Bio-prospecting Bacterial Diversity of Hot Springs in Northern Himalayan Region of India for Laccases

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Abstract Bacterial diversity of hot springs of northern Himalayan region of India was studied and explored for laccases, the multicopper enzymes applicable in a large number of industries due to their ability to utilize a wide range of substrates. 220 operational taxonomic units (OTUs) out of 5551 sequence reads for bacterial diversity and 3 OTUs out of 19 sequence reads for Laccase like multicopper oxidases (LMCOs) diversity were generated. Bacteroidetes (74.28%) was the most abundant phylum including genus *Paludibacter* (66.96%), followed by phylum Proteobacteria (24.53%) including genera *Chitinilyticum* (7.55%) and *Cellvibrio* (6.14%). In case of laccase diversity, three LMCO sequences showed affiliation with proteobacteria and one with two domain laccase from uncultivable bacteroidetes. LMCO sequences belonged to H and N families.

Keywords Hot springs · Bacteria · Laccase · Diversity

Introduction

Laccases are copper containing enzymes that catalyze the conversion of phenolic and non-phenolic compounds through a radical catalyzed reaction mechanism, using molecular oxygen. Laccase or laccase-like enzymes are found in all domains of life like plants, insects, fungi and bacteria and perform a number of biological functions viz. lignification, delignification, pathogenicity, detoxification, morphogenesis and sporulation, growth and development of rhizomorphs, protection of enzymes from oxygen, polymerization of melanin precursors, and spore coat resistance. Laccase like multicopper oxidases (LMCOs) is a term proposed in order to account for the potential multiplicity of their biological functions [1]. Laccases have potential applications in several industries and biotechnological units [2, 3]. But low production yields, low redox potential, requirement of costly mediators and instability at wide pH and temperature ranges are some of the hurdles in the journey of laccases from lab to commercialization scale. It is therefore necessary to explore diverse habitats for novel laccases [4].

Thermophilic bacteria have gained world-wide importance due to their ability to produce thermostable enzymes applicable in various pharma and industrial set-ups [5]. Hot springs are the natural habitats of thermophilic and hyperthermophilic microorganisms. Bacterial laccase producers viz. *Brevibacillus* sp. (Z1) and *Anoxybacillus gonensis*, showing high laccase catalytic activity, were isolated from hot springs [6, 7]. However, thermostable laccase producers identified till now are very few in comparison to total microbes present in hot springs. Despite previous effort [8], a comprehensive census of the bacterial community in hot springs of Nubra valley is still lacking as only culturable diversity was studied.

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Additionally, identifying sources of desirable quality laccases applicable in industry will help implement laccase based technology at pilot scale. Therefore, evidences of occurrence of laccases in hot springs and increasing demand of laccases for safe and ecofriendly technology propelled us to address these issues by exploring the bacterial diversity and bioprospecting it for novel laccases in the hot springs situated at the highest altitude studied so far in northern Himalayan region of India.

Materials and Methods

Sample Collection

Soil and water samples were obtained from hot sulphur springs of Leh (Nubra valley, Ladakh, India) located at latitude 34°7'9829"N and longitude of 77°28'43.5227"E and at elevation of 4475 m above sea level with temperature range of 60°–80 °C & pH 8.0–8.5. The water samples contained bicarbonate (290 mg/l), sulfate (258 mg/l) and sodium (355 mg/l) as the most abundant trace elements followed by chlorine (113 mg/l), boron (38 mg/l) and potassium (21 mg/l). eDNA was isolated and was further purified for removal of humic acids.

Phenol Oxidase (Laccase) Activity

Laccase activity in soil and water samples was measured as described by Floch et al. [9]. 1 g soil sample with 9 ml of hot spring water was shaken for 1 h with glass beads on a horizontal shaker. This soil suspension was centrifuged and supernatant was used for assaying the activity using 2 mM ABTS at different pH (5, 6, 7, 8) and temperatures (55–75 °C).

Amplification and Next Generation Sequencing of 16SrRNA Amplicon

The 16SrRNA gene amplification from eDNA was performed using universal primers 8F (5'-GAGTTT GATCMTGGCTCAG-3') and 1492R (5'-GGTT ACCTTGTTACGACTT-3') to yield a 1600 bp PCR amplicon. The purified DNA was further used to amplify the variable region of the 16S rRNA gene (V1-3) using 8F and 519R (5'-GTNTTACNGCGGCKGCTG-3') primers. These primers contained appropriate adaptors (adaptor 1: 5'-CCATCTCATCCCTGCGTGTCTCCGAC-3' and adaptor 2: 5'-CCTATCCCCTGTGTGCCTTGGCAGTC-3') and barcode sequences (7–11 bp long unique sequence) that were necessary for running the samples on the GS-FLX-Titanium (Roche) sequencer.

Assessment of Bacterial Community

Barcode, linker, and PCR primer sequences were trimmed before the assembly of sequences and removal of Chimeric sequences (UCHIME) [10]. The taxa were assigned to sequences using EzTaxon [11]. The OTUs were defined with the CD-HIT program. Shannon and Simpson indices were generated with CLcommunity software.

Amplification, Cloning and Sequencing of LMCO Genes

Conserved LMCO gene was amplified using degenerate primers Cu1AF (5'-ACMWCBGTYCAYTGGCAYGG-3') and Cu2R (5'-GRCTGTGGTACCAGAANGTNCC-3') [12]. T-Vector (INSTANT CLONING KIT, GeNei) and purified PCR amplicon were ligated by using T4 DNA ligase. *Escherichia coli* DH10 β electrocompetent cells were electroporated with ligated DNA using optimal settings (1.0 mm cuvette, 10 μ F capacitor and 1800 V).

Bioinformatics and Statistical Analysis of LMCO Sequences

Nucleotide sequences of clones were compared with the databases of the National Center for Biotechnology Information (NCBI) using the BLAST search algorithm. Further, sequences were used for construction of neighbor joining tree using MEGA5. The operational taxonomic units (OTUs) were established at 3% genetic distance. OTU based analysis (Shannon and Simpson diversity indices) was performed using Mothur software [13].

Accession Numbers

16SrRNA data reads obtained were stored with run accession SRR1997903 in NCBI. LMCO gene sequences obtained were deposited in the GeneBank nucleotide database of NCBI with accession numbers KT187934 to KT187937.

Results and Discussion

In this study, bacterial and LMCO diversities have been reported from Nubra valley situated at the highest elevation (4475 m) studied so far. High altitude, high temperature and undisturbed niche made this ecosystem a favorable site to study culturable and unculturable diversity of thermophiles and bioprospecting it for novel laccases.

Phenol Oxidase Activity

The culturable bacterial diversity isolated by using M162 medium [14] containing guaiacol (2 mM) and CuSO₄ (100 µM) at different temperatures did not show any laccase activity. However, phenol oxidase activity (10 nkat/g) was observed in soil/water suspension at 55 °C and pH 6 which suggested the presence of unculturable laccase positive microorganisms. pH of hot springs soils sample was alkaline (pH 8.0) however, maximum activity was observed at pH 6 which could be due to the use of non-phenolic substrate. The optimum pH for ABTS substrate has been reported in acidic range for bacterial and fungal laccases [15].

Bacterial Diversity

A total of 5551 16SrRNA sequence reads generated 220 OTUs. Diversity indices viz. Shannon–Wiener index (2.80) and Simpson index (0.143) were calculated at a genetic distance of 3% which represented moderate level of diversity and low evenness. Further, these sequences were classified from phylum to genus level using EzTaxon-e database [10]. The bacterial phylotypes detected are shown in Fig. 1. Bacteroidetes (74.28%) was the most abundant phylum consisting of *Paludibacter* (66.96%) as the most abundant genus followed by phylum Proteobacteria (24.53%) with genera *Chitinilyticum* (7.55%) and *Cel-livibrio* (6.14%). The order Bacteroidales (68%) was the most abundant followed by Cytophagales (4%) and Sphingobacteriales (1%) belonging to the phylum Bacteroidetes. In case of Proteobacteria, order Neisseriales (8%) belonging to class β-proteobacteria was the most abundant followed by Alteromonadales (6%), Burkholderiales (4%), Pseudomonadales (2%) and Enterobacteriales (2%). Orders representing less than 1% of sequences or phylotypes formed 3% of the total microbial community (Fig. 2).

Bacterial diversity observed in this study was different from previous studies (Table S1). Sequences in this study have shown similarity with those of genera viz *Acinetobacter*, *Pseudomonas*, *Chryseobacterium*, *Methylophilus*, *Ralstonia*, *Elizabethkingia*, *Delftia* and *Acidovorax* which were earlier found in ponds situated in Krishima geothermal area [16], and genera like *Leadbetterella*, *Rheinheimera*, *Thauera*, *Hydrogenophaga*, *Legionella* and *Azospirillum* observed in Siloam hot spring, South Africa [17]. Physicochemical conditions like temperature range, alkaline pH and trace elements of these hot springs were similar to those explored in this study except the altitude. Furthermore, majority of the bacteria were anaerobic or facultative anaerobic in comparison to other hot springs that were dominated by aerobic bacteria. This variation

may be due to high temperature and altitude of the studied hot spring where oxygen concentration is low. Another study [8] reported aerobic culturables only from Ladakh hot springs, and found Proteobacteria, Firmicutes and Actinobacteria but not Bacteroidetes. *Paludibacter* does not require oxygen, nitrate, sulfate, thiosulfate, elemental sulfur, sulfite, and Fe(III) nitrilotriacetate as terminal electron acceptors [18]. Limitation of all these compounds in studied hot springs correlates with the presence of *Paludibacter*. Sequence reads representing similarity with *Paludibacter* formed six different OTUs with different similarity levels (88–98%) which suggests the existence of various novel species related to this genus. Another major dominant group of sequences matched with *Chitinilyticum* which is known to have chitinolytic activity and also accumulate poly-beta-hydroxybutyrate [19]. Bacteria with chitinolytic activity have been reported from hot springs previously, however no *Chitinilyticum* has been isolated till now. Sequences that showed 89–97% similarity with *Cel-livibrio* suggested novel species and these species may harbor novel enzymes able to degrade organic matter present in hot springs. Above mentioned genera were formerly isolated from mesophilic environments but existence of these genera in hot springs shows the diverse range of temperature for their growth.

Malikia, another dominant group, is an aerobic poly-hydroxyalkanoate-accumulating proteobacterium [20]. It has been also found that oxygen limited conditions enhance PHA production [21, 22]. High temperature limits the solubility of oxygen and high altitude further caused marked decrease in oxygen in the studied hot springs which may have increased the occurrence of PHA producers. *Rhodococcus* found at this site is also known to produce PHA [23]. *Rhodococcus* was the only actinobacterium found from leh hot spring in comparison to other studies in which *Propionibacterium* sp., *Kocuria* sp. and *Cellulosimicrobium* sp. with or without *Rhodococcus* were observed (Table S1). Khalilova et al. [24] identified *Rhodococcus* as the major actinobacterium in Kizlyarskoye hot springs. pH and trace elements present in this hot spring were similar to Leh hot spring. It was observed that specific bacterial groups correlated with physicochemical conditions of the environment [15]. This can be the probable reason for finding *Rhodococcus* as the only or major actinobacterium in both the sites.

Pedobacter and *Enterobacter* were also the dominant genera in our study. These were also dominant in Big Spring in Thermopolis, Wyoming [25]. Volova et al. [26] reported PHA degradation potential of *Enterobacter* for the first time that may help in maintaining carbon cycle. *Aurantimonas*, *Paracoccus* sp., *Cellulosimicrobium*, *Kocuria*, *Staphylococcus* and *Brevundimonas* were the genera previously found in culturable diversity of

Fig. 1 Pie chart (a) representing the composition of bacterial community [phyla (b) and genera (c, d, e)]

Chumanthang hot spring [17]. Except *Aurantimonas* and *Brevundimonas*, all other genera were absent in our study and a new psychrotrophic genus *Janthinobacterium* was present. The presence of these genera suggests migration of bacterial communities from the sediments to the spring water aided by its flow. This variation may depend on

tolerance of bacteria and sampling time that varies with every study. Another group of sequences has shown low similarity (<90%) with reference sequences available in GeneBank. These sequences were classified under phyla bacteroidetes and proteobacteria representing unculturable groups.

possible that this hot spring may harbour bacteria from order bacteroidales having similarity with the genome of *Paludibacter* or *Acetothermus*. Three LMCO genes showed identity with those of species from class gammaproteobacteria. Two out of three clustered with LMCOs of *Shewanella*. Translated protein sequence of these LMCO genes clustered with laccase protein from *Halomonas*. This showed the affiliation of these sequences with class gammaproteobacteria but did not allow classifying them at genus level. We observed *Shewanella* in bacterial diversity but not *Halomonas*. It could be possible that this LMCO sequence may belong to *Shewanella* or belong to yet to be identified unculturable proteobacterium having similarity with the genome of *Halomonas*. The third LMCO did not cluster with any LMCO nucleotide sequence. However translated laccase protein sequence clustered with *Alisewanella* from the order Altermonadales. 16S rRNA sequence for *Alisewanella* was not found though 16S rRNA sequences for order Altermonadales were present. This suggested the affiliation of this laccase protein with genera from order Altermonadales.

Conclusion

Several bacterial sequences did not show any affiliation to known culturable bacteria, thus indicating novel diversity. Simultaneously, LMCOs observed in this study represented less than 96% similarity at nucleotide and amino acid level with known ones, thus indicating novel laccases which might have potential applications in industries like pulp and paper mills. Novel thermo-alkali stable laccases that do not require mediators to function are the future industrial requirements. Low bacterial diversity due to restricted environment in hot springs, absence of aromatic phenolic compounds because of less pollution and low oxygen concentration may contribute to low LMCO diversity. It would be interesting to explore these hot ecosystems for novel bacterial and LMCO diversity in different seasons and time spaces.

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References

- McCaig BC, Meagher RB, Dean JFD (2005) Gene structure and molecular analysis of the laccase-like multicopper oxidase (LMCO) gene family in *Arabidopsis thaliana*. *Planta* 221:619–636. doi:10.1007/s00425-004-1472-6
- Wang C, Zhang H, Ren D, Li Q, Zhang S, Feng T (2015) Effect of direct-current electric field on enzymatic activity and the concentration of laccase. *Indian J Microbiol* 55:278–284. doi:10.1007/s12088-015-0523-y
- Muthukumarasamy NP, Murugan S (2014) Production, purification and application of bacterial laccase: a Review. *Biotechnology* 13:196–205. doi:10.3923/biotech.2014.196.205
- Gupta V, Capalash N, Gupta N, Sharma P (2017) Bio-prospecting laccases in the bacterial diversity of activated sludge from pulp and paper industry. *Indian J Microbiol* 57:75–82. doi:10.1007/s12088-016-0624-2
- Turner P, Mamo G, Karlsson EN (2007) Potential and utilization of thermophiles and thermostable enzymes in biorefining. *Microb Cell Fact* 6:1–23. doi:10.1186/1475-2859-6-9
- Bozoglu C, Adiguzel A, Nadaroglu H, Yanmis D, Gulluce M (2013) Purification and characterization of laccase from newly isolated thermophilic *Brevibacillus* sp. (Z1) and its applications in removal of textile dyes. *Res J BioTechnol* 8:56–66
- Yannis D, Adiguzel A, Nadaroglu H, Gulluce M, Demir N (2016) Purification and characterization of laccase from thermophilic *Anoxybacillus gonensis* P39 and its application of removal textile dyes. *Rom Biotechnol Lett* 21:11485–11496
- Kumar M, Yadav AN, Tiwari R, Prasanna R, Saxena AK (2014) Evaluating the diversity of culturable thermotolerant bacteria from four hot springs of India. *J Biodivers Biopros Dev* 1:1–9. doi:10.4172/2376-0214.1000127
- Floch C, Alarcon-Gutierrez E, Criquet S (2007) ABTS assay of phenol oxidase activity in soil. *J Microbiol Methods* 71:319–324. doi:10.1016/j.mimet.2007.09.020
- Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R (2011) UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27:2194–2200. doi:10.1093/bioinformatics/btr381
- Kim OS, Cho YJ, Lee K, Yoon SH, Kim M, Na H, Park SC, Jeon YS, Lee JH, Yi H, Won S, Chun J (2012) Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *Int J Syst Evol Microbiol* 62:716–721. doi:10.1099/ijs.0.038075-0
- Kellner H, Luis P, Zimdars B (2008) Diversity of bacterial laccase-like multicopper oxidase genes in forest and grassland cambisol soil samples. *Soil Biol Biochem* 40:638–648. doi:10.1016/j.soilbio.2007.09.013
- Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Horn DJV, Weber CF (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* 75:7537–7541. doi:10.1128/AEM.01541-09
- Degryse E, Glansdorff N, Pierard A (1978) A comparative analysis of thermophilic bacteria belonging to the genus *Thermus*. *Arch Microbiol* 117:189–196
- Maestre-Reyna M, Liu WC, Jeng WY, Lee CC, Hsu CA, Wen TN, Wang AHJ, Shyur LF (2015) Structural and functional roles of glycosylation in fungal laccase from *Lentinus* sp. *PLoS ONE* 10(4):e0120601. doi:10.1371/journal.pone.0120601
- Satoh T (2013) Prokaryotic communities and biodiversity in the highly acidic hot springs. Ph.D. thesis for Soka University
- Tekere M, Lotter A, Olivier J, Jonker N, Venter S (2011) Metagenomic analysis of bacterial diversity of Siloam hot water spring, Limpopo, South Africa. *Afr J Biotechnol* 10:18005–18012. doi:10.5897/AJB11.899
- Qiu YL, Kuang XZ, Shi XS, Yuan XZ, Guo RB (2014) *Paludibacter jiangxiensis* sp. nov., a strictly anaerobic, propionate-producing bacterium isolated from rice paddy field. *Arch Microbiol* 196:149–155. doi:10.1007/s00203-013-0951-1

19. Chang SC, Chen WM, Wang JT, Wu MC (2007) *Chitinilyticum aquatile* gen. nov., sp. nov., a chitinolytic bacterium isolated from a freshwater pond used for Pacific white shrimp culture. *Int J Syst Evol Microbiol* 57:2854–2860. doi:[10.1099/ijs.0.65168-0](https://doi.org/10.1099/ijs.0.65168-0)
20. Spring S, Wagner M, Schumann P, Kampfer P (2005) *Malikia granosa* gen. nov., sp. nov., a novel polyhydroxyalkanoate- and polyphosphate-accumulating bacterium isolated from activated sludge, and reclassification of *Pseudomonas spinosa* as *Malikia spinosa* comb. nov. *Int J Syst Evol Microbiol* 55:621–629. doi:[10.1099/ijs.0.63356-0](https://doi.org/10.1099/ijs.0.63356-0)
21. Quillaguaman J, Hashim S, Bento F, Mattiasson B, Hatti-Kaul R (2005) Poly(beta-hydroxybutyrate) production by a moderate halophile, *Halomonas boliviensis* LC1 using starch hydrolysate as substrate. *J Appl Microbiol* 99:151–157. doi:[10.1111/j.1365-2672.2005.02589.x](https://doi.org/10.1111/j.1365-2672.2005.02589.x)
22. Singh M, Kumar P, Patel SKS, Kalia VC (2013) Production of polyhydroxyalkanoate co-polymer by *Bacillus thuringiensis*. *Indian J Microbiol* 53:77–83. doi:[10.1007/s12088-012-0294-7](https://doi.org/10.1007/s12088-012-0294-7)
23. Ray S, Kalia VC (2017) Microbial cometabolism and polyhydroxyalkanoate co-polymers. *Indian J Microbiol* 57:39–47. doi:[10.1007/s12088-016-0622-4](https://doi.org/10.1007/s12088-016-0622-4)
24. Khalilova EA, Nuratinov RA, Kotenko SC, Islammagomedova EA (2014) Hydrocarbon-oxidizing microorganisms of hot springs and their significance in the assessment of the biodiversity of microbial communities. *Arid Ecosyst* 4:25–30. doi:[10.1134/S2079096114010028](https://doi.org/10.1134/S2079096114010028)
25. Cheney C, Holley G, Law R, Smaglik SM (2013) Genomic study of microbes in thermopolis hot springs. Abstract book. University of Wyoming, Laramie, p 17
26. Volova TG, Boyandin AN, Vasilev AD et al (2011) Biodegradation of polyhydroxyalkanoates (PHAs) in the South China Sea and identification of PHA-degrading bacteria. *Microbiology* 80:252–260. doi:[10.1134/S0026261711020184](https://doi.org/10.1134/S0026261711020184)
27. Ausec L, Dirk J, Elsas V, Mandic-mulec I (2011) Two- and three-domain bacterial laccase-like genes are present in drained peat soils. *Soil Biol Biochem* 43:975–983. doi:[10.1016/j.soilbio.2011.01.013](https://doi.org/10.1016/j.soilbio.2011.01.013)
28. Wherland S, Miyazaki K, Pecht I (2016) Intramolecular electron transfer in the bacterial two-domain multicopper oxidase mgLAC. *Biochemistry* 55:2960–2966. doi:[10.1021/acs.biochem.6b00158](https://doi.org/10.1021/acs.biochem.6b00158)