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Variation of *Microcerotermes diversus* Silvestri (Isoptera: Termitidae) Gut Bacteria and Isolation of Gut Lignin-Degrading Bacteria

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Abstract

Lignin is the most abundant aromatic (phenolic) polymer and the second most plentiful raw material in the terrestrial environment. It is degraded by bacteria and fungi in the natural world. –To evaluate whether the role of symbiotic bacteria in lignin degradation among termites, four colonies of *Microcerotermes diversus* Silvestri (Termitidae) were collected from traps in Ahvaz, Bandar Abbas, and two islands of Khark and Kish. Sample suspensions were prepared from guts in 5 ml D.H2O. The samples were cultured on nutrient agar (NA) medium. The plates were incubated at a temperature of 27^{0}C for 24-72 h. Different types of bacteria with different colony morphologies were selected and purified. To differentiate lignin-degrading bacteria, all 47 bacterial isolates were grown on different extracted lignin agar containing MSM. In total, 45 isolates were positive for lignin degradation. Culture-independent 16S rDNA sequencing in combination with classical culturing methods were applied to identify the bacteria. These were assigned to genus/species *Stenotrophomonas maltophilia*, *Elizabethkingia anopheles*, *Ochrobactrum anthropic*, *Serratia marcescens*, *Lysinibacillus pakistanensis*, *Actinetobacter pitti*, *Pseudomonas* spp., and *Bacillus* spp. To specify the amount of ligninolytic activity, family selected bacteria were grown on lignin agar medium. Dye decolorization in culture plate assays with Methylene Blue was evaluated. Clear zones in agar around the isolates were measured.

Key Words: *Microcerotermes diversus*, endosymbionts, gut microbiota, lignin degradation 16S rRNA,

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Introduction

Lignin is an abundant, renewable, naturally aromatic compound that can potentially be utilized as a new option feedstock for various bio-based and value-added chemicals (Li et al., 2019). Lignin is a three-dimensional aromatic polymer composed of dimethoxylated, monomethoxylated, and non-methoxylated phenylpropanoid subunits (Li et al., 2019). Lignin is present in the secondary cell wall of plants, in areas that fill the spaces between the cellulose, hemicellulose and pectin components, making the cell wall stiffer and hydrophobic (Li et al., 2009). Lignin with its special properties provides plants with compressive strength and protection from pathogens (Li et al., 2009, 2019). Currently, millions of tons of lignin and lignin-related compounds are produced as waste effluent from the pulping and paper industries (Jablonsky and Haz, 2016). These values are expected to increase in the near future due to the recent developments aimed at replacing fossil raw materials with lignocellulosic biomass for the production of fuels and chemicals (Bandounas et al., 2011). Three groups of organisms are capable of biodegrading lignin. These are decay fungi, some soil and water micro-organisms and termites (Kato et al., 1998).

Termites are important decomposer insects, and are important pests of wooden and cellulosic products. They are often found in tropical forests and savannas as well as desert ecosystems (Brune and Ohkuma, 2010). Symbiotic microbial flora in the gut of termites play ~~s~~ important roles in lignocellulose digestion and nitrogen metabolism (Brune and Ohkuma, 2010), and especially in recent decades evidence is growing that these bacteria are capable of delignification (Gonzalo et al., 2016). Biodegradation of lignin by termites plays an important role in the carbon cycle because the world's termite population is 2.4 x 10^{17}, and the ability of termites to produce carbon dioxide is estimated at 5 x 10^{16} g (Kato et al., 1998).

Termites are composed of a variety of species, generally divided into two groups, higher and lower termites. Gastrointestinal microbiota of lower termites contain dense populations of prokaryotes and protists (single celled eukaryotes). Higher termites have only one apical family (Termitidae), but despite that they comprise 75% of all termite species. They also contain a diverse collection of prokaryotes, but lack protists in their gut (Ohkuma, 2003). The genus *Microcerotermes* is the largest genus of the Termitinae subfamily (Scheffrahn et al., 2010).

Microcerotermes diversus Silvestri is categorized among the phylogenetic group designated as higher termites, and is widespread within Iran (Ghayourfar, 1995). It is the most destructive termite in Iran, and is able to create secondary nests in walls, ceilings, and on trees, which makes it difficult to eradicate and control (Habibpour, 1994). Also, it is the most economically destructive termite in structures in Ahwaz and other southern regions of Iran, and consumes anything consisting of cellulose.

Wood and litter-feeding termites (Isoptera) are of global economic and environmental importance as lignocellulose decomposers (Kambhampati and Eggleton, 2000; Husseneder et al., 2009). To be able to digest lignocellulose effectively and use lignocellulose as the only source of nutrition, termites have a wide range of morphological and biological diversity in their gut. Density of microbial populations in termite intestines are as high as 1,012•ml^{-1} gut fluid, and therefore similar to other herbivorous and harmful invertebrates and even vertebrates (Bignell, 2000). In addition to protists and fungi, there is a remarkable community of prokaryotes from the domains of Archaea and Eubacteria, with densities of 109 to 1,011 per ml of gut fluid (Breznak, 2000, Husseneder et al., 2009).

It is well-documented application of antibiotics (Eutick et al., 1978b; Mauldin et al., 1978) or exposure to pure oxygen (Veivers et al., 1982) eradicates the termites' intestinal flora since these are-vital for termite survival (Husseneder et al., 2009).

The majority of microbes in *M. diversus*' gut are not fully known, and their role in termite feeding needs better understanding. There is a lack of information on the bacteria present in the guts of local Iranian termites. Therefore, the main objective of this study was to isolate and identify lignin-degrading bacteria in the gut of the termites.

MATERIALS AND METHODS

Sampling Sites

Termites (*M. diversus*) were collected in the Ahwaz, Bandar Abbas, and Khark and Kish Islands from infested wooden blocks previously buried in soil, and transferred to the laboratory. Rectangular beech wooden blocks used in the field to collect termites measured 20 20 x 6 20 x 2 cm. To equalize wood moisture content, these blocks were heated for 24 h at 60°C. They were then placed in the field.

To relieve stress on collected termites, they were placed in small containers and fed moistened filter paper for 24 h. Containers were kept in a dark incubator at 28 ± 2°C and 90 ± 5% relative humidity (RH). Only active, healthy worker caste were used in these tests.

Lignin Extraction

Plant sources used for extraction of lignin were dried and ground into powder (Sasikumar et al., 2014). In this study wheat straw was crushed by mills-to 0.5-cm length. Before use as a growth substrate, the wheat straw was extracted by boiling in deionized distilled water until the extraction water became discolored. Wet straw was then placed in oven for 24 h at 70°C to dry (Kerr et al., 1983). Lignin was then extracted from wheat straw by two different methods using either Alkali lignin, or Klason lignin.

Lignin-Rich Medium

Ten workers used from each of sampling sites were anesthetized. The whole body of termite workers were submerged in 70% ethanol with forceps and then gently swirled for approximately 10 s to remove surface contaminants. The specimens were taken out from the ethanol solution and allowed to dry for ~20 seconds. Sterile fine-tipped forceps were used to hold the worker's abdomen, and the tip of the abdomen was grabbed by another pair of forceps to gently pull the gut upward or downward at a 45-degree angle. If the gut is pulled at a straight angle and with too much force it is likely to break apart (Saidi et al., 2016).

Ten gut sample suspensions were enriched for 7 d at 30-45°C using a medium (MSM-L) in which lignin provided the sole carbon and energy source. MSM-L consisted of 1% lignin obtained from one of the lignin extraction methods with 'Minimal Salt Medium'(MSM) solution that contained 4.55-g K_2HPO_4, 0.53-g KH_2PO_4, 0.5-g $MgSO_4$, and 5.0-g NH_4NO_3 per liter of deionized water. These salt components were mixed together in the deionized water and autoclaved (Sasikumar et al., 2014, Kerr et al., 1983). Isolated bacteria were streaked onto fresh MSM-L agar plates to obtain pure cultures. Inoculated agar plates were incubated at 30°C for 7-d until bacteria colonies developed (Sasikumar et al., 2014).

Alkali Lignin

Ten grams of powdered wheat straw (lignin source), wheat straw already extracted with boiling water, then 5 ml of 1% sulfuric acid was added and to these components and the resulting mixture heated in hot air oven at 80°C for 20 min, and allowed to cool. Then 100 ml of 4% sodium hydroxide was added and this mixture boiled for 30 min. The resulting dark brown liquid Alkali lignin mixture was filtered and then autoclaved at 33-kg atmospheric pressure for 10 min, and then collected in a sterile polythene bag, transferred to the laboratory, and stored at -20°C. Lignin-degrading bacteria were streaked onto an agar plate containing MSM agar with Alkali lignin (MSM-AL) (Sasikumar et al., 2014).

Klason Lignin

0.25 g of extracted wheat straw was treated with 72% sulfuric acid at 15°C for 2 h. This mixture was then diluted with water to 3% acid concentration and refluxed for 4 h, and the straw residue was then washed thoroughly with water (Kerr et al., 1983). Lignin degrading bacteria were streaked onto an agar plates containing MSM and Klason lignin (MSM-KL).

Identification of Bacteria Isolates

Bacteria isolates were identified by morphological and biochemical tests such as gram staining test (Lay, 1994), motility test (Cappuccino and Sherman, 1992), fluorescent pigmentation on King's B Medium (Murray et al., 2003), oxidase test (Ijong, 2003), catalase test (Murinda et al., 2002), oxidative/fermentation glucose test (Leboffe et al., 2008; Hugh and Leifson, 1953), Levan production (Sangiliyandi et al., 1999), and a spore staining test (André et al., 2013).

Isolation and Screening of Lignin-Degrading Bacteria

For detection of lignin degraders, bacteria strains were grown and isolated at 30°C by streaking on different extracted lignin agar containing MSM. Methylene Blue was then used on bacteria isolates to see the halo zones around the colonies (Sasikumar et al., 2014; Kakkar et al., 2015; Umashankar et al., 2018). Plates were incubated at 30°C for 72 h, and the decolorized colonies were selected as lignin degraders (Umashankar et al., 2018). Morphologically different isolates with a surrounding halo zone were selected and purified for study of PCR analyses and ligninolytic activities.

Genomic DNA Extraction and 16S rRNA Gene PCR Amplification

Genomic DNA was extracted from all isolates using the boiling method, following the manufacture's protocol. DNA preparations were visualized after electrophoresis in a 1.0% agarose gel in 1x TBE buffer to assess their integrity, and then stored at -80°C prior to PCR amplification. The 16SrRNA gene was amplified by using the universal forward primer pair P1 (5′-ATATATAAGCGGCCGCAGAAAGGAGGTGATCC-3′) and reverse primer P6 (5′-ATATATAAGCGGCCGCAGAGTTTGATCATGCC TC-3') (Ramin et al., 2008).

PCR reactions were performed in a total volume of 25 μl using of master mix of 12.5 μl, 9.5 μl distilled water, 1 μl of each primer, and 1 μl of isolated DNA. PCR amplification was carried out in a PCR thermal cycler (Bio-Rad My cycler) using hot-start procedure (94°C for 4 min). Conditions consisted of: denaturation (94°C for 60 s), annealing (60°C for 45 s), and extension (72°C for 60 s) for 35 cycles followed by final extension step of 72°C for 7 min. PCR products were analyzed using 0.8% agarose gel in 1x TBE gel buffer electrophoresis.

DNA Sequencing and Phylogenetic Analysis

DNA sequencing of the purified PCR products were sent through Topaz Gene Research Company to Microsynth ("The Swiss DNA Company", Switzerland). The sequences were then trimmed with Chromas V 2.6.6 and DNA Baser Assembler V5.15.0 and compared with those in the GenBank database using the BLAST search program (http://www.ncbi.nlm.nih.gov/). Phylogenetic analysis was performed with the MEGA 6.0 program (Molecular Evolutionary Genetics Analysis, Version 5.0) (Tamura et al., 2011; Singh et al., 2015). The tree topologies were evaluated using bootstrap analyses based on 1,000 replicates and phylogenetic trees were inferred using the maximum likelihood method (Singh et al., 2015).

Antibiogram Test

Disk-diffusion methods were used for antibiotic tests. A colony of each bacteria was suspended into 5-ml distilled water. A loopful of each suspension was streaked on agar plates with the aid of sterile swab sticks. Antibiotic utilized in treated discs were Chloramphenicol (30 μg), Penicillin (10 μg), Amoxicillin (25 μg), Tetracycline (30 μg) and Azithromycin (15 μg). Using sterile forceps, antibiotic treated disks were applied to the surface of an agar plate containing the bacteria culture. Agar plates were allowed to absorb antibiotic while incubated at room temperature for 24-48 h. After overnight incubation, results were observed and inhibition zones were measured. The antibiogram were interpreted as resistant (R), intermediate (I), or sensitive (S) (Clinical Laboratory Standards Institute, 2015).

RESULTS

Enumeration and Characterization of Bacteria Isolates

A total of 47 isolates were identified (Table 1). Diagnosis of termite gut bacteria based on key morphological and biochemical tests are shown in Table 2. These identifications were confirmed by 16S rRNA gene sequence analyses. To the best of our knowledge some of these species are reported here for the first time from Iran. All 47 strains were belonged to 3 Phylum and 7 families: including Bacillaceae, Brucellaceae, Enterobacteriaceae, Flavobacteriaceae, Moraxellaceae, Pseudomonadaceae and Xanthomonadaceae. The family of Bacillaceae has two genus and the remaining families all have only one genus. The majority of the strains (n=19) are assigned to the family Bacillaceae. Psudomonadaceae and Xanthomonadaceae families each have 11 starins. Enterobacteriaceae and Moraxellaceae families each have 2 starins. Brucellaceae and Flavobacteriaceae each have 1 starin (Table 3).

Biochemical Tests

All eleven morphological and biochemical tests were determined. Results showed us that there were a significant differences among isolates (Table 2).

Halo-zone Decolorization Assay

Assays were done for all strains to determine delignification activity. Strains that digested lignin in the culture media produced decolorized halo-zones. Halo-zone diameters were measured, corresponding with the lignin degrading ability of each strain. Results indicated that the most activity of delignification belongs to the members of Bacillaceae, Xanthomonadaceae, and Pseudomonadaceae, in descending order, respectively.

The wheat straw carbon sources used for the isolation of lignin degrading-bacteria and total number of colonies screened and confirmed isolates are tabulated in Table 1. A total of 47 colonies were isolated by screening in the 2 different culture medias. From the 47 isolates, 45 isolates were confirmed as lignin degraders through conformational tests (Table 2). Decolorization of methylene blue has been previously demonstrated (Umashankar et al., 2018). A total of 42 isolates were identified from Klason lignin media culture, whereas 21 isolates from Alkali lignin media culture were positive.

Decolorization zones produced by isolates were measured. Seven isolates in Klason lignin and four isolates in Alkali lignin produced the largest decolorization zones (2.25 to 3 cm). Eight isolates in Alkali lignin and twelve isolates in Klason lignin produced halo zones of 1.5 to 2 cm. Six isolates in Alkali lignin and 19 isolates in Klason lignin media produced moderate decolorization zones (0.8 to 1 cm), and three isolates in Alkali lignin media and two isolates in Klason lignin media produced the smallest halo zones of 0.3 to 0.5 cm (Tables 4, 5).

DNA Sequencing, and Constructing Phylogenetic Tree

All 47 isolates were subjected to molecular identification using PCR amplification of the almost complete 16S rRNA gene. The size of the generated fragments was ~1.5 kb (Fig. 1). All sequences were deposited at the NCBI website. Table 1 shows the new strains and their accession numbers. Figures 2–5 provide family phylogenetic trees showing the relationships of 16S rRNA phylotypes affiliated with family Enterobacteriaceae, Xanthomonadaceae, Moraxellaceae, Psedumonadaceae and Brucellaceae in the phylum Proteobacteria. Also, we used *Staphylococcus succinus* in Firmicutes as the out-group in the phylogenetic tree for comparison between species (Figs. 2–5).

Antibiogram Tests

The results indicate that the species show a different reaction. In the genus *Bacillus* from the Bacillaceae family, the majority species were completely sensitive to Tetracycline, Chloramphenicol and Azithromycin, but the results were variable to Penicillin and Amoxicillin. *Pseudomonas* spp in family of Pseudomonadaceae showed that the majority of species were resistant to Amoxicillin, Azithromycin and Penicillin, while all species were completely intermediate towards Tetracycline and mostly were intermediate to Chloramphenicol and two species were sensitive and tow species were resistant. *Stenoterophomonas* spp. in family of Xanthomonadaceae , all species showed variable responses to each type of antibiotics. Two of *Serratia* spp. were completely resistant to Azithromycin, Penicillin and Tetracycline and were intermediate to Amoxicillin and Chloramphenicol. *Ochrobactrum* spp were resistant to Azithromycin, Amoxicillin, and Chloramphenicol, but they showed intermediate to Tetracycline. In tow *Actinetobcter* spp were resistant to Azithromycin, Amoxicillin and Penicillin, while they were quite sensitive to Chloramphenicol and intermediate to Tetracycline. *Elizabethkingia* spp from Flavobacteriaceae family showed resistant to Azithromycin, Chloramphenicol and Penicillin but were sensitive to Amoxicillin and Tetracycline (Table 3).

Table 1. Identity and accession number of bacteria species extracted from gut of *M. diversus* in NCBI.

AS Strains Submitted in NCBI	Sample ID of the Isolates in NCBI	Identity of Isolates	Genbank Accession Number	Location
A1-1	AB1	*Stenotrophomonas maltophilia*	MK963064	
A1-2	AB2	*Elizabethkingia anophelis*	MN080304	
A2-1	AB3	*Pseudomonas aeruginosa*	MK963065	
A8-1	AB4	*P. putida*	MK963070	
A6-1	AB5	*Ochrobactrum anthropi*	MK963069	Ahwaz
A2-2	AB6	*Bacillus Cereus*	MK963066	
A4-1	AB7	*B .pumillus*	MK963067	
A1S	AB8	*B. thuringiensis*	MK963071	
A5-1	AB9	*B. paramycoides*	MK963068	
A3-1	AB10	*B. paramycoides*	MN080305	
B2S	BB1	*S. maltophilia*	MK942627	
B1-2	BB1(1)	*S. maltophilia*	MN083283	
B5-1	BB1(2)	*S. maltophilia*	MN083284	
B1-1	BB2	*P. aeruginosa*	MK942630	
B2-2	BB2(1)	*P. aeruginosa*	MN087524	
B3-1	BB2(2)	*P. aeruginosa*	MN087525	
B3-2	BB2(3)	*P. aeruginosa*	MN087526	Bandar Abbas
B4-3	BB2(4)	*P. aeruginosa*	MN083282	
B5-2	BB2(5)	*P. aeruginosa*	MN087527	
B6-2	BB2(6)	*P. aeruginosa*	MN087528	
B4-1	BB3	*P. hibiscicola*	MK942629	
B2-1	BB4	*Actinetobacter pitti*	MK942628	
B6-3	BB5	*A. venetianus*	MK942631	
B1S	BB6	*B. cereus*	MK942632	
KH4-1	KHB1	*S. maltophilia*	MK949077	
KH3-3	KHB2	*Serratia marcescens*	MK949076	
KH3-2	KHB2(1)	*S. marcescens*	MN093337	
KH1-1	KHB3	*B. cereus*	MK949073	
KH4-3	KHB3(1)	*B. cereus*	MN093340	Khark Island
KH4-4	KHB3(2)	*B. cereus*	MN093341	
KH3-1	KHB4	*B. thuringiensis*	MK949075	
KH2S	KHB4(1)	*B. thuringiensis*	MN093338	
KH2-1	KHB5	*B. anthracis*	MK949074	
KH1S	KHB5(1)	*B. anthracis*	MN093342	
KH4-2	KHB6	*B. wiedmanii*	MK975805	
K3S	KB1	*S. maltophilia*	MK963013	
K2-1	KB1(1)	*S. maltophilia*	MN087219	
K3-1	KB1(2)	*S. maltophilia*	MN087220	
K4-1	KB1(3)	*S. maltophilia*	MN087214	
K5-1	KB1(4)	*S. maltophilia*	MN087221	Kish Island
K6-2	KB1(5)	*S. maltophilia*	MN087215	
K1S	KB2	*P. hibiscicola*	MK963012	
K1-1	KB3	*Lysinibacillus Pakestanensis*	MK963011	
K6-3	KB4	*B. cereus*	MK963015	
K2S	KB4(1)	*B. cereus*	MN087223	
K4S	KB5	*B. thuringiensis*	MK963014	
K6-1	KB5(1)	*B. thuringiensis*	MN087222	

Table 2. Morphological and biochemical characteristics of bacteria isolates.

		Morphhological Tests					Biochemical Tests					Hydrolysis Extraction	
Species	**Isolate**	**Gram Stain**	**Shape**	**Colony Color**	**Motility**	**Spore**	**King's B**	**Levan production**	**O/F**	**Oxidase**	**Catalase**	**Alkali Lignin**	**Klason Lignin**
B. cereus	AB6	+	Rod	White	+	+	-	-	F	-	+	+++	-
B. cereus	BB6	+	Rod	White	+	+	-	-	F	-	+	++	W
B. cereus	KHB3	+	Rod	White	+	+	-	-	F	-	+	++	+
B. cereus	KHB3(1)	+	Rod	White	+	+	-	-	F	-	+	++	+++
B. cereus	KHB3(2)	+	Rod	White	+	+	-	-	F	-	+	-	+
B. cereus	KB4	+	Rod	White	+	+	-	-	F	-	+	-	++
B. cereus	KB4(1)	+	Rod	White	+	+	-	-	F	-	+	-	++
B .thuringiensis	AB8	+	Rod	White	+	+	-	-	F	-	+	+++	-
B .thuringiensis	KHB4	+	Rod	White	+	+	-	-	F	-	+	-	+
B .thuringiensis	KHB4(1)	+	Rod	Yellow	+	+	-	-	F	-	+	++	W
B .thuringiensis	KB5	+	Rod	White	+	+	-	-	F	-	+	+	W
B .thuringiensis	KB5(1)	+	Rod	White	+	+	-	-	F	-	+	+++	-
B .pumilus	AB7	+	Rod	Cream	+	+	-	-	F	+	+	-	-
B.	AB9	+	Rod	White	+	+	-	-	F	-	+	-	++
paramycoides	AB10	+	Rod	White	+	+	-	-	F	-	+	-	++
B.	KHB5	+	Rod	White	W	+	-	-	F	-	+	-	+++
paramycoides	KHB5(1)	+	Rod	White	+	+	-	-	F	-	+	-	++
B. anthracis	KHB6	+	Rod	White	+	+	-	-	F	-	+	++	+++
B. anthracis	KB3	+	Rod	Cream	+	+	-	-	O	-	+	-	+
B. wiedmanii													
L. pakestanensis													
P. aeruginosa	AB3	-	Rod	Cream	+	-	-	-	O	+	+	-	+
P. aeruginosa	BB2	-	Rod	Cream	+	-	+	-	O	+	+	-	+
P. aeruginosa	BB2(1)	-	Rod	Yellow	+	-	+	-	O	+	+	+	+
P. aeruginosa	BB2(2)	-	Rod	Cream	+	-	+	-	O	+	+	-	++
P. aeruginosa	BB2(3)	-	Rod	Cream	+	-	+	-	O	+	+	-	+
P. aeruginosa	BB2(4)	-	Rod	Cream	-	-	+	-	O	+	+	+	+
P. aeruginosa	BB2(5)	-	Rod	White	+	-	+	-	O	+	+	-	+
P. aeruginosa	BB2(6)	-	Rod	White	+	-	-	-	O	+	+	++	++
P. hibiscicola	BB3	-	Rod	Cream	+	-	+	-	O	-	+	W	-
P. putida	AB4	-	Rod	Yellow	+	-	+	-	O	+	+	+++	+++
P. hibiscicola	KB2	-	Rod	Yellow	+	-	-	-	O	+	W	-	+
S. maltophilia	AB1	-	Rod	White	+	-	-	-	O	-	+	-	++
S. maltophilia	BB1	-	Rod	Yellow	+	-	-	-	O	-	+	-	+
S. maltophilia	BB1(1)	-	Rod	Cream	+	-	-	-	O	+	+	-	++
S. maltophilia	BB1(2)	-	Rod	Cream	+	-	-	-	O	-	+	-	+
S. maltophilia	KHB1	-	Rod	Cream	+	-	-	-	O	-	+	-	+
S. maltophilia	KB1	-	Rod	Cream	+	-	-	-	O	-	W	-	+
S. maltophilia	KB1(1)	-	Rod	Cream	+	-	-	-	O	+	+	-	+
S. maltophilia	KB1(2)	-	Rod	Cream	+	-	-	-	O	-	+	+	++
S. maltophilia	KB1(3)	-	Rod	White	+	-	-	-	O	-	+	W	+
S. maltophilia	KB1(4)	-	Rod	Cream	+	-	-	-	O	-	+	-	+++
S. maltophilia	KB1(5)	-	Rod	White	+	-	-	-	O	-	+	-	++
S. marcescens	KHB2	-	Rod	Cream	+	-	-	-	F	-	+	++	+++
S. marcescens	KHB2(1)	-	Rod	Cream	+	-	-	-	F	-	+	++	+
O. anthropi	AB5	-	Rod	Yellow	+	-	+	-	O	+	+	+	+++
A. pitti	BB4	-	Rod	Cream	-	-	-	-	O	-	+	+	+
A. venetianus	BB5	-	Rod	Yellow	+	-	-	-	O	-	+	W	+
E. anophelis	AB2	-	Rod	Yellow	+	-	-	-	O	+	+	-	+

***+ positive; ++ good positive; +++ very good positive; – negative; W Weak**

Table3. Classification of bacterial isolates.

Isolates	Species	Genus	Family	Phylum
AB6	*Bacillus cereus*	*Bacillus*	Bacillaceae	Fimicutes
BB6	*B. cereus*			
KHB3	*B. cereus*			
KHB3(1)	*B. cereus*			
KHB3(2)	*B. cereus*			
KB4	*B. cereus*			
KB4(1)	*B. cereus*			
AB8	*B. thuringiensis*			
KHB4	*B. thuringiensis*	*Lysinibacillus*		
KHB4(1)	*B. thuringiensis*			
KB5	*B. thuringiensis*			
KB5(1)	*B. thuringiensis*			
AB7	*B. pumillus*			
KHB5	*B. anthracis*			
KHB5(1)	*B. anthracis*			
AB9	*B. paramycoides*			
AB10	*B. paramycoides*			
KHB6	*B. wiedmanii*			
KB3	*Lysinibacillus pakestanensis*			
AB3	*Pseudomonas aeruginosa*	*Pseudomonas*	Pseudomonadaceae	Proteobacteria
BB2	*P. aeruginosa*			
BB2(1)	*P. aeruginosa*			
BB2(2)	*P. aeruginosa*			
BB2(3)	*P. aeruginosa*			
BB2(4)	*P. aeruginosa*			
BB2(5)	*P. aeruginosa*			
BB2(6)	*P. aeruginosa*			
AB4	*P. putida*			
KB2	*P. hibiscicola*			
BB3	*P. hibiscicola*			
AB1	*Stenotrophomonas maltophilia*	*Stenotrophomonas*	Xanthomonadaceae	Proteobacteria
BB1	*S. maltophilia*			
BB1(1)	*S. maltophilia*			
BB1(2)	*S. maltophilia*			
BB1(2)	*S. maltophilia*			
KHB1	*S. maltophilia*			
KB1	*S. maltophilia*			
KB1(1)	*S. maltophilia*			
KB1(2)	*S. maltophilia*			
KB1(3)	*S. maltophilia*			
KB1(4)	*S. maltophilia*			
KB1(5)	*S. maltophilia*			
KHB2	*Serratia marcescens*	*Serratia*	Enterobacteriaceae	Proteobacteria
KHB2(1)	*S. marccescens*			
BB4	*Actinetobacter pitti*	*Actinetobacter*	Moraxellaceae	Proteobacteria
BB5	*A. venetianus*			
AB5	*Ochrobacterum anthropic*	*Ochrobactrum*	Brucellaceae	Proteobacteria
AB2	*Elizabethkingia anophelis*	*Elizabethkingia*	Flavobacteriaceae	Bacteroidetes

Table 4. The enzymatic activity of the bacteria detected in Klason Lignin media culture by Methylene Blue assay test.

Species	Isolates	Klason Lignin Media Culture Halo Zone of Decolorization, cm
Bacillus cereus	BB6	**0.4**
B. cereus	KHB3	**0.8**
B. cereus	KHB3(1)	**2.6**
B. cereus	KHB3(2)	**0.7**
B. cereus	KB4	**1.7**
B. cereus	KB4(1)	**1.8**
B. thuringiensis	KHB4	**0.9**
B. thuringiensis	KHB4(1)	**0.3**
B. thuringiensis	KB5	**0.3**
B. paramycoides	AB9	**1.9**
B. paramycoides	AB10	**1.8**
B. anthracis	KHB5	**2.8**
B. anthracis	KHB5(1)	**2**
B. wiedmanii	KHB6	**2.4**
Lysinibacillus pakestanensis	KB3	**0.8**
Pseudomonas aeruginosa	AB3	**1**
P. aeruginosa	BB2	**0.7**
P. aeruginosa	BB2(1)	**0.6**
P. aeruginosa	BB2(2)	**1.3**
P. aeruginosa	BB2(3)	**0.8**
P. aeruginosa	BB2(4)	**0.7**
P. aeruginosa	BB2(5)	**0.6**
P. aeruginosa	BB2(6)	**1.5**
P. putida	AB4	**2.7**
P. hibiscicola	KB2	**0.8**
Stenotrophomonas maltophilia	AB1	**1.7**
S. maltophilia	BB1	**0.6**
S. maltophilia	BB1(1)	**1.5**
S. maltophilia	BB1(2)	**0.6**
S. maltophilia	KHB1	**0.7**
S. maltophilia	KB1	**08**
S. maltophilia	KB1(1)	**0.6**
S. maltophilia	KB1(2)	**1.3**
S. maltophilia	KB1(3)	**0.7**
S. maltophilia	KB1(4)	**2.6**
S. maltophilia	KB1(5)	**1.4**
S. maltophilia		
Serratia marcescens	KHB2	**2.3**
S. marcescens	KHB2(1)	**1**
Ochrobactrum anthropi	AB5	**2.7**
Actinetobacter pitti	BB4	**0.6**
A. venetianus	BB5	**1**
Elizabethkingia anophelis	AB2	**0.7**

Table 5. The enzymatic activity of the bacteria detected in Alkali Lignin media culture by Methylene Blue assay test.

Species	Isolates	Alkali Lignin Media Culture Halo Zone of Decolorization, cm
Bacillus cereus	AB6	**2.6**
B. cereus	BB6	**1.4**
B. cereus	KHB3	**1.5**
B. cereus	KHB3(1)	**2**
B. thuringiensis	AB8	**2.5**
B. thuringiensis	KHB4(1)	**2**
B. thuringiensis	KB5	**1**
B. thuringiensis	KB5(1)	**2.3**
B. wiedmanii	KHB6	**2**
Pseudomonas aeruginosa	BB2(1)	**0.8**
P. aeruginosa	BB2(4)	**1**
P. aeruginosa	BB2(6)	**1.4**
P. hibiscicola	BB3	**0.4**
P. putida	AB4	**3**
Stenotrophomonas maltophilia	KB1(2)	**0.7**
S. maltophilia	KB1(3)	**0.3**
Serratia marcescens	KHB2	**1.3**
S. marcescens	KHB2(1)	**2**
Ochrobactrum anthropic	AB5	**1**
Actinetobacter pitti	BB4	**0.9**
A. venetianus	BB5	**0.3**

Table 6. Antibiogram test of bacteria isolates in gut of *M. diversus*.

Species	Isolates	Antibiotics*				
		AMX	**AZM**	**C**	**P**	**TE**
B. cereus	AB6-A2-2	I	S	S	I	S
B. cereus	BB6-B1s	R	S	S	R	S
B. cereus	KHB3-KH1-1	R	S	S	R	S
B. cereus	KHB3(1)-KH4-3	R	S	S	R	S
B. cereus	KHB3(2)-KH4-4	R	R	R	I	R
B. cereus	KB4-K6-3	S	S	S	S	S
B. cereus	KB4(1)-K2S	R	R	I	R	S
B. thuringiensis	AB8-A1S	I	S	S	I	S
B. thuringiensis	KHB4-KH3-1	R	I	S	R	S
B. thuringiensis	KHB4(1)-KH2S	R	S	S	R	S
B. thuringiensis	KB5-K4S	I	S	S	I	S
B. thuringiensis	KB5(1)-K6-1	R	S	S	R	S
B. pumilus	AB7-A4-1	R	S	S	R	S
B. paramycoides	AB9-A5-1	I	S	S	I	S
B. paramycoides	AB10-A3-1	I	S	S	I	S
B. anthracis	KHB5-KH2-1	I	S	S	R	S
B. anthracis	KHB5(1)-KH1S	I	S	S	I	S
B. wiedmanii	KHB6-KH4-2	I	S	S	I	S
L. pakestanensis	KB3-K1-1	R	S	S	I	S
P. aeruginosa	AB3-A2-1	R	R	I	R	I
P. aeruginosa	BB2-B1-1	R	R	R	R	I
P. aeruginosa	BB2(1)-B2-2	R	R	I	R	I
P. aeruginosa	BB2(2)-B3-1	R	R	I	R	I
P. aeruginosa	BB2(3)-B3-2	R	R	I	R	I
P. aeruginosa	BB2(4)-B4-3	R	R	I	R	I
P. aeruginosa	BB2(5)-B5-2	S	S	S	I	S
P. aeruginosa	BB2(6)-B6-2	R	R	I	I	I
p. hibiscicola	BB3-B4-1	R	I	I	R	I
p. hibiscicola	KB2-K1S	R	R	S	R	I
P. putida	AB4-A8-1	R	R	R	R	I
S. maltophilia	AB1-A1-1	R	S	S	I	S
S. maltophilia	BB1-B2S	R	R	I	R	R
S. maltophilia	BB1(1)-B1-2	S	S	I	R	S
S. maltophilia	BB(2)-B6-2	S	S	I	R	S
S. maltophilia	KHB1-KH4-1	S	R	R	S	R
S. maltophilia	KB1-K3S	S	I	R	S	R
S. maltophilia	KB1(1)-K2-1	R	I	S	R	I
S. maltophilia	KB1(2)-K3-1	R	R	I	R	R
S. maltophilia	KB1(3)-K4-1	R	R	S	R	I
S. maltophilia	KB1(4)-K5-1	I	S	S	I	S
S. maltophilia	KB1(5)-K6-2	S	R	S	S	R
S. marcescens	KHB2-KH3-3	R	I	I	R	R
S. marcescens	KHB2(1)-KH3-2	R	I	I	R	R
O. anthropi	AB5-A6-1	R	R	R	R	I
A. pitti	BB4-B2-1	R	R	S	R	I
A. venetianus	BB5-B6-3	R	R	S	R	I
E. anophelis	AB2-A1-2	R	S	R	R	S

* R: Resistant; I: Intermediate; S: Sensitive.
AMX: Amoxicillin (25 μg); AZM: Azithromycin (15 μg); C: Chloramphenicol (30 μg); P: Penicillin (10 μg); TE: Tetracycline (30 μg)

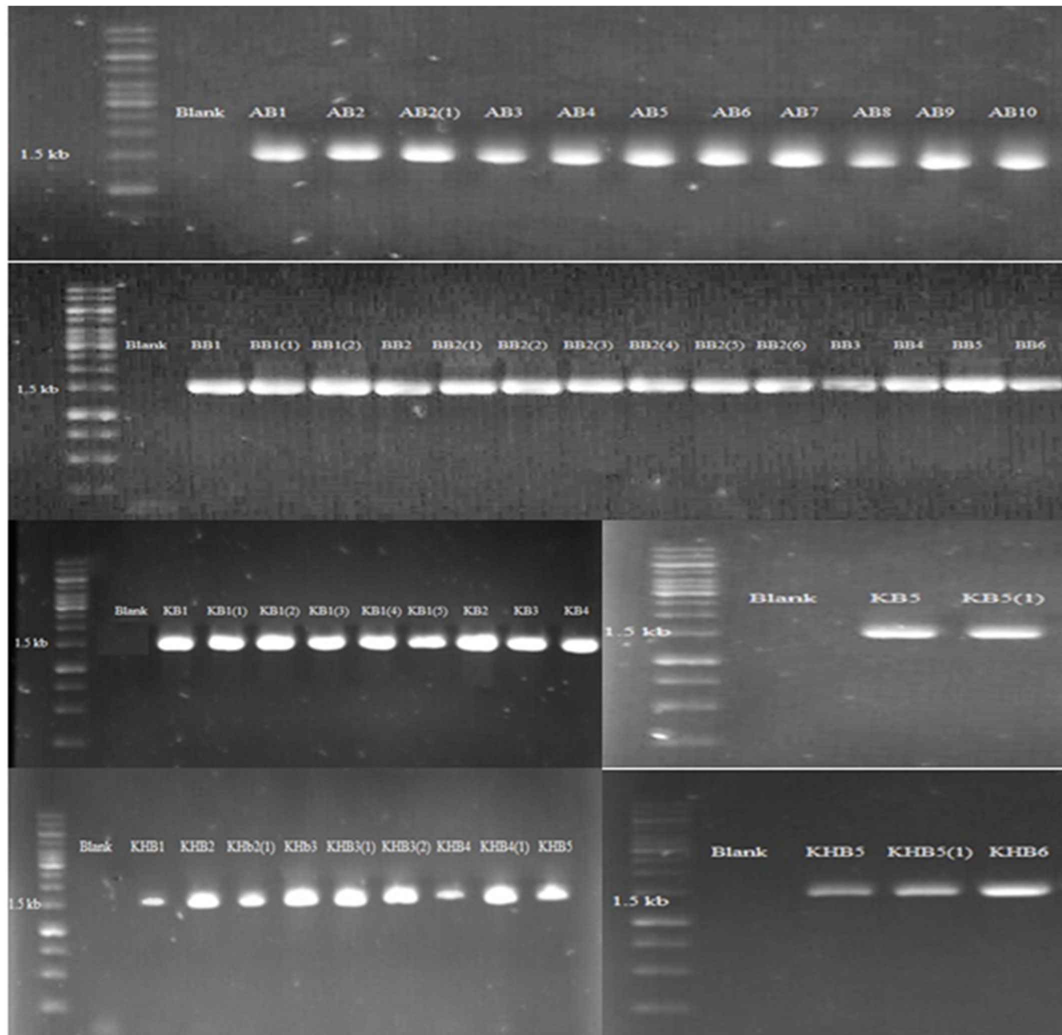

Fig. 1. PCR amplification of the 16S rRNA gene from bacteria isolates showing a single gene fragment in the range of 1.5 kb. Top to bottom figures: Lanes AB1 to KHB6 belong to bacteria isolates whose names are listed in Table 1. Lane 1 is a blank negative control. Top row: Lanes 2 to 11 [AB1 to AB10]; Second row: Lanes 2 to 15 [BB1 to BB6]: Third row left: Lanes 2 to10 [KB1 to KB4]; and Third row right: Lanes 2 to 3 [KB5 to KB5 (1)]. Bottom row left: Lanes 2 to 10 [KHB1 to KHB5]; and bottom row right: Lanes 2 to 4 [KHB5 to KHB6] were control + as DNA samples from gut. Size marker (1-kb DNA ladder, Promegarke).

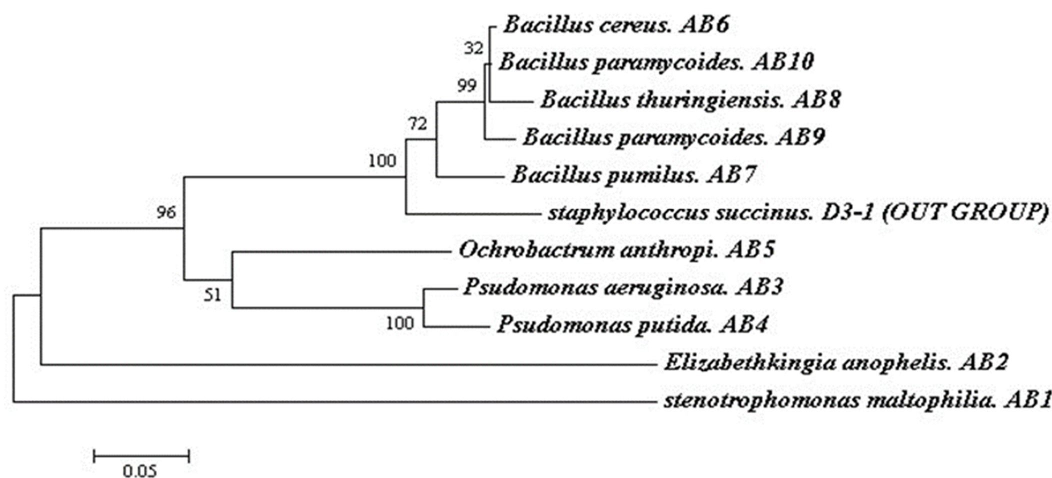

Fig. 2. Phylogenetic tree of bacteria species isolated from gut of *M. diversus* collected from Ahvaz area. The phylogenetic tree was constructed from rDNA sequences registered in GenBank, using MEGA6. The evolutionary history was inferred by using Maximum Likelihood Method based on Tamura-Nei model. The scale represents a relative evolutionary distance, and bootstrap values obtained after 1,000 replications (Tamura et al., 2013).

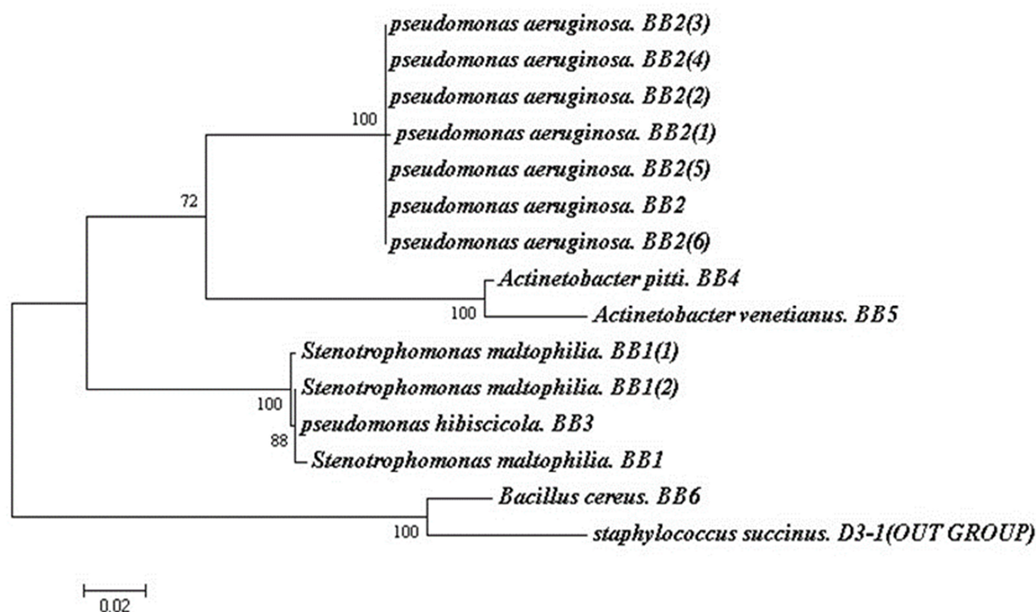

Fig. 3. Phylogenetic tree of bacteria species isolated from gut of *M. diversus* collected from Bandar Abbas area. The phylogenetic tree was constructed from rDNA sequences registered in GenBank, using MEGA6. The evolutionary history was inferred by using Maximum Likelihood Method based on Tamura-Nei model. The scale represents a relative evolutionary distance, and bootstrap values obtained after 1,000 replications (Tamura et al., 2013).

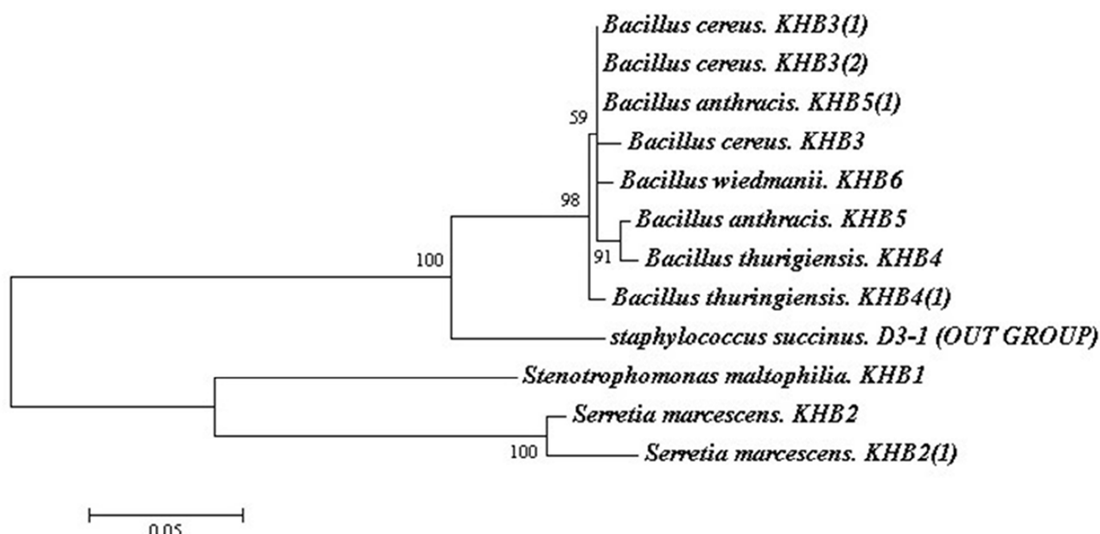

Fig. 4. Phylogenetic tree of bacteria species isolated from gut of *M. diversus* collected from Khark Island. The phylogenetic tree was constructed from rDNA sequences registered in the GenBank, using MEGA6. The evolutionary history was inferred by using Maximum Likelihood Method based on Tamura-Nei model. The scale represents a relative evolutionary distance, and bootstrap values obtained after 1,000 replications (Tamura et al., 2013).

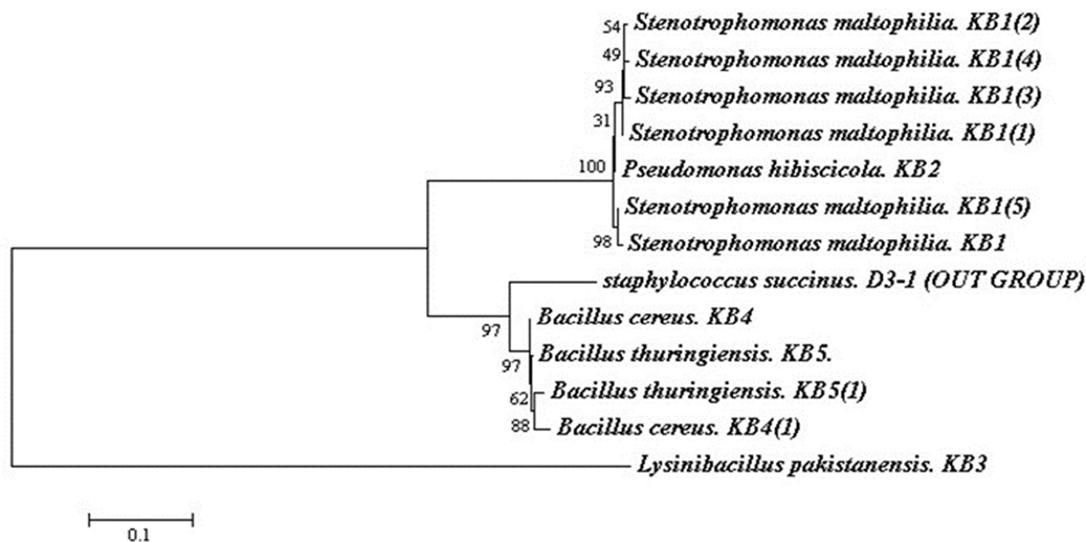

Fig. 5. Phylogenetic tree of bacteria species isolated from gut of *M. diversus* collected from Kish Island. The phylogenetic tree was constructed from rDNA sequences registered in GenBank, using MEGA6. The evolutionary history was inferred by using Maximum Likelihood Method based on Tamura-Nei model. The scale represents a relative evolutionary distance, and bootstrap values obtained after 1,000 replications (Tamura et al., 2013).

DISCUSSION

In the biosphere, several organisms are able to biodegrade lignin, including white rot fungi, some soil microorganisms and termites (Kato et al., 1998; Li et al., 2009). Most research emphasizes the role of bacteria and fungi in degrading lignin.

Bacteria are worthy of being studied for their ligninolytic potential because of their immense environmental adaptability and biochemical capabilities (Li et al., 2009). In addition, many bacteria species that have the ability to degrade lignin are also present in the digestive tract of termites. Therefore termites are excellent sources for isolation and study of lignin-degrading bacteria. About 2,000 species of termites are known, but only a few of their gut flora have been studied) O'Brien and Slaytor, 1982).

In the present study, termites were collected from four different regions in southern Iran. Results showed that microflora were diverse, and that the diverse bacteria species extracted were able to grow on all media containing lignin and wheat straw. Results based on molecular identification using PCR amplification showed a diverse community of bacteria in the termite gut. Based on morphological and biochemical tests, and antibiotic susceptibility tests, 47 bacteria isolates with different abilities of lignin digestion from very low activity to very high activity were identified.

Due to the degradability of lignin and biological degradation of wheat straw by selected bacteria, some isolates exhibit greater degradation ability. Out of 47 identified isolates, 17 species showed ability to grow and decompose lignin in tow specific culture media. In Flavobacteriaceae, *Elizabethkingia anopheles* had the ability to degraded lignin in Klason media, but not the ability to degrade lignin on Alkali media. Two *Actinetobacter* spp. of Moraxellaceae, had the ability of delignification in all media culture. *Ochrobactrum anthropi* from Brucellaceae, and two *Serratia* spp. from Enterobacteriaceae, also had the ability to degrade lignin in all two media.

Out of eleven isolates of *S. maltophilia* in the family Xanthomonadaceae, only two species (KB1(2) and KB1(3)) was capable of degrading lignin in all media, the remaining isolates exhibited very good positive degradation ability on Klason medium. Nine isolates had negative delignification activity in Alkali media culture, and one had weak activity. Of 11 Pseudomonadaceae isolates, 10 isolates showed very good lignin-positive digestion on Klason medium. Four of these isolates also showed good results on alkalin lignin medium. In Bacillaceae, 19 isolates were identified. These isolates have different delignification activity, six species had positive degradation activity in all media and 9 isolates were capable of delignification in Alkali lignin media. In addition, 12 species had very good positive and 3 species had very low delignification activity in Klason media culture.

Similar to these results, Butler and Buckerfield (1979) have determined that termites can degrade lignin, and also found significant degradation of lignin by *N. exitiosus*. In addition, Pasti et al. (1997) reported the delignification ability of *Actinomycetes* isolated from a termite (Termitidae) gut.

In the study performed by O'Brien and Slaytor (1982), some termite gut bacteria were identified and isolated, including *Actinomycetes*, *Alkaligenes*, *Bacillus*, *Citrobacter*, *Enterobacter*, *Serratia*, *Staphylococcus* and *Streptococcus*, which confirms the present study. Our study identified *Actinetobacter*, *Bacillus*, *Elizabethkingia*, *Ochrobactrum*, *Pseudomonas*, *Serratia* and *Stenotrophomonas*. A similar result by Li et al. (2009) showed that several genera of bacteria, including *Alcaligegnes*, *Arthrobacter*, *Nocardia*, *Pseudomonas*, and *Strepomyces* have delignification capabilities.

Similar results have been obtained by other researchers. Cookson, 1987 and Kerr et al., 1983 were determined lignin degradation ability of the bacteria on Klason lignin and modified medium (Cookson, 1987; Kerr et al., 1983).

Cookson (1987) examined *Nasutitermes exitiosus* (Hill), *Coptotermes acinaciformi*s (Froggatt), and *Mastotermes darwiniensis* Froggatt, using same medium for their ability to degrade lignin. Kato et al. (1998) showed presence of bacteria in the decomposition of lignin, confirming the present study.

Consistent with the present study, Borji et al. (2003) reported three isolates, of *Bacillus* sp., *Enterobacter* sp. and *Ocrobacterium* sp., from *Anacanthotermes vagans* that were capable of utilizing all three lignin preparations as well as extracted wheat straw as a sole source of carbon. Between these bacteria, *Enterobacter* had more and faster growth rate than the other two species. These bacteria could make biodegradation of wheat straw and other similar lignin-containing biological waste products commercially feasible.

Similar to our result, Azizi et al. (2016) reported that three bacteria, *Bacillus licheniformis, Ochrobactrum intermedium* and *Microbacterium paludicola* were isolated from termite guts, and evaluated in media containing different types of lignin and lignocellulosic materials. Results indicated that *Bacillus* sp. have very good positive delignification activity.

Other researchers also reported similar results. For example, Bandounas et al. (2011) reported that *Pandoraea norimbergensis*, *Pseudomonas* sp., and *Bacillus* sp. were isolated as potential lignin depolymerizing bacteria, and also indicated that the best lignin-like dye decolorizing capacity was observed for the *Bacillus* sp. Research by Li et al. (2019) found that a novel lignin degrading bacteria strain, *Pseudocitrobacter anthropi* MP-4, was successfully isolated from the gut of the wood-feeding termite species, *M. pakistanicus*. This bacterium exhibited a fast growth and efficient degradation rates in lignin medium, and had the ability to produce laccase and lignin peroxidase enzymes. Regarding the ability of termites to degrade lignin, Tarmadi et al. (2017) observed the required presence of lignin needed to maintain physiological activities of *C. formosanus* workers during their lignocellulose decomposition activities.

Multiple studies on microbial populations in termites from the point-of-view of numbers and variation reached roughly similar results. While many claims of lignin degradation by termite symbiotic bacteria have been reported, especially for *Bacillus* species improving in vitro fermentation parameters and the digestibility of organic matter, only during the past few decades have some bacteria enzymes involved in delignification been identified. Additional research in this area of termite and their bacteria would be beneficial.

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Author Contributions

This research has been done by the authors of this article. In this particular case, the results of this research have been done and evaluated under the supervision of Nader Hassanzadeh.

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شناسایی باکتری‌های روده گونه موریانه *Microcerotermes diversus* Silvestri (Isoptera: Termitidae) و جداسازی باکتری‌های تجزیه کننده لیگنین

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چکیده

لیگنین فراوان ترین پلیمر معطر (فنلی) و دومین ماده اولیه فراوان در محیط زیست است. لیگنین در طبیعت توسط باکتری‌ها و قارچ‌ها تخریب می‌شود. جهت ارزیابی نقش باکتری‌های همزیست موریانه‌ها در تخریب لیگنین، چهار کلنی از گونه موریانه *Microcerotermes diversus* Silvestri (Termitidae) که از طریق تله‌گذاری در مناطق اهواز، بندرعباس و دو جزیره خارک و کیش جمع‌آوری شدند. سوسپانسیون روده موریانه‌های هر منطقه در 5 میلی لیتر D.H2O تهیه شد. نمونه‌ها در محیط کشت مغذی آگار (NA) کشت داده شدند. پتری دیش‌های حاوی نمونه کشت داده شده در دمای $27^{0}C$ به مدت 72-24 ساعت درانکوباتور قرار گرفتند. کلنی باکتریایی متنوع با مورفولوژی‌های متفاوت انتخاب و خالص سازی شدند. برای تمایز باکتری‌های تجزیه کننده لیگنین، تمام 47 جدایه باکتریایی برروی محیط کشت حاوی آگار لیگنین مختلف استخراج شده حاوی MSM رشد داده شدند. در مجموع، 45 جدایه از نظر تجزیه لیگنین مثبت بودند. تعیین توالی 16S rDNAدر ترکیب با روش‌های کشت کلاسیک برای شناسایی باکتری‌ها استفاده شد. شناسایی شامل جنس / گونه *Serratia marcescens* ، *Ochrobactrum anthropic* ، *Elizabethkingia anopheles* ،*Stenotrophomonas maltophilia* ، *Lysinibacillus pakistanensis* ، *Actinetobacter pitti* ، *Pseudomonas* spp. و *Bacillus* spp بودند. برای تعیین میزان فعالیت لیگنینولیتیک ، باکتری‌ها از خانواده‌های شناسایی شده در محیط لیگنین آگار پرورش داده شدند. هیدرولیز لیگنین با استفاده از متیلن بلو بر روی محیط کشت مورد بررسی قرار گرفت. حاله‌های ایجاد شده دراطراف کلنی باکتری‌ها اندازه گیری شدند.

واژه‌های کلیدی: *Microcerotermes diversus* :، *endosymbionts*،میکروفلور روده ، تجزیه لیگنین، 16S rRNA

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