# Development of a novel biotechnological process for the production of high quality linen fibers

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## Summary

In this thesis a novel biotechnological process for the production of high quality linen fibers was established. Flax fibers, embedded in a woody matrix inside the stems and glued strongly together by polysaccharides (pectic material), are extracted with a process called retting. Dew retting is done in the fields and is highly weather dependent. If the weather is too wet, the fibers are damaged, making them weak and useless.

The process described in this work uses whole cells of the pectinolytic strain *Geobacillus thermoglucosidasius* PB94A and green decorticated fiber as starting material. In contrast to the whole stems, the raw fibers account only for about 30% of the total straw weight, which reduces costs and waste streams. The major advantage of using a whole cell treatment is the low cost of the bacterial broth and its reusability. *G. thermoglucosidasius* PB94A is a thermoalkaliphilic spore-forming bacteria. When grown on citrus pectin, the strain produced pectinolytic lyases, which were excreted into the medium. When flax fibers were incubated in the fermentation broth of *G. thermoglucosidasius* PB94A, the fineness of the fibers increased without damaging the main component of the fibers, the cellulose.

A 200 L scale pilot plant for the production of long flax fibers by the new process was designed, built and operated successfully. The fiber treatment process was optimized. The process developed for the production of high quality fibers consists of:

1. a mild alkaline incubation ($Na_2CO_3$),
2. incubation with the strain *G. thermoglucosidasius* PB94A,
3. a softening, shive removal wash with a $H_2O_2$-softener solution.

In experiments reusing the solutions from steps *1-3* for up to six times, the fiber quality was equally high for all batches. The fiber fineness was improved by 70% in comparison to the starting green fiber. No cellulases were detected and the fibers remained strong. More than 130 kg of high quality fibers were successfully produced in the pilot plant.

The long flax fibers produced with the new process are of high quality and can be used to produce fine yarns by wet spinning. Since all solutions can be reused, the process generates few waste streams. The fiber yield and quality are higher than those of dew retted fibers. The new biotechnological treatment eliminates the risks associated with dew retting completely and produces reliably fibers of consistent high quality. This thesis is the basis for the implementation of the new fiber retting alternative on a technical scale.

# Contents

# Nomenclature

| | |
|---|---|
| $A_{550}$ | absorbance at 550 nm |
| APS | ammonium persulfate |
| ATR | attenuated total reflectance |
| AU | absorbance units |
| $BOD_5$ | biochemical oxygen demand in mg/L |
| bp | base pairs |
| $C_{Av}$ | average cells in a large square of a Neubauer chamber |
| Cer | Ceramtec |
| COD | chemical oxygen demand in mg/L |
| CPD | critical point drying |
| $\Delta A$ | absorbance difference |
| DF | dilution factor of the original sample |
| DGGE | denaturing gradient gel electrophoresis |
| dH2O | distilled water |
| $\varnothing$ | diameter |
| DNA | deoxyribonucleic acid |
| DSM | Deutsche Sammlung von Mikroorganismen und Zellkulturen |
| dtex | mass in grams per 10 km |
| $\varepsilon$ | molar extinction coefficient |
| EA | enzymatic activity |
| EDTA | ethylene diamine tetra acetic acid |

Fig. figure

FTIR fourier transform infrared spectroscopy

× g g-force or relative centrifugal force

G. *Geobacillus*

IR infrared

ktex mass in kilograms per 1 km

LB Luria Bertani

$L_n$ volumetric flow at normal conditions (0°C and 1.013 bar)

MHDP meta-hydroxydiphenyl

min minute

m slope

MM master mix

M Molar

NIR near infrared spectroscopy

Nm metric yarns number: length in meters per 1 g of mass

NMR nuclear magnetic resonance

OD optical density

ORP oxidation reduction potential

PB94A *Geobacillus thermoglucosidasius* PB94A

PBR packed bed reactor

PBS phosphate-buffered-saline

PCR polymerase chain reaction

PE pectinesterase

PFA paraformaldehyde

PG polygalacturonase

PL pectate lyase

PLS partial least squares

PNL pectin lyase

Por Poraver glass beads

PP pilot plant

PUF polyurethane foam

$R^2$ correlation coefficient

$\rho$ density in $g/cm^3$

RMSEC root mean squared error of calibration

rpm revolutions per minute

RT room temperature

S standard deviation

sec seconds

SEM scanning electron microscopy

Sty Styropor

TAE tris-acetate-EDTA

$T_d$ fiber fineness in dtex

TEMED N,N,N',N'-tetramethylethylenediamine

TE tris-EDTA-buffer

tex mass in grams per 1 km

UV ultraviolet

v/v volume per volume

V volts

w/v weight per volume

# Chapter 1

# Introduction

## 1.1 The flax plant

Flax is the common name for members of the Linaceae, a family of annual herbs, especially for members of the genus *Linum*, and also for the fiber obtained from such plants. Several varieties of *Linum usitatissimum* have been cultivated for commercial purposes since prehistoric times. Flax is classified in the division Magnoliophyta, class Magnoliopsida, order Linales [69].

Flax is bred with the focus on producing seed (linseed) or fiber (flax). Its cultivation has expanded from its native region in Eurasia to most temperate zones of the world that provide a suitable habitat (a cool, damp climate) for its cultivation as a fiber plant. It is mainly grown in China, Russia, France, Belgium and Eastern Europe [46, 69].

Flax is a highly valuable plant used for textile and technical applications, such as specialty papers, composites and insulating material. Linseed on the other hand is grown in tropical as well as in temperate countries for its oil-bearing seeds [69]. It is one of the main crops in Canada for the production of linseed oil, which is used in food and chemical industries. The seeds are crushed to make linseed oil, and the remaining cake is used for fodder. Additionally, dried flaxseed has been used in various medicinal preparations [69].

Flax is an annual plant and grows up to 120 cm in height and produces blue or white flowers that mature into bolls (capsules) containing 10 oval seeds each. When grown for fiber, flax is sown densely to prevent branching and promote a taller plant. Side branches would produce lower quality fibers. Fiber flax must be gathered before maturity. On the contrary, branching is desirable for linseed, so the plant is sown sparsely and allowed to branch out to produce more seeds [69].

## 1.2 Flax products and byproducts

Plants that are not used for food purposes are a considerable source of renewable vegetable raw materials. They are used in industrial applications or as energy source. The so-called "industry plants" provide usable substances like starch, sugar, oils, colors, fibers, etc. Flax belongs to this category.

From 6 t/ha of harvested flax stems, 2 t/ha correspond to fibers, of which 1.1 t/ha are long flax fibers. The long flax fibers are at the top of the added-value chain. These long fibers are processed in the textile industry to 0.55 t of yarn and are used to produce fine yarns (wet spun) and strong threads [16, 69]. The short fiber, called tow, is a byproduct of the textile industry. It is used nowadays at the automotive industry as cushioning material for the seats, joined with sisal fiber as inside covering of the chassis and as replacement for asbestos in the brake pad [94]. Short flax fibers are used for composite material, textiles and paper. Further income is derived from the sale of linseed and shives [16]. Other uses of flax fibers or its byproducts include: acoustical and thermal insulation, animal bedding, composite fillers, composite reinforcement, sails, coarse yarns, fleeces, pots, geomats, interior auto parts, nonwovens, specialty pulp and papers, fiberboard, etc. [8, 62].

## 1.3 Flax fiber history

Flax is one of the oldest textile fibers known to humanity. It was used by the Egyptians to wrap mummies and the quality of the fabrics produced back then, is still today unsurpassable. Flax had an important role for clothing as well as for industrial purposes, such as sail material [46]. Traditional fiber production and primary processing techniques have been developed over centuries to produce high value long fiber material for the textile industry.

Until the 18th century, flax was the major source of cloth fiber, but driven by the industrial revolution, the cotton industry displaced the flax industry, which did not have such a well-developed process. The price difference was by then so large, that flax became a niche market for luxury goods, i.e. for bedding items because of its comfort characteristics. With the boom of the petrochemical industry, the growth of the synthetic fibers displaced the linen even more [46]. The competitive use of other natural fibers, such as jute affected the flax industry further [69]. The main reasons why flax culture is not so widespread are the complexity and the lack of knowledge for extracting the fiber from the plant. A deficit of infrastructure for further processing, the cost of the fiber production and the heterogeneity of the fibers are also deterrents for a more generalized use of flax [87].

## 1.4 Flax fiber structure

Flax is a bast plant, which means that its fibers are located in the inner bark of the stems, embedded in a woody matrix, as shown in Fig. 1.1. Inside the inner bark is a woody tissue (shive).

The fiber characteristics from the top, middle and bottom parts of the stem vary, e.g. the fibers from the bottom have more lignin. The stem is comprised of five main layers and a pith cavity as follows [51]:

**epidermis:** covered with a thin layer of wax, which prevents excessive evaporation and protects the plant.

**cortex:** not lignified circular cortical cells that contain pectin and coloring substances.

**fibers or bast layer:** the fiber bundles run throughout the length of the stem and are surrounded by parenchyma (fundamental tissue of plants).

**cambium layer:** tender growth tissue (thin-wall cells), that separates the fibers from the woody tissue.

**interior woody tissue (xylem):** thick-wall cells (exterior) and thin-wall cells (interior) that surround the pith cavity.

**pith cavity:** air chamber extending throughout the length of the stem.

The fibers account for less than 30% of the stem mass and must be separated from the bast layer for extracting the fibers. The fiber strands can be over one meter long (min-max: 0.1-1.3 m) and are composed of fiber bundles of overlapping single ultimate fibers [34, 97]. These long fibers are glued together into bundles by lignin, pectin and hemicellulose. The fibers act as a scaffold that confers the plant a great stability.

One general feature of natural fibers is their asymmetry. The flax stem is thicker near the root and thinner near the tip, and the elementary fibers in the flax are consequently irregular. Also the long stretched cross section of the elementary fiber is an irregular polygon. On average, a fiber is 19 $\mu$m wide (min-max: 8-31 $\mu$m). The elementary fibers are also heterogeneous longitudinally, they are in average 32 mm long with a minimum and maximum value of 8 and 69 mm respectively. Most fibers have a hollow core (lumen), which is bigger in the middle part of the elementary fiber and disappears toward the end of it. In an immature fiber, the lumen is larger and the cell walls are thinner compared to a mature fiber [51, 78]. In Fig. 1.2 the cross sectional view of fiber bundles embedded in a resin (most of them are individualized) is shown.

The cell walls of flax fibers contain numerous defects, known variously as nodes, slip planes, kink bands, dislocations or micro-compressive defects [21]. Fig. 1.3 shows flax

fibers observed with a light microscope, where some nodes at the surface of the fibers are present. Here, the nodes are located at the same position for all the fibers within the bundle. Some individualized fibers protrude out of the bundle.

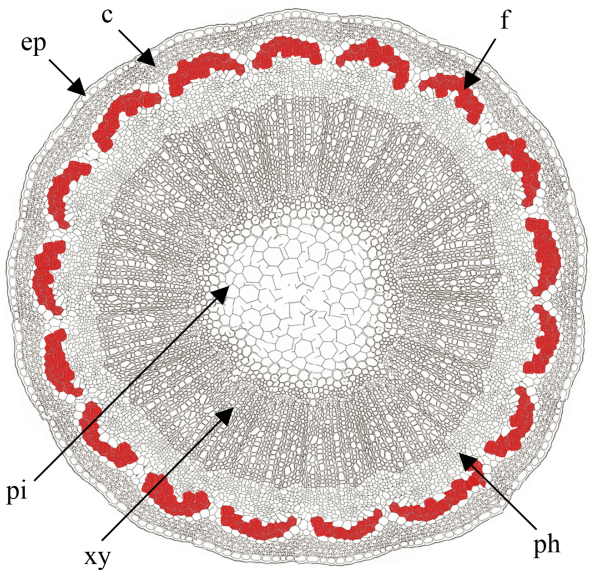



**Figure 1.1**: Cross sectional scheme of a flax stem. The fiber bundles (f) are highlighted and are located close to the periphery of the stem; (ep) epidermis; (c) cortical parenchyma; (ph) phloem; (xy) xylem; (pi) pith. Modified from [53].

## 1.5 Chemical composition of flax fibers

### 1.5.1 Composition of flax fibers

The elementary flax fiber consists of highly crystalline cellulose fibrils spirally wound in a matrix of amorphous hemicellulose and lignin. The fibrils are oriented in a slight tilt angle with respect to the axis of the fiber and hence display a unidirectional structure [21].

The three main polymers: cellulose, lignin and matrix polysaccharides (pectic substances and hemicelluloses), are joined to cellulose and lignins at the cell wall. Some minor components such as wax, inorganic salts, colors and nitrogen compounds are also present. The most important component is cellulose, which has a chain length between 2000 and 10 000 daltons. A description of the main components of the flax fiber is given below [44, 78]:

**cellulose:** linear homopolysaccharide of $\beta$-D-glucopyranoside units linked through $\beta$-(1-4) glycosidic bonds. The cellulose content of the flax fiber is 62.8%.

**hemicellulose:** amorphous, low molecular weight cell-wall polysaccharides, associated with cellulose and lignin, and located through the fiber. The monomeric units of

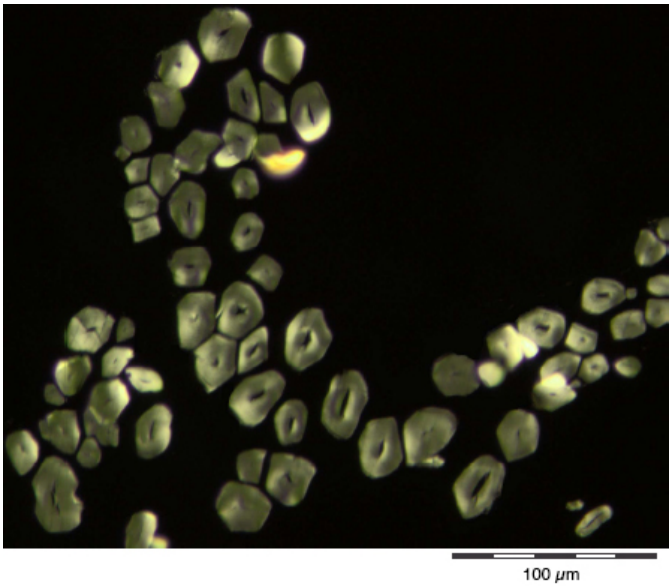



**Figure 1.2**: Cross sectional view of flax fiber bundles, most of them are individualized. A lumen is present in most of the fibers.

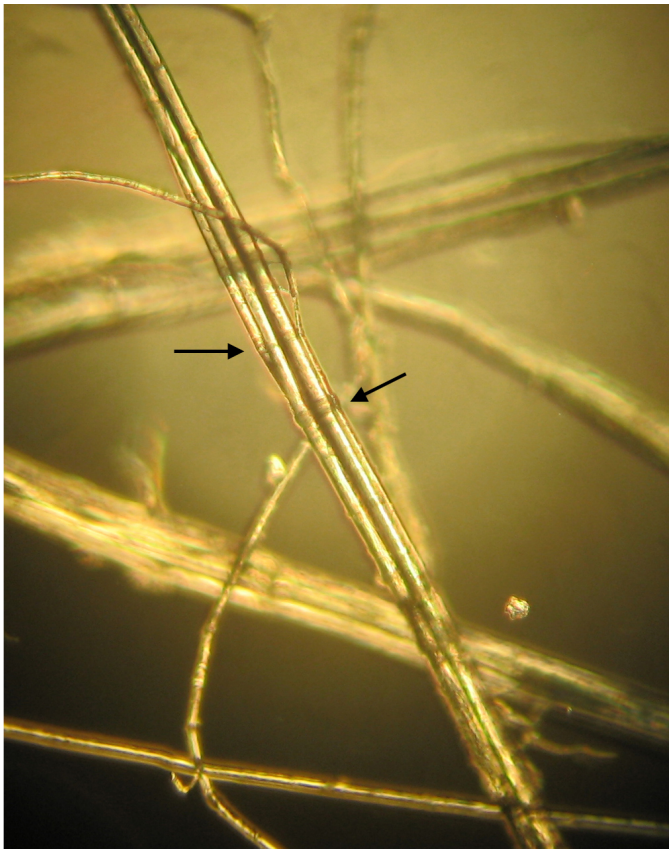


**Figure 1.3**: Longitudinal view of a flax fiber bundle, taken with a light microscope. Fiber defects (nodes) are marked with arrows.

the main chain are D-glucose, D-galactose, D-mannose, D-xylose and L-arabinose joined together in several combinations and by various glycosidic linkages. The hemicellulose content of the flax fiber is 17.1%.

**pectic substances:** complex branched heteropolysaccharides primarily containing (1-4) residues of $\alpha$-D-galacturonic acid. The residues can be esterified (randomly acetylated and methylated) mainly in the carboxylic group, other sugar units (rhamnose) can be intercalated in the chain, and branching of the main backbone with other sugar types can occur. The type depends on the maturity of the plant, position in it and many other factors. In flax, pectic substances are associated with hemicellulose as cementing material between the elementary flax cells. In Fig. 1.4, the basic pectin structure is shown. The pectin content of the flax fiber is 4.2%. Three different pectins have been isolated from plant cell walls [15]

- **-homogalacturonan:** composed of $\alpha$-(1-4) polygalacturonic acid backbone. Substituted homogalacturonans are modifications of this backbone with $\beta$-D-xylose branching at C3, or apiofuranose substitutions in the backbone with $\beta$-D-apiosyl-(1,3')-$\beta$-D-apiose branching.
- **-rhamnogalacturonan I:** alternating $\alpha$-(1-4) galacturonosyl and $\alpha$-(1-2) rhamnosyl residues, with primarily oligo $\alpha$-(1-3) arabinose and oligo $\beta$-(1-4) galactose branching.
- **-rhamnogalacturonan II:** composed of $\alpha$-(1-4) polygalacturonic acid backbone with complex branching composed of up to 11 different monosaccharide types.

**lignin:** stable cross-linked aromatic polymer resulting from radical polymerization of p-coumaryl, coniferyl and synapyl alcohols. The lignin content of the flax fiber is 2.8%.

Other components are fats and waxes (1.5%), and water soluble compounds (11.6%) [78].

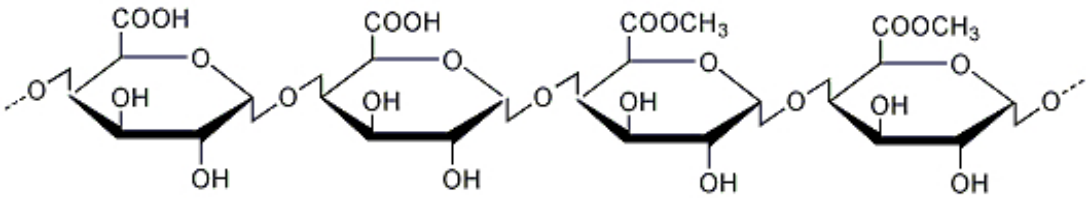



**Figure 1.4**: Homogalacturonan fragment of pectin, composed of $\alpha$-(1-4) polygalacturonic acid backbone. The residues found at the right are esterified with methanol.

### 1.5.2 Role of pectin in plant tissue structure

Pectic substances can comprise up to one third of the cell wall dry matrix of some plants, with the highest concentrations located in the middle lamella. They contribute to cell ad-

hesion via their gel-like properties and to cell wall mechanical strength. These substances are solubilized easier than other cell wall polymers and are more chemically active.

The composition and structure of the pectic substances varies with the different stages of plant development. Digestion of tissues with pectolytic enzymes (degradation of pectic acid or pectate) leads to the dissolution of the middle lamella and cell separation [113].

### 1.5.3 Pectin degrading enzymes

The three major classes of enzymes involved in the degradation of pectins in nature are pectinesterases (PE), polygalacturonases (PG) and pectin lyases (PL) [32].

**Pectinesterase** catalyzes the hydrolysis of the methyl esters of pectin to yield pectate and methanol [15]. Optimal fungal PE activity is in the pH range 4.0-5.2, while bacterial PE is in the pH range of 7.0-9.0 [30].

**Polygalacturonase** catalyzes the hydrolytic cleavage of the O-glycosyl bond of $\alpha$-D-(1-4) polygalacturonan. The pH optimum of endo-PG has been reported to be in the range of 3.6-5.5 [30].

The degradation pattern can proceed randomly (endo-polygalacturonase) or terminally (exo-polygalacturonase). When short reaction times were monitored, random cleavage of polygalacturonate resulted in a large decrease in viscosity with only a small percentage of the glycosidic bonds hydrolyzed, whereas terminal cleavage is characterized by little viscosity reduction with substantial percentage of glycosidic bonds degraded [30].

**Pectin lyase** degrades pectin and D-galacturonan polymers and oligomers. Lyases cleave the C-O-glycosidic bond of $\alpha$-D-(1-4) polygalacturonan by $\beta$-elimination. The way of action of a pectin lyase is shown in Fig. 1.5. The products contain a 4,5-unsaturated galacturonosyl residue on the nonreducing end of the cleaved substrate [30].

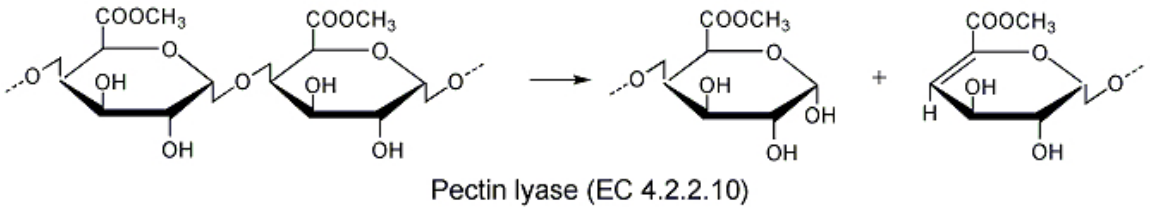



**Figure 1.5**: $\beta$-eliminative cleavage of methylated $\alpha$-D-(1-4) homogalacturonan by endo-pectin lyase.

The degradation pattern can be "endo" or "exo"- and the produced unsaturated oligomers absorb light at 235 nm. Products of $\beta$-elimination also react with perio-

date to form formylpyruvic acid, which reacts with thiobarbituric acid (TBA) and produces a red chromogenic product detectable at 545 to 550 nm [30].

The pH optimum of endo-pectate lyase (PL) has been reported to be in the range of 8.0-9.5, while the exo-PL is 8.0-8.5. For the endo-pectin lyase (PNL) it is between 4.9-6.5 [30].

Most lyases are produced and excreted in response to an induction event, like the presence of a suitable substrate [30].

Many pectin degraders are associated with raw agricultural products, and about 10% of them show some pectinolytic activity. They include bacteria, yeasts, molds, protozoa and nematodes, many of whom are plant pathogens [77].

## 1.6 Fiber extraction by traditional methods

Retting means the extraction of the fiber from the stem. It is normally done by microorganisms, but the term "retting" has also been used indistinctly for the chemical degradation of the pectic substances.

The fibers are glued strongly together by polysaccharides and lignins. First the fibers are obtained as bundles or long strands of overlapping fibers by mild retting. They can be further broken to elementary fibers by additional retting. The aim of the conventional long-line system (wet spinning) is to maintain the length of these long strands (around 1m) for as much as possible [70]. Natural bast fibers are quite variable in terms of strength, fineness and color within the same species and even within the same variety. The fiber extraction process can accentuate the fiber heterogeneity, which has been an obstacle to the large industrial scale application of the fibers [80].

Bast fibers are processed by various means that may include retting, breaking, scutching, hackling, and combing [34]. The quality and amount of extracted fibers depends on the retting process and the mechanical decortication made during the breaking and scutching. During retting, the pectic substances by which the fiber bundles are attached to the surrounding bark matrix and the woody core are degraded, allowing the flax fibers to loosen out of the plant core. Retting also helps to separate the bundles into individual fibers [51, 111].

The traditional retting methods used for the commercial fiber extraction are described below; other new or experimental methods, with mechanical, chemical, physical, enzymatic and biological steps (and combinations thereof) are described in Section 1.7.

### 1.6.1 Dew retting

After mechanical pulling (or cutting) and deseeding, the flax plants are spread in swaths evenly on the field, and left there for several weeks (3 to 7), depending on the weather conditions. The fungi colonize the stems and degrade the pectins and other organic compounds around the outer rind of the stalk. The moisture (rain and dew), followed by the mid-day drying action under warm temperatures, loosens the plant fibers. The swaths are turned regularly to maintain an uniform retting and to produce a uniform fiber quality. A very dry climate prevents that microorganisms colonize the stems, producing under-retted fibers, which are coarse and have many contaminating shives. On the contrary, if the weather is very humid, retting progresses too far and the cellulose of the fibers is damaged, making the fibers weak and of little value since they are not anymore usable for wet spinning processing [8, 51]. In Germany entire flax crops have been lost around once every 10 years because of adverse weather (high humidity), which has led to an almost complete extinction of the culture of the flax plant in this country (personal communication, Heger, E. 2006). Finding the optimum retting point is very important, the straw should be harvested at this stage. The end retting point is generally assessed subjectively with the color and the ease of shive-fiber detachment [8, 51].

The advantages of dew retting are many; it is mechanically simple (requires little labor); it uses natural air drying and it requires no chemicals and therefore produces fewer pollution compared to the other fiber extraction methods. However, it has some main drawbacks. Dew retting is weather dependent and is restricted to limited geographic areas. It produces a low fiber yield and inconsistent quality fibers in comparison to water retting. Because the fibers are left on the field for weeks, they pick up dust (contaminants), which is particularly problematic for textile processing [8, 51]. Another disadvantage is that the fields cannot be used for planting other crops while dew retting takes place [80].

Dew retting, also called field retting, accounts for the majority of commercial flax fiber production. It is the most common procedure for producing flax fibers in Western Europe, specifically in Belgium, France and the Netherlands [80].

### 1.6.2 Water retting

Water retting, also called pond retting, produces fibers of higher quality than dew retting. To obtain the fiber, the stems (stripped of the leaves and seeds) are tied in bunches and immersed in warm water (30-40°C) for a few days or in cold water for one or two weeks. After water retting, the stems are washed and dried generally in the fields forming cones [69]. Formerly, traditional methods of water retting were performed in dams, ditches or slow running streams and rivers, nowadays these methods have been replaced by modern tank retting. Tank design varies including open, closed and cascade systems. In all cases, chemical additives are used to improve the process, decrease energy cost and

lessen pollution [51].

Warm water retting uses the natural microorganisms of the plant. At the adequate temperature, the anaerobic bacteria thrive and start to ret the stems. Water retting starts with an aerobic phase, where bacteria decompose the pectic material of the stems releasing $CO_2$. Without aeration, the oxygen is soon depleted, about 15% of the carbon is metabolized by bacteria, and the rest remains in the retting liquor as fatty acids and other intermediates. Methane and hydrogen sulfide can be produced during anaerobiosis, and the volatile fatty acids (butyric acid) create an unpleasant smell. The waste retting liquor has a high oxygen demand. Because of the polluting and odorous retting liquors that water retting produces, it is not allowed anymore in Western Europe. This together with the high cost of drying, were the decisive factors why dew retting replaced water retting in Europe in the 1950s [50, 51, 67, 111, 114].

## 1.7 Alternative methods for bast fiber refinement

The technology of the traditional fiber extraction methods, dew and water retting, is well established. Dew retting is, as mentioned in Section 1.6, the most employed procedure for flax fiber extraction in Western Europe because of its economical benefit over other methods. In Section 4.8.1, its ecological advantages over other retting options will be discussed. However, dew retting is limited in Western Europe geographically to some regions of France and Belgium, where the weather conditions are the adequate for this process [50].

The chemical retting (e.g. NaOH boiling) and water retting methods are cheap and fast, but are not permitted in Europe due to the polluting effects of the soil and water bodies. Eventually, when the environmental regulation becomes tighter elsewhere in the world where these methods are employed, alternative methods will be required. Therefore, the interest in other ecological, weather-independent, controlled and cheap methods for producing fine bast fibers exists. Furthermore, if more homogeneous material were demanded for specific applications, other methods for fiber extraction would be needed. In general, the new methods for bast fiber extraction use the following principles and combinations thereof:

**enzymatic:** the use of polymer degrading enzymes (pectinase, hemicellulase and cellulase) [114].

**biological:** the use of microorganisms and its polymer degrading enzymes.

**mechanical:** the use of mechanical forces on dry stems.

**chemical:** the use of chemical agents.

**physical:** the use of physical phenomena to achieve fiber separation.

The bast fiber refinement methods and technologies described in this section, are alternative methods for producing long or short staple fibers. The majority of these methods are not applied in an industrial scale for the production of long bast fibers, which are the high quality-high priced application of the bast fibers.

### 1.7.1 Mechanical treatment of hemp bast straw

The mechanical fiber extraction method is probably the only procedure that is done on dry material and is often the first step of a process. However, it has also been proposed as the sole means for fiber extraction.

Hobson *et al.* report that the costs of decorticated green fibers could be lower than those of retted fibers. They developed a decorticator for unretted flax and hemp, that also could treat retted plants. The yield, length distribution and strength of fibers from unretted stems were the same as of fibers from retted stems. However unretted fibers were coarser and contained almost 4% impurities, compared with 2% for retted fibers. The unretted fibers were adequate for applications in pulp products and in reinforcement of composite materials [55].

A new machine line for bast fiber processing, which includes all process stages from pick-up and cutting of straw bales to the cleaning of the final products fibers and shives was established in pilot plant scale (1.5 t/h). The technology for natural fiber plants is based on a simple mechanical decortication principle. The low investment technology can be operated in a normal agricultural service company, and allows the manufacture of natural fibers at acceptable prices. Practical applications of the fibers are for automotive industry, geo-textiles, thermal blankets, fibrous insulating material and composites, building boards, coarse yarn, coarse textiles, and reinforcement of several composites and structural parts [82]. It has been described that mechanically treated hemp fibers have not been suitable for spinning on a OE rotor spinning machine [97].

The mechanical methods are harsh on the fibers and a low yield of long fibers is obtained. Therefore other methods are required for producing high quality long fibers.

### 1.7.2 *Tos*can*apa*–biological process for hemp fiber refinement

Within the *Tos*can*apa* project, the Italian Fibranova group proposed a fiber extraction and refinement process. Its primary objective was to establish a fully sustainable agro-industrial system focused on hemp processing and production in Tuscany. A pilot plant where scutched fiber is retted (bacterial/enzymatic treatment) under controlled conditions was planned. The process included: bacterial treatment, rinsing, drying by air-blowing, and softening stages [108]. A commercial production of this "bio-degummed" hemp, was

expected to be completed by 2007 or 2008, the obtained hemp could be then processed by ring or rotor spinning [31]. No information if this was accomplished could be found.

### 1.7.3 Biolin–enzymatic-chemical treatment of linseed and flax straw in pilot plant scale

The Canadian company Biolin Research Inc., tested enzyme-chelator systems for breaking down the straw from oilseed and fiber flax varieties grown in Saskatchewan. The stems had been treated with different mechanical harvesting and collection systems in the field [12].

Biolin Research Inc., calculated that cottonized flax fiber (short staple) could be produced at a price of CAN$1702/t (about 25% higher than the cost of cotton) in Saskatchewan. It was assumed that the market demand for cottonized flax could double or triple from the current estimated demand of 40 000 to 100 000 t/y [8].

### 1.7.4 Chemical and enzymatic process using anaerobic retting bacilli in an apparatus for treating flax stalks

A patent of 1915, describes an apparatus where baled flax stalks were retted using highly compressed material. An uniform result was reached with the advantage of handling large quantities of stems in a reduced volume. Three liquors were used in the process: anaerobic retting bacilli liquor, "retting fluid" and water. The amount of liquids used was minimal. The process was carried between 30-35°C. After a three-day treatment the used solutions were discarded. Constant pumping through the material was required. To remove the shives from the fibers, the retting fluid was supplemented with a "substance" that effectively dissolved the gums and eliminated this problem. The fibers produced had a high quality [109]. Reports of the commercial application of this process were not found.

### 1.7.5 Machinery for treating flax fibers, patented in 1924

A machinery for treating flax fibers and a process is described in a Patent of 1924. A method for de-gumming large quantities of fibers was proposed, which avoided the pitfalls of flax treatment. The failure so far, was that the fluid was not penetrating into the bulk of the fibers. The other problem was that the removed substances from the interior fibers were unable to escape to the liquid phase, re-adhering once the fibers were dried. This was avoided this by the addition of a "cleansing fluid" into the fiber bulk. The fibers were placed inside receptacles with adjustable walls and perforated floor. The flax fibers are buoyant and floated in the fluid. A gentle circulation motion was done upwards and was performed until all the gums were loosened out. The fibers were subjected to a gentle movement in one direction. The dirty solution was left back, so the fibers were being freed from the contaminant, always floating to aid mass transfer. This action was performed

until the pumped liquid contained neither "solvent fluid" nor "loosened substance". The fluid acting as a solvent could be a soap. The fibers were then immersed in water to wash out the undesired substances further. When the fibers were dried they did not adhere to each other. The duration of the process was of 1.5-2 h at 38°C. The machinery consisted of a series of receptacles with communicating and moving walls and a perforated bottom, hold by a cage. The vat had several compartments to separate the different streams [93].

### 1.7.6 Mechanical, chemical and enzymatic process for producing short flax fibers at Clemson University

The strategy followed by the USDA and the Center for American Flax Fiber at Clemson University, was to replace the anaerobic bacteria with enzyme mixtures in controlled conditions. In this approach they aimed to obtain short staple flax fibers that could be blended with cotton or other fibers. A combined mechanical, chemical, and enzymatic process for separating the flax fibers was developed. The flax stems were first passed through fluted rollers to mechanically break the stem to allow the enzymes to penetrate faster into the fiber. The procedure used a pectinase rich commercial enzyme (supplied by Novozymes) with a chelator (EDTA) to reduce the amount of enzyme used. The stems were incubated at 40°C for 24 h. Then the fibers were rinsed and dried with circulating heated air. Because all the processing steps were controlled, they could modify fiber properties (color, strength, fineness), and produce tailor made flax fibers [45]. The proposed retting process could be conducted in an enclosed facility, which would allow retting to occur year-round. The enzyme retting process was reduced from several weeks (i.e., dew retting process) to just 24 hours [8].

### 1.7.7 Chemical and enzymatic processing of flax fabrics

A non-ionic wetting agent (Lissapol N) in conjunction with an enzyme with hemicellulase and pectinase activities, were used to remove noncellulosic gummy materials from gray flax fabrics at 55°C with a 1:100 material to liquor ratio. For comparison, the conventional treatment was applied; a sample was treated with a 2% caustic soda solution at 80°C for 4 hours. During the conventional caustic soda treatment, noncellulosics were removed but no cracks were formed on the fiber surface. However, for the enzymatic treatment due to the presence of a small amount of cellulase activity, hydrolysis of cellulose took place along with removal of noncellulosics. These cracks and cavities were largely responsible for the higher dye uptake, increased absorbency, and decreased tensile tenacity of the treated flax fabric [24].

### 1.7.8 Biological treatment of green hemp

The use of decorticated hemp fibers in an aerobic process using naturally occurring alkaliphilic microorganisms present on the fibers (biological degumming) was proposed, with the aim of spinning a 100% hemp rotor yarn [73].

An alkaline aerobic biological process, carried out with the naturally occurring microorganisms of the fibers was used. The process took 3 days in laboratory scale [74].

The intention was to build a pilot scale prototype with two tanks, one for the aeration and mixing of the culture with the ideal conditions for it; the other tank for the submerged fibers, with the ideal conditions for the fiber treatment. A barrier was needed between the tanks so that the natural microorganisms of the bast fibers did not contaminate the seed culture. Other options for the barrier were to separate by filtering and/or centrifuging [74]. It was not possible to find out whether the pilot plant was built.

### 1.7.9 Enzymatic degradation of residual polysaccharides of flax roves

Back in 1987, there are reports on the use of commercial and laboratory produced enzymes for the refinement of flax roves. The roves had been produced from dew retted flax fibers and the aim was to upgrade the coarse flax fibers by enzymatic hydrolysis of the non-cellulosic polysaccharides in the fiber. The treatments were carried out at a range of pH 5.4-6.0 for 2 h at 45°C. Non-ionic surfactants were found to enhance the activity of the polysaccharide degrading enzymes. The enzyme treated roves produced higher quality yarns compared to the yarns spun from untreated roves. The enzymes did not affect the cellulose in the fibers. It was also mentioned that the method was applicable to green flax fiber, whose handling and spinning qualities were improved. However, it was also mentioned that the economical advantage of the method depended on an economical production of the degrading enzymes [98].

In 2005, the same author carried out an investigation to develop process recipes for the production of better quality yarn from lower grade fiber supplies. Treatments with EDTA at 40°C and two enzyme formulations containing pectinases, xylanase and laccase at 40°C were compared with the industry standard caustic boil (4% NaOH) treatment at 95°C. The selected treatments were scaled up in a twin pilot scale unit using rovings, followed by $H_2O_2$ bleaching and wet-spinning. The resulting yarns were evaluated for tenacity and regularity. The chosen process steps were: pre-treatment of fiber with EDTA followed by sequential treatments of a mixture of pectinases and xylanase enzymes (Pectinex treatment at 40°C) and $H_2O_2$ bleaching. An economic evaluation of the proposed method would be needed to test its feasibility [101].

### 1.7.10 Mechanical and chemical process for linseed fiber cottonization

The aim of the project was to convert bast fiber seed crops, linseed available in Australia, into a high value fiber that could be processed on existing cotton machinery [70].

The flax stalks were allowed to partially ret in the field. A mechanical separation by crushing the stems with fluted rollers followed. A chemical treatment was done, using 1.75 M NaOH with sodium dodecyl sulfate at 50°C for 20 hours in a flask with ball bearings (to promote mixing), followed by a neutral rinse and another rinse with 1% silicone softener. The silicon softener was used to avoid the fibers to stick together when drying [70].

The aim of the combined dew retting, mechanical and chemical treatment was to fully individualize the fibers. Although a fiber that was judged as nearly spinnable was produced, it was found that the fully individualized fibers were too short to be spun without blending with long cotton or synthetic fibers [70].

### 1.7.11 Texflax–mechanical and chemical process for fiber cottonization

The Texflax project aim was to produce high quality flax fiber that could be processed on short fiber cotton spinning systems, therefore enabling the production of yarns for use in the high-volume manufacture of fine fabrics in the UK. The Texflax project investigated flax growing by selecting the varieties whose fibers had properties that approached those required for cotton substitution. In the Texflax project, chemical retting (by desiccation) using glyphosate-based herbicides was examined, and it was found that the application of herbicide should be made no later than the mid point of flowering stage [52].

Using mechanically and post-processed fiber from the five best varieties, a yarn comprised of 50% flax and 50% cotton was spun. Also the percentage of flax that could be incorporated into a flax-cotton yarn of Nm 26 was increased from 30% (using current commercial varieties) to 50% [52].

### 1.7.12 Flasin–mechanical and chemical process for flax fiber cottonization

Flasin© is a patented process (and brand) for the production of linen fibers in cotton staple length (elementary fibers). With this mechanical and chemical process, a mixed yarn Nm 34 of 50% cotton and 50% Flasin (linen) could be produced. When combined with technical fibers, yarn counts up to Nm 50 could be spun.

A sliver of flax fibers was passed through a chemical treatment (borax and trisodium phosphate) and was then dried and cut. The fibers were carded with cotton machinery

and separated to elementary fibers. In contrast to NaOH treatments, this treatment did not damage the cellulose of the fiber. The treatment time was 200 min at 120°C. In 1998 the Flasin© bioprocess was launched, an enzymatic process that allowed a treatment time reduction to 60 min at 85°C [36]. In the year 2000, the Flasin process (Patent No. WO 97/19221) was used on an industrial scale in Germany [42]. Erpatex, the company that was operating the Flasin process, went bankrupt in 2001 and had to sell its production line to a company in the Czech Republic [23].

### 1.7.13 Korean–steam process for hemp fiber refinement

The use of steam to separate hemp fibers is a traditional method in Korea. In this process, the stems were subject to a dense steam for 24 h. Afterward the hemp fiber was so disintegrated that it could be easily separated. The method evolved over time, and in 1996, a steam-processing factory was built, which had a large pressurized tank accommodating two metal racks each containing 48 bundles of stalks [33].

### 1.7.14 Steam explosion for bast fiber refinement

Steam explosion is a process where a sample is treated with steam at high temperature and pressure followed by flashing. This process has been extensively studied in Japan, France, Germany, etc. as a pretreatment process to increase the accessibility and separate the main components of lignocellulosic biomass (cellulose, hemicelluloses, pectins and lignin). A mechanical separation of the bast fibers from the wood tissues is needed prior to steaming, due to their different behavior during the steam treatment [115].

At the Institute for Applied Research in Reutlingen, Germany, a steam explosion process was developed. In this process, decorticated fibers were impregnated in chemicals (alkali and auxiliaries) and steam-exploded (0-12 bar between 1-30 min). After the reaction time, the pressure was suddenly released and the sample discharged; this fast expansion opened the fibers. To eliminate the pectin and binding materials, the fibers were post-treated by rinsing, washing and bleaching. With this process, it was possible to treat green flax or hemp in one step, for many applications. The method could produce tailored materiasl by modifying the process parameters [63, 87].

Based on the results of the Interreg IIIa project “Regional hemp textile chain” the company Stextile BV was established in 2006, with the aim of developing a sustainable and commercially viable hemp production chain by the development of a commercial steam explosion facility. The refined hemp fibers would be spun in cotton spinning systems to produce 100% hemp yarns or blends [13]. However by 2008, the same company, Stextile BV, said that at that moment, the steam explosion technology was not on a commercial scale, and were waiting for approval and resources to build the steam explosion facility in the Netherlands [105].

### 1.7.15 The dry-line method in bast fiber production

The dry-line method in bast fiber plant harvesting consists of the combination of autumn harvesting of the oilseeds and spring harvesting of the fibers. During the winter the rest of the plant is left on the field to be retted and dried. The dry-line method uses the weather for its advantage. The repeated temperature changes above and below zero degrees Celsius, and the consequent formation and thawing of ice crystals are the core of the dry-line method. The frost detaches the bast fibers from the plant stems. Therefore freezing is an advantageous economical consideration for the production of bast fiber in Nordic latitudes [90].

Frost retting is a cost-effective way to facilitate fiber separation. The fiber is retted and relatively dry at harvest time in spring, which decreases the high costs for drying that otherwise are needed when harvesting in the autumn [68].

It was mentioned that the major challenges of the dry-line method were the properties of the spring harvested fibers, which differ from the specifications needed for the yarn and textile industry. The fibers obtained were cut and were used for composite reinforcing materials for substituting glass fiber. Other industrial applications were investigated, such as using linseed straw as a raw material for loose fill thermal insulation materials and hemp fiber pulping [90].

New technologies like the use of glyphosate to desiccate a standing crop and extract the fibers have proved to yield a more uniform product, but the costs and treatment time in this procedure made it economically nonviable [114].

### 1.7.16 Improving flax retting with genetic modification of flax to express pectinases

Flax plants that are more readily retted can be designed by genetic modification. Using the novel approach of transgenic flax plant generation with increased polygalacturonase (PGI) and rhamnogalacturonase (RHA) activities from *Aspergillus aculeatus* genes, a significant reduction in the pectin content in tissue-cultured and field-grown plants was obtained, which doubled the retting efficiency. While the flax fiber composition remained unaltered [83].

## 1.8 Flax processing for the production of yarns

Drying of the stems allows the fiber bundles to shrink away from the degraded brittle woody matrix, and to achieve some pre-separation. Breaking of the stems is done mechanically to separate the rotted woody matrix and loosen the fibers. The stalks are fed through fluted rollers that crush and break the woody matrix into pieces (shive). The

following step is scutching, where the dry stems are beaten by rotating turbine blades to separate the fibers from the other material and crush the pith while softening the fibers [51, 69].

The flax straw contains 25-30% of fibers based on the dry weight of retted stems. During scutching some fibers are broken and go to the scutching waste. Still, some short fibers (scutched tow) can be recovered from this waste by re-scutching. Coarse fabrics and cordage are the final products made with the scutched tow. Well-retted flax produces low amounts of scutched tow, which normally is between 30-35% of the overall fiber content of the stalk. The long fibers obtained after scutching are called "line", and are sorted according to their quality (smoothness, luster, hand and cleanliness). They are used for the spinning of fine yarns (wet spinning) and measure at this point of the process between 40-60 cm [51].

### 1.8.1 Long fiber processing

The process of converting flax line to yarn consists of hackling, preparing and spinning. The hackling (combing process) consists of splitting and separating the fibers that are glued together, then disentangling and parallelizing them since the fibers still contain coarse bundles, some shives and other impurities. Hackling is performed in several stages, the first of them is called roughing, in where a moving band with spiked bars works on the flax bundles (line). Successive stages with finer teeth are performed sequentially with the finest comb having about 2.4 pins/mm. The fiber bundles are combed first from the coarser end (stem root). The produced tow is moved from the bands by brushes and is used subsequently in the tow yarn manufacture. The yield of hackling is 55-65% of the initial weight and depends on the hackling degree and fiber quality. The main byproduct obtained is hackling tow (inferior quality) [51, 75].

The next step is denominated spreading, in which the hackled fibers are formed into a continuous and cohesive band called a sliver of 30-40 ktex[1]. By laying the fiber end over end in a conveyor belt and feeding them to spreading frames, the fiber sheets are straightened and overlapped (called gilling). It is usual to make successive doublings and draftings to produce a lighter and more homogeneous sliver. The final step of preparation is called roving, in where the sliver is slightly twisted while being wound onto rove bobbins for spinning preparation. For the production of finer yarns, the roves are boiled or bleached to eliminate residual pectic matter [51].

By the time the line reaches the spinning stage, the fibers have been further broken and measure about 30-38 cm [51]. Wet spinning is used for the production of finer yarns. It uses mainly long fibers as raw matter, though high quality tow can also be used [51].

The rove is fed to a receptacle containing hot water and the spinning is carried out wet,

[1]For an explanation of the textile units used throughout the text, refer to Table 2.1.

using ring spinning frames. The hot water helps to distribute and soften the gums (binding material) and to reduce friction. This allows the production of a more homogeneous yarn. Afterward, the yarns are dried by hot air. Often they are softened by twisting and untwisting them to eliminate the characteristic wiry stiffness that is acquired when the gums dry back on the yarn. Fine linen yarns are often bleached before weaving. The most common yarn counts produced by wet spinning are in the range of 41-28 tex (Nm 24.4-35.7) [51, 75].

Other post-spinning processes are often required, such as winding the fiber in special spinning bobbins; then the yarn is ready for weaving or knitting of apparel fabrics [51].

### 1.8.2 Short fiber processing

Tow is composed of shorter, broken and smoothed out fibers. For producing a yarn from tow, the impurities (woody material and others) have to be removed. Fiber opening and cleaning equipment is similar to that used for short-staple spinning. The tow is carded, to break up long fibers, split compounding fibers, eliminate fiber knots, remove dirt and to form a sliver. Depending on the end use of the fibers, the tow sliver can be further passed through the drawframe, combed and drawframed again, which improves sliver regularity. The obtained carded sliver is normally spun dry by ring spinning. This system is able to produce coarse yarns [51, 75]. Another more efficient spinning system is the open-end rotor (OE), with which cotton-type yarns are made. Dry-spun yarns are soft and open-structured, and are used for lower quality linen goods. However, dry spinning is less energy demanding than wet spinning since it does not require warm water for the yarn formation nor is necessary to dry the completed yarn.

## 1.9 Flax process economical overview

Conventional long flax processing is labor intensive and most of the processing is done on antiquated machinery, which has not been further developed in contrast to the cotton industry [70]. The price of a cotton yarn is five times cheaper that of a flax yarn. In addition, 72% of the total cost of the cotton yarn is the fiber cost, and the spinning and pre-spinning cost account together for only 28%, whereas the majority of the cost of a flax yarn, corresponds to the pre-spinning and spinning operations (77% of the total costs) [62].

The higher price of the flax yarn relegates it for small niche markets. Currently flax is seen as a fashionable fabric in the apparel sector, being used in blends to decrease wrinkling and obtain an easy care fabric. Linen is blended with synthetic as well as other natural fibers, to obtain stretchable, metallic and shining fabrics [62].

According to a report for the European Union made in 2000, the market potential for

high quality, high value textile products is limited. New uses for increasing production must be focused on the high volume but lower value markets. Moreover the limited markets for flax and hemp tend to be driven by fashion except in some Central and Eastern European Countries [5].

The worldwide production of all natural fibers has remained essentially constant over the last 20 years [8]. From 1970 to 2006, a slight increase of flax straw production in the EU was observed. Around 140 processing plants operated in the EU-27 in 2006, and scutched 635 589 t of flax straw into long fibers (112 914 t) and short fibers (61 775 t). This represents 53% of the world production of flax fibers. With a total turnover of about 235 million euro for long flax fiber and of about 32 million euro for short fiber [16]. Further transformation of flax fibers is largely located outside the EU. In 2006, more than 80% of European long flax fibers, mainly used in the textile industry, were exported, chiefly to China. Despite this strong export demand the price for long flax fibers decreased by 10% between 2001 and 2006, mainly as a result of the increase of value of the euro against the US dollar [16].

The average size of the European flax processing companies is small, with the exception of some French processors. For a considerable number of small enterprises, the EU government aid exceeds their gross margins. Without the aid from the EU, the smaller processors would face serious difficulties, specifically in the traditional production regions [16].

Cotton and synthetic fibers account for 48% and 45% of the world textile market. Both are related to environmental problems. Synthetic fibers deplete the non-renewable fossil resources. Cotton cultivation requires a high amount of fertilizers, pesticides and water [111].

Since the arrival of cotton and synthetic fibers, flax yarn has been considered as a niche product. To change this status, a new approach with more integrated and modern technologies in the production chain would be required [62]. In addition, new advertising strategies would be needed to make consumers aware of the advantages that bast fibers have.

## 1.10 Aim of this thesis: development of a novel biotechnological process for the production of high-quality linen fibers

Dew retting is cheap and is the most common procedure for producing flax fibers in Western Europe [80]. However this method is weather dependent and is restricted to limited geographic areas. In order to avoid the high risks of crop-loss due to unfavorable climatic conditions, other fiber extraction alternatives are needed.

Enzymatic treatments for fiber extraction are gaining popularity because of their beneficial effects on the environment. However the cost of the enzymes is still too high for the target product price. The great advantage of using whole cell treatments is the low cost of the pectinolytic bacterial broth and its eventual reusability.

The recently described strain *Geobacillus thermoglucosidasius* PB94A (DSM 21625), was isolated using hemp pectin as substrate. The strain PB94A, grew optimally at 60°C and pH 8.5. During its growth on citrus pectin, the strain produced pectinolytic lyases, which were excreted into the medium. In contrast to the commercially available pectinase Bioprep 3000 L, the enzymes from *G. thermoglucosidasius* PB94A converted pectin isolated from hemp fibers. In addition to hemp pectin, the culture supernatant also degraded citrus, sugar beet and apple pectin and polygalacturonic acid. When hemp fibers were incubated with the cell-free fermentation broth of *G. thermoglucosidasius* PB94A, the fineness of the fibers increased. The strain did not produce any cellulases, which is important in order to avoid damaging the fibers. Therefore, these bacteria or their enzymes can be used to produce fine high-quality bast fibers [112].

The proposal of this thesis is to set up a new process to produce long fine flax fibers using whole cells of *G. thermoglucosidasius* PB94A and green decorticated fiber as starting material. Green decorticated flax fibers account for only about 30% of the total straw weight, which would be an important economical advantage for reducing costs and waste streams.

# Chapter 2

# Materials and methods

## 2.1 Equipment

- cell growth and fermentation:
  - laboratory-scale fermentation:
    - Julabo SW23 (Seelbach/Black Forest, Germany) waterbath-shaker
    - New Brunswick shaker (New England, USA).
  - 2, 20 and 200 L bioreactor, Bioengineering (Wald, Switzerland)
  - 200 L main tank of the pilot plant, built by the TUHH workshop
- The equipment used in the pilot plant is described in detail in Table 3.7
- centrifugation:
  - Beckman J2-21 centrifuge with the rotors JA10 JA14, Beckman instruments (Munich, Germany)
  - Heraeus Biofuge 13, Heraeus (Osterode, Germany).
- pH determination: Schott Geräte, CG 822 (Hofheim, Germany)
- enzyme tests:
  - waterbath type W200, Memmert GmbH & Co. KG (Schwabach, Germany)
  - spectrophotometer Uvikon 930, Kontron (Eiching, Germany)
- polymerase chain reaction (PCR):
  - Primus 25 advanced thermocycler, Peqlab biotechnology (Erlangen, Germany)
  - UV fluorescent table 60-ECX-20M, Peqlab biotechnology (Erlangen, Germany)
  - gel documentation 60-DPII-X, Peqlab biotechnology (Erlangen, Germany)

  - Horizon 58 agarose gel chamber, Life Technologies, Gibco BRL (Gaithersburg, MD, USA)

- denaturing gradient gel electrophoresis (DGGE):
  - DCODE electrophoresis system, Biorad Laboratories (California, USA)
  - gradient mixer GM25, Scie-Plas Ltd., (Cambridge, UK)
  - pump model 323Du/D, Watson-Marlow Bredel (Cornwall, UK)

- Water purification system: Milli-Q water purification system, Millipore (Massachusetts, USA)

- Fourier transform infrared spectroscopy (FTIR):
  - Bruker Optics model Vertex 70, Bruker Optik GmbH (Ettlingen, Germany)
  - data acquisition software: Opus spectroscopy software version 6, Bruker Optik GmbH (Ettlingen, Germany)
  - data analysis software:
    * Matlab R2007a, The MathWorks (Massachusetts, USA)
    * N-way Toolbox for MATLAB, developed by the Food Technology, Chemometrics Group, The Royal Veterinary and Agricultural University (Frederiksberg, Denmark).
  - overhead ATR sampling unit model: PIKE MIRacle #1002612, PIKE Technologies (Wisconsin, USA)

- Near infrared spectroscopy (NIR):
  - ABB model MB 3600 FT-NIR with InGaAs detector, ABB Analytical (Quebec, Canada)
  - sampling accessory powder Sampl*IR*, ABB Analytical (Quebec, Canada)
  - HORIZON MB$^{TM}$FTIR software, ABB Bomem

- Chemical oxygen demand (COD):
  - photometer ISIS 9000, Dr. Lange GmbH (Berlin, Germany)
  - thermostat LT1W type LTG 037, Dr. Bruno Lange GmbH (Berlin, Germany)

- Biological oxygen demand (BOD): Respirometer BSBdigi,[1] Selutec GmbH (Hechingen, Germany)

- Scanning electron microscopy (SEM):

[1] kindly lent by the "Abwasserwirtschaft und Gewässerschutz" Institute of the TUHH

- Preparation for electron microscopy:
  - critical point drying, Bal-tec AG, model SCD 020, Balzers (Liechtenstein)
  - sputtering device, Bal-tec AG, model SCD 040, Balzers (Liechtenstein)
- Electron microscope: LEO Gemini 1530 scanning electron microscope, LEO Electron Microscopy Inc. (Oberkochen, Germany)

## 2.2 Chemicals

In general, reagents grade purum or puriss p.a. were utilized from the following companies: Merck (Darmstad, Germany), Fluka (Neu-Ulm, Germany), Sigma-Aldrich (Munich, Germany), Roth (Karlsruhe, Germany). Other chemicals used were:

- polymerase chain reaction (PCR):
  - Seakem LE Agarose, Cambrex biosciences (Rockland, Maine, USA)
  - primers (oligonucleotides), biomers.net (Ulm, Germany)
  - SYBR Green I Nucleic acid gel stain, Cambrex biosciences (Rockland, Maine, USA)
  - Taq DNA polymerase kit EP0401, Fermentas (Ontario, Canada)
  - GeneRuler$^{TM}$1kb SM0313, Fermentas (Ontario, Canada)
  - 6×DNA-loading dye R0611, Fermentas (Ontario, Canada)
  - Chelex-100 (200-400 mesh), Bio-Rad (Hercules, CA, USA)
- Invitrogen DAPI stain, Molecular Probes, Inc. (Oregon, USA)
- Adulcinol BUN and Cefasoft MIS, fiber conditioners, Zschimmer & Schwarz Mohsdorf GmbH & Co KG (Burgstädt, Germany)[2]
- Ceramtec E019, $\alpha-$alumina ceramic carrier, CeramTec AG (Plochingen, Germany)
- Poraver glass beads, Dennert GmbH, (Schlüsselfeld, Germany)
- COD measurement kit: LCK 514, Dr. Lange (Düsseldorf, Germany)

## 2.3 Water

The water used for the analysis and the media preparation was HPLC grade. The water used for the fiber treatment experiments in beaker, bench scale and pilot plant scale was tap water.

[2]kindly provided by the company Zschimmer & Schwarz

## 2.4 Flax fibers

For the experiments described in this work, flax fiber type *Flüh04* was grown in the summer of 2004 in Mielsdorf, Schleswig-Holstein by the company Holstein Flachs. The flax was harvested after flowering by pulling it out of the earth. The fresh green stems were mechanically decorticated and the fibers were baled out almost immediately and kept dry in a warehouse. The fibers contained chlorophyll and many shives.

Another fiber type, *Camino05* was produced as described above, with the difference that the fibers were left some days in the field and were slightly retted.

## 2.5 Strains

*Geobacillus thermoglucosidasius* PB94A (DSM 21625) was isolated from bast fiber raw material using hemp pectin as the sole carbon source. The 16S ribosomal DNA (rDNA) sequence was determined and submitted to the National Centre for Biotechnology Information (NCBI) [112]. *Geobacillus thermoglucosidasius* PB94A (DSM 21625) was the main microorganism used for this work. Other bacteria used as control were:

- *Geobacillus thermocatenulatus* PB94B
- *Geobacillus thermodenitrificans* PB1511 (DSM 21923)
- *Bacillus subtilis* sp.

## 2.6 Buffers and solutions

### 2.6.1 1×PBS, phosphate buffered saline

The phosphate buffered saline (PBS) was prepared according to Sambrook and Russell David [96] as follows: 8 g NaCl, 0.20 g KCl, 1.44 g $Na_2HPO_4$, 0.24 g $KH_2PO_4$ were dissolved in 800 mL $H_2O$. The pH was adjusted to 7.4 with 1 M HCl and the volume was completed to 1 L. The solution was sterilized by autoclaving.

### 2.6.2 50×TAE buffer

To prepare 1 L of 50×TAE, 242 g of Tris base were dissolved in 600 mL of water. 100 mL of 0.5 M EDTA and 57.1 mL glacial acetic acid were added. The mixture was brought to a final volume of 1 L with water and autoclaved [96].

### 2.6.3 Tris-EDTA buffer

The following stock solutions were prepared [96]:

- 1 M stock of Tris-Cl (pH 7.5) by dissolving 60.57 g of Tris (hydroxymethyl) aminomethane in 0.5 L HPLC water, adjusting the pH to 7.5 using HCl.
- 500 mM stock of EDTA (pH 8.0) by dissolving 18.6 g of diaminoethane tetraacetic acid in 100 mL HPLC water. The pH was adjusted to 8.0 with NaOH, at which point the EDTA solubilized.

Tris-EDTA buffer (TE) was prepared by mixing 10 mL of the tris solution and 2 mL of the EDTA solution. Water was added to a total of 1 L. The final pH was 7.5, and the final concentrations were: 10 mM Tris-Cl and 1 mM EDTA.

## 2.7 Liquid and solid media for bacterial cultivation

### 2.7.1 Pectin media

Pectin media were prepared using as basis the minimal media for denitrifying bacteria described in the Handbook of microbiological media from Atlas [20].

Minimal medium for cultivation of the isolates was prepared from 1 L of solution *A*, 10 mL of solution *B* and 10 mL of solution *C*. All solutions were autoclaved separately and added aseptically. The mixture of the solutions *A+B+C* was adjusted to pH 8.0 with 1 M NaOH. Solution *A* contained per liter of demineralized water: 1 g $(NH_4)_2SO_4$, 0.66 g $K_2HPO_4$, 0.54 g $KH_2PO_4$ and 5 g of citrus pectin as carbon source. Solution *B* contained per liter of demineralized water: 20 g $MgSO_4 \cdot 7H_2O$. Solution *C* contained per liter of 0.1 M HCl: 2 g $CaCl_2 \cdot H_2O$, 1 g $FeSO_4 \cdot 7H_2O$, 0.5 g $MnSO_4 \cdot H_2O$, 0.1 g $CaSO_4 \cdot 5H_2O$, 0.1 g $Na_2MoO_4 \cdot 2H_2O$. For plates, 10 g/L agarose were added.

### 2.7.2 Cellulose solid media

The cellulose media plates were prepared using the same minimal media described for pectin plates (Section 2.7.1), only substituting the pectin by carboxymethyl cellulose (CMC) and using 15 g/L of agar as solidifying agent.

### 2.7.3 Luria Bertani solid media

To prepare 1 L of LB complex media, 10 g tryptone, 5 g of yeast extract, and 10 g NaCl were added to water, 15 g/L of agar were added before autoclaving.

## 2.8 Cup plate test media for enzymatic activity determination

### 2.8.1 Plate test for cellulase activity

Cellulase tests were made with a modified technique that uses Congo Red to characterize cellulolytic bacteria [107] as follows: 0.8% (w/v) agar and 0.5% (w/v) carboxymethylcellulose were added to 100 mL demineralized water, boiled and poured into Petri dishes. Wells of 8 mm diameter were made into the solidified plates and 80 $\mu$L of culture supernatant or a control solution were filled into them. After incubation at 60°C, the plates were covered with 15 mL 0.1% Congo Red solution for 15 min at 4°C and washed with 1 M NaCl solution for 15 min. Cellulose degrading activity was detected by yellow halos around the wells in the stained cellulose plates.

### 2.8.2 Plate test for pectinase activity

The plate test for detecting pectinase activity were done with a modified assay for the quantification of polysaccharide degrading enzymes as follows [29]: 0.5% agarose (w/v) and 1% citrus pectin (w/v) were dissolved in 100 mL mineral medium for denitrifying bacteria [20]. The pH was adjusted to 8 with NaOH and the solution was boiled and poured into Petri dishes; 8 mm diameter wells were made into the solidified plates and filled with culture supernatant or a control solution. After incubation at 60°C, the plates were covered with 15 mL 0.05 % Ruthenium Red solution for 30 min at 4°C and then washed with distilled water. Pectinase degrading activity was detected by clear halos around the wells in the stained plates. Water was used as a negative control.

## 2.9 Cultivation of pectin degrading microorganisms

The cultivation of *G. thermoglucosidasius* PB94A was carried out in pectin minimal medium. Baffled shake flasks were used to grow the cultures when up to 1 L of bacterial broth was required. Otherwise, the fermentation of the strain was carried out in stirred bioreactors. The reactors were supplied with air and the pH was kept at 8 by the addition of NaOH.

When the required cell density in the seed fermentor was reached, the bacterial broth was used to inoculate a larger fermentor in a fermentation sequence: from 2 L to 20 L to 200 L. Once the required volume and cell density in the end fermentor was reached, the cell broth was harvested and used directly for the fiber treatment process or the broth was stored for subsequent use. The culture proved to be stable and enzymatically active when kept at 4°C for up to six months; freezing destroyed the enzymatic activity.

## 2.10 Determination of uronic acid content in solution

The determination of uronic acid content was done according to a modified procedure of Blumenkrantz and Asboe-Hansen [26]. The method for the quantitative assay of uronic is based upon the appearance of a color when uronic acid is boiled in concentrated sulfuric acid-tetraborate and further treated with meta-hydroxydiphenyl (MHDP). The uronic acid method was used to determine the pectin content.

Diluting the supernatant in water (1:20) was necessary. The following reagents were prepared:

- 12.5 mM $Na_2B4O_7$ solution: 0.48 g of $Na_2B_4O_7 \cdot 7H_2O$ were dissolved in 100 mL $H_2SO_4$ (97%)
- color reagent: 0.15% w/v MHDP were dissolved in 0.5% NaOH

For the determination, 200 $\mu$L of the supernatant were put into a glass test-tube, 1.2 mL of the $Na_2B_3O_7$ solution were added and mixed carefully. The mixture was then boiled for 10 min in a waterbath. The solution was carefully vortexed and cooled in an ice bath. The MHDP color reagent, (20 $\mu$L) was added and the solution was kept in ice for 5 min. Then the absorbance was measured at 520 nm. A solution of D-galacturonic acid (0.01 to 0.10 g/L) was used as standard.

For the determination of the uronic acid in fibers, the following procedure was applied: 10 g of dry fibers were put in a 250 mL Schott flask with 200 mL $H_2SO_4$ (1% v/v) and autoclaved. Once the flask had cooled to 60°C, the fibers were wringed to extract the most possible liquid. The warm solution was vacuum filtered, and the volume was completed to 250 mL with HPLC water. The obtained solution was diluted 1:20 with water and the uronic acid was determined as described above.

## 2.11 Determination of the pectin lyase activity

The determination of the pectin lyase activity was made with two methods, both based on the detection of the enzymatic $\beta$-elimination of the pectin chain (Section 1.5.3):

- Thiobarbituric acid method (TBA) reported as the absorbance at 550 nm ($A_{550}$).
- Lyase assay according to Collmer *et al.* Unless specified otherwise, this lyase assay was used and its lyase activity values were reported in activity units (AU).

### 2.11.1 Colorimetric test of lyase activity with thiobarbituric acid

The determination of the lyase activity with the colorimetric method thiobarbituric acid was done according to Nedjma *et al.*. This method is able to distinguish between polyga-

lacturonase and pectin lyase enzyme activity [88].

A solution of 0.04 M 2-thiobarbituric acid was prepared by dissolving the reagent in water and heating to 80°C for 15 min. This solution had to be crystalline and used within a month after its preparation or as long as no crystals or color change was observed.

The process (or culture) sample was centrifuged for 5 min at 13 000 rpm (16 060 × g). 500 $\mu$L of the supernatant and 50 $\mu$L 1 M NaOH were mixed and the test tubes were closed and incubated 5 min at 80°C. After this period, generally a yellow color appeared. The tubes were cooled in an ice bath for two minutes. 600 mL of 2-TBA solution and 500 mL of 1 M HCl were added and mixed well. The tubes were incubated for 5 min at 80°C. They were cooled for 2 min and in the case that precipitation or turbidity had appeared the samples were centrifuged prior to measuring. The determination was done at 550 nm in the spectrophotometer. Normally a dilution was required. A pink color indicated that lyase activity was present.

### 2.11.2 Lyase assay according to Collmer *et al.*

The pectin lyase activity was determined in the crude enzyme extract, which was obtained by centrifugation of the culture broth for 5 min at 13 000 rpm (16 060 × g). The activity was determined spectrophotometrically by measuring the increase of absorbance at 235 nm due to the eliminative cleavage of the pectin substrate (by $\beta$-elimination), according to a modified procedure of Collmer *et al.* [35]. Triplicates were determined, because of the high variability of the method. The solutions used for the method were:

- Glycine buffer was prepared by dissolving 0.7125 g glycine (15 mM) and 0.25 g $CaCl_2 \cdot 2H_2O$ (7 mM) in 200 mL of HPLC water. The pH was adjusted to 10 with 1 M NaOH. The volume was completed to 250 mL with water.

- Citrus pectin solution was prepared by dissolving 2.5 g of pectin in 250 mL of glycine buffer that was heated to 60°C. The solution was centrifuged for 25 min at 10 000 rpm (11 000 × g). The supernatant was used for the determination of the lyase activity. The solution proved to be stable for 3 months, when aliquoted before freezing.

- Stop-solution (0.02 M $H_3PO_4$ ) was used in the lyase assay to halt the reaction; it was prepared by adding 341 $\mu$L $H_3PO_4$ (85%) into 250 mL HPLC water.

For the control sample 100 $\mu$L of the enzyme supernatant was incubated at 60°C for 20 min; the same amount of pectin substrate was incubated in a separate tube. For the test sample, 100 $\mu$L of the enzyme supernatant and 100 $\mu$L of the pectin substrate solution were incubated together. Immediately after the incubation, the samples were cooled and 1 mL of stop-solution was added to stop the reaction. Finally 100 $\mu$L of the

pectin substrate solution was added to the control sample. After mixing, the samples were diluted with water (1:10) and measured at 235 nm in the spectrophotometer.

The activity unit (AU) was defined as the amount of enzyme producing 1 nanomol of unsaturated oligogalacturonides per minute. The activity was calculated according to the formula:

$$EA\left(\frac{AU}{mL}\right) = \frac{\Delta A}{t} \cdot 12 \cdot DF \cdot \frac{1}{\varepsilon} \tag{2.1}$$

where $\Delta A$ corresponds to the difference of absorbance between each sample and its control; all measurements were done in triplicate. The value was divided by the incubation time, $t$, which was 20 min. The number *12* was the dilution factor of the original sample supernatant within the final reaction volume (1200 $\mu$L/100 $\mu$L=12), DF was the dilution factor for measuring in the cuvettes (normally 10). The molar extinction coefficient, $\varepsilon = 5500\frac{1}{\mathrm{M \cdot cm}}$ was used [2].

## 2.12 Cell density determination with the Neubauer chamber

It is usually faster to determine the cell density of a suspension spectrophotometrically, but since the cell culture had pectin, this distorted the result. Therefore, the cell density of the culture of *G. thermoglucosidasius* PB94A was determined with a light microscope using a Neubauer counting chamber. Over the spectrophotometric determination, the microscopic method with the Neubauer chamber has the advantage that the cell morphology cell can be observed and the state and purity of the culture evaluated.

The chamber is etched with a fine grid of lines that allows estimating the size of objects seen under magnification. One entire grid of the Neubauer chamber could be seen at 40× magnification. Main divisions separate the grid into 9 large squares. Each large square is divided into 25 units (area of 0.0025 $\mathrm{mm}^2$ each). The depth of the chamber is 0.02 mm. The volume of a large square is $1.25\times10^{-3}\mathrm{mm}^3$ or $1.25\times10^{-6}$ mL. The cells in a total of four large squares were determined per sample and averaged. The cell suspension was diluted to have 20-200 cells in each large square (statistically significant count). The cell density was calculated with the following equation and the result is expressed in cells per unit volume, cells/mL.

$$Cell\ density\left(\frac{cells}{mL}\right) = \frac{C_{Av}}{1.25 \times 10^{-6}} \cdot DF \tag{2.2}$$

Where $C_{Av}$ is the average number cells in a large square, $DF$ is the dilution factor of the original sample.

## 2.13 DAPI staining

DAPI, $4', 6'$ diamino $-2-$ phenylindole $\cdot$ 2HCl, is a fluorescent stain that binds strongly to double-stranded DNA and is used in fluorescence microscopy to stain cells. DAPI staining is specific at pH 7, at other pH values, non-specific staining occurs.

For the fixation, it was required to incubate 1 volume of the cells with 3 volumes of 4% PFA (paraformaldehyde, pH 7.2) during 1 h at 4°C. This solution was used within one day and used under the fume hood. The cells were harvested (16 060 g and 10 min), washed twice with 1×PBS, and centrifuged at 13 000 rpm (16 060 g) and 3 min after each washing. The samples were resuspended in PBS-ethanol (1:1). If necessary, they were sonicated to disrupt clumping cells. The samples were incubated at -20°C for 15 min. Per slide well, 2.5 $\mu$L of cell solution were applied, air dried and dehydrated in an increasing ethanol series (50%, 80%, 100%) for 3 min each, then they were left to air-dry. The drained slides were put on towel paper, and DAPI staining solution (1 $\mu$g/mL) was given to the slides ($\sim$200 $\mu$L); they were incubated for 15 min in the dark. The excess solution was drained and cover slips were placed above the slides with mounting medium.

The morphology of the nuclei of the cells was observed using a fluorescence microscope at excitation wavelength 350 nm. Nuclei emit blue fluorescence and are considered to have the normal phenotype when glowing with homogeneous brightness [14].

## 2.14 Scanning electron microscopy of carriers

The scanning electron microscopy (SEM) was employed to observe if the cells of *G. thermoglucosidasius* PB94A had adhered to the different carriers tested and to observe the integrity of the carrier structure, see Section 3.5.

### 2.14.1 Preparation and drying of samples

SEM is done under vacuum, therefore the examined samples must be thoroughly dried with a special procedure. Biological samples are first fixed and then the water is exchanged with solvents; the last step is drying them in the critical point dryer.

Aldehydes are common fixatives in structural studies of biological specimens, where they stabilize the cell structure of the sample by chemical cross-linking of the proteins with glutaraldehyde. After the immobilization procedure, the carriers were washed with PBS buffer and then left overnight for fixation in 5% glutaraldehyde with 1×PBS buffer. Afterward, they were desiccated using a series of ascending concentration of ethanol-buffer solutions (20, 40, 60, 80% ethanol), each during 1-2 h contact time, finally the carriers were left in 96% ethanol overnight.

To prepare the carriers for drying, they were placed in a n-amylacetate-ethanol solution

(2:1) for 2 h and then placed in 100% n-amylacetate for 12 h. At this point the carriers were ready for critical point drying.

### 2.14.2 Critical point drying of samples

Critical point drying is a process for removing the liquid from a sample in a precise and controlled manner (supercritical drying). Otherwise, the strong capillary forces that the liquid exerts when migrating out of the material could damage the sample structure.

The samples were submerged in n-amylacetate inside the pressure chamber of the critical point dryer (CPD 020) and the mixer was started. The n-amylacetate was exchanged with high-pressure liquid $CO_2$ (50 bar). The $CO_2$ pressure was gradually decreased until it reached 25 bar. This procedure was done seven times. Then, the chamber temperature was set to 40°C, which increased the pressure to 85 bar. The pressure was slowly released until it reached 1 bar, in a time span of 10 minutes.

When the sample was dry, it was placed over a pin stub (small cup for holding the sample) which had an adhesive carbon disc for maintaining the sample in place. The samples were stored in a desiccator until they were sputtered.

### 2.14.3 Sputtering of samples with a conductive coating

The sputtering was done to prevent the accumulation of static electric fields on the sample during imaging with the scanning electron microscope and to obtain better images.

The samples were sputtered with a Bal-tec SCD 040 sputtering device for generating a gold layer as conducting material. The chamber was washed with argon in five cycles. Then the samples were treated in low vacuum (0.05 mbar) with a current of 15 mA for 120 s to produce the gold coat.

### 2.14.4 Scanning electron microscope observation

The electron microscope images (SEM) were produced at the central laboratory for electron microscopy of the Hamburg University of Technology with the LEO Gemini apparatus. The stubs with the samples were fixed to the stub holder of the microscope and placed in the vacuum chamber at the working vacuum of $1 \times 10^{-7}$ bar. The working distance was between 7 and 14 mm. The voltage (EHT) used was in the range of 2 to 10 kW. The detectors used were SE2 or InLens.

## 2.15 Denaturing gradient gel electrophoresis

Genetic fingerprinting techniques provide a pattern of the microbial community diversity, based on the physical separation of unique nucleic acid molecules. Denaturing gradient gel

electrophoresis (DGGE) is a molecular fingerprinting method that separates polymerase chain reaction-generated DNA products. DGGE has been used to study the diversity of eukaryotic microorganisms (18S rDNA) as well as prokaryotic microorganisms (16S rDNA) [84].

The polymerase chain reaction of environmental DNA can generate templates of differing DNA sequences that represent many of the dominant microorganisms. However, since PCR products from a given reaction are of similar size (bp), conventional separation by agarose gel electrophoresis results only in a single DNA band that does not allow distinguishing different strains. DGGE overcomes this limitation by separating PCR products based on sequence differences. That results in differential denaturing characteristics of the DNA. During DGGE, PCR products run through a polyacrylamide gel with increasing concentrations of the chemical denaturants urea and formamide, alternatively a linear temperature gradient could be used [84, 103].

The melting temperature of individual domains is sequence specific. When the melting temperature of the lowest melting domain is reached, the DNA will partially melt, creating branched molecules, which cannot migrate anymore. Different DNA sequences from different strains will denature at different denaturant concentrations resulting in a pattern of bands. Each band theoretically represents a different microorganism present in the community [103].

### 2.15.1 Pretreatment of samples with Chelex-100 for DNA extraction

The Chelex method has been recommended for extracting total DNA in view of its simplicity and cost-effectiveness [118]. Chelex-100 (iminodiacetic acid) is an anionic resin that can bind compounds that inhibit the PCR. The heavy-metal chelating resin Chelex-100, has been also reported to increase the PCR yield. The procedure followed was done with a modified method of Aldrich and Cullis described below [1].

The DNA containing sample (1 mL) was centrifuged, 700 $\mu$L of supernatant were removed and discarded. The pellet was resuspended in the remaining supernatant to obtain a concentrated sample. Then, 5 $\mu$L of the concentrated sample were mixed with 50 $\mu$L of 5% (w/v) Chelex-100. The mixture was incubated at 99°C and 700 rpm for 10 min. The samples were placed immediately on ice for 2 min. Then they were centrifuged at 13 000 rpm (16 060 g) for 5 min. Since Chelex-100 inactivates the PCR, only the supernatant was transferred to a new Eppendorf tube and 3 $\mu$L were used for the PCR reaction.

Note that the Chelex-100 stock solution should be prepared with sterilized HPLC water and kept sterile. Before using, the resin has to be resuspended by shaking.

### 2.15.2 DNA amplification with PCR

The polymerase chain reaction was done according to Muyzer *et al.* [85], with some slight modifications in the protocol. The PCR primers for 16S rDNA (bacteria specific) used were:

- 341F-GC clamp: 5' CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GCC TAC GGG AGG CAG CAG
- 907R: 5' CCG TCA ATT CMT TTG AGT TT

The polymerase chain reactions consisted of: 5 $\mu$L of 10×Taq Buffer with KCl, 0.25 $\mu$L of each primer (100 pmol/$\mu$L), 3.03 $\mu$L of 25 mM $MgCl_2$, 5 $\mu$L of 2 mM dNTPs, 0.3 $\mu$L of Taq enzyme (5 u/$\mu$L), 1.5 $\mu$L of DNA supernatant, 34.7 $\mu$L of $H_2O$, all summing 50 $\mu$L per reaction.

The touchdown cycling protocol consisted of a denaturing step at 94°C for 5 min, followed by 20 cycles of 94°C for 30 sec, 65°C for 1 min, 72°C for 1 min 30 sec, decreasing the annealing temperature by 1°C every second cycle until 56°C were reached. Then 10 cycles of 94°C for 30 sec, 55°C for 1 min, 72°C for 1 min 30 sec. Finally a 15 min extension at 72°C, the program ended with storage at 4°C.

Amplification products were mixed with 6×loading-dye (6:1) and analyzed by 1% (w/v) agarose gel electrophoresis. The gels were ran in 1×TAE buffer at 80 V for 1 h using a 1 kb ladder as reference. The gels were stained with a solution of Sybr green in 1×TAE (1 $\mu$L/1 mL) for 15 min. The expected fragments were about 500 bp long. If samples were not used right away, they were stored frozen in TE buffer (1:1).

### 2.15.3 Denaturing gradient gel electrophoresis protocol

Denaturing gradient gels were prepared using a gradient mixer and stock solutions of 6% polyacrylamide-bis-acrylamide (37.5:1) containing denaturant concentrations of 20% urea-formamide (back mixing chamber) and 80% urea-formamide (front mixing chamber). When a more detailed view of the bands pattern was required, gels with a gradient of 40-80% urea-formamide were used. The stacking or cap gel was made with a 0% denaturing solution. The gels dimensions were 1 mm × 16 cm × 20 cm [103].

The gradient gels were cast according to the D-Code Biorad instruction manual. The volume of loaded PCR-product depended on the DNA amount and the number of expected products. Generally, 20 $\mu$L of sample with 6×loading-dye (5:1) were put into each well with a Hamilton syringe. The gels were electrophoresed at 55°C for 20 hours at 100 volts using the DCode$^{TM}$universal mutation detection system. The gels were stained with SYBR Green diluted in 1×TAE (1$\mu$L/1 mL) for 30 min and observed on a UV transillumination table and documented.

## 2.16 Fiber treatment procedure

The fiber treatments in laboratory scale were done in glass beakers. Aeration was done with shaking or by injecting air, depending on the experiment volume. In the pilot plant, 1-1.5 bar of compressed air was applied during the bacterial incubation. The target fiber to liquid ratio was 1:15 (w/v), but in the pilot plant this value augmented depending on the amount of fibers used up to 1:25. The treatments done in bench scale were made in the water bath Julabo SW23 (Fig. 3.34) described in Section 3.6.3. The fiber treatment procedure was as follows:

1. The flax fibers were incubated in a 0.1-0.5% $Na_2CO_3$ solution at 90-95°C for 1-2 h.
2. After the fibers had cooled down to about 80°C the liquor was discarded carefully.
3. Bacterial broth of *G. thermoglucosidasius* PB94A, (cell density of more than $1 \times 10^8$ cells/mL) was added in 1:15 (w/v) ratio. During the incubation with the bacterial broth, the pH of the experiment was maintained at 8-9 by adding 1 M NaOH.
4. The fibers were incubated in the bacterial broth at 50-65°C for 15 to 24 h.
5. The fibers were washed for 30 min at 60°C, the washing water was discarded (optional step*).
6. The fibers were pressed to drain excess liquid (optional step*).
7. The fibers were bleached in solution of 1-3% hydrogen peroxide for 6 h (optional step**).
8. The fibers were immersed in a solution of 0.2-0.7% Adulcinol, which had been adjusted to pH 4-5 with acetic acid. The fibers were submerged in this solution for 1-7 h at a constant temperature of 50-60°C. The whitish color should have been absorbed by the fibers, leaving a clearer solution. In the pilot plant the fibers were left longer for operational ease.
9. The excess water was eliminated by pressing or wringing. In the pilot plant this was made with the help of the mangle described in Section 3.8.4.
10. The fibers were left to dry at room temperature. Once they had dried, they were combed in two stages, first with the coarse pin frame show in Fig. 3.49.
11. The second combing step was only applied when the fibers were send to the ITV-Denkendorf for analysis, using the wool hand card shown in Fig. 2.1

*The procedure followed at the pilot plant was similar, only steps 5 and 6 were not done. Another difference was that longer heating and cooling times were required. For

example, the cooking at the pilot plant, took about 3 h and the cooling as well. The machinery constructed for the purpose is shown in 3.45 and is described in Section 3.8.1.

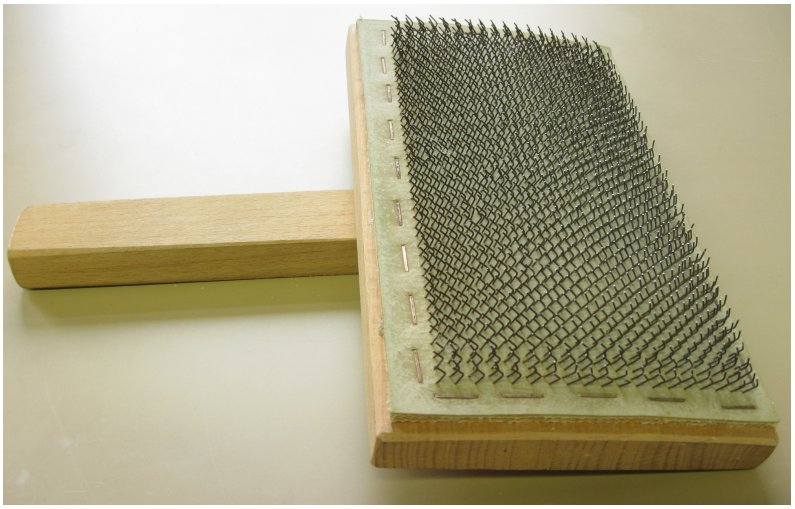


**Figure 2.1**: Fine-toothed card used for combing flax.

**The process step 7, was done only to those fibers that had many shives. The quantity of $H_2O_2$ used was a delicate balance that needed to be adjusted depending on the fiber, the amount of shives and also of the final tenacity (strength) and color goals. For some experiments, the bleaching and softening step were combined into one step, this allowed to save water, energy and to reduce the overall treatment time.

## 2.17 Fiber quality determination at the ITV-Denkendorf

The fiber quality determination was made by our project partners at the Institute Denkendorf Institut für Textil- und Verfahrenstechnik Denkendorf (ITV-Denkendorf). The parameters determined by conventional textile methods were fineness (dtex), resolution, tenacity (cN/tex) and elongation (%).

### 2.17.1 Fiber fineness and resolution

Fineness is the most important property of the fibers. It can be measured by the air permeation method, by a method called Index of Fineness Standards (IFS) or by the projection microscope method (image analysis). For this investigation, the later option was used.

The resolution and the fineness were determined via a projection microscope method with image analysis. A representative sample was combed and embedded in a resin. The cured specimen was cut with a microtome and placed onto a glass slide to observe it under a Olympus BX51 microscope. The obtained image was analyzed with the software from the company Olympus, Soft Imaging System (SIS). The determination of the fiber

cross sectional area requires the interaction of an analyst with the software, which then calculates the resolution value based on the distribution of fiber diameter in different area categories. It was necessary to measure at least 300 objects per sample.

According to the Ullmans Encyclopedia of Science and Technology [66], the fiber fineness is defined as a mass contained in a length. Some of the most important metric textile units used throughout the text, are shown in Table 2.1.

**Table 2.1**: Yarn numbering units [17].

| term | definition |
|---|---|
| tex (tex) | $\frac{1\ \text{g}}{1\ \text{km}} = \frac{1000}{\text{Nm}}$ |
| decitex (dtex) | $\frac{1\ \text{g}}{10\ \text{km}} = 1/10$ tex |
| kilotex (ktex) | $\frac{1\ \text{kg}}{1\ \text{km}} = 1000$ tex |
| metric yarn (Nm) | $\frac{1\ \text{m}}{1\ \text{g}} = \frac{1000}{\text{tex}}$ |

To calculate the fineness in decitex, the formula 2.3 was employed. The so-called diameter is a cross sectional area since the flax fiber is not circular. According to the ITV-Denkendorf, the target value for a good quality fiber was between 3-5 dtex.

$$T_d = \frac{\pi \varnothing^2 \rho}{400} \tag{2.3}$$

Where $T_d$ is the fineness (dtex); $\varnothing$ is the diameter ($\mu$m); $\rho$ is the density (g/cm$^3$); *400* is the unit correction factor. Concerning the resolution, the lower the value is, the better dissociated the fiber bundles are. The calculation of this value is performed by dividing the total number of fibers by the amount of fiber groups. A value of 1 would mean that only elementary fibers are present in the sample, however, this is not desirable because the elementary fibers measure 4-77 mm and this would not produce the long fibers needed for wet spinning. The ideal target value is about 2-3.

### 2.17.2 Fiber tenacity and elongation

For the tenacity (strength) and elongation determination, a stelometer of the company Uster Spinlab was used. Due to the variability of the properties of individual fibers, tensile tests are carried out on fiber bundles instead of individual fibers. The stelometer provides data for fiber strength. In this instrument, force is applied with the pendulum principle. A fiber clamp is used to hold the fiber until the stelometer breaks it and indicates the force required for that. It also determines the elongation using a graduated scale at the breaking point of the fiber sample. To calculate the fiber tenacity, it is necessary

to divide the breaking point number (which is indicated on the graduated scale) by the known weight of the sample [66, 71].

According to the ITV-Denkendorf an acceptable tenacity value for flax is $\geq$40 cN/tex, a weaker fiber would break in the spinning process. The elongation gives an idea of the fiber stiffness, and its values fall always between 1.5 and 3.5%, therefore this parameter was not used as a benchmark.

## 2.18 Fiber quality determination by infra-red with multivariate calibration

The fiber quality determination by the conventional methods is a slow and cumbersome process. The analysis of the flax samples at the ITV-Denkendorf took between 4-6 weeks. A faster method was desired to have a rapid quality assessment of the flax fiber. Moreover, the ability to measure a parameter that provided information about the fiber quality during the fiber treatment, would have been a great advantage for controlling the process.

One approach was to analyze the liquid phase (bacterial broth) for changes that could provide information about the fiber quality. The pH was monitored and UV/VIS spectra were measured (results not presented), but no correlation between them and the fiber quality was found. Therefore, the other option, the analysis of the fiber was further investigated. It was observed in a visit to a textile finishing company, that samples of the fibers were taken during the treatment, quick-dried and analyzed for its quality, and the process parameters were adjusted accordingly.

Infra-red (IR) techniques have been investigated for the prediction of the flax fiber quality [18, 22, 54, 57, 64]. Infrared spectroscopy is a useful method for checking the identity of chemical products.

Putting together IR technology with multivariate calibration methods, a new powerful method for calibration and prediction of unknown samples is obtained. According to the International Chemometrics Society, "Chemometrics is the science of relating measurements made on a chemical system or process to the state of the system via application of mathematical or statistical methods". According to Martens and Naes [76], multivariate calibration is one of the most valuable techniques in the field of chemometrics, used to enhance the use of quantitative measurement data within chemistry. Its objective is to extract quantitative and understandable information embedded in non-selective and apparently useless measurements. Multivariate calibration is the process of learning how to combine data from several channels, in order to overcome selectivity problems, gain new insight and allow automatic outlier detection. Multivariate calibration is the basis for the present success of high-speed Near-Infrared diffuse spectroscopy of intact samples. Multivariate calibration has also been applied to data of UV, Vis, IR spectrophotometry,

(transmittance, reflectance and fluorescence), x-ray diffraction, NMR and in chromatography.

Within the project, about 200 of the flax samples that had been produced in the experiments made at the TUHH were analyzed at the ITV-Denkendorf for the evaluation of the fiber quality (resolution, fineness, tenacity, elongation). The intention was to couple some of this quality data with the IR data sets for building a chemometrical model.

### 2.18.1 Fiber quality determination by FTIR

New technologies have allowed measuring the sample with IR without further preparation. Attenuated total reflectance (ATR) allows a direct sample examination, in solid or liquid state. FTIR spectra were recorded using the Bruker (Vertex 70) with the ATR diamond cell with a window diameter of 1.8 mm.

Before every FTIR measurement, an infrared background from the clean ATR crystal was collected. Then the fibers were positioned in the window and pressed with the clamp of the accessory in order to have reproducible measurements. The readings were made at three different positions of the sample per triplicate, 128 averaged transmission spectra were taken per sample in the wavenumber range from 500 to 3500 $cm^{-1}$ (wavelength range 20 000-2857 nm). The OPUS software data were exported and further analyzed with Matlab R2007a using the N-way Toolbox [3].

A total of 118 samples and their spectra were used for building the chemometric model. Several preprocessing strategies were used in different order and combinations for building the model, such as cutting, smoothing and deriving. These data pre-treatments are required to overcome problems associated with radiation scattering by a solid sample that was measured by reflectance and other phenomena that affect the spectrum baseline [91].

A full cross-validation strategy was followed in order to test the validity of the model. A model was built using a partial least square analysis (PLS) for correlating the spectra with the fineness and resolution (fiber qualities).

### 2.18.2 Fiber quality determination by NIR

An NIR-instrument of the company ABB, model MB 3600 FTIR with InGaAs detector (spectral wavenumber range: 3900-11 000 $cm^{-1}$ or wavelengths 2564-1111 nm) was used.[3] This instrument works on the basis of a polarization interferometer. Absorption spectra of the flax fibers were measured with the sampl*IR* accessory through a quartz window (⌀ 13 mm) using an optical fiber probe. The reflectance spectra were acquired between the wavenumbers of 3000 $cm^{-1}$ and 10 000 $cm^{-1}$ using a resolution of 8 $cm^{-1}$ and 32 scans. A total of 7 measurements per sample were made, considering that the sample is heterogeneous, this was considered necessary in order to build a robust model.

[3]kindly provided by ABB Germany

The strategy followed for building the NIR chemometric model was to divide the data in two sets: the calibration set (49 samples) and the validation set (24 samples).

The spectral data were then processed using the software Horizon MB$^{TM}$. In NIR, single compounds influence not only individual regions, but the whole spectrum. NIR spectra cannot be interpreted in a straightforward manner, as peak assignment to individual vibrations is not possible. Therefore the data obtained have to be analyzed by chemometry. The calibration data set was used to build a model (using PLS) which was later on used for predicting the values of the validation data set.

## 2.19 Chemical oxygen demand determination

According to the "Standard Methods for the Examination of Water and Wastewater", the chemical oxygen demand (COD) is a measure of the oxygen equivalent of the organic matter content of a sample that is susceptible to oxidation by a strong chemical oxidant.

The COD test uses a strong chemical oxidant in an acid solution and heat to oxidize organic carbon to $CO_2$ and $H_2O$. The test is not adversely affected by toxic substances, and test data are available in a couple of hours, providing faster assessment than the $BOD_5$ test [27]. The method basis is that oxidizable substances react with a sulfuric acid-potassium dichromate solution in the presence of silver sulfate as a catalyst. The green coloration of $Cr^{3+}$ is evaluated with the spectrophotometer.

The samples were measured diluted in water (1:5). A glucose solution was prepared as standard (2.5-10 mM). The determination of the COD was done with a Dr. Lange kit LCK 514 using the Dr. Lange photometer and thermostat, according to the manufacturer's instructions and following the classic COD cuvette test methodology [6].

## 2.20 Biochemical oxygen demand determination

Biochemical Oxygen Demand ($BOD_5$) is the amount of oxygen, expressed in mg/L or parts per million (ppm), required by bacteria for the biological oxidation of the organic matter in the wastewater. In this test, microorganisms consume organic compounds for food while consuming oxygen at the same time. The standard $BOD_5$ test measures the amount of oxygen consumed in a sample over a five-day period. The test is limited in some applications, like in industrial wastewater, that often contains toxic substances to the microorganisms, making them unable to oxidize waste [27, 65].

The $BOD_5$ of the samples, was be determined with the respirometer BSBdigi under aerobic conditions as a function of time [4]. The samples were diluted with water (1:1), the pH adjusted to 7, and 1 mL of fresh activated sludge and 250 $\mu$L of allylthiourea as nitrification inhibitor were added. The prepared samples were placed in a reaction vessel, and put to incubate at 20°C for five days. The $CO_2$ produced during the incubation was

absorbed chemically and the vacuum caused the activation of an electrochemical oxygen generator. The replenished oxygen was measured during five days (1 mg/L $O_2$=1 mg/L $BOD_5$).

## 2.21 Hydrogen peroxide detection with the active oxygen method

The determination of the hydrogen peroxide concentration was done according to the procedure proposed by Solvay Chemicals [10], with a detection range of 0.1-5%. The hydrogen peroxide reacts with the excess of potassium iodide in the presence of an ammonium molybdate catalyst to produce triiodide ions, which are then titrated with a standard solution of thiosulfate.

The determination of hydrogen peroxide was done as follows:

1. 3 g of sample were added to 200 mL of water, 20 mL of potassium iodide solution (10%), and 25 mL of an ammonium molybdate-acid mixture. The latter was prepared by adding 0.18 g of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ to 750 mL of water and 300 mL of concentrated $H_2SO_4$.

2. The solution was titrated with a 0.1 N sodium thiosulfate solution until the brown triiodide color had been shifted to a light straw color. A few drops of a starch solution (10 g/L) were added and the titration proceeded until the color of the solution changed from blue to colorless. The volume dispensed was recorded as $A$.

3. For a blank, the titration was repeated without the addition of a sample and the volume dispensed was recorded as $B$.

The calculation of the Hydrogen peroxide percentage (w/w) was done according to Eq. 2.4.

$$\% \ H_2O_2 = \frac{(A-B)(N)(1.7007)}{sw} \tag{2.4}$$

Where $A$ is the titration volume used for the sample (mL), $B$ is the titration volume used for the blank (mL), $N$ is the normality of $Na_2S_2O_3$, and $sw$ is the sample weight (g).

# Chapter 3

# Results

## 3.1 Optimization of the conditions for flax fiber treatment with *G. thermoglucosidasius* PB94A

### 3.1.1 Cultivation of *G. thermoglucosidasius* PB94A

A 200 L-scale fermentation of *G. thermoglucosidasius* PB94A in the bioreactor is shown in Fig. 3.1. The lyase activity and cell density values behave similarly. In Fig. 3.2 the parameters of the fermentation are shown. The initial pH was 9.0 and decreased because the degradation of pectin produced galacturonic acid; when the pH set point of 8.0 was reached, this value was further maintained by the addition of NaOH. When the fermentation was close to its end, the pH increased without further base addition, and this point was related to the loss of lyase activity and some cellular degradation. Another point worthwhile to observe is that even though the air flux was stable at 60 ($L_n$/min). By the end of the fermentation the dissolved oxygen increased, which meant that the oxygen demand by bacteria decreased. At this point the fermentation was stopped.

Some of the 200 L-scale fermentations were performed in the main tank of the pilot plant under non-sterile conditions (Fig. 3.3). The enzymatic activity and the cell number where lower than those obtained in the 200 L bioreactor. Since only 2 L of preculture were used to inoculate the 200 L tank, the fermentation had a larger lag phase and took longer than in the 200 L bioreactor. Nevertheless, the end values of the lyase activity and the cell density were within acceptable ranges.

It was important that the strain *G. thermoglucosidasius* PB94A produced pectinolytic enzymes, and no cellulolytic enzymes. Otherwise the structure of the flax fibers could be damaged. Therefore the supernatant of the culture of PB94A was tested for pectinase and cellulase activities. In Fig. 3.4a, a pectinase test was made with the broth of three different fermentations (all of *G. thermoglucosidasius*), the culture supernatant was put in the exterior wells and in the center well water was used as a control. As shown, the

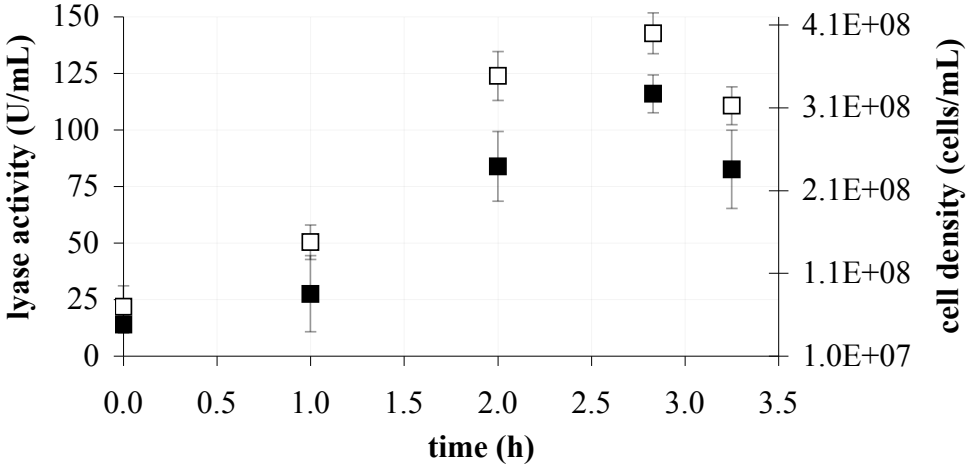



**Figure 3.1**: Lyase activity (■) and cell density (□) of *G. thermoglucosidasius* PB94A during the fermentation in the 200 L bioreactor.

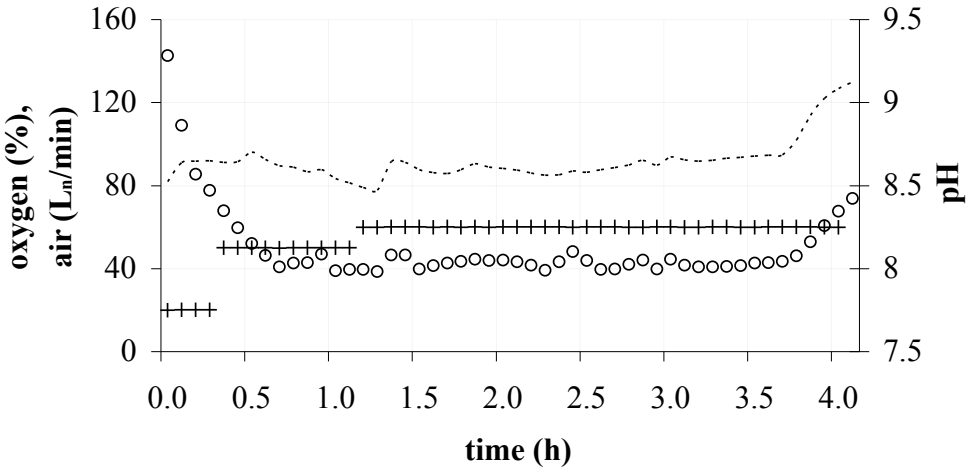



**Figure 3.2**: Dissolved oxygen (. . . ), amount of air supplied (+) and pH (∘) during the fermentation of *G. thermoglucosidasius* PB94A in the 200 L bioreactor.

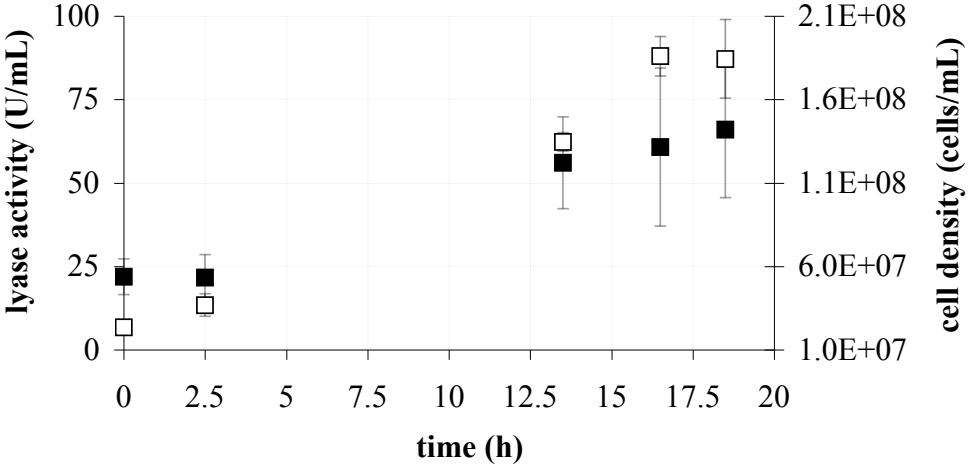
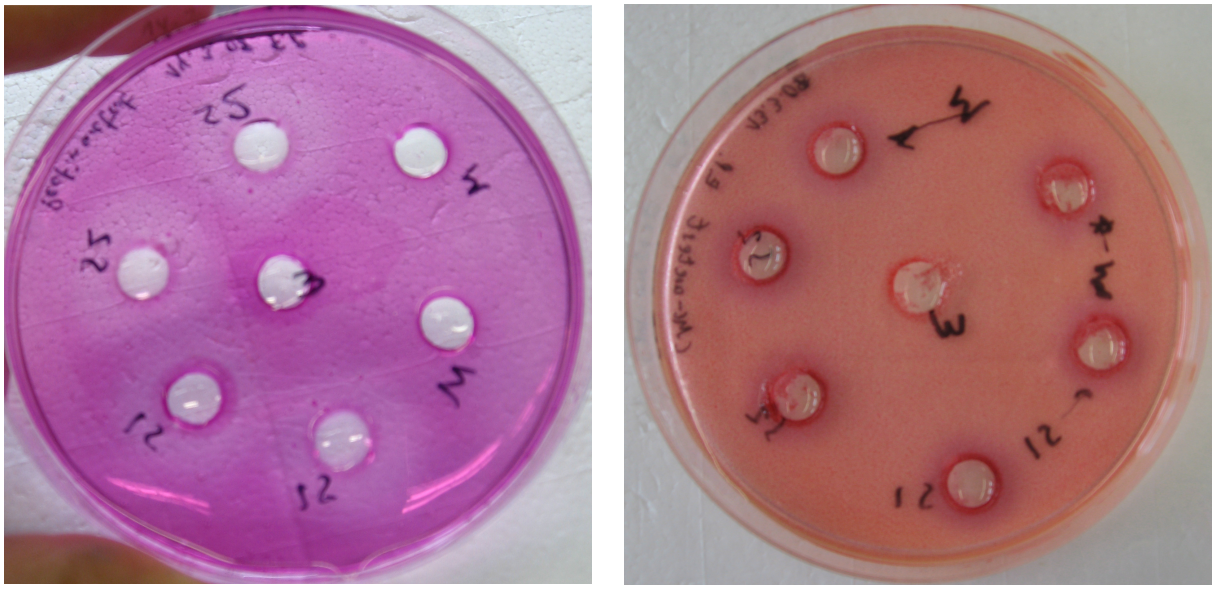



**Figure 3.3**: Lyase activity (■) and cell density (□) of *G. thermoglucosidasius* PB94A, during the fermentation in the 200 L-scale pilot plant.



(a) A clear halo was formed around the edge wells, indicating pectinolytic activity.

(b) No clear halo was formed around any well, therefore no cellulolytic activity was present.

**Figure 3.4**: Cup plate tests for detecting pectinolytic (a) and cellulolytic (b) activities in the culture of *G. thermoglucosidasius* PB94A, which was placed in the edge wells of each plate. At the center well, water was used as negative control.

clear halos are superposing each other, only in the center of the plate, and in the border of the plate, a darker pink coloration is seen, where no pectin was degraded.

Cellulase activity was not present in the *G. thermoglucosidasius* PB94A culture; no clear halo was formed on the outer wells were the culture was placed (Fig. 3.4b). The coloration seen around the wells is an oxidation of the components of the fermentation broth. As this phenomenon was gradual, the contours turned darker the longer the plate was left at ambient conditions, but there was no cellulase activity present.

### 3.1.2 Development of the fiber treatment procedure

Based on previous work made in the area of chemical bast fiber refinement [49, 59, 79, 92], some experiments were made to find an appropriate chemical pretreatment for the fibers. In order that *G. thermoglucosidasius* PB94A could reach and degrade the fiber binding substances readily, an alkaline chemical pretreatment was done. Moreover a $Na_2CO_3$ treatment would provide the pH (8-9) that the alkaliphilic strain *G. thermoglucosidasius* PB94A requires; in addition the pectin is more soluble in alkaline environments.

*Camino05* fibers were placed in beakers forming thin sheets between a net in a ratio of fiber to liquid 1:20. The experiments were performed varying the different process steps: water rinsing, incubation with $Na_2CO_3$ (0.5% or 1%) and treatment with *G. thermoglucosidasius* PB94A.

The best treatment was the (*2*), using the wash plus sodium carbonate 0.5% plus

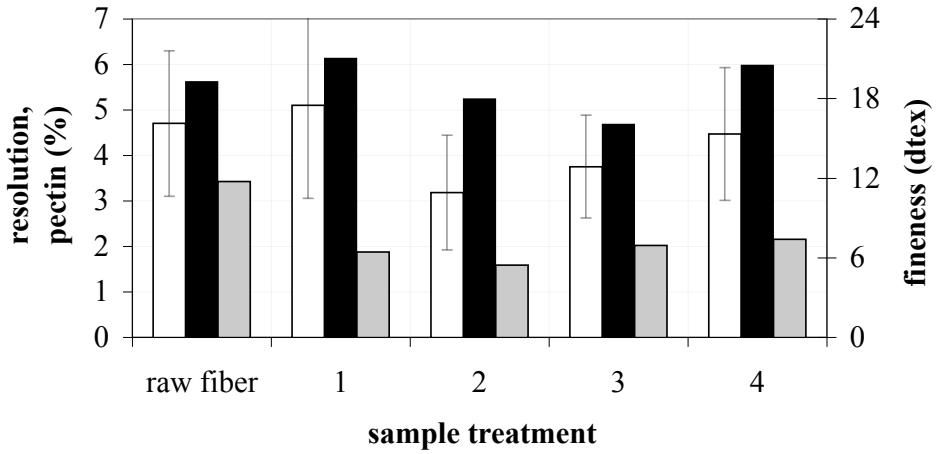



**Figure 3.5**: Resolution (□), fineness (■) and pectin content (■) of flax fiber when different treatment conditions were performed:
(*1*) water rinsing→0.5% $Na_2CO_3$ incubation
(*2*) water rinsing→0.5% $Na_2CO_3$ incubation→*G. thermoglucosidasius* PB94A
(*3*) water rinsing→1% $Na_2CO_3$ incubation
(*4*) water rinsing→*G. thermoglucosidasius* PB94A

the PB94A (see Fig. 3.5); the pectin content in the fiber was the lowest and also the resolution. Using only carbonate or bacteria decreased the pectin content but did not improve the fiber quality as much as the combined treatment.[1] It was decided that a value of 0.5% $Na_2CO_3$ was suitable for subsequent experiments.

It was expected that there would be a clear relation between the pectin content in the fiber and fiber quality, but there was no relationship between those values. Therefore the uronic acid (pectin content) was not determined anymore.

### 3.1.3 Softening of the fibers with conditioners

In general the wet fiber material after the fiber treatment procedure looked good but once it was dry, it glued again and became stiff. During the combing, the fibers were torn apart and further work with this material was not possible. This is a common phenomenon observed with bast fibers called "fiber stiffness" and can be solved by adding softeners, which help reduce the friction between the fibers and condition them [70, 119]. Initially some commercial conditioners were tried, and the texture and fiber quality improved dramatically, as it can be seen in the Table 3.1. The fibers were immersed in a 3% solution of the different conditioners.

Since the hair product Gliss Kur worked well, the following fiber softeners used in the textile industry were tested:

[1]Since the fiber fineness does not follow a normal distribution, the standard deviation is not a valid statistical parameter and is not reported.

- Adulcinol BUN: fatty acid condensation product
- Cefasoft MIS: amino-functionalized polysiloxane compound (microemulsion)

These products were applied in a solution of 5 g/L, the pH was adjusted between 4-5 with acetic acid. The fiber to solution ratio was 1:16.

**Table 3.1**: Quality of the flax processed by the ordinary fiber treatment compared to those treated additionally with the softeners Gliss Kur and Adulcinol.

| | no conditioner | Gliss Kur | Adulcinol |
|---|---|---|---|
| tenacity (cN/tex) | $47.6 \pm 6.1$ | $39.8 \pm 4.2$ | $39.5 \pm 3.6$ |
| elongation (%) | $2.4 \pm 0.5$ | $3.6 \pm 0.4$ | $3.4 \pm 0.4$ |
| resolution | $3.32 \pm 0.94$ | $1.94 \pm 0.69$ | $1.89 \pm 0.82$ |
| fineness (dtex) | 24.56 | 9.21 | 8.96 |

A sensory evaluation with the treated fibers was made, checking the splitting-pattern and the fiber hand, which means how soft the fiber feels. Adulcinol provided the softest fibers, and was chosen for the softening step.

### 3.1.4 Procedure for the removal of the remnant shives from the flax fiber

The treatment described above was useful for most of the green fibers used. However some lots had fibers that were not totally decorticated, and still had a considerable amount of woody material tightly attached to them. Even after the fibers were processed by the standard procedure (excluding step 7, of Section 2.16), it was not possible to separate the tightly bound wooden shives. This poses a great problem for the fabrication process of the flax yarn. The flax band breaks at this point when carded, low yields are obtained and the machinery can be damaged.

The bleaching process is a standard procedure in the textile industry and is widely used for several purposes effectively, such as shive removal, scouring, whitening and cleaning [25, 61, 115, 116, 117]. Based on the common practices in the textile industry the following chemicals were tested: sodium hypochlorite (10%), hydrogen peroxide (10%), Persil detergent (0.15% w/v) and $H_2SO_4$ (1%). The fibers had first been treated with sodium carbonate and *G. thermoglucosidasius* PB94A. The shive removal treatment was the last step.

A sensory evaluation was made with the fibers. The hydrogen peroxide and sodium hypochlorite allowed the shives to be detached by simple shaking once the fibers had been

dried. However, it was decided to use the hydrogen peroxide, because sodium hypochlorite is restricted by environmental regulations.

When dry fibers were moistened with water, the shives were removed partially. Presumably, the physical process of swelling and shrinking had an effect on making the shives loose. However, the moistening with water was not enough to clean the fibers out of shives. The bleaching agent, $H_2O_2$, was necessary to remove the shives, but the amount used seemed to be too high because the fibers were extremely white. At 10% $H_2O_2$ the fiber tenacity was negatively affected and the color was extremely white, therefore milder conditions were necessary. The application of $H_2O_2$ in the different steps of the process and lower dosages of it were tested.

The treatments *a*-*d* were performed as described in Table 3.2, using 0.5% $Na_2CO_3$ and a fiber to liquid ratio of 1:15. A sensory evaluation was made with the fibers, checking their strength and splitting pattern (correlated to the fineness) by breaking them by hand. The quantity of shives detaching from the fiber was also observed.

**Table 3.2**: Characteristics of the flax fibers treated by procedures *a*-*d*.

(a) $Na_2CO_3$ → PB94A → 4.2% hydrogen peroxide → Adulcinol

(b) $Na_2CO_3$ → 4.2% hydrogen peroxide → PB94A → Adulcinol

(c) 4.2% hydrogen peroxide → $Na_2CO_3$ → PB94A → Adulcinol

(d) $Na_2CO_3$ → PB94A → 0.9% hydrogen peroxide → Adulcinol

| | a | b | c | d |
|---|---|---|---|---|
| color | bright white | yellowish | yellowish | white |
| adhered shives | none | none-few | none-few | few |
| fiber fineness | high | high | high | high |
| splitting pattern | regular | good | good | good |

It was observed that the bacterial treatment and the sodium carbonate imparted a yellowish coloration to the fibers that had been previously treated with the hydrogen peroxide (*b*, *c*). The fibers where the hydrogen peroxide was used after the $Na_2CO_3$ and PB94A (*a*, *d*), had a desirable white color. The shive quantity was low for all of the four treatments. The fiber strength was affected by the harsh conditions of procedures *a* and *b*. Given that for *d*, fewer $H_2O_2$ was used, and the fibers had a good quality, this option was chosen. Treatment *d* was applied only when the fibers had shives.

### 3.1.5 Treatment of fibers with different concentration of *G. thermoglucosidasius* PB94A

To discard the possibility that the *G. thermoglucosidasius* PB94A was acting as a mere additional washing operation and to check the retting ability of *G. thermoglucosidasius* PB94A, *Flüh04* fibers were treated with *G. thermoglucosidasius* PB94A culture at different dilutions: 10, 40, 60 and 100%. The fibers were incubated in beakers at 60°C, 100 rpm for 50 h; the pH was maintained at 8. The fibers were treated with adulcinol and the results are shown in Table 3.3. Increasing concentration of PB94A had a positive effect on the characteristics of the fibers. Although, after only 3 h of incubation about 70% of the lyase activity had disappeared.

**Table 3.3**: Quality of the flax fibers treated with increasing concentration of *G. thermoglucosidasius* PB94A culture.

| PB94A (%) | tenacity (cN/tex) | elongation (%) | resolution | fineness (dtex) |
|---|---|---|---|---|
| 10 | $39.6 \pm 4.2$ | $3.0 \pm 0.4$ | $2.41 \pm 0.73$ | 16.11 |
| 40 | $42.8 \pm 3.6$ | $3.2 \pm 0.3$ | $2.41 \pm 0.96$ | 12.9 |
| 60 | $45.5 \pm 3.8$ | $3.0 \pm 0.4$ | $1.89 \pm 0.86$ | 7.6 |
| 100 | $40.4 \pm 9.1$ | $3.1 \pm 0.4$ | $1.74 \pm 0.52$ | 8.5 |

A yarn could be spun at the ITV-Denkendorf using a laboratory-scale rotor spinning machinery, with the fibers treated with pure culture of *G. thermoglucosidasius* PB94A (100%) (Fig. 3.6). The yarn was weak and coarse as expected because the rotor spinning technique uses short staple fibers like cotton, wool and flax tow, and produces coarse yarns. However, the rotor-spun yarn was promising because it used completely unretted green fiber as starting material. This encouraged proceeding with the project to a next step, the building of a pilot plant scale. Finer flax yarns are produced by wet spinning machinery that requires long fibers prepared in the form of a sliver. Since there are no small-scale spinning machines, a larger amount of fibers was needed to produce a yarn by wet spinning.

To prove if the supernatant of the culture of *G. thermoglucosidasius* PB94A was needed to obtain good quality fibers or if the bacterial biomass was enough, fiber treatments using PB94A biomass were made. The fresh *G. thermoglucosidasius* PB94A culture was centrifuged and the supernatant was discarded. The bacterial biomass was resuspended in water to a final volume of 1 L at different concentrations of 10, 40, 60 and 100% of *G. thermoglucosidasius* PB94A. The fibers obtained were of low quality and therefore

**Figure 3.6**: Flax yarn spun with fibers treated with undiluted culture of *G. thermoglucosidasius* PB94A (100%).

were not further characterized. The supernatant was needed in order to obtain good quality fibers. Therefore, the fiber treatments were made using the whole culture of *G. thermoglucosidasius* PB94A.

## 3.2 Detection of the conditions that promote the lyase activity of *G. thermoglucosidasius* PB94A

In Section 3.1.5, it was found that the best fibers were obtained when the whole culture of *G. thermoglucosidasius* PB94A was used. The enzymatic activity of the bacterial culture was presumably needed to obtain a good fiber quality. In the 200 L fermentation of Fig. 3.1, the lyase activity reached a maximum at 2.83 h fermenting time and after this point, it started to decrease. This pattern was found in the majority of the fermentations made and this maximum was the optimal fermentation time, from that point onward the lyase activity decreased steadily if incubated for a longer period of time at 60°C. The pH was maintained constant at 8.0 with the addition of 1 M NaOH, but after the maximum was reached, the pH increased steadily up to 8.5. If the fermentation culture was incubated longer at 60°C, it was observed that the lyase activity was lost after a couple of hours. This phenomenon had been described before for *G. thermoglucosidasius* PB94A [47, 49]. Therefore, for reusing the bacterial culture in multiple fiber treatments, it was needed to find a way to retain the lyase activity.

Several methods for the detection of the pectin lyase activity have been already investigated, such as the cyanacetamide method, the dinitrosalicylic acid method (DNS), the thiobarbituric acid method (TBA) and the lyase assay, but according to the reported

results, only the latter showed consistent results [47]. Nevertheless, in preliminary trials, the thiobarbituric acid test did show some correlation with the Collmer-lyase assay, and was investigated further to confirm the results obtained from the Collmer assay.

### 3.2.1 Storage conditions that maintain the lyase activity of the culture of *G. thermoglucosidasius* PB94A

In order to avoid the decrease of the lyase activity of the culture that was incubated at 60°C, it was important to find the causes for that phenomenon, since the fiber treatment was made also at these conditions. A full grown culture of *G. thermoglucosidasius* PB94A was incubated for 65.5 h at different conditions: -20°C, room temperature, 4°C, 60°C and 60°C with stirring. Samples were taken at 15, 42 and 65.5 h, and the lyase activity was measured by the thiobarbituric acid (TBA) and by the Collmer-lyase methods.

The best conditions for storing the culture were at -20°C or 4°C or at room temperature, where the initial level of lyase activity was maintained best (see Fig. 3.8). When the lyase activity was measured with the TBA, the best storage condition was either -20°C or 4°C, as shown in Fig. 3.7. The incubation of the sample at 60°C increased the lyase activity measured by both methods and the incubation at 60°C with stirring produced lower values of the lyase activity.

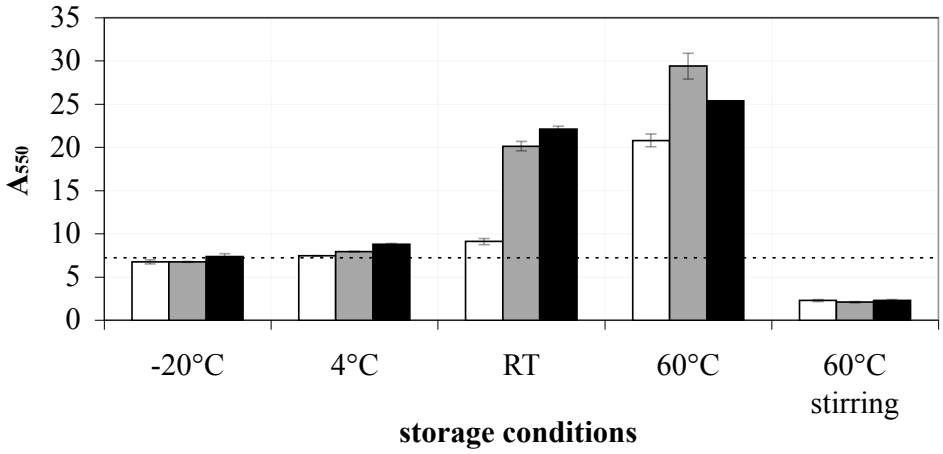



**Figure 3.7**: Lyase activity of the *G. thermoglucosidasius* PB94A culture subjected to different storage conditions measured by the thiobarbituric acid method. Initial value (. . . ) and value after incubation times: 15 (□), 42 (■) and 65.5 h (■) for all the samples.

The TBA method detects the unsaturated uronic esters (monomers, dimers, trimers, etc) by the formation of a pink fluorescent dye in the presence of the thiobarbituric acid and heat. It was assumed that the TBA method was detecting only the heat-degraded pectin of the sample incubated at 60°C and that for the sample incubated at 60°C with stirring, the metabolites were further degraded until they disappeared. The sample incubated at 60°C showed an elevated lyase activity (Collmer method) compared

to the initial culture. And for the sample incubated at 60°C with stirring, no activity was detected at all. It was concluded that the oxygen introduced to the sample at 60°C by stirring, damaged the pectin lyase activity.

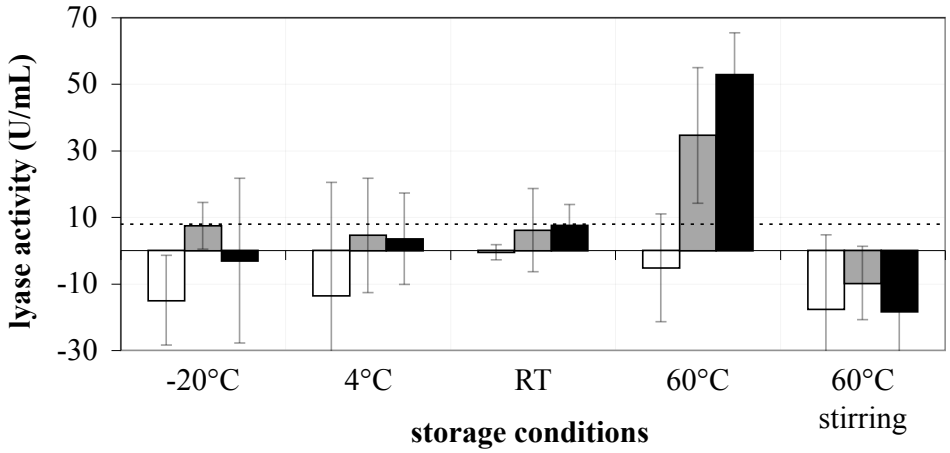



**Figure 3.8**: Lyase activity of the *G. thermoglucosidasius* PB94A culture subjected to different storage conditions measured by the Collmer-lyase assay. Initial value (. . . ) and value after incubation times: 15 (□), 42 (■) and 65.5 h (■) for all the samples.

Although the Collmer lyase assay was determined in triplicates, when the values of lyase activity are low, the error of the method is considerable. The values of the lyase activity of the samples are very similar to those of the their respective background samples, thus, the uncertainty on the difference becomes very large, i.e. Fig. 3.8.

### 3.2.2 Conditions that maintain the lyase activity of the culture of *G. thermoglucosidasius* PB94A when incubated with flax fibers

To investigate the effect of using a lower incubation temperature than 60°C over the lyase activity, full grown culture of *G. thermoglucosidasius* PB94A and a control of pectin mineral medium were put in contact with green fiber at 45°C. The fibers had been treated with sodium carbonate prior to the bacterial incubation.

As can be seen in Fig. 3.9, even though the lyase activity decreased, it was successfully retained for more than 28 hours. The control did not have lyase activity. It can be concluded that the pectinolytic enzymes are labile when incubated at 60°C for several hours. The lyase activities were measured with both the TBA method and the Collmer-lyase assay. The microorganisms grew in both the PB94A culture and the control during the incubation (Fig. 3.10). This phenomenon was expected, since the flax has its own microbial population and *G. thermoglucosidasius* PB94A was isolated from bast fibers as well.

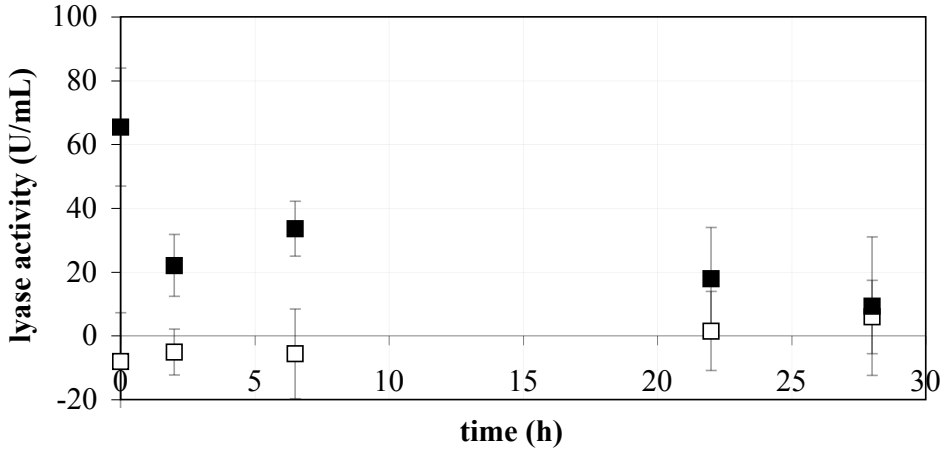



**Figure 3.9**: Lyase activity of the *G. thermoglucosidasius* PB94A culture (■) and of the control of pectin mineral medium (□) when incubated with flax fibers at 45°C.

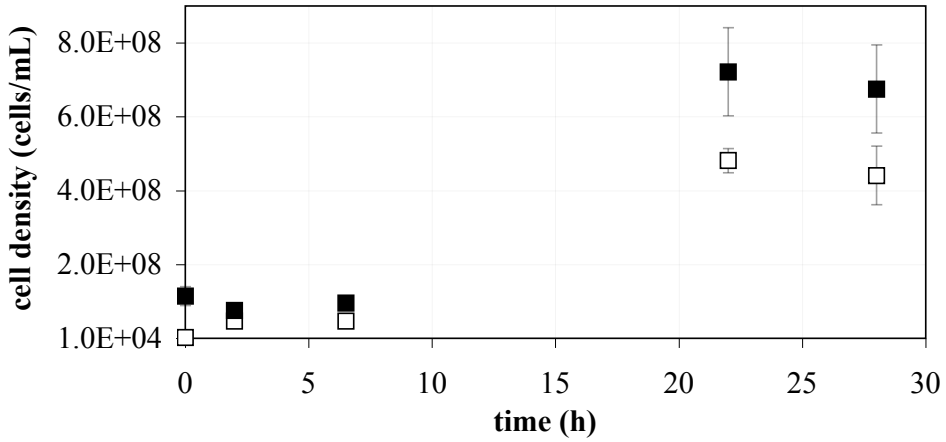



**Figure 3.10**: Cell density of the *G. thermoglucosidasius* PB94A culture (■) and of the control of pectin mineral medium (□) when incubated with flax fibers at 45°C.

The fiber resolution for the PB94A-treated flax was better than that of the control (see Table 3.4). With respect to the fineness parameter, both were quite similar. The tenacity was in the desired range for both samples.

**Table 3.4**: Quality of the fibers treated with a control-buffer or with *G. thermoglucosidasius* PB94A culture.

| | tenacity (cN/tex) | elongation (%) | resolution | fineness (dtex) |
|---|---|---|---|---|
| control | $41.8 \pm 5.9$ | $2.6 \pm 0.4$ | $2.90 \pm 1.40$ | 9.02 |
| PB94A | $39.2 \pm 4.1$ | $3.2 \pm 0.3$ | $1.8 \pm 0.62$ | 10.81 |

### 3.2.3 Reuse of the culture of *G. thermoglucosidasius* PB94A for seven flax fiber treatments

An experiment for testing the reusability of the bacterial culture was made for 7 batches in beaker scale. *G. thermoglucosidasius* PB94A culture, will be referred as "bacterial culture" once it was used for the fiber treatment; because from that point onward it is assumed that it is contaminated by other bacteria. The bacterial culture was used in the samples designated as *M1-M7*. A control (*C1-C7*) consisting of mineral medium without pectin was used to eliminate the possibility that the bacteria which appeared were originating from the citrus pectin. The fiber treatment procedure was repeated seven times as described in Section 2.16. The incubation was carried out at 50°C for 24 h per batch.

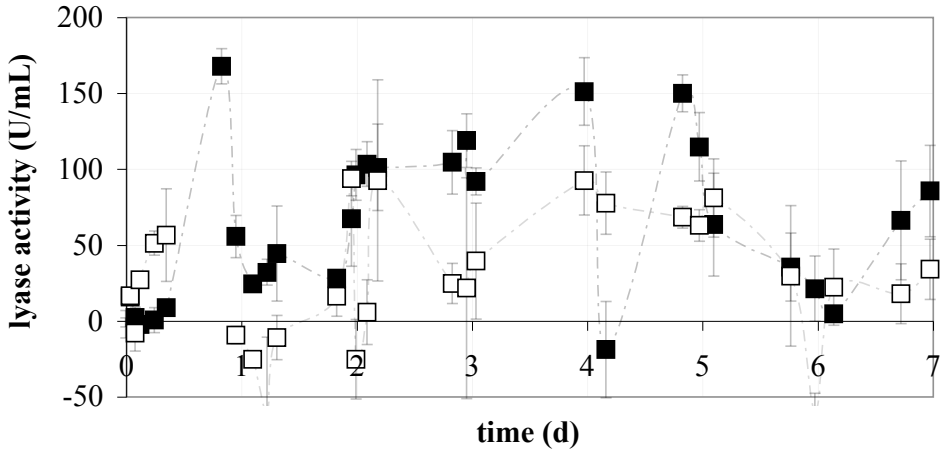



**Figure 3.11**: Lyase activity of the *G. thermoglucosidasius* PB94A culture (■) and of the control (□), when incubated with flax fibers for seven batch reuses. Every 24 h, fresh fibers were added to the process broth.

After each addition of the fresh fibers, the lyase activity and the cell density increased within the 24 h treatment time (Figs. 3.11 and 3.12). These values decreased when new fibers were introduced in the culture. This behavior was observed for the first four batches. The lyase activity was constantly higher for the PB94A sample than that of the control. Noteworthy was, that the control also acquired significant levels of lyase activity, even though it had no other pectin source than the pectin from the flax itself.

At the beginning of the cycle 1, the PB94A culture had a cell density of $5.9\times10^7$, and the control buffer had zero (it had been sterilized). As shown in Fig. 3.12, in both samples the cell density had considerably increased after 1 day, and after the end of the second cycle, both samples had reached almost the same cell number of $1\times10^9$. This value remained constant. When fresh fibers were added, some bacterial liquid was inevitably removed with them, causing a cyclic oscillation in the values of lyase activity and cell density. By the end of the cycle, the cell density had increased again.

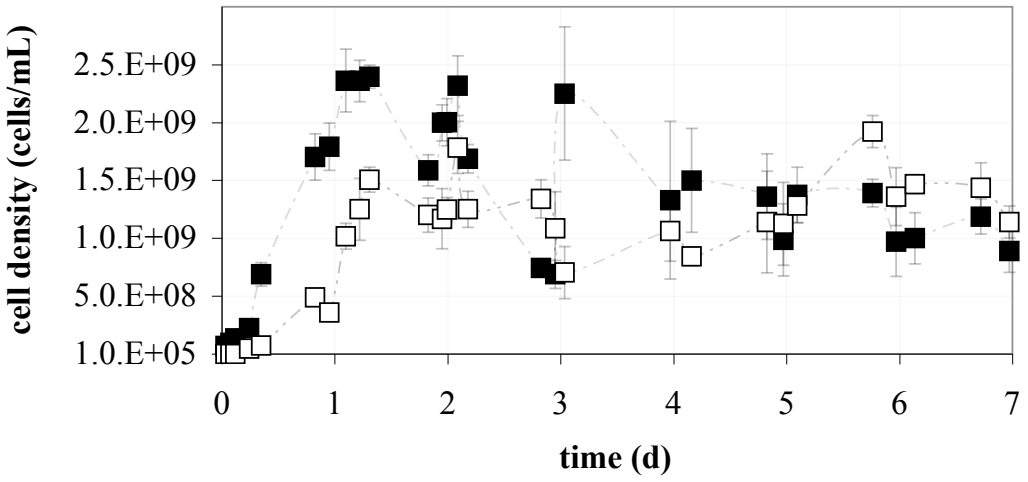



**Figure 3.12**: Cell density of the *G. thermoglucosidasius* PB94A culture (■) and of the control (□), when incubated with flax fibers for seven batch reuses. Every 24 h, fresh fibers were added to the process broth.

It was of interest to study what type of bacteria had grown in the control set (*C1-C7*), where many bacteria had grown and a considerable level of lyase activity was detected. A DGGE was made in the standard type of gel with a 20-80% denaturing gradient (Fig. 3.13). A 40-80% gradient (Fig. 3.14) was also cast to obtain more separated bands.

The lateral bands of the gel in Fig. 3.14 ran unevenly. This "smiling" phenomenon is common when the denaturing substance leaks from the gel out to the running buffer, causing distortion on the edges of the gel. However the profile can be seen clearly in the gel with the broader gradient in Fig. 3.13. In lanes 4 and 10 too little PCR product was loaded, therefore the bands were too faint for accurate comparison purposes.

In Fig. 3.13 most of the bands appear between 9 and 12 cm. In lane 9 the *G. thermoglucosidasius* PB94A profile is composed of four main bands at 9.8, 10.2, 10.6 and 10.8 cm. On lane 1, the lane at 10.6 is the only one conserved. From thereon, new bands appear and it is difficult to establish if PB94A was still present. The band at 10.2 cm appears again in lanes 6-8. In lanes 5-8 and lanes 12-17, common bands at 9.2 and 10.9 cm appeared.

In Fig. 3.14 the profile of *G. thermoglucosidasius* PB94A is shown in lane 9. It is composed of bands in positions 5, 5.5 and 6.3 cm. There is also a group of bands in the range of 3.5-4 cm. By the end of the second cycle with PB94A, in lane 3 the PB94A was still recognizable with bands in positions 3.5-4 and 5, and the band at 5.5 cm. was dim. New bands between 8.5 and 10 cm appeared. By the last cycles, many of the bands were common for samples PB94A and control. The PB94A series (lanes 5-8) and the control series (lanes 14-17), stabilized to similar populations. The profile of the PB94A strain was almost washed out. *G. thermoglucosidasius* PB94A was overgrown by the bacteria,

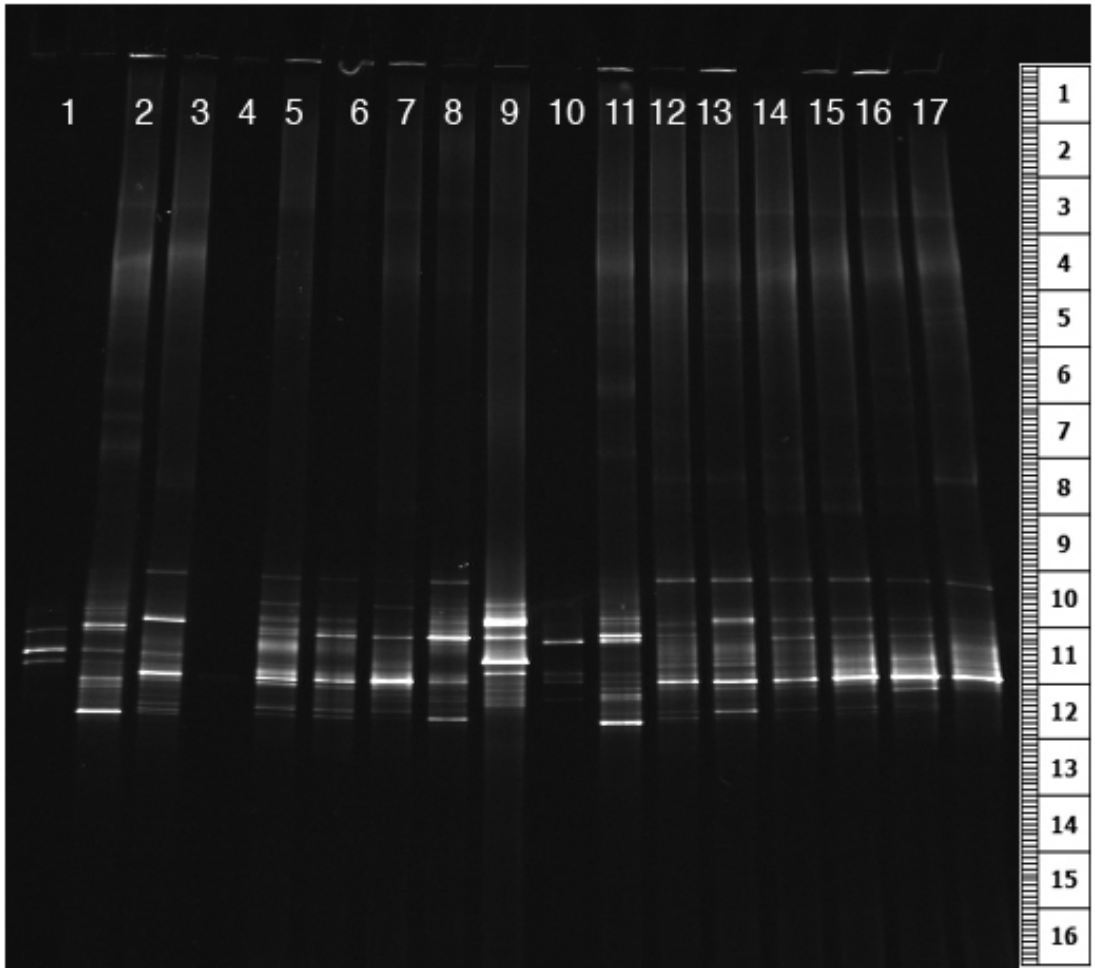



**Figure 3.13**: DNA profile by the DGGE technique of the bacterial culture (lanes 1-8) and of the buffer-control (lanes 10-17) samples, incubated with flax fibers; reusing each solution 7 times; in a 20-80% denaturing gradient gel. The profile of the strain PB94A is in lane 9.

Lane 1: *M1* (9.5 h); lane 2: *M1* (21 h); lane 3: *M3* (0 h); lane 4: *M3* (24 h); lane 5: *M4* (24.5 h); lane 6: *M5* (24 h); lane 7: *M6* (24 h); lane 8: *M7* (24 h); lane 9: *G. thermoglucosidasius* PB94A;

lane 10: *C1* (9.5 h); lane 11: *C1* (21 h); lane 12: *C3* (0 h); lane 13: *C3* (24 h); lane 14: *C4* (24.5 h); lane 15: *C5* (24 h); lane 16: *C6* (24 h); lane 17: *C7* (24 h). The scale in the right side of the Figure is a visual aid.

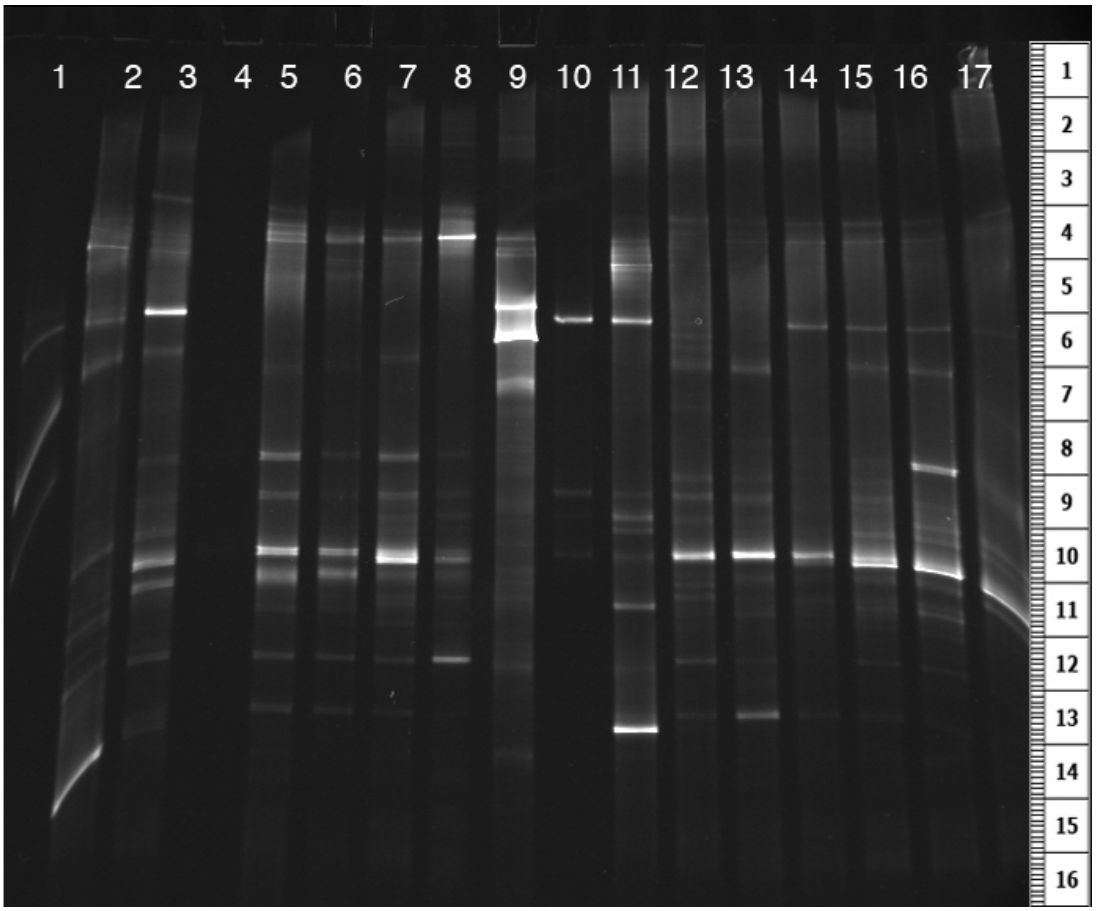



**Figure 3.14**: DNA profile by the DGGE technique of the bacterial culture (lanes 1-8) and of the buffer-control (lanes 10-17) samples, incubated with flax fibers; reusing each solution 7 times; in a 40-80% denaturing gradient gel.

Lane 1: *M1* (9.5 h); lane 2: *M1* (21 h); lane 3: *M3* (0 h); lane 4: *M3* (24 h); lane 5: *M4* (24.5 h); lane 6: *M5* (24 h); lane 7: *M6* (24 h); lane 8: *M7* (24 h); lane 9: *G. thermoglucosidasius* PB94A;

lane 10: *C1* (9.5 h); lane 11: *C1* (21 h); lane 12: *C3* (0 h); lane 13: *C3* (24 h); lane 14: *C4* (24.5 h); lane 15: *C5* (24 h); lane 16: *C6* (24 h); lane 17: *C7* (24 h). The scale in the right side of the Figure is a visual aid.

which probably originated from the flax fiber.

However it is important to notice that the fiber quality was good for all the samples, those incubated with PB94A, and those treated with the buffer only (see Fig. 3.15). The former felt much softer, and in general looked cleaner, with less woody parts. The quality parameters for both sets of samples (*M1-M7*) and (*C1-C7*) and of the green fibers used as starting material are shown in Table 3.5.

Lyase activity was detected in all the samples, even those that had not been inoculated with PB94A. It is important to note that a lower temperature 50°C instead of 60°C was used.

**Table 3.5**: Quality of the fibers treated with PB94A bacterial culture (*M1-M7*) vs. those treated with a control of mineral medium (*C1-C7*) in seven consecutive experiments. Green fibers were the starting material.

| | tenacity (cN/tex) | elongation (%) | resolution | fineness (dtex) |
|---|---|---|---|---|
| green fibers | 60.20 | 2.60 | 7.30 | 37.38 |
| *M1-M7* | $61.66 \pm 3.74$ | $2.80 \pm 0.16$ | $2.47 \pm 0.30$ | $10.87 \pm 1.78$ |
| *C1-C7* | $61.90 \pm 2.52$ | $3.01 \pm 0.23$ | $2.20 \pm 0.42$ | $9.73 \pm 2.26$ |

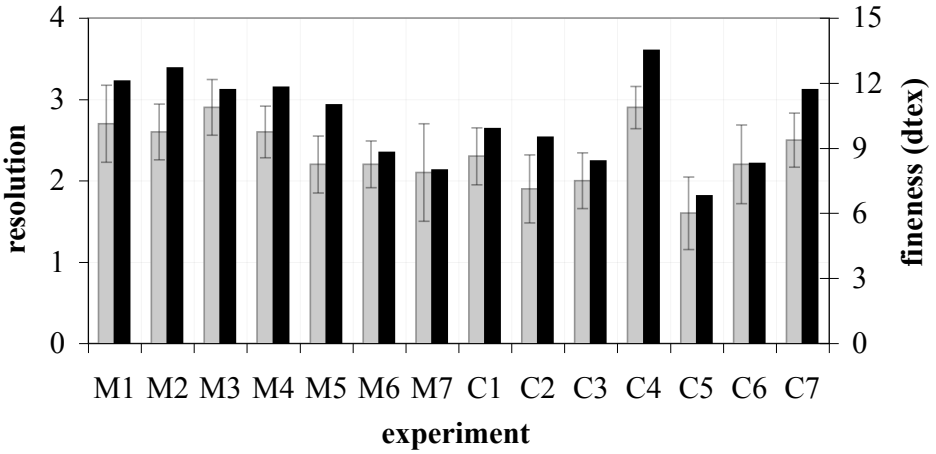



**Figure 3.15**: Fiber resolution (■) and fineness (■) of the fibers treated with PB94A bacterial culture (*M1-M7*) vs. those treated with a control of mineral medium (*C1-C7*) in seven consecutive experiments.

## 3.3 Fiber quality determination by IR spectroscopy

The analysis of the flax samples at the ITV-Denkendorf is laborious and took over a month to obtain the results. Therefore, a method to have a quick quality assessment of the flax fiber would be of great advantage. Even more if the fiber quality during the fiber treatment could be determined and the process controlled accordingly.

The fineness and the resolution are the parameters that describe the effectiveness of the fiber separation, and they were correlated to the IR data.

### 3.3.1 Fiber quality determination by FTIR-multivariate analysis

Flax fiber samples of different experiments with diverse qualities were measured by FTIR as described in Section 4.3.2. For building the chemometric model with partial least squares (PLS), a total of 10 factors were used. Several preprocessing strategies, such as: cutting, smoothing and deriving were made in different order and combination to look for the best model. However, no linear correlation was found for the fiber qualities (resolution and fineness); the cross-validation prediction made by the model was extremely poor and the data was highly scattered. An example of one of the models is shown in Fig. 3.16.

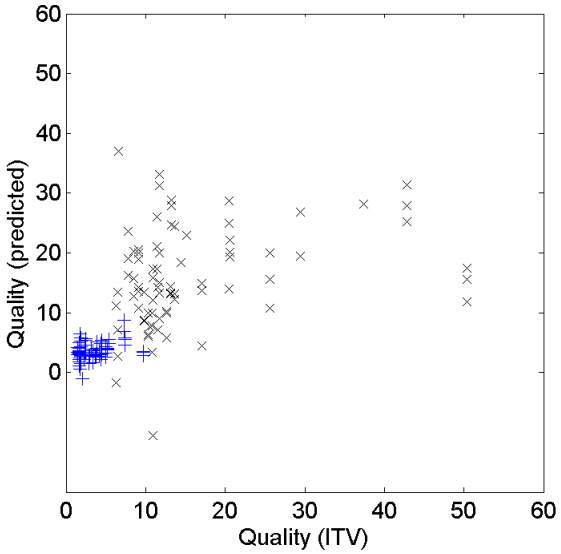



**Figure 3.16**: FTIR-ATR calibration statistics for fiber resolution (+) and fineness (dtex) (×) of the 118 samples showing no correlation using PLS analysis.

### 3.3.2 Fiber quality determination by NIR-multivariate calibration

Flax fiber samples of different experiments with diverse qualities were measured by NIR. The data were divided in two sets, a calibration data set composed of 49 items (resolution

$2.27 \pm 0.57$, fineness $9.66 \pm 2.59$ dtex) and a validation data set of 24 items (resolution $2.56 \pm 0.87$, fineness $11.43 \pm 2.73$ dtex).

For building the PLS chemometric model, 7 factors were used. Several preprocessing strategies were tested in different order and combination to look for a better model, such as: centering, normalizing, averaging, cutting, smoothing and deriving. The chosen strategy for preprocessing the spectra was a baseline correction, followed by the calculation of the second derivative (according to the differential quotient method). Finally the seven scans of the same samples were normalized and averaged. The models obtained for the fineness (RMSEC=0.6719 dtex, $R^2$=0.9341, m=0.9294) and resolution (RMSEC= 0.1497, $R^2$=0.9333, m=0.9285) are shown in Figs. 3.17a and 3.17b.

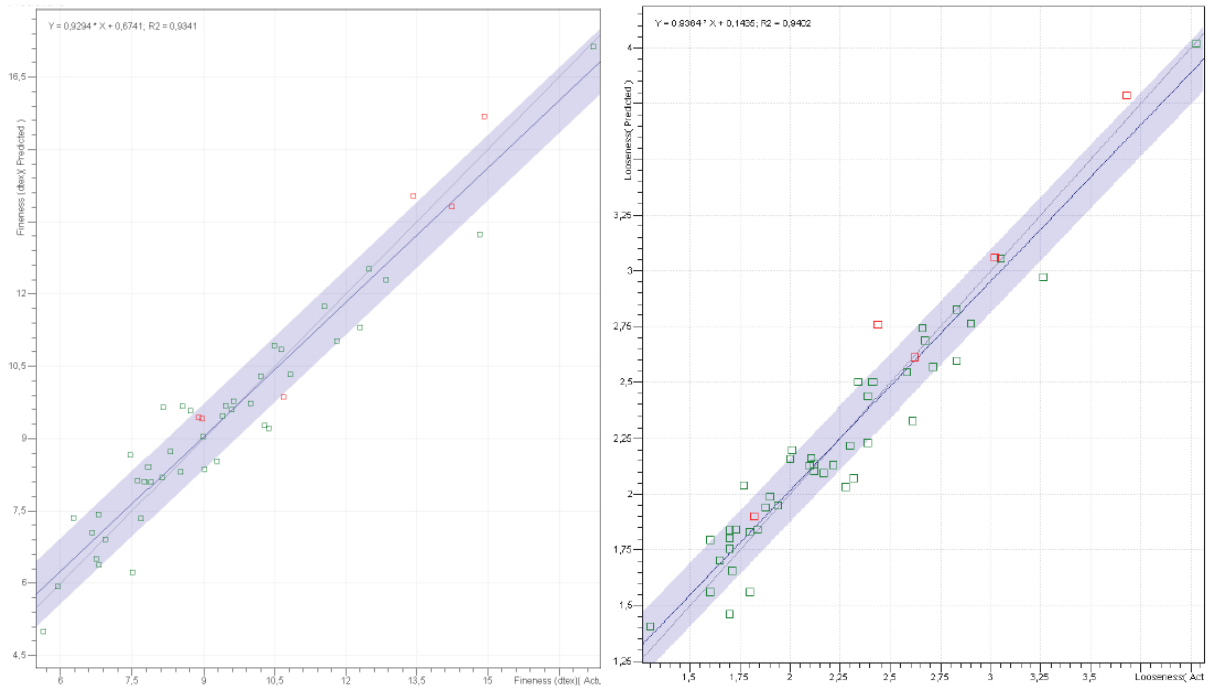
The models were tested against the independent validation data set, and the predictions obtained from them are shown in Figs. 3.18a and 3.18b. The broken line of slope m=1 with correlation coefficient $R^2$=1, represents an ideal model, but the data are spread in a cloud around this line.

The calibration models were further tested by a full cross-validation strategy (of the calibration data set). The fineness model had the following results; RMSEC= 1.7805 dtex, $R^2$=0.6685, m=0.5042, and the resolution PLS model had a RMSEC= 0.3808, $R^2$=6836, m=0.5372. The $R^2$ is the correlation coefficient and m is the slope of the model, being 1 the ideal value. The closest the root mean square error of calibration (RMSEC) is to zero, the better a model is.

The models were not accurate enough in predicting the fiber resolution and fineness of unknown samples reliably. Further refinement of the model is needed to use this tool for fiber analysis.

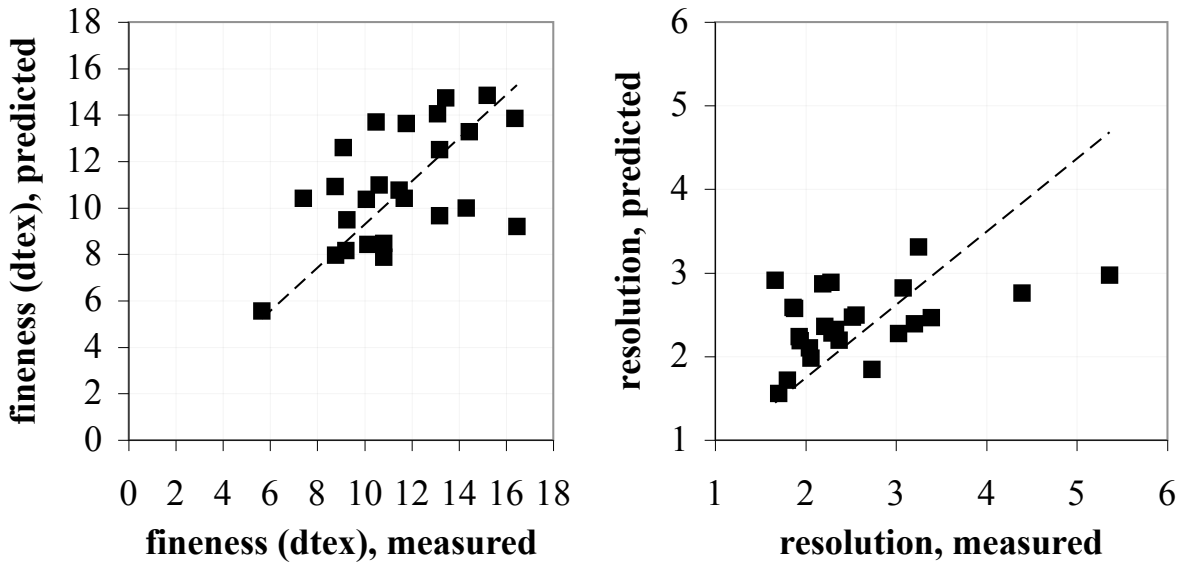
## 3.4 Analysis of the new strains appearing during the flax fiber treatment

As described in Section 3.2.3, a colonization with extraneous microorganisms, mainly bacilli, was observed (microscope) when the culture was incubated with flax fibers. This was even more pronounced when the culture was reused for several fiber treatments. In Fig. 3.19 a picture of a PB94A culture after its fifth reuse with fibers using the DAPI nucleic fluorescent stain is seen. Here the nuclei of the cells glow in blue color. Because they are not homogeneous, it was assumed that a mixed culture was present.



(a) fineness (b) resolution

**Figure 3.17**: Predicted (y-axis) vs. measured (x-axis) fineness (a) and resolution (b) of the 49 calibration samples. The PLS model was generated with 7 factors and is represented by the dark line, and the shadow area parallel to it, is the confidence level. The ideal model of slope of 1, is represented by the light gray line.




(a) fineness (b) resolution

**Figure 3.18**: Predicted vs. measured fineness (a) and resolution (b) of the 24 validation samples. The ideal correlation is depicted as a dashed line.

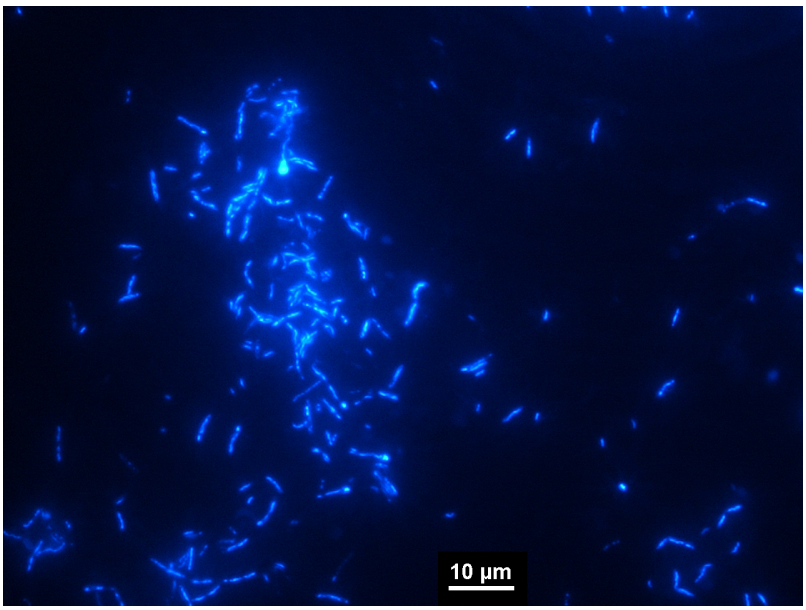



**Figure 3.19**: Microbial community after reusing the bacterial culture five times with flax fibers, stained with DAPI in 1000 magnification.

### 3.4.1 Monitoring the bacterial population change within one flax fiber treatment

To investigate the population change of the microorganisms during the fiber treatment, the metabolites and DNA profile were monitored during 133 h.

"Used" bacterial culture of *G. thermoglucosidasius* PB94A inhibited the growth of *G. thermoglucosidasius* PB94A. This was corroborated by immersing filter paper discs in used PB94A bacterial culture from different fiber treatment experiments, and incubating the discs on a pectin plate with freshly inoculated *G. thermoglucosidasius* PB94A. It was observed that the "used" culture caused inhibition to *G. thermoglucosidasius* PB94A, as seen in Fig. 3.20, where clear halos of no-growth-zones appeared around the filter-paper discs *3* and *7*.

A DGGE of some samples of the inhibition experiment showed that the strain PB94A was still present after 12 h of incubation (lane 2 of Fig. 3.21). However after 90 and 133 h incubation time (lanes 3-4), new bands appeared and the profile of *G. thermoglucosidasius* PB94A faded. Since the pattern of lane 3 and 4 is identical, it was assumed that the population had stabilized in the system.

### 3.4.2 Isolation of new pectinolytic strains appearing during the flax fiber treatment

To know the role and/or identity of the new strains detected in the broth reuse experiments (Section 3.2.3), bacterial cultures that had been used five times already were analyzed. The bacteria were selected by growth on pectin in liquid and solid cultures at 65°C.

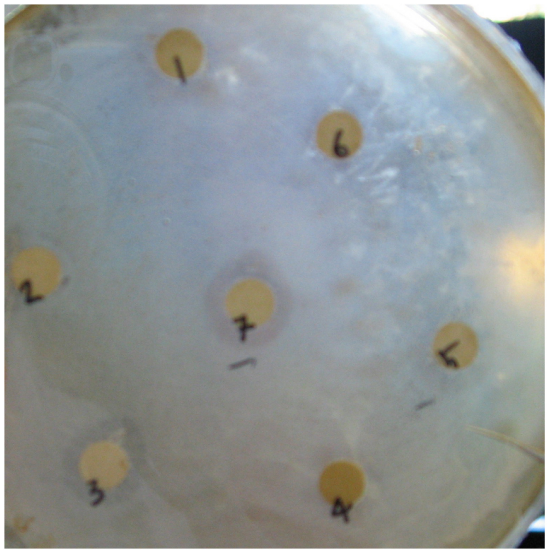



**Figure 3.20**: Pectin plate inoculated with *G. thermoglucosidasius* PB94A where filter paper discs immersed in PB94A culture that had been in contact with flax fibers, show an inhibition growth halo around some of them. Disc 1: 29h, disc 2: 39h, disc 3: 86h, disc 4: 90h, disc 5: 107h, disc 6: 115h, disc 7: 133h.

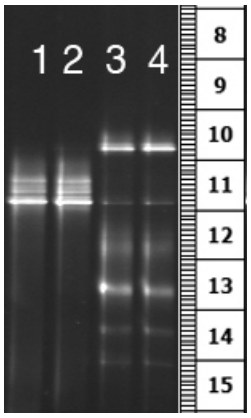



**Figure 3.21**: DNA profile of *G. thermoglucosidasius* PB94A (lane 1) and of samples of *G. thermoglucosidasius* PB94A incubated with flax fibers, after 12, 90 and 133 h contact time in lanes 2-4 consecutively; by the DGGE technique. The scale in the right side of the Figure is a visual aid.

Different colony morphologies were selected. The strains were named *3, 6, 8* and *88*. A DGGE analysis of the strains in a 40-80% denaturing gradient gel is shown in Fig. 3.22. The DGGE pattern of all the new isolated strains was identical to the *G. thermoglucosidasius* PB94A profile. The other strains used as reference were clearly different to PB94A. The strain PB94A was the only one isolated from the reused broth. The other bacteria that appeared during the broth reuse were not cultivable on pectin.

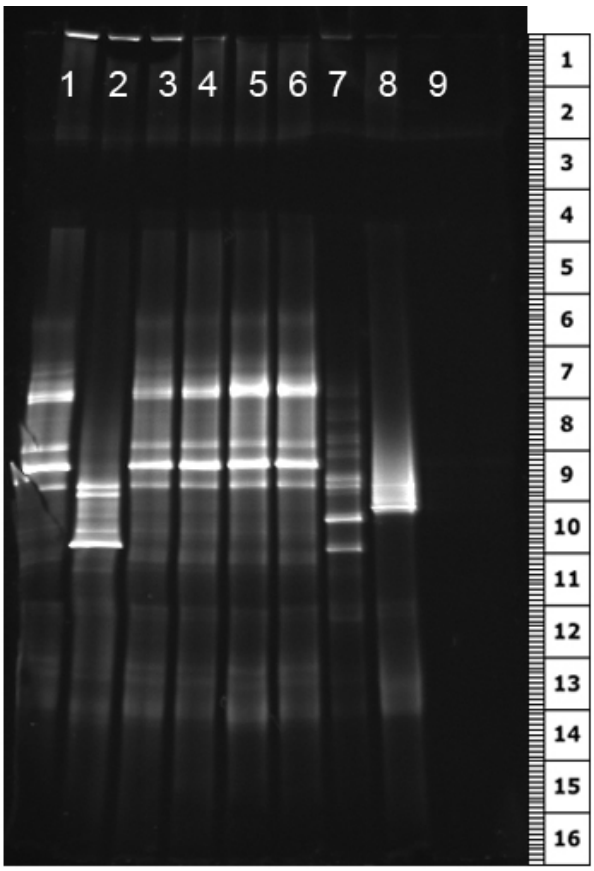



**Figure 3.22**: DNA profile by DGGE of *G. thermoglucosidasius* PB94A (lane 1) and of the new strains *3, 6, 8, 88* (lanes 3-6). Other bacteria used as control were: lane 2: *G. thermocatenulatus* PB94B, lane 7: *G. thermodenitrificans* PB1511 (DSM 21923), lane 8: *Bacillus subtilis* sp. lane 9: sterile water as negative control, in a 40-80% denaturing gradient gel. The scale in the right side of the Figure is a visual aid.

## 3.5 Immobilization of *G. thermoglucosidasius* PB94A on solid supports

New bacteria were appearing during the flax fiber treatment and were overgrowing the original inoculated strain, *G. thermoglucosidasius* PB94A (see Section 3.4.1). These new strains were possibly originating from the flax.

Several authors have reported the use of immobilized whole cells in cheap carriers for the degradation of pollutants and for the conversion of substances. Advantages include higher catalyst storage and operational stability, improved retention of the enzymatic activity and good possibilities for long-term use in continuous reactors [39]. To avoid the washing out of the *G. thermoglucosidasius* PB94A strain and to repel the rapid colonization of extraneous bacteria in the broth used for treating the flax fibers, it was proposed to make a seed culture for continuously providing the "right" bacteria, *G. thermoglucosidasius* PB94A, to the pilot plant.

Polyurethane foam (PUF) has acquired a great relevance being used as a carrier for the

removal of organic compounds, odor waste control, acetic acid fermentation, ferrous sulfate oxidation and hydrocarbon removal. PUF is inert and has good mechanical properties such as 97% porosity, easy oxygen diffusion because of its large pore size, high resistance and elasticity and all these at a low cost. Not all cells have the ability to fix themselves to solid surfaces but sometimes in the presence of a mixed culture, they could adhere better [40]. Four carriers whose price and manageability were apt for a flax agroindustrial facility were tested:

**Por:** Poraver (glass beads 0.5-1 mm diameter)

**Cer:** Ceramtec E109 ($\alpha$-aluminum)

**Sty:** Styropor (sliced polystyrene)

**PUF:** Polyurethane foam

The materials were washed in distilled water, degassed and sterilized by autoclaving. Only Styropor was sterilized by placing it under UV light in 70% ethanol.

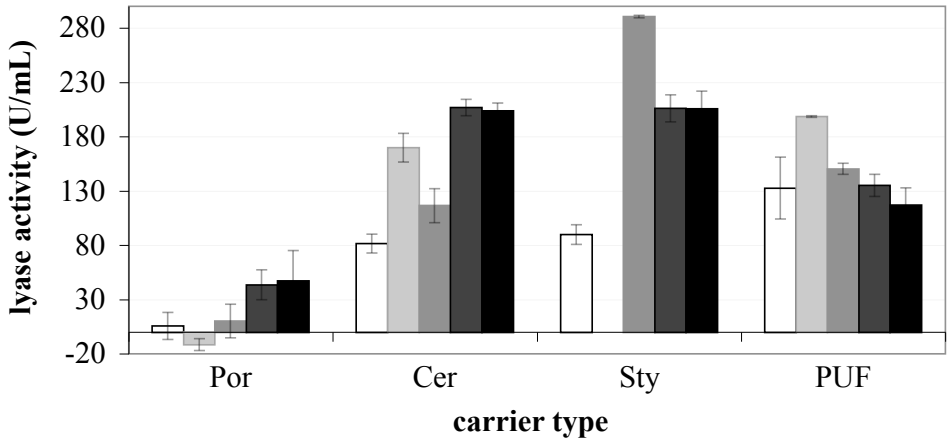



**Figure 3.23**: Lyase activity determined with the Collmer method, in immobilization cycles *1* (□), *2* (▒), *3* (▒), *4* (■), *5* (■), for all the carrier types: Poraver (Por), Ceramtec (Cer), Styropor (Sty), polyurethane foam (PUF).

Several immobilization cycles are required to obtain enough adherence of the cells to the carrier [40]. Five consecutive cycles of about 12 h each at 60°C were carried out in beakers. For cultivating PB94A in flasks, a maximum of 12 h incubation time was needed to reach the target activity values. Longer times caused a lost of the lyase activity. The used supernatant was discarded after the incubation time and fresh media was added to the carriers. The pH was maintained between 7 and 8.

The lyase activity by the Collmer assay and the TBA method (Figs. 3.23 and 3.24) and the cell density (Fig. 3.25) were determined after 12 h incubation for all the samples.

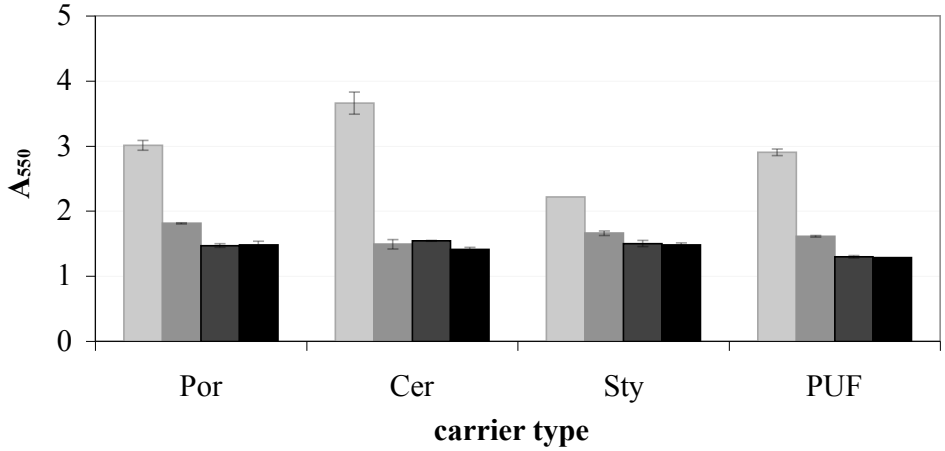



**Figure 3.24**: Lyase activity determined with the TBA assay for immobilization cycles *2* (■), *3* (■), *4* (■), *5* (■), for all the carrier types: Poraver (Por), Ceramtec (Cer), Styropor (Sty), polyurethane foam (PUF).

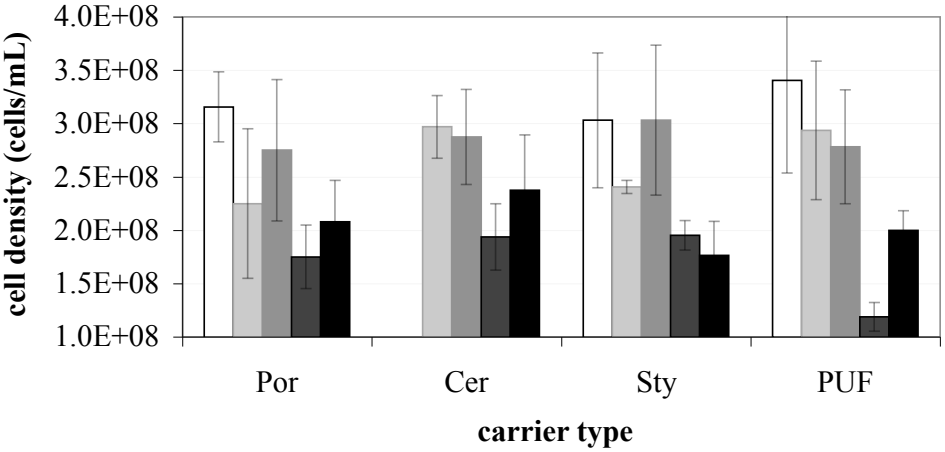
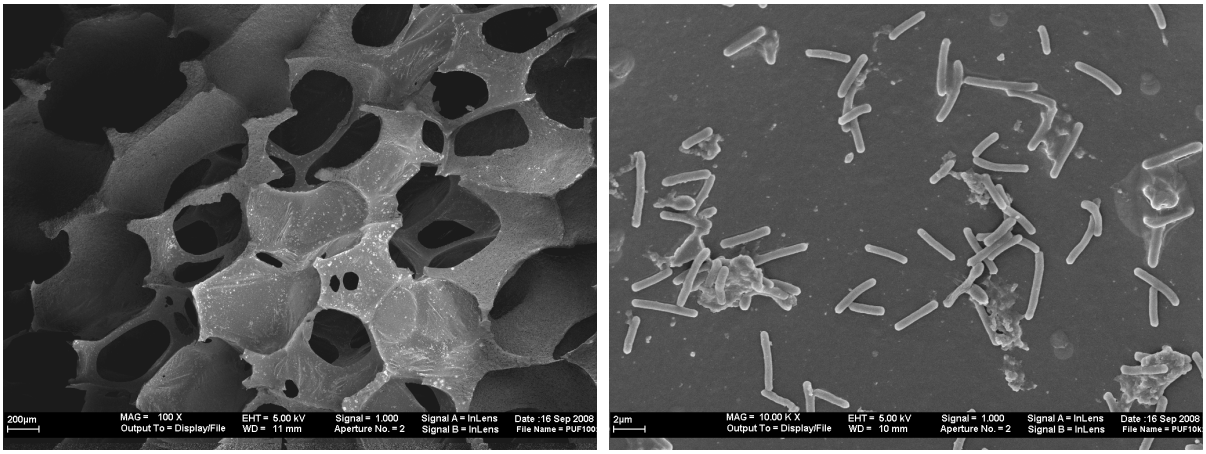



**Figure 3.25**: Cell density for immobilization cycles *1* (□), *2* (■), *3* (■), *4* (■), *5* (■), for all the carrier types: Poraver (Por), Ceramtec (Cer), Styropor (Sty), polyurethane foam (PUF).

The lyase activity increased after cycle *1* for the Ceramtec, Styropor and the PUF to levels never observed before in batch fermentation of PB94A. The cell number showed a decreasing tendency and was within 1-3×$10^8$ (after 12 h) for all cycles and carriers.

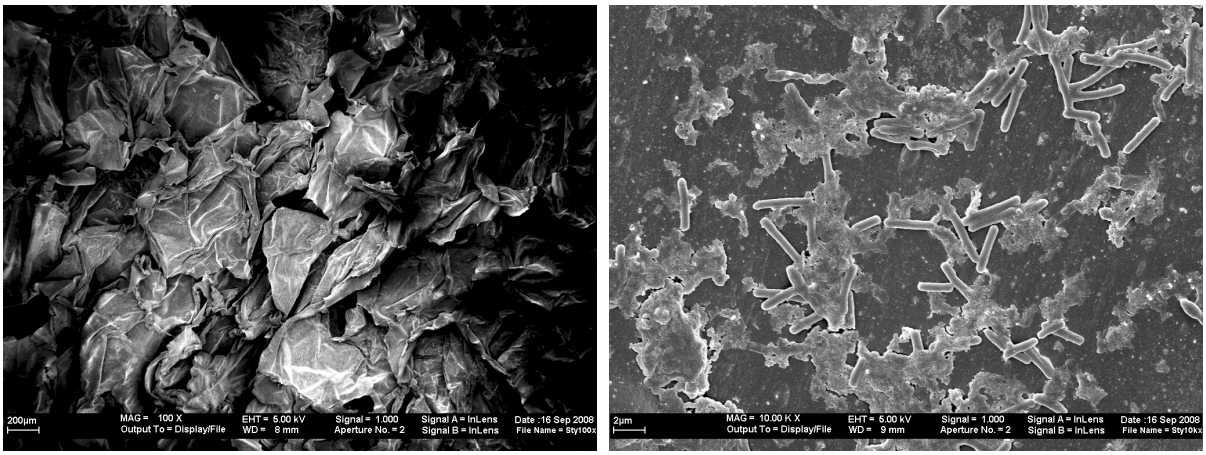
The TBA values in cycles *2-5* were decreasing, which could mean that the pectin degradation was becoming faster with each cycle; and fewer degradation products were observed after each cycle.

The colonization of the carrier particles was determined at the end of the experiment (after five immobilization cycles) with SEM microscopy. On the Ceramtec and Poraver carrier surface no cells were present (SEM pictures not presented). In PUF some cells are present (shown in Fig. 3.26), but not even a monolayer of cells was formed. Fig. 3.27 shows some scattered cells attached to the Styropor.




(a) 100 magnification (b) 10 000 magnification

**Figure 3.26**: SEM picture of the polyurethane foam used for immobilization of *G. thermoglucosidasius* PB94A.




(a) 100 magnification (b) 10 000 magnification

**Figure 3.27**: SEM picture of the Styropor used for immobilization of *G. thermoglucosidasius* PB94A

Not enough carrier colonization was reached for any of the carriers. Strain *G. thermoglucosidasius* PB94A seems not to have the mechanisms for attaching to the tested materials. Other immobilization concepts such as entrapment in a gel were not used because the gel aggregates would be too fragile for the operation conditions at the pilot plant.

Besides, a DNA analysis (DGGE) of the supernatant of the first three immobilization cycles, showed that other bacteria than PB94A were already present in the broth. Despite that extreme care had been taken to keep the system as isolated as possible from the environment. Lanes 1-3 correspond to cycles *1-3* using PUF (Fig. 3.28). Lanes 5 and 6 correspond the *G. thermoglucosidasius* PB94A. Lane 4 is *G. thermodenitrificans* PB1511 (DSM 21923) also used as control. As it can be observed, already in lane 1, new bands had appeared.

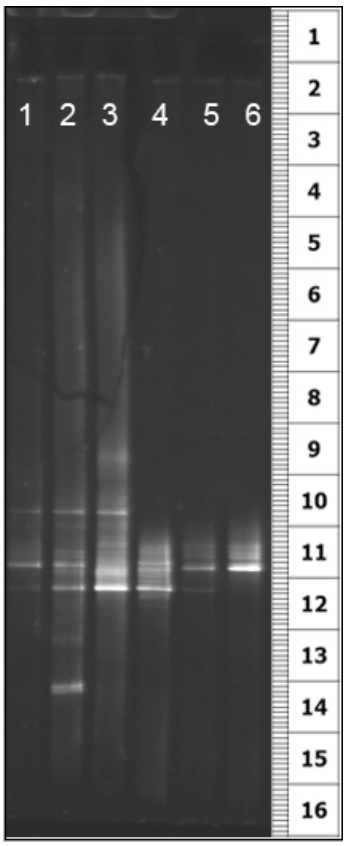



**Figure 3.28**: DGGE analysis of the immobilization cycles *1-3* (lanes 1-3) using polyurethane foam as carrier. *G. thermoglucosidasius* PB94A is in lanes 5 and 6. Lane 4: *G. thermodenitrificans* PB1511 (DSM 21923) control, in a 20-80% denaturing gradient gel. The scale in the right side of the Figure is a visual aid.

## 3.6 Laboratory and bench-scale tests for choosing the reactor concept for flax fiber treatment

After having thoroughly investigated the adequate conditions for strain *G. thermoglucosidasius* PB94A and after the procedure for the flax treatment process had been improved, it was necessary to find the best equipment for the fiber treatment. Several possibilities were tested in laboratory-scale before selecting the system to be used in the 200 L capacity pilot plant. The beaker scale treatment worked well for up to 50 g of fibers. With larger quantities of fibers it was necessary to introduce aeration, otherwise the system became anaerobic. Also the fibers tended to entangle, thus a way to maintain the fibers parallel was needed.

### 3.6.1 Beaker scale trials

The fibers were placed loosely in beakers and were floating in the liquid phase. They were coiled because they did not fit in parallel (see Fig. 3.29). In Section 3.2.3 the fiber treatment in beaker scale was described.


**Figure 3.29**: Beaker treatment of flax fibers using *G. thermoglucosidasius* PB94A.

### 3.6.2 Fiber treatment in a packed bed reactor

Another concept tested was to apply pressure over the fibers to improve the mass transfer. A 5 L jacketed vessel of 14.5 cm diameter by 30 cm height was tested (see Figs. 3.30 and 3.31). The total volume of the system including the tubing and pump was 6.5 L. The

vessel was fed through a 1 cm diameter tubing that distributed its flow among 120 outlet orifices. The ratio of the outlet to inlet area was 4.8. A strong pump had to be used to overcome this difference. The fibers were rolled tightly around a stainless steel cage and positioned inside the reactor. The liquid was continuously recirculated from the distribution tubing at center of the reactor outward through the fibers.

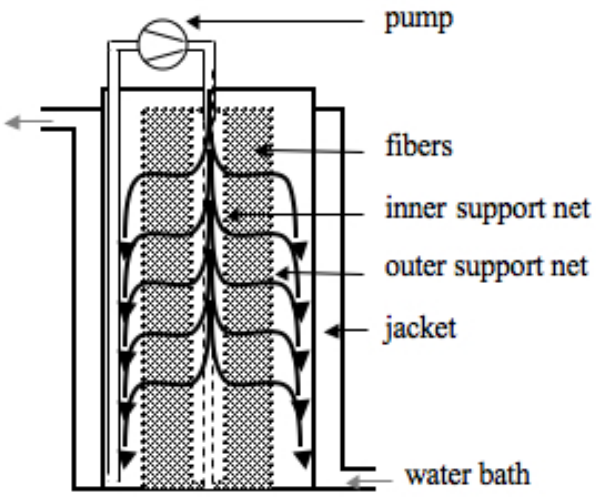



**Figure 3.30**: Schematic representation of the packed bed reactor used for flax fiber treatment.

Five trials were made in this reactor and it was consistently found that despite long treatment times of 5 to 10 days and high bacterial counts, the obtained fibers had a poor quality. The resolution was reduced from 3.33 to 2.48 and the fineness from 17.08 to 9.16 dtex. No lyase activity was detected.

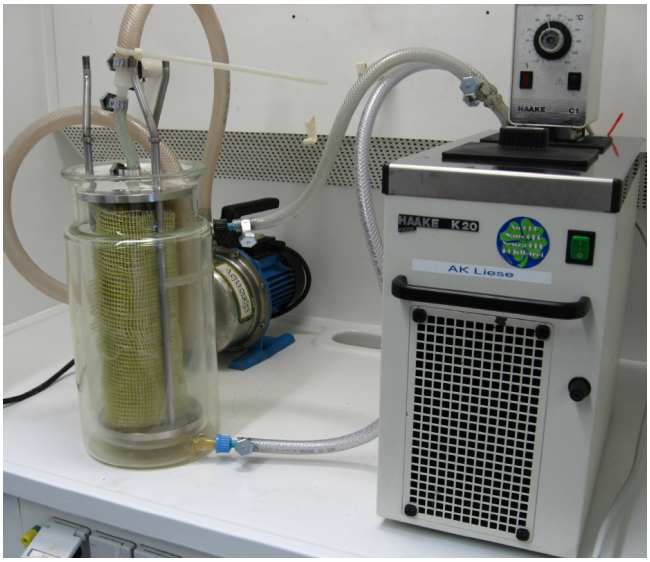



**Figure 3.31**: System setup for the packed bed reactor used for flax fiber treatment. The recirculation pump is on the back of the picture; the packed bed reactor has a jacket connected to a water bath located on the right.

The fiber packed bed reactor showed channeling and the fiber treatment was not uniform. This problem has also been described for unretted flax bobbins, when channeling occurred if the bobbins were not tight enough when bleached [61].

Another disadvantage of the packed bed reactor concept was the low fiber to liquid ratio of about 1:30 and that it was troublesome to roll the fibers around the net. This would make it highly impractical in view of an industrial process.

### 3.6.3 Floating fibers prototype in bench scale

The best quality fibers obtained so far were those from the beaker scale trials (Section 3.2.3), where the fibers were allowed to float freely in the liquid phase and a gentle rotary motion was applied. This gave the idea to test the same concept for the pilot plant. The scheme of this concept can be seen in Fig. 3.32. The experiments were made in a Julabo water bath SW23, which has a total capacity of 20 L, a recirculation pump, a temperature control and a shaking mechanism (Fig. 3.34). This water bath had the advantage that the area for oxygen exchange was larger than that of the beakers and therefore the anaerobiosis problems were reduced.

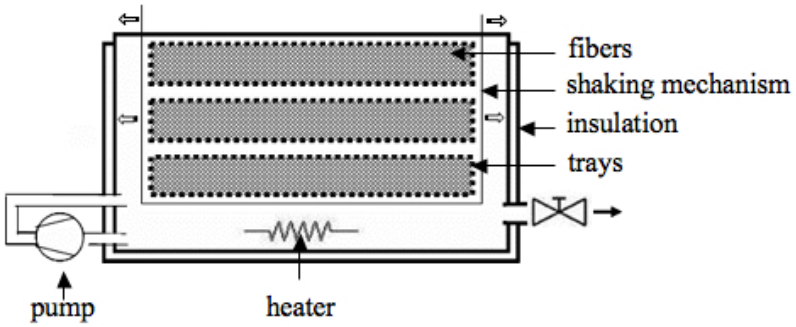



**Figure 3.32**: Reactor concept of free-floating fibers in a water bath.

To increase the capacity and effectiveness of the concept, the fibers were compartmentalized by a grid, which acted as a physical barrier to avoid fibers to float altogether to the surface and at the same time leave some free space between the bulk of them to facilitate mass and heat transfer. Here, the long parallel fibers were stacked in stainless steel trays (Fig. 3.33), and were subjected to a gentle motion. The process was carried out in the water bath and the solutions required for the process were transferred in and out as needed. The shaking velocity of the trays was adjusted to permit the fibers to remain parallel.

With the objective of testing the reusability of the bacterial culture of *G. thermoglucosidasius* PB94A, five batches were made using green fibers (*Flüh04* type) in the water bath.

This experiment was also accompanied by cup plat tests to detect cellulase activity. Figure 3.35 shows a plate with four wells. At the central well water was used as negative

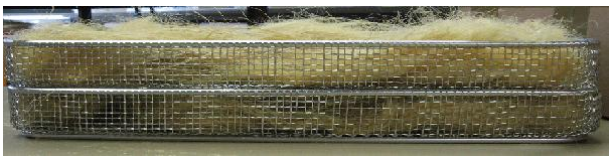


**Figure 3.33**: Trays filled with flax fibers used in the free-floating fibers prototype.

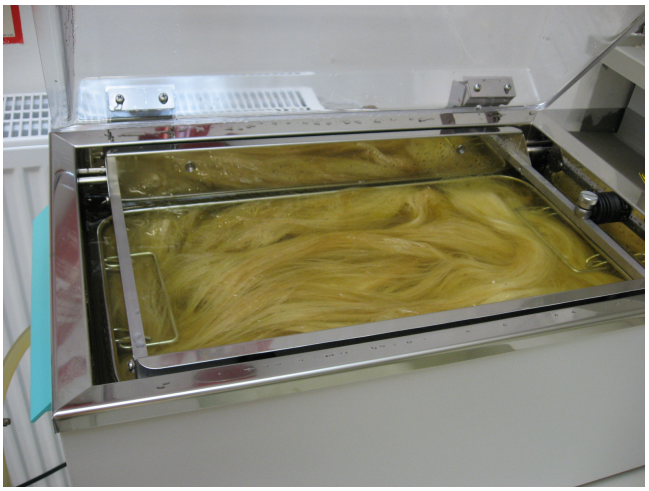


**Figure 3.34**: Water bath with parallel fibers used for testing the concept of free-floating flax fibers.

control and in the 3 wells around it, the supernatant of the first three batches was placed. No halo was formed for any sample, therefore no cellulase activity was present.

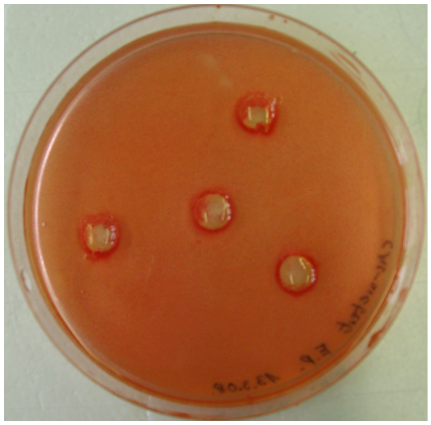


**Figure 3.35**: Cup plate test for detecting cellulolytic activity in the flax treatment in the "floating fibers" prototype. No halo was formed around any sample, therefore no cellulolytic activity was present.

The fiber quality was similar for the five batches made, yielding good fiber resolution, between 1.65 and 2.86 as well as fiber fineness within the range of 6.75 to 12.05 dtex. Figure 3.36 shows the fineness and resolution value for all the five samples. The fiber tenacity was $39.68 \pm 2.88$ cN/tex, which complies with the requirements. The visual appreciation of the fibers was positive for all the experiments. This was the main reason

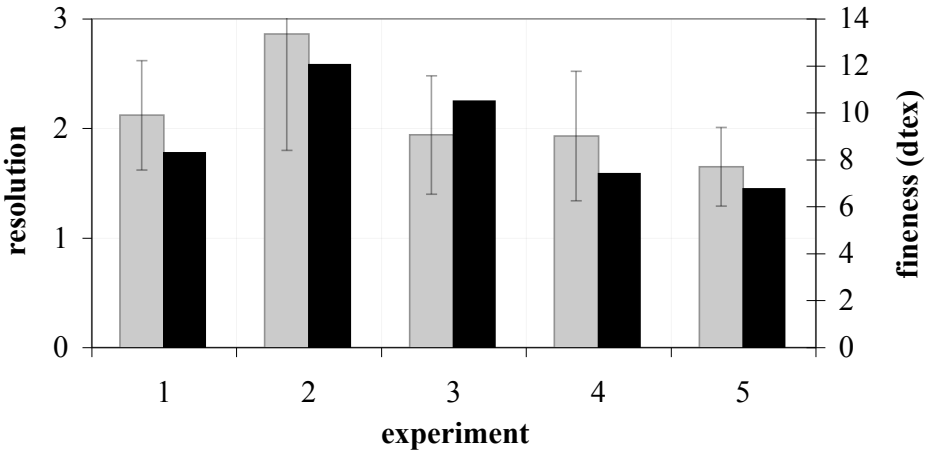



**Figure 3.36**: Fiber resolution (■) and fineness (■) for the five consecutive experiments performed at the "floating fibers" prototype.

why this concept was chosen to build the pilot plant.

## 3.7 Pilot plant tests using short flax fibers

Another objective of this thesis was to add extra value to the tow, a byproduct of the flax industry. The experiments using cut, green fibers in laboratory-scale, produced a yarn (Fig. 3.6, p. 48). To test the concept in a larger scale, green fibers (*Flüh04* type) were cut to 15 cm length, to simulate high quality tow (see Fig. 3.37). For the treatment of the fiber, the procedure of Section 2.16 was followed.

A total of six trials were performed in 200 L-scale (10 or 20 kg fibers per batch) at the company "Allgäuer Edel Färberei" in Memmingen, Germany.[2] A "loose stock dyeing machine", used commonly to dye flock, card sliver, or tow in batch mode, was used to make the tests (see Fig. 3.38). The apparatus is of the type "circulating machines" where the goods remain stationary and the liquor is circulated through them.

The equipment consisted of a 220 L capacity stainless steel rectangular tank with a 1.1 kW compartmentalized bottom propeller that directed the flow upward or downward. The tank had a clamping lid that prevented the fibers and the liquid to escape when the upward mode was in operation. Indirect vapor was the heating source. A feed water pipe was also connected to the tank to compensate for evaporation or losses. The tank had a temperature sensor and was drained from the bottom.

Figure 3.39a shows the washing step of the flax fibers at the tank in the Allgäuer Edel Färberei. In Fig. 3.39b the incubation of the short flax fibers with *G. thermoglucosidasius* PB94A in the 200 L-scale is shown. After the incubation, the fibers were dried in three steps at a temperature below 80°C (Fig. 3.40).

[2]The textile-dyeing expertise of Mr. Ernst Haug was of great help for performing the tests.

**Figure 3.37**: Green-shortened flax fibers (*Flüh04* type) used for the 200 L-scale experiments at the Allgäuer Edel Färberei

The dried fibers were further processed at the company Holstein Flachs, in Mielsdorf, Germany, where a card sliver (Fig. 3.41a) was produced. The yield of this operation was 84%, which is similar to the one obtained for carding of good quality retted flax. With the carded-sliver, an important objective of the project was achieved. Figure 3.41b shows the waste produced from the carding.

The treated fibers had a good resolution value and high tenacity. The fiber resolution as cross sectional view of the fibers embedded in a resin is shown in Figs. 3.42a, 3.42b and 3.42c corresponding to the green fibers, treated fibers and sliver, respectively. The green fibers were still inter-joined, forming fiber bundles, whereas the treated fibers and the sliver were more individualized and separated from each other. The resolution values for these fibers are shown in Table 3.6. The fiber area distribution of the samples is shown in Fig. 3.43. The green flax had a more shallow distribution, because there were many fiber bundles that had large cross sectional areas. Only 55% of the fibers were below 1640 $\mu$m, whereas for the treated flax and the sliver, 94 and 95% of the fibers fall under this category. The treated fibers had a major peak (70%) in the first category comprising fibers under 447 $\mu$m, in comparison to only 24% for the green flax.

The card sliver obtained from the 200 L-scale tests was sent to South Africa for spinning trials. But the fibers were too short for spinning with the machinery available there, and no yarn could be obtained.

**Figure 3.38**: 200 L-capacity apparatus used for treating the cut flax fibers.

(a) washing

(b) *G. thermoglucosidasius* PB94A incubation

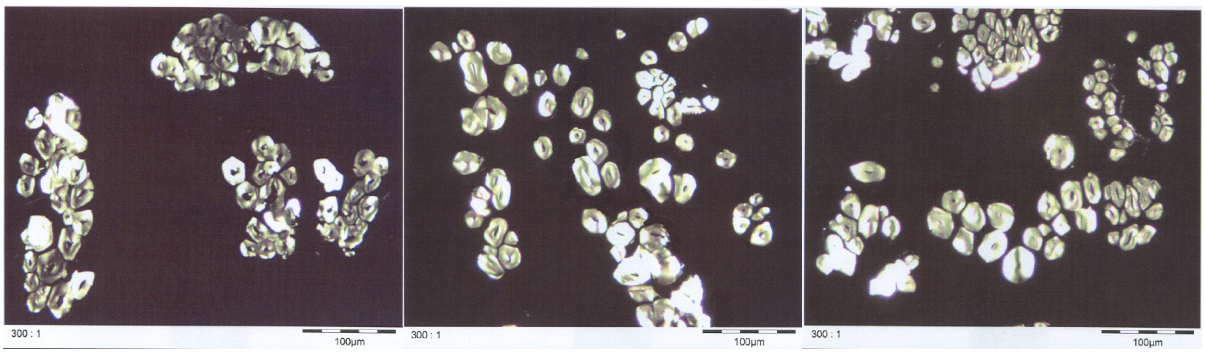
**Figure 3.39**: Treatment of short flax fibers in the 200 L tank.

(a) centrifugation (b) tray drying (c) comb-dryer

**Figure 3.40**: Drying of the treated flax fibers in three steps.

(a) sliver (b) sliver waste

**Figure 3.41**: Flax sliver production.




(a) green flax (b) treated flax (c) carded sliver

**Figure 3.42**: Microscopic view of a cross section of the flax fibers as raw green material and as processed products.

**Table 3.6**: Quality of the green fibers used as starting material, the treated fibers and the sliver, of the 200 L-scale tests.

| | Resolution | Fineness (dtex) |
|---|---|---|
| green flax | 7.30 | 37.38 |
| treated flax | 2.82 | 11.85 |
| sliver | 2.52 | 11.32 |

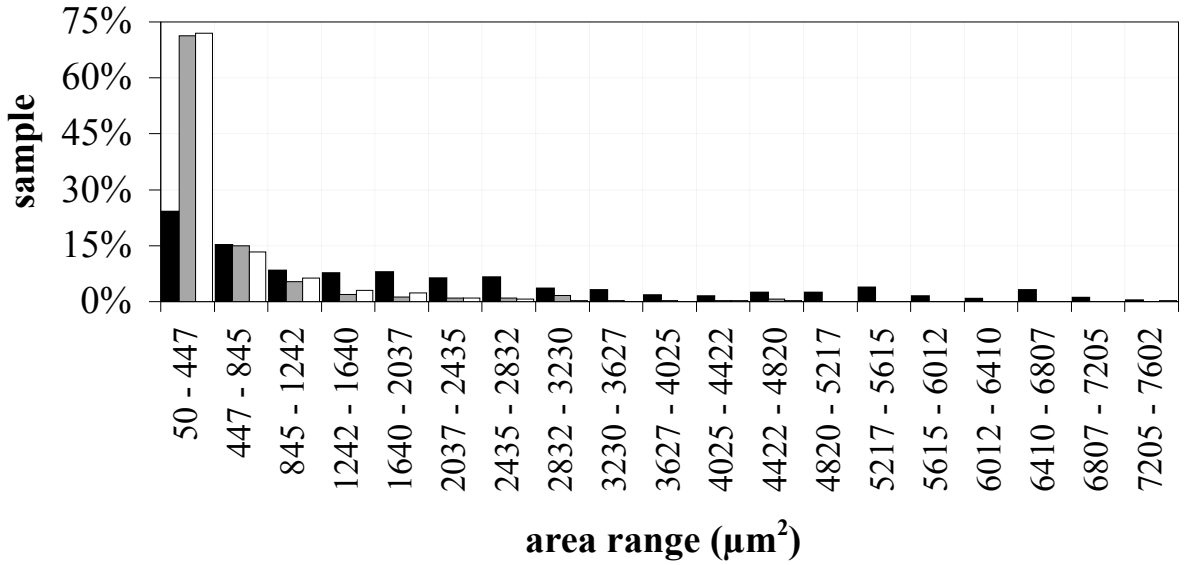



**Figure 3.43**: Fiber area distribution for the green fibers (■), treated fibers (■), and sliver (□) from the 200 L-scale tests with short fibers.

## 3.8 Design of a 200 L-scale pilot plant for the production of long flax fibers

### 3.8.1 Pilot plant concept

The concept of the pilot plant was developed considering the laboratory-scale tests (Section 3.6.3) as well as the technology used for textile dyeing. The textile material (e.g. flock, fibers, fabrics or roves) is referred to as "goods" in textile jargon. The textile goods can be subject to heating, mixing, absorption as well as reaction operations. Absorption is a common mass transport operation, where the solid phase needs to be left in contact with the liquid phase for enough time for absorption to take place.

The pilot plant process is similar to the textile dyeing operations. The Adulcinol softener wash is the absorption stage in the flax fiber treatment. The sodium carbonate cooking and hydrogen peroxide bleaching steps are reaction processes. The enzymatic

step is the *G. thermoglucosidasius* PB94A treatment.

In principle, two options regarding the material flow exist, to move the goods by cranes or transportation bands (i.e. so-called circulating-goods machinery) or to circulate the liquors through the textile machinery by pumping. The latter option was considered easier because of the requirement to keep the fibers in parallel.

For the dyeing process, it is reported that higher diffusion rates can be obtained by reversing the direction of the liquor flow intermittently [72]. The equipment of this type is run with a propeller housed in a separate compartment, which is communicated to the main tank where normally short fibers are placed. This movement is violent and causes the fibers to move around. This was not desirable for the long flax fibers because they would loose their orientation. A suitable pump that could smoothly invert the direction of the flow and could tolerate some fine fibers in the impeller was not found.

The concept for the pilot plant was to introduce two movement types, a unidirectional liquor pumping and a vertical movement of the fibers while keeping the material parallel. In the literature it was found that the so-called "star-shaped dyeing frames" are equipment that moves the goods gently up and down. The solids can be transferred to another vessel easily but the liquor can also be pumped out.

Since the specific surface area is inversely proportional to the adsorption rate and/or the reaction rate, compacting the fiber had to be avoided. That is why the laboratory-scale tests were made with trays separating the fibers. The pilot plant prototype was built similarly, with a vessel containing a basket holder with up to five adjustable height layers where the fibers were placed. This ensured high flexibility regarding the amount of fiber treated. In addition, it offered the possibility to test diffusion effects by manipulating the bed height.

A batchwise process was built for proving the fiber treatment concept, even though a continuous operation system would be the most economical way of production at industrial scale. Nevertheless the proposed concept could be translated to an industrial scale facility, where a semi-continuous operation can be achieved by having several batches in different times and process phases running in parallel. The fibers would then remain in the tanks and the different solutions transported among the tanks by pumping.

The flax pilot plant was built and assembled at the mechanical and electrical workshops of the TUHH.[3] Table 3.7 shows the parts list of the pilot plant and Fig. 3.44 shows the process diagram of it. The main tank and one of the three auxiliary tanks are shown in Fig. 3.45. The design of the main tank is shown in Fig. 3.46. Its total volume is 270 L but the operation volume is 210 L; the dead volume for housing the heating element accounts for 20 L and the remaining 50 L are from not completely filling the tank to avoid splashing.

[3]The coordination was done by Bernhard Pallaks of the "Institut für Mehrphasenströmungen". Gerhard Schietke of the "Institute of Technical Biocatalysis" and Cord Heineking of the "Forschungswerkstatt Maschinenbau" also collaborated with extremely valuable ideas.

**Table 3.7**: Pilot plant components.

| | Description |
|---|---|
| 1 | main tank (900 mm × 600 mm) × 510 mm, built by the TUHH workshop |
| 2 | sterilizable baskets (860 mm × 560 mm) × 40 mm, Würt. Celluloid- u. Drahtwarenfabrik (WCF) (Affalterbach, Germany) |
| 3 | mechanical mixer motor RW 28 D, IKA Werke (Staufen, Germany) |
| 4 | pump type CEA-V 80/5/A, Teknospeed, ITT-Lowara (Vicenza, Italy) |
| 5 | M700C modular transmitter, Mettler Toledo GmbH (Gießen, Germany) |
| 6 | pH/ORP measurement module for M700, Mettler Toledo GmbH (Gießen, Germany) |
| 7 | cabling, Mettler Toledo GmbH (Gießen, Germany) |
| 8 | pH-electrode model InPro3250, Mettler Toledo GmbH (Gießen, Germany) |
| 9 | oxygen measurement module for M700, Mettler Toledo GmbH (Gießen, Germany) |
| 10 | oxygen-electrode model InPro6800, Mettler Toledo GmbH (Gießen, Germany) |
| 11 | pump 323Du/D, Watson-Marlow Bredel (Cornwall, UK) |
| 12 | 200 L plastic tanks, ∅ 730 mm, height 670 mm |
| 13 | bridge circulator, Haake P2-H70, 3 kW, Thermo Electron GmbH (Karlsruhe, Germany) |
| 14 | drum pump, Grün-pumpen GmbH (Wertheim-Reichholzheim, Germany) |
| 15 | ∅ 32 mm hoses |
| 16 | 10 kW flange heater, Schniewindt GmbH & Co. KG (Neuenrade, Germany). Proportional-integral-derivative temperature controller (PID) 2132, Eurotherm Controls (Worthing, UK). Thermal interruption device, IMIT (Novara, Italy) |
| 17 | Others: valves, fittings, cage, motor mechanism |

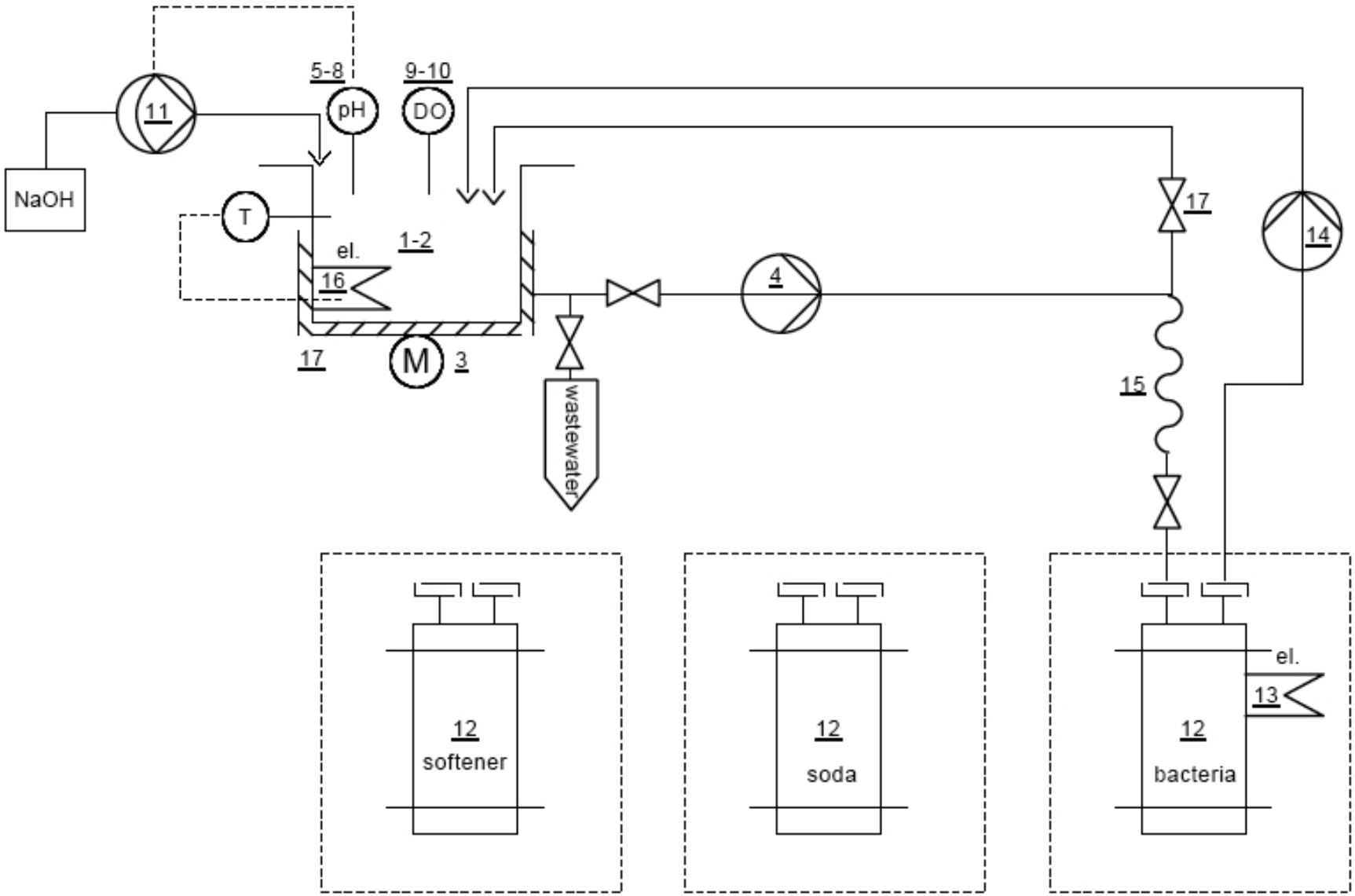



**Figure 3.44**: Process diagram of the pilot plant used for flax fiber treatment.

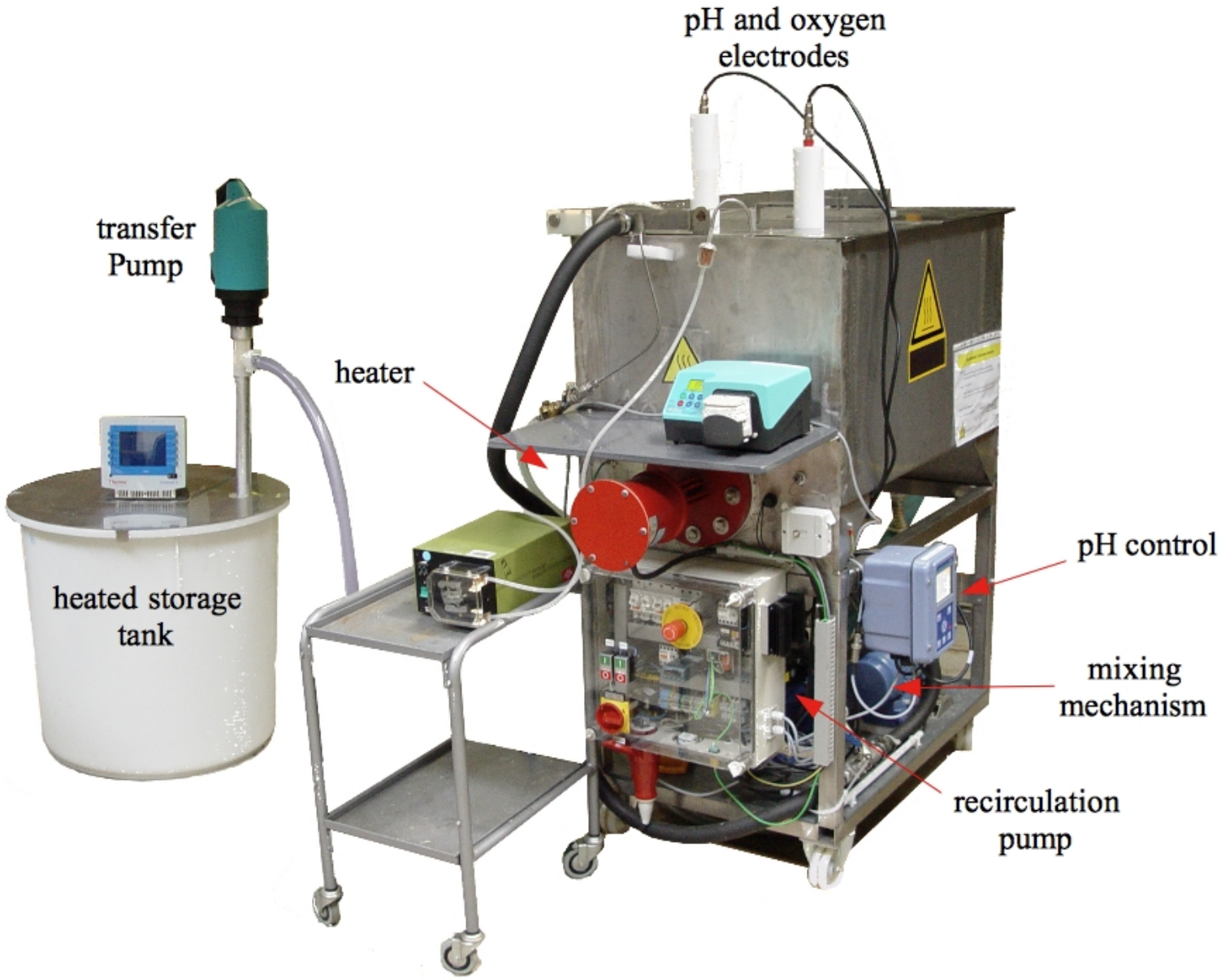



**Figure 3.45**: Pilot plant built for treating flax.

The up- and downward movement was effected by an eccentric shaft mechanism connected to the motor RW 28 D. The shaft was connected to a cage inside the tank, where the stainless steel trays were placed. The height of the trays could be adjusted by supports of diverse lengths. The plant capacity was adjustable between 2 and 10 kg. The 10-kW heater ran along the tank length at the bottom.

The recirculation pump and the shaking mechanism were located in the plate below the tank. The M700C modular transmitter was on the side of the tank. A drum pump was used for transferring the solutions between the auxiliary tanks and the main tank. A bridge circulator was used for maintaining the temperature of the stored solutions. The pump of the main tank had stainless steel sieve at the suction to avoid that fiber debris came into the impeller. An integrated potentiometer was used for regulating the flow of the pump from 30-100 L/min (head column 30-21 m), a halving of the range corresponded to a frequency of 25 Hz (maximum frequency 50 Hz). The hydraulic performance of the pump was proportional to the motor speed. The pump could operate at -10 to 110°C. The heater was controlled with the PID temperature controller and the temperature limit of the device was set to 90°C. The desired temperature set point could be adjusted; deviations from it were negligible. The heater was tested with water, and it took 2 h to increase the temperature from 20.5 to 88°C.

To avoid undesired anaerobic conditions, pressurized air was available for the main tank and for the bacteria-ancillary tank, where the bacterial culture was stored. The normal operating pressure in the main tank was 1 bar, but it was increased to 1.5 bar when needed.

A grid was placed upon the flax fibers because when the tank was filled with liquid, they tended to rise and float. This could lead to heterogeneous conditions in the vat and also that the bottom part of the tank was filled with liquid only. This phenomenon was even more pronounced when the hydrogen peroxide solution was used. In this case 5 kg extra weight were placed on top of the upper tray to ensure that all the fibers remained immersed.

### 3.8.2 Technical principles of the pilot plan design

Some parameters recommended for textile batch treatment, dyeing in particular, are [72]:

1. liquor ratio (solid to liquid) 1:4:-1:25
2. reactant concentration 0.8-5 g/L

These parameters were employed for the different steps of the flax treatment process. The distribution coefficient of a solute $K_D$ (L/kg) is independent of the liquor ratio in a given process. Hence the lowest possible ratio was used for the sake of the economy of the process and the environment welfare. It is defined in the following formula (3.1):

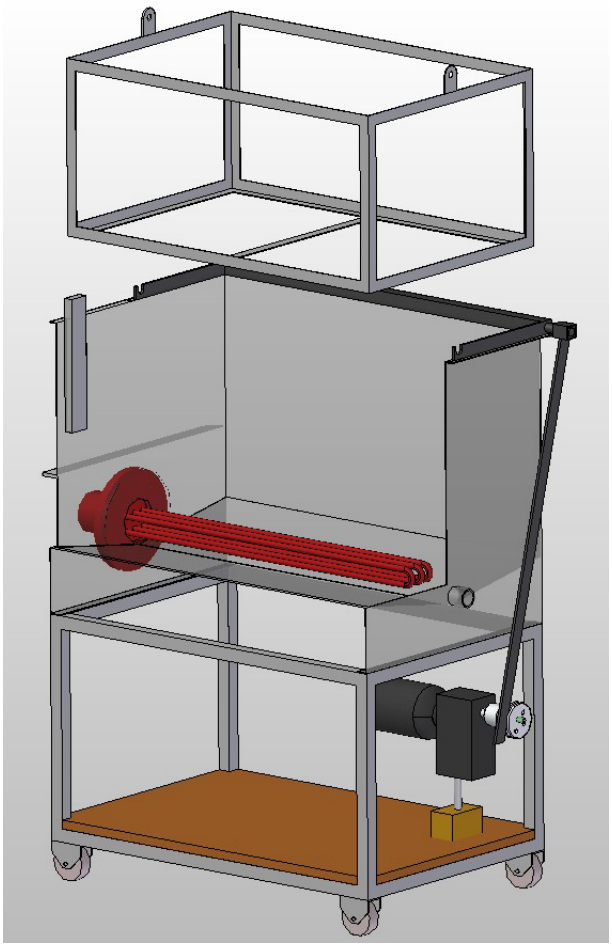


**Figure 3.46**: Schematic representation of the main tank of the pilot plant.

$$K_D \left( \frac{L}{kg} \right) = \frac{C_F}{C_L} \tag{3.1}$$

Where $C_F$ is the concentration of the solute in the fiber material in g/kg and $C_L$ is the concentration of the solute dissolved in the liquid phase in g/L.

Other disadvantages of using a high liquor ratio is that it leads to a low absorption rate and produces more wastewater. An adequate liquor ratio for the flax fibers was found to be 1:25, a lower ratio could not be used because the density of the fibers is too high and they would not be covered by liquid.

The following reactant concentrations were used at the pilot plant: 0.15% $Na_2CO_3$ (300 g in 200 L), 0.3% $H_2O_2$ (2 L of 30% $H_2O_2$ in 200 L), 0.3% Adulcinol (600 mL in 200 L).

The mass transport is often the rate-limiting step in pretreatment and finishing processes (wet processes) such as washing, dyeing, rinsing, de-sizing, scouring and bleaching. As a result, these processes require long residence times, large amounts of water and chemicals, and also demand much energy. In most of these processes, diffusion and convection in the inter- and intra-yarn pores of the fabric are the limiting mass transport mechanisms. Intensification of mass transport, preferentially in the intra yarn pores, is

key to the improvement of the efficiency of wet textile processes. Conventional methods for intensifying mass transport in textiles are operation at high temperature (not always feasible) and increasing the mass flow through the material (complex geometry of the textiles). New methods like ultrasound have proved useful for improving the diffusion [81].

The new fiber extraction process it is also a wet process, subject to diffusion limitation. But the decorticated flax fibers have the advantage that they are buoyant. Therefore, the bulk of the material is not that compact and fewer diffusion problems exist. The gentle push, made by the up- and downward movement in the main tank of the flax pilot plant helped to promote a better mass transport.

### 3.8.3 Measurement of pH and dissolved oxygen

The measurement of temperature was made by a sensor connected to the PID controller. The pH and dissolved oxygen concentration were measured by electrodes connected to a Mettler-Toledo M700 module, and the pH was controlled with it. The measuring electrodes were located in the cover of the tank and the fluid loop was fed with a peristaltic pump. With this design it was avoided that the electrodes were damaged by the moving parts and allowed to have a leak free operation. If the sensors had been positioned in the tank, a special bayonet sampling port would have been required, increasing the costs of the pilot plant.

### 3.8.4 Drying of the fibers with squeeze-rollers

For drying the fibers, they were pressed with the mangle (original Frieg) shown in Fig. 3.47. This is a machine used in former times for drying laundry by passing it between a set of wooden rollers to remove the excess liquid. The pair of drums had an adjustable spring, which helped to control the pressure and therefore the outlet humidity. After pressing the fibers "sheets" in the mangle, they were left to dry at ambient temperature. This process depended on the thickness of the fiber sheet, the temperature and the relative humidity. It usually took, depending on the ambient conditions, between 3 to 7 days for the fibers to be fully dried.

Because the fibers needed to stay parallel the commonly applied pre-drying by centrifugation could not be implemented. This concept works well for short staple fibers or for long fibers that are already in roves. Another option for accelerating the fiber drying was blowing hot air through the fibers. For this, the fibers were hung over a structure that hits the fibers with a scutching movement. Figure 3.48a shows the schematic representation of the dryer and Fig. 3.48b shows the device built at the TUHH workshop in operation with the flax fibers. The intention was to loosen the fibers and prevent the fiber stiffness with the hitting motion. This concept was discarded because the fibers tended to fall from the support and therefore became entangled. Besides, the treatment was not

**Figure 3.47**: Laundry mangle used for fiber squeezing.

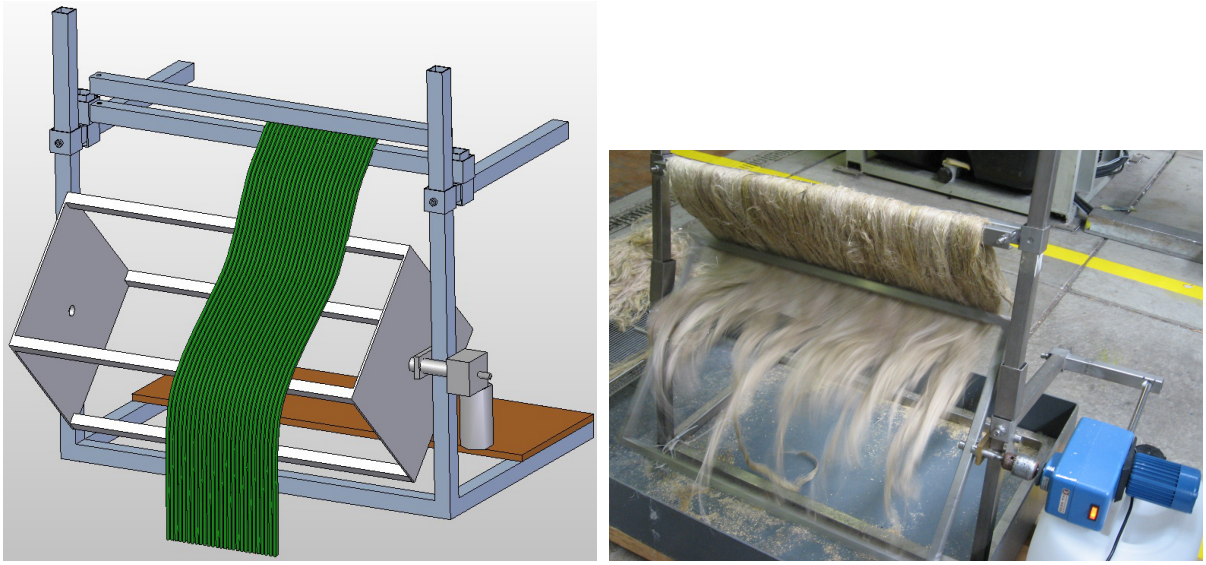
effective in the middle portion of the fibers, where they were pressed by the metal bar. Moreover the amount of shives that were detached from the fibers was similar to the ones allowed to dry at room temperature in fiber sheets without moving.

In general, the driving force for evaporation is proportional to the difference between the vapor pressure of the liquid being removed and the partial vapor pressure of the surrounding air. This gradient may be promoted in several means: by a temperature increase, since vapor pressure of the liquid will therefore increase; by decreasing the humidity of the drying medium, since this decreases partial pressure of the vapor; or by a combination of both methods. Another helpful strategy for drying is to increase the surface area-volume ratio for the material being dried. This accelerates heat transport to the center of the material, shortens the diffusing path, and provides more evaporating surface [28]. The residual humidity is the most difficult to remove. The drying speed depends on the material and is limited by the higroscopicity, the pore structure and the heat conductivity of it.

However for flax fibers is known that the fiber quality is reduced by artificial drying at high temperature above 80°C [99]. Another shortcoming of the drying operation is the huge energy consumption. Some authors have reported for flax stalks, that drying and threshing requires 100-130 kg/ha of fuel [56]. By using hot air blowing, the fibers became stiffer. That is why they were left to dry at room temperature over a grid.

### 3.8.5 Coarse fiber combing

In order for the fibers to be further processed for flax spinning preparation, they needed to be treated through a thick-toothed comb, similar to a rippling board used for separating the seed capsules from the fibers. But instead of using sharp spikes, bold ones were used



(a) Schematic representation (b) In operation

**Figure 3.48**: Flax fiber dryer.

to reduce fiber losses. This helped to dissolve the knots and to align the fiber strips that had been entangled. The comb was manufactured using dents of 6 mm diameter and a separation of 180 mm among the teeth with a total of 17 teeth as shown in Fig. 3.49. Once the fibers were combed, they were ready for processing into a sliver, which was required for wet spinning.

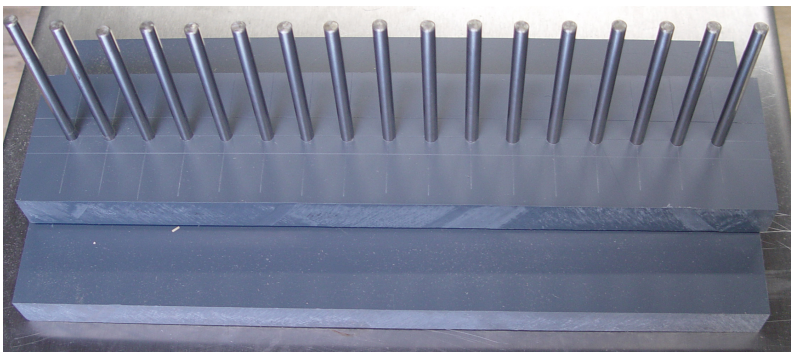


**Figure 3.49**: Thick-toothed comb used for aligning the flax fibers.

## 3.9 Mode of operation of the 200 L-scale pilot plant for long fiber treatment

A timetable of a typical experiment in the pilot plant is shown in Fig. 3.50. All the operations described were performed in the main tank, where the fibers were placed. The whole process lasted about 47 h, including the lag times of heating and cooling the liquors and of charging and discharging the fibers from the main tank. When the solutions were

reused, those that were not used at the time were stored separately in one of the three auxiliary tanks labeled as “softener”, “soda” and “bacteria” in Fig. 3.44.

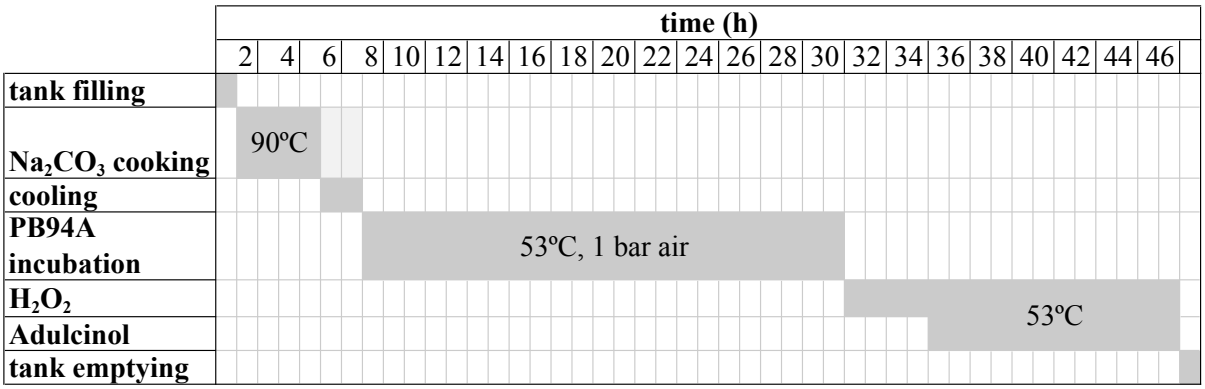



**Figure 3.50**: Timetable of a typical fiber treatment in the pilot plant.

**Table 3.8**: Conditions employed in the pilot plant experiments.

| | concentration (%) | temperature (°C) | time (h) |
|---|---|---|---|
| $Na_2CO_3$ | 0.05-0.17 | 90 | 6-7 |
| *G. thermoglucosidasius* PB94A | – | 53 | ∼22 |
| $H_2O_2$ and Adulcinol | 0.22 and 0.25 | 53 | 11-17 |

In comparison to the laboratory-scale experiments, some slight modifications were used:

- Cooking with $Na_2CO_3$ was done at 90°C because the heating element had this limit.
- The concentration of the reactants used in the process was lower (shown in Table 3.8). These values worked well in the pilot plant.

## 3.10 Liquor reuse experiments in the 200 L-scale pilot plant using long fibers

Experiments were performed at the pilot plant to test the feasibility of reusing all the three solutions required for the fiber treatment process:

1. $Na_2CO_3$ solution

2. *G. thermoglucosidasius* PB94A culture, which was referred as "bacterial culture" once it was used for the fiber treatment, because from that point onward it was assumed that it was contaminated by other bacteria.

3. hydrogen peroxide-Adulcinol solution

Per batch, 2.2 kg of green fibers, *Flüh* were used. The six experiments designated as *P1-P6* were made as described in Section 3.1.2.

Before reusing the exhausted liquor, the ingredient (i.e. $Na_2CO_3$, $H_2O_2$, adulcinol) was refilled. For example, in *P1* 134 g of $Na_2CO_3$ were initially used, and for all the other batches 60 g were added to obtain similar pH values (9-10).

The incubation with the bacterial culture was made at 53°C and for about 22 h per batch. Only the sixth batch received a different procedure in order to test the effect of a heat treatment on the bacterial culture. This culture was incubated at 90°C for a couple of hours and then the temperature was decreased to 53°C with a total incubation time of 42 h.

### 3.10.1 Characteristics of reused bacterial cultures

Figure 3.51 shows the lyase activity and cell density of the bacterial cultures, where *P0* corresponds to the freshly fermented *G. thermoglucosidasius* PB94A culture used at the beginning of the experiment. *P1-P4* were the experiments made afterward, measured at 22 h incubation time, *P5* sample was taken at 16.5 h and *P6* at 42 h after the addition of fresh fibers. The lyase activity, as well as the cell density, was relatively constant for all the batches.

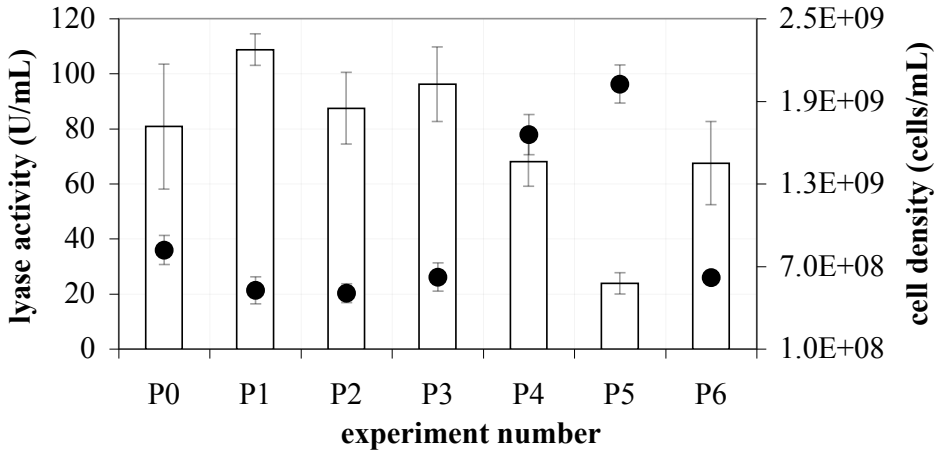



**Figure 3.51**: Lyase activity (□) and cell density (•) of the six experiments (*P1-P6*) made at the pilot plant reusing all the liquors. *P0* is the freshly fermented *G. thermoglucosidasius* PB94A culture.

For the *P6* culture, it was noticed that the majority of the bacteria disappeared after

the heat treatment, so the culture was left more time in contact with the fibers to see if the bacteria could recover from the harsh thermal treatment. The bacteria grew again and after 29 h of incubation, the culture was populated mainly by homogeneous bacilli, which looked like *G. thermoglucosidasius* PB94A when observed under the microscope and the culture had recovered its enzymatic activity.

### 3.10.2 Monitoring the bacterial population in the reused bacterial culture

A DGGE gel (20-80% denaturing gradient) of the bacterial cultures is show in Fig. 3.52. From experiment *P2* (0 h) until the beginning of *P6* (0 h), the bacterial population was stable (lanes 3-17). After the bacterial culture was heated *P6* (19 h) onward, the microbial population changed drastically. Bands corresponding to *G. thermoglucosidasius* PB94A appeared (lanes 18-20). Presumably the spore forming *G. thermoglucosidasius* PB94A was always present in the bacterial culture and was the only one to survive the heat treatment, while the other bacteria were killed. It is not known what would have happened if further experiments had been performed, but it is possible that the prevalent bacterial flora would show up again. However this is not a critical point since the quality of the fibers is satisfactory for all the six repetitions (see Fig. 3.57), and the fibers had a nice hand and appearance.

### 3.10.3 Behavior of the pH and dissolved oxygen of the bacterial culture

Figure 3.53 shows the oxygen concentration and pH of the *G. thermoglucosidasius* culture in contact with flax fibers during experiment *P5*. The initial pH was 9.2, and corresponded to the end pH value (pH 9.03) of the previous experiment *P4*. The culture had been kept in a tank with aeration at room temperature. The storage time (between uses) for the *G. thermoglucosidasius* PB94A culture was around 24 h. The pH and the amount of oxygen behaved similarly, indicating that when the bacteria were more active, at around 5 h of incubation, they produced acid metabolites.

### 3.10.4 Measurement of the hydrogen peroxide content in the hydrogen peroxide-Adulcinol solution

The measurement of the hydrogen peroxide concentration was done with the active oxygen method (detection range 0.1-5%) [10] . Since the added $H_2O_2$ was close to the lower detection limit (0.22%), it was difficult to measure accurately. Small peaks were detected when $H_2O_2$ was added, and the value fluctuated between 0.3% and 0.2% at the begin-

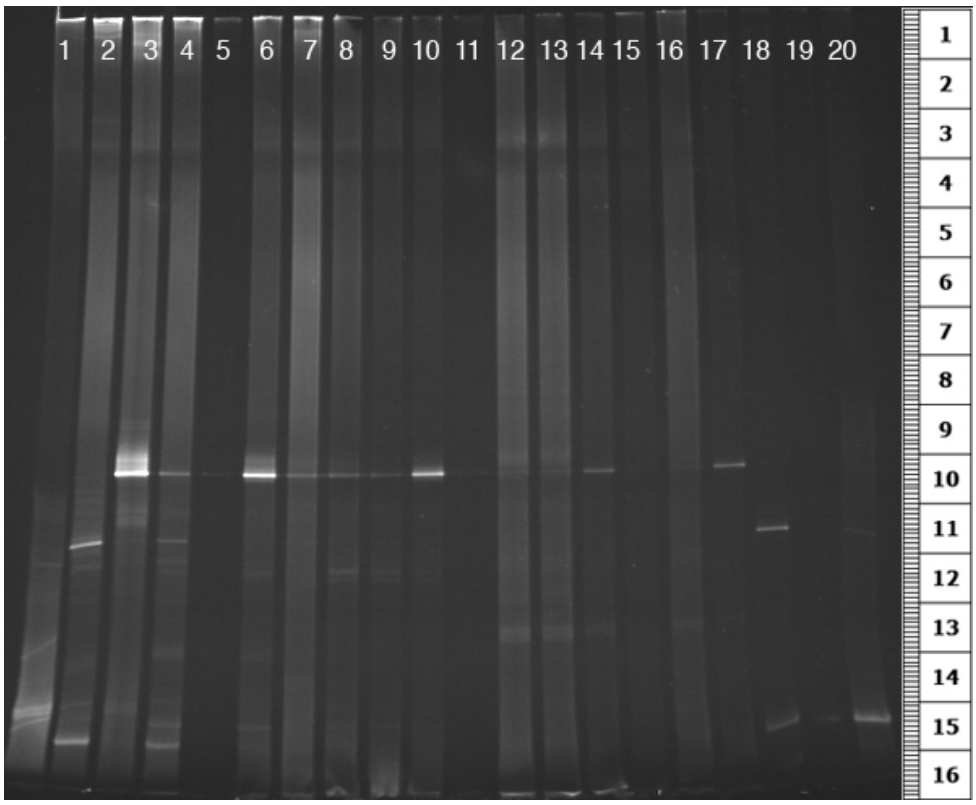



**Figure 3.52**: DNA profile by DGGE technique of the reused bacterial culture employed for the six experiments at the pilot plant; *P1-P6*.
Lane 1: freshly streaked *G. thermoglucosidasius* PB94A; lane 2: *P1* (15.5 h); lane 3: *P2* (0 h); lane 4: *P2* (16 h); lane 5: *P2* (22 h); lane 6: *P3* (15.5 h); lane 7: *P3* (13.5 h); lane 8: *P3* (18 h); lane 9: *P3* (22 h); lane 10: *P4* (0 h); lane 11: *P4* (14 h); lane 12: *P4* (19 h); lane 13: *P4* (22 h); lane 14: *P5* (0 h); lane 15: *P5* (4.5 h); lane 16: *P5* (16.5 h); lane 17: *P6* (0 h); lane 18: *P6* (19 h); lane 19: *P6* (29 h); lane 20: *P6* (42 h). The scale in the right side of the Figure is a visual aid.

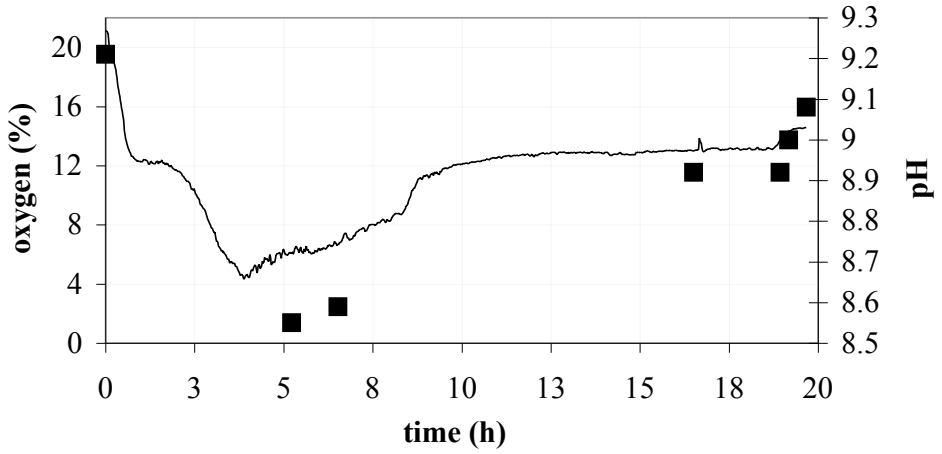



**Figure 3.53**: Oxygen concentration (–) and pH (■) of *G. thermoglucosidasius* culture in contact with flax fiber during experiment *P5* in the pilot plant.

ning and the end of the treatment. The peroxide was not exhausted after the bleaching process ended, ∼0.2% remained. In experiment *P5* (Figs. 3.54), the concentration of the $H_2O_2$ solution was measured regularly. In this experiment the peroxide was added at the beginning and again after 9 h, at which point a slight peak can be seen.

The fibers obtained were strong and were of similar color for the six batches (yellow-white). Therefore it was assumed that the quantity of $H_2O_2$ used was adequate. The Adulcinol in the mixed $H_2O_2$-Adulcinol solution was practically exhausted after the incubation with the fibers. This was noticed because the solution changed from milky to transparent. Therefore Adulcinol was always replenished to the initial value.

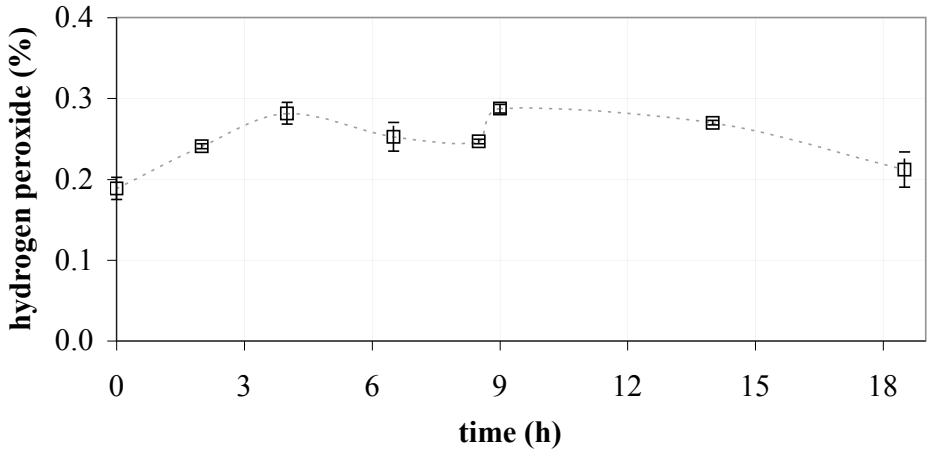



**Figure 3.54**: $H_2O_2$ concentration in the $H_2O_2$-Adulcinol solution of experiment *P5* made at the pilot plant.

### 3.10.5 Measurement of COD and $BOD_5$ of the reused solutions

The organic matter of the reused liquors, $Na_2CO_3$, bacterial culture and $H_2O_2$-Adulcinol, was monitored by the chemical oxygen demand (COD) and the biochemical oxygen demand ($BOD_5$).

The results of the COD and $BOD_5$ analysis for the $Na_2CO_3$ solutions are shown in Fig. 3.56. The $BOD_5$ increased steadily up to about 2700 mg/L at the sixth experiment *P6*-6h. The COD followed a similar pattern. The $BOD_5$/COD ratio is shown in the same Figure, most of the values are around 0.4.

The $BOD_5$ of the freshly fermented *G. thermoglucosidasius* PB94A (*P1*-0h) had the highest value, decreased after the second treatment (*P2*-22h) and slowly increased as it was used in the subsequent batches. The COD followed a similar pattern (Fig. 3.55). Theoretically COD values are always higher than $BOD_5$ values. The $BOD_5$/COD ratio should be less than one, as was the case for all samples.

From the third experiment (*P3*) onward, the COD values of the $H_2O_2$-Adulcinol solution were fairly constant at $4382 \pm 745$ mg/L. The $BOD_5$ measurement, gave zero for

all samples. Most probably the test was inhibited by the $H_2O_2$.

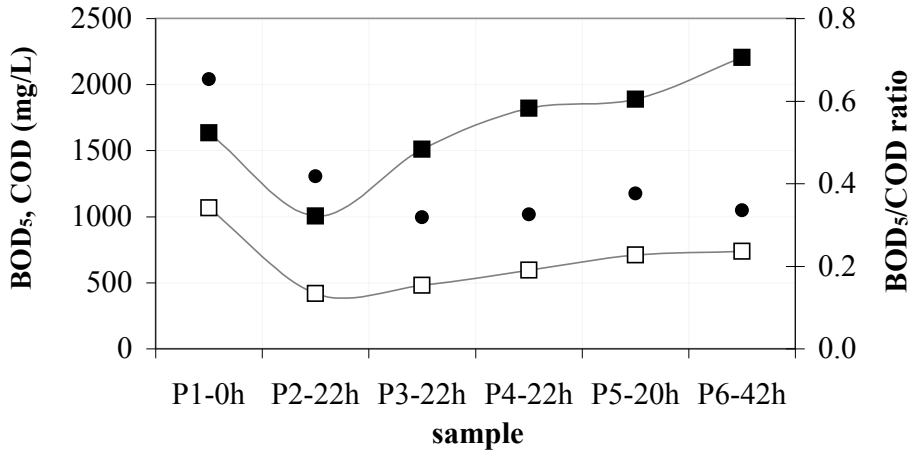



**Figure 3.55**: $BOD_5$ (□) and COD (■) of the bacterial cultures reused in the six experiments done at the pilot plant. The $BOD_5$/COD ratio (•) is also shown.

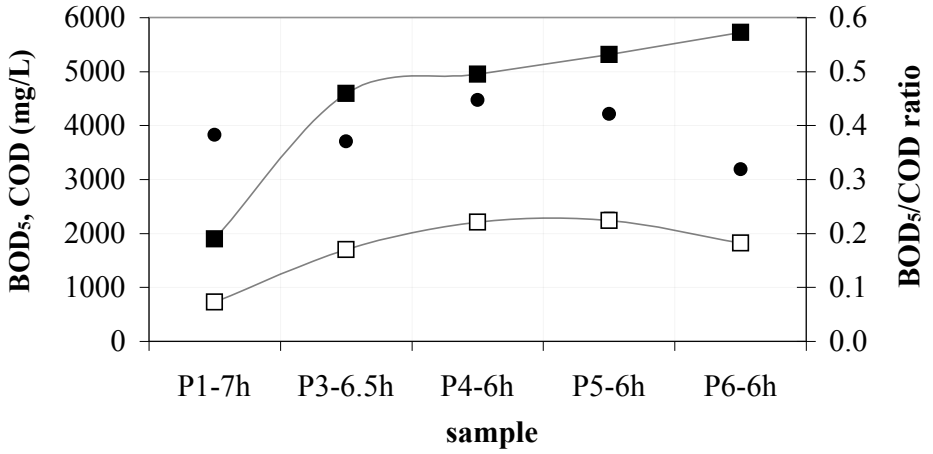



**Figure 3.56**: $BOD_5$ (□) and COD (■) of the $Na_2CO_3$ solution reused in the six experiments done at the pilot plant. The $BOD_5$/COD ratio (•) is also shown.

### 3.10.6 Quality of the fibers treated at the pilot plant

The fiber quality was similar for the six batches. The fiber resolution was between $2.73 \pm 0.37$ and the fiber fineness was $11.08 \pm 1.12$ dtex. Figure 3.57 shows the fineness and resolution value for all the six samples. The fiber tenacity was $62.03 \pm 5.19$ cN/tex, meaning that the fibers were stable. The fiber hand was good for all the six experiments. Pictures of some of the hackle-ready fibers produced in the pilot plant are shown in Figs. 3.58 and 3.59

Concerning the fiber yield, ∼3.3% of the weight loss corresponds to the shives detached from the fibers. The yield of fibers after the treatment at the pilot plant was $84 \pm 2\%$ and

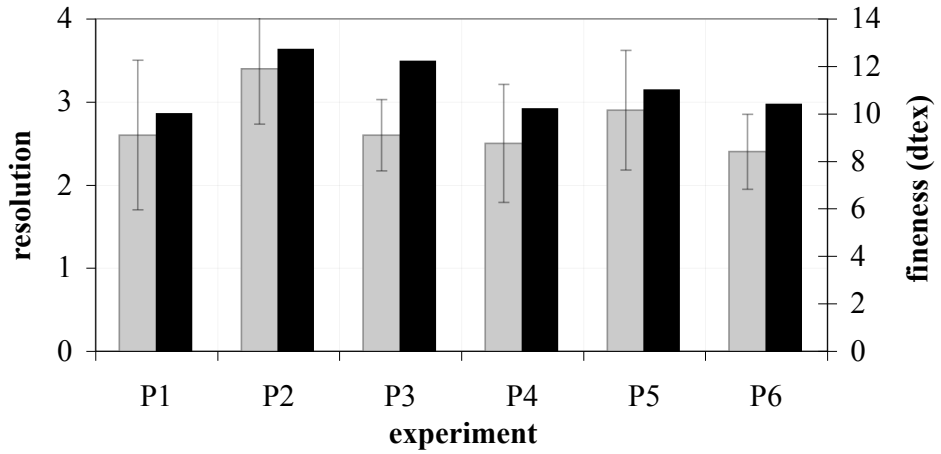



**Figure 3.57**: Fiber resolution (■) and fineness (■) for the six consecutive experiments performed at the pilot plant, where all the solutions were reused.

after combing was $70 \pm 5\%$. This yield is high, considering that in the conventional flax process, 40-50% of long flax is converted to the short byproduct tow. But this was not a mechanized regular technology, and still further carding steps are needed for making a sliver. The 14% lost during combing could seem too high. But this step was made per hand and the variability was not so high, considering that the overall yield of long line fibers in the flax processing chain is 55% [16].

## 3.11 Yarn production using the long fibers obtained in the 200 L-scale pilot plant

Only the last set of the experiments made in the pilot plant was presented in Section 3.10. However, the pilot plant was operated over a year, performing 4 series of reuse experiments. The overall yields are presented in Table 3.9. A total of 139 kg of long flax fiber were treated in total in the pilot plant. After the treatment, only ∼18% was lost, and after the coarse combing, ∼68% of combed long flax fibers were obtained. The fibers were combed and prepared for the next step, fiber hackling and sliver forming.

**Table 3.9**: Fiber yield of the different treatment steps.

| | starting material | treated fiber | combed fiber | waste |
|---|---|---|---|---|
| total (kg ) | 139 | 114 | 94 | 18 |
| yield (%) | – | 82 | 68 | 13 |

With this, the fibers would have been ready for wet spinning and then for weaving.

**Figure 3.58**: Pack of flax fibers treated in the pilot plant, after coarse combing.

**Figure 3.59**: Flax fibers treated in the pilot plant, ready for shipping.

Unfortunately, economical factors were a strong obstacle as the textile industry suffered significant setbacks during the years that this project was realized (2006-2010). Our project partner Lauffenmühle GmbH & Co. KG, required a sliver for the wet spinning procedure. The production of sliver composed of long flax fibers, is a specialized procedure performed mainly in China and Egypt. Only a couple companies in the Netherlands have still this technology. One of those, Van de Bilt Zaden en Vlas BV (Sluiskil, Netherlands), received about 80 kg of treated fibers at the pilot plant for making the sliver in the last quarter of 2009. Regrettably the economic recession caused that they did not receive

enough working volume to start the machinery, therefore the sliver was not produced.

Initially the plan was to make the spinning in Germany. The company located in south Germany, Füssener Textil AG, still had this technology when the project proposal was made. But it suspended its wet spinning operations in 2006. For all the mentioned reasons it was not possible to obtain a flax yarn by wet spinning, but still 94 kg of high quality, hackle-ready fibers (prepared for making a sliver) were obtained.

# Chapter 4

# Discussion

## 4.1 Development of the fiber treatment procedure

### 4.1.1 Procedure for treating the flax fibers

A process for hemp treating, called "bio-retting", developed by Fribranova group, utilizes a fiber to liquid ratio of 1:20 [111]. It is reported that for obtaining good quality fibers in traditional flax water retting, the first leach water must, after a couple of hours, be replaced by fresh water to remove soluble matter [79]. By using an alkaline solution for the removal of soluble oligosaccharides from the flax fibers at 25°C and isothermal conditions, the amount of extracted carbohydrates was independent on the contact time and was constant at about $10.9 \pm 0.5$%w. However the amount of extracted carbohydrates was time dependent for up to 4 hours treatment time when performed at 55°C, 85°C and 98°C; reaching a maximum weight loss of 30% at 98°C [92].

A combined chemical and enzymatic process for enhanced de-gumming of bast fibers can decrease the consumption of chemicals and energy [41]. Kashyap *et al.* found that for buel (*Grewia optiva*) bast fibers, it was better to wash them in a mild alkaline solution (2% w/v NaOH) prior to the enzymatic treatment. The chemical treatment of fibers made them more susceptible to the following enzymatic step by unmasking the pectic sites present in them thereby resulting in higher quality fibers [59].

Göpel studied the effect of pectinolytic enzymes over decorticated hemp fibers. A fiber to liquid ratio 1:28 in beaker scale was used for the tests. The hemp fibers were immersed in warm water, then rinsed with water and then put in contact with a pectinolytic broth (i.e. *G. thermoglucosidasius* PB94A) for 18.5 h at 60°C. After the incubation, the fibers were washed and dried at 37°C. With the treatment, the fiber width was reduced from 39 $\mu m$ to 29.1 $\mu m$. It was mentioned that a further fiber refinement could have been achieved if a culture with more lyase activity would have been used or if the mass transport would have been improved [49].

The fiber treatment used in this thesis is explained in Section 2.16. The fiber to liquid

ratio used was between 1:15 and 1:25 (w/v), depending on the setup of the experiment and on the amount of fibers used. The treatment included a mild alkaline cooking with $Na_2CO_3$ followed by the *G. thermoglucosidasius* PB94A step. The treatment with $Na_2CO_3$ provided the pH (8-9) that the alkaliphilic strain *G. thermoglucosidasius* PB94A required; in addition the pectin is more soluble in alkaline environments.

### 4.1.2 Use of *G. thermoglucosidasius* PB94A culture for fiber treatment

It was found in Section 3.1.5 that the best fibers were obtained when the undiluted *G. thermoglucosidasius* PB94A culture was used; only from those fibers a yarn could be spun. This experiment was made at 60°C, and after only 3 h of incubation about 70% of the lyase activity had disappeared.

In Section 3.2.3, a lower incubation temperature of 50°C was used to promote the lyase activity. The fibers were incubated either with *G. thermoglucosidasius* PB94A or with a sterile buffer. Both solutions were reused seven times. The results were encouraging, since the lyase activity was maintained, all the fibers had a good quality and were strong (∼60 cN/tex). The incubation at 50°C did not promote the emergence of cellulose degraders. The fiber hand was better and the shives were more easily detached for the fibers incubated with *G. thermoglucosidasius* PB94A. Therefore the *G. thermoglucosidasius* PB94A can be used several times, and it was better to incubate the fibers at 50°C than at 60°C.

### 4.1.3 Procedure for flax fiber softening after a wet treatment

It has been reported that after a wet treatment for producing fine flax fibers, the latter tend to stick together. Gruppo Fribranova reported that after the "bio-retting", the fibers were still glued together by some biofilm, which was removed by rinsing with pressurized water and mechanical softening. The dried fibers were then softened by shaking, where the remaining 5% of shives were removed [111]. In other report, the fibers were soaked in a solution of Fabrisoft CP (0.8 g/L) for 1 h at room temperature, to reduce fiber-to-fiber adhesion while drying. The fibers were soaked, squeeze dried, opened by hand and then dried overnight at 40°C [52].

Lamb and Denning mention that after making a mechanical and then a chemical wet treatment of the flax fiber, the sample did not break up well when no rinse with softener was given. The problem of the fibers sticking together on drying was diminished by adding a silicone softener, which was effective at preventing re-gluing of separated fibers [70].

In this thesis, Adulcinol was used for softening the fibers. Adulcinol is a fatty acid condensation product. It was applied in a solution of 5 g/L, the pH was adjusted between 4-5 with acetic acid. The fibers were soaked in this softener liquor after the bacterial

treatment without making an intermediate rinsing step, which helped to save water. The Adulcinol-treated fibers had an improved splitting pattern, the fiber hand was better, and also the quality of the fiber had improved (Table 3.1). Adulcinol was effective in preventing that the fibers glued again upon drying. When a combined $H_2O_2$-Adulcinol solution was used, the fiber quality was good; this helped to save more water by eliminating one process step (Section 3.10).

### 4.1.4 Procedure for the removal of the remnant shives from the flax fiber

To find the best procedure for the removal of the remnant shives from the flax fiber as mentioned in Section 3.1.4, an extensive literature research was made.

Kernaghan and Keikens [61] report that the absence of a retting step prior to scutching, causes tightly adhered shives. Thus the following hackling step must be intensive to remove these contaminants, reducing the yields in comparison to conventionally retted fibers. It is common that these type of fibers require bleaching processes to assure good spinning performance [61]. There are different bleaching processes for roves, all based on boiling or treatment with hydrogen peroxide, chlorite or hypochlorite.

Shive contains a higher amount of lignin than the rest of the fiber and if not removed it affects also the fiber dyeing, causing heterogeneous yarns. It has been said that hypochlorous acid is the only effective solution to remove the shives [51].

Vignon *et al.* investigated the effect of steam explosion and bleaching with sodium chlorite treatment of hemp bast fibers. A 95% pure cellulose was obtained and was used for composites [115].

Willis investigated several chemical treatments with green decorticated flax to make it apt for textile applications. The peroxide bleaching was part of the following procedure: *(1)* washing in water at about 25°C until the water was clear; *(2)* boiling for five hours in 1.5% caustic at 115°C; *(3)* washing until the water became neutral; *(4)* adding 1% sodium silicate to make it alkaline; *(5)* adding 2% peroxide and mixing for 4 hours. They reported that even though the non-soluble material could be removed with the bleaching, the fiber was stiff and could not be spun in cotton spinning machinery [117].

Wang *et al.* found that for hemp fibers, hydrogen peroxide bleaching after alkaline boiling removed more residual lignin than an acidic treatment, while increasing the fiber whiteness [116]. Hydrogen peroxide at a final concentration of 0.18% was used for the bleaching treatment, together with sodium hydroxide, sodium silicate and sodium lauryl sulfate. The temperature was 95°C and the bleaching time 45 minutes.

Bismarck *et al.* removed undesired "contaminants" from the flax fibers by boiling them in 5-10% NaOH for 30 min. Unlike the initial material, the treated one had more positive traits: shives could be removed easier, fibers were purified, unwanted fiber ingredients

were largely removed and fiber separation ability was increased [25].

It is reported that bleaching not only whitens the flax material, but also removes residual pectin and hemicelluloses, allowing to spin finer and stronger yarns. Bleaching is an essential operation for the production of fine yarns. The standard bleaching is done at high temperatures (90°C), and textile auxiliaries are a part of the procedure [111]. The majority of fine yarns made in Western Europe are wet spun from bleached roves [95].

For the new process described in this thesis in Section 3.1.4, the best treatment for removing shives from the flax fiber was:

- $Na_2CO_3 \longrightarrow$ PB94A $\longrightarrow$ hydrogen peroxide $\longrightarrow$ Adulcinol

In the pilot plant, 0.22% hydrogen peroxide was used, yielding a good shive detachment and strong fibers. In the experiments described in Section 3.10, the average fiber tenacity was 62±5 cN/tex. Considering that the green fibers used as starting material, had a tenacity of 60 cN/tex, it can be said that the fiber polymer was not damaged at all.

Another advantage of using hydrogen peroxide, is that it is used in wastewater treatment as an oxidation agent for elimination of COD from effluent [104]. Therefore this chemical had many important functions in the fiber treatment: shive removal, fiber bleaching and lowering the COD load of the wastewater. The $H_2O_2$ can decompose to water and oxygen.

Nevertheless, the hydrogen peroxide must be administered to the fibers with caution, because the super-oxide radical ion is hydrophilic and therefore works preferentially in the hydrophilic region of the fiber, attacking the fiber polymer. The hydrophobic associated material, such as the woody part of bast fibers can be attacked more efficiently with chlorine dioxide ($ClO_2$). A two-step hydrogen peroxide-chlorine dioxide bleaching would be a better option for removing the shives and bleaching of flax [9]. However the chlorine was not used because it yields chlorinated compounds, which are noxious to the environment and are restricted in the wastewater.

### 4.1.5 Effect of the moisture on the detachment of shives from the flax fibers

In the experiments for this thesis, the shives were removed from the treated fibers to a partial extent when dry fibers were moistened with water and left to dry again. It is presumed that wetting and drying caused swelling and shrinking of the materials, which in turn had an effect on making shives loose.

Trying to explain why some shive separation was also observed when moistening the fibers, the work of Pasila [90] provided some insight. A separation of the bast stem and the fiber occurred when the plants were frozen in the Finland winter. A thick ice mantle broke mechanically the bast fiber layer that surrounded the xylem, which is the core part

of the stem. During the winter moist bast fiber stems were frozen and thawed several times. Due to this mechanical stress and ice expanding movement on the stem surfaces the bast fibers were detached. The pressure or tension caused by the mechanical stress of the ice was enough to break the bonds and detach the fibers.

Bismarck *et al.* mention that since all natural fibers are hydrophilic materials, this is a major restriction in the successful use of natural fibers in composite applications [25]. Swelling can lead to micro-cracking of the composite and, therefore, to deteriorated mechanical properties.

It is well known that water absorption is less favorable the more hydrophobic a solid surface becomes. The water adsorption behavior of the shive is not the same as that of the fiber, and this phenomenon promoted the shive detachment.

## 4.2 Pectin lyase activity

### 4.2.1 Conditions that promote the lyase activity of *G. thermoglucosidasius* PB94A

Frank found for a shake flask fermentation of *G. thermoglucosidasius* PB94A at 60°C that after 10 h a maximum of the pectin lyase activity was reached. The pectin lyase activity was determined with the modified procedure of Collmer [35]. If the fermentation continued to 25 h, the activity decreased until it disappeared. The production of lyase took place and increased during the exponential phase of cultivation. If the culture was further incubated the lyase disappeared and at the same time the pH rose until no more lyase activity was detected. The lyase assay produced thereafter only negative values for the lyase activity. The cause for the lyase activity decrease, was attributed to proteolytic degradation [47]. Göpel also observed a similar behavior when fermenting *G. thermoglucosidasius* PB94A at 60°C in a 2 L bioreactor [49].

In the work made for this thesis, it was also found that if the fermentation broth was incubated longer after it had reached its maximum pectin lyase activity, the activity was lost after 5 hours when incubated at 60°C with shaking or stirring. But the lyase activity drop could be circumvented by decreasing the temperature as described in Section 4.1.2; which was important for the fiber incubation in the bacterial broth.

For this thesis, although the pectin lyase assay was determined in triplicates with the Collmer method [35], when the values of lyase activity were low, the error of the method was considerable. Since the values of the lyase activity of the samples were very similar to those of their respective background samples, the uncertainty on the difference became large. There are no negative values of the lyase activity, those values that are "negative" should be considered as the lack of pectin lyase activity.

### 4.2.2 Detection of the lyase activity of *G. thermoglucosidasius* PB94A by the thiobarbituric acid method

Nedjima *et al.* optimized the TBA method for the detection of the enzymatic $\beta$-elimination (pectin lyase activity), making it able to selectively differentiate between polygalacturonase and pectin lyase activities. According to Nedjima *et al.* the test was most selective when using 2-thiobarbituric acid substituted with a 1-pyridinyl group [88]. The unsubstituted acid was used for this thesis. As discussed in Section 3.2.1, the TBA method not necessarily detected the lyase activity present in the culture of *G. thermoglucosidasius* PB94A, but rather the presence of pectin metabolites. The method detects the unsaturated uronic esters (monomers, dimmers, trimers, etc) by the formation of a pink fluorescent dye when combined with the thiobarbituric acid and heat. The TBA method detected enzymatic activity for the samples incubated at -20°C, 4°C and room temperature (see Fig. 3.7) but with the alternate lyase detection method of Collmer [35], no lyase activity could be detected in those samples.

The TBA method was reliable for the detection of the pectin degradation products and the Collmer assay, though rather imprecise, was used to test if lyase activity was present. Therefore both methods were measured and used complementary.

## 4.3 Fiber quality determination

Farmers and traders have traditionally made the first flax quality assessment by subjective judgment. The qualitative methods for fiber quality determination are slow and time consuming. New technologies for testing fiber quality faster and/or non destructively would be of great value to allow the rapid evaluation of flax samples [38].

### 4.3.1 Fiber quality determination at the ITV-Denkendorf

Some authors affirm that due to the irregularity of the flax fiber structure, the fiber fineness determination is operator dependent and microscopic methods should not be used. More adequate methods are those based on air permeability and are [71]:

**For randomly oriented fibers** the difference of pressure over 1.2 g of flax (compressed in a measuring chamber) of a certain air flow is measured. This method is suitable for fibers in the form of flocks.

**In the reference method:** the pressure drop over a bundle of parallel fibers is measured. This method is suitable for fibers in the form of slivers.

The fiber fineness reported throughout this thesis was determined by analysis of microscopy images at the ITV-Denkendorf (Section 2.17).

### 4.3.2 Fiber quality determination with IR methods

There are many examples in the literature where infrared-multivariate calibration methods were used successful in predicting properties of plants or other natural products [18, 19, 22, 54, 57, 60, 64, 106, 110]. It is important that the samples used for the calibration are representative and accurately measured. The quality of the calibration obtained depends entirely on the accuracy of the reference data (see Section 4.3.1).

A minimum of six spectra per regression factor is recommended for building an IR-multivariate calibration according to the ASTM guidelines. For seven factors, as used in the NIR model, a minimum of 42 samples is required. And for the ten factors used in the FTIR calibration, at least 70 samples are necessary. Both calibrations produced in this work, complied with this criterion. An increase in the number of factors used for the PLS analysis, will always decrease the error produced by the model in the calibration set. This presents the risk that unwanted variability in the data set, such as random noise, may also be considered by the model. The model is over-fitted, and shows excellent results for evaluating samples belonging to the calibration set but failing to predict samples from an external validation set [91]. Therefore the minimum necessary number of factors must be used.

The calibration models generated with the NIR-data (Section 3.3.2), were much better than those obtained using the FTIR-data. However, when tested against an independent validation data set; they proved to be not so robust. Causes that could have affected the IR analysis are described below.

**Fiber heterogeneity:** the majority of flax samples produced during the laboratory scale and pilot plant scale experiments were line flax (long flax fibers). They were sent to the ITV-Denkendorf for analysis where different parts of the sample were measured. As mentioned in Section 1.4, the fiber structure varies at different parts of the stem. It is therefore highly probable that the same sample although correctly measured by the ITV-Denkendorf and by IR spectroscopy, was too heterogeneous. This impeded to get a good correlation between both measurements.

**Sampling window size:** in the FTIR-ATR device, the fiber sample is pressed against the diamond window (⌀ 1.8 mm). In a report from the literature, the quality of flax fiber determined by the airflow method was correlated with the reflectance Vis-NIRS data (400-2498 nm or 25 000-4000 $cm^{-1}$). The sample device for scanning natural products of 21 × 5 × 4 cm; allowed to sample 20 g of fibers cut in 20 cm length. A PLS regression method was used to establish relationships between fiber fineness and results from Vis-NIR spectra. This method produced a model of $R^2$ of 0.97 and a standard error for calibration (SEC) of 1.69 dtex. The work was based on an equation developed with a population of 462 dew retted and water retted samples

with a fineness range of 18.3-65.4 dtex, analyzed in the range from 1100 to 2500 nm (or 25 000 to 4000 $cm^{-1}$) [57, 100]. Additionally a model with thermogravimetric data was built, but the model was not so robust, because a larger data set of 100-250 of fiber samples was be necessary to generate a stable model [57].

The NIR sampling window diameter used in this thesis was 13 mm. In other work, NIR reflectance spectra of of a variety of cereal food products were acquired with a commercial dual diode-array (Si, InGaAs) spectrometer, which was able to measure a large area of the specimen surface of about 10 cm diameter. Interference from color, hydration effects, or irrelevant chemical variations was large and was not evenly distributed across the spectrum. It was found that selecting subsets of wavelength variables substantially improved the calibration performance. The calibration model obtained was able to determine the total dietary fiber accurately [19].

Therefore the scanning area needs to be larger to counteract the sample heterogeneity and to allow more information to be collected.

**Detection range:** the FTIR range and the NIR range were used in this work. Other works have used the visible NIR range and obtained good calibrations for flax fiber quality [57].

**Data:** the samples required to produce a robust model must include the natural variability of property of interest (e.g. fiber quality) whereas a number of samples uniformly distributed between the extremes values can give better results. The minimum range that a property or concentration value must vary in order to provide enough information for the calibration software has been recommended as $\pm$ 5 times (and not less than $\pm$ 3 times) the reproducibility of the reference method [91].

A larger data set of samples of fiber would be necessary to generate a stable model. Besides this, the error of the reference method done at ITV-Denkendorf, should have been quantified and replicate sampling would have been needed. This was not possible, due to the long time required for the fiber analysis.

**Regression type:** A PLS regression presupposes a linear relationship between the spectral data and the concentration or other property value to be determined. If nonlinearity (not easily accommodated by PCR and PLS) between the spectral data and the quantitative information of interest exists, "artificial neural networks" (ANN) have proved to be useful [91].

The models produced in this work, although not so accurate, can give a general idea of the sample quality in a fast way. But in order to use them for predictions, they need to be improved.

### 4.3.3 Fiber quality determination by measuring the uronic acid content

Meijer *et al.* found no clear relationship between uronic acid and fiber quality in flax retting experiments. Moreover, they found that water-retted fibers isolated from the stems having the lowest pectin content were the strongest and the high pectin samples of green flax fibers were the weakest [79]. In Section 3.1.2 the uronic acid content (pectin content) was compared to the fiber resolution and fineness, but there was no clear relationship between those values.

## 4.4 Isolation of the strains appearing during the flax fiber treatment

The bacterial culture of *G. thermoglucosidasius* PB94A was overgrown by other type of bacteria, when the culture was incubated with flax fibers. These new bacteria probably originated from the flax fibers. The DGGE analysis of those broths confirmed the appearance of new strains, while the original pattern of *G. thermoglucosidasius* PB94A almost disappeared (see Sections 3.2.3 and 3.4). The strains were isolated selectively in pectin media. But no new strains were found in the DGGE analysis, only strain *G. thermoglucosidasius* PB94A was detected in the experiments, some probable explanations are:

- A selection factor either in the sampling, or the PCR, or the DGGE was introduced, therefore isolating always *G. thermoglucosidasius* PB94A. This will be discussed in depth in Section 4.5.
- The other "new" bacteria were simply not cultivable with the employed method.
- The "new" bacteria were not degrading pectin, that is why they were not isolated in pectin media. The only pectin-degrading strain that could be detected was *G. thermoglucosidasius* PB94A, which was recurrently recovered from the broth of several experiments.

## 4.5 Analysis of the bacterial population by the DGGE technique

Although PCR-DGGE avoids some of the drawbacks of traditional culture-dependent methods, it can also have disadvantages [43, 86]:

- The first bias may already be introduced by sampling and how the sample is handled. Such biases are frequent in traditional as well as in molecular methods. Storage conditions, transport, delay in the analysis, etc., might affect the microbial population present in the sample originally.

- Another source of variability can be DNA extraction. Purification of nucleic acids from complex matrices can be difficult. Due to the differences of the cell wall, not all species can be lysed to the same extent. Reproducibility in cell lysis is important, as well as the removal of humic and exopolysaccharic substances, which may inhibit the PCR amplification.

- The different amounts of the species present in the original sample can also affect the concentration of the DNA extracted and its detectability. Furthermore, a complex matrix makes a good extraction and elimination of the impurities difficult, thus affecting the amplification step.

The samples from the pilot plant experiments performed for this thesis, can be considered complex because of the accumulation of a mixture of plant and microbial material. The inability to detect the profile of *G. thermoglucosidasius* PB94A in some the DGGE gels, could also be because the strain was present in such a low concentration, that the DGGE method failed to detect it. This hypothesis could be strengthened by the results of Section 3.10.2, when the thermal treatment of the bacterial culture destroyed much of the biomass and allowed PB94A to grow, making it detectable again.

Another problem in the use of PCR to amplify mixed target DNAs is the formation of chimeric molecules and/or the formation of heteroduplex molecules during the PCR reaction. These "artifact" molecules could contribute to overestimate the real number of community constituents, because more bands are present in the DGGE patterns, thus causing a wrong interpretation of the community complexity. However this problem can be solved by choosing the adequate conditions in the PCR reaction [86]. A microbial community pattern analysis using 16S rDNA PCR-DGGE showed an overestimation of the number of laboratory strains in the sample, whereas some strains were not represented at all. Therefore the 16S rDNA PCR-DGGE-based community analysis was limited by 16S rDNA heterogeneity [37].

The amplification efficiency of genes using whole bacterial cells as template instead of extracted DNA can be affected by the physiological state of the cells [86]. Since whole cell template was used for the PCR reactions made for this thesis, this could have been another bias factor that hampered detection.

Nevertheless we were able to prove with this method, that *G. thermoglucosidasius* PB94A was present in the bacterial broth even after two weeks of continuous operation of the pilot plant process under non sterile conditions.

## 4.6 Selection of the reactor concept for flax fiber treatment

### 4.6.1 Immobilization of *G. thermoglucosidasius* PB94A on solid supports

The application of immobilized microorganisms in a process has several advantages. The biochemical processes can be controlled easier, a phase separation between the solid phase of microorganisms and the liquid reaction mixture is achieved effortlessly and the amount of biomass in a particular phase can be altered by modifying the flow rate.

Polyurethane foam is a versatile carrier material. But not all cells have the ability to fix themselves on solid surfaces [40]. This was the case of *G. thermoglucosidasius* PB94A, which did not form a monolayer in any support tested (Section 3.5, 63). The colonization of the PUF was much better than that of the other supports tested, but was still not enough.

Other immobilization approaches like gel entrapment were not used since a stable material was needed to withstand the mixing regime at the pilot plant. This was corroborated in a preliminary test in lab scale, using the biphasic culture technique described by Gerhardt [48]. A solid agar pectin phase and a liquid phase were fermented together, resulting in a broken gel phase. In addition implementing a gel phase in the pilot plant would complicate its operability.

Other work attempted the immobilization of *G. thermoglucosidasius* PB94A on a silicon$-TiO_2$ porous carrier, and was also unfruitful. Few cells were resting on the carrier surface. However a positive effect on the cell density and the lyase activity was detected by the addition of carrier material [49]. The results of Section 3.5, also show the same effect over both factors. The lyase activity determined by the Collmer method, reached values never observed for the batch fermentation of *G. thermoglucosidasius* PB94A (shown in Fig. 3.23). An explanation could be that degradation catabolites that inhibit the lyase activity are trapped by the carriers. The inhibition by low weight catabolites for *G. thermoglucosidasius* PB94A was also described by Göpel. The implementation of dialysis fermentation allowed compartmentalizing the growth zone of the strain and the product zone. This resulted in a threefold lyase activity compared to an ordinary batch fermentation of the same strain [49].

It has been demonstrated that the enzyme production can be enhanced upon gel immobilization. *Bacillus subtilis* was entrapped in polyacrylamide for the production of $\alpha$-amylase. The yield of the produced enzyme tripled in comparison to the free cells, and the immobilized cells were longer active [89]. Immobilization can protect the cells from toxic or inhibitory substances or environments by the formation of a protective "micro-environment". Immobilization can also affect the cell morphology and the composition of

the outer membrane, which in turn, could make the cells more resistant to the environment and more productive. Another observation is that some cells are more resistant to changes in the pH, this was attributed to the slower diffusion in the immobilized cells as in comparison to the free cells [89].

A DNA analysis (DGGE) of the immobilization experiments showed that extraneous bacteria were already present in the culture that was being "immobilized" (shown in Fig. 3.28). If the immobilization had been successful and the system was to be implemented, this contamination would also be inevitable and the immobilization looses its purpose. It was clear by then that the flax fiber treatment was inevitably going to be a mixed culture process. Still, the fiber quality was good, although PB94A was not the predominant strain. If the fibers were incubated at 50°C, the enzymatic activity was conserved while strong and high quality fibers were obtained. The fear of the presence of cellulolytic microorganisms damaging the fibers at this temperature was hence discarded. The approach of using free cells was chosen for the scale up of the process at the pilot plant. However, it would be interesting to include carriers into the fiber treatment to test their effect over the lyase activity and the fiber quality.

### 4.6.2 Bench scale tests for choosing the reactor concept for flax fiber treatment

As described in Section 3.6.2, the fiber packed bed reactor tested showed channeling and the fiber treatment was not uniform. This problem is also described for unretted flax bobbins; when they were not tight enough, channeling occurred during bleaching [61]. This was the same problem that the packed bed reactor faced, but the construction did not allow to pack the fibers more tightly. Also this would have decreased the diffusion of the degraded polysaccharides out of the fiber bulk, making the fibers to stick and decreasing the treatment effectivity.

The other concept tested, the floating fibers prototype of Section 3.6.3, produced high quality fibers of reproducible quality. Therefore this concept was chosen for building the pilot plant. The description of a machinery for treating flax fibers that used a principle similar to the floating fibers prototype was found in a Patent of 1924, described in Section 1.7.5. The fibers were placed inside receptacles with adjustable walls and perforated floor. The flax fibers were buoyant and floated in the fluid. A gentle circulation motion was done upward and was performed until all the gums were loosened out. The fibers were subjected to a gentle movement in one direction, while the dirty solution was left back. This action was performed until the pumped liquid was clean. The fibers were then immersed in water to wash out further the undesired substances. The machinery consisted of a series of receptacles with communicating and moving walls and a perforated bottom, hold by a cage. The vat had several compartments to separate the different streams [93].

The system described in the Patent is complex and laborious to operate, but the working principle is correct, as it uses different washing steps operated at counter-current and a gentle movement for mixing. The floating-fibers process proposed in this thesis for an effective flax fiber treatment has some similarity to the Patent, but is easier to operate and has fewer moving parts.

### 4.6.3 Pilot plant test using short flax fibers

The quality of the card sliver from the experiments made at Memmingen and Mielsdorf, was good. A resolution of 2.52 and a fineness of 11.32 were obtained. A yarn would have been obtained if the fibers had been cut to the right size. The 15 cm length was too short for the machinery at the South African company. There were no other possibilities left for spinning in Europe. Regardless of that, it was positive that the fiber treatment process was successful in 200 L scale, which was an important factor for the design of the pilot plant for treating long flax fibers in this project.

## 4.7 Pilot plant results

### 4.7.1 Pilot plant process

The overall treatment time for one batch of fibers was about 47 h. This time could be shortened to half or less if more tanks were available. The hydrogen peroxide-Adulcinol solution was left overnight for operational ease. However this treatment time can be reduced to a couple of hours.

If an industrial plant were built, this process could operate in a battery of tanks, where the solutions are transferred to the other tanks where they are needed while the fibers remain in the tank. This would decrease the treatment time.

From the behavior of the *G. thermoglucosidasius* PB94A culture during the process (Fig. 3.53), it might be inferred that the incubation time can be shortened. The compressed air was fed at 1 bar, but the bacteria were consuming so much oxygen that they decreased the concentration of it to a minimum value of 4%. Even if the air pressure was increased to 1.5 bar, this had no effect on the oxygen concentration in the tank, most probably because the air bubbles were too big for an adequate mass transfer. At this point, the air pressure could not be further increased because the liquor spilled out of the tank. The pH decreased to 8.5 after only four hours of incubation. This decrease was due to the production of acidic groups by the degradation of pectin material. Afterward, the pH increased again most probably because of bacterial lysis, a similar phenomenon was observed in the 200 L fermentation of *G. thermoglucosidasius* PB94A (Fig. 3.2). When the bacteria are lysed, the protein content of the cell increases the pH of the medium.

Therefore, probably even 10 h would be enough time for the production of good quality fibers.

The weight loss reported for the flax treatment experiments is in the same range as reported in other studies. The yield of fibers after the treatment at the pilot plant was $84 \pm 2\%$ and after combing was $70 \pm 5\%$ (see Section 3.10.6). This yield value is high considering that in the conventional flax process, 40-50% of long flax is converted to the short byproduct tow.

A dew retted fiber can loose as much as 25% of its weight if boiled in a 2% NaOH solution, and a high proportion of residual hemi-celluloses and pectins have to be removed before bleaching the rove. Traditionally this has been done by boiling the fibers in NaOH or NaOH and ash, followed by a peroxide bleaching [98]. For rove bleaching of green flax, the presence of more than 10% of water-soluble substances results in about 20% weight loss during scouring [61]. Bleaching with acidic hypochlorite solutions can lead to 8.25% weight loss, depending on the desired color, which varies from cream to bright white. Flax is 70% $\alpha$-cellulose (native type) and the rest are hemicellulose and pectin impurities that can be removed by bleaching. However, nowadays only a 15% weight loss is acceptable for economical and technical reasons. If the whole hemicellulose would be removed, the fibers would become vulnerable and weak [51].

The conventional treatment of flax fabrics in India with 2% caustic soda solution at 80°C for 4 hours, causes a weight loss in the range of 10-16%. On the other hand, fabrics treated with an enzyme with hemicellulase and pectinase activities, could loose up to 25%, depending on the treatment conditions, but this was undesirable and damaged the flax fabric. Therefore, the fabric was treated so that the weight loss was similar to the conventional NaOH treatment. The extent of the residual content of noncellulosics remained in the range of 12-15%. Furthermore, a weight loss optimum of 12% was proposed, where the fabric properties like absorbency, whiteness, dyeability and tensile strength, were balanced [24]. The weight loss of 16% for the pilot plant process seems adequate. For a fair comparison with the conventional fabric treatment, the weight loss during the manufacturing of the fabric should also be considered.

### 4.7.2 Lyase activity during the fiber treatment in the pilot plant

Sharma described that the pectate-lyase activity of a commercial enzyme mixture had a maximum peak after 2 days of being in contact with the flax stems. Afterward the lyase activity decreased steeply, and by the fifth day, only one third of the maximum activity was left in the retting liquor. It is also probable that the enzyme was inhibited by the medium [102].

The lyase activity of the bacterial culture decreased when it was reused a couple of times, Section 3.10.1. A similar phenomenon was also observed in beaker scale experi-

ments of Section 3.2.3. This could be due to an inhibition process as commented already in Section 3.4.1, where the "aged" culture caused inhibition to freshly spread *G. thermoglucosidasius* PB94A.

Despite the inhibition phenomena, the lyase activity could be recovered with a heat treatment (see Section 3.10). The bacteria present in the broth, recovered the traits of *G. thermoglucosidasius* PB94A. A DGGE analysis showed that bands corresponding to PB94A were recovered. This indicates that the *G. thermoglucosidasius* PB94A can be used several times, and if some inhibition starts to manifest, this can be reverted by the thermal treatment.

### 4.7.3 Oxygen demand of the liquors used in the pilot plant process

One main environmental concern in the textile industry is the amount of water discharged and the chemical load it carries. Other important issues are energy consumption, air emissions, solid wastes and strong odors [9].

In the textile industry, the highest environmental loads come from salts, detergents and organic acids. Typical COD loads are between 40-80 g/kg fiber [9]. Typical values for batch dyeing are well above 5000 mg/L COD. For continuous finishing processes, the COD concentration can easily be in the range of 130 - 200 g/L [9].

**Sodium carbonate** is an inorganic substance that cannot be oxidized or biodegraded by microorganisms [7]. The COD and $BOD_5$ values of the $Na_2CO_3$ solution increased as it was used in the subsequent batches. The average $BOD_5$/COD ratio was 0.41 and remained fairly constant in all the batches.

***G. thermoglucosidasius* PB94A liquor:** the COD and $BOD_5$ values of the *G. thermoglucosidasius* PB94A solution used in the pilot plant, are shown in Section 3.10.5. The COD and $BOD_5$ of the fresh fermented bacteria initially had higher values. When the broth was put in contact with the fibers, the bacteria further degraded the components of the fermentation broth, reducing the carbon charge of it. After this point until the end of the experiment, the carbon load increased linearly. As long as air was injected, the pilot plant process was practically odor-free. Only a slight smell similar to corn being cooked was perceived. Continuous aeration of the solutions used in the pilot plant was beneficial in maintaining a low load of COD and BOD (average values were 1678 and 669 mg/L, respectively). This corresponds to a biodegradability ratio of $0.40 \pm 0.13$ ($BOD_5$/COD). A ratio $BOD_5$/COD of 0.5 or larger is characteristic of a readily biodegradable wastewater [104].

In a report from 1987, retting of flax straw by chemical (Trilon TB), enzymatic (Novozym 249) and water retting was compared at a semi-industrial scale. It was

suggested that the enzymatic liquor could be recycled if the suspended solids were removed. The $BOD_5$ (3520 mg/L) and COD (5950 mg/L) were highest in the effluent from enzyme retting (pH 4.8) compared to effluents from chemical or water retting. Aeration of the effluents for 2 weeks reduced the level of $BOD_5$ and COD by more than 50%. Aeration also changed the pH of the three samples to nearly neutral pH [102]. Literature data for hemp retting liquor, report COD and $BOD_5$ values of 1827 mg/L and 932 mg/L, respectively. The pH was 7.5 and the soluble oxygen concentration was zero [111]. The COD and $BOD_5$ values reported for this thesis, are similar to those of the literature.

**Hydrogen peroxide-Adulcinol liquor:** the COD of this solution remained constant from the third batch onward. However the COD test can be affected by residual $H_2O_2$, which despite its powerful oxidizing ability, when reacting with stronger oxidizing agents such as the potassium dichromate used in the COD test, could act as a reductant, liberating hydrogen gas and molecular oxygen. But for the experiments of this thesis, this phenomenon was not observed.

The $BOD_5$ measurement, gave for all samples zero-values. Most probably the activated sludge was inhibited by the residual $H_2O_2$. The samples should had been pretreated with peroxidase to destroy the hydrogen peroxide. There is no standard method for eliminating the hydrogen peroxide interference in wastewater analysis. Kang *et al.*, proposed to correct quantitatively the effect of the concentration of hydrogen peroxide on the chemical oxygen demand. The correlation equation valid in the hydrogen peroxide concentration range of 0-2000 mg/L, can be applied to the samples containing hydrogen peroxide and organic substances [58]. However, this correction was not applicable for the pilot plant samples because a heating pretreatment had been done to the samples and therefore their exact concentration of $H_2O_2$ was not known.

### 4.7.4 Cost of the new biotechnological process using *G. thermoglucosidasius* PB94A in comparison to conventional dew retting

In Table 4.1 a comparison between the conventional dew retting process and the biotechnological process is shown. The prices considered for the calculation were provided by the project partner Holstein Flachs and were taken from actual processing costs.

The value of the bacterial treatment in the pilot plant was taken considering the price of yarn dyeing, as provided by the company Eing Textilveredlung und Handelsgesellschaft GmbH & Co.KG (personal communication, 2008). Actual coloring prices were $\sim$3 €/kg for using the standard conditions. This value would make the biotechnological process

unprofitable.

A lower cost of the proposed bacterial treatment was considered (700 €/t) because the process would take place in a low-tech plant build specifically for flax retting. The yields shown in the Table 4.1 are actual process yields. The bold type numbers are the values that can be modified (assumptions), whereas the other values are calculated from them. The calculation considers a 12% risk harvest reduction bond, for avoiding the loss of the crop because of bad weather. The yields of the biotechnological process were taken from Table 3.9 of Section 3.11.

The positive consequences of using the biotechnological process, would be that more byproducts could be recovered and also more revenue from them. The yield and quality of seeds would be also higher. Since the shive would not be degraded by dew retting, more shives would be recovered and could be sold for animal bedding or composites. Also the treatment time would be reduced to a couple of days, instead of 4-6 weeks. The agricultural fields would be free in late summer and probably a second late crop could be also farmed. This could mean an important economical advantage to the farmer.

Fibers required for classical long spinning must have a uniform quality. Under-retting would produce a coarse yarn suitable only for coarse fibers or ropes. Over-retting would result in fiber loss during processing and a weak yarn with limited applications. The fiber length and fineness are critical and are heavily related to retting. The value of a batch can vary depending on its quality up to 100%. The prices fluctuate strongly year by year depending on supply and demand [11]. Also the fiber variety and processing method have a significant impact on fiber quality and therefore on price. In addition the fiber prices vary according to the target application [8]. Prices of short flax fibers are variable and ranged from around 300 €/t for the special paper industry, through 350-650 €/t for textile fibers, to 500-600 €/t for composite materials in 2005 [16].

However, for this exercise values close to the annual average were used. A price of 0.65 €/kg and 1.8 €/kg was considered for the dew retted tow and line. For the "biotechnologically" produced tow and line (using *G. thermoglucosidasius* PB94A), prices of 0.80 €/kg and 2.10 €/kg were used. It was assumed that the latter had a superior quality. In the case that the fiber quality was just the same as the dew retted fiber, the profit would disappear. The values depicted in Table 4.1, correspond already to a positive scenario.

The cost effectiveness of the process with *G. thermoglucosidasius* PB94A depends among other aspects, on how many times the solutions can be reused. Another advantage of reusing the solutions is that the need for effluent treatment would be minimized, sinking the costs even further.

**Table 4.1**: Flax fiber process cost comparison between dew retted fiber and the biotechnological treated fiber

| concept | dew retted | units | biotech. treated | units |
|---|---|---|---|---|
| land lease | **550** | €/ha | **550** | €/ha |
| growing (seeds, fertilizer, non-harvesting machinery) | **650** | €/ha | **650** | €/ha |
| harvesting (pulling, (2.5×turning), baling, transport) | **650** | €/ha | – | €/ha |
| harvesting (pulling, baling, transport) | – | – | 450 | – |
| **subtotal field costs** | 1850 | €/ha | 1650 | €/ha |
| unretted flax straw yield | – | – | 8 | t/ha |
| retted straw yield | **7.2** | t/ha | – | – |
| fiber in stem | **30%** | 0 | **30%** | 0 |
| processable fibre | 2.2 | t/ha | 2.6 | t/ha |
| fiber yield after treatment | **70%** | – | **82%** | – |
| fiber yield after raw combing | – | – | 68% | – |
| line flax | 1.5 | t/ha | 1.7 | t/ha |
| tow | 0.6 | t/ha | 0.4 | t/ha |
| seeds in food quality | – | – | 0.7 | t/ha |
| seeds for oil use | **0.1** | t/ha | – | – |
| scutching incl. tow scutching | **270** | €/t | **320** | €/t |
| swinging | 1944 | €/ha | 2560 | €/ha |
| bacterial treatment in the pilot plant | 0 | – | 700 | €/t |
| bacterial treatment and fine scutching | 0 | – | 1785 | €/ha |
| **subtotal processing costs** | 1944 | €/ha | 4345 | €/ha |
| **total field and processing costs** | 3794 | €/ha | 5995 | €/ha |
| line flax price | **1.8** | €/kg | **2.1** | €/kg |
| line flax revenue | 2722 | €/ha | 3641 | €/ha |
| price tow | **0.65** | €/kg | **0.80** | €/kg |
| tow revenue | 421 | €/ha | 286 | €/ha |
| straw yield | **50%** | – | **55%** | – |
| clean shives yield | 3.6 | t/ha | 4.4 | t/ha |
| clean shives price | **0.15** | €/kg | **0.25** | €/kg |
| clean shives revenue | 540 | €/ha | 1100 | €/ha |

continued on next page...

Table 4.1 - continued

| concept | dew retted | units | biotech. treated | units |
|---|---|---|---|---|
| seed price | **0.3** | €/kg | **0.5** | €/kg |
| seed revenue | 30 | €/ha | 350 | €/ha |
| **subtotal products revenue** | 3713 | €/ha | 5377 | €/ha |
| EC field subsidy | **350** | €/ha | **350** | €/ha |
| EC processing subsidy | **235** | €/ha | **275** | €/ha |
| risk harvest reduction bond | **0%** | – | **12%** | – |
| reduction bond × subtotal product revenue | 0 | €/ha | 446 | €/ha |
| **subtotal subsidies and premium revenue** | 585 | €/ha | 1071 | €/ha |
| **total product and subsidies revenue** | 4298 | €/ha | 6448 | €/ha |
| **profit balance** | 504 | €/ha | 453 | €/ha |

The government subsidies considered are quite important for the economy of the flax industry. For a considerable number of small enterprises, the EU government aid exceeds their gross margins. For the large flax processors in France and Belgium, the aid represented about 35% of their gross margins from 2002 to 2005. Many of those EU aids, are going to end. The additional processing aid for processors of flax grown in traditional areas (Belgium and the Netherlands) will cease from the 2009/2010 marketing year. The processing aid for short flax and hemp will also end from the 2009/2010 marketing year. On the other hand, the EU authorized a subsidy of 200 €/t from the 2009/2010 marketing year onward for long flax fiber. The EU government has considered a full decoupling of the aid in a "Single Payment Scheme" to help the small processors to shift to more profitable crops and adapt their production to the market [16]. In general, the economic balance of the flax growing and processing industry is quite delicate and is susceptible to the market volatility and government subsidies. At the current prices of flax, even the dew retting fiber is not economically attractive; therefore a new process for fiber production is at the moment not in sight.

## 4.8 Environmental impact of the flax plant cultivation

Flax and hemp cultures have a positive environmental impact. They consume less fertilizers and pesticides than other fiber and food crops. In addition, these cultures have positive effects on the biodiversity in agro-ecosystems and landscapes. Fiber crops provide

an environmental recovery period to the soil from more demanding crops. Flax cultures are located in some of the most intensively farmed areas of the EU where the current trend is in favor of simplifying rotation and increasing use of chemicals [16].

### 4.8.1 Comparison of the new biotechnological process for flax fiber production to other fiber extraction methods

A study named "Life Cycle Analysis of Hemp Textile Yarn", compared three hemp-fiber processing scenarios and a flax scenario to assess the environmental impact of the different technologies for producing hemp yarn. In this study the following scenarios for the comparison were used [111]:

**bio retting:** hemp green scutching followed by water retting using selected bacteria. The steps followed were: harvest, drying on the field, green scutching, bio-retting, drying, softening and hackling.

**baby hemp:** based on desiccation and stand-retting of premature hemp. The procedure was: desiccation, stand retting, harvesting, dew retting, drying on the field, scutching and hackling.

**traditional warm water retting of hemp:** the steps followed were: harvest, drying on the field, warm water retting (hot spring origin), drying on the field, scutching and hackling.

The study concluded, that the bio-retting option had the higher impact for climate change and energy use, due to the energy input in the fiber process, required for heating of the retting tanks and for drying the fibers. It was found that about 40% extra energy was needed for "bio retting" compared to the other retting options. The "bio retting" of hemp was made by Fibranova group and lasted 72 h. The process was not saving water in comparison to traditional water retting because the fibers were rinsed intensively in order to wash out the pectin from them. The drying was done merely by heating, without a mechanical pre drying step [111].

Another report of "bio retting" of jute with green scutching followed by retting, reduced the water consumption by half and the environmental pollution level to one fourth in comparison to traditional jute stem retting [111].

In the case of the retting process proposed in this thesis, it is similar to the "bio retting". However, some important differences presented below, give the biotechnological process of *G. thermoglucosidasius* PB94A an advantage:

1. all the solutions can be reused a minimum of 6 times.

2. A pre drying step by squeezing the excess water in a mangle, decreases the drying costs.

3. the fibers are not rinsed with pressurized water as in the "bio retting" process, but the rinsing happens in the combined bleaching-softening step.

4. the process is aerobic, whereas the "bio retting" and the warm retting used for comparison are anaerobic.

### 4.8.2 Implementation of the new biotechnological flax treatment

The best way that the new biotechnological process with *G. thermoglucosidasius* PB94A can be implemented is through cooperative structures, where many actors of the production chain are in contact. This would allow farmers and traders to join forces and develop high quality fibers in a bigger scale. Since the equipment needed for the biotechnological treatment is simple, the process could be carried out near the farms, in an enclosed facility, which would additionally reduce transport costs.

Fibers of consistent high quality can be produced with this new biotechnological method. The biotechnological process could work all year long, eliminating the risks associated with dew retting. The fibers would be cleaner (less dust, stones and debris) than dew retted ones. The fibers obtained would have a lighter color than the conventional dew retted fibers, while maintaining its strength.

# Chapter 5

# Conclusion and outlook

A new process to produce fine flax fibers using whole cells of *Geobacillus thermoglucosidasius* PB94A and green decorticated fiber as starting material was established. A 200 L scale pilot plant was designed, built and operated successfully.

The fiber treatment process was optimized. The process developed for the production of high quality fibers consists of:

1. a mild alkaline incubation with $Na_2CO_3$ at 90°C,
2. incubation with the strain *G. thermoglucosidasius* PB94A,
3. a softening, shive removal wash with a $H_2O_2$-softener solution at 53°C.

The treatment of the flax fiber with the alkaliphilic strain *Geobacillus thermoglucosidasius* PB94A was done at 50-55°C and pH 8-9.

In experiments reusing the solutions from steps *1-3* for up to six times, the fiber quality was equally high for all batches. The fiber fineness was improved by 70% and the resolution by 63% in comparison to the starting green fiber; while the fiber tenacity remained unchanged. The pectinolytic lyases were preserved after reusing the same bacterial culture 6 times. No cellulases were detected and the fibers remained strong. More than 130 kg of high quality fibers were successfully produced in the pilot plant.

The main advantage of the new biotechnological treatment is that it eliminates completely the risks associated with dew retting and produces reliably fibers of consistent high quality. Other advantages of the new method are:

1. The green decorticated flax fibers accounts for only ∼30% of the total straw weight. Therefore in this process only 30% of the weight has to be treated, which reduced costs and waste streams.
2. The pectinolytic bacterial culture is cheap can be used several times. This is a big advantage in comparison to other methods using commercial enzymes.

3. All solutions, $Na_2CO_3$, bacterial broth and $H_2O_2$-Adulcinol could be reused several times. In this way, very little waste was produced.

4. Since the process is aerobic, the waste streams produced had low pollution.

5. No intermediate rinsing steps were required, which helped to save water.

6. The pre drying step made by squeezing the excess water in a mangle decreased the drying costs.

7. The process can be easily implemented without the need of expensive equipment.

8. The process is reliable and the flax fibers can be produced in a controlled way.

9. The fiber yields and qualities obtained with the new process are higher compared to dew retting. Therefore, the farmers could obtain more revenues.

The new fiber retting alternative using *Geobacillus thermoglucosidasius* PB94A is reliable and ready for implementation on a technical scale. However the economic situation of the flax industry is critical at the moment. The prices paid by the market for dew retted flax fibers are too low, making this industry not profitable. To implement the new retting process is necessary to wait until the market of the flax fibers recovers.

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