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Anoxybacillus sp. Strain UARK-01, a New Thermophilic Soil Bacterium with Hyperthermostable Alkaline Laccase Activity

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Abstract We describe the isolation and characteristics of a novel thermophilic bacterium from soil. The organism is a member of the *Anoxybacillus* genus based on phylogenetic analysis of the 16S rRNA gene. The 16S rRNA of the organism shares >99% sequence identity with those of two species, *Anoxybacillus rupiensis* and *A. geothermalis*. We named this isolate as *Anoxybacillus* sp. strain UARK-01. UARK-01 grows optimally in the presence of oxygen at 55 °C and pH 8. It grew excellently in the presence of lignin as the sole carbon source. Culture supernatant from UARK-01 grown on lignin was rich in laccase activity. The laccase activity was optimal at 90 °C and pH 9, and there was comparable activity at 80 and 100 °C. The crude laccase decolorized approximately 75% of Congo Red in 7 h under optimal conditions. A single laccase gene was identified from the draft genome sequence of *Anoxybacillus* sp. UARK-01. The UARK-01 laccase (Anox_Lacc) was cloned and overexpressed in *Escherichia coli* and was partially purified. The partially purified Anox_Lacc decolorized approximately 1.64+/0.21 nanomoles of Congo Red per microgram protein in 30 min at 90 °C and pH 9. Anox_Lacc is a member of the multicopper polyphenol oxidoreductase laccase family (pfam02578 Cu-oxidase_4) and has novel characteristics. Multiple sequence analysis of Anox_Lacc with six homologs from the family revealed four conserved copper ligands and several new residues that are fully conserved. Anox_Lacc is enriched in leucine, glutamine, and lysine, and it contains fewer alanine, arginine, glycine, and serine residues. Skewed amino acid composition of Anox_Lacc likely contributes to the exceptional thermochemical properties of the laccase activity from UARK-01. Both lignin utilization and production of hyperthermostable alkaline laccase are new findings in the *Anoxybacillus* genus.

Introduction

Laccases (EC 1.10.3.2) are multicopper-containing polyphenol oxidases that oxidize a variety of substrates including phenols, polyphenols, amino phenols, methoxy phenols, and aromatic amines [17]. There is an ever-increasing demand for thermostable laccases for industrial processes and environmental bioremediation [16, 28]. One of the major applications of laccases includes the decolorization of dyes in textile effluents [25]. Every year up to 200,000 tons of dyes is released in effluents from textile industry. Toxic and mutagenic dyes in the effluents pose serious environmental and health concerns [25, 28]. Thermostable microbial laccases can be employed to effectively remove dyes from contaminated water [35]. Another important application for laccases includes the delignification of lignocellulose [10, 19]. Lignocellulose is the most abundant renewable resource for sustainable production of bioenergy and value-added chemicals [22]. However, the polyphenolic

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lignin component of lignocellulose is a major barrier in its bioconversion to valuable products [31]. Thermostable laccases can be utilized to effectively pretreat lignocellulose and remove phenolic inhibitors [10, 18, 31]. While laccases are produced by plants, fungi, and bacteria, most plant and fungal laccases are not very thermostable [16].

The genus *Anoxybacillus* is a relatively new genus of Gram-positive bacteria that was proposed in the year 2000 [24]. It is most closely related to the genus *Geobacillus* and belongs to the family *Bacillaceae*. The genus name, *Anoxybacillus*, which was derived from its first described aerotolerant anaerobic species, suggests that these organisms are anaerobic. However, mostly facultative anaerobic and strict aerobic members have been described since the first introduction of the genus [15]. To date, the genus is composed of 22 species and two subspecies. *Anoxybacillus* members are moderately thermophilic with optimum growth temperature (T_{opt}) around 50–60 °C, and their optimum pH (pH_{opt}) for growth can range from 5.5 to 9.7 [15]. Emerging data from biochemical and genomic studies are reinforcing the importance of *Anoxybacillus* genus as a potential new resource for thermostable enzymes. While starch, cellulose, xylan, and arabinofuran degradation capabilities have been identified in different *Anoxybacillus* species, lignin degradation capabilities remain to be studied in this genus [15]. Here, we describe the isolation and characteristics of *Anoxybacillus* sp. strain UARK-01 (referred to as UARK-01 below), a new thermophilic bacterium that utilizes lignin as a carbon source and produces hyperthermostable alkaline laccase activity.

Materials and Methods

Isolation of Microorganisms

In a search for new thermophilic lignocellulose-degrading microorganisms from soil, we used isolation media containing switchgrass as the sole carbon source. Isolation media contained mineral salts (per liter: 1.695 g Na_2HPO_4, 0.75 g KH_2PO_4, 0.125 g NaCl, and 0.25 g NH_4Cl) and 0.5% (w/v) switchgrass as the sole carbon source. Solid media were prepared by adding 2% agar (BD Difco); all media were sterilized using autoclave. Soil sample collected from 3 inches below a grass lawn on the university campus was inoculated into isolation media and incubated at 50 °C. After regular intervals, small aliquots were spread on solid media plates and incubated at 50 °C until microbial colonies developed. From the colonies, microorganisms were isolated into pure cultures through extensive successive culturing of single colonies on solid media plates. Phase contrast microscopy was used to also assess the segregation of microorganisms into pure cultures.

Media and Culture Conditions

UARK-01 was routinely cultured in mineral salts media (composition described above) at pH 8.0, 55 °C, and 250 rpm. Depending on the experiment, sole carbon source was provided in the form of either yeast extract, peptone, glucose, cellobiose, xylose, cellulose, carboxymethyl cellulose, beechwood xylan, or alkali lignin (all chemicals were purchased from Sigma-Aldrich). Prior to use, lignin, cellulose, and agar were washed extensively with deionized sterile water to remove any soluble contaminants. For determining optimum pH for growth, yeast extract (0.5% w/v) was used as the sole carbon source and the pH of the medium was adjusted using either sodium hydroxide or phosphoric acid. Bacterial growth in liquid media was measured using optical density at 600 nm.

Bacterial Identification

The 16S rRNA gene was amplified from the culture lysate of UARK-01, using polymerase chain reaction (PCR) and two bacteria-specific primers 8F and 1492R [14]. The PCR product was analyzed using agarose gel electrophoresis and DNA was purified from the gel using QIAquick Gel Extraction Kit (Qiagen). Purified DNA was sequenced from both ends at the DNA Resource Center on campus. The 16S rRNA gene sequence of UARK-01 was searched against the NCBI *nr* database using BLAST [1].

16S Phylogenetic Analysis

Nucleotide sequences of 16S rRNA genes from 24 bacteria were obtained from the GenBank database and were aligned with the 16S rRNA gene sequence of UARK-01. In order to make robust inferences, sequences were aligned separately using two independent programs, ClustalX and MUSCLE [9, 20]. Neighbor-joining trees were generated using ClustalX, and maximum-likelihood trees were generated using RAxML [30]. Non-parametric bootstrapping was performed with 1000 iterations and statistical bootstrap values were used to infer tree topology. Trees were visualized and annotated using the FigTree v1.4.2 software (http://tree.bio.ed.ac.uk/software/figtree/). The following 16S sequences were used for multiple sequence alignment and phylogenetic tree construction: *A. geothermalis* strain ATCC BBA-2555 (accession# KJ722458.1), *A. rupiensis* strain R270 (NR_042379.1), *A. tepidamans* strain GS5-97 (NR_025819.1), *A. kualawohkensis* strain ET10/3 (KJ722465.1), *A. eryuanensis* strain E-112 (NR_117229.1), *A. mongoliensis* strain T4 (NR_116097.1), *A. kamchatkensis* strain JW/VK-KG4 (NR_041915.1), *A. salavatliensis* strain A343 (NR_104492.1), *A. tengchongensis* strain T-11 (NR_116668.1), *A. ayderensis*

strain AB04 (NR_024837.1), *A. flavithermus* strain DSM 2641 (NR_026516.1), *A. contaminans* strain R-16222 (NR_029006.1), *A. amylolyticus* strain MR3C (NR_042225.1), *A. caldiproteolyticus* strain R-35652 (NR_116989.1), *A. voinovskiensis* strain IHB B 4422 (KF475881.1), *Anoxybacillus* sp. SK3-4 (GQ184213.1), *Anoxybacillus* sp. DT3-1 (GU129931.1), *A. gonensis* strain O9 (KM596794.1), *A. suryakundensis* strain JS1 (KC958552.1), *A. thermarum* strain A4 (KC310455.1), *Anoxybacillus* sp. P3H1B (NZ_LPUG01000026.1 contig000026 region 1–1460), *Anoxybacillus* sp. BCO1 (NZ_JRLC01000086.1 contig000086 region 11–1554), *Anoxybacillus* sp. KU2-6(11) (NZ_JPZN01000047.1 contig58 region 337–1884), and *Geobacillus thermodenitrificans* strain BGSC W9A38 (AY608964.1). The 16S rRNA gene from *G. thermodenitrificans* was used as the outgroup.

Laccase Activity Assay

Bacteria were cultured for 5 days in lignin-containing mineral salts media as described above. Cells were centrifuged at 10,000×*g* for 10 min at 4 °C to obtain culture supernatant. The culture supernatant was assayed for the presence of laccase activity using the dye decolorization method as described previously [13]. Briefly, 100 µl of the crude enzyme was added to 1 ml of 0.001% of Congo Red dye in sterile water and the reaction mixture was incubated at 55 °C with vigorous shaking at 250 rpm for 5 days. After each day, absorbance at 500 nm (Abs_{500}) was measured. Relative laccase activity was defined using the percentage of dye decolorization, which was calculated using the below equation. Absorbance at 0 h was used as initial Abs_{500}. In control reactions, supernatant from uninoculated media that had been incubated under the same conditions as the bacterial cultures was used. All assays were performed in triplicate. For biological replication, samples from three independently grown cultures were assayed. Dithiothreitol (DTT) was used at 0.2 mM as an inhibitor for the laccase activity (13).

$$\%\ \text{Decolorization} = \frac{\text{Initial Abs500} - \text{Measured Abs500}}{\text{Initial Abs500}} \times 100$$

Optimal Temperature and pH for Laccase Activity

To measure optimal temperature for laccase activity, reaction mixtures were incubated at different temperatures (30–100 °C) in water bath for 7 h and the % decolorization was measured. To study the optimal pH for the laccase activity, reactions were incubated at 90 °C (T_{opt}) for 7 h in various pH buffers. For pH 4.0–6.0, 50 mM sodium acetate buffer was used; for pH 7.0–8.0, 50 mM, phosphate buffer was used; and for pH 9.0–10.0 50 mM, borate buffer was used. The % decolorization was measured to determine the amount of laccase activity. All assays were performed in triplicate. For biological replication, samples from independently grown cultures were assayed.

Cloning of the UARK-01 Laccase

Draft genome sequence of UARK-01 [unpublished data] was used to identify the UARK-01 laccase (Anox_Lacc) gene. The *Anox_Lacc* gene was cloned into pET expression vector (Novagen). The UARK-01 genomic DNA was isolated using the DNeasy Blood and Tissue kit (Qiagen). The *Anox_Lacc* gene was amplified from the genomic DNA using PCR-engineered gene-specific primers carrying the NdeI and XhoI restriction sites at their 5′ ends (AACATATGCCAGAAATTTTCATCAAGATGGCGAAGAA and AACTCGAGCTATTCCTCCTTCCTGACGATAAATGCCATC; restriction sites are underlined). DNA was quantitated using the Qubit dsDNA BR Assay kit (Life Technologies) and analyzed using agarose gel electrophoresis.

Amplified gene was restriction-digested and ligated into pET-28a(+) expression vector (Novagen) that had been predigested with the same restriction enzymes. The ligation mixture was electroporated into subcloning efficiency electrocompetent *Escherichia coli* Rosetta™(DE3)pLacI competent cells (Novagen) using BioRad GenePulser using the manufacturer's preset protocol. Transformants were selected using Luria Bertani (LB) agar plates containing chloramphenicol (17 µg/ml) and kanamycin (25 µg/ml). Colonies were screened for the presence of the *Anox_Lacc* gene using the colony cracking method as well as using PCR to identify the clones carrying the *Anox_Lacc* gene.

Partial Purification of the Anox_Lacc protein

E. coli cells carrying the Anox_Lacc gene were grown in LB broth containing the above antibiotics. For production of the Anox_Lacc protein, cells were grown to an OD_{600} of 0.35 and induced for 4 h using 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) with vigorous shaking at 300 rpm. Cells were centrifuged and lysed using lysis buffer (50 mM Tris–Cl pH 8, 5% glycerol, 50 mM sodium chloride, 10 mg/ml lysozyme) at 37 °C for 20 min. Cell debris was removed using centrifugation and the clear lysate was obtained. The thermostable Anox_Lacc protein was partially purified by denaturing and precipitating the host proteins at 55 °C for 1 h; precipitated proteins were removed by centrifugation. Protein concentration was determined using Qubit Protein Assay kit (Life Technologies). Proteins were analyzed using sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE).

Bioinformatic Analysis of Anox_Lacc Sequence

Theoretical molecular mass and pI of the protein sequence of Anox_Lacc were calculated using the Compute pI/Mw tool from the ExPASy server (http://web.expasy.org/compute_pi/). The Anox_Lacc sequence was aligned with sequences of three biochemically characterized laccase homologs and three pfam02578 family homologs whose crystal structures have been determined. Alignment was done using ClustalX program with default parameters [20].

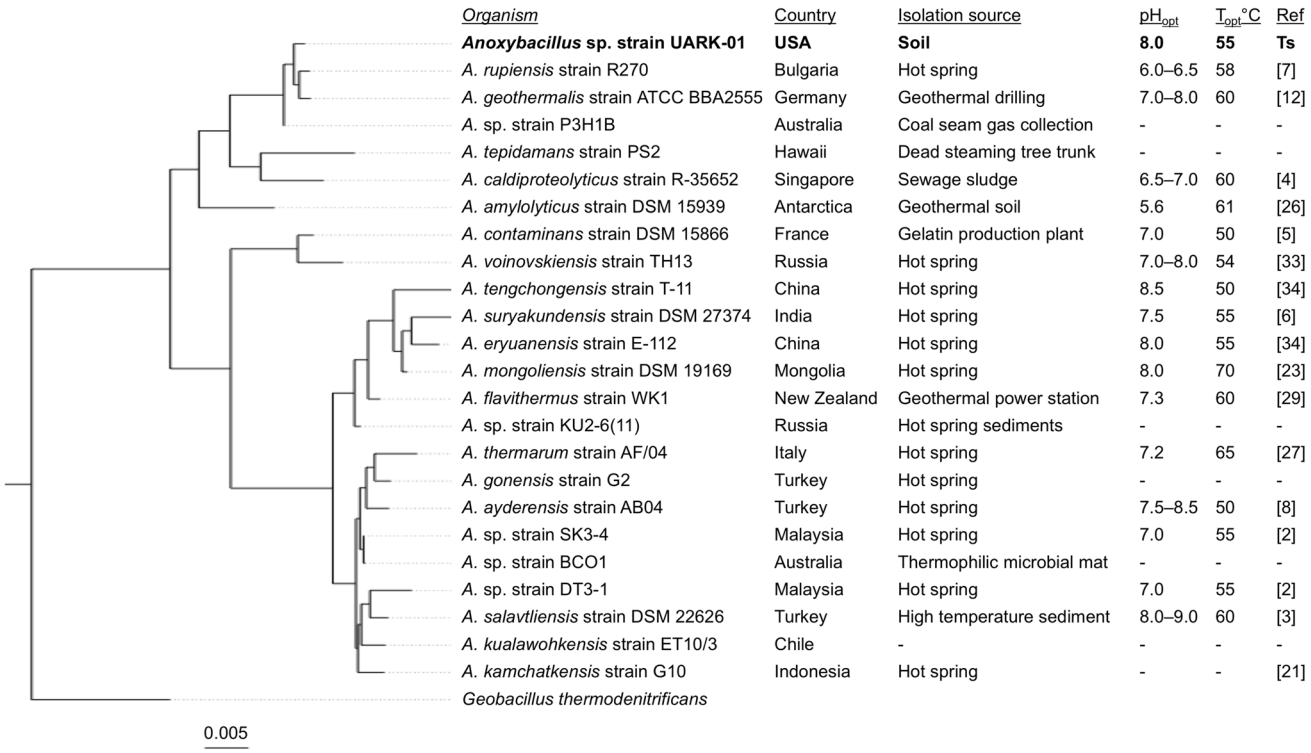
Results

Isolation and Identification of UARK-01

In our search for new lignocellulose-degrading microorganisms from soil, we isolated pure cultures of seven thermophilic microorganisms that grew on mineral salts media containing switchgrass as the sole carbon source (data not shown). Here, we describe the identification of one of the thermophilic bacteria. Using PCR, we amplified a ~1.5 kb fragment of the 16S rRNA gene of the bacterium (data not shown). After DNA sequencing, we obtained the sequence of a 1547 bp region of the 16S rRNA gene of the bacterium. Based on BLAST search of the sequence, the bacterium was identified as a member of the *Anoxybacillus* genus (data not shown). Phylogenetic analysis of the 16S rRNA gene sequence confirmed the bacterium groups within the *Anoxybacillus* clade (Fig. 1). It is phylogenetically most closely related to *A. geothermalis* and *A. rupiensis*. Its 16S rRNA gene sequence shares 99.676, 99.658, and 99.539% sequence identity with the 16S rRNA gene sequences of *A. geothermalis, Anoxybacillus* sp. strain P3H1B, and *A. rupiensis*, respectively. We named the new bacterium as *Anoxybacillus* sp. strain UARK-01.

Growth Characteristics of UARK-01

We determined the optimum pH and temperature for growth of the bacterium (Fig. 2a). While growth was maximal at pH 8.0, the organism also grew well at pH 7.0. Limited slow growth was observed at pH 9, and the organism failed to grow at pH 5. At pH 6, the organism grew well in the first 24 h but growth declined rapidly after that. The organism grew well at temperatures between 50 and 60 °C, with optimal growth at 55 °C (data not shown). To assess its preference for carbon sources, growth of UARK-01 was



Organism	Country	Isolation source	pH_{opt}	T_{opt}°C	Ref
***Anoxybacillus* sp. strain UARK-01**	**USA**	**Soil**	**8.0**	**55**	**Ts**
A. rupiensis strain R270	Bulgaria	Hot spring	6.0–6.5	58	[7]
A. geothermalis strain ATCC BBA2555	Germany	Geothermal drilling	7.0–8.0	60	[12]
A. sp. strain P3H1B	Australia	Coal seam gas collection	-	-	-
A. tepidamans strain PS2	Hawaii	Dead steaming tree trunk	-	-	-
A. caldiproteolyticus strain R-35652	Singapore	Sewage sludge	6.5–7.0	60	[4]
A. amylolyticus strain DSM 15939	Antarctica	Geothermal soil	5.6	61	[26]
A. contaminans strain DSM 15866	France	Gelatin production plant	7.0	50	[5]
A. voinovskiensis strain TH13	Russia	Hot spring	7.0–8.0	54	[33]
A. tengchongensis strain T-11	China	Hot spring	8.5	50	[34]
A. suryakundensis strain DSM 27374	India	Hot spring	7.5	55	[6]
A. eryuanensis strain E-112	China	Hot spring	8.0	55	[34]
A. mongoliensis strain DSM 19169	Mongolia	Hot spring	8.0	70	[23]
A. flavithermus strain WK1	New Zealand	Geothermal power station	7.3	60	[29]
A. sp. strain KU2-6(11)	Russia	Hot spring sediments	-	-	-
A. thermarum strain AF/04	Italy	Hot spring	7.2	65	[27]
A. gonensis strain G2	Turkey	Hot spring	-	-	-
A. ayderensis strain AB04	Turkey	Hot spring	7.5–8.5	50	[8]
A. sp. strain SK3-4	Malaysia	Hot spring	7.0	55	[2]
A. sp. strain BCO1	Australia	Thermophilic microbial mat	-	-	-
A. sp. strain DT3-1	Malaysia	Hot spring	7.0	55	[2]
A. salavtliensis strain DSM 22626	Turkey	High temperature sediment	8.0–9.0	60	[3]
A. kualawohkensis strain ET10/3	Chile	-	-	-	-
A. kamchatkensis strain G10	Indonesia	Hot spring	-	-	[21]
Geobacillus thermodenitrificans					

Fig. 1 Phylogenetic analysis of the 16S rRNA gene sequence of UARK-01. The 16S rRNA gene from *G. thermodenitrificans* was used as the outgroup. The *scale bar* corresponds to 0.005 changes per nucleotide. The country of origin, source of isolation, and the optimum temperature (T_{opt} in °C) and pH (pH_{opt}) for growth of the *Anoxybacillus* isolates were obtained from the literature (see 'Ref' column). *Ts* this study; '-' *indicates* that the data are not published

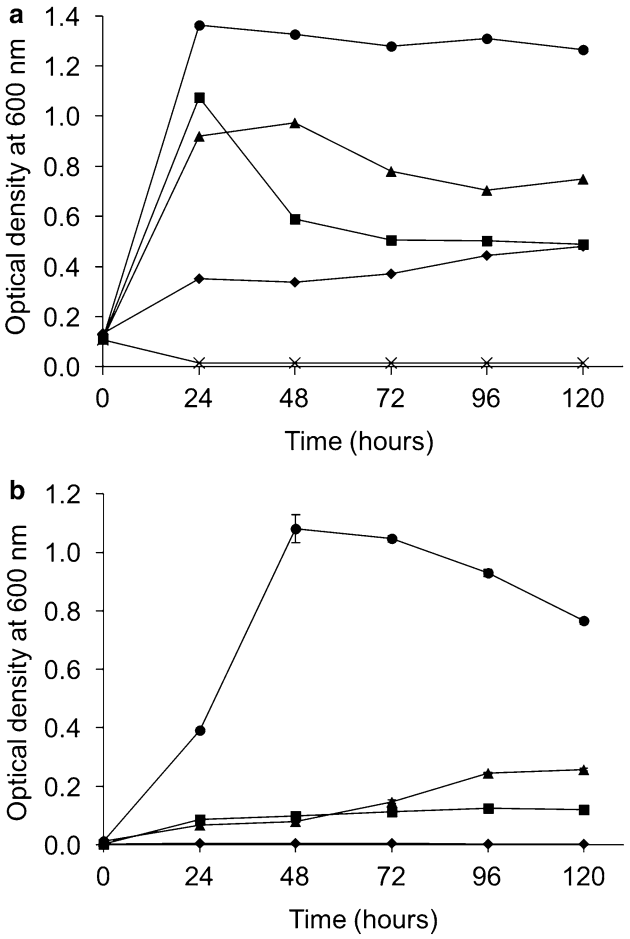


Fig. 2 Growth characteristics of UARK-01. **a** Growth at different pH concentrations in mineral salts media containing yeast extract (0.5% w/v) as the sole carbon. pH 5 (*X marks*), pH 6 (*squares*), pH 7 (*triangles*), pH 8 (*circles*), pH 9 (*diamonds*). **b** Growth in mineral salts media (pH 8.0) containing sole carbon source in the form of either 0.2% lignin (*circles*), 0.5% beechwood xylan (*triangles*), 0.5% cellulose (*squares*), or 0.5% carboxymethyl cellulose (*diamonds*). *Vertical error bars* represent standard deviation across three measurements

studied in mineral salts media containing yeast extract, peptone, and/or glucose as sole carbon sources (Table 1). The bacterium grows optimally in media A, which contained 0.5% yeast extract alone as the sole carbon source. Limited growth occurred in media B, which contained 0.1% yeast extract as the sole carbon source. Growth in media C (which contained 0.1% yeast extract and 0.25% D-glucose) was not significantly higher compared to media B suggesting that the organism does not utilize glucose as the carbon source. Compared to media C, more growth was observed in media D (which contained 0.1% peptone and 0.25% glucose) suggesting that the bacterium has a preference for utilizing amino acids as carbon source. The ability of UARK-01 to degrade and utilize major components of plant cell wall was studied (Fig. 2b). UARK-01 grew excellently in the presence of lignin as the sole carbon source,

Table 1 Summary of growth of UARK-01 in mineral salts media containing different sole carbon sources

Sole carbon source	Concentration	Growth[a]
Media A		++++
Yeast extract	0.5% (w/v)	
Media B		++
Yeast extract	0.1% (w/v)	
Media C		++
Yeast extract	0.1% (w/v)	
Glucose	0.25% (w/v)	
Media D		+++
Peptone	0.1% (w/v)	
Glucose	0.25% (w/v)	
Lignin	0.2% (w/v)	++++
Switchgrass	0.5% (w/v)	+
Beechwood xylan	0.5% (w/v)	+
D-xylose	0.5% (w/v)	+
D-glucose	0.5% (w/v)	+/−
Cellobiose	0.5% (w/v)	+/−
Cellulose	0.5% (w/v)	−
Carboxymethyl cellulose (CMC)	0.5% (w/v)	−

[a]Growth was determined in both liquid and solid media and relative growth is indicated; ++++ indicates excellent growth; +++ indicates good growth; ++ indicates limited initial growth, + indicates slow growth, +/−indicates poor growth, and − indicates no growth.

while it did not grow on either carboxymethyl cellulose or crystalline cellulose. The organism exhibited slow growth on xylan with growth increasing after 60 h. UARK-01 also grew slowly on xylose (Table 1) suggesting that it has weak ability to utilize xylan and/or its degradation products (e.g., xylose). Lack of growth on cellulose was confirmed on solid media. Additionally, no growth of the bacterium was observed in the presence of cellulose as the sole carbon source at pH 5, 6, 7, 8, or 9 (data not shown). UARK-01 grows extremely poorly on cellobiose or glucose (Table 1) suggesting that it does not have the ability to utilize cellulose and its degradation products (i.e., cellobiose, glucose).

Laccase Activity in UARK-01

Culture supernatants from UARK-01 grown on lignin showed steady increase in laccase activity after 24 h (Fig. 3a). The crude enzyme decolorized approximately 40% of the Congo Red dye after 5 days at 55 °C in water. The amount of laccase activity in culture supernatants of the bacteria after 3 and 5 days of growth on lignin was almost equal (data not shown). Control samples from uninoculated media did not decolorize the dye; also, Congo Red by itself showed no spontaneous decolorization under the same conditions. Addition of DTT, a known laccase

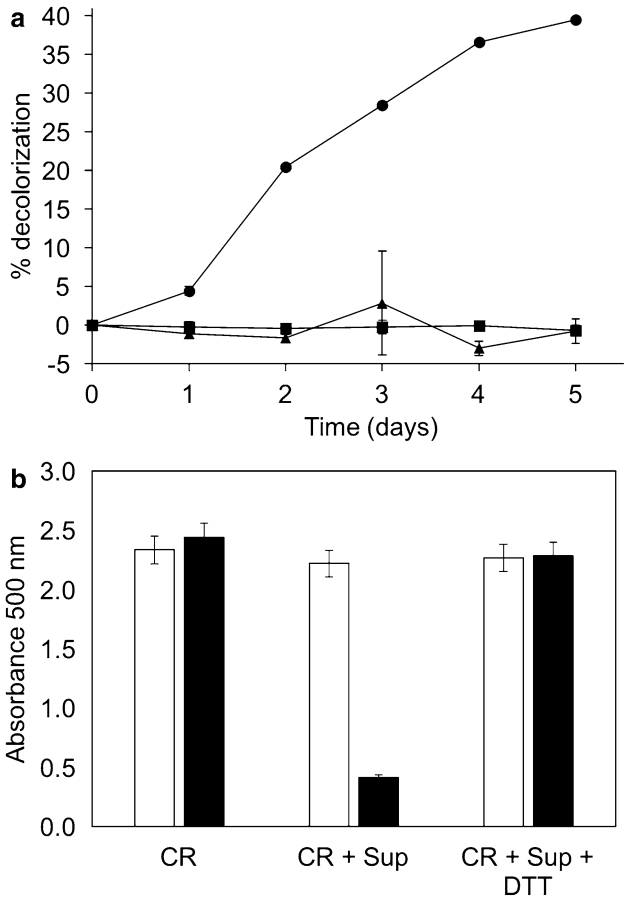


Fig. 3 Laccase activity in UARK-01. **a** Congo Red decolorization by UARK-01 culture supernatant at 55 °C in water (pH 7). Percentage of Congo Red decolorization was measured for 5 days in the presence of bacterial culture supernatant (*circles*). Samples from uninoculated media were used as control (*triangles*). Spontaneous decolorization of Congo Red alone was also measured (*squares*). **b** Inhibition of Congo Red decolorization by DTT. *CR* Congo Red alone, *CR + Sup* Congo Red incubated with UARK-01 culture supernatant, *CR + Sup + DTT* Congo red incubated with culture supernatant in the presence of 0.2 mM DTT. Culture supernatant that had been concentrated 100-fold and dialyzed was used in the inhibition assay. *Open bars* represent initial readings at 0 h, and *black bars* represent readings after 1 h of incubation at 90 °C in 50 mM sodium borate buffer (pH 9). *Vertical error bars* represent standard deviation across three measurements

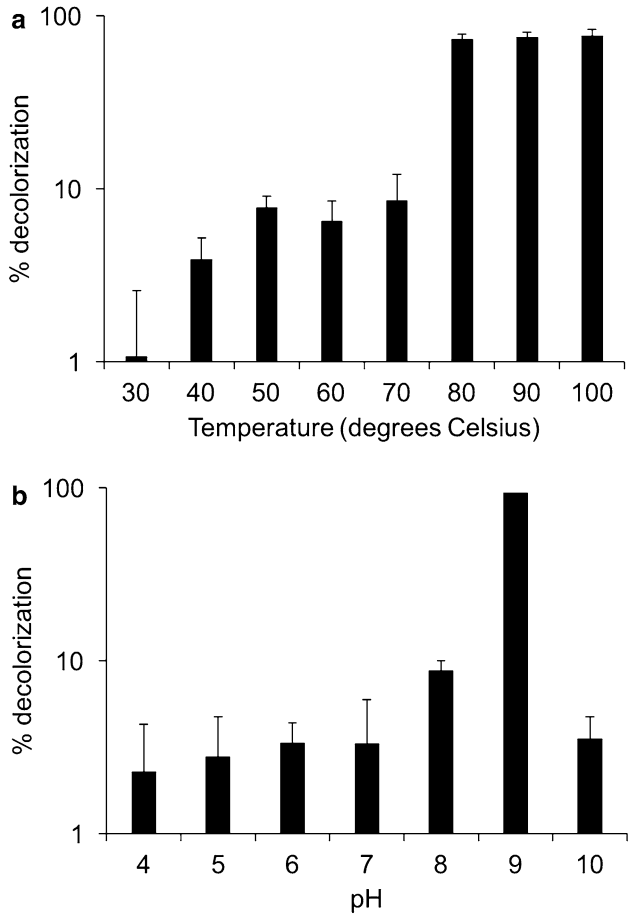


Fig. 4 Optimal temperature and pH for laccase activity. **a** Effect of temperature on laccase activity. **b** Effect of pH on laccase activity. Values on the *y axis* are in logarithmic scale. *Vertical error bars* represent standard deviation across three measurements

inhibitor, completely inhibited the decolorization of Congo Red (Fig. 3b) confirming the enzymatic decolorization of Congo Red. In order to determine the optimal conditions for activity, we studied the laccase activity at various temperatures and pH (Fig. 4). Approximately 75% of the dye was decolorized by the culture supernatant within 7 h at 80, 90, or 100 °C (Fig. 4a). Laccase activity was approximately tenfold lower at temperatures between 50 and 70 °C, approximately 20-fold lower at 40 °C, and 70-fold lower at 30 °C. Laccase activity was studied at various pH concentrations (i.e., pH 4–10) at 90 °C (Fig. 4b). Maximal activity was observed at pH 9 at 90 °C. Laccase activity was tenfold lower at pH 8, and approximately 25-fold lower at pH 6, 7, and 10. At pH 4 and 5, the laccase activity was 35- and 40-fold lower, respectively, compared to optimal activity.

Partial Purification and Activity of UARK-01 Laccase

Draft genome sequence of UARK-01 [unpublished data] revealed the presence of a single laccase gene (*Anox_Lacc*). The full-length (813 bp) gene encoding the Anox_Lacc protein (270 aa) was amplified using PCR (Fig. 5a). The gene was cloned and overexpressed in *E. coli* using the pET expression system and the Anox_Lacc protein was partially purified using heat denaturation of host proteins (data not shown). The partially purified Anox_Lacc actively decolorized Congo Red at 90 °C and pH 9 (Fig. 5b). Within 30 min, 1.64 ± 0.21 nanomoles of Congo Red was decolorized per microgram of the partially purified Anox_Lacc. Proteins from the host strain carrying the vector alone

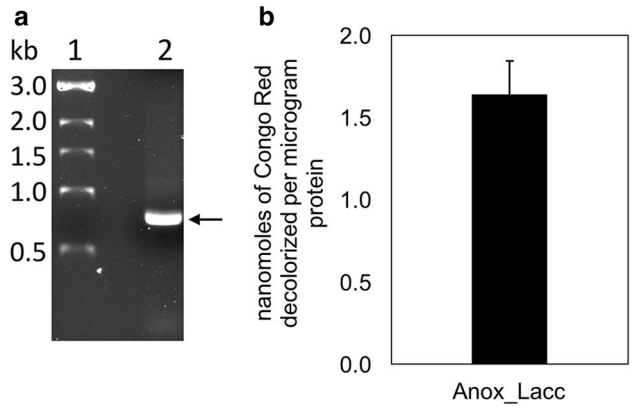


Fig. 5 Cloning and specific activity of *Anox_Lacc*. **a** Agarose gel electrophoresis showing the PCR amplified *Anox_Lacc* gene (813 bp). *Lane 1* TriDye 1 kb DNA ladder (New England Biolabs; molecular size in kb of bands is indicated on the *left*), *lane 2* PCR product from genomic DNA of UARK-01 using *Anox_Lacc* gene-specific primers. Position of the PCR band is indicated by an *arrow* on the *right*. **b** Congo Red decolorization activity of Anox_Lacc. Anox_Lacc was incubated with Congo Red at 90 °C and pH 9.0 for 30 min. The nanomoles of Congo Red decolorized per microgram of Anox_Lacc was calculated based on a Congo Red standard curve (data not shown)

(negative control) did not decolorize Congo Red (data not shown).

Bioinformatic Analyses of Anox_Lacc

Based on the sequence analysis, Anox_Lacc belongs to the multicopper polyphenol oxidoreductase laccase family (pfam02578 Cu-oxidase_4), which includes three experimentally characterized laccases [11]. Anox_Lacc shares 29–32% sequence identity with the three biochemically characterized laccases (data not shown). Specifically, it shares 32% sequence identity with the RL5 laccase from an uncultured microorganism from the microbial community of bovine rumen, 31% sequence identity with the laccase from *Bacteroides thetaiotaomicron*, and 29% sequence identity with the laccase from *Escherichia coli*. Multiple sequence alignment (Fig. 6) revealed full conservation of four amino acids that have been shown to be important for copper binding and laccase activity of RL5 [11]. These include Asn^{41}, His^{78}, Cys^{123}, and His^{140} in Anox_Lacc. Cys^{240} is fully conserved across the seven homologs, although mutation in this residue in the RL5 did not affect copper binding or enzyme activity [11]. Seven additional amino acids that are copper ligands in the RL5 laccase are not conserved across the homologs, while several other amino acids are fully conserved. Analysis of the overall amino acid composition of the laccase homologs exposed unique features of Anox_Lacc (Table 2). Particularly, Anox_Lacc is enriched in leucine and glutamine content (11.9 and 5.9%, respectively) compared to the other laccases, which contain 7.3–8.8% leucine and 1.6–3.8% glutamine residues. Also, Anox_Lacc contains more lysine (4.4%) compared to five other homologs (1.8–3.3% lysine). Similarly, in general, Anox_Lacc contains lower amounts of alanine, arginine, glycine, and serine residues compared to the other homologs. Currently, the GenBank database contains at least 19 non-redundant sequences from other *Anoxybacillus* isolates that share sequence homology with Anox_Lacc (Figs. S1 and S2); however, none of these putative proteins have been studied yet.

Discussion

Anoxybacillus species have been isolated and sequenced from around the world including from Europe, Asia, Australia, South America, and Antarctica (see Fig. 1). UARK-01 represents the first described isolate of *Anoxybacillus* from continental United States. Also, *Anoxybacillus* species described thus far have been isolated mostly from thermal environments, while UARK-01 was isolated from soil beneath a grass lawn where the temperature is 25–30 °C. The species designation of UARK-01 is unclear yet. Multiple characteristics distinguish it from the two phylogenetically closest species. In particular, growth on lignin and laccase production has not been reported in either *A. rupiensis* or *A. geothermalis*. In addition, both *A. rupiensis* and *A. geothermalis* can utilize glucose and cellobiose, while UARK-01 did not utilize either sugars from the medium under the conditions we tested. Moreover, the draft genome of UARK-01 [unpublished data] is significantly different compared to the published genome of *A. geothermalis* (data not shown). The genome sequence of *A. rupiensis* is not available. Future genomic investigations could clarify the taxonomic status of UARK-01.

Growth on lignin as well as hyperthermostable alkaline laccase activity from UARK-01 is new findings in the *Anoxybacillus* genus. Utilization of lignin as the sole carbon source has not been described for any member in this genus. Also, with the exception of one recent report, laccase activity has not been described in *Anoxybacillus*. Recently, moderately thermophilic and acidophilic laccase activity was described from *A. gonensis* strain P39, although ability of that bacterium to utilize lignin was not determined [32]. The *A. gonensis* laccase, which was optimally active at pH 5 and 60 °C, was reported to be a 40 kDa protein based on electrophoretic mobility (protein sequence of the *A. gonensis* laccase was not reported). Using BLAST search, we identified a pfam02578 family homolog (KGP60966) from a different strain of *A. gonensis*, strain G2 (data not shown). The *A. gonensis* protein shares 57% sequence identity with the Anox_Lacc and has a considerably different amino acid

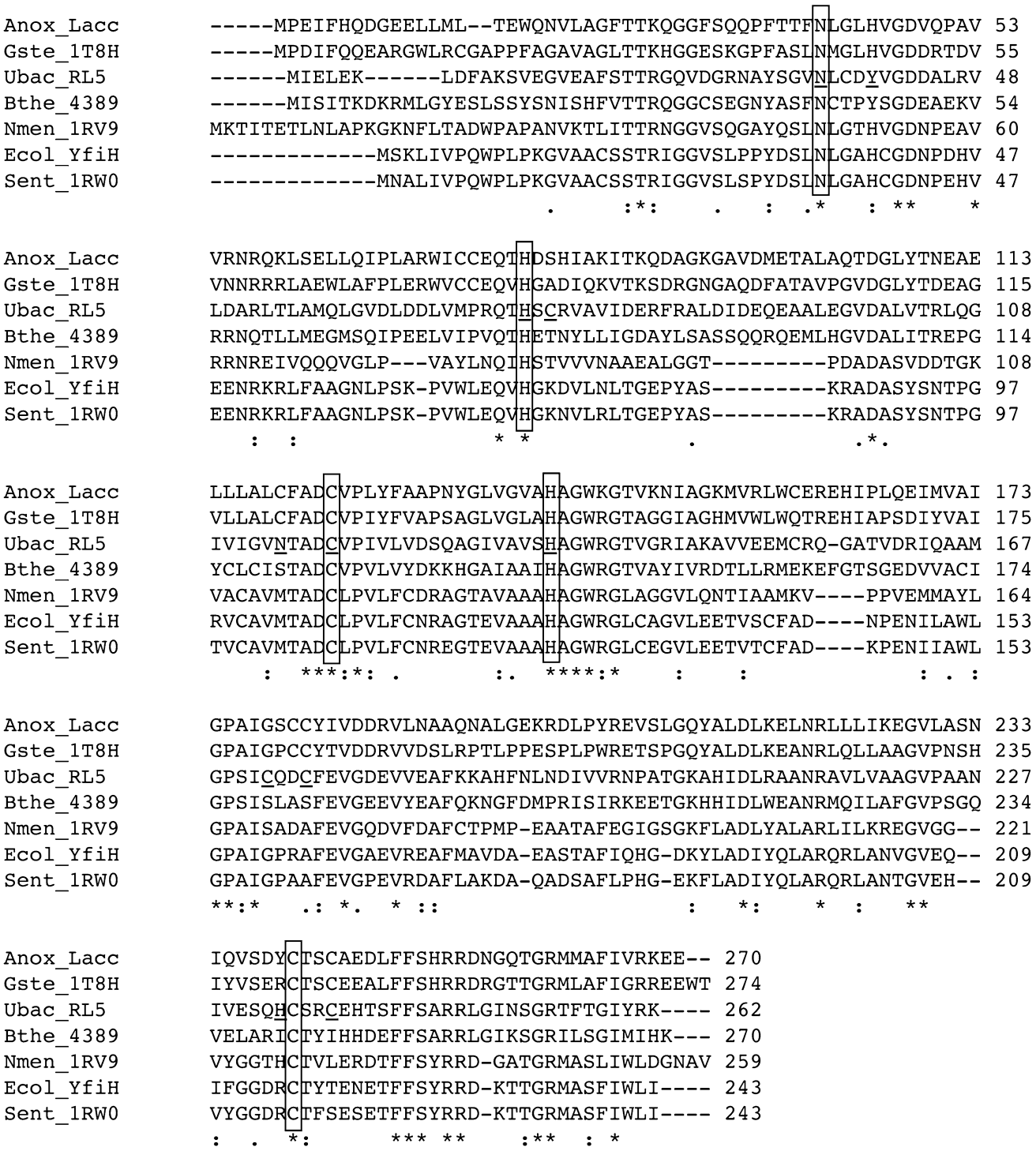


Fig. 6 Multiple sequence alignment of laccase homologs from the pfam02578 family. Anox_Lacc: UARK-01 laccase (this study); Ubac_RL5: laccase from an uncultured microorganism from the microbial community of bovine rumen (accession# CAK32504); Bthe_4389: *Bacteroides thetaiotaomicron* VPI-5482 laccase (NP_813300.1); Gste_1T8H: *Geobacillus stearothermophilus* protein (pdb|1T8H); Ecol_YfiH: *Escherichia coli* O157:H7 str. EDL933 laccase (AAG57706.1); Nmen_1RV9: *Neisseria meningitidis* protein Nmb0706 (pdb|1RV9); Sent_1RW0: *Salmonella enterica* serovar *typhi* protein (pdb|1RW0). The copper ligand residues in the RL5 laccase are *underlined*. Fully conserved asparagine, cysteine, and histidine residues are indicated with *boxes*

Table 2 Properties of laccase homologs from the pfam02578 family

Protein	Size (aa)	T_{opt} (°C)	pH_{opt}	Percentage of each amino acid																	
				A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
Anox_Lacc	**270**	**90**	**9.0**	9.3	4.8	4.1	5.2	3.3	5.9	7.0	7.8	2.6	5.2	**11.9**	**4.4**	2.6	3.7	3.3	3.3	4.8	1.5	2.6	6.7
Gste_1T8H	274	–	–	10.9	7.7	2.2	5.8	3.3	2.9	5.5	10.6	2.9	3.6	8.8	1.8	1.5	3.6	6.6	4.4	5.5	2.9	2.2	7.3
Ubac_RL5	262	60	4.0–5.0	12.2	8.0	3.4	7.6	3.1	3.8	5.7	8.0	2.3	5.7	7.3	2.7	1.9	3.4	1.9	4.6	4.6	0.4	1.1	12.2
Bthe_4389	270	52	4.5–6.0	6.7	5.9	2.6	4.1	2.6	3.7	8.1	8.5	3.7	8.5	7.4	4.1	3.3	3.3	3.0	8.5	5.2	0.7	4.1	5.9
Nmen_1RV9	259	–	–	14.7	4.6	4.2	5.8	1.9	3.1	3.9	10.8	1.5	3.5	8.5	3.1	2.7	3.5	4.6	3.1	7.7	1.2	2.3	9.3
Ecol_YfiH	243	44	5.5–8.4	12.3	6.2	4.5	4.9	3.3	2.5	6.2	8.6	2.1	3.7	8.2	3.3	1.6	4.1	5.8	5.3	4.9	2.1	2.9	7.4
Sent_1RW0	319	–	–	11.3	6.9	4.4	3.8	3.8	1.6	7.2	6.6	2.8	4.1	7.8	2.8	1.9	5.0	5.6	5.6	6.0	1.6	2.8	5.6

Proteins in this table are the same as those included in Fig. 5. The optimum temperature (T_{opt}) and pH (pH_{opt}) for enzyme activity of the biochemically characterized laccases were obtained from the literature [11]. The length of the protein in amino acids (aa) is provided in column 3. The percentage of each of the 20 amino acids was calculated based on the protein sequence (as shown in Fig. 5). Compared with the other homologs, amino acids that are over- and under-represented in Anox_Lacc are highlighted in bold and underline, respectively. '–' indicates that the data are not published

composition (data not shown), which may explain the differences in the thermochemical properties of the laccase activities from the two bacteria. The skewed amino acid composition of Anox_Lacc likely contributes to the thermochemical characteristics (high optimum temperature and pH) of the laccase activity from UARK-01. Mutational analyses in the future would define the amino acids that are a key to the extremophilic adaptations of Anox_Lacc.

Anox_Lacc is a novel member of the multicopper polyphenol oxidoreductase family (pfam02578 Cu-oxidase_4). Thus far, only three laccases belonging to this family have been studied, of which only RL5 has been studied in molecular detail using site-directed mutants [11]. Based on our analyses, the Anox_Lacc has very different properties than the RL5 laccase. Not only are the T_{opt} and pH_{opt} for the laccase activity from UARK-01 substantially higher than those for RL5, but also Anox_Lacc lacks seven of the twelve copper ligand residues of RL5. However, Anox_Lacc contains other conserved amino acids whose roles in copper binding and laccase activity await experimental validation. Structural and biophysical investigations of Anox_Lacc would provide novel insights into the pfam02578 family laccases.

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Compliance with Ethical Standards

Conflict of interest None.

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