ISOLATION OF EXCEEDINGLY LOW OXYGEN CONSUMING FUNGAL STRAINS ABLE TO UTILIZE LIGNIN AS CARBON SOURCE

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Lignin biodegradation is normally related to aerobic microorganisms, and it is often claimed that microbes do not metabolize lignin as a carbon source. In this work, several fungal strains were isolated from the sediment of a small stream located in a forest and tested on agar plates with lignin as the only carbon source. All identified strains were Ascomycetes, *Penicillium spinulosum*, *Pseudeurotium bakeri* and *Galactomyces geotrichum*. When cultivated in shaking flasks with lignosulphonate as a carbon source, the lignin was consumed, and cell free culture filtrates appeared to depolymerize lignosulphonate to some extent. It is suggested that the strains detected are part of a symbiotic community and live in a microbiological niche in which they are able to utilize lignin residues left from brown rot and humus having extremely low oxygen content.

Keywords: carbon source, extracellular enzymes, extremely low oxygen content, lignin biodegradation, soil microorganisms

INTRODUCTION

Lignins are the second or third most abundant polymer type in the cell walls of vascular plants and play an important role in the carbon cycle. Unlike most other biopolymers, they are racemic and have a partly random structure; their enzymatic depolymerization requires specific strategies from microorganisms. Lignins are branched and partly cross-linked and have several different types of inter-monomeric covalent bonds with the β-O-4 ether linkage being the most important (Fig. 1). There are also lignin carbohydrate linkages (LCCs) in the cell wall.[1,2] The lignified cell wall matrix is compact so that the large enzyme molecules cannot penetrate it. This is the reason why an immediate enzymatic hydrolysis cannot take place.[3]

Specialized wood degrading organisms, however, of which the white rot fungi are the most studied and efficient, have developed a special strategy for overcoming the steric hindrance. Their extracellular redox enzyme systems, such as lignin peroxidase, manganese peroxidase, laccase, and cellobiose dehydrogenase, generate reactive substances with low molecular weight – such as aromatic radicals, Mn(III), or hydroxyl radicals –, which act as specific cofactors and co-substrates.[4,5] In this way, steric barriers can be overcome and covalent bonds in lignin at a certain distance from the hyphae can be broken. The cell wall matrix is swollen and becomes accessible for degrading enzymes with larger dimensions.[3] All described efficient wood degrading organisms are aerobic because of the oxidative action of the first enzymatic attack. The integration of complex enzymatic systems in commercially feasible biotechnical applications is difficult as the cofactors complicate the process design. Nevertheless, such enzymes proved to be useful for pulping and bleaching both at a lab scale and in large-scale pilot plants.[6,7] In the case of lignolytic systems based on hydroxyl radicals, such as cellobiose dehydrogenase, the specificity is also poor, *i.e.*, the enzyme system also

depolymerizes cellulose and hemicelluloses.[8] The copper containing enzyme laccase is commercially available and used in several technical processes, but these are rather polymerizations and detoxifications than lignin degradation.[9,10]

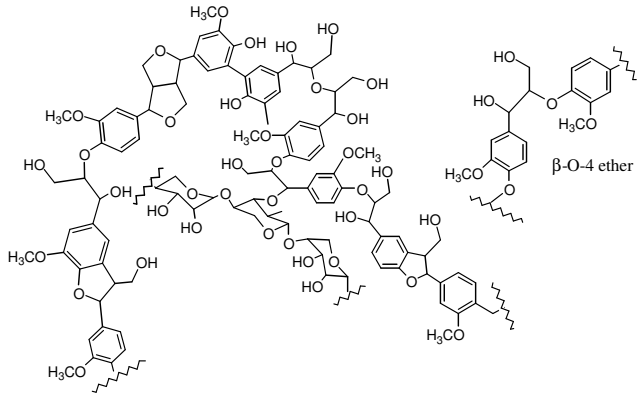

Figure 1: Example of a hypothetical softwood lignin structure. Structure of the most important intermonolignol bond, the β-O-4 ether is shown

Not all wood degrading organisms degrade the lignin totally. The brown rot fungi leave behind a structurally modified lignin residue.[11] Considering the fact that polysaccharides and extractives are the source of energy and carbon for wood degrading organisms, this finding is plausible. In the course of this strategy, lignin is just removed to get access to the polysaccharides for enzymatic degradation without being itself a source of energy and carbon.[12,13] Residual lignin does eventually end up in the ground as one of the main constituents of humus, the organic part of soil.[14] Soil is inhabited by a large microbiologic community and fungal strains, some of which might have lignolytic ability. A few of them possess oxidative enzymes similar to those from white rot fungi.[15] Reportedly, there are also available hydrolases that cleave β-O-4 ethers.[16] It should be underlined that lignin degradation in soil is different from that in wood, because in the former there are no steric obstacles. In soil, the 'classical enzymes' may bind directly to the substrate. Such enzymes have a high potential for technical applications. A recent study made by Bandounas *et al.* reported the isolation of three soil bacteria, *Pandoraea norimbergensis* LD001, *Pseudomonas sp.* LD002 and *Bacillus sp.* LD003, with ligninolytic potential.[17]

Recently, several strains were isolated from soil, which appear to be able to live on lignin as sole carbon source. Based on the analysis of cell free culture filtrates, it was suggested that these organisms cleave β-O-4 ethers by mechanisms that are different from those of oxidative ligninases in wood degrading organisms.[18] In continuation of the quoted study, the present paper describes the isolation of further strains, which live on materials under extremely low oxygen conditions, and are close to being anaerobic.

EXPERIMENTAL

Origin of chemicals and reagents. Agar gel was purchased from MERCK Company, Whitehouse Station, New Jersey, USA; lignosulphonate DP_401 (LS_{DP401}) was obtained from Boregaard Company, Sarpsborg, Norway.

The microorganism cultivation medium was based on a modified Vogel's medium[19] for bacteria, yeast and filamentous fungi cultivation (Table 1) and with LS_{DP401} as the sole carbon source in it. The selection medium was the same, but 12 g agar per liter medium was additionally added for preparation.

Soil samples were collected from approximately 1 dm down under the sediment of a small water stream. Samples of 1 g of the soil/mud sample were suspended in 9 ml milliQ water, and this liquid was used for inoculum in the purification of microorganisms.

Isolation and purification of microorganisms. The exceedingly low oxygen conditions were maintained in an incubator, which had been degassed by flushing with CO_2 for 10 min, after which no O_2 was detectable at the outlet. The tightly closed incubator was flushed daily with CO_2.

A diluted soil suspension (1 ml 10^{-2}) was placed on the selected medium plates. The plates were incubated for 4 to 7 days at room temperature in the incubator under conditions close to anaerobic until colonies appeared. After 4 cultivation days, a single colony was

transported to a new agar plate and when the appearance of the microorganism was homogeneous, it was reinoculated 3 times onto new plates. The strains were thereafter regarded to be pure.

Cultivation. The purified microbe strains were inoculated to agar plates after 5 days and then cultivated in 100 ml shake flask in a medium with LS_{DP401} as the sole carbon source. The medium was degassed by piping CO_2 into the shake flasks until no O_2 was detected above the flasks. The whole incubator was also degassed until no O_2 was left. The incubator was placed on a shaker with continuous agitation of 150 rpm at 25 °C and degassed every day.

An aerobic strain of *Penicillium thomii*[18] inoculated on an agar plate was cultivated along with the selected strains as a negative control. This should confirm the extremely low oxygen condition and that no fungal growth was possible.

Strain identification was performed by ribosome sequencing with Finnzymes' Phire® Plant Direct PCR Kit.[20] About 1 mm^2 size of each strain cultivated in LS_{DP401} medium was washed by Mili-Q water 3 times before PCR was applied for amplifying the stains' nuclear ribosomal DNA.

Table 1
Data on solution preparation

Components	Amount
Shake flask cultivation (pH 5.0).	
Vogel's stock solution	40 ml
Lignosulphonate DP 401	10 g
Vogel's trace element solution	0.1 ml
Deionized H_2O	960 ml
Vogel's stock solution	
Sodium citrate mono hydrate	62.5 g
H_2PO_4	125 g
NH_4NO_3	50 g
$MgSO_4 \cdot 7H_2O$	0.98 g
$CaCl_2 \cdot 2H_2O$	0.5 g
Deionized H_2O	1000 ml
Vogel's trace element solution	
$FeSO_4$	2.5 g
$MnSO_4 \cdot H_2O$	0.98 g
$ZnCl_2$	0.83 g
$CoCl_2$	1.0 g
HCl	5 ml
Deionized H_2O	95 ml

PCR procedures. Primers ITS1 (5'-TCCGTAGGTGAACCTTGCGG-3') and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3'), targeting the conversed ribosomal regions of 18 s and 5.8 s were used for PCR amplification in a 50 µl vessel. Parts of the washed mycelia (around 4-6 mm^2) of the different strains mentioned above were heated in 25 µl 2x Phire Plant PCR Buffer at 98 °C for 10 min. Then, a mixture of 22 µl distilled water, 1 µl of each primer (10 µM) and 1 µl of the Phire Hot Start II DNA Polymerase was added to each sample and the PCR was performed as follows: initial denaturation at 98 °C for 5 min; then 30 cycles of 98 °C for 20 s, 54-60 °C for 30 s (in every 2 cycles, the temperature was increased for 1 °C), 72 °C for 20 s; with a final incubation at 72 °C for 1 min.

PCR products were separated by agarose gel electrophoresis, purified with the QIAquick gel extraction kit (QIAGEN; Stockholm, Sweden) and sent for sequencing. The sequencing results were blasted in Genbank and the highest hits were chosen for identification.

Lignin consumption during cultivation was detected in the following way: samples with the same volume of shake flask medium before cultivation and of cell free filtrate after 10 days cultivation were analysed by SEC in alkaline medium. Vanillin was applied in both samples as internal standard. The samples with shake flask medium during cultivation were centrifuged at 14000 x g for 3 min. Cell free culture filtrates were obtained from the supernatant.

Size exclusion chromatography (SEC). Instrument: Rheodyne 7725 Manual Injector equipped with 20 µl sample loop, Waters 515 HPLC pump, was operated at a flow rate of 1 ml min^{-1}, equipped with the columns: 3 Tosoh TSKGel Columns (G3000PW-G4000PW-G3000PW) and a TSKGel Guard Column (PWL 7.5 cm x 7.5 mm). As detectors: Waters 2487 dual λ Detector operated at 254 and 280 nm and Waters 410 RI detector was applied. 10 mM NaOH in Milli-Q H_2O was the mobile phase. 20 µl of sample was injected for analysis.

Enzyme activity of lignosulfonate depolymerization was confirmed by running a reaction system with LS_{DP401} as substrate. The reaction system comprised 1 ml with 500 µl cell free culture filtrate, 400 µl reaction buffer (50 mM sodium acetate, pH 5), and 100 µl substrate (50 g l^{-1} LS_{DP401} dissolved in 50 mM sodium acetate). The reaction was performed at 25 °C overnight with continuous agitation at 600 rpm. Then, the reaction system was heated up to 90 °C to stop the reaction. The mixture was then injected into the SEC system. The shake flask cultivation medium that was not inoculated served as control.

RESULTS AND DISCUSSION

The three isolated and purified fungi are presented after re-inoculation and incubation under anaerobic conditions for 5 days in the selected medium plates, where LS_{DP401} was the sole carbon source, and the fungal growth was visible (Fig. 2). The aerobic strain *Penicillium thomii*, which grew well in aerobic conditions on this medium,[18] was inoculated on an agar plate for control to check the oxygen level inside the incubator. As there was no growth of *P. thomii* on the agar plate during the whole cultivation process, it can be safely concluded that the oxygen level was too low to support the growth of a regular aerobic strain. It seems therefore that there are several fungal strains that are able to live on LS_{DP401} as the sole carbon source under anaerobic or close to anaerobic conditions. This finding is, in a way, surprising, since it has been believed for a long time that no microorganisms are able to live on lignin as the sole carbon source, and that biological lignin degradation is always based on oxidation.[12] In an earlier work, we demonstrated that aerobic soil organisms seemed to be able to live on lignin as the sole carbon source, and that extracellular enzymes might be involved.[18] In another way, there is nothing surprising in that lignin is used as a carbon and energy source for microorganisms; lignin is abundant and energy rich, and there are multiple examples of microorganisms that can utilize phenols as a carbon source.[21] Using lignin as a carbon source for cultivation and investigating microorganisms' lignin degradation ability have also become more common trends in recent years. In 2011, Hainal *et al*. were able to cultivate some *Rhodotorula* species with lignin as a carbon source and showed structural changes of lignin caused by the microorganisms.[22] Furthermore, brown rot fungi and soft rot fungi produce a residual lignin when degrading wood, and if this material was not degraded, the coal of the biosphere should be accumulated in this residual lignin. Thus, it can be assumed that biological lignin degradation occurs in two biological niches, in wood biodegradation and in soil, brown rot and soft rot fungi degrading the remaining lignin (Fig. 3). The lignin degrading systems of wood degrading enzyme microorganisms are all redox enzymes,[4,12] and this can explain why wood degradation seems to be strictly aerobic. The lignin from wood is involved in a crosslinked network together with polysaccharides,[2] and the structure is so compact that molecules of the size of enzymes cannot penetrate the cell wall.[3] It is believed that that the lignin degrading enzymes by redox-reactions generate small reactive species, which are able to overstep steric barriers.

A B C

Figure 2: Strains on agar plates with lignin as a sole carbon source after 5 cultivation days; A: Strain *Penicillium spinulosum*, obtained from the bottom of a stream, in the mud area, described as white rot like; B: Strain *Pseudeurotium bakeri*, obtained from a root area, described as brown rot like; C: Strain *Galactomyces geotrichum*, obtained from the bank of a stream, in the mud area, described as transparent film like

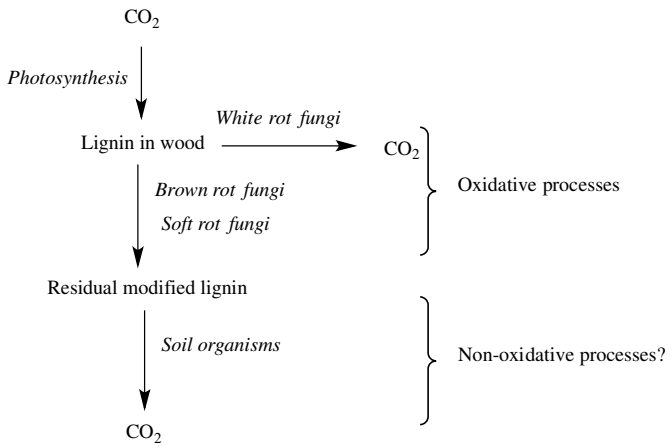

Figure 3: Hypothetical schedule for the part of carbon circulation in the biosphere that involves lignin. Different types of wood degrading fungi (white rot, brown rot, soft rot) use well-known oxidative enzymes for lignin degradation, but with exception of white rot fungi, they all produce residual lignin, which might be utilized by soil organisms. This process may use other non-oxidative processes for lignin biodegradation

Table 2
Data on purified microorganisms

Name	Isolated from	Description	Classification	NCBI Taxonomy ID
Penicillium spinulosum	Segment of a small stream	White rot like, white spores	Ascomycota	63822
Pseudeurotium bakeri	Root area	Brown rot like, white spores	Ascomycota	205925
Galactomyces geotrichum	Segment of a small stream	White film like, green spores	Ascomycota	27317

The low molecular weight reaction products of lignin degradation will be partly oxidized, and thereby less energy rich. This is not a major problem for wood degrading organisms, since they probably obtain most of their energy from carbohydrate degradation, and remove lignin in order to get access to the carbohydrates of wood. The degradation of residual lignin in soil has other goals and problems: the products need to energy rich, but on the other hand, the lignin polymer is readily accessible to enzymes. Thus, the enzyme systems for soil organisms might be different than the ones of wood degrading microbes, perhaps including non-oxidative degradation mechanisms. The latter is not unreasonable, since the mechanisms for lignin degradation in many technical processes are non-oxidative.[23]

Three of the isolated strains were identified by ribosome sequencing with Finnzymes´ Phire Plant Direct PCR Kit (Table 2). All of the strains were Ascomycetes. To our knowledge, this is the first demonstration that these strains are able to metabolize lignin as the sole carbon source.

The microorganisms also grow well on the shake flask medium with LS_{DP401} as the only carbon source. A blank control of cultivation medium without lignin had been performed and no growth was shown for any of the three strains. The purity of LS_{DP401} was very high, which contained a total of only 0.7% carbohydrate, which means that in 100 ml cultivation medium, there was only 7 mg carbohydrate contamination, and it could not support fungal growth. On the other hand, the three microorganisms were growing very well. The biomass of the strains after 10 days of cultivation was filtered off, and their dry weight amounted to 0.7 g, 0.9 g and 0.5 g. To further confirm LS_{DP401} had been used as a carbon source, the LS_{DP401} consumption is demonstrated by comparison of the SECs of the samples and the control (Fig. 4). Clearly, in the SEC chromatograms of the three strains, the area of the region with high molecular weight LS_{DP401} is lowered in all cases after cultivation in comparison with the control. All experiments had been repeated 3 times and the effects observed on the SECs were always very similar.

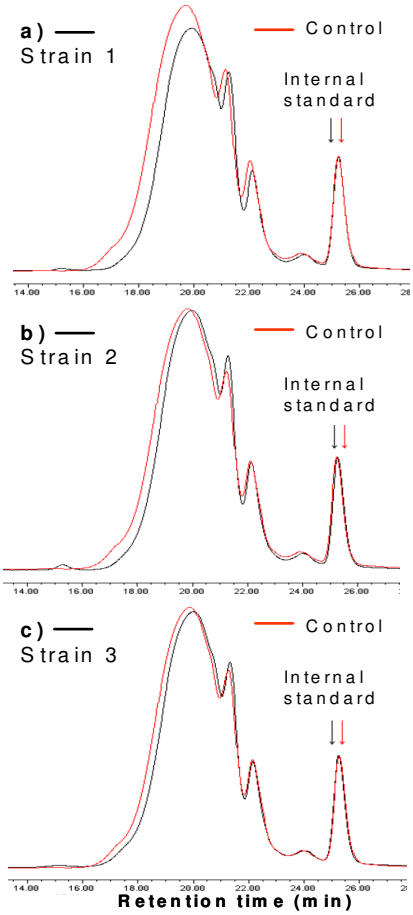

Figure 4: SEC chromatograms of the three strains' culture filtrates after 10 days of cultivation and of the medium control (red curves – medium control, black curves – strain culture filtrates after 10 days of cultivation; the earlier peaks are attributed to lignosulfonate and the later overlapping peaks correspond to internal standard vanillin); a), b) and c): *Penicillium spinulosum*, *Pseudeurotium bakeri* and *Galactomyces geotrichum bakeri* culture filtrates, respectively, in comparison with medium control

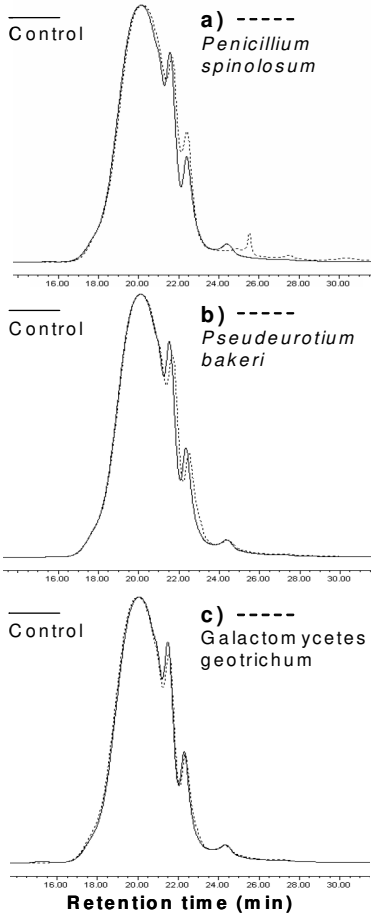

Figure 5: GPC data indicating that the cell free culture filtrate was able to cause the depolymerization of lignin (solid line – control experiment, dash line – incubation of 5 mg lignosulphonate with cell free culture filtrate overnight). Lignosulfonate depolymerization activity of a) *Penicillium spinulosum* culture filtrate; b) *Pseudeurotium bakeri* culture filtrate; and c) *Galactomyces geotrichum* culture filtrate

To answer the question whether the LS_{DP401} consumption was caused by extracellular enzymes or just by its absorption onto the mycelium, the enzyme activity test of lignosulfonate depolymerization was conducted. Filtrated cell free culture was incubated again together with fresh LS_{DP401} and phosphate buffer overnight and the samples were injected into SEC. Also, the cultivation medium – with fresh LS_{DP401}, after incubation, but without inoculation – served as a negative control sample (Fig. 5). In the SEC chromatograms of all three strains, the high molecular weight LS_{DP401} peak had slightly shifted to a lower molecular weight region. Especially for the *Penicillium spinulosum* strain (Fig. 5a), the lower molecular LS_{DP401} increased after incubation and there was even a new low molecular weight LS_{DP401} peak (around 26 minutes of retention time), showing the degradation of large Mw LS_{DP401} and the accumulation of low Mw LS_{DP401}.

CONCLUSION

All the experiments performed in this study – isolation, purification and cultivation of the three strains using LS_{DP401} as a sole carbon source and under exceedingly low oxygen environment, control growth experiment without LS_{DP401}, GPC analysis of LS_{DP401} consumption and GPC analysis of cell free degradation of LS_{DP401} – led to the same conclusion: the three isolated fungi – *Penicillium spinulosum*, *Pseudeurotium bakeri* and *Galactomyces geotrichum* – were able to grow on lignin as a carbon source under anaerobic or close to anaerobic conditions, and could produce extracellular enzymes to cause lignin degradation.

Thus, it seems that besides the organisms that consume residual lignin in aerobic soil, there is also an ecological niche with fungi that consume lignin under anaerobic or close to anaerobic conditions. The enzyme systems of these organisms present interest and may be the subject for further research.

REFERENCES

[1] J. Ralph, G. Brunow, P. J. Harris, R. A. Dixon, P. F. Schatz *et al*., in "Advances in Polyphenols Research", edited by F. Daayf, A. El Hadrami, L. Adam and G. M. Balance, Wiley-Blackwell, 2008, pp. 36-66.

[2] M. Lawoko, G. Henriksson and G. Gellerstedt, *Holzforschung*, **60**, 156 (2006).

[3] R. A. Blanchette, E. W. Kreuger, J. E. Haight, M. Akhtar and D. E. Akin, *J. Biotechnol*., **53**, 203 (1997).

[4] R. Ten Have and P. J. M. Teunissen, *Chem. Rev*., **101**, 3397 (2001).

[5] G. Henriksson, L. Zhang, J. Li, P. Ljungquist, T. Reitberger *et al*., *Biochim. Biophys. Acta*, **1480**, 83 (2000).

[6] H. P. Call and I. Mücke, *J. Biotechnol*., **53**, 163 (1997).

[7] S. Camarero, O. García, T. Vidal, J. Colom, J. C. del Río *et al*., *Enzyme Microb. Technol*., **35**, 113 (2004).

[8] G. Henriksson, P. Ander, B. Pettersson and G. Pettersson, *Appl. Microbiol. Biotechnol*., **42**, 790 (1995).

[9] N. Aktas and A. Tanyolac, *Bioresour. Technol*., **87**, 209 (2003).

[10] D. Areskogh, J. Li, G. Gellerstedt and G. Henriksson, *Biomacromolecules*, **11**, 904 (2010).

[11] D. J. Yelle, J. Ralph, F. Lu and K. E. Hammel, *Environ. Microbiol*., **10**, 1844 (2008).

[12] K. L. Eriksson, R. A. Blanchette and P. Ander, "Microbial and Enzymatic Degradation of Wood and Wood Components", Springer-Verlag, 1990, pp. 407.

[13] T. K. Kirk and R. L. Farrell, *Ann. Rev. Microbiol*., **41**, 461 (1987).

[14] I. Kögel, *Soil Biol. Biochem*., **18**, 589 (1986).

[15] T. Saito, P. Hong, K. Kato, M. Okazaki, H. Inagaki *et al*., *Enzyme Microb. Technol*., **33**, 520 (2003).

[16] Y. Otsuka, T. Sonoki, S. Ikeda, S. Kajita, M. Nakamura *et al*., *Eur. J. Biochem*., **270**, 2353 (2003).

[17] L. Bandounas, N. Wierckx, J. H. de Winde, H. J. Ruijssenaars, *BMC Biotechnol*., **11**, 94 (2011).

[18] R. Bi, O. Spadiut, M. Lawoko, H. Brumer and G. Henriksson, *Cellulose Chem. Technol*., **46**, 227 (2012).

[19] H. J. Vogel, *Am. Nat*., **98**, 435 (1964).

[20] Finnzymes Oy, "Instruction Manual for Phire® Plant Direct PCR Kit", 2010, Version 1.3.

[21] V. L. Santos, R. Valter and R. Linardi, *Process Biochem*., **39**, 1001 (2004).

[22] A. R. Hainal, A. M. Capraru, I. Volf and V. I. Popa, *Cellulose Chem. Technol*., **46**, 87 (2012).

[23] G. Gellerstedt, in "Pulp and Paper Chemistry and Technology", Vol. 2 "Pulping Chemistry and Technology", edited by M. Ek, G. Gellerstedt and G. Henriksson, De Gryyer, 2009, pp. 91-120.