 5.2.8) the *L*-erythrulose transamination with *L*-MBA achieved the highest product accumulation at pH 7.0 (Section 5.2.9). Therefore adjusting the pH to enhance the product yield was not an option.

Finally the stereo selective nature of catalysis was demonstrated by the investigation of the use of either the *D*- or *L*-isomer of MBA (Section 5.2.10). It was found that the *L*-isomer of this particular substrate was a desirable substrate whilst the *D*-isomer was not. Therefore the enzyme is discriminating between the substrate isomers.

Table 5.1: Summary of the characterisation of purified β-A: P TAm

The activity of purified β-A: P TAm was characterised for the native reaction with β-alanine and pyruvate, and the target transamination of *L*-erythrulose with the *L*-MBA amine donor.

Parameter investigated		Native reaction	Target reaction
Yield		30% in 100 minutes	27% in 75 hrs
Substrate inhibition		Not detected up to 40 mM	> 10 mM
Product inhibition		ND	Not detected to 20 mM
pH optimum	Initial rate	pH > 8.5	pH 7.0 and above
	Conversion	ND	pH 7.0
Operational Stability		19% decline in activity over 30 hrs	

Chapter 6 - Multi-step synthesis of 2-amino-1,3,4-butanetriol by coupled transketolase and β-alanine: pyruvate transaminase

The previous chapter described the characterisation of the candidate β-A: P TAm from *P.aeruginosa* for the synthesis of ABT from *L*-erythrulose. In this chapter the construction of a biocatalyst over-expressing the TK and β-A: P TAm is described. The synthesis of ABT using the TK and β-A: P TAm produced by the engineered strain is then demonstrated.

6.1 Introduction

The enzymes transketolase and transaminase, working in series have the potential to synthesise a product with two new chiral centres from achiral starting compounds. The model reaction, to demonstrate this, involves the transfer of a 2 carbon group from β-HPA onto glycolaldehyde by transketolase, forming an asymmetric carbon-carbon bond, followed by the stereospecific transfer of an amine on the carbonyl of the transketolase product by the transaminase, forming 2-amino-1,3,4-butanetriol (Figure 6.1).

In order to carry out this synthesis it is desirable to have the two enzymes over-expressed in a single strain. Compatible plasmids encoding the enzymes have already been constructed (Section 3.2.1, Section 3.4.1). These can be used to transform the host strain to create the dual plasmid biocatalyst. The use of a dual plasmid strain versus a mixed culture of the two single strains has two potential advantages. The first is the ability to predict the enzyme concentrations in the fermentation. If two strains each expressing one of the enzymes were to be used they either could be grown up separately and then mixed to obtain the desired enzyme ratio, this is laborious and requires two fermentations, or a fermentation containing both strains could be used but maintaining the ratio of enzymes between batches may be difficult.

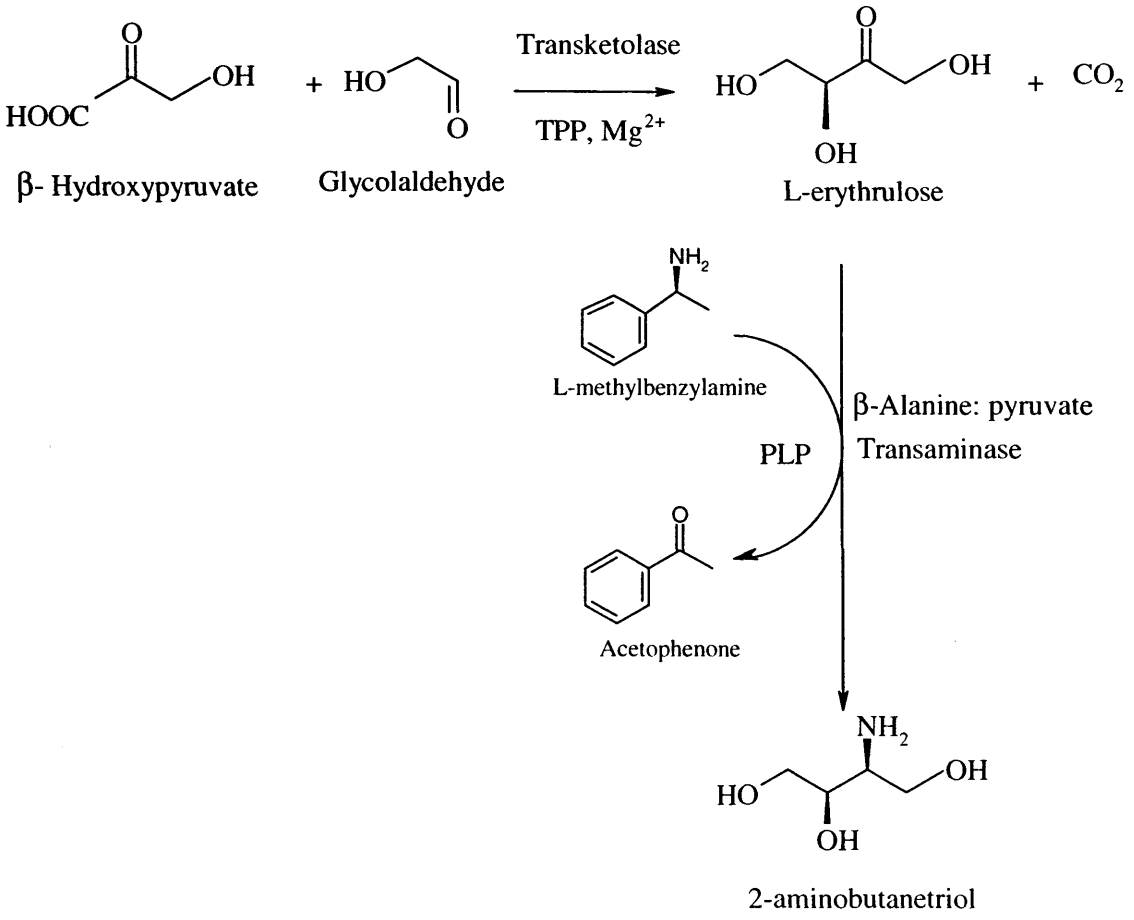


Figure 6.1: Reaction scheme for the synthesis of ABT by the TK-β-A: P TAm enzyme pathway

In the dual strain control of the expression levels of the individual enzymes can be achieved by the manipulation of the promoter. The second advantage may be concerning the transport of products. In this work lysate is examined as a catalyst but ultimately the evaluation of a whole cell biocatalyst would be desirable. If the synthetic enzymes are expressed in separate cells the membrane may impede the transfer of products from reactions to serve as substrates for subsequent reactions. The advantage of a single host strain for a synthesis involving multiple enzymes is exemplified by the synthesis of *D*-amino acids using *D*-hydantoinase and *N*-carbamoylase (Park, J.H. *et al.*, 2000). The authors demonstrate the increased yield of product from the biocatalyst over-expressing both enzymes compared to a process with separately expressed enzymes, achieving yields of 98 % and 71 % respectively over a 15-hour reaction period. Significant accumulation of the intermediate was detected in the process with the separately expressed enzymes but not with the co-expressed enzyme. This intermediate has low permeability through the cell membrane and, therefore, it is proposed that it is this transport that is limiting the reaction progress. If the intermediate is unstable the use of a dual strain may improve yields if the rate of the second reaction is sufficiently rapid to overcome accumulation of the unstable intermediate.

Once constructed, the growth and activity characteristics of the dual plasmid strain must be investigated in order to ensure the biocatalyst is producing an appropriate level of each enzyme. Lysate is used at this stage in order to remove any potential complications of the transport of reaction components into the cell since the transaminase reaction rates have been found to be very slow with isolated enzyme. Since there are many cellular components in the lysate it is necessary to evaluate the stability of the reaction components in the lysate before assessing the catalytic activity. Demonstration of the synthesis of the ABT product via the dual reaction can then be carried out and the monitoring of each component can provide a full profile of the bioconversion. The relative rates of reaction can then be evaluated and a strategy for improving the linked reaction proposed.

6.2 Construction of the dual TK-β-A: P TAm strain

In order to investigate the use of the multi-enzyme pathway produced in a single *E.coli* biocatalyst the plasmids pQR411 (Figure 3.1) and pQR426 (Figure 3.9), encoding the TK and β-A: P TAm respectively, were transformed into BL21gold(DE3) to form a dual plasmid strain over-expressing both enzymes (Section 2.11.1). This strain was cultivated in the presence of kanamycin and ampicillin to maintain selection for both plasmids. The over-production of both enzymes was verified by SDS-PAGE analysis (Figure 6.2). TK was found to make up ~ 24 % of the cellular protein and TAm ~ 33 %. These values are slightly lower than for the single plasmid strains where TK and TAm made up 33 % and 37 % respectively. The higher expression of the transaminase is probably due to the use of the T7 promoter in the pQR426 plasmid, which may be stronger than the native TK promoter in pQR411. The activity of both enzymes from the dual plasmid strain was confirmed with the model reaction for TK (Section 3.2.2) and the native reaction for β-A: P TAm (Section 3.4.3), as for the single strains.

The growth profile of the *E.coli* dual plasmid strain and the activity of both TK and β-A: P TAm throughout a typical fermentation are shown in Figure 6.3. The activity of TK was constitutive, increasing with cell density. The β-A: P TAm was induced with IPTG after 2 hours of growth. Retardation of growth upon induction led to the dual plasmid strain reaching 72% of the biomass of the wild-type strain, however the retardation was less than for the single plasmid strain BL21gold(DE3) pQR426 , which reached only 55% (Section 3.4.4). This may be due to the slightly lower overall production of the TAm in the dual strain as discussed above.

6.3 Stability of transamination reaction components

Stability of substrates and products becomes a particular issue over long reaction periods such as those observed for the transamination of *L*-erythrulose (Figure 5.5). The stability of the reaction components was therefore assessed by incubation of the individual reagents with lysate from the dual plasmid strain BL21gold(DE3) pQR411+pQR426 (Section 6.2), over-expressing both TK and β-A: P TAm under the standard reaction conditions (Section 2.10.2).

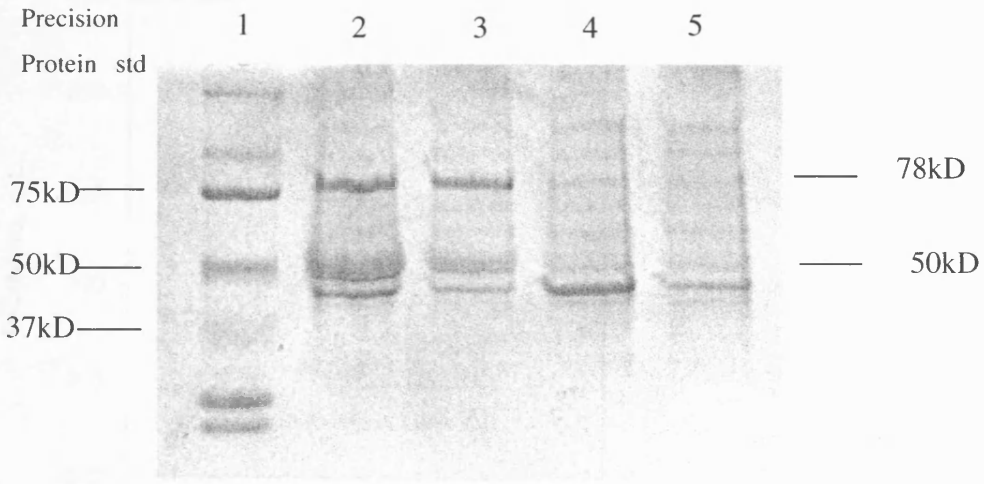


Figure 6.2: SDS page analysis of dual plasmid strain BL21gold(DE3) pQR411+pQR426

The expression of *Pseudomonas aeruginosa* β-A: P TAm and TK from the constructed *E.coli* strain BL21gold(DE3) pQR411+ pQR426 was analysed on a 12% v/v acrylamide SDS-PAGE gel and stained with commassie blue (Section 2.3). Sample lanes: 1= MW marker, lanes 2+3 = replicates of BL21gold(DE3) pQR411+ pQR426, lanes 4+5= repeats of wild-type BL21gold(DE3). The dual plasmid strain over-expressing both TK and β-A: P TAm shows prominent bands at 50 KDa and 78 kDa not present in the wild-type strain.

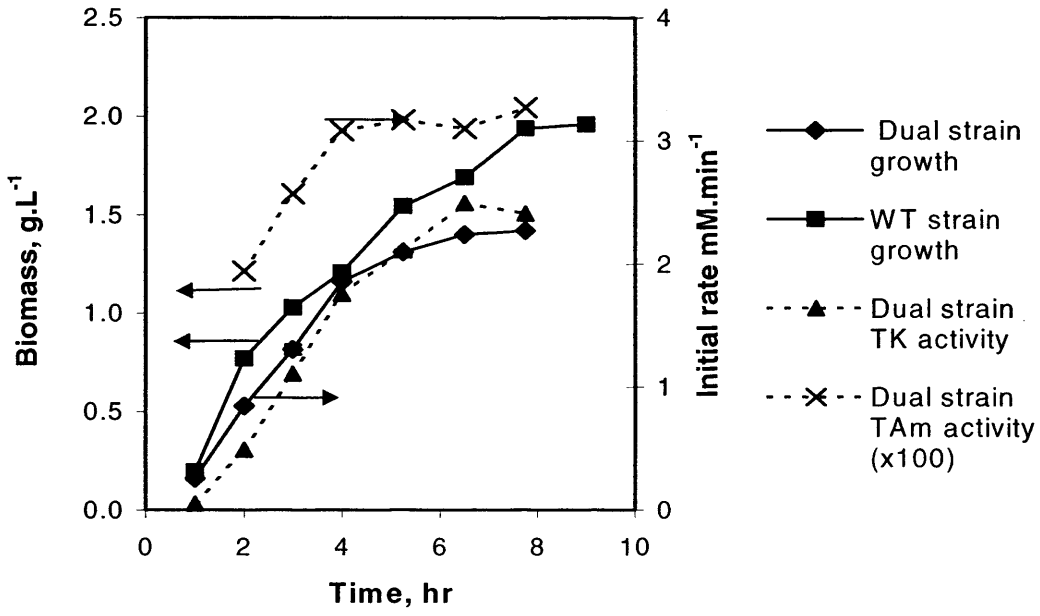


Figure 6.3: Growth and activity profile of dual plasmid strain BL21gold(DE3) pQR411+pQR426

Cells were cultured, as for the single β-A: P TAm strain, from a 1/20 inoculum, in 100 mL nutrient broth, in a baffled shake flask at 37°C, 200rpm (Section 2.4.3.5). Growth was monitored by OD 600 nm measurements, induction of β-A: P TAm was carried out by the addition of 1 mM IPTG 1 hour after inoculation. TK activity was measured by analysis of the initial rate of the model reaction *L*-erythrulose product formation at each time point (Section 2.4.1.3), β-A: P TAm activity was assessed by initial rate measurements with native substrates β-alanine and pyruvate (Section 2.4.3.5). TAm activity is presented at 100x actual measurement for ease of comparison of all data.

Substrate *L*-MBA, 25 mM, showed 3.7% depletion with corresponding acetophenone formation over a 110-hour incubation (Figure 6.4). This is in keeping with the previous result obtained for incubation with the single plasmid strain over-expressing β-A: P TAm in the absence of *L*-erythrulose (Section 5.2.2). This is attributed to the ability of the β-A: P TAm to accept other ketones present in the lysate as substrates for transamination. *L*-erythrulose, 15 mM, also exhibited some depletion in the presence of lysate from the dual plasmid strain with a loss of approximately 17%, over a 75-hour incubation period (Figure 6.5).

A similar experiment was set up with the pathway product ABT, synthesised 'in house' (Section 2.6.1). Loss of ABT, 5 mM, when incubated with the various constructed strains, was detected at a rate of 0.014 $mM.hr^{-1}$ in each case (Figure 6.6). At this rate over 75 hours a total of 1.05 mM of ABT could be lost although it is probable that the rate of consumption is concentration dependent. The rate of ABT loss was comparable between the strain over-expressing β-A: P TAm and the other strains. Therefore ABT product is being consumed by something other than the over-expressed β-A: P TAm. This was further confirmed by negligible degradation of the product upon incubation with purified β-A: P TAm enzyme. The appearance of a peak coincidental with ammonia in the chromatograms (not quantified) for the lysate stability experiments, suggests the activity degrading the product is possibly a deaminase. Deaminases are numerous in *E.coli* (10 are listed in the BRENDA database (Schomburg, I. *et al*, 2002)) and play important roles in metabolic processes. For example the tRNA adenosine deaminase has been found to play a role essential to cell survival, deaminating RNA to form site-specific inosine (deaminated adenosine) which can change the codon specificity enabling multiple protein products from a single mRNA (Wolf, J., Gerber, A.P. and Keller, W., 2002). Therefore a search to identify the pathway of ABT degradation by the cellular components would be tricky, and may result in the identification of an enzyme whose activity is essential to the cell. Thus, the enhancement of the rate of catalysis such that it far exceeds the rate of ABT consumption, is a more viable option than engineering a strain. Since ABT is the desired reaction product, this component is monitored to follow the transamination of *L*-erythrulose.

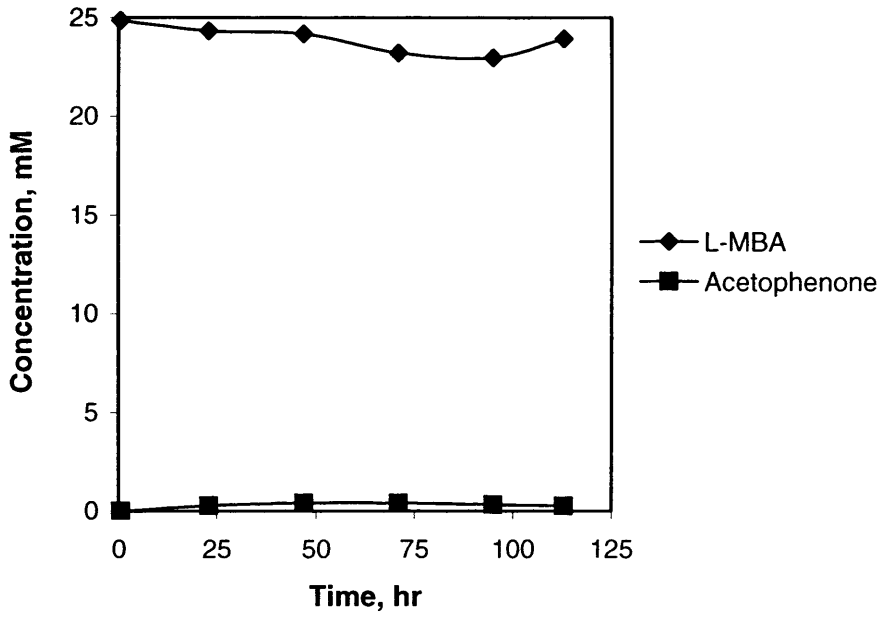


Figure 6.4: Stability of *L*-MBA with the cell lysate of E.coli BL21gold(DE3) pQR411+pQR426.

L-MBA, 25 mM, was incubated with the lysate of BL21gold(DE3) pQR411+pQR426 under the reaction conditions, 100 mM potassium phosphate buffer, pH 7.0 with 2 mM pyridoxal phosphate cofactor at ambient temperature (~25°C) and agitation at 200 rpm (Section 2.10.2). Monitoring of *L*-MBA and acetophenone by HPLC (Section 2.5.5) was carried out over a 110-hour period.

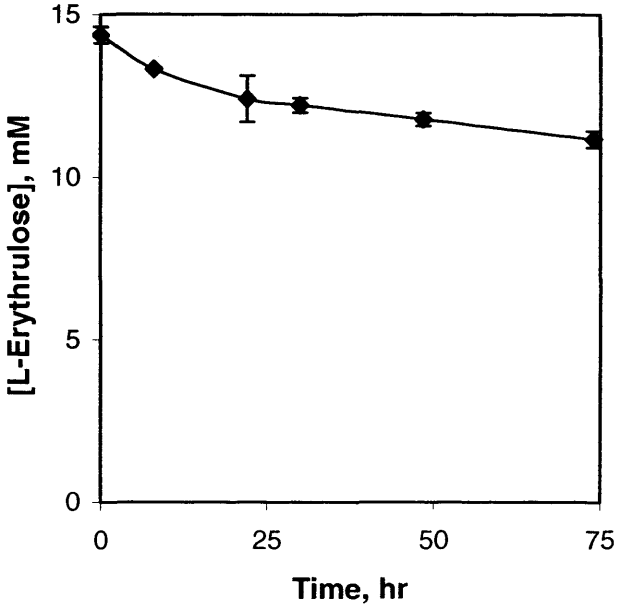


Figure 6.5: Stability of *L*-erythrulose with the cell lysate of E.coli BL21gold(DE3) pQR411+pQR426.

L-erythrulose, 15 mM, was incubated with the lysate of BL21gold(DE3) pQR411+pQR426 under the reaction conditions, 100 mM potassium phosphate buffer, pH 7.0 with 2 mM pyridoxal phosphate cofactor at ambient temperature (~25°C) and agitation at 200 rpm (Section 2.10.2). Monitoring of the concentration of *L*-erythrulose (Section 2.5.2) was carried out over a 75-hour period.

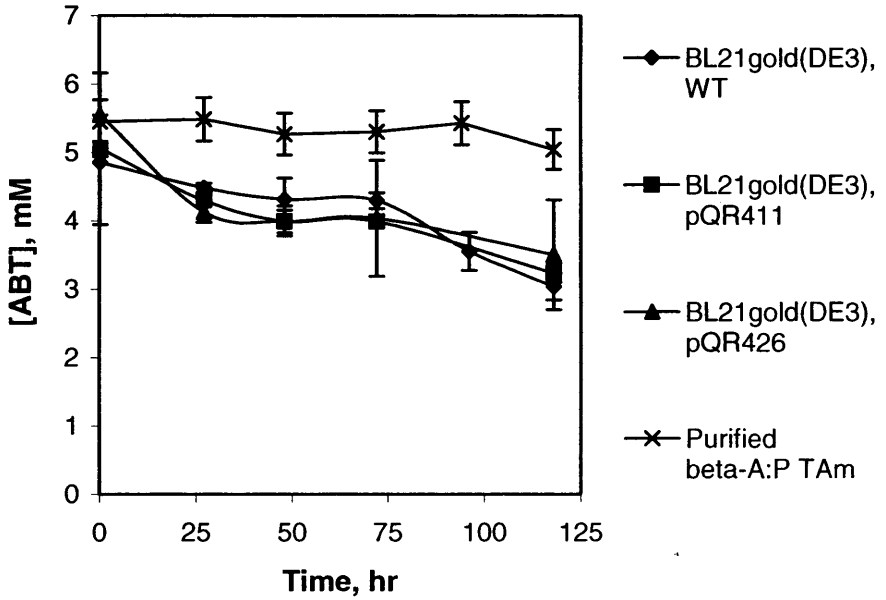


Figure 6.6: Stability of ABT with the cell lysate of E.coli BL21gold(DE3) pQR411+pQR426

ABT, 5 mM, was incubated with the lysate of either BL21gold(DE3) pQR411, BL21gold(DE3) pQR426 or BL21gold(DE3) wild-type under the reaction conditions, 100 mM potassium phosphate buffer, pH 7.0 with 2 mM pyridoxal phosphate cofactor at ambient temperature (~25°C) and agitation at 200 rpm (Section 2.10.2). The ABT concentration was monitored over a 120-hour period (Section 2.5.4). An additional reaction contained purified enzyme in place of lysate was also carried out. Error bars for reactions containing lysate represent 95 % confidence limits from triplicate samples and from triplicate reactions in the case of purified enzyme.

6.4 Demonstration of the model reaction

6.4.1 ABT formation in coupled TK and TAm reactions

The demonstration of the feasibility of the synthesis of ABT was carried out with the newly constructed dual strain, directly from achiral substrates glycolaldehyde and β-HPA along with *L*-MBA amine donor (Figure 6.1). Bioconversions with the dual plasmid strain lysate and TK substrates, β-HPA and glycolaldehyde, were set up in the presence of the transaminase amine donor substrate *L*-MBA and cofactors for both enzymes. All three substrates were charged at initial concentrations of either 10 mM, 15 mM or 20 mM (It should be noted that the substrate for reaction with 10 mM substrates was incorrectly charged at 20 mM *L*-MBA with 10 mM TK substrates). Reactions were monitored for final target reaction product ABT over a 110-hr period (Figure 6.7). Data was reproducible on separate occasions with different cell-lysate preparations.

Data is presented for whole-cell experiments conducted for reactions with 10 mM and 20 mM substrates. The transketolase reaction (data not shown) occurred at approximately half the rate of the lysate reactions, reaching approximately 50 % conversion over the 40 minute reaction period, where the lysate reaction reached >98% conversion. Unfortunately the data set for this experiment is incomplete due to analytical problems but the ABT accumulation shows similar trends to the reaction with lysate. This observed activity may be whole-cell activity although lysis could be occurring during the reaction period. This could be tested by assaying the supernatant for the activity of TK or β-TAm throughout the reaction.

The reaction profiles for each substrate concentration show the ABT concentration increasing throughout the 95-hour reaction period. Production of ABT is, therefore, occurring for longer than the 72-hour time period after which accumulation was seen to cease with purified β-A: P TAm enzyme due to inactivation (Figure 5.7). It is possible that the enzyme is more stable in the environment of the lysate than in the purified form. Protein in lysates is often subject to attack by endogenous proteases, however, the chosen host BL21gold(DE3) strain is lacking both the La and OmpT proteases which reduces such degradation (Studier, W.F., Moffatt, B.A., 1986).

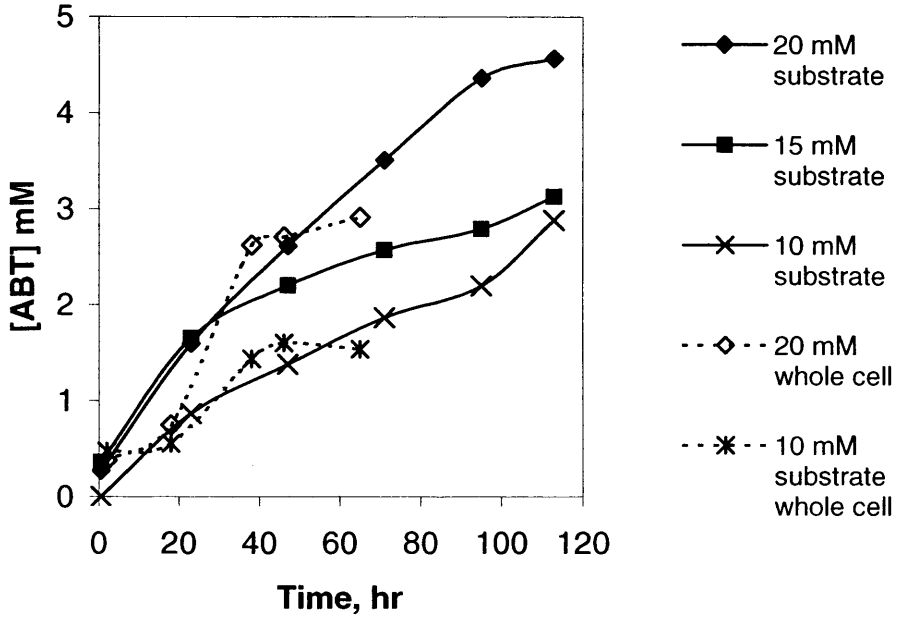


Figure 6.7: ABT production from one-pot reaction with dual strain expressing both TK and β-A: P TAm

The formation of ABT was analysed for reactions set up with dual plasmid lysate. Substrates β-HPA, GA and *L*-MBA were added in equal concentration (10 mM, 15 mM or 20 mM respectively) at the beginning of each reaction. Reactions contained 100 mM potassium phosphate buffer, pH 7.0 with cofactors thiamine pyrophosphate, 2.4 mM, $MgCl_2$, 9 mM, and pyridoxal phosphate, 2 mM, and were incubated at ambient temperature (~28°C) with agitation at 200 rpm (Section .2.10.2). Whole cell reactions contained freshly cultivated unlysed induced cells in place of lysate.

The structure of the closely related ω-amino acid pyruvate aminotransferase from *Pseudomonas sp.* F-126 has been determined (Watanabe, N. *et al.*, 1989) to be a tetramer comprised of two loosely associated dimers (Section 1.10.2). It is therefore possible that the increased instability of the purified β-A: P TAm enzyme may be due to the loss of the tetrameric conformation during purification. This could be investigated by examining the native form of the enzyme from both lysate and purified enzyme preparations.

The concentration of ABT produced increased with the increase substrate concentration. This result differs from those obtained for isolated enzyme (Section 5.2.4). The mechanism of substrate inhibition has not yet been determined but it may be that the substrates destabilise the enzyme. Therefore, the apparently increased stability of the enzyme in the lysate may reduce the detrimental effect of the substrate on the enzyme and enable conversion at higher substrate concentrations. Whilst the substrate concentrations investigated, selected based on the observations with purified enzyme (Section 5.2.4), are far below those required to utilise such a process on a commercial scale the results indicate the feasibility of a dual enzyme biocatalyst to carry out the synthesis of amino alcohols. Further investigation into the effect of increasing the substrate concentration using lysate and whole cell preparations is required.

For each concentration of substrate, the conversion of *L*-erythrulose to ABT reached ~21% yield over a 95-hour time period. However, it has previously been found that the ABT product is degraded by the lysate (Figure 6.6). Therefore, the observed concentration of ABT is the cumulative effect of formation and simultaneous degradation. Complete analysis of all reaction components was necessary to fully assess the reaction. The appearance of an additional peak in the dual conversion, and the control using dual lysate with just transaminase substrates, overlapping with the product peak requires further investigation (Appendix (E)). This peak occurs only where crude lysate is used. The identity of this peak could be determined using an LC-MS method but was not examined further here.

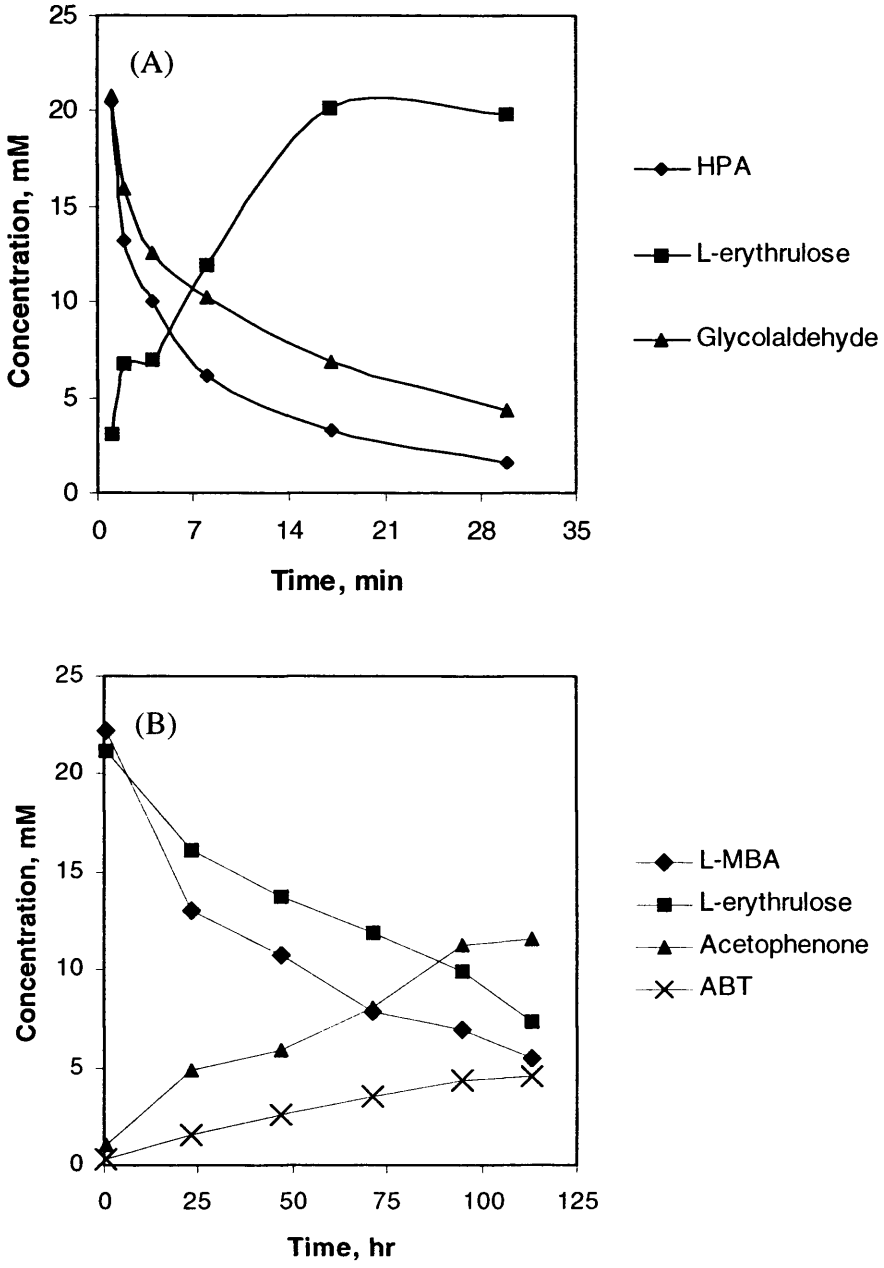


Figure 6.8: Complete analysis of target reaction of TK-β-A: P TAm pathway

(A) TK reaction analysis (B) β-TAm reaction analysis

Substrates HPA, GA and *L*-MBA were added each at 20 mM to reactions containing lysate from the dual plasmid strain over-expressing TK and β-A: P TAm in 100 mM potassium phosphate buffer, pH 7.0 with cofactors thiamine pyrophosphate, 2.4 mM, $MgCl_2$, 9 mM, and pyridoxal phosphate, 2 mM, and were incubated at ambient temperature (~28°C) with agitation at 200 rpm (Section 2.11.2). Analysis was carried out using 3 separate assays 1) HPA, GA and erythrulose (Section 2.5.2), MBA and acetophenone (Section 2.5.5) and 3) ABT (Section 2.5.4).

6.4.2 Substrate and product mass balance for the coupled ABT synthesis

In order to obtain a process mass balance of the TK β-A: P TAm reaction, analysis is required for all substrates and products (except CO_2) in a one-pot process, with all substrates present at the start of the reaction, using the dual plasmid strain. This was carried out at initial substrate concentrations of 15 mM and 20 mM. Analysis of the TK reaction progress was carried out to completion and the TAm reaction was monitored over a 110-hour period. The TAm reaction was monitored for the depletion of both *L*-erythrulose and *L*-MBA and the formation of ABT and acetophenone. The analysis of the dual enzyme reaction with 20 mM substrate (Figure 6.8) shows the TK reaction proceeding to >95% conversion within 30 minutes. The progression of the transaminase reaction is much slower with the production of ABT appearing at an initial rate of 0.043 $mM.hr^{-1}$ with accumulation levelling off around 100 hours to give a final overall yield of 21 %. The final yield of ABT was 45% lower than the yield of acetophenone generated from the *L*-MBA.

The rates of acetophenone formation and *L*-MBA and *L*-erythrulose depletion were comparable at around 0.1 $mM.hr^{-1}$ reaching a final conversion of ~ 60 %. Therefore the degradation of ABT by the lysate during catalysis is resulting in a large yield loss. Whilst the rate of degradation has been determined for 5 mM ABT at 0.014 mM/hr (Section 6.3), it is difficult to predict the degradation during the reaction as the rate of loss will be concentration dependent. It may be possible to model the degradation if the rate of degradation at several concentrations was obtained. This was not attempted due to time constraints. Therefore, the actual rate of catalysis of the reaction may be more accurately followed by assessing the acetophenone formation.

In order to compare the reactions with different initial substrate concentrations the data was normalised to percentage conversion (Figure 6.9). The experiments show good agreement with all the components of the transamination from both experiments exhibiting comparable trends. The rate of erythrulose consumption for the reaction at 15 mM is slightly higher than the rates for *L*-MBA and acetophenone. This can be attributed to the degradation of *L*-erythrulose by the lysate as determined by earlier stability experiments (Section 6.3).

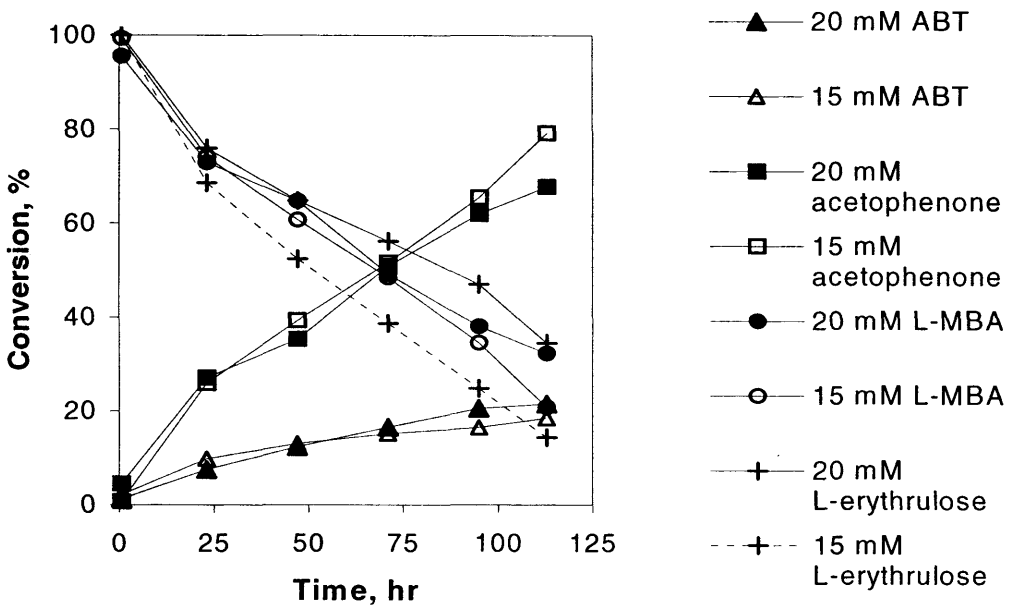


Figure 6.9: Comparison of TAm catalysis in the dual reaction pathway at different substrate concentrations

The data from the dual reaction experiment (Section 2.11.2) with 15 mM and 20 mM substrates can be compared with the conversion of each reaction component. Substrates HPA, GA and *L*-MBA were added in equal concentrations (20 mM or 15 mM) to reactions with 100 mM potassium phosphate buffer, pH 7.0 with cofactors thiamine pyrophosphate, 2.4 mM, $MgCl_2$, 9 mM, and pyridoxal phosphate, 2 mM, and were incubated at ambient temperature (~28°C) with agitation at 200 rpm (Section 2.11.2). Analysis was carried out using 3 assays 1) HPA, GA and erythrulose (Section 2.5.2) 2) MBA and acetophenone (Section 2.5.5) and 3) ABT (Section 2.5.4).

On comparison of the rates of catalysis of the two enzymes it is apparent that the transketolase is far more active than the transaminase in the catalysis of the model reaction (Figure 6.1), under the conditions investigated. At 20 mM substrate TK yielded erythrulose at an initial rate of 2.1 $mM.min^{-1}$. The rate of ABT accumulation was 0.043 $mM.hr^{-1}$. This is a difference of nearly 3000 fold. However it has already been discussed that the actual turnover of the TAm reaction may be more accurately assessed by the rate of acetophenone accumulation. Acetophenone was accumulated at a rate of 0.1 $mM.hr^{-1}$. This is still 1300 times slower than the rate of catalysis measured for TK in the reaction.

6.5 Confirmation of enzymatic 2-amino-1,3,4-butanetriol product synthesis

Further analysis of the reactions from the demonstration of the model synthesis using dual lysate was carried out by mass spectroscopy to corroborate HPLC data indicating the synthesis of the ABT product. The fingerprints of both ABT (Appendix G) and *L*-MBA (Appendix H) were obtained by MSMS. This was necessary, over a single fragmentation to distinguish between the two compounds as they are isobaric, therefore, have the same molecular ion. The determination of the presence of the ABT product in the enzyme-catalysed reactions was then possible (Appendix I).

6.6 Summary

An investigation into the stability of each of the reaction components (Section 6.3) under the reaction conditions with dual plasmid lysate, containing both the TK and β-A: P TAm catalysts, revealed the ABT product, at 5 mM, was consumed at a rate of 0.14 $mM.hr^{-1}$. Some loss of *L*-erythrulose was also experienced.

The model reaction was run by the addition of TK substrates β-HPA and GA, which are converted to *L*-erythrulose and the simultaneous addition of amine TAm substrate *L*-MBA, to reactions containing lysate from the constructed dual plasmid strain over-expressing both TK and β-A: P TAm (Section 6.4). The formation of ABT product from the linked enzymes was detected. Interestingly the reaction containing 20 mM substrate yielded much greater product concentration than with 10 mM substrates. This was unexpected as the experiments to investigate substrate concentration with the purified enzyme showed substrate concentrations of above 10 mM reducing

initial rate and product yield (Section 5.2.4). It is postulated that this result is due to reduced stability of the purified enzyme, which increases the susceptibility of the enzyme to negative effects from the substrate.

The analysis of each reaction component showed much greater accumulation of acetophenone than ABT. This was expected as it was previously determined that the ABT is degraded by the lysate (Figure 6.6). The formation of acetophenone was at a comparable rate to the depletion of *L*-MBA and *L*-erythrulose. It is therefore suggested that this rate is indicative of the actual rate of catalysis whilst the observed rate of ABT accumulation is a function of the production and simultaneous degradation. The rate of TK catalysis is 1300 fold greater than the observed rate of TAm activity (by acetophenone accumulation) under the conditions investigated. Therefore the reaction of the dual biocatalyst with the model substrate suffers from product degradation and does not reach completion (residual substrate present), within the 110-hour time period, due to the low rate of catalysis by the transaminase. The implications of these findings will be discussed in Chapter 7.

Chapter 7 - Project summary and future work

7.1 Summary of project achievements

The construction of a novel bacterial biocatalyst over-expressing both transketolase, from *E.coli* and a β-alanine: pyruvate aminotransferase, from *Pseudomonas aeruginosa*, has been achieved. This involved the expression and appraisal of transaminase candidates to function in series with the transketolase. This required the cloning of the genes into plasmid vectors for expression in *E.coli* BL21gold(DE3) the development of analytical procedures to assess the activity and substrate acceptance of the cloned enzymes and the chemical synthesis of the aminobutantriol product, the ultimate product of the model reaction for the TK-TAm pathway. Once the β-alanine: pyruvate aminotransferase, from *Pseudomonas aeruginosa* was identified as able to accept the TK product *L*-erythrulose as a substrate, characterisation of the enzyme activity was carried out. Finally the model reaction was demonstrated with the coupled TK and TAm using the lysate from the constructed dual plasmid *E.coli* strain.

7.2 Future work

Whilst the ultimate aim of the project was achieved, with the demonstration of the *de-novo* designed linked enzyme pathway (Section 6.4), the yield of the model reaction product, ABT, was low. This was due to the low activity of the β-A: P TAm with *L*-erythrulose (and the simultaneous degradation of the product where lysate was used (Figure 6.6). The low rate of catalysis led to long reaction times and the degradation of the purified enzyme activity (Section 5.2.6) apparently occurring before the reaction had run its course. Since it was found that addition of fresh enzyme, after product accumulation had ceased, led to the synthesis of further product (Figure 5.7), the reaction equilibrium had not been reached. Therefore, the potential to stabilise the enzyme may enable higher product yields to be achieved from reactions with the purified enzyme. The stability of the enzyme in the lysate preparation was not established, however, the longer duration of product turnover implied it was more stable than in the purified form (Section 6.4.1). The low reaction rates led to low ABT yield due to consumption of the product by other cellular components in the lysate. If the rate of production was higher the consumption of ABT may have a less significant impact on the yield.

Mutagenesis could be employed to both enhance the transamination of TK products and to increase the stability of the enzyme. At this stage the lack of availability of a high resolution crystal structure removes the option of rational mutagenesis. Therefore, a random mutagenesis strategy would be required. A mutant library could be screened for both enhanced stability and increased activity with *L*-erythrulose. The mutagenesis strategy could be the wpepPCR, which was partially investigated (but shelved) during this work (Section 4.4.3). Alternatively the immobilisation of the enzyme may enhance stability enabling higher levels product accumulation.

An alternative approach would be to find a related transaminase to be expressed alongside TK that has a higher affinity for *L*-erythrulose. This requires the screening of large numbers of enzymes and may require cloning of candidate enzymes in order to produce enough catalyst to assess the activity. The acceptance of products from TK reactions with different substrates (Table 1.1), such as aromatics, could be investigated. Since the TK reaction is so much more efficient than the TAm reaction for the model reaction it may be that compromising on TK activity may enable synthesis of a product more favourable as a TAm substrate. This could lead to an enhanced yield from the transaminase and therefore increase the overall yield of an amino alcohol pathway product. Alternatively the TK enzyme could be modified to produce a product, which is a more favourable substrate for ω-transaminases. This may be easier than modifying the activity of the β-A: P TAm since TK is a well studied protein, therefore enabling a more rational approach to mutagenesis.

Favourable changes to the TK product may be to replace the 1-hydroxyl with a methyl group by the substitution of β-hydroxypyruvate with pyruvate. This would require modification of the TK enzyme which has no wild-type activity with pyruvate (Miller, O.M., 2004). The resulting product may be a more favourable substrate for the transaminase as indicated by the *Pseudomonas sp. F-126* ω-transaminase having lower activity with hydroxylated substrates than their non-hydroxylated counterparts (Yonaha, K.a.T., M., 1978) The use of an alternative enzyme such as DoxP synthase (Kuzuyama, T. *et al.*, 2000), which similarly synthesises carbon-carbon bonds from achiral substrates but naturally uses pyruvate

and glyceraldehyde-3-phosphate to form xylulose-3-phosphate, creating a phosphorylated, less hydroxylated product than TK, could also be investigated. Another approach is the production of an aromatic product by transketolase. An aromatic compound may be a more favourable substrate to an enzyme of the ω transaminase subclass, based on the commercial production of a variety of aromatic amine products from Celgene using ω-transaminases from various sources (Stirling, D.I., 1992). The native TK has lower activity with aromatic (Humphrey, A.J. *et al*, 2000; Morris, K.G. *et al.*, 1996) substrates than for glycolaldehyde (Table 1.1). However the mutagenesis of TK has already been carried out to produce TK mutants which require further characterisation (Bacon, S., 2001).

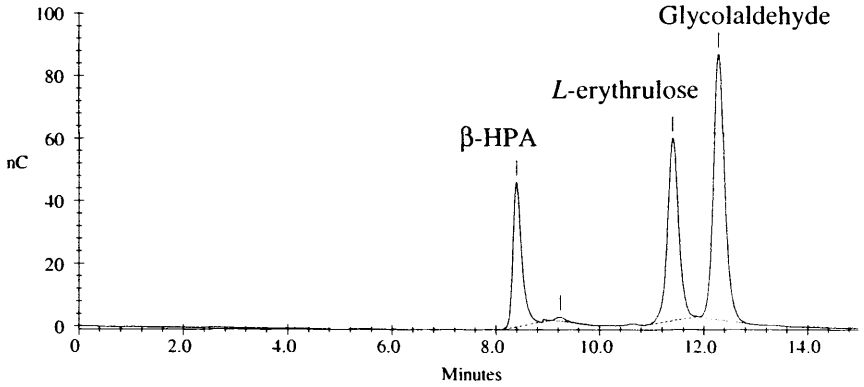
.

Finally a practical limitation on this work was the availability of analytical assays for the efficient monitoring of enzyme activity. This led to the TAm reaction requiring three separate assays to fully assess the transamination of *L*-erythrulose. This is not a feasible option when high throughput is required such as mutagenesis studies. An alternative analytical technique could be LC-MS. This may enable both the detection of all the reaction components by one technique and the identification of any side products yielded from the reaction in a single assay. Additionally the development of assays, to determine the chirality of reaction products from different substrates, are needed.

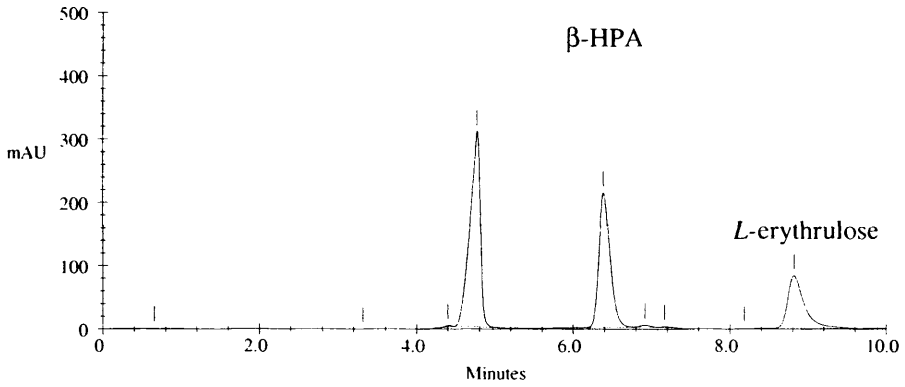
In summary, possible extensions to this project to further optimise the linked pathway are:

- Mutagenesis of β-A: P TAm to enhance the reaction rate with *L*-erythrulose.
- Investigation into alternative transaminase candidates.
- Exploration into the acceptance of alternative TK products by the ω-transaminases.
- Modification of TK to produce a less hydroxylated or an aromatic product.

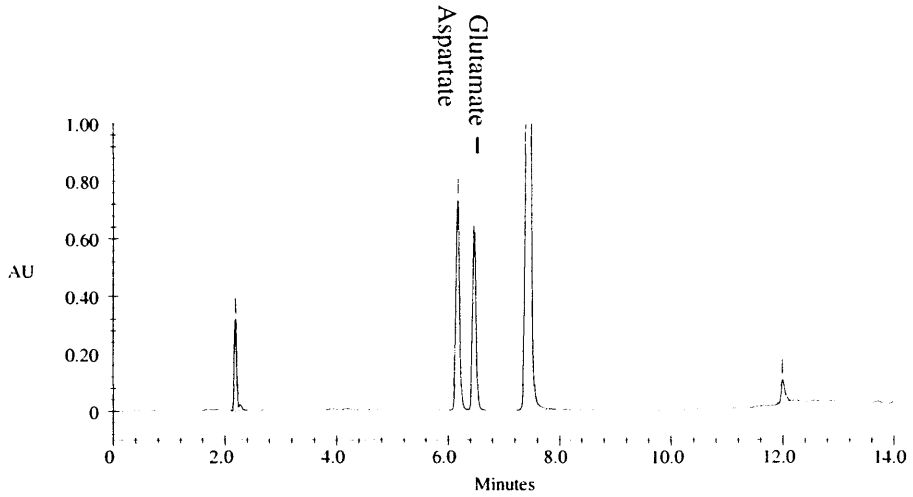
Appendix



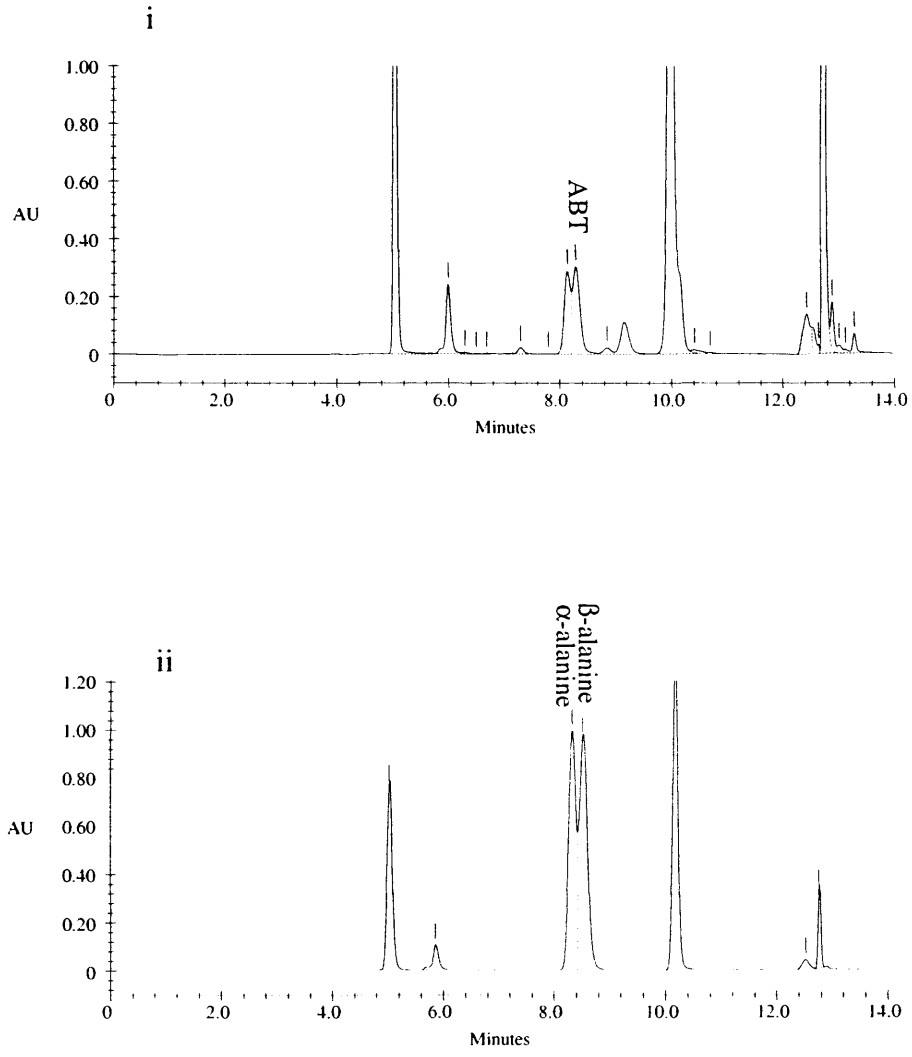
(A) Standard chromatogram of assay for TK native activity (ECD) (Section 2.5.2)



(B) Standard chromatogram of assay for TK native activity (UVD) (Section 2.5.2)

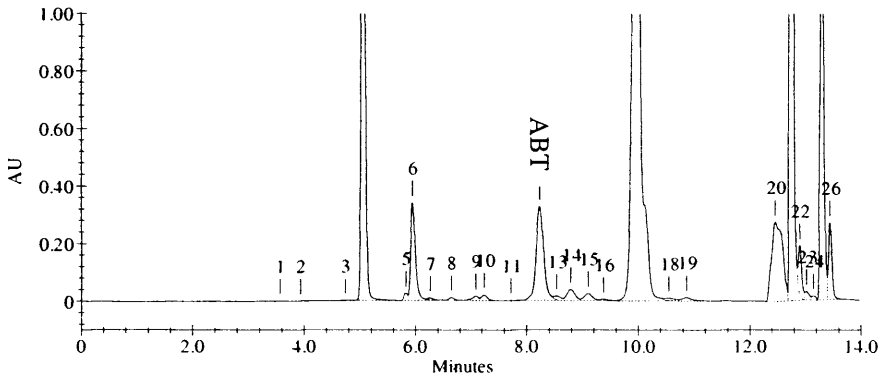


(C) Standard chromatogram of assay for ArTAm native activity (Section 2.5.3)

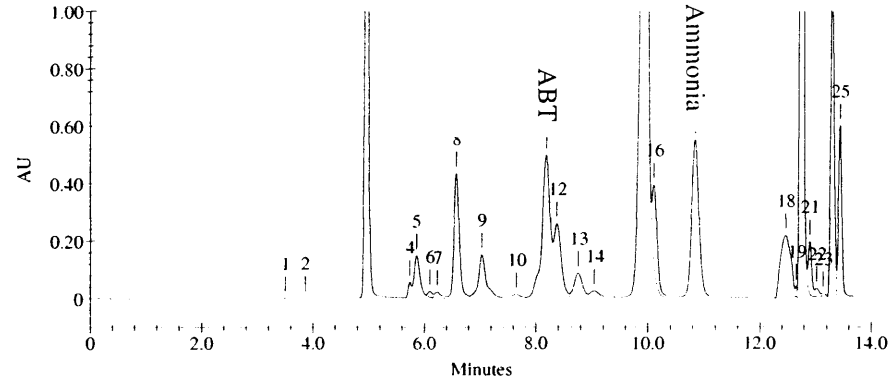


(D) Standard chromatogram of assay for native β-A: P TAm activity (Section 2.5.4) i ABT diastereomers, ii α- alanine and β- alanine

i

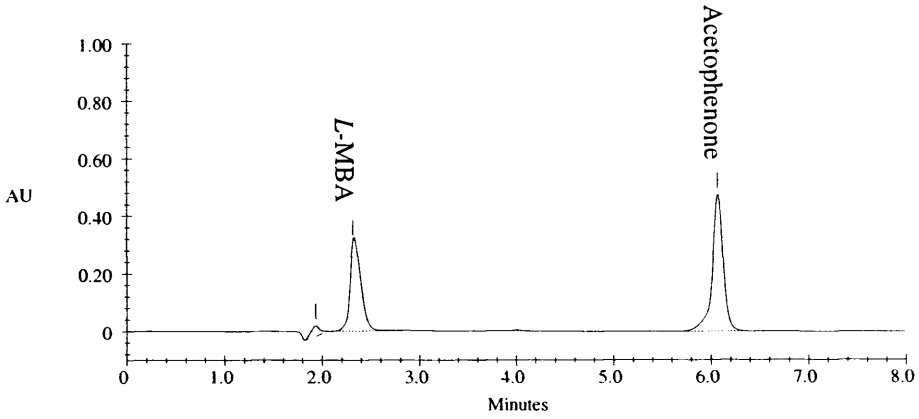


ii

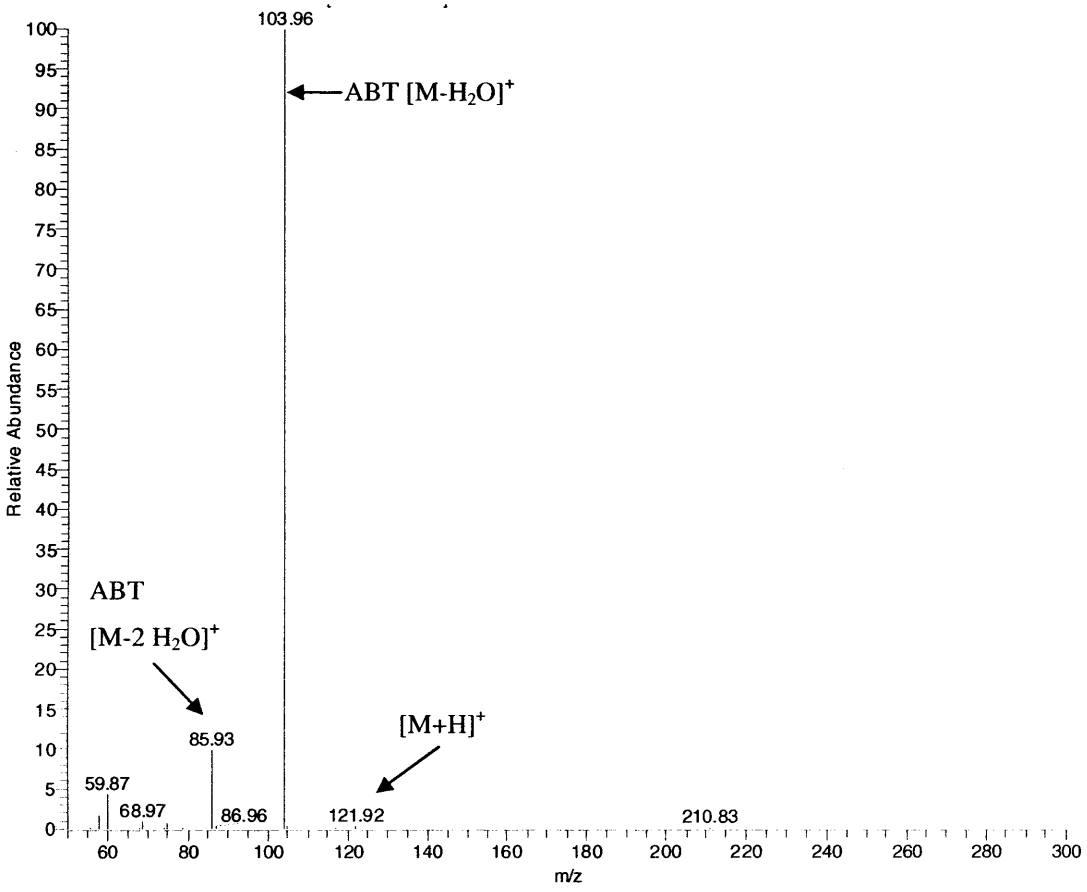


(E) ABT product generated by biocatalysis

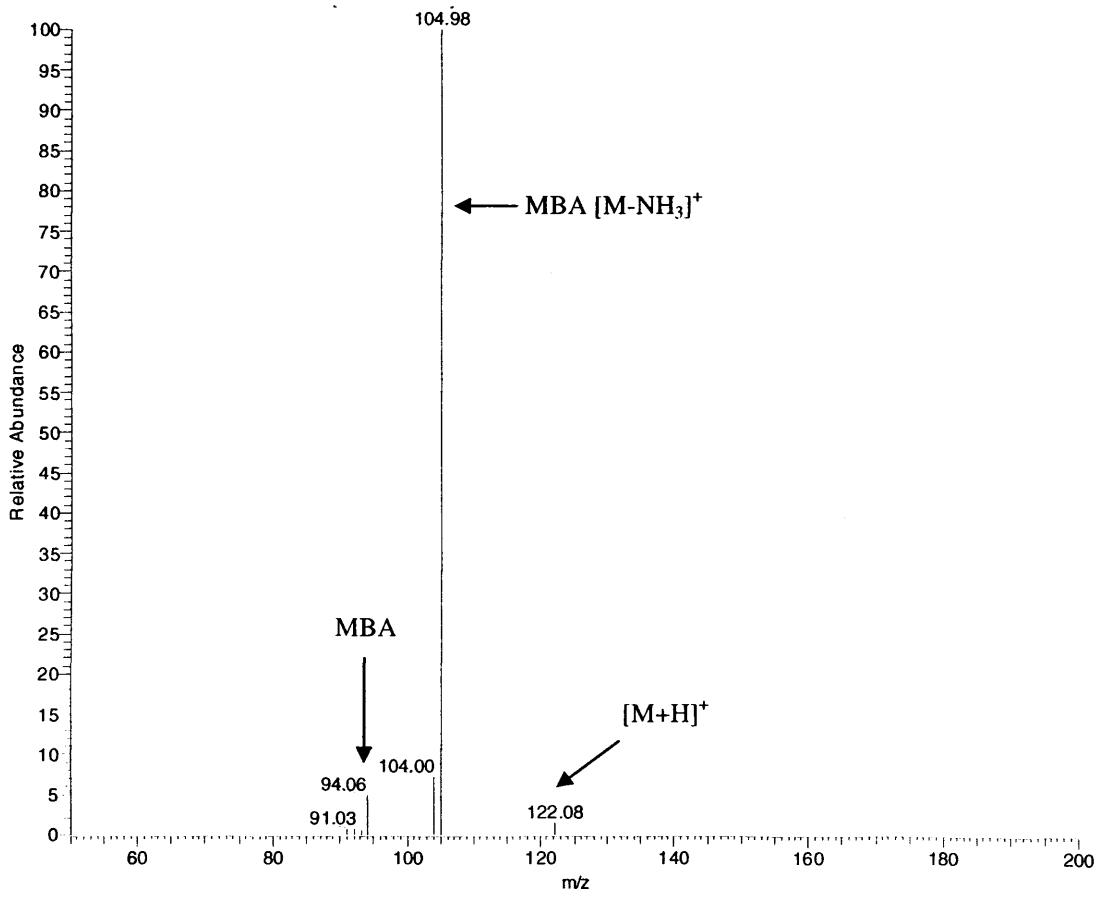
i purified enzyme reaction (Section 5.2.7) ii Lysate reaction (Section 6.4)



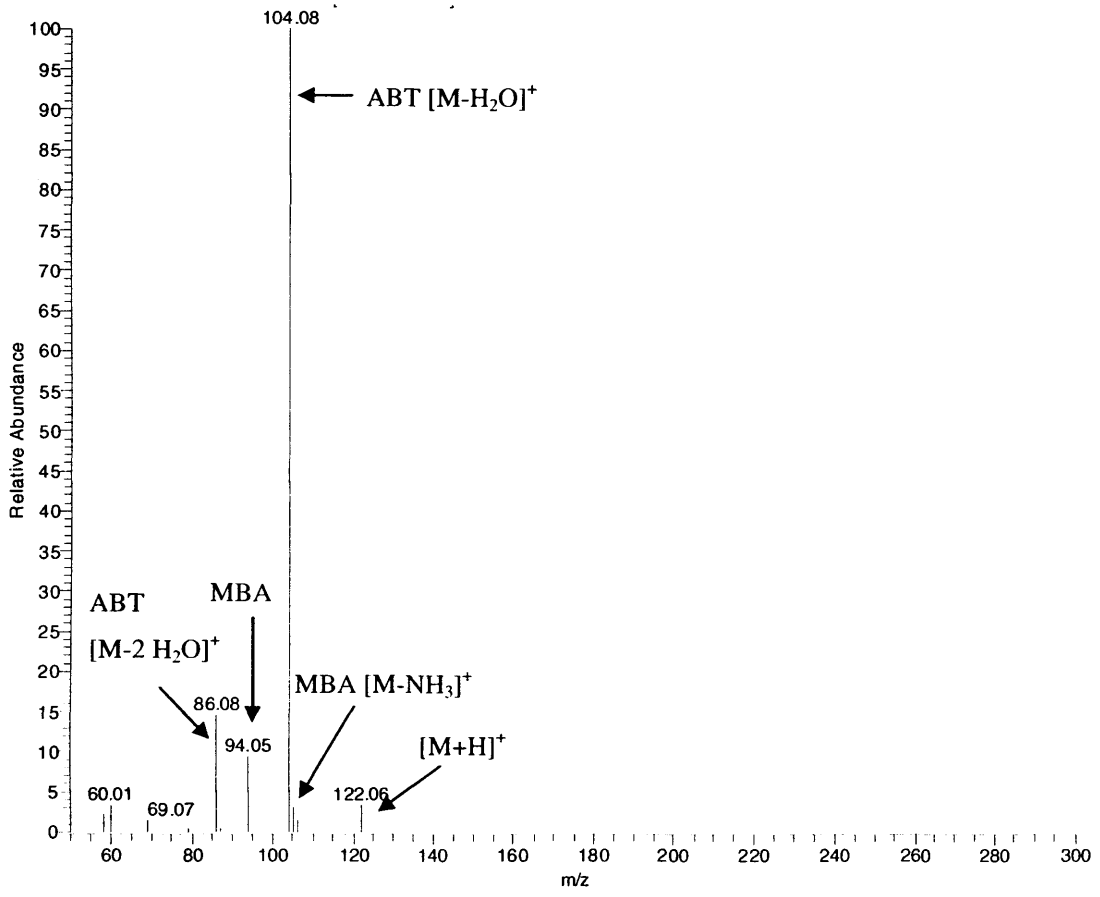
(F) Standard chromatogram of assay for transamination of aromatics (Section 2.5.5)


103.96
ABT [M-H2O]+
ABT
[M-2 H2O]+
85.93
[M+H]+
59.87
68.97
86.96
121.92
210.83
Relative Abundance
m/z

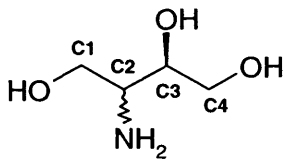
(G) MSMS of ABT

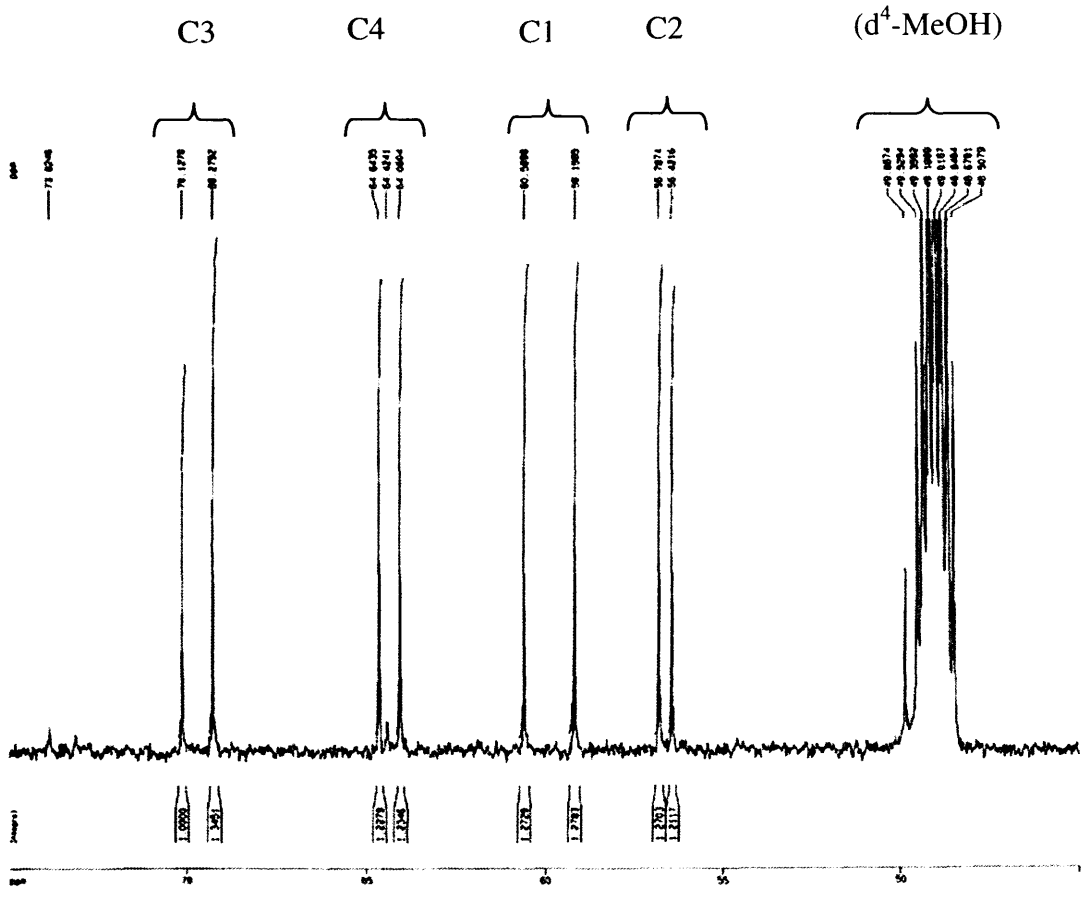

104.98
MBA [M-NH3]+
MBA
[M+H]+
104.00
94.06
91.03
122.08
Relative Abundance
m/z

(H) MSMS of MBA



(I) MSMS of enzyme catalysed reaction





(J) ^{13}C NMR of 2-amino-1,3,4-butanetriol product standard (mixture of diastereomers (Figure 4.1, **5**))

References

Andre, C., Guerard, C., Hecquet, L., Demuynck, C., Bolte, J. (1998). Modified *L*-Threose and D-Erythrose as Substrates of Transketolase and Fructose-1,6-Bisphosphate Aldolase, Application to the Synthesis of New Heptulose Analogues, *Journal of Molecular Catalysis B: Enzymatic* **5**, 459-466.

Antony, C. (1982). *in* "The Biochemistry of Methylotrophs" (C. Antony, Ed.), Pp. 95-136. Academic Press, London.

Arnone, A., Rogers,P.H., Hyde, C.C., Briley, P.D., Metzler, C.M., Metzler, D.E. (1985). "Transaminases". John Wiley and Sons, New York.

Bacon, S. L. (2001). Recombinant Transketolases with increased Synthetic Potential, PhD Thesis, *Department of Biochemistry and Molecular Biology*, University College London

Bajusz, S., *Et Al.* (1990). Highly Active and Selective Anticoagulants: D-Phe-Pro-Arg-H, a Free Tripeptide Aldehyde Prone to Spontaneous inactivation, and Its Stable N-Methyl Derivative, D-Me-Phe-Pro-Arg-H., *J Med Chem* **33**, 1729-1735.

Bommerius, A., Drauz, K., Krix, G., Kula, R.-M.,. (1996). Novel Optically Active *L*-Amino Acids with Bulky Side Chains and their Uses, *Chemical Abstracts* **124**, 173602.

Bornscheuer, U. T., Altenbuchner, J., Meyer, H.H. (1998). Directed Evolution of an Esterase for the Stereoselective Resolution of a Key intermediate in the Synthesis of Epothilones, *Biotechnol. Bioeng.* **58**, 554-559.

Bradford, M. (1976). A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding., *Analytical Biochemistry* **72**, 248-254.

Breuer, M., Ditrich, K., Habicher, T., Hauer, B., Kesseler, M., Sturmer, R., Zelinski, T. (2004). industrial Methods for the Production of Optically Active intermediates, *Angew Chem int Ed Engl* **43**, 788-824.

Brocklebank, S., Mitra, R. K., Woodley, J. M. and Lilly, M. D. (1996). Carbon-Carbon Bond Synthesis: Preparation and Use of Immobilised Transketolase, *Ann.N.Y.Acad.Sci.* **799**, 729-736.

Burnett, G., Yonaha, K., Toyama, S., Soda, K., Walsh, C. (1980). Studies On the Kinetics and Stoichiometry of inactivation of *Pseudomonas* ω-Amino Acid:

pyruvate Transaminase By Gabaculine, *The Journal of Biological Chemistry* **255**, 428-432.

Cheng, S., Fockler, C., Barnes, W., Higuchi, R. (1994). Effective Amplification of Long Targets From Cloned inserts and Human Genomic DNA, *Proc. Natl. Acad. Sci. Usa* **91**, 5695-5699.

Chin, D. T., Goff, S.A., Webster, T., Smith, T., Goldberg, A.L. (1988). Sequence of the Lon Gene in *Escherichia Coli.* A Heat-Shock Gene Which Encodes the Atp-Dependent Protease La., *J.Biol. Chem.* **263**, 11718-28.

Cho, B.-K., Cho, H.-J., Yun, H., Kim, B.-G. (2003a). Simultaneous Synthesis of Enantiomerically Pure *(S)*-Amino Acids and *(R)*-Amines Using Coupled Transaminase Reactions, *Biotechnol. Bioeng.* **81**, 783-789.

Cho, B.-K., Cho, H.-J., Yun, H., Kim, B.-G. (2003b). Simultaneous Synthesis of Enantiomerically Pure *(S)*-Amino Acids and *(R)*-Amines Using Alpha/Omega-Aminotransferase Coupling Reactions with Two-Liquid Phase Reaction System, *J. Mol. Cat. B: Enzymatic* **26**, 273-285.

Cho, B.-K., Park, H.-Y., Seo, J.-H., Kinnera, K., Lee, B.-S., Kim, B.-G. (2004). Enzymatic Resolution for the Preparation of Enantiomerically Enriched D-Beta-Heterocyclic Alanine Derivatives Using *Escherichia Coli* Aromatic *L*-Amino Acid Transaminase, *Biotechnol. Bioeng.* **88**, 512-519.

Chotani, G., Dodge, T., Hsu, A., Kumar, M., Laduca, R., Trimbar, D., Weyler, W., Sanford, K. (2000). The Commercial Production of Chemicals Using Pathway Engineering, *Biochemica et Biophysica Acta* **1543**, 434-455.

Cline, J., Hogrefe, H.H. (2000). Randomized Gene Sequences with New PCR Mutagenesis Kit, *Strategies* **13**, 157-161.

Cohen, S. A., Michaud, D.P. (1993). Synthesis of A Fluorescent Derivatizing Reagent, 6-Aminoquinolyl-N-Hydroxysuccinimidyl Carbamate, and Its Application for the Analysis of Hydrolysate Amino Acids Via High-Performance Liquid Chromatography., *Anal Biochem* **211**, 279-287.

Collier, R. H. and Kohlhaw, G. (1972). Non-Identity of the aspartate and the Aromatic Aminotransferase Components of Transaminase A in *Escherichia Coli, J Bacteriol* **112**, 365-71.

Cowan, D., Meyer, Q., Stafford, W., Muyanga, S., Cameron, R. and Wittwer, P. (2005). Metagenomic Gene Discovery: Past, Present and Future, *Trends Biotechnol* **23**, 321-9.

Cronin, C. N., Kirsch, J.F. (1988). Role of Arginine-292 in the Substrate Specificity of aspartate Aminotransferase as Examined By Site-Directed Mutagenesis, *Biochemistry* **27**, 4572 - 4579.

Danishefsky, A. T., Onnufer, J.J., Petsko, G.A., Ringe, D. (1991). Activity and Structure of the Active-Site Mutants, R386Y and R386F of *Escherichia Coli* aspartate Aminotransferase, *Biochemistry* **30**, 1980 - 1985.

Datta, A. G., Racker, E. (1961). Mechanism of Action of Transketolase I. Properties of Crystalline Yeast Enzyme., *J Biol Chem* **236**, 617-623.

De La Haba, G. and Racker, E. (1952). Metabolism of Ribose-5-Phosphate and Ribulose-5-Phosphate, *Fed.Proc.* **11**, 201.

Dequeker, E., Compernolle, F., Toppet, S., Hoornaert, G. (1995). Diastereoselective Conversion of *L-(S)*-Erythrulose to 2-Amino-2-Deoxy-*L*-Erythritol, *Tetrahedron* **51**, 5877-5890.

Deroode, B. M., Van Beek, J., Van Der Padt, A., Franssen, M.C.R., Boom, R.M. (2001). The integrated Enzymatic Production and Downstream Processing of Hexyl Glucoside, *Enz. Microb. Tech.* **29**, 513-520.

Devroom, E. (1999). The Central Role of Penicillin Acylase in Antibiotics Production, *Chim. Oggi.* **17**, 65-68.

Doran, P. M. (2000). "Bioprocess Engineering Principles". Academic Press Limited, London.

Draths, K. M., Pompliano, D. L., Conley, D. L., Frost, J. W., Berry, A., Disbrow, G. L., Staversky, R. J. and Lievense, J. C. (1992). Biocatalytic Synthesis of Aromatics From *D*-Glucose: the Role of Transketolase, *Journal of the American Chemical Society* **114**, 3956-3962.

Effenberger, F., Null, V. and Zeigler, T. (1992). Preparation of Optically Pure *L*-2-Hydroxyaldehydes with Yeast Transketolase, *Tetrahedron Letts.* **33**, 5157-5160.

Eichhorn, E., Roduit, J.-P., Shaw, N., Heinzmann, K. and Kiener, A. (1997). Preparation of *(S)*-Piperazine-2-Carboxylic Acid, *(R)*-Piperazine-2-Carboxylic Acid, and *(S)*-Piperidine-2-Carboxylic Acid By Kinetic Resolution of the Corresponding Racemic Carboxamides with Stereoselective Amidases in Whole Bacterial Cells, *Tetrahedron: asymmetry* **8**, 2533-2536.

Ensley, B. D., Ratzkin, B.J., Osslund, T.D., Simon, M.J., Wackett, L.P., Gibson, D.T. (1983). Expression of Naphthalene Oxidation Genes in *Escherichia Coli* Results in the Biosynthesis of indigo, *Science* **222**, 167-169.

Fadnavis, N. W., Sharfuddin, M., Kumara Vadivel, S. (2001). Porcine Pancreatic Lipase Mediated Regio- and Stereoselective Hydrolysis: Chemoenzymatic Synthesis of (2*S*,3*S*)-2-Amino-3,4-Dihydroxybutyric Acid, *Tetrahedron: asymmetry* **12**, 691-693.

FDA. (1992). FDA's Policy Statement for the Development of New Stereoisomeric Drugs, *Http://Www.FDA.Gov/Cder/Guidance/Stereo.Htm.*

Fessner, W.-D. and Helaine, V. (2001). Biocatalytic Synthesis of Hydroxylated Natural Products Using Aldolases and Related Enzymes, *Current Opinion in Biotechnology* **12**, 574-586.

Fotheringham, G., Dacey, S.A., Taylor, P.P., Smith, T.J., Hunter, M.G., Finlay, M.E., Primrose, S.B., Parker, D.M., Edwards, R.M. (1986). The Cloning and Sequence Analysis of the *AspC* and *Tyrb* Genes From *Escherichia Coli* K12, *Biochem. J.* **234**, 593-604.

Fotheringham, G., Grinter, N., Pantaleone, D. P. and Senkpeil, R. F. (1999). Engineering of a Novel Biochemical Pathway for the Biosynthesis of *L*-2-Aminobutyric Acid in *Escherichia Coli* K12, *Bioorganic and Medicinal Chemistry* **7**, 2209-2213.

Fotheringham, G. I. (2001). Transaminase Biotransformation Process, 6197558, Usa

French, C., Ward, J.M. (1995). Improved Production and Stability of *E.Coli* Recombinants Expressing Transketolase for Large-Scale Biotransformation, *Biotechnol. Lett.* **17**, 247-252.

Furste, J. P., Pansegrau, W., Frank, R., Blocker, H., Scholz, P., Bagdasarian, M., Lanka, E. (1986). Molecular Cloning of the Plasmid Rp4 Primase Region in a Multi-Host Range *Tac*p Expression Vector, *Gene* **48**, 119-131.

Galkin, A., Kulakova, L., Yoshimura, T., Soda, K. and Esaki, N. (1997). Synthesis of Optically Active Amino Acids From Alpha-Keto Acids with *Escherichia Coli* Cells Expressing Heterologous Genes, *Applied and Environmental Microbiology* **63**, 4651-4656.

Gao, H.-J., Qiong, W.U., Chen, G.-Q. (2002). Enhanced Production of *D*-(-)-3-Hydroxybutyric Acid By Recombinant *Escherichia Coli*, *Fems Microbial Lett* **213**, 59-65.

Gefland, D. H., Rudo, N. (1977a). Mapping of the aspartate and Aromatic Amino Acid Aminotransferase Genes *TyrB* and *AspC.*, *J.Bacteriol.* **130**, 141-144.

Gefland, D. H., Steinberg, R.A. (1977b). *Escherichia Coli* Mutants Deficient in the Aspartate and Aromatic Amino Acid Aminotransferases., *J.Bacteriol.* **130**, 429-440.

Gleider, A., Farinas, E.T., Arnold, F.H. (2002). Laboratory Evolution of a Soluble, Self-Sufficient, Highly Active Alkane Hydroxylase, *Nat. Biotechnol.* **20**, 1135-1139.

Goldfine, H., Stadtman, E.R. (1960). Propionic Acid Metabolism, *The Journal of Biological Chemistry* **235**, 2238-2245.

Goswami, A., Guo, Z., Parker, W. L. and Patel, R. N. (2005). Enzymatic Resolution of *Sec*-Butylamine, *Tetrahedron: asymmetry* **16**, 1715-1719.

Greener, A., Callahan, M, Jerspeth, B. (1997). an Efficient Random Mutagenesis Technique Using an *E.Coli* Mutator Strain, *Mol. Biotechnol.* **7**, 189-195.

Greingl, H., Klempier, N., Pochlauer, P., Schmidt, M., Shi, N.Y., Zabelinskakamackova, A.A. (1998). Enzyme Catalysed formation of (*S*)-Cyanohydrins Derived From Aldehydes and Ketones in a Biphasic Solvent System, *Tetrahedron*, **54** 14477-14486.

Grodberg, J., Dunn, J.J. (1988). Ompt Encodes the *Escherichia Coli* Outer Membrane Protease That Cleaves T7 RNA Polymerase During Purification, *J.Bacteriol.* **170**, 1245-53.

Hagishita, T., Yoshida, T., Izumi, Y. and Mitsunaga, T. (1996). Cloning and Expression of the Gene for Serine-Glyoxylate Aminotransferase From an Obligate Methylotroph *Hyphomicrobium Methylovorum Gm2*, *Eur.J.Biochem.* **241**, 1-5.

Hanson, R. L., Schwinden, M.D., Banerjee, A., Brozozowski, D.B., Chen, B-C., Patel, B.P., Mcnamee, C.G., Kodersha, G.A., Kronenthal, D.R, Pater, R.N., Szarka, L.J. (1999). Enzymatic Synthesis of *L*-6-Hydroxynorleucine, *Bioorganic and Medicinal Chemistry* **7**, 2247-2252.

Hanson, R. L., Howell, J., Laporte, T., Donovan, M., Cazzulino, D., Zannella, V., Montana, M., Nanduri, V., Schwarz, S. Eiring, R., Durand, S., Wasylyk, J., Parler, L., Liu, M., Okuniewicz, F., Chen, B.-C., Harris, J., Natalie, K., Ramig, K., Swaminathan, S., Rosso, V., Pack, S., Lotz, B., Bernot, P., Rusowicz, A.,Lust, D., Tse, K., Venit, J., Szarka, L., Patel, R.N. . (2000). Enzymatic Synthesis of Allysine Ethylene Acetal, *Enzyme and Microbial Technology* **26**, 348-358.

Hayaishi, H., Mizuguchi, H., Miyahara, I., Islam, M.M., Ikushiro, H., Nakajima, Y., Hirotsu, K., Kagamiyama, H. (2003a). Strain and Catalysis in aspartate Aminotransferase, *Biochimica Et Biophysica Acta* **1647**, 103-109.

Hayaishi, H., Mizuguchi, H., Miyahara, I., Nakajima, Y., Hirotsu, K., Kagamiyama, H. (2003b). Conformational Change in aspartate Aminotransferase On Substrate Binding induces Strain in the Catalytic Group and Enhances Catalysis, *The Journal of Biological Chemistry* **278**, 9481-9488.

Hayaishi, O., Nishizuka, Y., Tatibana, M., Takeshita, M., Kuno, S. (1960). Enzymatic Studies On the Metabolism of β–Alanine, *the Journal of Biological Chemistry* **236**, 781-790.

Hayashi, H., inoue, K., Nagata, T., Kuramitsu, S. and Kagamiyama, H. (1993). *Escherichia Coli* Aromatic Amino Acid Aminotransferase: Characterization and Comparison with Aspartate Aminotransferase, *Biochemistry* **32**, 12229-12239.

Hayashi, H., inoue, K., Mizuquchi, H., Kagamiyama, H. (1996). Analysis of the Substrate-Recognition Mode of Aromatic Acid Aminotransferase By Combined Use of Quasisubstrates and Site-Directed Mutagenesis: Systematic Hydroxy-Group Addition/Deletion Studies to Probe the Enzyme-Substrate interactions, *Biochemistry* **35**, 6754-6761.

Hecquet, L., Bolte, J. and Demuynck, C. (1996). Enzymatic Synthesis If 'Natural-Labelled' 6-Deoxy-*L*-Sorbose Precursor of an Important Food Flavour, *Tetrahedron* **52**, 8223-8232.

Hilker, I., Alphand, A., Wohlgemuth, R., Furtoss, R. (2004). Preparative Scale asymmetric Baeyer-Villiger Oxidation Using a Highly Productive "Two-in-One" Resin-Based *in Situ* SFPR Concept, *Adv. Synth. Catal.* **346**, 203-214.

Hobbs, G. R. (1994). The Production and Use of Transketolase as a Catalyst for Carbon-Carbon Bond formation, PhD Thesis, *Department of Biochemical Engineering*, University College London

Hobbs, G. R., Lilly, M. D., Turner, N. J., Ward, J. M., Willets, A. J. and Woodley, J. M. (1993). Enzyme Catalysed Carbon-Carbon Bond formation; Use of Transketolase From *E.Coli*, *J.Chem Soc.Perkin Trans.* **1**, 165-166.

Hobbs, G. R., Mitra, R.K., Chauhan, R.P., Woodley, J.M., Lilly, M.D. (1996). Enzyme-Catalysed Carbon-Carbon Bond formation: Large-Scale Production of *Escherichia Coli* Transketolase, *Journal of Biotechnology* **45**, 173-179.

Hochuli, E., Dobeli, H. and Schacher, A. (1987). New Metal Chelate Adsorbent Selective for Proteins and Peptides Containing Neighbouring Histidine- Residues, *Journal of Chromatography* **411**, 177-184.

Humphrey, A. J., Parsons, S.F., Smith, M.E.B., Turner, N.J. (2000). Synthesis of a Novel N-Hydroxypyrrolidine Using Enzyme Catalysed asymmetric Carbon-Carbon Bond Synthesis, *Tetrahedron Letters* **41**, 4481-4485.

Ikeda, M., Katsumata, R. (1999). Hyperproduction of Tryptophan By *Corynebacterium glutamicum* with the Modified Pentose Phosphate Pathway, *Appl. Environ. Microbiol.* **65**, 2497-2502.

Inoue, K., Kuramitsu, S., Aki, K., Watanabe, Y., Takagi, T., Nishigai, M., Ikai,A., Kagamiyama, and H. (1988). Branched-Chain Amino Acid Aminotransferase of *Escherichia Coli*: Overproduction and Properties, *J. Biochem. (Tokyo),* **104**, 777-784.

Inoue, Y., Kuramitsu, S., inoue, K., Kagamiyama, H., Hiromi, K., Tanase, S., Morino, Y. (1989). Substitution of a Lysyl Residue for Arginine 386 of *Escherichia Coli* Aspartate Aminotransferase, *The Journal of Biological Chemistry* **264**, 9673 - 9681.

Islam, M. M., Hayashi, H., Mizuguchi, H., Kagamiyama, H. (2000). The Substrate Activation Process in the Catalytic Reaction of *Escherichia Coli* Aromatic Amino Acid Transferase, *Biochemistry* **39**, 15418-15428.

Izumi, Y., Yoshida, T., Yamada, H. (1990). Purification and Characterisation of Serine-Glyoxylate Aminotransferase from a Serine Producing Methylotroph, *Eur.J.Biochem.* **190**, 285-290.

Jacques, J., Collett, A., Wilen, S. (1980). "Enantiomers, Racemates and Resolutions", New York.

Jager, J., Moser, M., Sauder, U., Jansonius, J.N. (1994). Crystal Structures of *Escherichia Coli* aspartate Aminotransferase in Two Closed Confirmations - Comparison of an Unliganded Open and 2 Liganded Closed forms, *J.Mol.Biol.* **239**, 285 - 305.

Jensen, P. R. and Hammer, K. (1998). Artificial Promoters for Metabolic Optimization, *Biotechnol Bioeng* **58**, 191-5.

Jeong, S., Hwang, B.-Y., Kim, J., Kim, B.-G. (2000). Lipase-Catalysed Reaction in the Packed Bed Reactor with Continuous Extraction Column to Overcome a Product inhibition, *Journal of Molecular Catalysis B: Enzymatic* **10**, 597-604.

Johansson, A. (1995). Methods for the Asymmetric Preparation of Amines, *Contemp. Org. Synth.* **2**, 393-407.

Kaldor, W. K., Kalish, V.J., Davies, J.F., Shetty, B.V., Fritz, J.E., Appelt, K., Burgess, J.A., Campanale, K.M., Chirgadze, N.Y., Clawson, D.K., Dressman, B.A., Hatch, S.D., Khalil, D.A., Kosa, M.B., Lubbehusen, P.P., Muesing, M.A., Patick, A.K., Reich, S.H., Su, K.S., Tatlock, J.H. (1997). Viracept (Nefinavir Mesylate, Ag1343): A Potent, Orally Available inhibitor of HIV-1 Protease, *J.Med.Chem.* **40**, 3979-3985.

Kelley, M. T., Walson, P. D., Edge, J. H., Cox, S. and Mortensen, M. E. (1992). Pharmacokinetics and Pharmacodynamics of Ibuprofen Isomers and Acetaminophen in Febrile Children, *Clin Pharmacol Ther* **52**, 181-9.

Kempf, D. J., Codacovi, L. M., Norbeck, D. W., and Plattner, J. J. (1991). Retroviral Protease inhibiting Compounds, 5,354,866, Usa

Kim, K.-H. (1963). Purification and Properties of a Diamine α-Ketoglutarate Transaminase from *Escherichia Coli*, *The Journal of Biological Chemistry* **239**, 783-786.

Kim, M.-J., Whitesides, G.M.,. (1998). *L*-Lactate Dehydrogenase: Substrate Specificity and Use as a Catalyst in the Synthesis of Homochiral 2-Hydroxy Acids, *J.Am Chem Soc* **110**, 2959-2964.

Kirsch, J., Eichele, G., ford, G.C., Vincent, M.G., Jansonius, J.N., Gehring, H., Christen, P. (1984). Mechanism of Action of *E.Coli* Aspartate Aminotransferase On the Basis of Its Spatial Structure, *J.Mol.Biol.* **174**, 497 - 525.

Ko, T.-P., Wu, S.-P., Yang, W.-Z., Tsai, H., Yuan, H.S. (1999). Crystallisation and Preliminary Crystallographic Analysis of the *Escherichia Coli* Tyrosine Aminotransferase, *1999* **D55**, 1474-1477.

Kondo, K., Wakabayashi, S., Kagamiyama. (1987). Structural Studies on Aspartate Aminotransferase From *Escherichia Coli*. Covalent Structure, *J.Biol. Chem.* **262**, 8648-8657.

Kuzuyama, T., Takagi, M., Takahashi, S. and Seto, H. (2000). Cloning and Characterization of 1-Deoxy-D-Xylulose 5-Phosphate Synthase from Streptomyces Sp. Strain Cl190, Which Uses Both the Mevalonate and Nonmevalonate Pathways for Isopentenyl Diphosphate Biosynthesis, *J Bacteriol* **182**, 891-7.

Kwon, S. J., Ko, S.Y. (2001). Orthagonally Protected Enantiopure Syn-2-Amino-1,3,4-Butanetrols: A General Building Block for *Syn*-Amino Alcohols, *J.Org.Chem.* **66**, 6833-6835.

Kwon, S. J., Ko, S.Y. (2002). Synthesis of Statine Employing a General *Syn*-Amino Alcohol Building Block, *Tetrahedron: Letters* **43**, 639-641.

Li, K., Frost, J.W. (1999). Microbial Synthesis of 3-Dehydroxyshikimic Acid: A Comparative Analysis of *D*-Xylulose, *L*-Arabinose and *D*-Glucose, *Biotechnol. Prog.* **15**, 876-883.

Liu, S.-J., Steinbuchel, A. (2000). A Novel Genetically Engineered Pathway for the Synthesis of Poly-Hydroxyalkanoic Acids in *Escherichia Coli*, *Appl Environ. Microbiol.* **66**, 739-743.

Lundberg, K. S., Shoemaker, D. D., Adams, M. W., Short, J. M., Sorge, J. A. and Mathur, E. J. (1991). High-Fidelity Amplification Using a Thermostable DNA Polymerase Isolated From *Pyrococcus Furiosus*, *Gene* **108**, 1-6.

Lye, G. J., Woodley, J.M. (1999). Application of *in Situ* Product-Removal Techniques to Biocatalytic Processes, *Trends Biotechnol* **17**, 395-402.

Machado, G. D. C., Gomes, J., Marlito, Antunes, O. A. C. and Oestreicher, E. G. (2005). Enzymic Resolution of D/*L*-Phenylglycine, *Process Biochemistry* **40**, 3186-3189.

Maier, T., Winterhalter, C. (2000). Fermentative Production of *L*-Cysteine with a Recombinant *Escherichia Coli* Strain, *Chemical Abstracts* **133**, 361976.

Matsumara, I., Ellington, A.D. (2001). *in* "Methods in Molecular Biology, Vol 182: *in vitro* Mutagenesis" (J. Braman, Ed.), Pp. 261-269.

May, O., Nguyen, P. T. and Arnold, F. H. (2000). inverting Enantioselectivity By Directed Evolution of Hydantoinase for Improved Production of *L*-Methionine, *Nat.Biotechnol.* **18**, 317-320.

Mehta, P., Hale, T.I., Christen, P. (1993). Aminotransferases: Demonstration of Homology and Division into Evolutionary Subgroups, *Eur. J. Biochem.* **214**, 549-561.

Merino, P., Fransco, S., Merchan, F.L., Revuelta, J., Tejero, T. (2002). Efficient Synthesis of (2*R*,3*S*)-and (2*S*,3*S*)-2-Amino-1,3,4-Butanetriols Through Stereodivergent Hydroxymethylation of D-Glyceraldehyde Nitrones, *Tetrahedron Letters* **43**, 459-462.

Meyers, A. I., Drauz, K., Schwarm, M., Mckennon, M. (1994). Process for Reduction of Carboxylic Acids or Derivatives, and New Compounds, *Chemical Abstracts* **121**, 10000.

Miller, O. M. (2004). Directed Evolution of Transketolase, a Carbon-Carbon Bond-forming Enzyme, PhD Thesis, *Department of Biochemical Engineering*, University College London

Mitra, R. K. (1997). Caron-Carbon Bond formation Reactor Selection for Transketolase-Catalysed Biotransformations, PhD Thesis, *Department of Biochemical Engineering*, University College London

Mitra, R. K. and Woodley, J. M. (1996). A Useful assay for Transketolase in asymmetric Synthesis, *Biotechnology Techniques* **10**, 167-172.

Mitra, R. K., Woodley, J. M. and Lilly, M. D. (1998). *E.Coli* Transketolase-Catalysed Carbon-Carbon Bond formation: Biotransformation Characterisation for Reactor Evaluation and Selection, *Enzyme and Microbial Technology* **22**, 64-70.

Mizuguchi, H., Hayashi, H., Okada, K., Miyahara, I., Hirotsu, K., Kagamiyama, H. (2001). Strain Is More Important Than Electrostatic interaction in Controlling the Pk_a of the Catalytic Group in aspartate Aminotransferase, *Biochemistry* **40**, 353-360.

Monache, G. D., Misiti, D., Zappia, G. (1999). A Stereo Synthesis of (-)-Detoxinine From *L*-ascorbic Acid, *Tetrahedron: asymmetry* **10**, 2961-2973.

Morita, Y., Aibara, S., Yonaha, K., Soda, K. (1979). Preliminary Crystallographic Study of ω-Aminotransferase from *Pseudomonas Sp.* F-126, *J.Mol.Biol.* **130**, 211-213.

Morris, K. G., Smith, M. E. B., Turner, N. J., Lilly, M. D., Mitra, R. K. and Woodley, J. M. (1996). Transketolase from *E.Coli:* A Practical Procedure for Using the Biocatalyst for Asymmetric Carbon-Carbon Bond Synthesis, *Tetrahedron: asymmetry* **7**, 2185-2188.

Nagasawa, T. and Yamada, H., (1990). Application of Nitrile Converting Enzyme for the Production of Useful Compounds. *Pure Appl. Chem.* **62**, 1441-1444.

Okamoto, A., Nakai, Y., Hayashi, H., Hirotsu, K., Kagamiyama, H. (1998). Crystal Structures of *Paracoccus Denitrificans* Aromatic Amino Acid Aminotransferase: A Recognition Site Constructed Hydrogen Bond Network, *J.Mol.Biol.* **280**, 443 - 461.

Onuffer, J. J., Kirsch, J.F. (1995). Redesign of Substrate Specificity of *Escherichia Coli* aspartate Aminotransferase to that of *Escherichia Coli* Tyrosine Aminotransferase By Homology Modelling and Site Directed Mutagenesis, *Protein Sci.* **4**, 1750-1757.

Otten, L. G., Sio, C.F., Vrielink, J., Cool, R.H., Quax, W.J. (2002). Altering the Substrate Specificity of Cephalosprin Acylase by Directed Evolution of the Beta-Subunit, *J.Biol. Chem.* **277**, 42121-42127.

Panke, S., Held, M., Wubbolts, M. (2004). Trends and innovation in industrial Biocatalysis for the Production of Fine Chemicals, *Current Opinion in Biotechnology* **15**, 272-279.

Park, J.-H., Kim, G.-J., Kim, H.-S. (2000). Production of *D*-Amino Acid Using Whole Cells of Recombinant *Escherichia Coli* with Separately and Co-Expressed *D*-Hydantointase and *N*-Carbomylase, *Biotechnol. Prog.* **16**, 564-570.

Park, J.-H., Kim, G.-J., Kim, H.-S. (2002). Complete Conversion of *D,L*-5-Monosubstituted Hydantoins with a Low Velocity of Chemical Racemisation into *D*-Amino Acids Using Whole Cells of Recombinant *Escherichia Coli*, *Biotechnol. Prog.* **18**, 1201-1206

Park, J. H., Oh, K. H., Lee, D. C. and Kim, H. S. (2002). Modelling and Kinetic Analysis of the Reaction System Using Whole Cells with Separately and Co-

Expressed *D*-Hydantoinase and *N*-Carbamoylase, *Biotechnol.Bioeng.* **78**, 779-793.

Patel, R. N. (2001). Enzymatic Synthesis of Chiral intermediates for Omapatrilat, an Antihypertensive Drug, *Biomolecular Engineering* **17**, 167-182.

Porath, J., Carlsson, J., Olsson, I. and Belfrage, G. (1975). Metal Chelate Affinity Chromatography, a New Approach to Protein Fractionation, *Nature* **258**, 598-9.

Racker, E. (1948). Enzymatic formation and Break Down of Pentose Phosphate, *Fed.Proc.* **7**, 180.

Rowe, L. A., Geddie, M.L., Alexander, O.B., Matsumura, I. (2003). A Comparison of Directed Evolution Approaches Using the Beta Glucuronidase Model System, *J.Mol.Biol.* **332**, 851-860.

Rozzell, J.D. (1999) Commercial Scale Biocatalysis: Myths and Realities, Bioorg. and Med. Chem. **7**, 2253-2261

Rudman, D., Meister, A. (1953). Transamination in *Escherichia Coli*, *J.Biol. Chem.* **200**, 591-604.

Ruhdal, P., Hammer, K. (1998). Artificial Promoters for Metabolic Optimisation, *Biotechnol Bioeng* **58**, 191-195.

Sadikot, R. T., Blackwell, T. S., Christman, J. W. and Prince, A. S. (2005). Pathogen-Host interactions in *Pseudomonas Aeruginosa* Pneumonia, *Am J Respir Crit Care Med* **171**, 1209-23.

Sambrook, J., Fritsch, E.F., Maniatis, T. (1989). "Molecular Cloning: a Laboratory Manual". Cold Spring Harbour Laboratory Press, Cold Spring Harbour Ny.

Samuelson, J. C., Xu, S.Y. (2002). Directed Evolution of Restriction Endonuclease *Bstyi* to Achieve increased Substrate Specificity, *J.Mol.Biol.* **319**, 673-683.

Scherzinger, E., Bagdasarian, M.M., Scholz, P., Lurz, R., Ruckert, B., Bagdasarian, M. (1984). Replication of the Broad Host Range RSF1010: Requirement for Three Plasmid Encoded Proteins., *Proc. Natl. Acad. Sci. USA* **81**, 654-658.

Schmid, A., Dordick, J. S., Hauer, B., Kiener, A., Wubbolts, M. and Witholt, B. (2001). Industrial Biocatalysis Today and Tomorrow, *Nature* **409**, 258-268.

Schmidt, E., Blaser, H.-U. (2003). "Asymmetric Synthesis On Industrial Scale: Challenges, Approaches and Solutions", New York.

Schmidt, E., Ghisalba, O., Gygax, D. and Sedelmeier, G. (1992). Optimization of a Process for the Production of *(R)*-2-Hydroxy-4-Phenylbutyric Acid--an

Intermediate for Inhibitors of Angiotensin Converting Enzyme, *J Biotechnol* **24**, 315-27.

Schneider, G. and Lindqvist, Y. (1993). Enzymatic Thiamine Catalysis: Mechanistic Implications From Three Dimensional Structure of Transketolase, *Bio-Organinc Chemistry* **21**, 109-117.

Schoemaker, H. E., Mink, D. and Wubbolts, M. G. (2003). Dispelling the Myths--Biocatalysis in Industrial Synthesis, *Science* **299**, 1694-1697.

Scomburg, I., Chang,A., Hofmann, O., Ebeling, C., Ehrentreich, F., Schomburg, D., (2002) BRENDA: a resource for enzyme data and metabolic information, *Trends in Biochemical Sciences* **27**(1), 54-56

Schultze, B. M. W., M.G. (1999). Biocatalysis for the Production of Fine Chemicals, *Current Opinion in Biotechnology* **10**, 609-615.

Shin, H. S., Rogers, P.L. (1996). Production of *L*-Phenylacetylcarbinol (*L*-Pac) From Benzaldehyde Using Partially Purified Pyruvate Decarboxylase, *Biotechnol Bioeng* **49**, 52-62.

Shin, J.-S. and Kim, B.-G. (1998). Kinetic Modelling of ω-Transamination for Enzymatic Kinetic Resolution of α-Methylbenzylamine, *Biotechnol Bioeng* **60**, 534-40.

Shin, J.-S., Kim, B.-G. (1999). Asymmetric Synthesis of Chiral Amines with Omega-Transaminase, *Biotechnol Bioeng* **65**, 206-211.

Shin, J.-S., Kim, B.-G. (2001). Comparison of the ω-Transaminases From Different Microorganisms and Application to Production of Chiral Amines, *Biosci. Biotechnol. Biochem.* **65**, 1782-1788.

Shin, J.-S. and Kim, B.-G. (2002a). Substrate inhibition Mode of ω-Transaminase From *Vibrio Fluvialis* JS17 is Dependent On the Chirality of Substrate, *Biotechnol Bioeng* **77**, 832-7.

Shin, J.-S., Kim, B.-G. (2002b). Exploring the Active Site of Amine: pyruvate Aminotransferase On the Basis of the Substrate Structure-Reactivity Relationship: How the Enzyme Controls Substrate Specificity and Stereoselectivity, *J.Org.Chem.* **67**, 2848-2853.

Shin, J.-S., Yun, H., Jang, J.-W., Park, I., Kim, B.-G. (2003). Purification, Characterization, and Molecular Cloning of a Novel Amine: pyruvate

Transaminase From *Vibrio Fluvialis* Js17, *Appl Microbiol Biotechnol* **61**, 463-471.

Silbert, D. F., Jorgensen, S. E. and Lin, E. C. (1963). Repression of Transaminase A By Tyrosine in *Escherichia Coli*, *Biochim Biophys Acta* **73**, 232-40.

Simon, E. S., Plante, R. and Whitesides, G. M. (1989). *D*-Lactate Dehydrogenase. Substrate Specificity and Use as a Catalyst in the Synthesis of Homochiral 2-Hydroxy Acids, *Appl Biochem Biotechnol* **22**, 169-79.

Sprenger, G. A., Schorken, U., Sprenger, G. and Sahm, H. (1995). Transketolase A of *E.Coli* K12. Purification and Properties of the Enzyme From Recombinant Strains, *Eur.J.Biochem.* **230**, 525-532.

Sprenger, G. A., Schorken, U., Wiegert, T., Grolle, S., De Graaf, A. A., Taylor, S. V., Begley, T. P., Bringer-Meyer, S. and Sahm, H. (1997). Identification of a Thiamine-Dependent Synthase in *Escherichia Coli* Required for the formation of the 1-Deoxy-*D*-Xylulose 5-Phosphate Precursor to Isoprenoids, Thiamine, and Pyridoxal, *Proc Natl Acad Sci U S A* **94**, 12857-62.

Steinbuchel, A., Lutke-Eversloh, T. (2003). Metabolic Engineering and Pathway Construction for Biotechnological Production of Relevant Polyhydroxyalkanoates in Microorganisms, *Biochem Eng Journal* **3734**, 1-16.

Stewart, J. D. (2001). Dehydrogenases and Transaminases in Asymmetric Synthesis, *Current Opinion in Chemical Biology* **5**, 120-129.

Stinson, S. C. (1994). Chiral Drugs, *Chemical Engineering News*, 38-72.

Stirling, D. I. (1992). *in* "Chirality in Industry" (A. N. Collins, Sheldrake, G.N., Crosby, J., Ed.), Pp. 209-222. Wiley & Sons Ltd, New York.

Stirling, D. I., Zeitlin, A. I., Matcham, G. W. and Rozell, J. D. (1992). Enantiomeric Enrichment and Stereoselective Synthesis of Chiral Amines, 5169780, USA

Straathof, A., A.J.J., Panke, S., Schmid, A. (2002). The Production of Fine Chemicals By Biotransformation, *Current Opinion in Biotechnology* **13**, 548-556.

Studier, W. F., Moffatt, B.A. (1986). Use of Bacteriophage T7 RNA Polymerase to Direct Selective High-Level Expression of Cloned Genes, *Journal of Molecular Biology* **189**, 113-130.

Tao, J., Mcgee, K. (2002). Development of a Continuous Enzymatic Process for the Preparation of *(R)*-3-(4-Fluorophenyl)-2-Hydroxy Propionic Acid, *Org. Process Res. Dev.* **6**, 520-524.

Turner, N. J. (2000). Applications of Transketolase in Organic Synthesis, *Current Opinion in Biotechnology* **11**, 527-531.

Van Der Werf, M. J., Van Den Tweel, W.J.J., Kamphuis, J., Hartmans, S., De Bont, J.A.M. (1995). The Potential of Lyases for the Production of Optically Active Compounds, *Trends Biotechnol* **12**, 95-103.

Vries, T., Wynberg, H., Vanecten, E., Koek, J., Tenhoeve, W., Kellog, R.M., Broxterman, Q.B., Minnaard, A., Kaptein, B., Van Der Sluis, S., Hulshof, L., Kooistra, J. (1998). The Family Approach to the Resolution of Racemates, *Angew Chem int Ed Engl* **37**, 2349.

Wainer, I. W. (1993). Stereoisomers in Clinical Oncology: Why It Is Important to Know What the Right and Left Hands Are Doing, *Ann Oncol* **4**, 7-13.

Wang, M.-X., Liu, J., Wang, D.-X. and Zheng, Q.-Y. (2005). Synthesis of Optically Active [α]-Methylamino Acids and Amides Through Biocatalytic Kinetic Resolution of Amides, *Tetrahedron: asymmetry* **16**, 2409-2416.

Warren, P. V. and Swanson, R. V. (1999). Transaminases and Aminotransferases, 6268188, USA

Watanabe, N., Sakabe, K., Sakabe, N., Higashi, T., Sasaki, K., Aibara, S., Morita, Y., Yonoha, K., Toyama, S., Fukutani, H. (1989). Crystal Structure Analysis of ω-Amino Acid: pyruvate Aminotransferase with Newly Developed Weissenberg Camera and an Imaging Plate Using Synchrotron Radiation, *J.Biochem* **105**, 1-3.

Wilms, B., Wiese, A., Syldatk, C., Mattes, R. and Altenbuchner, J. (2001). Development of an *Escherichia Coli* Whole Cell Biocatalyst for the Production of *L*-Amino Acids, *J Biotechnol.* **86**, 19-30.

Wolf, J., Gerber, A.P., Keller, W. (2002). *TADA*, an Essential tRNA-Specific Adenosine Deaminase from *Escherichia Coli*, *The Embo Journal* **21**, 3841-3851.

Wu, T.-K. A. G., J.H.,. (2002). Conversion of Plant Oxidosqualene-Cycloatenol Synthase to an Oxidosqualene-Lanesterol Cyclase By Random Mutagenesis, *Biochemistry* **41**, 8238-8244.

Yano, T., Oue, S., Kagamiyama, H. (1998). Directed Evolution of an Aspartate Aminotransferase with New Substrate Specificities, *Proc. Natl. Acad. Sci. Usa* **95**, 5511-5515.

Yazbeck, D. R., Martinez, C.A., Hu, S., Tao, J. (2004). Challenges in the Development of an Efficient Enzymatic Process in the Pharmaceutical industry, *Tetrahedron: asymmetry* **2004**, 2757-2763.

Yonaha, K., Nishie, M., Aibara, S. (1992). The Primary Structure of ω-Aminoacid: pyruvate Aminotransferase, *The Journal of Biological Chemistry* **267**, 12506-12510.

Yonaha, K., Toyama, M., Kagamiyama, H. (1983). Properties of the Bound Coenzyme and Subunit Structure of ω-Aminoacid: pyruvate Aminotransferase, *The Journal of Biological Chemistry* **258**, 2260-2265.

Yonaha, K., Toyama, M., Yasuda, M. and Soda, K. (1976). Purification and Crystallisation of Bacterial W-Aminoacid-Pyruvate Aminotransferase, *Febs Letters* **71**, 21-24.

Yonaha, K., Toyama, S., Yasuda, M., Soda, K. (1977). Properties of Crystalline ω-Amino Acid: pyruvate Aminotransferase of *Pseudomonas Sp.* F-126, *Agricul. Biol. Chem.* **41**, 1701-1706.

Yonaha, K., Toyama, S., Yasuda, M., Soda, K. (1997). Properties of Crystalline ω-Amino Acid: pyruvate Aminotransferase of *Pseudomonas Sp.* F-126, *Agricul. Biol. Chem.* **41**, 1701-1706.

Yonaha, K., Toyama, S. (1978). Monoamine Transamination Catalysed By ω-Amino Acid;Pyruvate Aminotransferase of *Pseudomonas Sp F-126, Agricultural and Biological Chemistry* **42**, 2363-2367.

Zhao, G., Winkler, M.E. (1994). An *Escherichia Coli* K-12 *TktA TktB* Mutant Deficient in Transketolase Activity Requires Pyridoxine (Vitamin B_6) as Well as the Aromatic Amino Acids and Vitamins for Growth, *J.Bacteriol.* **176**, 6134-6138.