

## Effect of different wavelengths of light on laccase, cellobiose dehydrogenase, and proteases produced by *Cerrena unicolor*, *Pycnoporus sanguineus* and *Phlebia lindtneri*

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Three species of white rot fungi: *Cerrena unicolor*, *Phlebia lindtneri* and *Pycnoporus sanguineus* were cultured in two different media under five different lighting conditions: dark, white, red, blue, and green light. Laccase, cellobiose dehydrogenase, and protease activities were examined in the samples. Blue light efficiently boosted laccase synthesis in *C. unicolor* and *P. sanguineus*, whereas the highest activities (20654 nkat/l) of *P. lindtneri* laccase were observed when this fungus was maintained in green light. On the contrary, the green light allowed obtaining the highest activities of cellobiose dehydrogenase of *C. unicolor* and *P. lindtneri*, while CDH of *P. sanguineus* seems to be dependent on white light. It is clearly visible that differences in protease activities are noticeable not only between the lights variants but also among the media used. However, high proteases activities are correlated with light variants inducing laccase in Lindeberg and Holm medium. Contrary to the cellulose-based medium, where they are weak in light variants that lead to high CDH activities.

**Key words:** white rot fungi, submerged cultures, visible light wavelengths

**Received:** 07 January, 2016; revised: 08 January, 2016; accepted: 11 January, 2016; available on-line: 17 February, 2016

### INTRODUCTION

During millions years of evolution, light became a crucial factor for most living organisms in terms of both the beneficial and harmful effects; moreover, it is considered as a main limiting condition for successful competition and survival in nature (Tisch & Schmoll, 2010). In the past decade, explosion of interest in fungal photosensory abilities was observed considering their responses and molecular basis. It is worth noting that these analyses are significantly accelerated by the advances in genome sequencing, which provide information about the gene content in numerous and phylogenetically diverse fungal species. In a natural habitat, the ability to sense light for fungi means recognizing and anticipating conditions unfavourable for vegetative growth. Moreover, light is often needed for appropriate timing of production and dissemination of conidia (Tisch & Schmoll, 2010). In the light of research conducted over the world, it should be mentioned that light influences many other fungal physiological responses such as asexual conidiation, the circadian clock, secondary metabolism, pigmentation, and sexual development. Therefore, it is not surprising that fungi are capable of sensing light over a broad-spectrum

range, from ultraviolet to far-red light. Hence, a variety of photoreceptors are conserved in fungi, some of which have been analysed in the past few years (Purschwitz *et al.*, 2006).

Numerous publications have been produced indicating that the numbers of genes regulated by light vary between organisms. For *Trichoderma atroviride*, an estimated 2.8% of the genome is light-regulated. Microarray studies on *Neurospora crassa* suggest light regulation in 3–7% genes; in contrast, less than a quarter of a percent (0.25%) of the *Cryptococcus neoformans* genome was regulated by light (Rosales-Saavedra *et al.*, 2006; Dong *et al.*, 2008; Chen *et al.*, 2009a; Idnurm & Heitman, 2010). It has been proved many times that light is engaged in regulation of fungal carotenoid metabolism, polysaccharide and carbohydrate metabolism, fatty acid metabolism, nucleotide and nucleoside metabolism, and in regulation of production of secondary metabolites. Consequently, enzyme and metabolite synthesis is influenced via transcription of genes initiated within minutes in order to cope with the harmful effects of light or to prepare for reproduction (Tisch & Schmoll, 2010).

Interestingly, many enzymes engaged in wood decomposition by fungi are also regulated by cAMP levels. During evolution, fungi became the most effective degraders of ligninocellulosis: in favourable conditions, they decompose wood mainly by fast mycelium growth. They are capable of production of hyphae penetrating into the wood and diffusing a variety of enzymes and low molecular compounds called mediators (Smith & Berry, 1974; Kirk, 1987). Extensive research efforts have been dedicated to characterize regulation of the expression of wood degrading enzymes in numerous fungal species. However, until now only lignin peroxidase has been proved to be induced by light in *Phanerochaete chrysosporium* (Ramirez *et al.*, 2010). Analysis of available genomes of white rot fungi (*Phanerochaete chrysosporium*, *Coprinopsis cinerea*, *Lentinula edodes*) showed existence of homologous sequences to white collar proteins which are widely found in moulds. Therefore, it should be examined how different light conditions (dark and light lengths) will influence enzyme production in different fungal species. In this paper, we present the results of studies of the influence of light on the activity of laccase, cellobiose dehydrogenase, and proteases in three different fungal

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**Abbreviations:** FCL, Fungal Culture Collection of Lublin; DCIP, 2,6-dichloroindophenol; CDH, cellobiose dehydrogenase; WWC, white collar complex

species: *Cerrena unicolor*, *Pycnoporus sanguineus* and *Phlebia lindtneri*.

## MATERIAL AND METHODS

**Culture conditions.** The white rot fungi: *Cerrena unicolor* strain FCL139, *Phlebia lindtneri* strain FCL22, and *Pycnoporus sanguineus* strain FCL199 were obtained from the culture collection of Regensburg University, the Agriculture Academy in Cracow, and the Agriculture University, Tokyo, Japan (FCTUA), respectively. The fungi were maintained on 2% (w/v) malt agar slants. As an inoculum, pieces of fungal mycelia were grown in Lindenberg and Holm medium (Lindeberg & Holm, 1952) in non-agitated conical flasks for 7 days at 28°C.

Mycelial mats were subsequently collected, broken in a Waring blender (three times for 15 s at 10000×g), and homogenates were used as inocula in shaken microplates. After inoculation with 10% (v/v) mycelial suspension, the shaken cultures were run up to 13 days at 28°C in 24-well microplates (each well with 2 ml of Lindenberg and Holm medium or cellulose-based medium placed in an orbital rotary shaker at 120 rpm). The cellulose-based medium had the following composition (1l): 2 g Avicel, 10 g (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, 1 g KH<sub>2</sub>PO<sub>4</sub>, 0.3 g MgSO<sub>4</sub>×7H<sub>2</sub>O, 0.08 g CaCl<sub>2</sub>, 5 mg ZnSO<sub>4</sub>×7H<sub>2</sub>O, 1.5 mg MnSO<sub>4</sub>×4H<sub>2</sub>O, 1.5 mg CoCl<sub>2</sub> z 6H<sub>2</sub>O, 5 mg FeSO<sub>4</sub>×7H<sub>2</sub>O, 100 mg yeast extract, and 0.1 mg thiamine. The pH was adjusted to 6.5 with 5 M HCl (Fang *et al.*, 1999). Every strain and light variant was run in four replications. The samples (40 µl) were collected every 24-hours.

All analysed strains were grown under five different conditions of lighting: dark, white, blue (325–495 nm), green (450–590 nm), and red (600–700 nm). As the dark variants microplates were completely covered with aluminium foil to prevent light penetration. Terrarium lamps ReptiGlo 2.0 (Exoterra, Montreal, Canada) were used as a light source (26 Watt, 1600 Lumen) (one for each microplate). To achieve different spectral ranges Tair MTO photographic filters (72 mm diameter) were used. The filters spectral ranges were determined by using UV-160A spectrophotometer (Shimadzu, Japan). Light intensity (5 µmol/m<sup>2</sup>×s) was measured with Optel FR-10 (Optel, Poland).

**Determination of enzymatic activities.** Laccase activity in the culture fluid was measured spectrophotometrically at 525 nm in a Shimadzu UV-Vis 160A spectrophotometer (Tokyo, Japan) using syringaldazine (4-hydroxy, 3,5-dimethoxybenzaldehyde) as a substrate (Leonowicz & Grzywnowicz, 1981). The reaction mixture (1 ml) contained 100 µl of diluted enzyme, 500 µl of 0.1 M McIlvaine buffer, pH 5.5, 450 µl H<sub>2</sub>O, the reaction was started by addition of 50 µl of 0.5 mM syringaldazine in ethanol. One nano katal (nkat) of laccase activity was defined as the amount of enzyme catalysing the production of one nano mol of the coloured product (quinone, ε<sup>M</sup>=65000 M<sup>-1</sup>cm<sup>-1</sup>) per second at 25°C and pH 5.5, and expressed as nano katal per litre of culture (nkat/l).

Cellobiose dehydrogenase activity was measured by following the decrease in absorbance of the electron acceptor 2,6-dichloroindophenol (DCIP) (Sigma Chemical Co., St. Louis, MO, USA) at 520 nm (ε<sub>520</sub>=6.8 mM<sup>-1</sup>cm<sup>-1</sup>), pH 4.5 and 30°C (Baminger *et al.*, 2001; Karapetyan *et al.*, 2006) with a Shimadzu UV-160A (Shimadzu, Tokyo, Japan) spectrophotometer. This assay measures the activity of an intact enzyme as well as of the catalytically active flavin domain. The reaction mixture (1 ml)

contained 50 µl of 3 mM DCIP (solution in water containing 10%, v/v ethanol), 100 µl lactose (300 mM in 100 mM sodium acetate buffer, pH 4.5), 50 µl NaF (80 mM NaF) in water, and an appropriate amount of the same buffer. After temperature adjustment, the reaction was initiated by addition of a diluted CDH sample (100 µl) and the decrease in absorbance was monitored during the first 60 s. The final enzyme activity was expressed as nkat per liter. This assay was used for determination of the activity of the native enzyme as well as for the catalytically active flavin domain.

For the zymographic analysis of proteolytic activities, the samples of the culture medium were centrifuged (10 min at 4°C at 10000×g), concentrated, and separated using Microcon Centrifugal Filter Units, 3000 NMWL (Millipore). Samples were applied to 10% separating gel containing 0.3% gelatin and 4% stacking gel (Laemmli, 1970). A mixture of samples and loading buffer were deposited into each line (6 µl, 5 µg of protein). Protein concentration was determined according to Bradford (1976). The gels were run at alkaline pH under non-denaturing conditions in 4°C at 145 V. After electrophoretic separation, the gels were incubated for 18 h at 37°C in 0.1M citrate-phosphate (pH 3.5) buffer. Next, the gels were stained with a dye Coomassie Brilliant Blue (R-250). Protease activity was visualized as white bands.

All presented results are expressed as the mean ±S.D. from three independent experiments (n=3). The mean values as well as standard deviation were calculated by the Excel program (Microsoft Office 2010 package) and only values of *p*≤0.05 were considered as statistically significant.

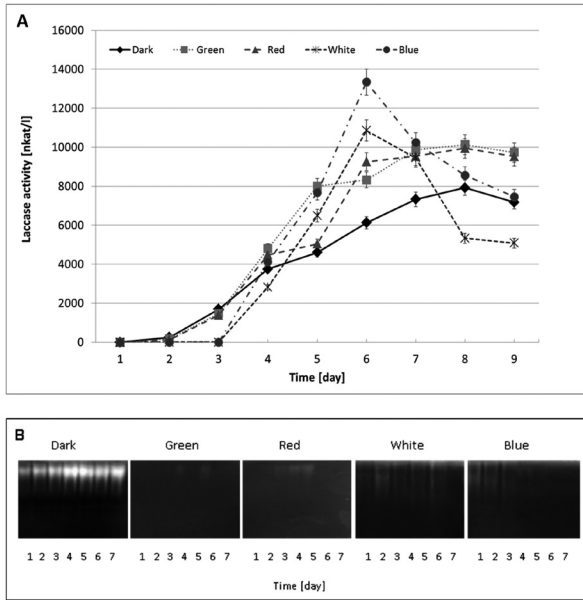
## RESULTS

### Effect of different light wavelengths on laccase activities

Laccase production was evaluated in Lindenberg & Holm (1952) medium which was already successfully used to optimize this multicopper oxidase synthesis in *Cerrena unicolor* and *Rhizoctonia praticola* cultures (Janusz *et al.*, 2006; Janusz *et al.*, 2007). Quadruplicate cultures were used for each treatment at each wavelength evaluated (dark, white light, blue, red, and green). All three analysed fungal species reacted positively to light induction, however, to a varied extent. Blue light efficiently boosted laccase synthesis by *Cerrena unicolor* (Fig. 1) and *Pycnoporus sanguineus* (Fig. 2), whereas the highest activities (20654 nkat/l) of *P. lindtneri* laccase were observed when this fungus was maintained in the green light (Fig. 3). It should be mentioned that blue light seems to be the only wavelength inducing laccase production in *P. sanguineus* cultures. Even if laccase activities differed slightly among the light variants in *Cerrena unicolor*, green light enhances laccase production in *P. lindtneri* over four times comparing to dark experiment.

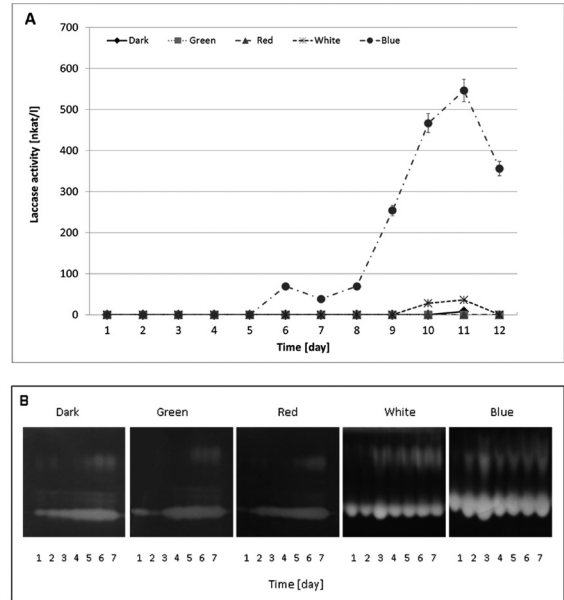
### Influence of light on cellobiose dehydrogenase activities

Production of CDH was analysed in cellulose-based medium, which was already used for production of cellobiose dehydrogenase by *Pycnoporus sanguineus* (Sulej *et al.*, 2013a) and *Phlebia lindtneri* (Sulej *et al.*, 2013b). It may be clearly observed that cellobiose dehydrogenase produced by the analysed fungal strains was induced by different light wavelengths. The highest activities of CDH in cul-



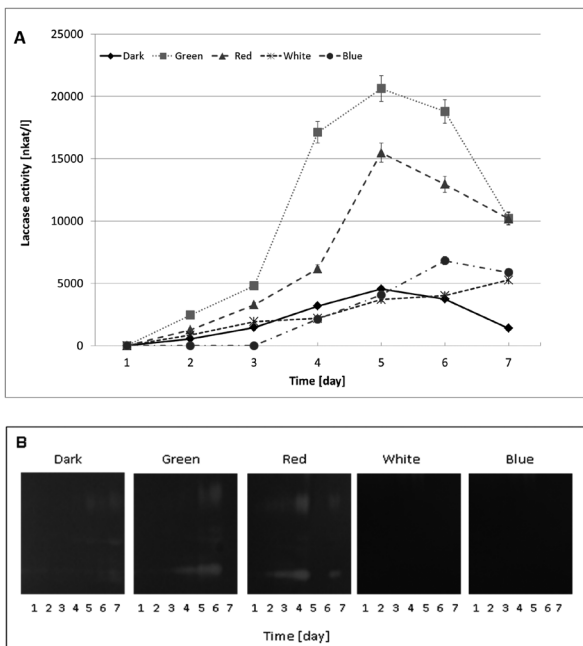
**Figure 1.** *C. unicolor* grown in Lindeberg and Holm medium under white, green, blue, red lights and in the dark. (A) laccase activity. Proteolytic activities (B) were analyzed by gelatin zymography of the extracellular media. 6 µl of samples (5 µg of protein) were loaded on a polyacrylamide gel containing 0.3% of gelatin. After electrophoresis, proteins were renaturated in the gel and the proteolytic activity was revealed after a 16-h incubation required for gelatin degradation

tures of *C. unicolor* (Fig. 4) and *P. lindtneri* (Fig. 5) where observed when green light was applied. Whereas *P. sanguineus* (Fig. 6) only under white light induction was able

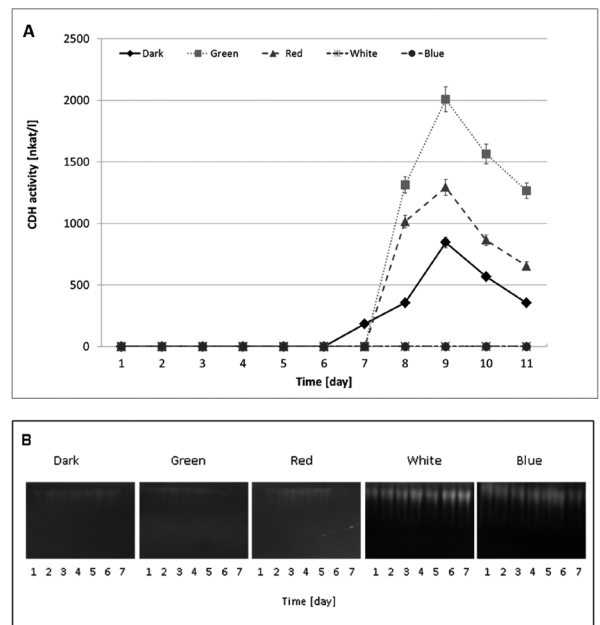


**Figure 2.** *P. sanguineus* grown in Lindeberg and Holm medium under white, green, blue, red lights and in the dark. (A) laccase activity. Proteolytic activities (B) were analyzed by gelatin zymography of the extracellular media. 6 µl of samples (5 µg of protein) were loaded on a polyacrylamide gel containing 0.3% of gelatin. After electrophoresis, proteins were renaturated in the gel and the proteolytic activity was revealed after a 16-h incubation required for gelatin degradation

to synthesize this enzyme efficiently. It is worth noting that green light stimulated CDH synthesis with an al-

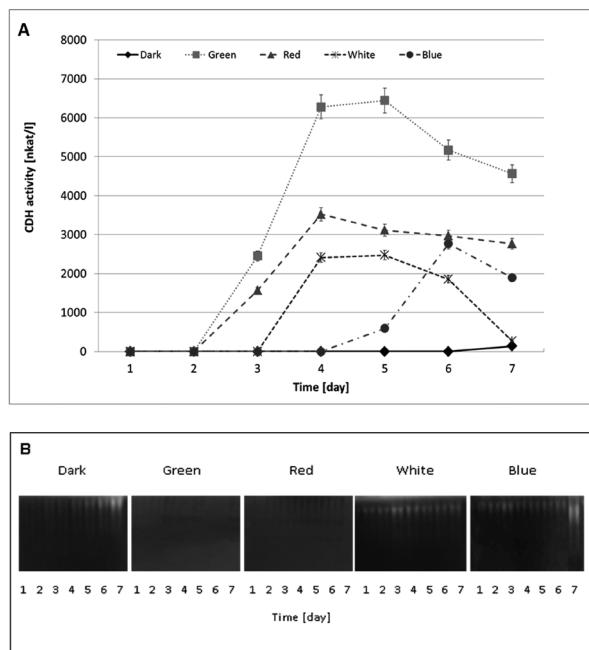


**Figure 3.** *P. lindtneri* grown in Lindeberg and Holm medium under white, green, blue, red lights and in the dark. (A) laccase activity. Proteolytic activities (B) were analyzed by gelatin zymography of the extracellular media. 6 µl of samples (5 µg of protein) were loaded on a polyacrylamide gel containing 0.3% of gelatin. After electrophoresis, proteins were renaturated in the gel and the proteolytic activity was revealed after a 16-h incubation required for gelatin degradation



**Figure 4.** *C. unicolor* grown in cellulose-based medium under white, green, blue, red lights and in the dark. (A) CDH activity. Proteolytic activities (B) were analyzed by gelatin zymography of the extracellular media. 6 µl of samples (5 µg of protein) were loaded on a polyacrylamide gel containing 0.3% of gelatin. After electrophoresis, proteins were renaturated in the gel and the proteolytic activity was revealed after a 16-h incubation required for gelatin degradation





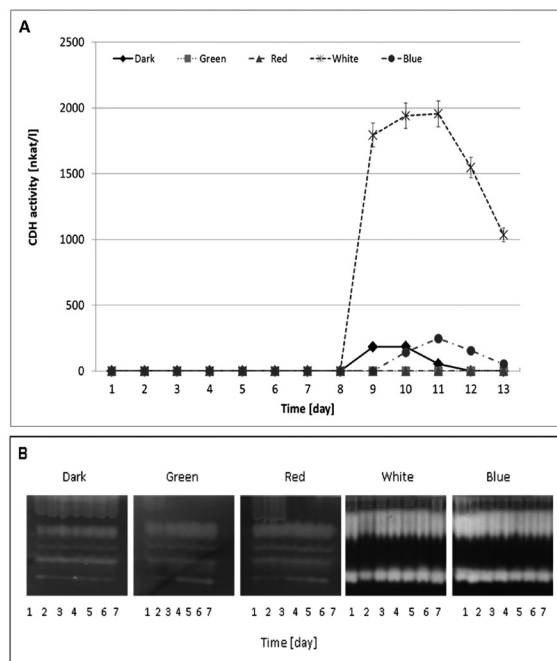
**Figure 5.** *P. lindtneri* grown in cellulose-based medium under white, green, blue, red lights and in the dark.

(A) CDH activity. Proteolytic activities (B) were analyzed by gelatin zymography of the extracellular media. 6  $\mu$ l of samples (5  $\mu$ g of protein) were loaded on a polyacrylamide gel containing 0.3% of gelatin. After electrophoresis, proteins were renatured in the gel and the proteolytic activity was revealed after a 16-h incubation required for gelatin degradation.

most two-fold (*C. unicolor*) and over six-fold (*P. lindtneri*) higher efficiency comparing to dark experiments.

#### Protease activities dependence on light

In order to analyse a possible influence of proteases on laccase and CDH activities, their synthesis was compared in both medium variants mentioned above under all the light wavelengths. It is clearly visible that the differences are noticeable not only between the light variants but also among the media used. Moreover, analysis of protease activities is complicated by the number of groups that are synthesized by these fungal strains. *Phlebia lindtneri* tend to produce a few proteases and those with low migration are rather observed in cellulose based medium (Fig. 5) or in Lindeberg and Holm medium (Fig. 3) induced by dark, light, or blue conditions. The electrophoretic analysis of *C. unicolor* proteases proved that this strain was able to secrete only a slow migrating fraction whose high activities may be observed mainly in the dark (Lindeberg and Holm medium — Fig. 1) or blue and white light (cellulose medium — Fig. 4). The most complicated picture is presented in the case of *P. sanguineus*, which produced even up to five protease fractions. The main difference is once again the slow migrating fraction of proteases, which is almost absent in Lindeberg and Holm medium (Fig. 2) in all lighting conditions except darkness and the last days of culture in white light. It should be pointed out that the same fractions tend to disappear in cellulose medium induced by green (day 3 of culture) or red light (4 day of culture) (Fig. 6). Moreover, the intensity of bands differs among mediums and it seems that the cellulose-based medium is better to obtain higher activities of slower migrating proteases than Lindeberg and Holm medium, which proved to be inducing faster migrating ones.



**Figure 6.** *P. sanguineus* grown in cellulose-based medium under white, green, blue, red lights and in the dark.

(A) CDH activity. Proteolytic activities (B) were analyzed by gelatin zymography of the extracellular media. 6  $\mu$ l of samples (5  $\mu$ g of protein) were loaded on a polyacrylamide gel containing 0.3% of gelatin. After electrophoresis, proteins were renatured in the gel and the proteolytic activity was revealed after a 16-h incubation required for gelatin degradation.

#### DISCUSSION

Lighting conditions exert an impact on life on earth comparably to water or oxygen. For many years it was believed that fungal organism are poorly influenced by lighting conditions in comparison to plants. Thus, it may be surprising to what extent fungal metabolism and development is based not only on presence or absence of light but also on its length (Tisch & Schmoll, 2010). Recently the explosion of papers considering light influence on *Ascomycetes* metabolism is observed. Until now, most papers analysing this subject are focussed on species belonging to the phylum *Ascomycetes*. At the same time, the phylum *Basidiomycetes*, which is equally advanced metabolically, is hardly examined in terms of the influence of light. However, in recent years, molecular techniques have been increasingly used to localize homologues of photosensory proteins in some basidiomycetous genomes (Idnurm *et al.*, 2010). Among them, a number of species engaged in wood decomposition (*Phanerochaete chrysosporium* or *Lentinula edodes*) may be found as parasites or saprophytes living mainly in forests habitats. In the available genomes of *Phlebia brevispora* and *Cerrena unicolor*, photoreceptor proteins: phytochromes or PAS (<http://genome.jgi-psf.org/>) may be found. It is well known that life in forests depends not only on light, due to the sunlight angle, changing seasons or time of the day, but also on shadows cast by taller organisms. Therefore, it seems natural that life of wood decomposing fungi may be to some extent influenced by lighting conditions. Moreover, many papers have proved the influence of light on metabolism of moulds comprising a number of enzymes engaged in amino acid or carbohydrate metabolism (including cellulases) (Bayram *et al.*, 2008; Fox & Howlett, 2008; Tisch & Schmoll, 2010). As indicated by the re-

sults presented above, wood parasites/saprophytes face the problem of degradation of the complex biopolymer – lignocellulose, which is successfully undertaken by engaging a number of enzymes decomposing carbohydrates or proteins. It seems obvious that their synthesis may be somehow regulated by light; therefore, we have examined the ability of three species to produce proteases, cellobiose dehydrogenase (an enzyme that together with lytic polysaccharide monoxygenase starts cellulose degradation) and, finally, laccase — the best described lignin-degrading enzyme. Moreover, the activity of extracellular proteases may also regulate the levels of CDH and laccase, which cooperate and to some extent are dependent on each other in lignocellulose decomposition (Henriksson *et al.*, 2000). Until now, only Ramirez *et al.* (2010) have found green light to boost lignin peroxidase production in *Phanerochaete chrysosporium*. Similarly, our results showed that green light stimulated *P. lindtneri* laccase and CDH produced by *P. lindtneri* and *C. unicolor*. It should be mentioned that any induction of the synthesis of both enzymes may be altered by simultaneous regulation by presence/absence of medium components (carbon or nitrogen source). Similar results were obtained by Schmoll *et al.* (2005), who proved that light must be an additional external factor influencing cellulase expression in *Hypocrea jecorina* beside the enzyme synthesis inducer (cellulose). Therefore, production of the cellulose degrading enzyme — cellobiose dehydrogenase by the species used should be analysed in terms of the presence of cellulose in culture medium. In recent years, light was proved to regulate the levels of cAMP in *N. crassa* via WWC (white collar complex); at the same time, cAMP is responsible for regulation of expression of a number of enzymes engaged in lignocellulose degradation (Chen *et al.*, 2009b; Smith *et al.*, 2010; Schmoll *et al.*, 2012; Janusz *et al.*, 2013). Moreover, blue light is responsible for increased levels of cAMP-dependent kinase in cultures of *Trichoderma atroviride* (Casas-Flores *et al.*, 2006). It should be mentioned that CRE sites were found in promoter regions of enzymes engaged in wood degradation. They are responsible for reaction to carbon repression through cAMP and moreover it was already proved that WCC in *N. crassa* acts on *cre1* which also regulates expression of cellulases and hemicellulases (Chen *et al.*, 2009b; Janusz *et al.*, 2013). It seems light may regulate enzyme expression by controlling cAMP levels and through carbon catabolite repressor protein. WCC in fungi is responsible for reaction to blue light exposure; therefore, it helps understand the enhanced laccase activities in cultures of *C. unicolor* and *P. sanguineus* illuminated by blue wavelength. Moreover, laccase is considered as a stress-response enzyme in fungi and 39 out of 178 light-regulated genes in *Trichoderma* are related to the response to stress factors (Schmoll *et al.*, 2010; Yang *et al.*, 2012). Both laccase and cellobiose dehydrogenase as extracellular proteins may undergo maturation and therefore their activity is dependent on the presence/absence of proteases (Habu *et al.*, 1993; Palmieri *et al.*, 2000; Staszczak *et al.*, 2000). Considering the results obtained by Mayer (2006), who described changes in transport and protease dependent maturation of tomato polyphenol oxidase influenced by light, we examined changes in protease activities in both described mediums. The results obtained showed changes in not only laccase or cellobiose dehydrogenase activities but also protease activities, which are correlated to each other to some extent. The differences in light wavelengths that induce certain enzymes may be dependent on the kind of photosensory proteins expressed in the fungal organism. Analysis of fungal genomes indi-

cated not only quantitative differences but also changes in the gene copy number from one up to five. Apparently, as a result of the differences in the complexity of fungal response to light, still unknown photosensory proteins are suspected to exist (Idnurm *et al.*, 2010). In the light of the presented results, there are opportunities to use light to obtain high activities of biotechnologically interesting enzymes without the need to use chemical inducers, particularly in the case of laccase. Beside the biotechnological value of the presented results, the studies on the dependence of wood decay fungi on light should be analysed in detail in future, because it seems that light may be more crucial in the life of white rot fungi than it was suspected.

## FIGURE LEGENDS

### Acknowledgements

This work was financially supported by the National Science Centre (Poland) based on the decision number DEC-2013/09/B/NZ9/01829.

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