ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Lignin Biodegradation by Thermophilic Bacterial Isolates from Saudi Arabia

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ABSTRACT

Lignin next to cellulose, it is the second most abundant compound in the plant biomass. Partial decay of lignin provides numerous monomers that have attracted attention as renewable resources of certain chemicals traditionally derived from petroleum. Bioconversion of lignin is one of the most important trends in biotechnology. Decomposition of the lignin-cellulose complex in fodder plants improves the digestibility and nutritional value of the materials for livestock feeding, enriching it with physiologically active substances. The objective of this research was to biodegradation of lignin, where found in pulp mill effluent (450 mg/l) and agriculture wastes: leaves tree, corn stalks and wheat straw by selected strains of thermophilic bacteria and optimize physiological conditions required for better lignin degradation by bacteria. Biodegradation experiments were carried out in L-MSM (1g KL /L. MSM) at pH 7.5. Kraft lignin (KL) concentration is greater than that found in pulp mill effluent (450 mg/l). Samples were withdrawn periodically at one day intervals for seven days and determine the bacterial growth, reduction of colour and residual KL content. It was found that, colour reduction ratio for the best two thermophilic strains *Bacillus subtilis, Bacillus licheniformis* were 86.6 and 75.4 %, respectively and lignin degradation ratio were 73.0, and 64.2 %, respectively.

Keywords: Biotechnology, Biodegradation, Kraft lignin, 16S rDNA, *Bacillus subtilis,* and *Bacillus licheniformis*

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ISSN: 0975-8585

INTRODUCTION

Environmental pollution was increased by increasing the industry development all over the world and especially in Kingdom of Saudi Arabia; increment of these pollution caused many hazards for all organisms, even for humans such as carcinogenicity and toxicity. Also there has been increasing pollution with hydrocarbon compounds, many of these hydrocarbons considered to be a potential health hazard [1, 2]. Wastewater discharged by industrial activities is often contaminated by a variety of toxic or otherwise harmful substances which have negative effects on the water environment. Historically, pulp and paper production has been recognized as a significant point source of pollution [3]. It is the sixth largest polluter (after the oil, cement, leather, textile and steel industries), discharging a variety of gaseous, liquid and solid wastes into the environment which can cause considerable damage to the receiving waters if discharged untreated [4]**.** They block the passage of light to the lower depths of the aquatic system resulting in cessation of photosynthesis, leading to anaerobic conditions, which in turn result in the death of aquatic life causing foul smelling toxic waters [5].

In order to manufacture good quality paper, the pulp and paper industry discards lignin as an unwanted constituent of wood and raw materials and therefore discharges a significant amount of effluent or wastewater containing lignin and other hazardous materials. Black liquor is one of the main byproducts discarded as waste which has a high level of chemical oxygen demand (COD) and contains lignin from 10 to 50% by weight [6]. 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 color.

For several decades, a number of methods for black liquor treatment have been developed, e.g., adsorption of organic pollutants from Kraft pulp mill wastewater using activation carbon and polymer resin and chemical coagulation of lignin from pulp and paper wastewater using synthetic and natural coagulants. However, these processes are not very effective, but are costly. Furthermore, in these processes lignin compounds are not degraded, but are just transferred from a water-soluble state into a solid state. Biological treatment is found to be more efficient, less costly than previous mentioned methods and has no hazard impact on the ambient environment. In biological treatment systems a wide variety of microorganisms including fungi, actinomycetes and unicellular bacteria have been implicated in lignin biodegradation and decolourization of pulping effluent [7].

Thermophilic bacteria, in particular, deserve to be studied for ligninolytic potential because of their immense environmental adaptability and biochemical versatility. In addition the application of fungi in bioleaching of raw pulp is not feasible due to its structure hindrance caused by fungal filament .Several studies have investigated biological treatment of black liquor by using various pure bacterial strains [8].

Increased knowledge about the environmental effects of industrial activities has led to a need for developing better techniques and more efficient waste management systems in order to reduce their environmental impact [9]. Therefore, the main objective of this study was to overcome the problems of contamination of water with paper manufacturing wastes (black liquor) using genetics and biotechnology methods.

MATERIALS AND METHODS

Soil Samples Collection

Soil samples were collected in Polyethylene bags from rich places of lignin source (agriculture area) of different sites at beside Road of Taif-Jeddah (El-Hada road) at western area of Saudi Arabia (Taif, Macca and Jeddah), and others soil samples near waste disposal sites in Taif, Macca and Jeddah were transferred to laboratory and stored at 4 °C.

The industry uses the Kraft process for pulping of raw materials, mainly rice straw. Subsequently this pulp is bleached by multistage chlorination. The effluent generated by this process contain high concentrations of dissolved Kraft Lignin (KL), Chlorophenol and other soluble components of the raw material, which subsequently undergo activated sludge treatment after filtration of residual wood fibers. The sludge of the

treatment plant remains rich with ligninolytic bacteria. Hence soil and the activated sludge samples were collected for the isolation of potential ligninolytic bacteria.

Media Composition, Bacterial Isolation and Screening

All chemicals used in this study were obtained from Sigma and Fisher Companies. Alkali Kraft Lignin (KL) degrading bacteria were isolated from water, soil and the effluent sludge by enrichment culture technique [10]. 5 ml of sludge, 5 ml of contaminated water and 5 gm of soil sample separately were inoculated to 100 ml sterile mineral salt medium (MSM) containing KL 500 mg/l (designated here after L-MSM). MSM (pH 7.5) consisted of (g/l de-ionized water): Na_2HPO_4, 2.4; K_2HPO_4, 2.0; NH_4NO_3, 0.1; $MgSO_4$, 0.01; $CaCl_2$, 0.01 and Trace elements solution 1.0 ml. The latter solution composed of (mg/l): $ZnCl_2$, 70; $MnCl_2.4H_2O$, 100; $CoCl_2.6H_2O$, 50; $NiCl_2.6H_2O$, 50; $CuCl_2.2H_2O$, 25; $NaMoO_4.2H_2O$, 50; $NaSeO_3.5H_2O$, 26; $NaVO_3.H_2O$, 10; $NaWO_4.2H_2O$, 30 and HCl 25%, 1.0 ml.

The bacterial strains could not grow using KL as single carbon source due to its high molecular weight. Therefore, through nutrient optimization process, 1.0 % glucose and 0.5% peptone (w/v) were added in L-MSM-broth and L-MSM-agar as growth supportive substrates. The flasks were incubated for 6 days on rotary shaker 120 rpm under aerobic conditions at 50 °C. Samples from flasks exhibiting decolourisation were serially diluted and spread on L-MSM agar plates and incubated in dark at 50^{o}C for 6 days. Four phenotypically different bacterial colonies were picked and purified by repeated sub-culture in order to obtain pure isolates. The purity of isolates was checked by microscope and these isolates were named as Isolates 1, 2, 3 and 4.

Bacterial Isolates Identification

DNA Extraction and Purification

Extraction and purification of total genomic DNA was carried out according to Abdulamir et al. [11] and Leonard *et al*., [12]. Four isolates were cultured routinely at 30 ºC on luria-Bertani (LB) medium. Bacterial cultures for DNA extraction were grown in broth at 30ºC.Total bacterial genomic DNA was extracted as follows: five ml of overnight cultured bacterial cells was centrifuged for 10 minutes at 10000 rpm (High-speed centrifuge sorvall RC 285, USA) subsequently, the resulted pellets were resuspended in 467 µl lyses buffer containing: 30µl of 10% sodium dodicyl sulphate (SDS) and 3 µl proteinase K (20 mg/ml), mixed well and incubated for one hour at 37 °C. The protein/DNA mixture was then subjected to phenol/chloroform extraction, and the upper aqueous phase was transferred to a new tube. Directly, for DNA precipitation an equal volume of absolute ethanol (about 0.6 ml) and 1/10 volume 3 M sodium acetate at pH5.2 (about 0.1 ml) were added and incubated at -20°C at least one hour .After centrifugation, the resulted pellets of DNA were washed twice with 70% ethanol then air dried, dissolved in 20 µl sterile distilled water, and stored at -20 °C until used.

Sequence Analysis of 16S rDNA Genes

Though the 16S rRNA gene is found conserved on evolutionary scale, it is still diverse enough to identify and classify the eubacteria [13]. 16S rRNA gene sequencing involves amplification of target sequences using universal primers to yield a 1.5 kb amplicon followed by sequencing and homology generation using ribosomal DNA database.

The 16S rRNA gene was amplified using universal eubacterial primers [14]. Polymerase chain reaction (PCR) reactions were performed using eppenorff 9700 thermocycler under the following conditions: Initial denaturation at 94 °C (5 min), 35 cycles of denaturation at 94 °C (1 min), annealing at 57 °C (1 min), extension at 72 °C (2 min), final extension at 72 °C (10 min) and stored at 4 °C. Product was amplified using the universal primer (forward primer 5\-AGAGT TTGATCMTGGCTCAG-3\ and reverse primer 5\-TACGGYTACCTTG TTACGACTT-3\).

Reaction Preparation

For each reaction the following reagents were added to a separate 0.2 ml PCR tube: 5 µl buffer 10x, 1 µl Template DNA, 2µl of forward & reverse universal Primers, 5µl dNTPs, 0.4µl (5units/µl) Taq Polymerase (Sigma), De-ionized water was added up to a total volume 50µl, then, the mixture was mixed well.

Electrophoresis of Sequencing Reaction

Two µl of loading dye was added prior to loading of 10 µl per well. Electrophoresis was performed at 80 volt (mini-sub DNA electrophoresis gel 170-4307 and power supply 1000/500 from Bio-Rad, USA) with 1× Tris boric acid EDTA(TBE) buffer in 1.5% Agarose gels and then the gel was stained in 0.5µg/ml (w/v) ethidium bromide solution and destained in deionized water. Finally the gel was visualized and photographed by using gel documentation system (Alpha Imager TM1220, Canada). Size of the target band was estimated using DNA marker (100 base-pair ladder).

Sequencing Alignments and Phylogenetic Analysis

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 1500 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 [15].

Kraft Lignin De-Colourisation and Degradation

Biodegradation experiment was carried out in 100 ml flask containing 50 ml sterile L-MSM (1000 mg/l KL) at pH 7.6, which is greater than that found in pulp mill effluent. Culture suspension 1% (v/v) having an inoculum size (CFU)/ml 105 × 10^4 were inoculated into triplicate flasks. The inoculum was grown overnight in 100-ml flasks containing 50 ml luria-Bertani (LB) broth. The flasks were incubated for seven days on rotary shaker incubator under aerobic conditions at 55^{o}C and 200 and 250 rpm. Uninoculated medium was used as control in all cases. Samples were withdrawn periodically at one-day intervals for seven days and analyzed for bacterium growth, reduction of colour and residual KL content.

Bacterium Growth

Cell growth was determined by measuring absorbance of inoculated sample at 620 nm (A_{620}) on spectrophotometer (UV-visible Cintra 40-GBC) using uninoculated medium as blank.

Colour Reduction

The colour reduction of the effluent was determined according to Morii *et al,*[10],[16]. For the measurement of color, samples were centrifuged at 8000g for 30 min (High-speed centrifuge, Sorvall RC 285, USA) to remove the biomass. Supernatant (1 ml) was diluted by adding 3 ml phosphate buffer (pH 7.6) and absorbance measured at 465 nm for colour reduction. The absorbance at 465 nm against distilled water was measured using a spectrophotometer (Shimadzu, UV-2200). The absorbance values were then transformed into colour units (CU) according to the following formula.

$$CU = 500 \times (A_2/A_1)$$

Where A_1 corresponds to the A_{465} of a 500-CU platinum cobalt standard solution; and A_2 is the absorbance of the effluent sample. The color removal (%) was defined as the ratio of CU of the culture supernatant to that of the initial medium.

$$\text{Colour removal \%} = (A\text{-}B)\backslash A \times 100$$

Where A: is colour units of uninoculated sample, and B is a colour unit of inoculated sample.

ISSN: 0975-8585

Lignin Content

For the estimation of residual lignin, centrifuged supernatants from control and inoculated were acidified with 12 M HCl to pH 1–2 and then centrifuged at 12,000g for 10 min. Residual KL was obtained after each precipitate had been washed with de-ionised water and dried at 60 °C for 48 h and weighed [17]. Kraft lignin loss (%) in the supernatants decolourised by the strain was determined daily for seven days as dry weight (estimating 100% as the KL present in the same volume of uninoculated medium). All experiments were carried out in triplicates. The values are presented as mean ± standard deviation (n = 3).

RESULTS AND DISCUSSION

Bacterial Isolation and Screening

In order to obtain the largest possible collection of lignin-degrading bacteria soil, water and pulp paper sludge were added directly to L-MSM as the source of ligninolytic bacteria with the medium containing glucose and peptone as additional carbon and nitrogen sources to stimulate bacterial growth and hence the decolourization of KL was observed. Also Vicuna [18] reported that, as glucose and nitrogen were essential as co-substrate in media to promote bacterial growth for degradation of lignin. After incubation for 7 days when samples in flasks showed visible decolourization were plated on L-MSM agar, four bacterial isolates were selected for detail characterization and biodegradation assay on the basis of their growth on tested lignin concentration (1000 mg/l), colour reduction and lignin degradation.

Bacterial Isolates Identification

Sequencing Alignments and Phylogenetic Analysis

The selected isolates were identified by partial sequencing of the PCR amplified 16S rDNA gene. The obtained sequences were submitted to the BLAST in order to find a homology with other 16S rDNA sequences. Comparing the sequence of the 16S rDNA gene of the isolates with the sequences in Gendata Bank (http://www.ncbi.nlm.nih.gov/) revealed that the isolates were similar to *Bacillus subtilis*, *Bacillus licheniformis*, *Staphylococcus pasteuri* and *Bacillus cereus* with similarity as shown in table 1 and Fig. 2.

The phylogeny of the bacterial strains and closely related species was analyzed using the multi-sequence alignment program and the results are presented in Fig. 1, where the phylogenetic tree displayed that the four strains were grouped in two clusters, one of them includes strain *Bacillus cereus* and the other cluster is divided into two sub-clusters, one of the Them includes two strains *Bacillus subtilis* and *Bacillus licheniformis*, while Staphylococcus *pasteuri* included in the remaining in the other sub cluster.

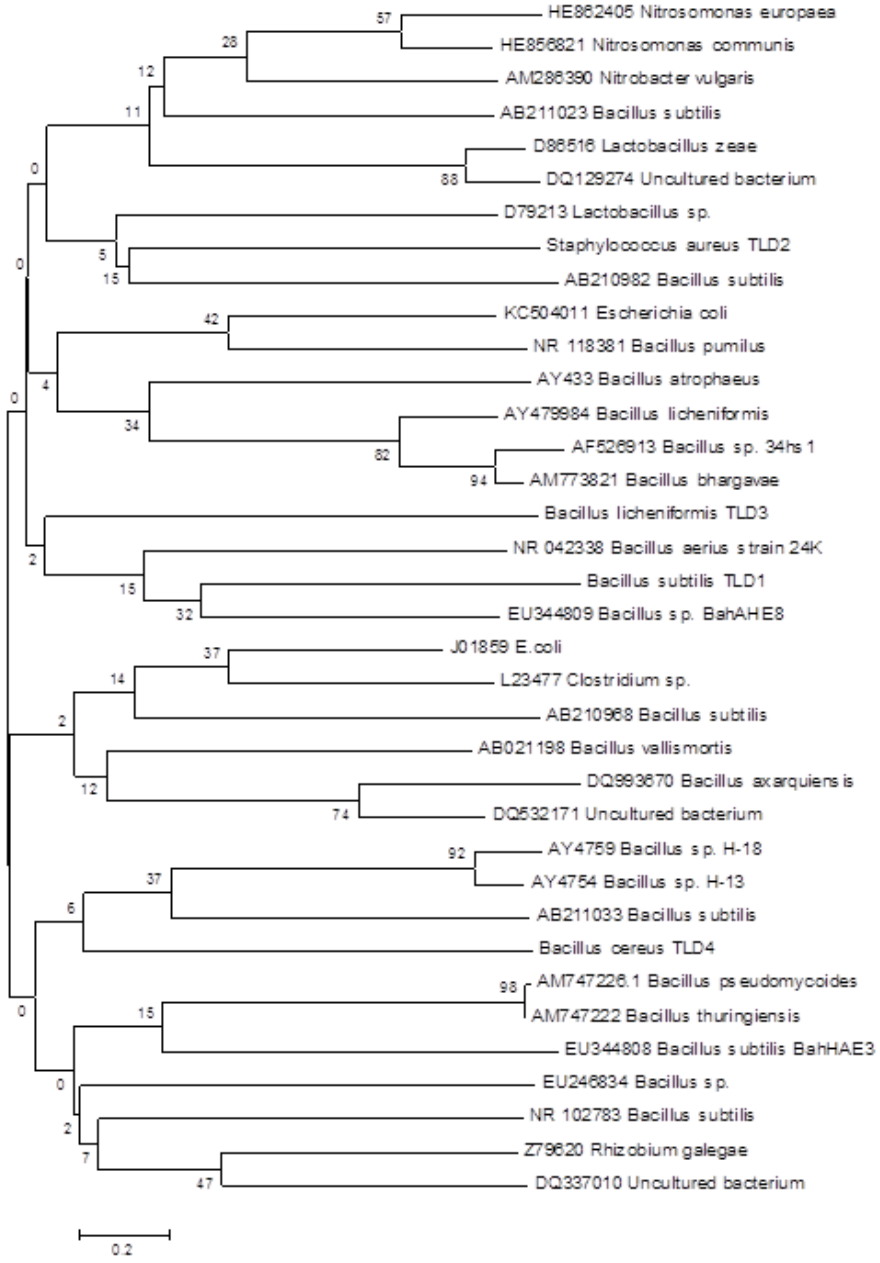
Figure 1: Map illustrated Macca zone and soil samples sites with (X) spots.

ISSN: 0975-8585

Table 1: Similarity percentage of 16S rDNA sequences for the selected isolates compared to those obtained from database.

Isolate	Organism	Identity (%)	Accession NO.
Bacillus subtilis	*Bacillus subtilis strain 168* *Bacillus subtilis SBMP4*	99 99	NR_102783.1 NR_118383.1
Bacillus licheniformis	*Bacillus licheniformis DSM 13* *Bacillus licheniformis BCRC 11702*	99 99	NR_118996.1 NR_116023.1
Staphylococcus pasteuri	*Staphylococcus pasteuri* *Staphylococcus pasteuri ATCC 51129*	99 99	NR_121749.1 NR_024669.1
Bacillus cereus	*Bacillus cereus ATCC 14579* *Bacillus cereus CCM 2010*	99 99	NR_074540.1 NR_115714.1

Figure 2: Phylogenetic tree of *Bacillus Subtilis*, *Bacillus licheniformis*, *Staphylococcus pasteuri*, and *Bacillus cereus* strains and their related genera have been linked based on partial 16S rDNA sequence comparisons



ISSN: 0975-8585

Growth Optimization Selected Strains

The optimization growth of the highly two selected thermophilic lignin degrading bacterial strains were studied with some parameters such as incubation temperature, medium acidity (pH value), agitation speed and salinity concentration (NaCl %), demonstrated to obtain the best optimum conditions for highly growth and ligninolytic activity of both *Bacillus subtilis* and *Bacillus licheniformis.*

Effect of Incubation Temperature on Growth Rate

To study the optimum temperature for growth rate of each selected thermophilic lignin degrading bacterial isolates (*Bacillus subtilis* and *Bacillus licheniformis*). Different incubation temperatures (25, 30, 35, 40, 45, 50, 55, 60, and 65ºC) were used at pH 7.0 and incubated in shaker incubator water bath for 6 hrs at 120 rpm. The growth rate of both *Bacillus subtilis* and *Bacillus licheniformis* as shown in Fig. 3 were increased with increasing temperature of the incubator, the *Bacillus subtilis* recorded the highest growth rate ($O.D_{620}$: 1.287) at 55 ºC, while the growth rate was dramatically decreased with increased temperature over 55 ºC, where recorded at 65ºC optical density 0.608. Also, the same trend was found with respect of *Bacillus licheniformis* where, the highest growth rate was recorded when the incubation temperature was 55 ºC ($O.D_{620}$: 1.236), while the growth rate was decreased with increased the incubation temperature up to 65ºC to recorded optical density 0.88 at 65 ºC.

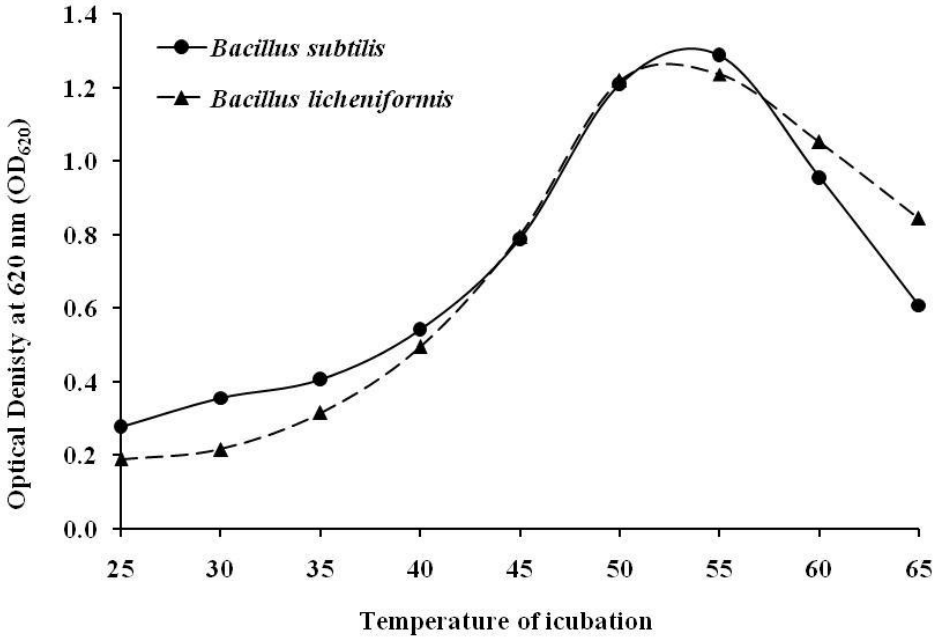


Figure 3: The relationship between temperature of incubator and growth rate of both thermophilic degrading bacterial isolates: *Bacillus subtilis* and *Bacillus licheniformis* after incubation for 6 hrs at different temperature degrees.

Effect of Medium Acidity (pH Value) on Growth Rate

The influence of acidity degree (pH value) on the growth rate of the two selected thermophilic lignin degrading bacterial isolates (*Bacillus subtilis* and *Bacillus licheniformis*) was assessed by using LB medium. The pH medium was adjusted in range from pH 5.0 up to pH 9.0 by using either, 1.0 M HCl or 1.0 M NaOH and incubation after inoculation the bacterial strains for 6 hrs at 55°C. The growth of bacteria was measured through measured the turbidity at optical density ($O.D_{620}$) 620 nm. The growth rate of both *Bacillus subtilis* and *Bacillus licheniformis* as showed in Fig. 4 were increased with increasing pH value (decreasing the acidity) of the LB medium up to pH 7.5 for *Bacillus subtilis*, where the maximum growth ($O.D_{620}$: 1.158) was found at this degree of the acidity while decreasing dramatically in the medium growth from pH 8 up to pH 9.0 to be 0.394. While in respect of *Bacillus licheniformis,* the optimum pH was 8.0. Where, the maximum growth rate ($O.D_{620}$: 1.041) as illustrated in Fig. 4, while the growth decreased gradually by increasing the pH to record $O.D_{620}$: 0.748 at 9.0. pH value.

ISSN: 0975-8585

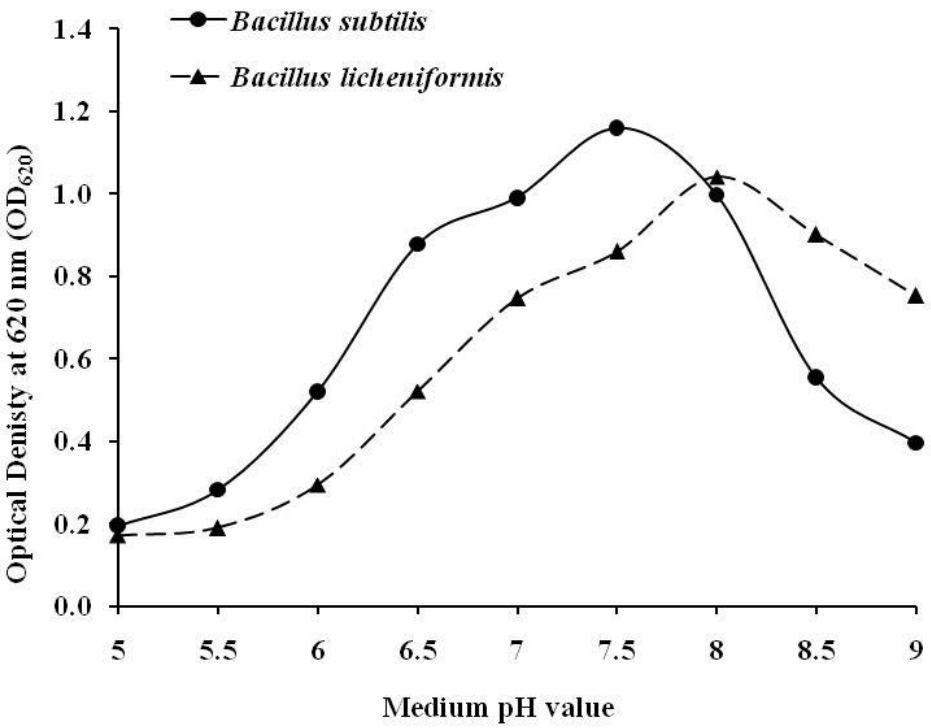


Figure 4: The relationship between the acidity of growth medium (pH) and the growth rate of both thermophilic degrading bacterial isolates: *Bacillus subtilis* and *Bacillus licheniformis* after incubation for 6 hrs at 55°C.

Effect of Agitation Speed on Growth Rate

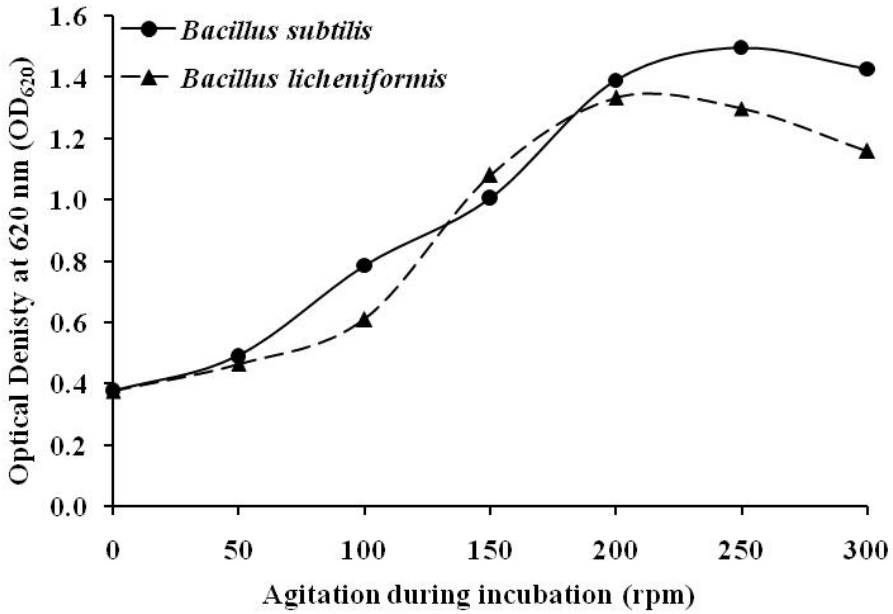


Figure 5: The relationship between agitation speed (rpm) and the growth rate of both thermophilic degrading bacterial isolates: *Bacillus subtilis* and *Bacillus licheniformis* after incubation for 6 hrs at 55°C with optimum pH value.

The agitation speed was studied through the effect on the growth rate of the two selected thermophilic lignin degrading bacterial isolates (Bacillus subtilis and Bacillus licheniformis), through changed the speed of agitation from the static stage (zero shaking) to 300 rpm. In general, the growth rate of both *Bacillus subtilis* and *Bacillus licheniformis* as showed in Fig. 5 were increased with increasing agitation speed. In respect of *Bacillus subtilis* isolate, the growth rate increased approximately twofold ($O.D_{620}$: 1.496) at speed 250 rpm compared with the growth rate ($O.D_{620}$: 0.785) at agitation speed 100 rpm, then slow gradually decreased the growth rate at agitation speed 300 rpm. While, in respect of *Bacillus licheniformis* the growth

rate was increased twofold at ($O.D_{620}$: 1.333) at agitation speed 200 rpm compared with the growth rate ($O.D_{620}$: 0.611) at agitation speed 100 rpm then, slightly decreased ($O.D_{620}$: 1.159) with increase agitation speed up to 300 rpm.

Effect of Salt Concentration on Growth Rate

Effect of salt stress (NaCl %) concentration at different rates (from 0.5 to 6.0 %) in LB medium on the growth rates of *Bacillus subtilis* and *Bacillus licheniformis* isolates were used to determine the best salt concentration produce highly growth rates of the two selected strains, at stability both of pH, agitation and temperature as previously mentioned and incubated for 6 hrs in 100 ml Erlenmeyer conical flask, and the O.D of the growth rate was measured at 620 nm against control of the same media.

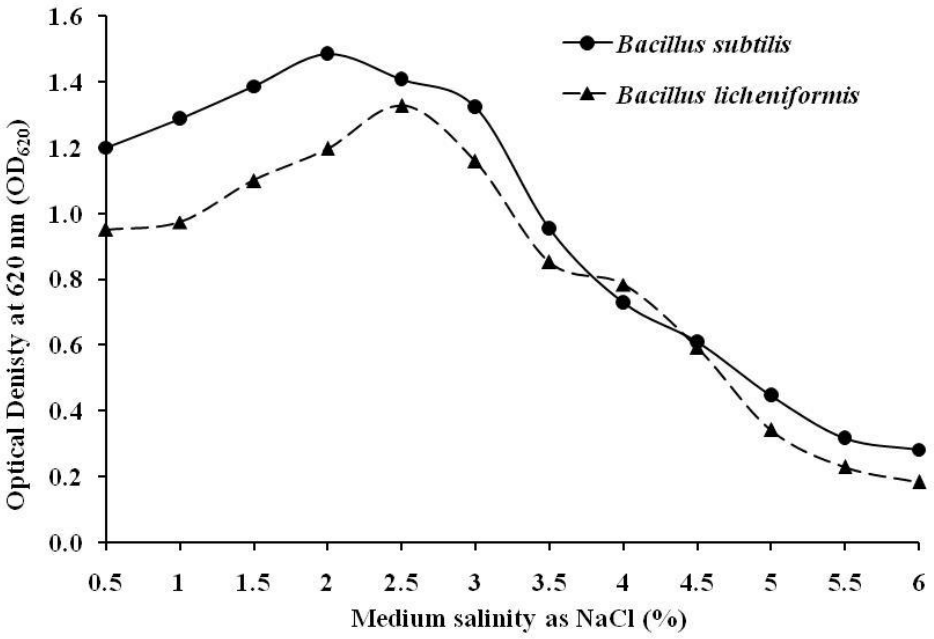


Figure 6: The relationship between salt concentration as sodium chloride (%) and the growth rate of both thermophilic degrading bacterial isolates: *Bacillus subtilis* and *Bacillus licheniformis* after incubation for 6 hrs at 55°C with optimum pH value.

As shown in the Fig. 6, the *Bacillus subtilis* and *Bacillus licheniformis* recorded the highest growth rate $O.D_{620}$: 1.484 and 1.328 when the salinity was 2% and 2.5% respectively in the growth medium. While, decrease gradually the growth rate with increasing sodium chloride concentration in the growth medium, where the growth rate became 0.281 and 0.183 respectively, as $O.D_{620}$ at 6% of sodium chloride concentration.

Kraft Lignin de-colourization and degradation

When the decolourization assay was carried out in 100 ml conical flasks contain L-MSM with optimum pH 7.5 and 8.0 for *Bacillus subtilis* and *Bacillus licheniformis* respectively, and incubated at 55 °C, in shaking incubator at 250 and 200 rpm respectively, a marked increase in optical density growth at 620 nm (OD_{620}) revealed that growth reached maximum at 4^{th} days for the two isolates and thereafter declined (Fig. 7). However there was less reduction of colour, lignin and total substrate present in the culture media of flask at initial 48 h incubation as shown in Figs. 8 and 9, possibly because bacteria initially utilized glucose for growth and subsequently utilized lignin as a co-metabolite[19] after that, there were greater colour and lignin reduction (Figs. 8 and 9). The colour reduction and lignin degradation reached a maximum by 5^{th} day for *Bacillus Subtilis* (86.6 %, 73 %), and *Bacillus licheniformis* (75.4 %, 64.1%) respectively. The reduction of colour resulting from lignin biodegradation has been assumed to be due to de-polymerization of lignin polymers by bacterial ligninolytic systems [20].

ISSN: 0975-8585

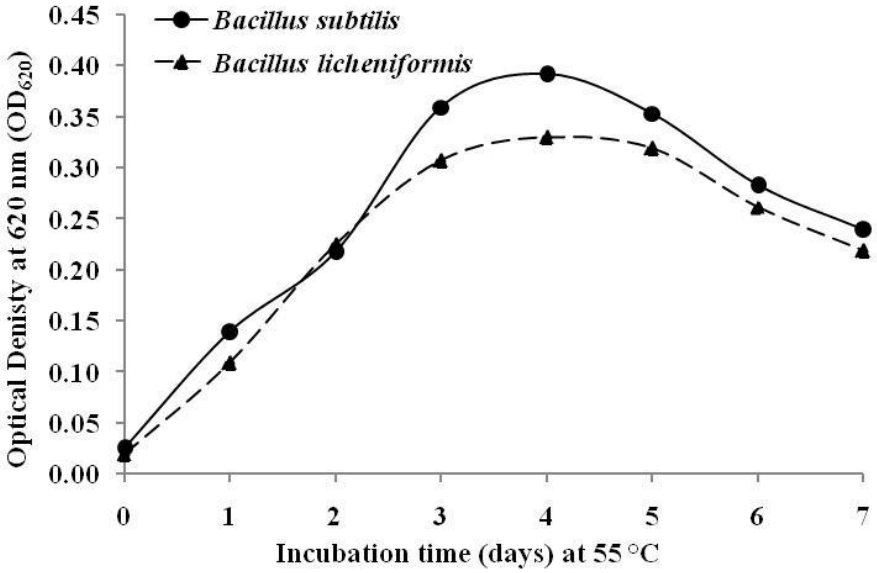


Figure 7: The relation between incubation time (days) and the growth rate of both thermophilic degrading bacterial isolates: *Bacillus subtilis* and *Bacillus licheniformis* after incubation for 6 hrs at 55°C with optimum pH value

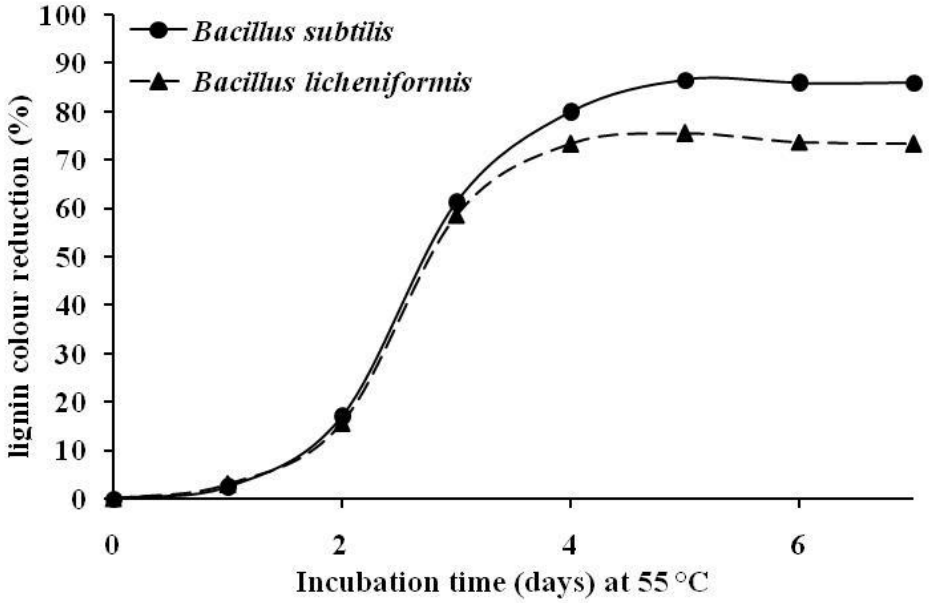


Figure 8: The relation between incubation time (days) and lignin colour reduction (%) rate of both thermophilic degrading bacterial isolates: *Bacillus subtilis* and *Bacillus licheniformis* after incubation for 6 hrs at 55°C with optimum pH value

ISSN: 0975-8585

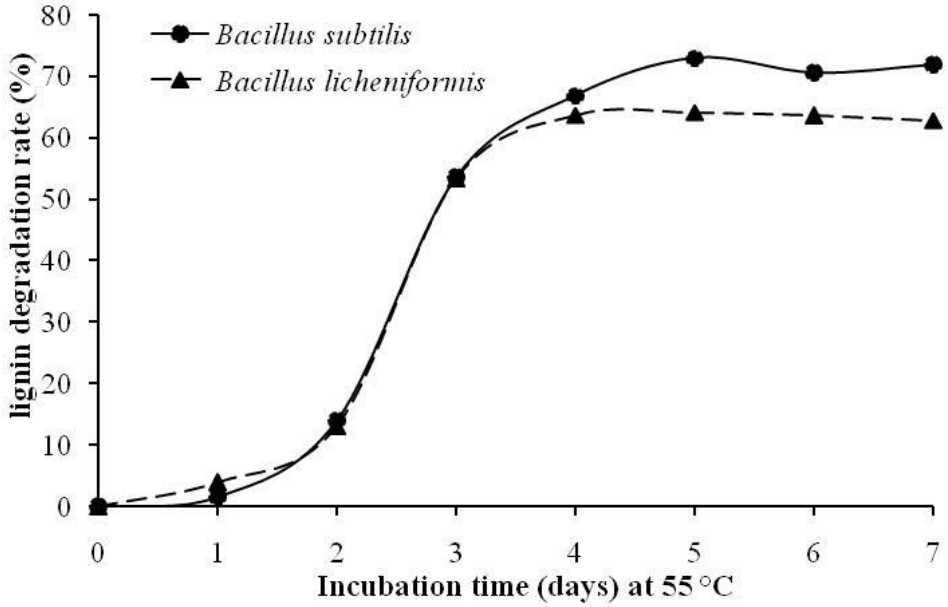


Figure 9: The relation between incubation time (days) and the alkali lignin degradation rate (%) of both thermophilic degrading bacterial isolates: *Bacillus subtilis* and *Bacillus licheniformis* after incubation for 6 hrs at 55°C with optimum pH value

CONCLUSION

In a screening thermophilic lignin degrading bacteria in kingdom of Saudi Arabia environment, 36 native bacterial strains were isolated from different contaminated soil sites from Macca region, four potent bacterial candidates were characterized and identified by biochemical and PCR techniques, based on 16S rRNA sequencing. Two of the thermophilic lignin degrading bacterial isolates recorded highest lignin degradation rate, Phylogenetic analysis revealed its closeness to the thermophilic *Bacillus subtilis* and *Bacillus licheniformis*. The maximum growth rate of the highly ligninolytic activity of the thermophilic bacterial strains *Bacillus subtilis* and *Bacillus licheniformis*, was studied with both temperature of incubation growth, acidity of growth medium (pH), salinity concentration in the growth medium (NaCl %) and agitation rate (rpm) of incubation growth were evaluated. Temperature influences on the growth rate of both *Bacillus subtilis* and *Bacillus licheniformis,* where the maximum growth was found at 55ºC. So, we can conclude that the two bacterial strains: *Bacillus subtilis* and *Bacillus licheniformis* are thermophilic bacteria. Kraft lignin biodegradation and colour reduction reached a maximum by 5th day after inoculation KL-MSM with *Bacillus Subtilis* and *Bacillus licheniformis*.

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