Flitsch Annemarie

Antimicrobial and bleaching systems based on cellobiose dehydrogenase

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Guebitz Georg Dr. Professor
Nyanhongo, Gibson Stephen, Dr. techn.
Institut für Umweltbiotechnologie
Technische Universität Graz

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1. Abstract

Cellobiose dehydrogenase (CDH) is a fast emerging enzyme in the field of biotechnology recognized for its multifaceted applications and flexibility in regard to its electron transfer mechanism. In this study, CDH from *Myriococcum thermophilum* (MtCDH) was investigated for its ability to generate hydrogen peroxide for bleaching and antimicrobial purposes. The substrate specificities of MtCDH were investigated on a large variety of soluble and insoluble substrates, using oxygen as an electron receptor and optimized for maximum production of hydrogen peroxide. In the second phase of the study, the ability of MtCDH to produce hydrogen peroxide from oligosaccharides contained in cotton desizing effluents was assessed and different hydrogen peroxide stabilizers were comparted. This study aimed at replacing hydrogen peroxide with an in situ source of hydrogen peroxide during the bleaching of cotton with an additional advantage of recycling the water.

The results of screening of possible substrates indicated the possibility of MtCDH to use sugars like maltose, glucose and higher carbohydrates for hydrogen peroxide production without significant discrimination for glucose. MtCDH was able to use carbohydrates in desizing water for bleaching of cotton and brightness increases 92 (0 = black, 100 = perfect white) were obtained. Similarly, the ability of the MtCDH to produce hydrogen peroxide was successfully exploited in the development of an antimicrobial system. For both applications future investigation should involve engineering of the catalytic activity of MtCDH towards improved acceptance of oxygen as electron acceptor.

2. Introduction

2.1. Cellobiose Dehydrogenase (CDH)

Cellobiose dehydrogenase [(cellobiose:acceptor) 1-oxidoreductase, EC 1.1.99.18] was discovered by Ulla Westermark and Karl-Erik Eriksson in the two white rot fungi *Trametes versicolor* and *Phanerochaete chrysosporium* (Westermark, Eriksson, 1974). This enzyme reduced stable quinones in the presence of cellobiose. Due to this reaction the enzyme was named cellobiose:quinone oxidoreductase (CBQ), which was later abandoned. Another name coined cellobiose oxidase as it was assumed to prefer dioxygen to other electron acceptor. Further studies refuted the preference of dioxygen and the name was changed to cellobiose dehydrogenase (CDH).

CDH is produced by wood degrading, phytopathogenic white rot fungi and some soft rot fungi and molds under cellulolytic conditions in the presence of limiting amounts of cellobiose. This enzyme is a flavocytochrome and it is a member of the haemoflavoprotein family which includes flavocytochrome b_2, mandelate dehydrogenase, fumarate dehydrogenase, nitric oxide synthase and bacterial cytochrome P-450. These proteins have a distinct flavodehydrogenase domain (DH) and a cytochrome domain (CYT). The flavodehydrogenase domain belongs to the glucose-methanol-choline oxidoreductase super family with FAD as prosthetic group, which is attached by a serine and threonine rich flexible linker to a cytochrome domain carrying a haem b. Molecular masses are within 80 to 100 kDa and the mass percentage of the glycosylation is about 10 %(Gorton et al. 2010). This glycosylation is responsible for the high stability of the enzyme in the pH range of 3-10. Crystallization of the whole protein was hindered by the interdomain peptide linker. The crystal structure of both domains from *P. chrysosporium* was determined separately by Divne et al. (2000) (Figure 1).

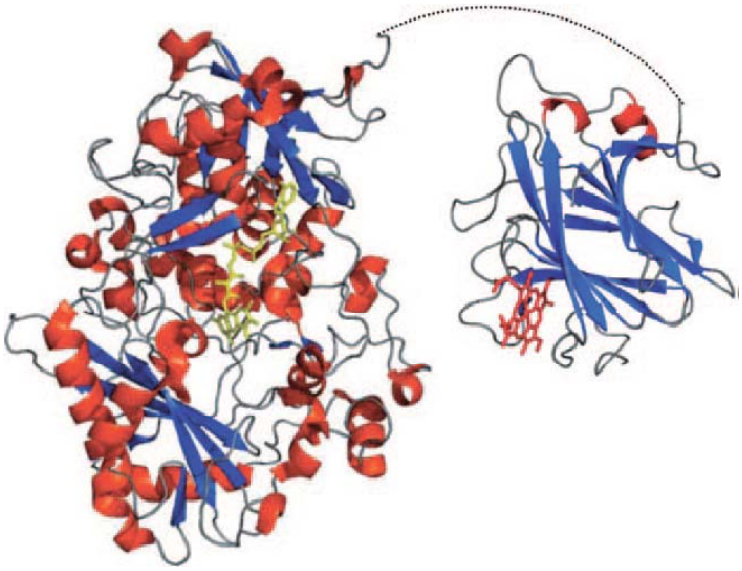

Figure 1: Crystal structure of flavodehydrogenase domain (left, PDB entry 1KDG) and cytochrome domain (right, PDB entry 1D7C). The interdomain linker is indicated by dots. Both domains are twisted from their face-to-face position by 45° towards the observer to give a better view of the FAD (yellow) and haem b (red) (Gorton et al. 2010).

CDH exist in different forms or isoforms and can be divided into two major classes based on amino acid sequence and catalytic properties. The genes of the flavodehydrogenase domain and cytochrome domain are in both classes well conserved. Class I stands for CDH produced by basidiomycetes. Sequences of this class are shorter with a highly conserved linker region. Class II involves CDHs produced by ascomycetes. These CDHs are more complex with a C-terminal cellulose binding domain and a less conserved linker region. Class I shows a strong substrate inhibition for glucose. In contrast, class II shows a broader substrate spectrum with a little glucose discrimination (Gorton et al., 2010).

2.1.1. Production of CDH

CDH is produced by diverse fungi, which can be fermented up to lab fermenter scale (Pricelius et al. 2009, Saha et al. 2008, Baminger et al. 2001, and Nakagame et al. 2006). Production of wild-type CDH has disadvantages. Different cellulolytic enzymes are produced simultaneously with CDH, the enzyme is produced in low amounts and the purification is more difficult.

Organism

CDH from *Phanerochaete chrysosporium* has been widely studied. Yoshida et al. (2001) used the CDH gene from this organism for the establishment the heterologous expression of CDH in *Pichia pastoris*. CDH from *Trametes hirsuta* (Nakagame et al. 2006), *Trametes pubescens* and *Trametes villosa* (Ludwig et al. 2004) are also well studied organism producing CDH. Harreither et al. (2009) and Sygmund (unpublished data) are using *Myriococcum thermophilum* for producing CDH.

Basic media requirements for production of wild type CDH

For biomass production the fungi was fermented in precultures without cellulose. Malt extract, glycerol and glucose were used as carbon source, yeast extract, peptone from meat, soybean, and casein were used as nitrogen source. Magnesium sulfate and trace element solution were added to the medium. For the main fermentation/CDH production, cellulose was used as carbon source. During the hyphal growth the formation of CDH is induced in the attendance of cellulose or cellobiose (Gorton et al. 2010). The other compounds of the media are mentioned above. The pH of the media for pre and main cultures was adjusted to 5 (Harreither et al. 2009, Ludwig et al. 2004, and Baminger et al. 2001). Concentration of each compound can vary. Saha et al. (2008) prepared a low concentrated medium; Nakagame et al. (2006) added thiamine as growing factor to the medium. The media for fermentation in the bioreactor are containing a higher amount of cellulose (Harreither et al. 2009) than the media for shaking flask cultures (Saha et al. 2008, Nakagame et al. 2006, and Yoshida et al. 2001).

Genetic engineering strategies

Heterologous expression is more advantageous to obtain a large amount of CDH without contaminations (i.e. cellolytic enzymes). Various expression systems are known, for the expression of proteins into prokaryotic or eukaryotic organism. The choice of expression systems is very important for the heterologous expression of proteins with posttranslational modifications (i.e. glycosylated proteins). Prokaryotic organisms, like *E. coli* are not able to glycoslylate proteins. CDH is a glycosylated protein, which is important for stability of the enzyme. For the expression of CDH the *Pichia pastoris* expression system was developed.

Pichia pastoris as eukaryotic organism is able to perform posttranslational modifications. *Pichia pastoris* expressions kits are commercially available (i.e. Invitrogen). A well known expressions vector for the *Pichia pastoris* expression system is pPIC. The cDNA encoding CDH from *Phanerochaete chrysosporium* K-3 has already been cloned by Raices et al. (1995). For the ligation into the *Eco*RI cutting site of the expression vector pPIC9K two oligonucleotide primers were prepared based on the sequence of the CDH cDNA (Yoshida et al.2001).

For the production of MtCDH used in this work, the *mtcdh* gene was codon-optimized for expression in *Pichia pastoris* corresponding to the method described in Abad et al. (2010) and synthesized by Gen-script. cDNA encoding for *Mt*CDH was cut out of the plasmid pMTSopt using the cutting sites *Eco*RI and *Not*I and cloned into the expression vector pPICZαA along with its native signal sequence (Sygmund, unpublished data). An example for construction of the expression vector is shown in figure 2.

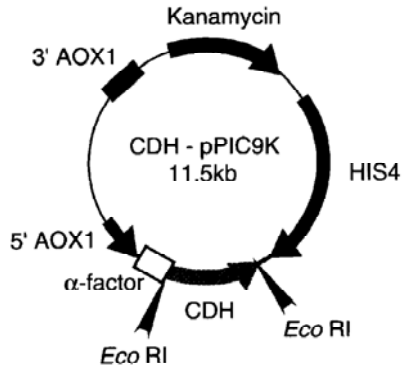

Figure 2: Example of construction of CDH-pPIC9K. α-factor: a secretion signal adhered to the CDH sequence; 5'AOXI: promoter fragment; HIS4: *Pichia pastoris* wild-type gene encoding histidinol dehydrogenase (Yoshida et al. 2001)

These are two examples for construction of an expression vector for the heterologous expression of CDH in *Pichia pastoris.* Correct incorporation of the gene and the lack of mutations were tested by DNA sequencing. The *Sac*I linearized expression plasmid was transformed into electro competent cells and transformants were selected on YPD plates containing the right antibiotics (Sygmund, unpublished data, Yoshida et al. 2001).

Media requirement for heterologous expressed CDH

For production of heterologous expressed CDH, the media composition is similar to the media requirements for production of wild type CDH. For biomass production YPG medium (Yoshida et al. 2001) or Yeast Basal Medium (Sygmund unpublished data) was used. YPG medium is containing 10 g/l yeast extract, 20 g/l peptone and 10 g/l glycerol. Yeast Basal Medium is an undefined medium for production of microorganism. For production of heterologous expressed CDH in the host organism *Pichia pastoris*, methanol was added as inducer.

Conditions

Shaking flask cultures are incubated at 30 °C under constant shaking at about 150 rpm for 17 days (Saha et al. 2008).

For production of CDH in a fermenter a preculture has to performed, which is incubated in flasks at 30 °C under constant shaking at about 120 rpm for 7 days (Ludwig et al. 2004) up to 30 days (Harreither et al. 2009). Fermentation and production of CDH is carried out in a stirred tank reactor, which is inoculated with the preculture. The bioreactor fermentations are performed at constant agitation at 100 rpm (Ludwig et al. 2004) up to 400 rpm (Baminger et al. 2001), with constant aeration up to 100 % (volume air/volume media) per minute (Harreither et al. 2009).

Purification

Purification of CDH depends on the required use of the enzyme, industrial application, food or medicine. Sygmund (unpublished data) purified CDH in one step purification via preparative chromatography (Phenyl-Sepharose Fast flow column). Before application on the column the sample was mixed with ammonium sulfate to a saturation of 20 %. After washing and eluation, fractions were tested for CDH activity with the DCIP assay (see point 5.1.) and pooled. Purified enzymes were concentrated and diafiltrated.

The purification procedure of other groups (Yoshida et al. 2001, Nakagame et al. 2006, Saha et al. 2008, Ludwig et al. 2004, and Harreither et al 2009) contains further chromatography steps.

Yoshida et al. (2001) incubated the concentrated enzyme solution with bentonite and removed the bentonite by centrifugation. The supernatant was purified by preparative

chromatography step (DEAE-Sepharose Fast flow). After eluation and concentration the enzyme solution was fractionated by anion exchanger and this step was repeated twice to obtain CDH in a high purity.

The purification procedure described by Nakagame et al. (2006) involves freezing, thawing and centrifugation steps to remove polysaccharide produced by fungi before the concentration step by ultrafiltration. The concentrated enzyme solution was purified by preparative chromatography (POROS HP 2 column). After washing and eluation, fraction with CDH activity were pooled were dialyzed, ultrafiltrated and purified by 2 preparative chromatography steps (Hi Load Superdex-200, QHR5/5). Active fractions were pooled, dialyzed and concentrated.

The purification of CDH as described by Saha et al. (2008) concentrated the enzyme solution by ultrafiltration, purified the enzyme by preparative chromatography (Sepharose 4B). Active fractions were pooled, washed by passing through a PM 30 cut off membrane and concentrated. This concentrated solution was purified by chromatography step using Phenyl Sepharose column. After eluation, active fraction were pooled and subjected to HPGPLC (Protein Pak 300 SW). Fractions with CDH activity were pooled.

The purification described by Ludwig et al. (2004) also involves centrifugation and ultrafiltration steps. After a second centrifugation step, the sample was diafiltrated against water and aliquots were purified using preparative chromatography (DEAE Sepharose fast flow column). After eluation, fractions were tested and fractions with CDH activity were pooled. The enzyme was applied to a Sephacryl S300 column for the next purification step. After eluation, active fractions were pooled and purified with a third chromatography step (QSource). The enzyme was eluated and tested for the activity. The active fractions were pooled and stored.

The first steps of purification of CDH as described by Harreither et al. (2009) were the same. After centrifugation and ultrafiltration, the concentrated enzyme was purified by preparative chromatography using DEAE-Sepharose fast flow column. After eluation, fractions containing CDH activity were pooled. Ammonium sulfate (to a 20 % saturation) and loaded onto a PHE Sepharose fast flow column. After eluation, the fractions with CDH activity were pooled and diafiltrated. The sample was purified in five consecutive

runs on a Mono Q column. After eluation, active fractions were pooled, aliquoted and frozen.

2.1.2. Natural catalytic activities of CDH in relation with other enzymes

The suggested *in vivo* functions of CDH are multifaceted, according to the quantity of potential CDH substrates that are attendant in lignocellulose. One of the first proposed functions is to weaken the substrate repression of cellulases by reaction of cellobiose to the corresponding lactone. Cellobionolactone is converting spontaneously to cellobionic acid, this product assists the fungus to reduce the pH and chelate metal ions present in wood. The reduction of toxic quinones to diphenols is a defense mechanism of the fungi. Polymerization reactions in areas of naked cellulose were inhibited via reduction of phenoxy radicals created by lignin peroxidase or laccase (Gorton et al. 2010).
CDH can assist manganese peroxidase by generating cellobionic acid to chelate Mn^{3+} ions, generation of hydrogen peroxide and reduction of MnO_2 and toxic quinones formed by manganese peroxidase activity. The generation of Fe^{2+} and hydrogen peroxide for a Fenton type reaction to produce hydroxyl radicals, which can straightly affect the lignocelluloses framework, is the most acknowledged biological function of CDH (Gorton et al. 2010). The natural catalytic activities of CDH in relation with other enzymes are shown in figure 3.Recently, synergistic cellulose depolymerization together with glycosidehydrolase 61 was also demonstrated (Langston et al. 2011)

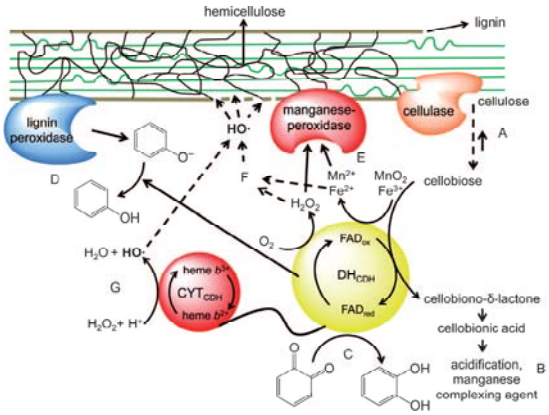

Figure 3: Suggested interaction of CDH in lignocelluloses degradation by: lowering substrate inhibition of cellulases (A), acidification an solubilisation of metal ions (B), reduction of toxic quinones (C), interaction with lignin peroxidase or laccase (D) and manganese peroxidase (E), production of hydroxyl radicals (OH•) via Fenton reaction (F) and direct production of reactive oxygen species (G) (Gorton et al. 2010)

2.1.3. Reaction mechanism

The dehydrogenase reaction which is typical for CDH can be divided into an oxidative and reductive half reaction. The oxidative half reaction is the oxidation of a saccharide in C 1 position to a lactone. The lactone hydrolyzes spontaneously to a carboxylic acid. The oxidative half reaction is shown in figure 4.

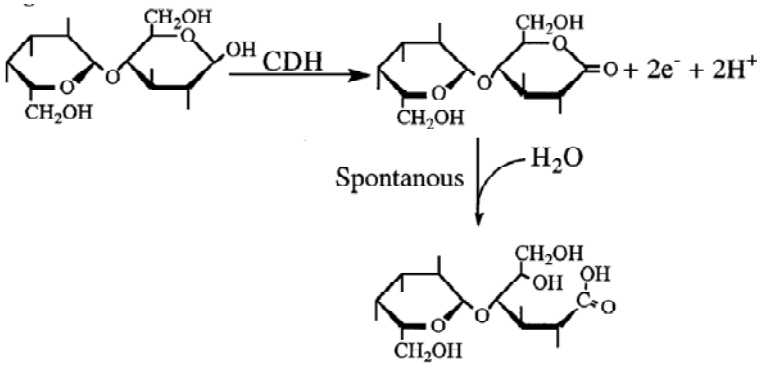

Figure 4: Oxidative half reaction of CDH. CDH achieve a two electron oxidation of C1 of cellobiose to the corresponding lactone. The electrons are taken up by FAD. The lactone will in aqueous environment spontaneously to a carboxylic acid (Henriksson et al. 2000).

The enzyme collects the two electrons and delivers to one two-electron acceptor or two one-electron acceptor (Henriksson et al. 2000). The electron acceptor is reduced. A list of possible electron acceptors is shown in table 1.

Table 1: List of possible electron acceptors

One electron acceptor	Two electron acceptor
Cytochrome c	2,6-dichloroinophenol sodium salt (DCIP)
Horseradish peroxidase comp 2	Several benzoquinones
Ferric ion complexes	Methyleneblue
semiquinones	O_2
	NADH/NADPH

Mono-, di- or oligosaccharides like cellobiose, lactose, maltose, and glucose act as electron donor. CDH exhibits a good activity with di- or oligosaccharides with β-linked glucose or mannose residue at the reducing end with high k_{cat}. The α-1,4 linked disaccharide maltose and monosaccharides shows lower k_{cat} than i.e. lactose or cellobiose. A binding of a β-linked disaccharide to the active site produces an induced fit that assist catalysis or maltose binds to the catalytic site in a fewer convenient way for catalysis could be an explanation for this difference (Henriksson et al. 2000).

Further studies have focused on the role of the prosthetic groups FAD and haem b. (Jones and Wilson 1988; Samejima et al. 1992; Rogers et al. 1994; Cohen et al. 1997) Preparations of the fragments can be done in vitro following proteolytic cleavage with papain (Canevascini et al. 1991; Henriksson et al. 1993). The results suggest that the oxidation of the electron donor is carried out by FAD which is converted to $FADH_2$ (Henriksson et al. 1991).

A schematic representation of the pathway of electron transfer of cellobiose, reducing quinones and producing hydrogen peroxide is shown in figure 5.

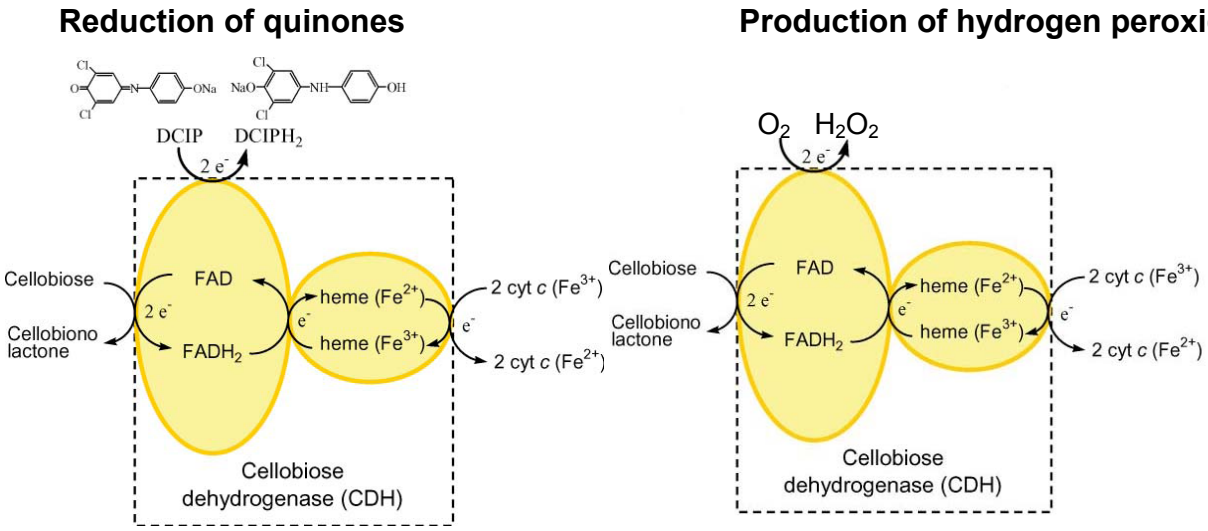

Figure 5: Schematic representation of the pathway of electron transfer of cellobiose and production of hydrogen peroxide and reduction of DCIP (Fujita et al. 2009)

The catalytic mechanism and the role of the cytochrome domain are at the present not well understood.

Positions relating the next destination of the electrons differ. Several groups suggested mechanism like the electron transfer chain and electron sink model (Canevascini 1991; Hallberg et al. 2000; Henriksson et al. 1998, Samejima and Eriksson 1992) to describe the role of the cytochrome domain. The studies demonstrated that one electron acceptors are generally reduced more slowly by the FAD fragment than by the intact CDH, while the reduction of two electron acceptors were unaffected by the loss of the haem function. The role of the haem could be that of an electron sink, to further the reaction rate of one electron acceptors (electron sink, figure 6).

The alternative model is the electron chain model, where the haem directly reduces one electron acceptors after conversion of FAD to $FADH_2$ (Henriksson et al. 1993).

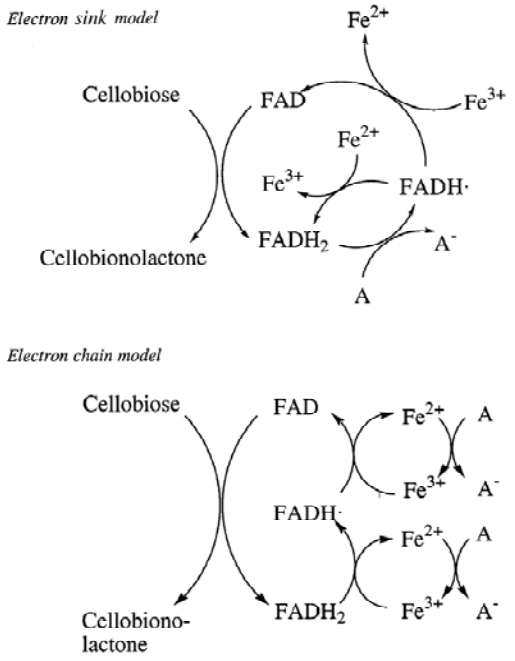

Figure 6: Mechanistic models for reduction of one electron acceptors by CDH: Electron sink and electron chain model. Fe stands for the haem iron and A represents one electron acceptor. The electron acceptor reacts directly with the reduced flavin, $FADH_2$ in the electron sink model. This model is based on the acceptation that the generated FADH• reacts more unchaste with the electron acceptor than does the fully reduced enzyme. Electron substitution between FADH radical and haem working as an electron sink will rise the time of fully reduced and oxidized stages of flavin. In the electron chain model the electrons transported to the haem and eventually to the electron acceptor. The electron sink model is facilitated by the fact that all known electron acceptors are reduced by the FAD containing fragment of CDH (Henriksson et al. 1993).

2.1.4. Applications

CDH has interesting properties that might find technical application. Few applications are described in the following sections.

Biosensors

CDH is intensively investigated for application in biosensors for the detection of cellobiose, cellodextrins, maltose, lactose, diphenolic compounds and catecholamines, such as dopamine, adrenaline and noradrenaline, as well as in biofuel cell anodes fuelled by glucose, lactose or cellobiose. While CDH is fixed on the surficial area of electrode and in touch with a substrate containing solution, it will catalyze the oxidation of the substrate, whereby two electrons are delivered from the substrate to the oxidized FAD cofactor of DH_{CDH} and the FAD cofactor will react into its fully reduced state.

Direct electron transfer (DET) by redox mediators and by redox polymers which were in solution ensures the electric contact between its catalytic site and the electrode. The electron transfer from an aldose via CDH to an electrode is shown in figure 7 (Gorton et al. 2010). This electron flow can be used for the detection of aldoses.

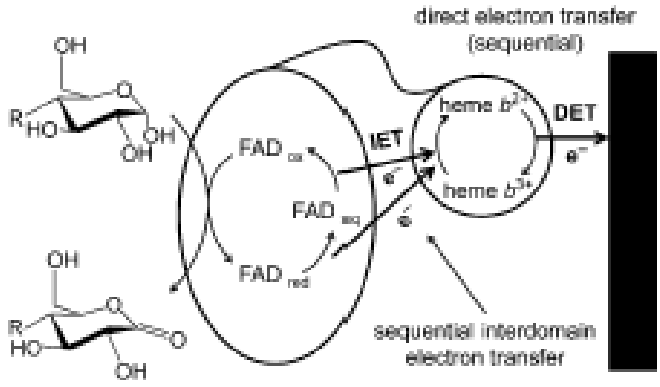

Figure 7: Electron transfer from an aldose via CDH to an electrode

Combined with a glucose oxidase electrode and a reverse-phase column, CDH affords a powerful analytical tool for determining cellodextrins. Highly specific biosensors can be generated by immobilizing flavin-containing oxidases in a redox polymer on an electrode surface. By combining one glucose oxidase electrode and one cellobiose oxidase electrode in a flow cell Nordling et al. (1993) made a sensor for flow-injection analysis, or post column quantification of glucose, cellobiose and higher cellodextrins in an HPLC system. Different concentrated solutions of glucose and cellobiose, alone or in a mixture were injected into the mobile phase and the directly response was recorded synchronistically from both electrodes. The recorded response peak heights could be used for calibration curves. The usable measuring ranges were 50 µM-50mM for glucose and 5 µM-80mM for cellobiose. Soluble cellodextrins (Glucose $_{1\text{-}6}$) could be separated on a C_{18} column by isocratic elution and detected by the sensor (Nordling et al. 1993).

Identification for cellobiose or lactose CDH can also be used in colorimetric assays. The formation of Prussion Blue, a very intense blue colored mixed salt of Fe^{2+} and Fe^{3+} was used by Canevascini (1988) used to get a high sensitivity and Henriksson (1995) used

triiodide ion. Samejima et al. (1998) used and electrode system to monitor the reduction of Fe^{3+} to Fe^{2+}.

CDH qualification to reduce quinones and phenoxy radicals can serve as a way to adjust the breakdown and polymerizations process of lignin and lignin degradations products, during white-rot fungal action (Ander et al. 1996). The lignin related quinones are established in photo-yellowing of lignin carrying paper, CDH has a potential to control the yellowing of pulp and paper (Ander et al. 1996).

An action of fungal laccase, cellobiose dehydrogenase and chemical mediators for the removal of various classes of textile dyes was studied by Ciullini et al. (2008). The combined action of laccase and CDH can use as an alternative to the expensive chemical mediators. CDH shows ferrireductase activity which is important for the generation of hydroxyl radicals (Hyde and Wood 1997).

The iron containing haem group of CDH is able to generate in a Fenton type reaction hydroxyl radicals which are very powerful oxidants (Kremer and Wood, 1992). CDH producing brown rot fungi secrete enzymes like CDH and oxalic acid to lower the local pH of the fungi. Oxalic acid is an exceptional strength organic acid and moderate iron chelator which prevents the fungi of the self damage by reactive oxygen species. It is recommended that the diffusion of ferric iron into areas of higher pH promotes the autooxidation to ferrous iron which forces the production of reactive oxygen species via Fenton's reaction (Hyde and Wood 1997).

The addition of CDH yields an improvement of decolorization in Reactive Red 272, Reactive Blue 69 and Acid yellow 49. The improved reduction of color intensity is much higher than with laccase activity alone (Cameron and Aust, 1999; Henriksson et al. 2000).

The hydroxyl radicals which were produced by the haem group of CDH and the hydrogen peroxide can result the demethoxylation and/or hydroxylation of diverse aromatic nonphenolic structures to phenolic ones, thus executing the molecule easily oxidized by laccases or peroxidases (Hilden et al. 2000). The reaction rate of the reaction of hydroxyl radicals produced by CDH and dyes has been reported reliant on the basic molecular structure and on the nature of auxiliary groups attached to the aromatic core of the dyes (Galindo and Kalt 1999). In this study, the addition of CDH

yields in substantial rise in removal for all the dyes in comparison to the action of laccase alone.

2.2. Application as antimicrobials

Several chemical substances are known as antibacterial agents, like peracetic acid, hydrogen peroxide, silver ions, iodine, alcohol, ozone etc. These are summarized in figure 8.

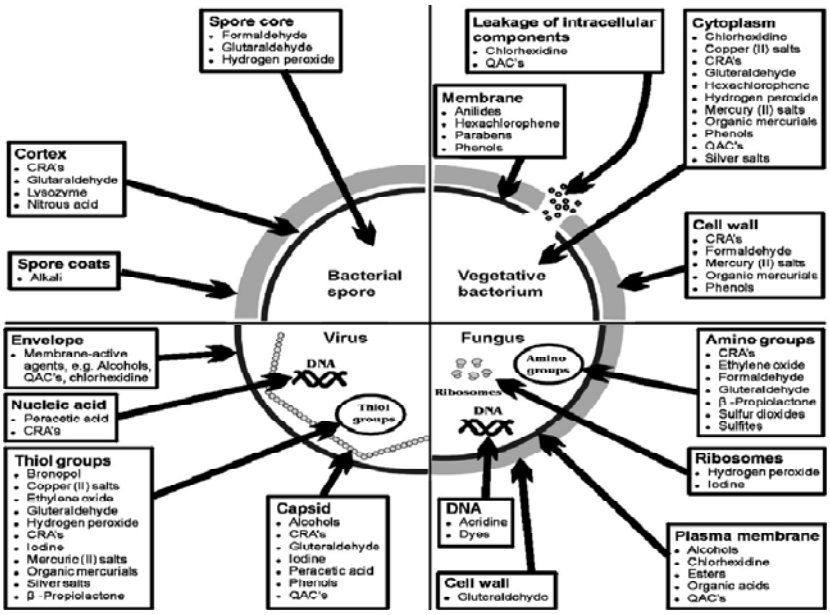

Figure 8: Mechanism of microorganism inactivation by biocides (Russel et al., 1997), CRA's = chlorine releasing agents, QAC's = quaternary ammonium compounds

The hydrogen peroxide producing properties of CDH can be adopted for the inhibition of microbial growth. The haem group of CDH, which contains iron, is involved in the production of reactive oxygen species. These reactive oxygen species were also produced by incomplete reduction of oxygen during the respiratory chain where haemoproteins were involved (Imlay and Linn, 1986).

2.2.1. Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is a well known antiseptic since it quickly acts to kill microorganisms and has no long-term or conserving effect (Lück and Jager 1997). This short-lived action is due to hydrogen peroxide's rapid decomposition to oxygen and water upon contact with organic material. The antimicrobial action of hydrogen peroxide is not due to its oxidative properties as a molecule, but primarily in the production of other powerful oxidants such as singlet oxygen, superoxide radicals, and the hydroxyl radical (Davidson and Harrison 2002). Nonreversible damage to enzymes, membrane constituents and DNA were caused by reactive oxygen species. Hydrogen peroxide alone will not cause protein, lipid or nucleic acid modification in aqueous solution without the presence of radicals which acts as catalysts (Juven and Pierson 1996). The hydroxyl radicals play the major role in the toxicity of hydrogen peroxide (Imlay and Linn 1988). Hydroxyl radicals add to DNA bases add to DNA bases and abstract H-atoms from the DNA helix (Cadenas 1989). Hydroxyl radicals may also damage cell membranes. In a study of model membrane systems, Anzai et al. (1999) found that hydroxyl radicals increased lipid peroxidation as well as the ion permeability of model membrane systems, though via independent mechanisms. A prevalently cited example is the Fenton reaction whereby a reducing agent such as the superoxide radical reduces $Fe^{3+}$to Fe^{2+}, which then reacts with $H_2O_2$to produce hydroxyl anions, hydroxyl radicals, and Fe^{3+}(Juven and Pierson 1996). Accordingly, growing *Staphylococcus aureus* cells in broths of increasing iron concentrations was found to increase killing by H_2O_2, whereas addition of radical interceptors had a protective effect against such killing (Repine et al. 1981).

2.2.2. Peracetic acid

Peracetic acid is a strong oxidant and disinfectant. The oxidation potential of peracetic acid is larger than that of chlorine or chlorine dioxide (Alasri et al. 1992; Gehr and Cochrane 2002). Its disinfectant activity is based on the release of active oxygen (Liberti and Notamicola 1999). Sensitive sulfhydryl and sulfur bonds in proteins, enzymes and

metabolites were oxidized by the action of peracetic acid. Peracetic acid disturbs the chemiosmotic function of the lipoprotein cytoplasmic membrane and transport through dislocation or rupture of cell walls (Baldry and Fraser 1988; Leaper 1984). The increasing organic content in the wastewater due to acetic acid and the potential for microbial regrowth is the major disadvantage of the usage of peracetic acid for disinfection. In peracetic acid mixtures acetic acid is present and formed after decomposition of peracetic acid. Due to its high cost because of the limited production capacity worldwide is another drawback (Kitis 2004).

The strong inhibitory and antibacterial effects of Silver ions have been known long time (Berger et al. 1976). Feng et al. (2000) suggested the following bacterial mechanism of silver ions against *Escherichia coli* and *Staphylococcus aureus*:

- As a reaction against the denaturation effects of silver ions, DNA molecules become condensed and lose their replication abilities.
- Silver ions interact with thiol groups in protein, which induce the inactivation of the bacterial proteins.

2.3. Cotton bleaching systems

The textile industry comprises a diverse and fragmented group of establishments that produce and/or process textile-related products (fiber, yarn and fabric) for further processing into apparel, home furnishings and industrial goods. Textile establishments receive and prepare fibers; transform fibers into yarn, thread or webbing; convert the yarn into fabric or related products and dye and finish these materials ant various stages of production (Ghosh and Gangopadhyay 2000). The process of converting raw fibers into finished textile products for clothing and non-clothing is complex. There is little difference between knitting and weaving in the production of man-made cotton and wool fabrics (Hashem et al. 2005). Textiles go through different steps of production that include yarn formation, fabric formation, and wet processing and textile fabrication. These processing steps are shown in figure 9.

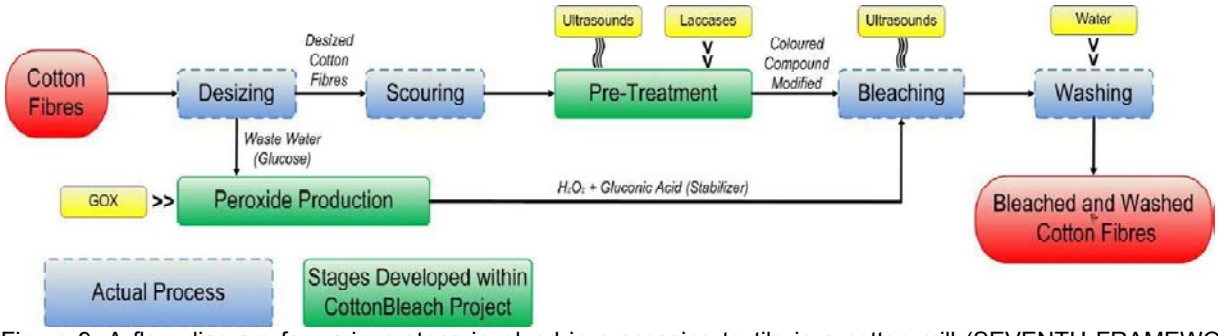

Figure 9: A flow diagram for various steps involved in processing textile in a cotton mill (SEVENTH FRAMEWORK PROGRAMME, 2009)

2.3.1. Weaving

The fabric consists of yarn, the warp and the weft. During the weaving process the warp is mechanically loaded (periodically extensions, abrasions, folding). An unprotected warp is no match for these mechanically stress and has to be coated with a viscous, abrasion-proof protection film.

2.3.2. Sizing

During the sizing process the warp is coated with a protection film. A multiplicity of substances is used as sizing agent, which are described by Goswami et al. 2004.

There are quantities of desirable characteristics which a warp size should exhibit. A good sizing material should have most of these properties; however, sizes that lack in some of these properties still may be used.

- Environmentally friendly
- Reasonable use economics
- Elasticity
- Good specific adhesion
- Transparency
- Reasonable strength
- Water soluble or water dispersible
- Uniformity
- Improves weaving efficiencies
- Reasonable extensibility
- Good film former
- Penetration of yarn bundle
- Good film flexibility
- Good frictional properties (lubricity)
- Bacterial resistance (mildew)
- Controllable viscosity (fluidity)
- Good hygroscopicity characteristics
- Clean split at bust rods
- No effect on drying
- Recoverable and reusable (or treatable)

- Low static propensity
- Easily removed (desized)
- Lack of odor
- Compatible with other ingredients
- Neutral pH
- Insensitive to high heat (overdrying)
- No build-up on dry cans
- Rapid drying
- No skimming tendency
- Easily prepared
- No beam blocking
- Good abrasion resistance
- High fold endurance
- Low BOD
- Reduced shedding
- No redeposition of size

In other words the convenience of a size material for a specific application will depend onto the nature, the type of the fibers, the type of the applied weaving machine applied and the characteristics of the woven fabric.

Starch

Starch was at one time the primary sizing agent for textiles. It is still used in large amounts alone or in blends. Starch is produced by plants but it is high concentrated in seeds, roods and cereals. In the US part of the corn is used for the application in the textile industry.

The linear form of the starch polymer chain is called amylose and the highly branched chain form is called amylopectin which is containing several thousand glucose units. Amylopectin is a high branched molecule. The branching out occurs every 9–20 glucose units of the molecule, prevents rapid gelling, i.e., retrogradation or microcrystallization of starch pastes, of the cooled size. And amylose, because of its linear nature, significantly plays a part to the size film strength. High amylose starches require cooking temperatures of greater than 150 °C for dissolution but retrogrades (gels) rapidly if cooled.

The potency of hydrogen bonds the coagulation of starch is high when cooked. The temperature, which can break these bonds, is called gelatinization or paste temperature. This temperature will depend upon the ratio of amylose/amylopectin and the arrangement of these molecules within the starch granule structure.

When the solution is cooled, the potential for building up intermolecular chain is high and the coupled chains composed microcrystalline micelles is developed and the starch mixture coagulate. When this is applied to the yarns, it dries and forms a film around the yarn. At high starch concentrations causes a high viscosity of the gel and heavy for

further application. By application of the hot gel on the yarn, following by drying, a protective film is formed around the fiber bundle.

Modification of starch

The bias of raw starches to produce high viscous gels can be reduced or eliminated by modification the starch by any one of the following processes:

- Acid modification
- Conversion to gums
- Enzyme treatments
- Derivatization (chemical modification)

Acid modification

The starch granules were suspended in an aqueous solution and incubated with hydrochloric acid to reduce the size of the starch chains, while no obviously change of the starch granules occurs. The reaction occurs under diverging conditions (time, temperature and hydrochloric acid concentration). The incubation is performed below the gelatinization temperature. The viscosity of the cooked starch can be reduced by the shortening the chain lengths. Modification of starch prevents the textile mill with high viscous size solution with high solids content.

Conversion to gums

The degree of polymerization of starch chains is decreased during the roasting of starch granules but it aggregates to build highly branched systems that are solvable in cold water. These aggregates are called gums. Most of it has low film strengths but they are used for the transformation of stronger size materials and provide differential viscosity of sizes for more efficient degrading processing. Further thermal degradation gives dextrins that do not have any sizing uses. Gums are easily desized in warm water.

Enzyme Modification

Some mills start with cheap pearl corn starch and modify the starch themselves during the cooking cycle. The modification can be performed by adding heat resistant fungal or bacterial enzymes to the cooking chamber. The enzyme degrades the starch chain structure to decrease the viscosity during the heating of the size. Care must be taken to program the cook cycle to provide the same rate of temperature rise from batch to batch in order to achieve reproducible results. At temperatures near boiling the enzyme activity is destroyed and the chain cleavage stops to prevent continuing breakdown during storage and use.

Derivatized Starches

Each glucose unit of the starch molecule has three hydroxyl groups that can react analogous to those in cellulose. Derivatization reactions which can be successfully performed for cellulose can be done with starch. Although diverse starch derivatives are known, few were used as sizing agent. Their retrogradation and gelling tendencies are reduced to a minimum or eliminated. The gelatinization temperature is reduced, the water holding ability is improved and the desizing is simplified. Water holding ability can be providing an advantage as it can help reduce the humidity in the weave room. The films do not shrink during the drying and tearing and fluffing of the size film is reduced. Typical derivatives used for sizing include oxidation, esterification, cross-linking, and cationic starches.

Polyvinyl alcohol

Polyvinyl alcohol (PVA) has been one of the most multifunctional size materials available for warp sizing formulations since it was commercialized as a textile warp sizing agent sometime around 1965. PVA can be used alone or in combination with other size materials (starch, polyester resins, acrylic copolymers, CMC) or as binding agent with fibers (glass, acrylic, polyester, cellulose acetate, nylon) which enhance the potential of PVA. The PVA chain can be tailored for specific needs by the control of the length of the chain, the degree of hydrolysis of the acetate groups. The breaking of the PVA film increases as the chain length of each grade increases and as the percentage of acetyl removal increases. PVA improve the performance of weaker sizes like starch.

Carboxymethylcellulose

Carboxyl methyl cellulose (CMC) is the cold water–soluble carboxymethyl (ether) derivative of cellulose. CMC a renewable natural product is an excellent film former and has numerous textile and nontextile uses. The nontextile uses have virtually supplanted its markets, primarily for price considerations. The raw material for CMC manufacture is wood pulp or cotton linters or similar cotton waste. In either case the cellulose must be purified. The carboxymethyl group primarily forms at the cellulose chain at the C6 primary hydroxyl a stable primary ether linkage. The CMC derivate is more stable against removal than the acetate ester bond on the partially hydrolyzed PVA chain. CMC is more advantageous in the sizing of shrink sensitive fabrics like acetate and viscose silk because removal of other sizes in the finishing step is the usage of hot water necessary. Hot water, enzymes, and chemical additions are not required for desizing of CMC. CMC is generally used in textile sizing as a more water-soluble sodium salt derivative.

Acrylics

The term ‘‘acrylic’’ is a generic term for sizing materials that contain polyacrylic acid (PAA) and its derivatives as a homopolymer or copolymer. Acrylic size is used in some cases alone or as binder of the size for the improvement of the adhesion of the base sizing agent and the fiber. Acrylic sizes do not require cooking but can be heated up to 100 °C for dispersing wax or lubricants as well as with other sizes, e.g., starch, with which it may be blended. The major advantage is the high binding strength to a number of synthetic fibers under weave room conditions, yet it has low dry bonding strength on the slasher, facilitating easy splitting at the bust rods. The acrylic sizes can be divided into water or alkali soluble of water insoluble classes. The alkali soluble types will have *acrylic acid* or its ammonia or sodium salt or other water-soluble derivative as a major component of the polymer chain. The insoluble size materials contain acrylic esters or acrylonitrile derivatives. The copolymers can vary widely in composition (ester, amide, nitrile, acid, etc.) to give a large family of ‘‘acrylic’’ polymers useful as sizing materials. Typically temperature range for sizing is from 80 to 105 °C.

2.3.3. Desizing

Dyeing, printing and finishing of the fabrics were hindered by the presence of sizing agents. For dyeing of fabrics the dye has to penetrate the fiber and the presence of starch hinder the performance of textile dyes. Starch is removed or converted into simple water-soluble products either by enzymatic or chemical hydrolysis or by oxidation (by sodium bromide, sodium chloride, etc.) (Batra 1985).Enzymes degrade starch into compounds that are soluble sugars and dextrins, oxidizing agents oxidize starch into compounds that are soluble in alkaline solution, while acids hydrolyze starch into water soluble compounds (Rucker and Smith 2006).

During the acid desizing the fabric is treated with diluted sulfuric acid in the concentration of 5-10 g/l at a temperature of about 40 °C for 3-4 h. Dilute acid attacks the polymer chain of starch and due to chain cleavage of starch molecule short water soluble or decomposable chain segments are formed. With sulfuric acid higher than 10 g/l and above 50 °C there is always the possibility of weakening the cloth or causing holes.

Use of sodium hypochlorite, sodium chlorite and sodium bromite as desizing agents are reported by Holbrook et al. (1966). Sodium chlorite is an excellent bleaching agent but it force to ‘fix’ rather than remove size in single stage bleaching. The performance of sodium bromite oxidizes starch in the presence of cellulose. Sized cotton fabric consumes more bromite than that of unsized cotton (Freytag 1961). Sodium bromite does not act as a bleaching agent. Bromite treatment can performed in continuous process hot with a dwell time of 20 min. The advantage of the usage of hypochlorite and chlorite is the whitening of the fabrics which reduces the oxidant concentration in subsequent bleaching (Karmakar 1999).

Enzymes are specific in their action in that they do not attack cotton, while oxidizing agents and acids can degrade cotton in addition to starch. Process parameters that affect enzymatic desizing are in general those affect the activity of the enzyme. They include pH, temperature and compounds which enhance or decrease activity. Compounds which reduce enzyme activity should not be allowed to contaminate the desize bath include copper and zinc ions, some anionic wetting agents, chelating agents, oxidizing and reducing agents. The washing step is very important in removing

the dextrinized starch from the fabric (Rucker and Smith 2006). The introduction of enzymatic systems in the preparation of cotton is advantageous in terms of decreased water-, chemicals- and energy consumption, reuse of desizing waste baths, milder process conditions and environmentally friendly processes (Tzanov et al. 2001).
Desizing waste water is rich in carbohydrates and sugars which can be used as substrates for CDH to produce hydrogen peroxide for the bleaching step. For this study, MtCDH is used to develop an enzymatic process for bleaching of cotton. CDH is able to use different sugars like glucose, maltose, maltotriose etc. as substrates which favorable for recycling of desizing waste water.

2.3.4. Scouring

A conventional process of removing non-cellulose substances from cotton fibers is called alkaline scouring and is usually carried out by using sodium hydroxide (Etters 1999). These conventional scouring processes of cotton are executed at temperatures up to 130 °C in a very alkaline medium (pH 10-12) with sodium hydroxide. This process does not remove pigments and seed coat fragments (Fortre Tavčer et al. 2006) and this is the reason why scouring is almost always followed by bleaching in order to remove residual substances. Scouring is carried out at a high temperature and at a high pH value. Although non-cellulose substances are efficiently removed or discolored, but the aggressive treatment conditions damage the cellulosic fiber. After the treatment at this high pH values the fabric has to be rinsed intensively to neutralize the pH of the fabric (Preša, Fortre Tavčer; 2007). This gives the textile industry the reason for the introduction of biological methods to reduce the damage of the fiber and the environment Positive effects of removing of non-cellulose substances have been obtained with the enzyme pectinase. Pectinases catalyze the hydrolysis of pectin substances. After the break down the non-cellulose substances can be removed from cotton by using surfactants and by mechanical action. Scouring with pectinases is carried out in acidic or alkaline media, depending on the type of pectinases. The temperature of treatment is 50-60 °C and the time of treatment is 30-60 min. (Aly et al. 2004; Hartzell-Lawson and Durant 2000).

2.3.5. Bleaching

The next step in the textile processing is the bleaching process. Classification of bleaching agents is shown in figure 10.

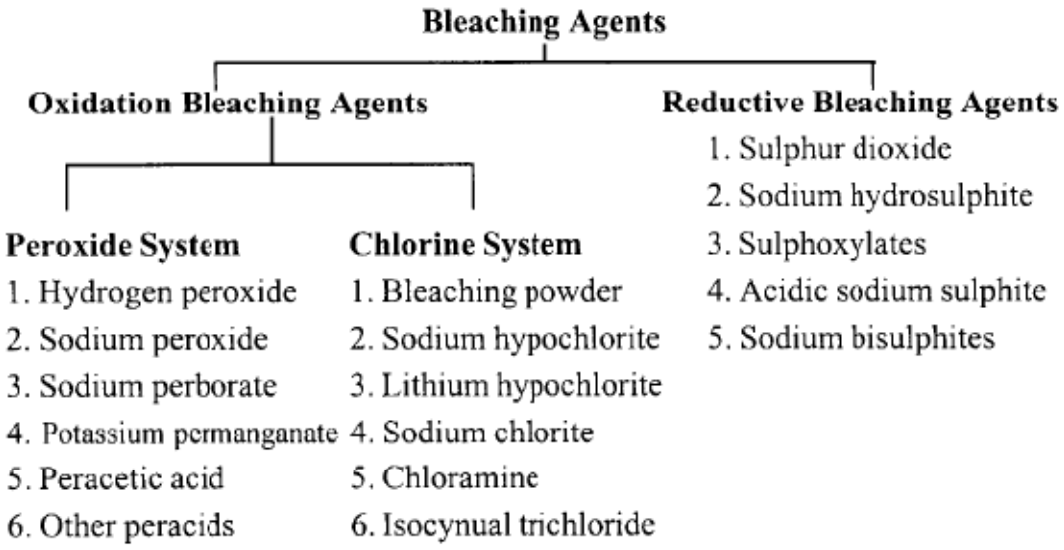

Figure 10: Classification of bleaching agents (Karmakar 1999)

Chlorine system

A solution of bleaching powder contains calcium hypochlorite [$Ca(OCl)_2$•4 H_2O]. calcium chloride [$CaCl_2$], lime hypochlorous acid formed by the hydrolysis of hypochlorite, possibly free chlorine and other soluble impurities lice calcium chlorate, calcium permanganate etc. Calcium hypochlorite is mainly prepared from bleaching powder or from liquid chlorine and slaked lime suspension., the temperature must not increased above 55 °C, because for the formation of chlorate and chloride at higher temperatures. The oxidizing action of calcium hypochlorite is due to hypochlorous acid (HOCl, shown in figure 11).

$$Ca(OCl)_2 + HCl = CaCl_2 + 2HOCl$$
$$Ca(OCl)_2 + H_2SO_4 = CaSO_4 + 2HOCl$$
$$CaCl_2 + H_2SO_4 = CaSO_4 + 2HCl$$
$$2HOCl + 2HCl = 2H_2O + 2Cl_2$$

Figure 11: Decomposition of calcium hypochlorite (Karmakar 1999)

The conditions (pH) which induce the formation of hypochlorous acid increase the oxidizing power of bleaching powder. This acidic hypochlorous acid is more powerful bleaching agent than the alkaline hypochlorite and is not safe as this causes degradation of the cellulosic material due to the formation of oxycellulose.

Peroxide system

The most common bleaching agent is hydrogen peroxide. In the attendance of sulphuric acid and phosphoric acid is pure hydrogen peroxide is fairly stable in if stored away from sunlight in a perfectly smooth bottle. The stability of hydrogen peroxide decreases in alkaline medium and even traces of alkali (NaOH...) degrade aqueous solution of hydrogen peroxide. The addition of alcohol, glycerin or berbituric acid also stabilize hydrogen peroxide. The presence of finely divided heavy metals such as copper, iron, manganese, nickel, chromium etc. destabilizes hydrogen peroxide or their oxides with release of oxygen.

Though hydrogen peroxide is stable in acidic medium, but bleaching occurs by the addition of alkali or by increased temperature. Hydrogen peroxide releases perhydroxyl ion (HO_2^-) in aqueous medium and chemically performance like a weak dibasic acid. The perhydroxyl is very instable. In the presence of oxidisable compounds like impurities in cotton perhydroxyl ion decomposes and bleaching the cotton. Sodium hydroxide activates hydrogen peroxide because H^+ ion is neutralized by alkali which is favorable for release of HO_2^- (shown in figure 12).

$$H_2O_2 \rightleftharpoons H^+ + HO_2^-$$

$$H_2O_2 \rightarrow H^+ + HO_2^- \xrightarrow{OH^-} HO_2^- + H_2O$$

Figure 12: Production of perhydroxyl ion (Karmakar 1999)

Higher pH destabilizes the release of perhydroxyl ion with the formation of oxygen without bleaching property. If the release of the perhydroxyl ion is too high, the unutilized perhydroxyl ion may damage the fiber. A safe and optimum pH for cotton bleaching lies between 10.5 and 10.0 where the rate of evolution of perhydroxyl ion is equal to the rate

of consumption for bleaching (Karmakar 1999). The bleaching process is performed at high pH and at high temperature containing bath additives. A modern bleach bath is containing wetting agents, detergents, emulsifiers and stabilizer. The most important, very frequently used stabilizer systems are organic stabilizers based on phosphoric acids, aminocarboxylates and sodium silicate (waterglass) in combination with alkaline earth metal ions, particularly magnesium ions. In the bleaching liquor, sodium silicate and magnesium ions produce colloids, which act as buffers and keep liquor alkalinity constant. Sodium silicate not only helps to buffer the pH, but also inactivates metallic impurities (i.e. Fe, Cu) which could cause extensive fiber damage by catalytic decomposition of hydrogen peroxide. The difficult removals of silicate stabilizers during washing, formation of precipitates on the fiber and on processing machinery, harsh handle etc. are disadvantages of these stabilizers. This is the reason that nowadays organic stabilizers are mostly employed, such as aminocarboxylates (Rucker and Smith 2006). The hydroxide bleaching process consumes high amounts of chemicals, water and energy. In practice cotton bleaching with hydrogen peroxide is carried out at 90-100 °C, but the temperature may be increased to 120 °C in the case of pressurized equipment with a corresponding reduction in process time. The rate of bleaching increases with the increase in temperature, but at the same time solution becomes unstable and degradation of cotton increases. Below 80 °C, the evolution of perhydroxyl ion is very slow so also the rate of bleaching. The optimum concentration of hydrogen peroxide depends on number of factors namely liquor ratio, temperature and class of fiber. Very high concentration may damage the fiber. The time required to bleach with hydrogen peroxide depends on temperature, class of fiber and equipment used for bleaching. In general, the time of bleaching is inversely proportional to the temperature of bleaching bath. Cotton may be bleached in open kiers by circulating heated hydrogen peroxide solution (88-95 °C) for 6-10 hours.

Peracetic acid

The mechanism of reaction is somewhat similar to that of hydrogen peroxide. Recently, peracetic acid is used as a substitution of hypochlorite in multi-stage bleaching process of cotton. Peracetic acid is most effective as a bleaching agent of cotton in the pH range of 6 to 7. The desirable bleaching temperature range is between 50-80 °C and bleaching time of 20-60 min depending on the temperature. The degree of brightness rises proportionately with the concentration of bleaching agent. To avoid the damage of cloth, a complexing agent may be added to remove to those catalytically active ions such as copper, iron etc. which can be absorbed by fiber (Karmakar 1999).

Enzymatic bleaching

The alternative for bleaching with chemically produced hydrogen peroxide is the usage of hydrogen peroxide producing enzymes. Cellobiose dehydrogenase and glucose oxidase are known for their ability to produce hydrogen peroxide. Several studies used glucose oxidase to develop an enzymatic bleaching process. The disadvantage of GOX is the substrate spectrum which is limited to monosaccharides. CDH can use di-, tri- or higher saccharides for hydrogen peroxide production. Due to this fact MtCDH was used for this study to produce hydrogen peroxide from desizing waste water.

3. Aim

This study is aimed at developing bleaching and antimicrobial systems based on CDH.

4. Objectives

The specific objectives of this study were:

- To screen different substrates of MtCDH for the production of hydrogen peroxide
- To optimize conditions for the production of MtCDH and produced hydrogen peroxide with GOX using glucose as substrate to compare the results with MtCDH
- To develop an enzymatic bleaching system based on MtCDH using desizing water as possible substrate for MtCDH from cotton processing
- To develop antimicrobial systems for pathogenic bacteria(*Escherichia coli, Staphylococcus aureus)*

5. Materials and Methods

5.1. Cellobiose dehydrogenase (CDH) activity assay using DCIP

A method developed by Baminger et al. 2002 and Ludwig et al. 2003 was used as a standard method for determination of the Cellobiose dehydrogenase activity in crude extracts or partially purified preparations. The natural substrate of CDH cellobiose was replaced with lactose. The substrate cellobiose acts as electron donor and exhibits substrate inhibition. Lactose is a much cheaper and easy to handle substrate. In this reaction the reduction of 2,6-dichloroindophenol (DCIP) is monitored.

Before the start of the assay following stock solutions were prepared:

DCIP solution was prepared by dissolving of 87.03 mg 2,6-dichloroindophenol (DCIP) in 10 ml 96 % ethanol by stirring for 30 min at 50° C in a volumetric flask. After complete dissolution the flask is filled up to 100 ml with distilled water. This solution can be stored at 4° C in the dark.

Lactose solution was prepared by dissolving of 10.81 g lactose monohydrate in 100 ml distilled water by stirring.

Sodium fluoride solution was prepared by dissolving of 84 mg sodium fluoride is dissolved in 10 ml distilled water. The addition of this solution eliminates the laccase activity, which will cover part of the CDH activity by reoxidising DCIP.

Sodium acetate buffer pH 4 was prepared by pouring of 3 g or 2.86 ml of 99 % of acetic acid in 400 ml of distilled water and is titrated with freshly prepared sodium hydroxide solution to pH 4.0. After the adjustment of the pH the buffer was filled up with distilled water to 500 ml.

Cellobiose dehydrogenase solution was provided from Dr. Roland Ludwig's Group, BOKU Vienna.

Table 2: Pipetting protocol

Solution	Concentration [M]	Volume [µl]
DCIP solution	0.003	100
Lactose solution	0.3	100
Sodium fluoride	0.2	20
Sodium acetate buffer	0.1	760
Enzyme/sample		20

All solutions except the enzyme are pipet in a 1.5 ml Eppendorf tube. The mixture was equilibrated to 30 °C for least 20 min in an Eppendorf thermomixer. The reaction solution is transferred into a 1 ml microcuvette and placed in the sample holder. The reaction was started by adding the enzyme and absorption is recorded at 520 nm. Samples were measured in triplicate.

5.2. Screening different carbohydrates as substrates of MtCDH

Cellobiose dehydrogenase activity assay using DCIP was used for screening of different carbohydrates for possible substrates of MtCDH. Lactose was replaced by following substrates:

Table 3: Substrate for screening

Substrate	Concentration [mg/ml]
Cellobiose	300 – 2
lactose	300 – 2
Glucose	300 – 2
Maltose	300 – 2
Amylopectin	10
Dextrane	10
Carboxymethylcellulose	20 - 2

Amylopectin, dextrane and carboxymethylcellulose were tested in this lower concentration range due to their low solubility in water. Carboxymethylcellulose swells

intensely in water and makes it impossible to test this substrate in a higher concentration range.

5.3. Measurement of Hydrogen peroxide

The assay of hydrogen peroxide production was based on a sensitive assay modified from the published methods from Ling-Su Zhang (Zhang and Wong 1994) and Nutt (Nutt et al. 1997). The assay measures the oxidation of leuco-crystal violet by hydrogen peroxide in the presence of horseradish peroxidase. To measure the hydrogen peroxide production at pH 6.5 with different substrates, the reaction mixture contained 555 µl of 50 mM sodium-phosphate buffer pH 6.5, 100 µl of substrate (300 mM cellobiose, lactose, maltose, glucose) 100 µl of desferrioxamine mesylate (1 mM) and 0.5 u/ml of enzyme (enzymatic activity determined at pH 6.5). The reaction mixture was incubated at 40 °C in an Eppendorf Thermomixer for 30 min. Since the activity of MtCDH interferes with the peroxidase activity, the assay was boiled for 2 min, which does not influence the hydrogen peroxide level to any measurable extent. The colour reaction was initiated by the addition of 50 µl peroxidase (1 mg/ml), 50 µl of leuco crystal violet (1 mM dissolved in 0.06 M HCl) and 500 µl sodium acetate buffer (100 mM, pH 4). The absorbance of oxidized crystal violet was measured at 592 nm with a UV/visible spectrophotometer. The absorbance achieved is linearly correlated to the concentration of hydrogen peroxide. Blank reaction was prepared without MtCDH and the calibration curve was obtained by using hydrogen peroxide in concentrations from 0 to 200 µM (Pricelius et al. 2009).

5.4. Amylase Assay using DNS

α-Amylase from *Aspergillus niger* was used for desizing of the raw cotton fabrics. The assay as described by Bernfeld (1955) was used for the determination of the activity of this enzyme. For the procedure of the assay following solutions were prepared;

Starch Solution was prepared by adding of 1.0 g Starch (soluble) in 100 ml 20 mM Sodium Phosphate Buffer pH 6.9 with 6.7 mM sodium chloride. The starch was solubilised by heating the solution in a glass beaker directly on a heating plate using constant stirring. The solution was boiled for 15 min and cooled down to room temperature with constant stirring. Final volume (100 ml) was controlled and filled up with deionised water if it was needed.

For the standard solution a 0.2 % (w/v) Maltose solution in 10 ml deionised water was prepared using Maltose Monohydrate.

DNS Solution was prepared by dissolving of 13.98 g Sodium hydroxide and 216.1 g Potassium sodium tartrate in 800 ml deionised water. 5.155 g Phenol was added, followed by 7.48 g 2-Hydroxy-3,5-dinitrosalicylic acid and 5.86 g Sodium meta bisulfite. Filled up to 1000 ml with deionised water and stored at 4 °C.

For the determination of the released maltose a calibration has to be made using a dilution series of the standard solution.

Table 4: Pipetting protocol for the dilution of the standard solution

Volume Standard Solution [µl]	Volume ddH_2O [µl]
20	180
40	160
60	140
80	120
100	100

For the blank 200 µl of deionised water were pipetted in an Eppendorf reaction tube. Dilution series and blank were put on ice. 100 µl of DNS solution were added to all reaction tubes and heated at 95 °C for 15 minutes via Eppendorf Thermomixer. The reaction solutions were cooled down on ice before the addition of 900 µl of deionised water.

For the determination of the enzyme activity 100 µl of starch solution and 100 µl of the enzyme were pipetted into a reaction tube. 100 µl of the starch solution were added into a reaction tube for the blank. Samples und blank were incubated for 3 min at 20 °C by shaking (300 rpm). After the incubation the reaction tubes were cooled down on ice. 100µl of DNS solution were added to the samples and 100 µl of enzyme solution were added to the blank. This mixture were heated at 95 ° C for 15 minutes and cooled down on ice. 900 µl deionised water were added before the measurement.

For the measurement with the Plate reader 200 µl of calibration solution, samples and blanks were pipette in a 96 well plate and measured at 540 nm. For the measurement with the Spectrophotometer the total volume were transferred into a cuvette and measured at 540 nm.
This assay can be used for the determination of the pectinase activity. The starch solution was replaced by pectin solution. D-(+)-galacturonic acid monohydrate was used for the standard solution for the calibration.

5.5. Antimicrobial effect of hydrogen peroxide

Procedure developed by Macomber et al. (2007) and Thomas et al. (1994) tested the antimicrobial activity of hydrogen peroxide. The procedures were modified for the test of antimicrobial activity of in situ produced hydrogen. The Gram negative organism *Escherichia coli* and the Gram positive organism *Staphylococcus aureus* were used as model organism. Lactose and maltose were used as substrates for the enzyme MtCDH to start the in situ hydrogen peroxide production.

An overnight culture of the tested organism was prepared in 20 ml 2xTY media at 37 °C, 125 rpm (Infors HT, Multitron shaker). Before the preparation of the test solution, the OD of the overnight culture was determined.

Lactose solution in the concentration range from 80 to 300 mg/ml was used for this study. 10 ml of the tested sugar solution was sterile filtered into a sterile 50 ml tube (Sarstedt). Afterwards 15 ml of sterile 2xTY media was added and well mixed. The reaction solution was incubated with the overnight culture to an OD of 0.3 (measured at 600 nm). The addition of 5 ml of Cellobiose dehydrogenase (MtCDH) started the in-situ production of hydrogen peroxide. A solution without MtCDH was prepared as control. Aliquots of 100 µl were taken out after 0, 10, 20, 30, 45 and 60 min and diluted in 900 µl of 1 % sterile sodium chloride solution. This dilution was further diluted to get countable amount of colonies on the plates. The dilutions 1:100.000-1:10.000.000 was plated out, incubated at room temperature. The survival colonies were enumerated after 2 days.

To get a growth inhibition rate, different concentrations of the tested sugar solution were used. The concentration range of the tested sugar solution was between 300 mg/ml and 80 mg/ml.

5.6. Biofilm formation assay

The biofilm formation assay as described by Croeset al. (2009) was used for the test of the effect of in situ produced hydrogen peroxide. Overnight grown cultures of *Staphylococcus aureus* are diluted to an OD^{560} of 0.1 in fresh TSB supplemented with 0.5 % glucose and 3 % sodium chloride.

Two considerations concerning the incubation time of *Staphylococcus aureus* were taken:

First is incubation of the microorganism for 24 h therewith a stable biofilm can be built up. Afterwards the in situ production of hydrogen peroxide was started. The effect of hydrogen peroxide on the existing biofilm and on the growth of further biofilm was analyzed.

Second is the in situ production of hydrogen peroxide before addition of the microorganism. The microorganisms were incubated in the reaction solution. For this study the effect of hydrogen peroxide on the formation of biofilm were examined.

Sample 1 was prepared by transferring of 150 µl of diluted overnight culture in triplicate into each well of a micro titer plate (polystyrene). The cultures were incubated for 24 h at 37° C without shaking.
Afterwards 80 µl of lactose solution and 40 µl MtCDH were added to the culture and were incubated for 20 min at 40°C without shaking.

Sample 2 was prepared by transferring of 150 µl of diluted overnight culture in triplicate into each well of a micro titer plate (polystyrene). The cultures were incubated for 1 h at 37° C without shaking. Afterwards 80 µl of lactose solution and 40 µl of MtCDH were added to the culture and were incubated for 20 min at 40° C without shaking.

Two blanks were prepared for each sample. One blank were prepared without MtCDH, the second blank was just the culture.

Further procedure (for all samples and blanks):
After the incubation time, the culture/reaction solutions were removed. Each well of the micro titer plate were washed 3 times with PBS. The adherent cells were fixed with 200 µl of 100 % ethanol for 10 min. Ethanol was removed and the cells were air dried for 2 min.
The adherent cells were stained for 2 min with 200 µl of 0.41 % Crystal violet solution (w/v in 12 % ethanol).
After the removal of the staining solution the cells were washed 3 times with PBS. The wells were dried on air and the stained cells were eluted with 200 µl of 100 % ethanol.
The absorbance was measured at 595 nm using a plate reader.

5.7. In vitro bleaching systems

The ability of enzymatically produced hydrogen peroxide to bleach pigments of cotton was investigated. For this study the pigments morin, isoquercitrin, rutin and quercetin were used which are phenolic compounds. Pereira et al. 2005 developed the oxidation of these phenolic compounds using the enzyme laccase. The procedure was modified for this study.

Various experiments were done to determine the bleaching effect of these phenolics. For the first experiment 20 µl of 5 mM stocksolution of phenolic compounds, 50 µl of MtCDH (14 u/ml), 100 µl of cellobiose and 830 µl of 0.1 M sodium acetate buffer pH 4 were incubated at 60 °C for 1 h. The same amount of enzyme was used for each experiment excluded experiment five. The second experiment was the incubation of the phenolic compounds with MtCDH without substrate. As third experiment the phenolic compounds were incubated in the same way than for experiment one but 100 µl of 1 mM desferrioxamine mesylate solution were added to exclude Fenton's reaction. The fourth experiment was performed in the same way than experiment one but 5 µl of 5 mM $FeSO_4$-solution were added to the reaction solution to induce Fenton's reaction. The last experiment was performed with chemical hydrogen peroxide which was added into the reaction solution at the same concentration level which was produced enzymatically. The reaction tubes were put on ice to stop the enzymatic reaction. The bleaching of the different phenolic compounds were monitored using UV/vis spectroscopy in wavelength scan mode within the range 200 – 500 nm at time 0 and after 1 h. Samples without enzyme were prepared and monitored in the same way for time 0. A dilution series (0.01 µM – 0.1 µM) of each pigment in 0.1 M sodium acetate buffer pH 4 was prepared and measured at the wavelength of the peaks which were disappeared during the oxidation to convert absorption difference into concentration difference.

5.8. Cotton treatment

The procedure described by Tzanov et al. 2001, Preša and Forte Tavčer(2007) and Opwis et al. 2005 studied the enzymatic cotton treatment was used with modifications. For this study the procedures were modified to develop an enzymatic bleaching system using MtCDH for the production of hydrogen peroxide. In the experiments starch sized, plain woven 100 % cotton fabric (137 g/m^2) was used. The fabric was cut into square pieces and weight before treatment. Diverse treatment conditions were tested to realize a good result after treatment. The first experiments were performed in smaller scale to screen different treatment conditions. Cotton treatments were achieved as single bath experiment in order to test the possibility to save resources.

5.8.1. Desizing

In the desizing procedure described by Tzanov et al. (2001), the fabric was desized by a commercial amylase (Rapidase L140). This amylase was for this study not available. Instead of that the sized fabrics were treated with commercial α-amylase from *Bacillus amyloliquefaciens.* Before the usage of the enzyme the activity was tested with the Amylase assay using DNS. The average activity of α-amylase was 2.56 u/ml or 273 mu/mg. Varying volume and different time period were tested to obtain the complete removal of the starch and to optimize the desizing liquor as substrate solution for the bleaching step. A part of the desizing water was used as substrate for the MtCDH activity assay using DCIP and for the determination of the hydrogen peroxide concentration. The activity of MtCDH obtained with desizing water (2 h incubation) was with 0.7 U/ml higher than the activity obtained with maltose. The hydrogen peroxide concentration determined with the desizing solution was with 40 µM. The sugar (glucose or maltose) concentration of the desizing solution was not analyzed. Due to this fact the comparison of the hydrogen peroxide concentration could not compared.

The constants in the desizing conditions were the temperature of 60 °C and the absence of shaking. Variables of the desizing condition were mentioned in following table:

Table 5: Variables of the desizing conditions The table shows the used amount of enzyme, the ratio of enzyme amount to volume buffer, the period of desizing and possible second treatment of the desizing liquor. If the desizing liquor is treated for a second time, the same amount of enzyme was used and the same conditions were used (except period). The fabrics were taken out of the liquid before start of the second treatment.

samples	Enzyme amount [u]	Enzyme amount/ volume buffer [u/ml]	Period [h]	Second treatment of the desizing liquor
10 - 17	0.32	0.128	1	
18 - 29	0.32	0.128	1	GOD 1-4 + 9-10 for 0.5 h
30 - 38	0.32	0.128	2	
39 - 41	0.32	0.128	1	for 1 h
42 - 45	3.20	0.128	2	
48	6.40	0.512	1	
49 - 53	6.40	0.427	2	

5.8.2. Scouring

Almost the half of the samples was scoured with commercial pectinase from *Aspergillus aculeatus* instead of the two different pectinases, which were used in the study of Tzanov et al. (2001). The usage of one type of pectinase is a simplification of the method. For comparison of the influence of scouring on the quality of the treated cotton, the second half of the samples was bleached without previous scouring. Before usage of the enzyme the activity was checked with the modified Amylase activity assay using DNS (see point 5.3.). The activity of the used enzyme amount was 65.35 u/ml. Pectinase was added to the desizing liquor to start the scouring process.

Table 6: Scouring conditions The table shows the used amount of enzyme, the ratio enzyme amount to volume buffer, period of scouring and scouring temperature.

samples	Enzyme amount [u]	Enzyme amount/ volume buffer [u/ml]	Period [h]	Temperature [°C]
10 - 17	0.82	0.33	2	40
18 – 21, 23 - 25	0.82	0.33	2	40
35	0.82	0.33	2	60
51	0.016	0.001	0.5	60
52 - 53	13.07	0.871	2	60

For the comparison of enzymatic souring with standard scouring, one sample was treated via boiling in 0.2 M sodium hydroxide for half an hour.

5.8.3. Bleaching

Bleaching procedure described by Tzanov et al. (2001) was used with modification to use MtCDH. The study of Preša and Forte Tavčer (2007) was an 'inspiration' for the usage of peracetic acid for bleaching. After desizing or scouring procedure bleaching of the fabrics were carried out in the same bath. Main part of the samples was bleached with the enzyme MtCDH (20.50 u/ml) and for comparison of the performance of the enzyme the other part of the samples was bleached with the enzyme glucose oxidase (2.5 u/ml). Two samples were bleached with the standard alkali bleaching process. The fabric was boiled in 0.2 M sodium hydroxide and 2.5 ml/g cotton of 35 % hydrogen peroxide for 0.5 h. For comparison of the bleaching performance of enzyme produced hydrogen peroxide and added hydrogen peroxide a bleaching experiment with fixed hydrogen peroxide concentration was carried out. The fabric was incubated in 0.1 M sodium acetate buffer pH 4.0 and 70 µM hydrogen peroxide at 60 °C for 0.5 h. Experimental set up for the enzymatic bleaching process are shown at table 7.

Table 7: Experimental set up for bleaching

Samples	Size of the fabric [cm^2]	Enzyme amount [u]	Enzyme amount/ total volume [u/ml]	Final substrate concentration [mg/ml]	Final concentration of activators/ stabilizer [mM]
10, 11, 31	2.25	5.125 MtCDH	1.464	Lactose: 42.9	-
12, 13	2.25	5.125 MtCDH	1.367	Lactose: 40.0	DCIP: 0.549
14, 15, 32, 35	2.25	5.125 MtCDH	1.367	Lactose: 40.0	Gluconic acid: 209.6
16, 17	2.25	5.125 MtCDH	1.281	Lactose: 37.5	Gluconic acid: 199.0, DCIP:0.188
18, 22, 26, 28, 36, 39	2.25	0.625 GOX	0.185	Glucose: 22.2	Gluconic acid: 209.6
19, 23, 29, 37, 40	2.25	0.625 GOX	0.200	Glucose:24.0	-
20, 21, 24, 27, 38, 41	2.25	0.625 GOX	0.238	-	-
25	2.25	0.625 GOX	0.217	-	Gluconic acid: 273.4
30, 34	2.25	5.125 MtCDH	1.783	-	-
33	2.25	5.125 MtCDH	1.640	-	Gluconic acid: 251.5
42	60	51.25 MtCDH	1.519	Lactose: 44.4	-

43	60	51.25 MtCDH	1.414	Lactose: 41.4	Gluconic acid: 216.6
44	60	51.25 MtCDH	1.414	Lactose: 41.4	EDTA: 0.069
45	60	6.25 GOX	0.185	Glucose:44.4	-
48	60	51.25 MtCDH	2.181	Lactose: 63.8	-
49, 50	60	51.25 MtCDH	1.898	Lactose: 68.2	Tetrasodium pyrophosphate: 15.52
51 - 53	60	51.25 MtCDH	1.956	Lactose:57.3	TAED: 14.9 (sample 51) 9.7 (sample 52) 85.9 (sample 53)
54	2.25	2.5 MtCDH	1.0	-	-
55	2.25	2.5 GOX	1.0	-	-
56	2.25	2.5 MtCDH + 2.5 GOX	1.0/enzyme	-	-

After treatment, the fabrics were taken out of the bath, rinsed with deionised water and were dried in a drying oven at 60 °C for about 24 h.

5.8.4. Monitoring of the bleaching results

After complete drying of the fabrics, the bleaching results were monitored via determing the weight loss, Iodine test, measuring the wetting time and measuring the brightness, the whiteness and color difference.

For the determination of the weight loss, fabrics were weighted before and after treatment after complete drying. The iodine test was used to determine the residual starch on the fabrics. 40 µl of Lugol'sche Solution was pipetted on the fabrics, after 5 min the fabrics were photographed and compared with an untreated sample.

The wetting time was monitored by dropping of 17 µl of deionised water on the fabric and the time for complete sinking of this drop was stopped. The residual parameters were measured using ColorLite Sph850 Spectrophotometer (d/8, D_{65}/10°).

6. Results

6.1. Screening of different carbohydrates for substrates of CDH

Different mono- and disaccharides were screened as possible substrates of CDH.The method is described at point 5.1.
The disaccharides cellobiose, lactose and maltose and the monosaccharide glucose were used for the determination of MtCDH activity. MtCDH did not show discrimination against glucose.
MtCDH showed the highest specific activity with lactose with 12. 7 U/mg protein which was two times higher than the specific activity achieved with the natural substrate cellobiose. The kinetic parameters were determined by linearization using the Lineweaver-Burk plot. The lowest K_m was obtained with the natural substrate cellobiose with 23.59 µM. The highest v_{max} was determined with lactose. Specific activity and kinetic parameters are shown in table 8 and the results of the screening are shown in the appendix.

Table 8: Kinetic parameters of MtCDH

Substrate	**Specific activity [U/mg]**	**K_m [µM]**	**V_{max} [U/mg]**
Cellobiose	6.32	23.59	0.099
Lactose	12.74	335.7	0.795
Maltose	0.56	3,928	0.027
Glucose	1.70	159,640	0.070

6.1.1. Other substrates

Higher carbohydrates were screened for possible substrates of CDH. CDH was not very active with dextrane or amylopectin as substrate, the achieved result was 0.02 to 0.04 units/ml. The obtained hydrogen peroxide concentration was about 0.47 mM. The results are shown in table 8.

Table 8: Determination of the activity of MtCDH using different carbohydrates as substrates

Substrate	Substrate Concentration [mg/ml]	Activity [U/mg]
Dextrane	10	0.0135
Amylopectin	10	0.0264
Carboxymethylcellulose	10	0.5871

6.2. Determination of hydrogen peroxide concentration

For better comparison of the substrates, the substrate concentration and the enzyme activity were fixed. The enzymatic produced hydrogen peroxide was measured with the assay (see point 5.3.). The highest hydrogen peroxide concentration was obtained with the natural substrate cellobiose with 18 µM followed by lactose. The results are shown in figure 13.

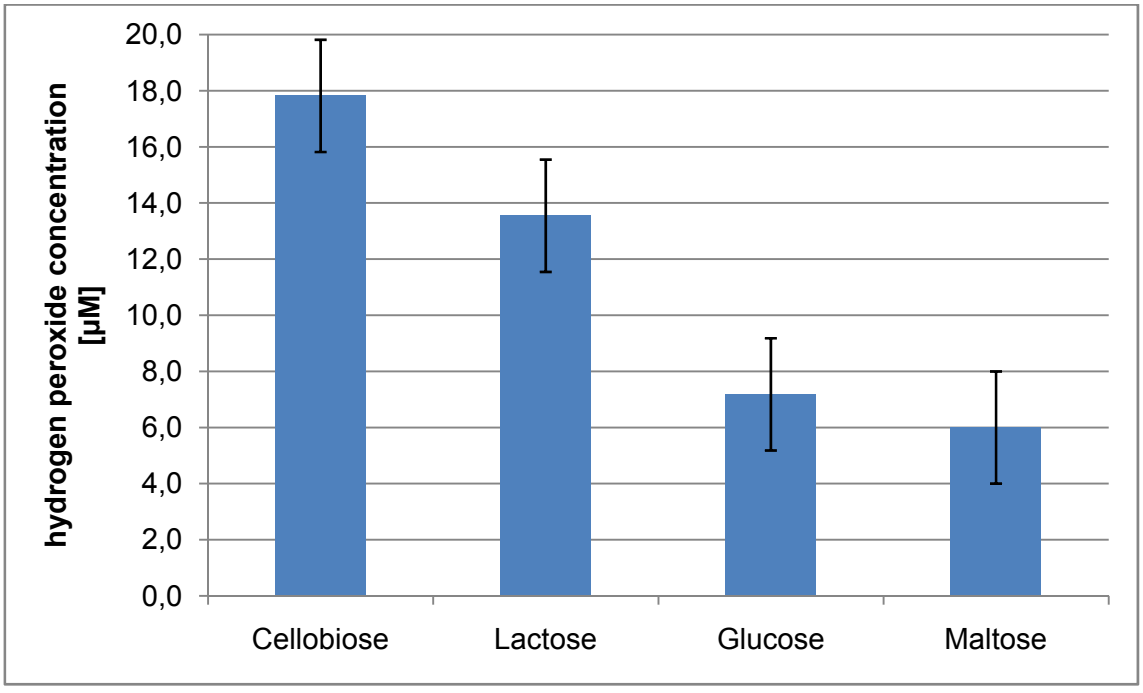

Figure 13: Measurement of enzymatic produced hydrogen peroxide

6.3. Antimicrobial effect of hydrogen peroxide

Antimicrobial effect of *in situ* produced hydrogen peroxide was tested with the model organism *Staphylococcus aureus* and *Escherichia coli*. The procedure is described at point 5.6.

The growth of *Staphylococcus aureus* was inhibited with both substrates in the same level. The lowest amount of survival was reached at 300 mg/ml for lactose with 21.2% and at 200 mg/ml for maltose with 23.6 %.

Less survival were after the incubation of *Escherichia coli* with lactose as substrate for MtCDH. The lowest % of survival was at 300 mg/ml for lactose with 13.7 %. The percentage of survival in the experiments with maltose as substrate for MtCDH was with 47 % quiet higher (Figures 14 and 15).

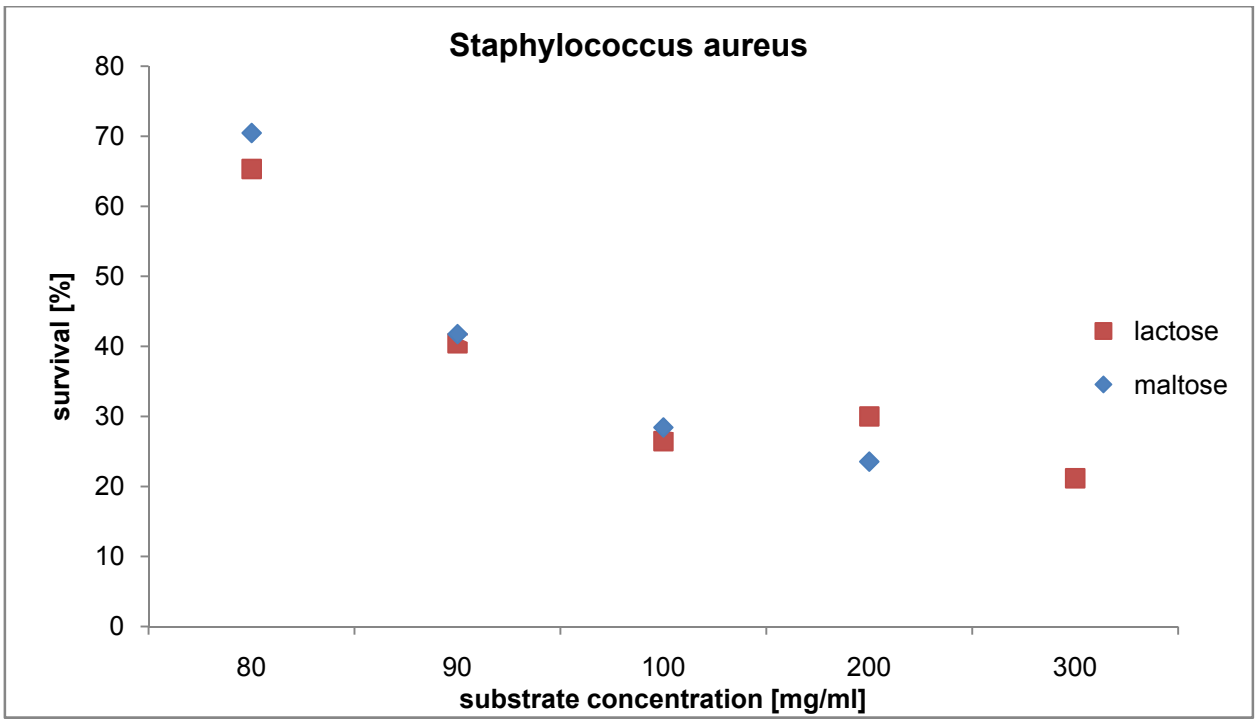

Figure 14: Antimicrobial effect of in situ produced hydrogen peroxide using *Staphylococcus aureus*

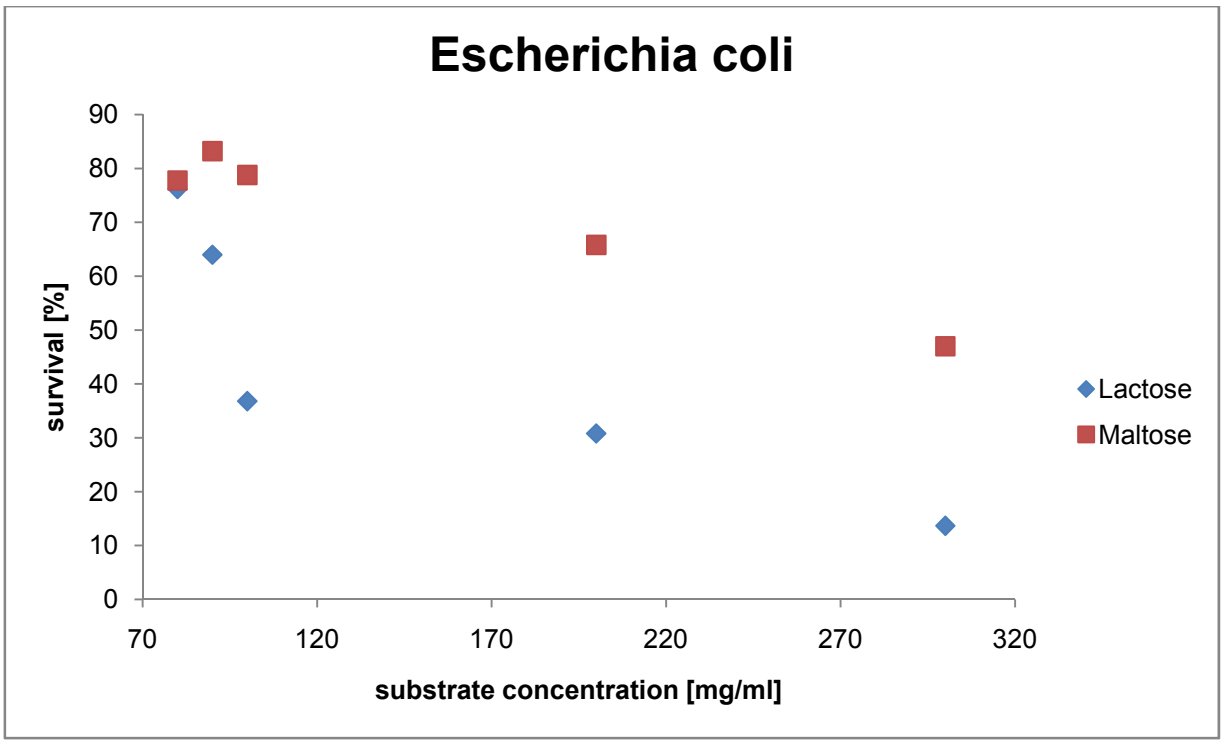

Figure 15: Antimicrobial effect of in situ produced hydrogen peroxide using *Escherichia coli*

6.4. Biofilm formation assay

The biofilm formation assay described by Croes et al. (2009) was used for the test of the effect of *in situ* produced hydrogen peroxide. The method is described at point 5.7. The highest inhibition of biofilm formation was obtained in the experiment with lactose as substrate for MtCDH with 52.7 % and in the experiment with cellobiose as substrate with 45.5 %. The inhibition of biofilm formation increased with the increasing concentration of substrate as summarized in Fig 16. At substrate concentration higher than 100 mg/ml, the inhibition of biofilm formation decreased.

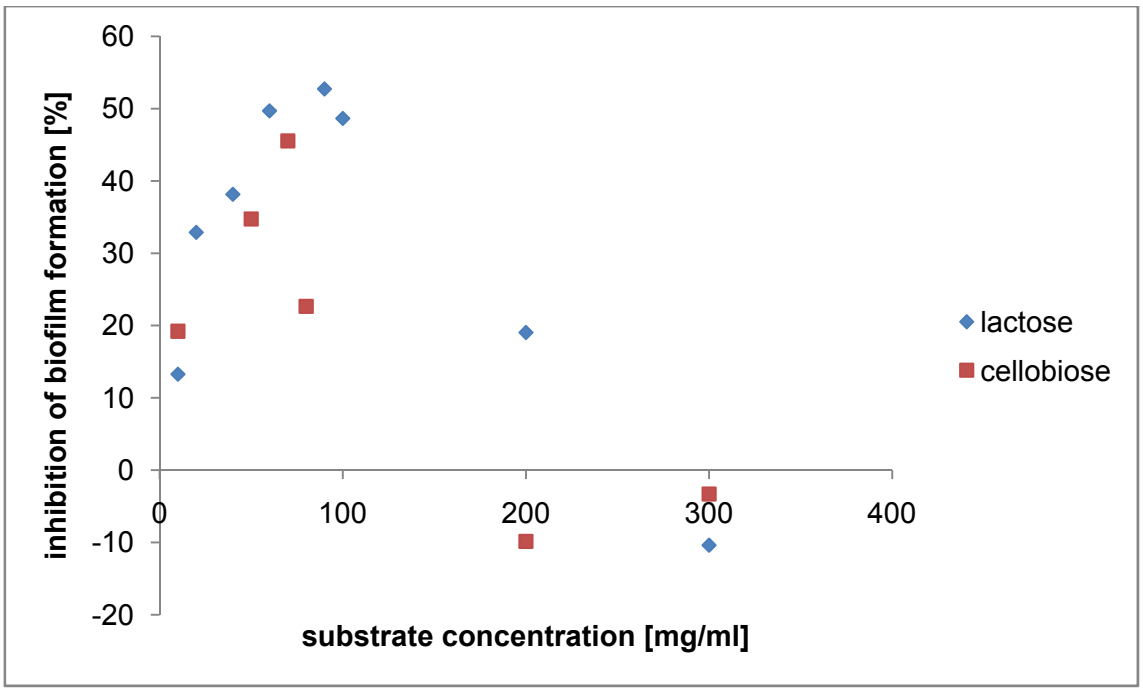

Figure 16: Biofilm formation assay using lactose or cellobiose as substrate and *Staphylococcus aureus* as test organism

6.5. In vitro bleaching systems

For this study different cotton pigments were incubated under different conditions. The method is described at point 5.8.

6.5.1. Rutin

Rutin (molecular structure of rutin is shown in Figure 17) was incubated with MtCDH and cellobiose or lactose.

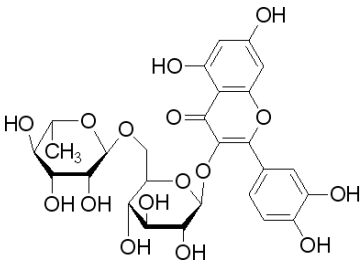

Figure 17: Structure of rutin (Sigma Aldrich)

The reaction was monitored spectrophotometrically in wavelength scan mode. The reaction solution was scanned before and after addition of the enzyme/hydrogen peroxide. The major differences in the spectra were marked with arrows. In both cases, the peak at about 230 nm was rising after addition of the enzyme, hydrogen peroxide or desferrioxamine mesylate. The addition of ferrous iron containing solution caused an absorption shift from more than 1.4 to lower than 0.4 at about 260 nm and disappearance of the peak at about 430 nm. This peak (430 nm) was also removed in the other experiments except the experiment two (MtCDH without substrate).The scans are shown in figure 18.

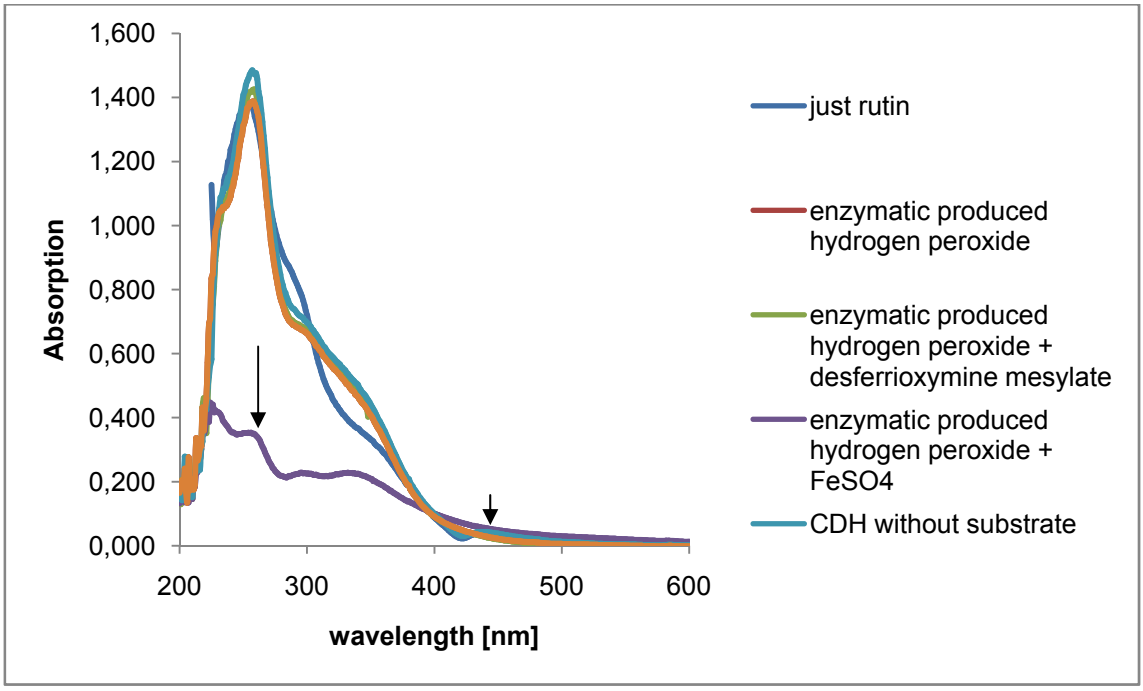

Figure 18: Bleaching of rutin under different conditions

6.5.2. Morin

The other cotton pigment was incubated and the reaction was monitored in the same way as rutin. The molecular structure of morin is shown in figure 24.

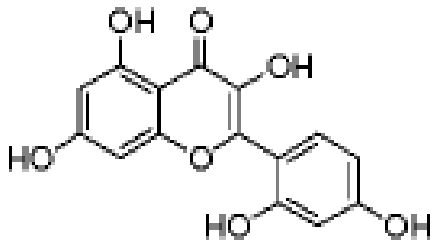

Figure 19: Structure of morin (Sigma Aldrich)

The spectra were showing bigger differences than the spectra of rutin. The incubation of morin with MtCDH and cellobiose as substrate effected a better oxidation than with rutin. All experiments yielded in an absorption shift at about 260 nm and 346 nm. A third peak at about 430 nm disappeared during the reaction excluding experiment 2 (MtCDH without substrate). A dilution series of morin was prepared and measured at 346 nm for the determination of the concentration difference of morin after 60 min. The conversion of absorption into concentration showed that 9 % of morin was oxidized by MtCDH alone, 43 % was oxidized with enzymatic produced/chemical hydrogen peroxide and after addition of desferrioxamine mesylate and 61 % of morin was oxidized after addition of ferrous containing solution after 60 min reaction. The scans are shown in figure 20.

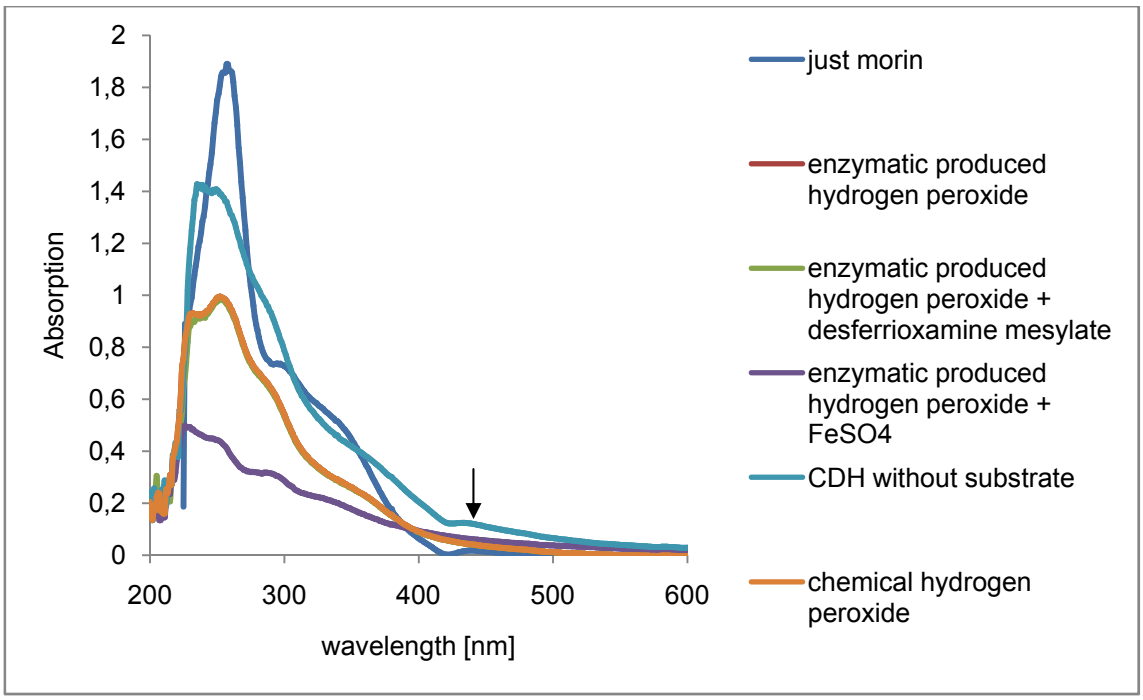

Figure 20: Bleaching of Morin under different conditions

6.5.3. Quercetin

The third tested cotton pigment was quercetin. The experiment was done in the same way than with the other pigments and for monitoring the results a wavelength scan was done. The molecular structure is shown in figure 27.

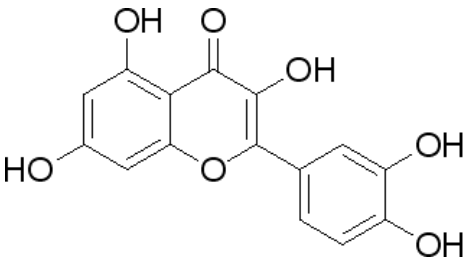

Figure 21: Structure of quercetin (Sigma Aldrich)

The in vitro bleaching of quercetin caused a decrease of the peak at about 430 nm excluded experiment 2 (MtCDH without substrate). An incubation of quercetin with ferrous iron containing solution yielded into a decrease of the peak at about 360 nm and a shift of the absorption in the UV-range to higher absorption. The scans are shown in figure 22.

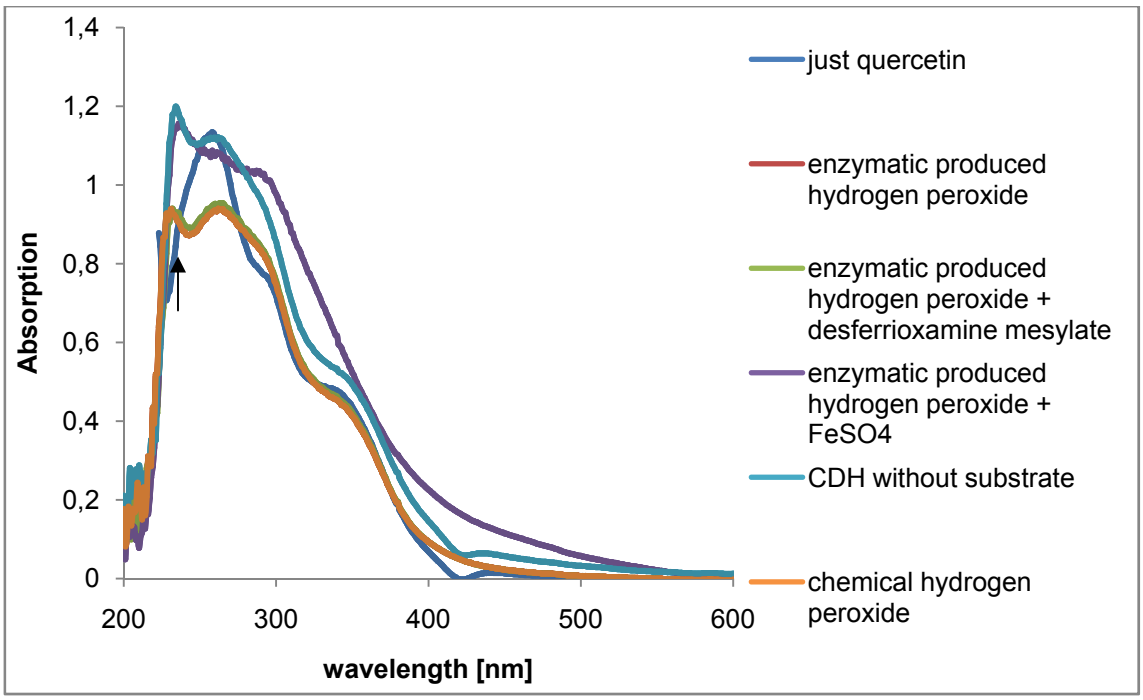

Figure 22: Bleaching of Quercetin under different conditions

6.5.4. Isoquercitrin

Isoquercitrin as last phenolic compound was tested for the oxidation with MtCDH and cellobiose and lactose as substrate. The molecular structure of isoquercitrin is shown in figure 23.

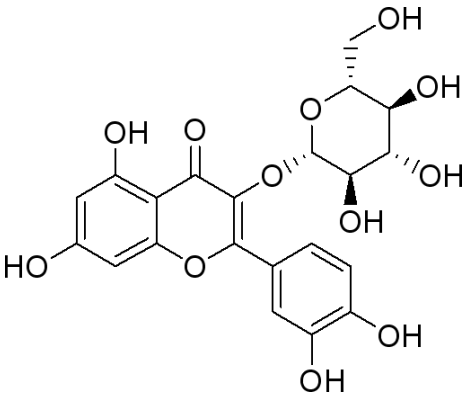

Figure 23: Structure of isoquercitrin (Sigma Aldrich)

The oxidation of isoquercitrin with MtCDH was weak except the incubation with ferrous iron containing solution. The difference in the wavelength scans between 0 and 60 min

was unincisive. The incubation with ferrous iron containing solution caused a large absorption shift in the UV-range. The peak at about 430 nm also disappeared after 60 min incubation excluded experiment two (MtCDH without substrate). The scans are shown in figure 24.

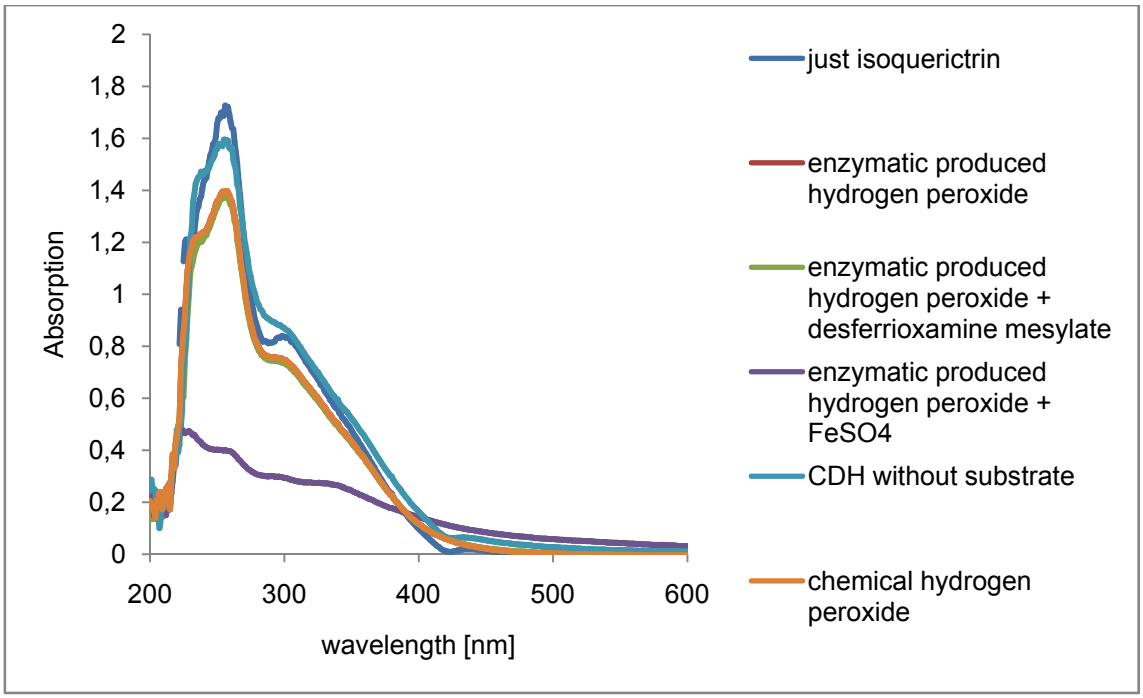

Figure 24: Bleaching of Isoquercitrin under different conditions

6.6. Results of cotton treatment

After the treatment of the cotton fabric the results were monitored as described at point 5.9.4. Sized cotton fabrics were desized with alpha amylase from *Aspergillus niger* with the activity of 2.56 U/ml (273 mU/mg). Several samples were treated 0.5 hour, 1 hour or 2 hour at 60° C without shaking. Highest weight loss was achieved after 1 hour desizing with 20 %. This value was determined after the whole treatment process, including desizing, scouring and bleaching. About 10 % of the fabric is the starch size. During the enzymatic cotton treatment were other components like pectins, waxes and parts of the cellolytic surface removed. This can influence the weight loss. The percentage of

residual starch was determined via Iodine starch test. Raw cotton fabrics were used as blank (= 100 % residual starch). The highest amount of starch was removed from the samples which were desized for one hour. Longer desizing did not remove more starch from the cotton fibers but degraded the carbohydrate containing liquid into smaller sugars. Results are shown in figure 24 and 26.

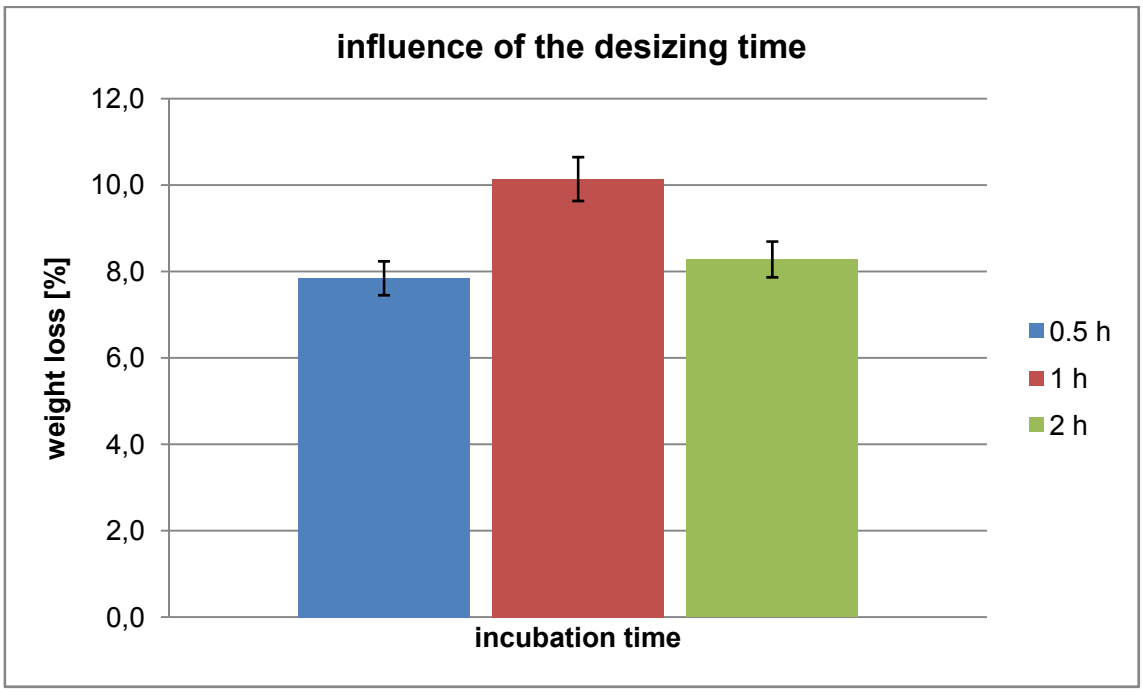

Figure 25: Influence of the desizing time on the weight loss of the fabrics. The fabrics were weighted before treatment and after treatment if they were complete dried.

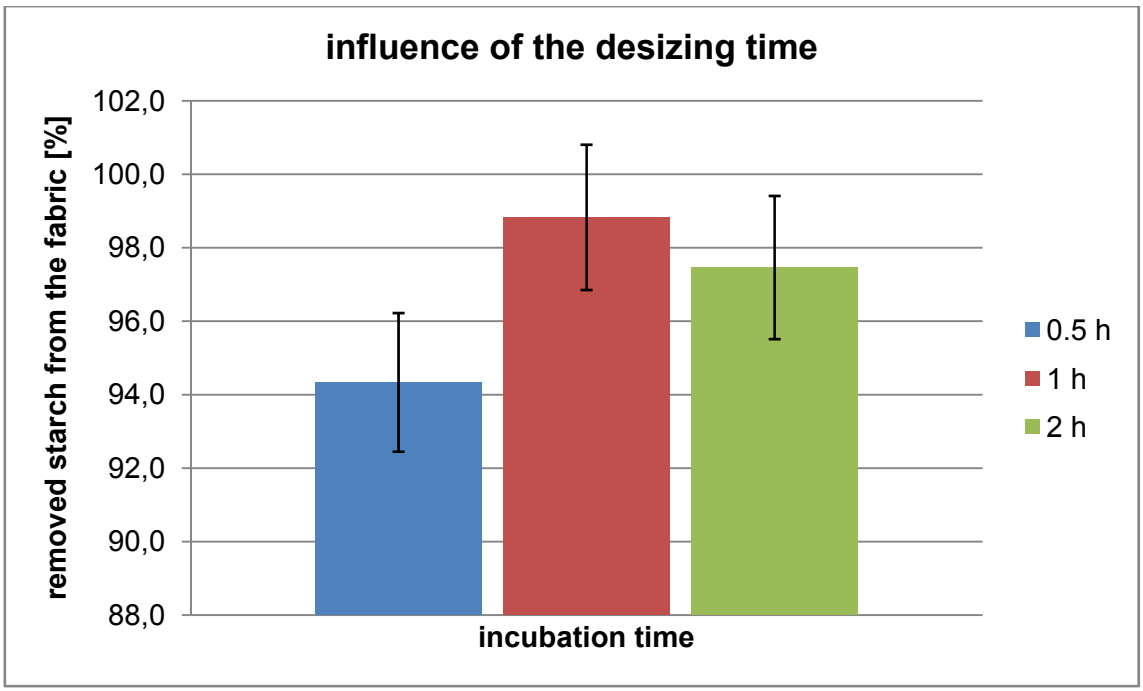

Figure 26: Influence of the desizing time on the removal of starch

For determination of the influence of scouring a part of the samples were scoured with pectinase from *Aspergillus aculeatus*. The other part of the samples was not scored before bleaching. The aim of scouring was to remove pectins, waxes etc. from the raw cotton. The shortest wetting time was 2 seconds and the longest 568 seconds. The results are shown in figure 27.

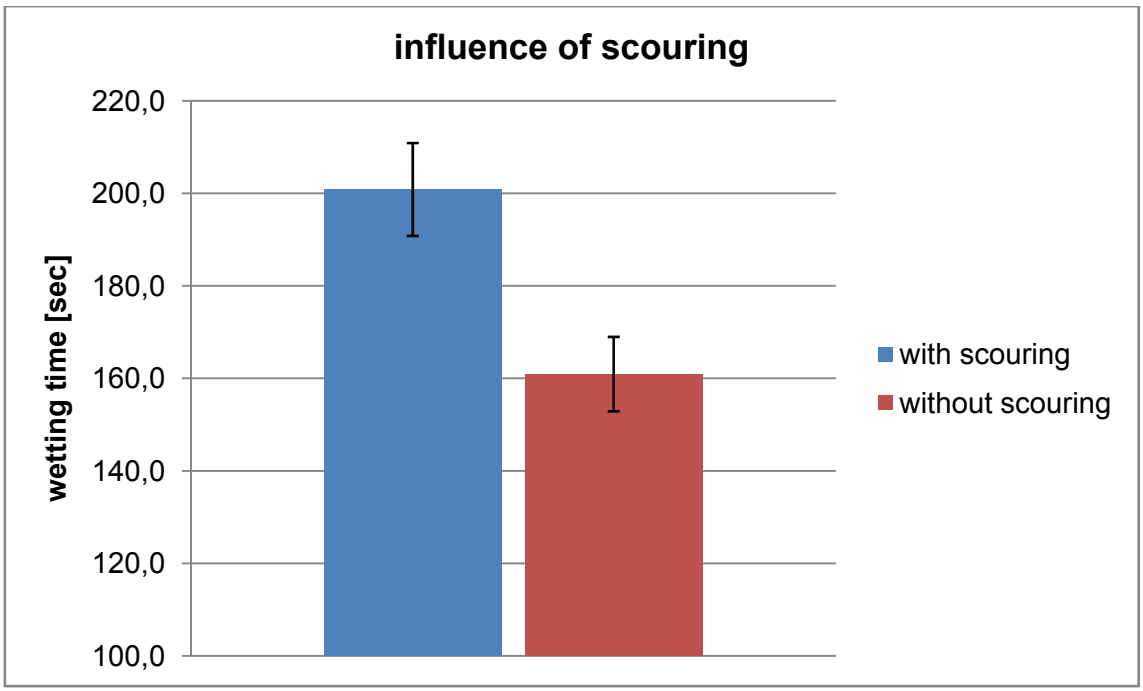

Figure 27: Influence of scouring on the wetting time. The wetting time is an indicating factor for the hydrophobicity of the surface. The longer the wetting time is the more hydrophobic is the surface.

The influence of addition of stabilizer or activator on the resulting lightness of the fabrics is shown in figure 28. Addition of stabilizers like gluconic acid or sodium pyrophosphate resulted in 94.8 (0 = black, 100 = white) a higher brightness of the fabric.

The activation of hydrogen peroxide via shifting of the pH to 8 was more effective because the same results were obtained after bleaching of a half of the time.

The influence of additional substrate for MtCDH was low. The samples without additional substrate showed a brightness of 91 whereas the samples with additional substrate exhibit a brightness of 92.

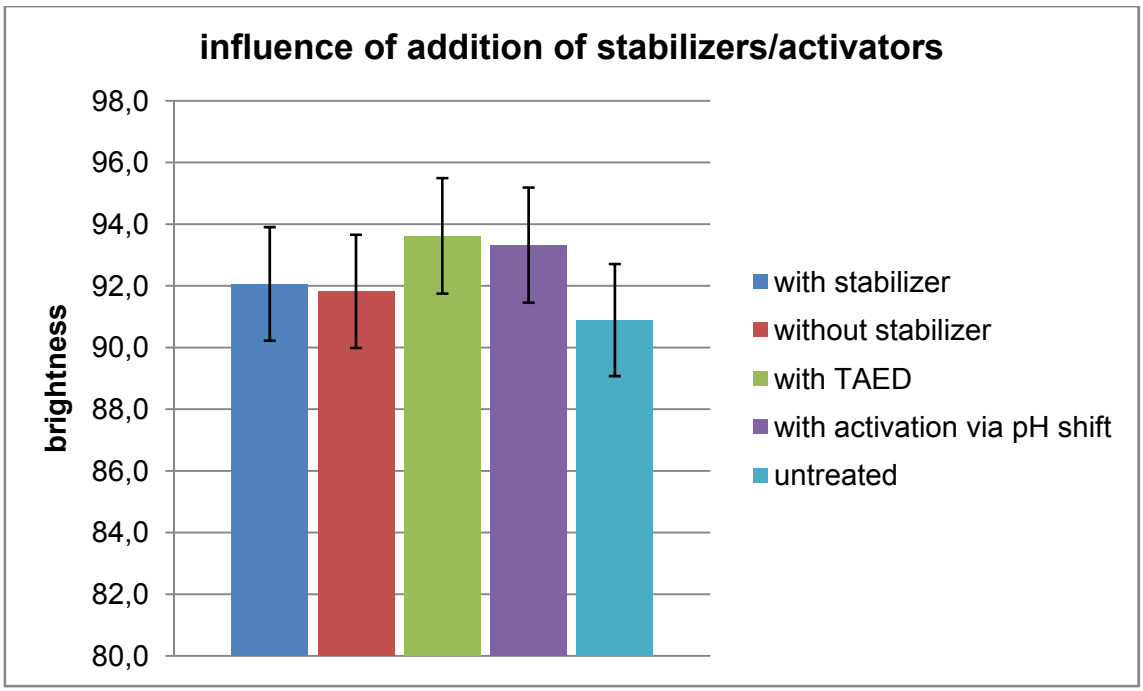

Figure 28: Influence of the addition of stabilizer or activator on the resulting brightness of the fabrics after treatment

For the comparison of the bleaching results a part of the samples were bleached with the enzyme glucose oxidase. The bleaching results obtained with MtCDH were slightly higher than the results with glucose oxidase. The results are shown in figure 28.

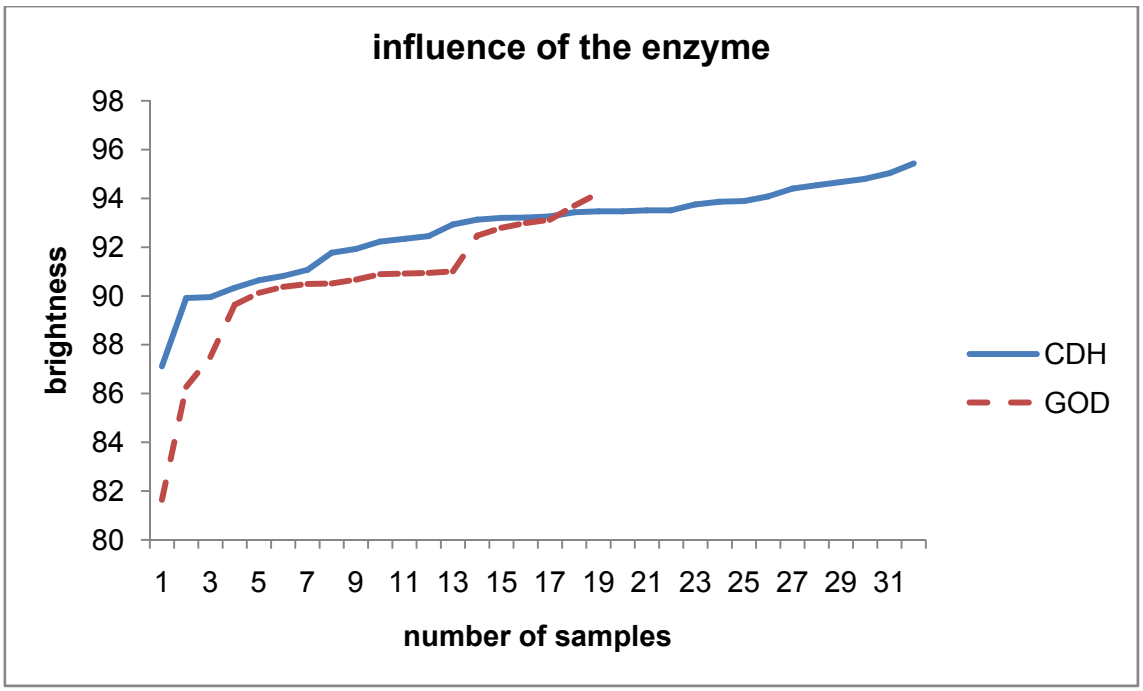

Figure 28: Influence of the usage of different enzymes for the bleaching result

Bleaching of the samples was performed in different ways. In one experiment the hydrogen peroxide production and bleaching was performed simultaneously. For the second experiment the hydrogen peroxide production and the bleaching of the fabrics were performed in two separate steps. The third experiment was similar to the first but after a certain time, activators or stabilizing agents was add to the bleaching bath. 2 samples were bleached with the standard bleaching process (see point 5.9.3.).

The results of these bleaching processes were similar. Addition of activators/stabilizators shortened the bleaching time to half an hour. Bleaching with the standard process took the same time. The highest obtained brightness of the fabrics were 93 (standard bleaching process), 91 (simulate and 2 steps) and 92 (with additional activators/stabilizer). For comparison a bleaching experiment with the same hydrogen peroxide concentration at the same conditions was started. A brightness of 76 was obtained in both experiments. The difference of the brightness of the last experiments and the experiments before was due to the limitation of the enzyme activity to 1 u/ml in the last experiments which led to less hydrogen peroxide. The exact experimental set up is mentioned at point 5.9.3. The results are shown in figure 29.

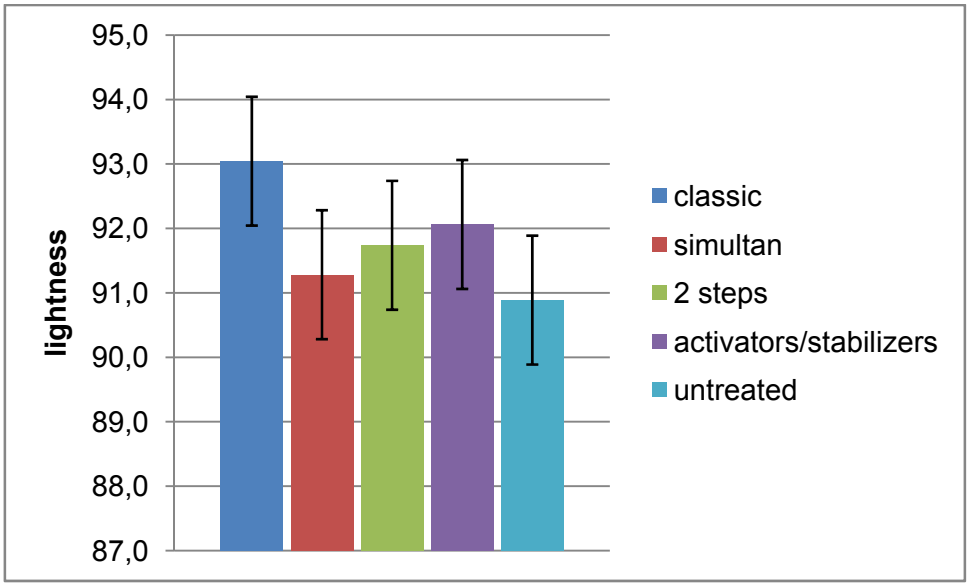

Figure 29: Influence of different bleaching types (classic = bleaching with the standard bleaching process, simulate = hydrogen peroxide production and bleaching was performed simultaneously, 2 steps = hydrogen peroxide production and bleaching was performed in 2 steps, activators/stabilizer = similar to simulate but addition of activators or stabilizers, untreated = raw cotton fabric)

7. Discussion

7.1. Screening of different carbohydrates for substrates of CDH

Different mono-, di- and oligosaccharides were screened as possible substrates for MtCDH. The results of the kinetic studies showed that lactose was the best substrate for MtCDH followed by cellobiose. The disaccharide maltose was a poor substrate because of the difference of the linkage between the glucose subunits. Cellobiose a β-1,4 linked disaccharide and maltose an α-1,4. The results of this study confirmed that the orientation of the connection between the sugar subunit at the reducing end and the next sugar subunit is important for the enzyme.

The enzyme produced during the study of Pricelius et al. 2009 had a lower specific activity (3.5 U/mg) than the MtCDH used in this thesis (12.7 U/mg). A reason of this difference could be the difference of the production of the enzyme. In the study of Pricelius et al. 2009 the enzyme was produced in the natural producing organism using cellulolytic conditions. The MtCDH produced for this study was cloned into *Pichia pastoris.* Production of cloned MtCDH led to a higher specific activity than MtCDH produced by the natural organism. However, this comparison is based on a comparison with published data for the native enzyme while purity and assay conditions might also play a role.

7.2. Comparison of hydrogen peroxide concentration

Previously, the catalytic activity of CDH with oxygen as electron acceptor was shown to be at least 100 times lower than that with other electron acceptors (Gorton et al. 2010).This was also the reason why long time ago the name cellobiose oxidase for this enzyme has been changed to CDH (Gorton et al. 2010).Also the bleaching with CDH occurred in the presence of oxygen as an electron acceptor. The assay described by Pricelius et al. (2009) was used to determine the hydrogen peroxide concentration with fixed enzyme activity, substrate concentration and oxygen as electron acceptor.

Highest hydrogen peroxide concentration was obtained with cellobiose. The results of this assay were differing from the results of the study of Pricelius et al. (2009). In this study the highest hydrogen peroxide concentration was obtained with cellobiose and lactose which were up to 6 times lower than the obtained hydrogen peroxide in this study although the same activity level was used. In the study of Pricelius 0.14 mg of enzyme protein was used for the determination of the hydrogen peroxide concentration which was about 3 times higher than the used protein amount of MtCDH. The specific activity of MtCDH obtained during the study of Pricelius was with 3.5 U/mg about 3 times lower than the specific activity of the recombinant MtCDH used during this thesis (12.7 U/mg).

The difference of the specific activity of the native and recombinant MtCDH was not really explainable, because the amino acid sequences of both enzymes were the same. The glycosylation pattern was due to the different production organism slightly different but this could not explain the difference. Maybe there was a mistake in the determination of the protein amount or a mistake in the determination of the purity of the enzyme.

7.3. Antimicrobial effect of hydrogen peroxide

The antimicrobial effect of *in situ* produced hydrogen peroxide was tested with the model organism *Staphylococcus aureus* and *Escherichia coli*. The results of the incubation of *Staphylococcus aureus* indicated a decreasing of the percentage of the survival with the increase of substrate. *Staphylococcus aureus* was not eliminated during the incubation with CDH and lactose or maltose as substrate but more as 2/3 of the cells of *Staphylococcus aureus* was killed.

The results of incubation of *Escherichia coli* with CDH with lactose as substrate are similar to the results obtained with *Staphylococcus aureus*. The killing of *Escherichia coli* using maltose as substrate is not as effective as lactose. The reason is the

microorganisms can metabolite maltose easily and the uptake rate to the organism is higher than the reaction rate of the enzyme. Other reason could be that a hydrogen peroxide concentration greater than 100 µM is initially bacteriostatic or lethal for *Escherichia coli* (Demple and Halbrook1983) and the hydrogen peroxide concentration obtained during the incubation was lower than 100 µM.

Bacteria like *Escherichia coli* produce catalase, superoxide dismutase and peroxidase which detoxify hydrogen peroxide; *Staphylococcus* lacks this enzyme (Thomas et al. 1994, Imlay and Linn 1986). This confirms with the results of this study, that hydrogen peroxide is more toxic for *Staphylococcus aureus* than for *Escherichia coli*. Hydrogen peroxide has a greater activity against gram negative than gram positive bacteria. In the presence of iron, hydrogen peroxide will be converted into the more toxic hydroxyl radicals. The hydroxyl radical is highly reactive and can attack membrane lipids, DNA and other essential cell components. It is described that the antimicrobial action of hydrogen peroxide due to the oxidation of sulfhydryl and double bonds in proteins, lipids and surface membranes (Block 2001).

7.4. Biofilm formation assay

The biofilm formation assay described by Croes et al. (2009) was used for the test of the effect of in situ produced hydrogen peroxide on the formation of biofilms. The method is described at point 5.7. *Staphylococcus aureus* is known as biofilm producing microorganism. Biofilm formation plays an important role in the pathogenesis of staphylococcal infection (Beenken et al. 2003). Formation of staphylococcal biofilm was inhibited by the incubation of CDH and lactose or cellobiose as substrate for CDH which was tested in this study for the first time. The results divide from the expectation. An increase of the inhibition of the biofilm formation with the increase of the substrate for CDH was expected. The inhibition of biofilm formation decreases at a substrate concentration of 90 (cellobiose) to 100 mg/ml (lactose).

The reason could be the metabolization of lactose by the cells which are embedded in the staphylococcal biofilm is faster than the enzymatically reaction. This is the main

problem of in situ produced hydrogen peroxide, because substrates of CDH are sugars and carbohydrates which can metabolize by microorganism easily. A test without additional substrate could solve the problem because biofilms are rich in polysaccharides (Götz 2002). These polysaccharides could be substrate for CDH to produce hydrogen peroxide.

7.5. In vitro bleaching systems

Flavonoids like rutin, morin, quercetin and isoquercitrin are present in cotton and are responsible amongst other components for dark color. The aim of this study was the possible oxidation of these flavonoids and the in vitro bleaching of these cotton compounds. Pereira et al. (2005) tested the oxidation of flavonoids (morin, luteolin, rutin and quercetin) using laccase. The results of the study of Pereira et al. were in the similar range than the results of this study but not really comparable because the used enzymes were different. In this study MtCDH was used for this experiment for the first time.

The results of the incubation of rutin with MtCDH with substrate or added desferrioxamine mesylate caused a weak reaction in the range of UV light but the peak at about 430 nm which is in the range of visible light disappeared in the most experiments. The results yielded into an oxidation but it seems that after the oxidation of the C3 group a reactive species were generated which could polymerize (Kurisawa et al. 2003).

The results of the incubation of morin with MtCDH and cellobiose confirm the expectations. The results show an oxidation and a bleaching of rutin. Peaks at about 340 and 430 disappeared after 60 min. It seems that there was a reaction between morin and MtCDH but in a lower dimension than the reactions containing enzymatic produced hydrogen peroxide or chemical hydrogen peroxide.

The incubation of quercetin yielded in a weak reaction excluded the reaction with ferrous iron containing solution. The peak at about 430 nm disappeared in all hydrogen peroxide containing reactions. Incubation with ferrous iron containing solution yielded an absorption shift in the range of UV light and the peak at about 360 nm disappeared. It seems that the reactive oxygen species which were produced during the Fenton's reaction reacted another position than the reaction with hydrogen peroxide.

The results of incubation of isoquercitrin indicated a weak reaction excluded the reaction with ferrous iron containing solution. The peak at 430 nm disappeared in the hydrogen peroxide containing reaction solutions. It seems that isoquercitrin was not oxidized during the reaction with cellobiose as substrate. The glycosyl group could sterically hinder the oxidation at the C3 group or too less hydrogen peroxide or reactive oxygen species were produced to oxidize isoquercitrin completely.

After comparison of all tested flavonoids indicates that the presence of a hydroxyl group at the C3 position is more favorable than the presence of bigger glycosyl groups because small hydrogen is easier to abstract than a big glycosyl group. This confirm with the results of the study of Pereira et al. (2005). The results of in vitro bleaching indicated that an addition of ferrous iron induces a greater absorption shift due to a possible oxidation of these compounds.

The results confirmed the expectation that enzymatic produced hydrogen peroxide and reaction with chemical hydrogen peroxide which was added in the same concentration led to the same effect.

7.6. Cotton treatment

For this study sized raw cotton was desized, scoured and bleached using enzymes in a single bath process. The method is described at point 5.9.

Desizing

Sized cotton samples were desized with α-amylase from *Aspergillus niger.* Highest weight loss of the fabrics and the lowest amount of residual starch on the fabric was achieved after 1 hour incubation.

A high weight loss and the lowest amount of residual starch were obtained after 1 but the desizing water after 2 hour incubation was a better substrate for CDH. The action of amylase resulted to the production of smaller sugars (i.e. maltose or glucose) which were better substrates than bigger carbohydrates. The aim of this study was the reuse of desizing water for bleaching as the fabrics were desized for 2 hours to obtain more substrate for CDH.

Scouring

A part of the samples were scoured with pectinase from *Aspergillus aculeatus* to study the influence of scouring on the characteristics of the surface of the fabrics. The wetting time of scoured fabrics was lower than the wetting time of the unscoured fabrics. The scouring step was carried out without surfactants because the possibility to get the right surfactant was not given. “The surfactant is a necessary component in the enzymatic composition for scouring and seemed to have a high impact in the removal of waxes and fats, while the enzyme facilitates the removal of the pectin substances” (Tzanov et al. 2001). This statement confirms with the results of this study. The surface of the samples was more hydrophilic than the sample of the study of Tzanov et al. (2001).

Bleaching

After desizing and scouring, the samples were enzymatically bleached. Main part of the samples was bleached with CDH which were used for bleaching of cotton fabrics for the first time. For comparison of the bleaching results residual samples were bleached with glucose oxidase. The experimental set up for bleaching is described at point 5.9.3. and table 7.

Bleaching was carried out in different ways. In the first experiment hydrogen peroxide production and bleaching were performed simultaneously. For the second experiment hydrogen peroxide production and bleaching were separated into two steps. Third experiment was performed similar to the first experiment with additional activators or stabilizers. For comparison 2 samples were bleached with the standard alkali bleaching process.

Addition of stabilizer did not improve the bleaching results very well. The activation via pH shift to pH 8 and the addition of TAED improved the bleaching results. The bleaching time was divided in half and the obtained lightness was nearly the same. The pH shift did not influence the enzyme activity negatively because MtCDH is in a broad pH range active.

The addition of further substrate for CDH did not improve the bleaching result. It seems that the desizing water contains enough substrate for CDH to produce hydrogen peroxide.

Bleaching with CDH obtained better results than the bleaching with glucose oxidase. CDH can use a broader spectrum of substrates for the hydrogen peroxide production than glucose oxidase.

The results of different ways of bleaching were similar. Division of hydrogen peroxide production and bleaching led to better results than the simultaneous hydrogen peroxide production and bleaching.

Bleaching with the standard alkali bleaching process was unfortunately better than the bleaching with enzymes. Catalytic activity of CDH with oxygen is at least 100 times

lower than that with other electron acceptors (Gorton et al. 2010) which make it the rate limiting step during the production of hydrogen peroxide. Other electron acceptors are colored substances (i.e. methylen blue, ABTS, Baminger et al. 2001). The reaction solution after complete reaction of the enzyme is not colorless. This residual color influences the bleaching results.

8. Conclusions and future recommendations

Various carbohydrates and sugars were screened for possible substrates for newly produced recombinant CDH from *Myriococcum thermophilum*. CDH could oxidize a broad spectrum of carbohydrates. The results indicated the possibility of using desizing water to develop an enzymatic bleaching system. CDH was able to use desizing water for bleaching of cotton. For further application of CDH in enzymatic bleaching of cotton, the procedure and the performance of CDH has to be optimized. Especially the catalytic activity of CDH with oxygen as electron acceptor has to be improved to obtain a more effective system.

The enzymatic production of hydrogen peroxide to develop an antimicrobial system yielded into promising results. To develop the antimicrobial system for application further studies have to carried out to exclude the effect of the CDH substrates to support growth of microorganism.

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10. References

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11. Appendix

11.1. List of chemicals

Chemicals	**Company/Tradename**
2,6-Dichloroindophenole sodium salt hydrate	Sigma-Aldrich/Fluka
Lactose monohydrate	Carl Roth GmbH + Co KG
Acetic acid	Carl Roth GmbH + Co KG
Ethanol	Carl Roth GmbH + Co KG
D(+)-Cellobiose	Carl Roth GmbH + Co KG
Sodium fluoride	Sigma-Aldrich/Fluka
Glucose oxidase f. Aspergillus niger	Sigma-Aldrich
Sodium dihydrogenphosphate dehydrate	Carl Roth GmbH + Co KG
Sodium chloride	Carl Roth GmbH + Co KG
D(+)-Maltose monohydrate	Carl Roth GmbH + Co KG
Starch (potatoe)	Sigma-Aldrich/Fluka
Acetic acid anhydride	Sigma Aldrich/Riedel de Haen
α-amylase f. Bacillus amyloliquefaciens	Sigma Aldrich
α-D(+)-glucose monohydrate	Carl Roth GmbH + Co KG
Sodium acetate trihydrate	Carl Roth GmbH + Co KG
EDTA disodiumsalt dihydrate	Carl Roth GmbH + Co KG
Crystal violet	Merck
Potassium dihydrogenphosphate	Sigma Aldrich
Hydrogen peroxide	Carl Roth GmbH + Co KG

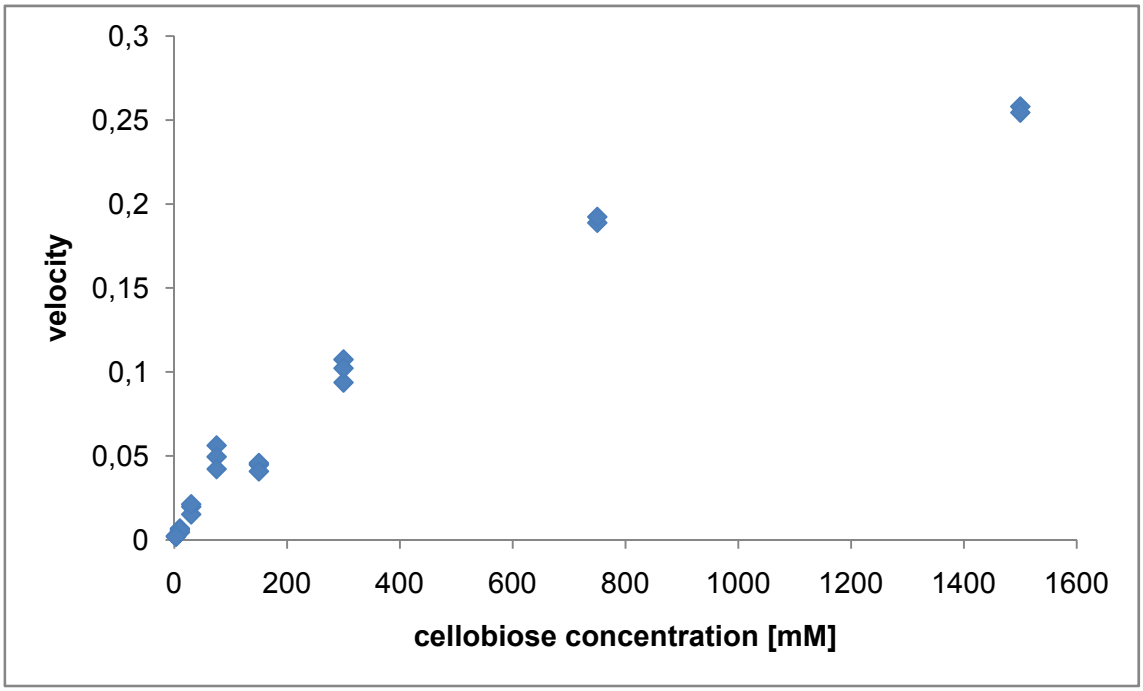
D-gluconic acid lactone	Sigma Aldrich
Dextrane f. Loconostoc. Ssp.	Sigma Aldrich/Fluka
Cellulast 1,5 L (Cellulase)	Novozymes
CASO bouillon	Carl Roth GmbH + Co KG
Potassium dihydrogenphosphate	Carl Roth GmbH + Co KG
Potassium chloride	Carl Roth GmbH + Co KG
di-sodium hydrogenphosphate	Carl Roth GmbH + Co KG
Tetra-sodiumdiphosphate 10 hydrate	Merck
Sodium carbonate	Carl Roth GmbH + Co KG
Peroxide 100 Test strips	Machete-Nagel
Pectinase from Aspergillus aculeatus	Sigma Aldrich
N,N,N',N'-Tetraacetylethylendiamine (TAED)	Sigma Aldrich
Bio-Rad reagent f. determination of the protein concentration	Bio-Rad
Bovine serum albumin	Sigma Aldrich
Carboxymethylcellulose sodium salt	Serva Feinbiochemica
Manganese(II)-sulfate-1-hydrate	Merck
Copper(II)-sulfate pentahydrate	Merck
Magnesium sulfate heptahydrate	Carl Roth GmbH + Co KG
Calcium sulfate dehydrate	Merck
Zinc sulfate heptahydrate	Merck
Ferrous sulfate heptahydrate	Merck
Dipicolinic acid	Merck
Rutin hydrate	Sigma Aldrich
Quercetin	
Morin	Riedel de Haën
Peracetic acid	Fluka
Alginate	
Sodium persulfate	Riedel de Haën

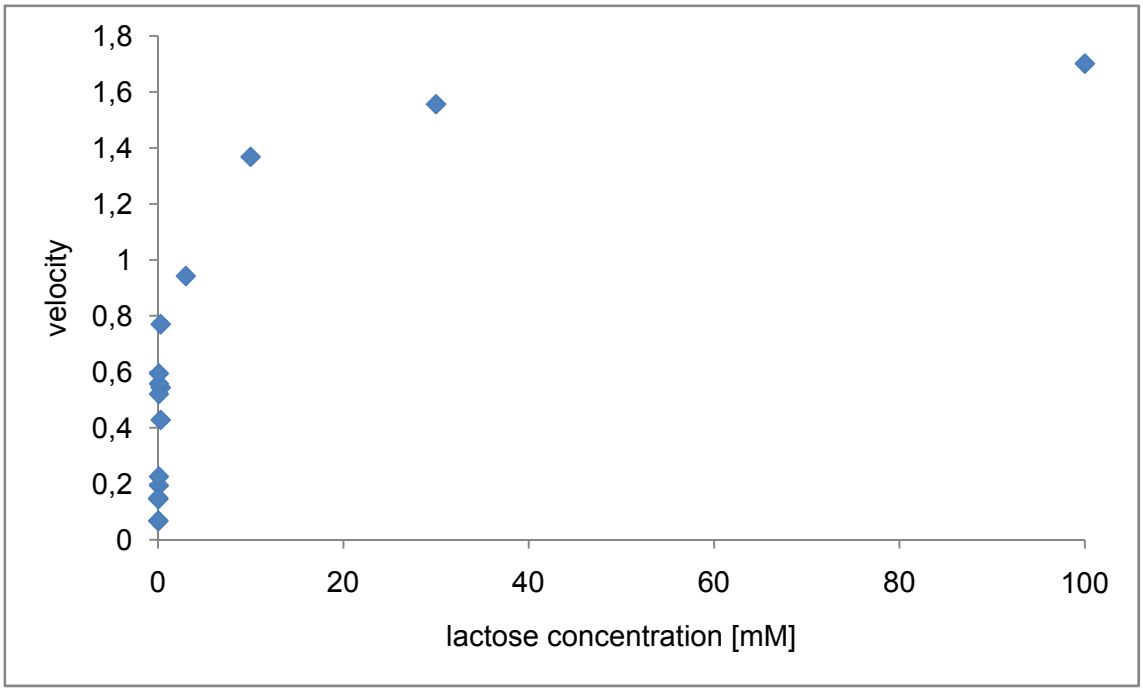
11.2. List of equipment

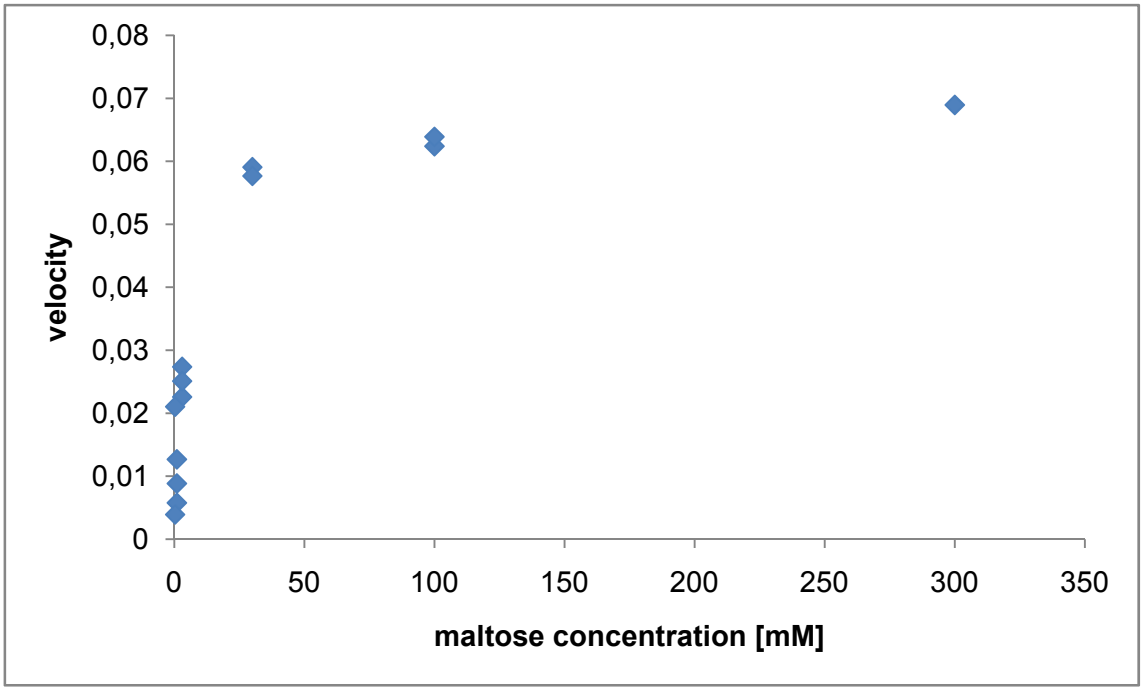
List of equipment

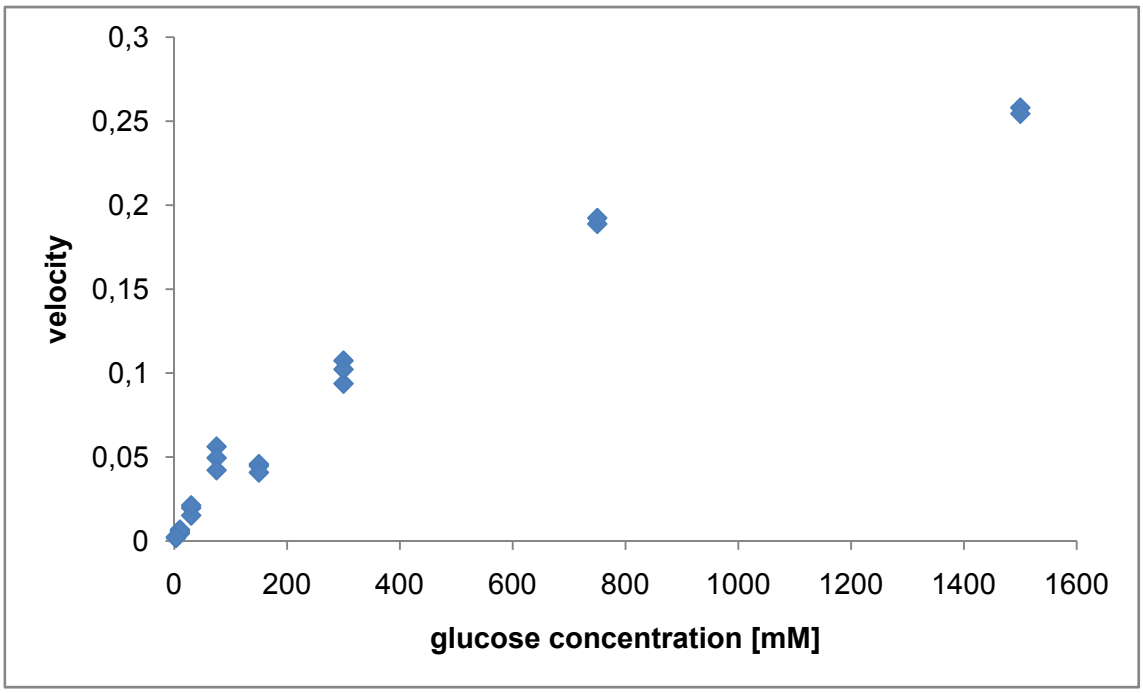
Eppendorf Thermomixer comfort
U/2001 Hitachi, spectrophotometer, INULA Austria
Eppendorf BioPhotometer
ColorLite sph850 spectrophotometer, ColorLite
Multitron INFORS HT shaker, Bartelt
TECAN Platereader, infinite M200
Eppendorf MiniSpin desk centrifuge

11.3. Results of substrate screening






0,08
0,07
0,06
0,05
0,04
0,03
0,02
0,01
0
velocity
0
50
100
150
200
250
300
350
maltose concentration [mM]


0,3
0,25
0,2
0,15
0,1
0,05
0
velocity
0
200
400
600
800
1000
1200
1400
1600
glucose concentration [mM]

11.4. Cotton treatment results

summary cotton treatment

size: 10 x 6 cm

samples	weight loss [%]	wetting time [sec]	Whiteness (Berger)	Iodine Test	L	a	b	ΔE
42	7,7	72	32,2	0	93,13	1,00	8,98	5,24
43	7,5	262	30,5	0	93,43	1,06	8,82	5,49
44	6,4	21	30,6	0	92,34	2,20	11,8	2,48
48	10,9	2	22,9	3	90,33	1,38	9,84	4,65
49	5,9	14	28,6	2	92,23	1,30	10,60	3,81
50	6,9	2	39,4	2	93,51	1,46	9,68	4,97
51	7,7	36	28,0	2	93,26	2,88	10,6	3,94
52	6,7	175	36,4	2	94,40	1,33	9,60	5,40
53	7,0	45	34,4	0	93,20	1,91	10,9	3,70
45	6,4	9	n.d.	0	90,92	1,61	9,63	4,68
46	7,9	4	65,5	0	93,12	1,30	3,80	10,55
47	7,9	6	57,2	0	92,97	1,27	4,50	9,84
untreated		4200	23,0	100	90,89	2,48	15,5	

size: 1.5 x 1.5 cm

samples	weight loss [%]	wetting time [sec]	Iodine Test	L	a	b	ΔE
30	7,1	24	3	93,86	0,79	8,89	5,72
31	7,1	25	3	92,45	1,01	8,57	5,26
32	6,5	165	5	94,8	0,95	9,2	6,08
33	12,5	134	5	90,82	0,73	9,12	4,99
34	8,7	15	5	93,51	0,98	9,36	5,11
35	9,7	191	5	91,93	0,95	8,34	5,33
36	6,9	568	3	90,89	0,81	7,58	5,91
37	14,3	37	2	90,37	0,99	9,3	5,15
38	6,9	27	2	90,49	0,86	7,91	5,56
39	15,4	205	2	90,12	0,79	8,79	4,74
40	15,6	16	5	93,7	0,73	8,92	5,62
41	20,0	34	10	87,5	1,05	7,82	6,35

size: 1.5 x 1.5 cm

samples	weight loss [%]	wetting time [sec]	Iodine Test	L	a	b	ΔE
10	9,7	245	0	91,77	0,66	10,11	4,88
11	14,8	305	0	92,93	0,78	9,61	5,62
12	16,7	91	2	87,11	1,36	8,79	6,98
13	6,9	210	2	89,91	1,77	8,19	6,48
14	13,3	12	0	91,07	0,85	9,01	5,78
15	12,1	22	2	93,47	0,9	8,62	6,67
16	9,1	10	2	90,64	1,49	9,66	5,00
17	9,1	15	2	89,95	1,45	9,02	5,71
18	5,9	490	0	92,79	0,44	8,11	7,02
19	3,6	110	0	94,21	0,61	10,14	5,83
20	8,3	280	0	81,64	0,56	4,43	13,85
21	7,7	90	0	90,67	0,86	9,25	5,55
22	3,7	1110	0	92,99	0,37	8,11	7,10
23	11,1	485	0	90,94	0,91	8,21	6,53
24	7,4	243	0	89,64	1,14	8,30	6,52
25	3,1	24	0	91,01	0,28	6,72	8,14
26	9,7	30	0	90,51	1,06	8,58	6,16
27	9,4	145	0	86,27	-0,85	3,96	12,03
28	12,1	350	0	93,12	0,89	8,78	6,40
29	8,6	170	0	92,47	0,97	8,99	5,98
untreated		4200	negative	90,89	2,48	15,46	