



Isolation of Lignin-Degrading Bacteria from Different Sources and Testing of Their Ligninolytic Activities

Farklı Kaynaklardan Lignin Parçalayıcı Bakterilerin İzolasyonu ve Lignolitik Aktivitelerinin Test Edilmesi

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Abstract

Nine lignin-degrading bacteria were isolated from petroleum-contaminated soil and animal manure samples and characterized by 16S rRNA sequence analysis. Three isolates were identified as *Enterobacter cancerogenus*, two as *Enterobacter ludwigii*, one as *Citrobacter sedlakii*, one as *Citrobacter farmari*, one as *Klebsiella pneumoniae*, and one as *Citrobacter murliniae*. These bacteria used ligno sulphate as the sole carbon source but did not utilize kraft lignin (KL) as the sole source of carbon and energy. For this reason, basic nutrients, such as 1.0% glucose (w/v) and 0.5% peptone (w/v), were used as additional carbon and nitrogen sources to stimulate bacterial growth for KL decolorization. Under these conditions, the isolates *Enterobacter cancerogenus* L1, *Enterobacter cancerogenus* L2, *Enterobacter ludwigii* L3, *Enterobacter ludwigii* L4, *Enterobacter cancerogenus* PT21, *Citrobacter farmari* PT22, *Citrobacter sedlakii* PT41, *Klebsiella pneumoniae* G1, and *Citrobacter murliniae* C1 degraded kraft lignin by 37 %, 14

Özet

Petrol bulaşmış toprak ve gübre örneklerinden dokuz adet lignin parçalayan bakteri izole edilmiş ve 16S rRNA analizleri yapılmıştır. Bu izolatlardan üç tanesi *Enterobacter cancerogenus*, iki tanesi *Enterobacter ludwigii*, bir tanesi *Citrobacter sedlakii*, bir tanesi *Citrobacter farmari*, bir tanesi *Klebsiella pneumoniae*, biri *Citrobacter murliniae* olarak tanımlanmıştır. İzolatlar, tek karbon kaynağı olarak ligno sülfat kullanırken, tek karbon ve enerji kaynağı olarak kraft lignini (KL) kullanmamışlardır. Bu nedenle kraft lignin parçalanmasında bakteri çoğalmasını teşvik etmek için ilave karbon ve azot kaynakları olarak % 1.0 glukoz (a / h) ve % 0.5 pepton (a / a) gibi ek karbon ve azot kaynakları kullanılmıştır. Bu koşullar altında, *Enterobacter cancerogenus* L1, *Enterobacter cancerogenus* L2, *Enterobacter ludwigii* L3, *Enterobacter ludwigii* L4, *Enterobacter cancerogenus* PT21, *Citrobacter farmari* PT22, *Citrobacter sedlakii* PT41, *Klebsiella pneumoniae* G1 ve *Citrobacter murliniae* C1 izolatları ile kraft lignini sırasıyla

%, 20%, 43%, 48%, 51%, 28%, 60%, and 99%, respectively. Remazol Brilliant Blue R (RBBR) decolorization potentials of the isolates were determined. The isolates were decolorized RBBR by 20-90 % , respectively.

Keywords: Lignin, Bacteria, 16S rRNA, Decolorization

Abbreviations: KL:Kraft Lignin, LiP: Lignin peroxidase, MnP: Manganese peroxidase

1. INTRODUCTION

The aromatic polymer lignin is well known for its resistance to microbial degradation because of its high molecular weight and the presence of various biologically stable carbon-to-carbon and ether linkages. The microorganisms that degrade plant lignin through an oxidative process are fungi (Tien & Kirk, 1983), actinomycetes (Hernandez et al., 2001), and to a lesser extent, bacteria (Trojanowski et al., 1977). Among them, white rot fungi have attracted widespread attention because of their powerful lignin-degrading enzymatic systems (Hatakka, 1994; Janusz et al., 2017). However, the use of fungi in industrial applications is not feasible due to the structural hindrance caused by fungal filaments; the requirement of particular culture conditions, such as humidity, aeration, temperature, and pH, that are not compatible with industrial processing environments; the requirement of a long lag period, which thus results in very slow lignin degradation; the need for additional food sources, such as glucose and nitrogen, to support the fungal growth; and the instability of fungi in practical treatment under extreme environmental and substrate conditions, such as higher pH, oxygen limitation, and high lignin concentrations

% 37,% 14,% 20,% 43,% 48,% 51,% 28, %60 ve% 99 oranında parçalamışlardır. İzolatların ayrıca Remazol Brilliant Blue R (RBBR) dekolorizasyon oranı analiz edilmiştir ve sırasıyla % 20-90 oranında RBRR dekolorizasyonu tespit edilmiştir.

Anahtar kelimeler: Lignin, Bakteri, 16S rRNA, Dekolorizasyon

(Bholay et al., 2012). In contrast, bacteria are worth studying for their ligninolytic potential due to their immense environmental adaptability and biochemical versatility (Chandra et al., 2007).

The ligninolytic system is an extracellular enzymatic complex that includes peroxidases, laccases, and oxidases responsible for the production of extracellular hydrogen peroxide (H₂O₂)(Ruiz-Duenas & Martinez, 2009). Subject to the species, strains and culture conditions those enzymes display differential characteristics (Kirk & Farrel, 1987). Ligninolytic enzymes have potential applications in a large number of fields, including the chemical, fuel, food, agricultural, paper, textile, cosmetic, and other industries (Maciel et al., 2010). The enzymes reported to be involved in bacterial lignin degradation are laccases, manganese peroxidase, lignin peroxidase, glutathione S-transferases, ring-cleaving dioxygenases (Masai et al., 2003; Allocati et al., 2009), monooxygenases, and phenol oxidases (Perestelo et al., 1989). Such enzymes are also involved in degradation of polycyclic aromatic hydrocarbons (PAHs), which have the same structural properties and resistance to microbial degradation as lignin (Allocati et al., 2009; Perestelo et al., 1989).

Dyes and dyestuff are also used in the textile, cosmetic, pharmaceutical, and leather industries but are of primary importance to textile manufacturing, the wastewater of which may contain a variety of these pollutants (McKay, 1979). The release of dyes has caused concern because of their environmental and health effects. Bioremediation is still seen as an attractive solution due to its reputation as a low-cost and environmentally friendly technology compared to chemical and physical treatment processes (McMullan et al., 2001). As mentioned above, ligninolytic enzymes are used in dye decolorization; however, although bacteria are more efficient than fungi, the latter are widely used. For this reason, there is still a need for a continued search for more efficient ligninolytic bacterial strains for bioremediation and other applications (Raj et al., 2007). Therefore, in the present study, we isolated several bacterial strains which have a great lignin-degrading potential from fuel-contaminated soil and manure and we evaluated their dye decolorization and bioremediation capacity. The aim of this study is to determine the usability of isolated bacteria for various industries. Considering that alternative ways are being sought for mechanical and chemical methods used in waste dye decolorization and paper production, this study will contribute to the fulfillment of these needs.

2. MATERIALS AND METHODS

2.1. Isolation of Lignin-Degrading Bacteria

The contaminated soil and manure samples were collected with polyethelene bags from different

areas in Arsin (Trabzon) were combined with lignin medium [(1-1): 5 g lignosulphonate, 9 g NaCl at pH 7.0] in a 250-ml Erlenmayer flask and then shaken at 37°C for 2 days to enrich the culture. After 2 days, the samples were inoculated into lignin agar plates that contained (1-1): 5 g lignin, 9 g NaCl, and 17 g agar at pH 7.0. This medium was used for screening the lignin-degrading bacteria. The best lignin-degrading colonies were selected according to their colony morphology and their growth potential on lignin agar. Cells of ligninolytic strains were Gram-stained using the method of Dussault (1955) and the Gram type was also determined using the KOH test (Powers, 1995). Cell morphology was examined by using phase-contrast microscopy (Nikon Eclipse E600; Olympus) on an exponentially growing liquid culture.

2.2. DNA Isolation and 16S rRNA Gene Sequence Analysis

The 16S rRNA genes were selectively amplified from purified genomic DNA (according to the users manual of Promega Wizard® Genomic DNA Purification Kit) with the use of oligonucleotide primers designed to anneal to conserved positions in the 3' and 5' regions of the bacterial 16S rRNA genes. The forward primer, UNI16S-L(5'-ATTCTAGAGTTTGATCATGGCTTCA), corresponded to positions 11 to 26 in Escherichia coli 16S rRNA, and the reverse primer, UNI16S-R (5'-ATGGTACCGTGTGACGGGCGGTGTTGTA), corresponded to the complement of positions

1411 to 1393 in *Escherichia coli* 16S rRNA (Somogyi, 1952). PCR reaction conditions were carried out according to Beffa et al. (1996), and the PCR product was cloned into the pGEM-T vector system. After the PCR amplification and the cloning of the 16S rRNA genes of our isolates, the 16S rRNA sequences were determined with the use of an Applied Biosystems 373A DNA sequencer with an ABI PRISM cycle sequencing kit (Macrogen, Holland). Sequences consisting of about 1400 nt of 16S rRNA genes were determined and compared with those in the EzTaxon database. The 16S rRNA gene sequences of the species most closely related to our isolates were retrieved from the database. The retrieved sequences were aligned with the use of the Clustal X program (Thompson et al., 1997) and manually edited. Phylogenetic trees were constructed by the neighbor-joining method with the use of the Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 (Tamura et al., 2007).

2.3. Enzyme Assays

For enzyme assays bacterial supernatant was used. The isolates were grown in lignin medium for seven days at 37°C and 120 rpm. Then the cultures were centrifuged at 10000 rpm for 10 minutes and the bacterial supernatant was used as enzyme solution.

Laccase activity was determined by the oxidation of 2,2'-azinobis - (3-ethylbenzthiazoline-6-sulphonate) (ABTS). The reaction mixture contained 0.5 mM ABTS, 0.1 M sodium acetate

buffer (pH 5.0), and a suitable amount of enzyme. The oxidation of ABTS was followed by an absorbance increase at 420 nm (More et al., 2011).

Manganese peroxidase (MnP) activity was measured by monitoring the formation of Mn(III)-malonate complex at 270 nm. The reaction mixtures contained MnSO₄ (0.2 mM), H₂O₂ (0.1 mM), and enzyme in 50 mM sodium malonate (pH 4.5) (Paice et al., 1993).

Lignin peroxidase (LiP) activity was measured by monitoring the oxidation of veratryl alcohol at 310 nm. The reaction mixtures contained 50 mM Na tartrate buffer (pH 2.5 or 4.5), 0.1 mM H₂O₂, and 2 mM veratryl alcohol in a 1.0-ml reaction volume (Tien and Kirk, 1983).

2.4. The Ratio of Lignosulphonate Degradation

The percentage of lignosulphonate degradation of the isolates was tested in a 500-ml erlenmeyer flask with 100 ml of lignin degradation medium (LDM) consisting of (l-1): 1 g KH₂PO₄, 4 g NaHPO₄, 0.2 g NaCl, 0.2 g MgSO₄.7H₂O, 0.05 g CaCl₂, 2 g yeast extract, and 5 g lignin. The sterilized medium was inoculated with 0.5 ml of a 24-h culture of bacteria in nutrient broth. Controls consisting of uninoculated flasks were also prepared for comparison. The cultures were incubated for 1 week at 120 rpm and 37°C (El-Gammal et al., 1997).

After 1 week, the cultures were sterilized and passed through filter paper, the weight of which had been previously determined. After

filtration, the papers were dried in Pasteur's furnace to determine the residual lignin ratio. The lignin degradation ratio of different isolates was expressed according to the following equation:

$$\text{Degradation (\%)} = \frac{L_I - L_F}{L_I} \times 100$$

where L_I = initial amount of lignin, and L_F = the amount of lignin after 1 week of culture.

2.5. Kraft Lignin Decolorization

Biodegradation experiments were carried out in 250-ml Erlenmeyer flasks containing 100 ml of sterile mineral salt medium (MSM, g l⁻¹: 2.4 g Na₂HPO₄, 2 g K₂HPO₄, 0.1 g NH₄NO₃, 0.01 g MgSO₄, 0.01 g CaCl₂, 10 g D-glucose, 5 g peptone. The trace element solution (1 ml l⁻¹, pH 7.6) contained 2 g l⁻¹ kraft lignin. The sterilized medium was inoculated with 0.25 ml of a 24-h culture of bacteria in nutrient broth. The Control and inoculated erlenmeyer flasks were incubated for six days in a rotary shaker at 120 rpm under aerobic conditions at 30°C (Raj et al., 2006). The biodegradation of kraft lignin was determined in terms of the loss of total kraft lignin from the treated sample. Uninoculated (control) and bacteria-inoculated samples (30 ml) were centrifuged at 8,000 g for 15 min to remove biomass. Supernatants were acidified to pH 1–2 with concentrated HCl. The precipitate was collected in tared centrifuge tubes by centrifugation at 8,000 g for 30 min, dried at 60 ± 5°C for 48 h, and then weighed. The kraft lignin loss (%) in the sample treated with bacteria was

determined as dry weight (assuming kraft lignin present in identical volume of control as 100%).

2.6. Ligninolytic Activity Tests

Isolates were subjected to different plate assays for screening of several lignin-degrading enzymes (laccase and peroxidase (LiP and MnP)). An extracellular peroxidase (LiP and MnP) test was done according to a modification of the method proposed by Rayner & Boddy (1988). The bacteria were grown on NA at 37°C for 2 days. The addition of equal parts of 0.4% (v/v) H₂O₂ and 1% pyrogallol in water gave a yellow-brown color to colonies with peroxidase activity.

Laccase activity was determined with the use of ABTS agar. This colorless agar medium turns green due to the oxidation of ABTS (2,2'azino-bis(3-ethylbenzthiazoline-6-sulphonate) to ABTS-azine in the presence of laccase. For preparation of ABTS agar LME basal medium (g-l in distilled water: 1 g KH₂PO₄, 0.5 g C₄H₁₂N₂O₆, 0.5 g MgSO₄•7H₂O, 0.01 g Yeast Extract, 0.001 g CuSO₄•5H₂O, 0.001 g Fe₂(SO₄)₃, 0.01 g CaCl₂•2H₂O, 0.001 g MnSO₄•H₂O) medium supplemented with 0.1 % w/v ABTS and 1.6 % w/v agar and autoclave. To every 100 ml of the growth medium prepared, 1 ml of a separately sterilized 20 % (w/v) aqueous glucose solution was aseptically added. The production of laccase was shown by the formation of a green color in the growth medium.

2.7. Utilization of Lignin Monomers

To monitor the growth on lignin monomers, a mineral medium (MM: g-l in distilled water: 1.55

g KH_2PO_4 , 0.85 g $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 2 g $(\text{NH}_4)_2\text{SO}_4$, 0.1 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 10 mg EDTA and 5 ml trace element solution) was supplemented with 5 mM lignin monomers (vanilic acid, vanillin, 4-hydroxybenzoic acid, syringic acid, phenol, veratryl alcohol, guaiacol) as the sole carbon and energy source. Growth was considered positive if observed after successive transfers to fresh medium (Bogdan et al., 2018).

2.8. Decolorization of Azo, Triphenylmethane and Heterocyclic Dyes

The decolorization of dyes was monitored at the maximum visible absorbance of each dye. Unless otherwise stated, the reaction mixture consisted of 80 μM of dye (see Table 1), 0.1 U of lignin peroxidase, and 0.4 mM of H_2O_2 in 50 mM sodium tartrate in a total volume of 1 ml. The reaction was initiated by the addition of H_2O_2 , and absorbance was measured 15 min after the initiation (Ollikka, 1993).

Table 1. Conditions for dye decolorization by lignin peroxidase

Dye	Absorbance maximum
BPB	590
Methylene blue	662
Methyl orange	502
Toluidine blue	625
Malachite green	620

2.9. Dye Decolorization Determination

The dye decolorization by the isolated microorganisms was tested in 250 ml erlenmayer flasks with 25 ml of basal liquid medium (BLM) consisting of (1-1): 10 g glucose, 5 g peptone, 2 g

yeast extract, and 70 ml of trace element solution (in g l-1: 1.5 g nitrilacetic acid at 99%, 3 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 1 g NaCl, 0.1 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g CoSO_4 , 0.1 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.01 g $\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$, 0.01 g H_3BO_3 , and 0.01 g $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$) supplemented with 200 mg l-1 of Remazol Brilliant Blue R (RBBR). The sterilized medium was inoculated with 0.25 ml of a 24-h culture of bacteria in nutrient broth. Controls consisting of uninoculated flasks were also prepared for comparison. Triplicate flasks were used for each isolate and control. The cultures were incubated for 7 days at 120 rpm and 37° C and then centrifuged at 13,000×g for 5 min at 4° C. The cell-free supernatant color was read with a spectrophotometer at the maximum absorbance spectra (λ_{max}) of the dyes used, i.e., 595 nm for rbbbr. the uninoculated dye-free medium was used as blank. the uninoculated dye-containing controls were used as reference to correct abiotic color disappearance (Chantarasiri and Boontanom, 2017). The decolorization efficiency of different isolates was expressed according to the following equation:

$$\text{decolorization}(\%) = \frac{A_{\lambda, \text{initial}} - A_{\lambda, \text{final}}}{A_{\lambda, \text{initial}}} \times 100$$

where $a_{\lambda, \text{initial}}$ = initial absorbance; and $a_{\lambda, \text{final}}$ = absorbance after 7 days of culture.

3. RESULTS AND DISCUSSION

3.1. Ligninolytic Microorganisms

As a result of isolation and screening, nine lignin-degrading bacteria were selected on the basis of their growth degree on lignin agar. The medium contained lignin as the sole carbon source. The isolates designated as L1, L2, L3, L4, PT21, PT22, PT41, G1, and C1 used lignin as the carbon source and developed colonies within 24 h of incubation. The cells of the strains were gram-negative and rod-shaped. The isolate G1 had a bacterial capsule. On the agar plates, all isolates had white and mucoid colonies.

3.2. 16S rRNA Gene Sequence Analysis

A total of 1400 nucleotides of the 16S rRNA from 9 isolates were aligned and compared to sequences of related bacteria. A phylogenetic tree was constructed with the use of the neighbor-joining method (Figure 1). On the basis of the 16S rRNA gene sequence analysis, the isolates were identified as members of the Enterobacteriaceae family. Five of them belong to the genus of Enterobacter, three to the genus Citrobacter, and one to the genus Klebsiella. The accession numbers of the 16S rRNA gene sequences of the 9 isolates had been assigned by GenBank (Tables 2 and 3). According to the data obtained, the isolates L1, L2, and PT21 are different strains of Enterobacter cancerogenus; L3 and L4 are different strains of Enterobacter ludwigii; PT22 is a strain of Citrobacter farmeri C1 is a strain of Citrobacter murlinae; PT41 is a

strain of Citrobacter sedlakii and G1 is a strain of Klebsiella pneumoniae.

Table 2. The percentage similarity of 16S rRNA of Enterobacter species and the isolates

	L1	L2	L3	L4	PT21
<i>Enterobacter cancerogenus</i>	99	98,8	96,6	98,9	98,7
<i>Enterobacter asburiae</i>	98,6	98,2	96,5	98,4	98
<i>Enterobacter ludwigii</i>	98,5	98	97,3	99,7	97,8
<i>Enterobacter cowanii</i>	97	98,1	95,7	97,7	98,2

Table 3. The percentage similarity of 16S rRNA of Citrobacter and Klebsiella species and the isolates

	PT22	PT41	C1	G1
<i>Citrobacter sedlakii</i>	97,2	99,2		
<i>Citrobacter farmeri</i>	99,2	98,1		
<i>Citrobacter rodentium</i>	97,8	98,4		
<i>Citrobacter murlinae</i>			97,4	
<i>Klebsiella pneumoniae</i>				99,8
<i>Klebsiella variicola</i>				99
<i>Klebsiella granulomatis</i>				98

3.3. Lignin Degradation

Figure 2 shows the rate of lignosulphonate degradation after one week of incubation. As shown in the figure, the lignin degradation of the isolates is considerably high. Lignins are very complex natural polymers with many random couplings, but their exact chemical structure is not known. The physical and chemical properties of lignin differ depending on the extraction technology. For example, whereas lignosulphonates are hydrophilic (dissolve in water), kraft lignins are hydrophobic (do not dissolve in water). However, the kraft lignin is very important in the paper and pulp industry. Thus, the kraft lignin degradation of the isolates was investigated in this study. The isolates do not

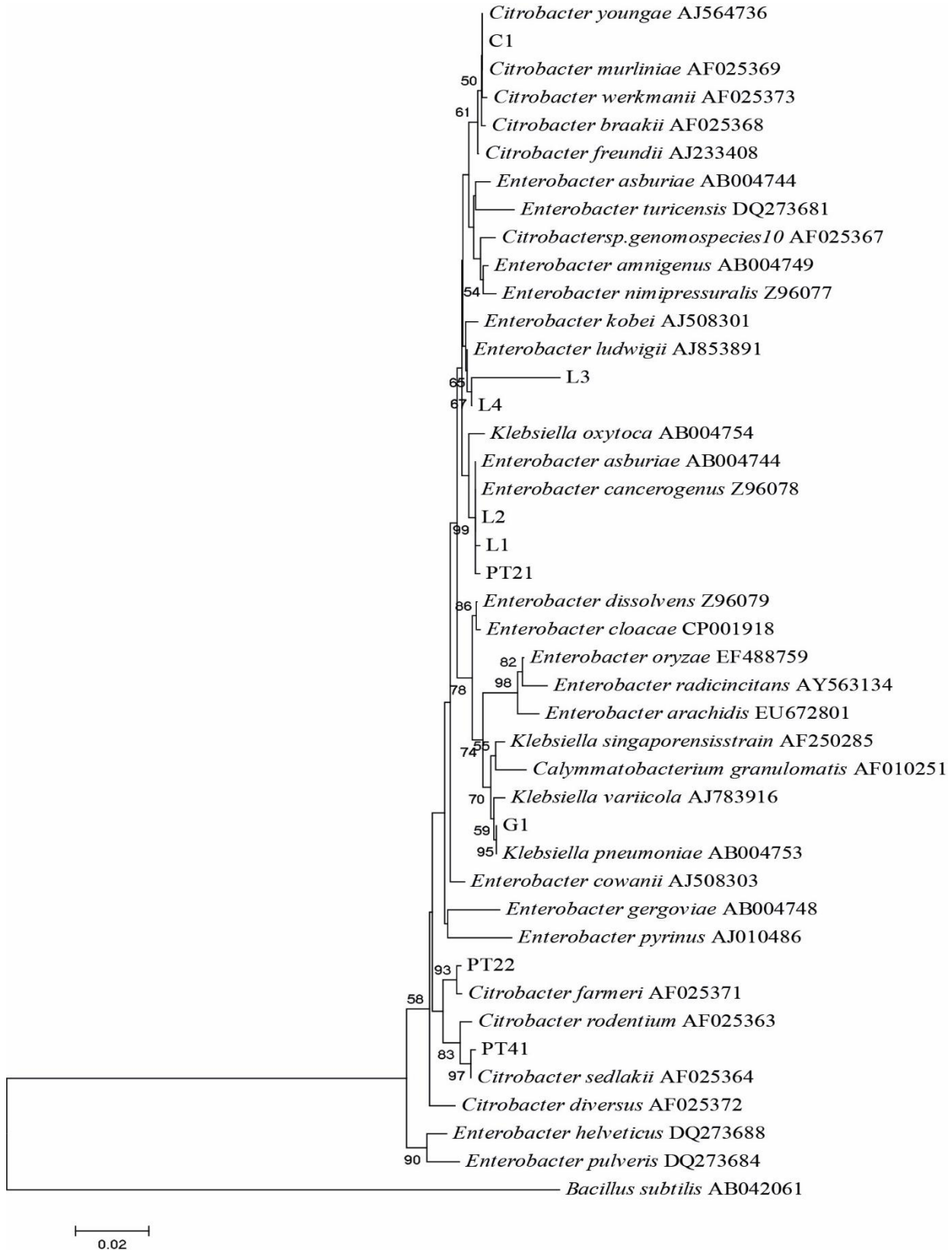


Figure 1. Dendrogram estimated phylogenetic relationship on the basis of 16S rRNA gene sequence data of the bacteria isolated from different sources and some reference strains, using the neighbor-joining method. The accession numbers are given in parentheses. The scale bar represents 2% divergence.

use kraft lignin as the sole carbon source. For this reason, basic nutrients, such as 1.0% (w/v) glucose and 0.5% (w/v) peptone, were used as additional carbon and nitrogen sources to stimulate bacterial growth for KL decolorization.

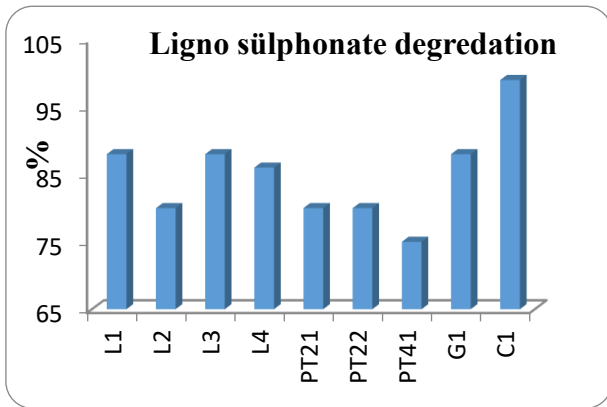


Figure 2. The percentage of lignin biodegradation

Figure 3 provides the data obtained from the kraft lignin biodegradation. The results are in accordance with those obtained by El-Hanafy et al. (2008) but are much better because the KL concentration is higher. El-Hanafy et al. (2008) found that the isolated strains BahHAE3 and BahHAE8 reached a maximum lignin degradation of 76.3% and 67.1%, respectively, on the sixth day. The lignin concentration in our study (2 g lignin/l) is much higher than the 0.7 g used by Chandra et al. (2007), who reported that *Paenibacillus* sp., *Aneurinibacillus aneurinilyticus*, and *Bacillus* sp. achieved lignin degradation rates of 37, 33, and 30%, respectively, during the incubation period.

Deschamps et al. (1980) used an industrial kraft lignin (1 g lignin/l) as the sole carbon source in their study. They found that *Aeromonas* sp. degraded 98% of the kraft lignin after 5 days of

incubation. The differences in the lignin degradation rates obtained between previous studies could be explained by the variations in bacterial strains, incubation conditions, and lignin structures used.

The results of the current study support the finding that many bacterial strains degrade and assimilate lignin (Chandra et al., 2007; Bal et al., 1989; Nishimura et al., 2006; Odier et al., 1981; Pometto et al., 1986). Furthermore, the capability of the soil-isolated bacteria to effectively degrade and assimilate lignin as the sole carbon source complied with the findings of Morii et al. (1995), who reported that three bacterial species isolated from compost soil, namely, *Azotobacter*, *Bacillus megatarium*, and *Serratia marcescens*, are capable of degrading lignin. Regarding the *Enterobacter* species isolated in the current study, many studies have pointed out the ability of the species to degrade lignin (Chandra et al., 2007; Morii et al., 1995).

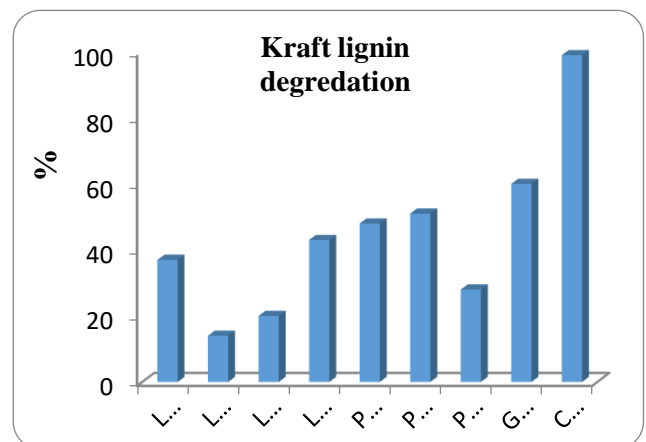


Figure 3. The percentage of kraft lignin biodegradation

3.4. Ligninolytic Enzymes of The Isolates

In this study, all the isolates showed extracellular peroxidase activity. To determine whether the peroxidase was MnP or LiP, enzyme assays were done. With the use of spectrophotometric methods, we determined that the isolates showed lignin peroxidase activity. Only the C1 isolate showed manganese peroxidase activity.

According to the ABTS agar laccase test, only the isolate PT41 showed laccase activity. As a result, the isolated bacterium *Citrobacter sedlakii* PT41 contained both laccase and lignin peroxidase. Previous studies have reported that the *Bacillus* species contains laccase (Canas et al., 2007; Reiss et al., 2011; Franc et al., 2001); in addition, Oliviere et al. (2009) found that *Bacillus pumilus* and *Paenibacillus* sp. contain manganese peroxidase. The *Streptomyces* species has also been found to contain lignin peroxidase (Gottschalk et al., 1999; Nascimento & Silva, 2008).

3.5. Utilization of Lignin Monomers

There is a wide spectrum of lignin monomers that could be used to stimulate the growth of the isolates *Enterobacter cancerogenus* L1, *Enterobacter cancerogenus* L2, *Enterobacter ludwigii* L3, *Enterobacter cancerogenus* PT21, and *Citrobacter sedlakii* PT41 (Table 4). In this study, these isolates used all of the tested monomers in their growth. C1 was the only isolate that did not grow on vanillic acid; neither did it grow on vanillin and 4-hydroxybenzoic acid. *Enterobacter ludwigii* L4, *Citrobacter*

farmeri PT22, and *Klebsiella pneumoniae* G1 also did not grow on vanillin. Remarkably, the isolated strains have the ability to oxidize aromatic alcohols or aldehydes to their corresponding carboxylic acid form. Bandounas et al. (2011) found that the spectrum of lignin monomers that could be utilized for growth was relatively limited for all of the isolates and that the alcoholic forms of the aromatic monomers (veratryl alcohol and guaiacol) were not metabolized by any of the isolates. In contrast, the isolates in this study metabolized the aromatic monomers well.

3.6. Decolorization of Azo, Triphenylmethane and Heterocyclic Dyes

In this study, we examined the decolorization of several dyes by crude lignin peroxidase. Only the lignin peroxidase obtained from *Klebsiella pneumoniae* G1 decolorized dyes significantly. It decolorized BPB, methylene blue, methyl orange, and toluidine blue by 70%, 40%, 19%, and 58%, respectively (Figure 4). These results are in accordance with those obtained by Ollikka et al. (1993), who tested the dye decolorization of *Phanerochaete chrysosporium* and found that the best decolorization (93%) was obtained for bromophenol blue, as shown in this study. Ollikka et al. found that the decolorization ability of the enzymes was increased when veratryl alcohol was present in the reaction mixtures. In the present study, veratryl alcohol was not used in the reaction mixtures. Taking into consideration the previous findings, we can predict that the

Table 4 : Growth of bacterial isolates on lignin monomers

Aromatic compound	L1	L2	L3	L4	PT21	PT22	PT41	G1	C1
Vanilic acid	+	+	+	+	+	+	+	+	-
Vanilin	+	+	+	-	+	-	+	-	-
4-Hydroxybenzoic acid	+	+	+	+	+	-	+	+	-
Syringic acid	+	+	+	+	+	+	+	+	+
Phenol	+	+	+	+	+	+	+	+	+
Veratryl alcohol	+	+	+	+	+	+	+	+	+
Guaiacol	+	+	+	+	+	+	+	+	+

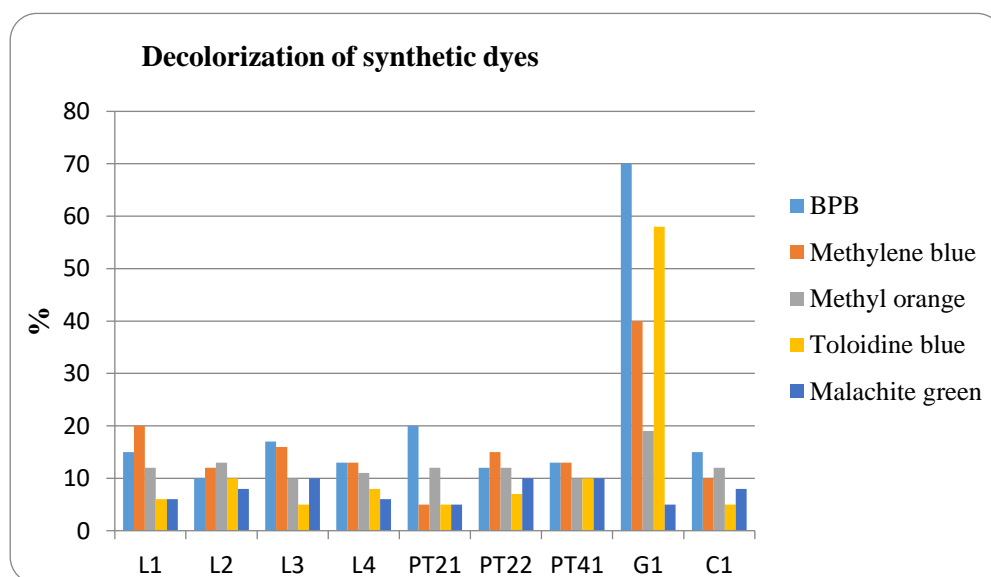


Figure 4. The percentage of dye decolorization

decolorization capacity of *Klebsiella pneumoniae* G1 lignin peroxidase will be increased.

3.7. Dye Decolorization Studies

The nine isolates were further tested by measuring their efficiency of RBBR decolorization (Figure 5). All of the strains were able to decolorize RBBR to some extent, and four of them achieved a 90% decrease in dye color. In the present study, this showed that the ability to decolorize RBBR dye is correlated to ligninase production. Previous works also found a correlation between decolorization of polymeric dyes and lignin degradation activity among several wood- or soil-inhabiting fungi (Falcon et al., 1995; Pasti and Crawford, 1991). However, there are very few studies about such correlation among bacteria. In this research, we showed that some bacteria have as much ability to degrade polymeric dyes as fungi.

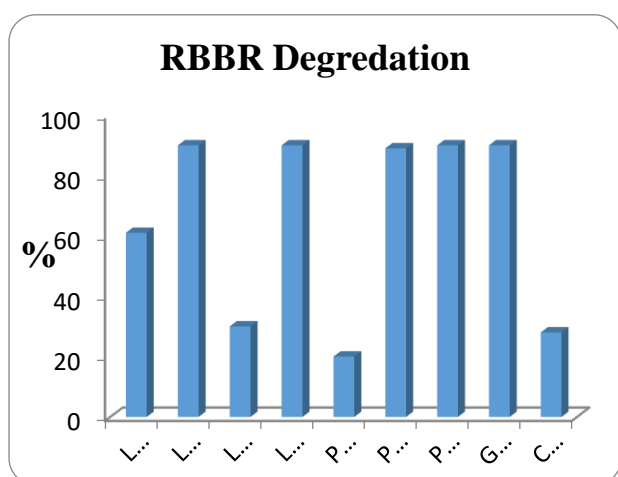


Figure 5. The percentage of RBBR degradation

M. J. Lopez et al. (2006) tested the dye decolorization of bacteria and fungi that they had isolated, and they found that RBBR was difficult

to decolorize. Twenty-five strains showed some decolorization of RBBR, but the efficiency was around 15–30% except for three mesophilic fungi, whose decolorization rates were greater than 50%. Rodriguez et al. (1999) found that *Pleurotus ostreatus* strains exhibited high laccase and manganese peroxidase activity and decolorized different industrial dyes very efficiently. An earlier study by Özsoy et al. (2005) revealed that *F. trogii* could effectively decolorize RBBR dye by up to 90% within 24 h. They used an initial dye concentration of about 10-50 mg/l, compared to the RBBR concentration of 200 mg/l used in this study. Kiran et al. (2019) found that when synthetic dye wastewater concentration was increased from 0.01-0.05%, the percent decolourization decreased from 80.93% to 24.705%. Taking into consideration these previous findings and the dye concentration, we can say that our isolates were more effective at dye decolorization.

In conclusion, many lignin-degrading organisms are reported in this study, as are their lignin degradation and dye decolorization rates. The isolation and identification of environmentally friendly bacteria for lignin degradation have become essential because all previous researchers concentrated on the use of fungal treatments. The importance of ligninolytic bacteria has increased because lignin-degrading bacteria have a wider tolerance for temperature differences, pH variations, and oxygen limitation compared to fungi. This study found that the strains *Klebsiella pneumoniae* G1 and C1 have a strong potential for KL degradation. In particular,

the *Klebsiella pneumoniae* G1 strain is very effective in RBBR decolorization. Additional studies have to be made to determine the optimum conditions (nutrients, temperature, etc.) for maximum lignin degradation and dye decolorization by pure or mixed cultures of the strains. In addition, ligninolytic enzymes from these strains could be extracted and evaluated.

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