THE PERFORMANCE OF CUTINASE AND PECTINASE IN COTTON SCOURING

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THE PERFORMANCE OF CUTINASE AND PECTINASE IN COTTON SCOURING

DISSERTATION

to obtain
the doctor's degree at the University of Twente,
on the authority of the rector magnificus,
prof. dr. W.H.M. Zijm,
on account of the decision of the graduation committee,
to be publicly defended
on Friday 30 September 2005 at 15.00 hrs.

by

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born on 19 February 1975
in Soundad, India

Dit proefschrift is goedgekeurd door de promotor
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en de assistent-promotor
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To my family…

Contents

Summary

Advances in biotechnology and enzymology have brought new lines of research and have accelerated the development of enzymatic applications in wet textile processing for now nearly one decade. Amongst the various stages of cotton preparation, wet textile processing is a highly energy, water and chemicals consuming step. Enzymes are known for their specificity, high efficiency and ability to work under mild conditions and provide a promising solution to these challenges. It is clear that enzyme technology can be used to develop a usable, more environmental friendly, economical competitive scouring process. Several attempts were made to develop an enzymatic cotton scouring process. Still this process faces several problems like a long incubation time, high enzyme doses, sometimes non-uniform enzyme action, uneven dyeing behaviour, high temperature wax removal and overall slow process speeds. The most important aspect identified was the inability to remove cotton fibre waxes. The aim was to explore the potential of enzyme technology to design an efficient and low-temperature scouring process for grey cotton fabrics.

A rational approach is adopted to design a new efficient enzymatic scouring process. Several aspects were considered such as the specificity of enzymes, the complexity of the cotton fibre substrate and mass transfer. To compare the different treatments, the structural contact angle θ was measured with an auto-porosimeter. A model fabric was selected having uniform pore volume distribution (PVD). Since the un-uniform PVD in fabric negatively affects the structural contact angle measurements.

Essential knowledge concerning cotton fibre structure has been gathered upon which the bioscouring process development was based. Wax and pectin removal are identified to be the most important step in scouring process. Removal of non-cellulosic material is translated in terms of change in the structural contact angle. For proper benchmarking, the structural contact angle has been measured for the untreated fabric, for solvent extracted fabric and for alkaline scoured fabric.

Different commercial as well as specially produced pectinases, were tested for bioscouring performance. Alkaline pectinases (PL and Bioprep 3000L) work better than acidic pectinases (PGs). The pectin removal efficiency of specially produced PL was comparable to commercial Bioprep 3000L. The most important parameters such as enzyme concentration, pH, temperature, ionic strength, chelators etc. for the bioscouring process have been evaluated.

The waxy layer contributes significantly to the hydrophobicity of the cotton fibre. Scouring materials have to cross this first barrier in order to attack the primary wall components of the fibre. In case of enzymatic scouring, this thin waxy layer hinders the performance of enzymes targeting for the primary wall. Therefore, it is necessary to recognise the importance of the removal of the waxy layer in cotton scouring. We confirmed our hypothesis, that the removal of the outermost waxy layer will facilitate pectin removal with the pectinases. A wax removal step has been introduced to reduce the pectinase incubation time and to increase the pectinase hydrolytic rate.

The challenge was to remove cotton waxes at low-temperature without organic solvents. We have shown that a cutinase from *Fusarium solani pisi* degrades and removes cotton waxes at a low-temperature (30°C). Within 15 minutes cutinase can achieve almost the same degree of wax removal compared to solvent (n-hexane) extraction.

Surfactants play an important role in the scouring process. Hence, the compatibility of cutinase with a surfactant was investigated. Experiments were done in pH-stat to demonstrate that increased substrate surface area for cutinase action counteracts the negative effect of increased hydrophilicity caused by a surfactant. We have demonstrated that cutinase also removes cotton waxes in presence of high concentrations of Triton X-100. The results clearly demonstrate that cutinase was able to increase pectinase kinetics in terms of pectin removal, equivalent to the n-hexane followed by the pectinase treatment, as a benchmark. Various parameters were evaluated such as pH, temperature, ionic strength, enzyme concentration, and incubation time. The adsorption and catalytic mechanism of cutinase has been explained. Cutinase from *F. solani pisi* has great industrial potential together with pectinase to achieve low-temperature scouring. Cutinase application for a low-temperature wax removal from cotton fibre is a breakthrough towards achieving low-temperature scouring.

Even though cutinase and pectinase can make fabric hydrophilic, the incubation time required to achieve the desired hydrophilicity is longer compared to conventional scouring. Improving mass transfer in the diffusion-controlled process is important for the successful new enzymatic scouring process. Important aspects such as mass transfer improvement via mechanical action and wetting were considered. The wedge apparatus seems to be an excellent tool to study the effect of mass transfer during various stages of the scouring process. The effect of mechanical action was more pronounced when used after pectinase treatment.

At lab scale, sequential and one-step low-temperature enzymatic scouring process was developed using cutinase, pectinase, Triton X-100 as wetting agent and mechanical action. We demonstrate that one-step scouring with cutinase and pectinase is possible at 30°C for 15 minutes. That is half of the time required for the sequential process. From the structural contact angle measurements and SEM pictures, we concluded that pectinase alone is not able to achieve the desired hydrophilicity even though it can remove up to 75% of the pectin from the cotton fibre. To achieve the needed hydrophilicity, the removal of the outermost waxy layer is important. Therefore, to measure only pectin removal as enzymatic scouring efficiency is not a good decisive factor. Confirmations of the scouring results were made by re-evaluating structural contact angle for selected fabric samples, after solvent extraction. Re-evaluation was also done to evaluate the effect of various treatments on the removal of degraded components from the cotton fibres. Results are explained using stagnant core and convective shell model.

We can conclude that, scouring is related to hydrophilicity and can be achieved by uncovering the outer surface layers. The generated knowledge allows a good basis for the development of a fast and continuous bioscouring process. In the future, this enzymatic scouring process can be combined with low-temperature desizing and bleaching to develop an overall energy efficient wet textile process.

Samenvatting

Vooruitgang in enzymologie en biotechnologie heeft nieuw onderzoek doen ontstaan en heeft in het afgelopen decennium de ontwikkeling van enzymatische toepassingen in natte textielprocessen versneld. Natte textielprocessen gebruiken in de verschillende fases in de behandeling van katoen veel energie, water en chemicaliën. Enzymen staan bekend om hun specifieke werking, hoge efficiëntie en de mogelijkheid om gebruikt te worden onder milde omstandigheden en ze zijn veelbelovend om alle uitdagingen in natte textiel processen het hoofd te bieden. Het is duidelijk dat enzymtechnologie gebruikt kan worden om een bruikbaar, milieuvriendelijk, economisch concurrerend scouring-proces te realiseren. Verschillende pogingen zijn gedaan om enzymtechnologie toe te passen voor het scouren van katoen. Dit proces kent echter nog verschillende problemen, zoals een lange incubatietijd, hoge enzymdoseringen, soms niet-uniforme enzymwerking, niet-uniforme verfverspreiding, hoge temperatuur bij de verwijdering van was en langzame processnelheden. Het bleek dat het onvermogen om was van katoenvezels te verwijderen het belangrijkste aspect was. Daarom was de doelstelling om de mogelijkheden van enzymtechnologie te onderzoeken voor het ontwerp van een efficient scouring-proces bij een lage temperatuur voor grijze katoendoeken.

Een rationele aanpak is gekozen om een nieuw efficiënt enzymatisch scouring-proces te ontwerpen. Verschillende aspecten worden onder de loep genomen, zoals de specifieke werking van enzymen, de complexiteit van het katoenvezel-substraat en massatransport. Om de verschillende behandelingen te vergelijken werd de structurele contacthoek θ gemeten met een auto-porosimeter. Er werd een modeldoek met een uniforme porie volume distributie (PVD) uitgekozen. Een niet-uniforme PVD in het doek heeft immers een negatief effect op de structurele contacthoek-metingen.

Essentiële kennis betreffende de vezelstructuur van katoen is vergaard, waarop de ontwikkeling van het bioscouring-proces is gebaseerd. Verwijdering van was en pectine is de belangrijkste stap gebleken in het scouring-proces. Verwijdering van niet-cellulose materialen wordt gerelateerd aan de verandering van de contacthoek. Voor de juiste benchmarking werd de structurele contacthoek van verschillende soorten doek gemeten: onbehandeld doek, in alkalisch milieu gescourd doek en met oplosmiddel geëxtraheerd doek (om het was te verwijderen).

De prestatie van verscheidene commerciële en specifiek geproduceerde pectinases zijn getest in het gebruik voor bioscouring. Pectinases gebruikt in een alkalische omgeving, PL en Bioprep 3000, hebben een betere werking dan pectinases die gebruikt worden in een zure omgeving, PG's. De efficiëntie van de verwijdering van pectine door specifiek geproduceerd PL is vergelijkbaar met het commercieel geproduceerde Bioprep 3000L. De belangrijkste parameters zoals enzymconcentratie, pH, temperatuur, ionsterkte, chelaten, etc., die een grote uitwerking hebben op het bioscouring-proces, zijn geëvalueerd.

De waslaag draagt voor een groot deel bij aan de hydrofobiciteit van de katoenvezel. De chemicaliën die voor het scouren gebruikt worden, moeten eerst deze barrière passeren, voordat de componenten in de primaire katoenstructuur kunnen worden aangepakt. In het geval van enzymatisch scouren belemmert deze dunne waslaag de doorgang van de enzymen naar de primaire katoenstructuur. Het is daarom noodzakelijk het belang van de verwijdering van de waslaag bij het scouren van katoen te onderkennen. Onze hypothese is bevestigd: de verwijdering van de buitenste waslaag zal de verwijdering van pectine door pectinases vergemakkelijken. De stap om eerst de was te verwijderen is geïntroduceerd om de tijd van de incubatie met pectinase te verkorten en om de hydrolyse-snelheid van pectinase te verhogen.

De uitdaging is om het was van het katoen te verwijderen bij lage temperatuur zonder gebruik te maken van organische oplosmiddelen. Wij hebben laten zien dat een cutinase van *Fusarium solani pisi* bij lage temperatuur (30°C) het was degradeert en verwijdert. Cutinase kan binnen 15 minuten nagenoeg dezelfde hoeveelheid was verwijderen als met extractie met een oplosmiddel (n-hexaan) verwijderd wordt.

Oppervlakte actieve stoffen, surfactanten, spelen een grote rol in het scouringproces. Om deze reden is de compatibiliteit van cutinase met een surfactant onderzocht. Er zijn experimenten uitgevoerd met een pH-stat om te demonstreren dat een groter substraatoppervlak het negatieve effect van verhoogde hydrofiliciteit, veroorzaakt door een surfactant, neutraliseert. Wij hebben laten zien dat cutinase ook was verwijdert in combinatie met hoge concentraties van Triton X-100. De resultaten tonen duidelijk aan dat cutinase in staat is om de kinetiek van de pectinase, in termen van pectineverwijdering, te verhogen. Dit was gelijk aan het uitgangspunt: de verwijderde hoeveelheid pectine bij extractie met n-hexaan gevolgd door behandeling met pectinase. Verschillende parameters zijn onderzocht, zoals pH, ionsterkte, enzymconcentratie en incubatietijd. De adsorptie en het katalytische

mechanisme van cutinase wordt uitgelegd. Cutinase van *F. solani pisi* heeft groot industrieel potentieel in combinatie met pectinase om bij lage temperatuur te scouren. De toepassing van cutinase bij de verwijdering van was van katoen bij lage temperatuur is een doorbraak om een scouring-proces bij lage temperatuur te bereiken.

Ondanks dat cutinase en pectinase het doek hydrofiel kunnen maken, is de incubatietijd om een bepaalde graad van hydrofiliciteit te behalen langer, dan bij het conventionele scouring-proces. Het is belangrijk dat het massatransport verbeterd wordt in het, door diffusie beheerste, proces om een succesvol nieuw enzymatisch scouring-proces te ontwikkelen. Er is gekeken naar mechanische actie en bevochtiging om het massatransport te versnellen. Het wig-apparaat blijkt een efficiënt middel om het effect van massatransport tijdens verschillende stappen in het scouring-proces te demonstreren. Het principe van het apparaat is de vervorming van het doek door compressie en het strekken van het doek. Het toepassen van de mechanische actie was duidelijker zichtbaar bij doek dat voorbehandeld was met pectinase.

Een experiment met meerdere stappen en een alles-in-één enzymatisch scouring-experiment bij lage temperatuur is ontwikkeld, waarbij gebruik is gemaakt van cutinase, pectinase en Triton X-100 als wetting agent en mechanische actie. Wij laten met het alles-in-één experiment zien dat het mogelijk is om bij 30°C en in 15 minuten doek te scouren; dit is de helft van de tijd die nodig is om te scouren bij het experiment met meerdere stappen. Wij hebben geconcludeerd met behulp van contacthoekmetingen en SEM-foto's, dat pectinase alleen niet in staat is de gewenste hydrofiliciteit te bereiken, ondanks dat 75% van de pectine van de katoenvezel verwijderd is. Om de gewenste hydrofiliciteit te bereiken, moet de buitenste waslaag eerst verwijderd worden. Alleen de verwijdering van pectine als graad voor het enzymatisch scouren gebruiken, is niet afdoende. Bevestigingen van de resultaten zijn gemaakt door opnieuw de contacthoekmetingen van geselecteerde stukken katoen te bekijken, nadat deze met oplosmiddel behandeld zijn. Tevens is er opnieuw gekeken naar het effect van verschillende behandelingen op de verwijdering van gedegradeerde componenten van de katoenvezels. De resultaten worden verklaard met behulp van het stagnante kern- en convectieve schil-model.

Wij kunnen hier afsluiten met de conclusie dat scouring gerelateerd is aan de hydrofiliciteit en dat een hoge hydrofiliciteit kan worden bereikt door de poriën in het doek bloot te leggen door middel van de verwijdering van was en andere niet-cellulose materialen. De opgedane kennis is een goede basis voor de ontwikkeling

van een snel en continu bioscouring-proces. In de toekomst kunnen energetisch efficiënt scouren en ontsterken bij lage temperatuur gecombineerd worden met bleken en zo een energetisch efficiënt nat textielproces vormen.

1

Introduction

This chapter starts with some information on the history of cotton and presents a journey from cotton fields to one of the most important textile materials. Various stages of textile pre-treatments are discussed. An important step in these pre-treatments, of which effectiveness, efficiency and environmental impact can be improved, is scouring. The state of the art of scouring is presented including traditional alkaline methods and the current situation of scouring with enzymes. The main problems of enzymatic scouring have been identified and defined. Finally, the scope of the thesis is presented.

1.1 Introduction

When human life began on the earth, food and shelter were the two most important necessities. Immediately, thereafter, came clothing. The first materials used for clothing were fur, hide, skin, and leaves. All of them were sheet like, two-dimensional structures, not too abundantly available and somewhat awkward to handle. A few thousand years ago, a very important invention was made to manufacture two-dimensional systems – fabrics – from a simple mono-dimensional element – fibres. It was the birth of the textile industry based on fibre science and technology. Fibres were readily available everywhere; they came from animals (wool, hair, silk etc.) or from plants (cotton, flex, hemp, reeds, etc.). Amongst these natural fibres, cotton is the most used fibre until today.

1.1.1 Cotton history

The word 'cotton' comes from an Arabic word *'qutun'* or *'kutun'* used to describe any fine fabric. Archaeologists found cotton fabric nearly 5000 years ago at 'Mohenjo Daro', an ancient town in the Indus River Valley of India (now West Pakistan) [1, 2]. Around 300BC, the army of Alexander the Great brought cotton goods into Europe, but the cloth was so expensive that only the rich could afford it [2]. In the early 17th century, the southern American colonies began harvesting cotton and making a coarse fabric for their own use. The development of the cotton industry took a dramatic upward turn in the 18th century as Britain acquired colonies suitable for the harvesting of cotton. Moreover, improvements in textile machinery made it possible to spin stronger yarns.

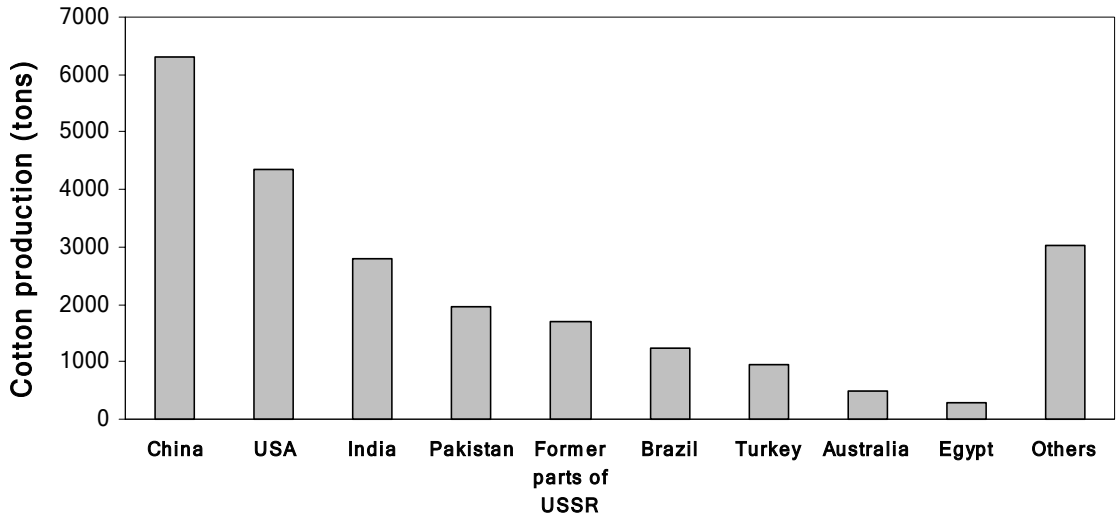

Figure 1.1: World cotton production for the year 2004-2005 [4]

In the early 19th century the southern American states became the biggest single supplier of cotton to the now thriving English textile mills. By the end of the 1920s, the United States was producing more than half the world's cotton [3]. Since then many other countries have increased their production, with manufacturing being carried out mainly in Europe and Asia. Today, cotton is the most widely used fibre. Almost one-half of the total world fibre demand for cotton. Figure 1.1, presents data on world cotton production for the year 2004-2005 [4].

1.1.2 Cotton: from fields to textiles

The word *Textile* comes from the Latin word '*Texere' that* means to weave, and was originally only applied to woven fabrics. It has become, however, a general term for materials made of fibres and yarns. Today the textile industry is one of the largest and basic industries worldwide. The modern textile industry covers different consumer sectors such as apparel textiles, household textiles, medicinal textiles, and technical textiles. The production of fabrics includes many steps starting from raw cotton. A simple illustration of the journey of cotton from fields to consumer is presented in Figure 1.2.

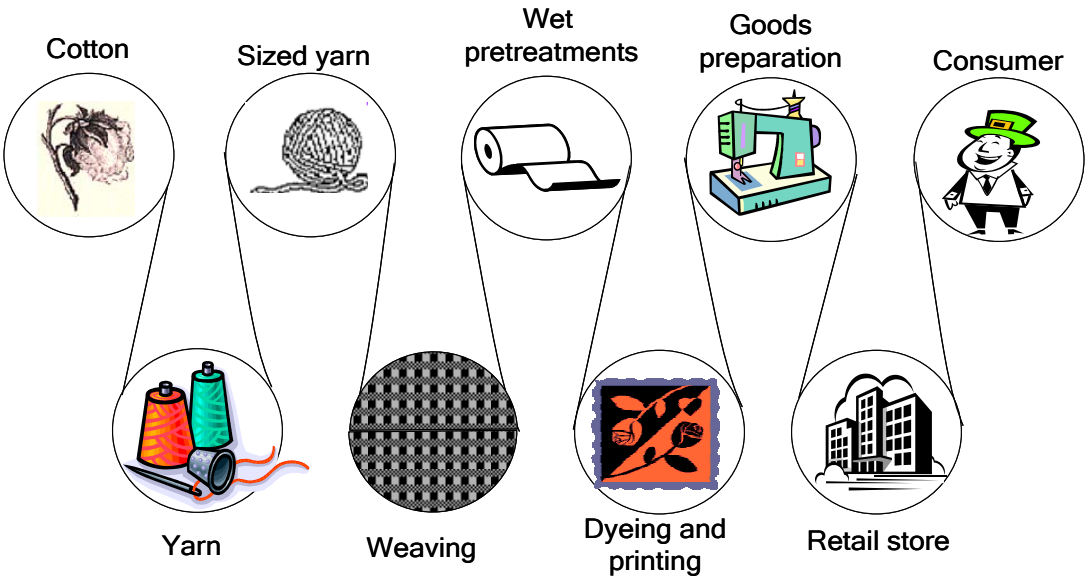

Figure 1.2: Schematic representation of cotton processing chain from fibre preparation to the consumer.

The production of cotton starts with cotton harvesting and converting it into yarns by processes like ginning and spinning. Then the yarns can be converted to stronger yarns by sizing [5]. Sizing makes the wrap yarns stronger and reduces friction during weaving. The resulting textiles are known as grey fabrics [6]. Grey

fabrics are not ready to use, because of their hydrophobic nature (water repellent) and unwanted colours [5, 6]. Therefore, grey fabrics undergo a wet-pretreatment consisting of a chain of chemical treatments that alters the properties of cotton fabric, for example – texture, converting fabrics from hydrophobic to hydrophilic and making them brighter in terms of colour [5]. Thereafter, fabric is dyed and or printed before the final apparel production. Finally the cloths go to consumer via the outlets. Amongst the various stages of cotton preparation as given in Figure 1.2, wet pre-treatment is a highly energy, water and chemicals consuming step [5].

1.2 Wet-pretreatment process for the cotton textiles

Wet-pretreatment textile finishing forms the most important stage in the textile processing chain as shown in Figure 1.3. Wet textile processes are called '*wet' because* they use water as the medium for transport of mass and heat across textile materials. Wet pre-treatment consists of desizing, scouring and bleaching [5, 6]. A short description of these three stages is given below.

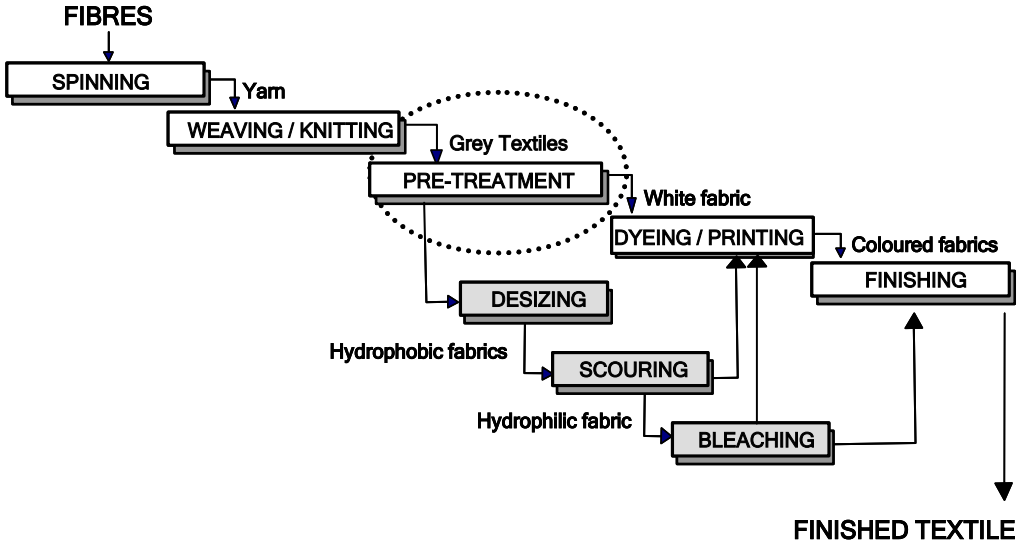

Figure 1.3: An illustration of textile processing chain.

The *desizing* process involves the removal of starch from the fabric. Starch is added to yarns before weaving to strengthen them. Traditionally desizing was carried out using hydrogen peroxide (H_2O_2) and sodium hydroxide (NaOH), however since the 1950's, enzymatic desizing processes based on α-amylases have been widely introduced and implemented successfully in the textile industry. Nowadays

thermostable α-amylases produced by bacteria, especially from *Bacillus subtilis,* are widely available and active in the temperature range of 40-110°C [7].

Scouring - also known as boil off, kiering, kier boiling or caustization is the first process step in which the fibre surface is treated [5]. The objective of a scouring process is to make the material hydrophilic, before it undergoes other processes like bleaching, dyeing and printing (Figure 1.3). A desired hydrophilicity during the scouring can be achieved by removing non-cellulosic material from the cotton fabric, especially from the cuticle (waxes and fats) and the primary wall (e.g. pectin, protein and organic acids). More precisely scouring not only removes non-cellulosic material from cotton fibres but also removes substances that have adhered to the fibres during the production of the yarn or fabric. Substances like, dirt, lint, pesticides, oils, and any sizing agent applied to yarns to facilitate weaving [5, 6, 8-12]. Effective scouring is essential for subsequent processing of any cotton made substrate, regardless of its natural source.

Bleaching is the last chemical process before dyeing that eliminates unwanted coloured matter from fibres, yarns or fabrics (Figure 1.3). Bleaching of cotton fabric needs intense reaction conditions, to make the fabric free from seed coat fragments, and to achieve a required degree of whiteness [5, 6]. Traditionally, a bleaching process is performed oxidatively at an elevated temperature (90°C to 100°C) and at high concentrations of H_2O_2 and NaOH. In the past the enzyme laccase has been tried for bio-bleaching but the results have not been promising [13]. Tzanko *et al.,* [14] claimed that, under controlled laboratory conditions, a whiteness equivalent to industrial bleach can be achieved by H_2O_2, generated via the enzyme glucose oxidase. However, till date no bio-bleaching process has been introduced on the industrial level. Recently the focus has been shifted towards the use of low-temperature oxidative catalyst for the bleaching of cotton fabrics [15].

Wet-pretreatment steps for fabrics involve the addition or removal of solid-liquid substances to or from the fibre surfaces. Therefore, mass transfer is an important phenomenon in wet-pretreatment of textile finishing [16]. In order to improve existing wet pre-treatment processes, knowledge of several scientific disciplines such as enzyme technology, biochemistry, fibre science, polymer technology, colour chemistry, mechanical engineering, chemical engineering and applied physics is required. Thus, wet textile processing can be seen as the most knowledge-intensive area of the entire textile processing chain. The work in this thesis is focused on overall improvement in the wet pre-treatment process by developing a new low-temperature, fast enzyme based scouring process.

1.3 Overview of the alkaline scouring for grey cotton

One of the earliest techniques for the scouring of cotton fabric involved the use of potash, the by-product of wood combustion [5, 6]. A major component of wood ash is potassium oxide, and when wood ash is added to water, the potassium oxide reacts to produce potassium hydroxide, a strong alkali. Early textile chemists prepared their fabric by treating the fabric in hot slurries of potash, followed by neutralisation of the treated fabric in solutions of buttermilk [5, 6]. Although it can be argued that the chemicals used by ancient textile chemists were of *'natural'* origin, the release of these natural substances did not have a positive impact on the environment. Soap is an example of a supporting scouring agent, before the introduction of synthetic detergents. The major drawback with soap as a supporting agent is its tendency to form insoluble calcium salts or *'scum'* in hard water [17].

Even today, alkaline scouring of cotton is still the most widespread commercial technique for removing or rupturing the fibre cuticle to make the fibre absorbent for the cotton processing. Various scouring agents used in the textile industry are listed in the Table 1.1 [6]. Although sodium hydroxide is used generally for the scouring, sodium carbonate and calcium hydroxide are also mentioned as a scouring agent [5, 6, 17-19]. Scouring of cotton fabric is typically done with a hot solution (90°C to 100°C) of sodium hydroxide (± 1 mol/L) for up to one hour [5]. The concentration of alkali used and the time and temperature conditions needed depend on the condition of the starting materials and the desired quality of the scoured fabric. Reducing agents are added during the scouring process to prevent oxidation of cellulose by air oxygen at high pH.

Other chemicals for instance, wetting agents, emulsifying agents and chelating agents [17-19] are also included in typical preparation baths for scouring. Wetting agents act by reducing the surface tension of water enabling improved penetration of the chemicals into the cotton fabric. Emulsifying agents assist in removing waxy materials. Chelating agents remove polyvalent metal ions such as calcium, magnesium, iron or other salts that can have a harmful effect on subsequent wet-processing operations. Polymeric materials can also act as chelating agents or as pickup enhancing agents for the application in continuous preparation processes. These various chemicals tend to be used in excessively high amounts [5, 6, 18].

The key factors for a successful industrial scouring process are the concentration of the NaOH, the treatment temperature, the reaction time and the exclusion of air to avoid weakening the fibre by the formation of oxy-cellulose [6]. In

view of the high extracting action of the scouring process, the final effect obtained also depends on the efficiency of the subsequent rinsing steps.

SN	Scouring agent	Chemicals
1	Alkaline agents	NaOH, KOH, Na_2CO_3, Liquid NH_3 sod. metasilicate, sod. silicate, sod. phosphate, trisodium phosphate, tetrasodium phosphate, sodium tripolyphosphate and borax
2	Surfactants	Anionic activator, non-ionic activator
3	Organic solvent	Chlorine system: Carbonate trichloride, trichloroethylene, perchloro ethylene, methyl chloroform, trichloro methane, fluorine
		Hydrocarbon system: Benzene, industrial gasoline, white spirit, solvent naphtha

Table 1.1: Classification of traditional scouring agent [6].

1.3.1 Scouring with NaOH - mechanism of action

Cotton fabrics arrive at mills with number of impurities. This includes motes, seed coat fragments, pesticides, dirt, chemical residues, metallic salts of various kinds, and immature fibres. The clear mechanism of action of alkaline scouring on various non-cellulosic materials in the cotton fibre is given in Table 1.2.

Scouring with NaOH cleans by physical loosening fragments from the fabric and by dissolution of metallic salts and chemical residues. It softens and preconditions the seed coat fragment materials entrapped in the yarns and the fabrics. The dilute alkali swells the seed coat fragment material and opens up the cell structure to access the hydrogen peroxide in bleaching that takes place later. The swelling process helps to loosen the attachment of the seed coat fragments from the yarn and fibres. High temperature in the presence of sodium hydroxide melts some of the waxy material and converts some of it to a water-soluble form. It also converts non-cellulosic material (pectins, hemicelluloses and proteins) in the cuticle-primary wall to water soluble forms to effect removal. The cotton fibre morphology and detailed structure is given in Chapter 2.

More precisely, the scouring process is based on the reaction between cotton impurities and alkali hydroxide. Traditional scouring implies a certain alkali consumption that determines the minimum concentration of sodium hydroxide to be used. When sodium hydroxide is brought into contact with the cotton fabric, some of

the alkali absorbs, since the hydroxyl groups of cellulose have a weak acidic character [5]. So, at pH around 13-14, cellulose absorbs about 1% or 10 g/kg of sodium hydroxide. Alkali is also required to neutralise the carboxyl group of the pectins. About 0.5% of the sodium hydroxide concentration is required to change the pectins into water soluble salts of pectic or meta-pectic acid [6]. Neutralisation of the amino acids obtained by hydrolysis of the protein used around 1% of sodium hydroxide. It is evident that around 3% to 4% (±1 mol/L) of sodium hydroxide is necessary for the saponification of waxes and to maintain sufficient alkalinity [5, 6]. Literature reveals that fats are esters of fatty acids with glycerol and constitute around 37% to 47% of the total fat constituents. They have low melting points and are hydrolysed into soaps and glycerol (saponification reaction) quite easily using an aqueous solution of NaOH [7, 18, 19].

So, during the scouring process, the intra and intermolecular hydrogen bridges of the cellulose are cleaved and the polar hydroxyl groups of the polysaccharides are solvated. The fabric swells, and this facilitates transport of the impurities from the interior of the fibre to the outside.

Impurities	Mechanism of impurity removal
Fats and waxes	• Saponification: The saponifiable parts of waxes (fatty acid, glycerides, and esters) are converted in to soap. • Emulsification: The non-saponifiable parts of the waxes such as alcohols and hydrocarbons are emulsified by the soap formed. • High temperature: melts some of the waxy materials and converts some of it to a water soluble form. • In extreme cases the use of solvent is necessary.
Pectin and related substances	• Solubilisation: by the action of alkali, which also acts as a swelling agent to facilitate removal • Pectins are converted to water soluble salts of pectic or meta-pectic acid
Proteins and amino acids	• Hydrolysis: Proteins are hydrolysed with the formation of soluble sodium salts of amino acid.
Hemicelluloses	• Dissolution: Hemicelluloses with low DP are dissolved in NaOH.
Inorganic substances, minerals and heavy metals	• Partially dissolve in NaOH • By producing more soluble salt e.g. acid demineralisation • By use of sequestering or chelating agents.

Table 1.2: The removal of impurities of cotton fibre during alkaline scouring process [5, 6, 17-19].

1.3.2 Drawbacks associated with the alkaline scouring

The scouring process requires large quantities of chemicals, energy and water and is rather time consuming [7]. Owing to the high sodium hydroxide concentration and its corrosive nature, intensive rinsing is required that leads to a high water consumption. The use of high concentrations of sodium hydroxide also requires the neutralisation of wastewater, which requires additional acid chemicals. Furthermore, the alkaline effluent requires special handling because of very high BOD and COD values. Apart from the above wet processing problems, the biggest drawback of alkaline scouring is a non-specific degradation of cellulose that produces fabrics of lower tensile strength and therefore of lower quality. Moreover alkaline scouring is hazardous to the workers and creates an unpleasant work atmosphere. Although, alkaline scouring is effective and the costs of NaOH are low, this process can be improved considerably to meet today's energy and environmental demands.

1.4 Scouring with solvents

Solvent scouring appears to be an alternative to the aqueous scouring and is particularly suitable for polyester or woollen fabrics. Solvent processing has been developed due to the reduced water pollution, reduced energy consumption and costs apart from an effective removal of the impurities. Solvent scouring gives excellent results in terms of uniformity, reproducibility and high absorbency [5, 6, 17, 20]. The most widely used solvents for textile processing are the chlorinated hydrocarbons, e.g. tetrachloroethylene (perchloroethylene), trichloroethylene and 1,1,1-trichloroethane [21]. Usually stabilisers and booster solvents are added during the process to stabilise the solvent and to make the process more efficient. The use of a detergent is also reported for enhancing the detergency of the scouring process. Examples of such solvent detergents are mono-ethanolamine, alkyl-benzene sulphonate, alkyl poly-glycol ether and alkyl pyridine chloride [22]. The use of solvent scouring is limited because of the increasing governmental and environmental restrictions. Several drawbacks are associated with the solvent scouring. Only waxes are removed by this method and therefore some form of alkaline scouring is still required [23]. Most of the scouring solvents are flammability and/or carcinogenic in nature. Moreover, there is the need of the system to recover the solvent from the fabric after processing. This is why solvents have very limited applications for cotton scouring.

1.5 Overview of enzymatic scouring

Enzymes are substrate specific bio-catalysts; they operate best at ambient pressures, mild temperatures and often at a neutral pH range. Enzymes are gaining an increasingly important role as a tool in various wet textile pre-treatment and finishing processes [7, 8, 20]. Biocatalysts have proven to be a flexible and reliable tool in wet textile processing and a promising technology to fulfil the expected future requirements. Enzymatic scouring has been investigated extensively by various institutes and laboratories now for nearly one decade [9-11, 24-25]. Initial investigations explored the possibility of cotton scouring with enzymes, to see if cotton could be made hydrophilic in a reasonable time. Extracellular enzymes involved in the degradation of the plant cell wall's outer layer during the invasion of the plant, excreted by phyto-pathogenic fungi and by bacteria have been considered as candidates.

Different enzymes like pectinases such as lyases (EC 4.2.2.2); polygalacturonase endo acting type (EC 3.2.1.15) and polygalacturonase exo acting type (EC 3.2.1.67), proteases (EC 3.4.21-25), cellulases such as endoglucanases (EC 3.3.1.4); cellobiohydrolases (EC 3.2.1.91), xylanases (EC 3.2.1.8), lipases (EC 3.1.1.3) and recently cutinases (EC 3.1.1.74) have been examined to degrade and subsequently remove the natural component present in the outer layer of cotton fibres [9-11, 25-28]. These studies incorporated staining tests, scanning electron microscopy (SEM), weight loss analysis, cotton wax residue and nitrogen content analysis.

A schematic representation of enzymatic scouring adopted by various researchers in the past is presented in Figure 1.4 [8-13]. The scheme essentially contains the impregnation of cotton fabric with one or more enzymes in presence or absence of surfactants and chelators, followed by a high temperature rinsing step. The enzyme incubation time used, was up to 24 hours depending on other process conditions and the density of the fabric.

Lipases, were found to be less effective in fulfilling this task [10]. Proteases were found to be efficient to improve whiteness rather than hydrophilicity [9]. Cellulases were the only enzymes reported to improve the wettability efficiently when applied without any other treatment or in combination with other enzymes. However, cellulase also cause a decrease in fibre strength and hence a decrease in fabric quality [25, 26, 29-34]. The best results have been obtained by alkaline pectinases or pectinases in combination with cellulase. Especially bacterial alkaline pectinase, a pectate lyase (EC 4.2.2.2) has been proven to be effective [10, 11]. Hardin [8]

postulated that pectin acts as cement in the primary wall of cotton fibres. After enzymatic destabilisation of a pectin structure, the different components present in the primary wall layer can be removed easily in subsequent rinsing steps. A proper interpretation of the enzymatic action on cotton fibres on a molecular basis was not possible because of the lack of structural knowledge of cotton fibre.

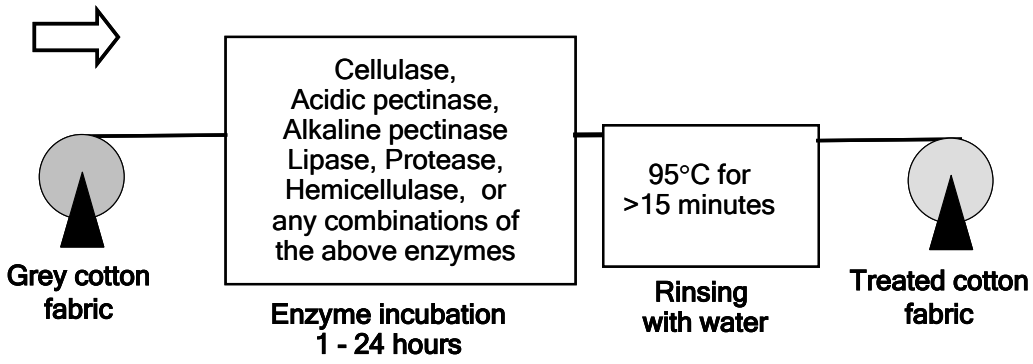

Figure 1.4: A typical adopted approach in past towards enzymatic scouring process of cotton fabrics [8-11, 30, 33, 34].

From the literature, it can be concluded that enzymatic scouring of cotton fibre is possible [8-13, 30, 33, 34]. However, it still faces several problems such as longer incubation time with enzymes, non-uniformity of enzyme treatment and sometimes a lack of fundamental knowledge to explain the obtained results. The potential advantages that can make enzymatic scouring commercially attractive include better quality (texture and tensile strength), less waste water, energy savings and compatibility with other processes, machinery and materials.

1.6 Problem definition, research strategy and scope of the thesis

Several attempts were made to develop enzyme technology for cotton scouring. Still this process faces several problems like a need for long incubation time, high enzyme doses, sometimes non-uniform enzyme action, uneven dyeing behaviour, high temperature water treatment before enzyme incubation and overall slow process speeds [8-13, 30, 33, 34]. A rational approach is necessary to design a new efficient enzymatic scouring process. Several aspects such as the specificity of enzymes, the complexity of the substrate (cotton fibre) and mass transfer, need to be considered for a successful and efficient enzymatic scouring process. A systematic approach will lead to the design of a new cotton scouring process in which the

selected enzymes are efficiently used to remove specific unwanted components to achieve hydrophilic fabric.

The aim of this research is to study, the potential of enzyme technology to design an efficient and fast low-temperature scouring process for grey cotton. In other words, the aim is to obtain a hydrophilic cellulosic fibre surface, which is well accessible for chemicals that are used in textile treatment steps like bleaching and dyeing. Typical problems encountered with the existing scouring process are addressed throughout the thesis.

1.6.1. The research strategy and scope of the thesis

The present work was carried out as part of the European project GRD1-1999-10671: BPT, which stands for continuous bio-pre-treatment of cellulosic fibres. The aim of the project was to develop new environmentally friendly and economic methods for cotton pre-treatment. A clear strategy is required to achieve sufficient hydrophilicity at lower temperature and without using hazardous chemicals. The proposed research strategy for the new enzymatic scouring process (for converting hydrophobic cotton fabric in to the hydrophilic fabric) is presented below and illustrated in Figure 1.5.

Step I: To design a new enzymatic scouring process on a rational basis, knowledge of cotton fibre structure and morphology is essential. This is important because most enzymes are substrate specific, so the selection of enzymes depends on the composition of the cotton fibre. Detailed knowledge of all constituents in the waxy layer and primary wall (see also section 2.2.1 and Figure 1.5) and how unwanted cotton constituents in those layers are interconnected with one another is vital for this research. The detailed structure of cotton fibre is presented in Chapter 2.

Step II: To evaluate the new scouring process, we need a proper benchmark. The removal of particular components from the fibres must be related to a measurable change in hydrophilicity and performance. As explained in section 2.6 and shown in Figure 1.5, wax and pectin removal are essential during enzymatic scouring. It is important to translate the scouring task into removal of particulate components (waxes and pectins) from the fibre. After enzyme treatment, the cuticle and primary wall are removed, so the fibre left with underneath hydrophilic cellulosic layer. All the mentioned aspects in this step are covered in Chapter 4.

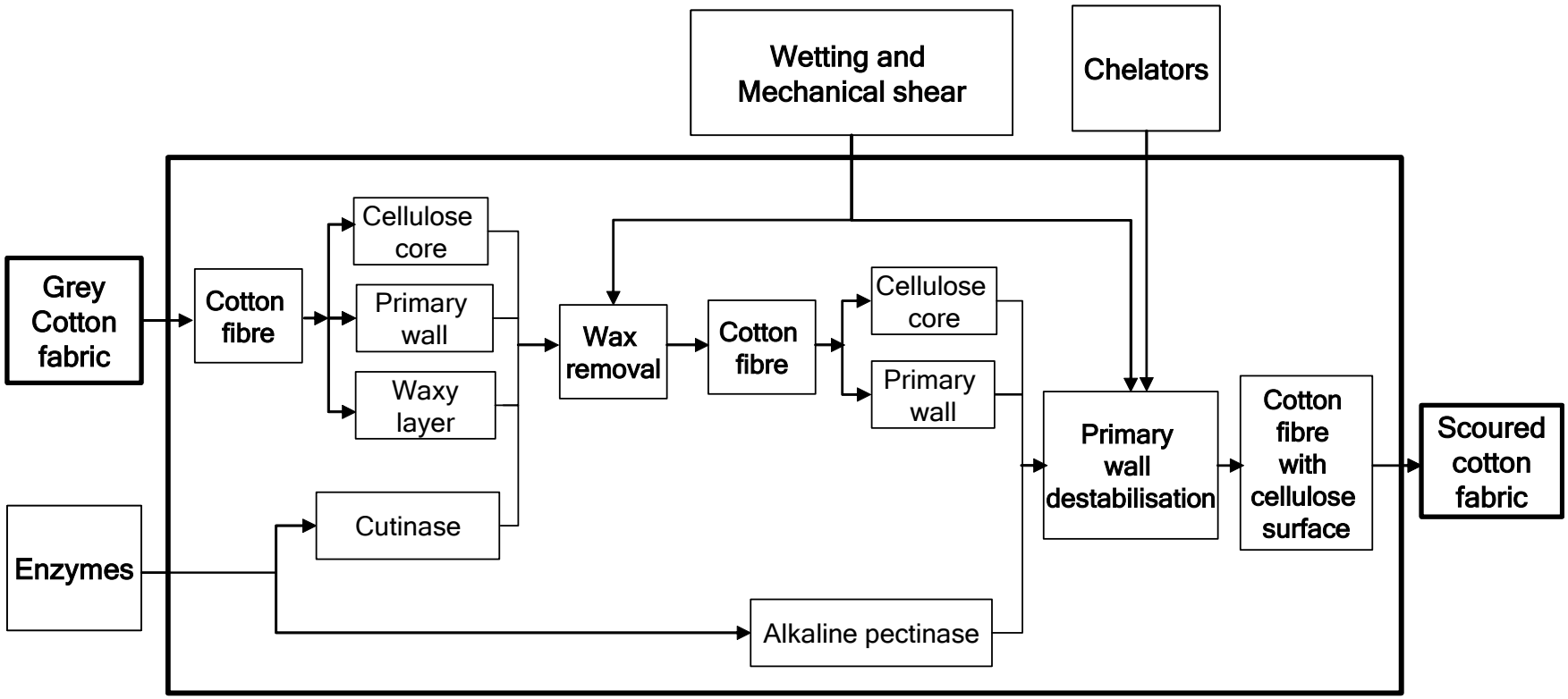

Figure 1.5: An illustration of various steps identified to achieve fast and efficient enzymatic cotton scouring process.

Step III: Based on structural information gathered in step I, the most appropriate enzyme for the removal of cuticle and primary wall will be identified. As discussed in section 3.1 and illustrated in Figure 1.5, it is clear that cutinase and alkaline pectinase are most promising enzymes for the cuticle and primary wall destabilisation respectively. Apart from discussing various experimental tools, Chapter 3 also covers all the information related to step III.

Step IV: From step I, II and III it is clear that the removal of the waxy layer is the first most logical step in the scouring process. In Chapter 4, we confirm our hypothesis, which states that the removal of outermost waxy layer will facilitates pectin removal with the pectinases. To confirm our hypothesis, n-hexane - a non-polar solvent will be used for the wax removal. This will lead to improved pectinase performance in terms of increased hydrophilicity as well. Chapter 4 covers the results related to pectin degradation with pectinases and optimisation of process parameters.

Step V: The challenge is to remove cotton waxes in an efficient and environmentally friendly way. Cotton wax degradation with cutinase is studied and data are presented in Chapter 5. To set a benchmark, wax removal efficiency of cutinase is compared with solvent extracted fabrics. As discussed in Chapter 5, we have proven that efficient wax removal with cutinase treatment improves the performance of pectinase.

Step VI: Even though cutinase and pectinase can make fabric hydrophilic, the incubation time required to achieve the desired hydrophilicity is longer compared to conventional scouring. Fast wetting, mass transfer improvement and the influence of mechanical action, are issues that need to be considered for a faster and more efficient enzymatic cotton scouring process. As illustrated in Figure 1.5, diffusion governed processes can be converted to faster processes by applying mechanical action and efficient wetting. Efficient wetting (Chapter 5), mechanical action together with cutinase and pectinase will result in an efficient destabilisation of waxy layer and primary wall. This will lead to the desired hydrophilicity that needs to be achieved during scouring.

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2

Cotton Fibre Structure and Composition in Relation to Enzymatic Scouring Process

To design a new environmentally acceptable and industrially viable enzymatic scouring process, it is necessary to have insight of the structure and composition of a cotton fibre. This is important because most enzymes are substrate specific. So the selection of enzymes depends on the composition of the substrate i.e. fibre. The structure and composition of the cotton fibre has been established on the basis of thorough literature study. The detailed structure and morphology of the cotton fibre forms a good reference for any process that deals with its surface modification.

2.1 Introduction

Most of the research done to develop an innovative enzymatic scouring process was focused on the potentials of different enzymes [1-10]. In this study a rational approach is adopted to design a new efficient enzyme based cotton scouring process. The main aim is to find suitable enzymes for the scouring purpose and to use it efficiently in the process. To do so, detailed knowledge of cotton fibre structure is required. A question that needs to be answered for designing a new scouring process is, what to modify in cotton fibres to make them hydrophilic. Related questions are which specific components need to be removed and how to remove these unwanted components from the fibre. Apart from studying, each constituent in detail, the knowledge of how these unwanted cotton components are interconnected is vital. Knowledge of the interactions between cotton fibres and enzymes/chemicals/additives is essential for proper interpretation of the obtained results. All these issues are directly related to the fundamental knowledge about cotton fibre structure and morphology.

A thorough literature review has been done on this issue, including the latest insights from plant science. That reveals structural information that directly and strongly influences the selection of enzymes for developing a new scouring process. The gathered information about the cotton fibre is focused on those aspects that can affect the performance of an enzymatic scouring process. To complete the cotton fibre structure, some additional information on the secondary wall, colouring matters and the metal contents as well have been given.

2.2 Cotton fibre structure

Cotton is a seed hair fibre of the plant belonging to *Gossypium,* the botanical genus of the *mallow* family [11]. After flowering, an elongated capsule or boll is formed in which the cotton fibres grow. Once the fibres grow completely, the capsule bursts and fibres come out. A cotton capsule contains about 30 seeds and each seed hosts around 2000 to 7000 seed hairs (fibres). Depending on the cotton type and growing conditions, the colour of the fibre is usually creamy white or yellowish. The average fibre length of different kinds of cotton varies from 22 to 50 mm, and its diameter, from 18 to 25 µm. The higher quality fibres are known as long-staple fibres or extra-long staple.

2.2.1 Cross section of cotton fibre

The cell wall is a dynamic structure which composition and form can change markedly, not only during cell growth but also after the cells have become matured [11]. The cotton fibre is structurally built up into concentric zones and a hollow central core known as the lumen. The mature fibre essentially consists of (from outside to inside) - the cuticle i.e. the outermost layer, the primary cell wall, the secondary wall and the lumen [11, 12]. Figure 2.1 systematically shows the different layers present in the cotton fibre with the compositions of each layer. Cotton contains nearly 90% of cellulose and around 10% of non-cellulosic substances, which are mainly located in the cuticle and primary wall of the fibre. Typical components in dry mature cotton fibres are given in Table 2.1. From this table it is clear that most of the non-cellulosic materials are present in the outer layers of cotton fibre.

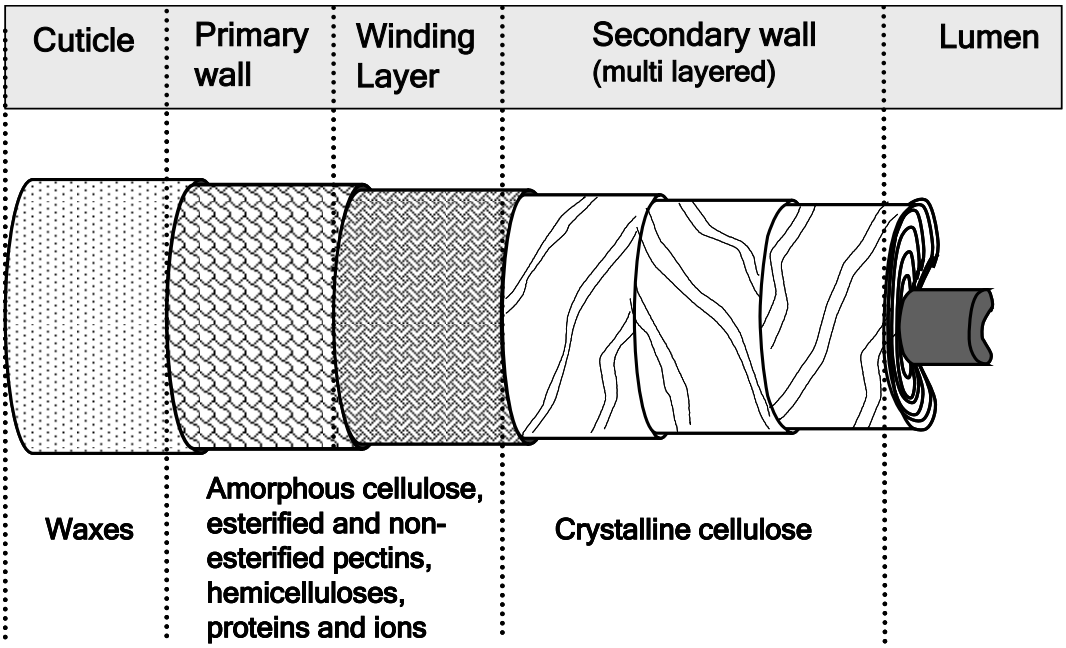

Figure 2.1: A schematic representation of mature cotton fibre showing its various layers.

Figure 2.2 illustrates schematically the distribution of cellulose and other non-cellulosic materials in the various layers of cotton fibre. Figure 2.3 shows some SEM photos of different layers of the mature cotton fibre. The outermost layer is the cuticle (Figure 2.3b). It is a thin film of mostly fats and waxes. Figure 2.3b shows the waxy layer surface with some smooth grooves. The waxy layer forms a thin sheet over the primary wall that forms grooves on the cotton surface. The primary wall (Figure 2.3c), comprises of non-cellulosic materials and amorphous cellulose in which the fibrils are arranged in a criss-cross pattern [13, 14]. Owing to non-structured orientation of

cellulose and non-cellulosic materials, the primary wall surfaces is unorganised and open. This gives the flexibility to the primary wall, which is required during the cell growth. The basic ingredients, that are responsible for complicated interconnections in the primary wall (Figure 2.3c), are cellulose, hemicelluloses, pectins, proteins and ions. These components are present throughout the primary wall. The only difference is the concentration and nature of each component, as when approaching the secondary wall. In the secondary wall, only crystalline cellulose is present, which is highly ordered and has compact structure (Figure 2.3d). As seen from Figure 2.3d, the cellulose fibrils in the secondary wall are laying parallel to one another.

Constituents	Composition (%)	
	Whole fibre	Outer layer
Cellulose/xylo-glucan	94	54
Waxes	0.6 – 1.3	14
Pectic substances	0.9 – 1.2	9
Protein (nitrogen substances)	0.6 – 1.3	8
Ash	1.2	3
Organic acids	0.8	-
Others	1.4	12

Table 2.1: Typical composition of dry mature cotton fibre [11, 26].

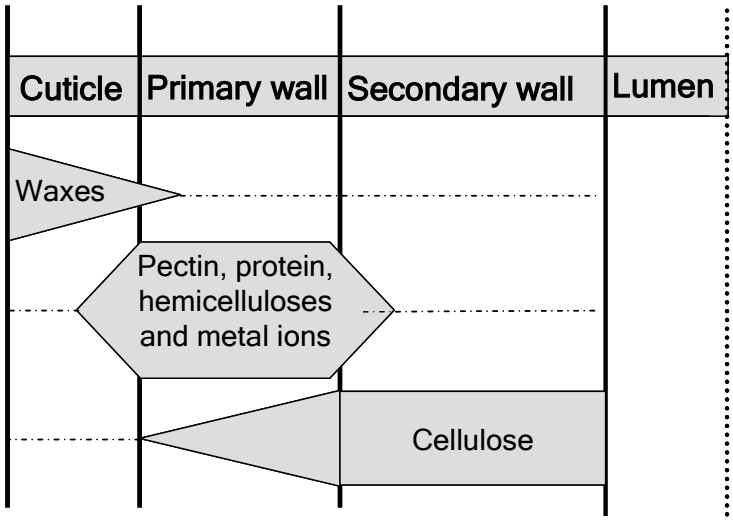

Figure 2.2: A schematic representation of the cellulosic and non-cellulosic materials in the cotton fibre.

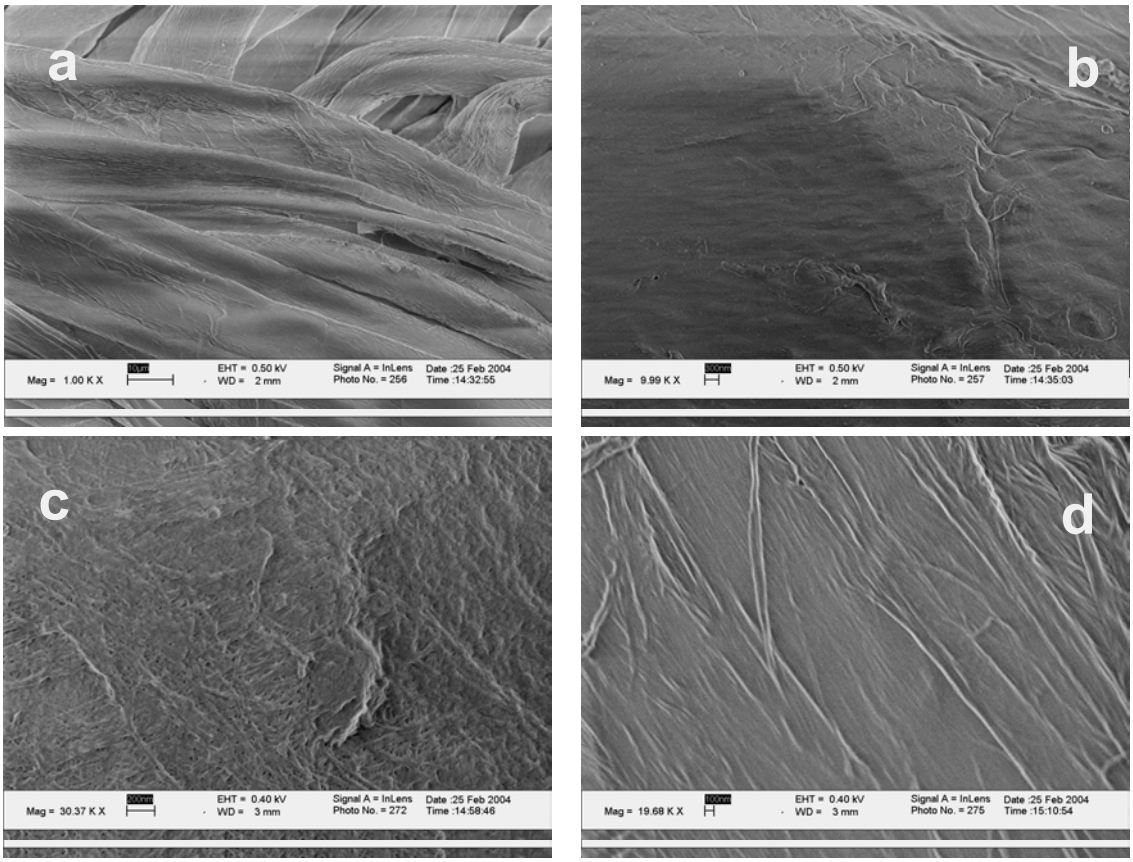

Figure 2.3: SEM pictures of various parts of cotton fibre, a) fibres from desized cotton fabric, b) amorphous wax surface of the desized cotton fibre, c) complicated network of primary wall of the cotton fibre, and d) the secondary wall of the cotton fibre, in which crystalline cellulose is visible in the form of parallel microfibrils.

2.2.2 Cotton fibre development

Each fibre consists of a single cell that grows from the epidermis of the cottonseed over four overlapping stages: 1) initiation, 2) elongation, 3) secondary wall thickening, and 4) maturation as describe below.

Initiation: As shown in Figure 2.4A, fibre growth initiates from the epidermis of the cotton seed. The initiation stage of cell starts two days prior to the day of anthesis (flowering) and then the cell elongates until it reaches its final length.

Elongation: Cell elongation is crucial for fibre growth and development and determines the length and fineness of the fibre. Cotton fibres are unicellular so there is no cell division [15]. Figure 2.4B schematically shows the growth of a cotton fibre. It also shows the cross section of a fibre with only outer layer (cuticle and primary

wall) and the inner lumen. The secondary wall is not developed at this stage. This stage takes about 20-25 days. The cuticle and primary cell wall that mainly contains non-cellulosic materials forms during these first two stages of cotton fibre growth.

Secondary wall thickening: The next stage in the development of the fibre is secondary wall thickening that occurs during the 35 to 50 days after anthesis. Cell elongation and secondary wall thickening are overlapping stages in the cotton fibre development. As shown in figure 2.4C the secondary wall is deposited from the outside to the centre of the fibre. The fibre becomes thicker as the cellulosic layers are formed and deposited from outside to inside. A cotton fibre expands via the diffuse growth and is is driven by turgor pressure [15-17]. As shown in Figure 2.4C, the turgor pressure is the force exerted outward on a cell wall by water preventing the cell wall from collapse [18]. The primary wall of a cotton cell owns a remarkable combination of strength and flexibility to sustain the large mechanical forces that arise from cell's turgor pressure. Plant cells also release expansins- a cell wall loosening protein, in the primary wall to hydrolyse the cross-linking of wall polysaccharides. This facilitates turgor-driven cell expansion during the active growth period [19]. At the same time, the primary wall permits a controlled polymer extension to create space for the secondary wall enlargement [18].

Maturation: All three stages initiation, elongation and secondary wall thickening occur in a closed cotton fruit capsule (see Figure 2.4A, B and C respectively). After completion of secondary wall thickening, the capsule breaks, opens and the young fibres undergo a drying process. Until this stage, the cotton fibre has a cylindrical shape. Removal of water from the fibre causes the internal layer to twist and collapse producing wrinkles and moulds to the under laying layers [20]. The primary wall, is less able to shrink because of its network structure, produces folds, twists and compression marks on the fibre surface. Paralikar and Hussain [21] proved that the fibre collapses characteristically to form the well-known kidney shape cross section.

Figure 2.4D shows the schematic representation of collapsed cotton fibre. The cross section of a mature dry fibre has a convex and concave side. Concisely, these differences in fibrillar packing densities around the perimeter of the fibre originate from the shape of lumen. According to Boylston [22], the dry mature fibre can be divided into four different accessibility zones namely neutral zone (N), concave zone (C), convex zone (B) and tight zone (A). The accessibility (from maximum to minimum) for chemicals and other scouring additives are in an order of N > C > B >

A. For this reason during enzymatic hydrolysis of the fibre, the disordered concave region is broken down preferentially [21, 22]. The remaining convex region (Zone A and Zone C) are only dissolved at advanced stages of hydrolysis. Probably this difference in the packing densities in the dry mature cotton fibres is responsible for a different dyeing behaviour after a mild treatment like enzymatic scouring [11].

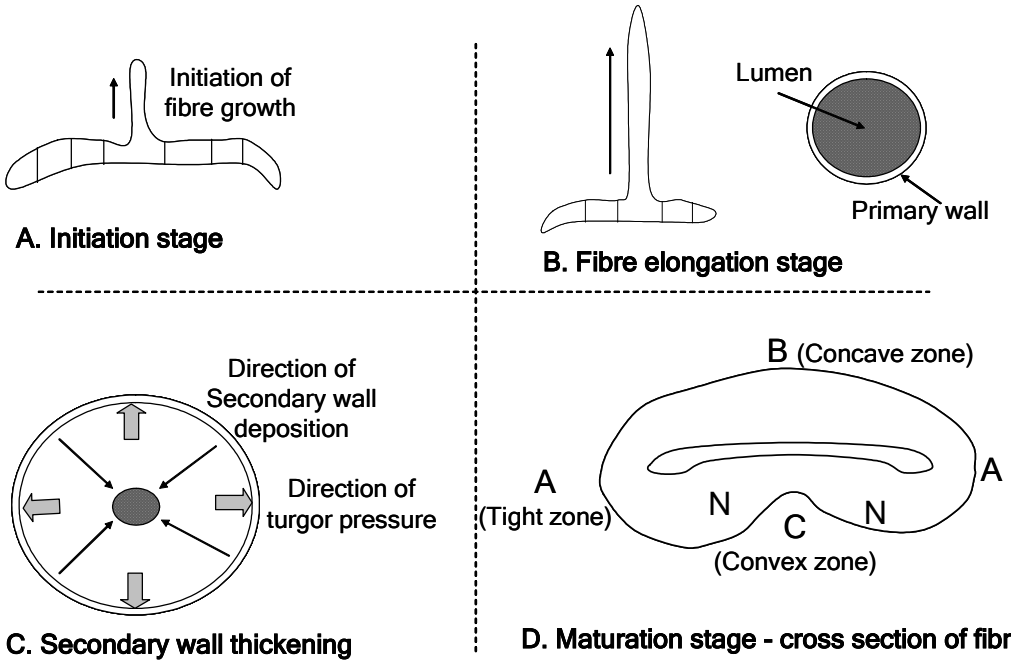

Figure 2.4: An illustration of various stages of cotton fibre growth. A: Initiation, B: Elongation. C: Secondary wall thickening and D: a cross section of cotton fibre after the maturation. Mature cotton fibre has different accessibility zones. Accessibility is in an order of (from maximum to minimum) N > C > B > A. Where, N is neutral zone, C is concave zone, B is convex zone and A is tight zone [11, 21].

2.3 The cuticle – the outermost layer

The aerial surfaces of vascular plants are covered with an extracellular layer called the cuticle or cuticular membrane that overlays the cell wall of epidermal cells. The term "cotton waxes" has been used for all lipid compounds found in the cuticle of fibre [17]. The main function ascribed to the cuticle is to minimise water losses from cotton fibre. Other functions are, to limit the loss of substances from fibres internal tissues, and to protect the fibre against physical, chemical and biological aggressions [23]. The cuticle contains primary alcohols, higher fatty acids, hydrocarbons, aldehydes, glycerides, sterols, acyl components, resins, cutin and suberin, which are called waxes [24]. The cuticle gives a soft touch to the fibre and reduces the friction forces during spinning. At the same time, presence of these waxy materials is

detrimental in chemical processing of cotton yarn because it gives the fibre surface a high hydrophobicity.

The cotton waxes are solid substances with rather high and wide range of melting points (64°C to 214°C) and constitute around 0.4 to 0.8% dry weight of raw cotton. Table 2.2 gives a detailed description of the composition of the waxy material. As shown this in Table, the waxy contents can be divided into two categories, a saponifiable part (nearly 40% of total wax content) and a non-saponifiable part, which is around 60% of the total wax. Alcohols such as gossypol ($C_{30}H_5OH$), montanyl ($C_{28}H_{57}OH$) and ceryl ($C_{28}H_{53}OH$) are high molecular weight monohydric alcohols and belong to the category of non-saponifiable waxes [25]. These n-primary alcohols (C_{26}-C_{36}) combined with the fatty acids (C_{16}-C_{36}) are the main components of wax from the mature white cotton fibre. After treatment with boiling NaOH, waxes are hydrolysed into a sodium salt of the fatty acid and alcohol [16, 26, 27]. Apart from the above mentioned components in the cuticle, there are also some complex biopolymers present.

2.3.1 Complex biopolymers

Suberin and cutin are insoluble, lipophilic biopolymers also called as bio-polyesters. Together with complex mixtures of soluble lipids, suberin and cutin form the protective layers of higher plants. These cell layers are diffusion barriers for water and other small, polar compounds [28]. Suberins and cutins, are closely related to each other, the only difference is their chain length and substitution patterns. These, complex bio-polyesters are present in large amounts in the un-matured or green cotton fibre. Therefore, green fibres are suitable to study the nature of these bio-polyesters. There are some differences between the un-matured (green) and matured (white) fibres. The waxy composition of green fibres is roughly 10-16 times higher compared with white matured fibres [29-31]. Ryser [29] reported the unidentified high molecular weight compounds are the major constituents of the green fibre. Wax extracted from green fibres also contains some yellow-green pigments probably flavonoid in nature [30]. Another difference is the absence of suberised inner layers in the mature white fibres of cultivated cotton species (*Gossypium hirsutum*) [28-30].

Type	Main Constituents	% Dry Weight	Details of each constituents				
			Constituent	Chemical formula	Molecular Weight	Melting Point (°C)	Remark
Unsaponifiable part 52 – 62 %	High molecular weight alcohol C_{23} to C_{34}	40-52	n-Triacontanol	$C_{30}H_{61}OH$	438.8	87	Major
			Gossypol (colouring matter)	$C_{30}H_{30}O_8$	518.5	184 – 214	Major
			Montanyl (1-octacosanol)	$C_{28}H_{58}O$	410.8	83	Major
			Octacosanol	$C_{28}H_{58}O$	410.7	83	Traces
			Other alcohol	C_{28} -C_{30}	-	-	-
			Glycerol (low molecular weight alcohol, esterified with fatty acid)				
	Saturated and Unsaturated hydrocarbons	7-13	Heptaconsane	$C_{27}H_{56}$			
			Triacontane	$C_{30}H_{60}$			
			Untriacontane	$C_{31}H_{64}$			
			Dotriacontane	$C_{32}H_{66}$			
	Phytosterol, Sterol, Glucoside and Polyterpens	3-8	β -Sitosterol	$C_{20}H_{50}$	414.7	140	
			γ-sitosterol	$C_{20}H_{50}$	414.7	147	
			Sitosterol glucoiside (sitosteroline)	$C_{35}H_{60}O_6$			
			α& β- Amyrin	$C_{30}H_{50}O$			
Saponifiable part 37 – 47 %	Fatty acid (with even number of carbon atoms) in free or esterified form	23-47	n-tetracosanic acid (Lignoseric acid)	$C_{21}H_{43}O_{20}$	368.6	84	Major
			n-pentadecanoic acid	$C_{15}H_{30}O_2$	242.0	69.6	Traces
			Hexadecanoic acid (Palmatic)	$C_{16}H_{32}O_2$	256.4	64	Major
			cis-9-octadecanoic acid (Oleic)	$C_{18}H_{34}O_2$	282.5	04	Traces
			Octadecanoic acid (Steric)	$C_{18}H_{36}O_2$	284.5	70	Major
			Iso-behenic acid	$C_{22}H_{44}O_2$	340.6	80	Traces
			n-pentadecanoic acid	$C_{15}H_{30}O_2$	242.0	69.6	Traces
			Hexadecanoic acid (Palmatic)	$C_{16}H_{32}O_2$	256.4	64	Major
	Resins	?	Coloured resinous products				

Table 2.2: Detail wax composition of the mature dry cotton fibre [11, 24-26, 29, 30].

Structure and composition of suberin: Suberin from cotton fibre predominately comprises of C_{16} and C_{18} compounds [31]. The moderately hydrophobic suberin polymer is thought to function as a scaffold to enable the deposition of the more hydrophobic suberin-associated waxes. Moire *et al.,* [28] and Ryser [29, 31] concluded that suberin is also found within the layers of the secondary wall of green cotton fibre (*G. hiresutum* cv. Green lint). Suberin in green fibres is deposited in concentric layers, alternating with cellulose. Each concentric layer is lamellated with periodicity of about 4.2 nm. Up to 20 alternating layers of suberin and cellulose are characterised [29, 30]. Treatment with aqueous KOH or NaOH at elevated temperatures (90°C to 100°C) removes most of the stainable material in the concentric layers of the green fibre cell wall [31-33].

Structure and composition of the cutins: Cutin is high molecular weight polyester comprises of various inter-esterified C_{16} and C_{18} hydroxy and hydroxy-epoxy fatty acids [34, 35]. As shown in Figure 2.5, the cutin biopolymer in the cuticle is formed by cross-linking hydroxylated fatty acids by intermolecular ester bonds leading to a three-dimensional structure [36-39]. Luque *et al.*, [36] proved on the basis of Fourier-transform infrared (FT-IR) spectroscopical analysis and X-ray diffraction analysis, that cutin has an amorphous structure. A study on the waxy composition of white cotton fibre supports the presence of cutin in the cuticle of cotton fibre. Ryser [29, 31] and Schemutz *et al.,* [30], based on electron microscope examination, showed the existence of an extremely thin cuticle of approximately 12 nm. SEM pictures of cotton surface, as shown in Figure 2.3b, reveals that the distribution of wax on the surface is uniform and amorphous in nature. Such a composition is typical for waxes that are embedded in plant cuticular membranes, the so-called intra-cuticular waxes [29].

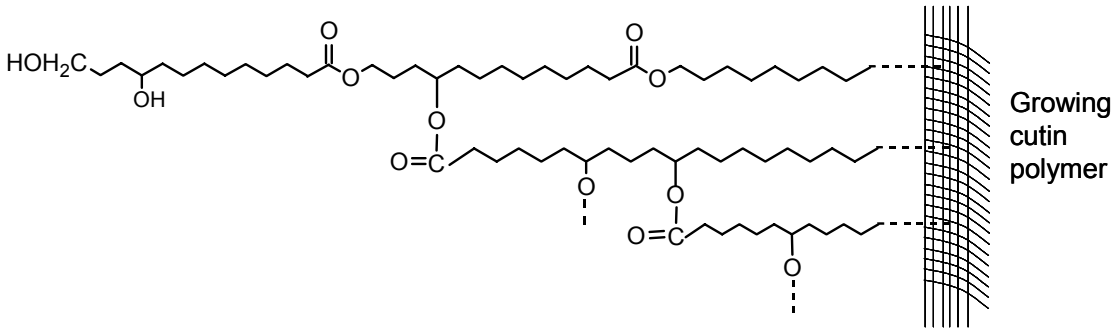

Figure 2.5: A schematic representation of the cutin polymerisation in the plant cell wall [37, 39].

2.4 The primary wall

The primary wall in a cotton fibre is a thin film with a thickness of about 0.5 µm [11]. It serves as the exterior surface of the fibre. In the primary wall, apart from amorphous cellulose, most of the constituents are non-cellulosic materials. This layer is flexible and swells uniformly in all directions. Table 2.3 gives the typical composition of the primary wall with details of each component. As seen from Table 2.3, the primary wall contains amorphous cellulose, pectins, proteins, hemicellulose and colouring components. It is important to study the individual component and interconnections in the primary wall, for attacking specific components with enzymes to destabilise the primary wall efficiently. A brief description of the primary wall components, followed by a description of their interconnections is given below.

SN	Structure	Compound	Mol. Wt.	Melting Point (°C)
1	Cellulose and its constituents	Glucose	180.2	83 (monohydrate) 146 (anhydrous)
		Cellobiose	342.3	225
		Cellulose		320
2	Pectins	Rhamnose	164.2	82 - 92
		Galactose	180.2	118 - 120 167 (anhydrous)
		Arabinose	150.1	157 - 160
		Galactouronic acid	194.1	159 (α - form) 166 (β - form)
3	Proteins	Proline	115.1	190
		Hydroxyproline	131.1	274
4	Hemicelluloses	Mannose	180.2	133
		Fucose	164.2	140
		Xylose	150.1	144-146
		Glucouronic acid	194.1	16
5	Colouring components	Morin (3,5,7,2',4'-pentahydroxyflavone)	302.2	-
		Gossypetin (3,5,7,8,3',4' - hexahydroxyflavon)	-	-
		Gossypol (2,2'-bis(8-Formyl-1,6,7-trihydroxy-5-isopropyl-3-methylnaphthalene)	518.6	184-214
		Tannins and pigments		

Table 2.3: Detail composition of the primary wall component in mature dry cotton fibre [11, 26]

2.4.1 Details of each constituent in the primary wall

I. Cellulose: Cellulose in the primary wall is heterogeneous and has a low degree of polymerisation (DP up to 2000 glucose units) compared with cellulose in the secondary wall [40, 41]. The amorphous region of cellulose in a cotton fibre is characterised by its ability to swell in water [11, 41]. The orientation of cellulose macromolecules in the primary wall is low, that means that individual macromolecules are not arranged in any definite order. The cellulose microfibrils in the primary wall are surrounded by a matrix of other non-celluloses. Microfibrils of cellulose are crystalline aggregates of β(1-4)-linked glucose polymers. They are omnipresent elements of the plant cell wall and are responsible for much of its tensile strength.

II. Hemicelluloses: Hemicellulose is the name of a heterogeneous group of branched matrix forming polysaccharides. Hemicelluloses bind non-covalently to the surface of cellulose microfibrils in the primary wall. They form a coating over the cellulose microfibrils and are able to cross-link them into a complex network of the primary wall [13, 42]. There are several classes of hemicelluloses with an average 50 glucose units that are linearly β(1-4)-linked to one another. The difference between various classes of hemicelluloses is expressed in terms of oligosaccharide side chains.

III. Glycoproteins: Glycoproteins also known as extensins account up to 15% of the primary cell wall mass [11]. Glycoproteins contains a protein backbone with extended rod like carbohydrates that protrude outwards. The carbohydrates in the glycoproteins account for ~65% of the total structure. For a cotton fibre, these rod shape extensins are made up of roughly 300 amino acids, and abundantly contain hydroxyl-proline (Hyp). Most of the hydroxyl-prolines are glycosylated with chains of three or four sugar residues e.g. arabinose and galactose. The carboxyl-terminal peptides in the glycoprotein molecule are covalently linked by disulfide bonds and often contain an oligosaccharide chain. These oligosaccharides chains are also interconnected with other polysaccharides in the primary wall.

IV. Pectins: Pectins are acidic polysaccharides, which are found in fruits, fibres and vegetables [43]. Pectin being a non-cellulosic material in cotton fibres plays several

important roles. It contributes to the firmness and structure of cotton fibre, both as a part of the primary cell wall and as a component of the winding layer (see section 2.4.3) [43]. Pectin acts as cementing material for the cellulosic network in the primary wall [44, 45]. Pectin, as a hydrating agent, controls the movement of water and other plant fluids through the rapidly growing fibre [13, 43].

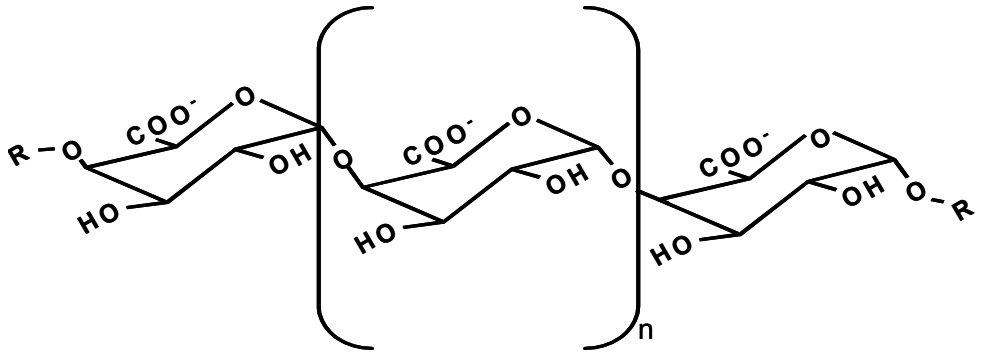

Figure 2.6: Backbone of pectin molecule, which is chemically α-(1,4)-linked D-galacturonan chain. Where 'n' is the number of D-galacturonan units [46, 47].

Pectin has a complex structure. Figure 2.6 shows the backbone of pectin structure. Pectin is comprised of an α-(1,4)-linked D-galacturonan backbone, occasionally interrupted by a α-(1,2)-linked α-L-rhamnopyranose residue. In cotton fibres, up to 60% of the galacturonic acid residues of the backbone are methyl esterified [45-47]. The homo-galacturonan parts of the polymer are referred as "smooth" regions, while the rhamnose rich zones are called "hairy" regions. A schematic representation of smooth and hairy regions in the pectin backbone is given in Figure 2.7.

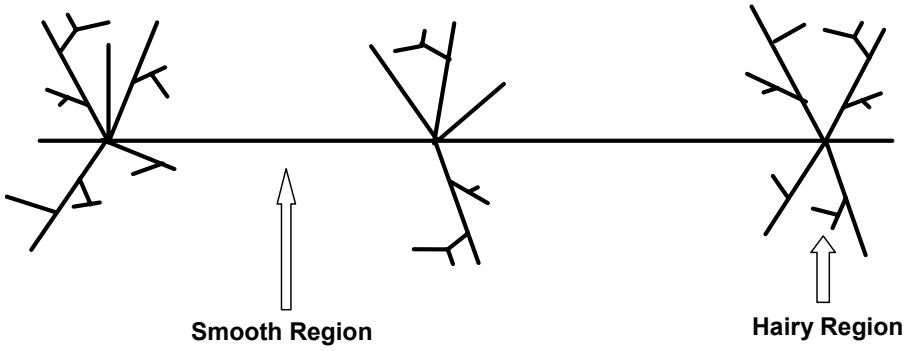

Figure 2.7: Schematic structure of pectin with its hairy and smooth regions [47, 48]

In the smooth region D-galacturanic acid (Figure 2.7), is either methyl esterified or non-esterified, depending on its location in the cotton fibre. The hairy region or rhamnose parts are made up of 1 to 20 residues of mainly D-xylose, L-fucose, D-glucuronic acid, D-apiose, L-arabinose and D-galactose units [47, 48].

Esterified or high methylated pectins (HM pectins): Esterified pectins are located at the outer layers of the primary wall. Pectic substances in the primary cell wall have a higher percentage of oligosaccharide side chains on their backbone. The side chains of the esterified pectins are much longer than acidic pectins, which are located in the winding layer (a layer between secondary and primary wall) [46]. These long side chains on the pectin backbone in the primary wall are required for the wall relaxation. Cell wall relaxation is necessary for expansion during the fibre growth [43]. Esterified pectin is less stable to moisture and heat because of its tendency to de-esterify in a humid atmosphere. In an alkaline solution at a low-temperature, saponification of methyl ester group occurs more readily [49-51]. Degradation of pectin proceeds with β-elimination cleavage of the glycosidic linkage (Figure 4.3). This reaction only occurs at glycosidic bonds adjacent to methyl esterified carboxyl groups. This phenomenon shows that esterified pectin is prone to de-polymerisation. Because of β-elimination reactions, the rate of pectin degradation will be higher when the degree of methylation is higher [51].

Non-esterified or low methylated pectin (LM pectin): The winding layer (see section 2.4.3) of a cotton fibre contains non-esterified pectin. Non-esterified pectins or acidic pectins contain many negatively charged galacturonic acid residues. Because of these negative charges on their backbone, highly hydrated non-esterified pectins are strong binding cations. A pectin molecule with an increased number of charged groups is straighter than esterified ones and therefore more likely to form a Ca^{2+} bridge. This phenomenon is demonstrated by egg box model as proposed by Grant *et al.,* [52], As shown in Figure 2.8, Ca^{2+} ions form cross-links with non-esterified pectins, thereby holding cell-wall components together. This bond between two non-esterified pectin molecule is so strong that it ultimately prevents secondary wall expansion. Krall [49] systematically describes the strong interaction between Ca^{2+} and other oxygen atoms and carboxyl groups in the pectin backbone.

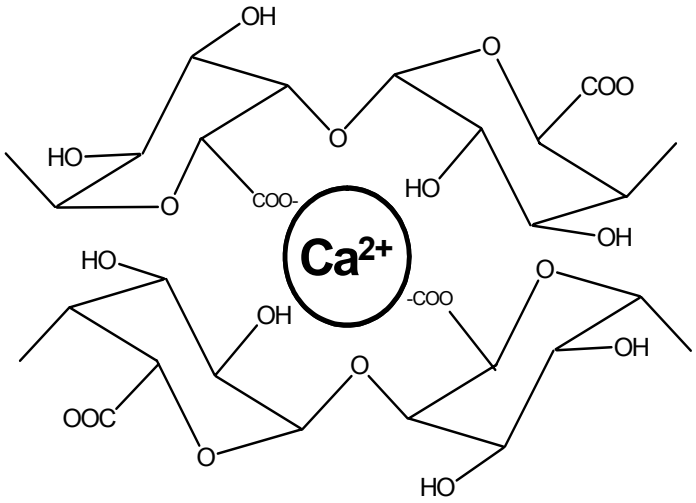

Figure 2.8: Egg box model showing bonding of non-methylated pectins with Ca^{2+} ions in the winding layer of cotton fibre [43].

V. Colouring matters: Colouring matter (pigments) in cotton fibres is rarely studied [53, 54]. In the natural state, cotton is off-white, cream, brownish or greyish green, depending on the source and growing conditions. Sadov *et al.,* [53] reported that a cotton fibre contains a considerable amount of polyphenols such as gossypol [2,2'-bis(8-Formyl-1,6,7-trihydroxy-5-isopropyl-3-methylnaphthalene], flavon and tanning substances (Table 2.3). The content of these substances depends on the maturity of the fibre. It has been established that cotton pigments consist of mainly morin [3,5,7,2',4'-pentahydroxyflavone or gossypectin (3,5,7,8,3',4'-hexahydroxyflavon)]. Gossypol and morin are mainly responsible for the cotton colour. Sadov *et al.,* [53] stated that naturally coloured cotton fibres contain the substances with phloroglucinol, resorcinol and pyrocatechol groups. However these colouring components do not take part in the scouring process.

VI. Metal contents: The primary wall of a cotton fibre contains different quantities of metal depending on their growing conditions and source [11]. Potassium is the most abundant metal ion in cotton fibres followed by magnesium and calcium [26]. Other metal ions which are present in traces are sodium, iron, magnese, copper and zinc. Removal of calcium is essential during the scouring process for better primary wall destabilisation. However, removal of rest of the metals from cotton fibres is also important, because they can contribute to problems during further wet-pretreatment processes like oxidative bleaching [11].

2.4.2 Interconnections in the primary wall

To destabilise the primary wall with enzymes, it is essential to study the interconnections of cellulosic and non-cellulosics. As illustrated in Figure 2.9, cellulose microfibrils are embedded in and linked to a matrix that contains hemicellulose, acidic pectins, esterified pectins and fibrous glycoproteins (extensins). Hemicellulose molecules (e.g. xyloglucans) are linked by hydrogen bonds to the surface of the cellulose microfibrils. The backbone of hemicellulose is similar to that of cellulose while, xylose, galactose and fucose (the polysaccharides) are the additional residues present on hemicellulose backbone. Some of these hemicellulose molecules are cross-linked to acidic pectin molecules (e.g. rhamnogalacturonan) through short and esterified pectin molecules (e.g. arabinogalactan). Rod shape glycoproteins are tightly woven into the matrix [11, 13, 14, 42-44].

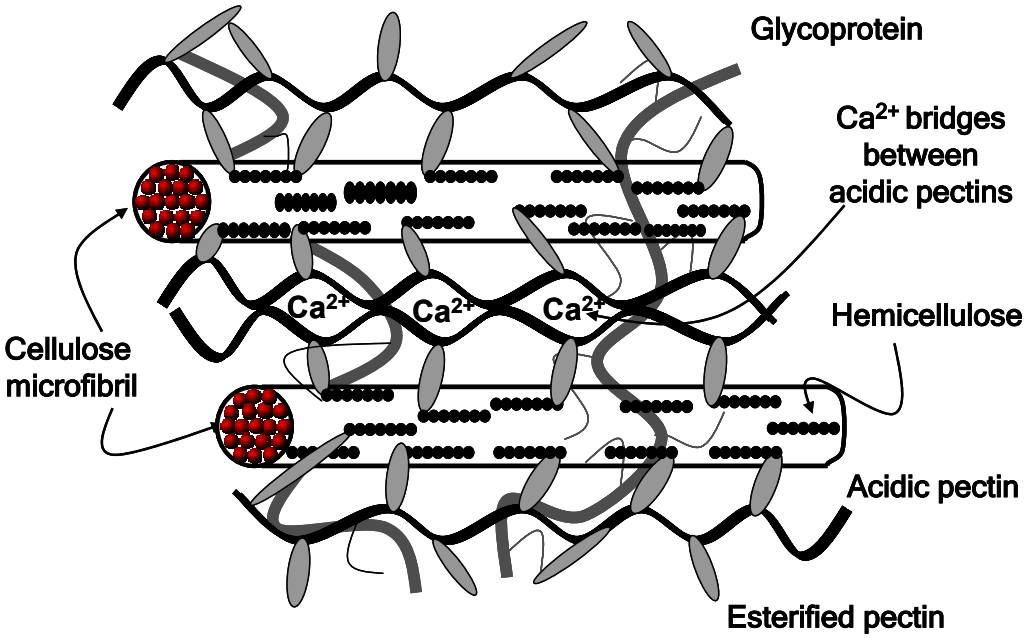

Figure 2.9: Interconnections between cellulose and other non-cellulosic components in the primary wall of the cotton fibre.

2.4.3 The winding layer

The thin layer directly adjoining the primary wall is called the winding layer or also known as immediate outer layer of the secondary wall or primary wall II. The winding layer consists of cellulose microfibrils (with a crystallinity higher than in the primary wall) that are deposited in a lacy network, with its microfibrils oriented more along the fibre axis. In the winding layer the interconnections between cellulose, hemicellulose and pectin (non-esterified) are mostly stabilised by Ca^{2+} ions. The interconnections in the winding layer are stronger than the outer layers. Thus, the

winding layer provides dynamic covering that allows limited swelling within the secondary wall and makes the secondary layer less accessible to the external damage [11, 12].

2.5 The secondary wall

Cellulose in the secondary wall is characterised by a higher degree of polymerisation (nearly 5000 units) compared with cellulose in the primary wall. Table 2.4 shows the various units and sub-units of cotton cellulose with their diameter. In the secondary wall of a cotton fibre, two cellulose molecules can form a long planar chain of β(1-4)-linked glucose units, resulting in a ribbon like structure [41]. These two cellulose chains forms a sheet, which is called an elementary fibril. Microfibrils of cellulose are crystalline aggregates of approximately 21 elementary cells (Table 2.4). Intermolecular hydrogen bonds play an important role to stabilise chains of elementary fibril to become a microfibril [11, 41]. Finally, a large numbers of such microfibrils are laying in parallel direction forms the various layers of the secondary cell wall [11, 13, 20].

Substructure	Diameter (nm)	Number of cellulose polymers
Cellulose molecule	0.5	1
Element cell/ unit	0.6	2
Microfibril	3.5	42 (or 21 element cell)
Cotton fibre	10000	0.5×10^9 (nearly 1500 microfibrils)

Table 2.4: Information on various subunits of cellulose fibre in the cotton fibre secondary wall [11, 20].

The direction of the fibrils reverses at random intervals along the fibre length. Reversals represent the zone of variation in breaking strength and are more likely to break under stress [55]. Looking deeply inside the cotton fibre, the elementary fibrils which are positioned in a parallel way, show different contact points. Either they are crystallographically aggregated, or they are held together by a non-covalent physical interaction (hydrogen bond, Van der Waals forces). The higher the number of these lateral contacts, the lower the possibility of one elementary fibril slipping off from another when subjected to tensile stress or compression load [11, 20]. The strength of the fibre essentially depends on the regularity and on the numbers of contacts between the elementary fibrils.

2.6 Discussion

2.6.1 Destabilisation of the waxy layer

Even today, NaOH is used in the textile industry to remove the outermost waxy layer during cotton scouring process. NaOH at high temperatures (90°C to 100°C) removes cotton waxes by saponification and emulsification (Table 1.2). The waxy layer contributes significantly to the hydrophobicity of the cotton fibre. Scouring materials have to cross this first barrier in order to attack the primary wall components of the fibre. In case of enzymatic scouring, this thin waxy layer hinders the performance of enzymes targeted for the primary wall. Therefore, it is necessary to recognise the importance of the removal of the waxy layer in cotton scouring. This can be achieved by studying the effect of wax removal on hydrophilicity of the cotton fibre. It has been postulated that the removal of waxy layer will make the primary wall better accessible for the enzymes, resulting in an improved performance. An experimental evidence of this hypothesis will be presented in Chapter 4.

The challenge now is how to remove this waxy layer efficiently in an environmental friendly way. Based on the cotton structure, it is postulated that for an efficient removal of the waxy layer, cutin degradation is a pre-requisite. Cutin forms a three-dimensional network structure in which other amorphous waxy materials are embedded. Therefore, destabilisation or degradation of cutin will lead to an efficient removal of the waxy layer. This can be done by enzymes. The selection of wax degrading enzymes and its motivation is discussed in Chapter 3.

2.6.2 Destabilisation of the primary wall

The primary wall is one of the most complicated parts of the cotton fibre, where cellulosic and several non-cellulosic materials are interconnected in criss-cross pattern (Figure 2.3 and Figure 2.9). In traditional scouring, the primary wall is removed by swelling and hydrolysis with NaOH at elevated temperatures. After studying the non-cellulosic components and their interconnections in the primary wall, it is clear that this layer needs to be removed during cotton scouring. In order to design an enzymatic scouring process, the destabilisation of the primary wall has to be carried out with selected enzymes. To minimise the number of different enzymes involved in the scouring process, it is desirable to select one that is most suitable for destabilisation of the primary wall.

The first choice for the primary wall destabilisation is the enzyme cellulase. However, the use of cellulase can hydrolyse the cellulose in the secondary wall of

cotton fibre, that reduces the degree of polymerisation (DP). Literature confirms that cellulose affects the tensile strength of the cotton fibre [8, 10]. Therefore, the use of cellulases for the destabilisation of the primary wall is not an attractive alternative to the traditional scouring.

As mentioned before, hemicellulose forms a coating on the cellulose microfibrils in the primary wall (Figure 2.9). The bond between cellulose and hemicellulose is strong, that makes it difficult to be degraded efficiently with enzymes. Additionally, hemicellulases also have some cellulose degrading properties and can influence the tensile strength of the cotton fibre. Therefore, also the uses of hemicellulose degrading enzymes such as xylanases or hemicellulases are not a good choice for the primary wall destabilisation.

Glycoproteins or extensins can be removed by the enzyme proteases. Even though, glycoproteins are tightly woven into the primary wall matrix, its removal would not greatly affect the complicated cellulose – hemicellulose – pectin structure (Figure 2.9). However, the removal of proteins or extensins would help to remove mainly nitrogen containing substances that are associated with the glycoproteins. This will lead to a fibre surface, which is brighter but not hydrophilic enough. Literature supports that proteases improve the whiteness of the fabric rather than the hydrophilicity [2, 3] and therefore proteases are not a good candidate for cotton scouring.

From the discussion on pectin, it is apparent that pectin is one of the most complicated non-cellulosic constituent in the primary wall. In the outer layers of the primary wall, pectin is responsible for holding other non-cellulosic component together. In the winding layer, Ca^{2+} ions hold the acidic pectin together. Hardin [18] has proposed that pectin is a cementing material in the primary wall of the cotton fibre. After studying the interconnection in the primary wall, it is clear that the removal of pectin from the primary cell wall of cotton fibre can destabilise the other non-cellulosic materials. Breaking pectin bonds will isolate them from proteins, hemicelluloses and cellulose (Figure 2.9). Therefore, the degradation and removal of pectin is essential for an efficient enzymatic scouring process.

There are several pectinases available for pectin degradation. Proper knowledge of types of pectin in the primary wall of cotton fibre with the information on other non-cellulosic material is essential to choose the best enzyme for the scouring purpose. Esterified pectins are mainly located in the outer primary wall of the cotton fibre. Therefore, hydrolytic enzymes that can degrade these esterified pectins would be desirable. Pectin in the winding layer is mainly held by Ca^{2+} ions. Therefore, the

use of chelators during a later stage of the scouring process can help to remove these acidic pectins. The selection of a suitable enzyme for the degradation of esterified pectin is discussed in the Chapter 3. The experimental results related to pectin degrading enzymes are presented in the Chapter 4.

2.7 Conclusions

A study of cotton fibre structure is essential for a proper understanding and designing of a new efficient enzymatic scouring process. A thorough literature survey reveals many clues, which has direct influence on designing an efficient enzymatic scouring process. From the structure of cotton we hypothesized that more attention should be given to a wax removal step prior to the enzymatic treatment to remove pectin. The disruption and removal of the outermost waxy layer is of prime importance to allow enzymes to react efficiently with the substrate. Wax and pectin removal are identified to be the most important step in the scouring process.

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3

Enzyme Selection and Experimental Overview

An overview of the enzyme selection procedure, experimental techniques, and substrate characterisation towards developing a new efficient enzymatic scouring process is given. A motivation and description of promising enzymes for cotton scouring is presented. Various tools to evaluate the modification in cotton fabric have been briefly described. The determination of the structural contact angle, θ and pore volume distribution (PVD) with an auto-porosimeter is discussed. Other techniques discussed briefly are, deformation of textile materials with a wedge apparatus, surface tension measurement with a bubble tensiometer, tensile strength measurements for the treated fabric samples, pectin analysis and activity assays for various enzymes used. A selection of cotton samples for scouring experiments were made on the basis of an uniform PVD.

3.1 The selection of enzymes

Based on the morphological structure of cotton fibre, we deduced that sufficient hydrophilicity during cotton scouring could be achieved by removing the outermost waxy layer, followed by destabilising the primary wall (Chapter 2). Therefore, wax and pectin removal are considered the most important steps in the enzymatic scouring process (Chapter 1).

3.1.1 Cutinase: an enzyme for cotton wax removal

Cotton wax removal is possible in a non-polar solvent or boiling the fabric with a surfactant solution. As explained in chapter 1, these techniques are not a good alternative to NaOH scouring. There is need to have an environmental friendly process that should degrade waxes at low temperature and in an aqueous environment. An enzyme that can hydrolyse the ester bonds of triglycerides and cutin, to destabilise the waxy layer in cotton fibre is desired. Enzymes from the carboxylic ester hydrolyse group (EC. 3.1.1) are promising candidates. Esterase (EC 3.1.1.1), lipase (EC 3.1.1.3) and cutinase (EC 3.1.1.74) belong to this group [1-4].

Carboxyl esterases or esterases (EC 3.1.1.1), are enzymes that hydrolyse ester bonds by the addition of a water molecule [1]. Esterases typically show the highest activity towards less aggregated form of its substrate, the so-called soluble form [1, 5, 6]. For that reason, esterase was not considered to be a good candidate for the cotton wax degradation.

Lipases and cutinase are all lipolytic enzymes that show a high activity towards the substrate in their aggregated form, hence they are suitable candidates for the cotton wax degradation [6]. Cutinase has some advantages over lipases for cotton wax degradation. Lipases, in general, require interfacial activation at the lipid water interface, whereas cutinase does not. The interfacial activation is an extra energy need to expose the active site of Lipase enzymes to the substrate. Another important characteristic of cutinase is that, it can hydrolyse waxes in the absence of Ca^{2+} ions [5]. Lipases need Ca^{2+} ions for their hydrolytic action [1, 6]. Since it is known that the presence of Ca^{2+} ions can interfere negatively with the pectinase performance, it will be difficult to combine lipases with pectinases during enzymatic cotton scouring [7]. The selection of suitable pectinases for the destabilisation of the primary wall of the cotton fibre is discussed in section 3.1.2. It is possible to combine cutinase and pectinase, because similar to pectinases, cutinase also does not require Ca^{2+} ions for its action. Degani *et al.,* [8] also reported the potential of

cutinase from *Pseudomonas mandocino* for the cotton wax degradation. Based on all this information cutinase was selected as a promising candidate for the cotton wax degradation.

Cutinase: Cutinase is released by pathogenic fungi [2], plant pollen [3] and bacteria [4]. The cutinase used in this study is an extracellular enzyme from the phytopathogenic fungus *Fusarium solani pisi*. Cutinase bridges functional properties between lipase and esterase [5]. Cutinase from *F. solani pisi* is a one domain enzyme, there is no quaternary structure. It is one of the smallest members of the serine hydrolase family, with 197 residues (Mw 22 kDa). The isoelectric point (pI) of cutinase is between the pH 7.6 and 7.8. Its dimensions are 45 × 30 × 30 Å [5].

Figure 3.1, shows a three-dimensional representation of cutinase [9]. In contrast to other lipases, cutinase does not have a characteristic lid domain (that covers the active site) and thus cutinase is always present in the so called "open confirmation". Therefore, the cutinase active site is directly accessible to water [5, 6], although there is a small helix loop located near the active site that can partially block the entrance (Figure 3.1). The active site Ser120 is located in a rigid part of the protein backbone, whereas the other two active site residues namely Asp175 and His188 are situated in a more flexible binding loop [5]. In contrast to lipases, the so called oxy-anion hole in crystalline cutinase is flexible [5, 10]. In cutinase, the oxy-anion hole stabilises intermediates and lowers the activation energy for catalysis. Because of the flexible binding loop and the oxy-anion hole cutinase can accept a wide range of substrates, though conversion rates differ depending on the substrate. The natural function of cutinase is to catalyse the hydrolysis of cutin. Cutinase also catalyses the hydrolytic reaction of triglycerides via inter- and intra-transesterification reactions efficiently [11-14]. It shows high specificity towards the Sn-3 position in triglycerides [5]. Cutinase is less sensitive to the other two acyl chains, they remain mobile while the Sn-3 chain is bound to the cutinase enzyme [5].

Granulated *F. solani pisi* cutinase was kindly provided by Unilever R&D laboratory in Vlaardingen, the Netherlands. The production of this specific cutinase from the *Fusarium solani pisi* in the yeast *Saccharomyces cerevisiae* has been described by Mannese *et al.,* [14]. To obtain the enzyme in its free form approximately 238 mg of granulated cutinase was stirred in a 25 mL beaker with a small magnetic stirrer for 10 minutes, with 10 mL of a 50 mM Tris-HCl buffer at pH 8. The resulting emulsion was centrifuged (Hettig Zentrifugen, Mikro 12-24) at 9000 rpm

for 15 minutes. Finally, the transparent yellow solution containing the solubilised cutinase was stored at 4°C. The extracted cutinase solution was used within a few hours to prevent enzyme deactivation.

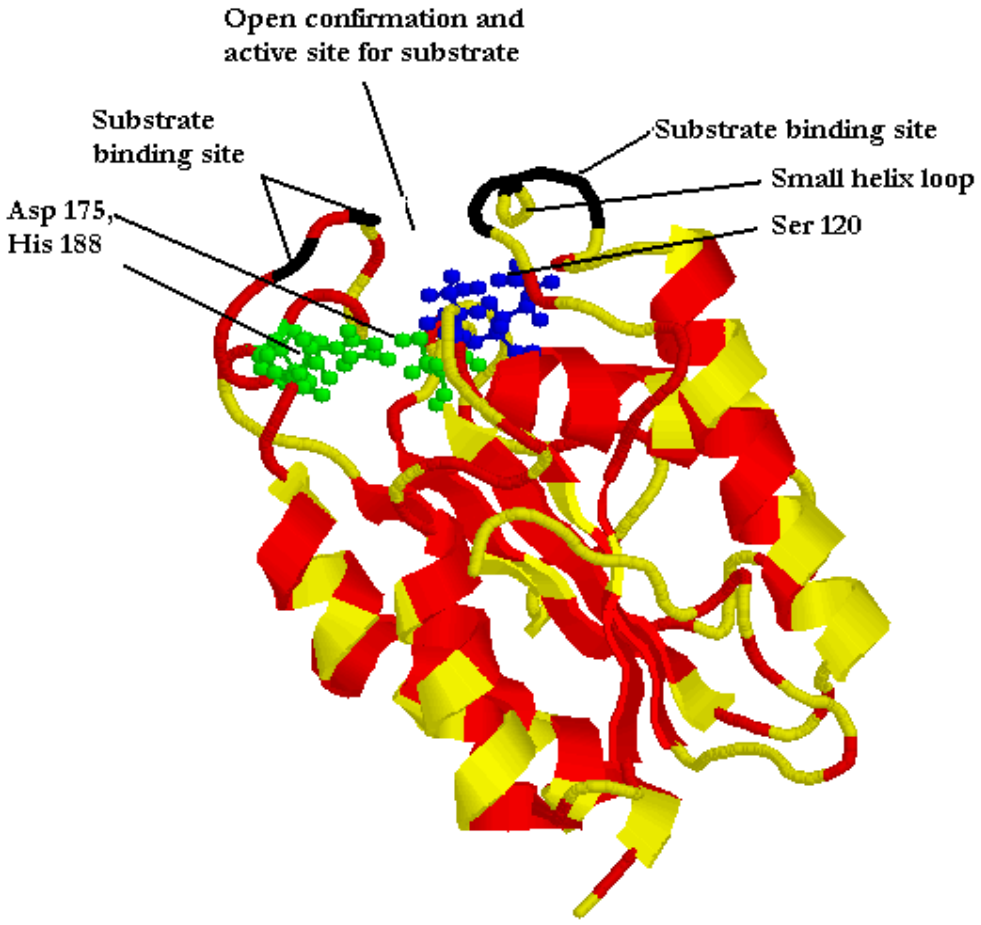

Figure 3.1: Structural representation of cutinase from *F. Solani pisi.* Picture developed using 'raswin' program from Protein Data Bank entry number 1CEX [9].

3.1.2 Pectinases for primary wall destabilisation

Pectinase can degrade pectins in cotton fibres. Pectinolytic enzymes are produced by many plant-associated micro organisms. In nature, there are three major classes of pectin degrading enzymes: pectin esterases, polygalacturonases and pectin lyases [7]. Pectin esterase (EC 3.1.1.11) catalyses the de-esterification reaction of the methyl group of pectin (polymethylgalacturonate), producing pectic acid (polygalacturonate). Polygalacturonases (PGs) are a group of enzymes which hydrolyse the α-1-4 glycosidic linkages in pectin. Pectin lyase cleaves polygalacturonate or the pectin chains via a β-elimination mechanism, which results

in the formation of a double bond between C_4 and C_5 at the non-reducing end (Chapter 4).

There are three major types of pectin lyases namely, endo-polygalacturonate lyase (EC 4.2.2.2), exo-polygalacturonate lyase (EC 4.2.2.9) and endo-polymethylgalacturonate lyase (EC 4.2.2.10). The endo-polygalacturonate lyase (EC 4.2.2.2) hydrolyses polygalacturonate chains randomly. The exo-polygalacturonate lyase (EC 4.2.2.9) cleaves polygalacturonate chains at the end and yields unsaturated galacturonic acid. Finally, the endo-polymethylgalacturonate lyase (EC 4.2.2.10) cleaves pectin randomly [7]. Pectinases like endo-arabinases, pectate lyases and polygalacturonases that solubilise a water-insoluble form of pectin (protopectin) are called protopectinases [15, 16].

Since pectin esterase only catalyses the de-esterification of the methyl group of pectin, we do not consider this enzyme as a suitable candidate for cotton scouring. Considering their mode of action on pectin, we have chosen polygalacturonases (acidic pectinase) and pectate lyase (alkaline pectinases) as promising candidates for cotton scouring.

Alkaline pectinases: In this study, Bioprep 3000L (Novozymes), ammonium sulphate precipitated Bioprep 3000L, and specially screened, isolated and purified Pectate Lyase have been selected for the degradation of cotton pectin [17, 18].

Bioprep 3000L: This commercial product from Novozymes [19] contains mono-component pectate lyase, which is active in the alkaline pH range (pH 8-9). Apart from exploring the potential of Bioprep 3000L on cotton pectin, it was used as a benchmark to evaluate the potential of the specially screened and purified Pectate Lyase. Bioprep 3000L was used as supplied by Novozymes after precipitation with ammonium sulphate. To limit the so-called impurities in enzyme solution, precipitation of Bioprep 3000L was carried out with ammonium sulphate. In commercial enzyme products, unknown stabilisers, emulsifiers and or surfactants are added to improve its performance. This complicates comparing other pectinases with Bioprep 3000L. Precipitation of Bioprep 3000L and the production of specially screen, isolated and purified Pectate Lyase and was carried out by Klug [17] of the Institute of Environmental Biotechnology, Technical University of Graz (TUG), Austria as a partner in this European project.

The Pectate Lyase (PL): An alkalophilic bacterium was isolated from *Bacillus pumilus BK2* producing an extra-cellular endo-pectate lyase PL (EC 4.2.2.2). The molecular mass of PL was 37.3 ±4.8 kDa. PL showed an optimum activity at pH 8.5 in a Tris-HCl buffer. The half-life time of PL was 75 hours at 30°C. The enzyme does not require Ca^{2+} ions for its action, and strongly inhibited by EDTA and Co^{2+}. The activity of PL decreases when the degree of esterification of the pectin backbone was above 50%. PL cleaved polygalacturonic acid via a β-elimination mechanism as shown by NMR analysis [17, 18].

Acidic pectinases: Polygalacturonases (PGs) can be divided into two groups, endo-polygalacturonase (EC 3.2.1.15) and exo-polygalacturonase (EC 3.2.1.67). Endo-polygalacturonase (EC 3.2.1.15) randomly hydrolyses the polygalacturonic acid backbone. Exo-polygalacturonase (EC 3.2.1.67) cleaves the polygalacturonic acid backbone starting from the non-reducing end. The potential of the three different PGs from both endo- and exo-polygalacturonase class were evaluated for pectin removal. The selected PGs were specially screened, isolated, purified and supplied by Schnitzhofer [20], of the Institute of Environmental Biotechnology, TUG, Austria. All three PGs were isolated from the fungal strains of *Sclerotium rolfsii.* Schnitzhofer [20] tested two different media viz., glucose and cellulose for the PGs production. Finally, three different PGs (PG-glucose, PG-cellulose, and PG-precipitated) have been used for the scouring experiments. PG-glucose hydrolyses the pectin backbone via endo-mechanism and PG-cellulose, and PG-precipitated cleaves polygalacturonase backbone via exo- mechanism [20]. The half-life times of PGs at 50°C were 10 hours at pH 5 and 20 minutes at pH 8.

3.2 Analytical methods applied

The catalytic activities of cutinase and pectinases were measured in an activity assay. A brief description of the activity assays is given below. All the chemicals used in study are of analytical grade from Merck and Sigma with a minimum purity of 99%.

3.2.1 Activity assay for the cutinase in a pH-stat

The activity of the cutinase was determined with a tributyrin oil assay as proposed by Gupta *et al.,* [21] and Beisson *et al.,* [22]. Depending on the pH at which the activity was tested, 1 mM Tris-HCl buffer (pH 7-8) or 1 mM borax buffer was used (pH 8.5-10.8). A buffered tributyrin-water emulsion containing the final concentration

of 67 mM tributyrin was titrated for 20 minutes with a 0.01 M NaOH solution using a Schott TritoLine alpha pH meter with a Schott Blueline gel electrode. In this stirred pH-stat, the temperature was kept at 30°C. A small nitrogen flow was run over the solution to prevent air oxygen, which stabilises the weak buffer, hence the stable readings. The pH-stat measurements were also used to study the effect of different non-ionic surfactants on the cutinase activity, using tributyrin and cotton fabric as substrate. To study the release of fatty acids from the fabric, experiments were conducted at pH 9.5 with 0.5 g of cotton fabric, using 2 mM borax buffer, at 30°C for 15 minutes (Chapter 5). The pH 9.5 is above the pK value of most C_{16} fatty acids present in cotton fibre. At this pH, the liberated fatty acids have negative charge which is easy to monitor by the pH-stat.

3.2.2 Activity assay for pectinases (PL, Bioprep 3000L and PGs)

PL, Bioprep 3000L and PG activity were determined measuring the increase of reducing sugars released from the substrate polygalacturonate [23]. A 50 µl aliquot of diluted enzyme solution was incubated in the presence of 450 µl of 0.25% (w/v) polygalacturonic acid (degree of esterification 0%). The incubation was conducted in 50 mM of acetate buffer, pH 5 (for PGs) or Tris-HCl buffer, pH 8 (for PL and Bioprep 3000L) for 5 minutes. The reducing sugars were assayed with an addition of 750 µl dinitrosalicylic acid reagent (DNS). Subsequently the solution was boiled for 5 minutes, cooled on ice and centrifuged, thereafter the absorbance at λ=540 nm (Hitachi UV 2001 spectrophotometer) was read. D-galacturonic acid (0.5-3 g/L) was used for the calibration curve. 1U was defined as the amount of enzyme that released 1 mol of reducing groups per second.

3.3 Materials and methods

Various experimental setups and techniques were applied in the enzymatic scouring experiments. The fabric used was an industrially desized 280 g/m^2 plain-woven 100% cotton fabric, supplied by the Ten Cate Technical Fabrics B.V. in the Netherlands. All experiments were performed with demineralised water.

3.3.1 The general experimental setup for scouring experiment

Scouring experiments where performed in 1 L beaker in which three fabric samples of 5 × 12 cm were treated in an enzyme solution of about 500 mL, with the 50 mM Tris-HCl buffer (pH 8). The beaker was placed in a temperature controlled water bath at 30°C or 50°C. After the treatment, the fabric samples were rinsed in

500 mL of water at 90°C for 15 minutes, to inactivate the enzymes. Thereafter the samples were rinsed twice for 5 minutes in water at room temperature. Finally, the samples were kept on a flat acrylic surface to be dried at air for at least 24 hours before evaluating the fabric samples. All the experiments were performed in duplicate. Other details or modifications of this protocol are explained in the corresponding chapters.

3.3.2 Wedge apparatus – for stretching deforming action on fabric

Mechanical action applied on fabric helps to increase mass transfer [24, 25]. It was expected that the performance of enzymes would also be sensitive to the mechanical action. However, it is difficult to measure the amount of mechanical energy applied on the cotton fabric. An innovative device to produce a controlled mechanical action on a fabric, the so-called wedge apparatus is used. This wedge apparatus was designed, developed and kindly provided by Unilever Research and Development, Vlaardingen, the Netherlands. A schematic representation of this system for mechanical action is given in the Figure 3.2. The principle action of the wedge apparatus is compression and stretching deformation of the fabric, when it moves along a wedge (Figure 3.2).

So, the compression plus stretching deforming device in the system is a metal wedge over which a strip of fabric can oscillate forward and backward (Figure 3.2). At one side (left side of the drawing), the strip was fixed to one of the rollers. The other end of the strip was led freely over the other roller and a known weight (to stretch the fabric) was attached. A pneumatic system regulated the number of strokes and hence the time the fabric strip was being deformed. The wedge was mounted in a bath filled with demineralised water. The force exerted on the textile at the wedge tip could be varied by varying the suspended weight at the free end of the strip or by varying the sharpness of the wedge radius. The formula, to quantify the mechanical action (N/m^2) exerted on the fabric to produce stretching deforming action is given by equation 3.1.

$$\text{Mechanical action} = \frac{m \times g}{2\pi r l\left(\theta / 360\right)} \qquad (3.1)$$

Where, m is the weight (kg) applied to one end of the fabric, g is the gravitational constant (m/s^2), r is the wedge radius (m), l is the wedge length (0.05 m), and θ is the angle formed between the tip of the wedge and the fabric surface

(degrees). To study different levels of mechanical action on a fabric surface, three deforming weights of 104 g, 275 g and 444 g were used with two wedges having a radius of 2 mm and 3 mm. The water level in the bath was adjusted such that it just touches the tip of the wedge. The deforming action exerted on the fabric sample was only at the edge of the wedge. The treated area of the sample was 5 × 6 cm.

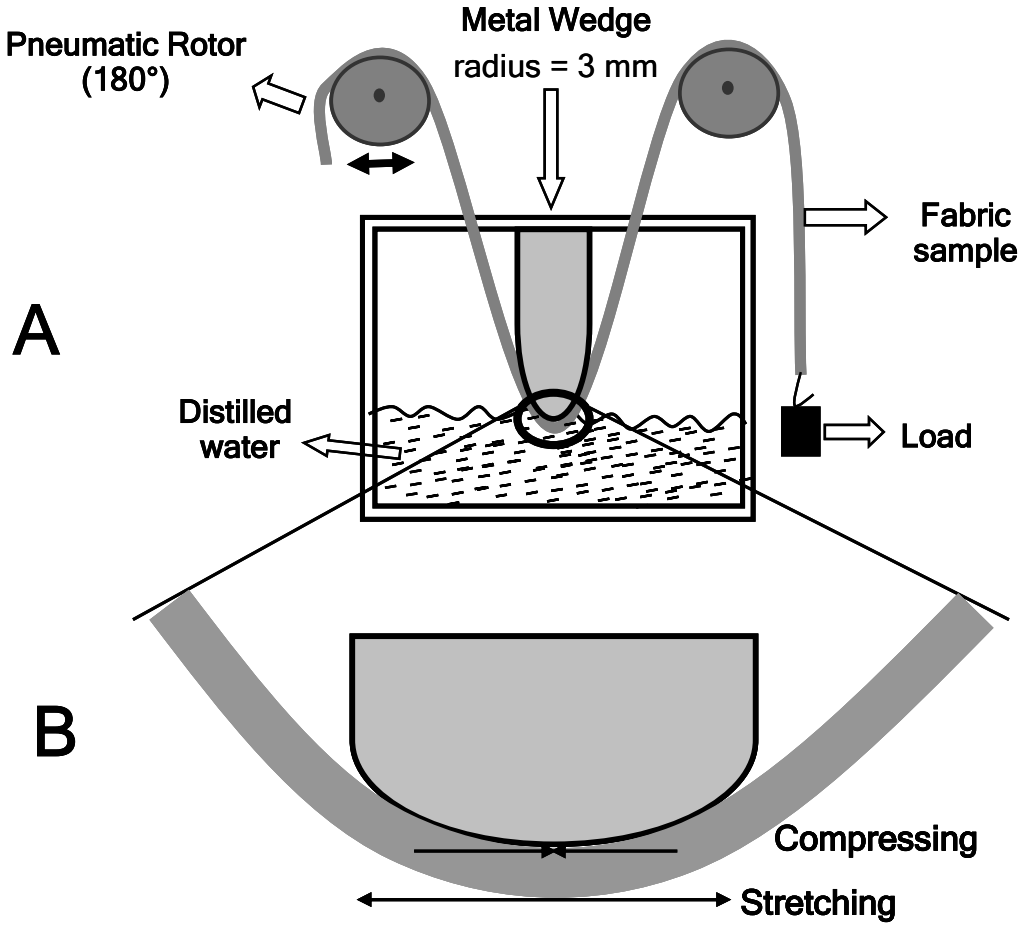

Figure 3.2: A schematic representation of A) all parts of the wedge apparatus that can produce a controlled mechanical action for the specified time, B) schematically illustrates the principle mechanism of the wedge apparatus.

3.3.3 A bubble tensiometer – for the surface tension measurements

A bubble tensiometer was used to measure the equilibrium and dynamic surface tension of various surfactant solutions used in this study. By changing the gas flow rate, it was possible to alter the bubble formation time and consequently the adsorption time of the surfactants (from 0.03 seconds to 60 seconds). The bubble tensiometer was a Sita online T60 (Sita, Germany).

3.4 Evaluation techniques

The properties of the fabric were determined by pore volume distribution (PVD) measurements, structural contact angle measurements θ, pectin removal measurements, and by tensile strength measurements. To visually characterise the fabric surface, scanning electron microscope (SEM) pictures were made.

3.4.1 The structural contact angle θ measured with liquid porosimetry

To study the change in hydrophilicity (wetting) of fabric sample, two methods are widely used e.g., the so-called 'drop test' and another is the 'water absorption test'. In the AATCC standard drop test, a water drop is placed on to the fabric and the time until the drop is fully disappeared is measured [26]. Both the tests have some disadvantages. Results from 'drop test' are greatly affected by the change in pore radius of fabric samples. Pore radius might be affected by the different treatment that is why 'drop test' values give large standard deviations. Water absorption test is influenced if the pore size changes because of given treatment. Moreover, this test does not give any information on the pore size distribution (PVD) in a fabric that allows us to monitor pore changes. Since textile fibres do not have ideal surfaces, their wetting phenomena are complicated by surface roughness and structure heterogeneity [27]. The contact angle of a moving liquid drop into the fabric is different from the contact angle formed by the static liquid [28, 29]. The drop on fabric surface is dynamic and hence depends on the structural contact angle [29].

A TRI auto-porosimeter [30] was used to measure the structural contact angle of fabric together with information on the inter- and intra-yarn pore size distribution. The pore size is determined via its effective radius, and the contribution of each pore size to the total free volume. The advantage of an auto-porosimeter above other wettability experiments is that the results obtained are independent of the fabric density, and of the structure of the fabric (e.g. knitted or woven). Miller [30] described the operating procedure for liquid porosimetry in detail, which requires quantitative monitoring of the movement of liquid into or out of a porous structure. The effective radius R_{eff} of a pore is defined by the Laplace equation.

$$R_{eff} = \frac{2\gamma \cos\theta}{\Delta P} \qquad (3.2)$$

Where γ is the surface tension (mN/m), θ is the advancing or receding contact angle of the liquid and ΔP is the Laplace pressure difference across the liquid/air

meniscus (N/m^2). The structural contact angle within a porous network can be determined from two plots of the liquid uptake *vs.* the effective radius. The first measurement is done with test liquid (e.g. double distilled water) and the other with a reference liquid (e.g. 0.1% Triton X-100). It is assumed that $(\cos\theta)_{ref} = 1$ for the reference liquid and that the test liquid does not form a zero contact angle. In that case, it is possible to calculate $(\cos\theta)_{test}$ value for the test liquid [30] by:

$$(\cos\theta)_{test} = \frac{2\gamma_{ref}}{(\Delta P)_{ref}} = \frac{2\gamma_{test}}{(\Delta P)_{test}} \qquad (3.3)$$

It should be kept in mind that for equation (3.3) to hold, the porous structure of the specimen must be unchanged during switching from one liquid for the other.

3.4.2 Pectin analysis with the ruthenium red dye method

Residual pectin on the fabric was analysed by staining with ruthenium red [31]. This method was well adopted to evaluate pectin removal in scouring [32, 34]. To summarise the method, two fabric samples of which the pectin content has to be determined were sewed together with a grey fabric sample and an alkaline scoured fabric sample to form a circular shaped fabric with a diameter of 8 cm. The pectin content of grey fabric is assumed as 100% and that of scoured fabric is 0%. The composite fabric was treated with ruthenium red (0.2 g/L) with 100 mL/g fabric in the presence of each 1 g/L of Silwet L-77 and Tergitol 15-S-12. A treatment was applied for 15 minutes at room temperature followed by rinsing with demineralised water for 10 minutes at 60°C in a Linitest. The Linitest is a small temperature controlled washing device. After line drying, the reflectance at 540 nm was measured and K/S values calculated. Where, K is the absorption coefficient of sample and S is the measured substrate scattering. The K/S values for the swatches are transferred into percentage residual pectin by the Kubelka Munk equation 3.4 [31, 34]:

$$\%\ \text{residual pectin} = \frac{100 \times (K/S - K/S_0)}{(K/S_{100} - K/S_0)} \qquad (3.4)$$

3.4.3 Tensile strength meter

The tensile strength measurements were performed to evaluate the effect of different treatments on cellulose polymers of the cotton fabric. Important information related to the fabric samples was gathered, for example, the average force in N to

break the fabric sample (F-break) and the percentage elongation of the fabric sample at F-break. A Zwick/Roell Z500™ tensile strength meter was used for the various measurements. All fabric samples were kept overnight to have a uniform humidity and temperature, the relative humidity was 38% and the temperature was 19.6°C.

3.4.4 SEM (Scanning Electron Microscopy) pictures

SEM is a widely used technique to study surface topography. Various fabric samples were subjected to SEM analysis, to visualise changes of the surfaces. The magnification was varied from 1000× up to 30000×. The analysis was carried out at the Central Materials Analysis Lab (CMAL/MESA+) at the University of Twente, the Netherlands.

3.5 Uniformity of cotton fabrics – a substrate selection

As a model, we have selected a plain-woven cotton fabric. A woven fabric is a bi-porous material with pores between the fibres - the intra-yarn pores, and pores between the yarns - the inter-yarn pores (Figure 3.3). It is important that the substrate has an uniform PVD for a proper determination of the structural contact angle. The substrate was characterised by measuring the PVD using an auto-porosimeter [30].

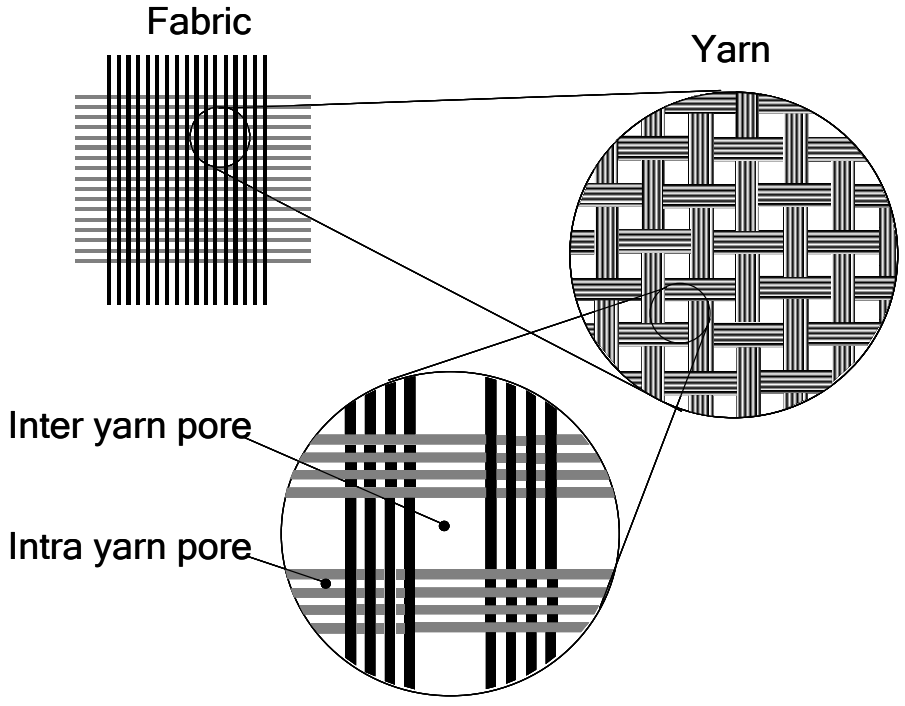

Figure 3.3: Schematic representation of structure of a woven textile material showing dual porosity an inter-yarn and intra-yarn pores [25].

Two swatches of 6 × 6 cm were randomly cut from a cloth with a minimum mutual distance of 0.5 m. Two PVDs for the Ten Cate industrially desized fabric are presented in Figure 3.4. Apparently, the intra-yarn pores are in the range of 3 – 8 µm. And inter-yarn pores are in the range of 38 – 55 µm, with a mean radius of 46 µm. The PVD distributions of both samples are identical (Figure 3.4). The PVD of fabric samples from a much lighter cotton fabric (120 g/m^2) were also measured. The PVD of these samples were found to be irregular, so the structural contact angle could not be determined correctly. Considering this, the Ten Cate fabric was selected as the standard cloth used in this study, because it was enough homogenous to determine the wetting characteristics as a result of different treatments.

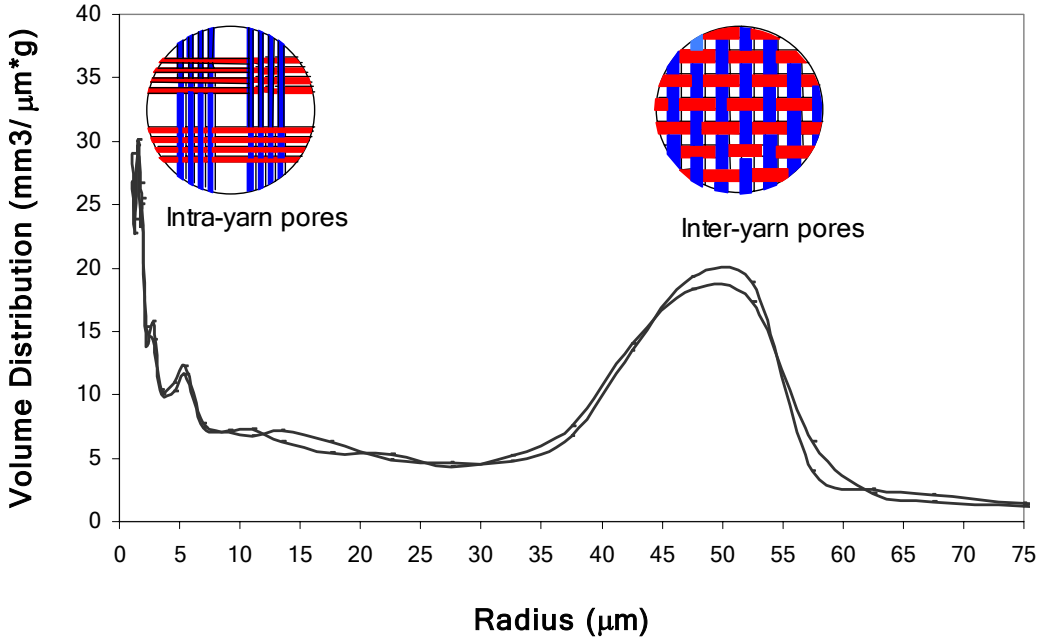

Figure 3.4: A PVD analysis for the Ten Cate industrially desized fabric (~280 g/m^2) in receding mode using Triton X-100 (0.1 g/L).

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4

Application of Alkaline Pectinase in the Bioscouring of Cotton Fabrics

Different commercial and specially produced pectinases were evaluated for an optimal bioscouring performance on grey cotton. The result of this treatment has been quantified in terms of percentage pectin removal and the structural contact angle of the treated fabric. It has been found that the pectinolytic capability of alkaline pectinases on cotton pectin is nearly 75% higher than that of acidic pectinases. To use pectinase efficiently in the scouring process, we explored the effect of wax removal on the pectinase performance. It has been found that a treatment of the cotton fibre to remove or to disrupt the waxy layer improves the affectivity of the pectinase treatment. Additives such as surfactants and chelators were used to optimise the enzymatic scouring process step. Parameters such as temperature, ionic strength and pH have been studied for best possible bioscouring performance.

4.1 Introduction

The aim was to explore the best-suited pectinase for cotton scouring and to use it efficiently for its maximum action towards pectin removal. Three main issues are discussed in this chapter. Firstly, a benchmarking of the existing alkaline scouring process was done by measuring the hydrophilicity after scouring in terms of the structural contact angle and the percentage pectin removal. Secondly, an evaluation was made for several selected acidic and alkaline pectinases for cotton scouring. It has been deduced that the pectinolytic ability of alkaline pectinase would be higher compared to acidic pectinases (Chapter 2). Finally, we present the proof for our hypothesis, which states that wax removal prior to pectinase treatment improves the enzyme performance. In this way pectinase can be used efficiently in the scouring process. N-hexane - a non polar solvent was used to remove the wax from cotton in order to measure the full potential of the chosen pectinase. It must be noteed that the purpose of the n-hexane treatment was to prove the significance of a wax removal step prior to the enzymatic treatment and not to establish n-hexane as pre-treatment agent.

4.2 Benchmarking

A proper benchmarking of the current alkaline scouring is essential to establish a clear relationship with other treatments. Miller [1], showed that the structural contact angle θ is directly proportional to the hydrophobicity of cotton fibre. Therefore, the structural contact angle and pectin removal have been measured for untreated fabric ($\theta_{untreated}$), after the wax removal ($\theta_{primary\ wall}$), and after scouring with NaOH ($\theta_{scoured}$) fabric. The contact angles for untreated fabric, primary wall and cellulose are in the following order $\theta_{untreated} > \theta_{primary\ wall} > \theta_{scoured}$ [1, 2]. The structural contact angle was used to benchmark the performance of the current alkaline scouring process and to compare the results of other treatments.

The results of the treatments are shown in Figure 4.1. Sample A was the blank, which was not treated at all. Sample B was extracted with n-hexane to remove the waxes. Sample C was scoured conventionally with NaOH. The structural contact angle of untreated cotton (sample A) was about 83°, the structural contact angle of the conventionally scoured fabric (sample C) was around 53°. These values are the benchmark values of which the latter must be achieved in the new scouring process. The average standard deviation in the structural contact angle measurements is in

between 3-7°. As shown in Figure 4.1, the pectin removal for untreated fabric was considered as 0% and for the NaOH scoured fabric 100%. To benchmark the effect of wax removal, we extracted desized fabric with n-hexane in soxhlet apparatus for 30 minutes at 75°C (sample B). Such a non-polar solvent extraction treatment removes almost all the cotton waxes [3]. The structural contact angle of ~68° was achieved after the solvent extraction. Solvent extraction treatment targeted to the wax removal, as a result, less than 7% pectin was removed (Figure 4.1).

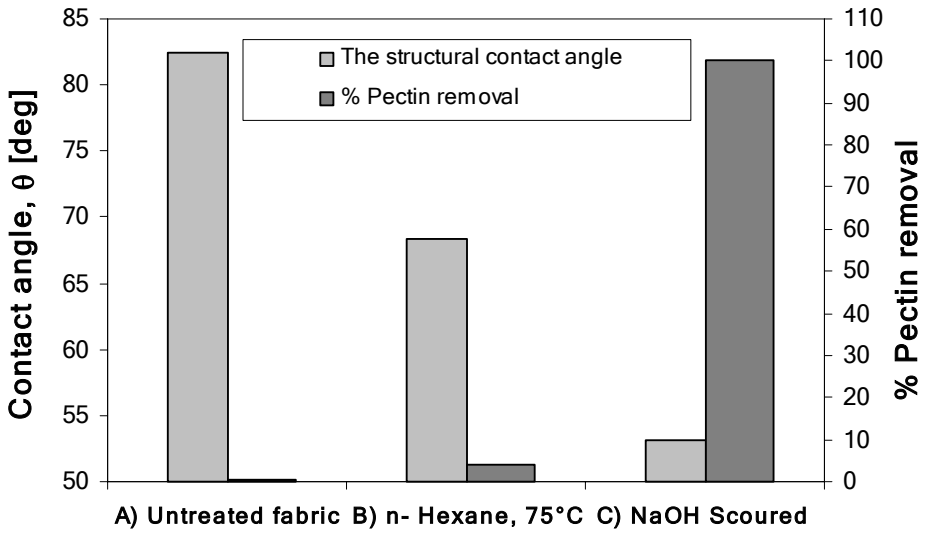

Figure 4.1: Change in the structural contact angle and percentage pectin removal with different treatments and its comparison with NaOH scoured fabric. Various treatments are given, A is the standard fabric as a blank sample, B) is solvent extraction with n-hexane for 30 minutes at 75°C to remove surface waxes and C) is NaOH scoured sample as a benchmark.

4.3 Potential of different pectinases

Effects of six selected pectinases (three different acidic and three alkaline pectinase) were studied on pectin removal from cotton fabric. To be sure that only the enzymatic effect of pectinase was determined, all samples used here have been treated first with n-hexane at 75°C for 30 minutes. It was assumed that in this way, the wax layer is removed and the primary wall that contains the pectin layer was fully exposed to the enzymes. The concentration of all the pectinases used was 100 U/g. Experiments with acidic pectinases (PGs) were performed at pH 5; while for the alkaline pectinase buffered solution of pH 8 was used (recommended pH). All the treatments lasted 30 minutes at 50°C (recommended temperature). Figure 4.2A shows the results obtained with the acidic pectinases and Figure 4.2B shows the results with the alkaline pectinase treatments.

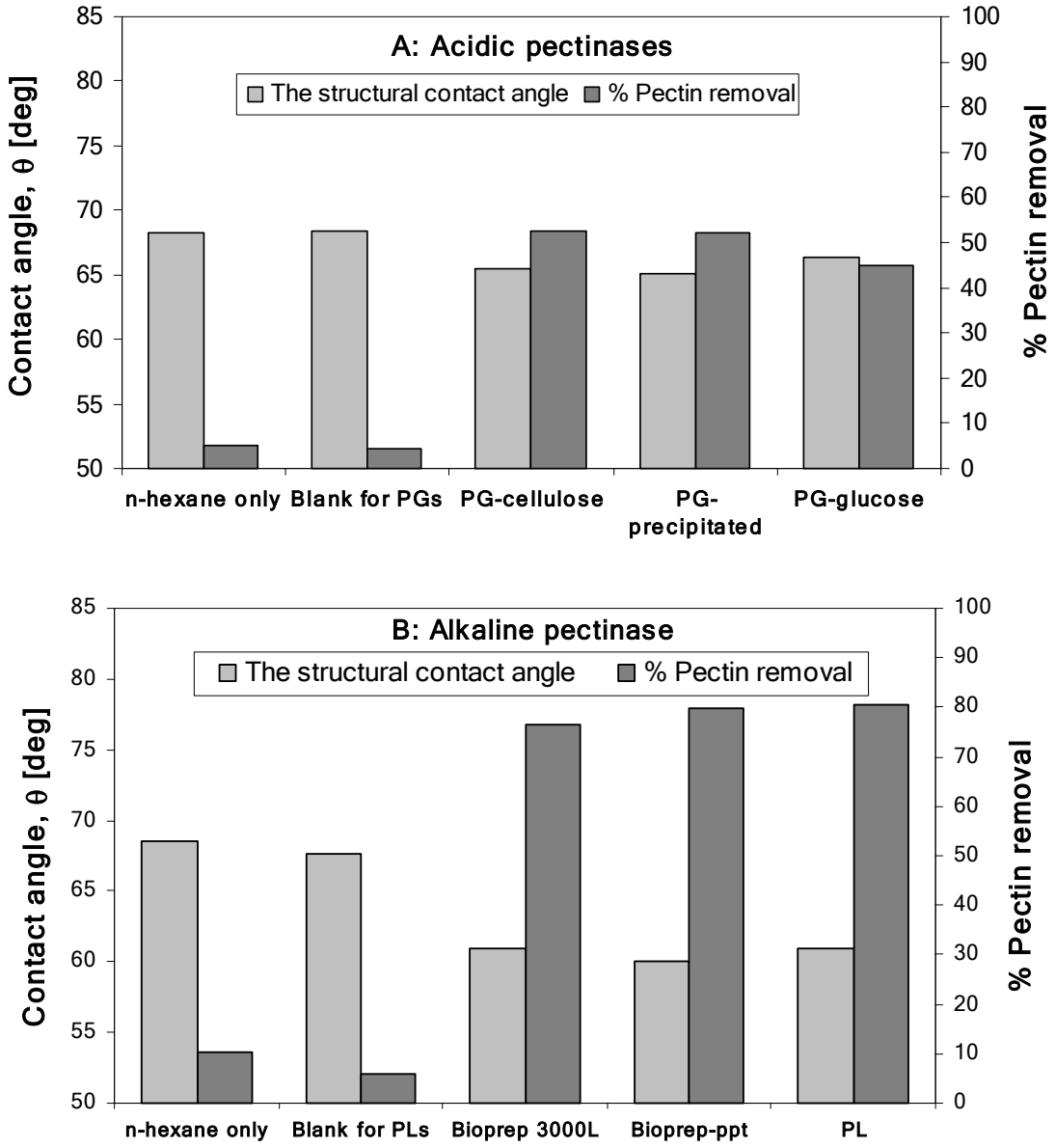

Figure 4.2: Effect of six different pectinases on the structural contact angle and percentage pectin removal. A) Fabric samples were treated with three different specially produced acidic pectinases (PGs) at pH 5, in 50 mM acetate buffer B) fabric samples were treated with Bioprep 3000L, precipitated Bioprep 3000L and specially produced PL at pH 8, 50 mM Tris-HCl buffer. Experiments were performed at 50°C, for 30 minutes using 100 U/g of pectinase.

The acidic pectinases (PGs) remove 45% to 52% of the pectin while with alkaline pectinases (PL or Bioprep 3000L), 76% to 83% of the pectin was removed. That means the performance of alkaline pectinase (PL) was 75% higher compared to the acidic pectinases (PGs). As far as the structural contact angle θ was concerned, the treatment with PGs give a contact angle of ~66° that was higher than with PL's, $\theta \approx 60°$. Blank samples without any pectinase treatment had a structural contact angle

of ~69°. Figure 4.2B shows that there was almost no difference between the performances of three selected alkaline pectinases in terms of the structural contact angle or pectin removal. Three conclusions can be drawn here; firstly, the performance of the alkaline pectinase is better than acidic pectinases (PGs). Secondly, the pectinolytic capability of specially produced PL was equivalent to that of commercial Bioprep 3000L and finally, the precipitated Bioprep 3000L has a similar performance as commercial Bioprep 3000L.

4.3.1 Mechanism of action of alkaline and acidic pectinase on cotton pectin

It has been found that, the pectinolytic capability of alkaline pectinase (PL) is higher than that of acidic pectinase (PG) on cotton pectin. Figure 4.3 schematically shows different cleavage mechanisms of PG and PL on a pectin molecule as proposed by Klug *et al.,* [4, 5]. The PG cleaves the galacturonic acid by an acidic hydrolysis reaction, while PL degrades pectin by a β-elimination reaction. The same authors found that PG cleaves α-1-4 glycosidic linkages in pectin that are not esterified (acidic pectins). PL cleaves the partially esterified pectin backbone and the long methylated chains of rhamnogacturonan, which results in the formation of a double bond between C4 and C5 at the non-reducing end. For more detailed information on the structure of pectin see Chapter 2.

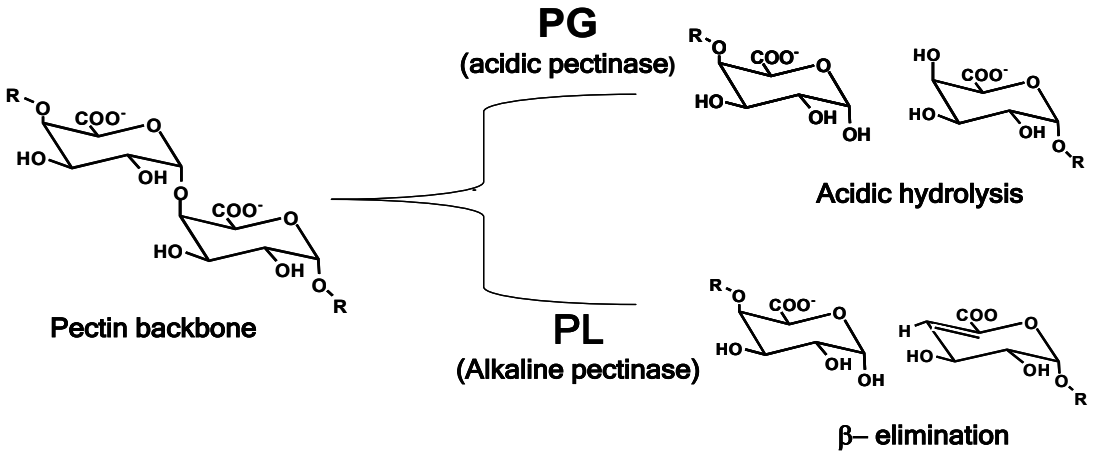

Figure 4.3: Schematic representation of action of pectinolytic enzymes - PG (polygalactouronase) and PL (pectate lyase) on pectin backbone [4, 5].

In Chapter 2, it is reported that the degree of pectin esterification has an opposite effect on the rate of degradation by acid hydrolysis compared to its effect on the rate of β-elimination. Alkaline pH also promotes the β-elimination reaction and

de-esterification process of methyl-esterified pectin. This helps to break the pectin polymer interaction between two esterified pectin molecules at the outer surface of the cotton fibre. The presence of partially esterified pectins on the outer layers of the cotton fibre and the alkaline pH increases the performance of alkaline pectinase compared to acidic pectinase.

Results prove our postulation about the superior performance of alkaline pectinase on cotton pectin. We have already explained in Chapter 2, based on the cotton fibre morphology that partially esterified pectins are abundantly available on the outer surface of the primary wall; hence, alkaline pectinase would perform better than acidic pectinase. From this point on, all alkaline pectinases used in the experiments i.e., Bioprep 3000L or PL, will be named 'pectinase'.

4.4 The effect of the waxy layer on the pectinase performance

In chapter 2, it was hypothesized that the presence of waxy layer hinders the penetration of pectinase to the pectin layer of the cotton fibre and thereby affecting its performance negatively. Therefore, we evaluated the effect of waxy layer on the pectinase performance towards improved hydrophilicity. The aim was to determine if either wax removal or the presence of surfactants improves the performance of pectinase. To verify this, two different set of experiments were done with the standard fabric. In the first set various treatments were given to the fabric followed by pectinase treatment. It has been deduced that boiling with water or buffer solution would melt some low melting cotton waxes (Table 2.2), resulting in an improved pectinase penetration in the primary wall. These different treatments are, a) blank or untreated fabric, b) boiling with demineralised water at 100°C for 2 minutes, c) boiling with 50 mM, Tris-HCL buffer, pH 8 at 100°C for 2 minutes and d) n-hexane extraction 75°C for 30 minutes. In the second set of experiments the same wax removal treatments (samples a, b, c and d) are given but this time the pectinase treatment was done in presence of 1 g/L Triton X-100. Before using Triton X-100 with pectinases, it has been established that Triton X-100 is compatible with the pectinase by conducting the activity assay (Chapter 3).

The results of first and second set of experiments are shown in Figure 4.4A and 4.4B respectively. Figure 4.4A shows that a treatment to remove waxes has a positive influence on the structural contact angle. However, the change in the structural contact angle was small, $\theta \approx 77°$ for the untreated fabric and $\theta \approx 68°$ with n-hexane treatment (both blank experiments). Comparing these structural contact angle values with that of pectinase treated fabric (Figure 4.4A), it was noticeable that

the difference in structural contact angle was higher for n-hexane extracted fabric (θ difference >7°), compared to any other treatment. The decrease in the structural contact angle for the solvent extracted fabric can be ascribed to an increased surface area accessible for pectinase treatment.

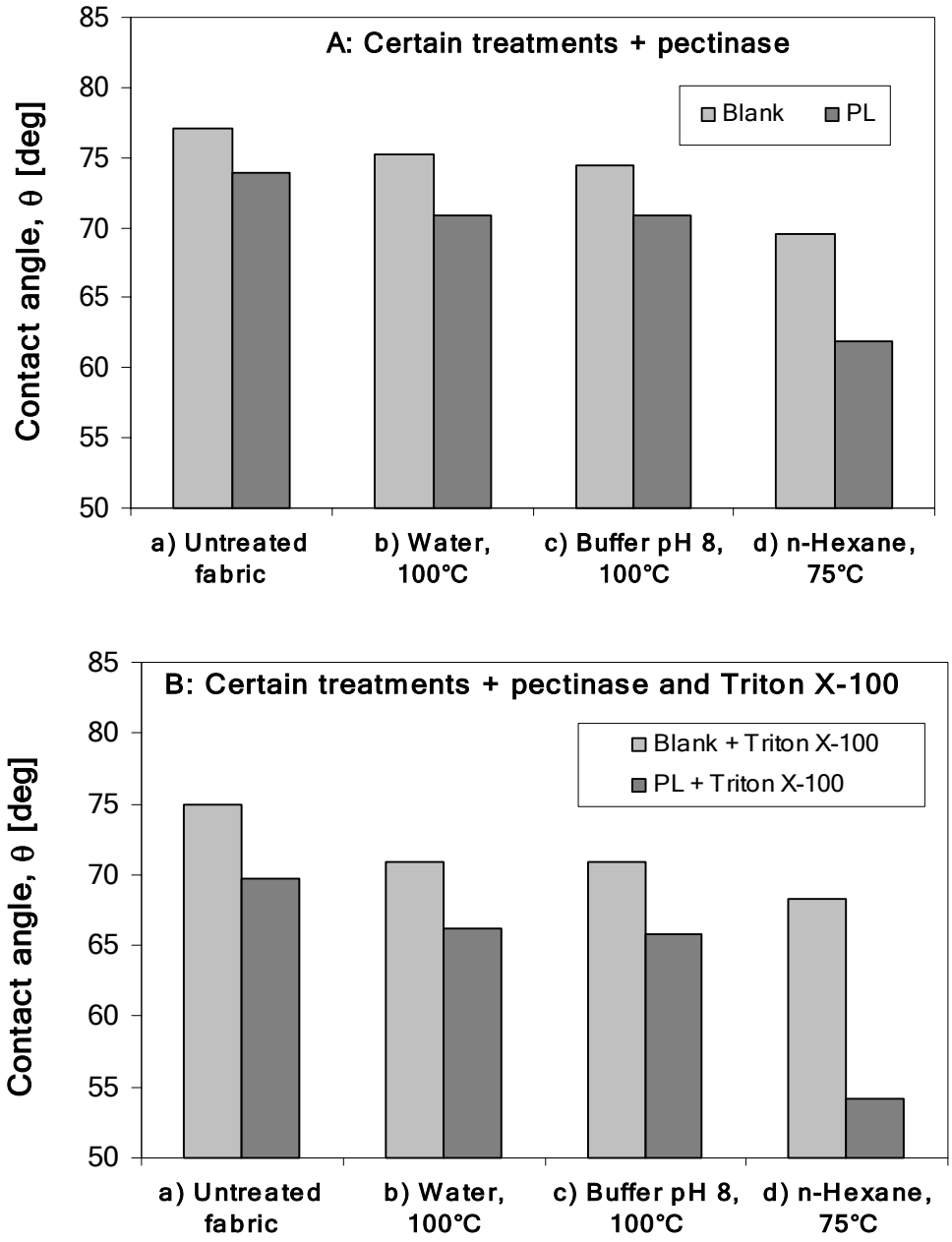

Figure 4.4: Effect of wax removal on pectinase performance in terms of the structural contact angle. Various wax removal treatments followed by 100 U/g PL incubation at 50°C for 30 minutes, in 50 mM Tris HCl buffer, pH 8, and the appropriate blanks. A) A certain treatment followed by blank and pectinase incubation, B) A certain treatment followed blank and pectinase incubation in presence of 1 g/L Triton X-100.

Figure 4.4B presents the combined effect of Triton X-100 and pectinase on grey cotton fabric. The structural contact angle improved marginally from ~73° to ~69° for the fabric without any additional treatment. The demineralised water and buffer treated fabric (sample b and sample c) shows almost the same structural contact angle ~65°. For n-hexane treated fabrics, a significant difference in the structural contact angle ~12° was achieved comparing the blank with the pectinase treated sample. A structural contact angle of ~54°, which is close to the desired value was obtained using surfactants in combination with Bioprep 3000L.

The biggest shift in structural contact angle was found with the samples that were extracted first with n-hexane. This was because solvent removes waxes in a much more uniform and efficient way and exposes the non-cellulosic interconnections to the pectinase. This was confirmed by the SEM picture as shown in Figure 2.3c. The picture shows the exposed, rough and open primary wall in which the non-cellulosic components are arranged in criss-cross pattern. N-hexane treated fabrics have the smallest standard deviation (± 3°), indicating that efficient wax removal leads to an uniform pectinase action on the cotton fibre surface.

From Figure 4.4, it can be concluded that the incorporation of a treatment that removes surface waxes like the n-hexane treatment leads to an increased hydrophilicity of the fabric, making pectin surface of the fabric more accessible to pectinase. Moreover, it can be concluded that for n-hexane treated fabrics, a non-ionic surfactant such as Triton X-100 has a positive impact on the pectinase performance because of the better surfactant penetration inside the yarns.

4.5 The effect of the waxy layer on the pectinase performance in terms of pectin removal

In section 4.4, it has been demonstrated that pectinase treatment after wax removal leads to an improved hydrophilicity. Therefore the effect of wax removal on the pectinase hydrolytic rate was evaluated. We hypothesised that wax removal from cotton fibre surface will result in more pectin surface available for the pectinase action, and hence the kinetics of pectin removal will be faster. The evaluation was done in terms of pectin removal as a function of time for different treatment. Standard fabric was considered as a control sample with an intact waxy layer on its fibre surface. Another sample was the n-hexane extracted fabric followed by the pectinase treatment. It was assumed that the n-hexane treated fabric (75°C for 30 minutes) is almost free from waxes.

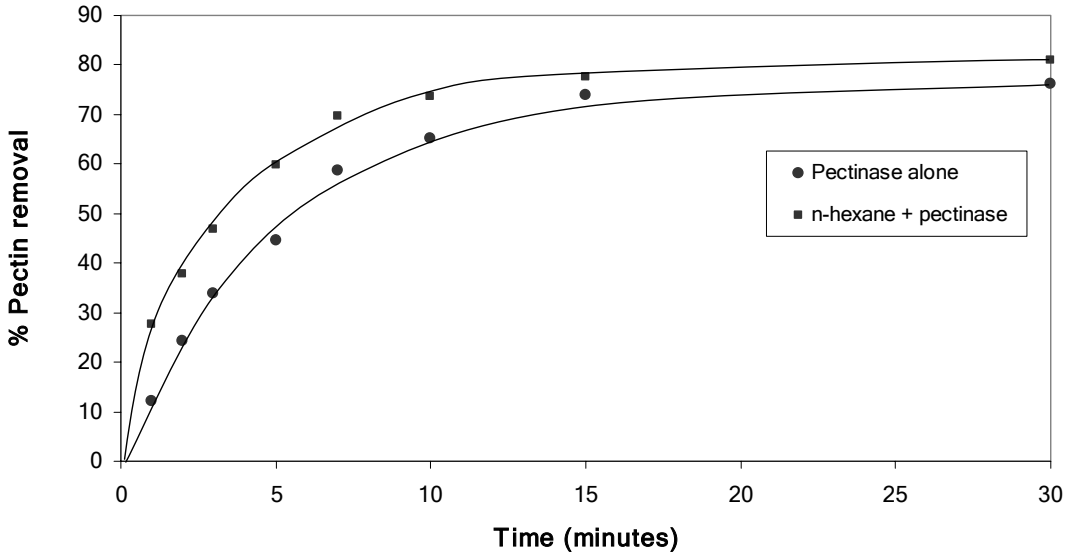

Figure 4.5: Performance of PL (13 U/g of fabric) in terms of pectin removal before (•), and after wax extraction (■). Lines are tentatively drawn. Desized fabric was subjected to n-hexane extraction at 75°C for 30 minutes prior to pectinase incubation.

Figure 4.5 shows that rate of pectin removal increases considerably if a wax removal treatment was applied prior to the pectinase treatment. It is apparent from the figure that the rate of pectin removal follows first order kinetics. After 30 minutes of pectinase incubation, 76-80% of pectin can be removed. It is clear that the kinetics of pectin removal is faster for the fabric sample which was previously treated with the n-hexane. The time constant for these two different treatment can be calculated by the 63% method. The time constant for a first order process gives insight into how the kinetics of product formation changes because of different treatment. Pectinase treatment alone takes nearly 9 minutes to remove 63% pectin; however for n-hexane treatment followed by pectinase needed only 5.5 minutes for the same amount of pectin removal. Therefore, the rate of pectin removal for the additional n-hexane extracted fabric is nearly 1.65 times higher than the pectinase treatment alone.

The simplest general equation for single-substrate enzyme catalysed reaction (in which there is just one substrate-binding per site per enzyme) can be written as

$$S + E \underset{K_2}{\overset{K_1}{\rightleftharpoons}} [SE] \xrightarrow{K_3} P + E \qquad (4.1)$$

Where, K_1, K_2 are the adsorption and desorption constant respectively, K_3 is the reaction rate constant. E, S, and ES represent the concentrations of enzyme, free substrate and the enzyme-substrate complex respectively. The Michaelis-Menten kinetics is not applicable for the heterogeneous system; however, our interest is to

know the relationship between the rate of the reaction and the substrate concentration which can be done by Michaelis-Menten equation. According to Michaelis-Menten kinetics, the initial conversion rate can be written as:

$$\left(\frac{dp}{dt}\right)_{t=0} = \frac{K_3.[E_0][S]}{[S]+Ks} \qquad (4.2)$$

Where, $[E_0]$ is the initial enzyme concentration and Ks is the dissociation constant of enzyme-substrate [ES] complex. From equation 4.2, it is clear that initial product formation is dependent on the initial enzyme concentration $[E_0]$ and available free substrate [S]. In our experiments $[E_0]$ is not varied for both treatments; hence the rate of pectin removal is completely dependant of the available substrate for pectinase action. The larger surface area was available when the fabric previously extracted with n-hexane, which facilitates exposing the non-cellulosic interconnections in the primary wall to the pectinase. As a result the rate of product formation increases by ~1.65 times.

Interestingly, from the structural contact angle measurements (Figure 4.4A), it has been found that $\theta \approx 72°$ was achieved for only pectinase treated fabric, while $\theta \approx 63°$ was obtained when fabric was first treated with n-hexane followed by pectinase. The difference in the structural contact angle was ~9°. However, the amount of pectin removal is nearly same (76% - 80%) with both treatments after 30 minutes. It can be concluded that the structural contact angle is influenced by both wax and pectin removal. In case of n-hexane followed pectinase treatment, the waxes are removed efficiently, but that was not the case with only pectinase treatment. This issue will be discussed in Chapter 6 in more detail. To conclude, addition of efficient wax removal step improves the performance of pectinase in terms of pectin removal and hydrophilicity.

4.6 Scouring parameters

Owing to the density of the fabric and its compactness of the woven structure, the fabric used in this study was difficult to scour, conventionally as well as enzymatically. This explains why this fabric produces substantial differences between enzymatically treated and blank samples. For less dense and open fabrics, this difference was less pronounced (unpublished results). Therefore it is expected that the selected fabric will reveal the influence and significance of different process variables needed for optimising the scouring process. After different treatments with pectinase (PL) all the fabrics underwent the standard rinsing and drying protocol

(Chapter 3). Scouring parameters, such as pectinase concentration, pH, temperature, ionic strength, and chelator concentration were investigated. Results from scouring parameters are shown in Figure 4.6.

4.6.1 Pectinase concentration

The pectinase concentrations were varied from 1 U/g to 20 U/g. All the experiments were done at 50°C for 5 minutes in a 50 mM Tris-HCl buffer at pH 8 with mild shaking. The purpose of the mild shaking was to distribute the pectinase uniformly in the bulk medium and not to create mechanical shear in the system. It is apparent form Figure 4.6A that pectin removal increases with increasing pectinase concentration. 1 U/g of pectinase can remove up to 6% of the pectin in 5 minutes, at 5 U/g 30% is removed, at 10 U/g 41% is removed, while almost 46% of the pectin has been removed using 13 U/g of pectinase in 5 minutes. Increasing the pectinase concentration above 13 U/g had no positive influence on the pectin removal. That means above 13 U/g concentrations the increase in pectinase hydrolytic rate is restricted because of substrate limitations. Such a trend in pectin removal was achieved without any additional ingredients or modification of the overall scouring protocol as mentioned in general experimental set up (Chapter 3). Therefore for all subsequent experiments 13 U/g of pectinase was used.

4.6.2 pH

Experiments with PL were performed at pH 6, 7, 7.5, 8, 8.5, 9, 9.5, and 10 using 50 mM acetate, Tris-HCl or borate buffer. All other parameters are identical to the ones in the paragraph above. The optimum pH range documented for Bioprep 3000L is pH 8.0 to 9.0 [6], which corresponds to the obtained results. Figure 4.6B illustrates typical bell shape curve for the pH optimum for the PL. At pH 6 (blank experiment with double distilled water instead of Tris-HCl buffer) only 11% of the pectin could be removed, while at pH 7 and 7.5, 24% to 29% of the pectin was removed. 45% of the pectin can be removed when the pH was between 8 and 9. Klug [4] reported that the isoelectric point for PL is at pH 8.5 which coincides with its maximum activity. The pectin removal efficiency of pectinase reduced drastically above pH 10 (20% pectin removal). Therefore for all other scouring experiments pH 8 was chosen.

4.6.3 Temperature

Experiments were performed at different temperatures from 20°C to 80°C with an interval of 10°C. All other parameters are identical to the ones in the paragraph

above. The temperature has a large impact on the overall performance. According to Novozymes the optimum operating temperature range for the Bioprep 3000L was 50°C to 60°C [6]. From Figure 4.6C it is evident that at the low temperatures ranging from 20°C to 30°C, only 15% to 36% of the pectin can be removed owing to the low reaction rate in first 5 minutes. Pectin removal with PL increases drastically to 46% when the temperature was raised to 50°C. The performance remains more or less constant up to 60°C. At 70°C only 27% of the pectin was removed. This can be attributed to the higher denaturation rate of the pectinase at high temperatures. Klug [4] reported that at 60°C, the PL is stable for less than 4 minutes. The operating temperature is often a compromise between reaction rate and enzyme stability. Therefore, a temperature of 50°C was selected for all other scouring experiments with the PL.

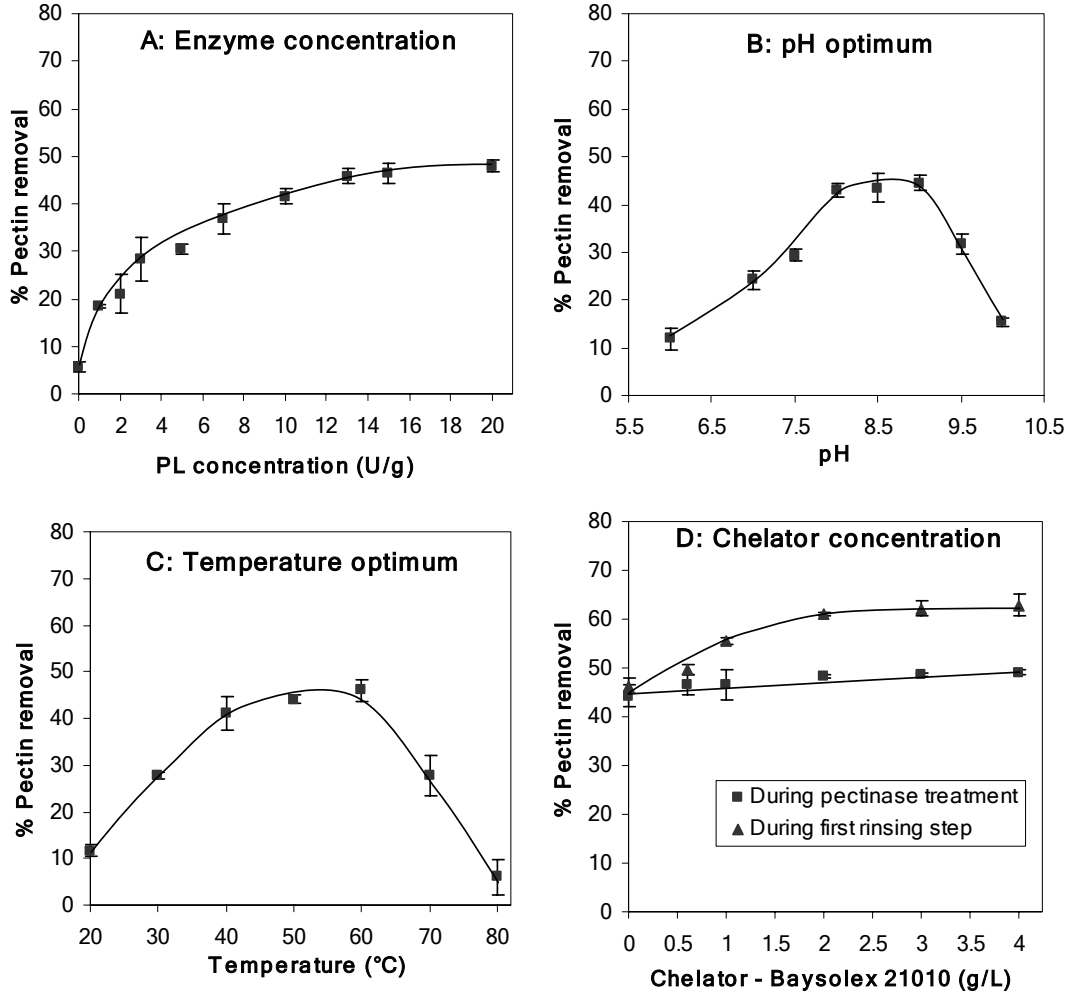

Figure 4.6: Evaluation of various parameters for the optimal pectinase (PL) action using standard fabric as substrate: A) pectinase concentration, B) effect of pH, C) effect of temperature, and D) effect of chelators.

4.6.4 Ionic strength

The ionic strength of the Tris HCL buffer solution (pH 8) was varied from 10 mM to 100 mM at the time of the PL incubation, to study its effect on the pectin removal. The pectinase concentration and other experimental conditions were identical to the ones mentioned before. The pectin removal increases with increasing ionic strength of the buffer solution. PL in 10 mM buffer solution can remove up to 16% of the pectin, in 20 mM buffer solution 29% removal is possible, in 50 mM buffer nearly 46% of pectin was removed, while almost 47% of the pectin can be removed using PL in 100 mM buffer solutions. This suggests that pectin removal is not only influenced by the pH of the buffer but also substantially by to the ionic strength of the media. The buffer solutions itself (10 mM to 100 mM) not have any effect on the pectin removal (blank experiments). Considering this, 50 mM ionic strength was selected for all other experiments.

4.6.5 Chelators

Chelators or sequestering agents have a complex structure usually heterocyclic in nature and are known for their binding capacity of ions such as Ca^{2+} and Mg^{2+} by removing them from their native location. The complex structure of the chelators can inhibit enzyme's action toward the target substrate. The use of chelators was desired in cotton scouring for the removal of Ca^{2+} ions from the winding layer of cotton fibre. That will help to remove other non-cellulosic materials from the primary wall and therefore a hydrophilic fabric (Chapter 2).

Two different experiments were performed. In the first set, the experiment was conducted to study the compatibility of two commercially available chelators with the pectinases (Bioprep 3000L and PL). Chelators used in this study are Baysolex 21010 (from Bayer) and EDTA (Ethylene Diamine Tetra-acetic Acid). Commercial chelator Baysolex 21010 predominantly contains imidodisuccinate apart from some unknown additives [7]. Results confirm that there was no influence of Baysolex 21010 at all the concentrations (from 0.5 to 2 g/L) on pectinase (Bioprep 3000L or PL) activity. Whereas EDTA, even in the small amount (0.5 g/L) inactivates the action of both the pectinases almost completely. Considering this, Baysolex 21010 was selected for the further study as a choice of chelator.

In the second set of the experiment, different concentrations of the Baysolex 21010 were used (0.2, 0.4, 0.6, 1, 2, 3 and 4 g/L) to evaluate the effect on pectin removal during pectinase treatment. The results from second set of experiments are presented in Figure 4.6D. The chelator was not much effective on pectin removal

when added with pectinase incubation. The change in pectin removal was marginal (from 45% to 49%), when comparing the results with the blank experiment (no chelator).

However, we also studied the effect of chelators when added after pectin incubation or during first rinsing step at 90°C for 15 minutes. From Figure 4.6D; it is apparent that the chelators have significant impact when added during the first rinsing step. Pectin removal changes from 45% for no chelator to nearly 63% after adding 2 g/L Baysolex 21010. The results obtained with chelators can be explained in terms of the cotton fibre structure (Chapter 2). It is known that chelators act on acidic pectins or on non-esterified pectins that are cross-links with Ca^{2+} ions to hold the cell wall components together. These acidic pectins are deeply located in the winding layer (Chapter 2). It was expected that the chelators would bind the Ca^{2+} ions and would remove additional pectins. From the cotton fibre morphology, we deduced that alkaline pectinase will hydrolyse partially esterified pectin located in the outer layers of cotton fibre by a β-elimination reaction. In the later stage of the scouring process, chelators can help to remove Ca^{2+} and other deeply located ions. As predicted that the removal of these deeply located Ca^{2+} ions and acidic pectin was possible only after wax removal and removal of esterified pectins.

That was the reason why the performance of chelator during pectinase treatment was poor compared to its performance during the first rinsing step. The incubation of chelator during the first rinsing step has two advantages. Firstly, there will be no possibility of enzyme inactivating owing to chelator and secondly, there will be enough time and sufficiently higher temperature (~90°C) for the chelator to act on deeply located pectin especially after the pectinase incubation. From Figure 4.6D, it can be concluded that addition of chelating or sequestering agent has significant impact on pectin removal and hence on overall scouring process. The addition of chelator can improve overall scouring effect if it was added at the right stage of the process.

4.7 Conclusions

Benchmarking of the existing NaOH scouring process was done by measuring the structural contact angle θ and pectin removal for untreated ($\theta_{untreated}$), after wax removal ($\theta_{primary\ wall}$), and for alkaline scoured fabric ($\theta_{scoured}$). A structural contact angle of less than 53° is needed to achieve sufficient hydrophilicity in the scouring process.

Six selected acidic and alkaline pectinases were tested for their scouring potential after wax removal. The difference in performance can be deduced form the cotton structure. The pectin removal performance of alkaline pectinases (PL and Bioprep 3000L) is nearly 75% higher than that of acidic pectinase (PG). Moreover, alkaline pectinase improves the hydrophilicity ($\theta \approx 60°$) better than the acidic pectinase ($\theta \approx 66°$). The specially produced pectate lyase from *Bacillus Pumilus* (PL) was, like the commercial Bioprep 3000L, a potential enzyme for an industrial bioscouring process.

It is essential to use pectinases efficiently for an improved scouring process. Results validate an important hypothesis, which states, that the disruption and removal of the outermost waxy layer is of prime importance to allow pectinase to react efficiently with the substrate. This should result in a more efficient process because of improved hydrophilicity. N-hexane extracted fabrics followed by the pectinase incubation in the presence of surfactant produces the best results, $\theta \approx 54°$ that was close to alkaline scoured fabric. The rate of pectin removal was nearly 1.65 times faster when the fabric was treated with n-hexane followed by the pectinase. From these results it was concluded that wax removal is a prerequisite for higher pectinase performance to achieve sufficient hydrophilicity in the enzymatic scouring process.

Various scouring parameters such as enzyme concentration, pH, temperature, ionic strength, and chelator concentrations have been evaluated using alkaline pectinase. The optimal conditions for PL are, 13 U/g of enzyme concentration at 50°C, 50 mM, Tris-HCl buffer at pH 8. Such experiments provide a good basis for process optimisation. The efficient use of chelator at rinsing stage of the scouring process has been justified in terms of maximum pectin removal. The chelator at this stage can help to remove extra 10-15% of the pectin, which would not possible to remove otherwise with the help of enzymes.

References

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5

Cutinase Application for Low-Temperature Wax Removal During Cotton Scouring

The challenge is to remove cotton waxes at low-temperature without using organic solvents. The relevance and potential of a cutinase from phytophathogenic fungus Fusarium solani pisi for cotton wax degradation is presented. Since, surfactants are essential for the faster migration of the scouring enzymes into the fabric, compatibility of cutinase with surfactants is an important aspect. The effect of cutinase treatment on pectinase performance was investigated. The optimum pH, temperature, ionic strength, enzyme concentration and time were determined for cutinase action. It has been concluded that cutinase is an effective tool for the degradation and removal of cotton waxes at low-temperature.

5.1 Introduction

The cuticle of the cotton fibre is cross-linked to the primary cell wall by esterified pectic substances, which hinder pectinase action on the pectin backbone. Therefore it is necessary to remove these cuticle waxes from the cotton fibre for an efficient enzymatic scouring process. The challenge is to do this at low-temperature under mild conditions. A thorough literature survey was done to find low-temperature aqueous based methods. The enzyme cutinase from fungus *Fusarium solani pisi* was identified as potential candidate (see Chapter 3). Degani *et al.,* [1] were the first to report about the potential of cutinase from a bacterial source *Pseudomonas mandocino* for wax degradation in cotton scouring. As a proof for wax degradation, Degani *et al.,* [1] confirmed the release of C_{16} and C_{18} fatty acids in the bulk medium, by means of advance techniques such as reverse phase- high pressure liquid chromatography (RP-HPLC) and gas chromatography-mass spectroscopy (GC-MS). They successfully demonstrate that combination of cutinase and pectinase with surfactants gives a synergistic effect in cotton scouring. To measure the change in hydrophilicity after the various treatments, the 'drop test' was applied by them. These results are encouraging; although, their enzyme incubation times were between 10 to 20 hours, which is too long to allow its industrial implementation.

A systematic study is conducted to characterise cutinase for the improvement of wettability of raw cotton fabrics, by specific hydrolysis of the ester bonds of triglycerides and cutin in the cuticle. Cotton wax degradation at low-temperature and within short time is attempted, which is a pre-requisite for the successful implementation of cutinase technology at an industrial scale. The effect of cutinase alone as well as in combination with pectinase is studied on cotton fabrics. The experimental approach is the same as described in Chapter 4. By the specific degradation of the cuticle, an increase in pectinase action on the pectin backbone is expected. This will result in an improved destabilisation of the primary wall and consequently increased hydrophilicity.

5.2 Wax removal with cutinase

To explore the potential of cutinase from *Fusarium solani pisi* for degrading cotton waxes, experiments were performed with the standard fabric as a substrate. Three different treatments were performed with: A) a cutinase, B) n-hexane extraction, as a benchmark, and C) a blank for sample A. The structural contact angle θ was measured, which is less sensitive to the effect of structural changes in the fabric sample due to the different treatments (Chapter 3). Figure 5.1 shows the

results of these treatments in terms of the structural contact angle θ and the pectin removal. From this figure it is clear that cutinase is capable to hydrolyse cuticle waxes. A structural contact angle of ~70° is obtained, which is close to the desired benchmark of θ ≈68°, the value obtained with n-hexane extraction. The average standard deviation in the structural contact angle measurements is in the range of 3° to 7°. The results also show that the blank treatment has no effect on wax removal θ ≈80°. All treatments were targeted towards wax removal, and as expected, pectin removal was negligible i.e. less than 7%.

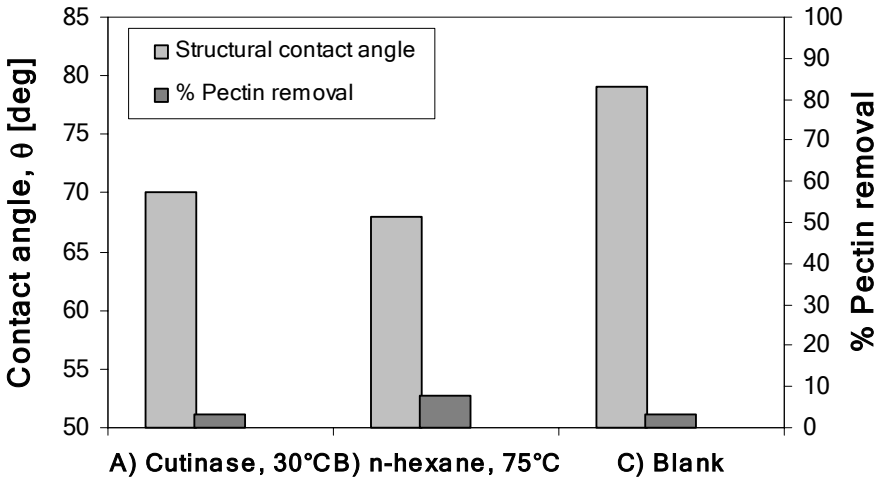

Figure 5.1: Comparison of cutinase treatment with n-hexane treated fabric. Various treatment were done, A) cutinase incubation, 100 U/g of fabric at 30°C for 30 minutes (pH 8 in 50 mM, Tris-HCl buffer, B) n-hexane extraction in Soxhlet apparatus at 75°C for 30 minutes, as benchmark, and C) blank for sample A.

To visualise the effect of these three treatments, SEM pictures were taken as shown in Figure 5.2. Treatments with cutinase Figure 5.2A and n-hexane Figure 5.2B remove surface waxes and unveil the primary wall of the cotton fibre. In these figures, the rough and open primary wall surface is visible, the smooth waxy layer is absent. A complicated network in which non-cellulosic materials are arranged in criss-cross pattern can be seen. The surface of the cotton fibre of the blank treatment shows the smooth waxy layer with grooves, caused by the underneath complicated primary wall, Figure 5.2C.

To conclude, cutinase is capable of achieving the same structural contact angle results as the benchmark. Thereby we confirm the potential of cutinase for the low-temperature cotton wax removal as proposed by Degani *et al.,* [1]. Even under non-optimised conditions, the treatment time to remove waxes was just 30 minutes,

which is much shorter then the 10-20 hours reported by Degani *et al.* This can be due to the use of different cutinase and different experimental conditions. This low-temperature fast wax removal treatment with cutinase from *F. solani pisi* has the potential to be applied in industrial cotton scouring process. Thus, it was decided to explore the treatment of cutinase together with surfactants and pectinase for an efficient cotton scouring process.

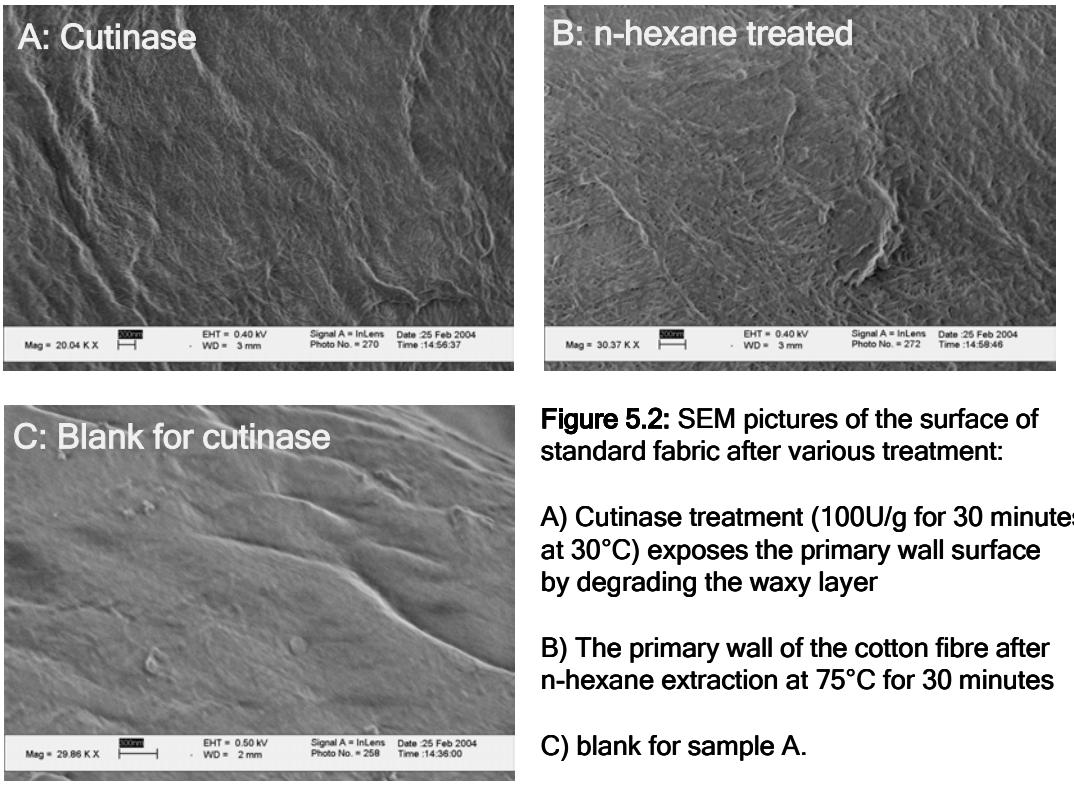

Figure 5.2: SEM pictures of the surface of standard fabric after various treatment:

A) Cutinase treatment (100U/g for 30 minutes at 30°C) exposes the primary wall surface by degrading the waxy layer

B) The primary wall of the cotton fibre after n-hexane extraction at 75°C for 30 minutes

C) blank for sample A.

5.3 Surfactants and their effect on cutinase action

In Chapter 4 it has been found that surfactants facilitate the penetration of pectinase into the yarns, thereby improving hydrolysis of pectin. It has also been established that wax removal with n-hexane helps to expose the pectin surface to the pectinase resulting into a faster hydrolysis. So, for a good pectinase performance the waxy layer has to be removed and surfactant are needed for good wetting conditions. As we have demonstrated, waxy layer can be removed by cutinase. Therefore in order to apply cutinase and pectinase in one step, the compatibility of cutinase with surfactants is essential.

Egmond [2] and Pocalyko [3] reported that a hydrophilic environment due to anionic surfactants negatively affects the adsorption of cutinase and the catalytic activity on its substrate. However, the same authors concluded that adding a non-

ionic surfactant such as Triton X-100 reverses the negative effect of anionic surfactants on cutinase. In other words, refolding of the cutinase structure can be achieved by the addition of non-ionic surfactants that are inert towards cutinase [2-4]. Degani *et al.,* [1] found a 70 fold decrease in wetting time of the drop test in the presence of the non-ionic surfactant Triton X-100 or with a mixture of different non-ionic and anionic surfactants. However, results from the 'drop test' can be influenced by changes in pore radii caused by the treatments of fabric samples (Chapter 3). So, it is not fully clear whether the results of Degani *et al.,* are caused by enhanced wetting or by a change in the pore size distribution.

Non-ionic surfactants are known to be very well compatible with enzymes and only a low concentration is required. Therefore, to systematically study the effect of surfactants on cutinase performance, a homologues series of four different non-ionic surfactants were selected. These surfactants are distinguished by different numbers of oxy-ethylene (EO) units as a head group, e.g. EO 6, EO 8, EO 10 and EO 12. In Chapter 4 we have shown that EO 10 (Triton X-100) is compatible with pectinase and improves the enzyme affectivity towards the pectin surface. The chemical names and some relevant properties of the surfactants are presented in Table 5.1.

SN	Non-ionic surfactant	Number of oxy-ethylene units (EO)	HLB number	Cloud point (°C)	Mol. wt.
1	Polyoxyethylene 6 myristyl ether	6	11.7	< 30	478.7
2	Polyoxyethylene 8 lauryl ether	8	12.3	35-38	538.7
3	Triton X-100 or Octylphenol ethoxylate	9-10	13.8	65	625
4	Tergitol15-S-12 or Secondary alcohol ethoxylate	12	14.5	89	728

Table 5.1: Details of different non-ionic surfactants used in study [5, 6].

Factors that have to taken into account for the selection of surfactants are the cloud point, the critical micelle concentration (CMC) and the hydrophilic lipophilic balance (HLB). The cloud point is the temperature at which micelle aggregation starts. Kissa [5] reported that surfactants are most active at a temperature close to its cloud point. For an optimum action, the surfactant concentration should be above the CMC. The HLB number reflects the characteristic properties of a surfactant to form

O/W or W/O emulsion [6]. A HLB number of less than 8 results in a W/O emulsion, while a HLB number above 12 results in a O/W emulsion. In cotton scouring, logically the latter is desired. The selected homologues series of surfactants (Table 5.1) meet these criteria.

5.3.1 Wetting

For the selected surfactants the equilibrium surface tension and liquid uptake of the standard fabric were measured. The structural contact angles θ for the selected surfactant were calculated from the liquid uptake data and are related to wetting characteristics (Chapter 3). For the surface tension measurements the bubble tensiometer was used (Chapter 3). At a bubble life-time of 55 seconds, the generation of new surface is slow enough to assume that the surface tension approaches the equilibrium value. All the selected surfactants achieve the CMC value at a concentration less than 0.2 g/L. As shown in Table 5.2, at CMC, the equilibrium surface tension was 27 mN/m for EO 6, 27.7 mN/m for EO 8, 28.1 mN/m for EO 10, and 31.2 mN/m for EO 12. The structural contact angle was measured for the four selected surfactants with two liquid uptake plots of test and reference liquid (Chapter 3). A structural contact angle of $\theta \approx 0°$ was found for EO 6, EO 8 and EO 10. The structural contact angle formed by EO 12 was nearly $\theta \approx 17°$ (Table 5.2). Summarising, the wetting with EO 6, EO 8 and EO 10 of fabric sample is better than that with EO 12.

SN	Surfactants	Surface tension (γ) mN/m	Structural contact angle, θ
1	EO 6	27	0
2	EO 8	27.7	0
3	EO 10 (Triton X-100)	28.1	0
4	EO 12	31.2	17

Table 5.2: Equilibrium surface tension data and the obtained structural contact angle θ for the selected non-ionic surfactants

5.3.2 Effect of surfactants on cutinase hydrolytic rate

As already mentioned, Degani *et al.,* [1] measured the wetting time using the 'drop test', in evaluating the final effect of surfactants on cutinase for enzymatic scouring. To systematically evaluate the effect of surfactants on cutinase kinetics, model experiments with fabric were done in the pH-stat. An O/W emulsion was

produced with tributyrin oil drops and 1 mM Tris-HCl buffer solution. The effect of cutinase hydrolytic rate on tributyrin drops were evaluated in the presence of selected surfactants. The concentrations of all surfactants were 1 g/L, which were well above their CMC values. Six experiments were performed: A) blank (no cutinase and no surfactant), B) only cutinase, C) cutinase + EO 6, D) cutinase + EO 8, E) cutinase + EO 10, and F) cutinase + EO 12.

The results of these experiments are shown in Figure 5.3. In this figure the hydrolytic rate of cutinase (case B), after 15 minutes has been taken as point of reference and considered as 100% activity. From the figure it is clear that all surfactant applied increases the hydrolytic rate of cutinase. The highest rate increase has been found with EO 8, line D and with EO 10, line E. The lowest rate increase is obtained with EO 6, line C. The increase of the hydrolytic rate is caused by the fact that the tributyrin drop size became smaller in the presence of surfactants, creating a higher substrate surface for the cutinase.

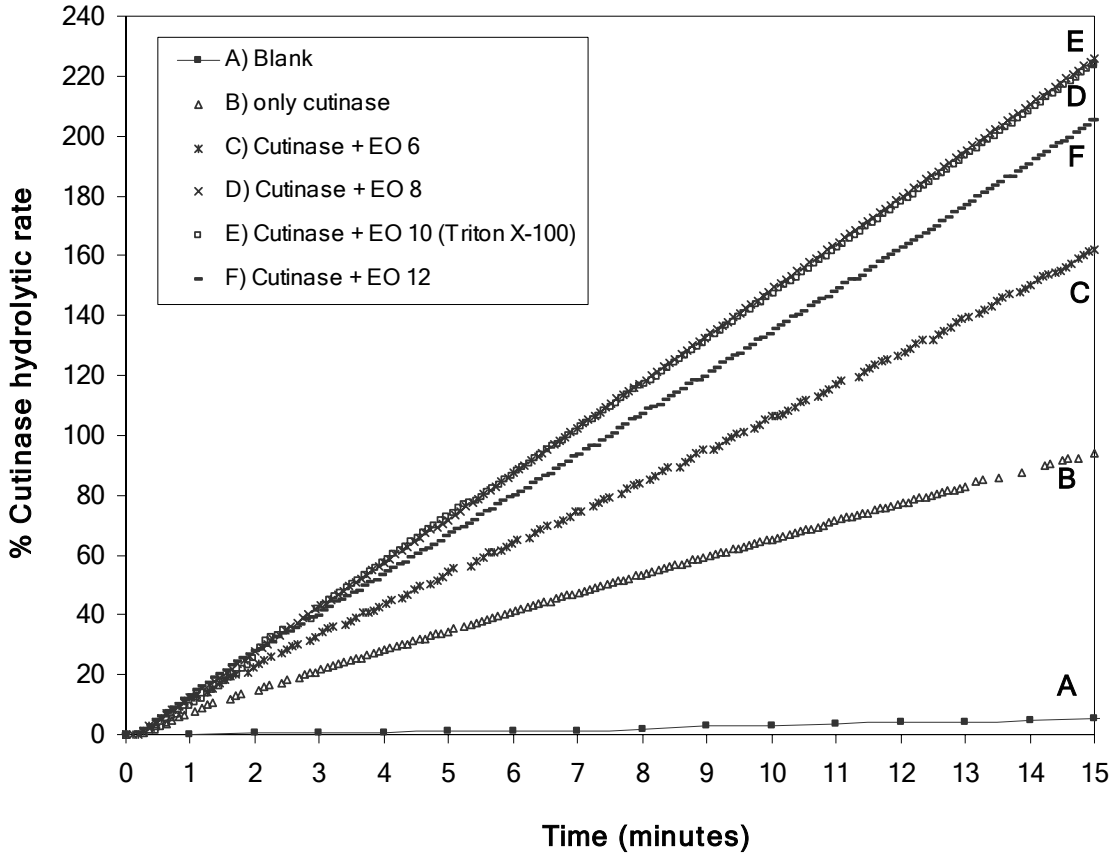

Figure 5.3: Effect of different non-ionic surfactants on cutinase performance with tributyrin oil drops as substrate. Experiments were performed in the pH-stat, with 1 mM, Tris-HCl buffer at pH 8, 30°C, with 50 U cutinase and 0.5 mL tributyrin.

From the pH-stat and equilibrium surface tension measurements and from structural contact angle data, EO 8 and EO 10 (Triton X-100) appear to be the better candidates for further experiments with cutinase. Triton X-100 has a cloud point of 65°C, which is higher than the cloud point for EO 8 (Table 5.1). For this reason EO 10 (Triton X-100), performs well in broader temperature. Additionally, the HLB number of Triton X-100 exceeds that of EO 8. Therefore, we have selected Triton X-100 for further study.

5.4 Cutinase and Triton X-100

In the previous section it has been shown that the hydrolytic rate of cutinase on tributyrin oil drops increases in the presence of surfactants. Surfactants reduce the interfacial tension of the oil-water surface, and thus the radius of the tributyrin oil droplets. This consequently increases the substrate surface area available for cutinase, and hence improves the tributyrin hydrolysis. However, it is known that the hydrophilicity of the substrate surface negatively affects the cutinase action [1, 7, 8]. So here we have to deal with two counteracting mechanism: the increase of the cutinase action by an increase of the substrate area and decrease of the cutinase action by an increased hydrophilicity of the substrate surface. To confirm this, two different sets of experiments were performed: The first set is to show that the presence of surfactant negatively affects cutinase action, and the second is to show that an increase in surface area of the substrate improves the cutinase hydrolytic rate.

5.4.1 Cutinase action – effect of different Triton X-100 concentrations

In the first set as mentioned above, three experiments were done in the pH-stat at a constant cutinase concentration and for different amounts of tributyrin. The Triton X-100 concentration was increased up to 1 g/L. The results of the first set of experiment are shown in Figure 5.4. In this figure we used the same reference value as in the former experiments. All curves in Figure 5.4 shows two distinct parts; in the first part of the curve the hydrolytic rate decreases because of an increase in surfactant concentration or higher hydrophilicity of the substrate surface. In the second part of the curves the hydrolysis rate increases. This effect was more pronounced when the amount of tributyrin was higher. For example in the case of 1 mL of tributyrin the cutinase hydrolysis rate increased after the initial decrease up to 190%. In the case of 0.1 mL tributyrin the hydrolytic rate increased only up to 40% after the initial decrease. In both the cases initial decrease in cutinase hydrolytic rate corresponds to initial increase in Triton X-100 concentrations till its CMC value i.e.

0.13 g/L, thereafter, the cutinase hydrolytic rate is proportional to the increase in Triton X-100 concentration. To conclude, the increase in hydrophilicity affects cutinase action negatively, the initial decrease in the curve. This negative effect of enhanced hydrophilicity was counteracted by an increase in the amount of tributyrin (1 mL). So, in this system the cutinase kinetics is substrate limited.

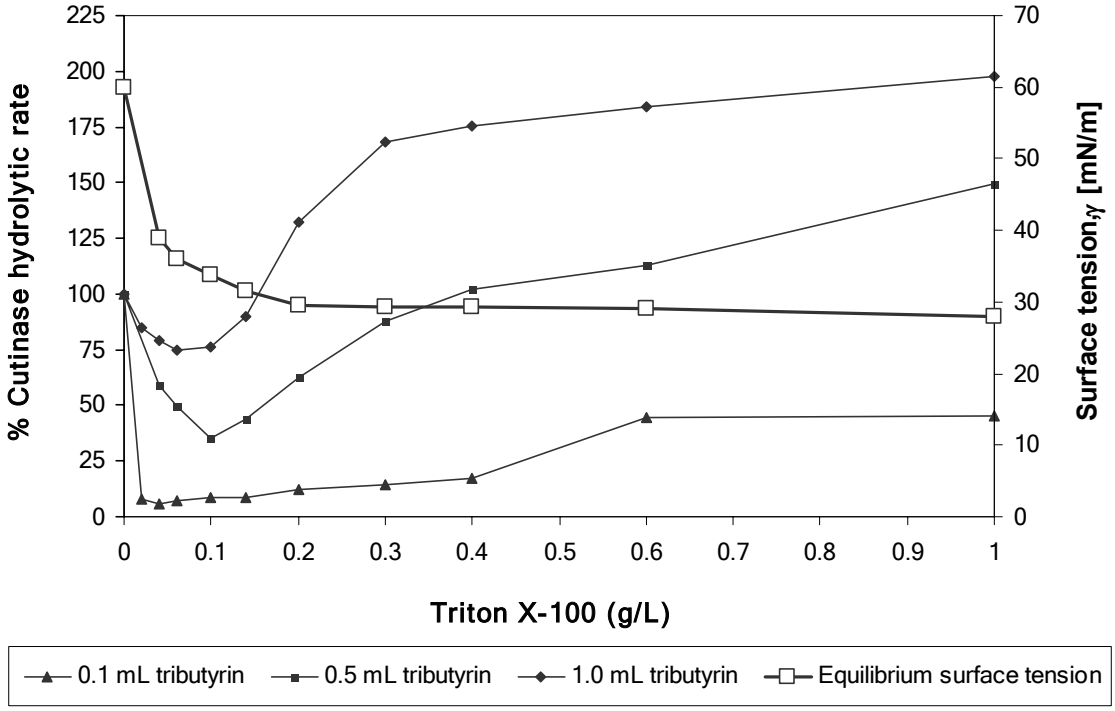

Figure 5.4: Effect on various concentration of Triton X-100 on cutinase hydrolytic rate. In the pH-stat experiment, 50 U of free cutinase and three different amounts of tributyrin substrate (0.1 mL, 0.5 mL and 1 mL) were used.

5.4.2 Cutinase action – effect of stirring speed and of Triton X-100

In the second set of experiments we investigated the effect of surfactants and the tributyrin drop size on the cutinase hydrolytic rate. Three different experiments were done in the pH-stat with increasing stirring speed (0-1200 rpm), to change the tributyrin drop size. The only difference was the presence or absence of Triton X-100. Experiments with; A) no surfactant, B) Triton X-100, below the CMC, and C) Triton X-100, well above the CMC, were done. To visually inspect the effect of the stirring speed and or surfactant on the change in tributyrin drop size, video recordings were made.

The experimental results are shown in Figure 5.5. Also in this figure we applied the reference of 100% cutinase hydrolytic rate after 15 minutes without stirring. In curve A, the effect of stirring speed on cutinase hydrolytic rate is shown. Up to 300 rpm, tributyrin droplets are just suspended in the bulk medium, resulting in

a slow increase in cutinase hydrolytic rate. Increase of the stirring speed from 300 rpm up to 1000 rpm results in the formation of dispersion with tiny tributyrin droplets. In this phase a maximum increase in cutinase hydrolytic rate was observed. Above 1000 rpm the drop size remains approximately constant and as a result the hydrolytic rate as well. To conclude, the cutinase kinetics depends on the amount of available substrate.

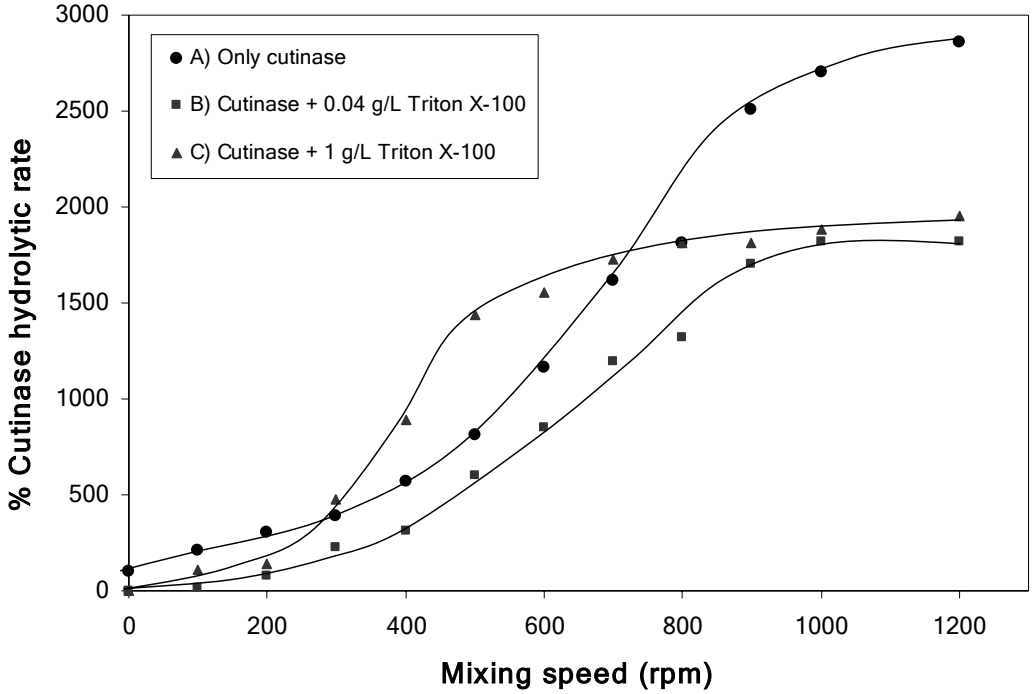

Figure 5.5: Effect stirring speed (rpm) on cutinase (50 U) hydrolytic rate (substrate 0.5 mL of tributyrin oil drops) in the pH-stat. The initial activity of cutinase was set to 100% (without surfactant and with no stirring).

The curve B shows, the hydrolytic rate of tributyrin is smaller compared to the treatment given in experiment A. The reason for this behaviour is that the concentration of Triton X-100 is below its CMC level. Since the interfacial tension is higher below the CMC, as a result, the drop size will be larger compared to the drop size above the CMC. The negative effect of the increase in hydrophilicity on the hydrolysis rate of cutinase is predominant and counteracts the smaller effect of the increase in tributyrin surface. That means, in experiment B, the negative effect of Triton X-100 is dominating.

In curve C, is a result of the negative effect of hydrophilicity and the positive effect of decrease in drop radius caused by the surfactant. High concentrations of Triton X-100 reduce the tributyrin drop size even at a low stirring speed, and hence a faster increase in cutinase kinetics. However, at 600 rpm, the maximum reduction in drop size was already achieved; and hence no further increase in the hydrolytic rate

was measured. The difference in the hydrolytic rate in experiment A and C, at high stirring speeds is a consequence of the increase in hydrophilicity caused by the surfactant. Therefore, the surfactant concentration is a compromise between these counteracting positive and negative effects.

5.5 Cutinase with cotton fabric – effect of Triton X-100

So far the experiments were done with a model substrate of tributyrin oil drops instead of cotton fabrics. Therefore to evaluate the effect of the surfactant on the performance of cutinase on the standard fabric, two experiments were done. In the first experiment the hydrolytic rate of cutinase on cotton fabrics in a pH-stat was measured. In the second experiment the structural contact angle was determined after the treatment of the standard fabric with cutinase and Triton X-100 in a beaker glass.

5.5.1 Experiments in the pH-stat

Experiments were performed with cutinase and various concentrations of Triton X-100 with 0.5 g of the standard fabric in the pH-stat. The results are shown in Figure 5.6. An initial decrease of the cutinase hydrolytic rate was obtained until 40% of the original value. This was due to the increased hydrophilicity of the substrate surface. 70% of original activity is retained at the higher surfactant concentration (above 0.3 g/L, Triton X-100). It is known that the small intra-yarn pores affect the mass transfer and wetting rates [5, 6, 9]. With the help of better wetting using the surfactant a larger substrate surface become available, therefore, partially counteracting the negative effect of increased hydrophilicity. The obtained results are similar as described as in section 5.3.2.

5.5.2 Scouring with cutinase and Triton X-100

To evaluate the potential of the cutinase and Triton X-100 together, the structural contact angle θ was determined and compared with the n-hexane extracted fabric as a benchmark. Three different treatments were done: A) cutinase alone, B) cutinase with Triton X-100 and, C) n-hexane extraction 30°C for 30 minutes as a benchmark. The results are shown in Figure 5.7. Apparently, both the treatments (sample A and B) are comparable with n-hexane treated fabrics. A structural contact angle of ~68° was achieved. Interestingly, n-hexane extraction followed by cutinase treatment yields a contact angle of ~66°, compared to ~68° for only n-hexane extracted fabric. In sample C (n-hexane followed by cutinase), we have removed all

waxes and the result from the treatment of cutinase and Triton X-100 (sample B) is comparable to that.

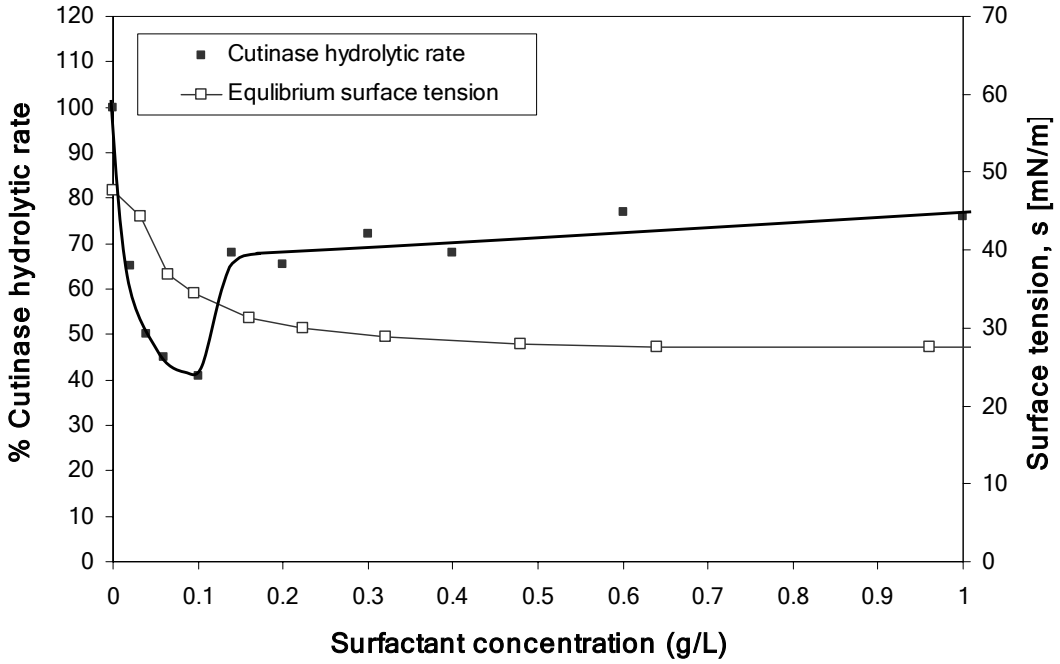

Figure 5.6: Effect of various concentrations of Triton X-100 on cutinase hydrolytic rate in pH-stat. Cutinase hydrolytic rate is related with the different Triton X-100 concentration and accomplished equilibrium surface tension. Cutinase concentration was 50 U, and 0.5 g standard fabric as a substrate.

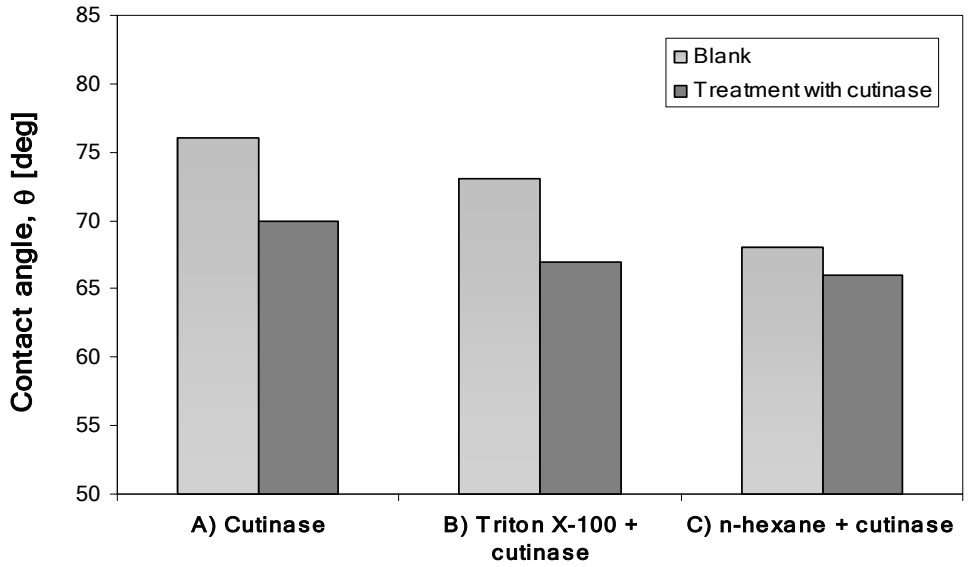

Figure 5.7: Comparison of cutinase treatment (with and without Triton X-100) with n-hexane treated fabric, measured in terms of the structural contact angle. Fabrics were subjected to various treatments: A) only cutinase (100 U/g, 30°C for 30 minutes at pH 8, in 50 mM Tris-HCl buffer), B) 1 g/L Triton X-100 and cutinase in one bath, C) n-hexane extraction at 75°C for 30 minutes as benchmark.

We demonstrated that low-temperature wax removal is possible with cutinase and in combination with Triton X-100. Degani *et al.,* [1] reported up to 70 fold decrease in wetting time when fabrics are treated with cutinase in presence of Triton X-100 or a combination of anionic and non-ionic surfactants. The time needed to achieve that result was 10-20 hours [1]. We demonstrate that less than 30 minutes is enough to remove nearly all the waxes from the cotton fibre surface, which is far shorter than the 10-20 hours reported by Degani *et al.,* [1]. It was complicated to compare our results with the reported results [1], owing to different measurement techniques, different fabric, and the different cutinases. To conclude, the results obtained are a breakthrough in cotton wax removal at low-temperature. Cutinase has great potential to be used in low-temperature industrial cotton scouring process.

5.6 Effect of cutinase on pectinase kinetics

In Chapter 4, we have established that wax removal prior to pectinase treatment improves the hydrophilicity and the pectin removal. To demonstrate the importance of wax removal for better scouring process, various wax removal techniques were used. We have developed a low-temperature wax removal technique based on enzyme technology, using cutinase. To explore whether an increased hydrophilicity due to wax removal with cutinase also leads to an improved pectinase performance, the removal of pectin from the cotton fibre was determined as function of time. Four different experiments were done:

A) the untreated standard fabric with an intact waxy layer on its fibre surface,
B) n-hexane extracted fabric assumed as sample without waxy layer (benchmark),
C) cutinase followed by pectinase treatment, and
D) treatment with cutinase and Triton X-100 together followed by pectinase.

The results of these experiments are given in Figure 5.8. In Chapter 4, we showed that the rate of pectin removal increases nearly 1.65 times if wax removal with n-hexane (treatment B) was applied before the pectinase treatment. The results from treatments A and B are already explained in section 4.5. For treatments C and D, 56% - 58% of the pectin is removed in the first 5 minutes, which is slightly less than with n-hexane extraction. After treatment with cutinase alone and in combination with Triton X-100, the rate of pectin removal is increased nearly 1.65 times. This is comparable with the benchmark. The total amount of pectin removed (~ 80%) for treatment C and D is the same as for n-hexane extracted fabrics. So, it can be concluded that cutinase remove waxes in an efficient way, thereby exposing the non-

cellulosic interconnections to the pectinase. The structural contact angle measurements and SEM pictures support these results.

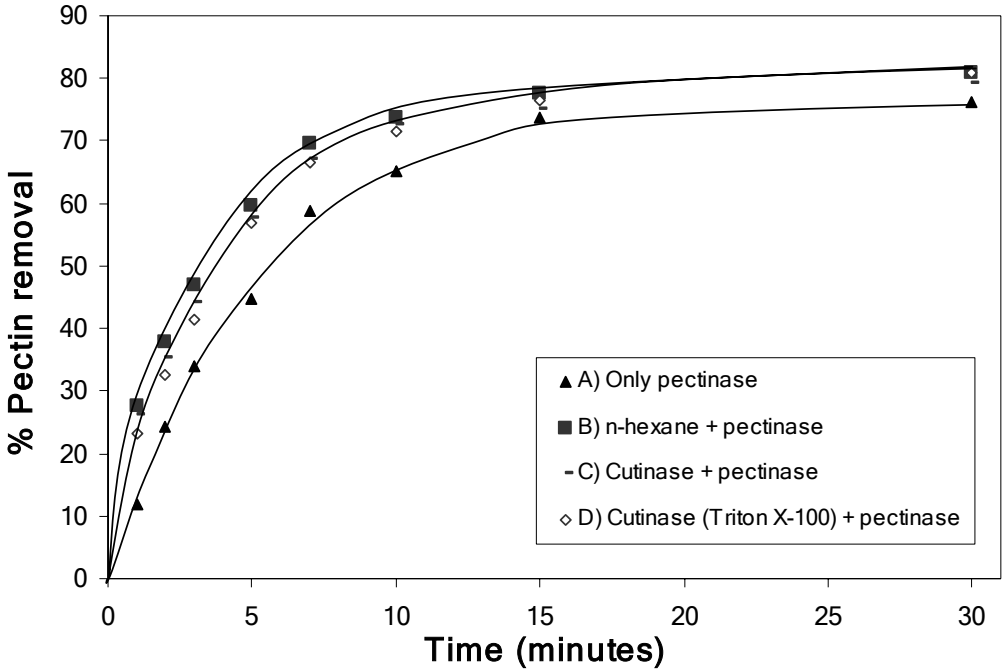

Figure 5.8: Effect of various treatments on the pectinase performance, in terms of pectin removal 13 U/g pectinase was used at 50°C, in 50 mM Tris-HCl buffer pH 8. 100 U/g cutinase concentration was used at 30°C at pH 8 in 50 mM Tris-HCl buffer pH 8, and 1 g/L Triton X-100 a non-ionic surfactant was used.

5.7 Parameters evaluation for cutinase

The sensitivity of parameters such as pH, temperature, ionic strength, enzyme concentration, and incubation time for cotton fabric were determined on enzymatic cotton scouring. The effect of the pH and temperature was also determined in the case of cutinase action on tributyrin drops in the pH-stat.

5.7.1 pH

Experiments were performed at pH 7, 7.5, 8, 8.5, 9, 9.5, and 10 using 100 U/g of freshly prepared cutinase. 50 mM Tris-HCl buffer was used for pH 7-8.5, and 50 mM borax buffer was used up to a pH of 10. All experiments were done at 30°C, and lasted 30 minutes. Figure 5.9A1 shows the structural contact angle as a function of pH. At optimum pH (8-9), the structural contact angle was ~70°. For the blank experiments, the structural contact angle was nearly 78°. The pH optimum for cutinase in the pH-stat shows a broader optimum, with considerable deviations in the data points, notably at higher pH (Figure 5.9A2). At higher pH, NaOH hydrolysis for

the blanks becomes more significant. The high deviation in the data points, especially for the varying pH, can be attributed to the high sensitivity of the activity assay. Instead of the broad optimum shown in Figure 5.9A2, the data points could also indicate two pH optima. However Carvelho *et al.,* [10] and Peterson *et al.,* [11] reported a single optimum pH, the former in the range of pH 9-10 towards triolein as substrate, and latter at a pH of approximately 8.5 towards tributyrin, the same substrate as in this study. This suggests that cutinase has broad optimum pH 8-9.5 rather than two pH optima. The catalytic mechanism of cutinase at alkaline pH is explained in section 5.8.

5.7.2 Temperature

To determine the optimum temperature, experiments were performed at different temperatures from 20°C to 50°C at an interval of 5°C. It is evident from Figure 5.9B1 that the optimum temperature for cutinase action is at 30°C. At this temperature, the obtained structural contact angle was ~71°. As shown in Figure 5.9B1, above 40°C there was no difference between the structural contact angle for the blanks ($\theta \approx 78°$) and the cutinase treated fabric. Similar results were obtained with pH-stat when determining temperature optimum for cutinase using tributyrin as substrate. The temperature optimum was around 25°C to 30°C (Figure 5.9B2). Above 35°C, a rapid decrease in activity occurs. The temperature optimum found with pH-stat is very well coinciding with the temperature optimum found with the fabric. Lucia *et al.,* [12], demonstrated irreversible deactivation of cutinase from *F. solani pisi* at high temperature. Deactivation of cutinase occurs because of precipitation and folding which starts above 40°C. Summarising, cutinase is sensitive to temperature, and therefore the process needs temperature control.

5.7.3 Ionic strength

The buffer strength was varied from (10 mM to 100 mM) using Tris-HCl buffer at pH 8.0. Increasing buffer strength has a positive impact on cutinase performance. For cutinase at low buffer strength (10 mM) the obtained structural contact angle was ~75°. The optimum buffer concentration for cutinase action was found to be 50 mM that corresponds to a structural contact angle of ~70°. The structural contact angle for the blank treatment was $\theta \approx 77°$. Increasing buffer strength above 50 mM has no additional improvement in the structural contact angle. Cutinase action on triglyceride releases free fatty acids in the bulk medium. Since these liberated fatty acids are negatively charged (above pK value), this may reduce the pH in the system, hence the stronger buffer is desired.

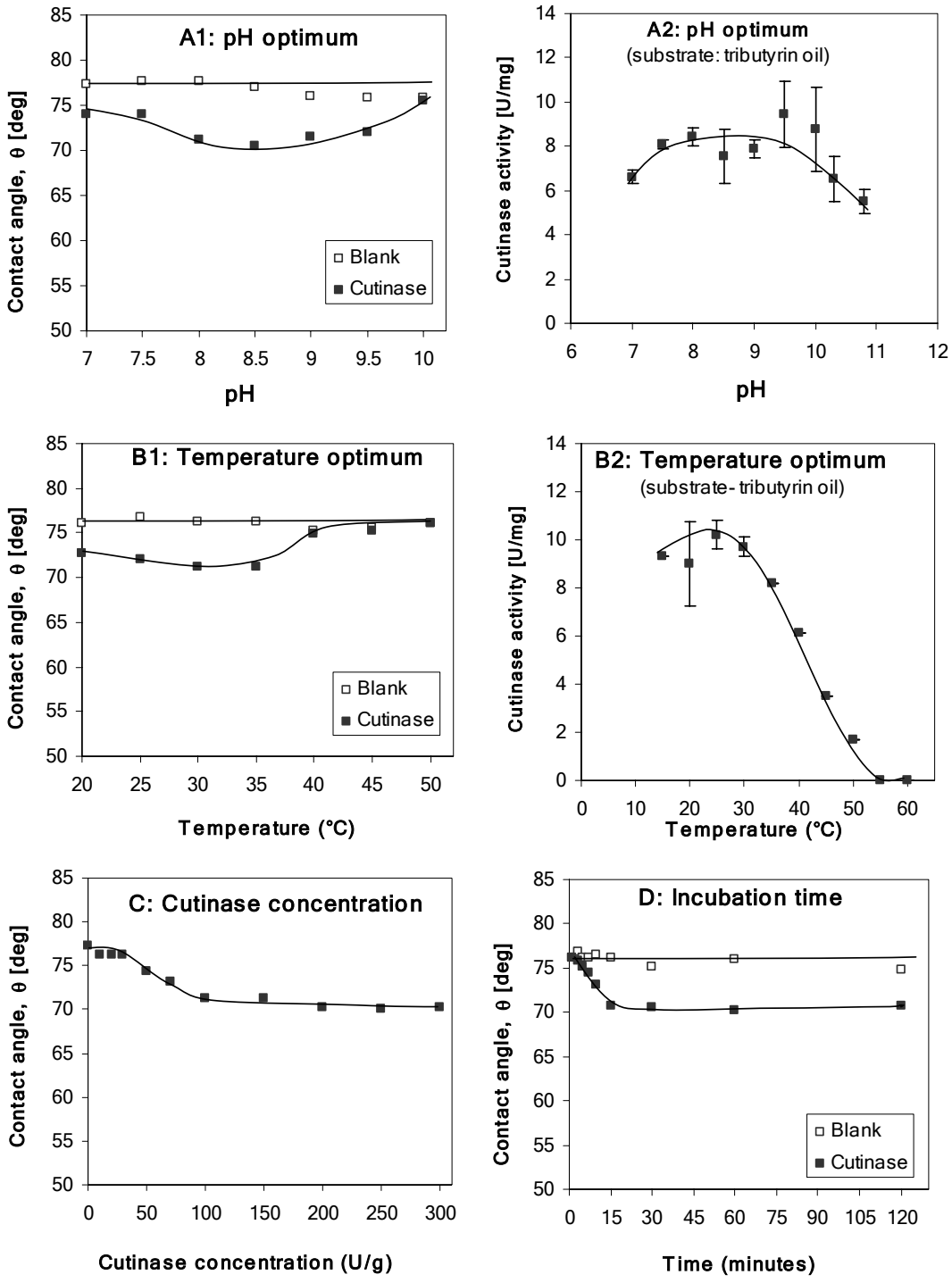

Figure 5.9: Evaluation of various parameters for the optimal cutinase action: A1) effect of pH (substrate- standard fabric), A2) effect of pH (substrate – tributyrin), B1) effect of temperature (substrate – standard fabric), B2) effect of temperature (substrate – tributyrin), C) enzyme concentration, and D) incubation time. Except for graphs A2 and B2, the structural contact angles θ of cotton fabric were measured after each treatment.

5.7.4 Cutinase concentration

The effect of cutinase concentration on cotton wax removal has been studied by changing its concentrations from 10 U/g to 300 U/g of cotton fabric. Results are shown in Figure 5.9C. Cutinase concentration up to 30 U/g has little effect on the structural contact angle. At 50 U/g cutinase concentrations, the improvement in the structural contact angle was $\theta \approx 73°$ from $\theta \approx 78°$ (blank). A structural contact angle of ~70° was achieved at 100 U/g of cutinase concentration. An increase in cutinase concentration up to 300 U/g did not improve the structural contact angle. The benchmark treatment achieves a structural contact angle of ~68°. To conclude 100 U/g cutinase is capable of removing almost all waxes.

5.7.5 Incubation time

To determine the incubation time that needs to remove the waxes from cotton fibre was essential for possible industrial application of cutinase in the scouring process. The effect of the incubation time on cutinase activity has been studied for 1, 3, 5, 7, 10, 15, 30, 60, and 120 minutes and the results are shown in Figure 5.9D. The optimal conditions such as 30°C temperature, 50 mM Tris-HCl buffer at pH 8 and 100 U/g of cutinase was used in this experiment. Results show that the structural contact angle changes rapidly from $\theta \approx 77°$ to $\theta \approx 71°$ in the first 15 minutes. A longer incubation time does not lead to an improved structural contact. After determination of the optimal conditions for cutinase we were able to reduce the incubation time to just 15 minutes.

5.8 Adsorption and catalytic mechanism of cutinase

Various parameters were evaluated for optimum cutinase action on its substrate, to fully explore its potential for cotton scouring. Structural information on cutinase is presented in Chapter 3 and an illustration is shown in Figure 3.1. Cutinase has two different adsorption mechanisms as function of the pH. At acidic pH, cutinase has a net positive charge due to the ionization of Asp175 and Glu44 amino acids. At this pH, adsorption is mainly governed by the electrostatic attraction, a positive charge on cutinase molecule and a negative charge on substrate [2, 11], which is not the case for cotton fibre. So, at acidic pH cutinase adsorption is only possible if there is a net negative charge on the substrate surface. Egmond [2] reported that, in the alkaline pH range, adsorption of the cutinase is predominantly governed by the hydrophobic interaction. There is no net charge on the cutinase

molecule in the alkaline pH range. However, as shown in chapter 3, the enzyme cutinase has large hydrophobic and non-polar patches at its surface near the active site [2, 8]. Therefore, at alkaline pH, at a hydrophobic substrate surface, there is a minimum intramolecular and/or lateral repulsion of adsorbed cutinase.

Our results show that cutinase has a maximum activity at pH 8-9. Petersen *et al.,* [11, 13] proposed 'the electrostatic catapult model' to explain cutinase activity towards its substrate. At alkaline pH, the negative electrostatic potential inside and the surrounding of the active site of the cutinase molecule is closely linked to the efficient catalysis [11, 13]. Cutinase can easily adsorb on hydrophobic substrates such as triglycerides, because triglycerides do not have a charge. However, the reaction products - free fatty acids and diglycerides, most likely carry negative charges. As a result, the released products experience a strong repulsion force from the active site of cutinase molecule [8, 11]. The negative charge in the active site cleft provides this repulsion force for the liberated product. At acidic pH, a cutinase molecule has a net positive charge; hence the free fatty acid is actually attracted to the active site and blocks it for catalysis. This causes the enzyme to be inaccessible for another substrate molecule and the product itself act as an inhibitor. However, when the potential in the active site becomes extremely negative at high alkaline pH (above pH 10), the activity drops because of deprotonation of active sites and reduced structural stability [8]. Thus, for optimum cutinase adsorption and catalytic activity a hydrophobic substrate surface at pH 8-9 is desired.

5.9 Conclusions

Cutinase application for a low-temperature wax removal from cotton fibre is a breakthrough towards achieving low-temperature scouring. We have shown that a cutinase from *Fusarium solani pisi* degrades and removes cotton waxes at a low-temperature (30°C). Cutinase can achieve almost same degree of wax removal compared to solvent extraction in 15 minutes, which is far better than 10-20 hours as reported by Degani *et al.,* [1]. The results were obtained by measurement of structural contact angle via auto-porosimeter, pectin removal and SEM pictures.

Surfactants play an important role in the scouring process. Hence, the compatibility of cutinase with a surfactant was investigated. Experiments were done in pH-stat to demonstrate that the increased surface area for cutinase action counteracts the negative effect of increased hydrophilicity caused by surfactant. We have demonstrated that cutinase removes cotton waxes even in presence of high concentrations of Triton X-100.

An increase in hydrolytic rate of pectinase was achieved after treating it with cutinase (with and without Triton X-100). The results clearly demonstrate that cutinase was able to increase pectinase kinetics in terms of pectin removal, equivalent to the n-hexane followed by the pectinase treatment. Various parameters were evaluated such as pH, temperature, ionic strength, enzyme concentration, and incubation time. The adsorption and catalytic mechanism of cutinase has been explained. To conclude, cutinase is an effective tool in degrading and removing cotton wax at low-temperature without organic solvents. Cutinase from *F. solani pisi* has great industrial potential together with pectinase to achieve low-temperature scouring.

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6

Towards Low-Temperature Enzymatic Souring Process for the Cotton Fabrics

The objective is to develop an improved enzymatic cotton scouring process on the basis of a fast enzyme reaction to efficiently remove the cuticle and primary wall compounds from the cotton fibre. In this chapter the role of mass transfer for improving the enzymatic scouring process is discussed. Beside this, the difference between a sequential process and an all-in-one process is investigated. To study the mass transfer, the mechanical action with the wedge apparatus was applied before and after the enzyme treatment. The effect of surfactants and mechanical action was evaluated for a faster scouring process with cutinase and pectinase. A lab scale sequential (two-step) and combined (one-step) low-temperature cotton scouring process is presented. Re-evaluation of the fabric samples were done after n-hexane extraction - to confirm the obtained results and to explain the results in terms of 'stagnant core and convective shell model' as proposed by Warmoeskerken [1].

6.1 Introduction

As described in Chapter 1, woven cotton fabric has dual porosity, the smaller inter-yarn pores and larger intra-yarn pores. For the standard fabric in this study the intra-yarn pores are in the order of 2 μm, and inter-yarn pores are in the order of 47 μm (Figure 3.4). The migration of the enzyme molecules into the intra-yarn pores is necessary for a good enzymatic treatment of the fibres within a yarn. This can be achieved by flowing through of the fabric with an enzyme solution. However, the flow resistance in the intra-yarn pores is much higher than the resistance in the inter yarn-pores, the bulk of the liquid will flow along the yarns instead of through the yarns [1, 2]. Based on this assumption, Warmoeskerken [1] proposed a stagnant core and convective shell model. The flow pattern of a liquid flowing along a yarn is schematically drawn in Figure 6.1, showing the stagnant core and the convective shell near the periphery of the yarn. The stagnant core of the yarn is the area in which there is no flow at all. The convective shell is the outer area of the yarn in which the flow penetrates to some extent. The transfer processes in the stagnant core are based on molecular diffusion, while the transport processes in the outer convective shell are driven by convective diffusion. Since convective diffusion is much faster than molecular diffusion, the rate of mass transfer in the yarn is determined by the size of the stagnant core [1, 2]. This means that the migration time of enzymes into the intra yarn pores is determined by molecular diffusion in the stagnant core, which is a relatively slow process.

A clear strategy for the development of a fast and efficient scouring process is presented in Chapter 1 and illustrated in Figure 1.5. Apparently, cotton scouring with enzymes is a diffusion controlled process. As a consequence enzymes need relatively long time to penetrate completely into the yarns. Therefore, the focus is on a fast enzymatic incubation and efficient removal of the cuticle and primary wall components from the cotton fibre. As discussed in Chapter 4 and 5, surfactants form an effective tool to improve the mass transfer during the scouring process by facilitating the penetration of enzymes into the fabric i.e. a better wettability of the fibres.

In this chapter, we explore the possibilities of improving the mass transfer during enzymatic scouring process through mechanical action on fabric materials with wedge apparatus. Beside this, the question was if the fabrics have to be treated sequentially with the enzymes cutinase and pectinase or that we could work with a combination of the enzymes in one single step. In these model processes, sequential as well as single

steps, also the effect of mass transfer, i.e. mechanical energy and of wetting i.e. surfactant have been investigated.

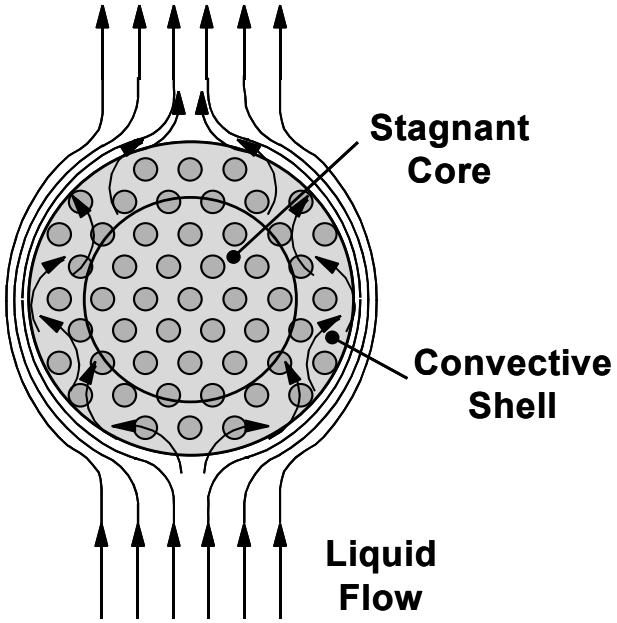

Figure 6.1: Liquid flow around a through a textile yarn. The dots represent the fibres in a yarn. The flow in the inter-yarn region may penetrate to a small extent inside the yarn, thus giving rise to a convective shell near its periphery. The central core of the yarns remains a stagnant zone where the mass transfer occurs by diffusion only [1,2].

6.2 Wedge apparatus – a tool for mechanical action

When a mechanical action on fabric is applied, the fabric will be deformed and the fabric-pores become smaller resulting in a flow of the pore liquid to the bulk. If thereafter the mechanical action is released, the fabric relaxes and the pores recover to their original shape resulting in a liquid flow from the bulk into the pores [2, 3]. This so-called squeezing effect is occurring in yarns when they are compressed and stretched. This mechanism contributes to a large extend to the mass transfer rate in the fabric yarns [1-4]. In Chapter 3, the wedge apparatus has been described for producing mechanical action on a fabric sample. As explained in that chapter, the compression and stretching deforming action is the principle working mechanism of the wedge apparatus [1, 4].

The effect of mechanical action was evaluated during two different stages of enzymatic scouring process. For this, two sets of experiments were done: In the first set, mechanical energy was applied before the pectinase treatment. In the second set the mechanical energy was applied after the pectinase incubation. Unfortunately it was not possible to combine pectinase treatment and mechanical action at once,

due to the small volume available inside the wedge apparatus (6 cm^3) for producing mechanical action on the fabric sample. Therefore it was not possible to work at the same fabric-liquid ration as applied in the beaker glass experiments. Moreover, it was not possible to control the temperature in the wedge system. Results from both sets of experiments are presented separately.

However, to be sure that the mechanical energy applied did not damage the fabrics we had to determine the maximum mechanical action that could be applied. The applied mechanical energy on fabric samples should be high enough to facilitate the removal of enzyme-degraded materials but low enough to prevent fibre damage. To study this, the fabric samples were subjected to varying mechanical action by changing the weight and the number of strokes in the wedge apparatus. In this study, three deforming weights 104 g, 275 g and 444 g were used along with the 3 mm radius wedge. The evaluation was done with a tensile strength meter by measuring the force (N) required to break different fabric test samples. The various treatments given to the fabric samples and data from the tensile strength meter are given in Table 6.1.

Test SN	Wedge radius 3 mm (Weight / Strokes)	Treatment	Tensile strength measurements		
		Mechanical energy applied by wedge (N/m^2)	Average F-break* (N)	Standard Deviation (%)	Elongation at F-break (%)
1	Blank (standard fabric)	0	626.2	52	7.68
2	104 g / 25 times	3468	650.5	52	7.82
3	104 g / 50 times	3468	644.1	11	7.63
4	104 g / 100 times	3468	605.8	57	7.76
5	275 g / 25 times	9710	616.1	47	7.69
6	275 g / 50 times	9710	613.5	14	7.24
7	275g / 100 times	9710	624.3	48	7.94
8	444 g / 25 times	15258	532.5	44	7.32
9	444 g / 50 times	15258	530.5	48	7.28
10	444 g / 100 times	15258	486.5	54	7.55

Table 6.1: Data from tensile strength measurement after mechanical action applied to different test fabric samples. F-break* is the force (N) required to break the fabric.

It is clear form the table that the force equivalent to 626 N, is required to break the standard fabric (test sample 1), and is considered as the reference value. For test samples 2-7, the applied mechanical energy was varied from 3468 N/m^2 to 9710 N/m^2. The data show that, the force required to break the test fabrics are still in the range of 600 – 650 N with an average standard deviation of ± 50 N. It can be said that, for all these fabric samples there was no negative influence of the mechanical action in terms of their tensile strength when compared with the standard fabric (test sample 1). However, increasing the mechanical energy from 9710 N/m^2 to 15258 N/m^2 (test samples 8-10) results in a decreased tensile strength (average F-break 500 – 550 N). From the data it is also clear that the number of strokes in the wedge apparatus did not affect the tensile strength of the samples. On the basis of these results we have chosen for a maximum mechanical force, corresponding to a pressure of 9710 N/m^2 and for 100 strokes per treatment.

6.2.1 Effect of mechanical action before pectinase treatment

The effect of mechanical action on the pectinase performance was evaluated in terms of the pectin removal. The fabric sample was subjected to the mechanical action according to the settings as given above followed by the pectinase treatment (13 U/g for 5 minutes at 50°C, pH 8). The other sample was also given a similar treatment, but it was pre-soaked with 1 g/L of Triton X-100 for 2 minutes, to study the additional effect of the surfactant. The results are shown in Figure 6.2. From the results it can be concluded that, there was no improvement in pectin removal even after mechanical action. The fabric sample that was pre-soaked with Triton X-100 showed only a marginal improvement in pectin removal, from 37% for the blank (no pectinase) to 43% after the mechanical action followed by the pectinase.

From Chapter 4, we know that the wax removal from fibre surface results in an increased pectin surface area thereby improving the pectinase hydrolytic rate. However, in this experiment there was no increase in the pectin removal. Therefore, it can be concluded that under the experimental conditions applied here the mechanical action did not disturb the fibres of the standard fabric. So the mechanical action has no effect on the structure or interconnections in the primary wall of the cotton fibre.

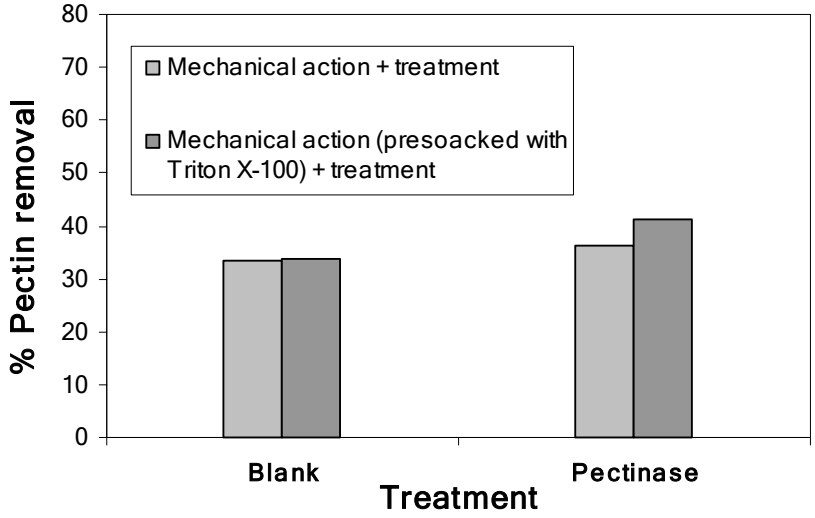

Figure 6.2: The effect of mechanical action on the pectinase performance.

6.2.2 Mechanical action after the enzyme treatment

In this second set of experiments, in which the mechanical energy was applied after the enzyme treatment, it was expected that the removal of degraded non-cellulosic material from the cotton fibre will be improved. To confirm this, various treatments were given to the fabric samples prior to the mechanical action (~9710 N/m^2 and 100 strokes). These treatments are:

A) blank – standard fabric,

B) 100 U/g cutinase at 30°C for 30 minutes,

C) n-hexane extraction, 75°C for 30 minutes, and

D) 13 U/g pectinase (PL) treatment at 50°C for 15 minutes.

All the samples (A-D) were also produced without mechanical action to study the effect of an individual treatment. After the treatments the amount of pectin removal and the structural contact angle were measured. The results of these experiments are shown in Figure 6.3. It is evident from this figure that the treatments B and C have a marginal improvement in the structural contact angle (~4°) after the mechanical action. Moreover, the amount of pectin removal was only up to 9% after these treatments. For the standard fabric (treatment A), no change in the structural contact angle was measured, $\theta \approx 83°$. Two inferences were drawn: firstly, the properties of n-hexane and cutinase treated fabric were identical with and without mechanical action. Secondly, the marginal effect of mechanical action was because the treatments were aimed to remove the waxy layer and hence there was no effect

on the undisrupted primary wall. This is also reflected in the negligible pectin removal, which was not more than 9%.

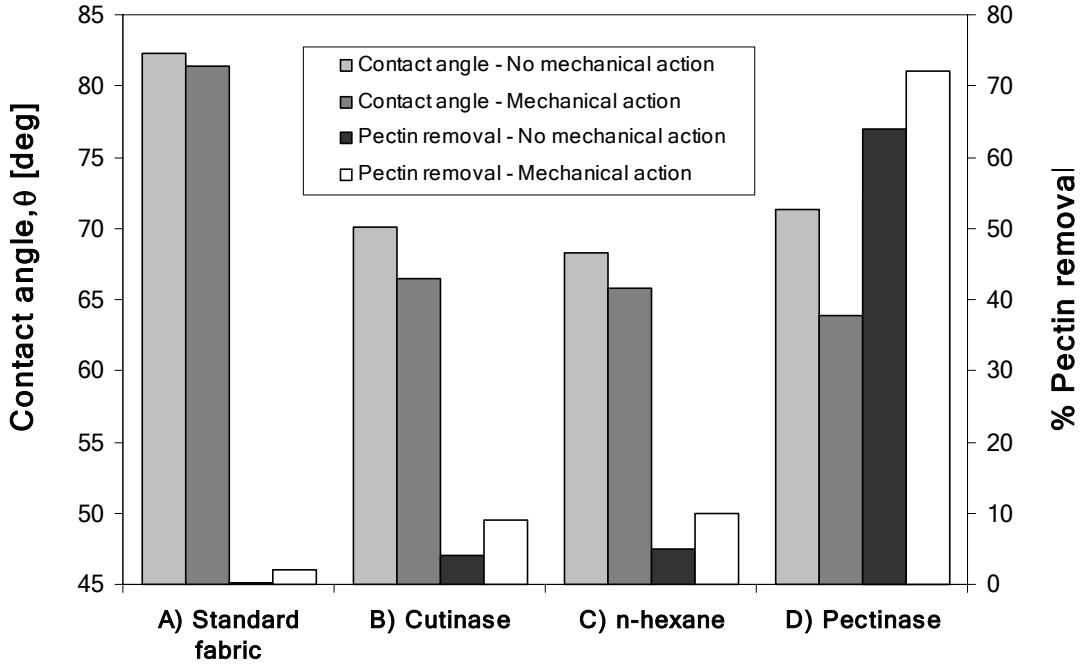

Figure 6.3: Effect of mechanical action in terms of stretching deforming action via wedge apparatus after various treatments. Various treatments are: A) standard fabric, B) cutinase, 100U/g at 30°C for 30 minutes, C) n-hexane extraction in Soxhlet for 30 minutes at 75°C and D) 13 U/g PL treatment at 50°C for 15 minutes.

The effect of mechanical action became really apparent after the pectinase treatment, sample D, here the structural contact angle shifted from $\theta \approx 71°$ to $\theta \approx 64°$ after mechanical action. The pectin removal was increased from 62% to 71%. It is worthwhile to note that, the additional 9% pectin removal is significant, once nearly 60% of the pectin has been removed. It can be said that that the pectinase treatment destabilises the primary wall and mechanical action facilitates the removal of degraded product. The cutinase and n-hexane treatment are only targeted towards wax removal, while, pectinase degrades whole primary wall, generating more degraded products that have to removed from the yarns by the mass transfer. This justifies why the effect of mechanical action is more pronounced after the pectinase treatment compared to the cutinase treatment. To conclude, the mechanical action on fabric samples facilitates the removal of only weakened products and has no effect on the structure or interconnections in the primary wall of cotton fibre.

6.3. Low-temperature scouring with cutinase and pectinase

Even though cutinase and pectinase can make fabric hydrophilic, the incubation time required to obtain the desired hydrophilicity is longer than compared to the NaOH scouring. It has been postulated that wetting and mechanical action, together with cutinase-pectinase will result in an efficient destabilisation of waxy layer and primary wall. This will lead to the desired hydrophilicity that needs to achieve during scouring process. On the basis of this we developed the two steps and single step enzymatic scouring process.

6.3.1 Sequential scouring

Sequential or two-step enzymatic scouring experiments were designed because of the different optimal temperatures for the cutinase and the pectinase action. The cutinase and the pectinase were used under their optimum process conditions as determined in Chapter 4 and Chapter 5. The scouring experiments were done in the following order:

Step I – 100 U/g cutinase treatment at 30°C

Step II – 13 U/g pectinase treatment at 50°C

followed by the mechanical action, and a standard rinsing and drying procedure (Chapter 2). Treatment with each enzyme was conducted fro 15 minutes using 50 mM Tris-HCl buffer at pH 8. Five different treatments were applied:

A) blank (step I and II) - no cutinase and no pectinase treatment,
B) cutinase treatment (step I) + blank for the pectinase (step II),
C) blank for the cutinase (step I) + the pectinase treatment (step II),
D) cutinase treatment (step I) + pectinase treatment (step II), and finally
E) cutinase and 1 g/L Triton X-100 treatment in one bath (step I) + pectinase treatment (step II).

The results from these treatments are given in Figure 6.4. It is clear from the figure that an individual enzyme influences the structural contact angle θ. The cutinase (treatment B) and the pectinase (treatment C) treated fabric obtain nearly same the structural contact angle of ~67° which was higher than with the blank treatment, $\theta \approx 75°$. However, the big difference is in the amount of pectin removed after these treatments; for the cutinase treated fabric this was only 9% and for the pectinase treatment it was nearly 70%. Interestingly, even though the structural contact angle was similar after these treatments B and C, the hydrophilicity achieved was obtained by different ways. In case B, the increase in hydrophilicity was

achieved by wax removal because it has been established that cutinase has no effect on the primary wall. In case C, the increase in hydrophilicity was because of the pectin removal and thereby destabilising the primary wall of cotton fibre. It may be possible that, waxes are still present on the cotton fibre surface even after the partial removal of the underlying pectins and other non-cellulosic materials. Evidence of a remaining waxy layer on the cotton fibre surface after a pectinase treatment is visible in Figure 6.7, giving the results of some SEM pictures. In picture 6.7a, the waxes are visible, covering the underlying primary wall interconnections and cellulose microfibrils. When we compare this picture, 6.7a with picture 2.3b in Chapter 2, it can be concluded that the fibre surface after pectinase treatment is much different than the smooth and intact waxy layer. The remaining waxy layer affects the structural contact angle results in a more hydrophobic surface. Therefore, the removal of a waxy layer is essential to obtain sufficient hydrophilicity during cotton scouring. So it is important here to mention that the removal of the waxy layer is not only to improve the pectinase action by a larger pectin surface area available but also contributes substantially towards an improved hydrophilicity.

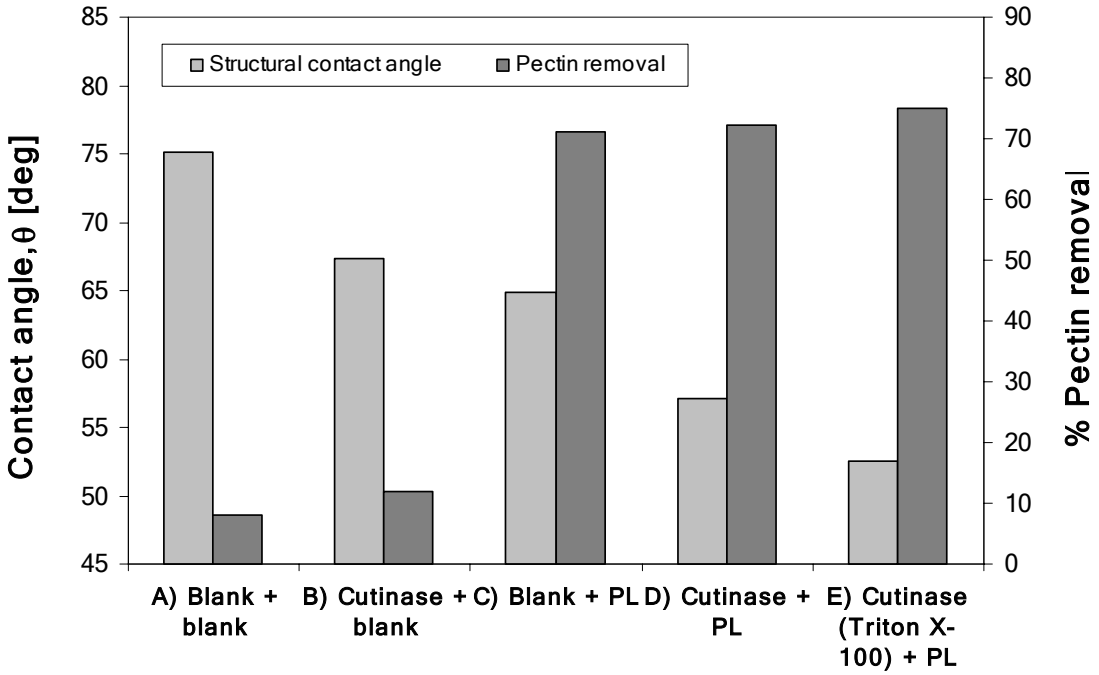

Figure 6.4: Sequential or two-step experiments with the cutinase at 30°C and the pectinase at 50°C for 15 minutes each using 50 mM, Tris-HCl buffer at pH 8. Various treatments are given (A-E) followed by mechanical action equivalent to ~9710 N/m^2.

Figure 6.4 shows that, a combination of cutinase and pectinase together with mechanical action (treatment D) produces a structural contact angle of ~57° and leads to nearly 72% pectin removal. This clearly indicates that the cutinase and

pectinase together have a positive effect on the hydrophilicity. Finally, the combination of cutinase, Triton X-100 followed by the pectinase treatment and mechanical action (treatment E) gives the desired structural contact angle of ~51°. Now the obtained hydrophilicity in terms of the structural contact angle is equivalent to the NaOH scoured fabric. Roughly 75% (±5) of pectin removal is achievable with this treatment. Although in NaOH scouring all the pectin is removed, now it is clear that is not really necessary for obtaining the desired structural contact angle.

6.3.2 One-step scouring

The single step or combined enzymatic scouring experiment was done with cutinase and pectinase in one bath. Before doing so, it was necessary to confirm that both cutinase and pectinase are compatible with each other. As an experimental proof, the effect of cutinase on pectinase was measured by conducting an activity assay of pectinase with D-galacturonic acid as a substrate (Chapter 3). From the activity assay it was confirmed that cutinase and pectinase are compatible with each other. Moreover as explained in Chapter 3, both the enzymes do not need Ca^{2+} for their actions. Another pre-requisite for the one-step scouring was to lower the pectinase incubation temperature from 50°C to 30°C, the optimum temperature for the cutinase action. In Chapter 4, Figure 4.6A, we found a maximum pectin removal for a pectinase concentration of about 13 U/g at 50°C. Repeating this experiment at 30°C gave the results as shown in Figure 6.5. From this graph it is clear that the concentration of pectinase has to be increased with a factor of four to get the same performance at 30°C compared to 50°C. This is due to a lower activity of the enzymes at a lower temperature as indicated in Figure 4.6B in Chapter 4. Of course another option was to do the cutinase-pectinase incubation at 50°C. However, from Figure 5.9b, it is clear that the cutinase action diminishes above 40°C. Moreover in terms of a low-temperature scouring process the choice for 30°C is more logic.

All the given treatments for the one-step scouring were similar to the sequential scouring experiments (treatments A-E in the previous section). Unlike sequential scouring experiments, one-step scouring was performed in one bath with cutinase and pectinase at 30°C for 15 minutes. Figure 6.6 shows the results of these treatments. All the results obtained are in line with the results from the sequential experiments. Also in the case of treatment B and C gives the same contact angle for different pectin removals. This phenomenon is explained already in the section 6.3.1.

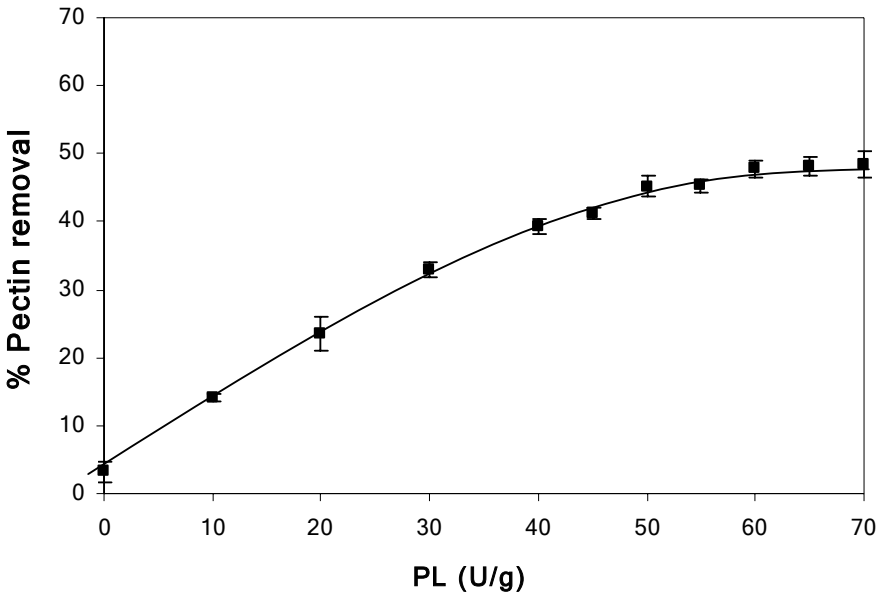

Figure 6.5: Pectinase (PL) optimization at 30°C for 5 minutes by measuring percentage pectin removal as a function of pectinase concentration. An experiment is similar as shown in Figure 4.6A, but that was at 50°C.

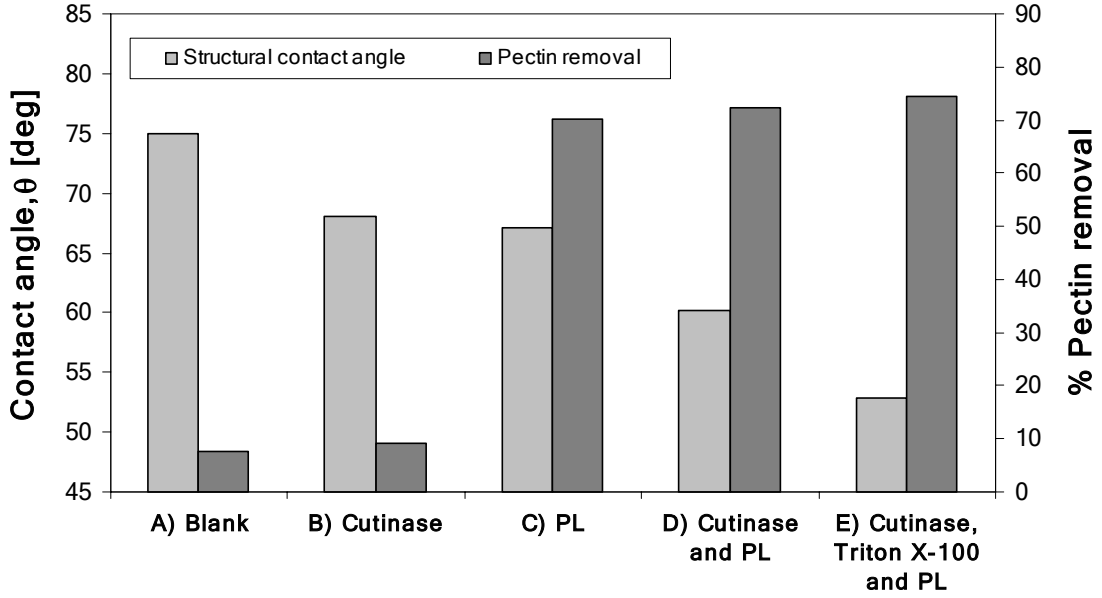

Figure 6.6: Combine or one-step scouring experiments with cutinase and pectinase at 30°C for 15 minutes at pH 8 using 50 mM Tris-HCl buffer. Various treatments are given (A-E) followed by mechanical action equivalent to ~9710 N/m^2.

The combined scouring experiment with cutinase and pectinase, sample D, produces a structural contact angle of $\theta \approx 60°$, which was far better than the results with an individual enzyme, sample B and C. Cutinase and pectinase act together, in which cutinase degrades the waxy layer at the surface and pectinase destabilises the primary wall. The overall pectin removal was similar to treatment C. However, unlike

treatment C, the improved structural contact angle indicates that waxes are also removed. This is also clear from SEM picture in Figure 6.7b. This figure shows the surface of the cotton fibre after treating with cutinase and pectinase together (treatment D). A rough and open primary wall surface becomes visible, in which the smooth waxy layer is absent. The primary wall surface in this figure seems degraded because of the enzyme treatment and looks different from the intact primary wall as shown in Figure 2.3c.

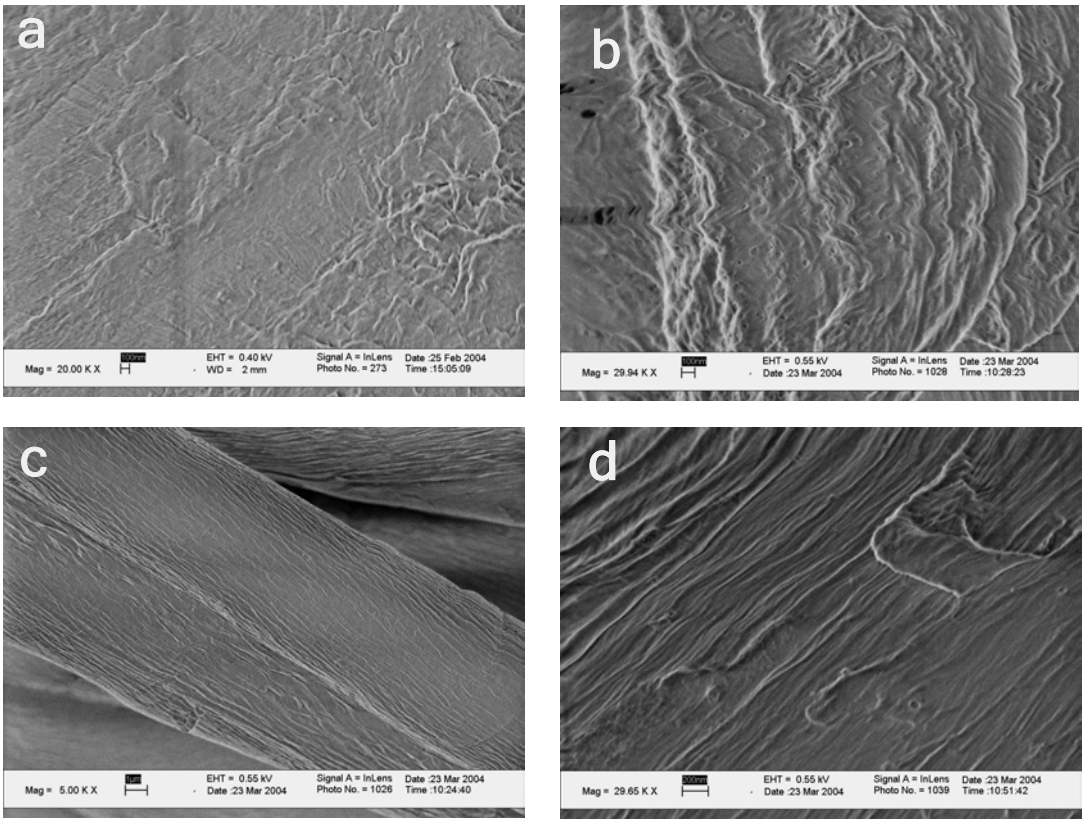

Figure 6.7: SEM pictures of various treatments given to standard fabric. a) surface of PL treated fabric (13 U/g, 30°C for 15 minutes, 50 mM Tris-HCl buffer, pH 8), b) surface of cutinase and PL treated fabric during one-step scouring experiments, c and d) cotton fibre surface finest treatment with cutinase, pectinase, Triton X-100 and mechanical action.

Finally, cutinase, pectinase, together with Triton X-100 and followed by mechanical action (treatment E), produces the structural contact angle of ~52° that is equal to the value of NaOH scoured fabric. The SEM pictures for this treatment are shown in Figure 6.7c and 6.7d. These figures show the surface of enzymatic scoured cotton fibre, in which the cellulosic layer is clearly visible. The cellulose fibrils are laying parallel to one another in one direction, (Figure 6.7d). In these figures the smooth waxy layer and complicated primary wall are absent. This becomes clearer if

Figure 2.3c and 2.3d are compared to Figure 6.7d. In Figure 2.3c the complex structure of the primary wall is visible while in this is absent in Figure 2.3c and 6.7d. To conclude, because of the combine effect of cutinase-pectinase in one bath, the overall enzyme incubation time can be reduced to just 15 minutes compared to 30 minutes in the sequential case.

6.3.3 Re-evaluation of the obtained results

To confirm the conclusions we have drawn on the basis of all the experimental results, we have done a re-evaluation. With this re-evaluation we also want to see if our postulations regarding the removal of degraded components from the cotton fibres are correct. By comparing the structural contact angle of a particular fabric sample before and after the solvent extraction it is possible to judge whether there was some still wax present at the fibre surface. So, the obtained data of the re-evaluation were used to judge the performance of the previously given treatments.

The list of selected samples for the re-evaluation experiments is given in Table 6.2 and the results are shown in Figure 6.8. The figure shows the structural contact angle data before and after solvent extraction of the selected samples (A-E, Table 6.2). The results can be compared with the structural contact angle obtained by the NaOH scouring procedure. The NaOH treated fabric shows no effect of additional n-hexane extraction, indicating that there were no waxes left after this strong treatment.

SN	Treatment	Description
A	Triton and wedge	Pre-soaked with 1 g/L Triton X-100 for 2 minutes followed by mechanical action (~9710 N/m^2 for 100 strokes)
B	Cutinase and PL	Cutinase and pectinase in one bath at 30°C for 15 minutes
C	Wedge + cutinase and PL	Mechanical action (~9710 N/m^2 for 100 strokes) followed by Cutinase and pectinase in one bath at 30°C for 15 minutes
D	Cutinase (Triton) and PL	Cutinase and pectinase together with 1 g/L Triton X-100 in one bath at 30°C for 15 minutes
E	Cutinase (Triton) and PL + wedge	Cutinase and pectinase together with 1 g/L Triton X-100 in one bath at 30°C for 15 minutes followed by mechanical action (~9710 N/m^2 for 100 strokes)
	NaOH scoured	NaOH scouring resembling to the industrial conditions

Table 6.2: List of treatments applied to the fabric samples for re-evaluating in terms of remaining wax contents.

Solvent extraction of the sample A shows a shift in the structural contact angle from $\theta \approx 74°$ to $\theta \approx 67°$. The latter obtained structural contact angle was typical for a fabric from which only the wax layer was removed by n-hexane extraction or by

cutinase treatment. Results from sample A also suggests that pre-soaking in a surfactant solution or applying mechanical action has no affect on wax removal. These results are in agreement with confirm the data in section 6.2, where the structural contact angle (Figure 6.3), and the pectin removal (Figure 6.2) were measured.

After the extraction of sample B, the structural contact angle shifted from $\theta \approx 61°$ to $\theta \approx 56°$. That indicates that there was some wax still left inside the yarns. The results obtained suggest that the treatment with cutinase and pectinase does not reach all the fibres of the yarn due to poor wettability. That is also the reason why sample B shows an increase in hydrophilicity after solvent extraction. However, even after the solvent extraction, the obtained structural contact angle was not equivalent to the value of the NaOH scoured fabric, the benchmark. This phenomenon strongly suggests that inside the yarn there are some fibres of which the primary wall is still intact. By comparing the results of sample B and C, it can be concluded that additional mechanical action before the enzyme treatment (cutinase and pectinase) has no added impact.

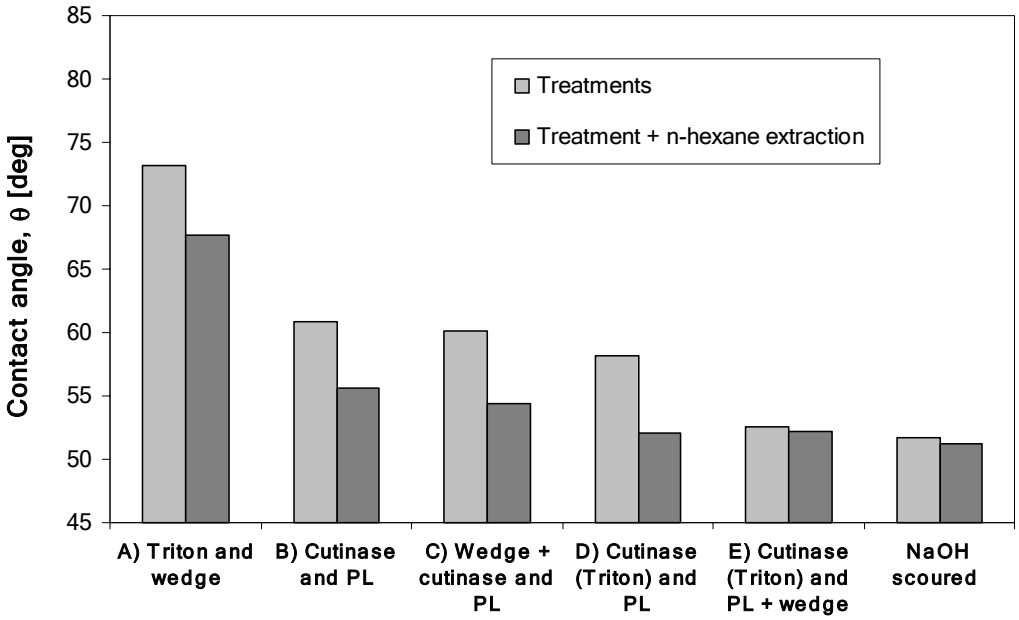

Figure 6.8: Selected fabric samples are extracted with n-hexane at 75°C for 30 minutes to study the remaining waxes on cotton fibre after selected treatments as given in Table 6.2. (wedge = mechanical action ~9710 N/m^2, 100 strokes Triton = 1 g/L Triton X-100, PL = pectate lyase).

A treatment with cutinase and pectinase in the presence of Triton X-100 (sample D) shows similar results as obtained with sample B and sample C. The only difference is that structural contact angle obtained after n-hexane extraction is

equivalent to the NaOH scoured fabric. This is because an improved penetration of the scouring enzymes inside the yarns due to the presence of surfactant. Consequently, all the fibres in the yarn are treated with the enzymes and hydrolysis occurs at all the fibre surfaces within the yarn. In this treatment all the non-cellulosic materials are degraded, however some of them are still inside the yarns and responsible for a less hydrophilic fibre surface compared to the NaOH scoured fabric.

For sample E (cutinase, pectinase together with surfactant and mechanical action), there was almost no difference in the structural contact angle before and after the solvent extraction. The targeted structural contact angle of ~52° was obtained. The hydrophilic fabric was achieved in two stages: in the first stage the enzymes in presence of surfactant are penetrated efficiently to the interiors of the yarn due to a better wettability. In the second stage the mechanical action facilitates the removal of degraded or weakened products from the yarns to the bulk liquid. To conclude, the wetting and mechanical action has a positive effect on the cutinase and the pectinase treatment that helps to degrade and to remove the cuticle and primary wall of the cotton fibre and hence to achieve the desired hydrophilicity equivalent to NaOH scoured fabric.

The obtained results can also be explained by the stagnant core and convective shell model as proposed by Warmoeskerken [1]. Schematic illustrations of the proposed removal mechanisms of degraded non-cellulosic materials are presented in Figure 6.9. The letters A-E, in this scheme corresponds to the samples as given in Table 6.2. In the figure the outer circles represent the periphery of the yarn. The inner thick circles symbolise the untreated fibres with intact waxy layer and the primary wall. The thin circle represents the hydrophilic fibre surface. The small dots are the degraded non-cellulosic products. The dots outside the yarn indicate removed products and the dots on the fibre surface resemble to the adhered degradation products.

Figure 6.9A shows that either mechanical action or pre-soaking in a surfactant solution has no any effect on the removal of non-cellulosic materials from the fibre surface. Rather it has been established that a high mechanical shear above 15000 N/m^2 can damage the standard fabric used in this study.

As discussed earlier, both cutinase and pectinase has a capability to degrade non-cellulosic materials; however their penetration inside the yarn is restricted due to the poor wettability. A schematic illustration of this phenomenon is presented in Figure 6.9B. Here only the outer fibres of the yarn are cleaned (lighter circles) while primary wall and wax layer of the inner fibres are still intact. Some of the products are

removed during the treatment and others are still adhered to the fibre surface. The end result of sample C, Figure 6.9C, is similar to that of sample B. The same reasoning as done for sample B applies to sample C. Additional mechanical action before enzyme treatment has no positive impact on the hydrophilicity.

A schematic illustration of treatment D is presented in Figure 6.9D. It illustrates that because of the surfactant, now enzymes can penetrate better to the interiors of the yarn and degrade non-cellulosic materials from all the fibre surfaces. However, some of the reaction products are removed while the rest is still adhered to the fibre surface and responsible for the hydrophobicity found. Finally Figure 6.9E, systematically shows the scouring results obtained in two steps: In the first step cutinase-pectinase in the presence of a surfactant degrade all the non-cellulosic materials. In the second stage the mechanical action removes all the degraded non-cellulosic materials resulting in a hydrophilic fibre surface.

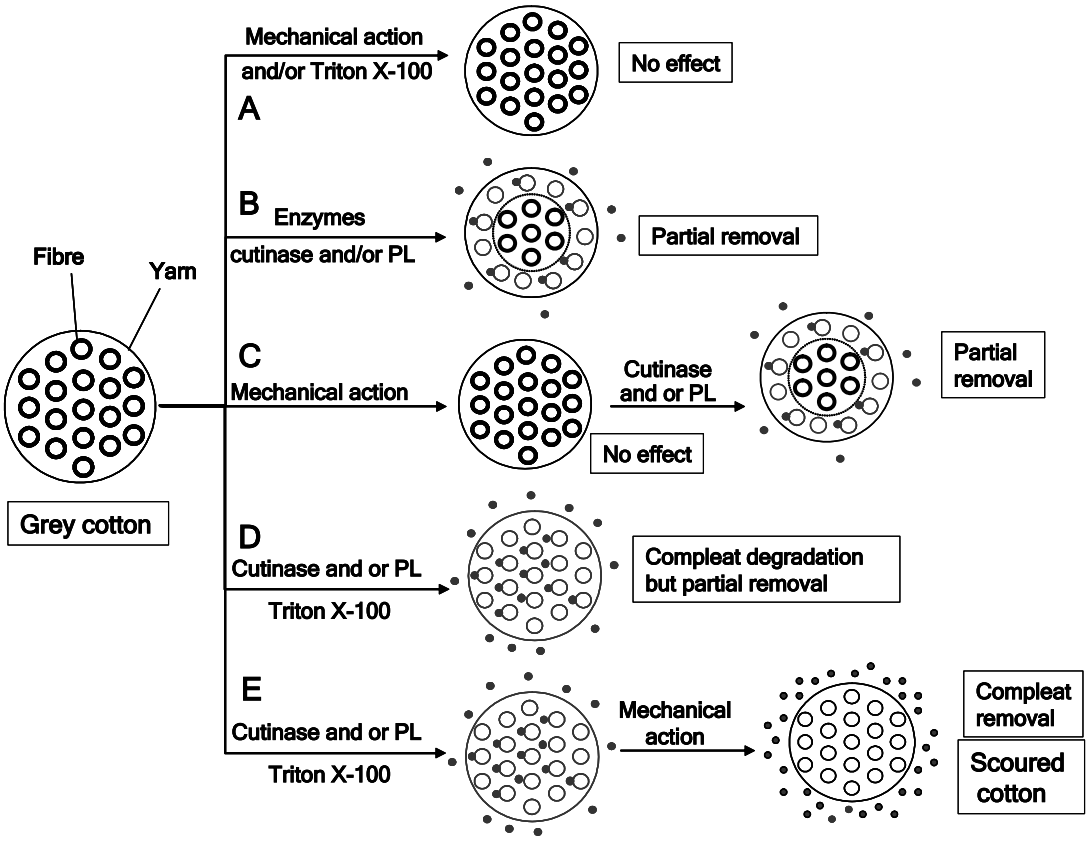

Figure 6.9: An illustration showing non-cellulosic removal mechanism during enzymatic scouring of the cotton fabrics. All the treatments from A to E are listed in Table 6.2. In this figure the outer circles represent the periphery of the yarn. The inner thick small circles symbolise the untreated fibres with the intact waxy layer and the primary wall. The inner thin circles represent the hydrophilic fibre surface. The small dots are the degraded non-cellulosic impurity from the cotton fibre.

6.4 Conclusions

An efficient wax removal with the cutinase before or during pectinase treatment is key to achieve a low-temperature enzymatic scouring process of grey cotton fabrics. Improving the mass transfer in the diffusion-controlled process is important for the new enzymatic scouring process. The wedge apparatus seemed to be an excellent tool to show the effect of the mass transfer during various stages of the scouring process. The compression and stretching deforming action is the principle mechanism of this system. The effect of mechanical action was more pronounced when it was applied after the pectinase treatment. Due to the limitations of the wedge system we could not measure the effect of mechanical energy during enzyme incubation.

A lab scale, sequential and one-step low-temperature enzymatic scouring process was developed using cutinase-pectinase together with the surfactant Triton X-100 and mechanical action with the wedge apparatus. One-step scouring with cutinase and pectinase is possible at 30°C for 15 minutes which is half of the time required for the sequential process. From the structural contact angle results and SEM pictures, it is concluded that pectinase alone is not able to achieve the desired hydrophilicity even though it can remove up to 75% of the pectin from the cotton fibre. To achieve the needed hydrophilicity, the removal of the outermost waxy layer is important. Therefore, to measure only the pectin removal as enzymatic scouring efficiency is not a good criterion. Confirmations of the scouring results were made by re-evaluating the structural contact angle for selected fabric samples after solvent extraction. Re-evaluation was also done to show the effect of various treatments on the removal of degraded components from the cotton fibres. Results could be explained with the help of the stagnant core and convective shell model.

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7

Conclusions and Outlook

In this chapter the general conclusions and our point of view for future research are presented. Research has been carried out now for a decade to replace conventional scouring with enzymes. Until today continuous enzymatic scouring is not widely accepted in textile industry. In this chapter some recommendations are given to develop an enzymatic scouring process that has a better potential to be implemented in the industry.

7.1 Conclusions

The aim of this research was to study, the potential of enzyme technology to design an efficient and low-temperature scouring process for the grey cotton fabric. Scouring is related to hydrophilicity and can be achieved by uncovering the pores that are already present in the fibres, by removing waxes and other non-cellulosic materials in the primary wall. The technical feasibility of enzymatic scouring has been recognised by many researchers over the last decade. However, continuous enzymatic scouring process has not yet been widely implemented by textile industries. The most important reason identified was the inability to remove cotton fibre waxes during enzymatic souring. The pre-rinsing in hot water above 90°C with a surfactant helps to reduce the wax impurity load and renders a better subsequent enzyme effect towards primary wall destabilisation. However, the introduction of such an additional high temperature step complicates the development of a low-temperature scouring process and limits the actual value offered by the enzymes. Therefore, the main challenge was to remove these cotton waxes with enzymes at low-temperature.

The work presented in this thesis shows that cutinase from *Fusarium solani pisi* degrades and removes cotton waxes at a low-temperature (30°C). Cutinase can achieve within 15 minutes almost the same degree of wax removal as solvent (n-hexane) extraction. The results clearly demonstrate that cutinase is able to increase the pectinase kinetics in terms of pectin removal, equivalent to the n-hexane followed by the pectinase treatment. By this we confirmed our hypothesis, which states that the removal of the outermost waxy layer will facilitate pectin removal with the pectinases. The wax low-temperature removal step with cutinase has been introduced to reduce the time during pectinase incubation and to increase the pectinase hydrolytic rate. We prove on the basis of experiment and as deduced from the cotton fibre morphology, that alkaline pectinases (PL and Bioprep 3000L) performs better than acidic pectinases (PGs). Cutinase from *Fusarium solani pisi* has great industrial potential together with pectinase to achieve low-temperature scouring. Various parameters were evaluated for cutinase and pectinase such as pH, temperature, ionic strength, enzyme concentration, and incubation time.

In chapter 1, we have presented a clear strategy towards the development of enzymatic scouring process. We showed that mass transfer improvement is a pre-requisite to convert the diffusion-controlled scouring process in to industrially viable enzymatic scouring process. A lab scale, sequential and one-step low-temperature enzymatic scouring was developed using cutinase-pectinase together with wetting

with Triton X-100 and mechanical action via wedge apparatus. With these results we have reduced the usual scouring temperature from 100°C for NaOH, to 55°C for pectinase, to ultimately 30°C for a mixture of cutinase-pectinase. We obtained good results with one-step scouring with cutinase and pectinase at 30°C for 15 minutes. We have established the knowledge needed to develop a new, innovative scouring process. It is expected that this knowledge forms a good basis for the development of a fast and continuous bioscouring process.

7.2 Outlook

Compared to conventional alkaline boiling off, the advantages of bioscouring are apparent: it can save energy by lowering the treatment time from boiling to now around 30°C, super soft handle, no fibre damage, and less COD and BOD in the effluent. We have given clear guidelines for the successful scouring process, which we have proved on the laboratory scale. These guidelines are:

A) Wax removal is a pre-requisite, since scouring is related to hydrophilicity and not how much pectin is removed.
B) Efficient wax removal facilitates fast pectinase incubation and improves pectin removal rate and hence fast primary wall destabilisation, and
C) Efficient wetting and mass transfer improvement are essential to convert slow diffusion based scouring process into faster process.

In order to develop an industrial scouring process for cotton fabric based upon the guidelines we have given, more research is needed to be done. Special attention has to be given to:

1) The one-step scouring with cutinase and pectinase was developed at 30°C by using optimum enzyme concentration of each enzyme. We have evaluated optimum enzyme concentration for both the enzymes. In the one-step scouring we utilised the optimum concentration of both the enzymes, which may not be required. Therefore, there is a need to optimise the cutinase-pectinase concentration ration for the system.

2) The mass transfer improvement is necessary for the faster and efficient scouring process. In our study we have improved mass transfer by applying mechanical action after the enzyme treatment. Research on the effect of mechanical action applied during the enzymatic incubation is needed. More studies are necessary to design a device to produce mechanical action at an

industrial level. Other mass transfer tools need to be explored that will work at an industrial level.

3) Cutinase has proven its importance for cotton scouring process. The cutinase has it optimum activity up to 35°C, as a result there a need of high pectinase dose for the one-step scouring process, because the optimum concentration of pectinase is 50-60°C. Further studies are necessary to increase the cutinase working temperature. This may be possible by immobilising the enzyme on suitable surfaces or finding a new thermostable class of cutinase. Other alternative is to find suitable alkaline pectinase that ideally works at a temperature near 30°C.

4) Even though we have evaluated important scouring parameters for cutinase and pectinase, there is a need to re-establish few important parameters at an industrial level e.g. enzyme residence time and cutinase-pectinase concentration ratio. Both the factors will be greatly influenced by the speed at which the fabric is moving and the fabric pickup ratio.

5) Further developments for the enzymatic cotton scouring process should be focused on the assessment of economical and environmental parameters.

6) The developed low-temperature scouring process at the lab scale has a great potential to be integrated with desizing and bleaching operations. Future research should focus on the continuous desizing-scouring-bleaching process for the cotton textiles at low-temperature with enzymes.

List of abbreviations

AATCC	American association of chemist and colorist
Asp	Aspartic acid (an amino acid)
CMC	Critical micelle concentration of a surfactant
DNS	Dinitrosalicylic acid (reagent)
DP	Degree of polymerisation of cellulose
EC	Enzyme commission numbers assigned by IUPAC and IUBMB
EDTA	Ethylene diamine tetra-acetic acid (a chelator)
EO	Oxy-ethylene units as a head group on non-ionic surfactant
FT-IR	Fourier-transform infrared
GC-MS	Gas chromatography-mass spectroscopy
His	Histidine (an amino acid)
HLB	Hydrophilic Lipophilic Balance number
Hyp	hydroxyl-proline (an amino acid)
O/W	Oil in water emulsion
PG	Polygalacturonases (acidic pectinase)
pI	isoelectric point of the enzyme
PL	Pectate lyase (alkaline pectinase)
PVD	Pore volume distribution
RP-HPLC	Reverse phase high pressure liquid chromatography
rpm	Rotation per minute with the magnetic stirrer
SEM	Scanning electron microscopy
Ser	Serine (an amino acid)
W/O	Water in oil emulsion
Glu	Glutamic acid (amino acid)

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Pramod

Résumé

Pramodkumar Bhagwandas Agrawal was born on February 19, 1975 at Soundad, India. After completing his higher education from Secondary Education Society, high school, Sakoli (Maharashtra, India), in July 1992, he joined Smt. Kusumtai Wankhede Institute of Pharmacy, Katol to obtain his Diploma in Pharmaceutical Sciences. In August 1994, he joined Bachelor course in Pharmaceutical Sciences at the Department of Pharmaceutical Sciences, Nagpur which is affiliated with the University of Nagpur, India. As a part of his bachelor study he worked as trainee in Cadila Pharmaceuticals Limited, Ahmedabad, India.

After the graduation, he received a Junior Research Fellowship (JRF), from the University Grand Commission, New Delhi. In August 1998, he joined Master of Bio-process Technology program at University Department of Chemical Technology (UDCT), Mumbai, India. During his master studies he worked on dissertation titled "Protein/Enzyme Isolation by Adsorption" with the guidance of Prof. A.B. Pandit.

On 1st December 2000, he joined a PhD project in the Technology of Structured (Textile) Materials group at University of Twente, The Netherlands. This European Commission funded project was aim to conduct research on the development of continuous bio-pretreatment of cellulosic materials. During his PhD, he is supervised by Prof. dr. ir. M.M.C.G. Warmoeskerken and dr. ir. V.A. Nierstrasz. The results of his research are descried in this thesis.