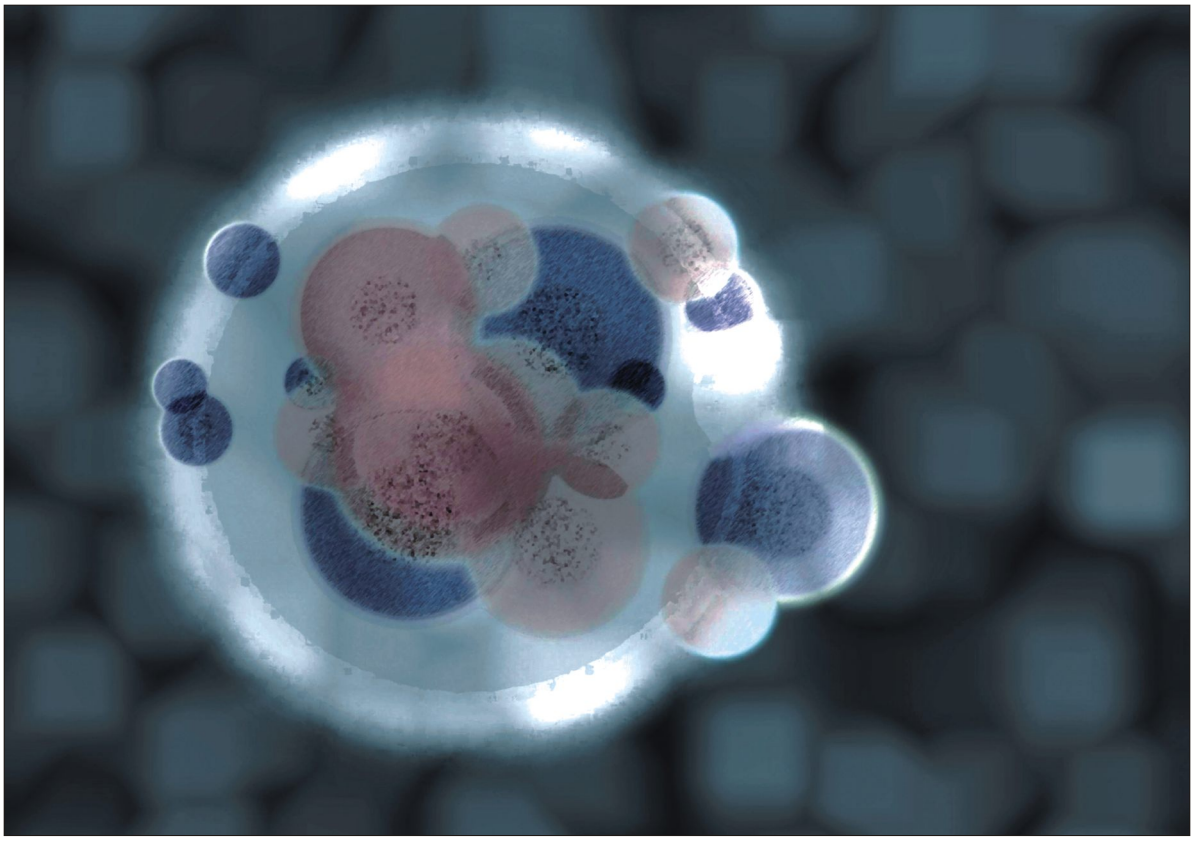

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Research Article
Characterization of Fungi that Able to Degrade Phenol from Different Contaminated Areas in Saudi Arabia

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Abstract

Background and Objective: Aromatic compounds, such as phenols, occur in the wastewaters of a number of industrial sectors, petroleum refining, pesticide, chemicals and plastics production, pharmaceutical industries, steel industries and dye manufacturing products. **Materials and Methods:** Various fungal strains were isolated from different contaminated sites in Saudi Arabia and screened for phenol degradation. **Results:** Three fungal isolates demonstrated a high degradation for phenol. These isolates were microscopically and molecularly identified. Partial sequence of 18S rRNA identified these strains as *Aspergillus niger* (S1), *Penicillium griseofulvum* (S2) and *Aspergillus terreus* (S3). The factors that affected the degradation rate of phenol were studied in this work such as (pH, temperature, shaking and incubation time). The selected strains can degrade phenol at pH 5-7, they degrade (19-29% of phenol). However, the optimum temperature was at 20-30°C and optimum degradation occurs at static condition until 100 rpm. Different incubation periods were studied and it appeared that the degradation started after 3 days. *Aspergillus niger*, *Penicillium griseofulvum* and *Aspergillus terreus* degrade (13, 3 and 8% of phenol), respectively but the optimum degradation occur after 15 days, they degrade (86, 63 and 77% of phenol), respectively. **Conclusion:** This study revealed that *Aspergillus niger*, possessed greater potential to degrade phenol when compared with other fungal strains.

Key words: Phenol, biodegradation, characterization, *Aspergillus* sp., *Penicillium* sp.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Phenol is the common name of hydroxybenzene, an aromatic compound having one hydroxyl group attached to the benzene ring. Phenol is the structural unit for a variety of synthetic organic compounds. It is a white crystalline solid which is soluble in most organic solvents[1-3]. It is a listed priority pollutant by the U.S. Environmental Protection Agency and Agency for Toxic Substances and Disease Registry. Phenolic pollutants are generated from several sources, like the partial degradation of phenoxy herbicides, the use of wood preservatives and the generation of wastes by petroleum-related industries such as petroleum refineries, gas and coke oven industries, pharmaceuticals, explosive manufacture, phenol-formaldehyde resin manufacture, plastic and varnish industries and related metallurgical operations, etc.[4-7].

Long and high doses of exposure to phenol by the oral route leads to damage to blood, liver, kidney and cardiac toxicity, including weak pulse, cardiac depression and reduced blood pressure[8]. Various physicochemical methods like ionization, adsorption, reverse osmosis, electrolytic oxidation, photocatalysis have been used for the elimination of phenol from contaminated waters[9]. Phenol removal by biological methods is preferred to physicochemical methods because of its eco-friendliness and cost effective nature[10,11]. It was demonstrated that biological treatment of phenol is very effective[12]. Phenol has aromatic structure so, it is resistant to natural biodegradation and phenolic compounds have been reported to have high steadiness, due to the difficulty of cleaving the benzene ring. Nevertheless, several microbes can tolerate phenol and make use of it as carbon and energy source[12]. Many micro-organisms capable of degrading phenol through the action of variety of enzymes. These enzymes may include oxygenases, hydroxylases, peroxidases, tyrosinases and oxidases. These enzymes participate in the oxidative metabolism of phenol[13]. Many micro-organisms including bacteria, fungi and algae are capable of degrading phenol[14]. Fungi play a critical part in the re-using of aromatic compounds in the biosphere and several investigations have demonstrated that different fungi are capable of mineralize a wide variety of carbon sources by producing enzymes, thus providing possibilities for metabolizing phenols and other aromatic derivatives[14]. There are numerous factors that can influence the degradation rate of micro-organisms by either preventing or stimulating growth of the organisms. These factors may include temperature, pH, agitation and physical properties of contaminants[8,15-17].

The objective of the this study was to isolate, screen, identify of phenol degrading fungi from different contaminated area in Saudi Arabia (Taif and Jeddah Governorate), obtain the optimum (pH, temperature, shaking and incubation time) for biodegradation and to decide the degree of biodegradation of the selected fungal strains.

MATERIALS AND METHODS

Samples collection: Surface water and topsoil samples (10 samples) were collected from Taif and Jeddah Governorate (Saudi Arabia) from different contaminated sites containing several pollutants including phenol. The samples were collected during May, 2017, these samples were used for the study of phenol degradation capabilities of fungi. The samples were stored in sterile glass bottles, packed in plastic bags and transferred to the laboratory for isolation and maintained at 4°C.

Isolation of fungal isolates that showed ability to degrade phenol: Fungi were isolated on potato dextrose agar medium (PDA) with typical formula (g L^{-1}): Potato pieces[18] 200.0; dextrose 20.0; agar 16.0 and distilled H_2O to 1000 mL. Soil samples were suspended by vortexing in sterile distilled water and allowed to stand for several minutes. The supernatants were then subjected to serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}). Also, in water samples it make serial dilution until 10^{-5}. A 0.1 mL portion from each dilution was plated onto the PDA and Sabouraud Dextrose Agar (SDA), containing 12 μg mL^{-1} tetracycline and streptomycin solution, respectively in order to suppress the growth of bacterial colonies then incubated at room temperature for 7 days. The sample was subcultured three times to obtain a pure culture, which was transferred to a PDA slant and stored at 4°C.

Screening for phenol degradation in liquid culture: All chemicals used in this study were obtained from Sigma and Fisher Companies (Germany). Mineral Salt Medium (MSM) was used as a general medium for isolation of strains capable of growing on phenol as sole carbon source according to Hadad *et al.*[19], the typical formula (g L^{-1}) was: NH_4NO_3, 1.0; $MgSO_4.7H_2O$, 0.2; K_2HPO_4, 1.0; $CaCl_2.2H_2O$, 0.1; KCl, 0.15 and yeast extract, 0.1; in addition to 1.0 mg L^{-1}, of each of the following micro-elements: $FeSO_4.6H_2O$, $ZnSO_4.7H_2O$ and $MnSO_4$. The final pH was adjusted at 5.8. Biodegradation tests were performed with supplemented the medium with 10 and 20 μg L^{-1} liquid phenol as sole carbon and energy source. The medium was prepare in flasks, each containing 100 mL of mineral medium and 1 mL of spore suspension. The medium was incubated at 25±2°C for 1 week[20].

Selection of isolates with highest degradative potential: The isolates with highest degradative potential for phenol were selected by measuring phenol content by using the folin-ciocalteu phenol reagent. To the 0.1 mL of supernatant liquid add 0.1 mL of 2% of sodium carbonate (Na_2CO_3), 0.1 mL of Folin-ciocalteau reagent and add 2 mL of distilled water. Then it was kept aside for 60 min at 20°C. Then the absorbance was measured at 727 nm against a distilled water and reagent blank[21]. All samples were measured by the spectrophotometer, then phenol concentration was determined. The bio-removal efficiency of the isolates was then calculated according to the following equation:

$$\text{Phenol removal efficiency (\%)} = \frac{c_i - c_f}{c_i} \times 100$$

where, c_i is the initial concentration of phenol (mg L^{-1}) and c_f is the final concentration of phenol.

Characterization and identification of the highly degrading fungi using molecular tools

DNA extraction and PCR amplification conditions: Extraction and purification of total genomic DNA from fungi was carried out according to Saghai-Maroof *et al.*[22]. Polymerase Chain Reaction (PCR) reactions were performed under the following conditions: 34 cycles of denaturation at 94°C (1 min), annealing at 59°C (1 min) and extension at 72°C (1 min). Amplification was done using forward primer 5'-GTAGTCATAT GCTTGTCTC-3' and reverse primer 5'-TCCGCAGGTTCACCTAC GGA-3'. The PCR product was analyzed in 2% agarose gel stained with ethidium bromide. Gels were photographed by Gel documentation system. 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. Phylogenetic and molecular evolutionary analyses of the phenol degrading fungi based on 18S r-RNA genes were conducted[23] using MEGA version 4.

Factors affecting biodegradation rate of phenol: The selected isolates that could degrade phenol were subjected to various pH value, incubation temperature, agitation and different incubation time to obtain the best optimization conditions for highly degradation rate.

Effect of pH on the growth and degradation rate: The influence of pH on the growth of the selected isolates was assessed using MSM. The pH medium was adjusted at 3.0, 5.0, 7.0 and 9.0 using 1.0 M HCl or 1.0 MNaOH. The prepared medium was autoclaved and inoculated with different fungal strains, the medium was then supplemented with liquid phenol[24], then incubated at 25±2°C for 1 week. The activity of isolates for degradation was monitored by measuring phenol content by using the folin-ciocalteu phenol reagent[21].

Effect of temperature on the growth and degradation rate: Different incubation temperatures were used at 20, 25 and 30°C. The MSM liquid medium was prepared and the optimum pH was adjusted as previously mentioned, the medium was autoclaved and the medium was then supplemented with liquid phenol[24], then inoculated with the selected isolates and incubated at previously temperature for one week. The activity of isolates for degradation was monitored by measuring phenol content by using the folin-ciocalteu phenol reagent[21].

Effect of agitation on the growth and degradation rate: Different agitation speeds (100, 150 and 200 rpm) were used to determine the best one for high growth rates of the selected isolates. The MSM was prepared and the optimum pH and temperature were adjusted as previously mentioned. The medium was autoclaved and inoculated with the selected isolates, the medium was then supplemented with liquid phenol[24] and incubated for one week at the different speed. The activity of isolates for degradation was monitored by measuring phenol content by using the folin-ciocalteu phenol reagent[21].

Effect of different incubation periods on the growth and degradation rate: Experiments were performed in three of 250 mL Erlenmeyer flask containing 100 mL of MSM (for each sample). The medium was then supplemented with liquid phenol after medium sterilization. Fungal disk of 5 days old culture, with 5 mm diameter from selected isolates was used as inoculum on the tested medium[24]. Upon incubation of the flasks at 25°C for different incubation periods (3, 6, 9 and 15 days). For the determination of phenol content, the folin-ciocalteu phenol reagent was used.

RESULTS

Isolation of fungal isolates that showed ability to degrade phenol: Eight fungal isolates were obtained as shown in (Fig. 1).

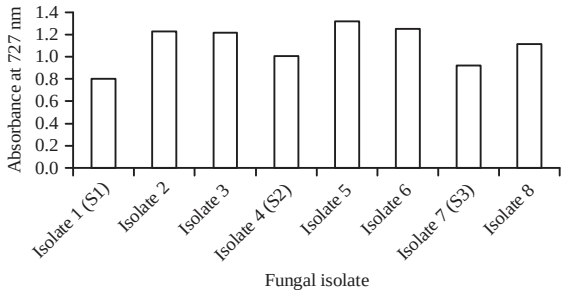

Fig. 1: Isolation and selection of fungal isolates that able to degrade phenol
S1: *Aspergillus niger*, S2: *Penicillium griseofulvum* and S3: *Aspergillus terreus*

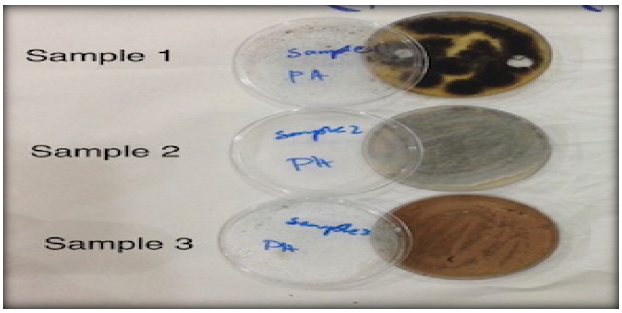

Fig. 2: Morphological shape of three selected fungal isolates

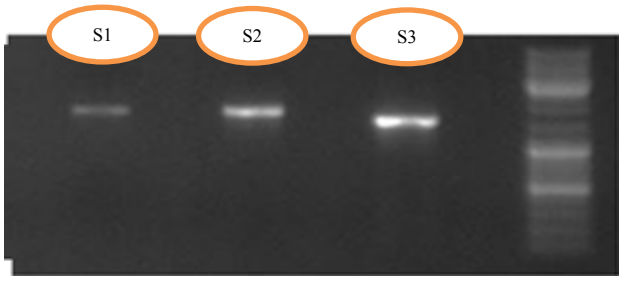

Fig. 3: PCR amplification of the 18S rDNA gene from the fungal isolates
S1: *Aspergillus niger*, S2: *Penicillium griseofulvum* and S3: *Aspergillus terreus*

Selection of isolates with highest degradative potential: The best degradations appeared as reduction in absorbance at 727 nm. The fungal isolates (S1, S2 and S3) were selected as the most potent for phenol degrading fungi (Fig. 1, 2).

Characterization and identification:

- Morphological
- Molecular tools

Sequence analysis of 18S rDNA genes: The amplicon was subjected to DNA sequence using 377 sequencer Applied Biosystem. The data presented in Fig. 3 showed the PCR amplification bands appeared.

Alignments and phylogenetic tree: The phylogeny of the fungal isolates and closely related species was analyze using the multi-sequence alignment program and the results were presented in phylogenetic tree Fig. 4 showed the similarity between selected isolates and isolates obtained after comparing the sequence of the tested isolates to the submitted sequences in Gen Bank.

Factors affecting biodegradation rate of phenol

Effect of pH on the growth and degradation rate: The results presented in Fig. 5 showed a relationship between the tested strains and their degradation rate of phenol at tested pH values. Figure 5 presented that the isolates (S1) showed higher degradation rate at pH 5 and S2 and S3 showed higher degradation rate at pH 7.

Effect of temperature on the growth and degradation rate: The obtained results in Fig. 6 indicated high phenol degradation rate with fungal isolates S1 and S3 at temperature 20-25 and 25-30°C for S2 that appeared as reduction in absorbance.

Effect of agitation on the growth and degradation rate: The data presented in Fig. 7 indicated high degradation rate of phenol for the fungal strains (S1, S2 and S3) occurred at static condition until 100 rpm good results appeared as a reduction in absorbance of phenol.

Effect of different incubation periods on the growth and degradation rate: Data presented in Fig. 8 showed that the degradation rate of phenol was increased with increasing the incubation time (3, 6, 9 and 15 days) for fungal strains (reduction in absorbance).

DISCUSSION

Phenols are common pollutants in waste waters produced from oil refineries, compound plants, coke manufactures, coal gasification[25]. Hence, phenol is utilized similarly an anti-microbial agent[26]. A large portion of microbes, including bacteria and fungi can degrade phenolic compounds and use them as a source of carbon and energy[27]. The bioremediation of phenol in domestic

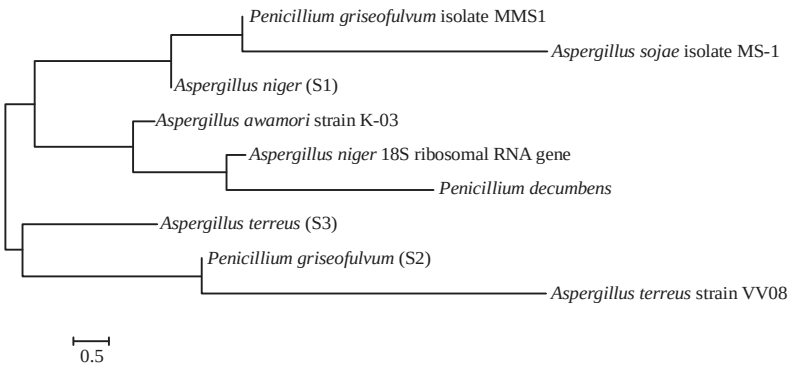

Fig. 4: Molecular phylogenetic analysis showed the similarity between selected isolates and isolates obtained after comparing the sequence of the tested isolates to the submitted sequences in Gen Bank by minimum evolution method

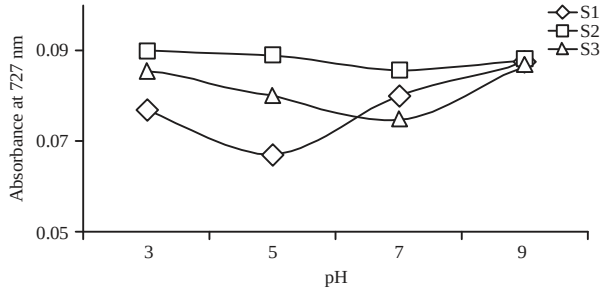

Fig. 5: Effect of pH values on phenol degradation rate using fungal isolates (S1, S2 and S3) measured as absorbance at 727nm
S1: *Aspergillus niger*, S2: *Penicillium griseofulvum* and S3: *Aspergillus terreus*

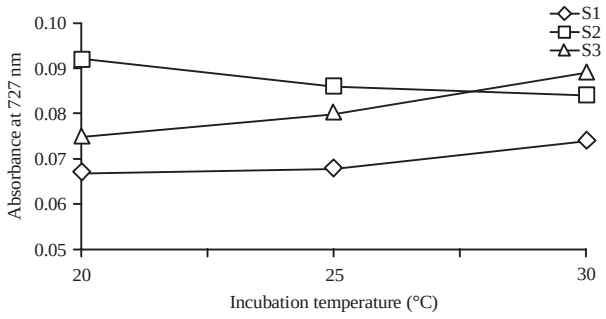

Fig. 6: Effect of different temperature on the degradation rate of phenol (measured as absorbance at 727 nm)
S1: *Aspergillus niger*, S2: *Penicillium griseofulvum* and S3: *Aspergillus terreus*

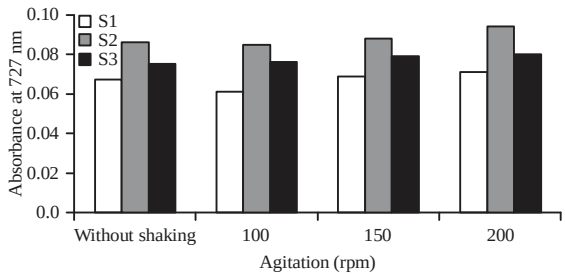

Fig. 7: Effect of agitation values on phenol degradation rate using fungal isolates (S1, S2 and S3) measured as absorbance at 727 nm
S1: *Aspergillus niger*, S2: *Penicillium griseofulvum* and *Aspergillus terreus*

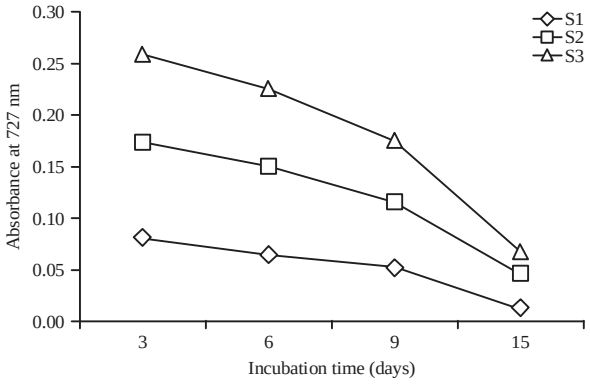

Fig. 8: Effect of different incubation time of fungal isolates (S1, S2 and S3) on the degradation rate of phenol (measured as absorbance at 727 nm)
S1: *Aspergillus niger*, S2: *Penicillium griseofulvum* and S3: *Aspergillus terreus*

and professional effluents is very important because of its persistent and harmful effect[15]. As reported by alternative authors, fungal micro-organisms were eminent for removal of cyclic waste in batch scales[26,27].

Fungi exhibited an important role in the recycling of aromatic chemical substances in the biosphere and several studies have shown that diverse fungi are capable of phenols mineralization. They are really capable of utilize numerous carbon sources by enzymatic mechanisms, thus providing

possibilities for metabolizing phenols and other aromatic derivatives. Fungi are microbes that they can tolerate the occurrence of high concentration of various toxic materials, even at a decreased bioavailability exploiting their powerful extracellular oxidative enzymatic system[15,28-30]. Fungi with success will be used for phenol degradation since they're less sensitive to inhibition[31] and because of active production of assorted enzymes[32], which are able to degrade cyclic ring compounds. Accordingly, the present work was initiated by collecting water and soil samples from different locations contaminated with phenol. The enrichment culture technique was used to isolate fungi[18] that can grow on phenol as a sole carbon and energy source. Enrichment technique is reported by Arutchelvi *et al.*[33] and Tachibana *et al.*[34] to save the long time (lag phase) in getting native population up to effective levels. Polluted locations revealed high fungal count able to degrade phenol.

From this study, three isolates of phenol degrading fungi were chosen and identified using molecular techniques[18,35], DNA sequences were examined for each sample. The selected experimental fungal isolates were identified by partial sequencing the PCR amplified 18S rRNA genes. The results are presented in phylogenetic tree, It was recognized in the displayed tree that the experimental isolate S1 is closely related to *Aspergillus niger* and included in one cluster whereas, the isolates S2 is closely related to *Penicillium griseofulvum* and isolate S3 is closely related to *Aspergillus terreus* and included in one cluster. These results were in arrangement with many investigators, *Aspergillus niger* a filamentous fungus isolated from waste of paper manufacture in India was capable to break down phenol[28]. Among the commonly genera that was isolated from soil are known as *Fusarium*, *Alternaria*, *Penicilium* and *Graphium*[28,29]. The growth of selected fungal strains (*Aspergillus niger*, *Penicillium griseofulvum* and *Aspergillus terreus*) on carbon free media with phenol as a sole carbon source reflects the use of phenol by the strains and these final result was agreement with Basha *et al.*[36], who studied the biodegradation performance of phenol by using free cell of *Aspergillus niger* under different temperature, pH, different incubation times under the aerobic condition.

Results obtained in this study demonstrated the highest growth was at pH 5-7 for the selected fungal strains. Similar results were obtained by Sharma and Gupta[37], they noticed that there was maximum phenolic degradation of *A. niger* occurs at natural pH due to maximum utilization of carbon source. Optimal pH of medium reported in literature for phenol biodegradation with fungi[37,38] is pH 5-6. Results obtained in this study showed the maximum growth at temperature 20-30°C for the chosen fungal strains, which matches with our results, Limiting temperature effect on phenol biodegradation in various fungal bioreactors started[27] from 30-34°C. Similar results were obtained by Sharma and Gupta[37]. They observed that maximum phenol degradation will take place at room temperatures to 30°C and by further increase in temperatures, the rate of biodegradation reduces because the catalytic activity of the enzymes was starts to decrease over that temperature. Therefore, the optimum temperature for the ideal enzymatic activity was 20-28°C. In the present study, shaking don't effect growth rate for all the tested strains.

Results obtained in this study showed that maximum phenolic degradation was occurred after 15 days due to maximum utilization of phenol was occurred at this time. Similar results were obtained by Sharma and Gupta[37], they were showed that maximum phenolic degradation was took place by *A. niger* after 120 h due to maximum utilization of phenol after that time, it starts to decrease due to saturation of active sites of utilizing enzymes. As well this is explained by the long acclimation period, which was carried out for the organism to get started on potential growth with phenol and utilize it as only carbon and energy source. Similar results were obtained by Al-Fawwaz *et al.*[39], who indicated that the phenol removal was found to be increased little by little with time from the beginning of bio-removal by the two types of fungi *Rhizopus* sp. and *Mucor* sp. at 100 mg L^{-1} after 25 days.

CONCLUSION

Based on our study and survey, it can be concluded that phenol is very useful in our day to day life. It can be used for in cleaning products and even in your furniture varnish, in medicines. The list of everyday products that contain phenol is endless. Phenol has many toxicity effects so, various phenol degradation methods are available but the cheapest, eco-friendly and acceptable method is the degradation using microbes. Fungal strains are shown to be an efficient degrader of phenol. *A. niger*, showed a high degradation rate of phenol. The optimum temperature for *A. niger* was found to be 20-25°C and pH was found to be 5 and occurred at static condition until 100 rpm. Biodegradation efficiency for the removal of phenol was increase with increasing incubation time until 15 days.

SIGNIFICANCE STATEMENT

This work is the first work to get rid of phenol wastes by fungi with optimum condition in the Kingdom of

Saudi Arabia. In this work, different samples were collected from various contaminated sources with phenols and other wastes and then isolating different strains of fungi that have the ability to degrade phenol and then select the best conditions for phenol degradation using fungi.

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