Detection and identification of bacterial isolates from lignin-rich environments

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Overview

Lignin is a cross-linked alkyl-aromatic polymer found in plant cell walls which confers rigidity and impermeability to vascular plants. It is one of the most abundant biopolymers in the world and possesses a very high energy density in a considerable variety of covalent bonds. Due to the heterogeneity and stability of its bonds it is extremely resistant to the attack of microorganisms, being one of the most recalcitrant biopolymers in nature. Despite its robustness, many microbes have evolved enzymatic systems to catalyze its biodegradation in different environments. Currently, in the pulp and paper industry, most part of the large amount of lignin produced is either burnt to generate process energy or converted to lignosulfonates, which have different applications as binding or dispersant agents. However, the real potential of lignin as a source of aromatic compounds and other chemicals is still clearly untapped.

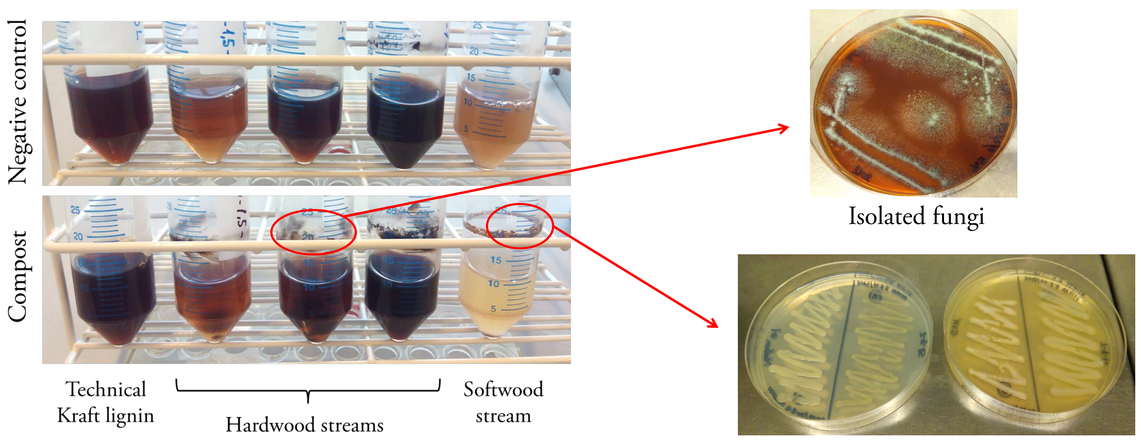
Finding (or engineering) microorganisms that are able to use lignin as a source of carbon and energy will open new paths towards an efficient biological valorization of lignin. Considerable efforts have been directed in the last decades to study the numerous mechanisms of lignin degradation in basidiomycetous fungi, the fastest and most abundant lignin degraders in natural environments, but until relatively short time ago not much was known about the role of bacteria in these processes. Now we know that bacteria can metabolize a huge diversity of low molecular weight lignin-derived compounds, and some species can even carry out the depolymerization of lignin, but a lot is still to be known about lignin degradation by prokaryotes.

Screening from compost samples

Vegetable compost is an interesting environment, in which the prolonged action of soil microorganisms has resulted in a range of aromatic compounds originating from lignin depolymerization, such as humic substances. Bacteria dwelling in this environment are very resistance to this kind of compounds, which are inhibitory to many microorganisms, and some of them are even able to metabolize and grow on these lignin-related aromatic molecules.

Sysav Waste management plant, Malmö, Sweden

For this set of experiments we did and enrichment culture in liquid **M9 mineral medium** supplemented with **hardwood** (Birch) or **softwood** (Spruce) **lignin-enriched streams** from a Kraft pulping process. Another tube was supplemented with **technical Kraft lignin**. These media were inoculated with extracts of mature compost and the growth of microorganisms after 6 days of incubation at 30° C was assessed.



Isolated fungi

Isolated bacteria

The hardwood streams gave rise mainly to **fungal growth**, producing abundant mycelium as can be observed in the figures. In the media enriched with softwood lignin, however, only **bacterial biofilm** was detected, accompanied by a strong discoloration of the medium. In the case of technical Kraft lignin, bacterial growth produced a noticeable darkening of the culture.

16S rRNA sequencing results

Isolate name	BLAST	Identity (%)	EzTaxon	Similarity (%)	RDP-II	Score
A	*Klebsiella pneumoniae* DSM 30104	99	*Klebsiella variicola* DSM 15968	99.79	*Klebsiella* sp. WR20	0.998
	Klebsiella variicola F2R9	99	*Klebsiella pneumoniae* DSM 30104	99.58	*Klebsiella variicola* GL6	0.997
	Klebsiella pneumoniae ATCC 13883	99	*Klebsiella quasipneumoniae* 01A030	99.57	*Klebsiella variicola* XF7	0.997
B	*Pseudomonas plecoglossicida* NBRC 103162	99	*Pseudomonas monteilii* NBRC 103158	99.79	*Pseudomonas putida* IARI-RP28	1.000
	Pseudomonas taiwanensis BRCR 17751	99	*Pseudomonas plecoglossicida* NBRC 103162	99.79	*Pseudomonas mosselii* L27	1.000
	Pseudomonas monteilii CIP 104883	99	*Pseudomonas taiwanensis* BRCR 17751	99.79	*Pseudomonas plecoglossicida* R4	1.000
C	*Pseudomonas plecoglossicida* NBRC 103162	99	*Pseudomonas plecoglossicida* NBRC 103162	100	*Pseudomonas plecoglossicida* P-9	0.998
	Pseudomonas plecoglossicida FPC951	99	*Pseudomonas monteilii* NBRC 103158	99.86	*Pseudomonas putida* TP0701	0.997
	Pseudomonas taiwanensis BCRC 17751	99	*Pseudomonas taiwanensis* BCRC 17751	99.86	*Pseudomonas monteilii* SB 3067	0.996
Sigma	*Pseudomonas chengduensis* MBR	99	*Pseudomonas alcaliphila* AL15-21	99.83	*Pseudomonas mendocina* PC6	0.994
	Pseudomonas alcaliphila NBRC 102411	99	*Pseudomonas chengduensis* MBR	99.83	*Pseudomonas toyotomiensis* SW237	0.994
	Pseudomonas oleovorans subsp. *lubricantis* RS1	99	*Pseudomonas toyotomiensis* HT-3	99.83	*Pseudomonas pseudoalcaligenes* C70b	0.994

Looking into the taxonomy of the genus Pseudomonas

The genus *Pseudomonas* is a very large bacterial group with around 200 species described. They have in common a remarkable metabolic versatility, and its taxonomy is quite complex; due to this, sequencing of the 16S rRNA is not enough to accurately determine the species in particular, so other more or less conserved phylogenetic markers need to be sequenced to this end. For a more reliable identification of the *Pseudomonas* isolates found in both screenings, we sequenced a fragment of the *gyrB* gene, encoding a subunit of the DNA gyrase, with the goal of obtaining a better discrimination power between species.

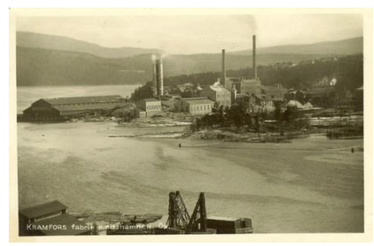
Comparison between 16S and *gyrB* sequencing results

		16S BLAST results			*gyrB* BLAST result		
	Strain	Species	Query cover (%)	Identity (%)	Species	Query cover (%)	Identity (%)
Compost	Isolate B	*P. plecoglossicida* NBRC 103162	99	99	*P.* sp. G4R	99	99
		P. taiwanensis BRCR 17751	99	99	*P. putida* KT2440	99	99
		P. monteilii CIP 104883	99	99	*P. putida* JCM 6156	99	99
	Isolate C	*P. plecoglossicida* NBRC 103162	99	99	*P. putida* A10L	98	98
		P. plecoglossicida FPC951	99	99	*P.* sp. FGI182	98	98
		P. taiwanensis BCRC 17751	99	99	*P. plecoglossicida* NyZ12	98	98
	Isolate Sigma	*P. chengduensis* MBR	100	99	*P. sihuensis* KCTC 32246	100	95
		P. alcaliphila NBRC 102411	100	99	*P. chengduensis* MBR	100	95
		P. oleovorans subsp. *lubricantis* RS1	100	99	*P. alcaliphila* LMG 23134	100	94
Pulp & paper mill	Isolate 8.1	*P. donghuensis* HYS	100	98	*P. syringae* pv. *delphinii*	99	88
		P. putida F1	100	98	*P. syringae* RM12EL_22A	99	88
		P. putida KT2440	100	98	*P. avellanae* CIP 105176	99	88
	Isolate 9.1	*P. deceptionensis* M1	99	99	*P. fluorescens* PF02	99	99
		P. fragi ATCC 4973	99	99	*P. fragi* ATCC 27362	99	99
		P. psychrophila E-3	99	99	*P. deceptionensis* CECT 7677	99	94
	Isolate 9.2	*P. umsongensis* Ps 3-10	99	99	*P. mohnii* IpA-2T	98	97
		P. baetica a390	99	99	*P. moorei* RW10T	98	97
		P. vancouverensis DhA-51	99	99	*P. putida* P-2	98	97

The difference between the results obtained from 16S and *gyrB* sequencing is notable, as shown in the table, this fact makes clear the need to choose several markers for reliable determination of the species, and points out the importance of considering the different strains used in research, which might be very close taxonomically but display very different metabolic properties.

Screening from pulp and paper mill sediments

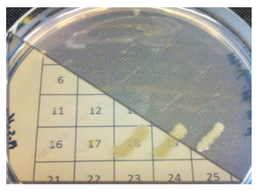
In this occasion we screened a very different but equally relevant environment, the sediments of a shore in the Baltic Sea (near Kramfors, Sweden), where the waste from a **pulp and paper mill** (enriched in lignin degradation products) has been spilled for decades, polluting the area to a considerable extent. Throughout these years, the microorganisms inhabiting these sediments have been adapted to this kind of environment, which constitutes a promising source of bacteria with interesting metabolic capabilities.



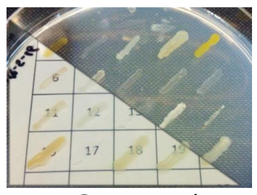
Kramfors pulp factory, 1934

A different approach was devised for these experiments, the sediments were **washed** with saline solution and the microorganisms in this wash were **revitalized** in a rich medium plate, prior to their collection and **screening** in a battery of **lignin-related lignin model compounds**, again in mineral **M9 medium**. To avoid the growth of fungi, **cycloheximide** was added to the plates in the first stage of the screening. The model compounds used included cinnamic acid, vanillin, *p*-coumaric acid, vanillic acid, sinapic acid, guaiacol, lignosulfonates and an experimental sample of depolymerized lignin.

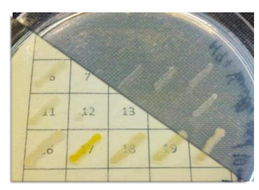
Cinnamic acid



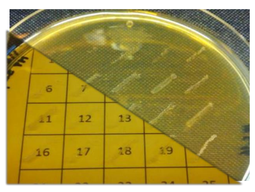
Vanillin



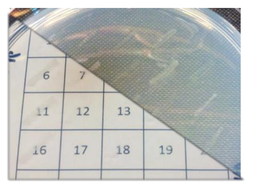
p-Coumaric acid



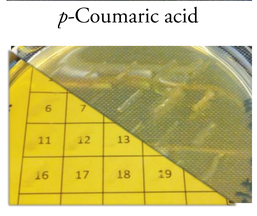
Vanillic acid



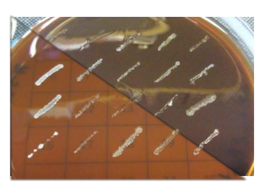
Sinapic acid



Guaiacol



Lignosulfonate



Depolymerized lignin

Screening on lignin model compounds, qualitative results

Isolate name	Cinnamic acid	Vanillin	*p*-Coumaric acid	Vanillic acid	Sinapic acid	Guaiacol	Lignosulfonate	Depolymerized lignin	Origin
8.1			++						Ferulic acid
8.2			+						
9.1			++				+	+	
9.2	+		++			+		+	
19	++	+	++	++	+	++	++	++	
47.1			++		+	++	+	+	Guaiacol
47.2			++	+	+	+	++	+	
49	++		++	+	+	++	++	+	Softwood WS
3B	+		++	+	+	+	++	+	
Cupriavidus necator JMP134			++		+		+		Culture collection
Candida tropicalis KW2	++		+	+	++	+	+	++	
Rhodococcus opacus DSM1069	++	+	++	++	+	++	++	+	
Sphingobium SYK-6			+	++	+				
Pseudomonas putida KT2440	+	++	++	++	+		+	++	

A variety of bacterial species from different taxonomic groups were isolated and identified by this method. A predominance of γ-proteobacteria was observed, particularly of Pseudomonads, but also a few species from the Bacillaceae family and a very robust *Rhodococcus* strain were found.

16S rRNA sequencing results

Isolate	BLAST Species	Query cover (%)	Identity (%)	EzTaxon Species	Similarity (%)	Completeness (%)	Taxonomic group	Gram
8.1	*Pseudomonas donghuensis* strain HYS	100	98	*Pseudomonas alcaligenes* TPID2 (Roco 2016)/JYOC 5	98.86	100	γ-proteobacteria, Pseudomonads	-
	Pseudomonas putida F1 strain F1	100	98	*Pseudomonas alkylphenolia* KL28 (Lim 2014)	98.37	100		
	Pseudomonas putida KT2440	100	98	*Pseudomonas putida* ATH-43	98.22	100		
8.2	*Lelliottia amnigena* strain JCM1237	99	99	*Lelliottia amnigena* strain JCM1237	99.79	99	γ-proteobacteria, Enterobacteriaceae	-
	Kluyvera intermedia strain 256	99	99	*Kluyvera intermedia* ATCC 33110	99.13	96.4		
	Kluyvera intermedia strain NBRC 102594	99	99	*Raoultella terrigena* ATCC 33257	98.86	99.3		
9.1	*Pseudomonas deceptionensis* strain M1	99	99	*Pseudomonas deceptionensis* strain M1	99.79	99.5	γ-proteobacteria, Pseudomonads	-
	Pseudomonas fragi strain ATCC 4973	99	99	*Pseudomonas psychrophila* strain E-3	99.64	100		
	Pseudomonas psychrophila strain E-3	99	99	*Pseudomonas fragi* strain ATCC 4973	99.43	99.4		
9.2	*Pseudomonas umsongensis* Ps 3-10	99	99	*Pseudomonas moorei* RW10	99.78	100		-
	Pseudomonas baetica strain a390	99	99	*Pseudomonas mohnii* Ipa-2	99.64	100		
	Pseudomonas vancouverensis DhA-51	99	99	*Pseudomonas umsongensis* Ps 3-10	99.58	99.7		
19	*Rhodococcus erythropolis* PR4	99	99	*Rhodococcus erythropolis* NBRC 100887	99.71	100	Actinobacteria, Nocardiaceae	+
	Nocardia coeliaca strain DSM 44595	99	99	*Rhodococcus erythropolis* DSM 43066 (T)	99.71	100		
	Rhodococcus erythropolis strain N11	99	99	*Rhodococcus jialingiae*	99.07	100		
47.1	*Acinetobacter lwoffii* strain DSM 2403	99	99	*Prolinoborus/Aquaspirillum fasciculus* CIP 103579	98.92	96.5	γ-proteobacteria, Pseudomonads, Moraxellaceae/ β-proteobacteria, Chromobacteriaceae (Prolinoborus)	-
	Acinetobacter lwoffii strain JCM 6840	99	99	*Acinetobacter lwoffii* NCTC 5866	98.58	100		
	Prolinoborus fasciculus strain CIP 103579	98	99	*Acinetobacter harbinensis* HITLi 7	98.44	100		
47.2	*Lysinibacillus macroides* LMG 18474	100	99	*Lysinibacillus macroides* LMG 18474	99.72	100	Firmicutes, Bacillaceae	+
	Lysinibacillus boronitolerans NBRC 103108	100	99	*Lysinibacillus xylanilyticus* XDB9	99.33	91.5		
	Lysinibacillus pakistanensis NCCP-54	99	99	*Lysinibacillus boronitolerans* T-10a	99.23	91.5		
49	*Bacillus licheniformis* DSM 13	100	99	*Bacillus licheniformis* ATCC 14580	99.76	100		+
	Bacillus licheniformis ATCC 14580	100	99	*Bacillus aerius* 24K	99.69	100		
	Bacillus licheniformis BCRC 11702	100	99	*Bacillus sonorensis* NBRC 101234	99.61	100		
3B	*Bacillus safensis* NBRC 100820	100	99	*Bacillus safensis* FO-36b	100	100		+
	Bacillus safensis FO-36b	99	99	*Bacillus pumilus* ATCC 7061	99.79	100		
	Bacillus pumilus NBRC 12092	100	99	*Bacillus* sp. DW5-4	99.79	100		

Acknowledgements

The authors would like to thank Adrian Pratt and Ellen Lindblad from Sysav (South Scania Waste Company, Malmö, Sweden) for arranging the visit to their waste management plant and providing the compost samples, Olla Wallberg and Basel Al-Rudainy from the Department of Chemical Engineering (Lund University) for supplying the lignin-enriched streams, Catherine Paul from Applied Microbiology (Lund University) for gently donating the sediment samples from the pulp and paper mill and Domsjö for supplying the lignosulfonates.

This project is funded by the Swedish Foundation for Strategic Research (SSF)

Stiftelsen för Strategisk Forskning