Investigating the Ligninolytic Potential of Bacteria Isolated from a Decayed Wood Sample in Egypt

Prince Hotor*, Ahmed Hassanin, Mitsuo Yamamoto, Yukie Saito, and Ahmed Abdel-Mawgood

***Abstract*—Lignin is a natural polyphenol material found in complex with cellulose and hemicellulose in every terrestrial and some aquatic plant species. They are recalcitrant and prevent easy access to cellulose; an energy-rich, versatile, promising biomaterial for several eco-friendly and cheap products. The lignin-degrading potential of microbes isolated from degraded wood in an environment in Egypt was investigated. In this study, four bacteria were screened using a methylene blue decolorization test, growth on media with lignin as carbon source, and cellulolytic screening using CMC-Congo red test. The screened isolates were used to treat sorghum stem stalks to ascertain the level of degradation of the lignin content. The four bacterial isolates were positive to the decolorization of methylene blue with percentage decolorization: (23%, 8%, 34%, and 24%) after 24 hrs. respectively. Similarly, the four isolates were able to grow on minimal salt media with lignin as carbon source. The optical densities values measured ranged from (0.030–0.165, −0.040–0.080, 0.040–0.121, −0.039–0.054) after 12 h for the four bacterial isolates, respectively. Also, the four bacterial isolates showed negative results for the cellulase test. FT-IR spectrum for the bacterial-treated sorghum stalks revealed a reduction in peaks at wavelengths indicative of lignin functional groups. Similarly, proximate analysis of the lignocellulose content after treatment recorded were extractives (E) (9, 9, 13.5, 9.5, and 10) %, hemicellulose content (H) (29.5, 26.0, 31, 26, and 30) %, Klason lignin (L) (39, 31, 33, 24, and 26) %, and cellulose (C) (22.2, 33.5, 21.3, 40.5, and 33.5) % for control and the four bacterial isolates, respectively. The p-values estimated from the average values for the extractive and hemicellulose content were not significantly different, whiles that of Lignin and Cellulose was significantly different. The four bacterial isolates showed potential for having ligninolytic enzymes for lignin depolymerization.**

***Index Terms*—Depolymerization, lignin, decolorization of methylene blue, Kraft lignin, ligninolytic ability**

I. Introduction

The quest to move from petroleum-based products to bio-based materials have warranted extensive research into raw biomaterials and their applications into renewable, biodegradable, and easily transformed products [1]. Plant materials provide cheap natural sources for products, cellulose, lignin, chromogens, etc. that are non-persistent, eco-friendly, and biodegradable [2]. Bioplastics, ethanol production, epoxy-resins [3], carbon fibers [4], bio-based fuels [5], chromogens [6], quantum dots [7], among others, are some examples of bio-based products. The ability to convert plant materials into useful products creates many challenges as these plant-based sources are in conjunction with other secondary substances. Cellulose, the most abundant natural biomass on earth, is a straight-chain polysaccharide with carbohydrate, whose monomeric units are held by 1,4 β glycosidic bonds [8]. Cellulose's physical, biological, mechanical, and chemical properties make it a good natural material for several bio-based products [9]. However, cellulose comes in complex with lignin (second largest plant-based biomass on earth) [10], hemicellulose, and other natural materials. Lignin is a recalcitrant bio-polymeric, complex-phenolic natural material that acts as structural support for plants, protects cellulose from microbial attack, and forms the xylem and phloem tissues due to their hydrophobic nature [11]. This makes it imperative to depolymerize lignin to make it possible to utilize the energy-rich cellulose component of the plant for several applications. Chemical [12], mechanical, and high energy required approaches have been utilized to degrade lignin.

The above methods have some limitations; a) Not eco-friendly, b) expensive, c) alteration of physical and chemical properties of cellulose obtained d) Energy-intensive. Bio-depolymerization is another method that has been used. This method is eco-friendly, inexpensive, and easy to use. However, this method has not been fully exploited and has some demerits; a) time consuming, b) inefficient industrial scale due to the slow degradation process.

Nevertheless, there have been good microbes; *P. chrysosporium* [13], *T. Versicolor*, *Actinomycetes sp., Bacillus sp., Sphingobium sp.* **SYK-6,** *Pseudomonas putida mt-2, Robodocs sp*. [14], and *Citrobacter sp.* that have had very good ligninolytic abilities using Lignin peroxidase (LiP), Manganese peroxidase (MnP) [15], Laccase (Lac) [16], Versatile peroxidase (VP) [13], and Dye-decolorizing peroxidase enzymes (DyP) [17]. More microbes need to be isolated to get a greater pool to ascertain the higher degrading ability of microbes. The aim of this research; a) To isolate fungi and bacteria strains with lignin-degrading ability from several environments in Egypt. b) To qualitatively and quantitatively screen them to ascertain their degree depolymerization of lignin. The style will adjust your fonts and line spacing. underline.

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II. Method

A. Environmental Sample Collection

Decaying hard wood samples were collected from a farm in Wady El-Natron in Egypt, kept in a sterile bag, and transported to the Egypt-Japan University of Science and Technology, Biotechnology laboratory. Fig. 1 depicts the methodological approach used in this investigation.

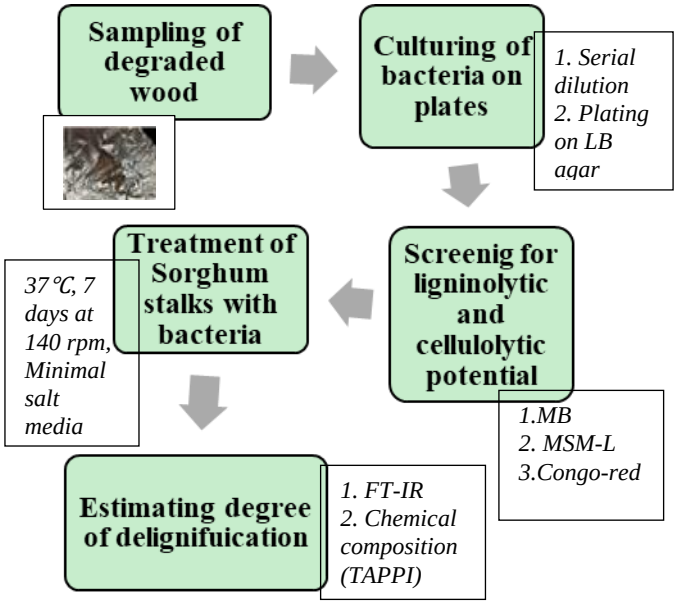

Fig. 1. Graphical overview of the methodology.

B. Isolation of Bacteria

Degraded hard wood sample (5.00±0.01 g) was weighed and placed into sterile 15 mL Falcon tubes containing 10 mL of distilled water and was vortexed gently to mix the content. Serial dilutions were performed to the order of 105 where each falcon tube contained 9 mL of sterile distilled water supplemented with 1 mL from each tube. After which 10 µL of solution from these five falcon tubes were plated on 25 cm^3 petri dishes containing solidified and dried nutrient agar (NA) which was composed of tryptone 5.0 g/L, beef extract 3.0 g/L, agar 15 g/L. The inoculated plates were kept in an incubator at 37 ℃ for 48 h. Individual bacterial strains were streaked on new agar plates several times till pure cultures were seen for each strain. The pure bacterial strains were grown overnight (16 h) in 7 mL sterile Luria Bertani broth (LB) composed of $NaCl_2$ 10 g/L, yeast extract 5 g/L, tryptone 10 g/L. Storage and working stock from each pure isolate were made by taking 1 mL of culture in (2:3, v/v) sterile LB glycerol mixture in sterile cryovials and kept at −80 ℃.

C. Primary Screening of Bacteria

1) Plate assay

The pure isolates (20 µL) from the working stock were grown in (LB) overnight and screened for their potential to degrade lignin using a method in [18]. In this method, spot inoculation (10 µL of culture aliquot) was used to inoculate the strains on (LB) agar supplemented with methylene blue (25 mg/L). E. coli DH5α was used as the negative control. Plates were incubated at 37 ℃ for 48 h. Similarly, the purified strains were spot inoculated on minimal salt media supplemented with Kraft lignin (MSM-L) to ascertain their growth on agar with lignin as a carbon source. Plates were incubated at 37 ℃ for one week. MSM-L was composed of K_2HPO_4 0.3 g/L, $NaCl_2$ 0.5 g/L, KH_2PO_4 0.4 g/L, $MgCl_2$ 0.24 g/L, NH_4Cl 0.5 g/L, yeast 1 g/L, agar 15 g/L, and 1% Kraft lignin. Bacterial isolates were also spot inoculated on MSM supplemented with 0.188% carboxymethylcellulose sodium salts (CMC), 0.2% gelatin and, 0.02% Congo-red. MSM supplemented with CMC was used to test for cellulase activity. E. coli DH5α was used as the negative control. After which the plates were incubated at 37 ℃ for one week.

2) Liquid dye decolorizing assay

The pure isolates (20 µL aliquot) from overnight culture each were used to treat MB (25 mg/L) broth in sterile LB (50 mL, 500 mL sterile conical flask). These flasks were incubated in a shaker (37 ℃, 140 rpm) for 24 h in the dark to prevent photocatalytic decolorization. A treatment with no cell culture was used as a negative control to account for any decolorization due to environmental factors. Aliquots (2 mL) from each treatment were taken at 0 h and 6 h intervals for 24 h. These aliquots were centrifuged (10,000 g, 5 min), and the absorbance of the supernatants were measured at 664 nm using Hitachi U-3900 single beam spectrophotometer. Absorbance readings were done in technical triplicates for each strain, and blanking was performed using LB broth.

3) Utilization of Kraft lignin for growth

Similarly, aliquots (20 µL) from an overnight culture of each bacterial isolate were inoculated into sterile minimal salt media supplemented with 1% Kraft lignin as a carbon source. The technique was modified from [18]. The treatment was incubated in a shaker (37 ℃, 140 rpm) for 24 hours. Aliquots (2 mL) were taken from each flask at an interval of 6 hours, at 0 h to 24 h. The absorbance of each aliquot was measured at 600 nm using Hitachi U-3900 single beam spectrophotometer to ascertain their optical density (OD_{600}). Readings were done in technical triplicates, and MSM-L broth was used as blank for the measurement.

D. Small-Scale Pre-treatment of Lignocellulose Biomass

Fresh sorghum stalks (5.001±0.001 g) were surfaced sterilized (ethanol, 70%) and were placed in a 500 ml flask containing 200 ml of sterilized MSM broth. Aliquots (20 µL) of an overnight culture of each of the bacterial isolates were inoculated into each flask and were incubated in a shaker (37 ℃, 140 rpm) for one week. A blank treatment containing no bacterial isolate was also incubated. After treatment, the sorghum stalks were removed washed thoroughly under running water. Their wet mass was measured, after which they were dried in an oven (24 h, 105–110 ℃). The masses of the stalks were measured until a constant mass was attained.

E. Fourier Transform Infrared-Spectroscopy (FT-IR) of Sorghum Stalks

Samples of the treated sorghum stalks were cut into smaller pieces and grind using a blender. After, the samples were dried again overnight (105 ℃) to remove moisture. The dried samples were then analyzed using FT-IR to ascertain the differences in the spectrum between the treated samples using Bruker Vertex 70.

F. Lignocellulosic Content Determination

1) Extractives

The treated sorghum stalk (0.500±0.001 g) for each isolate was cut into significantly smaller sizes. After which, each was treated with 30 ml of acetone (98%) in flask and covered. The treatment was done at (90 ℃, 2.5 h) whiles stirring with a magnetic stirrer using a hot plate. After each sample was filtered, the residue was washed with distilled water until the filtrate became colorless. The residue was dried (24 h, 105–110 ℃) until constant mass was attained [19]. The extractive treatment was done in duplicates for each treatment. The extractive component is the difference between the mass before and after the treatment.

2) Hemicellulose

Extractive free sorghum stalk from each bacterial treatment (0.500 ± 0.001 g) was treated with (75 mL, 0.5 M NaOH) in a covered flask and was kept on a hot plate at 80 ℃ for 4 h. The stalk residue was washed with distilled water until pH 7 was noticed for the filtrate using a pH meter. The residue was dried in an oven (24 h, 105–110 ℃) until a constant mass was attained. All hemicellulose determination was done in duplicates for each treatment. The difference between the mass before and after the treatment was the hemicellulose content.

3) Klason Lignin

Extractive free sorghum stalks for each bacterial treatment (0.500±001 g) were treated with (5 mL, 72% H_2SO_4) in a covered 250 ml flask and were stirred using a magnetic stirrer until content began to dissolve. The treatment content was then placed in a water bath at (30±0.5) ℃ for 1 h. The treatment was diluted with 140 mL of hot distilled water to make about 3% H_2SO_4. After, each of the treatments were autoclaved (121 ℃, 1 h, 1.5 kPa). The autoclaved samples were filtered hot (around 80 ℃) to collect the residue. The residues were washed thoroughly with distilled water till the pH of the filtrate drops closer to pH 7. The neutral residue was then dried overnight in an oven (24 h, 105–110 ℃). The Klason lignin determination was duplicated for each isolate treatment [20]. The Klason lignin content was measured from the constant mass of the residue.

III. Results and Discussions

A. Isolated Strains and Their Plate Assay Reaction (MB, CMC-Congo red, and MSM-L)

Four bacterial isolates showed positive results towards the MB and the MSM-L plate test. However, they all showed negative tests towards the CMC-Congo red plate assay. The E. coli, a negative test, also showed negative results with the MB and CMC-Congo red. The decolorization of MB and the cell growth on MSM-L by the four bacterial isolates indicate their potential to have ligninolytic enzymes and their ability to use lignin as a carbon source for cell growth. While their negative reaction towards the CMC-Congo red test explains the absence of cellulolytic enzymes. The results of the plate assay are seen in Table I and Fig. 2 below.

TABLE I: Test Results for the Various Plate Assays

		Test results				
Test	**Test Condition**	P1	P2	P3	P4	*E. coli DH5α*
1	MB + isolate	++	+	++	++	-
2	CMC-Congo red + isolate	-	-	-	-	-
3	MSM +Kraft lignin + isolate	+	+	+	+	N/A

NB: (++), (+), and (-) indicate very high, high, and no decolorization of **MB** for **test 1**, respectively. Similarly, (+), and (-) indicate positive and negative cellulase activity for **test 2**, respectively. (+) and (-) indicate ability and inability to use lignin as carbon source for **test 3,** respectively. N/A represent no test for that isolate. P1, P2, P3, and P4 are the four bacterial isolates used for the tests. MB= methylene blue, CMC= carboxymethylcellulose, MSM= minimal salt media.

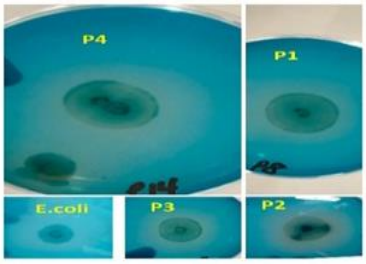

Fig. 2. Methylene blue decolorizing plate assay.

B. Methylene Blue Decolorization Assay

The four bacterial strains showed decolorization of the MB in the LB broth after 24 h P3 (34%) recorded the highest decolorization percentage, while the lowest was P2 (8%). The decolorization percentages as seen in Table II for P1 and P4 were (23%) and (24%), respectively as seen in Table II. The extent of decolorization by the four isolates can be seen visually in Fig. 3. The highest percentage of decolorization of P3 may indicate a higher ligninolytic enzyme activity. The negative values may indicate full dissolution of methylene blue particles in the solution during the 0-hr time.

TABLE II: Percentage Decolorization of Methylene Blue

		Decolorization of MB (%)				
Time/hours	**Media composition**	**C**	**P1**	**P2**	**P3**	**P4**
0		0.0	0.0	0.0	0.0	0.0
6	LB+MB+ isolate	−4.0	2.0	−8.0	3.0	2.0
12		3.0	3.0	8.0	5.0	10.0
24		1.0	23.0	8.0	34.0	24.0

NB: LB= Luria broth, MB=, methylene blue. C, P1, P2, P3, and P4 represents no isolate treatment and the four bacterial treatments respectively.

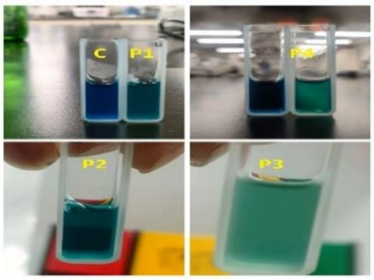

Fig. 3. Visual final color after 24 hrs treatment of the methylene blue in a cuvette. NB: C = is negative control with no cell treatment. 0 h, 6 h, 12 h, and 24 h.

C. Cell Growth Pattern in MSM-L Broth

The four bacterial strains were able to grow in MSM-L broth where the only carbon source was lignin after 12 h. The strain with the highest average optical density value was P1 (0.165±0.005), followed by P4 (0.121±0.008), P2 (0.119±0.003), and P3 (0.107±0.006) as seen in Table III. The peak in growth was recorded at the 12 h for P1 and P4. Whiles that of P2 and P3 was recorded in the 6 h. The difference in the peaks for the growth may be due to different growth pattern of the four bacterial isolates.

TABLE III: ABSORBANCE READINGS AT 600 NM FOR THE VARIOUS ISOLATES IN MSM-L

		Average ± Standard deviation (OD_{600})			
Time/ hours	**Test composition**	**P1**	**P2**	**P3**	**P4**
0	MSM + Kraft lignin + isolate	0.030 ± 0.008	-0.040 ± 0.005	-0.045 ± 0.003	0.040 ± 0.009
6		0.054 ± 0.002	0.120 ± 0.003	0.107 ± 0.006	0.120 ± 0.002
12		0.165 ± 0.005	0.082 ± 0.007	0.067 ± 0.004	0.121 ± 0.008
24		0.084 ± 0.003	0.038 ± 0.003	0.054 ± 0.007	-0.193 ± 0.059

NB: P1, P2, P3, and P4 represents the four bacterial isolates. MSM-L= minimal salt media supplemented with Kraft lignin.

D. Small Scale Pre-treatment of Lignocellulosic Biomass

1) FT-IR Spectrum of Treated Sorghum Stalks

The Peaks at wavelengths (3447.32 cm^{-1}) represent -OH functional groups [21]. The peaks from wavelengths (1637.46–623.15) cm^{-1} represent lignin functional groups of the sorghum stalks (Fig. 4). The reduction in the peaks at wavelengths (1637.46 and 1380.95) cm^{-1} representing the lignin functional groups for the four bacterial strains indicates the degradation of the lignin components of the sorghum stalks. The reduction in the peaks representing the -OH group for the four isolates indicates the removal of hemicellulose components as well as some phenolic components of the lignin in the sorghum stalks.

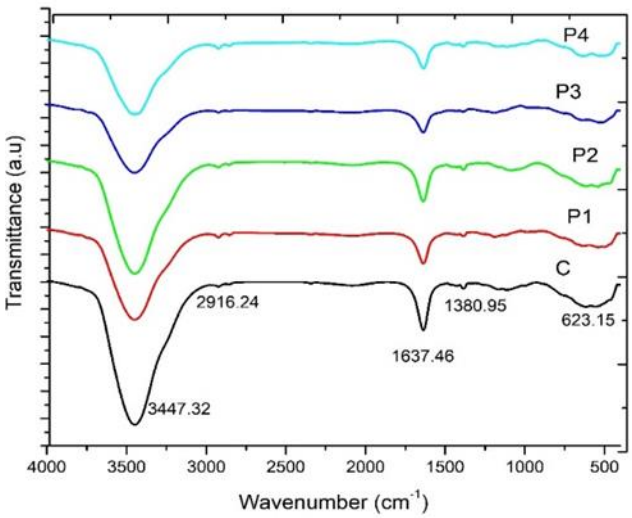

Fig. 4. FTIR spectrum of the treated sorghum stem stalks. (C) indicates no isolate treatment of sorghum stalks, while P1, P2, P3, and P4 represent the four bacterial treated sorghum stalks.

2) Lignocellulose chemical composition of pre-treated sorghum stalks

After treatment with the four bacterial isolates, the lignocellulosic content of the sorghum stalks showed the highest lignin depolymerization of the P3 with (24%) lignin content after treatment. At the same time, the lowest was recorded by P2 treated stalk with (33%) lignin content after treatment as seen in Table IV. Table IV also shows the average mass and percentages in grams of the lignocellulosic content of the four bacterial treated sorghum stalks. The p-values estimated (ANOVA) show that the lignin (L) and cellulose (C) content measured across the treatment was significantly different p-value (0.010) and (0.021), respectively. The p-values estimated for extractives (E) and hemicellulose were not significantly different. The four bacterial isolates could degrade the lignin contents in the sorghum stalk.

TABLE IV: LIGNOCELLULOSE CHEMICAL COMPOSITION OF THE SORGHUM STALKS AFTER BACTERIAL PRE-TREATMENT

	Average mass (g) ± standard deviation (% composition)					
Lignocellulose content	C	P1	**P2**	**P3**	**P4**	**p-values**
Extractives	0.0450±0.0210 (9.0)	0.0450±0.0070 (9.0)	0.0680±0.0040 (13.5)	0.0475±0.0040 (9.5)	0.0500±0.0140 (10)	0.403
Hemicellulose	0.1475±0.0040 (29.5)	0.1300±0.0070 (26.0)	0.1550±0.0280 (31.0)	0.1300±0.0070 (26.0)	0.1525±0.0040 (30.5)	0.306
Klason Lignin	0.1965±0.0020 (39.3)	0.1575±0.0040 (31.5)	0.1650±0.0000 (33.0)	0.1315±0.0160 (24.0)	0.1300±0.0280 (26.0)	0.010
Cellulose	0.1110±0.0230 (22.2)	0.1675±0.0110 (33.5)	0.1130±0.0320 (22.5)	0.1910±0.0130 (40.5)	0.1675±0.0180 (33.5)	0.021

NB: Values in Bracket '()' represents the % chemical composition of the content. C, P1, P2, P3, and P4 indicate Control and the four bacterial treatments, respectively.

IV. CONCLUSION AND FUTURE WORK

The four (4) bacterial strains (P1, P4, P3, and P2) isolated and screened, all showed positive reaction towards lignin-degrading enzyme through the methylene blue assay and cell growth on media with Kraft lignin as carbon source. Also, their negative reaction towards the cellulolytic enzyme test shows that they lack cellulase enzymes. The four isolates also showed significant depolymerization of lignin when they were used to treat sorghum stalks. Hence, all the isolates screened have ligninolytic abilities. Optimization with different treatment media, change in pH, and consortium will be conducted to access a higher depolymerization process. Molecular characterization of the isolates will be done via 16S rRNA and sequencing. After degradation, lignin

degraded products will be assessed using reverse HPLC, LC-MS, and GC-MS

Conflict of Interest

This research was conducted towards development of further work and to also provide knowledge in the subject area. There are no conflicts of interest among the authors.

Author Contributions

Prince Hotor conducted the experimental work and the data analysis and drafting of the paper; Ahmed Hassanin assisted in providing the technical advice relating to material science aspect and reviewing of the paper draft; Ahmed Abdel-Mawgood also helped in providing technical help in the biological aspect and reviewing of the paper draft; Yukie Saito and Mitsuo Yamamoto helped in the reviewing of the paper draft. All contributions from all authors helped to make the research work and paper submission a success.

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