Indian Journal of Biochemistry & Biophysics
Vol. 58, April 2021, pp. 148-155

Isolation and identification of lignin degrading bacteria residing in mandeepkhol cave of Chhattisgarh: A RAPD Study

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Received 01 January 2020; *revised 02 August 2020*

Lignin is biopolymer synthesized by plants and it consists upto 30% dry mass weight of any woody tissue. It is also known as second most abundant source of carbon after cellulose but still has a major problem, *i.e.* very tough to degrade. Thus, in present work authors were studied about the degradation of lignin by bacteria. The paper reports isolation, screening and identification of lignin degrading bacteria from collected samples. The bacterial strains were isolated from a cave that is located in the Chhattisgarh state of India in the mountain range of Salewara which have a temperature range between 26-28°C. A total of 32 bacterial isolates were studied and their RAPD analysis was performed and it was observed that *Brevundimonas diminuta*, *Bacillus thuringiensis* and *Bacillus cereus* were isolated in lignin- rich media which showed evidence of high lignin degradation.

Keywords: 16S rDNA, Bioleaching, Lignin, RAPD

Biodegradation is the best way of breaking down organic matters into nutrients that can be used by other organisms and hence help in recycling waste. This can be used for managing waste and environmental remediation. It has been noticed that many species of Bacteria are known for the process of degradation which is essential for natural recycling also. Nearly 80-90% of carbon sources in different forms are locked by plants with approximate ratio of 35% cellulose, 25% hemicellulose including pectin, and up to 35% lignin, depending on the plant species[1]. As lignin is very hard to degrade by both chemical and biological ways, so scientists in the recent scenario are exploring potential microorganisms that can degrade lignin. The importance of ligninolytic bacteria with their enzymes is appreciated globally as they are for the pretreatment of lignocellulosic materials for the production of liquid and gaseous fuels. Currently, other applications of such organisms and enzymes have been envisaged which includes bio pulping, bioleaching in paper industries, fading of industrial effluents, fabrication of aromatics from lignin, generation of improved cattle feed, and removing of heavy metals from wastewater *etc*[2,3]. It is effective in the delignification of lignocellulosic materials[4].

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On the other hand, farmers are moving towards organic fertilizers rather than practicing chemical fertilizers. Organic farming requires a huge amount of manure to cope up with the demand. Manure formation is the result of degradation and decomposition of plant and animal wastes and dead bodies that is been carried out by bacteria and fungi. Mostly it has been noticed that biodegradation is carried out in thermophilic conditions and becomes slow when conditions are not thermophilic. So by taking this in view, the present study has focused in search of mesophilic ligninolytic bacteria which can degrade this complex material at a faster speed in a mesophilic conditions also. The selected Mandeepkhol cave is an adit cave with several branched tunnels which has inhabitants of thousands of bats. It is located in a deep forest where there is not much access to humans. These bats mostly feed on fruits or other parts of plants. The guano of bats falls over the floor of the caves in large amounts which contains an abundant amount of organic matter including lignin. They cannot fully digest those plant materials and hence comes out through guano. The cave is totally dark with high humid and temperature of around 25°C throughout the year. The cave receives a huge amount of plant parts as dried wood, dried leaves *etc*. along with heavy water streams flowing from surrounding hillocks. The guano deposits and accumulated plant parts contain a large amount of cellulose, lignin

and other organic matters which get decomposed at a very faster rate by mesophilic fungi thriving inside the cave.

Methodology

Sample collection and isolation

The sample were collected from various locations of Mandeepkhol cave such as guano deposits, log and twig deposits and leaf litter deposits in a different seasons like rain, winter and summer. Isolation of bacteria was performed by standard serial dilution method[5] for which one gram of each sample was added in distilled water thereafter sample with dilution factor 10^{-6} and 10^{-7} were taken for bacterial isolation. The media used was a nutrient agar medium; the composition of which is described elsewhere[6]. The plates were kept at 26°C ± 2°C for 24 h in incubator. The composition of culture media is given in (Table 1). Individual bacterial colonies were observed after 24 h of growth which then analyzed by simple and gram staining method. Thereafter, the selected microorganism was then identified from an authentic centre, GENEI group, Bangalore.

Evaluation of lignin degradation by bacterial isolate

The isolates were purely cultured for further evaluation of lignin degradation. The isolated culture were kept in lignin media that contain lignin as sole carbon source (Table 2) and was analyzed for their growth and ligninase activity as described by Karkun *et al.*[7]. Furthermore, colonial dry weight method was used to ascertain the bacterial growth[8].

Table 1 — Component and concentration nutrient agar media

Component	Concentration
Peptone	5 g
Beef Extract	3 g
NaCl	5 g
Agar	15 g
Distilled Water	1000 mL

Table 2 — Culture media having lignin as sole carbon source

Component	Concentration
Lignin sulphonic acid sodium salt	10 g
KH_2P0_4	1.0 g
$MgS0_4$	0.5 g
NaCl	0.5 g
$FeSO_4.7H_2O$	0.01 g
$MnSO_4.H_2O$	0.01 g
NH_4NO_3	0.3 g
Agar	12 g
Distilled water	1000 mL

RAPD analysis of bacterial samples

Genomic DNA was isolated from selected bacterial culture using GeneiUltrapure™ bacterial genomic DNA purification kit (KT 162 Cat #61211620 0021730). The experimental design and condition ofthe PCR study was shown in (Tables 3 & 4), respectively. The amplification of ~1.5 kb 16S rDNA fragment of genomic DNA was achieved using consensus primer and *Taq* DNA polymerase. Thereafter, PCR product was bi-directionally sequenced using the forward, reverse and an internal primer. Sequence data was aligned and analyzed for finding the closest homologous microbes. The PCR products were loaded in 1.0% agarose gel along with StepUp™ 500 bp DNA ladder (#612651970501730).

Results and Discussions

Bacterial isolation

Total of 32 bacterial species were isolated from different deposits of Mandeepkhol cave and their growth pattern has been summarized in (Table 5). The morphology of cultured bacterial cell and its colony has been listed in (Table 6). Furthermore, microscopic appearance of selected microorganisms is shown in (Fig. 1). In the present, study it was observed that the maximum diversity was found in Guano deposits followed by leaf litter deposits and log and twig deposits out of 32 bacterial species. Growth pattern study reveals that the cream colony I, *Brevundimonas diminuta*, *Bacillus thuringiensis*, *Bacillus cereus*, transparent colony II, whitish cream colony, creamish yellow colony II, lemon yellow colony I and transparent colony 4 were found dominant and selected from all three deposit's samples as mentioned above.

Authors believes that the finding of the present work has reported first time which explain the degradation

Table 3 — Experimental design of PCR analysis

Component	Concentration
DNA	1.0 µL
Taq Buffer A (10X)	2.5 µL
dNTP mix (2.5 mM each)	1.0 µL
Forward Primer	1.0 µL
Reverse Primer	1.0 µL
Taq DNA polymerase enzyme	3 U
Glass distilled water	To make up the volume to 25 µL

Table 4 — Temperature and timing condition of each PCR cycle

Temperature	94°C	94°C	55°C	72°C	72°C
Time	5 min	1 min	45 sec	1.30 min	10 min
			35 cycles		

Table 5 — Growth pattern of all bacterial isolates collected from different cave deposits in different seasons

S. No.	Bacterial Isolate	Summer season Number of colony			Post rainy season Number of colony			Winter season Number of colony		
		Guano deposits	Leaf litter deposits	Log and twig deposits	Guano deposits	Leaf litter deposits	Log and twig deposits	Guano deposits	Leaf litter deposits	Log and twig deposits
1.	*Bacillius cereus*	++	+	++	++	++	+	++	+	
2.	*Bacillus thuringiensis*	++	+++		++	++++				
3.	*Brevundimonas diminuta*	++++	++	+	+	+++	+	++	++	++
4.	Brown smooth colony	+			++			+		+
5.	Cream colony 4				+		+			
6.	Cream colony I	++	++	++	+++	+	+	+	++	+
7.	Cream colony II	+++	+			++	+	+		
8.	Cream colony III	+			+++				++	
9.	Cream rough colony I	++				+		+++	+	
10.	Cream rough colony II	+	+	+		++	+	++		
11.	Cream rough colony III	++	+	++	+++	++			++	+
12.	Creamish yellow colony	+	++	+	++++		+++		+	
13.	Creamish yellow colony II				+	+	++		++	
14.	Lemon yellow colony I		++	++++	+	++	+	+		
15.	Lemon yellow colony II	++	+	++	+			+	+	
16.	Light pink colony				+				+	
17.	Light yellow colony I	+				+				
18.	Light yellow type II			+++	+		+	++	+	+
19.	Milky white colony			++			++	++		
20.	Orange colony	+	+	+	++	++				
21.	Pink colony				+	+		+		
22.	Transparent colony 4	+	++	+	+	+	+	+	+	+
23.	Transparent colony I		++			++		+		+
24.	Transparent colony II	++	+++		++	++	+	++	+	
25.	Transparent colony III				+		++	++		+
26.	White colony I		++		+		+		+	
27.	White colony II	++	+++		++	++				
28.	White colony III	++				+		++	++	+
29.	White rough colony				+	+		++	+	
30.	Whitish cream colony		++	++	+	+	++	++	+	
31.	Yellowish rough colony	++	++		++	+		+	++	+
32.	Yellowish smooth colony	+	+		+	++	+	+	+	

Note: + = poor, ++ = moderate, +++ = good, ++++ = excellent

Table 6 — Morphology of cultured bacterial cells and its colony

S. No.	Bacterial Isolate	Colony morphology				Cell morphology		
		Colour	Size	Shape	Texture	Cell shape	Cell grouping	Gram reaction
1)	*Bacillus cereus*	Dull white	Small	Irregular	Rough	Rod	In chain	Positive
2)	*Bacillus thuringiensis*	White	Medium	Rounded	Smooth	Rod	Single	Positive
3)	*Brevundimonas diminuta*	Light brown	Small	Rounded	Smooth	Rod	In chain	Negative
4)	Brown rough colony	Brown	Large	Irregular	Rough	Rod	In chain	Positive
5)	Brown smooth colony	Brown	Small	Rounded	Smooth	Spiral	Irregular colony	Negative
6)	Cream colony I	Cream	Small	Rounded	Smooth	Rod	In chain	Negative
7)	Cream colony III	Cream	Small	Irregular	Rough	Round	Paired	Positive
8)	Cream colour II	Cream	Medium	Irregular	Rough	Rod	Irregular colony	Negative

(*Contd*.)

Table 6 — Morphology of cultured bacterial cells and its colony (*Contd*.)

		Colony morphology				Cell morphology		
S. No	Bacterial Isolate	Colour	Size	Shape	Texture	Cell shape	Cell grouping	Gram reaction
9)	Cream rough colony I	Cream	Large	Irregular	Rough	Round	Single	Negative
10)	Cream rough II	Cream	Large	Irregular	Rough	Round	Single	Negative
11)	Cream rough III	Cream	Small	Irregular	Rough	Cocci	In chain	Positive
12)	Creamish yellow colony	Creamish yellow	Small	Rounded	Smooth	Round	In chain	Negative
13)	Creamish yellow colony II	Creamish yellow	Small	Rounded	Smooth	Elliptical	Irregular colony	Negative
14)	Lemon yellow colony I	Lemon yellow	Small	Rounded	Smooth	Rod	Irregular colony	Negative
15)	Lemon yellow colony II	Lemon yellow	Medium	Rounded	Rough	Rod	Paired	Negative
16)	Light pink colony	Light pink	Medium	Irregular	Rough	Round	Paired	Positive
17)	Light yellow colony I	Light yellow	Small	Irregular	Smooth	Round	Single	Positive
18)	Light yellow colony II	Light yellow	Medium	Rounded	Smooth	Rod	In chain	Negative
19)	Milky white colony	White	Large	Rounded	Smooth	Rod	Irregular colony	Negative
20)	Orange colony	Orange	Medium	Rounded	Smooth	Round	Tetrad	Positive
21)	Pink colony	Pink	Small	Rounded	Smooth	Rod	In chain	Negative
22)	Transparent 4	Transparent	Medium	Irregular	Smooth	Cocci	Irregular colony	Positive
23)	Transparent colony I	Transparent	Small	Rounded	Smooth	Round	Irregular colony	Negative
24)	Transparent colony II	Transparent	large	Rounded	Smooth	Rod	Single	Negative
25)	Transparent colony III	Transparent	Small	Rounded	Smooth	Rod	In chain	Negative
26)	White colony I	White	Medium	Rounded	Smooth	Rod	Single	Negative
27)	White colony II	White	Large	Rounded	Rough	Rod	Single	Positive
28)	White colony III	White	Small	Rounded	Smooth	Round	Irregular colony	Negative
29)	White rough colony	White	Medium	Irregular	Rough	Round	Single	Positive
30)	Whitish cream colony	Whitish yellow	Large	Irregular	Rough	Rod	In chain	Positive
31)	Yellowish rough colony	Yellowish	Medium	Rounded	Rough	Round	Single	Positive
32)	Yellowish smooth colony	Yellowish	Small	Rounded	Smooth	Round	In chain	Negative

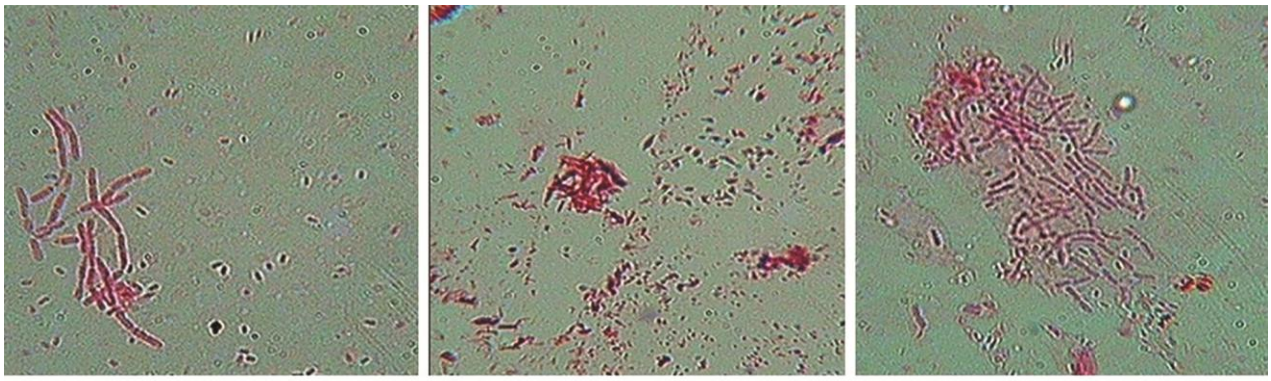

Fig. 1 — Microscopic appearance of three microorganisms selected out of 32 bacterial isolates of Mandeepkhol cave

of lignin by lignolytic bacteria isolated from Mandeepkhol cave of Chhattisgarh. Authors have already reported on microbial diversity of Mandeepkhol caves[9], while several microorganisms have also been isolated previously from different caves like Kotumsar cave[10], Borra cave[11].

Evaluation of Lignin degradation

The organisms were grown on basal agar plates containing lignin sulphonic acid sodium salt as a sole carbon source to study colonial growth and then grown in basal media containing lignin as an only carbon source to obtain colonial dry weight. Incubation periods of 7 days were given for all the test organisms and its results have been shown in (Table 7).

Out of 32 species, 6 species grown well on media containing lignin as sole carbon source, thus giving the impression that 6 species of bacteria are potent degradersas shown in (Table 7). According to molecular analysis and identification report of sample B1, B2 and B3 bacterial strain isolated from Mandeepkhol cave were *Brevundimonas diminuta*

Table 7 — Colonial growth and dry weight of bacterial isolates in lignin media

Bacterial Isolate	Growth rate	Colonial dry weight per 50 mL	Colonial dry weight per 1000 mL
Bacillus cereus (sample B3)	+	0.03 g	0.6 g
Bacillus thuringiensis (sample B2)	+	0.04 g	0.08 g
Brevundimonas diminuta (sample B1)	+	0.06 g	1.2 g
Cream colour I	+	0.02 g	0.4 g
Creamish yellow colony II	+	0.02 g	0.4 g
Transparent colony II	+	0.01 g	0.2 g

Note: + = poor, ++ = moderate, +++ = good, ++++ = excellent

Fig. 2 — Growth of selected microorganism in lignin containing medial after 7 day incubation period

(B34), *Bacillus thuringiensis* (YY23) and *Bacillus cereus* (DS 16), respectively (Fig. 2). *Brevundimonas diminuta*, *Bacillus thuringiensis*, *Bacillus cereus* were found to grow best in lignin media and showed the highest dry weight. *Brevundimonas diminuta* has shown maximum efficiency for degrading lignin followed by *Bacillus thuringiensis* and *Bacillus cereus*. Gonzalez and his co-workers used lignin as an only carbon source[12]. They successfully isolated bacterial strains and checked their ability to multiply in lignin media. Deschamps[13] in his research work has isolated lignin degrading bacteria in which kraft lignin acted as the sole carbon source. Their result revealed that *Aeromonas* sp. degraded 98% of lignin (1 g/L) after 5 days of incubation whereas our result revealed that *Brevundimonas diminuta* degraded lignin upto 6 g/L after 7 days of incubation. Yingjie Su[14] and co-workers had screened sixty- three strains of Bacteria for treatment of lignocellulose biomass of corn stover. They found three basic enzymes secreted by some bacterial strains which are laccase, lignin peroxidaseand manganese peroxidase. Hassan and Hanafy[8] during their study had noticed biodegradation of lignin by *Bacillus* species in which they had used lignin as only carbon source. Lai and coworkers[14]had isolated thermophillic lignin degrading bacteria from oil palm empty fruit bunch compost. During their work, they had isolated ten different species of bacteria from EFB compost. Naz[16] also has worked in lignin degrading bacteria where the isolation was from both mixed and black soil samples of Kuthrel agro fields of Bhilai & Durg Region. The result showed that out of four different species two species (*Bacillus and Streptomyces*) are potential in lignin degradation. Karkun *et al.*[7] has isolated lignin degrading fungi from Mandeepkhol cave where they used lignin as sole carbon source. They had isolated fungal species and screened potent lignin degraders. Waang *et al*[17] has worked on lignin degradation where they isolated bacterial species from coal samples. In their work, the result revealed that *Hauera*, *Arthrobacter* and *Rhizobium*are potent lignin degraders. Maryam and co-workers[18] also used lignin and isolated 19 microorganisms from seven genera and according to which *Bacillus*, *Pseudomonas*, *Streptomyces*, *Beauveria*, *Paecilomyces*, *Trichoderma and Trichosporon* grew on the tested media. *P. aurantiaca S-1* has only shown to grow well in lignin media.Yadav and Yadav[19] has studied enzymatic characteristics of ligninperoxidases from *Penicillium citrinum*, *Fusarium oxysporum* and *Aspergillus terrus.* In similar study, Anita and Somvirhas studied degradation of cellulose from the enzymatic activity produces from thermophilic fungus

Thermoascus aurantiacus[20]. Shanmugam *et al*[21] has isolated and screened lignin peroxidase activity of *Phanerochaete chrysoporium*. Researchers have also purified catecholase from the brinjal plant where they used lignin as a natural affiant[22]. Patel *et al* have identified bacterial consortium which consists of *Bacillus* sp., *Proteus mirabilis Alcaligenes faecalis* and *Bacillus cereus* which are capable of removing dyes and other contaminants from effluents. A recent study has also shown that the *Bacillus* group is a potential degrader that reflects the unique properties of enzymes present on it[23-24].

RAPD analysis of bacterial samples

16S rDNA fragment of three bacterial isolates collected from Mandeepkhol cave was amplified and the PCR construct was made with a length of 1500bp as shown in (Fig. 3). The sequencing of these three constructs was also performed and its result has been shown in (Fig. 4). According to the sequencing report the three bacterial species B1, B2 and B3 are actually *Brevundimonas diminuta*, *Bacillus thuringiensis*, *Bacillus cereus*, respectively. Furthermore, the RAPD study reveals that the *Bacillus thuringiensis* and *Bacillus cereus* are phylogenetically closely related, as shown in (Fig. 5). The Dendrogram of the three samples were plotted using the Neighbour-Joining cluster analysis method produced from the Jaccard estimate and RMSD coefficient (Fig. 6).

Molecular analysis is the major tool for the identification and characterization of micro-organisms[25]. Various molecular techniques based on protein, nucleic acid and lipids are available which are able to analyze structural and functional properties among microorganisms. To study genetic diversity several techniques are used such as RFLP, RAPD SSR *etc*. The PCR technique with RAPD analysis[26,27] has been well appreciated for determining the genetic diversity

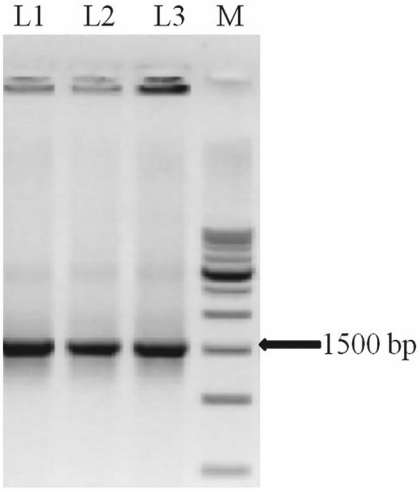

Fig. 3 — PCR construct of bacterial isolates collected from Mandeepkhol cave. L1, L2 and L3 represent the sample of *Brevundimonas diminuta*, *Bacillus thuringiensis* and *Bacillus cereus*, respectively. M represent the marker lane loaded with StepUpTM 500bp DNA ladder (Cat# 612651970501730)

Bacillus cereus

CGGGGGGGGCGGGCGTGCTATACATGCAAGTCGAGCGAATGGATTGAGAGCTTGCTCTCAAGAAGTTAGC
GGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAA
TACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGAC
CCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGG
TGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCA
ATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTT
AGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACT
ACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGC
AGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACT
TGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAG
TGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGA
TACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAG
TTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCC
CGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTC
TGAAAACCCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTC
GTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTAAGTTG
GGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTT
ATGACCTGGGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATC
TCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGCTAGTAATC
GCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTT
GTAACACCCGAAGTCGGTGGGGTAACCTTTTGGAGCCAGCCGCCTAAGGGGACAGAAGGG

Brevundimonas diminuta

TAGAGTTTGATCATGGCTCAGAGCGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAACGGACCCTTC
GGGGTTAGTGGCGGACGGGTGAGTAACACGTGGGAACGTGCCTTTAGGTTCGGAATAGCTCCTGGAAACG
GGTGGTAATGCCGAATGTGCCCTTCGGGGGAAAGATTTATCGCCTTTAGAGCGGCCCGCGTCTGATTAGC
TAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATCAGTAGCTGGTCTGAGAGGATGACCAGCCACACTG
GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATCTTGCGCAATGGGCGAAAGCCTG
ACGCAGCCATGCCGCGTGAATGATGAAGGTCTTAGGATTGTAAAATTCTTTCACCGGGGACGATAATGAC
GGTACCCGGAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTG
CTCGGAATTACTGGGCGTAAAGGGCGCGTAGGCGGATCGTTAAGTCAGAGGTGAAATCCCAGGGCTCAAC
CCTGGAACTGCCTTTGATACTGGCGATCTTGAGTATGAGAGAGGTATGTGGAACTCCGAGTGTAGAGGTG
AAATTCGTAGATATTCGGAAGAACACCAGTGGCGAAGGCGACATACTGGCTCATTACTGACGCTGAGGCG
CGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGATTGCTAGTTGT
CGGGCTGCATGCAGTTCGGTGACGCAGCTAACGCATTAAGCAATCCGCCTGGGGAGTACGGTCGCAAGAT
TAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGC
AGAACCTTACCACCTTTTGACATGCCTGGACCGCCACGGAGACGTGGCTTTCCCTTCGGGGACTAGGACA
CAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC
TCGCCATTAGTTGCCATCATTTAGTTGGGAACTCTAATGGGACTGCCGGTGCTAAGCCGGAGGAAGGTGG
GGATGACGTCAAGTCCTCATGGCCCTTACAGGGTGGGCTACACACGTGCTACAATGGCAACTACAGAGGG
TTAATCCTTAAAAGTTGTCTCAGTTCGGATTGTCCTCTGCAACTCGAGGGCATGAAGTTGGAATCGCTAG
TAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGG
AGTTGGTTCTACCCGAAGGCGGTGCGCTAACCAGCAAGGAGGCAGCCGACC

Bacillus thuringiensis

GCTCTTGCGGCGTCCTATACATGCAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCG
GACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACC
GGATAACATTTTGAACTGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGC
GTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGAT
CGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGG
ACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGG
AAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGT
GCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGT
GGTTTCTTAAGTCTGATGTGAAAGCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGT
GCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCG
AAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCC
TGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAAC
GCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCAC
AAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGAAA
ACCCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTC
GTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTAAGTTGGGCAC
TCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGAC
CTGGGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATCTCATA
AAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGCTAGTAATCGCGGA
TCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAAC
ACCCGAAGTCGGTGGGGTAACCTTTTTGGAGCCAGCCGCCTAACGTGAAC

Fig. 4 — Sequences of PCR amplified fragment of three bacterial species B1, B2 and B3 *i.e*., *Brevundimonas diminuta*, *Bacillus thuringiensis* and *Bacillus cereus*, respectively

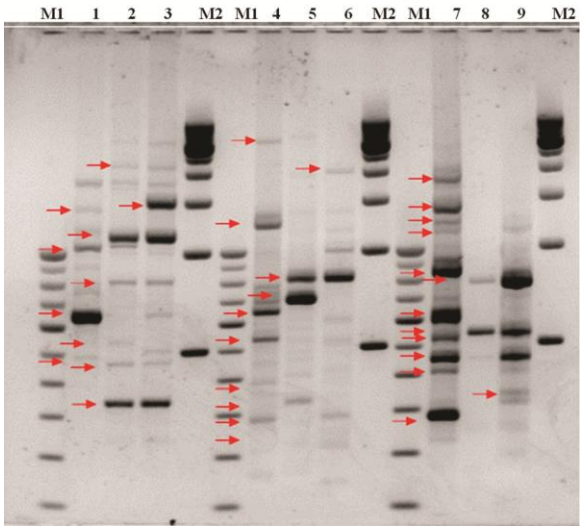

Fig. 5 — RAPD profile of three bacterial samples generated using RAPD primers. M1- StepUpTM 100 bp DNA ladder (Cat# 612652670501730), M2- StepUpTM 500 bp DNA ladder (Cat# 612651970501730), Lane 1-3, 4-6 and 7-9 are loaded with sample B1, B2 and B3 with primer B10, B12 and B14, respectively

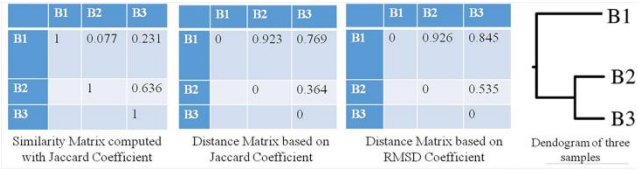

Fig. 6 — Dendrogram of the three samples using Neighbour-Joining cluster analysis method produced from Jaccard estimate and RMSD coefficient

of various fungi and bacteria and which further relates genetic diversity and identify strains[28-30]. The RAPD profiles are produced by the PCR amplification of DNA segments using arbitrary primers of usually 9 to 10 nucleotides long[31]. RAPD technique is extensively used as it is easy to implement. The nucleotide sequence of 16S rDNA has widely been accepted for discerning phylogenetic relationships among prokaryotes. Hwang *et al*[32] has termed that rRNA sequences are the first and foremost technique used biological phylogenetic nomenclature including microorganisms. Nowrouzian with co-researchers[33] has designed an RAPD typing method for the identification of E. coli strains. Hassan and Hanafy[7] have performed 16s rDNA of two species of bacteria *viz Bacillus subtilis* and *Bacillus* sp. Hanafy *et al*[34] worked on RAPD analysis of 21 bacterial isolates isolated from four Egyptian types of soil.

Conclusion

Study of the present study reveals that the three isolated bacterial strain named *Brevundimonas diminuta*, *Bacillus thuringiensis and Bacillus cereus* were found potential candidate which can degrade lignin biomass. Even phylogenetic relationships between the above stated three organisms are scientifically proved. Thus we conclude that the cave is having abundant microbial diversity regardless of speleological conditions. This is an indication of old existence of the cave which needs further geological investigation. In future, authors are interested to investigate and analyze the function of enzymes associated with lignin degradation.

Conflict of interest

All authors declare no conflict of interest.

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