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Bio-scouring of Linen Fabrics with Laccase Complex from *Cerrena unicolor*

Abstract
Presently biotechnology plays an important role especially in the field of environmental protection. In the textile industry enzymes are often used in many technological processes as they are ecological. Flax fibres are special among textile raw materials due to their properties. Flax fibres, besides cellulose, contain non-cellulosic substances such as hemicellulose, lignin, pectins, waxes and fats. Lignin is the substance not desired in fibres. Enzyme such as laccase is active during the decomposing of lignin-cellulose complex. Hence, the research task attempts to apply laccase complex in the treatment of woven fabrics made of flax fibres. The aim of the research was to test the possibility and effectiveness of applying laccase complex produced by Cerrena unicolor strain in the scouring processes of linen fabrics. The tests performed proved that the pre-treatment with laccase complex from Cerrena unicolor provides a high level of water sorption capabilities in of linen fabrics. The results obtained confirm that linen fabric pre-treatment with laccase can be an alternative to traditional chemical scouring.

Key words: *flax, linen, bio-scouring, enzymes, laccase.*

Introduction

Natural fibres mainly formed out of cellulose are surrounded by a hydrophobic layer inhibiting their wetting. This hydrophobic layer constituted from so called "natural impurities" (pectin, hemicellulose, lignin, proteins, waxes, fats and mineral compounds) must be removed to render a valuable hydrophilic property to natural fibres. Flax fibres, which together with ramie, jute, hemp belong to the grow-up of bast fibres, are special among textile raw materials due to their properties. Flax fibres, besides cellulose (65 - 80%) contain non-cellulosic substances such as hemicellolose and lignin.

Lignin – being a constituent of this non-cellulosic matter - is a large, cross-linked macromolecule with molecular mass in excess of 10,000 amu. It is relatively hydrophobic and aromatic in nature. In primary flax fibre, lignin occurs in the primary wall and in the outer part of the secondary wall. Lignin is resistant to mineral acid activity and it is sparingly soluble. It can dissolve when it is initially transformed into derivatives by chlorination and oxidation and then by leaching. Lignin presence in fibre affects its rigidity due to incrustation in amorphous areas of cellulose. Lignin is the substance not desired in fibres.

Traditionally these "impurities" are effectively removed by chemical scouring in water solutions of sodium hydroxide at an elevated temperature of 98 °C, and time of 60 min.), which is harmful for the environment. Application, for this purpose, of equally efficient ecological biotechnological methods eliminates these problems.

Our experience in the application of enzymatic pre-treatment of fabrics made of natural fibres showed a possibility of substituting traditional alkali scouring of cotton woven fabrics. In particular, the employment of pectinolytic enzymes happened to be effective in removing non-cellulosic substances from cotton and linen fabrics [1, 2].

Enzyme such as laccase is active during the decomposing of the lignin-cellulose complex. Hence, the research task attempts to apply laccase complex in the treatment of woven fabrics made of flax fibres. Laccase (EC 1.10.3.2, p-diphenol oxidase) is an extracellular blue oxidase capable of oxidizing phenols and aromatic amines by reducing molecular oxygen to water by a multicopper system [3]. Laccase occurs in certain plants and bacteria, but the enzyme is particulary abundant in white-rot fungi and it is assumed to comprise a lignin biodegradable complex [4]. From among other microorganisms it is the best lignin degrader [5]. It degrades wood by a simultaneous attack of lignin and cellulose/hemicellulose or selectively degrades lignin far more than polysaccharides [6, 7]. Laccase seems to be one of the most important enzymes in lignin degradation [8] since it can attack polymeric lignin, degrade the framework structure loosely, introduce additional hydrophilic groups, and produce water-soluble material [9]. In the presence of suitable redox mediators (e.g. 1-hydroxybenzotriazole), laccase is even able to oxidize recalcitrant non-phenolic lignin units [10]. This capability has generally extended their use to a series of biotechnological applications, all of them related to the degradation of structurally diverse aromatic compounds. Laccase is currently being investigated by many researchers with respect to litt-er mineralisation [11], dye detoxification and decolorisation [12, 13], the bleaching of paper pulp [14, 15] and bio-scouring of flax fibre [16, 17]. Sharma et all [17] conducted tests on enzyme application for scouring dew-retted flax rovings. From literature analysis it turns out that research performed up to now has referred mainly to flax roving [16]. Former research carr-ied out by the Textile Research Institute concerned the removal of impurities from fabrics made of natural fibres applying pectinolytic enzyme complex [18]. The aim of the research described was to test the possibility and effectiveness of applying laccase complex produced by *Cerrena unicolor* strain in the scouring processes of fabrics made of flax fibres.

In traditional alkali treatment, the unwanted effects of sodium hydroxide application are degradation of cellulose fibre, and generation of highly polluted wastewaters. Hence, the search for new solutions which could form an ecological alternative. The quality of the results obtained after bio-pre-treatment was mainly evaluated with reference to the sorption properties of textile fabrics.

Materials and methods

Textile fabrics

Raw linen woven fabric (plain weave), mass per unit area 223 g/m^2.

Fungal strain and culture conditions

In the enzymatic treatment of flax fibres, laccase enzyme produced by *Cerrena*

unicolor (Bull. Ex Fr.) strain 137, which belongs to white rot fungi, was used (culture collection of the Department of Biochemistry, Maria Curie-Sklodowska University, Lublin, Poland). Stock cultures were maintained on 2% malt extract agar (MEA) at 4 °C, and inoculation material was pregrown on MEA plates at 25 °C for 10 - 14 days. For laccase production, Lindeberg-Holm liquid media were prepared which contained per liter: glucose 10 g; L-asparagine 1.5 g; KH_2PO_4 0.47 g; $MgSO_4 \times 7\ H_2O$ 0.5 g; $Na_2HPO_4 \times 12\ H_2O$ 0.48 g; yeast extract 0.1 g and microelements: $Mn(CH_3COO)_2 \times 4\ H_2O$ 12 mg; $Zn(NO_3)_2 \times 6\ H_2O$ 3.14 mg; $CuSO_4 \times 5\ H_2O$ 3.19 mg; $Ca(NO_3)_2 \times 4\ H_2O$ 50 mg; $FeCl_3 \times 6H_2O$ 3.2 g; and thiamine 50 µg. The pH of the medium was adjusted to 5.6, then were autoclaved in 500-ml flasks and inoculated with homogenised fungal mycelium from overgrown MEA plates. Under aseptic conditions 2.5 ml of the mycelial suspension were transferred into each 500-ml Erlenmeyer flask containing 100 ml of medium. Cultures were incubated on a rotary shaker (100 r.p.m.) at 25 °C for 14 days.

Preparation of enzymatic complex

After 14 days of fungal growth, the cultures were harvested and laccase activity reached its maximum. The culture grown was then filtrated to remove the mycelia and the solution was centrifuged at 9500 r.p.m. for 30 minutes. The clear supernatant was collected and concentrated to the required volume at 4 °C with a Pellicon XL ultrafiltration system equipped with a Biomax membrane with a cut-off 10-kDa (Millipore, Billerica, USA).

Analytical procedures

Enzyme assays

Laccase activity was determined in the culture liquid by measuring the oxidation of 0.5 mM syringaldazine dissolved in ethanol buffered with 0.1 M citrate phosphate (pH 5.6, ε_{525} = 65 $mM^{-1}\ cm^{-1}$) [19]. All spectrophotometric measurements were carried out using a UV-300 spectrophotometer (Unicam, Cambridge, UK). Enzyme activities were expressed in units defined as 1 µmol of product formed per 1 minute.

Enzyme stability, temperature and pH optima

Thermal stability and the optimum temperature of laccase were determined using 2.2'-azino-bis(3-ethylthiazoline-6-sulfonate) (ABTS) as a substrate in citrate-phosphate buffer (pH 4.5). For stability measurements, laccase was incubated for 1 hour at different temperatures (25 - 80 °C) in 100 mM citrate-phosphate buffer (pH 4.5), and afterwards, the residual activity was measured at 25 °C. Optimum temperature was determined by varying the cuvette temperature in the spectrophotometer between 5 °C and 75 °C using an integrated Peltier element. To estimate the pH optimum of the enzyme, activity was measured with syringaldazine as a substrate, and the pH of the citrate-phosphate buffer (50 mM) was changed within the range from pH 2.5 to pH 7.5; the pH stability was tested by storing the purified enzymes for one hour at pH 3.7 and pH 10 in 100 mM phosphate buffers.

Determination of hemicellulose contents

Determinig hemicellulose content was done according to Ermakov's method in which hemicelluloses were sugared by a 2% solution of sulphuric acid, and then the amount of created sugars was determined by the Somogyi-Nelson method [20].

Determination of lignin contents

Evaluation of changes in lignin composition in linen fabric after bio-treatment was done according to the weight method. The principle of this method is based on polysaccharide saccharification by sulfuric acid and on heating up the sample with this acid. Then the weight of residuals obtained (incinerated) is determined [21].

Methods of evaluating linen woven fabrics

Liquid (water) sorption by fibres was examined according to the method developed at The Textile Research Institute, Łódź, determining liquid sorption coefficients by SORP-3 instrument [22].

Pre-treatment of linen woven fabric

Enzymatic pre-treatment

Linen woven fabric before enzymatic treatment was washed in water bath at 60 – 65 °C for 60 minutes in order to remove sizing agents. Linen woven fabric was subjected to pre-treatment in Linitest laboratory dyeing apparatus, using different amounts of laccase enzymes produced by *Cerrena unicolor*. Enzymatic pre-treatment of woven fabric was performed in baths of varying concentration of laccase enzymes from 2.4 to 7.5 U/g fabric in optimal treatment conditions: pH 5.3 (acetate buffer), temperature of 60 °C, time 30 - 120 minutes; liquid ratio 10:1. Enzymes inactivation occurred in water bath at a temperature of 98 °C for 5 minutes.

Traditional alkali treatment

Traditional alkali-scouring pre-treatment was performed in Linitest laboratory dyeing apparatus at the liquid ratio of 10:1 in a bath containing sodium hydroxide 1.8 g/l. Process conditions: temp. of 98 °C, time of 60 minutes; rinsing: temp. of 80 °C, time of 10 minutes.

Bleaching process

Linen woven fabrics after the bio- and chemical - scouring were subjected to two-stage bleaching in baths containing:

- hydrogen peroxide 35%, 10.0 ml/l
- stabiliser, anionic agent 0.7 g/l
- sodium hydroxide 2.0 g/l

Process conditions: temp. of 98 °C, time of 60 minutes, liquid ratio of 10:1

Results and discussion

Laccase production

Applied Lindeberg-Holm medium resulted in high amounts of laccase (3600 $U1^{-1}$). After filtration and concentration high activity (24000 $U1^{-1}$) was obtained.

Enzyme stability, temperature and pH optima

Laccase from *Cerrena unicolor* was found to be relatively thermostable (Figure 1). Surprisingly, at 50 °C, the enzyme did not lose almost any activity within 1 hour and at 70 °C still 10% of its activity remained after 60 minutes of incubation. A higher temperature of 80 °C, however, caused the rapid inactivation of laccase (95% activity loss within 10 min).

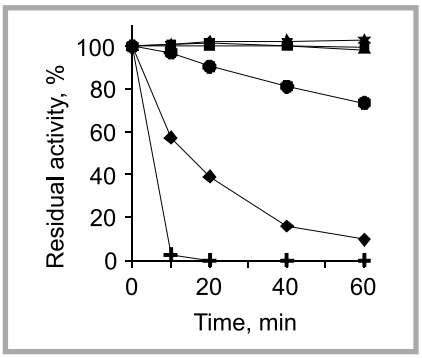

Figure 1. *Stability of Cerrena unicolor laccase at different temperatures: 25 °C (triangles), 40 °C (stars), 50°C (squares), 60 °C (circles), 70 °C (diamonds), 80 °C (crosses). Measurements were carried out in triplicate (standard deviations <5%).*

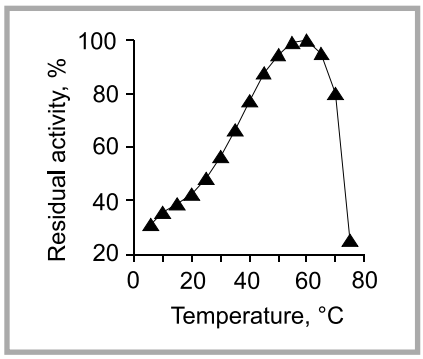

Figure 2. *Effect of temperature on the activity of Cerrena unicolor laccase with ABTS. Measurements were carried out in triplicate (standard deviations <5%).*

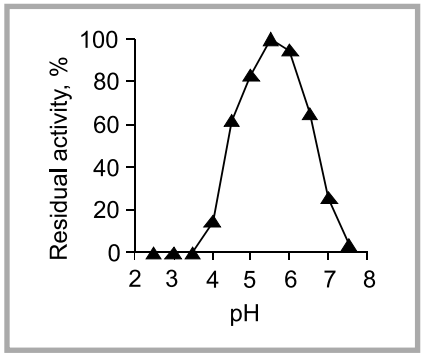

Figure 3. *Effect of pH on the activity of Cerrena unicolor laccase with syringaldazine. Measurements were carried out in triplicate (standard deviations <5%).*

Laccase also seems to be relatively stable during long time storage in a refrigerator at 4 °C. After 6 months, the activity of laccase was almost unchanged.

The influence of temperature on the activity of the enzyme (Figure 2) was found to be quite predictable. Activity increased constantly with the temperature and reached the maximum at 60 °C. At higher temperatures, the enzyme activity declined rapidly reaching only 20% of the maximum level at 75 °C. Laccase showed relatively high activity at 5 °C amounting to 30% of the maximum at 60 °C.

The effect of pH on laccase activity (Figure 3) showed distinct activity maxima at pH 5.5. With decreasing pH, the laccase activity decreased constantly until it became zero at pH 3.5. Below this value (pH 3.5) syringaldazine was not oxidised at all. With increasing pH, the laccase activity again decreased constantly until it became almost zero at pH 7.5.

Laccase was stable at neutral pH and lost almost no activity during 24 h of storage in a respective phosphate buffer (data not shown). At an alkaline pH of 10, the enzyme was also stable while an acidic pH of 3 caused the partial inactivation of laccase. Within 10 min, it lost about 30% of activity; after one hour at pH 3, however, the residual activity still amounted to 64% of the initial activity indicating the slowing down of the inactivation process.

Liquid sorption capability of tested sample of linen woven fabric

The water sorption ability of linen fabrics was determined on the basis of sorption coefficients defined by the method of sorption curve analysis. For comparison purposes the tests of raw woven fabrics and fabrics after traditional alkali boiling-off were performed.

It has been stated that woven fabric made of flax fibres after enzymatic pretreatment (using laccase from *Cerrena unicolor*) are characterised with higher sorption values when compared to woven fabrics after alkali boiling-off (Figure 4).

Evaluation of changes of chemical composition of linen fabric after enzymatic treatment

Studying the contents of hemicellulose in tested samples of linen woven fabric it was found that the samples subjected to alkali or enzymatic treatment have smaller amounts of this ingredient as compared to the sample of raw fabric with no treatment at all. Hemicellulose contents in samples after alkali or enzymatic treatment is at a similar level (Figure 5).

Enzymatic treatment caused a substantial decrease in lignin content in linen fabrics. It was obvious, as it is known that laccase causes the removal of lignin from lignin-cellulose complex existing in flax fibre (Figure 6).

Bleaching of fabrics after enzymatic treatment applying laccase

For woven fabrics made of flax fibres and bleached after enzymatic pre-treatment, comparable or even higher whiteness degree was obtained when compared to fabrics bleached after traditional alkali treatment (Table 1). This confirms the effectiveness of applied bio-treatment.

Conclusions

Applied Lindeberg-Holm medium resulted in high concentration of laccase (3600 $U1^{-1}$). After filtration and concentration, high activity (24000 $U1^{-1}$) was obtained and successfully applied in the bio-scouring of linen fabrics.

Pre-treatment with laccase complex from *Cerrena unicolor* provides a high level of water sorption capabilities in linen fabrics. As is known, the ability of fibres to absorb liquids is an important parameter of flat textile fabrics during their processing (bleaching, dyeing).

The tests performed have confirmed the usefulness of laccase produced by *Cerrena unicolor* in purifying woven fabrics made of flax fibres. Efficient removal of lignin from flax fibre facilitates the penetration of oxidizing whitening agents into fibre structure. After bio-treatment, comparable whiteness degrees are obtained as compared to the ones after alkali scouring.

The reasearch performed is only an initial study. Yet first results confirm that linen woven fabric treatment with laccase enzymatic complex from *Cerrena unicolo*r can be a better alternative to traditional chemical treatment.

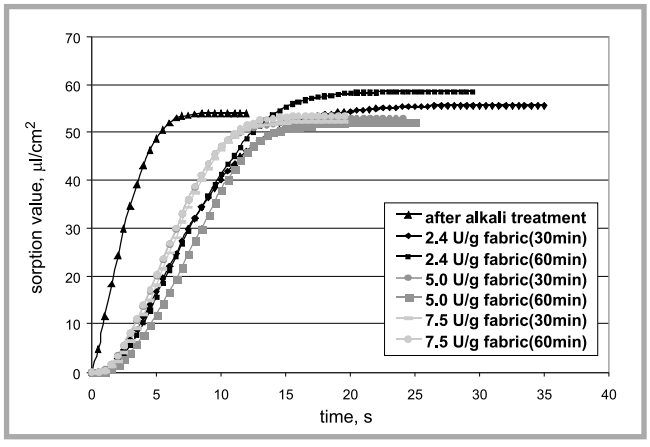

Figure 4. *Kinetic curves of H_2O sorption on linen woven fabric after treatment with laccase enzyme*

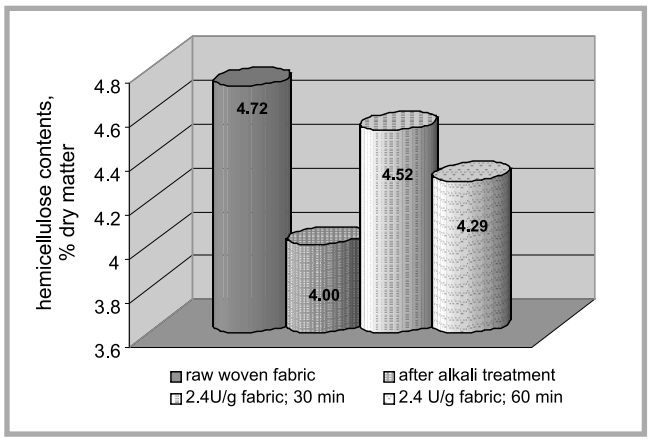

Figure 5. *The influence of enzymatic treatment of linen woven fabric onto the contents of hemicellulose dry matter.*

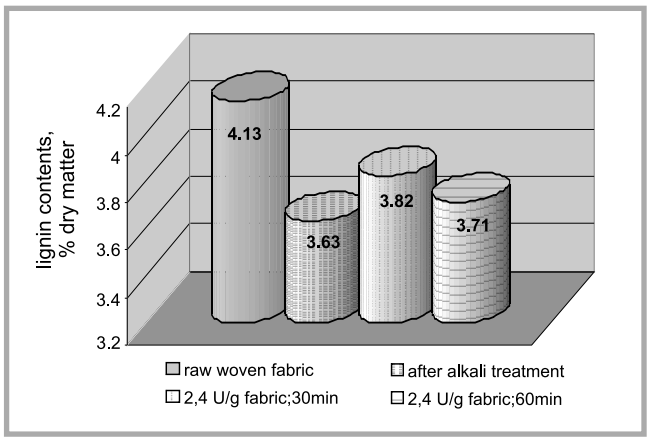

Figure 6. *The influence of enzymatic treatment of linen woven fabric onto the contents of lignin dry matter.*

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Table 1. *Test results of whiteness degree (after bleaching H_2O_2) of linen woven fabric depending on the type of applied pre-treatment; W - whiteness coefficient; TV- whiteness digital assessment; * PN-EN ISO 105-J02:2002 – Textiles – tests for colour fastness – Part J02: Instrumental assessment of relative whiteness.*

Conditions of pre-treatment of linen woven fabric		Whiteness degree according to PN-EN ISO 105 J02:2002*	
		W	TV
Fabric after alkali scouring		36.5	-3.5
Bio-pre-treatment 2,4 U/g fabric;	30 minutes	37.2	-3.1
	60 minutes	37.9	-3.0
	90 minutes	38.1	-3.0
Bio-pre-treatment 5,0 U/g fabric;	30 minutes	37.9	-3.0
	60 minutes	35.6	-3.2
	90 minutes	38.3	-3.0

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