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Lignin Biodegradation with Ligninolytic Bacterial Strain and Comparison of *Bacillus subtilis* and *Bacillus sp.* Isolated from Egyptian Soil

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Abstract: Lignin is the most abundant aromatic polymer in nature. It is synthesized by higher plants, reaching levels of 20-30% of the dry weight of woody tissue, next to cellulose, is the second most abundant compound in plant biomass and a partial decay of lignin provides numerous aromatic monomers such as ferulic and vanillic acids. These aromatic compounds have attracted attention as renewable resources for the production of chemicals traditionally derived from petroleum. An Isolation and identification environmental friendly bacteria for lignin degradation becomes an essential, because all the previous researches concentrated on using fungal treatments. The importance of ligninolytic bacteria raised, because lignin-degrading bacteria have wider tolerance of temperature, pH and oxygen limitation than fungi. In addition, the application of fungi in bioleaching of raw pulp is not feasible due to its structural hindrance caused by fungal filament. One bacterial strain isolated from Egyptian soil in Kafr El-Dawar (latitude 31.1397 and longitude 30.1292) area was molecularly and physiologically identified. Potential aerobic bacterial strains KafAH19 was found capable to effectively degrade synthetic lignin (lignin Alkali) and utilize it as a sole carbon source. Further, this potential strain (KafAH19) was biochemically characterized as Gram-positive rod. Subsequently, partial sequence of 16S rDNA identified these strains as *Bacillus sp*. (EU978470). In batch 6 days degradation experiments this strain utilized lignin as a sole carbon source and achieved maximum lignin degradation on the sixth day at pH 6 (81.4%), however the lowest lignin degradation rate was observed at pH 13 (34.2%) at the end of the incubation time.

Key words: *Bacillus sp*. · Biodegradation · Egyptian soil · Lignin alkali · 16S rDNA

INTRODUCTION

Lignin is the major noncarbohydrate, polyphenolic structural constituent of wood and other plant material that encrusts the cell walls and cements the cells together, A highly polymeric substance, with a complex, cross-linked, highly aromatic structure of molecular weight about 10,000 derived principally from coniferyl alcohol ($C_{10}H_{12}O_3$) by extensive condensation polymerization. The aromatic polymer lignin is well-known for resistance to microbial degradation because of its high molecular weight and presence of various biologically stable carbon-to-carbon and ether linkages. Microorganisms that degrade plant lignin via an oxidative process, are fungi [1], actinomycetes [2] and to a lesser extent, bacteria [3].

Plant cell wall material is composed of three important constituents: cellulose, lignin and hemicellulose. Lignin is particularly difficult to biodegrade and reduces the bioavailability of the other cell wall constituents. Lignin is the well-known complex substance covalently bound to side chains of xylans of cell-walls. It represents an obstacle to microbial digestion of structural carbohydrates both because it is a physical barrier and because of the depressing effect on microbial activity, due to the phenolic compounds it contains [4]. Mechanical and chemical treatment of lignocelluloses in agricultural wastes and poor quality roughages has been frequently

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used for improving the quality of such materials, however these methods are either costly or have hazardous impact on ambient environment. In contrast, biological treatment is a novel trend, which is not only more efficient in improving the quality of these materials through degradation of their lignin and indigestible fiber contents, but also is of less cost and environmental friendly. In addition, pulp and paper industry use wood and non-wood materials in the production of chemical pulp, wherein lignin is degraded and dissolved almost completely (90-95%) in black liquor. If not removed from the treated wastewater, the lignin presents a serious pollution and toxicity problem in aquatic ecosystems, owing to its low biodegradability and high range of colour [5]. A number of physico-chemical methods have been developed for the treatment of lignin from paper mill wastewater, however, However, these processes are not very effective and costly [6]. Biological treatment has the advantage of being more efficient and less costly than previous mentioned methods. Furthermore, it has no hazardous impact on the ambient environment.

In biological treatment systems a wide variety of microorganisms including fungi, actinomycetes and bacteria have been implicated in lignin biodegradation and decolourization of pulping effluent [7, 8, 9]. Among them, white rot fungi have received extensive attention due to their powerful lignin-degrading enzymatic systems [10]. However, fungi are not stable in practical treatment under extreme environmental and substrate conditions such as higher pH, oxygen limitation, high extractive and lignin concentration [11]. Bacteria, in particular, deserve to be studied for ligninolytic potential because of their immense environmental adaptability and biochemical versatility [6]. Several bacterial strains were found to degrade and assimilate lignin [6, 12, 13, 14, 15]. Lignin induced peroxidases, both extracellular and cell-associated, were identified and characterized in *Streptomyces spp*. [16]. Perestelo *et al.* [9] and Morii *et al.* [17] have studied lignin degradation with unicellular bacteria. Bacteria isolated from compost soil, viz. *Azotobacter*, *Bacillus megatarium* and *Serratia marcescens*, were capable of decolourizing, or solubilizing lignin [17]. *S. marcescens* produced laccase and its activity correlated positively with lignin mineralization and solublization [18]. The contributions of bacteria have also been reported to the utilization of low-molecular weight lignin oligomers as the sole source of carbon and energy that produce enzymes catalyzing cleavage of intermonomeric linkages [19].

The present study is conducted for biodegradation of lignin alkali with *Bacillus sp.* that isolated from Egyptian soil and studies its molecular and physiological characterization in order to maximize the bacterial lignin degradation and justification to use this ligninolytic bacterial strain in industrial and agricultural applications.

MATERIALS AND METHODS

Isolation of Lignin Degrading Bacteria: Four soil samples collected from different areas in Egypt (Bahig, El-Etr, Abu El-Matamer and Kafr El-Dawar) were used for preparing 0.5% soil suspensions in mineral salt media (MSM). One gram of wheat straw was added to 50 ml of different soil suspensions in 100 ml flasks and incubated at 30°C with shaking for 90 days. Bacterial colonies from solidified media were selected according to their shape and growth and used to inoculate LB enriched medium and incubated at 30°C for 72 h to achieve maximum growth rate and subsequently used for selection of bacterial isolates capable to utilize lignin as a sole carbon source [20]. The best 21 isolates were used for phylogeny study. Based on the results of the phylogeny study number of these isolates were chosen to be molecular identified by partial sequencing of 16S rDNA gene.

DNA Isolation and PCR Amplification of 16S rDNA: DNA was extracted from one bacterial isolate that considered the best selected isolates from Kafr El-Dawar area (KafAH19), according to the method described by Araujo *et al.* [21]. From the polymorphic region of the 16S rDNA gene, a fragment of 350 bp was amplified using specific primers. PCR reaction was performed under the following conditions: 34 cycles of denaturation at 94°C (1 min), annealing at 58°C (1 min) and extension at 72°C (1 min). A 350 bp fragment was amplified using the forward primer (5`-AACTGGAGGAAGGTGGGGAT-3`) and the reverse primer (5`-AGGAGGTGATCCAACCGCA-3`). PCR product was analyzed on 2% agarose gel stained with ethidium bromide. Gels were photographed by Gel Doc system (Alpha Imager TM1220, Canada).

Sequencing steps were performed at Gene Analysis unit, VACSERA. Cycle sequencing was done by using a Bigdye terminator cycle sequencing kit (Applied Biosystems, Foster City). Sequencing products were purified by using Centri-sep spin Column and were resolved on an applied Biosystems Model 310 automated genetic analyzer. Approximately 350 bp was sequenced and phylogenetic and molecular evolutionary analyses of the lignin degrading bacteria based on 16S rDNA gene were conducted using software *MEGA* ver 4 [22].

Lignin Alkali Degradation Study in Pure Culture: A 50 ml flasks containing MSM supplemented with 0.1% lignin (Lignin Alkali, Sigma) at pH (6-13) were used. Pure cultures (1%) of strain KafAH19 were incubated at 30°C on a rotary shaker (200 rpm) for 6 days. Bacterial cell growth in liquid medium was monitored at 620 nm [6]. For the measurement of lignin degradation, 1 ml of samples were centrifuged at 15000 rpm for 5 min. Supernatant (250 µl) was diluted by adding 2.5 ml phosphate buffer (pH 7.6) and absorbance measured at 280 nm [23] for lignin degradation on a UV-visible spectrophotometer (Perkin Elmer Lambda EZ201 UV/VIS Spectrometer).

Table 1: Lignin residue during the incubation time with KafAH19 (EU978470) at pH from 6 to 13

Acidity (pH)	Residue of lignin (mg lignin/l) at incubation times (h)				
	0	24	48	96	144 h
6	1000	557.4949	402.4641	357.8029	185.8316
7	1000	879.4554	818.0693	754.9505	399.7525
8	1000	774.9077	546.1255	510.7011	470.8487
9	1000	886.166	810.2767	773.386	622.6614
0	1000	589.1287	497.2022	413.2694	383.693
11	1000	859.8726	737.7919	703.8217	560.5096
12	1000	809.9174	657.6151	645.8087	563.1641
13	1000	824.7863	700.8547	675.2137	658.1197

RESULTS AND DISCUSSION

16S rDNA Gene Sequence: The investigation of 16S rDNA partial sequence alignment identified KafAH19 as *Bacillus sp.* (Accession no. (EU978470). The phylogenetic tree in Fig. 1 demonstrated the linkage distance of this isolate with other bacillus species; this was constructed using 28 different Bacillus isolates from GenBank. Similar data can be generated using the parial 16S rDNA sequence data set for *Bacillus sp.* The phylogenetic tree generated from *MEGA* version 4 and ClustalW for isolate KafAH19 indicated that there are two clusters, one of them contains *Bacillus sp.* (EU978470), which isolated from the soil sample of Kafr El-Dawar area and *Bacillus sp.* (EU344809) that obtained from Bahig area in two groups, while *Bacillus subtilis* (EU344808) lay on the other cluster. *Bacillus sp.* (EU978470) showed 10% homology with *Bacillus thuringiensis* strain (Accession no. AM292317.1) and 6% with two other strains, *Bacillus axarquiensis* strain CIP (DQ993670.1) and *Uncultured bacterium* (DQ532171.1).

Bacterial Growth and Lignin Degradation: A marked increase in optical density (OD) growth at 620 nm revealed that strain KafAH19 was reached a maximum growth at 4th day and thereafter declined (Data are not shown). Similar results were found with strains BahHAE3 (EU344808) and BahHAE8 (EU344809) previously isolated from Egyptian soils [24]. Figure 2 represents lignin degradation rate for strain KafAH19 during incubation time in lignin-MSM (1g lignin/l MSM) at pHs from 6 to 13. It was obvious from this figure that lignin degradation rates gradually increase during incubation time to reach its maximum values during the sixth day at different pHs.

In respect to the effect of pH on lignin degradation, it is obvious from the Fig. 2 that during the sixth day of the experiment the maximum lignin degradation rate occurred at pH 6 was 81.4%, while the minimum lignin degradation rate occurred at pH 13 was34.2% at this time. These results are in accordance with the previous results obtained by EL-Hanafy *et al*. [24]. They found that isolated strains BahHAE3 and BahHAE8 reached the maximum lignin degradation during the sixth day for both of the strains at pH 6 and represents 76.3% and 67.1%, respectively. Also, they indicated that the minimum lignin degradation rates during the end of incubation period occurred at pH 12 for BahHAE3 and at pH 13 for BahHAE8, however BahHAE8 (EU344809) kept higher lignin degradation rate at pH 8 to 11 than BahHAE3 (EU344808). The results of the present study confirmed the matter that many of bacterial strains were found to degrade and assimilate lignin [6, 12, 13, 14, 15].

Moreover, the capability of the soil-isolated bacteria to degrade efficiently and assimilate lignin as a sole carbon source agreed with the findings of Morii *et al.* [17] as they reported that the three bacterial species isolated from compost soil, viz. *Azotobacter*, *Bacillus megatarium* and *Serratia marcescens* were capable of decolourizing, or solubilizing lignin. In respect to Bacillus species isolated in the present study, many studies indicate the ability of Bacillus species to degrade lignin [6, 17, 25].

Data in Table 1 represent lignin Alkali residues during the incubation time with KafAH19 (EU978470) at pH from 6 to 13.The initial concentration of lignin was 1000 mg lignin/l and it is obvious from the table that lignin degradation start slowly at the first 24 hours of the incubation period then increase gradually to reach its maximum rate during the sixth day of the experiment at the various pHs. On the other hand, minimum lignin residue was observed at pH 6 at the end of the incubation time which reflects the highest rate of degradation at this pH. The concentration of lignin used in our study (1g lignin/l) remains much higher than the concentrations used by Chandra *et al.* [6] as they used lignin concentration up to

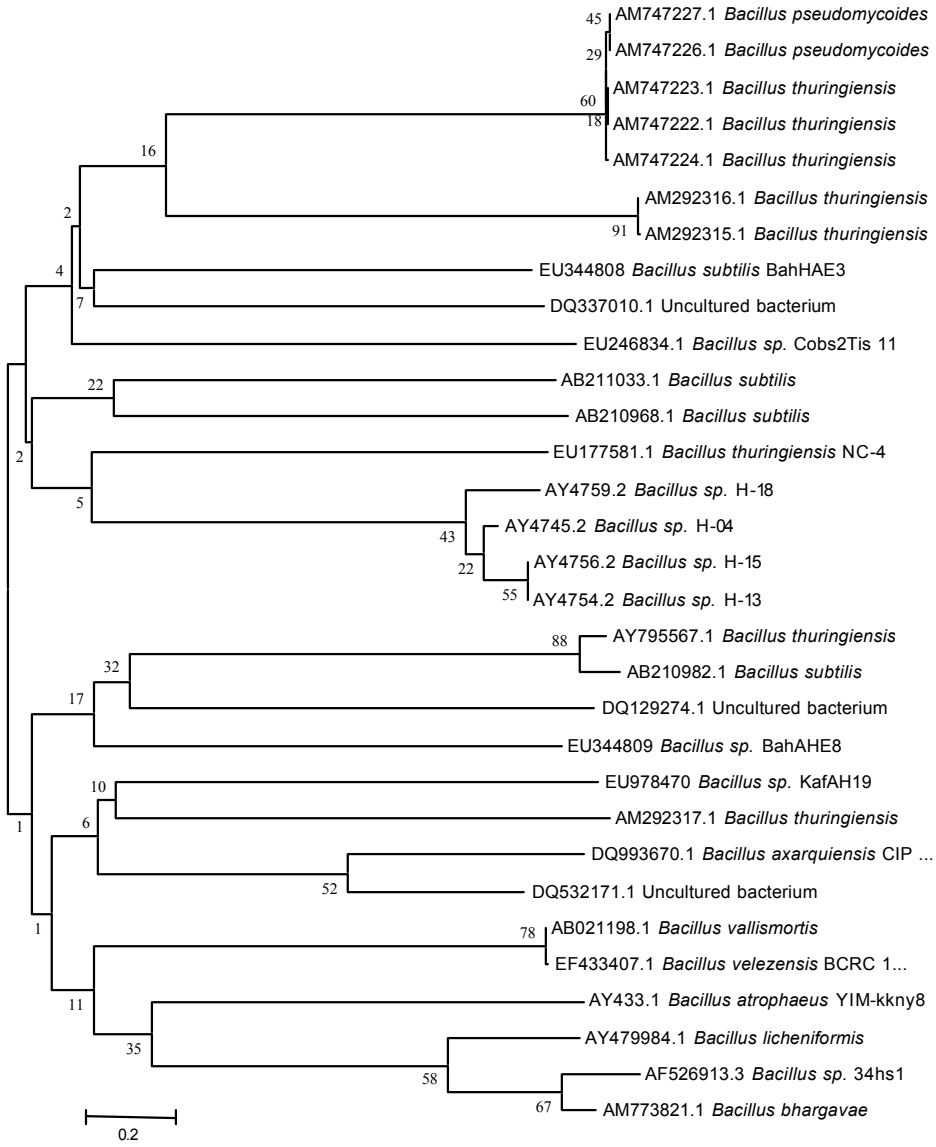

Fig. 1: Phylogenetic tree of the *Bacillus sp*. isolate and their related genera has been linked based on partial 16S rDNA sequence comparisons. Their names and respective accession numbers are given in the tree

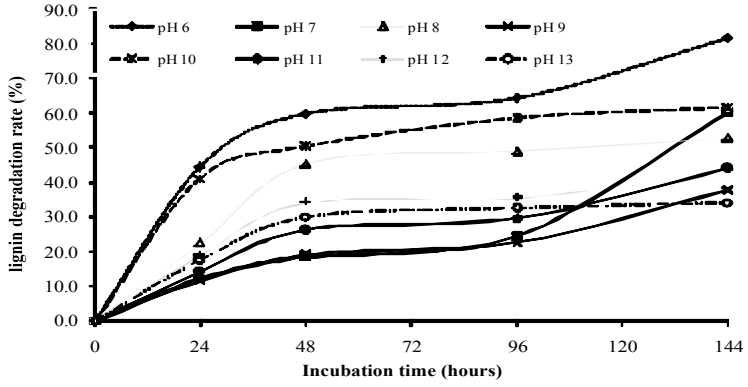

Fig. 2: Lignin alkali degradation rate for KafAH19 (EU978470) at pH from 6 to13

0.7 g lignin/l and they reported 37, 33, 30% Kraft lignin degradation for the *Paenibacillus sp*., *Aneurinibacillus aneurinilyticus* and *Bacillus sp*., respectively during the incubation period. Also, Chandra *et al*. [6] used glucose (1%) as additional carbon source for ligninolytic bacteria, however in the present study lignin was used as a sole carbon source and obtained 81.4% lignin degradation rate at the end of the incubation period at pH 6. Similar findings were obtained by EL-Hanafy *et al.* [24] at the same incubation conditions in which BahHAE3 and BahHAE8 degraded 76.3% and 67.1% of lignin added as a sole carbon source during the last day of incubation period at pH6. The higher degradation rate obtained in the present and last mentioned studies may be attributed to long adaptation period (40 days) with lignin as a sole carbon source during the isolation and selection of ligninolytic bacterial strains [20]. Earlier study by Deschamps *et al.* [26] reveal bacterial lignin degradation much higher than was obtained in the present study. They used an industrial kraft lignin (Indulin AT) as the sole carbon source and they found that *Aeromonas sp* had degraded 98% of 1 g lignin/l after 5 days of incubation. Also, Odier *et al.* [14] selected Eleven gram-negative aerobic bacteria (Pseudomonadaceae and Neisseriaceae) out of 122 soil isolates for their ability to assimilate poplar dioxane lignin without a cosubstrate. Dioxane lignin and milled wood lignin degradation rates ranged between 20 and 40% of initial content after 7 days in mineral medium, as determined by a loss of absorbance at 280 nm. The variation of lignin degradation rates obtained by the previous studies could be referred in part to the variation of bacterial strains, incubation condition, lignin structure and concentration used in these studies.

The low lignin degradation rate at the first 24 hours of the incubation may be explained by the low growth rate for both of the strains because of lack of adaptation on lignin. On the other hand, the decline in the culture growth rate during the fifth may be due to inhibitory effect of some low molecular weight lignin fragments as described by Perestelo *et al.* [18]. They reported that when *S. marcescens* was grown in the presence of 0.1% kraft lignin, as sole carbon and energy source, the bacterial population declined until total kill after 4 days incubation. This inhibitory effect was not present when medium was supplemented with 1 and 5 g glucose/l. This trend also agreed with the finding of Chandra *et al.* [6] who revealed that growth reached maximum at 3rd day for strain ITRC S8, *Bacillus sp*. and 4th day for strains S6 and S7 and thereafter declined.

Data represented in Fig. 2 indicated that maximum lignin degradation occurred on the sixth day of the experiment at pH 6, while the minimum value of lignin degradation rate was obtained at pH13. Also, EL-Hanafy *et al*. [24] found that isolated strains BahHAE3 and BahHAE8 reached the maximum lignin degradation during the sixth day for both of the strains at pH 6, however BahHAE8 (EU344809) kept higher lignin degradation rate at pH 8 to 11 than BahHAE3 (EU344808). This finding revealed high tolerance of this strain in this pH range. Raj et al. [25] found that increase the pH during the growth of *Bacillus sp*. (AY952465) till it reached an alkaline pH did not alter the culture growth significantly. On the other hand, Damiano *et al*. [27] isolated Alkalophilic *Bacillus licheniformis* capable to bleaching of eucalyptus Kraft pulp at pH 9. Maximum lignin degradation occurred at pH 6 in the current work may be interpreted by increasing of pH during the growth of ligninolytic bacteria, which led to reach the alkaline range suitable for lignin biodegradation. This attitude has been described in Bacillus species [6, 25].

CONCLUSION

One local Egyptian ligninolytic bacterial strain was isolated from the soil and identified as *Bacillus sp*. (Accession no. EU978470). This strain utilized lignin as a sole carbon source and achieved maximum lignin degradation on the sixth day at pH 6 was 81.4% which reflect higher lignin degradation rate than previously isolated Bacillus species (BahHAE3 and BahHAE8), however the lowest lignin degradation rate was observed at pH 13 was 34.2% at the end of the incubation time Additional studies have to be made to reveal the optimum conditions (nutrients, temperature, gaseous pressure, etc.) require to maximum lignin degradation by pure or mixed cultures of these strains. In addition, ligninolytic enzymes from these strains would be extracted and evaluated.

REFERENCES

1. Tien, M. and T.K. Kirk, 1983. Lignin-degrading enzymes from himenomycete Phanerochaete chrysosporium Burds. Sci., 221: 661-663.
2. Hernandez, M., M.J. Hernandez-Coronado, M.D. Montiel, J. Rodriguez and M.E. Arias, 2001. Analysis of alkali-lignin in a paper mill effluent decolorised with two *Streptomyces* strains by gas chromatography-mass spectrometry after cupric oxide degradation. J. Chrom., 919: 389-394.

3. Trojanowski, J., K. Haider and V. Sundman, 1977. Decomposition of 14C-labelled lignin and phenols by a Nocardia sp. Arch. Microbiol., 114: 149-153.
4. Antongiovann, M.I. and C. Sargentini, 1991. Variability in chemical composition of straws. Options Méditerranéennes, 16: 49-53.
5. Berryman, D., F. Houde, V. DeBlois and M. O'Shea, 2004. Nonylphenolic compounds in drinking and surface waters downstream of treated textile and pulp and paper effluents: a survey and preliminary assessment of their potential effects on public health and aquatic life. Chemosphere, 56: 247-255.
6. Chandra, R., A. Raj, H.J. Purohit and A. Kapley, 2007. Characterisation and optimization of three potential aerobic bacterial strains for Kraft lignin degradation from pulp paper waste. Chemosphere, 67: 839-846.
7. Modi, D.R., H. Chandra and S.K. Garg, 1998. Decolorization of baggasebased paper mill effluent by the by white-rot fungus Trametes versicolor. Bioresour. Technol., 66: 79-81.
8. Nagarathnamma, R., P. Bajpai and P.K. Bajpai, 1999. Studies on decolourization, degradation and detoxification of chlorinated lignin compounds in Kraft bleaching effluents by Ceriporiopsis subvermispora. Process Biochem., 34: 939-948.
9. Perestelo, F., A. Rodriquez, R. Perez, A. Carnicero, G. Fuente and M.A. Falcon, 1996. Isolation of a bacterium capable of limited degradation of industrial and labeled natural and synthetic lignins. World J. Microbiol. Biotechnol., 12: 111-112.
10. Hatakka, A., 1994. Lignin-modifying enzyme from selected white-rot fungi: production and role in lignin degradation. FEMS Microbiol. Rev., 13: 125-135.
11. Daniel, G. and T. Nilsson, 1998. Developments in the study of soft rot and bacterial decay. In: A. Bruce, J.W. Palfreyman, Eds. Forest Products Biotechnology. Taylor & Francis, London, UK, pp: 37-62.
12. Ball, A.S., W.B Betts and A.G. McCarthy, 1989. Degradation of lignin-related compounds by Actinomycetes. Appl. Environ. Microbiol., 55: 1642-1646.
13. Nishimura, M., O. Ooi and J. Davies, 2006. Isolation and characterization of *Streptomyces* sp. NL15-2K capable of degrading lignin-related aromatic compounds. J. Biosci. Bioeng, 102: 124-127.
14. Odier, E., G. Janin and B. Monties, 1981. Poplar lignin decomposition by gram-negative aerobic bacteria. Appl. Environ. Microbiol., 41: 337-341.
15. Pometto, A.L. and D.L. Crawford, 1986. Effect of pH on lignin and cellulose degradation by *Streptomyces viridosporus*. Appl. Environ. Microbiol., 52: 246-250.
16. Ramachandra, M., D.L. Crawford and A.L. Pometto 1987. Extracellular enzyme activities during lignocellulose degradation by *Streptomivces* spp.: A comparative study of wild-type and genetically manipulated strains. Appl. Environ. Microbiol., 53: 2754-2760.
17. Morii, H., K. Nakamiya and S. Kinoshita, 1995. Isolation of lignin decoloursing bacterium. J. Ferment. Bioeng., 80: 296-299.
18. Perestelo, F., M.A. Falcon, A. Carnicero, A. Rodriguez and G. de la Fuente, 1994. Limited degradation of industrial, synthetic and natural lignins by Serratia marcescens. Biotechnol. Lett., 16: 299-302.
19. Vicuna, R., B. Gonzalez, D. Seelenfreund, C. Ruttimann and L. Salas, 1993. Ability of natural bacterial isolates to metabolize high and low molecular weight lignin-derived molecules. J. Biotechnol., 30: 9-13.
20. EL-Hanafy, A.A., H.E. Abd-Elsalam and E.E. Hafez, 2007. Fingerprinting for the lignin degrading bacteria from the soil. J. Appl. Sci. Res., 3: 470-475.
21. Araujo, W.L., D.A. Angellis and J.L. Azevedo, 2004. Direct RAPD evaluation of bacteria without conventional DNA extraction. Brazilian Arch. Biol. Technol., 47: 375-380.
22. Tamura, K., J. Dudley, M. Nei and S. Kumar, 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biol. Evolution, 24: 1596-1599.
23. Lara, M.A., A.J. Malaver-Rodriguez, O.J. Rojas, O. Holmquist, A.M. Gonzalez, J. Bullon, N. Penaloza and E. Araujo, 2003. Black liquor lignin biodegradation by Trametes elegans. Int. Biodeterior. Biodegrad., 52: 167-175.
24. EL-Hanafy, A.A., H.E. Abd-Elsalam and E.E. Hafez, 2008. Molecular characterization of two native Egyptian ligninolytic bacterial strains. J. Appl. Sci. Res., 4(10): 1291-1296.
25. Raj, A., M.M.K. Reddy, R.Chandra, H.J. Purohit and A. Kapley, 2007. Biodegradation of Kraft-lignin by *Bacillus* sp. isolated from sludge of pulp and paper mill. Biodegrad, 18: 783-792.
26. Deschamps, A.M., G. Mahoudeau and J.M. Lebeault, 1980. Fast degradation of Kraft lignin by bacteria. Appl. Microbiol. Biotechnol., 9: 45-51.
27. Damiano, V.B., D.A. Bocchini, E. Gomes and R. Da Silva, 2003. Application of crude xylanase from Bacillus licheniformis 77-2 to the bleaching of eucalyptus Kraft pulp. World J. Microbiol. Biotechnol., 19: 139-144.