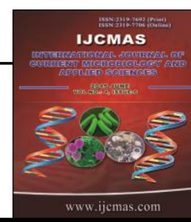

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Original Research Article

Isolation and identification of newly effective bacterial strains exhibiting great ability of lignin and Rice straw biodegradation

Abdelaal Shamseldin[1*] and Ahmed A. Abdelkhalek[2]

[1]Environmental Biotechnology Department, Genetic Engineering and Biotechnology Research Institute, Egypt
[2]Department of plant Protection and Biomolecular Diagnosis (ALCRI), City for Scientific Research and Technological Applications, New Borg El-Arab, Alexandria, Egypt

**Corresponding author*

A B S T R A C T

Nowadays, degradation of lignin and rice straw has great attention especially in Egypt and worldwide due to the accumulation of rice straw as agricultural wastes that causing many different environmental problems. Consequently, thirty-eight bacterial isolates were isolated from the warehouse of rice straw and their ability to degrade cellulosic materials and lignin were tested in minimal medium free from carbon source. Among them six strains were able to degrade cellulose, filter paper, cotton fiber and CMC while only strains LDBI1 and LDBI 5 were effective for degrading lignin and rice straw. These two strains produced the maximum value of Laccase enzyme (810 ul^{-1} and 915 ul^{-1}) after three days while the maximum activity of MnP was of 1720 ul^{-1} and 1750 ul^{-1} after 4 days. They could also produce maximum activity of ligninase 190 to 220 ul^{-1} after 5 days on respectively. The two effective strains were identified as *Acinetobacer baumanii* based on phylogenetic tree of 16S rRNA sequences with similarity of 99%. These findings provide important bacterial strains capable of degrading rice straw and lignin in very short time through producing three degradable enzymes.

Keywords

Lignin, rice straw wastes, manganese peroxidase, lignin peroxidase, Laccase and bacterial strains

Introduction

Great amounts of lignin and lignin-related compounds such as rice straw are produced as wastes of agriculture activity or effluent from the pulping, and paper industries (de La Santoes *et al.*, 2009). Lignin is the second abundant biomass that is associated with cellulose and hemicellulose in plant cells. It is a poly polymer and high molecular weight that gave it rigidity and resistant against decomposition by different kinds of microorganisms, (Wang *et al.*, 2011).

Much more benefits may be obtained if lignin could be utilized as feedstock for value-added chemicals such as substituted aromatics (Ragauskas *et al.*, 2006; Zaldivar *et al.*, 2001). Such valorization would require controlled de-polymerization of lignin, which is hampered by its high

resistance towards chemical and biological degradation (Martinez *et al*., 2005). Lignin can be de-polymerized by thermo chemical methods such as pyrolysis (thermolysis), chemical oxidation, hydrogenolysis, gasification, and hydrolysis under super critical conditions (Pandey *et al*., 2011). However, many of these processes are environmentally difficult, unsafe and occur under specific conditions that requiring large amounts of energy (Ward and Singh, 2002). Consequently, it is necessary for searching to find out the microorganisms that can be used for lignin biodegradation or bioremediation.

White-rot and brown-rot fungi are the well known for having obvious lignin degradation and functional extracellular oxidative enzymes abilities. Nonetheless, fungi have high capability of lignin degradation, but they are sometimes not stable in practical treatment under extreme environmental and substrate conditions (Hatakka, 1994). Bacteria, in particular, are considered to be a better bio-resource for lignin degradation because of their immense environmental adaptability and biochemical versatility (Daniel and Nilson, 1998; Maki *et al*., 2009). Lignin can be degraded by different bacterial species such as those belong to *Pseudomonas*, *Cellulomonas*, and other different genera within the order *Actinomycetales* (Ramachandra *et al*., 1988; Lynd *et al*., 2002; Perez *et al*., 2002). They are likely producing extracellular enzymes such as laccases and peroxidases. Furthermore, the use of bacteria as bioremediation agent has other several advantages as they have short generation time, can be easily cultured and lignocellulolytic bacteria could also allow better separation of lignin from cellulose and hemicellulose.

Several studies have been focused on isolation of highly efficient bacterial strains with more specific enzyme activities such as cellulose, xylanase, laccase, manganese peroxidase and lignase-producing bacteria (Maki *et al*., 2009; Maki *et al*., 2011; Sizoza *et al*., 2011) and Phenol oxidase and chitinase (Woo *et al*., 2014). In Nile Delta region of Egypt rice straw is generated at an annual rate of about 2.4 million tons, and this of course causing a huge environmental problem due to the use of burning process to eliminate this waste of agricultural process from environment.

Therefore, in this study, the purposes were to isolate efficient lignin degrading bacterial strains more adopted to Egyptian environment in order to optimize the use of these bacteria for safe disposal of rice straw and lignin, test their ability to produce lignocellulolytic enzymes and identify them using molecular biology tools.

Materials and Methods

Source of cellulosic materials

Filter paper Whatman no. 1, Carboxymethyl cellulose (CMS), lignin powder were purchased from Sigma Company). Medical Cotton obtained from Pharmacy and rice straw was collected of soil samples that were taken from Egyptian Kafr El-Dawar sites and used as warehouse for Rice straw for long time until to send it to the manufacture for reuse in paper pulping.

Treatment of rice straw before experimental use

Raw rice straw was cut into about 10 cm in length and treated with 1% NaOH for 24h, then washed with distilled water several times until neutrality and dried at 60 °C. All cellulosic materials were of analytical or molecular biology grade and were autoclaved at 121 °C for 15 min before use.

Isolation of Lignin Degrading Bacteria isolates (LDBI):

Different soil dilution were made from soil samples of the warehouse till 10^{-6}, then 200 µl were spread on the surface of minimal salt agar medium (MSAM) as previously described by Hamouda *et al.* (2014) supplemented with cellulose sources such as filter paper, Carboxymethyl cellulose (CMS), lignin and treated rice straw and amended with 0.1g glucose l^{-1}. Strains able to use these materials as carbon source were selected and clearing zone around colonies was an indication on cellulose and lignin degrading bacteria.

Pure colonies from MSAM were moved to 200 ml broth culture of MSM with one of the above mentioned cellulose material source and incubated on rotary shaker at 30ºC for 5 to 15 days. Strains that able to assimilate the cellulosic compounds as a carbon source with good growth were kept at -80 °C till further analysis. The pure isolated strains were designated as LDBI as abbreviation of (Lignin degrading bacterial isolates).

Measuring the degradation rate of lignin and cellulosic compounds

To measure the ability of strains to degrade cellulose, filter paper, cotton fiber, CMC, lignin or Rice straw, each material was supplemented to MSM at a concentration of 0.1% (w/v), and cultures were incubated on rotary shaker at 30 ºC and 180 rpm for 5 to 7 days. After incubation of cellulosic material with bacterial strains the culture was filtered. Then solid filtration was suspended in 100 ml acetic acid/nitric acid reagent (Feng *et al*., 2010) and heated at 100 °C for 30 min to remove the biological cells and re-filtered. The remaining of cellulosic material washed several times with distilled water and dried at 80 °C, the powder weighted and the reduction in weight of cellulosic material used to estimate the degradation rate of these solid material using the following equation:

$$\text{Degradation ratio } \% = Mt - Mr \div Mt \times 100$$

Mt is total weight of the cellulosic materials before degradation, while Mr is the residual weight after degradation. The best strain for degrading cellulosic compound was selected, to measure the change in optical density and account the CFU of bacteria.

Enzymes assay

Laccase activity determined according to the method described by Paszczynski *et al.* (1988) that depend on the oxidation of Dimethoxy phenolic compound (DMP) by laccase enzyme. Six hundred µl of samples mixed with 250 µl of 250 mM sodium malonate buffer (pH 4.5) and 50 µl of 20 mM DMP then kept on water bath at 30ºC for 2 min. The reaction was stopped by incubating on ice for 10 min. The enzyme assayed by measuring the absorbance at 468 nm using 3UV.vis spectrophotometer.

Lignin-peroxidase (LiP) activity measurement was based on the oxidation of veratryl alcohol (VA) to veratraldehyde in the presence of H_2O_2 as strong oxidant reagent (Bonnen *et al.,* 1994). The assay solution contained 2 mM VA in 25 mM tartrate buffer, pH 2.5 and was initiated by the addition of 0.4 mM H_2O_2. The change in absorbance was noticed at 310 nm. One unit of enzyme oxidizes 1 µmol min^{-1} VA to veratraldehyde.

Manganese Peroxidase (MnP) activity was estimated based on the oxidation of phenolic compounds such as phenol red by H_2O_2 oxidant to phenoxy radicals (that can be degraded spontaneously to different aromatic compounds) and measuring change

in absorbance at A610 (Kuwahara *et al*., 1984). Reaction mixture contained 500 µl enzyme extract, 100 µl phenol red solution (1.0 g l^{-1}), 100 µl sodium lactate pH 4.5 (250 mmol l^{-1}), 200 µl bovine serum albumin solution (0.5%), 50 µl manganese sulphate (2 mmol l^{-1}) and 50 µl H_2O_2 (2 mmol l^{-1}) in sodium succinate buffer pH 4.5 (20 mmol l^{-1}). Activity is expressed as increase in absorbance at 610nm per minute per milliliter. One unit of enzyme activity is defined as the amount of enzyme oxidizing 1 µmol of substrate per minute.

DNA isolation and 16S rRNA amplification

DNA extracted from bacterial isolates using INTRON DNA extraction kit, then the crude DNA was used to amplify the complete region of 16S rRNA using forward fd1 and rD1 reverse primers published by Weisburg *et al*. (1991) according to the standard PCR program at annealing temperature of 55°C. The generated fragments were analyzed on a 1% agarose gel and the PCR products were purified using INTRON-PCR purification Kit as manufacture recommended before to digest with restriction enzyme.

RFLP and sequencing of 16S rRNA and phylogenetic analysis

The PCR products were purified using INTRON-PCR purification Kit as manufacture recommended before to digest with restriction enzyme. The product of PCR (1500 bp) was digested with *HinfI* enzyme and then analyzed on 2.0% agarose gels to quick differentiate among LDBI and gels were normalized using 100 bp ladder. All the obtained sequences were submitted to the BLAST network service of NCBI (http:// www.ncbi.nlm.nih.gov/blast/) to identify the examined strains and to know their genetic relatedness with standard bacterial strains. Sequences of 16S rRNA of LDBI were deposited in the Gene bank under accession numbers KJ746504 and KJ746505. Multiple alignments of 16S rRNA of LDBI were compared with sequences of reference strains using CLUSTAL X. The phylogenetic tree was constructed based on a neighbor-joining algorithm software and MEGA 4.0 program. The strains under this study were written in bold and the accession numbers of reference strains were included between brackets. The confidence values of branches in the phylogenetic tree were determined using bootstrap analysis based on 1000 iterations and bootstrap values more than 50% were included.

Results and Discussion

Selection of effective bacterial strains degrading lignin and rice straw

Lignocellulose is the major component of plant material that consists of three types of polymers such as cellulose, hemicellulose, and lignin. These plant materials are strongly intermeshed and chemically bonded by non-covalent forces and by covalent cross-linkages that made them difficult to be degraded by soil microorganisms (Wang *et al*., 2011).

A variety of bacterial species have been reported to be able to degrade lignin and cellulosic materials such as *Bacillus*, *Pseudomonas* and *Acintobacteria* (Maki *et al*., 2011; Bandounas *et al*., 2011); *Bacillus* and *Clostridium* (Wang *et al*., 2011), *Acintobacteria* (Woo *et al*., 2014; Ahmad *et al*., 2010). Therefore, bioremediation of lignocellulosic materials has considerable importance for cleaning up environments and to use the end product of degradation of such materials in production of biocompost and/or biofuel and to use the lignin for

producing dyes and generated silica from rice straw.

Among 38 bacterial strains that isolated from warehouse of Rice straw, there are six strains (from LDBI 1 to LDBI 6) were able to degrade different cellulosic and lignocellulolytic materials such as cellulose, filter paper, cotton fiber, lignin and Rice straw (Table 1).

All of these strains were able to degrade all the previous mentioned materials except lignin and Rice straw, only strains LDBI 1 and LDBI 5 that had the ability to biodegrade them. Both of these two examined strains were having equal ability to biodegrade cotton fiber (% of Bio-degradation was 89%), while strain LDBI 5 was better than LDBI 1 to biodegrade cellulose, filter paper, lignin and Rice straw with biodegradation rate of 95%, 88%, 84% and 81% on respectively (Table 2). Strain LDBI 1 was higher efficient to biodegrade CMC than strain LDBI 5.

The potential of the isolated strains to biodegrade treated rice straw was assessed by testing their ability to utilize it as carbon source. The best two strains for degrading lignin and Rice straw (LDBI 1 and LDBI 5) were selected from previous experiments to examine their growth on MSM broth through measuring the optical density (OD) Figure 1. The growth of the two examined strains showed an obvious increase in the OD with the elongation of incubation time and results of the two strains LDBI 1 and 5 were completely similar, as they gave the highest OD after 5 days of incubation.

Strains LDBI 1 and LDBI 5 were highly effective strains for degrading lignin and rice straw with degradation rate of 83% to 84% and 75% to 81% on respectively. These results are in same line with results obtained by Wang *et al*. (2011) who could isolate bacterial strains capable of bioremediating rice straw with degradation rate from 60% to 75%.

Analysis of enzymes contributed to lignin or rice straw degradation

To confirm the ability of selected strains LDBI 1 and LDBI 5 on the degradation of lignin and Rice straw, the three different enzymes that reported previously to play vital role in lignin degradation such as Manganese peroxidase (MnP), Laccase enzyme (Lac) and lignin peroxidase LiP have been determined. The activity of MnP from the two effective lignin-degrading bacterial strains LDBI 1 and LDBI 5 were shown in Figure 2. MnP activity increased significantly to reach the maximum value 1720 ul^{-1} and 1750 ul^{-1} after the third day with the two examined strains on respectively and decreased to reach constant value after the sixth day, while the activity of Lac enzyme reached the maximum value after the fourth day (Figure 3) to 810 ul^{-1} and 915 ul^{-1}. It was retardation in the production of ligninase enzyme (LiP), where it gave the highest value after the fifth day on contrast with previous two enzymes (Figure 4).

Our results were in agreement with results obtained by Shi *et al*. (2013) who reported the ability of bacterial strain *Cupriavidus basilensis* B-8 to produce both of Mn peroxidase and Laccase enzyme during the degradation of lignin after the third day of inoculation. On the contrary to the results obtained by Shi *et al*. (2013), as they could not detect activity of LiP, while in our study we could detect the ability of strains LDBI 1 and 5 to produce maximum LiP enzyme activity after the fifth day (Figure 4). Thus results indicated that these two selected strains produce three enzymes to biodegrade lignin and rice straw.

Table.1 Degradation of different sources of cellulose and lignin by native Egyptian bacterial strains

Strain name	Cellulose	Filter Paper	Cotton fibber	CMC	lignin	Rice straw
LDBI 1	+	+	+	+	+	+
LDBI 2	+	+	+	+	-	-
LDBI 3	+	+	+	+	-	-
LDNI 4	+	+	+	+	-	-
LDBI 5	+	+	+	+	+	+
LDBI 6	+	+	+	+	-	-

CMC carboxymethyl cellulose

Table.2 Degradation rate % of different cellulosic compounds by effective bacterial strains

Strain name	Day of incubation	Cellulose	Filter Paper	Cotton fibber	CMC	lignin	Rice straw
LDBI 1	5	93%	86%	89%	92.5	83%	75%
LDBI 5	5	95%	88%	89%	90.4	84%	81%

Figure.1 Growth curves of the lignin degrading bacterial isolates (LDBI) measured as optical density

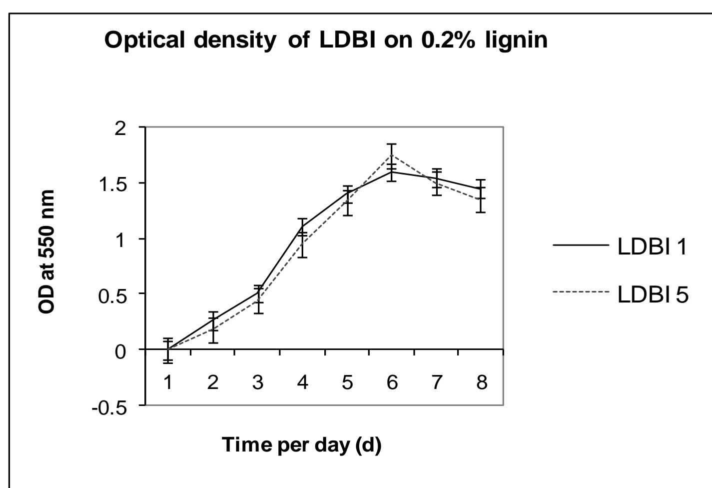


Figure.2 Activity of manganese peroxidase enzyme by lignin degrading bacterial isolates (LDBI)

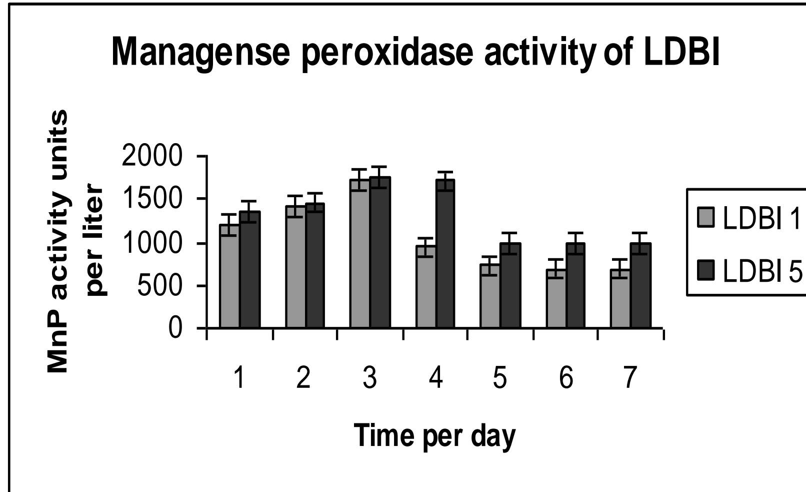


Figure.3 Activity of laccase enzyme by lignin degrading bacterial isolates (LDBI)

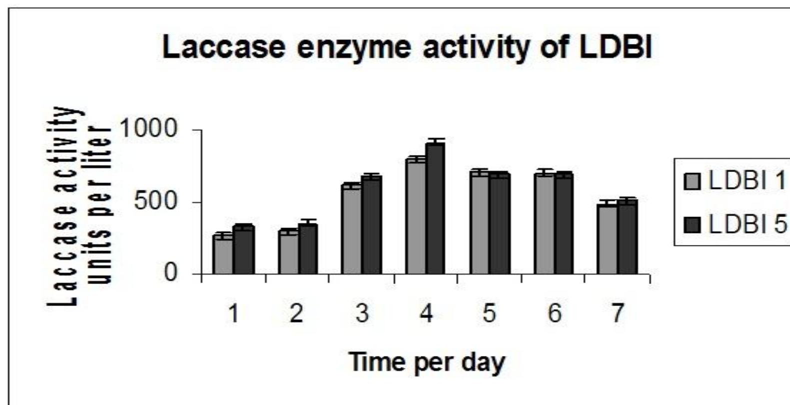


Figure.4 Activity of lignin peroxidase by the most effective LDBI strains

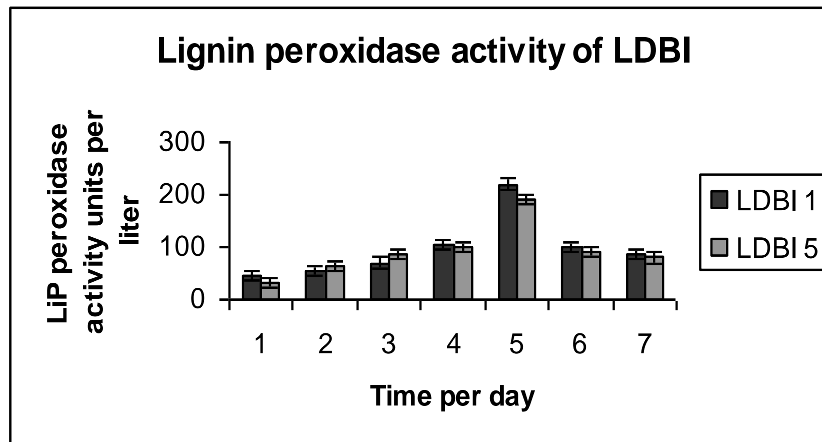


Figure.5 RFLP of 16S rRNA (1450 bp) with restriction enzyme Hinf1 of LDBI for quick grouping of bacterial isolates

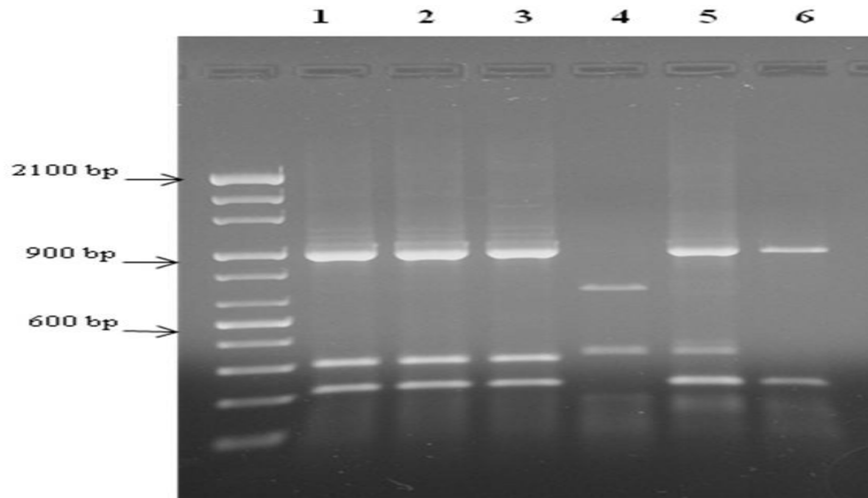
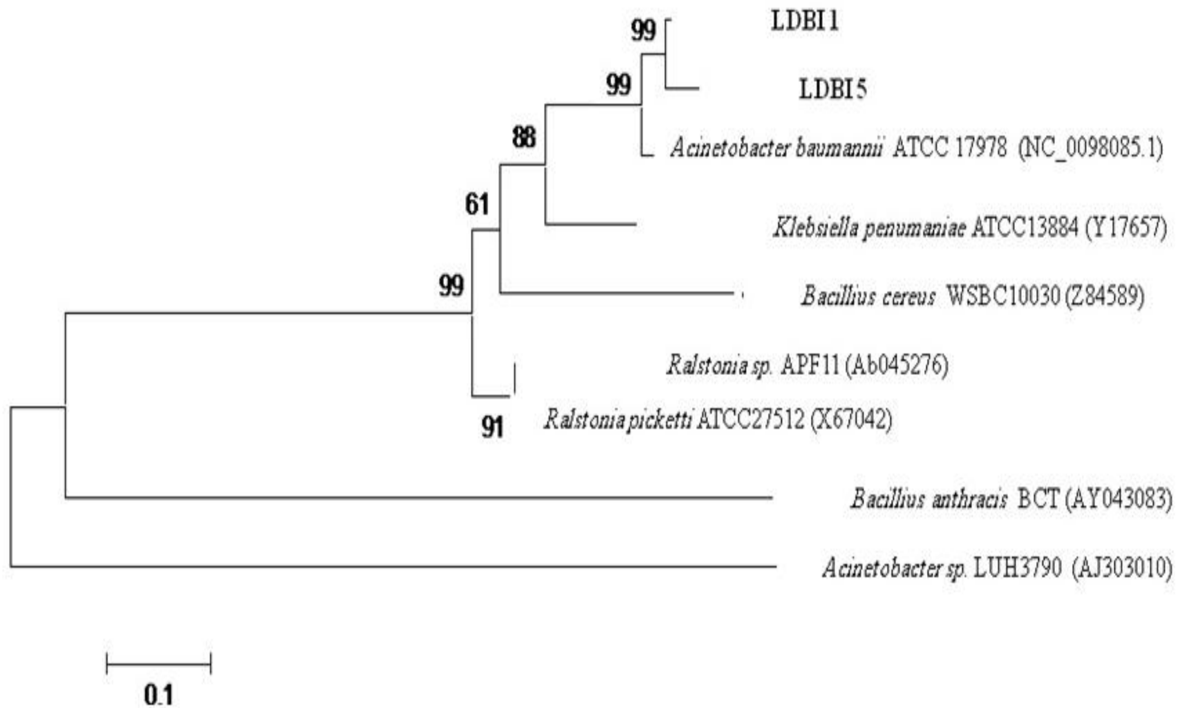


Figure.6 Neighbour-joining phyllogenetic tree of 1450 bp of 16S rRNA of lignin degrading bacterial isolates (LDBI) compared with standard strains, values of bootstrap probability more than 50% were included at branching points



Identification of LDBI using RFLP and phylogenetic analysis of 16S rRNA

To quick differentiate and identify the six strains that exhibited high ability to degrade cellulosic materials, then extracted genomic DNA was used as a template to amplify the entire region of 16S rRNA using primers rD1 and fD1 as mentioned previously. An expected fragment of about 1500 bp could be generated from all the examined strains (data not shown). This amplified fragment subjected to digest with the enzyme *Hinf*1, strains produced three RFLP patterns using this enzyme. Strains LDBI 1, 2, 3 and 5 occupied one RFLP group, while strains LDBI 4 and 6 had two separate groups (Figure 5). Strains LDBI 1 and 5 selected from these strains due to their ability to degrade lignin and Rice straw to do sequencing of their 16S rRNA. When their 16S rRNA sequences compared to the sequences from the Gene bank using BLAST research, results revealed that these two strains had similarity of about 99% with *Acinetobacter baumanii*.

The sequence of the strains was used to draw a phyllogenetic tree (Figure 6), both of these strains shared the genetic clade with strain *Acinetobacter baumanii* ATCC 17978 with bootstrap probability of 99%. Several authors (Maki *et al*., 2011;Bandounas *et al*., 2011; Wood *et al*., 2014; Ahmad *et al*., 2010) noted the ability of bacterial strains belong to different species of genus *Acinetobacter* to degrade the lignin and cellulosic materials. We can conclude that the present work led to the identification of two novel and native Egyptian bacterial isolates that identified as *Acinetobacter baumanii* and exhibited high ability to degrade both of lignin and rice straw through producing three ligninolytic enzymes. These two strains could degrade more than 80% of lignin and 75% of rice straw within maximum five days. The strains gave high enzyme activity of MnP and Lac enzyme after three days while the maximum activity of LiP enzyme was after the fifth day. We will direct our research in the future to do genome analysis of these two strains for selection the genes responsible for the production of these enzymes and cloning them in *E. coli* and studying the ability of *E. coli* strain to degrade lignin and rice straw on a large scale using 10 liter bioreactor fermentor.

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