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Original Article

Bioremediation of Phenol by Mutated and Immobilized *Aspergillus* and *Penicillium* Species

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Abstract

Phenol and its chemical derivatives are essential for production of polycarbonates epoxies, bakelite, nylon, detergents, herbicides, and numerous pharmaceutical drugs. In order to increase the biodegradation of phenol by fungi, fungal strains (*Aspergillus niger*, *Penicillium griseofulvum* and *Aspergillus terreus*), were isolated from different contaminated sites in Saudi Arabia such as Jeddah Governate, the second industrial city of Jeddah, some garbage collection places, gas stations and Red Sea), then screened for phenol degradation. For the first time in Saudi Arabia, biodegradation of phenol by fungi is improved by mutation as well as immobilization of fungi above calcium alginate. The isolated fungal strains (*Aspergillus niger*, *Penicillium griseofulvum* and *Aspergillus terreus*), were mutated physically (UV) and chemically (Ethidium bromide), also immobilized in alginate beads and its phenol degradation efficiency was observed. The degradation was increased many fold after immobilization, but after mutation some mutants appeared highly degradation rate for the phenol such as *Aspergillus niger*, and *Penicillium griseofulvum* but *Aspergillus terreus* appeared highly degradation rate for the phenol only after exposure to UV for 5 and 10 mins only than the wild strains. In addition, phenol degradation was increased with increase the fungal disk size of the tested strains.

Keywords: biodegradation; enhancement; immobilization; mutation; phenol

Introduction

Phenol is a structure unit for the production of pharmaceuticals, such as, aspirin (Busca *et al*., 2008; Acosta *et al*., 2018). Phenol can be used with chloroform (a commonly-used mixture in molecular biology for DNA & RNA purification from proteins), also used for cell disruption and lysis purpose (Sambrook and Russell, 2001).

It can be absorbed through the skin, inhalation and by swallowing (Germain *et al*., 2019). The typical main absorption way is the skin, through which phenol is resorbed rapidly; simultaneously causing burns on the area of skin (Rappoport, 2003).Contact with phenol may result in irritation of eye, conjunctional swelling, corneal whitening and finally blindness. The continuous exposure to phenol can induce renal damage (Germain *et al*., 2019). Other effects include fizzing from mouth, nose and subsequently headache. Chronic exposure may result in rashes, vomiting, weakness; lose in weight, pain in muscles and nervous (Downs and Wills, 2019). Due to muscle weakness, paralysis can be also being followed. It is also suspected that cancer and striation can also be caused. Phenol and its derivatives are poisonous and classified as dangerous materials (Zumriye and Gultac, 1999; ATSDR, 2007). Ingestion of phenol for a prolonged period of time causes mouth sore, diarrhea, excretion of dark urine and impaired vision at concentrations levels ranging between 10 and 240 mg/L (Álvarez-Torrellas *et al*., 2017).

Due to presently increasing concern of pollution and its effect, the pollution control methods are getting more attention and are equally more important because all the pollutants released in atmosphere are very harmful to human in form of some disease or disorder in the environment. From the list of many pollutants, one of the pollutants is phenol which is 11th most toxic compound out of 126 toxic compounds given by environmental protection agency (EPA) (Saluja, 2015). Phenol released from industries in free form or in the form of phenol derivative is main problematic cause for pollution of soil and water (Saluja, 2015).

Phenol is degraded by so many microbes which utilizes phenol as the sole carbon source for their growth (Rudzanova *et al*., 2019). Several microbes both anaerobic

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and aerobic microorganisms degrading phenol are isolated and characterized (Nair *et al*., 2008), while microorganisms capable of aerobic phenol degradation were defined as early as 1908 (Van Schie and Young, 1998).

Along with bacteria, fungi are known for their multiplicity and notable ability in degrading phenolic compounds. Conflicting to bacteria, fungi can grow under ecologically stressed environments such as low nutrient availability, low water activity and at low pH values where bacterial growing might be inadequate (Saluja, 2015).

Cell immobilization has been well-defined as the physical confinement or localization of viable microbial cells to a certain defined region of space in such a way as to limit their free movement and exhibit hydrodynamic characteristic which differ from those of the surrounding environment while retaining their catalytic activities for repeated and continuous use (Freeman and Lilly, 1998; Covizzi *et al*., 2007; Amim *et al*., 2010; Mrudula and Shyam, 2012). Immobilization technique could increase the efficiency of the microbial cell viability and/or growth (Doran and Baily, 1986).

To increase and enhancement the efficiency of biological treatment of phenol, the present study focused on mutation chemically and physically and immobilization of fungal strains that were isolated previously (Ibrahim and EL-Gamdi, 2019), using Ca-alginate beads.

Materials and Methods

Phenol determination

For the determination of phenol content, the Folin-Ciocalteau phenol reagent was used. To the 0.1 ml of supernatant liquid add 0.1 ml of 2% of sodium carbonate Na_2CO3, 0.1 ml of Folin- Ciocalteau reagent and add 2 ml of distilled water. Then it is kept aside for 60 minutes at 20 °C. Then the absorbance was measured at 727 nm against distilled water and reagent blank (Leonard and Lindley, 1998). All samples are measured by the spectrophotometer, and then phenol concentration was determined. The bio-removal efficiency of the strains was then calculated according to the following formula:

% Phenol Removal Efficiency (PRE) = $(C_i\text{-}C_f/C_i) \times 100$

Where c_i is the initial concentration of phenol (mg/L) and c_f is the final concentration of phenol

Effect of different fungal disk size on the growth and degradation rate

Experiments were performed in three of 250 ml flask containing 100 ml of mineral salt medium (MSM), containing liquid phenol as sole carbon and energy source after medium sterilization, fungal disk of 5 days old culture (*A. niger*, *P. griseofulvum* and *A. terreus*), with (2-5 and 7 mm) was used as inoculum on the tested medium (Abd El-Zaher *et al*., 2011), then incubation of the flasks at 27 ± 2 °C for one week. After incubation, the determination of phenol content was measured by using Folin-Ciocalteau phenol reagent.

Effect of different concentration of phenol on the growth and degradation rate

Experiments were performed in three of 250 ml Erlenmeyer flask containing 100 ml of MSM, the medium containing phenol at concentration ranging (0.01, 0.05, 0.08, 0.1, 0.2, 0.5 and 0.8 g/100 ml), which were added by micro filter (0.45 μm) after medium sterilization, as sole carbon and energy source then add fungal disk of 5 days old culture, with 7 mm diameter from fungal strains (*Aspergillus niger*, *Penicillium griseofulvum* and *Aspergillus terreus*), were used as inoculum on the tested medium (Abd El-Zaher *et al*., 2011),then incubation of the flasks at 27± 2 °C for one week. After incubation, the determination of phenol content was measured by using Folin-Ciocalteau phenol reagent.

Increasing the biodegradation rate of phenol by different methods

A-Physical mutation by UV

For UV-mutagenesis, spores obtained from 5-day old cultures of *A. niger*, *P. griseofulvum* and *A. terreus* were suspended in sterile distilled water and exposed to UV-light (254 nm) from Philips TUV-30 W lamp source at a distance of 25 cm for 5, 10 , 15 ,30 and 60 minutes. The treated conidia were put in dark for one hour, and transferred to dishes with potato dextrose agar (PDA) supplemented with liquid phenol (El-Bondkly and Keera, 2007),and one ml of the the treated conidia transferred into 250 flasks, each flask contains 100 ml mineral medium (MSM). The medium was then supplemented with liquid phenol as sole carbon and energy source (Jacob and Sohail, 2010). All samples are incubated in incubators at 27 ± 2 °C for 1 week. For the determination of phenol content, the Folin-Ciocalteau phenol reagent was used.

B-Chemical mutation by ethidium bromide (EtBr)

A. niger, *P. griseofulvum* and *A. terreus* were grown as previously described and pelleted by centrifugation at 5000 rpm for 10 min. The resulted pellets were suspended in solution of 3% NaCl (w/v). Subsequently, various ethidium bromide (EtBr) concentration (50, 100, 150 and 200 μg/ml), were added to the fungal cells. After the examined exposure period (24 hours), cells were pelleted again, washed by phosphate buffer saline at pH 6.8, and inoculated into mineral salt agar containing phenol, then incubated for 2-7 days at 27 ± 2 °C. The survived cells were then selected and cultivated on PDA broth (Katrcolul *et al*., 2003). The activity of fungal strains for degradation was monitored by measuring phenol content by using the Folin-Ciocalteau phenol reagent.

C-Immobilization of the fungal strains in alginate beads

Spore suspension of our strains from potato dextrose agar plates was suspended in a previously autoclaved solution of sodium alginate to a final concentration of 40 g/L and 100 mL/L biomass. Beads without fungi, served as the controls. The alginate-fungi mixture was added drop wise with a sterile syringe (20 mL) fitted with a wide pore needle (1 mm diameter) from a height of about 20 cm into an autoclaved solution of calcium chloride (30 g/L, adjusted to pH = 7.0), where beads formed immediately. The beads were left in this hardening solution overnight at 4 °C before being harvested by filtration and washed thoroughly with fresh culture medium to remove the un-entrapped free hyphae under aseptic conditions (Mohanty and Jena, 2016).

Storage stability and reusability of immobilized cells

Free and immobilized cells were tested for phenol degradation. Cells were grown at 27 ± 2 °C for one week in MSM (pH = 7), containing phenol as sole carbon source, and the phenol degradation rate was detected. The immobilized cells were incubated in MSM for 1 week (Mohanty and Jena, 2016). For the determination of phenol content, the Folin-Ciocalteau phenol reagent was used.

Results

Effect of different size of fungal disk on the growth and degradation rate

It was noticed that phenol degradation increased with the increasing the size of fungal disk for all tested isolates that appeared with reduction in absorbance as shown in Fig. 1.

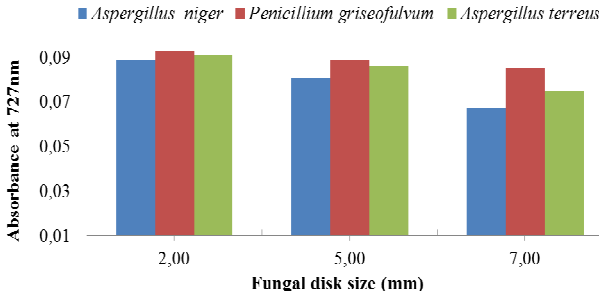

Fig. 1. Effect of fungal disk size on the degradation rate of phenol measured as absorbance at 727 nm

Effect of different concentration of phenol on the growth and degradation rate

Data presented in Fig. 2 and Table 1, showed that the degradation rate of phenol was decrease with increasing the concentration of phenol (0.01, 0.05, 0.08, 0.1, 0.2 , 0.5 and 0.8 g/100 ml) for fungal strains , the best degradations appear as reduction in absorbance at 727 nm as mentioned before in the materials and methods by using Folin-Ciocalteau reagent.

Increasing the biodegradation rate of phenol by different methods

A- Physical mutation by UV

The results were as follows: increases the growth of *A. niger* as the time of exposure to UV increases, decreases the growth of *P. griseofulvum* and *A. terreus* as the time of exposure to UV increases as shown in Fig. 3.

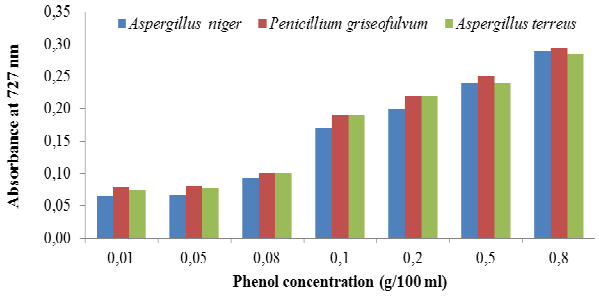

Fig. 2. Effect of different concentration of phenol on the degradation rate of fungal strains measured as absorbance at 727 nm

Table 1. Degradation rate (%) of the fungal strains with different concentration of phenol using spectrophotometer at 727 nm

Phenol concentration (g/100 ml)	Degradation rate of phenol (%)		
	Aspergillus niger	*Penicillium griseofulvum*	*Aspergillus terreus*
0.01	28	13.3	17
0.05	27	13.1	15
0.08	16	11	11
0.1	15	4.4	6
0.2	13	4.3	4
0.5	4	0.4	3.9
0.8	2	0.3	1.5

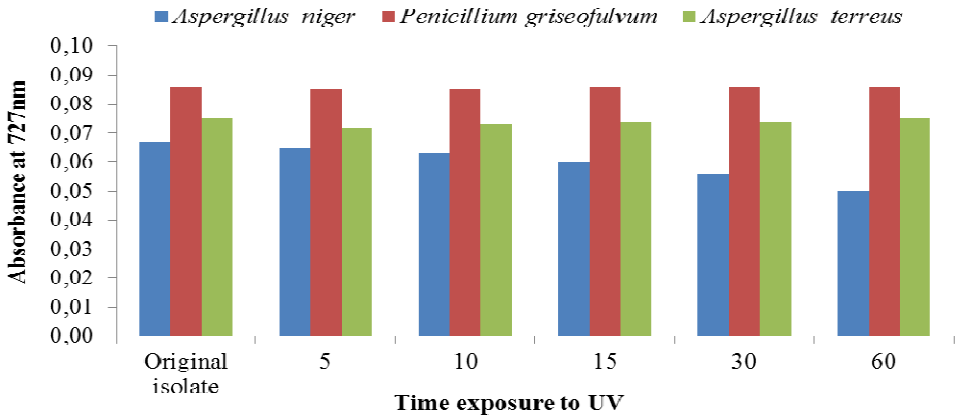

Fig. 3. Effect of exposure of fungal strains to different time of UV (5, 10, 15, 30 and 60 min.) on phenol degradation rate using spectrophotometer which appeared as reduction in absorbance at 727 nm

B- Chemical mutation by ethidium bromide (EtBr)

As shown in Fig. 4, the results were as follows, increases the growth of *A. niger*, *P. griseofulvum* as the time of exposure to EtBr increases, and decreases the growth of *A. terreus* as the time of exposure to EtBr increases.

C- Immobilization of the fungal strains in alginate beads

Encapsulation of *A. niger*, *P. griseofulvum* and *A. terreus* into Ca- alginate beads were performed and the results are shown in Table 2 and Fig .5, which revealed that the degradation rate of phenol was increased by immobilized cells more than the free cells that appeared with reduction in absorbance. Controls (Ca-alginate beads without fungal cells in MSM medium containing phenol), demonstrating that phenol cannot adsorb by these beads and therefore no degradation was occurred.

Discussion

Phenolic compounds are public waste product in the production of industrial and agricultural products. Particularly, phenolic compounds are found in wastewaters from coal processing plants, oil refineries, pulp and paper manufacturing plants, resins and coke manufacturing, steel industries, pharmaceutical industries, plastic and varnish industries, textile units, pesticide plants, tannery, smelting and related metallurgical operations (Marrot *et al*., 2006; Bodalo *et al*., 2008; Jayachandran and Kunhi, 2008).

Phenol may present in air, sea water or surface water, soil or sewage. Now, the associated problem due to phenol is that when it is persist in waste water, even in low concentrations, it can be poisonous to some aquatic species (Rittmann and McCarty, 2001).

Table 2. Effect of free and immobilized fungal strains on phenol degradation rate measured as absorbance at 727 nm by spectrophotometer

Fungal strains	Degradation rate of phenol (%)	
	Free cells	Immobilized cells
Aspergillus niger	29	40
Penicillium griseofulvum	12	21
Aspergillus terreus	17	31

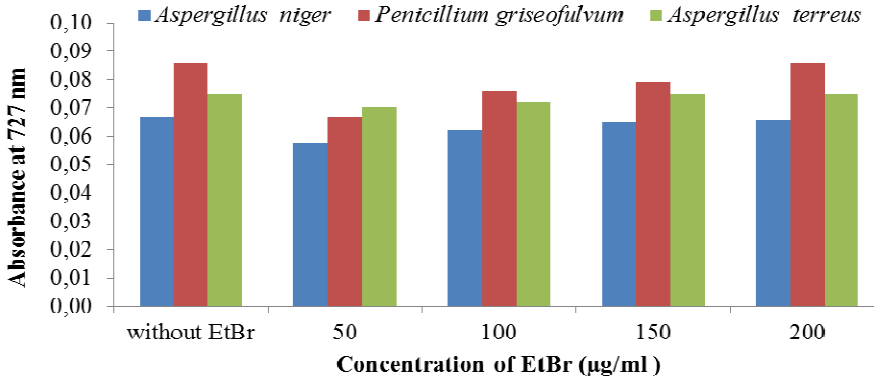

Fig. 4. Effect of exposure of fungal strains to different concentration of EtBr (5,100,150 and 200 µg/ml) on phenol degradation rate using spectrophotometer which appeared as reduction in absorbance at 727 nm

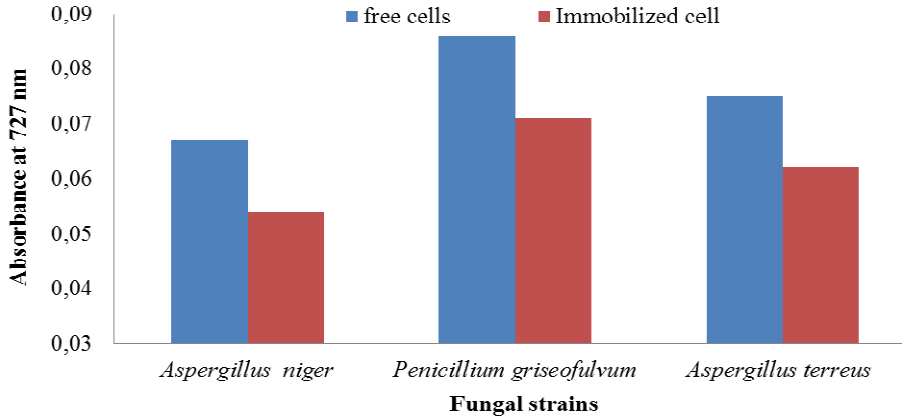

Fig. 5. Effect of free and immobilized selected fungal strains on phenol degradation rate using spectrophotometer which appeared as reduction in absorbance at 727 nm

Fungi are capable of metabolizing phenol by enzymatic mechanisms (Agarry *et al*., 2008). Biodegradation of phenol by fungi is influenced by number of factors; the substrate concentration is an important factor in the phenol biodegradation, as phenol itself, is well known to inhibit microbial growth, especially at greater concentrations. This phenomenon is commonly known as substrate inhibition. High concentration of a phenol can be preventing the growth of microorganisms, and the concentration at which inhibition occurs varies from compound to another (Alexander, 1985).

Adjei and Ohta (2000) reported that phenol was completely inhibitory for cyanide utilization by the bacteria *Burkholderia cepacia* strain C-3. (Wei *et al*., 2008), studied the influence of substrate concentration on the biodegradation efficiency by varying initial phenol concentration from 100 mg to 1000 mg /l.

In the present study, high concentration of a phenol can be prevent the the growth of fungal strains, that the degradation rate of phenol was decrease with increasing the concentration of phenol (0.01, 0.05, 0.08, 0.1, 0.2, 0.5 and 0.8 g/100 ml). Similar results were obtained by (Supriya and Neeha, 2014), they showed that maximum phenolic degradation by *A. niger* was observed at 300 mg/l, this due to the fact that the phenol degrading enzymes activity is optimum at this concentration.

Phenol removal at lower concentrations is attributed to the presence of more available sites on the adsorbent (fungi biomass) than the number of phenol ions which are available in solution. The bio-treatment in our study was proportional to the concentration. This agrees with the results of (Moyo *et al*., 2012), who showed that the yeast *Saccharomyces cerevisiae* used phenol as the sole energy and carbon source and tolerated up to 120 mg/L phenol.

Results obtained in this study revealed that phenol degradation increased with the increasing the size of fungal disk for all tested strains (2, 5 and 7 mm). An adequate number of inoculums were necessary to minimize the period of the lag phase, increase the degradation rate, and prompt the exponential growth phase after seeding. Similar results were obtained by (Balamurugan *et al*., 2012), who showed that sufficient quantity of inoculums of *A. fumigatus* ensures rapid proliferation and biomass synthesis in cultivation. Phenol degradation at an initial concentration of 400 mg/l was achieved at an inoculum concentration of 313% (v/v) at pH 7.5 and 30 °C. At low inoculums concentrations (3-5%), microbial growth had a prolonged lag phase when the inoculums concentration was increased to 7-10% (v/v), the lag phase was largely eliminated.

Physical (UV-light) and chemical (EtBr) mutagens proved to be powerful in inducing a wide range of genetic variability. The results in this study proved that *P. griseofulvum* and *A. terreus* were sensitive to UV radiation. Lethality was increased with radiation time and reached 95% at 15 minutes, after which it become constant. Thus, 5 and 10 minutes appeared to be an appropriate radiation time for further studies. But the mutant strain *A. niger* show a higher phenol-degrading efficiency increased with radiation time, these results showed that the mutant strain *A. niger*, has more tolerant than the wild strain until after 60 minutes, and the mutant strains *P. griseofulvum* and *A. terreus* has more tolerant than the wild strain until after 5 and 10 minutes only. The enhanced phenol-degrading efficiency of mutant strains may result from the increase in the activity of the related enzymes.

Many papers reported that UV mutagenesis has a positive effect on increasing the biodegradation ability of strains (Chen *et al*., 2011; Joshi *et al*., 2016). Yet, another study showed that UV mutagenesis had no influence on degradation efficiency (Sekar *et al*., 2009). Similar results were obtained by (Zhen *et al*., 2015), who indicated that the mutant strain *Pseudochrobactrum* sp. XF1 has more tolerant over a wide range of pH values than the wild strain (EL-Bondkly and Keera, 2007), who observed that UV-irradiation and (EtBr) were applied in different doses for inducing mutations in the *P. roquefortii*. Results show the gradual increase of the lethality which was associated with the increase of the mutagen dosage. On the other hand, the survival percentages decreased by increasing (EtBr) doses.

Immobilization technique could increase the efficiency of the microbial cell viability and/or growth (Doran and Baily, 1986). Entrapment of fungal cells using insoluble calcium alginate is a rapid method, non-toxic, inexpensive and versatile. More than 80% of cell immobilization process is still carried out using alginate (Thu *et al*., 1996).

Immobilization could increase the efficiency of the microbial cell viability, growth and some properties (Doran and Baily, 1986). Also (Wiesel *et al*., 1993), reported that immobilization is considered to promote better survival and activity of some microorganisms. These observations confirmed our results on phenol degradation with immobilized and free fungal cells, as well as control (Ca-alginate beads without fungi). Phenol cannot adsorb by control (beads without fungi), while the immobilized cells showed higher degradation rate as compared with free cells.

Conclusions

It can be concluded that phenol is so widely found and used in everyday activities; some factors that affect the degradation rate of phenol were studied in this work and revealed that, phenol degradation increased with the increase the size of fungal disks of the tested cultures. Also, phenol degradation increased as the incubation period increased. UV irradiation showed that the growth and degradation increases as the time of exposure to UV increases for *A. niger* and the growth and degradation decreases as the time of exposure to UV increases for *P. griseofulvum* and *A. terreus*. Also phenol degradation increased with immobilization of fungal strains.

Conflict of Interest

The authors declare that there are no conflicts of interest related to this article.

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