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EXPERIMENTAL ARTICLES

Ligninolytic Activity of Bacteria of the Genera *Azospirillum* and *Niveispirillum*

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Abstract—Capacity of associative soil bacteria of the genera *Azospirillum* and *Niveispirillum* for degradation of lignin model compounds was demonstrated. Lignin and Mn peroxidases were detected in the culture liquid of the type strains of these genera. The data on involvement of nonspecific bacterial peroxidases in lignin degradation were obtained.

Keywords: Azospirillum, Niveispirillum, lignolytic activity, lignin degradation, Mn peroxidase, lignin peroxidase

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Lignin is a complex polymer consisting of monomeric phenylpropane units resistant to the impact of microorganisms. Until recently, only basidiomycetous fungi were shown to be capable of oxidative destruction of lignin. However, the ability to degrade lignin has been shown recently for some representatives of soil bacteria: *Pseudomonas, Rhodococcus,* and *Serratia* (Bugg et al., 2011). The enzymes analogous to fungal ligninolytic enzymes are supposed to play the key role in the enzymology of bacterial degradation of lignin-like substances (Ahmad et al., 2010). The major enzymes of basidiomycetes involved in oxidative destruction of lignin are Mn peroxidase and lignin peroxidase.

Prior to the onset of our research there had been absolutely no literature data on the ability of associative bacteria from the genera *Azospirillum* and *Niveispirillum* to depolymerize lignin compounds. In our previous works we demonstrated that these bacteria can produce a complex of phenol-oxidizing enzymes including, together with other enzymes, Mn and lignin peroxidases (Nikitina et al., 2010).

The aim of this study was to reveal the ability of azospirilla and niveispirilla to degrade lignin and the involvement of Mn and lignin peroxidases in this process.

MATERIALS AND METHODS

Organisms and cultivation conditions. The following bacterial strains were used in the work: *Azospirillum brasilense* Sp7, *A. brasilense* SR 80, *A. brasilense* Sp245, *A. brasilense* Sp107, *A. picis* TAR-3, *A. lipoferum* Sp59b, *A. tiophilum* Bv-S, *Niveispirillum irakense* KBC-1, and *N. irakense* KA-3 from the collection of microorganisms of the Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences. The bacteria were cultivated in Erlenmeyer flasks (250 mL) in a liquid malate–salt medium containing the following (g/L): KH_2PO_4, 0.1; K_2HPO_4, 0.4; NaCl, 0.1; $Na_2MoO_4 \cdot 7H_2O$, 0.002; $MgSO_4 \cdot 7H_2O$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.02; malic acid, 5.0; NaOH, 1.7; NH_4Cl, 1.0; $CaCl_2$, 0.02; pH 6.8. The medium was sterilized for 30 min at 121°C. A 12-h culture grown in the same medium was used as inoculum. Bacteria were cultivated in a thermostat at 30°C.

Screening for the ligninolytic ability of azospirilla. Bacterial cells were sedimented for 15 min at 7000 *g* in a K-24 centrifuge (MLV, German Democratic Republic). The supernatant was used for further studies. The lignin-degrading ability of microorganisms was assayed by the method of Ahmad (Ahmad et al., 2010). The experiment was carried out using two model Klason lignin preparations obtained from native (not subjected to methanolysis) and methanolyzed oak sawdust kindly provided by researchers of the Laboratory of Biochemistry, Penza State Agricultural Academy (Penza, Russia). The lignin-degrading ability was determined by spectrophotometry in 96-well polystyrene plates. Optical density of the samples was measured with a Multiskan Ascent Microplate Reader; the results were processed using Ascent Software for Multiskan Ascent (Thermo Electron, Finland) at the Simbioz Center for Collective Use (CCU) of scientific equipment in the field of physicochemical biology and nanobiotechnology, Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences.

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The reaction mixture contained nitrated lignin solution, 160 μL; sample, 30 μL; and 40 mM H_2O_2, 10 μL. The total volume of the reaction mixture was 200 μL. The reaction was started by adding peroxide. Detection was performed at 414 nm for 20 min, with 1-min measurement intervals. As the control, 30 μL of synthetic medium was added to the reaction mixture instead of the culture liquid. The control values were subtracted from the experimental values.

Nitrated lignin preparation. Glacial acetic acid (1 mL) was added dropwise into 5 mg of the model lignin compound; the resultant suspension was mixed at room temperature and filtered to remove unsolved particles. Then 0.2 mL of concentrated nitric acid was added to the mixture. The reaction was carried out under continuous stirring for 1 h; then 2 mL of distilled water was added, and the mixture was titrated with 1 M NaOH to pH 7. The resultant solution (yellow orange) was diluted 100 times with 750 mM Tris–HCl buffer, pH 7.4, containing 50 mM NaCl.

Determination of enzymatic activity. The enzyme activities were determined by spectrophotometry with a Specord M 40 (Carl Zeiss, Germany) by the rate of 2,6-dimethoxyphenol oxidation ($\varepsilon = 30.5\ mM^{-1}\ cm^{-1}$) at 30°C for Mn peroxidase (Paszczynski et al., 1988) and by the rate of veratryl alcohol oxidation to veratrylaldehyde at 310 nm ($\varepsilon = 9.3\ mM^{-1}\ cm^{-1}$) for lignin peroxidase (Orth et al., 1993). The reaction was started by adding H_2O_2.

The amount of the enzyme catalyzing conversion of 1 μM substrate in 1 min was taken as a unit of activity. Specific activity was expressed in units per 1 mg of protein. Protein concentration was assayed by the Bradford method (Bradford, 1976).

Enzyme isolation and purification. Mn peroxidase was isolated from the culture liquid of *A. brasilense* Sp245 according to the scheme proposed previously (Kupryashina et al., 2012). Extracellular lignin peroxidase was isolated from a 24-h culture of *A. brasilense* Sp245 grown in a liquid malate–salt medium. The cells were precipitated by centrifugation; the supernatant was used for further purification including fractional precipitation of culture liquid proteins with ammonium sulfate; selective sorption of admixtures on the CaF_2 gel (based on the method proposed by Novakovsky; Novakovsky et al., 2006); gel filtration in a PD10 column (Sigma-Aldrich, Sweden) equilibrated with 0.05 M Tris–HCl buffer, pH 7.5; and ion exchange chromatography in a Toyopearl DEAE-650M column (Tosoh, Japan) equilibrated with the same buffer, with elution in a NaCl step gradient. The resultant enzyme preparations were dialyzed against water.

Determination of the lignin-degrading function of the enzymes. The lignin-degrading functions of lignin and Mn peroxidase preparations were detected by the method of Ahmad (Ahmad et al., 2010). As a control, 30 μL of distilled water was added to the reaction mixture instead of the enzyme solution.

RESULTS AND DISCUSSION

The process of lignin depolymerization by bacteria has been studied insufficiently. Recently, it has been hypothesized that the ability of some soil bacteria to oxidize aromatic compounds mediates their predisposition to the degradation of complex polyphenols including lignin (Bugg et al., 2011; Bholay et al., 2012). Based on the previously shown ability of azospirilla to oxidize compounds such as pyrocatechol and syringol (the components of lignin depolymerization) (Nikitina et al., 2010; Kupryashina et al., 2012), we supposed that these bacteria may have a ligninolytic activity and the ability to induce destructive changes in the complex polyphenol molecule of lignin. At the same time, we speculated that it would be logical to consider the species specificity of this property of azospirilla and niveispirilla.

In this work we studied epiphytic and endophytic representatives of the genera *Azospirillum* and *Niveispirillum*. At the first stage, the method of Ahmad was used for screening the ligninolytic activity of culture liquids of 9 strains: *A. brasilense* Sp245, *A. brasilense* Sp107, *N. irakense* KBC 1 (endophytic), *A. brasilense* Sp7, *A. brasilense* SR 80, *A. picis* TAR-3, *A. lipoferum* 59b, *A. tiophilum* Bv-S, and *N. irakense* KA 3 (epiphytic). It should be noted that the strains *N. irakense* KBC 1 and *N. irakense* KA 3 belonged to the genus *Azospirillum* but were reclassified in 2014 as *N. irakense* on the basis of taxonomic studies (Lin et al., 2014). The oxidation of lignin preparations is confirmed by the changes in optical density of the solution during 20 min. As it has been shown previously (Ahmad et al., 2010), the increase in optical density observed during this test is associated with the release of phenolic products formed during the degradation of nitrated lignin.

As Fig. 1 shows, all strains under study were more or less able to degrade model lignin compounds obtained from both methanolyzed and native sawdust. Methanolyzed sawdust is a modified substrate with the high content of methoxyl groups in the lignin molecule; as a result, the latter is less condensed (has a more loose structure) and contains more monomeric, dimeric, and oligomeric phenol derivatives. The methanolyzed lignin-containing substrates are known to be more liable to the degradation by xylotrophic basidiomycetes than native lignin (Ilyina, 2011). Many authors noted that bacterial depolymerization of lignin is a very slow process affecting mainly the lateral portions of the molecule, off the main chain (Yan and Yang, 2008). As Fig. 1 shows, the preparation from methanolyzed sawdust was oxidized by the bacteria to a greater extent, except for *A. brasilense* Sp245 and *N. irakense* KBC-1; at the same time, the optical density values were maximal for the strains *A. brasilense*

SR 80, *A. picis* TAR-3, and *A. lipoferum* Sp59b. The strains *A. thiophilum* Bv-S, *N. irakense* KBC-1, and *N. irakense* KA-3 had the lowest lignin-degrading potential against model lignin compounds obtained from both native and methanolyzed sawdust. It seems interesting that lignin degradation of the native preparation by strains *A. brasilense* Sp245 and *N. irakense* KBC-1 was more effective compared to the degradation of the methanolyzed preparation. This was most probably due to the ecological and physiological peculiarities of the studied bacteria. These strains are able to establish endophytic symbiosis with herbaceous plants, mainly cereals. The lignin molecules of herbaceous plants are known to be characterized by a low degree of methoxylation (Dalimova and Abduazimov, 1994). Most soil bacteria possessing the ligninolytic activity are capable of endophytic existence in plant tissues (Bholay et al., 2012). Therefore, it would be logical to assume that endophytic strains must have a higher lignin-degrading potential compared to associative strains, which results from their penetration capability. We have revealed no significant differences in the ability to degrade lignin between the groups of endophytic and epiphytic representatives of azospirilla and niveispirilla. However, it was shown that among the strains studied, only *A. brasilense* Sp245 had a reliably higher lignin-degrading potential against the complex molecule of native lignin than against the methanolyzed one. It should be noted that the strain *A. brasilense* Sp245 is a model for studying endophytic symbiosis, which can colonize root filaments and intercellular spaces of the conducting system of the root (Schloter and Hartmann, 1998).

Bugg et al. and Ahmad et al. used a similar method to test the lignin-degrading ability of the white and brown rot fungi, as well as of some soil bacteria (Bugg et al., 2011; Ahmad et al., 2010). Comparison of our results with the data from the cited works shows that the ligninolytic activities of azospirilla and niveispirilla were lower by several orders of magnitude than in the major lignin destructor, the basidiomycete *Phanerochaete chrysosporium*, but were of the same order as in the polypore fungus *Trametes versicolor* and some mycorrhizal fungi. Comparison of the lignin-degrading potentials of soil bacteria from the genera *Pseudomonas, Rhodococcus*, and *Acinetobacter* revealed that, unlike azospirilla and niveispirilla, representatives of these genera could not degrade wood lignin but were active only against the lignin from herbaceous plants.

At present, the following three extracellular enzymes are supposed to play the key role in lignin depolymerization by fungi: lignin peroxidase, Mn peroxidase and, to a lesser extent, laccase (Wong, 2009). Some laccase-producing fungi and bacteria are incapable of depolymerizing lignin-like compounds (Leisola et al., 2012). Rahman et al. have shown that the degradation of kraft lignin by *Bacillus* sp. SHC1, *Ochrobactrum* sp. SHC2 and *Leucobacter* sp. SHC3 is accompanied by the increase in Mn peroxidase activity (Rahman et al., 2013).

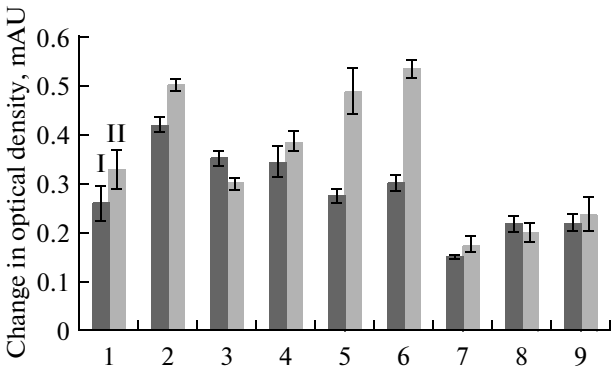

Fig. 1. The change in optical density from the 1st to 20th min during the degradation of model lignin preparations by the strains *A. brasilense* Sp7 (1); *A. brasilense* SR 80 (2); *A. brasilense* Sp245 (3); *A. brasilense* Sp107 (4); *A. picis* TAR-3 (5); *A. lipoferum* Sp59b (6); *A. tiophilum* (7); *N. irakense* KBC-1 (8); and *N. irakense* KA-3 (9). Preparation I is the Klason lignin; preparation II is the Klasson lignin with increased content of methyoxy groups.

When screening the ligninolytic activity, we noted that the absence of hydrogen peroxide in the reaction mixture resulted in a decreased difference in optical density, as was observed for most of the strains under study (data not shown). These results demonstrated decreased degradation of the lignin molecule. Hence, we supposed that ligninolytic peroxidases, rather than oxidases, played a more important role in the structural changes in lignin caused by azospirilla and niveispirilla.

The Mn and lignin peroxidase activities were screened to reveal a correlation between the ligninolytic activity of bacteria and the presence of extracellular nonspecific peroxidases (Figs. 2, 3). There are very few data on these enzymes of bacterial origin in the scientific literature. Our investigation revealed the presence of lignin and Mn peroxidases in the culture liquid of the type strains of bacteria from the genera *Azospirillum* and *Niveispirillum*. Figure 3 shows that the maximum specific activities of lignin peroxidase against all of the studied strains were recorded for the endophyte *A. brasilense* Sp245. At the same time, the activity of other two endophytic strains (*A. brasilense* Sp107 and *N. irakense* KBC-1) was lower by 50% than in Sp245. The Mn peroxidase activities were different in all endophytic strains under study by 5–10% (Fig. 3). Under these conditions, the maximal Mn peroxidase activity was demonstrated by the strains *A. lipoferum* Sp59b and *A. tiophilum*, while the minimal activity was demonstrated by *A. picis* TAR-3 and *N. irakense* KA-3.

Analysis of the data obtained demonstrated that the maximal production of the studied enzymes in experimental strains did not always correlate with the degree of lignin degradation. For example, the strain *A. brasilense* SR 80 had a high lignin-degrading poten-

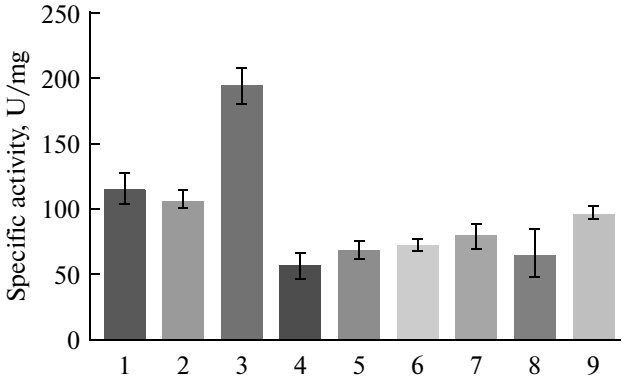

Fig. 2. The activity of Mn peroxidase in the culture liquid of the strains *A. brasilense* Sp7 (1); *A. brasilense* SR 80 (2); *A. brasilense* Sp245 (3); *A. brasilense* Sp107 (4); *A. picis* TAR-3 (5); *A. lipoferum* Sp59b (6); *A. tiophilum* (7); *N. irakense* KBC-1 (8); and *N. irakense* KA-3 (9).

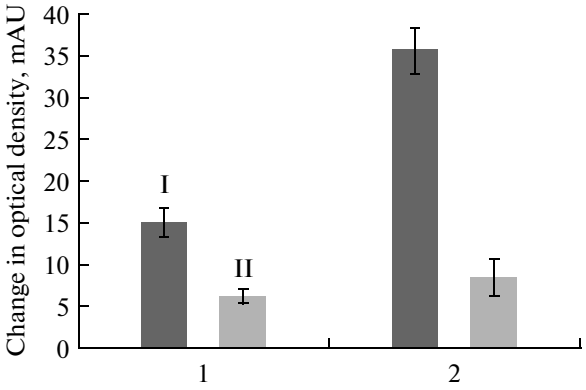

Fig. 4. Degradation of the model lignin compounds by electrophoretically homogeneous preparations of Mn peroxidase (1) and lignin peroxidase (2) from *A. brasilense* Sp245. Preparation I is the Klason lignin and preparation II is the Klason lignin with increased content of methoxy groups.

tial against both model lignin preparations; however, the values of enzymatic activity in this strain were average. At the same time, *A. brasilense* Sp245, which was more capable of degrading native lignin than methanolyzed lignin, had the highest lignin-peroxidase activity among all the studied strains. The maximal ability to degrade the modified lignin preparation in the strain *A. lipoferum* 59b correlated with the maximal activity of extracellular Mn peroxidase. However, no such regularity was shown for other strains.

The function of the major ligninolytic enzyme in the polyphenolic complex of fungi is attributed to lignin peroxidase, while Mn peroxidase is believed to play a key role in detoxification of the formed degradation products. However, in the scientific literature there are absolutely no data on the ability of lignin and Mn peroxidases of bacterial origin to degrade complex polyphenolic substances, including lignin.

It was necessary to isolate electrophoretically pure enzyme preparations in order to establish whether bacterial Mn and lignin peroxidases can degrade the model lignin compounds. Toward this end, extracellular lignin and Mn peroxidases were isolated from the culture liquid of the strain *A. brasilense* Sp245 and purified to the electrophoretically homogeneous state. The ligninolytic functions of the preparations were also determined by the method of Ahmad (Ahmad et al., 2010) using two model Klason lignin preparations (from native and methanolyzed sawdust). The data (Fig. 4) show that both of the isolated enzymes can oxidize the model lignin compounds. This fact is demonstrated by the change in optical density during 20 min, which is associated with the release of phenolic products of lignin degradation. The homogeneous lignin peroxidase preparation was more destructive for the model compounds taken for the experiment.

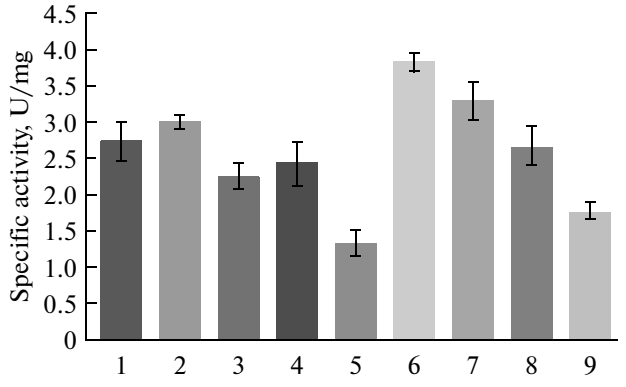

Fig. 3. The activity of lignin peroxidase in the culture liquid of the strains. Designations are as on Fig. 2.

The dynamics of oxidation of nitrated lignin preparations shows that peroxidases under study more actively oxidize the model compounds obtained from native sawdust. These results are in agreement with the previous data showing the more effective degradation of the lignin preparation from native sawdust by the strain *A. brasilense* Sp245 compared to the lignin from methanolyzed sawdust.

When degrading the Klason lignin from modified sawdust, both enzymes showed similar activities. However, the lignin-degrading potential of lignin peroxidase during the oxidation of the unmodified sample was higher by 42% compared to that of Mn peroxidase. It is most likely indicative of the ability of lignin peroxidase to degrade more complex polyphenolic structures. Thus, we have shown for the first time the ability of bacterial Mn and lignin peroxidases to degrade the model lignin compounds.

As a result of this study, the enzymatic nature of lignin degradation by bacteria was confirmed by the example of the strain *A. brasilense* Sp245. The involve-

ment of its own lignin and Mn peroxidases in this process was shown. As follows from the data obtained, the ability of azospirilla and niveispirilla to produce extracellular nonspecific peroxidases is universal. The fact that these enzymes are found in both endophytic and epiphytic representatives of bacterial nitrogen fixers suggests that the action of these enzymes is associated not only with the penetration capability of the strains but also, most likely, with overcoming the phenolic barrier appearing in the rhizosphere, which is mediated by the secondary metabolites of the plants, as well as by lignin-like compounds and substances formed during their degradation. The physiological role of these enzymes needs further investigation.

The biotechnologies for lignin degradation have been actively developed worldwide during the last 20 years and, hence, our results are of considerable practical value and the study of lignin degradation by bacteria may open up new prospects in lignin bioconversion.

REFERENCES

Ahmad, M., Roberts, J.N., Hardiman, E.M., Singh, R., Eltis, L.D., and Bugg, T.D.H., Identification of DypB from *Rhodococcus jostii* RHA1 as a lignin peroxidase, *Biochemistry*, 2011, vol. 50, no. 23, pp. 5096–5107.

Ahmad, M., Taylor, C.R., Pink, D., Burton, K., Eastwood, D., Bending, G.D., and Bugg, T.D.H., Development of novel assays for lignin degradation: comparative analysis of bacterial and fungal lignin degraders, *Mol. Biosyst.*, 2010, no. 6, pp. 815–821.

Bholay, A.D., Borkhataria, B.V., Jadhav, P.U., Palekar, K.S., Dhalkari, M.V., and Nalawade, P.M., Bacterial lignin peroxidase: a tool for biobleaching and biodegradation of industrial effluents, *Univ. J. Environ. Res. Technol.*, 2012, vol. 2, no. 1, pp. 58–64.

Bradford, M.M., A rapid and sensitive method for the quantitation of microorganisms qualities of protein utilizing the principle of protein-dye binding, *Anal. Biochem.*, 1976, vol. 72, pp. 248–254.

Bugg, T.D.H., Ahmad, M., Hardiman, E.M., and Singh, R., The emerging role for bacteria in lignin degradation and bio-product formation, *Curr. Opin. Biotech.,* 2011, vol. 22, pp. 394–400.

Dalimova, G.N. and Abduazimov, Kh.A., Lignins of herbaceous plants, *Khim. Prirod. Soed.*, 1994, no. 2, pp. 160–177.

Il'ina, G.V., Ecological and physiological potential of environmental isolates of xylotrophic basidiomycetes, *Doctoral (Biol.) Dissertation*, Srartov, Saratov Gos. Univ., 2011.

Kupryashina, M.A., Selivanov, N.Yu., and Nikitina, V.E., Isolation and purification of Mn-peroxidase from *Azospirillum brasilense* Sp245, *Appl. Biochem. Microbiol.*, 2012, vol. 48, no. 1, pp. 17–20.

Leisola, M., Pastinen, O., and Axe, D.D., Lignin-designed randomness, *BIO-Complexity*, 2012, vol. 3, pp. 1–11.

Lin, S.Y., Hameed, A., Shen, F.T., Liu, Y.C., Hsu, Y.H., Shanina, M., Lai, W.A., and Young, C.C., Description of *Niveispirillum fermenti* gen. nov., sp. nov., isolated from a fermentor in Taiwan, transfer of *Azospirillum irakense* (1989) as *Niveispirillum irakense* comb. nov., and reclassification of *Azospirillum amazonense* (1983) as *Nitrospirillum amazonense* gen. nov., *Antonie van Leeuwenhoek*, 2014, vol. 105, pp. 1149–1162.

Nikitina, V.E., Vetchinkina, E.P., Ponomareva, E.G., Gogoleva, Yu.V. Phenol oxidase activity in bacteria of the genus *Azospirillum, Microbiology* (Moscow), 2010, vol. 79, no 3, pp. 327–333.

Novakovskii, M.E., Drozhdenyuk, A.P., Epshtein, T.V., Dubovskaya, L.S., Khalimonchik, S.V., Fil'chenkov,N.A., Vshakevich, I.I., and Sviridov, O.V., A new nonchromatographic method for streptavidin isolation from the culture liquid of *Streptomyces avidinii*: comparison with affine chromatigraphy on iminobiotin–sefarose, *Biotekhnologiya*, 2006. vol. 42, no. 1, pp. 68–75.

Orth, A.B., Royse, D.J., and Tien, M. Ubiquity of lignin-degrading peroxidases among various wood-degrading fungi, *Appl. Environ. Microbiol.*, 1993, vol. 59, pp. 4017–4023.

Paszczynski, A., Crawford, R., and Huynh, V.B., Manganese peroxidase of *Phanerochaete chrysosporium*: purification, *Methods Enzymol.*, 1988, vol. 161, pp. 264–270.

Rahman, N.H.A., Rahman, N.A., Abd Aziz, S., and Hassan, M.A., Production of ligninolitic enzymes by newly isolated bacteria from pail oil plantation soil, *Bio Res.*, 2013, vol. 8, no. 4, pp. 6136–6150.

Wong, D.W.S., Structure and action mechanism of ligninolytic enzymes, *Appl. Biochem. Biotechnol.*, 2009, vol. 157, pp. 174–209.

Yan, J.L. and Yang, J., Bacteria and lignin degradation, *Front. Biol. China*, 2008, vol. 4, pp. 29–38.

Schloter, M. and Hartmann, A., Endophytic and surface colonization of wheat roots (*Triticum aestivum*) by different *Azospirillum* brasilense strains studied with strain specific monoclonal antibodies, *Symbyosis*, 1998, vol. 25, pp. 159–179.

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