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Maria Teresa Gonçalves de Macedo Matamá Enzymatic Treatment of Acrylic and Cellulose Acetate Fibres

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Universidade do Minho
Escola de Engenharia

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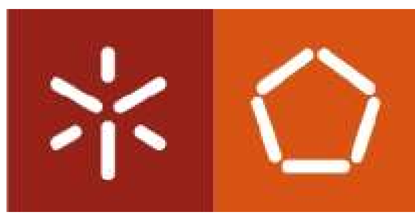

Universidade do Minho
Escola de Engenharia

Maria Teresa Gonçalves de Macedo Matamá

Enzymatic Treatment of Acrylic and Cellulose Acetate Fibres

Thesis for Doctoral degree in Textile Engineering - Textile Chemistry

Elaborated under the supervision of
Professor Doutor Artur Cavaco-Paulo

Julho de 2008

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

Maria Teresa Gonçalves de Macedo Matamá

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« *One of the wonders of human nature is our ability to hope... The great biologist Edward O. Wilson calls us "the future-seeking species" and suggests that natural selection has made hopefulness a unique human quality, "a necessary companion of intelligence".*

Still more human, perhaps, is our capacity for acting on our hopes. We not only dream, we strive to achieve the dreams we imagine. Behind all human achievement, from the creative acts of artists to the building of communities, from the making and trading of goods to the work of nations, there is aspiration, resolve, and action. »

By William McDonough, a “thinking green” architect
2001

ABSTRACT

The background theme of the present thesis is the multidisciplinary area of textile biotechnology, which is of major importance for the textile industry and its sustainable development. The work here described was devoted to the treatment with enzymes of two man-made fibres - acrylic and cellulose acetate. The thesis is divided in several chapters being the first one a general introduction. Biocatalysis is addressed, especially in the context of textile industry and surface modification of polymers, followed by a general description on the properties and applications of both fibres. The enzymes used throughout the work – nitrilase (EC 3.5.5.1) and cutinase (EC 3.1.1.74), are briefly mentioned as well as the major methods to manipulate and improve enzymes. The general aim of the work is the formation of reactive and/or hydrophilic groups at the surface of acrylic and cellulose acetate fibres by enzymatic hydrolysis of their pendent groups. As follows, the purpose is to preserve the desirable bulk properties of the fibres acting only at the surface by using eco-friendly catalysts.

In chapter 2, the modification of the surface of acrylic fabric with a commercial nitrilase is reported. The enzymatic conversion of nitrile groups into the carboxylic groups, on the fibre surface, was monitored for 36 hours by the release of ammonia to the media and by the improvement in the affinity of the treated fabric for a basic dye. The steady release of ammonia along the enzymatic treatment showed that the adsorption of nitrilase to the acrylic led to an increase in its operational stability, resembling the immobilization procedures used to stabilize proteins. A maximum affinity for the basic dye was observed for a treatment period of 8 hours, which corresponded to a relative K/S of 135% when the colouration of acrylic was performed at 70 ºC. A surface erosion phenomenon took place causing the “oscillatory” behaviour of the amount of dye uptake with the time of treatment. Polyacrylic acid was determined in solution as a non desirable, secondary product of the modification of acrylic with nitrilase. These results showed that the outcome of nitrilase application is closely dependent on reaction parameters like time, enzyme activity and media formulation.

The chapter 3 describes the modification of the comonomer vinyl acetate of the acrylic used with two enzymes: cutinase from the fungus *Fusarium solani pisi* and a commercial esterase (Texazym PES). The effect of acrylic solvents and stabilizing polyalcohols on cutinase operational stability in solution was studied. The influence of these additives and mechanical agitation on the enzymatic modification of acrylic fabric was also investigated. The hydroxyl groups produced on the fibre surface reacted with the dye Remazol Brilliant Blue R, C.I. 61200, increasing the colour of treated fabric. The best colour level was obtained with a high level of mechanical agitation and with the addition of 1% (v/v) N,N-dimethylacetamide. Under these conditions, the increase in the acrylic fabric colour depth was around 30% for cutinase and 25% for Texazym, comparing to the respective controls. The crystallinity degree, determined by wide angle X-ray scattering, was not significantly changed between control samples and samples

treated with cutinase. The results showed, once more, that the success of the application of enzymes, in this case cutinase and a commercial esterase, depends closely on the conditions in which the treatment takes place.

Cutinase was also chosen to modify the surface of cellulose diacetate and triacetate fibres. This work is reported in chapter 4. The enzymatic hydrolysis of acetyl groups on the fibre surface was evaluated by the release of acetic acid and by the specific chemical colouration of the fabrics with Remazol Brilliant Blue R. The treatment for 8 hours, at 30 ºC and pH 8, resulted in an acetyl esterase activity of 0.010 U and 0.0072 U on cellulose diacetate and triacetate, respectively. The colour levels for samples treated with cutinase for 24 hours increased 25% for cellulose diacetate and 317% for cellulose triacetate, comparing to the controls. Cross-sections of both fibres were analysed by fluorescence microscopy and the superficial action of cutinase was confirmed. Comparing to other enzymes described in literature, cutinase is a catalyst to consider for the superficial regeneration of cellulose hydrophilicity and reactivity on highly substituted acetates.

For further improvement of cutinase activity on cellulose modified fibres, chimeric cutinases were produced, by recombinant DNA technologies, and used to treat cellulose acetate fabrics, as described in chapter 5. Two distinct carbohydrate-binding modules were fused independently to the C-terminal of cutinase: the carbohydrate-binding module of cellobiohydrolase I, from the fungus *Trichoderma reesei*, and the carbohydrate-binding module of endoglucanase C, from the bacterium *Cellulomonas fimi*. Both chimeric cutinases had a more efficient performance than the wild type enzyme, but the interaction of these bifunctional enzymes with cellulose acetate needs to be further characterized for a better assessment of the nature and yield of the observed modifications.

The chapter 6 is dedicated to the general discussion, final remarks and future perspectives. In this thesis, evidences are presented showing that enzymes, more specifically, nitrilase and cutinase are important tools for the acrylic and cellulose acetate surface functionalization. This work also evidenced that this is only the first step towards the efficient utilization of these resources that Nature provide us.

RESUMO

O tema de fundo deste trabalho é a área multidisciplinar da biotecnologia têxtil que tem vindo a afirmar-se como uma área de grande importância para a indústria têxtil e para o seu desenvolvimento sustentável. O trabalho aqui apresentado consistiu no tratamento com enzimas de duas fibras, a acrílica e o acetato de celulose. A tese encontra-se dividida em vários capítulos consistindo o primeiro numa introdução geral a diversos tópicos abordados pelo trabalho. A biocatálise é referida, especialmente, no contexto da indústria têxtil e modificação superficial de polímeros, seguida de uma descrição geral das propriedades e aplicações das duas fibras. As enzimas utilizadas ao longo do trabalho – nitrilase (EC 3.5.5.1) e cutinase (EC 3.1.1.74), são mencionadas de forma sucinta assim como os métodos principais de manipulação da actividade enzimática. O objectivo geral do trabalho assentou no desenvolvimento de metodologias não poluentes conducentes à formação de grupos reactivos/hidrofílicos à superfície das fibras, via hidrólise dos grupos laterais dos respectivos polímeros de forma a preservar as propriedades nucleares e desejáveis das fibras.

No capítulo 2 é reportada a modificação da superfície da fibra acrílica por uma nitrilase comercial. A conversão enzimática dos grupos nitrilo em grupos carboxílicos à superfície da fibra foi avaliada durante 36 horas através da libertação de amoníaco para a solução e através do aumento da afinidade do tecido tratado para um corante básico. O aumento linear do amoníaco, libertado durante o tratamento, mostrou que a adsorção da nitrilase à fibra acrílica conduziu a um aumento da sua estabilidade operacional assemelhando-se aos procedimentos de imobilização usados para estabilizar enzimas. Um valor máximo de K/S foi observado para um período de incubação de 8 horas, o que corresponde a um K/S relativo de 135% quando se fez uma coloração a 70 ºC. Teve lugar um fenómeno de erosão superficial que determinou o comportamento oscilatório da quantidade de corante fixada com o tempo de tratamento. Foi determinado ácido poliacrílico nas soluções de tratamento como produto secundário não desejado da modificação da acrílica pela nitrilase. Estes resultados revelam que o efeito final da aplicação da nitrilase no tratamento da acrílica está intimamente dependente dos parâmetros da reacção como o tempo, a actividade da enzima e a composição do meio.

O capítulo 3 descreve a modificação do comonómero acetato de vinilo da fibra acrílica usada pela acção da cutinase do fungo *Fusarium solani pisi* e de uma esterase comercial (Texazym PES). Foi estudado o efeito de solventes da acrílica e de poli-álcoois na estabilidade operacional da cutinase em solução, bem como o impacto desses aditivos e da agitação mecânica na modificação enzimática do tecido de acrílica. Os grupos hidroxilo produzidos na superfície da fibra reagiram com o corante Remazol Brilliant Blue R, C.I. 61200, aumentando a cor do tecido tratado. O melhor nível de coloração foi obtido com elevada agitação mecânica e com a adição de 1% (v/v) de N,N-dimetilacetamida. Sob estas condições, o aumento da intensidade de cor, em relação aos controlos, foi de 30% para o tratamento com a cutinase e

25% para a Texazym. O grau de cristalinidade determinado por difracção de raios-X não foi alterado significativamente entre amostras controlo e amostras tratadas com cutinase. Uma vez mais, os resultados mostraram que o sucesso da aplicação de enzimas, neste caso a cutinase e uma esterase comercial, depende muito das condições em que se realiza o tratamento.

A cutinase foi escolhida para modificar também a superfície de fibras de diacetato e triacetato de celulose, trabalho este que é abordado no capítulo 4. A hidrólise enzimática dos grupos acetilo na superfície da fibra foi monitorizada pela libertação de ácido acético e pela coloração específica dos tecidos com o corante Remazol Brilliant Blue R. O tratamento do tecido durante 8 horas a 30 ºC e pH 8 resultou numa actividade acetil esterase de 0.010 U e 0.0072 U tendo como substrato a fibra de diacetato e triacetato de celulose, respectivamente. Os níveis de cor das amostras tratadas com cutinase durante 24 horas aumentaram 25% para o diacetato e 317% para o triacetato, comparando com os controlos. Foram analisadas secções transversais de ambas as fibras, por microscopia de fluorescência, e confirmou-se a acção superficial da cutinase. Por comparação com outras enzimas já descritas, a cutinase é um catalizador a ter em consideração para a regeneração superficial da hidrofilicidade e reactividade da celulose em acetatos com grau de substituição elevado.

Para melhorar a actividade da cutinase nas fibras modificadas de celulose foram produzidas, através de tecnologias de ADN recombinante, cutinases quiméricas que foram, posteriormente, usadas no tratamento dos tecidos de acetato de celulose descrito no capítulo 5. Dois módulos distintos de ligação a carbohidratos foram fundidos, independentemente, ao terminal carboxílico da cutinase: o módulo da celobiohidrolase I, do fungo *Trichoderma reesei* e o da endoglucanase C, da bactéria *Cellulomonas fimi*. Ambas cutinases quiméricas tiveram uma performance mais eficiente que a cutinase nativa, mas a interacção destas enzimas bifuncionais com os acetatos de celulose carece de mais estudos para melhor caracterizar a natureza e extensão destas modificações.

O capítulo 6 é dedicado a uma discussão geral, observações finais e perspectivas futuras. Nesta tese são apresentadas evidências que mostram que as enzimas, mais concretamente, a nitrilase e a cutinase são ferramentas importantes para a funcionalização da superfície das fibras acrílicas e de acetato de celulose. Com este trabalho também fica claro que apenas se deu o primeiro passo num percurso que eventualmente nos conduzirá a um aproveitamento eficiente destes recursos que a Natureza nos providencia.

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AXE II – acetyl xylan esterase II

C.I. – Colour Index

CBH I – cellobiohydrolase I from *Trichoderma reesei*

CBM – carbohydrate-binding module

CD – crystallinity degree

CDA – cellulose diacetate

CenA – endoglucanase A of *Cellulomonas fimi*

CenC – endoglucanase C from *Cellulomonas fimi*

CTA – cellulose triacetate

DMA – N,N-dimethylacetamide

DMF – N,N-dimethylformamide

DNA – deoxyribonucleic acid

DRIFT – diffuse reflectance infrared Fourier transform spectroscopy

DS – degree of substitution or acetylation

EG – ethylene glycol

FITC – fluorescein isothiocyanate

FT-IR – Fourier transform infrared spectroscopy

I_C – crystallinity index

IR – infrared

K/S – Kubelka-Munk relationship: K is the adsorption coefficient and S is the scattering coefficient

LCD – liquid crystal display

nd – non detected

NMR – nuclear magnetic resonance

o.w.f. – of weight of fabric

PA – polyamide

PAA – polyacrylic acid

PAN – polyacrylonitrile

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PEG – polyethylene glycol

PET – poly(ethylene terephthalate)

PLA – polylactic acid

***p*-NP** – *p*-nitrophenol

***p*-NPB** – *p*-nitrophenyl butyrate

r – radius

rpm – rotations per minute

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM – scanning electron microscopy

T_d – dyeing transition temperature

T_g – glass transition temperature

WAXS – Wide angle X-ray scattering

XRD – X-ray diffraction

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1 INTRODUCTION

The present thesis, entitled *Enzymatic Treatment of Acrylic and Cellulose Acetate fibres*, connects topics from different areas of science that will be the subject of a brief bibliographic revision throughout this chapter.

The first topic to be addressed is biocatalysis which is the background theme of this thesis and its importance will be emphasized in the context of the textile industry and surface modification of synthetic polymers. A general description of the two fibres used in this work, acrylic and cellulose acetate, will be given as well as their most common end-uses and reported biomodifications. The properties and general applications of the two main enzymes used to modify the fibres will be referred next. Finally, a summary of the methods used to manipulate enzymes is made before a concise description of the two carbohydrate-binding modules chosen to be fused with the C-terminal of cutinase.

1. INTRODUCTION

1.1 BIOCATALYSIS

1.1.1 INDUSTRIAL ENZYMES

Biocatalysis is present in some of the oldest transformations known to humans: descriptions of various beer recipes were found in Sumerian writings (Ball, 2001). More recently, in the beginning of the 19th century, acetic acid was produced industrially from ethanol by an *Acetobacter* strain (Wandrey *et al.*, 2000). Studies of fermentation processes led to a *big bang* in the knowledge of life's chemistry. In the 19th century, Louis Pasteur came to the conclusion that the fermentation of sugar to alcohol by yeast was catalyzed by a vital force contained within the yeast cells called "ferments", which were thought to function only within living organisms. Wilhelm Kühne was the first to use the term "enzyme" and, years later, Emil Fisher proposed the "Lock and Key Model" to visualize the substrate and enzyme interaction (Cabral et al., 2003).

Enzymes are the subgroup of proteins that catalyse the chemistry of life, transforming both macromolecular and small molecules; they are the focus of present biocatalysis research (Walsh, 2001). The chiral nature of enzymes results in a remarkable chemical precision seen as different types of selectivity: substrate, stereo-, regio- and functional group selectivity (Rozzell, 1999). Most enzymes operate at room temperature, under neutral aqueous conditions and in the absence of functional group-protection (Koeller and Wong, 2001). Enzymes are environmental friendly catalysts not just because they are biodegradable themselves but also because of their mild operating conditions. They can result in processes that generate fewer waste disposal problems and that require lower energy input, leading to lower costs and lower emissions of greenhouse gases to the environment (Rozzell, 1999). These qualities make enzymes remarkable catalysts.

Nowadays, it is widely recognized that enzyme-catalysed chemical transformations are convenient alternatives to traditional (non-biological)

transformations and suitable solutions to difficult synthetic problems (Koeller and Wong, 2001). The enzyme can be used as the sole catalyst in a reaction, in combination with other enzymes, or with inorganic reagents. Besides, many enzymes accept unnatural substrates, and genetic, pathway and medium engineering can improve further their stability and specific activity as well as modulate their substrate specificity (Koeller and Wong, 2001). Biocatalysis is accomplished by either using isolated enzymes or using whole cells which are more common in synthesis reactions that require cofactors (Schmid *et al.*, 2001). The majority of currently used industrial enzymes are hydrolases (figure 1.1) from which proteases remain the dominant enzyme type, followed by amylases and cellulases (Kirk *et al.*, 2002).

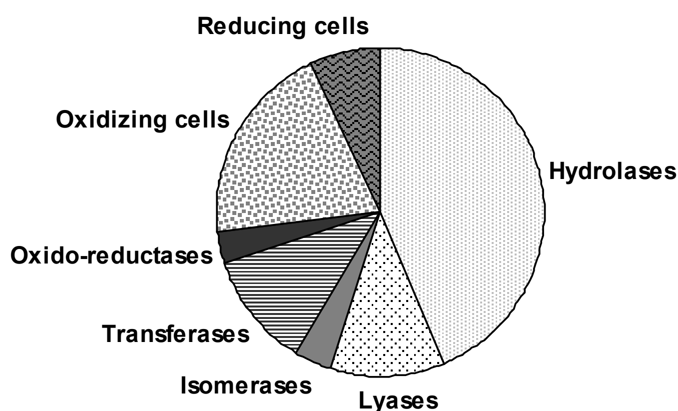
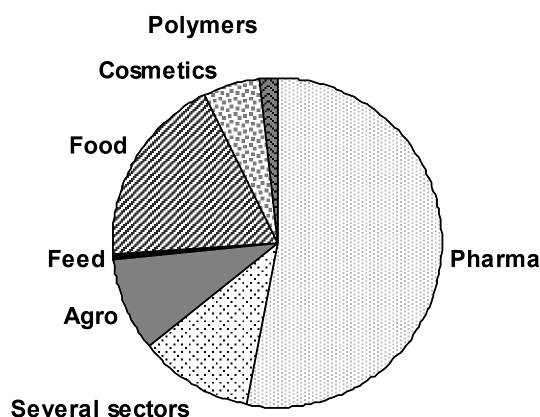


Figure 1.1 – Biocatalysts used in industrial transformations (adapted from Straathof *et al.*, 2002).

Biocatalysis is a tool of increasing importance for industries which aim a sustainable development. Successful industrial applications of enzymes are growing rapidly over the past decade (Straathof *et al.*, 2002). The major traditional consumers of enzymes are the food, feed, agriculture, paper, leather and textile industries (van Beilen and Li, 2002; Schäfer *et al.*, 2007). However, the largest growth seen, in terms of number of occurrences (figure 1.2), is in the application of enzymes to the industrial chemical synthesis, especially in the pharma and agro sectors (Straathof *et al.*, 2002; Schmid *et al.*, 2001). Another sector that is becoming important includes the companies that provide enzymes. Besides producing the

enzymes, they also invest in the research, using molecular technologies to discover and design new biocatalysts or to improve available ones (Straathof *et al.*, 2002).



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1.1.2 Biocatalysis in Textile Industry

In the textile industry, the impact of biotechnology has been observed at three main levels: the introduction of enzymes in manufacturing wet processes and laundry detergents, the design of new and biodegradable fibres and the treatment of textile effluents.

The use of enzymes as detergent additives represents one of the largest applications of biocatalysis in industry (Kirk *et al.*, 2002; Schäfer *et al.*, 2007). Different classes of hydrolases are used to cover as much as possible all kinds of stains: proteases, amylases, lipases and, more recently, mannanases and pectate lyases (Kirk *et al.*, 2002; Schäfer *et al.*, 2007). Cellulases are also used in laundry detergents in order to renew the cellulosic fabric surfaces damaged with microfibrils, fuzz, loose fibres and to improve their colour brightness (Durán and Durán, 2000; Gübitz and Cavaco-Paulo, 2001). The typical advantage of including enzymes in detergent formulations is to clean clothes at lower washing temperatures. However, these benefits are ultimately achieved if consumers are willing to accept low temperature washing (Schäfer *et al.*, 2007).

The first application of enzymes to industrial textile processes began with the desizing of cotton fabric with amylases present in the malt extract used around 1857 (Cegarra, 1996; Etters and Annis, 1998). Today, a variety of commercial products are available, in particular for the wet processing of cellulosic fabrics. The protein fibres have also been the focus of industrial enzyme applications, mainly proteases (Cegarra, 1996; Etters and Annis, 1998). The main enzymes used in commercial textile processing are summarized in table 1.1, as well as their general conditions of use.

Table 1.1 – Main enzymes used in commercial textile processing (adapted from Schäfer *et al.,* 2007)

Fibre	Process stage	Main enzyme	Typical treatment conditions pH/ T/ t
Cotton	Desize	Amylase	pH 5-10/ 20-115 ºC/ 0.2-16 h
		Lipase	pH 7-9/ 20-70 ºC/ 10-30 min
	Scour	Pectinase	pH 8-9/ 45-65 ºC/ 10-30 min
	Bleach clean-up	Catalase	pH 6-7/ 30-60 ºC/ 10-20 min
	Depilling and softening, Denim abrasion	Cellulase	pH 5-8/ 30-60 ºC/ 30-60 min
	Reactive dye rinse	Peroxidase	pH 6-9/ 40-80 ºC/ 10-30 min
	Denim decolourization	Laccase	pH 4-6/ 60-70 ºC/ 15-30 min
Lyocell	Defibrillation	Cellulase	pH 5-8/ 30-60 ºC/ 30-60 min
Ramie	Preparation/degumming	Pectinase/xylanase	pH 8-9/ 50-60 ºC/ 1-2 h
Wool	Scour	Lipase	pH 7-9/ 20-70 ºC/ 10-30 min
	Softening	Protease	pH 7-9/ 40-50 ºC/ 30-60 min
Silk	Degumming	Protease	pH 8-9/ 50-60 ºC/ 0.5-2 h

New biodegradable synthetic fibres are being developed from renewable sources of biomass. A prime example is polylactic acid (PLA) made by fermenting a sugar feedstock into lactic acid which is then chemically

transformed into a polymer fibre. PLA based materials have properties similar to other synthetic fibres; they are durable, with a silky feel and may be blended with wool or cotton. PLA was launched in 2003 as the first man-made fibre derived from renewable resources under the commercial name *Ingeo* (Burke, 2008). Another commercial fibre named *Sorona*, made by DuPont, results from the polymerization of 1,3-propanediol, derived from a fermenting process, and a petrochemical-based monomer (Burke, 2008). New fibres are also being derived from natural materials like chitin, collagen and alginate. These materials are used for medical applications in wound dressings and investigated for drug-releasing systems (Lu and Chen, 2004; Qin, 2008; Rinaudo, 2008).

The textile industry is under substantial environmental pressure. Considering the volume discharged and effluent composition, the wastewater generated by this industry is classified as one of the most polluting among all industrial sectors (Vandevivere *et al.*, 1998). Biocatalysis can be applied as a tool for the textile effluents treatment. Possible bioremediation processes can be divided in whole cell microbial systems and isolated enzymes (Gübitz and Cavaco-Paulo, 2001; Ramalho, 2005; Soares, 2000). Biodegradation through activated sludge is widely used in treatment plants but is still ineffective is decolorizing textile effluents (Vandevivere *et al.*, 1998). It is necessary to integrate other chemical and physical technologies in order to increase the efficient treatment and reuse of this kind of effluents. Several laboratory-scale investigations have illustrated the potential of sequential anaerobic/aerobic biotreatment steps but a current use is still missing (Vandevivere *et al.*, 1998).

Although the widespread academic efforts in the textile biotechnology, the actual application of such work is modest. Hopefully in a near future this reality will be changed by both the academic and industrial communities.

1.1.3 BIOCATALYSIS IN SYNTHETIC POLYMER SURFACE MODIFICATION

In the preparation of wide-ranging synthetic polymer materials, sometimes it is desirable for the properties at the surface to be different from the bulk properties. Surfaces that promote cell adhesion, biocompatibility, hydrophobic/ hydrophilic character or chemical resistance are some examples of the properties that can be changed (Hutchings *et al.*, 2008).

One important field where surface modification of polymers has a great impact is the materials science. The biocompatibility can be accomplished by immobilizing certain bioactive molecules on functionalized surfaces of the materials to be used. The superficial immobilization of molecules is also important for the manufacturing of specific analytical assays like microarrays and biosensors. The immobilization of industrial enzymes into the surface of solid supports is also a widespread process (Goddard and Hotchkiss, 2007).

Textiles are one of the major and oldest subjects for surface modifications. Textile finishing is the final stage in the fabric manufacturing process and includes all the processes that modify the surface of fibres to add useful qualities to the fabric, ranging from interesting appearance and fashion aspects to high performance properties for industrial needs (Schindler and Hauser, 2004).

The methods to accomplish distinct surface properties may involve the application of a coating layer or chemical modification of the surface (Hutchings *et al.*, 2008). The modification of the surface can be performed by ionized gas treatments, like plasma and Corona discharge, and UV irradiation (Goddard and Hotchkiss, 2007). The wet chemical modification is the most classical and easy approach for the functionalization of polymer surfaces, offering advantages for porous materials, besides, it does not require very specialized equipment (Goddard and Hotchkiss, 2007). In the textile industry both chemical and physical methods are used, but chemical finishing has always been an important component of textile processing and its importance is growing in recent years with the trend to 'high tech' products (Schindler and Hauser, 2004). As the use of high performance textiles grows, the need for chemical finishes to provide the fabric properties required in these special applications has grown accordingly. Nowadays, more than 20 different types of chemical finishers exist for both natural or man-made fibres (figure 1.3) (Schindler and Hauser, 2004).

Textile materials made from synthetic fibres are, in general, uncomfortable to wear because they are hydrophobic. This means that these materials can not absorb the perspiration and the water vapour can not easily be transported away from the body. The hydrophobic nature also leads to their characteristic static cling and stain retention during laundering (Gübitz and Cavaco-Paulo, 2008). There are several methods for the surface modification of synthetic polymers with the purpose of increasing the hydrophilicity. The methods currently used

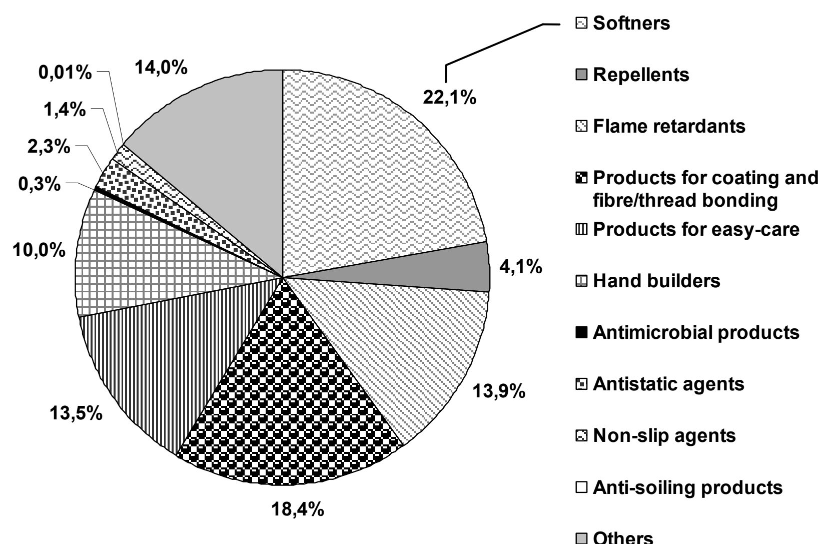


Figure 1.3 – Distribution of textile chemical finishers by amount in 2001 (adapted from Schindler and Hauser, 2004).

are plasma treatments and chemical finishers, including alkaline and acid hydrolysis. In addition to the environmental issues, these methods are difficult to control, sometimes they are of reduced technical life-time and in some cases severe fibre weight losses and yellowing occur (Gübitz and Cavaco-Paulo, 2008, 2003). On the other hand, enzymes are well-suited for targeted surface functionalization of polymers. Besides the advantages referred before, they are macromolecules and for that reason their action is normally restricted to the most superficial layers of polymer fibres.

At the research level, there are several reports of successful surface enzymatic modifications of the major three synthetic textile fibres: polyester (poly(ethylene terephthalate)-PET), polyamide (PA) and polyacrylonitrile (PAN). The targets for enzyme catalysis are the ester and amide bonds of the polymer backbone of PET and PA, respectively, and the side chain nitrile groups of PAN (Gübitz and Cavaco-Paulo, 2008). PET was hydrolysed by cutinases, from different microorganisms, lipases, serine and nitro-benzyl esterases (Gübitz and Cavaco-Paulo, 2008). The limited surface hydrolysis led to an increase in hydrophilicity and a depilling effect was also observed (Gübitz and Cavaco-Paulo, 2008; O'Neill *et al.*, 2004, 2007). PA fibres have also been hydrolysed superficially by proteases, cutinases and amidases. As a consequence of the enzymatic treatment there was a good improvement in the hydrophilicity (Gübitz and Cavaco-Paulo, 2008; Silva

et al., 2005b, 2007). The enzymatic modification of PAN with nitrilases and nitrile hydratases led to the formation of more hydrophilic carboxylic and amide groups (section 1.2.5) (Gübitz and Cavaco-Paulo, 2008).

The broad substrate specificity and catalytic promiscuity, exhibited by some enzymes, uncover a new range of possible biocatalytic transformations (Bornscheuer and Kazlauskas, 2004). The ability of enzymes to use textile synthetic fibres as substrates under mild conditions, which are known for their stability and chemical inertia, is an evidence of the vast potential of these catalysts in industrial processes.

1.2 ACRYLIC FIBRE

1.2.1 GENERAL DESCRIPTION

Acrylic fibres are defined, according to the Federal Trade Commission of United States, as manufactured fibres in which the fibre forming substance is any long-chain synthetic polymer composed of at least 85% by weight of acrylonitrile units (Guillen, 1987).

Although the acrylonitrile was synthesised by Moreau in 1893, only in the 1940s, suitable solvents were found that allowed the polyacrylonitrile processing, for the reason that its polymer decomposes prior to melting (Frushour and Knorr, 1998). The first appearance of acrylic fibres was in the year 1950, when DuPont introduced them in the market under the commercial name Orlon®. During the 1950s, the acrylic industry experienced an impressive growth with at least 18 companies producing acrylic goods (Frushour and Knorr, 1998).

During the year 2005, global production of acrylic staple fibre reached 2 791 thousand tons and acrylic accounted for 8% of all chemical fibre produced in the world (figure 1.4). The share has fallen dramatically from 15% in early 1980s. During the period 2000-2005, the acrylic staple fibre production increased at a rate of 1.25% per annum which was the slowest growth rate among all chemical fibres production (www.yarnsandfibers.com/ir/report/acrylic_chain_report2006.html). In the year of 2006, the acrylic fibre production decreased to 6.8% of the total chemical fibres. In terms of the contribution, Asia holds a major share of 59.5% in global production, where China has emerged as the leading

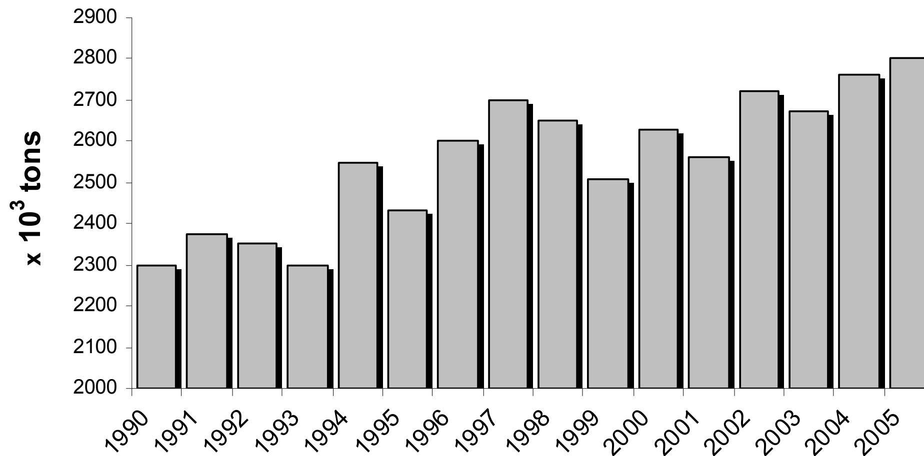


Figure 1.4 – World acrylic production between 1990 and 2005 (from www.yarnsandfibers.com/ir/report/acrylic_chain_report2006.html).

producer of acrylic staple fibre in the world and also as the major consumer (figure 1.5) (www.yarnsandfibers.com/revamp_ir/report_fullstory.php3?id=401&p_type=62&source _id=15&source=YarnsandFibers%20Paid&story_type=F&BF=Special&report_show=First).

Acrylic fibre covers a broad range of products, more diverse in composition than any other synthetic fibre (Masson, 1995). The major reason for this is that acrylonitrile can copolymerise with many different monomers with an ethylene unsaturated group. In fact, the PAN homopolymer is rarely used in fibre manufacturing with the exception of some industrial applications (Frushour and Knorr, 1998). The homopolymer is difficult to spin and dye and therefore virtually all commercial acrylic fibres are made from acrylonitrile and at least one

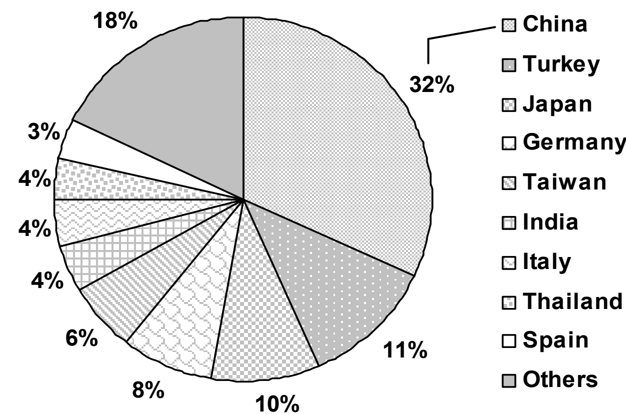
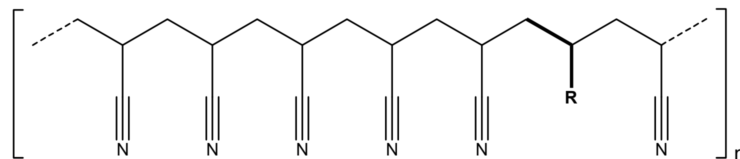


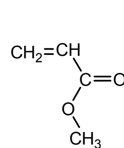
Figure 1.5 – The most important world acrylic fibre producers in 2006 (from www.yarnsandfibers.com/revamp_ir/report_fullstory.php3?id=401&p_type=62&source_id=15&source=YarnsandFibers%20Paid&story_type=F&BF=Special&reportshow=First).

other monomer (figure 1.6). The comonomers are used in order to increase the solubility of the polymer in the spinning solvents and improve the rate of dye diffusion into the fibres (Frushour and Knorr, 1998). Acid and basic comonomers are also used to create additional sites for dye fixation and to provide a hydrophilic component in water-reversible crimp bicomponent fibres (Frushour and Knorr, 1998; Masson, 1995). Halogenated comonomers, usually vinylidene chloride, vinyl bromide and vinyl chloride, can be used to impart flame resistance to the acrylic textiles (Frushour and Knorr, 1998; Masson, 1995). Modacrylic fibres are composed of 35% to 85% of polyacrylonitrile and they are produced to be fire retardant by incorporation of halogenated comonomers (Masson, 1995).

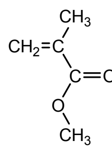
The copolymer is formed through free radical polymerization. There are four polymerization methods: bulk, aqueous dispersion, solution and emulsion polymerization (Frushour and Knorr, 1998; Masson, 1995). The industrial methods of choice are the aqueous dispersion and solution polymerization. After



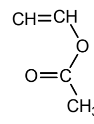
R = neutral comonomers



methyl acrylate

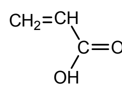


methyl methacrylate

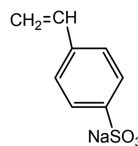


vinyl acetate

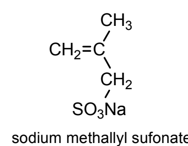
R = acid comonomers



acrylic acid

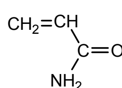


sodium styrene sufonate

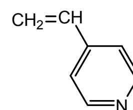


sodium methallyl sufonate

R = basic comonomers



acrylamide



4-vinyl pyridine

Figure 1.6 – The acrylic polymer structure and some examples of its common comonomers.

precipitation the copolymer is dried and dissolved in an appropriate organic solvent, mainly dimethylformamide (DMF) or dimethylacetamide (DMA), and wet or dry spun (Capone, 1995; von Falkai, 1995).

After the spinning stage, the acrylic fibre properties are unsuitable for their *end-use*; the fibres contain residual amounts of the organic solvent, the tenacity and elastic modulus are low while the plastic elongation is high, the fibre lacks the crimp needed to provide cohesion and bulk to the yarn (von Falkai, 1995). Thus, the fibre is subjected to further treatments in order to develop the desirable balance of processing and performance properties (Frushour and Knorr, 1998). During this pos-spinning stage, the fibre undergoes a series of steps along a production line. The essential steps of washing, orientation drawing, drying and relaxation are common to all acrylic fibre spinning processes, but the sequences as well as a number of possible variations in the manufacture of acrylic fibres are protected and the details are not available (Frushour and Knorr, 1998). The improvement of the acrylic fibre properties by manipulation of the conditions of spinning and pos-spinning processes is an important issue and it is the subject of several research groups (Bahrami *et al.*, 2003; Wu *et al.*, 2003; Chen and Harrison, 2002). The figure 1.7 is a schematic representation that illustrates a production line for acrylic fibres in the tow and staple forms.

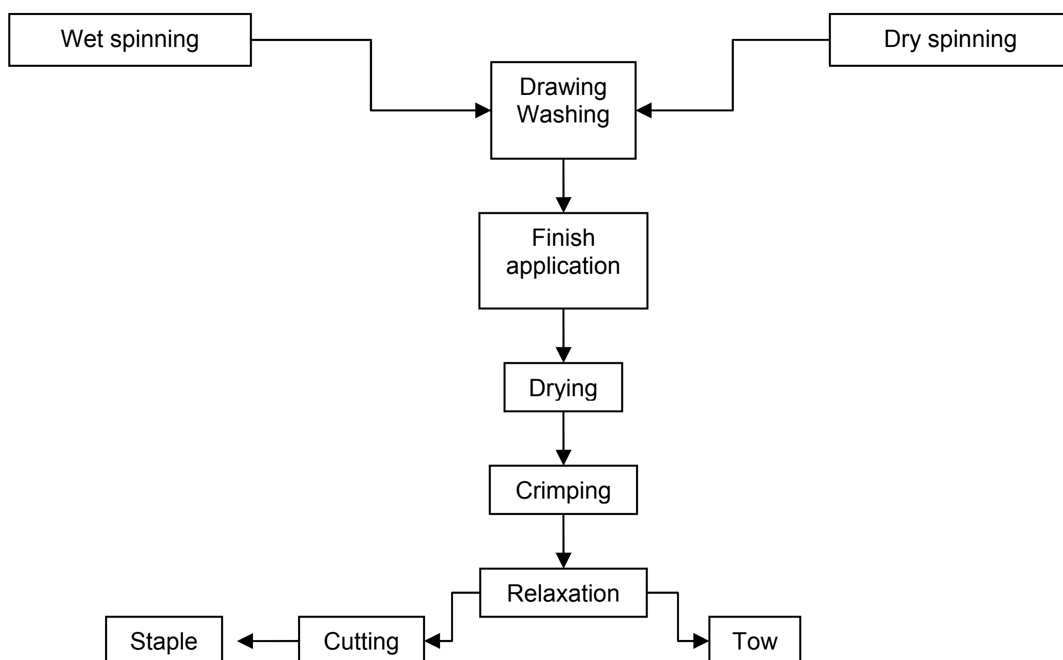


Figure 1.7 – Conventional manufacture process of acrylic fibres (adapted from Frushour and Knorr, 1998).

The nature of the comonomer in acrylic fibres affect the overall dyeability and the classes of dyes that may be used (Needles, 1986). The cationic or basic dyes are the most used in acrylics given the good fastness properties obtained. The fixation occurs with the salt bridge between dye cations and anionic sites (mainly sulphate from the initiator radicals). Acrylic can be dyed using disperse dyes, in particular the smaller ones, for pastel and light shades where dyeing uniformity may be hard to obtain with other dyes (Emsermann and Foppe, 1995). Acrylics can be dyed by either batch or continuous processes, in the fibre, yarn, fabric or garment form (Emsermann and Foppe, 1995).

1.2.2 Structural characterization

Textile fabrics are planar structures produced by interlacing or entangling yarns or fibres in a particular manner (Needles, 1986). Textile yarns are made up of fibres and each individual fibre is, in turn, made up from millions of individual long molecular chains of discrete chemical composition (Needles, 1986). The molecular structure of the long polymer chains determines the basic chemical and physical properties of the fibre. Although special treatments and changes in yarn and fabric production parameters can alter the fabric properties to some degree, the basic properties are inherent to the structure of the polymer from which the fibre is produced (Needles, 1986).

Most textile fibres have a morphology that can be described by the two-phase model for semicrystalline polymers (Frushour, 1995). According to this model, discrete crystalline domains of several hundred angstroms (Å) are mixed with amorphous domains of similar size. The individual polymer chains have lengths in the order of 1000 to 2000 Å, so a single chain can span two or more crystalline domains and its assembly in the intercrystalline regions forms the amorphous domains (Frushour, 1995). When a synthetic fibre is drawn, the molecules, in most cases, orientate themselves in crystalline domains parallel to the fibre axis. The degree of crystallinity is dependent on the total forces available for chain interaction and the stereo-regularity of adjacent chains (Needles, 1986; Stevens, 1990).

Commercial vinyl polymers are in general atactic (with no stereo-regularity) and form amorphous glasses that have no long-range crystalline

order (Frushour, 1995). Fibres can be melt-spun from these glassy polymers but they can not be used for textile applications because of the lack of crystallinity (Frushour, 1995). Crystallinity is a very important characteristic because it confers good tensile properties (Frushour, 1995). PAN is atactic, but the fact that functional synthetic fibres can be made from it suggests that some degree of order must be present in PAN fibres. The other major factor that contributes to the formation of a crystalline phase is the ability of interchain bonding. In fact, some atactic polymers like PAN possess an unexpected high degree of order or pseudocrystallinity due to a substitutent group capable of strong interactions (Frushour, 1995).

The pendent group in PAN molecules is the nitrile group. The distinguishing feature of the nitrile group is the large dipole moment, turning it one of the most polar organic functional groups (Frushour, 1995). The interaction between two nitrile groups can be either attractive or repulsive, depending upon the spatial orientation of the nitriles, while the magnitude depends upon the distance of separation (Frushour, 1995). In the PAN isolated chain, the potential energy will be minimized by placing the adjacent nitrile groups as far as possible, since they have parallel orientation between each other and this will lead to repulsive interactions (Frushour, 1995). To model the configuration with the lowest potential energy, the initial proposal was the helical conformation for the chain backbone (figure 1.8A). In such conformation all the nitrile groups would be pointing away from the helical axis. If several chains are packed together, then some of the nitrile groups on adjacent chains will be in an anti-parallel orientation leading to a net attraction between PAN polymer chains (Frushour, 1995). More recent models, having in account the density of the fibres, propose stretches of more extended atactic molecular chains, where the nitrile groups are pointing normally to the backbone axis along directions which are displaced from each other ≈120º (Rizzo *et al.*, 1996; Liu and Ruland, 1993).

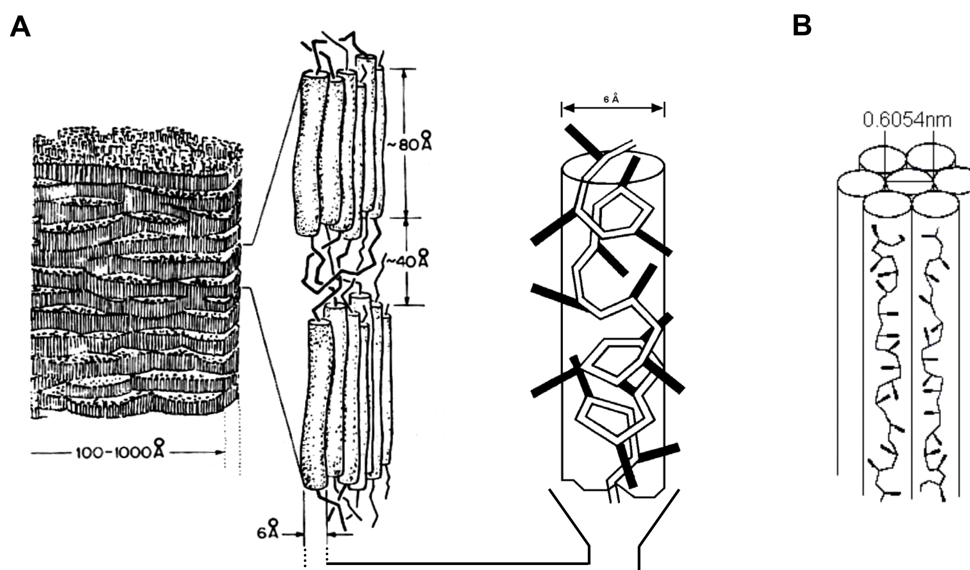


Figure 1.8 – A) Model of the oriented acrylic fibres morphology with emphasis for the assumed irregular helical conformation of PAN polymer chain (adapted from Frushour, 1995). **B)** Pictorial representation of hexagonally packed chains of PAN (adapted from Bashir and Rastogi, 2005).

Bohn *et al.* (1961) reported the first detailed model of the PAN fibre morphology using wide angle X-ray scattering (WAXS) and by studying the thermal behaviour of polyacrylonitrile. The diffraction pattern was indexed to a two-dimensional hexagonal lattice with an interchain distance of 6 Å, given by the most intense reflection at 2θ of 17° (Bohn *et al.*, 1961; Frushour, 1995). A single polymer chain resembles a rod with a diameter of 6 Å where some of the nitrile groups extend beyond the confines of the cylinder (figure 1.8B) (Bohn *et al.*, 1961; Frushour, 1995). It is believed that these protuberant nitrile groups are responsible for the net attraction between adjacent PAN polymer chains and the ability of this vinyl polymer to form fibres with structural order.

The differences in the order between the paracrystalline and amorphous phase are much lower than in conventional crystalline polymers, not only because there seems to be a lack of a true three-dimensional order in the crystalline phase but also because the amorphous phase may be quite stiff and extended due to intrachain dipole repulsions (Frushour, 1995). Despite the investigation performed for more than 50 years, polyacrylonitrile is still a very controversial polymer, because it belongs to an unusual type of material that

cannot be obtained in proper crystalline or fully amorphous state (Bashir, 1994, 2001; Bashir and Rastogi, 2005).

The effect of the comonomers on the PAN morphology was studied by several authors. In all cases, the addition of comonomers lowers the crystallinity, here referred to the two-dimensional order (Frushour, 1995). This decrease in the crystallinity of PAN copolymers affects the dynamic-mechanical properties as well as the melting behaviour and glass transition temperature (Frushour, 1982). Incorporation of comonomers, as methyl acrylate and vinyl acetate, disrupts the laterally bonded structure in a way dependent on the comonomer molar fraction and on the molar volume of its side chain (Frushour, 1995). The polymer becomes more soluble, making the preparation and storage of spinning dopes easier. The resultant fibres are also more extensible, less prone to fibrillation and more easily dyed, albeit a decrease in the hot-wet strength and modulus (Frushour, 1995). These main advantages provided by the comonomer incorporation are the reason for virtually all commercial acrylic fibres being spun from PAN with 5% - 10% comonomer.

1.2.3 ACRYLIC FIBRE PROPERTIES

The wet spinning technology is the most used to produce acrylic fibres (Lulay, 1995). This process leads to a round or bean shaped in contrast with the dog-bone shaped fibre produced by the dry spinning method. The macro-structure of the fibre formed in the spin bath influences the mechanical properties of the final product. The values of stiffness predict softer yarn at a comparable fibre denier for the dry spun fibres (Lulay, 1995). The dog-bone shaped fibres also lead to a less dense and bulkier yarn, which in turn contributes to increase the comfort of the final products allowing a faster perspiration removal (Frushour and Knorr, 1998). The acrylic fibre densities may vary from 1.14 to 1.19 g cm^{-3} and the filament denier can range from 1.2 to 15 (Collier and Tortora, 2001).

In spite of all the differences among the existing commercial fibres, the general idea is that acrylics have moderate strength and elongation at break (Needles, 1986). The standard breaking strength (tenacity) varies from 2.0 to 3.6 g

per denier and the breaking elongation varies from 20% to 64% (Collier and Tortora, 2001; Frushour and Knorr, 1998).

At 2% elongation the elastic recovery is 99% and at 5% it decreases to 50% - 95% (Needles, 1986). The acrylic fibre is, therefore, moderately stiff, but its resilience ranges from good to excellent; the crimped fibres have good compression and bending recovery (Collier and Tortora, 2001). Comparing to the other two major synthetic fibres, acrylics have a lower breaking elongation and considerably lower tenacity, which make them inappropriate for end-uses requiring high strength (Frushour and Knorr, 1998).

Acrylic fibres are less moisture absorbent than cellulose acetate, cotton, wool and rayon and more absorbent than nylon and polyester (Frushour and Knorr, 1998). The standard moisture regain varies from 1.0% to 2.5% and the fibres swell ≈5% when saturated with water (Collier and Tortora, 2001; Frushour and Knorr, 1998). At room temperature, there is a slight decrease in the tenacity when the acrylic fibres are wet, however when the temperature of water is raised above the glass transition temperature, the reduction in the modulus becomes significant (Frushour and Knorr, 1998). Raising the water temperature from 20 ºC to 95 ºC, the tenacity and the fibre modulus are reduced 65% and 98%, respectively (Frushour and Knorr, 1998). The hot-wet strength of acrylic is very low, compared to other man-made fibres like cellulose acetate, nylon and polyester, and is attributed to the plasticizing effect of water and the lack of a true crystalline phase (Frushour and Knorr, 1998). The acrylic fibres have low electrical conductivity, even so they dissipate static charge more readily than most other synthetic fibres because they are more hydrophilic (Frushour and Knorr, 1998).

The acrylic fibres have good colour and heat stability bellow 130 ºC (Frushour and Knorr, 1998). In a dry state, the fibre decomposes before melting at 315 ºC. Acrylic fibres are moderately flammable; untreated acrylic fibres ignite, burn and melt, leaving a hard black bead residue at the edge of the fabric as other synthetic fibres (Collier and Tortora, 2001). The glass transition temperature for the oriented dry commercial fibres is between 85 ºC and 95 ºC (Emsermann and Foppe, 1995). Acrylic fibres can be heat-set under appropriate conditions acquiring good dimensional stability (Collier and Tortora, 2001).

The chemical stability of acrylic is good; it is resistant to weak acids, weak alkalis, organic solvents, oxidizing agents and dry cleaning solvents. These

fibres are sensitive to strong bases and highly polar organic solvents (Frushour and Knorr, 1998). Regarding environmental factors, acrylics show very good resistance to sunlight and to all biological agents.

The acrylic fibres impart warm, natural-like aesthetics to most fabrics as opposed to the cold, plastic handle of polyester and nylon fibres (Lulay, 1995). According to a study performed to evaluate qualitatively the properties of several fibres, consumers considered the acrylic as having moderate performance on highly desirable properties like abrasion, wrinkle and pill resistances, strength and wash-wear. Nylon and polyester were considered to have a better performance for those properties. Therefore a continuous effort to improve acrylic properties is being taken in order to compete with the other synthetic fibres. Several methods were already tested, consisting in the incorporation of comonomers, modification of the spinning process and/or finishing treatments (Frushour and Knorr, 1998). Success has been achieved mostly regarding the improvement on the pilling behaviour as well as in the strengthening of the already good properties of acrylic fibres (Frushour and Knorr, 1998).

1.2.4 PRODUCTS AND APPLICATIONS

Acrylic fibres are commonly produced in the tow and staple forms and the main use is the replacement of wool. Acrylic has many of the desirable wool properties: warmth, softness of hand and generous bulk qualities. It is also less costly, more resistant to abrasion, light, heat, chemical and biological attacks and it has lower tendency to shrink than wool (Frushour and Knorr, 1998). Acrylic fibres are fabricated into woven and specially knitted constructions in a variety of textures and weights, according to the end-use. The major market sectors are the apparel and home furnishing. The apparel uses are sweaters, sleepwear, knit-accessories, fleece fabrics and hand-knitted garments (Collier and Tortora, 2001). In the home furnishing sector, the main uses are blankets, upholstery, draperies and carpets (Masson, 1995).

There are several product variants that constitute less than 25% of total acrylic fibre sales. Acrylic fibres can be made in bicomponent varieties by extruding two different types of acrylic polymer as one fibre. The differences in

the properties of the two lead to the formation of moisture-sensitive or heat-sensitive crimp, most used in knitted apparel (Collier and Tortora, 2001). Acid dyeable-fibres are obtained by the incorporation of basic monomers (pyridines, tertiary amines and quaternary ammonium salts) and blended with cationic dyeable-acrylics to produce certain colour effects (Masson, 1995). Acrylic fibres can be also pigmented for a particularly better light fastness as well as better colour stability regarding the exposure to a variety of chemicals (Masson, 1995). These variants are more important for outdoor applications and work clothes.

More technical applications include the reinforcing fibres, made from polyacrylonitrile homopolymer, used as asbestos replacement, and the carbon fibre precursor. Carbon fibres are produced by the thermal decomposition of polyacrylonitrile copolymers in the absence of oxygen. More recently, polyacrylonitrile has been used in reverse osmosis gas separation, ion exchange, ultrafiltration and dialysis (Frushour and Knorr, 1998; Masson, 1995).

1.2.5 POLYACRYLONITRILE AS AN ENZYMATIC SUBSTRATE

As described previously, the modification of the surface of textile polymers with enzymes is focused mainly on natural fibres. In the case of synthetic polymers the methods currently used involve the action of chemical or physical agents. Acrylic fibres are treated with strong reactive chemical agents, like concentrated bases, or with radiation and plasma in order to add functional groups or transform the existent nitrile groups (Battistel *et al.*, 1995; Gübitz and Cavaco-Paulo, 2008).

Several research groups have already demonstrated that is possible to modify the acrylic fibre with nitrile metabolizing enzymes. These enzymes can convert the nitrile groups into carboxylic or amide groups, depending on the type of enzyme system present (figure 1.9). The modification of nitrile surface groups into amide groups was accomplished by the action of nitrile hydratases from *Brevibacterium imperiale* and *Corynebacterium nitrilophilus* (Battistel *et al.*, 2001 and 1995). The newly formed amides were assessed by X-ray photoelectron spectroscopy and the treated acrylic fibres and powder were coloured with acid dyes, while the untreated controls were inert to the same dyes. In another work it was reported a nitrile conversion to the carboxylic acid with the release of

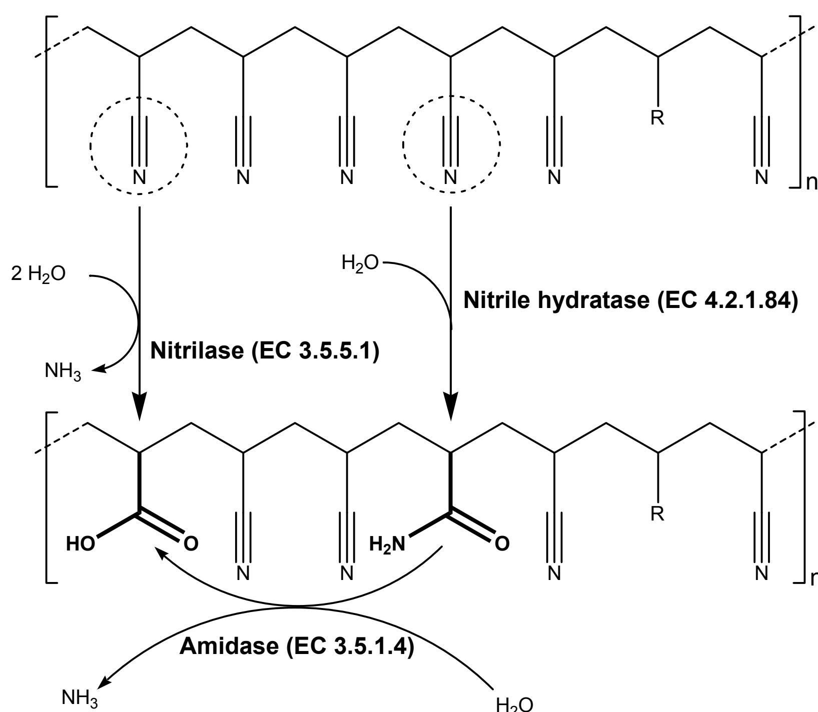


Figure 1.9 – Possible chemical transformations on PAN surface catalysed by nitrile metabolizing enzymes.

ammonia, on granular PAN as substrate, and to the amide on acrylic fibres surface, with a nitrile hydratase and amidase from *Rhodococcus rhodochrous* (Tauber *et al.*, 2000). It was also reported that the molecular weight of PAN influenced negatively the enzymatic action. A nitrile hydratase, from *Arthrobacter* sp. *Ecu* 1101, was used to selectively transform the nitrile into amide groups (Wang *et al.*, 2004). The modified acrylic fibre became more hydrophilic, antistatic and acid-dyeable. The authors concluded that the use of non-ionic surfactants improved the enzyme activity while DMF did not. Another microorganism, *Agrobacterium tumefaciens*, was found to grow on polyacrylonitrile as a carbon source, converting it to polyacrylic acid (PAA) as shown by solid state nuclear magnetic resonance (NMR) (Fischer-Colbrie *et al.*, 2006). The detected enzyme activities were nitrile hydratase and amidase, although this amidase was, once more, less efficient in using acrylic fabric as a substrate, like amidases from other species.

More recently, a membrane-bound nitrilase was isolated from *Micrococcus luteus BST20* (Fischer-Colbrie *et al.*, 2007). Nitrile groups from both polymers and fabrics of PAN were converted to carboxylic acid groups, with the associated

release of ammonia. The nitrilase preferred the less crystalline polymer among seven polymers with different comonomer compositions, as generally observed for other enzymes acting on solid substrates.

1.3 CELLULOSE ACETATE FIBRES

1.3.1 GENERAL DESCRIPTION

Acetate fibres are defined as manufactured fibres by the Federal Trade Commission from United Stated, in which the fibre-forming substance is cellulose acetate (Needles, 1986). Cellulose acetates are classified as derivative cellulose fibres, distinct from rayon and lyocell which are regenerated cellulose fibres, because the chemical composition of cellulose acetate is not cellulose but an ester of cellulose (Collier and Tortora, 2001). Each anhydroglucose repeating unit of cellulose (figure 1.10) has three hydroxyl groups located at the positions 2, 3 and 6 (La Nieve, 2007). These sites are available for acetylation to produce the acetate fibres. The degree of acetylation or substitution (DS) is the average number of acetylated positions per anhydroglucose unit. Commercial cellulose triacetate has a DS of 2.91-2.96 while cellulose acetate has a DS of ≈2.4 (La Nieve, 2007). Therefore, the denomination of cellulose acetate fibres is used to refer all commercial acetylated cellulose fibres, but it is also the common name for the cellulose acetate fibre with a DS ≈2.4. To avoid misinterpretations, throughout this text, the name cellulose acetate will be used to refer all types of fibres and cellulose diacetate will be used to refer the acetate with a DS ≈2.4.

Cellulose diacetate (CDA) was first synthesized by Schützenberger in

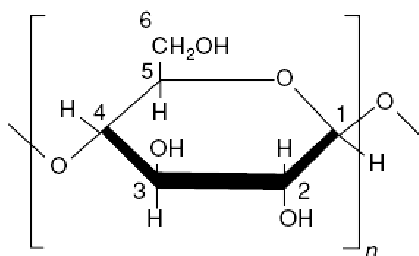


Figure 1.10 – Chemical structure of the anhydroglucose repeating unit of cellulose (from La Nieve, 2007).

1865 and, 14 years later, Franchimont found that sulphuric acid acted as a catalyst, allowing for the CDA production at room temperature (Rustemeyer, 2004a). In 1902-1905, several patents appeared for the application of cellulose triacetate (CTA) as photographic film, artificial silk and hornlike plastic material (Rustemeyer, 2004a). The first important application of CDA was the coating for the fabric wings of airplanes in World War I. The Dreyfus brothers were invited to found CDA plants in Europe (1914) and in United States (1917). In 1924, they developed a successful spinning process of a silk-like yarn textile called “Celanese”, for the Celanese Co. (Rustemeyer, 2004a). CTA textile fibre was commercialized later in the 1950s (La Nieve, 2007).

Cellulose diacetate flake is mainly used to produce tow used in cigarette filters. Other uses include the manufacture of textile fibres, films, sheets and moulded objects. The combined CDA and CTA textile fibre world production reached a maximum in 1971 with 426 000 tons (La Nieve, 2007). Since then the textile fibre production has been decreasing gradually (figure 1.11). An exception is CTA film used for LCD manufacture, which is expected to grow continuously (www.sriconsulting.com/CEH/Public/Reports/580.0400). CDA tow was introduced in 1952 and has been applied successfully in the production of cigarette filters (La Nieve, 2007). In contrast to the textile fibre, the world production of CDA tow has increased steadily (figure 1.11).

The decline in the sales volume of CDA has been felt by all major world

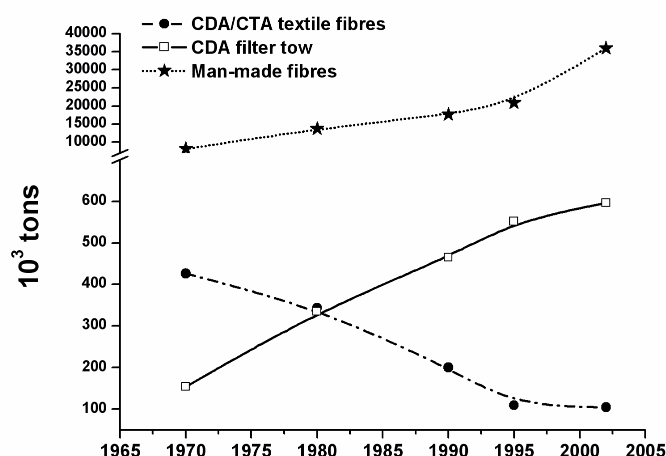


Figure 1.11 – World production of cellulose diacetate (CDA) and triacetate fibres (CTA) compared with the cellulose diacetate tow and total man-made fibres (adapted from La Nieve, 2007).

areas except China, Central Europe and Russia. In these developing countries the trend in cigarettes consumption is increasing and it is expected that it will offset the declining number of smokers in North America and Western Europe (www.sriconsulting.com/CEH/Public/Reports/580.0400). Global market for CDA is highly concentrated with major producers representing over 80% of global capacity: Celanese Corp, Eastman Chemical Company, Daicel Chemical Industries, Mitsubishi Rayon and Rhodia Acetow (www.industrialnewsupdate.com/news/manufac turing/archives/2008 /04/cellulose_aceta.php).

Cellulose diacetate and triacetate are produced from high quality cellulose, such as cotton linters and wood pulps, with an α-cellulose content above 95% (Saka and Matsumura, 2004). The initial stages of both CDA and CTA formation are the same. Purified and shredded cellulose pulp is pretreated to increase its reactivity and to disperse it uniformly in the reaction media (La Nieve, 2007). The most used commercial acetylation process is the acetic acid system where the acetic acid serves as solvent for the cellulose acetylation and acetic anhydride and sulphuric acid as catalysts (La Nieve, 2007). The esterification of cellulose is

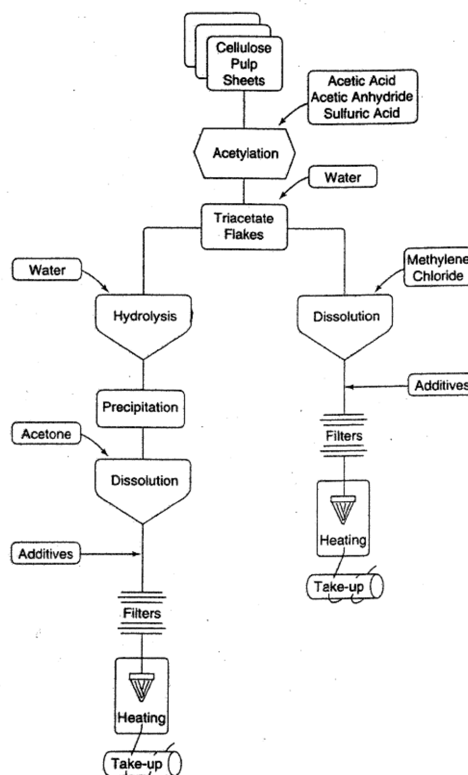


Figure 1.12 – Schematic representation of the manufacturing line of cellulose acetate fibres (from Collier and Tortora, 2001).

allowed for 7-8 hours till the CTA, also known as primary acetate, is formed (Collier and Tortora, 2001). After this point the procedure differs depending on which DS is being produced (figure 1.12).

To obtain the CTA, water is added to the acetylating media and CTA is precipitated as a white flake, which is then collected, and dried (Collier and Tortora, 2001). To obtain the CDA, also known as secondary acetate, water is added in an amount and for a period of time that allow partial hydrolysis of the primary acetate. The CDA is then precipitated, collected, washed and dried (Collier and Tortora, 2001). The spinning dope preparation is basically the same for both CDA and CTA except for the solvent used (La Nieve, 2007). CDA is usually dissolved in 95% aqueous acetone while CTA is dissolved in methylene chlorine containing 5% to 15% of methanol. At the dope stage, titanium dioxide pigment can be added if dull yarns are to be produced. Dope dying is possible for both fibres by the addition of pigments or dyes soluble in the particular solvent system used for the spinning dope (La Nieve, 2007). The CDA and CTA fibres are produced by the dry-spinning technology. The fibres are lubricated and crimp is also introduced for further processing. Besides the continuous filament, acetate fibres are also produced in the tow and staple forms (La Nieve, 2007).

Cellulose diacetate and triacetate are not satisfactorily dyed by soluble dyes; instead disperse dyes are the ones preferred for these fibres (La Nieve, 2007; Needles, 1986). Disperse dyes are insoluble or slightly soluble in hot aqueous baths, so dispersing agents and carriers are currently used to enhance the uniformity and penetration of the dye molecules into the fibres. In the case of CDA, carriers are not necessary and the dyeing temperatures range between 70º and 80 ºC. CTA has a slower relative dyeing rate, the carriers are needed as well as dyeing temperatures in the range of 96 ºC - 100 ºC (La Nieve, 2007; Steinmann, 1998).

Although triacetate and diacetate are produced in a very similar way and have similar structures, the small difference in the DS value provides significant differences in the properties of these fibres (Needles, 1986).

1.3.2 STRUCTURAL CHARACTERIZATION

While cellulose, either from cotton linters or wood pulp, is highly crystalline, the substitution of the hydroxyl by acetyl groups disrupts its original structure in such way that dry-spun cellulose acetates show very low crystalline order (La Nieve, 2007). The structure order of cellulose disappears during the normal process of esterification as observed by Robert Work (Work, 1949). CTA appears to have a certain amount of crystallinity but the amorphous component is predominant. He observed that by raising the CTA to a temperature slightly bellow its melting point there was a molecular rearrangement leading to a more crystalline material. Robert Work also verified that the same was not true for the less substituted CDA, due to the steric disorder provided by heterogeneous side groups (acetyls and hydroxyls) randomly placed along the polymer chain. Stretching the swollen CDA yarns improved the orientation but there was not a great increase in crystallinity. To prove his hypothesis, he hydrolyzed completely the CDA obtaining regenerated cellulose with a high degree of structural order.

Two structural polymorphisms exist for CTA designated by CTA I and CTA II (Zugenmaier, 2004; Sprague *et al.*, 1958). Commercial CTA, which is produced normally by homogeneous acetylation of cellulose, has a crystalline structure that corresponds to the polymorph CTA II.

The unit cell dimensions of both CTA polymorphs and the structural models are depicted in figure 1.13. The crystal structure of CTA I is assumed to have a orthogonal unit cell composed of chains packed with parallel polarity (Stipanovic and Sarko, 1978; Zugenmaier, 2004). The cell unit of CTA II is also orthorhombic but the chains pack in pairs with an antiparallel orientation (Roche *et al.*, 1978; Zugenmaier, 2004).

In both CTA and CDA, hydrogen bonding between cellulose chains is substantially decreased and the bulky acetyl group prevents the close packing of cellulose chains (Needles, 1986). The van der Waals forces are the major associative forces between the polymer chains and their lower magnitude is the reason for cellulose acetate being considerably weaker than cellulose fibres.

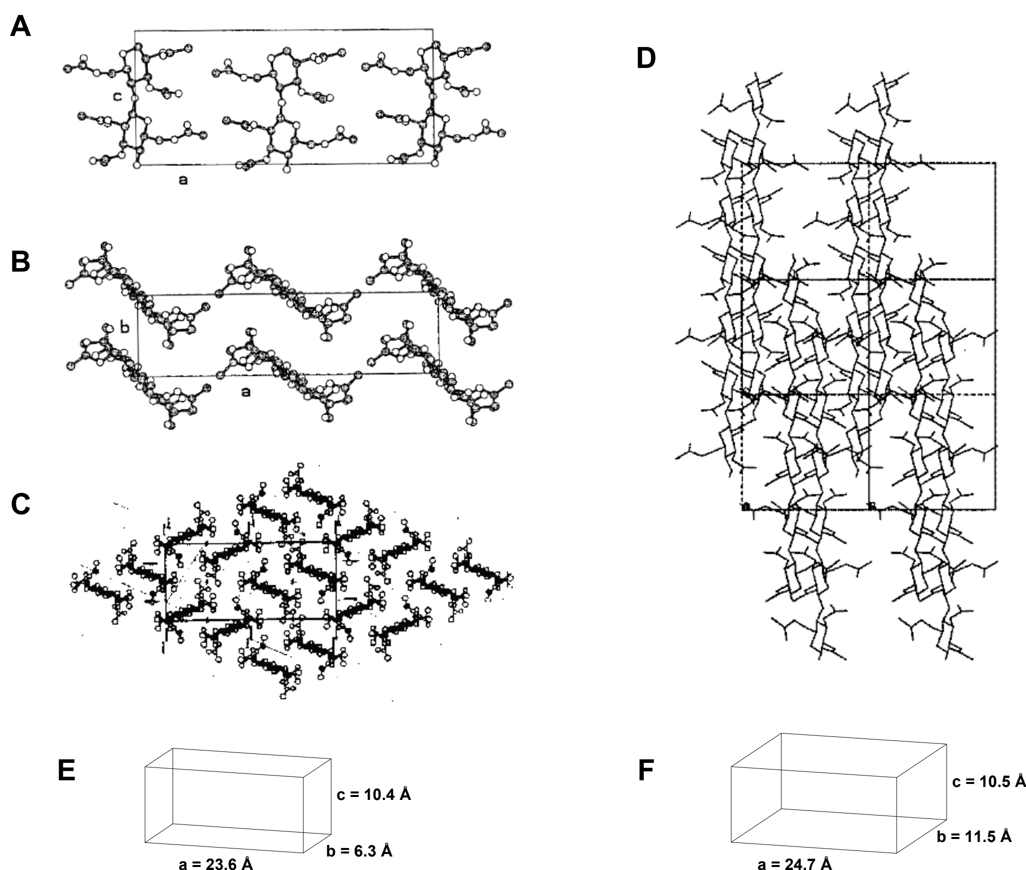


Figure 1.13 – Structural models of CTA I and CTA II in two projections: **A)** CTA I perpendicular to the *ac* plane, **B)** CTA I along the c axis (the chain axis), **C)** CTA II perpendicular to the *ac* plane, **D)** CTA II along the c axis, **E)** unit cell dimensions of CTA I and **F)** unit cell dimensions of CTA II (adapted from Zugenmaier, 2004).

The increase in crystallinity of CTA by heat treatment has beneficial effects on the fibre and fabric properties, resulting in "ease-of-care" and "wash-and-wear" characteristics (Steinmann, 1998).

1.3.3 Cellulose acetate fibre properties

The fibre cross section of both CTA and CDA is irregular with as many as 5 to 6 lobes and the longitudinal views show striations along the fibre length (Needles, 1986). Both fibres have white bright appearance and good lustre if not dyed or treated with a delustering agent (Collier and Tortora, 2001). CDA and CTA have densities of 1.30-1.35 g cm^{-3} and the filament denier ranges from 3 to 60 (Needles, 1986; Steinmann, 1998).

Both CDA and CTA have a very low strength, with standard tenacities of 1.2-1.4 g per denier and standard elongations at break of 25% - 45%, however, for their end-uses, strength is not a primary consideration (Steinmann, 1998; Collier and Tortora, 2001). At 2% elongation the fibres have recoveries ranging from 80% to 99%, but above 5% elongation the recovery is very low, in particular for CDA. CDA and CTA are moderately stiff fibres with good resilience on bending and deformation.

The moisture regains for CDA and CTA is 6.5% and 3.5%, respectively (Steinmann, 1998; La Nieve, 2007). The CDA has a moisture regain close to the value 7% of natural cotton yarn, while, the CTA has a lower value but still higher than the commercial synthetic fibres. Wetting the cellulose acetate fibres decreases their tenacity to 0.8-1.0 g per denier and the elongation at break increases more in the case of CDA (35% - 50%) than in the case of CTA (30% - 40%). CTA is more hydrophobic and the water has lower impact on the CTA crystalline structure when compared to CDA. This imparts a better dimensional stability to CTA during washing and drying cycles, whereas CDA may exhibit relaxation shrinkage on laundering (Collier and Tortora, 2001). Both fibres have a high electrical resistance and, as a consequence, static build-up is a problem unless antistatic treatments are applied (Needles, 1986).

The CDA and CTA are good heat conductors and they are cool to the touch (Needles, 1986). These fibres are thermoplastic so they soft and melt with the application of heat. CDA is more heat sensitive than CTA, it softens in the range of 190 ºC - 205 ºC and it melts at ≈260 ºC with discolouration (Steinmann, 1998; La Nieve, 2007). The softening temperature for heat-treated CTA is ≈240 ºC and the melting point is ≈305 ºC with discoloration and decomposition. If ignited, CDA and CTA burn with melting, leaving a small, hard, beadlike residue at the edge of the burning area (Collier and Tortora, 2001).

The chemical stability of cellulose acetates is poor. They are attacked by a number of organic solvents capable of dissolving esters, strong acids and bases, which result in saponification of acetyl groups (La Nieve, 2007; Collier and Tortora, 2001). CDA is more susceptible of being attacked by strong oxidizing agents than CTA, however both can be bleached. They can be damaged by microorganisms under certain conditions, but CTA is considerably more

resistant to biological attack (Needles, 1986; Collier and Tortora, 2001). In general, the CTA fibre is more resistant to sunlight exposure and aging, while CDA looses strength upon extended exposure to sunlight. Neither CDA nor CTA are resistant to abrasion (La Nieve, 2007; Collier and Tortora, 2001).

Acetate fibres are soft, cool, have silk-like aesthetics, good drape and they can be easily blended with other fibres like silk, rayon, nylon, cotton and polyester (Law, 2004). Their unique attributes remain desirable and they are responsible for the survival of acetate production in the competitive market of man-made fibres. In a study made in 1999 by the Institute of Environmental Research from Kansas State University to determine the perceived difference between acetate and polyester linings, a group of consumers felt that acetate was more comfortable (less sticky, clammy and damp) and lighter in weight than polyester (www.acetateworld.com). Another attribute that is gaining importance is the fact that cellulose acetate fibres are environmental friendly compared to the major synthetic fibres.

Some methods were developed to improve the strength, abrasion resistance and dimension stability of acetate fibres, in particular of CDA (Steinmann, 1998). One approach was to add to CDA spin dope polymer additives. Several were tested but, unless their concentration was bellow 5%, the phase compatibility was poor (Steinmann, 1998). To improve the compatibility, some polymers were graft on CDA. In the case of acrylonitrile, the graft copolymer increased the compatibility of PAN and cellulose acetate and the resulting fibres possessed improved thermal and chemical stabilities (Steinmann, 1998). It was also investigated the effect of crosslinking agents on cellulose diacetate, though the improved properties were still not equal to those of heat-treated CTA (Steinmann, 1998).

1.3.4 PRODUCTS AND APPLICATIONS

The properties of acetate fibres led to their largest use in women's apparel and apparel linings. The yarns are evenly distributed between woven and knit constructions. Principal items of clothing are blouses, dresses, lingerie, robes and linings for suits, skirts and pants. They are also used in household textiles like draperies, bedcovers, curtains, etc (La Nieve, 2007; Collier and Tortora, 2001; Law,

2004). Other textile applications include sportswear, medical bandages and tape (Law, 2004).

Staple fibres and nonwovens are used in wound dressings, personal hygiene, wipes and speciality papers (Law, 2004).

CDA is also used for the production of cigarette filters. The CDA crimped tow presents several advantages like the ability to produce uniform and firm filters, it selectively removes phenols, nitrosamines, quinolines and other undesired smoke components, it is non toxic, tasteless, odourless and it is biodegradable. These attributes account for the preference for filters made from CDA which represents 90% of the consumed cigarettes in the world (Rustemeyer, 2004b).

Other non textile applications include solvent-cast diacetate film that is produced by dissolution of CDA flake in acetone and the addition of certain additives like plasticizers that impart the film attributes. The films are applied in a variety of end-uses like adhesive tapes, labels, general packaging, photo negative sleeves, and graphic arts, among others. They are known for their high transparency and gloss, although they can be produced with irregular matt surfaces (Law *et al.*, 2004). The CTA film has unique properties exploited in its application as photographic film, protective film for polarizing plates or optical compensation film for liquid crystal displays (Sata *et al.*, 2004). CDA is also used to produce plastic products whose use implies direct and prolonged contact with humans, like fashion accessories, tooth brushes, protective glasses, casino chips, etc (Carrolo and Grospietro, 2004).

Cellulose acetate is extensively applied in the separation technology. Acetate membranes are used in desalting (by reverse osmosis), hemodialysis, drinking-water purification (by ultrafiltration) and filters for laboratory use. Cellulose acetate is also used as stationary phase in chromatography separations (Shibata, 2004).

The manufacture of products made from cellulose acetate and other esters, has a promising future due to their renewable resource and properties. Cellulose acetate is becoming the raw material of choice for a new generation of high-tech products for the separation technology, the pharmaceutical industry, the controlled release and biopolymer fields (Glasser, 2004).

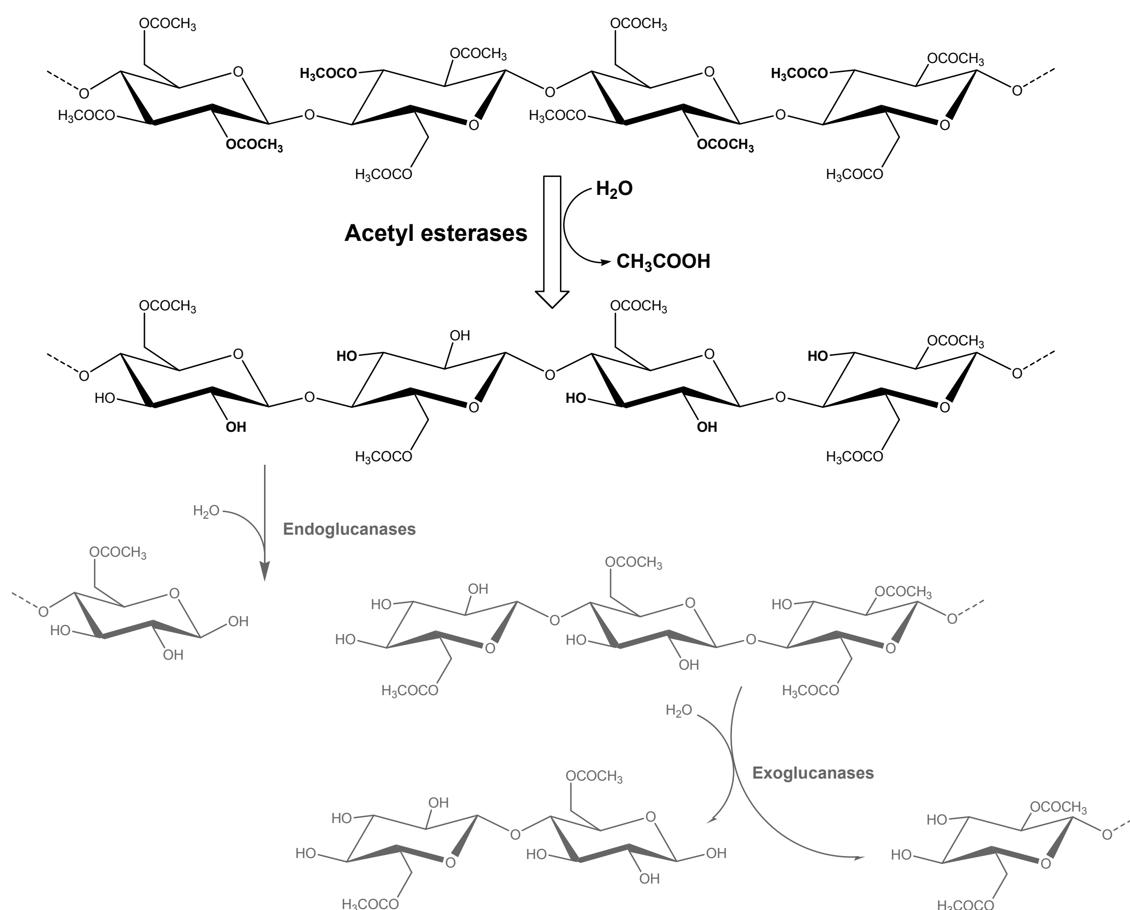


Figure 1.14 – Main reactions occurring during biodegradation of cellulose acetate.

1.3.5 CELLULOSE ACETATE AS AN ENZYMATIC SUBSTRATE

Work on the modification of cellulose acetate with enzymes has been done in the context of its biodegradation (Puls *et al.*, 2004). The figure 1.14 summarizes the main reactions expected to occur during the biodegradation of cellulose acetate with special emphasis to the deacetylation reaction. The degradation of cellulose and hemicellulose is naturally carried out by microorganisms and requires the concerted action of many enzymes for their complete destruction. Among those carbohydrate-active enzymes, there is the group of carbohydrate esterases which hydrolyse the ester linkage of polysaccharides substitutents, allowing for the exo- and endoglycoside hydrolases to break the polymer chains.

Cellulose acetate was found to be a carbon source for several microorganisms and a substrate of several acetyl esterases in cell-free systems (Gardner *et al.*, 1994; Samios, 1997; Sakai *et al.*, 1996; Altaner *et al.*, 2003a, 2003b). It was

found a negative correlation between the degree of substitution and the biodegradability of cellulose acetate (Samios, 1997). The deacetylation efficiency of carbohydrate esterases is decreased when the degree of substitution of cellulose acetate increases and consequently its hydrophobicity and crystallinity.

1.4 NITRILASE (E.C. 3.5.5.1)

1.4.1 GENERAL DESCRIPTION, STRUCTURE AND FUNCTION

The formation and cleavage of carbon-nitrogen bonds are essential in biology. Peptide bonds are the most obvious class of carbon-nitrogen bonds, but there are others whose metabolism is less well understood (Brenner, 2002). Such less obvious bonds include nitriles (R-C≡N), amides [R-C(=O)-NH_2], secondary amides [R-C(=O)-NH-R'], N-carbamyl amides [R-NH-C(=O)-NH_2]. They are hydrolytic substrates for 9 of 13 branches of the nitrilase superfamily (Brenner, 2002; Pace and Brenner 2001). Nitrile-degrading activity is found in three of the 21 plant families and in a limited number of fungal genera, while it is relatively frequent in bacteria (Banerjee *et al.*, 2002).

Nitrilases belong to the first branch of the Nitrilase superfamily, which is

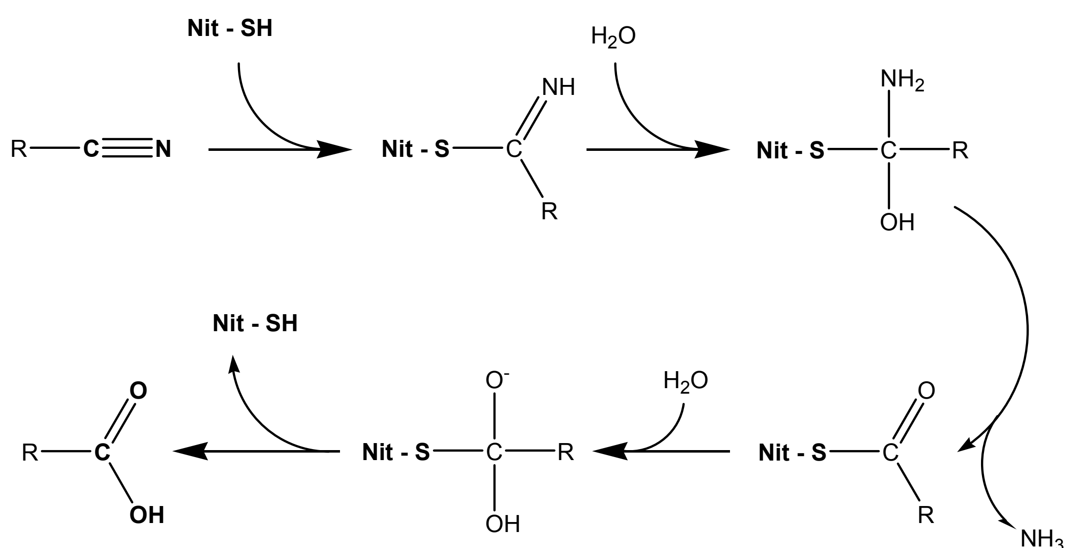


Figure 1.15 – Mechanism of the nitrile hydrolysis catalysed by nitrilase (adapted from Banerjee *et al.*, 2002).

the only one displaying true nitrilase activity (Brenner, 2002; Pace and Brenner, 2001). Nitrilases are thiol enzymes that perform the hydrolysis of the nitrile group to the corresponding acid and ammonia. The reaction starts with the nucleophilic attack on the nitrile carbon atom by the thiol group of a catalytic cysteine residue (figure 1.15). Subsequent steps involve the attack by two water molecules, protonation of the nitrogen atom, which is released as ammonia, and regeneration of the enzyme, through tetrahedral intermediates (Banerjee *et al.*, 2002).

The nitrilase was first isolated from barley leaves, in 1964, where it catalyses the conversion of indole-3-acetonitrile to the auxin indole-3-acetic acid (Mahadevan and Thimann, 1964). In the same year, a nitrilase was also isolated from a soil bacterium by selection for growth on a natural occurring nitrile – ricinine (Robinson and Hook, 1964; Hook and Robinson, 1964). Since then, several nitrilases were isolated and characterized but they represent a rather small fraction compared to the 137 nitrilases that the Diversa Corporation claimed to discover recently (Robertson *et al.*, 2004). From the available information it is clear that nitrilases are a very heterogeneous group regarding their biochemical characteristics: substrate specificity, optima pH/temperature, and quaternary structure (O'Reilly and Turner, 2003; Harper, 1977a, 1977b, 1985; Stalker and McBride, 1987; Kobayashi *et al.*, 1990; Nagasawa *et al.*, 2000; Khandelwal *et al.,* 2007).

Most nitrilases show highest activities for aromatic nitriles and, in general, they prefer the *meta* and *para* substitutions with poor or no activity with *ortho* substituted substrates (Harper, 1977a, 1977b, 1985; Stalker and McBride, 1987). Some nitrilases have a preference for arylacetonitriles and others for aliphatic nitriles (Kobayashi *et al.*, 1990). The relative low optimum temperature of nitrilases (30 ºC - 45 ºC) reflect the growth optimum temperature of the source organisms and the intracellular nature of nitrilases, nevertheless few nitrilases have been isolated from thermophilic microorganisms (Cowan *et al.*, 1998; Khandelwal *et al.*, 2007). In this last case, the optimum temperature can vary from 50 ºC to 65 ºC. Regarding the pH, nitrilases have highest activities in the neutral to alkaline range (Cowan *et al.*, 1998; O'Reilly and Turner, 2003).

So far, the described nitrilases consist of a single folded polypeptide, which, in general, aggregates to form the active enzyme. The oligomeric form, in some cases, is subjected to substrate activation or is dependent on the pH,

temperature and enzyme concentration (Harper, 1977a; Nagasawa *et al.*, 2000; O'Reilly and Turner, 2003). The number of polypeptides in the aggregates can range from 6 to as much as 26 subunits with the addition of subunits taking place in pairs (Sewell *et al.*, 2003).

The crystal structures of three members of the nitrilase superfamily are available (Kumaran *et al.*, 2003; Nakai *et al.*, 2000; Pace *et al.*, 2000). Their domains have similar topology and dimerization modes (Kumaran *et al.*, 2003). Each subunit is a compact αββα sandwich and it forms dimers with an eight layer αββα:αββα structure (figure 1.16A). The subunit is well defined, globular and, in all the three structures, the dimer interface is through interactions between α layers. These dimers combine further to form tetramers in two of the three studied proteins through contacts between the exposed edges of the β-layers (Nakai *et al.*, 2000; Pace *et al.*, 2000). From the structures, mutagenesis and gene sequence comparison, it is assumed that the entire superfamily uses a catalytic triad consisting of conserved glutamic acid, lysine and cysteine residues (figure 1.16B) (Brenner, 2002; Robertson *et al.*, 2004; Kumaran *et al.*, 2003; Nakai *et al.*, 2000; Pace *et al.*, 2000).

The enzymes of the nitrilase superfamily play diverse and important roles in biology. The known functions include synthesis of signalling molecules, vitamins and coenzymes metabolism, small molecules detoxification and protein post-translation modifications (Brenner, 2002).

1.4.2 APPLICATIONS

Nitrilases have attracted the attention and interest in the past two decades and they are been recognised as important "green" catalysts (Kaul *et al.*, 2007; Singh *et al.*, 2006). The conversion of nitriles into carboxylic acids is a key organic reaction in the synthesis of many intermediate compounds and final products. The chemical hydrolysis of nitriles requires drastic conditions such as strong bases or acids and/or elevated temperatures, besides, it originates undesirable by-products and inorganic wastes (Zhu *et al.*, 2007). The advantages presented by these enzymes, besides the eco-friendly biotransformation is their inherent enantio- and, in particular, regio-selectivity.

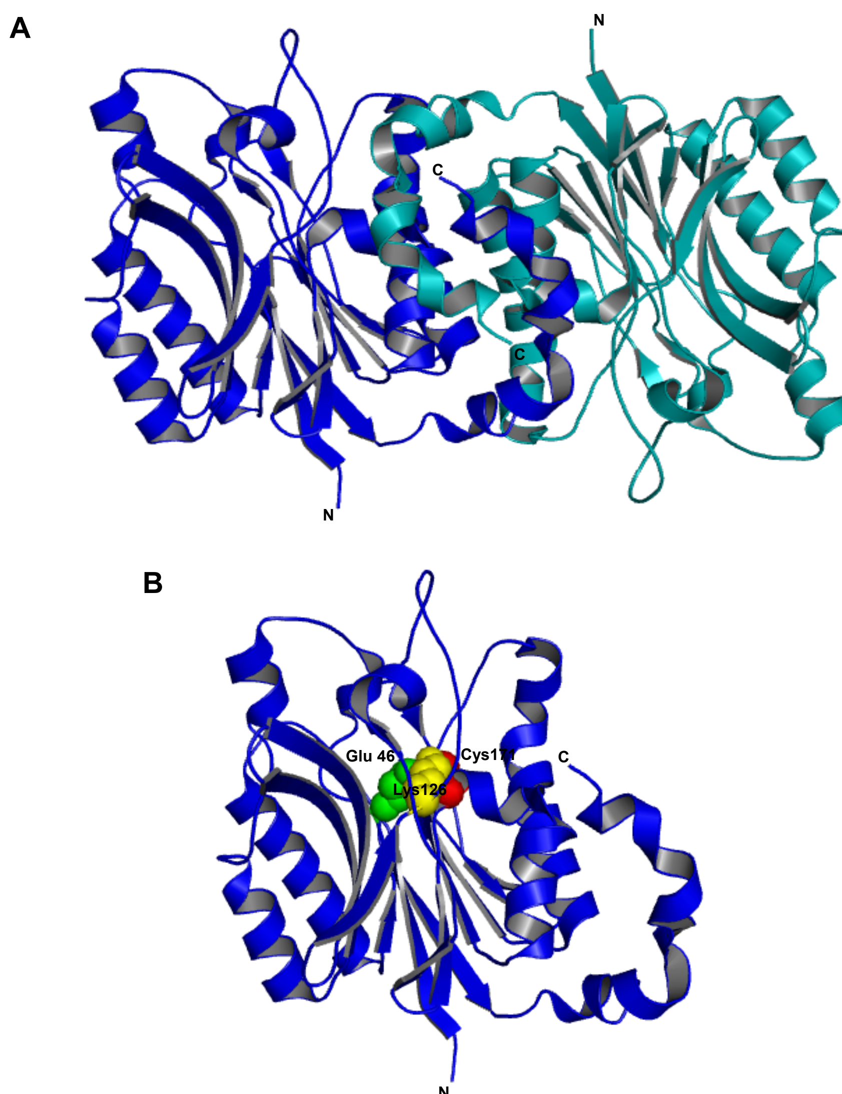


Figure 1.16 – Schematic ribbon diagrams depicting the overall structure of N-carbamyl-D-amino acid amidohydrolase from *Agrobacterium* sp. (Protein Data Bank ID: 1erz). **A)** View of the dimer where each polypeptide has a different colour and a labelled N- and C-terminal. **B)** View of one subunit where the catalytic triad, composed by a cysteine, lysine and glutamic acid, is shown as spheres.

There are several examples of industrial conversions of pyridine-nitriles, pyrazine-nitriles and regio-selective transformation of dinitriles using nitrilases (Kaul *et al.*, 2007). The nitrilase from *Rhodococcus rhodochrous J1* was applied in the production of *p*-aminobenzoic acid, the vitamin nicotinic acid and the antimycobacterial agent pyrazinoic acid, with a substrate conversion of 100% (Kobayashi and Shimizu, 1994). Acrylic and methacrylic acid are also produced by the same nitrilase using acrylonitrile as substrate. The acrylic homo- and copolymers are important as print-thickeners, dispersants in pigment

suspensions, detergent builders, and disposable diapers, among others (Hughes *et al.*, 1998).

One of the most important industrial applications of nitrilase (from *Alcaligenes* strains) is the transformation of racemic mandelonitrile to (R)-(-)-mandelic acid. This compound is a key intermediate in the production of semi-synthetic cephalosporins, penicillins, and it is also important in the synthetic process of antitumor and antiobesity agents (Kaul *et al.*, 2007).

Transgenic crop plants expressing the nitrilase gene from *Klebsiella ozaenae* were obtained in order to survive to the herbicide bromoxynil which is a photosynthesis inhibitor (Kobayashi and Shimizu, 1994). The nitrilase destroys the herbicide by hydrolysis of its nitrile group.

Another important application of nitrilase or nitrilase-producing organisms is the decontamination of acrylonitrile waste effluents and aqueous polymer emulsions containing this highly toxic and carcinogenic compound (Battistel *et al.*, 1997; Wyatt and Knowles, 1995).

1.5 CUTINASE (E.C. 3.1.1.74)

1.5.1 GENERAL DESCRIPTION, STRUCTURE AND FUNCTION

Cutinases are carboxylic ester hydrolases that degrade cutin which is the structural component of the outer envelope (the cuticle) of higher plants (Purdy and Kolattukudy, 1975a; Kolattukudy, 2002). Cutin is a natural polyester composed mainly of C_{16} and C_{18} hydroxyl and epoxy fatty acids as monomers. The cuticle constitutes an efficient barrier against desiccation and entry of pathogens in plants (Kolattukudy, 2002). The enzymatic degradation of the cuticle, as a consequence of the secretion of cutinases by fungi, was proved to be one of the first steps in the infectious process of plants (Carvalho *et al.*, 1998).

Cutinases have been purified from different sources, especially fungi but also from pollen and bacteria (Carvalho *et al.*, 1998). The first and most studied cutinase is from the fungal pathogen of peas, *Fusarium solani pisi* (Purdy and Kolattukudy, 1975a, 1975b). This is a small ellipsoid protein (≈22 KDa, 45x30x30 Å) that bridges functional properties of lipases and esterases, because it hydrolyses both soluble esters and emulsified triacylglycerols (Mannesse *et al.*,

1995; Egmond and de Vlieg, 2000). Studies with triglyceride analogues revealed that cutinase activity is very sensitive to the length and distribution of the acyl chains, with highest activities observed for short chain lengths of three to five carbons (Mannesse *et al.*, 1995; Egmond and de Vlieg, 2000). The optimum pH for the hydrolysis is around 8.5, depending on the particular substrate used, and the maximum thermal stability is obtained for the pH range 6-9 (Petersen *et al.*, 1998, 2001b).

Cutinase is a serine esterase that shares the basic catalytic features with serine proteases (Köller and Kolattukudy, 1982). The essential feature is the catalytic triad involving the hydroxyl group of a serine, the imidazole side chain of a histidine and a carboxylic side chain of an acidic residue which, in cutinase, is an aspartic residue (Martinez *et al.*, 1994). The function of the serine is to participate in a transacylation reaction with the substrate to form an acyl-enzyme intermediate (figure 1.17). The intermediate is hydrolysed to release the first product. The serine acyl ester reacts then with a water molecule to

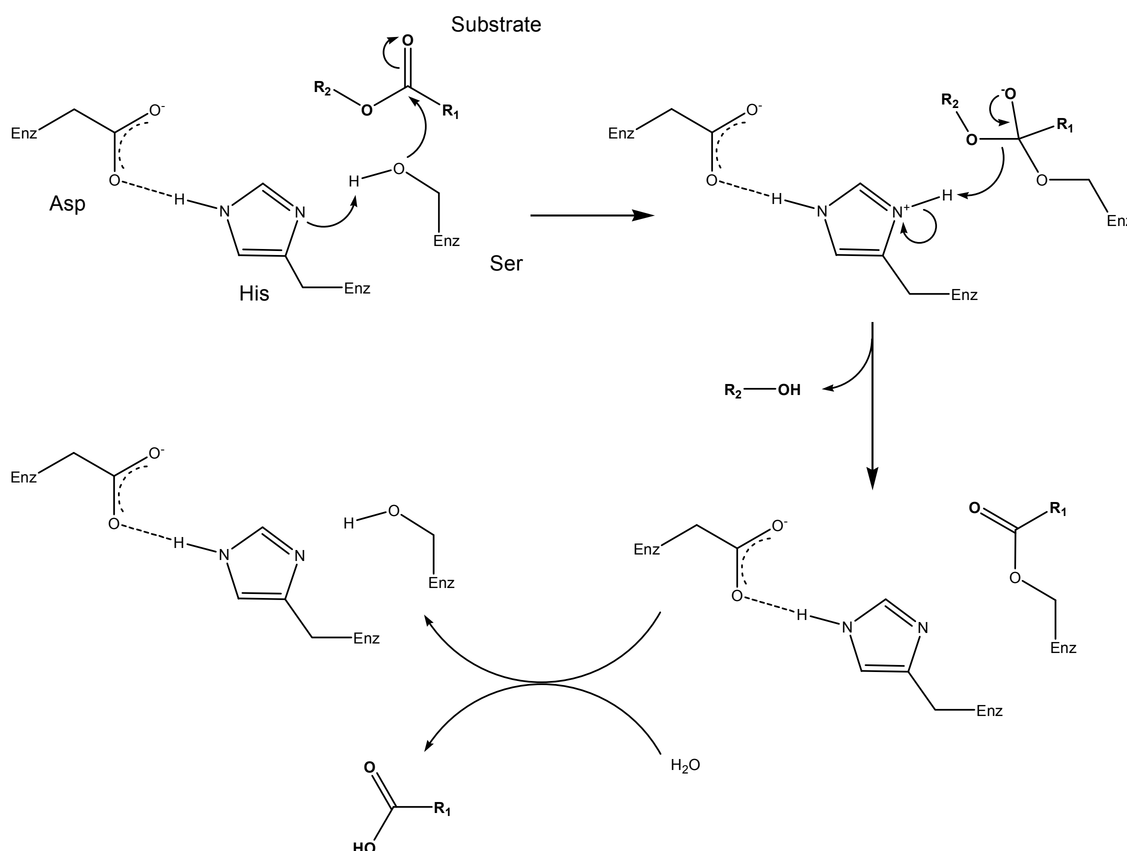


Figure 1.17 – Mechanism of the carboxylic ester bond hydrolysis catalysed by cutinase (adapted from Lau and Bruce, 1999).

release the fatty acid and to regenerate the catalytic hydroxyl group (Lau and Bruce, 1999; Petersen *et al.*, 2001a). The role of the imidazole ring is to act as a base to remove the proton from the serine hydroxyl group in concert with the nucleophilic attack of the serine oxygen on the carbonyl carbon of the substrate. The aspartic residue seems to orient the imidazole ring properly and to stabilize the local structure around the active site (Lau and Bruce, 1999).

The *F. solani pisi* cutinase belongs to the superfamily of α/β-hydrolases, to which lipases also belong. Cutinases have a central slightly twisted β-sheet consisting of five parallel strands covered by two α-helices on either side of the sheet (figure 1.18A) (Longhi and Cambillau, 1999; Egmond and de Vlieg, 2000). The nucleophilic serine is located in an extremely sharp turn between a β-chain and an α-helice, named nucleophilic elbow. The amino acid sequence around the catalytic serine (Gly-Tyr-Ser-Gln-Gly) matches the consensus sequence commonly present in lipases (Gly-His/Tyr-Ser-X-Gly). The catalytic triad is located at one edge of the ellipsoid protein (figure 1.18B), it is rather accessible to the solvent, in contrast with lipases, and it is surrounded by two loops with

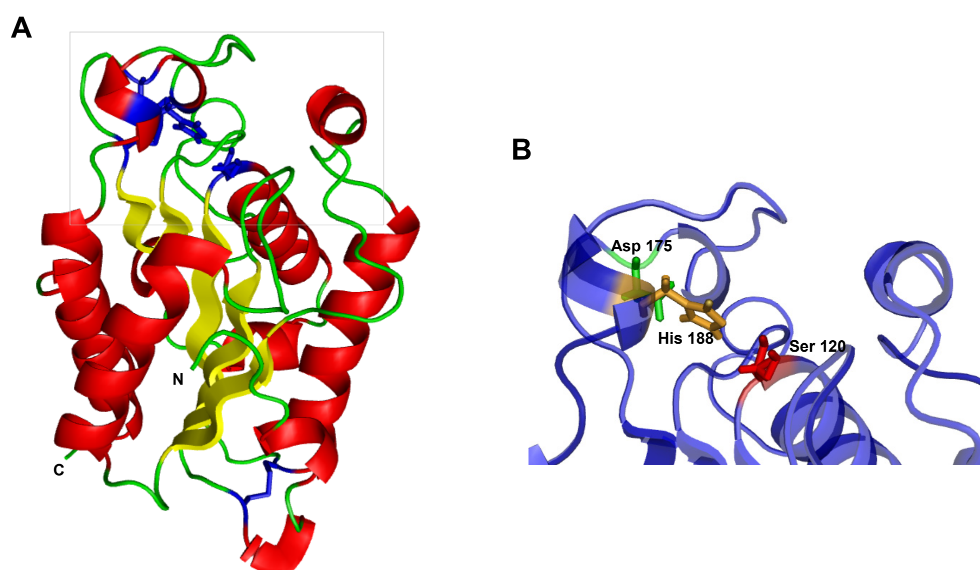


Figure 1.18 – **A)** Schematic ribbon diagrams depicting the overall structure of cutinase from *Fusarium solani pisi* (Protein Data Bank ID: 1cex); the secondary structural elements are represented in different colours (β-strands are in yellow, α-helixes are in red and connecting loops are in green; the N- and C-terminal are labelled; the two disulfide bridges and catalytic residues are depicted in blue by the stick model. **B)** Closed view of the catalytic triad composed by a serine, histidine and aspartic acid, shown by the stick model; the two possible locations of the Ser120 side chain are both represented.

high mobility (Prompers *et al.*, 1999; Longhi *et al.*, 1997). These loops delimit the catalytic site and the substrate binding cleft which is hydrophobic, reflecting the lipolytic nature of cutinases (Egmond and de Vlieg, 2000).

The *F. solani pisi* cutinase is also classified as a member of the family 5 of carbohydrate esterases (www.cazy.org/fam/CE5.html), sharing a very similar 3D-structure with other two members with known structure: the acetyl xylan esterase (E.C. 3.1.1.72) from *Trichoderma reesei* and the acetyl xylan esterase II (AXE II) from *Penicillium purpurogenum* (Hakulinen *et al.*, 2000; Ghosh *et al.*, 2001). Although they present very similar overall structures, the conformation of the active site is different. These esterases have more exposed catalytic residues than cutinase, reflecting their specificity towards nonlipidic polar substrates (Ghosh *et al.*, 2001). There is also an additional loop, delimiting the active site, in the AXE II from *P. purpurogenum*, which is responsible for the preference for short substrates. When this loop is deleted, AXE II is also able to hydrolyse long chain fatty acidic esters, up to at least 14 carbons, resembling cutinase specificity (Colombres *et al.*, 2008).

1.5.2 APPLICATIONS

Cutinase is a very versatile enzyme: it can utilise a broad range of substrates, it is rather stable and at low water activities cutinase catalyses the reverse reaction – trans-esterification of fats and selective esterification of alcohols (Carvalho *et al.*, 1998). Both, hydrolytic and synthesis reactions have potential use in food processing, beverages, perfume industries, pharmaceutical industries, agriculture, chemical industries and others like pulp/paper, textile and leather industries (Panda and Gowrishankar, 2005). Some of its potentialities are already applied in industry, but many others are still at a research level. Some cutinase preparations have been produced by Genencor and Unilever for detergent and surfactant formulations (Carvalho *et al.*, 1998). A lot of research is devoted to the study and optimization of cutinase activity in non conventional media (organic media, supercritical fluids and gas/solid systems) and in the stabilization of the enzyme through immobilization, micro-encapsulation and lyophilized preparations (Carvalho *et al.*, 1999a).

Another important application of cutinase is the degradation of plastics. The synthetic polyester, polycaprolactone, was hydrolysed to soluble products that were used by *F. solani* wild type strains as source of carbon and energy (Murphy *et al.*, 1996).

1.6 Tailoring the properties of biocatalysts

1.6.1 Protein and media engineering

The Nature's biodiversity provides us with a large collection of enzymes well suited for supporting life, however, they may not always be well suited for our technological interests (Arnold, 2001). Therefore, the impact of enzymes is dependent on our ability to tailor their properties according to the demands of a particular technological process (Arnold, 2001). Some important issues that often need to be addressed are limited substrate range, limited stability to temperature, pH and solvent, limited enantioselectivity and limited turnover number (Powell *et al.*, 2001). Although screening the biodiversity continues to be an important approach to find better biocatalysts, the manipulation of the protein molecule itself and/or the reaction media are gaining increasing importance.

Reaction media engineering is important to optimize and modulate biocatalysts activity and stability. Biocatalysis in non-aqueous media offers unique advantages compared to aqueous enzymology (Castro and Knubovets, 2003). There are drastic changes in the enantioselectivity of catalysed reactions, reversal of the thermodynamic equilibrium of certain reactions, suppression of water-dependent side-reactions, improved solubility of hydrophobic substrates or products and resistance to bacterial contamination (Klibanov, 2001; Castro and Knubovets, 2003). Homogeneous biocatalysis in water-organic mixtures is also used and major changes in enzyme catalytic activity and initial rates have been reported, depending on the type and amount of the cosolvent (Castro and Knubovets, 2003). In several cases, at low concentration of the organic cosolvent (between 5% and 25%) the initial rate and stability of the enzyme are maximized regarding aqueous buffers (Castro and Knubovets, 2003).

The stability of enzymes *in vitro* is a very critical issue in biotechnology and both protein and media engineering strategies are used to increase it.

However, it is not always possible to resort to recombinant DNA technologies to obtain more stable biocatalysts. Besides, the chemical modifications that entrap enzymes into larger aggregates or immobilize them on supports for better half-life times are not always adequate. They create diffusion and accessibility barriers which, in some situations like a solid substrate, may be problematic. Stabilizing additives are low-molecular weight compounds that interact with the enzyme molecules in a way that the unfolding is thermodynamically disfavoured (Ó'Fágáin, 2003). Sugars, polyalcohols and surfactants are examples of common compounds used to improve the stability of enzymes (Melo *et al.*, 2001, 2003; Matsumoto *et al.*, 1997).

A revolution in the biocatalysts design at the molecular level was provided by the establishment of the recombinant DNA technology (Arnold, 2001). It allowed the manipulation of DNA sequences in a highly specific manner and the expression of their protein products in a variety of organisms, from animal cells to bacteria (Arnold, 2001). If *a priori* there is the knowledge of the protein structure, function and catalytic mechanism, it is possible to rational redesign enzymes through molecular site-specific changes. These changes can be a single point mutation, several point mutations or they can consist in the exchange/addition of a whole structural domain. It is also common to fuse two enzymes or an enzyme with a non-catalytic domain (Nixon *et al.*, 1998). A good example of the benefits of such rational modelling of enzymes is provided by the work of Araújo *et al.* (2007). Computational modelling and site-directed mutagenesis were used to enlarge the active site of cutinase from *Fusarium solani pisi* to better fit larger substrates like a synthetic polymer chain. Several mutations were performed and one of these, cutinase L182A, showed an activity increase of five-fold when compared to the native enzyme, using PET as substrate (Araújo *et al.*, 2007). Another procedure is the random redesign of enzymes through directed evolution which consists in repeated cycles of random mutagenesis and/or gene recombination, followed by screening and selection of the functionally improved mutants (Rubin-Pitel and Zhao, 2006).

Besides recombinant DNA technology, biocatalysts can also be altered by physical and chemical modification procedures, involving the side chain reactive groups of the amino acid residues, allowing, for example, different types of enzyme immobilization (Ó'Fágáin, 2003; Illanes, 1999; Sheldon *et al.*, 2005). Silva

(2005) modified a commercial serine protease by covalent coupling to a soluble-insoluble polymer of high molecular weight. The enzyme conjugated to such a carrier, in its soluble form, may be used as a catalyst to modify the scales of wool fibres surface, with the purpose of providing resistance to felting and shrinkage without significant loss in fibre resistance. Then, it can be recovered via the insoluble state, presenting good operational and storage stabilities which are quite interesting from an industrial point of view (Cavaco-Paulo and dos Santos Martinho da Silva, 2003).

1.6.2 Fusion proteins with charbohydrate - binding modules and applications

In 1950, it was proposed that the initial stage of enzymatic degradation of cellulose involved the action of two components: C_1 was the non-hydrolytic component and C_x was the catalytic component (Din *et al.*, 1994). The C_1 was thought to be responsible for the nonhydrolytic disruption of cellulose, making the substrate more accessible to the C_x (Shoseyov *et al.*, 2006). Decades later, the C_1 component, named cellulose-binding domain, was isolated from the fungus *Trichoderma reesei* and the bacterium *Cellulomonas fimi* by proteolytic cleavage of the linker that connected this domain to the catalytic domain of cellulose-hydrolytic enzymes (figure 1.19) (van Tilbeurgh *et al.*, 1986; Langsford *et al.*, 1987; Tomme *et al.*, 1988; Gilkes *et al.*, 1988). The name cellulose-binding domain evolved to carbohydrate-binding module (CBM) to reflect the diverse ligand specificity of these non-catalytic polysaccharide-recognizing domains.

More than 500 putative sequences from bacteria, fungi, plants and

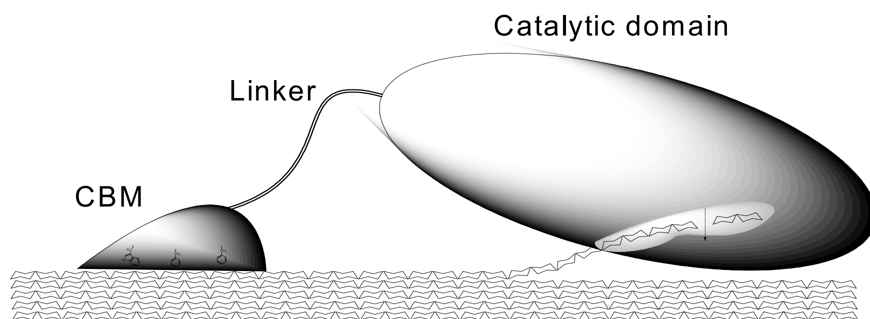


Figure 1.19 – Schematic representation of a cellulase based on the cellobiohydrolase I from *Trichoderma reesei* (adapted from Hildén and Johansson. 2004).

animals have been identified and the CBMs have been classified into 52 different families based on the amino acid sequence, binding specificity and structure (www.cazy.org/fam/acc_CBM.html#1). The CBMs are a contiguous amino acid sequence from 30 to ≈200 residues, with a discrete fold and exist as a single, double or triple domain in one protein. They can be localised, within the protein, at the N- or C-terminal and seldom in the middle (Shoseyov *et al.*, 2006).

In general, CBMs can have three functions in the parental protein (Shoseyov *et al.*, 2006; Boraston *et al.*, 2004). The proximity effect increases the concentration of the enzyme on the surface of the substrate. Several authors reported a reduction in the catalytic activity when the CBM is removed, but only for solid polysaccharides. The targeting effect, meaning substrate binding and selectivity, is also attributed to the CBM. The interaction of aromatic amino acid side chains with the ligand is ubiquitous to CBM carbohydrate recognition; the key determinant of the binding specificity is the binding-site topography. The CBMs can be grouped in two three general classes according to structural and functional similarities. The type A CBMs bind to surfaces of crystalline polysaccharides and they have flat or platform-like binding sites. The type B CBMs interact with single chains, thus with the amorphous phases of the polysaccharides, and they have binding sites in the shape of a groove or cleft of varying depth. The type C CBMs bind to small sugars having one to three units. Finally, the last but the most controversial role attributed to CBMs is the non-hydrolytic disruptive effect of the polysaccharide structure. This effect was described only for a few CBMs. The first evidence was found for the CBM from the endoglucanase A (CenA) from *C. fimi,* which alone or attached to the linker region led to a rough surface on cellulose fibres and to the release of small particles (Din *et al.*, 1991).

The application of CBMs in several areas of biotechnology has been growing in the recent years due to the fact that CBMs are independently folded domains and *a priori* they can function perfectly when fused to other proteins. Moreover, their ligands are abundant and renewable materials with good properties and normally low-priced (Shoseyov and Warren, 1997). One of the most important areas is bioprocessing, because large-scale purification and recovery of biological molecules continues to be a challenge. CBMs can be expressed as an affinity tag for protein immobilization, processing and purification using

cellulose as a matrix in many formats, from affinity chromatography to two phase liquid separations (McCormick and Berg, 1997). Targeting of compounds to polysaccharides that are present in many daily products, especially cellulose, is also an important application of CBMs. This area includes oral care products, denim stonewashing, the targeting of enzymes that do not possess natural affinity to cotton, in laundry detergents, and other chemicals, like fragrances (von der Osten *et al.*, 2000a,b; Cavaco-Paulo, 1995; Kalum and Andersen, 2000; Berry *et al.*, 2001; Fuglsang and Tsuchiya, 2001). Other applications include whole cell immobilization, enzyme immobilization and modification of polysaccharide fibres by non-hydrolytic disruptive activity (Shoseyov *et al.*, 2006; Pinto *et al.*, 2004). The application of CBMs is faraway from being exhausted and will be further expanded with the increasing knowledge on these binding domains.

1.6.3 Carbohydrate-binding module of cellobiohydrolase I from *Trichoderma reesei*

In the 1980s, major steps were taken in the understanding of how cellulolytic enzymes work. Many studies were conducted with the fungus *T. reesei* and it was evident that the four cellulases, known at that time, shared a common structural organization. They consisted in a catalytic domain (≈400-500 residues), a highly conserved terminal domain (≈40 residues), either at the C- or N-terminal and a heavily O-glycosylated linker region (≈30 residues) connecting the two domains (Fägerstam *et al.*, 1984; Bhikahbhai and Pettersson, 1984; van Tilbeurgh *et al.*, 1986). The limited proteolysis of the cellobiohydrolase I from *T. reesei* (CBH I) led to the first clue on the function of the conserved C-terminal domain (van Tilbeurgh *et al.*, 1986). The core protein was fully active against small, soluble substrates, while the whole cellulase, with the C-terminal peptide was active against Avicel (insoluble microcrystalline cellulose). The implication of the C-terminal peptide of CBH I in the adsorption process and enhancement of cellulase activity towards solid substrates was confirmed later on and it was demonstrated that the C-terminal domain and the linker interacted directly with insoluble cellulose independently of the presence of the core domain (Tomme *et al.*, 1988; Stahlberg *et al.*, 1988). This cellulose-binding activity was eliminated by

reduction of disulfide bridges in the C-terminal peptide, pointing to the existence of a defined 3D-structure (Johansson *et al.*, 1989).

The structure of the CBM from CBH I was first determined in 1989 by NMR (Kraulis *et al.*, 1989). Subsequent directed mutagenesis of the CBM and sequence alignment studies determined the most important residues for the adsorption and cast some light on the mechanism of interaction with cellulose (Reinikainen *et al.*, 1992, 1995; Linder *et al.*, 1995a, 1995b; Mattinen *et al.*, 1997a, 1997b; Hoffrén *et al.*, 1995). The CBM from CBH I belongs to the CBM family 1 and folds into a wedge-shape structure with overall dimensions of 30x18x10 Å (figure 1.20). One face of the wedge is flat, hydrophilic and it is the one that interacts with crystalline cellulose. The main secondary structure is a small anti-parallel β-sheet composed by three strands which is stabilized by hydrogen bonds and two disulfide bridges. The residues that compose the flat binding surface are well

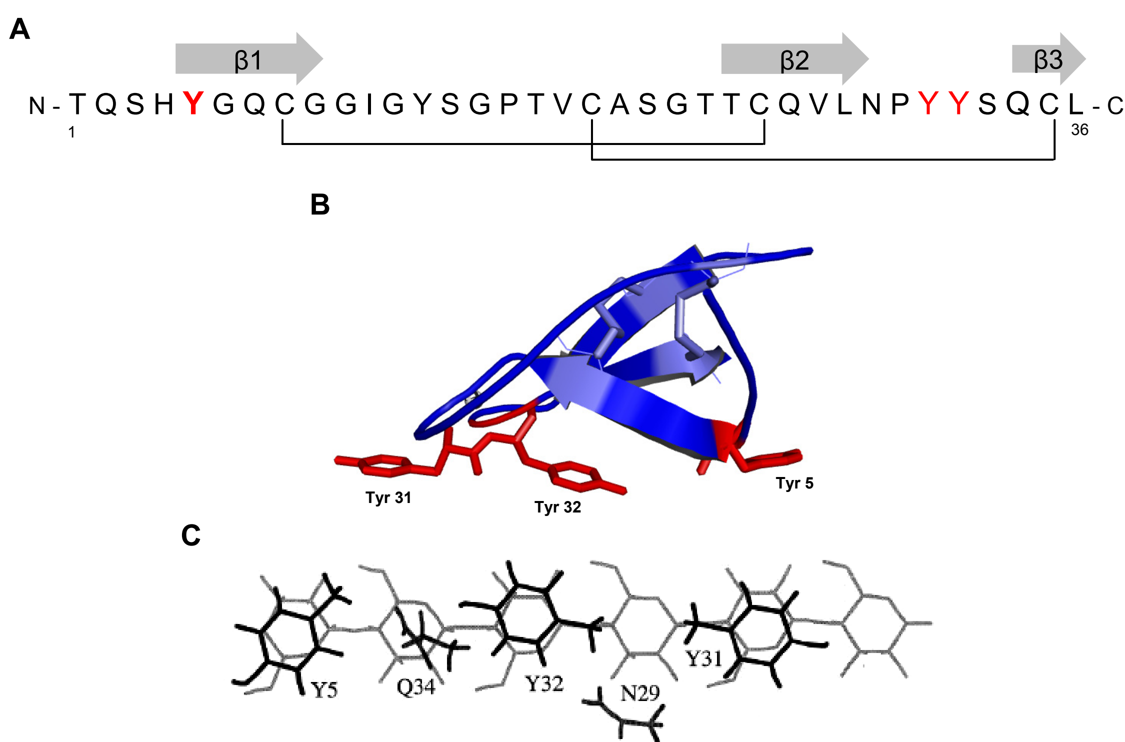


Figure 1.20 – A) Primary sequence and secondary structures of the CBM of CBH I from *Trichoderma reesei*; the two disulfide bridges are represented by the sticks connecting the cysteine residues; the tyrosines involved in the interaction with the ligand are in red. **B)** Ribbon schematic representation of the overall 3D-structure (Protein Data Bank ID: 2cbh); the two disulfide bridges and ligand-binding tyrosines are depicted by the stick model. **C)** Binding of the flat surface of CBM to an oligosaccharide of 6 glucose units (from Mattinen *et al.*, 1997b).

conserved among fungal CBMs. In the CBM from CBH I, the three tyrosines Tyr5, Tyr31 and Tyr32 are involved in the interaction with the glucose rings of cellulose, mainly through van der Waals forces and their substitution leads to a significant decrease in the binding affinity.

The construction of a bifunctional CBM, using the CBM from CBH II and CBH I spaced by a small linker, showed that the connection affects the binding of the individual CBMs (Linder *et al.*, 1996). The double CBM exhibits higher affinity to the solid substrate than the isolated CBMs. Another work demonstrated the reversibility with a high exchange rate of the CBM from CBH I which is crucial for the mobility of CBH I on the cellulose surface and its continuous activity (Linder and Teeri, 1996).

1.6.4 Carbohydrate-binding module N1 of endoglucanase C from *Cellulomonas fimi*

The gene and its product, the endoglucanase C (CenC), from the bacterium *C. fimi*, were first identified in 1989, after other cellulases from the same organism had already been characterized (Moser *et al.*, 1989). The complete sequence of *cenC* gene and the characterization of the cellulase CenC were accomplished later, revealing an unusual organization that did not resemble the other cellulases from *C. fimi* found previously (Coutinho *et al.*, 1991). The identification of the cellulose-binding domain was possible by the dissection of the gene and independent expression of the different sequences (Coutinho *et al.*, 1992). It was found that CenC has two CBMs (N1 and N2) in tandem at the N-terminal, with distinct structures and affinities (Coutinho *et al.*, 1993). The CBM_{N1} binds preferentially amorphous cellulose and can also bind Avicel weakly but not crystalline cellulose (Tomme *et al.*, 1996a).

The structure of the CBM_{N1} was first determined in 1996 by NMR (Johnson *et al.*, 1996a, 1996b). Subsequent directed mutagenesis and structure determination of the CBM_{N1} in complex with an oligosaccharide by NMR and X-ray crystallography have elucidated the interaction of this CBM with a single chain of cellulose and revealed the most important residues (Kormos *et al.*, 2000; Johnson *et al.*, 1999; Boraston *et al.*, 2002). The CBM_{N1} belongs to the CBM family 4 and it folds into jelly-roll β-sandwich (figure 1.21). There are no α-helices, the

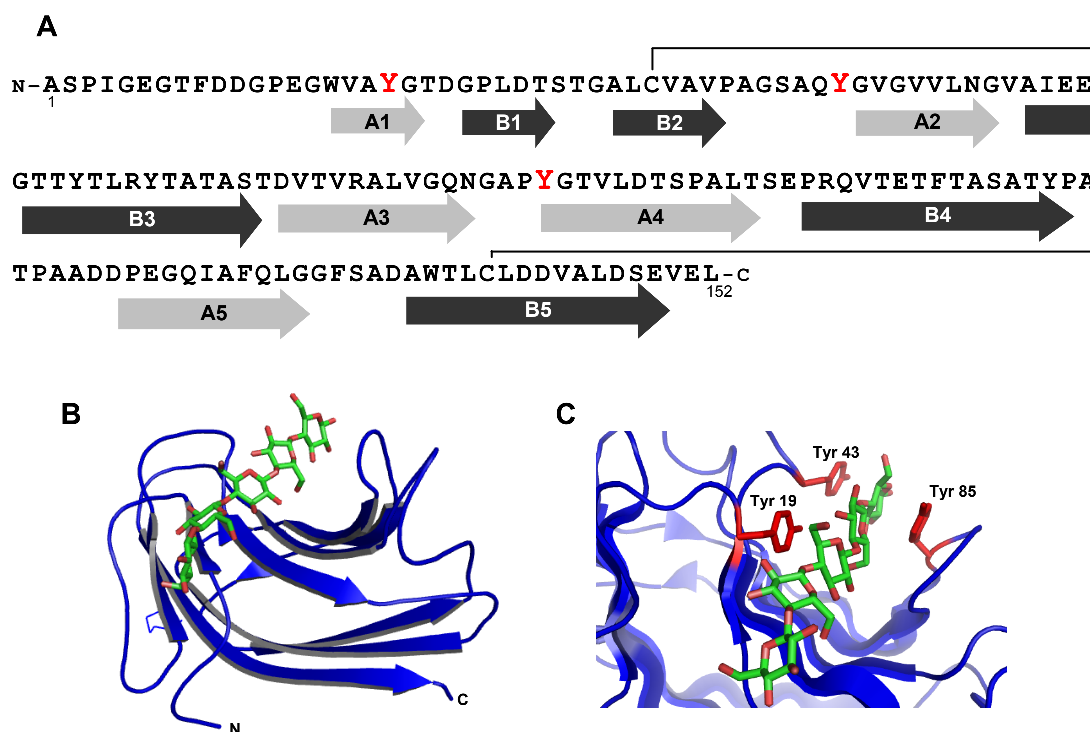


Figure 1.21 – A) Primary sequence and secondary structures of the CBM_{N1} of CenC from *Cellulomonas fimi*; the disulfide bridge is represented by the line connecting the cysteine residues; the tyrosines involved in the interaction with the ligand are in red. **B)** Ribbon schematic representation of the overall 3D-structure showing a ligand with 5 glucose units (Protein Data Bank ID: 1gu3); the N- and C-terminal are labelled. **C)** Close up view of the binding-site where the tyrosines involved are represented by the stick model.

sandwich comprises two anti-parallel β-sheet, A and B, of five strands each and there is one disulfide bridge connecting two neighbour strands in the β-sheet B. The binding site of CBM_{N1} is a cleft that runs across the β-sheet A and consists of a central strip of hydrophobic side chains, flanked on both sides by polar and aromatic residues. The side chains of the residues Arg75, Asn50, Gln124 and Gln128 form hydrogen bonds with the hydroxyl groups of the cellopentose and the three tyrosines Tyr19, Tyr43 and Tyr85 are involved in the interaction with the glucose rings of cellulose, mainly through van der Waals forces. The substitution of these residues by alanine leads to a significant decrease in the binding affinity, specially the aromatic residues.

1.7 AIMS OF THE WORK

In the industrial processing of natural textile fibres, several enzyme applications have been introduced. The same did not happen regarding the processing of most man-made fibres, specially the synthetic ones. Conventional modifications of such fibres require high amounts of energy and chemicals in order to obtain the desired end-product properties.

The general purpose of the present work is the development of eco-friendly methodologies to produce value added fibres with new surface functionalities, through the application of enzymes for the specific and targeted modification of the surface of acrylic and cellulose acetate fibres. More precise objectives are the characterization of the fibre modifications by physicochemical methods and the improvement of the enzymes performance through the manipulation of the treatment media and recombinant DNA technologies.

2 Using a Nitrilase for the Surface Modification of Acrylic Fibres

The modification of nitrile surface groups into carboxylic groups by the action of a commercial nitrilase, is reported in this chapter. The enzymatic modification of acrylic fibres is a heterogeneous process and, for that reason, requires longer treatments than one-phase catalysis. The reaction media was manipulated with the purpose of improving nitrilase efficiency. The nitrilase catalysis was further studied without additives for a better understanding of the chemical modifications that were occurring at the acrylic fibre surface.

The direct enzymatic modification of nitrile into carboxylic groups constitutes a promising approach to make the acrylic fibre more hydrophilic, enhancing the basic dye uptake of PAN treated fabrics and producing surface reactive chemical sites that may be the target of other finishing processes.

2. USING A NITRILASE FOR THE SURFACE MODIFICATION OF ACRYLIC FIBRES

2.1 MATERIALS AND METHODS

2.1.1 REAGENTS AND ENZYMES

The enzyme used in this work was a commercial nitrilase (EC 3.5.5.1), Cyanovacta Lyase, supplied by Novacta Byosystems Ltd, Hatfield, Herts, UK.

The acrylic taffeta fabric used was produced from a copolymer of PAN and 7% vinyl acetate, with 82 g m^{-2} and 36/36 ends/picks per cm, supplied by Fisipe, Lavradio, Portugal.

The cationic dye Basic Blue 9, C.I. 52015, was from Carlo Erba, Milan, Italy. All other reagents were laboratory grade reagents from Sigma-Aldrich, St. Louis, USA, unless stated otherwise. The buffer used throughout the work was a phosphate buffer having a concentration of 50 mM and pH 7.8. The exceptions are specified when necessary.

2.1.2 NITRILASE ACTIVITY ASSAY

Nitrilase activity was determined by the quantification of the ammonia released into the reaction media, using benzonitrile as the substrate. The reaction was started by the addition of 0.25 mL of a buffered nitrilase solution containing 0.08 mg mL^{-1} of total protein to 0.25 mL of 19.4 mM benzonitrile. The mixture was incubated for 1 to 10 min in a water bath at 30 ºC. The reaction was stopped by adding 0.1 mL of 2 M hydrochloric acid. The ammonia was quantified using an enzymatic kit from Sigma Diagnostics, St. Louis, USA (cat. No 171-A). All the assays were performed in triplicate. One unit of nitrilase initial activity was defined as one µmol of ammonia released per minute.

2.1.3 pH AND TEMPERATURE PROFILE

Nitrilase solutions with 0.08 mg ml^{-1} of total protein were incubated for 10 min at 30 ºC in 300 mM Britton-Robinson buffer of constant ionic strength. The pH range tested was 4.5 to 10.6.

The effect of temperature was investigated within the range 20 ºC - 60 ºC. Preparations of buffered nitrilase solution with 0.08 mg mL^{-1} total protein were incubated for 10 minutes, at the tested temperatures.

The nitrilase activity was measured as described previously.

2.1.4 STABILITY OF NITRILASE

The operational stability of nitrilase was investigated in the presence of two organic solvents and several polyalcohols. The buffered enzyme was incubated at a final total protein concentration of 0.08 mg mL^{-1} in different media compositions: 4%-15% v/v of DMA, 4%-15% DMF or 1 M of a polyalcohol. The polyalcohols studied were glycerol, sorbitol and ethylene glycol (EG). The enzyme was also incubated at the same final concentration with 4% v/v DMA and 1 M sorbitol. For each assay, the final volume was 25 mL, kept in 50 mL polypropylene containers. The control was identically prepared except that buffer substituted the enzyme. The incubation took place in a water bath at 30 ºC, with an orbital agitation, until the drop in nitrilase activity was above 50%. The enzyme activity was assayed at proper time intervals as previously described.

2.1.5 EFFECT OF ADDITIVES IN THE ENZYMATIC TREATMENT OF ACRYLIC FABRIC

All samples of acrylic fabric used were washed prior to use in order to remove possible impurities from manufacture and from human handling. The washing was performed at 60 ºC with an aqueous solution of 1.0 g L^{-1} Lutensol AT25, a non-ionic detergent (BASF, Ludwigshafen, Germany). After removing the detergent, the fabric was washed with distilled water and allowed to air dry.

The treatment of acrylic fabric was carried out in stainless steel pots of 450 mL in capacity, housed in a laboratory scale machine Rotawash MKIII (vertical agitation simulating European washing machines, from SDL International Ltd.), operating at 30 ºC and 20 rpm, for 116 hours. The samples

of acrylic fabric, having an average weight of 9 g, were incubated with 24 U of nitrilase per gram of fabric, in a final volume of 360 mL of buffer. Three different media were tested: no additives, 4% v/v DMA plus 1 M sorbitol and the control without enzyme and without additives.

After the enzymatic treatment, all fabric samples were immediately washed to remove the adsorbed protein. The samples were left under running tap water for several minutes, then they were washed with 2 g L^{-1} sodium carbonate and distilled water, for 60 min each, at 60 ºC.

2.1.6 Enzymatic treatment of acrylic fabric

All samples of acrylic fabric used were previously washed, as already described. The acrylic fabric was treated with 412 U of nitrilase per gram of fabric in buffer, at 40 ºC. The acrylic fabric samples were treated in independent containers, one piece of fabric and bath solution for each time determination, in the Rotawash MKIII at 20 rpm. Samples of acrylic fabric with an average weight of 2 g were incubated in a final volume of 100 mL of treatment solution for 0, 2, 4, 8, 24, and 36 hours.

After enzymatic treatment, all fabric samples were washed twice in 2 g L^{-1} sodium carbonate for 20 min, once in 4 g L^{-1} Lutensol AT25 for 20 min and three times in distilled water for 25 min, in the Rotawash at 70 ºC. Before and in between the changes of the washing solution, the samples were left under running tap water.

2.1.7 Quantification of total protein concentration

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

2.1.8 Determination of ammonia concentration

Detection of ammonia in the reaction media of the first acrylic fabric treatment (section 2.1.5) was performed using the enzymatic kit from Sigma Diagnostics.

For the second nitrilase treatment (section 2.1.6), the Nessler method was adapted and applied to quantify ammonia (Greenberg *et al.*, 1992). The protein was

previously precipitated using trichloroacetic acid and the pH of supernatants was neutralized with 250 mM sodium hydroxide. The standard solutions were prepared using anhydrous ammonium chloride and were submitted to the same protein precipitation procedure and pH adjustment. For the Nessler determination, 0.5 mL of samples/standards were diluted with 4.5 mL of distilled water in glass tubes and 10 µL of Rochelle salt (aqueous solution: 50% w/v potassium sodium tartrate tetrahydrated) were added. After mixing well, 0.2 mL of Nessler reagent was added to each tube and again mixed. The colour was allowed to develop for 10 min and the absorbance was registered at 425 nm. All the assays were performed at least in duplicate.

2.1.9 Determination of polyacrylic acid concentration

Detection of PAA in the reaction media was performed by an absorption-colorimetric method using a kit from Hach Company, USA (cat. No. 22252-00 and No. 22257-00). The standards were prepared from aqueous solutions of PAA (average molecular weight of 2000 g mol^{-1}). All the assays were performed in duplicate.

2.1.10 Determination of fabric weight differences

The fabric weight differences were determined by the difference between the weight of the dry fabric before and after the enzymatic treatment. The fabric pieces were whipstitched with the same acrylic yarn before the treatment. After the washing procedures, the samples were dried at 60 ºC for three hours in a laboratory oven and they were then conditioned in a glass desiccator for 30 min. The drying and desiccation were repeated until differences in the measures were not significant (<5%).

2.1.11 Acrylic fabric colouration with a basic dye

After the enzymatic treatment, samples were competitively coloured in duplicate with 1% o.w.f. (of weight of fabric) Basic Blue 9, C.I.52015. The colouration was performed in a lab-scale dyeing machine (AHIBA Spectradye, from Datacolor International) with a temperature gradient of 4 ºC min. For a more detailed procedure, see table 2.1.

Table 2.1 – Conditions for the colouration of acrylic fabrics with Basic Blue 9, C.I. 52015, according to each enzymatic treatment performed

Enzymatic treatment	Temperature/ ºC	Fabric weight/ g	Colouration bath volume/ mL	Time/ min
1st treatment	80	1	100	75
2nd treatment	70, 75, 80, 90	1.7	100	90

After colouration, all samples were washed twice with 4 g L^{-1} Lutensol AT25, for 20 min each and several times with water at 70 ºC in Rotawash, until no more colour could be detected in the solution. The colour measurements (5 for each sample) were carried out with a reflectance spectrophotometer having a standard illuminant D65 (Spectraflash 600 Plus, from Datacolor International). The colour strength was evaluated as K/S at the maximum absorption wavelength (660 nm). 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 (Kuehni, 1997).

2.2 RESULTS AND DISCUSSION

2.2.1 PH AND TEMPERATURE PROFILE OF NITRILASE ACTIVITY

Enzymes as proteins have properties that are quite dependent on the environment. Among other factors, slight changes on pH and temperature can affect the performance of an enzyme. The effect of the solution pH on the initial activity of nitrilase was studied within the pH range 4.5-10.6. Nitrilase was active in a wide range of pH and it kept more than 75% of its maximum activity between 7.5 and 10.6. The maximum initial activity of nitrilase was observed in the pH range 8-10, being pH 9 the optimum pH value for which the activity was 15 U per mg of total protein. Nitrilases are in general very labile enzymes, especially for high purity levels (Harper, 1977a, 1985; Bandyopadhyay *et al.*, 1986), with

optimum pH values ranging between 7.5 and 8 (Banerjee *et al.*, 2002; O'Reilly and Turner, 2003). The commercial enzyme used throughout this work is supplied as cell preparation and according to the results obtained, this nitrilase has a relative high optimum pH, resembling nitrilases from fungi and from the bacterium *Klebsiella ozaenae* (Harper, 1977b; Stalker *et al.*, 1988). According to the suppliers, the enzyme solution should be maintained in the pH region 7-8, thus the pH 7.8 was chosen to perform all the enzymatic treatments.

The temperature effect on nitrilase initial activity was studied between 20 ºC and 60 ºC. The maximum formation of ammonia occurred between 40 ºC and 45 ºC. The highest value of activity was 33 U mg^{-1}, obtained at 45 ºC. Above this temperature, there was a sharp decrease in the nitrilase activity. In the literature, the optimum temperature for mesophilic nitrilases varies considerably but there is some agreement in considering this group of enzymes very sensitive to this parameter (Cowan *et al.*, 1998). The range of temperatures for which enzymes are stable is often below the optimum value.

2.2.2 STABILITY OF NITRILASE

Operational stability, defined as the persistence of enzyme activity under the conditions of use, was studied before applying the enzyme to acrylic fabric treatment (Ó'Fágáin, 2003). The effect of the addition of two organic solvents and polyalcohols on nitrilase activity was studied by comparing the half-life time, as a measure of stability. The values of half-life time were calculated from first order exponentional decay fitting of data, using OriginPro 7.5 SR0 (OriginLab Corporation, Northampton, USA). The decay of nitrilase activity obeyed to this exponential model of enzyme deactivation.

The addition of both organic solvents, DMA and DMF, to the enzyme solution decreased the initial activity and the half-life time of nitrilase. For many enzymes, deactivation in homogeneous water-organic solvent mixtures may be due to the disruption of the quaternary structure, disruption of the protein hydrophobic core and/or to the water stripping (Castro and Knubovets, 2003). This effect was more pronounced as the organic cosolvent concentration increased. The decrease in the half-life time is almost linear with the increase in the organic solvent concentration (table 2.2). The decrease of nitrilase activity was

faster and more pronounced when DMF was added. Therefore, DMA should be used instead of DMF regarding the enzymatic treatments of acrylic fibre. The investigated organic solvents were chosen because of their action on the fibre structure. They are known solvents of PAN and they are commonly used in industrial production of acrylic fibre (Burkinshaw, 1995; Capone, 1995). Their plasticizer function disturbs the regular structure of the polymer, reducing the magnitude of interchain bonding, which should aid the accessibility to the enzyme, improving its action on the fibre.

Table 2.2 – Influence of organic solvents and polyalcohols on initial activity and half-life time of Nitrilase, at 30 ºC and pH 7.8. An initial activity and a half-life time of 100% correspond to 12.6 U mg^{-1} and 15 hours, respectively. The half-life time was calculated as $t_{1/2} = \ln 2 / k$, from the 1st order exponential decay fit of data ($a_t = a_{t=0} . e^{-kx}$)

Assay conditions		**Initial activity[a] (%)**	**Half-life time[a] (%)**
Control	No additives	100	100
	4 %	87	30
DMF	10 %	78	19
	15 %	59	9
	4 %	84	90
DMA	10 %	89	55
	15 %	87	19
	Glycerol	122	101
Polyalcohols	Sorbitol	122	294
	EG	107	160
4% DMA + 1 M sorbitol		105	249

[a] The standard deviations for the initial activity and half-life time values were ≤ 5% and ≤ 7%, respectively.

The initial activity of nitrilase increased in the presence of all the studied polyalcohols, particularly, sorbitol and glycerol (table 2.2). It cannot be excluded that this effect could be due to a better solubility of the substrate benzonitrile in

the media containing these compounds. The highest half-life time values were obtained with the addition of sorbitol and EG. Sorbitol showed the best performance as a nitrilase stabilizer for long-term treatments. The half-life time increased almost three times (from 15 to 44 hours) in the presence of sorbitol. It has been found that the addition of high concentrations (usually higher than 1 M) of some low molecular weight compounds in solution stabilizes the native conformation of globular proteins (Xie and Timasheff, 1997; Timasheff, 1995).

A combined effect of the organic solvent and the stabilizer was studied in order to check the possibility of a compromise between an increase in the accessibility of the nitrilase to acrylic fibre and a good operational stability of the enzyme. The least deleterious condition studied was 4% (v/v) of DMA. The addition of 1 M of sorbitol to this medium proved to be beneficial (table 2.2). The initial activity was slightly improved and the half-life time, although shorter than the one obtained only in the presence of sorbitol, was still 249% of the half-life time for the control (no additives). The half-life time of nitrilase without additives increased from 15 to 38 hours in the presence of the additives. Therefore, these conditions were chosen for an enzymatic treatment of acrylic fabric.

2.2.3 EFFECT OF ADDITIVES ON THE MODIFICATION OF ACRYLIC SURFACE WITH NITRILASE

The hydrolysis of nitrile groups from the acrylic fibre should result in the formation of carboxylic groups at the fibre surface and in the release of ammonia to the treatment solution.

The formation of carboxylic groups at the fibre surface was evaluated by colouring the fabric with a basic dye, which has a cationic group able to establish ionic bonds with anionic groups on the fibre. If nitrilase is able to modify some of the nitrile groups into carboxylic groups, at the surface, then more dye can be absorbed into the fibre and this could be seen as an increase in K/S. The colouration methodology was already reported and proved to be a valuable and a very sensitive qualitative method (Silva *et al.*, 2005a, 2005b).

For samples treated with nitrilase without additives, the K/S value increased 156% at the dyeing temperature of 80 ºC (table 2.3). The dye uptake was even more pronounced by the addition of sorbitol and DMA to the

enzymatic treatment. The increase of the colour level was 17%, when compared with the nitrilase treatment without additives, which can be attributed to the partial swelling of the fibre by DMA and to an improvement in nitrilase performance provided by an increase in the fibre surface area available for the enzyme attack.

Table 2.3 – Effect of the additives on the enzymatic modification of acrylic fabric. The acrylic samples were treated with 24 U of nitrilase per gram of fabric, at pH 7.8 and 30 ºC, for 116 hours

	Control	Enzymatic treatment	
		No additives	DMA + sorbitol
K/S_{660nm}[a]	9.8	25.1	29.3
Weight loss[a] (%)	0.3	0.4	0.9
Ammonia[a] (mM)	0	15.3	nd

[a] The standard deviations for the K/S, weight loss and ammonia concentration values were ≤ 10%, ≤ 5% and ≤ 11%, respectively.

Evidence of hydrolysis was also obtained by the formation of ammonia. The ammonia concentrations are reported in table 2.3. It was not possible to determine the ammonia in the solution from the treatment with additives, because they interfered with the kit reagents.

From the expected reaction products, the weight of acrylic fabric should, in theory, increase. The results obtained showed the opposite (table 2.3). There was a slight weight loss instead of a weight gain.

The above results suggested that some modified polymer chains could be solvated and could become soluble in the form of PAA or a copolymer of acrylic acid and acrylonitrile. To verify this hypothesis, another assay was performed and samples of acrylic fabric and treatment solutions were collected at different times of incubation. This study is reported in the next section.

2.2.4 STUDIES OF ACRYLIC SURFACE MODIFICATION WITH NITRILASE

As in all heterogeneous catalysis, it is necessary to consider two general steps in the modification of acrylic: the physical adsorption of nitrilase to the

fibre surface and the formation of the enzyme/substrate complex and consequent hydrolysis of the nitrile group.

The protein adsorption was indirectly calculated by a decrease in total protein remaining in the treatment solution (figure 2.1). In the first 8 hours of treatment, there was a sharp decrease in the protein content in the treatment solution. Afterwards, the equilibrium around an average value was reached, with some oscillations due to alternating adsorption and desorption phenomena (Azevedo *et al.*, 2000). The equilibrium value of protein concentration in the solution was around 9 mg L^{-1}, meaning a total protein adsorption of 80%, at 40 ºC.

There was no significant ammonia release before 8 hours of treatment (figure 2.1). It is interesting to notice that the release of ammonia into treatment solutions was coincident with the achievement of the adsorption equilibrium. This lag phase is also seen in homogeneous catalysis of nitrilases whose activity onset depends on the assembly of its oligomeric active form (Harper, 1977a). The ammonia release is a good indicator of successful nitrilase catalysis, which should render an improvement of the acrylic dyeability. It also shows that a certain amount of those 80% of total protein adsorption must be nitrilase and that the enzyme is able to recognize the nitrile groups of acrylic fabric as the substrate.

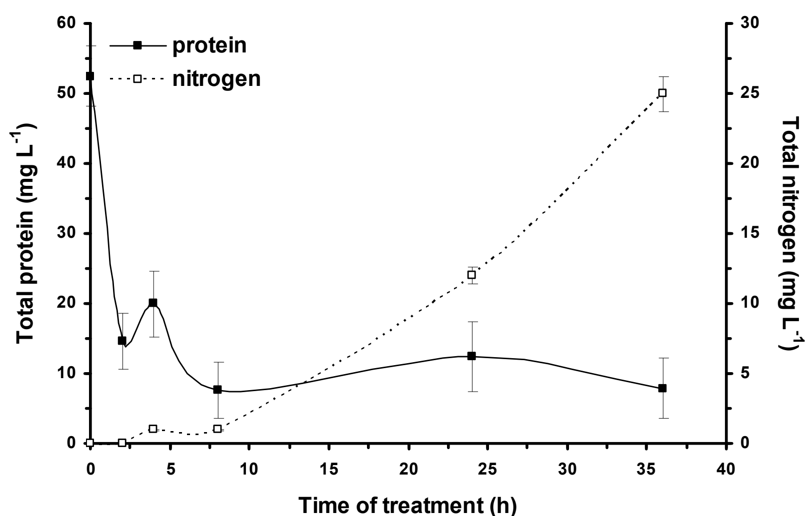


Figure 2.1 – Total protein and nitrogen concentrations in the treatment solutions. The acrylic samples were treated with 412 U of Cyanovacta Lyase per gram of fabric, at pH 7.8 and 40 ºC.

The linear release of ammonia also implies the absence of activity decay during the 36 hours of treatment. Since its half-life time in solution was 15 hours, it is feasible to assume that the adsorption of the enzyme to acrylic led to an increase in its stability, resembling the immobilization procedures employed to stabilize proteins.

In the figure 2.2, the values of K/S are represented as a percentage of the initial value. The relative K/S had a waving behaviour with incubation time. This behaviour was attenuated with the increase in the colouration temperature, as expected. Enzymes are large molecules and they will not penetrate inside the tight structure of PAN fibres. Therefore, the chemical changes catalysed by nitrilase are located at the surface. At lower temperatures, the adsorption of dye occurs mostly at the fibre surface where the enzymatic catalysis takes place, producing different K/S values between samples treated with enzyme and controls. The significant raise in dye diffusion that occurs at or about the glass transition temperature (T_g) is often referred as the dyeing transition temperature (T_d). Both transition temperatures, T_g and T_d, indicate the onset of segmental mobility of the polymer chains and the consequent increase of the free volume within the polymer (Burkinshaw, 1995). Approaching these temperatures, the colour depth increases sharply for both enzymatic treated and untreated

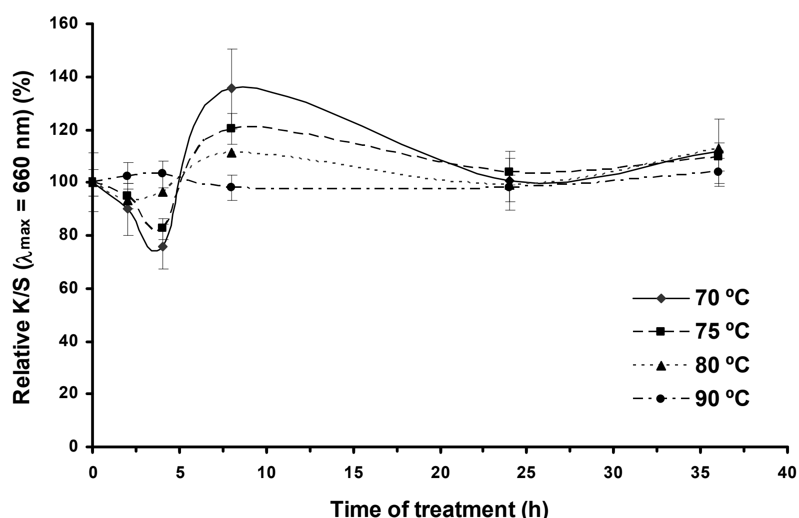


Figure 2.2 – Relative K/S values for acrylic fabric treated with 412 U of Cyanovacta Lyase, at pH 7.8 and 40 ºC. Samples and controls were competitively coloured at 70, 75, 80 and 90 ºC. Relative K/S was calculated as $K/S_{enzyme} / K/S_{control}$ (%).

samples and camouflages the differences in K/S due to superficial changes. This is the reason for the observed decrease in the amplitude of the oscillation of relative K/S with increasing colouration temperatures (70 ºC to 80 ºC). At 90 ºC, which is above T_d, the differences are absent.

The oscillation of relative K/S values with time of treatment could be explained assuming that above a threshold conversion of nitrile groups into carboxylic groups, the polymer chains would be more stable in solution and would be detached from the surface of the fibre. The modification of CN into COOH groups could create some instability in the arrangement of PAN chains at the fibre surface, mainly due to steric hindrance and pH dependent charge repulsion. It was possible to detect increasing concentrations of PAA in treatment solutions (figure 2.3). The removal of carboxylic groups from the surface of the fabric could be the cause of the lower points on the K/S curve. When the PAA macromolecules leave the surface, the underneath PAN chains are exposed to further nitrilase catalysis. This leads to an increasing number of carboxylic groups, thus, to an increase of K/S, until the threshold value for chain solubilization is again achieved (figure 2.4).

A maximum K/S value was observed for the treatment of 8 hours and it corresponded to a relative K/S of 135% (for the lower colouration temperature).

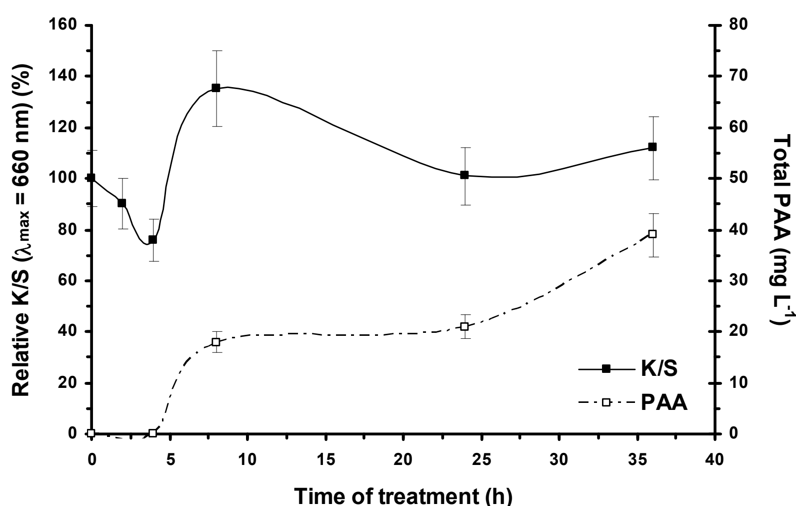


Figure 2.3 – Relative K/S for acrylic fabric coloured at 70 ºC and polyacrylic acid concentration in the treatment solutions. The acrylic fibre was treated with 412 U of Cyanovacta Lyase per gram of fabric, at pH 7.8 and 40 ºC.

For longer incubation periods, the "wave amplitude" tends to decrease, as the "length" tends to increase with time. This could be related to modifications in the fibre surface, since there was not an activity decay of the enzyme. However, the particular phenomenon that explains why this happens is still unclear.

The hypothetical model of "surface erosion" of acrylic fibre, here described (figure 2.4), is very similar to the one applicable for synthetic polymers biodegradation (Marten *et al.*, 2003, 2005). The acrylic fibre surface is depleted layer by layer, depending on factors such as micro-structural properties of PAN copolymers, molar mass of the polymer chains, enzyme adsorption, nitrilase deactivation, removal and dissolution of products.

2.3 Concluding remarks

In spite of the increasing interest in the nitrilase enzymatic system and its successful industrial applications using whole cells (Kobayashi and Shimizu, 1994; Hughes *et al.*, 1998), there is still much space to fulfil in the understanding and improvement of the nitrile conversion by purified or semi-purified nitrilases. The work reported evaluates the stabilization efficiency of various additives and the influence of two organic cosolvents in the activity of a commercial nitrilase. From the additives studied, the best stabilizing performance was induced by sorbitol. Another important aspect is the fact that the organic solvent DMA, used in acrylic fibre industry, revealed no significant loss in enzyme initial activity, when used in small amounts.

It was also demonstrated in this study that a successful application of nitrilase to the acrylic surface modification would be dependent on the control of important factors like time and enzyme activity. The nitrilase action seems to induce "surface erosion" of PAN fibres, by the dissolution of the modified polymer molecules. This hypothesis is confirmed by the detection of PAA in the treatment solutions and the increase of its concentration with the period of incubation of acrylic fabric with nitrilase.

Further studies will be necessary to evaluate the impact of nitrilase activity in the physical properties of the fabric and to assess if some amide groups were also produced as by-products of nitrilase hydrolysis.

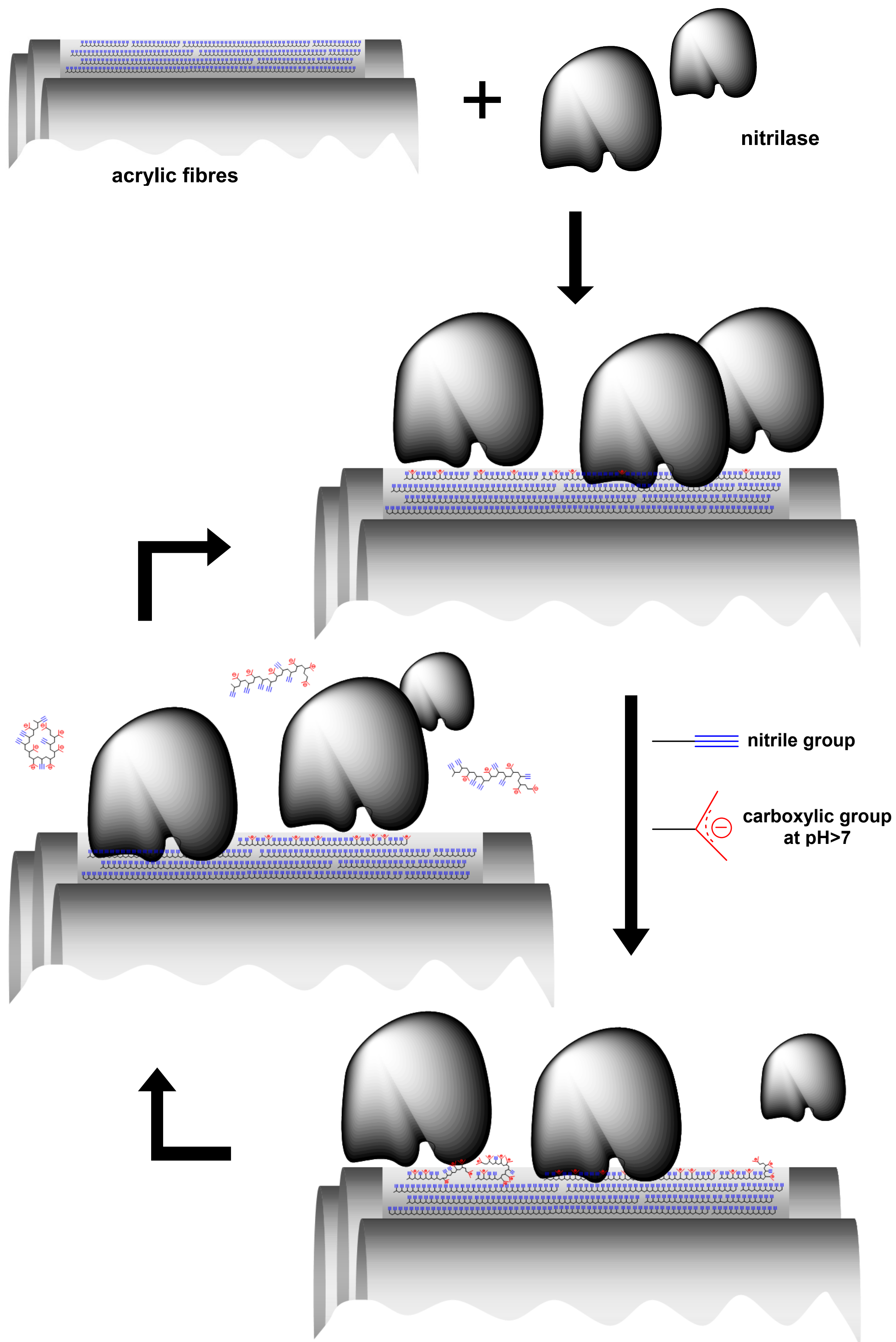


Figure 2.4 – Surface erosion model for acrylic fibres treated with nitrilase at alkaline pH (for simplification, the comonomer vinyl acetate is not represented).

From the above considerations, it could be suggested that the stabilized nitrilase has potential in textile industry for the modification of PAN fibres. The wet spinning would be the most adequate industrial process for the introduction of such enzymatic treatment, though it would demand a very fast enzymatic modification (a few minutes). The accessibility of the fibre would be greater than that of the woven fabric used in this study. If the application of the enzyme occurred when the fibre is still in a gel state, a considerable reduction in the time needed to maximize the enzymatic conversion into carboxylic groups could occur. In addition, after coagulation, there are other possible stages for the application of an enzymatic treatment, during which several finishing processes normally take place. The nitrilase stability would not be so compromised by the amount of solvent present as in earlier stages. In any case, a careful study is needed to evaluate the feasibility of such application, but the work here reported gives encouraging perspectives.

3 The Effect of Additives and Mechanical Agitation in Surface Modification of Acrylic Fibres by Esterases

A previous work reported that cutinase could be used to modify the acrylic surface. After a long enzymatic treatment, the hydrolysis of ester bonds from the comonomer vinyl acetate produced acetic acid and hydroxyl groups on the surface of the fibre that could be detected by reactive colouration (Silva *et al.*, 2005a).

The major purpose of this work described in this chapter was to reduce the treatment time of acrylic fabric with cutinase and to improve its catalysis efficiency by means of reaction media manipulation. In order to do that, the influence of known acrylic solvents and the influence of known stabilizers on cutinase operational stability were investigated. The impact of mechanical agitation and additives on vinyl acetate enzymatic hydrolysis by cutinase and by a commercial esterase was also studied.

3. THE EFFECT OF ADDITIVES AND MECHANICAL AGITATION IN SURFACE MODIFICATION OF ACRYLIC FIBRES BY ESTERASES

3.1 MATERIALS AND METHODS

3.1.1 REAGENTS AND ENZYMES

The enzymes used in this work were a cutinase (EC 3.1.1.74), from *Fusarium solani pisi*, and a commercial esterase Texazym PES, from inoTEX Ltd, Dvur Kralove nad Labem, Czech Republic. The recombinant wild type cutinase was over-expressed in *Saccharomyces cerevisiae* SU50 strain and supplied as culture medium, with 50% to 70% of purification degree in respect to total protein (Silva *et al.*, 2005a; Calado *et al.*, 2002). It was a generous gift from Centro de Engenharia Biológica, Instituto Superior Técnico, Lisbon, Portugal.

The acrylic taffeta fabric used was produced from a copolymer of PAN and 7% vinyl acetate, with 82 g m^{-2} and 36/36 ends/picks per cm, supplied by Fisipe, Lavradio Portugal.

The reactive dye Remazol Brilliant Blue R, C.I. 61200, was acquired from Sigma. All other reagents were laboratory grade reagents also from Sigma-Aldrich, St. Louis, USA, unless stated otherwise. The buffer used throughout this work was a phosphate buffer having a concentration of 50 mM and pH 8.

3.1.2 ESTERASE ACTIVITY ASSAY

Esterase activity was determined following the product release (*p*-nitrophenol, *p*-NP) through the increase in the absorbance at 400 nm. The activity assay conditions for cutinase were described previously (Silva *et al.*, 2005b; Shirai and Jackson, 1982). The esterase activity of Texazym PES was also determined by using *p*-nitrophenyl butyrate (*p*-NPB) as substrate, but using slightly different conditions that were optimized for this enzyme. The enzymatic reaction was started with the addition of 0.1 mL of 10 mg L^{-1} Texazym PES to a final volume of 2 mL of phosphate buffer, containing 75 µM *p*-NPB and 5% v/v

ethanol. The mixture was incubated for 1.5 min in a water bath, at 35 ºC. The hydrolysis was stopped by the addition of 2 mL of acetone. All the assays were performed in triplicate. Standard solutions of *p*-NP were used to obtain the calibration curve. One unit of esterase initial activity was defined as one µmol of *p*-NP released per minute.

3.1.3 STABILITY OF CUTINASE

The operational stability of cutinase was investigated in the presence of two organic solvents and several polyalcohols. The enzyme was incubated at a final concentration 1 mg L^{-1} in phosphate buffer containing 0.05% w/v sodium azide. The concentrations of DMA or DMF used in this study were 7.5%, 15%, 25% and 50% v/v. The final concentration of glycerol, sorbitol, xylitol and EG used was 1 M. For each assay, the control was identically prepared except that buffer substituted the enzyme. The incubation took place in a water bath at 35 ºC with orbital agitation until the drop in esterase activity was above 50%.

3.1.4 ENZYMATIC TREATMENT OF ACRYLIC FABRIC

All samples of acrylic fabric used were previously washed to remove impurities. The washing consisted in several steps, all performed in a laboratory scale machine, the Rotawash MKIII (vertical agitation simulating European washing machines, from SDL International Ltd.), at 60 ºC and 20 rpm. The fabric was washed twice for 30 min with 0.1 g L^{-1} Lutensol AT25 (non-ionic detergent, BASF, Ludwigshafen, Germany) and left for 10 min under running tap water. Then, the fabric was washed once for 30 min with 2 g L^{-1} sodium carbonate and left for another 10 min under running tap water. Finally, the fabric was washed three times in distilled water for 20 min each and was left to dry at room temperature.

Two sets of experiments were carried out for each enzyme taking into account the degree of mechanical agitation. For both sets, the treatment of acrylic fabric was performed in stainless steel pots of 450 mL capacity in Rotawash machine, in the case of cutinase, and in Washtec-P 05/99A (vertical agitation simulating European washing machines, from Roaches International Ltd.), in the case of Texazym. All the treatments were performed in phosphate

buffer at 30 ºC and 20 rpm, for three hours. To increase the mechanical agitation, stainless steel discs (19.2 g, 32 x 3 mm) were added to the reaction mixture.

In the cutinase treatment, samples of acrylic fabric, having an average weight of 1.57 g, were incubated with 9 U mL^{-1} of cutinase, in a final volume of 100 mL. Five different media were tested: no additives, 1% and 15% v/v of DMA, 1 M glycerol and 1% DMA + 1 M glycerol. In a second set of assays, these conditions were repeated and 4 stainless steel discs were added to each assay.

In the treatment with Texazym PES, samples of acrylic fabric, having an average weight of 3.05 g, were incubated with 3.4 U mL^{-1} of the enzyme, in a final volume of 200 mL. The reaction media were the same as described for cutinase. The number of discs added was raised to 9 per assay. For each reaction media, a control was run in parallel in which buffer substituted the enzyme.

After enzymatic treatment, all fabric samples were washed twice in 2 g L^{-1} sodium carbonate for 30 min, once in 0.25 g L^{-1} Lutensol AT25 for 30 min and three times in distilled water for 15 min, in the Rotawash or Washtec-P at 70 ºC.

3.1.5 Quantification of total protein concentration

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

3.1.6 Determination of acetic acid concentration in the bath solutions

Detection of acetic acid in reaction media was performed with a kit from Boehringer Mannhaim R-Biopharm (cat. No.10148261035), Darmstadt, Germany. Protein was previously precipitated using perchloric acid according to the manufacture instructions. The samples pH was neutralized using 1 M potassium hydroxide and the subsequent salts were removed by centrifugation.

3.1.7 ACRYLIC FABRIC COLOURATION WITH A REACTIVE DYE

After enzymatic treatment, samples were competitively coloured in duplicate with 2% o.w.f. Remazol Brilliant Blue R. The colouration bath consisted in a 10 g L^{-1} sodium carbonate solution. The colouration was performed at 70 ºC and 80 ºC, in a lab dyeing machine (AHIBA Spectradye, from Datacolor International), for 90 min at 20 rpm. The total fabric average weight per colouration assay was 2.87 g and 2.18 g for samples treated with cutinase and Texazym PES, respectively.

After colouration, all samples were washed once with 0.25 g L^{-1} Lutensol AT25 and several times with water at 70 ºC in Rotawash, until no more dye could be detected in the solution. The colour measurements (5 for each sample) were carried out with a reflectance spectrophotometer having a standard illuminant D65 (Spectraflash 600 Plus, from Datacolor International). The colour strength was evaluated as K/S at maximum absorption wavelength (590 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 (Kuehni, 1997).

3.1.8 WIDE ANGLE X-RAY SCATTERING

The X-ray diffraction (XRD) patterns were obtained for the acrylic fabric samples treated with cutinase without and with stainless steel discs (the later for the samples treated in the presence of the additives). The WAXS experiments were undertaken in a Philips PW1710 apparatus, using Cu Kα radiation and operating at a 40 KV voltage and 30 mA current. The patterns were continuously recorded in the diffraction angular range 2θ from 5º to 40º, with a step size of 0.02º at 0.6º min^{-1}.

The degrees of crystallinity were obtained according to the method of El-Zaher (El-Zaher, 2001).

3.2 RESULTS AND DISCUSSION

3.2.1 OPERATIONAL STABILITY OF CUTINASE

From the biotechnological point of view, both storage and operational stabilities greatly influence the usefulness of enzyme-based products (Ó'Fágáin, 2003). Operational stability, defined as the persistence of enzyme activity under the conditions of use, was studied for cutinase before applying the enzyme to acrylic fabric treatment. The media conditions, such as buffer, pH and temperature were chosen based on preliminary studies performed in the laboratory, using the esterase activity determination methodology described earlier. The conditions chosen, which maximize the hydrolysis of *p*-NPB by cutinase, were phosphate buffer at pH 8 and temperatures comprised between 30 ºC and 40 ºC. These preliminary results were in agreement with those described in literature (Petersen *et al.*, 1998, 2001b; Melo *et al.*, 1997). Under the reported conditions, the specific activities of cutinase and Texazym PES were 253 ± 51 and 11 ± 1 U mg^{-1}, respectively.

The half-life times obtained for cutinase, incubated under different conditions, are shown in table 3.1, as a measure of operational stability. The values of half-life time were calculated whenever the experimental data was adequately fitted with a first order exponential decay, using OriginPro 7.5 (Origin Lab Corporation, USA). This was the case for the assays where cutinase was incubated with 7.5% and 15% of both organic solvents and with EG and PEG. The half-life times for cutinase incubated with glycerol, sorbitol, xylitol, and both organic solvents at 25% and 50% (v/v) were obtained from the second order exponential decay fitting data. Many protein deactivation models are non first order (Ó'Fágáin, 2003; Aymard and Belarbi, 2000; Baptista *et al.*, 2000 and 2003; Melo *et al.*, 2001). Cutinase has already been described as displaying non-first order exponential decay for acid values of pH (Melo *et al.*, 1997; Baptista *et al.*, 2003).

The values attained suggest that cutinase tolerates well the two organic solvents tested if their concentration remains bellow 15% (v/v). The stability of cutinase was improved with 15% DMA, being the half-life time 3.5 times higher than that of the control. Considering organic solvent contents above 15%, the

Table 3.1 – Influence of two organic solvents and several polyalcohols on operational stability of cutinase. The cutinase was incubated at 35 ºC, pH 8, in a water bath with agitation, under different media compositions. The half-life time was calculated as $t_{1/2} = \ln 2 / k$, in the cases of a 1st order exponential decay of activity ($a_t = a_{t=0}.e^{-kx}$)

Assay conditions		Half-life time/ days
No additives		45 ± 7
DMF	7.5%	45 ± 4
	15%	46 ± 7
	25% [a]	0.6
	50% [a]	0.01
DMA	7.5%	48 ± 2
	15%	159 ± 24
	25% [a]	4
	50% [a]	0.4
Polyalcohols	Glycerol [a]	134
	Sorbitol [a]	113
	Xylitol [a]	31
	EG	29 ± 8
	PEG	23 ± 8

[a] The half-life time was obtained from the 2nd order exponential decay fitting curve $a_t = a_{t1}.e^{-x.k1} + a_{t2}.e^{-x.k2}$

half-life time was drastically reduced in the presence of both DMA and DMF. Cutinase is one among other enzymes reported in literature that exhibits an increase in its stability as well as in its maximal activity (results not shown), in the presence of low concentrations of organic cosolvents, (Ó'Fágáin, 2003; Castro and Knubovets, 2003; Klibanov, 2001; Soares *et al.*, 2003).

The use of some low molecular weight compounds in solution has been found to stabilize native conformations of globular proteins like cutinase, when added at high concentrations (≥ 1 M) (Xie and Timasheff, 1997). From the polyalcohols studied, glycerol and sorbitol were the only ones improving the stability of cutinase. With glycerol, the half-life time increased by three fold. The xylitol, EG and PEG had the opposite effect, the half-life time was reduced more than 30% (table 3.1).

3.2.2 Enzymatic modification of acrylic surface

The modification of a solid substrate, like acrylic fibres, with enzymes constitutes a heterogeneous biocatalysis, since enzyme and substrate are in different phases. Therefore, it is necessary to consider two general steps in order to make the catalysed reaction occur: the physical adsorption of cutinase/Texazym to acrylic fibre surface and the formation of the enzyme/substrate complex and consequent hydrolysis of the ester bond between vinyl acetate and the backbone of the polymer chain.

During the treatment, aliquots were taken at different time intervals to follow the protein adsorption. In figure 3.1, the total protein is plotted for the cutinase treatment with different amounts of DMA, under low and high mechanical agitation. These conditions were chosen as an example. It is clear from the results that there is no significant protein adsorption under the conditions tested, taking into account the experimental error. When the discs are present, the greater mechanical agitation did not affect the adsorption behaviour of this enzyme into acrylic fibre. In addition, it was not possible to see a clear effect of the organic solvent on protein adsorption. The lack of significant adsorption was also verified in the acrylic treatment with Texazym PES for both degrees of mechanical agitation.

These results were not surprising since polyacrylonitrile polymers are polar materials and they are known low-protein-adsorbing polymers, often used to produce inert membranes and supports for bioprocessing technologies (Belfort and Zydney, 2003).

In theory, if the hydrolysis of vinyl acetate happens on the surface of the fibre, according to the 'electrostatic catapult' model (Petersen *et al.*, 2001a, 2001b) at pH 8, cutinase should release the product – acetate anion. It was not possible to detect acetic acid in the aliquots collected during the treatment of acrylic with both enzymes. The detection limit, according to the kit suppliers, is 1.5 mg L^{-1} acetic acid. The average acrylic weight used was 15.5 g L^{-1} of treatment solution. Thus, it means that less than 0.14% of the total available vinyl acetate was hydrolysed (in average, 7% w/w of 15.5 g L^{-1}). According to the model proposed by Warner *et al.*, they are organized in fibres of strong laterally bound chains (Frushour and Knorr, 1998). Assuming that the enzyme

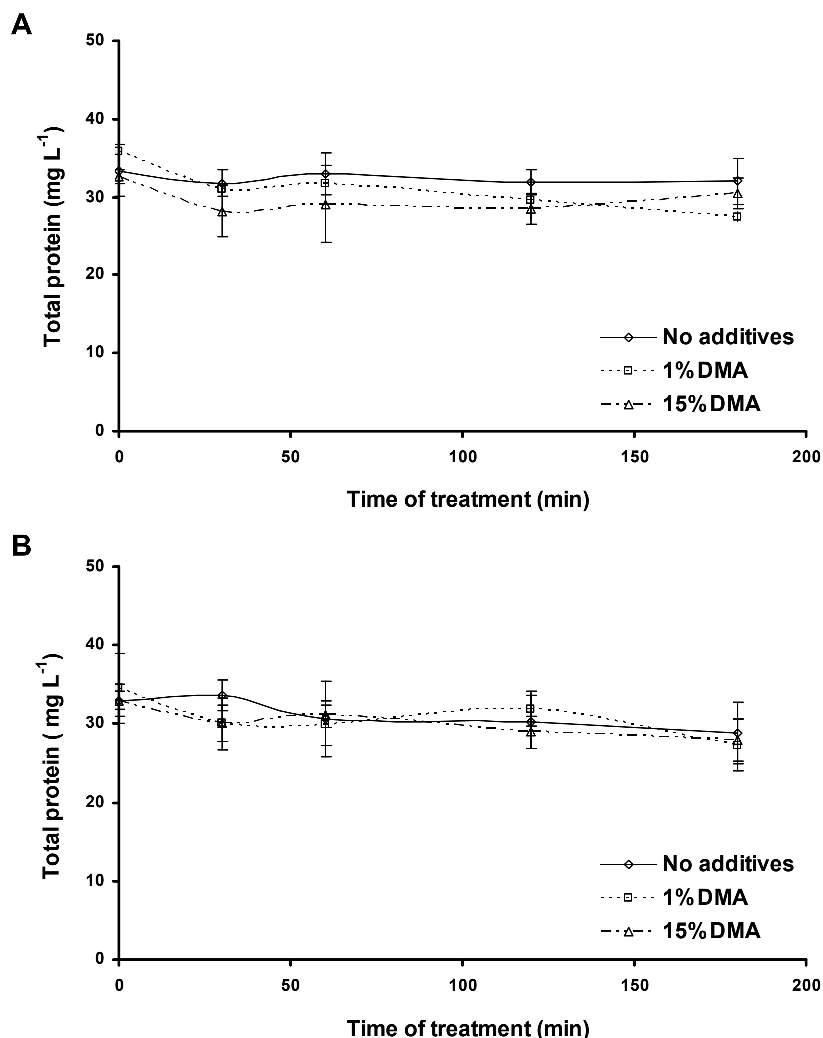


Figure 3.1 – Total protein concentration in bath treatment solutions during the 3 hours incubation of acrylic with 9 U mL^{-1} of cutinase and different amounts of DMA, (**A**) in absence and (**B**) in presence of stainless steel discs.

cannot penetrate inside the fibres, the reason area/volume of these fibres determines the vinyl acetate available for enzyme attack. Relating the area/volume (2/r) between a single polymer chain (6 Å) - maximal accessibility, and acrylic fibres (diameter: 100-1000 Å) - <<100% accessibility, the acetic acid concentration produced would be between 6.5 and 65 mg L^{-1}.

Factors like the use of textile fabric instead of free fibres and the lower efficiency of enzymatic catalysis in heterogeneous system could explain the failure to detect acetic acid by this method.

A methodology was developed (Silva *et al.*, 2005a) to measure the hydroxyl groups that result from the enzymatic hydrolysis of the ester linkage of vinyl

acetate and that remain attached to the polymer backbone. The basic principle is the specific reaction between a vinylsulphonic group from a reactive dye, in this case Remazol Brilliant Blue R, and the hydroxyl group at the fibre surface. The specificity of the method is increased by the fact that this kind of reactive dye was designed for cellulose fibre dyeing, thus it has low affinity for synthetic fibres. The sensitivity is also very high due to the large molar absortivities of dye molecules.

A higher level of mechanical agitation was of crucial importance in the acrylic fabric treatment with both enzymes. Without the addition of stainless steel discs, it was not possible to measure any difference in K/S between treated samples and controls. The relative increase in K/S, obtained for the set of experiments where the discs were used, is represented in figure 3.2. Treating acrylic fabric, either with cutinase or with a commercial esterase, only led to the formation of hydroxyl groups when the stainless steel discs were introduced into the incubation vial. The experiments were performed in lab machines reproducing the vertical agitation of European washing machines, where the mechanical work involves the fibre-fibre and fibre-metal friction as well beating effects. The introduction of metal discs increases the beating effects and the fibre-metal friction (Vasconcelos and Cavaco-Paulo, 2006). The finishing and washing effects produced by cellulases also depend highly on strong mechanical work delivered to cotton fabrics (Cavaco-Paulo, 1998; Azevedo *et al.*, 2000). Heterogeneous catalysis implies adsorption of the enzyme which is in itself a complex process depending, among other issues, on transport toward the surface by convection and diffusion. Accordingly, it is likely that an increase in mechanical agitation is helpful for the outcome of acrylic biotransformation, increasing the accessibility. In addition, in a comparable way to cellulose, the increased friction has a micro-pilling effect on the acrylic fabric, therefore, increasing the surface area available for enzymatic attack.

DMA is a known solvent of PAN and it is commonly used in acrylic fibre industrial production. Its plasticizer function disturbs the regular structure of the polymer, reducing the magnitude of inter-chain bonding, which should aid the penetration of the enzyme, improving its action on the fibre. The acrylic fabric sample treated, both with cutinase and with Texazym PES, in the presence of 15% DMA did not show any difference in respect to controls (figure 3.2). The

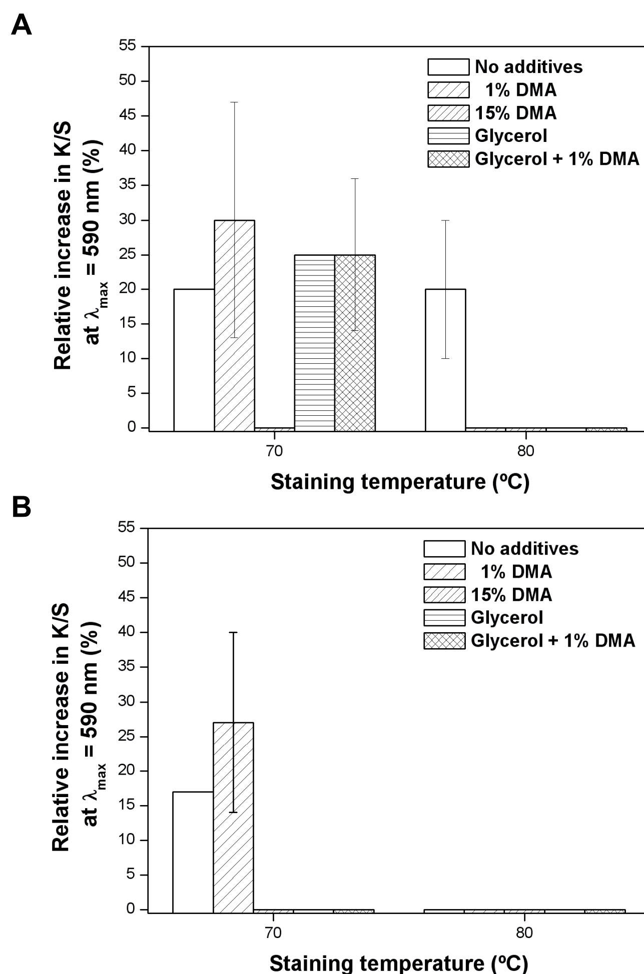


Figure 3.2 – Percentage of increase in K/S for acrylic samples treated (**A**) with cutinase (573 U per gram of fabric) and (**B**) with a commercial esterase (223 U per gram of fabric). To increase the mechanical agitation, stainless steel discs were added to all the assays plotted. Samples were competitively coloured at 70 ºC and 80 ºC, and the relative increase in K/S was calculated as $(K/S_{enzyme} - K/S_{control}) / K/S_{control}$.

increased accessibility, as the consequence of swelling of fibres by the organic solvent, does not therefore render an enhancement in cutinase catalysis on acrylic surface. If the observed increase in cutinase stability is due to the preferential hydrating phenomena induced by DMA, most probably the interactions between enzyme and polymer are not sufficiently strong to displace the tightly bound water. This 15% DMA content has no improving effect upon catalysis on the contrary to just 1%, where the relative K/S increase is clear, at the colouration temperature of 70 ºC. From all the conditions tested, both cutinase and Texazym PES have shown the highest activity with 1% DMA, in spite of the error margin.

For 1 M glycerol and a combination of 1 M glycerol with 1% DMA, the two enzymes showed different behaviours (figure 3.2). In spite of the similar increase of K/S for the absence of additives and for 1% DMA, the units of esterase activity from cutinase were more than twice the units from Texazym were, in all assays. This could mean a saturation of cutinase at the referred media conditions. When glycerol was present in the reaction media, Texazym treatment did not lead to a better colouration of acrylics, while, cutinase performance was improved, in respect to the treatment without additives. The presence of 1% DMA could not raise the colour level provided by glycerol.

The differences in K/S were observed when the samples were coloured at 70 ºC. At 80 ºC, the method failed somehow to detect the action of both enzymes on the surface of the acrylic, except for the treatment with cutinase without additives. The raise from 70 ºC to 80 ºC, which is very near the glass transition temperature (T_g) for commercial acrylic fibres, has a major impact in segmental mobility of polymer chains. As the dye molecules penetrate more deeply in the fibre, the differences in K/S are diluted. Dye molecules can also react with other chemical groups present in the fibre, such as the initiator molecules used in acrylic polymerisation. The yield of this side reaction will be enhanced by temperature since more of these groups will be exposed causing the colour depth to be higher. The addition of dye molecules to hydroxyl groups that result from enzymatic catalysis is not improved in an equal extension because they are located on the surface of the fibre. Thus, the observed increase in K/S, at 80 ºC, for the cutinase treatment without additives is an exception and is not fully understood. To confirm the dependency of relative increases in K/S with dyeing temperature, more studies are required.

3.2.3 WIDE ANGLE X-RAY SCATTERING

The XRD patterns obtained for the different samples showed the characteristic reflection peaks of polyacrylonitrile homopolymer described in literature (JCPDS card no 48-2119) (Bashir, 1992 and 1994; Liu and Ruland, 1993). The XRD pattern of the control sample for the treatment without discs or additives is given as an example (figure 3.3). The main peaks are characterized by a well defined sharp and intense peak positioned at $2\theta \approx 16.8º$, characteristic

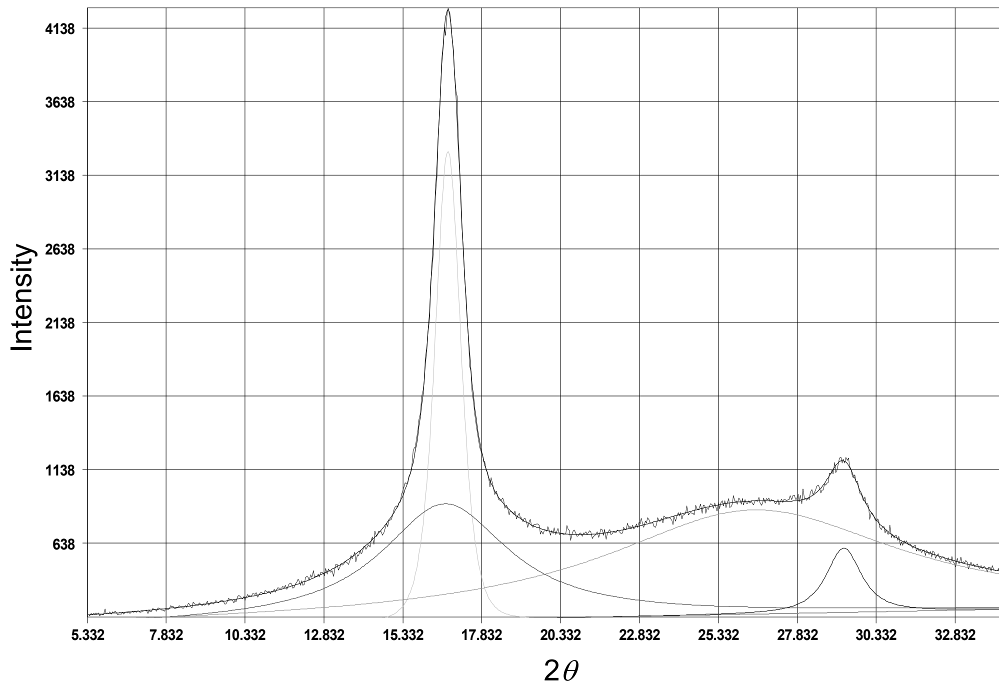


Figure 3.3 – X-ray diffraction pattern of control sample for the treatment performed in the absence of additives and stainless steal discs. The XRD data was analyzed by profile fitting of the scans with the Pearson VII function, using the software WinFit! beta release 1.2.1, 1997.

of (010) plane, and a second one, less intense at $2\theta \approx 29.4°$, from the (300) plane. A third diffuse peak was also observed in all samples, located at $2\theta \approx 26.5°$, attributed to (210) plane. This peak was found to be a very broad and diffuse one, commonly associated to amorphous phases. In order to obtain an accurate fitting profile, it was always necessary to consider a fourth significantly diffuse peak, at an angular position $2\theta \approx 16.8°$, which is in fact very similar to that of the previously mentioned peak. Although unusual, similar behaviour was also found by Causin *et al*. (2005). This peak appearance might result from the introduction of the comonomer vinyl acetate, which probably induces some defects in the lateral packaging of polymer chains and thus being responsible for this second “amorphous type” phase. In a systematic study of homologous series of amorphous polymers, Miller and Boyer (1984a, 1984b) found in the XRD patterns two types of amorphous halos. The position of the first type corresponded to the close packaging of atoms ($2\theta \geq 18°$) and its position was dependent on the size of the pendent group, while the second type

Table 3.2 – Crystallinity degrees (CD) for acrylic fabric samples treated for three hours with 573 U of cutinase per gram of fabric, at pH 8 and 30 ºC. The values were obtained according to the equation $CD = \frac{I_{(010)} - I_{(300)}}{I_{(010)}}(\%)$ where $I_{(010)}$ and $I_{(300)}$ are the intensities of the peaks at 2θ 16.8º and 29.4º, respectively

Sample			Crystallinity degree (%)
No discs	**No additives**	control	86,5
		enzyme	86,3
Discs	**No additives**	control	85,5
		enzyme	86,2
	DMA 1%	control	85,1
		enzyme	85,0
	DMA 1% + Glycerol	control	85,7
		enzyme	86,4

corresponded to regions within the samples which exhibit some near-range, intersegmental order (2θ <18º).

No significant differences (below 1%) in the values of crystallinity index were found between acrylic control samples and samples treated with the enzyme (table 3.2). This was expected given the superficial nature of the enzymatic treatment of the fabric and, in the particular case of acrylic fibres, the preservation of an intact carbon skeleton of polymer chains.

The WAXS and scanning electronic microscopy (SEM) studies were initially intended as negative controls on the modification of physical properties of acrylic fibres. The figure 3.4 shows the photos taken by SEM to fabric from one of the treatments that did not led to colour differences between control and treated samples and to the treatment with 1% DMA and stainless steel discs, which produced the highest colour improvement due to the cutinase treatment. In fact, there were no qualitative significant differences on the morphology between controls and enzymatic treated acrylic samples, analysed by SEM. The chemical modifications catalysed by cutinase/esterase do not affect significantly the physical properties of acrylic fibres.

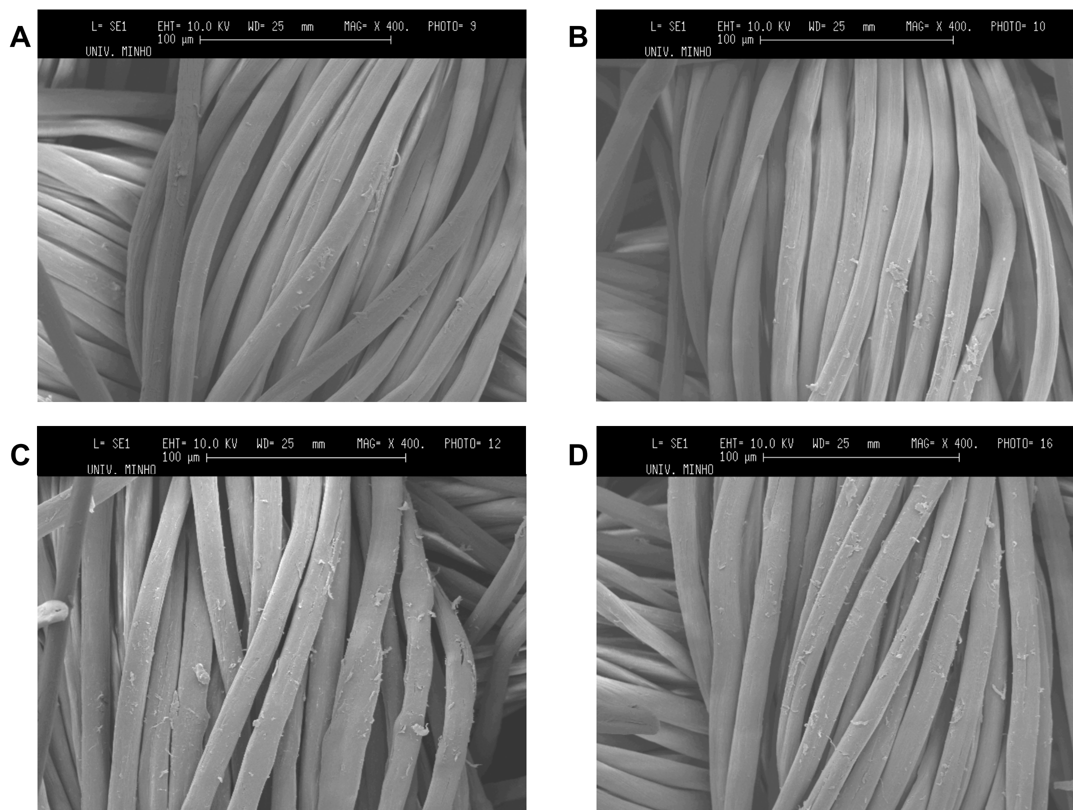


Figure 3.4 – SEM photographs of acrylic fabric (**A**) control and (**B**) cutinase treated sample without stainless steel discs, and (**C**) control and (**D**) cutinase treated sample treated with discs and 1% DMA. All images were acquired under the same conditions with a total magnification of 400x, in a scanning electronic microscope Leica S360.

3.3 Concluding remarks

In literature, a great amount of work has been published in respect to cutinase stability (Silva *et al.*, 2005b; Petersen *et al.*, 2001b; Baptista *et al.*, 2000, 2003; Melo *et al.*, 2001, 2003; Gonçalves *et al.*, 2003; Carvalho *et al.*, 1999b; Pocalyko and Tallman, 1998; Crevel *et al.*, 2001). Special attention has been devoted to trehalose and surfactants, given the industrial interest in this enzyme for detergents formulation, for enzyme immobilization and micro-encapsulation. The work reported in this paper contributes to expand the pool of information regarding stabilization additives for cutinase, in aqueous media, which is still of great importance in biotechnological applications.

The catalytic efficiency of both enzymes studied on acrylic surface modification, although not considerably significant, was proved to be enhanced by DMA at low concentrations, a solvent of PAN fibres commonly used in its spinning process. Besides, the reduction in incubation time with cutinase from 90 hours (Silva *et al.*, 2005a) to just three hours, due to a higher mechanical agitation, represents a great advance in the application of these enzymes to acrylic surface modification, especially in wet spinning lines of production and at stages of a more “exposed and accessible” surface of the acrylic fibre. It is expected that the impact of esterase activity on fibre surface modification will be greater when used in acrylic or other textile fibres with higher contents in hydrolysable ester monomers in their compositions.

4 Surface Modification of Cellulose Acetate with cutinase

Work on the modification of cellulose acetate with enzymes has been mostly done in the context of its biodegradation (Puls *et al.*, 2004). This chapter describes the hydrolysis of acetate surface groups of CDA and CTA fabrics using *Fusarium solani pisi* cutinase. The preference for hydrophobic substrates, as well as the versatility in respect to soluble and insoluble substrates makes cutinase an attractive esterase for highly substituted cellulose acetates.

The hydrolysis of surface acetyl groups from CDA and CTA with a cutinase constitutes a promising approach for the partial regeneration of cellulose reactivity and hydrophilicity in these fibres, here demonstrated by the enhanced reactive dye uptake of treated fabrics.

4. SURFACE MODIFICATION OF CELLULOSE ACETATE WITH CUTINASE

4.1 MATERIALS AND METHODS

4.1.1 REAGENTS AND ENZYMES

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

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

The reactive dye Remazol Brilliant Blue R, C.I. 61200, was from Sigma. All other reagents were laboratory grade reagents also from Sigma-Aldrich, St. Louis, USA, unless stated otherwise. The buffer used throughout this work was a phosphate buffer having a concentration of 50 mM and pH 8.

4.1.2 ESTERASE ACTIVITY ASSAY

Esterase activity was determined following the product release (*p*-NP) continuously through the increase in the absorbance at 400 nm at 30 ºC. The assay conditions for the determination of cutinase activity were described previously (section 3.1.2). All the assays were performed at least in triplicate. Standard solutions of *p*-NP were used to obtain the calibration curve. One unit of esterase activity was defined as one µmol of *p*-NP released per minute.

4.1.3 TREATMENT OF CELLULOSE DI- AND TRIACETATE FABRIC WITH CUTINASE

All samples of cellulose acetate fabric were washed prior to use in order to remove possible impurities from manufacture and from human handling. Washing was performed at 35 ºC and 20 rpm, in stainless steel pots of 450 mL in capacity and housed in a laboratory scale machine, the Rotawash MKIII

(vertical agitation simulating European washing machines, from SDL International Ltd.). The fabric was washed twice for 30 min in 40 mg L^{-1} Lutensol AT25 (non-ionic detergent, BASF, Ludwigshafen, Germany), then rinsed four times with distilled water for 30 min each and left to dry at room temperature.

Several sets of experiments were carried out taking into account the amounts of enzyme, fabric and time of incubation. For all experiments, the treatment of cellulose acetate fabric was performed in phosphate buffer with vertical agitation, in the Rotawash machine operating at 30 ºC and 20 rpm. To evaluate the effect of enzyme concentration, samples of CDA and CTA fabric, with an average weight of 0.1 g, were incubated in duplicate for 8 hours with 0, 25, 50, 75 and 100 U mL^{-1} of cutinase, in a total volume of 5 mL. To obtain a progress curve, samples of CDA and CTA fabric, with an average weight of 0.1 g, were treated with 50 U mL^{-1} of cutinase, in a final volume of 10 mL for different periods of time. For each sample a control was run in parallel in which the buffer substituted the corresponding volume of enzyme. In another treatment, the average weight of both type of fabric was increased to 0.5 g and the incubation extended to 24 hours. The initial activity of cutinase was 25 U mL^{-1} in a final volume of 25 mL. For each sample a control was run in parallel without the enzyme.

After enzymatic treatment, all fabric samples were washed at 35 ºC, in the Rotawash machine, to remove the adsorbed protein, according to the order: 250 mg L^{-1} Lutensol AT25 for 30 min, 70% ethanol for 20 min, 15% isopropanol for 15 min, three steps of increasing concentrations of sodium chloride for 10 min each, three steps in distilled water for 20 min each. Between the detergent/alcohol and alcohol/salt steps the fabric was rinsed under running cold tap water for 5 min.

4.1.4 Quantification of total protein concentration

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

4.1.5 DETERMINATION OF ACETIC ACID CONCENTRATION IN THE TREATMENT SOLUTIONS

Detection of acetic acid in the reaction media was performed with the acetic acid UV test from Roche (Darmstadt, Germany) as described previously (section 3.1.6).

4.1.6 CELLULOSE ACETATE FABRIC COLOURATION WITH A REACTIVE DYE

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

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

The colour measurements (5 for each sample) were carried out according to the procedure described previously (section 3.1.7).

4.1.7 FLUORESCEIN ISOTHIOCYANATE LABELLING

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

4.1.8 FLUORESCENCE MICROSCOPY

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

Leica DFC350 FX digital Camera and Leica Microsystems LAS AF software, version 2.0 (Leica Microsystems GmbH, Wetzlar, Germany).

4.1.9 Fourier transform infrared spectroscopy (FT-IR)

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

4.1.10 Wide angle X-ray scattering

The XRD patterns were obtained for the CDA and CTA fabric samples treated during 24 hours with cutinase and respective controls. The WAXS experiments were undertaken in a Philips PW1710 apparatus, using Cu Kα radiation and operating at a 40 KV voltage and 30 mA current. The patterns were continuously recorded in the diffraction angular range 2θ from 4º to 40º, with a step size of 0.02º at 0.6º min^{-1}. The non linear fitting of the diffraction patterns was performed using the Pseudo-Voigt peak function from OriginPro 7.5 (Origin Lab Corporation, USA) considering the CTA structural polymorphism II (Cerqueira *et al.*, 2006). The peaks that were considered crystalline were at the diffraction angles 2θ: 10º, 17º and 21º for CDA, and 8º, 10º, 13º, 17º, 21º and 23º for CTA (Chen *et al.*, 2002; Hindeleh and Johnson, 1972).

4.2 RESULTS AND DISCUSSION

4.2.1 EFFECT OF CUTINASE CONCENTRATION ON THE MODIFICATION OF CELLULOSE DI- AND TRIACETATE

The media conditions, such as buffer, pH and temperature, were chosen based on earlier studies performed in our laboratory, using the esterase activity determination methodology already described (section 3.1.2). The conditions chosen were phosphate buffer pH 8 and the lowest optimum temperature 30 ºC. The hydrolysis of the acetate groups in cellulose ester substrates leads to the formation of hydroxyl groups at the fibres surface and to the release of acetic acid to the treatment solution. The effect of cutinase concentration was analysed by measuring the acetic acid in the treatment solutions, after a period of 8 hours (figure 4.1).

The acetic acid release was not linearly proportional to all the tested enzyme concentrations as it would be expected (Tipton, 2002; Lee and Fan, 1982). The higher level of acetic acid released from the less substituted cellulose acetate was according to the irreversible relation between the degree of substitution and the degree of bio-deacetylation (Samios *et al.*, 1997; Altaner *et al.*, 2001, 2003a; Moriyoshi *et al.*, 1999, 2002). Steric hindrance and crystallinity are

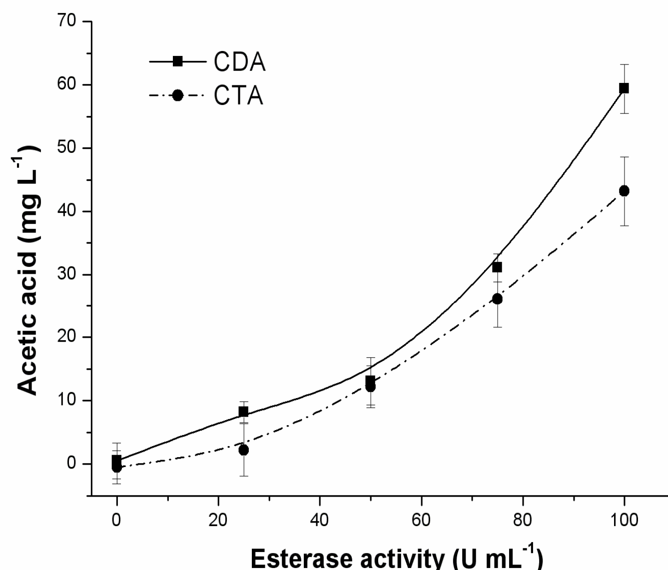


Figure 4.1 – Effect of cutinase concentration on the acetic acid release. The CDA and CTA fabrics were treated during 8 hours, at pH 8 and 30 ºC, with several concentrations of cutinase expressed as esterase initial activity in U mL^{-1}.

considered important factors in the adsorption and mostly in the effectiveness of the adsorbed enzyme to promote the hydrolysis (Lee and Fan, 1982). These factors should favour CDA over CTA. At the maximum concentration used, the enzyme activity was 0.010 U and 0.0072 U (μmol of acetic acid per min) while only 0.54% and 0.36% of the acetyl groups were released from CDA and CTA, respectively. These values were obtained considering a DS 2.4 for CDA and a minimum DS 2.7 for CTA commercial fibres (Steinmann, 1998; Zugenmaier, 2004). A very low yield in deacetylation is not uncommon for highly substituted cellulose acetates treated with cell-free enzymes (Puls *et al.*, 2004). By comparison, in view of the fact that at least one of the cellulose acetate used has a higher DS, cutinase demonstrated potential as cellulose acetate esterase. Altaner *et al.* (2001) reported that acetylesterases from 13 different commercial origins could significantly use cellulose acetates with DS ≤1.4 as substrates. Only one enzyme from *Humicola insolens* was able to release a small amount (10%) of acetyl groups from a cellulose acetate DS 1.8, after 220 hours (Altaner *et al.*, 2001). Another enzyme purified from a commercial preparation, derived from *Aspergillus niger*, was able to hydrolyse 5% of the existing acetyl groups on a cellulose acetate DS 1.8 after 140 hours (Altaner *et al.*, 2003a). Considering the values found in the literature, the percentage of acetic acid released obtained with cutinase was not insignificant, mainly because the final purpose of this modification is not biodegradation of the substrate, but the modification of the fibre surface.

4.2.2 PROGRESS CURVES FOR THE MODIFICATION OF CELLULOSE DI- AND TRIACETATE FABRICS AND PROTEIN ADSORPTION

Samples of CDA and CTA fabric (1% w/v) were treated with 50 U mL^{-1} of cutinase for different periods of incubation. The action of cutinase was evaluated by indirectly measuring the hydroxyl groups formed at the fibres. Since the cellulose acetates used in this work were insoluble, the enzyme adsorption to the substrate was a prerequisite for the formation of the enzyme-substrate complex. The protein adsorption was indirectly calculated by the decrease in total protein remaining in the treatment solution. For CDA (figure 4.2A), an equilibrium level of relative protein adsorption of 45% (2.8 mg g^{-1} of

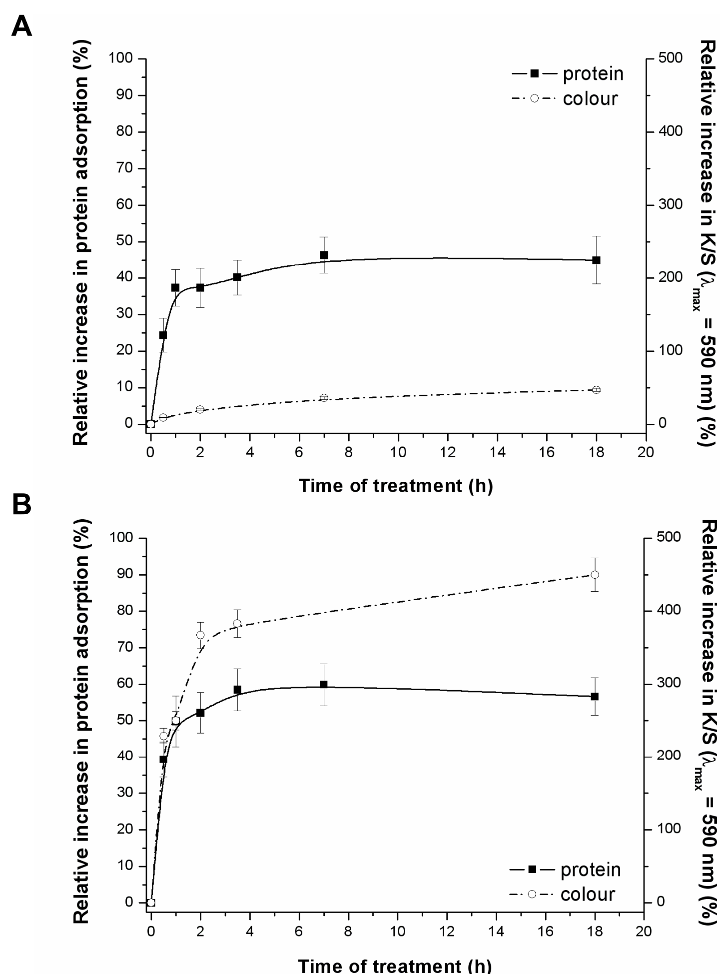


Figure 4.2 – Progress curves for the formation of hydroxyl groups at the fibres surface, measured as relative increase in K/S values, and total protein adsorption for (**A**) CDA and (**B**) CTA. All the samples were treated with cutinase (5000 U per gram of fabric) at pH 8 and 30 ºC. Samples and controls were competitively coloured at 60 ºC. The relative increase in K/S was calculated as $(K/S_{enzyme} - K/S_{control}) / K/S_{control}$ (%) and the relative protein adsorption as $(P_{0h} - P_t) / P_{0h}$ (%), where *P* is the total protein in solution.

protein per fabric weight) was reached. For CTA (figure 4.2B), the equilibrium level of protein adsorption was higher, with 57% of relative protein adsorption (3.5 mg g^{-1}). The hydrophobic character of the substrate should not be a problem for cutinase adsorption since this enzyme is a lipolytic enzyme and its natural substrate, cutin, is hydrophobic (Egmond and Vlieg, 2000; Mannesse *et al.*, 1995; Kolattukudy, 2002).

The formation of hydroxyl groups at the fibre surface was evaluated by colouring the fabric with a cotton reactive dye. If the cutinase is able to

hydrolyse some of the acetyl groups at the surface, then more dye can be chemically linked to the fibre, resulting in an increase in K/S.

In the case of CDA fibre, the sensitivity is not as good as for the CTA fibre. The dye has more affinity for diacetate and, as a result, the controls are very coloured while the triacetate controls are very faint. This is the reason for the observed differences between the two fibres in the relative increase in the colour strength values (figure 4.2). After 18 hours, the relative difference in colour strength between treated samples and controls was around 50% for CDA and 450% for CTA. For both fabrics, the relative K/S increased rapidly in the first hours of treatment and slowed down as the protein adsorption equilibrium was being settled. In the particular case of these modifications, a very slow enzymatic reaction occurs. We believe that the fast initial increases in colour are an artefact created by an incomplete protein removal during the washing procedure at the end of each treatment. It seems that the dye is also able to react with hydroxyl groups present in the protein molecules not removed from the fabric. If this argument is correct the actual relative K/S increase is bellow the observed values.

4.2.3 SURFACE MODIFICATION OF CELLULOSE DI- AND TRIACETATE FABRICS WITH CUTINASE

Samples of CDA and CTA fabric were treated with 25 U mL^{-1} of cutinase for 24 hours. Table 4.1 shows the values of increase in colour strength and acetic acid liberated to the reaction medium for both cellulose acetates (the control values were subtracted).

Table 4.1 – Hydrolysis of CDA and CTA fabrics by cutinase. The samples were treated with 1250 U per gram of fabric, at pH 8 and 30 ºC, for 24 hours. Samples and controls were competitively coloured at 50 ºC

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

Evidence of hydrolysis was obtained by the increase in K/S for both fabrics and by the formation of acetic acid for CDA. It was not possible to detect this product in the treatment medium of CTA. Compared to previous results, the levels of acetic acid are lower than the observed for the same esterase activity per weight of the substrate.

The DRIFT technique was used to collect the infrared (IR) spectra of CDA and CTA fabric samples and respective controls in order to obtain further evidence of the hydrolysis of the ester linkage at the surface of treated fibres. This technique allows examining the IR absorption by rough surfaces. The figure 4.3 shows the IR spectra in the region of 1800-1720 cm^{-1} which is the wavenumber region for the stretching vibration of the carbonyl group (Krasovskii

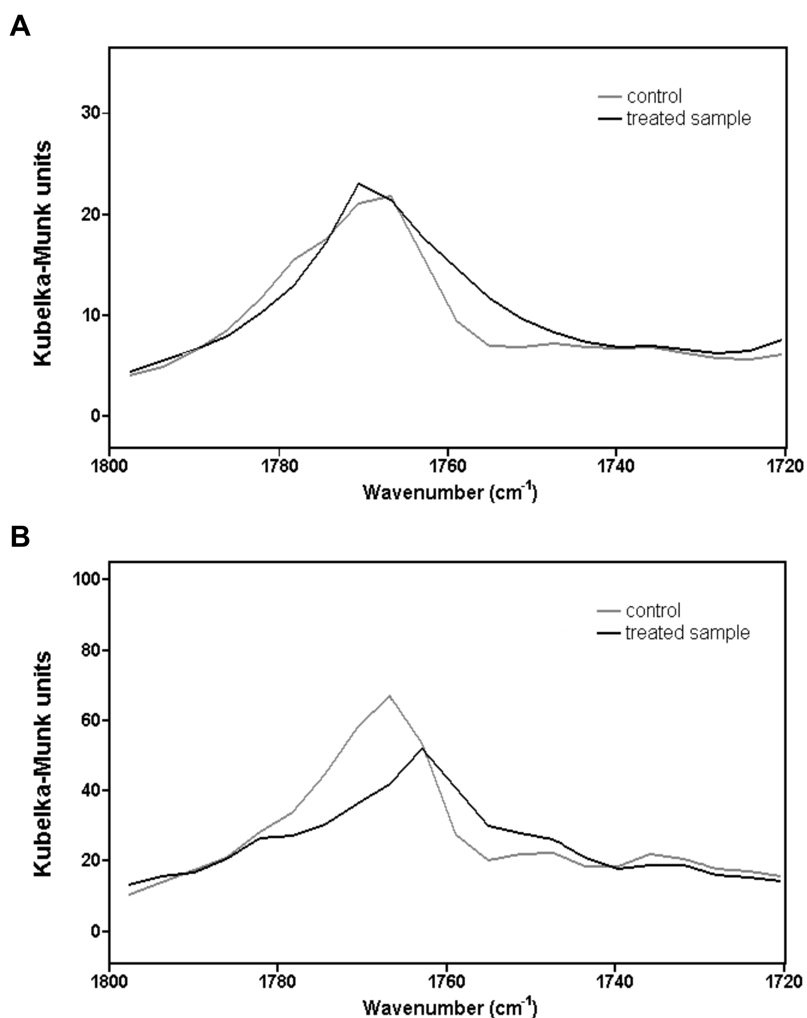


Figure 4.3 – DRIFT spectra showing the carbonyl group stretching band of (**A**) CDA and (**B**) CDT controls and samples. The samples were treated with cutinase (1250 U per gram of fabric), at pH 8 and 30 ºC, for 24 hours.

et al., 1996).

For CTA (figure 4.3B) there was a clear difference in both the intensity and shape of the carbonyl stretching band between the treated sample and the control. There was a decrease in the intensity after the cutinase treatment and there was also a shift of the band to lower wavenumbers. The decrease in the intensity was correlated to the enzymatic hydrolysis of some ester linkages at the surface of the samples. The displacement could be caused by the formation of intermolecular hydrogen bridges between the remaining carbonyl groups and the newly formed hydroxyl groups (Ilharco and Barros, 2000) or it could be due to a preferential hydrolysis of the carbonyl groups at C_2 e C_3 positions (Krasovskii *et al.*, 1996). Regarding CDA (figure 4.3A), the observed differences between the control and sample were considered not significant. The absence of a significant difference was unexpected because in the treatment liquor it was possible to detect acetic acid while for CTA it was not.

Cross sections of fibres treated with cutinase conjugated with FITC were observed by fluorescence microscopy (figure 4.4). The fluorescence signal is located mainly around the fibres of both CDA and CTA. The fibres core does not emit fluorescence indicating that the labelled protein was found at the fibre surface of both fabrics and confirming the superficial action of cutinase on these fibres.

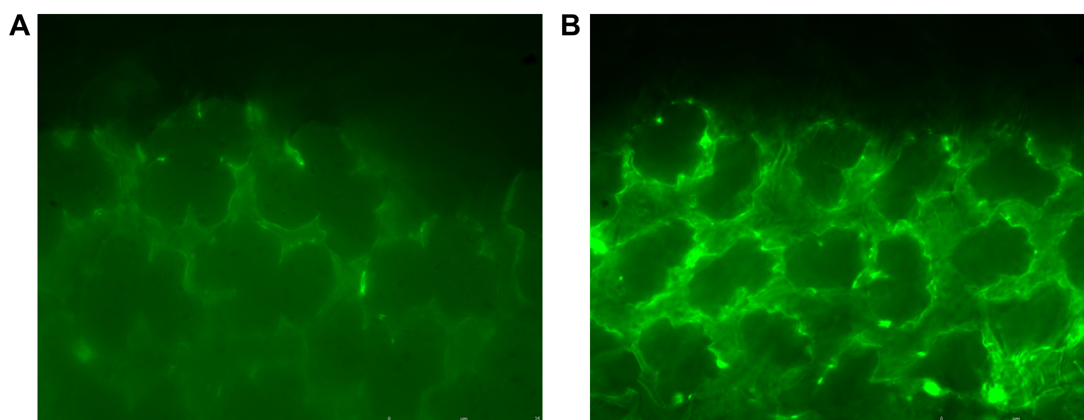


Figure 4.4 – Epifluorescent photographs of **c**ross-sections from (**A**) CDA and (**B**) CTA samples. The samples were treated with 10 mg of FITC-conjugated cutinase per gram of fabric, at pH 8 and 30ºC, for 15 hours. Both images were acquired with a total magnification of 1000x.

SEM images were also obtained for both fabrics treated for 18 hours with 50 U mL^{-1} cutinase (figure 4.5). The surface of CDA was not apparently altered by the enzymatic treatment while a slight fibrillation of the triacetate surface was visible after the cutinase treatment.

The impact of the hydrolysis of acetyl groups should be more drastic on the highly ordered structure of CTA than on the more disordered CDA. From the

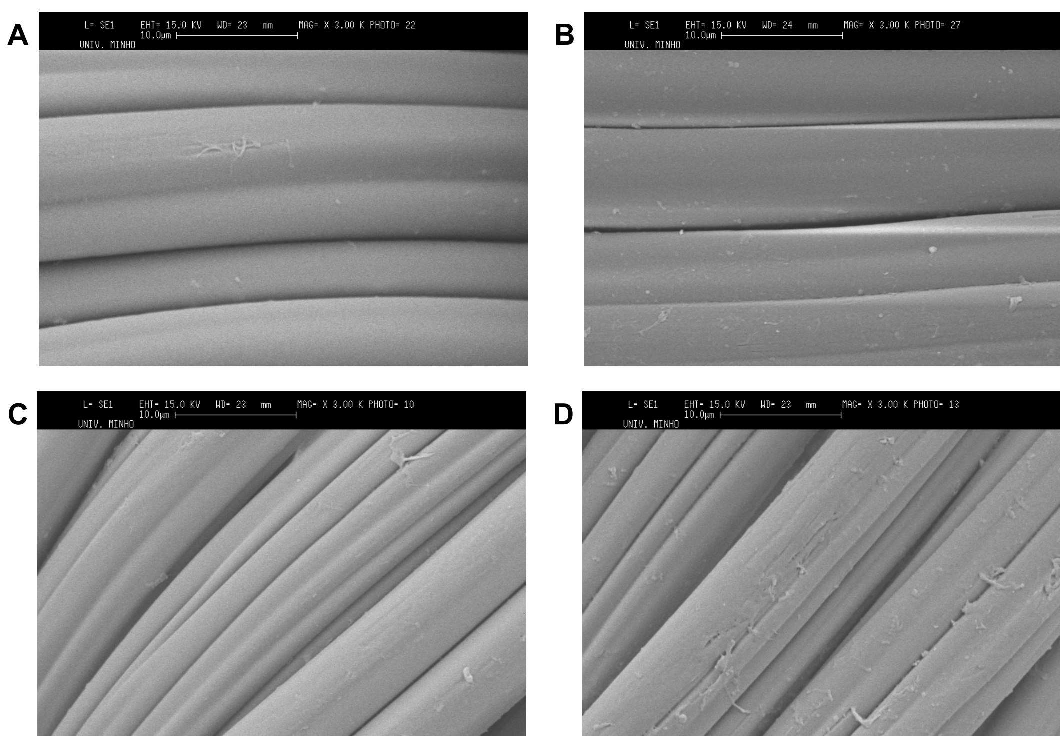


Figure 4.5 – SEM photographs of CDA (**A**) control and (**B**) treated sample, and CTA (**C**) control and (**D**) treated sample. The samples were previously treated with cutinase (5000 U per gram of fabric), at pH 8 and 30ºC, for 18 hours. All images were acquired under the same conditions with a total magnification of 3000x, in a scanning electronic microscope Leica S360.

mathematical fitting of XRD patterns (figure 4.6), crystallinity indexes were determined for CDA and CTA, samples and respective controls (table 4.2). There was a small decrease in the crystallinity index after the enzymatic treatment, for both fibres. CTA was most affected, with a decrease of 12% while CDA had a decrease of 8%.

Cutinase was able to modify the surface of the cellulose acetate fabrics, increasing the number of hydroxyl groups and consequently the hydrophilic

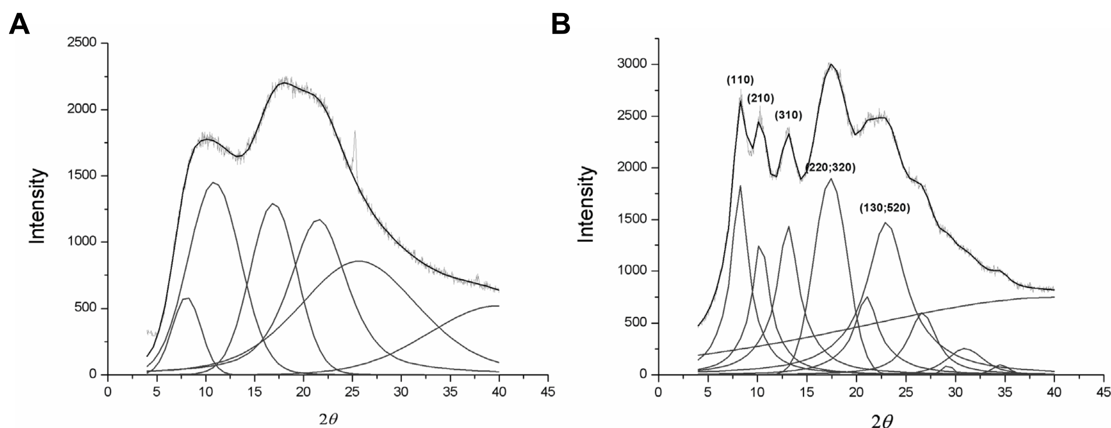


Figure 4.6 – X-ray diffraction pattern obtained for (**A**) CDA sample and (**B**) CTA sample, both treated with cutinase (1250 U per gram of fabric), at pH 8 and 30 ºC, for 24 hours. The XRD data was analyzed by profile fitting of the scans with the Pseudo-Voigt peak function from the software OriginPro 7.5 (Cerqueira *et al.*, 2006)

character and the dye affinity. Since there were changes on the crystallinity index, other physical properties should be tested for a better evaluation of the impact of such surface modifications on the textile performance of these fibres.

Table 4.2 – Crystallinity indexes for CDA and CTA. The samples were treated with cutinase (1250 U per gram of fabric), at pH 8 and 30 ºC, for 24 hours. The crystallinity index (I_C) was determined according to the equation $I_C = A_C/(A_C + A_A)$, where A_c is the total area of the crystalline peaks and A_A is the total area of the amorphous peaks

	CDA	CTA
control	0.58	0.68
treated sample	0.57	0.60

4.3 Concluding remarks

The biomodification of the surface of cellulose acetate with high degree of substitution with cutinase was demonstrated by the acetic acid release and the improvement in the chemically specific colouration of the fabrics with a reactive dye. From the acetic acid release, the hydrolysis yield is higher for the less substituted cellulose acetate fabric, but the consequences of the acetyl hydrolysis are more pronounced for CTA, as shown by the differences in colour,

morphology of the fibres surface and crystallinity between controls and treated samples. Further studies will be necessary to evaluate the impact of cutinase activity in the physical properties of the fabrics and to assess the contribution of the incomplete protein removal and of the physical, rather than chemical, modifications on the differences seen upon enzymatic treatment.

5 Production of Cutinase Fused to Carbohydrate-binding Modules for the Modification of Cellulose Acetate

The enzymatic modification of highly substituted cellulose acetate fibres is a heterogeneous process, therefore, an attempt was made to increase cutinase efficiency towards this substrate by mimicking other carbohydrate-active enzymes with modular nature. Two different carbohydrate-binding modules (CBMs) were fused to the C-terminal of cutinase. The CBMs act synergistically with the catalytic domains by increasing the effective enzyme concentration at the substrate surface and, for some CBMs, by physical disruption of the solid substrate. Two types of CBM were chosen on the basis of ligand affinity, since the two cellulose acetate fibres used in this work are structurally different from cellulose (the native ligand) and different between themselves, presenting two different overall crystallinities.

The production of fusion cutinases with new functionalities is here described and a comparison with cutinase regarding its efficiency for CDA and CTA modification is presented.

5. PRODUCTION OF CUTINASE FUSED TO CARBOHYDRATE-BINDING MODULES FOR THE MODIFICATION OF CELLULOSE ACETATE

5.1 MATERIALS AND METHODS

5.1.1 REAGENTS AND ENZYMES

The cellulose diacetate and triacetate plain woven fabrics used were kindly supplied by Mitsubishi Rayon Co. Ltd., Tokyo, Japan. Their characteristics were described in a previous section (section 4.1.1).

All reagents were laboratory grade reagents from Sigma-Aldrich, St. Louis, USA, unless stated otherwise. The buffer used for the enzymatic treatment of both fabrics was a phosphate buffer having a concentration of 50 mM and pH 8. Other buffers are specified in the text when necessary.

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

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

5.1.2 BACTERIA, PLASMIDS AND GENES

The bacterial hosts used for cloning and expression of cutinase fusion genes were the *Escherichia coli* strain XL1-Blue and strain BL21 (DE3), respectively. The plasmid pGEM®-T Easy (Promega Corporation, Madison, USA) was used to clone and sequence the products of the polymerase chain reaction (PCR). The plasmid pCWT (pET25b(+) carrying native cutinase gene

from *F. solani pisi*, (Araújo *et al.*, 2007) was used to insert the genes coding for the CBMs at the 3' end of the cutinase gene and to express the fusion proteins.

The DNA coding the wild type linker and a CBM of *T. reesei* CBH I, wtCBM$_{CBHI}$, was synthesized and purchased from Epoch Biolabs (Missouri City, USA), as well as, the DNA fragment coding for a smaller linker and the CBM, sCBM$_{CBHI}$. The plasmid pTugN1 containing the gene coding the CBM$_{N1}$ from *C. fimi* CenC was kindly provided by Professor Anthony Warren (Department of Microbiology, University of British Columbia, Vancouver, Canada) (Johnson *et al.*, 1996a).

5.1.3 Plasmid construction

Standard techniques were used for all the DNA manipulations. The wtCBM$_{CBHI}$ and sCBM$_{CBHI}$ were amplified by PCR, using the primers supplied by Epoch Biolabs, and cloned directly into pGEM®-T Easy. Transformants were selected and the gene sequences were confirmed by DNA sequencing, following the method of Sanger (Sanger *et al.*, 1977). The constructs pGEM::wtCBM$_{CBHI}$ and pGEM::sCBM$_{CBHI}$ were digested with *Sac*I and *Sal*I, the DNA fragments were extracted and purified from agarose gels and cloned into the *Sac*I/*Sal*I restricted and dephosphorilated pCWT, resulting in the final pCWT::wtCBM$_{CBHI}$ and pCWT::sCBM$_{CBHI}$ vectors. The CBM$_{N1}$ sequence was PCR-amplified from pTug, with the primers CBM N1 for (5'-ataagaat**gcggccgc**tagcccgatcggggagggaacgt) and CBM N1 rev (5'-accg**ctcgag**ctcgacctcggagtcgagcgc) containing the *Not*I and *Xho*I sites (in bold). The PCR product was cloned into pGEM®-T Easy and a positive clone was selected and confirmed by DNA sequencing. The construct pGEM::CBM$_{N1}$ was restricted with *Not*I and *Xho*I, the DNA fragment was extracted and purified from agarose gel and cloned into the *Not*I/*Xho*I restricted and dephosphorilated pCWT, resulting in the final pCWT::CBM$_{N1}$ construct. The DNA coding the linker PT$_{box}$ of *C. fimi* CenA (Shen *et al.*, 1991) was obtained by PCR amplification of two overlapping primers (underlined sequence) containing the *Sal*I and *Not*I sites (in bold): PTbox for (5'-ctcgagctca**gtcgac**ccgacgccaaccccgacgcctacaactccgactccgacgccgaccccgactc) and PTbox rev (5'-gagggactgcgtc**gcggccgc**ggtaggggtcggtgttggagtcggggtcggcgtcggagtcggagttg). The amplification by PCR consisted in

30 cycles of 20 s at 94 ºC and 20 s at 72 ºC for the Accuzyme extension. The PCR product was cloned into pGEM®-T Easy and a positive clone was selected and confirmed by DNA sequencing. The plasmid pGEM::PT_{box} was restricted with *Sal*I and *Not*I, the DNA fragment was extracted and purified from agarose and cloned into the *Sal*I/*Not*I restricted and dephosphorilated pCWT::CBM_{N1}, resulting in the final pCWT::PT_{box}::CBM_{N1} vector.

5.1.4 EXPRESSION AND PURIFICATION OF CHIMERIC CUTINASES

The constructs pCWT::wtCBM_{CBHI}, pCWT::sCBM_{CBHI}, pCWT::CBM_{N1} and pCWT::PT_{box}::CBM_{N1} were first established in *E. coli* strain XL1-Blue. Medium-scale purifications of plasmid DNA were made and used to transform the *E. coli* strain BL21(DE3). Clones harbouring the constructs were grown, at 15 ºC and 200 rpm, in 2.5 L Luria-Broth medium supplemented with 100 µg mL^{-1} ampicillin until an absorbance $A_{600\ nm}$ of 0.3-0.5 was reached. Cells were induced with 0.7 mM isopropyl-1-thio-β-D-galactopyranoside, and further incubated for 16 hours at 15 ºC. The cells were harvested by centrifugation at 4 ºC (7500 x*g*, 10 min), washed with phosphate buffered saline (PBS) pH 7.4 and frozen at -80 ºC. The ultrasonic disruption of the bacterial cells was accomplished on ice with a 25.4 mm probe in an Ultrasonic Processor VCX-400 watt (Cole-Parmer Instrument Company, Illinois, USA). The lysate was centrifuged for 30 min at 16000 x*g* and 4 ºC. The supernatant was collected, pH was adjusted to 7.6 and imidazole was added to a final concentration of 25 mM. Protein purification was performed with the affinity chromatography system HiTrap Chelating HP (GE Healthcare Bio-Sciences Europe GmbH, Munich, Germany) coupled to a peristaltic pump. The 5 mL column was loaded with 100 mM nickel(II) and equilibrated with the binding buffer (20 mM phosphate buffer pH 7.6, 500 mM sodium chloride, 25 mM imidazole). The samples were loaded and washed with 10 column volumes of binding buffer followed by buffers with 50 and 100 mM imidazole. The fusion proteins (figure 5.1) were eluted with 550 mM imidazole buffer.

The fractions obtained were monitored by SDS-PAGE with Coomassie Brilliant Blue staining. The elution buffer was changed to 50 mM phosphate buffer pH 8 with HiTrap Desalting 5 mL columns (GE Healthcare Bio-Sciences

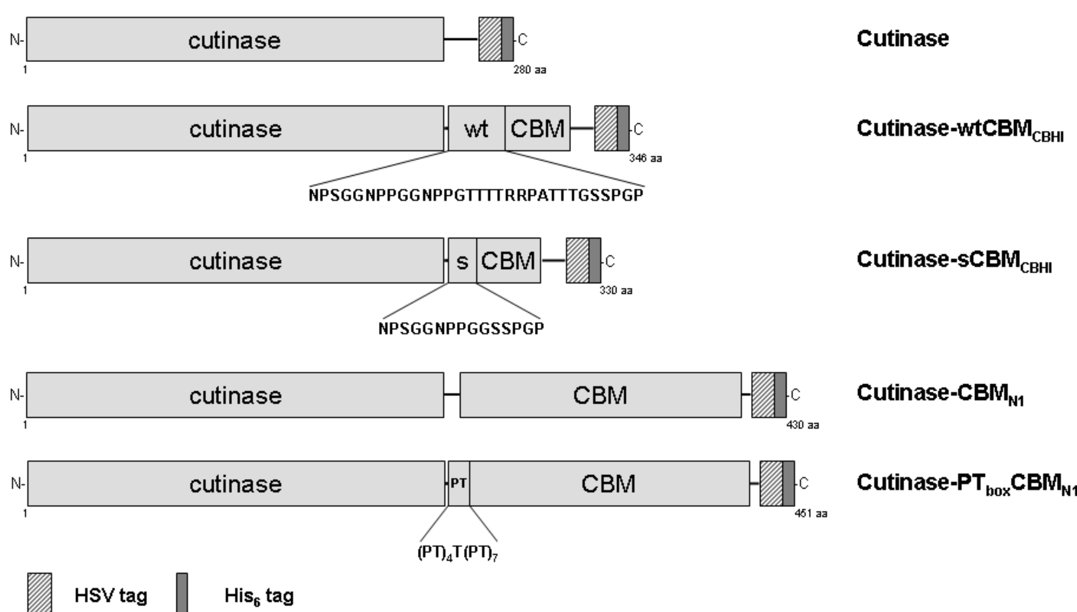


Figure 5.1 – Schematic representation of the recombinant wild-type cutinase from *F. solani pisi* (Araújo *et al.*, 2007) and its new chimeric proteins with the fungal carbohydrate-binding module of CBH I, from *T. reesei*, and the bacterial carbohydrate-binding module N1 of CenC, from *C. fimi*. The amino acid sequence of the linkers is specified in the figure using the one letter code.

Europe GmbH, Munich, Germany). Prior to the 2.5 L culture scale up, western blotting was performed with monoclonal Anti-polyHistidine-Peroxidase Conjugate from mouse to confirm the expression of the fusion proteins. The detection was made with ECL western blotting reagents and analysis system (Amersham Biosciences Europe GmbH, Freiburg, Germany).

5.1.5 Esterase activity assay

Esterase activity was determined following the product release (*p*-NP) continuously through the increase in the absorbance at 400 nm at 30 ºC. The assay conditions for the determination of cutinase activity were described previously (section 3.1.2). All the assays were performed at least in triplicate. Standard solutions of *p*-NP were used to obtain the calibration curve. One unit of esterase initial activity was defined as one µmol of *p*-NP released per minute.

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

All samples of cellulose acetate fabric were washed prior to use in order to remove possible impurities from manufacture and from human handling, as described previously (section 4.1.3).

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

After enzymatic treatment, all fabric samples were washed at 35 ºC, in the Rotawash machine, to remove the adsorbed protein, according to the procedure described previously (section 4.1.3).

5.1.7 Quantification of total protein concentration

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

5.1.8 Cellulose acetate fabric colouration with a reactive dye

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

After the colouration, all samples were washed and the colour was measured as described previously (section 4.1.6).

5.2 RESULTS AND DISCUSSION

5.2.1 CELLULOSE DI- AND TRIACETATE TREATMENT WITH CUTINASE FUSED TO CARBOHYDRATE-BINDING MODULES

For further improvement of cutinase catalysis, several fusion proteins with known and well characterized CBMs were produced. The inclusion of spacers between the cutinase and the CBMs was performed in three of the fusion proteins. The importance of these spacers was studied by several authors mainly through deletion studies. It was demonstrated that linker peptides, connecting the catalytic domains of carbohydrate-active enzymes and the CBMs, are necessary for the synergistic activity between the two domains (Srisodsuk *et al.*, 1993; Shen *et al.*, 1991). The wild-type linker of CBHI was included in the fusion protein with the CBM from the same enzyme. A smaller linker was also used to connect cutinase to the fungal CBM (figure 5.1). The initial purpose was to increase the levels of expression in *E. coli* of the soluble cutinase fused to CBM_{CBHI}, by removing from the wild-type linker a sequence of residues that constitute possible sites for O-glycosylation. Since *E. coli* does not possess the machinery necessary for this post-translation eukaryotic modification, removing those residues could promote correct folding of the fusion protein. The expression levels were very low for soluble cutinase-$wtCBM_{CBHI}$ and were not significantly improved in the case of cutinase-$sCBM_{CBHI}$. The bacterial linker used was the proline-threonine box $(PT)_4T(PT)_7$ present on the CenA from *C. fimi* (Shen *et al.*, 1991). This type of PT linker is also naturally glycosylated, but when it is not, the conformations of catalytic domain and CBM are preserved, since only a partial increase in the linker flexibility seems to occur (Poon *et al.*, 2007).

Protein quantification after the cellulose acetate treatment with cutinase-CBM_{N1} and cutinase-$PT_{box}CBM_{N1}$ was unviable due to the turbidity of solutions. This turbidity happened only for the referred assays, where protein adsorption might be underestimated. The turbidity could be precipitated protein or it could be due to non-hydrolytic disruption of cellulose acetate fibres, in particular, of CDA for which this phenomenon was most visible. This mechanical disruption was already described for cellulose and cotton in the presence of CenA, Cex

and isolated CBMs (Din *et al.*, 1991, 1994; Cavaco-Paulo *et al.*, 1999). Comparing the amount of protein adsorbed and relative K/S between chimeric proteins and cutinase, there was a clear difference between the two cellulose acetates studied (figure 5.2). The fusion of cutinase to the CBMs had a more pronounced effect for the less substituted acetate, independently of the CBM type. The

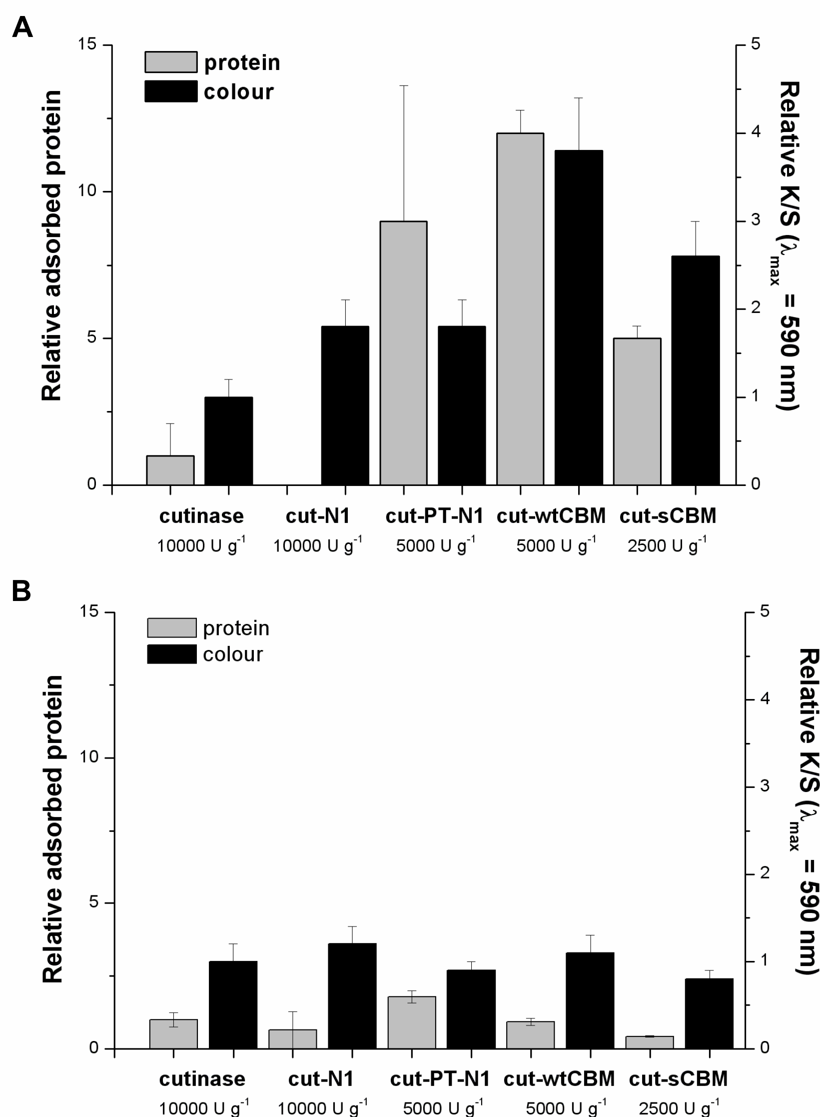


Figure 5.2 – Protein adsorption and relative increase in K/S values for the (**A**) CDA and (**B**) CTA treated with cutinase and cutinase fused to CBMs (initial concentration is expressed in units per gram of fabric). The samples were incubated during 18 hours with cutinase, cutinase-CBM_{N1} (cut-N1), cutinase-$PT_{box}CBM_{N1}$ (cut-PT-N1), cutinase-wt$CBM_{T.reesei}$ (cut-wtCBM), and cutinase-s$CBM_{T.reesei}$ (cut-sCBM), at pH 8 and 30 ºC. Samples and control were competitively coloured at 60 ºC. Relative protein adsorption was calculated as $\frac{P_{0h} - P_{18h}}{P_{0h_{cutinase}} - P_{18h_{cutinase}}}$ and relative K/S as $\frac{K/S_{chimeric\,cutinase} - K/S_{control}}{K/S_{cutinase} - K/S_{control}}$.

steric constrains should be stronger in the triacetate fibre given its higher DS than in the diacetate fibre. The backbone is more fully ornamented with the acetyl groups and consequently the interactions necessary for the recognition of the anhydroglucose units by the CBM should be more impaired on this fibre surface regarding the diacetate fibre. Due to the fact that different initial amounts of protein were used, is not possible to compare directly the protein adsorption behaviour of the several constructs. But it is possible to see for this particular treatment that there was no obvious relation between the colour differences and the amount of protein adsorbed.

Taking into account the different esterase activities used, the cutinase-wtCBM$_{CBHI}$ and cutinase-sCBM$_{CBHI}$ seem the most efficient catalysts under the treatment conditions used. For CDA, the relative K/S was improved 3.8 and 2.6 fold by cutinase-wtCBM$_{CBHI}$ and cutinase-sCBM$_{CBHI}$, respectively, regarding cutinase alone. For treated CTA, the relative increase in K/S was not different between cutinase alone and fused to the fungal CBMs, but the initial esterase activity of cutinase was higher (figure 5.2). The differences in relative K/S were also improved with the fusion of the bacterial CBM to cutinase. For CDA, cutinase-CBM$_{N1}$ improved the relative K/S by 1.8 fold, the same as cutinase-PT$_{box}$CBM$_{N1}$.

The treatment was performed at pH 8 which was the optimum pH for the cutinase. The optimum pH for binding of most CBMs corresponds to the optimum pH for the catalytic domain of the respective carbohydrate-active enzyme and it is in the range of acidic to neutral. The better performance on cellulose acetate fibres of the fungal CBM could be explained by the affinity of CBM$_{CBHI}$ to insoluble ligands being relatively more insensitive to pH than the affinity of CBM$_{N1}$ (Tomme *et al.*, 1996b). Other reason could be the difference in size of both CBMs. The activity of cutinase could be more constrained by the bigger bacterial CBM than by the smaller fungal CBM. Indeed, using half the esterase activity in the treatment with cutinase-PT$_{box}$CBM$_{N1}$, the increase in K/S obtained was in the same range of that with cutinase-CBM$_{N1}$, for both fabrics (figure 5.2). Further studies, aiming at a better characterization of the action of chimeric cutinases on the surface modification of cellulose acetates, would contribute to clarify these issues.

5.3 CONCLUDING REMARKS

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

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

General Discussion, Final Remarks and Future Perspectives

6. GENERAL DISCUSSION, FINAL REMARKS AND FUTURE PERSPECTIVES

6.1 GENERAL DISCUSSION

The textile industry presents well succeed examples of enzyme applications on the processing of natural fibres. For cotton, enzymes can be used almost along all steps of its wet processing, nearly replacing conventional textile practices like the stone washing of denim. Regarding man-made fibres, in particular the synthetic fibres, the scenario is by far different. Nature did not design efficient ways to use and integrate them as part of ecosystems and they are not natural substrates for enzymes. Besides, due to their chemical structure man-made fibres require high amounts of energy and chemicals for their modification in order to achieve the desired properties. Taking into account that they have a market share of more than 50% (Aizenshtein *et al.*, 2003), it is imperative to address the environmental and safety issues brought by their processing and disposal. Finding eco-sustainable alternatives to traditional practices for man-made fibres in an extremely competitive market, such as the textiles, is a hard challenge. The first and critical step is to find, to develop and to improve methodologies for the enzymatic treatment of a given fibre (based on the target chemical bounds of the polymer molecules). However it may not be enough. The manipulation of the structure of enzymes through recombinant DNA technologies may be crucial to the competitiveness of a bioprocess by improving the ability of an enzyme to recognize and use the synthetic fibre as a substrate.

The work presented in this thesis was focused on the application of enzymes to specifically modify two man-made fibres – acrylic and cellulose acetate, producing new functional groups on their surfaces. The enzymatic modification of both acrylic and cellulose acetate fibres was targeted to the pendent groups of the backbone chain of their polymers, which were the nitrile and acetyl groups of acrylic, and the acetyl groups of cellulose acetate.

Chapter 2 reports the employment of a commercial nitrilase on the treatment of acrylics, leading to the superficial conversion of nitrile into carboxylic groups. It was found that nitrilase was able to use acrylic fibre as a substrate with a steady release of ammonia to the reaction media, during 36 hours, at 40 ºC. First important outcome of this work was the improved stability of the enzyme under operational conditions. When incubated in solution, without any substrate, at 30 ºC, nitrilase lost half of its activity after 15 hours. When the acrylic fabric samples were present in the system, there was an adsorption of 80% of total protein and the nitrilase adsorbed did not loose activity during the 36 hours of treatment, since it was not observed a slowing down on the release of ammonia. Second, it can be inferred that the adsorption that takes place is a dynamic process. Either an alternating adsorption and desorption take place, or nitrilase has the ability to move over the acrylic surface like other processive enzymes. Otherwise the release of ammonia would eventually stop and a plateau would be established with time.

Looking to the other and most important product of the biocatalysed reaction – the carboxylic groups at the fibre surface, the results were somehow unforeseen. The treatment of acrylic with nitrilase presented an interesting case study due to the fact that while ammonia was steadily produced the same was not verified for the acidic groups. The increase in K/S of treated acrylic fabric samples was not regular along the 36 hours of treatment. Instead, it was observed a succession of colour strength values higher and lower than the controls, which created an oscillating pattern. The hypothesis was that in some way the groups could leave the surface of the fabric. This was confirmed when the polyacrylic acid was determined in the treatment solutions. The important consequence of this finding is that the nitrilase biomodification of acrylic needs to be properly controlled in order to maximize the concentration of the surface acidic groups.

In order to achieve a proper control over the acrylic biomodification is important to understand better how nitrilase acts on such substrate. Is the enzyme very sensitive to the crystallinity degree of the fibre? How does crystallinity affect the release of polyacrylic acid? How the molecular size of the polymer is related to the released amount of polyacrylic acid? Is there any difference in treating the fibre before and after orientation? It would be

interesting to treat polymers with different compositions, crystallinities and molecular size distributions, as well as, from different stages of the manufacture process to have a more complete picture of the modifications catalysed by nitrilase and to minimize the treatment time.

The acrylic samples, non treated and treated for 4 and 24 hours, were evaluated by WAXS, but no significant difference between the degrees of crystallinity of samples was observed (less than 1%). In a previous work, where alkaline and enzymatic treatments of synthetic fibres were compared, the nitrilase did not affect significantly the breaking strength of the acrylic fabric (Silva, 2002). Once the main chain of each molecule of acrylonitrile copolymer is not chemically altered and because enzymatic hydrolysis is in general superficial, major consequences of such modifications on the bulk properties of these fibres are not expected.

The acrylonitrile represents 93% of the total monomer composition of the acrylic used in this work. The remaining 7% is vinyl acetate which constitutes a possible substrate for another class of enzymes – esterases. Chapter 3 describes the experiments performed with an esterase that has the natural ability to hydrolyse both soluble and insoluble substrates – the cutinase from *Fusarium solani pisi*. The enzyme was very stable and active but the modifications were faint, since only 7% of the surface groups were possible substrates for cutinase. The protein adsorption was practically absent, in contrast to the prior observed nitrilase adsorption, indicating that significant unspecific interactions were not occurring, but also indicating lack of specific binding sites for cutinase. The introduction of stainless steel discs in the reaction media allowed the evaluation of changes, in terms of colour, on the fibre treated for short periods. Besides increasing the mass transfer, the discs may have increased the surface area available for enzymatic hydrolysis by a micropilling effect. Nevertheless, the observation that small amounts of a common industrial acrylic solvent (DMA) improved the activity of the enzyme lead to the hypothesis that the accessibility of this substrate can be higher in early stages of its wet-spinning manufacture. As long as the stability of the biocatalyst is not to much compromised, the industrial tanks after coagulation, where the washing and orientation of the acrylic fibre begin, may be a good starting point for the application of biocatalysts.

In a similar way, cutinase was used to hydrolyse the acetyl groups esterified to the main chains of cellulose acetate (chapter 4). Commercial CDA and CTA fabrics, having different degrees of substitution and, consequently, different degrees of crystallinity and hydrophilic character, were tested. From the formation of the soluble product (acetic acid), during 8 hours, it was possible to conclude that cutinase, like most enzymes, slightly preferred the less crystalline and more hydrophilic substrate, the CDA. When the solid product of the enzymatic reaction (hydroxyl groups) was evaluated through colouration and DRIFT, there was an apparent opposite preference. This result could be due to the hydroxyl groups that are already present at the CDA fibre comparing to their almost absence at CTA fibre. The CDA controls had a much higher capacity to fix the cotton reactive dye than the CTA controls; the K/S values for CDA were around 8 times higher than the ones obtained for CTA. This meant a lower sensitivity of the colouration methodology and maybe a lower sensitivity of DRIFT for the differences in the concentration of hydroxyl groups between controls and cutinase treated samples of CDA fabric. The differences between the K/S and the intensity/shape of the carbonyl stretching band of controls and treated samples of CTA were clearer than the differences obtained for CDA.

The chapter 5 describes the production of cutinases with new functionalities by recombinant DNA technologies and the study of their efficiency for CDA and CTA modification in comparison with the native enzyme. Since the two cellulose acetate fibres used are structurally different from cellulose (the native ligand) and different between themselves, presenting different overall crystallinities, two different CBMs were fused to the C-terminal of cutinase. The idea was to mimic other carbohydrate-active enzymes which also have CBMs that improve their activity. In terms of protein adsorption, the purpose was attained: the fusion with both CBMs increased the affinity of native cutinase for the fibres. In terms of increase in the hydroxyl groups at the fibres surface the results of the colouration were preliminary but there are strong evidences that the chimeric cutinases are more efficient than the native cutinase.

The adsorption of cutinase to the cellulose acetate fibres was higher than the one observed for acrylic, obviously reflecting a higher concentration of recognizable substrates at the fibre surface. However, because the washing

procedure after the enzymatic treatments was milder, in terms of pH and temperature, and because the cellulose acetate is less polar and more hydrophobic than the acrylic taffeta used, it is not possible to exclude unspecific binding of cutinase to the cellulose acetates. It will be necessary to have controls like null mutated or inhibited cutinases for a better assessment of this phenomenon.

6.2 FINAL REMARKS

The general conclusions that can be drawn from the work here described are:

- nitrilase is an adequate biocatalyst for the modification of nitrile groups of polyacrylonitrile copolymers; using a single enzyme, (which is an advantage regarding the enzymatic systems of nitrile hydratase and amidase, reported in the literature) at mild conditions of pH and temperature, acid groups were formed at the fibre surface;
- cellulose acetate and triacetate can have more reactive surfaces using a lipolytic enzyme - cutinase, which is a new and important finding; the surface ‘regeneration’ of cellulose composition can impart these yarns a bicomponent character that would be very interesting for the sportswear field;
- the affinity of cutinase for cellulose acetates can be improved by adding CBMs, resembling natural carbohydrate esterases.

The application of enzymes to the surface modification of acrylic and cellulose acetate fibres, and the resultant new functionalities may lead to eco-friendly finishing steps of these textile materials compared to the traditional ones, saving on water, energy and reducing the amounts of hazardous compounds.

In spite of these findings, many questions remain to be answered which give space for future work.

6.3 FUTURE PERSPECTIVES

In order to transform the biomodifications here reported into cost competitive industrial processes, a considerable amount of work will be needed.

The modification of acrylic by nitrilase needs to be carefully studied in order to maximize the formation of acid groups on its surface, in terms of time and without compromising too much the nitrilase activity. The production of a know nitrilase that can be redesigned to use more efficiently acrylic as a substrate is, beyond doubt, needed. It would be interesting to look for naturally more stable nitrilases, like the ones found in extremophile microorganisms (Cowan et al., 1998; Mueller *et al.*, 2006; Khandelwal *et al.*, 2007), because the presence of high temperatures and organic solvents during the wet-spinning process of acrylic fibres can have deleterious effects on the enzymatic activity.

A better biochemical characterization of cutinase fusion proteins is needed in addition to their interaction with the cellulose acetates. The optimization of the expression system also is needed for higher yields of protein production which is essential for the economical point of view.

It also would be interesting to compare cutinase with natural acetyl esterases in the hydrolysis of cellulose acetates surface, like the acetyl esterase II from *Trichoderma reesei* and *Penicillium purpurogenum* (Margolles-Clark *et al.*, 1996; Hakulinen *et al.*, 2000; Ghosh *et al.*, 2001; Colombres *et al.*, 2008). These enzymes have acetyl esters as natural substrates and their structures are very similar to cutinase.

Finally it would be necessary to study the feasibility of upgrading the optimized processes to an industrial scale and the application of such modified fibres for the production of speciality-properties textile products.

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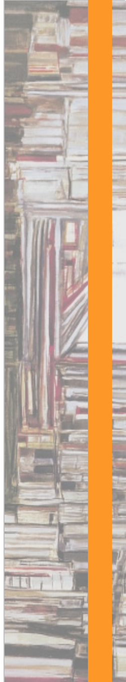

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