OVEREXPRESSION AND PARTIAL CHARACTERIZATION OF A MODIFIED FUNGAL XYLANASE IN *Escherichia coli*

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FINAL COPY APPROVED FOR SUBMISSION

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DECLARATION

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

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T.K. Wakelin

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ABSTRACT

Protein engineering has been a valuable tool in creating enzyme variants that are capable of withstanding the extreme environments of industrial processes. Xylanases are a family of hemicellulolytic enzymes that are used in the biobleaching of pulp. Using directed evolution, a thermostable and alkaline stable xylanase variant (S340) was created from the thermophilic fungus, *Thermomyces lanuginosus.* However, a host that was capable of rapid growth and high-level expression of the enzyme in large amounts was required. The insert containing the xylanase gene was cloned into a series a pET vectors in *Escherichia coli* BL21 (DE3) pLysS and trimmed from 786 bp to 692 bp to remove excess fungal DNA upstream and downstream of the open reading frame (ORF). The gene was then re-inserted back into the pET vectors. Using optimised growth conditions and lactose induction, a 14.9% increase in xylanase activity from 784.3 nkat/ml to 921.8 nkat/ml was recorded in one of the clones. The increase in expression was most probably due to the removal of fungal DNA between the vector promoter and the start codon. The distribution of the xylanase in the extracellular, periplasmic and cytoplasmic fractions was 17.3%, 51.3% and 31.4%, respectively. The modified enzyme was then purified to electrophoretic homogeneity using affinity chromatography. The xylanase had optimal activity at pH 5.5 and 70°C. After 120 min at 90°C and pH 10, S340 still displayed 39% residual activity. This enzyme is therefore well suited for its application in the pulp and paper industry.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Through man's endeavours to harness the powers of nature as well as make use of all the resources nature affords us, he has inadvertently begun poisoning that very system to his own detriment. Problems have begun arising as a result of this, including global warming and dwindling reserves of fossil fuels. In order to combat these and other future problems, steps must be taken now in order to prevent and/or minimise them. One way of doing this would be to focus and strive towards a more sustainable industrial model. The World Commission on Environment and Development (Brundtland, 1987) describes industrial sustainability as, "meeting the needs of the present without compromising the ability of future generations to meet their own needs."

The current focus of biotechnology is in implementing a paradigm shift from a chemical-based manufacturing industry, to a one using sustainable, biotechnological processes. The chemical industry has essentially been solely responsible for the quality of life we have today, however, a cleaner more efficient technology has begun to pay dividends, and is poised to replace the chemical industry. Many industries that had previously never considered biological sciences as impacting on their business have begun exploring the possibility of using biotechnology to their benefit. As well as industries, the aforementioned environmental concerns have helped drive the use of biotechnology to

not only remove pollutants from the environment, but prevent the actual pollution in the first place (Gavrilescu & Christi, 2005). Biotechnology does not seek to overthrow the chemical industry; rather, it strives to form a productive union between the two. In fact, 5% of chemical sales depend directly on biotechnological procedures. This is expected to increase to 10 – 20% by 2010 (Arnold & Glieder, 2003).

The pulp and paper industry has benefited greatly from the advances in enzyme technology and is a perfect illustration of how biotechnology seeks to improve the ecological and economical problems associated with conventional pulping technologies (Shatalov & Pereira, 2006). These conventional methods use chlorine or chlorine-containing compounds to bleach the pulp, which have been shown to be detrimental to the environment. One way of circumventing this problem would be to use xylanase, an enzyme capable of enhancing the bleaching of pulp, whilst saving up to 25% on chlorine-containing chemicals (Viikari *et al.,* 1986).

There are two main problems facing researchers in establishing this technology. Firstly, the industrial process of pulping would require xylanases to retain stability from 60^{o}C – 90^{o}C at pH 8 – 10 for up to three hours (Techapun *et al.,* 2003). And secondly, to find an organism that is capable of producing the enzyme quickly and efficiently, with minimal cost. The gram-negative bacterium, *Escherichia coli,* is a quick-growing organism that is capable of expressing heterologous proteins very efficiently, with an added benefit of having genetic machinery that is easy to manipulate (Makrides, 1996). Taking the above two problems into account, the focus of this research project, was to overexpress a

thermo- and alkaline-stable xylanase in *E. coli*. The xylanase gene was modified in a previous study by Stephens (2006), by the directed evolution of a *Thermomyces lanuginosus* xylanase, to confer both thermo- and alkaline-stability. The gene was cloned into several expression vectors, and the expression parameters were optimised to increase expression. The xylanase was also purified and characterised allowing us to ascertain the optimum conditions for its use.

1.1 Nature's biological catalysts

Enzymes are nature's way of increasing the rate or velocity of biochemical reactions. They are usually made up of proteins and are not changed in the reactions that they catalyse. The sequence of amino acids that makes up the protein, determines the three dimensional shape of the protein, which in turn determines its function. This is a very important property of proteins, as even a change in one amino acid could alter the characteristics of the protein. Mutations in proteins can either be beneficial or detrimental to an organism, and researchers are currently exploiting this fact by purposefully mutating enzymes to enhance and/or change certain characteristics of a particular enzyme (Mathews *et al.,* 2000).

The use of enzymes in many processes is by no means a new concept. For thousands of years people have used them for the baking of bread and the brewing of beer. These processes relied either on the enzymes being produced by growing microorganisms or as added preparations (e.g., calves' rumen or papaya fruit). Therefore, the enzymes were not used in any pure or characterized form (Kirk *et al*., 2002). As our knowledge of microorganisms and biochemistry has grown, so has our understanding of the complex interplay between enzyme and substrate, allowing us to discover novel enzymes, as well as novel reactions that can be catalysed enzymatically (Haki & Rakshit, 2003). Today, enzymes are commonly used in many industrial applications and the demand for more stable and highly active enzymes is growing rapidly. The world market for industrial enzymes was in the range of 1.7 and 2 billion US dollars in 2000 and this value is

expected to increase several fold by 2010 (Bhat, 2000). Enzymes are now used in a variety of chemical reactions/processes. A few examples of this can be seen in Figure 1.1.

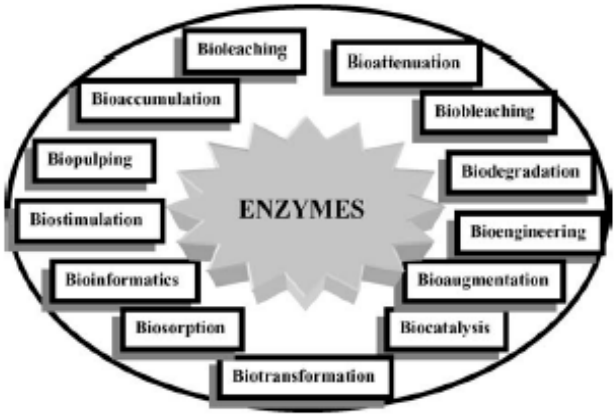

Figure 1.1 An overview of the various processes industrial being catalysed by enzymes (Whiteley & Lee, 2005).

1.2 Extremozymes

Enzymes are increasingly being used in industrial processes whereby they are out-competing traditional methods because they offer the following useful characteristics: they are biodegradable, so they do not persist in the environment; they are extremely selective for the substrates they bind, allowing for the correct product to be produced (enantioselectivity); as well as being able to function at mild temperatures and pH's (Arnold *et al.*, 2001). Unfortunately, most well-established industrial processes take place at conditions of high temperatures and pHs and it would be too costly to change the entire process. One solution is to look for enzymes in organisms that are found in extreme

environments and modify the already established chemical process by using these enzymes as biocatalysts. Since these organisms are able to grow and reproduce in these harsh conditions, there is a very high probability that their enzymes will be more or even completely stable at these extreme conditions.

As man's search for life in every environment on the planet continued, it became increasingly clear that there were organisms that not only lived in extreme environments, but actually thrived there. Since this discovery, microbial communities have been found at extremes of salinity, acidity, alkalinity, temperature and pressure. In the last decade, organisms gave been isolated from a wide range of environments, including; hot springs and deep sea hydrothermal vents (Niehaus *et al.*, 1999). Some members of the domain Archaea and are loosely termed "extremophiles" and therefore the enzymes which they produce are called "extremozymes." These extremozymes are very similar in three-dimensional structure to their enzyme counterparts found in mesophilic organisms. However, they have randomly attained useful mutations over millions of years that allowed them to have enhanced capabilities (Arnold *et al.*, 2001). The structure of many thermophilic extremozymes have been elucidated and compared to their homologs in mesophiles using structural alignment and homology modeling, and some interesting data has been uncovered. The ultimate goal of this research is to determine an underlying mechanism for thermostability. According to Palardini *et al.* (2002), there were three main factors that worked together that seemed to promote thermostability:

(i) an increased surface charge;

(ii) increased protein core hydrophobicity; and

(iii) the replacement of exposed ‘thermolabile’ amino acids.

As well as these important factors, there are several other adaptations shown to be important for thermostability, including the use of chaperonins, which help in the refolding of proteins into their native, active form (Everly & Alberto, 2000) and the presence of a reverse DNA gyrase, that introduces positive supercoils into the DNA structure, increasing its overall melting temperature (Lopez, 1999).

Some of the uses of archaeally-derived materials include: chemically stable lipids of archaeal membranes as a novel drug delivery system (Patel & Sprott, 1999); haloarchaeal polymers have been considered as raw materials for biodegradable plastics (Fernandez-Castillo *et al.*, 1986); and methanogenic archaea for their ability to degrade compounds and as an inexpensive energy source (Reeve *et al.,* 1997). Although many of these products carry significant commercial value, the biotechnologically useful extremozymes are of highest industrial interest. As a result of these enzymes ability to function at the temperature and pH limits of life, they have been considered for a variety of uses in biotechnology (Eichler, 2001). The following few examples of enzymes isolated from extremophiles, was adapted from Shiraldi and De Rosa (2002), and can be found in Table 1.1.

Table 1.1 Examples of enzymes isolated from extremophiles and some of their applications

Microorganisms	Enzymes	Application
Thermophiles		
(50 – 110^{o}C)	Amylases & glycosidases	Starch processing, saccharification
	Lipases	Detergent formulations
	Xylanases	Paper bleaching
	Proteases	Food processing, detergents
	DNA polymerases	Genetic engineering
	Esterases	Stereo-specific reactions
Psychrophiles		
(0 – 20^{o}C)	Amylases, proteases, lipases	Polymer-degrading agents
	Dehydrogenases	Biosensors
Alkaliphiles		
pH ≥ 9	Cellulases, proteases	Polymer-degrading agents
	Amylases, lipases	Food additives

The use of these extremozymes as opposed to conventional methods, confer the following benefits to industrial processes:

(i) it reduces the overall risk of contamination;

(ii) improves transfer rates of the reactions involved; and

(iii) lowers the viscosity and increases the solubility of the substrates (Egorova & Antranikian, 2005).

As more and more enzymes are discovered, or indeed 'designed,' researchers are improving current industrial processes, or even designing new ones. At the moment, there are over 500 commercial products using these enzymes in their synthesis. Two of the more popular are the low–calorie sweetener aspartame and the antibiotic amoxicillin (Schoemaker *et al.*, 2003). The majority (65%) of the industrial enzymes are used in the hydrolysis of polymers, and have found value in the textile, pulp and paper and detergent industries. The remainder of the enzymes are mostly used in either the food processing industry (25%), or in the supplementation of animal feed (10%) (Cherry & Fidantsef, 2003).

It is nothing short of astonishing to witness the amazing array of enzymes that nature has afforded us. Over many millennia, mutations have been collecting in enzymes allowing them to bind to countless substrates and perform a myriad of different functions. High-throughput screening technology has allowed millions of enzymes to be screened, to look for variants with different or enhanced capabilities. However, the time for simple screening for variants is being replaced by a new way of thinking. Instead of looking to nature, man is taking it upon himself to design the enzymes himself. By doing this he can fill in the gap between what nature has given us, and what is actually needed from an enzyme to retain functionality under the conditions of most industrial processes (Johannes & Zhao, 2006).

1.3 Enzyme engineering

The preceding sections have painted a rather one-sided view on the reality of enzymes today. Rather than being flawless in every facet, there are still some inherent problems that need to be addressed. These limitations usually come into play when enzymes are used on an industrial scale. Some of the more concerning issues are: the laboured catalysis on unnatural substrates, the low tolerance for changes in operating parameters and the poor activity in nonaqueous media (Schmid *et al.*, 2001). One way to address these shortcomings would be to tirelessly screen hundreds, if not thousands of variants, until one that worked optimally in the desired conditions was found. The other strategy would be to use protein engineering techniques to identify and change specific amino acid residues within a protein sequence, to generate a desired change in enzyme performance (Farinas *et al.*, 2001). Protein/enzyme engineering can be separated into two main categories depending on the methods employed and the desired outcome. They are rational design and directed evolution.

1.3.1 Rational design

When looking to modify a specific protein, researchers have been exceedingly more successful at modifying the existing properties or catalytic activities of that protein, as opposed to engineering an entirely novel function into the protein. Therefore, any attempt to alter the specific properties of a protein would require a thorough understanding of the protein's primary, secondary and tertiary structure. Moreover, when using a rational

design approach to altering an enzyme's catalytic site, extensive knowledge of that enzyme's catalytic mechanism and structure is a prerequisite (Shao & Arnold, 1996). This can sometimes become quite a problem, as there is information available for a few well-studied systems, however, very little is known about the vast majority of potentially attractive enzymes. This fact alone can potentially dissuade a researcher from continuing work on a new, yet-to-be sequenced protein. The current amount of knowledge on structure-function relationships is insufficient to predict the individual or cumulative effects of point mutations on enzymes (Liese & Filho, 1999). However, rational design is still a very powerful technique, which allows researchers to determine exactly which amino acids are important in determining the enzyme's structure, and ultimately function.

The main technique used in rational design is site-directed mutagenesis (SDM). SDM allows for the substitution of a single, specific amino acid residue. By using data generated by computer-assisted modeling, it can be determined which amino acids are most likely to cause a structural change allowing for the overall functional change (Blackburn, 2000). Depending where the amino acid residue is situated, the single substitution may have little effect, and it may increase or even hinder the enzymes ability to function optimally. If the catalytic activity of an enzyme needs to be altered, the researcher would study the residues closest to the enzyme active site, as this area would most likely be involved in catalyzing the substrate. The research involving penicillin acylase (PA), which is an enzyme used in the industrial synthesis of β-lactam antibiotics, is a perfect illustration of this technique. Analysis of the crystal structure of PA showed that two residues on the active site of the enzyme were involved in binding the substrate.

This suggested that they would possibly be good targets for mutagenesis aimed at improving catalytic performance (Jager *et al.*, 2007). The variants were screened and a few of the mutants displayed a 15-fold increase in catalytic activity. SDM involves designing oligonucleotide primers containing the desired mutations, flanked by bases complementary to specific sequences on either side of the desired mutation point on the enzyme (Ge & Rudolph, 1997).

1.3.2 Directed evolution

Directed evolution is a new and exciting protein engineering technique that is helping shape the enzyme landscape of tomorrow. It does not rely on a detailed understanding between enzyme structure and function; rather, it relies on the Darwinian principles of mutation and selection. The starting point is a specific gene or set of genes. These undergo random mutagenesis and/or gene recombination in order to create a library of mutant genes. The library is then cloned into an expression vector and transformed into a host microorganism for protein expression. Functionally improved mutant proteins are identified through a carefully designed screening or selection process, and are often used as the starting genes for further rounds of mutagenesis. This process can be repeated several times until the desired improvement is attained or no further improvement is possible. The screening step is often the most important step, and ultimately decides on the success of the experiment (Johannes & Zhao, 2006).

Directed evolution has become a powerful tool in tailor-making enzymes, especially for industrial processes. The laborious task of screening organisms for the possibility of finding enzymes with increased activities and novel properties, is being replaced by inducing the changes yourself, albeit blindly. It has already had many successes in improving various enzymes, as researchers have concentrated on improving specificity, activity, stability and solubility (Roodveldt *et al.,* 2005). The enantioselectivity of an *Aspergillus niger* epoxide hydrolase was improved two-fold using only one round of error-prone PCR (epPCR), whereby three amino acid substitutions were made, two of them were far away from the active site (Reetz *et al.,* 2004). With regards to activity, Castle *et al.* (2004) were able to undertake eleven rounds of DNA shuffling on the enzyme glyphosate *N*-acetyltranferase (GAT), which resulted in a variant with a 10000-fold improvement in catalytic efficiency. DNA shuffling is a technique used to create novel genes by the recombination of closely-related DNA sequences (Stemmer, 1994b).

Directed evolution has been shown to be a useful technique in improving the various properties of the xylanase enzyme. The xylanases that have been isolated from many different microbial sources from nature have not been able to function at the optimal conditions for industrial processes. However, using the above-mentioned techniques of directed evolution, much progress has been made in this respect, and the future possibilities look endless. In a study conducted by Stephens *et al.* (2007), the thermostability of a xylanase from *T. lanuginosus* was increased using ep-PCR. One of the resulting mutants, 2B7-10, retained 71% of its activity after treatment at 80^{o}C for 60 min.

1.4 Xylanases

1.4.1 Xylan

Xylan is the second most abundant polysaccharide in nature, as it is the major component of the plant cell wall. Plant biomass is usually comprised of about 23% lignin, 40% cellulose and 33% hemicellulose. Xylans (hemicelluloses) are a hardening component in the cellulose structure, which cover the lignin component (Techapun *et al.*, 2002). The structure of xylan is extremely complex. This makes its enzymatic degradation into small xylose monomeric units very difficult and therefore requires the action of a specific group of hemicellulases (Zimmerman, 1992). Xylan is a highly branched heteropolysaccharide, with a homopolymeric backbone composed of 1,4-linked β-D-xylopyranosyl units, which are substituted to varying degrees with glucuronopyranosyl, 4-*O*-methyl-D-glucuronopyranosyl, α-L-arabinofuranosyl, acetyl, feruloyl and/or *p*-coumaroyl side-chain groups (Kulkarni *et al.*, 1999). As esterification and substitution increase, the digestibility by hemicellulases decreases. Due to this inherent heterogeneity, it requires the help of several enzymes that act in synergy for its complete hydrolysis, as can be seen in Figure 1.2.

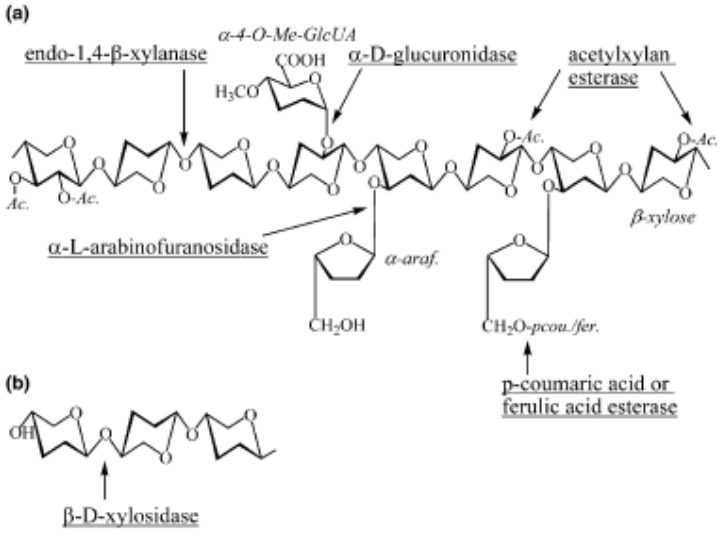

Figure 1.2 (a) Structure of xylan and the points of enzymatic cleavage (b) The hydrolysis of xylo-oligosaccharides by β-D-xylosidase (Collins *et al.,* 2005).

Endo-1,4-β-D-xylanases randomly cleave the xylan backbone, whilst β-D-xylosidases cleave the monomeric xylose residues from the non-reducing end. The removal of the side groups is catalysed by α-L-arabinofuranosidases, α-D-glucuronidases, acetylxylan esterases, ferrulic acid esterases and *p*-coumaric acid esterases. The complete set of the xylanolytic enzyme system has been found in many different species of fungi and bacteria, and are typically found in environments where plant material has accumulated and is being decomposed (Collins *et al.,* 2005).

1.4.2 Classification and structure of xylanases

As a result of the heterogeneity of xylan, an abundance of extremely diverse xylanases with varying specificities, amino acid sequences and overall 3-D shape have been discovered. In order to minimise confusion, xylanases were originally grouped into six

families (A-F), generally called the glycoside hydrolases. They are grouped according to primary structure comparisons of their catalytic domains (Henrissat *et al.*, 1989). However, as more sequences have been elucidated, there are now over 96 families of glycoside hydrolases. The enzymes within a particular family have a similar 3-D structure and a similar molecular mechanism. Within this classification system, xylanases make up families 10 (F) and 11 (G) (Subramaniyan & Prema, 2002).

The glycoside hydrolase family 10 consists of endo-1,4-β-D-xylanases, endo-1,3-β-D-xylanases and cellobiohydrolases. Members of this family typically have a high molecular mass, acidic pI and display an $(\alpha/\beta)_8$ barrel fold structure. This structure has been likened to a 'salad bowl', with one face of the molecule having a large radius, with the catalytic site near the narrower end, closest to the carboxyl terminus of the β-barrel (Fig. 1.3) (Harris *et al.*, 1994). The glycoside hydrolase family 11 is monospecific, as it consists solely of 'true xylanases' that are exclusively active on D-xylose containing substrates. They have an alkaline pI, a much lower molecular mass and a β-jelly roll fold structure. Their structure consists of β-pleated sheets formed into a two-layered trough that surrounds the catalytic site. This structure has been likened to a palm and fingers of a right hand (Fig. 1.3) (Torronen & Rouvinen, 1997). All xylanases retain the anomeric configuration of the glycosidic oxygen following the hydrolysis of xylan. This shows that they use a double-displacement mechanism in which the reactive intermediate is bound to the enzyme (Davies & Henrissat, 1995).

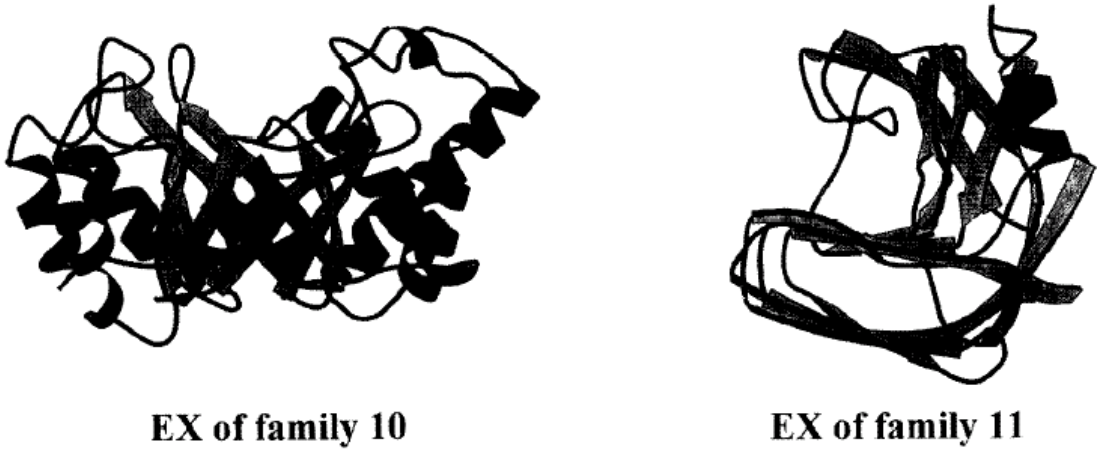

Figure 1.3 Ribbon representations of the 3D structure of the endoxylanase (EX) families 10 and 11 (Davies and Henrissat, 1995).

1.4.3 Commercial applications of xylanases

The global market for industrial enzymes grew from €1 billion in 1995, to an estimated €2 billion in 2001 and is continuing to grow at a rapid pace as new enzymes are being discovered frequently (Collins *et al.,* 2005). Hydrolases amount to 75% of the market for industrial enzymes, with the glycosidases, cellulases, amylases and hemicellulases constituting the second largest group after proteases (Bhat, 2000).

The use of hemicellulases in biotechnology first started in the early 1980's, where they were used in the animal feed and food processing industries (Voragen, 1992). In the animal feed industry, xylanases are used in conjunction with β-glucanases to increase the digestibility and absorption of cereal-based feeds for monogastric animals, by breaking-down the cellulose and hemicellulose fractions present within the cell wall (Kirk *et al.,*

2002). In the food processing industry, xylanases are used in conjunction with α-amylases in the prevention of bread staling. Current research shows that these two enzymes are critical for maintaining softness and elasticity, by increasing the bread's water-binding capacity (Andreu *et al.*, 1999). Subsequently, xylanases have found uses in the textile, laundry and pulp and paper industry, some of their other applications can be found in Table 1.2.

Table 1.2 Examples of xylanases and other hemicellulases and their applications in industry (Bhat, 2000)

Enzyme(s)	Function	Application
Arabinoxylan modifying enzymes (endoxylanases)	Modification of cereal arabinoxylans	Improvement in the texture, quality and shelf-life of bakery products
Macerating enzymes (hemicellulases)	Hydrolysis of plant cell wall polysaccharides	Improvement in skin maceration in grapes and filtration and clarification of wines
Cellulases and hemicellulases	Partial hydrolysis of lignocellulosic materials	Improvement in the nutritional quality of animal feed
Xylanases, mannanases & β-glycosidases	Removal of xylan from lignin-carbohydrate complexes	Bio-bleaching of kraft pulps, reducing the chlorine requirement
Mixture of cellulases and hemicellulases	Solubilisation of plant and/or fungal cell walls	Production of plant or fungal protoplasts

1.4.4 The pulp and paper industry

According to the Food and Agriculture Organization (FAO), the demand for paper will rise from 210 million tons in 1988, to an estimated 350 million tons by 2010 (Roncero *et al.,* 2003). Over two million tons of chlorine and chlorine derivatives are used annually in the USA alone in the bleaching of pulp, and the chlorinated lignin derivatives generated by this process amount to an extremely large environmental problem (Mcdonough, 1992). Recent research has illustrated the use of xylanases and other hemicellulases as an enzymatic pre-treatment as a suitable alternative to chlorine bleaching for the removal of residual lignins from pulp (Daneault *et al.,* 1994; Yang *et al.,* 1994; Chauhan *et al.,* 2006; Khandeparkar & Bhosle, 2006).

Lignin is deposited in wood as a polyphenolic matrix surrounding and encapsulating the cellulosic and hemicellulosic polysaccharides that make up the major constituent of plant cell walls. Removal of this lignin from the wood is notoriously difficult due to its unreactive nature, however current pulping technologies, such as alkaline extraction at high temperatures, are capable of removing nearly 90% of the polymer (Chen *et al.*, 1997). This process of cooking wood pulp under conditions of high temperature and alkaline pH's is referred to as kraft cooking, resulting in kraft pulp. This process, however, still leaves 10% of the lignin in the pulp, giving it a dark brown colour. Traditional processes designed at removing this residual lignin include chlorine bleaching, which tend to accumulate and pollute the environment. The residual lignin maintains a close association with the cell wall hemicelluloses, particularly the xylans,

allowing for enzymatic degradation by xylanases. Therefore, by treating kraft pulps with xylanases, the release of the xylan component and the residual lignin can be accomplished, without losing any of the other pulp structural constituents. The xylanase treatment opens up the cell wall structure, facilitating the lignin removal in subsequent bleaching stages, reducing up to 25% of the elemental chlorine needed (Chen *et al.,* 1997; Christopher *et al.,* 2005.). This was the first step in continuing research, that ultimately led to making the entire process elemental chlorine-free (ECF) and then totally chlorine-free (TCF) (Christov *et al.,* 1996). However, TCF pulp bleaching accounts for only 15% of the total pulp production worldwide (Atik *et al.,* 2006).

The use of xylanases as a pretreatment step has been termed biobleaching, as it helps in increasing the brightness of the pulp. Xylanases are only useful in the aforementioned process, if they are free from contaminating cellulases, as the cellulosic fibre should not be affected (Viikari *et al.,* 1994). Xylanases should ideally have an alkaline pH optimum and a high temperature optimum to withstand the inherently tough conditions found in the pulp and paper mills (Beg *et al.,* 2001). The search for thermoalkalophilic xylanases has ultimately led to the screening of thermophilic and hyperthermophilic organisms and modification of their xylanases using protein engineering techniques. However, many stable xylanases have been found in a wide variety of organisms including *Arthrobacter* sp. (Khandeparkar & Bhosle, 2006), *Bacillus coagulans* (Raha *et al.,* 2006), *Rhodothermus marinus* (Karlsson *et al.,* 1999), *Streptomyces cyaneus* (Ninawe & Kuhad, 2006), *Thermomyces lanuginosus* (Stephens *et al.,* 2007), *Thermotoga maritima* (Chen *et al.,* 1997), and *Trichoderma reesei* (Fenel *et al.,* 2004). The stability of some

thermostable xylanases has been improved by introducing more disulphide bridges, single residue substitutions and alterations near the N-terminal region of the protein (Shibuya *et al.,* 2000; Fenel *et al.,* 2004). Finding a suitable host to express the final protein still remains a challenge, although there has been plenty of success in this area. The current strategy entails using a mesophilic host to express the thermophilic enzyme, and will be discussed in the following section (Zhou *et al.,* 2007).

1.5 Protein expression

1.5.1 *Escherichia coli* – the original prokaryotic expression system

Heterologous gene expression first announced itself to the world and put biotechnology on the map back in 1977, when Itakura *et al.* (1977) successfully cloned and expressed the mammalian peptide hormone somatostatin in *E. coli.* The dollar value of producing this bioactive compound in a liquid medium was comparable to extracting it from the brain of 500 000 sheep, and was regarded as a milestone in genetic engineering. Among the many available expression systems available today, the little Gram-negative bacterium *E. coli* remains one of the most attractive systems for the following reasons:

(i) it can grow very rapidly at a high density on cheap substrates;

(ii) it has well-characterised genetic machinery; and

(iii) there is a large availability of cloning vectors and mutant host strains (Baneyx, 1999).

There is still no guarantee that the recombinant protein will accumulate in high amounts, and will be the correct length and shape and be in a biologically-active form. However, an enormous amount of research has been directed at improving the performance of this workhorse microorganism, whilst eliminating some of its limitations (Makrides, 1996).

1.5.1.1 Factors involved in E. coli-based expression

The plasmid copy number and stability are important factors to consider when choosing the expression system and depends upon the level of expression required as well as the nature of the protein itself (whether it is toxic to the cell or not). Low copy number plasmids (e.g., pMB1/ColE1 derivatives) replicate in a fairly relaxed fashion and are found from 15-60 copies per cell, whilst medium copy number plasmids (e.g., the pUC series) are found at a few hundred copies per cell (Baneyx, 1999). High copy number plasmids (e.g., the pET series) are found at several hundred copies per cell, sometimes causing plasmid loss due to a reduction in the growth rate (Summers, 1998). The maintenance of plasmid stability is done by creating a selective pressure, usually by using an antibiotic-resistance marker. This ensures that only the cells carrying the plasmid, and therefore the resistance marker survive, whilst those without die or have their growth inhibited.

Many of the promoters used today to drive the transcription of heterologous genes are based on the *E. coli* lactose utilisation (*lac*) operon. The *lac* promoter, however, is fairly weak, but can be used for sufficient levels of protein expression when using the non-

hydrolysable lactose analog isopropyl-β-D-thiogalactopyranoside (IPTG) (Hashemzadeh-Bonehi *et al.,* 1998). The synthetic *tac* (e.g., Amersham Biosciences pGEX) and *trc* (e.g., Amersham Biosciences pTrc) promoters are made up of different regions of the *trp* and *lac* promoters. They are both fairly strong and can allow the accumulation of proteins to about 15-30% of the total cell protein. The cost and toxicity of IPTG can remain an issue, unless the products formed are of high value (Sorensen & Mortensen, 2005). The pET vectors (Novagen) are the most used vector in recombinant protein preparations. Target genes are placed downstream of the bacteriophage T7 promoter on medium to high copy number plasmids. This system has been shown to synthesise large amounts of mRNA and of the desired protein at concentrations as high as 40-50% of the total cell protein. Furthermore, any deleterious effects of expressing such large amounts of protein, can be overcome by using *E. coli* strains such as BL21(DE3) pLysS or BL21(DE3) pLysE. They have been developed to overexpress phage T7 lysozyme to degrade T7 RNA polymerase as well as incorporation of a *lac* operator sequence downstream of the T7 promoter to help reduce leaky transcription (Miroux & Walker, 1996). These strains are also deficient in the outer membrane protease, OmpT, which reduces the risk of degradation of the heterologous proteins (Georgiou & Segatori, 2005).

The degree of success with any cloning strategy for the overexpression of a target gene will ultimately depend on the above mentioned factors, as well as the degree of post-translational modification needed for a particular protein and its final destination (cytoplasm, periplasm or extra-cellular environment). Prokaryotic expression systems do not have the necessary genetic machinery to undertake extensive post-translational

modifications (e.g., N- and O-linked glycosylation, fatty acid acylation, phosphorylation and disulphide bond formation) that are needed for a properly folded protein (Jung & Williams, 1997). The final destination within the *E. coli* cell also determines the solubility of the protein as well as its level of expression and ease of downstream processes like purification and the some of the fates of these proteins can be seen in Figure 1.4. Overexpression of proteins in the cytoplasm can lead to the formation of inclusion bodies (insoluble protein aggregates) and leave the protein susceptible to proteolytic degradation (Choi & Lee, 2004). A better strategy to employ entails the secretion of the protein into the periplasm, which is an oxidizing environment that contains the necessary enzymes for the formation and rearrangement of disulphide bonds. This is especially useful when cloning eukaryotic proteins (Aslund & Beckwith, 1999).

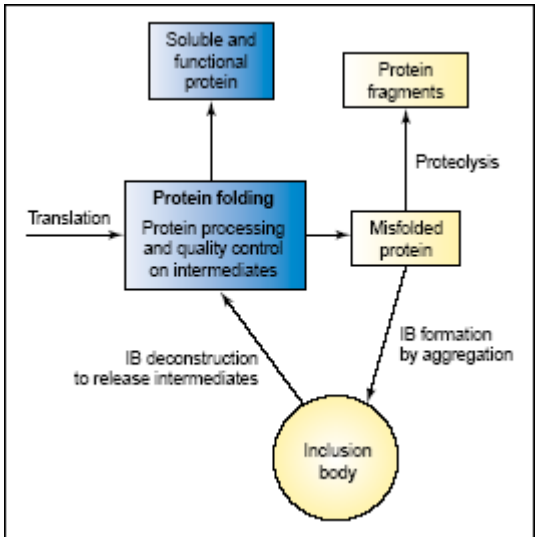

Figure 1.4 An overview of the fate of intracellular proteins (Middelberg, 2002).

1.5.1.2 Overcoming the limitations of E. coli-based expression

The *E. coli*-based expression system is still the most popular system used today for the expression of heterologous proteins. However, there are a few inherent problems associated with it. Proteolytic degradation and protein folding are common problem that are encountered, especially if the protein is soluble and is found in the cytoplasm. The host cell's catabolism is highly efficient at conserving cellular resources, and therefore it recycles improperly folded or irreversibly damaged proteins into their constituent amino acids (Baneyx, 1999). In the cytoplasm, almost all of the degradation is carried out by five ATP-dependant heat-shock proteins (Hsps)(Gottesman, 1996). There are a number of strategies that can be employed to combat the destructive nature of these proteases. One solution is to use mutant host strains that are deficient in some of these proteases, especially the Lon protease and the OmpT outer membrane protease. *E. coli* BL21 and its derivatives are examples of these mutant strains that are able to accumulate proteins at a high rate and are much less likely to degrade these proteins (Henderson *et al.,* 1994). Another solution would be to target your heterologous protein into the periplasm. This would have a two-fold effect, firstly it would reduce the chance of proteolytic degradation as there are much less proteases present in the periplasm, and it would help with the protein folding as this is where most disulphide bonds are formed (Choi *et al.*, 2006). Proteins can be targeted to the periplasm by the use of signal sequences, including those from OmpA, OmpF and PelB (Wong *et al.,* 2003). The use of protease inhibitors may be considered, if it is found to be economically feasible. Ramchuran *et al.* (2002)

also noted that the presence of proteases is also dependant on the induction time and inducer employed during protein production.

The formation of inclusion bodies (IBs) in *E. coli* seems to be a ubiquitous problem associated with the overproduction of recombinant proteins. They have long been regarded as unorganized masses of polypeptide chains. However, recent research has revealed that they actually have defined conformations within this aggregated state (Ventura & Villaverde, 2006; Rinas *et al.,* 2007). They are in fact refractile, insoluble, intracellular protein aggregates that are devoid of any biological activity. Strictly speaking they are an accumulation of folding intermediates rather than the native or unfolded protein (Carrio & Villaverde, 2001). These facts make it a significant challenge to overcome, if large amounts of soluble heterologous proteins are needed. The first solution to this problem is to solubilise and refold the IBs into an active conformation. This is by no means a straightforward process and requires an extensive trial-and-error approach (Tsumoto *et al.,* 2003). The most common solution to the problems of IBs is fairly simple and entails a simple reduction in the cultivation temperature. Usually a drop from 37^{o}C to 30^{o}C is sufficient to drastically increase the concentration of soluble proteins (Schein, 1989). Several researchers today, however, are actually purposefully directing protein production towards inclusion body formation since IBs are easier to purify (usually a simple centrifugation step is required). Also, they have no biological activity toxic proteins can be produced without killing the host and they are entirely resistant to proteolysis (Choi *et al.,* 2006). So, the choice to keep the desired protein

within the soluble fraction or to direct it towards IB formation would depend on the downstream application.

Another important limiting factor that needs to be overcome is the problem of codon bias or rare codon usage. One example would be human proteins, that contain codons that are rare in *E. coli* [e.g., Arg (AGG/AGA/CGA), Leu (CUA) and Pro (CCC)], leading to their inefficient expression in *E. coli* (Kane, 1995). This problem can be circumvented by increasing the copy number of the limiting tRNA species. *E. coli* can be controlled to match the codon usage frequency in heterologous genes. Certain host strains like Rosetta (Novagen) are BL21 derivatives and are designed to enhance the expression of eukaryotic proteins that contain codons rarely used by *E. coli* (Clark, 1998). Taking the above limitations into account, one can see the flexibility of the *E. coli* expression system, as there are several solutions to every problem. In some cases, it can even be confusing as to which solution is most viable for the particular research problem.

1.5.2 Yeasts – the model for eukaryotic expression systems

Yeasts have been described as the expression system with the highest commercial value. Baker's yeast is the common name for *Saccharomyces cerevisiae* which has been used for years in making bread rise and in the production of beer and wine. More recently, it has been engineered to express many different heterologous proteins (Hitzeman *et al.*, 1981). It also satisfies the economic efficiency and biosafety regulations and it has been used to make human medicines, like the hepatitis B vaccine (DiMicelli *et al.*, 2006).

Another yeast that is becoming a popular expression host is *Pichia pastoris* (Cregg *et al.*, 2000). Yeasts in general share some of the same general advantages as *E. coli*, in that they have simple growth media, they grow rapidly and can generally express foreign proteins at a high level. They do have two advantages over *E. coli*, in that they can secrete intracellular proteins into the extracellular environment and are able to undertake all the necessary post-translational modifications (Daly & Hearn, 2005). Some of the currently used applications for the above mentioned expression systems are summarized in Table 1.3.

Table 1.3 Some common applications of popular expression systems (Yin *et al.,* 2007)

Host	**Advantages**	**Disadvantages**	**Applications**	**Commercial Products**
E. coli	Easy scale-up, low costs and minimal time required	Protein solubility and protein-specific optimisation	Structural analysis, functional assays and protein expression	pET Expression systems (Novagen), pQE (Qiagen), pTrc (Amersham)
Yeast	Eukaryotic protein processing and simple media	Fermentation requires high yield and growth optimisation	Structural analysis, antibody generation and protein expression	pPIC, pYC, pYES2 (Invitrogen), pYEX-BX (Clontech)

1.5.3 Cloning of xylanases in *Escherichia coli*

There has been much success over the years with the cloning of xylanases in *E. coli*. Many of the xylanases have been isolated from extremophiles, especially alkaliphiles and thermophiles. Many of the xylanases isolated from these thermophiles were cloned into the mesophilic host *E. coli* to either increase their expression, or to enhance them using protein engineering. Xylanases have been isolated and cloned into *E. coli*, from a wide variety of microorganisms, including *Aeromonas caviae* (Suzuki *et al.*, 1997), *A. niger* (Sriprang *et al.*, 2006), *A. usamii* (Zhou *et al.*, 2007), *Bacillus* sp. (Blanco *et al.*, 1999), *Bacteroides ruminicola* (Whitehead & Hespell, 1989), *Paenibacillus* sp. (Lee *et al.*, 2000), *Pichia stipitis* (Basaran *et al.*, 2001), *Trichoderma reesei* (Fenel *et al.*, 2004) and *T. lanuginosus* (Stephens *et al.*, 2007). Most of these xylanases had fairly high expression levels. As long as a suitable host-vector system is chosen for the particular xylanase, many of the associated problems with cloning in *E.coli* can be avoided (Lin *et al.*, 2001).

1.6 Rationale for the study

The use of xylanases in industry is expanding rapidly, especially due to the fact that protein engineering techniques are designing new, improved variants, whose characteristics are making them highly suitable for the pulp and paper industry. These xylanases are being mutated to alter their structure and hopefully their function, to increase both their thermostability and alkaline stability. The xylanase gene has been isolated and cloned from various organisms, with varying degrees of success. Finding an organism that is easy to work with, cheap and quick to grow and that expresses large amounts of heterologous proteins would be both a valuable tool and financially rewarding.

A thermostable and alkaline stable xylanase (S340) was created using directed evolution, more specifically, the staggered extension process (StEP), by Stephens (2006). This enzyme, however, was not able to be expressed at a very high level, as the focus of the research was not on overexpressing the protein, rather on improving its characteristics.

This project was an attempt to continue the work started by Stephens (2006). Firstly, the gene was amplified using conventional PCR and cloned into several expression vectors. The shake-flask expression parameters were optimised, and a baseline expression level was obtained for the different clones. The gene was then shortened using new primers and PCR, and re-cloned in to the same expression vectors. Expression levels were then

determined to check for an overall increase and the enzyme was then purified and characterized.

CHAPTER 2

CLONING OF THE XYLANASE GENE IN *Escherichia coli*

2.1 INTRODUCTION

The first step in any cloning protocol involves choosing a cloning strategy that suits the particular protein, as well as its downstream application. For instance, if a complex protein is needed for pharmaceutical use, then it might be better to use a eukaryotic expression system, to account for post-translational modifications. If the protein is needed in large amounts in a purified form, an expression vector with a tag for purification might be the best option. The many different types of plasmid vectors and engineered strains of *E. coli* illustrate the diversity and efficacy for its use in cloning procedures. Not all heterologous proteins can be successfully expressed in *E. coli*, as factors such as vector and host, the strength of the promoters used, the inducer concentration and time of induction, as well as the media composition will all influence its expression (Thangadurai *et al.*, 2008).

The choice of plasmid vector can be quite confusing as there is a wealth of choice currently on the market. The pET vectors (Novagen) are probably the most widely used, as they are fairly simple to manipulate and usually give positive results (Baneyx, 1999). The choice of *E. coli* strain is also of vital importance, as there are many different strains all tailored to suit different purposes. Conventionally, *E. coli* K12 strains such as,

NovaBlue (Novagen), XL1 Blue or DH5α (Invitrogen) are used specifically for cloning. Once the constructs have been assembled, they are cloned into an expression strain such as BL21 (DE3) pLysS or pLysE. These strains are able to overexpress toxic and non-toxic target proteins and are usually protease deficient, improving protein yields (Grossman *et al.*, 1998).

The gene of interest in this study was a mutated fungal xylanase gene that was initially isolated from *Thermomyces lanuginosus* DSM 5826 (Schlacher *et al.*, 1996), and made more thermostable using error-prone PCR (epPCR) (Stephens *et al.*, 2007). The *xyn*A gene was then used as a parent gene to further enhance both its thermostability and alkaline stability, using a type of DNA family shuffling, called the 'staggered extension process' (StEP) (Stephens, 2006). One of the mutant progeny, an enzyme variant called S340, showed an increase in both thermal and alkaline stability. There were, however, two aspects of this study that required further experimentation. Firstly, the overall activity of S340 was poor, below that of the parent *xyn*A (250 nkat/ml). Secondly, S340 was not purified but characterised in a crude cell lysate, possibly masking a true reflection of the enzyme's characteristics.

One of the main strategies of the current study was shortening of the S340 cloning fragment to bring the start codon (ATG) of the xylanase gene closer to the Shine-Dalgarno sequence on the plasmid, and therefore increase expression. There was a large amount of DNA present at both ends of the S340 gene, this excess DNA was derived from previous cloning procedures as well as consisting of the original *T. lanuginosus*

DNA. Because this DNA seemingly does not play an important role in the regulation of the S340 gene expression, its removal could possibly increase gene expression. The purpose of this chapter was therefore to employ two strategies to increase the expression of the S340 variant. Firstly, the S340 cloning fragment was cloned into four different expression vectors, and secondly, the S340 insert was shortened in the hope that it would increase expression levels.

2.2 MATERIALS AND METHODS

2.2.1 Construction of primary clones

2.2.1.1 Growth and maintenance of E. coli cultures

All *E. coli* cultures were grown and maintained on Luria Bertani (LB) medium (10 g/l bactopeptone, 5 g/l yeast extract powder, 5 g/l NaCl and 15 g/l technical agar) containing 100 µg/ml ampicillin at 37°C. For medium term storage, sub-culturing was done every two weeks, whilst for longer term storage cultures were supplemented with 15% glycerol and stored at -70°C.

2.2.1.2 Plasmids

The original plasmid used in this study was created by Stephens (2006), and consisted of a plasmid Bluescript SK vector (pBSK), containing the S340 xylanase gene as part of a 786 bp fragment cloned into the multiple cloning site (MCS). The open reading frame (ORF) of the gene is 678 bp long, and is flanked on either side by excess DNA and the MCS. A linear plasmid map depicting the S340 gene is shown in Fig. 2.1. The gene could therefore be amplified by PCR using the standard T3 (forward) and T7 (reverse) primers. Another option to recover the 786 bp insert was to use the restriction enzymes *Xho*I and *Bam*HI to excise it from the plasmid, leaving behind a 2928 bp fragment that corresponds to the pBSK vector.

Figure 2.1 A linear plasmid map showing the S340 gene, including the excess DNA and pBSK MCS. Restriction sites and locations on the polylinker of the plasmid used for cloning are also shown.

The four expression vectors used in this study were pET21a, b, c and pET22b (Novagen) and their plasmid maps can be seen in Fig. 2.2a and Fig. 2.2b, respectively. The pET21 series are identical, except for a decrease in size by 1 bp from 21a – 21c, to account for every reading frame. They also contain a C-terminal His·Tag sequence to facilitate purification. The pET22b vector is slightly larger (5493 bp) than the pET21a, b, c series (5443, 5442 and 5441 bp, respectively). It also contains a C-terminal His·Tag sequence, in addition to an N-terminal *pelB* signal sequence for potential periplasmic localization of expressed proteins. The pET system is currently the most powerful system yet developed for the heterologous cloning and expression of foreign proteins in *E. coli*. The target gene (S340) was cloned into the pET vectors' MCS under the control of a strong bacteriophage T7 transcription and translation signal. When fully induced, T7 RNA polymerase becomes fully activated and the cell's resources are directed towards target gene expression, which can comprise almost 50% of the total cell protein.

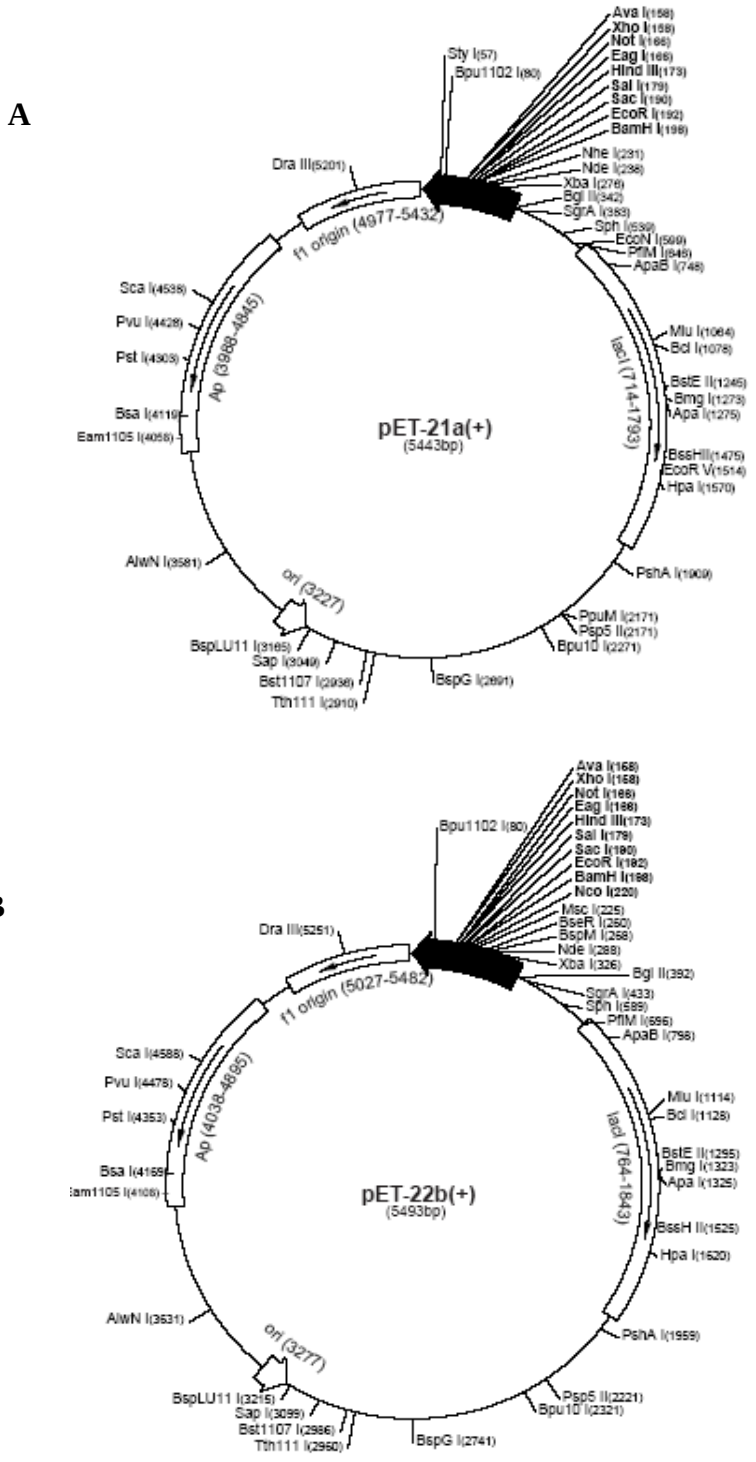

Figure 2.2 Plasmid gene maps of (a) pET-21a, b, c and (b) pET-22b showing the orientation of the plasmid, ampicillin resistance gene (Ap) and MCS.

Using the primers listed in section 2.2.1.7, the S340 gene was amplified using PCR, restricted (section 2.2.1.8) and ligated (section 2.2.1.9) into the four pET vectors (Fig. 2.2). The resulting clones, called the primary clones, were the first set of constructs used in this study.

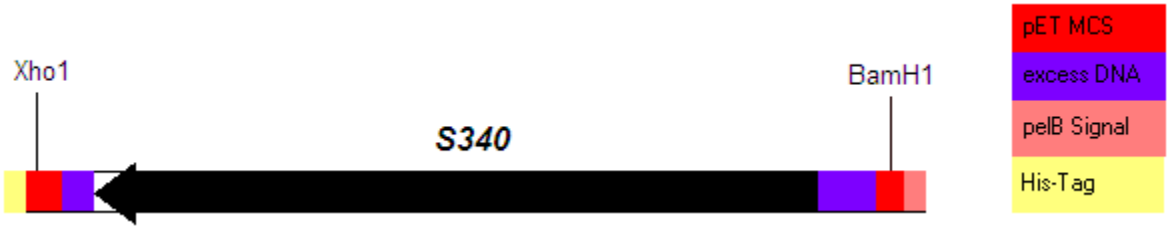

Figure 2.3 A linear plasmid map showing the S340 gene and flanking regions cloned into pET-22b, including the excess DNA and MCS. Restriction sites and locations on the polylinker of the plasmid used for cloning are also shown. Included are the N-terminal *pelB* signal sequence and the C-terminal His·Tag sequence. The black arrow, representing the S340 gene also shows the gene's orientation.

2.2.1.3 Plasmid DNA isolation

The alkaline lysis method of Birnboim and Doly (1979) was used to isolate plasmid DNA and modifications to this original method 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°C. Cells were harvested by centrifugation of broth cultures at 7000 *g* for 5 min. The pellets were resuspended in 100 µl of Solution A [25 mM Tris-HCl, 50 mM glucose, 10 mM EDTA (pH 8)] by vortexing and repeated pipetting. Solution A helps provide an osmotically-stable cell suspension, so the cells can be lysed gently. The cell suspension was then lysed by adding 200 µl of freshly prepared Solution B (0.2 N NaOH, 1% SDS) and mixed by inverting gently. Solution B contains the anionic detergent SDS which disrupts the cell

membranes and NaOH which denatures nucleic acids. One hundred and fifty microlitres of cold Solution C [3 M sodium acetate (pH 4.8)] was added and the tube was gently inverted and placed on ice for 5 min. Solution C precipitates chromosomal DNA and cellular proteins. The tube was then centrifuged at maximum speed for 15 min to pellet the cellular debris. The supernatant containing the plasmid DNA was removed and precipitated using two volumes of cold 100% ethanol and incubated at -70^{o}C for 30 min. The precipitated plasmid DNA was recovered by centrifugation at maximum speed for 15 min. The resulting pellet was washed once with 1 ml 70% ethanol, without resuspension, and was centrifuged for a further 3 min. The pellet was then air-dried and resuspended in 40 µl TE buffer [10 mM Tris-HCl, 1 mM EDTA (pH 8)] and was stored at -20^{o}C until needed. For specialized applications like PCR and molecular cloning, where DNA of a higher purity was necessary, the GeneJET Plasmid Miniprep Kit (Fermentas Life Sciences) was used according to the manufacturer's instructions.

2.2.1.4 DNA quantification

DNA concentration was determined using the NanoDrop Spectrophotometer ND-1000 (Thermo Scientific). A one microliter aliquot of the sample was loaded onto the stage, and its absorbance was measured at 260 nm. This was done in triplicate and the average value was used as the DNA concentration.

2.2.1.5 Preparation of λ DNA molecular weight marker

An accurate estimation of the size of DNA fragments after agarose gel electrophoresis may determine the success or failure of many molecular biology procedures. Thus, phage λ DNA (Roche Molecular Biochemicals) was restricted with *Eco*RI and *Hin*dIII restriction enzymes to yield 11 bands of known size. Forty seven microlitres of sterile distilled water and 10 µl restriction enzyme buffer B (Roche Molecular Biochemicals) were added to a sterile eppendorf, followed by the addition of 35 µl λ DNA (8.75 µg) and 4 µl each of *Eco*RI (10 U/µl) and *Hin*dIII (10 U/µl).

The mixture was incubated at 37°C for 1 h and then placed at 65°C for 10 min to deactivate the enzymes. Ten microlitres of a 6× 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°C. Aliquots were used as a molecular weight marker on all agarose gels.

2.2.1.6 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 1× TAE buffer, which was diluted from a 50× 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 45 s 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 (section 2.2.1.5) at 110 V for approximately 1 h. Gels were then stained in ethidium bromide (0.05 mg/ml) for 30 min and destained in distilled water for a further 5 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 CFW-1310M camera and Scion Image software (Scion Corporation).

2.2.1.7 Polymerase chain reaction (PCR)

Amplification of the S340 gene in pBSK was carried out using the two standard oligonucleotide primers T3 and T7 (Integrated DNA Technologies). They were used to bind to the template DNA strand and initiate DNA synthesis. The sites at which they bind can be seen in Fig. 2.1.

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

Ten nanograms of template pBSK-S340 plasmid DNA, 0.5 µM of the primers, 0.1 mM dNTPs, 1.5 mM $MgCl_2$, 10× PCR buffer and *Taq* DNA polymerase (Roche Molecular Biochemicals) were used for the PCR reaction, in a total volume of 50 µl.

The cycling conditions for amplification were as follows:

Denaturation	:	1 min at 94°C
Primer annealing	:	1 min at 42°C
Primer extension	:	2 min at 72°C

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

PCR products were separated by agarose gel electrophoresis (section 2.2.1.6) to determine if the target DNA was successfully amplified. Upon successful amplification, the PCR bands were excised from the gel and recovered using the DNA and Gel Band Purification Kit (GE Healthcare) according to the manufacturer's instructions. A small aliquot of the purified PCR products was run on an agarose gel to ascertain successful elution from the purification column.

2.2.1.8 Restriction digestion

Restriction endonucleases recognize and cleave at specific sequences of DNA, and were used for two main applications in this study. Firstly, restriction digestion of both the vector and the insert DNA with suitable restriction enzymes 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*. Such a restriction would yield two characteristic bands of different sizes: a large vector band and a significantly smaller insert band, confirming that a successful clone had been created.

Standard protocols were followed for restriction analysis (Sambrook *et al*., 1989; 2001). For digestion, 0.1 volumes of the corresponding restriction buffers (10×) were added to the DNA solution. Mixtures were incubated with the restriction enzymes (Roche Molecular Biochemicals) at 37°C for 1-2 h, depending on the DNA concentration (section 2.2.1.4). Restricted DNA was analyzed on 0.8% agarose gels.

The purified PCR product (section 2.2.1.7) was restricted using *Bam*HI and *Xho*I in order to create sticky ends compatible with the similarly digested pET vectors to facilitate ligation between both the vectors and insert. Restriction reactions were purified from solution using the DNA and Gel Band Purification Kit (GE Healthcare) according to the manufacturer's instructions. A small aliquot of the purified restriction products were run on a 0.8% agarose gel to ascertain successful purification.

2.2.1.9 Ligation

For the creation of recombinant clones, a molar vector:insert ratio of 1:3 was used to guarantee high ligation efficiency. The purified restricted PCR insert (S340) and purified restricted pET vectors were ligated using the volumes derived from the following equation (Cranenburgh, 2004):

$$V_V = \frac{T}{\left[\frac{V_c \cdot I_l \cdot I_r}{I_c \cdot V_l}\right] + 1} \quad \text{and} \quad I_v = T - V_V$$

where:

I_l = insert length (kb)

V_l = vector length (kb)

I_c = insert concentration (ng/µl)

V_c = vector concentration (ng/µl)

I_r = required vector : insert ratio

T = volume of total DNA solution component

V_v = vector volume (µl)

I_v = insert volume (µl)

The equation allows for the simple calculation of the exact volumes of insert and vector DNA required for a highly efficient ligation reaction. Once the volumes had been determined they were added in a vector:insert ratio of 1:3. The mixture was then heated to 65°C for 5 min to denature the dsDNA into ssDNA, and then rapidly chilled on ice to prevent re-annealing. One microliter of a 10× ligase buffer (Roche Molecular Biochemicals) and 1 µl of T4 DNA ligase (10 U/µl) was also added to the mixture. Sterilized, distilled water was added to a total volume of 10 µl. When performing blunt ended ligations (secondary clones), 1 µl of a 50% polyethylene glycol (PEG) 4000 was

added to increase inter-molecular crowding. The ligation mixture was incubated at 16^{o}C overnight, and then transformed into SEM-competent *E. coli* host cells.

2.2.1.10 Preparation of SEM-competent cells

Host cells were made 'competent' or capable of taking up DNA from their surrounding environment, by exposing them to Ca^{2+}, which interacts with their cell envelopes. Two strains of *E. coli* were used in the study. The first strain was *E. coli* XL1 blue MRF' (Strategene) and was used for all cloning procedures and plasmid storage, whilst *E. coli* BL21 (DE3) pLysS (Favorgen) was used exclusively for expression. Both were made competent using the Simple and Efficient Method, prior to transformation (Ausubel *et al*., 1989).

Both strains were 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°C and shaken overnight. One millilitre of this culture was used to inoculate 29 ml of fresh SOC medium and shaken at 37°C until it reached an OD of 0.375 at 590 nm. 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 $CaCl_2$

containing 10% glycerol. One hundred microlitres of the prepared competent cells were dispensed into eppendorfs, incubated at 4°C overnight, and then stored at -70°C. 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 were therefore incubated at 4°C overnight to enhance the effectiveness of the subsequent transformation procedure.

2.2.1.11 Transformation and screening

Two microlitres of the ligated DNA solutions (section 2.2.1.9) were added to one hundred microlitres of the SEM-competent cells and incubated on ice for 30 min and thereafter subjected to heat shock for 1 min at 42°C. After the heat shock, the cells were immediately incubated on ice for 5 min. Eight hundred microlitres of fresh SOC medium was added to each of the mixtures and they were then shaken at 37°C for 1 h at 200 rpm. The transformation mixtures were plated on RBB-xylan LB plates (0.4% RBB-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°C overnight. The dye, Remazol Brilliant Blue, was linked to birchwood xylan (Roth) for detection of xylanase producers during the transformation process. RBB-xylan was prepared according to the method of Biely *et al*. (1985; 1988).

This served as both a screening and selection method since only those cells that had taken up a plasmid were able to grow on the selective medium supplemented with ampicillin

and it further screened for clones expressing xylanase from those that merely contained self-ligated vectors.

2.2.2 Construction of secondary clones

2.2.2.1 Plasmids

A secondary set of plasmids were constructed, using the same four pET vectors from section 2.2.1.2. All the molecular methods used were identical to the ones already discussed, and any changes or modifications will be discussed. The only difference between the primary clones and the secondary clones was that in the secondary clones a shorter insert was used, which did not include excess DNA on either side of the gene. Using the primers designed in section 2.2.2.2, the S340 gene was amplified using PCR (section 2.2.2.3), restricted with *Bam*HI and *Xho*I, treated with Klenow polymerase (section 2.2.2.4), dephosphorylated with calf intestinal alkaline phosphatase (section 2.2.2.5) and ligated into the four pET vectors (Fig. 2.2). The resulting clones, the secondary clones, were the second set of constructs used in this study, and were called KW1, KW2, KW3 and KW4, respectively. The linear plasmid map of the pET22b vector with the shortened version of S340 (pKW4) is depicted in Fig. 2.4.

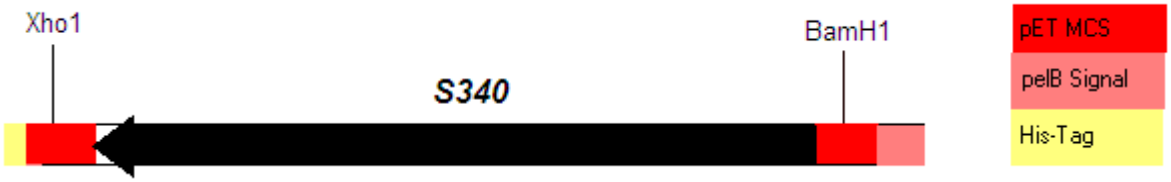

Figure 2.4 A linear plasmid map showing the S340 gene cloned into pET22b (KW4), excluding the flanking DNA and MCS. Restriction sites and locations on the polylinker of the plasmid used for cloning are also shown. Included are the N-terminal *pelB* signal sequence and the C-terminal His·Tag sequence. The black arrow, representing the S340 gene also shows the gene's orientation.

2.2.2.2 Primer design

Three primer sets were designed in order to account for each reading frame of the gene. The three forward primers have identical sequences, only differing in size by a single nucleotide, and were designated PR1, PR2 and PR3 whilst the reverse primer was designated PR4. All were designed using Vector NTI software (Invitrogen). The reason for designing new primers was to remove the fungal DNA at both ends of the gene. Primer set one (PR1 and PR4) shortened the insert from 786 bp to 693 bp, primer set two (PR2 and PR4) shortened the insert from 786 bp to 692 bp, whilst primer set three (PR3 and PR4) shortened the insert from 786 bp to 691 bp. Primer one (PR1) is a 20 bp long oligonucleotide and starts eight nucleotides upstream from the S340 start codon, primer two (PR2) is a 19 bp long oligonucleotide that starts seven nucleotides from the S340 start codon while primer three (PR3) is a 18 bp long oligonucleotide that starts six nucleotides from the S340 start codon. Primer four (PR4) is a 20 bp long oligonucleotide that starts seven nucleotides downstream from the S340 stop codon.

The primers were synthesized by Inqaba Biotechnical Industries. Lyophilized primers were diluted with sterile double distilled water to a final stock concentration of 100 µM and stored at -20°C in 10 µl aliquots. Working stock concentrations of 10 µM were subsequently prepared as required.

2.2.2.3 Polymerase chain Reaction (PCR)

Amplification of S340 in pBSK with newly designed primers PR1, PR2, PR3 and PR4 (section 2.2.2.2) was used to reduce the size of the cloning fragment in this study.

Forward primers:

PR1: (5` - TTGCAGTGATGGTCGGCTTT – 3`)

PR2: (5` - TGCAGTGATGGTCGGCTTT – 3`)

PR3: (5` - GCAGTGATGGTCGGCTTT – 3`)

Reverse primer:

PR4: (5` - TTACGTCTTAGCCCACGTCA – 3`)

Ten nanograms of template pBSK-S340 plasmid DNA, 0.5 µM of the primers, 0.1 mM dNTPs, 10× *Pfu* buffer (Fermentas Life Sciences) with $MgSO_4$ and *Pfu* DNA polymerase (Fermentas Life Sciences) were used for the PCR reactions in a total volume of 50 µl. *Pfu* DNA polymerase, unlike *Taq* DNA polymerase, lacks terminal transferase activity and

therefore generates blunt-ended PCR products, which can be used directly for blunt end ligation reactions.

The cycling conditions for amplification were as follows:

Denaturation	:	1 min at 94°C
Primer annealing	:	1 min at 55°C
Primer extension	:	2 min at 72°C

Three blunt-ended S340 gene inserts were created, differing in size by a single base pair. These inserts were then used in a ligation reaction with the blunt-ended vectors, to create the secondary clones (section 2.2.2.1).

2.2.2.4 Klenow treatment

The Klenow enzyme (Fermentas Life Sciences) was used to fill in the recessed 3´-termini of the four pET vectors restricted with *Bam*HI and *Xho*I. Three micrograms of restricted vector DNA was added to 10× Klenow reaction buffer, 2 mM dNTP mix, Klenow fragment and topped up with sterile deionized water in a total volume of 20 µl. The reaction mixture was incubated at 37°C for 10 min and directly purified using the DNA and Gel Band Purification kit (GE Healthcare) according to the manufacturer's instructions. A small aliquot of the purified filled-in products were run on an agarose gel to ascertain successful purification and then quantified (section 2.2.1.4).

2.2.2.5 Dephosphorylation

Dephosphorylation of the 5'-termini of the four pET vectors after Klenow treatment was undertaken using calf intestinal alkaline phosphatase (Fermentas Life Sciences). This prevented the vectors from self-ligating as they had been made blunt ended. Forty microlitres of restricted vector DNA from section 2.2.2.4 was added to 5 µl of 10× CIAP buffer (Fermentas Life Sciences). One microliter of CIAP was added and distilled water was added to a total volume of 50 µl. The mixture was then incubated at 37°C for 30 min. The reaction was then stopped by heating at 85°C for 15 min, then directly purified using the DNA and Gel Band Purification kit (GE Healthcare) according to the manufacturer's instructions.

2.3 RESULTS

2.3.1 Construction of primary clones

2.3.1.1 Plasmids

The four pET vectors described in section 2.2.1.2 were isolated as described in section 2.2.1.3. They were then linearized using *Eco*RI, in order to check that the plasmids were the correct size, using the procedure as described in section 2.2.1.8. The pET21 series (a, b and c) conformed to their described sizes of 5443, 5442 and 5441 bp, respectively, and the pET22b plasmid conformed to its size of 5493 bp, as can be seen in Fig. 2.5. The λ DNA molecular weight marker prepared in section 2.2.1.5 can be seen in lane 1. In lane 2, the expression vector pKK223-3 (4584 bp) was linearized with *Eco*RI and run alongside the pET vectors as an additional size marker. This gel confirmed the sizes of the pET vectors and showed that the isolated plasmid DNA was of sufficient quality and concentration for the subsequent cloning procedures.

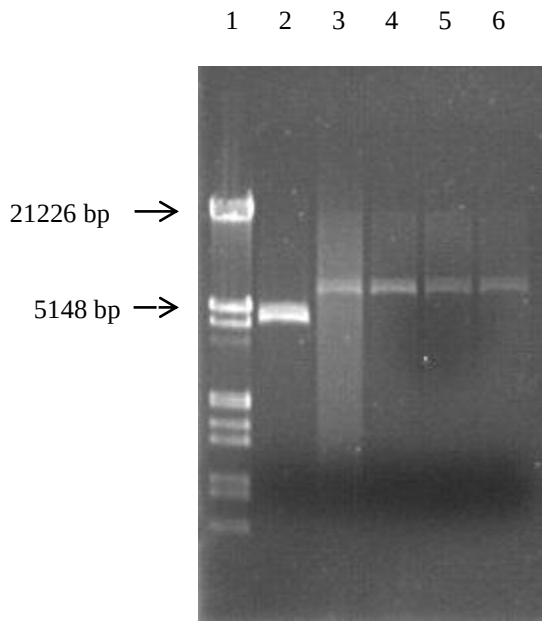

Figure 2.5 Restriction endonuclease digestions of pKK223-3, pET21a, b and c in lanes 2, 3, 4 and 5, respectively. Lane 6: pET22b plasmid; all plasmids were restricted with *Eco*RI. Lane 1: λ DNA molecular weight marker restricted with *Eco*RI and *Hin*dIII.

2.3.1.2 PCR

The pBSK vector containing the S340 gene was isolated as described in section 2.2.1.3, and then subjected to PCR under standard conditions using the T3 and T7 primers. A small aliquot of the PCR product was run on an agarose gel to determine whether or not the gene was successfully amplified and can be seen in Fig. 2.6. Amplification of the approximately 900 bp S340 gene fragment using the T3 and T7 primers is shown in lane 2, whilst a negative control containing all the reagents except the template DNA is shown in lane 3. The 900 bp amplification product contains the S340 cloning fragment (786 bp) as well as the T3 and T7 regions used for primer recognition. The band is very bright

compared to the marker, showing that PCR was successful in amplifying sufficient quantities of the insert.

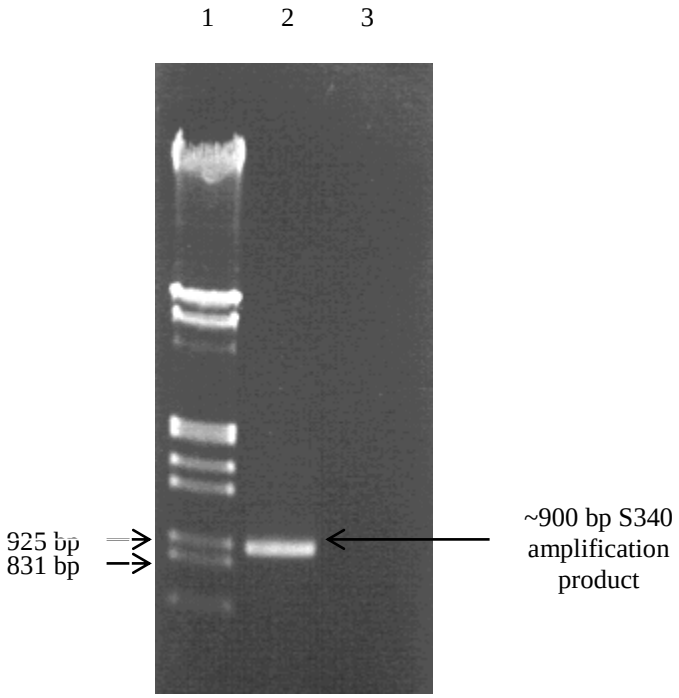

Figure 2.6 Agarose gel electrophoresis of the pBSK vector containing the S340 gene after PCR with T3 and T7 primers. Lane 1: λ DNA molecular weight marker restricted with *Eco*RI and *Hin*dIII. Lane 2: S340 amplification product. Lane 3: The negative control.

2.3.1.3 Screening transformants for xylanase production

The S340 gene insert was ligated into the four pET vectors (section 2.2.1.9) and was cloned into *E. coli.* After plating on RBB-xylan LB plates containing ampicillin (section 2.2.1.11), a few of the positive colonies were chosen and inoculated into 5 ml LB broth for plasmid isolation (section 2.2.1.3). Colonies producing xylanase hydrolyzed RBB-

xylan into colourless degradation products which are observed as a halo around the bacterial colony (Fig. 2.7).

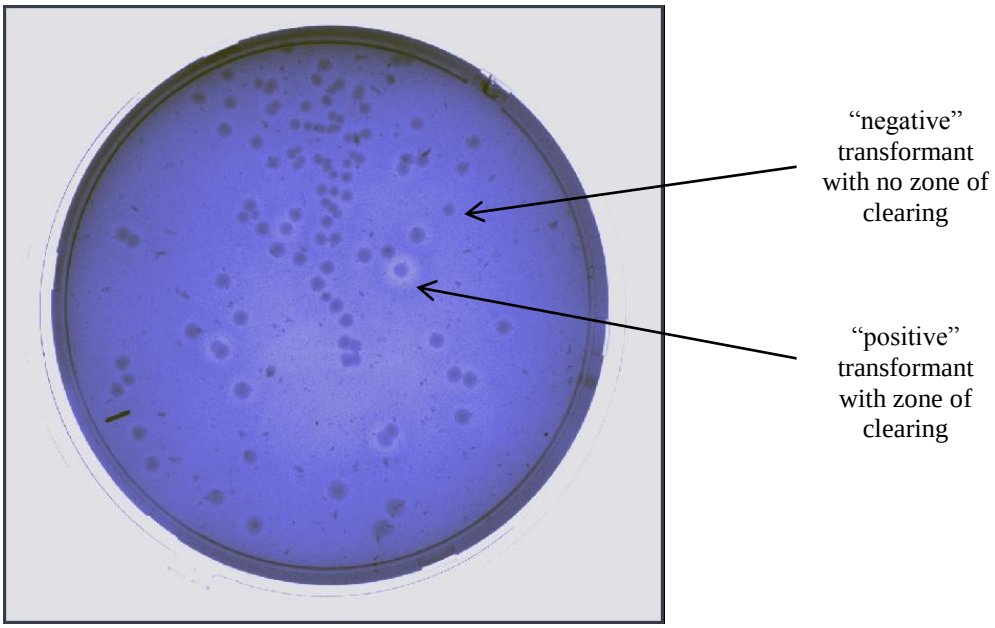

Figure 2.7 Recombinant *E. coli* BL21 colonies after transformation and incubation at 37°C for 16 h. Transformation mixtures were plated on LB medium supplemented with 0.4% RBB-xylan and ampicillin (100 μg/ml). The presence of a clear halo around the bacterial colony is indicative of xylanase production.

2.3.1.4 Restriction analysis of primary clones

The positive xylanase producing clones were grown in 5 ml LB broth supplemented with ampicillin (100 μg/ml) and their plasmids were isolated and subjected to restriction analysis using *Bam*HI and *Xho*I as described in section 2.2.1.8. Lanes 2, 3 and 4 show a large band of around 5400 bp corresponding to pET21a, b and c vectors, respectively,

and can be seen in Fig 2.8. The large band in lane 5 is about 5450 bp in size and corresponds to pET22b vector. The smaller band in lanes 2 – 5 correspond to the S340 gene and is 786 bp in size. This proves that the xylanase gene is indeed present within the vectors. There are a few very faint additional bands present in lane 5. This is probably due to incomplete cleavage products after restriction endonucleases digestion.

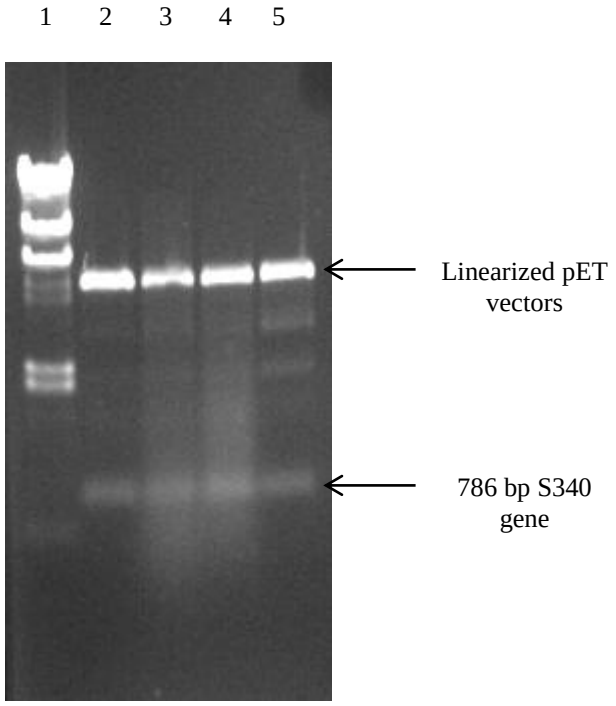

Figure 2.8 Agarose gel electrophoresis of the double digest of the four transformants using *Xho*I and *Bam*HI. Lane 1: The λ DNA molecular weight marker restricted with *Eco*RI and *Hin*dIII. Lane 2: pET21a-S340, lane 3: pET21b-S340, lane 4: pET21c-S340, lane 5: pET22b-S340.

2.3.2 Construction of secondary clones

2.3.2.1 PCR

The pBSK vector containing the S340 gene was isolated as described in section 2.2.1.3, and then subjected to PCR under standard conditions using the newly designed primers; namely PR1, PR2, PR3 and PR4 discussed in section 2.2.2.2. This resulted in three shorter blunt-ended S340 inserts that were used for cloning. Primer set one (PR1 and PR4) resulted in an insert (insert 1) of 693 bp (lane 3), primer set two (PR2 and PR4) resulted in an insert (insert 2) of 692 bp (lane 4) and primer set 3 (PR3 and PR4) resulted in an insert (insert 3) of 691 bp (lane 5). A small aliquot of the PCR products were run on an agarose gel to determine whether or not they were successfully amplified and can be seen in Fig. 2.9. Amplification of the ~900 bp S340 gene fragment using the original T3 and T7 primers is shown in lane 2 and was used as a comparison. The amplification of inserts 2 and 3 in lanes 4 and 5, respectively, was more efficient than that of insert 1 in lane 3. The intensity of their bands suggest they have been amplified to a higher concentration than insert 1. Figure 2.9 confirmed that all the new inserts (lanes 3 – 5) were successfully amplified and were ready for further cloning procedures.

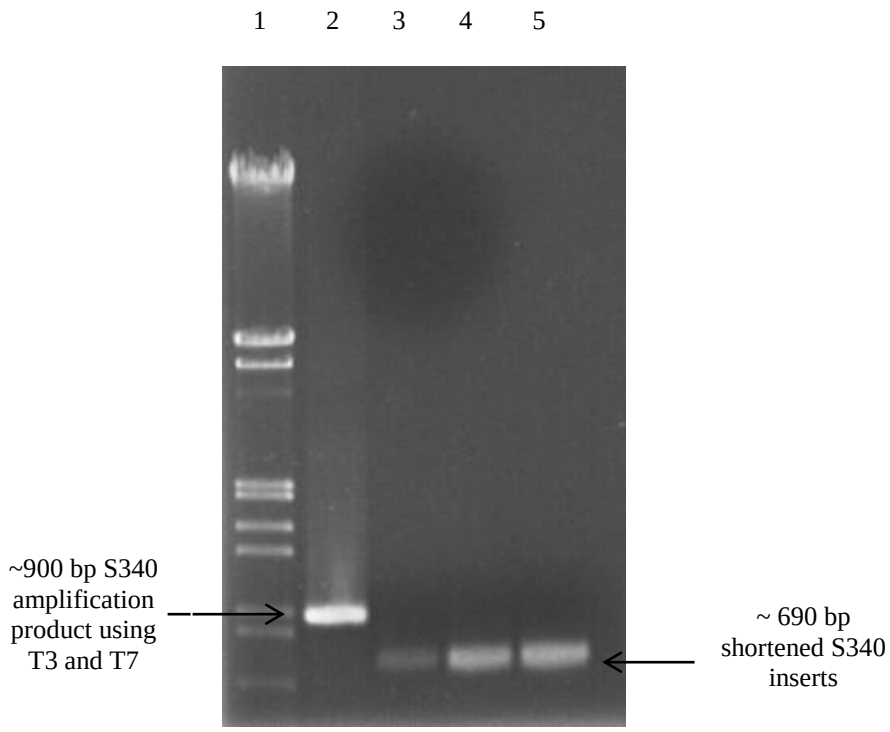

Figure 2.9 Amplification of shortened cloning inserts with newly-designed primers. Lane 2: PCR of pBSK – S340 with T3 and T7, Lane 3: PCR of pBSK – S340 with PR1 + PR4, Lane 4: PCR of pBSK – S340 with PR2 + PR4, Lane 5: PCR of pBSK – S340 with PR3 + PR4. The λ DNA molecular weight marker restricted with *Eco*RI and *Hin*dIII (lane 1).

2.3.2.2. PCR analysis of secondary clones

The newly designed inserts (section 2.2.2.3) were cloned into the four pET expression vectors after they had been blunt-ended by the Klenow fragment (section 2.2.2.4) and dephosphorylated (section 2.2.2.5). All positive transformants displaying xylanase activity were selected, as discussed in section 2.3.1.3. The positive xylanase producing clones were grown in 5 ml LB broth supplemented with ampicillin (100 μg/ml) and their plasmids were isolated and subjected to PCR analysis for confirmation of the presence of the xylanase inserts. PCR analysis was chosen over restriction analysis, as there were

problems finding intact restriction sites flanking the new constructs. PCR of the pET21 series was undertaken, using the new construct DNA as the template as can be seen in Fig. 2.10. The smaller bands at the bottom of lanes 2, 3 and 4 correspond to the S340 xylanase insert 3, the 691 bp insert that was constructed using primer set 3 (section 2.2.2.2). These bands would only have been formed if the insert was indeed present in the construct, as this would have allowed the primers to bind and the insert to be amplified. The larger band in lane 2 is a trace of the template DNA used in the reaction, and is therefore about 6000 bp in size which is approximately the size of the new construct. This confirms the success of the cloning procedures as the new constructs contain the xylanase insert. Amplification of the pET22b clone was also undertaken using the new construct DNA as the template, and can be seen in Fig. 2.11. Lane 2 in that figure shows an S340 insert, which was used for comparison, whilst lane 3 shows that the S340 insert 2 that was indeed present in the pET22b construct (pKW4). These figures both graphically confirm the construction of the secondary clones.

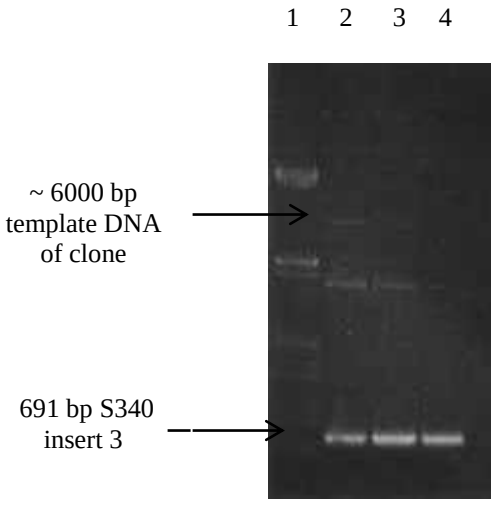

Figure 2.10 Agarose gel electrophoresis of PCR of pET21 series with S340 xylanase insert 3 amplified with PR3 + PR4. Lane 2: pET21a with insert 3 (pKW1), lane 3: pET21b with insert 3 (pKW2) and lane 4: pET21c with insert 3 (pKW3).

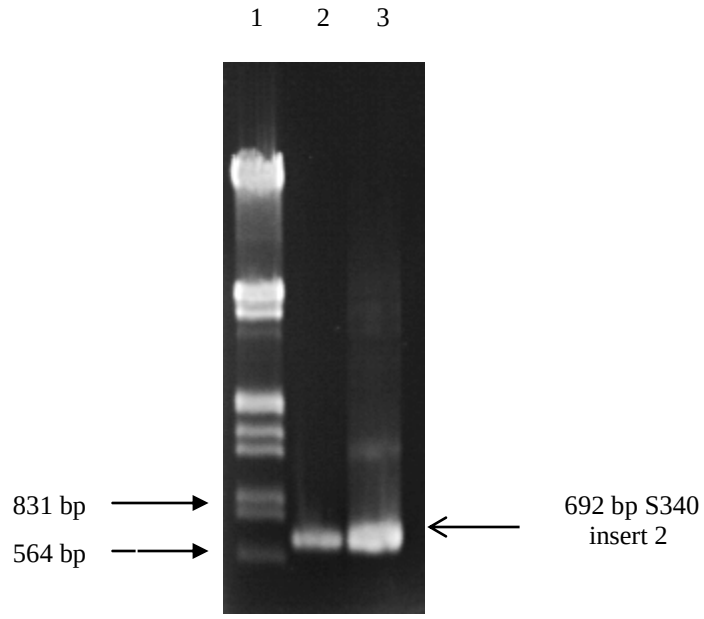

Figure 2.11 Agarose gel electrophoresis of PCR of pET22b vector with S340 xylanase insert 2 amplified with PR2 + PR4. Lane 2: purified S340 insert, lane 3: pET22b with insert 2 (pKW4).The λ DNA molecular weight marker restricted with *Eco*RI and *Hin*dIII (lane 1).

2.4 DISCUSSION

This chapter involved cloning of the modified fungal xylanase (S340) into a set of expression vectors. The goal we hoped to achieve was to have the inserts in vectors that were able to express the xylanase gene in large amounts and would also help in the downstream processing, in this case, purification. The first objective was to clone the insert into a set of three pET21 cloning vectors and a pET22b cloning vector. This initial S340 gene cloning fragment contained fungal DNA flanking either side of the actual open reading frame. Therefore, the initial insert cloned into the vectors was 786 bp in size. The second objective was to firstly shorten the insert with newly designed primers, by removing some of the DNA on either side of the ORF, and then re-clone a trimmed version of the S340 insert back into the four pET expression vectors. The S340 cloning fragment was originally cloned from a genomic DNA library from *T. lanuginosus* using a cosmid vector. Because genomic DNA was used instead of cDNA, there was some excess non-coding DNA left over on either side of the gene that presumably served no function for expression of this gene in *E. coli*. The new inserts created were much smaller after trimming this flanking DNA and range in size from 693-691 bp. It was hoped that there would be an increase in expression in the clones that contained the constructs with the shorter versions of the insert.

The original S340 gene containing the excess DNA both upstream and downstream from the gene was initially amplified using standard T3 and T7 primers (section 2.2.1.7) and the resulting 900 bp fragment can be seen clearly in Fig. 2.6. The four pET vectors were

isolated, restricted with *Bam*HI and *Xho*I as can clearly be seen in Fig. 2.5, and ligated with the similarly treated S340 insert, to form clones that would be selected for on the basis of their ability to degrade xylan (Fig. 2.7). Positive transformants, therefore, contain the xylanase gene that degrades the xylan linked to the RBB dye and therefore results in a zone of clearing indicative of xylan degradation. This method by Biely *et al*. (1985; 1988) is semi-quantitative as it allows one to determine which one of the clones is best expressing the xylanase gene, as the zones of clearing would be larger. In order to verify the incorporation of the gene within the vectors, restriction analysis was undertaken as can be seen in Fig. 2.8. The larger band of around 5400 bp corresponds to the linearised pET21a, pET21b and pET21c vectors, respectively. The large band in lane 5 of around 5450 bp corresponds to the linearised pET22b vector. The smaller band in all the above mentioned lanes corresponds to the 786 bp S340 insert. This confirms both the presence and location of the gene within the expression vectors. The additional bands in lane 5 that were presumably incomplete cleavage products could have been eradicated by extending the time of the digestion, to allow for complete digestion of the DNA. Although the initial insert is 786 bp long, the ORF is only 678 bp in length, which is identical in the size to the *Aspergillus niger* BCC14405 xylanase gene which was cloned into a pET28a vector in *E. coli* strain BL21 (DE3) pLysS (Sriprang *et al.*, 2006). The clones with the largest zones were assayed for xylanase expression in the proceeding chapter, and this was taken as the basal expression levels that we hoped to improve upon.

The construction of the secondary clones began with the design of new primers. The new primers were designed in order to trim the insert by removing the above mentioned

excess DNA from both ends of the gene in order to bring the start codon to within a closer proximity to the Shine-Dalgarno (SD) sequence on the plasmid. The presence and indeed, location of the Shine-Dalgarno sequence or ribosome-binding site is thought to be of paramount importance to the expression levels obtained, however, some authors have reported it not being overly important at all. Nakamoto (2006) showed that the initiation of protein synthesis would indeed take place, even in the absence of the SD sequence, however, how this absence would effect overall expression levels was not discussed in the article. Three sets of primers were designed to create shorter S340 inserts from 693-691 bp in size, to account for each reading frame. The inserts were created using *Pfu* polymerase and are compared to the original gene in Fig. 2.9. This figure clearly shows the decrease in size of the new inserts as compared to the original gene in lane 2. *Pfu* polymerase was used in order to ensure all the inserts contained blunt-ends, which would aid in their ligation reaction with the blunt-ended pET vectors. The secondary clones were transformed and selected for using the identical procedures discussed for the primary clones. With regards to the pET21 series of vectors, primer set 3 (PR3 and PR4) that made the shortest insert (insert 3) were used to clone into the three vectors. With the pET22b vector, all three inserts were cloned into the vector, with only one of them insert 2 (PR2 and PR4), producing clones that degraded xylan. This suggests that only this insert was in frame, and was therefore expressed. With all the secondary clones, restriction analysis of the constructs was unsuccessful, as a double digest would not reveal two bands (one large vector band and one smaller insert band). This could possibly be due to the destruction of the adjacent restriction sites as a result of cloning and therefore the restriction enzymes used could not cleave at their specific sites. So, in order

to confirm the presence of the gene, PCR analysis was undertaken on all the secondary clones. Fig. 2.10 shows the results of the PCR analysis of the pET21 series of clones. Some larger template DNA of around 6000 bp can be seen in some of the lanes, and this would be made up of the intact clone DNA comprised of the pET21 vector DNA and the S340 insert 3. This therefore confirmed the presence of the gene in the pET21 constructs and the fact that it also made a zone of clearing on the RBB-xylan LB plates confirmed that the gene was in the correct orientation. Similarly, Fig. 2.11 shows the results of the PCR analysis of the pET22b construct. Not much template DNA is present, but the smaller band in lane 3 is almost identical in size to the control S340 insert band in lane 2, once again confirming the presence of the gene in the construct.

The primary constructs created in this chapter served as the baseline expression levels in the following chapter, whilst the secondary clones were expected to show an increase in expression. The two sets of clones constructed were the basis for the rest of the study, and they allowed us to determine the effects that the shortening of the xylanase cloning fragment would have on cellular expression levels.

CHAPTER 3

XYLANASE EXPRESSION IN *Escherichia coli*

3.1 INTRODUCTION

E. coli is an extremely useful organism with regards to heterologous protein expression. The fact that it has well-characterised genetic machinery that is well understood, helps researchers in the process of troubleshooting during cloning or expression procedures. When ultimately looking at the feasibility of scaling up a particular process, *E. coli*-based expression systems can be particularly economical due to the high biomass-to-cost ratio (Xu and Foong, 2008). Provided all the process parameters for expression can be optimised, expression levels can be quite high. Most expression systems today are under control of a repressed promoter, this allows for a greater degree of control by allowing for the separation between the cell growth phase and the heterologous protein production phase (Gombert and Kilikian, 1998). An important factor to remember is that *E. coli* does not have a natural transporter system to translocate the majority of heterologous proteins into the extracellular environment, so enzyme activity is directly proportional to the cell concentration (Ramchuran *et al.*, 2002).

One of the more important factors that has received attention is the growth temperature. Conventionally, *E. coli* has always been grown at 37°C. However, it is a fairly well established fact that growth at 30°C almost always increases soluble protein yields. The

lowered growth temperature is thought to facilitate a decrease in inclusion body formation *in vivo,* resulting in an increase in soluble, bioactive heterologous protein (Schein, 1989; Yeh *et al.,* 2005). Not only does it affect the amount of soluble protein present, but the lowering of the growth temperature also reduces the nutrient uptake and growth rates, lowering toxic by-product formation. This also allows for a reduction in the cellular oxygen demand, allowing higher cell-densities to be obtained (Lee, 1996). The induction strategy is also of vital importance if the target gene is to be overexpressed. Conventionally, the non-hydrolysable lactose analog isopropyl-β-D-thiogalactopyranoside (IPTG) is used to induce *E. coli* cultures. However, IPTG remains very expensive, often becoming a limiting factor in the up-scaling of a fermentation process. Choi *et al.* (2000) also showed that high IPTG concentrations often result in the inhibition of cell growth and recombinant protein production. Recently, there has been much success in substituting IPTG for its natural analog, lactose. Lactose is not only cheaper to use, especially when up-scaling the fermentation, but it is not toxic to the *E. coli* cells themselves (Baneyx, 1999; Ramchuran *et al.,* 2005; Kim *et al.,* 2007). The use of lactose is more difficult when trying to establish the ideal induction conditions, because lactose will serve simultaneously as an inducer and as a carbon and energy source (Gombert and Kilikian, 1998).

E. coli cells essentially consist of both inner and outer membranes that are responsible in dividing the organism into three main compartments: the cytoplasm, the periplasm and the extracellular environment. Recombinant protein production can be targeted to one or more of these target areas (Choi *et al.,* 2006). The choice of a target destination depends

upon the characteristic advantages and disadvantages of that particular compartment, which are summarised in Fig. 3.1.

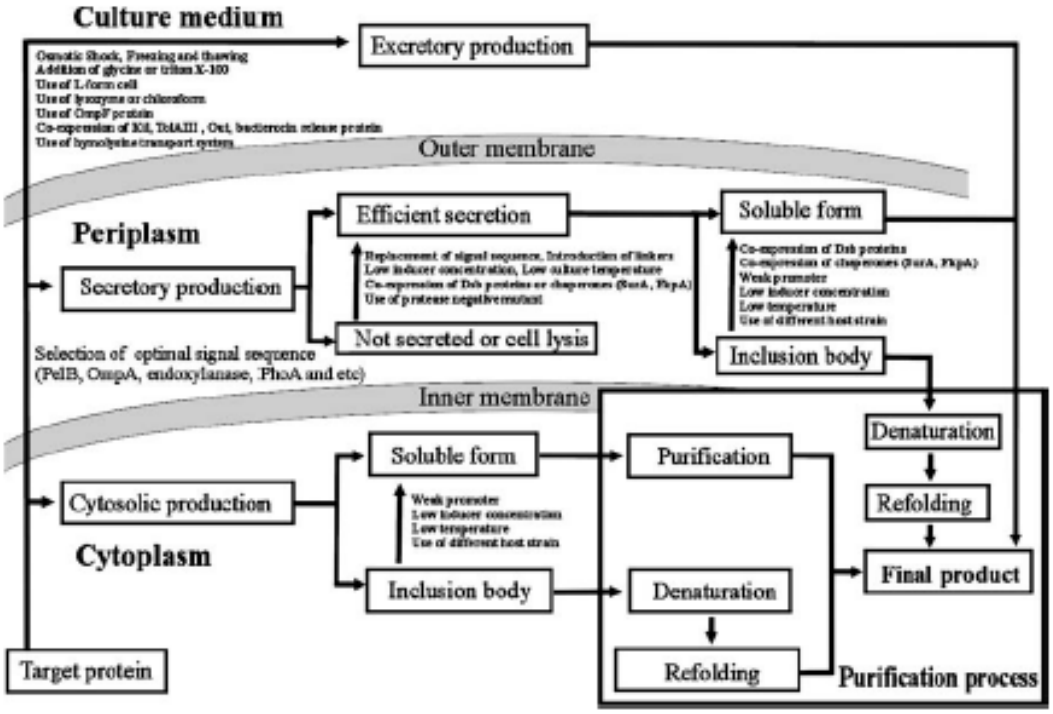

Figure 3.1 Strategies for the production of heterologous proteins in the three different compartments in *Escherichia coli* (Choi *et al.*, 2006).

The purpose of this chapter was to determine the xylanase expression levels in both the primary and secondary clones constructed in the previous chapter. Before this could be undertaken, three expression parameters were optimised. These were the optimal growth temperature for increased xylanase production, the most suitable inducer and the optimal induction time to use. Once this had been established, the expression levels of the primary and secondary clones were determined. Finally, two of the clones were fractionated to determine the localisation of the xylanase enzyme.

3.2 MATERIALS AND METHODS

3.2.1 Growth of primary clones and enzyme extraction

Two sets of growth conditions were tested in this section. The first growth temperature was 30°C and the second was 37°C. Besides changing the growth temperature, other conditions were identical, allowing the results obtained to be attributed only to the growth temperature. Only the primary clones were used in the optimisation of expression, as the effect of a shortened cloning fragment on the fermentation conditions were thought to be negligible. The four primary clones constructed in section 2.2.1 that exhibited the largest zones on RBB-xylan LB plates and therefore the highest xylanase activity, were picked with a sterile toothpick and streaked onto an LB plate containing 100 μg/ml ampicillin. Two controls were used; the first one contained the original S340 gene in the pBSK vector in *E. coli* XL1 Blue, whilst the second clone also contained the original S340 gene in the pBSK vector but in *E. coli* BL21. This was done so differences arising in expression could be attributed to specific characteristics of the host-vector system. Subsequently, a single colony from the overnight plates was inoculated into 20 ml LB broth containing 100 μg/ml ampicillin and incubated for 12 h at 30°C and at 37°C in a shaking incubator at 200 rpm. After 12 h the optical density (OD) at 600 nm was checked to ensure that all the tubes, had similar concentrations of cells. Tubes with higher cell concentrations were diluted appropriately in LB broth. The cells were then pelleted by centrifugation at 5000 *g* for 5 min. The cell pellets were then resuspended in 2 ml LB broth and inoculated into 1 L Erlenmeyer flasks containing 200 ml LB broth and 100

μg/ml ampicillin, resulting in a 10% (v/v) inoculum. OD_{600} readings were taken at time 0 min and at 30 min intervals. The flasks were placed in a shaking incubator at 200 rpm, until the OD_{600} value reached 0.65. Once this had been reached, the cultures were induced with 1 mM IPTG. The cultures were placed back in the shaking incubator for a further 3 h.

The cells were then harvested by centrifugation at 16 000 *g* for 10 min in 2 ml eppendorfs. The supernatant was decanted and the pellet allowed to dry. Five hundred microlitres of BugBuster Protein Extraction Reagent (Novagen) was added, as well as 5 μl of Benzonase nuclease and 10 μl of Protease Inhibitor Cocktail Set II (Calbiochem - 20 mM AEBSF hydrochloride, 1.7 mM bestatin, 200 μM E-64, 85 mM disodium EDTA, 2 mM pepstatin A). The cell pellet was then resuspended and incubated in a shaker at room temperature for 20 min at 40 rpm to gently lyse the cells. The insoluble cellular debris was pelleted by centrifugation at 16 000 *g* for 20 min at 4°C. The pellet was saved for inclusion body purification in section 3.2.3. The supernatant containing the soluble protein fraction was transferred into fresh eppendorfs and stored at -20°C until it was needed for the DNS assay in the following section.

3.2.2 Dinitrosalicylic acid (DNS) assay

The DNS method (Bailey *et al.*, 1992) is used to determine xylanase activity using 1% (w/v) birchwood 4-*O*-methyl glucuronoxylan (Roth) dissolved in 50 mM Na-citrate buffer (pH 6.5) as the substrate. One hundred microlitres of the suitably diluted crude

enzyme solution (section 3.2.1) was added to 900 µl of the substrate solution, mixed, and incubated for 5 min at 50°C. After this incubation, 1.5 ml of the DNS reagent (10 g/L 3,5-dinitrosalicylic acid, 16 g/L NaOH, 300 g/L sodium potassium tartrate) was added to stop the reaction. The solution was then boiled for 5 min and allowed to cool in cold water. Once cool, the absorbance was measured at 540 nm against a reagent blank. This absorbance was then corrected for background colour against an enzyme blank. The absorbance was then converted to nkat/ml or IU using a xylose standard curve. Each experiment was done in triplicate. One unit of xylanase activity was defined as the amount of enzyme that produced 1 µmol of xylose equivalents per minute.

3.2.3 Inclusion body extraction and solubilisation

The inclusion bodies are part of the insoluble cellular fraction obtained in section 3.2.1. Since the inclusion bodies are insoluble by nature, they need to be purified and then solubilised from the rest of the insoluble fraction before any further work can be done on them. The most popular methods to achieve this involve differential centrifugation and washing with denaturants or detergents. The pellet containing the insoluble cellular debris from section 3.2.1 was resuspended in 300 µl BugBuster Protein Extraction Reagent (Novagen, 2004). The suspension was mixed by gently vortexing the eppendorfs and incubated at room temperature for 5 min. Following this resuspension, 6 volumes of a 1:10 diluted BugBuster reagent (in deionized water) was added to the suspension and mixed by vortexing for 1 min. The suspension was then centrifuged at 7 000 *g* for 15 min at 4°C. This centrifugation sequestered the inclusion bodies in the pellet, so the

supernatant was discarded. The pellet was then resuspended in half the original volume of 1:10 diluted BugBuster reagent (150 µl), mixed by vortexing and centrifuged again at 7 000 *g* for 15 min at 4°C. This step was repeated twice. The pellet was then resuspended once more, centrifuged at 16 000 *g* for 15 min at 4°C and the supernatant was then removed.

The cell pellet contained the purified inclusion bodies that needed to be solubilised. The pellet was resuspended in 250 µl solubilisation buffer (phosphate buffered saline and 8 M urea) and incubated for 60 min at 4°C. The suspension was then centrifuged at 16 000 *g* for 20 min at 4°C. The supernatant containing the solubilised inclusion bodies was then removed and kept at 4°C until concentrated.

3.2.4 Precipitation of inclusion bodies

The supernatant containing the solubilised inclusion bodies obtained in the previous step underwent acetone precipitation to eliminate some of the soluble contaminants as well as to concentrate the protein sample. To the supernatants obtained in section 3.2.3, four volumes of cold acetone (-20°C) was added. The eppendorfs were then vortexed well and then incubated for 60 min at -20°C. After the incubation, the tubes were then centrifuged for 10 min at 16 000 *g*. The supernatant was then decanted and disposed of, whilst the pellet was allowed to air-dry for 30 min to allow the acetone to evaporate. The pellet was resuspended in 30 µl of solubilisation buffer (section 3.2.3).

3.2.5 SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the inclusion body proteins and to visualize the difference in protein concentration between the clones grown at 30°C and the ones grown at 37°C. A 12% bis-tris pre-cast gel (Bio-Rad) was used for separation. Five microlitres of the purified, solubilised inclusion body solution was then added to 5 µl of 2x SDS-PAGE sample buffer (Bio-Rad) and boiled for 2 min. The 10 µl samples were loaded into the wells and the gel was run at 110 V for approximately 3 h. Following SDS-PAGE, the gel was stained with Coomassie brilliant blue G-250. A low range molecular weight marker (Sigma-Aldrich) with a range from 6.5 – 66 kDa was used to determine the sizes of the various bands.

3.2.6 Growth and induction of primary clones

The induction optimisation was similar to the temperature optimisation in that all the clones were used, however, the temperature and other variables were kept constant at 30°C, and only the choice of inducer was changed. IPTG is the most commonly used inducer, however this compound is not only expensive, but is also toxic to the cells. So a much cheaper and less toxic option was chosen to compare it against: lactose. The length of induction period was also a parameter that needed to be assessed, so half hourly OD_{600} readings and 2 ml eppendorf samples were taken. The inoculum preparation and growth of organisms was identical to that in section 3.2.1. The only difference was that when

induction started, one set of the clones were induced with 1 mM IPTG, whilst the other set of clones were induced with 7.28 mM lactose, as this was determined to be the optimum lactose concentration by Sunitha *et al.* (2000). The induction was allowed to continue for 9 hours, taking half hourly samples, to determine the optimum induction time for the different inducers. The crude enzyme extracts were then obtained from all the samples collected as in section 3.2.1. These crude enzyme extracts were then assayed using the DNS assay as in section 3.2.2.

3.2.7 Optimised expression of primary and secondary clones

Since the optimum growth temperature, optimum inducer and induction time had been determined, both the primary and secondary clones could be assessed regarding their xylanase activity. Both sets of clones, primary and secondary, were grown as described in section 3.2.1, using the optimum growth temperature of 30°C and the optimum inducer, 7.28 mM lactose. The xylanase activities were measured using the DNS assay (section 3.2.2).

3.2.8 Cellular fractionation

In order to determine the localisation of xylanase in *E. coli*, it was important to fractionate the cells, and determine the xylanase content in each fraction. These are the extracellular fraction, periplasmic fraction and intracellular (cytoplasmic) fraction. The original method was described by Ames *et al.* (1984). However, this study used a

modified method by Huang *et al.* (2006). Both sets of clones were grown up in same way (section 3.2.1). Once induction was complete, 10 ml from each flask was removed and the cells were harvested by centrifugation at 5 000 *g* for 10 min. The supernatant was removed, filter sterilized and stored at -20°C for later measurement of the xylanase activity in the extracellular fraction. The periplasmic proteins were then released by incubation with 20 ml chloroform at room temperature for 20 min. The suspension was then centrifuged at 5 000 *g* for 20 min at 4°C. The supernatant containing the periplasmic proteins was withdrawn and stored at -20°C until its activity was measured. The cytoplasmic fraction was obtained by resuspending the pellet in 500 µl of BugBuster reagent (section 3.2.1). This fraction was also stored at -20°C until its activity could be measured by the DNS assay (section 3.2.2).

3.3 RESULTS

3.3.1 Optimisation of the growth temperature

The xylanase activities of all the clones were assayed using the DNS assay (section 3.2.2), the first set was grown at 30°C while the second set was grown at 37°C. Both sets contained six different strains, two controls containing the original S340 gene in pBSK, and the four primary clones from section 2.3.1.1. All the growth conditions were identical, except the growth temperature. As is clearly illustrated in Table 3.1, the growth temperature had a direct effect on the xylanase activity of every clone. Growth at 30°C resulted in clones that produced higher overall xylanase activities than those grown at 37°C. Since temperature was the only variable in the experiment, it follows that that xylanase expression is a function of the growth temperature. The BL21 [pET21a-S340] clone attained the highest overall xylanase activity of 465.3 ± 1.45 nkat/ml when grown at 30°C, however it was only able to achieve a xylanase activity of 392.33 ± 2.18 nkat/ml when grown at 37°C. The host *E. coli* strain also had an effect on the xylanase activity, as the original S340 gene in *E. coli* XL1 Blue yielded an activity of 254.3 ± 2.2 nkat/ml at 30°C, whilst the same construct in the *E. coli* BL21 strain yielded an activity of 374.45 ± 1.85 nkat/ml at 30°C, a 32% higher value. The choice of plasmid vector also had an effect on the xylanase activity. When the S340 gene in the pBSK vector was tested, a maximum value of 374.45 ± 1.85 nkat/ml at 30°C was attained. By changing the vector to the pET21a plasmid, the activity was increased by 19.4% to 465.3 ± 1.45 nkat/ml when grown at 30°C.

Table 3.1 The xylanase activities of the six different clones, grown at 30°C and 37°C

Strain (clone)	Xylanase activity (nkat/ml) after growth at 30°C	Xylanase activity (nkat/ml) after growth at 37°C
XL1 Blue [pBSK – S340]	254.3 ± 2.2	190.21 ± 2.9
BL21 [pBSK – S340]	374.45 ± 1.85	280.12 ± 1.01
BL21 [pET21a – S340]	465.3 ± 1.45	392.33 ± 2.18
BL21 [pET21b – S340]	401.89 ± 2.19	322.08 ± 3.45
BL21 [pET21c – S340]	411.8 ± 3.1	337.93 ± 2.85
BL21 [pET22b – S340]	443.76 ± 2.9	372.62 ± 2.15

3.3.2 SDS-PAGE

The insoluble fractions containing the inclusion bodies obtained from the four primary clones (section 3.2.1) were purified and solubilised (section 3.2.3) and concentrated by acetone precipitation (section 3.2.4). They were then loaded onto a polyacrylamide gel and separated by electrophoresis (Fig. 3.2). The first important observation was the disparity between protein concentrations between the clones grown at 30°C as opposed to those grown at 37°C. The highlighted band in lane 4 with a molecular weight of approximately 24 kDa which corresponds to the xylanase enzyme, is much darker than it is in lane 3 where it is barely visible. This pattern seems to prevail throughout all the pairs of clones at the two different growth temperatures; however it was not as pronounced in the other pairs.

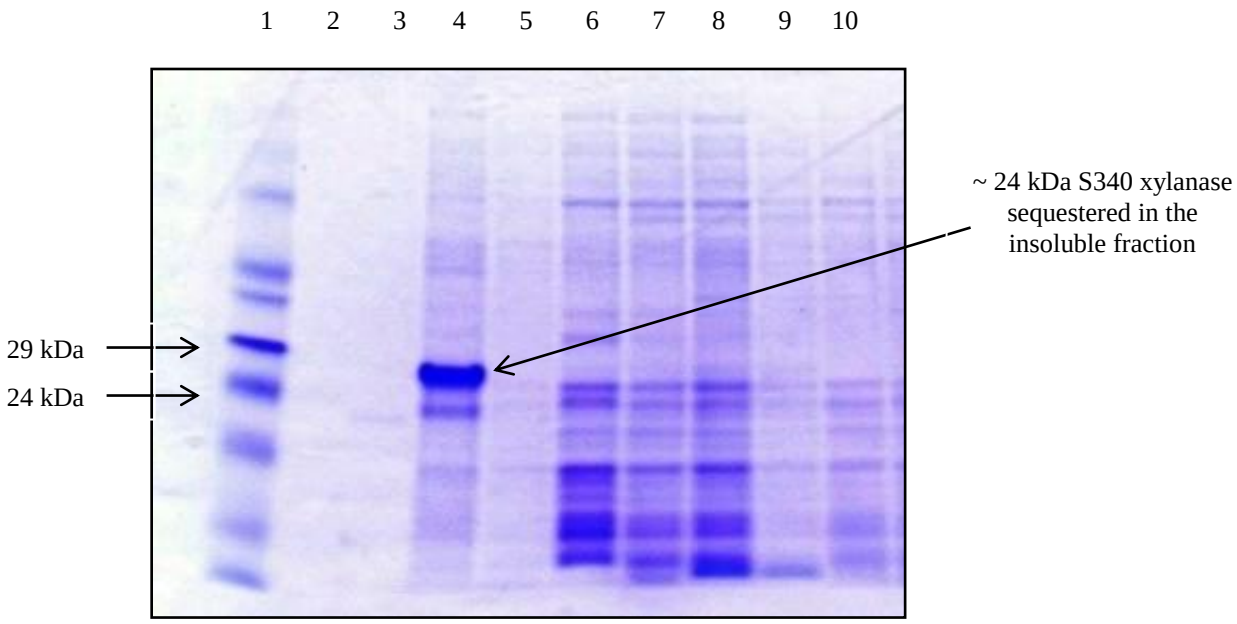

Figure 3.2 SDS-PAGE of the insoluble fractions of the four primary clones. Lane 1: Low range molecular weight marker (6.5 kDa – 66 kDa), Lane 2: empty, Lane 3: BL21 [pET21a-S340] (30°C), Lane 4: BL21 [pET21a-S340] (37°C), Lane 5: BL21 [pET21b-S340] (30°C), Lane 6: BL21 [pET21b-S340] (37°C), Lane 7: BL21 [pET21c-S340] (30°C), Lane 8: BL21 [pET21c-S340] (37°C), Lane 9: BL21 [pET22b-S340] (30°C), Lane 10: BL21 [pET22b-S340] (37°C).

3.3.3 Optimisation of induction

The four primary clones were grown in an identical manner (section 3.2.1) at the optimum temperature of 30°C (section 3.3.1). Two sets were grown; one set was subjected to induction with IPTG, whilst the other was induced with lactose (section 3.2.1). Half hourly samples were taken as well as half hourly OD_{600} measurements. The results of the differential induction with IPTG and lactose using the construct BL21 [pET21a - S340] can be seen in Fig. 3.3 – Fig. 3.5. When using IPTG as the inducer, as is shown in Fig. 3.2, there was a rapid increase in xylanase production post-induction, with

the maximum value of 674 nkat/ml being obtained in 5.5 h. When using lactose as the inducer, as is shown in Fig. 3.4, the gradient of the curve is much shallower, meaning that the maximum xylanase activity was achieved over a much longer period of time. It reached a maximum value of 843.2 nkat/ml after 8.5 h. The starting OD_{600} values were 0.65 at induction and were still very similar after 30 min of induction, in Fig. 3.3 (IPTG) the value was 0.72, whilst in Fig. 3.4 (lactose) the value was 0.727, showing that the increase in activity was not a consequence of a differing biomass. The final OD_{600} values were slightly different, with IPTG the value of 2.81 was obtained, whilst with lactose the value of 2.86 was slightly higher. When superimposing the xylanase activity graphs as is shown in Fig. 3.5, it is easy to see the difference in the rate of xylanase production between the two inducers. It is also interesting to note that after 5.5 h post-induction, the xylanase activities of IPTG and lactose are very similar, being 674 nkat/ml and 661.7 nkat/ml, respectively.

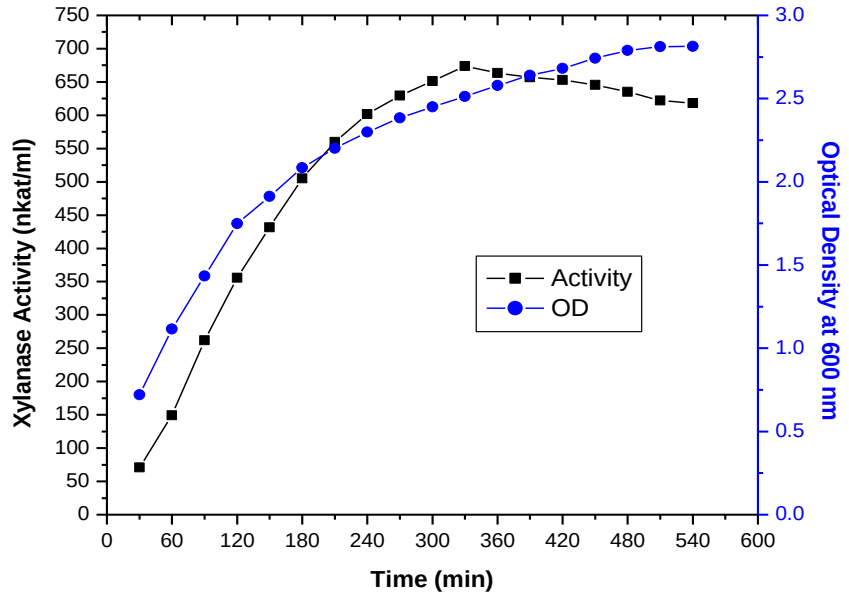

Figure 3.3 The relationship between xylanase activity (nkat/ml) and optical density after induction with IPTG over a 9 hour period, sampling half hourly.

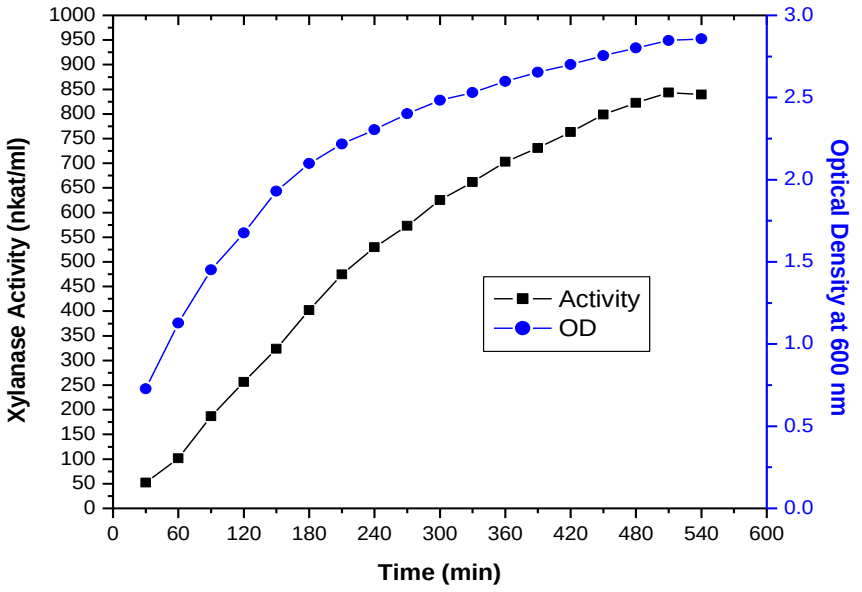

Figure 3.4 The relationship between xylanase activity (nkat/ml) and optical density after induction with lactose over a 9 hour period, sampling half hourly.

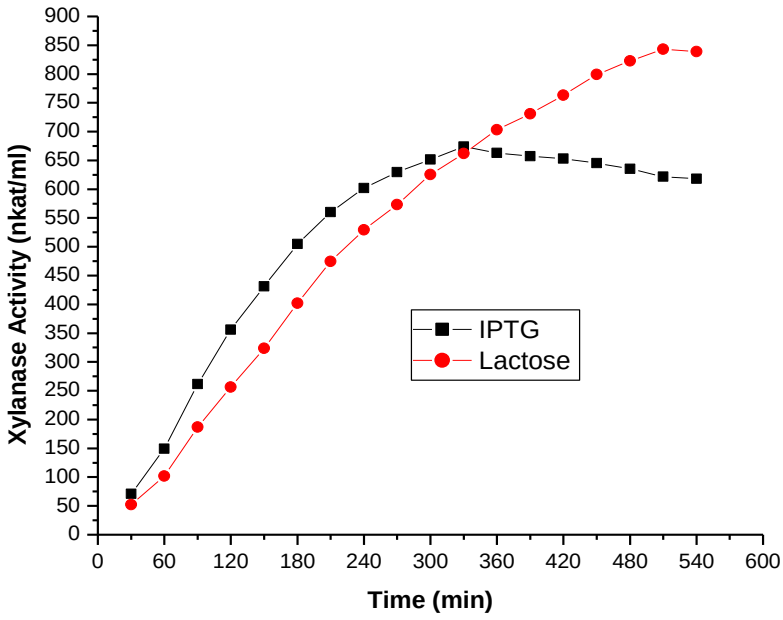

Figure 3.5 The xylanase activity (nkat/ml) after IPTG vs. lactose induction over a 9 hour period.

3.3.4 Expression of primary and secondary clones

The shake-flask fermentation conditions were optimised using the primary clones. The optimum temperature that had been in elucidated (section 3.3.1) and the choice of induction strategy (section 3.3.2) were used to grow both the primary and secondary clones. Taking the above optimisation into account, the two sets of clones were grown at 30°C using lactose as the inducer for an induction time of 8.5 h. The fact that all the conditions between the two sets were optimised as well as identical in every manner except for the S340 cloning fragment itself, allowed us to determine the direct effect that the shortening of the cloning fragment had on expression levels. As can be seen in Table 3.2, the secondary clones had higher xylanase activities than the primary clones using the

optimised expression conditions. It was clearly evident that the shortening of the insert did increase gene expression in every vector. The highest xylanase activity in the primary clones was 843.2 nkat/ml with BL21 [pET21a – S340], whilst in the secondary clones the highest value obtained was 921.8 nkat/ml with KW4. When comparing with the BL21 [pET22b – S340] primary clone its value of 784.3 nkat/ml was some 14.9% lower than that of KW4 at 921.8 nkat/ml.

Table 3.2 The xylanase activities (nkat/ml) of the primary and secondary clones after lactose induction for 8.5 h at 30°C

Clone	**Xylanase activity (nkat/ml) after lactose induction**
Primary clones	
BL21 [pET21a – S340]	843.2
BL21 [pET21b – S340]	692.34
BL21 [pET21c – S340]	723.5
BL21 [pET22b – S340]	784.3
Secondary clones	
KW1	867.51
KW2	744.17
KW3	767.3
KW4	921.8

3.3.5 Cellular fractionation

In order to determine the xylanase localisation, the different fractions were separated (section 3.2.4) and assayed independently using the DNS method (section 3.2.2). Only two of the secondary clones were chosen, the KW1 clone and the KW4 clone. These two clones were specifically chosen because only one of them, KW4, contained an N-terminal *pelB* leader sequence for periplasmic localisation, so any difference in the xylanase localisation was investigated. As can be seen in Table 3.3, both clones have a limited ability to transport the xylanase into the extracellular fraction or supernatant. Whilst, there is a difference in the ability of the clones to transport the xylanase into the periplasmic space, with KW1 36.2 ± 1.21% of the xylanase was found in this compartment, compared to the much higher 51.3 ± 1.17% of the xylanase in KW4. There was a corresponding difference between the amounts of cytoplasmic xylanase found between the KW1 and KW4 clones with values of 45.5 ± 1.78% and 31.4 ± 0.97%, respectively.

Table 3.3 The percentage of xylanase activity found in the extracellular, periplasmic and cytoplasmic fractions of the KW1 and KW4 clones

Fraction	**Clone**	
	KW1	**KW4**
Extracellular	18.3 ± 1.43%	17.3 ± 2.07%
Periplasmic	36.2 ± 1.21%	51.3 ± 1.17%
Cytoplasmic	45.5 ± 1.78%	31.4 ± 0.97%

3.4 DISCUSSION

The previous chapter dealt with the construction of a set of primary and secondary clones, only differing in the size of the S340 cloning fragment that had been cloned into them. This chapter was involved in all aspects of the expression of this gene from these clones. We began with optimising the expression strategy, in order to maximise heterologous protein expression as well as determine a standardised protocol, allowing the size of the insert in the constructs to be the only variable in the experiments. The first part of the optimisation involved determining the optimum growth temperature for protein production, using temperatures of 30°C and 37°C. The second part of the optimisation was involved the choice of inducer and induction time. Two different inducers were used, IPTG as well as lactose, and the optimal induction time was determined by taking half-hourly samples over the course of the post-induction phase. Once the optimal parameters had been elucidated, the expression levels of the primary and secondary clones were determined. Finally, the localisation of the xylanase enzyme was determined, in order to check for differential transport between two of the constructs.

The first part of this chapter dealt with determining the best growth temperature for expression of soluble proteins. As was previously mentioned, 37°C has been used as the optimum growth temperature in many studies. However, current research shows that a growth temperature of 30°C greatly increases the proportion of soluble to insoluble proteins (Yeh *et al.,* 2005). The fact is well illustrated in Fig. 3.2, whereby the bands in lanes 4, 6, 8, 10 where insoluble proteins grown at 37°C are darker than the bands in

lanes 3, 5, 7, 9 which were soluble proteins grown at 30°C. It follows that protein solubility seems to be a function of temperature. The results in Table 3.1 show that the BL21 [pET21a – S340] clone had a xylanase activity of 465.3 ± 1.45 nkat/ml when grown at 30°C, however it was only able to achieve a xylanase activity of 392.33 ± 2.18 nkat/ml when grown at 37°C. This correlation held true for all the clones grown 30°C, as they were all higher than the ones grown at 37°C. A possible reason for this is the increase in formation of proteases at elevated growth temperatures. Arnold and Ulbrich-Hofmann (2001), found that there is a partial elimination of heat-shock proteases upon reduction of the growth temperature, under overexpression conditions. In one study, however, there was no correlation between the presence of proteases and a reduction in xylanase activity. This fact was explained by the structural rigidity of thermostable enzymes helping them remain resistant to proteolytic degradation (Ramchuran *et al.*, 2002). Another factor that might have been involved in the xylanase activity reduction, was that of the formation of inclusion bodies at elevated growth temperatures. When overexpressing target proteins at higher growth temperatures, macromolecular crowding can result and this presents an unfavourable environment for protein folding (Sorensen & Mortensen, 2005). Mohorcic *et al.* (2009) reported a much higher production of soluble versatile peroxidase in *E. coli* at 25°C as opposed to 37°C. The results also illustrated the improvement in xylanase activity when changing the host *E. coli* strain. Generally, *E. coli* XL1 Blue is used for cloning and plasmid storage, whilst *E. coli* BL21 strains are used purely for expression purposes, validating the higher values attained when using the BL21 strain. Mohorcic *et al.* (2009) tested the expression levels of various different *E. coli* strains, with BL21 far outperforming the competitors. The reason for this was the

ability of the cells to grow to high densities and the fact that it is deficient in many cytoplasmic proteases.

The second part of the chapter dealt with optimising an induction strategy. Firstly, a choice between IPTG and lactose had to be made, and secondly the optimal induction period had to be determined by taking half-hourly samples and testing the xylanase activities at these intervals. It was clearly evident that when inducing with IPTG, enzyme induction was very fast and the maximum xylanase activity was reached relatively quickly after 5.5 h (Fig. 3.3). The lactose induction was much slower and more gradual, and only reached the maximal xylanase activity after 8.5 h. A possible reason for the laboured induction response to lactose is the fact that lactose has to be first converted to the true inducer allolactose, this could account for the slow induction period, and gradual but extended post-induction period (Karlsson *et al.*, 1999). To illustrate this point, Ramchuran *et al.* (2005) showed IPTG post-induction periods of 3 h and lactose post-induction periods of 6 h. These post-induction phases were slightly shorter than the current study reported. This could possibly be due to the fact that they used a growth temperature of 37°C, allowing the growth rate and enzyme production phases to be more rapid. When using lactose as the inducer, a maximum xylanase activity of 843.2 nkat/ml was attained, whilst when using IPTG, a maximum activity of 674 nkat/ml was attained. This was in contrast to what had been found by Gombert and Kilikian (1998) where they found similar expression levels when using lactose and IPTG to induce *E. coli* cultures to overexpress chicken muscle troponin C (TnC). However, similar differences in expression levels as a result of the inducer were found in another study by Ramchuran *et*

al., (2005), whereby lactose far outperformed IPTG when inducing a *Rhodothermus marinus* xylanase in *E. coli.* A possible reason for the decreased expression levels and lower levels of final cell density in the IPTG cultivations, is the fact that IPTG is in fact a synthetic analog that is toxic to the cells themselves (Baneyx, 1999). By being toxic at higher levels, IPTG can actually cause a stress response in *E. coli*, which can reduce the growth rate and in some cases cause elevated levels of stress response proteins (Andersson *et al.*, 1996).

Once the optimal conditions for xylanase expression had been determined, the 30°C optimal temperature and the use of lactose for an 8.5 h induction period were used on the both the primary and secondary clones constructed in chapter 2. This allowed us to ascertain the exact effect the shortening of the xylanase cloning fragment would have on xylanase overexpression. The secondary clones containing the shortened xylanase cloning fragment all outperformed the primary clones to varying degrees. The KW4 clone had a 14.9% increase in xylanase activity from 784.3 nkat/ml (47 U/ml) to 921.8 nkat/ml (55.3 U/ml). It is a well-established fact that for optimal expression levels when using a cloning vector, the closer the ATG start codon of the insert is to the Shine-Dalgarno (SD) sequence, the better. According to Hartley (2006), the ATG start codon should be within 5 – 13 bp downstream of the SD sequence in order to increase the recognition of the start codon by the ribosomes in *E. coli*. So, the fact that the size of the cloning fragment had been significantly trimmed probably helped the ribosomes in recognising the start codon, by eliminating interfering excess DNA. The highest level of xylanase production in this study was 921.8 $nkat.ml^{-1}$ or 55.3 $U.ml^{-1}$ after 8.5 h. These

results were very impressive when looking at xylanase expression in *E. coli*. Kubata *et al.* (1997) reported levels of 0.7 $U.ml^{-1}$ of xylanase activity when expressing an *Aeromonas caviae* ME-1 xylanase in *E. coli*. Whilst Lee *et al.* (2000) reported marginally higher activity of 1.5 $U.ml^{-1}$ when expressing a *Paenibacillus* sp. KCTC 8848P xylanase in *E. coli*.

The optimal expression conditions had been determined and the next step was to determine whether or not there was any difference in xylanase localisation between the two different types of vectors used in this study. With regards to the KW4 clone the xylanase distribution in the extracellular, periplasmic and cytoplasmic fractions was 17.3 ± 2.07%, 51.3 ± 1.17% and 31.4 ± 0.97%, respectively. This is very similar to the results reported by Huang *et al.* (2006), whereby they reported values of 22.4%, 49.6% and 28.0% when expressing a xylanase using a pUC18 plasmid in *E. coli*. This would tend to suggest that firstly, *E. coli* is capable of transporting a large portion of the xylanase enzyme across the cytoplasmic membrane into the periplasm and secondly, that it doesn't always need the *pelB* signal sequence to do so, since the pUC18 plasmid does not contain one. The KW1 clone (without a *pelB* signal sequence), was only able to transport 36.2 ± 1.21% of the total xylanase into the periplasm. The fact that both clones did not have a large amount of extracellular xylanase can be explained by the fact that *E. coli* lacks a natural secretion mechanism, so the only possible transport available out of the cytoplasmic fraction, is into the periplasm. The *pelB* signal sequence acts by directing the protein to the periplasm, where the membrane-anchored peptidase is found (Hauser &

Ryan, 2007). Once the protein has reached the periplasm, the signal sequence is rapidly removed by specific proteases and the protein begins to fold (Georgiou and Valax, 1996).

This chapter dealt with cloning of both the primary and secondary clones, beginning with an optimisation strategy and ending with a determination of localisation of xylanase activity. With regards to an optimal growth temperature, 30°C was better than 37°C as it produced a larger amount of soluble protein. This lowered growth temperature was also linked to lower levels of proteases, however, the fact that a protease deficient strain of *E. coli* was used and that proteases have been proven not to be destructive to thermostable enzymes, probably meant that increasing the proteins solubility was the greatest factor in decreasing the growth temperature. The better inducer was shown to be lactose. Although the induction times were longer in lactose than in IPTG, the lactose induction showed much higher xylanase activities than IPTG. Lactose also served as a carbon/energy source and is much cheaper than its synthetic analog IPTG, and circumvents the toxicity associated problems. When using the optimised expression parameters, the secondary clones all outperformed the primary clones, due to the shortened xylanase cloning fragment possibly helping the ribosomes find the start codon more efficiently. There was a difference in the total xylanase distribution between the two different vectors used in this study. The KW4 clone with the *pelB* signal sequence was able to export more of the xylanase into the periplasmic space. The xylanase produced in this clone was then used in the following chapter, for purification and characterisation.

CHAPTER 4

XYLANASE PURIFICATION AND CHARACTERISATION

4.1 INTRODUCTION

The purification of heterologous proteins is an important part of downstream processing. It allows researchers to ascertain important biochemical and structural information about that protein, without the influence of 'contaminating' proteins. With the current wealth of protein data and extensive protein engineering taking place, there is an ever expanding inventory of available proteins for researchers to work on. One current purification strategy that has been successful in high throughput screening approaches is the use of affinity tags. The tag allows for a common method to be used to purify different proteins, as opposed to specifically customizing purification procedures for ordinary chromatographic separation (Arnau *et al.*, 2006).

Polyhistidine tags are stretches of histidine residues (usually six) bound to either the N-terminus or the C-terminus of the desired protein, and bind to chelated metal ions that act as affinity ligands (Fig. 4.1). The two most common chromatography supports are nitrilotriacetic acid (NTA) for immobilizing metals like nickel in affinity chromatography (Ni-NTA) or Sepharose matrices (Arnau *et al.*, 2006). When using the Ni-NTA matrix, the proteins bind very tightly, allowing the protein preparation to be up to 95% pure in a single step (Qiagen, 2003).

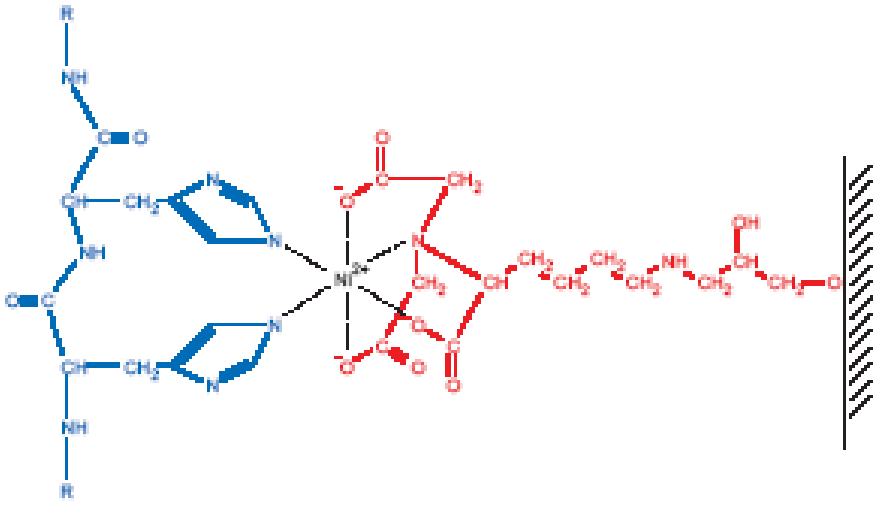

Figure 4.1 The interaction between the hexahistidine tagged protein and the Ni-NTA matrix (Qiagen, 2003).

Once the enzyme is purified, it is important to determine what its optimal operation conditions are. Enzymes are very specific for the reactions they catalyse and only work at optimal rates under certain conditions. This is especially important to know when wanting to use an enzyme in an industrial process, where it would be too expensive to change the design parameters. For instance, for the xylanase to be of any use to the pulp and paper industry, it would have to work fairly optimally from pH 8 – 10 and temperatures in the region of 60°C - 90°C for at least three hours (Techapun *et al*., 2003). Therefore, not only is it important to determine its optimal working conditions, but its stability at increasingly extreme conditions as well. In effect, how far the enzyme can be pushed before it loses its activity and as a result cannot perform its function.

Taking the above points into account, the first objective of this chapter was to purify the S340 xylanase enzyme using the polyhistidine tag present on the enzyme and the Ni-

NTA Fast Start Kit (Qiagen). Secondly, the pH and temperature for optimal xylanase activity were determined for both a crude enzyme extract (control) and the purified xylanase. And lastly, the effect alkaline pHs and high temperatures would have on the long-term stability of both enzyme preparations was investigated.

4.2 MATERIALS & METHODS

4.2.1 Xylanase purification

4.2.1.1 Growth of strain and xylanase expression

The *E. coli* clone producing the highest amount of xylanase from section 3.3.3 in the preceding chapter was chosen for xylanase purification. This was the pET22b-containing construct called KW4. Without having the DNA sequenced to ensure that the C-terminal His·Tag was in the correct reading frame, there was only a 1 in 3 chance of the tag being expressed and therefore the purification procedure working. The KW4 clone was grown up in an identical manner to section 3.2.1, however the enzyme extraction procedure using BugBuster Protein Extraction Reagent (Novagen) was not carried out. The 200 ml *E. coli* culture was decanted from the shake-flask into four 50 ml plastic centrifuge tubes. They cells were then pelleted by centrifugation at 5000 *g* for 5 min and were stored at -20°C until they were needed for purification.

4.2.1.2 Purification

The purification was carried out using the Ni-NTA Fast Start Kit (Qiagen). The recombinant xylanase contains a C-terminal affinity tag consisting of six consecutive histidine residues as a result of having being cloned into a pET vector. This hexahistidine tag binds selectively to the nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity

chromatography columns. The protein was purified under native conditions allowing it to maintain its three-dimensional conformation and therefore be biologically active at the end of the procedure. Before beginning, the supplied Lysis buffer was supplemented with lysozyme and Benzonase as per the manufacturer's instructions. The cell pellet from section 4.2.1.1 was allowed to thaw on ice for 20 min and was then resuspended in 10 ml of Lysis buffer. The suspension was incubated on ice for 30 min and gently mixed by swirling every 10 min. The lysate was transferred into 2 ml eppendorfs and centrifuged at 16 000 *g* for 30 min at 4°C in order to pellet the cellular debris. The supernatant contained the soluble fraction of the recombinant xylanase. A 10 µl aliquot of the supernatant was removed and added to 10 µl of 2 × SDS-PAGE sample buffer and stored at -20°C for SDS-PAGE analysis, as the cell lysate supernatant fraction. The resin in the Fast Start Column was resuspended by inverting it gently a few times. The seal at the outlet to the column was broken and the storage buffer allowed to drain out. The remainder of the cell lysate supernatant was applied to the column. The flow-through fraction was not discarded, but collected. A 10 µl aliquot of the flow-through was removed and added to 10 µl of 2 × SDS-PAGE sample buffer and stored at -20°C for SDS-PAGE analysis, as the flow-through fraction. The column was then washed twice with 4 ml of Wash buffer. Both wash fractions were collected and a 10 µl aliquot of each was removed and added to 10 µl of 2 × SDS-PAGE sample buffer and stored at -20°C for SDS-PAGE analysis. The tagged xylanase was then eluted in two separate steps, using 1 ml aliquots of Elution buffer. Ten microliter aliquots of each of the elution fractions were removed and added to 10 µl of 2 × SDS-PAGE sample buffer and stored at -20°C for

SDS-PAGE analysis. All fractions were analysed by SDS-PAGE, as described in section 3.2.5.

4.2.2 Xylanase characterisation

4.2.2.1 Determination of pH and temperature optima

The purified xylanase (section 4.2.1) as well as a crude xylanase extract harvested from *E. coli* XL1 Blue containing a pBSK vector were diluted in appropriate buffers over the pH range of 2 – 12 to determine their pH optima. The crude xylanase extract served as a control for comparison purposes. The buffer systems (50 mM) used were citrate-NaOH (pH 2 – 6.5), Tris-HCl (pH 7 – 9) and glycine-NaOH (pH 10 – 12). The substrate (1% birchwood xylan, Roth) was prepared in the buffers of the corresponding pH and assayed for xylanase activity as described in section 3.2.2.

The two enzyme preparations were then diluted in their optimal pH buffer and assayed for xylanase activity from 40°C - 100°C for 5 min as described in section 3.2.2 for determination of the temperature for optimal xylanase activity.

4.2.2.2 Determination of pH and temperature stability

The purified xylanase from section 4.2.2.1 and the crude xylanase extract were tested for stability in the alkaline pH range (pH 8 – 10) using 50 mM Tris-HCl (pH 8 and 9) and 50

mM glycine-NaOH (pH 10) from 60°C - 90°C for up to 180 min. Samples were removed every 15 min and treated as described in section 3.2.2.

The enzyme preparations were also diluted in their optimum pH buffer (pH 5.5) using 50 mM citrate buffer and incubated at 60°C for 90 min to determine their temperature stability at their optimum pH. Samples were taken every 15 min and treated as described in section 3.2.2.

4.3 RESULTS

4.3.1 Xylanase purification

The highest xylanase-producing clone was cultured and the cell suspension was pelleted by centrifugation as explained in section 4.2.1.1. The purification procedure (section 4.2.1.2) began with resuspending this pellet and lysing the cells to extract the proteins, and this crude extract was passed through the Ni-NTA metal affinity chromatography column. Different fractions were then removed at specific times during the purification procedure and analysed by SDS-PAGE as can be seen in Fig. 4.2. In lane 8, the xylanase enzyme appears highly purified, as most of the 'contaminating' proteins have been successfully removed. Both lane 7 and 8 the first and second elution fractions, respectively, show a dark band at the 24 kDa position, which corresponds to the size of the xylanase enzyme. There are a few accessory or contaminating bands in lane 8, but the there was a high degree of overall purification. When comparing the purified fraction (lane 8) with the cell lysate supernatant (lane 2 and 3), there are much less bands present illustrating the level of purification. A band corresponding to the 24 kDa xylanase is not present in the lanes containing the total cellular proteins, confirming that the xylanase is present at low concentrations. There are also very few differences between the negative control, uninduced and induced fractions (lanes 1 – 3, respectively), except for a dark band of low molecular weight present in lanes 2 and 3. The first wash fraction (lane 5) has a similar banding pattern to the induced fraction (lane 3), showing that many of the

‘contaminating’ proteins have been removed. The majority of these proteins are removed in the first wash, as the second wash (lane 6) is relatively clear.

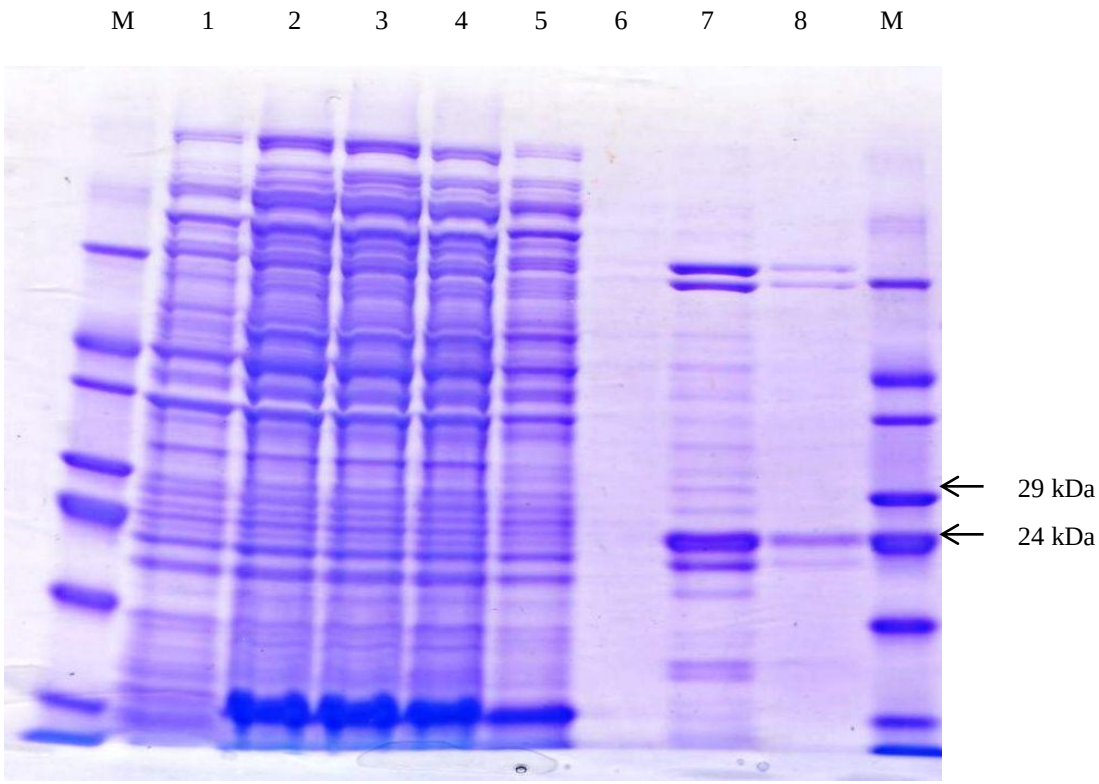

Figure 4.2 SDS-PAGE showing xylanase purification after affinity chromatography. Lane M: low range molecular weight markers (6.5 kDa – 66 kDa), lane 1: negative control, lane 2: uninduced cell lysate supernatant, lane 3: induced cell lysate supernatant, lane 4: flow-through fraction, lane 5: first wash fraction, lane 6: second wash fraction, lane 7: first elution fraction and lane 8: second elution fraction.

4.3.2 Xylanase characterization

4.3.2.1 Determination of pH and temperature optima

The pH and temperature optima were determined for both the purified S340 xylanase (section 4.2.1.2) as well as a crude xylanase extract from an *E. coli* XL1 Blue strain that carried the pBSK vector with the S340 xylanase gene present (control). This was done to check if the shortening of the cloning fragment and the purification procedure had any effect on the enzymes characteristics. As can be seen in Fig. 4.3, the pH optima of both preparations were 5.5, showing the typical bell-shaped pH profile. Although the pH optima was the same for both preparations, the crude extract had higher relative activity values overall. Interestingly, even the purified xylanase retained 64% and 39% of its initial activity at pH 2 and 12 respectively.

The temperature optimum for both preparations was 70°C as is illustrated in Fig. 4.4. Once again the enzyme performs fairly well whatever the temperature. The purified xylanase retains 50% of its original activity at 100°C. Once again the crude extract had higher relative activity values overall. There seems to be an increasing gap in activities between the two preparations as the temperatures would move towards each extreme, as exemplified by the fact that at 100°C there is a 9% difference in activity between the two.

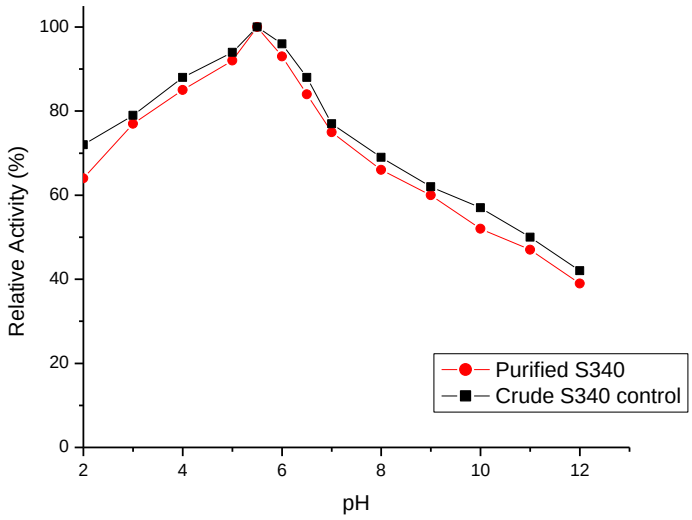

Figure 4.3 The effect of pH on activities of the crude S340 control and the purified S340 preparations from pH 2 – 12 at 50°C for 5 min. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

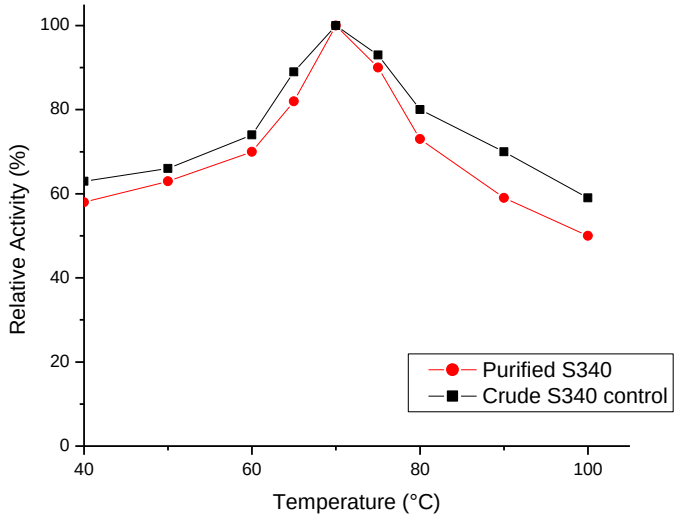

Figure 4.4 The effect of temperature on the activities of the crude S340 control and the purified S340 preparations from 40°C - 100°C at their optimum pH for 5 min. The remaining

activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

4.3.2.2 Determination of pH and temperature stability

At 60°C and pH 8 both preparations showed good stability, with the control and purified xylanase retaining 74% and 71% of their initial activities after 180 min of incubation as can be seen in Fig. 4.5. Their activities decreased as the temperature increased from 60°C - 90°C at pH 8 (Figs. 4.5 – 4.8), with activities of 56% and 55% at 90°C. There was a common trend between all the figures, the purified xylanase activities were on average always just below that of the crude xylanase. Once the pH was increased to 9 (Figs. 4.9 – 4.12), the stabilities decreased a lot quicker. For instance at pH 9 and 90°C (Fig. 4.12), after only 15 min of incubation, both the control and the purified xylanase had lost 26% and 28% of their initial activities, respectively. The gap between the two preparations had widened too, after 90 min of incubation there was a 5% gap in activities. This gap was illustrated even more clearly as the pH and temperature were increased to 10 and 90°C (Fig. 4.16), respectively. After 15 min incubation at these conditions, the activities of the control and the purified xylanase had decreased to 65% and 61%, respectively. However, after 120 min incubation at these rather extreme conditions, the control and the purified xylanase retained a respectable 43% and 39% of their initial activities, respectively.

When testing the stability of these two enzyme preparations at pH 5.5 (optimal pH), they both showed remarkable stability (Fig. 4.17). After 90 min at 60°C and pH 5.5, the residual activities of the control and purified xylanase were 94% and 93%, respectively.

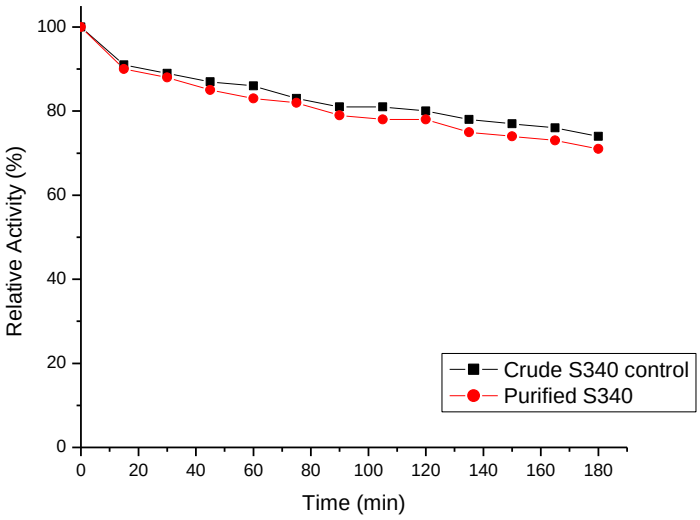

Figure 4.5 The effect of temperature and alkalinity on the stability of the crude S340 control and the purified S340 preparations at 60°C and pH 8. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

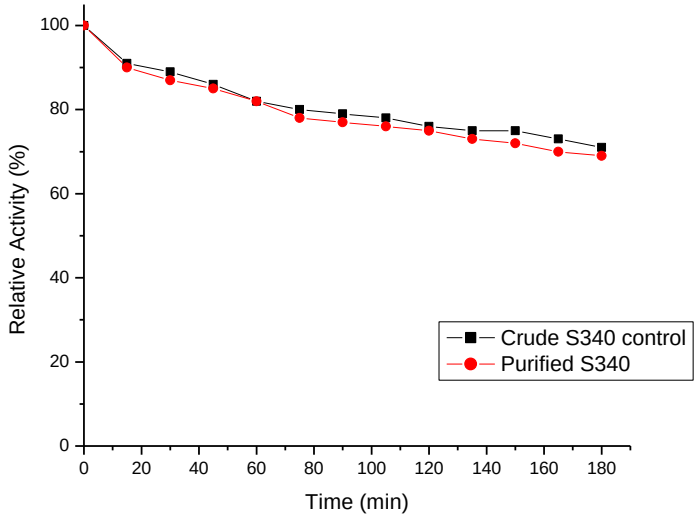

Figure 4.6 The effect of temperature and alkalinity on the stability of the crude S340 control and the purified S340 preparations at 70°C and pH 8. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

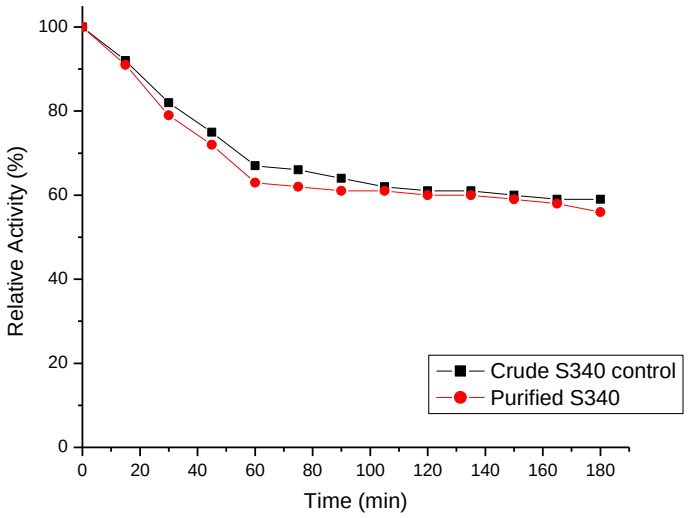

Figure 4.7 The effect of temperature and alkalinity on the stability of the crude S340 control and the purified S340 preparations at 80°C and pH 8. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

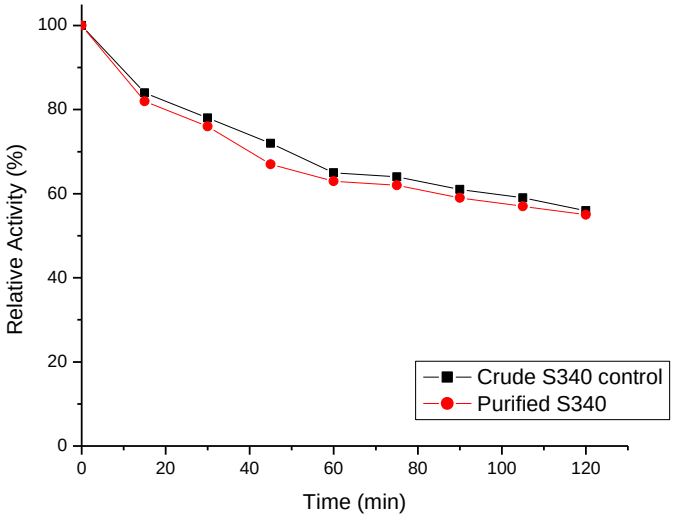

Figure 4.8 The effect of temperature and alkalinity on the stability of the crude S340 control and the purified S340 preparations at 90°C and pH 8. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

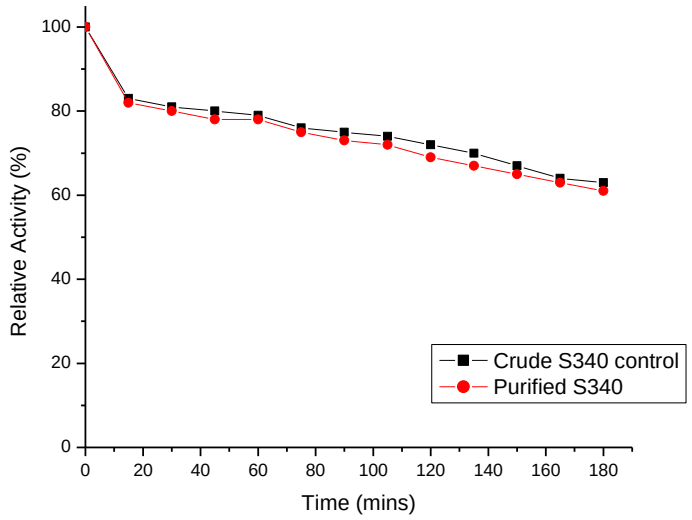

Figure 4.9 The effect of temperature and alkalinity on the stability of the crude S340 control and the purified S340 preparations at 60°C and pH 9. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

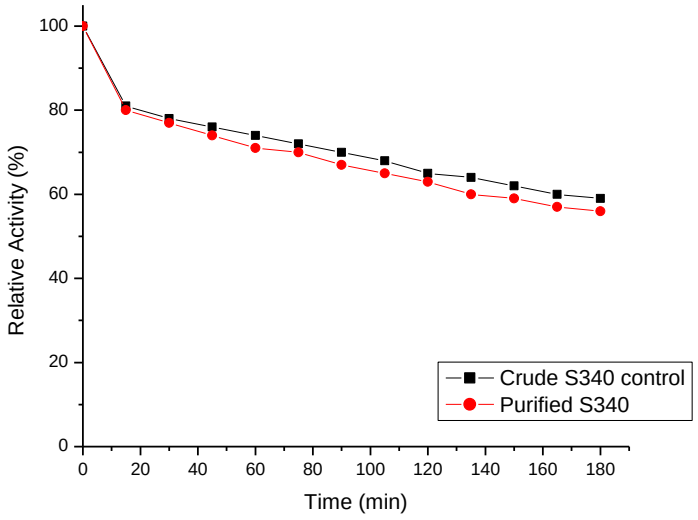

Figure 4.10 The effect of temperature and alkalinity on the stability of the crude S340 control and the purified S340 preparations at 70°C and pH 9. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

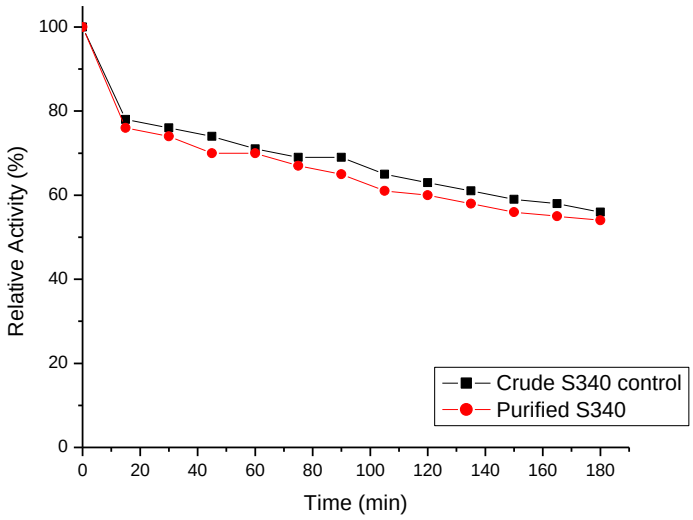

Figure 4.11 The effect of temperature and alkalinity on the stability of the crude S340 control and the purified S340 preparations at 80°C and pH 9. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

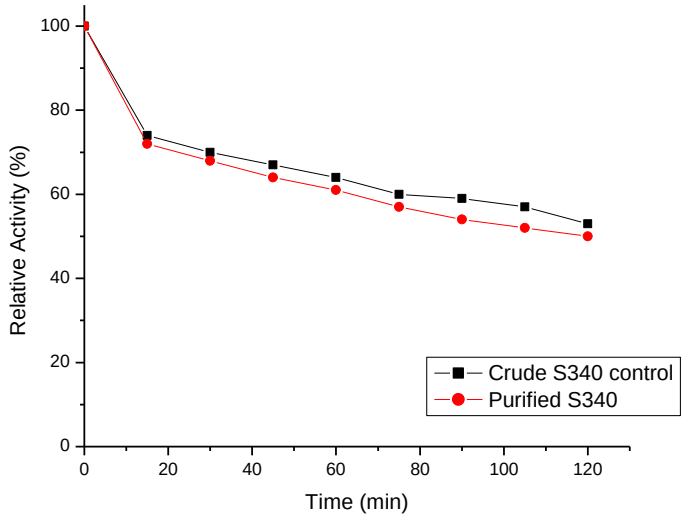

Figure 4.12 The effect of temperature and alkalinity on the stability of the crude S340 control and the purified S340 preparations at 90°C and pH 9. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

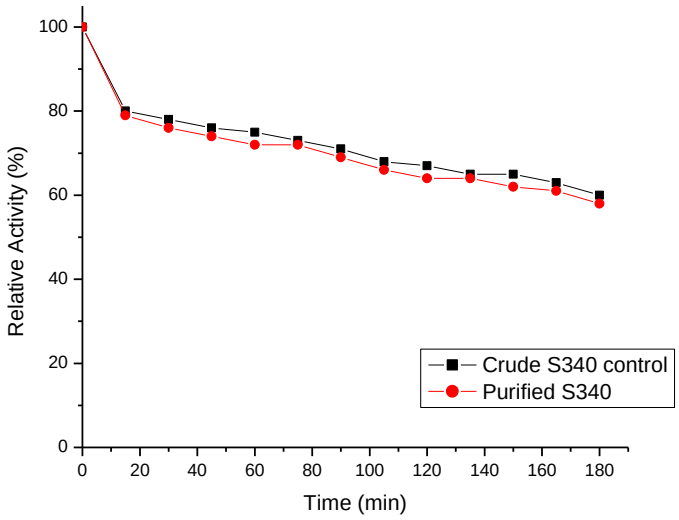

Figure 4.13 The effect of temperature and alkalinity on the stability of the crude S340 control and the purified S340 preparations at 60°C and pH 10. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

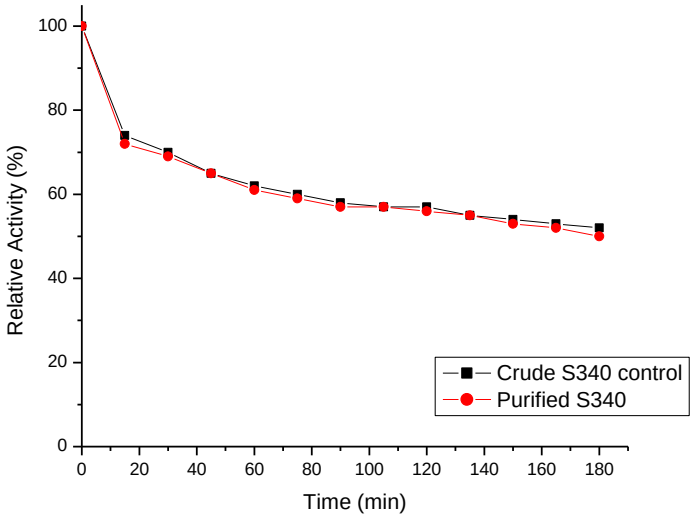

Figure 4.14 The effect of temperature and alkalinity on the stability of the crude S340 control and the purified S340 preparations at 70°C and pH 10. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

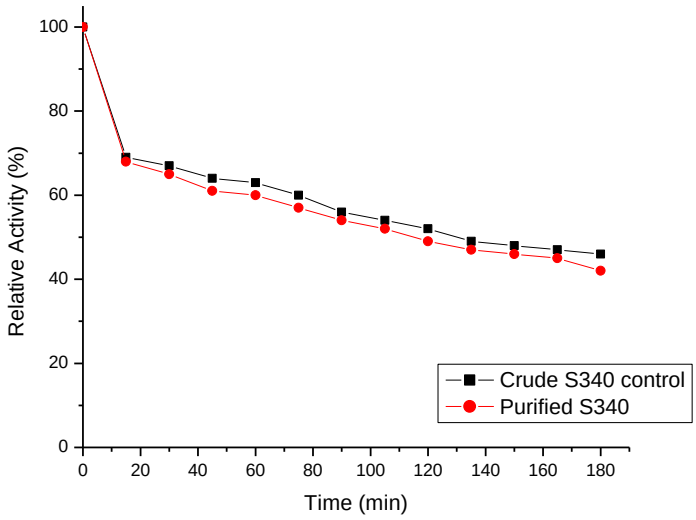

Figure 4.15 The effect of temperature and alkalinity on the stability of the crude S340 control and the purified S340 preparations at 80°C and pH 10. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

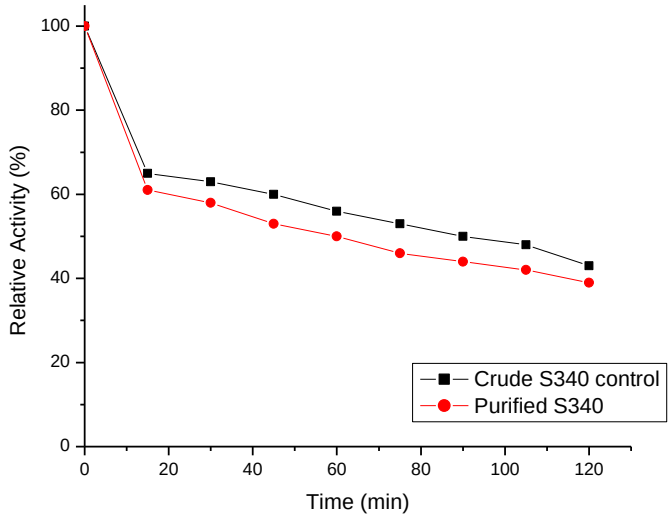

Figure 4.16 The effect of temperature and alkalinity on the stability of the crude S340 control and the purified S340 preparations at 90°C and pH 10. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

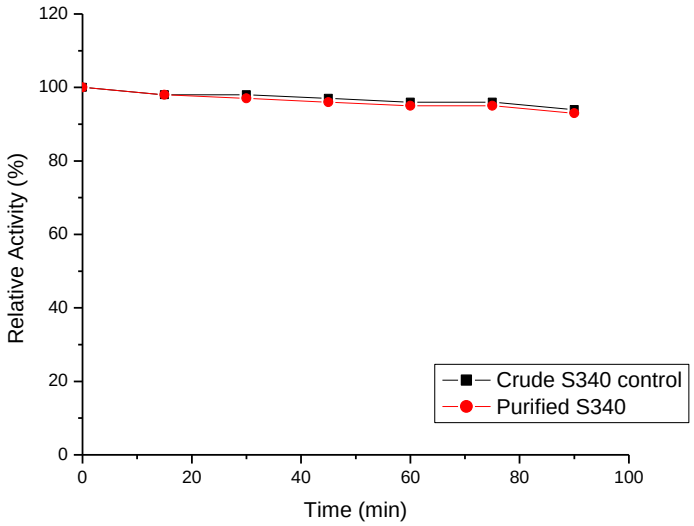

Figure 4.17 The effect of the optimum pH on the stability of the crude S340 control and the purified S340 preparations at 60°C and pH 5.5. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

4.4 DISCUSSION

The purpose of this chapter was to choose the best clone (from chapter 3) producing the highest amount of xylanase and to biochemically characterise that enzyme. Firstly, that enzyme had to be purified. This was done by making use of the polyhistidine tag on the C-terminus of the enzyme and running it through an affinity chromatography column. This purification procedure was in effect the beginning of the characterisation, as only by having a purified protein would we be able to ascertain the enzymes true performance and optimal conditions for use. Once this purification had been done, the optimal pH and temperature of the enzyme was determined. Throughout the characterisation procedures, the purified xylanase was compared to a crude xylanase extract (control) in order to determine the effect, if any, the purification had on the optimal working conditions of the enzyme. As well as optimal working parameters, the alkaline and temperature stability was determined from pH 8 -10 and temperatures from 60°C - 90°C. This was important as the upper extremes of these conditions are the requirement for an enzyme to be able to function optimally in the pulp and paper industry (Techapun *et al*., 2003). And lastly, the stability of the enzymes at their optimal pH was determined.

When looking at the results of the purification procedure (Fig. 4.2), we can see a clear decrease in the number of bands and therefore 'contaminating' proteins when comparing lane 3 (cell lysate supernatant) with lane 8 (second elution fraction). As well as the clear decrease in 'contaminating' bands, there is a clear increase in the intensity and therefore concentration of the two mains bands in lanes 7 and 8, which include the xylanase band.

Lane 3 contains hundreds of intracellular *E. coli* proteins, whilst in lane 8 there is only four clearly distinguishable proteins, one of which is the 24 kDa xylanase enzyme. Lane 5 is the first wash fraction that is responsible for washing away loosely-bound proteins with internal histidine residues out of the resin. By lane 6 (second wash fraction), there are hardly any bands, showing that the washing procedures were effective and cleaned the resin of the unwanted proteins. Lane 7 shows a very dark xylanase band at the 24 kDa position as well as a few other contaminating proteins. Lane 8 (second elution fraction) shows a similar banding pattern, but a lower number of contaminating proteins. What is important, however, is the obvious presence of the xylanase protein. The contaminating bands in lane 7 and 8 can probably be attributed to non-specific binding. When purifying proteins under native conditions, there is a much higher potential for non-specific binding (background contaminants) than under denaturing conditions (Qiagen, 2003). In C-terminally tagged proteins, the most common form of contamination are truncated polyhistidine proteins that are copurified on the column. These result from the internal initiation of translation or from protein degradation during expression and purification, and could possibly explain the presence of the bands that are smaller than 24 kDa. A possible solution for this would be the addition of low concentrations (10 – 20 mM) of imidazole to the lysis and wash buffers. The imidazole ring is part of the structure of histidine, and therefore binds to the nickel ions in the column and disrupts the binding of dispersed non-tagged background proteins (Qiagen, 2003). The reasons for purifying the xylanase enzyme under native conditions in this study were two-fold. Firstly, there was no problem with the accessibility of the tag at the C-terminus, and secondly, by purifying the enzyme under denaturing conditions its three-dimensional conformation and therefore

its bioactivity would have been lost and would have required renaturation and refolding procedures that could affect the enzymes characteristics.

Not much work has been reported on purifying xylanases using the Ni-NTA affinity chromatography setup. However, the work that has been done is very similar to the current study but differs by having certain pretreatment steps before loading the protein sample on the Ni column. Zhou *et al.* (2007) precipitated the proteins from their crude xylanase extract using 60% ammonium sulphate and then applied it to a Sephadex G-100 column, which was followed by Ni-chelating chromatography. These steps must have aided in the exclusion of background contamination. Li *et al.* (2009) purified their recombinant β-galactosidase by first heat-treating their cell lysate supernatant, followed by applying it to a Ni-NTA agarose metal chelate chromatography column. Purification of an enzyme is only necessary when wanting to determine a true reflection of the enzymes performance. In an industrial situation whereby purification procedures are too costly, a crude lysate or extracellular fraction is normally used.

When beginning the characterisation of the xylanase enzyme, an ideal place to start is to determine its optimal pH and temperature for its maximum xylanase activity. The crude xylanase extract served as the control throughout the characterisation. The optimal pH for the crude extract and the purified xylanase were both 5.5, as can be seen in Fig. 4.3. The optimal temperature for both the crude extract and the purified xylanase was 70°C, as can bee seen in Fig. 4.4. The fact that both enzyme preparations share the same optima for pH and temperature comes as no surprise, as they are the same enzyme. The optima seem to

be a characteristic of that particular enzyme, and are not affected by the host *E. coli* strain, plasmid, level of expression or level of purification. Interestingly, both preparations retain high levels of activity at both extremes of pH and temperature, showing that the enzyme is extremely versatile. Generally the optimal pH of xylanases isolated from *T. lanuginosus* range from 6 – 7 (Singh *et al.*, 2003), but this particular xylanase variant, S340, has undergone protein engineering, whereby three amino acid substitutions were responsible for lowering its optimal pH (Stephens, 2006). Once again the optimal temperature of 70°C was a feature of most xylanases isolated from *T. lanuginosus*, as this was the case when a xylanase from *T. lanuginosus* SSBP was tested (Lin *et al.*, 1999). In Fig. 4.4, there is a 9% gap between the two enzyme preparations at 100°C. The fact that the crude enzyme extract retains higher activity could be down the fact that it could be being protected from the destabilising effect of the increased temperature by the other proteins present or by culture medium components (Kim *et al.*, 2002).

When looking at the enzyme stability at high temperatures and alkaline pH, both preparations performed admirably, with the crude extract slightly outperforming the purified enzyme. For instance at pH 9 and 90°C (Fig. 4.12), after 90 min of incubation there was a 5% gap in activity. The gap seemed to become more pronounced as the temperature increased, presumably because the lack of a protective effect in the purified preparation, allowed the heat to denature the enzyme. The fact that the crude extract and the purified xylanase retain 43% and 39% of their respective activities at pH 10 and 90°C for 120 min is quite remarkable. This shows the robust nature of the enzyme and

ultimately how well-suited it is for its function in the pulp and paper industry. When determining the enzyme stability at its optimal pH at 60°C, it retained 93% of its original activity.

The S340 xylanase enzyme performs very well when comparing its biochemical characteristics to other published data. Li *et al.* (2005) tested a *T. lanuginosus* CBS 288.54 xylanase and reported that the enzyme retained more than 50% of its activity after heating at 85°C and pH 7 for 30 min. After the corresponding time of incubation, the crude extract and purified xylanase retained 63% and 58% of their respective activities at pH 10 and 90°C (Fig. 4.16). S340 outperforms that enzyme in both thermostability and alkaline stability. The only report to rival these specifications was from a thermotolerant *Streptomyces* sp. Ab 106 xylanase, which was able to retain more than 70% of its activity at 60°C and pH 9 with a half life of 6 h (Techapun *et al.,* 2002).

This chapter dealt with the purification and biochemical characterisation of the xylanase enzyme produced in chapter 3. It began by purifying the xylanase using Ni-NTA affinity chromatography column. The resultant SDS-PAGE analysis (Fig. 4.2) showed a high degree of purification in lane 8 of the gel. There was a bit of background contamination, which can be attributed to binding of non-specific proteins and truncated versions of the protein. This could possibly have been circumvented by the addition of imidazole. However, the end result of purification was good enough to get a true reflection of the characteristics of that enzyme. The fact that the enzyme was purified under native conditions probably also led to an increase in contamination. However, the fact that no

renaturation or refolding had to be done on the enzyme was an added benefit. After the purification, the optimum pH and temperature were determined for the enzyme, which was pH 5.5 and 70°C, respectively. This pH was slightly lower than the wild-type *T. lanuginosus* xylanases, but could have been explained by the amino acid substitutions made to it during protein engineering of the enzyme. The temperature is within the range that is usually expected from xylanases cloned from the thermophilic fungus, *T. lanuginosus*. The enzyme showed remarkable thermostability and alkaline stability from pH 8 – 10 and temperatures from 60°C - 90°C. This suggests that it is well-suited for the purpose of biobleaching in the pulp and paper industry. The fact that the crude enzyme extract slightly outperformed the purified enzyme is probably due to the fact that the crude extract was more than likely being protected from the destabilising effects of intense heat and alkaline conditions by the other proteins present in the preparation. Therefore, it could retain its conformation for longer, allowing the active site to remain in tact and retain its activity for longer. In summation, purification of the enzyme is important in being able to judge its unbiased characteristics; however, due to the costly nature of these procedures it would not be economically feasible in an industrial process. This may not be a problem at all, as was shown in the results; the crude extract continually outperformed the purified preparation, making it well-suited for an industrial process.

CHAPTER 5

GENERAL DISCUSSION

In order to combat the growing reliance on the chemical industry for our current way of life, a cleaner, more efficient technology has begun to work in unison and will eventually supplant that very industry. Biotechnology has begun entrenching itself in many industries all over the world, with new applications for its use being discovered continuously. A graphic illustration of its use is in the pulp and paper industry, whereby the enzyme xylanase is used in the biobleaching of pulp, used to treat the very paper that this current study is printed on. Several challenges in xylanase application exist, to make the overall process increasingly efficient and economical. The first challenge is in discovering or indeed 'manufacturing' a xylanase enzyme that is able to withstand the harsh industrial conditions of high temperatures and alkaline pH's. The second challenge is in determining the best enzyme production strategy, including; growth organism, growth conditions and enzyme inducer. Once these challenges have been overcome, a highly efficient enzyme being made in large amounts but economically, will result.

The main focus of this study was to overexpress the thermostable and alkaline-stable xylanase called S340 in a quick-growing strain of *E. coli*. Once the best xylanase-producing strain had been identified, the enzyme was purified and biochemically characterised, in order to determine its optimal conditions for use.

The first chapter involved cloning of the S340 xylanase gene into a set of four expression vectors. The S340 cloning fragment was also trimmed using newly-designed primers and re-cloned back into the four vectors. The excess DNA was trimmed off the insert in an effort to increase expression levels of the gene. All cloning procedures were successful and resulted in a set of primary clones with the original S340 insert, as well as a set of secondary clones containing a shortened S340 insert. The difference in the expression levels of the S340 gene would allow us to ascertain the effect that the shortening of the cloning fragment would have on overall expression. This chapter, therefore, served as the basis of the rest of the study.

The second chapter dealt with the expression of the two sets of clones. The expression strategy was first optimised by determining the best growth temperature, enzyme inducer as well as induction period. The study showed that the lower growth temperature of 30°C far outperformed the conventional *E. coli* growth temperature of 37°C. The reason for this result is no doubt multi-factorial, however, the fact that the proteins seemed to be sequestered in the insoluble fraction including the inclusion bodies when grown at 37°C, seemed to be the most likely cause for the lower expression levels of the enzyme. The inducer of choice in the study was lactose, as opposed to the conventional inducer, IPTG. Lactose post-induction periods were more gradual and slow, but always produced higher activity values. These two above-mentioned factors have important implications for scaling-up an *E. coli*-based fermentation. Firstly, the optimal growth temperature for heterologous protein production is 30°C as opposed to 37°C. This would save on energy and therefore money, especially when looking at large-scale bioreactors. Secondly,

lactose could be substituted for the expensive IPTG. This would make a scale-up process more economically feasible. Perhaps, the even cheaper option of using a lactose-containing by-product from the dairy industry exists. Once the expression strategy had been optimised, both the primary and secondary clones were assayed for xylanase activity. The secondary clones, with the shorter S340 gene insert, all showed higher relative xylanase activities, suggesting that the shortening of the cloning fragment did indeed increase xylanase expression. A plausible explanation for this could be that since the distance between the start codon, promoter and Shine-Dalgarno sequence was shortened significantly, it led to more efficient transcription and translation, respectively.

The fourth chapter dealt with xylanase purification and charcterisation. The S340 xylanase was purified on a Ni-NTA metal affinity column, allowing elimination of some of the 'contaminating' proteins. This was done to properly characterise the enzyme, without the experiment being biased by other proteins present in *E. coli*. This type of purification, however, would not be suitable when scaling-up this process, as the cost involved would be excessive. The cost of the columns themselves, coupled with the cost of imidazole to prevent non-specific binding of proteins to the resin, would not make this type of purification feasible on a large-scale. Ultimately, this would not be a problem though, as in most industries purified enzymes are not used. In fact, as was shown in the current study, the crude enzyme extract outperformed the purified enzyme, possibly due to the protective effect of the other proteins present in the extract. So, the costly nature of enzyme purification can be entirely circumvented, for the benefit of the industrial process. The results of the characterisation showed that the enzyme was impressively

both alkaline-stable from pH 8 – 10 and thermostable in temperatures from 60°C - 90°C. This makes it sufficiently suitable for its application in the biobleaching of pulp.

When looking at the study as a whole, it was successful in increasing the expression of a heterologous xylanase in *E. coli*. Various strategies were used to ultimately increase expression in *E. coli* by approximately 4.5-fold. Impressive expression yields of protein can be achieved using *E. coli* provided all the intricacies of cloning and expression can be worked out. There is also an abundance of new *E. coli* strains and plasmid vectors being designed almost daily, with some impressive results being attained using heat-inducible vectors. This study also showed the importance of understanding the relationship between the promoter and the start codon of the gene of interest, and how by manipulating their spatial configuration, expression levels can be increased. There are, however, eukaryotic expression systems that can essentially outperform *E. coli*, but its ease of use and quick expression times make it possibly more suitable for structural studies on proteins or for pharmaceuticals, where purified, high quality protein is needed.

REFERENCES

Ames, G.F.L., Prody, C. and Kustu, S. 1984. Simple, rapid and quantitative release of periplasmic proteins by chloroform. *Journal of Bacteriology* **160:** 1181 – 1183.

Andersson, L., Yang, S., Neubauer, P. and Enfors, S.O. 1996. Impact of plasmid presence and induction on cellular response in fed batch cultures of *Escherichia coli*. *Journal of Biotechnology* **46:** 255 – 263.

Andreu, P., Collar, C. and Martinez-Anaya, M.A. 1999. Thermal properties of doughs formulated with enzymes and starters. *European Food Research and Technology* **209:** 803 – 808.

Arnau, J., Lauritzen, C., Petersen, G.E. and Pedersen, J. 2006. Current strategies for the use of affinity tags and tag removal for the purification of recombinant proteins. *Protein Expression and Purification* **48:** 1 – 13.

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

Arnold, F.H. and Glieder, A. 2003. Chemistry and biotechnology: a productive union meets new challenges. *Current Opinion in Biotechnology* **14:** 567 – 569.

Arnold, U. and Ulbrich-Hofmann, R. 2001. Proteolytic degradation of ribonuclease A in the pretransition region of thermally and urea-induced unfolding. *European Journal of Biochemistry* **268:** 937.

Aslund, F. and Beckwith, J. 1999. The thioredoxin superfamily: redundancy, specificity and grey area genomics. *Journal of Bacteriology* **181:** 1375 – 1379.

Atik, C., Imamoglu, S. and Bermek, H. 2006. Impact of xylanase pre-treatment on peroxide bleaching stage of biokraft pulp. *International Biodeterioration and Biodegradation* **58:** 22 – 26.

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.

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. 1999. Recombinant protein expression in *Escherichia coli*. *Current Opinion in Biotechnology* **10:** 411 – 421.

Basaran, P., Hang, Y.D., Basaran, N. and Worobo, R.W. 2001. Cloning and heterologous expression of xylanase from *Pichia stipitis* in *Escherichia coli*. *Journal of Applied Microbiology* **90:** 248 – 255.

Beg, Q.K., Kapoor, M., Mahajan, L. and Hoondal, G.S. 2001. Microbial xylanases and their industrial applications: a review. *Applied Microbiology and Biotechnology* **56:** 326 – 338.

Bhat, M.K. 2000. Cellulases and related enzymes in biotechnology. *Biotechnology Advances* **18:** 355 – 383.

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

Birnboim, H.C. and Doly, J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* **7:** 1513 – 1523.

Blackburn, J. 2000. Engineering and design: rational versus combinatorial approaches. *Current Opinion in Structural Biology* **10:** 399 - 400.

Blanco, A., Diaz, P., Zueco, J., Parascandola, P. and Pastor, F.I.J. 1999. A multidomain xylanase from a *Bacillus* sp. With a region homologous to thermostabilizing domains of thermophilic enzymes. *Microbiology* **145:** 2163 – 2170.

Brundtland, G. 1987. Our common future. Oxford: Oxford University Press. pp. 229 - 235.

Carrio, M.M. and Villaverde, A. 2001. Protein aggregation as bacterial inclusion bodies in reversible. *FEBS Letters* **489:** 29 – 33.

Castle, L.A., Siehl, D.L., Gorton, R., Patten, P.A., Chen, Y.H., Bertain, S., Cho, H.J., Duck, N., Wong, J. and Liu, D.L. 2004. Discovery and directed evolution of a glyophosphate tolerance gene. *Science* **304:** 1151 – 1154.

Chauhan, S., Choudhury, B., Singh, S.N. and Ghosh, P. 2006. Application of xylanase enzyme of *Bacillus coagulans* as a prebleaching agent on non-woody pulps. *Process Biochemistry* **41:** 226 – 231.

Chen, C.C., Adolphson, R., Dean, J.F.D., Eriksson, K.-E.L., Adams, M.W.W. and Westpheling, J. 1997. Release of lignin from kraft pulp by a hyperthermophilic xylanase from *Thermotoga maritima. Enzyme and Microbial Technology* **20:** 39 – 45.

Cherry, J.R. and Fidantsef, A.L. 2003. Directed evolution of industrial enzymes: an update. *Current Opinion in Biotechnology* **14:** 438 – 443.

Choi, J.H., Jeong, K.J., Kim, S.C. and Lee, S.Y. 2000. Efficient secretory production of alkaline phosphatase by high cell density culture of recombinant *Escherichia coli* using the *Bacillus* sp. endoxylanase signal sequence. *Applied Microbiology and Biotechnology* **53:** 640 – 645.

Choi, J.H. and Lee, S.Y. 2004. Secretory and extracellular production of recombinant proteins in *Escherichia coli. Applied Microbiology and Biotechnology* **64:** 625 – 635.

Choi, J.H., Keum, K.C. and Lee, S.Y. 2006. Production of recombinant proteins by high cell density culture of *Escherichia coli:* A review. *Chemical Engineering Science* **61:** 876 – 885.

Christopher, L., Bissoon, S., Singh, S., Szendefy, J. and Szakacs, G. 2005. Bleach-enhancing abilities of *Thermomyces lanuginosus* xylanases produced by solid state fermentation. *Process Biochemistry* **40:** 3230 – 3235.

Christov, L.P., Akhtar, M. and Prior, B.A. 1996. Impact of xylanase and fungal pretreatment on alkali solubility and brightness of dissolving pulp. *Holzforshung* **50:** 579 – 582.

Clark, E.B. 1998. Refolding of recombinant proteins. *Current Opinion in Biotechnology* **9:** 157 – 163.

Collins, T., Gerday, C. and Feller, G. 2005. Xylanases, xylanase families and extremophilic xylanases. *FEMS Microbiology Reviews* **29:** 3 – 23.

Cranenburgh, R.M. 2004. An equation for calculating the volumetric ratios required in a ligation reaction. *Applied Genetics and Molecular Biotechnology* **65:** 200 – 202.

Cregg, J.M., Cereghino, J.L., Shi, J. and Higgins, D.R. 2000. Recombinant protein expression in *Pichia pastoris. Molecular Biotechnology* **16:** 23 – 52.

Daly, R. and Hearn, M.T.W. 2005. Expression of heterologous proteins in *Pichia pastoris*: a useful experimental tool in protein engineering and production. *Journal of Molecular Recognition* **18:** 119 – 138.

Daneault, C., Ledue, C., and Valase, J.L. 1994. The use of xylanases in kraft pulp bleaching: A review. *Tappi Journal* **77:** 125 – 131.

Davies, G. and Henrissat, B. 1995. Structures and mechanisms of glycosyl hydrolases. *Structure* **3:** 853 – 859.

DiMicelli, L., Pool, V., Kelso, J.M., Shadomy, S.V., Iskander, J., V.A.E.R.S Team. 2006. Vaccination of yeast sensitive individuals: review of safety data in the US vaccine adverse event reporting system (VAERS). *Vaccine* **24:** 703 – 707.

Egorova, K. and Antranikian, G. 2005. Industrial relevance of thermophilic Archaea. *Current Opinion in Microbiology* **8:** 649 – 655.

Eichler, J. 2001. Biotechnological uses of archaeal extremozymes. *Biotechnology Advances* **19:** 261 – 278.

Everly, C. and Alberto, J. 2000. Stressors, stress and survival: overview. *Frontiers in Bioscience* **5:** 780 – 786.

Farinas, E.T., Bulter, T. and Arnold, F.H. 2001. Directed enzyme evolution. *Current Opinion in Biotechnology* **12:** 545 – 551.

Fenel, F., Leisola, M., Janis, J. and Turunen, O. 2004. A *de novo* designed N-terminal disulphide bridge stabilizes the *Trichoderma reesei* endo-1,4-β-xylanase II. *Journal of Biotechnology* **108:** 137 – 143.

Fernandez-Castillo, R.F., Rodriguez-Valera, F., Gonzalez-Ramos, J. and Ruiz-Berraquero, F. 1986. Accumualtion of poly(β-hydroxybutyrate) by halobacteria. *Applied and Environmental Microbiology* **51:** 214 – 216.

Gavrilescu, M and Christi, Y. 2005. Biotechnology – a sustainable alternative for the chemical industry. *Biotechnology Advances* **23:** 471 – 499.

Ge, L. and Rudolph, P. 1997. Simultaneous introduction of multiple mutations using overlap extension PCR. *Biotechniques* **22:** 28 – 30.

Georgiou, G. and Segatori, L. 2005. Preparative expression of secreted proteins in bacteria: status report and future prospects. *Current Opinion in Biotechnology* **16:** 538 – 545.

Georgiou, G. and Valax, P. 1996. Expression of correctly folded proteins in *Escherichia coli*. *Current Opinion in Biotechnology* **7:** 190 – 197.

Gombert, A.K. and Kilikian, B.V. 1998. Recombinant gene expression in *Escherichia coli* cultivation using lactose as inducer. *Journal of Biotechnology* **60:** 47 – 54.

Gottesman, S. 1996. Proteases and their targets in *Escherichia coli*. *Annual Review of Genetics* **30:** 465 – 506.

Grossman, T.H., Kawasaki, E.S., Punreddy, S.R. and Osburne, M.S. 1998. Spontaneous cAMP-dependant derepression of gene expression in stationary phase plays a role in recombinant expression instability. *Gene* **209:** 95 – 103.

Haki, G.D. and Rakshit, S.K. 2003. Developments in industrially important thermostable enzymes: a review. *Bioresource Technology* **89:** 17 – 34.

Harris, G.W., Jenkins, J.A., Connerton, I., Cummings, N., Loleggio, L., Scott, M., Hazelwood, G.P., Laurie, J.I., Gilbert, H.J. and Pickersgill, R.W. 1994. Structure of the catalytic core of the Family F xylanase from *Pseudomonas fluorescens* and identification of the xylopentaose binding sites. *Structure* **2:** 1107 – 1116.

Hartley, J.L. 2006. Cloning technologies for protein expression and purification. *Current Opinion in Biotechnology* **17:** 359 – 366.

Hashemzadeh-Bonehi, L., Mehraein-Ghomi, F., Mitsopoulos, C., Jacob, J.P., Hennessy, E.S. and Broome-Smith, J.K. 1998. Importance of using *lac* rather than *ara* promoter vectors for modulating the levels of toxic gene products in *Escherichia coli. Molecular Microbiology* **30:** 676 – 678.

Henderson, T.A., Dombrosky, P.M. and Young, K.D. 1994. Artifactual processing of penicillin binding proteins 7 and 1B by the OmpT protease in *Escherichia coli. Journal of Bacteriology* **176:** 256 – 259.

Henrissat, B., Claeyssens, M., Tomme, P., Lemesle, L. and Mornon, J.P. 1989. Cellulase families revealed by hydrophobic cluster analysis. *Gene* **81:** 83 – 95.

Hitzeman, R.A., Hagie, F.E., Levine, H.L., Goeddel, D.V., Ammerer, G. and Hall, B.D. 1981. Expression of a human gene for interferon in yeast. *Nature* **293:** 717 – 722.

Huang, J., Wang, G. and Xiao, L. 2006. Cloning, sequencing and expression of the xylanase gene from a *Bacillus* strain B10 in *Escherichia coli*. *Bioresource Technology* **97:** 802 – 808.

Hauser, P.S. and Ryan, R.O. 2007. Expressed protein ligation using an N-terminal cysteine containing fragment generating *in vivo* from a pelB fusion protein. *Protein Expression and Purification* **54:** 227 – 233.

Itakura, K., Hirose, T., Crea, R., Riggs, A.D., Heyneker, H.L., Bolivar, F. and Boyer, H.W. 1977. Expression in *Escherichia coli* of a chemically synthesized gene for the hormone somatostatin. *Science* **198:** 1056 – 1063.

Jager, S.A.W., Shapovalova, I.V., Jekel, P.A., Alkema, W.B.L., Svedas, V.K. and Janssen, D.B. 2007. Saturation mutagenesis reveals the importance of residues αR145 and αF146 of penicillin acylase in the synthesis of β-lactam antibiotics. *Journal of Biotechnology* **133:** 18 – 26.

Johannes, T.W. and Zhao, H. 2006. Directed evolution of enzymes and biosynthetic pathways. *Current Opinion in Microbiology* **9:** 261 – 267.

Jung, E. and Williams, K.L. 1997. The production of recombinant glycoproteins with special reference to simple eukaryotes including *Dictyostelium discoideum. Biotechnology and Applied Biochemistry* **25:** 3 – 8.

Kane, J.F. 1995. Effects of rare codon clusters on high-level expression of heterologous proteins in *Escherichia coli. Current Opinion in Biotechnology* **6:** 494 – 500.

Karlsson, E.N., Holst, O and Tocaj, A. 1999. Efficient production of thermostable xylanases from *Rhodothermus marinus* in *Escherichia coli* fed-batch cultures. *Journal of Bioscience and Bioengineering* **87:** 598 – 606.

Khandeparkar, R. and Bhosle, N.B. 2006. Application of thermoalkalophilic xylanase from *Arthrobacter* sp. MTCC 5214 in biobleaching of kraft pulp. *Bioresource Technology* **98:** 897 – 903.

Kim, M., Elvin, C., Brownlee, A. and Lyons, R. 2007. High yield expression of recombinant pro-resilin: lactose-induced fermentation in *E. coli* and facile purification. *Protein Expression and Purification* **52:** 230 – 236.

Kim, S.A., Cheng, K.J. and Liu, J.H. 2002. A variant of *Orpinomyces joyonii* 1,3-1,4-β-glucanase with increased thermal stability obtained by random mutagenesis and screening. *Bioscience, Biotechnology and Biochemistry* **66:** 171 - 174.

Kirk, O., Borchert, T.V. and Fuglsang, C.C. 2002. Industrial enzyme applications. *Current Opinion in Biotechnology* **13:** 345 – 351.

Kubata, B.K., Suzuki, T., Ito, Y., Naito, H., Kawai, K., Takamizawa, K. and Horitsu, H. 1997. Cloning and Expression of xylanase I gene (xynA) of *Aeromonas caviae* ME-1 in *Escherichia coli. Journal of Fermentation and Bioengineering* **83:** 292 – 295.

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

Lee, H.J., Shin, D.J., Cho, N.C., Kim, H.O., Shin, S.Y., Im, S.Y., Lee, H.B., Chun, S.B. and Bai, S. 2000. Cloning, expression and nucleotide sequences of two xylanase genes from *Paenibacillus* sp. *Biotechnology Letters* **22:** 387 – 392.

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

Liese, A. and Filho, M.V. 1999. Production of fine chemicals using biocatalysis. *Current Opinion in Biotechnology* **10:** 595 – 603.

Li, L., Zhang, M., Jiang, Z., Tang, L. and Cong, Q. 2009. Characterization of a thermostable family 42 β-galactosidase from *Thermotoga maritima. Food Chemistry* **112:** 844 – 850.

Li, X.T., Jiang, Z.Q., Li, L.T., Yang, S.Q., Feng, W.Y., Fan, J.Y. and Kusakabe, I. 2005. Characterization of a cellulase-free, neutral xylanase from *Thermomyces lanuginosus* CBS 288.54 and its biobleaching effect on wheat straw pulp. *Bioresource Technology* **96:** 1370 – 1379.

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

Lin, W.J., Huang, S.W. and Chou, C.P. 2001. High-level extracellular production of penicillin acylase by genetic engineering of *Escherichia coli*. *Journal of Chemical Technology and Biotechnology* **76:** 1030-1037.

Lopez, G. 1999. DNA supercoiling and temperature adaptation: a clue to early diversification of life. *Journal of Molecular Evolution* **46:** 439 – 452.

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

Mathews, C.K., van Holde, K.E. and Ahern, K.G. 2000. Biochemistry (3rd Edition). Addison-Wesley Publishing Company, USA. pp. 360 – 363.

Mcdonough, T.J. 1992. A survey of mechanical pulp bleaching in Canada-single-stage hydrosulfite lines are the rule. *Pulp and Paper Canada* **93:** 57.

Middelberg, A.P.J. 2002. Preparative protein folding. *Trends in Biotechnology* **20:** 437 – 443.

Miroux, B. and Walker, J.E. 1996. Over-production of protein in *Escherichia coli:* mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. *Journal of Molecular Biology* **260:** 289 – 298.

Mohorcic, M., Bencina, M., Friedrich, J. and Jerala, R. 2009. Expression of soluble versatile peroxidase of *Bjerkandera adusta* in *Escherichia coli. Bioresource Technology* **100:** 851 – 858.

Nakamoto, T. 2006. A unified view of the initiation of protein synthesis. *Biochemical and Biophysical Research Communications* **341:** 675 – 678.

Niehaus, F., Bertoldo, C., Kahler, M. and Antranikian, G. 1999. Extremophiles as a source of novel enzymes for industrial applications. *Applied Microbiology and Biotechnology* **51:** 711 – 729.

Ninawe, S. and Kuhad, R.C. 2006. Bleaching of wheat straw-rich soda pulp with xylanase from a thermoalkalophilic *Streptomyces cyaneus* SN32. *Bioresource Technology* **97:** 2291 – 2295.

Novagen. 2004. *BugBuster Protein Extraction Reagent* – User protocol [online]. Available from http://wolfson.huji.ac.il/purification/PDF/Protein_Expression_Extraction/NOVAGEN_BugBuster_protein_extraction.pdf [Accessed 12 February 2008].

Palardini, A., Gianese, G., Bossa, F. and Pascarela, F. 2002. Structural plasticity of thermophilic serine hydroxymethyltransferases. *Proteins* **50:** 122 – 134.

Patel, G.B. and Sprott, G.D. 1999. Archaeobacterial ether lipid liposomes (archaeosomes) as novel vaccine and drug delivery systems. *Critical Reviews in Biotechnology* **194:** 317 – 357.

Qiagen. 2003. *The QIAexpressionist brochure* [online]. Fifth edition. Available from http://www.qiagen.com/literature/Default.aspx?Term=QIAexpressionist [Accessed 15 September 2008].

Raha, A.R., Chang, L.Y., Sipat, A., Yusoff, K. and Haryanti, T. 2006. Expression of a thermostable xylanase gene from *Bacillus coagulans* ST-6 in *Lactococcus lactis*. *Letters in Applied Microbiology* **42:** 210 – 214.

Ramchuran, S.O., Karlsson, E.N., Velut, S., de Mare, L., Hagander, P. and Holst, O. 2002. Production of heterologous thermostable glycoside hydrolases and the presence of host-cell proteases in substrate limited fed-batch cultures of *Escherichia coli* BL21(DE3). *Applied Microbiology and Biotechnology* **60:** 408 – 416.

Ramchuran, S.O., Holst, O. and Karlsson, E.N. 2005. Effect of postinduction nutrient feed composition and use of lactose as inducer during production of thermostable xylanase in *Escherichia coli* glucose-limited fed-batch cultivations. *Journal of Bioscience and Bioengineering* **99:** 477 – 484.

Reetz, M.T., Torre, C., Eipper, A., Lohmer, R., Hermes, M., Brunner, B., Maichele, A., Bocola, M., Arand, M. and Cronin, A. 2004. Enhancing the enantioselectivity of an epoxide hydrolase by directed evolution. *Organic Letters* **6:** 177 – 180.

Reeve, J.N., Nolling, J., Morgan, R.M. and Smith, D.R. 1997. Methanogenesis: genes, genomes and who's on first? *Journal of Bacteriology* **179:** 5975 – 5986.

Rinas, **U., Hoffmann, F., Betiku, E., Estape, D. and Marten, S.** 2007. Inclusion body anatomy and functioning of chaperone-mediated *in vivo* inclusion body disassembly during high-level recombinant protein production in *Escherichia coli. Journal of Biotechnology* **127:** 244 – 257.

Roncero, M.B., Torres, A.L., Colom, J.F. and Vidal, T. 2003. TCF bleaching of wheat straw pulp using ozone and xylanase. Part A: paper quality assessment. *Bioresource Technology* **87:** 305 – 314.

Roodveldt, C., Aharoni, A. and Tawfik, D.S. 2005. Directed evolution of proteins for heterologous expression and stability. *Current Opinion in Structural Biology* **15:** 50 – 56.

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

Sambrook, J., Fritsch, E.F. and Maniatis, T. 2001. Molecular cloning: a laboratory manual. 3rd edition. Cold Spring Harbour Laboratory, New York.

Schein, C.H. 1989. Production of soluble recombinant proteins in bacteria. *Biotechnology* **7:** 1141 – 1149.

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.

Schmid, A., Dordick, J.S., Hauer, B., Kiener, A., Wubbolts, M. and Witholt, B. 2001. Industrial biocatalysis, today and tomorrow. *Nature* **409:** 258 – 268.

Schoemaker, H.E., Mink, D. and Wubbolts, M.G. Dispelling the myths – biocatalysis in industrial synthesis. *Science* **299:** 1694 – 1697.

Shao, Z. and Arnold, F.H. 1996. Engineering new functions and altering existing functions. *Current Opinion in Structural Biology* **6:** 513 – 518.

Shatalov, A.A. and Pereira, H. 2006. Xylanase pre-treatment of giant reed organosolv pulps: Direct bleaching effect and bleach boosting. *Industrial Crops and Products* **25:** 248 – 256.

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.

Shiraldi, C. and De Rosa, M. 2002. The production of biocatalysts and biomolecules from extremophiles. *Trends in Biotechnology* **20:** 516 – 521.

Singh, S., Madlala, A.M. and Prior, B.A. 2003. *Thermomyces lanuginosus*: properties of strains and their hemicellulases. *FEMS Microbiology Reviews* **27:** 3 – 16.

Sorensen, H.P. and Mortensen, K.K. 2005. Advanced genetic strategies for recombinant protein expression in *Escherichia coli. Journal of Biotechnology* **115:** 113 – 128.

Sriprang, R., Asano, K., Gobsuk, J., Tanapongpipat, S., Champreda, V. and Eurwilaichitr, L. 2006. Improvement of thermostability of fungal xylanase by using site-directed mutagenesis. *Journal of Biotechnology* **126:** 454 – 462.

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

Stephens, D.E. 2006. Protein engineering of a fungal xylanase. D.Tech thesis. Durban University of Technology.

Stephens, D.E., Rumbold, K., Permaul, K., Prior, B.A. and Singh, S. 2007. Directed evolution of the thermostable xylanase from *Thermomyces lanuginosus*. *Journal of Biotechnology* **127:** 348 – 354.

Subramaniyan, S. and Prema, P. 2002. Biotechnology of microbial xylanases: enzymology, molecular biology, and application. *Critical Reviews in Biotechnology* **22:** 33 – 64.

Summers, D. 1998. Timing, self-control and a sense of direction are the secrets of multicopy plasmid stability. *Molecular Microbiology* **29:** 1137 – 1145.

Sunitha, K., Kim, Y.O., Lee, J.K. and Oh, T.K. 2000. Statistical optimisation of seed and induction conditions to enhance phytase production by recombinant *Escherichia coli. Biochemical Engineering Journal* **5:** 51 – 56.

Suzuki, T., Ibata, K., Hatsu, M., Takamizawa, K. and Kawai, K. 1997. Cloning and expression of a 58-kDA xylanase VI gene (xynD) of *Aeromonas caviae* ME-1 in *Escherichia coli* which is not categorized as a family F or family G xylanase. *Journal of Fermentation and Bioengineering* **84:** 86 – 89.

Techapun, C., Charoenrat, T., Poosaran, N., Watanbe, M. and Sasaki, K. 2002. Thermostable and alkali-tolerant cellulase-free xylanase produced by thermotolerant *Streptomyces* sp. Ab106. *Journal of Bioscience and Bioengineering* **93:** 431 – 433.

Techapun, C., Poosaran, N., Watanabe, M. and Sasaki, K. 2003. Thermostable and alkaline-tolerant cellulase-free xylanases produced from agricultural wastes and the properties required for use in pulp bleaching processes: a review. *Process Biochemistry* **38:** 1327 - 1340.

Thangadurai, C., Suthakaran, P., Barfal, P., Anandaraj, B., Pradhan, S.N., Boneya, H.K., Ramalingam, S. and Murugan, V. 2008. Rare codon priority and its position specificity at the 5` of the gene modulates heterologous protein expression in *Escherichia coli. Biochemical and Biophysical Research Communications* **376:** 647 – 652.

Torronen, A. and Rouvinen, J. 1997. Structural and functional properties of low molecular weight endo-1,4-β-xylanases. *Journal of Biotechnology* **57:** 137 – 149.

Tsumoto, K., Ejima, D., Kumagai, I. and Arakawa, T. 2003. Practical considerations in refolding proteins from inclusion bodies: a mini review. *Protein Expression and Purification* **28:** 1 – 8.

Ventura, S. and Villaverde, A. 2006. Protein quality in bacterial inclusion bodies. *Trends in Biotechnology* **24:** 179 – 185.

Viikari, L., Rauna, M., Kantelinen, A., Linko, M. and Sundquist, J. 1986. Bleaching with enzymes. *In: Proceedings of the 3rd International Conference on Biotechnology in the Pulp and Paper Industry, Stockholm*, pp. 67 – 69.

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

Voragen, A.G.J. 1992. Tailor-made enzymes in fruit juice processing. *Fruit Processing* **7:** 98 – 102.

Whitehead, T.R. and Hespell, R.B. 1989. Cloning and expression in *Escherichia coli* of a xylanase gene from *Bacteroides ruminicola* 23. *Applied and Environmental Microbiology* **55:** 893 – 896.

Whiteley, C.G. and Lee, D.-J. 2005. Enzyme technology and biological remediation. *Enzyme and Microbial Technology* **38:** 291 – 316.

Wong, W.R., Ali, A.B. and Ma, M.C. 2003. Cloning, expression and characterization of diuretic hormone Manduca diuresin from *Manduca sexta* in *Escherichia coli. Protein Expression and Purification* **29:** 51 – 57.

Xu, Y. and Foong, F.C. 2008. Characterization of a cellulose binding domain from *Clostridium cellulovorans* endoglucanase-xylanase D and its use as a fusion partner for soluble protein expression in *Escherichia coli. Journal of Biotechnology* **135:** 319 – 325.

Yang, J.L., Cates, D.H., Law, S.E. and Eriksson, K.-E.L. 1994. Bleaching of softwood kraft pulps with the Enzone process. *Tappi Journal* **77:** 243 – 250.

Yeh, M., Craig, S., Lum, M.G. and Foong, F.C. 2005. Effects of the PT region of EngD and HLD of CbpA on solubility, catalytic activity and purification characteristics of EngD-CBD_{CbpA} fusions from *Clostridium cellulovorans. Journal of Biotechnology* **116:** 233 – 244.

Zhou, C., Bai, J., Deng, S., Wang, J., Zhu, J., Wu, M. and Wang, W. 2007. Cloning of a xylanase gene from *Aspergillus usamii* and its expression in *Escherichia coli*. *Bioresource Technology* **10:** 391 – 397.

Zimmerman, W. 1992. Bacterial degradation of hemicellulases. *Metal Ions in Biological Systems* **28:** 357 – 398.