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Bioprospecting and characterization of laccase producing bacteria from paddy fields of Himachal Pradesh

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Laccase is a copper containing polyphenol oxidase that acts on a wide range of substrates, and thus used in several industrial and biotechnological applications. This enzyme is found in many plant species and is widely distributed in fungi and mostly fungal laccases are used in biotechnological applications. In contrast, little is known about bacterial laccases, although bioinformatic analysis revealed high diversity of bacterial genes for laccase like enzyme and suggests that the enzymes are widespread in bacteria. Since bacterial genetic tools and biotechnological processes are well established, developing bacterial laccases would be significantly important. Thus in the present study, significant high diversity of laccase producing bacteria from rhizosphere of rice plants from paddy fields of Himachal Pradesh was assessed. A total of 375 bacteria were isolated using Tryptone Yeast agar medium containing 5 mM guaiacol as substrate and 40 mg/L $CuSO_4$. Secondary screening for laccase activity based on their ability to oxidise tannic acid and laccase specific substrate dimethoxyphenol led to selection of 51 bacterial isolates. On the basis of morphological and biochemical characterization and laccase activity, nine bacterial isolates exhibiting maximum laccase activity were selected and molecular characterization was carried out using 16S *r*RNA gene technology. *In silico* analysis of 16S *r*RNA sequences identified these bacterial isolates as *Pseudomonas* and *Lysinibacillus* sp.

Keywords: 16S rRNA gene technology, Dimethoxyphenol, Green catalyst, Guaiacol, *Lysinibacillus*, *Pseudomona*s

Bioprospecting is exploration of biodiversity for economical biological and genetic resources using advanced technologies to develop new industrial enzymes and other valuable products. Such advances in laboratory-based biotechnology have increased the value of genetic resources and indigenous microbial wealth, providing important leads to commercially exploitable properties of resources. Bioprospecting and commercialization of bioresources by indigenous biological communities in India is moving towards the new paradigm. Though bioprospecting was largely centred on plants previously, in recent times various other forms of biodiversity, especially the microbial diversity, have been explored with considerable success. The opportunity of new industrial applications from microorganisms is as large as the variety of environments they confront. Microbes that evolve quicker provide increasing diversity of functions for industrial applications. Rapid evolution has been predicted based on great diversity and apparent plasticity of microbial products that have the characteristics of high chemical diversity, biochemical specificity and other molecular properties. Most of the microorganisms that contribute to the microbial diversity describe variability of the communities. Here, we explored the status of indigenous laccase producing bacteria from paddy field soil and roots of rice in the state of Himachal Pradesh (HP), India. The state HP harbours plenty of diverse forms of microbial life, which has remained largely unexplored with the need to search for novel forms of commercial uses of biodiversity and associated knowledge by indigenous communities.

Laccase enzyme has acquired the status of 'green catalyst' as it possesses remarkable bioremediation potential along with numerous applications in effluent detoxification, degradation of textile dyes, herbicide and insecticide degradation, wine clarification, enzymatic conversion of chemical intermediates, biosensors and organic synthesis. Laccases are exceptionally versatile enzymes and ubiquitous in nature being produced by wide variety of plants, fungi and bacteria. The majority of laccases characterized so far derived from fungi especially from white-rot *Basidiomycetes* that are efficient lignin degraders.

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Well-known laccase producing fungi are *Ascomycetes*, *Deuteromycetes*, *Basidiomycetes* and cellulolytic class. Galhaup and his co-workers[1] reported the enhanced formation of laccase by the white rot fungi *Trametes pubenscens* in the presence of copper. The important laccase producing white rot fungi are *Neurospora crassa*[2], *Agaricus bisporus*[3], *Pleurotus ostreatus*[4], *Phlebia radiata*[5], *Trametes versicolor*[6], *Cyathus stercore* [7], *Pycnoporus cinnabarinus*[8], *Pycnoporus sanguineus*[9], *Coriolus hirsutus* and *Coriolus sonatus*[10], *Bjerkandera adusta*, and *Panus tigrinus*[11]. Recently, Khan *et al*.[12] have isolated laccase from *Pleurotus sajor-caju* and studied their fermentative conditions and influence of nitrogen source, and Chaudhary and his co-workers[13] too isolated laccase from medicinal mushroom (*Ganoderma lucidum*).

Laccase in bacteria is present intracellularly and as periplasmic protoplast[14,15]. Bacterial laccases have certain advantages over fungal laccases. They are less sensitive towards alkaline conditions, halides as well as have faster growth rate[16]. Spore coat laccase can withstand high temperature and extreme pH, and thus have a potential to be used for bioremediation or application in reactor[17]. The lack of commercially available robust and inexpensive laccase is a major barrier to the widespread applications of laccases in various industrial sectors. Isolating laccase from bacteria is of commercial importance since bacterial genetics and their use in industry is well established. The first bacterial laccase was found in the plant root associated bacterium, *Azospirillum lipoferum*[18-20], where it was shown to be involved in melanin formation[19-21].Thereafter laccase has been discovered in a number of bacteria including *Bacillus subtilis, Bordetella compestris, Caulobacter crescentus, Escherichia coli, Mycobacterium tuberculosum, Pseudomonas syringae, Pseudomonas aeruginosa* and *Yersinia pestis*[15,22,23]. *Stenotrophomonas maltophilia* strain is also found to be laccase producing, which was used to degrade synthetic dyes[15,24]. So, keeping in view the importance of bacterial laccases in pollution degradation, new bacterial strains displaying laccase activity are the need of the hour. Thus, the present study was carried out to explore new indigenous laccase producing strains from the paddy field samples of Himachal Pradesh.

Materials and Methods

Sample collection

Samples in the form of rhizospheric soil and rice root from 18 different paddy fields of Una, Hamirpur and Kangra districts of Himachal Pradesh were collected in sterilized screw capped vials and sterilized plastic bags. All the samples were stored at 4°C temperature for further use.

Enrichment and isolation of microorganisms

For isolation of laccase-producing bacterial strains, 100 mL tryptone yeast (TY) culture medium was enriched with 40 mg/L $CuSO_4$ and 5 mM guaiacol and then inoculated with 10 g of rhizospheric soil sample and incubated at 37°C on a rotary shaker (150 rpm) for 96 h. 5 mL of enriched culture was further transferred to 100 mL TY culture medium supplemented with 40 mg/L $CuS0_4$ and 5 mM guaiacol and incubated at 37°C at 150 rpm for 24-48 h. Enriched samples were appropriately diluted using serial dilution technique up to 10^{-10} dilutions and spread on TY agar medium plates containing 40 mg/L $CuSO_4$ and 5 mM guaiacol. Whereas in case of root samples these were surface sterilized by using 0.2% mercuric chloride ($HgCl_2$) for 3 min followed by repeated washing with sterilized distilled water. The surface sterility of roots was cross checked by incubating the surface sterilized roots in sterilized nutrient broth overnight. One gram of surface sterilized root samples were placed in 9.0 mL of sterilized distilled water and were ground to produce slurry using pestle and mortar under aseptic conditions. The root suspension was diluted in 10 fold series and spread plate on TY medium agar plate containing 40 mg/L $CuSO_4$ and 5 mM guaiacol. The plates were incubated at 28°C for 96 h. Individual colonies were streaked and restreaked repeatedly until axenic cultures were obtained. Colonies that produced reddish brown/brown colour on guaiacol containing medium were taken as putative positive laccase producing bacterial isolates.

Secondary screening of isolates

Isolated bacterial strains were further screened for laccase production using two different laccase substrates i.e. tannic acid and dimethoxyphenol. The isolates that were able to produce black zone on TY agar medium containing 0.5% tannic acid and produced orange colour on dropping with 2% dimethoxyphenol in 100 mM of phosphate buffer (pH 6.5) were further selected as laccase producing bacterial isolates.

Morphological and metabolic characterization

Isolated bacterial strains were investigated for various morphological, microscopic and biochemical

characters further. Different morphological characters like colony colour, shape, texture, elevation and pigment formation along with microscopic characters such as Gram's characteristics and cell morphology were determined. Biochemical metabolic ability of these isolates was determined using various biochemical descriptors viz. catalase, oxidase, urease, and IMVIC test and all the isolated bacterial strains were also studied for their ability to ferment five sugars viz. glucose, lactose, sucrose, salicin and dulcitol as well as for production of H_2S gas. All the assays were carried out in triplicates.

Laccase activity

Selected bacterial isolates were further screened for laccase activity. The culture supernatant was obtained by centrifugation of overnight cultures of selected bacterial isolates at 10000-×g, 4°C for 10 min and used as crude extracellular enzyme. Cells thus obtained were washed twice and suspended in 2.0 mL of 100 mM phosphate buffer (pH 6.5) and disrupted by ultrasonication using sonicator (MP Biomedical) giving 5-6 bursts of 1 min each at 100% power, with 5 min intervals. The cell debris was removed by centrifugation and cell free supernatant was obtained which was used as crude intracellular enzyme. The intracellular as well as extracellular laccase activity was measured spectrophotometrically at 420 nm using ABTS as substrate. Catalase was added to the assay solution and incubated for 1 h at 37°C to remove the possible effect of H_2O_2 produced by the bacteria. The reaction mixture contained 200 µL aliquots of crude intracellular/extracellular enzyme preparations and 0.2 mM ABTS in 0.1 M sodium acetate buffer (pH 4.5) making final volume to 1.0 mL. The reaction was held at 32°C for 10 min followed by addition of 0.5 mL of 80% trichloroacetic acid to stop the reaction. One unit of enzyme was defined as the amount of enzyme required to oxidize 1.0 µmol of ABTS per min. The molar extinction coefficient of ABTS was found to be 36000 M^{-1} cm^{-1}.

Isolation of genomic DNA and 16S rRNA sequences PCR amplification

Total genomic DNA of selected laccase producing bacterial strains was extracted using Genomic DNA extraction Mini-kit (Real Genomics) according to the manufacture instructions. The eluted genomic DNA was stored at −20°C until further use. The PCR amplification of the 16S *r*RNA sequence from purified genomic DNA was carried out in 0.2 mL PCR tubes with 20 µL reaction volume by using universal primers specific for 16S *r*RNA gene i.e. B27F 5′-AGAGTTTGATCCTGGCTCAG-3′ and U1492R 5′-GGTTACCTTGTTACGACTT-3′. All the amplifications were performed using thermal cycler (Biorad) and the amplification reaction consisted of an initial denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 50°C for 30 s and 72°C for 2 min, and a final extension step at 72°C for 10 min. The PCR products were analyzed by electrophoresis in 1.0% (w/v) agarose gel using Bangalore Genei power system. The gels were viewed and images were captured using gel documentation system (AlphaImager 2200, Alpha Infotech Corporation, USA). To counter possible stochastic effects of PCR, five amplifications were carried out on each sample and pooled prior to purification. Amplified PCR products were eluted from the gel using gel extraction kit (Hi Yield Gel/PCR DNA Extraction Kit from Real Genomics.

Nucleotide sequencing, alignment, and phylogeny

The PCR products obtained through amplification with universal primers specific for 16S*r*RNA gene sequenced, using same upstream and downstream primers, by a commercial sequencing facility (Xcelris lab). The sequences of these bacterial isolates after sequencing were analysed using online NCBI BLAST program, http://www.ncbi.nib.gov/blast. Phylogenetic analysis was used for comparative genomics to show evolutionary relationships. The analysis began with aligning of sequences using tools like CLUSTAL W and after alignment, phylogenetic tree was constructed using molecular evolutionary genetics analysis (MEGA); version 4[25]. The evolutionary history/phylogenetic analysis were inferred using the Neighbor-joining method[26].

Result and Discussion

Industrially important laccase enzyme (EC 1.1.3.2, p-diphenol, dioxygen oxidoreductase) belongs to a gene family named as the blue-copper proteins of the oxidases and is produced by 4 type of living organisms including bacteria, insects, higher plants and fungi. So far, very few bacterial origin laccase enzymes have been purified and characterized. The first prokaryotic laccase was derived from the *Azospirillum lipoferum*, a rizospheric bacterium[18]. Thus, the status of indigenous laccase producing bacteria was explored in the different rhizospheric soil and rice root samples of paddy fields in the state of

Himachal Pradesh, which harbours plenty of diverse forms of microbial life, which has remained largely unexplored and thus there is an urgent need to search for novel forms of commercial uses of biodiversity and associated knowledge by indigenous communities. Bacterial laccases as biocatalyst have received lots of importance because of their high capacity of oxidising phenolics and other aromatic compounds. This advantage makes laccase suitable for some biotechnological applications such as biodegradation of xenobiotic compounds including methoxyphenol, aniline and benzene thiols[27,28]. Since bacterial laccases have been found to possess certain advantages over their fungal counter parts, such as withstanding high temperature, extreme pH, less sensitivity towards halides and fast growth. It has been recognised that they possess better detoxification efficiency for pollutants and increase their industrial potential in pollution degradation.

Isolation of laccase producing bacteria

Laccase enzymes produced from different microorganisms show different properties. They are unique in both culture and environmental conditions. For enrichment and isolation, Tryptone Yeast (TY) culture medium containing 5 mM guaiacol and 40 mg/L $CuSO_4$ as a common inducer was standardized. Guaiacol is used as it is a substrate, which is chromogen in nature leading to successful screening of laccase producers, which were able to produce extracellular guaiacol oxidising enzymes, and it can also act as best source of carbon for laccase production whereas copper ions were also added to the medium during enrichment culture. Copper ions are toxic even at low concentrations to lots of bacteria. However, laccases are copper-containing enzymes and play important role in copper tolerance in some bacterial species; therefore, medium supplemented with copper ions augmented the isolation of potential laccase producing strains. Similarly, addition of 40 mg/L $CuSO_4$ to the TY medium has also been reported by Bally and his co-workers[29] for isolation of *Azospirillum lipoferum*, the first laccase producing bacteria, whereas Bains and his co-workers[30] isolated *γ-proteobacterium* JB using M162 medium containing 5 mM guaiacol. Mongkolthanaruk *et al*.[31] isolated *Rhodococcus* sp., *Enterobacter* sp., *Staphylococcus saprophyticus* and *Delftia tsuruhatensis* using M162 medium containing 0.1% guaiacol while Singh and co-workers[32] isolated *Bacillus* species using M162 medium with 5 mM guaiacol. We have also earlier isolated bacteria producing laccase using M162[33]. Furhter, Sondhi and co-workers[34] isolated extracellular thermo-alkali-stable laccase producing *Bacillus tequilensis* using M162 medium supplemented with 0.2% yeast extract, 0.2 % tryptone, 100 μM $CuSO_4$, and 2 mM guaiacol.

Two hundred sixty two bacterial isolates were obtained from 90 rhizospheric soil samples and 113 from 90 rice root samples using standardized TY medium supplemented with 5 mM guaiacol and 40 mg/L $CuSO_4$. From each sample, 3-7 different types of colony morphotypes were isolated. Out of 375 total bacterial isolates, 48 isolates found to possess reddish brown colonies, whereas 113 isolates produced brown coloured colonies, indicating positive potential for laccase activity. Thus, it has also been observed that 42.93% of the total 375 bacterial isolates of paddy field samples were putative laccase producers. After secondary screening, only 51 laccase producing bacterial strains were selected on the basis of orange colour development reaction to dimethoxyphenol and black colour zone formation in case of tannic acid test. Similarly, first report on laccase activity in *Azospirillum lipoferum* has been from the rhizosphere of rice where laccase plays a role in cell pigmentation, oxidation of phenolic compounds[21,35] and/or electron transport[36] followed by isolation of *Sinorhizobium melotii* possessing laccase activity has been reported from rice rhizosphere[18,35]. However, Bains and coworkers[30] isolated laccase producing bacteria *γ-proteobacterium* JB from industrial waste water drained soil samples, Dhiman and Shirkot[33] isolated laccase producing bacteria from paper mill effluents, soil sample and water sample, Sheikhi and co-workers[38] cultivated laccase producing bacteria from wastewater of a pulp and paper industry. Mongkolthanaruk and co-workers[31] also obtained laccase producing bacteria from soil and wastewater of the pulp and paper industry while Singh and co-workers[32] isolated bacteria from earthworm cast and isolation of bacterial species with significant laccase activity from seawater, river sludge/top-soil containing organic litter[39], soil contaminated with dye, textile industry effluent and lignocellulosic wastes[40], forest soil[41] and sludge samples from effluent treatment plant of paper and textile industry and soil samples from the areas where wood was decaying[34] have also been reported.

Characterization of bacterial isolates

Selected 51 laccase producing rhizobacterial strains were characterized morphologically, micro-scopically and biochemically. These bacterial strains produced cream/brown coloured colonies on medium without guaiacol with a size range of 0.85-2.55 and regular as well as regular texture with undulate/smooth margins. 80.39% were Gram negative and 21.57% were Gram positive in nature, majority were rod shaped and only five strains were coccus in nature, eight were spore formers, motility was shown by 41 strains and 10 were nonmotile in nature. Biochemical characterization showed that only one bacterial LHN12.2 was catalase and all strains were negative for oxidase test. Out of total 51 strains 24 were urease positive, and all 51 bacteria strains tested positive for MR test and citrate test whereas negative for VP test and indole test. All these strains showed significant variation to ferment sugars and H_2S production. Based on these morphological and metabolic characters, dendrogram were constructed using NTSYS version 2.0 program to find out relatedness among these isolates. Dendrogram based on the morphological separated these 51 isolates into two big clusters which further separated these 51 isolates into 13 groups on the basis of morphological characters whereas on the basis of biochemical descriptors these 51 bacterial isolates were also divided into 13 groups. These 51 selected laccase producing bacterial isolates were assessed for laccase enzyme activity using ABTS as substrate and bacterial isolates LHN12.2, LHN9.1, LHN8.1, LHB9.1, LHB7.1, LUD7.1, LUA14.1, LUA15.1 and LKM7.1 (Tables 1 and 2; Fig. 1) depicting maximum laccase activity were selected further for 16S *rRNA* sequences analysis.

16S *rRNA* sequences analysis

16S *rRNA* sequences of the selected ten bacterial isolates were amplified (Fig. 2) and sequenced.

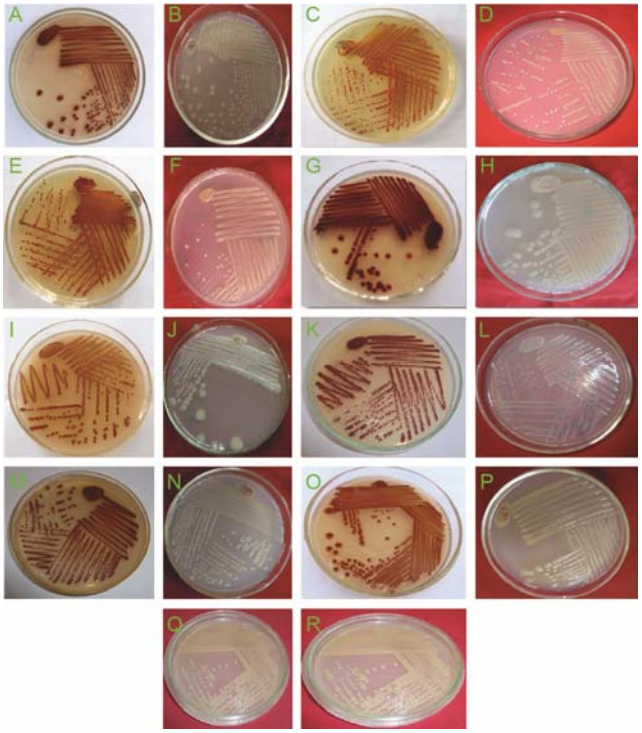

Fig. 1 — Selected laccase producing bacterial isolates of paddy fields on guaiacol medium and on without guaiacol medium. [(A,B) LUD7.1; (C,D) LHB7.1; (E,F) LHN9.1; (G,H) LHN8.1; (I,J) LHB9.1; (K,L) LUA14.1; (M,N) LUA15.1; (O,P) LUA14.1; and (Q,R) LKM7.1]

Table 2 — Microscopic characterization of nine selected bacterial isolates of paddy field samples

Isolate	Gram's reaction	Shape	Arrangement of cells	Spore formation	Motility	Pellicle formation
LHN8.1	-	Rods	Single	-	+	+
LHN9.1	-	Rods	Single	-	+	+
LHN12.2	-	Rods	Single	-	+	+
LUA14.1	-	Rods	Single	-	+	+
LUA15.1	-	Rods	Single	-	+	+
LUD7.1	-	Rods	Single	-	+	+
LHB7.1	-	Rods	Single	-	+	+
LHB9.1	-	Rods	Single	-	+	+
LKM7.1	+	Rods	Single	+	-	+

Table 1 — Biochemical characterization of nine selected bacterial isolates of paddy field samples

Isolate	Catalase	Oxidase	Urea	IMViC test				Fermentation of sugars						TSI
				Indole	MR	VP	Citrate	Glucose	Sucrose	Lactose	Dulcitol	Salicine	Manitol	
LHN8.1	+	+	-	-	+	-	+	+	+	+	-	+	+	-
LHN9.1	+	+	+	-	+	-	+	+	+	+	-	+	+	+
LHN12.2	+	+	-	-	+	-	+	+	-	-	-	-	-	-
LUA14.1	+	+	-	-	+	-	+	+	-	-	-	-	-	+
LUA15.1	+	+	-	-	+	-	+	+	+	+	-	+	+	+
LUD7.1	+	+	+	-	+	-	+	+	+	-	-	-	-	+
LHB7.1	+	+	-	-	+	-	+	+	-	-	-	-	-	-
LHB9.1	+	+	-	-	+	-	+	+	+	-	-	-	-	-
LKM7.1	+	+	+	-	+	-	+	-	-	-	-	-	-	-

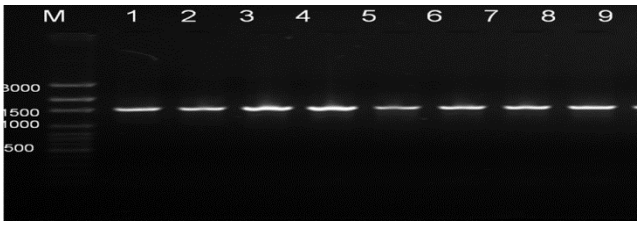

Fig. 2 — Amplicons of 16S *rRNA* sequences of selected five laccase producing bacterial isolates. [Lane 1: 100 bp DNA ladder; Lane 2: LUA15.1; Lane 3: LHB7.1; Lane 4: LUD7.1; Lane 5: LUA14.1; Lane 6: LHN9.1; Lane 7: LHN8.1; Lane 8: LHB9.1; Lane 9: LHN12.2; and Lane 10: LKM7.1]

The comparative analysis of DNA sequences with available NCBI database determined that LHN12.2, LHN8.1, LHN9.1, LHB9.1, LHB7.1, LUA14.1, LUA15.1 and LUD7.1 were found close to the members of genus *Pseudomonas*, and LKM7.1 was found close to the members of genus *Lysnibacillus*. The sequence similarity and phylogeny based on Clustal W indicated that the strain (Fig. 3 A-C) LUA15.1 bacterial isolate showed 99% similarity with *Pseudomonas putida* KT2440 strain KT2440, 16S ribosomal RNA, complete sequence, LUA14.1 bacterial isolate, showed 98% similarity with *Pseudomonas veronii* strain CP 104663, 16S ribosomal RNA, complete sequence. LUD7.1 bacterial isolate showed 99% similarity with *Pseudomonas chlororaphis* subsp. aurantiaca strain NCIB 10068, 16S ribosomal RNA, complete sequence. LHB7.1 bacterial isolate, showed 99% similarity with *Pseudomonas putida* F1 strain F1, 16S ribosomal RNA, complete sequence. LHB9.1 bacterial isolate, showed 99% similarity with *Pseudomonas umsongensis* strain Ps 3-10 16S ribosomal RNA, partial sequence. LHN12.2 showed 99% similarity with *Pseudomonas mohnii* strain: IpA-2 16S ribosomal RNA, partial sequence. LHN8.1 bacterial isolate showed 99% similarity with *Pseudomonas graminis* strain DSM 11363 16S ribosomal RNA, complete sequence and LHN9.1 bacterial isolate showed 97% similarity with *Pseudomonas jessenii* strain CIP 105274 16S ribosomal RNA, partial sequence. Whereas 16S *rRNA* sequences sequence analysis of bacterial isolate LKM7.1 bacterial isolate, showed 99% similarity with *Lysinibacillus fusiformis* strain DSM 2898 16S ribosomal RNA, complete sequence (Fig. 3 A-C). The 16S rRNA sequence of all these bacterial isolates were submitted to the National Centre for Biotechnology Information (NCBI) databases. The role of laccase enzyme in these isolated bacteria

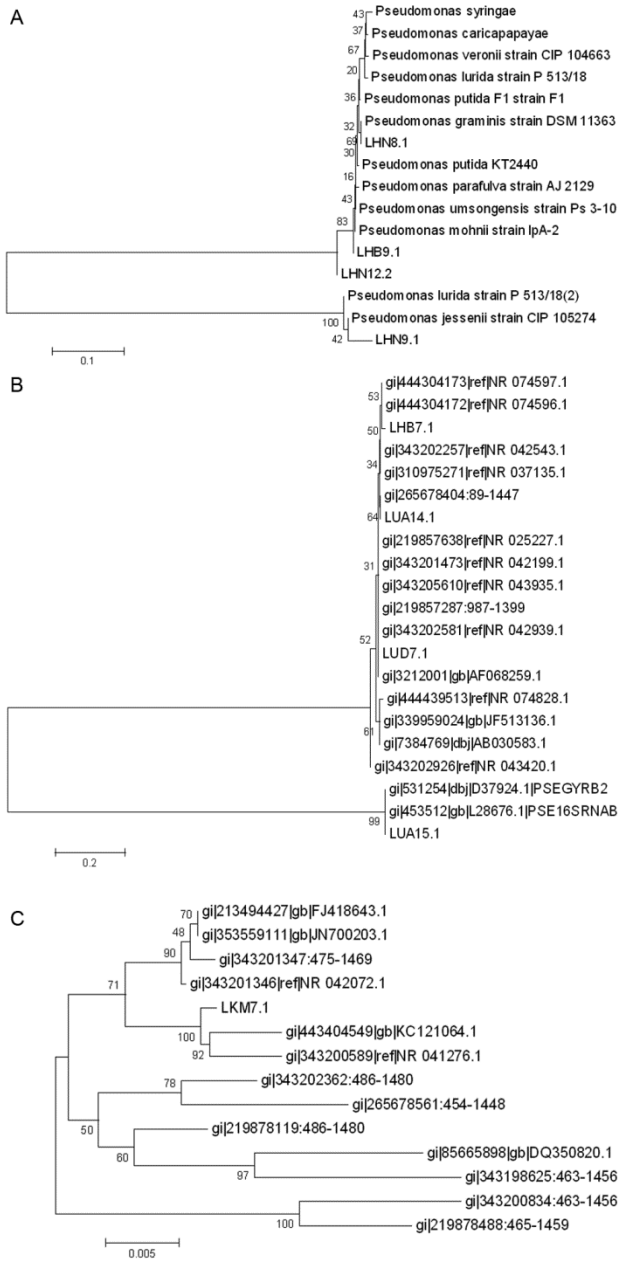

Fig. 3 — Phylogenetic tree of *Pseudomonas* species 16S *rRNA* sequences. (A) strains LHN8.1, LHB9.1, LHN12.2 and LHN9.1; (B) strains LHB7.1, LUA14.1, LUD7.1 and LUA15.1; and (C) strain LKM7.1. [GenBank accession numbers of the analyzed sequences are shown].

might be oxidation of phenolic compounds which is produced by the rice plants in the rhizospheric region. In another study, the first laccase producing bacteria *Azospirillum lipoferum* was reported to be isolated from rice rhizopshere.

Conclusion

It is concluded that nine bacterial isolates viz., exhibiting laccase activity have been screened

from rhizospheric soil and has been characterized. These strains showed activity of catalyzing canonical laccase substrates, guaiacol, tannic acid, dimethoxy-phenol and ABTS. Although gene encoding laccase protein sequence has been reported in the complete genome of all these bacterial isolates using prokaryotic genome annotation tool of NCBI but to the best of our knowledge, this is the first report to describe the isolation of culturable laccase producing *Pseudomonas jesseni*, *Pseudomonas graminis*, *Pseudomonas mohnii*, *Pseudomonas umsongensis*, *Pseudomonas veronii*, *Pseudomonas chlororaphis*, *Lysinibacillus fusiformis* and *Pseudomonas putida* from rice rhizospheric soil samples of paddy fields.

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