Directed evolution of amine oxidases for use in deracemisation reactions.

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A thesis submitted for the Degree of

Doctor of Philosophy

University of Edinburgh

May 2003

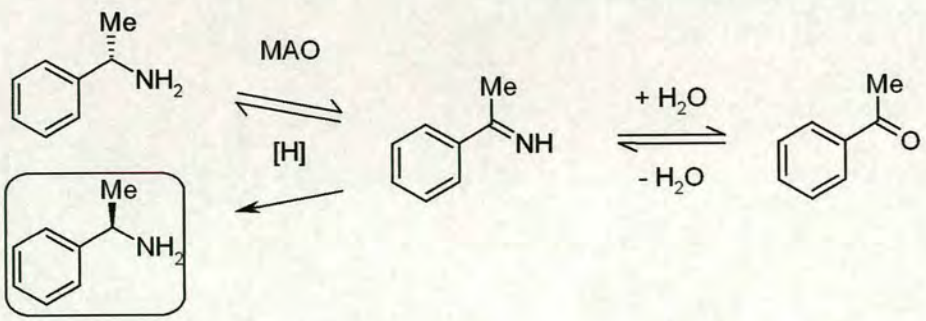
Abstract

Deracemisation is a process in which one enantiomer of a racemic mixture is converted to its optical isomer through a stereoinversion reaction. The aim of this project was to identify a novel enantioselective amine oxidase that would be suitable for application in deracemisation reactions of chiral amines.

The application of the technique of "directed evolution" allows changing certain amino acids in the enzyme so those new enzymes can be generated with altered properties. The real key to success in this area is the development of high-throughput assays that can be used to select the variant enzymes of interest.

Prior to these studies there were no reports in the literature concerning the application of amine oxidases for the deracemisation of chiral amines. In our initial studies we demonstrated that the amine oxidase from *Aspergillus niger* (MAO-N) possessed very low activity towards L-alpha-methylbenzylamine (L-AMBA) with even slower oxidation of the D-enantiomer (D-AMBA). The gene encoding for MAO-N was subjected to random mutagenesis using a *E. coli* mutator strain XL1-Red (Stratagene). This strain is deficient in the DNA repair system and permits the introduction of mutations randomly not only in the mutator genome, but also in foreign DNA, which has been transformed into the mutator strain. Using this approach it was possible to select a variant of MAO-N with activity towards L-AMBA 50 times higher and selectivity towards L-AMBA 6 times higher then the wild type.

The amine oxidase mutant was used for the deracemisation of racemic alpha-methylbenzylamine giving a 77% yield of the L-enantiomer with 93% *e.e.* These results show that new procedures of mutagenesis using a mutator strain can be simpler compared to more popular techniques such as error-prone PCR and DNA recombination. The efficiency of mutagenesis was achieved in conjunction with a novel high throughput screen.



(R)-AMBA, 77% yield, 93% e.e.

Acknowledgements

I would first of all like to thank Professor Nick Turner for all his encouragement, support and patience. Thanks also to Dr's Ian Fotheringham and Andrew Herbert for their valuable input and support.

Thanks to Dr Ian Fotheringham for proof reading this thesis and for pretending to enjoy it. I would also like to give thanks to everyone in the laboratories I have worked in, especially Dr Alexis Enright who made a significant contribution to this work as a chemist, and to the technical staff at the Chemistry Department for providing a wonderful service.

To my mum and dad I am eternally thankful: for loving me and believing in me through all my decisions. Thanks to my husband and my kids for their love and being beside me all the time.

Abbreviations

AA	n-amylamine
AAP	4-aminoantipyrine
AMBA	α-methylbenzylamine
AO	amine oxidase
APS	ammonium persulfate
A. niger	*Aspergillus niger*
BSA	bovine serum albumin
$BzNH_2$	benzylamine
CFE	cell free extract
CLERY	combinatorial libraries enhanced by recombination in yeast
CPM	concerted Polar Nucleophilic Mechanism
CSR	compartmentalized self replication
$CuSO_4$	copper (II) sulfate
CV	column volume
d	days
DAAO	D-amino acid oxidase from porcine kidney
DAO	Diamineoxidase
D_2 lamp	deuterium lamp
DMSO	dimethyl sulfoxide
DNA	deoxyribose nucleic acid
cDNA	complimentary deoxyribose nucleic acid
dsDNA	double stranded DNA
DTT	dithiothreitol

E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraaceticacid
e.e.	enantiomeric excess
eq.	equivalents
ESI-MS	electrospray ionisation mass spectrometry
Et	ethyl group
FAD	flavin adenine dinucleotide
FDH	formate dehydrogenase
FPLC	fast protein liquid chromatography
h	hours
HAT	hydrogen atom transfer
HF	high fidelity
HPLC	high performance liquid chromatography
HRP	horse radish peroxidase
imac	immobilised metal affinity chromatography
IPTG	isopropylthio-β-D-galactoside
ITCHY	incremental truncation for the creation of hybrid libraries
kb	kilobase
kDa	kiloDaltons
K. oxytoca	*Klebsiella oxytoca*
LAAD	L-amino acid deaminase
LAAO	L-amino acid oxidase
LC/MS	liquid chromatography-mass spectrometry
LeuDH	leucine dehydrogenase
LO	lysyl oxidase

MAO	monoamine oxidase
MAO-N	monoamine oxidase from *Aspergillus niger*
Me	methyl group
MeOH	methanol
mRNA	messenger ribonucleic acid
min	minute
MW	molecular weight
$(NH_4)_2SO_4$	ammonium sulfate
$NaCNBH_3$	sodium cyanoborohydride
$NaBH_4$	sodium borohydride
NMR	nuclear magnetic resonance
nt	nucleotide
OD_{600}	optical density at 600 nm
PAO	polyamine oxidase
PCR	polymerase chain reaction
Ph	phenyl group
PhCHO	benzaldehyde
PLP	pyridoxal-5'-phosphate
PMP	pyridoxamine-5'-phosphate
PMSF	phenylmethylsulfonylfluoride
RACHITT	Random chimeragenesis on transient templates
rt	room temperature
RPR	Random-priming *in vitro* recombination
S. cerevisiae	*Saccharomyces cerevisiae*
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis

SET	single electron transfer
Ser	serine
SHIPREC	sequence homology independent protein recombination
StEP	staggered extension process
ssDNA	single-stranded DNA
SSAO	semicarbazide sensitive amine oxidase
TEMED	N,N,N', N'-tetramethylethylenediamine
TBHBA	2,4,6-tribromo-3-hydroxybenzoic acid
TOPA	3-(2,4,5-trihydroxyphenyl)-1-alanine
TPQ	3-(2,4,5-trihydroxyphenyl)-1-alanine quinone
Tris	tris(hydroxymethyl)aminomethane
UV	ultra-violet
w/v	weight to volume ratio
ε	molar extinction coefficient

1. Introduction

Optically active amines are highly desired intermediates as starting materials for the production of enantiomericaly pure drugs. They are often used in asymmetric synthesis as chiral auxilliaries or catalysts, and also as building blocks for therapeutically important pharmaceutical and agrochemical compounds.[1,2,3] For example (*S*)-1-methoxy-2-aminopropane, which is produced by BASF AG in a multi-tonne scale, is used as the starting material for optically active corn-herbicides (FRONTIER X2® and OUTLOOK®) production.[1]

In the case of pharmaceutical or agrochemical compounds, generally only one enantiomer of a target molecule is active with the other showing no, or even detrimental, biological activity. Therefore the need to prepare compounds in an enantiopure form arises from both commercial and safety issues.

1.1. Biocatalysis

Traditional chemical catalysis generally refers to processes in which chemical reactions are catalysed by transition metals or organometallic compounds, sometimes under extreme pressure and temperature conditions and in the presence of toxic organic chemicals.[4-7]

An alternative approach to the conventional chemical synthesis of enantiomerically pure compounds lies in the area of biocatalysis. Enzymes are remarkable catalysts that perform their tasks under mild conditions and with a high degree of regio-selectivity and enantio-selectivity.

A large number of enzymes have become commercially available for organic synthesis. Of about 2500 identified so far, some 300 are available in a partially purified form. Because of advances in molecular biology, fermentation and

purification techniques, the fine chemical production costs are being reduced to industrially attractive levels.[8]

1.1.1. *Kinetic resolutions*

The process wherein one enantiomer of a racemate is more readily transformed into the product compare to its mirror image is known as a kinetic resolution (Figure 1).[9]

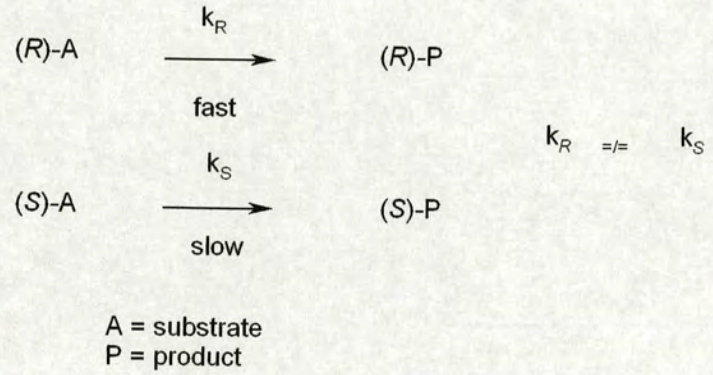


Figure 1: kinetic resolution.

The first kinetic resolution was documented by Pasteur in 1858, when he discovered that ammonium tartarate, fermented in the presence of *Penicillium glaucam,* resulted in faster metabolism of the D-enantiomer compare to the L-enantiomer.[9] Today either chemo- or biocatalysis can be used to bring about such resolutions, with reports of biocatalytic resolutions greatly outnumbering the chemocatalytic methods. The hydrolase enzymes, such as lipases and acylases, are the most popular class of enzyme reported for resolution processes.

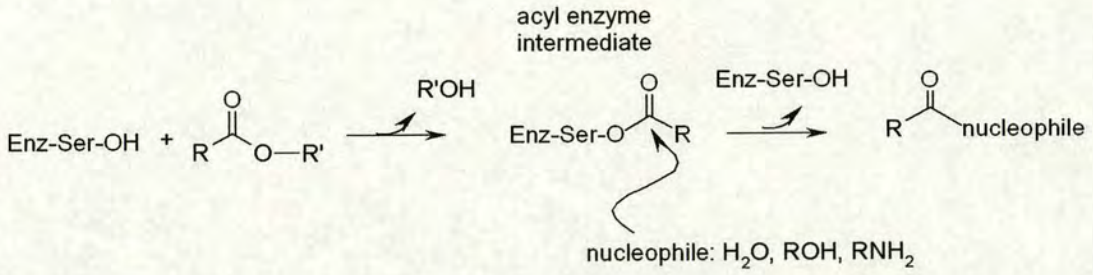


Figure 2: mechanism of lipase-catalysed acyl transfer to an alcohol or amine acceptor.

Lipases belong to a large class of enzymes that hydrolyse the ester bond between the fatty-acyl side chains and the lipid backbone. The lipase active side is composed of three different residues: serine, histidine and aspartate or glutamate. The hydrolysis of an ester involves formation of an acyl enzyme complex. The catalytic cycle is initiated by nucleophilic attack of the hydroxyl group of the serine side chain onto the carbonyl carbon atom of the ester bond followed by release of the alcohol. Thereafter nucleophilic attack by water liberates the carboxylic acid and the enzyme is regenerated (Figure 2).

As lipases are active in organic solvents, water can be replaced by other nucleophiles such as alcohols. The result of this reaction is a transesterification. This reaction is commonly used for kinetic resolution of amino acids.[10] Amines can also be used as nucleophiles. Racemic amines are efficiently resolved using ethylmethoxyacetate [1,11a] (Figure 3A) or ethyl acetate as the acylating agent.[11b] Due to the simplicity of lipase-catalysed reactions, a number of kinetic resolutions are now carried out on an industrial scale.[1,12] Nevertheless, it would seem that performing the enantioselective acylation of amines catalyzed by penicillin acylase from *Alcaligenes faecalis* in aqueous medium could be more efficient and practical (Figure 3B).[13]

One such process is the resolution of racemic primary amines using *Candida antarctica* lipase and ethyl acetate as the acyl donor.[11b] This method is used to resolve α-methylbenzylamine, with 50% recovery of the *S*-enantiomer and an *e.e.* of 90%.

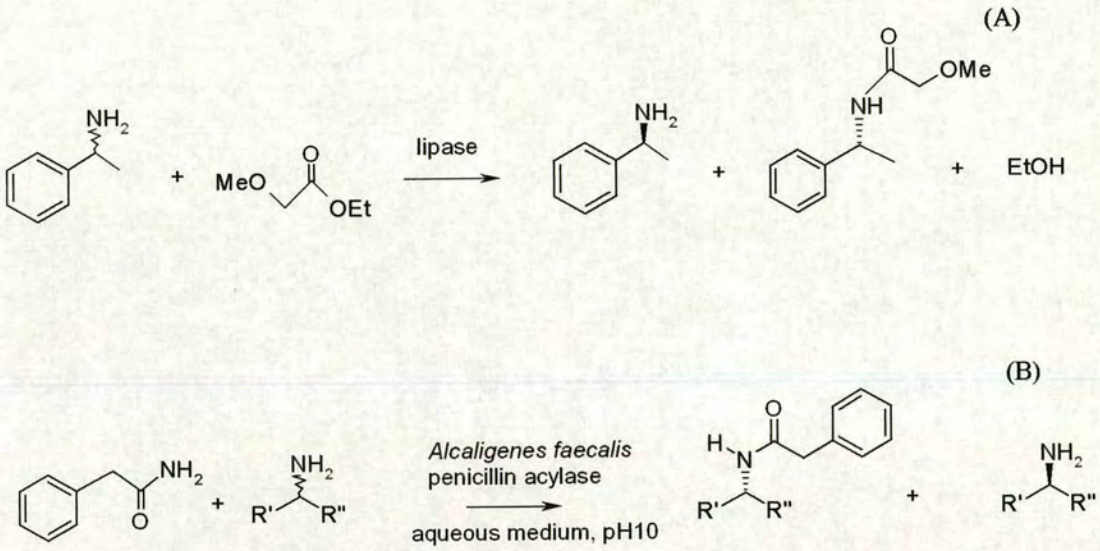


Figure 3: (A) lipase-catalysed synthesis of enantiomerically pure 1-phenylethylamine (BASF); (B) *Alcaligenes faecalis* penicillin acylase–catalysed acylation of amines, with phenylacetamide as an acyl donor.

Chiral amines *e.g.* α-methylbenzylamine (AMBA) have also been kinetically resolved using an ω-transaminase (Figure 4).[14] It was found that the enzyme was inhibited by the acetophenone product, which resulted in a decrease in the yield of the reaction. The problem was overcome by using a bi-phasic system, which offered two advantages: i) the inhibitory product acetophenone could be extracted from the aqueous phase and ii) AMBA formed an imine with cyclohexanone and resided primarily in the organic phase, thereby allowing the organic phase to act as a reservoir of AMBA.

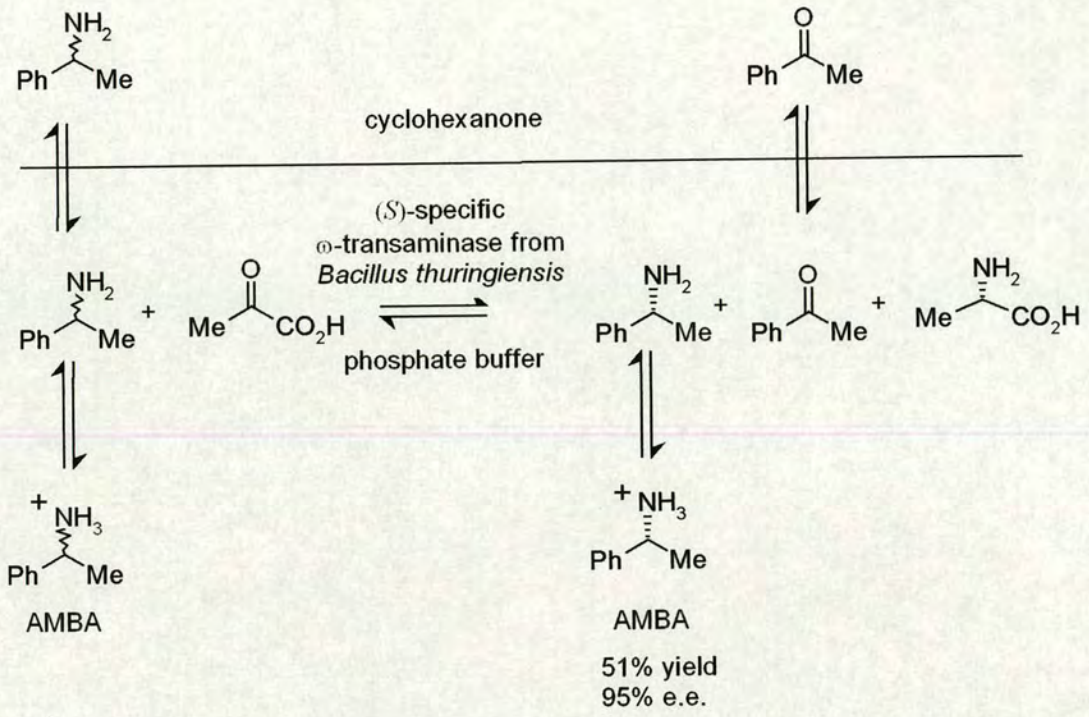


Figure 4: kinetic resolution of AMBA using ω-transaminase.[14]

However, no matter how efficient the resolution, classical kinetic resolutions suffer from three major disadvantages: (i) the maximum yield that can be obtained is only 50%, (ii) separation of the product from the unreacted starting material can be problematic and (iii) for kinetic reasons, the enantiomeric purity becomes depleted beyond 50% conversion.[15]

1.1.2. *Asymmetric biosynthesis*

Transaminases (aminotransferases) use the cofactor PLP (pyridoxal-5'-phosphate) to transfer an amine group by deamination from a donor amine, via PMP (pyridoxamine-5'-phosphate), to an acceptor α-keto acid. In the second stage of the reaction the amino group of PMP is transferred to α-keto acid to yield a new amine and regenerated PLP. The rate of both reactions is about equal ($K_{eq} = 1$), so that the substrate/product mixture is difficult to separate. This problem can be overcome by coupling the reaction with oxidative deamination of inexpensive (*S*)-amino acids

such as (*S*)-aspartate to oxaloacetate which decarboxylates spontaneously to pyruvate[16,17] (Figure 5).

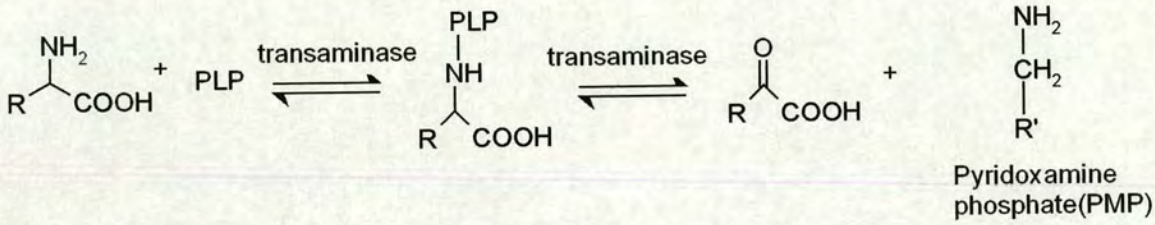


Figure 5: reaction of transamination catalysed by PLP-dependent transaminase.

The transaminase technology has been successfully developed by Celgene Corporation for the production of chiral amines by direct asymmetric synthesis from the corresponding prochiral ketone and amino donor (*e.g.* isopropylamine, 2-amino butane)[18] (Figure 6). In this way, pilot production of chiral amines to multi-100kg scale has been carried out at Celgene.

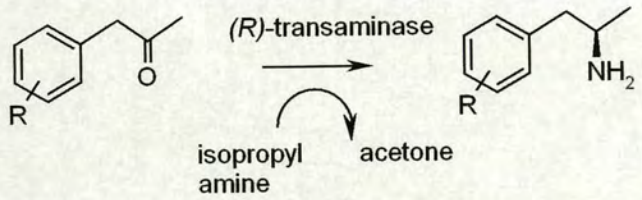


Figure 6: asymmetric synthesis of a chiral amine using an (*R*)-transaminase.

The main advantages of transaminase catalysed processes are the high turnover numbers of the enzymes and no requirement for external cofactor recycling. A number of compounds including L-2-aminobutyric acid and L–tertiary leucine have now been produced using transaminase-based biotransformations.[19,20] Enantiomerically pure L-2-aminobutyric acid was produced from L-threonine and L-aspartic acid in a whole cell biotransformation using recombinant *E. coli* cells

expressing cloned genes encoding threonine deaminase, tyrosine aminotransferase, and acetolactate synthase. The reversible nature of the reaction leads to the presence of a keto acid by-product, which limits the overall yield, and purity of product. To enhance the biosynthesis of L-2-aminobutyric acid, the keto acid was eliminated by incorporation of acetolactate synthase into the synthetic biochemical pathway of L-2-aminobutyric acid.[21,22] (Figure 7)

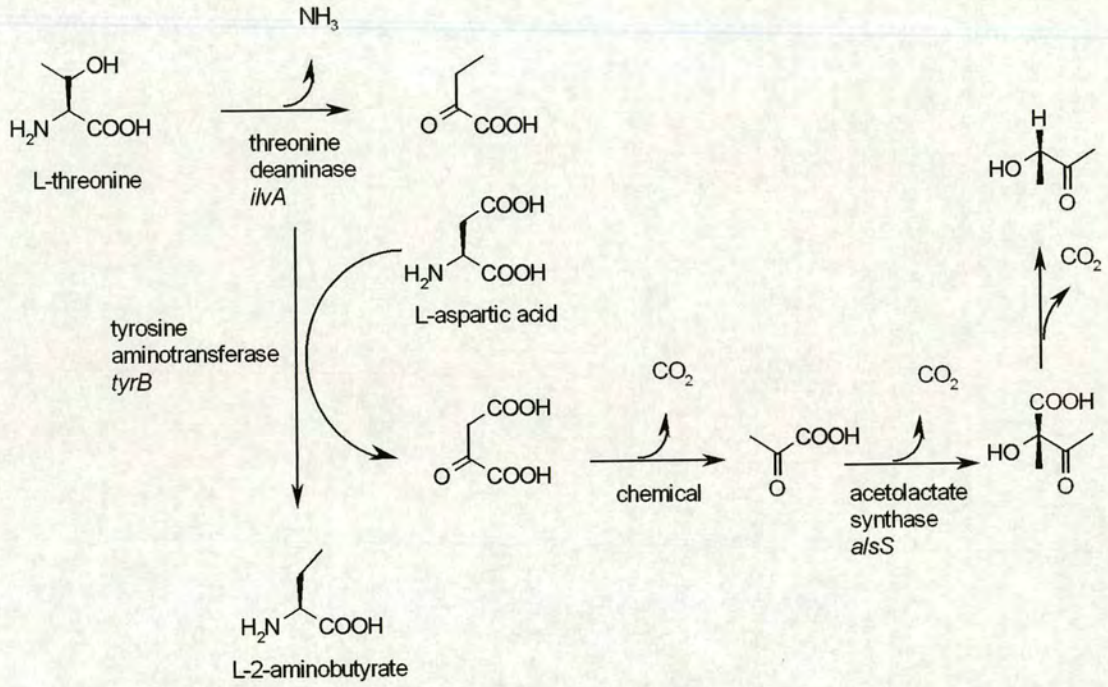


Figure 7: engineered biosynthetic pathway for production of L-2-aminobutyric acid (NSC Technologies).

The presence of acetolactate synthase increased the overall yield of the product from 68% to 92%. In the absence of threonine deaminase, the yield and purity are comparable to the three gene system but the commercial feasibility of the process is significantly diminished by the high cost and unavailability of 2-ketobutyrate.

In addition, α-keto acids can be reductively aminated to α-amino acids in a reaction with the equilibrium on the side of aminated products ($K_{eq} = 9 \times 10^{12}$ at pH 11.0), (Figure 8).[23]

Following conversion of the keto acid to the intermediate imine, the enzyme leucine dehydrogenase (LeuDH)[17] catalyses attack of hydride from NADH to give the optically pure amino acid as the product. Analogues of L-tert-leucine can also be prepared, which serve as chiral intermediates for the synthesis of antitumor, anti-inflammatory and antiviral drugs.[24]

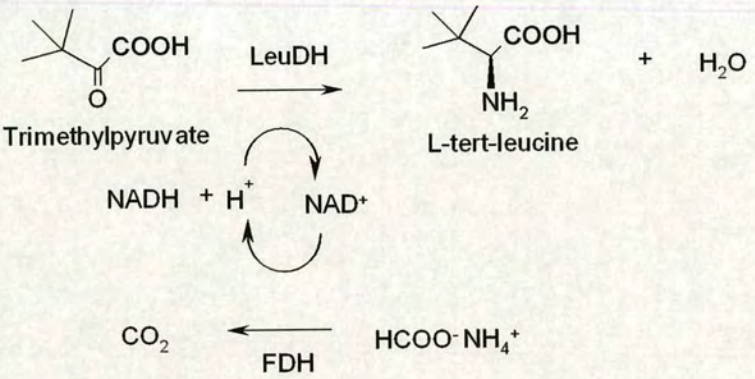


Figure 8: scheme for NADH recycling via synthesis of L-tert-leucine by reductive amination (Degussa-Hüls AG).

Enzymatic reductive amination with NADH as cofactor can only be operated on a large scale if the cofactor is regenerated (Figure 8).[17,24] The oxidised form of the cofactor NAD^+ is regenerated to NADH by employing formate dehydrogenase (FDH) and ammonium formate. The formate is oxidised irreversibly to CO_2, which can be easily removed.

1.1.3. Deracemisation

Deracemisation can be defined as a process that leads to the formation of a single enantiomer from a racemic mixture, with theoretical yields and enantiomeric excesses approaching 100%.[25]

1.1.3.1. Dynamic kinetic resolution

The non-static process in which the substrate can be racemised *in situ* is called 'dynamic kinetic resolution' (Figure 9).

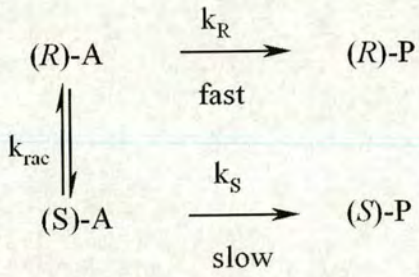


Figure 9: dynamic kinetic resolution.

If the rate of racemisation (k_{rac}) is much greater than the rate of product formation (k_R), then a theoretical yield of 100% is possible. For the maximum efficiency, product formation should be irreversible, the enantiomeric ratio (E) should be ≥ 20 (where $E = k_R/k_S$) and k_{rac} should be approximately ten times faster than k_R.[26]

A number of examples of dynamic kinetic resolutions have been published since 1980.[9, 26-30] This process very often requires conditions for racemisation that are incompatible with the enzyme used for resolution. A relatively new area in dynamic kinetic resolution uses enzymes and transition metal complexes in tandem,[31] where the transition metals that bring about racemisation are more compatible with biological systems.

Benzylic α-substituted primary amines can be racemised by palladium on charcoal at elevated temperatures, allowing for the preparation of enantiopure chiral amides in >60% yield.[32] Palladium is inserted into the N-H bond, followed by β-elimination of PdH to give a palladium-complexed imine. This is then non-selectively reduced by

palladium-catalysed hydrogenation to give the racemic starting material (Figure10).[32,33]

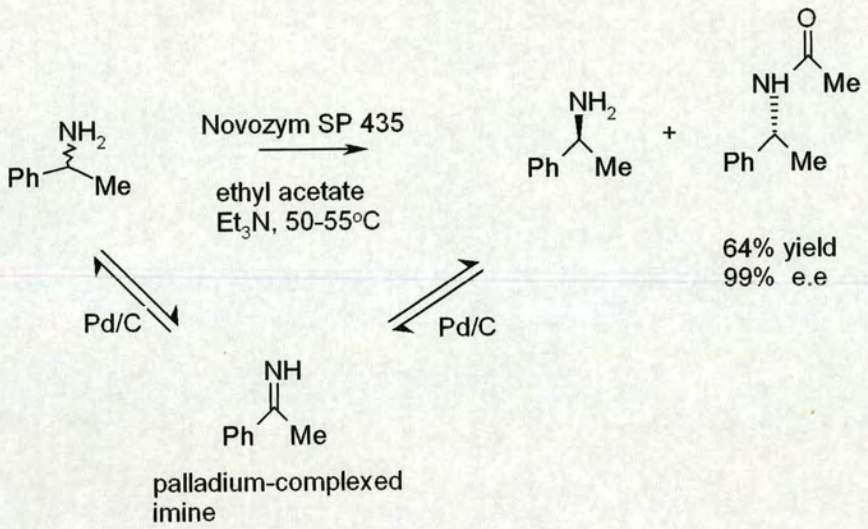


Figure 10: palladium-catalysed racemisatiopn of benzylic amines.

This process has been scaled-up by BASF Technologies with a capacity of 1000 tonnes per annum.[1]

Another approach for accessing optically pure amines was reported by Celgene via direct asymmetric synthesis from the corresponding prochiral ketone, or via resolution of the racemic amine, wherein the undesired enantiomer is converted back to the ketone (Figure 11).[18]

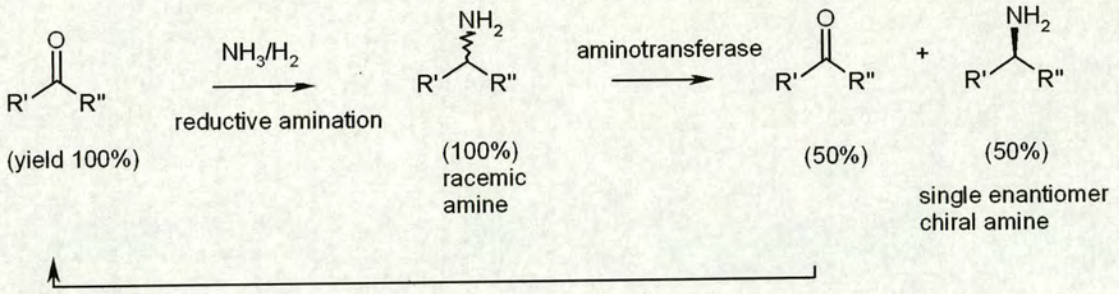


Figure 11: Celgene chiral amine production.

The selective ring opening of D-hydantoins by the enzyme D-hydantoinase is the basis for a successful industrial scale preparation of D-amino acids (Figure 12).[24] The spontaneous racemisation of hydantoins at pH 8.0 or above allows a dynamic kinetic resolution to occur.

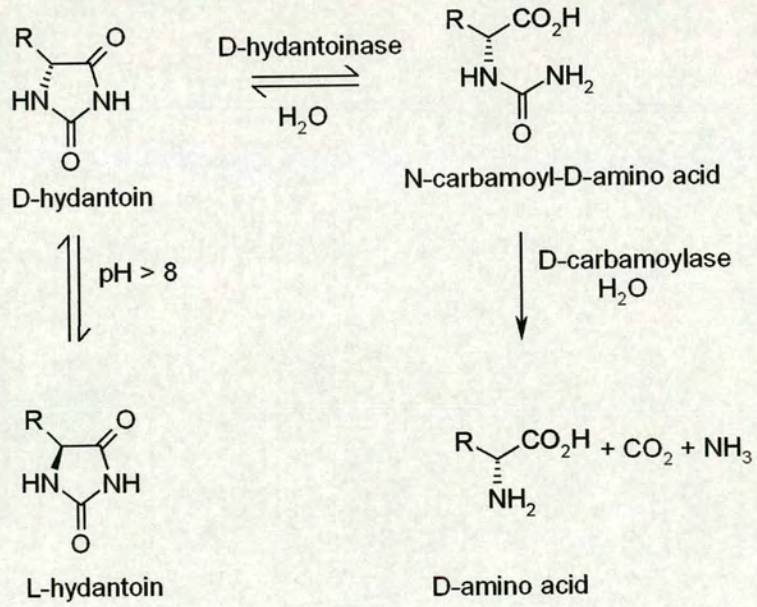


Figure 12: industrial preparation of D-amino acids by dynamic kinetic resolution (Degussa-Hüls AG).

A number of amino acid racemases have been employed for the dynamic kinetic resolution of chiral amino acids[34] (Figure 13).

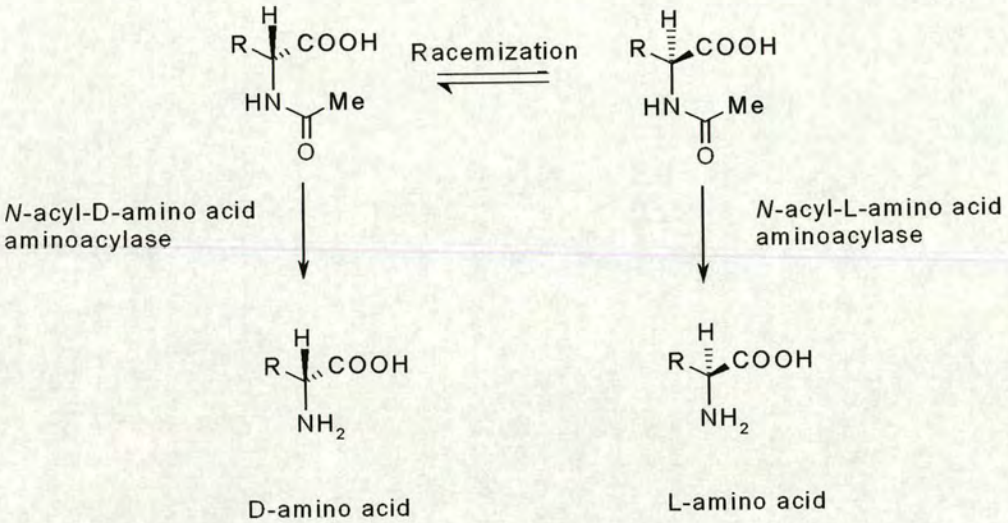


Figure13: production of optically active D-and L-amino acids from *N*-acyl-DL-amino acids by D- or L- aminoacylase and *N*-acylamino acid racemase.

Ideally the rate of racemization should be equal to or greater than the rate of hydrolysis and the racemase must be active only towards *N*-acylamino acids but not towards the free amino acids.

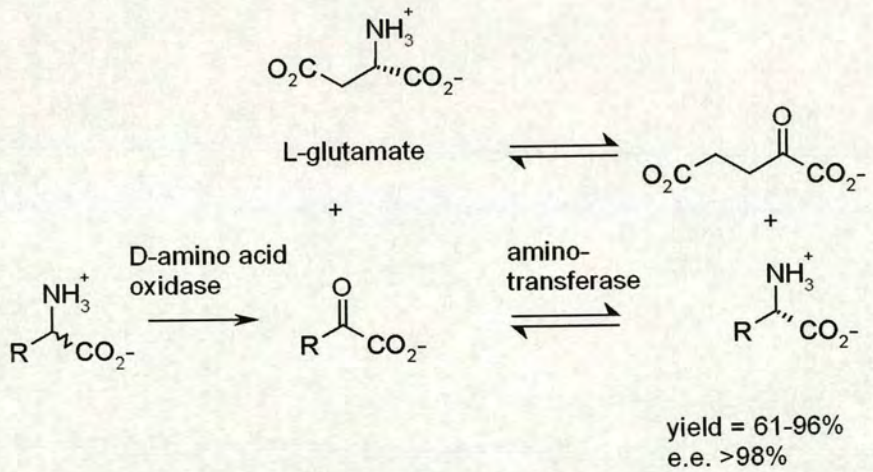
1.1.3.2 Deracemisation by stereoinversion

Deracemisation by stereoinversion is a cyclical process where only one enantiomer of a racemic mixture is oxidised to form an achiral intermediate which can be reduced by an enzyme with the opposite stereochemical preference.[35a] The net redox-balance of the system is zero and the necessary redox cofactors, *e.g.* nicotinamide adenine dinucleotide, may be recycled internally.[35b] The success of such systems relies on at least one of the redox reactions being irreversible. Biocatalytic

stereoinversions have been successfully used in the preparation of optically pure α-amino acids.[35c,d] The preparation of natural and unnatural α-amino acids *via* such a two-enzyme system is exemplified in Figure 14.[35c] D-Amino acid oxidase from porcine kidney (DAAO) selectively deaminates the D-amino acid from the racemic mixture. The α-keto acid produced from the reaction is then converted to the corresponding L-amino acid using an aminotransferase from *Escherichia coli*, with L-glutamate as the amine donor. Although excellent yields and enantioselectivities are observed, the system suffers from a few disadvantages:

i) ion-exchange chromatography is required to separate L-glutamate from the desired amino acid and

ii) the first step is a kinetic resolution and therefore the rate of oxidation of the D-enantiomer slows as the reaction proceeds.



R = -CH(CH$_3$)$_2$, -CH$_2$CH(CH$_3$)$_2$, -C(CH$_3$)CH(CH$_3$)$_2$, -CH$_2$SCH$_3$, -CH$_2$Ph, -CH$_2$CH=CH$_2$, -CH$_2$CH$_3$

Figure 14: stereoinversion of α-amino acids.

1.1.3.3. Deracemisation by a cyclic oxidation-reduction sequence

In 1971, Hafner and Wellner demonstrated the stereoinversion of D- to L- alanine by a cyclic oxidation-reduction sequence.[36a] In this approach, the starting racemic

mixture was subjected to enantioselective oxidation using a D-amino acid oxidase which selectively converted one enantiomer to the corresponding achiral imine. Addition of a reducing agent to the reaction resulted in conversion of the imine back to a 1:1 mixture of amino acids (Figure 15&16).

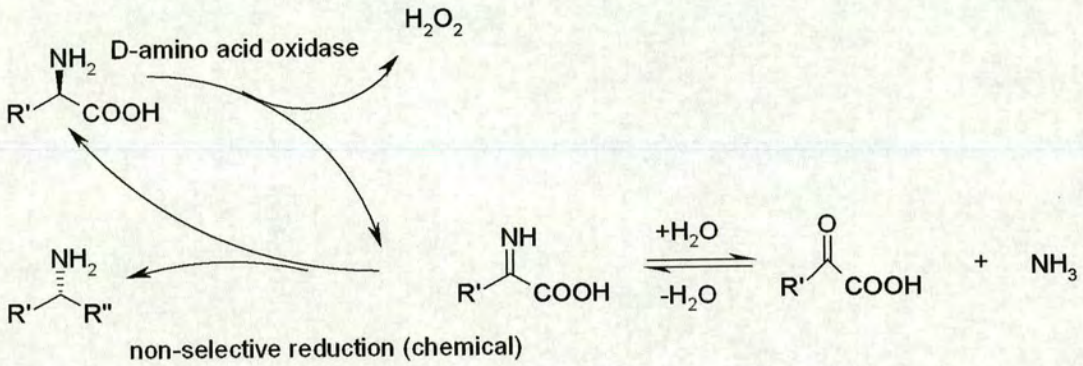
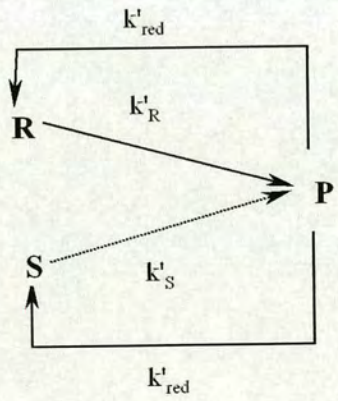
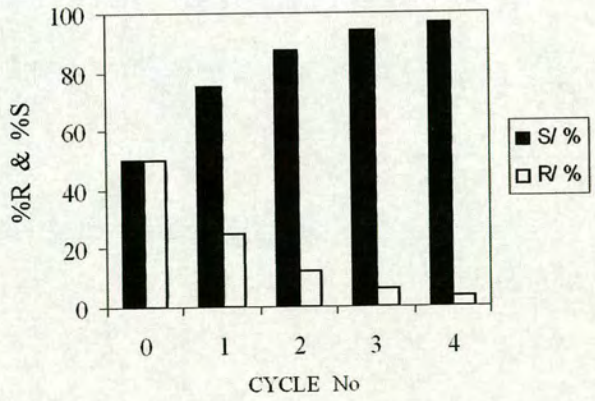


Figure 15: stereoinversion of α-amino acids by an oxidation-reduction cycle.

The first step of this method is a kinetic resolution with the limitation of 50% yield, and the second step bears no chiral induction at all.





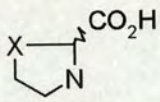
$k'_R > k'_S$

$k'_{red} = 2 \times k'_R$

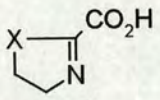
Figure 16: cyclic oxidation-reduction sequence.[36b]

However, a combination of the two reactions is potentially a very effective and economic technique. If this process undergoes a sufficient number of cycles then eventually the amino acid becomes enantiomerically pure (Figure16), provided that the oxidation is highly enantioselective and the intermediate imino acid is efficiently reduced before it undergoes hydrolysis to the ketone.

Soda *et al.*[37,38] extended this concept to the deracemisation of the cyclic α-amino acids proline and pipecolic acid in high yield and e.e. High chemical and optical yields were obtained due to the stability of the corresponding cyclic imine under aqueous conditions and their susceptibility to chemical reduction.



x = CH_2 - proline
x = CH_2CH_2 - pipecolic acid



imine

The major limitation of the reaction reported by Soda is the requirement for a large excess of $NaBH_4$ used for the reduction step (500 equivalents) because of competitive reaction with water at pH 7.0. Turner *et al.*[39] subsequently demonstrated only 6 equivalents of sodium cyanoborohydride ($NaCNBH_3$) were required for the complete deracemisation of DL-proline, due to the greater aqueous stability of $NaCNBH_3$ than $NaBH_4$ at pH < 8.0.[40] The method has since been further developed to produce optically pure acyclic α-amino acids in high yield (75-90%), using amine:boranes[41] as reducing agents.

The cyclic oxidation-reduction deracemisation protocol has also been investigated by the groups of Carnell[42] and Faber[43] and both groups have published examples of this type of reaction with reasonable success. However, in both cases separation of the achiral intermediate is required, before "reduction" can take place.

Deracemisation *via* repeated cycles of oxidation-reduction has been successfully applied to a range of D- and L-amino acids. However, to date there has been no report of using this method for the deracemisation of chiral amines. In principal deracemisation should be applicable to amines provided that suitable enantioselective amine oxidases can be identified.

1.2 Amine oxidases

Amine oxidases (AOs) are a heterogeneous family of enzymes, which metabolise various monoamines, diamines and polyamines that are either produced endogeneously, or absorbed as dietary or xenobiotic substances. These enzymes are widely distributed in nature and found in microorganisms (bacteria, yeast, and fungi) and in higher organisms such as mammals including humans. Amine oxidases catalyse the oxidative deamination of amines to yield the corresponding aldehyde, hydrogen peroxide and ammonia (Figure 17).

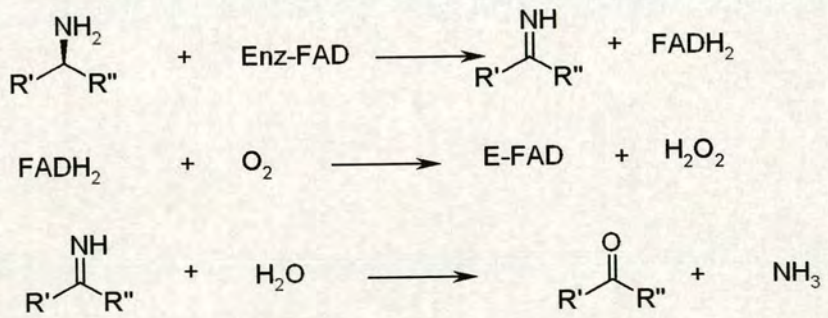


Figure 17: mechanism of oxidation of amines by amine oxidase.

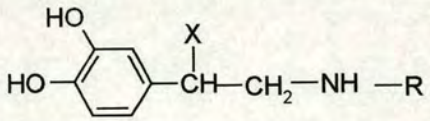
AOs are subdivided into two main classes based on the nature of their cofactors. The first class of amine oxidases are represented mainly by monoamine oxidases but also by DAO, PAO and LO (lysyl amine oxidase). The members of this first class [amine: oxygen oxidoreductase (deaminating), (copper-containing), EC 1.4.3.6] contain a

cofactor (TOPA) possessing a carbonyl group and also copper and are known to be inhibited by carbonyl reagents such as semicarbazide.[46] The second class of amine oxidases [amine: oxygen oxidoreductase (deaminating), (flavin containing), EC 1.4.3.4] consists of monoamine oxidase (MAO), diamine oxidase (DAO) and polyamine oxidase (PAO) and contain flavin adenine dinucleotide (FAD) as their cofactor. [44,45]

1.2.1. *Physiological importance and substrate specificity of AOs*

1.2.1.1. Flavin AOs: MAO-A and MAO-B

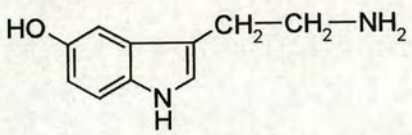
A large amount of research was undertaken on flavin MAOs in the late 50's because of the potential of MAO inhibitors to act as antidepressants (MAO controls the levels of serotonin and noradrenalin (norepinephrine) in the brain).



X=OH, R=CH_3 - epinephrine (adrenalin)

X =OH, R=H - norepinephrine (noradrenalin)

X=H, R=H - dopamine



Serotonin (5-hydroxytryptamine)

Figure 18: physiologically active amines.

Monoamine oxidases have been studied during 60 years. Since then, the enzyme was purified from a number of sources, providing evidence for the presence of two distinct forms, MAO-A and MAO-B.[47] Mammalian MAOs were found in the outer mitochondrial membrane within most cell types.[48] Since both forms are important for neurotransmitter regulation, different MAO activities may be associated with

neurological disorders such as depression, Parkinson's disease and perhaps Alzheimer's disease.[49]

MAO-A preferentially oxidises primary amines such as neurotransmitters noradrenaline (norepinephrine), 5-hydroxytryptamine (serotonin), secondary amines-such as adrenaline (epinephrine) (Figure 18), and is inactivated irreversibly by low concentrations of clorgyline.[50] MAO-B preferentially oxidises other primary amines *e.g.* benzylamine, β-phenylethylamine and is inhibited by low concentrations of L-deprenil.[51] Tyramine, tryptamine, and dopamine are common substrates for both enzymes (Figure 18a). MAO-B also has the ability to metabolise some tertiary amines such as xenobiotic 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). The neurotoxic oxidation product of the latter compound induces Parkinsonism in humans and subhuman primates.[52] MAO-A inhibitors are used clinically in the treatment of depression[53] and inhibition of MAO-B is used to treat Parkinson's disease.[54]

1.2.1.2. Flavin enzyme: MAO-N

In 1995 Schilling *et al.*[55] reported that *Aspergillus niger* expressed two different amine oxidases when grown on a range of amines. Purification by DEAE-Sepharose resulted in two fractions. The first, pink fraction was identical to the copper/TOPA enzyme previously described.[56a] The protein in the second pool (yellow), termed MAO-N, was a flavin containing enzyme which catalysed the oxidative deamination of aliphatic and aromatic monoamines, but not diamines (putrescine, cadaverine) or polyamines (spermine, spermidine, N-acetylspermidine). The flavin containing MAO-N was a soluble, tetrameric enzyme localized in the peroxysome, with a molecular mass of 55kDa. Upon extensive dialysis of the enzyme against 0.2M formic acid, FAD was irreversibly lost, which suggested that FAD was not covalently bound to MAO-N. By contrast, the FAD of mammalian MAOs is covalently bound to the active site cysteine residue.[56b]

The observations of MAO-N substrate specificity reported by different researchers were not the same. Thus, the data reported by Schilling *et al.*[55] was not in agreement with the results published by Sablin *et.al.*[57] The latter authors suggested that their data were more accurate because they obtained it by analysis of a purer enzyme. Schilling *et al.* reported that the rate of oxidation of aliphatic amines increased with the number of methylene groups, but Sablin *et al.* found that medium chain-length aliphatic amines were the best substrates for MAO-N. On comparing the substrate specificity of MAO-N with MAO-A and MAO-B, Sablin *et al.* reported that MAO-N, in common with MAO-A, exhibited the same properties towards MPTP and many of its analogues, but differed from MAO-A in not oxidising biogenic amines. Schilling *et al.* reported that noradrenalin, serotonin and dopamine were good substrates for MAO-N. However, both authors reported that substrates such as benzylamine and 2-phenethylamine were oxidised by both MAO-N and MAO-B.

MAO-N is not inhibited by the copper inhibitor semicarbazide. It was found that MAO-N is affected by low concentrations of clorgylin (best inhibitor for MAO-A), and (-)-deprenyl (specific inhibitor for MAO-B), but the fungal enzyme has the highest sensitivity to pargyline, which inhibits equally both MAO-A and MAO-B.[55] However, Sablin *et.al.*[57] reported that inactivation of MAO-N by the MAO-A specific inhibitor clorgyline was found to occur about five times faster than with (-)-deprenyl. Thus, in this regard, MAO-N behaves more like mammalian MAO-A than MAO-B.

Schilling *et al.*[55] compared the substrate specificities of flavin and copper containing AOs from *Aspergillus niger* and found them similar. However, copper AO had lower K_M values compared to MAO-N. This could indicate that the copper AO is active at low concentrations of amines as nitrogen sources. On the other hand, if copper is limited, MAO-N which has no metal ions dependence could be important as a source of nitrogen.[55]

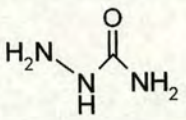
Despite the fact that several flavin-containing amine oxidases had been described in bacteria,[58] plants,[59] and fungi [44,60,61,62] MAO-N is the first non-vertebrate enzyme,

which is a true member of this class of MAO. MAO-N and the mammalian MAOs exhibit several structural and biochemical differences such as the cofactor attachment, the cellular localisation and lack of ability to distinguish between selective MAO-A and MAO-B substrates and inhibitors. Nevertheless, MAO-N can be proposed as an evolutionary prototype of MAO-A and B due to the many shared properties listed above. MAO-N can be used as a model to study both structural and catalytic properties. The lack of the FAD covalent attachment provides the possibility to acquire information on flavin-inhibitor adducts without proteolytic digestion, purification and interference of amino acids.[63]

1.2.1.3. Copper-containing amine oxidases

Copper containing monoamine oxidases were first described in 1928.[64] The copper amine oxidases are homodimers of subunit size approximately 70-95 kDa depending on the source. Each subunit contains one Cu(II) atom and a quinone cofactor[65] which has been identified as 2,4,5-trihydroxyphenylalanine quinone (TPQ or TOPA quinone) in the bovine serum enzyme.[66,67] Subsequently TPQ has been identified biochemically,[67] or its presence inferred from amino acid sequence homology in other copper-containing amine oxidases from *Hansenula polymorpha,*[68,69] *Klebsiella aerogenes,*[70] *Escherichia coli* K12,[71,72] lentil,[73,74] pea,[75] *Arthrobacter* strain P1,[76] *Arthrobacter globiformis,*[77] human kidney,[78] rat kidney,[79] and *A. globiformis* histaminase.[80]

To differentiate mammalial copper containing monoamine oxidases from flavin MAO, the former was referred to as SSAO due to its sensitivity to semicarbazide. SSAO is also called "benzylamine oxidase"[81] because of the relatively high activity towards benzylamine. SSAO exists in the plasma and tissue-bound forms in many species.[46]



semicarbazide

Tissue-bound SSAO is only active towards mono amines such as tyramine, tryptamine, dopamine and β-phenylethylamine, whereas plasma SSAO is also very active against polyamines such as spermidine and spermine.[82] The polyamine activity of plasma SSAO could be associated with polyamine metabolism in some animal species (*e.g.* ruminants).[83]

The fact that the substrate specificity of SSAO varies between species[84] and that benzylamine is the best substrate for SSAO, makes it difficult to propose a hypothesis about the physiological role of SSAO in amine metabolism *in vivo*. The copper monoamine oxidases isolated from different micro-organisms also display differences in substrate specificity.[85] So far, copper/TOPA amine oxidases are considered to be widely distributed in animal tissue. Low amounts of SSAO mRNA were detected in brain, heart, intestine, and kidney. An intermediate level of expression was seen in lung and skeletal muscle. SSAO is essentially present in smooth muscle cells in the blood vessel wall, but preferential SSAO gene expression was observed in adipose tissue and aorta.[86] Amine oxidase is expressed in high endothelial venuls from peripheral lymphatic tissue and appears to play an important role in lymphocyte trafficking.[87] The fact that SSAO from blood plasma plays an immunoregulatory role, is due to inhibition of lymphocyte transformation.[88] All of these findings underline the multiple physiological roles of SSAO according to the nature of tissue type.

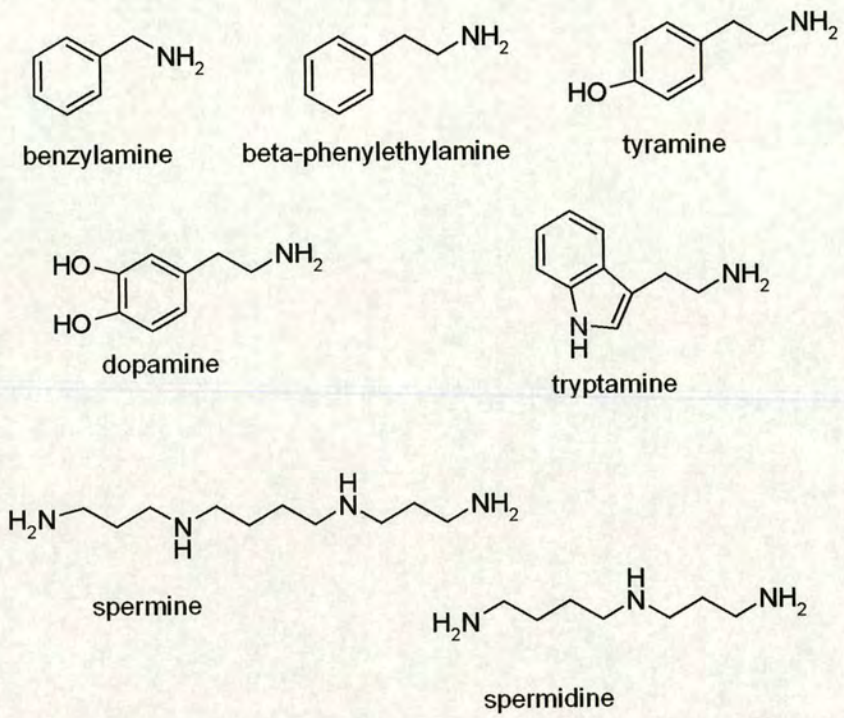


Figure 18a: chemical structures of endogenous and xenobiotic amines metabolized as substrates *in vitro* by amine oxidases.

1.2.1.4. Polyamine oxidases and diamine oxidases

It is worth noting that there are three other groups of AOs, namely diamineoxidases (DAOs), polyamineoxidases (PAOs) and lysyl oxidases(LOs).

Several flavin-containing polyamine oxidases have been described in mammals,[89] bacteria,[58] plants,[59] and fungi.[44,61,62] The intracellular concentration of polyamines is regulated by polyamine oxidases.[58] Inhibition of polyamine biosynthesis is associated with a decrease in cell proliferation, opening up the possibility of designing new antineoplastic agents.[90,91a,91b] In fact, several polyamine analogues, such as those with an ethyl or benzyl substituent on the terminal amino groups, are currently employed as antitumour agents.[92,93] Furthermore, it has been reported that accumulation of the products of polyamine catabolism resulting from the uptake of polyamine analogues leads to programmed cell death.[94,95,96]

Flavin-containing PAOs catalyses the oxidation of the secondary amino group of spermine, spermidine and their acetyl derivatives. In contrast to copper containing PAOs, which act only on the primary amine groups at the ends of the molecule,[83] the site of the enzymatic attack varies depending on the source of the enzyme. Thus, animal PAOs transforms spermidine and spermine into putrescine (1,4-diaminobutane) and spermidine, respectively, plus propionaldehyde and H_2O_2. Conversely, in plants and bacteria, the enzyme acts on the other side of the secondary amino group, so that the products resulting from oxidation of spermidine and spermine are 4-aminobutyraldehyde and 3-aminopropyl-4-aminobutyraldehyde, respectively[97,58,98b] (Figure 19).

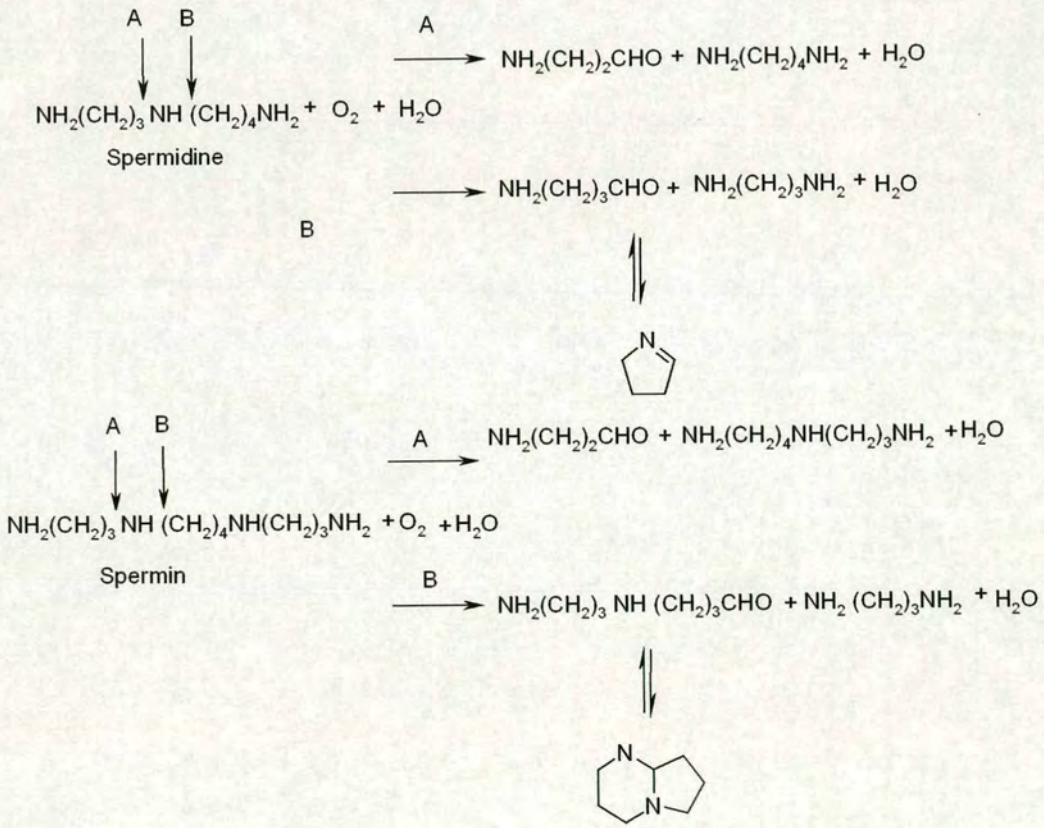


Figure 19: catalytic reaction and substrate specificity of PAO. A and B indicate the site of the enzymatic attack in the animal and plant enzyme, respectively. The plant enzyme oxidizes spermidine and spermine into 4-aminobutyraldehyde and 3-

aminopropyl-4-aminobutyraldehyde, which spontaneously cyclize to Δ^1-pyrroline and 1,5-diaza-bicyclononane, respectively.[98b]

Despite these differences, the plant, bacterial and animal proteins have essentially identical molecular properties. PAO is a monomeric soluble enzyme with a molecular weight of about 53 kDa. The prosthetic group is FAD, which is non-covalently bound to the protein.[99,58,100]

Diaminooxidases (DAOs) metabolise the diamines putrescine and cadaverine, but also deaminate histamine, hence sometimes they are called histaminases.[84] Flavin containing DAOs have been found in microorganisms *e.g. Micrococcus rubens.*[60] The highest level of DAO was found in swine kidney,[101] but also in small intestine and maternal placenta. It has been shown that the plasma of pregnant women consist of copper containing DAO, thought to be released from the placenta,[102] and which oxidatively deaminates histamine and short chain aliphatic diamines (*e.g.* putrescine and cadaverine). Flavin PAO activities were also found to be increased in the blood of pregnant women.[103] Copper DAO and flavin PAO activities have also been shown to appear in the blood plasma of pregnant rats.[104] By contrast, the level of SSAO in human plasma is not affected by pregnancy.[105] Blood plasma of pregnant women inhibits lymphocyte transformation when it is added to *in vitro* assays in the presence of spermine or spermidine.[88] This is thought to be due to the PAOs and DAOs immunoregulatory role in preventing rejection of the fetus by the mother.[106]

The final group of amine oxidases is the lysyl oxidases (LOs), which are a heterogeneous family of copper containing enzymes. LO was traditionally known as the extracellular catalyst of lysine-derived cross-links in fibrillar collagens and elastin.[107a,b] More recently it was shown that LO covers a spectrum of physiological functions such as development regulations, tumor suppression, cell motility, and cellular senescence. The LO has an intracellular, extracellular and intranuclear origin and its potential role in cell adhesion and cell growth has been suggested.[108]

1.2.2. Enantio-and stereo-selective properties of amine oxidases

Copper / TOPA amine oxidases display broad substrate specificity, exhibiting stereochemical heterogenity in the conversion of pro-chiral amines.[109] Amine oxidase from pea seed has been shown to catalyze the abstraction of the pro-*S* hydrogen from C1of benzylamine, tyramine and dopamine.[110a] Porcine plasma amine oxidase, on the other hand, is pro-*R* specific for tyramine and dopamine.[110b] Bovine plasma amine oxidase is pro-*S* specific for benzylamine, *p*-hydrohybenzylamine and 3-methylbutylamine, but is non-stereo specific for tyramine and dopamine.[110b] It has also been reported that amine oxidases from *E. coli* and the closely related enzyme isolated from *Klebsiella oxytoca,* possessed stereospecificity towards the pro-*S* hydrogen in the oxidation of pro-chiral substrates.[111b]

The enantioselectivity of amine oxidases from *E. coli* and *K. oxytoca* with respect to chiral amines have been investigated by Hacisalihoglu and co-workers.[111a] It was found that both enzymes catalyze enantioselective oxidation of amphetamine with E-values of 15. The preference for the (*R*)-enantiomer of amphetamine is in agreement with the pro-*S* specificity that has been observed for the conversion of 2-phenylethylamine. This enantioselectivity might be related to the higher potency of (*R*)-(-)- amphetamine over the (*S*)-(+)-enantiomer during the release of biogenic amines in rat brain.[112]

1.2.3. Cloning, expression and X-ray structural studies

1.2.3.1. Copper-containing amine oxidases

A number of Cu/TOPA amine oxidases have been cloned and over expressed. SSAO from murine adipocyte is a membrane associated enzyme, which has been cloned[86] and shown to have 95% amino acid sequence identity with SSAO from rat

adipocyte[113] and with the human placental amine oxidase (83% identity).[114] The level of identity is lower between murine SSAO and bovine serum amine oxidase (79% identity)[67] and human kidney diamine oxidase (35% identity),[78] especially in the N-terminal portion. This difference is because bovine serum amine oxidase and diamine oxidase are both secreted enzymes, while SSAO is membrane-associated. Cloned SSAO from adipocyte possesses activity against mono- poly- and diamines.
The cDNA of copper amine oxidase from pea seeding has been cloned[115] and expressed in *Pichia pastoris*, using the AOX1 promoter and the yeast α-factor secretion signal.[116] The 3D-structure of pea seeding amine oxidase has been solved and published.[117] The cDNA from human retina encoding copper amine oxidase was cloned and reveals the higest homology to bovine serum amine oxidase.[118] The *mao* operon of *Escherichia coli* W3350, which comprises the genes *mao-c* and *mao-a*, was cloned and appeared to be similar to that of *Klebsiella aerogenes.*[70] *E. coli* W 3350 gene that encodes aromatic amine oxidase was isolated, sequenced, and expressed in *E. coli* TG2. The purified enzyme exhibited properties of a copper/TOPA amine oxidase.[119] The 3D structure of the AO from *E. coli* W 3350 has been solved and published.[120]

Another gene encoding for copper amine oxidase has been cloned from yeast *Hansenula polymorpha*[69] and over expressed in *Saccharomyces cerevisiae*,[121] *E. coli*,[122] and the 3D structure solved.[123] Two open reading frames, *maox*I and *maox*II, which are >99% homologous, were cloned from the chromosomal library of *Arthrobacter* P1 strain and expressed in *E. coli.*[76] The gene of *Arthrobacter globiformis* encoding a quinoprotein, phenylethylamine oxidase, has also been cloned, sequenced and expressed in *E. coli.*[77] The 3D structure of the copper containing amine oxidase from this strain has been solved.[124]

All 3D structures reveal a high degree of structural similarity despite low sequence identity. An amino acid sequence alignment of 14 Cu-containing AOs identified only 33 conserved residues.[125] All listed sequencing studies showed the presence of a tetrapeptide sequence, Asn-Tyr-Asp-Tyr, which has been found to be highly conserved in other copper amine oxidases. Mutation of the Tyr-382 from the

sequence of Asn381-Tyr382-Asp383-Tyr384 of the recombinant enzyme from *Arthrobacter globiformis* into Phe resulted in the complete loss of catalytic activity and disappearance of the quinone compound that is specifically detected in the wild-type enzyme, suggesting that Tyr-382 is the precursor of the covalently-bound cofactor,[77] which is produced by the enzyme in post-translational modification. Expression of copper-containing AO from *Hansenula polymorpha* in *S. cerevisiae* has produced a functional recombinant enzyme.[121] Based on the fact, that *S. cerevisiae* is one of the few yeast species that does not have endogenous amine oxidase[126] it was concluded that the modification of the precursor tyrosine to TOPA cofactor happened in post-translational autoprocessing.[121]

1.2.3.2. Flavin-containing amine oxidases

In spite of their pharmacological importance, structural and mechanistic studies of flavin containing amine oxidases have been hindered due to the high hydrophobic properties of these enzymes. Most of the recombinant MAOs are from mammalian sources, but they are naturally tightly bound to the outer mitochondrial membrane, requiring detergent solubilization. To understand the mechanism and structure of mammalian MAOs and to develop enzymes for the deracemisation of racemic amines as discussed in this thesis, it is important to search for enzyme expression systems that produce soluble protein in large quantities.

A number of organisms have been reported to possess flavoprotein AOs, however almost all are either polyamine or diamine oxidases or have very narrow substrate specificities, *e.g.* tyramine oxidase. Amine oxidase activity was found in the mycelia of fungi belonging to *Aspergillus, Penicillum, Monascus* and *Fusarium,* grown on monoamines as the sole nitrogen source.[127] Frebort *et al.,* when trying to induce copper/TOPA amine oxidases in filamentous fungi, noted that some species possessed amine oxidase activities that were more sensitive to pargyline (flavin AO specific) than phenylhydrazine (copper AO specific), suggesting the presence of a flavin dependent amine oxidase. *Fusarium solani* TG-2 showed only one active band

that did not stain against copper amine oxidase, which clearly indicated it possessed only the flavin enzyme. *A. niger* GRR, *Penicillium chyrsogenum* AKU 3401, *Armilaria sp.*, *Trichoderma reesei*, *Monascus fuliginosus* – all of these species showed inhibition by both pargyline and phenylhydrazineine indicating that the species contained both the copper and flavin enzymes.[128] However the fungus *A. niger* is the only reported source to date which has a flavin dependent mono-amine oxidase (MAO-N).[55]

Schilling *et al.*[129] have cloned, sequence and expressed MAO-N from *A. niger* in *E. coli*. To generate a cDNA library, mRNA was isolated from culture of *A. niger* that had been induced with butylamine at concentration 0.1% (at 0.25% butylamine is toxic to the fungus).[55] The deduced primary amino acid sequence of MAO-N was subjected to sequence alignment with MAO-B and MAO-A. All three N-termini have the β–α–β protein fold necessary for binding the ADP moiety of flavin dinucleotide.[130] However, unlike the mammalian enzymes, MAO-N does not contain the highly conserved C- terminus pentapeptide, Ser-Gly-Gly-Cys-Tyr, responsible for the covalent binding of FAD.[131,132]

The MAO-A and MAO-B enzymes have an extended hydrophobic C-terminus compared to MAO-N, which is thought to be required for binding to the outer mitochondrial membrane. The lack of a membrane binding sequence in MAO-N should provide improved solubility, which may aid crystallisation studies.

MAO-N and MAO-A shares 25.5% sequence identity (49.1% similarity) and MAO-N and MAO-B have 23.7% identity (48.7% similarity) (Figure 20).[129] However, the highly conserved (95% identity) region proposed as an active site sequence in mammalian MAOs, is absent in MAO-N. MAO-N contains a C-terminal tripeptide Ala- Arg- Leu , which corresponds to the peroxisomal targeting signal found in many enzymes located in these organelles.[133]

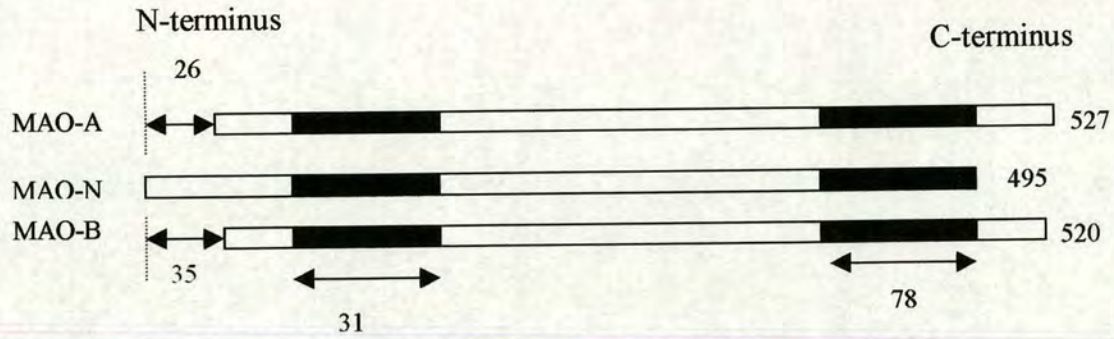


Figure 20: comparision of monoamine oxidases from mammalian and fungal sources (areas of high sequence identity/similarity are shown in bold).

Most of the studies have been carried out on MAO- A and B from human, rat and ox. The cDNA clones encoding for MAO-A and MAO-B were isolated from human liver. The molecular weight was determined as 59.7 kDa for MAO-A and 58.8 for MAO-B.[134] Comparison of the deduced amino acid sequences show 70 % sequence identity. Both sequences contain the pentapeptide Ser-Gly-Gly-Cys-Tyr localized in the C-terminal region where the cofactor FAD covalently binds to cysteine.[134]

The primary sequences of bovine MAO-B and MAO-A have been determined by cDNA cloning.[135] Both enzyme sequences possess an overall similarity of 41% and have >90% similarity for the flavin binding region of other enzymes.[135] The next MAO cDNA cloned was from rat liver and was of type B[136] and type A.[137] The latter report revealed further sequence similarity between different type of flavin monoamine oxidases from different sources. MAO-A from rat exhibited greater than 85% sequence identity with bovine and human MAO-A and 70% identity with rat MAO-B.[137]

Both mammalian MAO-A and MAO-B have some features in common. They are both homodimers and the molecular mass of both mammalian MAO-A and MAO-B is about 60 kDa. The prosthetic group of both enzymes from a variety of mammalian sources is 8α-S-cysteinyl FAD and the amino acid sequence around the flavin site is

Ser-Gly-Gly-cysteinyl FAD-Tyr. The flavin binding site is in the COOH-terminal region. There is a non-cleavable targeting signal in the 29 residue at the C-terminal end which directs the enzyme to the outer membrane, where it is anchored.[138]

Several attempts have been made to express human liver MAO-B in mammalian cell lines[139] but at very low yield. Shih and co-workers attempted expression in *E. coli*[140] but obtained far too low quantities of functional form. Both human MAO-A and MAO-B have been expressed in yeast[141] and mammalian cells,[142] but again with a low yield of the protein. The heterologous high-level expression of human liver MAO-A in *S. cerevisiae* was reported by Weyler and co-workers.[143] This recombinant system have been used in a number of mechanistic studies on recombinant human liver MAO-A.[144,145]

Attempts to over express human liver MAO-B in the same *S. cerevisiae* system have been unsuccessful despite the fact that both enzymes have 70% sequence identity. MAO-B was successfully expressed in methylotrophic yeast *Pichia pastoris* using the AOX1 promoter and the yeast α-factor secretion signal.[146] Approximately 200 mg of purified protein was obtained from a 2L fermentation culture. This enzyme exhibits many of the kinetic and structural properties of the well studied bovine enzyme[147] and was subjected to structural studies by X-ray crystallography. The crystal structure was solved to a resolution of 3.0Å by Binda and co-workers.[148] A search of the Protein Data Bank[149] reveals that the closest structural matches of MAO-B are L-amino acid oxidase[150] and polyamine oxidase.[98a]

MAO-A and MAO-B cDNAs were also obtained from some other sources. Chen *et al.*[151] cloned a trout liver MAO by screening a cDNA library with a human MAO-A cDNA probe. The deduced amino acid sequence of trout MAO showed 70% identity to human MAO-A and 71% identity to human MAO-B. This enzyme contains FAD covalently bound to the pentapeptide Ser-Gly-Gly-Cys-Tyr as with other MAOs listed above. Trout MAO displays substrate and inhibitor selectivities that are not identical to those of either MAO-A or MAO-B, so it represents a novel type of MAO.

There was an attempt to clone MAO from carp liver and muscle, but MAO-related sequences were not detected by the PCR method using PCR primers designed for rat MAO-A, MAO-B and trout MAO cDNAs. This indicates low homology of amino acid sequences among rat, trout and carp MAOs.[152]

Very recently, the gene for human PAO has been identified[89] and the protein further studied. Human PAO shares the sensitivity to the specific mammalian PAO inhibitor MDL 72,527, but is not sensitive to pargyline (MAO inhibitor) or to semicarbazide (copper/TOPA AO inhibitor).

Finally, the cDNA clone of maize polyamine oxidase was isolated from maize (*Zea mays* L. seeds) and characterised. The determination of the primary structure of maize PAO has revealed that the enzyme contains a Gly-X-Gly-X-X-Gly sequence characteristic of the residues forming the FAD binding site.[59,132] The absence of a Cys residue required for covalent binding of FAD in vertebrate MAOs confirms a non-covalent binding of the flavin,[153] reported also for MAO-N.[129]

Maize PAO is clearly homologous (sequence identity of about 20-30%) to vertebrate monoamine oxidase. PAO and MAO differ in their substrate specificity, since PAO oxidizes secondary amines whereas monoamine oxidase acts on primary amino groups. However, the sequence similarity indicates that the two enzymes form a group of related flavin-dependent amine oxidases, which are likely to share a similar mechanism of catalysis. Maize PAO was produced and purified from the natural source in quantities of 10mg per kilogram of plant cells[154] and subjected to X-ray crystallographic studies. The 3-D structure has been solved to 1.9Å resolution[98a] revealing structural similarity to L-amino acid oxidase from snake *Calloselasma rhodostoma* venom [150] and to human MAO-B.[148]

1.2.4. Mechanism of catalysis by AOs

1.2.4.1. Copper-containing AOs

Copper amine oxidases are homodimeric enzymes that catalyze two reactions: firstly, a self-processing reaction to generate the 2,4,5-trihydroxyphenylalanine quinone (TPQ) cofactor from an active site tyrosine by a single turnover mechanism; secondly, the oxidative deamination of primary amine substrates with the production of the corresponding aldehyde, hydrogen peroxide, and ammonia catalyzed by the mature enzyme. The importance of active site residues in both of these processes has been investigated by structural studies and site-directed mutagenesis of enzymes from various organisms. [117,120,123,124]

The TPQ is post-translationally synthesized by a single turnover, copper- and oxygen-dependent, processing event of an active site tyrosine residue.[155a] The Cu(II) atom is coordinated by three histidine imidazole groups and two water molecules, the Cu(II) atom has a distorted square-pyramidal geometry, and the TPQ cofactor is close to the Cu(II) atom but not coordinated to it. (Figure 21).

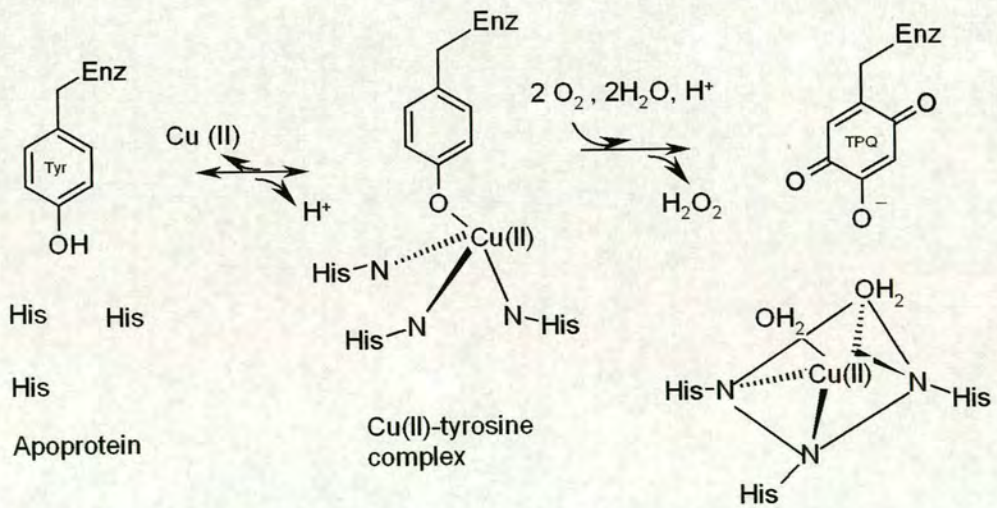


Figure 21: the formation of the cofactor TPQ.[155b]

Kinetic and spectroscopic studies have revealed that the unmodified (tyrosine-containing) apoprotein requires only copper and oxygen for stoichiometric TPQ formation. A preformed Cu(II) complex was shown to be competent for TPQ formation upon the addition of O_2[156] (Figure 22).

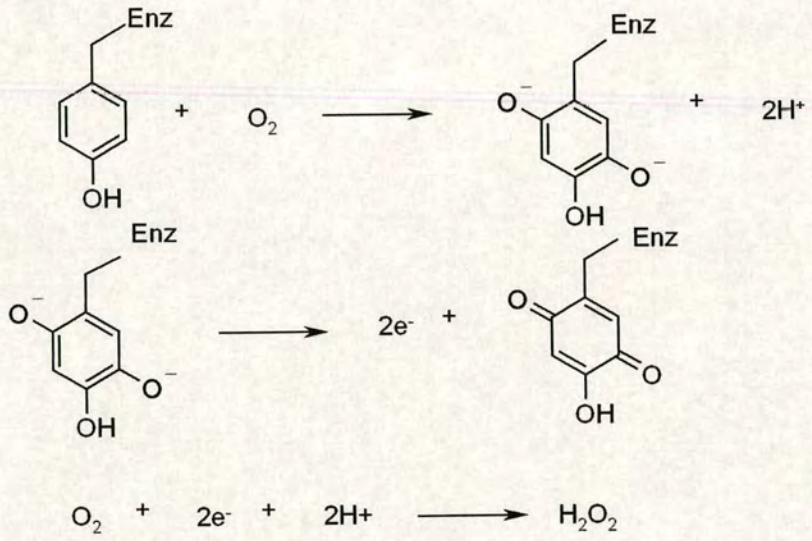


Figure 22: oxidation of tyrosine residue to TPQ cofactor.[124]

As a result of the availability of X-ray crystal structures, the catalytic mechanism of copper AO is well understood (Figure 23).

The first step is formation of the enzyme bound imine (I) from the primary amine and the carbonyl group of TPQ. The second step is hydrogen abstraction from the methylene group to generate (II) followed by tautomerisation and subsequent hydrolysis to the aldehyde (III). Reoxidation of TPQ by molecular oxygen then takes place to yield (IV), which undergoes hydrolysis to TPQ liberating hydrogen peroxide and ammonia by-products (V).

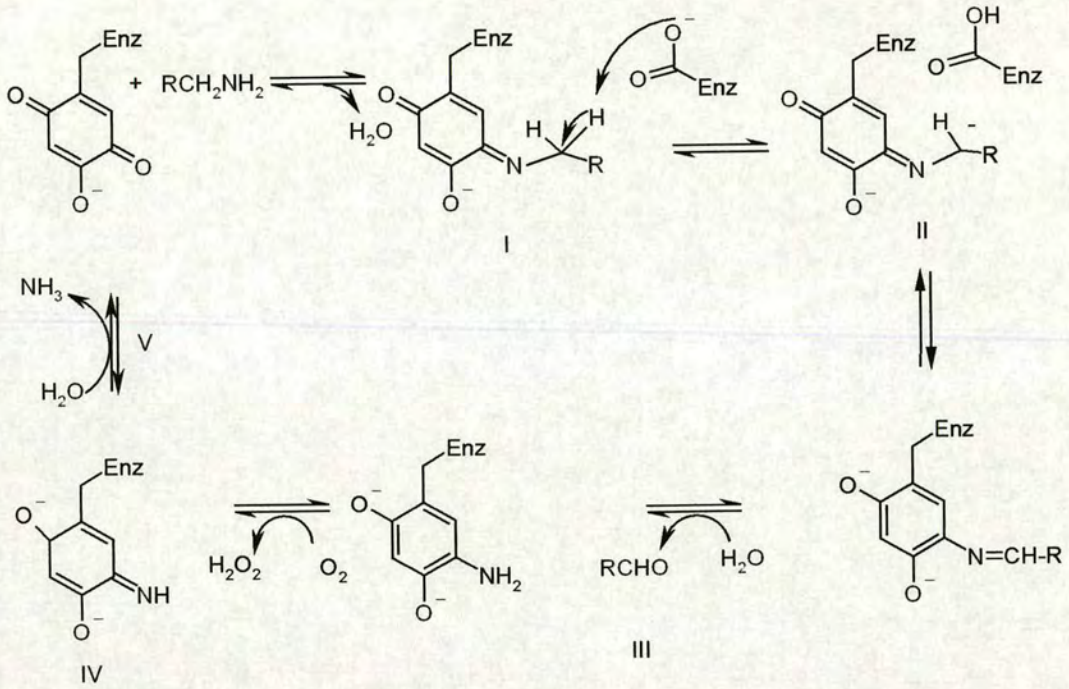


Figure 23: catalytic mechanism of copper AOs.

There is evidence in the literature that the copper/TOPA AO from *E. coli* and *K. oxytoca* carry out stereoselective and enantioselective transformations.[110a,111a] However, the copper AOs are not suitable enzymes for the deracemisation of amines, since the intermediate imine is not released from the active site.

1.2.4.2. Flavin-containing AOs

In 1995 the first direct evidence of an imine product from the reaction of an amine with MAO-B was reported.[157,158] Since then there has been much debate concerning the mechanism of imine intermediate production during the catalytic cycle.

The first mechanism was proposed by Silverman[159] and involves an initial single electron transfer (SET) from the nitrogen atom of amine (I) to N5 of the oxidized flavin cofactor (II) yielding an aminium radical cation (III) and flavin semiquinone

radical (IV). Subsequent deprotonation of (III) by an active side base residue would generate the iminium species (VI) and the reduced FAD (V) (Figure 24).[160]

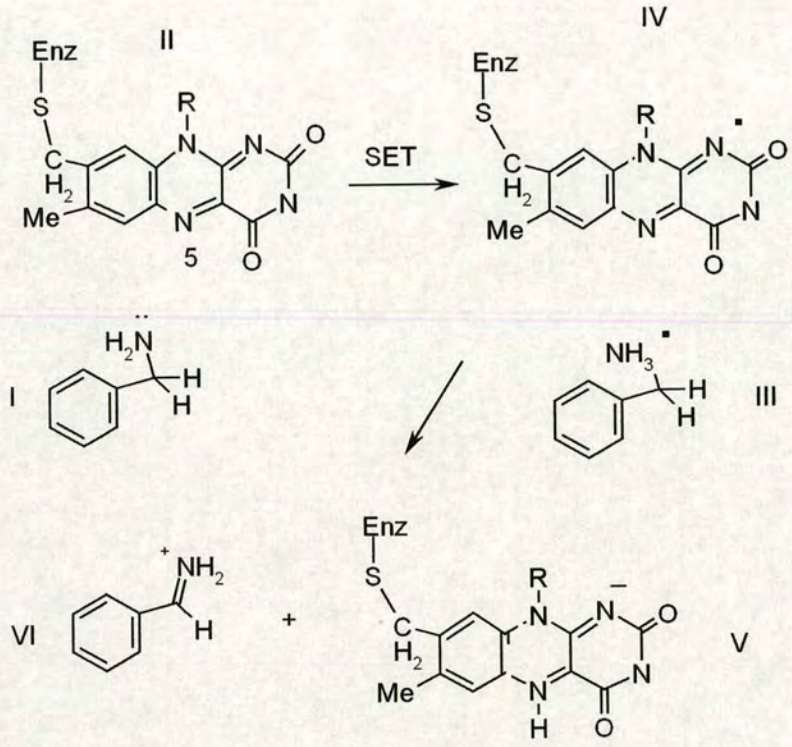


Figure 24: single electron transfer (SET) mechanism.[160]

Although this mechanism is mechanisticaly attractive, to date there is no evidence for any flavin radical intermediate through stopped flow studies, nor a characteristic peak for a free radical in the electron paramagnetic resonance (epr) spectrum.

A polar nucleophilic mechanism (also called the hydrogen abstraction mechanism: HAT) was originally suggested by Hamilton and co-workers.[161] Catalysis is proposed to occur initially via nucleophilic attack of the amine (I) onto the 4a position of flavin (II). Subsequent proton abstraction from the carbon atom of the amine-flavin adduct (III) is proposed to be facilitated by an active site base on the enzyme (polar nucleophilic mechanism) or directly by N5 of the flavin (concerted polar nucleophilic mechanisn, CPN)(Figure 25).

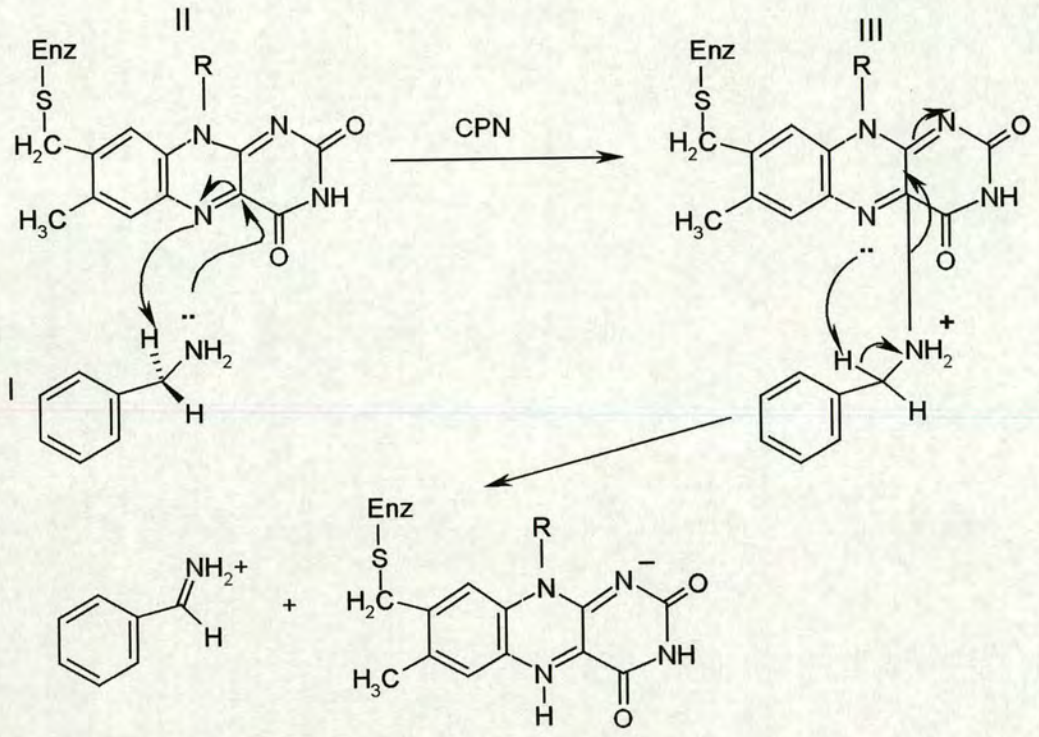


Figure 25: concerted polar nucleophilic mechanism for MAO catalysis.[145]

In this CPN mechanism, the formation of the 4a-alkylated isoalloxazine ring in a nucleophilic manner leads to a very strong base at N5 of the flavin with a pK_a of ~30 which is close to the pK_a of benzyl protons. Thus, a concerted transfer of the benzyl proton to N5 of the 4a-alkyl flavin should be facilitated. Following this α-C-H bond cleavage reaction, production of the protonated imine product will occur and the reduced flavin cofactor is reoxidized by O_2. Although the structural data [148] do not provide evidence for a CPN (or HAT) mechanism, recent quantitative structure-activity relationships (QSAR) data on MAO-A with a series of *para*-substituted benzylamine analogs[145] provides definitive evidence that α-C-H bond cleavage does occur by a proton abstraction mechanism. Current structural data on MAO-B[148] and PAO[98a] also show there to be no amino acid residue at the catalytic site that would be able to act as an active site base for any proposed H^+ abstraction mechanisms.

Flavin AOs are potentially suitable enzymes for deracemisation since the imine intermediate is not covalently bound to the cofactor, thereby allowing reduction back to the amine. Furthermore, there is some evidence that MAO-B shows stereospecific removal of the pro-*R* hydrogen for certain substrates.[159]

1.3. Enzyme directed evolution

Directed evolution of enzymes *in vitro*, also called molecular evolution, works by mimicking the process of natural "irrational" evolution (random mutation, recombination and selection). Directed evolution starts from a parental enzyme and involves random mutagenesis and screening offering a fast and effective way to generate new "improved" enzymes under artificial evolutionary conditions. Conditions for random mutagenesis can be created in a thermocycler using polymerase chain reaction (PCR), by treatment with chemicals such as nitrous acid, hydroxylamine, formic acid, and hydrazine, or by transformation of the gene into a bacterial mutator strain. To isolate the mutated enzyme with desired properties, the library of variants must be subjected to a high through put screening assay to assess the desired activity.

1.3.1 Construction of the libraries

Random mutagenesis makes it possible to generate a large number of randomly distributed nucleotide substitution mutations in a cloned DNA fragment. Various methods for constructing the libraries of mutants are now well established and are described below.

1.3.1.1. Random point mutagenesis

1) Random point mutagenesis in vitro using PCR.

In PCR, a DNA sample is separated into single strands and incubated with a cocktail of DNA polymerase, reaction buffer, dNTP's and two nucleotide primers whose sequences flank the DNA segment of interest.[162] The primers direct the DNA polymerase to convergentely synthesize complementary strands of the target DNA. Twenty cycles of PCR increases the number of copies of the target sequence by about a million fold with high fidelity (Figure 26).

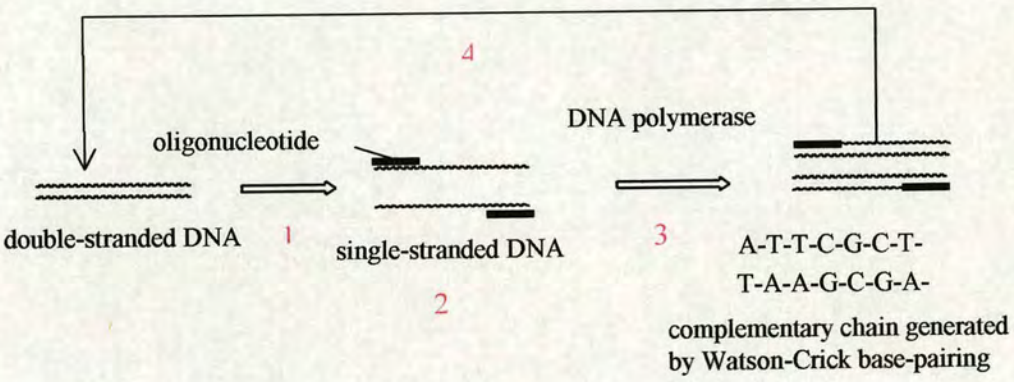


Figure 26: polymerase chain reaction (PCR) (1)- denaturation, 95° C; (2)- oligonucleotide annealing, ca. 50°C; (3)- elongation by DNA polymerase; (4)- DNA amplification by multiple PCR cycles.

Error prone PCR (epPCR) was developed by a modification of the standard procedure to decrease the fidelity of the PCR reaction by increasing the concentration of $MgCl_2$, adding $MnCl_2$, or unbalancing the concentration of the four dNTPs.[163,164] By changing the reaction conditions it is possible to introduce mutations at the rate of up to seven base pairs per 1000 during 30 PCR cycles. A low error rate (2–3 base substitutions or ~1 amino acid substitution per sequence per generation) accumulates beneficial mutations, whereas higher error rates tend to generate neutral and deleterious mutations, more frequently than beneficial ones. Beneficial mutations in multiple variants can be combined using recombination

methods such as Stemmer's DNA shuffling[165] or StEP (staggered extension process).[166] Recombination also removes neutral and deleterious mutations. The most common mutations are transitions (purine ↔ purine, pyrimidine ↔ pyrimidine) and the least common are transversions (purine ↔ pyrimidine). The method is not sufficient for small gene fragments (<1000 bp)[163]. epPCR can be optimised to introduce transitions (A ↔ G) and transversions (purine ↔ pyrimidine) at a similar level.[167]

Usually, optimal results are not obtained after one round of a mutagenesis cycle.[169] However, sequential error-prone PCR, where the output of one cycle is used as input for the next cycle, can accumulate greater level of mutation thereby increasing the opportunities to produce a beneficial mutant.

ii) Site-directed random mutagenesis.

Saturation mutagenesis. Saturation mutagenesis can be performed by overlap extension PCR.[168] In this method, two DNA fragments having overlapping ends are synthesized by PCR and combined in a subsequent fusion PCR reaction in which the overlapping ends anneal. The 3' overlap of each strand serves as a primer for the 3' extension of the complementary strand. The resulting fusion product is amplified further by PCR with template forward/reverse external primers (Figure 27). Specific alterations in the nucleotide (nt) sequence can be introduced into the overlapping oligo primers. Screening of mutant clones revealed at least a 98% efficiency of mutagenesis .The number of variants (N) in which one amino acid per enzyme is replaced by one of the remaining 19 amino acids can be calculated by the equation:

$$N = 19^{M}X! / [(X - M)!M!]$$

where X is the total number of amino acids per enzyme and M is the number of substituted amino acids.[169] The frequency of random nucleotide substitution has been estimated as approximately 1 in 4000 nt.[168] This method resulted in a significant improvement over standard methods of site-directed mutagenesis because it is much

faster, simpler and approaches 100% efficiency in the generation of mutated products.

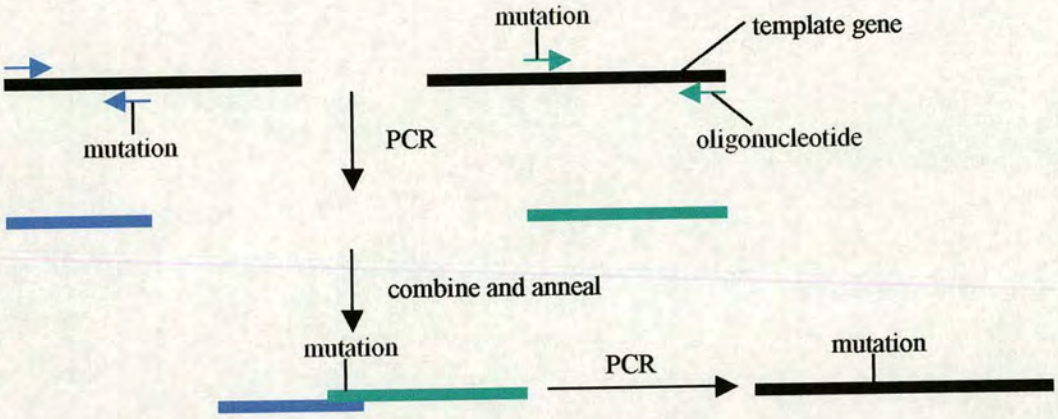


Figure 27: overlap extension PCR.[168]

Saturation mutagenesis has been applied to generate beneficial point mutations and also to explore active site "hot spot" sequences.

iii) Random point mutagenesis in vivo.

The alternative to *in vitro* PCR-based point mutagenesis is point mutagenesis *in vivo*. The latter method can be performed using the *E. coli* XL1-Red mutator strain.[170] The engineered genotype consists of mutations that inactivate three independent DNA repair pathways, namely in the loci *mut*S,[171] *mut*T,[172] and *mut*D.[173]

The *mut*S gene encodes for the MutS protein that recognises and binds to almost all base pair mismatches (exept C/C) despite their structural differences. MutS belongs to the methyl-directed MutHLS pathway in *E. coli* which corrects mismatches arising during replication (Figure 28). MutS initiates repair by binding to the mismatch (1) MutH endonuclease binds to hemimethylated *dam* sites (methylated adenines on 5'-GATC-3' sequence). MutH endonuclease activity is stimulated by MutS and MutL in the presence of ATP (2) MutH incises the newly synthesised unmethylated strand. The nicked unmethylated strand is degraded by 5'3' and 3'5' exonucleases followed

by resynthesis performed by DNA polymerase III and the remaining nick is sealed by a ligase (3).[174]

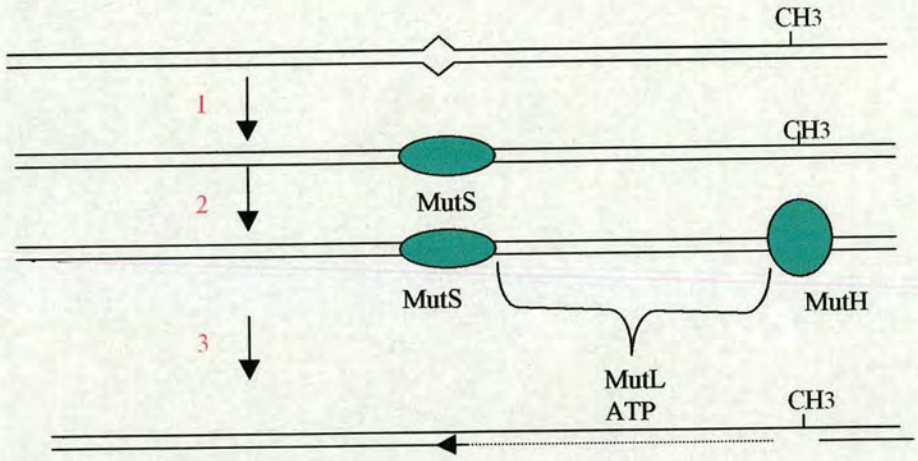


Figure 28: methyl-directed mismatch correction system in *E. coli*.[174]

The *mut*T gene encodes the MutT nucleoside triphosphatase, which hydrolyses 8-oxodGTP, produced by active oxygen species (superoxide, hydrogen peroxide and hydroxyl radical) generated as by-products of normal aerobic metabolism. 8-oxodGTP is hydrolysed to 8-oxodGMP in the nucleotide pool by MutT before it can be misincorporated opposite template adenines. A strain that lacks active MutT protein has elevated levels AT→GC transversions.[175]

Mutation of the gene encoding MutD (*dnaQ*) causes a lack of the proofreading and self-editing function of DNA polymerase III. The *dna*Q gene encodes a subunit of DNA polymerase III, which carries out a 3'→ 5' exonuclease (proofreading) function to remove incorrectly paired nucleotides during DNA replication. It was estimated that introduction of these three mutations into the wild type *E. coli* XL1-Red could increase the spontaneous mutation rate by approximately 5000 fold.[170] Greener *et al.*,[176] evaluated the random mutation rate after 30 generations and found that the spontaneous mutation rate on a pBluescript plasmid was 1 mutation per 2kb of cloned DNA after 30 generations of growth. The advantage of using XL1-Red for random mutagenesis, compared with chemical mutagenesis or a PCR based protocol,

is that the mutation rate can be carefully controlled. However a major disadvantage of the XL1-Red mutator strain is its genetic instability.

In contrast to XL1-Red mutator strains, a genetically stable *E. coli* mutator strain was engineered.[177] The target gene was propagated on a ColE1-type plasmid which uses DNA polymerase I for the initiation of replication. After replication of the initial 1kb of the plasmid insertion, polymerase I is replaced by polymerase III which completes the replication. The *E. coli* mutator strain expressed a mutated polymerase I with negligible proofreading activity, and the MutHLS repair system was disabled. As polymerase I replicates only 1% of the host chromosome, the target gene was mutagenized with minimal effect upon the genotype and phenotype. This makes it possible to produce 150 cell generations such that only ~10% of the cells in the culture become mutated. The limitation of this method is that the library diversity is limited by the low mutation rate and by the restriction that only 1 kb can be mutated.

Both methods (error-prone PCR and using a mutator strain) have their pros and cons. By using the mutator strain, mutations are not only introduced within the gene of interest, but may also occur on the entire plasmid. Some clones may contain defect in the protein expression sequence and, as a consequence, not all clones may produce the enzyme of interest. On the other hand, this method is much more simple compared to error-prone PCR. Bornscheuer and co-worker[178] reported serious problems in finding appropriate conditions for PCR itself as well as for the efficient ligation of PCR products.

Finally, the outcome of random mutagenesis for directed evolution depends highly on the mutation rate. A low mutation rate requires the screening of large enzyme libraries (because only a few clones bear mutations), but too high a mutation rate might lead to an increasing number of inactive enzyme variants. Bornscheuer and co-worker[178] have found with both methods that single mutations per gene is the optimum range of mutation rates to produce a beneficial mutation.

Here is a summary of the methods to produce point mutations in genes (Table 1).

Point mutations	*Advantage/Disadvantage*	*Nature of mutations*	*Mutations rate*
E. coli XL1-Red[170]	No special equipment required, fast, minimum optimisation required, no dependence on ligation-transformation efficiency, large gene size is applicable, easy to control a mutation rate. The presence of high population of wild type clones requires a very high throughput screening.	The most common T→ C; C→ T; G→ A, Least common A→ G; T→ G; G→ T; C→ G; G→ C	1 per 2kb per 30 cycles of growth
Error-prone PCR[163, 164]	Mutation rate optimisation required, ligation-transformation conditions optimisation required, 1kb size is optimum.	The most common mutations are A→ T, T→A and the least common are G →C, C→G	Up to 7 mutations per 1kb per 30 PCR cycles

Table 1: the methods for producing point mutations in genes.

1.3.1.2. In vitro methods for gene recombination

i). Recombination of genes with high DNA homology

DNA shuffling. This method was developed by Stemmer *et al.*[165,179] as a random *in vitro* DNA recombination method, enabling molecular mixing of randomly mutated genes. It involves random fragmentation of several mutated DNAs followed by their reassembly by PCR into full length molecules and introduces additional point mutations at a very low rate. The randomly mutated gene is digested with DNase1 to a pool of random DNA fragments followed by denaturation, self-priming and reassembly of the full-length gene by repeated PCR. The final library of full-length

recombinant DNA sequences is obtained by PCR amplification with forward and reverse oligonucleotides. The possible combination of the original mutations in the parental genes produces considerable molecular diversity and maximal combination of mutations to yield mutated enzymes. DNA shuffling allows the accumulation of beneficial point mutations by combining mutations resulting in two or more improved properties that have been evolved separately.[180]

Hybrid enzyme technique. Diverse gene libraries for laboratory evolution can be created by recombination of related genes.[181] This approach generates highly diverse sequences, but conserves function. Improved or altered enzymes can be identified by screening such hybrid protein libraries.[181] Genes from multiple parents, and even from different species with sequence similarity, can be shuffled in a single step.

The hybrid enzyme technique is similar to DNA shuffling and also has been termed molecular breeding by DNA shuffling or gene family shuffling (Figure 29). The method involves digesting target genes with DNase1 to a pool of random DNA fragments (1) followed by denaturing, self-priming and reassembling full -length genes by repeated PCR (2,3,4,5). The final library of full-length recombinant DNA sequences is obtained by PCR amplification with forward and reverse oligonucleotides.

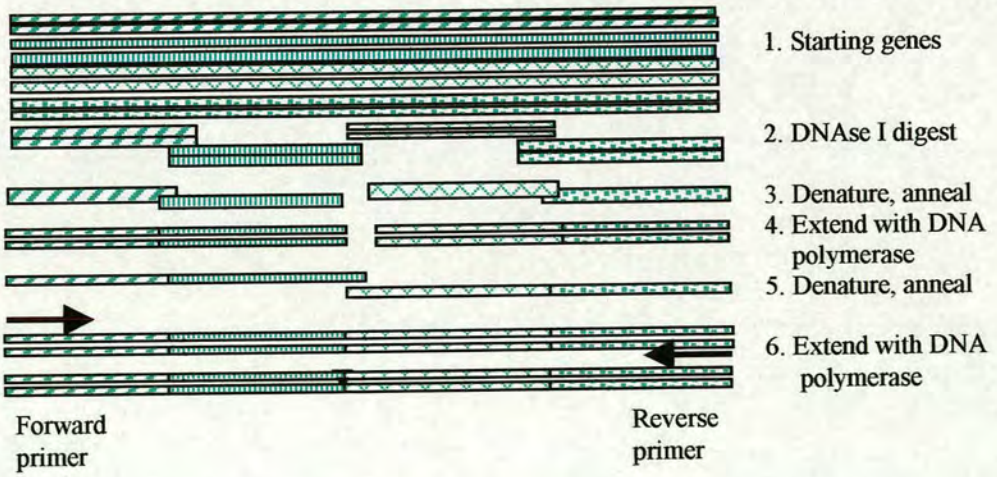


Figure 29: molecular breeding by DNA shuffling.

DNA shuffling is used widely to generate highly improved biocatalysts with features not present in the parent enzymes and not known in nature.[182,183]

Random-priming *in vitro* recombination (RPR).[184] This approach is an effective alternative to DNA shuffling. The principle of this technique is that a single-stranded DNA serves as a template for random priming DNA synthesis in PCR amplification. The primers used in the reaction are: (i) primers with random sequence and (ii) primers that are complementary to different sections of the template sequences. These primers are added to generate a large number of short DNA fragments. Due to base misincorporation and mispriming, these short DNA fragments also contain a low level of point mutations. The short DNA fragments can prime one another by homology, recombine and reassemble into full-length genes by a PCR-like technique. These sequences can be further amplified by PCR and cloned into an expression vector followed by screening or selection. RPR and screening or selection can be repeated using multiple cycles (Figure 30).

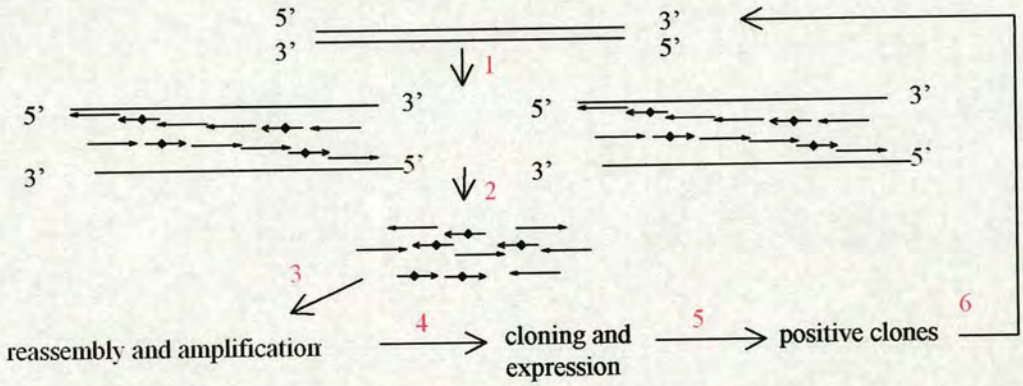


Figure 30: random-priming *in vitro* recombination (RPR). (1) random-priming synthesis; (2) template removal; (3) short fragments reassembly based on homology and amplification; (4) PCR with forward and reverse primers, subcloning and expression in suitable host; (5) screening for positive clones; (6) repeat cycle.[184]

RPR may allow access to a greater range of amino acid substitutions than error-prone PCR. In comparison to DNA shuffling, the RPR technique has several advantages:

(i) RPR can use single stranded polynucleotide templates without an intermediate step needed to synthesize the entire second strand. Potential mutations or crossovers can be introduced at the DNA level from single- or double-stranded DNA templates by using DNA polymerase, or directly from mRNA by using RNA-dependent DNA polymerase;

(ii) DNA shuffling requires fragmentation of the double-stranded DNA template (generally with DNase I), followed by complete removal of DNAse before the fragments can be reassembled into full length sequences;

(iii) Synthetic random primers have the same length and lack sequence bias. Every nucleotide of the template is copied or mutated at a similar frequency during extenuation;

(iv) RPR DNA synthesis is independent on the length of the DNA template.

Staggered extension process (StEP).[166] This method is based on a PCR-like reaction with very short annealing and extension steps. StEP consists of priming the template sequences followed by very short repeated cycles of denaturation and extremely abbreviated annealing/polymerase-catalyzed extention (Figure 31).

In each cycle the growing fragments can randomly anneal to different parental templates based on sequence complementarity and extend further to create recombinant sequences. Due to the template switching, the growing polynucleotides contain sequence information from different parental genes. StEP is continued until full-length genes are formed. It can be followed by a gene amplification step, if desired. The whole process can be performed with standard forward/reverse universal primers in a single tube.

This method has an advantage over DNA shuffling. Separation of parent templates from recombined products is unnecessary and there is no need to fragment and reassemble the genes. StEP recombination method can be applied for directed evolution of genes, operons, pathways and even whole bacterial/viral genomes.

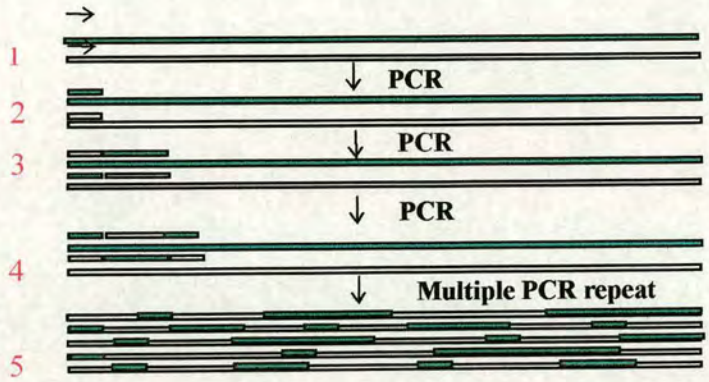


Figure 31: StEP recombination. Only one primer and single strands from two parent genes (templates) are shown. (1). Denatured templates are primed with one defined primer. (2) Short fragments are produced by brief polymerase-catalyzed primer extention. (3) Through another cycle of StEP, fragments randomly prime the templates (template switching) and extend further. (4) This process is repeated until full-length genes are produced. (5) Full-length genes are purified and (optionally) amplified in a PCR with external primers.

In vitro exon shuffling. Since exons represent only 1% of the human genome and introns about 24%, most of the crossovers occur between exons, rather than inside of them. The natural process of creating new combinations of exons by intronic recombination is called exon shuffling. Exon shuffling *in vitro,* which is similar to DNA shuffling, is an important tool for mutagenising and recombining much longer DNA sequences, especially eukaryotic genes. Exon shuffling is a natural way to create novel proteins from combinations of exons by homologous recombination inside the introns. This differs from DNA shuffling, where the recombination happens within all of the gene fragments. Recombination within introns is able to assemble independent exons into genes for novel proteins.[185] In this method, exons that encode domains are amplified using mixtures of chimeric oligonucleotides, thereby determining which exons are spliced together. The mixture of these PCR fragments are used as primers and templates in self-priming overlap extension PCR into the full-length gene.[186] Recombination occurs when an exon from one gene is connected to an exon from a different gene.

New methodological developments. Over the past two years there have been several approaches to improve *in vitro* recombination efficiency. *In vitro* recombination by DNA shuffling[179] creates libraries of chimeric genes with a strong tendency for reconstitution of parental structures by PCR-based reassembly methods. Priming among fragments of the same gene is favored and is known to decrease the number of crossovers and increase the proportion of non-chimeric parental structures in a recombinant library.

To decrease the contamination by parental structures, combinatorial libraries enhanced by recombination in yeast (CLERY) was developed.[187] This strategy for family shuffling in yeast expression vectors takes advantage of the unique properties of homologous recombination in yeast.

The classical Stemmer DNA shuffling step is followed by secondary shuffling obtained by *in vivo* recombination in yeast. Overall, this shuffling strategy allows direct expression and functional selection in eukaryotic cells without the need for intermediate cloning steps in *E. coli.* This procedure is most suitable for shuffling eukaryotic genes that cannot be expressed in bacteria. The statistical analysis of randomly selected clones reveals a high proportion of chimeric genes (86%) and around 4.4 crossovers per gene.

Random chimeragenesis on transient templates (RACHITT) has been developed by Coco *et al.*[188] The approach relies on the ordering, trimming, and joining of randomly cleaved parental DNA fragments annealed to a transient polynucleotide scaffold (Figure 32).

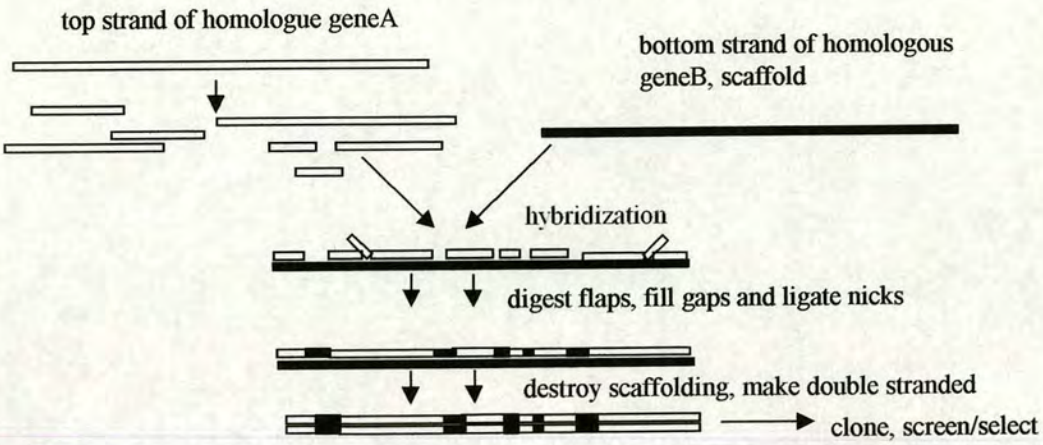


Figure 32: random chimeragenesis on transient templates (RACHITT).[188]

The genes A and gene B have 89.9% identity (differing by 127 nucleotides). Gene B was made as a single-strand to serve as a transient scaffold template (black bar). This molecule mediates the ordering of gene A top-strand fragments (white bars) and serves as a template for the filling of gaps. After hybrydization of the DNase I digested top strand of gene A, unhybridized 5' termini or "flaps" were cleaved by the flap endonuclease activity of Taq DNA polymerase. Ligation continued in a reaction in which 3' flaps were digested by the 3'-5' exonuclease activity of Pfu DNA polymerase, which also serves to fill the gaps.

RACHITT exploits a bottom-strand template from only one parent and only top-strand fragments of other parents. This prevents parental fragments from reannealing to their own complementary strands. The authors generated chimeric libraries with an average of 14 crossovers per gene, several fold higher than observed for other methods. 29% of crossovers occurred in the region of 10 or fewer bases of sequence identity, which is similar to Stemmer's approach (32%).[179] No unshuffled parental clones or duplicated "siblings" chimeras were detected.

Family shuffling using single-stranded DNA (ssDNA).[189] In order to improve the efficiency of hybrid formation in family shuffling, ssDNAs with 80% sequence identity have been used as templates. The only difference between the methods using

ssDNA and dsDNA was the template DNA for DNAse I fragmentation. The ssDNA preparation requires the genes to be cloned either in a phagemid vector or in a ssDNA phage such as M13, and ssDNA has to be prepared from filamentous phage particles. Although shuffling with ssDNAs requires one more step, it was worthwhile since chimeric genes were formed much more efficiently (chimerization rate 14%) than by shuffling with dsDNA (chimerisation rate <1%).

ii) Recombination of genes with low DNA homology.

All *in vitro* methods of gene recombination based on PCR are dependent on a stochastic process that reassembles homologous genes. All the available methods for DNA shuffling require high sequence similarity for recombination (>~60%). However, in shuffling parent sequences of lower identity or even with no sequence homology. When the level of similarity between the genes is too low for effective DNA shuffling by PCR, hybrid enzymes can be created by domain swapping *in vitro*.

Recently Sieber *et al.*,[190] introduced SHIPREC (sequence homology independent protein recombination). To maximize the abundance of functional hybrids in a library made by recombining two homologous genes of low sequence identity, the chimeras were selected by size to retain the length of the parent genes. SHIPREC therefore combines the N-terminal fragment of the parent protein with the appropriate C-terminal fragment and, in principle, makes all such possible combinations (Figure33).

(1) Two genes are connected to form a gene dimer through a linker sequence containing multiple restriction sites. (2) Blunt ended fragments are generated from the dimer by random fragmentation and nuclease treatment. (3) Fragments of the correct size are selected from the pool. (4) Selected fragments are circularised by blunt end ligation. (5) Circular DNA pieces are linearized by restriction digestion in the linker region to create a library of chimeric genes. (6) The chimeric genes are subcloned and expressed in a suitable host.

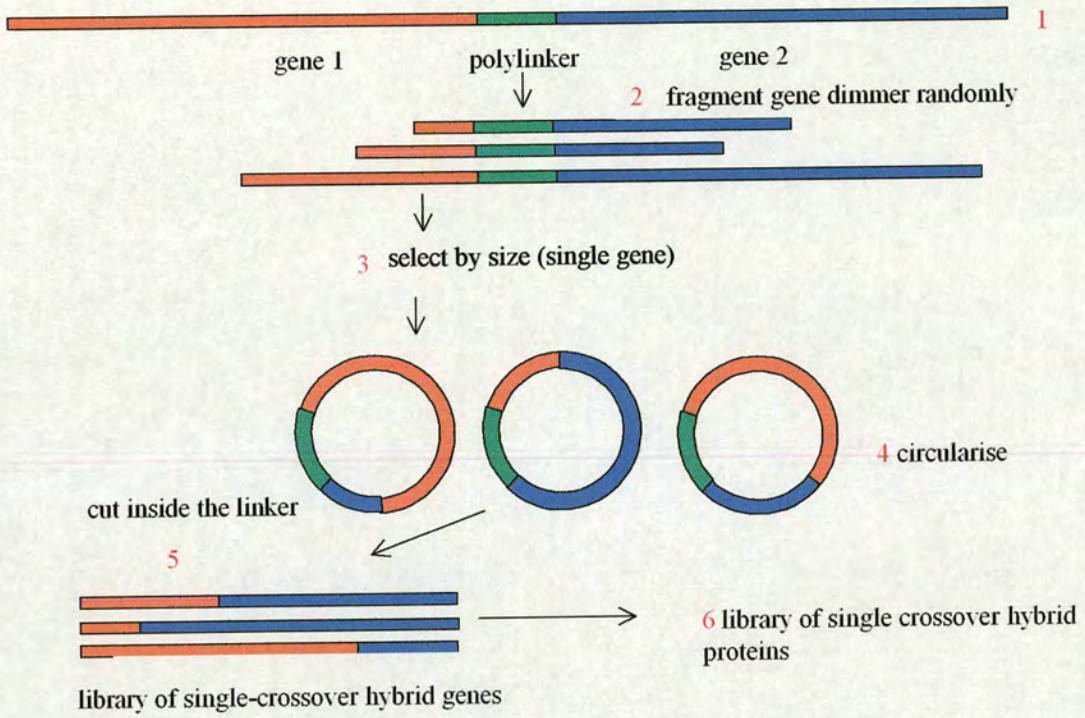


Figure 33: SHIPREC (sequence homology independent protein recombination).[190]

Incremental truncation for the creation of hybrid libraries (ITCHY).[191] In contrast to DNA shuffling and related methods, ITCHY does not rely on the parental genes containing regions of DNA sequence homology to create crossovers. The protein chimerization is driven by restriction enzyme fragmentation. A key step in the creation of these libraries is the digestion of the parental genes with 3' to 5' exonuclease (*e.g.*, Exonuclease III, Exo III) under conditions (*e.g.*, low temperature or in the presence of NaCl) which control the rate to ~10 bases/min or less. During Exo III digestion, small aliquots are removed frequently and quenched by addition to a low pH, high salt buffer. Theoretically, every single base deletion of the two gene fragments can be collected. Blunt ends are prepared by treatment with single-strand nuclease. Fusion of the truncated gene fragments by blunt end ligation then generates the ITCHY library (Figure 34).

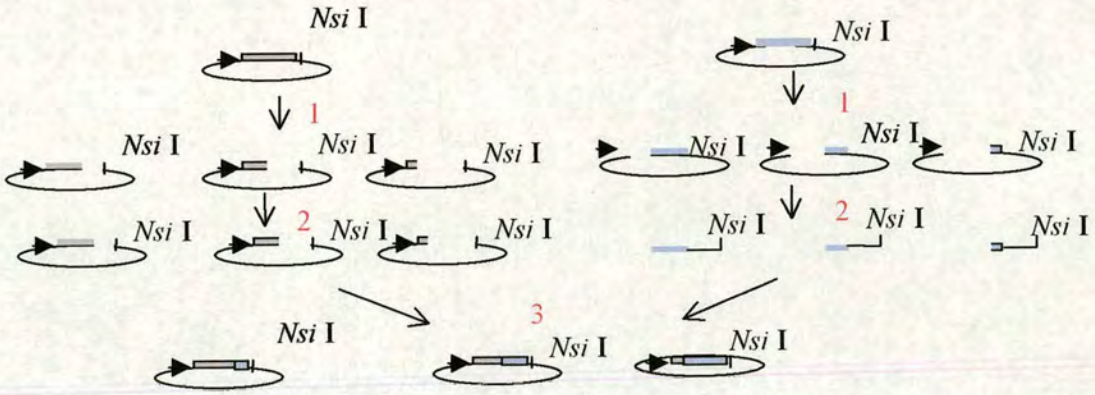


Figure 34: incremental truncation for the creation of hybrid enzymes (ITCHY).[191] (1) ExoIII random hydrolysis; (2) *Nsi*I digest; (3) blunt end ligation.

5' fragment of gene A and 3' fragment of gene B are cloned into the vector. *Nsi*I restriction site is designed for creation of incremental truncation libraries from the 3' end of gene A and 5' end of gene B.

This method was improved further to avoid extensive time for sampling.[192] The modified method is known as THIO-ITCHY the and key change is the random, low frequency incorporation (spiking) of the target DNA segment with alpha-phosphothioate nucleotides. These nucleotide analogues have been shown to protect the DNA from exonuclease digestion, thus leading to the desired variation in truncation length upon nuclease treatment.

The generation of diversity is based on the random distribution of the α-phosphothionate nucleotides and two targeted gene fragments can be combined into a single vector and processed for expression and screening.

Two overlapping gene fragments are cloned in the same vector with a unique restriction site between them. The THIO-ITCHY libraries were constructed in two different ways. Firstly, one was obtained by incorporation of phosphothioates in a primer extension reaction (Figure 35).

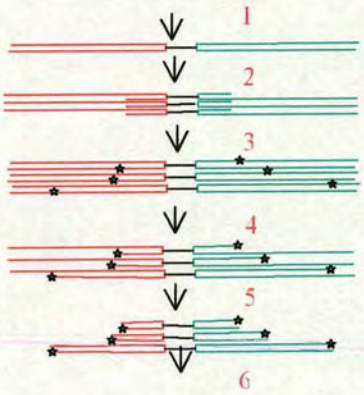


Figure 35:THIO-ITCHY using α--phosphothioate nucleotide incorporation by primer extention.[192]

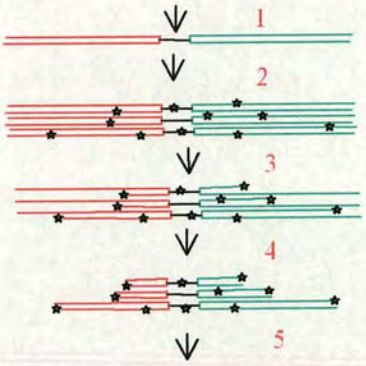


Figure 36: THIO-ITCHY using α–phosphothioate nucleotide incorporation by PCR amplification.[192]

(1) Linearization of the starting plasmid by restriction digestion at the unique site between two genes. (2) Treatment of the linearized plasmid with exonuclease III produces single-stranded overhangs, covering the entire length of the target region. (3) The single-stranded target region serves as the template for polymerase catalyzed synthesis of the complementary strand. The addition of small amounts of nucleotide analogues such as α-phosphothioate dNTPs to the reaction mixture results in random, low frequency incorporation of the analogue into the newly synthesised strand as indicated by stars. (4) A second incubation of the plasmid with exonuclease III results in hydrolysis of standard dNMPs while the dNMP analogues block enzymatic degradation. (5) The single-stranded portions of the plasmids are removed by mung bean nuclease. (6) The blunt-ended constructs are recircularised by intramolecular ligation.

The second library was produced by incorporation of phosphothioates in PCR amplification (Figure 36). (1) Linearization of the starting plasmid by restriction

digestion at the unique site between two genes. (2) PCR amplification of the entire linearized vector in the presence of a mixture of dNTPs and alpha-S-dNTPs. (3) Incubation of the plasmid with exonuclease III results in hydrolysis of standard dNMPs while the alpha-S-dNTPs will block enzymatic degradation. (4) The single-stranded overhangs of the plasmids are removed by mung bean nuclease. (5) The blunt-ended constructs are recircularized by intramolecular ligation.

The most important advantage of THIO-ITCHY is the possibility of combining incremental truncation with random mutagenesis, leading to further library diversity, which in turn can accelerate the identification of novel hybrid enzymes with improved function.

One of the main limitations with the methods described above (Figure 35&36) is the ability to create only a single crossover between the two parental genes. To overcome this problem, Benkovic and co-workers[193] developed a new non-homologous recombination method named SCRATCHY, which combines ITCHY with DNA shuffling. A new non-homologous recombination method based on modelling framework *e*SCRATCHY provides insight into the effect of sequence identity and fragmentation length on crossover statistics. Sequence analysis of the naive shuffled library identified members with up to three crossovers per gene, and modelling predictions are in good agreement with the experimental findings.

1.3.2. Library evaluation methodologies

Libraries of mutated genes can be generated by a variety of methods as described above and the methodology is rapidly becoming routine. The outcome of directed evolution experiments, however, is critically dependent on how a library is evaluated and this is a challenge that largely remains unmet.

1.3.2.1. Selection methods

Biological selection methods are based on complementation of auxotrophy or resistance to cytotoxic agents (*e.g.* antibiotics). Biological selection is a powerful method for screening large libraries and isolating variants of interest. In particular *in vivo* selection has been used very successfully to evolve microbiological pathways *in situ* and alter metabolic flux eg. Antibiotic or amino acid manufacture. However, *in vivo* methods can also be applied to specific enzymes. Oue and co-workers[194] altered the substrate specificity of aspartate aminotransferase using amino acid auxotrophs for the selection of mutants with increased catalytic activity against 2-oxovaline (non-native valine) at a rate $6.7x10^5$ greater then the wild type enzyme. However, the utility of phenotypic biological selection has limitations. Microbal genomes have evolved adaptation mechanisms to cope with changes in environmental conditions. The application of selective pressure can result in mutations that do not affect the catalytic activity of the recovered mutant. For example, selections may yield mutations that increase the expression level of a catalytically slow enzyme. Also, mutations in unrelated genetic loci may induce a different pathway unrelated to the enzyme of interest, or a general stress response may affect the growth level of the microorganism. The selection must therefore be designed very carefully.

The selection of large libraries is simplified by establishing a direct physical link between a gene, the protein that it encodes, and a desired function. This link can be established by *in vivo* and *in vitro* display technologies. For *in vivo* methods, the protein is displayed on the surface of the filamentous bacteriophage. In this method, a gene of interest is fused in-frame to phage genes encoding surface-exposed proteins, most commonly pIII. The gene fusions are translated into chimeric proteins in which the two domains fold independently. The phage displays a protein with binding affinity for the ligand immobilized on solid support. The selective adsorption of the ligand by the protein is known as "panning". The bound phage is desorbed from the surface, usually by acid elution, and amplified through infection of *E. coli*. Usually, four to six rounds of panning and amplification are sufficient to select for

phage displaying specific polypeptides from very large libraries with diversities up to 10^{11}.[195]

Nevertheles, this method has some limitations. Phage-display methods evolve the binding rather than catalytic capacity of the enzymes and provide information only for the clones that bind to a ligand. The method cannot be used for the quantitative analysis of an entire library. At the end of a phage panning experiment there is no data on the percentage of clones exhibiting a certain level of activity.

An *in vitro* selection method, which establishs a link between the gene, the protein and the function, was reported by Tawfik and Griffiths[196] and called compartmentalized self replication (CSR). In this method of *in vitro* protein translation, the protein activity does not interfere with cellular metabolism and can be distinguished from the background of all other cell reactions. The tiny aqueous droplets of water-in-oil emulsion serve as artificial compartments for *in vitro* protein synthesis and protein capture. Ribosomes, DNA, the corresponding protein and the reaction product are contained and kept physically separated within the droplet compartment (average one gene per compartment). The authors were able to extract the DNA encoding active enzyme again using product specific binders (Figure 37).

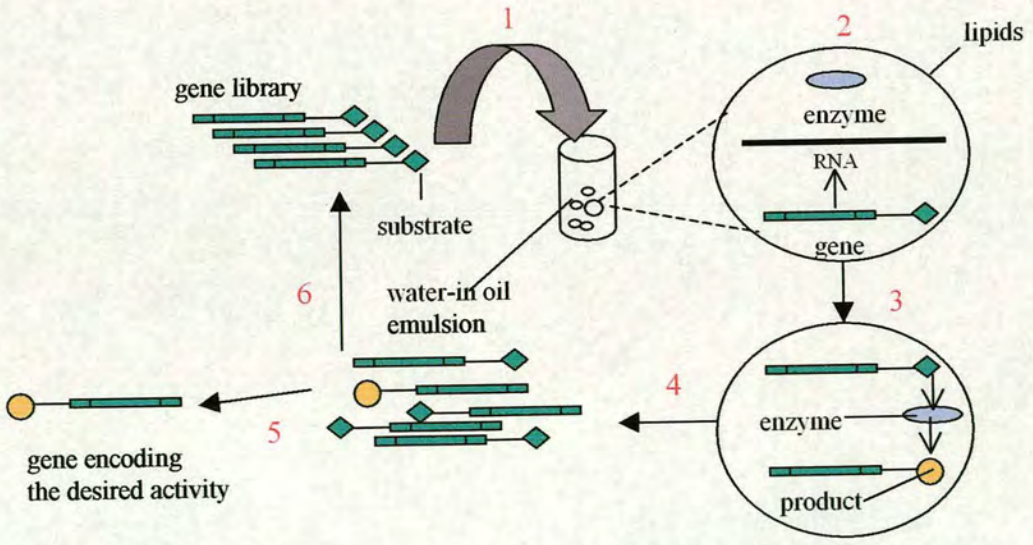


Figure 37: compartmentalized self replication (CSP).[197]

A gene library is expressed in an *in vitro* transcription/translation reaction (1) and distributed to the water-in-oil emulsion (2). In the proposed selection, active enzyme variants modify the DNA template to which they are coupled through compartmentalization in the droplet (3). The emulsion is broken (4) and the modified DNA templates enrich the gene coding for active enzymes (5). The entire cycle can be repeated to achieve higher enrichment (6).

One cycle was able to achieve an enrichment factor of at least 5000, and two cycles were enough to select the gene with the desired property (*Hae III* methyltransferase protected from digestion by *Hae III*) from a 10^7-fold excess of other DNA. The CSR method was also applied for directed evolution of *Taq* DNA polymerase.[198] The authors increased the polymerase thermostability by 11 fold and resistance to heparin by 130 fold.

1.3.2.2. Screening methods

Enzyme activities in libraries can be screened by means of a direct assay performed in microtiter plates that detects the formation of a coloured or fluorescent reaction product or the temperature change of an exothermic reaction. Some examples of enantioselectivity evaluation by UV/Vis- based, fluorescence-based, IR-thermographic systems and capillary array electrophoresis are described in a recent review.[199] However, quantitative colorimetric or fluorimetric assays performed directly in mictotiter plates are time consuming and expensive.

Solid-phase screening of colonies on agar plates or on filters allows rapid screening of large libraries. Typically, variants are identified on the basis of cleavage of a substrate present in the solid phase and detection of product release by a local change in pH using a pH indicator,[200a] formation of a coloured or fluorescent product,[201] development of a clearing zone or precipitation caused by the altered solubility of the released product in the solid phase[202] or monitoring the growth rate depending on the cleavage of an insoluble substrate.[203]

Most solid-phase screening methods lack sensitive quantification of catalytic activity and are typically qualitative screens suitable for a first round of directed evolution. However, a sensitive fluorescent high throughput digital imaging screen has been developed for P450 monooxygenase expressed in *E. coli.*[204]

Kairos Scientific Inc. has developed high through put screening methods, based on a solid phase system that allows the screening of the colonies (500/cm^2) that have been transferred onto an aporous assay disk. In this assay, each colony acts as a nanoliter reaction vessel and analysis is performed with a digital imaging spectrophotometer[205] (Figure 38).

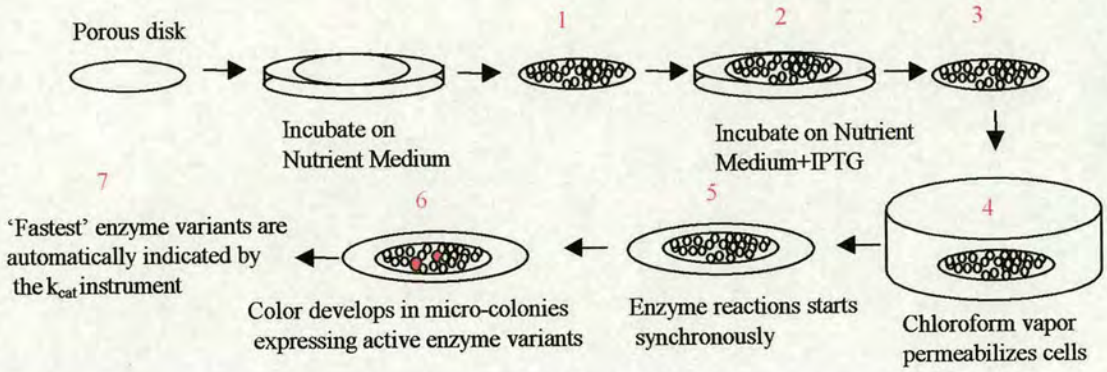


Figure 38: steps in the solid phase enzyme evolution assay for use in the K_{cat} instrument.The porous assay disk was inoculated with a library of mutant plasmid transformants and left for incubated on the nutrient medium. The disk containing the microcolonies was lifted (1) and transferred to an inducer plate (2). The disk was lifted again (3) and placed into the chloroform lysis chamber (4). The disk was then subjected to solid phase assay directly in the K_{cat} instrument (5). The K_{cat} instrument begins recording spectra as function of time (6). When coloured colonies develop, the fastest enzyme variants are automatically indicated by the K_{cat} instrument (7).[205]

Cell-surface and phage-display methods have been limited mostly to the evolution of binding rather than catalysis. Olsen *et al.*,[206] devised a strategy to screen large enzyme libraries displayed on bacterial cell surfaces by fluorescence-activated cell sorting (FACS). The *E. coli* cell-surface protease OmpT was evolved for novel

substrate specificity using a specially designed fluorescence resonance energy transfer substrate (FRET). The surface of a gramm-negative bacterium is highly negatively charged. The FRET substrate contained a fluorophore which consisted of a neutral FRET quenching partner released upon enzymatic conversion, and a positively charged fluorophore bound to the cell surface. Cleavage of the substrate by a surface-displayed enzyme disrupts intermolecular quenching, giving rise to a green fluorescent product that is surface retained via the polycationic tail. The amount of green fluorescence correlates with substrate turnover.

Cells expressing high green fluorescence emission are physically sorted from the library mixture by flow cytometry. Enzyme mutants that bound to the substrate but did not turn over efficiently exhibit enhanced quencher fluorescence (red fluorescence), presumably because the quencher fluorophore is secluded within the active site of the protein. Because of these features, protein surface display coupled with FRET/FACS screening is unique in being able to distinguish enzyme clones either by k_{cat} or K_M. The capacity of this method permits screening of up to 10^9 cells per hour (Figure 39).

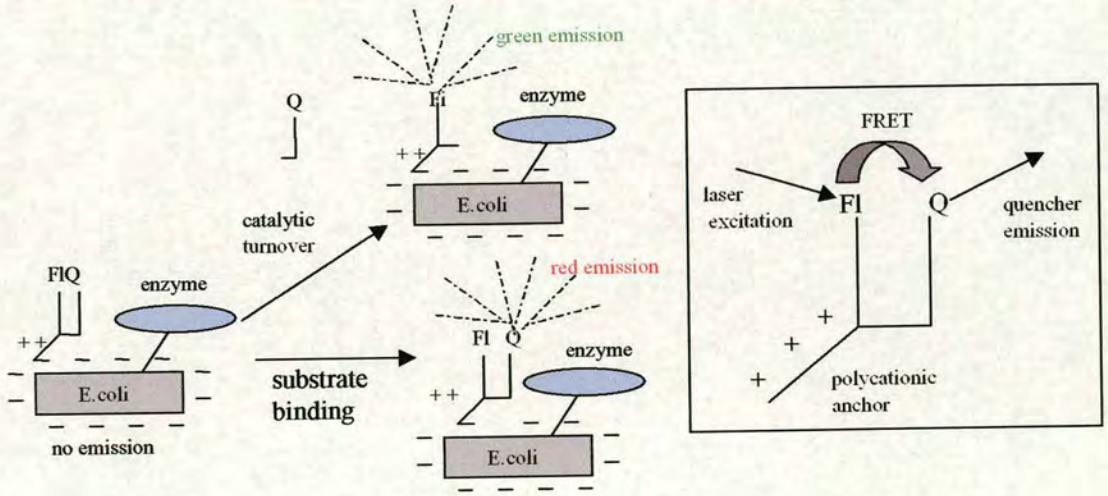


Figure 39: FACS-based enzyme evolution system. F: fluorophore; Q:quencher.[206]

Another strategy based on FRET/FACS screening of periplasmically expressed proteins in *E.coli* was developed by Chen *et al.*[207] This method allows the screening

of libraries of proteins that cannot be functional and stably displayed on a cell or phage surface. The authors found that under certain conditions, large ligands (up to 10kDa) could diffuse into the periplasmic space, allowing the screening of a number of binding proteins, including nucleic acid binding proteins as well as the screening of enzyme libraries.

For some activities, the reaction cannot be directly detected by light absorption or fluorescence. To overcome this problem, Firestine *et al.*[208] have developed a strategy based on a three-hybrid system called QUEST (querying for enzymes using the three-hybrid system) where substrate turnover *in vivo* is coupled to a transcriptional event to generate a signal (Figure 40).

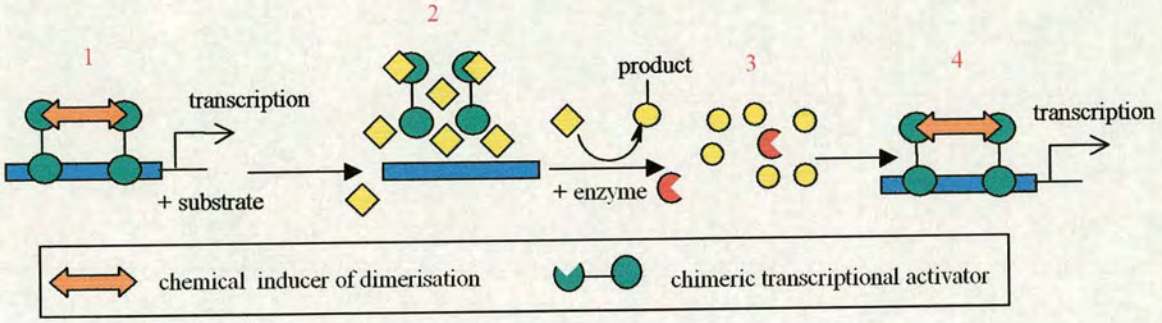


Figure 40: QEST system for enzyme evolution *in vivo*.[208]

The chimeric fusion protein (green) binds to a chemical inducer of dimerisation (orange), resulting in activation of transcription (1). The substrate (yellow squares) inhibits dimerisation and switches the transcription off (2). The target enzyme (red) converts the substrate into product (yellow circles) (3) and dimerisation again induces transcription (4). As the target activity is coupled to a transcriptional signal, the method is theoretically applicable to any enzymatic reaction.

1.3.3. Achievements by directed evolution in vivo

The techniques of directed evolution have been used to achieve several goals: (i) to change enzyme specificity and stereoselectivity, (ii) to change the stability of enzymes (thermostability, organic solvents tolerance), and (iii) to engineer new activities. For the purpose of this thesis, only the application of directed evolution in the synthesis of chiral compounds will be considered.

Control and optimisation of stereoselectivity in enzyme catalysed resolution and asymetric transformation has been a focus in recent years, because of the inefficiency of conventional methods for stereochemical control. Many highly stereoselective biocatalysts are already used in industry (Table 2).[3] These biocatalysts, particulaly the group of hydrolases, have been further improved by laboratory evolution.

Process	*Substrate*	*Product*	*Enzyme*	*Scale tonnes/ annum*	*Com pany*
Resolution	Racemic alcohols	Enantiomeric alcohols	Lipase	10^3	BASF
Resolution	Racemic amines	*R*- and *S*-amide	Lipase	10^2	BASF
Kinetic resolution	Racemic amino acid amides	L-amino acids	Amidase	10^2	DSM
Amination	Fumaric acid	L-acpartic acid	Aspartate ammonia lyase	10^2	DSM

Table 2: stereoselective large-scale biocatalytic process.[3,209]

The most popular members of the hydrolase group as stereoselective biocatalysts are esterases (carboxyl ester hydrolases) and lipases (triacylglycerol hydrolases). The most valuable properties of esterases and lipases are their high regio- and

stereospecificity towards a wide range of different substrates. No cofactor is required and the enzymes are typically rather stable and even active in organic solvents.

i). Esterases

Although a considerable number of microbial carboxyl esterases have been overexpressed in suitable hosts, only a few of them have been used for the synthesis of optically pure compounds. The major reason for this is their limited commercial availability and their frequently observed moderate enantioselectivity.

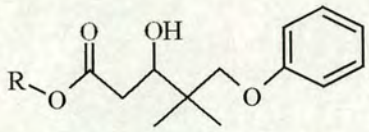
Bornscheuer *et al.*,[200a] attempted to resolve the 3-hydroxy esters(Figure 41, I&2), which show close structural similarity to a key building block in epothilones, a new class of anticancer drugs posessing cytotoxic activity at the stage of mitosis.[210] Previously, Bornscheuer *et al.*[200b,c] have demonstrated the successful resolution of aliphatic and arylaliphatic 3-hydroxy esters using lipases or esterases. However, the sterically hindered 3-hydroxy esters (Figure 41, I&2) were not accepted as substrates by 18 lipases and two esterases.[200a] The problem was overcome by evolving new enzymes by directed evolution. The substrate specificity of an esterase from *Pseudomonas fluorescens* (PFE) was altered by introduction of two point mutations by the mutator strain *Epicurian coli* XL1-Red.[200a,d] The key to the identification of improved variants was an agar plate assay system based on pH indicators, in which the colour changed upon hydrolysis of the ethylester. The best mutant showed a moderate improvement in enantioselectivity (E = 5,wild type E=0)* although possessed only 50% of the activity of the wild type.

* E value can be calculated from the V_{max}/K_M value for each enantiomer, but also determined from % *e.e.* and conversion using the equations developed by Chen *et al.*[211]

In the next step, the enantioselectivity of PFE was improved further. Mutant libraries were created by error-prone PCR and also by using the mutator strain. An extremely accurate determination of the enantioselectivity was achieved by using resorufin esters of (*R*)- or (*S*)-3-phenylbutyric acid (Figure 41, III), which allowed

measurement of fluorescence of resorufin in microtiter plates, avoiding problems with interfering compounds present in the culture medium. The measured enantioselectivity after a single round of error-prone PCR or passage in the mutator strain was approximately the same: E=5.2 - 6.6 (E=3.5 for wild type).[178]

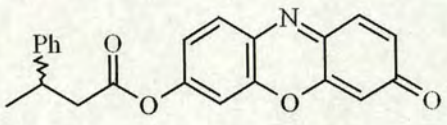
A further increase in the enantioselectivity up to E=12 was obtained by saturation mutagenesis at all three positions identified for the first generation variant (E.Henke, U.T.Bornscheuer, unpublished).[212] However, combination of the best variants by site-directed mutagenesis gave no further improvement.



3-hydroxy ester

I: R=CH_2CH_3

II: R=$CH_2CH(OH)CH_2OH$



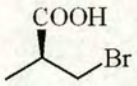
(R)- (S)- 3- Phenylbutyric acid resorufin ester

III

Figure 41: 3-hydroxy esters used as substrates for directed evolution of PFE.

Preliminary homology modeling of PFE based on the known structure of haloperoxidase from *Streptomyces aureofaciens* (55% identity) showed that all mutations produced by error-prone PCR or the mutator strain were neither near nor in the active site of PFE, but at the periphery of the enzyme.[200a]

Significantly higher increases in enantioselectivity were achieved for the resolution of methyl 3-bromo-2-methyl-propionate (U.T. Bornscheuer and R.J. Kazlauskas, unpublished).[212] Using a homology model of PFE, Trp29 and Phe199 were subjected to random mutagenesis and the pH indicators were used for screening. Mutant Trp29Leu was identified, which exhibited an E=90, compared to E=12 for wild type.



3-bromo-2-methyl-propionate

To make the application of enantioselective enzymes in industrial processes more efficient, directed evolution was successfully used to improve the stability and activity of an esterase from *B. subtilis*, which selectively cleaves the *p*-nitrobenzyl ester of Loracarbef, a cephalosporin antibiotic.[213] A combination of error-prone PCR and DNA shuffling led to the generation of a variant with 150 times higher activity compared to the wild type in 15% DMF. Moreover, directed evolution also resulted in an increase in the thermostability of this esterase by 14°C, thus making it more versatile in industrial application.[214]

ii) Lipases

Lipases are widely used as catalysts for enantioselective reactions, but many substrates react with unacceptably low enantioselectivities for industrial application.

Error-prone PCR was used for the random mutagenesis of a lipase from *Pseudomonas aeruginosa*, which catalyzes the hydrolytic kinetic resolution of 2-methyl decanoate-*p*-nitrophenol ester, with a slight preference for the (*S*)-acid with a low frequency of mutation (1 per gene). Approximately 1000 mutants were screened by UV/Vis spectroscopy (production of yellow hydrolysis product *p*-nitrophenol ester) in 96 well microtiter plates after each round of error-prone PCR. It was possible to improve the wild type enantioselectivity from *e.e.*=2% to *e.e* .= 81% at 25 % conversion (*E* value from 1.1 to 11) in four generations (Figure 42).[169]

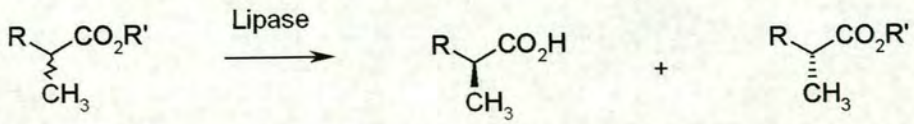


Figure 42: lipase catalysed hydrolysis of ester. R = *n*-C_8H_{17}; R' = *p*-NO_2-C_6H_4.

A combination of saturation mutagenesis and site-specific mutagenesis with a selected set of lipase variants produced by error-prone PCR increased the enantioselectivity further (*ee* = 90%, *E* = 26) for a mutant bearing five amino acid substitutions.[167] By modifying the combinatorial cassete mutagenesis[215] the enantioselectivity reached *E* = 51.[216a,b] It was recently shown that by screening approximately 45000 colonies, the direction of enantioselectivity could be reversed completely (from (*S*)- to (*R*)- with *E*=30) by several cycles of a combination of high error rate error-prone PCR and DNA shuffling. The best (*R*)-enantioselective mutant contained eleven amino acid substitutions.[217]

iii) Other enzymes.

Recently, Arnold and co-workeres[218] reported the inversion of enantioselectivity of an hydantoinase from *Arthrobacter* sp. DSM 9771. for improved L-methionine production from D,L-5-(2-methylthioethyl) hydantoin in the presence of L-carbamoylase and a racemase by recombinant *E. coli*. The D-selectivity (40% *ee*) was converted to moderate L-preference (20% *ee* at 30% conversion) by a combination of two rounds of error-prone PCR and saturated mutagenesis. Only one amino acid substitution was required to invert the enantioselectivity. The enzyme variant increased the productivity of the whole- cell process 5-fold to >90 % convertion.

D-2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase from *E. coli* catalyses asymmetric carbon-carbon bond formation via an aldol process. Fong *et al.*[219] evolved the aldolase to accept the nonphosphorylated analog of KDPG and L-glyceraldehyde (D-glyceraldehyde-3-phosphate is a substrate for the wild type enzyme in the aldol reaction). Wymer *et al.*[220] also performed directed evolution on KDPG aldolase, broadening its substrate range while retaining enantioselectivity.

DNA shuffling and selection were used to create an *E. coli* aminotransferase[221] that accepted β-branched substrates, which were poorly accepted by the wild type

enzyme. This aminotransferase activity compensated for a defective host *ilvE* gene (cells unable to make isoleucine, leucine and valine) and allowed cells to grow on minimal media in the absence of these amino acids. Four rounds of shuffling increased the activity of aspartate aminotransferase for valine and 2-oxo-valine (non-natural valine) by five orders of magnitude, while decreasing by 30-fold the activity towards the natural substrate aspartate.

1.4. General conclusions & Aims

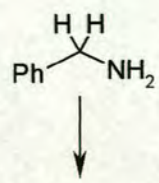
The extent of research into the synthesis of optically active amines over the last few decades has been extensive. However, there is still a need for more research and optimisation for the synthesis of enantiopure chiral amines, since a generic method for their production in high yields has not yet been found.

The recent work by Soda *et al.*[37,38] on the deracemisation of cyclic amino acids is a promising route to both enantiopure α-amino acids and amines in high yield. In order to successfully employ this deracemisation as a general method, oxidase enzymes with high enantioselectivity and substrate specificity are required. The deracemisation of racemic amines using a cyclic oxidation-reduction strategy is a potentially cheap and efficient method for their production, assuming an enantioselective monoamine oxidase can be found.

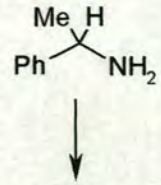
The aim of this work is to obtain variants of monoamine oxidases which possess activity and enantioselectivity towards substrates of interest by the methods of directed evolution.

2. Results & Discussion: Monoamine oxidase from *Aspergillus niger*. Cloning and expression

Evidence in the literature suggested that copper dependent AOs, from porcine plasma and kidney, pea seeding, soy bean and chick pea, catalysed stereoselective oxidation of amines[110] in addition to MAO-B.[159] Enantioselective oxidation of the (*R*)-enantiomer of amphetamine by copper-containing AO from *E. coli* and *Klebsiella oxytoca* was shown by A. Hacisalihoglu *et al.*[111a] However, the mechanism of the copper AO's makes them unsuitable enzymes for the deracemisation of amines, since the intermediate imine is not released from the active site. Attention therefore turned to the FAD containing monoamine oxidase recently identified by Schilling *et al.* from the fungus *A. niger* (MAO-N).[55] Although extensive characterization of the enzyme had been reported, the stereoselectivity of the enzyme towards chiral substrates had not been examined.[57] However, we reasoned that this enzyme had the potential to be a useful biocatalyst. It was soluble and did not require cofactor recycling. The hydrogen peroxide released in the oxidative reaction is easy to detect by colorimetric assay[222] and this suggested that there was great potential for the directed evolution of MAO-N to alter the enantioselectivity. Shilling *et al.* [55] reported that MAO-N had high activity towards benzylamine (k_{cat}=1400 min^{-1}). α-methylbenzylamine a chiral analogue of the achiral substrate benzylamine, was chosen as a target substrate for evolving altered substrate specificity, in view of its value as a chiral molecule. It is currently produced on a multi-ton scale via an enzyme-catalyzed resolution process.[1] Moreover it is often used as a model substrate in the development of new processes for the synthesis of enantiomerically pure amines.[11b, 32, 223, 224]



benzylamine



alpha-methylbenzylamine

2.1. Cloning into pET system

A construct containing the MAO-N gene cloned in the pET3a vector (pECME3) was kindly provided by Professor Schilling.[129] pET3a is one of a series of pET vectors supplied by Novagen. The pET vector is one of the most powerful systems for the cloning and expression of recombinant proteins in *E.coli.*[225] The target gene is cloned into the pET plasmid downstream of strong bacteriophage T7 transcription and translation signals (Figure 43).

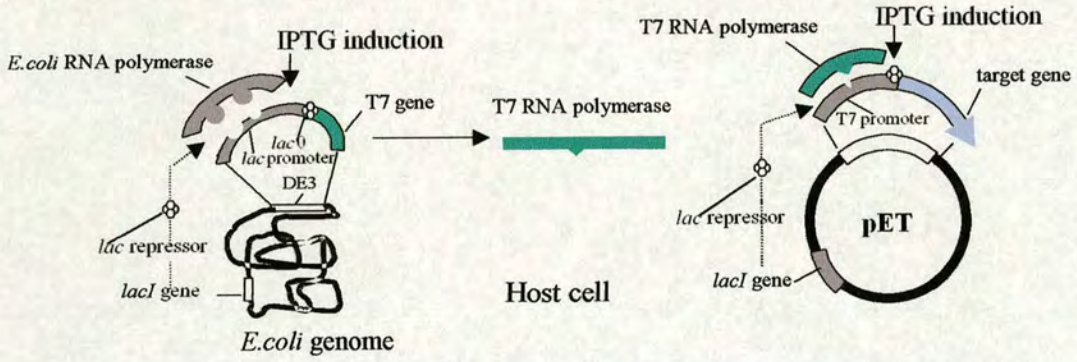


Figure 43: control of gene expression in the pET system.

The expression host contains a chromosomal copy of the bacteriophage T7 RNA polymerase gene under *lac UV5* promoter control. Transcription of T7 RNA polymerase is regulated by a protein called *lac*-repressor which is encoded by the gene *lac I*. An active repressor binds to the *lac*-operator (*lacO*) and blocks the transcription of mRNA. The expression of T7 RNA polymerase is induced by the addition of IPTG, which binds to the *lac*-repressor and rendering it unable to bind to DNA effectively. The expressed T7 RNA polymerase from the *lac* promoter then binds to the T7 promoter on the pET vector to enable transcription of the target gene in response to addition of IPTG. The IPTG is a useful expression inductor because the host cells do not metabolise it. *E. coli* BL21 (DE3) is used as an expression host

in pET system, because it carries a chromosomal copy of the gene for T7 RNA polymerase. This was accomplished using a lysogen of bacteriophage DE3, a lambda derivative that has the immunity region of phage 21 and carries a DNA fragment containing the *lacI* gene, the *lacUV5* promoter, and the gene for T7 RNA polymerase.[226]

The antibiotic ampicillin resistance is provided by the β-lactamase gene (*bla)* and the replication of the pET vector is controlled by ColE1 origin of replication, and the vector can be selected in growing cells by addition of ampicillin to the culture, due to its β-lactamase (*bla)* gene.

2.1.1. Cloning into the pET 16b vector

The key starting point for directed evolution of an enzyme is the availability of active enzyme, in order that the screening/ selection part of the process can be carried out efficiently. Expression of MAO-N in the pECME3 vector was carried out using the previously described protocol.[57] The authors reported that addition of IPTG in fact led to a decrease in MAO-N activity compared with uninduced conditions. Analysis of protein expression, in whole cell cultures, by SDS-PAGE showed that expression of MAO-N protein was poor. Therefore, in an attempt to improve expression of the protein, the gene encoding MAO-N was subcloned into the pET16b vector, which is similar to pET3a, but benefits from the native *lac* operator for tighter control of expression. In addition, a 10 histidine peptide tag is attached to the N-terminus of the target protein, allowing efficient purification on a Ni chelating affinity column.[225]

Mao-n gene amplification by PCR was performed with primers (§7.2.1.1) adapted from the native sequence to include codons more typical of highly expressed *E. coli* genes. This revision is shown in Table 3.

Amino acid	*DNA codon*		*Occurrence in E.coli genes (%)*	
	Actual	*Revised*	*Actual*	*Optimal*
Arg 4	CGA	CGT	7	36
Gly 6	GGA	GGT	13	34
Thr 10	ACA	ACC	17	40
Pro 11	CCC	CCG	12	49

Table 3: Primer revision according to codon preference in *E.coli* genes.[227]

Restriction sites were introduced into the primers according to the pET16b cloning requirement (*Nde*I at N terminus and *BamH*I at C terminus). A "Stop" codon was introduced at the C terminus to terminate the transcription. Amplification of the gene was performed with *taq* DNA polymerase or with *Hot start Herculase* high fidelity (HF) DNA polymerase (§7.2.1.1). The PCR product was digested with *Nde* I/ *Bam HI* restriction endonucleases and ligated into *NdeI/ Bam HI* digested pET16b (§7.2.1.2). The final construct was used to transform *E. coli* Top10 (§7.2.1.2). *E. coli* Top10 strain was chosen for *mao-n* gene cloning into the pET vector since it is recA$^-$ (mutation in gene responsible for general recombination) and helps to prevent unwanted recombination in cloned DNA. This strain also gives high transformation efficiencies and good plasmid yields. In each case a positive transformant was identified by restriction analysis and used for MAO-N expression in *E. coli* BL21 (DE3) (§7.2.2; ii). These were designated as mao(taq)pET16b and mao(HF)pET16b (pMAO-N).

2.1.2. Expression from pET16b

Expression of MAO-N from pET16b was carried out at 30°C and 37°C (§7.2.2; ii). *E. coli* BL21 (DE3) transformed with pET16b was used as a control reference. 1ml and 10ml aliquots of growing culture (§7.2.2; iv) were used for further studies. The hydrogen peroxide (§8.1.1) and benzaldehyde (§8.1.2) formation assays were used to assay the specific activity of MAO-N. The frozen 1ml pellets were used for volumetric specific activity determination (§7.2.4; i). The specific activity towards n-amylamine ($CH_3(CH_2)_4NH_2$) was determined as activity units produced per 1ml of culture (Table 4).

	Name of the construct	*Incubation Temperature(°C)*	*MAO-N specific activity (Uml^{-1})*
1	pECME3	37	0.03
2	pECME3	30	0.02
3	mao(taq)pET16b	37	0.0004
4	mao(taq)pET16b	30	0.01
5	mao(HF)pET16b	37	0.03
6	mao(HF)pET16b	30	0.1

Table 4: MAO-N specific activity in whole cells, cultured at different temperature with amylamine as substrate in the hydrogen peroxide formation assay; pECME3- supplied by Professor Schilling[129] (*mao-n* cloned into pET3a); mao(taq)pET16b- *mao–n* amplified by *taq* polymerase and cloned into pET16b; mao(HF)pET16b (also called pMAO-N).

A further set of 1ml frozen pellets was used to analyse the level of expressed MAO-N by SDS PAGE (§7.2.5; Figure 44).

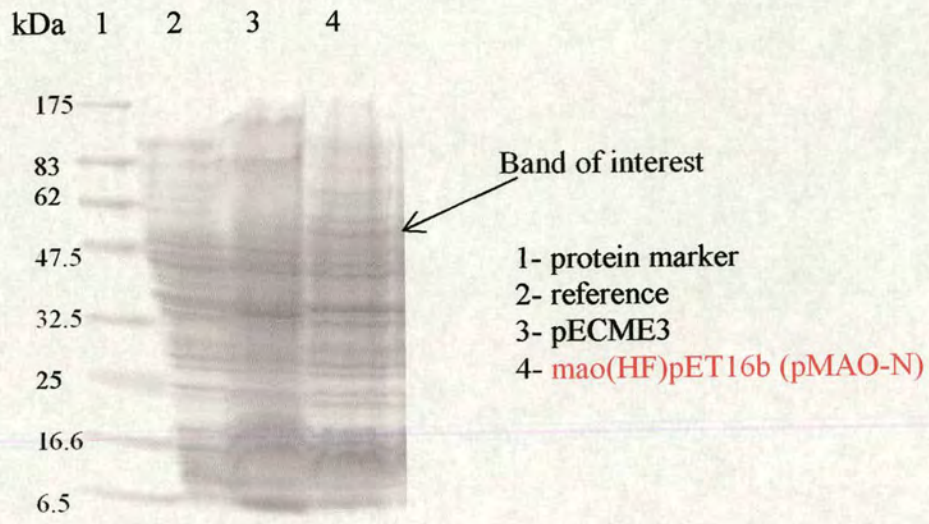


Figure 44: quantitative SDS PAGE analysis of MAO-N presence in *E. coli* BL21(DE3)/pMAO-N. The cells were cultured at 30°C and MAO-N express from the original construct pECME3[129] and as pMAO-N.

The frozen 10ml pellets were used to prepare a CFE (cell free extract; §7.2.3; i). The specific activity towards benzylamine was determined in the CFE as expressed as activity produced per 1mg of protein (Table 5). BL21(DE3) transformed with pET16b was used as a background reference.

	Construct	*MAO activity (Umg^{-1})*	*MAO activity ($U\ ml^{-1}$)*
1	pECME3[56]	0.2	-
2	pECME3	0.4	0.05
3	mao(HF)pET16b (pMAO-N)	**1.0**	**0.2**

Table 5: MAO-N specific activity in CFE (Umg^{-1}) and whole cell culture (Uml^{-1}) grown at 30°C incubation with benzylamine as a substrate in the benzaldehyde formation assay.

Two different substrates and assays were used to confirm the reproducibility of the obtained results. Additionally, two DNA polymerases with different levels of fidelity were used to amplify the *mao-n* gene. This was carried out to address the potential for error in the amplification.

The activity of MAO-N was about 10 fold lower when expressed from *mao-n* clone amplified by *taq* polymerase (Table 4; entry 3&4) compared to the activity derived from a *mao-n* clone amplified by the high fidelity *Hot start Herculase* DNA polymerase (Table 4; entry 5&6). However, the SDS PAGE revealed the same level of expressed MAO-N protein in each case (result not shown) and thus the reduced activity of the enzyme was possibly caused by errors introduced by the lower fidelity *taq* DNA polymerase.

The SDS PAGE also gave insight into the different levels of MAO-N protein expressed in cells cultured at 30°C or at 37°C (result not shown). However, the amount of active enzyme was found to be greatest when the gene amplified by high fidelity DNA polymerase (Table 4; entry 5&6) was expressed in cells cultured at 30°C (Table 4; entry 4& 6).

Intermolecular association of hydrophobic domains during folding is believed to play a role in the formation of inclusion bodies. The growth temperature often directly affects both expression levels and protein solubility. The lower temperature often reduces expression levels but yielding a higher amount of soluble protein.[228] In this case the activity of the enzyme expressed from pECME3 was not significantly affected by the incubation temperature, because of the low overall expression level.

Changing the codons specifying particular amino acids in MAO-N to ones more frequently used by *E. coli,* resulted in the production of higher levels of protein (Figure 44; lane 4) which was more active (Table 4; entry 6, Table 5; entry 3). Codon

usage affects translational efficiency since highly expressed *E. coli* genes use a characteristic set of codons that correspond to abundant tRNA molecules.[229]

The cell cultures were inoculated with a single colony rather than an over-night culture. The reason for this was to avoid the destruction of ampicillin by β-lactamase, secreted by an ampicillin resistant seed culture. Ampicillin is an unstable antibiotic and is rapidly depleted in growing cultures.[228] Subculturing from over night cell cultures leads to the initial presence of β-lactamase in the growth media, potentially resulting in the enrichment of a sub-population of cells without plasmid, thereby reducing the population of cells bearing the plasmid.

The set of conditions applied to improve the level of active MAO-N expression was efficient to produce five times more active enzyme compared to the original pECME3 construct (Table 5; entry 1,2&3).

2.2. Development of high-throughput screen

The development of a high throughput screen to validate the mutant library was the next crucial step in the enzyme directed evolution experiment. Most of the existing screening methods rely either on time consuming picking of colonies or on the use expensive robotic equipment. The current project required the development of a cheap, effective and widely applicable method, which could potentially deal with libraries of clones in excess of a million.

2.2.1 Detection of oxidase activity

At the outset of this work a variety of assay protocols, which allowed quantification of amine oxidase activities had been described.[222] The reaction catalysed by monoamine oxidase can summarised as follows:

$RCH_2NH_2 + H_2O + O_2 \rightarrow RCHO + NH_3 + H_2O_2$.

The enzyme activity can be measured by either measuring oxygen consumption or formation of aldehyde, ammonia or hydrogen peroxide. It is apparent that the identity of the aldehyde formed is dependent upon the identity of the substrate and, while some aldehydes can be detected directly by spectrophotometry (*e.g.* benzaldehyde), the technique is often unsuitable for use with crude extracts.[82] Ammonia (NH_3) production can be measured continuously with a coupled colorimetric assay [230a] and with Nestler reagent.[230b,c] A number of assays have been developed that allow measurement of enzymatic activities based upon hydrogen peroxide production.[222, 231, 232]

2.2.2. Soluble dye production assays

Most assays designed to detect hydrogen peroxide are based upon the formation of a coloured product. The production of a dye *via* an enzymatic reaction would allow quick and easy identification of active clones and could also be adapted to become a quantitative method using a spectrophotometer together with a kinetic-measuring program. A highly sensitive colorimetric assay for hydrogen peroxide was reported by Szutowitcz *et al.*[231] 2.2'- azino-*bis* (3-ethylbenzthiazoline-6-sulfonic acid, ABTS) was used as the chromogen although this causes inhibition of MAO at the concentrations used in the assay. Another hydrogen peroxide formation assay was described by Yamada *et al.*[232a] In this reaction, 4-aminoantipurine acts as the proton donor in the peroxidase reaction and then condenses with 2,4-dichlorophenol to form a red quinineimine dye. This reaction was used to measure sheep blood plasma amine oxidase activities[232b] and to assay porcine kidney diamine oxidase.[232c] The drawback in this method is the toxicity of 2,4-dichlorophenol towards MAO. Thus it was modified by Halt and co-workers[222] by replacing 2,4-dichlorophenol with vanilic acid. In this case the reaction was applicable for enzymatic activity measurement. In a modification of this approach, vanilic acid was replaced by 2,4,6-tribromo-3-

hydrobenzoic acid[233] to obtain a more intensely coloured dye. The mechanism of this reaction is presented on Figure 45. Hydrogen peroxide is reduced to water in the presence of horseradish peroxidase (HPR) and proton donor (*e.g.* 4-aminoantipurine, AAP). The proton donor for this peroxidase reaction is oxidised in the process and subsequently reacts with an acid, in this case 2,4,6-tribromo-3-hydrobenzoic acid, to form a soluble dye. The absorbance change at 510nm can be measured and used to calculate the activity of the oxidase enzyme (§8.1.1).

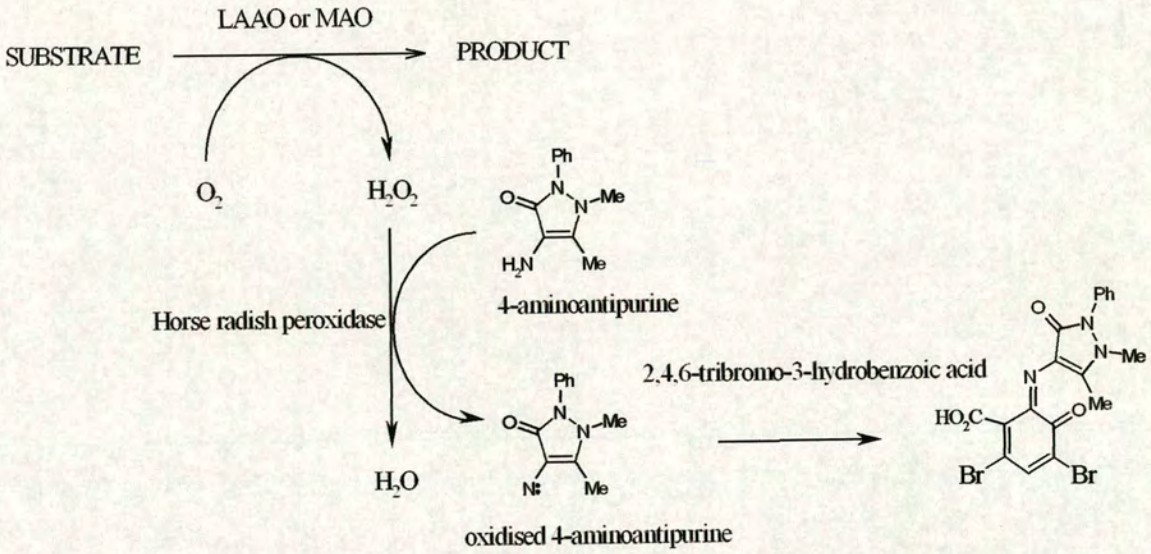


Figure 45: peroxidase-coupled soluble dye formation assay.

2.2.3. Detection of oxidase activity in bacterial colonies on agar plates

Initially a "solid phase assay" was developed to detect the enzyme activity directly in colonies grown on a nitrocellulose membrane placed on LB Amp agar. For optimal sensitivity it is necessary that the intracellular enzyme is released from the cell and available for detection. Another key point is to fix the dye produced by the colony to help identify the positive isolate for further analysis. In order to do that, the agarose was added to the assay mixture thereby fixing the dye corresponding a particular colony and making it available for further studies. The method is described in detail in §8.3.2; i and is featured in Figure 46.

2.2.4. Use of soluble dye

To demonstrate the practical application of the solid phase assay, two transformations were carried out in parallel using *E. coli* BL21(DE3) transformed with pMAO-N and *E. coli* BL21(DE3) transformed with pET16b. The cells from both transformations were mixed together and plated onto one membrane placed on LB Amp plate to perform the solid phase colorimetric assay *in situ* using amylamine as substrate (§8.3.2).

The cells were plated directly onto a nitrocellulose membrane to avoid the preparation of replica filters. This was done because colonies, which heavily expressed MAO-N *in situ* (without IPTG), developed a texture which significantly impeded their transfer onto a membrane. Also, the cells plated directly onto the membrane were easy to disrupt by freezing/thawing. Another advantage of avoiding colony replicating is the more precise identification of positive colony.

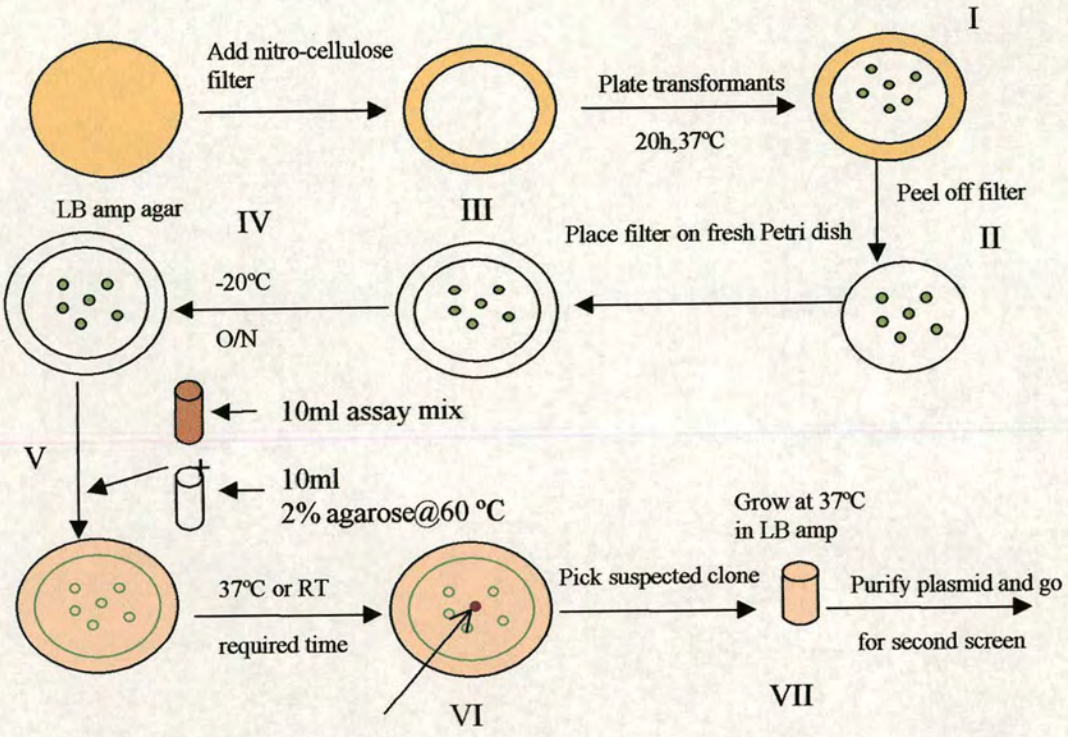


Figure 46: diagram of solid phase screening.(I)The cells were plated directly onto a nitrocellulose membrane to avoid the preparation of replica filters. (II) Peeled off filter placed into a fresh Petri dish (III) and subjected to freezing (IV) and thawing (V). (VI) Partially lyzed cells release the enzyme and the positive clone is picked (arrow pointing the positive clone). (VII) Recovery of the positive clone occurs via the partial lyzis of the cells.

The colonies harbouring the vector with *mao-n* began to develop a pink colour after 2 hours of incubation and the colonies with pET16b did not develop the colour at all (Figure 47).

However, after 4 hours incubation the pink colour was found to have diffused and hence we concluded that this soluble dye formation assay would not be applicable as a high throughput assay for high-density colony screening.

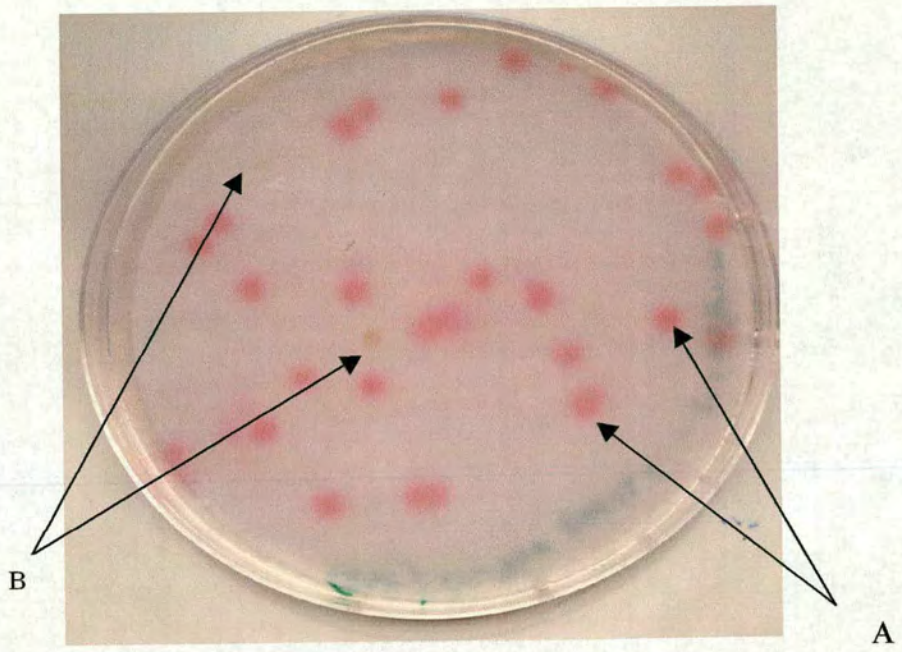


Figure 47: soluble dye formation solid phase assay (arrows A show positive colonies, arrows B show the negative colonies).

2.2.5. Use of insoluble dye

The problem with diffusion of the dye was overcome by switching to 3,3'-diaminobenzidin (DAB) as the substrate for HRP which gave rise to a dark pink insoluble product resulting in both very high definition and contrast of the active colonies. Oxidase activity was detected by incubation with HRP and DAB. The mechanism of this assay is shown in Figure 48.

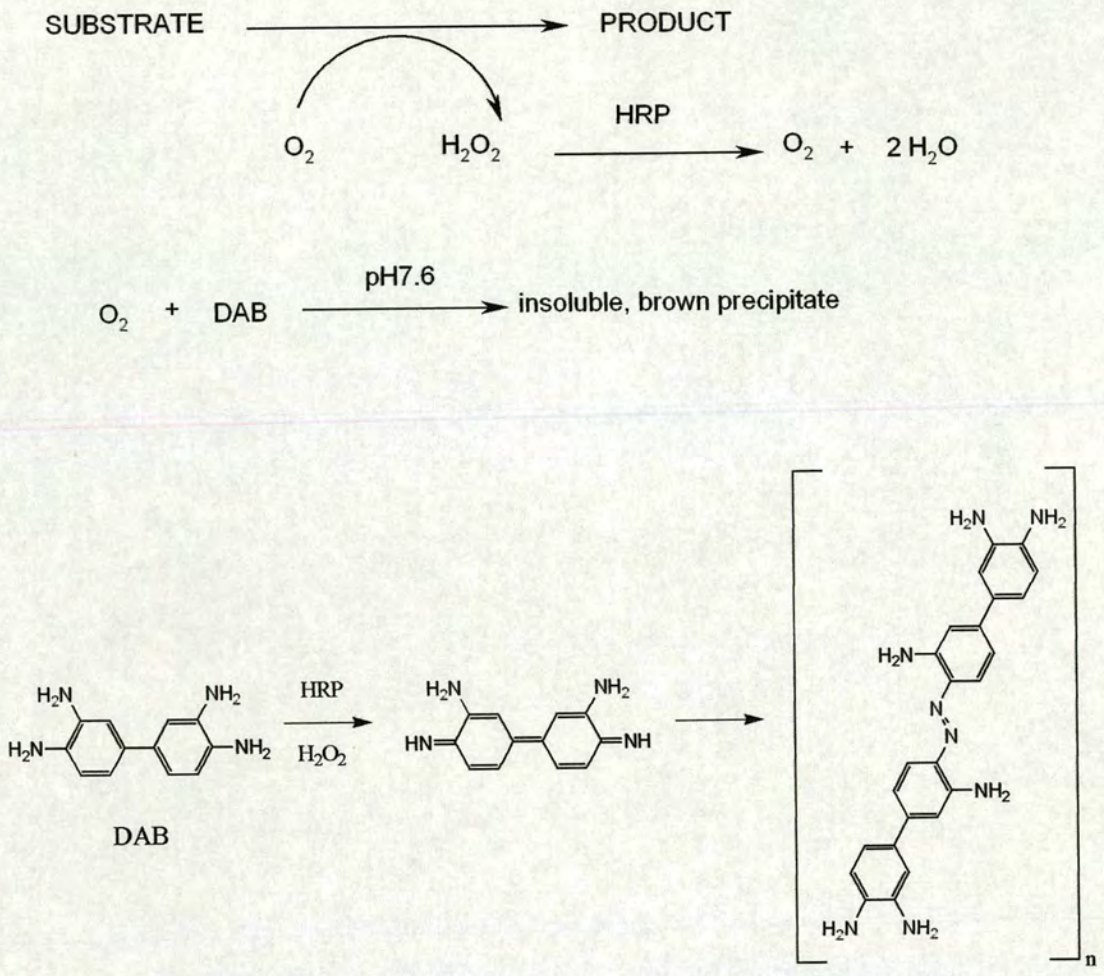


Figure 48: peroxidase-coupled insoluble dye formation assay.

The transformation and cell plating procedure was the same as for the soluble dye formation solid phase assay described above. Amylamine was used as a substrate. A membrane containing the colonies was incubated with the chromogenic solution containing 1% agarose (§ 8.2) at room temperature for several time intervals (§8.3.2; ii). On this occasion the coloured colonies began to develop significantly after 2 hours and the colour did not diffuse after prolonged exposure (Figure 49).

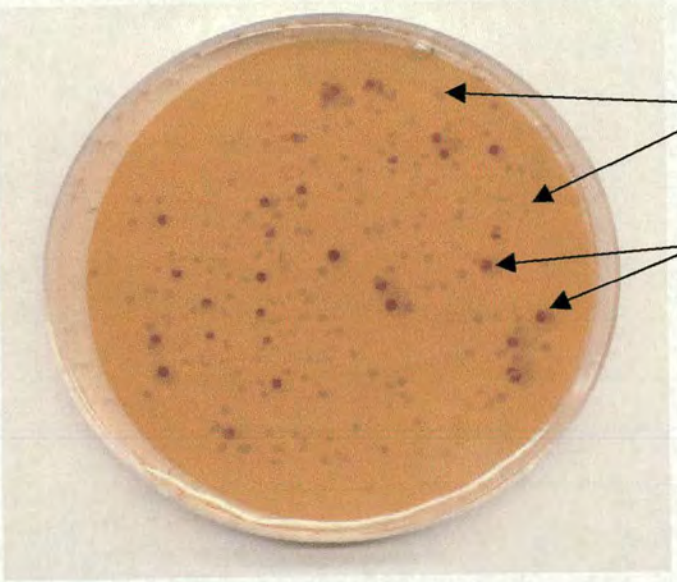
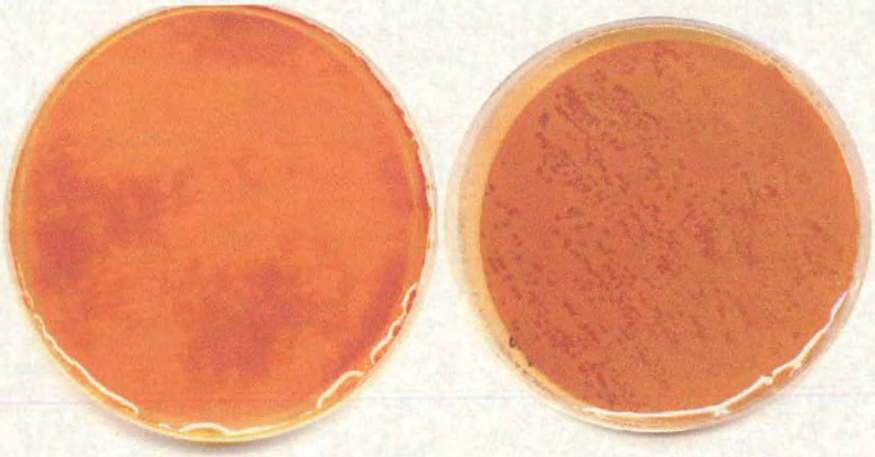


Figure 49: insoluble dye formation with solid phase assay (arrows A show positive colonies, arrows B show the negative colonies).

To further validate the screen, twenty positive colonies were picked from the membrane under the solid agarose gel with a yellow Gilson pipette tip. All of the colonies were recovered after 24 hours incubation at 30°C in 10ml LB Amp100 in 50ml Falcon tubes and the enzymatic activity was determined in the liquid phase soluble dye formation assay. The picked positive colonies were able to be recovered because some cells in the colony were left intact by freezing/thawing treatment.

Also, it was noticed that the colonies were able to be recovered under the agarose gel after several days of incubation at room temperature. This observation could be explained by a protective role of the agarose gel layer, which covers the colonies. In support of this protocol, we found that colonies grown on a membrane and not peeled from the LB agar plate prior to the freezing/thawing procedure, were subsequently not able to develop colour, presumably, because they were protected by the LB agar (Figure 50).



A B

Figure 50: different approaches to screen the MAO-N activity towards L-AMBA by the insoluble dye formation solid phase assay. The colonies on figure B were treated as shown on Figure 46. The figure A shows the result of assay if the nitrocellulose filter with the colonies was not peeled off from the LB agar prior to the freezing/thawing treatment.

2.2.6. *Application of high-throughput screening*

In the present work the two assays were applied to detect the activity of MAO-N, LAAO from snake venom (Sigma), DAAO from porcine kidney (Sigma) and LAAO from cyanobacterium *Synechococcus* PCC6301.[234] This demonstrates that both assays might be applied for other enzymes producing hydrogen peroxide in the catalytic reaction.

It should be noted that a similar solid phase assay has been used by Bylina *et al*[205] to detect the change of enzymatic properties of β-glucosidase from *Agrobacterium faecalis* in directed evolution experiments. The reaction involved the enzymatic

hydrolysis of an indolyl derivative used in the colorimetric solid phase assay. However, there are some important differences between the two procedures as shown in Table 6.

Reported protocol [205]	*Present protocol*
Enzyme expression obtained in two steps of growth (before IPTG induction and after)	Enzyme expression obtained in one step without IPTG induction.
Cells lysed by chloroform vapour	Cells partially lysed by freeze-thaw procedure
Peroxidase solution was spread manually on the surface of assay mix and allowed to diffuse into the gel over night at 4°C.	Peroxidase was mixed with warm (60°C) agarose
Membrane placed on the top of solid phase assay mix.	Membrane placed under the solid phase assay mix.

Table 6: difference between the reported[205] and present solid phase assay conditions.

We decided to compare the two procedures in practice to detect MAO-N activity towards amylamine. Freshly transformed *E. coli* BL21(DE3) cells were plated onto each of the two nitrocellulose membranes. The resulting colonies were treated by chloroform or by freezing/thawing according to either protocol. The chloroform treated membrane was placed on the top of the solid phase assay mixture and the latter was placed under the assay mixture. The rate of dye production in the published assay was faster, but the recovery of positive clones was a problem. Only one of three picked colonies was recovered after over night incubation in 10ml LB Amp100 at 30°C, whereas all three picked colonies were recoverable under the same conditions after incubation with the assay described in this thesis. This difference in recovery is probably due to the difference in cell lysis conditions *i.e.* freezing/thawing is milder then chloroform treatment.

Another experiment was set up to compare the two procedures. 10 month old *E. coli* BL21(DE3) colonies transformed with MAO-N and kept on a membrane at –20°C were subjected to both the published and solid phase assay described herein. Only one of three picked colonies again was recoverable from the published assay (membrane is "on" the gel) and all three colonies recovered from the present assay. This result may be possibly due to lower dye toxicity or to the protective role of agarose gel layer as was discussed in §2.2.5 (Figure 50).

2.3. Oxidation of D/L-α-methylbenzylamine by MAO-N

Initially the rate of oxidation of wild type MAO-N towards D/L-AMBA was investigated. pMAO-N was used to transform *E. coli* BL21(DE3) and to express MAO-N. *E. coli* BL21(DE3) transformed with pET16b was used as a reference. The oxidation reaction was carried out using whole cells grown in liquid media and by colonies grown on a plate.

2.3.1. Oxidation of DL-AMBA by whole cells

8 thawed whole cells pellets containing MAO-N and 8 reference pellets (§7.2.4; ii) were incubated with the chromogenic solution (§8.1.1) containing D-AMBA or L-AMBA at 22°C for 72 hours. At the end of the incubation period the cell suspension was centrifuged at 13,000 rpm for 5min and the absorption at 510nm was measured in both the reference samples (Table 7) and in the MAO-N samples (Table 8). The average Abs_{510nm} data for both the reference and for the MAO-N samples are in Table 9.

Sample	*L-AMBA (Abs 510nm)*	*D-AMBA (Abs 510nm)*
1	0.23	0.16
2	0.23	0.18
3	0.22	0.17
4	0.20	0.19
5	0.20	0.18
6	0.20	0.17
7	0.22	0.18

Table 7: L-and D- AMBA oxidation by reference samples (*E. coli* BL21(DE3) transformed with pET16b .

Sample	*L-AMBA(Abs 510nm)*	*D-AMBA (Abs 510nm)*
1	0.36	0.19
2	0.34	0.18
3	0.34	0.19
4	0.35	0.18
5	0.34	0.19
6	0.33	0.18
7	0.35	0.17
8	0.38	0.19

Table 8: L-and D- AMBA oxidation by MAO-N samples.

Sample name	*Average Abs510nm for L-AMBA oxidation*	*Average Abs510nm for D-AMBA oxidation*	*L/D oxidation ratio*
MAO-N	0.35 ±0.03	0.19 ±	1.90 ±0.3
Reference	0.20 ±0.03	0.18 ±	1.20 ±0.

Table 9: average data for L&D-AMBA oxidation activity and L- over D-AMBA oxidation ratio by MAO-N and by the reference samples.

The above data show that the whole cell culture can be used for initial activity studies as described in the present thesis. Even the small difference in activity between the sample culture and the reference culture can be detected. A 1.6 fold difference was detected towards L-AMBA oxidation and 1.6 fold difference towards the ratio of L-over D-AMBA oxidation. The initial 1.9 fold preference in activity of MAO-N towards L-AMBA was also established. The quality of this data was confirmed by solid phase assay (§2.3.2; Figure 51) and by assay of purified enzyme (§4.2.6.1; Table 18).

The specific activity of whole cells expressing MAO-N towards L-AMBA was calculated as 10^{-6} U/ml (U = μmol substrate oxidised per minute, ml - volume of whole cell culture).

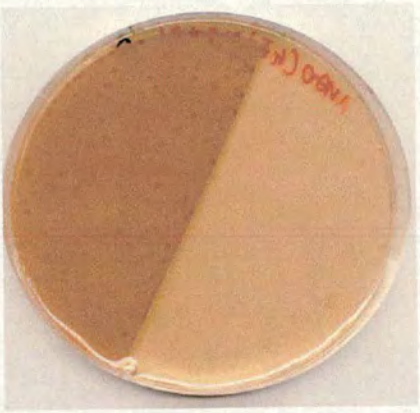
2.3.2. *Oxidation of DL-AMBA by colonies growing on a plate*

The membranes containing the thawed *E. coli* BL21(DE3) colonies transformed with MAO-N, and the reference colonies lacking the *mao-n* gene, were subjected to insoluble dye formation using the solid phase assay (§8.3.2; ii) in the presence of L-AMBA or D-AMBA. The membranes were cut in half to evaluate colonies under identical conditions. The color development was visualised over18 hours after which time it was clear that MAO-N preferentially oxidised the L-enantiomer of AMBA (Figure 51; entries 1&2). The reference samples showed only very small enantioselectivity. (Figure 51; entries 3&4)

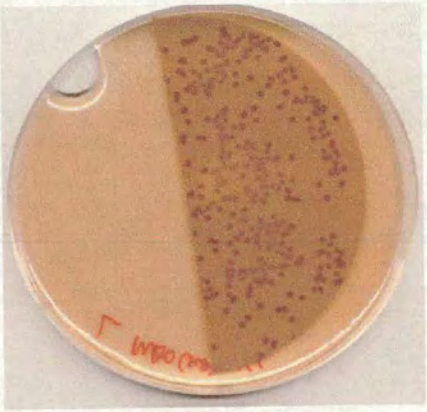
Both assays in whole cell culture (§2.3.1, Table 9) or using intact colonies of reference culture lacking *mao-n* gene (Figure 51, entries 3&4) indicated the presence of endogenous amine oxidase enantioselectivity in *E. coli* towards AMBA. This was presumed to be to the presence of an endogenous Type I amine oxidase in *E. coli*.

The Type I (copper-containing quinoprotein) enzyme expression was determined in *E. coli* K12 culture growing in presence of 2-phenylethylamine.[235]

The presence of the Cu(II) and TOPA was established by e.s.r. spectroscopy and by a characteristic peak at around 480nm in visible spectrum. The molecular weight of this enzyme was determined as 70kDa.

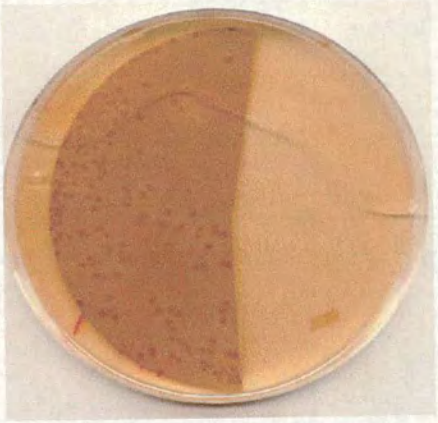


I. D-AMBA; MAO-N



II. L-AMBA; MAO-N

III. D-AMBA; reference



IV. L-AMBA; reference

Figure 51; application of the solid phase assay to assess the enantioselectivity of MAO-N towards L-and D-AMBA.

There is the evidence in the literature that the *E. coli* copper-containing quinoprotein amine oxidase is able to catalyse the enantioselective oxidation of amphetamine with preference for the (*R*)- enantiomer ($E \sim 15$).[111] The existence of copper and 3,4,6 trihydroxyphenylalanine quinone (TPQ) cofactor containing amine oxidase in *E. coli*

K-12 was established by Cooper and co-workwers.[72] The amine oxidase (AO) was found in the bacterial periplasm and the molecular weight of the enzyme was determined as 80kDa by SDS PAGE. The nucleotide sequence of the gene encoding AO from *E. coli* (*mao-A*) and the amino acid sequence of the mature enzyme (MW=81,295) were determined by Azakami and co-workers.[71]

The novelty of the above data is the establishment of enantioselectivity of the reference culture towards AMBA with preference for the (L)-enantiomer (Tables 7 & 9; Figure 51, entries 3 & 4) as well as of MAO-N towards L-enantiomer (Tables 8, 9 &18; Figure 51, entries 1&2).

The initial enantioselectivity of MAO-N towards L-AMBA can be a good starting point to develop this property further by enzyme directed evolution *in vitro*.

3. Results and Discussion: Random mutagenesis by *E. coli* XL1-Red

Recently, many laboratories have begun using the polymerase chain reaction to generate random mutations in genes of interest. This is of particular value with genes that have no selectable phenotype. This method exploits the inherent infidelity of *Taq* DNA polymerase during this reaction. By varying the reaction conditions ($MgCl_2$ concentration, adding $MnCl_2$, or unbalancing the concentration of the four dNTPs), the PCR process can yield random mutations at a rate up to seven base pairs per 1000 during 30 PCR cycles.[163,164]

A low error rate (2–3 base substitutions or ~1 amino acid substitution per sequence per generation) accumulates beneficial mutations, whereas higher error rates often generate neutral and deleterious mutations more frequently than beneficial ones. But there is a problem associated with this procedure. The gene of interest must be recloned with high efficiency into a suitable vector after the PCR reaction to generate

a highly representative library. The serious problems in finding appropriate conditions for the PCR itself as well as for the efficient ligation of PCR products have been encounted by researchers during directed evolution experiments.[178] This makes the method very time-consuming and expensive.

As an alternative, we used a commercially available mutator strain lacking DNA repair mechanism and called XL1-Red.[170,176] It has been demonstrated that if the clone of interest is propagated in XL1-Red in a high copy number plasmid (*i.e.*, pBluescript, pUC-derived), over 30 generations of growth (~ 2 hours needs to produce 1 generation), this will result in approximately one base change per 2000 nucleotides. If the gene of interest is 2000bp in length, then on the average, every single isolate should have one point mutation.

In addition, if the gene of interest is cloned in a lower copy number plasmid (i.g., pBR322, pACYC177), the XL1-Red strain can still be used in the manner described, except that the clone should be propagated in the mutator strain for additional generations to account for the lower relative gene dosage of the target sequence. The majority of obtained mutations were reported as transitions, but also some transversions and insertions occurred.

The process of propagating a clone in the mutator strain makes it extremely easy and cost-effective to generate random mutations in a gene that does not have any selectable or screenable phenotype. Alternatively, if there is a genetic screen or colorimetric screen to monitor mutations, the mutator strain makes it very easy to isolate mutants of interest.

A comparison of error-prone PCR and the mutator strain as mutagenesis tool is shown in §1.3.1.1; Table 1.

3.1. Production of libraries of plasmid mutants

Two different approaches were used to produce a library of mutated plasmids. The first method involved producing a library of MAO-N mutants cloned in pMAO-N and β-galactosidase mutants cloned into pUC18. The latter construct was used to establish the mutagenic efficiency of *E. coli* XL1-Red mutator strain using this approach. This strain contains mutations in the mismatch repair pathway (*mut*S), in the oxo-dGTP repair pathway (*mut*T), and in the 3'-5' exonuclease subunit of DNA polymerase III (*mut*D). The *mut* D mutation is more relevant to genes cloned into a non-colE1 derived vector which requires DNA polymerase III for replication. The PET16b and pUC18 vectors are colE1 derivatives and require mostly DNA polymerase I for replication. To establish the rate of spontaneous mutagenesis by combined action of *mut*S, *mut*T and *mutD* deficiency, pUC18 was used as a model.

3.1.1. First method of making mutations

Transformed cells were subjected to repeated over night growth with reinoculations. The published protocol[200d] was followed with some modifications (§9.1.2). The plasmid of interest (§9.1.1) was used to transform *E. coli* XL1-Red competent cells (Stratagene, La Jolla, CA) using the manufacturer's protocol. The suspension of transformed cells was used to inoculate LB Amp100 and the culture grown over night (18 hours) (Transformation 1). Approximately 12 generations of cells were produced (*E. coli* XL1-Red doubling time – 90 – 120 min).

After 12 generations of growth, the plasmid was purified (pMAO1 and pUC18.1 libraries). 20μl of this culture was then added to 10ml of fresh LB Amp100 media and subjected to another mutation cycle over 24 hours. From this sample a 1ml volume was centrifuged, the plasmids were isolated (pMAO2 and pUC18.2 libraries). This mutation cycle was repeated to produce the library of mutants after12,

24, 36,48, 60, 72, 84 and 96 cells generations (pMAO1 – pMAO8 and pUC18.1 – pUC18.8 libraries). The detailed protocol of this method is described in §9.1.2. Some of the plasmid libraries were used to transform expression host and subjected to screening.

3.1.2. Second method of making mutations

The second method was developed to minimise the production of sibling mutants during amplification. Another reason was to avoid the rapid accumulation of mutations in the host genomic DNA which, can lead to the loss of genotype specificity and viability.[176,236]

The second method differed from the first method in that retransformation of the fresh mutator strain was undertaken every two cycles of growth. The detailed protocol of this method is described in §9.1.3. Plasmid pMAO2 obtained in the first method was used to transform *E. coli* XL1-Red (Transformation II). The total transformed cell suspension was used to inoculate 20ml LB Amp and grown for 24h. The plasmid was purified and named pMAOretr1.1 library. The transformation II growing culture was used to inoculate LB Amp. The culture was grown for 24h, the plasmid purified (pMAOretr1.2 library) and used for Transformation III. The total transformed cell suspension was used to inoculate LB Amp, grown for 24h and the plasmid purified (pMAOretr2.1 library). The transformation III growing culture was used to inoculate LB Amp, grown for 24h, the plasmid purified (pMAOretr2.2 library) and used for Transformation IV. Total suspension of transformed cells was used to inoculate LB Amp, grown for 24h and the plasmid purified (pMAOretr3.1 library). The transformation IV growing culture was used to inoculate fresh 10ml LB Amp, grown for 24h and the plasmid purified (pMAOretr3.2 library). Each library was obtained after approximately 24 generations of growth of fresh cells. Some of the obtained plasmid DNA libraries (pMAOretr2.2, pMAOretr3.1, pMAOretr3.2) were used to transform *E. coli* BL21(DE3) cells to express mutated MAO genes and detect their activity towards the oxidation of L-α-methylbenzylamine (L-AMBA).

3.2. Determination of the mutagenic efficiency of the mutator strain

Seven pUC18 plasmid libraries (pUC18.1 – pUC18.7) obtained by the second mutagenesis method were subjected to white/blue colony screening (§9.2.1). The white colonies represented a lack of β-galactosidase activity caused by mutation. Seven plasmid libraries (pUC18.1 – pUC18.7) were used to transform *E. coli* Top 10 competent cells (Invitrogen) using the manufacturer's protocol with modification.

As described in §9.2.1, the transformed cells were plated on LB Amp100 agar supplemented with X-Gal (5-bromo-4-chloro-3-indolil-β-D-galactozide, substrate for β-galactosidase; §11.4). *E. coli* Top 10 cells do not require IPTG to induce expression from *lac* promoter. Using this method, approximately 2000 colonies per plate were obtained.

The efficiency of the mutator strain to mutate pUC18 plasmid which has the same origin of replication as pET16b, is clearly shown in Table 10. The frequency of inactivation of the β-galactosidase α-fragment produced by pUC18 was quantified by the number of white colonies per 1000 transformants. *E. coli* Top 10 cells transformed by non mutagenized pUC18 were used as a control.

Generations number		*White colonies per 1000 colonies*
PUC18.1	12	2
PUC18.2	24	8
PUC18.3	36	8
PUC18.4	48	12
PUC18.5	60	10
PUC18.6	72	26
PUC18.7	96	26

Table 10: the efficiency of *E. coli* XL1-Red mutator strain to inactivate β-galactosidase.

In conclusion, mutations in pUC18 plasmid DNA, propagated for 96 generations in XL1-Red, were generated at a frequency of at least 2.6%. Given that the β-gal α-fragment is tolerant of significant structural perturbations, the actual mutation frequency, including conservative and silent mutations, could be much greater. This was not quantitated due to the other priorities of this work.

3.3. `Screening of the libraries of MAO-N mutants

Two attempts were undertaken to screen libraries of the MAO-N mutants. The first attempt is referred to as "the scrubbed colonies screening". In this case the liquid phase whole cell biotransformation was performed on washed or scrubbed colonies from an entire plate prior to the solid phase assay. The second attempt is termed " the intact colonies screening". In this case the formation of solid phase insoluble product was performed directly on *in situ* colonies from an entire plate.

3.3.1. The scrubbed colonies screening

Schematically, this method can be presented as followed. The library of mutated plasmids is used to transform the expression host. The resulting colonies are washed or "scrubbed out" with LB medium and cultured in a shaking incubator. An aliquot of the pooled of whole cell cultures with expressed enzymes (some of them being amplified MAO-N mutants) is subjected for assay. If the pool contains amplified positive mutants, then the activity towards a substrate of interest can be detected. The plasmid DNA from this pooled positive whole cell culture is purified and used for retransformation and further screening either by solid phase colorimetric assay or by a moderate throughput assay as appropriate. Thus the "scrubbed colonies" assay can be applied as a high throughput screen even when a colorimetric assay is not available.

The diagram of this method is presented on figure 52.

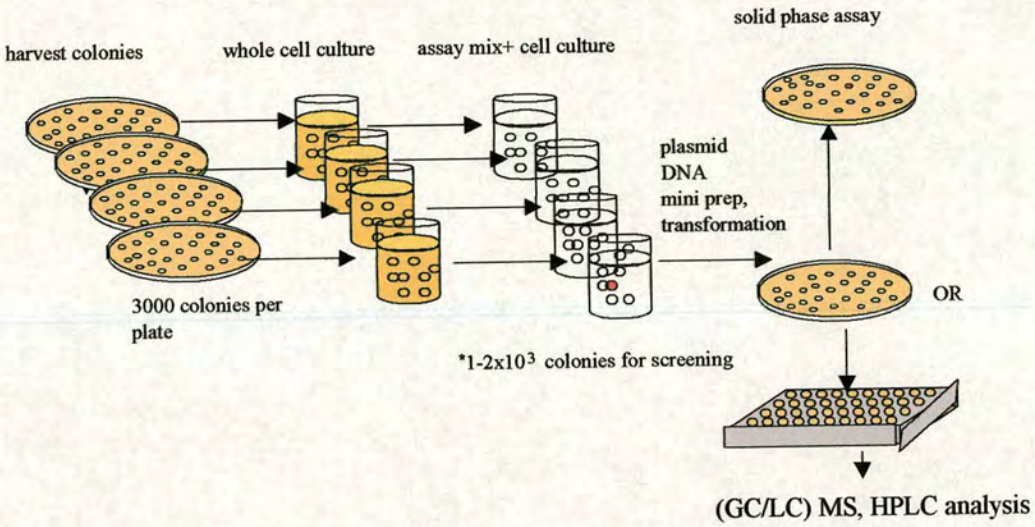


Figure 52: "The scrubbed colonies screening".

* also discussed in §4.1.2.

pMAO1, pMAO2, pMAO3, MAO8 (libraries produced by the first method) and pMAOretr3.1, pMAOretr3.2 (libraries produced by the second method) were subjected for the "scrubbed colonies screening" (§8.4). Each library was used to transform the expression host *E. coli* BL21(DE3) using the manufacturer's protocol with some modification as described (§8.4.1). Each library was plated on either two or three plates with 2000-3000 colonies on each. Additionally two plates containing cells transformed with pMAO-N served as baseline activity and two plates with cells harbouring pET16b served as the background activity reference or negative control. The colonies from each plate were washed out and subjected for assay as described in §8.4.2 in details.

Fifteen library cultures, two MAO-N scrubbed colonies as positive control and two reference negative control cultures were cultured over night and the OD_{600} was measured to confirm the identity of growth conditions. The OD_{600} varied from 13 to 7. The obtained cultures were assayed towards amylamine oxidation to confirm the enzyme expression. The average activity towards amylamine oxidation in the whole

cell cultures harbouring the mutated plasmid libraries and pMAO-N was 0.2 $U \cdot ml^{-1}$ compared to "no activity" in the reference negative control. The whole pellet from 1ml aliquots of culture was used to detect enzyme activity towards D/L-AMBA oxidation (§7.2.4; ii) and for plasmid purification. In order to do this, the frozen pellet was thawed and incubated with the chromogenic solution (§8.1.1) containing D-AMBA or L-AMBA. At the end of the incubation period the cell suspension was centrifuged and the absorption at 510nm in the supernatant was measured (Figure 53).

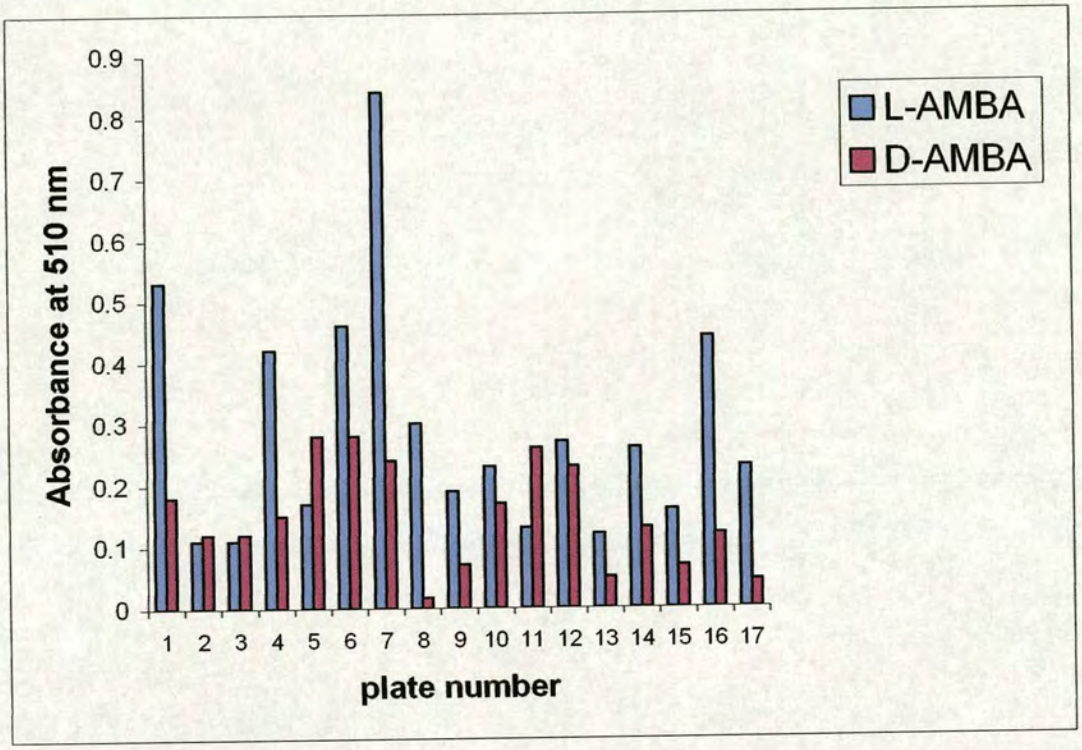


Figure 53: "Liquid phase" whole cell biotransformations performed on resuspended colonies from an entire plate. Blue line: L-AMBA as substrate, pink: D-AMBA. Plates number 8 and 14 are from wild type MAO-N.

As shown in Figure 53, the oxidation of L-AMBA by the culture from plate 7 (pMAOretr3.2, plate 3, $Abs_{510} = 0.80$) was significantly different to the control plates14 and 8 ($Abs_{510} = 0.26$ and 0.30 respectively). The OD_{600} value for culture 7 was 7.0 and for the control cultures – 10.0 and 13.0.

The plasmid from culture 7 (pMAO7a library) was purified and subjected to further screening using the "intact colonies solid phase assay".

3.3.2. The intact colonies screening

pMAO7a (library from scrubbed colonies), pMAO1, pMAO2 (libraries produced by first method), pMAOretr2.2, pMAOretr3.1, pMAOretr3.2 (libraries produced by the second method) were subjected to solid phase screening using insoluble dye formation (§8.3.1; §8.3.2; ii). Ten plates containing 2000-3000 colonies per plate were used to display the library(Figure 77,A). Positive clones were picked from the membrane and subjected to a second round of screening at low colony density (100-200 colonies per plate; §3.3.4, §8.3.3, Figure 77,B) to confirm activity. Plasmid DNA from the positive clone was isolated and used to confirm the enantioselectivity (Figure 77, C&D)

3.3.3. Identification of positive clones

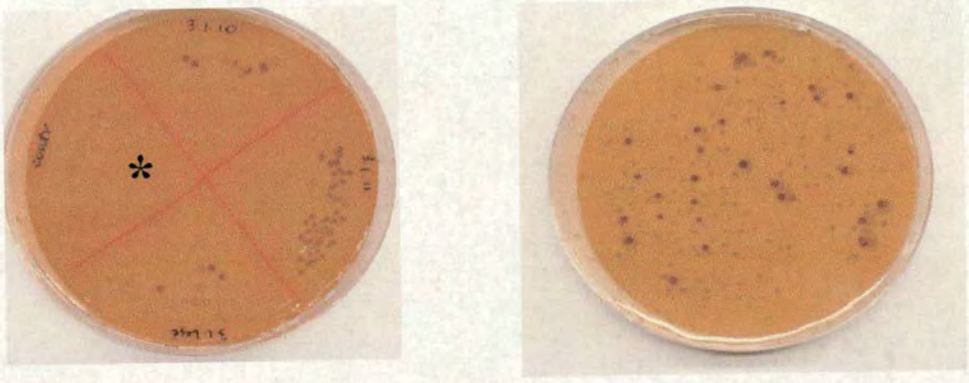
The library screens revealed a number of positive clones (Table11).

Library	*Positive colonies*	*Colonies subjected for second screening*	*Positive clones per 1000*
MAO7a	25	11	1
MAO1	2	2	0.08
MAO2	1	1	0.04
pMAOretr2.2	11	3	0.44
pMAOretr3.1	26	11	1.04
PMAOretr3.2	36	9	1.44

Table 11: identification of positive clones from the libraries.

3.3.4. Second screening of the libraries

The positive clones obtained from the first round screening were subjected to a second library screen (§8.3.3). In order to do that, *E. coli* BL21(DE3) was transformed by plasmid purified from the positive clone, obtained after the first screen. The transformed cells were transferred onto a membrane using a sterile loop (Figure 54; A) or by a spreading cell suspension onto the plate (approximately100 colonies per plate) (Figure 54; B).



A B

Figure 54: two different approaches for second round screening. Transferring positive colonies *via:* (A)-a loop; (B)-plate spreading. (*indicates a negative control (pMAO-N as the reference).

The first approach (Figure 54; A) was more economical because it allowed plating of three samples and one control cell cultures onto one membrane (£2 per membrane). Also, the presence of the control colonies together with sample ones on the same membrane allowed maintenance of the colonies under the same conditions during the assay. This was extremely beneficial to visualisation of the difference between the selected colonies.

Positive clones were picked from the membrane under the solid agarose using a yellow pipette tip and were subjected to a liquid phase whole cell assay with AA and D-,L-AMBA (§7.2.4; i,ii) using the chromogenic solution (§8.1.1).

4. Results and Discussion: Validation of positive clones

4.1. Validation of positive clones obtained from the "scrubbed colonies" screening

Six positive clones were selected after the second screening of the MAO7a library by the solid phase assay. All of them were subjected to further studies.

4.1.1. Validation of activity of positive clones in whole cell culture

Plasmid DNA from the clones was purified and used to transform *E. coli* BL21(DE3). Protein expression was performed in small scale (10ml) cultures (§7.2.2; i). A MAO-N wild type culture and the reference culture (BL21(DE3) transformed with pET16b) were grown at the same time. The reference culture was used to subtract the signal due to any background reaction.

The whole frozen/thawed cell pellet from the 1ml culture was used to perform a liquid phase assay with L-AMBA and D-AMBA as the substrates (§7.2.4; ii) for 96 hours, to confirm the positive nature of the clones. A 100 µl aliquot of the frozen/thawed pellet suspension (§7.2.4; i) was used to measure the initial rate of activity towards amylamine to detect the level of enzyme expression.

The activity of the mutants towards amylamine was comparable with the MAO-N wild type enzyme, although the activity towards L-AMBA was much higher. This observation supported the positive nature of the picked mutants (Figure 55). The activity towards D-AMBA also appeared to be higher, but the possibility of an artefactual result is high when measuring a low absorption reading. Moreover, the determination of enzyme activity in a whole cell is not a precise method at very low turnover numbers and allows qualitative, rather then quantitative differences to be detected.

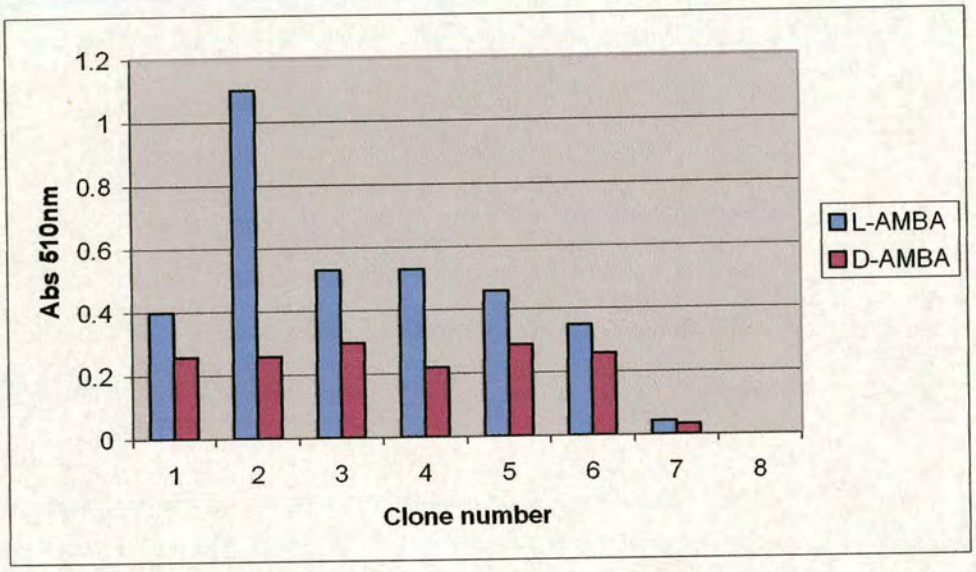


Figure 55: activity in whole cell culture of the six positive clones identified from MAO7a library screening. Blue line: L-AMBA as substrate; pink: D-AMBA; clone 7 is the MAO-N wild type control sample.

4.1.2. Validation of positive clones by sequencing

The MWG-BIOTECH facilities were used to perform sequencing reactions. The plasmids were prepared according to the MWG-BIOTECH requirement and provided together with forward (pETFor), reverse (pETRev) and internal (Int2) oligonucleotide primers (§10.1; §10.1.1;§10.1.2) .The sequencing analysis revealed

the same mutation in all six clones. Glycine (G) at position 403 had been replaced by glutamic acid (E) by changing the codon from GGG to GAG. The sequencing result (§4.2.4) also revealed the clone with this mutation (G403E) in the pMAOretr3.2 library, which was screened by "intact colonies screening" (§3.3.2).

The fact that all six positive clones had the same mutation indicated that the original increase in activity detected in the whole cell assay of scrubbed colonies was not an artefact. The occurrence of positive clones was one per 1000 colonies due to the original mutant clone amplification. This result suggests that the scrubbed colonies assay might be an alternative approach to "intact colonies" assay when the colorimetric screen is not available (Figure 52).

The "scrubbed colonies" method is more labour intensive compared to the colorimetric "intact colonies" assay, however it should be noted that this method required the manual screening of only 1000-2000 colonies to obtain a variant with desired property.

4.1.3. Validation of positive clones by purification studies

The protein of MAO-N mutant G403E was purified to homogeneity using metal chelating affinity chromatography (§10.4.1). The mechanism of protein affinity to the nickel chelating resin is described in §4.2.6.2. MAO-N wild type protein was purified at the same time.

The G403E MAO-N mutant was purified at a concentration of 0.14 mg/ml and MAO-N wild type at 0.10 mg/ml. 10 µl and 100 µl samples of the purified oxidases were subjected to the liquid phase soluble dye formation assay (§ 8.1.1) with amylamine (AA) and with L-AMBA respectively (Table 12).

Sample	*Measured parameters*				
	AA; Purified protein; Specific activity (U/m)g	*L-AMBA; Purified protein ; 5580min incubation (AU_{510nm} reading)*	*L-AMBA; Purified protein; Specific activity (U/mg)*	*D-AMBA; Purified protein; Specific activity (U/mg)*	*Activity ratio (L-AMBAover D-AMBA)*
MAO-N wild type	4.5	1.3	7.2×10^{-4}	4.4×10^{-5}	16
mMAO7a	4.0	3.6	1.6×10^{-3}	1.5×10^{-5}	106

Table 12: activity studies on partially purified mMAO7a mutant.

The activity towards amylamine was found to be unaffected, but the specific activity towards L-AMBA was increased approximately in 2.2 fold and was in agreement with the data obtained on whole cell culture (§4.1.1). Interestingly, the selectivity for L-versus D-AMBA was increased from *ca.* 16 to 106. The increase was due both to an increase in activity for the L-enantiomer and a decrease for the D-enantiomer.

The data in Table 12 allow assignment of this mutation as a "hot spot" and one which might affect the active site of the enzyme.

4.2. Validation of positive clones obtained by intact colonies screening

18 positive clones were selected after the second screening of pMAO1, pMAO2, pMAOretr2.2, pMAOretr3.1, pMAOretr3.2 libraries and subjected to the liquid phase soluble dye formation assay to confirm the positive nature of clones.

4.2.1 *Validation of the activity of the positive clones in the whole cell culture*

The small scale expression of the enzyme from each positive colony was performed (§7.2.2; i). The expression host transformed by MAO-N was cultured at the same time and served as a control. The activity assay towards amylamine (§8.1.1) was performed in whole cell culture (§7.2.4; i) to confirm the efficiency of protein expression.

The assay towards L-AMBA and D-AMBA (§8.1.1) in whole cell culture (§7.2.4; ii) was performed to confirm the positive activity of the isolated clones. The initial rate of reaction with amylamine was measured over 3 minutes and the OD_{510nm} was read after 96 hours incubation to find the activity towards L- and D-AMBA.

The results from the whole cell culture assay led to the identification of clones with improved activity towards L-AMBA compared to the wild type enzyme (Figure 56). Some clones also possessed increased activity towards amylamine. However two (identical) clones were clearly superior in terms of their selectivity and activity towards L-versus D-AMBA.

The activity of this "best" mutant against amylamine was significantly lower (~10 fold) compared to the wild type clone. This observation suggested that this mutant might possess interesting properties in terms of substrate specificity and improved enantioselectivity.

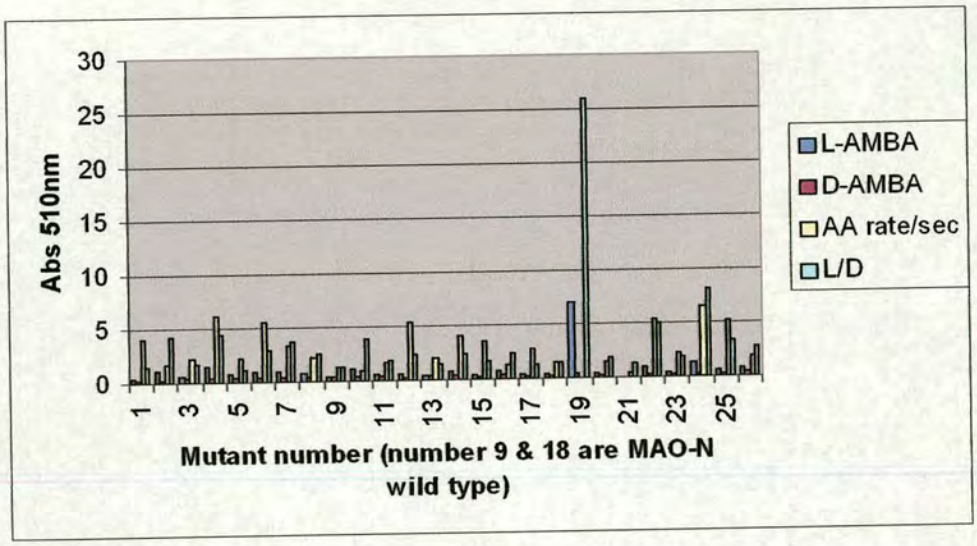


Figure 56: whole cell activity of the mutant clones identified by screening of intact (*in situ*) colonies. Blue line: L-AMBA as substrate, pink; D-AMBA, yellow, AA, light blue, ratio of activity towards L-versus D-AMBA. The data for L&D-AMBA oxidation was obtained by static absorption data measurement. The data for amylamine (AA) was measured as initial rate of oxidation reaction (v (AA)$10^{-3}s^{-1}$).

4.2.2. Determination of protein expression

Examination of the polyacrylamide gels (§7.2.5) showed that several of 24 positive clones appeared to be "expression" mutants: mutant 1 (clone 11 of pMAO7a library), mutant 4 (clone 6 of pMAOretr3.1 library), mutant 6 (clone1 of pMAO1 library), mutant 14 (clone 2 of pMAOretr2.2 library), mutant 22 (clone 7 of pMAOretr3.2 library), mutant 23 (clone 7 from library pMAOretr3.1), mutant 24 (clone 11 from pMAOretr3.1) and mutant 25 (clone 9 from library pMAOretr3.1). The "expression" nature of these mutants was evidenced as increased levels of MAO-N protein expression (Figure 57). All of the "expression" mutants possessed increased level of activity towards AA when examined in whole cell culture. However, mutant19 (the "best" mutant) (clone 10 from library pMAOretr3.1; Figure 57, entries 5&6 on a gel A and 3,4&10 on a gel B; Figure 56,entry 19) appeared not to have an increased

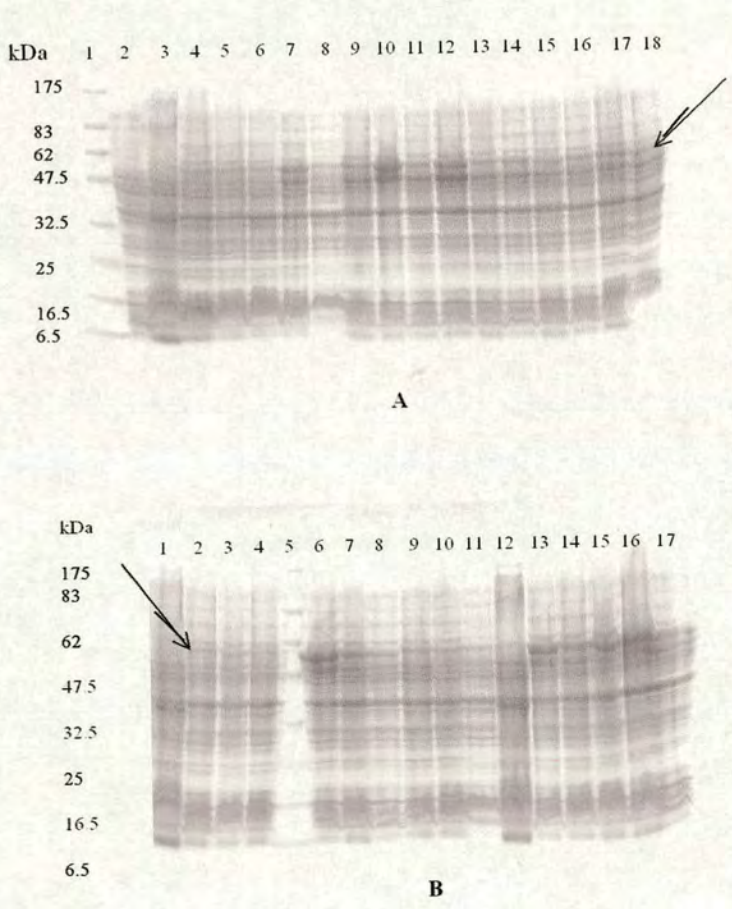
level of protein expression, suggesting that the mutation had affected the specific activity of the enzyme.

The high levels of protein expression in "expression" mutant clones could be due to a number of reasons:

(i) plasmid copy number mutants that have a higher gene dosage of the MAO-N wild type;

(ii) structural gene mutants which increase transcription/translation efficiency;

(iii) mutants in the gene regulatory signals for MAO-N activity that result in a greater quantity of the wild type MAO-N.



Fugure 57: SDS PAGE examination of the obtained mutants. Arrows point the band of interest (55kDa). * "Expression" mutants. MAOmut best is the mutant 19 (clone10 of pMAOretr3.1)

To distinguish among these possibilities, the DNA of these "expression" mutants was either analysed by agarose gel electrophoresis, stained with ethidium bromide, or subjected to the subcloning procedure described below (§4.2.3).

4.2.3 Validation of "expression mutants" by DNA manipulation

Some of the "expression" mutant clones (Figure 57) were chosen for further DNA examination by restriction digests: mutant 1, entry 7, gel A; mutant 4, entry 10, gel A; mutant 6, entry 12, gel A; mutant 14, entry 6, gel B; mutant 22, entry 13, gel B; mutant 24, entry15, gel B; mutant 25, entry 16, gel B.

Plasmid DNAs harbouring the MAO-N "expression" mutant genes were isolated and treated by restriction digest with restriction enzymes *Nde*I and *BamH*1 (§10.2.1) which are the restriction sites flanking MAO-N pET16b (§7.2.1).

The digested samples were examined by agarose gel electrophoresis. Mutant 1 (MAO7a library, clone11; Figure 58, entries 6&7) was found to have the same quantity of plasmid DNA compared to the wild type (Figure 58, entries 2-5).

This observation suggests that the increased level of protein expression was due to a mutation either within the structural gene or the genetic control region that results in the over expression of the native protein. The other mutants possessed more intense DNA bands, which could represent plasmid copy number "up" mutations.

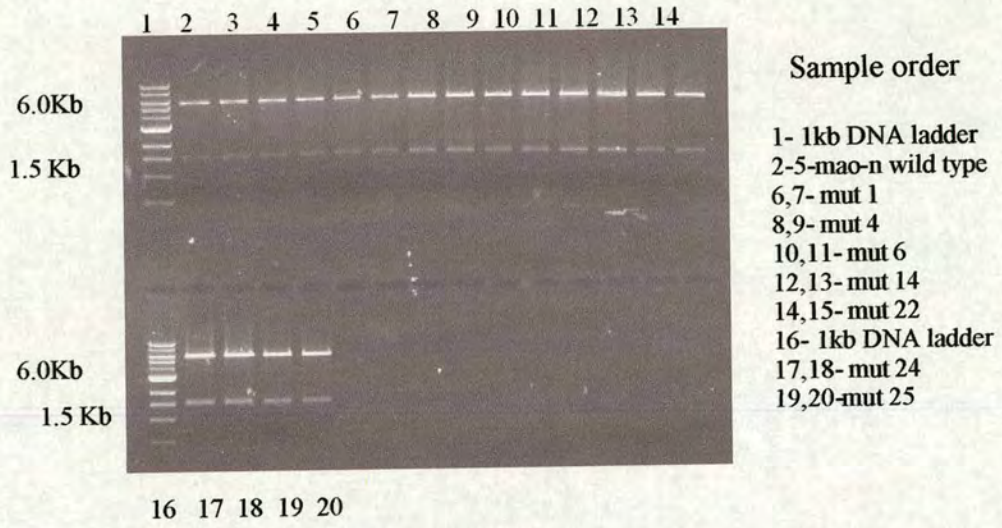


Figure 58: plasmid DNA analysis of "expression" mutants. (5.7kb-pET16b; 1.5kb-*mao-n*).

To identify which parts of the plasmid might contribute to the increased MAO-N expression level, a further subcloning procedure was undertaken. The vector portion of the mutated plasmids (Figure 58) was purified from the agarose gel. Wild type *mao-n* served as the insert to clone into the vector part of the mutants. If the increase in the mutant activity was due to the plasmid copy number, the total activity of MAO-N in whole cell culture should also increase.

Plasmid pMAO-N was purified to yield 115μg (OD_{260}=1.0 corresponds to approximately 50μg·ml^{-1} of DNA)[237] and the purity of the plasmid was determined by measuring the ratio of absorption reading at 260 nm versus 280 nm. These data provide an estimate of the purity of DNA. Pure preparation of DNA has an OD_{260}/OD_{280} value of 1.8. If there is contamination with protein or phenol, the ratio will be reduced and an accurate quantification of the amount of DNA is not possible[237]. The purified pMAO-N had an OD_{260}/OD_{280} value of 1.7.

pMAO-N was digested by *Nde*1 and *BamH*1 to separate the wild type *mao-n* gene from the vector and clone it into the vectors derived from the " expression" mutants (§10.2.2).

The following recombinants were obtained: pMAOrec1 (MAO-N wild type cloned in vector of mutant 1), pMAOrec4 (MAO-N wild type cloned into the vector of mutant 4), pMAOrec6, pMAOrec14, pMAOrec22, pMAOrec24, pMAOrec25 were obtained on the same manner. In each case, a single recombinant colony was used to perform the small scale expression of the enzyme (§7.2.2; i). At the end of induction the enzyme activity in whole cell culture towards AA was measured (§7.2.4; i). Two cultures of each recombinant and each "expression" mutant were grown at the same time to minimize results due to artifact. Two cultures of MAO-N wild type served as control (Figure 59).

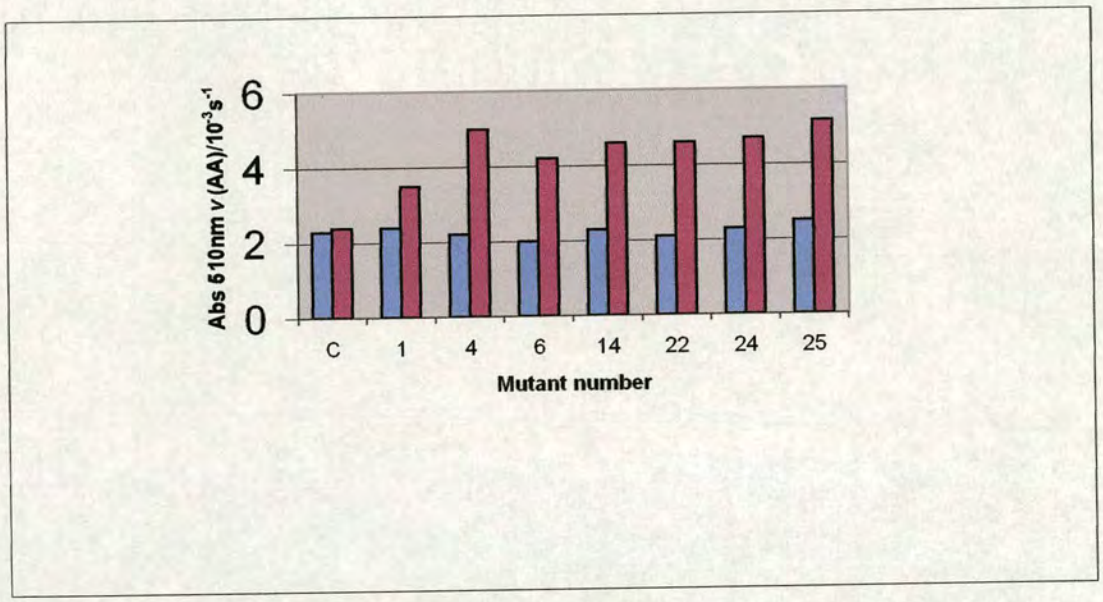


Figure 59: MAO-N activity in whole cell towards AA. Blue bars: MAO-N wild type cloned in "expression" mutant vector, red bars: "expression" mutants. C- MAO-N wild type.

The activity of the MAO-N cloned into the vectors derived from "expression mutants" was close to MAO-N wild type. This result suggested that the "expression" mutant's vectors were not affected by the mutator strain and that increasing level of the protein expression was due to mutations in the structural gene. This was supported by SDS PAGE examination of MAO-N cloned into "expression mutant"

vectors (§7.2.5) which showed no increase in the level of protein expression compared to MAO-N wild type (Figure 60).

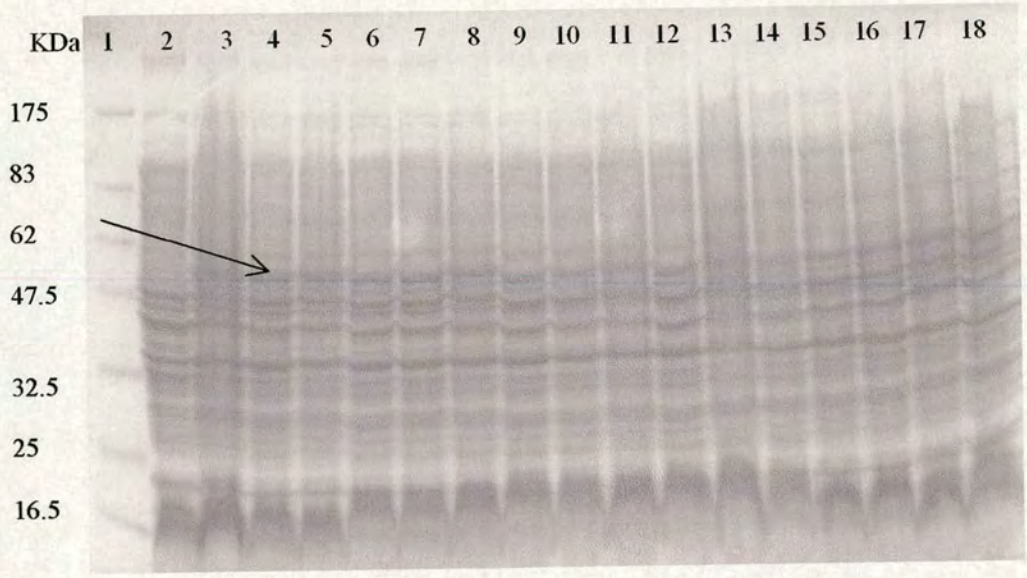


Figure 60: SDS PAGE examination of MAO-N expression level driven by vectors obtained from "expression" mutants. The arrow points to the band of interest. pMAOrec1 - MAO-N wild type cloned in vector of mutant 1, pMAOrec4 - MAO-N wild type cloned in vector of mutant 4; pMAOrec6 - MAO-N wild type cloned in vector of mutant 6, pMAOrec14 - MAO-N wild type cloned in vector of mutant 14, pMAOrec22 - MAO-N wild type cloned in vector of mutant 22; pMAOrec24 - MAO-N wild type cloned in vector of mutant 24; pMAOrec25 - MAO-N wild type cloned in vector of mutant 25.

The overall strategy used to study the "expression" mutants is schematically represented on Figure 61.

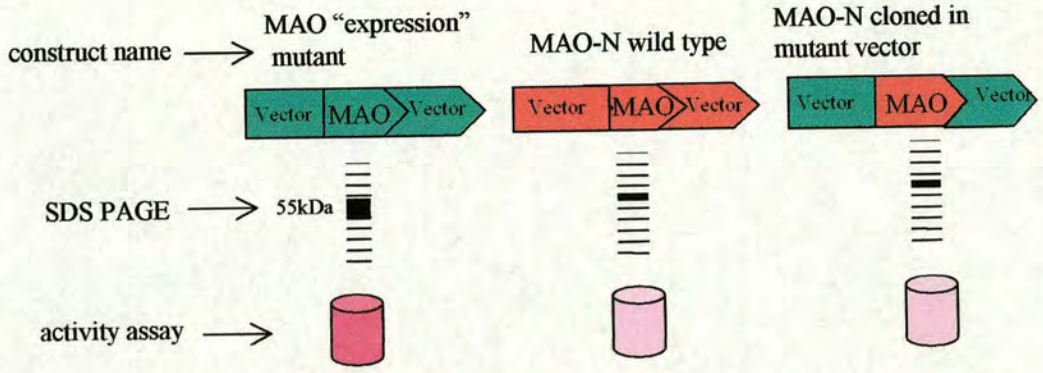


Figure 61: cloning, SDS PAGE examination and activity studies on MAO-N "expression" mutants.

The described DNA manipulation studies revealed that the plasmid bearing *mao-n* in the vectors chosen "expression" mutants were not involved in the over expression of protein and are not a subject for further studies.

4.2.4. *Identification of positive clones by DNA sequencing*

The *mao-n* gene DNA from the15 isolated mutants was subjected for sequencing studies. The MWG-BIOTECH facilities were used to perform the sequencing reactions. The plasmids were prepared according to the MWG-BIOTECH requirement and provided together with forward (pETFor), reverse (pETRev) and internal (Int2) primers (§10.1; §10.1.1; §10.1.2). The results of the sequencing analysis are in Table 13.

The sequencing results revealed that all of the "expression" mutants (4, 6, 22, 24 and 25) had the same mutation at position 260 wherein arginine was replaced by lysine. The MAO-N amino acid sequence has two arginines at positions 259 and 260. They are both encoded by codon (AGG) which has a low occurrence in *E. coli* genes (4%) The introduction of lysine instead of arginine in the MAO-N sequence does not change the charge of the protein but results in replacementof the low frequency codon for Arg AGG to the codon for Lys AAG (22 %). We concluded that this codon

change is preferential for higher protein expression in *E. coli* and decided to apply this mutation to achieve greater expression of the MAO-N wild type enzyme (§5.1).

The sequencing results also revealed the clone with a G403E mutation in pMAOretr3.2 library (mutant 11). This mutation is the same as that found in the pMAOretr3.2 library by sequencing of the "scrubbed colonies" (§4.1.2) lending further support to the efficiency of the latter method.

Library name	*Mutant name*	*Number of mutant*	*Genotype of the mutant*
pMAO1	MAO1.2	16	D385G(gat/ggt)
pMAO1	MAO1.1	6	R260K(agg/aag)
pMAO2	MAO2	8	A289V(gcg/gtg)
pMAOretr2.2	MAO2.2.1	26	D385A(gat/gct)
pMAOretr3.1	MAO3.1.6	4	R260K(agg/aag)
pMAOretr3.1	MAO3.1.1	7	M337R(atg/agg),
pMAOretr3.1	MAO3.1.2	10	D385G(gat/ ggt)
pMAOretr3.1	MAO3.1.5	5	G451S (ggt/agt)
pMAOretr3.1	MAO3.1.8	20	G451S(ggt/agt) R494C(cgt/tgt)
pMAOretr3.1	MAO3.1.9	25	R260K(agg/aag)
pMAOretr3.1	MAO3.1.11	24	R260K(agg/aag)
pMAOretr3.1	MAOmut"best"	19	N336S(aat/agt)
pMAOretr3.2	MAO3.2.6	11	G403E(ggg/gag),
pMAOretr3.2	MAO3.2.7	22	R260K(agg/aag)
pMAOretr3.2	MAO3.2.2	21	E145K(gag/aag)

Table 13: the mutations identified by sequencing studies.

Among the 15 sequenced clones, five mutants had the same mutation (R260K) and two had mutation D385G. These two mutations were introduced by the mutator strain after the first 18 generations (mutant 16, clone 2 from library pMAO1 and mutant 6, clone 1 from library pMAO1; Table 13) and then were amplified after

sequential transformations (mutants 4, 25, 24, 22 -R260K and mutant 10 – D385G). The other 8 sequenced mutants were produced throughout retransformations and revealed a great diversity of mutations (Table14).

Nucleotide changed	*Mutation name*
D385G(gat/ggt)	Transition
R260K(agg/aag)	Transition
A289V(gcg/gtg)	Transition
D385A(gat/gct)	Transversion
M337R(atg/agg),	Transversion
G451S (ggt/agt)	Transition
R494C(cgt/tgt)	Transition
N336S(aat/agt)	Transition
G403E(ggg/gag),	Transition
E145K(gag/aag)	Transition

Table 14: nature of mutations produced by the mutator strain

The frequency and quality of mutations produced by the mutator strain is in agreement with published results.[176,238] Approximately 1.2 mutations were produced per 1500bp gene after 12-24 generations of the mutator strain. Among 10 point mutations, transitions (80%) predominated over transversions (20%). The frequency of individual transitions was 30% for A → G, 40%, G →A, 20% C →T. There was one A →C and T →G transversion and no G →T, C→ G or G →C transversions. There were no insertions and deletions (Table 14).

Mutant 19, which produced the most intensely coloured colony in the solid phase assay screening and exhibited the highest activity in whole cell culture (Figure 56),

was named the MAOmut"best". This clone possessed a mutation at position 336 wherein asparagine had been replaced by serine.

4.2.5. Validation of MAOmut"best"

4.2.5.1. Validation of MAOmut"best" by site directed mutagenesis at position 336

To confirm the importance of the of Asn336Ser mutation, a site directed mutagenesis experiment was undertaken. The genes encoding for MAO-N wild type and MAOmut"best" were amplified by PCR using the external MAO For and MAO Rev primers (§7.2.1.1).

The internal primers MAOSer For and MAOSer Rev were designed to introduce serine at position 336 in the MAO-N wild type sequence. MAOmutAsn For and MAOmutAsn Rev were designed to introduce asparagine at position 336 in the MAOmut"best" sequence (§10.3.1.1).

Figure 62: solid phase assay screening of MAO-N wild type (N336), MAOmut"best"(S336) and their chimeras towards L-AMBA oxidation.

The introduction of the mutation was undertaken by serial PCR reactions (§10.3.1.2). Recombined genes were *Nde*I/*BamH*I digested, ligated into pET16b (§10.3.1.3) and transformed into *E. coli* Top10. 4 colonies from each ligation were chosen for plasmid DNA purification followed by transformation into *E. coli* BL21(DE3). The cells were plated on nitrocellulose membranes and subjected to solid phase screening to evaluate the resulting chimeras (Figure 62). The positive clones were sequenced to confirm the results of the site directed mutagenesis (§10.1).

The clone with the replacement of asparagine at position 336 to aspartate was produced accidentally and possessed lower activity towards AA compared to MAO-N wild type, and no activity towards L-AMBA. This clone (N336D) was also sequenced. Sequencing analysis revealed aspartic acid at position 336 in this clone, further confirming that position 336 is essential for the catalytic properties of MAO-N.

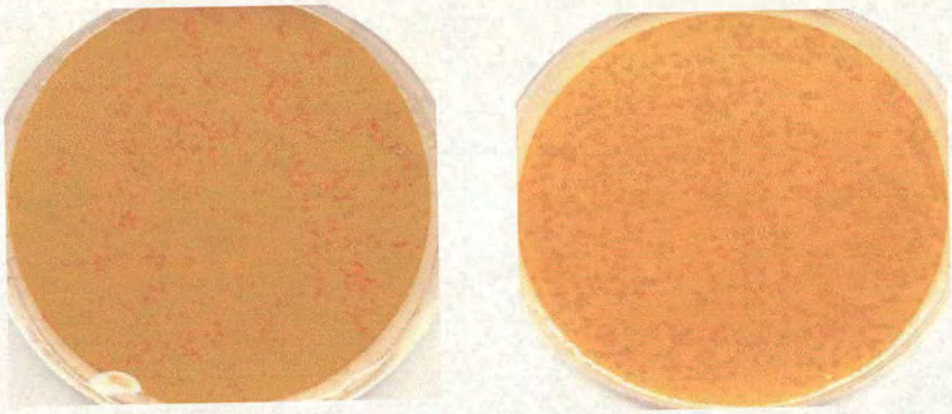
4.2.5.2. Confirmation of significance of N336S mutation by subcloning

To establish that the vector part of the plasmid pMAOmut"best" was not mutated by the mutator strain, the following additional subcloning procedure was undertaken. The c-DNA sequence encoding the amino acid at position 336 is located between *Not*I and *Mun*I restriction endonuclease sites. These restriction sites are unique in the *mao-n* sequence and not present in the pET16b sequence. Plasmid DNAs encoding MAO-N wild type and MAOmut"best" were therefore treated with *Mun*I/*Not*I restriction enzymes and crossover religated (§10.3.3). Ligation mixtures were used to transform *E. coli* BL21(DE3). The cells were plated onto nitrocellulose membrane and the desired constructs were obtained by solid phase screening. Positive clones were subjected to further study for determination of enzyme activity.

4.2.5.3. Activity studies on the site-directed MAO mutants and cloning chimeras at position 336

The site-directed mutants and cloning chimeras were subjected to small scale expression (§7.2.2; i) and assayed towards amylamine (§7.2.4; i) and L-AMBA (§7.2.4; ii) oxidation (Table 15). The activity against AA and L-AMBA was measured as an initial rate reading at λ=510nm. The data confirmed that the vector did not affect the activity of the MAOmut"best" towards L-AMBA but rather that the Asn336Ser mutation was the important one.

The presence of Asp at position 336 resulted in approximately a 10 fold decrease in activity towards amylamine and also the total absence of activity towards L-AMBA (Figure 63). The latter data confirmed the significance of the specific amino acid position 336 to the MAO-N phenotype.



MAO-N wild d type
A

MAO N336D mutant
B

Figure 63: solid phase assay screening of MAO-N wild type (A) and MAO N336D mutant (B) towards L-AMBA oxidation.

Enzyme	*Amylamine* ($\Delta A\ sec^{-1}$ @ 510_{nm})	*L-AMBA* ($\Delta A\ sec^{-1}$ @ 510_{nm})
MAO-N wild type (N336)	2.8×10^{-3}	as MAO-N wild type
MAOmut"best" (S336)	3.7×10^{-4}	4.8×10^{-5}
MAOmut"best" pcr chimera (S336 replaced to N)	2.0×10^{-3}	as MAO-N wild type
MAO WT pcr chimera(N336 replaced to S336)	8.0×10^{-4}	7.8×10^{-5}
MunI/NotI MAOmut"best" chimera (S336 replaced to N336)	2.1×10^{-3}	as MAO-N wild type
MunI/NotI MAO-N WT chimera (N336 replaced to S336))	9.0×10^{-4}	7.5×10^{-5}
MAO-N WT pcr chimera(N336 replaced to D336)	1.4×10^{-4}	No activity

Table 15: characterisation of MAOmut"best" by measurement of enzyme activity in whole cell culture. The data in blue denotes that the activity corresponds to the level of wild type activity; in red the activity corresponds to the level of MAOmut"best" activity; in black the activity which differs form both MAO wild type and MAOmut"best";

4.2.5.4. Mutagenesis at position 348

DNA sequencing of all the mutants, randomly obtained by using the mutator strain, revealed four differences in the amino acid sequence compared to the published sequence (*e.g.*. Schilling *et al.*[129] reported alanine at position 300 but valine was observed; L304 – published, V304 – observed; K348 – published, M 348 - observed and R450 – published, G 450 - observed). Two of these differences (K348M and R450G) had previously been noted by Sablin *et al.*[57], but had been missed by the author of the present thesis, so that K348M was initially considered as a "mutation" produced by the mutator strain. To investigate the significance of this change a mutant was constructed in which methionine at position 348 in the MAOmut"best" was reverted back to lysine. The gene encoding MAOmut"best" was amplified by PCR using the external MAOFor and MAORev primers (§7.2.1.1).

The internal primers MAOmutLys348FOR and MAOmutLys348REV were designed to introduce lysine at position 348 in the MAOmut"best" sequence (§10.3.2.1). The introduction of this mutation was undertaken as described in §10.3.2.2. The recombined gene was *NdeI*/*BamHI* digested, ligated into pET16b (§10.3.1.3) and used to transform *E. coli* Top10. 4 colonies were chosen for plasmid DNA purification followed by transformation in *E. coli* BL21(DE3). The cells were plated on nitrocellulose membrane and subjected to solid phase screening. The resulting chimeras were identified by solid phase assay as described in §4.2.5.1 (MAOmut"best" and MAO-N wild type were used as the controls). The presence of the double mutation (N336S and K348M) was confirmed by sequencing (§10.1).

The activity of one clone towards L-AMBA was clearly greater than MAOmut"best". A single colony of that clone was used to express the protein in medium scale culture (§7.2.2; ii) to determine the specific activity of a cell-free extract (§7.2.3; i) towards AA and L-AMBA by using the chromagenic solution (§8.1.1). MAOmut"best" and MAO-N WT were grown and purified under identical conditions (Table 16).

Enzyme (CFE)	*Protein (mg/ml)*	*Specific activity (U/mg)*	
		Amylamine	*L-AMBA*
MAO-N WT	5.0	0.30o	ND*
MAOmut"best"	5.0	0.06	3.0×10^{-3}
MAOmutN336S, M348K	7.0	0.05	4.0×10^{-3}

Table 16: specific activities of MAO-N WT and mutants at position 336 and 348. (ND*- not determined)

The data in Table 16 show that the double mutant produced higher specific activity (1.3 fold) towards LAMBA then MAOmut"best".

4.2.6. *Purification and characterisation of mutant enzymes*

4.2.6.1. *Purification of MAOmut"best"*

Both the wild type and MAOmut"best" enzymes were purified from *E. coli* BL21(DE3) harbouring pMAON or pMAOmut"best plasmid. *E. coli* is a known producer of Type I monoamine oxidase which was reviewed in §2.3.2. In order to eliminate possible interference from this endogenous enzyme, the CFE of a reference culture (*E. coli* BL21(DE3) harbouring pET16b was subjected to liquid chromatography purification at the same time. The reported protocol[57] was used for purification, but a ResourceQ column was used instead of a DEAE-sepharose (§10.4.2). Using this method the *E. coli* monoamine oxidase can be removed at the first stage of purification.[57] Purification of three CFE's was performed on the same day. The efficiency of purification of the two MAO enzymes was determined by measuring the specific activity of eluted samples towards amylamine (§10.4.4.1; Table 17). The highest activity was found in fractions 15 and 16 with MAO-N and MAOmut"best". There was no activity detected in fraction 15 and 16 with protein from the reference culture (Figure 64).

	Stage	Protein (mg/ml)	AA (Umg^{-1})	Yield [b] (%)	Fold [a] purification
MAO-N	CFE Resource Q	7.0 0.4	0.40 7.0	100 45	1 16
MAOmut"best"	CFE Resource Q	9.0 0.60	0.02 0.90	100 90	1 47
Reference	CFE Resource Q	2.0 0.03	0 0	- -	- -

Table17: efficiency of purification of MAO enzymes using anion exchange chromatography. U = μmol of amylamine oxidised per minute (§8.1.1).
[a]Data corresponds to the most active fraction.[b] Data corresponds to the two most active fractions.

The chromatography profiles of eluted proteins are shown in Figure 64.

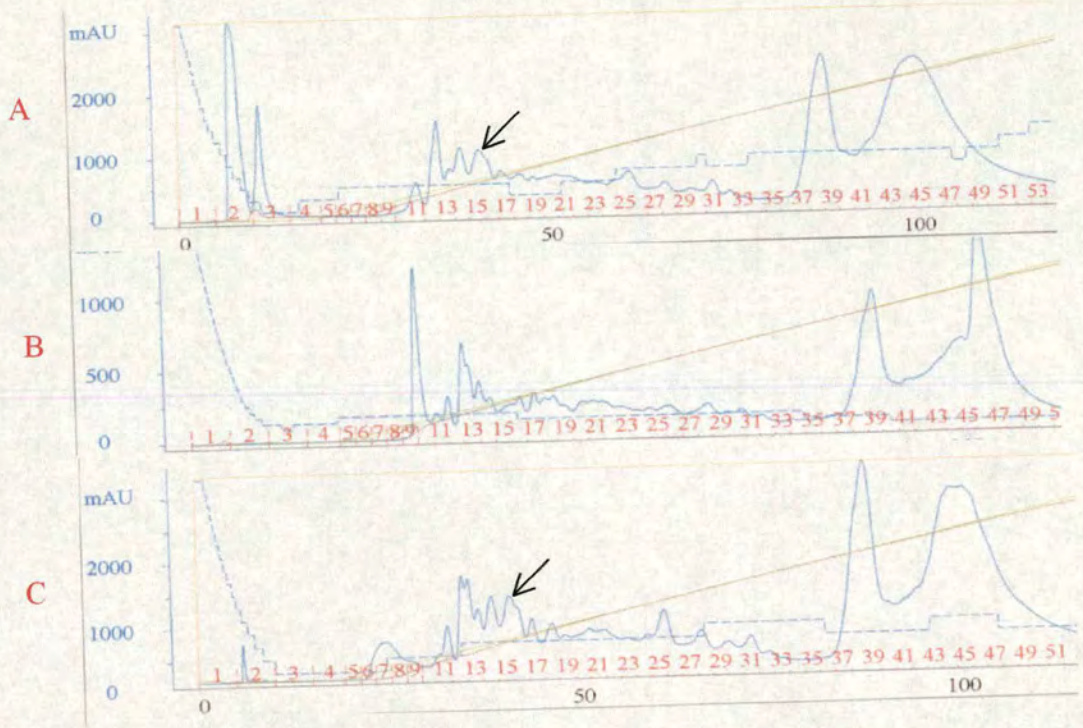
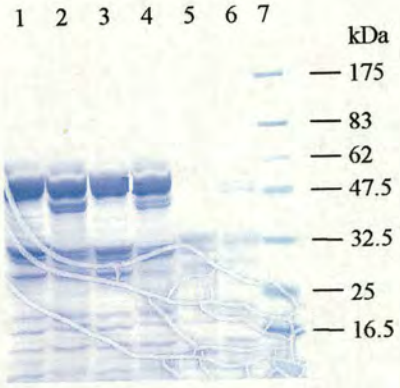


Figure 64: elution profiles of MAO-N from Resource Q column.
(A)-MAO-N wild type, (B) protein pool of reference culture (*E. coli* BL21(DE3) transformed by pET16b) and (C)-MAOmut"best"(N336S). Arrows point the elution peak of MAO-N and MAOmut"best".

The active fractions (15 & 16) were also analysed by SDS PAGE. SDS PAGE analysis showed a high concentration of MAO-N and MAOmut"best" in the purified active fractions at about 10% purity and the absence of a 70 kDa endogenous AO in any of preparations (Figure 65). Neither MAO-N protein nor endogenous AO were observed in the reference culture.



1) MAOmut"best" fraction 16
2) MAOmut"best" fraction15
3) MAO-N wild type fraction 16
4) MAO-N wild type fraction 15
5) BL21(DE3) [pET16b] fraction 16
6) BL21(DE3) [pET16b] fraction 15
7) molecular weight marker
MW(MAO-N) = 55.5kDa

Figure 65: SDS PAGE analysis of purified extracts (fractions 15&16) on Q-sepharose column.

Values of turnover numbers, k_{cat}, were measured per mol of flavoprotein subunit (§10.4.4.2; Table 18). The molar concentration of protein appeared to be 5 µM (50nM applied for assay) for MAO wild type and 7.2 µM (72nM applied for assay) for MAOmut"best".

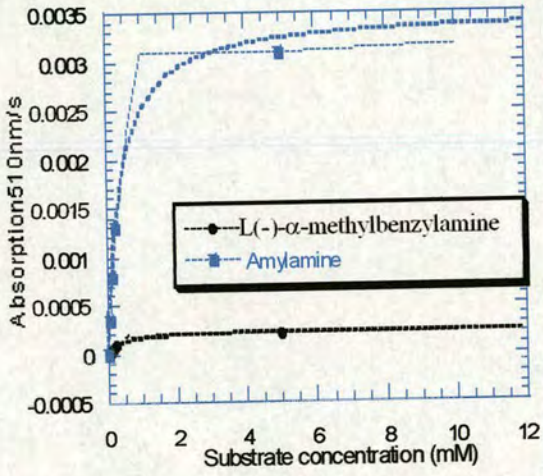
Values for K_M were measured for AA and L-AMBA as substrates. To determine K_M for MAOmut"best", activities were measured by determination of formation of H_2O_2 (§8.1.1) at different substrate concentrations (from 15nM to 10mM; §10.4.4.2).

The activity studies data is presented in Table 18.

Substrate	*MAO-N Wild type*		*MAOmut"best"*	
	K_M*(mM)*	k_{cat}*(min^{-1})*	K_M*(mM)*	k_{cat}*(min^{-1})*
L-AMBA	ND*	0.17†	0.4	8.0‡
D-AMBA	ND	0.01†	ND	0.08†
benzylamine	0.3**	370	ND	196
AA	0.25**	1000	0.4	116

Table 18: K_M, k_{cat} values for MAO-N wild type and MAOmut"best" using different substrates. * ND=not determined; **Published result[57]. † k_{cat} was obtained by measuring the initial

rate of oxidation to calculate the value for V_{max}, and protein concentration was obtained from molar extinction coefficient of FAD. ‡ k_{cat} was obtained from V_{max} calculated from measuring Abs $_{510nm}$ at the end point of 24h oxidation assuming linearity of reaction, protein concentration was obtained from molar extinction coefficient of FAD.



Value for L-(alpha)-methylbenzylamine		
	Value	Error
V_{max}	0.00023728	1.7497e-05
K_m	0.40082	0.084568

Value for Amylamine		
	Value	Error
V_{max}	0.0034997	0.000156
K_m	0.39598	0.050531

Figure 66: measurement of K_M and k_{cat} for MAOmut"best".

k_{cat} is the catalytic constant of an enzyme and reflects the number of enzymatic reactions (turnovers) that each active site catalyses per unit time: $k_{cat} = V_{max}$ / [Enz] V_{max} is the maximum velocity of a reaction, which occurs at high substrate concentration when the enzyme is saturated and [Enz]- molar enzyme concentration. The Michaelis constant K_M is the substrate concentration at which the reaction velocity is half maximum.

The eluted protein fraction from the reference culture did not show any activity towards the range of studied substrates. The data revealed that the activity of the MAOmut"best" towards L-AMBA (k_{cat}=8.0 min^{-1}) was 47-fold higher than the wild type enzyme (k_{cat}=0.17 min^{-1}). Moreover, the selectivity of the mutant for the L enantiomer versus D-AMBA (100:1) had also increased (5.8-fold) relative to the

wild-type enzyme (17:1). Thus, the outcome of the directed evolution experiments had been to simultaneously improve both the enantioselectivity and catalytic activity of the enzyme. For comparison, the activity towards the best substrate for the wild type, namely amylamine, and also benzylamine, is also presented.

4.2.6.2. Purification and characterisation of other mutants

The following mutant enzymes, presented in Table 13: number 4(R260K), 5 (G451S), 7(M337R), 8(A289V), 10(D385G), 11(G403E), 19(N336S; MAOmut"best"), 20(G451S;R494C), 21(E145K), 26(D385A), were subjected to purification by metal affinity chromatography as well as double mutant MAOmutN336S/ M348K, and MAO-N WT (§10.4.1). The procedure exploits the fact that many transition metal ions, *e.g.* zinc, nickel and copper, can coordinate to the amino acids histidine, cysteine, and tryptophan via Lewis basic groups on their respective amino acid side chains. In order to do so, the metal ion is first immobilised onto the chromatographic matrix and protein binding occurs at pH>7.0 to ensure that the amino groups are not protonated.

The high concentration of salt in the loading buffer prevents any ion-exchange effect. Elution of the protein can then be achieved by a decreasing pH gradient, which results in protonation of the metal binding group. An alternative elution protocol, which was employed here, involves the use of a competitive ligand (*e.g.* elution with an increasing concentration of imidazole).

The first attempt to express protein in medium scale culture (§7.2.2; ii) failed and we turned to the protocol for protein expression in large-scale culture (§7.2.2; iii). The protein expression was detected (§7.2.4; i, §8.1.1) and the bio-mass obtained (§ 7.2.2, iv) was subjected to purification (§10.4.1.1).

Such a difference in expression level was due to the procedure of inoculation of the (*ca.* OD600nm=1.0) rather then by a single colony in the case of the medium scale. The protein expression also suffered when the starting culture was used at stationary phase. This dependence of protein expression level on the age of a starting culture

could be explained by the presence of β-lactamase at the late stage of growth. The absence of antibiotic selection could lead to the large population of cells which are not able to express the protein of interest.

The eluted samples were diluted with 25mM Tris HCl pH 7.8 to lower the imidazole concentration to 75mM, in order to reduce the effect on enzyme activity. The diluted samples were concentrated using a 50K Vivaspin (2ml) filtration unit, stored over night on ice at 4°C, and then subjected to SDS PAGE analysis (Figure 67) and activity studies.

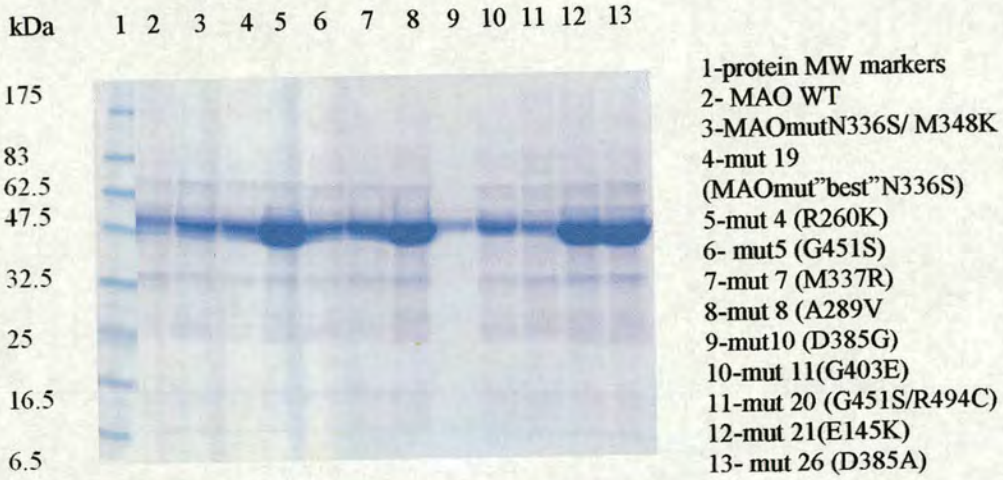


Figure 67: SDS PAGE of MAO and MAO variants purified by Ni affinity chromatography. All protein samples were purified at the same day to maintain the similarity of enzymes condition prior obtaining the activity data.

Protein concentration was determined by Bradford assay (§10.4.3) and the molar concentration of the enzyme was calculated from the flavin extinction coefficient and absorption measured at 458nm (§10.4.4.2). The activity towards AA was measured under standard conditions (§8.1.1) to establish the starting level of enzyme activity.

The k_{cat} towards AA was calculated as described in § 10.4.4.2. Specific activity was calculated as the amount of μmol (U) of substrate oxidised per minute and per

milligram of protein (§10.4.4.1). The volumetric activity (Uml^{-1}) was measured to reflect the amount of protein contributing to the activity (Table19).

Enzyme	*Protein (mg/ml)*	*Protein (μmols)*	k_{cat} *(min^{-1})*	*Volumetric activity (U/ml)*	*Specific activity (U/mg)*
Wild type	0.11	2	250	0.5	4.5
Mutant 20 G541S, R494C	0.14	2.5	208	0.5	3.7
Mutant 10 D385G	0.04	0. 7	150	0.1	2.7
Mutant 5 G451S	0.13	2.4	208	0.5	3.8
Mutant 7 M337R	0.17	3.1	325	1.0	5.9
Mutant 26 D385A	0.40	7.1	155	1.1	2.8
Mutant 11 G403E	0.14	2.5	245	0.6	4.0
Mutant 8 A289V	0.50	9.1	128	1.2	2.3
Mutant 4 R260K	1.0	18.0	156	1.1	1.0
Mutant 21 E145K	0.5	9.6	105	1.0	1.9
MAOmut"best" N336S	0.15	2.7	74	0.2	1.3
MAOmut N336S/ M348K	0.20	3.6	89	0.2	1.2

Table 19: preliminary activity studies on MAO-N enzyme mutants towards AA

The specific activities of MAO WT and MAOmut"best" towards AA were lower than after previous purification on the Resource Q column (§4.2.6.1;Table 17). This is presumably due to the loss of activity during the purification procedure and protein handling (protease inhibitors were not used at this time). Nevertheless, the data can

be used to compare the relative activity of the mutants since they were all purified at the same time, and under the same conditions as the MAO-N wild type.

The activity of the mutants 5,10,11,20 and the MAO-N wild type towards L-AMBA was calculated from the absorption reading at 510nm after incubation for 5580 minute (§7.2.4; ii, §8.1.1). The activity of all enzymes towards D-AMBA was measured on the same way. This reading was taken to calculate the V_{max} and k_{cat} (§10.4.4.2). A sample without enzyme was incubated in parallel and used as the reference.

The mutants 4,7,8, 21,26,19(MAOmut"best") and mut N336S/ M348K gave measurable initial rates using L-AMBA as a substrate and these values were used to determine the k_{cat} towards the latter substrate. The data obtained are shown in Table 20.

The final column in Table 20 gives the relative activity of the mutant enzymes towards L-AMBA compared to wild type. The data is in agreement with the previous studies using whole cell culture (§4.2.1; Figure 56), partially purified (§4.1.3; Table 12) and SDS PAGE analysis (§4.2.2; Figure 57).

The mutants 20, 5, 26, 8, 4 and 21 were initially detected (§3.3.2) due to increased level of protein expression and were designated as " expression mutants" by SDS PAGE (Figure 57). These mutants appear to have the same k_{cat} value as MAO wild type.

The mutants with higher turnover numbers (k_{cat}) than WT (10, 7, 19 (MAO mut"best") and double mutant N336S/ M348K), clearly possessed mutations, which affect the catalytic properties of MAO. These mutations allow the identification of "hot spots" within the enzyme, which appear to be important for activity. The data in the last two rows in Table 20 confirm that the MAOmutN336S/ M348K corresponds to an "expression" mutant of MAOmut"best".

Enzyme	*L-AMBA (k_{cat} min^{-1})* *I*	*D-AMBA (k_{cat} min^{-1})* *II*	*L-AMBA (U/ml)* *III*	*Data in I versus data in II* *IV*	*Data in IV relative to data for MAO wild type in IV* *V*	*Data in I relative to data for MAO wild type in I* *IV*
‡Wild type	3.0×10^{-2}	2.0×10^{-3}	8.0×10^{-5}	15	1.0	1.0
‡Mutant20 G541S, R494C	4.0×10^{-2}	1.0×10^{-3}	1.0×10^{-4}	40	2.7	1.3
‡Mutant 10 D385G	0.10	2.0×10^{-3}	1.0×10^{-4}	50	3.3	3.3
‡Mutant 5 G451S	3.0×10^{-2}	9.0×10^{-4}	1.0×10^{-4}	33	2.2	1.0
†Mutant 7 M337R	0.20	1.7×10^{-3}	6.0×10^{-4}	118	7.9	6.7
†Mutant 26 D385A	5.0×10^{-2}	1.0×10^{-3}	4.0×10^{-4}	50	3.3	1.7
‡Mutant 11 G403E	8.0×10^{-2}	7.0×10^{-4}	2.0×10^{-4}	114	7.6	2.7
†Mutant 8 A289V	1.5×10^{-2}	7.0×10^{-4}	2.0×10^{-4}	21	1.4	0.5
†Mutant 4 R260K	2.0×10^{-2}	4.0×10^{-4}	4.0×10^{-4}	50	3.3	0.6
†Mutant 21 E145K	3.5×10^{-2}	7.0×10^{-4}	4.0×10^{-4}	50	3.3	1.0
†"Best" N336S	4.5	1.0×10^{-2}	1.0×10^{-2}	450	30.0	150
†Mut N336S/ M348K	4.0	1;0 $\times 10^{-2}$	1.0×10^{-2}	400	27.0	133

Table 20: Activity of MAO mutants towards L- and D-AMBA. .† k_{cat} was obtained by measuring the initial rate of oxidation to calculate the value for V_{max}, and protein concentration was obtained from molar extinction coefficient of FAD. ‡ k_{cat} was obtained from V_{max} calculated from measuring Abs $_{510nm}$ at the end point of 24h oxidation assuming linearity of reaction, protein concentration was obtained from molar extinction coefficient of FAD.

4.3 Conclusion

The data obtained in this chapter clearly confirmed that the amine oxidase enzymatic activity could be detected by different approaches. The activity can be determined *in situ* by solid phase assay, by SDS PAGE, in whole cell culture and from purified enzyme. The determination of activity *in situ* was able to speed up the cloning procedure by eliminating the step of individual colony analysis. The activity in whole cell culture allowed analysis of a large number of samples in a short period of time. The importance of this multiple approach is that data obtained by one method can be supported by other approaches.

5. Results and Discussion: Application of MAO mutants obtained by directed evolution *in vitro*

5.1. Improvement of MAO-N WT expression

It has been shown in §4.2.4 (Table 13) that some of the higher expressing MAO mutants (6, 4, 24, 25, 22) contained a mutation at amino acid position 260, in which arginine has been replaced by lysine. The wild type MAO-N amino acid sequence has two arginines at position 259 and 260 both encoded by the codon (AGG), which has a low occurrence in *E. coli* genes (4%). Lysine and arginine are both basic amino acids and hence replacement of one by the other should not affect the charge of the protein, however the replacement of a low frequency codon for Arg AGG (4%) by a high frequency codon for Lys AAG (22 %) results in improved protein expression in *E. coli.* Thus we decided to replace the codons of both Arg 259 and Arg 260 with the codon CGT (38%) by site directed mutagenesis to evaluate the effect upon the expression level of MAO-N.

The genes encoding for MAO-N wild type were amplified by PCR by using the external MAO For and MAO Rev primers (§7.2.1.1). The internal primers MAOArg259/260 For and MAOArg259/260Rev (§10.5.1) were designed to introduce CGT codons at position 259 and 260 in the MAO-N wild type sequence. The site-specific mutations were introduced by the cloning protocol described in §10.5.1.

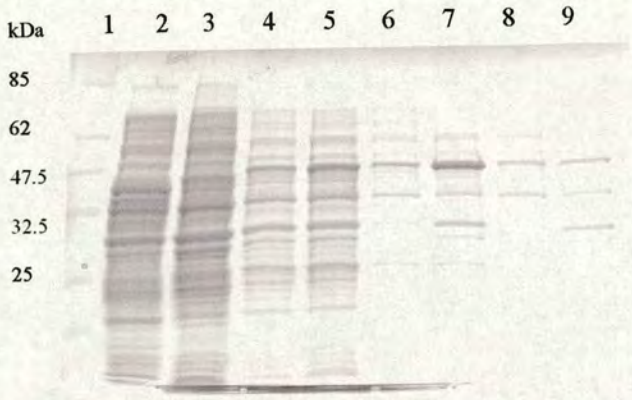
Four of the resulting colonies were analysed by the solid phase screening assay as described in (§4.2.5.1). One clone appeared to be significantly more active than MAO WT. This positive clone was named pMAOArg259/260 and subjected to further analysis. DNA sequencing of this clone revealed that the desired codon replacements had been achieved.

MAO WT and MAOArg259/260 were partially purified from recombinant *E. coli* BL21(DE3) harbouring a pET16b carrying the corresponding *mao-n* gene and subjected to small scale growth (§7.2.2.1; i). CFE's were prepared by sonication (§7.2.3; i). Protein concentration was determined by Bradford assay (§10.4.3) and the specific activities of MAO WT and MAOArg259/260 towards AA were measured for both the soluble and insoluble fractions (§10.5.2; Table 21).

Enzyme	*Protein concentration ($mg.ml^{-1}$)*	*Soluble fraction ($U.mg^{-1}$)*	*Insoluble fraction ($U.mg^{-1}$)*	*Total activity (U)*
MAO WT	1.20	0.20	0.30	0.60
MAOArg259/260	1.30	0.50	0.60	1.20

Table 21: specific activities of partially purified MAO WT and MAOArg259/260 (MAO wild type with replaced codons for Arg at positions 259 and 260) towards amylamine.

The total protein extracts from whole cell cultures of recombinant *E. coli* BL21(DE3) harbouring pECME3, pMAO-N and pMAOArg259/260 were analysed by SDS PAGE as well as soluble and insoluble fractions of MAO-N and MAOArg259/260 (Figure 69).



1- Protein MWmarkers
2 – BL21(DE3) [pET16b] reference (whole cells)
3 – pECME3 (whole cells)
4 - pMAO-N(whole cells)
5 - pMAOArg259/260 (whole cells)
6 - Soluble fraction from lane 5
7 - Insoluble fraction from lane 5
8 - Soluble fraction from lane 4
9 - Insoluble fraction from lane 4.

Figure 69: SDS PAGE analysis of protein expression in soluble and insoluble fractions. Protein expression was driven from different expression constructs: pECME3 (original plasmid from B.Shcilling), pMAO-N (*mao-n* geen with replaced codons at the beginning of the sequence (Table3, page 70) and MAO-N with replaced codons for Arg at position 259 and 260.

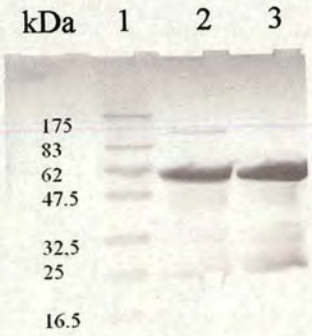
To confirm the increased level of the R260K mutant expression, CFEs of MAO WT, mutant 4 (R260K) and MAOArg259/260 were obtained (§7.2.3; iii) and assayed towards L-AMBA. This was achieved by incubating 50µl of each sample for 240 minutes and specific activities were determined (§10.4.4.1; Table 22).

Enzyme	ΔAbs_{510nm}	*Soluble fraction/* (Umg^{-1})
MAO WT	0.06	3.6×10^{-5}
MAOArg259/260	0.12	7.0×10^{-5}
MAO mutant 4 (R260K)	0.16	5.7×10^{-5}

Table 22 : specific activities towards L-AMBA of partially purified MAO WT, MAO mutant 4 (R260L) and MAOArg259/260.

The data in Tables 21 & 22 and in Figure 69 confirm the previous results obtained with whole cell cultures and by SDS PAGE analysis, namely that the activity increase towards L-AMBA is due to exchanging a low occurrence codon with a high one.

MAOArg259/260 was purified from a 4 litre culture inoculated with starting culture (§7.2.2, iii). A total 30g of biomass was used to obtain a CFE (§7.2.3, ii) and the latter was subjected to chelate affinity chromatography (§10.4.1.2; Figure 70). The concentration of pure protein was measured by Bradford assay and appeared to be 30 mg L^{-1} (3U.mg^{-1}) compared to a published result of 10 mg.L^{-1}.[57]



1- protein MWmarkers
2- after Ni column fraction 1
3-after Ni column fraction 2

Figure 70: purification of MAO Arg 259/260 by Ni chelating chromatography. The favorite codons for Arg 259 and Arg260 were introduced in MAO-N wild type gene to obtain larger level of protein expression.

Such an improvement in enzyme activity supported all the previous data regarding the "expression" mutant R260K obtained throughout the present directed evolution *in vivo* experiment.

5.2. Deracemisation of D,L-AMBA

The deracemisation of D/L-AMBA was achieved using MAOmut"best" enzyme. This experiment was performed by Dr. Alexis Enright. The substantial improvement in oxidase activity and selectivity of the mutant was confirmed by chiral HPLC (Chiralcel CrownPak CR+) in which the complete oxidation of the L-enantiomer of AMBA was achieved after 24 hours, whereas there was no detectable conversion of the D-enantiomer. This result facilitated the application of the mutant enzyme to the deracemisation of D/L-AMBA. A 77% yield of D-AMBA with an *ee* value of 93%

was obtained after deracemisation of 1mM D/L-AMBA in the presence of MAOmut"best" and an ammonia-borane complex as a reducing agent. The stereoinversion of L- to D-AMBA was also achieved in18% yield and 99% *ee* but under identical conditions there was no conversion of D- into L-AMBA.[239]

5.3. Studies on the active site of MAO-N

To assess the effects of the amino acid replacements in the mutant enzymes, a WU-Blast2 sequence alignment (EMBL-EBI) between MAO-N and MAO-B, the closely related enzyme with a published three-dimensional structure,[148] was performed. The amino acid sequence alignment revealed 24% sequence identity and 43% sequence similarity between the two proteins.

Figure 71 shows that L171, C172, F168, Y326 of the MAO-B active site correspond respectively to the identical amino acids in MAO-N (L213, C214, F210, Y365). Other similar amino acids possessing aromatic side chains include:

Y398 (MAO-B)/W430 (MAO-N); Y188 (MAO-B)/W230 (MAO-N); Y435 (MAO-B)/F466 (MAO-N); Y60 (MAO-B)/W94 (MAO-N).

The residue numbers on Figure 71 for MAO-B show one number difference compared to the numeration used in Figures 72 and 74.

Significant differences between MAO-B and MAO-N lie in residues that line the substrate cavity including F343 (MAO-B)/ T384 (MAO-N), Q206 (MAO-B)/ I246 (MAO-N) and two MAO-B isoleucines at positions 199 and 198 which do not have any corresponding residues in MAO-N sequence (Figure 72).

```
Query:   41 DVIVIGGGYCGLTATRDLTVAGFKTLLLEARDRIGGRSWSSNIDGYPY-EMGGTWVHWHQ 99
            DV+V+GGG  G+ A + L  +G   ++LEARDR+GGR+++        Y ++GG++V   Q
Sbjct:    5 DVVVVGGGISGMAAAKLLHDSGLNVVVLEARDRVGGRTYTLRNQKVKYVDLGGSYVGPTQ 64

Query:  100 SHVWR-----EITRYKMHNALSPSFNFSRGVNH-FQLRTNPTTS--TYMTHEAEDELLRS 151
            + + R      +  YK+ N +    +  +G ++ F+    P  +  TY+ H
Sbjct:   65 NRILRLAKELGLETYKV-NEVERLIHHVKGKSYPFRGPFPPVWNPITYLDH--------- 114

Query:  152 ALHKFTNVDGTNGRTVLPFPHDM-FYVPEFRKYDEMSYSERIDQIRDELSLNERSSLEAF 210
              + F       GR +   P D  +  P   ++D M+  E +D++     S  + ++L  F
Sbjct:   115 --NNFWRTMDDMGREI---PSDAPWKAPLAEEWDNMTMKELLDKLCWTESAKQLATL--F 167

Query:  211 ILLC-SGGTLENSSFGEFLHWWAMSGYTYQ--GCMDCLISYKFKDGQSAFARRFWEEAAG 267
            + LC +  T E S+   FL +    G T +     +     KF  G    + R  +
Sbjct:   168 VNLCVTAETHEVSALW-FLWYVKQCGGTTRIISTTNGGQERKFVGGSGQVSERIMDLLGD 226

Query:  268 TGRLGYVFGCPVRSVVNERDAARVTARDGREFAAKRLVCTIPLNVLSTIQFSPALSTER- 326
              +L      PV  +  R+   V   +  + AK ++  IP  +   I F+P L   R
Sbjct:   227 RVKLER----PVIYIDQTRENVLVETLNHEMYEAKYVISAIPPTLGMKIHFNPPLPMMRN 282

Query:  327 --ISAMQAGHVNMCTKVHAEV--DNKDMRSWTGIAYPFNKLCYAIGDGTTPAGNTHLVCF 382
              I+ +  G V  C   + E      KD      I      + Y + D T P GN +
Sbjct:   283 QMITRVPLGSVIKCIVYYKEPFWRKKDYCGTMIIDGEEAPVAYTLDD-TKPEGN-YAAIM 340

Query:  383 GTDANHIQPDEDVR----ETLKAVGQLAPGTFGVKRLV--FH----NWVKDEFAKGAWF- 431
            G    H +  + R    E LK + +L     G    H    NW +++++ G +
Sbjct:   341 GFILAH-KARKLARLTKEERLKKLCELYAKVLGSLEALEPVHYEEKNWCEEQYSGGCYTT 399

Query:  432 FSRPGMVSECLQGLREKHRGVVFANSDWALGWRSFIDGAIEEGTRAARVVLEELG 486
            +  PG++++  + LR+    + FA ++ A  W  +++GA+E G RAAR +L  +G
Sbjct:   400 YFPPGILTQYGRVLRQPVDRIYFAGTETATHWSGYMEGAVEAGERAAREILHAMG 454
```

Figure 71: sequence alignment of MAO-N and MAO-B from human liver (WU-Blast2). "Query" sequence corresponds to MAO-N, "Sbjct" sequence corresponds to MAO-B. Identical and similar residues belong to active site of MAO-B are in red and the residues from active site without similarity are in blue.

Figure 72(b), shows the position of an inhibitor bound at the active site of MAO-B together with most of the corresponding MAO-N amino acids that have aromatic side chains. The differences are mostly in the rear region where the substrate cavity is in van der Waals contact with the benzene ring of the inhibitor pargyline.

One of the mutations found in MAO-N that led to greater activity against L-AMBA (D385G) is next to Thr 384 which corresponds to Phe 343 in MAO-B and plays a role in separating the two cavities of the active site.

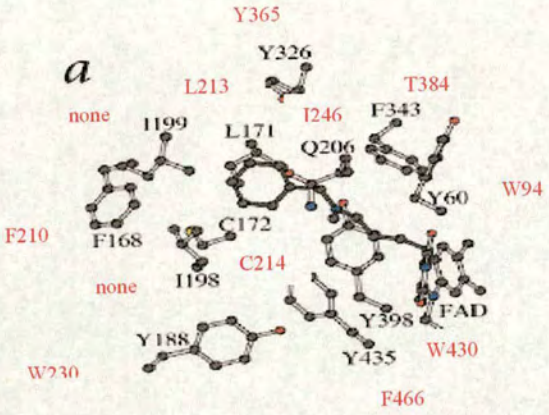
Phenylalanine at position 343 in MAO-B sequence corresponds to threonine at position 384 in MAO-N sequence. These amino acids clearly have different chemical structures. However, Thr 384 in MAO-N is next to aspartic acid, which was mutated twice to glycine and to alanine by the mutator strain. One of the mutations (D385G)

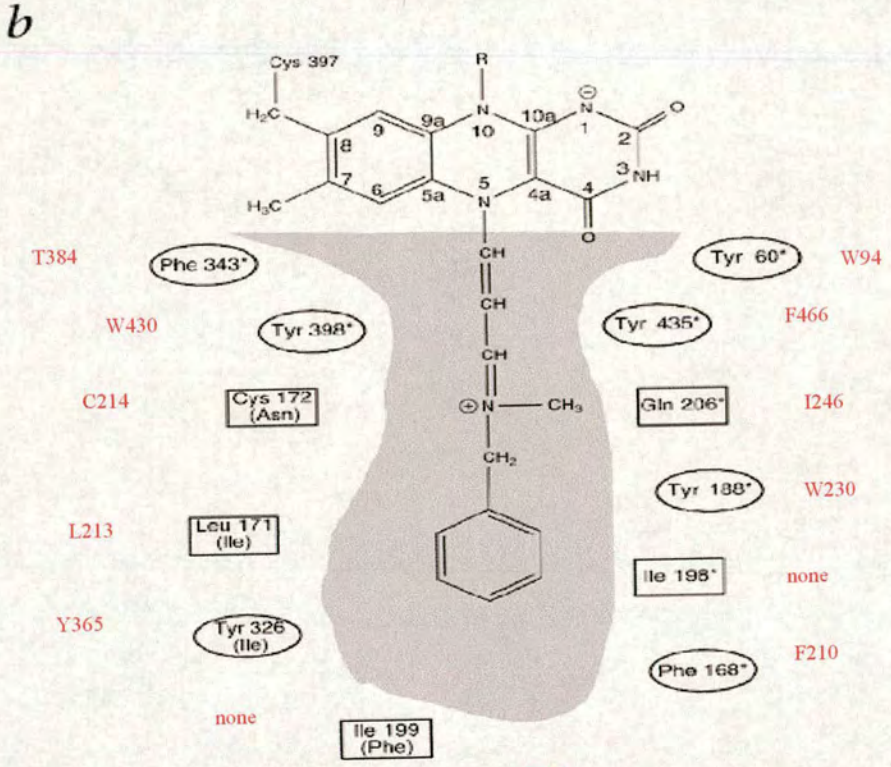
brought significant changes in the catalytic properties of MAO-N and another one (D385A) although lesser, also had some effect on the activity of enzyme (§4.2.6.2; Table 20). This observation can identify Asp385 as an important residue in MAO-N active site. The position of Asp385 next to Thr384 (corresponding to Phe343, active site residue in MAO-B) can also be considered as evidence of the importance of Asp385 in MAO-N active site architecture.

The recent determination of the crystal structures of plant PAO[98a] and human MAO-B[148] have revealed valuable insights into the structure-function of the flavin dependent amine oxidases. PAO is involved in catabolism of polyamines by catalyzing the oxidation of secondary amino groups of spermine or spermidine and their acetyl derivatives. It is a soluble enzyme with a non-covalently bound FAD cofactor. MAO-B oxidises the primary amino group of arylalkylamines. MAO-B has a C-terminal segment for membrane attachment, which is absent in PAO. PAO (472 residues) and MAO-B (520 residues) share about 20% amino acid sequence identity. Despite their overall structural folding similarity, PAO and MAO-B differ in overall topology of their substrate binding-sites (Figure 73).[160]

The PAO U-shaped tunnel and the MAO-B cavities follow entirely different pathways and are lined by residues that are not homologous in sequence. The only conserved features can be found in the sites for binding of the flavin and for recognition of the substrate amino group that undergoes oxidation.

In particular, Lys 300 of PAO is bridged to the N5 atom of the flavin through a water molecule and participates in addition of a hydrogen atom to the N5-position, which occurs during the cofactor reduction. This interaction is strictly conserved also in MAO B where Lys296 occupies a position identical to that of Lys300 in PAO (Figure 74, A& B). X-ray diffraction data on MAO-B have shown a water molecule acting as a bridge between Lys 296 and the flavin N5 atom. This structural feature is not unique to these amino acid oxidases and also been found in other flavoenzyme oxidases (e.g. L-amino acid oxidase).


a
Y365
Y326
L213
T384
none
I246
F343
I199
L171
Q206
W94
Y60
F210
F168
C172
C214
none
I198
FAD
Y398
Y188
Y435
W430
W230
F466


b
Cys 397
T384
Phe 343*
Tyr 60*
W94
W430
Tyr 398*
Tyr 435*
F466
C214
Cys 172 (Asn)
Gln 206*
I246
Tyr 188*
W230
L213
Leu 171 (Ile)
Ile 198*
none
Y365
Tyr 326 (Ile)
F210
Phe 168*
none
Ile 199 (Phe)

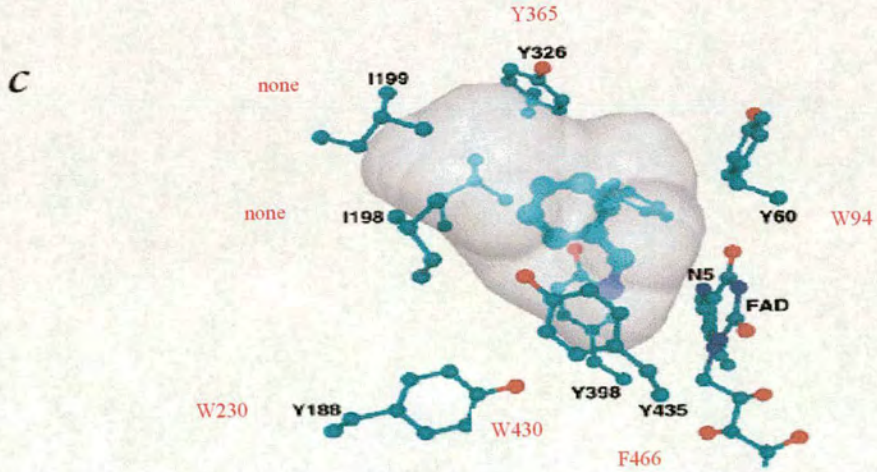

c
Y365
Y326
none
I199
none
I198
Y60
W94
N5
FAD
Y398
W230
Y188
Y435
W430
F466

Figure 72 :[148] the substrate binding site of human MAO-B.

a) Stereo view of pargyline inhibitor and the residues lining the binding site at the *re* side of the flavin. Carbon atoms are in black, nitrogen in blue, oxygen in red and sulphur in yellow. The inhibitor is outlined by shaded bonds. The corresponding MAO-N amino acids are in red. b) Schematic representation of the pargyline binding site. MAO-B residues that are conserved in human MAO-A are indicated by an asterisk. For non-conserved amino acids, the replacement side chains of MAO-A are in parentheses. Aromatic side chains are enclosed in ellipsoidal frames. Other residues are in rectangular boxes. The atoms of the flavin ring are numbered. The corresponding MAO-N amino acids are in red. c) A model for the binding of the substrate, benzylamine, to human MAO-B. For illustrative purposes, the atoms and bonds of the modeled substrate are shown in an increased size. The surface of the solvent inaccessible substrate-binding cavity is semitransparent. For clarity, only some of the residues lining the cavity are depicted. Carbon atoms are in cyan, nitrogen -in blue and oxygen -in red. The reactive N5 site on the twisted flavin is labelled. The corresponded MAO-N amino acids are in red.

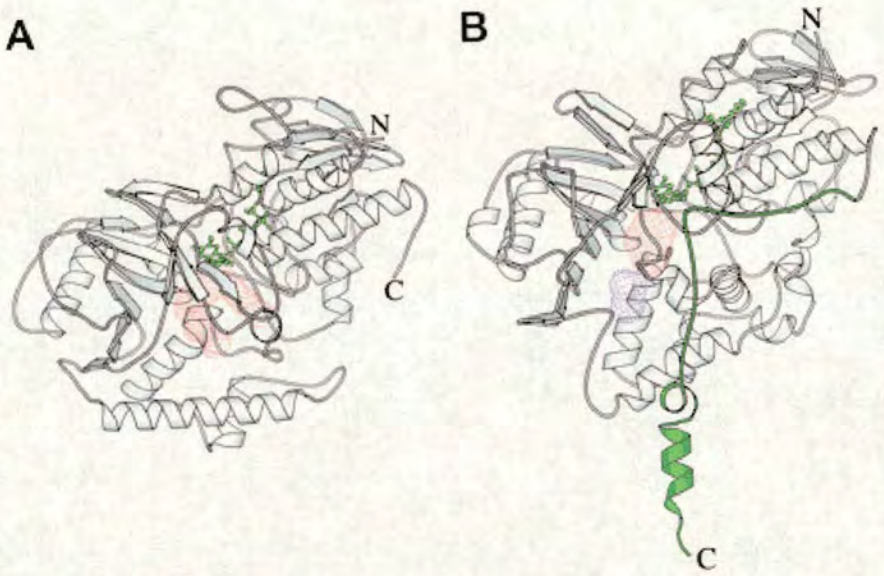


Figure 73 :[160] structures of PAO and of MAO-B.

A. Overall structure of maize PAO (Protein Data Bank entry 1B5Q). Labels *N* and *C* indicate the N terminus and C terminus, respectively. The FAD cofactor is shown in yellow, and the U-shaped catalytic tunnel is highlighted in red. **B**. Overall structure of human MAO-B (Protein Data Bank entry 1G0S) in the same orientation used for PAO in *A*. Labels *N* and *C* indicate the N terminus and C

terminus, respectively. The C-terminal membrane-binding region is in green, and the FAD cofactor is in yellow. The active site cavity is colored in red, whereas the entrance cavity is in blue.

Because MAO-N has 24% identity and 47% similarity to total MAO-B sequence (Figure 71) and 22% identity and 39% similarity to PAO (region of 279 to 483 amino acid, Figure 78) we decided to apply the structural information available for MAO-B and PAO to explore the MAO-N active site and thereby provide a partial rationalization of the mutations found through the directed evolution experiment. Thus, Lys 340 in MAO-N occupies a position identical to that of Lys 300 in PAO (Figure 74, A). However, Lys 296 in MAO-B corresponds to Met337 in MAO-N (Figure 74,B) which is the location of the mutation found in mutant 7 (M337R, §4.2.4, Table13). According to the preliminary activity studies (§4.2.6.2, Table 20), this mutation M337R was featured as a "hot spot" mutation. The position of methionine 337 next to asparagine 336, which was mutated to serine to produce MAOmut"best", can be used as evidence of the importance of both Met337 and Asn336 in MAO-N active site structure.

The PAO and MAO substrate-binding sites, where the flavin-dependent amine oxidation takes place, display several conserved features. In both proteins, two aromatic amino acids form an "aromatic sandwich" by facing each other in perpendicular orientation to the flavin. In PAO, residues Phe403 (Phe431in MAO-N) and Tyr439 (Phe466 in MAO-N) are positioned parallel to each other and perpendicular to the flavin plane (Figure 74,A). Likewise, in MAO-B, there is an aromatic pair formed Tyr 398 (Trp430 in MAO-N) and Tyr435 (Phe 466 in MAO-N) (Figure 74, B).

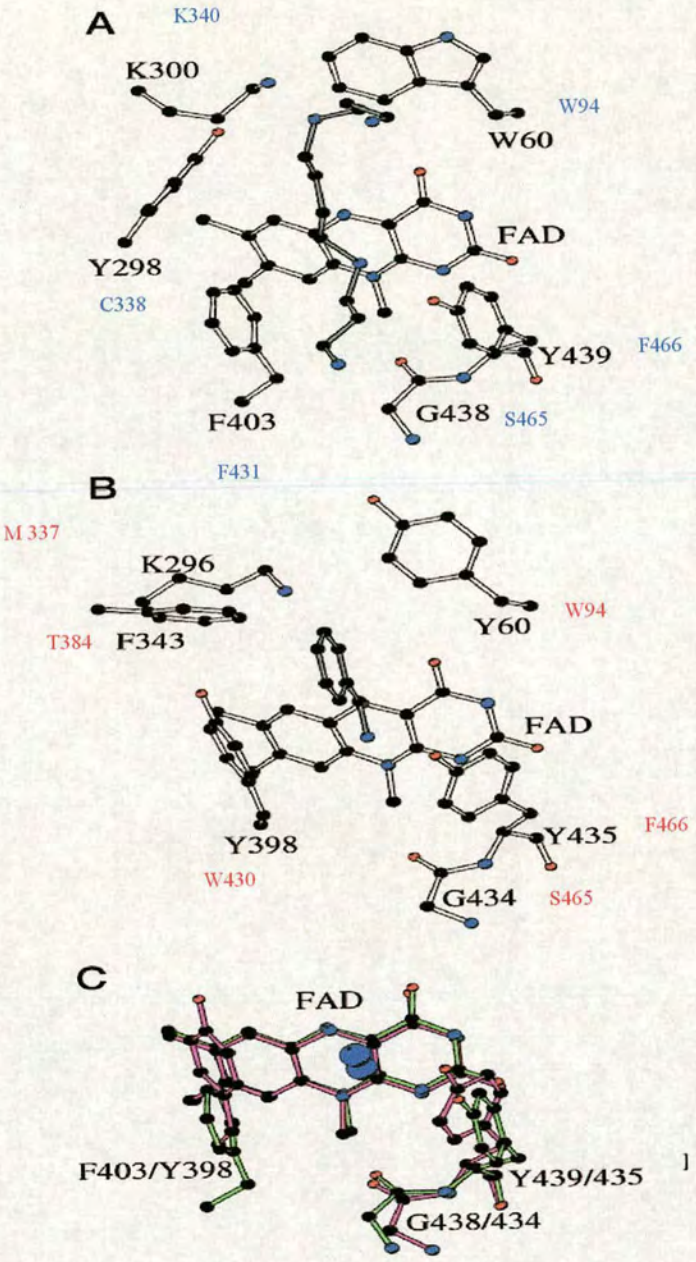


Figure 74:[160] catalytic sites of PAO and MAO B. **A**. Structure of the catalytic site of PAO with the substrate spermine modelled into it. Carbon atoms are in black, oxygen are in red, and nitrogen atoms are in blue. **B**. Structure of the catalytic site of MAO-B with the substrate benzylamine modeled into it. Atom color codes are as in A. **C**. Structure of the superposed PAO and MAO-B catalytic sites. PAO and MAO-B residues are outlined in green and magenta, respectively. Atom color codes are as in *A*. Residues are labeled as PAO/MAO B numbering. The larger blue spheres indicate the positions of spermine and benzylamine amino groups undergoing oxidation. The corresponding MAO-N amino acids are in red.

Furthermore, in both PAO and MAO-B the aromatic pairs are sheltered by additional aromatic residues. Trp60 in PAO (Trp 94 in MAO-N) and Tyr60 in MAO-B (Trp94 in MAO-N) are positioned at the top of the flavin ring, being coplanar to the respective pyrimidine rings of the flavin coenzymes. This aromatic residue was found to be conserved in almost all amine oxidases published in the data bank (WU-Blast2 alignment to MAO-N query). Tyr298 of PAO (Cys338 in MAO-N) and Phe343 of MAOB (Thr 384 in MAO-N) are both located on the same side of the flavin, in proximity of the lysine that is hydrogen bonded to the FAD.

The combination of the flavin and aromatic sandwich generates an "aromatic cage" that is suggested to recognize the deprotonated amine group of the substrate. The "aromatic sandwich" active site organization in MAO-N could be proposed as based on the aromatic side chain of the corresponding amino acids. This observation could account for the effects obtained after the amino acid replacements at position 336, 337 and 385 in MAO-N.

The origin of G403E "hot spot" mutation (§4.2.6.2; Table 20) is obscure due to the absence of a three-dimensional structure of MAO-N.

An overview of MAO-N amino acids which correspond to MAO-B and PAO active site residue and which were affected by random mutagenesis is given in Figure 75. The amino acid highlited in the second-row position is the corresponding residue in MAO-B (in red) and in PAO (in blue) sequences.

```
>MAO_cDNA

1     atg acc tcc cga gac gga tac cag tgg aca ccc gag aca ggg ctc   45
1     Met Thr Ser Arg Asp Gly Tyr Gln Trp Thr Pro Glu Thr Gly Leu   15

46    acg cag ggc gtc ccc tct cta gga gtc atc tcc ccg ccc act aat   90
16    Thr Gln Gly Val Pro Ser Leu Gly Val Ile Ser Pro Pro Thr Asn   30

91    atc gaa gac acg gac aaa gat ggt cca tgg gac gtg att gtc att   135
31    Ile Glu Asp Thr Asp Lys Asp Gly Pro Trp Asp Val Ile Val Ile   45

136   ggt gga ggg tac tgc ggg ttg act gcc act agg gac ttg act gta   180
46    Gly Gly Gly Tyr Cys Gly Leu Thr Ala Thr Arg Asp Leu Thr Val   60

181   gca ggc ttc aaa acc ctt ctc ctc gaa gcc cga gac cgc ata ggc   225
61    Ala Gly Phe Lys Thr Leu Leu Leu Glu Ala Arg Asp Arg Ile Gly   75

226   ggc cgc tcc tgg tcc tct aac atc gac ggc tat cct tac gag atg   270
76    Gly Arg Ser Trp Ser Ser Asn Ile Asp Gly Tyr Pro Tyr Glu Met   90
```

```
271  ggc ggc aca tgg gtc cac tgg cac caa tcg cac gta tgg cgc gaa   315
91   Gly Gly Thr Trp Val His Trp His Gln Ser His Val Trp Arg Glu   105
               W94 W94

316  atc acg cgc tac aag atg cac aac gcc cta tca ccc tcc ttc aac   360
106  Ile Thr Arg Tyr Lys Met His Asn Ala Leu Ser Pro Ser Phe Asn   120

361  ttc tcc cgc ggc gtg aat cac ttc cag cta cgg acc aac ccc acc   405
121  Phe Ser Arg Gly Val Asn His Phe Gln Leu Arg Thr Asn Pro Thr   135

406  aca tca acc tac atg act cac gaa gcc gag    gac gag ctc ctc cgc   450
136  Thr Ser Thr Tyr Met Thr His Glu Ala Glu/Lys Asp Glu Leu Leu Arg   150
                                       none

451  tcc gca ttg cac aag ttc acc aac gtg gat ggc acc aac ggc cgt   495
151  Ser Ala Leu His Lys Phe Thr Asn Val Asp Gly Thr Asn Gly Arg   165

496  act gtc ctg ccc ttc ccg cat gac atg ttc tat gtt cct gag ttc   540
166  Thr Val Leu Pro Phe Pro His Asp Met Phe Tyr Val Pro Glu Phe   180

541  agg aag tat gat gag atg tca tac tcg gag cgg att gat caa atc   585
181  Arg Lys Tyr Asp Glu Met Ser Tyr Ser Glu Arg Ile Asp Gln Ile   195

586  cgg gat gag ttg agc ctt aat gaa cgg agt tct ctg gaa gcg ttt   630
196  Arg Asp Glu Leu Ser Leu Asn Glu Arg Ser Ser Leu Glu Ala Phe   210
                                                        F168

631  ata ttg ctt tgc tct ggc gga acg ctg gag aat agc tca ttt gga   675
211  Ile Leu Leu Cys Ser Gly Gly Thr Leu Glu Asn Ser Ser Phe Gly   225
          L171 Cys172

676  gaa ttc ctg cat tgg tgg gcg atg agc gga tat acg tat cag gga   720
226  Glu Phe Leu His Trp Trp Ala Met Ser Gly Tyr Thr Tyr Gln Gly   240
                    Y188

721  tgc atg gac tgc ttg ata agt tat aag ttc aag gat ggg cag tct   765
241  Cys Met Asp Cys Leu Ile Ser Tyr Lys Phe Lys Asp Gly Gln Ser   255
                         Q206

766  gca ttt gcg agg agg     ttt tgg gag gag gcg gcc ggg acg ggg agg   810
256  Ala Phe Ala Arg Arg/Lys Phe Trp Glu Glu Ala Ala Gly Thr Gly Arg   270
                    Arg

811  ttg ggg tat gtg ttt ggg tgt ccg gtt agg agt gtt gtt aat gag   855
271  Leu Gly Tyr Val Phe Gly Cys Pro Val Arg Ser Val Val Asn Glu   285

856  aga gat gcg gcg     aga gtg acg gcg agg gat ggc agg gag ttc gct   900
286  Arg Asp Ala Ala/Val Arg Val Thr Ala Arg Asp Gly Arg Glu Phe Ala   300
                Val

901  gcg aag cgg ctg gtt tgc act att ccc ctc aat gtc ttg tcc acg   945
301  Ala Lys Arg Leu Val Cys Thr Ile Pro Leu Asn Val Leu Ser Thr   315

946  atc cag ttc tca cct gcg ctg tcg acg gag agg atc tct gct atg   990
316  Ile Gln Phe Ser Pro Ala Leu Ser Thr Glu Arg Ile Ser Ala Met   330



991  cag gca ggt cat gtg aat     atg tgc acg aag gtg cat gcc gaa gtg   1035
331  Gln Ala Gly His Val Asn/Ser Met Cys Thr Lys Val His Ala Glu Val   345
                          Ile  Met/ArgY298    K300
                                  Lys
```

```
1036  gac aat aag gat atg cgg tcg tgg acg ggc att gcg tac cct ttc   1080
346   Asp Asn Lys Asp Met Arg Ser Trp Thr Gly Ile Ala Tyr Pro Phe   360

1081  aat aaa ctg tgc tat gct att ggt gat ggg acg act ccc gcg gga   1125
361   Asn Lys Leu Cys Tyr Ala Ile Gly Asp Gly Thr Thr Pro Ala Gly   375
                      Y326

1126  aac acg cat ctg gtg tgt ttc ggg acg gat      gcg aat cat atc cag
376   Asn Thr His Leu Val Cys Phe Gly Thr Asp/Gly/ Ala Asn His Ile Gln   390
                                    F343 Ile Ala


1171  ccg gat gag gac gtg cgg gag acg ttg aag gcg gtt ggg     cag tta   1215
391   Pro Asp Glu Asp Val Arg Glu Thr Leu Lys Ala Val Gly/Glu Gln Leu   405
                                                      Cys

1216  gcg cct ggg aca ttt gga gtg aag cgg ttg gtg ttt cac aat tgg   1260
406   Ala Pro Gly Thr Phe Gly Val Lys Arg Leu Val Phe His Asn Trp   420

1261  gtg aag gat gag ttt gcg aag ggc gcg tgg ttc ttc tct agg cct   1305
421   Val Lys Asp Glu Phe Ala Lys Gly Ala Trp Phe Phe Ser Arg Pro   435
                                        Y398 F403

1306  ggg atg gtg agt gag tgt ttg cag ggg ttg agg gag aag cat cgc   1350
436   Gly Met Val Ser Glu Cys Leu Gln Gly Leu Arg Glu Lys His Arg   450

1351  ggt     gtt gtg ttt gcg aat tca gat tgg gcg ttg ggg tgg agg agc   1395
451   Gly/Ser Val Val Phe Ala Asn Ser Asp Trp Ala Leu Gly Trp Arg Ser   465
      R419                                                  G434 G438

1396  ttt att gat ggg gcg att gag gag ggg acg aga gct gct agg gtg   1440
466   Phe Ile Asp Gly Ala Ile Glu Glu Gly Thr Arg Ala Ala Arg Val   480
    Y435 Y439

1441  gtg ttg gag gaa ttg gga acg aag agg gag gtg aag gct cgt    ttg   1485
481   Val Leu Glu Glu Leu Gly Thr Lys Arg Glu Val Lys Ala Arg/CysLeu   495

1486   tga end
```

Figure 75: cDNA of MAO-N.

Light purple-"expression " mutation

Dark purple-"hot spot" mutation

Red – amino acid corresponded to amino acid in MAO B active site

Blue- amino acid corresponded to amino acid in PAO active site

Green- amino acid corresponded to amino acid in both PAO and MAO B active sites.

Bold black- no change

6. Conclusions and Future work

In conclusion, the "directed evolution" of an amine oxidase has been achieved to meet the specific requirement of a novel biotransformation. The decision to select the *A. niger* MAO-N gene for *in vitro* evolution was based upon the observation that the wild-type MAO-N enzyme showed inherent selectivity for L-over D-AMBA (*ca.* 17:1), although the overall rate was very low. An interesting aspect of the present work was the identification of a mutant with enhanced enantioselectivity by using a single enantiomer substrate in the screen (L-AMBA). There is currently much effort directed towards the development of truly enantioselective screens using both racemates, because the two enantiomers must compete for the enzyme in nature.[199,240,241,242a,b] However, the high throughput screen developed in the present work, allowed us to screen large library of variants and identify a MAO-N variant with improved enantioselectivity. The properties of obtained mutants are summarised below:

1. G403E No significant changes on the native substrate (amylamine). Minor activity increase on L-α-methylbenzylamine (L-AMBA) combined with major activity drop towards D-AMBA.
2. M337R No significant change on the native substrate. Minor improvement on L-AMBA, significant improvement in enantioselectivity
3. D385G No significant change on native substrate and minor improvement on L-AMBA, some improvement in enantioselectivity.
4. D385A No significant change on native substrate and minor improvement on L-AMBA, some improvement in enantioselectivity
5.N336S Strong improvement on L-AMBA and reduced activity towards native substrates (amylamine and benzylamine), dramatic improvement in enantioselectivity.

According to homology modelling, all sites are located close together in space and in close proximity to the putative active site. Though it is still possible that each

individual mutation affects reaction specificity from the distance as a result of conformational changes, it seems more likely that the mutated residues are involved directly. First, two mutations (G403E, M337R) bring moderate activity improvement towards L-AMBA which might probably be associated with the increase in polarizability of the active site by each of the first two mutations. Additional charges introduced by either of the two mutations might help to protonate the substrate amino group, but only in case of L-AMBA. It is not inconceivable to imagine that the neutral G403 and M337 are located at the opposite sides of the substrate binding pocket and the addition of a negative charge from one side (G403E) would have similar effect as the addition of a positive charge (M337R) to the other side of the binding pocket. Interestingly, these mutations affect only L-AMBA, but not the native substrate amylamine. The presence of the methyl group might drive the substrate towards G403 and M337 in the pocket.

Negative charge elimination of D385 by the next two mutations (D385G and D385A) did not affect the native substrate and resulted in a small improvement towards the methylated substrate. If compared to the first two cases, these mutations decrease the active site polarizability but improve the activity towards AMBA anyway.

The third mutation has the replacement of asparagine to serine at position 336. Both asparagine and serine are from the same group of amino acids with uncharged polar side chains. This mutation has much more interesting affect - it greatly improves the enzyme activity towards L-AMBA and reduces the activity towards the native substrate. The activity enhancement is stereo-selective improvement is much smaller for D-AMBA. It is plausible that the N336S shortening of the side chain (the amide group in Asn and hydroxyl group in Ser) introduces a spatial misfit between the enzyme and the native substrates amylamine and benzylamine (table 18, page 119) in the binding pocket but create more room for binding L-AMBA (Figure 75[a]).

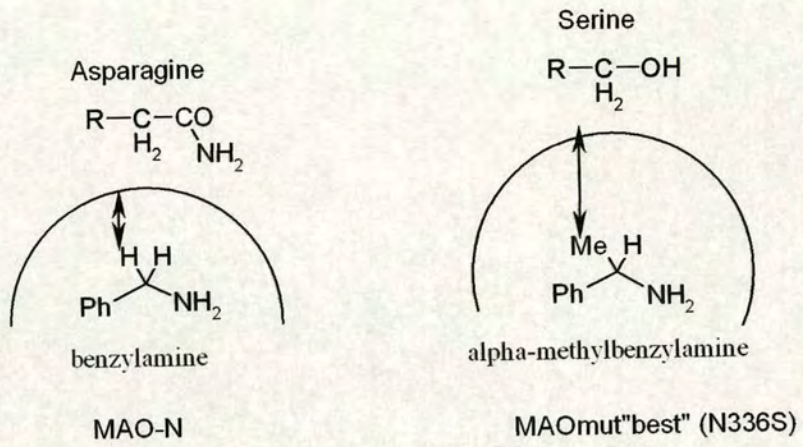


Figure 75[a] : the presumable model of MAO-N and MAOmut"best" active site. The size of the arrows corresponds to the size of the binding pocket in active site of the enzyme

It would be interesting to further investigate the mechanisms by which the mutations described, effect the function of MAO-N by X-ray structural studies of MAO-N wild type. Such studies may also shed light on the effect upon the altered substrate specificity of MAOmut"best".

The current activity of MAOmut"best" towards L-AMBA could potentially be improved further by either saturation mutagenesis at the specific residues identified in this work or by subjecting the MAOmut"best" variant to further rounds of random mutagenesis.

The application of MAOmut"best" for deracemisation of a range of chiral amines can be optimised further. In this case, the broadness of substrate specificity of MAOmut"best" would have to be investigated. The improved expression level of MAOmut"best" (as achieved for MAO-N wild type) will also be an advantage to provide suitable quantities of enzyme for further studies.

7. Experimental: Cloning and Expression

7.1. General Techniques

Plasmid (pECME3) carrying the full length of *A.niger* MAO-N gene was provided as a gift by Boris Schilling (Givaudan-Roure Research Ltd., Switzerland). Synthetic oligonucleotides were purchased from Interactiva Biotechnologie GmbH or Sigma-Genosys (Biotechnologies (Europe), Cambridge, UK). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs. PCR reaction 'Ready to go' beads containing *taq* DNA polymerase was purchased from Amersham Pharmacia Biotech and *Herculase Hotstart* DNA Polymerase was purchased from Stratagene. PCR reactions were performed in *Mastercycler personal* (Eppendorf).

Plasmid DNA was analysed by agarose gel electrophoresis (0.8% in TAE buffer) using equipment purchased from Anachem, Luton, UK. Agarose was obtained from Bio-Rad Laborarories, UK and DNA was extracted from agarose gels using the Qiaquick Gel Extraction Kit (Qiagen,UK, #28704). Plasmid DNA preparations were performed using the QIAprep Spin Miniprep Kit (Qiagen #27104) and GenElute Plasmid Midiprep Kit (Sigma-Aldrich, # PLD-35).

The expression vector pET16b was obtained from Novagen (CN Biosciences Ltd, Nottingham, UK) and *E.coli* competent cells of Top10, BL21(DE3) cell lines were obtained from Invitrogen (Paisley,UK). Expression media components were obtained from Becton Dickinson Microbiology Systems, USA. IPTG was purchased from Europa Bioproducts. Ampicillin was purchased from Boehringer Mannheim. Reagents for buffers, assay mixtures and cultures media were of standard laboratory grade and were used as supplied from commercial sources. Bacterial growth in liquid culture was performed in an orbital shaker (Innova 4430 or Innova 4000), set at the desired temperature. For static incubation a dry block heater (Anachem HBS-130) or a water bath set at the desired temperature were used.

Centrifugation of culture media and lysed cells was carried out in a Sorval RC5C plus centrifuge. Speed and rotor size follow in parentheses. Small-scale (<1.5ml) centrifugation was performed in either an Eppendorf 5G15C microfuge or a Hareaeus Biofuge *pico*.

Sterile procedures were performed either on the bench under flame, or in a SterilGard Class II Type A/B3 biological safety cabinet. Equipment and media were sterilised using a Monarch 745rh autoclave (121°C, 20min) or an Astell Scientific autoclave (121°C, 15min).

Optical density measurements and Bradford assay measurements were performed on a Cecil CE 1020s spectrophotometer (D_2 lamp). Kinetic measurements were carried out on a Hewlett Packard 8453 spectrophotometer (D_2 lamp), using HP 845xUV-visible system software for analysis.

SDS- PAGE equipment was purchased from Bio-Rad. Three different gels were used for SDS-PAGE analysis; I) Biorad Ready gels 10-20% acrylamide gradient, Tris/HCl, 19 wells, 30µl; ii) Biorad Criterion™ precast gel 10-20% acrylamide gradient Tris/HCl, 18 wells, 30µl; iii) self-poured 15% acrylamide gels (§11.9).

7.2. *mao-n* wild type cloning and expression in pET16b

7.2.1. Construction of the plasmid pMAO-N

All standard molecular biology procedures were performed as described by Maniatis *et al.*[237]

7.2.1.1. Gene amplification by PCR

A full-length *maoN* DNA (1502bp) was recovered by selective amplification (PCR) from the original plasmid pECME3. Two oligonucleotides were utilised in the reaction (both 5'-3') corresponding to the termini of the coding region. They were designed to contain unique restriction sites for subcloning into the expression vector pET16b.

MAOFor*Nde* primer (GCTCTAGA**CATATG**ACCTCCCGTGACGGTTACCAGT GGACCCCGGAG) contained a unique *Nde*I site (in bold, N-terminal methionine underlined).

MAORev-*BamH*I primer (ATA**GGATCC**ATCAATCACAAACGAGCCT) contained the unique *BamH*I site (in bold) and a *stop* codon (underlined). Nucleotide substitution from the native gene sequence included according to *E. coli* codon usage (§2.1.2, Table 3).

The PCR reaction was performed by using 'Ready to go' PCR beads with *taq* DNA polymerase or *Hot start Herculase* DNA polymerase. Template DNA (≈1ng) and primers (≈10pmol) were added to the reaction.

i). Amplification by taq DNA polymerase

One PCR bead was mixed with the solution containing 1 µl DNA template (usually from 1:10 dilution of plasmid DNA purified by Qiagen Plasmid Miniprep, unless otherwise stated), 0.5µl of each primer (from 0.1µg/ml primer solution) and 23µl of water to perform the PCR reaction.

ii). Amplification by Hot start Herculase DNA polymerase

1µl DNA template (made up as above), 0.5µl 1 of each primer (made up as above), 1µl of 10mM dNTPs, 5µl DNA polymerase buffer (supplied with kit), 0,5µl DNA polymerase and 41.5µl of water was mixed to perform the PCR reaction.

PCR reaction was performed using optimised cycling conditions:

1) Denaturing and polymerase activating – 94° C 2min 30sec
2) Denaturing – 94oC 40sec
3) Annealing – 50°C 45sec
4) Extension – 72°C 1min 20sec
5) Go to 2) and repeat for 29 cycles
6) Final extension – 72°C 2min

The presence of a PCR product of the correct molecular weight was determined by UV visualisation of ehidium bromide stain by agarose gel electrophoresis. The fragment was purified (approximately 1μg), eluted in 30μl of water and subjected to restriction digest.

7.2.1.2. ***Restriction digest of PCR product and construction of the plasmid pMAO-N.***

The amplified DNA fragment and the vector pET16b were each double digested with the restriction enzymes *Nde*I and *BamH*I (37°C, 4h) as followed:

28μl PCR product
2μl Nde1
2μl Bam H1
0.4μl BSA
4μl BamH1 buffer
2μl H_2O

The digested product was agarose gel purified in a total volume of 30μl, ligated into pET16b (vector/insert=1/10) by incubation with T4 DNA ligase in the buffer supplied (22°C-room temperature, 16 h). The ligated product was transformed into *E. coli* TOP 10 competent cells and colonies were screened for an Amp 100 resistance. Plasmid DNA was purified by using the QIAprep Spin Miniprep Kit (Quiagen), digested by *Nde*I */Bam H*I, mixed with loading buffer (§11.13) and analysed by agarose gel

electrophoresis at constant current of 80 mamp, in TAE buffer (§11.5). The DNA in agarose gel was stained with ethidium bromide (§11.15) and visualised in ultraviolet light.

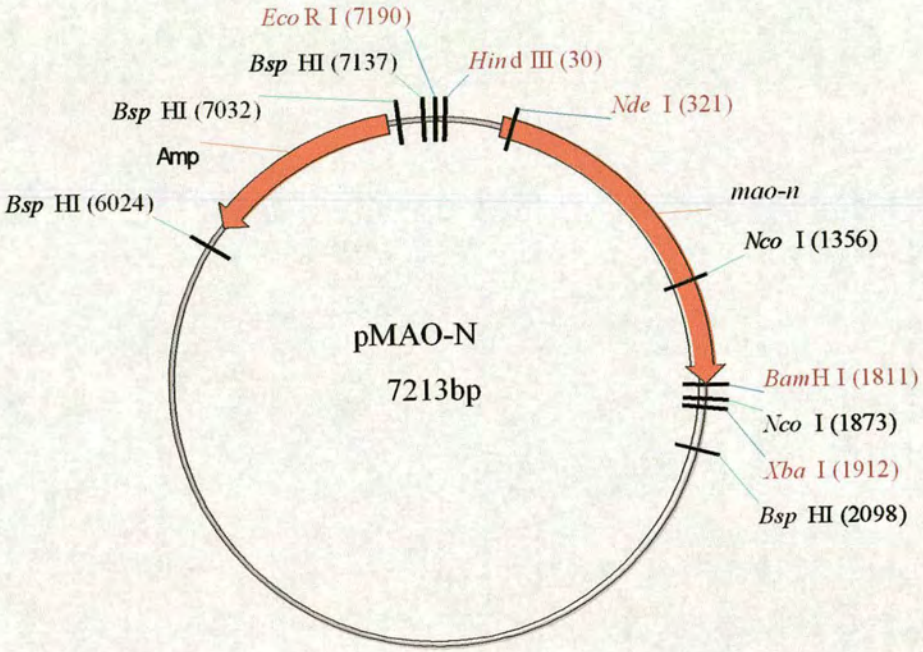


Figure 76: *mao-n* gene cloning into pET16b vector

7.2.2. *Expression of maoN gene in pET system*

Expression of *mao-n* gene in *E. coli* BL21(DE3) was performed with reference to the Novagen *pET System Manual.*[225]

E .coli BL21(DE3) transformed with vector pET16b without *maoN* gene was used as a background reference culture.

Transformation of *E.coli* BL21(DE3) (Invitrogen) by pMAON or by pET16b was performed according to the manufacturer's protocol.

i). *Small scale expression*

A freshly transformed colony was used to inoculated in 10ml LB Amp 100 and cultured in 50ml falcon tube by agitation for 24 hours at 30°C until cells density reached OD_{600}=4.0-5.0.

ii). Medium scale expression

A freshly transformed colony was used to inoculate into 100ml of LB Amp 100 and grown in 500ml baffled conical flasks by agitation for 23 hours at 30°C until an OD_{600}=4.0-5.0.

iii). Large scale expression

A freshly transformed colony was inoculated in 100ml LB Amp100 and cultured in 500ml conical flack by agitation either at 30°C over night or at 37°C during the day until cells density reached OD_{600}=0.8-1.0. This culture was spun at 4000 rpm, 5 minutes at room temperature, and the pellet resuspended in fresh the LB Amp100 media. 0.3ml of this suspension was then used to inoculate a total of 300ml LB Amp100 media, which was aliquated into 1L conical buffled flasks.
The culture was cultivated in a shaking incubator at 26°C for 24 hours until OD_{600}=4.0-5.0.

iv). Biomass recovery

1ml, 10ml aliquots or the entire volume of growing culture were spun down (1ml aliquots - at 13000rpm, 3min in microfuge; 10ml aliquots- at 4000rpm, 20min in bench top Sorvall Legend RT centrifuge; whole volume of culture in large scale expression–at 6000 rpm for 10min in Sorval RC5C plus) and the pellets were stored at – 20°C until required.

1ml frozen pellets were used for whole cell activity determination and for SDS PAGE analysis, 10ml frozen pellets were used for small scale CFE preparation and large scale culture pellets was used for large scale CFE preparation and purification.

7.2.3. *Cell free extract preparation*

i) Small scale

Lysis was performed as close to 4°C as possible. The frozen 10ml pellet (§ .2.2; iv) was thawed and resuspended in 5 ml of 25mM potassium phosphate buffer pH 7.6. The cell suspension was sonicated (Soniprep 150) on ice following an on-off protocol: (10 sec "on" 10sec "off ") for 2min. The resulting mixture was centrifuged at 4000 rpm, (Sorwal Legend RT, Kendro Ltd, USA) at 4°C for about 40 minute or until a clear supernatant was obtained. Cell free extracts were stored at –80°C.

ii) Large scale

The cell pellet obtained from large scale protein expression (§7.2.2; iv) was resuspended in buffer (1ml/1g cells) in the presence of lyzozyme at a final concentration of 1mg/ml. The cell suspension was incubated at 30°C for 30 minutes and was subjected to sonication. The sonication was performed on ice and in culture volume not greater then 15ml. The sonication conditions were: 30 sec "on", 1min "off" or until the culture viscosity disappeared. The resulting mixture was centrifuged at 20000rpm, Sorval SS-34 rotor (Kendro Ltd, USA) at 4°C until a clear supernatant was obtained. Cell free extracts were stored at –80°C.

iii). Medium scale

The cell pellet obtained from medium scale protein expression (§7.2.2; ii) was resuspended in 2ml buffer. The resulting culture was subjected to sonication performed on ice. The sonication conditions were: 10 sec "on", 20 sec "off" for 3 repeats. The resulting mixture was centrifuged at 20,000rpm using a Sorwal SS-34

rotor for 20min or until a clear supernatant was obtained. Cell free extracts were stored at –80°C.

7.2.4. Whole cell activity determination

i). Determination of the initial rate of enzymatic activity

The frozen pellets from 1ml culture were thawed and resuspended in 1ml of 25mM potassium phosphate buffer pH 7.6. A 0.1ml aliquot of this suspension was mixed with colorimetric solutions (§8.1.1 or §8.1.2) and used for the assays.

ii). Static measurement of enzymatic activity.

The whole frozen pellet from 1ml culture was resuspended in 1ml of assay mixture (§8.1.1 or §8.1.2) and subjected to static incubation at room temperature. *E. coli* BL21 (DE3) transformed with pET16b was used as a reference culture. At the end of incubation, the assayed samples were centrifuged at 13,000rpm, 5min. The spectrophotometer (Cecil CE 1020s) was zeroed using a reference culture supernatant and the absorption at 510nm in experimental supernatants was measured.

7.2.5. Protein analysis by SDS-PAGE

7.2.5.1. Protein in whole culture

The frozen pellet from 1ml culture was resuspended in 300µl of SDS-reducing loading buffer (§ 11.10), boiled for 5min and debris were removed by centrifugation on microfuge for 2 min at maximum speed. Prepared samples were loaded on acrylamide gel and run at a constant voltage on of 200V (Powerpac 200, Bio-Rad Laboratories Ltd, UK). The gel was placed in staining solution (§11.3) and agitated on

a rocking platform until the gel was dark blue in colour. The gel was de-stained in the same way using fresh de-stain solution (§11.4) until protein bands were visible and the \background gel was clear.

7.2.5.2. Purified protein

10µl of protein sample was mixed with 10µl of loading buffer, boiled for 5minutes and loaded on the acrylamide gel. The subsequent procedure was as described in §7.2.5.1.

8. Experimental: Development of high-throughput screen

8.1. Assay methods: soluble dye production assays

Commercially available L-amino acid oxidase (LAAO) purified from snake venom (Sigma # A8390, 1.0 Uml^{-1}) was used as the standard to develop reproducibility necessary for assay validation. L- Phenylalanine at 5mM was used as a substrate

8.1.1. Hydrogen peroxide coupled assay, using 2.4.6-tribromo-3-hydrobenzoic acid (TBHBA)

The standard chromogenic solution contained the following ingredients at final concentration:

i). 0.1M potassium phosphate buffer pH7.6 for monoamine oxidase or pH 7.0 for amino acid oxidase.

ii). 5mM substrate

iii). 0.75mM 4-aminoantipyrine (4-AAP)

iv). 0.02% TBHBA

v). 25U (50 μg/ml) HRP (horseradish peroxidase, Sigma # P-6782)

The dye produced by HRP from H_2O_2, 4-AAP, and TBHBA was detected at 510nm (ε_{510}= 29400$M^{-1}\cdot cm^{-1}$).[243]

The spectrophotometer was blanked against the chromogenic solution:

i).	Potassium phosphate buffer (1M)	5ml
ii).	TBHBA (2% in DMSO)	500μl
iii).	4-AAP (1M)	37.5 μl
iv).	Substrate from commercial flask	
	amylamine (8.6M)	30μl
	or	
	D,L-AMBA (7.7M)	40μl
	or	
	Benzylamine (9.2M)	28μl
	or	
	L-Phenylalanine 100mM	2.5μl
v).	HRP (Sigma # P-6782)	
	5mg/ml, 987U/mg solid	250μl
vi)	Water	up to 50ml

Purified enzyme (10 μl) or CFE (10 μl) or cell suspension (§7.2.4; i, ii) was then added to make the total volume of assay mixture up to 1ml. The dye produced by HRP from H_2O_2, 4-AAP, and TBHBA was detected at 510nm (ε_{510}= 29400$M^{-1}\cdot cm^{-1}$).[243]

The initial rate or standard reading was measured and the activity unit was defined as amount of enzyme that catalyses the formation of 1μmol of H_2O_2 per minute.

Activity calculation:

$(U \cdot ml^{-1}) = (Au \cdot s^{-1}) \times (1/\varepsilon) \times 60 \times (^{t.v}/_{s.v}) \times 1 \times 10^{6}$.

Where ε = molar extinction coefficient ($M^{-1} \cdot cm^{-1}$)

t.v. = total reaction volume

s.v. = sample volume

8.1.2. *Product formation assay*

The spectrophotometric assay was based on the difference between the molar extinction coefficients of benzylamine and benzaldehyde at 250nm. A quartz cuvette was used throughout this assay experiment. The spectrophotometer (Hewlett Packard 8453) was zeroed against 990µl of benzylamine solution: (20mM potassium phosphate buffer pH 7.2, 2mM benzylamine). 10µl of enzyme was mixed in benzylamine solution and the absorbance at 250nm was measured at 3 seconds interval at room temperature. The initial rate was measured and the activity was calculated as for method in §8.1.1 ($\varepsilon_{(PhCHO)\,250nm} = 12500\ M^{-1} \cdot cm^{-1}$).

8.2. Hydrogen peroxide coupled assay, using 3,3-diaminobenzidine

One tablet of DAB from *SIGMA FAST*™ kit (Sigma # D4418) was dissolved in 10ml of chromogenic solution (it takes approximately 20min to dissolve the pellet). The chromogenic solution contained the following ingredients at final concentration:

i). 100mM Potassium phosphate buffer pH 7.6.

ii). 10mM L-AMBA (or other required substrate)

iii). 50µg/ml of horseradish peroxidase HRP (Sigma # P-6782)

iv). Water up to 10ml

2% agarose (Bio-Rad # 161-3102) was melted in water, cooled down to 60°C and kept in water bath at 60°C until required. 10ml of 2% agarose solution was mixed with 10ml of chromogenic solution contained DAB immediately and poured over the membrane.

8.3. Detection of oxidase activity in bacterial colonies on agar plate (intact colonies screening)

8.3.1. Preparation the colonies for screening

A 5µl aliquot of each DNA library was used to transform a 45 µl cell suspention of expression host BL21(DE3) according to manufactures protocol with the following modifications. The transformed cells were incubated in 1ml of SOC medium in a shaking incubator for 1hour at 37°C to induce β-lactamase expression. 100 µl of cell suspension was then plated out directly onto nitro-cellulose HiBond-C Extra (Amersham Pharmacia # RPN82E) membranes placed on a LB Amp100 agar plate. Ten plates, containing 2000-3000 colonies per plate, were used to display the library. The plates were incubated for 24h at 37°C after which time the membranes containing the colonies were lifted from the plate, kept at –20°C for 24 hours and then incubated with the assay mixture (§8.1.1 or §8.2) at room temperature. Positive clones were picked from the membrane by using the yellow tip and subjected to a second round of screening as described below (§8.3.3).

0.5 µl of pMAO-N was used to transform into 5 µl of *E. coli* BL21(DE3) and incubated as described above but with 100 µl of SOC. The total volume (105.5µl) of cell suspension was plated onto nitro-cellulose HiBond-C Extra filter and used as a control in solid phase colony screening as described above.

8.3.2. Screening protocol

Each filter was transferred on a fresh petri-dish and stored at -20^0C for 24-72 hours in order to partially lyse the cells. Thereafter, each plate was treated with a cocktail containing both the assay mixture (§ 8.1.1 or § 8.2) and also 2% agarose as described in §8.2.

i) Use of soluble dye

10ml of chromogenic solution (§8.1.1) was mixed with 10ml of melted 2% agarose which was kept at 60°C in a water bath until required. Resulting mixture was then immediately poured onto the membrane carrying the colonies. The agarose mixture was allowed to polymerise on the membrane within 1-2 min. The plates were then left at room temperature for sufficient time to allow the assay to be developed.

ii). Use of insoluble dye

A DAB tablet was dissolved in 10ml of phosphate buffer, substrate and HPR mixture. Melted 2% agarose was kept at 60°C in a water bath until required. 10ml of 2% melted agarose was added to the 10ml of dissolved pellet. The resulting mixture was subjected to colonies screening as described above (§8.3.2; i).

8.3.3. Second screening of the libraries

5µl of *E. coli* BL21(DE3) was transformed using 1 µl of plasmid DNA purified from a positive clone, obtained from the initial screening of the library(§8.3.2). Transformed cells were incubated with 200 µl of SOC medium at 37°C and transferred onto a membrane using a sterile loop or by spreading 50µl of cell suspension per plate to obtain a low plating density of colonies.

8.4. Detection of oxidase activity in scrubbed bacterial colonies from the agar plate (scrubbed colonies screening.)

8.4.1. Transformation into expression host E. Coli BL21(DE3)

A 2 µl DNA aliquot from each library was used to transform a 20µl aliquot of expression host *E. coli* BL21(DE3) (Invitrogen) using the manufacturers protocol with the following modification. 150 µl of SOC medium was added to induce β-lactamase expression and 50 µl of cell suspension was plated on each LB Amp100 agarose plate. Two or three plates with 2000-3000 colonies on each plate represented each library. pMAO-N was used as a control (§8.3.1).

8.4.2. Scrubbing the colonies and whole cell culture assay

A 10ml volume of LB Amp100 was added to each plate to pool the colonies. The pooled colonies were incubated in shaking incubator in 50 ml Falcon tubes at 30°C for 16 hours. Two MAO-N two reference control cultures were prepared on the same way. At the end of incubation, the 10µl aliquots were used to measure the initial rate (3min) of amylamine oxidation to confirm expression of the enzyme (§7.2.4; i). Pellets obtained from centrifugation of 1ml aliquots (§7.2.2; iv) were collected and kept frozen until needed to assay oxidase activity opon D, L-AMBA (§7.2.4; ii) or for plasmid purification. The thawed pellets were incubated with chromogenic solution (§8.1.1) containing D-AMBA or L-AMBA at 22°C for 18 hours. At the end of this incubation the cell suspension was centrifuged at 13,000 rpm for 5min and absorption at 510nm in supernatant was measured. Plasmid DNA was purified from positive isolate and subjected to intact colony solid phase screening.

9. Experimental: Random mutagenesis by *E.coli* XL1-Red

9.1. Preparing libraries of mutated plasmids

9.1.1. Plasmids applied for mutagenesis

Plasmid DNA of pMAO-N was isolated using a QIAprep Miniprep kit (Quiagen, Hilden, Germany) from 2ml over-night cultures in LB Amp100 media. pUC18 was supplied with the "*Epicurian coli* XL1-Red competent cells" kit.

9.1.2. First method of making mutations

The plasmid of interest (5µl (~50 ng) of pMAO-N and 1µl (0.1ng) of pUC18) was used to transform cells of the *Epicurian coli* XL1-Red (Stratagene, La Jolla, CA) using the manufacturers protocol. A 0.7ml aliquot of transformed cell suspension was used to inoculate 20ml of LB Amp100 and grown over-night (18 hours) in a 50ml Falcon tube in shaking incubator at 37°C. These culture conditions were used throughout the experiment. Approximately 12 generations of cells were produced (*E.coli* XL1-Red doubling time is 90 – 120 min). Plasmid DNA was purified (QIAprep Miniprep kit) from 1ml of culture. A 20µl aliquot of this culture was then added to 10ml of fresh LB Amp100 media and subjected to another mutation cycle over 24 hours. From this sample a 1ml volume was centrifuged (13,000rpm, 5min) and the plasmid DNA isolated. Repetition of this mutation cycle produced libraries of mutants from 12, 24, 36,48, 60, 72, 84 and 96 generations of cells. Selected plasmid libraries were used to transform into the expression host (§8.3.1) and subjected to the solid phase screening assay (§8.3.2; ii).

9.1.3. *Second method of making mutations*

A 5µl (~50 ng) aliquot of pMAO-N plasmid DNA was used to transform *Epicurian coli* XL1-Red using the manufacturers protocol. 0.7ml aliquot of transformed cell suspension (Transformation 1) was used to inoculate 20ml of LB Amp100 and grown over-night (18 hours) in a 50ml Falcon tube in a shaking incubator at 37°C. These culture conditions were used throughout the experiment. About 12 generations of cells were produced. Plasmid was purified from 1ml of culture. A 20µ aliquot of this culture was added to 10ml of fresh LB Amp100 media and subjected to another mutation cycle over 24 hours. 1ml of this culture was centrifuged (13,000rpm, 5min) and the plasmid DNA isolated.

Isolated plasmid was used to transform *E. coli* XL1-Red (Transformation II). The total transformed cell suspension was used to inoculate 10ml of LB Amp and grown for 24h. Plasmid was purified from 1ml of culture. 100 µl aliquot of Transformation II culture was used to inoculate 10ml of LB Amp. The culture was grown for 24h and the plasmid DNA purified.

Isolated plasmid was used for Transformation III. Total transformed cell suspension (1ml) was used to inoculate 10ml of LB Amp, grown for 24h and plasmid purified. A 100 µl aliquot of Transformation III culture was used to inoculate the next 10ml of LB Amp. The culture was grown for 24h and the plasmid DNA purified.

Isolated plasmid was used for Transformation IV. Total suspension of transformed cells (1ml) was used to inoculate 10ml of LB Amp, grown for 24h and the plasmid DNA purified from 1ml culture. A 100 µl aliquot of Transformation IV culture was used to inoculate fresh 10ml LB Amp. The culture was grown for 24h and the plasmid purified.

Resulting plasmid libraries were used to transforme expression host (§8.3.1) and subjected to the solid phase screening assay (§8.3.2; ii).

9.2. Determination of the mutagenic efficiency of mutator strain

9.2.1. Screening for β- galactosidase activity (white/blue colonies screening)

A 2µl sample pUC18 plasmid DNA mutated by the first method of mutagenesis (§9.1.2) was used to transform 25µl of *E. coli* Top 10 competent cells (Invitrogen) using the manufacturers protocol with the following modifications. 125µl volume of SOC medium was used to induce β-lactamase expression and 50 µl was plated onto LB Amp100 agar supplemented with X-Gal (§11.14). After over night incubation at 37°C, the colonies producing active β-galactosidase are blue in colour.

10. Experimental: Validation and application of mutated clones.

10.1. Validation by sequencing

The MWG-BIOTECH facilities were used to perform DNA sequencing reactions. Plasmid DNA for sequence analysis was purified using GenElute Plasmid Midiprep Kit (Sigma # PLD-35). The plasmids and primers were provided in quantities according to the MWG-BIOTECH requirement (1-2µg of dry pellet of plasmid DNA (§10.1.1) and 10pmol/µl of each primer).

10.1.1. Preparation of plasmid DNA

Plasmids were purified from 15 ml of over-night *E. coli* Top10 culture. The purity of plasmid DNA was determined by the ratio of OD_{260}/OD_{280} (§10.2.2).

A dry DNA pellet was obtained by ethanol precipitation as follows. 500µl of DNA solution was mixed with 1ml of ethanol (molecular biology grade, Aldrich # E702-3) and 50µl of 3M sodium acetate pH5.2. The solution was kept on ice for 1hour and precipitated DNA was then collected by centrifugation using a microfuge (13,000rpm, 30min, room temperature). The ethanol was discarded and pellet was washed with 70% ethanol (500µl). The ethanol was removed and the pellet was then dried out on air at 37°C.

10.1.2. Primers used for sequencing reaction

i). T7 promoter primer (pETFor) was obtained from Novagen # 69348-1

5' TAA TAC GAC TCA CTA TAG GG 3'

ii). T7 terminator primer (pETRev) was obtained from Novagen # 69337-1

5' GGC GAC TCG TTA TTG ATC G 3'

iii). The internal primer (Int2) sequence consisted of 20 nucleotides of MAO-N wild type coding sequence beginning from nucleotide 510.

5' CCC GCA TGA CAT GAC ATG TTC TAT G 3'

10.2. Restriction analysis of mutated genes

10.2.1. *Analysis of plasmid DNA of "expression" mutants by agarose gel electrophoresis.*

Plasmids were used to transform *E. coli* XL1-blue (Stratogene) according to manufacturer's protocol and purified from 1ml of over-night culture.

A 20µl aliquot of plasmid DNA was mixed with 30µl of restriction enzyme digest cocktail stock (20µl *Nde*I, 20µl *BamH*I, 50µl *BumH*1 buffer, 205µl H_2O, 5 µl BSA) and was incubated for1hour at 37°C. The products of digestion were visualised by UV illumination of ethidium bromide stained agarose gels following electrophoresis (§7.2.1.2).

10.2.2. Subcloning of mao-n wild type gene into mutant vectors.

Plasmid pMAO-N was used to transformed into *E. coli* JM109 (Promega) according to manufacturers protocol and purified from a 50ml of LB Amp100 culture (GenElute Plasmid Midiprep Kit, (Sigma # PLD-35). The yield of plasmid was determined by OD_{260} measurement (OD_{260} =1.0 corresponds to approximately 50µg·ml^{-1} of DNA). The quality of the plasmid was determined by measuring the ratio of absorption reading at 260 nm versus 280 nm. Pure preparation of DNA has OD_{260}/OD_{280} value of 1.8.

A 140µl aliquot of plasmid DNA (50 µg) was mixed with a restriction digest cocktail (16µl *Nde*I, 16µl *BamH*I, 32µl *BamH*I buffer, 3.2µl BSA 96 µl H_2O) and the reaction was carried out at 37°C for 3 hours. The 1.5Kb *mao-n* band was purified from a preparative agarose gel. The mass ratio of vector/*mao-n* insert was determined by UV visualation after agarose gel electrophoresis to be 1:10. The ligation reaction was

performed with a quick ligation kit (Roche # 1 635 379) and was composed of 0.5 µl mutant vector DNA, 7.5 µl of *mao-n* insert DNA, 2 µl DNA dilution buffer, 10 µl ligation buffer and 1 µl T4 ligase. The reaction was incubated at room temperature for 20min. A 5 µl aliquot of ligation mix was used to transform BL21(DE3) in the presence of β-mercaptoethanol (2 µl from 0.5M stock).

10.3 Characterisation of MAOmut"best"

10.3.1. Validation by site directed mutagenesis at position 336

10.3.1.1. Primers used to introduce the mutations

The genes encoding MAO-N wild type and MAOmut"best" were amplified by PCR, using the external MAO For and MAO Rev primers (§7.2.1.1). The internal primers MAOSer For and MAOSer Rev were designed to introduce serine at position 336 in MAO-N wild type sequence (codon for serine is in bold).

MAOSer For
5' GGT CAT GTG **AGT** ATG TGC ACG AAG 3'
MAOSer Rev
5' CGT GCA CAT **ACT** CAC ATG ACC TGC 3'

The internal primers MAOmutAsn For and MAOmutAsn Rev were designed to introduce asparagine at position 336 in MAOmut"best" sequence.

MAOmutAsn336 For
5' GGT CAT GTG **AAT** ATG TGC ACG AAG 3'
MAOmutAsn336 Rev
5' CGT GCA CAT **ATT** CAC ATG ACC TGC 3'

10.3.1.2. Introduction of the mutations

Six PCR reactions (§7.2.1.1) were performed to introduce the desired mutation in *mao-n* wild type or in *mao-mut"best"* genes.

First PCR: *mao-n* as a DNA template, MAOSerFor and MAORev as the primers.

Second PCR: *mao-n* as a DNA template, MAOSerRev and MAOFor as the primers.

Third PCR (gene recombination): 1µl from solution (1µl of first PCR product, 1µl of second PCR product and 8µl water) as a DNA template, MAOFor and MAORev as the primers.

Fourth PCR: *mao-mut"best"* as DNA template, MAOmutAsn336For and MAORev as the primers.

Fifth PCR: *mao-mut"best"* as DNA template MAOmutAsn336Rev and MAOFor as the primers.

Sixth PCR (gene recombination): 1µl from solution (1µl of fourth PCR product, 1µl of fifth PCR product and 8µl water) as a DNA template, MAOFor and MAORev as the primers.

First, second, fourth and fifth PCR products were gel purified and recovered into 30µl of H_2O prior to subsequent PCR reactions.

10.3.1.2. Cloning protocol

Final PCR products (third and sixth) were gel purified and recovered in 30µl of H_2O and digested with *Nde*I and *BamH*I (1.5 µl each) in presence of *BamH*I buffer, in a volume of 40 µl for 4 hours. The digested PCR products were agarose gel purified in a total volume of 30µl of H_2O, ligated to pET16b digested with *Nde*I and *BamH*I (vector/insert=1/10) by incubation with T4 DNA ligase at room temperature, 16 h.

10.3.2. Mutagenesis at position 348

10.3.2.1. Primers used to introduce the mutations

The gene encoding MAOmut"best" was amplified by PCR, using the external MAOFor and MAORev primers (§7.2.1.1). The internal primersMAOmutLys348FOR and MAOmutLys348REV were designed to introduce lysine at the position 348 in MAOmut"best sequence (cogon for lysine is in bold).

MAOmutLys348FOR
5' GTG GAC AAT AAG GAT ATG CGG TCG 3'

MAOmutLys348REV
5' CCG CAT ATC CTT ATT GTC CAC TTC 3'

10.3.2.2. Introduction of the mutations

Three PCR reactions were performed as described in § 10.3.1.2 but using MAOmutLys348FOR and MAOmutLys348REV as internal primers.

10.3.3. Validation of MAOmut"best" by subcloning

A 10µl aliquot of plasmid DNA of MAO-N wild type (4 µg) and the same amount of MAOmut"best" were separately mixed with a restriction digest cocktail (2µl *Not*I, 2µl *Mun*I, 2µl buffer U (Stratagene) and 4µl H_2O) and the reaction carried out at 37°C for 2 hours. Upon agarose gel electrophoresis the resulting sizes of the bands were 6184bp and 1029bp. Band 1029bp contained the sequence encoding the amino acid at position 336 and the 6184bp band contained the sequence encoding pET16b and the remainder of the MAO gene. 4 bands were purified from the gel slices and the cross over ligation was undertaken. The 6184bp fragment of DNA of *mao-n* wild type in

pET16b was ligated with the1029bp DNA of *mao-mut"best"* and in addition the 6184bp fragment of DNA of *mao- mut"best"* in pET16b was ligated with 1029bp DNA of *mao-n* wild type. The mass ratio of vector/ insert was determined by visualization after agarose gel electrophoresis to be 1:10. The ligation reaction consisted of 1 µl of the vector part of the plasmid, 7 µl of *Mun*I/*Not*I insert DNA, 1 µl ligase buffer and 1 µl T4 ligase. The reaction was incubated at room temperature for 18 hours. Thereafter 5 µl of the ligation mix was used to transformed into BL21(DE3) in the presence of β-mercaptoethanol (2 µl from 0.5M stock).

10.4. Validation of positive clones by purification studies

10.4.1. Purification by metal chelate affinity chromatography

10.4.1.1. Purification on BoiCAD FPLC.

i). Purification procedure

The protein was purified by employing the BioCAD 700E FPLC system (PerSpective Biosystems) with "Scout Column Select". The column (POROS MC, 4.6mm width, 100mm width, 1.66ml volume) was self-packed with "Self Pack POROS 20 MC Media".

The whole procedure was performed as close to 4°C as possible. A cell pellet from a 300ml culture (§7.2.2; iii) was resuspended in starting buffer and CFE was obtained by sonication (§72.3; i.). A 5ml sample of CFE was filtered through a 0.45µm sterile membrane prior to loading on the column using the loop.

The purification was performed at 10ml/min flow rate. The eluted samples were diluted with 25mM Tris HCl pH 7.8 to decrease imidasol concentration to 75mM, because of the negative effect of imidazole on enzyme activity.

The diluted samples were concentrated on a 50K Vivaspin (2ml) filtration unit, and kept over-night on ice at 4°C, until they were subjected to SDS PAGE analysis and activity studies. Imidasol needs to be removed completely from protein samples by dialysis (25mM Tris HCl pH7.8) for long term storage of purified protein at 80°C.

ii). Buffers.

Charging buffer	0.1M $NiCl_2$ pH4.5-5.0.
Starting buffer	25mM Potassium phosphate , 300mM NaCl, 10mM Imidasole, pH 7.8.
Elution buffer	25mM Potassium phosphate, 0.3M NaCl, 1M Imidasole, pH 7.8.
Strip buffer	0.1M EDTA, 1M NaCl
H_2O	
NaCl	0.5M NaCl

The NaCl and Strip buffers were prepared from standard stock solutions of 4M NaCl and 0.5M EDTA blended in the appropriate concentrations.

All buffers were degassed and filtered (0.2μm).

The protein was eluted by a step gradient of imidasole in 3ml fractions.

iii). Basic method.

Strip Buffer	5CV
H_2O	10CV
Charge buffer	30CV
H_2O	10CV
NaCl	10CV
Starting buffer	10CV
Load loop	5ml
Flush loop + Wash unbound	10ml

25mM Imidasole	25CV
Elution: 300mM Imidasole	15CV
H_2O	10CV

10.4.1.2. Purification by metal chelating affinity chromatography on ÅCTA FPLC.

The protein was purified on "ÅCTA" FPLC protein purification system (AmershamPharmacia Biotech). The "HiTrap™ Chelating HP" (5ml CV) column was obtained pre-packed from AmershamPharmacia Biotech.

i). Purification procedure

The purification procedure was performed as close to 4°C as possible. A 30g quantity of cell pellet obtained from 4 liters culture (§7.2.2; iii, iv) was resuspended in buffer A and CFE was obtained by sonication (§7.2.3; ii). A 30ml sample of CFE was then filtered through a 0.45µm sterile membrane prior to loading on the column using the super loop. The eluted samples were diluted with 25mM Tris HCl pH 7.8 to decrease imidasol concentration to 75mM and active fractions were stored at +4°C over night without loss of activity. The residual imidasole was removed by dialysis against 25mM Tris/HCl pH7.8; 0.1mM PMSF; 1mMDTT and sample stored at –80°C.

ii). Buffers

Charging buffer	0.1M $NiCl_2$ pH4.5-5.0.
Buffer A	25mM TrisHCl, NaCl 300mM, 1mM PMSF20mM Imidasole, pH 7.8
Buffer B	Buffer A+ 1M Imidasole
Strip buffer	0.1M EDTA, 1M NaCl

All buffers were degassed and filtered (0.2µm).

The protein was eluted by step gradient of Imidasole in 3ml fractions.

iii). Basic method.

Strip Buffer	5CV
H_2O	10CV
Charge buffer	10CV
H_2O	10CV
NaCl	10CV
Buffer A	10CV
Load loop	30ml
Fraction collected	5ml
Wash unbound sample: 100% buffer A	2CV
First step of elution: 90% buffer A and 10% buffer B	10CV
Second step of elution: 67% buffer A and 33% buffer B	10CV
Column clean: 100% buffer B	4CV
Strip buffer	5CV
H_2O	5CV

10.4.2. Purification by anion exchange chromatography.

The protein was purified on "ÅCTA" FPLC protein purification system (AmershamPharmacia Biotech). The "ResourceQ" (6ml CV) column was obtained pre-packed from AmershamPharmacia Biotech.

i) Purification procedure

The purifcation procedure was performed as close to 4°C as possible. The cell pellet obtained from 300ml culture (§ 7.2.2; iii, iv) was resuspended in buffer A and CFE was obtained by sonication (§7.2.3; i.). A 5ml sample of CFE was filtered through a 0.45μm sterile membrane prior to loading on the column using the loop. Fractions

were assayed using a colorimetric solution (§8.1.1) and active fractions were stored at –80°C.

ii). *Buffers and basic method*

All buffers were degassed and filtered (0.2μm).

Buffer A	25mMTris/HCL, pH 7.8
Buffer B	Buffer A + 1M NaCl

Increasing gradient of NaCl was used to elute the protein.

Flow rate	$3ml \cdot min^{-1}$
Fraction to collect	2ml
Wash unbound sample: 100% buffer A	2CV
Elution: 100% buffer A to 100% buffer B	20CV
Column clean: 100% buffer B	4CV

10.4.3. Bradford assay

Protein concentrations were measured by mixing Bradford reagent (5ml, (§11.6) with enzyme solution (100μl) and standing for 2 minutes. The spectrophotometer was zeroed against Bradford reagent (5ml) and water (100μl), and then the absorbance of the protein sample at 595nm was measured. If the absorbance exceeded the calibration range then the enzyme was diluted accordingly and the assay repeated. The protein concentration was obtained by comparison with a pre-determined calibration curve derived from known dilutions of BSA.

10.4.4. Characterisation of mutant enzymes

Activities towards amylamine (§8.1.1) and benzylamine (§8.1.2) were taken as an initial rate measurement over 3 minutes. Slow rates of activities were measured after 17 hours incubation at 22°C. The spectrophotometer was blanked with chromogenic solution (§8.1.1).

10.4.4.1. Specific activity

The enzymatic activity units produced per 1ml of enzyme sample, were calculated as described in (§8.1.1). The protein concentration in mg/ml was determined by Bradford assay (§10.4.3). Values for specific activity were calculated per one milligram of protein in solution.

10.4.4.2. Michaelis coefficient (K_M) and turnover number (k_{cat}).

The rates of oxidation of various concentrations of L-AMBA and AA by enzyme were measured (§8.1.1) to determine K_M and V_{max} for these substrates.
The listed concentrations are in mM: 0.015; 0.03; 0.06; 0.08; 0.1; 0.12; 0.15; 0.2; 0.24; 0.3; 0.6; 1.0; 5.0. Values for K_M and V_{max} were determined using the program "KaleidaGraph for Windows, Version 309; Synergy Software".

Values of turnover numbers (k_{cat}) were calculated per mol of flavoprotein subunit and per minute. Protein concentration in sample was determined by ratio Abs $_{458nm}$ over flavin extinction coefficient (ε= 11.0/mM/cm)[221]. k_{cat} min^{-1} was calculated as the ratio of V_{max} over ε =29400 (extinction coefficient of dye) over protein concentration, multiply by 60.

10.5. Application of the obtained mutations.

10.5.1. Improvement of MAO-N wild type expression.

The gene encoding MAO-N wild type was amplified by PCR, using the external MAO-For and MAO-Rev primers (§7.2.1.1). The internal primers MAOArg259/260 For and MAOArg259/260Rev were designed to introduce CGT codon at position 259 and 260 into *mao-n* wild type sequence (cogon for arginine 259&260 is in bold).

Arg259Arg260For
5' CAG TCT GCA TTT GCG **CGT CGT** TTT TGG GAG GAG GC 3'

Arg259Arg260Rev
5' GC CTC CTC CCA AAA **ACG ACG** CGC AAA TGC AGA CTG 3'

The introduction of mutations and cloning protocol were the same as in §10.3.1& §10.3.2.

10.5.2. Validation of protein activity in soluble and insoluble fractions

The cell pellet was resuspended in detail buffer. The sonication was performed as described in §7.2.3. The supernatant was separated from the pellet and the latter was resuspended in the same volume of buffer as supernatant. The presence of active protein in supernatant and in pellet was analysed by liquid phase assay (§8.1.1) and by SDS PAGE (§7.2.5.2).

11. Stock recipes

11.1. LB medium

Per litre:	Triptone peptone	10g
	Sodium Chloride	10g
	Yeast extract	5g

11.2. LB agar medium

LB medium	100ml
Agar	1.5g

11.3. Coomassie blue staining solution

Methanol	450ml
Acetic acid	100ml
Water	450ml
Coomassie blueR-250	500mg

11.4. Coomassie Blue de-staining solution

Methanol	400ml
Acetic acid	100ml
Water	500ml

11.5. 50xTris-acetate buffer (TAE)

Tris base	242g
Acetic acid	57.1ml
0.5M EDTA (pH8.0)	100ml

11.6. Bradford reagent

Coomassie Brilliant Blue G-250	100mg
Ethanol	50ml
Phosphoric acid (85%)	100ml
Water	up to1L

Comassie Blue was dissolved in ethanol and phosphoric acid added to this solution. Water was added last.

11.7. Acrylamide solution

30% (w/v) acrylamide/*bis*-acrylamide (Anachem)

11.8. SDS stacking gel (4% acrylamide)

Per 10ml:	Acrylamide solution	1.3ml
	0.5M Tris HCl, pH6.8	2.5ml
	10% (w/v) SDS	0.1ml
	TEMED	20µl
	10% (w/v) APS	100 µl
	Water	6.1ml

TEMED and 10% APS (freshly made up) added immediately before pouring the gel.

11.9. SDS separating gel (15% acrylamide)

Per 10ml:	Acrylamide solution	5.0ml
	1.5M TrisHCl, pH8.8	2.5ml
	10% (w/v) SDS	0.1ml
	TEMED	10µl
	10% (w/v) APS	100µl
	Water	2.35ml

TEMED and 10% APS (freshly made up) added immediately before pouring the gel.

11.10. 4xSDS reducing buffer

0.5M TrisHCl, pH6.8	2.0ml
Glycerol	1.6-2.0ml
10% (w/v) SDS	3.2ml
2-β-Mercaptoethanol	0.8ml
0.05%(v/v) Bromophenol Blue	0.4ml

11.11. 0.05(w/v) Bromophenol blue

0.04% in 20% Methanol, pH2.8-4.6

11.12. 10x SDS running buffer

Per litre	Tris base	30g
	Glycine	144g
	SDS	10g

11.13. 5 x DNA loading buffer

0.05% bromophenol blue in 4% glycerol

11.14. X-Gal solution

Stock solution – 20mg/ml in dimethylformamide.

80μg/ml final concentaration

11.15. Ethidium bromide

Stock solution	10mg/ml
Final concentration	0.5 μg/ml

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13 APPENDICES

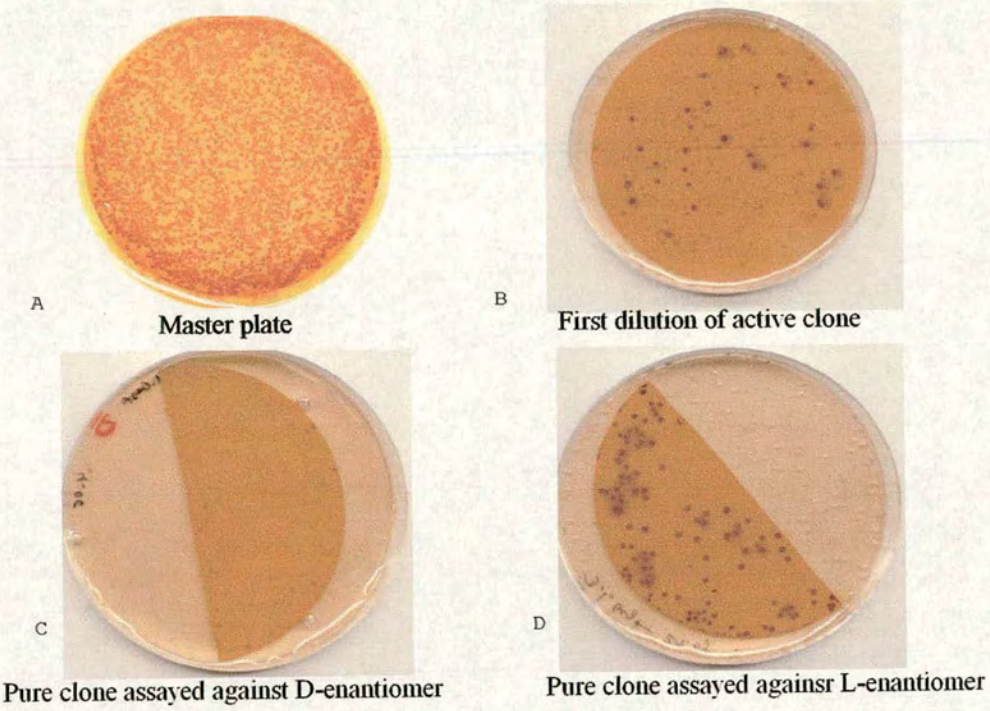


Figure 77: Strategy for solid phase assay. A. Colonies growing directly on nitrocellulose membrane at density 2000-3000 per plate. B. Active clone must be separated from the neighbour ones by subsequent dilution. C&D. Active clone plasmid DNA transformed into expression host and segmented membrane subjected to detect the enantioselectivity by solid phase assay.

```
>SWALL:PAO_MAIZE O64411 Polyamine oxidase precursor (EC 1.5.3.11).
        Length = 500

 Score = 121 (47.7 bits), Expect = 2.1e-07, Sum P(2) = 2.1e-07
 Identities = 28/59 (47%), Positives = 35/59 (59%)

Query:     38 GPWDVIVIGGGYCGLTATRDLTVAGFKTLL-LEARDRIGGRSWSSNIDGYPYEMGGTWV 95
              GP  VIV+G G  G++A + L+ AG    LL LEA D IGGR   +N  G   E+G  WV
Sbjct:     32 GP-RVIVVGAGMSGISAAKRLSEAGITDLLILEATDHIGGRMHKTNFAGINVELGANWV 89

 Score = 79 (32.9 bits), Expect = 2.1e-07, Sum P(2) = 2.1e-07
 Identities = 50/221 (22%), Positives = 87/221 (39%)

Query:    279 VRSVVNERDAARVTARDGREFAAKRLVCTIPLNVLST--IQFSPALSTERISAMQAGHVN 336
              VR +         V   D    ++A  ++ +  L VL +  IQF P L T ++ A+    +
Sbjct:    265 VREIKYSPGGVTVKTEDNSVYSADYVMVSASLGVLQSDLIQFKPKLPTWKVRAIYQFDMA 324

Query:    337 MCTKVHAEVDNKDMRSWTG---IAYPFNKL-CYAIG---DGTTPAGNTHLVCFGTD--AN 387
              + TK+  +   K      G      Y  ++  Y +     +   P  N  LV   TD  +
Sbjct:    325 VYTKIFLKFPRKFWPEGKGREFFLYASSRRGYYGVWQEFEKQYPDANVLLVTV-TDEESR 383

Query:    388 HI--QPDEDVR-ETLKAVGQLAPGTF--GVKRLVFHNWVKDEFAKGAWFFSRPGMVSEC- 441
               I  Q DE  + E ++ + ++ PG         ++   W  D F KG  F + P  V+
Sbjct:    384 RIEQQSDEQTKAEIMQVLRKMFPGKDVPDATDILVPRWWSDRFYKGT-FSNWPVGVNRYE 442

Query:    442 LQGLREKHRGVVFANSDWALGWRSFIDGAIEEGTRAARVVL 482
               LR     V F     +  +  ++ GA   G  +A +++
Sbjct:    443 YDQLRAPVGRVYFTGEHTSEHYNGYVHGAYLSGIDSAEILI 483
              DV+V+GGG  G+ A + L  +G   ++LEARDR+GGR+++         Y ++GG++V   Q
```

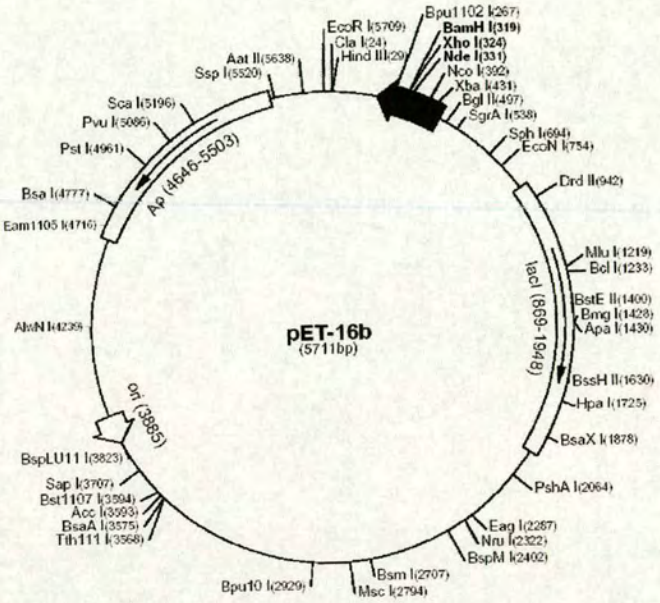
Figure 78: sequence alignment of MAO-N and PAO from maize (WU-Blast2). "Query" sequence corresponds to MAO-N, "Sbjct" sequence corresponds to MAO-B. Identical and similar residues belong to active site of MAO-B are in blue and the residues from active site without similarity are in yellow. The only amino acids belonging to the mature protein participe in sequences alignment (the mature PAO protein sequence starts with amino acid number 29).

TB046 2/00

pET-16b Vector

The pET-16b vector (Cat. No. 69662-3) carries an N-terminal His•Tag® sequence followed by a Factor Xa site and three cloning sites. Unique sites are shown on the circle map. Note that the sequence is numbered by the pBR322 convention, so the T7 expression region is reversed on the circular map. The cloning/expression region of the coding strand transcribed by T7 RNA polymerase is shown below.

pET-16b sequence landmarks	
T7 promoter	466–482
T7 transcription start	465
His•Tag coding sequence	360–389
Multiple cloning sites (*Nde* I–*BamH* I)	319–335
T7 terminator	213–259
lacI coding sequence	869–1948
pBR322 origin	3885
bla coding sequence	4646–5503



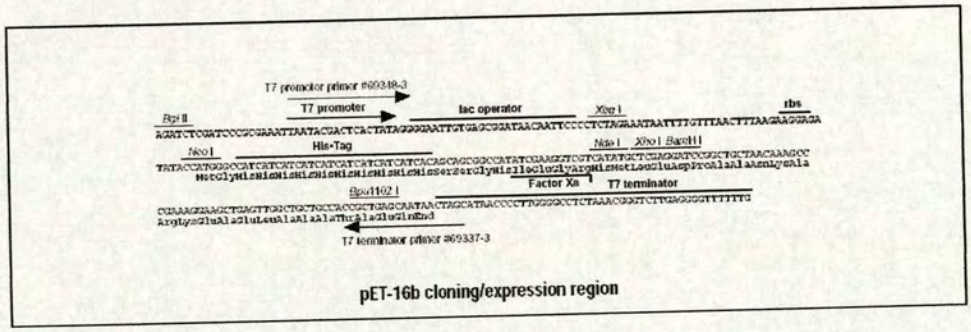


Figure 79: the map and the sequence of cloning/expression region of vector pET16b. The map obtained from the web site www.novagen.com.

pET16b restriction map

BioEdit version 5.0.9 Restriction Mapping Utility

5741 base pairs

```
1       TTCTCATGTTTGACAGCTTATCATCGATAAGCTTTAATGCGGTAGTTTATCACAGTTAAATTGCTAACGCAGTCAGGCAC   80
1       AAGAGTACAAACTGTCGAATAGTAGCTATTCGAAATTACGCCATCAAATAGTGTCAATTTAACGATTGCGTCAGTCCGTG   80
                                ClaI
                                    HindIII

81      CGTGTATGAAATCTAACAATGCGCTCATCGTCATCCTCGGCACCGTCACCCTGGATGCTGTAGGCATAGGCTTGGTTATG   160
81      GCACATACTTTAGATTGTTACGCGAGTAGCAGTAGGAGCCGTGGCAGTGGGACCTACGACATCCGTATCCGAACCAATAC   160

161     CCGGTACTGCCGGGCCTCTTGCGGGATATCCGGATATAGTTCCTCCTTTCAGCAAAAAACCCCTCAAGACCCGTTTAGAG   240
161     GGCCATGACGGCCCGGAGAACGCCCTATAGGCCTATATCAAGGAGGAAAGTCGTTTTTTGGGGAGTTCTGGGCAAATCTC   240
                                EcoRV
                                    BspEI

241     GCCCCAAGGGGTTATGCTAGTTATTGCTCAGCGGTGGCAGCAGCCAACTCAGCTTCCTTTCGGGCTTTGTTAGCAGCCGG   320
241     CGGGGTTCCCCAATACGATCAATAACGAGTCGCCACCGTCGTCGGTTGAGTCGAAGGAAAGCCCGAAACAATCGTCGGCC   320
                                                                                BamHI

321     ATCCTCGAGCATATGACGACCTTCGATATGGCCGCTGCTG361TGATGATGATGATGATGATGATGATGGCCCATGG     400
321     TAGGAGCTCGTATACTGCTGGAAGCTATACCGGCGACGAC361ACTACTACTACTACTACTACTACTACCGGGTACC     400
                                                                                NcoI
    XhoI    NdeI

401     TATATCTCCTTCTTAAAGTTAAACAAAATTATTTCTAGAGGGGAATTGTTATCCGCTCACAATTCCCCTATAGTGAGTCG   480
401     ATATAGAGGAAGAATTTCAATTTGTTTTAATAAAGATCTCCCCTTAACAATAGGCGAGTGTTAAGGGGATATCACTCAGC   480
                                XbaI                        BsrBI

481     TAT481TAATTTCGCGGGATCGAGATCTCGATCCTCTACGCCGGACGCATCGTGGCCGGCATCACC541GGCGCCACAGG     560
481     ATA481ATTAAAGCGCCCTAGCTCTAGAGCTAGGAGATGCGGCCTGCGTAGCACCGGCCGTAGTGG541CCGCGGTGTCC     560
                            BglII                       NgoAIV          NarI

561     TGCGGTTGCTGGCGCCTATATCGCCGACATCACCGATGGGGAAGATCGG601GCTCGCCACTTCGGGCTCATGAGCGCTT   640
561     ACGCCAACGACCGCGGATATAGCGGCTGTAGTGGCTACCCCTTCTAGCC601CGAGCGGTGAAGCCCGAGTACTCGCGAA   640
                NarI                                                        RcaI    Eco47III

641     GTTTCGGCGTGGGTATGGTGGCAGGCCCCGTGGCCGGGGGACTGTTGGGCGCCATCTCCTTGCATGCACCATTCCTTGCG   720
641     CAAAGCCGCACCCATACCACCGTCCGGGGCACCGGCCCCCTGACAACCCGCGGTAGAGGAACGTACGTGGTAAGGAACGC   720
                                        NarI                SphI

721     GCGGCGGTGCTC721AACGGCCTCAACCTACTACTGGGCTGCTTCCTAATGCAGGAGTCGCATAAGGGAGAGCGT781CG   800
721     CGCCGCCACGAG721TTGCCGGAGTTGGATGATGACCCGACGAAGGATTACGTCCTCAGCGTATTCCCTCTCGCA781GC   800
                                    BmrI

801     AGATCCCGGACACCATCGAATGGCGCAAAACCTTTCGCGGTATGGCATGATAGCGCCCGGAAGAGAGTCAATTCAGGGTG   880
801     TCTAGGGCCTGTGGTAGCTTACCGCGTTTTGGAAAGCGCCATACCGTACTATCGCGGGCCTTCTCTCAGTTAAGTCCCAC   880

881     GTGAATGTGAAACCAGTAACGTTATACGATGTCGCAGA901GTATGCCGGTGTCTCTTATCAGACCGTTTCCCGCGTGGT   960
881     CACTTACACTTTGGTCATTGCAATATGCTACAGCGTCT901CATACGGCCACAGAGAATAGTCTGGCAAAGGGCGCACCA   960
                        AclI
```

961 GAACCAGGCCAGCCACGTTTCTGCGAAAACGCGGGAAAAAGTGGAAGCGGCGATGGCGGAGCTGAATTACATTCCCAACC 1040
961 CTTGGTCCGGTCGGTGCAAAGACGCTTTTGCGCCCTTTTTCACCTTCGCCGCTACCGCCTCGACTTAATGTAAGGGTTGG 1040
DrdII EciI

1041 G1021CGTGGCACAACAACTGGCGGGCAAACAGTCGTTGCTGATTGGCGTTGCCACCTCCAGTCTGGCCCTGCACGCGCC 1120
1041 C1021GCACCGTGTTGTTGACCGCCCGTTTGTCAGCAACGACTAACCGCAACGGTGGAGGTCAGACCGGGACGTGCGCGG 1120

1121 GTCGCAAATTGTCGCGGCGATTAAATCTCGCGCCGATCAACTGGGTGCCAGCGTGGTGGTGTCGATGGTAGAACGAAGCG 1200
1121 CAGCGTTTAACAGCGCCGCTAATTTAGAGCGCGGCTAGTTGACCCACGGTCGCACCACCACAGCTACCATCTTGCTTCGC 1200
BmrI

1201 GCGTCGAAGCCTGTAAAGCGGCGGT1201GCACAATCTTCTCGCGCAACGCGTCAGTGGGCTGATCATTAACTATCCGCT 1280
1201 CGCAGCTTCGGACATTTCGCCGCCA1201CGTGTTAGAAGAGCGCGTTGCGCAGTCACCCGACTAGTAATTGATAGGCGA 1280
MluI BclI

1281 GGATGACCAGGATGCCATTGCTGTGGAAGCTGCCTGCACTAATGTTCCGGCGTTATTTCTTGATGTCTC1TGACCAGACA 1360
1281 CCTACTGGTCCTACGGTAACGACACCTTCGACGGACGTGATTACAAGGCCGCAATAAAGAACTACAGAG1ACTGGTCTGT 1360

1361 CCCATCAACAGTATTATTTTCTCCCATGAAGACGGTACGCGACTGGGCGTGGAGCATCTGGTCGCATTGGGTCACCAGCA 1440
1361 GGGTAGTTGTCATAATAAAAGAGGGTACTTCTGCCATGCGCTGACCCGCACCTCGTAGACCAGCGTAACCCAGTGGTCGT 1440
BmrI

1441 AATCGCGCTGTTAGCGGGCCCATTAAGTTCTGTCTCGGCGCGTCTGCGTCTGGCTGGCTGGCATAAATATCTCACTCGCA 1520
1441 TTAGCGCGACAATCGCCCGGGTAATTCAAGACAGAGCCGCGCAGACGCAGACCGACCGACCGTATTTATAGAGTGAGCGT 1520
ApaI

1521 ATCAAATTCAGCCGATAGCGGAACGGGAAGGCGACTGGAGTGCCATGTCCGGTTTTCAACAAACCATGCAAATGCTGAAT 1600
1521 TAGTTTAAGTCGGCTATCGCCTTGCCCTTCCGCTGACCTCACGGTACAGGCCAAAAGTTGTTTGGTACGTTTACGACTTA 1600

1601 GAGGGCATCGTTCCCACTGCGATGCTGGTTGCCAACGATCAGATGGCGCTGGGCGCAATGCGCGCCATTACCGAGTCCGG 1680
1601 CTCCCGTAGCAAGGGTGACGCTACGACCAACGGTTGCTAGTCTACCGCGACCCGCGTTACGCGCGGTAATGGCTCAGGCC 1680
BtsI BssHII

1681 GCTGCGCGTTGGTGCGGATATCTCGGTAGTGGGATACGACGATACCGAAGACAGCTCATGTTATATCCCGCCGTTAACCA 1760
1681 CGACGCGCAACCACGCCTATAGAGCCATCACCCTATGCTGCTATGGCTTCTGTCGAGTACAATATAGGGCGGCAATTGGT 1760
EcoRV HpaI

1761 CCATCAAACAGGATTTTCGCCTGCTGGGGCAAACCAGCGTGGACCGCTTGCTGCAACTCTCTCAGGGCCAGGCGGTGAAG 1840
1761 GGTAGTTTGTCCTAAAAGCGGACGACCCCGTTTGGTCGCACCTGGCGAACGACGTTGAGAGAGTCCCGGTCCGCCACTTC 1840

1841 GGCAATCAGCTGTTGCCCGTCTCACTGGTGAAAAGAAAAACCACCCTGGCGCCCAATACGCAAACCGCCTCTCCCCGCGC 1920
1841 CCGTTAGTCGACAACGGGCAGAGTGACCACTTTTCTTTTTGGTGGGACCGCGGGTTATGCGTTTGGCGGAGAGGGGCGCG 1920
PvuII NarI

1921 GTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTA 2000
1921 CAACCGGCTAAGTAATTACGTCGACCGTGCTGTCCAAAGGGCTGACCTTTCGCCCGTCACTCGCGTTGCGTTAATTACAT 2000
VspI PvuII BtsI VspI

2001 AGTTAGCTCACTCATTAGGCACCGGGATCTCGACCGATGCCCTTGAGAGCCTTCAACCCAGTCAGCTCCTTCCGGTGGGC 2080
2001 TCAATCGAGTGAGTAATCCGTGGCCCTAGAGCTGGCTACGGGAACTCTCGGAAGTTGGGTCAGTCGAGGAAGGCCACCCG 2080
BmrI

2081 GCGGGGCATGACTATCGTCGCCGCACTTATGACTGTCTTCTTTATCATGCAACTCGTAGGACAGGTGCCGGCAGCGCTCT 2160
2081 CGCCCCGTACTGATAGCAGCGGCGTGAATACTGACAGAAGAAATAGTACGTTGAGCATCCTGTCCACGGCCGTCGCGAGA 2160
Pf11108I NgoAIV Eco47III

```
2161  GGGTCATTTTCGGCGAGGACCGCTTTCGCTGGAGCGCGACGATGATCGGCCTGTCGCTTGCGGTATTCGGAATCTTGCAC  2240
2161  CCCAGTAAAAGCCGCTCCTGGCGAAAGCGACCTCGCGCTGCTACTAGCCGGACAGCGAACGCCATAAGCCTTAGAACGTG  2240

2241  GCCCTCGCTCAAGCCTTCGTCACTGGTCCCGCCACCAAACGTTTCGGCGAGAAGCAGGCCATTATCGCCGGCATGGCGGC  2320
2241  CGGGAGCGAGTTCGGAAGCAGTGACCAGGGCGGTGGTTTGCAAAGCCGCTCTTCGTCCGGTAATAGCGGCCGTACCGCCG  2320
                          AclI                                    NgoAIV      EagI

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2321  GCTGCGCGACCCGATGCAGAACGACCGCAAGCGCTGCGCTCCGACCTACCGGAAGGGGTAATACTAAGAAGAGCGAAGGC  2400
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2401  GCGGCATCGGGATGCCCGCGTTGCAGGCCATGCTGTCCAGGCAGGTAGATGACGACCATCAGGGACAGCTTCAAGGATCG  2480
2401  CGCCGTAGCCCTACGGGCGCAACGTCCGGTACGACAGGTCCGTCCATCTACTGCTGGTAGTCCCTGTCGAAGTTCCTAGC  2480
                                BspGI

2481  CTCGCGGCTCTTACCAGCCTAACTTCGATCACTGGACCGCTGATCGTCACGGCGATTTATGCCGCCTCGGCGAGCACATG  2560
2481  GAGCGCCGAGAATGGTCGGATTGAAGCTAGTGACCTGGCGACTAGCAGTGCCGCTAAATACGGCGGAGCCGCTCGTGTAC  2560
                                BspGI

2561  GAACGGGTTGGCATGGATTGTAGGCGCCGCCCTATACCTTGTCTGCCTCCCCGCGTTGCGTCGCGGTGCATGGAGCCGGG  2640
2561  CTTGCCCAACCGTACCTAACATCCGCGGCGGGATATGGAACAGACGGAGGGGCGCAACGCAGCGCCACGTACCTCGGCCC  2640
                          NarI

2641  CCACCTCGACCTGAATGGAAGCCGGCGGCACCTCGCTAACGGATTCACCACTCCAAGAATTGGAGCCAATCAATTCTTGC  2720
2641  GGTGGAGCTGGACTTACCTTCGGCCGCCGTGGAGCGATTGCCTAAGTGGTGAGGTTCTTAACCTCGGTTAGTTAAGAACG  2720
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2721  CCTCTTGACACTTACGCGTTTGGTTGGGAACCGTCTTGTATAGGTAGCGCAGGCGGTAGAGGTCGTCGGCGTGCGCCGCG  2800
                    FspI                                          EciI

2801  ATCTCGGGCAGCGTTGGGTCCTGGCCACGGGTGCGCATGATCGTGCTCCTGTCGTTGAGGACCCGGCTAGGCTGGCGGGG  2880
2801  TAGAGCCCGTCGCAACCCAGGACCGGTGCCCACGCGTACTAGCACGAGGACAGCAACTCCTGGGCCGATCCGACCGCCCC  2880
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2881  TTGCCTTACTGGTTAGCAGAATGAATCACCGATACGCGAGCGAACGTGAAGCGACTGCTGCTGCAAAACGTCTGCGACCT  2960
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  BspEI

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3121  GACTCACTAAAAAGAGACCAGGGCGGCGTAGGTATGGCGGTCAACAAATGGGAGTGTTGCAAGGTCATTGGCCCGTACAA  3200
                                                        AclI

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3201  GTAGTAGTCATTGGGCATAGCACTCGTAGGAGAGAGCAAAGTAGCCATAGTAATGGGGGTACTTGTCTTTAGGGGGAATG  3280

3281  ACGGAGGCATCAGTGACCAAACAGGAAAAAACCGCCCTTAACATGGCCCGCTTTATCAGAAGCCAGACATTAACGCTTCT  3360
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3361  CCTCTTTGAGTTGCTCGACCTGCGCCTACTTGTCCGTCTGTAGACACTTAGCGAAGTGCTGGTGCGACTACTCGAAATGG  3440
                          BspGI

3441  GCAGCTGCCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTG  3520
3441  CGTCGACGGAGCGCGCAAAGCCACTACTGCCACTTTTGGAGACTGTGTACGTCGAGGGCCTCTGCCAGTGTCGAACAGAC  3520
      PvuII

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3521  ATTCGCCTACGGCCCTCGTCTGTTCGGGCAGTCCCGCGCAGTCGCCCACAACCGCCCACAGCCCCGCGTCGGTACTGGGT  3600
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3601  CAGTGCATCGCTATCGCCTCACATATGACCGAATTGATACGCCGTAGTCTCGTCTAACATGACTCTCACGTGGTATATAC  3680
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3761  GCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATA  3840
3761  CGCGAGCCAGCAAGCCGACGCCGCTCGCCATAGTCGAGTGAGTTTCCGCCATTATGCCAATAGGTGTCTTAGTCCCCTAT  3840
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            EciI

4001  CCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTC  4080
4001  GGTCCGCAAAGGGGGACCTTCGAGGGAGCACGCGAGAGGACAAGGCTGGGACGGCGAATGGCCTATGGACAGGCGGAAAG  4080
                                  BssSI                                          EciI

4081  TCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTG  4160
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4161  CCGACACACGTGCTTGGGGGGCAAGTCGGGCTGGCGACGCGGAATAGGCCATTGATAGCAGAACTCAGGTTGGGCCATTC  4240
            ApaLI

4241  ACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCT  4320
4241  TGTGCTGAATAGCGGTGACCGTCGTCGGTGACCATTGTCCTAATCGTCTCGCTCCATACATCCGCCACGATGTCTCAAGA  4320

4321  TGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGA  4400
4321  ACTTCACCACCGGATTGATGCCGATGTGATCTTCCTGTCATAAACCATAGACGCGAGACGACTTCGGTCAATGGAAGCCT  4400

4401  AAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTAC  4480
4401  TTTTCTCAACCATCGAGAACTAGGCCGTTTGTTTGGTGGCGACCATCGCCACCAAAAAAACAAACGTTCGTCGTCTAATG  4480

4481  GCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTT  4560
4481  CGCGTCTTTTTTTCCTAGAGTTCTTCTAGGAAACTAGAAAAGATGCCCCAGACTGCGAGTCACCTTGCTTTTGAGTGCAA  4560
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4561   AAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATC  4640
4561   TTCCCTAAAACCAGTACTCTAATAGTTTTTCCTAGAAGTGGATCTAGGAAAATTTAATTTTTACTTCAAAATTTAGTTAG  4640
                   RcaI                                     DraI                DraI

4641   TAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATT  4720
4641   ATTTCATATATACTCATTTGAACCAGACTGTCAATGGTTACGAATTAGTCACTCCGTGGATAGAGTCGCTAGACAGATAA  4720

4721   TCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTG  4800
4721   AGCAAGTAGGTATCAACGGACTGAGGGGCAGCACATCTATTGATGCTATGCCCTCCCGAATGGTAGACCGGGGTCACGAC  4800
                                                   Pfl1108I                  BmrI

4801   CAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGA  4880
4801   GTTACTATGGCGCTCTGGGTGCGAGTGGCCGAGGTCTAAATAGTCGTTATTTGGTCGGTCGGCCTTCCCGGCTCGCGTCT  4880

4881   AGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAA  4960
4881   TCACCAGGACGTTGAAATAGGCGGAGGTAGGTCAGATAATTAACAACGGCCCTTCGATCTCATTCATCAAGCGGTCAATT  4960
                         EciI           VspI

4961   TAGTTTGCGCAACGTTGTTGCCATTGCTGCAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCG  5040
4961   ATCAAACGCGTTGCAACAACGGTAACGACGTCCGTAGCACCACAGTGCGAGCAGCAAACCATACCGAAGTAAGTCGAGGC  5040
              FspI                  PstI
                  AclI

5041   GTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTT  5120
5041   CAAGGGTTGCTAGTTCCGCTCAATGTACTAGGGGGTACAACACGTTTTTTCGCCAATCGAGGAAGCCAGGAGGCTAGCAA  5120
                                                                                  PvuI

5121   GTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGT  5200
5121   CAGTCTTCATTCAACCGGCGTCACAATAGTGAGTACCAATACCGTCGTGACGTATTAAGAGAATGACAGTACGGTAGGCA  5200
                         BtsI                    BtsI

5201   AAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCC  5280
5201   TTCTACGAAAAGACACTGACCACTCATGAGTTGGTTCAGTAAGACTCTTATCACATACGCCGCTGGCTCAACGAGAACGG  5280
                                 ScaI

5281   CGGCGTCAACACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGA  5360
5281   GCCGCAGTTGTGCCCTATTATGGCGCGGTGTATCGTCTTGAAATTTTCACGAGTAGTAACCTTTTGCAAGAAGCCCCGCT  5360
              BsbI                                  DraI                AclI

5361   AAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTT  5440
5361   TTTGAGAGTTCCTAGAATGGCGACAACTCTAGGTCAAGCTACATTGGGTGAGCACGTGGGTTGACTAGAAGTCGTAGAAA  5440
                                                            BssSI
                                                             ApaLI

5441   TACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAAT  5520
5441   ATGAAAGTGGTCGCAAAGACCCACTCGTTTTTGTCCTTCCGTTTTACGGCGTTTTTTCCCTTATTCCCGCTGTGCCTTTA  5520

5521   GTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTT  5600
5521   CAACTTATGAGTATGAGAAGGAAAAAGTTATAATAACTTCGTAAATAGTCCCAATAACAGAGTACTCGCCTATGTATAAA  5600
                                 SspI                                 RcaI
                                                                          BsrBI

5601   GAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCAT  5680
5601   CTTACATAAATCTTTTTATTTGTTTATCCCCAAGGCGCGTGTAAAGGGGCTTTTCACGGTGGACTGCAGATTCTTTGGTA  5680
                                                                           AatII

5681   TATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTCTTCAAGAA  5741
5681   ATAATAGTACTGTAATTGGATATTTTTATCCGCATAGTGCTCCGGGAAAGCAGAAGTTCTT  5741
           RcaI                           BssSI
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Restriction table:

Enzyme	Recognition	Frequency	Positions
AatII	G_ACGT'C	1	5669
AclI	AA'CG_TT	5	900, 2280, 3179, 4973, 5346
ApaI	G_GGCC'C	1	1461
ApaLI	G'TGCA_C	3	3668, 4168, 5414
BamHI	G'GATC_C	1	320
BclI	T'GATC_A	1	1263
BglII	A'GATC_T	1	504
BmrI	ACTGGG	6	756, 1163, 1405, 2059, 3599, 4793
BsbI	CAACAC	2	3569, 5290
BspEI	T'CCGG_A	2	190, 3043
BspGI	CTGGAC	3	2437, 2515, 3380
BspLU11I	A'CATG_T	1	3854
BsrBI	CCG'CTC	3	456, 3786, 5587
BssHII	G'CGCG_C	1	1661
BssSI	C'ACGA_G	3	4030, 5414, 5718
BstZ17I	GTA'TAC	1	3625
BtsI	GCAGTG	4	1617, 1978, 5142, 5169
ClaI	AT'CG_AT	1	25
DraI	TTT'AAA	3	4613, 4632, 5324
DrdII	GAACCA	1	964
EagI	C'GGCC_G	1	2318
EciI	TCCGCC	5	1017, 2774, 3928, 4074, 4902
Eco47III	AGC'GCT	3	637, 2156, 3108
EcoRV	GAT'ATC	2	188, 1700
FspI	TGC'GCA	3	2737, 2835, 4969
HindIII	A'AGCT_T	1	30
HpaI	GTT'AAC	1	1756
MluI	A'CGCG_T	1	1249
MscI	TGG'CCA	1	2825
NarI	GG'CG_CC	5	552, 573, 690, 1890, 2585
NcoI	C'CATG_G	1	396
NdeI	CA'TA_TG	1	332
NgoAIV	G'CCGG_C	4	536, 2148, 2308, 2662
NruI	TCG'CGA	1	2353
Pfl1108I	TCGTAG	2	2137, 4764
PstI	C_TGCA'G	1	4992
PvuI	CG_AT'CG	1	5117
PvuII	CAG'CTG	3	1850, 1943, 3445
RcaI	T'CATG_A	4	630, 4574, 5582, 5687
ScaI	AGT'ACT	1	5227
SphI	G_CATG'C	1	707
SspI	AAT'ATT	1	5551
VspI	AT'TA_AT	3	1935, 1994, 4919
XbaI	T'CTAG_A	1	435
XhoI	C'TCGA_G	1	325

Table of unique sites:

Enzyme	Recognition	Frequency	Position
AatII	G_ACGT'C	1	5669
ApaI	G_GGCC'C	1	1461
BamHI	G'GATC_C	1	320
BclI	T'GATC_A	1	1263
BglII	A'GATC_T	1	504
BspLU11I	A'CATG_T	1	3854
BssHII	G'CGCG_C	1	1661
BstZ17I	GTA'TAC	1	3625
ClaI	AT'CG_AT	1	25
DrdII	GAACCA	1	964
EagI	C'GGCC_G	1	2318
HindIII	A'AGCT_T	1	30
HpaI	GTT'AAC	1	1756
MluI	A'CGCG_T	1	1249
MscI	TGG'CCA	1	2825
NcoI	C'CATG_G	1	396
NdeI	CA'TA_TG	1	332

NruI	TCG'CGA	1	2353
PstI	C_TGCA'G	1	4992
PvuI	CG_AT'CG	1	5117
ScaI	AGT'ACT	1	5227
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SspI	AAT'ATT	1	5551
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XhoI	C'TCGA_G	1	325

Enzymes that cut five or fewer times

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BamHI	G'GATC_C	1	320
BclI	T'GATC_A	1	1263
BglII	A'GATC_T	1	504
BsbI	CAACAC	2	3569, 5290
BspEI	T'CCGG_A	2	190, 3043
BspGI	CTGGAC	3	2437, 2515, 3380
BspLU11I	A'CATG_T	1	3854
BsrBI	CCG'CTC	3	456, 3786, 5587
BssHII	G'CGCG_C	1	1661
BssSI	C'ACGA_G	3	4030, 5414, 5718
BstZ17I	GTA'TAC	1	3625
BtsI	GCAGTG	4	1617, 1978, 5142, 5169
ClaI	AT'CG_AT	1	25
DraI	TTT'AAA	3	4613, 4632, 5324
DrdII	GAACCA	1	964
EagI	C'GGCC_G	1	2318
EciI	TCCGCC	5	1017, 2774, 3928, 4074, 4902
Eco47III	AGC'GCT	3	637, 2156, 3108
EcoRV	GAT'ATC	2	188, 1700
FspI	TGC'GCA	3	2737, 2835, 4969
HindIII	A'AGCT_T	1	30
HpaI	GTT'AAC	1	1756
MluI	A'CGCG_T	1	1249
MscI	TGG'CCA	1	2825
NarI	GG'CG_CC	5	552, 573, 690, 1890, 2585
NcoI	C'CATG_G	1	396
NdeI	CA'TA_TG	1	332
NgoAIV	G'CCGG_C	4	536, 2148, 2308, 2662
NruI	TCG'CGA	1	2353
Pfl1108I	TCGTAG	2	2137, 4764
PstI	C_TGCA'G	1	4992
PvuI	CG_AT'CG	1	5117
PvuII	CAG'CTG	3	1850, 1943, 3445
RcaI	T'CATG_A	4	630, 4574, 5582, 5687
ScaI	AGT'ACT	1	5227
SphI	G_CATG'C	1	707
SspI	AAT'ATT	1	5551
VspI	AT'TA_AT	3	1935, 1994, 4919
XbaI	T'CTAG_A	1	435
XhoI	C'TCGA_G	1	325

Enzymes that do not cut:

AarI, AflII, AscI, AvrII, BbvCI, BsrGI, BtrI, EcoRI, FseI, KpnI, MunI, NheI, NotI
NsiI, NspV, PacI, PinAI, PmeI, PmlI, PsiI, SacI, SacII, SalI, SbfI, SgfI, SmaI
SnaBI, SpeI, SrfI, StuI, SunI, SwaI

MAO-N Restriction Map
BioEdit version 5.0.9 Restriction Mapping Utility
1488 base pairs

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1     ATGACCTCCCGAGACGGATACCAGTGGACACCCGAGACAGGGCTCACGCAGGGCGTCCCCTCTCTAGGAGTCATCTCCCC  80
1     TACTGGAGGGCTCTGCCTATGGTCACCTGTGGGCTCTGTCCCGAGTGCGTCCCGCAGGGGAGAGATCCTCAGTAGAGGGG  80

81    GCCCACTAATATCGAAGACACGGACAAAGATGGTCCATGGGACGTGATTGTCATTGGTGGAGGGTACTGCGGGTTGACTG  160
81    CGGGTGATTATAGCTTCTGTGCCTGTTTCTACCAGGTACCCTGCACTAACAGTAACCACCTCCCATGACGCCCAACTGAC  160
                                      NcoI     BtrI

161   CCACTAGGGACTTGACTGTAGCAGGCTTCAAAACCCTTCTCCTCGAAGCCCGAGACCGCATAGGCGGCCGCTCCTGGTCC  240
161   GGTGATCCCTGAACTGACATCGTCCGAAGTTTTGGGAAGAGGAGCTTCGGGCTCTGGCGTATCCGCCGGCGAGGACCAGG  240
                                                                  NotI
                                                                  EagI
                                                                      BsrBI

241   TCTAACATCGACGGCTATCCTTACGAGATGGGCGGCACATGGGTCCACTGGCACCAATCGCACGTATGGCGCGAAATCAC  320
241   AGATTGTAGCTGCCGATAGGAATGCTCTACCCGCCGTGTACCCAGGTGACCGTGGTTAGCGTGCATACCGCGCTTTAGTG  320

321   GCGCTACAAGATGCACAACGCCCTATCACCCTCCTTCAACTTCTCCCGCGGCGTGAATCACTTCCAGCTACGGACCAACC  400
321   CGCGATGTTCTACGTGTTGCGGGATAGTGGGAGGAAGTTGAAGAGGGCGCCGCACTTAGTGAAGGTCGATGCCTGGTTGG  400
                                            SacII

401   CCACCACATCAACCTACATGACTCACGAAGCCGAGGACGAGCTCCTCCGCTCCGCATTGCACAAGTTCACCAACGTGGAT  480
401   GGTGGTGTAGTTGGATGTACTGAGTGCTTCGGCTCCTGCTCGAGGAGGCGAGGCGTAACGTGTTCAAGTGGTTGCACCTA  480
                                        SacI   BsrBI

481   GGCACCAACGGCCGTACTGTCCTGCCCTTCCCGCATGACATGTTCTATGTTCCTGAGTTCAGGAAGTATGATGAGATGTC  560
481   CCGTGGTTGCCGGCATGACAGGACGGGAAGGGCGTACTGTACAAGATACAAGGACTCAAGTCCTTCATACTACTCTACAG  560
          EagI                              BspLU11I

561   ATACTCGGAGCGGATTGATCAAATCCGGGATGAGTTGAGCCTTAATGAACGGAGTTCTCTGGAAGCGTTTATATTGCTTT  640
561   TATGAGCCTCGCCTAACTAGTTTAGGCCCTACTCAACTCGGAATTACTTGCCTCAAGAGACCTTCGCAAATATAACGAAA  640
          BsrBI   BclI

641   GCTCTGGCGGAACGCTGGAGAATAGCTCATTTGGAGAATTCCTGCATTGGTGGGCGATGAGCGGATATACGTATCAGGGA  720
641   CGAGACCGCCTTGCGACCTCTTATCGAGTAAACCTCTTAAGGACGTAACCACCCGCTACTCGCCTATATGCATAGTCCCT  720
        EciI                          EcoRI                    BsrBI      SnaBI

721   TGCATGGACTGCTTGATAAGTTATAAGTTCAAGGATGGGCAGTCTGCATTTGCGAGGAGGTTTTGGGAGGAGGCGGCCGG  800
721   ACGTACCTGACGAACTATTCAATATTCAAGTTCCTACCCGTCAGACGTAAACGCTCCTCCAAAACCCTCCTCCGCCGGCC  800
                                                                                  EagI
      NsiI                  PsiI

801   GACGGGGAGGTTGGGGTATGTGTTTGGGTGTCCGGTTAGGAGTGTTGTTAATGAGAGAGATGCGGCGAGAGTGACGGCGA  880
801   CTGCCCCTCCAACCCCATACACAAACCCACAGGCCAATCCTCACAACAATTACTCTCTCTACGCCGCTCTCACTGCCGCT  880
                                          BsbI

881   GGGATGGCAGGGAGTTCGCTGCGAAGCGGCTGGTTTGCACTATTCCCCTCAATGTCTTGTCCACGATCCAGTTCTCACCT  960
881   CCCTACCGTCCCTCAAGCGACGCTTCGCCGACCAAACGTGATAAGGGGAGTTACAGAACAGGTGCTAGGTCAAGAGTGGA  960
                                                                                AarI

961   GCGCTGTCGACGGAGAGGATCTCTGCTATGCAGGCAGGTCATGTGAATATGTGCACGAAGGTGCATGCCGAAGTGGACAA  1040
961   CGCGACAGCTGCCTCTCCTAGAGACGATACGTCCGTCCAGTACACTTATACACGTGCTTCCACGTACGGCTTCACCTGTT  1040
        SalI                                          ApaLI              SphI

1041  TAAGGATATGCGGTCGTGGACGGGCATTGCGTACCCTTTCAATAAACTGTGCTATGCTATTGGTGATGGGACGACTCCCG  1120
1041  ATTCCTATACGCCAGCACCTGCCCGTAACGCATGGGAAAGTTATTTGACACGATACGATAACCACTACCCTGCTGAGGGC  1120
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1121    CGGGAAACACGCATCTGGTGTGTTTCGGGACGGATGCGAATCATATCCAGCCGGATGAGGACGTGCGGGAGACGTTGAAG   1200
1121    GCCCTTTGTGCGTAGACCACACAAAGCCCTGCCTACGCTTAGTATAGGTCGGCCTACTCCTGCACGCCCTCTGCAACTTC   1200
          SacII                                                                 BtrI

1201    GCGGTTGGGCAGTTAGCGCCTGGGACATTTGGAGTGAAGCGGTTGGTGTTTCACAATTGGGTGAAGGATGAGTTTGCGAA   1280
1201    CGCCAACCCGTCAATCGCGGACCCTGTAAACCTCACTTCGCCAACCACAAAGTGTTAACCCACTTCCTACTCAAACGCTT   1280
                                                          MunI

1281    GGGCGCGTGGTTCTTCTCTAGGCCTGGGATGGTGAGTGAGTGTTTGCAGGGGTTGAGGGAGAAGCATCGCGGTGTTGTGT   1360
1281    CCCGCGCACCAAGAAGAGATCCGGACCCTACCACTCACTCACAAACGTCCCCAACTCCCTCTTCGTAGCGCCACAACACA   1360
                  DrdII          StuI                                                 BsbI

1361    TTGCGAATTCAGATTGGGCGTTGGGGTGGAGGAGCTTTATTGATGGGGCGATTGAGGAGGGGACGAGAGCTGCTAGGGTG   1440
1361    AACGCTTAAGTCTAACCCGCAACCCCACCTCCTCGAAATAACTACCCCGCTAACTCCTCCCCTGCTCTCGACGATCCCAC   1440
            EcoRI

1441    GTGTTGGAGGAATTGGGAACGAAGAGGGAGGTGAAGGCTCGTTTGTGA   1488
1441    CACAACCTCCTTAACCCTTGCTTCTCCCTCCACTTCCGAGCAAACACT   1488
          BsbI
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Restriction table:

Enzyme	Recognition	Frequency	Positions
AarI	CACCTGC	1	959
ApaLI	G'TGCA_C	1	1012
BclI	T'GATC_A	1	577
BsbI	CAACAC	3	844, 1354, 1443
BspLU11I	A'CATG_T	1	519
BsrBI	CCG'CTC	4	231, 450, 570, 701
BtrI	CAC'GTC	2	123, 1182
DrdII	GAACCA	1	1290
EagI	C'GGCC_G	3	226, 490, 795
EciI	TCCGCC	1	648
EcoRI	G'AATT_C	2	677, 1366
MunI	C'AATT_G	1	1255
NcoI	C'CATG_G	1	116
NotI	GC'GGCC_GC	1	226
NsiI	A_TGCA'T	1	725
PsiI	TTA'TAA	1	744
SacI	G_AGCT'C	1	444
SacII	CC_GC'GG	2	370, 1122
SalI	G'TCGA_C	1	967
SnaBI	TAC'GTA	1	711
SphI	G_CATG'C	1	1028
StuI	AGG'CCT	1	1303

Table of unique sites:

Enzyme	Recognition	Frequency	Position
AarI	CACCTGC	1	959
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BspLU11I	A'CATG_T	1	519
DrdII	GAACCA	1	1290
EciI	TCCGCC	1	648
MunI	C'AATT_G	1	1255
NcoI	C'CATG_G	1	116
NotI	GC'GGCC_GC	1	226
NsiI	A_TGCA'T	1	725
PsiI	TTA'TAA	1	744
SacI	G_AGCT'C	1	444
SalI	G'TCGA_C	1	967
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SphI	G_CATG'C	1	1028

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BclI	T'GATC_A	1	577
BsbI	CAACAC	3	844, 1354, 1443
BspLU11I	A'CATG_T	1	519
BsrBI	CCG'CTC	4	231, 450, 570, 701
BtrI	CAC'GTC	2	123, 1182
DrdII	GAACCA	1	1290
EagI	C'GGCC_G	3	226, 490, 795
EciI	TCCGCC	1	648
EcoRI	G'AATT_C	2	677, 1366
MunI	C'AATT_G	1	1255
NcoI	C'CATG_G	1	116
NotI	GC'GGCC_GC	1	226
NsiI	A_TGCA'T	1	725
PsiI	TTA'TAA	1	744
SacI	G_AGCT'C	1	444
SacII	CC_GC'GG	2	370, 1122
SalI	G'TCGA_C	1	967
SnaBI	TAC'GTA	1	711
SphI	G_CATG'C	1	1028
StuI	AGG'CCT	1	1303

Enzymes that do not cut:

AatII, AclI, AflII, ApaI, AscI, AvrII, BamHI, BbvCI, BglII, BmrI, BspEI, BspGI BsrGI, BssHII, BssSI, BstZ17I, BtsI, ClaI, DraI, Eco47III, EcoRV, FseI, FspI HindIII, HpaI, KpnI, MluI, MscI, NarI, NdeI, NgoAIV, NheI, NruI, NspV, PacI Pfl1108I, PinAI, PmeI, PmlI, PstI, PvuI, PvuII, RcaI, SbfI, ScaI, SgfI, SmaI, SpeI SrfI, SspI, SunI, SwaI, VspI, XbaI, XhoI