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Abstract

Enzymatic Treatment of Polyamide 6.6 Fibres

The controlled hydrolysis of polyamide fibres surfaces leads to the formation of amino and carboxylic end groups. The presence of these groups improves hydrophilicity and creates further starting points for covalent bonding of certain compounds. The coupling of flame retardants, proteins and other compounds bring extra added value to polyamide fibres. Chemical hydrolysis, tradicionally used to modify the surface of these fibres, is an “all-or-nothing” event leading always to yellowing and loss of fibres resistance. Given that enzymes are large globular proteins, their catalytic action remains at the surface of the fibres, therefore preserving their intrinsic structure. This dissertation successfully presents the use of enzymes to functionalise the surface of polyamide 6.6 fibres.

The ability of a cutinase from *Fusarium solani pisi* and a protease, a Subtilisin from *Bacillus* sp. to hydrolyse polyamide 6.6 fibres was evaluated. Different methodologies were developed in order to monitor the formation of the products resulting from enzymatic hydrolysis. The first one is based on the reaction of the compound 2,4,6-trinitrobenzenesulfonic acid (TNBS) with the amino groups released to the bath treatment and the other one is based on the reaction of a specific wool reactive dye with the terminal amino groups at the surface of the treated fabrics. In this work it was proved that the enzymatic hydrolysis with cutinase or protease leads only to surface modifications, being the reaction selective.

Studies were performed in order to reduce the treatment time and increase enzyme adsorption, hence increasing hydrolysis efficiency towards polyamide 6.6 fibres, by incubating polyamide 6.6 fabrics with cutinase in the presence of organic solvents. The organic solvents used were benzyl alcohol (BA) and dimethylacetamide (DMA), which are commonly used in polyamide processing as dyeing assistants. The stability of cutinase in the presence of these solvents was evaluated. It was observed that cutinase activity is preserved for several hours in the presence of low amounts of organic solvents (half-life time of 26 hours for 1.5% of benzyl alcohol and half-life time of 14 hours for 1.5% benzyl alcohol+10% dimethylacetamide). In the presence of these organic solvents, the polyamide structure became more suitable to be modified by enzymatic action. The results obtained confirmed an increase of terminal amino groups at the surface of the fabrics incubated with cutinase in the presence of the referred organic solvents.

Cutinase was presented as a versatile enzyme with unusual stereolytic activity towards polyamide substrates however turnover rates were very low. The analysis of the 3D structure of cutinase from *Fusarium solani pisi*, (PDB code 1CEX), showed that the external, but closed active site, was hindering the access to the fibre substrate. In order to overcome this difficulty, the genetic modification of native cutinase was performed. The site-directed mutagenesis was performed by changing specific amino acid residues around the active site by alanine (L81A, N84A, L182A, V184A and L189A) and five mutations were obtained. All mutations were done to create more space in order to fit the large inaccessible polymer in the active site of the cutinase. Molecular modelling studies were performed by

docking the synthetic model substrate of polyamide 6.6 at the cutinase active site. These studies predicted that L182A mutation provided the best stabilization of polyamide model substrate which support the experimental results obtained (+19% of amines in the bath solution treatment; 25% of protein adsorption).

Cutinase and protease were not designed by nature to interact with synthetic substrates like polyamide 6.6, therefore the accessibility of these enzymes to the fibre surface as well as their adsorption can be restricted by the compacted structure of polyamide fibres. As other authors have reported, the process of enzyme adsorption is of major importance to the enzymatic hydrolysis of synthetic fibres. Different studies have revealed that the adsorption of proteins follows different steps and that mechanical agitation plays an important role in all of them. In order to study the synergism among mechanical agitation, enzyme adsorption and enzyme activity, studies were performed using different levels of mechanical agitation. The results obtained revealed that the surface functionalisation of polyamide fibres with cutinases or protease should be performed using low levels of mechanical agitation for short periods of time (4 hours using a vertical agitation – Rotawash MKIII machine, in absence of discs).

A practical application was found for the functionalised surfaces obtained. The amine-enriched surfaces obtained by enzymatic action of a protease from *Bacillus* sp. were used to immobilise an enzyme (a laccase from *Trametes hirsuta*). An immobilisation procedure was developed to immobilise laccase onto woven polyamide 6.6 supports using glutaraldeyde as the crosslinking agent with the inclusion of a spacer (1,6-hexanediamine) in some cases. A 2^4 full factorial design was applied to study the influence of pH, spacer, enzyme and crosslinker concentration on the efficiency of immobilisation. The factors enzyme dosage and spacer have played a critical role in the immobilisation process. Under optimised working conditions (29 UmL^{-1} of laccase, 10% of glutaraldehyde, pH=5.5, with the presence of the spacer), the half-life time attained was about 78 h (18% higher than that of free enzyme), the protein retention was about 34% and the immobilisation yield was 2%. Laccase immobilisation onto polyamide 6.6 matrices can be a promising system for bioremediation of contaminated soils, wastewater treatment, wine and other beverage stabilisation, and even biosensor applications.

The results obtained reveal that cutinase and protease are able to modify the surface of polyamide 6.6 fibres and that these enzymes can be used in different steps of fibre processing. Higher added value products can be obtained by polyamide 6.6 functionalisation with enzymes, however the process of enzymatic hydrolysis needs to be characterized in more detail in order to be applied into an industrial process.

Resumo

Tratamento Enzimático de Fibras de Poliamida 6.6

A hidrólise controlada da superfície das fibras de poliamida conduz à formação de grupos terminais amina e carboxílicos. A presença destes grupos melhora a hidrofilidade das fibras e cria possíveis locais para a ligação de certos compostos. A ligação de agentes retardadores da chama, de proteínas e outros compostos conduz à obtenção de fibras com maior valor acrescentado. No entanto, a hidrólise química tradicionalmente usada para modificar a superfície destas fibras, é um mecanismo "tudo-ou-nada" que provoca o amarelecimento e perda de resistência das fibras. Dado que as enzimas são proteínas globulares grandes, a sua acção catalítica reduz-se somente à superfície das fibras, preservando assim a sua estrutura. Esta dissertação apresenta com sucesso o uso de enzimas na funcionalização da superfície de fibras de poliamida 6.6.

Foi testada a capacidade de uma cutinase, do fungo *Fusarium solani pisi*, e de uma protease, da bactéria *Bacillus* sp., para hidrolisar as fibras de poliamida 6.6. Foram desenvolvidas diferentes metodologias para monitorizar a formação dos produtos resultantes da hidrólise enzimática. Uma delas é baseada na reacção do composto 2,4,6 - ácido trinitrobenzenosulfónico (TNBS) com os grupos amina libertados para o banho de tratamento. A outra baseia-se na reacção de um corante reactivo específico para a lã com os grupos amina terminais que permanecem na superfície dos tecidos tratados após hidrólise. Neste trabalho, foi provado que a hidrólise enzimática com a cutinase ou com a protease, conduz apenas a uma modificação superficial, sendo a reacção selectiva.

Foram efectuados estudos para reduzir o tempo de tratamento e aumentar a adsorção da enzima, aumentando assim a eficiência hidrolítica sobre as fibras de poliamida 6.6. Para isso, os tecidos de poliamide 6.6 foram incubados com cutinase na presença de solventes orgânicos. Os solventes orgânicos usados foram o álcool benzílico e a dimetilacetamida, os quais são geralmente usados no processamento da poliamida como auxiliares no seu tingimento. Foi também avaliada a estabilidade da cutinase na presença destes solventes. Constatou-se que a actividade da cutinase é preservada durante algumas horas na presença de baixas concentrações de solventes orgânicos (tempo de meia-vida de 26 horas para 1.5% álcool benzílico e tempo de meia-vida de 14 horas para 1.5% álcool benzílico + 10% dimetilacetamida). Na presença destes solventes, a estrutura da poliamida torna-se mais apta a ser modificada por acção enzimática. Os resultados confirmam um aumento de grupos terminais à superfície da fibra nos tecidos incubados com cutinase na presença dos referidos solventes.

A cutinase foi assim apresentada como uma enzima versátil com invulgar actividade esteriolítica sobre substratos de poliamida, no entanto os níveis de modificação obtidos foram muito baixos. A análise da estrutura tridimensional da cutinase do fungo *Fusarium solani pisi* (PDB code 1CEX), mostrava que o centro activo externo, mas fechado, da enzima impedia o acesso ao substrato. De modo a ultrapassar esta dificuldade, foi efectuada a modificação genética da cutinase nativa. A técnica de mutagénese dirigida foi usada para efectuar modificações na

enzima, substituindo aminoácidos específicos perto do centro activo por alanina, obtendo-se cinco mutações (L81A, N84A, L182A, V184A e L189A). Todas as mutações foram feitas de modo a criar mais espaço no centro activo onde o substrato polimérico pudesse encaixar. Os estudos de modelação molecular foram efectuados incorporando o substrato modelo de poliamida 6.6 no centro activo da enzima. Estes estudos mostraram que a mutação L182A apresentou os melhores resultados de estabilização do substrato modelo, os quais são suportados pelos resultados experimentais obtidos (+19% aminas na solução do banho de tratamento; 25% de adsorção de proteína)

A cutinase e a protease não foram "desenhadas" pela natureza para interagir com substratos sintéticos como a poliamida 6.6, pelo que a sua acessibilidade à superfície das fibras assim como a sua adsorção pode ser restringida pela estrutura compacta das fibras de poliamida. Como referido por outros autores, o processo de adsorção enzimática é de extrema importância na hidrólise de fibras sintéticas. Diferentes estudos revelaram que a adsorção de proteínas segue diferentes passos e que a agitação mecânica tem um papel importante em todo o processo. De modo a estudar o sinergismo entre a agitação mecânica, adsorção de enzima e actividade enzimática, foram realizados estudos usando diferentes níveis de agitação mecânica. Os resultados revelaram que a funcionalização da superfície das fibras de poliamida com cutinases ou com a protease deve ser efectuada usando níveis baixos de agitação mecânica durante curtos períodos de tempo (4 horas usando agitação vertical – Rotawash MKIII, sem discos).

Foi posteriormente encontrada uma aplicação prática para os tecidos previamente funcionalizados. As superfícies dos tecidos ricas em grupos amina, obtidas por acção enzimática de uma protease do *Bacillus* sp., foram usadas para imobilizar uma enzima (lacase de *Trametes hirsuta*). Foi desenvolvido um procedimento para a imobilização da lacase nos suportes de poliamida 6.6 usando o glutaraldeído como agente bifuncional, incluindo a 1,6-hexanodiamina em alguns casos, de forma a aumentar a distância entre a matriz e a enzima. Neste estudo foi usado o design factorial (2^4) para estudar a influência do pH, da 1,6-hexanodiamina, da concentração de enzima e de glutaraldeído, na eficiência da imobilização. A dosagem de enzima e a presença de 1,6-hexanodiamina tiveram um papel determinante no processo de imobilização. Em condições the trabalho optimizadas (29 UmL^{-1} de lacase; 10% de glutaraldeído, pH=5.5, na presença de 1,6-hexanodiamina), o tempo de meia-vida obtido foi de cerca de 78 h (18% mais elevado do que o obtido para a enzima livre), a retenção de proteína foi de cerca de 34% e o rendimento de imobilização foi de 2%. A imobilização da lacase em matrizes de poliamida 6.6 pode ser um sistema promissor na bioremediação de solos contaminados, no tratamento de águas residuais, na estabilização de vinhos e outra bebidas e até mesmo na aplicação de biosensores.

Os resultados obtidos demonstram que a cutinase e a protease são enzimas capazes de modificar a superfície de fibras de poliamida 6.6 e podem ser usadas em diferentes etapas do processamento da fibra. Através da funcionalização da poliamida 6.6 com enzimas podem ser obtidos produtos de maior valor acrescentado, no entanto o processo de hidrólise enzimática necessita de ser mais detalhadamente caracterizado de forma a poder ser aplicado num processo industrial.

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Abbreviations

AAGR – average annual growth rate

ABTS – 2,2'-azino-bis-(3-ethyl benzothiazoline-6-sulfonic acid)

AOT – dioctyl sulfosuccinate sodium salt

Asn – asparagine

Asp – aspartic acid

ATR – attenuated total reflectance spectroscopy

BA – benzyl alcohol

BCC – business communications company

BSA – bovine serum albumin

CV –crystallinity value

Cys – cysteine

DMA – dimethylacetamide

DNA – deoxyribonucleic acid

DOE – design of experiments

DSC – differential scanning calorimetry

Eq. – equation

FTIR – Fourier transform infrared

Gln – glutamine

GTA – glutaraldehyde

HEX – 1,6-hexanediamine

His – histidine

HLT – half-life time

IR - Infrared

IY – immobilisation yield

K/S – Kubelka-Munk relationship (K-absorption coefficient; S-scattering coefficient)

L81A – Leucine81alanine

L182A – Leucine182alanine

L189A – Leucine189alanine

mM - milimolar

Mt – million tones

NMR – nuclear magnetic resonance spectroscopy

N84A – Asparagine84alanine

o.w.f. – of weight of fabric

OX – oxyanione-hole

PA – polyamide

PAN – polyacrylonitrile

PDB – protein data bank

PET – polyethylene terephthalate

***p*NP –** *p*-nitrophenol

***p*NPB –** *p*-nitrophenyl butirate

***p*NPP –** *p*-nitrophenyl palmitate

PR – protein retention

PRO – protein

PVC – polyvinyl chloride

Sd – standard deviation

SEM – scanning electronic microscopy

Ser – serine

TCA – trichloroacetic acid

Tg – glass transition temperature

TI – tetrahedral intermediate

T*m* – melting temperature

TNBS – 2,4,6 – trinitrobenzenesulfonic acid

UV – ultra-violet

V184A – Valine184alanine

WAXD – wide angle X-ray diffraction

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Chapter 1

GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

Polyamide 6.6 is an aliphatic semi-crystalline polymer made up of adipic acid and hexamethylenediamine, with a particular combination of strength, flexibility, toughness, stiffness, wear and abrasion resistance. It is also known for its low friction coefficient, low creep and good chemical resistance (Guillen 1986; Reimschuessel 1989; Brandrup and Immergut 1989). Applications of polyamide 6.6 include textile fibres, membranes, food packaging, electronics, automotive parts and others (Jia *et al.* 2006). Despite all of the excellent properties exhibited, polyamide fibres present low hydrophilicity and low reactivity making complex its functionalisation at mild treatment conditions (Reimschuessel 1989). Moreover, textile materials made of polyamide (PA) are uncomfortable to wear because perspiration cannot penetrate the materials and evaporate. This poor water permeability is due to the hydrophobicity of synthetic polymers, which also leads to static cling and stain retention during laundering (Guebtiz and Cavaco-Paulo 2008). The surface of these synthetic polymers are not easily wetted, thus difficulting the application of finishing compounds and colouring agents. In addition, a hydrophobic material hinders water from penetrating into the pores of the fabric. Classical methodologies to improve fibre hydrophilicity, like alkaline or acid hydrolysis, lead to the deterioration of fibres properties such as irreversible yellowing and loss of resistance (Miller and Wilmington 1958; Shukla *et al.* 1997; Silva 2002; Guebitz and Cavaco-Paulo 2003; Cribbs and Ogale 2003). These processes are based on an "all-or-nothing" mechanism where the adsorption of high concentrations of chemicals can destroy the surface of the polymers. Recent studies clearly indicate that the modification of synthetic polymers with enzymes is an environmentally friendly alternative to chemical methods using harsh conditions. The major advantages of enzymes in polymer modification, compared with chemical methods, are milder reaction conditions and highly specific non-destructive transformations, targeted to surfaces leading to lower fibres damage (Eriksson and Cavaco-Paulo 1998; Guebitz and Cavaco- Paulo 2003; Cavaco-Paulo and Guebitz 2003; Hsiey *et al.* 2003; Guebitz *et al.* 2004; Fischer-Colbrie *et al.* 2006).

Polyamide 6.6 performance can be improved by enzymatic hydrolysis resulting in an amine-enriched surface that can play an important role in processes like the removal of heavy metal ions from aqueous solutions, removal of dyes from wastewater, biofouling prevention, covalent immobilisation of proteins or coupling of flame retardants (Karimi and Amirshahi 2000; Jia *et al.* 2006). Nowadays, with the strong market demand the development of products with high added value can be a good strategy to overcome the difficulties that textile industry is facing.

1.1 POLYAMIDE FIBRES

Polyamide represents a family of synthetic polymers first produced in 1935 by Wallace Carothers at DuPont (Guillen 1986; Stevens 1999).

The first product was a polyamide-bristled toothbrush (1938), followed more famously by women's polyamide stockings (1940). Polyamide 6.6 was the first commercially successful polymer and it was intended to be a synthetic replacement for silk. This fibre was used for parachutes production after the USA entered World War II in 1941, making stockings hard to find until the war's end (Moncrieff 1975; Guillen 1986; Burkinshaw 1995).

Commonly, commercial polyamides are manufactured using processes related to either of three basic approaches, namely:

i) polycondensation of diamines and dibasic acids, as exemplified by hexamethylene diamine and adipic acid (polyamide 6.6);
ii) polycondensation of ω-amino acids, as typified by 11-aminoundecanoic acid (polyamide 11);
iii) ring-opening polymerisation of lactams, such as ε-caprolactam in polyamide 6 (Burkinshaw 1995).

The structure of polyamide fibres is defined by both chemical and physical parameters. The chemical ones are related mainly to the constitution of the polyamide molecule and are concerned primarily with its monomeric units, end-groups and molecular weight. The physical parameters are related essentially to

chain conformation, orientation of both polymer molecule segments and aggregates and to crystallinity (Reimschuessel 1989).

1.1.1 CHEMICAL AND PHYSICAL STRUCTURE

Polyamide 6.6 is a semi-crystalline polymer synthesized by the polycondensation of equal amounts of a diacid (adipic acid) and a diamine (hexamethylenediamine). On mixing their solutions in methanol, a 1:1 salt is formed and precipitates out, being rather insoluble in this solvent. This is dissolved in water to give the concentrated solution which is added to the sealed autoclave; thus exactly equal numbers of the monomers in the form of salt ions are present as the reaction mixture is heated to around 550 K. The polymerisation proceeds in the autoclave for 3-4 hours typically. Ultimately nitrogen gas is injected under pressure to force the molten polyamide 6.6 out through the valve or spinneret system at the base of the autoclave. An average degree of polymerisation in the range of 40-70 is desirable when the polyamide 6.6 is intended for fibre production (Campbell 2000). Figure 1.1 represents the simplified polycondensation reaction of polyamide 6.6.

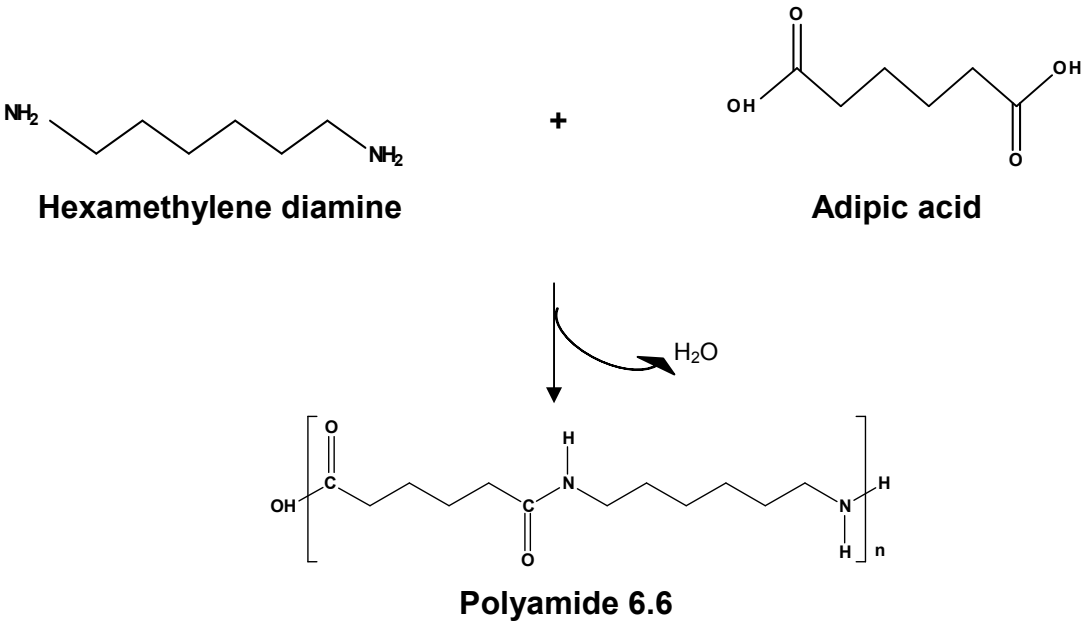


Figure 1.1 – Polycondensation reaction of polyamide 6.6 polymer (Guillen 1986).

Polyamide polymers are converted into fibres by melt-spinning processes, the yarn being produced either as continuous filament or converted into staple fibre; depending on the intended use of the yarn, it may also be texturised (Burkinshaw 1995).

The chemical characteristics of polyamide are determined by the functional end groups (-NH_2 and –COOH) and chain amide groups (-CONH-) present, the hydrocarbon chain portions of the polymer being relatively inert. Amide groups are the key units of many biological polymers (polypeptides). The differences in numbers of methyl units influence the profile properties of the different polyamides produced. The numbers 6.6 indicate how many –CH_2– units occur in the diacid and the diamine groups. The amount of the various groups present in a typical polyamide 6.6 sample shows that the quantities of acidic (-COOH) and basic (-NH_2) groups are not equivalent owing to the use of 'chain stoppers', such as acetic acid, to control the degree of polymerisation of the polymer (Burkinshaw 1995). The chemical reactivity of polyamide is located on terminal groups and on amide groups.

The number-average molecular weight of polymer suitable for textile fibre production ranges from 12.000 to 20.000 $gmol^{-1}$. If the molecular weight is below 6.000 $gmol^{-1}$ it is unlikely that the polymer will form fibres at all. Correspondingly, the molecular weight must not be allowed to become too high (over 20.000 $gmol^{-1}$), otherwise the polymer becomes difficult to melt or dissolve. Therefore, the process of polymerisation must not be allowed to go on indefinitely, but must be stopped at a given molecular weight (Moncrieff 1975).

The linear aliphatic homopolyamides are partially crystalline materials. Therefore they are characterized by both an unordered amorphous state and an ordered crystalline state. The latter may exhibit polymorphism.

The crystal structure of polyamide results from the conformation of the macromolecules and their packing. Generally, the packing of polymer chains will be such that the occupied volume is at a minimum, thereby minimising the potential energy of the structure, but maintaining appropriate distances for intermolecular forces between adjacent chain segments. In polyamides these

intermolecular forces are both Van der Waals bonds and hydrogen bonds (Reimschuessel 1989).

Polyamide 6.6 is a linear and partially amorphous material. It exists in two crystalline forms, α and β, the latter form representing an intermediate stage in the formation of the triclinic α form. The stable structure of polyamide 6.6 is the α structure that describes the 'even-even' polyamides. The term 'even-even' stems from the sequence of the methylene positions and on which side of the chain they are located. The chains in the α-structure are in a fully extended zigzag formation and form planar sheets of H-bonded molecules that are stacked upon one another. The crystal symmetry is triclinic with one chemical repeated unit per unit cell which differs in the c-axis dimension by a length proportional to the addition of methylene groups in the repeated unit. The only symmetry element is a center symmetry in both the diamine and the diacid portions and is preserved in the crystal structure. The even-even chains have no directionality so that parallel and anti parallel chains are equivalent (Reimschuessel 1989; Burkinshaw 1995).

In Figure 1.2 it is shown a schematic drawing of the unit cell and the principal crystallographic planes.

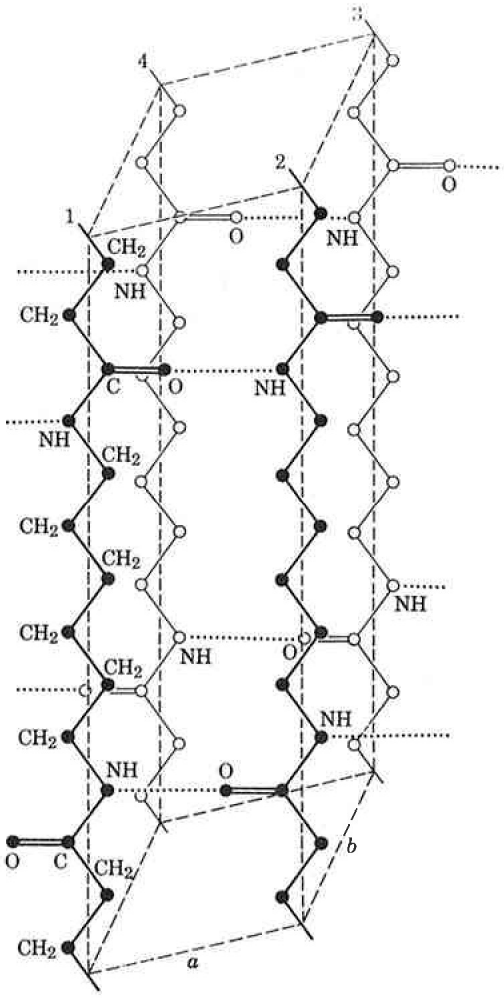


Figure 1.2 – Structure of α-cristal of polyamide 6.6, showing hydrogen-bonded sheets; a=4.9 Å, b=5.4 Å, c (fibre axis) = 17.2 Å (Mark *et al.* 1968; Guillen 1986).

The morphological structure of polyamide 6.6 being complex may involve paracrystals of any of the possible polymorphic forms, mesomorphic regions and essentially amorphous domains. This is indicated by a multiple melting phenomenon that was investigated by high-temperature x-ray techniques and differential scanning calorimetry (DSC), and has been explained on the basis of three phases: crystalline, intermediate and liquid amorphous (Reimschuessel 1989). The most important structural parameter of the noncrystalline (amorphous) phase is the glass transition temperature (Tg) since it has a considerable effect on both processing and the properties of the polyamide fibres. It is defined as the temperature, or temperature range, at which mobility of chain segments or

structural units commences. At 0% water, the Tg values for polyamide 6.6 are in the range of 40-57ºC as obtained by dilatometry or DSC, whereas those obtained by dynamic methods are in the range of 90-100ºC. Within these ranges the particular values are affected by other factors such as crystalline structure and orientation (Guillen 1986; Reimschuessel 1989).

Polyamide 6.6 is well conserved at low temperatures however it loses its resistance when exposed to high temperatures (above 150ºC) that cause the yellowing of fibres.

Above its melting temperature, T_m (265ºC) polyamide 6.6 is amorphous solid or viscous fluid in which the chains approximate random coils (Stevens 1999). Below T_m, amorphous regions alternate with regions which are lamellar crystals. The amorphous regions contribute to elasticity and the crystalline regions contribute to strength and rigidity. The planar amide (-CONH) groups are very polar, so polyamide forms multiple hydrogen bonds among adjacent strands. Because the polyamide backbone is so regular and symmetrical, especially if all the amide bonds are in the *trans* configuration, polyamides often have high crystallinity and make excellent fibres. The amount of crystallinity depends on the details of formation, as well as on the kind of polyamide. Apparently it can never be quenched from a melt as a completely amorphous solid.

Polyamide 6.6 can have multiple parallel strands aligned with their neighbouring amide bonds at coordinated separations of exactly 6 and 4 carbons for considerable lengths, so the carbonyl oxygen and amide hydrogen can line up to form interchain hydrogen bonds repeatedly, without interruption. When extruded into fibres through pores in an industrial spinneret, the individual polymer chains tend to align because of viscous flow. If subjected to cold drawing afterwards, the fibres align further, increasing their crystallinity, and the material acquires additional tensile strength. In practice, polyamide fibres are most often drawn using heated rolls at high speeds (Moncrieff 1975; Reimschuessel 1989; Campbell 2000).

1.1.2 CHEMICAL AND PHYSICAL PROPERTIES

Polyamide fibres are extremely chemically stable however they are influenced by other factors described further in the text.

The retention of water by polyamide 6.6 depends on the degree of orientation of the fibre, especially at high humidity, water absorption occurring predominantly at the strongly hydrophilic terminal amino groups and to those amide groups that are accessible. This property influences greatly the way fibres will dry, the comfort of handling and dyeability of polyamide fibres. The water absorption of polyamides prepared from diacids and diamines is proportional to the amount of amide groups per 100 atoms in the chain. It also depends on the fibre crystallinity being the values in the range of 3.5-4.5%. The electric properties of polyamide are also influenced by the capacity of humidity absorption. Electric conductivity is low due to the low water absorption and the insulating properties of polyamide are related with the facility to accumulate electrostatic charges. In conjunction with its relatively low moisture regain, polyamide 6.6 swells very little when immersed in water, but is hydrolysed at temperatures above 150ºC in the presence of water. Low swelling in aqueous media guarantees excellent dimensional stability when washed. The low density and low absorbency make them suitable for rainwear and swimwear. The polymers exhibit high resistance to aqueous alkalis, although severe hydrolysis of the amide link occurs at high temperatures. They are more sensitive to acids, being rapidly degraded by strong inorganic and organic acids. The diluted acids do not affect significantly polyamide 6.6 promoting however hydrolysis reactions. The acid degradation is of significance in dyeing as several types of dye are applied to the fibre under acidic conditions (Guillen 1986; Burkinshaw 1995). The basic products do not affect polyamide 6.6 fibres properties, although oxidants are able to alter them. The polyamide fibres are dissolved in formic acid, phenol, *m*-cresol, phenol derivatives and tricloroacetic acid (Araújo and Melo e Castro 1984).

Polyamide fibres are sensitive to light, heat, UV radiation, being resistant to several types of insects, fungi, mildew or bacteria and may undergo slight degradation in the body. When exposed to elevated temperatures, unmodified

polyamides undergo molecular weight degradation, which results in loss of mechanical properties. The degradation is highly time/temperature dependent (Moncrieff 1975).

The mechanical properties of polyamide 6.6 fibres are related to their molecular weight and polymer preparation. Polyamide 6.6 has an excellent combination of medium strength, rigidity, creep strength, high toughness and excellent tribological properties. It has the best overall properties of the aliphatic commercial polyamide resins, combining greatest hardness, rigidity and resistance to abrasion and heat deformation. Polyamide also possesses high tenacity, high relation strength/weight and high elongation resistance, as well as excellent deformation recovering and high resistance to bending (Guillen 1986). Some of the tensile properties of polyamide 6.6 are compared with the tensile properties of polyester fibres and are represented in Table 1.1. Comparing both fibres, polyamide 6.6 presents higher tenacity and higher work to rupture.

Table 1.1 – Tensile properties of polyamide 6.6 fibres (adapted from Guillen 1986 and from Guillen 1991)

	Polyamide 6.6	**Polyester**
Tenacity (N/tex)		
Normal	0.4-0.6	0.36-0.45
High tenacity	0.6-0.9	0.50-0.59
Breaking Extension (%)		
Normal	20-30	30-50
High tenacity	15-20	20-30
Initial modulus (N/tex)		
Normal	2-3.5	7.5
High tenacity	4.5-5.5	2.5-5.5
Work to rupture (mN/tex)		
Normal	60-70	55.4
High tenacity	50-60	-

The properties of aliphatic polyamide fibres depend on the molecular weight and number of methylene groups related to –CO-NH– groups. These properties are strongly affected by varying the processing parameters. The modification of the PA 6.6 fibres by using additives can be focused on improving antistatic properties, exhaust dyeability, increase of the modulus and recovery properties of industrial yarns, flame-resistant fibres and on light and heat-stabilised fibres (Zaikov and Lomakin 1998; Marcinčin 2002).

1.1.3 DYEABILITY PROPERTIES

Polyamide 6.6 fibres possess end groups –COOH and $–NH_2$, which exhibit polar and hydrophilic characteristics, therefore they are dyed with anionic dyes, disperse dyes, direct dyes or, in a less extent, with reactive dyes (Mark *et al.* 1968; Rocha Gomes 2001). The chemical structure, the concentration of amino groups, the crystallinity and molecular orientation as well as the fabric preparation are the main factors that influence dyeability of polyamide fibres. In their crystalline regions, polyamide 6.6 has a closed structure which disables the dye diffusion. Differences on the orientation grade of polyamide fibres would promote differences of dyeability along the fibres. Polyamide fibres can be coloured or dyed with a wide range of colours and the dyes can be applied to the molten mass of polyamide or to the yarn or finished fabrics (Moncrieff 1975; Guillen 1986; Burkinshaw 1995).

1.1.4 APPLICATIONS

Polyamide fibres are used in a huge number of applications. They are widely used in the manufacturing of clothing, tire cord, carpeting, ropes, tyre reinforcement and other specialised applications. In plastic form polyamides are used in making such items as gears, piping, wire insulation, zippers and brush bristles (Stevens 1999). Polyamide is the predominant fibre used in the carcasses of

truck, racing and airplane tyres due to the excellent strength but not favoured for radial tyres due to flatspotting tendency. Polyamide 6.6 is the principal fibre in hosiery, underwear and stretch fabrics. They also find applications in non-wovens as a substrate for artificial leather (PVC).

There has been a remarkable increase in the use of polyamide in the automotive industry in the 1990s. Heat stabilised polyamide 6.6 is used for cylinder head cam covers to protect valves, seal the oil and shield against noise. Heat aged grades are used for automotive connectors. Flame resistant grades are used in electronics and electrical parts such as cable ties, conductors and terminal blocks. Polyamide is also used in the production of air-bags and gas tanks. The production of parachutes is also made with polyamide fibres (Guillen 1986; Reimschuessel 1989).

1.2 CHARACTERISTICS AND PROPERTIES OF ENZYMES

Nature has designed enzymes to greatly accelerate the rates of chemical reactions. The catalytic power of enzymes facilitates life processes in essentially all life-forms from viruses to man (Copeland 1996). Most of the reactions in living organisms are catalysed by protein molecules that accelerate the rate of chemical reaction without themselves undergoing any permanent chemical change, i.e. they are true catalysts (Cavaco-Paulo and Guebitz 2003).

Many enzymes retain their catalytic potential after extraction from the living organism, and it did not take long to recognise and exploit the catalytic power of enzyme for commercial purposes. In fact, early civilizations have used enzymes for thousands of years without understanding what they were or how they worked. Earliest known references to enzymes are from ancient texts dealing with the manufacture of cheeses, breads and alcoholic beverages. Today enzymes continue to play key roles in many industries including agro-food, oil, animal feed, beverages, detergent, pulp and paper, textile, leather, petroleum and specially chemical and biochemical industry (Uhlig 1998). They also help to maintain an unpolluted environment through their use in waste management. Recombinant DNA technology, protein engineering, and rational enzyme design are the

emerging areas of research pertaining to environmental applications of enzymes (Ahuja *et al.* 2004). Enzymes are also of fundamental interest in the health sciences, since many disease processes can be linked to the aberrant activities of one or a few enzymes (Copeland 1996).

Some enzymes are still extracted from animal or plant tissues. Plant derived commercial enzymes include the proteolytic enzymes papain, bromelain and some other special enzymes like lipoxygenase from soybeans. Animal derived enzymes include proteinases like pepsin and rennin. Most of the enzymes are, however, produced by microorganisms in submerged cultures in fermentors (Copeland 1996; Silva 2005).

The selection of an industrial enzyme is based in their specificity, reaction rate, optimum pH and temperature, stability, effect of inhibitors and affinity to substrates (Copeland 1996; Uhlig 1998; Silva 2005). Enzymes used in industrial applications must be tolerant against various heavy metals and be used in absence of cofactors.

The application of enzymes to practical processes is based on the catalysis of the desired reaction with a gradual inactivation. The price of enzymes must be as low as possible in order to make them attractive economically.

Enzymes can also be applied by immobilizing them on several different supports overcoming some problems related to their operational lifetime, their non-reusability, their sensitivity to process conditions, and others (Taylor 1991). The immobilisation can be performed by using many different methods based on chemical reaction, entrapment, specific binding and adsorption (Taylor 1991).

The use of enzymes frequently results in many benefits that cannot be obtained with traditional chemical treatments that are generally non-specific, not always easily controlled and may create harsh conditions. The technical effect is obtained by controlling some parameters like temperature, pH, dosage and time. Enzymes are generally active at mild temperatures and pH's, in order to maintain their folded state (Cavaco-Paulo and Guebitz 2003).

The economic benefit of using technical enzyme preparations lies in lower process costs, often increasing product quality, reducing environmental impact by

generating less waste and fewer environmental pollutants and making use of renewable resources in technology (Uhlig 1998; Silva 2005).

1.2.1 PROPERTIES OF ENZYMES AS CATALYSTS

The quality of life on earth is inextricably linked to the overall quality of the environment. The increasing awareness of the environment we live in is bringing about a more intensive search for alternative cleaner technologies. Currently, there are two fundamental pollution-related problems: the disposal of large quantities of wastes that are continually being produced, and the removal of toxic compounds that have been accumulating at dump sites in the soils and in water systems over the last few decades. Biotechnology is an essential tool to deal with this problem because it can provide new approaches for understanding, managing, preserving and restoring the environment, transforming pollutants into benign substances, generating biodegradable materials from renewable sources and developing environmentally safe manufacturing and disposal processes (Ahuja *et al.* 2004).

Enzymes are natural catalysts for most reactions in living organisms and advances in biotechnology have led to their use for industrial processing of natural products. They not only work efficiently and rapidly, but are also biodegradable.

In biological systems, thousands of different types of reactions are known to be catalysed by different enzymes; many more are yet to be discovered. The diversity of enzymes is, therefore, enormous in terms of type of reactions they catalyse, and also in terms of structure (Price and Stevens 1999; Cavaco-Paulo and Guebitz 2003).

With environment and cost issues surrounding conventional chemical processes being subjected to considerable scrutiny, biotechnology is gaining rapidly due to the various advantages that it offers over conventional technologies. Industrial enzymes represent the heart of biotechnology processes.

At the present, nearly 4000 enzymes have been isolated and characterised, mainly from mesophilic organisms, and about 200 of these are in commercial use. Because of improved understanding of production biochemistry, fermentation processes and recovery methods, an increasing number of enzymes can be produced affordably.

The world enzyme demand is satisfied by 12 major producers and 400 minor suppliers. Around 60% of the total world supply of industrial enzymes is produced in Europe. At least 75% of all industrial enzymes are hydrolytic in action. Proteases dominate the market, accounting for approximately 40% of all enzyme sales (Sharma 2001).

According to a recent released report from Business Communications Company, Inc. (BCC 2004) the global market for industrial enzymes was estimated at US $2 billion in 2004. Volume growth of industrial enzymes is between 4% and 5% of the Average Annual Growth Rate (AAGR), which is accompanied by decreasing prices, due to the increase in the number of smaller players competing in the market. The industrial enzyme market is divided into three application segments: technical enzymes, food enzymes and animal feed enzymes. The following chart (Figure 1.3) shows the global enzyme markets by application sectors, through 2009 (Silva 2005; Hasan 2006).

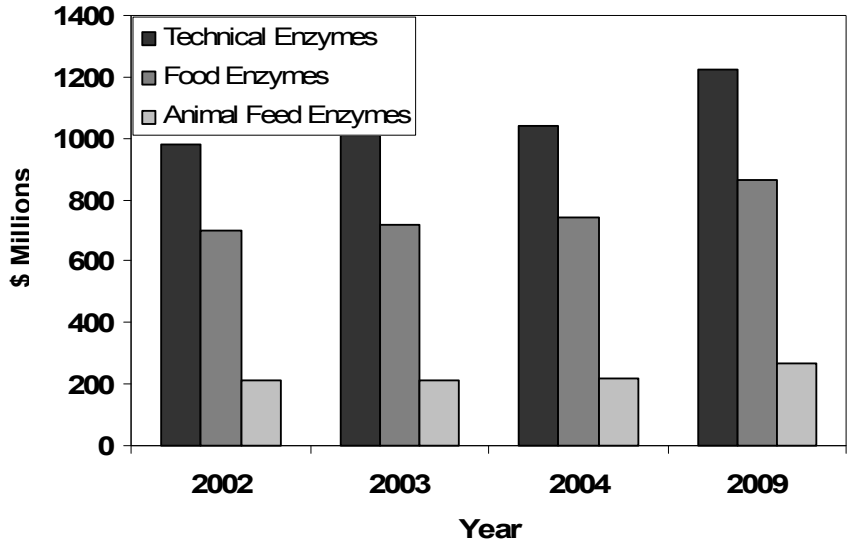


Figure 1.3 – Global enzyme markets by application sectors, through 2009 ($ Millions), according to BCC (2004).

Textile enzymes are the third most significant segment of the market of industrial enzymes, as can be seen on Figure 1.4 (BCC 1998). The major enzymes in this category are enzymes for processing cotton and cellulosic fibres, followed by enzymes for processing leather and fur.

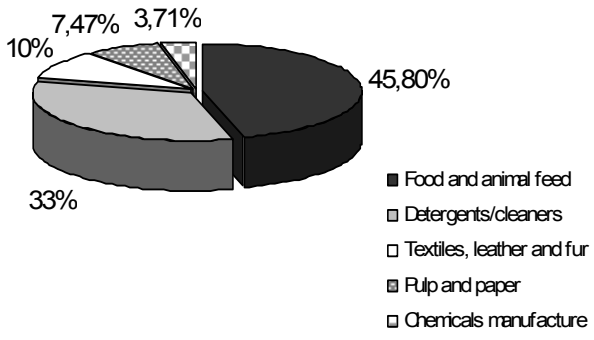


Figure 1.4 – Distribution of industrial enzymes: worldwide market forecast in 2002 ($ million), according to BCC (1998).

1.2.2 ENZYMES IN TEXTILE PROCESSING

Enzymes are increasingly being used in process industries for a wide variety of products in an attempt to create a "cleaner" technology and decrease the use of harsh chemicals that are not environmentally friendly (Ahuja *et al.* 2004). They are gaining an increasingly important role as a tool in various wet textile pre-treatment and finishing processes (Heine and Höcker 1995; Cavaco-Paulo 1998b). Conventional wet textile pre-treatment and finishing procedures applied in the textile industry are often characterised by high concentrations of chemicals, alkaline or acidic pH, and high temperatures with consequent high consumption of energy. Major advances in microbial technology and genetics allow the broad range of enzymatic applications in the industry. Cotton, wool, flax or starches are natural materials used in textiles that can be processed with enzymes. They have been used for desizing, scouring, polishing, washing, degumming, peroxide degradation in bleaching baths as well as for decolourisation of dye wastewaters, bleaching of released dyestuff and inhibiting dye transfer (Cavaco-Paulo and Guebitz 2003).

The most classical enzymatic application in the textile industry is the desizing of cotton fabrics using amylases (Nilsson and López-Ainaga 1996). The advantages of using amylases to remove the starch-based sizing agents, as opposed to chemical methods using oxidizing agents or sodium hydroxide, are a lower pollution load and assured fabric quality (Cegarra and Emer 1999; Gupta *et al.* 2003; Ahuja *et al.* 2004; Asgher *et al.* 2007).

Another step processing where enzymes have been applied is the scouring. The application of several enzymes such as pectinases, lipases, cellulases or proteases to perform a bio-scouring has been reported in literature (Tzanov *et al.* 2001_a; Karapinar and Sariisik 2004). Pectinases together with cellulases have been tested in the elimination of impurities in cotton and wool (Durán and Duran 2000; Tzanov *et al.* 2001_a; Jayani *et al.* 2005). Lipases are used in the textile industry to assist in the removal of size lubrificants and are also used in detergent formulations together with proteases and amylases, being responsible for removing fatty substances from clothing (Hasan *et al.* 2006). The ability of lipases to improve

hydrophilicity of polyester fabrics has also been reported (Alish-Mark *et al.* 2004). Studies performed by Sharma and co-workers reported the use of pectinases, xylanases and laccases on the enzymatic treatment of flax fibre (Sharma *et al.* 2005).

Enzymes have been used in laundry detergents since the 1960s. Proteases were the first enzymes to appear, followed by amylases, lipases and cellulases. The detergent industry has remained the largest market for industrial enzymes, and new enzyme products are constantly being developed for such use. Studies performed by Cavaco-Paulo and co-workers reported that there is also an increasing use of cellulases in domestic washing products (Cavaco-Paulo *et al.* 1999), where they are claimed to aid detergency and to remove damaged fibrillar material, improving appearance, softness and colour brightness. These enzymes are effective against various stains like grass, blood, egg, chocolate and other stains (Durán and Duran 2000; Ramachandran and Karthik 2004).

Cellulases have various effects on man-made cellulosic fabrics such as lyocell (Tencel), viscose and cellulose acetate. These enzymes can reduce the tendency of viscose to pill and reduce fibrillation of lyocell (Morgado *et al.* 2000).

Bio-stoning and the closely related process of biopolishing are perhaps attracting most current attention in the area of enzyme processing. Conventional stone-washing uses abrasive pumice stones in a tumbling machine to abrade and remove particles of indigo dyestuff from the surfaces of denim yarns and fabrics. Cellulases can cut through cotton fibres and achieve the same effect without damaging the fabrics (Heikinheimo *et al.* 2000; Tzanov *et al.* 2003_a). Cellulases are also used in bio-polishing to remove fine surface fuzz and fibrils from cotton, viscose and lyocell fabrics. The polishing action thus achieved helps to eliminate pilling and provides better print definition, colour brightness, surface texture, drapeability and softness, without any loss of absorbency (Cavaco-Paulo 1998_a; Ramachandran and Karthik 2004).

Carefully controlled treatments with proteolytic enzymes have been performed to reduce the buckling load and collapse energy of wool yarns. Proteases are also used in the textile industry for the degumming of silk and for producing sandwashed effects on silk garments (Freddi *et al.* 2003). The use of

proteases has been also reported for cotton scouring and to prevent backstaining in denim washing (Yoon *et al.* 2000) and in the bioantifelting of wool. Recently, proteases were added to detergent formulations for wool domestic washing (Vasconcelos *et al.* 2006). The pre-treatment with transglutaminases for increasing the longevity of the dyed wool fabrics after detergent action, was also recently an objectc of study (Cortez *et al.* 2005). The diffusion of serine proteases (free and immobilised) into wool fabrics and yarns was also studied (Silva and Cavaco-Paulo 2003; Silva *et al.* 2005) and other authors reported new process directions to obtain machine washable wool with acceptable quality (Lenting *et al.* 2006).

Another application established is the use of catalase enzymes to breakdown residual hydrogen peroxide after bleaching of cotton. Among the benefits listed in literature, there were a shorter washing process, savings in water consumption, reduced pollution and an increase in the efficiency and reproducibility of subsequent dyeings (Durán and Duran 2000; Tzanov *et al.* 2001_b; Costa *et al.* 2001). Another environmentally friendly approach studied has been the bleaching of cotton using laccases (Pereira *et al.* 2005).

The textile effluents, usually highly coloured, when discharged in open waters, present an obvious aesthetic problem. Moreover, the dyes without an appropriate treatment can persist in the environment for extensive periods of time. Several studies report the use of enzymes, namely, laccases, peroxidases or microorganisms for decolourization of textile effluents (Campos 2000; Ramalho *et al.* 2002; Zille *et al.* 2003; Soares *et al.* 2006). Enzymes either alone or in combination with other treatments, like ultrasound can be effectively used for effluent treatment (Basto *et al.* 2007).

Recently the effect of ultrasounds on the oxidative polymerisation of phenolic compounds in the presence of laccase was studied (Silva 2006). This enzyme has been also recently investigated for the enzymatic colouration of unbleached cotton (Kim *et al.* 2007).

1.2.3 ENZYME TECHNOLOGY IN SYNTHETIC FIBRES PROCESSING

Natural fibres are standard substrates in enzymatic processes. However, synthetic fibres have been explored in the context of their support properties for enzyme immobilisation and for potential special applications, such as biosensors or membranes. The majority of synthetic fibres are petroleum derivatives; they are designed in the chemical laboratory and created to achieve the most favourable properties at reasonable cost (Guillen 1986). About 90% of all synthetic fibres are made from polyethylene terephthalate (PET), polyamides (PA) and polyacrylonitrile (PAN). Synthetic fibres already hold a 54% market share in the fibre market and more than 25 million tones (Mt) of PET, 5Mt of PA and 2.6 Mt of PAN are produced annually (Guebitz and Cavaco-Paulo 2008). Manmade fibres tend to gain even more market share compared with natural fibres. The advantages of manmade fibres over natural fibres range from a lower specific weight to faster drying, better water transport properties and to better easy-to-clean-properties. With advances in polymer synthesis, recombinant technologies, engineering and fibre formation methods, more custom-made fibres are expected to enter in the market place (Cavaco-Paulo and Guebitz 2003; Fischer-Colbrie *et al.* 2006).

Polyamide, polyethyleneterephtalate and polyacrylonitrile fibres share as common features a high crystallinity and low moisture regain. They exhibit excellent physical properties of strength, flexibility, toughness, stiffness, wear and abrasion resistance. Beside these properties, they also demonstrate low friction coefficient, low creep and good chemical resistance. However, the poor wettability, high hydrophobicity, the build-up of static charges, not allowing 'breathing' and being difficult to finish are undesirable properties of these materials that make difficult the application of finishing compounds, colouring agents as well as the coupling of flame retardants or covalent immobilisation of proteins (Vertommen *et al.* 2005; Jia *et al.* 2006).

The classical chemical modification of synthetic polymers, using strong alkaline or acid agents, requires high amounts of energy and chemicals (binders, coupling agents, etc), which are partially discharged to the environment. Furthermore, some of the substances used are released from the end-products,

due to their weak bonding, presenting therefore potentially serious health risks and reducing the technical lifetime of the products (Guebitz and Cavaco-Paulo 2003). Despite the fact that alkaline products render synthetic fibres more hydrophilic, they also lead to the deterioration of other properties causing irreversible yellowing and loss of resistance. In addition, extremely high weight losses from 10-30% have been reported for these treatments (Zeronian and Collins 1989).

In order to reduce the use of such chemicals and their environmental impact, new technologies have been developed to replace classical methodologies. Two technologies suitable for this purpose are: plasma technology, where hydrophilic groups are generated at the surface and the grafting of specific groups, like poly(ethylene glycol) at the surface of the synthetic fibres (McCord *et al.* 2002; Tobiesen and Michielsen 2002; Grace and Gerenser 2003; Riccardi *et al.* 2003; Pappas *et al.* 2006; Shalaby *et al.* 2006). Chemical finishers based on hydrophilic carboxyl-containing polymers, are widely used to increase hydrophilicity of synthetic textiles and are continuously being improved, as evidenced by numerous patents filed (Soane *et al.* 2006).

By contrast, surface modification with enzymes has been considered a valuable tool to improve the quality and the processing properties of synthetic fibres. A bio-catalytic method would have the advantage of being performed under mild and environmentally friendly process conditions. Moreover, no complicated machinery would be required, as in the case of plasma treatments, and little or no additional chemicals would be needed. Furthermore, the direct control on the relative amount of specific groups' formation may be difficult to achieve by using physical techniques. Due to the selectivity of enzyme reactions and their occurrence only at the surface of the fibres, enzymatic hydrolysis is desirable when compared with traditional chemical methods.

Advances in enzymology, genetic engineering and industrial enzyme production make existing technologies using enzymes for fibre processing more attractive. They also open up new possibilities such as the improvement of synthetic fibres with higher added value and fibre materials functionalisation, hence improving environmental aspects of textile processing (Guebitz *et al.* 2006).

The enzymatic modification of synthetic materials has huge potential both in the functionalisation of bulk materials, such as polyamide, polyacrylonitrile or polyester and in the production of polymers for special applications such as medical devices, painting, anti-fogging, filtration and electronics (Deguchi *et al.* 1998; Tauber *et al.* 2000; Yoon *et al.* 2002; Silva *et al.* 2005_a).

The functionalisation of polyester has been extensively studied. A bio-catalytic method for polyester finishing would be performed at mild and environmentally friendly conditions (Taylor 1999). Compared with chemical hydrolysis, enzymatic hydrolysis has the advantage of maintaining mechanical stability as well as the natural colour of the polymer. Despite the fact that aromatic polyesters like PET have previously been considered resistant to hydrolytic action (Müller *et al.* 2001) some authors found changes in the physical properties of PET fibres and films by enzymatic action of *Cladosporium cladosporioides* (Sato 1983).

The enzymatic controlled surface hydrolysis of PET facilitates the attachment of cationic compounds (e.g. dyes) or the direct application of coatings for the production of technical fabrics with reduced consumption of coupling agents.

The hydrophilicity of polyesters can be increased by hydrolysis of ester bonds (Figure 1.5) (Genencor int 2001, Khoddami *et al.* 2001, Yoon *et al.* 2002; Nimchua *et al.* 2006). Treatment of PET with several lipases has been shown to improve wettability and absorbent properties of PET fabrics, while strength properties were maintained (Hsieh and Cram 1998; Hasan *et al.* 2006). Polyester hydrolysing bacterial and fungal esterases have been claimed to reduce the pilling properties of PET fabrics (Andersen *et al.* 1999). Other authors have claimed improved stain resistance, wetting and/or dyeing abilities of PET fabrics treated with so-called polyesterases (lipases, esterases or cutinases). Additionally, the fibres showed improved pilling and oil stain removal properties (Yoon *et al.* 2002; Alish-Mark *et al.* 2004; O'Neill and Cavaco-Paulo 2004; Fischer-Colbrie *et al.* 2004; Vertommen *et* al. 2005; Liebminger *et al.* 2007). The ability of cutinase to hydrolise PET cyclic oligomers was proved by Hooker and co-workers (Hooker *et al.* 2003).

Oxidative enzymes, such as laccases, have been also claimed to modify the PET surface (Miettinen-Oinonen *et al.* 2002). However, there is no detailed mechanism or application related data available yet.

McCloskey and Jump demonstrated that 100% polyester fabric can be treated with a cutinase to impart a biopolished finish (McCloskey and Jump 2005).

Recently the genetic modification of cutinase from *Fusarium solani pisi* was performed in order to obtain enzymes with higher activity/specificity towards polyester fibres and satisfactory results were achieved (Araújo *et al.* 2007). In addition, the synergism between mechanical agitation and enzyme activity was investigated using polyester fabrics as substrates, and the results revealed that a higher functionalisation was achieved when low levels of mechanical agitation were applied (O'Neill *et al.* 2007).

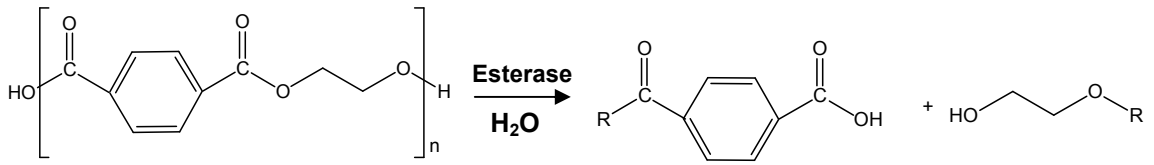


Figure 1.5 – Enzymatic hydrolysis reaction of polyester fibre by esterase action (adapted from Guebtiz and Cavaco-Paulo 2008).

Only a few studies on the modification of acrylic fibre with enzymes have been done, mainly reporting the use of nitrile hydratases (Tauber *et al.* 2000; Battistel *et al.* 2001; Banerjee *et al.* 2002). The modification of nitrile surface groups into amide groups was accomplished by the action of nitrile hydratase from *Rhodococcus rhodochrous* (Kobayashi *et al.* 1998; Tauber *et al.* 2000), from *Brevibacterium imperiale* (Battistel *et al.* 2001) and from *Arthrobacter* sp. ECU1101 (Wang *et al.* 2004). Recently it was shown that bacteria of *Micrococcus luteus* can degrade PAN fibres and the products of hydrolysis could be detected by nuclear magnetic resonance spectroscopy (NMR) analysis (Fischer-Colbrie *et al.* 2007). The direct modification of nitrile groups into carboxylic groups was performed by a nitrilase (Figure 1.6) (Matamá *et al.* 2007). Some of the carboxylic groups were released into the treatment solution as polyacrylic acid depending on the time of treatment. The affinity towards a basic dye was also higher after enzymatic treatment.

Recent studies revealed that cutinase from *Fusarium solani pisi* was also able to modify the surface of acrylic fibres, however in a lower extension compared with polyester modification (Figure 1.6) (Matamá *et al.* 2006). The enzymatic treatment in the presence of additives was studied in order to increase the enzyme efficiency towards acrylics. The results showed that the simultaneous action of enzyme and additives benefits fibre functionalisation. At the same time, the mechanistic parameters of enzymatic process were studied and it was possible to ensure that the functionalisation of acrylic fabrics was only possible when high levels of mechanical agitation were used (Matamá *et al.* 2006).

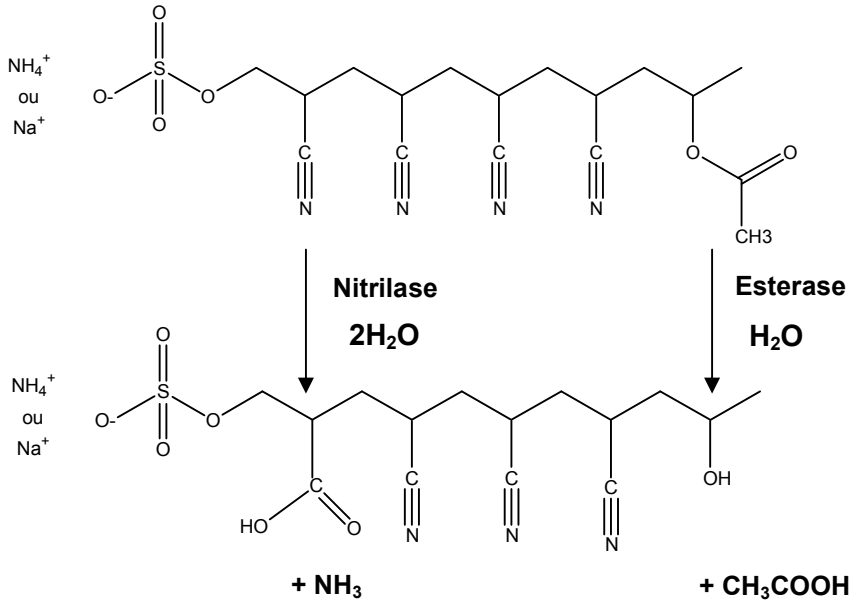


Figure 1.6 – Enzymatic hydrolysis of acrylic fibres (nitrilase hydrolises nitrile groups into carboxylic groups releasing ammonia; esterase hydrolises vinyl-acetate co-monomer into hydroxyl groups releasing acetic acid) (adapted from Guebitz and Cavaco-Paulo 2008).

Polyamide surfaces have been modified by physical and chemical methods. The first category includes activation of the surfaces through treatment with UV radiation (Shearer *et al.* 2000) or plasma activation (Foerch and Hunter 1992; McCord 2002; Pappas *et al.* 2006). The second includes the grafting of small molecules to the surface of the fibres in order to obtain products with special properties like anti-bacterial activity and others (Buchenska 1996; Tobiesen and Michielsen 2002; Saïhi *et al.*

2005; Shalaby *et al.* 2006). In the medical area the polyamide 6.6 fibres have been modified by incorporation of inorganic additives in order to improve their biocompatibility (Shamack *et al.* 2000).

While some of these methods are inherently clean, the disadvantages are that chemically well-defined surfaces cannot be designed and prepared and often functionalisation is accomplished by surface destruction. As mentioned before, a bio-catalytic method would be a valuable tool for the modification of polyamide.

Until now, few studies about functionalisation of polyamide fabrics with enzymes were reported. Some studies demonstrated that manganese peroxidase was able to modify the surface of PA 6.6 and PA 6 fabrics without reducing the fibre diameter (Deguchi *et al.* 1997; Deguchi *et al.* 1998; Friedrich *et al*. 2007). Heumann and co-workers demonstrated that polyamide substrates can be efficiently functionalised by esterase or protease action. A model substrate (adipic acid bishexyl-amide) was developed for screening polyamidase activity of a given enzyme. A protease from *Beauveria* sp., an amidase from *Nocardia* sp. and a cutinase from *Fusarium solani pisi* were used to hydrolise this model substrate (Heumann *et al.* 2006). Laccases in combination with a mediator have been claimed to increase the hydrophilicity of PA 6.6 fabrics (Miettinen-Oinonen *et al.* 2002). Polyamide degradation with a laccase from *Trametes versicolor* has also been investigated (Fujisawa *et al.* 2001). Partial hydrolysis of PA fibres and PA oligomers has been demonstrated with proteases (Kakudo *et al.* 1993; Fischer-Colbrie *et al.* 2004). Other authors showed the biodegradation of polyamide oligomers by isolating microorganisms of *Flavobacterium* sp. and *Pseudomonas* sp. (Prijambada *et al.* 1995; Negoro 2000). Oxidases from lignolytic fungi have been used to depolymerise polyamides (Klun *et al.* 2003).

1.2.4 CUTINASES

Cutinases are hydrolytic enzymes which are able to degrade the insoluble lipid polyester matrix i.e., cutin, the cuticular polymer of higher plants composed of hydroxyl and epoxy fatty acids. The fatty acids of cutin are usually *n*-C_{16} and

n-C_{18} and contain one to three hydroxyl groups. Ester bonds predominate in the cutins, although peroxide bridges and ether linkages have also been presented (Carvalho *et al.* 1998; Egmond and de Vlieg 2000). Cutin is a support biopolyester involved in waterproofing the leaves and fruits of higher plant cells and organs, and minimising the deleterious impact of pathogens (Heredia 2003). Cutin plays therefore a key role in protection against the entry of pathogens into plants, and its enzymatic degradation has proved to be one of the first steps in the infection process (Carvalho *et al.* 1998). The inhibition of cutinase could prevent fungal penetration of plants and thus prevent infection (Carvalho *et al.* 1998; Carvalho *et al.* 1999_a); Kolattukudy 2001).

Cutinases have been purified and characterised from several different sources, mainly fungi and pollen. Evidence of a bacterial cutinase produced by a phyllospheric fluorescent *Pseudomonas putida* strain, cohabiting with a nitrogen-fixing bacterium *Corynebacterium* sp., has been also demonstrated (Carvalho *et al.* 1998).

To date, the majority of the work has been done with the fungal pathogen of peas that was first purified in the 1970s from *Fusarium solani* f. sp. *pisi* grown on cutin as a sole source of carbon. Two forms of this enzyme were separated by ion exchange chromatography and no real differences in the catalytic capabilities were detected. More recently, an efficient production system for *Fusarium solani* f. sp. *pisi* cutinase in *Saccharomyces cerevisiae* was developed, and point mutations were introduced into the cutinase gene to optimise lipase activity (Carvalho *et al.* 1998; Verrips *et al.* 2000).

1.2.4.1 CUTINASE STRUCTURE

Cutinase, a compact one-domain molecule consisting of 197 residues, is an α/β-protein with a hydrophobic core comprising a slightly twisted five-parallel-stranded β-sheet, surrounded by four α-helices (Figure 1.7) (Martinez *et al.* 1994; Longhi and Cambillau 1999). This enzyme has a molecular weight around 22.000 daltons with highly conserved stretches, which include four invariant cysteins, forming two disulfide bridges. This enzyme has an isoelectric point of 7.8.

Cutinase is the smallest lipase/esterase structure solved so far. It belongs to the class of serine esterases and to the superfamiliy of the α/β hydrolases, being characterised by the triad Serine (Ser), Histidine (His), Aspartic acid (Asp) residues and by an oxyanion binding site that stabilises the transition state via hydrogen bonds with two main chain amide groups. The occurance of an α/β hydrolase fold and of a catalytic machinery composed by a nucleophile, an acid and a histidine, seems to be a common feature to all the esterases and lipases described so far. Two disulphide bonds are present in cutinase. The first, Cys31-Cys109, links the N-terminal to a β-turn and participates in the stabilization of the global molecular folding. The second disulphide bridge, Cys171-Cys178, can be assumed to play an important role in the stabilisation of the two consecutive β-turns on which the catalytic Asp 175 is located (Carvalho *et al.* 1998).

The catalytic triad, Ser120, Asp175 and His188, is accessible to the solvent; it is located at one extremity of the protein ellipsoid, is surrounded by the loop 80-87 and by the more hydrophobic loop 180-188. Furthermore, the active site presents appreciable flexibility that can explain the adaptation to different substrates (Carvalho *et al.* 1998; Carvalho *et al.* 1999_a).

The crystal structure of cutinase from *Fusarium solani f. pisi* indicates that this fungal cutinase constitutes a separate class of enzyme that may be regarded as a bridge between esterases and lipases. The free cutinase has a well defined active site and a performed oxyanion hole and that it does not need any rearrangements to bind its substrates (Figure 1.7) (Longhi and Cambillau 1999; Kolattukudy 2001). Contrary to lipases, with an activity greatly enhanced in the presence of a lipid-water interface, cutinases do not display, or display little interfacial activation, being active on both soluble and emulsified triglycerides (Longhi and Cambillau 1999).

Analogous to serine proteases, cutinase contains an oxyanion hole consisting of the main-chain nitrogens of Ser42 and Gln121 and the oxigen atom of Ser42, that stabilises the negative charge generated during the nucleophilic attack on the scissile bond of the substrate (Lau and Bruice 1999).

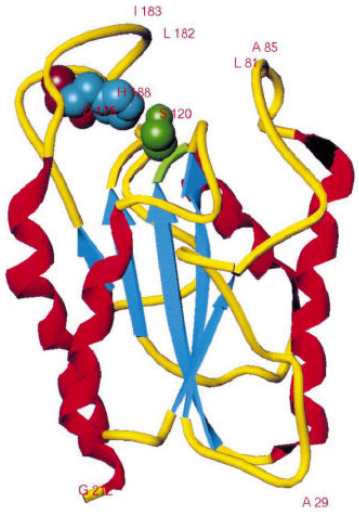


Figure 1.7 - Ribbon representation of cutinase: α-Helices (red), β- strands (blue) and coils (yellow) are shown. The residues of the catalytic triad (Ser120, green; His188, blue; Asp175, red) are represented in CPK. Residues of the loops delimiting the catalytic crevice (residues 80-88 and 180-188) have been labelled (Longhi and Cambillau 1999).

1.2.4.2 CUTINASE FUNCTION AND STABILITY

Cutinase is a very versatile enzyme that catalyses synthetic and hydrolytic reactions on a diversity of substrates from triglycerides to esters among others (Carvalho *et al.* 1999_b).

Fungal cutinase catalyse hydrolysis of model substrates and in particular *p*-nitrophenol esters of short chain fatty acids, providing a convenient spectrophotometric assay for this enzyme activity (Shirai and Jackson 1982). Hydrolysis of model esters by cutinase shows the highest specificity of this enzyme for primary alcohol ester hydrolysis. Wax esters and methyl esters of fatty acids are hydrolysed at low rates. Alkane-2-ol esters are hydrolysed much slowly than wax esters and esters of mid-chain secondary alcohols were not hydrolysed at significant rates. Triglycerides are hydrolysed by the purified fungal cutinases at slow rates and this activity is as sensitive as cutinase to active site-directed reagents, showing that both activities involve the same catalytic site. Trioleyl glycerol and tributyryl glycerol are hydrolysed 5–30 times more rapid than tripalmitoyl glycerol by several fungal cutinases. Time-course of formation of

products from the glycerides also show that the enzyme has a high degree of preference for hydrolysis of primary alcohol esters. Cutinase also catalyses hydrolysis of the oligomers to monomers (Osman *et al.* 1993; Kolattukudy 2001).

The catalytic ability of cutinase to hydrolyse ester bonds is represented in Figure 1.8.

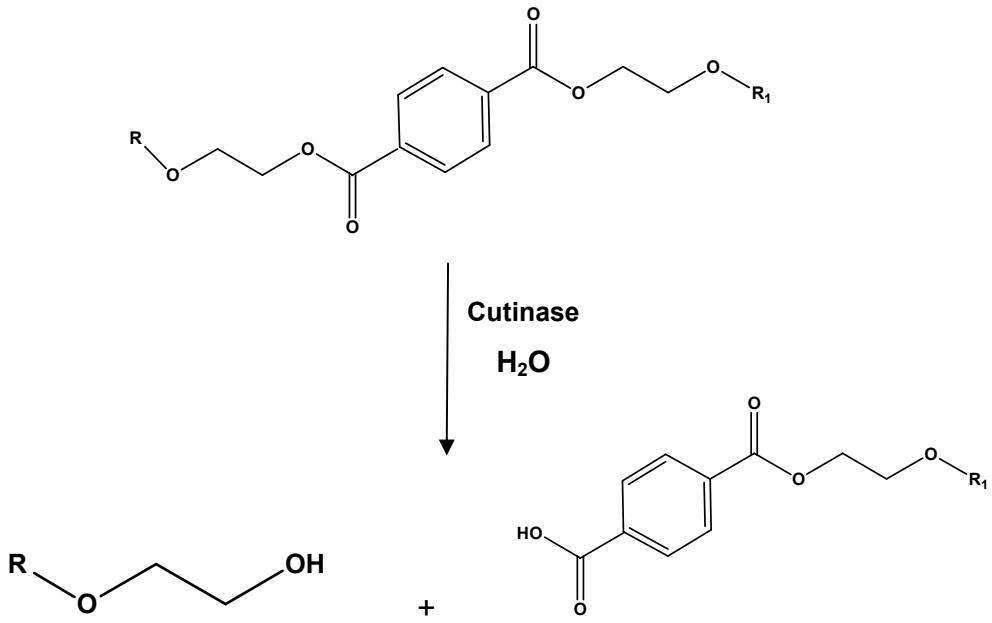


Figure 1.8 – Schematic representation of the cleavage of an ester bond by cutinase action (adapted from Guebitz and Cavaco-Paulo 2008).

Cutinase thermal and operational stabilities have been evaluated in different enzyme preparations and reaction conditions. This enzyme has been used in different forms in reaction media, namely dissolved in aqueous solution, suspended as a powder (solid form), microencapsulated in reversed micelles, and immobilised onto solid supports (Carvalho *et al.* 1999_a). The enzyme performance in the hydrolysis of triglycerides and analogs, namely in dioctyl sulfosuccinate sodium salt (AOT) reversed micelles and in biphasic system, was widely studied. Depending on the type of system, several authors stated optimal conditions for hydrolysis in terms of pH, temperature and buffer concentration. The kinetic constants were also optimised (Carvalho *et al.* 1999_a). The transesterification (alcoholysis) reaction of butyl acetate with hexanol in organic

media has been evaluated in several systems. The effects of the reaction conditions on the transesterification activity of cutinase microencapsulated in AOT reverse micelles were evaluated and it was stated that cutinase displayed high activities for 490 mM hexanol and for a temperature range from 40 to 50ºC. A pH value between 7 and 8 and a buffer molarity of 200 mM were adequate for the transesterification reaction. Other transesterification reactions were also evaluated with cutinase adsorbed on zeolites and other supports, achieving different reaction conditions. At the same time the alcoholysis of methyl propionate with propanol was studied in a gas/solid system (Carvalho *et al.* 1999_a); Melo *et al.* 2003).

Cutinase activity as a function of pH was also studied and Peterson and co-workers demonstrated that the thermal stability of cutinase versus pH displays the classical bell shaped appearance. Whereas the maximum stability is found in the pH range from 6-8.5, it decays rapidly at both flanking pH ranges (Petersen *et al.* 2001).

The assay conditions of enzymatic hydrolysis of synthetic fibres, namely polyester, were already established and reported in literature. Polyester fabrics are normally incubated with *Fusarium solani pisi* cutinase using phosphate buffer (pH 8.2) at 35ºC (Alisch-Mark *et al.* 2006).

Recently, pH and temperature profiles were studied and the results revealed the optimum conditions (pH: 7-8; temperature: 30-40ºC) (Matamá *et al.* 2006).

1.2.4.3 CUTINASE ACTIVITY IN ORGANIC MEDIA

Enzyme catalysis in nonaqueous media is nowadays an appealing issue as enzymes exhibit new properties in organic solvents. They are often more stable and can catalyse reactions that are impossible or difficult in water, like transesterification reactions. Other advantages of conducting enzymatic conversions in organic solvents as opposed to water are: high solubility of most organic compounds in nonaqueous media; relative ease of product recovery from organic solvents as compared to water; the insolubility of enzymes in organic media, which permits their easy recovery and reuse and thus eliminates the need

for immobilisation (Zacks and Klibanov 1985). Furthermore, enzyme selectivity can also differ from that in water and can change, or even reverse, from one solvent to another. In these media, the preservation of enzyme function is due to the fact that the enzyme essentially retains its native structure (Garcia *et al.* 2005). Enzyme activity in organic solvents depends dramatically on such parameters as water activity, pH control, enzyme form, and the nature of the solvent, which must be as hydrophobic as possible to maintain the activity of enzyme (Carrea and Riva 2000; Soares *et al.* 2003).

The effects of organic solvents on an enzyme are mainly due to the interactions with the essential enzyme-bound layer of water rather than with the enzyme itself. Water is a pivotal participant on enzyme instability, by promoting both conformational mobility of protein molecules and such major deleterious reactions as deamidation of Asn/Gln residues and hydrolysis of peptide bonds. Hence it would be expect that enzymes should be more thermostable in organic solvents than in water. In addition, when placed in organic solvents, enzymes become far more stable against another common cause of inactivation in water: proteolysis (Klibanov 2001; Gupta and Roy 2004).

The solvent may also affect the catalytic activity of the enzyme via direct molecular solvent/enzyme interactions, e.g. the binding of solvent molecules directly at the enzyme active site. This can cause changes in polarity and impact on the stabilisation of charged transition states. This fact occurs in the catalytic mechanism of serine hydrolases such as cutinases that require the establishment of a formal negative charge on the active site for full activity. Binding of solvent molecules at the active site can also result in competitive inhibition with the substrates (Garcia *et al.* 2005; Micaelo *et al.* 2005).

Several studies were described in literature respecting to the study of activity of cutinase in organic media and all of them have shown that enzyme activity is preserved in organic media, with a very low content of water (Vidinha *et al.* 2004; Garcia *et al.* 2005).

1.2.4.4 BIOCATALYTIC APPLICATIONS OF CUTINASE

Cutinase has been presented as a versatile enzyme showing several interesting properties for the application in industrial products and processes.

Hydrolytic and synthetic reactions catalysed by cutinase have potential use in the dairy industry for the hydrolysis of milk fat, in household detergents and in the oleochemical industry (Kolattukudy and Poulose 1996). In addition, cutinase can be applied in the synthesis of structured triglycerides, polymers and surfactants, in the synthesis of ingredients for personal-care products and in the synthesis of pharmaceuticals and agrochemicals containing one or more chiral centers. At low water activities transesterification of fats and oils or (stereo) selective esterification of alcohols can be achieved. Some of these processes are already applied in industry, while others are still under evaluation at research level (Carvalho *et al.* 1998). Another important application of cutinase pertains to degradation of plastics. Polycaprolactone, a synthetic polyester, has been hydrolysed to water-soluble products by cutinase (Carvalho *et al.* 1999_a).

In textile field, the use of cutinases to reduce backstaining during stone-wash processes in cotton denim fabrics was described (Uyama and Daimon 2001). Cutinases have also been extensively studied for the modification of polyester fibres in order to increase their hydrophilicity and dyeability (Kellis *et al.* 2001; O'Neill and Cavaco-Paulo 2004; Vertommen *et al.* 2005; Alish-Mark *et al.* 2006). Finishing effects such as biopolishing can be obtained using this enzyme (McCloskey and Jump 2005). The potential use of cutinase in other textile fibres has also been subject of study, namely the enzymatic scouring of cotton (Degani *et al.* 2002) or even the functionalisation of acrylic fibres (Matamá *et al.* 2006).

1.2.5 PROTEASES

Proteases have been used in food processing for centuries, like rennet obtained from calves' stomachs used traditionally in the production of cheese; and papain from the leaves and unripe of the pawpaw used to tenderise meats

(Warshel *et al.* 1989). Proteolytic enzymes catalyse the hydrolysis of certain peptide bonds in protein molecules. The general reaction is illustrated in Figure 1.9.

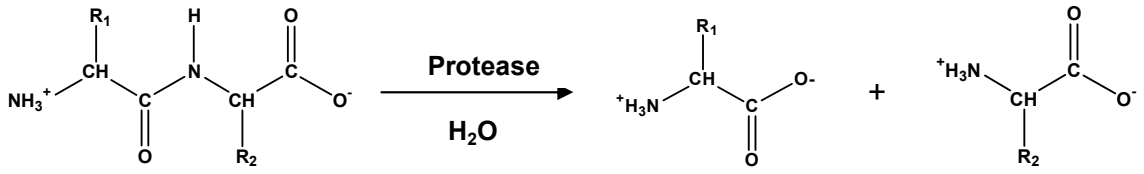


Figure 1.9 – Schematic representation of the cleavage of a peptide bond by a protease (adapted from Beynon and Bond 1989).

Microbial proteases are among the most important hydrolytic enzymes and have been studied extensively since the advent of enzymology (Gupta *et al.* 2002). Today, proteases account for more than 40% of the total enzyme sales in various industrial market sectors, such as detergent, food, pharmaceutical, leather, diagnostics, waste management and silver recovery (Anwar and Salermuddin 1998; Kumar and Takagi 1999; Sharma *et al.* 2003; Maurer 2004). However, until today, the largest share of the enzyme market has been held by alkaline proteases and different companies worldwide have successfully launched in the past few years several products based on these (Gupta *et al.* 2002). The largest application of alkaline proteases is as additives for detergents, where they help in removing protein based stains from clothing (Kumar and Takagi 1999; Gupta *et al.* 2002). In textile industry, proteases may also be used to remove the stiff and dull gum layer of sericine from the raw silk fibre to achieve improved lustre and softness (Freddi *et al.* 2003). Protease treatments can modify the surface of wool and silk fibres to provide new and unique finishes. Research has been carried out on the application of proteases to prevent wool felting (Heine and Höcker 1995; Silva *et al.* 2005). The bio-industrial viewpoints of microbial alkaline protease have been reviewed (Kumar and Takagi 1999; Gupta *et al.* 2002).

Proteases attack proteins via two models, yielding different products. Exopeptidase acts cleaving off single amino acids from either end of the peptide chain. Exoproteases are specific according to which end of the protein chain they attack: carboxypeptidases if they act the end with a free carboxylic acid (C-terminus) or aminopeptidases if they act the free amino end group (N-terminus).

Endopeptidases or proteinases attack peptide bonds in the interior of the peptide chain, yielding smaller polypeptides and peptides. The endoproteases are classified according to the mechanism of their active site. Four mechanistic classes: serine and cysteine proteases (which form covalent enzyme complexes) and aspartic and metallo-proteases (which do not form covalent enzyme complexes) are recognised by the International Union of Biochemistry (IUB 1992), and within these classes, six families of proteases are distinguished to date (Serine proteases I, Serine proteases II, Cysteine proteases, Aspartic proteases, Metallo proteases I and Metallo proteases II). Each family has a characteristic set of functional amino acid residues arranged in a particular configuration to form the active site (Rao *et al.* 1998).

The serine proteases are the most studied class of enzymes in the protease field. They include two distinct families: the mammalian serine proteases (for example chymotrypsin and trypsin) and the bacterial serine proteases (for example subtilisin). One of the most extensively studied families of serine proteases is the subtilisin (Neurath 1996). The structure of subtilisin is represented in Figure 1.10.

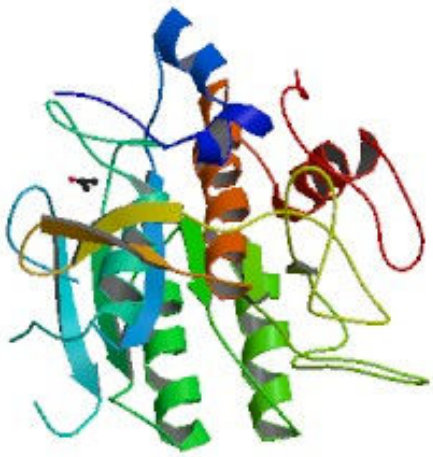


Figure 1.10 – Ribbon drawings of X-ray structure of a subtilisin from *Bacillus* sp. (from PDB, entry 2SBT). Arrows denote beta-sheets and spirals denote helices.

The optimum pH range of alkaline proteases is generally between pH 9 and 11, with a few exceptions. They also have high isoelectric points and are generally stable between pH 6 and 12. The optimum temperature ranges are from 50 to 70ºC. Alkaline proteases from *Bacillus* sp., *Steptomyces* sp. and *Thermus* sp. are quite stable at high temperatures, and the addition of Ca^{2+} further enhanced enzyme thermostability (Kumar and Takagi 1999).

1.3 ENZYME ADSORPTION *VS* MECHANICAL AGITATION

Protein adsorption plays a major role in a variety of important technological and biological processes. Moreover, protein adsorption is a fundamental problem that involves large competing energy scales and conformational statistics that may result in reversible and irreversible processes (Fang and Szleifer 2001).

Earlier investigations reveal that the adsorption of proteins, which can be serum proteins, enzymes, antibodies and foreign antigens, follows different steps. The first step involves diffusion of the protein molecule from the bulk onto the interface. A second step includes the adsorption and interfacial unfolding. A third step would involve aggregation within the interfacial layer, multilayer formation and even interfacial gelation. The different structure attained by the protein should be very dependant on the different steps of this mechanism (Maldonado-Valderrama *et al.* 2005).

In protein-surface interactions, the governing factors are determined both by the physical state of the material and protein surface and the intimate solution environment factors including bound ions, surface charge, surface roughness, surface elemental composition and surface energy, all have to be considered when defining the role of the solid-solution interface.

The process of enzyme adsorption is of key importance to fundamental knowledge of enzymatic hydrolysis of polymer surfaces. The interactions are mostly electrostatic or hydrophobic. In this case, the surface properties of the fabrics have an enormous effect on the mechanism, rate and extent of adsorption. The hydrophilicity of surface has generally been regarded as a very important factor: the more hydrophobic the surface is, the higher the extent of

adsorption (Palonen *et al.* 2004). Enzymes have hydrophobic amino acids exposed on the surface that can lead to binding to the hydrophobic polyamide surfaces.

Earlier studies performed using cellulases stated that enzyme adsorption and consequently the extent of hydrolysis are directly related with the mechanical agitation provided (Cavaco-Paulo and Almeida 1996; Radhakrishnaiah *et al.* 1999; Azevedo *et al.* 2000; Cortez *et al.* 2001). Mechanical abrasion has been indicated to synergistically cooperate with enzyme activity. The results attained also demonstrated that a careful balance between enzyme activity and mechanical action is required to achieve efficient fuzz-fiber and pill removal without excessive fabric strength and weight loss.

Furthermore, when substrates are textile materials with different structures such as woven, knitted fabrics and non-wovens, the mass transfer from the bulk solution to the intra or inter yarn pores would be easily achieved by using the adequate mechanistic parameters that include type of apparatus and mechanical agitation provided (Cavaco-Paulo and Guebitz 2003).

1.4 GENETIC ENGINEERING

Biotechnology is the application of living organisms and their components to industrial products and processes. The rapid developments in the field of genetic engineering have given a new impetus to biotechnology. This introduces the possibility of 'tailoring' organisms in order to optimise the production of established or novel metabolites of commercial importance and of transferring genetic material (genes) from one organism to another. With an improved understanding of how different genes are responsible for the various characteristics and properties of an organism, techniques have been developed for isolating these active components (in particular DNA) and manipulating them outside the cell (Ramachandran and Karthik 2004). If the amino acid is not identified, random mutagenesis is used. In this case many differente mutants are generated randomly and screened for improved stability. This technique is quite laborious and is less preferred when compared with site-directed mutagenesis. Using this technique, a mutation is created at a defined site of a DNA molecule, usually a

circular molecule known as a plasmid. In general, site-directed mutagenesis requires that the wild-type gene sequence to be known. The application of this technique allows the development of enzymes with higher specificity to certain substrates that would be more hardly modified with the native ones (Watson *et al.* 1992). In the last few years many research groups created mutations that improve enzyme properties such as selectivity, activity, alternate catalytic activity and thermal stability (Estell *et al.* 1985; Takagi *et al.* 1988; Egmond and de Vlieg 2000; Priyadharshini and Gunasekaran 2007).

In textiles, genetic engineering has also been used to obtain biosensitive materials that are used to produce intelligent filter media, smart textiles, and protective clothing. Attempts have been made to transfer certain advantageous textile properties into micro-organisms where they can be more readily reproduced by bulk fermentation processes. As an example, the spider DNA is transferred into bacteria with the aim of manufacturing proteins with the strength and resilience of spider silk for use in bulletproof vests. Using biotechnological process routes, several possibilities exist for producing entirely new fibres. Research and development work is under progress in genetically modified micro-organisms and dyestuffs for the textile field (Ramachandran and Karthik 2004).

1.5 ENZYME IMMOBILISATION

Enzymes exhibit a number of features that make their use advantageous when compared with conventional chemical catalysis methods. However, an important factor determining their use in a technological process is their cost as well as their non-reusability, the instability of their structures and their sensitivity to process conditions. Many of the undesirable limitations may be overcome by using the enzyme immobilisation processes (Taylor 1991; Cao 2005). Nevertheless, immobilisation of enzymes often incurs in additional expense and is only undertaken if there is a solid economic or process advantage in the use of immobilised rather than free enzymes. The interest in the use of immobilised enzymes in industry is based on the potential advantages that they confer over

their soluble counterparts. The application of immobilisation processes allows enzymes to be recovered from solution and reused. Moreover, the enzyme stability to temperature, pH and organic solvents as well as the ability to run a process on a continuous basis can be enhanced (Taylor 1991; Nazari 1998; Drevon 2002). Compared with the free enzyme, the immobilised enzyme usually presents lower activity and the Michaelis constant increases (Durán *et al.* 2002). These alterations result from structural changes introduced to the enzyme by the applied immobilisation procedure and from the creation of a microenvironment in which the enzyme works. Steric hindrance and diffusion limitations can also be two parameters that hamper the enzyme immobilisation process.

There is a variety of methods by which enzymes can be immobilised, mainly based on chemical and/or physical mechanisms. They can be classified as follows:

1) Adsorption of the enzyme into insoluble matrices (e.g. by Van der Waals forces, ionic binding or hydrophobic forces);
2) Covalent binding of the enzyme to a support material;
3) Entrapment by intermolecular cross-linking of enzyme molecules using multi-functional reagents;
4) Encapsulation by membrane confinement of the enzyme inside a water-insoluble polymer lattice or semi-permeable membrane (Hartmeier 1986; Taylor 1991).

Among the different methods for immobilisation considered, covalent coupling of the enzymes to matrices is an extensively researched technique due to its benefits in the repeated use of biocatalysts in bioconversions and down-stream processing. This method involves the formation of a covalent bond between the enzyme and the support material, or matrix, using generally a crosslinking agent. Glutaraldehyde is a very useful bifunctional reagent which may be used to cross-link enzymes or link them to supports. It is relatively cheap, available in industrial quantities and has the additional advantage of being a biocide and sanitise the biocatalyst (Onyezili 1987; Migneault *et al.* 2004; Betancor *et al.* 2006; Silva *et al.* 2006_b).

Covalent immobilisation is more effective than the other methods of immobilisation in improving enzymes resistance to heat, chemical disruption and pH changes (Bickerstaff 1997).

1.6 DESIGN OF EXPERIMENTS

Experimental design, more frequently called design of experiments (DOE), is an important statistical tool. It is a systematic set of experiments that allows one to evaluate the impact, or effect, of one or more factors without concern for extraneous variables or subjective judgements. Thus, it is a structured, organised method for determining the relationship between the factors affecting a process and the output of that process (Box *et al.* 1978; Barros Neto *et al.* 1995). Factors are the variables under investigation that are set to a particular value (level) during the experiment. These variables may be quantitative or qualitative. Response variables are the results from the experimental run. An understanding of the relationship between the response variables and the factors is the desired outcome of the entire DOE effort. Once the relationship is understood, the response variable can usually be optimised by setting the factors to their optimal levels (Silva 2005).

The DOE process consists of four primary phases:
- the planning phase;
- the screening phase;
- the optimisation phase;
- the confirmation phase.

The DOE strategy has been widely used when evaluating the effects of several variables and for the optimisation of numerous technological processes, such as immobilisation, production of enzymes and production of food components. However, this methodology is rarely implemented in textile processes. In a recent work performed by Tzanov *et al.* (2003_b), the effects of the process variables on the wool enzymatic treatment were evaluated, using a 2^3 factorial design. Also Bruno *et al.* (2004) studied the variables that affect the

immobilisation of *Mucor miechei* lipase on polyamide membrane, using a 2^3 full factorial design. Some other works using this methodology have been published by Jovančić *et al.* (1993). Some authors have also performed the optimisation of alkaline protease production from *Bacillus* sp. by response surface methodology including factorial design and regression analysis (Puri *et al.* 2002). The application of factorial design to study of transesterification reactions using cutinase in AOT-reversed micelles was performed by Carvalho *et al.* (1997). Recently the optimisation of a serine protease coupling to Eudragit S-100 was performed by experimental design techniques (Silva *et al.* 2006_a).

1.7 RESEARCH OBJECTIVES AND THESIS OUTLINE

The classical chemical methods to improve the hydrophilicity of polyamide fabrics and other synthetic fibres and hence to promote its functionalisation impose damage to the fibres and a negative environmental impact. Many attempts have been made to replace these strong agents by an environmentally friendly process, without fibres damaging or weight loss.

The aim of this thesis is to develop an enzyme-based treatment of polyamide 6.6. The specific objectives intend to functionalise the surface of the polyamide 6.6 fabrics in order to obtain amine-enriched surfaces that can play an important role on the covalent immobilisation of proteins and others compounds.

In agreement with the aims of the present thesis, the first part (chapter 1) presents a brief bibliographic review concerning the most relevant topics related to: polyamide fibre properties, enzyme applications and specificities, state-of-the-art about enzymatic modification of synthetic fibres, stability of cutinases on organic solvents, immobilisation of proteins, genetic engineering and design of experiments. The second part contains the major results obtained within the scope of this thesis. The third part presents a general discussion, the major conclusions and some future work perspectives for continuing this research field.

Thus, the organization of this thesis consists of 3 major parts:

- **1st part:** Theoretical considerations related to the topics of thesis outline – **Chapter 1**;

- **2nd part:** Major results in the scope of this thesis. This section contains the experimental procedures, as well as the results obtained and respective discussion (chapters 2-6):

- **Chapter 2**, **Monitoring Biotransformations in Polyamide 6.6 Fibres**, describes the development of two methodologies to monitor the formation of the hydrolysis products during enzymatic hydrolysis;

- **Chapter 3**, **Influence of organic solvents on cutinase stability and accessibility to polyamide 6.6 fibres**, is focused on the influence of organic solvents, such as benzyl alcohol and dimethylacetamide on cutinase stability and accessibility to polyamide fibres;

- **Chapter 4**, **Tailoring cutinase activity towards polyamide 6.6 fibres**, describes the genetic studies performed on cutinase and the application of the mutations obtained in the polyamide 6.6 fibres functionalisation;

- **Chapter 5**, **Influence of mechanical agitation on cutinases an protease activity towards polyamide substrates**, investigates the influence of mechanical agitation on cutinases and protease performance to functionalise the surface of polyamide substrates;

- **Chapter 6**, **Laccase immobilisation on enzymatically functionalised polyamide 6.6 fibres**, describes a practical application for the previously functionalised samples; in this chapter, the immobilisation of a laccase from *Trametes hirsuta* on the enzymatically functionalised polyamide 6.6 fibres was studied;

- **3rd part:** General discussion, conclusions and future perspectives (**Chapters 7 and 8**). This part presents the general discussion about the results obtained and the major conclusions of the work. The future perspectives part describes the future work that can follow this thesis outline.

Chapter 2

MONITORING BIOTRANSFORMATIONS IN POLYAMIDE 6.6 FIBRES

2. MONITORING BIOTRANSFORMATIONS IN POLYAMIDE 6.6 FIBRES

2.1 INTRODUCTION

The present chapter describes the behaviour of cutinase from *Fusarium solani pisi* (E.C. 3.1.1.74) on the surface modification of 100% polyamide fabric samples.

The aim of this work is to report new methodologies to monitor the extension of enzymatic hydrolysis and therefore to quantify functional groups formed after enzymatic treatment. The measurement of the presence of amino groups released to the liquid treatment, as soluble oligomers, can be a direct way to prove enzymatic hydrolysis. For this purpose 2,4,6 – trinitrobenzenesulfonic acid (TNBS) was used, as it reacts with the amino groups forming a coloured complex that can be measured spectrophotometrically (Morçöl *et al.* 1997). The amino end groups at the surface of the treated fibres are detected by specific reaction with the α-bromoacrylamido wool reactive dye (Lewis 1992). The colour intensities of both samples and control are determined after a competitive staining procedure below glass transition temperature of polyamide fibres (Silva and Cavaco-Paulo 2004).

2.2 MATERIALS AND METHODS

2.2.1 ENZYMES AND REAGENTS

Commercial polyamide (100% PA 6.6) woven fabric, obtained from Rhodia (Switzerland), taffeta, with 30/28 yarnscm^{-1} and 63 gm^{-2}, was used in all experiments.

The enzyme used in this study was a cutinase (E.C. 3.1.1.74) from *Fusarium solani pisi* (GCI 2002/1410), generously supplied by Genencor International (USA). The wool reactive dye, Lanasol Red 5B – reactive red 66; C.I.: 17555, was generously supplied by CIBA (Switzerland) and the 2,4,6 – trinitrobenzenesulfonic acid (TNBS) was obtained from SIGMA (St. Louis, USA).

Lutensol AT25, a non-ionic agent, was acquired from BASF (Ludwigshafen, Germany). All other reagents used were of analytical grade.

2.2.2 ENZYMATIC HYDROLYSIS OF POLYAMIDE 6.6

To remove some impurities from the fabrics, before enzymatic treatment, all polyamide fabric samples were washed with 2 gL^{-1} of a non ionic agent, Lutensol AT25 (10 gL^{-1}) and with 2 gL^{-1} of Na_2CO_3, both at 50ºC for 1 hour and with distilled water for 1 hour. Finally, the fabric was left to dry at room temperature. All washes were performed in a laboratory scale machine (Rotawash MKIII).

In this study, two sets of experiments were carried out. In the first set, 0.20 g of polyamide fabric, cut in small pieces (± 0.5 cm each), was incubated in a glass recipient with different concentrations of cutinase (1.58×10^3 UL^{-1} to 20.10×10^3 UL^{-1}), using borate buffer (pH = 8.5; 50 mM), with a bath ratio of 1:50, at 30ºC for 24 hours. Aliquots were taken at different periods of incubation (6 and 24 hours) for each enzyme concentration assayed. A shaker bath with orbital agitation (70 rpm) was used and all the experiments were performed in duplicate.

In the other set of experiments, 1.00 g of polyamide fabric (10×14 cm) was incubated with 12.98×10^3 UL^{-1} of cutinase, using borate buffer (pH=8.5; 50 mM), at 30ºC for 70 hours. A bath ratio of 1:200 was used. The enzymatic treatment was performed in sealed, stainless steel dye pots of 250 cm^3 in a laboratory scale machine, Rotawash MKIII (vertical agitation simulating European washing machines, from SDL International Ltd) at 20 rpm. Samples of fabric were taken at 4 and 70 hours of incubation for posterior staining. All experiments were performed using duplicate samples.

After enzymatic treatment, all samples were washed with a 2 gL^{-1} Na_2CO_3 solution for 1 hour at 50ºC followed by washing with 2 gL^{-1} of Lutensol AT25 (10gL^{-1}) for 1 hour at 50ºC. After this, the samples were washed with distilled water until no protein was detected in the bath washing. The washes were performed in a Rotawash MKIII machine.

2.2.3 DETERMINATION OF THE AMINO GROUPS IN THE BATH SOLUTION TREATMENT

To follow the formation of amino groups and quantify these groups present in the liquid of treatment, the TNBS method was adapted from a methodology described in literature (Morçöl *et al*. 1997).

This method is based on the reaction of the primary amino groups with the sodium salt of TNBS. The product of this reaction is a complex between the amino groups and the TNBS, which can be read spectrophotometrically at 420 nm. The first step of the experiment was the precipitation of the protein present in the bath treatment solutions. This precipitation was done with trichloroacetic acid (TCA) (110 mM) with the total elimination of the protein content (Lowry *et al*. 1951). After protein precipitation, the pH of the supernatant was adjusted to 8.0 with a borax solution.

Afterwards the assays were done by mixing 4 mL of the supernatant with 100 µL of aqueous TNBS solution (30 mM); the mixture was incubated for 30 minutes at room temperature. The blank for the assay consisted of 4 ml of borate buffer (pH=8.0). The absorbance was read against the blank at 420 nm in a Heλios alfa spectrophotometer (Unicam). The calibration curve was performed with different concentrations of hexamethylenediamine. All measurements were performed using at least triplicate samples (Silva and Cavaco-Paulo 2004).

2.2.4 DETERMINATION OF THE AMINO END GROUPS ON THE FIBRE SURFACE BY REACTIVE STAINING

The amino end groups at the surface of polyamide fabrics were analysed by competitive staining of treated samples and controls with a wool reactive dye (Lanasol Red 5B – reactive red 66; C.I. 17555) (Silva and Cavaco-Paulo 2004). Standard reactive dyeing fixes the chromophoric structures to hydroxyl groups of the fibres. In the case of polyamide, the free amino groups at the surface of the

fibres are detected by the specific reaction with the α-bromoacrylamido dye reactive group (Figure 2.1) (Lewis 1992).

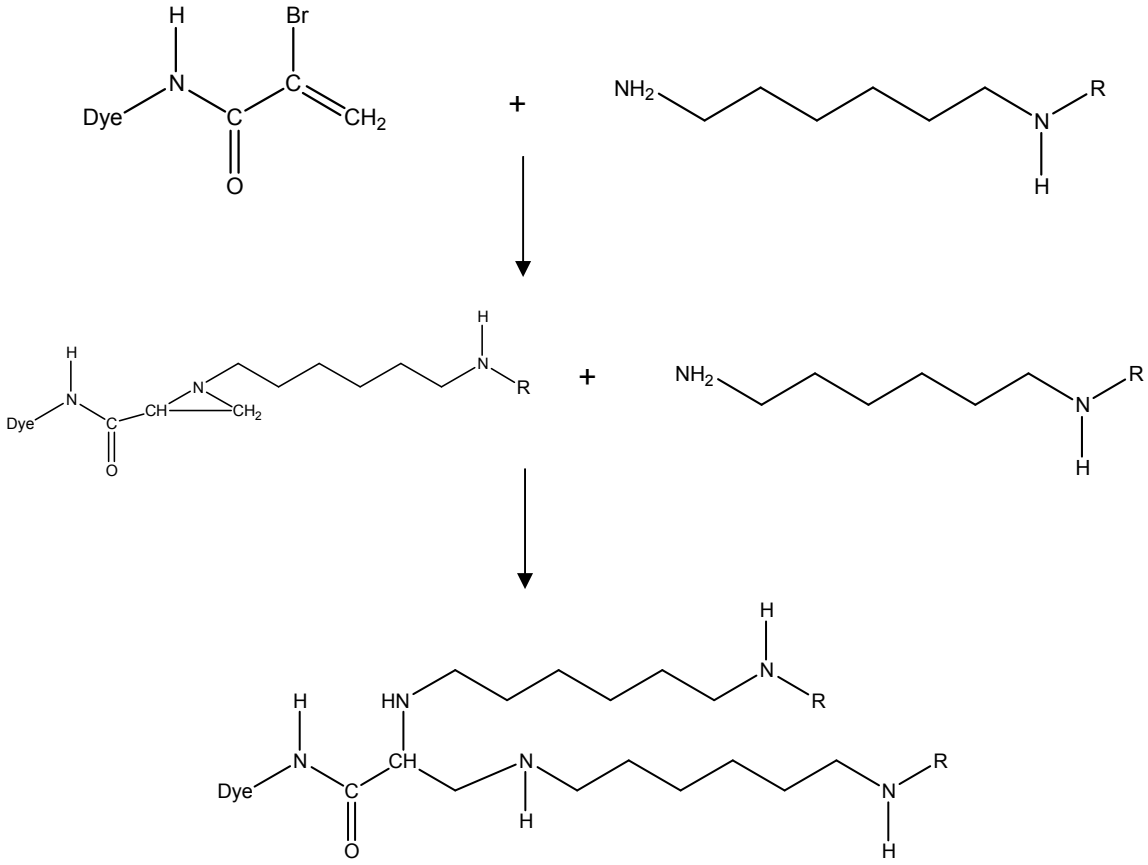


Figure 2.1 – Reaction of the α-bromoacrilamido wool reactive dye with the amino groups of polyamide oligomers resulting from enzymatic hydrolysis (adapted from Lewis 1992).

The fabric samples of polyamide were submitted to a competitive staining with 2% (o.w.f.) of reactive dye at different temperatures, 50, 60 and 70ºC. The staining was performed in sealed, stainless steel dye pots of 120 cm^3 capacity in a laboratory scale dyeing machine (AHIBA Spectradye, from Datacolor International) at 30 rpm with a gradient of 2ºCmin^{-1} for 90 minutes. A bath ratio of 1:50 was used. After staining, the samples were washed with 2 gL^{-1} of Lutensol AT25 solution (10 gL^{-1}) for 1 hour at 50ºC and then with distilled water at 50ºC until no more dye could be detected in the washing solution. The colour measurements were carried out using a spectrophotometer (illuminant D_{65} at

570 nm) (Spectraflash 600 Plus, from Datacolor International) coupled to a computer. The colour strength was evaluated as K/S at maximum absorption wavelength (570 nm). The ratio between absorption coefficient (K) and scattering coefficient (S) is related to reflectance data by applying Kubelka-Munk's law (Equation 2.1) at each wavelength, and it is proportional to dye concentration (Harold 1987; Mc Donald 1987). All measurements were performed using at least triplicate samples.

$$\frac{K}{S} = \frac{1 - R^2}{2R}$$

Equation 2.1 – Determination of K/S using the simplified Kubelka-Munk's law (R – light reflection; K – absorption coefficient; S – scattering coefficient).

The relative K/S was calculated for each sample treated, comparing the K/S value of sample with the K/S value of control, using Equation 2.2.

$$\frac{K/S_{enzyme} - K/S_{control}}{K/S_{control}} \times 100$$

Equation 2.2 – Determination of relative K/S for each treated sample.

2.2.5 DETERMINATION OF THE PROTEIN CONCENTRATION AT DIFFERENT PERIODS OF INCUBATION

To study the relationship between the adsorbed protein and the time of incubation, the determination of protein concentration was performed by following the Bradford method using bovine serum albumin (BSA) as standard. The amount of bounded protein was determined indirectly by the difference between the amount of protein placed into the reaction mixture and the amount of protein in the supernatant after enzymatic treatment and in the washing solutions. All measurements were performed using at least triplicate samples (Bradford 1976).

2.3 RESULTS AND DISCUSSION

The cutinase treatment of polyamide yields primary amino groups and carboxylic groups resulting from the cleavage of the amide bonds.

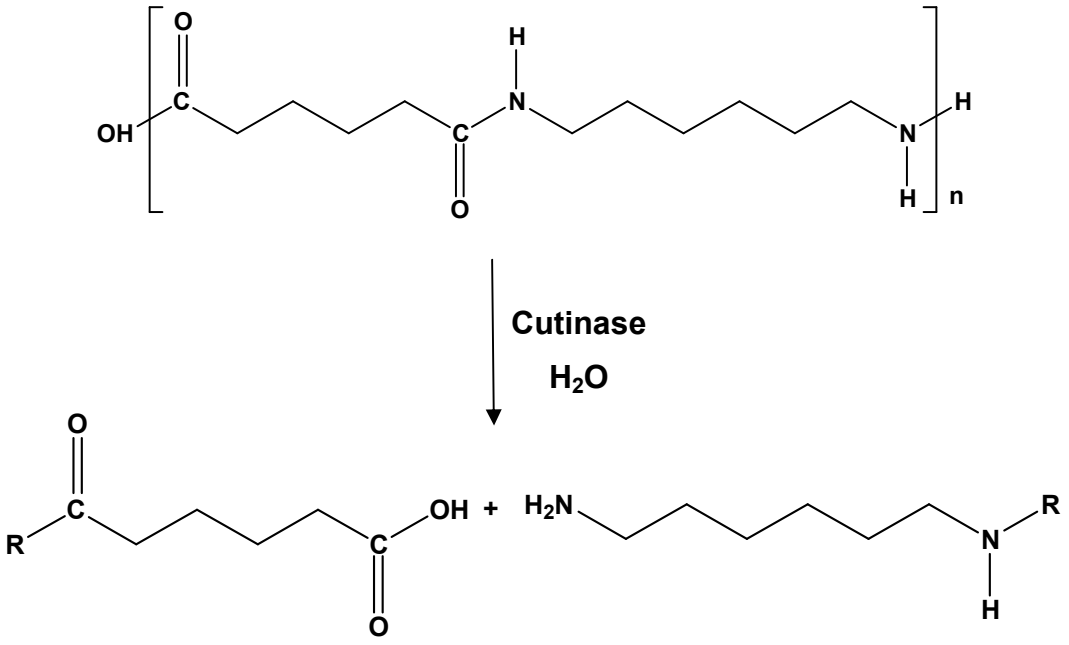


Figure 2.2 – Reaction of polyamide 6.6 hydrolysis with cutinase by cleavage of amide bonds.

These groups can be found in the liquid bath treatment or as amino end groups at the fibre surface. The amount of the soluble amino groups produced after 6 and 24 hours of enzymatic treatment is shown in Figure 2.3. These results confirm that very low levels of soluble amines are produced after the enzymatic hydrolysis process of the polyamide. This also confirms our previous findings that 1 U of cutinase measured towards *p*NPP (*p*-nitrophenyl palmitate) yield only 2.30×10^{-3} U of activity over the polyamide fibre (Silva *et al.* 2005_a; Cavaco-Paulo *et al.* 2005).This is mainly due to an heterogeneous reaction where the enzyme can only access the amide groups at the fibre surface. The results of Figure 2.3 show that saturation levels can be observed for very high dosages of enzyme in the range of 2×10^{4} UL^{-1}, confirming the somewhat superficial treatment mode. Two sets of experiments were performed. In the first set (Figure 2.3) the treatment of

polyamide fibres was done in a shaker bath and therefore very low quantities of soluble amino groups would be expected. The results obtained confirm previous work with cotton fibres (Cavaco-Paulo and Almeida 1994). The enzymatic treatment of cotton fibres is also restrained to fibre surface and therefore a synergistic action was found with high levels of mechanical agitation and enzyme activities (Cavaco-Paulo 1998_a). In the second set of experiments (Figure 2.4), higher levels of mechanical agitation were provided in a Rotawash machine (vertical agitation simulating European washing machines) and a saturation enzyme dosage of 12.98×10^3 UL^{-1} was used. Despite the high levels of mechanical agitation, a level-off of amines produced was found after 4 hours of treatment with a maximum in the range of 0.01 mM. These experiments also monitored the protein adsorption levels, obtaining a maximum of adsorption levels of 15% after 4 hours of treatment. These results can be explained considering that, in the first hours of incubation, the protein is adsorbed to the fibre raising a saturation level (Figure 2.4). It seems evident that, after 4 hours of incubation, lower amounts of protein (15%) are adsorbed and almost no more amino groups are formed. The results obtained show that there is a relation between maximum adsorption levels and amino groups formation.

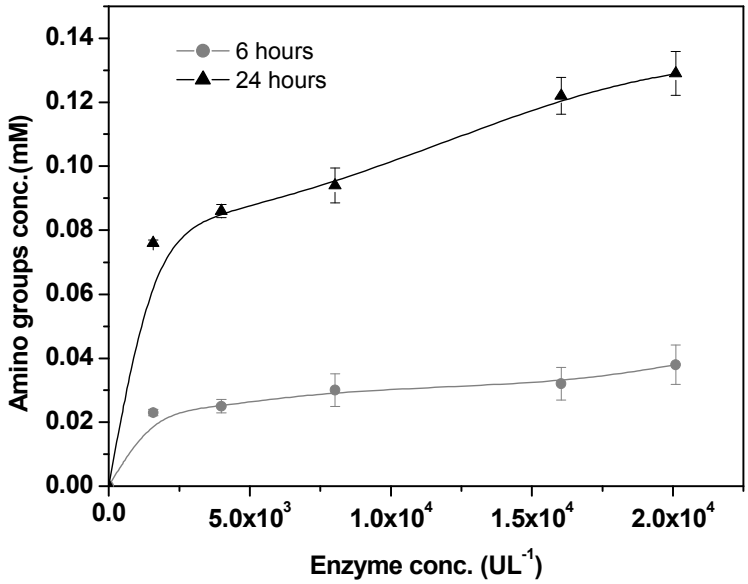


Figure 2.3 – Amino groups concentration in the bath after treatment of polyamide samples with cutinase for 6 and 24 hours of incubation in a shaker water bath with orbital agitation.

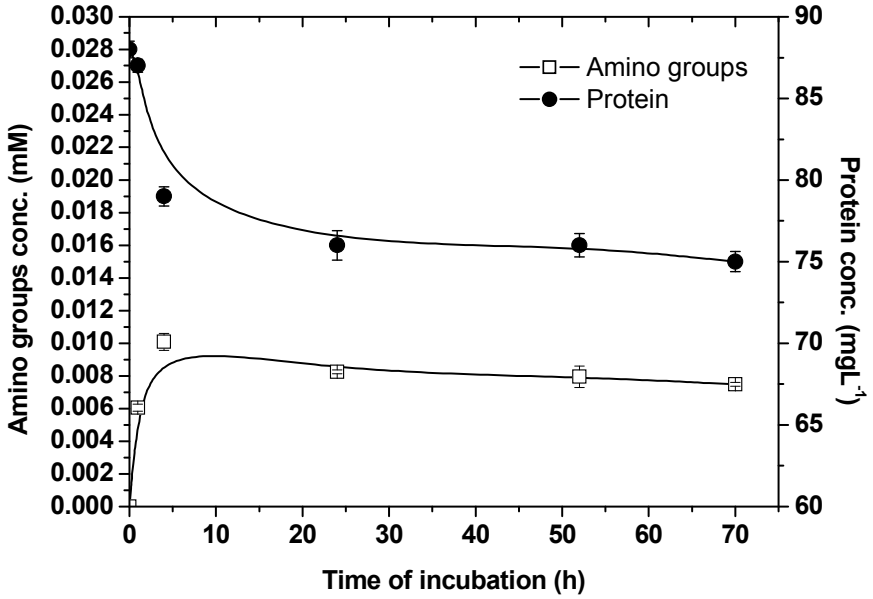


Figure 2.4 – Variation of protein and amino groups concentration in the bath treatment *vs* time of incubation of polyamide samples treated with 12.98×10^3 UL^{-1} of cutinase in a Rotawash MKIII machine.

The percentage of K/S increase of samples treated with cutinase and stained with a wool reactive dye, by comparison with the controls is shown in Figure 2.5. The reactive dye reacts with the primary amino groups present at the fibre surface and the colour intensities are proportional to the amount of those free groups formed at the surface of the fibres. Results from Figure 2.5 seem to show the formation of amino end groups after 4 hours of treatment, while after 70 hours there are no more end groups present due to the substrate saturation with enzyme after the first hours of incubation. After a certain period of incubation (4 hours), the substrate (fabric) was totally covered with enzyme that filled all the available sites for enzymatic attack. Therefore, no more hydrolysis products could be produced. This fact is of extreme importance regarding the future application of this enzyme in textile industry, where the time of incubation is one of the most important factors to be taken in account. The results presented in Figure 2.5 show that after 70 hours of incubation it was difficult to detect the amino groups at the surface of the fabric. This is mainly due to the rearrangement of the formed chains during this long period of incubation. The amino groups might be hidden and less accessible to the reactive dye.

The best staining results were obtained at 50ºC (4 hours of treatment), below glass transition temperature of fibre. The dye fixation occurs only at the surface of the fibre, where it is believed that cutinase can act. Above glass transition temperature, in the range of 57ºC, the polyamide structure is more open and the amino groups, formed by enzymatic action, might be hidden as suggested by the dyeing results at 70ºC, due to the groups rearrangement.

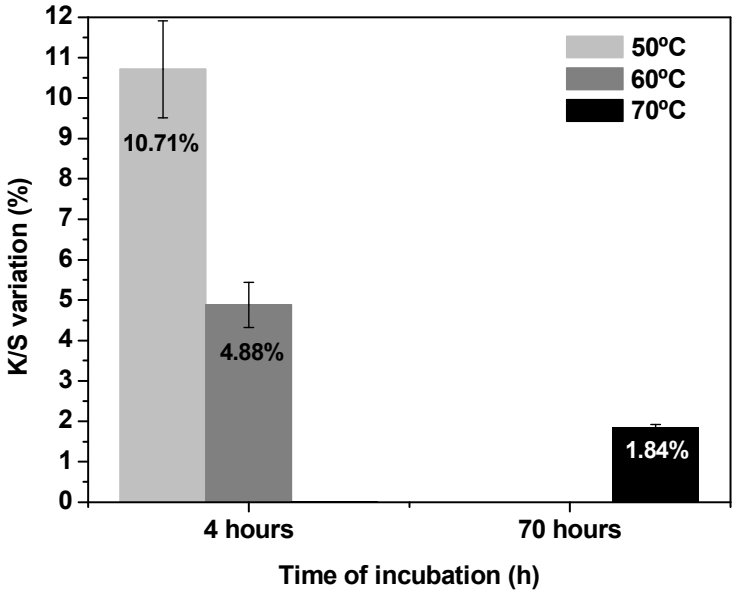


Figure 2.5 – Percentage of increase in K/S (λ= 570 nm) for polyamide samples treated with cutinase and competitively stained with a wool reactive dye (Lanasol Red 5B). The relative K/S was calculated using Equation 2.2. The controls were identically prepared for each assay conditions, except that buffer substituted the enzyme.

2.4 CONCLUSIONS

The results obtained proved that enzymatic hydrolysis with cutinase can modify the surface of polyamide fabrics by means of hydrolysis of the amide linkages with the formation of amino and carboxylic groups.

It was also shown that the enzymatic hydrolysis can be followed by determination of amino groups in the solution and at the fibre surface. Using the TNBS method amino groups were found in the liquid treatment of polyamide fabrics, which proves the enzymatic hydrolysis. When analysing the staining results, it was evident that after enzymatic hydrolysis, some groups resulting from the cleavage of the amide bonds remain at the surface of the fibre. These groups react with the reactive dye, and their presence can be detected as an increase of the coloration levels. Both methods were very useful tools to prove the enzymatic

hydrolysis and to predict the biotechnological phenomenon that occurs when fibres are submitted to the enzymatic action.

Chapter 3

INFLUENCE OF ORGANIC SOLVENTS ON CUTINASE STABILITY AND ACCESSIBILITY TO POLYAMIDE 6.6 FIBRES

3. INFLUENCE OF ORGANIC SOLVENTS ON CUTINASE STABILITY AND ACCESSIBILITY TO POLYAMIDE 6.6 FIBRES

3.1 INTRODUCTION

In the previous chapter it was demonstrated that polyamide 6.6 fibres can be efficiently modified by enzymatic action using a cutinase from *Fusarium solani* sp. *pisi.* Two methodologies to quantify the hydrolysis products, the amino groups in the bath treatment and at the surface of the treated fabrics, were also developed.

Since enzymes are large molecules, their diffusion inside the fibre core is difficult to occur. That implies that the reaction is site selective, involving only the modification at the fibre surface, which has the advantage of avoiding the loss of strength of the treated materials (O'Neill and Cavaco-Paulo 2004; Silva and Cavaco-Paulo 2004; Matamá *et al.* 2006).

In order to reduce the treatment time and improve enzyme adsorption as well as hydrolytic efficiency, treatments with cutinase in presence of organic solvents, like benzyl alcohol (BA) and dimethylacetamide (DMA) were performed in this part of the work. These organic solvents are well known for the swelling ability of fibres and are used as dyeing assistants of polyamide 6.6 fibres, accelerating dye uptake (Burkinshaw 1995; Nechwatal and Rossbach 1999; Domingues *et al.* 2003). The stability of cutinase in presence of these solvents was at the same time evaluated.

By performing the enzymatic treatment with cutinase in presence of different concentrations of these two solvents, our purpose was to "open" the compacted structure of polyamide 6.6 fabric increasing enzyme adsorption towards the insoluble substrate, hence increasing enzyme action. The presence of organic solvents on the reaction medium would allow an increase of enzyme stability, which is a main requirement for a continuous enzymatic process application.

This chapter describes the stability studies of cutinase in presence of organic solvents, benzyl alcohol (BA) and dimethylacetamide (DMA). The extent of hydrolysis when this enzyme and the organic solvents are used simultaneously was measured as well as the amount of enzyme adsorbed. It was also intended to achieve the optimum organic solvent concentration for further applications on polyamide samples (Silva *et al.* 2005_b).

3.2 MATERIALS AND METHODS

3.2.1 ENZYMES AND REAGENTS

Commercial polyamide (100% PA 6.6) woven fabric, obtained from Rhodia (Switzerland), taffeta with 30/28 yarnscm^{-1} and 63 gm^{-2}, was used in all experiments. Cutinase from *Fusarium solani pisi* (GCI 2002/1410) was generously supplied by Genencor International (USA). The wool reactive dye used, Lanasol Red 5B – reactive red 66; C.I.: 17555, was generously supplied by CIBA (Switzerland) and the acid dye (C.I. Acid Red 1-18050) was obtained from Sigma (St. Louis, USA). Lutensol AT25 was acquired from BASF (Ludwigshafen, Germany). The organic solvents, benzyl alcohol and dimethylacetamide were also obtained from Sigma (St. Louis, USA). All other reagents used were of analytical grade.

3.2.2 STUDY OF THE STABILITY OF CUTINASE IN PRESENCE OF BENZYL ALCOHOL AND DIMETHYLACETAMIDE

3.2.2.1 PROCEDURE FOR DETERMINATION OF CUTINASE STABILITY

The operational stability of cutinase was investigated in the presence of two organic solvents. The enzyme was incubated at a final concentration of 1 mgL^{-1} in 50 mM phosphate buffer, pH 8.0 with 0.05% of sodium azide, in the presence of several amounts of benzyl alcohol (BA) and dimethylacetamide (DMA) at 35ºC using a shaking water bath at 72 rpm. For each assay, the final volume was 25 mL and 50 mL closed tubes were used as containers. The amounts of organic solvents tested were 1.5%, 5%, 10% and 15% of BA and 5% and 10% of DMA and a combination of both solvents (1.5% BA + 10% DMA; 10% BA + 10% DMA). Measures were taken until activity was below 50%.

The half-life time was calculated as ln2/k, from the first order exponential decay fit of data using equation 3.1.

$$\frac{a}{a_0} = Ae^{-kx}$$

Equation 3.1 – Determination of half-life time of cutinase using the first order exponential decay.

3.2.2.2 DETERMINATION OF CUTINASE ACTIVITY

Cutinase activity was determined using *p*-nitrophenyl butyrate (*p*-NPB) as a substrate (Shirai and Jackson 1982). For assays with this substrate, 20 µL of *p*-NPB 6 mM were added to 1880 µL of 50 mM phosphate buffer, pH 8.0 containing 0.05% of sodium azide, and then 100 µL of bath treatment aliquots were incubated in this reaction media for 1 min, at 35ºC. The hydrolysis was stopped by addition of 2 mL of acetone. Activity was measured as absorbance increase at 400 nm. 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. The assays were done in triplicate.

3.2.3 POLYAMIDE SAMPLES PREPARATION

To remove some impurities from the fabrics, before enzymatic treatment all polyamide fabric samples were washed with 2 gL^{-1} of a non ionic agent, Lutensol AT25 (10 gL^{-1}) and with 2 gL^{-1} of Na_2CO_3, both at 50ºC for 1 hour and finally with distilled water for 1 hour. Finally, the fabric was left to dry at room temperature. All washes were performed in a laboratory scale machine (Rotawash MKIII).

3.2.4 STUDY OF THE INFLUENCE OF ORGANIC SOLVENTS ON THE POLYAMIDE STRUCTURE

To study the influence of organic solvents on polyamide structure modification, samples of polyamide were stained with an acid dye in presence of organic solvents. The conditions of enzymatic treatment in presence of organic solvents were simulated in order to measure the extension of the fibre modification caused by the organic solvents. The assays were performed using 2.00 g of polyamide samples that were stained for 4 hours at 35ºC using a bath ratio of 1:100. The dye used was an acid dye (C.I. Acid Red 1-18050) and the organic solvents used were 1.5% of benzyl alcohol (BA) and 1.5% of benzyl alcohol (BA) + 10% of dimethylacetamide (DMA). The staining was performed in sealed, stainless steel dye pots in a laboratory scale textile treatment machine (Rotawash MKIII) at 20 rpm. After staining the samples were washed with 2 gL^{-1} of a Lutensol AT25 solution (10 gL^{-1}) at 50ºC for 1 hour followed by washing with distilled water, until no more dye could be detected in the washing water. The colour measurements were carried out using a spectrophotometer (illuminant D_{65} at 570 nm) (Spectraflash 600 Plus, from Datacolor International) coupled to a computer. The colour strength was evaluated as K/S at maximum absorption wavelength (570 nm). The ratio between absorption (K) and scattering (S) is related to reflectance data by applying Kubelka-Munk's law at each wavelength,

and it is proportional to dye concentration. All measurements were performed using at least triplicate samples.

3.2.5 ENZYMATIC TREATMENT OF POLYAMIDE FABRICS IN PRESENCE OF ORGANIC SOLVENTS

In order to measure the hydrolysis extent of enzymatic action, samples of polyamide fabric were incubated with cutinase in presence of organic solvents.

The assay was performed using 1.00 g of polyamide fabric that was incubated with 2×10^3 UL^{-1} of enzyme and different concentrations of organic solvents (1.5% of benzyl alcohol and 1.5% of benzyl alcohol + 10% of dimehylacetamide) at 35ºC using phosphate buffer (pH 8.0; 50 mM) for 4 hours. Aliquots were taken at different periods of incubation (1 and 4 hours) for each enzyme concentration assayed for posterior chemical analysis and samples of fabric were taken at 4 hours of treatment for posterior staining. A bath ratio of 1:200 was used. The enzymatic treatment was performed in sealed, stainless steel dye pots of 250 cm^3 in a laboratory scale dyeing machine (vertical agitation simulating European washing machines – Rotawash MKIII) at 20 rpm. Samples treated only in presence of buffer and in presence of organic solvents were used as controls. After enzymatic treatment, all samples were washed with a 2 gL^{-1} Na_2CO_3 solution for 1 hour at 50ºC followed by washing with 2 gL^{-1} of Lutensol AT25 solution (10 gL^{-1}) for 1 hour at 50ºC. After this, samples were washed with distilled water until no protein was detected in the bath washing. The washes were performed in a Rotawash MKIII machine. All experiments were performed in duplicates.

3.2.6 DETERMINATION OF THE AMINO GROUPS IN THE BATH SOLUTION TREATMENT

To follow the formation of amino groups and quantify these groups present in the treatment liquid, the trinitrobenzenesulfonic acid (TNBS) method was adapted from a methodology described in detail in the chapter 2 (section 2.2.3).

3.2.7 DETERMINATION OF THE AMINO END GROUPS AT THE FIBRE SURFACE BY REACTIVE STAINING

The amino groups resulting from enzymatic hydrolysis at the surface of the treated fabrics were measured as previously described in chapter 2 (section 2.2.4)

3.3 RESULTS AND DISCUSSION

3.3.1 CUTINASE STABILITY IN PRESENCE OF ORGANIC SOLVENTS

In order to measure the cutinase stability in presence of organic solvents (benzyl alcohol and dimethylacetamide), the enzyme activity at different periods of incubation was measured and the half life time was determined (Table 3.1).

Table 3.1 – Influence of the two organic solvents on operational stability of cutinase. Cutinase was incubated at 35ºC, pH 8.0 in a water bath with agitation, under different media conditions. The half-life time was calculated using Equation 3.1

ORGANIC SOLVENTS	HALF-LIFE TIME (hours)
Control (buffer)	35 ± 3.0
1.5% Benzyl Alcohol	26 ± 2.0
5% Benzyl Alcohol	< 1
10% Benzyl Alcohol	< 1
15% Benzyl Alcohol	< 1
1.5% Dimethylacetamide	12 ± 3.0
5% Dimethylacetamide	11 ± 2.0
10% Dimethylacetamide	12 ± 2.5
1.5% Benzyl Alcohol + 10% Dimethylacetamide	14 ± 2.0
10% Benzyl Alcohol + 10% Dimethylacetamide	< 1

Analysing the results of Table 3.1, it can be observed that in presence of percentages of benzyl alcohol above 1.5%, cutinase enzyme loses all activity in

the first hour of incubation. For 1.5% of benzyl alcohol the activity decreases when compared with the value of control but this amount of solvent does not denature the enzyme. It can also be observed that the half-life time of the enzyme in presence of benzyl alcohol (1.5%) is higher than in presence of dimethylacetamide. When we tested the two solvents simultaneously, the half-life time decrease, although in the case of 1.5 % of BA + 10 % of DMA, the half-life time is higher, compared with the value obtained with the other combination, and corresponds to a half of the value obtained for 1.5 % of BA. The results obtained are not fully understood yet but the probable explanation can be based on the hydrophobicity of the pair enzyme/organic solvent. Hydrophobic solvents are usually more superior to hydrophilic ones when used as enzymatic reaction media because the latter have a greater tendency to strip tightly bound water (which is essential for catalytic activity) from the enzyme molecules (Klibanov 2001). Low percentages of benzyl alcohol (< 1.5%) have a great affinity to the active site of the cutinase enzyme that is also hydrophobic. However, the use of low amounts of benzyl alcohol in the system does not affect the enzyme activity, which is maintained in acceptable values. When higher amounts of benzyl alcohol are used, the great affinity of this solvent to the active site of the enzyme might affect the internal structure of the enzyme, causing its denaturation. On the other hand, when dimethylacetamide is used, the decrease of activity is probably due to the tendency of this solvent to strip tightly bound water from the enzyme molecules. Regarding this, the enzyme becomes more rigid and looses its absolute activity but the stability is somehow maintained.

3.3.2 POLYAMIDE FIBRES ACCESSIBILITY IN PRESENCE OF ORGANIC SOLVENTS

To measure the accessibility of the organic solvents to the polyamide structure, fabric samples were stained for 4 hours at 35ºC with an acid dye (CI Acid Red 1 - 18050) in presence of organic solvents (Figure 3.1). Analysing the results it can be seen that the combined action of the benzyl alcohol and dimethylacetamide has the most pronounced effect on polyamide structure. In

presence of benzyl alcohol the samples stained with the acid dye show an increase above 100% of K/S respecting to the control (buffer). The samples stained in presence of a combined action of benzyl alcohol and dimethylacetamide show an increase of K/S above 150% respecting to the control (buffer). The organic solvents act on the polyamide fibre like swelling agents, opening the structure that becomes more accessible to the dye that penetrates into the fibre structure.

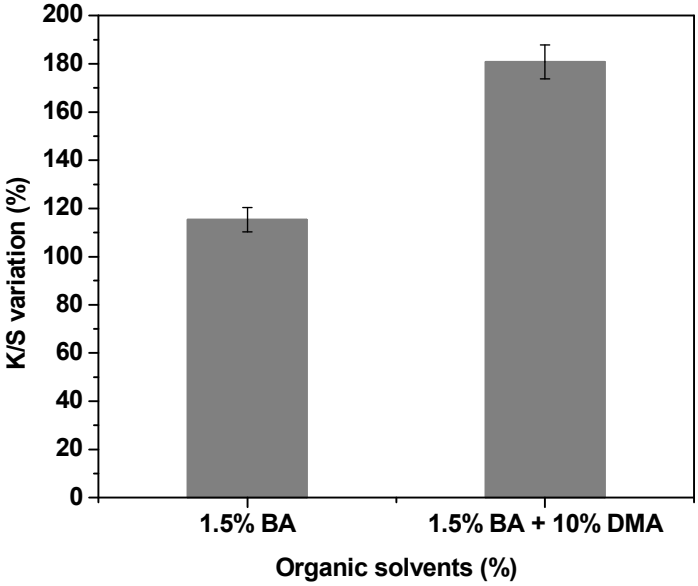


Figure 3.1- Colour level variation of fabrics treated with solvents (4 hours) relatively to the fabrics treated with buffer (4 hours) at 35ºC; the staining was performed with the Acid Red 1-18050.

3.3.3 SOLUBLE AMINO GROUPS IN THE BATH SOLUTION TREATMENT

The cutinase treatment of polyamide yields carboxylic and amino groups resulted from the cleavage of the amide bonds of the fibre chains. These groups can be found on the surface of the treated fabric or in the liquid of treatment. The amino groups resulting from the polyamide hydrolysis were measured spectrophotometricaly as described before. The amount of soluble amino groups produced after 1 and 4 hours of incubation is shown in Figure 3.2. After 4 hours

of incubation a maximum of 0.15 mM of amines was achieved. As expected, the organic solvents action increased enzyme accessibility into the fibre, which can be seen as an increase of amino groups produced. The amount of amino groups formed in these cases (1.5% of BA and 1.5% of BA + 10% of DMA) is higher than the one obtained only with cutinase enzyme.

The results also demonstrate that when cutinase and BA were used simultaneously, there is an increase of amino groups after 1 hour of treatment, but after 4 hours the level of amines reached is the same as the one obtained when the samples were treated with cutinase (without solvents). This can be due to the higher enzyme deactivation along the time in the presence of solvents. Solvents have beneficial effect in the promotion of the accessibility of the dye or enzyme to the fibre, but they are detrimental for the stability of the enzyme. An equilibrium between the two effects must be reached using short periods of treatment.

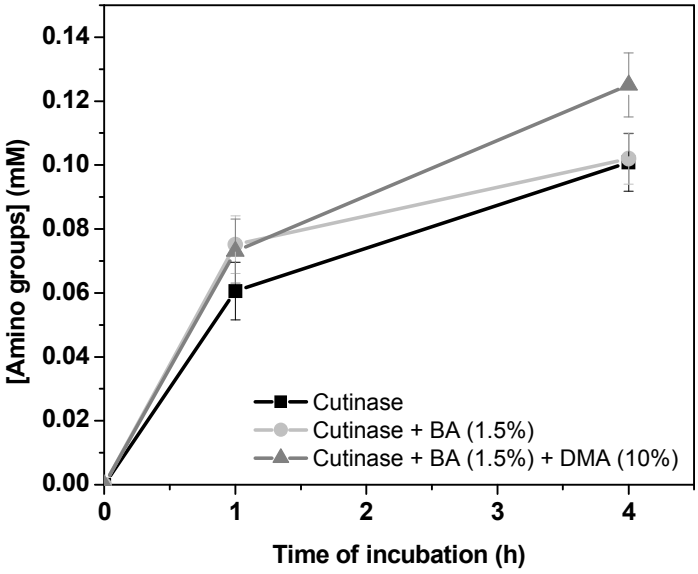


Figure 3.2 – Concentration of primary amino groups in the liquid of treatment of polyamide samples (2×10^3 UL^{-1} of protein; 35ºC using a Rotawash MKIII machine).

3.3.4 AMINO END GROUPS AT THE FIBRE SURFACE IDENTIFIED BY REACTIVE STAINING

In a previous work we have shown that the grade of conversion by cutinase is low in synthetic substrates (Silva *et al.* 2005_a).The polyamide hydrolysis was promoted using cutinase in the presence of organic solvents for 4 hours and the treated samples were stained at 50ºC with a reactive dye, specific to react with the primary amino groups formed. The reason for which a low temperature (50ºC) was chosen is due to the fact that above this value the polyamide structure is more open and the amino groups formed by enzymatic action might be hidden in the interior of the fibre, as it was concluded in chapter 2. The spectral values obtained are shown in Figure 3.3. Comparing the K/S values of samples treated with cutinase and the samples treated with cutinase in presence of benzyl alcohol + dimethylacetamide, it can be seen that organic solvents action can improve the enzymatic hydrolysis. The accessibility of the enzyme to the fibre increases, and the enzymatic action might occur, not only at the surface of the fibre, but also at a more hidden part of the structure.

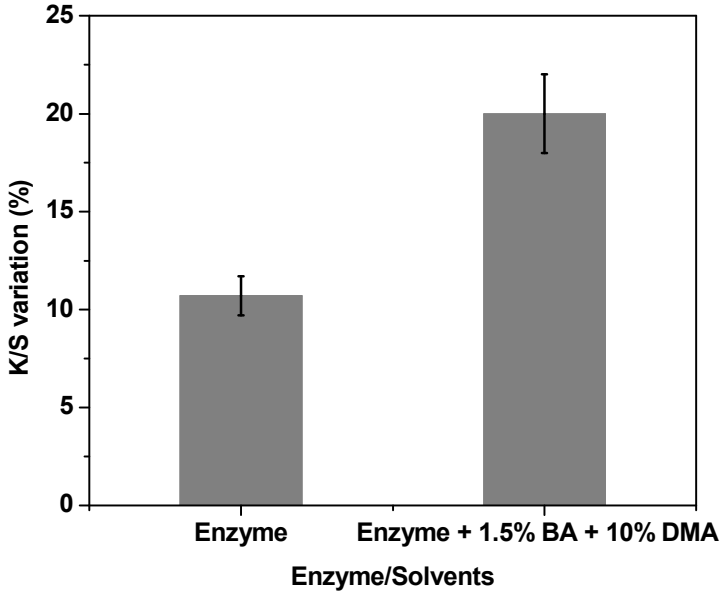


Figure 3.3 – Percentage of increase in K/S for polyamide samples treated for 4 hours with cutinase and organic solvents, competitively stained with Lanasol Red 5B (C.I. Reactive Red 66) at 50ºC. The K/S was calculated using Equation 2.2. The controls consisted on samples treated only with buffer and samples treated only with organic solvents. K/S values of samples treated only in presence of organic solvents were subtracted to the total K/S value.

3.4 CONCLUSIONS

It can be concluded that the activity of cutinase enzyme is preserved in the presence of low amounts of benzyl alcohol and dimethylacetamide. Polyamide fibres become more “open” structures and more suitable to be modified by cutinase action. The percentage of benzyl alcohol and dimethylacetamide was optimised in order to improve the enzymatic treatment of the fibres. The presence of these solvents produces an increase of cutinase accessibility to the polyamide structure and an increase of the amino groups formed both in solution and at the fibre surface

The low amounts of solvents used 10% for DMA and 1.5% for BA will not present environmental concerns in the face of achieved benefits like low process time and high amount of amino groups produced.

Chapter 4

TAILORING CUTINASE ACTIVITY TOWARDS POLYAMIDE 6.6 FIBRES

4. TAILORING CUTINASE ACTIVITY TOWARDS POLYAMIDE 6.6 FIBRES

4.1 INTRODUCTION

In the previous chapters it was demonstrated that cutinase is a versatile enzyme. It shows unusual stereolytic activity towards the non natural polyamide substrates although turnover rates are very low. The analysis of the three-dimensional structure of cutinase from *Fusarium solani pisi*, (PDB code 1CEX) (Longhi *et al.* 1997), shows that the external, but closed active site, is hindering the access to the fibre substrate. In order to overcome this difficulty, the genetic modification of native cutinase was performed.

This chapter describes the studies of genetic engineering on cutinase. The modelling studies were conducted on the native enzyme structure aiming enlarging the area around the active site to better accommodate the polyamide substrate. The site-directed mutagenesis was performed by changing specific amino acid residues around the active site by alanine (Leucine81Alanine, Asparagine84Alanine, Leucine182Alanine, Valine184Alanine and Leucine189Alanine) in order to fit a larger substrate in the active site. Five mutations were obtained.

A comparative discussion was made about the biocatalytic ability of the several mutations, based on modelling data, enzyme activity and protein adsorption levels from the polymeric substrate. 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 activity of cutinase was also discussed (Araújo *et al.* 2007).

4.2 MATERIAL AND METHODS

4.2.1 ENZYMES AND REAGENTS

The experimental part of genetic modification of native cutinase was performed in other laboratory and it was based in a methodology described by Araújo *et al.* (2007).

For biodegrading experiments a commercial polyamide (PA 6.6) woven fabric, taffeta, with 30/28 yarnscm^{-1} and 63 gm^{-2}, supplied by Rhodia, Switzerland was used. All other reagents used were laboratory grade reagents.

4.2.2 MODELLING STUDIES

The modelling studies were performed in the Universidade Nova de Lisboa, Institute of Chemical and Biological Technology and the methodologies applied are described by Araújo *et al.* (2007).

4.2.3 PLASMID CONSTRUCTION AND PROTEIN EXPRESSION

The plasmid construction was performed as described by Araújo *et al.* (2007).

4.2.4 CUTINASE ACTIVITY TOWARDS *P*-NITROPHENYL BUTIRATE (*P*- NPB)

The stereolityc activity of cutinase was determined using the methodology described in chapter 3 (section 3.2.2.2).

4.2.5 CUTINASE ACTIVITY TOWARDS POLYAMIDE 6.6 FIBRES

1.00 g of polyamide fabric cut in pieces (± 0.5 cm) was incubated with 20 mgL^{-1} of native and genetically modified mutant enzymes, in 300 mL of phosphate buffer (0.10 M NaOH, 0.10 M KH_2PO_4, pH 7.5) at 37ºC. Samples were incubated in erlenmeyers flasks using a shaking bath with orbital agitation (90 rpm) for 48 hours. After treatment all samples were washed with 2 gL^{-1} of Na_2CO_3 for 2 hours at 50ºC, in order to stop the enzymatic reaction, followed by washing with 2 gL^{-1} of Lutensol AT25 solution (10 gL^{-1}) of at 50ºC for 1 hour. The hydrolysis products were quantified as previously described in chapter 2 (section 2.2.3).

4.2.6 DETERMINATION OF PROTEIN ADSORPTION

The total protein concentration was estimated by the Bradford quantitative protein determination assay using bovine serum albumin as standard (Bradford 1976).The protein adsorption was obtained by measuring the difference between the amount of protein introduced into the reaction mixture and the amounts of protein in the supernatant and washing solutions.

4.2.7 CUTINASE ACTIVITY TOWARDS ALIPHATIC POLYESTERS

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 UmL^{-1} of cutinase in a 3 mL phosphate buffer bath (pH=7.5) at 37ºC for 5 hours. The incubations took place in a Rotawash machine MKII at 20 rpm. The activity towards aliphatic substrates was quantified by measuring the succinic acid formation, using the succinic acid UV kit method (UV-method; Cat. Nº10176281035; Boehringer Mannheim) obtained from Biopharm (Germany).

4.3 RESULTS AND DISCUSSION

Molecular modelling studies were performed by docking the synthetic model substrate of PA 6.6 at the cutinase active site (Figure 4.1). All mutations were done to create more space in order to fit the large inaccessible polymer in the active site of the cutinase. The modelling studies show that mutations L182A, L189A, L81A and V184A provide a better stabilization of the tetrahedral intermediate (TI) of the model substrate relatively to the native enzyme (Table 4.1), while the N84A mutation fails in stabilising the TI model substrate due to the favourable interaction of the aspargine with the oxyanion hole. This is in accordance with the experimental activity obtained for *p*-NPB and PA 6.6 (Table 4.2). Higher stabilization is achieved with L182A as shown by the experimental results (Table 4.2). The modelling results suggest that L189A, L81A and V184A also stabilise TI, but in a lower extent. Experimentally, a higher hydrolytic activity was obtained with L182A form (119%) while L189A, V184A and L81A displayed a slight decrease (Table 4.2). 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 stabilisation of model substrate than the native enzyme. The modelling studies also predict that the longer polymer chain in PA 6.6 fabrics will also be more stabilised by this modified enzyme, which is corroborated by the experimental results, since there is a wider channel in the active site for the polymer to go through.

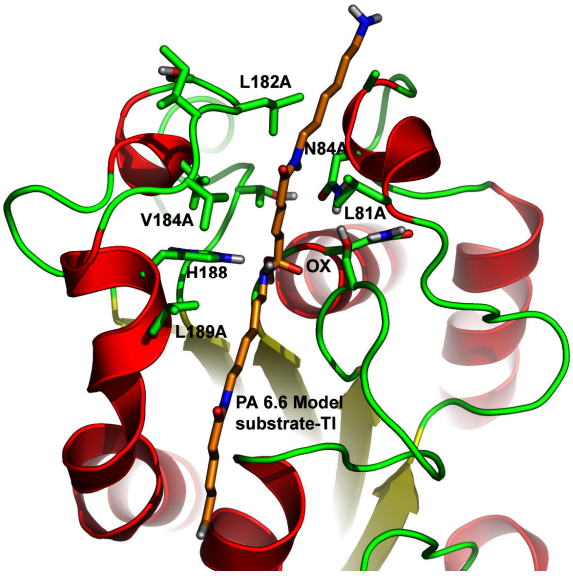
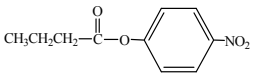
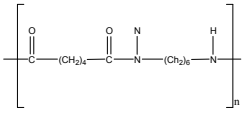


Figure 4.1 - Detail of the active site X-ray structure of cutinase with the energy minimized structure of the TI PA 6.6 model substrate. The catalytic histidine (H188) and oxyanion-hole (OX) are shown. Residues mutated in this study are labelled as: L81A, N84A, L182A, V184A and L189A (Araújo *et al.* 2007).

Table 4.1 - Stabilization free energy of the TI model substrate of PA 6.6 estimated for the mutated enzymes. Free energies are calculated relatively to the native enzyme (Araújo *et al.* 2007).

Mutation	$\Delta\Delta G$ (kJmol^{-1})
L182A	-3.80
L189A	-2.38
V184A	-2.44
L81A	-1.77
N84A	15.95

Table 4.2 - Activity of cutinases towards soluble substrate (*p*-NPB) and towards PA 6.6; protein adsorption levels after fabrics incubation with 20 mgL^{-1} of each cutinase mutant during 48 hours (values are normalised to the native enzyme)

Mutation	***p*- nitrophenyl butyrate (*p*-NPB)**  **% (Umg^{-1})(±sd)**	**Amines formed in solution after hydrolysis of PA66 fibres (48 h)**  **% (mM)(±sd)**	**Protein adsorption levels after 48 h of incubation (%)(±sd)**
Native	100 (210)	100(0.094)	30 (± 1.5)
L182A	465 (±1.8)	119 (± 2.5)	25 (± 2.0)
L189A	147 (±1.3)	94 (± 3.0)	37 (± 2.0)
V184A	289 (±2.2)	98 (± 3.0)	21 (± 2.5)
L81A	125 (±1.8)	98 (± 2.8)	43 (± 2.0)
N84A	45 (±2.6)	83 (± 3.0)	15 (± 3.0)

The activity of cutinases towards PA 6.6 fibres was expressed as mM of soluble amines, obtained after a certain period of time. Due to the fact that the substrate is solid, no proper Michaelis-Menten kinetic could be calculated. Since the objective was to compare performances of each mutant enzyme based on equal amounts of protein, it were expressed those estimated activities as 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 modelling studies on basis of the favourable interaction of the aspargine with the oxyanion hole (Table 4.1). Modified cutinases L182A and V184A have shown a remarkable increase in activity on *p*-NPB. Hydrolytic activity of L182A form increased more than 4-fold. This seems to be a promising mutation to modify the hydrophobic surface of polyamide fibres.

Cutinase is able to biodegrade polyamide 6.6 substrates, but the designed mutations failed to give a clear increase of activity (Table 4.2). Just 19% of

increase of amines was found for L182A mutant. These results tend not to be in good agreement with the modelling studies of the free energy of stabilisation of TI for all mutant enzymes for polyamide 6.6 (Table 4.1). Given that PA 6.6 fibres are mostly hydrophobic, the adsorption properties of native and mutant unbound enzymes were modelled considering an analysis based on the total hydrophobic surface of the enzyme. The tested modified enzymes have an equal or lower percentage of hydrophobic surface in comparison with the native, as it was expected (Table 4.3), taking into account 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 fibres, but V184A and L189A show a decrease in hydrophobic area, suggesting that fibre adsorption is reduced in this order. The modelling studies predictions are not in total agreement with the experimental results. N84A, L81A, V184A, and L189A displayed different behaviour of protein adsorption levels when compared with the modelling studies. On the other hand the L182A seems to present a slight decrease.

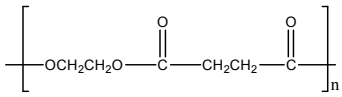
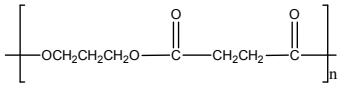
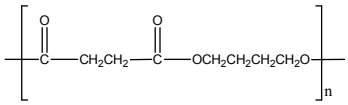
Considering that the biotransformation of a fibre is a heterogeneous reaction, therefore a pre-adsorption of the enzyme on the solid substrate is assumed before the catalysis can proceed. Despite the stabilisation of the TI model substrate for several mutant cutinases, other adsorption and substrate recognition issues seem to play a major role on the enzymatic hydrolysis of solids substrates in presence of cutinase. According to these results, L182A was considered to be the most promising enzyme for future studies.

Table 4.3 - Hydrophobic surface percentage (hydrophobic surface/total surface) with Sd of native and mutated enzymes (Eisenhaber *et al.* 1995; Araújo *et al.* 2007)

Enzyme	Hydrophobic surface (hydrophobic/total) (%)	Standard deviation (sd)
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

The activity of the native and L182A cutinases towards aliphatic polyesters, which resemble the original cutin substrate, was also measured. Apparently, the native form seems to have a decrease of activity and protein adsorption levels as the hydrophobicity of the substrate increases (Table 4.4). The opposite seems to happen with L182A, which presents a lower hydrophobic area (Table 4.3). 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 recognise aliphatic chains, being one of the reasons why this enzyme shows activity towards the aliphatic structure of polyamide 6.6.

Table 4.4 - Activity of cutinases towards aliphatic polyesters and protein adsorption levels

Aliphatic Polyesters	Enzymes			
	Native		L182A	
	Succinic acid (mM)	Protein adsorption (%)	Succinic acid (mM)	Protein adsorption (%)
Poly(ethylenesuccinate) 	728	50	127	20
Poly(1,3 – propylene succinate 	626	41	330	29
Poly(1,4 – butylene succinate) 	432	43	339	24

4.4 CONCLUSIONS

Regarding polyamide treatment, the findings suggest that these fibres 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 fibres, showing however slow enzymatic kinetics.

The results presented shared that genetic engineering is a powerful tool for developing enzymes with higher specific activity towards polyamide substrates which are non natural substrates of cutinase.

Chapter 5

INFLUENCE OF MECHANICAL AGITATION ON CUTINASES AND PROTEASE ACTIVITY TOWARDS POLYAMIDE SUBSTRATES

5. INFLUENCE OF MECHANICAL AGITATION ON CUTINASES AND PROTEASE ACTIVITY TOWARDS POLYAMIDE SUBSTRATES

5.1 INTRODUCTION

The technical and mechanistic complexity of the enzymatic process is high since cutinases or proteases have not been "designed by nature" to interact with insoluble fibre substrates. Similarly, the compacted fibre structure of the synthetic materials severely restricts the mass transfer and the accessibility of the enzyme to the fibre surface hence restricting enzyme adsorption.

In this chapter new insights about the influence of mechanical agitation on protein adsorption and cutinase (wild-type and L182A mutant) and protease hydrolysis efficiency towards different polyamide substrates (trimmer and fabric) will be provided (Silva *et al.* 2007_a).

For this purpose a protease, subtilisin from *Bacillus* sp. (E.C. 3.4.21.62), was used. This protease promotes the hydrolysis of proteins with a broad specificity for peptide bonds. It also hydrolyses peptide amides. This subtilisin is a serine endopeptidase that contains no cystein residues. This enzyme consists of a single polypeptide chain of about 275 aminoacid residues and possesses the "catalytic triad" of Asp, His and Ser residues that is conserved among serine proteases as the hallmark of its active site (Kumar and Takagi 1999).

The cutinases used in this part of the work were already described in the previous chapter.

5.2 MATERIALS AND METHODS

5.2.1 ENZYMES AND REAGENTS

Commercial polyamide (100% PA 6.6) woven fabric, obtained from Rhodia (Switzerland), taffeta, with 30/28 yarnscm^{-1} and 63 gm^{-2}, was used in all experiments.

The polyamide model substrate (trimmer: adipic acid bishexyl-amide), generously supplied by the Technical Institute of Graz (Austria), was synthesised as described by Heumann and co-workers (Heumann *et al.* 2006). The cutinase, native and mutant forms, from *Fusarium solani pisi* were obtained from the Department of Biology, University of Minho. The genetic modification of cutinase was performed as previously described in chapter 4 (section 4.2.3). The protease (Esperase), 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). Lutensol AT25 was acquired from BASF (Ludwigshafen, Germany). All other reagents used were laboratory grade reagents.

5.2.2 DETERMINATION 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.

5.2.3 DETERMINATION OF PROTEIN ADSORPTION

The protein adsorption was obtained by measuring the difference between the amount of protein introduced into the reaction mixture and the amounts of protein in the supernatant and washing solutions.

5.2.4 DETERMINATION OF THE AMINO GROUPS RELEASED TO THE BATH TREATMENT

To quantify the amino groups released to the bath treatment during enzymatic hydrolysis, the trinitrobenzenesulfonic acid (TNBS) method was adapted from a methodology already described in chapter 2 (section 2.2.3).

5.2.5 DETERMINATION OF THE AMINO END GROUPS AT THE FIBRE 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 (Silva and Cavaco-Paulo 2004). The reaction occurred only at the surface of the fabric as can be depicted from Figure 5.1 and the free amino groups were detected by the specific reaction with the α-bromoacrylamido wool reactive dye (Lewis 1992).

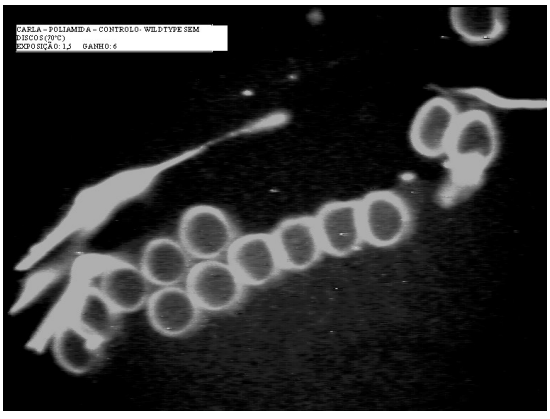


Figure 5.1 - Microscopic image of the polyamide stained samples (the reactive dye is linked only at the surface of the fibre).

All stainings were carried out in a 150 cm^3 capacity sealed stainless steel dyepots, housed in a dyeing machine (AHIBA Spectradye, from Datacolor International). Stainings of 4% (o.w.f.) were obtained using a bath ratio of 1:100 at different temperatures (50, 60 and 70ºC) for 90 minutes with a temperature

gradient of 4ºCmin^{-1}. After staining, the samples were washed with 2 gL^{-1} of a Lutensol AT25 solution for 1 hour and then rinsed in cold distilled water until no more dye could be detected in the washing water. Two independent staining experiments were performed and the results represent the mean of these experiments.

The colour measurements were carried out as already described in chapter 2 (section 2.2.4).

5.2.6 DETERMINATION OF THE WETTABILITY OF THE POLYAMIDE TREATED SAMPLES

In order to obtain the degree of wettability of the untreated and treated polyamide fabrics, a water-drop test was applied according to an internal procedure based on the AATCC standard method (AATCC-39 1980). This method allows an easy and fast measurement of the wettability of fabrics. 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 evaluation was used as a comparative method.

5.2.7 ENZYMATIC HYDROLYSIS OF POLYAMIDE MODEL SUBSTRATE (TRIMMER)

5.2.7.1 ENZYMATIC TREATMENT WITH 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 4.0 UmL^{-1} (measured against *p*-NP) of native cutinase and the second solution contained the same amount of buffer and 4.7 UmL^{-1} of cutinase mutant (L182A). Both experiments were performed at 35ºC 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 5.2.2. After 8 hours of incubation, a protein precipitation step was performed (the solution was mixed with perchloric acid in a ratio of 1:2; the mixture was centrifuged and the supernatant was neutralised with 2 M of KOH for subsequent analysis) and the primary amino groups resulting from enzymatic hydrolysis were quantified by the TNBS method (as described in section 2.2.3).

5.2.7.2 ENZYMATIC TREATMENT WITH PROTEASE

On this set of experiments, 10 mL of Tris-HCl buffer (pH=7.6) containing 1.8 UmL^{-1} (measured against tyrosine) of protease were incubated with 0.01 g of model substrate at 37ºC 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 5.2.2. 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 (as described in section 2.2.3).

5.2.8 ENZYMATIC HYDROLYSIS OF POLYAMIDE FABRIC SUBSTRATE

5.2.8.1 PRE-TREATMENT OF POLYAMIDE SAMPLES

To remove some impurities from the fabrics, before the enzymatic treatment all polyamide fabric samples were washed with 2 gL^{-1} of a non ionic agent, Lutensol AT25 (10 gL^{-1}) and with 2 gL^{-1} of Na_2CO_3, both at 50ºC for 1 hour and with distilled water for 1 hour. Finally, the fabric was left to dry at room temperature. All washes were performed in a laboratory scale machine (Rotawash MKIII).

5.2.8.2 ENZYMATIC TREATMENT WITH CUTINASES – VERTICAL AGITATION

In 500 mL stainless steel pots of a laboratory Rotawash MKII machine, from SDL International Ltd, rotating at 20 rpm, 1.00 g of pre-treated polyamide fabric was incubated with 8 UmL^{-1} and 28 UmL^{-1} of native cutinase in 300 mL of phosphate buffer (0.1 M NaOH, 0.1 M KH_2PO_4, pH 7.8) at 37ºC 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 19.10 g, 32 mm x 3 mm) into the reaction mixture. The L182A cutinase mutation was also tested, where 15 UmL^{-1} 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 bath treatment at 0.5, 1, 2, 3 and 4 hours. After 4 hours of incubation, the fabrics were removed from the bath and rinsed in Na_2CO_3 solution (2 gL^{-1}) for 2 hours at 50ºC, to stop the enzymatic reaction and remove the protein adsorbed, followed by washing with 2 gL^{-1} of Lutensol AT25 solution for 1 hour at 50ºC. After that, the samples were rinsed with distilled water until no more protein could be detected in the washing water and allowed to dry at open air. The washes were performed in a Rotawash MKIII machine. Two independent experiments were

done for each treatment, and the results represent the mean value of these experiments.

5.2.8.3 ENZYMATIC TREATMENT WITH CUTINASE – ORBITAL AGITATION

1.00 g of pre-treated polyamide fabric was incubated in 300 mL of phosphate buffer (0.1 M NaOH, 0.1 M KH_2PO_4, pH 7.8) with 8 UmL^{-1} of native cutinase at 37ºC for 48 hours under continuous orbital agitation. The low level of mechanical agitation was achieved using an Erlenmeyer flask held in a shaking water bath operating at 90 rpm. For protein and amino groups determination, aliquots were taken from the bath 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.

5.2.8.4 ENZYMATIC TREATMENT WITH PROTEASE – VERTICAL AGITATION

In this set of experiments, 1.00 g of pre-treated polyamide fabric were incubated with 4 UmL^{-1} of subtilisin in 300 mL of Tris-HCl buffer (0.3 M Tris, 3M HCl, pH=7.5) at 37ºC for 4 hours under continuous vertical agitation. To achieve a higher level of mechanical agitation, experiments were also performed by adding 5 stainless steel discs (each disc with average weight of 19.10 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 rpm. For protein and amino groups determination, aliquots were taken from the bath 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.

5.2.9 WIDE-ANGLE X-RAY DIFFRACTION (WAXD)

WAXD patterns of the PA 6.6 fabrics were obtained for the samples treated with cutinases and protease, both in the presence and absence of stainless steel

discs. The X-ray diffraction experiments were undertaken in a Philips PW1710 apparatus, using Cu Kα radiation source (λ=0.154 nm) and operating at 40 kV voltage and 30 mA current. The patterns were continuously recorded in the diffraction angular range of 5º to 35º (2θ). The scan speed was 0.01º s^{-1}.

The WAXD data were analysed by profile fitting of the obtained wide-angle x-ray equatorial diffraction scans using the WinFit 1.2 programme. 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 equation 5.1, available in literature where d_{100} and d_{010} are the interplanar distances related to the planes (100) and (010), respectively (Dismore and Statton1966; Botelho *et al.* 2002).

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

Equation 5.1 - Determination of crystallinity of polyamide 6.6 based on the interplanar distances.

Equation 5.1 can be simplified and expressed as equation 5.2, where the θ_{100} and θ_{010} 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.

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

Equation 5.2 - Determination of crystallinity of polyamide 6.6 polymers based on the interplanar distances and the total area (crystalline and amorphous).

5.2.10 ATTENUATED TOTAL REFLECTANCE SPECTROSCOPY - INFRARED (ATR-IR)

Multi bounce HATR Avatar 360 (OMNIC 5.2) IR spectrophotometer (Nicolet Instrument Corporation, Waltham, MA, USA) using an attenuated total reflectance accessory (ATR) was used to record the IR spectra of control and treated samples. The HATR-IR consists of a composite ZnSe-diamond crystal that allows collection of the IR spectra directly from the sample without any special preparation. The IR spectra were collected at a spectrum resolution of 32 cm^{-1}, over the range of 800-4000 cm^{-1} at room temperature. The number of scans was 60 and the sample gain 8.0. A background scan with no samples and no pressure was acquired before collecting spectra of samples.

5.2.11 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.

5.3 RESULTS AND DISCUSSION

5.3.1 ENZYMATIC ACTIVITY OF CUTINASE AND PROTEASE TOWARDS POLYAMIDE MODEL SUBSTRATE

One of the objectives of this work was to confirm that cutinases (native and mutant) and protease were able to hydrolyse the surface of polyamide substrates. Before the enzymatic treatment of the main substrate (polyamide fabric), a smaller substrate was studied. The purpose was to prove that if these enzymes were able to work on the hydrolysis of small polyamide substrates, they could act also on bigger ones. For that reason a small amount of PA 6.6 model substrate (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 (Table 5.1), it can be observed that the protein adsorption, as well as the enzymatic activity, expressed as mM of amines in solution, are 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 Leucine amino acid by a smaller (Alanine) amino acid, close to the active site, resulted in a more "open" enzyme structure. This allows 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 hydrolyse amide bonds.

The data obtained for PA 6.6 trimmer shows relative ability to hydrolyse 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 6.6 fabric).

Table 5.1 - Protein adsorption and enzyme activity (measured as amines formation) after incubation of polyamide trimmer for 8 hours with native cutinase, L182A cutinase mutant and protease

Enzyme	Protein adsorption (%)	Amines (mM)
Native (4.0 UmL^{-1})	18.2 ± 2.1	0.073 ± 0.006
L182A mutant (4.7 UmL^{-1})	53.1 ± 1.5	0.105 ± 0.005
Protease (1.8 UmL^{-1})	54.2 ± 1.7	0.323 ± 0.005

5.3.2 ENZYMATIC ACTIVITY OF CUTINASE AND PROTEASE TOWARDS POLYAMIDE FABRIC SUBSTRATE

5.3.2.1 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 enzyme activity. The activity of native cutinase, measured as amino groups released in the bath bath treatment, increased when higher mechanical agitation was used (stainless steel discs addition) (Figure 5.2). The findings suggest that mechanical agitation influences greatly the enzyme hydrolysis on the polyamide fabric. Comparing the experiments performed on the Rotawash machine (vertical agitation), it clearly shows 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 leads to an increase of the fibre-metal friction, as well as an increase of the beating 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 itself. 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 microfibrils and consequently more sites for possible enzyme attack. This phenomenon was accomplished by the mechanical abrasion of the fabric surface where the amino end groups formed by enzymatic action were released to the bath bath treatment and could be spectrophotometrically quantified (Figure 5.2). In order to measure the hydrolysis extent, a fabric reactive staining was performed (Figure 5.3). In the absence of the stainless steel discs an increase of the staining values was obtained, 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 57ºC the polymer structure is more exposed and the dye does not link only at the surface of the fabric (Figure 5.1) but is also able to penetrate into the fibres. Moreover, the increase of temperature promotes the rearrangement of the chains and the amino groups are not so easily accessible to the reactive dye. 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 observed 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 colour intensity compared to the other part of the treated fabric. However, these results were not considered because the spectrophotometrically measurement is technically difficult to obtain. The fibrils resulting from strong beating effects are too small and dispersed on the fabric sample surface. The mechanical action with discs promotes by itself the abrasion of the terminal end chains from the surface of the treated fabrics restricting therefore the fabric functionalisation.

In the same set of experiments described a shaking bath with orbital agitation was used. In this apparatus the polyamide samples were incubated for a long period (48 hours) at 90 rpm. A slow kinetic of enzymatic activity, measured in terms of amino groups formed, in the first 24 hours of incubation was obtained. After this period the activity of cutinase, measured as amines formation, increased reaching the same level of the one obtained on the Rotawash machine (vertical agitation), without discs (Figure 5.2). However, the K/S values obtained demonstrate that it was only possible to detect amino groups at the surface of the treated fabrics in the first 4 hours of incubation. The oligomers formed by enzymatic action were released to the bath treatment during the long period of incubation (24 hours).

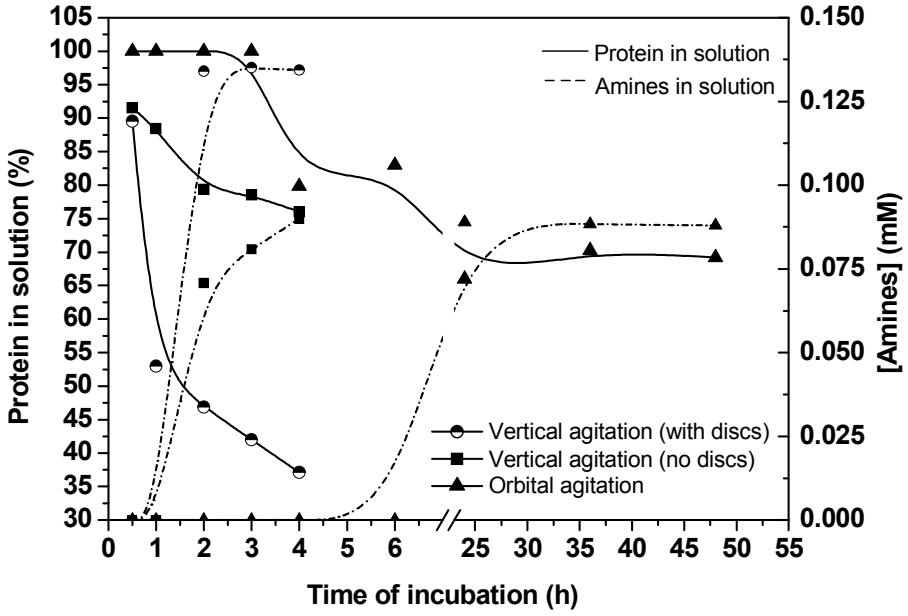


Figure 5.2 - Native cutinase (8 UmL^{-1}) activity (measured as amines released to the bath treatment) *vs* protein adsorption.

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 5.2 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%. The results show that mechanical agitation increases enzyme adsorption hence increasing the enzymatic action until a certain level. However, the main goal of the work, the surface functionalisation of the fabrics, is not achieved because the functional groups are released to the bath treatment. The results also demonstrate that the higher protein adsorption, the higher amine concentration in the bath treatment. Moreover, when protein adsorption stops, no more amines increase is achieved. These facts are related with the saturation levels obtained. After a certain period of incubation (first 4-5 hours), the fabric became totally covered by the enzyme and no more enzymatic action is detected.

The results of reactive staining obtained for vertical and orbital agitation show that the incorporation of discs on the system increased the release of the amines to the bath of treatment (Figure 5.3). The K/S values decrease when higher level of mechanical agitation was applied due to the released of the formed groups to the bath treatment. In order to achieve functionalized surfaces, the results of Figure 5.3, demonstrate that low levels of mechanical agitation should be provided and short periods of incubation should be used. After the first hours of incubation (4 hours), the formed oligomer chains are released to the bath treatment and at the same time, the ones that remain at the surface of the treated fabrics can rearrange and became inaccessible to the reactive dye.

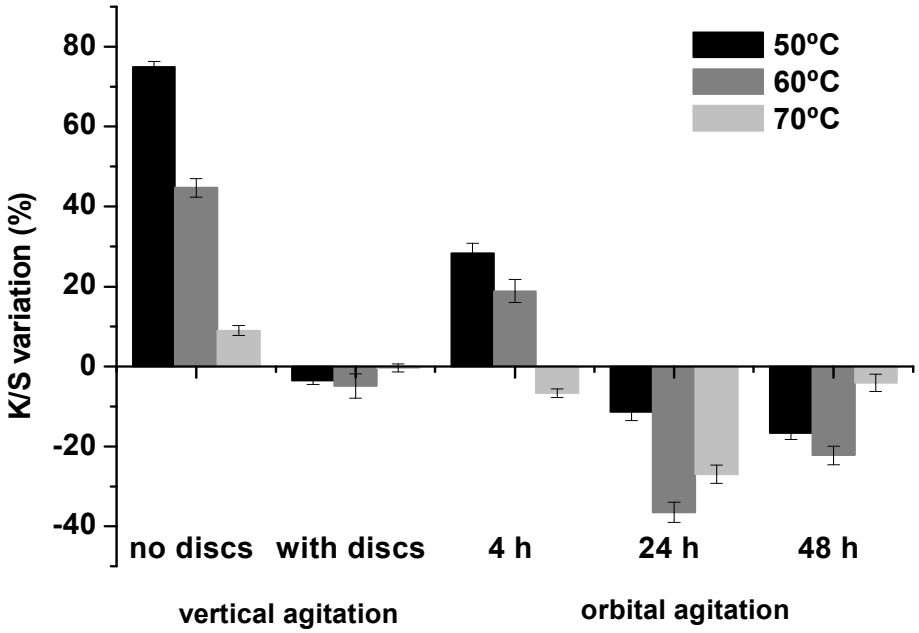


Figure 5.3 – Percentage of K/S variation related to control (buffer), proportional to the amino groups at the surface of the treated fabric with native cutinase (8 UmL^{-1}; 4% (o.w.f.) of reactive dye; bath ratio 1:100 for 90 minutes.

As obtained for polyamide trimmer substrate (Table 5.1) on fabric the L182A cutinase mutant presented a considerably higher activity when compared with the native one (Figures 5.4 and 5.5). Independently of the protein concentration used for fabrics treatment, the protein adsorption values are similar for both enzymes and presented higher values when stainless steel discs were included on the enzymatic system. In heterogeneous substrates like polyamide fabric, the values of protein adsorption achieved are generally about 30% when no additional mechanical agitation is provided. Higher levels of adsorption can be obtained by improving the agitation of the process, however the main goal of the work can be affected, because the formed groups are release from the fibre surface and moreover fibre damage can be obtained.

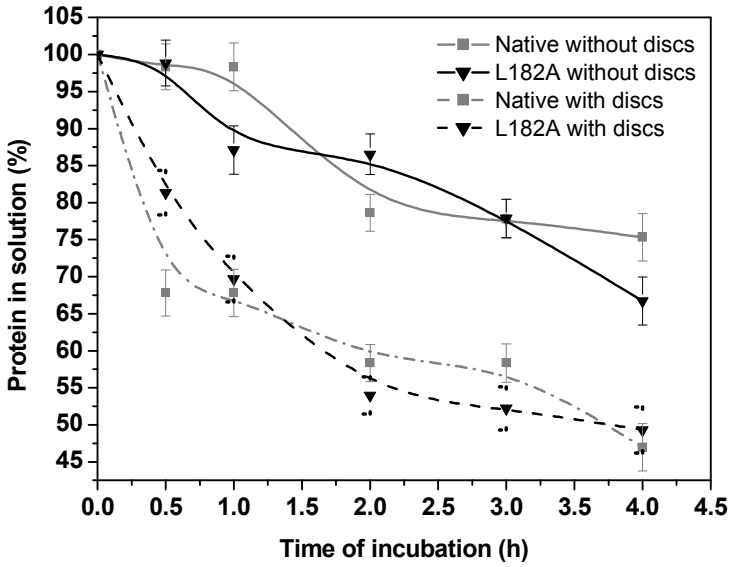


Figure 5.4 - Protein content in the bath treatment after 4 hours of incubation with native (28 UmL^{-1}) and cutinase mutant (15 UmL^{-1}).

The results presented on Figure 5.5 show that even when using higher concentration of native enzyme, the amine concentration is lower, when compared with the levels obtained for cutinase mutant. The enzymatic activity is more related with the interactions of the pair enzyme/substrate than with the concentration of protein used. In addition, the results also demonstrate a higher level of amines formed when a higher level of mechanical agitation was provided.

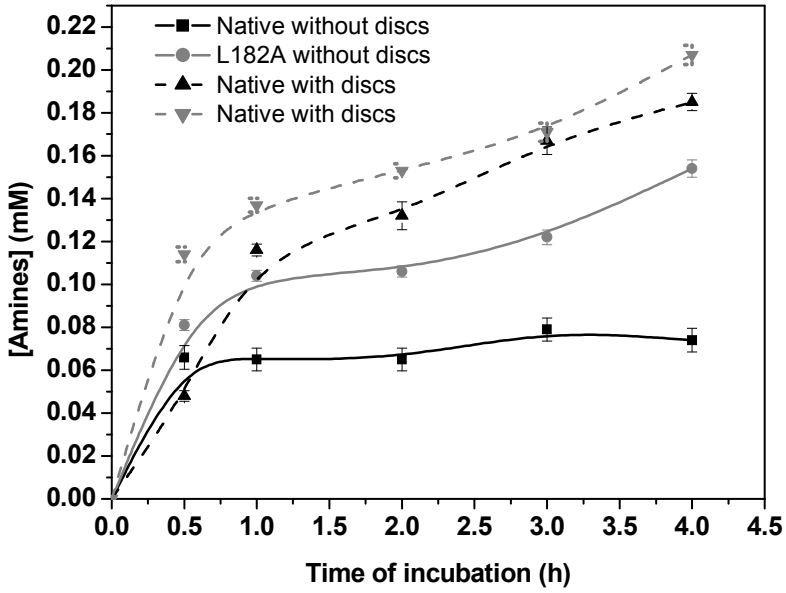


Figure 5.5 - Amino groups concentration in the bath treatment after 4 hours of incubation with native (28 UmL^{-1}) and cutinase mutant (15 UmL^{-1}).

5.3.2.2 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 to synergistically cooperate with protease activity. This is explained by the fact that mechanical agitation causes more fibrillation. In this situation, the lost fibrils (pills) formed represent a more exposed specific surface area for enzyme attack. The synergistic action of the enzyme specificity and the mechanical agitation leads to a higher activity, measured as amino groups released, compared with the cutinases (Figure 5.6). The amino groups concentration, when protease was used, reached 1.0 mM, a value which is five times higher when compared to that obtained for cutinases (0.2 mM) (Figure 5.7). Spectral values obtained after reactive staining of treated fabric samples increased when compared with controls, which can be correlated with an increase of the amino groups at the surface of the treated fabrics (Figure 5.8). However, the inclusion of stainless steel discs on the system promoted the

decrease of K/S values, being the mechanical action responsible for the surface abrasion, with the release, to the bath, of the amino groups formed. 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 occurs a rearrangement of the chains and the amino groups are not so easy accessible to the reactive dye. Differences between samples are not so easily detected, when analysing the obtained data. It can be concluded 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. The values obtained when samples were dyed at 60ºC, in absence of discs should be higher than the ones obtained with discs. This result is not fully understood yet.

Despite the fact that protease presents a more pronounced activity towards polyamide substrates, this enzyme shows less ability to functionalise these substrates, presenting lower values of K/S than the ones obtained on samples treated with cutinases. These results can be explained by the high specificity of this enzyme towards polyamide 6.6 substrates that is directly related with a higher activity. This activity can produce by itself the breakage and the release of the amino groups formed to the bath of treatment.

Since the main purpose of the work is to functionalise the fabric surface the use of protease should be carefully studied in order to obtain a balance between enzyme concentration and functionalisation degree.

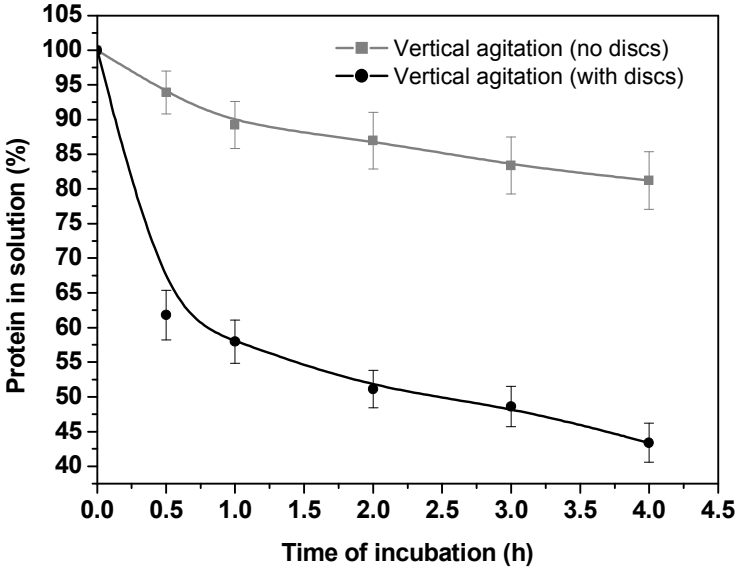


Figure 5.6 - Protein adsorption after 4 hours of incubation with protease (4 UmL^{-1}).

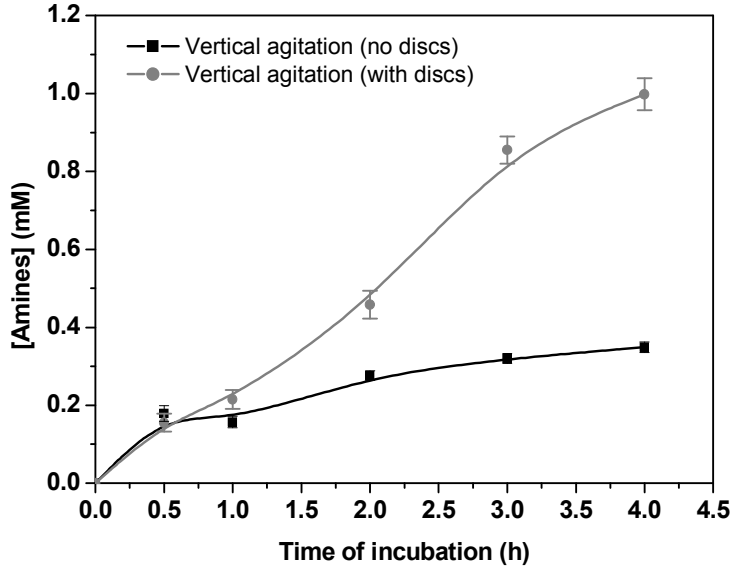


Figure 5.7 - Amino groups concentration in the bath treatment after 4 hours of incubation with protease (4 UmL^{-1}).

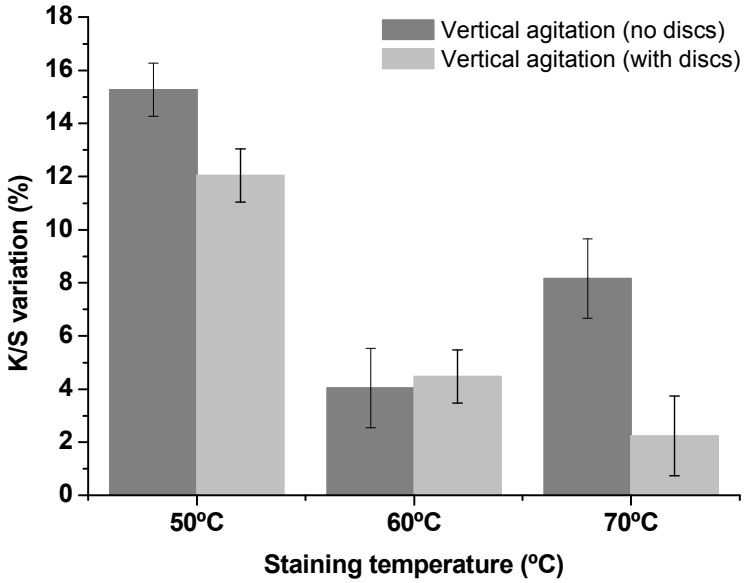


Figure 5.8 – Percentage of K/S variation related to control (buffer), proportional to the amino groups at the surface of the fabric samples treated with protease (4 UmL^{-1}); 4% (o.w.f.) of reactive dye; bath ratio 1:100; 90 minutes.

5.3.3 WETTABILITY OF THE TREATED FABRIC SAMPLES

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 has increased. The samples without treatment presented a hydrophobic behaviour (>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 5.2), approximately. The surface of polyamide fabrics became more hydrophilic and probably easier for finishing treatments application.

Table 5.2 - Time of water drop absorption of fabrics treated with different enzymes

Enzymes	Time of water drop absorption (min)
Control	> 10
Native (28 UmL^{-1})	5.34 ± 0.20
L182A mutant (15 UmL^{-1})	4.67 ± 0.20
Protease (4 UmL^{-1})	5.00 ± 0.40

5.3.4 CRYSTALLINITY DETERMINATION TROUGH WAXD

WAXD studies of treated polyamide samples show two strong diffraction peaks, one located at 2θ = 20.2º and the other one at 2θ = 23.4º (Botelho *et al.* 2002). The crystallinity value (CV) of the control and the treated samples was calculated as defined in Eq. 5.2. As expected, no significant changes were observed between samples incubated with the same level of mechanical agitation. 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 5.3 - 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

5.3.5 INFRARED STUDIES

Infrared spectra taken for PA 6.6 samples are shown in Figure 5.9 and 5.10, as an example. In this study the different spectra obtained for control and each treated sample were compared. All the spectra were collected using the region of 800-4000 cm^{-1}, although the band region used for comparison was 1500-1800 cm^{-1} where it shows some relative intensity differences. This region corresponds to the amide bands, where a decrease of the peak intensity can be correlated with the breakage of some amide linkages at the surface of the fabric, as theoretically predicted. The carbonyl amide stretching vibrations (1663 – Amide I band of secondary amide; 1558 – Amide II band: N-H bending and –N stretching) presented different intensities depending on the enzyme.

Regarding the spectra obtained for samples treated with the native cutinase, L182A mutant cutinase and protease without stainless steel discs, it can be concluded that there is a decrease of the absorbance values 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 5.11) and consequently the hydrophilic groups formed by enzymatic hydrolysis can not be so easily detected by reactive staining or by infrared studies.

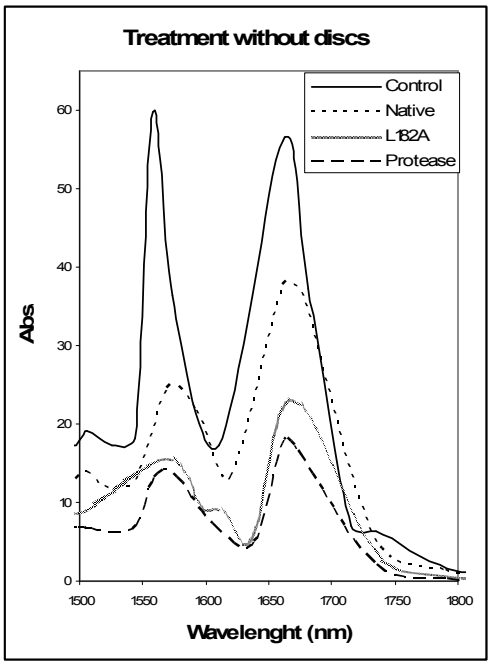


Figure 5.9 – Infrared spectra of samples treated with Native cutinase, L182A cutinase mutant and protease in the absence of stainless steel discs (most significant band).

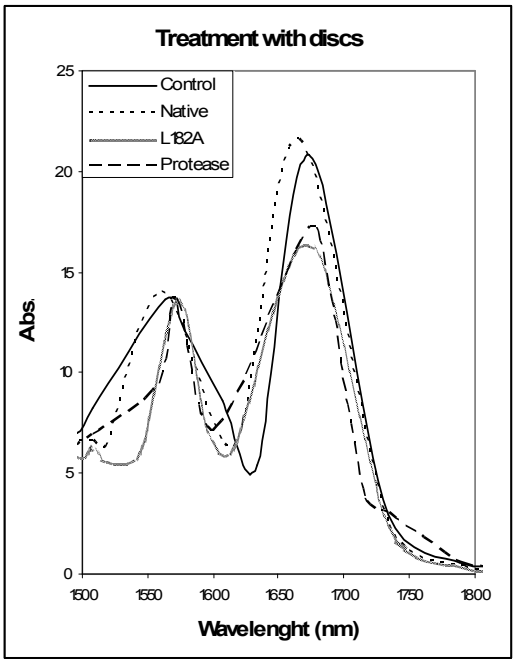


Figure 5.10 - Infrared spectra of samples treated with Native cutinase, L182A cutinase mutant and protease in the presence of stainless steel discs (most significant band).

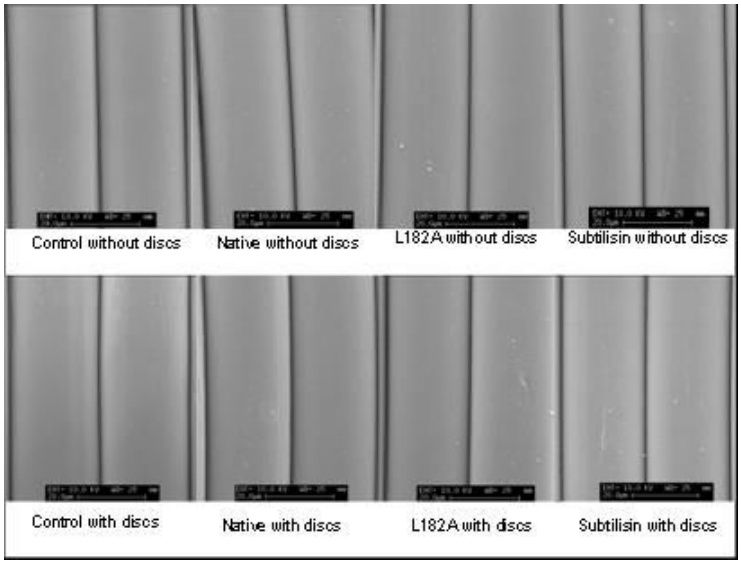


Figure 5.11 – SEM microphotographs of polyamide 6.6 fabrics samples treated with native cutinase, L182A mutant cutinase and protease in the absence and in the presence of stainless steel discs (×500 of magnification).

5.4 CONCLUSIONS

This study provided new insights about the influence of mechanical agitation on cutinases 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 at the surface of the fabrics, short times of incubation must be used as well as vertical agitation without discs. More studies have to be performed in order to predict and better control the polyamide finishing.

Chapter 6

LACCASE IMMOBILISATION ON ENZYMATICALLY FUNCTIONALISED POLYAMIDE 6.6 FIBRES

6. LACCASE IMMOBILISATION ON ENZYMATICALLY FUNCTIONALISED POLYAMIDE 6.6 FIBRES

6.1 INTRODUCTION

The present chapter presents a practical application for the functionalised fabrics obtained in the previous parts of the work.

The immobilisation of enzymes onto synthetic non-woven supports like polyester and polyamide has been applied for the gentle treatment of sensitive surfaces, like sensitive skin regions, wounds with difficult healing, valuable documents or paintings (Isgrove *et al.* 2001; Vaillant *et al.* 2002; Moeschel *et al.* 2003). A severe drawback, however, is the frequently unfavourable interaction between the enzyme and the surface of the support, which must fulfil some specific criteria such as chemical reactivity, compatibility with the enzyme, insolubility and stability in process solutions (Nazari 1998; Nouaimi-Bachmann *et al.* 2007). In addition, several studies report the immobilisation of various enzymes such as proteases, glucosidases, endocellulases and laccases onto different polyamide matrices, like membranes or non-wovens (Lozano and Iborra 1997; Nazari 1998; Isgrove *et al.* 2001, Vaillant *et al.* 2002; Bruno *et al.* 2004; Couto *et al.* 2004). However, until now, the state-of-the-art does not report the immobilisation of enzymes on woven polyamide supports. The application of woven polyamide as an immobilisation matrix could offer several advantages for enzyme immobilisation when compared with other materials such as polyamide membranes. Woven polyamide is inexpensive, chemically inert, non-toxic, mechanically stable, insoluble in water, readily available and can be obtained in a number of forms. In addition, the immobilisation takes place only in the external surface of the support, allowing a better expression of the enzymatic activity (Bruno *et al.* 2004).

Laccase immobilisation was extensively studied with a wide range of different methods and substrates (Ruiz *et al.* 2000; Durán *et al.* 2002; Al-Adhami *et al.* 2002; Durante *et al.* 2004).

The low substrate specificity of this enzyme, associated with its good intrinsic stability properties, has prompted interest for application in biobleaching, wastewater treatment, dye decolourisation, cathode fuel cells and biosensors (Mayer and Staples 2002; Zille *et al.* 2003; Couto *et al.* 2004; Claus 2004; Zille *et al.* 2005; Brandi *et al.* 2006). Immobilisation can protect laccase from denaturation by organic solvents, extend its half-life time and allows enzyme reuse in several reaction cycles (D'Annibale *et al.* 2000; Durán *et al.* 2002; Brandi *et al.* 2006). The laccase immobilisation onto polyamide matrices can be a promising system for the bioremediation of contaminated soils, wastewater treatment, wine, other beverages stabilisation and even on biosensors applications.

This chapter will report the development of a procedure to immobilise *Trametes hirsuta* laccase onto woven polyamide supports using glutaraldehyde as the crosslinking agent (Silva *et al.* 2007_b).

A two-level factorial design was adopted in this study for a complete understanding of the effects of pH, spacer, glutaraldehyde and enzyme concentration in the immobilisation procedure, and their possible interactions. This method is ideal for the identification of the vital variables that significantly affect the immobilisation process and has been applied successfully to study and optimise a different number of biocatalytic and bioseparation processes (Silva and Roberto 1999; Silva and Roberto 2001; Moyo *et al.* 2003; Serralha *et al.* 2004; Silva *et al.* 2004; Mayerhoff *et al.* 2004;Cortez *et al.* 2004; Silva *et al.* 2006_a).

6.2 MATERIALS AND METHODS

6.2.1 ENZYMES AND REAGENTS

Commercial polyamide (100% PA 6.6) woven fabric, obtained from Rhodia (Switzerland), taffeta, with 30/28 yarnscm^{-1} and 63 gm^{-2}, was used in all experiments. Esperase, a subtilisin from *Bacillus* sp. (E.C. 3.4.21.62), was a commercial protease purchased from SIGMA (St. Louis, USA) and the laccase from *Trametes hirsuta* (E.C. 1.10.3.2) was obtained from Institute of

Biotechnology of Graz (TUG) (optimum pH: 5.0; optimum temperature: 60ºC). Glutaraldehyde 50% solution in water and the azine-2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS) were purchased from SIGMA (St. Louis, USA) and Lutensol AT25 was acquired from BASF (Ludwigshafen Germany). All other reagents used were of analytical grade.

6.2.2 SUPPORT PREPARATION AND ACTIVATION

To remove some impurities from the fabrics, before enzymatic treatment all polyamide fabric samples were washed with 2 gL^{-1} of a non ionic agent, Lutensol AT25 (10 gL^{-1}) and with 2 gL^{-1} of Na_2CO_3, both at 50ºC for 1 hour and with distilled water for 1 hour. Finally, the fabric was left to dry at room temperature. All washes were performed in a laboratory scale machine (Rotawash MKIII).

6.2.2.1 ENZYMATIC FUNCTIONALISATION OF POLYAMIDE

Cleavage of the amide bonds of the polymer surface was achieved by hydrolysis with a protease, 0.70 g (5×5 cm^2) of washed polyamide fabrics were incubated in 100 mL of Tris-HCl buffer (0.3 M Tris; 3 M HCl; pH=7.5) with 18 UmL^{-1} of Esperase from *Bacillus* sp. at 37 ºC under continuous orbital agitation using an erlenmeyer held in a shaking water bath, operating at 90 rpm. After 24 hours of incubation, the fabrics were removed from the bath and washed in Na_2CO_3 solution (2 gL^{-1}) for 2 hours to stop the enzymatic reaction, followed by washing with 2 gL^{-1} of Lutensol AT25 solution for 1 hour. After that, the samples were rinsed in distilled water until no protein could be detected in the washing water, and then allowed to air dry (Silva *et al.* 2007_a).

6.2.2.2 ACTIVATION OF POLYAMIDE

Woven polyamide pieces (0.23 g) were placed in different glutaraldehyde solutions (2%, 15% and 28%) in borate buffer (0.1 M; pH=9.0) at room temperature (25ºC) for 2 hours. The non reacted glutaraldehyde was removed by washing several times with a 0.1 M of potassium phosphate (pH=7.5).

6.2.2.3 INTRODUCTION OF A SPACER (1,6-HEXANEDIAMINE)

After activation of polyamide samples with glutaraldehyde, half of the samples were placed in 40 mL of 0.5 M 1.6-hexanediamine in 0.1 M Na_2CO_3/$NaHCO_3$ buffer, pH=9.5, for 4 hours at room temperature. Then, the samples were washed thoroughly with distilled water followed by washing with 0.1 M of potassium phosphate (pH=7.5).

6.2.2.4 REACTIVATION OF POLYAMIDE

All the samples were reactivated with different glutaraldehyde solutions (2%, 15% and 28%) in borate buffer (pH=9.0) at room temperature (25ºC) for 2 hours. The non reacted glutaraldehyde was removed by washing with a 0.1 M of potassium phosphate (pH=7.5) buffer.

6.2.3 LACCASE IMMOBILISATION

Treated woven polyamide pieces were incubated with laccase solutions containing 10, 49 or 88 UmL^{-1} of enzyme at different pHs (acetate buffer 0.2 M for pH=4.0 and 5.5; phosphate buffer 0.1 M for pH=7.0). Polyamide fabric was maintained in laccase solutions overnight at 4ºC. After that, the supernatant was kept for protein measurements and the polyamide samples were washed three times with 0.1 M of potassium phosphate (pH=7.5) buffer. The solutions produced from the three washing steps were stored for protein determination.

In Figure 6.1 it is possible to observe all the steps described and the possible crosslinking reactions between glutaraldehyde, spacer and protein, in the immobilisation procedure.

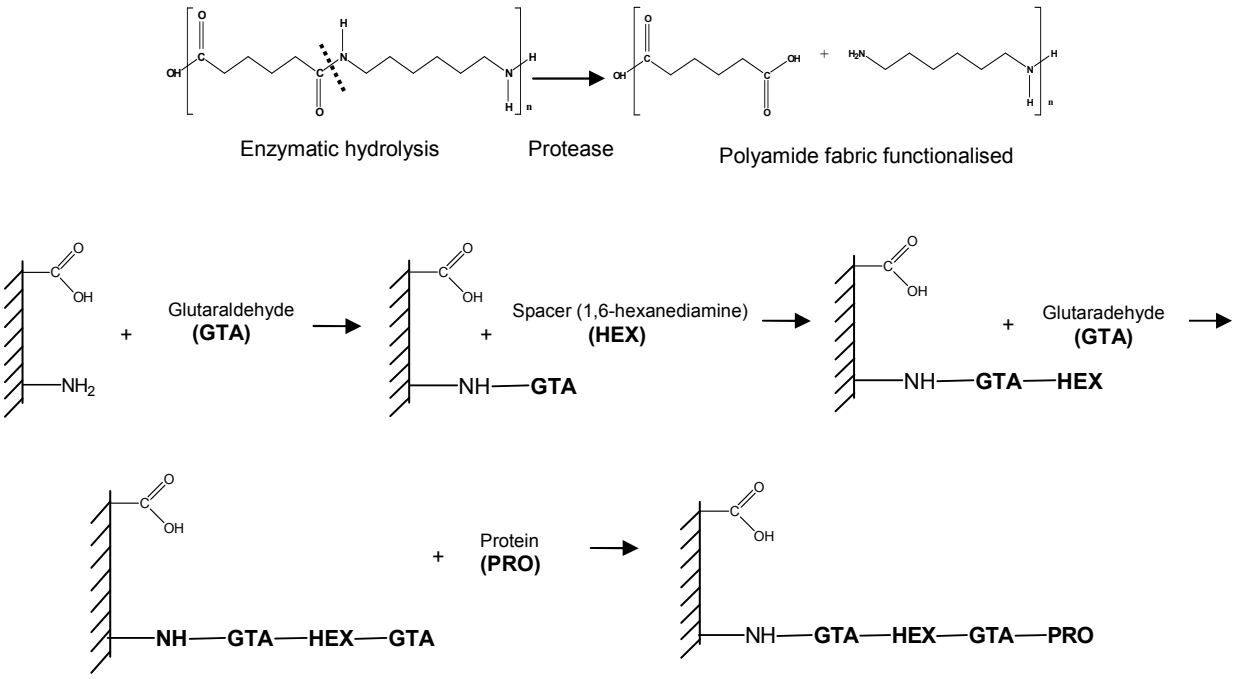


Figure 6.1 - Possible crosslinking reactions among glutaraldehyde, spacer and protein, in immobilisation procedure (Silva *et al.* 2007_b).

6.2.4 EXPERIMENTAL DESIGN

The influence of pH (A), spacer (B), glutaraldehyde concentration (C) and enzyme concentration (D) on laccase immobilisation was studied using a 2^4 full factorial design with four repetitions at the central point (Table 6.2). The variable (B) was categorical and therefore two conditions were tested: without the spacer and with the spacer. The range and the levels of the variables investigated in this study are given in Table 6.1 and were chosen based on preliminary studies. For statistical calculations, the variables were coded according to Eq. 6.1:

$$xi = \frac{Xi - X0}{\Delta Xi}$$

Equation 6.1 – Determination of statistical variables.

Where *xi* is the independent variable coded value, *Xi* the independent variable real value, *X0* the independent variable real value on the centre point and ΔXi is the step change value.

Table 6.1 - Factor levels used according to the 2^4 factorial design

Variable	Level		
	-1	**0**	**1**
A: pH	4	5.5	7
B: Spacer	without	without/with	with
C: Glutaraldehyde (%)	2	15	28
D: Enzyme (UmL^{-1})	10	49	88

The Design-expert version 7.0 – Free evaluation version (Stat-Ease Inc., Minneapolis, USA) was used for regression and graphical analysis of the data. The half-life time of immobilised laccase (*HLT*, hours), the protein retention (*PR*, %) and the immobilisation yield (*IY*, %) were taken as the responses of the design experiments. The statistical significance of the regression coefficients was determined by Student's *t*-test and the model equation was determined by Fischer's test. The proportion of variance explained by the model obtained was given by the multiple coefficient of determination, R^2. The optimum conditions were obtained by the graphical analysis using the Design-expert program.

Table 6.2 - Values for half-life time of immobilised laccase (*HLT*), protein retention (*PR*) and immobilisation yield (*IY*), according to the 2^4 factorial design

Assay	Variables				Responses		
	A	B	C	D	*HLT* (h)	*PR* (%)	*IY* (%)
1	-1	-1	-1	-1	38	47.2	2.2
2	1	-1	-1	-1	35	39.2	2.4
3	-1	1	-1	-1	70	37.5	4.3
4	1	1	-1	-1	60	32.5	4.2
5	-1	-1	1	-1	41	36.4	2.2
6	1	-1	1	-1	36	26.0	2.1
7	-1	1	1	-1	61	30.2	5.2
8	1	1	1	-1	65	32.1	5.1
9	-1	-1	-1	1	50	22.6	0.2
10	1	-1	-1	1	70	15.3	0.1
11	-1	1	-1	1	63	33.3	0.2
12	1	1	-1	1	78	20.4	0.2
13	-1	-1	1	1	41	10.0	0.3
14	1	-1	1	1	43	16.5	0.3
15	-1	1	1	1	134	13.8	0.5
16	1	1	1	1	139	5.3	0.5
17	0	0	0	0	62	18.5	0.8
18	0	0	0	0	95	17.4	0.8
19	0	0	0	0	45	16.4	0.1
20	0	0	0	0	63	14.4	0.5

6.2.5 LACCASE ACTIVITY AND PROTEIN DETERMINATION

Laccase activity was measured in presence of 2,2'-azino-bis-(3-ethyl benzothiazoline-6-sulfonic acid) (ABTS) according to the method described by Childs and Bardsley (1975). In the case of free enzyme solution, 1 mL of diluted enzyme (in acetate buffer, pH=5.0, 0.1 M) was mixed with 1 mL of ABTS

(0.5 mM) solution in water, in a disposable cuvette. The increase in absorbance was followed at 420 nm (ε=36 $mM^{-1} \times cm^{-1}$) for 2 minutes. The spectrophotometer was zeroed with the $ABTS_{zero}$ sample, which contained 1 mL of acetate buffer (0.1 M, pH=5) and 1 mL of ABTS solution. The experiment was performed at 25ºC. One unit of activity was defined as the amount of enzyme required to oxidise 1 µmol of ABTS per minute (Childs and Bardsley1975).

A similar procedure was adopted to measure the activity of laccase bounded to the woven polyamide. The immobilised sample (0.073 g) was soaked in a stirred cell, outside the range of the laser light, containing 1 mL of acetate buffer (0.1 M, pH=5.0). This solution was mixed with 1 mL of ABTS and the absorbance of the supernatant was measured at 420 nm (ε=36 $mM^{-1} \times cm^{-1}$) for 10 minutes. The final activity of immobilised laccase in UmL^{-1} was converted to activity in Umg^{-1} of fabric used in the assay. The immobilisation yield was determined by dividing the activity value of immobilised laccase, obtained immediately after the immobilisation procedure, by the value of activity of the initial laccase solution, converted to Umg^{-1}. A conversion factor of 0.086 $mLmg^{-1}$ was used, considering the ratio of total volume of the immobilisation solution (20 mL) by the total weight of polyamide fabric used to immobilise the enzyme (0.233 g).

The half-life time for the native and immobilised enzymes was determined by measuring the remaining activity of the fabrics after 24, 48 and 96 h of incubation at 25ºC. The decay curves attained fitted well a first-order exponential curve, and the time at which half of the initial activity was lost was assigned as half-life time.

The protein concentration was determined by the Bradford method using bovine serum albumin (BSA) as a standard (Bradford 1976). The amount of bound protein was determined indirectly by the difference between the amount of protein introduced into the reaction mixture and the amounts of protein in the supernatant and washing solutions.

6.3 RESULTS AND DISCUSSION

Laccase from *Trametes hirsuta* was immobilised on woven polyamide supports, previously hydrolysed with a protease for amine activation based in a methodology described by Silva *et al.* (2007_a). It is known that the cleavage of the secondary amide linkages reduces the mechanical strength of the woven polyamide. Therefore, the extent of amide bond cleavage represents a compromise between the adequate supply of free amino groups and the structural integrity of the polymer (Goldstein *et al.* 1974). In the case studied, the total strength loss obtained after enzymatic surface treatment was lower than 3% (data not shown), consequently the integral structure of the polymer was well preserved. Moreover the weight loss was also insignificant (<2%) (data not shown).

In the immobilisation procedure, the concentrations of laccase and glutaraldehyde were varied, as well as the pH. In addition, two different approaches were used by immobilising the enzyme in the absence and in the presence of a spacer (1,6-hexanediamine). The introduction of a spacer enables a decrease in the steric hindrance effects and consequently increases enzymatic activity.

After the immobilisation procedure and according to the variations of the factors imposed by the design (Table 6.2), the activity of the immobilised laccase was measured as previously described. As expected, the immobilised enzyme presented absolute activity values lower than those obtained for the free enzyme, presenting also a slower kinetics (data not shown) for the oxidation of the soluble substrate ABTS. This evidence can be explained by the mass transfer limitations of the substrate. In this system, where the enzyme is covalently incorporated onto a polyamide matrix, the consumption of substrate and the release of the product resulting from enzymatic activity to the aqueous medium, is limited by external and internal constrains that can be related with the steric hindrance effect of the woven polyamide support (Nouaimi-Bachmann *et al.* 2007). The distribution of the immobilised enzyme is completely different compared with homogeneous solutions, the catalytic efficiency of the enzyme can be induced by the covalent

fixation and the free diffusion of substrate to and from the catalytic site may be constricted. Therefore, the activity of the immobilised laccase on woven polyamide depends on several variables such as: area distribution of immobilised enzyme, level of support activation, density of enzyme binding sites, the surface charge, the hydrophilicity of the support, the bulk mass transfer and local diffusion of the system (Nouaimi-Bachmann *et al.* 2007). The polymeric microenvironment and the covalent interactions connecting the biocatalyst to the support usually lead to a reduction in the enzyme mobility. Despite these drawbacks, the half-life time of immobilised laccase could be improved when compared with the free enzyme. The inclusion of a spacer, 1,6-hexanediamine, was essential to improve enzyme conformational flexibility and enhance enzymatic activity.

Table 6.2 shows the designed experiment matrix, together with the experimental results. Regression analysis was performed to fit the response functions (half-life time, *HLT*; protein retention, *PR* and immobilisation yield, *IY*) with the experimental data (Table 6.3).

Table 6.3 - Estimated coefficients, standard errors and Student's *t*-test for half-life time (*HLT*), protein retention (*PR*) and immobilisation yield (*IY*) using the 2^4 full factorial design

Factors	*HLT* (h)			*PR* (%)			*IY* (%)		
	Effects	Standard errors	*t* values	Effects	Standard errors	*t* values	Effects	Standard errors	*t* values
Intercept	64.000	±3.71	---	26.14	±0.77	---	1.87	±0.079	---
A: pH	1.75	±3.71	0.47	-2.730	±0.77	-3.55[c]	-0.007	±0.079	-0.09
B: Spacer	19.75	±3.71	5.32[b]	-0.500	±0.77	-0.65	0.660	±0.079	8.35[c]
C: Glutaraldehyde	6.000	±3.71	1.62	-4.85	±0.77	-6.30[b]	0.160	±0.079	2.03
D: Enzyme	13.25	±3.71	3.57[c]	-9.000	±0.77	11.69[a]	-1.58	±0.079	-20.00[a]
AB	0.000	±3.71	0.00	-0.320	±0.77	-0.42	-0.014	±0.079	-0.18
AC	-1.000	±3.71	-0.27	1.420	±0.77	1.84	-0.014	±0.079	-0.18
AD	3.500	±3.71	0.94	-0.039	±0.77	-0.05	0.015	±0.079	0.19
BC	10.000	±3.71	2.69[c]	-0.440	±0.77	-0.57	0.160	±0.079	2.03
BD	6.500	±3.71	1.75	1.560	±0.77	2.03	-0.590	±0.079	-7.47[c]
CD	6.000	±3.71	1.62	-0.890	±0.77	-1.15	-0.046	±0.079	-0.58
ABC	1.500	±3.71	0.40	-0.002	±0.77	-0.003	0.021	±0.079	0.27
ABD	-0.250	±3.71	-0.07	-2.240	±0.77	-2.90[c]	0.016	±0.079	2.03
ACD	-2.500	±3.71	-0.67	0.860	±0.77	1.12	0.025	±0.079	0.32
BCD	11.000	±3.71	2.96[c]	-2.460	±0.77	3.19[c]	-0.120	±0.079	-1.52
Centre point (1)	9.25	±11.73	0.79	-9.200	±2.43	-3.78[c]	-0.760	0.25	-3.04
Centre point (2)	-4.75	±11.73	-1.28	-9.720	±2.43	-4.00[c]	-1.850	0.25	-7.40[c]

[a.] ***P* < 0.0005**; [b.] ***P* < 0.0050**; [c.] ***P* < 0.0500**

Both the Student's *t*-test and *p*-value statistical parameters were used to verify the significance of the considered factors. In this study, factors having a confidence level higher than 95% were considered to a further analysis of the responses in the area studied.

According to the Student's *t*-test results, the most significant parameter for the responses *PR* and *IY* was the amount of enzyme, which was significant at a probability level of less than 0.0005. Analysing the values attained for *IY* in Table 6.2, it is clearly seen that the highest values were attained in assays 1-8 (were the enzyme dosage was in the lowest level) comparing to assays 9-16. Also, the presence of the spacer (factor B) increased the *IY* (assays 3-4 and 7-8), confirming therefore its positive effect. The response half-life time was also

increased by the presence of the spacer, where the highest values for this response were attained when the factors glutaraldehyde concentration and enzyme dosage were in the highest level (assays 15-16).

Surprisingly, for the response *HLT*, the most influencing parameter was the presence/absence of the spacer. This variable had a positive effect, meaning that its presence could significantly increase the half-life time for the immobilised enzyme. This fact can be justified by the increase in the stability of the immobilised laccase. It is known that immobilisation often brings an increase in the operational stability of enzymes (Al-Adhami *et al.* 2002; Sharma *et al.* 2003). Silva *et al.* found an increase in half-life time of 46 days at room temperature, when a protease (Esperase) was immobilised on the polymer Eudragit S-100 (Silva *et al.* 2006_a). Enhanced stability seems to depend on the rigid conformation of the enzyme modified by the crosslinker and/or by covalent binding to the polymer, where the introduction of a spacer can further help in stabilising the tertiary structure of the enzymes

The effect of the spacer was also positive for the immobilisation yield, basically for the same reasons. Note that without the spacer, the maximum half-life time attained for immobilised laccase was 70 hours, when the maximum amount of enzyme was used, which is quite close to the half-life time of native enzyme (64 hours) in the same working conditions. With the introduction of 1,6-hexanediamine, the half-life time increased significantly, and it reached 139 hours when factors C and D were in the highest level.

The concentration of crosslinker was significant only for *PR*. This response also presented as significant the amount of enzyme added, both showing negative effects. Glutaraldehyde is an effective crosslinker of proteins, able to link the amino groups of the enzyme to the fabric and moreover it can promote self-oligomerisation reactions (Migneault *et al.* 2004). If the polyamide surface is saturated with enzyme and crosslinker, the recovery will be lower, as the study confirms.

The other factor studied, pH (factor A), showed no significance at less than 95% confidence level for the responses *HLT* and *IY* and it showed a small effect in the response *PR*, at less than 95% confidence level.

6.3.1 EFFECT OF PARAMETERS ON HALF-LIFE TIME

The model expressed by Eq. 6.2, where the variables take their coded values, represents the half-life time (*HLT*) of the prepared immobilised conjugates as a function of the spacer (B), glutaraldehyde concentration (C) and enzyme dosage (D).

$$HLT\ (\text{h}) = 64 + 19.75B + 13.25D + 10BC + 11BCD$$

Equation 6.2 – Determination of the half-life time of the immobilised enzyme based on the ANOVA model.

The statistical significance of the linear model equation (Table 6.4) was evaluated by the *F*-test analysis of variance (ANOVA), which revealed that this regression is statistically significant ($P < 0.0001$) at a 99% confidence level. The model did not show lack of fit and presented a determination coefficient of R^2=0.82, that explains 82% of the variability in the response.

Table 6.4 - Analysis of variance (ANOVA)[a] for the representative model of half-life time for immobilised laccase at 25ºC, in the area studied

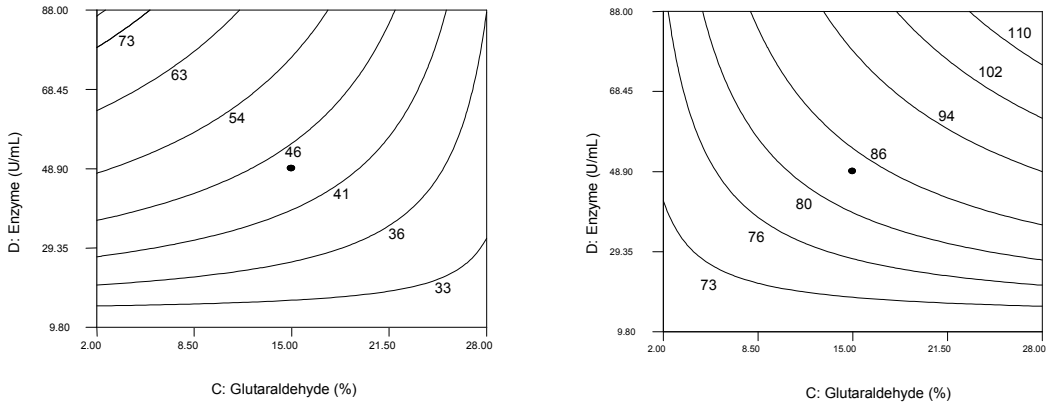
Source	SS	d.f.	MS	*F*-value	P
Model	13079.45	4	3269.86	14.73	<0.0001
Curvature	173	2	86.50	0.39	0.6850
Residual	2886.50	13	222.04		
Lack of Fit	*2230*	*11*	*202.73*	*0.62*	*0.7581*
Pure error	*656.50*	*2*	*328.25*		
Total	16138.95	19			

[a] R^2=0.82; CV=23.12% SS=sum of squares; d.f.= degrees of freedom; MS=mean square

The contour plot attained for the half-life time in the area studied is presented in Figure 6.2. It shows a different behaviour when the spacer is either present (Figure 6.2 b) or absent (Figure 6.2 a). Without the spacer, the maximum half-life time predicted by the model is around 73 hours, in a good agreement with

the measured data, which was attained when the lowest level of glutaraldehyde and the highest level of enzyme were used. With the introduction of the spacer, the profile of this response changes and the predicted half-life times increase considerably, varying from 73 h to 110 h. The best conditions to maximize this response are therefore attained when both enzyme and glutaraldehyde are used in the highest levels.

Analysing the contour plots obtained for this response (Figure 6.2), it is possible to see, by the shape of the curve, that the interaction effect among enzyme and glutaraldehyde becomes significant for a higher concentration of crosslinker.



a) **b)**

Figure 6.2 - Contour plot showing the effect of laccase and glutaraldehyde concentration, (a) without the spacer and (b) with the spacer, on the half-life time of the immobilised enzyme at 25ºC. The other factors were kept at the central level.

6.3.2 EFFECT OF PARAMETERS ON PROTEIN RETENTION

The response *PR*, which measures the retained protein in the polyamide fabric, was also measured. According to the Students *t*-test, factor D (enzyme dosage) was extremely significant for this response, having a negative effect. The

concentration of glutaraldehyde was also significant at less than 95% of confidence level. Although pH was the less significant factor, it was decided to include it in the model to minimise the error. Again, the interaction of the spacer, glutaraldehyde and the enzyme was significant. It seems that these three factors are extremely dependent on each other, justifying the third order interaction as significant. The model to represent *PR* in the studied region is therefore:

$$PR\ (\%) = 26.14 - 2.73A - 4.85C - 9D - 2.24ABD - 2.46BCD$$

Equation 6.3 – Determination of the protein retention of the immobilised enzyme based on the ANOVA model.

Table 6.5 shows the analysis of variance for the response *PR*. It shows that the considered model is extremely significant in the area studied (P<0.0001) and presents a high determination coefficient (R^2=0.94), thus explaining 94% of the total variation in the response, the rest (6%) being explained by the residues. This is proof that the model describes the studied region well.

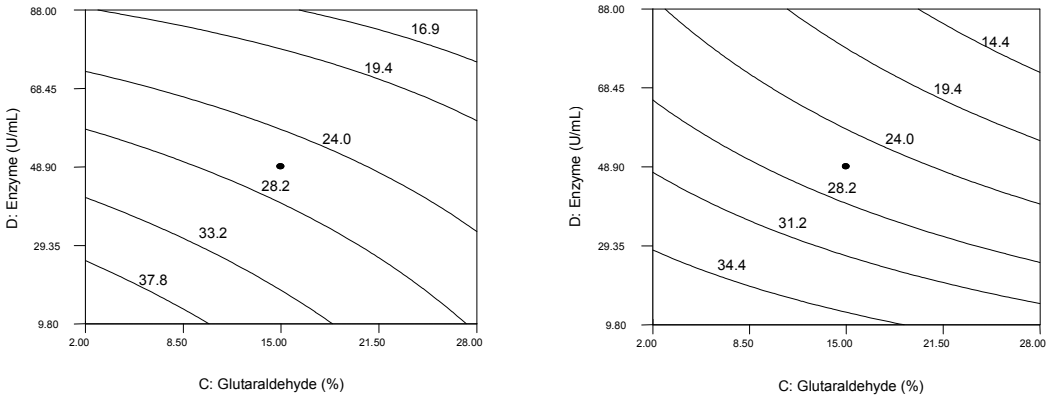
Although this model presented significant curvature (*P*=0.001), showing that the studied area should be extended to perform a correct analysis, our goal was to study the influence of these parameters on the three evaluated responses (*HLT*; *PR*, *IY*) and to maximise them in this range, so the model was accepted. Another proof that validates the model, as can be seen from the ANOVA table, is that it presents no lack of fit and its significance (*P*<0.0001) is much higher than the curvature's probability level (*P*=0.001), having also the residuals distributed along a well-randomised straight line.

Table 6.5 - Analysis of variance (ANOVA)[a] for the representative model of protein retention, in the area studied

Source	SS	d.f.	MS	*F*-value	p
Model	1967.52	5	393.50	35.54	<0.0001
Curvature	288.78	2	144.39	13.04	0.001
Residual	132.88	12	11.07		
Lack of Fit	*126.13*	*10*	*12.61*	*3.74*	*0.2295*
Pure error	*6.75*	*2*	*3.38*		
Total	2389.18	19			

[a] R^2=0.94; CV=13.72%
SS=sum of squares; d.f.= degrees of freedom; MS=mean square

The contour plots attained for this model are shown in Figure 6.3. The protein retention was similar for the two considered situations: with and without the spacer, confirming the non significance of factor B. This response showed higher values when both glutaraldehyde concentration and enzyme dosage were in the lowest levels. Factor pH was negative and therefore, lower pHs increase protein retention. This fact might be explained by the increase in the electrostatic interactions of the enzyme and crosslinkers with polyamide fabric, therefore increasing the amount of protein retained.



a) **b)**

Figure 6.3 - Contour plot showing the effect of laccase and glutaraldehyde concentration, (a) without the spacer and (b) with the spacer, on the protein retention at 25ºC. The other factors were kept at the central level.

6.3.3 EFFECT OF PARAMETERS ON IMMOBILISATION YIELD

Finally, the last response studied was the immobilisation yield (*IY*). From Table 6.6 it can be seen that the main effects B and D and their interaction were significant at less than 5% probability. The linear model then obtained was:

$$IY\ (\%) = 1.87 + 0.66B - 1.58D - 0.59BD$$

Equation 6.4 – Determination of the immobilisation yield of the immobilised enzyme based on the ANOVA model.

Analysing the ANOVA table it can be seen that the model was highly significant, presenting no lack of fit and the R^2 value, being the measurement of the goodness of the fit, indicates that 97% of the total variation is explained by the model.

Nevertheless, this model also presented significant curvature. By the same reasons stated above, the model will be accepted to optimise the immobilisation

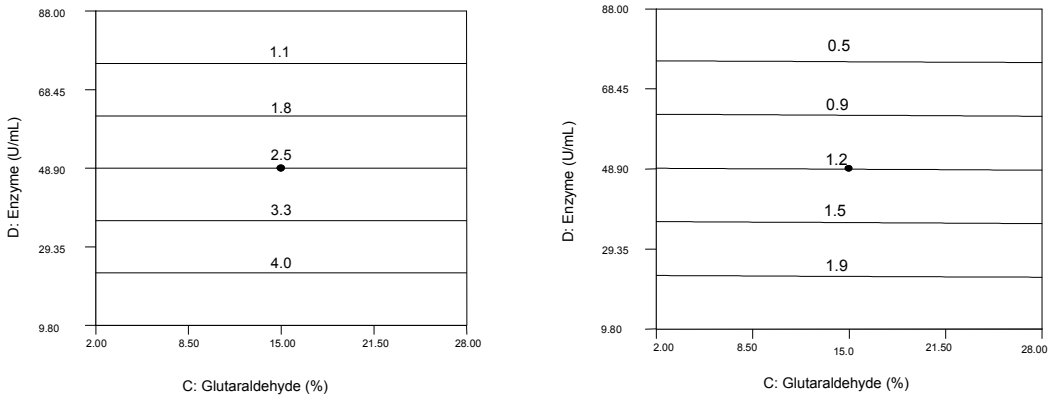
methodology in the area studied. A rotation of the experimental plan had to be considered to account for curvature, if the objective of the study was the modelling of the responses. In that situation, a quadratic model would be attained.

Table 6.6 - Analysis of variance (ANOVA)[a] for the representative model of immobilisation yield, in the area studied

Source	SS	d.f.	MS	*F*-value	p
Model	51.69	3	17.23	173.12	<0.0001
Curvature	6.42	2	3.21	32.24	<0.0001
Residual	1.39	14	0.100		
Lack of Fit	*1.10*	*12*	*0.092*	*0.63*	*0.7576*
Pure error	*0.29*	*2*	*0.15*		
Total	59.50	19			

[a] R^2=0.97; CV=19.61%
SS=sum of squares; d.f.= degrees of freedom; MS=mean square

The contour plots attained for the immobilisation yield are presented in Figure 6.4. The graphs clearly show the dependence of this response on enzyme dosage, since glutaraldehyde concentration was not significant and therefore not included in the model. The contour plots, by a matter of uniformity, were built with the same two factors (C and D). Again, factor D, enzyme dosage, has a negative effect and consequently, the immobilisation yield is improved at lower levels of enzyme added to the media. The influence of the spacer is positive, as previously observed in the other analysed responses. As a result, higher immobilisation yields are attained in the presence of the spacer.



a) **b)**

Figure 6.4 - Contour plot showing the effect of laccase and glutaraldehyde concentration, (a) without the spacer and (b) with the spacer, on the immobilisation yield at 25ºC. The other factors were kept at the central level.

Taking into account that the main purpose of the study was to optimise the immobilisation procedure in order to maximise all the analysed responses, the graphical optimisation of the statistical program 'Design-expert' was performed. The method basically consists of overlaying the curves of the models according to the criteria imposed (Silva and Roberto 2001). Based on the three models obtained, a graphical optimisation was conducted using the statistical program 'Design-expert', defining the optimal working conditions to attain high half-life times, protein retention and immobilisation yields. The criteria imposed for the preparation of the enzyme conjugates were: (a) the half-life time at 25ºC should be no less than 75 hours, (b) the protein retention should be more than 30% and (c) the immobilisation yield should be above 3%. The overlay plot attained (Figure 6.5) shows a shaded area where all these criteria are satisfied simultaneously. The pH was kept at the central level and the optimisation was investigated when the spacer was present, since this effect was always positive for the three responses evaluated.

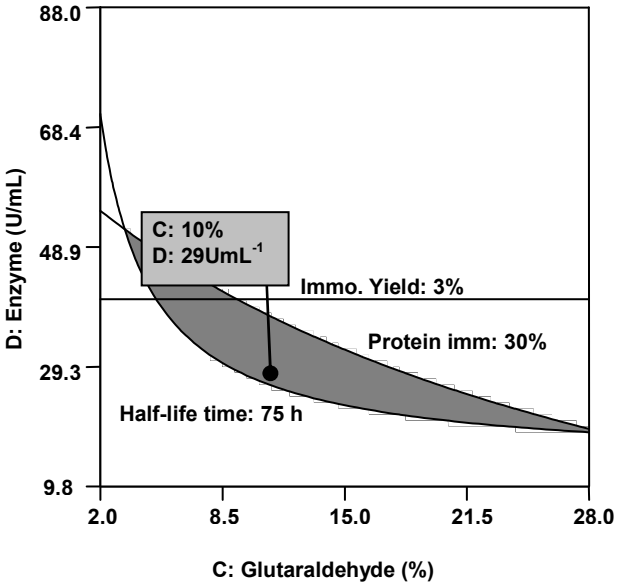


Figure 6.5 - The optimum region by overlay plots of the three responses evaluated (half-life time, protein retention and immobilisation yield) as a function of glutaraldehyde and enzyme concentration. Factor pH was kept at the central level and factor spacer was in the upper level.

Thus, a point was chosen on the graph (marked by the circle), which was assigned as optimum point corresponding to 10% (v/v) of glutaraldehyde and 29 UmL^{-1} of laccase. Analysing the plot attained for the graphical optimisation, it can be observed that the same responses can be attained using medium levels of enzyme and lower levels of glutaraldehyde, or contrarily, using lower levels of enzyme and higher amounts of crosslinker. These factors were chosen assuming a compromise between their lowest possible values, for economic reasons. Under the optimised conditions, the models attained predicted the following values for the responses, together with the experimental error in the 95% confidence interval:

Half-life time (HLT): 78 hours [64-87]

Protein retention (PR): 34% [30-34]

Immobilization Yield (IY): 3.4% [3.4-3.9]

To confirm these results, a validation assay was conducted in the conditions imposed as the optimum, i.e., immobilisation conducted in the presence of the spacer, using 10% of glutaraldehyde, 29 UmL^{-1} of laccase and at pH 5.5. In this assay, a half-life time of 78 hours, protein retention of 34% and immobilisation yield of 2% were attained. Figure 6.6 shows the activity decay for native and laccase immobilised at optimized conditions.

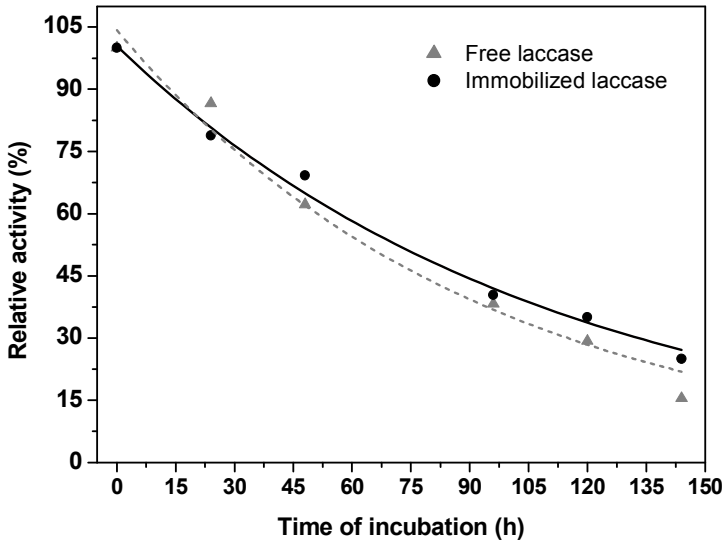


Figure 6.6 - Half-life time for native laccase (*HLT*=64 h) and laccase immobilised at the optimised conditions onto a polyamide support (*HLT*=78 h), measured at different periods of incubation at 25ºC, pH=5.0; the half-life time was calculated using equation 3.1.

The values reached in the validation assay for *HLT* and *PR* are in good agreement with the predicted values for the analysed responses, validating the mathematical linear models attained in the studied region, except for *IY*. For the response immobilisation yield, the attained value was only 2%, which was out of the interval of prediction in the 95% confidence level. This fact can be explained by the high determination errors in the analysis of this parameter. First, it represents a ratio of the immobilised enzyme at the fabric surface to the native enzyme initially present in solution. Secondly, the model shows a high significant curvature (see Table 6.6) and consequently the linear model is not adequate.

Finally, the method of analysis has to be considered. The initial activity was measured with the enzyme free in the solution, completely capable of interacting with the soluble substrate, ABTS. After the immobilisation of the laccase, the method to determine the remaining active enzyme consisted of using a small piece of polyamide fabric, which was introduced into the sample cell in the spectrophotometer, therefore enabling the determination of the activity of the laccase in Umg^{-1}. The release of polyamide micro fibrils to the medium can interfere with the spectrophotometer measurements, weakening the signal (~1%), which in turns might induce higher values for the activity of the immobilised laccase. Therefore, to overcome this systematic error, all the activity measurements were expressed in terms of relative activity.

6.4 CONCLUSIONS

In this study a multi-step procedure to immobilise laccase, from *Trametes hirsuta*, onto a woven polyamide matrix was developed. Four variables that could influence the immobilisation procedure, namely pH, spacer, enzyme and glutaraldehyde concentration were studied and an optimum work region was achieved.

Woven polyamide matrix seems to have high potential as an immobilisation support since it is inexpensive and readily available. At the same time glutaraldehyde, used as a crosslinking agent, is much less hazardous and easy to handle, when compared with other reticulating agents.

The application of a heterogeneous biocatalysis via immobilisation can lead to a reduction of downstream processing costs and the solid biocatalyst can be reusable. Moreover, the laccase showed a high ability to be used as biocatalyst and its immobilisation onto woven polyamide presents new insights about future applications. The laccase immobilisation onto polyamide matrices is a promising system for bioremediation of contaminated soils, wastewater treatment, wine and other beverages stabilization. Polyamide woven can also probably be applied as support of laccase, used on designing biosensors for monitoring of Kraft lignin and sulphate pine lignin (Shleev *et al.* 2006).

In conclusion, woven polyamide matrices show a high ability to act as supports for immobilising several enzymes like laccases or proteases and other different products such as perfumes or medical drugs. In this area a more exhaustive investigation is being carried out.

Chapter 7

GENERAL DISCUSSION

7. GENERAL DISCUSSION

The development of textile products via surface functionalisation is an issue with an increasing interest for several areas, since it can lead to high added value textile products and less environmental concerns about the wastes generated. The market demands require the research for new products with “higher added value” and new applications. There are several attempts refered in literature to functionalise polyamide surfaces which report the efficiency of harsh chemicals, like acids or basis, however they also mention considerable fibre damage and high environmental impact of these reagents.

In this work, the chemicals normally used for fibre functionalisation were replaced by an environmental friendly process with enzymes (cutinases and protease), which act only at the surface of the fibre, therefore avoiding the damage of the fibres.

The work done and presented in this thesis is a step towards the study and implementation of the enzymatic process on polyamide 6.6 fibres. Each chapter presents the discussion of the respective results and conclusions. As only now was possible to have an overall prespective of the work, some aspects need to be further discussed.

First of all, it was necessary to prove the concept underlying this thesis. Chapter 2 was the starting point for the experimental work. In this chapter were presented the results of the polyamide 6.6 fabrics surface modification using cutinase from *Fusarium solani* sp. *pisi*. Despite the fact that this enzyme degrades natural substrates and hence would not normally be able to degrade synthetic substrates, it showed good activity towards polyamide 6.6. This activity can be explained by the low specificity of cutinase as well as its ability to degrade aliphatic substrates similar to polyamide. Moreover, this enzyme shares the catalytic triad with other proteases enabling the possibility to hydrolyse amide bonds.

In order to confirm enzymatic hydrolysis of polyamide 6.6 fibres it was necessary to develop appropriate methodologies to monitor the formation of the resulting hydrolysis products. In this part of the work two different methodologies

for this purpose were developed. The first was based on the reaction of the primary amino groups resulting from polyamide surface hydrolysis with the TNBS compound. These groups could be found in the bath treatment as soluble oligomers. The other technique was based on the reaction of the α-bromoacrylamido wool reactive dye with the terminal amino groups at the surface of the modified fabrics. After studying the performance of the above mentioned methods it was observed that after incubation of polyamide 6.6 fabrics with cutinase, the soluble amino groups resulting from surface hydrolysis were detected by the TNBS method in the bath treatment. Nevertheless, the heterogeneity of the reaction, the compacted structure of the fibre and the size of the enzyme, disabled the penetration of the enzyme in the fibres core. Therefore the enzymatic reaction occurs only at the surface of the fibres and the concentration of amines levelled off, after 4 hours of incubation, reaching only 0.01 mM. Another aspect to take into account is the saturation level attained after a certain period of incubation (4 hours). After this period, there were no more sites for enzymatic attack, because the fabric substrate was totally covered by the enzyme and hence disabling the enzymatic action. The results showed a direct correlation between enzyme adsorption levels and amino groups formation.

Since the main goal of the thesis was the functionalisation of the polyamide 6.6 fabrics, it was necessary to obtain permanent amine-enriched surfaces, with the majority of the hydrolysis products staying at the surface of the treated fabrics. Results showed an increase of the colour levels (10.71%) after reactive staining, proportional to the terminal amino groups at the surface of the fabrics. The results also demonstrated that the stainings should be performed below the glass transition temperature (57ºC). Above this temperature, the resulting groups can rearrange and be less accessible for the reactive dye. An important conclusion can be drawn based on the results presented in this chapter: both methodologies were demonstrated as direct ways to confirm what actually occurs when polyamide 6.6 fabrics were treated with enzymes. However, due to the hydrophobic compacted structure of the non-natural substrate (polyamide 6.6.) and the large size of the enzyme, the turnover rates obtained were very low. Thus, in order to overcome these difficulties, another approach was assayed. In

the enzymatic system two different organic solvents (benzyl alcohol and dimethylacetamide) were included, that were hypothesised to 'open' the polyamide structure and hence increase enzyme adsorption.

Chapter 3 presented some of the results obtained when polyamide 6.6 fabrics were treated with cutinase in the presence of the above mentioned solvents, normally used in textile processing. The enzyme stability in the presence of these solvents was studied and the amount of protein adsorbed was quantified. The extent of hydrolysis was also quantified using the methodologies described in the previous chapter. The results obtained showed that cutinase half-life times decreased, relatively to buffer, in the presence of low amounts of organic solvents (35 hours in the presence of buffer; 26 hours in the presence of 1.5% of benzyl alcohol; 14 hours in the presence of 1.5% of benzyl alcohol + 10% of dimethylacetamide). The enzyme remains stable for a period of time that is sufficient for textile process applications. Moreover, the results showed a significant increase of enzyme adsorbed by the fabric substrate when the organic solvents were added to the system. This is perhaps due to the more open structure of the fibre, obtained as a result of organic solvents action that allows the adsorption of higher amounts of protein. At the same time, the simultaneous action of enzyme and organic solvents promoted more efficient surface modification of polyamide 6.6 fabrics. The samples treated with this combination showed an increase of 10% of K/S when compared with samples treated only with enzyme. The major conclusion that was deduced from this part of the work was that the low amounts of solvents used will not present an environmental impact in respect to the higher functionalisation level attained in a short period of time (4 hours).

Afterwards, modelling of the 3D structure of cutinase from *Fusarium solani pisi*, (PDB code 1CEX) (Longhi *et al.* 1997) showed that the external, but closed active site, was hindering the access to the fibre substrate. In order to overcome this, the genetic modification of native cutinase was performed.

In chapter 4, the cutinase from *Fusarium solani* sp. *pisi* was genetically modified near the active site, by site-directed mutagenesis, to enhance its activity towards polyamide 6.6 fibres. The mutations L81A, N84A, L182A, V184A and

L189A were performed to enlarge the active site in order to better fit a larger polymer chain. The molecular modelling studies performed by docking the synthetic model substrate of polyamide 6.6 at the cutinase active site showed that a higher stabilization was achieved with the mutant L182A, which was in accordance with the experimental results attained. Experimentally, a higher hydrolytic activity was also achieved with L182A form (+19% of amines). The modelling studies also predicted that this mutant form would not have significantly affected the adsorption by the hydrophobic fibres (25% of protein adsorption). The experimental activity measured towards a soluble substrate (*p*-NPB) showed also remarkable increase compared with the native one, when L182A mutant was used (365%).

Studies performed by incubating aliphatic substrates with native and L182A form, indicated that cutinase is designed to recognise aliphatic chains, as the mutant form L182A is more proficient to efficiently hydrolyse the bigger aliphatic substrates. Given these results, the most important conclusions to take into account are that L182A mutant form seems to be a promising mutation to modify the hydrophobic surface of polyamide 6.6 fibres and that the similarity of polyamide structure with cutin and the diversified substrate recognition of cutinase, might explain the cutinase ability to modify the polyamide surface.

Other approaches were further explored in order to overcome the low enzyme adsorption by the polyamide fibre, caused by the compacted polymer structure and by the low affinity of the enzyme with these synthetic substrates. Different levels of mechanical agitation were provided in order to study their effect on enzyme adsorption and hydrolysis efficiency.

The technical and mechanistic complexity of the enzymatic process is high since cutinases or proteases have not been “designed by nature” to interact with the insoluble fibre substrates. Similarly, the compacted fibre structure of the synthetic materials severely restricts the accessibility of the enzyme at the fibre surface, hence restricting enzyme adsorption. The results obtained lead to several assumptions:

- Cutinase (native and mutant) and protease were able to hydrolyse a small polyamide 6.6 substrate;

- When higher levels of mechanical agitation were provided, it was observed an increase of hydrolysis products in the bath treatment for all the enzymes tested;
- A higher level-off concentration of amines was achieved in the bath treatment when protease was used to treat polyamide fabric, showing that this enzyme has higher catalytic efficiency towards polyamide substrates, probably due to the fact that this enzyme acts towards peptide structures and amides; however its action must be controlled in order to achieve fabrics with higher levels of functionalisation, since the hydrolysis products were mainly released to the bath treatment (K/S increase was only 15%, in contrast with the K/S values obtained after treatment with cutinase, almost 80%);
- The wettability of fabrics increased; the samples treated with cutinases and protease became more hydrophilic, the samples changed from hydrophobic (time of water drop absorption higher than 10 minutes) to hydrophilic with values of water absorption near to 5 minutes;
- The crystallinity of fabrics was not changed by the enzymatic treatment due to the fact that this treatment is only superficial and does not influence the intrinsic properties of the polymer;
- A careful balance among mechanical agitation, time of incubation and enzyme concentration has to be taken in account in order to achieve the highest level of functionalisation.

Despite all the results attained, the main goal of the work, the functionalisation of the fabric, has to be taken in account. In order to achieve this, a vertical agitation must be used for a short period of time (4 hours), however no discs should be included in the system. The presence of the discs would promote the mechanical abrasion of the fabrics, raising the hydrolysis products through the bath treatment. Moreover, this abrasion would increase the weight loss (±4%) (data not shown). In the absence of discs, the treated fabrics presented values of weight loss lower than 2%. The fabrics resistance was also tested after enzymatic

treatments and the values attained showed loss of resistance lower than 3% (data not shown).

Finally, it was the thesis objective to achieve a feasible application for the functionalised samples attained. Thus, the immobilisation of a protein seemed to be a promising approach for industrial applications, such as waste-water treatment, bioremediation, medical devices, etc.

In chapter 6 it was reported the development of a procedure to immobilise *Trametes hirsuta* laccase onto woven polyamide supports, previously functionalized with protease, using glutaraldehyde as the crosslinking agent. Four variables, pH, presence of spacer, concentration of glutaraldehyde and concentration of laccase, were studied using a two-level factorial design. An optimum working region was achieved and the results attained, after a validation assay performed using 10% of glutaraldehyde, 29 UmL^{-1} of enzyme at pH 5.5 with spacer, revealed a half-life time of 78 h, a protein retention of 34% and an immobilisation yield of 2%. The values reached for half-life time (HLT) and protein retention (PR) were in good agreement with the ones predicted by the mathematical model, however IY failed this prediction. This lower value can be attributed to the high determination errors of the analysis method which measures the enzyme in a heterogeneous medium, differing from the free enzyme activity determination which is measured in a homogeneous medium. In addition, the surface of polyamide fabric was not homogeneously functionalised, having only some specific places where the crosslinker, and consequently the enzyme, can bind. Therefore the activity per mg of functionalised fabric was low.

This work allowed to conclude that woven polyamide can be used as support for protein immobilisation via crosslinking method and that the immobilisation of laccase onto polyamide woven surfaces can be a promising approach for future applications in wastewater, bioremediation of contaminated soils, wine and beverage stabilisation, perfumes or medical drug release and others, at least at laboratory scale.

Chapter 8

CONCLUSIONS AND FUTURE PERSPECTIVES

8. CONCLUSIONS AND FUTURE PERSPECTIVES

8.1 CONCLUSIONS

The enhancement of the hydrophilicity of synthetic polymers is a key requirement for many applications ranging from electronics to functional textile manufacture. Until now, few studies about the functionalisation of polyamide 6.6 fabrics with enzymes have been published. This work was a significant step for the replacement of the chemical functionalisation by the enzymatic one. It was proven that enzymes like cutinases or protease can be environmentally friendly tools for the surface hydrolysis of polyamide 6.6. The bulk properties of the polymers are not affected and the process can be controlled, in face of chemical methods which are based in an “all-or-nothing” mechanism. The practical evidences of enzymatic hydrolysis were achieved by developing two main methodologies, namely the determination of amines released to the bath treatment and at the surface of the treated fabrics.

The study of process parameters, like time of incubation, type and level of agitation or even the presence of agents such as organic solvents in the reaction medium, was of extremely importance for the comprehension of the enzymatic mechanism. The results corroborate the conclusion that a careful balance between mechanical agitation and time of incubation should be taken in account in order to achieve a higher level of fabric functionalisation. The inclusion of organic solvents can be a favourable tool to increase enzyme accessibility to the heterogeneous substrate and hence increase fabric functionalisation, however the amounts of these products should be controlled.

The genetic engineering approach showed some promising results but has to be more exhaustively exploited. A better understanding of the interaction of the enzyme with the heterogeneous substrate with regard to factors such as sorption, movement of the polymer surface and the role of hydrophobins or binding modules, will be necessary to develop enzymes with further enhanced activity.

The immobilisation of a protein, namely laccase from *Trametes hirsuta* was a big step in what concerns the immobilisation area, where polyamide fabric was a support with low levels of application. The development of an immobilisation procedure allows for a great variety of applications that were already mentioned in the discussion part. At least in a laboratory scale, polyamide fabrics can be an excellent support for the immobilization of proteins or other products, such as perfumes, drugs, etc.

Despite the several difficulties felt through this work, the results presented in this thesis represent a successful prolongation of some works already published in this area.

Consequently, the innovative aspects of this thesis are:

- the development of a promising environmental friendly alternative to the conventional chemical methods;
- the possibility to obtain functionalised fabrics via enzymatic action;
- the possibility of performing a controlled enzymatic hydrolysis, including other agents on the enzymatic system;
- the possibility of proteins immobilisation on the previously functionalised fabrics.

8.2 FUTURE PERSPECTIVES

Additional studies must be performed in order to better understand the mechanism of enzymatic hydrolysis of polyamide 6.6 fabrics and explain all the factors that affect the enzymatic reaction. Therefore, the future perspectives are:

- Continue this study, upgrading the enzyme modification technology from laboratory scale to a large-scale process, allowing for a “green industrial process” to be developed and implemented for the functionalisation of polyamide 6.6 fabrics with higher added-value.
- Study the influence of ultrasounds on the mass transfer of enzyme through the polymeric surface;
- Find the optimal industrial process parameters for the enzymatic functionalisation where weight loss, tensile strength and environmental impact will be minimised;
- Optimise the surface modification of different polyamide structures, such as knitted fabrics or even woven with different constructions.
- Develop new enzyme mutations on cutinase or on other enzymes in order to obtain catalysts with higher specificity towards polyamide 6.6 fibres;
- Produce new enzymes with higher specificity to hydrolyse synthetic substrates, such as polyamidases or other esterases;
- Search for new practical applications for the functionalised fabrics, such as the immobilisation of laccase in the process of polyoxometalates reoxidation;
- Search for new applications of PA functionalised fabrics in the areas of painting, inking, anti-fogging, filtration, electronics, biomedical field, drug control release and others.

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