Directed evolution of β-xylanase from *Thermomyces lanuginosus*

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DECLARATION

I hereby declare that this dissertation is my own, unaided work. It is being submitted for the degree, Master of Technology, to the Durban Institute of Technology, Durban, South Africa. It has not been submitted before for any degree or dissertation to any other institution.

Dawn Elizabeth Stephens

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"..But those who hope in the Lord will find their strength renewed.
They will soar on wings like eagles;
They will run and not get weary;
They will walk and not grow weak."

Isaiah 40: 31

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Undoubtedly, the most gratitude goes to the Almighty, whose love and patience guides me through every triumph and disappointment. Thank you for being my 'footprints in the sand'.

It is not the strongest of the species that survive, nor the most intelligent, but the one most responsive to change.

Charles Darwin

CONTENTS

SUMMARY

Most natural enzymes may be unsuitable for biotechnological processes since they have evolved over millions of years to acquire their specific biological functions. Such enzymes are often genetically altered to suit the rigours of industrial processes. Directed evolution is one such strategy and makes use of iterative rounds of random mutagenesis, screening and recombination to enhance the existing properties of enzymes. *Thermomyces lanuginosus* is a thermophilic fungus that produces high levels of a thermostable xylanase. The xylanase gene from *T. lanuginosus* DSM 5826 (*xynA*) was functionally expressed in *E. coli* as a LacZ-fusion protein (Schlacher *et al.*, 1996) and later crystallized (Gruber *et al.*, 1998). In this study, it was undertaken to improve the thermostability and catalytic activity of *xynA* using error-prone PCR with different concentrations of $MnCl_2$. The first step prior to mutagenesis was to determine the levels of xylanase that could be attained by the wild type XynA, both in the presence and absence of an inducer. IPTG, a lactose analogue, was used since *xynA* was expressed with a *lac* promoter. High amounts of IPTG were found to adversely affect xylanase production, whilst a low amount (0.1 mM) enhanced xylanase production. This amount was used to later induce xylanase production by the variants obtained after mutagenesis. IPTG was found to increase the rate and production of xylanase. After random mutagenesis of *xynA*, transformed colonies were first selected for xylanase production on 0.4% Remazol Brilliant Blue xylan and then screened at different temperatures for improved stability and activity. After the first round of screening, four variants, *viz.*, 1B5, 1B7, 1B11 and 1D2, showed slight improvement in both stability and activity and were subjected to further mutagenesis, using low concentrations of $MnCl_2$. Three variants, *viz.*, 2B7-10, 2B7-6 and 2B11-16, with markedly enhanced stability, were obtained. Variant 2B7-10 exhibited a five fold higher activity (3430 nkat/μg total protein) than the wild type XynA (657 nkat/ μg total protein). It retained 71% of its activity after treatment at 80^0C for 60 min and had a $t_{1/2}$ of 215 min at 70^0C, which is higher than that attained by XynA. Long-term thermostability screening at 70, 80, 90 and 100^0C revealed that variants 2B7-6 and 2B11-16 were, however, the most stable enzymes generated in this study, although their activities were lower or almost comparable with their parents. Sequence analysis of variant 1D2 revealed 4 amino acid substitutions within the α-helix of the protein. This region was strongly conserved with the more stable variant xylanases generated in this study. The most profound mutation seen with variant 2B7-10 was the disruption of the disulphide bridge. Most of the mutants obtained in this study displayed a trade-off between stability and activity, the exception being mutant 2B7-10. Currently, DNA shuffling techniques are being used to recombine these traits in a single xylanase.

LIST OF FIGURES

LIST OF TABLES

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

As man makes significant advancement in science, he has become more aware of the importance of the conservation of energy and natural resources, after repeatedly ravaging the environment for commercial gain. The depletion of natural resources, the advent of the threat of the greenhouse effect and other such catastrophes, led to the idea of utilizing lignocelluloses and other agricultural wastes for bioconversion. As lignocellulose is such an abundant and renewable resource, it is being exploited for the generation of numerous products. In addition, microbial degradation of this complex polymer has the capability of expanding the potential for this biomass. The products of such hydrolysis may be converted subsequently into liquid fuels, single cell protein, solvents and other chemicals by the selective use of specific fermentative microorganisms and can also contribute to the elimination of agricultural wastes (Thomson, 1993; Ahsan *et al.*, 2001). Increasing environmental concerns and governmental mandates have encouraged the development of greener chemistry to perform catalytic tasks in a variety of industrial processes.

Consequently, enzymes have been introduced into consumer products such as laundry detergents and are finding increasing application in industrial processes in the food, textile and pulp and paper industries (Kulkarni *et al.*, 1999). Initially, suitable enzymes were identified by screening strains from various culture collections. However, relatively poor enzyme stabilities, decreased catalytic activities under the conditions that characterize industrial processes, insufficient enzyme production and the availability or cost of producing the enzyme itself have proven to be formidable impediments to their large-scale application (Schellenberger, 1998).

As the field of molecular biology grew, so too did the scientists' desire to tinker with the basic building blocks of an enzyme: the amino acid sequence. This resulted in the birth of a fascinating field of research called protein engineering. As a result, the past few decades have seen the emergence of 'rational design', relying on the structural analysis and site-directed mutagenesis for the engineering of improved properties. This approach, however, has its drawbacks because the effects of multiple mutations on protein function are not easily predictable, even with structural data in hand (Tobin *et al.*, 2000).

However, investigators have been reporting better success by following an alternative approach, known as directed evolution, to alter the functions of complex molecules such as proteins and nucleic acids. Directed evolution, does not require information about how enzyme structure relates to function (Stemmer, 1994a; Kuchner and Arnold, 1997). This technique employs a random process in which error-prone PCR is used to create a library of mutagenized genes. Genetic selection or high throughput screening subsequently identifies the mutants that possess improved properties. The sorted genes might be subjected to further cycles of mutation and screening to enhance the original beneficial mutations. Directed evolution has been significantly improved using *in vitro* recombination or DNA shuffling (Stemmer, 1994b). These methods rapidly combine beneficial mutations that arise from random mutagenesis and significantly expand the sequence diversity derived from small pools of heterologous genes.

Second only to cellulose, hemicelluloses represent an enormous renewable resource, and as such, have immense potential to be utilized as alternative sources of energy (Ahsan *et al.*, 2001). Xylans are heteropolysaccharides that represent the most abundant type of hemicellulose synthesized in the biosphere. In recent years, there has been a growing interest in xylanases due to their potential application in the pulp, paper and fibre-processing industries. Biologically, xylanases are synthesized by microorganisms and secreted to degrade the surrounding xylan as food supply. Xylan degradation has been reported in bacteria, fungi and yeasts (Dekker and Richards, 1976). Xylanase-producing microorganisms include the thermophilic Deuteromycete, *Thermomyces lanuginosus*, which is reported to rank amongst the best cellulase-free xylanase producers in nature (Singh *et al.*, 2000a). Endo-β-1,4-xylanase from *T. lanuginosus* is not only thermostable but also displays other advantages, notably it's preference for slightly acidic to neutral pH optima (Gomes *et al.*, 1993a).

While chlorine-free bleaching is carried out at acidic pH, chlorine bleaching is preceded by a hot, caustic treatment of the wood. Desirable properties of a biotechnologically-useful xylanase therefore include stability and activity at high temperatures and extreme pH values. For the commercial realization and economic viability of xylanase production, it is necessary to identify organisms which can hyper-produce the enzymes. Therefore, improvement in the hydrolyzing efficiency of xylanases has long been anticipated and trials, aided by modern recombinant DNA technologies, have been undertaken at an ever-increasing pace over the last few years (Ahsan *et al.*, 2001). Clearly, the best route for the improvement of xylanases to withstand the rigours of pulping processes (alkaline pH, high temperatures) would be to genetically tailor the xylanase gene to overcome such bottlenecks.

Most investigations have focused on the external parameters (environmental pH, temperature, etc.) that maximize xylanase production, but improving the enzyme on a genetic level seems to be a much more feasible approach for the over-production of a multi-faceted enzyme with unique capabilities. Directed evolution seems to be the appropriate vehicle of choice for the proposed improvement of the xylanase since it requires no structural information, knowledge of deactivation mechanisms or understanding of the molecular basis for thermostability or catalytic efficiency. Thus, the focus of this study was to improve the inherent thermostability and catalytic efficiency of the xylanase produced by *T. lanuginosus*.

1.2 ENZYMES – BIOCATALYSTS FOR THE FUTURE

Biological systems are the masters of chemical synthesis. The remarkable specificity of their enzyme catalysts allows hundreds of reactions to proceed simultaneously inside the tiny reactor that is a living cell. An enzyme's ability to carry out complex chemical reactions under very mild reaction conditions with virtually no waste products has earned them the admiration of chemists and biochemists. It is easy to envision that a future chemical industry sensitive to energy needs and the environment could be fashioned after these highly efficient chemical factories. The molecules responsible for this remarkable performance are the enzymes. Enzymes are proteins that consist of linear chains of typically hundreds of amino acids that fold up into unique well-defined three-dimensional structures (Arnold, 1996).

During the last 15 years, enzymes have been introduced into consumer products such as laundry detergents and are increasingly used in large-scale industrial processes in the food, textile and pulp and paper industries (Schellenberger, 1998). Enzymes of a given function can exhibit widely different properties (stability, solubility, tolerance to different pH, etc.) depending on where they are found (Arnold, 1996).

Mounting social, political and environmental pressures on industry to provide alternatives to chemical-based methods, have given added impetus to the search for novel enzymes with unique and industrially-significant traits. Thus, numerous organisms have been isolated from extreme environments. From the frosty Antarctic ice fields to the fiery volcanic pools, these organisms possess unique survival kits that allow them to survive under these extreme conditions. In most cases, adaptation to such extreme environments has not required completely new molecular machinery: in fact many 'extremophilic' enzymes are similar to their mesophilic counterparts found in more

hospitable environments. Sequence comparisons indicate that these enzymes are derived from a common ancestral enzyme and have accumulated mutations that allowed them to adapt over millions of years (Jaenicke and Bohm, 1998).

Even though there are so many advantages to using enzymes as substitutes for chemical catalysts, practical applications of enzyme catalysis are few and far between. This is largely due to their relatively poor stabilities and catalytic activities under the conditions that characterize industrial processes: high temperatures, extremes of pH or non-aqueous solvents. Enzymes evolved for the survival benefit of an organism may not exhibit features essential for *in vitro* application (Chen and Arnold, 1993).

Chemical engineers who try to design real industrial processes using biological catalysts are constantly stymied by a simple fact: biological systems have evolved over millions of years to perform very specific biological functions and to do so within the context of a living organism. Some of the features required for function in a complex chemical network are undesirable when the catalyst is lifted out of context. Conversely, many of the properties that are desired for an industrial enzyme clash with the needs of the organism, or at least were never required. The biotechnologist is hardly impressed by a catalyst whose inability to tolerate the most common of industrial conditions necessitates the use of complicated hardware and large reactors to facilitate their function. Catalysts that are stable to high temperatures, able to function in solvents other than water, tolerate wider ranges of pH, catalyze reactions on substrates not encountered in nature and catalyze reactions not found in nature, are required for industrial processes (Arnold, 1996).

1.3 PROTEIN ENGINEERING – A BROAD PERSPECTIVE

An enzyme is defined by a unique sequence of amino acids, which in turn is dictated by the organism's DNA code (the gene) and assembly in the cell. This amino acid sequence determines how the chain folds, and ultimately, how the enzyme functions. By modifying the amino acid sequence, the enzyme's function can be altered. This field dealing with protein redesign is known as protein engineering (Arnold, 1996).

The application of enzymes in industrial processes often calls upon properties not found in enzymes isolated from their natural environments. Natural enzymes are poised on the brink of conformational

instability, with native structures that walk a tightrope between large stabilizing and destabilizing forces. The molecular origins of enzyme stability are critical to understanding how proteins fold into their unique three-dimensional structures as well as to understanding the limits of life. The challenge, however, for elucidating such mechanisms, is daunting (Giver *et al.*, 1998).

A primary goal of protein engineering is to generate proteins with new or improved properties as a means to complement the shortcoming of known natural enzymes. The three main targets for optimization of enzymes as industrial biocatalysts are the volumetric productivity or activity of the enzyme, the stability of the enzyme under process conditions, and the availability or cost of producing the enzyme itself (Marrs *et al.*, 1999). The key to protein engineering is to understand how enzymes attained their eccentricities, i.e., how did enzymes adapt to their unique niches? If these adaptive mechanisms are understood, then this insight could be used to engineer enzymes for non-natural conditions. Unfortunately, this understanding has proven elusive because these mechanisms are both numerous and complex. Sometimes, it is impossible to determine if differences between enzymes from polar extremes are the results of adaptation or the results of neutral drift. Another serious difficulty facing comparative studies is the identification of which enzyme properties have evolved under selective pressure. In addition, organisms are subject to complex combinations of selective pressures. Furthermore, not all differences in enzyme properties reflect adaptation (Miyazaki and Arnold, 1999), and biological relevance plays an important role in the selection of thermostable enzymes (Jaenicke, 1981; Adams and Kelly, 1998).

Although evolution is a superb method to design proteins, it is slow. A process that naturally occurs over time scales of millions of years is infeasible for laboratory work and, even mutation in rational protein design has its drawbacks. Despite many advances in protein engineering, altering the specificity of an enzyme proves to be very difficult. When amino acids are altered to engineer a desired change, it is nearly impossible to predict all the small structural changes that occur to neighbouring amino acids. Even alteration of amino acids far from the active site has been shown in numerous systems to have large effects on enzyme performance. Orbital steering, which postulates that very small changes in the orientation of active site residues and substrates play a large role in substrate binding and catalysis, can explain some of these difficulties (Doyle *et al.*, 2000). Also, proteins are surprisingly resilient toward mutation. Protein tolerance is defined as the ability of a protein to undergo mutation without disrupting its fitness or structure. Within a protein there is a distribution of tolerances. Some sites that are essential for function may not accept any mutations, while other

positions can accept any amino acid substitutions with little effect. Functional tolerance is a significant factor for the success of protein engineering. A protein that is functionally tolerant allows many mutations without disrupting its structure, making it more likely that there is a connected path in sequence space of single mutations that leads to regions of higher fitness. Tolerance also affects the quality of the mutant library. If the protein is functionally intolerant, the mutant library will consist of mostly inactive proteins (Voigt *et al.*, 2001).

Enzyme engineering is undergoing the most profound and exciting transformation in its history. It promises unprecedented expansion in the scope and applications of modified or improved enzymes with desired physical and catalytic properties. Two complementary strategies are currently available: rational design and directed evolution. Although both approaches have been met with great success, each has its own limitations (Chen, 2001).

1.4 RATIONAL DESIGN

In rational design precise changes in amino acid sequence are preconceived based on detailed knowledge of protein structure, function and mechanism, and are then introduced using site-directed mutagenesis (Fig. 1.1). This technology holds much promise for optimizing the desired properties of enzymes for commercial applications. It also greatly enhances the basic understanding of enzyme binding and catalytic mechanisms, thus increasing the success of future enzyme engineering efforts and lays the foundation for functional prediction of new protein sequences in databases (Chen, 1999). The power of rational design has been demonstrated by the complete inversion of coenzyme specificities for isopropylmalate dehydrogenases (Chen *et al.*, 1996), where individual amino acid substitution or secondary structure engineering generated enzymes with desirable properties.

However, despite many other spectacular examples, numerous attempts at redesigning enzymes have failed. These failures might result, to some extent, from an incomplete understanding of the underlying mechanisms required to enhance the desired enzyme properties. Many original 'rational' engineering endeavours failed since a significant number of attempts were based on primary amino acid sequence homologies as the only criterion for amino acid replacements. In many instances, these substitutions were made without regard to the structural properties of the protein. Such 'homology-based' engineering frequently leads to substituting rigidly conserved amino acids that do not affect the desired enzyme properties and renders the enzyme inactive because of changes in protein conformation.

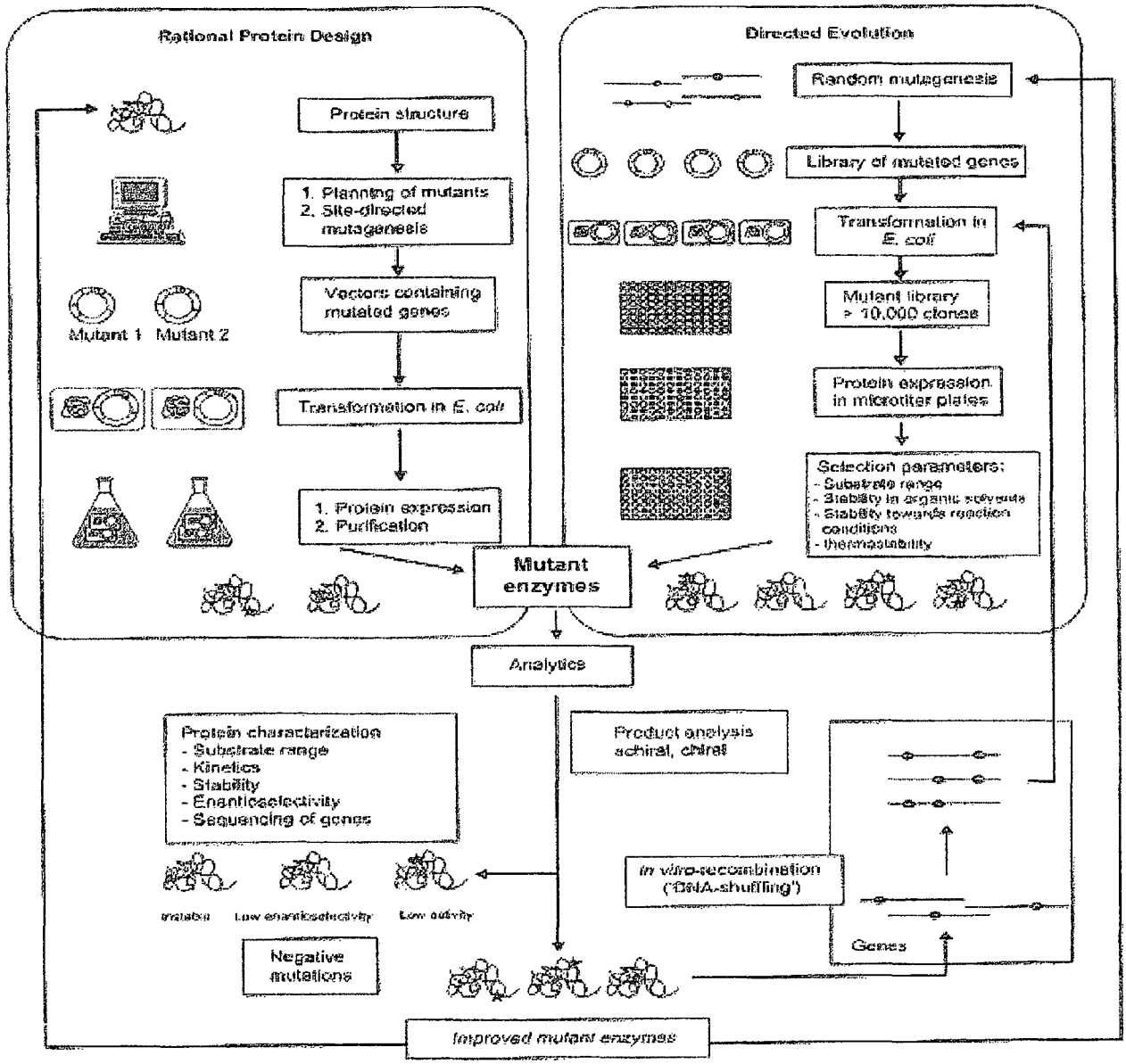

Fig. 1.1 Comparison of rational protein design and directed evolution strategies. During rational protein design, mutants are planned on the basis of their protein structure and then prepared by site-directed mutagenesis. After transformation in the host organism (e.g. *E. coli*), the variant is expressed, purified and analyzed for desired properties. Directed evolution starts with the preparation of mutant gene libraries by random mutagenesis, which is then expressed in the host organism. Protein libraries are usually screened in microtitre plates using a range of selection parameters. Protein characterization and product analysis sort out desired and negative mutations. *In vitro* recombination by DNA shuffling, for example, can be used for further improvements. Both protein engineering approaches can be repeated or combined until biocatalysts with desired properties are generated (Bornscheuer and Pohl, 2001).

This process overlooks key amino acid residues, particularly in the comparison of highly divergent enzymes (Chen *et al.*, 1996). The conventional approach of rational design requires confirmation of the mutation by sequencing and subsequent purification of the mutant enzymes following each round of mutagenesis so that kinetic and functional properties can be determined.
Such an approach is tedious and expensive, and might be impractical for multiple cycles of mutagenesis. An efficient strategy for identifying beneficial mutants using kinetics has been put forward to greatly facilitate the rational design of enzymes that require many cycles of mutagenesis to improve their properties (Chen, 1999).

Despite intense research into fundamental features governing protein folding and function, there are enormous gaps in the understanding of two critical processes: the relationship between sequence and structure and the relationship between structure and function. As a result, the rational design of new proteins by the classical 'reductionist' approach can be an extremely frustrating exercise (Arnold, 1996). The directed evolution of enzymes, however, is described as a new and elegant approach to generate and identify new enzyme variants.

1.5 DIRECTED EVOLUTION – AN OVERVIEW

Many clues as to how to engineer better enzymes came from studying how nature has created enzymes. By studying the evolution of natural proteins, it has been learned that they are highly adaptable, constantly changing molecules, at least over evolutionary time scales. They can adapt to new environments and they can even take on new tasks. It is now known that many enzymes catalyzing very different reactions have come about by divergent evolution from a common ancestral protein of the same general structure, acquiring diverse capabilities by processes of random mutation, recombination and natural selection (Arnold, 1996).

Since the beginning of human existence, man has relied on evolution to improve his chances of survival. Corn was bred for bigger size and more kernels; yeast was bred to improve its beer and fermenting properties and livestock were bred for desirable traits and applications. Throughout, man's ancestors were not in a position to understand, for example, that the structure of the corn growth factor receptor was a way of improving the final product. Function came first and form followed function – a seemingly 'rational' approach that was made possible by genetic recombination resulting from natural

sexual inheritance. This simple formula has resulted in a long history of success using directed evolution (Tobin *et al.*, 2000).

Directed evolution does not require information about how enzyme structure relates to function. Experiments that facilitate the evolution of enzymes in the laboratory under controlled conditions and well-defined selection pressures can help clarify the confusion introduced by natural evolution. Directed evolution allows for the generation of functional changes of enzymes with only minimal changes in sequence. This approach also allows for the generation of different evolutionary scenarios and the monitoring of the adaptation process, with access to all intermediates. Moreover, it can be determined whether the mutations parallel those that are found in natural homologous enzymes or whether there are multiple pathways that lead to the same functional result. With laboratory evolution, apart from the requirement that the enzyme be functionally expressed in the host organism, it is free of biological constraints and free to access all physically possible enzymes that the search algorithm (mutation, recombination and screening or selection) can generate. Furthermore directed evolution can attempt to create enzymes for which there are no natural counterparts (Arnold *et al.*, 2001).

Directed enzyme evolution generally begins with the creation of a library of mutated genes. Gene products that show improvement with respect to the desired property or set of properties are identified by selection or screening and the gene(s) encoding those enzymes are subjected to further cycles of mutation and screening in order to accumulate beneficial mutations (Fig. 1.1). This evolution can involve few or many generations, depending on how far one wishes to progress and the effects of the mutations observed in each generation (Kuchner and Arnold, 1997).

1.5.1 Pre-requisites for successful directed evolution

The main requirements for successful directed evolution are:

(a) the functional expression of the enzyme in a suitable microbial host,

(b) the availability of a screen (or selection) sensitive to the desired properties and

(c) identifying a workable evolution strategy.

The vast majority of possible evolutionary paths lead to inferior enzymes, thus the strategic challenge is to identify a path that will result in the improvement of the desired feature(s). The number of possible variants increases rapidly with the size of the enzyme and with the number of amino acids that

are allowed to vary simultaneously. Even for a small protein, an impossibly large number of variants can be generated when multiple mutations are introduced. Because most mutations are deleterious, the chances of identifying improved enzymes in libraries containing large numbers of mutations are very small indeed. Therefore, the mutation rate must be tuned to the power of the screen or selection (Arnold *et al.*, 2001).

1.5.2 Generation of molecular diversity

Mutation is required for the introduction of gene diversity and such diversity can be introduced into genes of interest by various methods. One efficient approach is to synthesize degenerate oligonucleotides by mixing nucleotides at each step. Accordingly, highly mutated synthetic oligonucleotide libraries can be prepared. Such a strategy is almost ideal in that it allows the researcher to precisely define the randomly mutagenized window, to mutagenize each nucleotide with a given probability and to select the bases introduced at each position. However, this method is limited to DNA fragments containing no more than 100 residues (Arnold and Volkov, 1999).

Alternative methods are based on the use of chemical mutagens like sodium bisulphate, nitrous acid, formic acid, hydrazine or hydroxylamine. However, these methods do not yield highly mutagenized fragments and cannot generate all the possible base substitutions. Exposure of DNA to ultraviolet light and propagation of a particular gene in mutator strains can be used to produce a particular genetic segment containing multiple mutations allows one to mutagenize a gene of interest. Alternatively, 'poisoned' primers can be used to introduce mutations into a small region of a gene (Fromant *et al.*, 1995). Random mutations can be enzymatically introduced through misincorporation of nucleotides by polymerases. In such an approach, the rate of mutations may be increased by using polymerase species lacking proof-reading activity, by mixing unequal concentrations of the four deoxynucleoside triphosphates (dNTPs) or by using PCR to perform successive runs of polymerization, which accumulate misincorporations (Cadwell and Joyce, 1992).

Random point mutagenesis by error-prone PCR (ep-PCR) involves a modified PCR protocol that uses variations in magnesium chloride ($MgCl_2$) and manganese chloride ($MnCl_2$) concentrations to achieve a mutation level of 2-5 base substitutions per gene, corresponding to an average exchange of one amino acid per mutated protein (Fig. 1.2). Because of the inherent mutation bias of ep-PCR and the restrictions imposed by the genetic code, this method is not suitable for introducing all 20 amino acid

residues at each position of the protein, i.e., the mutations are not truly random. In fact, an average of 5.7 amino acid substitutions is accessible for any given amino acid residue using this method (Miyazaki and Arnold, 1999).

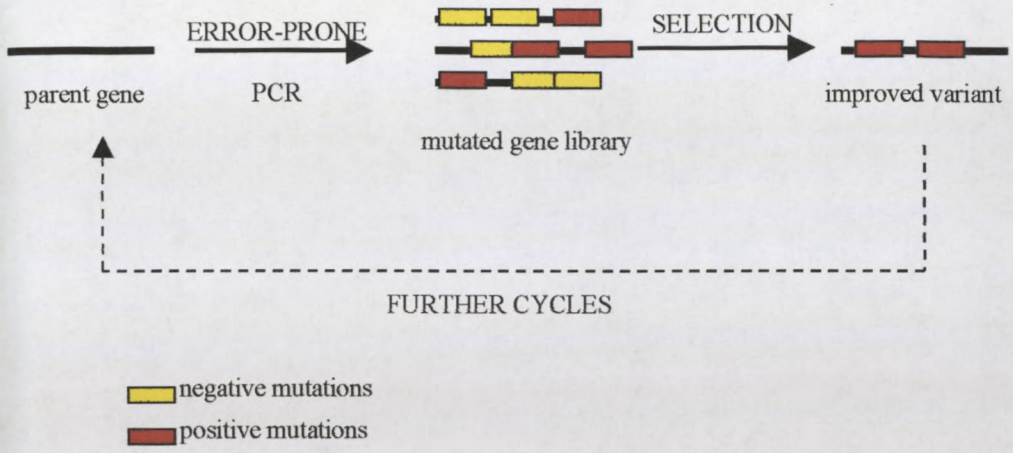

Fig. 1.2 The asexual evolution of a starting gene through, for example, error-prone PCR or application of mutator strains introduces point mutations. The resulting enzyme library is then screened for improved enzyme variants (Bornscheuer, 1998).

The beginning of the 'modern era' of directed evolution can be essentially defined by the invention of DNA shuffling (Stemmer, 1994a; 1994b). This technology accesses an important facet of natural evolution that was lacking in previous formats: the ability to recombine mutations from individual genes akin to natural sexual recombination.

A salient difference between recombination in DNA shuffling and natural sexual recombination is that shuffling accesses not only natural pair-wise recombination, but also pool-wise (multiple parent) recombination of parent molecules. The benefit of pool-wise recombination is obvious: an enormous combinatorial potential is realized, enabling access to vast areas of sequence space. DNA shuffling provides a method for the rapid functional evolution of DNA, including genes encoding enzymes and enzyme families and synthetic and degradative pathways, multi-gene traits, vectors, viruses and chromosomes. It has proven to be an especially powerful method for the evolution of single gene products with enhanced activity (Chang *et al.*, 1999), altered substrate specificity (Zhang *et al.*, 1997), improved protein folding (Crameri *et al.*, 1996) and pathways with improved function (Crameri *et al.*, 1997). This technique has recently been further refined and termed DNA family shuffling or molecular breeding.

1.5.3 Identification of improved variants

A major challenge in the application of directed evolution is to establish a technique for isolating improved variants from a population of random clones. These techniques can be divided into two categories: selection and screening.

Selection is normally used for evolving intracellular enzymes, for which the desired link between cell survival and enzyme activity is easier to implement. The following example depicts a selection where cell growth was linked to enzyme activity. The serine protease subtilisin, produced by various *Bacillus* species, is used as an additive in most modern laundry detergents, where it helps to dissolve proteinaceous stains. Cells were grown in hollow fibres at an average density of one clone per fibre, using bovine serum as the sole source of nitrogen. Cells secreting more subtilisin or more active enzyme should be able to obtain more nitrogen and grow faster. One round of mutagenesis and enrichment produced a clone with fivefold increased protease production, which arose from mutations outside the target gene. This is a good example of how selection, as a method of identifying improved variants, was successful (Naki *et al.*, 1998).

Applying selection techniques to very large populations of random mutants significantly increases the probability of identifying useful single or double mutations that are too rare to be found in small populations. Most selection methods are based either on surface display or on functional complementation. Selection is normally used for evolving intracellular enzymes, for which the required link between cell survival and enzyme activity is easier to implement. Selection also requires that the target enzymatic activity does not interfere with cellular metabolism and that it can be distinguished from the background of all other cell reactions (Arnold and Volkov, 1999).

Display techniques start from constructs in which the protein of interest and its coding gene are structurally linked to one another, typically by fusing the gene of interest to a protein in the outer coat of a phage, virus or cell (Burton, 1995). Alternatively, the gene of interest may be fused to promoters of a plasmid. Improved variants can be isolated from large libraries by binding them to an immobilized ligand, a method known as biopanning. Because the gene encoding the target protein is carried within a virus, plasmid or cell, the selected mutations can be easily identified and the enriched population can be subjected to further rounds of evolution (Mandecki *et al.*, 1995). Display techniques are extremely

powerful for improving the binding properties of antibodies or enzyme inhibitors (Schellenberger, 1998).

When an enzyme's activity is essential for growth of an organism, functional complementation provides a straightforward means for selecting enzymes with improved catalytic properties. A prominent example is antibiotic resistance: in many cases, such resistance reflects the catalytic conversion of the antibiotic into compounds that are no longer toxic to the targeted microorganisms. In such circumstances, selection may enrich for even those mutations that only slightly improve a function (Schellenberger, 1998).

Selection is an attractive technique for searching larger libraries of variants, but is difficult to devise for enzymes that are not critical to the survival of the host organism. Furthermore, organisms are notoriously adept at evading imposed selective pressures by unexpected mechanisms. Less-stringent functional complementation can be very useful for identifying useful variants that retain biological activity in libraries generated using relatively high mutagenic rates. In choosing an assay or selection, it is useful to remember the first law of random mutagenesis: 'you get what you screen for!' (Arnold and Volkov, 1999).

Screening is by far the most flexible approach. It can be performed on agar or microtitre plates, and the assay conditions can be readily adjusted to improve the performance of the affected population. Furthermore, screening can be performed under conditions that are compatible with growth or even survival of the host organism, making this approach feasible for investigating industrial enzymes that are used under harsh conditions of elevated temperature or in the presence of solvents or surfactants. A screen is required when the desired activity or feature cannot be linked easily to cell survival. In this regard, screens are more versatile than selections (Arnold and Volkov, 1999). The throughput, however, is generally relatively low and inversely proportional to the sensitivity of the screen used to detect the desired property. Screening commonly relies on visual detection and much ingenuity has gone into the design of protocols that couple some function to a visual signal, commonly via activation of a reporter gene, such as β-galactosidase. A quantitative colorimetric pH-based assay was developed for screening the enantioselectivity of hydrolytic reactions. Seventy-two commercial lipases and esterases were screened using this approach in order to identify the enzyme with the best enantioselectivity toward solketal butyrate, an important building block in the synthesis of pharmaceuticals and biologically active compounds (Janes, *et al.*, 1999).

However, screening can be very labour intensive, which limits its use to small numbers of samples. Rapid screening of very large libraries generated by the different types of mutagenesis has been possible for only a few enzymatic reactions in which the desired function can be linked to a selectable phenotype (MacBeath *et al.*, 1998).

1.5.4 Successful applications of directed evolution

The surge in new directed evolution technologies is rapidly finding its way to commercial applications. Evolved green fluorescent protein (Crameri *et al.*, 1996; sold by Clonetech) and an evolved lipase (NovoNordisk's Lipoprime) have been marketed commercially. New proteases (Ness *et al.*, 1999) and peroxidases (Cherry *et al.*, 1999) suitable for the global enzyme market are being engineered for commercial use. There have been many successful examples of directed evolution (Table 1.1). But, for any new enzyme to gain commercial acceptance, its manufacturers will have to keep down the costs of production by ensuring that the domesticated microbes used as the enzyme-producing factory will reliably generate large quantities of the protein. The difficulties in perfecting manufacturing techniques, and the reluctance of industries to change systems that already work reasonably well, could slow the entry of new enzymes into the marketplace. It seems inevitable, however, that the many advantages of evolved enzymes will prove irresistible for application.

Table 1.1 Examples of enzymes that have been improved by directed evolution engineering.

Evolved enzyme	Altered property	Application	Reference
Green fluorescent protein	Fluorescence	Biosensor	Crameri *et al.*, 1996
ρ-Nitrobenzyl esterases	Stability in organic solvents	Effluent treatment	Spiller *et al.*, 1999
Serine protease subtilisin	Stability and activity in organic solvents	Laundry detergents	Kuchner and Arnold, 1997
Lipase	Refined enantioselectivity	Laundry detergents	Liebeton *et al.*, 2000
Esterase	Substrate specificity	Organic solvents	Bornscheuer *et al.*, 1998
Horseradish peroxidase	Enzyme thermal and oxidative stability	Laundry wash water	Lin *et al.*, 1999b
Ribonuclease H1	Enzyme activity	Molecular biology	Hirano *et al.*, 2000
Barley α-amylase	Thermostability	Syrup production	Joyet *et al.*, 1992

1.5.5 Challenges and prospects for directed evolution

Challenges for future directed evolution include developing workable strategies for the evolution of new catalytic functions, evolving complex biosynthetic or degradative pathways, evolving single enzymes and new enzyme pathways for large-scale chemical production, and evolving enzymes that

are difficult to handle in terms of functional expression, stability and assay development. As a reliable catalyst-improvement technology, directed evolution will play a major role in removing bottlenecks to biocatalysis process development (Schmidt-Dannert and Arnold, 1999).

Laboratory evolution is a promising new tool for studying enzyme function and adaptation, which facilitates the observation of adaptation under controlled conditions. In addition, with entire lineages, and not just the endpoints of evolution, there is a unique vantage point for studying fundamental questions of protein structure and function. Moreover, because the evolutionary pressures can be defined, it should be possible to explore unnatural functions to distinguish what is biologically relevant from what is physically possible. This approach should prove useful for studying protein adaptation in a variety of contexts, both natural and artificial (Arnold *et al.*, 2001).

1.6 β-XYLANASES

1.6.1 Xylan and the xylanolytic system

Hemicelluloses are non-cellulosic polysaccharides that are found in plant tissues and are the major constituents of plant cell wall polysaccharides. The structure of various types of hemicelluloses depends on the type of plant, and may even vary between different parts of the same plant. Hemicelluloses are usually classified according to the main sugar residues in the backbone, e.g., xylans, glucomannans, galactans and glucans (Woodward, 1984).

In the cell walls of land plants, xylan is the most common hemicellulosic polysaccharide, representing more than 30% of the dry weight (Joseleau *et al.*, 1992). Xylans are composed of β-1,4-linked xylopyranosyl residues (Whistler and Richards, 1970). Most xylans occur as heteropolysaccharides, containing different substituent groups in the backbone and the side chains (Biely, 1985; Puls and Poutanen, 1989). The common substituents found on the backbone of xylan are acetyl, arabinosyl and glucuronosyl residues (Whistler and Richards, 1970). Homoxylans, on the other hand consist exclusively of xylosyl residues. This type of xylan is not widespread in nature and has been isolated from esparto grass (Chanda *et al.*, 1950), tobacco stalks (Eda *et al.*, 1976), and guar seed husk (Montgomery *et al.*, 1956).

Due to the heterogeneity of xylan, its hydrolysis requires the action of a complex enzyme system. This is usually composed of β-1,4-endoxylanase, β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, acetylxylan esterase and phenolic acid esterases. All these enzymes act cooperatively to convert xylan to its constituent sugar (Fig. 1.3). The presence of such multifunctional xylanolytic enzyme systems is quite widespread among bacteria and fungi (Woodward, 1984; Wong *et al.*, 1988; Coughlan and Hazlewood, 1993). The degradation of complex heteroxylans may involve synergistic action between the different components of the xylanolytic enzyme system (Biely *et al.*, 1986; Kleupfel *et al.*, 1992).

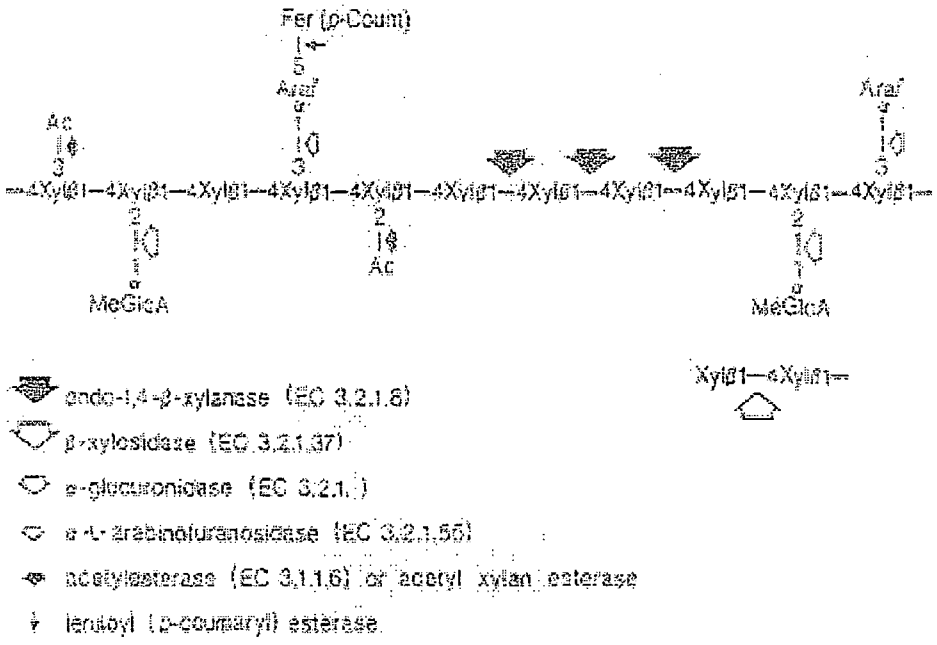

Fig. 1.3 Xylan structure and enzymes involved in xylan degradation (Biely, 1985).

1.6.2 Mechanism of action of xylanases

Endo-1,4-β-D-xylanases catalyze the random hydrolysis of 1,4-β-D-xylosidic linkages in xylans. Endoxylanases show the highest activity against polymeric xylan, and the rate of the hydrolysis reaction normally decreases with the decreasing chain length of the oligomeric substrates. They do not hydrolyze xylobiose, and the hydrolysis of xylotriose is in most cases negligible or at least limited. The main products formed from the hydrolysis of xylan are xylobiose, xylotriose, and substituted oligomers of two to four xylosyl residues (Fig. 1.3).

The length and the type of substituted products depend on the mode of action of the individual xylanases. Most of the enzymes studied cleave the xylan backbone, leaving the substituent on the non-reducing end of the xylosyl chain of the oligosaccharide. Some xylanases were reported to leave the substituent on the reducing end and in the middle of the oligosaccharide chain (Dekker, 1985; Coughlan, 1992). Of the end products, at least xylotriose has been reported to inhibit the action of xylanases (Royer and Nakas, 1989). In addition to hydrolytic activity, transferase activity has been detected in several xylanases (Coughlan, 1992; Viikari *et al.*, 1993).

1.6.3 Occurrence of xylanases

Xylanases are widespread in nature and they have been reported to be present in marine and terrestrial bacteria, rumen and ruminant bacteria, fungi, marine algae, protozoa, snails, crustaceans, insects and seeds of terrestrial plants (Dekker and Richards, 1976). The published information on the induction (constitutive or induced) as well as regulation (under separate or common regulatory control system with cellulases) of xylanases varies. Both inductory and regulatory systems seem to vary amongst the different organisms. Some reports demonstrate that xylanases are inducible and that the specific induction can occur independently of cellulase synthesis (Coughlan and Hazlewood, 1993).

1.6.4 Properties of xylanases

Fungal xylanases of *Aspergillus* and *Trichoderma* spp., and bacterial xylanases of *Bacillus* spp., *Streptomyces* spp., and *Clostridium* spp. have been intensively studied (Wong *et al.*, 1988; Eriksson *et al.*, 1990). Several of the xylanases purified fall within the molecular mass range of between 16 and 38 kDa and have basic isoelectric points (pI 8 - 9.5). They also exhibit a great homology on the molecular level and belong to the family G (or 11) of glycosyl hydrolases. The other xylanases with higher molecular mass and lower pI values belong to family F (or 10) (Henrissat, 1991; Henrissat and Bairoch, 1993).

The optimum pH for xylan hydrolysis is around pH 5.0 for most fungal xylanases, and they are normally stable between pH values of two and nine. The pH optima of bacterial xylanases are generally higher than the pH optima of fungal xylanases. Alkalophilic *Bacillus* spp. (Nakamura *et al.*, 1993) and alkalophilic actinomycetes (Tsujibo *et al.*, 1990) have been reported to produce xylanases with high activity at alkaline pH values. Most of the fungi and bacteria produce xylanases, which

tolerate temperatures below 40 – 50^{0}C. Xylanases have also been characterized from thermophilic organisms. The most thermostable xylanase described is that of an extremely thermophilic *Thermotoga* spp. with a half-life of 20 min at 105^{0}C (Bragger *et al.*, 1989). Xylanases with half lives of a few minutes up to 90 min at 80^{0}C have been produced by *Thermoascus aurantiacus*, *Bacillus stearothermophilus*, *Caldocellum saccharolyticum*, *Clostridium stercorarium* and *Thermomonospora* spp. (Lüthi *et al.*, 1990; Zamost *et al.*, 1991).

1.6.5 Commercial potential of xylanases

To really appreciate the rationale behind current studies of microbial xylanases, it is necessary to take a broad view that takes into account not just their intrinsic interest, but also their undoubted commercial potential. The structural polysaccharides cellulose and hemicellulose together account for greater than 50% of plant biomass and are consequently the most abundant terrestrial organic molecules. The value of plant biomass as a renewable resource is thus immediately apparent (Coughlan, 1985).

Xylan-degrading enzymes, especially xylanases, have considerable potential in several biotechnological applications. In some processes, the use of purified enzymes is required. However, in other applications, the presence of additional enzyme activities is desired. Commercial applications suggested for xylanases involve the conversion of xylan, which is present in wastes from the agricultural and food industry, into xylose (Biely, 1985). Similarly, xylanases could be used for the clarification of juices, for the extraction of coffee, plant oils and starch (Wong and Saddler, 1993), and for the production of fuel and chemical feedstocks (Linko *et al.*, 1989).

Another application of xylanases is the use of this enzyme in poultry diets. Depression in weight gain and feed conversion efficiency in rye-fed broiler chicks has been associated with intestinal viscosity (Van Paridon *et al.*, 1992). Incorporating the xylanase from *T. longibrachiatum* into a rye-based diet of broiler chickens resulted in reduced intestinal viscosity, thus, improving both the weight gain of the chicks and their feed conversion efficiency (Bedford and Claasen, 1992). The efficiency of xylanases in improving the quality of bread has also been demonstrated. The introduction of *A. niger* var. *awamori* xylanase into bread dough resulted in an increase in specific bread volume. This is further enhanced when amylase in combination with xylanase is used (Maat *et al.*, 1992).

Over the last two decades, much research has been conducted on xylanases due to their potential application in the pulp, paper and fibre-processing industries. In the jute fibre industry, the xylanase pre-treatment of low grade fibres before milling may be an alternative to conventional chemical softening because the former enables the selective removal of xylan, which is assumed to be an economic and environmentally safe process (Gosh and Dutta, 1983; Hoq *et al.*, 1992). Jute fibre is a natural biodegradable product and could replace the usage of plastics and synthetic fibres.

Since biobleaching with xylanases was first reported (Viikari *et al.*, 1986), a number of mill trials in both Europe and North America have been conducted (Koponen, 1991; Lavielle *et al.*, 1992; Tolan and Canovas, 1992; Turner *et al.*, 1992). The first industrial application of xylanases was carried out in Finland in 1991 (Koponen, 1991). In 1994, it was reported that 18 bleaching mills in Canada had run xylanase trials and six are regular users of the enzyme to treat 750 000 tons of pulp, representing 8% of Canada's bleached kraft pulp production (Tolan *et al.*, 1996). The benefits of using xylanases have been mostly the economic and environmental advantages, which include:

(i) savings in bleaching chemicals;
(ii) increased throughput;
(iii) improved pulp properties such as brightness and strength;
(iv) marketing advantage;
(v) easy adaptation to different bleaching sequences with minimal investment costs; and
(vi) improved effluent with reduced AOX content (Christov and Prior, 1998).

1.6.6 Limitations of commercial xylanases

It is apparent that plant biomass provides an extensive renewable resource: however, its exploitation has been limited by the cost of hydrolyzing plant structural polysaccharides to their component sugars. Although microbial xylanases can be produced by some organisms at very high levels, the enzymes have extremely low turnover rates when hydrolyzing crystalline substrates. Furthermore, hydrolysis of the designated substrate is invariably incomplete. Thus, the production of enzymes that exhibit a relatively high specific activity against xylan is crucial if the potential agricultural and industrial benefits of plant biomass are to be fully realized. The development of such enzymes is not beyond the scope of current technology, but will be possible only when a detailed understanding of the structure and catalytic mechanisms of existing xylanases is available.

In the pulp and paper industry, some problems have been encountered associated mainly with corrosion of equipment, maintenance of residence time of xylanase treatment, pulp yield loss and bleach plant control (Tolan *et al.*, 1996). Thus, the xylanase bleaching technology is being further developed towards increasing the pH and temperature stability of xylanases, the utilization of enzyme mixtures with supplementary hemicellulolytic accessory enzymes to enhance xylanase efficiency, improvement of the competitiveness of xylanases with respect to enzyme price and the optimization of elemental chlorine-free (ECF) and total chlorine-free (TCF) bleaching in conjunction with xylanases (Viikari *et al.*, 1994).

1.6.7 Cloning and expression of xylanase genes

For the commercial realization and economic viability of xylanase production it is necessary to identify organisms which can hyper-produce the enzymes. Recombinant DNA techniques offer the means to enhance protein production. Xylanase genes have been cloned from different microbial genera into various suitable hosts, of which *E. coli* features most commonly. The expression in *E. coli* is generally found to be lower than the parent organism, and confined to the cytoplasmic or periplasmic fractions. The absence of post-translational modifications such as glycosylation in *E. coli* and the intracellular accumulation of the recombinant xylanases have been suggested to be the key reasons for low levels of activity (Kulkarni *et al.*, 1999).

In the last five years, a few attempts were made to improve xylanases at the genetic level. A few successful examples are outlined below. In an attempt to improve the thermostability and activity of xylanase for industrial biobleaching, the *N*-terminal region of xylanase B from *S. lividans* was replaced with the corresponding region from *T. fusca* xylanase A (Shibuya *et al.*, 2000). The latter xylanase retains 96% of its activity after 18 h at 75^0C and its catalytic domain has a 72% sequence homology to that of *S. lividans*. The stability of the *S. lividans* xylanase, however, decreases gradually above 37^0C. DNA shuffling of both xylanases however yielded two promising mutants that exhibited significant thermostabilities at 70^0C and had markedly higher activities than the parent enzymes. This study was a hallmark in the study of recombination between xylanases since it proved the theoretical concept that random shuffling between a mesophilic enzyme and its thermophilic counterpart is a promising approach for the improvement of the thermostability of a mesophilic enzyme.

Another highly successful example of modern recombinant DNA technology being used to improve xylanases was when the catalytic domain of the xylanase from the anaerobic fungus, *Neocallimastix patriciarum* was made more alkalophilic through directed evolution using ep-PCR and site-directed mutagenesis (Chen *et al.*, 2001). Alkalophilic variants, grown on LB agar, produced large clear zones when overlaid with alkaline, xylan-containing agar. Whereas the wild-type xylanase exhibited no activity at pH 8.5, the relative and specific activities of six alkalophilic mutants were higher at pH 8.5 than pH 6. Eight amino acid substitutions were identified in the selected mutant xylanases and seven of these substitutions were assembled in a single enzyme (xyn-CDBFV) by site-directed mutagenesis. This composite xylanase not only had a relatively high specific activity, but was also more thermostable at 60^0C and alkaline tolerant at pH 10 than the wild-type xylanase. The composite mutant xylanase was a promising and suitable candidate for pulp biobleaching.

Site-directed mutagenesis was also used to study the key amino acid residues responsible for the alkali-tolerance of the *B. pumilis* xylanase (Liu *et al.*, 2002). Asparagine 71 was observed to be highly conserved in the alkaline family 11 xylanases. Substitution of this residue led to a decrease in the specific activity of the xylanase, especially in the alkaline pH range. It was noted that although other residues could also influence the pH optimum of the xylanase, they did not affect their activity in the alkaline pH range as much as that of asparagine 71. In addition, all mutant xylanases studied, changed their pH optima to a more acidic value. Thus, it was found that this residue was crucial for alkali-tolerance of the *B. pumilis* xylanase.

Thus, the xylanolytic genes of many microorganisms have been sequenced and cloned in a bid to further understand the basic building blocks that contribute to the overall structure and function of these enzymes. Knowledge of the tertiary structure of an enzyme can facilitate the understanding of its function and its relationship with substrates and inhibitors. The advent of sophisticated and advanced molecular genetics heralded the beginning of a new era in enzyme technology. The availability of suitable methodology and the improved prospects for commercialization of the hemicellulases have prompted the application of recombinant DNA techniques to the study of xylan-degrading enzymes, with the result that a number of genes have been cloned and fully sequenced, to further improve them for eventual industrial use.

1.7 THE ATTRACTION OF THERMOSTABLE HEMICELLULASES

Given the natural abundance of hemicellulose, it is not surprising that many microorganisms have enzyme systems for its hydrolysis (Hazlewood and Gilbert, 1993). Moreover, given the variety and complexity of hemicelluloses, several biocatalytic steps are required to hydrolyze specific polysaccharides completely into simpler sugars that can be readily used as carbon and/or energy sources by particular microorganisms. Thermophilic microorganisms are sources of thermostable saccharolytic and hemicellulolytic enzymes which could either replace those currently used at less than optimal processing temperatures or be used in new biocatalytic applications (Duffaud *et al.*, 1997).

The rapid growth of the enzyme industry reflects the advantages of using enzymes as industrial catalysts, and the real and potential advantages of using enzymes from thermophiles have been well documented. The properties that allow thermostable enzymes to withstand high temperatures also confer resistance to denaturing agents (Fujita *et al.*, 1976; Cowan and Daniel, 1982), solvents (Zaks and Klibanov, 1982) and proteolytic enzymes (Daniel *et al.*, 1982). In general, the higher the growth temperature of the organism, the more stable are its enzymes (Singleton and Amelunxen, 1973). Thus, the most stable enzymes are likely to be extracellular enzymes from the most extreme thermophiles. Extracellular enzymes are more stable than their intracellular counterparts, since they are not limited by the requirements for rapid turnover as a mechanism for controlling metabolite fluxes. Cells producing extracellular enzymes may also be subject to evolutionary pressure to minimize the loss of exported carbon; high specific activities and enhanced molecular stability would be positive consequences of such pressures (Cowan, 1997).

The concept of thermodynamic stability applies to the equilibrium between the native and unfolded state of a protein. Thermostability is a desired property in biotechnological applications for a number of reasons. Substrate solubility may be increased, the risk of microbial contamination may be minimized and the reaction rates may not only be increased in general, but may favour some side reactions over others (Cowan, 1997). Biotechnological processes may require extremes of pH, or the presence of chelators, proteases and detergents. Stability in aprotic environments would make protein catalysts interesting for a wide range of applications. Unfortunately, most proteins denature only a few degrees above the physiological temperature and this is frequently an irreversible process that rapidly draws folded protein out of the equilibrium and into the unfolded state. In general, the reason for

irreversible inactivation at high temperatures or under other adverse environmental conditions is aggregation of the unfolded state (Van den Berg *et al.*, 1998).

Biotechnological processes, such as pulping, are best carried out at elevated temperatures. The increase in temperature has a significant influence on the bioavailability and solubility of organic compounds, and is accompanied by a decrease in viscosity and an increase in the diffusion coefficient of organic compounds. Consequently, higher reaction rates due to smaller boundary layers are expected (Krahe *et al.*, 1996; Becker *et al.*, 1997). The bioavailability of insoluble environmental pollutants can also be improved dramatically at elevated temperatures allowing efficient bioremediation.

Since the xylanases of mesophilic fungi, e.g., strains of *Gliocladium* (Gomes *et al.* 1989; 1992) and *Schizophyllum* (Steiner *et al.*, 1987), are not thermostable at 50^0C or above, the hydrolytic efficiencies of these enzymes are low. Moreover, these fungi produce appreciable amounts of cellulase activity. The use of xylanase in biobleaching of wood pulp produced in the sulphite (acid) and kraft (alkaline) processes has received much attention (Christov and Prior, 1997). As the cooking of wood is conducted at temperatures above 145^0C (Bierman, 1993), thermostable xylanases would be particularly useful in biobleaching directly after cooking (Yu *et al.*, 1987). Many of the xylanases produced by mesophilic fungi lack thermostability and this limits their industrial application. Xylanases produced by thermophilic fungi are usually more thermostable than those of mesophilic fungi (Steiner *et al.*, 1987). The use of thermostable xylanases for enzymatic hydrolysis or pre-treatment of pulp at high temperatures might help in achieving technical and economic feasibilities. Moreover, as the thermophilic fungus is grown at high temperatures, there is less risk of microbial contamination and diminished cooling requirements for the fermentation process (Gomes *et al.*, 1993b; Singh *et al.*, 2000b).

The most thermostable xylanase reported to date is that from the extremely thermophilic anaerobic bacterium *Thermotoga*, which has a half life of more than 20 min at 105^0C (Bragger *et al.*, 1989). Despite offering a potentially rich source of a variety of very stable enzymes, the commercial development of xylanase production by archaebacteria has been impeded by difficulties in the cultivation of these organisms. Therefore, thermophilic fungi such as *T. lanuginosus* are an attractive alternative source of thermostable xylanases (Singh *et al.*, 2000b; 2000c).

1.8 *T. lanuginosus*

Thermophilic hyphomycetes and their distribution in diverse habitats is well documented (Cooney and Emerson, 1968). *T. lanuginosus* is one such thermophilic Deuteromycete which thrives at temperatures up to 60^{0}C, the upper temperature limit for growth of true fungi, and fails to grow below 20^{0}C. The worldwide distribution of *T. lanuginosus* is a result of the common occurrence of self-heating masses of organic debris (Emerson, 1968). It has been isolated from a wide range of geographical regions across the world. It has been associated with self-heating grains of barley and wheat, the atmosphere around silos, pecans, tobacco products, various composting materials and dung (Cooney and Emerson, 1968).

Colonies of *T. lanuginosus* are fast-growing, reaching 2.5 to over 5 cm on various media at 37^{0}C - 50^{0}C in 2 days. Colonies appear white and felty at first, less than 1 mm high, but soon turn grey, beginning at the centre of the colony. Gradually the colony turns purplish-brown, with the agar substratum staining a deep pink to red colour, due to diffusible substances being secreted by the fungus into the medium. Mature colonies are dark brown to black (Cooney and Emerson, 1968).

T. lanuginosus has an exceptional ability to produce extremely high levels of xylanase. These levels are comparable to those produced by commonly studied mesophilic fungi (Sunna and Antranikian, 1997). The xylanase produced by this fungus is not only remarkably thermostable, but is also active over a wide pH range. These properties have elicited numerous groups to investigate the xylanase-producing ability of strains isolated from nature and obtained from culture collections. A notable variability in the production of xylanase by *T. lanuginosus* strains has been observed (Anand *et al.*, 1990; Alam *et al.*, 1994; Puchart *et al.*, 1999; Singh *et al.*, 2000a).

1.8.1 *T. lanuginosus* DSM 5826

Strain DSM 5826 is a well-researched *T. lanuginosus* strain. It was isolated by Dr I. Gomes from self-heated (65 – 70^{0}C) jute stacks at the Jute Research Institute, Dhaka, Bangladesh, in 1988. It was the highest xylanase-producing *T. lanuginosus* strain with an activity of 32 500 nkat/ml (Purkarthofer *et al.*, 1993a). However, a unique strain (designated SSBP) was found to be a hyper-producer of xylanase having an activity of 59 600 nkat/ml (Singh *et al.*, 2000b; 2000c).

The DSM 5826 xylanase is a protein with an apparent molecular weight of 24-26 kDa (Purkarthofer *et al.*, 1993b), which is slightly larger than the weight of xylanases of other isolates of *T. lanuginosus* (Anand *et al.*, 1990; Lin *et al.*, 1999a). Numerous studies have shown that the xylanase produced by this strain is highly selective for xylan-containing substrates, especially those of a lignocellulosic nature (Gomes *et al.*, 1993a; 1993b; Purkharthofer *et al.*, 1993a; 1993b).

Biochemical characterization of this xylanase revealed that it was stable in the pH range of 3 to 9 (Gomes *et al.*, 1993a) whilst another study showed that its xylanase retained 80% of it activity at 70^0C for 10 min when tested in the pH range of 4 to 12 (Lischnig *et al.*, 1993). A separate study by Singh *et al.* (2000b), showed the xylanase from this strain to have a $t_{1/2}$ of 201 min at 70^0C. The xylanase gene from this fungal strain was purified and successfully cloned into plasmid Bluescript (pBSK) and functionally expressed in *E.* as a LacZ-fusion protein. The *xynA*-LacZ-fusion protein was produced intracellularly with a tendency to form inclusion bodies (Schlacher *et al.*, 1996).

The crystal structure analysis of this enzyme and a comparison of its structure with other family 11 xylanases were made by Gruber *et al.* (1998). It was found that the most prominent feature of the enzyme is a long cleft that spans the whole molecule and contains the active site. The structure is dominated by two heavily twisted β-sheets, designated sheets A and B (Fig. 1.4). Such sheets are commonly found in thermophiles and are responsible for intrinsic enzyme stability (Chakravarty and Varadarajan, 2000).

Sheet A forms the outer surface of the enzyme and consists of five antiparallel strands. Its hydrophilic, solvent-accessible surface contains a large number of serine and threonine residues. Sheet B consists of nine mostly antiparallel strands. One face forms the active site of the enzyme, whilst the other is packed against sheet A to form the hydrophobic core of the protein. There is only one α-helix in this structure, which follows strand A6, consists of ten residues, and is packed against the hydrophobic face of sheet B (Fig. 1.4B). The overall shape of the molecule resembles a right-hand with the two β-sheets and the α-helix forming the 'fingers' and the 'palm' and two loop regions forming the 'thumb' and a 'cord' (Fig. 1.4A). In the crystal structure, the thumb is well-ordered by interactions with sheet B and crystal contacts. Molecular dynamics simulations, however, indicate that it is one of the most flexible parts of the molecule.

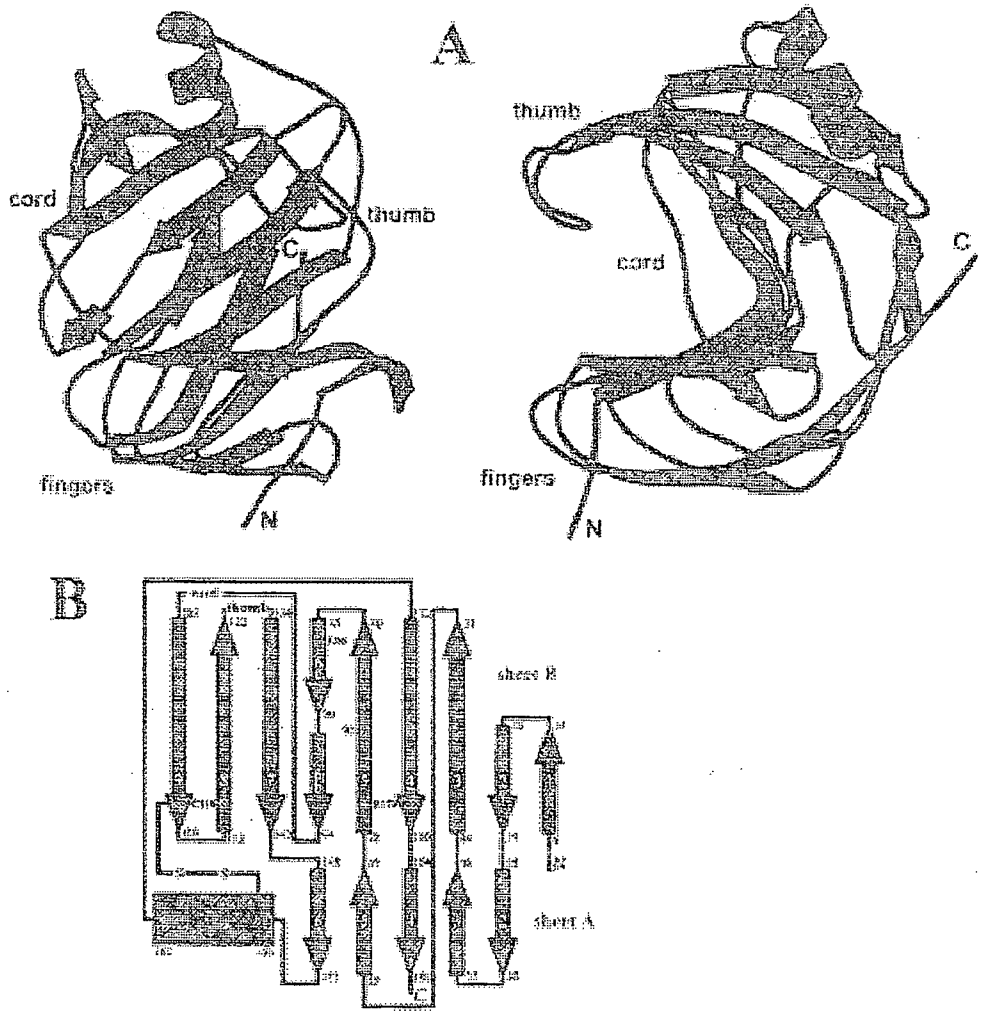

Fig. 1.4 Molecular structure of *xynA* from *T. lanuginosus* DSM 5826. (A) Two perpendicular views of the structure of *xynA* after 3D protein modelling. (B) Topology diagram depicting the arrangement of the β sheets and α-helix within *xynA* (Gruber *et al.*, 1998).

The thermostability of this xylanase was largely attributed to the presence of a disulphide bridge plus an extension of an existing network of charged residues, which were responsible for the stabilization of the most sensitive region of the molecule. Thermostability is further enhanced by an overall increase in the number of ion pairs, without targeting a specific area. Interestingly enough, *T. lanuginosus* has the largest number of ion pair interactions amongst the family 11 xylanases. Disulphide bridges have been shown to enhance the fast and correct back folding of proteins after heat-induced perturbation (Tatu *et al.*, 1990). Ion pairs are usually present in small numbers in proteins but represent a significant stabilizing force in folded proteins. These electrostatic interactions enable the thermostable protein to unfold at a much slower rate but render it more sensitive to pH variations. Thus, ion pairs at key surface positions play a kinetic role in protein unfolding (Vetriani *et al.*, 1998).

High homology to other xylanases was found when the protein sequence was compared with sequences contained in the SwissProt database. Two totally conserved glutamate residues seen in the multiple alignments of eight different xylanases have been reported to be actively involved in enzyme catalysis (Ko *et al.*, 1992; Miao *et al.*, 1994; Wakarchuk *et al.*, 1994a). Variations surrounding the region of the second glutamate residue are thought to be responsible for different pH stability values found with the enzymes (Törrönen *et al.*, 1994). The cloning of this gene heralded the birth of a new approach to adapt it for the rigours of biotechnological processes: recombinant DNA technology.

1.8.2 *T. lanuginosus* in industry

Directed evolution is an innovative type of recombinant DNA technology that is revolutionizing protein engineering. Directed evolution has met with much success and was used on a lipase produced by *T. lanuginosus* (Okkels, 1997; Okkels *et al.*, 1997). Laundry applications make tough demands on enzymes and this lipase was successfully evolved to improve its performance in laundry detergents. A combination of random mutagenesis of the entire gene and site-directed mutagenesis of selected regions of the protein, followed by *in vivo* recombination in *S. cerevisiae*, produced a variant with the desired wash properties (Arnold and Volkov, 1999). Thus, by performing directed evolution on the *T. lanuginosus* xylanase, the catalytic efficiency, thermostability and other industrially-significant properties of the enzyme could possibly be improved without resorting to the traditional approaches of protein engineering.

1.9 SCOPE OF THE PRESENT STUDY

The large-scale industrial use of xylanases is impeded by many factors; one of which is its long-term thermostability. Rational protein engineering, although a suitable application in theory, often fails because of the tremendous hindsight required to alter precise amino acids and to then predict its effect on the overall three-dimensional structure of the protein. Directed evolution, on the other hand, uses a combination of powerful search techniques to generate proteins with improved properties. The weighing of parameters associated with mutation, recombination and screening to achieve the maximum protein improvement is the beginning of rational evolutionary design. By subjecting the xylanase gene to successive rounds of mutagenesis and selection of improved variants, it is hoped that the gene will accumulate beneficial mutations that would pave the way for its eventual large-scale use in industry.

1.9.1 Hypotheses to be tested

It was hypothesized that the *T. lanuginosus* DSM 5826 xylanase is a functionally tolerant protein that would allow numerous mutations without disrupting its fitness and in doing so, allow for the production of a high quality mutant library. It was further hypothesized that at least a few of these mutations would improve the temperature stability and activity of the enzyme.

1.9.2 Objectives

The following objectives were established to test the above hypotheses:

I) to induce high levels of xylanase production in recombinant *E. coli* using IPTG;

II) to introduce random mutations into *xynA* using error-prone PCR;

III) to develop a screening method to detect xylanase-expressing mutants with enhanced thermostability;

IV) to evaluate the long-term thermostability of promising mutants; and

V) to sequence these mutants and analyze their genes for beneficial mutations.

1.9.3 Experimental design

Phase I:

a) to determine the maximum amount of xylanase that could be produced by recombinant *E. coli* X with and without IPTG induction; and

b) to determine the amount of IPTG required for xylanase production.

Phase II:

a) to isolate plasmid pX3 from *E. coli*;

b) to determine the ep-PCR condition that produced the best mutants; and

c) to develop a screening method to detect thermostable mutants.

Phase III:

a) to further mutate promising parent mutants using the optimal ep-PCR conditions;

b) to further screen these progeny mutants for improved stability and activity at 80^0C; and

c) to carry out long-term thermostability testing on the improved enzyme variants at different temperatures.

Phase IV:

a) to sequence the genes of improved mutants and align the mutant protein sequences to *xynA* and determine the amino acid substitutions.

CHAPTER TWO: INDUCTION OF XYLANASE EXPRESSION

2.1 INTRODUCTION

The choice of an expression system for the high-level production of recombinant proteins depends on many factors. These include: cell growth characteristics; expression levels; intracellular and extracellular expression; post-translational modifications; biological activity of the protein of interest; and regulatory systems crucial for the production of the target protein (Goeddel, 1990; Hodgson, 1993). In addition, the selection of a particular expression system requires a cost breakdown in terms of process design and other economic considerations. Among the many systems available for heterologous protein production, the Gram-negative bacterium *E. coli* remains one of the most attractive because of its ability to grow rapidly and at high density on inexpensive substrates, its well-characterized genetics and the availability of an increasingly large number of cloning vectors and mutant host strains (Makrides, 1996).

However, in spite of the extensive knowledge on the genetics and molecular biology of *E. coli*, not every gene can be expressed efficiently in this organism. This may be due to the unique and subtle structural differences of the gene sequence, the stability and translational efficiency of mRNA, the ease of protein folding, degradation of the protein by host cell proteases, major differences in codon usage between the foreign gene and native *E. coli*, and the potential toxicity of the protein to the host (Hodgson, 1993). The major drawbacks of *E. coli* as an expression system include the inability to perform many of the post-translational modifications found in eukaryotic proteins, the lack of a secretion mechanism for the efficient release of protein into the culture medium, and the limited ability to facilitate extensive disulphide bond formation (Gold, 1988; 1990).

Recombinant processes are mostly based on the use of strong expression systems that are regulated at the level of transcription. Strong inducible promoters are used and for many years, the *E. coli* lactose utilization *(lac)* operon has served as one of the paradigms of prokaryotic regulation. It is therefore not surprising that many of the promoters used to drive the transcription of heterologous genes have been constructed from *lac*-derived regulatory elements. The *T. lanuginosus xynA* used in this study was cloned in *E. coli* as a LacZ-fusion protein and was produced intracellularly with a tendency towards inclusion body formation (Schlacher *et al.*, 1996). Such a *lac*-derived promoter is rather weak and tends to have leaky expression (low levels of the target protein are secreted into the medium). Thus, it is rarely used for the high-level production of recombinant proteins. Its expression can be strengthened when induced with the non-hydrolyzable lactose analogue, isopropyl-β-D-thiogalactopyranoside (IPTG), which binds to the *lac* repressor and induces synthesis of the target protein well above the

normal regulated levels within the cell. IPTG serves as a gratuitous inducer since it is capable of protein synthesis but remains in the cell for a long time because it cannot be hydrolysed by any enzymes (Jacob and Monod, 1961; Burcin *et al.*, 1998).

Over-expression of recombinant proteins is desired for experiments that improve the protein for further possible industrial applications. *E. coli*, in addition to producing the target protein, also manufactures many other accessory or "housekeeping" proteins. Consequently, a competition exists between target product synthesis and the synthesis of native host cell proteins. The addition of suitable inducers allow for the channelling of the host's metabolism to produce high levels of the target product.

After cloning of *xynA* into *E. coli*, the resultant enzyme was poorly expressed and no studies were conducted into improving its production and expression (Schlacher *et al.*, 1996). Thus, prior to any attempts to mutate the gene for enhanced properties and improved efficiency, it was vital to establish the maximum production levels that could be attained by the native enzyme with induction by IPTG; and also, the amount of IPTG required for optimal xylanase production.

2.2 MATERIALS AND METHODS

2.2.1 Strains, plasmids and media

The *E. coli* SURE clone that contained the *xynA* gene from *T. lanuginosus* DSM 5826 (Schlacher *et al.*, 1996) was designated as *E. coli* X. A second *E. coli* strain contained only plasmid Bluescript and was used as a control for this study and was designated as *E. coli* B. Both strains were obtained from the Institute of Biotechnology, Technical University of Graz, Austria. Strains were maintained on Luria Bertani (LB) agar medium (10 g/l bactopeptone, 5 g/l yeast extract, 5 g/l sodium chloride, 15 g/l technical agar). Ampicillin (100 µg/ml) was added after autoclaving the medium and cooling to 55^{0}C. Both strains were sub-cultured every 3 weeks and stored on plates at 4^{0}C. Long-term preservation of both strains was accomplished by freezing concentrated cultures containing LB broth and 15% glycerol, and stored at -20^{0}C.

2.2.2 Effect of different concentrations of IPTG on recombinant xylanase production

Both strains were grown for 12-16 hours at 37^{0}C on LB-ampicillin plates and single colonies were picked and used to inoculate 5 ml LB-ampicillin medium and grown for a further 12-16 hours at 37^{0}C in a shaking incubator. One hundred microlitres of the overnight *E. coli* X culture was used to inoculate 45 X 500 ml Erlenmeyer flasks containing 100 ml LB medium, while the overnight *E. coli* B culture was inoculated into 18 such flasks containing the same amount of medium. LB medium was supplemented with 100 µg/ml ampicillin and incubated with shaking at 37^{0}C until the OD_{600} of all flasks were 0.5 absorbance units. IPTG was added aseptically to 36 flasks containing *E. coli* X culture to various final concentrations (0.1 mM, 0.25 mM, 0.5 mM and 1 mM) from a 1 M stock solution. The remaining nine flasks containing *E. coli* X culture served as negative controls (no IPTG addition). Nine flasks containing *E. coli* B were inoculated with 1 mM IPTG, while the remaining nine served as negative controls. The flasks were then incubated in a shaking incubator at 37^{0}C. A flask representing each experimental condition was removed at the following time intervals (0, 1, 2, 3, 4, 5, 24, 48, 72 h) and immediately placed on ice throughout the enzyme extraction procedure. All experiments were conducted in duplicate.

2.2.3 pH measurement

The pH of *E. coli* X cultures induced with 0.1 mM IPTG was monitored over 72 h with a pH 211 Microprocessor pH Meter (HANNA Instruments) prior to centrifugation and enzyme preparation. This

was done to determine if there were any significant changes in the pH of the culture medium with the flasks induced with 0.1 mM IPTG.

2.2.4 Enzyme extraction

The cultures from each of the flasks were centrifuged at 5000 *g* for 10 min and the medium discarded. The resulting pellet was completely resuspended in 5 ml of cold breaking buffer [6.80 g/l KH_2PO_4, 0.61 g/l $MgCl_2.6H_2O$, 0.77 g/l dithiothreitol, 0.37 g/l EDTA - pH of the solution was adjusted to 6.8 prior to the addition of 0.10 g/l phenylmethylsulfonylfluoride (PMSF)]. Cell suspensions were sonicated 3 times for 30 seconds at 10 seconds per cycle (5000 Hz, constant pulse) using a VirSonic sonicator (Virtis). Suspensions were chilled on ice during the entire procedure. After sonication, the suspensions were centrifuged at 15 000 *g* for 45 min at 4^0C to prevent denaturation of the enzyme. The clear supernatant was decanted into clean eppendorf tubes and stored at -20^0C for further analysis.

2.2.5 Xylanase activity

Xylanase activity was determined using the method of Bailey *et al.* 1992. One unit of xylanase activity was defined as the amount of enzyme releasing 1 μmole of reducing sugar (xylose) per second per ml from xylan under the standard assay conditions and expressed in nkat/ml.

Protein concentration was determined by the Folin-Lowry method (Lowry *et al.*, 1951) and expressed as μg total protein/ml. Bovine serum albumin (BSA) was used as a standard for protein determination. Activity of all test samples was expressed as micromoles of xylose released from birchwood xylan per minute per microgram of total protein under the above assay conditions and is expressed as nkat/μg total protein.

2.3 RESULTS

2.3.1 Effect of different concentrations of IPTG on recombinant xylanase production

Small amounts of IPTG are known to improve the production of recombinant proteins of interest because they bind to *lac* repressors and increase production of the target protein (Schweder *et al.*, 2002). This concept was reinforced with the results obtained. The lowest level of IPTG used in this study (0.1 mM) was found to induce the highest levels of xylanase (2797 nkat/µg total protein) after 48 h. Higher concentrations (0.5 mM and 1 mM) negatively impacted xylanase production. IPTG addition increased the rate and amount of product synthesis (Fig. 2.1). *E. coli* X without IPTG addition produced appreciable levels of the enzyme (1108 nkat/µg total protein) after 48 h. Predictably, the control strain *E. coli* B (no *xynA* gene), showed no xylanase expression (Fig. 2.2). All test conditions displayed a drop in activity after 48 h. This effect was more pronounced with the culture induced with 0.1 mM IPTG, which experienced a 24% drop in xylanase activity. All data shown represents an average of duplicate results that are fully reflected in Appendix IA.

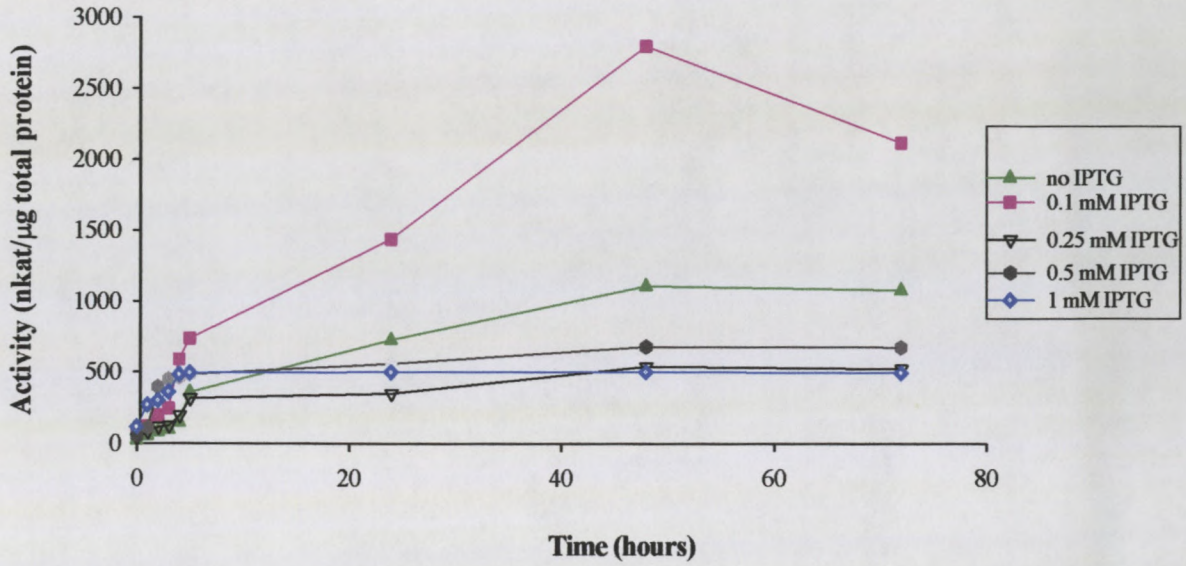

Fig. 2.1 Effect of varying levels of IPTG on xylanase production by *E. coli* X. Activity was expressed as micromoles of xylose equivalents released from xylan per microgram total protein per second under standard assay conditions (section 2.2.5). Each point represents the mean of duplicate determinations.

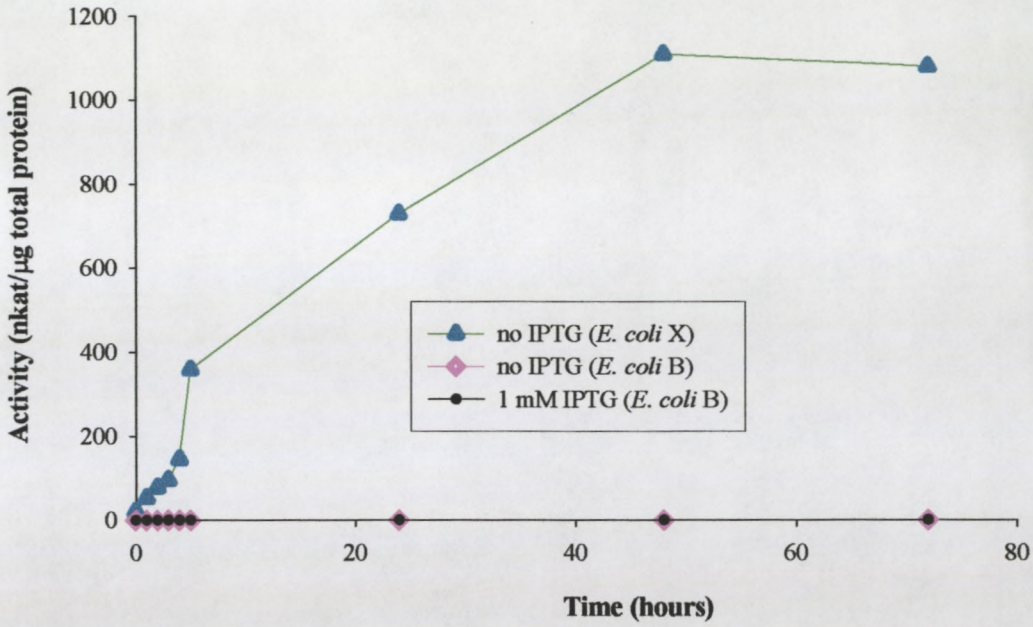

Fig. 2.2 Effect of IPTG on xylanase production by *E. coli* X and *E. coli* B. Activity was expressed as micromoles of xylose equivalents released from xylan per microgram total protein per second under standard assay conditions (section 2.2.5). Each point represents the mean of duplicate determinations.

2.3.2 pH measurement

Since *E. coli* X induced with 0.1 mM IPTG exhibited a plunge in activity after 48 h, its pH was monitored to ascertain whether there were any significant changes in the pH. The pH of the culture medium before *E. coli* X addition was 5.75 and dropped to 5.25 during growth to 0.5 absorbance units at OD_{600nm}. Twenty four hours after induction, it dropped to pH 4.88 and decreased to 4.65 after a further 24 h. The culture medium pH was 3.95 at 72 h. All figures shown in Fig. 2.3 represent an average of duplicate results and are fully reflected in Appendix IB.

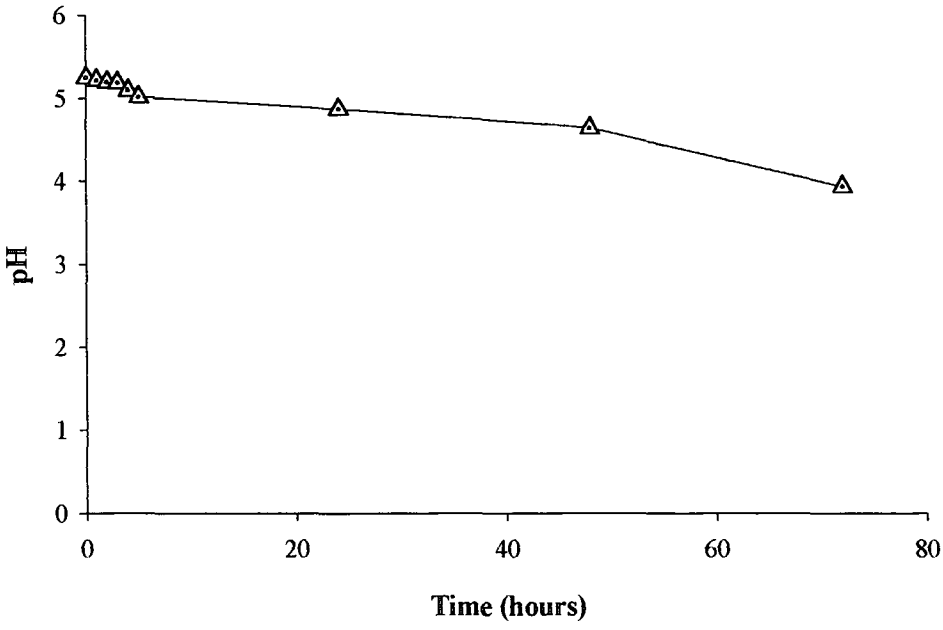

Fig. 2.3 pH of the culture medium of *E. coli* X induced with 0.1 mM IPTG monitored over 72 h. Each point represents the mean of duplicate determinations.

2.4 DISCUSSION

The advent of recombinant DNA techniques confers on bacteria the possibility to produce novel products of high biotechnological value in bacteria. The high potential also lies in the ability to grow bacteria to elevated cell densities and for them to produce the protein product as a large portion of their final biomass. The major task in any recombinant *E. coli* experiment is to obtain a high level of genetic protein expression, which presents a formidable task primarily because it is unable to properly fold recombinant proteins into their native conformations and the fact that recombinant processes compete strongly with host cell metabolism. Consequently, the over-expression of heterologous genes is influenced by several factors like plasmid stability, plasmid copy number, stability of mRNA, availability of ribosomes, transcription and translation efficiency, post-translational modification, the stability and solubility of the recombinant protein itself, as well as host cell and culture conditions (Baneyx, 1999a). Recombinant processes are mostly based on the use of strong expression systems that are controlled at the transcription level. Strong inducible promoters are commonly used to drive the transcription of the target protein forward.

Although the *xynA* was cloned into *E. coli* (Schlacher *et al.*, 1996), and later crystallized (Gruber *et al.*, 1998), the recombinant xylanase activity was not quantified. It was thus imperative to quantify its activity prior to any mutagenesis of the native gene. Since the enzyme was functionally expressed in *E. coli* as a Lac-Z fusion protein, the growth medium of *E. coli* X was supplemented with IPTG. This lactose analogue has been widely used to improve the production of recombinant proteins by *E. coli*. It binds to the lactose repressor and allosterically reduces its affinity for the operators responsible for metabolic control within the cell, and in doing so, induces the synthesis of *lac* mRNA and consequently, the target protein. An added advantage is that IPTG is such a strong inducer that it is only required in minute quantities in order to effect induction of the recombinant protein (Burcin *et al.*, 1998).

It was reported that *E. coli* X had a tendency towards inclusion body formation, (Schlacher *et al.*, 1996). Over-production of heterologous proteins in the cytoplasm of *E. coli* is often accompanied by misfolding and segregation into insoluble aggregates known as inclusion bodies. There are several strategies to minimize the formation of inclusion bodies and improve protein folding. These include the co-production of molecular chaperones, which promote the proper isomerization of target proteins, and fusion protein technology (Baneyx, 1999b). Thus, after growth, all cultures were resuspended in breaking buffer (section 2.2.4) and sonicated to lyse the host cells and release the produced xylanase from their inclusion bodies.

As seen in Fig. 2.1, a low concentration of 0.1 mM IPTG is required to increase heterologous protein expression in *E. coli* X, which attained an activity of 2797 nkat/μg total protein after 48 h. Without induction, *E. coli* X is capable of attaining a maximal activity of 1108 nkat/μg total prot after 48 h. This is significant because it implies that xylanase can be produced without induction, albeit at a lower amount. Small amounts of the xylanase are secreted into the culture medium and were measured at 25 nkat/ μg total protein (results not shown). This proved to be an opportune discovery, as the tendency of *E. coli* X to secrete small amounts of xylanase into its surrounding medium was exploited for the development of the screening procedure (chapter 3).

Although, *E. coli* X induced with the highest concentration of IPTG (1 mM) displayed an almost immediate production of xylanase, overall, it still attained the lowest activity (503 nkat/μg total protein). This is because high levels of IPTG are known to be toxic to the host cell and affects central metabolism, accordingly decreasing recombinant protein production (Makrides, 1996). It was important to also establish that the control strain B did not display any xylanase activity. This was confirmed as *E. coli* B displayed no xylanase activity with or without IPTG addition. Thus, *E. coli* B served as a suitable control, since it was devoid of xylanase activity (Fig. 2.2).

The pH of the culture medium also plays a crucial role in any shake flask cultivation of *E. coli*. It is well documented in literature that relatively small deviations from the controlled pH can be expected to have physiological effects on the cells. It was observed that the pH of the culture medium of *E. coli* X supplemented with 0.1 mM IPTG became more acidic over time (Fig. 2.3). Only this condition was tested since it displayed the most decline in activity. However, the exact relationship between the pH of the culture medium and the diminished xylanase activity observed with this culture was not determined. Nevertheless, according to literature, this observed decrease in pH is due to the formation of the lipophilic agent, acetate. Over time, as the density of the *E. coli* culture increases, the amount and availability of dissolved oxygen drops. Concomitantly, carbon dioxide levels increase due to cellular respiration and this stimulates acetate formation, which is detrimental to cell growth and recombinant protein production (Lee, 1996). For future fermentation processes, adjustment of the culture medium pH using a suitable buffer would perhaps suffice to maintain the integrity of the recombinant *E. coli* culture.

Thus, by using an IPTG-based regulatory system, it is possible to achieve high induction of target genes, as seen in Figs. 2.1 and 2.2. By including 0.1 mM IPTG in the growth medium of *E. coli* X, it was possible to drastically increase the production of xylanase to almost three times the level obtained without induction. An unequivocal disadvantage of using IPTG for the production of recombinant proteins is its high cost, although this rarely presents a problem for high-added-value products, such as

hormone and antibiotic production (Baneyx, 1999b). However, for the mass production of recombinant xylanases, a cheaper means of induction needs to be investigated once an industrially-suited enzyme is created. Another drawback to using IPTG is that it is toxic to most cells, thereby limiting the scope of its application (Yarranton, 1992). However, it remains a potent inducer that is still extensively used for basic research, and was used in this study for the induction of xylanase variants in chapter three.

There are thus many factors that influence recombinant protein production, all of which could not be properly elucidated for this study as it reaches beyond the scope of the project. However, these studies proved that *E. coli* X could be induced with a low concentration of the inducer IPTG (0.1 mM) to produce relatively high levels of xylanase. After 48 h, a drop in xylanase production was seen for all cultures and was more pronounced with cultures induced with 0.1 mM IPTG. It seems that after this time frame, the host's regulatory systems act against recombinant protein production to conserve its energy for the manufacture of house-keeping proteins, especially after depletion of nutrients in the surrounding medium. Such studies could provide the foundation for future scale-up fermentations of *E. coli* X, once a suitable xylanase has been genetically evolved for industrial use.

Recent advances in the understanding of the function, regulation and interactions of cellular gene products, together with the availability of new genetic tools, are making *E. coli* a more attractive host than ever for the production of heterologous proteins. Future challenges in the use of *E. coli* for gene expression will involve the following factors. The first is the achievement of enhanced yields of correctly folded proteins by manipulating the molecular chaperone machinery of the cell. The second is the realization of a "true" and robust secretion mechanism for the efficient release of protein into the culture medium. The third is the endowment of the prokaryotic cell with the ability to perform post-translational modifications found in eukaryotic proteins, such as glycosylation. All these considerations would make the engineering of industrially-significant proteins a far less daunting task, than it presently is.

Once the maximum production levels that could be attained by the native enzyme with induction by IPTG were determined, it was possible to continue with the random mutagenesis of *xynA*. A low concentration of IPTG (0.1 mM) was thus used to improve the production of the mutant xylanases by the *E. coli* transformants. The activity of *xynA* served as the unmutated control for thermostability testing of the different variants.

CHAPTER THREE: DIRECTED EVOLUTION OF *xynA*

3.1 INTRODUCTION

In nature evolution has ensured a steady adaptation of the organism and of the proteins involved in their biological function over billions of years. However, enzymes isolated from natural sources do not always fulfil the requirements of an efficient biotechnological process. Thus, there exists a need for enzymes that have been genetically altered to improve their performance under defined, application-specific conditions (Arnold *et al.*, 2001; Arensdorf *et al.*, 2002).

Xylanases are finding an increasing number of applications, both alone and in combination with other enzymes. Among such applications are cellulose pulp biobleaching (Buchert *et al.*, 1994), bread-making (Courtin *et al.*, 1999) and saccharification of lignocellulosic biomass (Lee, 1997). These applications require enzymes capable of operating under specific and often unnatural conditions. Enzymes are generally quite vulnerable structures and are sensitive to environmental changes such as pH and temperature fluctuations since they affect its equilibrium. The denaturation of such proteins at such environmental extremes is usually the result of unfolding, which is followed by an irreversible process, most often aggregation (Van den Berg *et al.*, 1998). Parameters of particular interest for enzyme improvement at the molecular level include thermostability and pH stability. Biobleaching, for instance, requires thermostable and alkali-stable enzymes. Family 11 xylanases may be of particular interest in biobleaching due to their smaller size; a fact which may facilitate penetration in the cellulose fibre network (Sapag *et al.*, 2002).

The major drawback of rational protein engineering is that it is not only labour-intensive, but also does not guarantee an efficient mutant even after years of research. Efforts to improve the thermostability of a *B. circulans* xylanase, via site-directed mutagenesis, were only partly successful (Wakarchuk *et al.*, 1994a), whilst aims to improve the alkalophilicity of the *N. patriciarum* xylanase using directed evolution were much more promising (Chen *et al.*, 2001). The cloning of the *T. lanuginosus* DSM 5826 *xynA* gene into *E. coli* (Schlacher *et al.*, 1996), and its subsequent crystal structure analysis (Gruber *et al.*, 1998), heralded the first steps towards its genetic optimization for industrial use.

Directed evolution – in which enzyme variants are generated and screened for such improved performance in an iterative fashion – arguably offers the fastest and most effective means of creating improved enzymes for industrial applications. In directed enzyme evolution, DNA carrying the gene for a specific enzyme is randomly mutated by chemical treatment, creating a

library of mutated genes. The mutated genes are transformed into bacteria and used to produce the individual new proteins. The bacteria are then screened for the property of interest. By alternating mutation and selection, enzymes with higher and higher activity sometimes evolve, depending on the degree and location of the mutations.

Mutations are often described as errors, but, from the perspective of evolution, it increases the diversity among descendants thus allowing them to flourish and survive even as the environment presents challenges and opportunities (Caporale, 2000). Random mutagenesis of the target gene is the first step of a directed evolution strategy. Exposure of DNA to UV light often compromises the entire gene and not just its active site. Alternatively, mutator strains with defects in their DNA repair mechanism can be used, which leads to the introduction of mutations during replication. It is much simpler than ep-PCR but, the mutation rate cannot be adjusted. Ep-PCR is the most important method for asexual evolution. When non-optimal conditions (Mn^{2+}, excess Mg^{2+}, excess nucleotides or nucleotide imbalance) are used during PCR, the fidelity of *Taq* polymerase is affected (Bornscheuer, 1998; Matsumura and Ellington, 2001). It is the method most preferred by researchers when mutating whole genes. It allows a series of libraries to be made, which differ in mutation frequency simply by altering PCR conditions, which is important, because libraries with too many wild-type or over-mutated clones decrease the effective throughput of the screen (Matsumura and Ellington, 2001). For all these reasons, ep-PCR was chosen for the random mutagenesis of *xynA* in this study. The number of enzyme variants which can be generated by directed evolution grows exponentially with the size of the enzymes and the numbers of simultaneously exchanged amino acids. Consequently, an efficient screening system to detect promising transformants from those that carry genes which have been genetically compromised, is crucial to any directed evolution experiment.

The creation of a library of mutated *xynA* genes, using ep-PCR and its transformation into *E. coli* were the primary aims of this chapter. Another important objective was to develop an appropriate screening method to identify transformants exhibiting xylanase activity. A thermostability screening assay was used to identify enzymes with enhanced thermostability. The long-term thermostabilities and kinetics of selected mutants, which displayed improved thermostability under standard conditions, were evaluated. Several of the mutated genes were sequenced and aligned to *xynA* to assess the amino acid changes at the molecular level that contributed to the enhanced stability and activity of these mutants.

3.2 MATERIALS AND METHODS

3.2.1 Plasmids

The *E. coli* strain used in this study contained the cloned *T. lanuginosus* DSM 5826 *xynA* (Schlacher *et al.*, 1996) and was designated *E. coli* X. A plasmid map of pX3 (plasmid pBSK containing *xynA*) with some of the restriction and gene sites is depicted in Fig. 3.1. The cloned *xynA* is 678 bp in size and was cloned into a plasmid Bluescript vector (pBSK) as a LacZ-fusion protein. The gene is amplified by PCR or sequenced using standard T3 (forward) and T7 (reverse) primers. The *xynA* gene is excised from the plasmid in a 1003 bp fragment using the restriction enzymes *Xho*I and *Eco*RI, leaving behind a 2928 bp fragment that corresponds to the pBSK vector. The different plasmids used or constructed in this study are listed in Table 3.1.

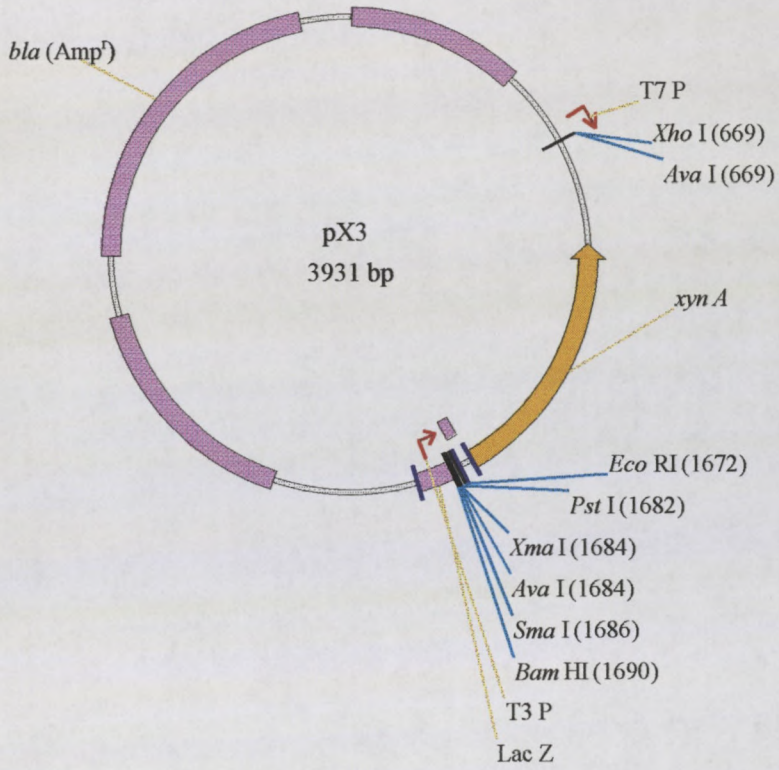

Fig. 3.1 Map of pX3 showing location of the *xynA* gene from *T. lanuginosus* DSM 5826. pX3 contains the β-lactamase (*bla*) gene and *xynA*. Restriction sites on the polylinker of the plasmid are shown and their exact positions indicated in brackets. T3P and T7P designate the positions of the T3 and T7 promoters, respectively. LacZ indicates the position of a short section of the LacZ gene that was used in the cloning of *xynA* as a LacZ-fusion protein.

Table 3.1 Plasmids used or constructed in this study.

Plasmid	Characteristics	Source/Reference[*]
pX3	3931 bp plasmid containing 678 bp *xynA* gene	(Schlacher *et al.*, 1996)
pBluescript	2980 bp plasmid cloning vector, Amp^r, LacZ	W. Steiner
Recombinant plasmids		
p1A1	3931 bp plasmid containing *xynA* variant from first round of random mutagenesis (0.1 mM Mn^{2+})	This study
p1B5	3931 bp plasmid containing *xynA* variant from first round of random mutagenesis (0.2 mM Mn^{2+})	This study
p1B7	3931 bp plasmid containing *xynA* variant from first round of random mutagenesis (0.2 mM Mn^{2+})	This study
p1B8	3931 bp plasmid containing *xynA* variant from first round of random mutagenesis (0.2 mM Mn^{2+})	This study
p1B11	3931 bp plasmid containing *xynA* variant from first round of random mutagenesis (0.2 mM Mn^{2+})	This study
p1D2	3931 bp plasmid containing *xynA* variant from first round of random mutagenesis (0.5 mM Mn^{2+})	This study
p2B5B-1	3931 bp plasmid containing *xynA* variant from second round of random mutagenesis (0.1 mM Mn^{2+})	This study
p2B7-6	3931 bp plasmid containing *xynA* variant from second round of random mutagenesis (0.1 mM Mn^{2+})	This study
p2B7-10	3931 bp plasmid containing *xynA* variant from second round of random mutagenesis (0.1 mM Mn^{2+})	This study
p2B7-16	3931 bp plasmid containing *xynA* variant from second round of random mutagenesis (0.1 mM Mn^{2+})	This study
p2B11-16	3931 bp plasmid containing *xynA* variant from second round of random mutagenesis (0.1 mM Mn^{2+})	This study

[*] W. Steiner: Technical University of Graz, Austria

3.2.2 Plasmid DNA isolation

The boiling lysis method of Sambrook *et al.* (1989) was used to isolate plasmid DNA and modifications to the original protocol are described. *E. coli* clones were inoculated in 5 ml LB-medium containing 100 µg/ml ampicillin and grown for 12-16 h at 37^0C. Cells were harvested by centrifugation of broth cultures at 5000 *g* for 5 min. The pellets were resuspended in 400 µl of STET-buffer [8% Sucrose, 5% Triton X-100, 50 mM EDTA (pH 8), 50 mM Tris-HCl (pH 8)] and transferred to 1.5 ml microcentrifuge tubes. Ten microlitres of lysozyme/RNase mix [10 mg/ml lysozyme and 1 mg/ml RNase in 50 mM Tris-HCl (pH 8)] was added and the mixture was incubated for 10 min at room temperature. The tube was transferred to a boiling water bath set at 95°C for exactly 3 min. The tube and its contents were placed on ice for 5 min and then centrifuged in a microcentrifuge at 10 000 *g* for 10 min. The white pellet was removed with a sterile toothpick and discarded. Four hundred microlitres of ammonium acetate/isopropanol solution (1 vol 5 M ammonium acetate and 2 vols isopropanol) was added to the supernatant and centrifuged at 10 000 *g* for 10 min. The resulting pellet was washed with 700 µl 70% ethanol without resuspension and centrifuged at 10 000 *g* for 5 min. The pellets were air-dried for 10 to 15 min at room temperature. The DNA pellet was dissolved in 20 to 50 µl of sterile distilled water.

For specialized applications like PCR and DNA sequencing where DNA of a high purity was necessary, the High Pure Plasmid DNA Isolation kit (Roche Molecular Biochemicals) was used, according to the manufacturer's instructions.

3.2.3 DNA quantification

DNA concentration was estimated spectrophotometrically at 260 nm and calculated on the premise that an absorbance of 1 at $OD_{260\ nm}$ corresponds to approximately 50 µg DNA/µl. Purity of the DNA sample was calculated using the ratio of DNA concentrations measured at both $OD_{260\ nm}$ and $OD_{280\ nm}$, respectively. DNA purity between a calculated ratio of 1.7 – 1.8 is considered ideal for stringent procedures like sequencing.

3.2.4 Restriction analyses

Type II restriction endonucleases were used for specific digestion of plasmid DNA for three different applications in this study. Firstly, restriction digestion of both the vector and the insert DNA with *Xho*I and *Eco*RI was used to create compatible sticky ends for ligation with DNA ligase. Secondly, recombinant plasmids were restricted with the same enzymes to determine if they

contained the insert after transformation into *E. coli*. This was done because restriction of the plasmids from xylanase-producing clones with both *Xho*I and *Eco*RI yields two characteristic bands of different sizes, which corresponds to that of the vector (2928 bp) and the insert DNA (1003 bp). Finally, restriction endonucleases were also used for preparation of the λ DNA molecular weight marker (section 3.2.5).

Standard protocols were followed for restriction analyses (Sambrook *et al.*, 1989). For digestion, 0.1 volumes of the corresponding restriction buffers (10X) were added to the DNA solution. Mixtures were incubated with the restriction enzymes (Roche Molecular Biochemicals) at 37^{0}C for 2 h or more. Restricted DNA was analyzed on 0.8% agarose gels.

3.2.5 Preparation of λ DNA molecular weight marker

An accurate estimation of the size of restricted plasmid DNA after agarose gel electrophoresis is used to determine the success or failure of a restriction analysis. Thus, λ DNA (Roche Molecular Biochemicals) was restricted with *Eco*RI and *Hin*dIII to yield approximately 11 bands of known size. Forty seven microlitres of sterile distilled water and 10 µl restriction enzyme buffer B were added to a sterile eppendorf, followed by the addition of 35 µl λ DNA and 4 µl each of *Eco*RI and *Hin*dIII

The mixture was incubated at 37^{0}C for 1 h and then placed at 65^{0}C for 10 min to deactivate the enzymes. Ten microlitres of gel loading buffer (0.0375 g bromophenol blue, 4 g sucrose, 1.5 ml 10% SDS, 3 ml 0.5 M EDTA in a total volume of 15 ml) was added and then stored at 4^{0}C. Aliquots were used as a molecular weight marker on all agarose gels.

3.2.6 Agarose gel electrophoresis

DNA molecules were separated on the basis of size using 0.8% agarose gels. The desired amount of agarose was placed in an Erlenmeyer flask together with the required amount of 1X TAE buffer, which was diluted from a 50X TAE stock (242 g Tris, 57.1 ml acetic acid, 100 ml of 0.5 M EDTA, pH 8). The contents of the flask were then microwaved for 1 min and poured into a casting tray with well combs and allowed to set. Gel loading buffer was added to the DNA samples in a ratio of 1:5 which was then loaded into the agarose gel wells. Samples were run alongside the λ DNA molecular weight marker, prepared in section 3.2.5, at 90 V for 1 h. Gels were then stained in ethidium bromide (0.05 mg/ml) for 20 min and destained in distilled water for a further 5 - 10 min. Stained gels were then viewed on a UV transilluminator and the band sizes compared to the DNA

marker. The interaction of double-stranded DNA with ethidium bromide results in a strong, UV-excitable orange fluorescence, which shows the location of the DNA bands. Gel images were captured using a Scion digital imaging system.

3.2.7 Error-prone PCR

Ep-PCR was used for random mutagenesis of *xynA*, where Mn^{2+}, excess amounts of nucleotides and Mg^{2+} hamper the fidelity of *Taq* DNA polymerase (Matsumura and Ellington, 2001). A broad range of conditions were investigated to determine which of the mutagenic PCR conditions might be suitable for the directed evolution strategy. Two separate rounds of mutation were carried out and are outlined below.

Besides a control PCR reaction, 8 different mutagenic conditions (Table 3.2) were chosen from literature (Cadwell and Joyce, 1994; Shafikani *et al.*, 1997) for the initial mutation of *xynA*. Concentrations of Mg^{2+}, nucleotides and Mn^{2+} were varied throughout the mutagenic PCR conditions.

Table 3.2 Mutagenic PCR conditions used for random mutagenesis of *xynA*.

Condition	$[MgCl_2]$	$[MnCl_2]$	[dNTPs]
Control	1.5 mM	-	0.1 mM each
A	7 mM	-	1 mM each
B	7 mM	0.1 mM	1 mM each
C	7 mM	0.2 mM	1 mM each
D	7 mM	0.5 mM	1 mM each
E	7 mM	1 mM	1 mM each
F	7 mM	1.5 mM	1 mM each
G	7 mM	2 mM	1 mM each
H	7 mM	2.5 mM	1 mM each

Four thermostable mutants were identified after initial screening and testing and then subjected to a second round of mutagenesis as outlined in Table 3.3. These thermostable parent clones were named as follows: the number one implies that the clone was obtained from the first round of mutagenesis; the alphabet designates the ep-PCR condition from which the mutant was acquired and the last number is merely given to differentiate between the positive transformants for each condition.

Table 3.3 Mutagenic PCR conditions for iterative mutagenesis of first generation *xynA* variants.

Condition	[$MgCl_2$]	[$MnCl_2$]	[dNTPs]
Control	1.5 mM	-	0.1 mM each
1B5B	7 mM	0.1 mM	1 mM each
1B5C	7 mM	0.2 mM	1 mM each
1B7	7 mM	0.1 mM	1 mM each
1B11	7 mM	0.1 mM	1 mM each
1D2	7 mM	0.1 mM	1 mM each

The PCR mixtures for both rounds of mutation were prepared according to Tables 3.2 and 3.3. pX3 containing the *xynA* gene served as the template DNA for the first round of mutagenesis whilst the 4 mutant plasmids were the templates for the sequential round. The following primers (IDT, USA) were used for the PCR reactions:

Forward primer, T3 (5`- ATTAACCCTCACTAAAGGGA- 3`)
Reverse primer, T7 (5`- TAATACGACTCACTATAGGG-3`)

The forward primer is responsible for synthesis of the DNA in the forward direction whilst the reverse primer synthesises DNA on the opposite DNA strand, thus accomplishing synthesis of the double-stranded DNA molecule. The amount of template DNA (10 ng) and the primers (200 ng) were kept constant in all PCR reactions in which the total volume was always 50 µl. The temperature program for amplification was as follows:

Denaturation	:	1 min at 95^0C
Primer annealing	:	1 min at 42^0C
Primer extension	:	1.5 min at 72^0C

Each PCR reaction comprised a total of 30 cycles and the reactions were performed using a thermal cycler, PCR Genius (Techne).

Five microlitres of each PCR product was separated by agarose gel electrophoresis to determine if the target DNA was successfully amplified. Those conditions that did not yield a PCR product were discarded. The remaining PCR products were recovered using the GFX PCR DNA and Gel Band Purification kit (Amersham) and restricted with *Xho*I and *Eco*RI to create sticky ends compatible with the pBSK vector to facilitate ligation between both the vector and insert. After agarose gel electrophoresis, the restricted bands were excised and purified from the agarose using the same kit, according to the manufacturer's instructions.

3.2.8 Ligation

The restricted PCR inserts and restricted pBSK vector were ligated using T4 DNA ligase (Roche Molecular Biochemicals) in a total reaction volume not exceeding 10 µl. For the creation of mutant libraries, a molar vector:insert ratio of 3:1 was used to guarantee high ligation efficiency. In addition, a control ligation containing restricted vector only was performed to determine the efficiency of the ligation reaction. In this study, it was fortuitous that the vector (2980 bp) was almost three times the size of the amplified inserts (1003 bp). Consequently, three times the concentration of the vector was added to the inserts to adjust the molar ratio to approximately 3:1. Mixtures containing the appropriate amounts of vector and insert DNA were heated to 65^{0}C for 5 min. After chilling on ice for 5 min, 1X Ligase buffer, 1 µl 100 mM ATP and 1 µl T4 DNA ligase were added to the mixture. Ligations were performed for at least 4 h at room temperature or 16^{0}C overnight. Ligation allows for circularization of the plasmid DNA to facilitate transformation back into the host. The ligation mixtures were directly transformed back into SEM-competent host *E. coli* cells for screening and expression of the mutant enzymes.

3.2.9 Preparation of SEM-competent cells

Host cells are made 'competent' or capable of taking up DNA from their surrounding environment, by exposing them to Ca^{2+}, which interact with their cell envelopes. *E. coli* XL1 blue MRF' (courtesy of W.H. van Zyl, University of Stellenbosch, South Africa) was used for all cloning procedures and were made competent using the Simple and Efficient Method prior to transformation (Ausubel *et al.*, 1989).

E. coli XL1 Blue was cultured on LB-medium. A single colony was used to inoculate 5 ml sterile SOC medium (20 g/l tryptone, 5 g/l yeast extract, 40 mM glucose, 20 mM NaCl, 20 mM $MgCl_2$, 20 mM $MgSO_4$, 5 mM KCl), incubated at 37^{0}C and shaken overnight. One millilitre of this culture was used to inoculate 29 ml of fresh SOC medium and shaken at 37^{0}C until it reached an $OD_{600\ nm}$ of 0.5. The culture was immediately placed on ice and kept cold for the duration of the procedure. The cells were pelleted at 5000 *g* for 10 min and the supernatant was discarded. The cells were resuspended in 10 ml cold 100 mM $CaCl_2$ and recentrifuged at the same speed and resuspended in 10 ml cold 100 mM $CaCl_2$. The entire mixture was incubated on ice for 20 min and then centrifuged. The competent cells were subsequently resuspended in 2 ml 100 mM $CaCl_2$ and 10% glycerol. One hundred and fifty microlitres of the prepared competent cells were dispensed into eppendorfs, stored at 4^{0}C overnight, and then transferred to -70^{0}C, after freezing in liquid nitrogen. According to the protocol followed (Ausubel *et al.*, 1989), it is postulated that SEM-competent

cells are most efficient when prepared 24 h prior to transformation. The preparations are incubated at 4^0C overnight to allow for better binding of the Ca^{2+} to the negatively-charged *E. coli* cell envelopes. This step significantly enhances the effectiveness of the subsequent transformation procedure by increasing the competency of these cells.

3.2.10 Transformation

The ligated DNA solutions were added to one hundred and fifty microlitres of the SEM-competent cells and incubated on ice for 30 min and thereafter subjected to heat shock for 30 sec at 42^0C. Rapid addition of 800 µl of fresh SOC medium to each of the mixtures followed the heat shock procedure and they were then shaken at 37^0C for 1 h. Aliquots of the transformation mixtures (50 µl, 100 µl and 100 µl of concentrated sample obtained after centrifugation and resuspension in residual medium) were plated on RBB-xylan-LB plates (0.4% Remazol Brilliant Blue-xylan, 10 g/l bactopeptone, 5 g/l yeast extract, 5 g/l sodium chloride, 15 g/l technical agar) containing 100 µg/ml ampicillin and incubated at 37^0C overnight.

3.2.11 Screening transformants for β-xylanase production

Initially, LB-plates containing ampicillin (100 µg/ml) and different concentrations of RBB-xylan were inoculated with crude *E. coli* X xylanase to determine the concentration of dye-labelled substrate required to produce the clearest halo. The clearest zones of hydrolysis were produced on 0.4% RBB-xylan-LB plates; therefore this concentration was used for the screening procedure.

Transformation mixtures obtained in section 3.2.10 were directly plated on RBB-xylan-LB plates containing 100 µg/ml ampicillin and screened for β-xylanase production. This served as both a screening and selection method since only those cells that had taken up a fully functional plasmid were able to grow on the selective medium supplemented with ampicillin and it further screened for recombinant plasmids (expressing β-xylanase) from those that were merely self-ligated vectors.

The dye, Remazol Brilliant Blue, was linked to birchwood xylan (Roth) for detection of β-xylanase producers during the transformation process. RBB-xylan was prepared according to the method of Biely *et al.* (1985; 1988).

3.2.12 Growth of mutants and enzyme extraction

All positive transformants, i.e., those exhibiting β-xylanase activity were picked up with a sterile toothpick and first streaked onto an LB plate containing 100 µg/ml ampicillin and then inoculated into 5 ml LB broth containing 100 µg/ml ampicillin and incubated for 12-16 h at 37^{0}C. One hundred microlitres of these overnight mutant starter cultures were used to inoculate 100 ml LB medium containing 100 µg/ml ampicillin in 500 ml Erlenmeyer flasks and shaken at 37^{0}C until the OD_{600} of all flasks were 0.5 absorbance units. The cells were induced for xylanase production by adding 0.1 mM IPTG to the flasks and further incubated at 37^{0}C for a further 24 h. Thereafter the samples were centrifuged and treated in the same manner as described in section 2.2.4. All mutants were analyzed in duplicate.

3.2.13 Thermostability screening assay

The clear lysate obtained after enzyme extraction contained the crude enzyme and was used to test the thermostability of the xylanase variants. The protocol followed was a combination of the methods used by Giver *et al.* (1998) and Matsuura *et al.* (1999). Prior to incubation at 80^{0}C in a water bath, 0 min samples were removed from the clear cell lysates and placed on ice. Five hundred microlitres of crude enzyme were subsequently removed from the samples in the water bath every 10 min for 40 min, chilled on ice for 15 min and incubated for 30 min at room temperature to prevent low temperature denaturation of the enzymes. The samples were centrifuged and the supernatants assayed for residual activity of the xylanase (section 2.2.5). Activities of the 0 min samples were considered as 100%, and activities at the other incubation times were expressed as percentages of this control to determine the thermal stability profiles of the enzymes. The wild type XynA served as the control.

3.2.14 Long-term thermostability

The enzyme variants were initially screened at 80^{0}C for 40 min since the wild type enzyme was inherently stable at 70^{0}C. Those that were considerably stable after this time frame (2B7-6, 2B7-10 and 2B11-16) were analyzed at 70, 80, 90 and 100^{0}C for up to 360 min, using the same method outlined in section 3.2.13. The wild type enzyme served as the control.

3.2.15 First order kinetics of the thermostable XynA variants

The first order constants of inactivation for each of the 3 mutants and the control were determined by constructing Arrhenius plots. The constants were calculated from the semilogarithmic plots of the percentage of remaining xylanase activity over different time intervals at 70, 80, 90 and 100^{0}C. The Arrhenius equation (1) was used to calculate the activation energy of the reaction.

$$k = Ae^{(Ea/RT)} \qquad (1)$$

k is the inactivation rate constant, A is the pre-exponential factor, R is the universal gas constant (8.314 J/Kmol), T is the absolute temperature in Kelvin and E_a is the activation energy for the reaction (Price and Dwek, 1979).

The half-lives ($t_{1/2}$) of the 4 above enzymes were calculated using equation 2, to determine the time frame in which the enzyme retained exactly 50% of their initial activities under the different conditions tested. For first order reactions, the $t_{1/2}$ is independent of the concentration of the substrate. If half of the substrate reacts, the ratio inside the log is always equal to ln 2, no matter what the initial concentration. The time needed for half of the reagent to react is called the half-life of the enzyme and is derived from the following equation:

$$\ln 2 = kt_{1/2}$$

$$t_{1/2} = \frac{\ln 2}{k}$$

$$t_{1/2} = \frac{0.693}{k} \qquad (2)$$

3.2.16 Automated DNA sequencing and sequence analysis of *xynA* variants

Automated sequencing of *xynA* variants 1B5, 1B7, 1B8, 1B11, 1D2 and the best progeny mutant, 2B7-10, were performed by Mr. Carel van Heerden, Department of Genetics, University of Stellenbosch, using an ABI PRISM ® 3100 Genetic Analyzer. Chain terminating sequence reactions were performed using the BigDye ® Terminator Ver3 Sequencing kit (Applied Biosystems). All sequencing reactions were performed according to the manufacturer's instructions.

DNA sequencing data was initially processed using the DNAMAN software package and both DNA strands were edited to yield the complete gene sequences. The DNA sequences were then translated into their protein counterparts and compared to the wild type parent using the CLUSTALW (version 1.81) alignment program on the GenomeNet server (www.clustalw.genome.ad.jp).

3.3 RESULTS

3.3.1 Plasmid isolation

After performing plasmid isolation on recombinant *E. coli* X, a small aliquot of the isolation was run on an agarose gel. Confirmation of the plasmid isolation of pX3 is depicted in Fig. 3.2. The 3 darkest bands in lane 1 probably correspond to the 3 conformations of plasmid DNA molecules. The additional bands seen correspond to aggregates of plasmid molecules and possibly chromosomal DNA contamination. The λ DNA molecular weight marker prepared in section 3.2.5 can be seen in lane 2.

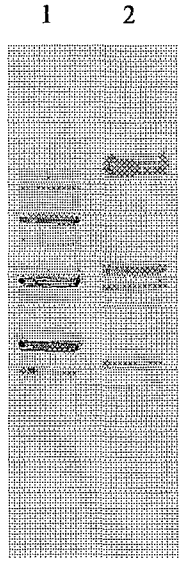

Fig. 3.2 Agarose gel electrophoresis of pX3 plasmid isolation using High Pure Plasmid DNA Isolation kit (Roche Molecular Biochemicals). Lane 1: pX3 plasmid isolation; Lane 2: λ DNA molecular weight marker restricted with *Eco*RI and *Hin*dIII.

3.3.2 Error-prone PCR

3.3.2.1 Random mutagenesis of xynA

Five microlitres of each PCR product, corresponding to each of the 8 conditions tested (Table 3.2), was separated by agarose gel electrophoresis. A single band with a size of approximately 1000 bp for the first 5 reactions (lanes 9 to 5), can be observed in Fig. 3.3, which corresponds to the control and conditions A to D, respectively. Conditions E to H (lanes 4 to 1) did not yield any PCR products; hence these reactions were eliminated from further consideration. An interesting observation was that the intensity of the bands decreased with increasing Mn^{2+} concentration

demonstrating that these ions negatively influence the reaction with regard to the amount of PCR product obtained. Although not clearly visible in the figure, the bands with lower Mn^{2+} concentration (lanes 6, 7 and 8) were markedly brighter and more dense than the bands that were amplified with a higher Mn^{2+} concentration (lanes 1 to 5).

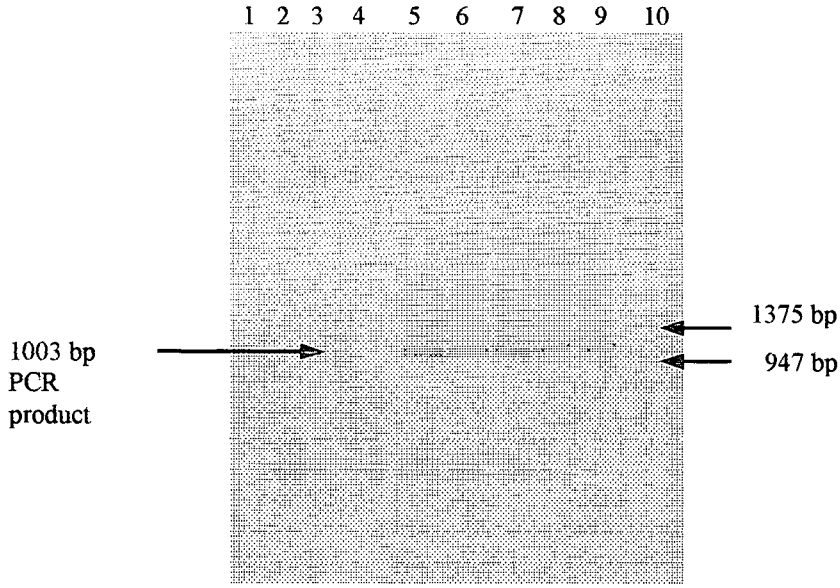

Fig. 3.3 Agarose gel electrophoresis of *xynA* variants after exposure to different mutagenic PCR conditions (A to H – refer to Table 3.2). Lane 1: H (2.5 mM Mn^{2+}); Lane 2: G (2 mM Mn^{2+}); Lane 3: F (1.5 mM Mn^{2+}); Lane 4: E (1 mM Mn^{2+}); Lane 5: D (0.5 mM Mn^{2+}); Lane 6: C (0.2 mM Mn^{2+}); Lane 7: B (0.1 mM Mn^{2+}); Lane 8: A (no Mn^{2+} but high concentrations of dNTPs and Mg^{2+}); Lane 9: Control (amplified under normal PCR conditions without Mn^{2+}); Lane 10: λ DNA molecular weight marker restricted with *Eco*RI and *Hin*dIII.

3.3.2.2 Mutation of selected first generation xynA variants

After the initial round of screening mutants for enhanced activity and thermostability, four promising variants *viz.*, 1B5, 1B7, 1B11 and 1D2, were identified (refer to Figs. 3.8 and 3.11). Three of the 4 best mutants arose from condition B, i.e., the addition of 0.1 mM Mn^{2+} to the PCR reaction (Table 3.2). This represents the condition with the least amount of Mn^{2+} incorporated into the PCR reaction. It was postulated that the thermostability of these mutants could be further augmented by subjecting them to an additional round of mutagenesis, this time using low amounts of Mn^{2+} in the reactions. A single band equivalent to the 1003 bp fragment containing the *xynA* gene was amplified in all of the 6 reactions tested as shown in Fig. 3.4. The products in lanes 2 and 5 were less dense *vis-à-vis* the other bands.

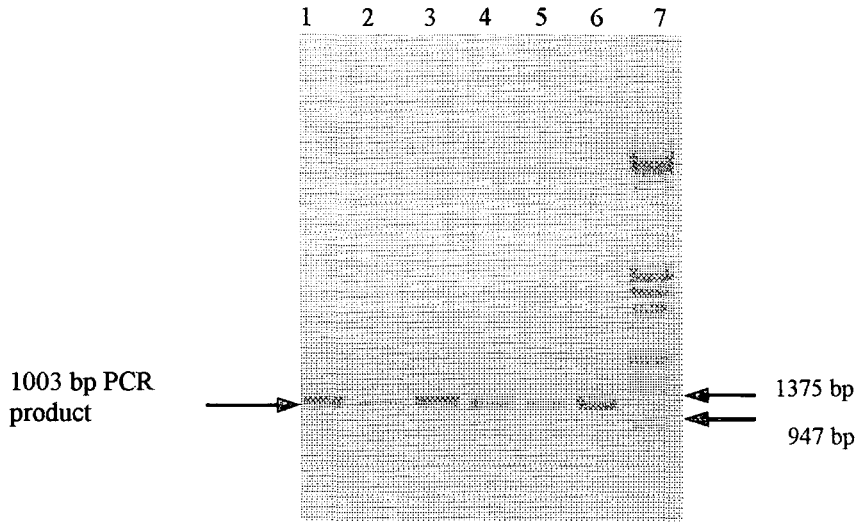

Fig. 3.4 Agarose gel electrophoresis of parent *xynA* variants after exposure to different mutagenic PCR conditions (refer to Table 3.3). Lane 1: 1B5B (0.1 mM Mn^{2+}); Lane 2: 1D2 (0.1 mM Mn^{2+}); Lane 3: 1B7 (0.1 mM Mn^{2+}); Lane 4: 1B11 (0.1 mM Mn^{2+}); Lane 5: 1B5C (0.2 mM Mn^{2+}); Lane 6: Control (amplified under normal PCR conditions without Mn^{2+}); Lane 7: λ DNA molecular weight marker restricted with *Eco*RI and *Hin*dIII.

3.3.3 Restriction analysis of pX3 and its variants

Prior to mutagenesis, it was essential to determine the restriction pattern of pX3 when restricted with both *Xho*I and *Eco*RI. This was used as a point of reference for the future restriction of recombinant plasmids as well as an indication of the band sizes obtained after pX3 restriction (Fig. 3.5). After restriction of pX3 with the above enzymes, 2 fragments of molecular weights 2928 bp (vector) and 1003 bp (fragment containing *xynA*) were produced (lane 6). However, the extra bands in lane 6 (pX3), correspond to incomplete or partial restriction products of the plasmid DNA. Lane 5 depicts the restriction of pBSK with the same two enzymes and yielded a single band corresponding to the size of the vector (2928 bp). The restriction pattern of the bands in the first 4 lanes corresponds to the bands in lane 6. They represent the 4 first generation mutant clones chosen for additional mutation, and contain the 1003 bp insert which confirms their possession of the *xynA* gene.

The restriction profile of the mutants obtained after the second round of random mutagenesis (Fig. 3.6) showed a similar trend. The extra band visible in lane 5 also indicates incomplete restriction of p2B7-10, which had a high initial plasmid DNA concentration.

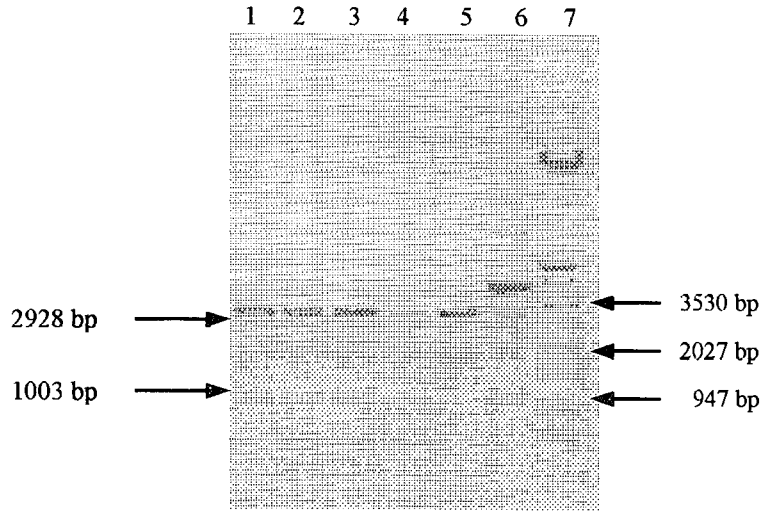

Fig. 3.5 Agarose gel electrophoresis of first generation mutant plasmids after restriction with *Xho*I and *Eco*RI. Lane 1: 1D2; Lane 2:1B11; Lane 3: 1B7; Lane 4: 1B5; Lane 5: pBSK; Lane 6: pX3; Lane 7: λ DNA molecular weight marker restricted with *Eco*RI and *Hin*dIII.

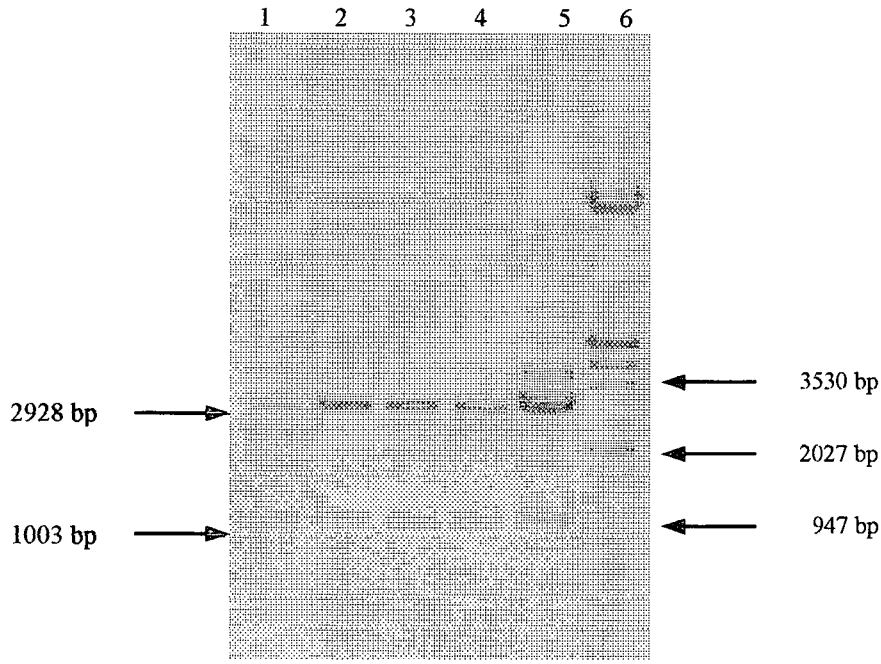

Fig. 3.6 Agarose gel electrophoresis of second generation mutant plasmids after restriction with *Xho*I and *Eco*RI. Lane 1: 2B5B-1; Lane 2:2B11-16; Lane 3: 2B7-16; Lane 4: 2B7-6; Lane 5: 2B7-10; Lane 6: λ DNA molecular weight marker restricted with *Eco*RI and *Hin*dIII.

3.3.4 Screening transformants for β-xylanase production

True or positive transformants were identified by zones of clearing around the colonies since β-xylanase splits RBB-xylan into colourless degradation products (Fig. 3.7). The clones were then numbered for easy identification according to the PCR conditions from which they were generated, the size of their colonies and more importantly, the extent to which they degraded the RBB-xylan. A colony designated as "one" after the alphabet of its mutagenic condition, produced the largest halo on RBB-xylan for that specific mutagenesis parameter. This was done to later determine, after thermostability assessment, if any correlation existed between the size of the zone of clearing and the stability and activity of the respective variant. Sixty four clones were screened from the initial round of mutagenesis and 168 clones were screened after iterative mutagenesis for thermostable xylanase production.

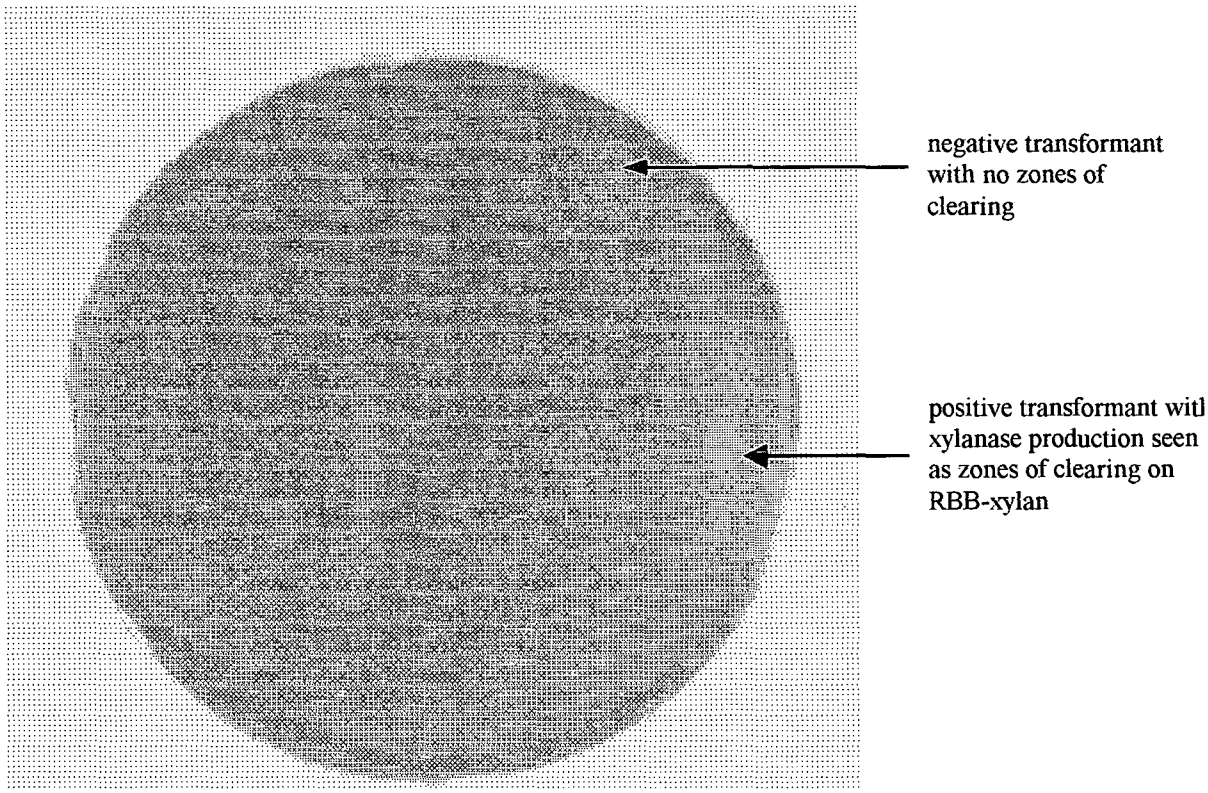

Fig 3.7 Recombinant *E. coli* XynA variants after transformation and plating on LB medium supplemented with 0.4% RBB-xylan and ampicillin (100 μg/ml). The plate was photographed after 16 h incubation at 37°C.

3.3.5 Activity of XynA and its enzyme variants

Since the objectives of this study were to improve both the activity and thermal stability of XynA, these results are shown separately. All figures shown represent an average of duplicate determinations and are fully reflected in Appendices IIA and B.

The activities of some of the mutants were lower than that of the wild type xylanase, which had an initial activity of 657 nkat/μg total protein. Enzyme 1A1 is an example of a first generation variant that was negatively affected by the random mutagenesis procedure as seen in Fig. 3.8. It had an initial activity of 116 nkat/μg total protein, which is much lower than XynA (657 nkat/μg total protein). Xylanase variants 1B5 (819 nkat/μg total protein), 1B7 (979 nkat/μg total protein) and 1B11 (687 nkat/μg total protein) displayed higher activities than the wild type enzyme. Of all the variants generated from the first round of mutation, variant 1D2 exhibited the highest activity of 3564 nkat/μg total protein, an activity more than five fold greater than XynA.

After the second round of mutagenesis, variant 2B7-10 exhibited a five-fold higher activity (3430 nkat/μg total protein) than XynA (657 nkat/μg total protein) and a 3.5 fold higher activity than its first generation parent 1B7 (979 nkat/μg total protein) (Figs. 3.8 and 3.9). The overall activity of mutant 2B5B-1 was severely compromised (298 nkat/μg total protein) and much lower than its parent, 1B5 (819 nkat/μg total protein). A comparison of the activities of the second generation progeny thermostable variants and their first generation mutant parents is depicted in Fig. 3.10. From the figure, it is evident that the activities of some second generation mutants were lower than that displayed by their first generation mutant 'parents', e.g., 2B5B-1 (Fig. 3.10A). Variants 2B7-6 (438 nkat/μg total protein) and 2B7-16 (179 nkat/μg total protein) had significantly lower activities than parent 1B7 (Fig. 3.10B). Variant 2B11-16 had an activity of 621 nkat/μg total protein, which is almost similar to that of its parent 1B11 (687 nkat/μg total protein; Fig. 3.10C).

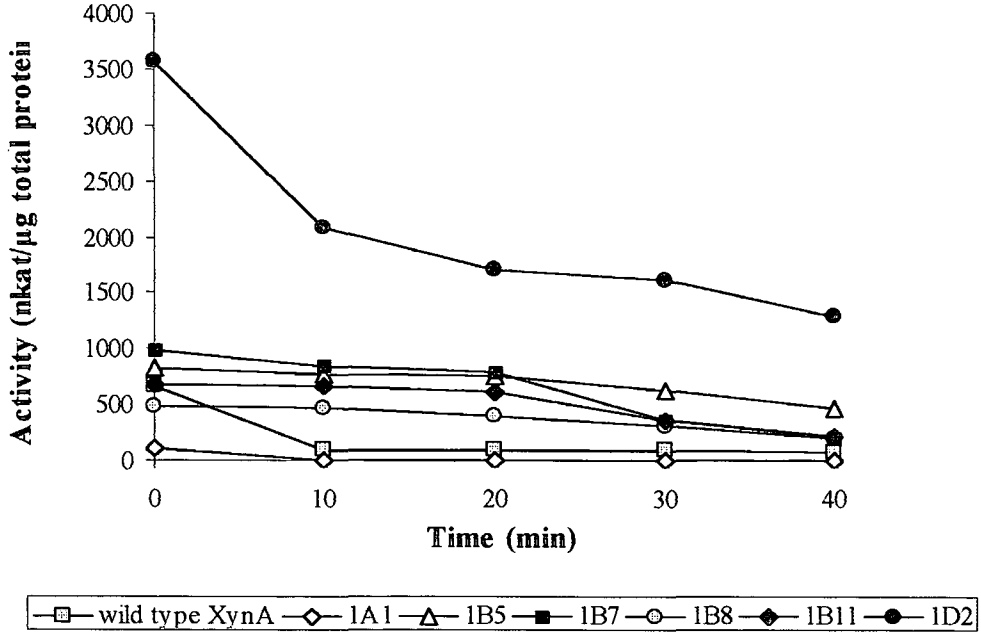

Fig. 3.8 Effect of temperature on the activity of wild-type XynA and XynA variants after the first round of random mutagenesis. The crude enzymes were incubated at 80°C for 40 min. Each point represents the mean of duplicate determinations.

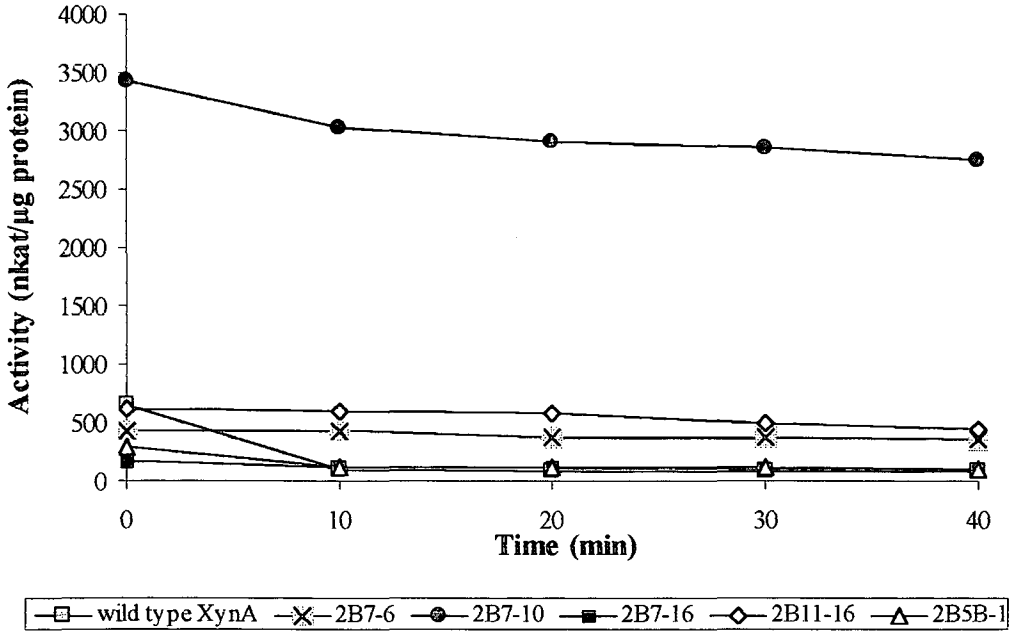

Fig. 3.9 Effect of temperature on the activity of wild-type XynA and second generation mutants. The crude enzymes were incubated at 80°C for 40 min. Each point represents the mean of duplicate determinations.

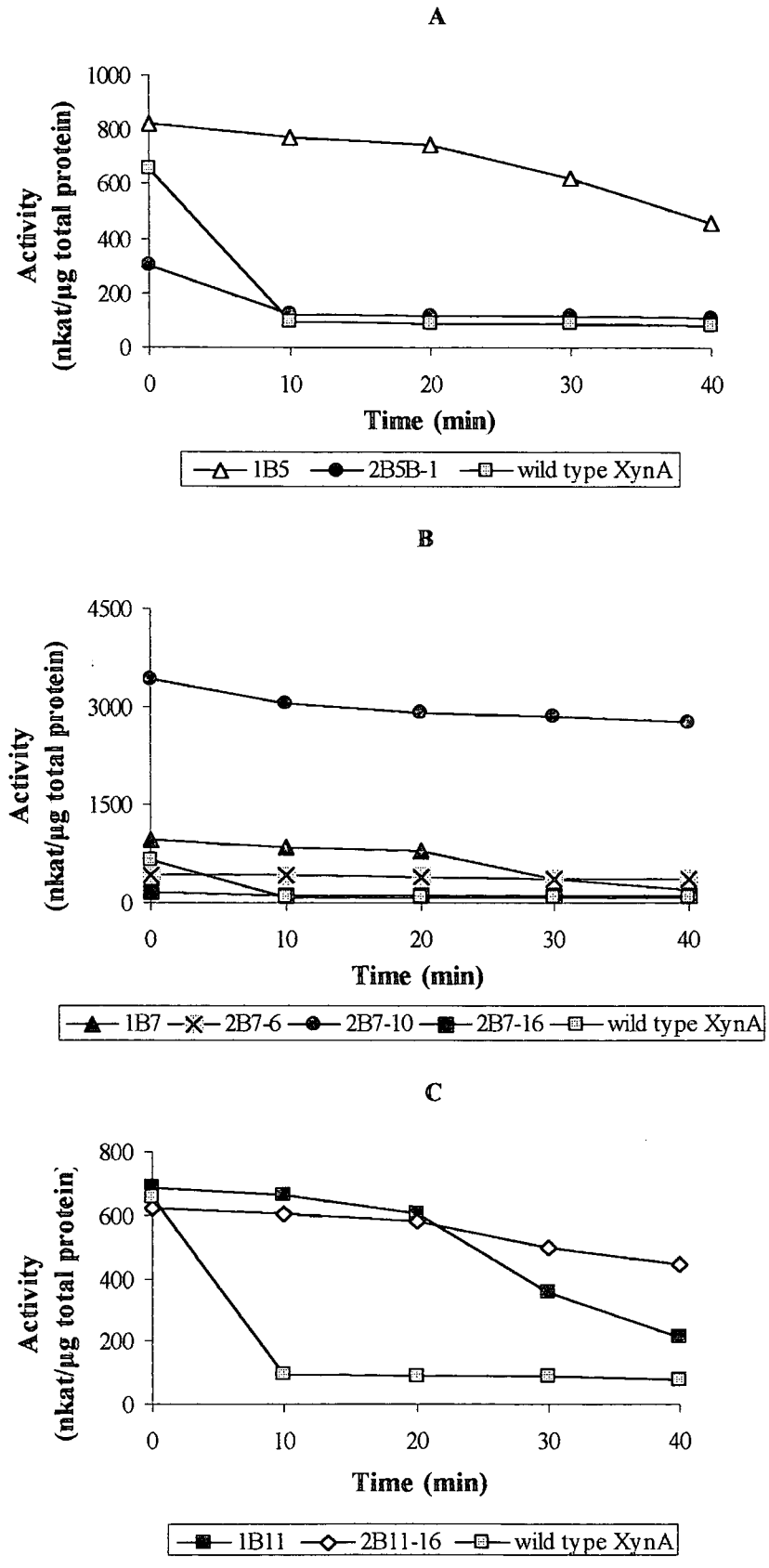

Fig. 3.10 Comparison of the effect of random mutagenesis on the activities of parent and progeny XynA variants. XynA served as the unmutated control for experiments A, B and C. Parent mutants are marked 1, whilst progeny mutants are marked 2. The crude enzymes were incubated at 80^{0}C for 40 min. Each point represents the mean of duplicate determinations.

3.3.6 Thermostability of XynA and its enzyme variants

The clones that underwent amplification under the control or standard conditions displayed a similar thermostability profile to XynA and are not reflected in the graphs. Some of the variants were adversely mutated and displayed a lower stability than the wild type enzyme as seen with variant 1A1, whilst variant 1B5 displayed the best overall thermal stability from all the first generation variants tested (Fig. 3.11). The wild type xylanase lost 80% of its initial activity after exposure to 80^0C for 10 min, whilst variant 1A1 lost almost 94% of its activity under the same conditions. In comparison, variant 1B5 lost only 6% of its activity when treated similarly. The 5 mutants that were improved after mutagenesis exhibited a marked increase in stability at 80^0C when compared to the wild type enzyme. Even though the mutants showed a loss in stability after 20 min of heat treatment, their stability was still greater than the wild type enzyme. The 4 'parent' mutants that displayed the best overall stability were selected for an additional round of mutagenesis to possibly improve on this stability.

After the second round of mutagenesis, a large library of mutants was generated. From Fig. 3.12, it can be seen that four "progeny" mutants (2B7-6, 2B7-10, 2B7-16, and 2B11-16), demonstrated a higher thermostability than the wild type xylanase. The fifth mutant, 2B5B-1 had a much lower stability and activity than its parent, 1B5, implying that the second round of mutagenesis had a negative effect on the enzyme (Fig. 3.13A). Mutant 1B5 retained 56% of its activity after a 40 min exposure at 80^0C, whilst 2B5B-1 had only 4% of its original activity when treated in the same manner. Parent clone, 1B7, after initial mutagenesis, retained 21% of its activity after 40 min at 80^0C and had sufficiently good activity as well. However, one of its progeny mutants, 2B7-10, exhibited an admirable thermostability (81% activity after 40 min at 80^0C) and activity (5 fold greater than XynA and 3 fold greater than 1B7). Mutant 2B7-6 retained 83.44% of its activity after 40 min at 80^0C. Another mutant, 2B7-16, although more thermostable than the parent (56% activity after 40 min at 80^0C; Fig. 3.13B), had a slightly lower activity (Fig. 3.10B). Progeny mutant, 2B11-16, also exhibited superior thermostability (71% activity after 40 min at 80^0C; Fig. 3.13C) when compared to its parent, 1B11 (31% activity after 40 min at 80^0C), however, the overall activities of both were much lower than the 1B7 progeny clones (Figs. 3.10B and C).

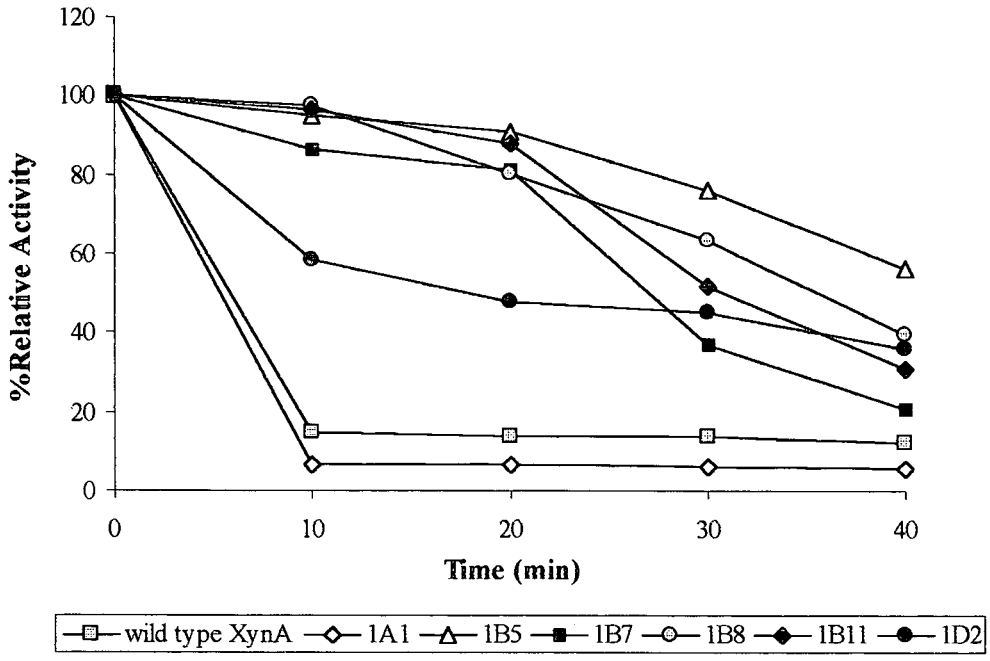

Fig. 3.11 Effect of temperature on the stability of wild-type XynA and XynA variants after the first round of random mutagenesis. The crude enzymes were incubated at 80^{0}C for 40 min. Each point represents the mean of duplicate determinations.

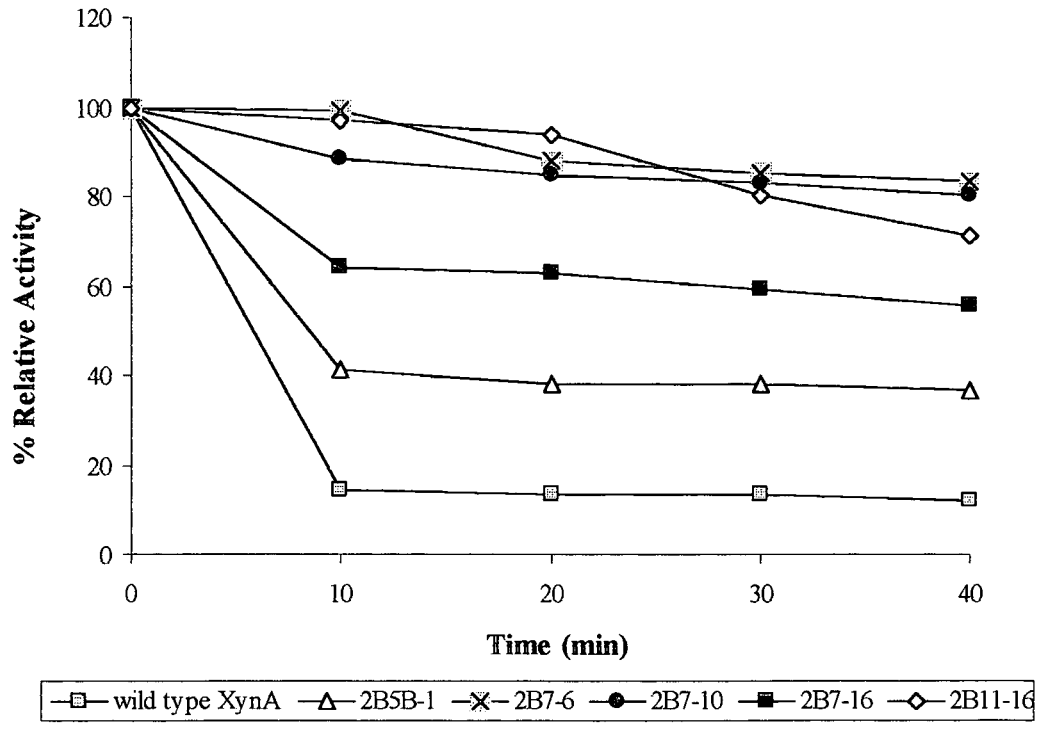

Fig. 3.12 Effect of temperature on the stability of wild-type XynA and XynA variants after the second round of random mutagenesis. The crude enzymes were incubated at 80^{0}C for 40 min. Each point represents the mean of duplicate determinations.

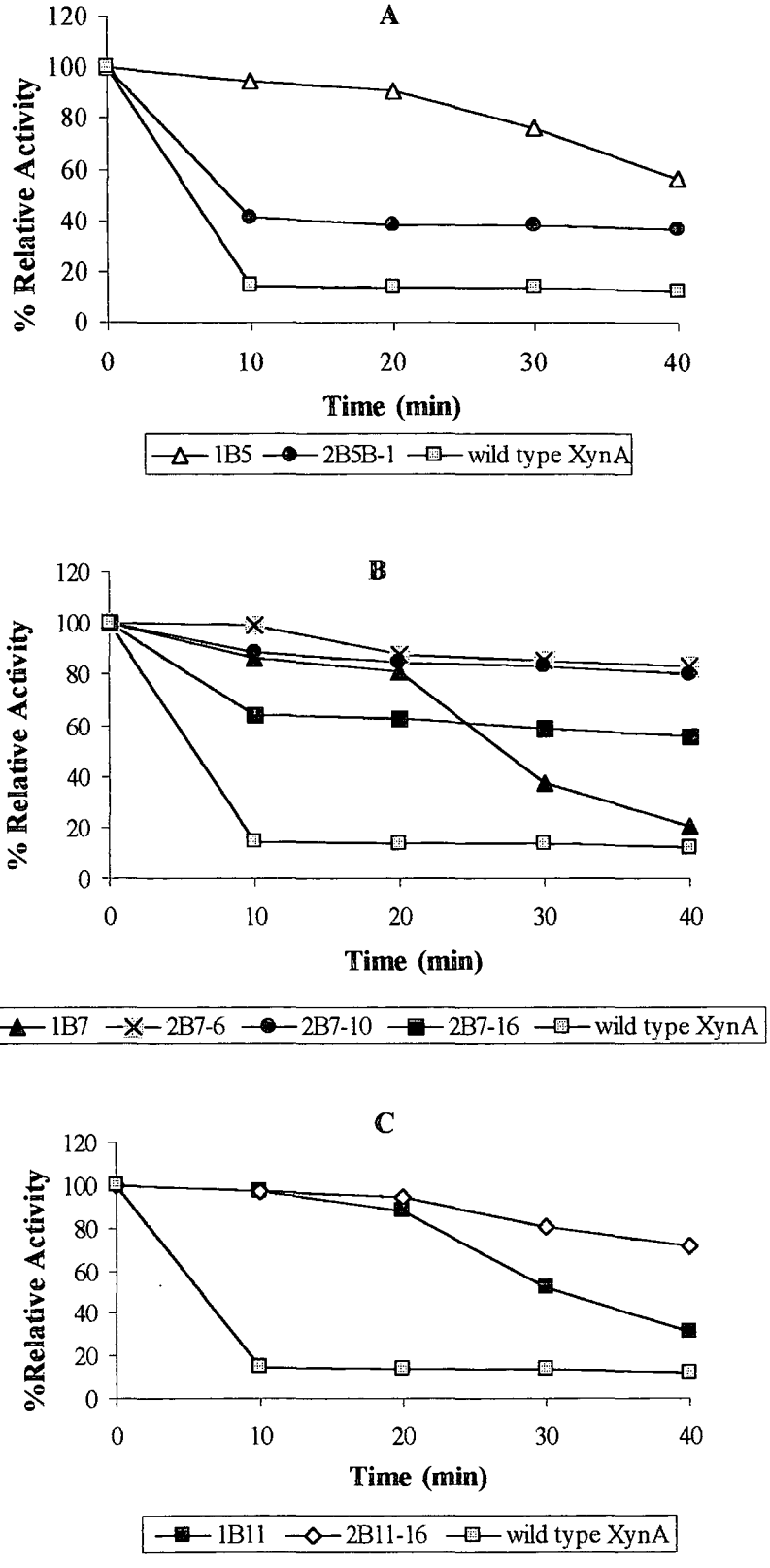

Fig. 3.13 Comparison of the effect of random mutagenesis on the thermostability of parent and progeny XynA variants. Wild type xylanase served as the unmutated control for experiments A, B and C. Parent mutants are marked 1, whilst progeny mutants are marked 2. The crude enzymes were incubated at 80^{0}C for 40 min. Each point represents the mean of duplicate determinations.

3.3.7 Long-term thermostability and first order kinetics of XynA and its variants

The stabilities of the wild type and thermostable mutants at 70^{0}C are shown in Fig. 3.14. From the graphs, it is observed that the mutants were considerably stable at this temperature and closely fit the regression curves whilst the wild type has a greater fluctuation, which implies that it has a lower stability. At 80^{0}C, variants 2B7-6 and 2B11-16 fit well with the regression curve whilst 2B7-10 shows some deviation after remaining stable for 60 min at this temperature (Fig. 3.15). The control experiences a major loss of activity after 10 min at 80^{0}C. The control enzyme and 2B7-10 show strong indications of being irreversibly damaged at 90 and 100^{0}C (Figs. 3.16 and 3.17) after 10 min exposure to these temperatures. This is evident from the steep Arrhenius plots and the observation that the plots exhibit a great deviation from the stability curves, indicating a faster inactivation of the enzyme. Thermostable variants 2B7-6 and 2B11-16 exhibited similar stabilities at both 90 and 100^{0}C, with variant 2B11-16 showing a better overall stability. Variants 2B7-6 and 2B11-16 were considerably stable, especially at 70 and 80^{0}C and their regression plots fit well with the stability curves. Although both enzymes experienced a loss of stability at 90 and 100^{0}C, their inactivation was still considerably lesser than that seen with the wild type and the 2B7-10 xylanases. Percentage remaining activities are fully reflected in Appendix IIC.

Using the Arrhenius equation (section 3.2.15), it was possible to calculate the $t_{1/2}$ and energy of activation for each of the enzymes, which are shown in Table 3.4. From this table it can be seen that the wild type XynA has a lower $t_{1/2}$ and greater inactivation rate (k) than the thermostable mutant xylanases for the different temperatures tested. Mutant 2B7-10 has the greatest $t_{1/2}$ at 70^{0}C (215 min), whilst mutants 2B11-16 and 2B7-6 had higher half-lives than either XynA or 2B7-10 at 80, 90 and 100^{0}C. Enzymes that had reduced stability were found to have a higher inactivation rate and a lower $t_{1/2}$. From the kinetic data, it is evident that variants 2B7-6 and 2B11-16 are considerably stable at all temperatures tested. Variant 2B11-16 had the lowest activation energies whilst XynA had the highest k value. This implies that less stable enzymes expend more energy for a given reaction than their thermostable counterparts.

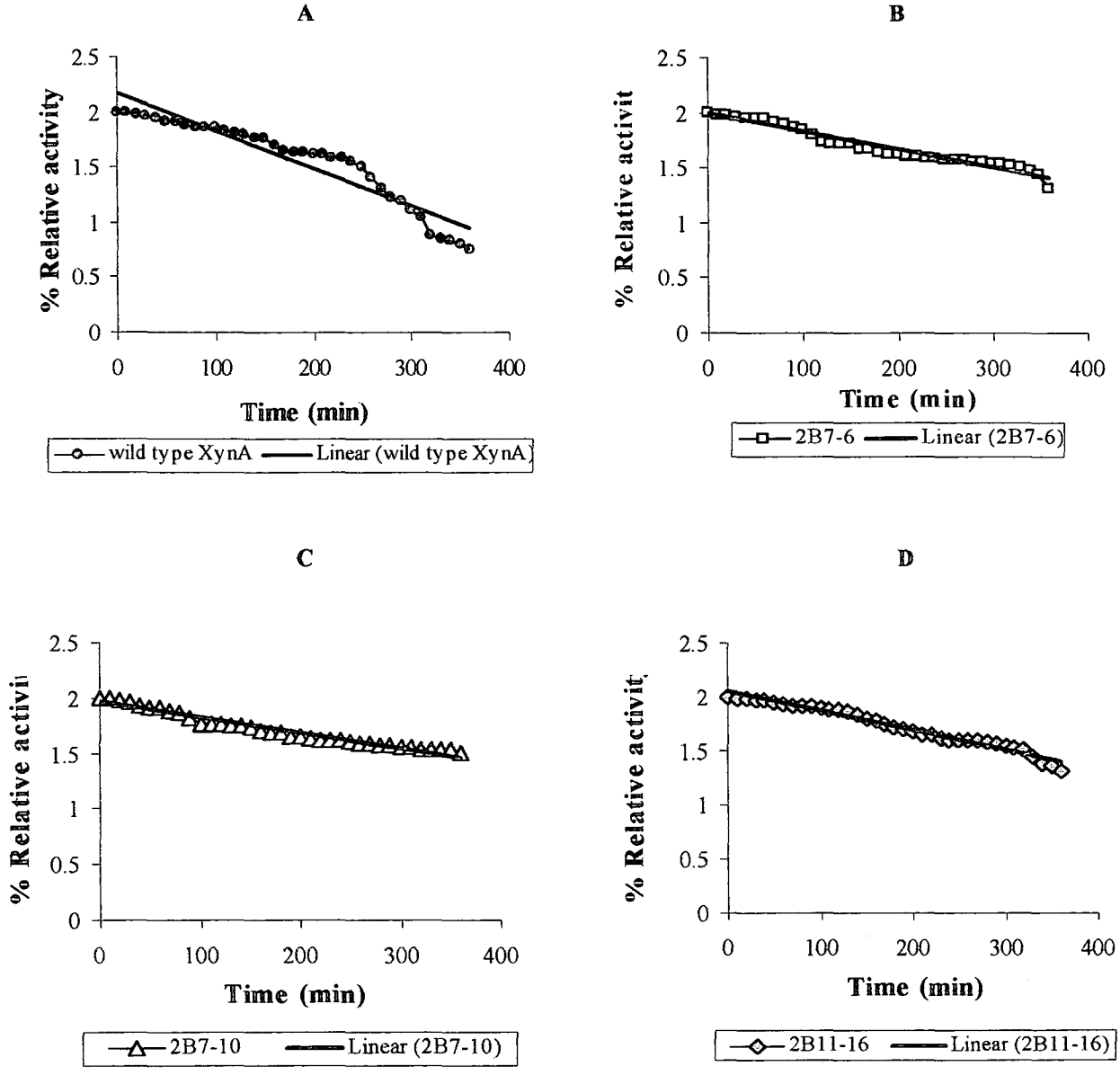

Fig. 3.14 Thermal inactivation curves for the wild type and thermostable mutants of XynA. Linear black lines indicate Arrhenius plots or regression of the semilogarithmic plots as follows: A: wild type control XynA; B: mutant 2B7-6; C: mutant 2B7-10 and D: 2B11-16. The crude enzymes were treated at 70^{0}C for 360 min. The remaining activities were expressed as percentages of the original activities and their log values were plotted. Each point represents the mean of duplicate determinations.

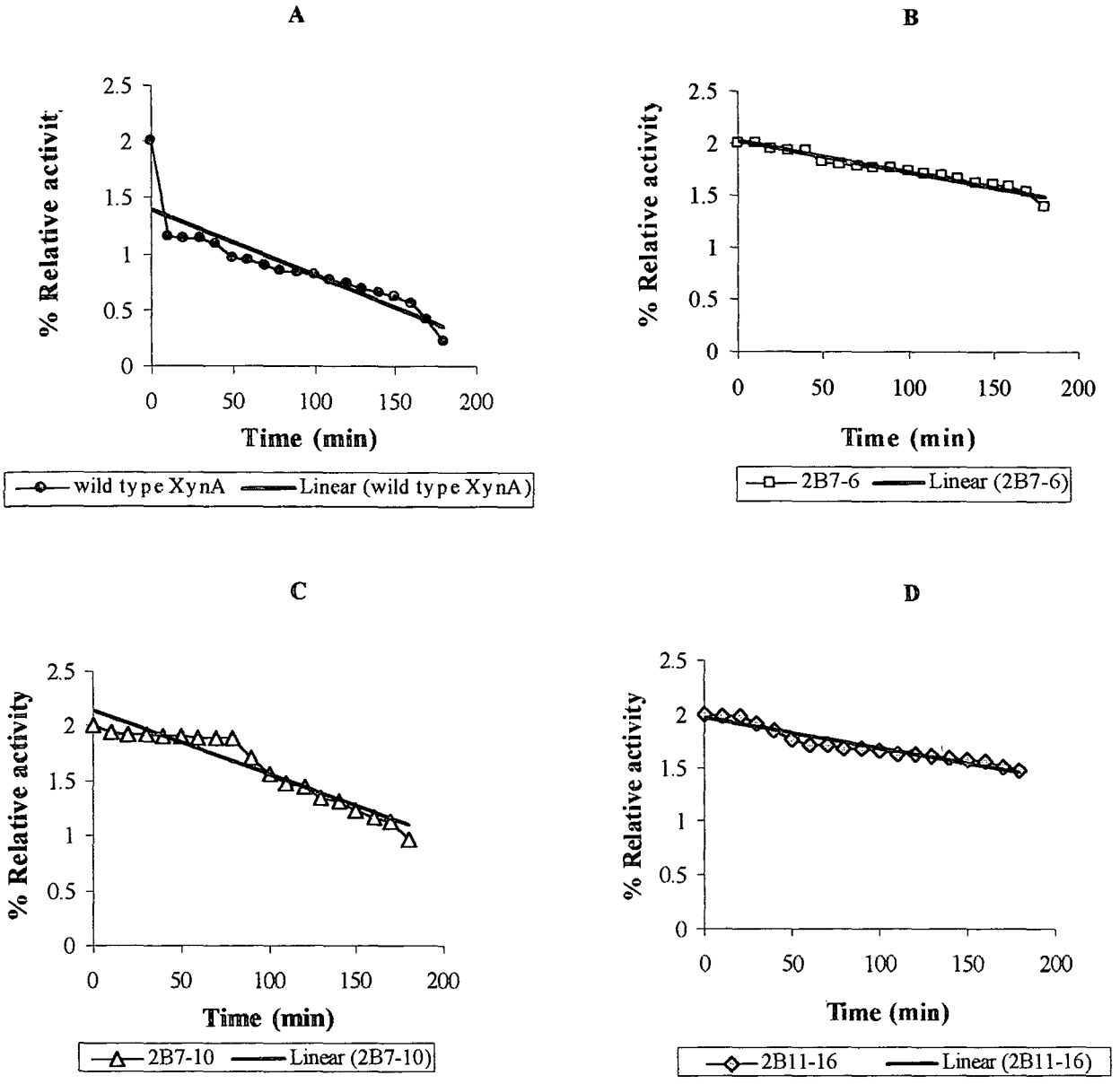

Fig. 3.15 Thermal inactivation curves for the wild type and thermostable mutants of XynA. Linear black lines indicate Arrhenius plots or regression of the semilogarithmic plots as follows: A: wild type control XynA; B: mutant 2B7-6; C: mutant 2B7-10 and D: 2B11-16. The crude enzymes were incubated at 80^0C for 180 min. The remaining activities were expressed as percentages of the original activities and their log values were plotted. Each point represents the mean of duplicate determinations.

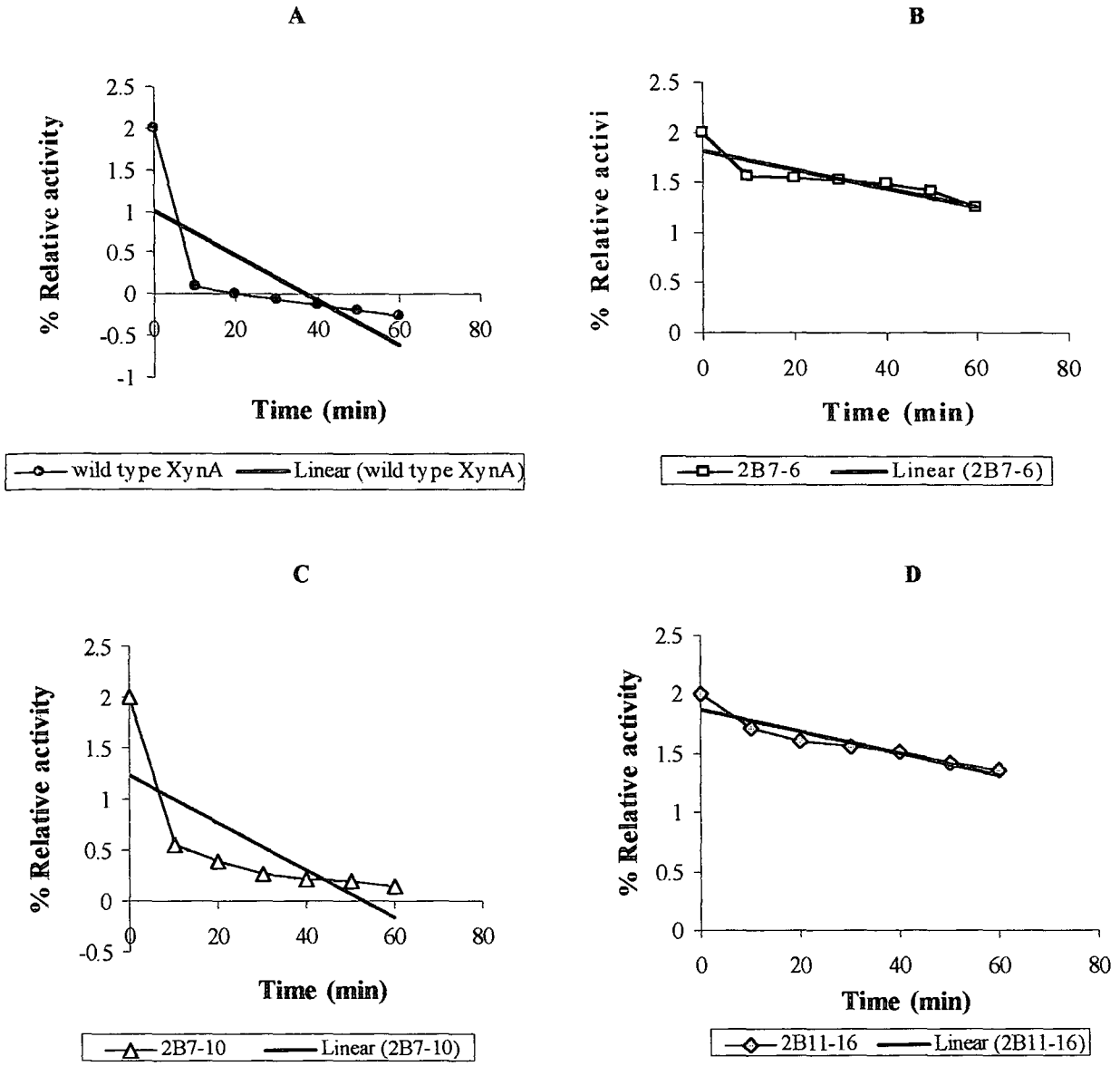

Fig. 3.16 Thermal inactivation curves for the wild type and thermostable mutants of XynA. Linear black lines indicate Arrhenius plots or regression of the semilogarithmic plots as follows: A: wild type control XynA; B: mutant 2B7-6; C: mutant 2B7-10 and D: 2B11-16. The crude enzymes were incubated at 90^{0}C for 60 min. The remaining activities were expressed as percentages of the original activities and their log values were plotted. Each point represents the mean of duplicate determinations.

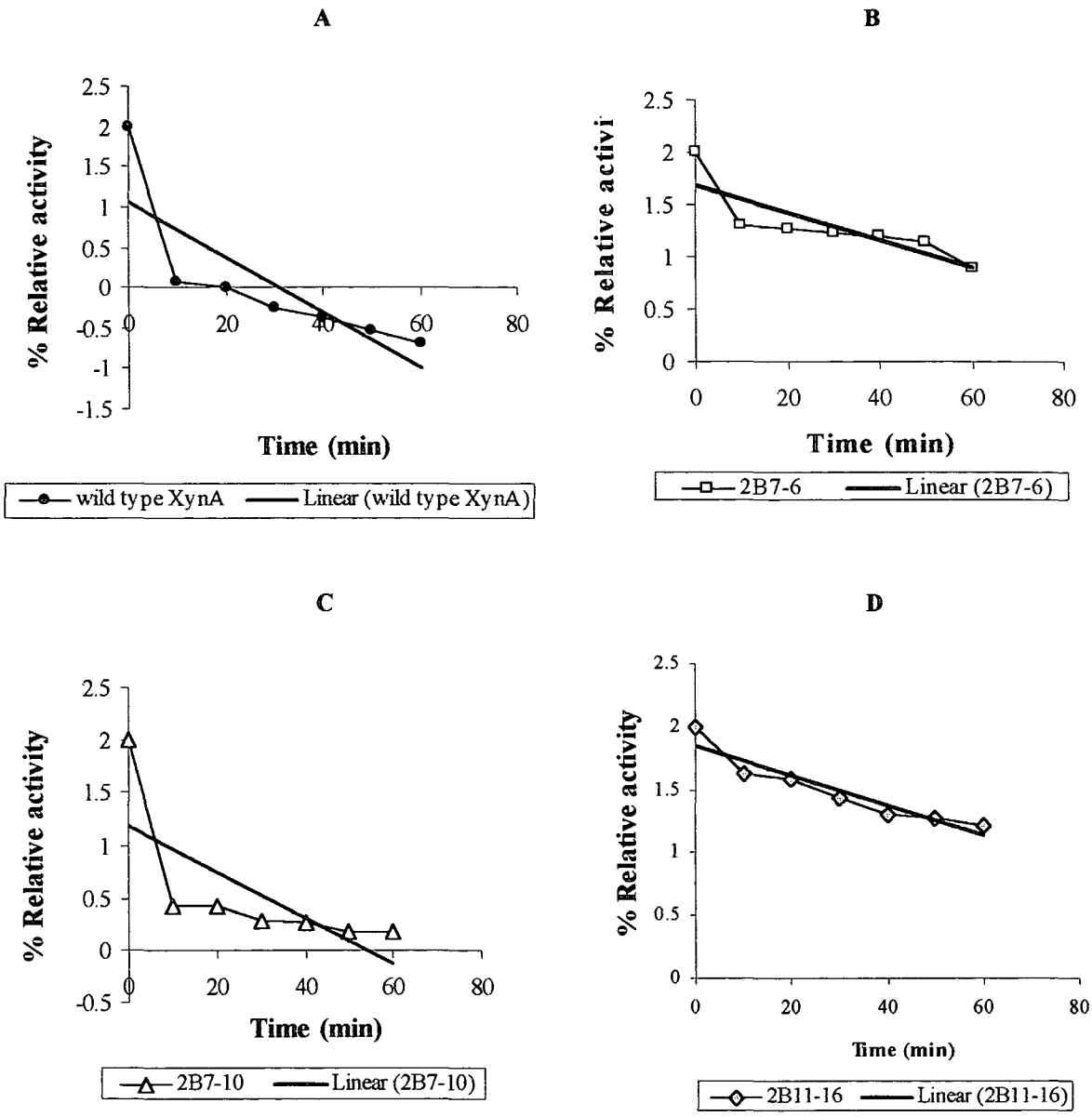

Fig. 3.17 Thermal inactivation curves for the wild type and thermostable mutants of XynA. Linear black lines indicate Arrhenius plots or regression of the semilogarithmic plots as follows: A: wild type control XynA; B: mutant 2B7-6; C: mutant 2B7-10 and D: 2B11-16. The crude enzymes were incubated at 100^{0}C for 60 min. The remaining activities were expressed as percentages of the original activities and their log values were plotted. Each point represents the mean of duplicate determinations.

Table 3.4 Estimated $t_{1/2}$ in min, inactivation rates (k) and activation energies (E_a) of wild type *xynA* and second generation thermostable mutants at different temperatures.

	Temperature (^{0}C)								E_a
	70		80		90		100		
	k	$t_{1/2}$	k	$t_{1/2}$	k	$t_{1/2}$	k	$t_{1/2}$	(kJ/mol)
wild type	0.008	89	0.013	31	0.062	11	0.080	9	97
2B7-6	0.004	188	0.007	104	0.021	32	0.030	23	80
2B7-10	0.003	215	0.013	52	0.053	14	0.051	13	84
2B11-16	0.004	168	0.006	109	0.022	32	0.027	25	74

3.3.8 Sequence analysis of *xynA* variants

3.3.8.1 First generation protein alignments

Five of the most promising first generation mutants (1B5, 1B7, 1B8, 1B11 and 1D2) were sequenced. After sequence editing, their protein sequences were aligned to that of *xynA* and the alignment scores are shown in Table 3.5. These alignment scores indicate the similarity of the respective protein sequences to each other. For example, mutant 1B5 has 98% protein sequence similarity to 1B7; 98% similarity to 1B8; 96% identity with mutant 1B11 and shows the least similarity with mutant 1D2 (89%). All the sequenced mutants displayed the least similarity to mutant 1D2. Mutants 1B5 and 1B7 had two amino acid substitutions each; mutant 1B8 had three; mutant 1B11 had six and mutant 1D2 had 22 such substitutions. All individual amino acid substitutions are reflected in Table 3.6. The position of the original amino acid in the wild type gene and the subsequent amino acid replacements are shown. From the total number of substitutions, it can be seen that mutant 1D2 was mutated to the greatest extent, in comparison with the other sequenced mutants. This mutant was mutated with a high concentration of Mg^{2+} (0.5 mM), implying that the *Taq* polymerase was induced to make more errors during PCR amplification of *xynA*.

The resultant protein alignment of all the first generation mutants with the wild type xylanase is shown in Fig. 3.18. The amino acids are shown in their actual positions within the structural motifs of *xynA*. From the alignment, it is clear that the mutations were not restricted to any particular part of the protein, since all the substitutions are fairly random. Most of the mutations are restricted to the beta sheet B, as opposed to beta sheet A. Four amino acid replacements were made in the α-helix of mutant 1D2.

Table 3.5 Percentage alignment scores of protein sequences of first generation mutants with each other and with wild type *xynA*.

	Mutants				
	1B5	1B7	1B8	1B11	1D2
xynA/WT[¤]	99.	99	99	97	90
1B5	100	98	98	96	89
1B7	98.	100	98	96	89
1B8	98	98	100	96	89
1B11	96	96	96	100	90
1D2	89	89	89	90	100

[¤]Wild Type

Table 3.6 Amino acid substitutions of the improved first generation mutants obtained after random mutagenesis of *xynA*.

Mutant	Amino acid		
	Position	Original	Substitution
1B5	3	glycine	glutamic acid
	218	arginine	proline
1B7	62	glutamic acid	glutamine
	150	lysine	glutamic acid
1B8	38	glutamic acid	glycine
	148	leucine	arginine
	167	glutamine	histidine
1B11	29	glutamic acid	aspartic acid
	34	threonine	glycine
	118	tyrosine	histidine
	127	tyrosine	asparagine
	154	valine	glycine
	210	glycine	phenylalanine
1D2	26	threonine	isoleucine
	27	glutamic acid	aspartic acid
	29	glutamic acid	valine
	34	threonine	glycine
	37	serine	alanine
	70	tryptophan	leucine
	73	glycine	cysteine
	95	phenylalanine	tyrosine
	103	serine	arginine
	114	proline	alanine
	117	glutamic acid	tyrosine
	118	tyrosine	glutamic acid
	129	proline	glycine
	142	aspartic acid	glutamic acid
	143	glycine	alanine
	153	arginine	glycine
	154	valine	leucine
	158	serine	glycine
	186	histidine	leucine
	187	phenylalanine	isoleucine
	193	alanine	asparagine
	194	glycine	arginine

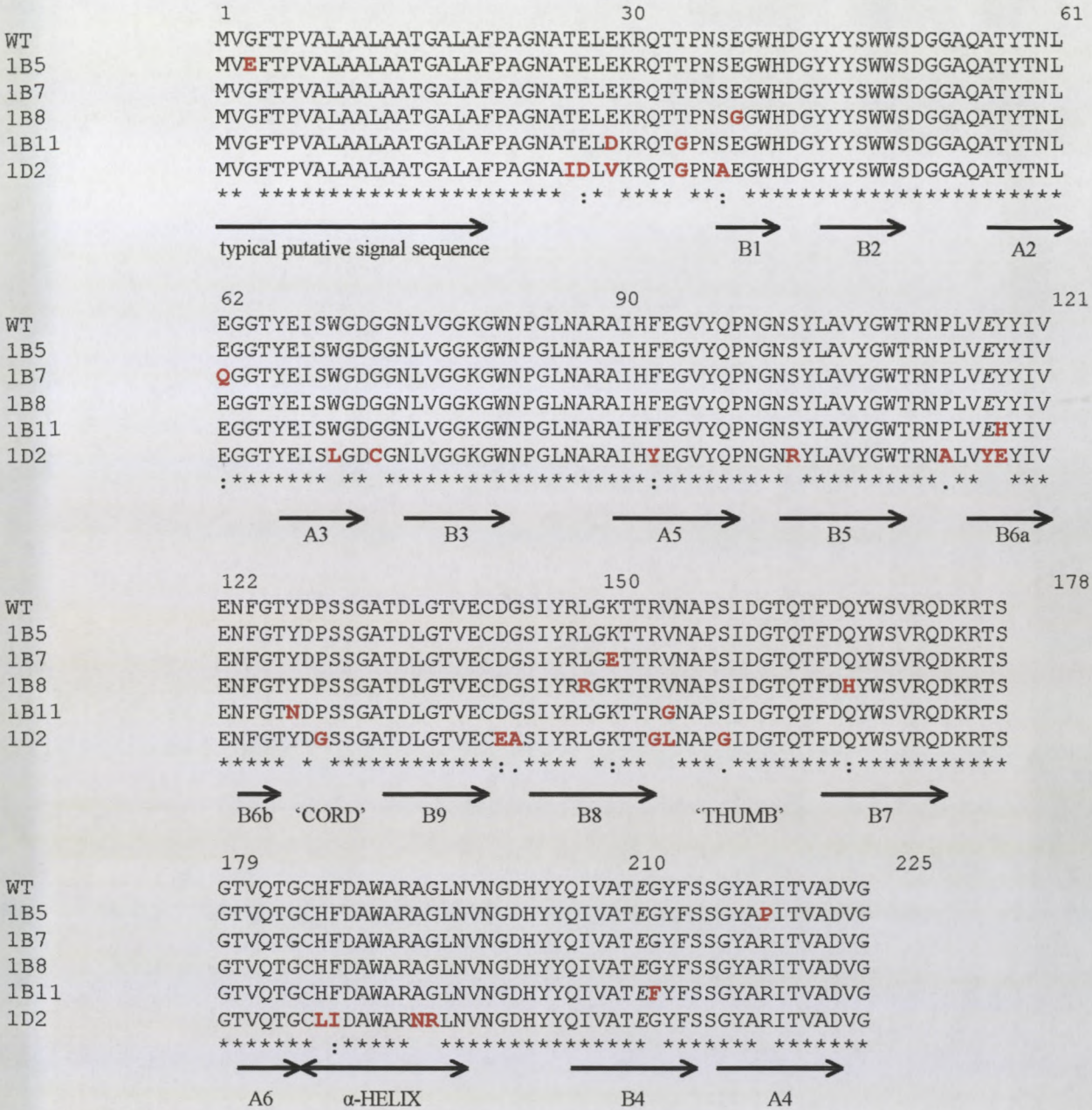

Fig. 3.18 Protein sequence alignment of first generation mutants with each other (1B5, 1B7, 1B8, 1B11, 1D2) and with wild type *xynA* (WT). The alignment was done by using the CLUSTALW (version 1.81) alignment program on the GenomeNet server (www.clustalw.genome.ad.jp). Alignment sequence characters are indicated as follows: '*' indicates positions which have a single, fully conserved residue; ':' shows conservation within a strong group of amino acids; '.' indicates conservation within weaker groups of amino acids. The absence of an alignment character implies that an unrelated amino acid was substituted. Amino acid substitutions that differ from *xynA* are indicated in boldface. Catalytic residues are shown in italics. Arrows indicate the position of the amino acids within the enzyme structure (refer to Fig. 1.4).

3.3.8.2 Second generation protein alignments

Since mutant 2B7-10 displayed enhanced activity and stability under the conditions tested, it was selected for sequence analysis. It was obtained from the mutation of first generation variant, 1B7. Its protein sequence was subsequently aligned to its parent, 1B7 and the wild type *xynA* and the alignment scores are shown in Table 3.7. The wild type *xynA* displayed a greater similarity to 1B7, a first generation mutant (99%), than 2B7-10 (94%).

Amino acid substitutions are shown in Table 3.8 and by the number of mutations seen, it is evident that 2B7-10 was mutated to a greater extent than its parent, 1B7, since it has a greater degree of substitution. From the protein alignment shown in Fig. 3.19, it can be seen that in comparison with 1B7, mutant 2B7-10 had 14 amino acid substitutions. Mutant 2B7-10 displayed 12 amino acid differences from the wild type *xynA*. Two significant substitutions occurred at positions 62 and 150. Mutant 1B7 differed from the wild type xylanase in only these two amino acids, *viz.*, glutamic acid 62 became glutamine and lysine 150 was replaced by glutamic acid after mutagenesis. However, mutant 2B7-10, which was obtained by the mutation of 1B7, also had substitutions at these positions. Glutamine 62 and glutamic acid 150 were substituted by glutamic acid and lysine respectively, which is identical to *xynA*. Another very significant substitution occurred at position 185. Here, a glycine residue disrupted one of the cysteine components of the disulphide bridge, which is reported to be important for the thermal stability of *xynA* (Schlacher *et al.*, 1996; Gruber *et al.*, 1998).

Table 3.7 Percentage alignment scores of protein sequences of second generation mutant 2B7-10 with its parent 1B7 and with wild type *xynA*.

	Mutants	
	1B7	2B7-10
xynA/WT[¤]	99	95
1B7	100	94
2B7-10	94	100

[¤]Wild Type

Table 3.8 Comparison of the amino acid substitutions of the improved first generation parent mutant 1B7 with second generation mutant 2B7-10.

Mutant	Amino acid		
	Position	Original	Substitution
2B7-10	11	alanine	aspartic acid
	58	tyrosine	phenylalanine
	62	glutamine	glutamic acid
	67	glutamic acid	lysine
	92	histidine	proline
	121	valine	aspartic acid
	128	aspartic acid	tyrosine
	140	glutamic acid	glutamine
	150	glutamic acid	lysine
	173	glutamine	proline
	178	serine	isoleucine
	185	cysteine	glycine
	192	arginine	leucine
	217	alanine	proline

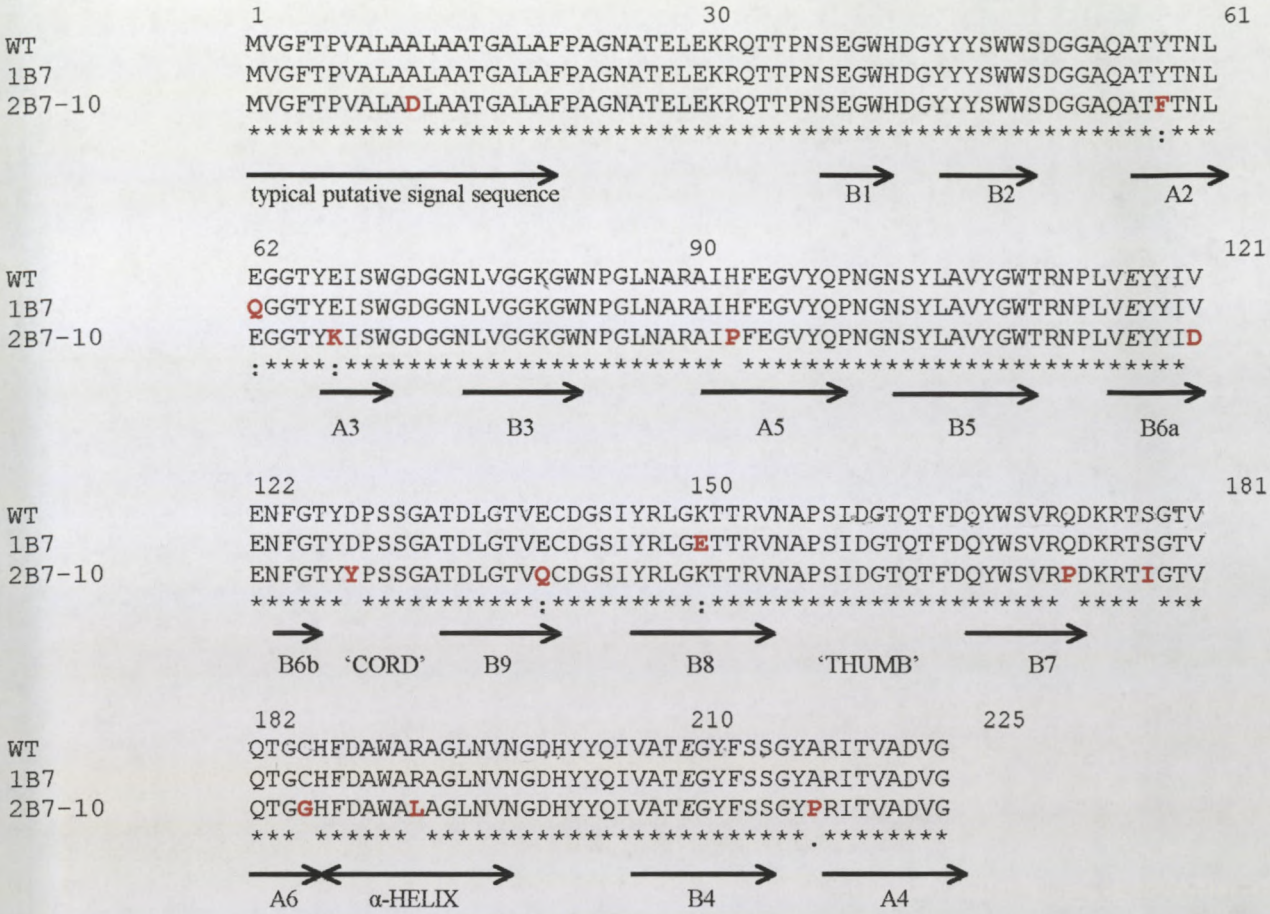

Fig. 3.19 Protein sequence alignment of second generation mutant 2B7-10 with its parent 1B7 and wild type *xynA* (WT). The alignment was done by using the CLUSTALW (version 1.81) alignment program on the GenomeNet server (www.clustalw.genome.ad.jp). Alignment sequence characters are indicated as follows: '*' indicates positions which have a single, fully conserved residue; ':' shows conservation within a strong group of amino acids; '.' indicates conservation within weaker groups of amino acids. The absence of an alignment character implies that an unrelated amino acid was substituted. Amino acid substitutions that differ from *xynA* and 1B7 are indicated in bold-face. Catalytic residues are shown in italics. Arrows indicate the position of the amino acids within the enzyme structure (refer to Fig. 1.4).

3.4 DISCUSSION

The findings presented in this chapter outline the numerous steps required to successfully mutate a gene using directed evolution, from a typical plasmid isolation of pX3 (Fig. 3.2) to cloning the variant genes back into a suitable host for expression and subsequent screening for the desired property. In this study, ep-PCR was used to create genetic diversity by the inclusion of different concentrations of Mn^{2+}, excess $Mg^{2+}$and nucleotides within the PCR reaction. For the first ep-PCR reaction, a broad range of mutagenic PCR conditions was initially investigated from literature (Cadwell and Joyce, 1994; Shafikani *et al.*, 1997), since at the commencement of this study no xylanases were improved using the directed evolution strategy. Both these studies focussed on optimizing ep-PCR conditions for the directed evolution of a wide range of enzymes and these conditions are outlined in Table 3.2.

During ep-PCR (Fig. 3.3), it was noted that the intensity of the bands decreased with increasing Mn^{2+} concentration, until for some conditions (E to H); there were no PCR bands. These conditions contained the highest concentrations of mutagen (1 – 2.5 mM Mn^{2+}) in their PCR reactions. This phenomenon was attributed to a decrease in the normal functioning of *Taq* polymerase, which is highly sensitive to PCR reaction conditions. A low initial transformation efficiency of the ep-PCR products with high Mn^{2+} concentration was also evident. Low ligation proficiency is a common bottleneck when using ep-PCR for the creation of mutant libraries, as found in previous studies (Spee *et al.*, 1993).

Developing a suitable screen to detect mutants with enhanced thermostability proved to be a major stumbling block in this study, a setback experienced by many other researchers seeking to improve thermostability using directed evolution. Initially, a replica-plating method using nitrocellulose membranes was devised to screen mutants, following an *in situ* cell-lysis step, for thermostability at high temperatures. Nitrocellulose membranes were blotted onto the transformants, which were grown on LB agar. These blotted membranes were subsequently placed on Whatman no.1 filter paper that was soaked with breaking buffer, pH 6.8 (section 2.2.4) for 45 min. This was done to lyse the cells and release the intracellularly produced xylanase. The same membrane was then placed on filter paper soaked in 50 mM Tris-HCl (pH 8) to prevent denaturation of the enzymes. The membranes were partially dried and then placed on plates with 0.4% RBB-xylan for different time intervals at various temperatures. The method was initially very promising since many variants produced large zones of hydrolysis on RBB-xylan after exposure to the different temperatures. However, the method yielded many false positives probably because the nitrocellulose cosseted the blotted enzymes at high temperatures because these so-called "positive"

enzymes displayed poor stability when their filtrates were exposed to the same temperatures. Eventually, the clones were selected for after plating transformation mixtures on LB plates supplemented with ampicillin and 0.4% RBB-xylan (Fig. 3.7).

After the mutants were labelled for easy identification (section 3.2.11), grown and their enzymes extracted, the enzyme variants were exposed to a temperature of 80^0C for 40 min. This time frame was chosen because wild type XynA lost almost 85% of its activity after just 10 min of exposure to this temperature as shown in Figs. 3.8 to 3.13. This temperature was selected because *T. lanuginosus* DSM 5826 xylanase exhibited long-term stability at 70^0C (Gomes *et al.*, 1993a). Thus, it was decided to attempt to evolve this stability at 80^0C.

Thirty percent of the first generation mutants tested had a lower activity and stability than the wild type XynA and an example of such a mutant was 1A1 (Figs. 3.8 and 3.11). This mutant lost almost 89% of its initial activity after a 10 min exposure to 80^0C and its stability profile was very similar to the wild type XynA. Although ep-PCR condition A lacked Mn^{2+}, it still had abnormally high concentrations of $Mg^{2+}$and dNTPs, which are known to decrease the fidelity of *Taq* polymerase when included in a PCR reaction (Matsumura and Ellington, 2001).

The greatest number of mutants was generated by condition B (0.1 mM Mn^{2+}), with most mutants having enhanced stability in comparison with the wild type enzyme. A few of them, although more stable than the wild type enzyme, however, displayed a lower activity. Mutant 1B8 was more stable than wild type XynA but had a slightly lower activity, whilst mutant 1D2 had an activity almost 5 times greater than XynA (Fig. 3.8), yet experienced a 42% loss in stability after 10 min (Fig. 3.11). These findings seemed to endorse previous beliefs that thermostability and activity were negatively correlated. Meiering *et al.* (1992); were able to stabilize barnase by 0.15 – 0.64 kJ/mol for 30 min at 60^0C, whilst compromising its activity by 15 – 99% after site-directed mutagenesis. In another study, the T4 lysozyme was mutated using site-directed mutagenesis and its stability was enhanced by 1.2 – 1.5 kJ/mol for 60 min at 55^0C, however, its activity was reduced by 2.5 – 5 fold (Schoichet *et al.*, 1995).

An interesting observation was that the best first generation mutants (1B5 and 1B7) emerged from the condition with the least amount of mutagen and not only exhibited good stability but a higher activity as well. Mutant 1B11 however, attained good thermostability for 20 min without compromising its activity. These results reinforce the fact that there need not be a trade-off between thermostability and activity and they both are at least partially independent properties

which can be optimized together in the same enzyme, as found in other studies (Giver *et al.*, 1998; Van den Berg *et al.*, 1998).

For the screening procedure, the mutants were labelled according to the size of their zones of hydrolysis on RBB-xylan (section 3.3.4) to determine if any correlation existed between the size of the zone of clearing and the stability and activity of the respective mutants subsequent to thermal stability measurements. No such correlation existed because although the less-stable mutants 1B1 and 1D1 produced large zones of clearing on RBB-xylan and had high activities, mutants 1B5, 1B7 and 1D2 had even better activities and stabilities yet produced smaller clearings on RBB-xylan. This could possibly be attributed to a large portion of the enzyme being intracellularly located. Thus, the size of the zone of hydrolysis is not indicative of the stability or activity of the enzyme produced by the transformant and, cell lysis is necessary to liberate much of the produced enzyme.

Since the most thermostable mutants arose from condition B, (mutants 1B5, 1B7 and 1B11), it was decided to further mutate the selected clones using a concentration of 0.1 mM Mn^{2+}. An additional round of mutagenesis was carried out in an attempt to further augment the mutants' increased thermostability since the clones were stable for a maximum of 20 min at 80^0C. Mutant 1B5 not only produced the best thermostability profile, but also had an activity 1.4 times greater than XynA and retained 56% of its activity after 40 min at 80^0C. In addition to mutating this clone with condition B, it was also mutated with 0.2 mM Mn^{2+} (condition C). The first generation mutants that displayed incomparable activity after preliminary thermostability screening were generated from condition D (0.5 mM Mn^{2+}). Mutant 1D2 was included in the additional PCR procedure because of its extraordinarily high activity. These 4 mutants were referred to as the "parent" mutants and were mutated as described in Table 3.3.

Overall in terms of activity, most of the second-generation variants tested exhibited a decrease in activity (Fig. 3.9), with one exception. The thermostability profiles of the variants however, were more interesting. Exposure of the second generation or "progeny" mutants to 80^0C for 40 min yielded 3 thermostable mutants, *viz.*, 2B7-6, 2B7-10 and 2B11-16 (Fig. 3.12). All these mutants were subjected to 0.1 mM Mn^{2+} for both rounds of ep-PCR. It was essential to determine whether the progeny mutants had overall better stabilities and activities than the parent mutants and Figs. 3.10A, B and C, and Figs. 3.13A, B and C illustrate such a comparison.

In Figs. 3.10A and 3.13A, the parent mutant, 1B5 obtained from the initial round of mutation, had better stability and an activity (819 nkat/μg total protein) 3 times that of its progeny clone, 2B5B-1 (298 nkat/μg total protein). Whilst 1B5 retained 76.46% of its activity after 30 min at 80^0C, 2B5B-

1 had only 38.17% of its activity under the same conditions. Thus, 2B5B-1 was deleteriously mutated, in terms of both stability and activity.

Two promising mutants were generated from the mutagenesis of parent 1B7 (Fig. 3.10B). Upon closer examination, it was noted that progeny mutant 2B7-6 displayed a marked increase in stability, conserving 83.44% of its activity after 40 min at 80^0C, making it the most thermostable variant at 80^0C for the second round of mutation. However, its activity (438 nkat/μg total protein) dropped 2 fold lower than its parent, 1B7 (979 nkat/μg total protein). In this instance, there seemed to be a trade-off between thermal stability and activity. Parent 1B7 experienced a 14% drop in stability after 10 min at 80^0C and 2B7-16 had a 36% decrease in stability over the same period. In the case of this latter enzyme variant, both enzyme stability and activity (243 nkat/μg total protein) were compromised. It even had a lower activity than XynA (657 nkat/μg total protein). Progeny mutant 2B7-10 proved to be the most successful variant in the second round of random mutagenesis as it not only retained 80.59% of its activity after 40 min at 80^0C but, its activity (3430 nkat/μg total protein) was 3.5 fold greater than 1B7 and 5 fold greater than XynA.

Variant, 2B11-16, produced an activity of 621 nkat/μg total protein that was almost identical to its parent, 1B11 (687 nkat/μg total protein), but was more stable as shown in Figs. 3.10C and 3.13C. Although, variant 2B11-16 experienced a 10% drop in activity in comparison with 1B11, it was more thermostable, retaining 71.37% of its activity after 40 min at 80^0C. The parent mutant retained only 31% of its activity for the same parameters. No promising mutants were identified in the second-generation clones of 1D2 and 1B5C. They were most likely mutated to such an extent, that they lost much of the stability and activity of their first generation parent mutants. All the variants that arose from these mutagenic conditions were considerably less stable and less active than their parent variants.

Most of the second generation mutants obtained in this study were deleteriously mutated. Variant 2B11-16 exhibited an increase in stability but its activity remained the same. Variant 2B7-6, although highly thermostable, displayed a much reduced activity in comparison with its parent, 1B7. These findings correlate with previous findings that mutations, which improve both stability and activity simultaneously, are rare. Thus, the process by which a trait is acquired becomes particularly important in determining the product enzyme. Since most mutations are deleterious or neutral, an increase in any one property is likely to evolve at the cost of another if the experiment, natural or laboratory, does not constrain both (and the two properties are not positively correlated). Thus, two properties need not be physically coupled to show an apparent trade-off during evolution (Giver *et al.*, 1998).

Although sometimes stabilization of an enzyme is achieved with a significant loss of catalytic activity (Schoichet *et al.*, 1995), one mutant in this study, *viz.*, 2B7-10, exhibited both increased activity and thermostability (Figs. 3.9 and 3.12). Molecular flexibility of an enzyme is essential for both binding to substrates and catalysis. It has been proposed that thermophilic and mesophilic proteins exhibit similar degrees of flexibility at their respective optimum temperatures (Song and Rhee, 2000), although thermophilic proteins are thought to be less flexible than mesophilic proteins at lower temperatures. It has been frequently reported that thermostability and catalytic activity are not mutually exclusive, suggesting that they are both partially independent properties and that they are not incompatible in an enzyme. For instance, six generations of random mutagenesis, recombination and screening increased the melting temperature of *B. subtilis* ρ-nitrobenzyl esterase by more than 14^0C without compromising its catalytic activity at 30^0C (Giver *et al.*, 1998). Similarly, five generations of random mutagenesis, recombination and screening increased the temperature optimum of *B. subtilis* subtilisin E by 17^0C and, at the same time, increased the catalytic efficiency (k_{cat}/K_m) by a factor of 5 to 6 (Zhao and Arnold, 1999). Thus, if an evolutionary engineering experiment is not limited to one property but allows the researchers to determine both properties, thermostability and catalytic activity can be enhanced simultaneously in an enzyme, as with variant 2B7-10.

Enzymes are much more efficient catalysts than any model catalysts yet devised, and in addition, the catalytic activity of enzymes is often very sensitive to experimental conditions, such as temperature, pH and ionic strength. Enzyme kinetics is important for the biochemist because it provides valuable information about the mechanism of an enzyme-catalyzed reaction and allows further insight into the likely properties of an enzyme in its environment *in vivo* (Price and Dwek, 1979). It was thus important to study the kinetics and long-term stability of the thermostable second-generation mutants at different temperatures. Three mutants from the initial thermostability screening (*viz.*, 2B7-6, 2B7-10 and 2B11-16) were found to be thermostable and along with the control, they were subjected to long-term thermostability testing. The cloned wild type *xynA* had a $t_{1/2}$ of 88.5 min at 70^0C (Table 3.4 and Fig. 3.14A). This is in stark contrast with the original fungal enzyme, which was reported to have a $t_{1/2}$ of 201 min at the same temperature (Singh *et al.*, 2000b). There are many reasons as to why the cloned xylanase differs from the fungal enzyme, in terms of stability and activity, the most significant being that their expression systems are different. Bacterial systems are unable to properly express cloned fungal enzymes and fold them into their proper conformations. This finding is very common with heterologous cloning. The expression of cloned enzymes in *E. coli* is generally found to be lower than the parent organism, and confined to the cytoplasmic or periplasmic fractions. The absence of post-translational modifications such as glycosylation in *E. coli* and the intracellular accumulation of the recombinant xylanases have been

suggested to be key reasons for the observed differences between the original and cloned enzymes (Kulkarni *et al.*, 1999).

The three thermostable mutants exhibited much higher half-lives than the control at 70^{0}C (Table 3.4; Fig. 3.14). Mutant 2B7-10 had a $t_{1/2}$ of 215 min at 70^{0}C. This value was greater than that obtained for the original fungal enzyme, which was reported to have a half-life of 201 min at 70^{0}C (Singh *et al.*, 2000b). At 80^{0}C, all 3 mutants display a higher enzyme half-life than the control (Fig. 3.15). Mutants 2B7-6 and 2B11-16 exhibited half-lives of 104 and 109 min respectively. These mutants also have higher half-lives and stabilities than mutant 2B7-10 and the control at both 90 (Fig. 3.16) and 100^{0}C (Fig. 3.17), which make them the mutants with the highest stabilities in this study. The rate constants for all reactions tested increase with an increase in temperature. This is evident from Table 3.4, where it is seen that with each enzyme tested, the k value is higher at each temperature, which implies an increase in the rate of the reactions. However, for each temperature tested, it was observed that the most stable enzyme at that temperature had the lowest k value. Thus, the more stable an enzyme, the lower its reaction rate. The activation energies are directly correlated with the stabilities of the tested enzymes. XynA has the highest activation energy of 97 kJ/mol, whilst the stable enzymes, 2B7-6 and 2B11-16, had energy levels of 80 and 84 kJ/mol respectively. This means that more energy is required to activate XynA than the other mutant enzymes, which have a higher stability.

Increasing the thermostability of an enzyme is known to usually require a combination of multiple amino acid changes, each of which slightly increases the unfolding temperature of the protein. The critical and difficult task in this endeavour is the identification of stable amino acid replacements (Lehmann *et al.*, 2000). Sequence alignment is one of the approaches for obtaining useful information about functional and evolutionary relationships. Residues occupying equivalent positions are believed to have the same biological roles.

The alignment scores of the first generation mutants in comparison with the wild type enzyme are shown in Table 3.5. All of the sequenced first generation mutants displayed a greater than 90% sequence similarity with *xynA* and each other, with the exception of 1D2. All of the mutants showed less than 90% identity with this mutant. Mutants that displayed improvements in their stabilities had only a few amino acid substitutions. Mutant 1D2, which had a total of 22 substitutions, displayed a marked increase in activity whilst its stability at 80^{0}C was severely reduced, with reference to the other mutants (Figs. 3.8 and 3.12).

The protein sequence alignment of the first generation mutants with the *xynA* is shown in Fig. 3.18 and it is noticeable that there are only a few differences, with respect to amino acid changes in comparison with *xynA*. Most of the substitutions occurred within the beta sheet B of the proteins, which form the hydrophobic region of the enzyme. Few mutations were observed in sheet A, which forms the solvent-accessible, hydrophilic portion of the enzyme. The individual amino acid changes in comparison with wild type *xynA* are listed in Table 3.6. Mutants 1B5, 1B7, 1B8 and 1B11 displayed slightly higher activities and markedly higher stabilities than *xynA* (Figs. 3.8 and 3.11). With these mutants, it is significant that their substituted amino acids are mostly those belonging to the charged amino acids (glutamic acid and arginine). Arginine residues are commonly found in protein active or binding sites and because of their propensity to form ionic interactions with negatively charged amino acids where they reportedly increase the stability of many proteins (Das and Gerstein, 2000). Since these substitutions are charged, they could possibly have an impact on the overall electrostatic interactions of the protein, which in turn could be responsible for the observed improvement of the enzyme's stability. From all the family 11 xylanases studied, *xynA* was reported to have the highest number of ion pair interactions and these electrostatic interactions were reputed to account for much of the thermal stability of the enzyme (Gruber *et al.*, 1998).

With mutant 1B11, tyrosine 118 and 127 were substituted in the protein. In *xynA*, tyrosine residues are crucial for substrate recognition and binding (Gruber *et al.*, 1998). This could possibly account for the slightly lower activity seen with the mutant in comparison to the wild type enzyme. The substitutions were more charged than the wild type enzyme and it is possible that this increased the electrostatic interactions, within the protein. This in turn may have lead to the improvement in the stability of 1B11 as compared to XynA (Fig. 3.11).

A crucial substitution occurred at position 153 in mutant 1D2. Arginine 153 was replaced by glycine. This arginine is an important participant in the formation of hydrogen bonds with the substrate. A change to glycine is bound to have an effect on the catalytic mechanism of the enzyme since this substitution is highly unfavoured (Betts and Russell, 2003). This substitution occurs within the cord region of the enzyme, which is a very flexible part of the molecule. The variability in this region of the family 11 xylanases is thought to be responsible for their differences in stability (Gruber *et al.*, 1998). Furthermore, the close proximity of the strongly positive guanidinium group of this arginine to glutamic acid 117 appears to be important for the stabilization of the negative charge on the glutamic acid, which must be unprotonated for the catalytic mechanism of *xynA* to come into effect (Gruber *et al.*, 1998).

Another interesting observation was made when examining the amino acid substitutions in mutant 1D2. Four substitutions occur within the α-helix of the protein, *viz.*, leucine, isoleucine, asparagine and arginine. The α-helix is one of the most common secondary structural motifs found in proteins and *xynA* is reported to have only one α-helix made up of 10 highly hydrophobic amino acids (Gruber *et al.*, 1998). The substitutions within this helix could have been, in part, responsible for the lower stability of 1D2 in comparison with the other mutants, where this helix is strongly conserved. It is often observed that the stability of the constituent α-helices also appears to contribute to the overall thermal stability of proteins (Kumar *et al.*, 2000).

The alignment of the *xynA* protein sequence to first generation parent mutant 1B7 and second-generation mutant 2B7-10 is depicted in Fig. 3.19. The latter mutant displayed enhanced stability and activity when compared to both its parent and the wild type enzyme (Figs. 3.11B and 3.13B). Mutant 2B7-10 exhibited 12 amino acid substitutions over its entire protein length. This was the most promising of the second-generation mutants since it displayed both increased thermostability and activity.

Mutant 2B7-10 has a 95% similarity to *xynA* and a 94% similarity to its parent 1B7 (Table 3.7). The amino acid substitutions that occurred over the entire protein are reflected in Table 3.8. Especially noteworthy are the substitutions that occurred at positions 62 and 150. Mutant 1B7 was altered at these positions, from glutamic acid 62 to glutamine and from lysine 150 to glutamic acid. After further mutation of 1B7, it seems that these residues were changed back to the original amino acids in the second-generation mutant 2B7-10, i.e., glutamine 62 reverted back to glutamic acid and glutamic acid 150 was substituted by lysine. It is possible that the negative charge of glutamic acid 62 and the positive charge of lysine at these positions are strongly favoured by the protein.

Three proline substitutions were seen in mutant 2B7-10: proline was substituted for histidine 92, glutamine 173 and alanine 217. The histidine and alanine residues are located in the hydrophilic outer surface regions of the enzyme, which is suitable for proline. These proline substitutions could be very significant since it was previously reported that an increased occurrence of proline residues in thermophilic proteins could contribute to their stability (Kumar and Bansai, 1998). At the molecular level, thermostability is considered to be closely linked to molecular flexibility (Cowan, 1997). Conformational stability can be affected by altering the stability of the unfolded as well as of the native form of the protein. Thus, a mutation which decreases the conformational freedom of the unfolded state will raise its free energy and increase the difference in free energy between the folded and unfolded states, thus stabilizing the native protein (Daniel *et al.*, 1996). The substitution of proline for other amino acid residues has been proposed to have a general stabilizing effect, in

some cases due to the restriction of the conformational freedom of the unfolded state due to the presence of their bulky side-chain (Matthews *et al.*, 1987). However, this is clearly not a universal stabilization rule (Kumar *et al.*, 2000) and the position of proline in the structure is probably a dominating factor, since the occurrence of proline residues within α-helices results in destabilization and distortion of these structures (Barlow and Thornton, 1988).

A vital amino acid substitution occurred when cysteine 185 was interchanged with glycine, disrupting the disulphide bridge. The thermophilic nature of *xynA* was partly attributed to the presence of a disulphide bridge, which connected the C-terminus of the β-strand B9 (residue 141) with the *N*-terminus of the α-helix (residue 185), (Gruber *et al.*, 1998). Disulphide bridges are believed to stabilize proteins mostly through an entropic effect, by decreasing the entropy of the protein's unfolded state. The entropic effect of the disulphide bridge increases in proportion to the logarithm of the number of residues separating the two cysteines bridged (Matsumura *et al.*, 1989). Introducing disulphide bridges into the *B. circulans* strain D3 xylanase using site-directed mutagenesis improved the stability by 15^0C, but hindered the catalytic activity of the enzyme (Wakarchuk *et al.*, 1994a; 1994b). Gruber *et al.* (1998) reported that the disulphide bridge enhances the thermostability of *xynA*, but other factors, such as electrostatic interactions, play a greater role in its stabilization. This is evident from the fact that the *T. lanuginosus* xylanase outperforms, with respect to its thermostability, the xylanase from *S. commune*, despite 55% sequence homology and the presence of a disulphide bridge in the latter protein. Thus, disulphide bridges do not always improve the stability of enzymes. The enzyme has a sharp turn at the point where this mutation occurred, and the small structure of glycine allows it to reside within this tight region, where it could possibly confer a degree of flexibility to the enzyme. The insertion occurs between the two loop regions, which form the 'thumb' and 'cord' of the enzyme, next to sheet B9. Molecular dynamics simulations indicate that it is one of the most flexible parts of *xynA* (Gruber *et al.*, 1998).

Electrostatic interactions involve amino acid side chains that carry formal electrostatic charge at physiological pH. Electrostatic interactions affect and are affected by protein flexibility. Flexibility implies movement of atoms, residues and fragments of the protein with respect to one another. In folded proteins, pairs of neighbouring, oppositely charged residues often interact to form very stable interactions known as salt bridges (Kumar and Nussinov, 1999). Four such salt bridges are found in *xynA*, but the amino acid compositions and location of these bridges were not reported (Gruber *et al.*, 1998). No mention was made of their effect on the stability on the protein, if any at all. Salt bridges are more frequent in proteins of thermophilic origin than of mesophiles and they

are alleged to have important roles in protein structure and function, with particular emphasis on thermostability and flexibility of proteins (Barlow and Thornton, 1983; Kumar *et al.*, 2001).

Although explanations were delivered in an attempt to rationalize the various amino acid substitutions that occurred within the different mutant proteins, it is imperative to be aware that thermostability may be influenced by structural components which are spatially distant from the active site, such as the immobilization of *N*-terminal sequences (Blake *et al.*, 1991) and that there are no 'rules' governing amino acid substitutions. Every amino acid contributes to some extent, to its overall stability. Consequently, it would be of advantage to 'optimize' a considerable number of residues concomitantly in a one-step procedure, or apply a reliable concept over the entire sequence and structure of a protein. Every amino acid forms a delicate network of multiple interactions with its neighbours. Consequently, there is a high risk of introducing deleterious interactions (e.g., steric or charge repulsions) when changing multiple residues along the entire sequence of the protein. It is often seen that with random mutagenesis, destabilizing mutations seem to be much more prevalent than stabilizing ones (Daniel, 1996). Numerous problems were encountered when some of the more thermostable second-generation mutants were sequenced. For some inexplicable reason, their sequences were unreadable. Sequencing by two independent laboratories yielded the same results.

Since the mutations were scattered and not restricted to any particular area of the mutant proteins, it was difficult to determine which substitutions affected catalytic activity and which affected stability without embarking on protein modelling studies, which was not an objective of this study. Most of the mutants generated in this study had either high activity or improved thermal stability, but not both. Point mutations, which increase enzyme stability often, lower activity, and *vice versa*. There is, therefore, strong evidence for the contention that enzyme stability, flexibility and activity are closely inter-related, and that a balance between stabilizing and destabilizing interactions is required to meet the conflicting demands of stability on the one hand, and catalytic function and cellular turnover on the other (Daniel *et al.*, 1996). Variant 2B7-10, however, was the only exception to the rule and displayed increases in both properties. DNA or family shuffling is normally implemented after directed evolution to combine both desirable traits in the same enzyme, i.e., high thermal stability and enhanced activity. This type of 'sexual recombination' involves the digestion of the improved genes with Dnase and reassembly PCR, during which the fragments are recombined in a random manner to generate the complete enzyme. The resultant recombinant enzymes display improvements in both properties. It is very rare to accomplish both improvements in one enzyme with the directed evolution strategy only.

CHAPTER FOUR: GENERAL DISCUSSION

4.1 THE RESEARCH IN PERSPECTIVE

Several applications of xylanases are being developed for the food and paper industries, which are based on the partial hydrolysis of xylan. The long-term application of xylanases such as conversion of renewable biomass into liquid fuels, where xylanases play a crucial role in conjunction with cellulases, is not yet economically feasible. However, stringent environmental regulations and awareness to reduce the emission of greenhouse gases have added an incentive for future research developments in the study of xylanases (Kulkarni *et al.*, 1999).

Thermostable xylanases are of considerable biotechnological importance since their enhanced stability could greatly reduce enzyme replacement costs or permit processes to be executed at high temperatures. But perhaps most of all, they offer an environmentally benign alternative to chemical catalysts in many commercial and industrial applications. The major disadvantage of employing such enzymes for industrial use is their poor stability and performance under harsh industrial conditions. Thus, for enzymes to have a competitive edge over traditional chemical catalysts, they need to be genetically altered to improve their performance in biotechnological applications. Directed evolution is a molecular technique that requires no knowledge of protein structure-function relationships, as with rational design, and evolves improved variants within a very short time frame. It is thus a powerful tool for both the study and engineering of thermostability and catalytic activity of enzymes (Song and Rhee, 2000).

It was thus undertaken to improve the thermostability of *T. lanuginosus* xylanase (cloned into *E. coli* SURE). This enzyme had many features, which make it an attractive industrial biocatalyst. It was reported by Schlacher *et al.* (1996), that although *E. coli* produced the enzyme intracellularly, it had a tendency to aggregate within insoluble aggregates called inclusion bodies. Such aggregates require sonication to release the enzymes. Another stumbling block was that no investigations, regarding its activity and expression, were conducted. Therefore, there was no control study to which mutants could be compared. Consequently, the first step prior to mutagenesis was to determine the levels of xylanase that could be attained by the wild type XynA, both in the presence and absence of an inducer. IPTG, a lactose analogue, was used for induction since *xynA* was expressed as a fusion protein from a *lac* promoter.

High amounts of IPTG were found to adversely affect xylanase production, whilst a low amount (0.1 mM) sufficiently enhanced xylanase production. This amount was used to later induce

xylanase production by mutants. IPTG was found to increase the rate and production of xylanase. *E. coli* X containing the wild type XynA produced appreciable levels of the enzyme, and did secrete some enzyme into the medium, an observation that was used to later develop the screening method.

Genetic variation, in this study, was created by mutagenic or ep-PCR. This form of random mutagenesis is preferred by most scientists when mutating whole genes, because it allows a series of libraries to be constructed, which differ in mutation frequency simply by altering PCR conditions, which is important, because libraries with too many wild type or over-mutated clones decrease the effective throughput of the screen. Manganese chloride is the most common mutagen used for ep-PCR. However other mutagens such as dithiothreitol, Triton X-100 and mercaptoethanol have also been used with equal success. Thus, adding more mutagen to a PCR reaction increases the number of mutations and hence the odds that a particular mutation will change the shape of the enzyme. A single DNA nucleotide error can change the overall structure of the enzyme enough to affect its activity or stability. However, the trade-off with increasing Mn^{2+} concentration in a PCR reaction is that as the number of mutations increases, so will the likelihood that that enzymes will not be functional after the changes due to detrimental mutations, as seen with most of the enzyme variants in this study.

Screening was performed at 2 stages. The first stage was performed at the level of transformation, where transformation mixtures were directly plated onto 0.4% RBB-xylan supplemented with LB medium and 100 μg/ml ampicillin. Since a low level of xylanase was secreted by the transformants into the medium, which was seen as a zone of hydrolysis, it was possible to differentiate between functional and non-functional enzyme producers. The second stage of screening separated the variants according to their thermal stabilities by subjecting their crude lysates containing the enzymes to 80^0C for 40 min. Condition B (0.1 mM Mn^{2+}) was found to produce the variants with the best improved stability, whilst variants generated from condition D (0.5 mM Mn^{2+}) displayed a marked increase in their activities, albeit with a lower thermal stability. After the first round of mutation and screening, 4 "parent" mutants that were much more stable and active than XynA, were subjected to additional mutagenic PCR, this time only with low levels of Mn^{2+}.

The second round of mutation and screening yielded some encouraging results. After comparison with their parent mutants, most "progeny" variants exhibited a trade-off between stability and activity, i.e., while the stabilities of the variants increased; their activities plummeted well below the levels attained by their parents. Mutant 2B7-10 was the only mutant to display significant increases in both its activity and stability.

The trade-off between stability and activity observed in the results of this study are possibly explained by the continued debate of biological relevance versus physical chemistry. It is possible that natural selection actively avoids highly stable, highly active enzymes. An enzyme that is very stable, for example, might be resistant to degradation within the cell. For thermophilic organisms, high activity at moderate temperatures could translate into catalysts that are so active at elevated physiological temperatures, that they hinder cellular function. Thus, stable enzymes can lose their high activity if they are mutated to have improved stability (Arnold, *et al.*, 2001). Perhaps the most important fact to remember in directed evolution is that the mutations are completely random and it is quite impossible to foresee the effect mutations will have on the catalyst.

The advantage of using the RBB-xylan screening method is that it is not limited for the selection of a single property only. Rather, it allows for the determination of many properties in a single enzyme. For example, if alkaline pH were to be the desired property to be screened for, then it would be very simple to test for this property. The disadvantage of the method, however, is that it only allowed for the selection of colonies that produced a zone of hydrolysis on RBB-xylan. It was therefore possible that variants with greatly enhanced stability were ignored because they did not have any activity, and therefore did not produce a discernible zone of clearing on RBB-xylan plates. It should also be taken into account that the amount of extracellular activity was very low (refer to chapter two). For many mutants, it would also be possible that no extracellular enzyme was present or detectable on RBB-xylan, although it was still produced and accumulated intracellularly.

Long-term thermostability screening at 70, 80, 90 and 100^{0}C revealed that mutants 2B7-6 and 2B11-16 were the most stable enzymes generated in this study, although their activities were lower or almost comparable with their parents. The tested enzymes obeyed first order kinetics and Arrhenius plots indicated that mutants 2B7-6, 2B7-10 and 2B11-16 were considerably stable at 70^{0}C. The inactivate rate or Arrhenius constants for each of the enzymes tested increased as the temperature of the reaction increased. The more stable enzymes exhibited a lower energy of inactivation, as dictated by first order kinetics. This means that less energy was required for the reaction to proceed at optimal conditions. The gradients of the Arrhenius plots become steeper as the enzymes become more denatured and less stable at higher temperatures. In this study, it was found that enzymes that were less stable had higher inactivation rates, lower half-lives and higher energies of activation.

The advent of recombinant DNA technology and the increasing availability of high-resolution three-dimensional structures of thermostable proteins have lead to an increasingly sophisticated

understanding of the role and diversity of mechanisms of protein stabilization. Such information provides valuable guidelines for the design of mutational strategies for the stabilization of labile proteins (Cowan, 1997). The function of biomolecules, especially peptides and nucleic acids is pre-determined significantly by their tertiary structure in space. Active residues of these molecules are kept in precise position by a huge, spatially organized framework of interacting residue-side chains and backbone. A large fraction of amino acid residues can be exchanged without changing the coarse-grained structures of polypeptide backbones, which are apparently relevant in an evolutionary context (Forst, 1998).

Five first generation mutants and one second generation mutant were sequenced to determine the genetic mutations that contributed to the increased stability and activity displayed by these mutants. The mutations were random and not restricted to a particular region of the protein and it was impossible to correlate amino acid changes with the different trends displayed by the mutants in terms of thermal stability and activity. A very significant change though was seen with mutant 1D2 where 4 amino acid substitutions occurred within the α-helix of the protein. This region was strongly conserved with the more stable mutant xylanases generated in this study, implying that it is entirely possible that this structural motif plays a more significant role in the stability of *xynA* than previously thought. The most profound mutation was observed with mutant 2B7-10, where a glycine residue disrupted the disulphide bridge. This xylanase was more stable and active than the wild type xylanase and its first generation parent, 1B7, proving that the disruption of the disulphide bridge did not have a deleterious effect on the enzyme, and somehow contributed to the improvement in its overall stability and catalytic activity.

Many studies were undertaken in an attempt to improve the thermostability of xylanases for biotechnological application by increasing the number of disulphide bridges within the enzymes (Keskar *et al.*, 1989; Deshpande *et al.*, 1990). Introducing such bridges into proteins has met with limited success, since their use is restricted to regions where disulphide bonds are stable (Kulkarni *et al.*, 1999). Interestingly, xylanases without disulphide cross-links are known to be more stable than those with disulphide bonds, of which *B. circulans* is an example (Oku *et al.*, 1993). This could also offer an explanation as to why mutant 2B7-10 displayed good stability, even with disruption of its disulphide bridge. Also, in nature, thermostability of the enzymes from hyperthermophiles appears to be the result of the reduction of water-accessible hydrophobic surfaces rather than the disulphide cross-link strategy (Adams, 1993). The substitution of 3 proline residues could also have contributed to the stability of the enzyme (Kumar and Bansai, 1998).

A huge discrepancy exists between the activity of both the native enzyme and the heterologous enzyme, even after mutation. This raises the question of the viability of studying the heterologous enzyme in *E. coli* with this huge decrease in activity even if the thermostability of the enzyme is improved. However, cloning of the enzyme and expression in a bacterial host makes it considerably easier to improve the enzyme on a genetic level through mutagenesis. Also, the many advantages of *E. coli* have ensured that it remains a valuable host for the efficient and cost-effective production of heterologous proteins. The primary aim of this study was to attempt to engineer a highly thermostable xylanase suitable for industrial application. Only two rounds of random mutagenesis were carried out. For directed evolution procedures, five or six rounds of classical mutagenesis are deemed suitable for sufficient exploration of sequence space and the accumulation of beneficial mutations. Thus, further rounds of ep-PCR are required to fully evolve the enzyme for industrial use. Once that is accomplished, alternative hosts and promoters will be evaluated for improving enzyme expression.

Most of the mutants obtained in this study displayed a trade-off between stability and activity, the only exception being mutant 2B7-10. DNA shuffling is commonly used to combine traits within families of related genes to improve enzyme performance (family shuffling) or to recombine properties or novel functions between proteins with a low sequence homology (DNA recombination). Directed evolution thus, offers a fast, effective means of generating mutants with superior traits. The significant improvements in NMR spectroscopy, X-ray crystallography techniques, as well as the availability of advanced software technologies in recent years have vastly broadened the horizons of rational design. Thus, rational design could further augment evolution studies since most industrial maladies are elucidated by a combination of approaches (Zhao *et al.*, 2002).

4.2 FUTURE PROSPECTS FOR RESEARCH

Cleaner biobleaching technology for the pulp and paper industry is currently concentrated in the developed countries, whereas renewable energy generation from agricultural waste has more relevance for the developing nations. Much work needs to be done to bring these research ideas to fruition. The xylanases that are commercially available today for possible application in the pulp and paper industry, e.g., pulpzymes HA, HB and HC (NOVO), do not meet the ideal criteria identified for enzymatic activity, i.e., optimum activity at pH 10 and temperatures greater than 90^0C. Hence, it is necessary to acknowledge the potent xylanase producers by screening for extremophiles in nature or to design a tailor-made enzyme by the application of protein engineering (Kulkarni *et al.*, 1999).

Biotechnological applications of xylanases require thermostable enzyme preparations with a wide pH and temperature range. Since the availability of the ideal enzyme preparation is limited, the application of protein engineering studies to xylanases has gained momentum. Protein engineering is also one of the principal means of examining the active site of an enzyme to identify the roles of specific residues in catalysis. The identification of active site residues by chemical modification, X-ray crystallographic data and site-directed mutagenesis has provided basic information regarding the structure-function correlation of the xylanases. These studies have formed the basis for protein engineering of xylanases for specific manipulation of the gene for desired enzymatic properties (Liu *et al.*, 2002; Sapag *et al.*, 2002).

The latter approach has gained much momentum in recent years, primarily due to the elegant and sophisticated technologies available. Mutation and selection have become the key components of the emerging field of directed evolution to fashion enzymes with novel or advanced properties. Directed molecular evolution is an alluring alternative to rational design because it does not require knowledge of protein structure or structure-function relationships. It relies, however, on two critical assumptions. The first is that proteins can generally tolerate a certain level of amino acid residue substitutions without severely compromising on folding or stability (Axe *et al.*, 1996); the second is that natural evolution has screened only a subset of the potentially useful sequences and that the unexplored sequence space may disclose different or better solutions to specific biological problems (Zaccolo and Gherardi, 1999).

A few of the mutants generated in this study are currently being characterized to determine if random mutagenesis shifted their pH optima or improved their stability at alkaline pH. The most

thermostable and alkalophilic mutant xylanases will be partially purified and included in a pulping reaction to determine their effectiveness on the pulp.

It would also be of interest to investigate random mutagenesis of *xynA* using one of the many elegant commercial mutagenesis kits now available (e.g., Diversify ^{TM} PCR Random Mutagenesis Kit). Such mutagenesis kits increase the rate of ligation efficiency, which is often low with error-prone PCR and allow for the precise monitoring of the frequency of amino acid substitutions within a given gene. Commercial kits also have the advantage of conserving the relevant regions of the gene of interest and this could perhaps limit the trade-off often observed between stability and catalytic activity during the random mutagenesis of a gene. In addition, further rounds of random mutagenesis are being carried out to properly evolve the *xynA* gene and improve on the desired properties.

Another aim is to find new ways to explore *xynA* sequence space more effectively using sexual evolution. In ep-PCR, no exchange of genetic material occurs between parent sequences. Sexual evolution or DNA recombination methods rely on the mixing and concatenation of genetic material from a number of parent sequences, and are the most successful of all *in vitro* evolution techniques. One method that will be further explored is DNA shuffling since it recombines a large number of mutations within a few selection cycles quickly yielding functional blocks with combinations of beneficial mutations (Stemmer *et al.*, 1994a; 1994b). Since most of the mutants generated in this study were either thermostable or very active, DNA shuffling will be used to combine both these traits in a single enzyme. Work has already commenced on the further improvement of the mutant xylanases using two methods of DNA shuffling. One focuses on the traditional method of family shuffling using Dnase I digestion, whilst the other focuses on the use of restriction enzymes to create gene fragments for recombination.

Once the fitness and stability of the enzyme has been completely evolved using ep-PCR and much larger avenues of sequence space have been explored, the expression of the mutated gene will be optimized for large-scale application. This will be attempted with the employment of different expression vectors, the use of diverse promoters and also cloning the evolved enzyme into other bacterial or eukaryotic hosts. Another highly attractive alternative would be to use the modified *xynA* gene(s) and test the activity in the native host through homologous gene recombination of the native gene. Homologous gene recombination is carried out by targeted disruption of the native *xynA* in the fungal host using site-directed mutagenesis or even through targeted 'knock-out' of the gene. The evolved gene is then cloned back into the fungal host. The fungus should then be able to produce the evolved enzyme for industrial use. This option is far more appealing since the fungal

host has all the appropriate mechanisms at its disposal to fold the enzymes into their proper conformations and secrete them, which would tremendously facilitate downstream processing of the enzyme and eliminate the need for cell lysis.

Industrial biocatalysis is on the verge of significant growth. Directed evolution has rapidly become the method of choice for developing enzyme- and microorganism–based biocatalysts. The recent improvement in new screening technologies will further accelerate biocatalyst development. Furthermore, the ever-expanding capabilities of rational design will lead to more powerful biocatalyst design strategies that combine the best of both approaches. Advances in other fields such as bioinformatics, functional genomics and functional proteomics will also extend the applications of directed evolution and rational design to more industrial biocatalysts.

It would thus seem that the speed and quality of evolutionary solutions to protein engineering problems is truly impressive because ever less knowledge is required about the system being optimized. This is a good indication that evolutionary enzyme engineering is rapidly moving biotechnology into a new phase. Thus, the solutions obtained by directed evolution offer a remarkable opportunity to unravel the mysteries of these amazing molecular machines. With evolution, it is clear that the future is no longer limited by ignorance, but rather only by imagination.

REFERENCES

Adams, M.W.W. 1993. Enzymes and proteins from organisms that grow near and above 100^{0}C. *Annual Reviews in Microbiology* **47:** 627-658.

Adams, M. and Kelly, R. 1998. Finding and using hyperthermophilic enzymes. *Trends in Biotechnology* **16:** 329-332.

Ahsan, M.M., Kaneko, S., Wang, Q., Yura, K., Go, M. and Hayash, K. 2001. Capacity of *Thermomonospora alba* XylA to impart thermostability in family F/10 chimeric xylanases. *Enzyme and Microbial Technology* **28:** 8-15.

Alam, M., Gomes, I., Mohiuddin, G and Hoq, M.M. 1994. Production and characterization of thermostable xylanases by *Thermomyces lanuginosus* and *Thermoascus aurantiacus* grown on lignocelluloses. *Enzyme and Microbial Technology* **16:** 298-302.

Anand, L., Krishnamurthy, S. and Vithayathil, P.J. 1990. Purification and properties of xylanase from the thermophilic fungus, *Humicola lanuginosa*. *Archives in Biochemistry and Biophysics* **276:** 546-553.

Arensdorf, J.J., Loomis, A.K., DiGrazia, P.M., Monticello, D.J. and Pienkos, P.T. 2002. Chemostat approach for the directed evolution of biodesulfurization gain-of-function mutants. *Applied and Environmental Microbiology* **68:** 691-698.

Arnold, F.H. 1996. Directed evolution: creating biocatalysts for the future. *Chemical Engineering Science* **51:** 5091-5102.

Arnold, F.H and Volkov, A.A. 1999. Directed evolution of biocatalysts. *Current Opinions in Chemical Biology* **3:** 54-59.

Arnold, F.H., Wintrode, P.L., Miyazaki, K. and Gershenson, A. 2001. How enzymes adapt: lessons from directed evolution. *Trends in Biochemical Sciences* **26:** 100-106.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Siedman, J.G., Smith, J.A. and Struhl, K. 1989. Current protocols in molecular biology, Vol. 2. Greene Publishing and Associates, Wiley-Interscience, New York.

Axe, D.D., Foster, N.W. and Fersht, A.R. 1996. Active barnase variants with completely random hydrophobic cores. *Proceedings of the National Academy of Sciences, USA* **93:** 5590-5594.

Bailey, M.J., Biely, P. and Poutanen, K. 1992. Interlaboratory testing of methods for assay of xylanase activity. *Journal of Biotechnology* **23:** 257-270.

Baneyx, F. 1999a. *In vivo* folding of recombinant proteins in *Escherichia coli.* **In:** Manual of industrial microbiology and biotechnology. pp. 551-565. Davies, J.E., Demain, A.L., Cohen, G., Hershberger, C.L., Forney, L.J. and Sherman, D.H. (eds.). American Society for Microbiology, Washington, USA.

Baneyx, F. 1999b. Recombinant protein expression in *Escherichia coli. Current Opinion in Biotechnology* **10:** 411-421.

Barlow, D. and Thornton, J.M. 1983. Ion pairs in proteins. *Journal of Molecular Biology* **168:** 867-885.

Barlow, D. and Thornton, J.M. 1988. Helix geometry in proteins. *Journal of Molecular Biology* **201:** 601-619.

Becker, P., Abu-Reesh, I., Markossian, S., Antranikian, G. and Märkl, H. 1997. Determination of the kinetic parameters during continuous cultivation of the lipase-producing thermophile *Bacillus* sp. IHI-91 on olive oil. *Applied Microbiology and Biotechnology* **48:** 184-190.

Bedford, M.R. and Classen, H.L. 1992. The influence of dietary xylanase on intestinal viscosity and molecular weight distribution of carbohydrates in tey-fed broiler chicks. **In:** Xylans and xylanases. pp. 361-370. Visser, J., Beldman, G., Kusters van Someren, M.A. and Voragen, A.G.J. (eds.). Elsevier, Amsterdam.

Betts, M.J. and Russell, R.B. 2003. Amino acid properties and consequences of substitutions. **In:** Bioinformatics for geneticists. pp. 48-84. Barnes, M.R. and Gray, I.C. (eds.). Wiley Publishers, New York.

Biely, P. 1985. Microbial xylanolytic enzymes. *Trends in Biotechnology* **11:** 286-290.

Biely, P., Mislovicova, D. and Toman, R. 1985. Soluble chromogenic substrates for the assay of endo-β-1,4-xylanases and endo-β-1,4-glucanases. *Analytical Biochemistry* **144:** 142-146.

Biely, P., Mackenzie, C.R., Puls, J. and Schneider, H. 1986. Co-operativity of esterases and xylanases in the enzymatic degradation of acetyl xylan. *Bio/Technology* **4:** 731-733.

Biely, P., Mislovicova, D. and Toman, R. 1988. Remazol brilliant blue xylan: a soluble chromogenic substrate for xylanases. *Methods in* Enzymology **160:** 536-541.

Bierman, D.J. 1993. Essentials of papermaking. pp. 48-59. Academic Press, New York.

Blake, P.R., Park, J.B., Bryant, F.O., Aono, S., Magnuson, J.K., Eccleston, E., Howard, J.B., Summers, M.F. and Adams, M.W.W. 1991. Determinants of protein hyperthermostability – purification and amino acid sequence of rubredoxin from the hyperthermophilic archaebacterium *Pyrococcus furiosus* and secondary structure of the zinc adduct by NMR. *Biochemistry* **30:** 10855-10895.

Bornscheuer, U.T. 1998. Directed evolution of enzymes. *Angewande Chemie – International edition* **37:** 3105-3108.

Bornscheuer, U.T., and Pohl, M. 2001. Improved biocatalysts by directed evolution and rational protein design. *Current Opinion in Chemical Biology* **5:** 137-143.

Bragger, J.M., Daniel, R.M., Coolbear, T. and Morgan, H.W. 1989. Very stable enzymes from extremely thermophilic archaebacteria and eubacteria. *Applied Microbiology and Biotechnology* **31:** 556-561.

Buchert, J., Tenkanen, M., Kantelinen, A. and Viikari, L. 1994. Application of xylanases in the pulp and paper industry. *Bioresource Technology* **50:** 65-72.

Burcin, M.M., O'Malley, B.W. and Tsai, S.Y. 1998. A regulatory system for target gene expression. *Frontiers in Bioscience* **3:** 1-7.

Burton, D.R. 1995. Phage display. *Immunotechnology* **1:** 87-94.

Cadwell, R.C. and Joyce, G.F. 1992. Randomization of genes by PCR mutagenesis. *PCR Methods and Applications* **2:** 28-33.

Cadwell, R.C. and Joyce, G.F. 1994. Mutagenic PCR. *PCR Methods and Applications* **3:** 136-140.

Caporale, L.H. 2000. Mutation is modulated: implications for evolution. *BioEssays* **22:** 388-395.

Chakravarty, S. and Varadarajan, R. 2000. Elucidation of determinants of protein stability through genome sequence analysis. *FEBS Letters* **470:** 65-69.

Chanda, S.K., Hirst, E.L., Jones, J.K.N. and Percival, E.G.V. 1950. The constitution of xylan from esparto grass (*Stipa tenacissima*, L.). *Journal of Chemistry* **1950:** 1289-1297.

Chang, C.C., Chen, T.T., Cox, B.W., Dowes, G.N., Stemmer, W.P.C., Punnonen, J. and Patten, P.A. 1999. Evolution of a cytokine using DNA family shuffling. *Nature Biotechnology* **17:** 793-797.

Chen, K. and Arnold, F.H. 1993. Tuning the activity of an enzyme for unusual environments: sequential random mutagenesis of subtilisin E for catalysis in dimethylformamide. *Proceedings of the National Academy of Sciences, USA* **90:** 5618-5622.

Chen, R., Greer, A. and Dean, A.M. 1996. Redesigning secondary structure to invert coenzyme specificity in isopropylmalate dehydrogenase. *Proceedings of the National Academy of Sciences, USA* **93:** 12171-12176.

Chen, R. 1999. A general strategy for enzyme engineering. *Trends in Biotechnology* **17:** 344-345.

Chen, R. 2001. Enzyme engineering: rational design versus directed evolution. *Trends in Biotechnology* **19:** 13-14.

Chen, Y.L., Tang, T.Y. and Cheng, K.J. 2001. Directed evolution to produce an alkalophilic variant from a *Neocallimastix patriciarum* xylanase. *Canadian Journal of Microbiology* **47:** 1088-1094.

Cherry, J.R., Lamsa, M.H., Schneider, P., Vind, J., Svendson, A., Jones, A., and Pederson, A.H. 1999. Directed evolution of a fungal peroxidase. *Nature Biotechnology* **17:** 379-384.

Christov, L.P. and Prior, B.A. 1997. Biobleaching response of sulphite pulps to pre-treatment with xylanases. *Biotechnology Progress* **13:** 695-698.

Christov, L.P. and Prior, B.A. 1998. Research in biotechnology for the pulp and paper industry in South Africa. *South African Journal of Science* **94:** 195-200.

Cooney, O.G. and Emerson, R. 1968. Thermophilic fungi: An account of their biology, activities and classification. pp. 80-88. W.H. Freeman and Company.

Coughlan, M.P. 1985. Cellulases: production, properties and applications. *Biochemical Society Transactions* **13:** 405-406.

Coughlan, M.P. 1992. Towards an understanding of the mechanism of action of main-chain hydrolyzing xylanases. **In:** Xylans and xylanases. pp. 111-139. Visser, J., Beldman, G., Kusters van Someren, M.A. and Voragen, A.G.J. (eds.). Elsevier, Amsterdam.

Coughlan, M.P. and Hazlewood, G.P. 1993. β-1,4-D-Xylan-degrading enzyme systems: biochemistry, molecular biology and applications. *Biotechnology and Applied Biochemistry* **17:** 259-289.

Courtin, C.M., Roelants, A. and Delcour, J.A. 1999. Fractionation-reconstitution experiments provide insight into the role of endoxylanases in bread-making. *Journal of Agricultural Food Chemistry* **47:** 1870-1877.

Cowan, D.A. and Daniel, R.M. 1982. Purification and some properties of an extracellular protease (caldolysin) from an extreme thermophile. *Biochimica et Biophysica Acta* **705:** 293-305.

Cowan, D.A. 1997. Thermophilic proteins: stability and function in aqueous and organic solvents. *Comparative Biochemistry and Physiology* **118:** 429-438.

Crameri, A., Whitehorn, E.A., Tate, E. and Stemmer, W.P.C. 1996. Improved green fluorescent protein by molecular evolution using DNA shuffling. *Nature Biotechnology* **14:** 315-319.

Crameri, A., Dawes, G., Rodriguez, E., Silver, S. and Stemmer, W.P.C. 1997. Molecular evolution of an arsenate detoxification pathway by DNA shuffling. *Nature Biotechnology* **15:** 436-438.

Daniel, R.M., Cowan, D.A., Morgan, H.W. and Curran, M.P. 1982. A correlation between protein thermostability and resistance to proteolysis. *Biochemical Journal* **207:** 641-644.

Daniel, R.M., Dines, M. and Petach, H.H. 1996. The denaturation and degradation of stable enzymes at high temperatures. *Biochemical Journal* **317:** 1-11.

Daniel, R.M. 1996. The upper limits of enzyme thermal stability. *Enzyme and Microbial Technology* **19:** 74-79.

Das, R. and Gerstein, M. 2000. The stability of thermophilic proteins: a study based on comprehensive genome comparison. *Functional Integrated Genomics* **1:** 76-88.

Dekker, R.F.H. and Richards, G.N. 1976. Hemicellulases: their occurrence, purification, properties and mode of action. *Advanced Carbohydrate Chemistry and Biochemistry* **32:** 277-352.

Dekker, R.F.H. 1985. Biodegradation of the hemicelluloses. **In:** Biosynthesis and degradation of wood components. pp. 505-533. Higuchi, T. (ed.). Academic Press, Orlando.

Deshpande, V., Hinge, J. and Rao, M. 1990. Chemical modification of xylanases: evidence for essential tryptophan and cysteine residues at the active site. *Biochimica et Biophysica Acta* **1041:** 172-177.

Doyle, S.A., Fung, S.Y.F. and Koshland, D.E. 2000. Redesigning the substrate specificity of an enzyme: isocitrate dehydrogenase. *Biochemistry* **39:** 14348-14355.

Duffaud, G.D., McCutchen, C.M., Leduc, P., Parker, K.N. and Kelly, R.M. 1997. Purification and characterization of extremely thermostable β-mannanase, β-mannosidase and α-galactosidase from the hyperthermophilic eubacterium *Thermotoga neapolitana* 5068. *Applied and Environmental Microbiology* **63:** 169-177.

Eda, S., Ohnishi, A., and Kato. K. 1976. Xylan isolated from the stalk of *Nicotiana tabacum. Agricultural and Biological Chemistry* **40:** 359-364.

Emerson, R. 1968. Thermophiles. **In**: The fungus - an advanced treatise. pp. 105-128. Answorth, G.C. and Sussman, A.S. (eds.). Academic Press, London.

Eriksson, K.E.L., Blanchette, R.A. and Ander, P. 1990. Microbial and enzymatic degradation of wood and wood components. Springer-Verlag, Berlin **3:** 540-561.

Forst, C.V. 1998. Molecular evolution: a theory approaches experiments. *Journal of Biotechnology* **64:** 101-118.

Fromant, M., Blanquet, S. and Plateau, P. 1995. Direct random mutagenesis of gene-sized DNA fragments using the polymerase chain reaction. *Analytical Biochemistry* **224:** 347-353.

Fujita, S.C., Oshima, T. and Imahori, K. 1976. Purification and properties of D-glyceraldehyde-3-phosphate dehydrogenase from an extreme thermophile, *Thermus thermophilus* strain HB 8. *European Journal of Biochemistry* **64:** 57-68.

Giver, L., Gershenson, A., Freskgard, P.O. and Arnold, F.H. 1998. Directed evolution of a thermostable esterase. *Proceedings of the National Academy of Sciences, USA* **95:** 12809-12813.

Goeddel, D.V. 1990. Systems for heterologous gene expression. *Methods in enzymology* **185:** 3-7.

Gold, L. 1988. Post-transcriptional regulatory mechanisms in *Escherichia coli*. *Annual Reviews in Biochemistry* **57:** 199-233.

Gold, L. 1990. Expression of heterologous proteins in *Escherichia coli*. *Methods in Enzymology* **185:** 11-14.

Gomes, I., Gomes, J., Steiner, W. and Esterbauer, H. 1992. Production of cellulase and xylanase by a wild strain of *Trichoderma viride*. *Applied Microbiology and Biotechnology* **36:** 701-707.

Gomes, J., Gomes, I., Esterbauer, H., Kreiner, W. and Steiner, W. 1989. Production of cellulases by a wild strain of *Gliocladium virens*: optimization of the fermentation medium and partial characterization of the enzymes. *Applied Microbiology and Biotechnology* **31:** 601-608.

Gomes, J., Gomes, I., Kreiner, W., Esterbauer, H., Sinner, M. and Steiner, W. 1993a. Production of a high level of cellulase-free and thermostable xylanase by a wild strain of *Thermomyces lanuginosus* using beechwood xylan. *Journal of Biotechnology* **30:** 283-297.

Gomes, J., Purkarthofer, H., Hayn, M., Kapplmüller, J., Sinner, M. and Steiner, W. 1993b. Production of a high level of cellulase-free xylanase by the thermophilic fungus *Thermomyces lanuginosus* in laboratory and pilot scales using lignocellulosic materials. *Applied Microbiology and Biotechnology* **39:** 700-707.

Gosh, B.L. and Dutta, A.K. 1983. The enzymatic softening and upgrading of lignocellulosic fibres. *Journal of the Textile Institute* **74:** 83-91.

Gruber, K., Klintschar, G., Hayn, M., Schlacher, A., Steiner, W. and Kratky, C. 1998. Thermophilic xylanase from *Thermomyces lanuginosus*: high resolution x-ray structure and modelling studies. *Biochemistry* **37:** 13475-13485.

Hazlewood, G.P. and Gilbert, H.J. 1993. Molecular biology of hemicellulases. **In:** Hemicellulose and hemicellulases. pp. 103-126. Coughlan, M.P. and Hazlewood, G.P. (eds.). Portland Press Research Monograph. Portland Press Ltd., Chapel Hill, N.C.

Henrissat, B. 1991. A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochemical Journal* **280:** 309-316.

Henrissat, B. and Bairoch, A. 1993. New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochemical Journal* **293:** 781-788.

Hirano, N., Haruki, M., Morikawa, M. and Kanaya, S. 2000. Enhancement of the enzymatic activity of ribonuclease H1 from *Thermus thermophilus* HB8 with a suppressor mutation method. *Biochemistry* **39:** 13285-13294.

Hodgson, J. 1993. Expression systems: a user's guide. *Bio/Technology* **11:** 887-893.

Hoq, M.M., Hempel, C. and Deckwer, W. 1994. Cellulase-free xylanase by *Thermomyces lanuginosus* RT9: Effect of agitation, aeration and medium components on production. *Journal of Biotechnology* **37:** 49-58.

Jacob, F. and Monod, J. 1961. Genetic regulatory mechanisms in the synthesis of proteins. *Journal of Molecular Biology* **3:** 318-356.

Jaenicke, R. 1981. Enzymes under extreme physical conditions. *Annual Reviews in Biophysics and Bioengineering* **10:** 1-67.

Jaenicke, R. and Bohm, G. 1998. The stability of proteins in extreme environments. *Current Opinions in Structural Biology* **8:** 738-748.

Janes, L.E., Lowendahl, A.C. and Kazlauskas, R.J. 1999. Quantitative screening of hydrolase libraries using pH indicators: identifying active and enantioselective hydrolases. *European Journal of Chemistry* **43:** 1-11.

Joseleau, J.P., Comtat, J. and Ruel, K. 1992. Chemical structure of xylans and their interaction in the plant cell walls. **In:** Xylans and xylanases. pp. 361-370. Visser, J., Beldman, G., Kusters van Someren, M.A. and Voragen, A.G.J. (eds.). Elsevier, Amsterdam.

Joyet, P., Declerck, N. and Gaillardin, C. 1992. Hyperthermostable variants of a highly thermostable alpha-amylase. *Biotechnology* **10:** 1579-1583.

Keskar, S.S., Srinivasan, M.C. and Deshpande, V. 1989. Chemical modification of xylanase from a thermotolerant *Streptomyces*: evidence for essential tryptophan and cysteine residues at the active site. *Biochemical Journal* **261:** 49-55.

Kleupfel, D., Daigneault, N., Morosoli, R. and Shareck, F. 1992. Purification and characterization of a new xylanase (xylanase C) produced by *Streptomyces lividans* 66. *Applied Microbiology and Biotechnology* **36:** 626-631.

Ko, E.P., Akatsuka, H., Moriyama, H., Shinmiyo, A., Hata, Y., Katsube, Y., Urabe, I. and Okada, H. 1992. Site-directed mutagenesis at aspartate and glutamate residues of xylanase from *Bacillus pumilis*. *Biochemical Journal* **288:** 117-121.

Koponen, R. 1991. Enzyme systems prove their potential. *Pulp and Paper International* **33:** 20-25.

Krahe, M., Antranikian, G. and Märkl, H. 1996. Fermentation of extremophilic microorganisms. *FEMS Microbiological Reviews* **18:** 271-285.

Kuchner, O. and Arnold, F.H. 1997. Directed evolution of enzyme catalysis. *Trends in Biotechnology* **15:** 744-749.

Kulkarni N., Shendye, A. and Rao, M. 1999. Molecular and biotechnological aspects of xylanases. *FEMS Microbiology Reviews* **23:** 411-456.

Kumar, S. and Bansai, M. 1998. Geometrical and sequence characteristics of α-helices in globular proteins. *Biophysical Journal* **75:** 1935-1944.

Kumar, S. and Nussinov, R. 1999. Salt bridge stability in monomeric proteins. *Journal of Molecular Biology* **293:** 1241-1255.

Kumar, S., Tsai, C.J. and Nussinov, R. 2000. Factors enhancing protein thermostability. *Protein Engineering* **13:** 179-191.

Kumar, S., Wolfson, H.J. and Nussinov, R. 2001. Protein flexibility and electrostatic interactions. *IBM Journal of Research and Development* **45:** 499-512.

Lavielle, P., Koljonen, M., Piiroinen, P., Koponen, R., Reid, D. and Frederiksson, R. 1992. Three large-scale uses of xylanases in kraft pulp bleaching. **In:** Proceedings of the 4th International Conference on New Available Techniques and Current Trends, Bologna, pp. 203-215.

Lee, J. 1997. Biological conversion of lignocellulosic biomass to ethanol. *Journal of Biotechnology* **56:** 1-24.

Lee, S.Y. 1996. High cell-density culture of *Escherichia coli*. *Trends in Biotechnology* **14:** 98-105.

Lehmann, M., Pasamontes, L., Lassen, S.F. and Wyss, M. 2000. The consensus approach for the thermostability engineering of proteins. *Biochimica et Biophysica Acta* **1543:** 408-415.

Liebeton, K., Zonta, A., Schimossek, K., Nardin, M., Lang, D., Dijkstra, W., Reetz, M.T. and Jaeger, K.E. 2000. Directed evolution of an enantioselective lipase. *Chemistry and Biology* **7:** 709-718.

Lin. J., Ndlovu, L.M., Singh, S. and Pillay, B. 1999a. Purification and biochemical characteristics of β-D-xylanase from a thermophilic fungus, *Thermomyces lanuginosus*-SSBP. *Biotechnology and Applied Biochemistry* **30:** 73-79.

Lin, Z., Thorsen, T. and Arnold, F.H. 1999b. Functional expression of horseradish peroxidase in *E.coli* by directed evolution. *Biotechnology Progress* **15:** 467-471.

Linko, M., Poutanen, K. and Viikari, L. 1989. New developments in the application of enzymes for biomass processing. **In:** Enzyme systems for lignocellulose degradation. pp. 331-346. Coughlan, M.P. (ed.). Elsevier, London.

Lischnig, T., Purkarthofer, H. and Steiner, W. 1993. Thermostability of endo-β-xylanase from the thermophilic fungus *Thermomyces lanuginosus*. *Biotechnology Letters* **15:** 411-414.

Liu, X., Qu, Y., You, F. and Liu, Y. 2002. Studies on the amino acid residues responsible for the alkali-tolerance of the xylanase by site-directed or random mutagenesis. *Journal of Molecular Catalysis* **18:** 307-313.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, J.R. 1951. Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193:** 265-275.

Lüthi, E., Jasmat, N.B. and Berquist, P.L. 1990. Xylanase from the thermophilic bacterium *Caldocellum saccharolyticum*: over-expression of the gene in *Escherichia coli* and characterization of the gene product. *Applied and Environmental Microbiology* **56:** 2677-2683.

Maat, J., Roza, M., Verbakel, J., Stam, H., Santos da Silva, M.J., Bosse, M., Egmond, M.R., Hagemans, M.L.D., van Gorcom, R.F.M., Hessing, J.G.M., van Der Hondel, C.A.M.J.J. and van Rotterdam, C. 1992. Xylanases and their application in bakery. **In:** Xylans and xylanases. pp. 349-360. Visser, J., Beldman, G., Kusters van Someren, M.A. and Voragen, A.G.J. (eds.). Elsevier, Amsterdam.

MacBeath, G., Kast, P. and Hilvert, D. 1998. Redesigning enzyme topology by directed evolution. *Science* **279:** 1958-1961.

Makrides, S.C. 1996. Strategies for achieving high level expression of genes in *Escherichia coli*. *Microbiological Reviews* **60:** 512-538.

Mandecki, W., Chen, Y.C. and Grihalde, N. 1995. A mathematical model for biopanning (affinity selection) using peptide libraries on filamentous phage. *Journal of Theoretical Biology* **273:** 544-551.

Marrs, B., Delagrave, S. and Murphy, D. 1999. Novel approaches for discovering industrial enzymes. *Current Opinions in Microbiology* **2:** 241-245.

Matsumura, I. and Ellington, A.D. 2001. Mutagenic PCR of protein-coding genes for *in vitro* evolution. **In:** *In vitro* mutagenesis. pp. 261-269. Braman, J. (ed.). Humana Press Inc., Totowa, New Jersey.

Matsumura, M., Signor, G. and Matthews, B.W. 1989. Substantial increase of protein stability by multiple disulphide bonds. *Nature* **342:** 291-293.

Matsuura, T., Miyai, K., Trakulnaleamsai, S., Yomo, T., Shima, Y., Miki, S., Yamamoto, K. and Urabe, I. 1999. Evolutionary molecular engineering by random elongation mutagenesis. *Nature Biotechnology* **17:** 58-61.

Matthews, B.W., Nicholson, H. and Becktel, W.J. 1987. Enhanced protein thermostability from site-directed mutations that decrease the entropy of unfolding. *Proceedings of the National Academy of Sciences, USA* **84:** 6663-6667.

Meiering, E.M., Serrano, L. and Fersht, A.R. 1992. Effect of active site residues in barnase on activity and stability. *Journal of Molecular Biology* **225:** 585-589.

Miao, S.C., Ziser, L., Aebersold, R. and Withers, S.G. 1994. Identification of glutamic acid 78 as the active-site nucleophile in *Bacillus subtilis* xylanase using electrospray tandem mass spectroscopy. *Biochemistry* **33:** 7027-7032.

Miyazaki, K. and Arnold, F.H. 1999. Exploring non-natural evolutionary pathways by saturation mutagenesis: rapid movement of protein function. *Journal of Molecular Evolution* **49:** 716-720.

Montgomery, R., Smith, F. and Srivastava, H.C. 1956. Structure of corn hull hemicellulose. *Journal of American Chemical Society* **78:** 2827-2839.

Nakamura, S., Wakabayashi, K., Nakai, R., Aono, R. and Horikoshi, K. 1993. Production of alkaline xylanase by a newly isolated *Bacillus* sp. strain 41M-1. *World Journal of Microbiology and Biotechnology* **9:** 221-224.

Naki, D., Paech, C., Ganshaw, G. and Schellenberger, V. 1998. Selection of a subtilisin-hyperproducing *Bacillus* in highly structured environments. *Applied Microbiology and Biotechnology* **49:** 290-294.

Ness, J.E., Welch, M., Giver, L., Bueno, M., Cherry, J.R., Borchert, T.V., Stemmer, W.P.C. and Minshull, J. 1999. DNA shuffling of sub-genomic sequences of subtilisin. *Nature Biotechnology* **17:** 883-893.

Okkels, J.S. 1997. Method for preparing polypeptide variants. *Patent:* WO9707205.

Okkels, J.S., Thellersen, M., Petersen, D.A., Svendsen, A., Patkar, S.A. and Borch, K. 1997. *Patent:* WO9707202.

Oku, T., Roy, C., Watson, D.C., Wakarchuk, W., Campbell, R., Yaguchi, M., Jurasek, L. and Paice, M.G. 1993. Amino acid sequence and thermostability of xylanase A from *Schizophyllum commune*. *FEBS Letters* **334:** 296-300.

Price, N.C. and Dwek, R.A. 1979. The kinetics of enzyme-catalyzed reactions. **In:** Principles and problems in physical chemistry for biochemists. 2nd edition. pp. 132-204. Clarendon Press, Great Britain.

Puchart, V., Katapodis, P., Biely, P., Kremnický, L., Christakopoulos, P., Vrsanská, M., Kekos, D., Macris, B.J. and Bhat, M. 1999. Production of xylanases, mannanases and pectinases by the thermophilic fungus *Thermomyces lanuginosus*. *Enzyme and Microbial Biotechnology* **24:** 355-361.

Puls, J. and Poutanen, K. 1989. Mechanisms of enzymic hydrolysis of hemicelluloses (xylans) and procedures for determination of the enzyme activities involved. **In:** Enzyme systems for lignocellulose degradation. pp. 151-165. Coughlan, M.P. (ed.). Elsevier, London.

Purkarthofer, H., Sinner, M. and Steiner, W. 1993a. Cellulase-free xylanase from *Thermomyces lanuginosus*: optimization of production in submerged and solid-state culture. *Enzyme and Microbial Technology* **15:** 677-682.

Purkarthofer, H., Sinner, M. and Steiner, W. 1993b. Effect of shear rate and culture pH on the production of xylanase by *Thermomyces lanuginosus. Biotechnology Letters* **15:** 405-410.

Royer, J.C. and Nakas, J.P. 1989. Xylanase production by *Trichoderma longibrachiatum. Enzyme and Microbial Technology* **11:** 405-410.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular cloning: a laboratory manual. 2nd edition. Cold Spring Harbor Laboratory, Cold Spring, New York.

Sapag, A., Wouters, J., Lambert, C., de Ioannes, P., Eyzaguirre, J. and Depiereux, E. 2002. The endoxylanases from family 11: computer analysis of protein sequences reveals important structural and phylogenetic relationships. *Journal of Biotechnology* **95:** 109-131.

Schellenberger, V. 1998. Directed evolution can improve subtilisin-secreting *Bacillus* strains. *ASM News* **64:** 634-638.

Schlacher, A., Holzmann, K., Hayn, M., Steiner, W. and Schwab, H. 1996. Cloning and characterization of the gene for the thermostable xylanase XynA from *Thermomyces lanuginosus. Journal of Biotechnology* **49:** 211-218.

Schmidt-Dannert, C. and Arnold, F.H. 1999. Directed evolution of industrial enzymes. *Trends in Biotechnology* **17:** 135-136.

Schoichet, B.K., Baase, W.A., Kuroki, R. and Matthews, B.W. 1995. A relationship between protein stability and protein function. *Proceedings of the National Academy of Sciences, USA* **92:** 452-456.

Schweder, T., Lin, H.Y., Jürgen, B., Breitenstein, A., Riemschneider, S., Khalameyzer, V., Gupta, A., Büttner, K. and Neubauer, P. 2002. Role of general stress response during strong over-expression of a heterologous gene in *Escherichia coli. Applied Microbiology and Biotechnology* **58:** 330-337.

Shafikani, S., Siegel, R.A., Ferrari, E. and Schellenberger, V. 1997. Generation of large libraries of random mutants in *Bacillus subtilis* by PCR-based plasmid multimerization. *Biotechniques* **23:** 304-310.

Shibuya, H., Kaneko, S. and Hayashi, K. 2000. Enhancement of the thermostability and hydrolytic activity of xylanase by random gene shuffling. *Biochemical Journal* **349:** 651-656.

Singh, S., Pillay,B., Dilsook, V. and Prior, B.A. 2000a. Production and properties of hemicellulases by a *Thermomyces lanuginosus* strain. *Journal of Applied Microbiology* **88:** 1-9.

Singh, S., Pillay,B., and Prior, B.A. 2000b. Thermal stability of β-xylanase produced by different *Thermomyces lanuginosus* strains. *Enzyme and Microbial Technology* **26:** 502-508.

Singh, S., Reddy, P., Haarhof, J., Biely, P., Janse, B., Pillay, B., Pillay, D. and Prior, B.A. 2000c. Relatedness of *Thermomyces lanuginosus* strains producing a thermostable xylanase. *Journal of Biotechnology* **81:** 119-128.

Singleton, R. and Amelunxen, R.E. 1973. Proteins from thermophilic microorganisms. *Bacteriological Reviews* **37:** 320-342.

Song, J.K. and Rhee, J.S. 2000. Simultaneous enhancement of thermostability and catalytic activity of phospholipase A_1 by evolutionary molecular engineering. *Applied and Environmental Microbiology* **66:** 890-894.

Spee, J.H., de Vos, W.M. and Kuipers, O.P. 1993. Efficient random mutagenesis method with adjustable mutation frequency by use of PCR and ITP. *Nucleic Acids Research* **21:** 777-778.

Spiller, B., Gershenson, A., Arnold, F.H., and Stevens, R.C. 1999. A structural view of evolutionary divergence. *Proceedings of the National Academy of Sciences, USA* **96:** 12305-12310.

Steiner, W., Lafferty, R.M., Gomes, I. and Esterbauer, H. 1987. Studies on a wild strain of *Schizophyllum commune*: cellulase and xylanase production and formation of the extracellular schizophyllan. *Biotechnology and Bioengineering* **30:** 169-178.

Stemmer, W.P.C. 1994a. DNA shuffling by random fragmentation and reassembly: *in vitro* recombination for molecular evolution. *Proceedings of the National Academy of Sciences, USA* **91:** 10747-10751.

Stemmer, W.P.C. 1994b. Rapid evolution of a protein *in vitro* by DNA shuffling. *Nature* **370:** 389-391.

Sunna, A., and Antranikian, G. 1997. Xylanolytic enzymes from fungi and bacteria. *Critical Reviews in Biotechnology* **17:** 39-67.

Tatu, U., Murthy, S.K. and Vithayathil, P.J. 1990. Role of a disulphide cross-link in the conformational stability of a thermostable xylanase. *Journal of Protein Chemistry* **9:** 641-646.

Thomson, J.A. 1993. Molecular biology of xylan degradation. *FEMS Microbiology Reviews* **104:** 65-82.

Tobin, M.B., Gustafsson, C. and Huisman, G.W. 2000. Directed evolution: the 'rational' basis for 'irrational' design. *Current Opinions in Structural Biology* **10:** 421-427.

Tolan, J.S. and Canovas, R.V. 1992. The use of enzymes to decrease the chlorine requirements in pulp bleaching. *Pulp and Paper - Canada* **93:** 39-42.

Tolan, J.S., Olson, D. and Dines, R.E. 1996. Survey of mill usage of xylanases. **In:** Enzymes for pulp and paper processing. pp. 25-35. Jeffries, T.W. and Viikari, L. (eds.). American Chemical Society, Washington, D.C.

Törrönen, A., Harrki, A. and Rouvinen, J. 1994. Three-dimensional structure of endo-1,4-beta-xylanase II from *Trichoderma reesei*: two conformational states in the active site. *EMBO J* **13:** 2493-2501.

Tsujibo, H., Sakamoto, T., Nishino, N. and Hasegawa, T. 1990. Purification and properties of three types of xylanases produced by an alkalophilic actinomycete. *Journal of Applied Bacteriology* **69:** 398-405.

Turner, J.C., Skerker, P.S., Burns, B.J., Howard, J.C., Alonso, M.A.and Andres, J.L. 1992. Bleaching with enzymes instead of chlorine-mill trials. *Tappi Journal* **75:** 83-89.

Van den Berg, B., Vriend, G., Veltman, O.R., Venema, G. and Eijsink, V.G. 1998. Engineering an enzyme to resist boiling. *Proceedings of the National Academy of Sciences, USA* **95:** 2056-2060.

Van Paridon, P.A., Boonman, J.C.P., Selten, G.C.M., Geerse, C., Barug, D., de Bot, P.H.M. and Hemke, G. 1992. The application of fungal endoxylanase in poultry diets. **In:** Xylans and xylanases. pp. 371-378. Visser, J., Beldman, G., Kusters van Someren, M.A. and Voragen, A.G.J. (eds.). Elsevier, Amsterdam.

Vetriani, C., Maeder, D.L., Tolliday, N., Yip, K.S., Stillman, T.J., Britton, K.L., Rice, D.W., Klump, H.H. and Robb, F.T. 1998. Protein thermostability at above 100^0C: a key role for ionic interactions. *Proceedings of the National Academy of Sciences, USA* **95:** 12300-12305.

Viikari, L., Kantelinen, A., Sunquist, J. and Linko, M. 1994. Xylanases in bleaching: from an idea to the industry. *FEMS Microbiological Reviews* **13:** 335-350.

Viikari, L., Ranua, M., Kantilinen, A., Linko, M. and Sundquist, J. 1986. Bleaching with enzymes. **In:** Biotechnology in the Pulp and Paper Industry. pp. 67-69. Stockholm.

Viikari, L., Tenkanen, M., Buchert, J., Rättö, M., Biely, M., Siika-aho, M. and Linko, M. 1993. Hemicellulases for industrial applications. **In:** Bioconversion of forest and agricultural plant residues. pp. 131-182. Saddler, J.N. (ed.). CAB International, Oxford.

Voigt, C.A., Kaufmann, S. and Wong, Z.G. 2001. Rational evolutionary design: the theory of *in vitro* protein evolution. *Advances in Protein Chemistry* **55:** 79-160.

Wakarchuk, W.W., Campbell, R.L., Sung, W.L., Davoodi, J. and Yaguchi, M. 1994a. Mutational and crystallographic analyses of the active-site residues of the *Bacillus circulans* xylanase. *Protein Science* **3:** 467-475.

Wakarchuk, W.W., Sung, W.L., Campbell, R.L., Cunningham, A., Watson, D.C. and Yaguchi, M. 1994b. Thermostabilization of the *Bacillus circulans* xylanase by the introduction of disulphide bonds. *Protein Engineering* **7:** 1379-1386.

Whistler, R.L. and Richards, E.L. 1970. Hemicelluloses. **In:** The carbohydrates – chemistry and biochemistry. pp. 447-449. Pigman, W. and Horton, D. (eds.). Academic Press, New York.

Wong, K.K.Y. and Saddler, J.N. 1993. Applications of hemicellulases in the food, feed and pulp and paper industries. **In:** Hemicellulose and hemicellulases. pp. 127-143. Coughlan, M.P. and Hazlewood, G.P. (eds.). Portland Press, London.

Wong, K.K.Y., Tan, L.U.L. and Saddler, J.N. 1988. Multiplicity of β-1,4-xylanases in microorganisms: functions and applications. *Microbiological Reviews* **52:** 305-317.

Woodward, J. 1984. Xylanases: functions, properties and applications. *Topics in Enzyme and Fermentation Biotechnology* **8:** 9-30.

Yarranton, G.T. 1992. Inducible vectors for expression in mammalian cells. *Current Opinion in Biotechnology* **3:** 506-511.

Yu, E.K.C., Tan, L.U.L., Mana, K.H., Chan, L.D. and Saddler, N.J. 1987. Production of a thermostable xylanase by a thermophilic fungus, *Thermoascus aurantiacus*. *Enzyme and Microbial Technology* **9:** 16-24.

Zaccolo, M. and Gherardi, E. 1999. The effect of high frequency random mutagenesis on *in vitro* protein evolution: a study on TEM-1 β-lactamase. *Journal of Molecular Biology* **285:** 775-783.

Zaks, A. and Klibanov, A.M. 1982. Enzymatic catalysis in organic media at 100^{0}C. *Science* **224:** 1249-1251.

Zamost, B.L., Nielsen, H.K. and Starnes, R.L. 1991. Thermostable enzymes for industrial applications. *Journal of Industrial Microbiology* **8:** 71-82.

Zhang, J.H., Dawes, G. and Stemmer, W.P.C. 1997. Directed evolution of a fucosidase from a galactosidase by DNA shuffling and screening. *Proceedings of the National Academy of Sciences, USA* **94:** 4504-4509.

Zhao, H. and Arnold, F.H. 1999. Directed evolution converts subtilisin E into a functional equivalent of thermitase. *Protein Engineering* **12:** 47-53.

Zhao, H., Chockalingum, K. and Chen, Z. 2002. Directed evolution of enzymes and pathways for industrial biocatalysis. *Current Opinion in Biotechnology* **13:** 104-110.

APPENDICES

Appendix I: Induction of xylanase expression

A: Effect of different concentrations of IPTG on recombinant xylanase production

Time (h)	Activity (nkat/μg total protein)													
	E. coli X										*E. coli* B			
	no IPTG		0.1 mM IPTG		0.25 mM IPTG		0.5 mM IPTG		1 mM IPTG		no IPTG		1 mM IPTG	
0	20.17	22.09	68.20	72.48	32.57	40.01	44.87	53.91	112.36	117.04	0.03	0.05	0.039	0.041
1	47.80	59.26	118.96	121.04	79.64	84.80	101.19	112.17	260.39	277.21	0.012	0.048	0.036	0.044
2	68.44	89.08	171.11	221.17	109.55	120.05	361.28	430.62	292.58	306.98	0.015	0.045	0.025	0.035
3	93.67	98.23	227.16	261.12	113.36	134.98	425.36	473.04	348.22	372.80	0.028	0.032	0.017	0.023
4	141.18	148.94	575.11	606.21	172.93	216.65	457.63	467.95	479.22	488.66	0.025	0.035	0.029	0.031
5	349.66	367.28	733.95	742.89	306.91	324.13	480.15	489.91	492.35	498.57	0.015	0.065	0.013	0.027
24	721.05	735.61	1407.23	1467.01	337.41	362.77	516.73	604.01	476.29	527.31	0.017	0.063	0.026	0.034
48	1032.90	1182.50	2685.99	2908.43	518.92	563.18	666.58	693.06	459.32	547.10	0.026	0.054	0.028	0.052
72	1049.90	1107.70	2055.33	2178.95	502.38	543.94	659.28	692.54	461.98	530.38	0.035	0.045	0.033	0.047

B: pH of the culture medium of *E. coli* X induced with 0.1 mM IPTG monitored over 72 hrs

Time (hrs)	pH	
0	5.25	5.26
1	5.22	5.24
2	5.20	5.18
3	5.19	5.21
4	5.08	5.02
5	5.00	5.01
24	4.87	4.89
48	4.66	4.64
72	3.92	3.93

Appendix II: Thermostability screening

A: Effect of temperature (80^0C) on the activity and thermostability of wild type XynA and first generation XynA variants.

Mutant	Time (min)	Activity (nkat/μg total protein)		Mutant	Time (min)	Activity (nkat/μg total protein)		Mutant	Time (min)	Activity (nkat/μg total protein)		Mutant	Time (min)	Activity (nkat/μg total protein)	
Wild	0	650.12	663.52	1B3	0	1592.74	1669.94	1B8	0	476.18	496.24	1B13	0	769.35	807.33
type	10	92.38	97.76		10	246.91	257.85		10	469.58	478.26		10	85.66	92.90
XynA	20	87.68	91.18		20	241.53	245.59		20	385.69	397.17		20	85.15	88.69
	30	84.00	94.00		30	210.36	215.04		30	308.92	311.20		30	82.41	83.83
	40	75.00	85.00		40	196.12	198.52		40	192.55	194.75		40	81.74	82.64
1A1	0	115.75	116.27	1B4	0	194.26	198.38	1B9	0	1084.63	1113.91	1B14	0	1209.47	1132.63
	10	6.95	7.97		10	87.65	93.35		10	86.65	93.53		10	169.33	270.43
	20	6.99	7.93		20	89.35	90.81		20	84.48	93.00		20	152.61	191.93
	30	6.81	6.83		30	88.21	91.87		30	82.59	84.95		30	132.70	147.12
	40	6.25	6.55		40	77.42	80.36		40	72.49	74.39		40	113.65	130.79
1A2	0	1022.63	1060.71	1B5	0	804.57	833.29	1B10	0	647.52	657.72	1D1	0	1396.72	1500.72
	10	132.73	136.99		10	759.44	793.86		10	49.66	52.04		10	860.13	892.85
	20	128.67	141.97		20	744.82	749.10		20	45.86	51.94		20	826.91	845.61
	30	121.75	134.31		30	620.17	632.11		30	43.22	46.88		30	180.33	187.39
	40	118.56	121.68		40	451.33	470.29		40	41.15	44.85		40	15.07	17.07
1B1	0	1355.26	1410.26	1B6	0	502.11	674.89	1B11	0	666.67	707.19	1D2	0	3287.57	3839.71
	10	460.12	484.32		10	267.26	271.68		10	655.10	674.72		10	1998.62	2151.48
	20	360.25	373.63		20	255.33	260.51		20	589.26	618.72		20	1619.23	1801.71
	30	361.23	371.55		30	242.13	254.07		30	350.14	361.84		30	1567.34	1673.84
	40	323.66	330.34		40	237.34	249.96		40	211.17	215.49		40	1100.25	1487.05
1B2	0	275.36	302.42	1B7	0	966.25	992.63	1B12	0	736.92	760.06	1D3	0	2051.44	2284.70
	10	113.88	115.32		10	840.16	856.44		10	61.32	74.36		10	1099.83	1452.55
	20	111.43	113.01		20	792.63	799.07		20	57.43	76.77		20	187.64	191.58
	30	95.28	100.04		30	363.55	365.23		30	56.28	76.40		30	142.57	175.63
	40	74.61	78.05		40	201.54	205.00		40	55.23	67.69		40	134.58	142.14

B: Effect of temperature (80^{0}C) on the activity and thermostability of wild-type XynA and second generation mutants.

Mutant	Time (min)	Activity (nkat/μg total protein)		Mutant	Time (min)	Activity (nkat/μg total protein)		Mutant	Time (min)	Activity (nkat/μg total protein)		Mutant	Time (min)	Activity (nkat/μg total protein)	
2B5B-1	0	290.61	305.93	2B5B-7	0	1800.72	2178.72	2B7-2	0	550.32	647.76	2B7-8	0	330.41	334.39
	10	121.47	126.15		10	588.77	628.95		10	542.36	644.94		10	315.24	316.88
	20	113.67	115.07		20	260.33	298.85		20	20.35	22.83		20	280.36	282.88
	30	111.25	116.47		30	190.67	216.93		30	19.89	22.89		30	246.36	251.62
	40	108.65	112.11		40	90.62	98.08		40	15.69	17.49		40	230.51	241.53
2B5B-2	0	311.55	316.65	2B5B-8	0	182.23	191.81	2B7-3	0	240.15	254.55	2B7-9	0	250.63	266.37
	10	23.20	27.80		10	101.47	107.09		10	76.24	78.24		10	59.62	62.08
	20	20.86	21.86		20	62.35	97.43		20	76.01	80.41		20	50.05	53.81
	30	17.45	19.19		30	72.12	76.94		30	71.44	73.96		30	49.38	51.38
	40	15.14	17.70		40	71.24	73.14		40	70.56	74.20		40	48.77	49.51
2B5B-3	0	210.34	220.48	2B5B-9	0	347.92	372.04	2B7-4	0	30.23	32.29	2B7-10	0	3396.25	3464.53
	10	201.33	209.87		10	111.32	115.48		10	14.53	16.81		10	3000.49	3079.31
	20	38.66	43.26		20	99.65	105.87		20	10.74	11.86		20	2871.64	2958.56
	30	24.53	28.91		30	97.88	102.56		30	10.52	11.08		30	2800.39	2908.69
	40	23.99	30.47		40	96.43	102.85		40	10.10	11.24		40	2699.83	2829.21
2B5B-4	0	260.39	271.83	2B5C-1	0	230.16	238.00	2B7-5	0	20.45	22.07	2B7-11	0	2700.56	2775.48
	10	46.99	50.83		10	25.02	28.56		10	3.99	4.03		10	2685.67	2737.69
	20	30.87	33.55		20	14.92	16.12		20	3.65	3.95		20	364.23	376.47
	30	30.04	33.92		30	10.58	12.16		30	3.42	3.62		30	209.88	210.78
	40	29.13	29.71		40	9.68	10.82		40	2.78	3.08		40	190.32	194.56
2B5B-5	0	166.32	168.78	2B5C-2	0	268.22	272.80	2B7-6	0	427.59	449.33	2B7-12	0	142.12	146.92
	10	21.05	25.41		10	77.31	79.47		10	430.02	439.22		10	93.12	95.72
	20	9.61	11.25		20	73.20	76.70		20	347.36	423.50		20	91.17	93.39
	30	9.28	11.98		30	72.45	76.51		30	339.62	409.20		30	85.55	88.21
	40	8.71	9.35		40	72.35	77.81		40	326.59	405.09		40	79.62	80.64
2B5B-6	0	328.56	332.86	2B7-1	0	24.23	27.57	2B7-7	0	250.14	263.70	2B7-13	0	359.86	379.10
	10	32.16	34.20		10	23.87	27.77		10	51.25	58.85		10	125.67	127.67
	20	19.25	21.97		20	17.22	20.20		20	48.26	50.74		20	105.31	109.63
	30	11.96	12.68		30	12.62	14.80		30	48.00	54.40		30	81.32	100.20
	40	9.03	10.01		40	10.98	11.54		40	41.53	47.25		40	80.99	102.85

Mutant	Time (min)	Activity (nkat/μg total protein)		Mutant	Time (min)	Activity (nkat/μg total protein)		Mutant	Time (min)	Activity (nkat/μg total protein)		Mutant	Time (min)	Activity (nkat/μg total protein)	
2B7-14	0	313.60	314.96	2B7-20	0	11.13	11.87	2B7-26	0	38.13	40.75	2B7-32	0	62.54	65.14
	10	84.12	86.74		10	4.75	5.13		10	8.96	9.18		10	21.53	22.73
	20	75.45	77.07		20	3.67	4.05		20	6.63	7.15		20	11.00	11.42
	30	62.96	64.90		30	3.74	3.84		30	6.30	6.74		30	9.86	10.56
	40	63.54	64.66		40	3.72	4.18		40	5.63	6.17		40	8.35	8.85
2B7-15	0	211.17	213.83	2B7-21	0	92.16	95.50	2B7-27	0	62.95	66.25	2B7-33	0	16.12	17.26
	10	95.12	96.26		10	134.62	135.90		10	11.86	12.38		10	3.73	4.23
	20	95.11	96.19		20	132.46	134.44		20	11.83	12.41		20	2.10	2.54
	30	82.00	83.76		30	81.15	83.35		30	9.53	10.03		30	2.38	2.42
	40	81.23	83.93		40	76.12	79.00		40	8.60	9.28		40	2.20	2.34
2B7-16	0	178.22	180.34	2B7-22	0	20.31	21.05	2B7-28	0	34.36	36.76	2B7-34	0	19.64	21.40
	10	114.67	116.01		10	4.60	4.80		10	14.32	15.36		10	4.23	4.77
	20	111.33	113.57		20	4.59	4.79		20	8.26	9.22		20	3.42	3.66
	30	104.28	107.70		30	3.75	4.75		30	6.53	9.03		30	2.95	3.17
	40	97.63	102.25		40	3.70	4.18		40	5.81	8.13		40	2.83	2.99
2B7-17	0	240.19	245.11	2B7-23	0	31.00	31.38	2B7-29	0	40.32	42.90	2B7-35	0	6.83	8.39
	10	118.64	121.06		10	6.70	6.76		10	10.25	10.75		10	1.69	1.89
	20	100.25	103.25		20	6.34	6.66		20	8.53	10.41		20	1.23	1.69
	30	98.63	102.67		30	5.52	5.68		30	7.63	10.19		30	1.17	1.49
	40	98.55	104.65		40	5.26	5.42		40	7.59	8.61		40	1.00	1.58
2B7-18	0	188.67	190.37	2B7-24	0	24.12	29.84	2B7-30	0	82.12	85.06	2B7-36	0	20.67	21.63
	10	84.12	87.48		10	15.93	17.47		10	30.72	32.80		10	10.25	11.17
	20	77.32	80.10		20	9.32	10.32		20	26.13	29.83		20	10.20	11.08
	30	68.53	70.57		30	7.22	7.26		30	26.01	29.13		30	8.00	8.46
	40	60.31	63.55		40	7.22	7.22		40	21.87	23.17		40	7.65	8.49
2B7-19	0	457.98	469.86	2B7-25	0	47.26	50.00	2B7-31	0	76.39	78.17	2B7-37	0	174.38	179.22
	10	125.34	126.70		10	13.00	14.04		10	46.31	49.33		10	101.12	105.26
	20	80.33	81.75		20	11.12	11.56		20	45.62	47.16		20	63.28	66.62
	30	79.96	81.20		30	8.98	9.14		30	37.46	39.22		30	62.95	64.87
	40	75.35	78.13		40	7.03	8.91		40	35.68	36.70		40	50.44	54.44

Mutant	Time (min)	Activity (nkat/μg total protein)		Mutant	Time (min)	Activity (nkat/μg total protein)		Mutant	Time (min)	Activity (nkat/μg total protein)		Mutant	Time (min)	Activity (nkat/μg total protein)	
2B7-38	0	22.13	25.99	2B11-6	0	12.02	12.84	2B11-12	0	8.07	8.97	2B11-18	0	34.51	38.81
	10	5.36	5.92		10	3.26	3.86		10	1.45	1.75		10	8.25	8.93
	20	5.28	5.80		20	3.00	3.58		20	1.40	1.54		20	6.52	6.96
	30	4.76	5.14		30	2.63	2.85		30	1.26	1.34		30	5.64	5.82
	40	4.98	5.08		40	1.35	2.37		40	1.25	1.39		40	5.46	5.86
2B11-1	0	219.45	222.31	2B11-7	0	22.43	33.25	2B11-13	0	82.15	94.23	2B11-19	0	26.58	29.02
	10	116.98	118.74		10	5.53	5.81		10	40.68	42.78		10	7.64	8.54
	20	5.36	7.26		20	5.21	5.89		20	39.80	42.16		20	7.61	8.07
	30	5.04	6.00		30	5.19	6.15		30	39.65	40.039		30	7.21	7.57
	40	4.95	5.93		40	5.15	5.45		40	35.22	37.16		40	7.19	7.65
2B11-2	0	79.32	81.06	2B11-8	0	19.58	22.58	2B11-14	0	42.17	44.63	2B11-20	0	15.00	15.26
	10	20.53	22.41		10	5.12	5.54		10	7.50	7.74		10	4.21	4.69
	20	14.74	15.90		20	5.02	5.60		20	7.02	7.18		20	3.49	4.05
	30	9.63	10.55		30	4.76	4.96		30	6.32	6.38		30	3.39	3.83
	40	7.28	7.62		40	3.88	4.30		40	6.25	6.57		40	2.51	2.83
2B11-3	0	48.52	50.24	2B11-9	0	100.23	118.29	2B11-15	0	15.32	19.24	2B11-21	0	23.35	34.07
	10	39.62	41.80		10	69.32	72.34		10	3.50	4.00		10	5.41	5.49
	20	32.10	34.08		20	57.22	60.40		20	3.23	3.45		20	5.31	5.33
	30	18.76	20.76		30	50.01	52.43		30	3.00	3.14		30	5.28	5.29
	40	16.35	18.29		40	44.93	47.95		40	2.87	3.21		40	4.30	4.44
2B11-4	0	50.64	53.02	2B11-10	0	16.19	18.09	2B11-16	0	618.35	623.27	2B11-22	0	16.57	19.97
	10	14.27	16.01		10	3.29	3.89		10	600.15	606.19		10	3.82	3.98
	20	14.02	15.90		20	2.93	3.29		20	577.42	588.72		20	3.80	3.86
	30	10.32	13.62		30	2.49	2.67		30	486.59	511.23		30	3.51	3.73
	40	9.32	12.34		40	1.19	1.21		40	440.18	446.02		40	3.25	3.49
2B11-5	0	110.67	115.99	2B11-11	0	160.13	176.11	2B11-17	0	37.32	41.64	2B11-23	0	15.10	18.70
	10	30.21	31.99		10	90.25	97.91		10	11.49	12.67		10	3.67	4.09
	20	21.34	24.00		20	51.14	53.98		20	8.28	8.36		20	3.64	4.10
	30	20.31	25.11		30	45.06	47.64		30	7.73	7.95		30	3.52	4.00
	40	16.25	19.21		40	40.12	42.52		40	7.67	7.99		40	3.36	3.56

Mutant	Time (min)	Activity (nkat/μg total protein)		Mutant	Time (min)	Activity (nkat/μg total protein)		Mutant	Time (min)	Activity (nkat/μg total protein)		Mutant	Time (min)	Activity (nkat/μg total protein)	
2B11-24	0	80.02	80.66	2B11-30	0	289.66	326.22	2B11-36	0	9.47	9.59	2B11-42	0	102.59	105.99
	10	78.46	79.74		10	193.52	196.92		10	4.58	4.72		10	57.65	59.97
	20	75.80	76.58		20	171.25	178.74		20	4.30	4.48		20	42.57	45.71
	30	74.19	77.61		30	129.65	131.77		30	4.29	4.37		30	40.05	42.81
	40	62.29	64.47		40	126.93	127.89		40	3.80	3.98		40	28.61	30.19
2B11-25	0	40.05	44.15	2B11-31	0	23.39	27.85	2B11-37	0	34.51	37.99	2B11-43	0	18.36	21.10
	10	7.14	7.44		10	6.99	7.03		10	10.61	10.83		10	10.69	11.55
	20	6.03	6.21		20	6.13	6.25		20	10.26	10.60		20	3.86	4.00
	30	5.97	5.99		30	4.78	7.12		30	10.23	10.63		30	3.72	4.06
	40	5.80	5.90		40	4.83	6.97		40	8.95	10.41		40	2.93	3.55
2B11-26	0	31.42	35.16	2B11-32	0	26.35	27.69	2B11-38	0	25.64	29.74	2B11-44	0	180.23	193.19
	10	9.28	9.60		10	11.85	12.85		10	9.91	12.05		10	73.05	78.47
	20	8.10	8.40		20	11.46	11.48		20	8.76	8.94		20	72.12	78.06
	30	6.48	6.62		30	11.33	11.51		30	8.42	8.60		30	61.32	77.78
	40	6.45	6.47		40	10.59	11.69		40	7.59	8.03		40	59.42	79.90
2B11-27	0	20.08	22.84	2B11-33	0	26.28	28.50	2B11-39	0	61.02	65.42	2B11-45	0	19.25	21.61
	10	10.60	10.96		10	10.24	12.44		10	26.39	28.01		10	4.51	4.75
	20	8.94	10.79		20	6.43	8.71		20	24.87	27.01		20	4.08	4.34
	30	8.63	9.30		30	6.40	8.48		30	24.17	26.75		30	3.76	4.40
	40	8.52	8.68		40	6.12	8.14		40	22.81	24.77		40	3.50	4.12
2B11-28	0	23.38	27.60	2B11-34	0	172.89	174.45	2B11-40	0	71.28	83.24	2B11-46	0	15.26	20.00
	10	10.28	10.76		10	51.16	53.68		10	7.30	7.54		10	4.60	4.66
	20	9.23	9.49		20	43.67	45.63		20	7.29	7.33		20	4.52	4.62
	30	8.81	9.13		30	40.95	42.87		30	6.90	7.16		30	4.47	4.53
	40	8.47	8.81		40	39.62	42.68		40	6.85	7.01		40	4.37	4.57
2B11-29	0	17.84	19.24	2B11-35	0	18.54	22.64	2B11-41	0	70.02	73.68	2B11-47	0	161.44	168.24
	10	5.16	5.62		10	6.47	6.79		10	12.12	12.74		10	57.29	60.35
	20	4.76	5.48		20	5.38	6.70		20	10.26	11.74		20	50.00	52.22
	30	4.48	5.57		30	5.33	5.47		30	10.13	10.71		30	47.82	49.90
	40	4.46	5.52		40	5.16	5.60		40	8.74	9.48		40	46.33	49.55

Mutant	Time (min)	Activity (nkat/μg total protein)		Mutant	Time (min)	Activity (nkat/μg total protein)		Mutant	Time (min)	Activity (nkat/μg total protein)		Mutant	Time (min)	Activity (nkat/μg total protein)	
2B11-48	0	200.67	203.39	2D2-5	0	271.54	274.80	2D2-11	0	303.42	350.98	2D2-17	0	644.25	743.41
	10	96.25	99.29		10	19.75	21.07		10	9.25	9.75		10	250.16	318.10
	20	89.10	91.84		20	19.03	19.35		20	8.10	9.04		20	132.64	140.38
	30	88.78	89.40		30	15.74	17.00		30	8.02	9.04		30	48.75	49.77
	40	82.24	84.02		40	12.65	14.89		40	5.13	8.21		40	45.18	48.28
2B11-49	0	44.36	46.22	2D2-6	0	730.16	744.60	2D2-12	0	261.31	296.83	2D2-18	0	24.19	28.63
	10	17.96	18.12		10	573.26	592.23		10	70.45	73.33		10	9.62	12.16
	20	17.20	17.68		20	512.43	516.15		20	19.28	22.00		20	6.35	6.83
	30	16.73	16.91		30	355.28	365.58		30	13.92	15.56		30	6.24	6.28
	40	15.90	15.96		40	199.35	201.85		40	7.41	8.25		40	5.68	6.40
2D2-1	0	147.35	149.91	2D2-7	0	349.12	353.88	2D2-13	0	348.77	366.47	2D2-19	0	20.54	23.44
	10	35.76	37.24		10	203.21	210.47		10	77.63	81.27		10	6.88	7.46
	20	25.33	26.87		20	180.53	182.05		20	40.06	45.56		20	6.80	7.00
	30	18.07	20.37		30	178.65	183.35		30	36.95	38.81		30	6.50	7.02
	40	15.99	16.69		40	126.37	130.55		40	27.34	31.18		40	5.73	6.19
2D2-2	0	126.35	131.09	2D2-8	0	350.62	358.75	2D2-14	0	67.58	72.34	2D2-20	0	447.32	487.44
	10	43.67	46.21		10	19.35	21.97		10	56.23	59.55		10	303.26	306.64
	20	21.61	24.33		20	15.51	15.77		20	45.26	48.48		20	300.62	303.18
	30	14.56	15.86		30	15.30	15.34		30	38.21	41.29		30	295.67	306.65
	40	9.65	9.97		40	12.49	13.65		40	25.33	32.09		40	273.55	276.55
2D2-3	0	274.31	295.53	2D2-9	0	179.35	198.05	2D2-15	0	37.55	43.83	2D2-21	0	27.96	28.86
	10	17.29	17.61		10	9.67	10.67		10	24.38	25.70		10	6.88	7.48
	20	16.60	17.40		20	7.81	8.03		20	20.14	23.44		20	2.41	2.53
	30	15.35	16.05		30	7.30	7.64		30	1.12	2.60		30	2.40	2.48
	40	15.22	16.04		40	7.20	7.38		40	0.14	0.18		40	2.24	2.36
2D2-4	0	371.64	394.44	2D2-10	0	63.87	64.57	2D2-16	0	1500.44	2013.84	2D2-22	0	18.87	21.27
	10	53.62	55.86		10	16.80	18.80		10	113.62	121.76		10	3.65	4.13
	20	39.21	42.59		20	10.19	10.41		20	79.63	83.85		20	2.79	2.93
	30	29.64	31.20		30	8.00	8.16		30	50.17	54.41		30	2.60	2.70
	40	23.81	24.77		40	6.98	7.32		40	42.10	49.32		40	2.15	1.99

Mutant	Time (min)	Activity (nkat/μg total protein)		Mutant	Time (min)	Activity (nkat/μg total protein)		Mutant	Time (min)	Activity (nkat/μg total protein)		Mutant	Time (min)	Activity (nkat/μg total protein)	
2D2-23	0	21.37	27.19	2D2-29	0	23.64	27.86	2D2-35	0	54.81	56.45	2D2-41	0	22.35	25.91
	10	7.00	7.74		10	19.04	19.62		10	13.24	13.98		10	5.52	5.94
	20	1.76	1.60		20	12.04	12.50		20	10.20	10.38		20	5.14	5.42
	30	1.60	1.66		30	10.42	12.08		30	10.13	10.17		30	4.92	5.20
	40	1.39	1.53		40	10.64	11.22		40	9.86	10.14		40	4.87	5.09
2D2-24	0	137.65	155.33	2D2-30	0	19.84	22.04	2D2-36	0	36.94	38.96	2D2-42	0	26.49	28.53
	10	38.56	39.98		10	5.32	5.56		10	4.03	4.19		10	5.87	6.15
	20	30.25	31.81		20	5.10	5.20		20	3.63	3.95		20	5.14	5.50
	30	25.57	27.31		30	4.76	5.16		30	3.46	3.58		30	5.11	5.17
	40	22.17	24.61		40	4.56	5.08		40	2.71	2.95		40	4.85	5.13
2D2-25	0	80.14	86.92	2D2-31	0	28.35	31.83	2D2-37	0	39.21	41.79	2D2-43	0	779.29	787.63
	10	40.08	47.38		10	6.00	6.32		10	5.58	6.54		10	217.54	244.62
	20	14.50	14.58		20	5.82	6.00		20	4.50	4.74		20	180.14	196.24
	30	14.20	14.28		30	5.71	5.81		30	4.30	4.40		30	168.94	176.26
	40	11.93	13.95		40	5.47	5.61		40	4.23	4.31		40	134.16	140.88
2D2-26	0	50.23	53.45	2D2-32	0	237.94	250.72	2D2-38	0	26.38	28.78	2D2-44	0	18.24	19.78
	10	32.21	47.59		10	66.20	66.40		10	5.51	5.87		10	9.50	9.82
	20	21.53	24.59		20	66.10	66.36		20	5.32	5.50		20	9.18	9.28
	30	19.14	21.24		30	65.61	65.93		30	4.87	5.37		30	8.94	9.26
	40	16.22	17.096		40	62.59	65.21		40	3.12	3.92		40	8.51	8.89
2D2-27	0	39.61	41.09	2D2-33	0	219.74	249.38	2D2-39	0	17.62	21.00	2D2-45	0	91.10	103.82
	10	10.24	11.48		10	42.85	43.85		10	7.98	9.32		10	47.91	49.69
	20	10.10	10.40		20	40.74	41.00		20	5.82	6.10		20	45.67	46.39
	30	10.00	10.18		30	40.27	40.55		30	5.51	5.81		30	42.15	44.77
	40	9.50	10.44		40	39.60	40.68		40	4.59	5.35		40	37.21	40.93
2D2-28	0	26.50	27.54	2D2-34	0	263.91	273.53	2D2-40	0	29.35	34.23				
	10	16.24	17.58		10	70.23	72.87		10	7.86	8.76				
	20	16.12	16.16		20	62.35	64.73		20	7.79	8.15				
	30	15.52	16.00		30	61.57	62.15		30	7.19	8.03				
	40	12.05	13.03		40	60.1	61.91		40	6.71	7.07				

C: Long-term thermal inactivation of XynA and thermostable mutants

Mutant	Time (min)	Activity (nkat/μg total protein)							
		70°C		80°C		90°C		100°C	
Wild type	0	611.97	613.79	650.12	663.52	650.12	663.52	650.12	663.52
XynA	10	609.34	613.58	92.38	97.76	7.94	8.50	7.34	8.22
	20	575.20	561.96	87.68	91.18	6.21	7.33	6.14	7.02
	30	558.72	566.08	84.00	94.00	5.13	6.13	3.37	4.01
	40	529.36	545.22	75.00	85.00	4.73	4.97	2.76	2.92
	50	499.62	507.00	56.14	62.12	4.01	4.45	1.71	2.25
	60	486.70	501.30	54.32	60.50	3.42	3.86	1.15	1.49
	70	451.94	481.26	50.19	53.61	-	-	-	-
	80	445.80	448.98	46.67	47.95	-	-	-	-
	90	444.01	445.15	43.33	45.51	-	-	-	-
	100	438.21	449.69	43.00	43.36	-	-	-	-
	110	400.77	431.61	37.24	39.02	-	-	-	-
	120	390.78	403.08	35.94	36.44	-	-	-	-
	130	382.47	388.03	30.83	32.73	-	-	-	-
	140	358.60	364.12	29.26	30.08	-	-	-	-
	150	342.16	357.16	27.54	28.60	-	-	-	-
	160	303.41	305.13	22.51	24.73	-	-	-	-
	170	270.45	279.15	16.59	17.99	-	-	-	-
	180	265.38	269.26	10.67	11.35	-	-	-	-
	190	259.81	269.89	-	-	-	-	-	-
	200	256.67	258.91	-	-	-	-	-	-
	210	256.31	259.27	-	-	-	-	-	-
	220	233.60	235.36	-	-	-	-	-	-
	230	225.80	236.38	-	-	-	-	-	-
	240	212.49	218.17	-	-	-	-	-	-
	250	188.64	198.82	-	-	-	-	-	-
	260	131.29	179.29	-	-	-	-	-	-
	270	118.71	131.01	-	-	-	-	-	-
	280	100.35	108.57	-	-	-	-	-	-
	290	94.82	98.88	-	-	-	-	-	-
	300	74.36	85.98	-	-	-	-	-	-
	310	60.33	77.19	-	-	-	-	-	-
	320	46.18	48.64	-	-	-	-	-	-
	330	42.89	43.47	-	-	-	-	-	-
	340	39.06	45.02	-	-	-	-	-	-
	350	37.24	39.88	-	-	-	-	-	-
	360	30.14	37.64	-	-	-		-	-

Mutant	Time (min)	Activity (nkat/μg total prot.)							
		70°C		80°C		90°C		100°C	
2B7-6	0	380.54	395.72	431.16	445.76	412.54	439.68	405.38	472.46
	10	369.87	376.65	430.59	438.65	149.61	160.09	84.57	88.59
	20	358.26	370.80	384.61	386.25	143.26	146.26	76.55	83.27
	30	341.33	358.35	373.26	375.56	141.37	142.83	71.17	78.49
	40	336.12	339.08	362.51	369.17	118.75	141.49	66.42	69.64
	50	335.91	338.93	285.67	287.01	100.43	116.41	55.38	66.46
	60	330.61	336.49	281.45	285.53	61.24	89.00	30.21	38.01
	70	316.67	319.33	269.12	275.68	-	-	-	-
	80	302.14	318.64	258.91	262.99	-	-	-	-
	90	279.35	290.85	254.69	258.01	-	-	-	-
	100	271.63	266.05	226.55	239.83	-	-	-	-
	110	238.15	252.25	218.47	226.67	-	-	-	-
	120	209.44	212.78	211.67	216.99	-	-	-	-
	130	201.53	208.47	190.14	206.56	-	-	-	-
	140	199.30	200.84	179.44	189.36	-	-	-	-
	150	191.41	206.43	170.89	177.35	-	-	-	-
	160	177.20	179.78	159.32	176.20	-	-	-	-
	170	177.10	179.54	127.68	169.26	-	-	-	-
	180	165.90	168.74	91.36	124.66	-	-	-	-
	190	160.14	167.54	-	-	-	-	-	-
	200	157.48	162.26	-	-	-	-	-	-
	210	153.29	155.17	-	-	-	-	-	-
	220	153.08	156.82	-	-	-	-	-	-
	230	150.80	152.42	-	-	-	-	-	-
	240	147.16	151.16	-	-	-	-	-	-
	250	144.90	146.28	-	-	-	-	-	-
	260	143.06	143.28	-	-	-	-	-	-
	270	143.00	143.10	-	-	-	-	-	-
	280	137.46	140.00	-	-	-	-	-	-
	290	136.25	137.55	-	-	-	-	-	-
	300	135.42	136.00	-	-	-	-	-	-
	310	131.42	139.22	-	-	-	-	-	-
	320	125.10	131.06	-	-	-	-	-	-
	330	119.23	126.67	-	-	-	-	-	-
	340	111.40	113.94	-	-	-	-	-	-
	350	104.72	111.54	-	-	-	-	-	-
	360	64.45	94.01	-	-	-	-	-	-

Mutant	Time (min)	Activity (nkat/μg total prot.)							
		70°C		80°C		90°C		100°C	
2B7-10	0	2769.31	2783.91	2948.65	3912.13	3100.72	3486.68	3284.17	3371.25
	10	2697.24	2719.82	2940.13	3139.67	101.34	129.80	88.50	90.76
	20	2614.33	2631.07	2900.41	2929.79	76.53	86.31	85.46	87.56
	30	2481.77	2680.19	2815.36	2893.72	60.22	63.04	61.37	65.69
	40	2341.60	2363.16	2704.12	2824.92	50.41	57.17	57.64	64.00
	50	2306.11	2337.69	2683.21	2792.83	47.62	54.86	47.61	50.45
	60	2271.33	2327.47	2671.86	2751.50	43.78	46.54	47.02	50.52
	70	2107.51	2205.49	2666.37	2750.99	-	-	-	-
	80	1968.03	2171.05	2637.64	2649.30	-	-	-	-
	90	1740.56	1932.78	1507.28	2054.84	-	-	-	-
	100	1641.97	1656.65	1100.69	1378.69	-	-	-	-
	110	1628.35	1639.11	987.31	1105.97	-	-	-	-
	120	1606.40	1628.12	916.23	1007.15	-	-	-	-
	130	1567.34	1580.70	735.44	780.02	-	-	-	-
	140	1549.30	1572.66	656.83	743.95	-	-	-	-
	150	1500.17	1516.93	561.33	627.27	-	-	-	-
	160	1376.59	1389.39	481.22	522.24	-	-	-	-
	170	1342.56	1362.58	450.60	484.10	-	-	-	-
	180	1300.46	1377.64	300.53	343.17	-	-	-	-
	190	1253.65	1261.59	-	-	-	-	-	
	200	1233.80	1256.54	-	-	-	-	-	
	210	1199.38	1228.54	-	-	-	-	-	
	220	1167.19	1171.53	-	-	-	-	-	
	230	1159.04	1162.62	-	-	-	-	-	
	240	1139.51	1139.51	-	-	-	-	-	
	250	1125.30	1133.26	-	-	-	-	-	
	260	1088.49	1099.69	-	-	-	-	-	
	270	1068.29	1076.81	-	-	-	-	-	
	280	1039.78	1048.64	-	-	-	-	-	
	290	1017.31	1038.97	-	-	-	-	-	
	300	995.28	1000.00	-	-	-	-	-	
	310	980.37	1002.79	-	-	-	-	-	
	320	977.54	980.78	-	-	-	-	-	
	330	973.16	979.16	-	-	-	-	-	
	340	961.33	968.83	-	-	-	-	-	
	350	943.25	966.67	-	-	-	-	-	
	360	894.67	908.77	-	-	-	-	-	

Mutant	Time (min)	Activity (nkat/μg total prot.)							
		70°C		80°C		90°C		100°C	
2B11-16	0	710.44	729.02	607.83	633.79	600.35	690.13	508.16	687.74
	10	682.31	718.77	591.38	614.96	300.70	361.32	236.57	265.81
	20	680.25	690.91	572.49	593.65	251.34	270.20	200.71	254.57
	30	671.24	679.52	467.23	530.59	224.37	248.45	157.24	169.62
	40	664.32	676.26	400.15	486.05	189.31	226.67	114.22	129.04
	50	630.51	658.47	340.69	378.41	154.83	179.57	109.75	115.57
	60	619.48	627.62	316.87	327.25	132.01	164.05	90.06	104.96
	70	598.67	610.21	303.56	323.74	-	-	-	-
	80	590.11	594.69	295.16	304.84	-	-	-	-
	90	579.82	586.64	293.71	301.53	-	-	-	-
	100	567.84	574.28	274.98	295.72	-	-	-	-
	110	546.28	571.92	266.30	269.50	-	-	-	-
	120	538.62	542.52	263.51	267.59	-	-	-	-
	130	519.48	521.56	250.77	262.31	-	-	-	-
	140	472.35	494.21	239.85	256.09	-	-	-	-
	150	447.31	461.75	228.17	240.17	-	-	-	-
	160	420.89	460.45	218.45	231.83	-	-	-	-
	170	380.72	414.62	199.62	203.88	-	-	-	-
	180	386.95	353.49	174.60	195.52	-	-	-	-
	190	352.16	370.46	-	-	-	-	-	-
	200	338.56	348.24	-	-	-	-	-	-
	210	318.70	326.96	-	-	-	-	-	-
	220	311.12	322.22	-	-	-	-	-	-
	230	291.63	302.05	-	-	-	-	-	-
	240	287.55	291.49	-	-	-	-	-	-
	250	286.93	292.11	-	-	-	-	-	-
	260	283.91	284.79	-	-	-	-	-	-
	270	278.26	289.56	-	-	-	-	-	-
	280	269.58	277.20	-	-	-	-	-	-
	290	259.30	266.04	-	-	-	-	-	-
	300	250.14	258.06	-	-	-	-	-	-
	310	237.60	249.86	-	-	-	-	-	-
	320	227.48	243.00	-	-	-	-	-	-
	330	199.53	206.35	-	-	-	-	-	-
	340	170.96	177.66	-	-	-	-	-	-
	350	159.32	176.74	-	-	-	-	-	-
	360	130.61	167.25	-	-	-	-	-	-