DECOLORIZATION OF POST-INDUSTRIAL LIGNIN
BY MUTANTS OF Bjerkandera adusta CCBAS 930
WITH ENHANCED LIGNINOLYTIC ACTIVITY

DEKOLORYZACJA LIGNINY POPRZEMYSŁOWEJ
PRZEZ MUTANTY Bjerkandera adusta CCBAS 930
O ZWIĘKSZONEJ AKTYWNOŚCI LIGNINOLITYCZNEJ

Abstract: The paper describes the mutagenesis of the anamorphic fungus Bjerkandera adusta CCBAS 930 capable of decolorization and biodegradation of post-production lignin with participation of extracellular peroxidase. Seven mutants were isolated, out which 6 were characterized by shortened time of decolorization of model solutions of post-industrial lignin, from 21 to 7 or 14 days. The effect was caused by faster decomposition of the lignin polymer, as measured by decrease in the content of total phenols and of metoxyphenols. The process of biodegradation of lignin was catalyzed by an extracellular peroxidase that was not fully characterized. Two of the mutants studied, R59-2 and R59-5, were characterized by increased biosynthesis of that enzyme, coupled with accelerated transition of the fungus from the trophophase (primary metabolism) to the idiophase (secondary metabolism). This was accompanied by a drop in pH of the substrate that was more pronounced compared with the parental strain and to the other mutants.

Keywords: decolorization, lignin, Bjerkandera adusta, mutants, peroxidases
Methods used so far for the decolorization of sewage containing post-industrial lignin are mainly of physicochemical character and involve adsorption with the use of activated carbon [4] and coagulation of lignin in the pulp and in paper industry sewage [5]. Those methods lead only to the transition of lignin from a water-soluble form to a solid form, and are considered to be costly and of low efficiency [6]. Decolorization of lignin-containing sewage with the method of chlorination, on the other hand, is conducive to the formation of colorless but resistant to biodegradation and strongly toxic and mutagenic chlorinolignins and other chlorinoaromatic complexes, including carcinogenic dioxins [1, 3]. The application of toxic chlorine can be limited through the use, for the purpose of decolorization of lignin-rich sewage, of selected effective microorganisms, mainly white rot fungi and ligninolytic enzymes - laccase and peroxidases [2, 7]. Until now, the greatest attention has been paid to the ligninolytic capabilities of such species of wood white rot fungi (Basidiomycota) as Phanaerochaete chrysosporium, Trametes (Coriolus) versicolor and Pleurotus ostreatus [6, 8-10]. Less researched in this respect are white rot fungi representing the genus Bjerkandera [11,12]. Our own studies [13] indicate that in stationary cultures, the anamorphic strain Bjerkandera adusta CCBAS 930, isolated from soil, decolorizes 0.2% solutions of lignin originating from the process of wood pulp pulping (alkaline fraction I). The process of decolorization was of enzymatic character and was dependent on the presence of extracellular peroxidase. Decolorization of lignin by the fungal strain Bjerkandera adusta CCBAS 930 was coupled with the production of aerial mycelium, which corresponded with the secondary metabolism of that fungus. Its maximum, observable as complete brightening of the substrate, became evident after 25 days of culturing [13].

The objective of the study presented here was an attempt at increasing the efficiency of post-industrial lignin decolorization by the above-mentioned strain of Bjerkandera adusta through mutagenesis. The criteria applied in the estimation of the decolorization activity of the mutants included the rate of decolorization of post-industrial lignin, changes in the total content of phenolic and methoxyphenolic substances (CH₃O-phenols), and the activity of extracellular peroxidase as compared with the parental strain.

Material and methods

Fungus strain

The anamorphic strain Bjerkandera adusta CCBAS 930 was isolated from samples of a soil (Phaeozems acc. FAO) collected in the region of south-east Poland. The isolation, identification and morphological and taxonomic characterization of the fungus have been described in the paper by Kornillowicz-Kowalska et al [14].

Lignin

Lignin (LG) precipitated from the first alkaline fraction (from wood extract) by acidification with H₂SO₄ was obtained from InterCell S.A. Ostroleka (Poland). Before
usage the lignin was dissolved in sterile 0.1 M NaOH and adjusted to pH = 7 with 0.2 M HCl. Lignin contained (g · kg⁻¹ d.m.) 408.2 of carbon, 40.4 of hydrogen, 0.2 of nitrogen and no ashes.

Isolation and selection of mutants of B. adusta CCBAS 930

Induction of mutants of B. adusta CCBAS 930 was conducted with the use of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and UV radiation in accordance with the method of Kornillowicz-Kowalska and Iglik [15]. Selection of mutants was performed in the test of decolorization of 0.2% lignin in the Park and Robinson substrate [16]: 0.07% glucose, 0.05% MgSO₄, 0.02% KH₂PO₄, 0.01% NH₄NO₃, agar 2%, pH 6.5, after inoculation of plates (ø = 9 cm) with a circle of mycelium (ø = 0.5 cm) obtained from 7-day culture on glucose-potato agar (20% potato, 2% glucose, 2% agar). The alkaline lignin concentration applied corresponded to the content of that waste as encountered in the first fraction of wood pulp pulping with lye, called the black liquor. The culture was incubated at 26°C, measuring the decolorization zone after 3, 7, 10 and 14 days. Mutants that decolorized agarized lignin faster than the parental strain were selected for further experiments.

Culture conditions

Liquid cultures of the parental strain and of the selected mutants were set up in 100 cm³ Erlenmayer flasks with 50 cm³ of mineral medium for ligninolytic fungi [17] enriched with 0.2% of lignin and 0.25% of glucose. The inoculum were 3 circles of mycelium, 1 cm in diameter, obtained from 7-day culture on glucose-potato agar. Non-inoculated medium was used as control. The cultures and the control medium were incubated at 26°C for 30 days under static conditions, with 3 parallel replications.

Analytical methods

Clear post-culture fluids, obtained after straining of mycelium and centrifuging at 3000 revs min⁻¹ for 5 minutes, were used to determine the following:

- Degree of decolorization of lignin (LG) at λ = 430 nm (maximum of absorbance), calculated from the formula [18]:
  \[
  \text{% decolorization} = \frac{A_{\lambda i} - A_{\lambda f}}{A_{\lambda i}} \times 100
  \]
  where: \% decolorization = degree of decolorization in %, \( A_{\lambda i} \) - initial absorbance, \( A_{\lambda f} \) - final absorbance.

- Content of phenolic (λ = 400 nm) and metoxyphenolic (λ = 500 nm) substances, with the method of Malarczyk [19], using standard curves prepared for protocatechuic acid and vanillic acid, respectively.

- Peroxidase activity, according to Maehly and Chance, with \( o \)-dianisidine as substrate [20].

- Laccase activity, according to Leonowicz and Grzywnowicz [21], using syringaldasine as substrate.

- Protein content in the substrate, with the method of Lowry [22] with beef albumin as protein standard.

- pH of medium.
Culture observations

Microscope observations of mycelium growth and development and of changes in the substrate comprised the morphology of the vegetative mycelium, formation of spore-producing aerial mycelium, substrate color changes, and mycelium color changes.

Evaluation of results

All results obtained are given as means from 3 replications for which standard deviations were calculated.

Results

Selection of mutants with enhanced post-industrial lignin decolorization activity

UV irradiation (UV-C, $\lambda = 200÷280$ nm) and treatment with nitrosoguanidine (MNNG) (0.01%, 5 min) of $10^5$ cfu $\cdot$ cm$^{-1}$ B. adusta CCBAS930 resulted in the survival of $44 \cdot 10^1$ cfu after treatment with MNNG and $0.6 \cdot 10^1$ cfu after ultraviolet irradiation. This indicated greater survivability of the fungus under study in the presence of MNNG than under UV irradiation, at 0.04% and 0.006%, respectively. Among the 50 colonies grown, seven were characterized by notable structural changes, consisting in “felting” of the mycelium, which produced colonies more flattened and compacted compared with the parental strain which grows in the form ofuffy colonies, with loose and wooly aerial mycelium.

Subsequent post-mutation selection took into account the rate of decolorization of post-industrial lignin (alkaline lignin) in cultures on Park and Robinson solid medium containing 0.2% of that substrate. That selection revealed that only three (R59-5, R59-9 and R59-14) out of seven morphological mutants decolorized lignin faster than the parental strain (Table 1). A characteristic phenomenon is that initially (cultures 1-3 days old) the clones mentioned above caused an increase in the intensity of coloring of the substrate (dark brown), and only afterwards its brightening. The most active decolorizer of lignin in the solid substrate was mutant R59-5 which, after 7 days, brightened ca 80% of surface area of substrate with 0.2% of alkaline lignin (Table 1).

<table>
<thead>
<tr>
<th>Strains</th>
<th>Days of culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>CCBAS 930 (parent strain)</td>
<td>$10^4$ / 0**</td>
</tr>
<tr>
<td>R59-2</td>
<td>35 / 0</td>
</tr>
<tr>
<td>R59-5</td>
<td>35 / 0</td>
</tr>
<tr>
<td>R59-9</td>
<td>20 / 0¹</td>
</tr>
<tr>
<td>R59-14</td>
<td>40 / 0¹</td>
</tr>
<tr>
<td>R59-28</td>
<td>0 / 0</td>
</tr>
<tr>
<td>R59-29</td>
<td>15 / 0</td>
</tr>
<tr>
<td>R59-30</td>
<td>20 / 0</td>
</tr>
</tbody>
</table>

Explanations: * - growth diameter in mm; ** - diameter of decolorization zone [mm]; ¹ - substrate darkening in growth zone of 15.10 and 10 mm; plate diameter - 9 cm
Decolorization of lignin by mutants of \textit{B. adusta} CCBAS 930 in liquid cultures

Among the 7 mutants tested, only one (R59-30) decolorized 0.2% solution of post-industrial lignin at a rate similar to that of the parental strain (Fig. 1). The other mutants removed the coloring caused by the presence of that biopolymer at rates two- to three-fold faster than the parental strain. That effect became noticeable after 7-10 days of culturing, as 52\pm83\% (7 days) and 51\pm85\% (10 days) of substrate decolorization (measured by drop in absorbance), respectively. During the same time, the parental strain removed only 26\pm27\% of the coloring, and mutant R59-30 decolorized 17\pm34\% of lignin contained in the solution. Decolorization of solution containing 0.2\% of alkaline lignin proceeded the fastest in cultures of mutants R59-2 and R59-29. After 7 days of culturing, both of those clones removed 73\pm83\% of the coloring. Mutant R59-5, that decolorized lignin the fastest in the solid substrate, was less efficient here - after 7 days of growth it removed 52\% of the color.

![Fig. 1. Decolorization of 0.2% lignin by mutants \textit{B. adusta} CCBAS 930](image)

In culturing time of the mutants under study longer than 10 days, a recurring though less pronounced increase was observed in the substrate coloring as measured with increase of absorbance (Fig. 1). However, no biosorption of lignin was observed, either in the mutant cultures or in the non-mutated strain; their mycelia remained colorless (white).

Changes in the content of phenolics in the culture medium

In the course of decolorization of 0.2\% solutions of alkaline lignin in the cultures of the mutants and of the parental strain of \textit{B. adusta} CCBAS 930 the content of phenolic substances (\(\lambda = 400\) nm) was decreasing (Fig. 2A). In the mutant cultures that decrease was 2-3-fold stronger than in the cultures of the parental strain. The exception were mutants R59-28 and R59-30 which displayed low effectiveness in the removal of phenolics (maximum 20\%) - similar to that of the control (parental strain). The reduction of the total level of phenols was the fastest between days 7 and 14 (18) of culturing. That effect was the most pronounced in the cultures of mutant R59-2: 47\% reduction of the concentration of phenolics after 7 days of growth. Whereas, the greatest decrease in the total phenolic
content was observed in the case of the culture of R59-29 - 53% after 14 days of growth of the strain. The slowest decrease in the level of phenolic substances was observed in the cultures of mutants R59-28 and R59-30, with decrease rates of 21% and 24%, respectively, after 18 days of culturing (Fig. 2A).

Initially, changes in the content of methoxyphenolics in the substrate had a run similar to that in the total content of phenols, but were more pronounced (Fig. 2A,B). The content of methoxyphenolic complexes decreased the fastest between days 7 and 14 (18). The most effective in this respect was the mutant R59-2 which, after 7 days, removed 58% of CH$_3$O-phenols, while the parental strain removed only 18%. Also relatively effective were mutants R59-29 and R59-5 - removal rates of 43% and 35%, respectively. The level of CH$_3$O-phenols-decreased the most slowly in cultures of the mutant R59-30 - 14% after 7 days (Fig. 2B).

Changes in peroxidase activity in the culture medium

Extracellular peroxidase activity of the parental strain of *B. adusta* CCBAS 930 and its 7 mutants became observable on the 3rd day of culturing on the substrate with 0.2% lignin and gradually increased from then on. The maximum of activity of that enzyme became evident in cultures of 10-14 (18) days old. However, the strain under study was notably varied in the level and dynamics of activity of that enzyme (Fig. 3). The highest levels of peroxidase activity were observed in cultures of mutants R59-2 and R59-5. The mean specific peroxidase activity in the cultures of those strains was 101.0 and 125.5 mU · mg$^{-1}$ of protein, respectively. Those values were 20-25-fold higher with relation to the peroxidase activity of the parental strain. Considerably lower values of activity of the phenoloxidase under study were observed in the case of mutants R59-29 and R59-30 (12.5 and 46.9 mU · mg$^{-1}$ of protein, respectively). Peroxidase activity of the remaining 3 clones, R59-9, R59-14 and R59-29, was at the same low level as in the culture of the parental strain (Fig. 3).
Decolorization of post-industrial lignin by mutants of *Bjerkandera adusta* CCBAS 930 …

Laccase activity

In the experimental design adopted in this study neither the parental strain nor its mutants produced any laccase.

Increase and changes in pH of the culture fluids

Under the conditions of stationary culture on substrate with 0.2% lignin, mutants of *B. adusta* CCBAS 930 were characterized by faster rate of growth and by the production of aerial mycelium, as compared with the parental strain. The process of aerial mycelium production (reflecting the transition of the fungus to the phase of secondary metabolism) in the mutant cultures began on the 7th day, while in the cultures of the parental strain on the 10th day of culturing (Tab. 2).

Table 2

<table>
<thead>
<tr>
<th>Strains</th>
<th>Days of culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>CCBAS 930 (parent strain)</td>
<td>- / -</td>
</tr>
<tr>
<td>R59-2</td>
<td>- / -</td>
</tr>
<tr>
<td>R59-5</td>
<td>+ / -</td>
</tr>
<tr>
<td>R59-9</td>
<td>+ / -</td>
</tr>
<tr>
<td>R59-14</td>
<td>- / -</td>
</tr>
<tr>
<td>R59-28</td>
<td>- / -</td>
</tr>
<tr>
<td>R59-29</td>
<td>+ / -</td>
</tr>
<tr>
<td>R59-30</td>
<td>- / -</td>
</tr>
</tbody>
</table>

Explanations: - no growth; + mycelium growth 20%; ++ mycelium growth 50%; +++ mycelium growth 80÷100%; + / + ratio of vegetative mycelium to aerial mycelium

Fig. 3. Peroxidase activity in culture fluids of mutants of *B. adusta* CCBAS 930 containing 0.2% of lignin

![Peroxidase activity in culture fluids of mutants of *B. adusta* CCBAS 930 containing 0.2% of lignin](image)

![Graph showing peroxidase activity over time](image)
This was accompanied by visually observable gradual brightening of the substrate that progressed with the passage of the time of culturing. In the mutant cultures, its maximum was observed on the 14th day. Within a longer time-frame, a slight re-colorization of substrate was observed. Whereas, in the cultures of the parental strain, visually observable brightening of substrate was apparent as late as on the 21st day of culturing (Table 2).

In the course of the growth of the parental strain and the mutants of *B. adusta* CCBAS 930, pH of the substrate (6.33±6.40) decreased by ~0.7±1.3 units, attaining values of 5.02±5.59. The process of acidification was the fastest within the first week of culturing, which should be attributed to assimilation of glucose. Beginning with day 10 in the mutant cultures, and day 14 in the parental strain cultures, a gradual re-increase in pH values was observed, most pronounced in the case of the parental strain and of the mutant R59-14. The weakest re-increase of pH was noted in cultures of mutants R59-2 and R59-5, by as little as 0.15±0.29 of a unit after 18 days of culturing (Fig. 4).

![Fig. 4. Changes of pH in culture fluids of mutants of B. adusta CCBAS 930 containing 0.2% of lignin](image)

**Discussion**

Decolorization of lignin solutions in stationary cultures of *Bjerkandera adusta* CCBAS 930 is an enzymatic process catalyzed by a non-fully characterized extracellular peroxidase of that fungus [13]. That extracellular peroxidase of *B. adusta* CCBAS 930 is also responsible for biodegradation and, in consequence, for decolorization of daunomycin (an anthracyclin antibiotic) and of pigments with aromatic structure, including anthraquinonic pigments [14, 15]. That enzyme is an enzyme of the secondary metabolism of *B. adusta* CCBAS 930, and the processes of decolorization and of biodegradation of lignin and of related compounds (humic acids, daunomycin, anthraquinonic pigments) are of cometabolic character, as they require the presence of an easily available source of carbon, such as glucose [14, 15].

In the present study, out of the 50 morphological mutants isolated after mutagenesis of homogenate of mycelium of *B. adusta* CCBAS 930 (1 x 10^5 cfu · cm⁻³) under the effect of UV and nitrosequanisidine (MNNG) treatment, 7 mutants were selected that had increased capacity for decolorization of post-industrial lignin (first alkaline fraction). UV exerted a stronger lethal effect compared to MNNG - out of the 50 clones isolated only 6 were
Decolorization of post-industrial lignin by mutants of *Bjerkandera adusta* CCBAS 930…

obtained after UV irradiation. Stronger fungicidal effect of UV radiation was also observed by Miura et al [23] during induction of mutants of non-sporification stage of white rot basidiomycete (des. as IZU-154) with increased ligninolytic activity. Out of the seven selected mutants of *B. adusta* CCBAS 930 (1 after UV mutagenesis, 5 after MNNG mutagenesis), two: R59-2 and R59-5 (both after treatment with MNNG) were characterized, on average, by 20-25x greater peroxidase activity compared with the parental strain. Stimulation of peroxidase activity (manganese-dependent peroxidase MnP) of mutants of non-sporifying fungus IZU-154 after mutagenesis of its ancestors with UV radiation and with MNNG has been reported earlier by Miura et al [23] who applied their capacity to decolorize synthetic melanin as the criterion of selection. Higher ligninolytic activity of mutants (UV induction) compared with the parental strain of the white rot fungus *Phanerochaete chrysosporium* was also observed by Kakar et al [24]. Those authors applied decolorization of chromatophores from the group Poly-R (polymeric pigments) as the criterion for the estimation of ligninolytic activity.

In our study, using the degree of decolorization of alkaline lignin as the index of ligninolytic activity of *B. adusta* CCBAS 930, we observed similar relationships. The selected mutants of the fungus decolorized water solutions of post-industrial lignin faster 3x compared with the parental strain. After 7 days of growth, the most efficient clones - R59-2, R59-5 and R59-29 - caused color reduction by 73%, 52% and 83%, respectively, while the parental strain rate of decolorization was 26%.

Our earlier study [13] showed that decolorization of alkaline lignin in stationary cultures of *B. adusta* CCBAS 930 was caused by biodegradation of that polymer, which was accompanied by a reduction in the level of total phenolics and of methoxyphenolic in the substrate. The decrease in the content of methoxyphenolic indicated demethylation of the lignin polymer under the effect of peroxidase [13]. Whereas, in cultures of *B. adusta* CCBAS 930 containing lignin no presence of laccase was detected. Laccase, like peroxidase, can catalyze the reaction of demethylation [25]. Also mutants of *B. adusta* CCBAS 930 did not synthesize laccase in stationary cultures containing lignin. Mutants (but not the parental strain) of *B. adusta* CCBAS 930, on the other hand, synthesized laccase in cultures containing synthetic dyes: carminic acid, erythrosine, and brilliant green, which was reported in the work by Kornillowicz-Kowalska and Iglik [15]. The process of decolorization of lignin in mutant cultures, as in cultures of the parental strain, proceeded along the cometabolic path, as indicated by its dependence on the presence of glucose [13]. Whereas, the mutants did not decolorize 0.2% solutions of lignin under conditions of absence of glucose (unpublished data). It was found, however, that the selected mutants R59-2 and R59-5, in cultures containing lignin and glucose, were characterized by faster growth of vegetative mycelium (trophophase) and transition to idiophase (secondary metabolism) compared with the parental strain. This involved faster absorption of glucose, causing increased acidification. The glucose metabolism in the parental strain cultures was notably weaker [13].

The study reported herein indicates that MNNG mutagenesis, increasing the biosynthesis of extracellular peroxidase, accelerates the process of biodegradation of lignin. This was indicated by stronger than in parental strain cultures decrease in the content of phenolic substances, which caused faster decolorization of solutions of post-industrial lignin. The production of peroxidase by the parental strain and the mutants began on the 3rd day, but only in the case of the mutants R59-2 and R59-5 it intensified rapidly and remained
at a considerable level throughout the period of culturing. The observed dynamics of peroxidase production in the cultures of mutants R59-2 and R59-5 was similar to that of peroxidase activity (lignin peroxidase, LiP) in cultures of mutants obtained after UV mutagenesis of conidia of \textit{P. chrysosporium}, observed by Kakar et al \[24\].

The results of this study, therefore, indicate a possibility of improving the efficiency of decolorization and biodegradation of post-industrial lignin by the strain \textit{B. adusta} CCBAS 930 through mutagenesis, as it leads to stimulation of growth and to biosynthesis of peroxidase catalyzing that process.

References


DEKOLORYZACJA LIGNINY POPRZEMYSŁOWEJ PRZEZ MUTANTY Bjerkandera adusta CCBAS 930 O ZWIĘKSZONEJ AKTYWNOŚCI LIGNINOLITYCZNEJ

Pracownia Mikologiczna, Katedra Mikrobiologii Rolniczej, Uniwersytet Przyrodniczy, Lublin


Słowa kluczowe: dekoloryzacja, lignina, Bjerkandera adusta, mutanty, peroksydazy