Biodegradation of Hydroquinone Compound in Pharmaceutical Wastewater using the *Penicillium Citrinum* AUMC14751 Isolate

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Abstract

Hydroquinone compounds are the most common pollutant in pharmaceutical and industrials wastewater and these have been known as health risks to human and aquatic organisms even at low concentrations. Hydroquinone is toxic for aquatic organisms at the concentration level of 4ppm/L. Therefore, it is very important to remove hydroquinone from contaminated water before discharge into any natural water. The present study focused on the isolation of fungi from samples collected from different location in the hydroquinone production. Surveying the fungal species showed that 49 out of 432 samples (11.3%) were positive for fungal growth given three genera of fungi (*Aspergillus sp, Penicillium sp,* and *Fusarium sp*). The collected samples were enriched in sterile Minimal Salt Medium (MSM). Hydroquinone used as the sole carbon source. Fungal growth was screened to determine their ability to grow and degrade Hydroquinone at 500 ppm concentration. A total of 15 isolates that have the capability to growth in MSM supplemented with hydroquinone were selected to identify at Mycological Center, Assiut University. The results demonstrate that the

genus of *Aspergillus* was the most predominant being represented by 40 % of all fungal isolates, followed by *Penicillium* species 33 % and *Fusarium* species 27 %. Out of these 15 isolates, *Penicillium citrinum* AUMC14751 was selected to determination the biodegradation rate of hydroquinone by HPLC analysis. The rate of hydroquinone degradation due to the fungal consumption of hydroquinone as a sole carbon source was increased gradually depending on the time. HPLC results indicated that the rate began gradually increased in the presence of isolate of *P. citrinum* AUMC14751 starting from 0.63% after the second day up to the tenth day. The maximum percentages of the hydroquinone degradation rate appeared after 10 days at 97.02%.

Keywords: ***Penicillium citrinum; biodegradation; hydroquinone; HPLC analysis***

Introduction

Industrial development has caused a huge increase in the release of potentially toxic materials **[1].** Hydroquinone (Hydroxy aromatic, HQ) was chosen as source of high toxicity. It is widely existed in the effluents of many industries such as textile, paper, pulp, steel, petrochemical, petroleum refinery, rubber, dyes, plastics, pharmaceutical. As a result, HQ is extensively present in the effluents of their manufactures, and hence introduced into water inevitably. However, HQ is a primary pollutant in the wastewater and consequently, removal of HQ from wastewater has attracted significant environmental concerns **[2].**

Hydroquinone (HQ) is one of the most harmful metabolites of phenolic substances. HQ has several toxic effect on environment, animal as well as human health. It is very toxic to aquatic organism, shellfish and fish at the concentration level of part-per-million. It is the most toxic dihydroxybenzene which decreases the cultivable microorganisms with increasing concentration. At higher dose, HQ has inhibitory effect on mouse and human bone marrow cells **[3-6]**.

Meanwhile, HQ is widely applied as inhibitor, rubber antioxidant, and food antioxidant. Therefore, it exists widely in industrial effluents, such as the waste from oil refineries, rubber,

and pharmaceutical industries **[7]**. Owing to its high-potential for toxic effect on aquatic organisms and persistence in water, the discharge of HQ to natural water causes different threats to human health and the ecological environment. Therefore, there is an urgent need to treat HQ in wastewater**[8]**. Despite the toxic properties, a number of microorganisms can utilize hydroquinone, especially under aerobic conditions as ***Aspergillus fumigatus, Candida parapsilosis, Tyromycespalustris, Gloeophyllumtrabeum, Penicillium chrysogenum,*** and ***Phanerochaete chrysosporium*** .Which has led to the development of low-cost treatment of polluted effluents **[9]**.The aims of this study were to isolate and screen hydroquinone-degrading fungi. The degradation rate of hydroquinone by selected fungus using HPLC was investigated.

Materials and methods

Materials and equipment's

Different size flasks, pipettes, sterile cotton swabs, petri plates, Test tubes and chemicals for different tests, autoclave, laminar flow hood, Incubator, hot-air oven and orbital shaker.

Sample collection.

Along a period of 12 months, a total of 432 samples were collected from different location in the production area (Semi solid Section) using four different methods at the end of every week (operation only). The samples were collected into sterile bottles, and then transferred to lab through ice tank for analysis within 12 hours.

Table 1: Total collected samples

Location	Washing room	Preparation room	Filling room
	No of samples		
Settle plates	48	48	48
Final rinse after cleaning	32	32	32
Sample from drain Sewage	32	32	32
Swabs from equipment's	32	32	32
Total	144	144	144

Preparation of Hydroquinone stock solution.

The stock solution of Hydroquinone was prepared by adding 10 g of Hydroquinone to purified water and the volume was complete to 1000 ml. A serial dilution were done to obtain the concentration of 100 ppm, 250 ppm and 500 ppm. The stock solution was sterilized by filtration it through a 0.22 µm membrane filter.

Minimal Salt Medium (MSM).

The obtained fungal isolates were cultivated in minimal salt medium as showed in **Table 2**. The MSM composition used in the study is as per **[10].** The composition of the MSM containing the following: -

Table 2: Chemical composition of the Minimal Salt Medium (MSM).

Chemical composition	g/L
KH2PO4	0.5 g
K2HPO4	0.5 g
CaCl2	0.1 g
NaCl	0.2 g
MgSO4_7H2O	0.5 g
MnSO4_7H2O	0.01 g
FeSO4_7H2O	0.01 g
NH4NO3	1.0 g

Hydroquinone (analytical grade) was used as a sole carbon source and sterilized hydroquinone solution was added directly to MSM after cooling. The media were sterilized by autoclaving and the hydroquinone was used after sterilization by filtration method.

The collected samples were suspended into peptone buffer solution and inoculated in 250 ml conical flasks contain potato dextrose broth medium. The flasks were incubated on orbital

shaker incubator at 150 rpm at 27ºC for 7days. After the incubation period, the fungal growth was observed and harvested to subject to further studies.

Screening of hydroquinone degrading fungi:

All the obtained fungal isolated by enrichment technique were individually inoculated into 10 ml of the minimal salt Hydroquinone medium contains 150 ppm of Hydroquinone. The flasks were incubated on orbital shaker incubator at 250 rpm at 27 ºC for 7 days. The isolates which showed growth in the broth medium were plated individually into minimal salt Hydroquinone medium with 250 ppm of Hydroquinone. The same procedure was repeated with 500 ppm Hydroquinone containing minimal salt medium. The culture which showed growth in minimal salt Hydroquinone medium with 500 ppm of Hydroquinone were selected as the Hydroquinone degrading strains. The selected cultures were kept at 20ºC in 30% glycerol stock. Working cultures were maintained by sub-culturing every two weeks on minimal salt agar slant plates and broth containing Hydroquinone.

Identification of fungal isolates

Fungi were identified based on their macro- and microscopic features with the aid of the following references **[11-14].**

Determination of Biodegradation of Hydroquinone by HPLC.

The degradation of the hydroquinone was confirmed by HPLC analysis. The culture of the selected isolate was separated by centrifugation at 13,000 rpm for 5 min. Then, the culture supernatant was filtered through a 0.22 μm pore-sized filter membrane and subjected to Hydroquinone determination on an HPLC system using an Equisil BDS. C18 column (5 μm 250 × 4.6 mm). Sample (20 μL) was injected into the HPLC system standard solution: weight 500 mg of hydroquinone in 250 ml Aceto nitrile and complete to 1000 with mobile phase with a flow rate of 1.2mL/min at 270 nm. A hydroquinone concentration standard curve was created based on HPLC and used to quantify the Hydroquinone concentration.

Result and discussion

Isolation, identification and selection of an efficient hydroquinone - degrading by isolated fungi

The chances of isolating the microbial strains with high ability to metabolize hydroquinone are brighter from the contaminated site **[15].** Hence, the pharmaceutical manufacturing environment moreover the wastewater contaminated with hydroquinone was chosen as the source of microorganisms' isolation in this study. The samples were collected from the pharmaceutical production area (Semi solid Section) along a period of 12 months from November 2018 to October 2019 were enriched in sterile Minimal Salt medium (MSM) using hydroquinone as the sole carbon source. The sample was further treated with hydroquinone to ensure that only utilized hydroquinone strain would be selected.

Surveying the fungal species associated with the Hydroquinone manufacturing environment showed that 49 out of 432 samples (11.3%) were positive for fungal growth given three genera of fungi (*Aspergillus sp*, *Penicillium sp* and *Fusarium sp*). A total of 15 fungal isolates that able to utilize hydroquinone as sole of carbon source were obtained from the enriched population grown in MSM medium supplemented with hydroquinone.

Enrichment culture technique has earlier been used to isolate several fungi capable of hydroquinone degradation**[16]**. Hydroquinone was metabolized, but no formation of products was observed. Indeed, the same authors described the effective mineralization of aromatic by the brownbasidiomycetes Tyromycespalustris and Gloeophyllumtrabe umled to the formation of hydroquinone, which it is further metabolized **[16].** The hydroxylated intermediate was also found as product of phenol metabolismoffungi. Theascdomycetousfungi, *Penicilliumchrysogenum* var. *halophenoli cum* (previously known as *Penicillium chrysogenum* is able to complete mineralization of phenol in single and combined phenol and glucose cultures**[17]**. However, during the conversion of phenol in the combined phenol and glucose cultures, hydroquinone

was accumulated in the early stages of incubation and disappeared after 80 hours of culture, indicating that hydroquinone was a metabolic intermediate, but it is not a dead-end product**[17].**It has been also detected in the biodegradation of 4-ethylphenol by *Aspergillus fumigates*, another ascomycetous fungi. According to these authors, hydroquinone was obtained by hydrolysis of 4-hydroxyphenylacetate, which undergoes further hydroxylation to form 1,2,4-trihydroxybenzene followed by ring fission substrate to produce maleylacetate**[18].**

The hydroquinone degradation was measured by estimating the residual hydroquinone remaining in the broth medium. Among the fungal isolates were recovered from the collected samples from washing room, preparation room and filling room of semi-solid section, a total of 15isolates that have the capability to growth in MSM supplemented with hydroquinone were selected to identified at Mycological Center, Assiut University. The results demonstrate that the genus of filamentous fungi *Aspergillus* were the most predominant being represented by 40 % of all fungal isolates, followed by *Penicillium* species 33 % and *Fusarium* species 27 % as showed in Table 3.

Table 3: Morphological identification of fungal isolates.

Isolate NO	AUMC	Identification
1	14751	***Penicilliumcitrnum*Thom**
2	14752	***Fusariumsolani* (Martius) Saccardo**
3	14753	***Penicilliumcitrnum***
4	14754	***Aspergillusflavus* Link**
5	14755	***Penicilliumcitrnum***
6	14756	***Fusariumsubgiutinans* (Wollenweber&Reinking)**
7	14757	**White sterile mycelium**
8	14758	***Aspergillusflavus***
9	14759	***Aspergillusflavus***
10	14760	***Aspergillusflavus***
11	14761	***Aspergillusflavus***
12	14762	***Penicilliumcitrnum***
13	14763	***Penicilliumcitrnum***
14	14764	***Fusariumsubgiutinans***
15	14765	***Aspergillusflavus***

Confirmatory experiments and tests (HPLC analysis).

In this study, the confirmatory experiments on the biodegradation of hydroquinone were done using HPLC analysis for the isolate of (*Penicillium citrnum* AUMC 14751) Calibration curve and Spectrum of HPLC analysis of hydroquinone degradation by the selected Fungal strain are shown in the Table 4. The rate of hydroquinone biodegradation due to the fungal consumption of hydroquinone as a sole carbon source was increase gradually depending on the time. Along the experiment, the

standard sample was kept in the refrigerator and the sample was incubated in the incubator at the optimum conditions.

The results show that the peak area for the standard sample (hydroquinone only at the concentration of 500 mg/L) at zero time was 100%. The biodegradation rate began gradually increase in the presence of the isolate which the results demonstrated the rate of biodegradation of hydroquinone in the sample was 0.63 % increased to 1.4 %, after second day While the maximum percentages of hydroquinone degradation rate was showed after 10 days reached to 97 % as showed in **Figures 1-6.**

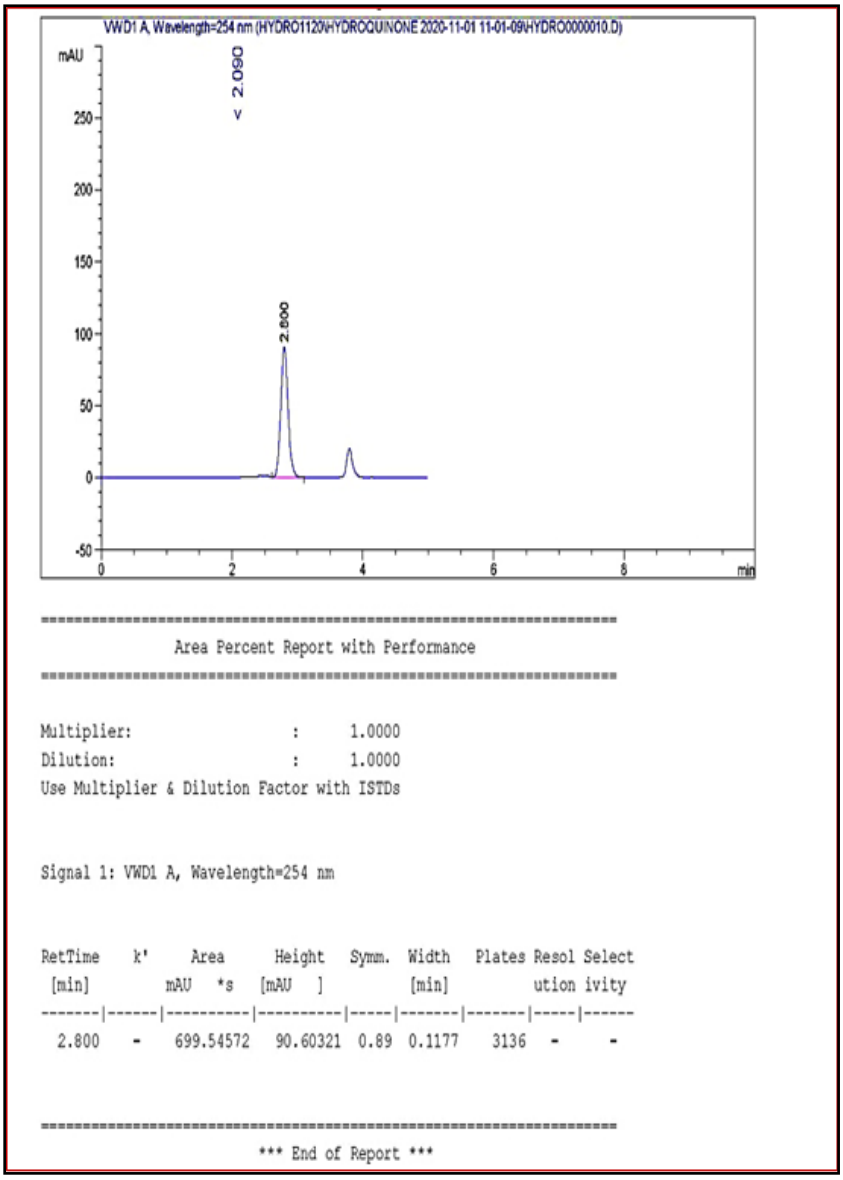
Table (4): Hydroquinone degradation rate by *Penicilliumcitrinum* AUMC14751 at selected concentrations of hydroquinone (500 mg/L^{-1}) using HPLC analysis.

Time	Date	Response Peak area		Assay %	Biodegradation %
		Sample	Standard		
zero time	01/11/2020	700.71204	699.54572	100.326%	0 %
24 hr.	02/11/2020	683.72864	689.20453	99.3634%	0.6366%
48 hr.	03/11/2020	682.46869	694.20386	98.4661%	1.4750%
72 hr.	04/11/2020	637.05200	693.75812	91.8262 %	8.17038 %
96 hr.	05/11/2020	622.67303	693.85510	89.88401%	10.11599 %
5 days	06/11/2020	542.27539	694.11853	78.24875 %	21.75125 %
7 days	08/11/2020	329.17059	694.13586	47.56569 %	52.43431 %
9 days	10/11/2020	211.43378	695.20166	30.46174 %	69.5775 %
10 days	11/11/2020	26.422500	822.07220	2.97557 %	97.02443 %

Assay calculation formula: Assay = Test / Standard * Potency Standard * weight Standard / weight test

Data in Table 4 showed that the biodegradation rate of hydroquinone in the sample was 0.63%, and after the second day the biodegradation rate of hydroquinone was 1.47 %, and it increased to 8.17, 10.11, 21.75, 52.43, 69.57 and 97.02% after 3,4, 5, 7, 9 and 10 days of incubation, respectively. Our results revealed that the maximum hydroquinone breakdown rate was 97.02 % after 10 days of incubation. The rate of hydroquinone assay (concentration) due to the fungal consumption of hydroquinone as a sole carbon source was decreasing gradually biodegradation depending on the time. Fungi are known to be capable of transforming or mineralizing hydroquinone; *Aspergillus fumigatus*, *Candidaparapsilosis*, *Tyromycespalustris*, *Gloeophyllum trabeum*, *Penicillium chrysogenum*, and *Phanerochaetechrysosporium* are examples of fungi able to degrade hydroquinone **[16, 17, 19].**

Assay formula of Standard sample (Hydroquinone only)



Area Percent Report with Performance

Multiplier: : 1.0000
Dilution: : 1.0000
Use Multiplier & Dilution Factor with ISTDs

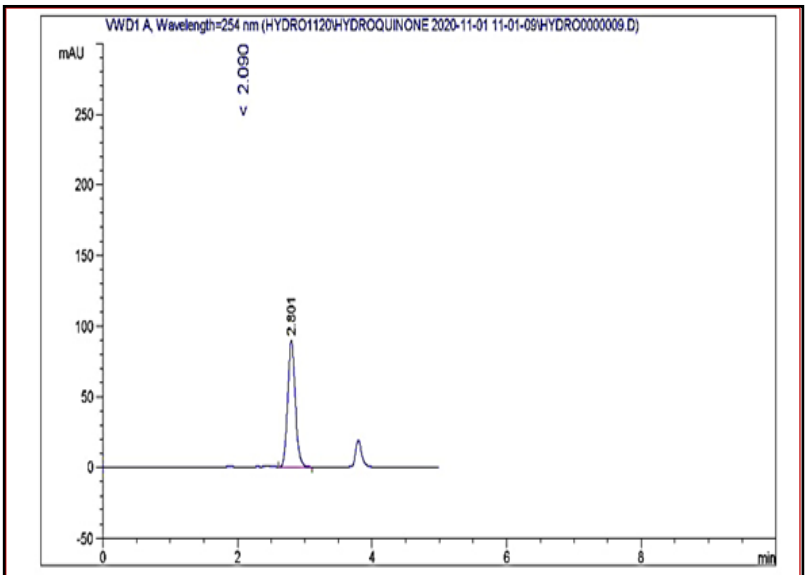
Signal 1: VWD1 A, Wavelength=254 nm

RetTime [min]	k'	Area mAU *s	Height [mAU]	Symm.	Width [min]	Plates	Resolution	Selectivity
2.800	-	699.54572	90.60321	0.89	0.1177	3136	-	-

*** End of Report ***

Fig 1: Biodegradation of Hydroquinone Standard at zero time

Assay of the tested sample at zero time.
700.71204/699.54572*99.8*501.8/500 = 100.326%



Area Percent Report with Performance

Multiplier: : 1.0000
Dilution: : 1.0000
Use Multiplier & Dilution Factor with ISTDs

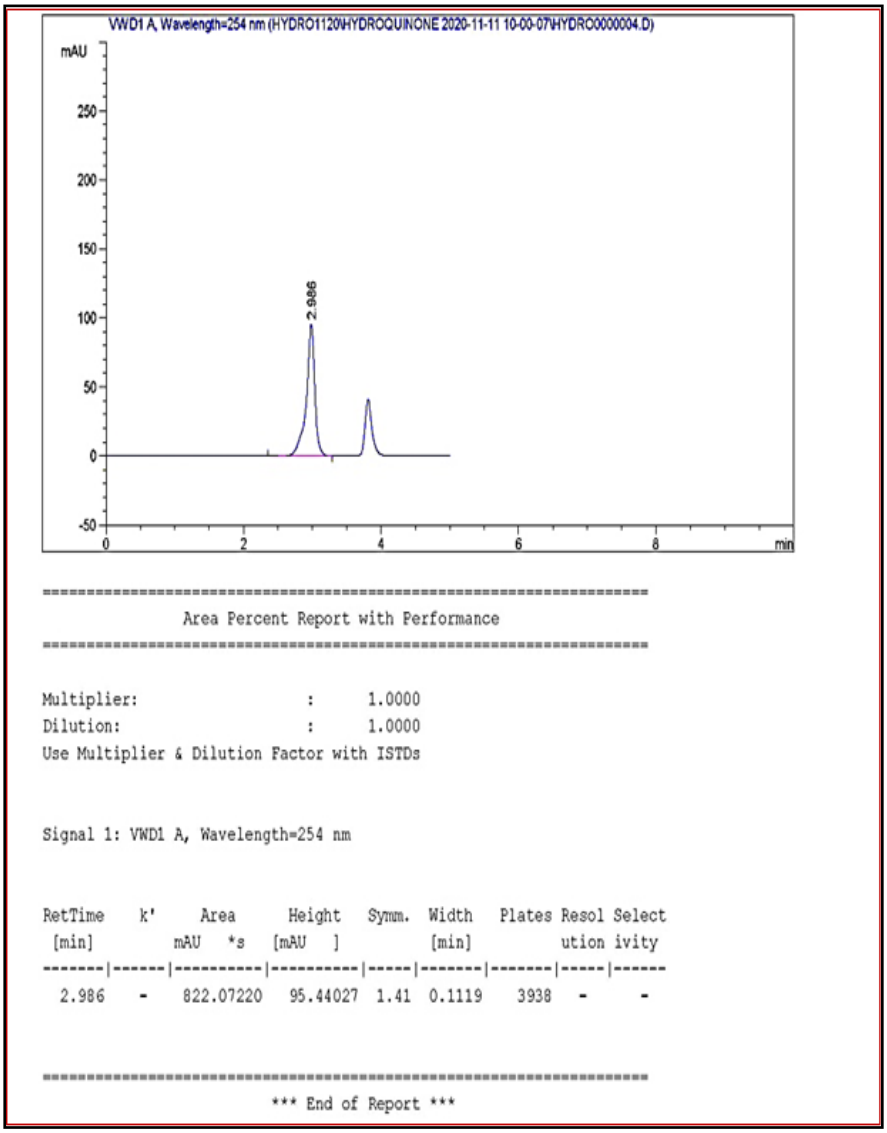
Signal 1: VWD1 A, Wavelength=254 nm

RetTime [min]	k'	Area mAU *s	Height [mAU]	Symm.	Width [min]	Plates	Resol ution	Select ivity
2.801	-	700.71204	89.82789	0.90	0.1186	3090	-	-

*** End of Report ***

Fig 2: Biodegradation of Hydroquinone by *Penicilliumcitrinum* AUMC14751at conc. of 500 ppm zero time

Assay of the standard after 10 days.



Area Percent Report with Performance

Multiplier: : 1.0000
Dilution: : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: VWD1 A, Wavelength=254 nm

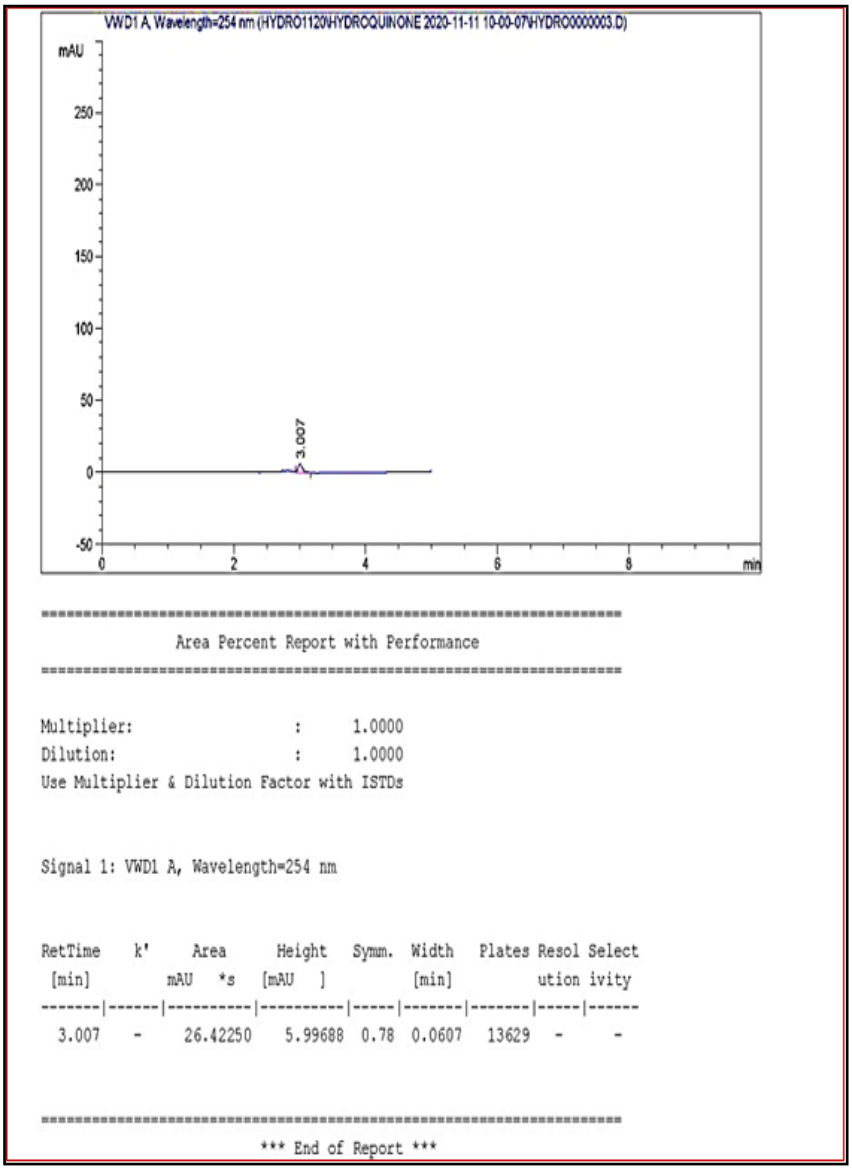
RetTime [min]	k'	Area mAU *s	Height [mAU]	Symm.	Width [min]	Plates	Resol ution	Select ivity
2.986	-	822.07220	95.44027	1.41	0.1119	3938	-	-

*** End of Report ***

Fig 3: degradation of Hydroquinone Standard at conc. of 500 ppm after 10 days.

Assay of the standard after 10 days

26.42250/822.0720*99.8*501.8/500 = 2.97557%



Area Percent Report with Performance

Multiplier: : 1.0000
Dilution: : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: VWD1 A, Wavelength=254 nm

RetTime [min]	k'	Area mAU *s	Height [mAU]	Symm.	Width [min]	Plates	Resol ution	Select ivity
3.007	-	26.42250	5.99688	0.78	0.0607	13629	-	-

*** End of Report ***

Fig 4: Biodegradation rate of Hydroquinone by PenicilliumcitrinumAUMC14751at conc. of Hydroquinone 500 ppm after 10 days

Assay formula of Standard sample (Hydroquinone only

26.42250/822.0720*99.8*501.8/500 = 2.97557%

Table (5): Correlation of Assay % and Biodegradation % with time (days) from zero time to 10 days.

Group	Assay %		Biodegradation %	
	r-value	P-value	r-value	P-value
Biodegradation of hydroquinone using HPLC analysis for the isolate of *Penicillium citrnum* AUMC 14751	-0.957	0.000*	0.956	0.000*

Biodegradation of hydroquinone using HPLC analysis for the isolate of *Penicillium citrnum* AUMC 14751 indicated r-value -0.957 and p-value 0.000* of assay.

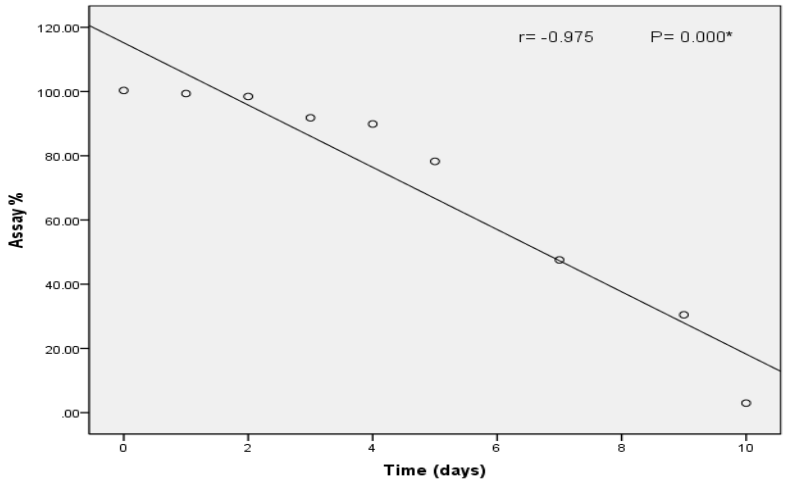


Fig 5: Comparison between the assay of hydroquinone and time (days) for the isolate of (*Penicillium citrnum* AUMC 14751).

Biodegradation of hydroquinone using HPLC analysis for the isolate of *Penicillium citrnum* AUMC 14751 indicated r-value 0.956 and p-value 0.000* of Biodegradation %.

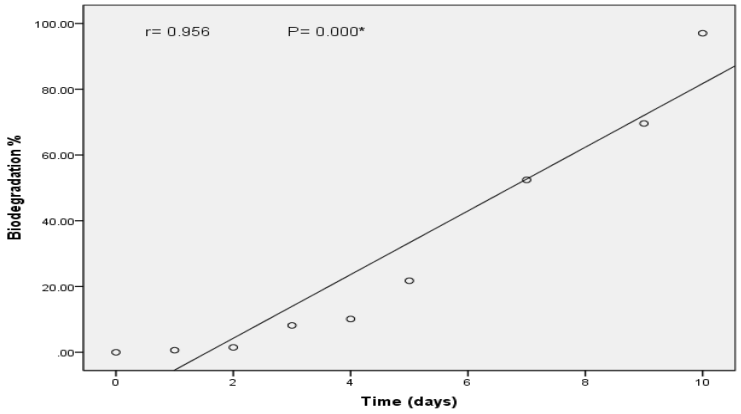


Fig 6: Comparison between the biodegradation of hydroquinone and time (days) for the isolate of (*Penicilliumcitrnum* AUMC 14751).

Conclusion

Hydroquinone compounds are the most common pollutants in pharmaceutical and industrials wastewater and it has been known as health risks to human and aquatic organisms even at low concentrations. Therefore, it is very important to remove Hydroquinone from contaminated water before discharge into any natural water. Enrichment techniques are a powerful way to obtain the promising strains that could utilize Hydroquinone as the sole carbon source. In this study a powerful fungal strain *Penicillium citrnum* AUMC 14751exhibited a high ability for Hydroquinone degradation HPLC results indicated that the biodegradation rate began gradually increased in the presence of (hydroquinone only at the concentration of 500 mg/L) at zero time was 100%. which the results demonstrated the rate of biodegradation of hydroquinone in the sample was 0.63 %, after second day and the maximum percentages of hydroquinone degradation rate (97.02%) was showed after 10 days of the incubation the with optimal conditions.

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الملخص العربي

التحلل البيولوجي لمركب الهيدروكينون من مياه الصرف الدوائي عن طريق فطر بنسليوم سيترانيوم AUMC14751

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أصبح تلوث البيئة بالمركبات العضوية من صنع الإنسان وخصوصاً مياه الصرف الناتجة عن القطاعات الدوائية والصناعية مشكلة رئيسية. فوجود كميات هائلة من المركبات العضوية الصناعية والتي تجد طريقها إلى المسطحات المائية مثل مركبات الهيدروكينون أصبحت من أكثر الملوثات شيوعاً في مياه الصرف الصناعي و الدوائي وقد عُرفت بالمخاطر الصحية على الكائنات الحية البشرية والمائية حتى عند التركيزات المنخفضة فهي شديدة السمية للكائنات المائية عند مستوى تركيز ٤ جزء في المليون لكل لتر .لذا تم اختيار مركب الهيدروكينون ليكون محل الدراسة, حيث أظهر مسح الأنواع الفطرية المعزولة من بيئة تصنيع الهيدروكينون أن ٤٩ عينة من أصل أجمالي ٤٣٢ عينة (١١,٣٪) كانت إيجابية لنمو الفطريات حيث شملت على ثلاثة أجناس من الفطريات هي (أسبرجيلس و بينيسيلوم وفيوزاريوم). تم تقدير معدل تحلل الهيدروكينون عن طريق تقدير الهيدروكينون المتبقي في الوسط الغذائي السائل.حيث تم اختيار ١٥ عزلة فطريه مختلفة أظهرت القدرة على النمو في الوسط الغذائي المضاف إليه الهيدروكينون وتم تعريفها بناءً على خصائصها المورفولوجية في مركز الفطريات بجامعة أسيوط. حيث أظهرت النتائج أن جنس الفطريات الخيطية اسبرجيلس كان الأكثر انتشارًا حيث مثلت نسبة ٤٠٪ من إجمالي جميع العزلات الفطرية ، تليها أنواع البنسليوم بنسبة ٣٣٪ ثم أنواع فيوزاريوم بنسبة ٢٧٪. من بين هذه العزلات الـ ١٥، تم اختيارفطربنسليوم سيترانيومAUMC14751 لتقدير معدل التحلل البيولوجي للهيدروكينون باستخدام تقنية High performance liquid

Chromatography (HPLC). حيث أظهرت النتائج قدرة العزلة الفطرية علي تكسير الهيدروكينون واستخدامه كمصدر وحيد للكربون وكان التحلل يزداد تدريجياً بزيادة فترة التحضين. حيث وصلت نسبة تكسير مركب الهيدروكينون إلي ٠,٦٣٪ بعد اليوم الثاني، في حين وصلت الى اقصى معدل تحلل لمركب الهيدروكينون خلال ١٠ أيام حيث تم تكسير حوالي ٩٧,٠٢٪ من مركب الهيدروكينون بواسطة الفطر.من خلال نتائج هذه الدراسة يمكن التوصية باستخدام سلالة بنسيليوم سترينوم AUMC14751 لإزالة مركب الهيدروكينون من مياه الصرف الصناعي والدوائي في المصانع الصناعية والدوائية كمعالجة بيولوجية ذات تأثير قوي وفعال.