Biodegradation of Lignin Monomers and Dimers by Bacteria

Wheeler, Kyle
Microbiology and Molecular Genetics
Faculty Sponsor: Dr. Babu Fathepure
Second Reader: Dr. John Gustafson

December 2019

Abstract

In natural soil, many species of bacteria are actively involved in the degradation of lignin, a complex biomolecular matrix that makes up a portion of plant cell walls. This activity is of great interest to researchers in the biofuel industry who are attempting to unlock the secret to a biologically-derived biofuel, in which no costly chemical processes are necessary in order to produce biofuels like ethanol. However, the natural degradation of lignin is a complicated process that has not yet been efficiently executed in a controlled setting. In this study, I attempted to better understand the ability of three bacterial strains, *Pseudomonas* sp. strain YS-1p, *Alcaligenes* sp. strain -3K, and *Arthrobacter* sp. strain RT-1, to degrade lignin-derived monomers (ferulic acid and p-hydroxy-transcinnamic acid) and a dimer (benzyl-phenyl ether). Cultures were prepared with mineral salts medium and lignin-compounds as the sole carbon source and lignin degradation was monitored indirectly by measuring growth in CFU/mls. Experimental results indicated that both monomers inhibit growth of strain YS-1p and drastically reduce growth of strain 3K. On the other hand, strain RT-1 grew well with benzyl-phenyl ether as a carbon source. Further research with these bacteria on different lignin-derived aromatic compounds could shed more light on their possible roles in lignin degradation.

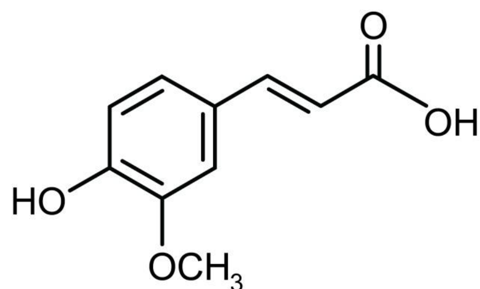
Introduction

Biofuels are combustible fuels that are created by fermenting plant biomass to produce ethanol (Bugg et al., 2011). First-generation biofuels, such as those developed from sugar cane, are created by amylase first breaking down sucrose into glucose and fructose and then fermenting the produced glucose to produce usable fuels (Bugg et al. 2011). This type of biofuel production however, has disadvantages. The crops utilized in this process are resource intensive and they also utilize fertile lands which could be used for food production instead. On the other hand, second-generation biofuels can be produced from a large variety of plants that do not necessarily require as much resource investment and can be grown on less fertile land. Second-generation biofuels utilize energy contained within molecules present in plant cell walls, specifically cellulose and hemicellulose, rather than energy present within the cells themselves as in first-generation biofuels (Bugg et al. 2011). Plant cell walls are made up of 3 major biomolecules: cellulose, hemicellulose, and lignin. Cellulose and hemicellulose are plant polysaccharides that are easily converted to sugar monomers by enzymes such as cellulases and hemicellulases and thus these sugar monomers can be easily fermented to bioethanol or biobutanol. The term lignin encompasses a large group of biomolecules synthesized by plants through the oxidation and coupling of 4-hydroxyphenylpropanoids in successive reactions to form large matrices (Vanholme et al. 2010). These lignin matrices interfere with the process of biofuel production by cross-linking with cellulose and hemicellulose in plant cell walls, making hydrolysis of plant polysaccharides and fermentation of sugars by enzymes difficult (Zeng et al. 2014). In order to combat this problematic biomolecule, second-generation biofuel production involves three main steps. First, lignin is depolymerized or degraded to release cellulose and hemicellulose. In the second step cellulose and hemicellulose are hydrolysed by cellulases and hemicellulases to sugar monomers. In the third step, sugar monomers are fermented to usable fuels such as ethanol and butanol (Bugg et al. 2011). Thus far in the biofuel industry, mostly chemical or physical pretreatment methods of lignin degradation have been utilized successfully, however, these methods are inefficient due to their high cost, high requirements for energy input, and release of chemicals that can inhibit the fermentation process (Huang et al. 2013; Feofilova et al. 2016; Yang and Wyman

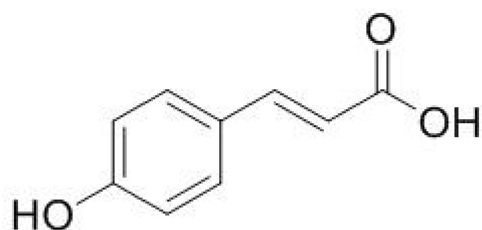
2008). As lignin is regularly deposited in natural soil, one can conclude that there must be a variety of fungal and bacterial organisms which might participate in the metabolism of plant lignin. Recently, there is increasing interest in the isolation and characterization of microorganisms from soil for their ability to degrade lignin or lignin-derived aromatic compounds and how these organisms can be utilized for the pre-treatment of plant biomass for biofuel production.

In this study, three bacterial strains previously isolated from decaying wood and temite gut were used to study lignin degradation. In our studies we used two monomers (ferulic acid and trans-4-hydroxycinnamic acid) and one dimer (benzyl phenyl ether) as sole carbon sources (Fig. 1). Degradation of monomer trans-4-hydroxy-3-methoxycinnamic acid (Ferulic acid) is of great interest due to its presence as an intermediate in the degradation of coniferyl alcohol, a highly represented lignin monomer (Andreoni et al. 1994). Trans-4-hydroxycinnamic acid also exists as an intermediate in lignin degradation pathways (Katase & Bollag 1991). Bacterial growth on benzyl-phenyl ether (BPE) is also of great interest since it contains an α-O-4 linkage which is lowest bond dissociation energy of the common interunit linkages of lignin degradation pathways (Zhu et al. 2017). Before bacteria became a major focus in second-generation biofuel research, some fungi, like white-rot fungi, were shown to be capable of breaking down lignin using secreted enzymes such as peroxidase and laccase (Bugg et al. 2011). Recently, bacterial strains have become the focus of developing biological techniques to degrade lignin because bacteria can grow in a variety of environmental conditions including in extreme environments and they are easier to genetically manipulate then fungi (Bugg, et al. 2011; Bandounas et al., 2011).

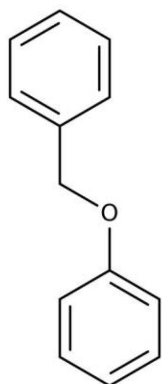
Figure 1. The three lignin-compounds studied, trans-4-hydroxy-3-methoxycinnamic acid, trans-4-hydroxycinnamic acid, and benzyl-phenyl ether



trans-4-hydroxy-3-methoxycinnamic acid



trans-4-hydroxycinnamic acid



Benzyl phenyl ether

We selected three bacterial strains that were previously isolated in our laboratory on the basis of their ability to degrade lignin monomers, dimers, and alkali lignin as the sole carbon sources. *Pseudomonas* sp. strain YS-1p was isolated from a wood sample taken from Yellowstone National Park which was then enriched through growth in media which selected for organisms that grow on decaying plant matter (Jackson et al. 2017; Prabhakaran et al. 2015). *Alcaligenes* sp. strain 3K was isolated from a petroleum contaminated soil in Nigeria (Adetitun et al. 2019). *Arthrobacter* sp. strain RT-1 was isolated from the termite gut and bovine rumen fluid (Couger et al. 2019. Unpublished results). Previous studies have also shown that various species of the genera *Pseudomonas, Alcaligenes,* and *Arthrobacter* have been shown to degrade single ring aromatic compounds that are common as lignin intermediates (Singh et al. 2011). In this

study, I have selected the above mentioned organisms to further evaluate their ability to degrade lignin monomers and dimers.

Methods

Biodegradation of lignin monomers by *Pseudomonas* sp. Strain YS-1p and *Alcaligenes* sp. Strain 3k:

Culture flasks (250 ml) containing 100 ml of mineral salts medium with 0.02% yeast-extract (MSM-YE) and supplemented with 0.02% ferulic acid or 0.02% trans 4-hydroxycinnamic acid as a carbon source (other carbon sources were present due to use of yeast extract) were prepared and sterilized by autoclaving. The various media were then inoculated with approximately 10^3 CFU/ml of strain YS-1p or strain 3k. For the inoculation, strain YS-1p and strain 3k were first grown in 1/10 strength LB broth for 1 –2 days at 30°C and plated on LB agar to determine the inoculum size. Un-inoculated autoclaved control flasks were also prepared. All flasks were incubated at 37°C in a shaking incubator at 60 rpm. Samples were then withdrawn at various time points and plated on LB agar. The plates were then incubated at 37°C for 1-2 days before CFUs/ml were determined. Detection of contamination was based on comparative colony morphology during weekly CFU/ml assays.

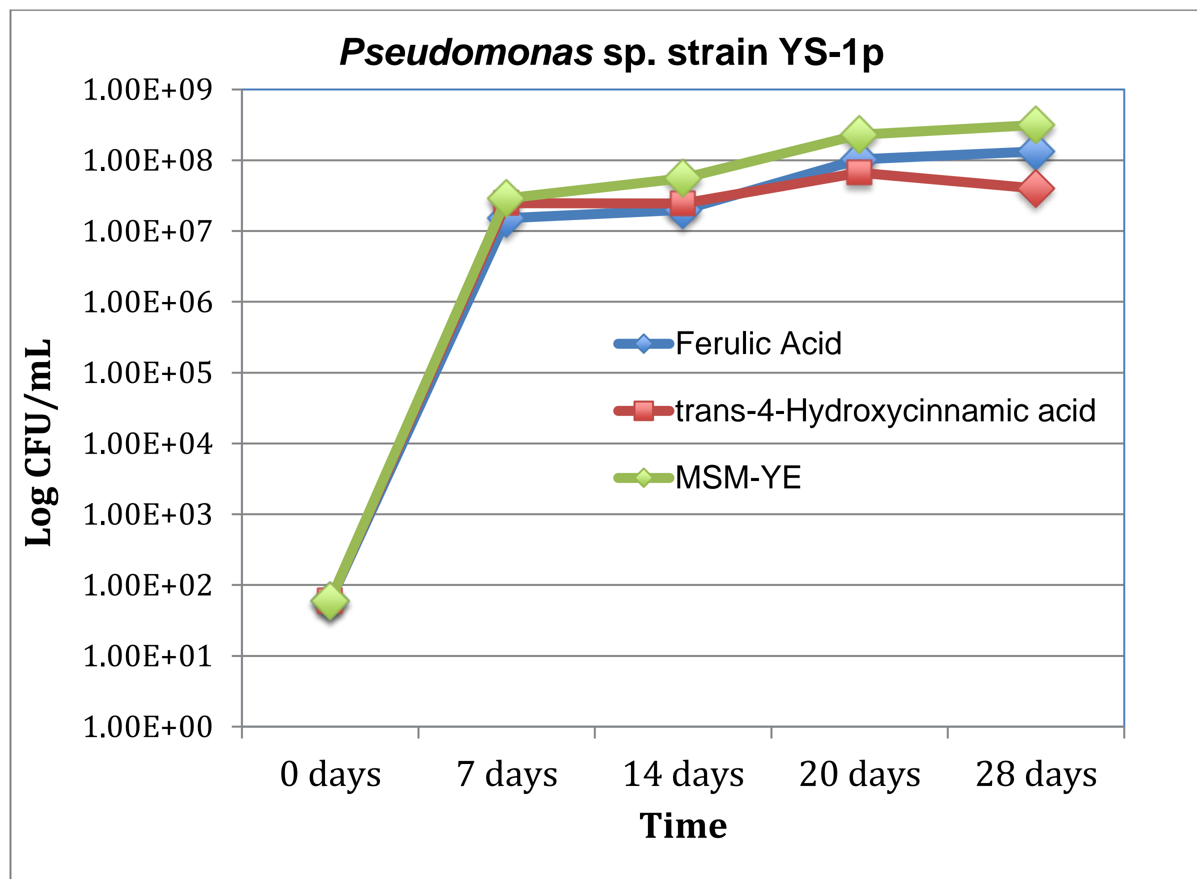
Biodegradation of lignin dimer by *Arthrobacte*r sp. Strain RT-1:

Experimental flasks were prepared as above except the MSM medium was devoid of yeast extract. The medium was supplemented with 0.02% benzyl phenyl ether as the sole carbon source and flasks were inoculated with approximately 10^3 CFU/ml of strain RT-1. Un-inoculated autoclaved flask controls were also prepared. Cultures were incubated at 37°C in a shaking incubator at 60 rpm and samples were withdrawn at various time points and plated on LB agar. The plates were incubated at 37°C for 2 days and CFUs/ml was determined. Detection of contamination was based on comparative colony morphology during weekly CFU/ml assays.

Results and Discussion

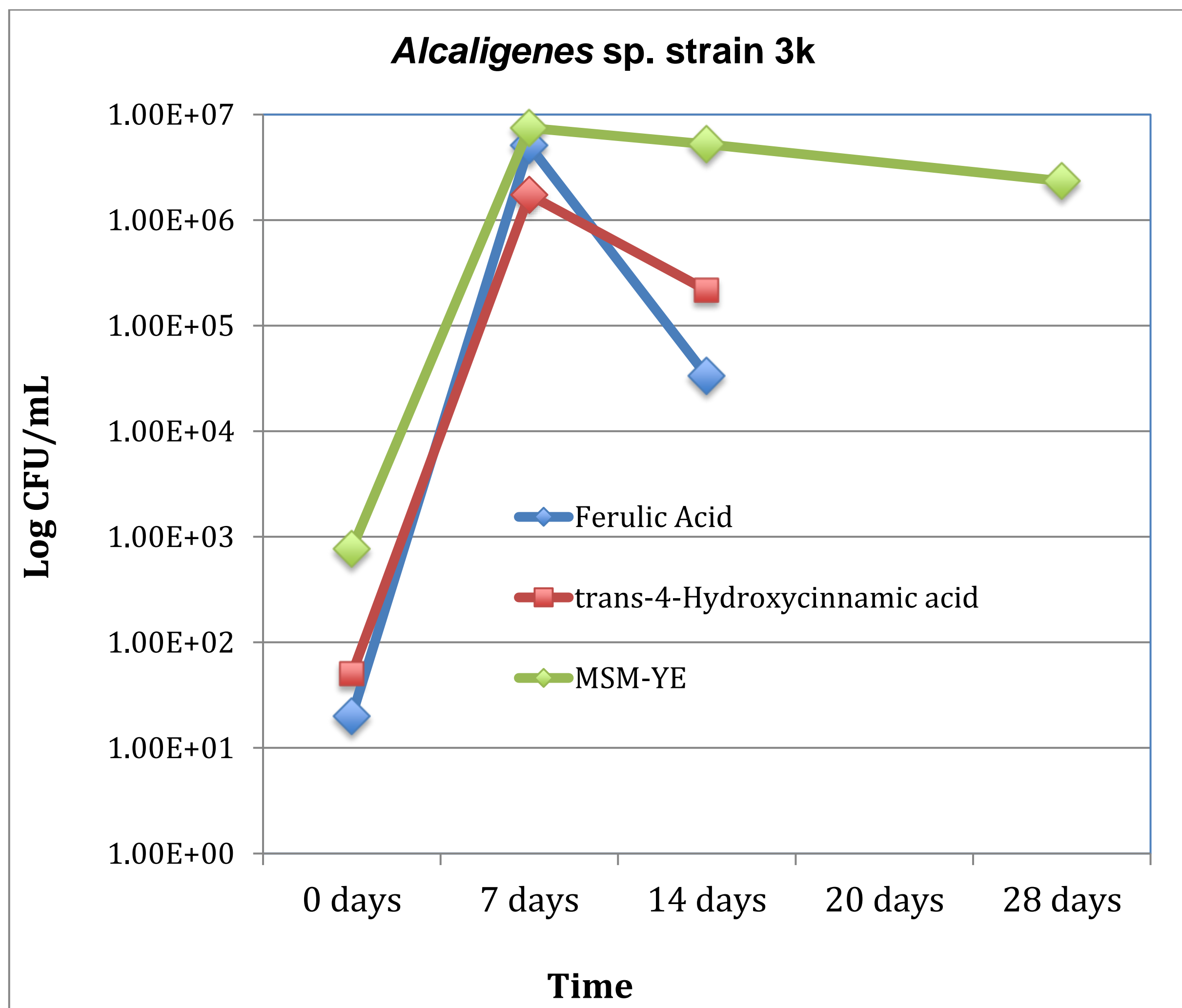
Results in Fig. 2 show that strain YS-1p grew rapidly initially by > 5 log units in the first 7 days of incubation on all substrates and then growth entered stationary phase. Overall, our results show that growth was superior when grown on MSM with 0.02% yeast extract (YE) compared to flasks containing YE + ferulic acid or YE+ 4-hydroxy cinnamic acid. For example, strain YS-1p grew by 8.5 log CFUs/ml in the presence of YE alone compared to 8.1 log CFUs/ml and 7.6 log CFUs/ml in the presence of YE + ferulic and or YE + 4-hydroxy cinnamic acid, respectively at the end of 4 weeks. These results clearly show that addition of ferulic acid and trans-4-hydroxycinnamic acid did not improve the growth of strain YS-1p. We hypothesize that ferulic acid and 4-hydroxy cinnamic acid were perhaps slightly toxic to strain YS-1p growth. These results are consistent with other studies that showed the toxic nature of these monomers (Brink et al. 2019; Campillo et al. 2014; Zhu et al. 2017). Also, from our studies, it is not clear whether strain YS-1p was able to metabolize lignin monomers since growth was much higher when grown in the presence of YE alone compared to the two lignin monomers. In our future studies, we will use GC-MS or HPLC to determine the degradation of ferulic acid and 4-hydroxycinnamic acid.

Figure 2. Growth curves of *Pseudomonas* on ferulic acid, trans-4-hydroxycinnamic acid, and standard MSM-YE.



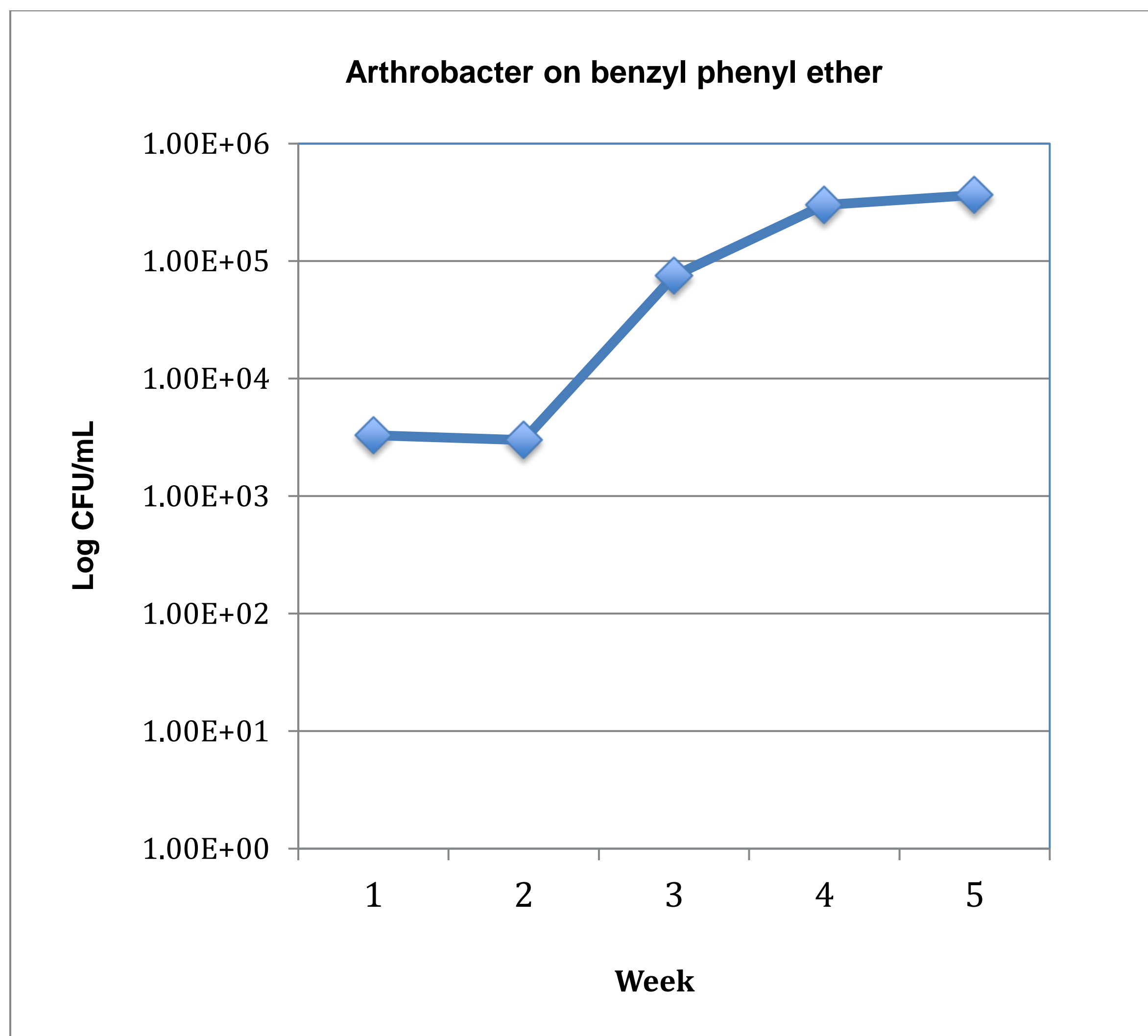
I also tested the ability of strain 3k to degrade ferulic acid and 4-hydroxycinnamic acid and utilize them as carbon sources. Fig. 3 shows the growth of strain 3k in MSM supplemented with YE, MSM + YE + ferulic acid or MSM + YE + 4-hydroxycinnamic acid. Results show that strain 3k grew rapidly initially reaching a cell density of 4 to 4.5 log units in the first 7 days, after which cell numbers declined in a way that has been noted as normal for the strain, however, strain 3k did show abnormal declination in cultures containing ferulic and 4-hydroxycinnamic acid. We hypothesize that the carbon sources available in YE allowed these two cultures to grow well during the first week, but began to suffer from the toxic monomers during the following weeks. These results also show the toxic effects of lignin monomers.

Figure 3. Growth curves of *Alcaligenes* on ferulic acid, trans-4-hydroxycinnamic acid, and standard MSM-YE.



I have also tested the ability of strain RT-1 to degrade a dimer, benzyl phenyl ether (BPE) as the sole source of carbon. Fig. 4 shows the growth of strain RT-1 in MSM supplanted with BPE as the sole source of carbon (no YE added). Results show that after a lag phase of 2 weeks, the culture population grew from log CFU 3.5/ml to log CFU 5.56 CFU/ml after the next three weeks suggesting an increase in cell density by 2 log units. These results clearly show the organism's ability to degrade lignin dimers and utilize them as carbon sources.

Figure 4. Growth curves of *Arthrobacter* on Benzyl Phenyl Ether



Conclusion

Some of the results of this study are inconclusive, especially with respect to the organisms' (Strain YS-1p and Strain 3K) ability to degrade ferulic acid and 4-hydroxycinnamic acid. In order to confirm that these compounds cannot be degraded by strain YS-1p and strain 3k, we need to set up experiments without YE and see if the cells grow on the monomers as the sole source of carbon. Also, analysis of culture samples for the formation of degradation intermediates using GC-MS or HPLC is

necessary to confirm the degradation of monomers by our strains. On the other hand, our studies have clearly demonstrated that strain RT-1 has the ability to degrade the lignin dimer BPE. These are encouraging results since ether-bond containing lignin dimers are harder to degrade and ether bonds are the dominant bond in native lignin.

Acknowledgments

The author would like to thank Dr. Fathepure for the tremendous amount of time, energy, and support that he has offered me during this project, Dr. Gustafson for sacrificing his time to be my second reader, Ms. Hadia Mukhtar for her teachings, advice, and assistance, Mr. Nick Hazell for his time, energy, and encouragement, Mr. Will Marsh for his advice and exerptise, and everyone who has encouraged me along this journey.

Literature Cited

Adetitun DO, Fathepure B, Hugh H, Kolawole OM, Olayemi AB. 2019. Degradation of Hydrocarbons and Lignin-like compounds by Alcaligenes sp. strain 3k isolated from Ilorin. Pollution. 5(2): 269-277. DOI: 10.22059/poll.2018.264288.505

Andreoni V, Bernasconi S, Bestetti G. 1994. Biotransformation of ferulic acid and related compounds by mutant strains of Pseudomonas fluorescens**.** Appl Microbiol Biotechnol (1995) 42:830-835. https://doi.org/10.1007/BF00191177

Bandounas L, Wierckx NJP, de Winde JH, Ruijssenaars HJ. 2011. Isolation and characterization of novel bacterial strains exhibiting ligninolytic potential. BMC Biotechnology. 11:94. https://doi.org/10.1186/1472-6750-11-94

Brink DP, Ravi K, Lidén G, Gorwa-Grauslund MF. 2019. Mapping the diversity of microbial lignin catabolism: experiences from the eLignin database. Applied microbiology and biotechnology. 103: 3979-4002.

Bugg TDH, Ahmad M, Hardiman EM, Rahmanpour R. 2011. Pathways for degradation of lignin in bacteria and fungi. Nat. Prod. Rep. 28, 1883-1896. DOI: 10.1039/C1NP00042J

Campillo T, Renoud S, Kerzaon I, Vial L, Baude J, Gaillard V, Bellvert F, Chamignon C, Comte G, Nesme X, Lavire C. 2014. Analysis of hydroxycinnamic acid degradation in *Agrobacterium fabrum* reveals a coenzyme A-dependent, beta-oxidative deacetylation pathway. Appl. Environ. Microbiol. 80:3341-3349.

Couger MB, Grahm C, Fathepure BZ. 2019. Genome sequence of the lignin degrading *Arhrobacter* sp. Strain RT-1 isolated from the termite gut and rumen fluide. Microbiological Resource Announcement (Submitted).

Feofilova EP, Mysyakina IS. 2016. Lignin: Chemical Structure, Biodegradation, and Practical Application. Applied Biochemistry and Microbiology. 52 (6) 573-581.

Huang XF, Santhanam N, Badri, William DV, Hunter J, Manter DK, Decker SR, Vivanco JM, Reardon KF. 2013. Isolation and Characterization of Lignin-Degrading Bacteria from Rainforest Soils. Biotechnology and Bioengineering. 110: 1616–1626. DOI 10.1002/bit.24833

Jackson CA, Couger MB, Prabhakaran M, Ramachandriya KD, Canaan P, Fathepure BZ. 2017. Isolation and characterization of Rhizobium sp. strain YS-1r that degrades lignin in plant biomass. Journal of Applied Microbiology. 122(4), 940-952. https://doi.org/10.1111/jam.13401

Katase T, Bollag JM. 1991. Transformation of Trans-4-Hydroxycinnamic Acid by a Laccase of the Fungus Trametes Versicolor: Its Significance in Humification. Soil science. Vol.151(4), p.291

Prabhakaran M, Couger MB, Jackson CA, Weirick T, Fathepure BZ. 2015. Genome Sequences of the Lignin-Degrading Pseudomonas sp. Strain YS-1p and Rhizobium sp. Strain YS-1r Isolated from Decaying Wood. Genome Announc 3(2):e00019-15. doi:10.1128/genomeA.00019-15.

Singh S, Singh B, Mishra BK, Pandey AK, Nain L. 2011. Microbes in Agrowaste Management for Sustainable Agriculture. Microorganisms in Sustainable Agriculture and Biotechnology. p 127-151. https://doi.org/10.1007/978-94-007-2214-9_8

Vanholme R, Demedts B, Morreel K, Ralph J, Boerjan W. 2010. Lignin Biosynthesis and Structure. Plant Physiology. 153, 895-905. https://doi.org/10.1104/pp.110.155119

Yang B. and Wyman CE. 2008. Pretreatment: the key to unlocking low-cost cellulosic ethanol. Biofuels, Bioproducts and Biorefining: Innovation for a sustainable economy. *2*(1), 26-40.

Zeng Y, Zhao, Yang S, Ding SY. 2014. Lignin plays a negative role in the biochemical process for producing lignocellulosic biofuels. Current Opinion in Biotechnology. 27, 38–45 https://doi.org/10.1016/j.copbio.2013.09.008

Zhu C, Cao JP, Zhao XY, Xie T, Ren J, Wei XY. 2017. Mechanism of Ni-catalyzed selective CeO cleavage of lignin modelcompound benzyl phenyl ether under mild conditions. Journal of Energy Institute. 91 (2019) 74-81. https://doi.org/10.1016/j.joei.2017.11.004

Zhu D, Zhang P, Xie C, Zhang W, Sun J, Qian WJ, Yang B. 2017. Biodegradation of alkaline lignin by Bacillus ligniniphilus L1. Biotechnology for biofuels. 10: 44.