

Short Communication

Screening for Lignin Degrading Bacteria by Means of ^{14}C -Labelled Lignins

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Abstract. Several *Nocardia* and *Pseudomonas* spp., as well as some unidentified bacteria, isolated from lake water containing high loads of waste lignin, were tested for their capacity to release $^{14}\text{CO}_2$ from specifically ^{14}C -labelled dehydropolymer of coniferyl alcohol (DHP) or corn stalk lignins. The bacteria were selected according to their ability to degrade phenolic compounds. However, only some of them could release significant amounts of $^{14}\text{CO}_2$ from the labelled lignin. The tested *Nocardia* spp. were more active than the *Pseudomonas* spp. and the unidentified bacteria. The most active strains belonged to *N. autotrophica*. These strains released CO_2 significantly from the methoxyl group and transformed the other carbons from the phenylpropane skeleton of lignin also into CO_2 . Other less demethylating strains also released little CO_2 from the other carbons of the lignin molecule. From corn stalk materials which were specifically labelled in the lignin part, only small amounts of labelled CO_2 were released.

Key words: Lignin biodegradation — Bacteria — *Nocardia* spp. — *Pseudomonas* spp.

The role of bacteria in lignin degradation is still a matter of conjecture. Some authors conclude that there is no direct evidence to implicate any particular species of bacteria which is essentially active in the breakdown of lignin in situ (Jaschhof, 1964; Greaves, 1971; Cartwright and Holdom, 1973). Other authors, however, demonstrated that either mixed (Sundman et al., 1968) or pure cultures of bacteria (Sørensen, 1962) can grow on lignin as a carbon source. *Pseudomonas* spp.

were claimed by Kawakami (1976) and Odier and Monties (1977) to degrade plant lignins. Odier and Monties also indicated several other bacterial strains that can use within seven days time more than 50% of the lignin supplied in a mineral medium containing glucose. In a recent note in Chemical and Engineering News (Anon., 1977), some bacteria were reported which showed considerable activity in lignin degradation.

The ability of bacteria to degrade phenols with a structural relationship to lignin and to cleave arylglycerol- β -aryl ether bonds may be of importance as a criterion to screen for the ability to degrade lignin (Crawford et al., 1973; Fukuzumi and Katayama, 1977). However, there is not yet a direct evidence for a correlation between the ability to degrade monomer phenols and the ability to degrade lignin (Crawford et al., 1973).

Trojanowski et al. (1977) described a *Nocardia* sp. (DSM 1069) from soil which released CO_2 from methoxyl, side chain or ring carbons from specifically ^{14}C -labelled DHP or corn stalk lignins. This species also degraded numerous phenols with structural relationships to lignins. A great number of other *Nocardia* were characterized by Kutzner (1977) and Hammann (1977) by their capacity to metabolize phenols. Several of these strains were obtained from the German Collection of Microorganisms and were tested along with some well-characterized *Pseudomonas* spp. for their capacity to release $^{14}\text{CO}_2$ from carbon-labelled lignin. Furthermore, a number of unidentified bacteria, isolated from lake water containing a high load of waste lignin from paper mills, were checked by the same method. These bacteria were selected from a greater number by their ability to utilize several phenols or lignin sulfonate as the only carbon source. Some of these bacteria also responded positively in a plate test for lignin degradation developed by Sundman and Näse (1971).

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Non-Common-Abbreviation Used. DHP = dehydropolymers of coniferyl alcohol

Methods

The preparation of the labelled DHP by polymerizing labelled coniferyl alcohol and the preparation of the corn stalk material labelled in the lignin component followed the same methods as described by Haider and Trojanowski (1975) and Trojanowski et al. (1977). These labelled lignins were incubated with the bacteria in liquid culture media and the CO₂ released was collected and measured for its radioactivity. The *Nocardia* strains were obtained from the German Collection of Microorganisms (DSM). They were grown in a medium No. 65 of the DSM-Catalogue (1977). The medium consisted of 4 g glucose, 4 g yeast extract, 10 g malt extract dissolved in 1 l H₂O. Replicate flasks with 50 ml of the medium were inoculated and after incubation for one day, 3 mg of labelled DHP or 50 mg of the corn stalk material was added. The *Pseudomonas* spp. were cultivated in a medium consisting of 1 g p-hydroxybenzoic acid, 1 g casein hydrolysate (Merck), 0.01 g FeSO₄ dissolved in 1 l H₂O. 50 ml of this medium were inoculated with the following *Pseudomonas* spp.: *P. putida* (ATCC 17433), *P. putida* (DSM 50906), *P. sp.* (Inst. Pasteur No. 6323, probably *P. putida*, it degrades cinnamic acid derivatives by o-cleavage), *P. sp.* (isolated by Dr. Reber, FAL Braunschweig, utilizes phenol as only carbon source), *P. testosteroni* (DSM 50244), *P. acidovorans* (described by Reber, 1973). The bacteria isolated from lake water were cultivated in a medium consisting of 2 g NH₄Cl, 2.9 g K₂HPO₄, 0.1 g NaCl, 0.3 g MgSO₄ · 7 H₂O, 1 g yeast extract, 1 g vanillic acid dissolved in 1 l H₂O and adjusted to pH 6.5. The incubation experiments were made at 30° C and the cultures were shaken with 110 rpm for 10–15 days.

Results and Discussion

Table 1 shows the ¹⁴CO₂-release by several of the tested *Nocardia* spp. and *Pseudomonas* spp. from the 3 mg of added methoxyl labelled DHP within a 10 and 15-day period. The figures indicated the CO₂ release in per cent of the added activity and the standard deviation for 3 replicate flasks. According to the table, the *Nocardia* spp. released more ¹⁴CO₂ than the *Pseudomonas* spp. The most active strains were representatives of *N. autotrophica*. Some *N. spp.*, not shown in Table 3, released only about 2–3% of the added activity as ¹⁴CO₂ in 10 days. These were *N. autotrophica* (DSM 43083 and 43100), *N. opaca* (43203, 43204 and 43135) and *N. aurantia* (DSM 43287). However, most of them were more vigorous than the pseudomonads. Among

these less active *N. strains* in this latter group, the *N. autotrophica* strains also released more ¹⁴CO₂ than the other species.

The *Nocardia* spp. were screened before by Kutzner (1977) and Hammann (1977) for their ability to grow on benzoic, p- and m-hydroxybenzoic and protocatechuic acids or on catechol. They also determined whether catechol or protocatechuic acid was metabolized through o- or m-cleavage. Generally, there was a correlation between the findings of Hammann (1977) and Kutzner (1977) about the ability to utilize the indicated phenols and the ability to release ¹⁴CO₂ from DHP. For example the two *N. autotrophica* strains (DSM 43083 and 43100) and *N. corallina* (DSM 43230) could not utilize several of the compounds tested by Hammann (1977). Others, however, which readily metabolized the phenols were not very active in demethylating DHP. Furthermore, the degradation rates of p-hydroxybenzoic and vanillic acid were tested in our laboratory and it was found that *N. autotrophica* strains metabolized these compounds not as rapidly as some of the other *N. spp.*

The *Pseudomonas* spp. were selected according to their ability to utilize compounds such as p-hydroxybenzoic, vanillic, veratric or anisic acids. However, most of them released only small amounts of CO₂ from the methoxyl group of DHP lignin. Among the most active ones was *P. testosteroni* which cleaves aromatic compounds through m-cleavage. However, *P. acidovorans*, which also degrades aromatic compounds through the m-pathway, released similar amounts of ¹⁴CO₂ as some of the *P. spp.* which cleave aromatic compounds through o-cleavage.

Some characteristics of the bacteria which were isolated from lake water containing high loads of waste lignin are shown in Table 2. This table also indicates the ¹⁴CO₂ release from the methoxyl-labelled DHP-lignin within a 10 and 15 day period. The bacteria showed a negative Gram stain only RP 40 was Gram variable. The bacteria released relatively little CO₂ from the

Table 1. Release of ¹⁴CO₂ from ¹⁴C-methoxyl labelled DHP-lignin by several *Nocardia* and *Pseudomonas* spp. and within a 10- and 15-day period. Figures indicate the accumulative release of ¹⁴CO₂ in % of the applied radioactivity

<i>Nocardia</i> spp.	10 days	15 days	<i>Pseudomonas</i> spp.	10 days	15 days
<i>N. autotrophica</i> DSM 43089	8.2	13.8 ± 1.1	<i>P. testosteroni</i> DSM 50244	1.8	2.2
<i>N. autotrophica</i> DSM 43099	7.5	12.5 ± 0.7	<i>P. sp.</i> (anisate) ^a	1.8	2.3
<i>N. autotrophica</i> DSM 43088	6.5	11.3 ± 0.9	<i>P. sp.</i> (phenol) ^b	2.1	2.5
<i>N. corallina</i> DSM 43001	4.1	5.3 ± 0.5	<i>P. putida</i> ATCC 17433	0.9	1.0
<i>N. opaca</i> DSM 43202	4.0	4.8 ± 0.2	<i>P. putida</i> DSM 50906	0.5	0.8
<i>N. asteroides</i> DSM 43003	3.8	4.2 ± 0.2	<i>P. sp.</i> Inst. Past. 6232	0.7	0.9
<i>N. globerula</i> DSM 43273	3.8	4.2 ± 0.1	<i>P. acidovorans</i> (Reber) ^c	0.8	0.9

^a Utilizes anisate by o-cleavage

^b Utilizes phenol

^c Described by Reber (1973)

Table 2. Characteristics of several bacteria isolated from lakewater with high load of waste lignin. Utilization of aromatic compounds and ability to release $^{14}\text{CO}_2$ from ^{14}C -methoxyl labelled DHP-lignin within a 10- and 15-day period (in % of the applied activity)

Characteristics	RP 26	RP 40	RP 88	RP 126	RP 137	RP 146
Fluorescence at 350/254 nm	++/+++	-/-	++/+++	-/-	±/+++	-/++
Growth on benzoic acid	+	+	+	-	-	+
p-OH-benzoic acid	+	+	+	+	+	-
vanillic acid	+	+	+	+	+	-
ferulic acid	+	-	-	+	+	-
Na-peritane	+	+	+	-	-	+
Plate test with lignin ^a	-	-	-	+	+	-
$^{14}\text{CO}_2$ -release from O $^{14}\text{CH}_3$ -DHP-lignin ^b	2.1; 2.6	1.1; 1.3	1.9; 2.4	2.4; 3.4	2.3; 3.8	1.0; 1.3

^a According to Sundman and Näse (1971)^b CO_2 -release within 10 and 15 days, respectively**Table 3.** Release of $^{14}\text{CO}_2$ by several *Nocardia* and *Pseudomonas* spp. from DHP-lignin labelled by ^{14}C in the methoxyl, C₂ of the side chain or in the benzene ring, respectively, within a 10- and 15-day period. Figures indicate mean values of the accumulative release of $^{14}\text{CO}_2$ in % of the applied activity of 2 replicate flasks. The deviation was mostly less than 1%

Bacteria	O $^{14}\text{CH}_3$		$^{14}\text{C}_2$		$^{14}\text{C}_{\text{ring}}$	
	10 d	15 d	10 d	15 d	10 d	15 d
<i>N. autotrophica</i> DSM 43089	7.5	14.1	6.8	9.5	5.3	7.6
<i>N. autotrophica</i> DSM 43088	6.3	12.1	4.8	6.5	4.0	5.3
<i>N. autotrophica</i> DSM 43099	6.9	12.5	5.3	7.9	5.4	6.1
<i>N. corallina</i> DSM 43001	4.4	6.1	1.8	2.1	2.5	3.1
<i>N. globerula</i> DSM 43273	2.9	4.0	1.1	1.7	1.1	1.5
<i>N. opaca</i> DSM 43202	3.9	4.5	1.4	1.7	0.9	1.0
<i>P. putida</i> DSM 50906	0.8	-	0.7	-	0.2	-
<i>P. testosteroni</i> DSM 50244	2.1	-	1.1	-	1.1	-
<i>P. sp.</i> (phenol)	2.2	-	1.8	-	1.1	-

methoxyl group. The strains RP 126 and RP 137, however, which were positive in the lignin degradation test by Sundman and Näse (1971), also showed the highest release of $^{14}\text{CO}_2$ from the labelled lignin. Several authors (Sundman et al., 1968) reported a decrease in the methoxyl contents of lignin or some detachment of side chain by bacteria (Jaschhof, 1964). Trojanowski et al. (1977) found with *N. sp.* (DSM 1069) a more rapid release of CO_2 from the methoxyl group also, however, the significant CO_2 -release from other carbons of labelled DHP lignin indicated a considerable attack of the carbon skeleton. Several tests made with some of the *Nocardia* and *Pseudomonas* spp. for their ability to release $^{14}\text{CO}_2$ from the side chain or ring carbons of specifically labelled DHP-lignin are shown in Table 3. This table indicates that the tested strains of *N. autotrophica* attacked vigorously the carbon framework of DHP-lignins. The other *N. spp.* and the *P. spp.* which had little demethylating activity were also not very active in degrading the carbons of the side chain and of the rings.

The experiments with the *Nocardia* spp. were made in a culture medium which contained carbohydrates as

the main carbon sources. Preliminary experiments where the carbohydrates were partly substituted by vanillic acid showed with labelled DHP-lignins that the CO_2 -release from the labelled groups was significantly inhibited. Further experiments conducted with corn stalk materials labelled in the lignin part showed that the *N. spp.* released only small amounts of $^{14}\text{CO}_2$ from the labelled lignin if it was located in an organized cell wall. As with the DHP-lignin, the plant lignin was most actively degraded by strains of *N. autotrophica*. Experiments with cultures of mixed cellulolytic bacteria or fungi and the lignolytic *Nocardia* spp. are in progress.

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References

Anonymous: Bacteria that degrade lignin are isolated. Chem. Eng. News 8, (Nov. 21, 1977)

- Cartwright, N. J., Holdom, K. S.: Enzymic lignin, its release and utilization by bacteria. *Microbios* **8**, 7–14 (1973)
- Crawford, R. L., Mc Coy, E., Harkin, J. M., Kirk, T. K., Obst, J. R.: Degradation of methoxylated benzoic acids by a *Nocardia* from a ligninrich environment: Significance to lignin degradation and effect of chloro substituents. *Appl. Microbiol.* **26**, 176–184 (1973)
- Deutsche Sammlung von Mikroorganismen: Catalogue of strains (D. Claus, C. Schaab-Engels, eds.). München: Gesellsch. f. Strahlen- u. Umweltforschung mbH 1977
- Fukuzumi, T., Katayama, Y.: Bacterial degradation of dimer relating to structure of lignin. I. β -Hydroxypropiovanilline and coniferylalcohol as initial degradation products from guaiacylglycerol- β -coniferylether by *Pseudomonas putida*. *Mokuzai Gakkaishi* **23**, 214–215 (1977)
- Greaves, H.: The bacterial factor in wood decay. *Wood Sci. Technol.* **5**, 6–16 (1971)
- Haider, K., Trojanowski, J.: Decomposition of specifically ^{14}C -labelled phenols and dehydropolymers of coniferyl alcohol as models for lignin degradation by soft and white rot fungi. *Arch. Microbiol.* **105**, 33–41 (1975)
- Hamann, R.: Untersuchungen zur Stoffwechselfysiologie der Ordnung Actinomycetales Buchanan 1917: Bildung organischer Säuren und Neutralprodukte, Oxidation von C_1 -Verbindungen und Abbau von Aromaten. Diss., Univ. Darmstadt (1977)
- Jaschhof, H.: Preliminary studies of the decomposition of lignin by bacteria isolated from lignite. *Geochim. Cosmochim. Acta* **28**, 1623–1638 (1964)
- Kawakami, H.: Bacterial degradation of lignin. 1. Degradation of milled wood lignin by *Pseudomonas ovalis*. *Mokuzai Gakkaishi* **22**, 252–257 (1976)
- Kutzner, H. J.: Abbau von Kohlenwasserstoffen im Boden durch Actinomyceten. Kurzfassung der Vorträge des 89. VDLUFA-Kongresses in Aachen, 1977, S. 96–97. Darmstadt: VDLUFA 1977
- Odier, E., Monties, B.: Activité ligninolytique in vitro de bactéries isolées de paille de blé en décomposition. *C. R. Acad. Sci. Paris* **284** Série D, 2175–2178 (1977)
- Reber, H.: Comparative studies with two pseudomonads on the sequential degradation of aromatic substances metabolized via different pathways. *Arch. Microbiol.* **89**, 305–315 (1973)
- Sørensen, H.: Decomposition of lignin by soil bacteria and complex formation between autoxidized lignin and organic nitrogen compounds. *J. Gen. Microbiol.* **27**, 21–34 (1962)
- Sundman, V., Kuusi, T., Kuhanen, S., Kilpi, S., Sederholm, H.: Observations on bacterial utilization of the lignin from brown rotted spruce wood and Brauns' native lignin. *Fin. Kemists. Medd.* **77**, 70–86 (1968)
- Sundman, V., Näse, L.: A simple plate test for direct visualization of biological lignin degradation. *Pap. Puu* **2**, 67–71 (1971)
- Trojanowski, J., Haider, K., Sundman, V.: Decomposition of ^{14}C -labelled lignin and phenols by a *Nocardia* sp. *Arch. Microbiol.* **114**, 149–153 (1977)

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