Universidade do Minho
Escola de Ciências

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Molecular Biotechnology Approaches Towards the Optimization of Enzymes for Advanced Textile Applications

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Molecular Biotechnology Approaches Towards the Optimization of Enzymes for Advanced Textile Applications

Thesis for Doctoral degree in Sciences

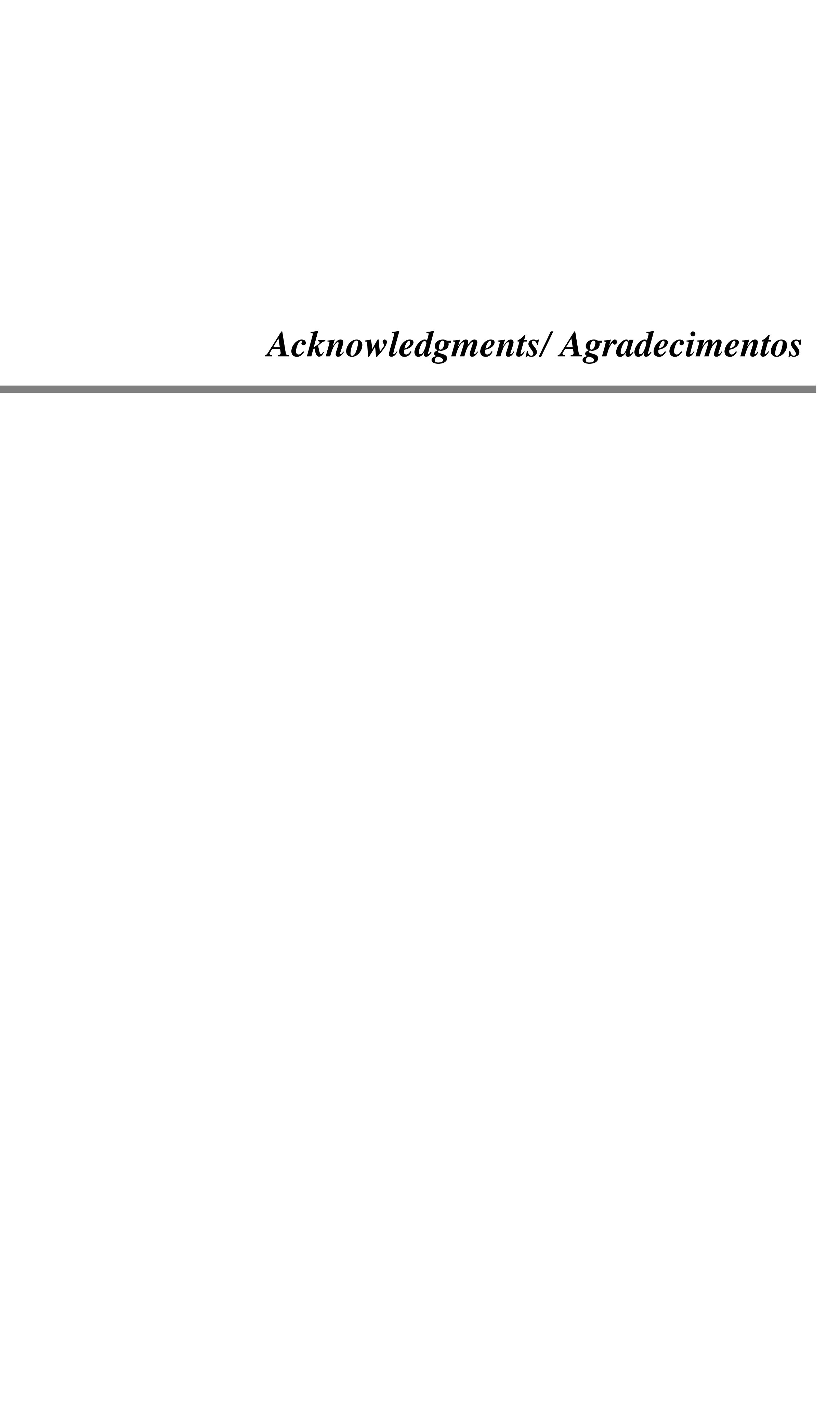
Elaborated under the supervision of
Professora Doutora Margarida Casal
Professor Doutor Artur Cavaco-Paulo

Junho 2008

É AUTORIZADA A REPRODUÇÃO PARCIAL DESTA TESE,
APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO
INTERESSADO, QUE A TAL SE COMPROMETE.

Rita Isabel Lima de Araújo

We are made wise not by the recollection of our past, but by the responsibility for our future." George Bernard Shaw



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Abstract

Abstract

The challenges facing the textile finishing industry have intensified during the last decade. Current awareness of the negative environmental impact of chemical processing in textile industry, combined with increased strict legislation on industrial effluents, has led to the search for advanced, non-polluting processes, for treating both natural and synthetic fibre fabrics. Enzymes can represent good alternatives for the traditional textile processes allowing not only the reduction of costs, the protection of the environment, and increasing safety of employees but also contributing for the improvement of the quality and functionality of the final products.

In the present work biotechnological approaches and genetic engineering methods were used aiming at the development and optimization of enzymatic eco-friendly processes for surface modification of synthetic and natural fibres.

A general introduction is presented in Chapter 1 where an extensive bibliographic revision concerning the use of enzymes in textile industry is presented, through the identification and description of the major commercial processes, and the most recent developments obtained in this field.

Chapter 2 deals with the surface modification of synthetic fibres by recombinant cutinase from the phytopathogenic fungus *Fusarium solani pisi* produced by molecular genetics tools. The Subchapter 2.1 is an introduction to the synthetic fibres utilized in the scope of this work. Subchapter 2.2 reports the structural modulation studies that allowed the identification of the aminoacids L81, N84, L182, V184 and L189 as targets to be substituted by Alanine allowing a better fit of large susbtrates in the active site of cutinase. All the mutations were obtained by site-directed mutagenesis and heterologously expressed in *Escherichia coli*. The genetically modified cutinase L182A presented higher stabilization on polyamide 6,6 (PA 6,6) and polyethylene terephthalate (PET) model substrates, a mutant variant that was chosen for further studies concerning the design and optimization of processes for functionalization of both fibres (Subchapters 2.3 and 2.4, respectively). Optimization of native enzyme was also performed, for the surface modificationof cellulose acetate, by creating chimeric fusions of the cutinase DNA coding sequence with either the fungal carbohydrate-binding

module (CBM) of Cellobiohydrolase I, or the bacterial CBM of Endoglucanase C. The new recombinant cutinase fused to the fungal CBM presented higher hydrolysis of cellulose diacetate and improved the colour levels of the treated fabrics (Subchapter 2.5).

The Chapter 3 describes the design of enzymatic-based technologies for wool fibres finishing industrial applications. Wool has the intrinsic characteristic to felt and shrink due to its scaly structure; the chlorine-Hercosett is the commercial process used to modify the scales of wool fibres with the purpose of providing resistance to felting and shrinkage. There have been several attempts to replace this chlorine process by proteases, in order to degrade scales, providing wool with anti-felting and anti-shrinkage characteristics. However, proteases commercially available can diffuse inside the fibre causing unacceptable damages. In this thesis two novel approaches were followed to increase molecular weight of the subtilisin E by genetic engineering. Poly-enzymes composed of two and four subtilisin E coding sequences fused in frame were constructed. Additionally, another chimeric subtilisin was obtained by 3´-terminus fusion with the nucleotidic sequence coding for the human neckdomain of surfactant protein D. All chimeric subtilisins were cloned and overexpressed into *E. coli* but the soluble and active forms were not attained, regardless the expression system or the strain used, under the culture conditions tested (Subchapter 3.2). Subchapter 3.3 describes the fusion of subtilisin E gene in frame with the DNA coding an elastin-like polymer containing 220 repeats of the monomer VPAVG. With this strategy the construction of a chimeric enzyme presenting a molecular weight above 116 kDa was achieved. Wool yarns treated either with commercial or chimeric enzyme showed a size-dependent diffusion process: the commercial enzyme penetrated into wool cortex while the chimeric one was retained at the surface, in the cuticle layer. These results represent a major achievment: the production of a recombinant high molecular weight protease, for wool surface controlled-hydrolysis, is reported for the first time.

Chapter 4 presents a general discussion, the major conclusions and gives some perspectives for continuing the work in this research field.

Resumo

Resumo

Os desafios que a indústria têxtil enfrenta têm-se intensificado na última década, especialmente devido ao impacto ambiental provocado pelos químicos derivados dos acabamentos das fibras têxteis. Concomitantemente, a legislação cada vez mais restrita no que respeita aos efluentes têxteis levou a uma procura de soluções avançadas, não-poluentes, para o tratamento de fibras naturais e sintéticas. A aplicação de enzimas representa uma alternativa promissora aos processos químicos tradicionais, permitindo uma redução de custos e representando simultaneamente, vantagens no que concerne à protecção ambiental, ao aumento da segurança dos trabalhadores e à melhoria da qualidade e da funcionalidade do produto final.

No presente trabalho seguiu-se uma abordagem biotecnológica, com recurso às técnicas de engenharia genética, com vista ao desenvolvimento e optimização de métodos enzimáticos, eco-sustentáveis, com aplicação na modificação da superfície de fibras têxteis naturais e sintéticas.

O capítulo 1, correspondente à introdução geral, apresenta uma revisão bibliográfica do uso de enzimas na indústria têxtil através da identificação e descrição dos processos enzimáticos já implementados ao nível comercial, bem como da investigação e dos resultados mais recentemente obtidos nesta área.

No Capítulo 2 apresentam-se as estratégias de modificação da superfície de fibras sintéticas por acção da enzima cutinase, originária do fungo fitopatogénico *Fusarium solani pisi,* produzida por expressão heteróloga em *Escherichia coli*. No Subcapítulo 2.1 apresenta-se uma introdução teórica às fibras sintéticas utilizadas no âmbito deste trabalho. No Subcapítulo 2.2 descrevem-se os estudos de modelação estrutural da cutinase que permitiram a identificação dos resíduos aminoacídicos L81, N84, L182, V184 e L189 como alvos de substituição pelo aminoácido Alanina, viabilizando acomodar substratos de maior dimensão no centro activo. As mutações referidas foram obtidas recorrendo à técnica de mutagénese dirigida. A cutinase geneticamente modificada L182A apresentou uma maior estabilidade com os substratos modelo da poliamida 6,6 (PA 6,6) e do polietileno tereftalato (PET), tendo sido seleccionada para estudos ulteriores com vista ao desenho e optimização de processos de funcionalização

das fibras sintéticas referidas (Subcapítulos 2.3 e 2.4, respectivamente). A cutinase nativa foi ainda utilizada para a modificação da superfície da fibra acetato de celulose. Foram efectuadas duas construções quiméricas pela fusão do gene da cutinase com o módulo de ligação a carbohidratos (CBM) de origem fúngica, presente na enzima *Cellobiohydrolase* I e com o CBM de origem bacteriana, presente na enzima *Endoglucanase* C. Verificou-se que a enzima recombinante, fundida com o CBM de origem fúngica, apresentou maior actividade hidrolítica sobre o diacetato de celulose e aumentou os níveis de tingimento das fibras (Subchapter 2.5).

O Capítulo 3 descreve o desenho de tecnologias baseadas em processos enzimáticos para aplicação industrial do acabamento da lã. A tendência da lã para feltrar e encolher é devida principalmente à sua estrutura em forma de escamas. O tratamento anti-feltragem, normalmente utilizado para modificar as escamas das fibras de lã, utiliza cloro, pelo que, têm sido conduzidas abordagens para substituir este processo por uma alternativa mais ecológica, nomeadamente pelo recurso a proteases. Contudo, devido ao seu tamanho, as proteases difundem-se no interior da fibra, atacando não só a cutícula mas também o córtex, o que provoca danos inaceitáveis do ponto de vista comercial. Os Subcapítulos 3.2 e 3.3 descrevem as estratégias utilizadas para aumentar o peso molecular da protease subtilisina E através de técnicas de engenharia genética. Foram construídas duas poli-enzimas compostas por duas e por quatro repetições da unidade codificante, clonadas na mesma fase de leitura. Além disso, construiu-se ainda uma fusão da sequência nucleotídica da subtilisina E com a sequência codificante do *neckdomain* da proteína humana surfactante D. Todas as proteínas recombinantes referidas foram sobre-expressas mas, independentemente do sistema de expressão ou da estirpe usada, não foi conseguida a sua recuperação na forma solúvel e activa nas condições testadas (Subcapítulo 3.2). No Subcapítulo 3.3 descreve-se a fusão da sequência codificante da subtilisina E com a sequência nucleotídica que codifica um polímero elastomérico contendo 220 repetições do monómero VPAVG. A enzima quimérica obtida apresentou um peso molecular superior a 116 kDa. Quando comparada com uma protease comercial constatou-se que a difusão das enzimas no interior da lã era dependente do peso molecular das mesmas: a de origem comercial, de baixo peso molecular, penetrou no córtex, por outro lado a enzima quimérica, de elevado peso

molecular ficou retida à superfície na cutícula da fibra. Estes resultados representam um grande avanço para o objectivo deste trabalho uma vez que constitui o primeiro caso de sucesso, referido na literatura, em que foi consegida a produção de uma protease recombinante de elevado peso molecular, aplicável à hidrólise controlada da superfície da lã.

O Capítulo 4 apresenta a discussão geral, as principais conclusões e algumas perspectivas que direccionam para a continuidade do trabalho desenvolvido nesta linha de investigação.

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LIST OF ABBREVIATIONS AND SYMBOLS

AFM– Atomic force microscopy
Ala/A– Alanine
Asn/N– Aspartate
ATR– Attenuated total reflectance
BOD– biological oxygen demand
BSA– Bovine serum albumine

CATs– Catalases
CBD– Carbohydrate binding domain
CD– Circular dichroism
CM– Complete Minimal
COD– chemical oxygen demand
CPs– Cysteine proteases
CV– Crystallinity value
Cys/C– Cysteine
DFP– Diisopropyl-fluorophosphate
DLS– Dynamic light scattering
DNA– Deoxyribonucleic acid
EDTA– Ethylenediamine tetraacetic acid
EGTA– Ethylene glycol tetraacetic acid
ELP– Elastin-like polymer
FT-IR– Fourier-transform infrared
Gly/G– Glycine
His/H– Histidine
HPAEC– High-performance anion-exchange chromatography
HSV– Herpes simplex virus
IMAC– Immobilized metal affinity chromatography
IPTG– Isopropyl-1-thio-β-galactopyranoside
LB – Luria Bertani
Leu/L– Leucine
Lys/K– Lysine
MD/MM– Molecular dynamics/ molecuçar mechanics
Met/M– Metionine
MTGase– Microbial transglutaminase
MW– Molecular weight
NHase– Nitrile hydratase
NMR– Nuclear magnetic resonance
OD– Optical density
OS– Osmotic solution
OX– Oxianion-hole
PA– Polyamide
PAN– Polyacrylonitrile
PBS – Phosphate buffered saline
PCR– Polymerase chain reaction
PDB– Protein data base
PEs – Pectin esterases
PET– Polyethyleneterephthalate

PGLs – polygalacturonate lyases
PGs– polygalactutonases
PMSF– Phenyl methyl sulphonyl fluoride
RNA– Ribonucleic acid
SDM– Site directed mutagenesis
SDS-PAGE– Sodium dodecyl sulphate-polyacrilamide gel electrophoresis
SEM – Scanning electron microscopy
Ser/S– Serine
SP-D– Surfactant protein D
TB – Terrific broth
TCA– Trichloroacetic acid
TE – Tris EDTA
TGs–Transglutaminases
Thr/T– Threonine
TI– Tetrahedral intermediate
TNBS– Trinitrobenzenesulfonic acid
Trp/W– Tryptophan
Tt – Transition temperature
Tyr/Y– Tyrosine
UV– Ultra violet
Val/V – Valine
WAXD– Wide-angle X-ray diffraction
ρ-NP– ρ-nitrophenol
ρ-NPB– ρ-nitrophenyl butyrate

Chapter 1

General Introduction:
Application of Enzymes for Textile Fibres Processing

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Application of Enzymes for Textile Fibres Processing

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Abstract

The aim of this paper is to highlight the use of enzymes in textile industry through the identification of the already commercial existing processes, as well as, the research that has been done in this field. Amylases have been used for desizing since the middle of last century. Enzymes used in detergent formulations have also been successfully used for the past 40 years. The application of cellulases for denim finishing and laccases for decolourization of textile effluents and textile bleaching are the most recent advances in the commercial sector. New developments rely on the modification of natural and synthetic fibres. The advances in enzymology, molecular biology and screening techniques provide good direction for the development of new enzyme-based processes for a more ecological approach for textile industry.

1. Biotechnology in textile industry

Biotechnology is the application of scientific and engineering principles to the processing of materials by biological agents and/or their components to provide goods and services. White or industrial biotechnology is biotechnology applied to industrial processes. An example is the use of enzymes in textile industry which allows the development of environmentally friendly technologies in fibre processing and in strategies to improve the final product quality. The consumption of energy and raw-materials, as well as, an increasing awareness with environmental concerns related to the use and disposal of chemicals into landfills, into water, or release in air during chemical processing of textiles are the principal reasons for the application of enzymes in finishing of textile materials (O'Neill *et al.,* 1999).

2. Production of enzymes: searching for efficient production systems

Commercial sources of enzymes are obtained from any biological source: animal, plants and microbes. These naturally occurring enzymes are quite often not readily available in sufficient quantities for industrial use. With the advances in genomics, proteomics and bioinformatics, the number of proteins being produced using recombinant techniques is exponentially increasing. Screening approaches are being performed to rapidly identify enzymes with a potential industrial application (Korf *et al.,* 2005). For this purpose, different expression hosts (*Escherichia coli*, *Bacillus* sp., *Saccharomyces cerevisiae*, *Pichia pastoris*, filamentous fungi, insect and mammalian cell lines) have been developed to express heterologous proteins (Makrides, 1996; Silbersack *et al.,* 2006; Li *et al.,* 2007; Ogay *et al.,* 2006; Huynh and Zieler, 1999; Chelikani *et al.,* 2006). Among the many systems available for heterologous protein production, the enteric Gram-negative bacterium *Escherichia coli* remain one of the most attractive. Compared with other established and emerging expression systems, *E. coli*, offers several advantages including its ability to grow rapidly and at high density on inexpensive carbon sources, simple scale-up process, its well-characterized genetics and the availability of an increasingly large number of cloning vectors and mutant host strains (Baneyx, 1999). However, the use of *E. coli* is not always suitable because it lacks some auxiliary

biochemical pathways that are essential for the phenotypic expression of certain functions, e.g. degradation of aromatic compounds, antibiotic synthesis, sporulation, so there is no guarantee that a recombinant gene product will accumulate in *E. coli* at high levels in a full-length and biologically active form (Makrides, 1996). In such circumstances, the genes have to be cloned back into species similar to those from which they were derived. In these cases bacteria from the unrelated genera Bacillus, (Silbersack *et al.,* 2006; Biedendieck *et al.,* 2007) *Clostridium* (Girbal *et al.,* 2005) *Staphylococcus* and the lactic acid bacteria *Streptococcus* (Arnau *et al.,* 2006) *Lactococcus* (Miyoshi *et al.,* 2002) and *Lactobacillus* (Miyoshi *et al.,* 2004) can be used.

If heterologous proteins require complex post-translational modifications and are not expressed in the soluble form using prokaryotic expression systems, yeasts can be an efficient alternative once they provide several advantages over bacteria for the production of eukaryotic proteins. Among yeast species, the methylotrophic yeast *Pichia pastoris* is a particularly well suited host for this purpose. The use of this organism for expression offers a number of important benefits: i) high levels of recombinant protein expression are reached under the alcohol oxidase1 gene (aox 1) promoter; ii) this organism grows to high cell densities; iii) scaled-up fermentation methods without loss of yield have been developed; iv) efficient secretion of the recombinant product together with a very low level of endogenous protein secretion represents a very simple and convenient pre-purification step; v), accurate post-translational modifications are allowed (such as proteolytic processing and glycosylation). Furthermore, the existence of efficient methods to integrate several copies of the expression cassette carrying the recombinant DNA into the genome, eliminating the problems associated with expression from plasmids, is making this yeast the microorganism of choice for an increasing number of biotechnologists (Cereghino and Cregg, 2000; Hollenberg and Gellissen, 1997).

Once fermentation is completed, the microorganisms are destroyed; enzymes are isolated and further processed for commercial use.

3. Role of enzymes in textile industry

Textile processing has benefited greatly on both environmental and product quality aspects through the use of enzymes. From the 7000 enzymes known, only about 75 are commonly used in textile industry processes (Quandt and Kuhl, 2001).

The principal enzymes applied in textile industry are hydrolases and oxidoreductases. The group of hydrolases includes amylases, celulases, proteases, pectinases and lipases/esterases. Amylases were the only enzymes applied in textile processing until the 1980's. These enzymes are still used to remove starch-based sizes from fabrics after weaving. Cellulases have been employed to enzymatically remove fibrils and fuzz fibres and have also successfully been introduced to the cotton textile industry and later for lyocell processes. Further applications have been found for these enzymes to produce the aged look of denim and other garments. The potential of proteolytic enzymes was assessed for the removal of wool-fibre-scales resulting in improved anti-felting behaviour. Despite the fact that investigations in this area are still on going, an industrial process has not yet been achieved. Esterases have been successfully studied for the partial hydrolysis of synthetic fibres surface, improving their hydrophilicity and further finishing steps. Besides hydrolytic enzymes, oxidoreductases have also been realized as powerful tools in various textile-processing steps. Catalases have been used to remove H_2O_2 after bleaching reducing in this way water consumption. A recent book edited by Wolfgang Aehle (2007), contains an excellent chapter dealing with enzyme technology application in the textile processing industry. A more detailed description of both the most common group of enzymes applied in textile industry and the processes where they are applied will be given in this review.

4. Amylases

Amylases are enzymes which hydrolyse starch molecules to give diverse products including dextrins and progressively smaller polymers composed of glucose units (Windish and Mhatre, 1965). This starch hydrolysing enzymes are classified according to the type of sugars produced by enzymatic reaction: α-amylases and β-amylases. α-Amylases are produced by a variety fungi, yeasts and bacteria, however enzymes from

filamentous fungal and bacterial sources are the most used in industrial sectors (Pandey *et al.,* 2000).
Molecular weights of microbial α-amylases range from 50 to 60 KDa, with few exceptions, like a 10 KDa α-amylase from *Bacillus caldolyticus* and a 210 KDa α-amylase from *Chloroflexus aurantiacus* (Grootegoed *et al.,* 1973; Ratanakhanokchai *et al.,* 1992). α-Amylases from most bacteria and fungi are quite stable over a wide range of pH from 4 to 11. *Alicyclobacillus acidocaldarius* α-amylase present an acidic pH optima of 3 in contrast to α-amylases from several alkalophilic and extreme alkalophilic *Bacillus* sp. with pH optima of 9 to 10.5 and 11 to 12, respectively (Schwermann *et al.,* 1994; Krishnan and Chandra, 1983; Lee *et al.,* 1994; Kim *et al.,* 1995).
Optimum temperature for the activity of α-amylases is also related to the growth of the producer microorganism (Vihinen and Mantsala, 1989). Temperatures from 25 to 30 ºC were reported for Fusarium oxysporum α-amylase (Chary and Reddy, 1985) and higher temperatures of 100 and 130 ºC for *Pyrococcus furiosus* and *Pyrococcus woesei*, respectively (Laderman *et al.,* 1993; Koch *et al.,* 1991).
Addition of Ca^{2+} can, in some cases, enhance α-amylases thermostability (Vihinen and Mantsala, 1989; Vallee *et al.,* 1959). They are extremely inhibited by heavy metal ions, sulphydryl group reagents, EDTA and EGTA (Mar *et al.,* 2003; Tripathi *et al.,* 2007).
In general microbial α-amylases display highest specificity towards starch followed by amylase, amylopectin, cyclodextrin, glycogen and maltotriose (Vihinen and Mantsala, 1989).

4.1 Textile Desizing

Amylases are the most successful enzymes used in textile industry for desizing. For fabrics made from cotton or blends, the warp threads are coated with an adhesive substance know as “size” to lubricate and protect the yarn from abrasion preventing the threads to break during weaving. Although many different compounds have been used to size fabrics, starch and its derivatives have been the most common sizing agents because of their excellent film forming capacity, availability, and relatively low cost (Feitkenhauer *et al.,* 2003).

After weaving, the applied sizing agent and the natural non-cellulosic materials present in the cotton must be removed in order to prepare the fabric for dyeing and finishing. Before the discovery of amylases, this process (desizing) used to be carried out by treating the fabric with chemicals such as acids, alkali or oxidising agents at high temperatures. The chemical treatment was not totally effective in removing the starch (which leads to imperfections in dyeing) and also resulted in a degradation of the cotton fiber conducting to destruction of the natural, soft feel of the cotton.
Nowadays amylases are currently commercialized (MAPS, India) and preferred for desizing due to their high efficiency and specific action. Amylases bring about complete removal of the size without any harmful effects on the fabric (Etters and Annis, 1998; Cegarra, 1996). The starch is randomly cleaved into water soluble dextrins that can be then removed by washing. The utilization of harsh chemicals in the textile desizing was substituted by amylases resulting in a lower discharge of waste chemicals to the environment and improved the safety of working conditions for textile workers.

5. Pectinases

Pectin and other peptic substances are complex polysaccharides present in plant cell wall as a part of middle lamella. Pectinolytic enzymes or pectinases are a complex group of enzymes involved in the degradation of pectic substances. They are primarily produced in nature by saprophytes and plant pathogens (bacteria and fungi) for degradation of plant cell walls (Lang and Dörenberg, 2000; Bateman, 1966). There are three major classes of pectin degrading enzymes: pectin esterases (PEs), polygalacturonases (PGs) and polygalacturonate lyases (PGLs).
Pectin esterases are mainly produced in plants such as banana, citrus fruits and tomato and also by bacteria and fungi (Hasunuma *et al.,* 2003). Pectin esterase catalyzes deesterification of the methyl group of pectin, forming pectic acid. The enzyme acts preferentially on a methyl ester group of galacturonate unit next to a non-esterifed galacturonate unit. The molecular weight of most microbial and plant PEs varies between 30 – 50 kDa (Hadj-Taieb *et al.,* 2002; Christensen *et al.,* 2002). The optimum pH for activity varies between 4.0 and 7.0. The exception is PE from *Erwinia* whose

optimum pH is in alkaline region. The optimum temperature ranges between 40 and 60 ºC and pI between 4.0 and 8.0.

Polygalacturonases are a group of enzymes which hydrolyze α-1,4 glycosidic linkages in pectin by both exo and endo splitting mechanisms. Endo PGs are widely distributed among fungi, bacteria and yeast. These enzymes often occur in different forms having molecular weights in the range of 30 – 80 kDa and pI between 3.8 and 7.6. Their optimum pH is in the acidic range of 2.5 – 6.0 and the optimum temperature between 30 and 50 ºC (Singh and Rao, 2002; Takao *et al.,* 2001). Exo PGs are widely distributed in *Aspergilus niger*, *Erwinia* sp. and in some plants such as carrots, peaches, citrus and apples (Pressey and Avants, 1975; Pathak and Sanwal, 1998). The molecular weight of exo PGs vary between 30 – 50 kDa and their pI ranges between 4.0 and 6.0.

The polygalacturonate lyase cleaves polygalacturonate or pectin chains via a β-elimination mechanism which results in the formation of a double bond between C4 and C5 at the non-reducing end and an elimination of CO_2. Endo-polygalacturonate lyase cleaves polygalacturonate chains arbitrarily and exo-polygalacturonate lyase splits at the chain end of polygalacturonate which yields unsaturated galacturonic acid (Sakai *et al.,* 1993). The molecular weight of PGLs varies between 30–50 kDa except in the case of PGL from *Bacteroides* and *Pseudoalteromonas* (75 kDa) (McCarthy *et al.,* 1985; Truong *et al.,* 2001). The optimum pH ranges between 8.0 and 10.0 although PGL from *Erwinia* and *Bacillus licheniformis* were still active at pH 6.0 and 11.0 respectively. The optimum temperature for PGL activity is between 30 and 40 ºC. However, certain PGL from thermophiles have an optimum temperature between 50 and 75 ºC. The potential of some pectate lyases for bioscouring has been exploited.

5.1 Enzymatic scouring

Greige or untreated cotton contains various non-cellulosic impurities, such as, waxes, pectins, hemicelluloses and mineral salts, present in the cuticle and primary cell wall of the fibre (Batra, 1985; Etters *et al.,* 1999). These non-cellulosic materials are responsible for the hydrophobic properties of raw cotton and interfere with further aqueous chemical processes on cotton, like dyeing and finishing (Freytag and Dinze,

1983). Therefore, before cotton yarn or fabric can be dyed, it needs to be pretreated to remove materials that inhibit dye binding. This step, named scouring, contributes to the wettability improvement of fabric that can be then bleached and dyed successfully. Highly alkaline chemicals such as sodium hydroxide were normally used for scouring. These chemicals not only remove the impurities but also attack the cellulose, leading to a reduction in strength and loss of fabric weight. Furthermore, the resulting wastewater has a high COD (chemical oxygen demand), BOD (biological oxygen demand) and salt content (Buschle-Diller *et al.,* 1998). On the other hand, the enzymatic scouring, bioscouring, leaves the cellulose structure almost intact, so it prevents cellulose weight and strength loss. Bioscouring has a number of potential advantages over traditional scouring. Bioscouring is performed at neutral pH which reduces total water consumption, the treated yarn/fabrics retain their strength properties, the weight loss is reduced or limited compared to processing in traditional ways and increases cotton fibres softness. Several types of enzymes, including pectinases (Li and Hardin, 1997; Karapinar and Sariisik, 2004; Tzanov *et al.,* 2001; Choe *et al.,* 2004; Ibrahim *et al.,* 2004), cellulases (Li and Hardin, 1997; Karapinar and Sariisik, 2004), proteases (Karapinar and Sariisik, 2004), and lipases/cutinases, alone or combined, (Deganil *et al.,* 2002; Sangwatanaroj and Choonukulpong, 2003; Buchert *et al.,* 2000; Hartzell and Hsieh, 1998) have been studied for cotton bioscouring, pectinases seems to be the most effective for that purpose.

Besides all the research done to develop an efficient bioscouring process, there is no broad commercial application yet on industrial scale. There is still a demand for a pectinase with higher activity and stability at hight temperatures and alkaline conditions. A new bio-scouring pectate lyase from *Bacillus pumilus* BK2 was reported by Klug-Santner and collaborators with optimum activity at pH 8.5 and around 70 ºC (Klug-Santner *et al.,* 2006). The new isolated pectate lyase was assessed for bio-scouring of cotton fabric. Removal of up to 80% of pectin was proven by means of ruthenium red dyeing and HPAEC. Liquid porosimetry was used to evaluate the increasing hydrophilicity of fabrics based on changes of the structural contact angle (Bernard and Tyomkin, 1994). Using this methodology the authors found that, upon enzyme treatment, hydrophilicity of the fabrics was dramatically enhanced (Klug-

Santner *et al.,* 2006). Solbak and collaborators developed a novel pectate lyase, by Directed Evolution, with improved thermostability. The new enzyme contained eight point mutations (A118H, T190L, A197G, S208K, S263K, N275Y, Y309W, and S312V). Compared to the wild-type, it presented a 16 °C higher melting temperature and exhibited better bioscouring performance at low enzyme dosage in a high temperature bioscouring process (Solbak *et al.,* 2005).

More recently, Agrawal and collaborators performed a wax removal step prior to enzymatic scouring of cotton. The authors hypothesized that removal of outer waxy layer would allow access and efficient reaction of pectinase with the substrate. They demonstrated that pre-treatment of fibres with n- hexane (for wax removal) improved alkali pectinase performance in terms of hydrophilicity and pectin removal (Agrawal *et al.,* 2007).

Characterization of chemical and physical surface changes of fabrics, after bioscouring, and identification of suitable methods, for surface analysis, are also subject of great interest in order to better understand the bioscouring mechanism and evaluate its effects on fabrics. Fourier-transform infrared (FT-IR) attenuated total reflectance (ATR) spectroscopy was used for the first time, by Chung and collaborators, for fast characterization of cotton fabric scouring process (Chung *et al.,* 2004). Later, Wang combined FT-IR ATR spectroscopy with scanning electron microscopy (SEM) and atomic force microscopy (AFM) to characterize bioscoured cotton fibres (Wang *et al.,* 2006). SEM had been used before for this purpose (Li and Hardin, 1997), however, this technique did not provide information about height and roughness of sample surface. On the other hand, the authors demonstrated that AFM, which can generate fine surface topographies of samples at atomic resolutions, is a useful supplement to SEM in characterizing cotton surfaces (Wang *et al.,* 2006).

6. Cellulases

Cellulases are hydrolytic enzymes that catalyse the breakdown of cellulose to smaller oligosaccharides and finally glucose. Cellulase activity refers to a multicomponent

enzyme system combining at least three types of cellulases working synergistically together (Teeri, 1997).

Endoglucanases or endocellulases cleave bonds along the length of cellulose chains in the middle of the amorphous region. Cellobiohydrolases or exo-cellulases start their action from the crystalline ends of cellulose chains, producing primarily cellobiose. Cellobiohydrolases act synergistically with each other and with endoglucanases, thus mixtures of all these types of enzymes have greater activity than the sum of activities of each individual enzyme alone. Cellobiose and soluble oligosaccharides, produced by exo-cellulases, are finally converted to glucose by β-4-glucosidase (Teeri, 1997).

These enzymes are commonly produced by soil-dwelling fungi and bacteria, being the most important *Trichoderma*, *Penicillium* and *Fusarium* (Verma *et al.,* 2007; Jorgensen *et al.,* 2005; Kuhad *et al.,* 1999). Many of the fungal cellulases are modular proteins consisting of a catalytic domain, a carbohydrate-binding domain (CBD) and a connecting linker. The role of CBD is to mediate the binding of the enzyme to the insoluble cellulose substrate (Mosier *et al.,* 1999).

Cellulases are active in a temperature range from 30 to 60 ºC. Based on their sensitivity to pH, they are classified as acid stable (pH 4.5-5.5), neutral (pH 6.6-7) or alkali stable (pH 9-10). The application of cellulases in textile processing started in the late 1980s with denim finishing. Currently, in addition to biostoning, cellulases are also used to process cotton and other cellulose-based fibres.

6.1 Denim finishing

Many garments are subjected to a wash treatment to give them a slightly worn look, an example is the stonewashing of denim jeans. In the traditional stonewashing process, the blue denim is faded by the abrasive action of pumice stones on the garment surface. However, thanks to the introduction of cellulase enzymes, the jeans industry can reduce or even eliminate the use of stones. The use of less pumice stones results in less damage to garment, machine and less pumice dust in the laundry environment. Productivity can also be increased because laundry machines contain fewer stones or no stones at all and more garments. Denim garments are dyed with indigo, which adheres to the surface of

the yarn. The cellulase molecule binds to an exposed fibril on the surface of the yarn and hydrolyses it in a process know as 'Bio-Stonewashing', leaving the interior part of the cotton fibre intact. When the cellulases partly hydrolyse the surface of the fibre, the indigo is partly removed and light areas are created. There are a number of cellulases available, each with its own special properties. These can be used either alone or in combination in order to obtain a specific look. Heikinhemo and collaborators demonstrated that *Trichoderma reesei* endoglucanase II was very effective removing color from denim, producing a good stonewashing effect with the lowest hydrolysis level (Heikinheimo *et al.,* 2000). Later Miettinen-Oinonen and collaborators developed new genetically engineered *T. reesei* strains able to produce elevated amounts of endoglucanase activity. Production of endoglucanase I and II was increased fourfold above that of the host strain, without any production of cellobiohydrolases. Cellulase preparations derived by the new *T. reesei* overproduction strains proved to be more efficient for stonewashing process than the ones produced by parental strain (Miettinen-Oinonen and Suominen, 2002). Application research in this area is mainly focused on preventing or enhancing backstaining depending on the style required. Backstaining is defined as the redeposition of released indigo onto the garments. Cavaco-Paulo and collaborators were the first group studying in detail the nature of indigo-cellulase-cellulose interactions (Cavaco-Paulo *et al.,* 1998). These authors attribute the effect of backstaining to the high affinity between indigo and cellulase and proved that the strong ability of cellulase enzymes to bind to cotton cellulose is the major cause of backstaining (Cavaco-Paulo *et al.,* 1998). Later, the affinity of cellulases from different fungal origins for insoluble indigo dye in the absence of cellulose was compared. The authors reported that acid cellulases from *T. reesei* have higher affinity for indigo than neutral cellulases of *Humicola insolens* (Campos *et al.,* 2000). The same group studied the interactions of cotton with CBD peptides from family I and family II and they provided new highlights for tailoring cellulases when they found that truncated cellulases without CBDs caused less backstaining than entire enzymes (Cavaco-Paulo *et al.,* 1999; Andreaus *et al.,* 2000). These authors had previously studied the effect of temperature on the cellulose binding ability of cellulases from *T. reesei* and the

influence of agitation level on the processing of cotton fabrics with cellulases having CBDs from different families (Cavaco–Paulo *et al.,* 1996; Andreaus *et al.,* 1999).

In order to overcome the lack of methods to access the performance of small quantities of enzymes, Gusakov and collaborators have developed a model microassay to test the abrasive and backstaining properties of cellulases on a "test-tube scale" (Gusakov *et al.,* 2000). Using these microassays, the same group identified an endoglucanase from *Chysosporium lucknowense* with a high washing performance and a moderate level of backstaining (Sinitsyn *et al.,* 2001).

Knowing that backstaining process could be significantly reduced at neutral pH range, neutral cellulases started to be screened in order to minimize backstaining. Miettinen-Oinonen and collaborators reported the purification and characterization of three novel cellulases of *Melanocarpus albomyces* for textile treatment at neutral pH: a 20 kDa and 50 kDa endoglucanases and a 50 kDa cellobiohydrolase. The 20 kDa endoglucanase had a good biostoning performance. Combining the 50 kDa endoglucanase, or the 50 kDa cellobiohydrolase with the 20 kDa endoglucanase, it was possible to decrease the backstaining levels (Miettinen-Oinonen *et al.,* 2004). The respective genes were further cloned and efficiently expressed at adequate levels for industrial applications in *T. reesei* by the same group (Haakana *et al.,* 2004; Pazarlioglu *et al.,* 2005; Anish *et al.,* 2007). Nowadays due to availability of effective anti-backstaining agents based on chemicals or enzymes, like proteases and lipases, backstaining problems can be minimized. The combination of new looks, lower costs, shorter treatment times and less solid waste have made abrasion with enzymes the most widely used fading process today.

6.2 Pilling and fuzz fibre removal

Besides "biostoning" process, cotton and other natural and man-made cellulosic fibres can be improved by an enzymatic treatment called "biopolishing". The main advantage of this process is the prevention of pilling. A ball of fuzz is called a 'pill' in the textile trade. These pills can present a serious quality problem since they result in an unattractive, knotty fabric appearance. Cellulases hydrolyse the microfibrils (hairs or

fuzz) protruding from the surface of yarn because they are most susceptible to enzymatic attack. This weakens the microfibrils, which tend to break off from the main body of the fibre and leave a smoother yarn surface. After treatment, the fabric shows a much lower pilling tendency. Other benefits of removing fuzz are a softer, smoother feel and superior colour brightness. Unlike conventional softeners, which tend to be washed out and often result in a greasy feel, the softness-enhancing effects of cellulases are washproof and non-greasy.

Optimization of biofinishing processes has been an important matter of research. Azevedo and colleagues (2001) studied the desorption of cellulases from cotton which can be applied for recovering and recycling of cellulases (Azevedo *et al.,* 2001). Lenting and collaborators came up with guidelines to minimize and prevent loss of tensile strength that can result from cellulase application. The choice of enzyme, enzyme concentration, incubation time, as well as, application of immobilized enzymes, use of liquids with different viscosities, use of foam ingredients and hydrophobic agents to impregnate clothes can prevent the drawbacks of cellulases action (Lenting and Warmoeskerken, 2001). Yamada and collaborators reported the action of cellulases on cotton dyed with reactive dyes which have an inhibitory effect on cellulase activity (Yamada *et al.,* 2005). Ultrasound technology application can be an efficient way to improve enzymatic action in bioprocessing of cotton (Yachmenev *et al.,* 2002).

For cotton fabrics, polishing is optional for upgrading the fabric. However, this step is almost essential for the fibre lyocell, invented in 1991. It is made from wood pulp and is characterised by a tendency to fibrillate easily when wet (fibrils on the surface of the fibre peel up). If they are not removed, finished garments made from lyocell will end up covered with pills. Lyocell fabric is then treated with cellulases during finishing not only to avoid fibrillation, but also to enhance its silky appearance. There are several reports in literature concerning lyocell treatment with cellulases and the elucidation of its mechanism of action (Morgado *et al.,* 2000; Valldeperas *et al.,* 2000). Cellulases were reported not only for processing of lyocell but also for viscose type regenerated celluloses like viscose and modal (Carrillo *et al.,* 2003).

7. Serine proteases: subtilisins

Subtilisins are a family of alkaline serine proteases, generally, secreted by a variety of *Bacillus* species (Siezen and Leunissen, 1997). These enzymes catalyze the hydrolysis of peptide and ester bonds through formation of an acyl-enzyme intermediate. Subtilisins are biosynthesized as preproprotein precursors (Wells *et al.,* 1983). The NH2-terminal prepeptide, of 29 amino acid residues is the signal peptide required for secretion of prosubtilisin across the plasma membrane. The propeptide of 77 amino acids, located between the prepeptide and mature sequence, acts as an intramolecular chaperone required for the correct folding of mature enzyme in active form (Stahl and Ferrari, 1984; Wong and Doi, 1986; Ikemura *et al.,* 1987; Ikemura and Inouye, 1988). Subtilisins are characterized by a common three-layer α/ β/ α tertiary structure. The active site is composed of a catalytic triad of Aspartate, Histidine and Serine.

Molecular weight of subtilisins is generally between 15 to 30 kDa, but there are few exceptions, like a 90 kDa subtilisin from *Bacillus subtilis* (*natto*) (Kato *et al.,* 1992).

The optimum temperature of alkaline proteases ranges from 50 to 70 ºC but these enzymes are quite stable at high temperatures.

The presence of one or more calcium binding sites enhances enzyme thermostability (Paliwal *et al.,* 1994). Phenyl methyl sulphonyl fluoride (PMSF) and diisopropyl-fluorophosphate (DFP) are able to strongly inhibit subtilisins (Gold and Fahrney, 1964; Morihara, 1974). Most subtilisin protein engineering continues to involve enhancement of catalytic activity (Takagi *et al.,* 1988; Takagi *et al.,* 1997), and thermostability, (Takagi *et al.,* 1990; Wang *et al.,* 1993; Yang *et al.,* 2000a; Yang *et al.,* 2000b), as well as, substrate specificity and oxidation resistance (Takagi *et al.,* 1997).

7.1 Attempts for enzymatic treatment of wool

Raw wool is hydrophobic due to epicutical surface membranes containing fatty acids and hydrophobic impurities like wax and grease. Different harsh chemicals are commonly used for removal of these impurities: alkaline scouring using sodium carbonate, pre-treatment using potassium permanganate, sodium sulphite or hydrogen peroxide.

Wool fabric has the tendency to felt and shrink on wet processing. The shrinkage behaviour of wool can be regulated by various chemical means. The most successful commercial shrink-resistant process available is the chlorine-Hercosett process developed more than 30 years ago (Heiz, 1981). Although the excellent advantages of this method (good antifelt effect, low damage and low weight loss), there are some important drawbacks (limited durability, poor handle, yellowing of fibres, difficulties in dyeing and environmental impact by the release of absorbable organic halogens to the effluents) (Julia *et al.,* 2000; Schlink and Greeff, 2001).

Several authors have suggested the use of benign chemical processes such as low temperature plasma to treat wool (Kan *et al.,* 1998; Kan *et al.,* 1999; Kan *et al.,* 2006a; El-Zawahry *et al.,* 2006; Kan *et al.,* 2006b). Plasma treatment is a dry process, in which the treatment of wool fibre is performed by electric gas discharges (plasma). It is regarded as an environmentally friendly process, as no chemicals are used and can be applied as an effective technique for modifying the surface properties of wool without much alteration of the interior part of the fibre. However, costs, compatibility and capacity are obstacles to commercialization of a plasma treatment process and the shrink-resist properties obtained do not impart a machine-washable finish, which is one of the main objectives (McDevitt and Winkler, 2000). The posterior application of a natural polymer, such as chitosan, was also investigated to improve wool shrink-resistance or anti-felting properties (Onar and Sariisik, 2004).

More recently, mainly for ecological reasons, proteases, namely subtilisin type, are being studied as an alternative for chemical pre-treatment of wool. Several studies reported that pre-treatment of wool fibres with proteases, before the dyeing process, has been shown to improve anti-shrinkage properties, to remove impurities and to increase dyeing affinity (Levene *et al.,* 1996; Parvinzadeh, 2007).

However, due to its small size, the enzyme is able to penetrate into the fibre cortex which causes the destruction of the inner parts of wool structure (Shen *et al.,* 1999). Several reports show that increasing enzyme molecular weight by chemical crosslinking with glutaraldehyde or by the attachment of synthetic polymers like polyethylene glycol, is possible to avoid enzyme penetration and the consequent reduction of strength and weight loss (Silva *et al.,* 2004; Schroeder *et al.,* 2006). Some of these processes

have been tested at the scale of industrial process (Shen *et al.,* 2007). Pre-treatment of wool fibres with hydrogen peroxide, at alkaline pH in the presence of high concentrations of salts, also targets enzymatic activity on the outer surface of wool, by improving the susceptibility of cuticle for proteolytic degradation (Lenting *et al.,* 2006). Some authors describe methods to improve the shrink resistance of wool by treating wool previously with a smoother oxidizing agent, like H_2O_2, instead of the traditional oxidizers, NaClO or $KMnO_4$ and then with a protease (Yu *et al.,* 2005). The strong oxidation power of NaClO or $KMnO_4$ is always difficult to control. Besides, reaction of NaClO with wool produces halogenide. On the other hand, H_2O_2 seemed to provide a more controlled, cleaner and moderate oxidation. Zhang and collaborators used an anionic surfactant to promote the activities of proteases on wool (Zhang *et al.,* 2006). Other authors refer processes to achieve shrink-resistance by treating wool with a protease followed by a heat treatment (Ciampi *et al.,* 1996). The screening for new protease producing microorganisms with high specificity to cuticles is being investigated as an alternative for the existing proteases (Erlacher *et al.,* 2006).

8. Cysteine proteases: papain

Cysteine proteases (CP´s) catalyse the hydrolysis of peptide, amide, ester, thiol ester and thiono ester bonds. More than twenty families of cysteine proteases have been described (Barrett, 1994). The CP family can be subdivided into exopeptidases (e.g. cathepsin X, carboxypeptidase B) and endopeptidases (papain, bromelain, ficain, cathepsins). Exopeptidases cleave the peptide bond proximal to the amino or carboxy termini of the substrate, whereas endopeptidases cleave peptide bonds distant from the N- or C-termini (Barrett, 1994).

CPs are proteins of molecular mass in the range of 21 - 30 kDa. They are synthesized as inactive precursors with an N-terminal propeptide and a signal peptide. Activation requires proteolytic cleavage of the N-terminal propeptide that also functions as an inhibitor of the enzyme (Otto and Schirmeister, 1997; Grzonka *et al.,* 2001).

Papain is the best known cysteine protease. It was isolated in 1879 from the fruits of *Carica papaya* and was the first protease with the crystallographic structure determined

(Drenth *et al.,* 1968; Kamphuis *et al.,* 1984). Papain is a 212 amino acids protein with a molecular weight of 23.4 kDa. The enzyme has three internal disulphide bridges and an isoelectric point of 8.75.
The optimal activity of papain occurs at pH 5.8 - 7.0 and at temperature 50 - 57 ºC when casein is used as the substrate (Light *et al.,* 1964; Kamphuis *et al.,* 1984). The general mechanism of papain action has been very well studied. The catalytic triad is formed by Cys25, His159 and Asn175 residues. Asn175 is important for orientation of the imidazolium ring of the histidine in the catalytic cleft. The reactive thiol group of the enzyme has to be in the reduced form for catalytic activity. Thus, the cysteine proteases require a rather reducing and acidic environment to be active (Theodorou *et al.,* 2007). Generally, papain can cleave various peptide bonds and possesses therefore fairly broad specificity.

8.1 Degumming of silk

Papain is reported to be used for boiling off cocoons and degumming of silk. Raw silk must be degummed to remove sericin, a proteinaceous substance that covers the silk fibre. Degumming is usually performed in an alkaline solution containing soap, a harsh treatment that also attacks fibrin structure. Several alkaline, acidic and neutral proteases have been studied as degumming agents since they can dissolve sericin but are unable to affect silk fibre protein. Alkaline proteases have a better performance removing sericin and improving silk surface properties like handle, shining and smoothness (Freddi *et al.,* 2003; Arami *et al.,* 2007), but there is no commercial application yet on this field.
In the past, papain was also used to ‘shrink-proof’ wool. A successful method involved the partial hydrolysis of the scale tips. This method also gave wool a silky lustre and added to its value. The method was abandoned a few years ago for economic reasons.

9. Transglutaminases (TGs)

Transglutaminases are a group of thiol enzymes that catalyse the post-translational modification of proteins mainly by protein to protein cross-linking, but also through the

covalent conjugation of polyamines, lipid esterification, or the deamidation of glutamine residues (Folk and Cole, 1966; Folk *et al.,* 1968; Folk, 1969; Folk, 1980; Lorand and Conrad, 1984). Transglutaminases are widely distributed among bacteria, plants and animals.

The first characterized microbial transglutaminase (MTG) was that of the bacterium *Streptomyces mobaraensis* (Ando *et al.,* 1989). This enzyme is secreted as a zymogen that is sequentially processed by two endogenous enzymes to yield the mature form (Zotzel *et al.,* 2003). The mature enzyme is a monomeric protein with a molecular weight of 38 kDa. It contains a single catalytic cysteine residue (Cys64) and an isoelectric point (pI) of 9 (Pasternack *et al.,* 1998; Kanaji *et al.,* 1993).

The optimum pH for MTG activity was found to be between 5 and 8. However, MTG showed some activity at pH 4 or 9, and was thus considered to be stable over a wide pH range (Ando *et al.,* 1989). The optimum temperature for enzymatic activity was 55 °C; it maintained full activity for 10 min at 40 °C, but lost activity within a few minutes at 70 °C. It was active at 10 °C, and retained some activity at near-freezing temperatures.

MTG does not require calcium for activity, shows broad substrate specificity and can be produced at relatively low cost. These properties are advantageous for industrial applications.

9.1 Attempst for treatment of wool and leather

The study of TGs for the treatment of wool textiles has been shown to improve shrink resistance, tensile strength retention, handle, softness, wettability and consequently dye uptake, as well as, reduction of felting tendency and protection from the damage caused by the use of common detergents (Cortez *et al.,* 2004; Cortez *et al.,* 2005).

Treatment of leather with TG, together with keratin or casein, has a beneficial effect on the subsequent dyeing and colour properties of leather (Collighan *et al.,* 2002). The application of TG for leather and wool treatment seems to be a promising strategy, but is still in the research level. There is no industrial application.

10. Lipases/ Esterases: Cutinase

Esterases represent a diverse group of hydrolases that catalyze the cleavage and formation of ester bonds. They are widely distributed in animals, plants and microorganisms. These enzymes show a wide substrate tolerance, high regio- and stereospecificity, which make them attractive biocatalysts for the production of optically pure compounds in fine-chemicals synthesis. They do not require cofactors, are usually rather stable and are even active in organic solvents (Bornscheuer, 2002). Two major classes of hydrolases are of most importance: lipases (triacylglycerol hydrolases) and 'true' esterases (carboxyl ester hydrolases). Both classes of enzymes have a three-dimensional structure with the characteristic α/β-hydrolase fold (Ollis *et al.,* 1992; Schrag and Cygler, 1997). The catalytic triad is composed of Ser-Asp-His (Glu instead of Asp for some lipases) and usually also a consensus sequence (Gly-x-Ser-x-Gly) is found around the active site serine (Ollis *et al.,* 1992).

The mechanism for ester hydrolysis or formation is essentially the same for lipases and esterases and is composed of four steps: First, the substrate is bound to the active serine, yielding a tetrahedral intermediate stabilized by the catalytic His and Asp residues. Next, the alcohol is released and an acyl-enzyme complex is formed. Attack of a nucleophile (water in hydrolysis, alcohol or ester in transesterification) forms again a tetrahedral intermediate, which after resolution yields the product (an acid or an ester) and free enzyme (Stadler *et al.,* 1995). Lipases can be distinguished from esterases by the phenomenon of interfacial activation (which was only observed for lipases). Esterases obey classical Michaelis-Menten kinetics; lipases need a minimum substrate concentration before high activity is observed (Verger, 1998). Structure elucidation revealed that this interfacial activation is due to a hydrophobic domain (lid) covering lipases active site and only in the presence of a minimum substrate concentration, (a triglyceride phase or a hydrophobic organic solvent), the lid moves apart, making the active site accessible (Derewenda *et al.,* 1992). Furthermore, lipases prefer water-insoluble substrates, typically triglycerides composed of long-chain fatty acids, whereas esterases preferentially hydrolyze 'simple' esters (Verger, 1998). Lipases and esterases were among the first enzymes tested and found to be stable and active in organic

solvents, but this characteristic is more apparent with lipases (Schmid and Verger, 1998).

A comparison of amino acid sequences and 3D-structures of both enzymes reported that the active site of lipases displays a negative potential in the pH-range associated with their maximum activity (typically at pH 8); esterases show a similar pattern, but at pH values around 6, which correlates with their usually lower pH-activity optimum (Fojan *et al.,* 2000).

Cutinases are extracellular esterases secreted by several phytopathogenic fungi and pollen that catalyse the hydrolysis of ester bonds in cutin, the structural polyester of plants cuticle (Soliday and Kolattukudy, 1975). Cutinases are also able to hydrolyse a wide variety of synthetic esters and triacylglicerols, as efficiently as lipases, without displaying interfacial activation (Egmond and Van Bemmel, 1997; Martinez *et al.,* 1992). Therefore cutinases are suitable enzymes to be applied in laundry industry, dishwashing detergent composition to remove fats, in the synthesis of structured triglycerides, polymers and agrochemicals and in degradation of plastics (Flipsen *et al.,* 1998; Murphy *et al.,* 1996; Carvalho *et al.,* 1999).

Among cutinases, the one from the phytopathogenic fungus *Fusarium solani pisi* is the best studied example of a carboxylic ester hydrolase. *F. solani* cutinase is a 22 kDa enzyme and it was shown to be present at the site of fungal penetration of the host plant cuticle (Purdy and Kolattukudy, 1975a; Purdy and Kolattukudy, 1975b; Shaykh *et al.,* 1977). Specific inhibition of cutinase was shown to protect plants against fungal penetration and consequently infection (Koller *et al.,* 1982). The enzyme belongs to the family of serines esterases containing the so-called α/ β hydrolase fold. The active site of cutinase is composed of a catalytic triad involving serine, histidine and aspartate. *F. solani pisi* cutinase has an isoelectric point of 7.8 and an optimum pH around 8. The enzyme contains two disulfide bonds which are essential for structural integrity and catalytic activity (Egmond and de Vlieg, 2000).

10.1 Surface modification of synthetic fibres

Synthetic fibres represent almost 50% of the worldwide market of textile fibres. Polyethyleneterephthalate (PET), polyamide (PA) and polyacrylonitrile (PAN) fibres show excellent features like good strength, high chemical resistance, low abrasion and shrinkage properties. However synthetic fibres share as common disadvantages high hydrophobicity and crystallinity which affect, not only wearing comfort, (making these fibres less suitable to be in contact with human skin), but also processing of fibres, impeding the application of finishing compounds and colouring agents. Most of finishing processes/agents are water-dependent which require an increase in hydrophilicity of fibre surface (Jaffe and East, 1998; Yang, 1998; Frushour and Knorr, 1998; Burkinshaw, 1995).

Currently, chemical treatments with sodium hydroxide are used in industry to increase hydrophilicity and improve flexibility of fibres. However, chemical treatment extension is hard to control, which leads to unacceptable losses of weight and strength and to irreversible yellowing in the case PAN and PA fibres. Besides, this is not an environmentally appealing process since it requires high amounts of energy and chemicals that are further discharged to the environment.

A recently identified alternative under study is the use of enzymes for the surface modification of synthetic fibres (Gübitz and Cavaco-Paulo, 2003). The use of cutinase on vinyl acetate, (a comonomer in acrylic fibre) was reported for the first time by Silva and collaborators (Silva *et al.,* 2005). Lipases and esterases are mainly used for biomodification of PET. Enzymatic hydrolysis of PET fibres with different lipases revealed to increase hydrophilicity, measured in terms of wettability and absorbent properties (Hsieh and Cram, 1998; Hsieh *et al.,* 1997). A polyesterase was reported by Yoon et al (2002), for surface modification of PET and polytrimethyleneterephthalate (PTT). The authors reported that formation of terephthalic acid, (a hydrolysis product), could be monitored at 240 nm. The enzymatic treatment resulted in significant depilling, efficient desizing, increased hydrophilicity and reactivity with cationic dye and improved oily stain release (Yoon *et al.,* 2002). The production of polyester-degrading hydrolases from a strain of *Thermomonospora fusca* was investigated and

optimized (Gouda *et al.,* 2002). Later, Alisch and collaborators reported biomodification of PET fibres by extracellular esterases produced by different strains of actinomycetes (Alisch *et al.,* 2004). Fischer-Colbrie and collaborators found several bacterial and fungal strains, able to hydrolyse PET fibres, after screening using a PET model substrate (bis-benzoyloxyethyl terephthalate) (Fischer-Colbrie *et al.,* 2004). O´Neill and Cavaco-Paulo came up with two methods to monitor esterase hydrolysis of PET fibres surface, as alternative to the detection of terephthalic acid release at 240 nm. Cutinase hydrolysis of PET, will cleave ester bonds, releasing terephthalic acid and ethylene glycol and remaining hydroxyl and carboxyl groups at the surface. The terephthalic acid is quantified, after reaction with peroxide, by fluorescence determination of the resulting hydroxyterephthalic acid. Colouration of PET fibres with cotton reactive dyes, specific for hydroxyl groups, allows direct measurement of hydroxyl groups that remain at fibre surface (O'Neill and Cavaco-Paulo, 2004). Given the promising results obtained with cutinase and other PET degrading enzymes, several authors performed important comparison studies between different class/activity types of enzymes. All of the studies confirmed that cutinase from *F. solani pisi* exhibits significant hydrolysis on PET model substrates, as well as, on PET fibres resulting in an increased hydrophilicity and dyeing behaviour (Vertommen *et al.,* 2005; Alisch-Mark *et al.,* 2006; Heumann *et al.,* 2006).

Despite the potential of cutinase from *F. solani* to hydrolyse and improve synthetic fibres properties, these fibres are non-natural substrates of cutinase and consequently turnover rates are quite low. By the use of site-directed mutagenesis recombinant cutinases, with higher specific activity to large and insoluble substrates like PET and PA, were developed (Araújo *et al.,* 2007). The new cutinase, Leu181Ala mutant, was the most effective in the catalysis of amide linkages of PA and displayed a remarkable hydrolytic activity towards PET fabrics (more than 5-fold compared to native enzyme) (Araújo *et al.,* 2007). This recombinant enzyme was further used to study the influence of mechanical agitation on the hydrolytic efficiency of cutinase on PET and PA in order to design a process for successful application of enzymes to synthetic fibres (Silva *et al.,* 2007; O'Neill *et al.,* 2007). The use of cutinase, open up new opportunities for

targeted enzymatic surface functionalisation of PET and PA, polymers formerly considered as being resistant to biodegradation.
Recently, Nechwatal and collaborators have tested several commercial lipases/esterases for their ability to hydrolyse oligomers formed during manufacture of PET (Nechwatal *et al.,* 2006). These low-molecular-weight molecules are insoluble in water and can deposit themselves onto the dye apparatus, damaging it. The authors found that lipase from *Triticum aestivum* removed 80 wt % of oligomers from liquor bath treatment, however the observed decrease seems to be more related with adsorption of oligomers on the enzyme than with catalytic hydrolysis of ester groups (Nechwatal *et al.,* 2006).

11. Nitrilases and Nitrile Hydratases

Nitrilase was the first nitrile-hydrolysing enzyme described some 40 years ago. It was known to convert indole 3-acetonitrile to indole 3-acetic acid (Thimann and Mahadevan, 1964; Kobayashi and Shimizu, 1994). The nitrilase superfamily, constructed on the basis of the structure and analyses of aminoacid sequence, consists of 13 branches. Members of only one branch are known to have true nitrilase activity, whereas 8 or more branches have apparent amidase or amide condensation activities (Pace and Brenner, 2001; Brenner, 2002). All the superfamily members contain a conserved catalytic triad of glutamate, lysine and cysteine, and a largely similar α-β-β-α structure.
Nitrilases are found relatively frequently in nature. This enzyme activity exists in 3 out of 21 plant families (*Gramineae, Cruciferae* and *Musaceae*) (Thimann and Mahadevan, 1964), in a limited number of fungal genera (*Fusarium, Aspergillus, Penicillium*) (Harper, 1977; Šnajdrová *et al.,* 2004; Vejvoda *et al.,* 2006; Kaplan *et al.,* 2006) but it is more frequently found in bacteria. Several genera such *Pseudomonas, Klebsiella, Nocardia* and *Rhodococcus* are known to utilize nitriles as sole sources of carbon and nitrogen (Dhillon *et al.,* 1999; Kiziak *et al.,* 2005; Bhalla and Kumar, 2005; Hoyle *et al.,* 1998; Bhalla *et al.,* 1995). Manly due to the biotechnological potential of nitrilases different bacteria and fungi capable of hydrolysing nitriles were isolated (Singh *et al.,* 2006).

Most of nitrilases isolated consist of a single polypeptide with a molecular mass between 30 and 45 kDa, which aggregate to form the active holoenzyme under different conditions. The prevalent form of the enzyme seems to be a large aggregate composed of 6 to 26 subunits. Most of the enzymes show substrate dependent activation, though the presence of elevated concentrations of salt, organic solvents, pH, temperature or even the enzyme itself may also trigger subunit association and therefore activation (Nagasawa1 *et al.,* 2000).

Nitrile hydratase (NHase) is a key enzyme in the bienzymatic pathway of the conversion of nitriles to amides, which are further converted to the corresponding acid by amidases. Several microorganisms (*Rhodococcus erythropolis, Agrobacterium tumefaciens*) having NHase activity have been isolated and the enzymes have been purified and characterized (Stolz *et al.,* 1998; Hirrlinger *et al.,* 1996; Trott *et al.,* 2001; Okamoto and Eltis, 2007). NHases are composed of two types of subunits (α and β) complexed in varying numbers. They are metalloenzymes containing either cobalt (cobalt NHases) or iron (ferric NHases).

11.1 Surface modification of Polyacrylonitrile (PAN)

PAN fibres exhibit excellent properties like high chemical resistance, good elasticity and natural-like aesthetic properties, which contribute to the increased use of these fibres, representing nowadays about 10% of the global synthetic fibre market. However, the hydrophobic nature of PAN fabrics also confers undesirable properties resulting in a difficult dyeing finishing process (Frushour and Knorr, 1998). Chemical hydrolysis of PAN fibres at the surface generally leads to irreversible yellowing of fibres. Thus, similarly to other synthetic fibres, selective enzymatic hydrolysis of PAN could represent an interesting alternative to chemical processes.

The surface of PAN was modified by nitrile hydratase and amidase enzymes from different sources (*R. rhodochrous* and *A. tumefaciens*). After enzymatic treatment the fabric became more hydrophilic and the adsorption of dye was enhanced (Tauber *et al.,* 2000; Fischer-Colbrie *et al.,* 2006). Similarly, in a work developed by Battistel and collaborators treatment of PAN with nitrile hydratases from *Brevibacterium imperiale*,

Corynebacterium nitrilophilus and *Arthrobacter* sp. resulted in an increase of amide groups on the PAN surface which leaded to increased hydrophilicity and dyeability (Battistel *et al.,* 2001). In another study it was reported for the first time the use of a new *Micrococcus luteus* strain BST20 which produces membrane-bound nitrile hydrolysing enzymes. The enzymes were shown to hydrolyze the nitrile groups on the PAN surface by determining the NH3 release from PAN powder, and measuring the depth of shade of enzyme treated fabric after dyeing with a basic dye (Fischer-Colbrie *et al.,* 2007).

The biomodification of acrylic fibres using a nitrilase, instead of nitrile hydratases/amidases, was introduced for the time, by Matamá and collaborators. The addition of 1 M sorbitol and 4% N,N-dimethylacetamide to the treatment media enhanced nitrilase catalysis efficiency (Matamá *et al.,* 2006).

Although there is no industrial application yet, the results of research demonstrate that enzymatic treatment of PAN would give advantages in the quality of treated fibres as well as in energy saving and pollution control.

12. Laccases

Laccases are extracellular, multicopper enzymes that use molecular oxygen to oxidize phenols and various aromatic and nonaromatic compounds by a radical-catalysed reaction mechanism (Thurston, 1994). They belong to a larger group of enzymes termed the blue-multicopper oxidase family. Laccases have been found in plants, insect, bacteria, but are most predominant in fungi (Claus, 2004; Benfield *et al.,* 1964; Baldrian, 2006).

Laccase activity has been demonstrated in more than 60 fungal strains (Gianfreda *et al.,* 1999). Typical fungal laccase is a protein of approximately 60 – 70 kDa with pH optima in the acidic pH range. The optima temperature ranges between 50 and 70 ºC. Few enzymes with optima temperature below 35 ºC have been described, for example the laccase from *Ganoderma lucidum* with the highest activity at 25 ºC (Ko *et al.,* 2001). The range of substrates with which laccases can react is very broad, showing a remarkable nonspecific activity regarding their reducing substrate.

12.1 Decolourization of dyes and textile bleaching

Laccases are widely researched for the decolourization of textile effluents. Due to their ability to degrade dyes of diverse chemical structure, including synthetic dyes, laccases can be used as a more ecological alternative to treat dye wastewater (Abadulla *et al.,* 2000; Hou *et al.,* 2004; Hao *et al.,* 2007; Salony *et al.,* 2006; Couto *et al.,* 2006). They are also studied for textile bleaching. Bleaching of cotton is achieved by the decolourization of natural pigments giving cotton fibres a white appearance. The most common industrial bleaching agent is hydrogen peroxide usually applied at temperatures close to boiling. However high temperatures and alkaline pH can cause sever damage to the fibres, besides, high amounts of water are needed to remove hydrogen peroxide from fabrics that can interfere with dyeing.

Laccases can improve whiteness of cotton by oxidation of flavonoids. The substitution or combination of chemical bleaching with an enzymatic bleaching system leads not only to less fibre damage but also to significant savings of water (Tzanov *et al.,* 2003a). Pereira and collaborators isolated a new strain of *Trametes hirsuta* for cotton bleaching. Laccases of this organism were responsible for oxidation of the flavonoids morin, luteolin, rutin and quercetin. The authors reported that pretreatment of cotton with *T. hirsuta* laccases resulted in an increase of whiteness (Pereira *et al.,* 2005).

Later ultrasound was used to intensify the efficiency of enzymatic bleaching. The authors found that low intensity of ultrasound improved diffusion of the enzyme from the liquid phase to the fibres surface, acting synergistically with the enzyme in the oxidation of natural pigments (Basto *et al.,* 2007). Regarding denim finishing, there are already some successful industrial applications of laccases like DeniLite® commercialized by Novozyme (Novo Nordisk, Denmark) and Zylite from the company Zytex (Zytex Pvt. Ltd, Mumbai, India).

The application of laccases for the coating of natural and synthetic fibres is under study. Tzanov and co-workers developed a laccase-assisted dyeing process of wool. With this new process wool is dyed using low temperatures without dyeing auxiliaries which permit saving water and energy (Tzanov *et al.,* 2003b; Tzanov *et al.,* 2003c). More

recently, Kim and collaborators described the utilisation of the natural flavonoids to dye cotton by an enzymatic process catalyzed by laccases (Kim *et al.,* 2007).

13. Catalases

Catalases (CATs), more correctly hydroperoxidases, catalyse the degradation of H_2O_2 to H_2O and O_2. Catalases are produced by a variety of different microorganisms including bacteria, moulds and yeasts (Mueller *et al.,* 1997). Most of the known catalases have their activity optimum at moderate temperatures (20 - 50 °C) and neutral pH.
CATs from animal sources (bovine liver) are generally cheap, therefore the production of microbial CATs will only be economically advantageous when recombinant strains and cheap technology is used specially for the production of CATs with special properties for instance to work at high or low temperatures or at alkaline or acidic pH.

13.1 Treatment of bleach liquor

In the textile industry, bleaching with H_2O_2 is performed after desizing and scouring, but before dyeing. Normally a reducing agent is used to destroy the hydrogen peroxide, or water to rinse out the hydrogen peroxide bleach. CAT is now used to decompose excess H_2O_2 to water and oxygen (Fraser, 1986). With the use of catalase, the reducing agent can be eliminated and the amount of rinse water can be dramatically reduced, resulting in less polluted wastewater and lower water consumption. The cost of enzyme for degradation of hydrogen peroxide in bleaching effluents could be reduced by the introduction of immobilised enzymes. Immobilization will allow not only the recovery of enzyme but also the reuse of treated bleaching effluents for dyeing (Fruhwirth *et al.,* 2002; Costa *et al.,* 2001; Paar *et al.,* 2001).

14. Enzyme use in related market segment: the detergent industry

Most of the enzymes previously reported can be used in detergent formulations. In fact the most successful and largest industrial application of enzymes is in detergents. The first use of enzymes in detergents goes back to the use of pancreatic extracts by Roehm

in 1913. However, the use of enzymes from animal sources led to few successes, as those enzymes were not suited to prevailing washing conditions. The first detergent containing a bacterial enzyme was introduced into the market in the 1960s (Maurer, 2004). Due to environmental concerns detergent manufacturers have replaced since the early 1980s phosphate with other detergent builders such as zeolite and silicates, and developed and incorporated bleach activators. New proteases that were stable at alkaline pH, show good washing performance at low temperatures, also in the presence of sequestering agents, bleach and surfactants were sought. The bacterial subtilisins were identified as being the most suitable for detergent applications (Saeki *et al.,* 2007).

At present only proteases and amylases are commonly used. Proteases are the major component and used to facilitate the removal of proteinaceous stains. Likewise, amylases are able to facilitate the removal of stains of starchy food. More recently, cellulases are being incorporated in detergents to remove pills, reducing the fuzzy appearence and restoring lustre. Lipases are under research and can be used to remove fatty stains, especially at low temperatures and on blends of cotton/polyester.

The most recent introduction of a new class of enzyme into detergent formulation is the addition of a mannanase. This enzyme helps removing various food stains containing guar gum, a commonly used stabilizer agent in food products (Bettiol and Showell, 1999). The most recent innovation in detergent industry is the use of psychrophilic enzymes able to work effectively in cold water, allowing the save of energy (Cavicchioli *et al.,* 2002).

Currently, the majority of enzymes used in detergents are subtilisins isolated from *Bacillus licheniformis*, *B. lentus*, *B. alcalophilus* or *B. amyloliquefaciens*. They can now be generated by recombinant techniques (heterologous expression) and engineered in any aspect, as already described. Products like Purafect xP (Genencor), Everlase, Savinase, Esperase (Novozymes), were created and used as detergent additives which have been on the market for several years (Maurer, 2004).

15. Conclusions and future prospects

As already mentioned, enzymes can be used in order to develop environmentally friendly alternative processes regarding almost all steps of textile fibres processing. There are already some commercial successful applications, like amylases used for desizing, cellulases and laccases for denim finishing and proteases incorporated in detergent formulations. Further research is still required for the implementation of commercial enzyme based processes for the biomodification of synthetic and natural fibers. Another field of research is the search for new enzyme-producing microorganisms and enzymes extracted from extremophilic microorganisms (Schumacher *et al.,* 2001).

Although some types of enzymes already play an important role in some textile processes, their potential is much greater and their applications in future processes are likely to increase in the near future.

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Chapter 2

Surface Modification of Synthetic Fibres

Subchapter 2.1

Introduction

1. Synthetic fibres

Synthetic fibres are defined by the International Organization for Standardization (ISO) as fibres manufactured from polymers built up from chemical elements or compounds, in contrast to fibres made from naturally occurring fibre-forming polymers.
Synthetic fibers represent almost 50% of the worldwide market of textile fibers. Common synthetic fibres include: acetate, nylon, modacrylic, olefin, acrylic and polyester. This chapter will focus on molecular biotechnology approaches aiming at the surface modification of poly(ethylene) terephthalate, polyamide 6,6 and acrylic.

2. Polyester

2.1. Poly(ethylene terephthalate) (PET)

Polyester fiber, specifically poly(ethylene terephthalate) (PET), is the largest volume synthetic fiber produced worldwide. The total volume produced in 2002 was 21 million metric tons which corresponds to 58% of synthetic fiber production worldwide. Poly(ethylene naphthalate) (PEN); poly(butylene terephthalate) (PBT); poly(propylene terephthalate) (PPT); and poly(lactic acid) (PLA) are examples of other polyesters commercially produced in fiber form, however with lower volumes of production compared to PET.
PET is the condensation product of terephthalic acid (TA) and ethylene glycol. The key to successful PET polymerization is monomer purity and the absence of moisture in the reaction vessel (Brown and Reinhart, 1971).
The technology that allowed for the cost-effective polymerization of PET was the development of low-cost and pure TA from mixed xylenes in the mid-20th century (McIntyre, 2003). The alternative to TA, and the monomer of choice before the availability of low-cost TA, was dimethyl terephthalate (DMT). While direct esterification of TA is the preferred method of PET synthesis, ester interchange between DMT and ethylene glycol is still utilized in some PET manufacture, partially because of local choice and partially because DMT is a product of polyester recycling by methanolysis or glycolysis (Milgrom, 1993). The second monomer, ethylene glycol, is a

major material of commerce, produced by the oxidation of ethylene followed by ring opening with water. The large-scale production of all PET monomers assures low-cost polymers and makes competition from new compositions of fiber-forming polymers very difficult.

2.2. PET fibres properties

PET fiber has excellent properties like convenient processability and tailorable performance, associated with low cost production. Undesired characteristics of the most widely used synthetic fibres based on polyethyleneterephthalates (PET) include difficulties in finishing, build-up of electrostatic charge, the tendency to pilling, insufficient washability and wearing comfort due to low water absorbency (East, 2005). Although alkaline treatment of PET can easily increase hydrophilicity, favourable properties such as strength are negatively influenced (Zeronian and Collins, 1989).

3. Polyamides

3.1. Aliphatic polyamides

Aliphatic polyamides are macromolecules whose structural units are characteristically interlinked by the amide linkage –NHCO-. The nature of the structural unit constitutes a basis for classification. Aliphatic polyamides with structural units derived predominantly from aliphatic monomers are members of the generic class of nylons, whereas aromatic polyamides in which at least 85% of the amide linkages are directly adjacent to aromatic structures have been designated aramids (Reimschuessel, 1998).

3.1.1. Polyamide 6,6

Among various nylon compositions, nylon-6,6 is by far the most important polyamide for the commercial production of fibers. Nylon 6,6 is a copolymer of diamine (hexamethylene diamine) and diacid (adipic acid). These molecules alternate along the chain, each donating 6 carbons to the polymer.

In the laboratory, nylon 6,6 can also be made using adipoyl chloride instead of adipic. It is difficult to get the proportions exactly correct, and deviations can lead to chain termination at molecular weights less than a desirable 10,000 Daltons. To overcome this problem, a crystalline, solid "nylon salt" can be formed at room temperature, using an exact 1:1 ratio of the acid and the base to neutralize each other. Heated to 285 °C, the salt reacts to form nylon polymer. Above 20,000 daltons, it is impossible to spin the chains into yarn, so to combat this, acetic acid is added to react with a free amine end group during polymer elongation to limit the molecular weight. In practice, and especially for 6,6, the monomers are often combined in a water solution. The water used to make the solution is evaporated under controlled conditions, and the increasing concentration of "salt" is polymerized to the final molecular weight (Kohan, 1992).

3.2. Polyamide 6,6 fibres properties

Despite of all the excellent properties exhibited, nylon fibers present low hydrophylicity and low reactivity with the most usual finishing and colouring agents. Coating finishing effects are difficult to obtain when hydrophobic polyamide fabrics are used. Recent studies clearly indicate that the modification of synthetic polymers with enzymes is an effective and environmentally friendly alternative to chemical methods using alkaline products (Silva *et al.*, 2004).

4. Acrylic

Acrylic fibers are synthetic fibers made from a polymer (polyacrylonitrile) with an average molecular weight of approximatly 100,000 Dalton. To be called acrylic the polymer must contain at least 85% acrylonitrile monomer (Greenley, 1989). Typical comonomers are vinyl acetate or methyl acrylate. The polymer is formed by free-radical polymerization. The fiber is produced by dissolving the polymer in a solvent such as N,N-dimethylformamide or aqueous sodium thiocyanate, metering it through a multi-hole spinnerette and coagulating the resultant filaments in an aqueous solution of the same solvent. Washing, stretching, drying and crimping complete the processing.

End uses include sweaters, hand-knitting yarns, rugs, awnings, boat covers, and beanies; the fiber is also used as a precursor for carbon fiber.

4.1. Polyacrylonitrile fibres Properties

Polyacrylonitrile (PAN) fibres are characterised by a combination of desirable properties such as high resistance to outdoor exposure and chemicals, excellent elasticity, natural-like aesthetics properties and colour fastness. However, due to its hydrophobic nature, PAN fibres also exhibit undesirable properties such as uncomfortable hand and static charge accumulation (Lulay, 1995; Frushour and Knorr, 1998). The quality and properties of acrylic fibres can be improved by chemical and physical means using co-monomers, additives and polymer blends (Burkinshaw, 1995). The surface modification is also considered an important tool to improve the quality and the processing properties of PAN fibres (Battistel *et al.*, 2001). Traditional processes used to modify polymer surfaces are based on the addition of strong chemical agents. The application of enzymes towards the modification of this polymer has major advantages compared to those agents, such as milder reaction conditions, easier control, specific non-destructive transformations, and environmental friendly processes.

5. Surface modification of synthetic fibres

Major characteristics of synthetic fibers are their excellent strength, high hydrophobicity and low reactivity with most common chemical agents. The suitability of fibers for a given end-use application depends on several factors including mechanical behavior, chemical resistance, dimensions, and surface characteristics. For instance, the low hydrophobicity makes those fibers less suitable to be in contact with the human skin, and the low reactivity makes the fiber unsuitable to act as carrier to other chemical finishing agents. The interest in fiber surface modification is related to the introduction of specific properties required for a certain application. The desired properties may range from improved adhesion to response to stimuli from the environment.

Fibre surface modification has been one of the main areas of research in the development of functional fibres. In addition to research in developing/synthesizing new fibre forming polymers with specialized properties, surface modification offers many new opportunities. Properties of fibres such as anti-microbial, anti-odor, anti-fungal, anti-static, wicking, soil resistance, adhesion, and biocompatibility are among fibre function surface properties.

There are various techniques available for surface modification including hot and cold plasma irradiation (Cioffi et al., 2003; Xu and Liu, 2003), enzymatic treatment (Silva *et al.*, 2005; Vertommen *et al.*, 2005; Yoon *et al.*, 2002; Battistel *et al.*, 2000; Tauber *et al.*, 2000), chemical finish (Cai *et al.*, 2001) and metal vapor deposition (Jeon *et al.*, 2008). Among various surface modification techniques strong alkaline treatments can improve hydrophilicity and chemical reactivity of synthetic fibers but the treatment extension is hard to control, leading to unacceptable levels of strength loss.

Enzyme treatment techniques are attractive to implement due to ecological and economic reasons however, the application of enzymes in textile processes often requires properties or performances not found in enzymes isolated from natural sources. Molecular biotechnology can be used to introduce desired changes in catalytic activity, thermal stability and molecular recognition behaviour of enzymes. The following subchapters describe the work developed in the scope of this thesis aiming at the optimization of cutinase activity for the surface modification of synthetic fibres polyethilene therephthalate, polyamide and cellulose acetate.

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Subchapter 2.2

Tailoring Cutinase Activity Towards Polyethylene Terephthalate and Polyamide 6.6 Fibers

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Tailoring Cutinase Activity Towards Polyethylene Terephthalate and Polyamide 6,6 Fibers

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Abstract

Cutinase from *Fusarium solani pisi* was genetically modified near the active site, by site-directed mutagenesis, to enhance its activity towards polyethylene terephthalate (PET) and polyamide 6,6 (PA 6,6) fibers. The mutations L81A, N84A, L182A, V184A and L189A were done to enlarge the active site in order to better fit a larger polymer chain. Modeling studies have shown enhanced free energy stabilization of model substrate tetrahedral intermediate (TI) bound at the enzyme active site for all mutants, for both model polymers. L81A and L182A showed an activity increase of four- and five-fold, respectively, when compared with the wild type, for PET fibers. L182A showed the one- and two-fold higher ability to biodegrade aliphatic polyamide substrates. Further studies in aliphatic polyesters seem to indicate that cutinase has higher ability to recognize aliphatic substrates.

1. Introduction

Cutinase from the fungus *Fusarium solani pisi* is a secreted enzyme that degrades cutin, the structural polyester of plants cuticle, being a versatile serine hydrolase showing unusual stereolytic activity (Carvalho *et al.,* 1998). *In vitro*, cutinases display hydrolytic activity towards a broad variety of esters including triglycerides (Carvalho *et al.,* 1998). Synthetic activities of cutinases have also been described for the production of triglycerides, polymers and agrochemicals containing one or more chiral centers (Carvalho *et al.,* 1999).

Our group showed for the first time the ability of cutinase to biodegrade polyamide 6,6 (PA 6,6) and vinyl acetate (co-monomer of acrylic fiber) fibers (Silva *et al.,* 2005) and we also confirmed that cutinase is an enzyme with a high potential to hydrolyze and improve the surface properties of polyethylene terephthalate (PET) fibers in an environmentally friendly way (Silva *et al.,* 2005). However, these synthetic fibers are non-natural substrates and, despite the broad specific activity of cutinase, turnover rates are very low.

The analysis of the 3D structure of the cutinase from *F. solani pisi* (PDB code 1CEX) (Longhi *et al.,* 1997) shows that the external, but closed active site is hindering the access to the fiber substrate. In the present work, site-directed mutagenesis was performed on selected residues allowing the fit of a larger substrate in the active site.

A comparative discussion is made on the biodegradation ability of the several mutations based on modeling data, enzyme activity and protein adsorption levels from the polymeric substrates. The cutinase ability to biodegrade polyamide aliphatic substrates was confirmed by measuring the activity on hydrophobic aliphatic polyesters, which present a similar structure to cutin. Amidase and esterase activity of cutinase is also discussed.

2. Materials and methods

2.1. Fibers and reagents

Oligonucleotides (0.05 μmol scale) were purchased from MWG Biotech, Germany. Restriction and modification enzymes were supplied by Roche Applied Science, Germany. Accuzyme DNA Polymerase was obtained from Bioline, Germany. The *Escherichia coli* strain BL21(DE3) and the plasmid vector pET25b (+) were purchased from Novagen, Madison, WI, USA. The succinic acid kit Cat No. 10 176 281 035 was obtained from R-Biopharm, Germany. For biodegrading experiments it was used commercial polyamide woven fabric, a plain woven structure with 63 g/m^2 and commercial polyester taffeta fabric with 62 g/m^2, both supplied by Rhodia, Switzerland. All other reagents used were laboratory grade reagents.

2.2. Modeling studies

Modeling studies were performed using the cutinase X-ray structure of Longhi *et al.,* (1997), PDB code 1CEX, solvated dodecahedral water box (minimum distance between the protein and box wall of 0.8 nm), with the model substrates tetrahedral intermediate (TI) bound at the enzyme active site. These model substrates, 1,2-ethanodiol dibenzoate and PA 6,6 mimics the polyester and polyamide hydrophobic properties, respectively, and are suitable models for simulation and experimental studies.

The formation of the TI is known to be the rate limiting step in the catalytic mechanism of serine proteases (Warshel *et al.,* 1989). This model system was chosen in order to evaluate the free energy of stabilization of the TI provided by selected mutations to alanine of residues located at the enzyme active site (Figure 1): L182A, V184A, L189A, L81A and N84A according to Scheme 1. These mutations were initially designed as possible changes leading to a better fitting of the 1,2-ethanodiol dibenzoate and PA 6,6 TI in the active site. Molecular dynamics/molecular mechanics (MD/MM) simulations (van Gunsteren and Berendsen, 1990) were performed with the GROMACS package (Berendsen *et al.,* 1995; Lindahl *et al.,* 2001) Version 3.1.4, using the GROMOS96

force field (Scott *et al.,* 1999) with an integration time step of 2 fs. Five simulations (with different initial velocities) were made, both with the free enzyme and enzyme bound to the TI. Bond lengths of the solute were constrained with LINCS and the ones of water with SETTLE. Non-bonded interactions were calculated using a twin-range method with short and long range cut-offs of 8 and 14 A°, respectively. The SPC water model was used (Hermans *et al.,* 1984).

A reaction field correction (Tironi *et al.,* 1995; Barker and Watts, 1973) for electrostatic interactions was applied, considering a dielectric of 54 for SPC water (Smith and van Gunsteren, 1994). The solute and solvent were coupled to two separate heat baths (Berendsen *et al.,* 1984) with temperature coupling constants of 0.1 ps and reference temperatures of 300 K. The pressure control was implemented with a reference pressure of 1 atm and a relaxation time of 0.5 ps (Berendsen *et al.,* 1984).

Free energy calculations (Beveridge and Dicapua, 1989) were made using thermodynamic integration by slowly changing the selected residues to alanine using 11 equally spaced sampling points 100 ps each. Five replicates based on the five different trajectories were made for each mutation. The trajectories were run for 2 ns prior to the free energy calculations.

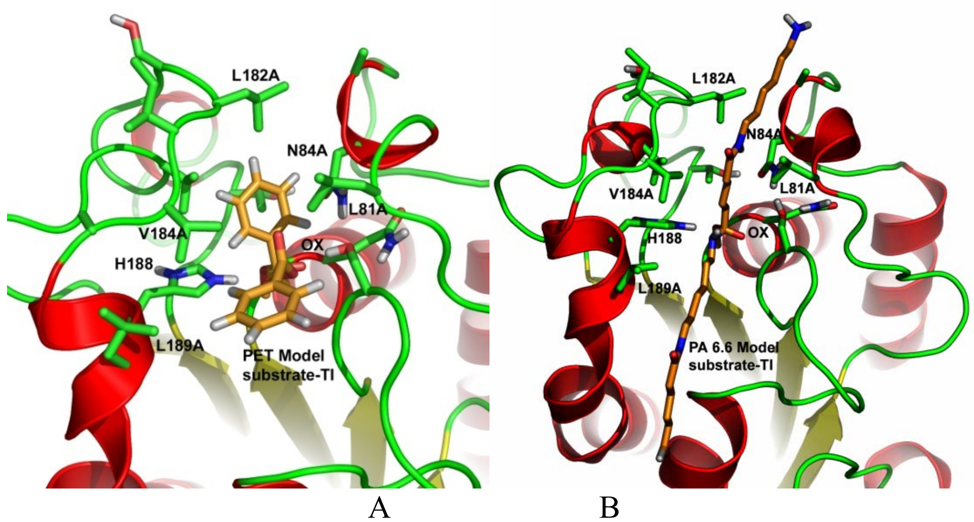
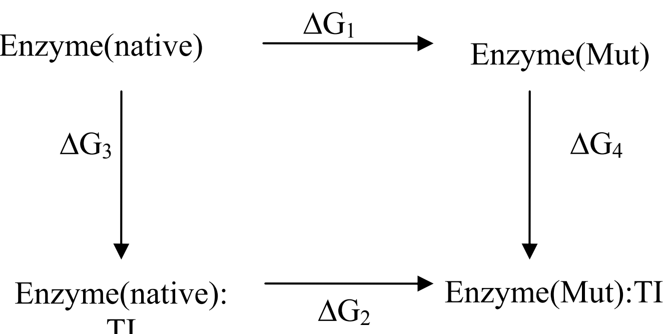


Figure 1. Detail of the active site X-ray structure of cutinase with the energy minimized structure of the TI of 1,2-ethanodiol dibenzoate (PET model substrate) (A) and PA 6.6 (B). Catalytic histidine (H188) and oxianion-hole (OX) are shown. Residues mutated in this study are labelled as: L81A, N84A, L182A, V184A and L189A.



Scheme 1. Thermodynamic cycle employed in the calculation of the relative free energy of stabilization of the model substrate TI between native and genetically modified (Mut) enzyme, $\Delta\Delta G$native-Mut = ΔG_2 - ΔG_1. This thermodynamic cycle evaluates the preferential stability of the model substrate TI to be bound to the native or to the genetically modified enzyme. This is achieved through the calculation of ΔG_1 and ΔG_2 by thermodynamic integration (Beveridge *et al.,* 1989).

2.3. Plasmid construction and protein expression

The native cutinase gene sequence was PCR-amplified with the primers CutFor (5´-CGGGATCCCATGAAACAAAGCACTATTGCACTG- 3´) and CutRev (5´-CGAGCTCGCAGCAGAACCACGGACAGCC- 3´) from the vector pDrFST (kindly provided by Professor G. Georgiou, Institute for Cell and Molecular Biology, University of Texas, Austin, USA) (Griswold *et al.,* 2003). The PCR product was restricted with *BamH*I and *Sac*I and cloned into the *BamH*I and *Sac*I restricted and dephosphorilated pET25b(+), resulting in the final pCWT vector. The plasmid construct was verified by DNA sequencing. The sequencing was performed following the method of Sanger *et al.,* (1977), using an ABI PRISM 310 Genetic Analyzer.

Site-directed mutagenesis was performed using recombinant PCR technique (Ansaldi *et al.,* 1996). This approach is based on the PCR amplification of an entire plasmid by mutagenic primers (Table I) divergently oriented but overlapping at their 5´ends. The mutagenic nucleotides are located only in the reverse primer.

Table I. Primers used for site-directed mutagenesis of cutinase gene from *Fusarium solani pisi*. The codons corresponding to the specific mutations introduced are indicated in bold. The overlapping regions (22 bp) of primers forward (F) and reverse (R) are underlined.

Mutation	Primer (5´→ 3´)	Bp	GC %
L81A	F1 CTCTCCCTCGCGGAACCTCTAGCGCCGCAATCAGGGAGA	39	64.1
	R1 CTAGAGGTTCCGCGAGGGAGAGCATTGTCTCC**GGC**AGTGGCTCGGTAGGCAC	52	63.5
N84A	F2 GCGGAACCTCTAGCGCCGCAATCAGGGAGATGCTCGGTC	39	64.1
	R2 ATTGCGGCGCTAGAGGTTCCGCGAGGGAGAGC**GGC**GTCTCCAAGAGTGGCTC	52	65.4
L182A	F3 CACCTCACTTGGCTTATGGTCCTGATGCTCGTGGCCCTG	39	59.0
	R3 GGACCATAAGCCAAGTGAGGTGCAGCAACGAT**GGC**GCTACCAGTACAAACGA	52	53.8
V184A	F4 ACTTGGCTTATGGTCCTGATGCTCGTGGCCCTGCCCCTG	39	61.5
	R4 GCATCAGGACCATAAGCCAAGTGAGGTGCAGC**GGC**GATCAAGCTACCAGTAC	52	55.8
L189A	F5 CTGATGCTCGTGGCCCTGCCCCTGAGTTCCTCATCGAGA	39	61.5
	R5 GGGGCAGGGCCACGAGCATCAGGACCATAAGC**GGC**GTGAGGTGCAGCAACG	51	66.7

The pET25b (+) carrying native and genetically modified cutinases were first established in *E. coli* strain XL1 Blue, according to the SEM method (Inoue *et al.,* 1990), and the presence of each specific mutation was confirmed by sequencing. DNA cloning and manipulation were performed according to the standard protocols (Sambroock *et al.,* 1989). T7 expression host strain BL21(DE3) was used for protein expression.

Strains were grown at 37 ºC in Luria-Broth medium, supplemented with 50 µg/ml ampicillin until an absorbance A600 nm of 0.6 was reached. Cells were then induced by adding isopropyl-1-thio-β-galactopyranoside (IPTG) (final concentration 1 mM), followed by 16 h at 18 ºC. The cells were harvested by centrifugation (5000 rpm for 10 min) and washed twice with phosphate buffered saline solution (10 mM Na_2HPO_4, 2 mM KH_2PO_4, 137 mM NaCl, 3 mM KCl, pH 7.4), supplemented with a mixture of protease inhibitors. Ultrasonic treatment of bacterial cells was performed at 20 KHz with a 13-mm probe in an Ultrasonic Processor GEX 400. Four 2-min pulses with 2 min in ice between each pulse were performed. The lysate was centrifuged for 30 min at

14000 rpm at 4 ºC. The supernatant, periplasmatic fraction was decanted and reserved for cutinase purification.

2.4. Protein purification by immobilized metal affinity chromatography (IMAC)

An IMAC system was performed with the XK 16 column (Amersham Pharmacia Biotech) containing 2.5 ml Chelating Sepharose Fast Flow (Amersham Pharmacia Biotech). The XK 16 column was linked to an AKTA P900 workstation (Amersham Pharmacia Biotech). After loading with 3 ml 0.1M $NiSO_4$ in H_2O, equilibration was performed with 10 mM imidazole, 0.5 M NaCl and 20 mM phosphate buffer pH 7.6. Samples were applied onto the column at a flow rate of 2 ml/min, followed by washing with the equilibration buffer until the UV baseline was reached. Elution was performed with a buffer containing 500 mM imidazole, 0.5 M NaCl and 20 mM phosphate buffer, pH 7.6. The activity-containing fractions were collected and used as the pure enzyme for polyester enzymatic hydrolysis.

Sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) electrophoresis, using a Tris–SDS–glycine buffer system, was used to monitor the fractions obtained from IMAC (Laemmli, 1970). Protein detection was done by Coomassie Brilliant Blue R250, as well as by InVision His-tag In-gel Stain (Invitrogen, California, USA). The total protein concentration was estimated by the Bradford quantitative protein determination assay (Bradford, 1976) using bovine serum albumine as standard.

2.5. Cutinase activity towards p-nitrophenyl butyrate (p-NPB)

The stereolityc activity of cutinase was determined spectrophotometrically following the hydrolysis of p-nitrophenyl butyrate (p-NPB) at 400 nm (Shirai and Jackson, 1982). One unit of activity was defined as the amount of enzyme required to convert 1 µmol of p-nitrophenyl butyrate to p-nitrophenol (p-NP) per minute. All the activity assays were done in triplicate.

2.6. Cutinase activity towards polyethylene terephthalate (PET) and polyamide 6,6 (PA 6,6) fibers

One gram of polyamide fabric was incubated with 20 mg/l of native and genetically modified mutant enzymes, in 300 ml of phosphate buffer (0.1 M NaOH, 0.1 M KH_2PO_4, pH 7.5) at 37 ºC. For this experiment, the samples were incubated in Erlenmeyers using a shaking bath with orbital agitation (90 rpm) for 48 h. After treatment all samples were washed with 2 g/l of Na_2CO_3 for 2 h, in order to stop the enzymatic reaction, followed by washing with 10 g/l of Lutensol at 25 ºC for 1 h. The same procedure was followed for PET fabrics with an incubation period of 24 h. The hydrolysis products were quantified as previously described (O'Neill and Cavaco-Paulo, 2004; Silva and Cavaco-Paulo, 2004).

2.7. Determination of protein adsorption

The protein adsorption was obtained measuring the protein content in the incubation solutions before and after the enzymatic treatment of PET and PA 6,6 fabrics. The difference between the values obtained for these two periods measures the protein adsorbed by the substrates.

2.8. Cutinase activity towards aliphatic polyesters

In order to measure the activity of native and L182A cutinase mutant towards aliphatic substrates, 0.05 g of each substrates, poly(ethylene succinate), poly(1,3-propylene succinate) and poly(1,4-butylene succinate) were incubated with 1 U (μmol of p-NP per min)/ml in a 3 ml phosphate buffer bath (pH 7.5) at 37 ºC for 5 h. The activity towards aliphatic substrates was quantified by measuring succinic acid formation, using the succinic acid UV kit method.

The total protein concentration was estimated by the Bradford quantitative protein determination assay (Bradford, 1976) using bovine serum albumine as standard.

3. Results and discussion

Molecular modeling studies were performed by docking the synthetic model substrates of PET and PA 6,6 at the cutinase active site (Figure 1). All mutations were done to create more space in order to fit the large inaccessible polymer in the active site of the cutinase.

The modeling studies show that mutations L182A, L189A, L81A and V184A provide a better stabilization of the TI of the model substrates relatively to the native enzyme (Table II), while the N84A mutation fails in stabilizing the TI model substrates due to the favourable interaction of the aspargine with the oxianion hole (Longhi and Cambillau, 1999). This is in accordance with the experimental activity obtained for p-NPB and PA 6,6 (Table III). Higher stabilization is achieved with L182A as shown by the experimental results (Table III). The modeling results suggest that L189A, L81A and V184A also stabilize both TI, but in a lower extent. Of all these four mutations found to stabilize the TI theoretically, experimentally L182A, L81A and V184A displayed an increased activity for PET fibers, while L189A showed a decreased activity. Experimentally, in the case of PA 6,6 fibers, a higher hydrolytic activity was obtained with L182A form (119%) while L189A, V184A and L81A displayed a slight decrease (Table III).

Structural analysis of the enzyme active site suggests that, replacing the bulky side chain of L182 by a smaller residue such as alanine (the L182A mutant) provides a less restrained active site, allowing a better accommodation of the model substrate, which gave the best enzyme activity improvement. This mutation allows the opening of the hydrophobic cleft of the enzyme active site, providing a better fit and stabilization of both model substrates than the native enzyme. The modeling studies also predict that the longer polymer chain in PET and PA 6,6 fabrics will also be more stabilized by this modified enzyme, which is corroborated by the experimental results, since there is a wider channel in the active site for these polymers to go through (Figure 2).

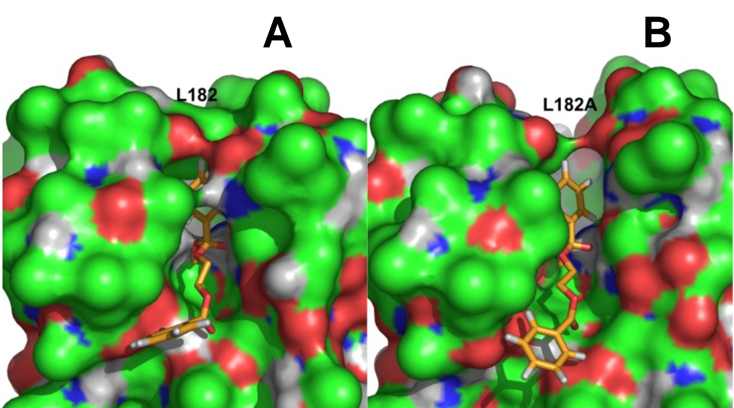


Figure 2. Cutinase surface rendering of a representative configuration of an equilibrated simulation: Native (A), and genetically modified cutinase L182A (B). L182A mutation is responsible for the highest increase of TI stabilization and enzymatic activity toward the 1,2-ethanodiol dibenzoate substrate. The 1,2-ethanodiol dibenzoate TI model is rendered in sticks.

Table II. Stabilization free energy of the model substrate TI estimated for the genetically modified enzymes. Free energies are calculated relatively to the native enzyme.

	ΔΔ*G (kJ/mol)*	
Mutation	**PA 6.6**	**PET**
L182A	-3.80	-4.81
L189A	-2.38	-2.83
V184A	-2.44	-1.81
L81A	-1.77	-2.35
N84A	15.95	14.64

Table III. Activity of cutinases towards PET and PA 6,6 and protein adsorption levels. Values are normalized to the native enzyme.

	PET			PA 6,6	
Mutation	p- Nitrophenyl butyrate (p-NPB) $CH_3CH_2CH_2-C(=O)-O-C_6H_4-NO_2$ % (Umg^{-1})	Terephthalic acid formed after incubation with PET fibers (24 h)* $[-C(=O)-C_6H_4-C(=O)-O-CH_2CH_2-O-]_n$ % (mM)	Protein adsorption levels after incubation (24h) (%)	Amines formed in solution with PA66 fibers (48 h)* $[-C(=O)-(CH_2)_4-C(=O)-N-(CH_2)_6-N(H)-]_n$ % (mM)	Protein adsorption levels after incubation (48h) (%)
Native	100 (210)	100 (0.024)	95	100 (0.094)	30 (± 1.5)
L182A	465 (± 1.8)	528	95	119 (± 2.5)	25 (± 2)
L189A	147 (±1.3)	78	45	94 (± 3)	37 (± 2)
V184A	289 (± 2.2)	203	95	98 (± 3)	21 (± 2.5)
L81A	125 (± 1.8)	399	75	98 (± 2.8)	43 (± 2)
N84A	45 (± 2.6)	170	95	83 (± 3)	15 (± 3)

*See references (O'Neill *et al.,* 2004) (Silva *et al.,* 2004) for experimental details.

The activity of cutinases towards PET and PA 6,6 fibers was expressed as mmolar (mM) of soluble terephthalic acid and soluble amines, respectively, obtained after a certain period of time. Due to the fact that these substrates are solid, no proper Michaelis–Menten kinetic could be calculated. Since we wanted to compare performances of each mutant enzyme based on equal amounts of protein, we expressed those estimated activities in mM of hydrolysis products.

The experimental hydrolytic activity on p-NPB was higher for all the mutant enzymes, when compared with the native cutinase, with the exception of N84A, which is explained by the modeling studies on basis of the favorable interaction of the aspargine with the oxianion hole (Table III). Modified cutinases L182A and V184A have shown a remarkable increase in activity on p-NPB. Hydrolytic activity of L182A form increased more than four-fold. This seems to be a promising mutation to modify the hydrophobic

surface of polyamide and polyester fibers. Concentrations of PET hydrolysis products were calculated after 24 h of PET fabric incubation with the enzyme, in the linear area of substrate conversion. Again L182A was the most active enzyme. The relative ratios of activities were similar for PET fibers and p-NPB, except for L189A and N84A.

Cutinase is also able to biodegrade polyamide 6,6 substrates, but the designed mutations failed to give a clear increase of activity (Table III). Just 19% of increase was found for L182A. These results tend not to be in agreement with the modeling studies of the free energy of stabilization of TI for all mutant enzymes for polyamide 6,6 (Table II).

Given that PET and PA 6,6 fibers are mostly hydrophobic, the adsorption properties of native and mutant unbound enzymes were modeled considering an analysis based on the total enzyme hydrophobic surface. Modified enzymes tested have an equal or lower percentage of hydrophobic surface in comparison with the native, as it was expected (Table IV), given that large hydrophobic residues were changed by smaller ones (with the exception of N84, which is polar). Our studies predict that L182A, N84A and L81A do not significantly affect the adsorption by the hydrophobic fibers, but V184A and L189A show a decrease in hydrophobic area, suggesting that fiber adsorption is reduced in this order. The modeling studies predictions are not in total agreement with the experimental results. N84A, L81A, V184A and L189A displayed different behavior of protein adsorption levels for both fibers when compared with the modeling studies.

On the other hand, the L182A seems to maintain the same adsorption properties as the native enzyme for PET fibers and a slight decrease in the case of PA 6,6.

Given that the biotransformation of a fiber is a heterogeneous reaction, a pre-adsorption of the enzyme on the solid substrate is assumed before the catalysis can proceed. By looking at adsorption data it is possible to verify that cutinase adsorption levels are higher for PET than for PA 6,6 (Table III). For the same concentrations of native cutinase, PET fibers seems to be fully covered with enzyme (± 95%) while this level was not reached for PA 6,6 fibers (± 30%).

Despite the stabilization of TI for several mutant cutinases, other adsorption and substrate recognition issues seem to play a major role on the enzymatic hydrolysis of solids substrates by cutinase. According to these results, L182A was considered to be the most promising enzyme for future studies.

Table IV. Hydrophobic surface percentage (hydrophobic surface/total surface) with SE of native and genetically modified enzymes (Eisenhaber *et al.,* 1995).

Enzyme	**Hydrophobic surface (hydrophobic/total) (%)**	**Standard error (SE)**
Native	40.710	0.096
L81A	40.780	0.192
N84A	40.650	0.243
L182A	40.610	0.147
V184A	40.560	0.154
L189A	40.410	0.185

We further measured the activity of the native and L182A cutinases towards aliphatic polyesters, which resemble the original cutin substrate. Apparently, the native form seems to have a decrease of activity and protein adsorption levels as the hydrophobicity of the substrate increases (Table V). The opposite seems to happen with L182A, which presents a lower hydrophobic area (Table IV). However, the space created by the substitution of leucine by alanine close to the active site, appears to be enough to better "accommodate" hydrophobic aliphatic substrates. These results seem to indicate that cutinase is designed to recognize aliphatic chains, being one of the reasons why this enzyme shows activity towards the aliphatic structure of polyamide 6,6.

In summary, we have obtained cutinases with enhanced activity towards polyester fibers, namely L81A and L182A. The increase in activity of these mutations can be explained by a higher stabilization of TI and a better accommodation of the substrate, as has been shown by our modeling studies. Furthermore, the L182A mutation does not affect the adsorption levels. Regarding polyamide treatment, our findings suggest that these fibers can be more efficiently modified when L182A cutinase is used. Being cutinase an esterase, it seems unlikely that it will biodegrade polyamide substrates. However, our findings suggest that the similarity of polyamide structure with cutin and the diversified substrate recognition of cutinase, might explain the ability of this enzyme to modify the surface of these fibers, showing however a slow enzymatic kinetics (Silva and Cavaco-Paulo, 2004).

Table V. Activity of cutinases towards aliphatic polyesters and protein adsorption levels.

	Enzymes			
	Native		**L182A**	
Aliphatic Polyesters	**Succinic acid (mM)**	**Protein adsorption (%)**	**Succinic acid (mM)**	**Protein adsorption (%)**
Poly(ethylenesuccinate) $[-OCH_2CH_2O-C(=O)-CH_2CH_2-C(=O)-]_n$	728	50	127	20
Poly(1,3-propylenesuccinate) $[-OCH_2CH_2CH_2O-C(=O)-CH_2CH_2-C(=O)-]_n$	626	41	330	29
Poly(1,4 -butylene succinate) $[-C(=O)-CH_2CH_2-C(=O)-OCH_2CH_2CH_2CH_2O-]_n$	432	43	339	24

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Subchapter 2.3

Influence of Mechanical Agitation on Cutinases and Protease Activity Toward Polyamide Substrates

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Influence of Mechanical Agitation on Cutinases and Protease Activity Towards Polyamide Substrates

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Abstract

Two polyamide 6,6 substrates with different construction, namely a model substrate and a fabric, were hydrolyzed using native cutinase and L182A cutinase mutant (from *Fusarium solani pisi*) and a protease (subtilisin from *Bacillus* sp.). The catalytic efficiency of these enzymes, measured in terms of hydrolysis products release, was measured for both substrates and the protease released 5 times more amines to the bath treatment. The L182A cutinase mutant showed higher activity when compared with the native enzyme. All enzymes have shown activity additive effects with higher levels of mechanical agitation for polyamide fabrics. The results achieved are of paramount importance on the design of a process of enzymatic functionalization of polyamide

1. Introduction

Polyamide 6.6 (nylon 6.6) is an aliphatic semi-crystalline polymer made up of adipic acid and hexamethylenediamine. The amide groups -(-CO-NH-)- provide hydrogen bonding among polyamide chains, giving high strength properties at high temperatures, toughness at low temperatures, as well as stiffness, wear and abrasion resistance, low friction coefficient and good chemical resistance.

Nylons are therefore one of the strongest synthetic fibers commonly used, with an extensive range of applications such as clothing, apparel, carpets, tyre reinforcement, parachutes and many other applications (Guillen, 1986; Reimschuessel and Herbert, 1998). Despite of all the excellent properties exhibited, nylon fibers present low hydrophylicity and low reactivity with the most usual finishing and colouring agents (Silva *et al.,* 2004; Vertommen *et al.,* 2005). Coating finishing effects are difficult to obtain when hydrophobic polyamide fabrics are used. Recent studies clearly indicate that the modification of synthetic polymers with enzymes is an effective and environmentally friendly alternative to chemical methods using alkaline products (Guebitz and Cavaco-Paulo, 2003). New processes using cutinases have been developed for the surface modification of polyamide fibers and quite satisfactory results were obtained (Silva *et al.,* 2004; Vertommen *et al.,* 2005; Guebitz and Cavaco-Paulo, 2003; Silva *et al.,* 2005).

Cutinase from *Fusarium solani pisi* is a α/β hydrolase that degrades cutin, the cuticular polymer of higher plants, which is an insoluble hydrophobic polyester composed of hydroxyl and epoxy fatty acids (Carvalho *et al.,* 1998; Heredia, 2003; Osman *et al.,* 1993). This enzyme has a catalytic mechanism similar to that presented by serine proteases. It is characterized by the triad Ser, His, Asp residues and by an oxyanion binding site that stabilizes the transition state via hydrogen bonds with two main chain amide groups (Carvalho *et al.,* 1998; Heredia, 2003; Osman *et al.,* 1993; Nicolas *et al.,* 1996; Lau and Bruice, 1999; Longhi *et al.,* 1997).

Serine proteases such as subtilisin have a structural homology however they do not recognize the same substrates (Gupta *et al.,* 2002).

Being cutinase an esterase, it seems improbable that it will hydrolyze polyamide substrates. Although, polyamide has a structure quite similar to the cutin, that is an

aliphatic polyester. This similarity and the diversified substrate recognition of cutinase makes it able to modify the polyamide surface, showing however a slow enzymatic kinetics (Silva *et al.,* 2004).

The high crystallinity of the polyamide structure and the low affinity of the enzyme to the non-natural substrate are the main factors responsible for that. A more detailed study is needed in a way to know which process variables are the most significant in determining the efficiency of enzymatic hydrolysis. These variables may include different genetic modifications as well as the more usual operating conditions such as enzyme concentration, liquor ratio, treatment time, temperature, pH and mechanical agitation (Azevedo *et al.,* 2000).

Site direct mutagenesis is a way of developing the cutinase in order to obtain a higher specific activity to insoluble substrates like polyamide fiber. The modeling studies are based on the substitution of the specific amino acid residues close to the active site of cutinase, resulting in a modified enzyme with different properties and bigger binding sites. A previous work was performed in order to increase cutinase activity towards polyamide substrates (Araújo *et al.,* 2007). Site-directed mutagenesis of cutinase was performed and five genetically modified enzymes were obtained by changing specific amino acids residues around the active site by alanine (L81A, N84A, L182A, V184A and L189A). The L182A mutant form was the most efficient in the catalysis of the amide linkages (Araújo *et al.,* 2007).

The process of enzyme adsorption is also of main importance to the enzymatic hydrolysis of polyamide. Different studies reveal that the adsorption of proteins follows different steps and that the mechanical agitation plays an important role in all of them (Cavaco-Paulo *et al.*, 1996; Maldonado-Valderrama *et al.,* 2005). Earlier investigation with cellulases proved that higher mechanical agitations increased greatly the enzyme performance at the fibers surface, although it can lead to an increase of the weight loss (Azevedo *et al.,* 2000; Araújo *et al.,* 2007; Cavaco-Paulo and Almeida, 1994; Maldonado-Valderrama *et al.,* 2005; Cavaco-Paulo, 1998; Cavaco-Paulo and Almeida, 1994).

Different levels of mechanical agitation might lead to different levels of protein adsorption and enzymatic hydrolysis. The surface properties have an enormous effect

on the mechanism, rate and degree of adsorption. The hydrophilicity of the surface has generally been regarded as a very important factor: the hydrophobicity of the surface increases the adsorption degree (Palonen *et al.,* 2004). Cutinases, as well as proteases, have hydrophobic amino acids exposed on the surface which can increase the binding to the hydrophobic surface of polyamide fibres.

The purpose of the present work is to provide new insights about the influence of mechanical agitation on cutinase and protease activities towards polyamide substrates. The interaction between the activity of the genetically modified cutinases, the activity of a protease and the mechanical agitation were studied.

2. Materials and methods

2.1. Enzymes and reagents

Commercial polyamide (PA 6.6) woven fabric, a plain woven structure with 63 gm^{-2}, was supplied by Rhodia (Switzerland). The polyamide model substrate (trimmer) was synthesized as described (Heumman *et al.,* 2006). The genetic modification of cutinase was performed as previously described (Araújo *et al.,* 2007). The protease, subtilisin from *Bacillus* sp. (E.C. 3.4.21.62), was a commercial enzyme purchased from SIGMA (St. Louis, USA). The reactive dye used, Lanasol Red 5B (C.I. Reactive Red 66 – 17555), was generously supplied by CIBA (Switzerland). All other reagents used were laboratory grade reagents.

2.2. Quantification of protein concentration

Total protein concentration was determined by the Bradford methodology using bovine serum albumin (BSA) as standard (Bradford, 1976). For each sample three determinations were made.

2.3. Determination of the amino groups in the liquor treatment

To quantify the amino groups released to the liquor treatment during enzymatic hydrolysis, the trinitrobenzenesulfonic acid (TNBS) method was adapted from a methodology already described (Silva *et al.,* 2004).

2.4. Determination of the amino end groups at the fiber surface by reactive staining

The amino groups at the surface of the polyamide fabric resulting from the enzymatic hydrolysis were detected by staining polyamide with a wool reactive dye, specifically designed to react with the primary amino groups. The reaction occurred only at the surface of the fabric as can be depicted from Figure 1 and the free amino groups were detected by the specific reaction with the α-bromoacrylamide dye reactive group (Lewis, 1992).
All stainings were carried out in a 150 cm^3 capacity sealed stainless steel dyepots, housed in a dyeing machine (AHIBA Spectradye, from Datacolor). Stainings of 4% o.w.f (weight of fabric) were obtained using a liquor ratio of 1:100 at different temperatures (50, 60 and 70 ºC) for 90 minutes with a temperature gradient of 4 $ºCmin^1$. After staining, the samples were washed with 2 g L^{-1} of a Lutensol AT 25 solution and then rinsed in running cold tap water for 10 min and air dried. Two independent staining experiments were performed and the results represent the mean of these experiments. Colour differences of the stained fabrics were measured by using a reflectance-measuring apparatus, Spectraflash 600 Plus, from Datacolor International according to the CIELab colour difference concept, at standard illuminant D65. The colour strength was evaluated as K/S at maximum absorption wavelength (570 nm) and the results were summarized by the overall K/S differences (Harold, 1987).

2.5. Determination of the wettability of the polyamide treated fabric samples

In order to obtain the degree of wettability (hydrophilicity) of the untreated and treated polyamide fabrics, a water-drop test was applied according AATCC standard method, (1980). The wetting time was determined by placing a drop of distilled water on the stretched fabric sample (5*5 cm) from a burette held 1 cm from the fabric. The time of

disappearance of the water-mirror on the surface (in other words the time for the water drop to lose its reflective power) was measured as the wetting time. This procedure was applied to both untreated and treated fabrics.

2.6. Enzymatic hydrolysis of polyamide model substrate (trimmer)

2.6.1. Cutinases

In the first part of the work, a native and a mutated cutinase (L182A) were used to incubate the polyamide trimmer. Two sets of experiments were performed where 0.01 g of a polyamide model substrate was incubated in two different solutions. The first solution contained 10 mL of phosphate buffer (pH 7.5) and 35 U (μmol of p-NP per min)/mL of native cutinase and the second solution contained the same amount of buffer and 47 U (μmol of p-NP per min)/mL of cutinase mutant (L182A). Both experiments were performed at 35 ºC for 8 hours under continuous shaking (using an AHIBA Spectradye, from Datacolor, with vertical agitation). At different periods of incubation, the total protein content in the solution was determined as described in section 2.2. After 8 hours of incubation, a protein precipitation step was performed and the primary amino groups resulting from enzymatic hydrolysis were quantified by the TNBS method (Silva *et al.,* 2004).

2.6.2. Protease

On this set of experiments, 10 mL of Tris-HCl buffer (pH 7.6) containing 18 U (μmol of Tyrosine per min)/mL of protease were incubated with 0.01 g of model substrate under the same conditions already described for cutinases. At different periods of incubation, the total protein content in solution was determined as described in section 2.2. After 8 hours of incubation, a protein precipitation step was performed and the primary amino groups resulting from enzymatic hydrolysis were quantified by the TNBS method (Silva *et al.,* 2004).

2.7. Enzymatic hydrolysis of polyamide fabric

Pre-treatment

All samples of polyamide fabric used on this work were subjected to a previous washing with 2 gL^{-1} of a non ionic agent, Lutensol AT 25 (10 gL^{-1}) and water for 1 hour, followed by washing with a 2 gL^{-1} of Na_2CO_3 solution for 1 hour, both at 50 ºC.

2.7.1. Cutinase - vertical agitation

In 500 mL stainless steel pots of a laboratory Rotawash MKII machine, from SDL International Ltd, rotating at 20 rev/min, 1 g of pre-treated polyamide fabric was incubated with 78 U (µmol of p-NP per min)/mL and 282 U (µmol of p-NP per min)/mL of native cutinase in 300 mL of phosphate buffer (0.1 M NaOH, 0.1 M KH_2PO_4, pH 7.8) at 37 ºC for 4 hours under continuous vertical agitation. A higher level of mechanical agitation was achieved by adding 5 stainless steel discs (each disc with average weight of 19.1 g, 32 mm x 3 mm) into the reaction mixture. The L182A cutinase mutation was also tested, where 1524 U (µmol of p-NP per min)/mL were incubated using the same conditions of the native one, as already described.

The experiments were performed in the presence of the discs as well as in their absence. For protein and amino groups quantification, aliquots were taken from the liquor treatment at 0.5, 1, 2, 3 and 4 hours. After 4 hours of incubation, the fabrics were removed from the liquor and rinsed in sodium carbonate solution (2 g L^{-1}) for 2 hours to stop the enzymatic reaction, followed by washing with 2 g L^{-1} of Lutensol AT25 solution for 1 hour. After that, the samples were rinsed in running cold tap water for 5 min and allowed to dry at open air. Two independent experiments were done for each treatment, and the results represent the mean of these experiments.

2.7.2. Cutinase - orbital agitation

In 300 mL of phosphate buffer (0.1 M NaOH, 0.1 M KH_2PO_4, pH 7.8), 1 g of pre-treated polyamide fabric was incubated with 78 U (µmol of p-NP per min)/mL of native cutinase at 37 ºC for 48 hours under continuous orbital agitation. The low level of mechanical agitation was achieved using an Erlenmeyer held in a shaking water bath

operating at 90 strokes min^{-1}. For protein and amino groups determination, aliquots were taken from the liquor treatment at 4, 6, 24, 36 and 48 hours of incubation. After 48 hours of incubation, the same procedure already described for the enzymatic treatment using vertical agitation was followed.

2.7.3. Protease - vertical agitation

In this set of experiments, 3 g of pre-treated polyamide fabric were incubated with 36 U (μmol of Tyrosine per min)/mL of subtilisin in 300 mL of Tris-HCl buffer (0.3 M Tris, 3 M HCl, pH 7.5) at 35 ºC for 4 hours under continuous vertical agitation. A higher level of mechanical agitation was achieved by adding 5 stainless steel discs (each disc with average weight of 19.1 g, 32 mm x 3 mm) to the reaction mixture contained in 500 mL stainless steel pots of a Rotawash MKII machine, rotating at 20 rev/min. The experiments were performed both in the presence and absence of stainless steel discs. For protein and amino groups determination, aliquots were taken from the liquor treatment at 0.5, 1, 2, 3 and 4 hours. After 4 hours of incubation, the same procedure already described for the enzymatic treatment with cutinase using vertical agitation was followed.

2.8. Wide-angle X-ray diffraction (WAXD)

WAXD patterns of the PA 6.6 fabric were obtained for the samples treated with cutinases and protease, both in the presence and absence of stainless steel discs. The X-ray diffractometer used was the model PW1710, from Philips. The Cu Kα radiation source (λ=0.154 nm) was operated at 40 kV and 30 mA. The WAXD spectra were continuously recorded in the diffraction angular range of 5 º to 35 º (2θ). The scan speed was 0.01º s^{-1}.

The WAXD data were analyzed by profile fitting of the obtained scans. The Pearson VII functions were applied and several simulations were performed in order to provide the best fit. At the end several parameter values were obtained such as pick intensities, pick positions, full width at half-maximum and others.

The crystallinity value (CV) of the different assayed samples was obtained using eq. (1), available in literature where d100 and d010 are the interplanar distances related to the planes (100) and (010), respectively (Dismore and Sttaton, 1966; Botelho *et al.,* 2002).

$$\text{CV} = \frac{[d_{010} / d_{100}] - 1}{0.189} \times 100 \qquad (1)$$

Equation (1) can be simplified and expressed as eq. (2), where the θ100 and θ010 are the angles related to the (100) and (010) interplanar distances (d); 546.7 is a constant related with polyamide crystallinity and 0.50 is the other constant obtained by calculating the reason between the crystalline area and the total area (crystalline and amorphous) of each sample.

$$\text{CV} = \left(\frac{2\theta_{010}}{2\theta_{100}} - 1 \right) \times 546.7 \times 0.5 \qquad (2)$$

2.9. Fourier transformed infrared spectroscopy (FT-IR)

Infrared spectra were taken with a Bomem M Series Spectrophotometer. All the spectra reproduced were collected using an attenuated total reflectance accessory (ATR). Before colleting, the background scanning was performed using KBr powder. The fabric samples were placed in a large sample cup in top of KBr. At least 32 scans were obtained to achieve an adequate signal to noise ratio. The spectra were taken in the region of 800-4000 cm^{-1} with a resolution of 8 cm^{-1} at room temperature.

2.10. Scanning electronic microscopy (SEM)

The scanning electronic microscopy (SEM) pictures were obtained in a scanning electronic microscope model LEICA S360 with a backscattered and secondary electrons detector.

3. Results and discussion

3.1. Enzymatic activity of cutinase and protease towards polyamide trimmer

One of the objectives of this work was to prove that cutinase and protease were able to hydrolyze polyamide surface substrates. Therefore, before the enzymatic treatment of the main substrate (polyamide fabric), a smaller substrate was studied. Our purpose was to prove that if these enzymes were able to work on the hydrolysis of small polyamide substrates, they could probably act also on bigger ones. For that reason a small amount of PA 6.6 trimmer was incubated with native, mutated cutinase (L182A) and protease and their activity was measured as the amino groups formation. The protein adsorbed during the enzymatic process was also quantified for all the enzymes assayed. Comparing the obtained data for both cutinases assayed, it can be observed (Table I) that the protein adsorption, as well as the enzymatic activity, expressed as mM of amines in solution, is higher when L182A mutant was used. These values are explained based on the assumption that the site-directed mutagenesis, that consisted on the substitution of the Leu amino acid by a smaller (Ala) amino acid, close to the active site, resulted in a more “open” enzyme structure. This allows for a better “accommodation” of the bigger polyamide substrate into the active site. The protein adsorption values for protease were quite similar to those obtained for the L182A mutant but the enzyme activity was higher. This result can be attributed to the specificity of this enzyme to hydrolyze amide bonds.

The data obtained for PA trimmer shows relative ability to hydrolyze small substrates of polyamide. A posterior study was performed to confirm these results and the ability of these enzymes to modify the surface of a bigger substrate (PA fabric).

Table I. Protein adsorption and enzyme activity (measured as amines formation) of native cutinase, L182A cutinase mutant and protease.

Enzyme	*Protein adsorption (%)*	**Amines (mM)**
Native (35 UmL^{-1})	18	0,0728
L182A mutant (47UmL^{-1})	53	0,1053
Protease (18 UmL^{-1})	54	0,3228

3.2. Enzymatic activity of cutinase and protease towards polyamide fabric

3.2.1 Cutinase

The ability of cutinase to modify bigger polyamide substrates was determined as well as the interaction of mechanical agitation with enzymatic activity.

Different levels of mechanical agitation were applied on the described experiments in order to measure its influence on enzymes activity.

The activity of native cutinase, measured as amino groups released in the liquor bath treatment, increased when higher mechanical agitation was used (stainless steel discs addition) (Figure 2). The findings seem to suggest that mechanical agitation influences greatly the enzyme hydrolysis on the polyamide fabric. Comparing the experiments performed on the Rotawash machine (vertical agitation), it seems clear that the increase of the native cutinase activity was due to the incorporation of the stainless steel discs on the treatment pots. This process variable lead to an increase of the fibre-metal friction, as well as an increase of the betting effects during enzymatic incubation. The higher mechanical agitation used increased the action of cutinases. The combined action of the enzyme and the mechanical agitation lead to a more pronounced effect compared with the enzyme action it self. The additive effects of the enzyme and the mechanical agitation created more superficial cuts along the polymer, corresponding to the breakage of the amide linkages. Mechanical action raised these broken ends, creating micro fibrils and consequently more sites for possible enzyme attack. This phenomenon was

accomplished by the mechanical abrasion of the fabrics' surface where the amino end groups formed by enzymatic action were released to the liquor bath treatment and could be spectrophotometricaly quantified (Figure 2). In order to measure the hydrolysis extent, a fabric reactive staining was performed (Figure 3). In the absence of the stainless steel discs was obtained an increase of the staining values corresponding to an increase of the amino groups at the surface of the treated fabrics since they were not released to the bath treatment. The K/S values decreased when temperatures above Tg were applied. Above 57 ºC the polymer structure is more exposed and the dye does not link only at the surface of the fabric (Figure 1) but is also able to penetrate into the interior of the fibres. It is important to notice that, to measure the hydrolysis extent at the surface of the treated fabric, the reactive staining should be performed below glass transition temperature. Regarding the other values obtained, it can be seen that mechanical agitation preferentially removed microfibrillar material with a high content of end groups which can not be detected by reactive staining. High levels of mechanical agitation are aggressive and cause fabric fibrillation. The formed fibrils (pills) represent a more exposed specific surface area of enzyme attack and will present a more pronounced color intensity compared with the other part of the treated fabric. However, these results were not considered because the spectrophotometrically measure is technically difficult to obtain.

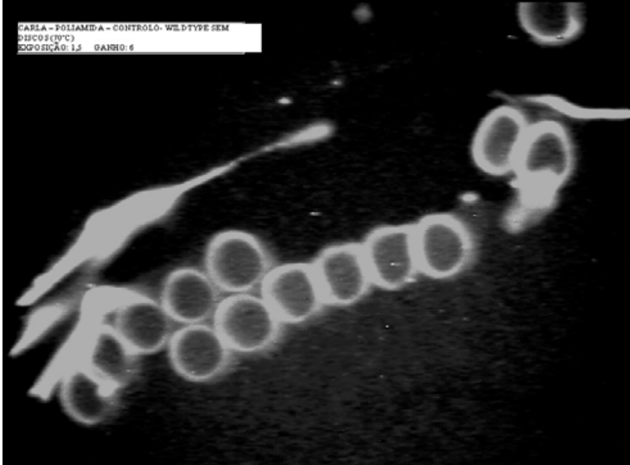

Figure 1. Microscopic image of the polyamide stained samples (the reactive dye is linked only at the surface of the fabric).

In the same set of experiments described was used a shaker bath with orbital agitation. In this apparatus the polyamide samples were incubated for a long period (48 hours) at 90 strokes per minute. A slow kinetic of enzymatic activity in the first 24 hours of incubation was obtained. After this period the activity of cutinase increased reaching the same level of the one obtained on the Rotawash machine (vertical agitation), without discs (Figure 2).

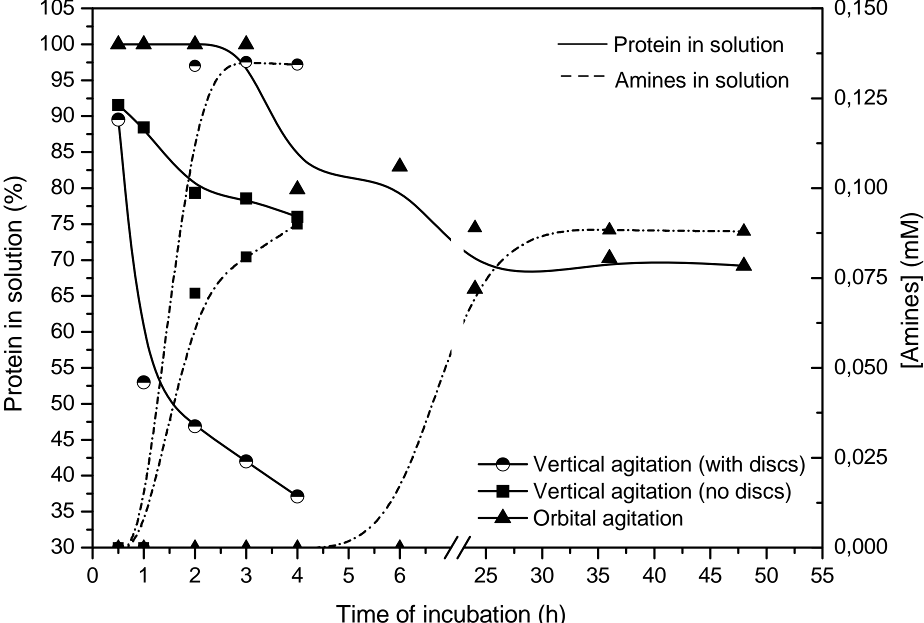

Figure 2. Native cutinase (78 U mL^{-1}) activity (measured as amines released to the bath treatment) vs protein adsorption.

The results of reactive staining obtained for vertical agitation, as well as the results obtained for orbital agitation show that the incorporation of discs on the system increased the release of the amines to the bath system (Figure 3). The K/S values decreased when a higher level of mechanical agitation was applied.

Adsorption studies were also performed in order to measure the influence of mechanical agitation on the protein adsorption on the fibres. The results given in Figure 2 show that protein adsorption increased when stainless steel discs were included in the system,

reaching a high level of about 60% of adsorption. Orbital agitation provided values of protein adsorption in the order of 30%.

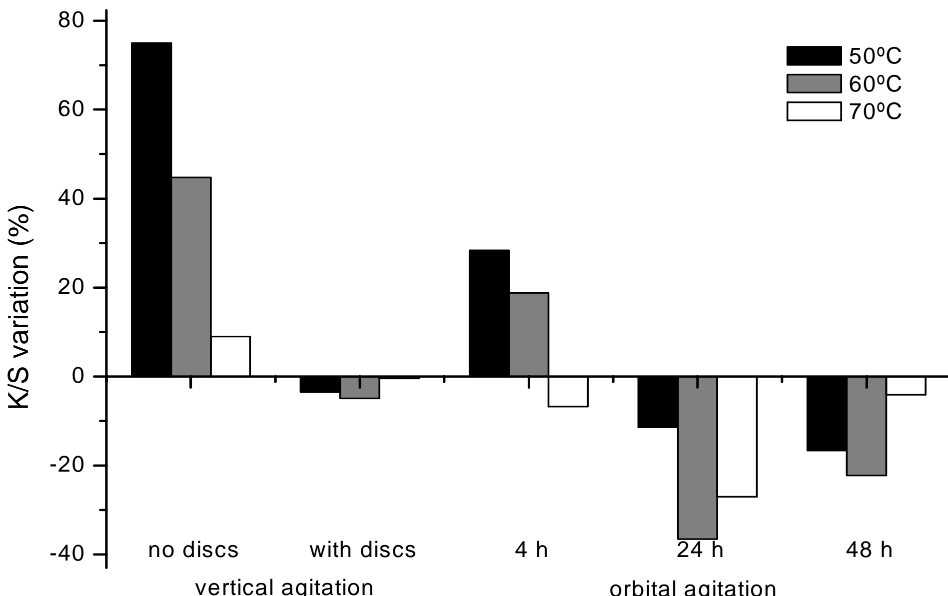

Figure 3. K/S variation, proportional to the amino groups at the surface of the treated fabric (4% of reactive dye; liquor ratio 1:100; 90 minutes).

As obtained for polyamide model substrate (Table I), on fabric the L182A cutinase mutant presented a considerably higher activity when compared with the native one. (Figures 4 a) and 4 b)) Protein adsorption values are similar for both enzymes and presented higher values when stainless steel discs were included on the enzymatic system.

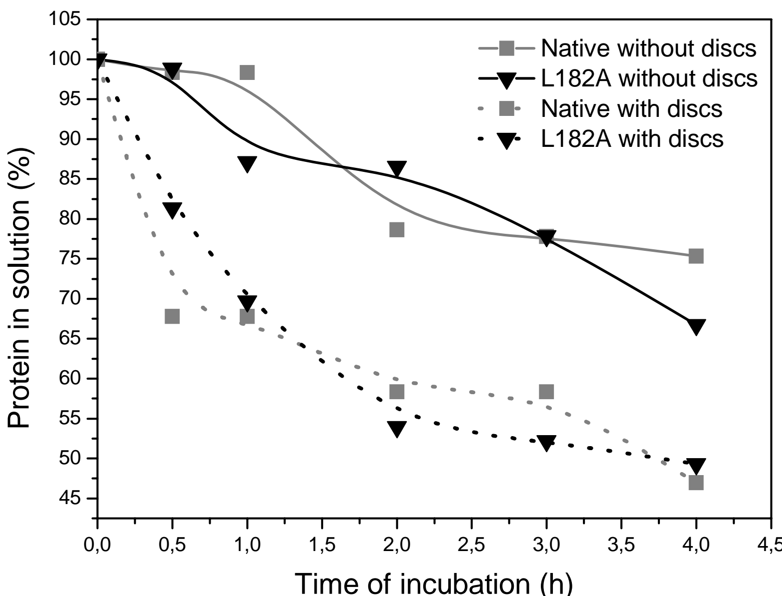

Figure 4 a). Protein content in the liquor treatment after 4 hours of incubation with native (282 U mL^{-1}) and cutinase mutant (1524 U mL^{-1}).

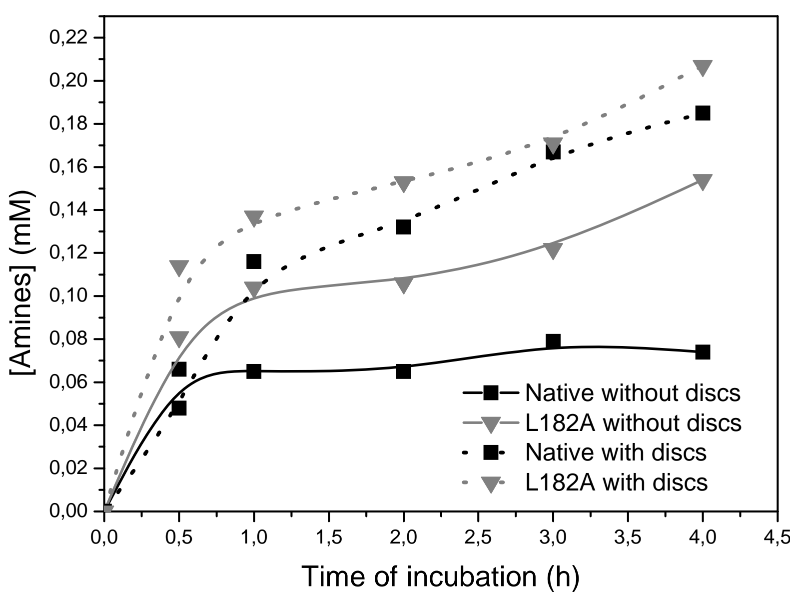

Figure 4 b). Amino groups concentration in the liquor treatment after 4 hours of incubation with native (282 U mL^{-1}) and cutinase mutant (1524 U mL^{-1}).

3.2.2. Protease

Similarly to the results obtained for cutinases, the protease activity as well as the protein adsorption, increased when high levels of mechanical agitation were used. Mechanical abrasion has been indicated as to synergistically cooperate with protease activity. This is explained by the fact that mechanical agitation causes more fibrillation. In this situation, the loose fibrils (pills) formed represent an increased and more exposed specific surface area for enzyme attack. The synergistic action of the enzyme specificity and the mechanical agitation lead to a higher activity, measured as amino groups released, compared with the cutinases (Figure 5 b). The amino groups concentration, when protease was used, reached 1 mM, a value which is five times higher when compared with that obtained for cutinases (0.2 mM) (Figure 4 b). Spectral values obtained after reactive staining of treated fabric samples increased which can be correlated with an increase of the amino groups at the surface of the treated fabrics (Figure 6).

The increase of the staining temperature lead to a lower increase of the K/S values due to the fact that above glass transition temperature the polymer structure is more open and the dye penetrates in the interior of the fibre. Differences between samples are not so easily detected when analyzing the obtained data it can be seen that when a higher level of mechanical action was used, the amino groups at the surface were partially removed and consequently the K/S values decreased. Protease hydrolysis was efficient on surface fabric modification.

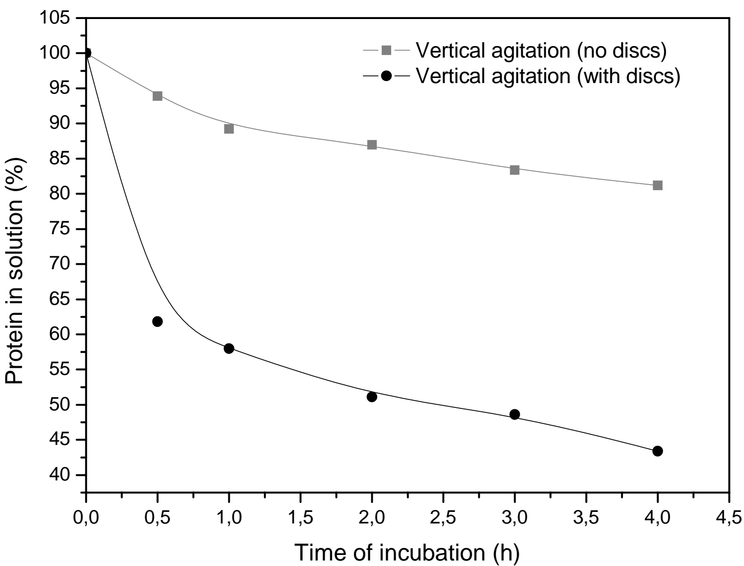

Figure 5 a). Protein adsorption after 4 hours of incubation with Protease (35 U mL^{-1}).

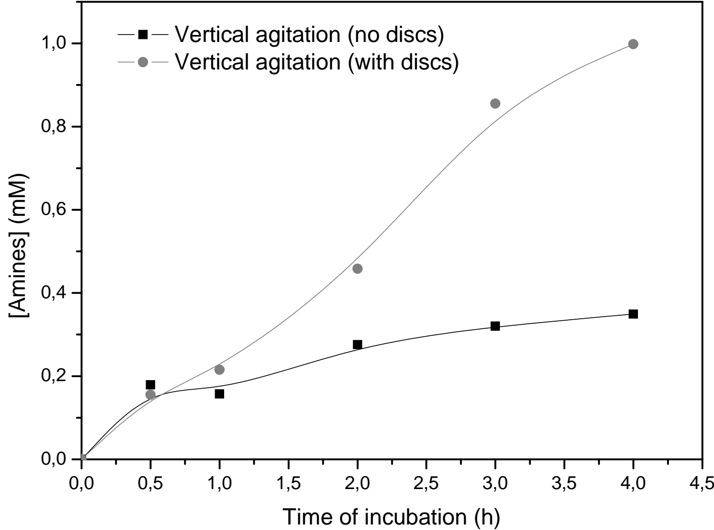

Figure 5 b). Amino groups concentration in the liquor treatment after 4 hours of incubation with Protease (35 U mL^{-1}).

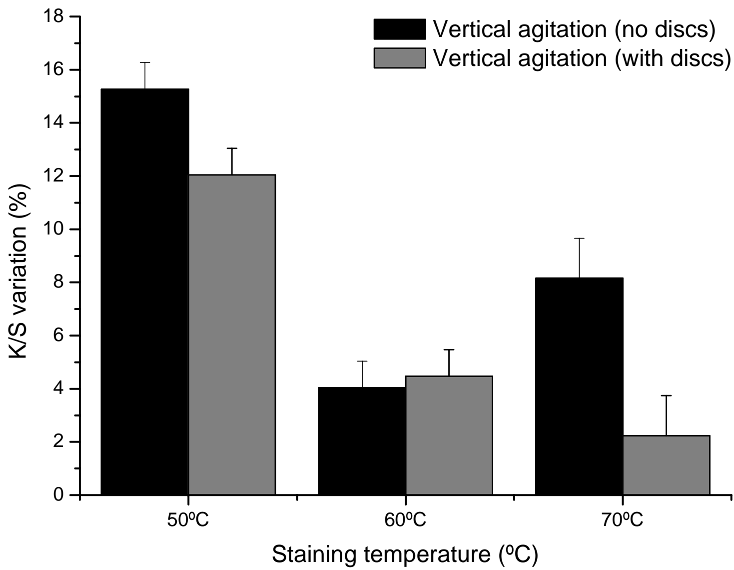

Figure 6. K/S variation, proportional to the amino groups at the surface of the fabric samples treated with protease (4% of reactive dye; liquor ratio 1:100; 90 minutes).

3.3. Wettability

Polyamide fabric samples were tested in terms of water absorption after enzymatic incubation procedure. This measurement is an evidence that the content of hydrophilic groups at the surface have increased. The samples without treatment presented a hydrophobic behavior (>10 min. of absorption). The enzymatic hydrolysis with cutinases and protease were able to modify the surface of polyamide fabrics with a consequent decrease of the time of water drop absorption to 5 min (Table II). The surface of polyamide fabrics became more hydrophilic and probably more able to be finished.

Table II. Time of water drop absorption of fabrics treated with different enzymes.

Enzymes	**Time of water drop absorption (min.)**
Control	> 10
Native (282 UmL^{-1})	5.34 ± 0.20
L182A mutant (1524 UmL^{-1})	4.67 ± 0.20
Protease (36 UmL^{-1})	5.00 ± 0.40

3.4. Crystalline measurements

WAXD studies of treated polyamide samples show, as expected, two strong diffraction peaks, one located at $2\theta = 20.2^{\circ}$ and the other one at $2\theta = 23.4^{\circ}$ (Botelho *et al.,* 2002). The crystallinity value (CV) of the control and the treated samples was calculated as defined in Eq. (2). As expected, no significant changes were observed. Enzymatic action occurs only at the surface of the fabric and the formation of hydrophilic groups by hydrolysis does not influence the intrinsic physical properties of polyamide polymer.

Table III. Crystallographic results of polyamide samples treated with cutinases and protease.

Sample	**% of crystallinity**
Control (no discs)	42.837
Native	42.964
L182A mutant	42.911
Protease	42.957
Control (with discs)	43.037
Native	43.419
L182A mutant	43.723
Protease	43.838

3.5. Infrared studies

Infrared spectra, taken for PA 6.6 samples are shown on figure 7 a) and 7 b), as an example. In this study the different spectra obtained for control and each treated sample were compared. The band region used for comparison was 1700-1650 cm^{-1} where it seams to show some relative intensity differences. The carbonyl amide stretching vibration in the region of 1680-1630 presented different intensities depending on the enzyme.

Regarding the spectra obtained (band 1660 cm^{-1}) for samples treated with the native, L182A mutant cutinase and protease without stainless steel discs it seems clear that there is a decrease of the absorbance value relatively to the control. The breakage of the amide linkages of the polyamide polymer and the increase of the other groups at the surface of the fabric might influence the amide vibrations and consequently its absorbance intensity. The spectra obtained for the samples treated with all the enzymes in presence of stainless steel discs does not show significant changes on the absorbance intensity value.

These results can be correlated with the spectral results already described. Samples treated in the absence of discs presented higher K/S values than samples treated in their presence. The simultaneous action of enzyme and mechanical agitation lead to the surface abrasion of the superior layer of polyamide fabric (Figure 8) and consequently the hydrophilic groups formed by enzymatic hydrolysis can not be so easily detected by reactive staining.

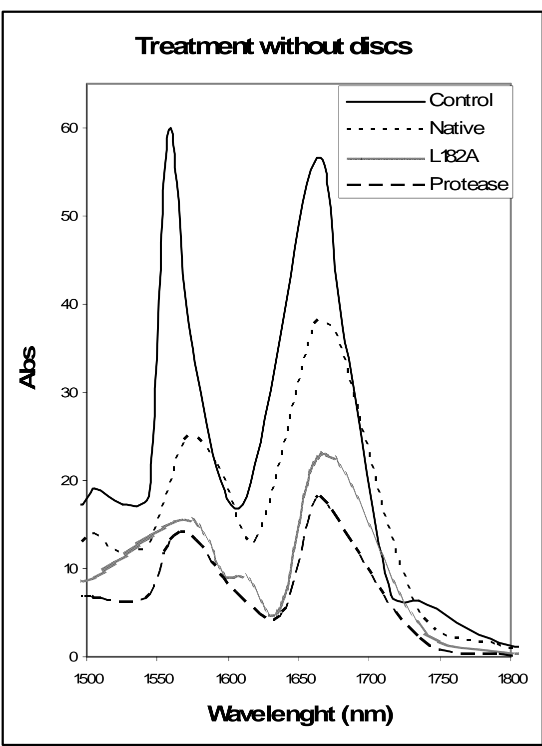

Figure 7a). Infrared spectra of samples treated with Native, L182A cutinase mutant and protease in the absence of stainless steel discs (most significant band).

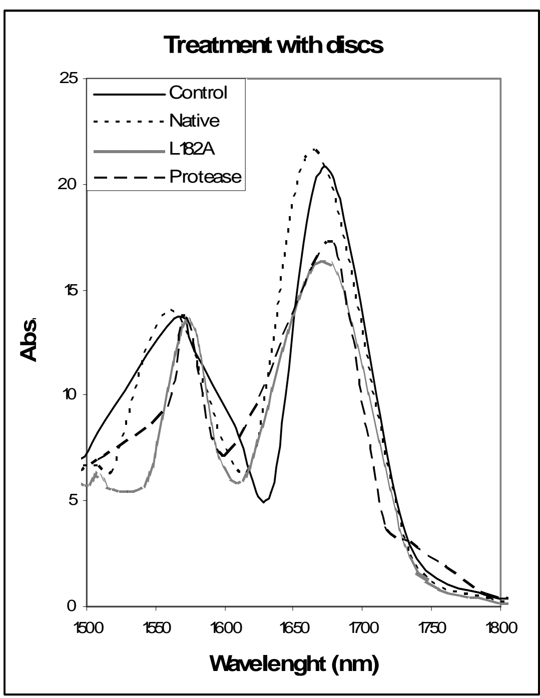

Figure 7b). Infrared spectra of samples treated with Native, L182A cutinase mutant and protease in the presence of stainless steel discs (most significant band).

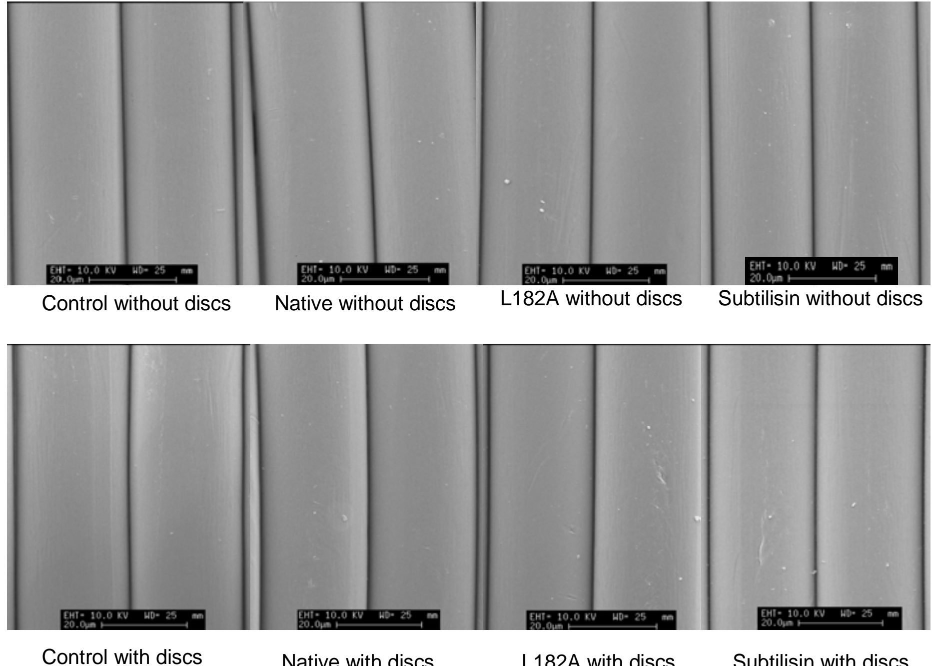

Figure 8. Scanning electronic microscopy (SEM) of samples treated with native, L182A mutant and proteases in the absence and in the presence of stainless steel discs.

4. Concluding remarks

This study provided new insights about the influence of mechanical agitation on cutinase and protease activities towards polyamide substrates. The cutinase mutant (L182A) showed more ability to modify the surface of polyamide substrates when compared with the native one. However, the higher catalytic efficiency was obtained for protease due to its enzymatic specificity. The results obtained support the idea that when higher levels of mechanical agitation were introduced on the system the level of surface modification increased. The simultaneous action of the enzymes and the stainless steel discs lead to an increase of the enzymatic conversion, although a careful balance between the enzyme activity and the mechanical agitation is required to achieve higher level of hydrolysis without excessive fabric strength and weight loss.

For a future industrial application of this process it is necessary to find this equilibrium. To produce the large amount of amino groups, short times of incubation must be used as well as vertical agitation. More studies have to be performed in order to predict and better control the polyamide finishing.

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Subchapter 2.4

Effect of the Agitation on the Adsorption and Hydrolytic Efficiency of Cutinases on Polyethylene Terephthalate Fibres

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Effect of the Agitation on the Adsorption and Hydrolytic Efficiency of Cutinases on Polyethylene Terephthalate Fibres

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Abstract

The effect of agitation on adsorption, desorption and hydrolytic efficiency of native and the genetically modified cutinase (L182A) on polyethylene terephthalate fibres is reported in this paper. The effect of mechanical agitation was studied using a shaker bath with orbital agitation and a Rotawash machine with vertical agitation with and without extra steel discs inside the reaction pots. The results obtained indicate that mechanical agitation combined with enzymatic action enhances the adsorption and activity of cutinases towards PET (polyethylene terephthalate) fibres. L182A showed higher adsorption than the native enzyme for all the levels of mechanical agitation. Lower units of L182A lead to similar yields of terephthalic acid formed in all levels of mechanical agitation. The highest increase of hydroxyl surface groups was found for the genetically modified L182A at the lowest level of mechanical agitation with a shaker bath. These results indicate that enzymatic functionalization of PET is favoured with a process with lower levels of mechanical agitation.

1. Introduction

Advances in biotechnology enabled the design of enzymes with improved catalytic activities towards the substrates of interest and better stabilities than those previously available (Araújo *et al.*, 2007). The use of genetic engineering stands as a powerful tool that has been used to modify an enzyme to improve its action on polyester fibres (Araújo *et al.*, 2007). It is known that cutinases are able to catalyse the hydrolysis of ester bonds in polyester, resulting in the generation of hydroxyl and carboxyl groups at the surface and in the formation of terephthalic acid and ethylene glycol as reaction products (Carvalho *et al.*, 1999; Silva *et al.*, 2005). The cutinase from *Fusarium solani pisi* is an extracellular enzyme that is naturally designed to catalyse the hydrolysis of cutin, the structural polyester from the cuticle of plants. This enzyme was modified, by site directed mutagenesis, around the active centre in order to fit a larger polymer substrate and therefore improve its activity towards a non-natural substrate such as polyester (PET) fibre (Araújo *et al.*, 2007). The change of specific amino acid residues was performed based on the native enzyme structure. The modified cutinase L182A has been shown in a previous work to have a higher affinity and to be more effective for polyester hydrolysis than the native one (Araújo *et al.*, 2007).

The process of enzyme adsorption is of key importance concerning fundamental knowledge of enzymatic hydrolysis of water insoluble fibre substrates (Araújo *et al.*, 2007; Cavaco-Paulo and Almeida, 1996; Azevedo *et al.*, 2000; Palonen *et al.*, 2004). Enzyme adsorption and desorption on polyester is a pre-requisite for the hydrolysis process to occur (Azevedo *et al.*, 2001; Cavaco-Paulo *et al.*, 1998). The adsorption of proteins starts with the formation of various contacts between the adsorbing protein molecule and the sorbent surface (Stuart, 2003; Norde, 2003). Protein adsorption is very complex and involves different steps that have been subject of earlier studies in order to understand this process (Kim and Yoon, 2004; Nicolau and Nicolau 2004: Maldonado-Valderrama *et al.*, 2005). The hydrophobic amino acids exposed on the surface of the enzyme will lead to binding to the hydrophobic surface of the PET fibre. The important substrate characteristics that influence the enzymatic hydrolysis are: accessibility, degree of crystallinity and degree of polymerization (Palonen *et al.*, 2004).

A balance between enzyme activity and mechanical agitation is required to achieve the desired effect on the textile substrates. For cotton fibres, it was observed that high mechanical agitation lead to an increased accessible surface area. Consequently higher levels of enzyme adsorption were measured (Cortez *et al.*, 2001). The mechanical action on these fibres is expected to protruding fibres and therefore more sites in the fibre for enzymatic attack. In short treatment times, the enzyme attacks what is more exposed, i.e., the pills or raised microfibrils at the fabric surface (Cavaco-Paulo, 1998; Morgado and Cavaco-Paulo, 2000).

In this work we studied the influence of the level of mechanical agitation on the adsorption and activity of cutinases on PET fabrics. Industrially suitable conditions such as short processing times were reproduced at both low and high level of mechanical agitation during the enzymatic process. The low level was reproduced with orbital agitation in a shaker bath and the high level with vertical agitation in a laboratory washing machine with and without metal disks added.

2. Experimental

2.1 Materials

The fabric used was 100% polyester, 107 g/m^2, 18 yarns/cm, 29 Tex (warp and weft) obtained from Rhodia. Activity of cutinases was assayed towards p-Nitrophenylpalmitate (pNPP) as described in literature (Quyen *et al.*, 1999). The enzymes used in this work were cutinases from *Fusarium solani pisi* (native cutinase with a specific activity of 221.0 U (μmol of pNPP per minute)/mg^{-1} and a genetically modified cutinase L182A with a specific activity of 125.0 U (μmol of pNPP per minute)/ mg^{-1} produced and purified as previously described (Araújo *et al.*, 2007). The dye used for staining was C.I. Reactive Black 5 (RB5) from Ciba, Switzerland. All other chemicals used were laboratory grade reagents.

2.2. Enzymatic treatment

The polyester fabric was washed with 2% v/v of Lutensol AT25 at 50 ºC during 60 min, with distilled water for 60 min at 50 ºC and dried at oven at 40 ºC for 24 h. The enzymatic treatments were performed in sealed, stainless steel dye pots of 400 cm^3 in a Rotawash machine (laboratory scale dyeing machine) with and without stainless steel discs (10 discs were used in each pot, each disc with an average weight of 19 g, 32mmx3mm) with vertical agitation of 40 rpm. For comparing different agitation modes, samples were also incubated in 250 mL glass flaks in a shaker bath with orbital agitation of 80 rpm. The enzyme concentration used was 100 $mgprotL^{-1}$ of both purified enzymes corresponding to esterase activity if 22.1 U (μmol of pNPP per minute) L^{-1} of native and 12.5 U (μmol of pNPP per minute) L^{-1} of L182A. The enzymatic treatments were performed at 35 ºC using phosphate (KH_2PO_4 0.1 M, NaOH 0.1 M) buffer 0.1 M pH 8.5. All samples were incubated for 5 h. For each incubation period (1, 2, 3, 4 and 5 h) a fabric sample and an enzymatic solution sample were taken for further analysis. Five PET fabric samples, each with 0.2 g, were incubated with enzyme in 150 mL of phosphate buffer. Control samples were incubated in the same buffer solution but without enzyme and samples removed after each incubation period. After enzymatic treatment, all samples were washed first with tap water, then with a 2 g L^{-1} sodium carbonate solution for 60 min at 50 ºC (to remove the remaining proteins) and finally with distilled water at 50 ºC for one hour.

2.3. Staining with reactive dye

The staining was performed at 60 ºC, below the glass transition temperature (Tg) of PET fibre, which is approximately 69 ºC. Polyester fabric samples after enzymatic treatment were stained all together in the same sealed, stainless steel dye pot of 120 mL capacity in an Ahiba machine (laboratory scale dyeing machine). The dyeing was performed with RB5 (10%) owf, bath ratio of 100:2 (100 mL of liquor for 2 g of fabric), at pH 11.0 using 20 gL^{-1} sodium carbonate and 60 gL^{-1} sodium sulphate at 60 ºC during 90 min with 30 rpm of agitation. After the dyeing process, samples were all washed in a

flask with stirring with water at 50 ºC for 1 h and then dried in an oven at 40 ºC for 24 h.

2.4. Terephthalic acid (TPA) determination by fluorescence

We measured the TPA in the enzymatic treatment solution for the different incubation periods in order to study the enzymatic hydrolysis efficiency, since TPA is one of the hydrolysis reaction products. Fluorescence scans were performed with a luminescence spectrometer between 300 and 600 nm wavelengths after reaction of terephthalic acid solution with hydrogen peroxide 30% at boiling temperature and using an excitation wavelength of 315 nm. The presence of hydroxy-terephthalic acid (HTA) (O'Neill and Cavaco-Paulo, 2004) is detected by analyzing the wavelength of 425 nm. If the concentration of TPA increases, there will be a higher intensity at this wavelength will be observed due to the presence of more HTA ions. One mL of solution was added to 2 mL of hydrogen peroxide and heated at 90 ºC for 30 min. After cooling down to room temperature, samples were measured by fluorescence and the intensity of the peak at 425 nm was used for the determination of TPA concentration (O'Neill and Cavaco-Paulo, 2004).

The calibration curve was determined using standard solutions with different concentrations of TPA (0.006, 0.03, 0.06 and 0.12 mM) dissolved in a 0,05 M NaOH solution. The calibration curve attained shows a linear fit between 0.006 and 0.12 mM TPA with a standard deviation below 4% and R^2=0.995. All measurements were performed using duplicate samples and results represent the mean. Values had a standard deviation below 10%.

2.5. Colour measurements

The K/S increase after the enzymatic treatments and after staining with a reactive dye (RB5) was measured in order to detect differences of hydroxyl groups formed at the surface (Araújo *et al.*, 2007). The colorimetric data (K/S) of the dyed samples with Reactive Black 5 was collected using a spectrophotometer Spectraflash 600 plus interfaced to a PC using an illuminant D65 at the wavelength of maximum absorption

(600 nm) as an average of five readings. The control samples were incubated in phosphate buffer solution.

2.6. Protein adsorption and desorption

The remaining protein in the enzymatic treatment solution for different incubation periods was measured to study the adsorption and desorption phenomena (Araújo *et al.*, 2007). For this adsorption/desorption studies the protein in solution was determined by the Bradford method using bovine serum albumin (BSA) as standard (Bradford, 1976). Different time samples of enzymatic treatment solution were measured (0, 1, 2, 3, 4 and 5 h). After 5 h of incubation, the enzymatic treatment solution was diluted (1:2) with the same previously specified phosphate buffer and the mixture was subsequent incubated for another 60 minutes, with the same agitation and temperature conditions, to verify the desorption phenomena. Values had a standard deviation below 9%. The percentage of adsorption was calculated by the following expressions as described in literature (Azevedo *et al.*, 2000):

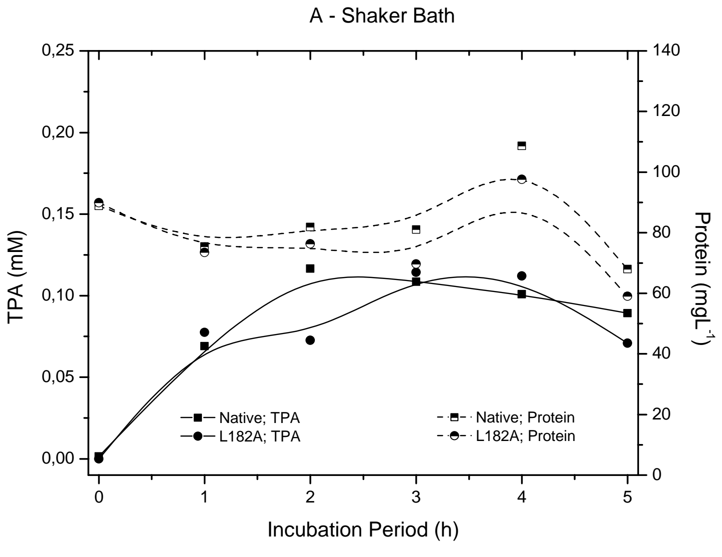
$$\%\ \text{adsorption} = \frac{A - B}{A} \times 100 \qquad (1)$$

$$\%\ \text{desorption} = \frac{D - C}{A} \times 100 \qquad (2)$$

Where: A – initial protein concentration (at 0 h); B – protein concentration at time t (1, 2, 3, 4 and 5 h); C – dilution (dilution 1:2 with buffer solution); D – final protein concentration (after the dilution and 60 more minutes of incubation).

3. Results and Discussion

The formation of terephthalic acid (TPA) and the protein adsorption for the two studied cutinases, with a low agitation level (shaker bath, 2A) and strong agitation level (Rotawash, 2B), is shown in figure 1. In table 1 the values of percentage of protein adsorbed for the different incubation periods are shown for both modes of mechanical agitation.



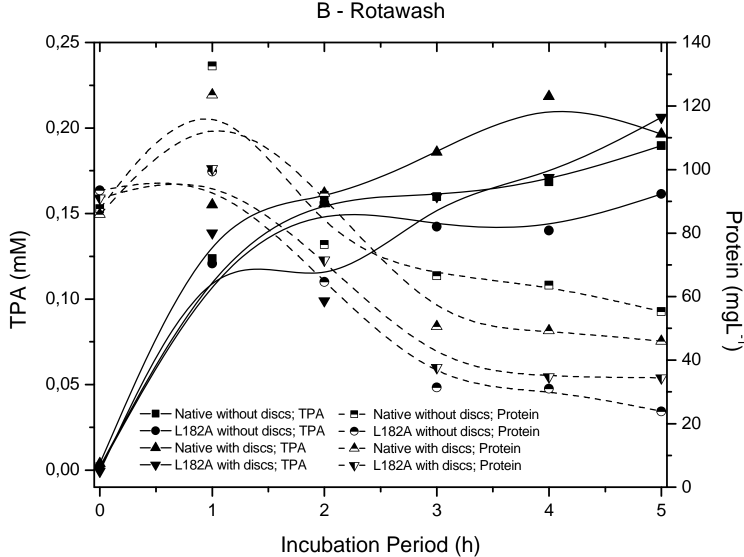


Figure1. Terephthalic acid concentration and remaining protein concentration in the incubation liquor in a shaker bath (A) and a Rotawash machine with and without stainless steel discs (B) for different incubation periods. Initial cutinase dosed is 100 mgprotL^{-1}.

Table 1. Values of percentage of protein adsorbed after 1, 2, 3, 4 and 5 hours of incubation with native and genetically modified cutinases with different modes of mechanical agitation (Shaker bath with 80 rpm and Rotawash machine with 40 rpm) at 35 ºC.

	Protein Adsorbed (%)					
	Native cutinase			**Genetically modified cutinase L182A**		
Incubation Time (h)	Shaker bath (80 rpm)	Rotawash without discs (40 rpm)	Rotawash with discs (40 rpm)	Shaker bath (80 rpm)	Rotawash without discs (40 rpm)	Rotawash with discs (40 rpm)
1	15	--	--	18	--	--
2	8	13	--	15	31	21
3	9	24	41	23	66	59
4	--	28	43	--	67	62
5	24	37	47	34	75	62

In all cases, no desorption was detected after the dilution 1:2 and 1 hour of incubation at 35 ºC. Therefore, this data is not shown.

The highest amount of TPA formed in solution was obtained under higher levels of mechanical agitation used for both enzymes (native and genetically modified cutinase). In case of applying orbital agitation (shaker bath), the concentration of TPA in solution was nearly 2 fold lower.

The higher amount of TPA (hydrolysis reaction product) in the enzymatic treatment solutions with high levels of mechanical agitation (Rotawash) can be due to the mechanical agitation action on the PET fibres, raising the broken ends and creating microfibrils that result in more sites in the fibre for cutinase attack (Cavaco-Paulo, 1998; Morgado and Cavaco-Paulo, 2000). However, no TPA was detected in solution without enzyme with low or high levels of mechanical agitation.

After 5 hours of incubation, almost the same amount of TPA was formed in solution at a lower activity of the genetically modified when compared to the native one (22.1 UL^{-1}

of native cutinase and 12.5 UL^{-1} of L182A). Since the same amount of protein was used at the beginning of the incubation for both enzymes, this result indicate that using less units of activity on pNPP with the genetically modified cutinase, the same activity on PET is obtained. The activity on a soluble substrate is different on an insoluble one as PET fibre, since with only 12.5 UL^{-1} of L182A the same amount of TPA was detected in the enzymatic treatment liquor as for the 22.1UL^{-1} used for the native enzyme.

This behaviour was obtained for both types of mechanical agitation (≈ 0.10 mM – native and L182A in shaker bath and ≈ 0.20 mM – native and L182A in Rotawash). A constant value, corresponding to a saturation level, could be expected if longer incubation periods were used. That specific profile was not observed since only short periods were used in this study.

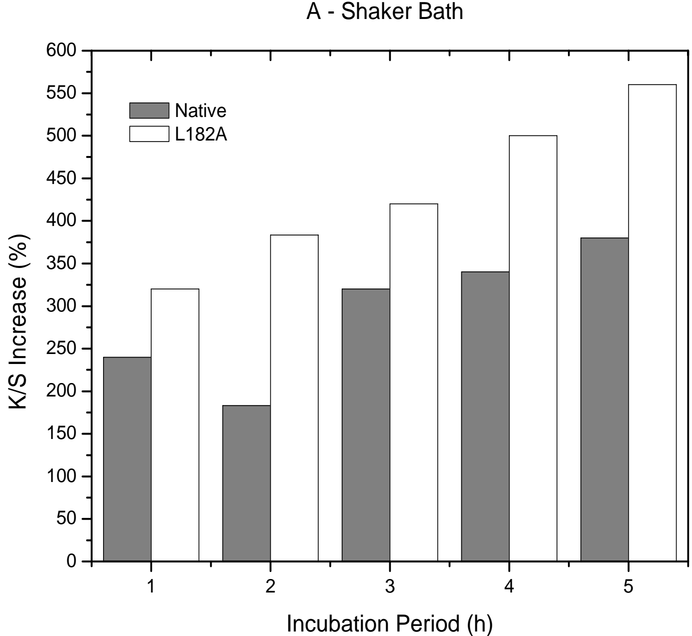
Both enzymes had the highest adsorption when the strongest mechanical agitation was used (Rotawash). At the end of the incubation period, the adsorption levels were 37% for Rotawash without discs, 47% for Rotawash with discs and only 24% for the shaker bath, all for the native cutinase. For the genetically modified cutinase L182A, the difference between the several mechanical agitation levels was even bigger, with 75% for Rotawash without discs, 62% for Rotawash with discs and only 34% for the shaker bath. When the strongest mechanical agitation was used, the maximum adsorption values were obtained with the genetically modified cutinase L182A.

The effect of the presence of the stainless steel discs is not so pronounced in the genetically modified cutinase (L182A) compared to the native enzyme. This result was already expected since the mutant L182A was specially designed for better accommodation of the PET fibre as a substrate. Therefore, the native enzyme seems to be more influenced by the action of agitation. The change of Leu 182 by an Ala resulted in an enlargement of the area around the active site and consequently, better accommodation of the substrate (Araújo *et al.*, 2007). Due to this, the effect of the stainless steel discs is not so pronounced for the genetically modified cutinase (L182A) as for the native. The surface displayed hydrophobic amino acids influence the adsorption behaviour of proteins. In this case, the change was one amino acid (Ala) into another (Leu), which is more hydrophobic. As the primary goal of this change was enlargement of the active site, via change of one amino acid, changes to the adsorption

behaviour were not expected, however, some influence to this was noted as detailed previously.

The vertical agitation using the Rotawash machine was by itself much more effective for hydrolysis (TPA formation) and protein adsorption than the lower agitation level (orbital agitation) for both enzymes (native and genetically modified cutinases). This fact is due to the beating effects of the type of Rotawash agitation (vertical). Also, the abrasion provoked by fibre-metal friction of the stainless steel discs increases the effect of this type of agitation (Silva *et al.*, 2007).

The enzymatic hydrolysis at the surface of the polyester fabric generates not only terephthalic acid (product of the hydrolysis) but also hydroxyl end groups. The hydroxyl end groups can be detected by reaction with cotton reactive dyes and their amount by quantification as K/S. After the enzymatic treatment, control samples (incubated without enzyme in buffer solution) and enzymatically treated samples were dyed with reactive dye (Reactive Black 5) and the K/S increase in percentage relative to control is shown in Figure 2 (A and B).



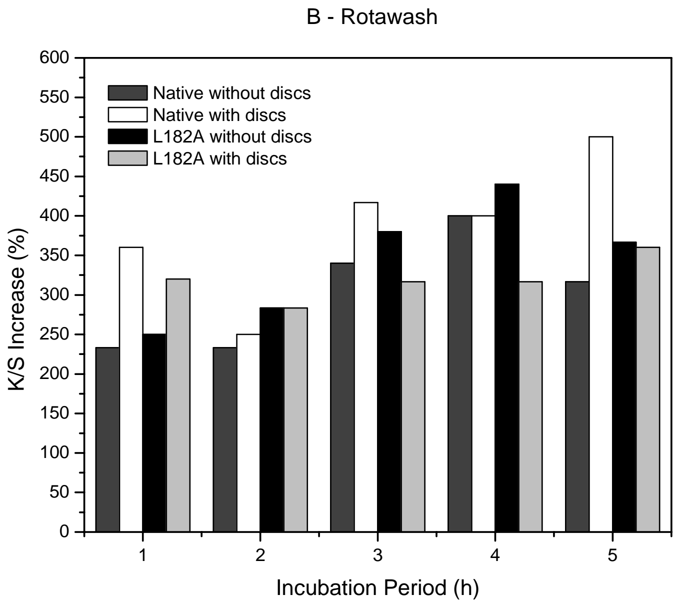


Figure 2. K/S values at 600 nm, in percentage relative to stained control samples, for polyester after staining with reactive black 5 (10% v/v). Fabric samples were treated with 100.0 mgprotL^{-1} of cutinase (native and genetically modiefied L182A) for indicated incubation periods, both in a shaker bath (A) and Rotawash (B).

At lower level of agitation a systematic increase of hydroxyl end groups is verified for both enzymes. On the Rotawash machine, the increase of hydroxyl end groups is inconsistent as whilst the strong mechanical agitation enhances hydrolysis, it also results in the release of soluble and/or insoluble hydroxyl end groups to the bath. No visible debris is formed in the pots during the treatments with the Rotawash and it can be assumed that only soluble oligomers and/or monomers are released to the solution. The higher level of agitation results in greater amount of hydrolysis in terms of TPA in solution. Since PET is produced from polyethylene glycol and TPA, the hydrolysis of ester bonds will generate equal amounts of hydroxyl (OH) and carbonyl (COOH) groups. However, this hydrolysis also generates oligomeric products, either in solution or on the fabric surface. With the highest level of mechanical agitation, these products are removed from the fabric surface and released to the enzymatic treatment solution. Therefore, the highest K/S increase was obtained for the sample treated with the genetically modified cutinase (L182A) at the lowest mechanical agitation (shaker bath).

Additionally, FT-IR spectra were obtained for both control and enzymatically treated samples. However, no significant differences were detected with this method.

4. Conclusions

Mechanical agitation greatly enhances the hydrolysis process of PET fibres, measured as TPA formation. Turnover yields are very low for both cutinases, as no significant changes are verified in fabric appearance. The major advantage of enzyme treatment seems to be the formation of hydroxyl end groups at the surface of the fibres. These groups can be used to attach surface finishing agents to the fibre. The results of our work indicate that surface functionalisation is better done at low levels of mechanical agitation. Our results have important application to the design of an industrial process to functionalize PET with enzymes, where padding processes can be chosen.

Acknowledgements

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Subchapter 2.5

Surface Modification of Cellulose Acetate with Cutinase and Cutinase Fused to Carbohydrate-binding Modules

The work presented in this chapter has been submitted:
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Surface modification of cellulose acetate with cutinase and
cutinase fused to carbohydrate-binding modules.
Biotechnology & Bioengineering (submitted)

Surface Modification of Cellulose Acetate with Cutinase and Cutinase Fused to Carbohydrate-binding Modules

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Abstract

The surface of commercial cellulose diacetate and triacetate fabrics was modified with a cutinase (EC 3.1.1.74). The enzymatic hydrolysis of acetyl groups on the fibre surface was evaluated by the release of acetic acid and by the specific chemical staining of the fabrics with the cotton reactive dye Remazol Brilliant Blue R. The fabric treatment, during 8 hours at 30 ºC and pH 8, led to an acetyl esterase activity of 0.17 nkat and 0.12 nkat on cellulose diacetate and triacetate, respectively. The colour levels for samples treated with cutinase for 24 hours increased 25% for cellulose diacetate and 317% for cellulose triacetate, comparing to the control samples. Cross-sections of both fibres were analysed by fluorescence microscopy and confirmed the superficial action of cutinase. Crystallinity of both fibres was slightly decreased as a result of the enzymatic treatment. For further improvement of cutinase catalysis, fusion cutinases at the C-terminal ends were produced with either the carbohydrate-binding module of Cellobiohydrolase I, from the fungi *Trichoderma reesei*, or the carbohydrate-binding module of Endoglucanase C, from the bacteria *Cellulomonas fimi*. The knew recombinant cutinase fused to the fungal CBM was better than the bacterial one in improving the colour levels of treated fabrics, in particular for cellulose diacetate.

In this work, evidences are provided showing that cutinase is a good candidate for superficial regeneration of cellulose hydrophilicity and reactivity on highly substituted cellulose acetates, although further studies will be necessary to better characterize the nature of the fibre transformations induced by the modular cutinases.

Nomenclature:

C.I. - Colour Index
CBH I - Cellobiohydrolase I of *Trichoderma reesei*
CDA - Cellulose diacetate
CenA - Endoglucanase A of *Cellulomonas fimi*
CenC - Endoglucanase C of *Cellulomonas fimi*
CTA - Cellulose triacetate
DS - degree of substitution
K/S - Kubelka-Munk relationship: K - adsorption coefficient, S - scattering coefficient
o.w.f. - of weight of fabric

1. Introduction

Cellulose is the most abundant natural polymer and it is a valuable renewable resource (Steinmann, 1998; Kamide, 2005). However, it presents major drawbacks in its applicability due to its insolubility in most solvents and its decomposition prior to melting (Braun and Kadia, 2005). Since the end of the 19th century, the attained development on cellulose chemistry allowed new processes and a continuous expansion of the applications of cellulose derivatives, particular the cellulose esters. Cellulose acetate is used as a raw material for plastics, textiles, filter tows, films or membranes (Rustemeyer, 2004). In the textile field, cellulose acetates are characterised by a combination of desirable and unusual properties like soft and silk-like hand, good textile processing performance and higher hydrophilicity than synthetic fibres, thus being more comfortable to use (Steinmann, 1998; Law, 2004). The native cellulose properties of cellulose esters can be partially recovered by chemical hydrolysis of the acyl groups (Braun and Kadia, 2005). The surface hydrolysis is also considered an important tool to improve the surface reactivity and hydrophilic character without radically change the tensile properties of the fibres and films (Braun and Kadia, 2005). Traditional processes used to modify polymer surfaces are based on the addition of strong chemical agents. The major advantages of using enzymes in polymer modification compared to chemicals are milder reaction conditions and easier control. In addition, they are environmental friendly and perform specific non-destructive transformations on polymer surfaces (Guebitz and Cavaco-Paulo, 2003, 2007).

Work on the modification of cellulose acetate with enzymes has been mostly done in the context of its biodegradation (Puls *et al.,* 2004). The degradation of cellulose and hemicellulose is naturally carried out by microorganisms and requires the concerted action of many enzymes. Among these carbohydrate-active enzymes, there is the group of carbohydrate esterases which hydrolyse the ester linkage of polysaccharides substitutents, allowing the exo- and endoglycoside hydrolases to cleave the polymer chains. Cellulose acetate was found to be a carbon source for several microorganisms and a substrate of several acetyl esterases in cell-free systems (Gardner *et al.,* 1994; Samios, 1997; Sakai *et al.,* 1996; Altaner *et al.,* 2003a, 2003b). A negative correlation between the degree of substitution and the biodegradability of cellulose acetates was

identified (Samios, 1997). The deacetylation efficiency of carbohydrate esterases decreases with the increase in the degree of substitution of cellulose acetate and consequently its hydrophobicity.

In the work here reported, the hydrolysis of acetate surface groups of cellulose diacetate (CDA) and cellulose triacetate (CTA) fabrics was investigated using *Fusarium solani pisi* cutinase (E.C. 3.1.1.74). It is an extracellular enzyme able to degrade cutin, the lipid-polyester natural coating of plants, thus conferring phytopathogenicity to the fungus from which it originates. This enzyme is a small ellipsoid protein (~22 KDa, 45x30x30 Å) that belongs to the class of serine esterases and to the superfamily of α/β-hydrolases (Longhi and Cambillau, 1999). The *F. solani pisi* cutinase also belongs to the family 5 of carbohydrate esterases (www.cazy.org/fam/CE5.html), sharing a very similar 3D-structure with other two members with known structure: the acetylxylan esterase (E.C. 3.1.1.72) from *Trichoderma reesei* and the acetylxylan esterase II from *Penicillium purpurogenum* (Hakulinen *et al.,* 2000; Ghosh *et al.,* 2001). Although they present very similar overall structures, the conformation of the active site is different, reflecting the lipid nature of the cutinase substrates (Ghosh *et al.,* 2001). The preference for hydrophobic substrates, as well as the versatility in respect to soluble, insoluble and emulsified substrates makes cutinase an attractive esterase for highly substituted cellulose acetates.

The enzymatic modification of highly substituted cellulose acetate fibres is a heterogeneous process. An attempt was made to increase cutinase efficiency towards this substrate by mimicking other carbohydrate-active enzymes with modular nature. Two different carbohydrate-binding modules (CBMs) were fused to the C-terminal of cutinase. The CBMs act synergistically with the catalytic domains by increasing the effective enzyme concentration at the substrate surface and, for some CBMs, by physical disruption (Linder and Teeri, 1997; Boraston *et al.,* 2005). Two types of CBMs were chosen on the basis of ligand affinity, since the two cellulose acetate fibres used in this work are structurally different from cellulose (the native ligand) and different between themselves, presenting two different overall crystallinities. Type A, the CBM of Cellobiohydrolase I (CBHI) from *T. reesei* belongs to the family CBM1, has preference for crystalline or microcrystalline regions of cellulose while type B, the

CBM of Endoglucanase C (CenC) from *Cellulomonas fimi*, which belongs to the family CBM4, is able to bind amorphous cellulose (Boraston *et al.,* 2005).

To our knowledge, this is the first report of the hydrolysis of surface acetyl groups from CDA and CTA with a cutinase. It constitutes a promising approach for the partial regeneration of cellulose reactivity and hydrophilicity in these fibres, here demonstrated by the enhanced reactive dye uptake of treated fabrics. The production of fusion cutinases with new functionalities is here described and a comparison with cutinase regarding its efficiency for CDA and CTA modification is presented.

2. Materials and methods

2.1. Reagents and enzymes

The cellulose diacetate and triacetate plain woven fabrics used were kindly supplied by Mitsubishi Rayon Co. Ltd., Tokyo, Japan. The CDA fabric has 41/27 ends/picks per cm and 64 g m^{-2}. The CTA fabric has 45/31 ends/picks per cm and 98 g m^{-2}.

All other reagents were laboratory grade reagents from Sigma-Aldrich, St. Louis, USA, unless stated otherwise.

The cutinase (EC 3.1.1.74) from *F. solani pisi* used in this work was expressed and purified as previously reported by Araújo *et al.,* 2007.

Restriction enzymes were purchased from MBI Fermentas (Vilnius, Lithuania) and from Roche Diagnostics GmbH (Penzberg, Germany). Accuzyme^{TM} DNA polymerase was purchased from Bioline GmbH (Luckenwalde, Germany) and recombinant *Taq* DNA polymerase was purchased from MBI Fermentas. T4 DNA ligase was purchased from Roche Diagnostics GmbH (Penzberg, Germany).

2.2. Esterase activity assay

Esterase activity was determined following the product release (p-nitrophenol) continuously through the increase in the absorbance at 400 nm at 30º C. The assay conditions for the determination of cutinase activity were described previously (Matamá *et al.,* 2006). All the assays were performed at least in triplicate. Standard solutions of

p-nitrophenol were used to obtain the calibration curve. One unit of esterase activity was defined as one μmol of p-nitrophenol released per minute.

2.3. Treatment of cellulose di- and triacetate fabric with cutinase

All samples of cellulose acetate fabric were washed prior to use in order to remove possible impurities from manufacture and from human handling. Washing was performed at 35º C and 20 rpm, in stainless steel pots of 450 mL in capacity and housed in a laboratory scale machine, the Rotawash MKIII (vertical agitation simulating European washing machines, from SDL International Ltd.). The fabric was washed twice for 30 min in 40 mg L^{-1} Lutensol AT25 (non-ionic detergent, BASF, Ludwigshafen, Germany), then rinsed four times with distilled water for 30 min each and left to dry at room temperature.
Several sets of experiments were carried out taking into account the amounts of enzyme, fabric and time of incubation. For all experiments, the treatment of cellulose acetate fabric was performed in 50 mM phosphate buffer pH 8 with vertical agitation, in the Rotawash machine operating at 30 ºC and 20 rpm. To evaluate the effect of enzyme concentration, samples of CDA and CTA fabric, with an average weight of 0.1 g, were incubated in duplicate for 8 hours with 0, 25, 50, 75 and 100 U mL^{-1} of cutinase, in a total volume of 5 mL. To obtain a progress curve, samples of CDA and CTA fabric, with an average weight of 0.1 g, were treated with 50 U mL^{-1} of cutinase, in a final volume of 10 mL for different periods of time. For each sample a control was run in parallel in which the buffer substituted the corresponding volume of enzyme. In another treatment, the average weight of both type of fabric was increased to 0.5 g and the incubation extended to 24 hours. The initial activity of cutinase was 25 U mL^{-1} in a final volume of 25 mL. For each sample a control was run in parallel without the enzyme.
After enzymatic treatment, all fabric samples were washed at 35 ºC, in the Rotawash machine, to remove the adsorbed protein, according to the order: 250 mgL^{-1} Lutensol AT25 for 30 min, 70% ethanol for 20 min, 15% isopropanol for 15 min, three steps of increasing concentrations of NaCl for 10 min each, three steps in distilled water for

20 min each. Between the detergent/alcohol and alcohol/salt steps the fabric was rinsed under running cold tap water for 5 min.

2.4. Determination of acetic acid concentration in the treatment solutions

Detection of acetic acid in the reaction media was performed with the acetic acid UV test from Roche (Darmstadt, Germany). Protein was previously precipitated using perchloric acid according to the manufacture instructions. The samples pH was neutralized using 1 M potassium hydroxide and the subsequent salts were removed by centrifugation.

2.5. Quantification of total protein concentration

Total protein in solution was quantified following Bradford methodology (Bradford, 1976) using BSA as standard. All samples were measured at least in duplicate.

2.6. Cellulose acetate fabric staining with a reactive dye

After enzymatic treatment, samples were competitively stained in 50 mM phosphate buffer pH 8 with 2% o.w.f. (of weight of fabric) Remazol Brilliant Blue R, C.I. 61200, in duplicate. The staining was performed at 50º C or 60º C, for 90 min at 20 rpm, in sealed stainless steel beakers of 140 mL in capacity and housed on a lab-scale dyeing machine (AHIBA Spectradye, from Datacolor International).

After staining, all samples were washed once with 0.25 g L^{-1} Lutensol AT25 and several times with distilled water in Rotawash, until no more dye could be detected in the solution. The washing temperature was 5º C higher than the staining temperature.

The colour measurements (5 for each sample) were carried out with a reflectance spectrophotometer having a standard illuminant D65 (Spectraflash 600 Plus, from Datacolor International). The colour strength was evaluated as K/S at the maximum absorption wavelength (660 nm) which is proportional to the dye concentration in the samples. The ratio between absorption (K) and scattering (S) is related to reflectance (R) data by applying Kubelka-Munk's law at each wavelength (Kuehni, 1997).

2.7. Fluorescein Isothiocyanate (FITC) labelling

Enzymes were incubated with FITC (33:1 w/w) in 0.5 M sodium carbonate buffer pH 9.5, for one hour at room temperature. The unconjugated FITC was removed with HiTrap Desalting 5 mL columns (GE Healthcare Bio-Sciences Europe GmbH, Munich, Germany) while the carbonate buffer was exchanged by the 50 mM phosphate buffer pH 8.

2.8. Fluorescence microscopy

Thin strips of CDA and CTA fabric samples were embedded in an epoxy resin (Epofix kit, Struers, Copenhagen, Denmark) and cross sections were cut with 20-25 µm thickness. The samples were observed under a Leica Microsystems DM5000 B epifluorescence microscope equipped with a 100 W Hg lamp and an appropriate filter setting. Digital images were acquired with Leica DFC350 FX digital Camera and Leica Microsystems LAS AF software, version 2.0 (Leica Microsystems GmbH, Wetzlar, Germany)

2.9. Fourier Transformed Infrared Spectroscopy

The diffuse reflectance (DRIFT) technique was used to collect the infrared spectra of CDA and CTA fabric samples treated during 24 hours with cutinase and respective controls. The spectra were recorded in a Michelson FT-IR spectrometer MB100 (Bomem, Inc., Quebec, Canada) with a DRIFT accessory. The fabric pieces were placed and hold on top of the sample cup, previously filled with potassium bromide powder that was used to collect the background. All the spectra were obtained under a nitrogen atmosphere in the range 4000 - 800 cm^{-1} at 8 cm^{-1} resolution and as the ratio of 32 scans to the same number of background scans. The spectra were acquired in Kubelka-Munk units and baseline corrections were made using Bomem Grams/32R software, version 4.04.

2.10. Wide Angle X-ray Scattering

The X-ray diffraction patterns were obtained for the CDA and CTA fabric samples treated during 24 hours with cutinase and respective controls. The X-ray diffraction experiments were undertaken in a Philips PW1710 apparatus, using Cu Kα radiation and operating at a 40 KV voltage and 30 mA current. The patterns were continuously recorded in the diffraction angular range 2θ from 4º to 40º, with a step size of 0.02º at 0.6ºmin^{-1}. The non linear fitting of the diffraction patterns was performed using the Pseudo-Voigt peak function from OriginPro 7.5 (Origin Lab Corporation, USA) considering the cellulose acetate structural polymorphism II (Cerqueira *et al.,* 2006). The crystallinity index was determined according to the equation (1)

$$I_C = A_c / (A_c + A_a) \qquad (1)$$

A_c is the total area of the crystalline peaks and A_a is the total area of the amorphous peaks. The peaks that were considered crystalline were at the diffraction angles 2º 11º and 17º, for CDA, and 8º, 10º, 13º, 17º, 21º and 23º, for CTA (Chen *et al.,* 2002; Hindeleh and Johnson, 1972).

2.11. Cloning and expression of cutinase fused to carbohydrate-binding modules

2.11.1. Bacteria, plasmids and genes

The bacterial hosts used for cloning and expression of cutinase fusion genes were the *Escherichia coli* strain XL1-Blue and strain BL21 (DE3), respectively. The plasmid pGEM®-T Easy (Promega Corporation, Madison, USA) was used to clone and sequence the PCR products. The plasmid pCWT (pET25b(+) carrying native cutinase gene from *F. solani pisi*, (Araújo *et al.,* 2007) was used to insert the genes coding for the CBMs at the 3' end of the cutinase gene and to express the fusion proteins.

The DNA coding the wild type linker and wild type CBM of *T. reesei* CBH I, $wtCBM_{CBHI}$, was synthesized and purchased from Epoch Biolabs (Missouri City, USA), as well as, the DNA fragment coding for a smaller linker and the wild type CBM,

$sCBM_{CBHI}$. The plasmid pTugN1 containing the gene of CBM_{N1} from *C. fimi* CenC was kindly provided by Professor Anthony Warren (Department of Microbiology, University of British Columbia, Vancouver, Canada) (Johnson *et al.,* 1996).

2.11.2. Plasmid construction

Standard techniques were used for all the DNA manipulations. The $wtCBM_{CBHI}$ and $sCBM_{CBHI}$ were amplified by PCR, using the primers supplied by Epoch Biolabs, and cloned directly into pGEM®-T Easy. Transformants were selected and the gene sequences were confirmed by DNA sequencing, following the method of Sanger (Sanger *et al.,* 1977). The constructs pGEM::$wtCBM_{CBHI}$ and pGEM::$sCBM_{CBHI}$ were digested with *Sac*I and *Sal*I, the DNA fragments were extracted and purified from agarose gels and cloned into the *Sac*I/*Sal*I restricted and dephosphorilated pCWT, resulting in the final pCWT::$wtCBM_{CBHI}$ and pCWT::$sCBM_{CBHI}$ vectors. The CBM_{N1} sequence was PCR-amplified from pTug, with the primers CBM N1 for (5'-ATAAGAAT**GCGGCCGC**TAGCCCGATCGGGGAGGGAACGT) and CBM N1 rev (5'-ACCG**CTCGAG**CTCGACCTCGGAGTCGAGCGC) containing the *Not*I and *Xho*I sites (in bold). The PCR product was cloned into pGEM®-T Easy and a positive clone was selected and confirmed by DNA sequencing. The construct pGEM::CBM_{N1} was restricted with *Not*I and *Xho*I, the DNA fragment was extracted and purified from agarose gel and cloned into the *Not*I/*Xho*I restricted and dephosphorilated pCWT, resulting in the final pCWT::CBM_{N1} construct. The DNA coding the linker PT_{box} of *C. fimi* CenA (Shen *et al.,* 1991) was obtained by PCR amplification of two overlapping primers (underlined sequence) containing the *Sal*I and *Not*I sites (in bold): PTbox for (5'CTCGAGCTCA**GTCGAC**CCGACGCCAACCCCGACGCCTA<u>CAACTCCGACT CCGACGCCGACCCCGACTC</u>) and PTbox rev (5'GAGGGACTGCGTC**GCGGCCGC**GGTAGGGGTCGGTGTTG<u>GAGTCGGGGT CGGCGTCGGAGTCGGAGTTG</u>). The PCR amplification consisted in 30 cycles of 20 s at 94 ºC and 20 s at 72 ºC for Accuzyme extension. The PCR product was cloned into pGEM®-T Easy and a positive clone was selected and confirmed by DNA sequencing. The plasmid pGEM::PT_{box} was restricted with *Sal*I and *Not*I, the DNA fragment was

extracted and purified from agarose and cloned into the *Sal*I/*Not*I restricted and dephosphorilated pCWT::CBM_{N1}, resulting in the final pCWT::PT_{box}::CBM_{N1} vector.

2.11.3. Expression and purification of cutinase fusion proteins

The constructs pCWT::wtCBM_{CBHI}, pCWT::sCBM_{CBHI}, pCWT::CBM_{N1} and pCWT::PT_{box}::CBM_{N1} were first established in *E. coli* strain XL1-Blue. Medium-scale purifications of plasmid DNA were made and used to transform the *E. coli* strain BL21(DE3). Clones harbouring the constructs were grown, at 15º C and 200 rpm, in 2.5 L Luria-Broth medium supplemented with 100 µg mL^{-1} ampicillin until an absorbance $A_{600\ nm}$ of 0.3-0.5 was reached. Cells were induced with 0.7 mM isopropyl-1-thio-β-D-galactopyranoside, and further incubated for 16 hours at 15º C. The cells were harvested by centrifugation at 4º C (7500 x*g*, 10 min), washed with PBS pH 7.4 and frozen at -80º C. The ultrasonic disruption of the bacterial cells was accomplished on ice with a 25.4 mm probe in an Ultrasonic Processor VCX-400 watt (Cole-Parmer Instrument Company, Illinois, USA). The lysate was centrifuged for 30 min at 16000 x*g* and 4 ºC. The supernatant was collected, pH was adjusted to 7.6 and imidazole was added to a final concentration of 25 mM. Protein purification was performed with the affinity chromatography system HiTrap Chelating HP (GE Healthcare Bio-Sciences Europe GmbH, Munich, Germany) coupled to a peristaltic pump. The 5 mL column was loaded with 100 mM Ni^{2+} and equilibrated with the binding buffer (20 mM phosphate buffer pH 7.6, 500 mM NaCl, 25 mM imidazole). The samples were loaded and washed with 10 column volumes of binding buffer followed by buffers with 50 and 100 mM imidazole. The fusion proteins (figure. 1) were eluted with 550 mM imidazole buffer.
The fractions obtained were monitored by SDS-PAGE with Coomassie Brilliant Blue staining. The elution buffer was changed to 50 mM phosphate buffer pH 8 with HiTrap Desalting 5 mL columns (GE Healthcare Bio-Sciences Europe GmbH, Munich, Germany). Prior to the 2.5 L culture scale up, Western blotting was performed with monoclonal Anti-polyHistidine-Peroxidase Conjugate from mouse to confirm the expression of the fusion proteins. The detection was made with ECL Western blotting

reagents and analysis system (Amersham Biosciences Europe GmbH, Freiburg, Germany).

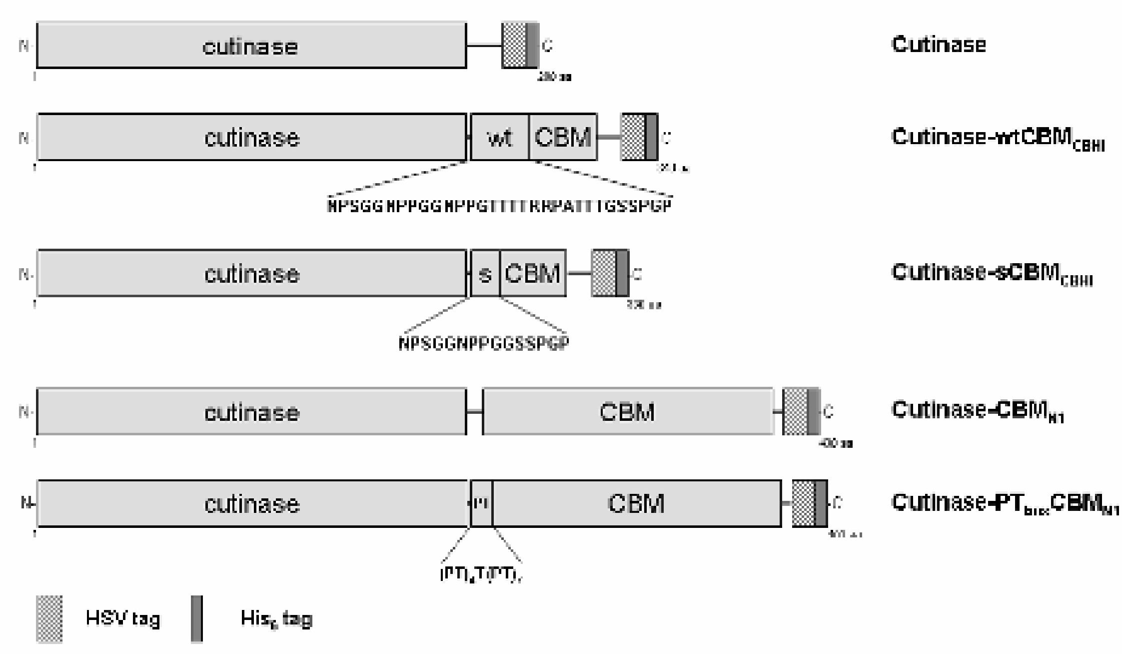

Figure 1. Schematic representation of the recombinant wild-type cutinase from *F. solani pisi* (Araújo *et al.*, 2007) and its new fusion proteins with the fungal carbohydrate-binding module of CBH I, from *T. reesei*, and the bacterial carbohydrate-binding module N1 of CenC, from *C. fimi*. The primary sequences of the linkers used are specified in the figure.

2.12. Treatment of cellulose di- and triacetate fabric with cutinase fused to carbohydrate-binding modules

All samples of cellulose acetate fabric used were previously washed as already described in 2.3.

Cellulose acetate fabric samples with an average weight of 0.1 g were incubated with 100 U mL^{-1} of cutinase and cutinase-CBM_{N1}, 50 U mL^{-1} of cutinase-$PT_{box}CBM_{N1}$ and cutinase-$wtCBM_{T.reesei}$, 25 U mL^{-1} of cutinase-$sCBM_{T.reesei}$ in 10 mL of 50 mM phosphate buffer pH 8 with 0.01% sodium azide, under continuous vertical agitation at 30º C and 20 rpm, for 18 hours. A control for both types of fabric was run in parallel in

which the buffer substituted the enzyme. After enzymatic treatment, all fabric samples were washed according to the procedure described earlier (see 2.3).

3. Results and discussion

3.1. Effect of cutinase concentration on the modification of cellulose di- and triacetate

The media conditions, such as buffer, pH and temperature, were chosen based on earlier studies performed in our laboratory (Matamá *et al.,* 2006), using the esterase activity determination methodology described earlier. The conditions chosen were phosphate buffer pH 8 and the lowest optimum temperature 30º C. The hydrolysis of the acetate groups in cellulose ester substrates leads to the formation of hydroxyl groups at the fibres surface and to the release of acetic acid to the treatment solution. The effect of cutinase concentration was analysed by measuring the acetic acid in the treatment solutions, after an incubation period of 8 hours (figure. 2).

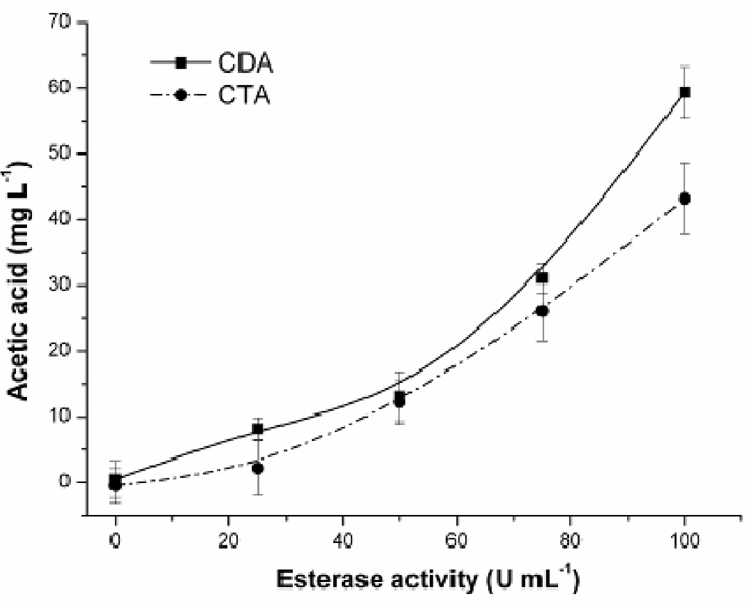

Figure 2. Effect of cutinase concentration on the acetic acid release. The CDA and CTA fabrics (2% w/v) were treated during 8 hours, at pH 8 and 30º C, with several concentrations of cutinase expressed as esterase activity in U mL^{-1} (see 2.2).

The acetic acid release was not directly proportional to all the tested enzyme concentrations as it would be expected (Tipton, 2002; Lee and Fan, 1982). The observed upward curvature could be caused by several factors like inadequate sensitivity of the method used to quantify the acetic acid and/or, most probably, by a very slow enzyme reaction. The higher level of released acetic acid from the less substituted cellulose acetate was according to the irreversible relation between the degree of substitution and the degree of bio-deacetylation (Samios *et al.,* 1997; Altaner *et al.,* 2001, 2003b; Moriyoshi *et al.,* 1999, 2002). Steric hindrance and crystallinity are considered important factors in the adsorption and mostly in the effectiveness of the adsorbed enzyme to promote the hydrolysis (Lee and Fan, 1982). These factors should favour CDA over CTA. At the maximum concentration used, the enzyme activity was 0.17 nkat and 0.12 nkat (nmol/s of acetic acid) while only 0.54% and 0.36% of the acetyl groups were released from CDA and CTA, respectively. These values were obtained considering a DS 2.4 for CDA and a minimum DS 2.7 for CTA commercial fibres (Steinmann, 1998; Zugenmaier, 2004). A very low yield in deacetylation is not uncommon for highly substituted cellulose acetates treated with cell-free enzymes (Puls *et al.,* 2004). By comparison, in view of the fact that at least one of the cellulose acetate used has higher DS, cutinase showed potential as cellulose acetate esterase. Altaner *et al* (2001) reported that acetylesterases from 13 different commercial origins could significantly use cellulose acetates with DS ≤1.4 as substrates. Only one enzyme from *Humicola insolens* was able to release a small amount (10%) of acetyl groups from a cellulose acetate DS 1.8, after 220 hours. Another enzyme purified from a commercial preparation, derived from *Aspergillus niger*, was able to hydrolyse 5% of the existing acetyl groups on a cellulose acetate DS 1.8 after 140 hours (Altaner *et al.,* 2003b). Considering the values found in the literature, the percentage of acetic acid released obtained with cutinase was not insignificant having in consideration that the final purpose of this modification is not biodegradation of the substrate, but the modification of the fibre surface. The amount of cutinase was a limiting factor, therefore amounts of enzyme used in subsequent treatments were ≤50 U mL^{-1}.

3.2. Progress curves for the modification of cellulose di- and triacetate fabrics and protein adsorption

Samples of CDA and CTA fabric (1% w/v) were treated with 50 U mL^{-1} of cutinase for different periods of incubation. The action of cutinase was evaluated by indirectly measuring the hydroxyl groups formed at the fibres. Since the cellulose acetates used in this work were insoluble, the enzyme adsorption to the substrate was a prerequisite for the formation of the enzyme-substrate complex. The protein adsorption was indirectly calculated by the decrease in total protein remaining in the treatment solution. For CDA (figure. 3A), an equilibrium level of relative protein adsorption of 45% (3 mg g^{-1} of protein per fabric weight) was reached. For CTA (figure.3B), the equilibrium level of protein adsorption was higher, with 57% of relative protein adsorption (3.5 mg g^{-1}). The hydrophobic character of the substrate should not be a problem for cutinase adsorption since this enzyme is a lipolytic enzyme and its natural substrate, cutin, is hydrophobic (Egmond and Vlieg, 2000; Mannesse *et al.,* 1995; Kolattukudy, 2004).

The formation of hydroxyl groups at the fibre surface was evaluated by staining the fabric with a cotton reactive dye. The basic principle is the specific reaction between a vinylsulphonic group from a reactive dye, in this case Remazol Brilliant Blue R, and the hydroxyl group at the fibre surface. The sensitivity is high due to the large molar absortivities of dye molecules. If the cutinase is able to hydrolyse some of the acetyl groups at the surface, then more dye can be chemically linked to the fibre, resulting in an increase in K/S. The staining 'titration' methodology was already reported (Silva *et al.,* 2005; Matamá *et al.,* 2006, 2007; O'Neill *et al.,* 2007) and proved to be a valuable and a very sensitive qualitative method.

In the case of CDA fibre, the sensitivity is not as good as for the CTA fibre. The dye has more affinity for diacetate and, as a result, the controls are very coloured while the triacetate controls are very faint. This is the reason for the observed differences between the two fibres in the relative increase in the colour strength values (figure 3).

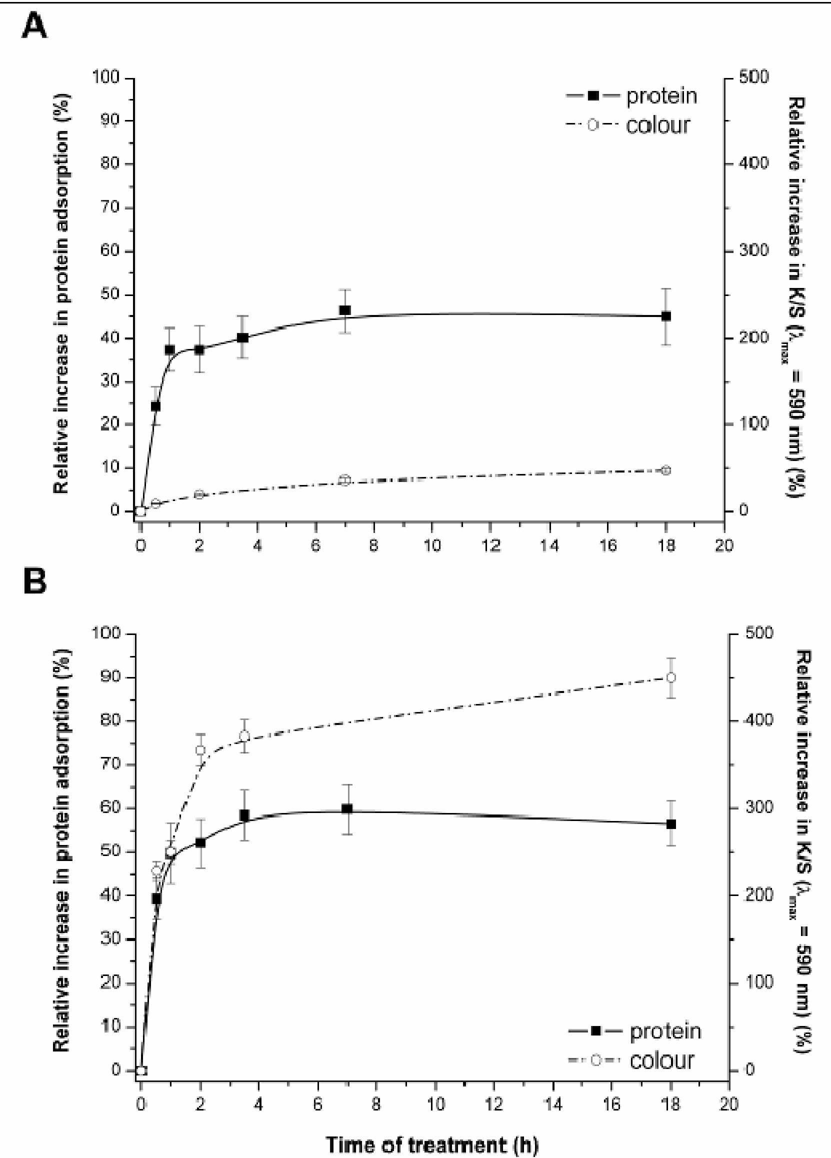

Figure 3. Progress curves for the formation of hydroxyl groups at the surface of fibres, measured as relative increase in K/S values, and protein adsorption for the (**A**) CDA and (**B**) CTA. All the samples (1% w/v) were treated with 50 U mL^{-1} of cutinase, at pH 8 and 30º C. The controls were treated under the same conditions except for the enzyme. Samples and controls were competitively dyed at 60º C. The relative increase in K/S is calculated as $(K/S_{enzyme} - K/S_{control}) / K/S_{control}$ (%) and the relative protein adsorption as $(P_{0h} - P_t) / P_{0h}$ (%), where *P* is the total protein in solution.

After 18 hours, the relative difference in colour strength between treated samples and controls was around 50% for CDA and 450% for CTA. For both fabrics, the relative K/S increased rapidly in the first hours of treatment and slowed down as the protein adsorption equilibrium was being settled. In the particular case of these modifications, a very slow enzymatic reaction occurs. We believe that the fast initial increases in colour are an artefact created by an incomplete protein removal during the washing procedure at the end of each treatment. It seems that the dye is also able to react with hydroxyl groups present in the protein molecules not removed from the fabric. If this argument is correct the actual relative K/S increase is bellow the observed values.

3.3. Surface modification of cellulose di- and triacetate fabrics with cutinase

Samples of CDA and CTA fabric (2% w/v) were treated with 25 U mL^{-1} of cutinase for 24 hours. Table I shows the values of increase in colour strength and acetic acid liberated to the reaction medium for both cellulose acetates (the control values were subtracted).

Table I. Hydrolysis of CDA and CTA fabrics by cutinase. The parameters evaluated were the amount of hydroxyl groups at the fibre surface, measured as relative increase of K/S values at 590 nm, and the acetic acid release to the liquor. The samples (2% w/v) were treated with 25 U mL^{-1} of cutinase, at pH 8 and 30º C, for 24 hours. The controls were subjected to the same conditions except the for enzyme. Samples and controls were competitively dyed at 50º C.

	CDA	CTA
K/S_{590nm} (%)	25 ± 9	317 ± 32
Acetic acid (mg L^{-1})	1.9 ± 0.2	Nd

nd – non detected

Evidence of hydrolysis was obtained by the increase in K/S for both fabrics and by the formation of acetic acid for CDA. It was not possible to detect this product in the

treatment medium of CTA. Compared to previous results, the levels of acetic acid are lower than the observed for the same esterase activity per weight of the substrate.
The diffuse reflectance (DRIFT) technique was used to collect the infrared spectra of CDA and CTA fabric samples and respective controls in order to obtain further evidence of the hydrolysis of the ester linkage at the surface of treated fibres. This technique allows examining the IR absorption by rough surfaces. Figure 4 shows the IR spectra in the region of 1800-1720 cm^{-1} which is the wavenumber region for the stretching vibration of the carbonyl group (Krasovskii *et al.,* 1996).
For CTA (figure. 4B) there was a clear difference in both the intensity and shape of the carbonyl stretching band between the treated sample and the control. There was a decrease in the intensity after the cutinase treatment and there was also a shift of the band to lower wavenumbers. The decrease in the intensity was correlated to the enzymatic hydrolysis of some ester linkages at the surface of the samples. The displacement could be caused by the formation of intermolecular hydrogen bridges between the remaining carbonyl groups and the newly formed hydroxyl groups (Ilharco and Barros, 2000) or it could be due to a preferential hydrolysis of the carbonyl groups at C_2 e C_3 positions (Krasovskii *et al.,* 1996). Regarding CDA (figure. 4A), the observed differences between the control and sample were considered not significant. The absence of a significant difference was unexpected because in the treatment liquor it was possible to detect acetic acid while for CTA it was not.

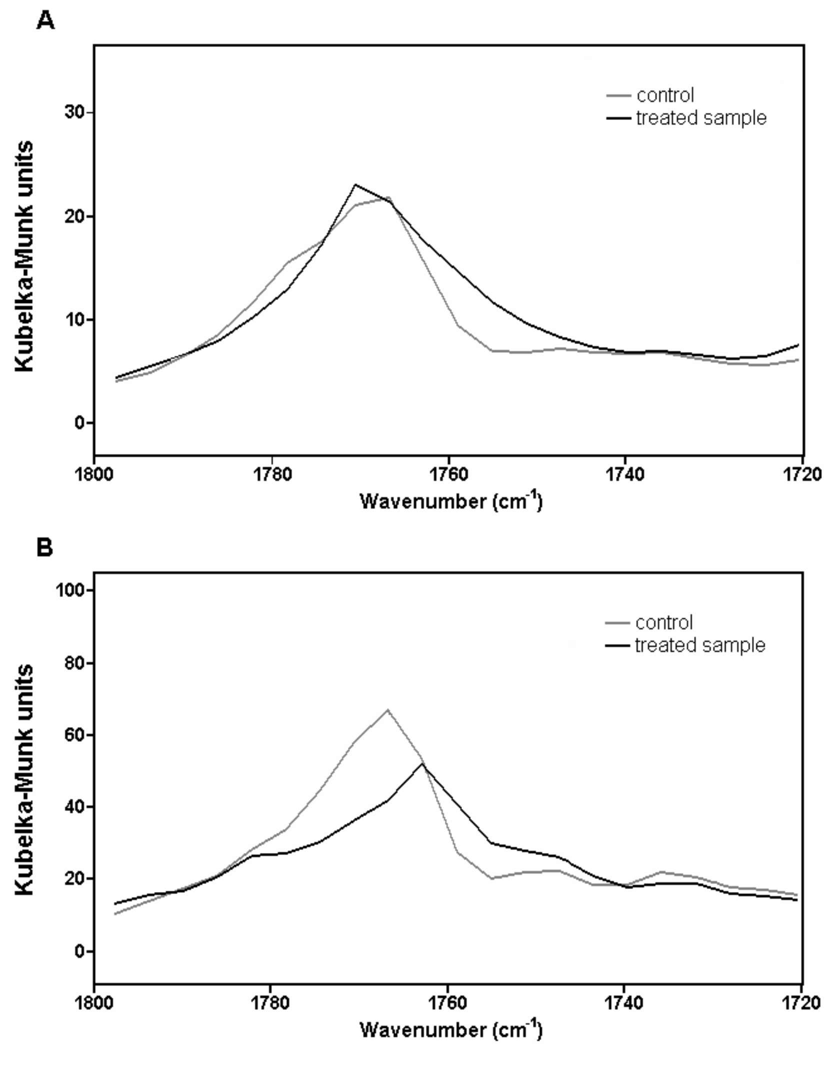

Figure 4. DRIFT spectra showing the carbonyl group stretching band of (**A**) CDA and (**B**) CDT controls and samples. The samples (2% w/v) were treated with 25 U mL^{-1} of cutinase, at pH 8 and 30º C, for 24 hours. The controls were treated under the same conditions but without enzyme.

After the fabric samples and controls were competitively stained, cross sections of fibres were made and observed by Fluorescence microscopy to assess the diffusion of the dye inside the fibre and indirectly to confirm the surface action of cutinase.

The inherent affinity of Remazol Reactive Blue R for CDA did not allow any significant difference in the dye distribution in the fibres (results not showed). The

differences between controls and treated samples were more evident for CTA (figure. 5).

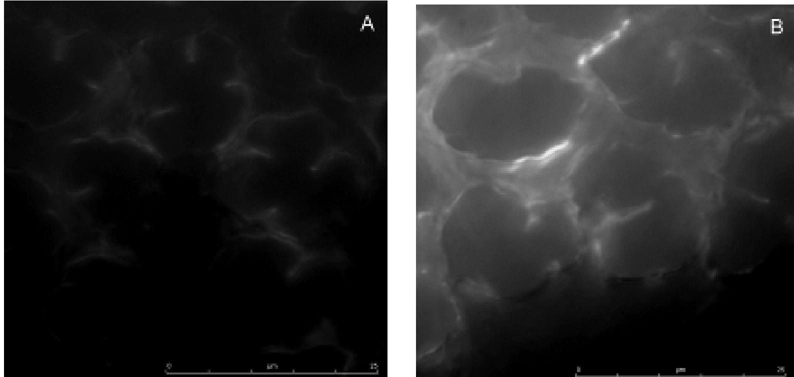

Figure 5. Epifluorescent photographs of cross-sections from (**A**) control and (**B**) treated sample of CTA, competitively stained with Remazol Reactive Blue R, C.I. 61200. The samples (2% w/v) were previously treated with 25 U mL^{-1} of cutinase, at pH 8 and 30º C, for 24 hours. The controls were subjected to the same conditions except for the enzyme. Both images were acquired under the same conditions with a total magnification of 1000x.

The hydroxyl groups appeared to be located mainly on the fibre surface where the dye was chemically fixed, which could be attributed to a superficial action of cutinase. Therefore, cross sections of fibres treated with cutinase conjugated with FITC were also observed by Fluorescence microscopy (figure. 6).

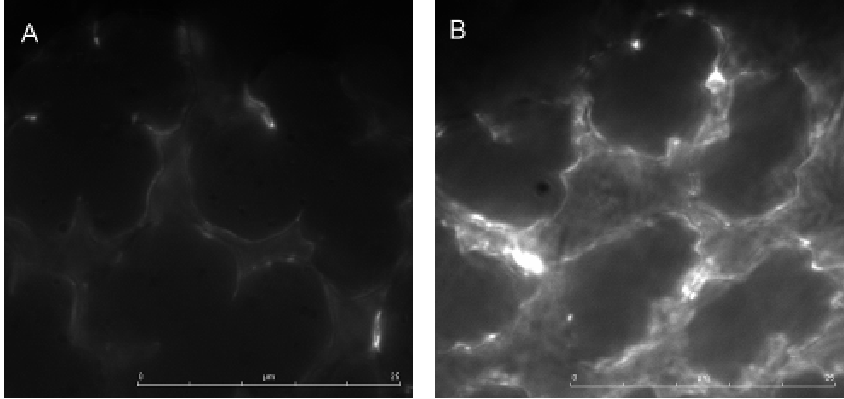

Figure 6. Epifluorescent photographs of **c**ross-sections from (**A**) CDA and (**B**) CTA samples. The samples (2% w/v) were treated with 10 mg g^{-1} of FITC-conjugated

cutinase per fabric weight, at pH 8 and 30º C, for 15 hours. Both images were acquired with a total magnification of 1000x.

The protein was found at the fibre surface of both fabrics and it was also possible to see some lack of uniformity as for the reactive dye staining. Scanning electronic microscopy images were also obtained for both fabrics treated for 18 hours with 50 U mL^{-1} cutinase (results not shown). The surface of CDA was not apparently altered by the enzymatic treatment while a slight fibrillation of the triacetate surface was visible after the cutinase treatment. The impact of the hydrolysis of acetyl groups should be more drastic on the highly ordered structure of CTA than on the more disordered CDA. From the mathematical fitting of X-ray diffraction patterns, crystallinity indexes were determined for CDA and CTA, samples and respective controls (table II). There was a small decrease in the crystallinity index after the enzymatic treatment, for both fibres. CTA was most affected, with a decrease of 12% while CDA had a decrease of 8%.

Table II. Crystallinity indexes for CDA and CTA. The samples (2% w/v) were treated with 25 U mL^{-1} of cutinase, at pH 8 and 30º C, for 24 hours.

	CDA	CTA
Control	0.38	0.68
treated sample	0.35	0.60

Cutinase was able to modify the surface of the cellulose acetate fabrics, increasing the number of hydroxyl groups and consequently the hydrophilic character and the dye affinity. Since there were changes on the crystallinity index, other physical properties should be tested for a better evaluation of the impact of such surface modifications on the textile performance of these fibres.

3.4. Cellulose di- and triacetate treatment with cutinase fused to cellulose-binding modules

For further improvement of cutinase catalysis, several fusion proteins with known and well characterized CBMs were produced. The inclusion of spacers between the cutinase and the CBMs was performed in three of the fusion proteins. The importance of these

spacers was studied by several authors mainly through deletion studies. It was demonstrated that linker peptides, connecting the catalytic domains of carbohydrate-active enzymes and the CBMs, are necessary for the synergistic activity between the two domains (Srisodsuk *et al.,* 1993; Shen *et al.,* 1991). The wild-type linker of CBHI was included in the fusion protein with the CBM from the same enzyme. A smaller linker was also used to connect cutinase to the fungal CBM (figure. 1). The initial purpose was to increase the levels of expression in *E. coli* of the soluble cutinase fused to CBM_{CBHI}, by removing from the wild-type linker a sequence of residues that constitute possible sites for O-glycosylation. Since *E. coli* does not possess the machinery necessary for this post-translation eukaryotic modification, removing those residues could promote correct folding of the fusion protein. The expression levels were very low for soluble cutinase-$wtCBM_{CBHI}$ and were not significantly improved in the case of cutinase-$sCBM_{CBHI}$. The bacterial linker used was the proline-threonine box $(PT)_4T(PT)_7$ present on the CenA from *C. fimi* (Shen *et al.,* 1991). This type of PT linker is also naturally glycosylated, but when it is not, the conformations of catalytic domain and CBM are preserved, since only a partial increase in the linker flexibility seems to occur (Poon *et al.,* 2007).

In the treatment of CDA and CTA with cutinase and its fusion proteins, it was not possible to detect acetic acid as previously. For longer treatments, the quantification of acetic acid was somehow impaired. The reasons could be some volatility, microbiological contamination (in spite of the sodium azide) and/or different efficiencies of cutinase from different batches.

Protein quantification after treatment with cutinase-CBM_{N1} and cutinase-$PT_{box}CBM_{N1}$ was unviable due to the turbidity of solutions. This turbidity happened only for the referred assays, where protein adsorption might be underestimated. The turbidity could be precipitated protein or due to non-hydrolytic disruption of cellulose acetate fibres, in particular, of CDA for which this phenomenon was most visible. This mechanical disruption was already described for cellulose and cotton in the presence of CenA, Cex and isolated CBMs (Din *et al.,* 1991 and 1994, Cavaco-Paulo *et al.,* 1999). Comparing the amount of protein adsorbed and relative K/S between chimeric proteins and cutinase, there was a clear difference between the two cellulose acetates studied (figure.

7). The fusion of cutinase to the CBMs had a more pronounced effect for the less substituted acetate, independently of the CBM type. The steric constrains should be stronger in the triacetate fibre and consequently the interactions necessary for the ligand recognition by the CBM should be more impaired on this fibre surface regarding the diacetate fibre. Due to the fact that different initial amounts of protein were used, is not possible to compare directly the protein adsorption behaviour of the several constructs. But it is possible to see for this particular treatment that there was no obvious relation between the colour differences and the amount of protein adsorbed.

Taking in account the different esterase activities used, the cutinase-wtCBM$_{CBHI}$ and cutinase-sCBM$_{CBHI}$ seem the most efficient catalysts under the treatment conditions used. For CDA, the relative K/S was improved 3.8 and 2.6 fold by cutinase-wtCBM$_{CBHI}$ and cutinase-sCBM$_{CBHI}$, respectively, regarding cutinase alone. For treated CTA, the relative increase in K/S was not different between cutinase alone and fused to the fungal CBMs, but the initial esterase activity of cutinase was higher (figure. 7). The differences in relative K/S were also improved with the fusion of the bacterial CBM to cutinase. For CDA, cutinase-CBM$_{N1}$ improved the relative K/S by 1.8 fold, the same as cutinase-PT$_{box}$CBM$_{N1}$.

The treatment was performed at pH 8. The optimum pH for binding of most CBMs corresponds to the optimum pH for the catalytic domain of the respective carbohydrate-active enzyme and it is in the range of acidic to neutral. The better performance on cellulose acetate fibres of the fungal CBM could be explained by the affinity of CBM$_{CBHI}$ to insoluble ligands being relatively more insensitive to pH than the affinity of CBM$_{N1}$ (Tomme *et al.,* 1996). In fact, lowering the treatment pH to neutral increased the adsorption of cutinase-CBM$_{N1}$ to CDA (results not shown). Other reason could be the difference in size of both CBMs. The activity of cutinase could be more constrained by the bigger bacterial CBM than by the smaller fungal CBM. Indeed, using half the esterase activity in the treatment with cutinase-PT$_{box}$CBM$_{N1}$, the increase in K/S obtained was in the same range of that with cutinase-CBM$_{N1}$, for both fabrics (figure. 7).

Further studies, aiming at a better characterization of the action of chimeric cutinases on the surface modification of cellulose acetates, would contribute to clarify these issues.

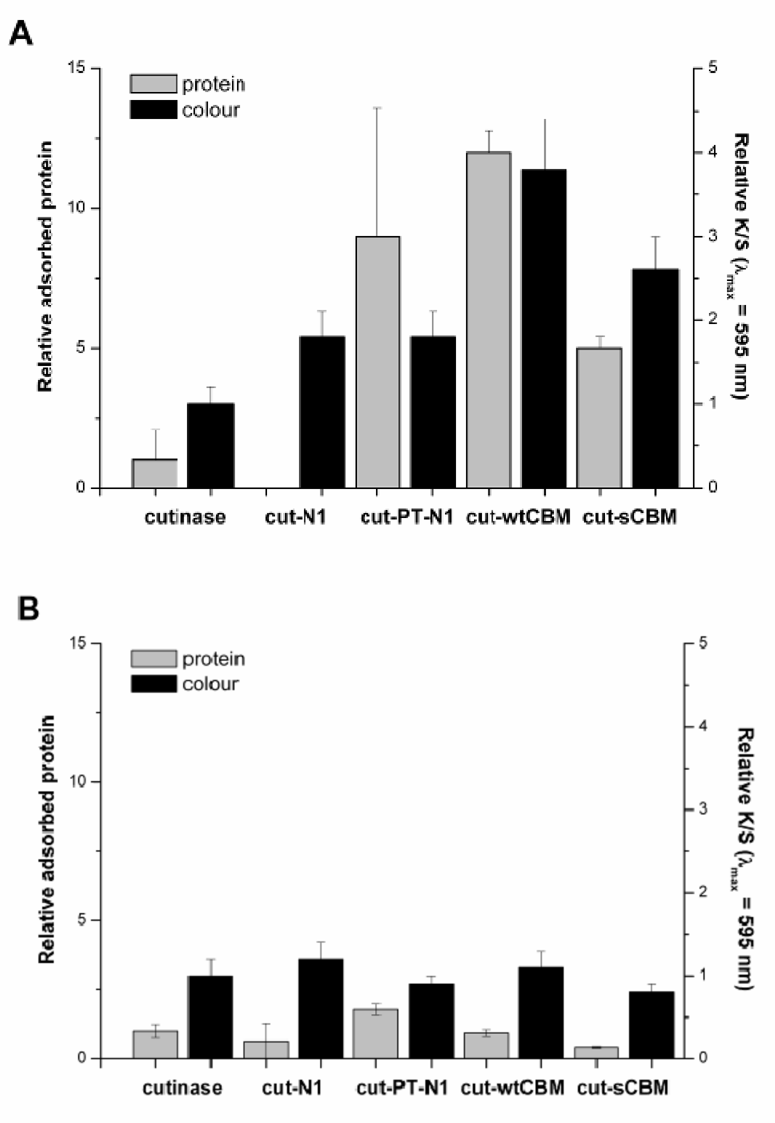

Figure 7. Protein adsorption and relative increase in K/S values for the (**A**) CDA and (**B**) CTA treated with cutinase and cutinase fused to CBMs. All the samples (1% w/v) were incubated during 18 hours with 100 U mL^{-1} of cutinase and cutinase-CBM_{N1} (cut-N1), 50 U mL^{-1} of cutinase-$PT_{box}CBM_{N1}$ (cut-PT-N1) and cutinase-$wtCBM_{T.reesei}$ (cut-wtCBM), 25 U mL^{-1} of cutinase-$sCBM_{T.reesei}$ (cut-sCBM), at pH 8 and 30º C. A control was treated under the same conditions but without any enzyme. Samples and control

were competitively dyed at 60º C. Relative protein adsorption was calculated as $\frac{P_{0h} - P_{18h}}{P_{0h_{cutinase}} - P_{18h_{cutinase}}}$ and relative K/S was calculated as $\frac{K/S_{enzyme} - K/S_{control}}{K/S_{cutinase} - K/S_{control}}$.

4. Concluding remarks

The biomodification of the surface of cellulose acetate with high degree of substitution with cutinase was demonstrated by the acetic acid release and the improvement in the chemically specific staining of the fabrics with a reactive dye. From the acetic acid release, the hydrolysis yield is higher for the less substituted cellulose acetate fabric, but the consequences of the acetyl hydrolysis are more pronounced for CTA, as shown by the differences in colour, morphology of the fibres surface and crystallinity between controls and treated samples. Further studies will be necessary to evaluate the impact of cutinase activity in the physical properties of the fabrics and to assess the contribution of the incomplete protein removal and of the physical, rather than chemical, modifications on the differences seen upon enzymatic treatment.

The design of hybrid enzymes mimics the strategies that nature uses to evolve and it is a powerful tool in biotechnology. The production and application of the cutinase fused to CBMs, especially to the fungal CBM of CBHI of *T. reesei*, provided strong evidences of being an interesting strategy to pursuit. Future work is needed to improve the recombinant production of modular cutinases and to study in detail their affinities toward the cellulose acetates.

From the above considerations, it could be suggested that the cutinase has potential in textile industry for the surface modification and consequently on the “bicomponent yarns/fibres” production of cellulose acetate.

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Chapter 3

Enzymatic Modification of Wool Surface

SubChapter 3.1

Introduction

1. Properties of Wool

Wool is one of the oldest textile fibres known with unique natural properties like being easy to processe into yarn and as a consequence, the panoply of products into which it can be converted determines its commercial value as a textile fibre. Because it absorbs moisture vapour, wool clothing provides superior comfort in both hot and cold weather; it has excellent natural flame-resistance properties; good resilience, which makes it comfortable to wear by fitting the shape of the body; high static and dirty resistance; it can be easily dyed, and the range of colours is limitless. Wool is also a very good insulator against noise, by absorbing sound and reducing noise level considerably. Despite all the remarkable properties of wool, its laundry and durability performance are inferior to synthetic fibres which are the main causes of the research done in the wool industry.

2. The Morphological Structure of Wool

Sheep wool is the most important commercial fibre obtained from animal sources, (Rippon, 1992). Wool is produced in the fibre follicle in the skin of the sheep and the physical properties such as diameter, length and crimp, as well as, chemical composition can deeply vary depending of:

- Parts of the body of the sheep;
- Strains of the sheep within a breed;
- Age of the sheep;
- The diet and health of the sheep;
- Breeds;
- Environments (the climate, terrain, pasture, etc);
- Farming properties;
- Shearing regimes (timing, frequency, preparation procedures);
- Geographic regions;
- Seasons of the year (Pailthorpe, 1992).

Wool is a complex natural fibre being mainly composed of proteins (97%) and lipids (1%). 82% of total protein content of wool is keratinous proteins, which are characterized by a high concentration of cystine. Approximately 17% of wool is composed of proteins which have been termed non keratinous, because of their relatively low cystine content (Feughelman, 1997 Rippon, 1992).

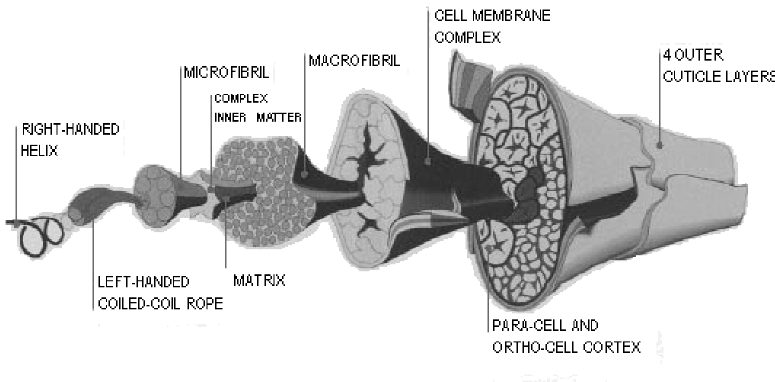

Figure 1. Cross-section diagram of a merino wool fibre showing the structure at progressive magnification (Feughelman, 1997).

Wool fibres have approximately the form of elliptical cylinders, with average diameters ranging from 15 μm to 50 μm and lengths depending of the rate of growth and the shearing regimes, as previous mentioned (Makinson, 1979). The wool fibre consists of two major morphological parts: cuticle and cortex. The cuticle (also referred as scale layer of wool) is composed of laminar and rectangular structures which form a sheath of overlapping scales enveloping the cortex (Speakman, 1985; Naik and Speakman, 1993). It is normally one cell thick and usually constitutes about 10% by weight of the total fiber. The cuticle is subdivided into three layers: exocuticle (which is subdivided into two main layers, A and B that differ mainly in the cystine content), endocuticle, and an outermost membrane called the epicuticle (Figure 2).

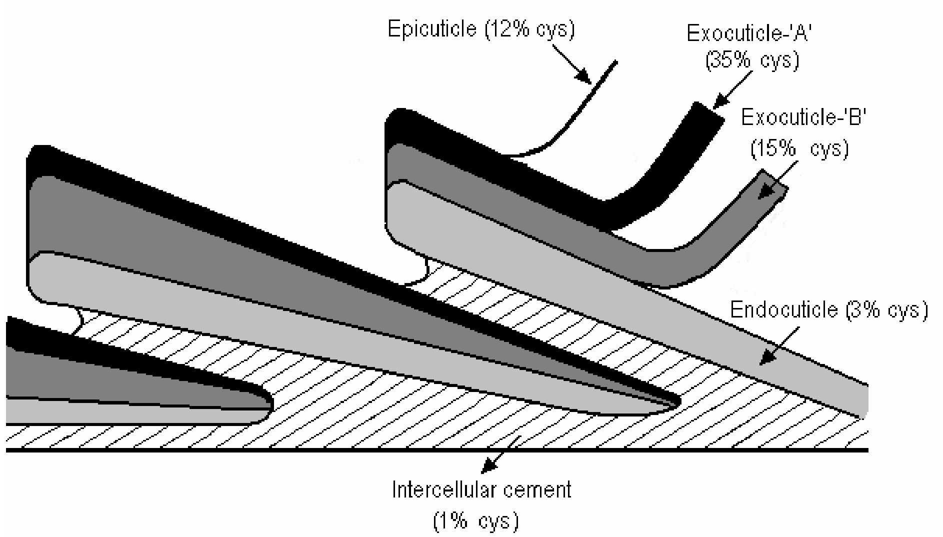

Figure 2. Schematic scale structure of the cuticle showing the major components (based on Rippon (1992).

The epicuticle, is very inert chemically, being resistant to acids, oxidising and reducing agents, enzymes, and alkalis (Makinson, 1979; Negri *et al.,* 1993).The epicuticle is known for its hydrophobicity, probably due to the lipid component 18-methylicosanoic acid (Negri *et al.,* 1993). This fatty acid is covalently bound to the protein matrix via cystine residues, forming a layer that can be removed by treatment with alkaline or chlorine solutions in order to enhance many textile properties such as wetability, dye uptake and polymer adhesion (Negri *et al.,* 1993; Brack *et al.,* 1999). Another important characteristic is the cross-linking of the exocuticle. The A-layer contains 35% cystine residues. In addition to the normal peptide bonds, the cuticle is cross-linked by isodipeptide bonds, (ε-(γ-glutamyl) lysine) (Rippon, 1992; Heine and Höcker 1995). The A and B layers are both resistant to boiling in diluted hydrochloric acid and to trypsin digestion; however they can be solubilised by trypsin treatment after oxidation or reduction. The endocuticle is preferentially attacked by proteolytic enzymes, and readily degraded in diluted boiling hydrochloric acid (Naik, 1994; Sawada and Ueda, 2001).

The cortex comprises the main bulk. Cortical cells are long, polyhedral, and spindle-shaped and consist of intermediate filaments (microfibrils) embedded in a sulfur-rich matrix.

Cuticle cells and cortical cells are separated by a continuous intercellular material, the cell membrane complex, which is mainly composed of non-keratinous proteins and lipids (Rippon, 1992; Makinson, 1979; Plowman, 2003; Negri *et al.,* 1993).

The composition and morphology of the wool surface is primarily modified in fibre pre-treatment processes (Brack *et al.,* 1999).

3. Conventional Finishing Processes for Wool Fibre

A variety of processes are available to improve the appearance, handle, performance and durability of the wool fabrics. Such processes include scouring, carbonizing, bleaching, dyeing, antimicrobial finishing and shrinkproofing.

3.1. Scouring

Raw wool contains 25-70% by mass of impurities. These consist of wool grease, perspiration products, dirt and vegetable matter such as burrs and seeds (Rippon, 1992; Pearson *et al.,* 2004). Before the more specialised finishing processes are applied, fabrics usually require cleaning (scouring) to remove these impurities (Pearson *et al.,* 2004; Lewis, 1992).

3.2. Carbonizing

If not completely degraded and removed from the textile goods, vegetable matter and skin residues will lead to uneven dyeing and printing. The vegetable matter is normally removed by carbonizing, a process where wool is impregnated with sulfuric acid and then baked to char the cellulosic impurities. The residuals are then crushed and extracted from the wool as carbon dust by brushing and suction.

3.3. Bleaching

Bleaching is a chemical process employed to destroy the natural creamy colourants in wool and produces a whiter wool. This operation is only performed when wool is intended to be white dyed or light dyed. Bleaching may take place at the sliver, top, yarn or fabric stages of production. Hydrogen peroxide based bleaching recipes are commonly employed although these compounds can damage wool fibres, due to progressive oxidation of disulfide bonds ultimately forming cysteic acid (Gacen and Cayuela, 2000).

3.4. Dyeing

Dyeing operations are used at various stages of production to add colour and sophistication to textiles and increase product value. Wool textiles are dyed using a wide range of dyestuffs, techniques, and equipment. Until fairly recently, most of the dyes used on wool were acid dyes. Nowadays, acid, chrome, metal-complex and reactive dyes may all be used for the dyeing of wool (Pailthorpe, 1992).

3.5. Antimicrobial Finishing

Natural fibres are more susceptible to microbial attack than synthetic fibres, once they provide the basic requirements for microbial growth (such as nutrients and moisture). In the carpet industry, the antimicrobial and/or mothproofing of wool fabric is an important finishing step. Various chemicals have been applied to wool to control microbial and larval attack, however, more recently, due to environmental concerns, restrictions have been placed on the type of agent which may be employed (Purwar and Joshi, 2004; Han and Yang, 2005). Magnesium hydroperoxide and related compounds, and chitin and chitosan based antimicrobial agents are the new generation of environmentally friendly antimicrobial agents (Purwar and Joshi, 2004). Non-toxic natural dyes have also been tested on the antimicrobial activity of wool with good results (Han and Yang, 2005).

3.6. Felting and shrinkage

One of the intrinsic properties of wool is its tendency to felting and shrinkage under a moisturizing environment, heat or mechanical agitation. Wool shrinkage is basically due to its scaly structure.

The shrinkage behaviour of wool can be regulated to a greater or smaller degree by various chemical means. There are various successful commercial shrink-resist processes available for textile industries that have been developed decades ago. These shrinkproofing processes aim at the modification of the fibre surface either by oxidative or reductive methods and/or by the application of a polymer resin onto the surface. These processes can be combined in 3 groups:

Subtractive processes – The first type of shrinkproofing treatment involves chemical attack on the cuticle of the fibres using a chlorine agent. Chlorination was introduced as a shrinkproofing treatment during the latter half of the nineteenth century (Makinson, 1979). The principal mode of action of subtractive antifelting treatment is that it making the cuticle cells softer in water than those of untreated fibres. This softening is the result of oxidation and scission of the numerous disulfide bonds in the exocuticle of the fibre and causes a reduction in the directional frictional effect (Makinson, 1979).

Additive processes – The second class of shrinkproofing treatments consists on the addition of a polymer to the wool. This treatment promotes fibres to stick among themselves at many points along their length, thereby preventing relative movement and thus shrinkage. The polymer can be applied to wool fabric or yarn as solutions or emulsions. The polymers contain reactive side-chains, which form cross-links between the polymer chains during a curing process and may form covalent links with the wool protein.

Combination of subtractive and additive processes- The most common surface specific treatment for wool is probably the chlorine-Hercosett process which renders the wool fibre shrink-resistant. The process uses chlorine gas generated *in situ* from sodium hypochlorite and sulphuric acid or chlorine gas dissolved in water. The treatment is

surface specific because the reaction with the cuticle takes place in less than 10 seconds. Chlorination results in the oxidation of cystine residues to cysteic acid residues in the surface of the fiber and allows the Hercosett 125 polymer, applied afterwards, to spread and adhere evenly along the fibre surface (Heine, 2002). This polymer, which is normally used to improve the wet strength of paper products, swells to 10x its normal thickness in water thus preventing the scale edges of adjacent fibres from interacting and causing felting during washing.

The chlorine-Hercosett process has been employed in the industry for the last 30 years, and about of 75% of the world's treated wool is processed by this route in one of its forms (Holme, 2003). As previously mentioned (Chapter 1) this process has excellent advantages (antifelt effect, low damage and low weight loss) but it also shows a number of drawbacks like limited durability, poor handle, yellowing of wool, difficulties in dyeing and the most important today, release of absorbable organic halogens (AOX) to the effluents (Heine, 2002; Schlink and Greeff, 2001). There is therefore an increasing demand for environmentally friendly alternatives.

4. Enzymatic Finishing Processes for Wool

Enzymes can be used in order to develop environmentally friendly alternative processes. Since wool mainly consists of proteins and lipids especially proteases and lipases have been investigated for wool fibre modification. However, the complex structure of natural fibres, especially of wool, brings complexicity to enzymatic fibre modification. Proteases and lipases can catalyse the degradation of different fibre components of wool preventing an accurate control of the reaction. If not controlled, this diffusion leads to a strong damage of the wool fibre, being crucial to restrict the enzymatic action to the wool fibre surface. The following subchapters describe the approaches developed in the scope of this thesis aiming to restrict subtilisin E hydrolysis to the surface of wool.

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Subchapter 3.2

Strategies Towards the Functionalization of Bacillus subtilis *Subtilisin E for Wool Finishing Applications*

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Strategies Towards the Functionalization of *Bacillus subtilis* Subtilisin E for Wool Finishing Applications

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Abstract

Subtilisin E is an alkaline serine protease secreted by the Gram positive bacteria *Bacillus subtilis* and widely used in industry as biocatalyst for various processes. The most common application of subtilisins is in laundry detergents but, due to environmental concerns, the application of subtilisins to treat wool is under study. There are some reports regarding the attempts to substitute the conventional chlorine treatment by an enzymatic process capable of providing the same characteristics to the fabric, like anti-shrinking and better uptake and fixation of the dyestuff. However, the uncontrolled hydrolysis degree due to diffusion of the enzyme inside the wool fibre causes unacceptable losses of strength. To overcome this fact, and taking advantage of the x-ray crystallographic structure, we have genetically modified subtilisin E, increasing its molecular weight, to restrict the hydrolysis to the surface of the wool fibres. Therefore, three genetically modified enzymes with a molecular weight 2-fold to 4-fold higher than the native subtilisin E were produced and assessed for activity. The prokaryotic expression systems, pET25b (+), pET11b and pBAD C were explored for the production of recombinant enzymes. The results demonstrated that regardless the expression system or strain used, chimeric subtilisins were not expressed with the correct folding. No active and soluble recombinant protein was recovered under the testing conditions. Despite this drawback, we have described here a novel approach to increase subtilisin molecular weight. The reported results are noteworthy and can indicate good guidelines for future work aiming the solubilization of recombinant chimeric subtilisins.

1. Introduction

Subtilisins are a family of alkaline serine proteases generally secreted by a variety of *Bacillus* species. There are also some reports of subtilisins production by Flavobacterium (Morita *et al.,* 1998). They are characterized by a common three-layer α/ β/ α tertiary structure and a catalytic triad of aspartate, histidine and serine residues (Graycar *et al.,* 1999). The molecular weight of subtilisins ranges from 15 to 30 kDa, with few exceptions, like a 90 kDa subtilisin from *Bacillus subtilis* (*natto*) (Kato *et al.,* 1992). They present optimum pH range between 10.0 and 12.5 and an isoelectric point (pI) near 9.0 (Rao *et al.,* 1998). Subtilisins from *Bacillus* sp. are quite stable at high temperatures and addition of Ca^{2+} enhances enzyme thermostability (Paliwal *et al.,* 1994). They are strongly inhibited by phenyl methyl sulphonyl fluoride (PMSF), diisopropyl-fluorophosphate (DFP) and potato inhibitor (Gold and Fahrney *et al.,* 1964; Mirihara, 1974).

Due to their widespread distribution, availability and broad substrate specificity, subtilisins are useful as biocatalysts for detergent industry, leather processing, silver recovery in photographic industry, for management of industrial and household waste, for food and feed processing, as well as, for medical purposes and chemical industry (Kumar and Takagi, 1999; Gupta *et al.,* 2002). Regarding the cited industrial applications, subtilisins have been extensively investigated as promising targets for protein engineering.

Among subtilisins, subtilisin E, from *B. subtilis*, is one example of the best studied alkaline serine proteases. Subtilisin E is first synthesized as a membrane-associated precursor preprosubtilisin (Wells *et al.,* 1983). The NH_2-terminal prepeptide, consisting of 29 amino acid residues is a typical signal peptide that is required for secretion of prosubtilisin across the plasma membrane. The propeptide located between the prepeptide and mature sequence has 77 amino acids and is essential for producing active subtilisin *in vivo*, as well as, *in vitro*. It acts as an intramolecular chaperone required for the correct folding of mature enzyme (Stahl and Ferrari, 1984; Wong and Doi, 1986; Ikemura *et al.,* 1987; Ikemura and Inouye, 1988). The mechanism of maturation by propeptide consists of three steps: (1) folding of mature region mediated by its propeptide; (2) cleavage of the peptide bond between propeptide and subtilisin and (3)

removal of the propeptide by an auto-proteolytic degradative process (Ikemura and Inouye, 1988; Zhu *et al.,* 1989; Shinde *et al.,* 1993; Li and Inouye, 1994; Yabuta *et al.,* 2001). Degradation is required, because the propeptide can inhibit the active site of subtilisin forming a stable and inactive propeptide-subtilisin complex (Li *et al.,* 1995; Fu *et al.,* 2000; Jain *et al.,* 1998). 3D structure of subtilisin E has been used to develop protein engineering strategies, aiming the enhancement of catalytic activity and thermostability, as well as, substrate specificity and oxidation resistance.

Catalytic efficiency of subtilisin E was 2-6 fold increased after changing the isoleucine at position 31 by a leucine, using site-directed mutagenesis (SDM) (Takahi *et al.,* 1988). The same group constructed a novel subtilisin E with high specificity, activity and productivity through three cumulative amino acid substitutions (Takagi *et al.,* 1997). Sroga and Dordick performed protein engineering to convert subtilisin E into an enzyme with broader esterase activity as opposed to its native amidase activity (Sroga and Dordick, 2001).

Improvement of thermal stability was first achieved by Takagi and collaborators by the introduction of an additional disulfide bond linkage between cysteines 61 and 98 in subtilisin E (Takagi *et al.,* 1990). SDM was used to introduce a N218S mutation that increased the thermostability of the enzyme (Wang *et al.,* 1993). SDM was also used by Yang and collaborators to generate a S236C mutant subtilisin E with a half-life, at 60 ºC, 4-fold longer than that of native subtilisin E. Using this mutant, thermostability could also be increased, by forming a disulfide bridge between two molecules of S236C subtilisin E (Yang *et al.,* 2000a). The same group used random mutagenesis PCR technique to develop a thermal stable and oxidation-resistant mutant. The new M222A/N118S subtilisin E was 5-fold more thermal stable than native enzyme (Yang *et al.,* 2000b). In another report, the thermal stability of subtilisin E was increased using directed evolution to convert *B. subtilis* subtilisin E into an enzyme functionally equivalent to its thermophilic homolog thermitase from *Thermoactinomyces vulgaris* (Zhao and Arnold, 1999).

Proteases, like subtilisin E can be used for wool fibre modification. Since wool mainly consists of proteins and lipids, proteases and lipases have been extensively studied in order to achieve more environmentally friendly processes (Schumacher *et al.,* 2001).

Wool cuticle treatment with subtilisin improves anti-shrinkage properties, leads to a reduced felting tendency and an increased dyeing affinity (Schumacher *et al.,* 2001). However, due to its small size, the enzyme is able to penetrate into the fibre cortex which causes the destruction of the inner parts of wool structure (Shen *et al.,* 1999). Several reports show that the increase of enzyme molecular weight, by attaching synthetic polymers like polyethylene glycol (PEG) or by crosslinking with glutaraldehyde (GTA), is effective avoiding enzyme penetration and the consequent reduction of strength and weight loss (Schroeder *et al.,* 2006; Silva *et al.,* 2004). Pre-treatment of wool fibres with hydrogen peroxide at alkaline pH in the presence of high concentrations of salts also targets enzymatic activity on the outer surface of wool, by improving the susceptibility of cuticle for proteolytic degradation (Lenting *et al.,* 2006).

Surfactant protein D (SP-D) is a member of the C-type lectin superfamily (Zhang *et al.,* 2001). It is synthesized and secreted by alveolar and bronchiolar epithelial cells and participates in the innate response to inhaled microorganisms and organic antigens. It also contributes to immune and inflammatory regulation within the lung (Zhang *et al.,* 2001). Each SP-D subunit (43 KDa) consists of four major domains: an N-terminal cross-linking domain, an uninterrupted triple helical collagen domain, a trimeric coiled-coil or neck domain and a C-type lectin carbohydrate recognition domain. The neck domain of SP-D is the unit responsible for driving the trimerization of the three polypeptide chains of SP-D and it was demonstrated that the presence of this sequence permits spontaneous and stable non-covalent association of a heterologous type IIA pro-collagen amino propeptide sequence (McAlinden *et al.,* 2002). SP-D neckdomain (SPDnd) was used for possible formation of subtilisin E trimers.

Increasing subtilisin molecular weight is crucial for its successful application in wool finishing. The main objective of this work was to provide an alternative to chemical modification of subtilisin, by expressing a genetically modified subtilisin E with increased molecular weight, to be used for wool finishing applications. Two novel approaches were followed, the construction of two polysubtilisins, (pro2subtilisin and pro4subtilisin) and the formation of a subtilisin trimer by fusion of native prosubtilisin with SP-D neckdomain. We were able to express the three modified enzymes although no activity was recovered for these enzymes yet. The expression systems tested, as well

as, the fermentation conditions that could increase the solubility of recombinant proteins are presented in great detail.

2. Materials and Methods

2.1. Bacterial strains, plasmids, and enzymes

The *Escherichia coli* strains BL21(DE3), BL21(DE3)pLysS and Tuner and the T7 plasmids pET25b (+) and pET11b were purchased from Novagen (Madison WI, USA). Plasmid pBAD C and *E. coli* strains TOP 10 and LMG194 were from Invitrogen, (Carlsbad, CA). The genetic sequences coding for native prosequence, subtilisinE and prosubtilisinE were PCR-amplified with the primers listed in table 1 using as template DNA, the vector pET11a containing the full sequence coding for pro-subtilisin E from *Bacillus subtilis* (kindly provided by Professor Masayori Inouye, Robert Wood Johnson Medical School, University of Medicine and Dentistry, Piscataway, New Jersey) (Hu *et al.,* 1994). Oligonucleotides (0.01 and 0.05 µmol scale) were purchased from MWG Biotech, (Germany). Restriction and modification enzymes were from Roche Applied Science, (Germany). The theoretical molecular masses of recombinant proteins were calculated using the Compute pI/Mw application from Expasy (http://www.expasy.ch/tools).

Unless specifically stated, all the other reagents were from Sigma-Aldrich (St. Louis MO, USA).

2.2. Transformation and DNA sequencing

All the vectors constructed were first established in *E. coli* XL1-Blue strain, according to SEM method (Inoue *et al.,* 1990). The correct plasmid constructs were verified by restriction map analysis followed by DNA sequencing with an ABI PRISM 310 Genetic Analyzer, using the method of Sanger (Sanger *et al.,* 1977). DNA cloning and manipulation were performed according to the standard protocols (Sambrook *et al.,* 1989).

2.3. Design of chimeric subtilisin genes and construction of expression vectors based on pET system

The PCR products purified from a 2% (w/v) agarose gel electrophoresis were first cloned into the p-GEM T-easy cloning system (Promega, USA), resulting in the following vectors: pGEM:prosequence, pGEM:subtilisinE and pGEM:prosubtilisinE. For the construction of the pro2subtilisinE chimeric gene, containing two subtilisin coding sequences cloned in frame, the BamHI/BglII fragment of pGEM:subtilisinE was ligated with the BglII linearized pGEM:proSubtilisinE (figure 1). Identical strategy was used for the construction of the chimeric gene pro4subtilisinE: the pGEM-pro2subtilisinE construction, linearized with BglII, and chimeric gene BamHI-2subtilisin-BglII were ligated.
The gene corresponding to prosubtilisinE-SPDnd was chemically synthesized by EpochBiolabs, Texas, USA.
Flanked by BamHI and BglII restriction sites, the entire DNA coding sequences for native and the three chimeric subtilisins, were subcloned into BamHI digested and dephosphorilated pET25b (+) and pET11b (table 2).

2.4. Site-directed mutagenesis of pBAD C plasmid and construction of pBAD expression vectors

The pBAD C plasmid was modified by site-directed mutagenesis (SDM), using recombinant PCR technique (Ansaldi *et al.,* 1996), in order to allow the introduction of the inserts in frame with C-myc and His6 tags. The primers were designed to remove an adenine at the end of pBAD C multiple cloning site (the overlapping regions are underlined and the mutation is indicated by an asterisk): BADmutF (5´-TCATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATC- 3´); BADmutR (5´-TTCAGATCCTCTTCTGAGATGAGTTTTTGTTC*AGAAAGCTTCGAATTCC-3´).
The mutation eliminates a recognition site for XbaI (TCTAGA → TCT*GA) which permitted to check the insertion of this mutation. Subtilisins coding sequences were recovered from cloning vectors by digestion with restriction enzymes BamHI and BglII, and further cloned into dephosphorilated pBAD C* linearized with BglII (table 2).

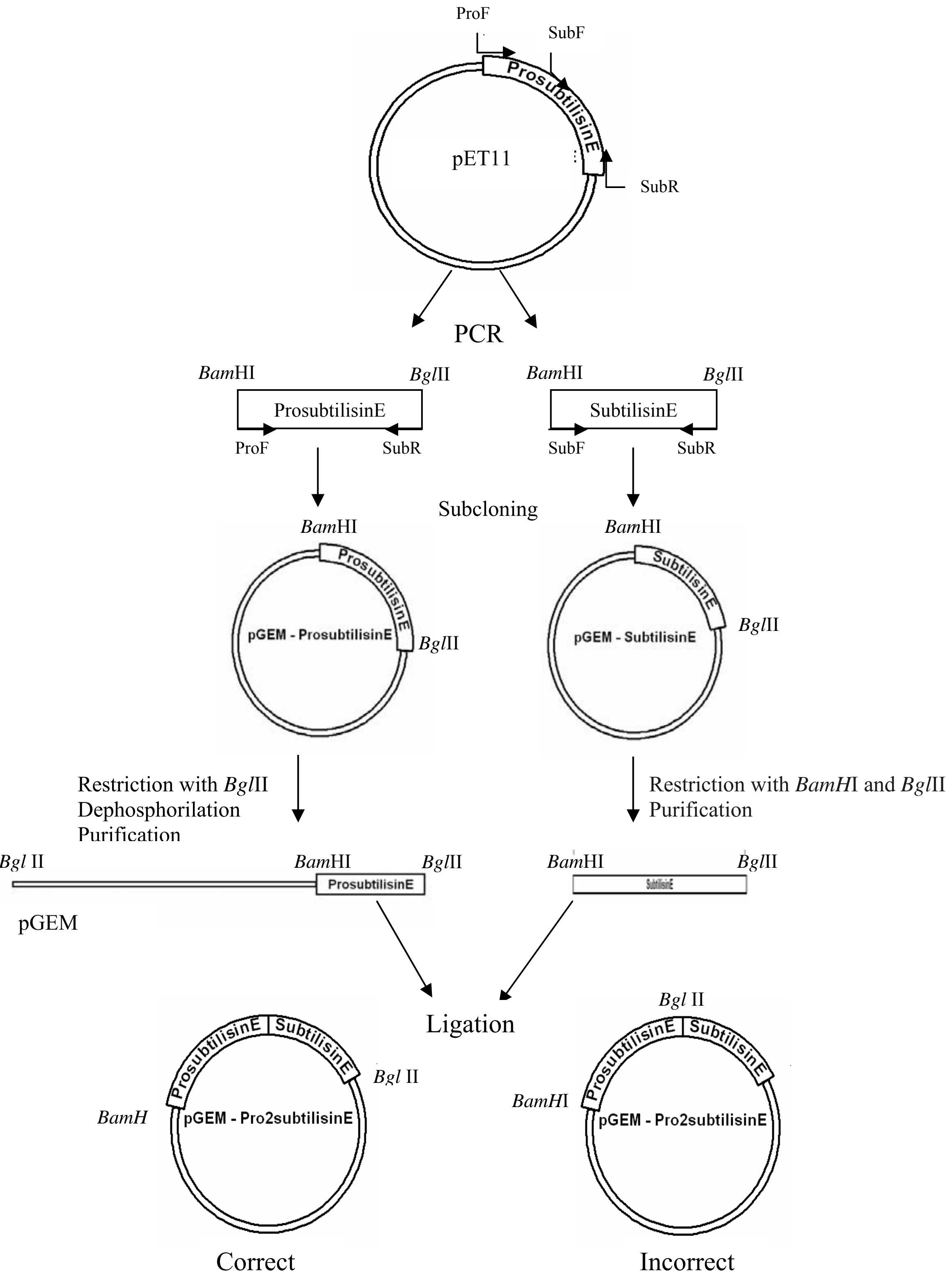
ProF
SubF
ProsubtilisinE
pET11
SubR
PCR
BamHI
BglII
ProsubtilisinE
ProF
SubR
BamHI
BglII
SubtilisinE
SubF
SubR
Subcloning
BamHI
ProsubtilisinE
pGEM - ProsubtilisinE
BglII
BamHI
SubtilisinE
pGEM - SubtilisinE
BglII
Restriction with BglII
Dephosphorilation
Purification
Restriction with BamHI and BglII
Purification
Bgl II
BamHI
BglII
ProsubtilisinE
pGEM
BamHI
BglII
Ligation
ProsubtilisinE
SubtilisinE
Bgl II
BamH
pGEM - Pro2subtilisinE
Correct
Bgl II
ProsubtilisinE
SubtilisinE
BamHI
pGEM - Pro2subtilisinE
Incorrect

Figure 1. Strategy used for the construction of chimeric gene pro2subtilisinE in pGEM T-easy. PCR was carried out with pET11a:prosubtilisin (Hu *et al.,* 1994) containing the native prosubtilisinE as template. PCR products were subcloned into pGEM T-easy. Construction pGEM-subtilisinE was digested with *BamH*I and *Bgl*II to recover the *BamH*I-subtilisinE-*Bgl*II fragment, which was then ligated to the *Bgl*II linearized pGEM-prosubtilisinE. *BamH*I and *Bgl*II recognize different restriction sites generating compatible sticky-ends.

The original *Bacillus subtilis* presequence, described by Ikemura and collaborators (1987) was synthesized in vitro using self-annealing oligonucleotides Fpres and Rpres, overlapping in 33 bp (table III). The full DNA sequence was obtained in a PCR of 20 s at 94 ºC and 20 s at 72 ºC, for Accuzyme (Bioline, Germany) extension, for 30 cycles. Primers Famp and Ramp (flanked with XhoI restriction site) were further used to amplify the presequence (table III). The PCR product was digested with XhoI, purified from a 2% (w/v) agarose gel electrophoresis and cloned into the XhoI digested and dephosphorilated pBAD C* constructions, resulting in the final expression vectors pBAD-pre-prosubtilisinE, pBAD-pre-pro2subtilisinE, pBAD-pre-pro4subtilisinE and pBAD-pre-prosubtilsinE-SPDnd (table 2).

Table I. Primers used to amplify the genes prosequence, subtilisinE and prosubtilisinE

Gene	Primer (5´→ 3´)	bp	GC %
Prosequence	ProF CGC GGA TCC CAT GGC CGG AAA AAG CAG TAC AG	32	59.4
	ProR GGA AGA TCT CCA TAT TCA TGT GCA ATA TGA T	31	35.5
SubtilisinE	SubF CGC GGA TCC CAT GGC GCA AAG CTT TCC TTA TG	32	56.3
	SubR GGA AGA TCT CCT TGT GCA GCT GCT TGT ACG TTG	33	51.5
proSubtilisinE	ProF CGC GGA TCC CAT GGC CGG AAA AAG CAG TAC AG	32	59.4
	SubR GGA AGA TCT CCT TGT GCA GCT GCT TGT ACG TTG	33	51.5

Table II. Heterologous protein expression systems used: *E. coli* strains and recombinant vectors.

***E. coli* strain/ vector**	**Constructs**
BL21(DE3)/ pET25b (+)	prosubtilisinE pro2subtilisinE pro4subtilisinE
Tuner/ pET25b (+)	prosubtilisinE pro2subtilisinE pro4subtilisinE
BL21(DE3)pLysS/ pET25b (+)	prosubtilisinE pro2subtilisinE pro4subtilisinE
BL21(DE3)/ pET11b	prosubtilisinE pro2subtilisinE pro4subtilisinE prosubtilisinE-SPDnd
TOP10/ pBAD C*	prosubtilisinE pro2subtilisinE pro4subtilisinE prosubtilisinE-SPDnd
LMG194/ pBAD C*	Prosequence prosubtilisinE pro2subtilisinE pro4subtilisinE prosubtilisinE-SPDnd pre-prosubtilisinE pre-pro2subtilisinE pre-pro4subtilisinE pre-prosubtilisinE-SPDnd

2.5. Induction conditions for protein expression

Expression host strains BL21(DE3), BL21(DE3)pLysS and Tuner, transformed with pET25 constructions, were used for protein expression. Cells were grown in Luria-Broth, (LB), medium containing 100 µg/µL ampicillin, and induced according to the conditions described in table IV.

Cells of the strain BL21(DE3) containing pET11 constructions were grown in LB medium/100 µg/µl ampicillin, at 37 ºC and induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) 1 mM at 18 ºC.

E. coli strains TOP10 carrying the pBAD C* constructions were grown in Complete Minimal (CM) medium supplemented with 20 amino acids (40 µg/mL) and vitamin B1 (5 mg/L). Glycerol was used at a concentration of 0.20% (w/v). Cells of the strain

LMG194 were grown in RM medium. In both cases ampicillin was used at the concentration of 100 µg/µL. Cells were induced according to conditions described in table IV.

Table III. Primers used to generate and amplify *B. subtilis* presequence. XhoI restriction site is underlined.

Gene	Primer (5´→ 3´)	bp	GC %
Presequence	Fpres CTC GAG TGA GAA GCA AAA AAT TGT GGA TCA GCT TGT TGT TTG CGT TAA CGT TAA TCT TTA CGA	63	38.1
	Rpres CTC GAG CCT GCG CAG ACA TGT TGC TGA ACG CCA TCG TAA AAG TTA ACG TTA AGC CAA ACA ACA	63	47.6
	Famp CCC TCG AGT GAG AAG CAA AA	20	50
	Ramp CCC TCG AGC CTG CGC AGA CA	20	70

Table IV. Fermentations conditions performed for *E. coli* pET25b (+) and pBAD vectors.

E. coli strain/ Vector	Temperature of growth (ºC)	[Inducer]	Temperature of induction (ºC) / time
BL21(DE3)/ pET25b (+)	37; 30; 25	IPTG 1.0; 0.5; 0.3 (mM)	30;18; 4 h
BL21(DE3)pLysS/ pET 25b (+)	30; 25	IPTG 1.0; 0.5; 0.3 (mM)	18 h
Tuner/ pET25b (+)	30; 25	IPTG 1.0; 0.5; 0.3 (mM)	18 h
TOP10 and LMG194/ pBAD C*	37; 30	Arabinose 0.2; 0.1(%)	18 h / ON and 3 h

2.6. Cell fractionation

Overnight cell cultures from all the strains transformed with pET25 and pBAD C* constructions were harvested by centrifugation (5000 rpm for 15 minutes) and resuspended in Osmotic Solution I (OS I: 20 mM Tris-HCl pH 8.0, 2.5 mM EDTA, 2 mM $CaCl_2$, sucrose 20%, w/v) to an OD600 of 5.00. Cells resuspended in OS I were incubated on ice for 10 min and centrifuged at 4 ºC. Supernatants were decanted and the cell pellets resuspended in the same volume of Osmotic Solution II (OS II: 20 mM Tris-HCl pH 8.0, 2.5 mM EDTA, 2 mM $CaCl_2$). The suspension was incubated for 20 min

on ice and centrifuged at 4 ºC. The supernatants (periplasmic fractions) were reserved. The pellet samples (cellular fractions) were resuspended in phosphate buffered saline (PBS) solution (10 mM Na_2HPO_4, 2 mM KH_2PO_4, 137 mM NaCl, 3 mM KCl, pH 7.4). Ultrasonic treatment of bacterial cells was performed at 20 KHz with a 13-mm probe in an Ultrasonic Processor GEX 400. Four 2 min pulses, with 2 min on ice between each pulse, were performed. The lysates were centrifuged for 30 min at 14000 rpm at 4 ºC.
The supernatants, soluble fractions, were decanted and reserved. The pellets, insoluble fractions, were resuspended in PBS solution and reserved.
Overnight cell cultures from BL21(DE3) strain transformed with pET11b constructions were harvested by centrifugation (5000 rpm for 15 minutes) and resuspended in PBS solution to an OD600 of 5.00. Cells were broken with ultrasonic treatment and lysates centrifuged for 30 min at 14000 rpm at 4 ºC and pellets reserved.

2.7. In vitro renaturation of recombinant enzymes

The pellets (inclusion bodies) from fermentations of *E. coli* carrying pET11 constructions were solubilized in urea 6 M. After overnight incubation at 4 ºC, the insoluble materials were removed by ultracentrifugation at 90000 x g for 40 min. The supernatants were then dialyzed against an excess of 50 mM sodium-potassium phosphate buffer (pH 5.0) containing 5 M urea at 4 ºC. Renaturation of recombinant proteins were performed by a stepwise dialysis procedure against 10 mM Tris-HCl (pH 7.0), 0.5 M $(NH_4)SO_4$, 1 mM $CaCl_2$, 5 mM β-mercaptoethanol and decreasing amount of urea. Buffer was changed every 24 h until urea was completely removed.

2.8. Isolation and purification of prosequence

For prosequence purification an Immobilized Metal Affinity Chromatography (IMAC) system was used with HiTrap Chelating HP 5 ml column containing 5 ml of Chelating Sepharose High Performance (Amersham Pharmacia Biotech). The HiTrap Chelating HP column was linked to a peristaltic pump. After loading with 2.5 ml 0.1 M $NiSO_4$ in H_2O, equilibration was performed with 10 mM imidazole, 0.5 M NaCl, 20 mM phosphate buffer pH 7.6.

Samples were applied onto the column at a flow rate of 5 ml.min^{-1}, followed by washing with the equilibration buffer. Elution was performed with a buffer containing 500 mM imidazole, 0.5 M NaCl and 20 mM phosphate buffer, pH 7.6.

2.9. Prosequence Mediated Folding

Prosequence mediated folding was performed by addition of IMAC purified prosequence, produced by *E. coli* LMG194, to native and chimeric enzymes, purified from BL21(DE3) inclusion bodies, in a 1:1 molar ratio. The mixture was allowed to incubate for 12 h at 4 ºC.

2.10. Analytical Methods for the Enzymes

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis, using a Tris-SDS-glycine buffer system was used to monitor the soluble and insoluble fractions (Laemmli, 1970). Protein detection was done by Coomassie Brilliant Blue R250. The total protein concentration was estimated by Bradford quantitative protein determination assay using bovine serum albumin as standard (Bradford, 1976).

2.11. Activity assay

Proteolytic activity was determined using azocasein as substrate, based on Sako and co-workers (Sako *et al.,* 1997). The reaction mixture containing 0.250 ml 50 mM Tris-HCl, pH 8.0, 2% (w/v) azocasein and 0.150 ml enzyme solution to a final volume of 0.400 ml, was incubated at 25 ºC for 30 minutes. The negative control was prepared replacing the enzyme solution with buffer. The reaction was stopped by the addition of 1.2 ml of 10% trichloroacetic acid (TCA). The solution was mixed thoroughly and allowed to stand for 15 minutes to ensure complete precipitation of the remaining azocasein and azocasein fragments. After centrifugation at 8000 × g for 5 min, 1.2 ml of the supernatant was transferred to a test tube containing 1.4 ml of 1 M NaOH solution. The absorbance of this solution was measured at 440 nm using a spectrophotometer Genesis 20 (Thermospectronic). The assays were performed in triplicate. One unit of protease

activity is defined to be the amount of enzyme required to produce an absorbance change of 1.0 in a 1 cm cuvette, under the conditions of the assay.

3. Results

3.1. Expression of recombinant proteins using pET25b (+) expression system

All the constructions reported in this work were originally cloned by PCR from the vector pET11a:pro-subtilisin (Hu *et al.,* 1994). The final constructions that are described in table 2 were transformed into the appropriate strains. Depending on the vector used, the expression of recombinant proteins was induced with IPTG or with arabinose, as well as, by varying the cell cultures temperature incubation, as described in Material and Methods.

E. coli BL21(DE3) cells transformed with pET25b (+) constructions (table II), grown at 37 ºC, were able to express prosubtilisinE, pro2subtilisinE and pro4subtilisinE in the presence of 1 mM IPTG at 30 ºC at a high level (figure 2). The molecular mass of prosubtilisinE, estimated as 45.0 kDa, is not in agreement with the expected molecular weight for mature subtilisinE, (30 kDa) (Ikemura *et al.,* 1987). The chimeric proteins pro2subtilisinE and pro4subtilisinE showed a molecular mass near 67 and 125 kDa, respectively, probably due to the unprocessed pelB-prosubtilisinE, pelB-pro2subtilisinE and pelB-pro4subtilisinE. The theoretical molecular mass of the processed proteins according to Expasy is near 58 and 116 kDa. pelB leader signal peptide directs the recombinant proteins to the periplasmic space, where they were mostly expected to be found. However, recombinant proteins were only found in the insoluble fractions (pellets) (figure 2). The same results were obtained for expression strains BL21(DE3)pLysS and Tuner using the same fermentation conditions (data not shown).

In order to increase the solubility of the recombinant proteins, all bacterial strains were grown under different temperatures (30 and 25 ºC) and induction phase was performed with lower concentrations of IPTG (0.5 and 0.3 mM) at lower incubation temperatures (18 and 4 ºC). It was assumed that combining decreasing of temperature with lower concentrations of inducer would prevent overloading the *E. coli* periplasmic transport system and recombinant enzymes would be able to fold properly. However, none active

subtilisin was secreted to the periplasmic space, (figures 3 and 4), which revealed that pET25b (+) pelB leader sequence was not a suitable signal sequence to export this protease, in the set of conditions tested. In figures 3 and 4 representative results obtained for strain BL21(DE3) are shown for all the parameters tested (culture temperature, inducer concentration and induction phase temperature). Similar results were obtained for the strains BL21(DE3)pLysS and Tuner, using the same fermentations conditions.

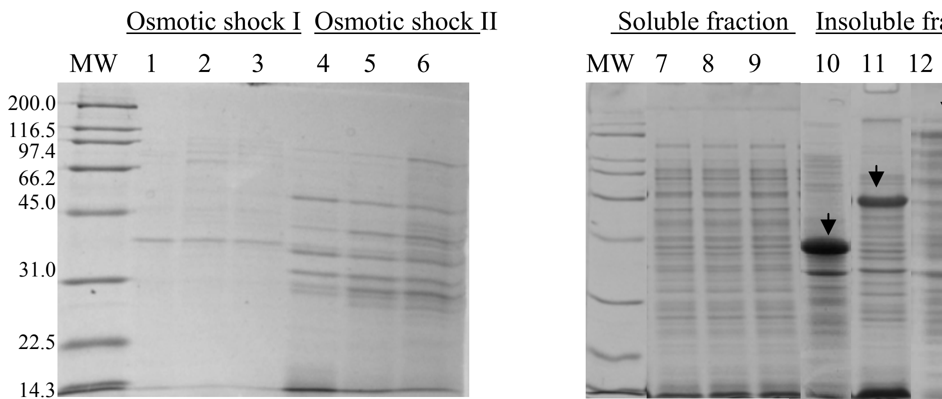

Figure 2. SDS-PAGE of proteins from *E. coli* BL21(DE3) cells grown at 37 ºC, induced with IPTG 1 mM at 30 ºC. *Lanes 1, 4, 7 and 10:* pET25pro-SubtilisinE. *Lanes 2, 5, 8 and 11:* pET25-pro2SubtilisinE. *Lanes 3, 6, 9 and 12:* pET25-pro4SubtilisinE. *MW*: SDS-PAGE Standard, Broad Range (Bio-Rad). The *solid arrowheads* indicate the position of recombinant proteins.

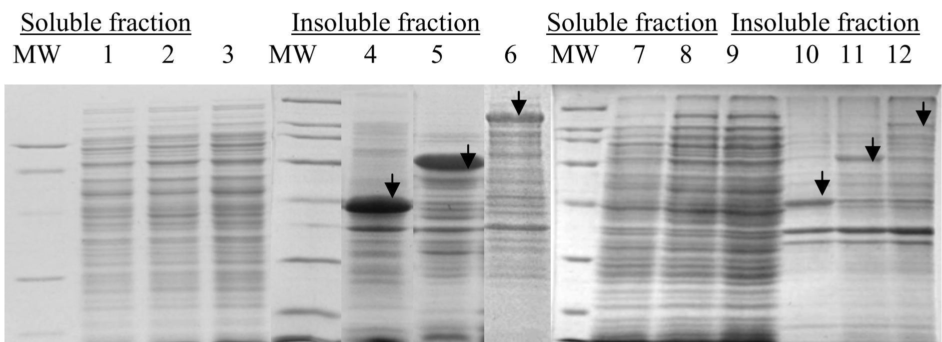

Figure 3. SDS-PAGE of proteins from *E. coli* BL21(DE3) cells grown at 30 ºC, induced with IPTG 0.5 mM at 18 ºC and grown at 30 ºC, induced with IPTG 0.3 mM at 4 ºC. *Lanes 1, 4, 7 and 10:* pET25-prosubtilisinE. *Lanes 2, 5, 8 and 11:* pET25-pro2subtilisinE. *Lanes 3, 6, 9 and 12:* pET25-pro4subtilisinE. *MW*: SDS-PAGE Standard, Broad Range (Bio-Rad). The *solid arrowheads* indicate the position of recombinant proteins.

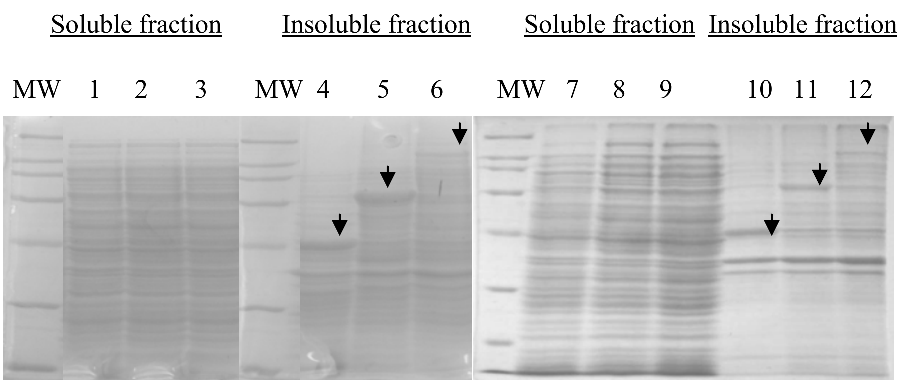

Figure 4. SDS-PAGE of proteins from *E. coli* BL21(DE3) cells grown at 25 ºC, induced with IPTG 0.5 mM at 18 ºC and grown at 25 ºC, induced with IPTG 0.3 mM at 4 ºC. *Lanes 1, 4, 7 and 10:* pET25-prosubtilisinE. *Lanes 2, 5, 8 and 11:* pET25-pro2subtilisinE. *Lanes 3, 6, 9 and 12:* pET25-pro4subtilisinE. *MW*: SDS-PAGE Standard, Broad Range (Bio-Rad). The *solid arrowheads* indicate the position of recombinant proteins.

3.2. Expression of recombinant proteins using pBAD expression systems

The *E. coli* pBAD expression system has been described to express *B. subtilis* subtilisinE (Sroga and Dordick, 2001; Sroga and Dordick, 2002), where the entire preprosubtilisinE gene was used and full functional enzyme was efficiently targeted to the periplasmic space. The pBAD plasmid has a geneIII signal sequence that can be used for periplasmic expression of recombinant enzymes. SDM by recombinant PCR was performed into pBAD C vector in order to allow in frame integration of inserts. The native and chimeric genes were cloned into pBAD C* expression system and *E. coli* strains TOP10 and LMG194 were used. TOP 10 *E. coli* did not express any of recombinant enzymes (data not shown). LMG194 cells, carrying pBAD C* constructions, produced the recombinant proteins in the insoluble fraction in all the conditions tested. Figure 5 shows representative results of these assays.

Unlike Sroga and its collaborators (2002) we were not able to produce active and soluble subtilsin E using pBAD expression system, since no azocasein activity could be detected in soluble fractions, derived both from cytoplasm or periplasmic space. As previously observed for pET25b (+) pelB leader sequence, also pBAD C geneIII leader sequence did not revealed to be a suitable signal peptide to export these recombinant proteins to the periplasmic space. It was assumed that these facts might be explained by the absence of native *B. subtilis* presequence. Following this hypothesis, in all the pBAD C* constructions, the original *B. subtilis* prepeptide was introduced in frame upstream the pBAD geneIII signal sequence. Cells LMG194 harboring the pBAD-pre-prosubtilisinE, pBAD-pre-pro2subtilisinE, pBAD-pre-pro4subtilisinE and pBAD-pre-prosubtilisinE-SPDnd, were grown and induced as previously described. The results demonstrated that no production of active and soluble recombinant protein using pBAD/gIII expression system was achieved, even in the presence of the original leader sequence of *B. subtilis* (figure 6).

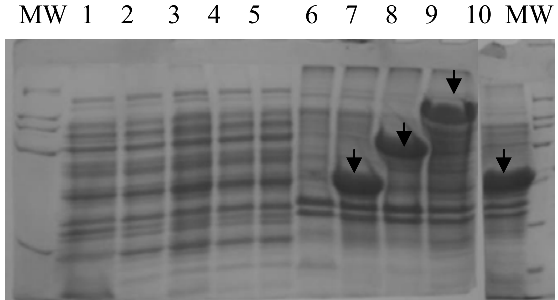

Figure 5. SDS-PAGE of proteins from *E. coli* LMG194 cells grown at 37 ºC, induced with 0.2% arabinose at 18 ºC. *Lanes 1* to *5*: soluble fractions of negative control pBAD, pBAD-prosubtilisinE, pBAD-pro2subtilisinE, pBAD-pro4subtilisinE and pBAD-prosubtilisinE-SPDnd respectively and *lanes 6 to 10*: insoluble fractions of negative control pBAD, pBAD-prosubtilisinE, pBAD-pro2subtilisinE, pBAD-pro4subtilisinE and pBAD-prosubtilisinE-SPDnd. *MW*: SDS-PAGE Standard, Broad Range (Bio-Rad). The *solid arrowheads* indicate the position of recombinant proteins.

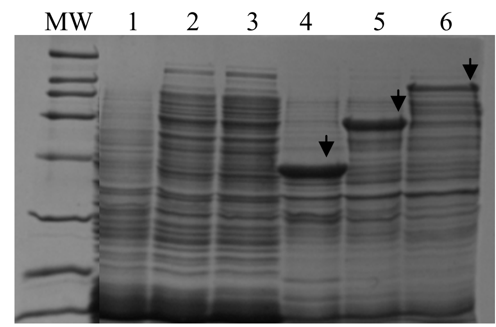

Figure 6. SDS-PAGE of proteins from *E coli* LMG194 cells grown at 30 ºC, induced 0.2% arabinose at 18 ºC. *Lanes 1* to *3:* soluble fractions of pBAD-pre-prosubtilisinE, pBAD-pre-pro2subtilisinE and pBAD-pre-pro4subtilisinE respectively and *lanes 4* to *6*: insoluble fractions of pBAD-pre-prosubtilisinE, pBAD-pre-pro2subtilisinE and pBAD-pre-pro4subtilisinE. *MW*: SDS-PAGE Standard, Broad Range (Bio-Rad).
The *solid arrowheads* indicate the position of recombinant proteins.

3.3. Expression of recombinant proteins using pET11b expression system

Since *B. subtilis* subtilsinE was previously expressed in *E. coli* pET11 system (Hu *et al.,* 1994), the genes coding for the native enzyme, as well as, the three chimeric enzymes, pro2subtilisinE, pro4subtilisinE and prosubtilisinE-SPDnd were cloned into this vector, using *E. coli* BL21(DE3) as host strain. The transformants carrying pET11 vectors were able to produce all the constructs at a level of almost 80 % of total cellular proteins in the presence of 1 mM IPTG (Figure 7).

After cell disruption, by ultra-sonic treatment, the products isolated in the pellets, were collected by low-speed centrifugation, indicating that the proteins aggregated to form inclusion bodies. Purification and refolding of native and chimeric enzymes were carried out with the isolated inclusion bodies as described under Materials and Methods. All samples were assayed for azocasein activity. Except for native subtilisinE, (3.2 U), no activity was detected for chimeric enzymes pro2subtilisinE, pro4subtilisinE and prosubtilisinE-SPDnd (data not shown).

Mature subtilisinE and pro2subtilisinE purified and renatured from inclusion bodies were used for circular dicroism analysis. Compared to active mature subtilisinE, chimeric pro2subtilisinE presented only 30% of secondary structure (data not shown) which suggests that in vitro renaturation of chimeric enzymes do not result in the correct folding necessary for enzymatic activity.

3.4. Prosequence mediated folding

E. coli LMG194 cells carrying the construction pBAD-prosequence, grown at 30 ºC and induced at 18 ºC, were able to express the prosequence at high level in the soluble, as well as, in the insoluble fractions (Figure 8).

Soluble fractions were purified by IMAC using a nickel column, and used for refolding procedures. Prosequence-mediated folding was performed by addition of prosequence to recombinant proteins purified from inclusion bodies in a 1:1 molar ratio and incubation at 4 ºC for 12 h. All the experiments were repeated three times. In previous work developed by Shinde and its collaborators (1993) the refolding of Gdn-HCl-denatured subtilisinE was achieved by incubation with prosequence in the same

conditions described above. In all the assays performed refolding of prosubtilisinE was attained, since activity was recovered, but refolding of chimeric pro2subtilisinE, pro4subtilisinE and prosubtilisinE-SPDnd enzymes was not achieved (data not shown).

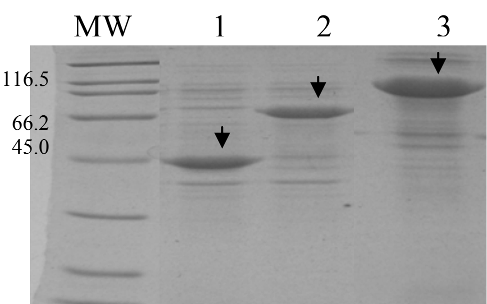

Figure 7. SDS-PAGE of insoluble fractions from *E coli* BL21(DE3) cell cultures grown at 37 ºC, induced with IPTG 1 mM at 37 ºC. L*ane 1:* pET11-prosubtilisinE*, lane 2*: pET11-pro2subtilisinE and *lane 3*: pET11-pro4subtilisinE. *MW*: SDS-PAGE Standard, Broad Range (Bio-Rad). The *solid arrowheads* indicate the position of recombinant proteins.

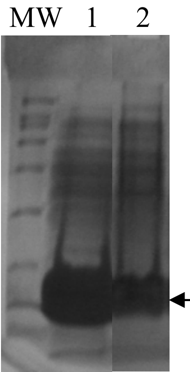

Figure 8. SDS-PAGE of proteins from *E. coli* LMG194 transformed with pBAD C*-prosequence. *Lane 1:* soluble fraction and *lane 2:* insoluble fraction. *MW*: SDS-PAGE Standard, Broad Range (Bio-Rad). The *solid arrowhead* indicates the position of recombinant prosequence.

4. Discussion

The results demonstrated that chimeric subtilisins were not expressed with the correct folding using three different *E. coli* expression systems, pET25b (+), pET11b and PBAD C.

The first objective was to choose an expression system for periplasmic secretion of recombinant proteins. Compared to cytosolic production, secretory production provides several advantages, for example, the possibility to obtain proteins with authentic N-termini, after cleavage of signal sequence by a specific signal peptidase; enhanced disulphide-bond formation, because periplasmic space provides a more oxidative environment than the cytoplasm; decreased proteolysis and minimization of harmful actions of recombinant proteins which are deleterious to the cell (Makrides, 1996). Furthermore, the periplasm contains only about 100 proteins as compared with about 400 proteins in the cytoplasm, so recombinant protein purification procedures can be more efficient when proteins are targeted to the periplasm (Blattner *et al.,* 1997).

Although active subtilisin has been expressed in *E. coli* periplasm using the IPTG inducible pIN-III-ompA vector, the amount of functional enzyme obtained was very low (Ikemura *et al.,* 1987). This vector has a strong Ipp promoter, as well as, a lac promoter operator fragment to ensure that expression is dependent on the addition of a lac inducer (Masui *et al.,* 1984). A disadvantage of this promoter is the absence of complete down-regulation under non-induced conditions, since an early overproduction of chimeric subtilisins could impair cell growth. Therefore we used a more tightly regulated IPTG-inducible system, the pET system, an alternative expression system for common lab-scale fermentations. pET vectors have a T7 promoter which is transcribed only by T7 RNA polymerase and must be used in strains carrying a chromosomal T7 RNA polymerase gene, that is under the control of a lac promoter (Studier, 1991; Studier *et al.,* 1990). pET25b (+) vector allowed to control the level of chimeric subtilisins expression. By having the pelB leader sequence it is possible to direct the proteins for periplasmic space which, in combination with the addition of a 6 x His tag to the enzyme´s C-terminus, improves recombinant proteins purification. It also allows easy immunological detection by adding the C-terminal HSV-epitope tag. Recombinant subtilisins were over expressed, but in a misfolded and inactive form, associated with *E.*

coli insoluble proteins. In order to test another signal sequence, different from pET25b (+) pelB leader, the genes were cloned into pBAD/gIII C expression vector. This vector, that contains the pBAD promoter from the arabinose operon, is tightly regulated and contains the gene III signal sequence utilized for secretion of the recombinant protein into the periplasmic space. Similarly to the results obtained for pET25b (+), also using pBAD C expression system none of the recombinant proteins were secreted to the periplasm. Since periplasmic expression did not revealed to be appropriated for the recombinant proteins under study, chimeric genes were subsequently cloned into pET11b. Using this system, proteins were expressed in the form of inclusion bodies. The dense inclusion bodies could be rapidly recovered by centrifugation and a high purity of proteins preparations obtained. The main disadvantage with inclusion bodies formation is the need for solubilization and refolding steps, necessary to achieve the correct folding and activity of recombinant proteins. This strategy was efficient to fold native subtilisin E correctly but not the chimeric enzymes. We are currently analysing the factors affecting solubility and studying alternative systems for chimeric enzymes expression. The co-expression of chimeric proteins with chaperons could be a strategy to promote the correct folding and to increase solubility of recombinant subtilisins. Different chaperone plasmid sets, able to express multiple molecular chaperons, have been successfully used to increase recovery of expressed recombinant proteins in the soluble fraction. Such proteins were hardly recoverable using conventional methods due to the formation of inclusion bodies (Nishihara *et al.,* 1998; Kim *et al.,* 2005; Schlapschy *et al.,* 2006). Addition of metal ions to culture medium could also have a positive effect on solubilization of recombinant proteins produced by *E. coli* (Yang *et al.,* 2003). If the expression of chimeric proteins in *E. coli* is found to be not effective, the genes could be cloned back into bacteria from the genera *Bacillus* (Silbersack *et al.,* 2006; Biedendieck *et al.,* 2007).

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Subchapter 3.3

Enzymatic Hydolysis of Wool with a Genetically Modified Subtilisin E

The work presented in this chapter includes parts of a manuscript to be submitted:
Araújo R, Machado R, Silva C, Cavaco-Paulo A, Casal M. 2008.
Enzymatic hydrolysis of wool with a genetically modified subtilisin E

Enzymatic Hydrolysis of Wool with a Genetically Modified Subtilisin E

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Keywords: Subtilisin E, protein engineering, protein-based polymer, wool hydrolysis

Abstract

In this work, we propose the construction of a novel high molecular weight subtilisin based on the fusion of prosubtitlin E DNA sequence from *Bacillus subtilis* with a DNA sequence that codifies to an elastin-like polymer. Wool yarns were treated with both commercial and chimeric subtilisins. It was shown that using the chimeric subtilisin there was a significant reduction of felting, pilling and tensile strength loss of wool yarns since the hydrolysis is restricted to the cuticle of wool.

The results stated here are of great importance once it is reported for the first time the microbial production of a chimeric high molecular weight protease for wool surface hydrolysis. This novel process of enzymatic-controlled hydrolysis overcomes the unrestrained diffusion and extended fibre damage which is the major obstacle on the use of enzymes for wool finishing applications.

1. Introduction

The morphology of wool is highly complex, not only in the fibre stem but also on the surface as well. It is in fact the surface morphology that plays an important role in the wool processing. Unwanted effects such as shrinkage, felting and barrier of diffusion are most probably due to the presence of wool scales on the fibre surface (Negri *et al.,* 1993).

Wool surface treatment with proteolytic enzymes seems to be a promissing alternative to the traditional pollutant Chlorine/Hercosett process. However, enzymatic reaction needs to be controlled, either by chemical or genetic modification, to avoid diffusion of enzyme into wool cortex and consequent fibre damage (Silva *et al.*, 2004; Silva *et al.*, 2005).

In contrast to chemical modification, the size and entire sequence of aminoacids of a recombinant high molecular weight protein can be precisely controlled bythe DNA coding sequence. In previous work developed by our group different cloning strategies were used to increase subtilisin E molecular weight. Although the recombinant enzymes were expressed, the soluble and active form of proteins was not achieved (Araújo *et al.*, 2008).

In this work we have used a protein based polymer to solubilize subtilisin E. A protein polymer is a polypeptide chain composed of amino acids sequences, commonly found in nature, which are arranged within a block or a set of blocks which are repeated in tandem, producing a high molecular weight repetitive protein (Urry, 2006). This type of polymers has been used for the solubilization and purification of hard proteins (Banki *et al.*, 2005, Trabbic-Carlson *et al.*, 2004). Since this is a high molecular weight polymer this may also function as an interesting possibility to functionalize subtilisin E for wool finishing applications.

The present work compares the behaviour of two proteases, the commercial subtilisin, Esperase, with low molecular weight, and a chimeric hight molecular weight subtilisin-GAG220, in the diffusion and hydrolytic attack to wool fibres.

2. Materials and Methods

2.1. Bacterial strains, plasmids, and enzymes

The *Escherichia coli* strain BL21(DE3), the T7 plasmid pET25b (+) and Overnight Express Instant TB Medium were purchased from Novagen (Madison WI, USA). Restriction and modification enzymes were from Roche Applied Science, (Germany). The commercial enzyme used in this study was the protease Esperase (E.C.3.4.21.62) from Sigma-Aldrich. Unless specifically stated, all the other reagents were from Sigma-Aldrich (St. Louis MO, USA).

2.2. Wool material

Untreated pure wool woven fabrics were provided by Albano Antunes Morgado Lda, Portugal.

2.3. Construction of chimeric prosubtilisin E-polimer

The strategy used for the construcion of polymeric gene was previously described (Girotti *et al.*, 2004; Rodríguez-Cabello *et al.*, 2005) and reported as the "Gutenberg Method". Briefley, the DNA coding for the peptide monomer containing 10 repetitions of VPAVG, was chemically synthesized and subjected to concatenation. The multimeric block genes (flanked by *Eam*1104I recognition sites) were obtained by recursive directional ligation in the cloning vector (Machado *et al. unpublished work*).

The construction/concatenation was performed in a modified cloning vector, pDrive (Qiagen) resulting in the construction pDrive:VPAVG220. Construction was confirmed with the restriction enzymes *Eam*1104I and *Eco*RI (Fermentas).

The construction pDrive-GAG220 was digested with *Mbo*II. The fragment *Mbo*II-VPAVG220-*Mbo*II was Klenow blunted and purified from a 1% (w/v) agarose gel electrophoresis. After DNA extraction the gene was cloned into the *Xho*I digested, Klenow blunted and dephosphorilated pET25b-prosubtilisinE (Araújo *et al.*, 2008), resulting in the final construction pET25-prosubtilisinE-GAG220. This vector was used to transform *E. coli* strain XL1 Blue, according to the SEM method (Inoue *et al.*, 1990).

The plasmid construct was verified by restriction with *Lgu*I and by DNA sequencing, following the method of Sanger (Sanger *et al.*, 1977), using an ABI PRISM 310 Genetic Analyzer. DNA cloning and manipulation were performed according to the standard protocols (Sambroock *et al.*, 1998). The recombinant plasmids were then transformed into the expression strain *Escherichia coli* BL21(DE3) (Novagen).

2.4. Protein expression and purification

Expression host strain BL21(DE3) transformed with pET25:proSubtilisin-VPAVG220 was used for protein expression. Bacterial cultures were grown at 30 ºC in Novagen Auto-induction medium, containing 100 μg/mL ampicilin. After fermentation time, (usually overnight) the cells were harvested by centrifugation, washed with phosphate buffered saline solution (10 mM Na_2HPO_4, 2 mM KH_2PO_4, 137 mM NaCl, 3 mM KCl, pH 7.4) and lysed by ultrasonic disruption (Ultrasonic Processor GEX 400). Supernantant was reserved for protein purification and the pellet, insoluble debris, was ressuspended in phosphate buffered saline solution and reserved for analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, SDS-PAGE.
Recombinant protein was purified using an IMAC system with a HisPrep FF 16/10 column (GE Healthcare) already prepacked with pre-charged Ni Sepharose 6 Fast Flow. Column equilibration was performed with 10 mM imidazole, 0.5 M NaCl, 20 mM phosphate buffer pH 7.6. Samples were applied onto the column at a flow rate of 2 $ml.min^{-1}$. Elution was performed with a buffer containing 80 mM imidazole, 0.5 M NaCl and 20 mM phosphate buffer, pH 7.6.
Proteins were detected by SDS-PAGE using a Tris-SDS-glycine buffer system (Laemmli, 1970). SDS PAGE gel images were acquired with Molecular Imager ChemiDoc XRS system and Quantity One software from Biorad. Protein detection was done by Coomassie Brilliant Blue R250.

2.5. Enzyme assay and protein concentration

The activity of commercial Esperase and chimeric subtilisin was measured, according to Silva *et al.*, (2004) using casein as substrate. One unit of activity is defined as the

amount of enzyme that hydrolyses casein to produce equivalent colour to 1 μmol of Tyrosine, per minute, at pH 7.5 and 37 ºC (colour by the Folin & Ciocalteu's reagent). The total protein concentration was determined by a modification of the micro Lowry method (Lowry *et al.*, 1951), using bovine serum albumin as standard and using Sigma test kit no. P5656.

2.6. FITC linkage to proteins

Enzymes (same Units of both enzymes were used) were linked to FITC (100/1, w/w) in sodium carbonate buffer pH 8.5. Free FITC was removed using HiTrap desalting columns (Amersham). Wool samples were treated in this solution at 37 ºC, 20 rpm, for 24 h.
Wool fibres cross-sections were analyzed on a Leica Microsystems DM-5000B epifluorescence microscope with appropriate filter settings using a 100× oil-immersion objective. Images were acquired with a Leica DCF350FX digital camera and processed with LAS AF Leica Microsystems software.

2.7. Pre-treatments performed on wool yarn

Before enzymatic treatment, wool yarns were subject to two different pre-treatment washings:
Surfactant washing (S): wool was washed with Lutensol ON 30 (non-ionic surfactant) 1 g/l, in a bath ratio 1:20, at pH 9.0 (Na_2CO_3 0.1 M and $NaHCO_3$ 0.1 M buffer), for 30 min, at 40 ºC, on a Rotawash machine. After the washing procedure, the surfactant was removed from wool first with tap water, followed by distilled water.
Bleaching washing (S + B): After the previous washing, wool was immersed in a bath with 1% (o.w.f.) H_2O_2, at pH 9.0 (Na_2CO_3 0.1 M and $NaHCO_3$ 0.1 M buffer), for 1 h at 55 ºC, on Rotawash machine.

2.8. Enzymatic treatment of wool yarns

Enzymatic treatment experiments were performed in plastic boxes each containing 0.5 g of 23 μm (mean diameter) wool yarns, subjected to the different pre-treatments

described above. Volumes of phosphate buffer solution (pH 7.6, 0.01 M) and protein stock solution were added to the sorbent so that every flask contained the same total volume (50 ml) and the same units of enzyme activity. Then, the flasks were closed and rotated at 90 rpm for 24 h at 37 ºC in a shaking water bath. Several controls were run simultaneously: a control test with wool without pretreatment and without protein (C), a control test with surfactant washed wool (C, S) and without protein and a control test with bleaching washed wool (C, S+B) and without protein. After incubation, wool yarns were removed and washed for tensile strength, felting and pilling evaluation.

2.8.1. Tensile strength

Tensile strength resistance was determined by using a tensile tester machine, accordingly to ASTMD5035-90. The samples were conditioned before testing in a standard atmosphere.
The tensile strength resistance values are given as the mean of an $n \geq 10$ replicates, together with the standard deviation (the coefficient of variation was bellow 10% for all cases).

2.8.2. Felting and pilling

Felting and pilling were visually evaluated after repeated washing (3 times) at 50 ºC, for 60 min and 20 rpm.

3. Results

3.1. Expression and purification of recombinant protein

E. coli BL21(DE3) transformants carrying pET25:proSubtilisin-GAG220 grown and induced at 30 ºC were able to express the quimeric enzyme at high level in the soluble fraction (Figure 1A). The SDS-PAGE analysis of the cellular lysate revealed the presence of a soluble chimeric protein with a high molecular weight (> 116 kDa). This chimeric protein was efficiently purified from all other contaminating proteins present

in the cellular lysate (Figure 1B). Preparations of purified chimeric enzyme were used for wool treatment experiments.

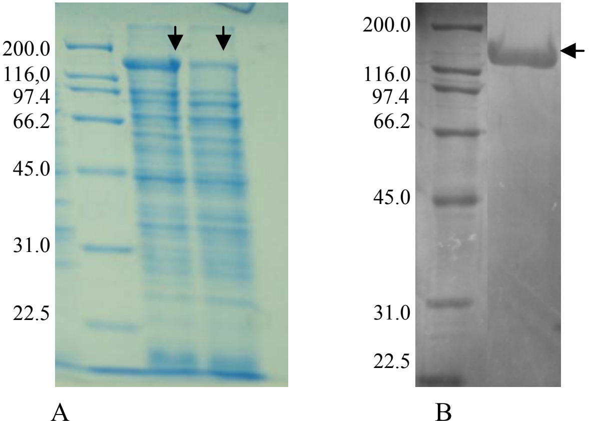


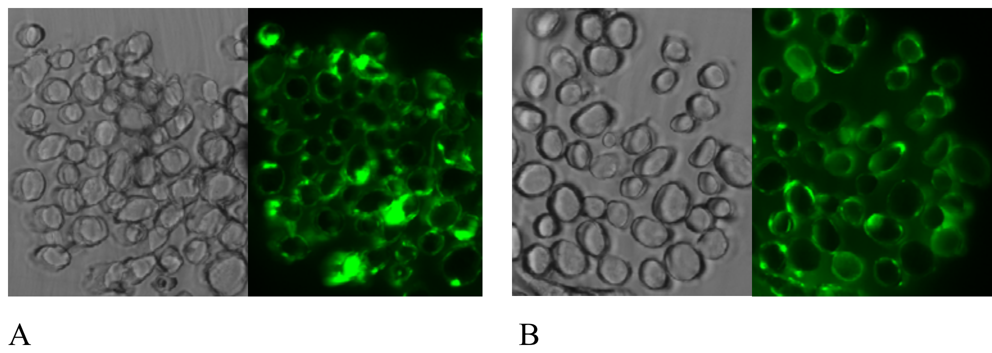
Figure 1. SDS-PAGE of A) soluble fraction from two different clones of *E. coli* BL21(DE3) transformed with pET25:prosubtilisin-VPAVG220 and B) purified subtilisin-VPAVG$_{220}$ polymer stained with copper chloride. The *solid arrows* indicate the position of recombinant protein.

3.2. Effect of subtilisin size

3.2.1. On diffusion trought the fibre

Native subtilisins have a molecular mass of approximately 30 kDa, which is the major drawback on the application of subtilisins for wool treatment. Due to their relatively small size the enzymes can diffuse into the fibre cortex causing the degradation of the internal parts of wool structure. We postulate that an increase of more than four-fold on subtilisin E molecular weight would prevent diffusion on enzyme into wool. To follow the diffusion of the enzymes into yarns, they were fluorescently labelled with a fluorescent dye, FITC. After the covalent coupling of enzymes to FITC, wool yarns were incubated in these solutions for 24 h. After enzymatic treatment, the yarns were entrapped in a non-fluorescent resin and sliced in thin layers with a microtome. Fluorescence microscopy images show that native subtilisin penetrates completely

inside the fibre cortex while chimeric subtilisinE-VPAVG220, with a molecular weight higher than 116 kDa, appears to be restricted to the surface of yarns (Figure 2A and B). Silva and collaborators have obtained similar results after chemical modification of subtilisins with PEG and Eudragit S-100. The chemically modified enzymes presented molecular weights higher than 97 KDa which appeared to be effective to limit the hydrolysis only at the wool cortex (Silva *et al.*, 2004; Silva *et al.*, 2005).
Fluorescence microscopy results provide good indication that chimeric subtilisin-VPAVG220 has the proteolytic activity restricted to wool surface.



A B

Figure 2. Fluorescence microscopy images of fibre cross-sections of wool yarns treated with FITC-labelled commercial Esperase (A) and chimeric subtilsin-VPAVG220 (B), (100x).

3.3.2. On yarns tensile strength

The wool fibre cuticle is covered by a covalently bound lipid layer, the main responsible for the hydrophobicity of wool. Alkaline pre-treatments can partially remove some of these lipids reducing its hydrophobic nature and enhancing at the same time protein diffusion inside the fibre (Brack *et al.*, 1999). Wool yarns were then subjected to two alkaline pre-treatments, a scouring washing (S) and a scouring washing followed by bleaching (S + B). Wool yarns previously pre-treated were incubated with the same units of commercial subtilisin and chimeric subtilisin-VPAVG220 for 24 h for tensile strength resistance valuation.
Figure 3 demonstrate that there are no significant differences in the tensile strength of yarns subjected to both pre-treatments compared to the control samples.

The maximum tensile strength supported by wool yarns was drastically lower for samples treated with commercial subtilisin. This treatment promoted more than 50% of reduction in the original tensile strength of yarns, indicating higher fibre degradation as a consequence of enzyme diffusion. On the other hand wool yarns incubated with chimeric subtilisin-VPAVG220 retained maximum tensile strength comparable to those of control samples (without enzyme). It seems that the high molecular weight subtilisin-VPAVG220 is retained at the surface of wool yarns. Since there is a strong reduction of diffusion of enzyme inside the wool fibres, only the cuticle is under proteolytic attack what can explain the higher tensile strength of yarns after enzymatic treatment.

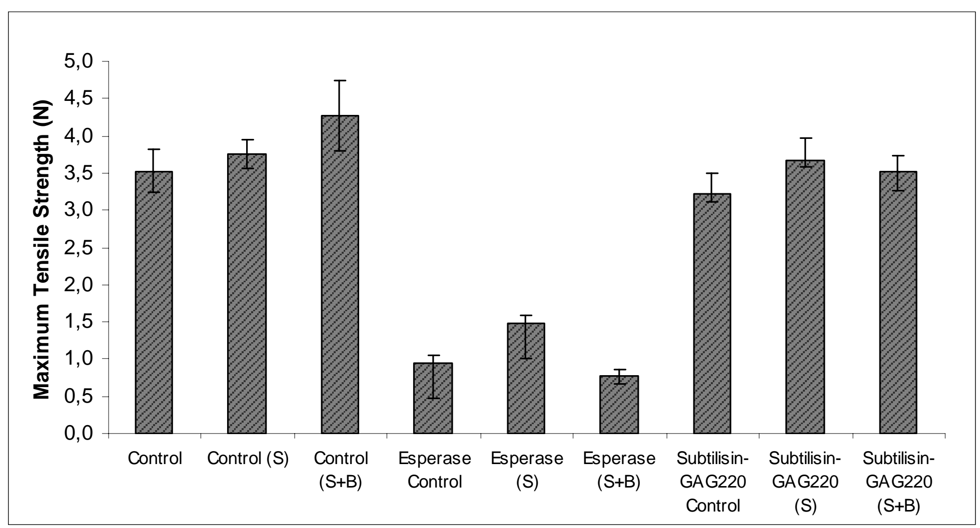


Figure 3. Maximum tensile strength (N) supported by wool yarns subjected to different pre-treatments without enzyme and yarns treated with the same enzyme units of commercial and chimeric subtilisin.

3.3.3. On yarns induced damage

To evaluate the damage of enzymatic treatment on wool yarns, samples pre-treated as previously described and incubated with both enzymes were washed for 3 consecutive cycles in a rota-wash machine. Felting and pilling were visually evaluated (Figure 4). Both pre-treatments seem to induce a slight degree of damage (although we found no differences on yarn´s tensile strength) (Figure 3). This degradation is higher when commercial Esperase is used. Wool yarns treated with Esperase presented a higher level

of felting and pilling than samples treated with chimeric enzyme. In fact it seems that yarns treated with subtilisin-VPAVG220 felted even less than the control samples what emphasize the idea that, due to its size, the hydrolytic activity of chimeric enzyme is restricted to the surface of wool yarns and also that the elastomeric polymer VPAVG220 can provide some kind of protection of yarns against excessive damage after 3 cicles of washing.

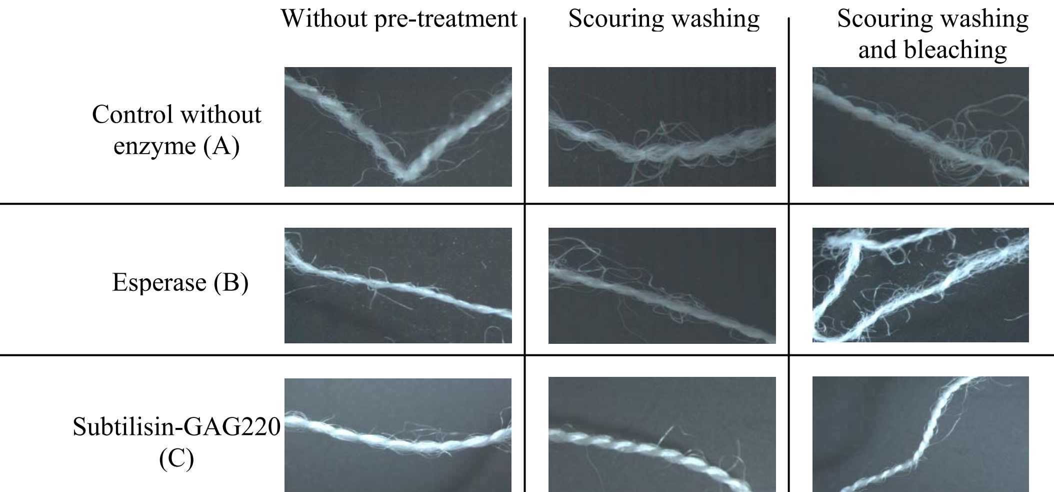


Figure 4. Visual damages on wool yarns after 3 cycles washing in a Rotawash machine. A) Wool yarns without enzyme; B) wool yarns treated with commercial subtilisin and C) wool yarns treated with subtilisin-VPAVG220.

4. Conclusion

In this work we have achieved the production of a recombinant high molecular weigh subtilisin through the fusion of subtilisin E DNA sequence with a sequence that codifies to an elastomeric polymer. The effect of chimeric high molecular weight subtilisin on wool yarns was compared to the commercial Esperase. It was found that, as already expected, due to its small size, the commercial subtilisin is able to penetrate inside the wool cortex, damaging the fibre.

Chimeric subtilisin-VPAVG220, the high molecular weight enzyme, hydrolyzed just the cuticle layer of wool. Yarns treated with this enzyme presented higher tensile strength and lower felting.

The recombinant hight molecular weight enzyme can be a promissing economical and ecological alternative to the tradicional chlorine treatment.

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Chapter 4

General Discussion and Future perspectives

General Discussion and Future perspectives

The introduction of synthetic fibers in textile industry, like polyethylene terephthalate, nylons or acrylics, had a significant impact on the quality of life. However, nowadays, there is an increased demand for natural fibers, especially for their properties including aesthetics, comfort, and biodegradability. Besides the traditional natural materials like cotton, wool and silk, many researches are also focused on exploring fibers from alternative sources like agricultural by-products, which are often underutilized (Collier *et al.*, 1992).

Independently on the use of natural or synthetic fibers, with old or new agricultural materials, the textile industry must search for sustainable technologies and develop methods of processing and finishing fabrics that meet customer expectations with the finished products performance, human health and environmental safety. Fiber surface modification has been one of the main areas of research in the development of functional fibers. In addition to research in developing/synthesizing new fiber forming polymers with specialized properties, surface modification offers many new opportunities.

Properties of fibers such as anti-shrinkage, anti-microbial, anti-odor, anti-fungal, anti-static, higher hidrophylicity, dyeing, soil resistance, adhesion, biocompatibility, are all function of fiber surface properties.

For the last years chemical modification has been employed with variable level of success. Chemical modification relates to an alteration of chemical structure. It can be conducted in the stage of synthesis of fibre-forming polymers by 3 methods: (1) copolymerization of the initial fibre-forming monomer with second comonomers containing the functional groups that carry new properties; (2) by addition of new side functional groups and (3) inclusion of new substances that react with the fibres during processing (Kozlowski, 1998; Rouette, 2001). Chemical modification is also possible in the stage of finishing materials being effective giving new properties to the fibres (Carr, 1992; Lee *et al.*, 2005; Freddy *et al.*, 2002; Freddy *et al.*, 1999). However, these treatments are not environmentally benign and frequently reduce the

quality of the fibres causing losts of fibre material during the process (Shukla *et al.*, 1997; Zeronian and Collins, 1989).

Nowadays, due to environmental concerns and especially due to more strict legislation and regulations on the wastewater discharges that were established and implemented, there is an incresed interest in the replacing of the chemical traditional textile processing for enzymatic processes. Enzymes can offer to the textile industry the ability to reduce costs, protect the environment, address health and safety and improve quality and functionality. Genetic engineering allowed the developement of many automated protocols for screening proteins with desirable properties and then provides the tools for amplification, cloning, expression of genes and purification of the recombinant selected proteins. However, in some cases, the technological application of enzymes under the demanding industrial conditions is not possible. In fact, among all the enzymes known, only a few are recognized as commercial products.

Molecular geneticsa associated with techniques of site-directed mutagenesis and/or random mutagenesis led to newer enzymes with altered functions that are desired for their application like improvement of catalytic efficiency, higher substrate specificity and increased stability.

For instance, the structure and function of cutinases are well studied, and genetic engineering was previously used to improve their properties for several applications as for example, fat stain removal in detergents (Carvalho et al. 1999; Longhi and Cambillau 1999).

In the first part f this work the potential of molecular genetics tools to modify cutinase from *Fusarium solani pisi* was demonstrated. Site-directed mutagenesis of cutinase was carried out to enlarge the active site, which could then better accommodate polymeric synthetic substrates like polyamide (PA) or polyethylene terephthalate (PET). Several cutinase mutants, all of which exhibited an enlarged active site were expressed. A single amino acid replacement, L182A, was shown to better stabilise the PET model substrate 1,2-ethanodiol dibenzoate tetrahedral intermediate at the enzyme active site (Subchapter 2.2- Araújo *et al.*, 2007). This mutant also showed increased PA-hydrolysing activity and up to five-fold higher

activity on PET when compared with the native enzyme (Subchapter 2.3- Silva *et al.*, 2007 and Subchapter 2.4- O'Neill *et al.*, 2007).

Similarly the activity of a nylon-oligomer hydrolysing enzyme EII' from *Flavobactererium* sp. was increased 200-fold by genetic engineering (Negoro, 2000). In addition to genetic engineering, reaction engineering (temperature and additives) seems to be an important factor and enzymatic hydrolysis of PA can be increased in the presence of solvents (Silva et al., 2005a).

Not only the structural design of the active site of the enzymes but also the regions required for sorption and for guiding the enzyme along the substrate, might be important for polymer hydrolysis. Cellulose-binding modules (CBM) accomplish this role in cellulases. For this reason, the biomodification of the surface of cellulose acetate was performed with cutinase fused with either the carbohydrate-binding module of Cellobiohydrolase I, from the fungi *Trichoderma reesei*, or the carbohydrate-binding module of Endoglucanase C, from the bacteria *Cellulomonas fimi*. The knew recombinant cutinase fused to the fungal CBM presented better performance hydrolysing cellulose diacetate and improving the colour levels of treated fabrics (Subchapter 2.5- Matamá *et al.*, 2008).

The second part of this thesis relates to the biomodification of wool, to accomplish total easy care wool i.e. machine washability plus tumble dryability, to compete with other fibres.

The traditional method to confer dimensional stability to wool articles uses chlorine which has various drawbacks. Several enzymatic methods have been attempted to replace this hazardous chemical finishing treatment, without great success mainly due to diffusion of enzyme into wool cortex (Shen *et al.,* 1999). Chemical modification of proteases proved to be effective for wool finishing, however the presence of small amounts of free enzyme still representes a drawback (Silva *et al.*, 2004; Silva *et al.*, 2005b). In our work we used subtilisin E from *Bacillus subtilis*, genetically modified, in order to avoid its penetration inside the fibre.

The first attempt was to create a chimeric poly-subtilisinE composed of 2, 3 and 4 subtilisin units. Chimeric subtilisins were overexpressed but no active and soluble recombinant proteins were recovered (Subchapter 3.2- Araújo *et al.*, 2008a). The

other approache describes the fusion of subtilisin E with a high molecular weight elastin-like polymer (Subchapter 3.3- Araújo et al, 2008b). The chimeric enzyme presented a molecular weight above 116 KDa. Wool yarns treated with the chimeric enzyme and subjected to several machine washings, presented a significantly lower damage than wool treated with the native enzyme, in the same conditions. Moreover yarns treated with chimeric high molecular weight subtilisin presented a tensile strength resistance comparable to the original one while yarns treated with commercial enzyme kept less than half of its initial resistance.

The results presented in this thesis prove that molecular biotechnology provides the approaches that make possible the genetic modification and production of enzymes either by site-directed mutagenesis or by fusion with functional domains or protein-based polymers, wich can represent promising alternatives for fibres bio-finishing processes at an industrial level. Here we developed effective ways of hydrolysing both synthetic and natural fibres surface and created environmentally friendly options to the conventional chemical treatments.

Nevertheless, the power of molecular genetics approaches linked to biotechnology has not yet been fully exploited and the processes developed here need to be further characterized for its complete understanding and optimization.

Further studies will contribute to a better understanding of the interaction of these enzymes with the substrates concerning factors such as sorption, movement on the fibre surface and the role of binding modules. It is also our intention to design new genetically modified enzymes and upgrading the enzymatic surface modification technology from laboratory to a large-scale process, contributing for new ecological industrial processes.

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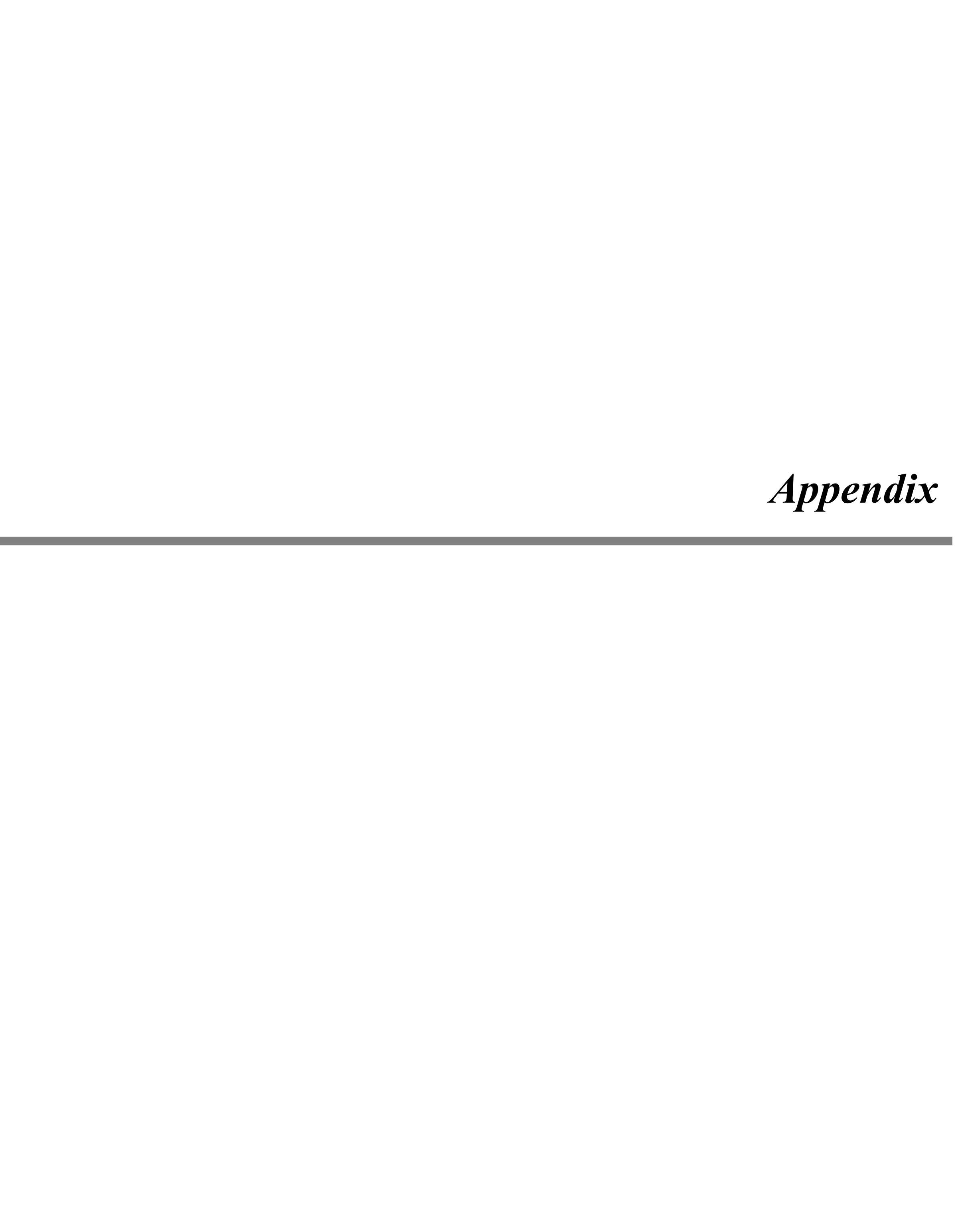
Appendix



Appendix I

Restriction enzyme(s) digestion of plasmid minipreps
(adapted from Sambrook *et al.,* 1989)

Add 2 µl of appropriate restriction enzyme buffer (10x), 11.5 (or 11) µl of ultra pure water, 0.5 µl restriction enzyme A (generally Roche 10 U/µl), (0.5 µl restriction enzyme B (generally Roche 10 U/µl) for double digestions) and 6 µl of plasmid DNA miniprep or midiprep. Incubate at appropriate temperature (37 ºC for most enzymes) for 3 h.
Analyse digestion fragments by gel electrophoresis.

Standard ligation of DNA fragments to plasmid vectores
(adapted from Sambrook *et al.,* 1989)

Digest DNA fragment and plasmid vector with appropriate restriction enzyme(s). Purify using QIAquick Gel Extraction Kit (QIAGEN).
To 50 ng of digested plasmid vector add digested DNA fragment enough to get a molar vector:insert ratio of 1:5 (insert quantity (ng) = (insert size x 50 x 5)/ plasmid size). Add 1 µl ligase buffer (10x), 1 µl of ligase T4 1 U/ µl (Roche) and, if necessary, ultra pure water to a final volume of 10 µl. Incubate overnight at 4 ºC.

Preparation and transformation of competent E. coli (XL1 Blue) SEM method
(adapted from)

Reagents

DMSO (Sigma)

IPTG (isopropyl-β-D-thiogalactopyranoside (Sigma)
40 mg/ml in sterilized destilled water

Luria-Bertani (LB)-Ampicillin agar

Tryptone	10 g/l
Yeast extract	5 g/l
NaCl	10 g/l
Agar	2%

Autoclave, cool to 50 ºC and add a stock solution of ampicillin 100 mg/ml to a final concentration of 75 mg/l, pour onto Petri plates.

LB medium (liquid)

Tryptone	10 g/l
Yeast extract	5 g/l
NaCl	10 g/l

Autoclave

SOB

Tryptone	2%
Yeast extract	0.5%
NaCl	10%

Autoclave, add sterilized $MgCl_2$ to a final concentration of 20 mM.

TE buffer

PIPES	10 mM
$CaCl_2$	5 mM
KCl	250 mM

Dissolve, adjust pH to 6.7 with KOH and add $MnCl_2$ to a final concentration of 55 mM, sterilize the solution by filtration and keep at 4 ºC.

X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)

40 mg/ml in dimethylformamide (DMF)

Preparation of competent *E. coli* (XL1 Blue)

Inoculate 250 ml SOB medium in a 2 L flask with 10 colonies;
Grow at 18 ºC with vigorous shaking (200-250 rpm) until an OD_{600} of 0.6.
Cool on ice for 10 min.
Spin cells down at 4ºC for 10 min at 2500 x g.
Resuspend cells in 80 ml of ice-cold TB buffer.
Cool on ice for 10 min.
Spin cells down at 4 ºC for 10 min at 2500 x g.
Gently resuspend pellet in 20 ml of ice-cold TB and add DMSO to a final concxentration of 7 %.
Leave on ice for 10 min
Distribute into 200 ml aliquots (into sterile, ice-cold 1.5 ml eppendorf tubes) and freeze in liquid nitrogen.
Store at -80 ºC.

Transformation of competent *E. coli* (XL1 Blue)

Thaw the competent *E. coli* cells on ice.
Add the experimental DNA (generally 1 µl of midi or mini and 10 µl of a ligation reaction) to 200 µl of competent cells.
Mix gently and incubate on ice for 30 min.

Heat-shock the tubes in a thermoblock, at 42 ºC for 30 s with gentle agitation.
Incubate the tubes on ice for 10 min.
Add 800 µl of SOC medium and incubate the tubes for at 37 ºC for 1 hour with vigorous shaking.
Spin cells down for a few seconds and discard about 950 µl of supernatant.
Ressuspend the pellet in the remaining 50 ul supernatant and plate on LB selective agar plates. For blue-white colour screening add X-Gal 40 mg/ml to a final concentration of 40 µg/ml and IPTG 40 mg/ml to a final concentration of 40 µg/ml. Allow the plates to dry before plating the transformation mixtures.
Incubate the plates at 37 ºC overnight.

Preparation of plamid DNA (Miniprep)
(adapted from Sambrook *et al.*, 1989)

Reagents

Alkaline lysis solution I
0.2 M NaOH
1% (w/v) SDS

Alkaline lysis solution II
NaAc 3 M, pH 5.2

Ethanol 100% and 70% (v/v)

Plate 8 *E coli* colonies, per Petri plate, on LB selective agar. Incubate the plates overnight at 37 ºC.
Resuspend ¾ of biomass, from each colony, in 200 µl of destilled water. Vortex for10 sec.
Add 200 µl of alkaline lysis solution I. Close the tubes tightly and mix the contents by inverting the tubes rapidly for four times. Do not vortex.
Add 200 µl of alkaline lysis solution II. Close the tubes tightly and disperse alkaline solution II through the viscous bacterial lysate by inverting the tubes rapidly for four times.
Centrifuge the bacterial lysate at maximum speed for 5 min at 4 ºC. Transfer the supernatant to a clean tube.
Precipitate nucleic acids from the supernatant by adding 500 µl of ethanol. Mix the solution by inverting four times.
Collect the precipitated nucleic acids by centrifugation at maximum speed for 10 min at 4 ºC.
Remove the supernatant and add 500 µl of 70 % ethanol to the pellet. Centrifuge at maximum speed for 5 min at 4 ºC.
Remove all of the supernatant and store the open tubes at room temperature until all the ethanol has evaporated.

Dissolve the nucleic acids in 30 µl of TE-RNase and incubate for 1 h at 37 ºC for RNA digestion.
Store nucleic acids at -20 ºC.

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BIOSYNTEX Biotechnical quality improvement of synthetic textile fibres GRD 2000-30110

ENZUP Enzymatic Up-grading of Wool Fibres 2005-032877

ELASTM Polímeros elastoméricos proteicos: da síntese biológica à moldação

FCT - POCI/CTM/57177/2004

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