COVALENT MODIFICATION OF CELLULASES FOR TEXTILE BIOFINISHING

by
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to my grandfather,

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ABSTRACT

Enzymes have been used for catalysis in diverse industrial applications such as food, energy and textile. Nowadays, the demand for modified enzymes in industry is constantly increasing. Cellulases, which have wide industrial application areas, have been extensively used for biopolishing of cellulosic fibers and fabrics. Cellulases are used to prevent pilling on the surface of cotton fabrics but this process causes losses of tensile strength and fabric weight. On the other hand, there is no cellulase formulation used in biopolishing of viscose fabrics since they have different structure than cotton fabrics. Enlargement of enzymes may be one alternative way to prevent these adverse effects on the fabrics.

In this study, commercial cellulases were crosslinked to increase the size of the enzymes while trying to keep the adverse impact on tensile strength and weight loss at minimum levels. Modified enzymes were characterized according to their activities against carboxymethyl cellulose and their effects on the properties of cotton and viscose fabrics were examined. The cross-linked aggregates of commercial enzymes were found to reduce losses of tensile strength and weight of both cotton and viscose fabrics while creating the desired biopolishing affect. This is the first study that reports use of enzymes for biopolishing of viscose fabrics effectively. Also this process is shown to be cost effective for biopolishing of cotton fabrics.

TEKSTİL BİYOPARLATMASI İÇİN SELÜLAZLARDA KOVALENT MODİFİKASYONLAR

Baran Canpolat

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ÖZET

Enzimler gıda, enerji ve tekstil gibi birçok endüstri alanında kullanılmaktadır. Günümüzde işlevce modifiye edilmiş enzimlere talep artmaktadır. Çok geniş endüstriyel uygulama alanlarına sahip selülazlar tekstil terbiyesinde selüloz fiberlerinin ve kumaşların biyoparlatılmasında kullanılmaktadır. Selülazlar pamuk kumaşlarda tüylenmenin önlenmesinde kullanılmakta, fakat bu işlem esnasında mukavemet ve ağırlık kayıplarına yol açmaktadır. Öte yandan, viskon kumaşların biyoparlatmasında kullanılan bir selülaz formülasyonu mevcut değildir. Enzimlerin boyutlarının büyütülmesi kumaşlardaki bu olumsuz etkilerin önlenmesi için alternatif bir yol olarak görülmektedir.

Bu çalışmada mukavemet ve ağırlık kayıplarını en aza indirgemek için ticari selülaz enzimleri çapraz bağlanmış, bu sayede büyüklükleri artırılmıştır. Modifiye edilmiş bu enzimler karboksimetilselüloza karşı aktivitelerine göre karakterize edilmiş, pamuk ve viskon kumaş üzerindeki etkileri test edilmiştir. Çapraz bağlanmış ticari enzimlerle işlem gören pamuk ve viskon kumaşlarda tüylenme probleminin önüne geçilmiş, aynı zamanda uygulama esnasında meydana gelen mukavemet ve ağırlık kayıplarında azalma olduğu gözlemlenmiştir. Viskon kumaşların biyoparlatılmasında kullanılamak üzere katma değerli ticari enzim üretimi ilk kez bu çalışmada raporlanmıştır. Ayrıca pamuk kumaşın biyoparlatılmasında uygulama maliyetleri aşağı çekilmiştir.

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ABBREVIATIONS

AATCC	Association for American Textile Chemists and Colorists
CBD	Cellulose binding domain
CBM	Carbohydrate binding module
CLE	Cross-linked dissolved enzyme
CLEA	Cross-linked enzyme aggregates
CLEC	Crosslinked enzyme crystals
CMC	Carboxymethyl cellulose
DNS	Dinitrosalicylic acid
Glu	Glutaraldehyde
LR	Liquor ratio
NaOAc	Sodium acetate
Rpm	Rotor per minute

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Chapter 1

1. INTRODUCTION

Enzymatic treatments have been a focus of interest for fabric finishing to attain fabric softness, good performance and looks as well as relatively cheap and simple manufacturing processes (Buchle-Diller et al, 1994). Biopolishing is a process that removes cellulose fibrils from the exterior surface of the fiber to reduce pilling through hydrolysis of the β-1,4 glycosidic bonds. Cellulase enzymes are used for biopolishing of cellulosic fabrics, such as cotton (Videbaek and Andersen, 1993). Particularly, controlled finishing with endoglucanase enzymes are routinely used for the removal of pills from cellulosic fabrics (Miettinen and Oionen, 2005). However, biofinishing processes usually cause decrease in fabric weight as well as tensile strength (Kumar et al, 1997).

Viscose, consisting of two-thirds amorphous and one-third crystalline cellulose, has less tensile strength when compared to cotton. Therefore biopolishing process is not convenient for viscose fabrics (Kumar et al, 1997). This is mainly due to the extremely aggressive action of biopolishing enzymes on the crystalline regions of viscose fibers. In biopolishing process, aggressive catalytic action of cellulases causes losses of fabric tensile strength and weight. One solution to this problem is to increase particle size of the enzymes in order to limit the catalyst diffusion into fiber, resulting in limitation of catalytic action to the fabric surface thereby using particular enzyme immobilization techniques. This can be either done by immobilizing the enzymes to a surface or to each other forming aggregates. Cross-linked enzyme aggregates (CLEA) technology offers a promising methodology specifically based on this phenomenon. In addition to that, there are also many possible approaches to reduce the aggressiveness of CLEA particles such as dilution of catalytic activity by introduction of non-catalytic additives to the enzyme preparations during CLEA preparation process(Serrano et al, 2002; Kumari et al, 2007) or subsequent encapsulation of CLEAs in carriers after CLEA synthesis process (Schoevaart et al, 2006). As an alternative solution, in last two decades, genetic modification approaches have been performed to obtain less aggressive biocatalysts. Removal of cellulose-binding domain (CBD), one of the most significant genetic

engineering studies, drastically decreases enzymes' effectiveness in the context of binding affinity (Zhou, 2013) and therefore reduces the weight and tensile strength losses of fabrics. However, even with such advancements in enzyme engineering, there is still no commercial enzyme formulation suitable for biopolishing of viscose rayon fabrics. Current research efforts focus on use of CBD free enzymes in cotton biopolishing but until now there are still no attempts in the literature aiming to solve the biopolishing problem for the viscose fabric without causing adverse impact on tensile strength.

Cross-linked enzyme aggregates (CLEAs) are produced by precipitation of the enzymes and subsequent chemical cross-linking of these aggregates with a bifunctional chemical reagent, has been proposed as an alternative immobilization method to conventional support-dependent immobilization methods in last two decades (Sheldon, 2011). Acetone is used for precipitation of enzymes. Acetone precipitation enables purification of the enzyme and the immobilization process to be carried out in a single stage. More importantly, the factors that influence CLEA particle size, including precipitant type, enzyme concentration, pH of cross-linker and enzyme/cross-linker ratio have been investigated in detail (Yu et al, 2006; Sheldon, 2011). Most important one of these factors is the enzyme/cross-linker ratio. By altering this ratio, one can obtain cellulase CLEA products with desired particle size.

In this work, I have performed the immobilization of two novel commercial cellulase enzyme formulations lacking functional CBD, as cross-linked enzyme aggregates and used the resulting products for biopolishing of cotton and viscose rayon fabrics. By doing so, I combined the advantages of both gene manipulation and covalent modification technologies into a single product and for the first time in literature; I obtained significant results in pilling notes, tensile strength and weight loss values. Therefore I expect the CLEAs that I produced would have a great impact in both cotton and viscose applications in textile industry.

Chapter 2

2. BACKGROUND

2.1 Cotton

Cotton, consisting of two-thirds crystalline and one-third amorphous cellulose, is a natural staple fiber that is cheap, biodegradable and that has good tensile strength and absorption properties. Cotton fiber has a length of 2.5 inches and its diameter ranges from 16 to 20 microns. It has a flat and twisted structure, having 125 convolutions per inch (Hatch, 1993). A cotton fiber consists of glucose molecules that are linked together by β-1,4 glycosidic bonds. These β-1,4 glycosidic bonds allow chains to rotate around the oxygen molecules providing the flexibility of cotton. Cotton fiber can form hydrogen bonds with water because of the existence of three hydroxyl groups per ring. These hydroxyl groups also provide hydrophilicity to the fiber and resistance to slippage during an applied force.

2.2 Viscose

Viscose, consisting of two-thirds amorphous and one-third crystalline cellulose, is made from the naturally occurring polymer cellulose that has high tenacity and extensibility. Viscose has less tensile strength when compared with cotton. Amorphous cellulose mostly takes place in the core region; on the other hand, outer region is composed of crystalline cellulose regions that are homogenously distributed throughout the fiber. Amorphous cellulose, which is provides flexibility to the fiber, is more prone to attack by cellulases when compared with crystalline cellulose. On the other hand, crystalline cellulose, which provides tensile strength to the fiber, is more rigid; and the loss of the tensile strength is the result of cellulase action on the highly ordered crystalline structure of the fiber.

2.3 Biopolishing

Biopolishing refers to removal of cellulose fibrils from the exterior surface of fiber to reduce pilling through the partial hydrolysis of the β-1,4 glycosidic bonds. Cellulases can react with natural or regenerated cellulose (Bazin et al., 1991; Asferg et al., 1990). There is an alternative method to reduce fibrillation: cross-linking the fibers. On the other hand, this also leads to decrease in fiber tenacity. Biopolishing is the finishing technique which applies cellulase enzymes to a cellulosic fabric to improve surface appearance by reducing loose micro fibrils that agglomerate on fabric surface. Biopolishing provides fabrics with

- better surface appearance
- improved flexibility
- improved drapability
- improved whiteness on full whites
- better color retention and lower cross staining
- reduced pilling and fuzz
- improved handling
- improved lustre

Biopolishing is carried out during the wet processing stages, mostly between bleaching and dying. The fabric becomes cleaner and more hydrophilic after bleaching. Hydrophilicity makes fabric prone to cellulase action (Wu and Li, 2008). Because of the risk of color fading and possibility of undesirable inactivation of enzymes as a result of chemical content of dyes, biopolishing is not performed after dyeing.

Enzyme activity and dose are the most significant parameters for biopolishing process. Enzyme dose is determined as a percentage of fabric weight. Usually, this percentage ranges from 0.5% to 1.5% enzyme over fabric weight. The process is performed at pH 4.5-5.5 for acid cellulases, and 5.5-6.5 for neutral cellulases; temperature between 40-60 ^{o}C for 30-60 minutes. Enzyme catalysis is inactivated by increasing the temperature above 80 ^{o}C and pH above 10 by adding calcium carbonate.

Controlled finishing with cellulase enzymes optimizes surface properties of the fabric but results in weight loss and reduction of tensile strength. Enzymatic treatment

of cotton fabric usually results in 3-6% weight loss and 10% loss in tensile strength (Buchle-Diller et. al, 1994).

2.4 Cellulases

Cellulase enzymes are produced by a wide variety of organisms, however, only few of these are capable of degrading cellulose effectively. In industrial applications, cellulases obtained from extremophilic microorganisms are preferred due to their stability and ability to operate at high temperatures and harsh conditions such as highly acidic or alkaline pHs as well as temperatures up to 90 ^{o}C (Lamed and Bayer, 1988).

Nowadays commercial cellulase preparations are available for use in biopolishing of cotton fabric. These enzymes seem to function over broad temperature and pH range. They also show diverse activity and stability profile. These enzymes are mostly originated from the filamentous fungi, *Trichoderma reesei*.

Cellulases are multicomponent enzymes divided into three major types: endoglucanases, 1,4-B-D-glucan 4-glucanohydrolases; cellobiohydrolases, 1,4-β-D-glucan cellobiohydrolases; and cellobiases, B-D-glucosidases. *Trichoderma reesei* secretes six endoglucanases, two cellobiohydrolases and two β-D-glucosidases. (Bhat, 1997; Heikinheimo, 2005) Table 1 indicates molecular weights and number of amino acids of some of these cellulase components. Cellulases belong to the glycosyl hydrolase family of enzymes that contains 96 subfamilies. 12 of these subfamilies contain cellulase.

Table 1: *Trichoderma reesei* cellulyotic system components (Vinzant et al, 2001)

Cellulase Components of Trichoderma reesei	Molecular Weight (kDa)	Number of amino acids
EG I	48,2	459
EG II	44,2	418
EG III	23,5	218
EG IV	35,5	344
EG V	24,5	242
CBH I	54	513
CBH II	49,6	471
B-D-glucosidase I	78,5	744

Cellulase components act synergistically on 1,4-B-glycosidic bonds of the cellulose. Endoglucanases, aggressively act on amorphous cellulose (Heikinheimo & Buchert, 2001). These enzymes randomly hydrolyze cellulose chains internally and results in production of new chain ends. Cellobiohydrolases hydrolyze crystalline cellulose chains from the ends (Sandgren, 2005). Cellobiohydrolase action produces cellobiose as the end product (Heikinheimo & Buchert, 2001). Cellobiose inhibits CBH and EG actions on cellulose (Gruno, 2004). By doing so, it slows down the enzymatic finishing process. On the other hand, cellobiose is hydrolyzed by β-glucosidases.

Cellulase has two domains linked by a short linker: catalytic domain and cellulose binding domain. The linker peptide is rich in Proline, Threonine and Serine residues. This peptide is often O-glycosylated and this protects the linker region against proteases. The role of CBD is to keep the cellulose in the vicinity of the catalytic domain (Zhou, 2013).

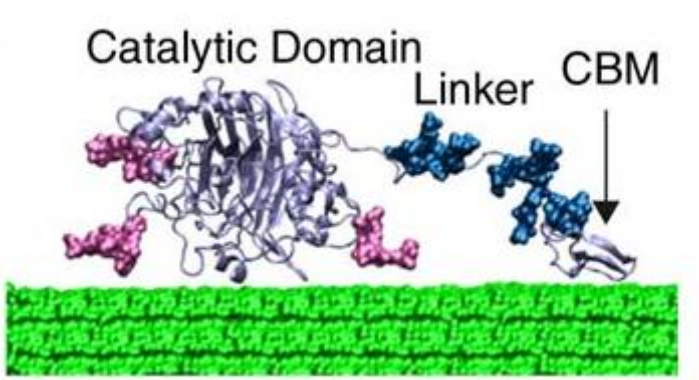

Figure 1: The *Trichoderma reesei* Family 7 cellobiohydrolase (Cel7A) acting on cellulose (Beckham et al., 2011).

The most recent and significant genetic modification on cellulases was the production of CBD truncated endoglucanase enzymes. A polypeptide that has endoglucanase activity but lacking a functional cellulose binding domain was produced, and that technology was used to produce new generation enzymes: Cellusoft 37500 L and Cellusoft CR (Zhou, 2013). In this study, I used Cellusoft 37500 L to in cotton biopolishing experiments and Cellusoft CR in viscose biopolishing experiments.

2.5 Cross-Linked Enzyme Aggregates (CLEA)

Immobilization methods are divided into two types: binding to a support (Boller et al., 2002), or cross-linking of pure enzymes with a bifunctional cross-linker (Cao et al., 2000). Covalent attachment to a support matrix is an intensely studied immobilization technique. There are several inorganic materials suitable for this process such as: silica, silicates, borosilicates, aluminosilicates, alumina and titania (Zucca, 2014). Some of the reaction types used in enzyme immobilization are diazotization, amide bond formation, alkylation and arylation, Schiff's base formation, amidation reaction, thiol-disulfide interchange and carrier binding with bifunctional reagents. In order to limit the adverse affects of cross-linking to activity one should choose the agent that does not bind to the amino acids in the vicinity of the active site. One way to prevent this inhibition would be to perform this reaction in the presence of a substrate. Since the substrate blocks the active site, this method assumes that residues around the

active site would not be available for cross-linking. As another alternative solution, reversible covalent attachment of an inhibitor to the enzyme would also be performed.

The technique of enzyme cross-linking -named CLE- by the use of glutaraldehyde with reactive amine residues on the protein surface was firstly developed in 1960s (Cao et al., 2003; Doscher and Richards, 1963). In this technique, pure enzymes are covalently attached to each other with the use of a bifunctional cross-linker. CLE technique has significant disadvantages such as low activity retention, poor reproducibility, low mechanical stability, not to mention the fact that, difficulty of handling due to its gelatinous structure. In order to overcome these disadvantages, Quiocho and Richards developed the technique of cross-linking of a crystalline enzyme. Subsequently, this application has been successfully commercialized as cross-linked enzyme crystals (CLEC) (Lalonde, 1997; Margolin, 1996). However, process of CLEC synthesis includes crystallization and purification which are cumbersome and costly processes, following research efforts focused on finding a more practical way of getting comparable results. Then, Cao and his friends came up with the idea of applying cross-linking on aggregated enzyme mass, and that led to the invention of the technique called cross-linked enzyme aggregates (CLEAs) (Cao et al, 2000; Sheldon et al, 2005). Within the CLEA technology, various methods of protein purification are applied such as the addition of precipitants such as salts, organic solvents, non-ionic polymers or acids (Hofland et al., 2000). Covalent attachment of aggregates results in drastic increase in catalytic activity of the enzymes on surface of the aggregates.

In cross-linking experiments, glutaraldehyde is the first reagent of choice. Glutaraldehyde exists in the monomeric form at lower concentrations. On the other hand, in high concentrations, it exists in polymerized form and leads to immobilization by forming Schiff's base bonds. Glutaraldehyde is a cross-linker that forms stable bonds with the amine groups of lysine residues (Weieser et al., 2014). Glutaraldehyde is commonly used in process of cross-linking, owing to its low cost, high reactivity and small size. Particularly, size of the cross-linker is significant due to the need of penetration into the interior of the physical aggregates. Glutaraldehyde is dissolved in acid solutions. At this pH, the aldehyde is stable and glutaraldehyde is in the monomeric form. In order to activate the glutaraldehyde, pH is elevated to 10 with the use of sodium hydroxide. After four hours, it is adjusted to 8 with acetic acid.

On the other hand, in the case of particular enzymes, low activity retention is observed after cross-linking with glutaraldehyde, due to the reaction of glutaraldehyde with lysine residues that are crucial for enzyme activity. These lysine amino acids are located around the active site of the enzyme. Therefore, in this case, other dialdehydes that involve less complicated chemistry are used as cross-linkers.

Precipitation is a widely used method of enzyme purification which involves aggregate formation of enzymes in acetone as a precipitant reagent. In the initial screening of precipitants, the amount of aggregates formed is a selection criterion showing the effectiveness of precipitation. Subsequently the aggregates are dissolved and the activity retention is measured. In fact, high activity retention of aggregates would not guarantee the activity retention after cross-linking all the time. For example, aggregates can fold into an unfavorable conformation upon cross-linking causing a reduction in the catalytic activity.

Enzyme: cross-linker ratio is another important factor. If the ratio is too high, too much cross-linking would occur and this may adversely affect the activity and the flexibility of the CLEA. If the ratio is too low, sufficient cross-linking may not occur resulting in decrease in the amount of insoluble CLEAs formed (Yu et al, 2006). Depending on the surface structure of the enzyme and the number of lysine residues that the enzyme contains; the optimum ratio varies for each enzyme. The enzyme: cross-linker ratio is also the most significant criterion in determining the particle size of CLEAs. From the point of view of large scale applications, particle size is one of the significant factors that affect mass transfer and filterability under operational conditions. Generally, CLEA particle size ranges from 5 to 50 micrometers, and that range is sufficient for the filterability of CLEA particles. For particular large-scale applications, it may be necessary to increase the particle size and mechanical stability of CLEA and one of the successful ways to achieve this goal is to encapsulate them in a polyvinyl alcohol matrix (Wilson, 2004). The most important advantage of CLEAs is that they can be synthesized from very crude enzyme abstracts (Sheldon, 2011), however, sometimes it would be difficult to form CLEAs from enzyme preparations that contain low enzyme content. In such of cases, the reactions would be performed in the presence of a proteic feeder such as bovine serum albumin (BSA).

In a successful application, activity recovery is expected to be very close to 100% (Sheldon, 2011). In the CLEA process, particularly, only the enzymes on the surface exhibit catalyst role. On the other hand, the enzymes in the core domain are involved in providing stability of the CLEA. Therefore, aggressiveness of the surface-enzymes determines the total CLEA activity.

2.6 Advantages & Disadvantages of CLEAs

Basically advantages of CLEAs are;

- No need for extra purification
- Low production cost
- No need for carriers
- Improved storage stability
- Improved operational stability
- High catalyst productivities
- High recycling capacity
- Possibility to co-immobilize more than one enzyme
- Ease of filtration
- Ease of particle size determination
- Possibility to use catalysts in water-free environments

CLEA particles have high catalytic activity when compared to that of monomeric enzymes. CLEA units are less mobile (less free to flex and vibrate, have less conformational possibilities per each cross-linked monomer), therefore; Gibbs energy state of CLEA is higher. However CLEAs cannot unfold due to the very high reorganizational energy constraints. When CLEAs dock to a substrate, the freedom lost is less when compared to than a monomer docking to a substrate. So the reaction system has a lower activation barrier.

On the other hand, due to their unique molecular structure CLEAs have also disadvantages such as:

- Loss of effectiveness due to diffusional limitation.
- Lack of accuracy in colorimetric assay results due to mechanical properties.

- Heterogeneous distribution in aqueous media.
- Gelatinous structure in aqueous media.

Small-sized substrates -like CMC- have diffusional limits in colorimetric assays. Small-scale assays are performed in 1.5 ml Eppendorf tubes in which all CLEA particles are clotted and settled on the bottom of the tube. Therefore, it is hard for CMC particles to diffuse into aggregates. Additionally, CLEAs are heterogeneously distributed in terms of particle size. This reflects heterogeneous distribution of CLEAs in aqueous media because bigger and heavier particles move faster. As a result of that, CLEAs may not exhibit their function equally on the surface of a larger substrate. Lastly, due to extensive glutaraldehyde cross-linking, CLEA particle conformation would be gelatinous and that makes it harder to handle CLEA particles and use them industrial applications.

Chapter 3

3. METHODS

3.1 Enzyme Characterization

All activity screening tests were performed in triplicate with a standard deviation of below 10%.

3.1.1 Effect of Temperature on Enzyme Activity

Activity of free enzyme samples at different temperatures (25 ^{o}C – 90 ^{o}C) were determined by 3,5-Dinitrosalicylic acid (DNS) method against 1% carboxymethyl cellulose (CMC) (w/v) in 50 mM sodium acetate buffer (pH 5) for Cellusoft 37500 L and in 100 mM potassium phosphate buffer (pH 6) for Cellusoft CR. 3,5-Dinitrosalicylic acid is an aromatic compound that reacts with free carbonyl group (C=O), which is so-called reducing sugars. DNS method was performed in order to test for the presence of reducing sugars as the end products of cellulase action on CMC substrate. Enzymes were preincubated for 5 minutes at 55 ^{o}C. Subsequently enzymes and CMC substrates were incubated in thermo-shaker for 10 minutes in 1000 rpm. Reducing sugars produced were measured at 550 nm.

3.1.2 Effect of pH on Enzyme Activity

Activity of free enzyme samples at different pHs (ranging from pH 3 to pH 8) were determined by DNS method against 1% CMC (w/v). Enzymes were preincubated for 5 minutes at 55 ^{o}C. Subsequently enzyme and CMC substrate were incubated in the thermo-shaker for 10 minutes in 1000 rpm. Reducing sugars produced were measured at 550 nm.

3.2 CLEA Preparation Protocols

3.2.1 CLEA Preparation from Cellusoft 37500 L

Step	Action
1	Add 160 ml acetone to beaker with a magnetic stirrer bar.
2	Add 40 ml of enzyme solution drop by drop
3	Add 10 ml of 0.1 M potassium phosphate buffer (pH 7.3) containing 4ml of 25% glutaraldehyde to the mixture.
4	Stir the suspension for 30 minutes at 1000 rpm.
5	Add 40ml of 1 M Tris solution at pH 8 in order to quench the reaction.
6	Centrifuge the suspension 5.000 rpm for 5 minutes
7	Remove supernatant.
8	Wash CLEA particles with 0.1 M potassium phosphate buffer
9	Freeze CLEA particles with liquid nitrogen and put them in lyophilizer
10	Ground CLEA particles using TissueLyzer for 1 minute at a frequency of 1/30 (1/sec)

3.2.2 CLEA Preparation from Cellusoft CR

Step	Action
1	Add 25 ml of enzyme to 50 ml Eppendorf tube, then add 25 ml of acetone, flip the tube up and down
2	Transfer 25 ml of the suspension to another 50 ml Eppendorf tube, then add 25 ml of acetone, flip the tube up and down
3	Repeat the second step and finally 1:7 enzyme acetone ratio is obtained, then transfer the suspension to a 5 lt beaker
4	Repeat the first three steps until 800 ml of final suspension volume is obtained
5	Add 50 ml of 0.1 M potassium phosphate buffer (pH 7.3) containing 8 ml of 25% glutaraldehyde.
6	Stir the suspension by a mechanical stirrer at 1000 rpm for 30 minutes.
7	Add 100ml of 1 M Tris solution at pH 8 in order to quench the reaction.
8	Centrifuge the suspension at 5.000 rpm for 5 minutes
9	Remove supernatant.

10	Wash CLEA particles with 0.1 M potassium phosphate buffer
11	Freeze CLEA particles with liquid nitrogen and put them in lyophilizer
12	Ground CLEA particles using TissueLyzer for 1 minute at a frequency of 1/30 (1/sec)

3.3 Enzymatic Biofinishing Protocol

Step	Action
1	Place the fabric samples in standard atmosphere for at least 12 hours, weigh each fabric swatch.
2	Pre-heat Gyrowash machine to 55 ^{o}C. Place 20 steel balls in each test beaker.
3	Add 200 ml buffer solution to each beaker.
4	Add 1 swatch of standard fabric (10 g)
5	Place beakers in Gyrowash.
6	Set the timer to 60 minutes
7	After 60 minutes, remove the beakers.
8	Leave the beakers for 5 minutes before opening to avoid aerosols
9	Add 1-2 ml of 30% (w/v) sodium carbonate into test beakers
10	Remove the swatches and wash them in a 5 lt beaker for three times
11	Dry the swatches
12	Place the samples in standard atmosphere for at least 12 hours
13	Weigh the samples

3.4 Fabric Tests

3.4.1 Pilling Test

Pilling tests were performed in Ak-Kim Chemicals textile laboratory. Martindale 2000 pilling machine was used at 200 rpm. The reference photographs used were evaluated according to AATCC (Association for American Textile Chemists and Colorists) standards. Pilling values are determined by taking averages of five measurements. Pilling notes were reported based on the scale ranging from 5 to 1 (no pilling to very severe pilling).

3.4.2 Bursting Strength Test

Bursting strength tests were performed in Ak-Kim Chemicals textile laboratory. Textile strength values were evaluated according to AATCC standards. The fabric swatch is placed between annular clamps, and is subjected to an increasing pressure by a needle. Bursting strength is expressed in kilopascal (kPa). Triple measurements were taken for each fabric swatch and the average of three were taken.

Chapter 4

4. RESULTS

4.1 Enzyme Characterization

4.1.1 Effect of Temperature on Enzyme Activity

Figure 2 and Figure 3 show the activity results for native Cellusoft 37500 L and Cellusoft CR at different temperatures. The temperature activity profiles shown in each figure exhibited peak at 55 ^{o}C for both enzyme formulations. Both graphics show similar patterns, namely the activity of both enzymes follow a rising trend up to 55 ^{o}C. With the temperature 75 ^{o}C activities decrease drastically. Both enzymes seem to function over a broad temperature range (45-65 ^{o}C).

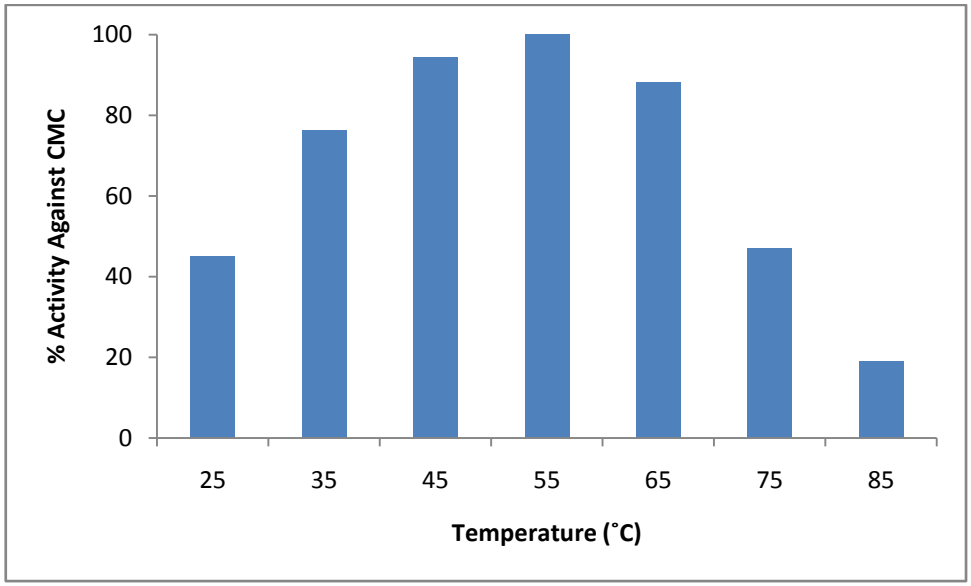

Figure 2: Catalytic activity results for native Cellusoft 37500 L at different temperatures.

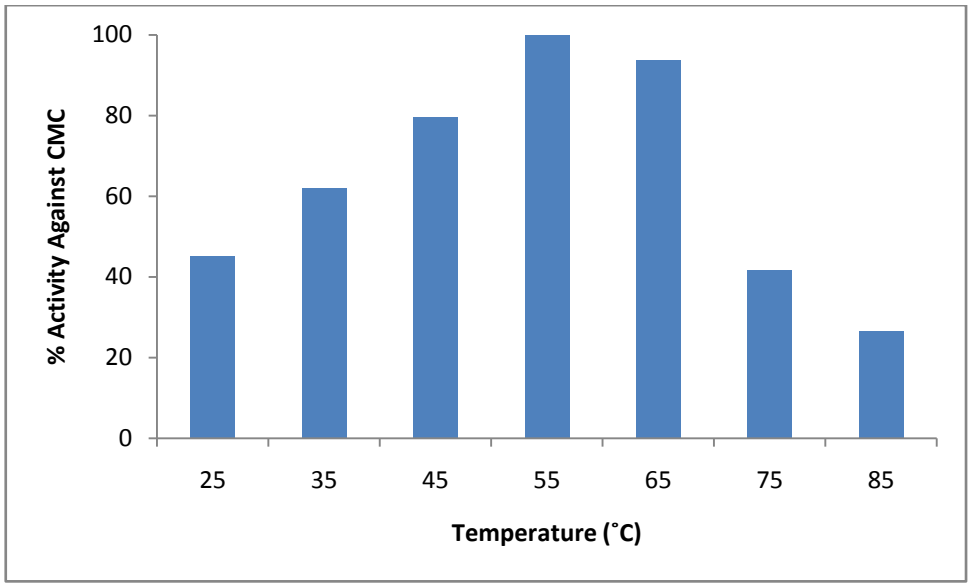

Figure 3: Catalytic activity results for native Cellusoft CR at different temperatures.

4.1.2 Effect of pH on Enzyme Activity

Optimum pH for Cellusoft 37500 L was found to be pH 5 and for Cellusoft CR it was found to be pH 6. Figure 4 indicates that Cellusoft 37500 L seems to function over a narrow pH range (pH 4-5) since it has 90% activity at pH 4. Additionally, Cellusoft CR activity profile ranges from pH 5 to pH 7 since it retains at least 80% of its activity at these pHs (Figure 5).

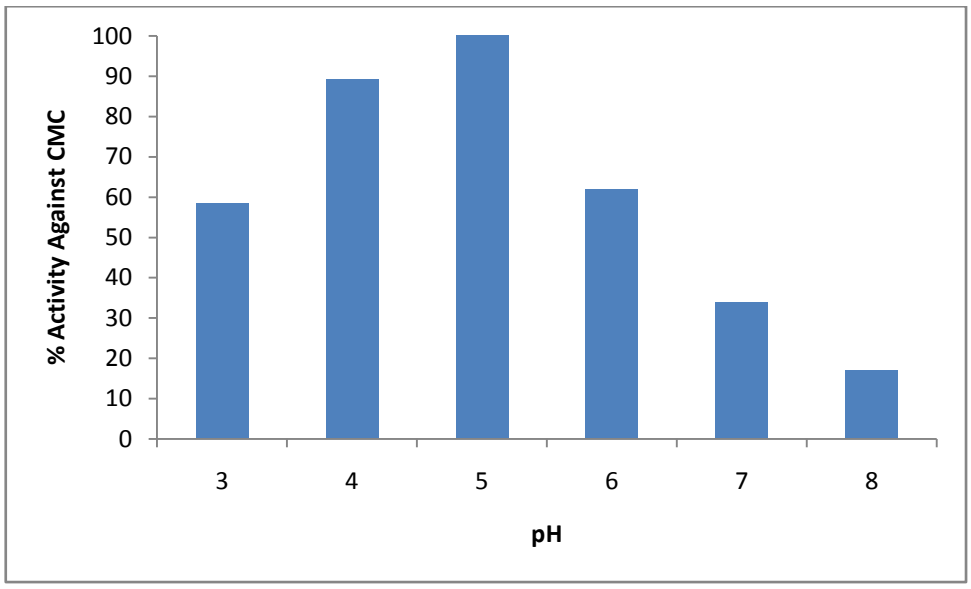

Figure 4: pH activity profile for Cellusoft 37500 L.

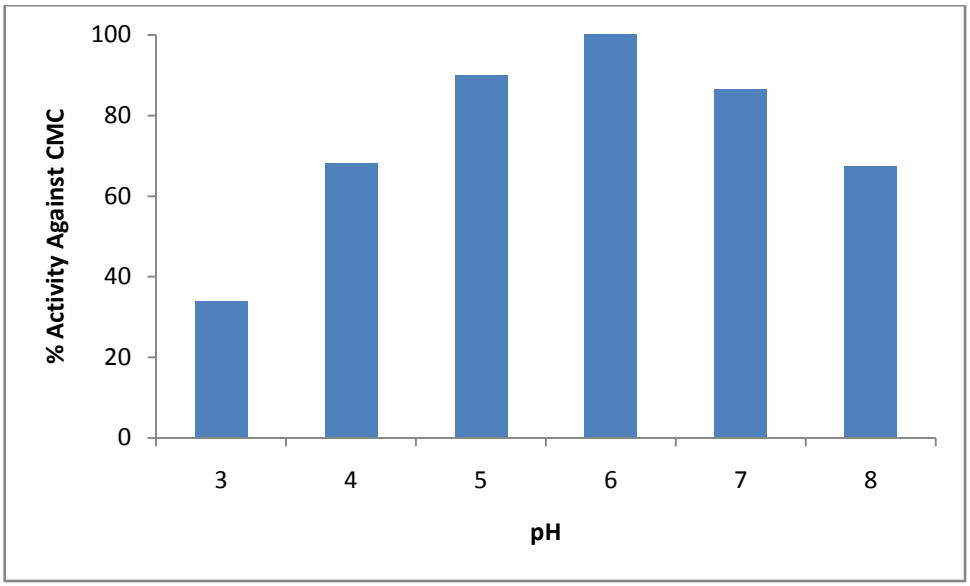

Figure 5: pH activity profile for Cellusoft CR.

4.2 CLEA Preparation

4.2.1 CLEA Preparation from Cellusoft 37500 L

-20 ^{o}C was selected as precipitation temperature for CLEA synthesis from Cellusoft 37500 L enzyme formulation. At -80 ^{o}C, CLEAs exhibited similar activity profile, but the end product weight was too low. With the increase of precipitation temperature, supernatant activity also increases, which means acetone starts to dissolve some of the enzymes. A cooling bath mixture of NaCl and ice was prepared and placed around the exterior surface of the plastic beaker in which the synthesis was performed. Afterwards, a centrifugation step was performed at 4 ^{o}C. After centrifugation, light brown-colored aggregates were obtained, however they became darker in 24 hours. According to Figure 6, CLEA activity profile exhibited a peak at 100 mM glutaraldehyde concentration. Contrary to expectations, CLEA activity did not show a decreasing trend with the increase in glutaraldehyde concentration.

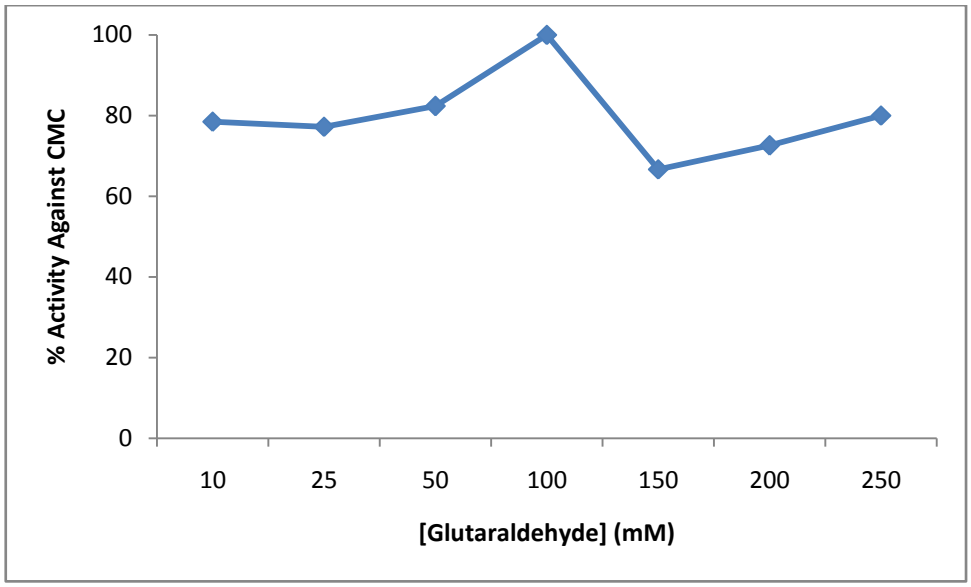

Figure 6: Effect of glutaraldehyde concentration on CLEA activity against carboxymethyl cellulose.

I also examined the effect of optimum glutaraldehyde concentration on pellet and supernatant at room temperature. Aggregates exposed to optimum glutaraldehyde concentration retained 93% of their activity; however there was no significant difference between supernatant activities (Figure 7).

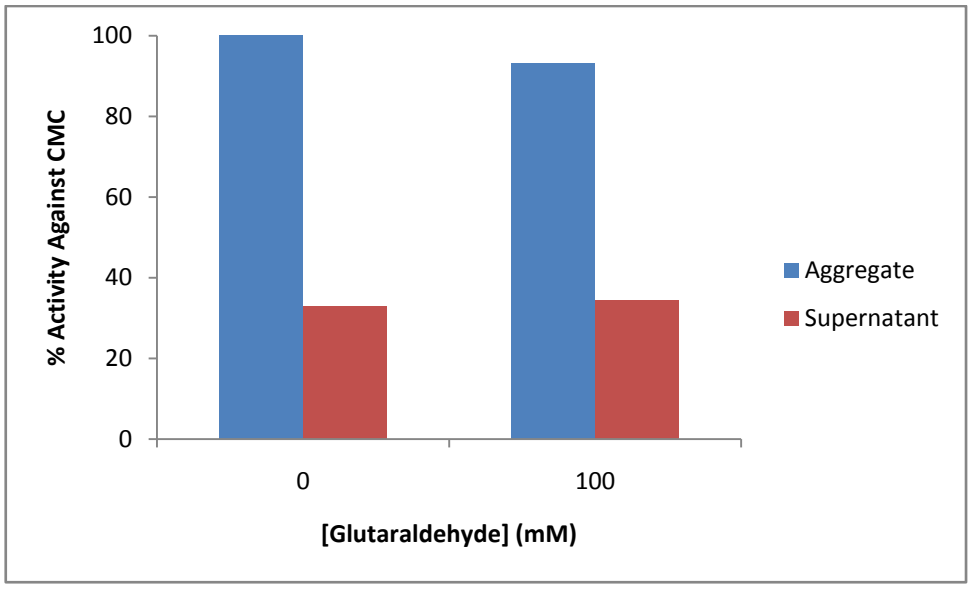

Figure 7: Comparison of pellet and supernatant activities for Cellusoft 37500 L with 0mM and 100 mM glutaraldehyde concentrations.

In order to observe diffusional limits of CMC into CLEA particles, a little portion of CLEAs were lysed for less than 1 minute in TissueLyzer to obtain 1000µm sized CLEA particles. Moreover, we compared CLEA activities at -20 ^{o}C with the ones at -80 ^{o}C. CLEAs showed higher activity results at -20 ^{o}C. In addition to that, CLEAs with bigger particle size exhibited lower activity results when compared to that of small-sized CLEAs (Figure 8).

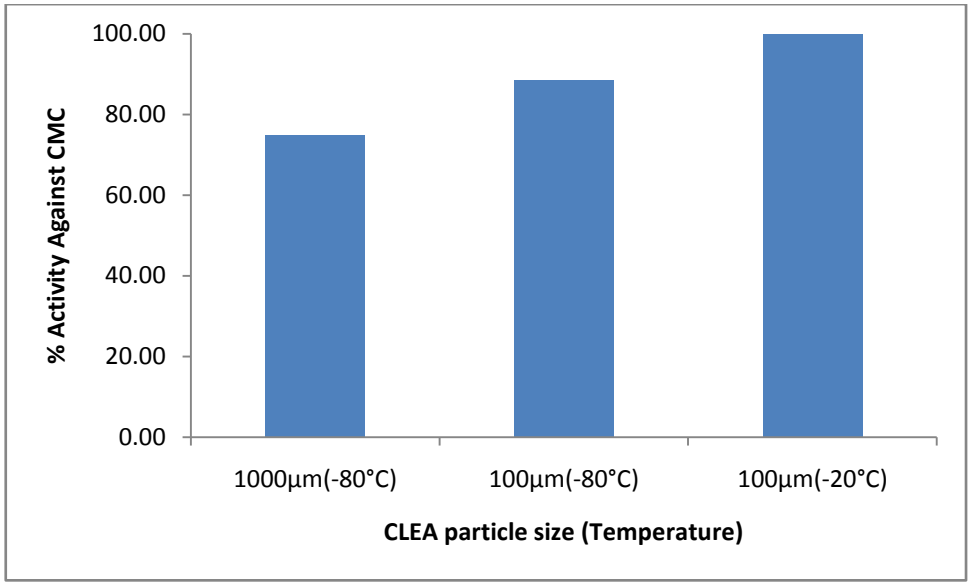

Figure 8: Catalytic activity results for Cellusoft 37500 L CLEAs synthesized in different conditions.

4.2.2 CLEA Preparation from Cellusoft CR

At first, I followed the same protocol for the synthesis of Cellusoft CR CLEA however, I obtained 1 gram of CLEA from 100 ml of Cellusoft CR using this protocol. Therefore, I changed specifically the precipitation part of the protocol. I performed a gradient precipitation with acetone in 50 ml Eppendorf tubes. Unlike common precipitation procedures, I added the acetone on to the enzyme solution. By that way, I obtained 7 grams of CLEA from 100 ml of Cellusoft CR. Additionally, after centrifugation I obtained light brown-colored aggregates and this color remains the same all the time. According to Figure 9, CLEA activity profile exhibited a peak with 100 mM glutaraldehyde. CLEA activity showed a slightly decreasing trend with the increase in glutaraldehyde concentration.

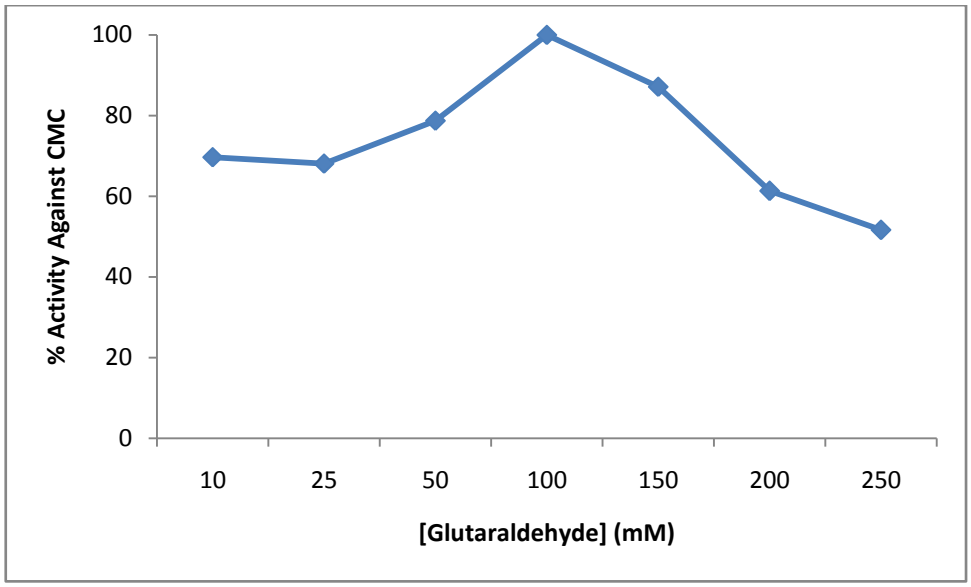

Figure 9: Effect of glutaraldehyde concentration on CLEA activity against carboxymethyl cellulose.

I also examined the effect of optimum concentration of glutaraldehyde on pellet and supernatant at room temperature. Aggregates exposed to optimum glutaraldehyde concentration retained 98.5% of their activity however there was no significant difference between supernatant activities (Figure 10).

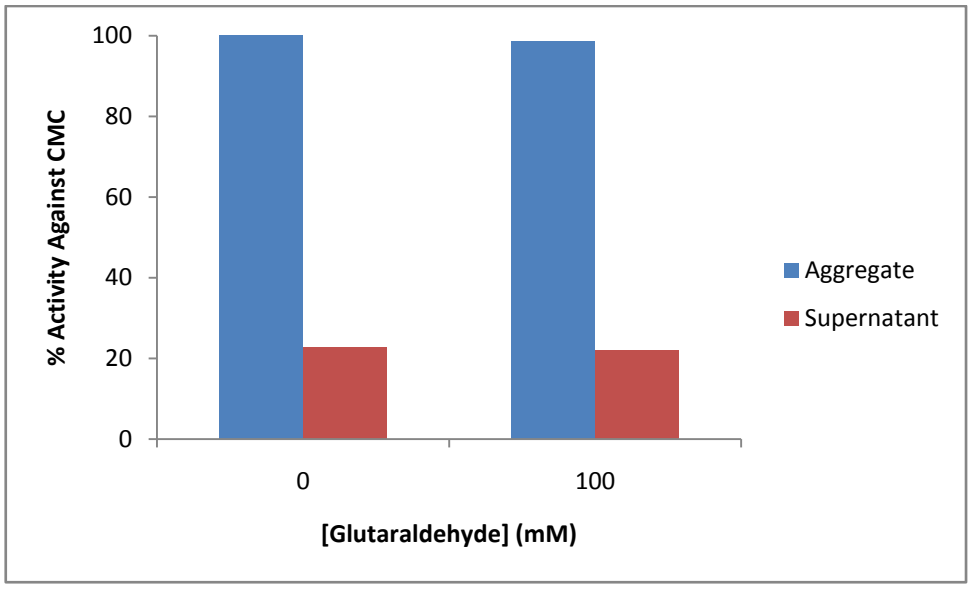

Figure 10: Comparison of pellet and supernatant activities for Cellusoft CR with 0mM and 100 mM glutaraldehyde concentrations.

4.3 Fabric Tests

Effects of native and cross-linked forms of Cellusoft 37500 L on cotton and that of Cellusoft CR on viscose biopolishing were examined, pilling and bursting strength test results were evaluated. I applied different amounts of enzyme formulations in order to analyze effect of enzyme dose on pilling and fabric strength values. In the preliminary studies, CLEA-Cellusoft 37500 used for viscose, and CLEA-Cellusoft CR was also used in varying amounts for cotton fabric biopolishing however the results were not promising, therefore we cancelled further studies for these cases.

4.3.1 Cotton Fabric Test Results

We used Cellusoft 37500 L formulation for enzymatic treatment of cotton fabric. Biopolishing of cotton fabrics with native enzyme and CLEA samples were performed in Gyrowash under optimum temperature (55 ^{o}C) and pH (5) conditions of the enzyme. Liquor ratio was 1:20 (10 g fabric sample and 200 ml buffer solution), and 20 steel balls (d: 14mm, 11g) were used to provide the mechanical effect. Pilling tests were performed in Ak-Kim Chemicals textile laboratory. Martindale pilling machine was used at 200 rpm. The reference photographs used were evaluated according to AATCC (Association for American Textile Chemists and Colorists) standards (Figure 10). Pilling values are determined by taking averages of five measurements. For pilling measurements, a scale from 1 to 5 is used. 1 refers to intense pilling and 5 refers to no pilling.

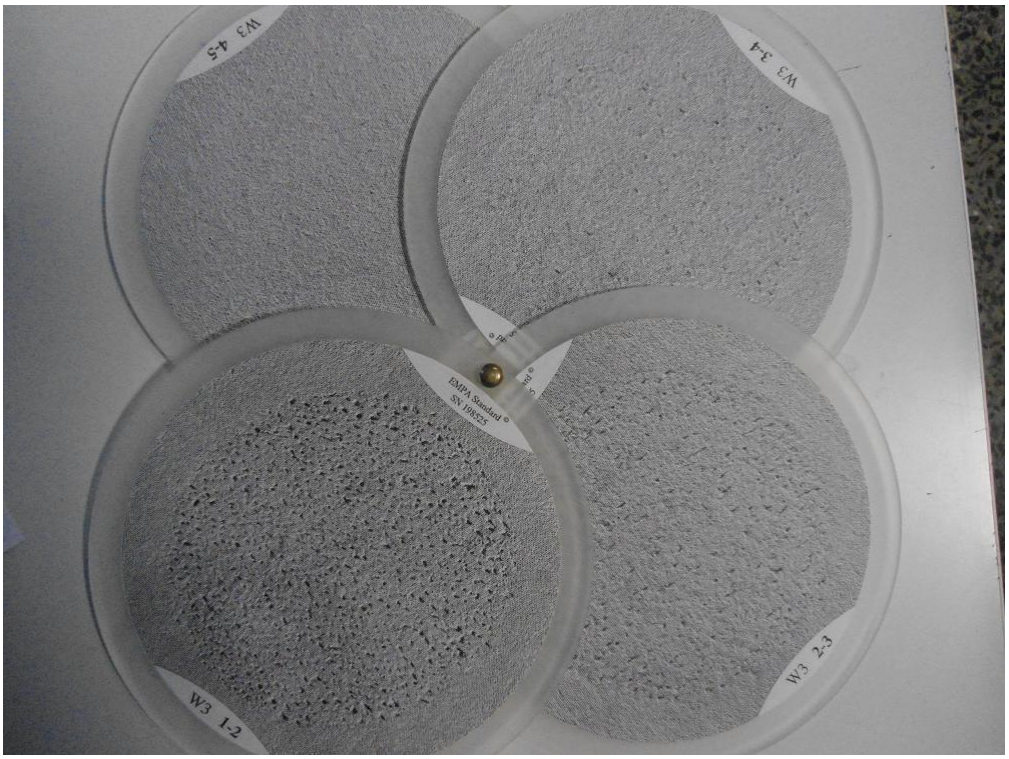

Figure 11: Martindale Pilling Test Standards. Top left: 4-5, top right: 3-4, bottom right: 2-3 and bottom left: 1-2.

CLEA dose was determined as 100 mg, and the same amount of native enzyme (347 µl) was used per 10 grams of cotton fabric. According to Table 2, both enzyme forms obtained best pilling notes. CLEA application on cotton fabric caused ~4.4% weight loss; on the other hand, native enzyme application resulted in loss of ~8.2%. Moreover, Cellusoft 37500 L CLEA decreased bursting strength values of the cotton fabrics to a much lesser extent when compared to the results of native formulation (Table 3).

Table 2: Pilling and fabric weight results for cotton fabrics treated with native Cellusoft 37500 L and CLEA-Cellusoft 37500 L.

Sample	Treatment	Fabric Weight (g) Before Treatment	Fabric Weight (g) After Treatment	Weight Difference(g)	% Weight Difference	Pilling Note
Control	Buffer treatment	10.668	10.763	0.095	0.891	1-2
Control	Buffer treatment	10.535	10.662	0.127	1.206	1-2
Control	Buffer treatment	10.624	10.748	0.124	1.167	1-2
CLEA	Fabric treated with 100 mg CLEA	10.548	10.15	-0.398	-3.773	4-5
CLEA	Fabric treated with 100 mg CLEA	10.585	10.174	-0.411	-3.883	4-5
CLEA	Fabric treated with 100 mg CLEA	10.530	10.032	-0.498	-4.729	4-5
Native	Fabric treated with 347 µl native enzyme	10.483	9.610	-0.873	-8.328	4-5
Native	Fabric treated with 347 µl native enzyme	10.647	9.769	-0.878	-8.246	4-5
Native	Fabric treated with 347 µl native enzyme	10.397	9.532	-0.865	-8.320	4-5

Table 3: Bursting strength and fabric weight results for cotton fabrics treated with native Cellusoft 37500 L and CLEA-Cellusoft 37500 L.

Sample	Treatment	Fabric Weigh (g) Before Treatment	Fabric Weigh (g) After Treatment	Weight (g) Difference	% Weight Difference	Pressure (kPa)
Control	Buffer treatment	10.644	10.729	0.085	0.799	232
Control	Buffer treatment	10.559	10.683	0.124	1.174	244.8
Control	Buffer treatment	10.633	10.743	0.11	1.035	230.9
CLEA	Fabric treated with 100 mg CLEA	10.430	9.916	0.514	-4.928	173.5
CLEA	Fabric treated with 100 mg CLEA	10.473	10.005	0.468	-4.469	187.9
CLEA	Fabric treated with 100 mg CLEA	10.437	9.948	0.489	-4.685	180.7
Native	Fabric treated with 347 µl native enzyme	10.466	9.618	0.848	-8.102	134.2
Native	Fabric treated with 347 µl native enzyme	10.391	9.546	0.845	-8.132	136.5
Native	Fabric treated with 347 µl native enzyme	10.468	9.587	0.881	-8.416	130.9

4.3.2 Viscose Fabric Test Results

We used Cellusoft CR formulation for enzymatic treatment of cotton fabric. Biopolishing of viscose fabrics with native enzyme and CLEA samples were performed in Gyrowash under optimum temperature (55 ^{o}C) and pH (6) conditions of the enzyme. Liquor ratio was 1:20 (10 g fabric sample and 200 ml buffer solution), and 20 steel balls (each d:14mm, 11g) were used to provide the mechanic effect. Pilling tests were performed in Ak-Kim Chemicals textile laboratory. Martindale pilling machine was used at 200 rpm. The reference photographs used were evaluated according to

Association for American Textile Chemists and Colorists (AATCC) standards. Pilling values are determined by taking averages of five measurements. For pilling measurements, a scale from 1 to 5 is used. 1 refers to intense pilling and 5 refers to no pilling.

Maximum CLEA dose was determined as 1000 mg, and the same amount of native enzyme (11.6 ml) was used per 10 grams of cotton fabric. First enzyme treatment trials showed that Cellusoft CR CLEAs did not distribute homogenously in buffer. That would be due to the moisture content of CLEA particles. We have further analyzed the effect of freeze-drying process in relation to biopolishing effectiveness of the catalyst. CLEA fraction that was synthesized without freeze-drying process was used firstly and results showed that pilling notes fluctuate from 2-3 to 4-5 points (Table 4). On the other hand, dried CLEA particles exhibited consistency in pilling results.

Table 4: Effect of moist CLEA particles on viscose biopolishing.

Sample No	Enzyme Dose	Pilling Note
No: 1	500mg	2-3
No: 2	500mg	3-4
No: 3	1000mg	4-5
No: 4	1000mg	2-3

Table 5: Effect of dried CLEA particles on viscose biopolishing.

Sample No	Enzyme Dose	Pilling Note
No: 1	500mg	2-3
No: 2	500mg	2-3
No: 3	1000mg	3-4
No: 4	1000mg	3-4

Effects of native and cross-linked Cellusoft CR on viscose biopolishing were examined; pilling and bursting strength test results were evaluated. According to Table 6, both enzyme forms obtained best pilling notes. Application of CLEA on 15 g viscose

fabrics caused ~3.3% weight loss; on the other hand native enzyme application resulted in loss of ~12%.

Table 6: Pilling and fabric weight results for cotton fabrics treated with native Cellusoft CR and CLEA-Cellusoft CR.

Sample	Treatment	Fabric Weigh (g) Before Treatment	Fabric Weigh (g) After Treatment	Weight (g) Difference	% Weight Difference	Pilling Note
Control	Buffer treatment	16.095	16.338	0.243	1.510	1-2
Control	Buffer treatment	15.872	16.108	0.236	1.487	1-2
Control	Buffer treatment	15.595	15.810	0.215	1.379	1-2
Native	Fabric treated with 17.4 ml native enzyme	15.833	13.936	-1.897	-11.981	3-4
Native	Fabric treated with 17.4 ml native enzyme	16.042	14.050	-1.992	-12.417	3-4
Native	Fabric treated with 17.4 ml native enzyme	16.090	14.214	-1.876	-11.659	4-5
CLEA	Fabric treated with 1500 mg CLEA	16.192	15.608	-0.584	-3.607	4-5
CLEA	Fabric treated with 1500 mg CLEA	15.935	15.430	-0.505	-3.169	3-4
CLEA	Fabric treated with 1500 mg CLEA	16.044	15.522	-0.522	-3.254	3-4

I further have performed the same experiment with another batch of Cellusoft CR enzyme that was kept at room conditions for 6 months. Therefore both native and CLEA forms were less aggressive, and this specifically affected pilling results. Pilling notes were reduced by 1 point (Table 7). Moreover, Cellusoft CR CLEA decreased the bursting strength values of the viscose fabrics to a much lesser extent when compared to the results of native formulation (Table 8).

Table 7: Pilling and fabric weight results for cotton fabrics treated with native Cellusoft CR and CLEA-Cellusoft CR.

Sample	Treatment	Fabric Weigh (g) Before Treatment	Fabric Weigh (g) After Treatment	Weight (g) Difference	% Weight Difference	Pilling Note
Control	Buffer treatment	10.891	10.858	-0.033	-0.303	1-2
Control	Buffer treatment	11.109	11.111	0.002	0.018	1-2
Control	Buffer treatment	10.664	10.660	-0.004	-0.038	1-2
Native	Fabric treated with 11.6 ml native enzyme	11.355	10.330	-1.025	-9.027	3-4
Native	Fabric treated with 11.6 m native enzyme	10.927	9.910	-1.017	-9.307	3-4
Native	Fabric treated with 11.6 m native enzyme	10.982	9.918	-1.064	-9.689	2-3
CLEA	Fabric treated with 1000 mg CLEA	10.863	10.417	-0.446	-4.106	3-4
CLEA	Fabric treated with 1000 mg CLEA	11.068	10.611	-0.457	-4.129	2-3
CLEA	Fabric treated with 1000 mg CLEA	10.948	10.486	-0.462	-4.220	2-3

Table 8: Bursting strength and fabric weight results for cotton fabrics treated with native Cellusoft CR and CLEA-Cellusoft CR.

Sample	Treatment	Fabric Weigh (g) Before Treatment	Fabric Weigh (g) After Treatment	Weight (g) Difference	% Weight Difference	Pressure (kPa)
Control	Buffer treatment	10.607	10.590	-0.017	-0.160	144
Control	Buffer treatment	10.752	10.724	-0.028	-0.260	138.7
Control	Buffer treatment	11.177	11.154	-0.023	-0.206	124.3
Native	Fabric treated with 11.6 ml native enzyme	10.702	9.749	-0.953	-8.905	102.1
Native	Fabric treated with 11.6 m native enzyme	11.119	10.100	-1.019	-9.164	101.7
Native	Fabric treated with 11.6 m native enzyme	10.911	9.886	-1.025	-9.394	104.4
CLEA	Fabric treated with 1000 mg CLEA	11.128	10.669	-0.459	-4.125	105.3
CLEA	Fabric treated with 1000 mg CLEA	10.651	10.216	-0.435	-4.084	110.5
CLEA	Fabric treated with 1000 mg CLEA	10.994	10.539	-0.455	-4.139	107.3

4.3.3 Screening of CLEA Dosage Effect on Biopolishing

Biopolishing experiments were repeated via using different amounts of CLEAs on 10 grams of cotton and viscose fabrics in order to analyze dose effect in enzymatic treatments. Table 9 reveals that, for cotton biopolishing, we can obtain highest pilling results while using 1mg CLEA of Cellusoft 37500 on 10 grams of Fabric. On the other hand, for biopolishing of viscose fabric, minimally 1000mg of Cellusoft CR CLEA must be used for highest pilling notes (Table 10).

Table 9: Pilling results for cotton fabrics treated with different amounts of CLEA-Cellusoft 37500 L.

Sample No	Enzyme Dose	Pilling Note
NO: 1	1mg	4-5
NO: 2	1mg	4-5
NO: 3	6.25 mg	4-5
NO: 4	6.25 mg	4-5
NO: 5	12.5 mg	4-5
NO: 6	12.5 mg	4-5
NO: 7	25 mg	4-5
NO: 8	25 mg	4-5
NO: 9	50 mg	4-5
NO: 10	50 mg	4-5
NO: 11	100 mg	4-5
NO: 12	100 mg	4-5
NO: 13	250 mg	4-5
NO: 14	250 mg	4-5
NO: 15	500 mg	4-5
NO: 16	500 mg	4-5
NO: 17	1000 mg	4-5
NO: 18	1000 mg	4-5

Table 10: Pilling results for viscose fabrics treated with different amounts of CLEA-Cellusoft CR.

Sample No	Enzyme Dose	Pilling Note
NO: 1	1mg	1-2
NO: 2	1mg	1-2
NO: 3	6.25 mg	1-2
NO: 4	6.25 mg	1-2
NO: 5	12.5 mg	1-2
NO: 6	12.5 mg	1-2
NO: 7	25 mg	1-2
NO: 8	25 mg	1-2
NO: 9	50 mg	1-2
NO: 10	50 mg	1-2
NO: 11	100 mg	2-3
NO: 12	100 mg	1-2
NO: 13	250 mg	2-3
NO: 14	250 mg	2-3
NO: 15	500 mg	2-3
NO: 16	500 mg	3-4
NO: 17	1000 mg	4-5
NO: 18	1000 mg	3-4

In the preliminary studies, CLEA-Cellusoft 37500 used for viscose, and CLEA-Cellusoft CR was also used in varying amounts for cotton fabric biopolishing however we could not obtain any promising results (Table 11) therefore we cancelled further studies for these cases.

Table 11: Pilling results for cotton fabrics treated with CLEA Cellusoft CR and pilling results for viscose fabrics treated with CLEA Cellusoft 37500 L.

Sample	Enzyme Dose (mg)	Pilling Note	Fabric
Control	0	1-2	Viscose
Control	0	1-2	Viscose
Control	0	1-2	Viscose
CLEA – Cellusoft 37500 L	100	1-2	Viscose
CLEA – Cellusoft 37500 L	250	1-2	Viscose
CLEA – Cellusoft 37500 L	500	1-2	Viscose
Control	0	1-2	Cotton
Control	0	1-2	Cotton
Control	0	1-2	Cotton
CLEA Cellusoft CR	100	1-2	Cotton
CLEA Cellusoft CR	250	2-3	Cotton
CLEA Cellusoft CR	500	2-3	Cotton

Chapter 5

5. DISCUSSION

5.1 Enzyme Characterization

5.1.1 Effect of Temperature on Enzyme Activity

Temperature activity profile of both commercial native enzymes show similar patterns so that catalytic activity follows a rising trend up to 55 ^{o}C. 55 ^{o}C is the optimum temperature of use for both enzymes. Subsequently, activity of both formulations slightly decreases up to 65 ^{o}C. After 65 ^{o}C, activity dramatically decreases so we can conclude that structural conformation of the proteins starts to disintegrate. This denaturation is an irreversible process. As a consequence, both enzymes seem to function sufficiently over a broad temperature range (45-65 ^{o}C). Moreover, all fabric trials were held at 55 ^{o}C, and there seems to be two sufficient ways to stop the enzymatic reaction on fabrics. One is to remove the fabric swatches and wash them with cold water. Another solution is to increase the temperature up to 75 ^{o}C and wait for 20 minutes. We preferred to use the first choice not to damage fibrillous structure of the fabric because with the increase of temperature, crystalline regions of both cotton and viscose fabrics would be deformed.

5.1.2 Effect of pH on Enzyme Activity

Optimum pH for native Cellusoft 37500 L was found to be pH 5 and Figure 4 indicates that Cellusoft 37500 L seems to function over a narrow pH range (pH 4-5). Activity of the native Cellusoft 37500 L follows a rising trend up to pH 5 and then it drops quickly. The enzyme formulation is most likely to be composed of EGI-enriched enzyme complex. As a consequence, cotton fabric trials would be held at pH 5.

Moreover, in order to stop the enzymatic reaction on fabrics, an additive (sodium carbonate) would be used in order to increase the pH up to 10.

Optimum pH for native Cellusoft CR was found to be pH 6, and Figure 5 indicates that Cellusoft CR activity profile ranges from pH 5 to pH 7. The enzyme formulation is most likely to be composed of EG-enriched enzyme complex since the enzyme still retains 60% of its activity at pH 4 and pH 5.

Intact form of endoglucanase enzyme is expected to function at pH 5 optimally; therefore, it is obvious that endoglucanase (EG) that involves in the formulation of Cellusoft CR is genetically modified. In order to change the pH range of the enzyme, the amino acid content of the EG has undergone a particular change by protein engineering methods. As a consequence, all the viscose fabric trials were held at pH 6.

5.2 CLEA Preparation

Cross-linked enzyme aggregates were prepared from commercial cellulase formulations according to two different protocols. Since CLEA synthesis from each enzyme is an individual case, routinely used protocol was altered for each case, respectively. As determined from enzyme characterization studies, DNS activity tests were performed at optimum temperature and pH values for each enzyme individually. Activity screenings were performed at pH 5 and 55 ^{o}C for Cellusoft 37500 L and at pH 6 and 55 ^{o}C for Cellusoft CR.

5.2.1 CLEA Preparation from Cellusoft 37500 L

Dielectric constant of a solution affects the solubility of protein. Solvent molecules that have large dielectric constants tend to favor protein-solvent interactions. On the other hand, acetone –as an organic solvent with small dielectric constant- tends to favor protein-protein interactions more than protein-solvent interactions. Dielectric properties also change with the temperature so that as the temperature decreases, solubility of the protein also decreases. Therefore, the most plausible way to increase the protein-protein interactions for effective precipitation is to perform the process at low temperatures. According to acetone precipitation protocols, protein precipitation is

optimally performed at -20 ^{o}C; on the other hand, -80 ^{o}C would decrease the precipitation time. However it would be hard to handle aggregates at this temperature since they tend to stick to the bottom of the beaker at this temperature. In general, cross-linking experiments are held in the room temperature, because low temperatures would decrease the reaction rate of cross-linking. Catalytic activity of CLEAs prepared at -80 ^{o}C was similar to that of the CLEAs prepared at -20 ^{o}C, however, at -80 ^{o}C; end product weight was too low. In addition, CLEAs that were produced at -80 ^{o}C had light brown color and the particles have retained this color therefore we may claim that the enzymes were perfectly isolated from the stabilizer. Another explanation to this is that tris solution completely quenched glutaraldehyde.

I have further examined diffusional limits of CMC while working with CLEAs having different particle size. The results indicated unequal distribution of CLEA activity on CMC particles. We can conclude that; diffusion of CMC substrate into CLEA particles influences the colorimetric activity test results. We encountered same problem in comparison of native enzyme activity with CLEA activity. On the other hand, in the large scale experiments, enzyme-substrate interaction would be different because a fabric swatch is a completely different substrate which is considerably larger than any enzyme form. Also in this case, CLEA effectiveness is intensively correlated with mechanical effect. In summary, in fabric tests, determination of CLEA amount based on its catalytic activity according to colorimetric results would be scientifically incorrect. We decided the CLEA amount based on the end product weight of the synthesized CLEA.

Cellusoft 37500 L CLEA activity profile exhibited a peak with 100 mM glutaraldehyde. However, contrary to expectations, CLEA activity did not show a decreasing trend with the increase in glutaraldehyde concentration. Moreover, in the process of synthesis, after centrifugation I obtained light brown-colored aggregates, however they became darker in 24 hours. Combining those two observations, we may conclude that after the centrifugation, there might be a very little amount of glutaraldehyde that is not quenched by tris solution. These glutaraldehyde molecules would continue to cross-link the enzymes. Moreover, centrifugation increases the proximity of enzymes and that would also help the crosslinking in the enzyme pellet. However the effectiveness of resulting end product is not influenced enormously. The most plausible reason is that the content of lysine residues on the enzyme surface would

be too low and those lysine residues would be placed far from the active site so that extra cross-linking occurs most possibly far from the active site of the catalytic domain.

I further have examined the effect of optimum concentration of glutaraldehyde on pellet and supernatant at room temperature. Aggregates exposed to optimum glutaraldehyde concentration retained 93% of their activity; however there was no significant difference between supernatant activities. We may conclude that, subsequent cross-linking after precipitation did not have adverse effect on catalytic activity of Cellusoft 37500 L which means, covalent attachments involved functional groups far from active site of the catalytic domain.

Before fabric tests, I examined the distribution of Cellusoft 37500 L CLEAs in sodium acetate in 1.5 ml Eppendorf tubes. After vortex, the CLEA particles subsided suddenly. After a month, I observed gel formation on the bottom of the tube due to the presence of small amount of enzyme stabilizer. Therefore, we consider that an unknown additive in the enzyme formulation would somehow interact with acetone and involve in the end product. In summary, distribution of CLEA particles in the 1.5 ml Eppendorf tube at the first moment is sufficient for application on fabric.

5.2.2 CLEA Preparation from Cellusoft CR

At first, I followed the same protocol for CLEA synthesis that was used in the case of Cellusoft 37500 L. However, with this protocol, end product weight was ~1 gram of CLEA from 100 mL of Cellusoft CR. Therefore, I specifically changed the precipitation part of the protocol. Gradient precipitation was performed with acetone in 50 ml Eppendorf tubes. Unlike common precipitation procedures, acetone was added on the enzyme solution. By that way, we obtained the CLEA products with an acceptable protein amount. In general, as I performed in Cellusoft 37500 L precipitation, enzyme solution is added drop by drop on a huge volume of acetone. In that case, each enzyme drop tends to discourage the precipitation of its interior region. Concentration of the enzyme stabilizer (i.e proxel) in the formulation of Cellusoft CR would be much more than that of Cellusoft 37500 L and this may render the problematic situation more likely to happen in the case of Cellusoft CR. Consequently, drop-wise addition of Cellusoft CR on acetone resulted in nearly a complete failure of precipitation. On the other hand,

when acetone was added on the same amount of protein, an equal protein distribution in acetone was observed. CLEA particles were relatively big-sized; therefore, the gradient precipitation process was performed repeatedly (3 times in total).

After centrifugation, light brown-colored aggregates were obtained. Aggregates retained their color which means; gradient precipitation efficiently removed enzyme stabilizer solution so that there was more space between the aggregates of Cellusoft CR than that of Cellusoft 37500 L. Additionally, there was no need to ground the CLEA particles of Cellusoft CR since washing step was properly performed.

CLEA synthesis from Cellusoft CR has been performed at room temperature because within this modified protocol, there is no chance to stabilize the temperature at -20 ^{o}C. Cellusoft CR CLEA activity profile at room temperature exhibited a peak with 100 mM glutaraldehyde. As we expected, CLEA activity showed a slightly decreasing trend with the increase in glutaraldehyde concentration.

I further have examined the effect of optimum concentration of glutaraldehyde on pellet and supernatant at room temperature. Aggregates exposed to optimum glutaraldehyde concentration retained 98.5% of their activity; however there was no significant difference between supernatant activities. Again, we may conclude that, subsequent cross-linking after precipitation did not have adverse effect on catalytic activity of Cellusoft CR aggregates which means, covalent attachments involved functional groups far from active site of the catalytic domain.

Before fabric tests, I examined the distribution of Cellusoft CR CLEAs in potassium phosphate buffer in 1.5 ml Eppendorf tubes. After vortex, I observed the CLEA particles subsided suddenly. After a month, I observed exactly the same distribution and subsidence of CLEA particles and there was no gel formation on the bottom of the tube. We can conclude that the removal of highly concentrated enzyme stabilizer was done perfectly. It would be one of the most significant impacts of the gradient precipitation on the enzyme efficiency in fabric tests. We consider that Cellusoft CR CLEA is the most efficient product discovered for biopolishing of viscose fabric.

5.3 Fabric Tests

Removal of pills gives aesthetic appearance to fabrics. Enzymatic application for biopolishing of cotton is widely used in the industrial processes. On the other hand, this process is not convenient for the viscose fabrics due to loss of tensile strength.

Effects of native and cross-linked Cellusoft 37500 L on cotton and that of Cellusoft CR on viscose biopolishing were examined, pilling and bursting strength test results were evaluated. Lastly, different amounts of CLEAs ranging from 1 to 1000 mg were applied on fabrics in order to analyze effect of enzyme dose on pilling and fabric strength values. CLEA dose effect on viscose fabric was analyzed. In the preliminary studies, CLEA-Cellusoft 37500 was used for viscose, and CLEA-Cellusoft CR for cotton was also used in varying amounts for cotton fabric biopolishing however we could not obtain any promising results therefore we cancelled further studies for these cases.

5.3.1 Cotton Fabric Test Results

Cotton fabric, consisting of two-thirds crystalline and one-third amorphous cellulose, has more tensile strength than viscose. Amorphous regions in the outer surface of cotton are easily degraded. Native enzyme also targets the crystalline cellulose inside core regions of the fiber and that results in extra decrease in the fabric tensile strength. However, catalytic action of the CLEA particles is limited on the fabric surface so that these particles do not exhibit function inside core regions.

Application of CLEA on cotton fabric caused ~4.4% weight loss. On the other hand, native enzyme application resulted in loss of ~8.2%. Moreover, native formulation of Cellusoft 37500 decreased bursting strength values more than CLEA did. The compared results indicate that I have attained my goal in terms of every aspect of biopolishing process such as fabric strength, weight loss and pilling notes.

5.3.2 Viscose Fabric Test Results

Viscose fabric is more prone to pilling than cotton due to its structure and fiber properties. In viscose fabric, amorphous cellulose mostly takes place in the core region; on the other hand, outer region is composed of crystalline cellulose that is homogenously distributed throughout the fiber. Amorphous cellulose is more prone to attack by cellulases when compared to crystalline cellulose. On the other hand, crystalline cellulose, which provides tensile strength to the fiber, is more rigid; and loss of the tensile strength is a result of cellulase action on the highly ordered crystalline structure of the fiber.

Effects of native and cross-linked Cellusoft CR on viscose biopolishing were examined, pilling and bursting strength test results were evaluated. According to Table 6, both enzyme forms obtained best pilling notes. Application of CLEA on 15 g viscose fabrics caused ~3.3% weight loss; on the other hand native enzyme application resulted in loss of ~12%. Moreover, native formulation of Cellusoft CR decreased bursting strength values more than CLEA did.

Degradation of amorphous regions results in easy access of cellulase enzymes to the crystalline regions in the outer region. Due to cellulase action on the crystalline regions, tensile strength of the fabric decreases. Native enzyme formulation also targets amorphous cellulose inside core regions of the fiber, resulting in an extra decrease in the fabric tensile strength. As catalytic action of the big-sized particles is limited on the fabric surface, CLEA do not damage fibrillous structure of fabric.

I performed biopolishing experiments with another batch of Cellusoft CR enzyme that was kept at room temperature for 6 months. Incubation of the enzyme at room temperature for 6 months had led to destabilization of enzymes. Therefore both native and CLEA forms were less aggressive, and it directly reflected to the results. Pilling notes were reduced by 1 point.

Shelf lives of both commercial cellulase formulations are 3 months at 4 ^{o}C. However, most of the time, these enzymes are kept more than 6 months at room temperature and that leads to irreversible enzyme denaturation. Pilling results in Table 7 indicate that CLEAs produced from Cellusoft CR that was kept at room conditions for 6 months have exhibited an acceptable performance on biopolishing of viscose fabrics. In conclusion, CLEA technology rendered denatured enzymes have a potential to exhibit

an acceptable performance namely; these enzymes would still have an acceptable market value when compared with its intact form.

5.3.3 Screening of CLEA Dosage Effect on Biopolishing

Screening results reveal that, for cotton biopolishing, one can obtain highest pilling results using 1 mg CLEA of Cellusoft 37500 on 10 grams of Fabric. On the other hand, for biopolishing of viscose fabric, minimally 1000mg CLEA of Cellusoft CR has to be used for the highest pilling notes.

In order to obtain the minimum dose of Cellusoft 37500 L CLEA for cotton biopolishing, smaller amounts than 1 mg have to be used. In order to obtain 0.5 mg of CLEA, a suspension of 10% CLEA (w/v) in 1 ml of potassium phosphate buffer has been prepared. Subsequently, activity screenings of 50 µl of the suspensions were performed repeatedly. The results fluctuated greatly indicating that we could not obtain equal amounts of CLEAs in the suspensions due to heterogeneous distribution of CLEAs in sodium acetate buffer. Therefore we cancelled further studies.

Chapter 6

6. CONCLUSION

There are many possible ways to alleviate over-aggressive catalytic activity of commercial enzymes inside the fabric. Genetic modification approaches have been performed to obtain less aggressive biocatalysts thereby altering binding affinity of the enzymes. CBD-truncated cellulase formulations are widely used in textile industry. However, even with such advancements in protein engineering, there is still no commercial enzyme formulation suitable for biopolishing of viscose fabrics. On the other hand, existing commercial enzyme formulations still cause adverse impact on tensile strength and fabric weight of cotton fabric. With the use of CLEA technology, I alleviated the problem of pilling formation, tensile strength loss and weight loss in both cotton and viscose fabrics. In viscose biopolishing experiments, I obtained highest pilling values with acceptable losses of fabric tensile strength and fabric weight.

CLEA methodology combines purification and immobilization techniques. I used acetone for precipitation and glutaraldehyde for cross-linking. Both are cheap, commonly used and sufficiently effective reagents. In general, CLEA synthesis is performed at room temperature however; enzymes cannot be completely precipitated in this condition. Although -20 ^{o}C seems to be very low temperature when compared with the temperatures in routinely used protocols of all CLEA approaches, I performed cross-linking of Cellusoft 37500 L at this temperature due to the high productivity at this temperature. Even though carrying out the precipitation stage at - 20 ^{o}C decreases the reaction rate of cross-linking action, and makes the industrial scaling up of the process infeasible, elevated precipitation efficiency may compensate for these drawbacks.

CLEAs prepared from Cellusoft 37500 L do not distribute homogenously in aqueous media. In order to solve this problem, gradient precipitation would be integrated into the CLEA preparation protocol of Cellusoft 37500 L. With the use of that technique, stabilizer would be completely removed from the CLEA particles, resulting in prevention of gel formation. More importantly, the factors that influence

CLEA particle size including precipitant type, enzyme concentration, pH of the cross-linker and enzyme: cross-linker ratio would be investigated in detail.

In the cross-linking experiments, glutaraldehyde is the first reagent of choice. Glutaraldehyde exists in the monomeric form at lower concentrations. On the other hand, in high concentrations, it exists in polymerized form and leads to immobilization by forming Schiff's base bonds. In order to increase the catalytic activity of the CLEA, a bisepoxy compound, glycerol diglycidyl ether (GDE) would be used as cross-linker. GDE is a cross-linker that forms stable bonds not only with the amine groups of lysine but also with the sulfur- and oxygen- containing residues of cysteine, tyrosine, aspartate or glutamate.

In industrial applications, in the process of cotton biopolishing, native enzyme dosage used in cotton biopolishing is 1% of the fabric weight. On the other hand, in the CLEA applications, this dosage was found to be 0.01% of fabric weight. We expect that will result in enormous economic benefits thereby reducing the catalyst amount to use. It is also known that in industrial processes, mechanical effect, which is the most significant factor that has an impact in CLEA effectiveness, is applied on fabric in much higher levels. Therefore CLEA dosage would decrease to lower percentage levels in industrial applications.

Removal of pills from viscose fabric was performed with huge amounts of CLEA synthesized from Cellusoft CR. The costs of CLEA synthesis and enzyme applications for viscose biopolishing are too high. One solution to reduce the production costs would be the innovation of preferential binding applications of particular cellulase domains onto a relatively cheap carrier. For example, cellulose binding domains have also antipilling action on fabric surface and there exist such subfamilies of CBDs consisting of ~40 amino acids including only one lysine residue. A ten amino acid long loop consisting of lysine and glycine residues can be introduced to CBD in order to create a critical hotspot for glutaraldehyde cross-linking. Then, immobilization of CBD onto a carrier would be performed and the resulting catalysts can be applied on viscose fabrics.

In this work, I performed the immobilization of two novel commercial cellulase enzyme formulations lacking functional CBD and synthesized cross-linked enzyme aggregates from these enzymes. Further, I used the resulting products for biopolishing

of cotton and viscose fabrics. By doing so, I combined the advantages of both gene manipulation and covalent modification technologies into a single product. The cross-linked aggregates of commercial enzymes were found to reduce losses of tensile strength and weight in biopolishing of both cotton and viscose fabrics. Additionally, this work is the first attempt to introduce a sufficient method for biopolishing of viscose fabrics. Therefore I expect the CLEAs that we produced would have a great impact in both cotton and viscose applications in textile industry.

APPENDICES

APPENDIX A: EQUIPMENTS

Equipment	Brand Name
Autoclave	Certoclav, Table Top Autoclave CV-EL-12L
	Hirayama, Hiclave HV-110, JAPAN
Balance	Sartorius, BP211D, GERMANY
	Sartorius, BP221S, GERMANY
	Sartorius, BP610, GERMANY
	Schimadzu, Libror EB-3200 HU, JAPAN
Burette	Borucam, TURKEY
Centrifuge	Eppendorf, 5415C, GERMANY
	Eppendorf, 5415D, GERMANY
	Eppendorf, 5415R, GERMANY
	Hitachi, Sorvall Discovery 100 SE, USA
	Hitachi, Sorvall RC5C Plus, USA
	Kendro Lab. Prod., Heraeus Multifuge 3L, GERMANY
Distilled Water	Millipore, Elix-S, FRANCE
	Millipore, MilliQ Academic, FRANCE
Eppendorf Tubes(1.5-2 ml)	Eppendorf
Falcon tubes(14-50 ml)	TPP
Freezer	-70 ^{0}C, Kendro Lab. Prod., Heraeus Hfu486 Basic, GERMANY
Glasswares	Schott Duran, GERMANY
Gyrowash	James Heal, ENGLAND
Ice Machine	Scotsman Inc., AF20, USA
Lyophilizer	
Magnetic Stirrer	ARE Heating Magnetic Stirrer, VELP

	Scientifica, ITALY
	Microstirrer, VELP Scientifica, ITALY
Micropipette	Eppendorf
Microscope	Olympos
Microtiter Plates (96-well)	TPP
Microtiterplate Reader	Model 680, BioRad
Multitube rotator	Labline
pH-meter	FisherBrand
Pipetteman	Hirschman Laborgate
Refrigerator (4 ^{o}C)	Bosch, TURKEY
Shaker	Forma Scientific, Orbital Shaker 4520, USA
	C25HC Incubator shaker New Brunswick Scientific, USA
	GFL, Shaker 3011, USA
	New Brunswick Sci., Innova 4330, USA
Tips	TPP
Thermal Heater	Bioblock Scientific
Thermomixer	Eppendorf

APPENDIX B: MATERIALS

Chemicals

Chemical	Supplier	Catalog Number
Acetic acid	Riedel de Haen	27225
Ammonium sulphate	Riedel de Haen	11225
Carboxymethyl cellulose	Acıselsan	ASEL SY100
Dinitrosalicylic acid	Fluka	42260
Dipotassium hydrogenphosphate	Riedel de Haen	04248
Ethanol	Riedel de Haen	32221
Hydrogen Cloride	Merck	100314
Liquid nitrogen	Karbogaz	-
Methanol	Riedel de Haen	24229
Na-K tartarate tetrahydrate	Riedel de Haen	25508
NaOH	Merck	106462
Potassium dihydrogenphosphate	Riedel de Haen	4243
Sodium acetate three hydrate	Riedel de Haen	32318
Tris	J. T. Baker	8079

Enzymes

Enzyme	Supplier	Origin
Cellusoft 37500 L	Novozymes	T. *reesei*
Cellusoft CR	Novozymes	T. *reesei*

Buffers

a. 0.05 M NaOAc buffer, pH 5
b. 0.1 M Potassium Phosphate buffer, pH 6
c. 0.1 M Potassium Phosphate buffer, pH 7.3
d. 1 M Tris pH 8

Solutions

a. 1% CMC in 0.05 M NaOAc buffer, pH 5
b. 1% CMC in 0.05 M KH_2PO_4 buffer, pH 6
c. DNS reagent (1% DNS, 1.6% NaOH, 30% Rochelle's Salt in ddH_2O (all w/v))
d. 1% DNS solution
e. 30% Rochelle's salt

f. 25% glutaraldehyde with 1% (v/v) phosphoric acid (pH is adjusted to 7.3 with NaOH)

Fabrics

Cotton

Fabric: 175 g/m^2, plain cotton fabric, 13 weft/cm- 14 warp/cm

Before and after enzymatic treatments, all cotton fabric swatches were placed in standard atmosphere for at least 12 hours.

Viscose

Fabric: 100% viscose supreme (single Jersey) knitted fabric

Fabric density: 137 g/m^2

Before and after enzymatic treatments, all viscose fabric swatches were placed in standard atmosphere for at least 12 hours.

APPENDIX C: Cellusoft 37500 L SAFETY DATA SHEET

SAFETY DATA SHEET

According to Regulation (EC) No 1907/2006

Revision date: *01/06/2011* **Version No:***1*

1. IDENTIFICATION OF THE SUBSTANCE/PREPARATION AND OF THE COMPANY/UNDERTAKING

Product name	Cellusoft® Prime 37500 L
Chemical Name	Enzyme preparation
Declared activity	Cellulase
Use of the substance/preparation	Novozymes' enzyme preparations are biocatalysts used in a variety of industrial processes within food manufacturing
Company/Undertaking Identification	Novozymes A/S Krogshoejvej 36 2880 Bagsvaerd Denmark Tel.: +45 44460000 Fax.: +45 44469999 E-mail: SafetyDataSheet@novozymes.com www.novozymes.com
Emergency Telephone Number	+45 44460000 (24/7)

2. HAZARDS IDENTIFICATION

Classification and labelling according to Directive 1999/45/EEC

Labelling Xn - Harmful

Classification

Symbol(s) Xn - Harmful
R42/43 - May cause sensitization by inhalation and skin contact

Physico-chemical properties The data available do not support any physical or chemical hazard

Environmental Effects The data available do not support any environmental hazard.

Human health effects Repeated inhalation of enzyme dust or aerosols resulting from improper handling may induce sensitization and may cause allergic type 1 reactions in sensitized individuals
Ingestion may cause gastrointestinal irritation, nausea, vomiting and diarrhea

Effects of overexposure See Section 4

See Section 11 for additional Toxicological information

3. COMPOSITION/INFORMATION ON INGREDIENTS

Hazardous Components

Chemical Name	CAS-No	EC No.	IUB No.	Weight %	EU Classification (67/548/EEC)
Cellulase	9012-54-8	232-734-4	3.2.1.4	30 - 50	Xn;R42
Proxel	2634-33-5	EEC No. 220-120-9	-	<5	Xn;R22 Xi;R38-41 R43 N;R50

**: Enzymes are defined as enzyme concentrate (dry matter basis)
For the full text of the R phrases mentioned in this Section, see Section 16

4. FIRST AID MEASURES

In case of unintended overexposure, the following measures apply

Inhalation

Effects May cause allergic respiratory reaction
Symptoms Shortness of breath, wheezing and coughing
The effect of inhalation may be delayed
First Aid Remove person to fresh air. If signs/symptoms continue, get medical attention
Show this safety data sheet to the doctor in attendance

Skin contact

Effects Unknown
Symptoms Unknown

First Aid	Remove and wash contaminated clothing before re-use. Wash off immediately with plenty of water. If symptoms persist, call a doctor. Show this safety data sheet to the doctor in attendance.

Eye contact

Effects	Unknown
Symptoms	Unknown
First Aid	Hold eye open and rinse slowly and gently with water for 15-20 min. Remove contact lenses, if present, after the first five minutes, then continue rinsing eye. If symptoms persist, call a doctor. Show this safety data sheet to the doctor in attendance

Ingestion

Effects	Ingestion may cause gastrointestinal irritation, nausea, vomiting and diarrhea.
Symptoms	Irritation
First Aid	Rinse mouth with water and drink plenty of water. If symptoms persist, call a doctor. Show this safety data sheet to the doctor in attendance.

5. FIRE-FIGHTING MEASURES

Suitable extinguishing media	Use water spray, alcohol-resistant foam, dry chemical or carbon dioxide
Unsuitable Extinguishing Media	none
Hazardous combustion products	none
Specific hazards arising from the chemical	May cause allergic respiratory reaction
Protective equipment and precautions for firefighters	Self-contained breathing apparatus

6. ACCIDENTAL RELEASE MEASURES

Personal precautions	For personal protection see section 8
Environmental precautions	No special environmental precautions required
Methods for cleaning up	Avoid formation of dust and aerosols Spilled preparation should be removed immediately to avoid formation of dust from dried preparation. Take up by mechanical means preferably by a vacuum cleaner equipped with a high efficiency filter. Flush remainder carefully with plenty of water. Avoid splashing and high pressure washing (avoid formation of aerosols). Ensure sufficient ventilation. Wash contaminated clothing.

For personal protection see section 8

7. HANDLING AND STORAGE

Handling	Avoid formation of dust and aerosols Ensure adequate ventilation

7. HANDLING AND STORAGE

Storage Keep tightly closed in a dry and cool place.Temperature 0-10°C (32°F-50°F)

8. EXPOSURE CONTROLS / PERSONAL PROTECTION

Occupational exposure controls

Ensure adequate ventilation, especially in confined areas

Personal Protective Equipment

Respiratory Protection	In case of insufficient ventilation wear suitable respiratory equipment
Eye protection	Safety glasses with side-shields
Skin Protection	Long sleeved clothing
Hand Protection	Protective gloves
General hygiene considerations	Handle in accordance with good industrial hygiene and safety practices
Environmental exposure controls	Local authorities should be advised if significant spillages cannot be contained

9. PHYSICAL AND CHEMICAL PROPERTIES

Physical state	liquid
Color	brown
Odor	Slight fermentation odor

10. STABILITY AND REACTIVITY

Chemical stability	Stable under recommended storage conditions
Conditions to Avoid	none
Materials to avoid	none
Hazardous Decomposition Products	none
Possibility of hazardous reactions	none

11. TOXICOLOGICAL INFORMATION

Acute toxicity

Oral rat LD50>2g/kg

Chronic toxicity

According to experience not expected

12. ECOLOGICAL INFORMATION

Ecotoxicity

LC50(fish) > 100mg/L, EC50(daphnia) > 100mg/L, IC50(algae) > 100mg/L

Environmental Effects	The data available do not support any environmental hazard.
Persistence/Degradability	The organic components of the product are biodegradable
Bioaccumulative potential	According to experience not expected
Other adverse effects	No information available

13. DISPOSAL CONSIDERATIONS

Waste Disposal Method	Dispose of in accordance with local regulations
Contaminated Packaging	Dispose of wastes in an approved waste disposal facility
Other information	Waste codes should be assigned by the user based on the application for which the product was used

14. TRANSPORT INFORMATION

Transport Regulations	No dangerous goods according to transport regulations No special precautions required

15. REGULATORY INFORMATION

Classification and labelling according to Directive 1999/45/EEC

Labelling	Xn - Harmful
Classification	
Symbol(s)	Xn - Harmful
	R42/43 - May cause sensitization by inhalation and skin contact
S-phrase(s)	S23 - Do not breathe gas/fumes/vapour/spray S24 - Avoid contact with skin S36/37 - Wear suitable protective clothing and gloves
WGK Classification	1

16. OTHER INFORMATION

Text of R phrases mentioned in Section 2&3
No information available

Disclaimer
The information provided on this SDS is correct to the best of our knowledge, information and belief at the date of its publication. The information given is designed only as a guide for safe handling, use, processing, storage, transportation, disposal and release and is not to be considered as a warranty or quality specification. The information relates only to the specific material designated and may not be valid for such material used in combination with any other material or in any process, unless specified in the text. Furthermore, as the conditions of use are beyond the control of Novozymes, it is the responsibility of the customer to determine the conditions of safe use of these products.

Training advice Details on the safe handling of this product can be found in the "Handling enzymes" on www.novozymes.com

End of Safety Data Sheet

1 / EU / English / 01/06/2011

APPENDIX D: Cellusoft CR SAFETY DATA SHEET

SAFETY DATA SHEET
According to Regulation (EC) No 453/2010

Cellusoft® CR Conc

Revision date: 01/16/2012 **Version No:** 2

1. IDENTIFICATION OF THE SUBSTANCE/MIXTURE AND OF THE COMPANY/UNDERTAKING

Product name	Cellusoft® CR Conc
Chemical Name	Enzyme preparation
Declared activity	Cellulase
Relevant identified uses of the substance or mixture and uses advised against	Novozymes' enzyme preparations are biocatalysts used in a variety of industrial processes and in certain consumer products. .
Details of the supplier of the safety data sheet	Novozymes A/S Krogshoejvej 36 2880 Bagsvaerd Denmark Tel.: +45 44460000 Fax.: +45 44469999 E-mail: SafetyDataSheet@novozymes.com www.novozymes.com
Emergency Telephone Number	+45 44460000 (24/7)

2. HAZARDS IDENTIFICATION

Classification of the substance or mixture

R-phrase(s)	R42/43 - May cause sensitization by inhalation and skin contact
Symbol(s)	Xn - Harmful
Physico-chemical properties	The data available do not support any physical or chemical hazard

Environmental Effects	The data available do not support any environmental hazard.
Human health effects	Repeated inhalation of enzyme dust or aerosols resulting from improper handling may induce sensitization and may cause allergic type 1 reactions in sensitized individuals Mild skin irritation May cause sensitization by skin contact Mild eye irritation
Effects of overexposure	See Section 4

Label elements

Symbol(s)	Xn - Harmful
R-phrase(s)	R42/43 - May cause sensitization by inhalation and skin contact
S-phrase(s)	S23 - Do not breathe gas/fumes/vapour/spray S24 - Avoid contact with skin S36/37 - Wear suitable protective clothing and gloves
Other hazards	The mixture does not meet the criteria for PBT or vPvB. See Section 11 and 12 for additional Toxicological information

3. COMPOSITION/INFORMATION ON INGREDIENTS

Hazardous Components

Chemical Name	CAS-No	EC No.	IUB No.	REACH Registration No.*
Cellulase	9012-54-8	232-734-4	3.2.1.4	-
Proxel	2634-33-5	220-120-9	-	-

*: REACH registration No. is given when the component is registered by Novozymes A/S as importer or manufacturer

Hazardous Components

Chemical Name	Weight % **	EU Classification (67/548/EEC)	CLP Classification (No 1272/2008)
Cellulase	5 - 10	R42	Resp. Sens. 1;H334

Proxel	<5	Xn;R22 Xi;R38-41 R43 N;R50	Acute Tox. 4; H302 Skin Irrit. 2; H315 Eye Dam. 1; H318 Skin Sens. 1; H317 Aquatic Acute 1; H400

Proxel = 20% Benz-iso-thiazoline-3-one, CAS-No 2634-33-5

**: Enzymes are defined as enzyme concentrate (dry matter basis)

Active enzyme protein (aep): < 5%

For the full text of the R/H phrases mentioned in this Section, see Section 16

4. FIRST AID MEASURES

Description of first aid measures, most important symptoms and effects, both acute and delayed and indication of any immediate medical attention and special treatment needed

Inhalation

Effects May cause allergic respiratory reaction
Symptoms Shortness of breath, wheezing and coughing
The effect of inhalation may be delayed
First Aid Remove person to fresh air. If signs/symptoms continue, get medical attention
Show this safety data sheet to the doctor in attendance

Skin contact

Effects May cause slight irritation. May cause an allergic skin reaction.
Symptoms Slight irritation. Allergic reactions.
First Aid Remove and wash contaminated clothing before re-use. Wash off immediately with plenty of water. If symptoms persist, call a doctor. Show this safety data sheet to the doctor in attendance.

Eye contact

Effects May cause slight irritation.
Symptoms Slight irritation
First Aid Hold eye open and rinse slowly and gently with water for 15-20 min. Remove contact lenses, if present, after the first five minutes, then continue rinsing eye. If symptoms persist, call a doctor. Show this safety data sheet to the doctor in attendance

Ingestion

Effects Ingestion may cause gastrointestinal irritation.
Symptoms Irritation
First Aid Rinse mouth with water and drink plenty of water. If symptoms persist, call a doctor. Show this safety data sheet to the doctor in attendance.

5. FIREFIGHTING MEASURES

Extinguishing media

Suitable extinguishing media	Use water spray, alcohol-resistant foam, dry chemical or carbon dioxide
Unsuitable Extinguishing Media	None
Hazardous combustion products	None
Specific hazards arising from the substance or mixture	May cause allergic respiratory reaction
Advice for firefighters	Self-contained breathing apparatus

6. ACCIDENTAL RELEASE MEASURES

Personal precautions, protective equipment and emergency procedures	For personal protection see section 8
Environmental precautions	No special environmental precautions required
Methods and material for containment and cleaning up	Avoid formation of dust and aerosols Spilled preparation should be removed immediately to avoid formation of dust from dried preparation. Take up by mechanical means preferably by a vacuum cleaner equipped with a high efficiency filter. Flush remainder carefully with plenty of water. Avoid splashing and high pressure washing (avoid formation of aerosols). Ensure sufficient ventilation. Wash contaminated clothing.
Reference to other sections	For personal protection see section 8

7. HANDLING AND STORAGE

Precautions for safe handling	Avoid formation of dust and aerosols Ensure adequate ventilation Liquid enzyme preparations are dustfree preparations. However, inappropriate handling may cause formation of dust or aerosols.
Conditions for safe storage, including any incompatabilities	Keep tightly closed in a dry and cool place.Temperature 0-10°C (32°F-50°F) In unbroken packaging - dry and protect from the sun. The product has been formulated for optimal stability. Extended storage or adverse conditions such as higher temperatures or higher humidity may lead to a higher dosage requirement.
Specific end use(s)	Handle in accordance with good industrial hygiene and safety practice

8. EXPOSURE CONTROLS / PERSONAL PROTECTION

Control parameters

Exposure controls
Ensure adequate ventilation, especially in confined areas

Personal Protective Equipment

Respiratory Protection	In case of insufficient ventilation wear an approved mask with a particle filter type P3 used according to the manufactures instruction
Eye protection	Safety glasses with side-shields
Skin Protection	Long sleeved clothing
Hand Protection	Protective gloves of e.g. nitrile rubber or neoprene (thickness > 0.3 mm) according to EN 374-3. Expected breakthrough time: > 4 hours. The recommendation is a qualified estimate based on the knowledge of the components in the mixture
General hygiene considerations	Handle in accordance with good industrial hygiene and safety practices
Environmental exposure controls	Local authorities should be advised if significant spillages cannot be contained Waste water should be discharged to sewage treatment plant

9. PHYSICAL AND CHEMICAL PROPERTIES

Information on basic physical and chemical properties

Physical state	liquid
Color	brown
Odor	Slight fermentation odor
Density (g/ml)	1.05
pH	Not relevant
Solubility	Active component is readily soluble in application-relevant solutions at all levels of concentration, temperature and pH which may occur in normal usage
Other information	No information available

10. STABILITY AND REACTIVITY

Reactivity	Not relevant
Chemical stability	Stable under recommended storage conditions
Possibility of hazardous reactions	None under normal processing
Conditions to Avoid	None
Incompatible materials	None
Hazardous Decomposition Products	None

11. TOXICOLOGICAL INFORMATION

Information on toxicological effects

Hazardous Components

Chemical Name	Acute oral toxicity	Respiratory sensitization	Genetic toxicity	Skin corrosion/irritation	Serious eye damage/eye irritation
Cellulase	LD50: > 2000 mg/kg bw (OECD TG 401, 420)	Sensitizer (Human experience)	No indication of mutagenic effects (OECD TG 471, 476)	Not irritating (OECD TG 404)	Not irritating (OECD TG 405)
Proxel	Harmful if swallowed	no data available	No mutagenic effect	Causes burns	Risk of serious damage to eyes

12. ECOLOGICAL INFORMATION

Toxicity

Chemical Name	Daphnia, acute	Algae, Acute	Acute fish toxicity =
Cellulase	EC50 (48 hours): >39.5 mg aep/l (OECD TG 202)	-	LC50 (96 hours): >39.5 mg aep/l (OECD TG 203)
Proxel	EC50 (48 hours): 2.94 mg/l (OECD TG 202)	ErC5 (72 hours): 0.11 mg/l (OECD TG 201)	2.18mg/l (OECD TG 203)

Persistence/Degradability

Chemical Name	Persistence and degradability	Partition coefficient (n-octanol/water)	Bioaccumulative potential
Cellulase	Readily biodegradable (OECD 301E/F)	LogPow: <0	Does not bioaccumulate
Proxel	Inherently biodegradable	-	Bioaccumulation is unlikely

Mobility in soil Not relevant

Results of PBT and vPvB assessment Components do not meet PBT or vPvB criteria according to REACH Annex XIII

Other adverse effects No information available

13. DISPOSAL CONSIDERATIONS

Waste treatment methods
Dispose of in accordance with local regulations
Waste water should be discharged to sewage treatment plant
Waste codes should be assigned by the user based on the application for which the product was used

14. TRANSPORT INFORMATION

Transport Regulations
No dangerous goods according to transport regulations
No special precautions required

UN number	not applicable
UN proper shipping name	not applicable
Transport hazard class(es)	not applicable
Packing group	not applicable
Environmental hazards	not applicable
Special precautions for user	not applicable
Transport in bulk according to Annex II of MARPOL 73/78 and the IBC Code	not applicable

15. REGULATORY INFORMATION

Safety, health and environmental regulations/legislation specific for the substance or mixture
The product complies with Regulation No. 689/2008 concerning the export and import of dangerous chemicals, Article 16

WGK Classification 1

No chemical safety assessment has been carried out

16. OTHER INFORMATION

Text of R/H phrases mentioned in Section 2&3
R42 - May cause sensitization by inhalation
R50 - Very toxic to aquatic organisms
R43 - May cause sensitization by skin contact
R41 - Risk of serious damage to eyes
R38 - Irritating to skin
R22 - Harmful if swallowed
R42/43 - May cause sensitization by inhalation and skin contact
H302: Harmful if swallowed.
H315: Causes skin irritation.
H317: May cause an allergic skin reaction
H318: Causes serious eye damage.
H400: Very toxic to aquatic life.

Further information	This SDS is in compliance with EU Regulation No. 453/2010
Training advice	Details on the safe handling of this product are located in the Novozymes Customer Center Document Library on www.mynovozymes.com

Disclaimer

The information provided on this SDS is correct to the best of our knowledge, information and belief at the date of its publication. The information given is designed only as a guide for safe handling, use, processing, storage, transportation, disposal and release and is not to be considered as a warranty or quality specification. The information relates only to the specific material designated and may not be valid for such material used in combination with any other material or in any process, unless specified in the text. Furthermore, as the conditions of use are beyond the control of Novozymes, it is the responsibility of the customer to determine the conditions of safe use of these products.

End of Safety Data Sheet

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