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Biodegradation of phenol by *Rhodococcus phenolicus* isolated from Kitchener wastewater drain, Kafr El-Sheikh, Egypt

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ABSTRACT

Water contamination and pollution present major environmental issues that face our world nowadays. One of the most harmful water pollutants are phenolic compounds, which are abundant in industrial wastewater, and can be accumulated in the water and sediment where aquatic organisms hunt and forage. Therefore, the aim of this study was to isolate an effective phenol-degrading microorganism and employ it in removing phenol from household wastewater. Thirty-one bacterial isolates were obtained from Kitchener wastewater drain, Kafr El-Sheikh, Egypt which is known for its highly industrial wastewater contamination sites able to utilize phenol as a sole carbon source at 1000 mg/L. Microbiological and physicochemical analyses were performed on the wastewater sample from the Kitchener drain. The indigenous phenol-degrading bacterial isolates were examined for their diversity using BOX-PCR fingerprinting and revealed a high genetic diversity. The bacterial isolates were evaluated for their ability to degrade phenol at 1000 mg/L for 11 days using mineral salt medium supplemented with phenol as a sole carbon source, monitoring the changes in their growth density, CFU, and the remaining phenol content after 0, 2, 4, 6, 8 and 11 days. The highest phenol biodegradation rate was recorded by a Gram-positive bacterial isolate, exhibiting 96% degradation ability. The selected isolate was identified using 16S rRNA gene sequence analysis, revealing 99.87% similarity with the species *Rhodococcus phenolicus*. The sequence was deposited in the NCBI GenBank database with accession number PP819386.

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Introduction

Water is essential to all of humanity's activities. The expanding human population results in the creation of tons of wastewater every day in households, agricultural, and industrial sectors (Abdel-Azeem et al. 2007; Ezugbe & Rathilal 2020).

Water contamination is a major problem in environmental degradation in 2022. In river basins and maritime nations, the suffering of fresh water from pollution is a major issue. More than 380 m^3 of wastewater is produced globally in 2022; this number will rise by 24% in 2030 and by 51% in 2050 (Gusti Wibowo et al. 2023).

Many of the Global Sustainable Development Goals SDGs of the UN for 2030 are connected to water and

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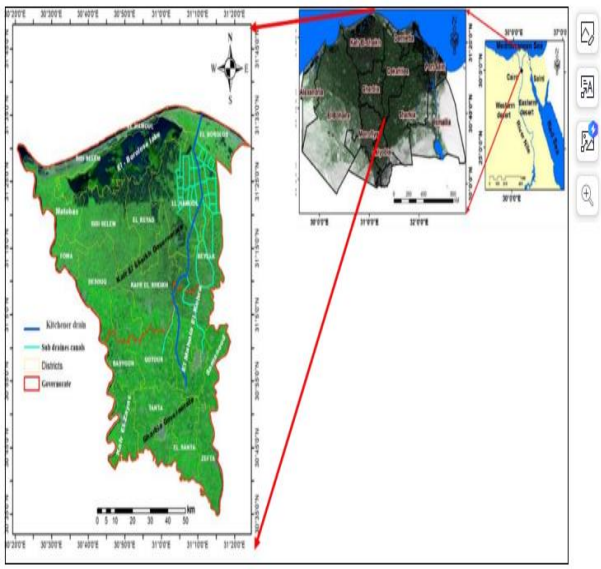
wastewater infrastructure, as well as to the welfare and sustainability of the planet and development. As a result, numerous studies have thoroughly examined SDG number (6), which aspires to guarantee accessibility and sustainable management of water and sanitation for everyone. Targets for SDG number (6) include enhancing freshwater supplies, wastewater treatment, and safe reuse; conserving and restoring water-related ecosystems; boosting water use efficiency; and ensuring freshwater supplies (Delanka-Pedige et al. 2021).

The Kitchener drain, one of Egypt's longest wastewater drains in the Delta region of Egypt, lies in El-Gharbia and Kafr El-Sheikh provinces and is drained into the Mediterranean Sea affecting the marine environment (Gebreil et al. 2018).

Kitchener drain is the longest in Egypt, with a total of 69 Km flows across the three governorates of Dakhalia, Gharbia, and Kafr El-Sheikh in the Delta region (Fig.1) according to Abd-Elfattah et al. (2021). Kafr El-Sheikh Governorate contains 46 km of the Kitchener drain, which empties water into the Mediterranean Sea.

Fig 1. Location map of Kitchener drain in Egypt.



The contamination in the Kitchener drain is among the worst in Egypt. Further to carrying agricultural wastewater, the drain also transports sewage from nearby villages and other regions and industrial effluent from different industrial activities, such as dyes and textiles, pharmaceuticals, petroleum, and pesticides. More than 6 million people, half of whom suffer from a lack of sanitary services, are affected by the drain. Presently, a significant portion of the water from the Kitchener drain is utilized for irrigation purposes in the area, which has a negative impact on the environment, creates health issues, and destroys the agriculture-based economy in the area. The Egyptian ministry of water resources and irrigation is currently most concerned with improving the health and environmental status of people in the governorates of Dakahlia, Gharbia, and Kafr El-Sheikh through large-scale funding projects from the European bank for reconstruction and development with 69 million Euros.

Several distinct harmful substances and elements play a risky role and pose a threat to human, plant, and animal life in the wastewater in the Kitchener drain and other various drains.

As a byproduct of petrochemical facilities, petroleum refineries, and the chemical and pharmaceutical industries (drugs, dyes, and pesticides), phenol and its derivatives are hazardous and carcinogenic pollutants that are frequently released into public wastewater systems (Basak et al. 2019).

Phenol (C_6H_5OH) is a crystalline solid that ranges in color from colorless to light pink and has an unpleasant scent. Exposure to phenol may cause irritation of the skin, eyes, nose, throat, and nervous system. Phenol exposure symptoms include weakness, discomfort in the muscles, weariness, weight loss, and lethargy. Severe exposure can cause liver and/or kidney damage in addition to skin burns, tremors, convulsions, and twitching (Centers for Disease Control and Prevention CDC)**.** According to the US Environmental Protection Agency (EPA), phenol is one of the most dangerous substances and is relatively resistant to natural degradation. It is listed as a priority organic pollutant (Hosseini et al. 2023).

Removal of phenol from aqueous media has been achieved using various methods, some of which are conventional procedures have been used, including chlorination, flocculation, distillation, absorption extraction, and biodegradation. While being widely used, these techniques have some disadvantages, including limited efficiency, the potential for the production of sludge and harmful byproducts, high energy and space needs, and high costs (de Farias et al. 2022)**.**

Thus, it was vital to develop a sustainable and efficient biodegradation technique for the remediation of phenolic compounds from industrial wastewater (Basak et al. 2019). Even at relatively high concentrations, many microorganisms can completely oxidize phenol and use it as a source of carbon and energy producing harmless compounds (Szilveszter et al. 2023)**.** Therefore, the current study focused on the isolation of efficient phenol-degrading bacteria to be used for phenolic compounds bioremediation.

Materials and Methods

Study area and Sampling

Wastewater sample was collected from phenol contaminated site in Kitchener drain in Kafr El-Sheikh, Egypt in 2019, at 30 cm depth, in a sterilized dark glass bottle. The sample was transferred immediately to the lab in an icebox and maintained at 4°C for further analysis.

Isolation of phenol-degrading microorganisms.

Isolation of phenol-degrading microorganisms was conducted by inoculating mineral salts medium (MSM) agar supplemented with different phenol concentrations: 500, 1000, 1500, 2000, 2500 and 3000 mg/L with 1ml of contaminated samples. Samples were diluted by adding 25 ml of the wastewater sample to 225 ml of 0.85% NaCl solution followed by serial decimal dilutions. One ml of each diluted sample was placed in a sterilized MSM petri plate, then incubated at 30 °C for 3-5 days. The obtained colonies were picked up and purified by repeated subculturing on MSM agar supplemented with phenol as a sole carbon source.

To confirm the capability of resulted colonies in phenol degradation, each isolate, obtained from the highest phenol concentration with positive results, was used to inoculate a tube containing 5ml of liquid MSM supplemented with the same phenol concentration and incubated at 30 °C for 3-5 days. After the incubation period, the inoculated and control MSM tubes were examined by visual observation of turbidity.

MSM was composed of (g/L): NH_4NO_3 1.0 g; $(NH_4)_2SO_4$ 0.5 g; NaCl 0.5 g; $MgSO_4 \times 7\ H_2O$ 0.5 g; K_2HPO_4 1.5 g; KH_2PO_4 0.5 g; $CaC1_2$ 0.01g; $FeSO_4 \times 7H_2O$ 0.01g; trace elements 1 ml; final pH 6.9 (Ehrhardt & Rehm 1985), supplemented with phenol as a sole carbon source, which was added to the sterilized MSM agar medium, as the concentrations described above, and cooled to 45°C.

Evaluation of the bacterial isolates for phenol degradation

Isolates, obtained from the confirmation test, were evaluated for their capability for phenol degradation to select the most potent phenol degrading isolate. Isolates were used to inoculate nutrient broth medium and incubated at 30°C for 24 hours. After incubation, the bacterial cultures were centrifuged for 15 min at 6000 × *g*; pellets were washed and re-suspended in sterilized 1 ml MSM culture medium then transferred to 30 ml of MSM culture medium containing phenol (1000 mg/L) as a sole carbon source and adjusted to final optical density of 0.10 (at OD_{600} nm), then incubated at 30°C in a shaking incubator (150 rpm) for 11 days. Phenol degradation (ppm), viable cell count, and bacterial growth (OD at 600 nm) were determined at 0, 2, 4, 6, 8, and 11 Days, as will be described.

Determination of phenol concentration

Phenol concentration was determined in the cell-free supernatants using the Folin-Ciocalteu reagents method (Singleton & Rossi 1965), by mixing 200 µl of each supernatant with 200 µl of Folin-Ciocalteu (10%) reagent in clean 2 ml Eppendorf tube. After allowing the mixture to stand for 5 minutes at room temperature, 800 µl of (10%) sodium carbonate was added, then incubated for 60 minutes at room temperature in dark conditions. At 725 nm, the absorbance was measured, using the phenol standard curve, and the phenol concentration was determined.

Molecular identification of the bacterial isolates

Bacterial isolate showing the highest level of phenol degradation was selected for identification, by molecular method, as described below:

DNA Extraction from the bacterial isolates

Bacterial isolates were grown in Tryptone Soy Broth TSB medium at 30°C for 24-48 hours then the bacterial cells were harvested by centrifugation at 12,000 x *g* for 5 min and washed three times using 0.85% NaCl solution The genomic DNA was extracted from bacterial isolates using Gene JET Genomic DNA purification Kit (Thermo Scientific, Lithuania) (Helal et al. 2022). DNA yields and purity were checked using agarose gel electrophoresis under UV light after staining with ethidium bromide. The concentration of the DNA was checked using NanoDrop spectrophotometer (NanoDrop 2000, Thermo Scientific, Germany). DNA aliquots were stored at -20 °C for further analysis.

Fingerprinting and genotype analysis

The BOX-PCR fingerprinting was performed for the bacterial isolates using BOX A1R primer (CTACGGCAAGGCGACGCTGACG) (Rademaker & De Bruijn 1997) Eight µl from each BOX-PCR product was separated using 1.5% agarose gel electrophoresis in 0.5 X TBE buffer for 4 hours (50 V). The BOX-PCR fingerprint patterns analysis was conducted using the Gel J software v.2.0 (Heras et al. 2015). The cluster analysis was performed using Pearson's correlation coefficients and the unweighted pair group method average (UPGMA) algorithm.

Molecular identification of the most potent isolate

16S rRNA gene sequencing was conducted for the most promising phenol-degrading bacterial isolate that has demonstrated a significant percentage of phenol degradation in the MSM broth compared to the remaining

bacterial isolates. The 16S rRNA gene for the bacterial identification was done using the universal primers F-27 (5′-AGAGTTTGATCMTGGCTCAG-3′) and R1494 (5′-CTACGGYTACCTTGTTACGAC-3′) (Lane D.J. 1991) and (Turner S. et al., 1999) using PCR thermal cycler instrument (Bio-Rad T100 thermal cycler). The PCR products were examined and checked using agarose gel electrophoresis (0.8%) for 45 min (80 v) followed by sequencing by Macrogen-Koria.

Phylogenetic analysis for the most promising phenol-degrading bacterial isolate

The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei 1987). The percentage of replicate trees, in the boot-strap test (1000 repetitions), showed the related taxa clustering together is displayed next to the branches (Felsenstein 1985) In terms of base substitutions per site, the evolutionary distances were calculated using the Maximum Composite Likelihood approach (Tamura et al. 2004) The analysis comprised 8 nucleotide sequences: one from this study and seven were selected to reflect the most similar hits found in the NCBI GenBank. Evolutionary analyses were conducted in MEGA5 (Tamura et al. 2011).

Results and Discussion

Physicochemical characteristics of the sample

The Kitchener industrial wastewater drain in Kafr El-Sheikh, Egypt, which is well-known for being heavily contaminated by industrial wastewater, provided a homogenized sample. To characterize of the traits and circumstances of the sampling source, physical-chemical analysis was performed on the sample, including pH, TDS, COD, nitrate (NO_3^-), phosphate (PO_4^{3-}), sulphate (SO_4^{2-}), nitrogen dioxide (NO_2^-), ammonium ion (NH_4^+), copper (Cu), zinc (Zn), iron (Fe), lead (Pb), and chromium (Cr), in that order, as well as the amount of viable bacteria present. The results of the sample's characteristics are illustrated in Tables (1 and 2)

High levels of mineral nitrate fertilizer can cause nitrate and phosphate to reach water sources. Alteration of other N-reduced forms (ammonia and nitrite) or organic N-substances (amino acids) can also generate it inside water in drains and water networks (Kidd 2011). According to (Ayers RS 1994) standards, all nitrite (NO_3^-) concentrations were under the allowable limit of 0.45 mgL^{-1},.whereas phosphate ($PO4_3^-$) concentration was 4.08 mgL^{-1} (Table 1). Also, nitrate concentration was within permissible limit of 0.06 mg/l according to (Ayers 1994). The main cause of eutrophication in water is high phosphate concentrations. (Addo et al. 2012)These findings concur with those of (Singh et al. 2004) who hypothesized that domestic, municipal, and agricultural waste discharge could be the cause of the high content of phosphate and sulphate in the drain water. TSS and TDS make up total solids (TS). Results show that all total solids concentrations fell under the 506.5 mgL^{-1} allowable range (Federal Republic of Nigeria Official Gazette 2011). Particulate matter suspended in water is measured as total suspended solids, or TSS. The prevalence of fecal coliform is one of the key indicators to evaluate the biological quality of water, along with chemical characteristics like COD and BOD_5 in water samples (Chigor et al. 2012). All levels of total and fecal coliform in El Gharbia Governorate were above the acceptable limit of 10 CFU/ml and < 10 CFU/ml, respectively (Health guidelines for the use of wastewater in agriculture and aquaculture). The high levels of total and fecal coliforms is possibly due the contamination by sewage water (Abakpa et al. 2013), where both *Shigella* and *Salmonella* were present in the sample.

The emphasis on microbial degradation capabilities in recent years has led to the isolation and screening of numerous phenol-degrading bacteria (Gong et al. 2021). Results of screening for phenol-degrading microorganisms showed that out of the 11 x 10^4 CFU/ml total bacterial count, 63.6% and 12.7% were able to degrade 500 and 1000 mg/L of phenol, respectively (Table 2), and out of the 53 isolates, 31 isolates showed the ability to grow in phenol-containing medium.

Isolation of phenol-degrading microorganisms.

Secondly, a total number of 53 colonies were picked up from the highest phenol concentration plates and inoculated, individually, in MSM broth tube supplemented with the same phenol concentration as a sole carbon source and incubated at 30^{o}C for 3-5 days. Out of the 53 isolates, only 31 isolates showed the ability to grow in phenol-containing liquid medium.

Evaluation of bacterial isolates for phenol degradation on concentration of 1000 mg/l

Results, illustrated in figures 2 and 3, show that phenol was degraded after 48 hours of incubation in the 700 mg/L to 1000 mg/L concentrations by all isolates with a positive correlation increase of the growth density at OD_{600} from an initial value of 0.1 to variable values 0.13 - 0.6 and log number of viable cells from 6 to 9 log number (figure 4). Continuous phenol degradation was achieved by only 5 isolates, which are 27, 28, 29, and 30 WKP to reach 327.9, 409.8, 305.3, and 632.9 mg/L, respectively, till the end of the incubation on day 11, however, isolate 31 WKP achieved the maximum phenol degradation, reaching 39.7 mg/L out of 1000 mg/L with 96% phenol degradation and reaching the highest growth (OD_{600} = 1.2)

with noticeable increasing in the log number of the isolate's viable cell from 6.5 to 8.47 on pH 6.9 at 30°C in shaking incubator (150 rpm).

Results of phenol degradation show that the highest degradation was achieved by the bacterial isolate 31 WKP, therefore this isolate was subjected to molecular identification by 16S rRNA sequencing, which showed to be *Rhodococcus phenolics*. Morphological characterization of the selected *Rh. phenolics* showed it is a Gram-positive with pleomorphic forming pale pink colonies when grown on MSM supplemented with phenol and pink colonies on Nutrient agar and TSA media. The early growth phase is characterized by long rods of varying lengths and extensive substrate mycelium on solid media. In the late stages of growth, mycelia split into short rods and coccoid bodies.

Table 1 Physicochemical analysis and metals analysis of Kitchener's drain sample.

Chemical Parameters												
pH	TDS (mg/L)	COD (mg/L)	NO_2^- (mg/L)	NO_3^- (mg/L)	PO_4^{-3} (mg/L)	SO_4^{-2} (mg/L)	NH_4^+	Cu (µg/L)	Zn (µg/L)	Fe (µg/L)	Pb	Cr
6.8	506.5	98.8	0.45	0.06	4.08	543.9	54.1	5	10	280	40	10

Table 2 Microbiological analysis of the Kitchener's drain sample.

Test				Microbial Count (CFU/mL)	
Total viable bacterial count				1.1×10^5	
Total phenol degrading bacteria (CFU/mL, %)					
500 mg/L	000 mg/L	500 mg/L	000 mg/L	500 mg/L	000 mg/L
7×10^3 (63.6%)	$.4 \times 10^3$ 12.7%)	Id	Id	Id	Id

The capability of *Rhodococcus* genus to degrade the phenol is due to its physiological metabolic activity with different biodegradation enzymes such as hydroxylase, 1,2-dioxygenase, 2,3-dioxygenase, and protocatechuate 3,4-dioxygenase (Torres et al. 2022).

There has been an increase in research pertaining to the bioremediation applications of the genus *Rhodococcus* that 6452 patent were granted from 1978 to July 2022 (https://worldwide.espacenet.com). In Scopus database (https://www.Scopus.com/home.uri) 48 articles covering the period from 2000 to July 2022 were published on bioremediation, biodegradation, bioaugmentation, biosorption, or decolorization by the genus *Rhodococcus*. (Torres et al. 2022) **.**

Previous study revealed that the highest phenol degradation of 96% in 11 days was achieved by *R. phenolics*, while taking into consideration the continuous increase in the growth density and viable cells which count of the *R. phenolics* led the surpass for the *R. phenolics* compared to the other isolates.

R. phenolics recorded a positive correlation between phenol degradation capability and viable cell count (figure 5), showing a noticeable increase in both cell density OD_{600} from 0.1 to 0.4 and log of the viable cell count increase from log 6.5 to 7.7 CFU/ ml with degradation of phenol at 300 mg/L concentration in 48 hours, and in the second 48 hours the concentration reached 88 mg/L with a slight increase in both cell density at OD_{600} to be 0.5 and viable cell count to log 7.73 CFU/ml, At the 3rd 48 hours, the noticeable increase in the phenol degradation, that phenol concentration reached 167 mg/L with a positive increase in both cell density OD_{600} to be 0.8 and log of the viable cell count to be log 8.24 CFU/ml. After 48 hours the *R. phenolics* recorded closely the same phenol degradation as the previous determination to be 169.8 mg/L and with increasing in both cell density at OD_{600} and log of the viable cell count reached log 1.2 and 8.33 respectively. The highest phenol degradation was recorded after 72 hours of incubation by the *R. phenolics* to reach 39.7 mg/L out of the initial phenol concentration1000 mg/L with a constant cell density at OD_{600} 1.2 and a slight increase of the log of viable cell count 8.47 CFU/ml.

R. phenolics is considered as a novel species of the genus *Rhodococcus* and this was reported in the few published research papers in the Scopus database (https://www.scopus.com/home.uri), which demonstrates the strain's ability to degrade not only phenol and phenolic compounds but also different types of environmental pollutants and xenobiotics (Rehfuss & Urban 2020; Nogina et al. 2020; Torres et al. 2022).

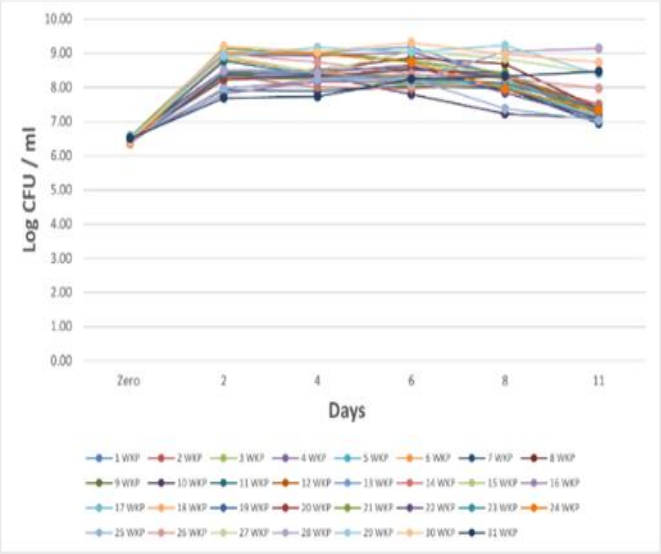


Fig 2. Phenol degradation by the 31 tested bacterial isolates at 1000 mg/L solution over 11 days incubation.

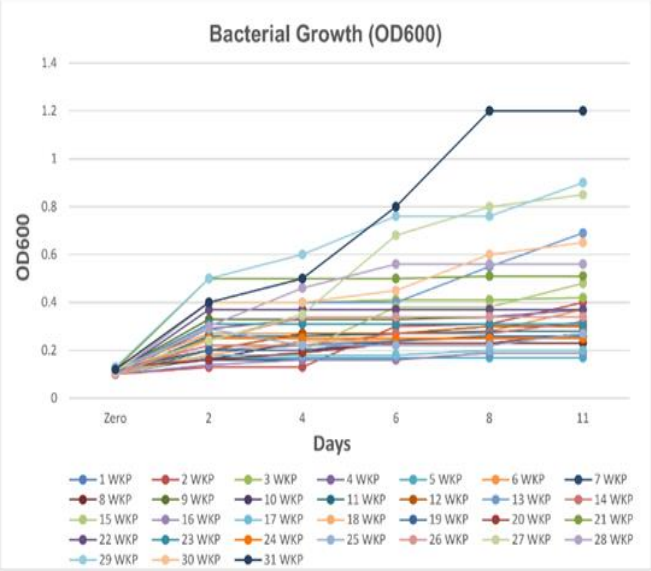


Fig 3. Optical Density OD_{600} of the 31 bacterial isolates growth in MSM medium supplemented with 1000 mg/L phenol as a sole carbon source in 11 days.

Bacterial Fingerprinting and genotypic analysis

BOX-PCR fingerprints were achieved on 29 out of 31 bacterial isolates (figure 6) obtained from the wastewater-contaminated sample. The fingerprint patterns revealed a high genetic diversity among tested isolates 7 clusters and 7 distinct fingerprint patterns were formed at 80% cutoff level.

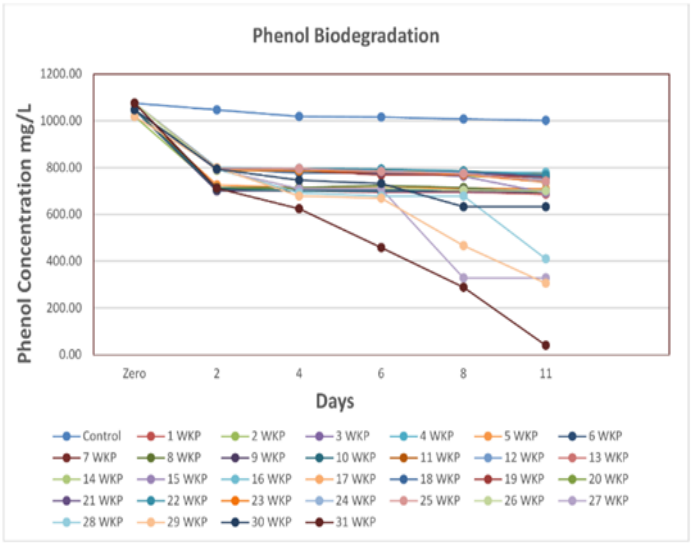


Fig 4. Growth of the 31 bacterial isolates in MSM medium at 30°C with the phenol degradation incubation time.

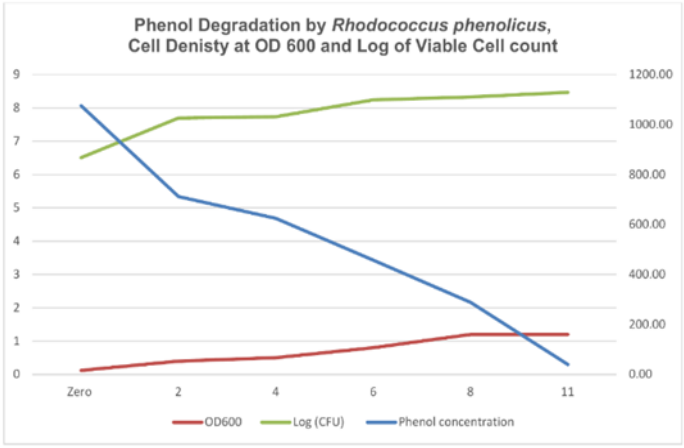
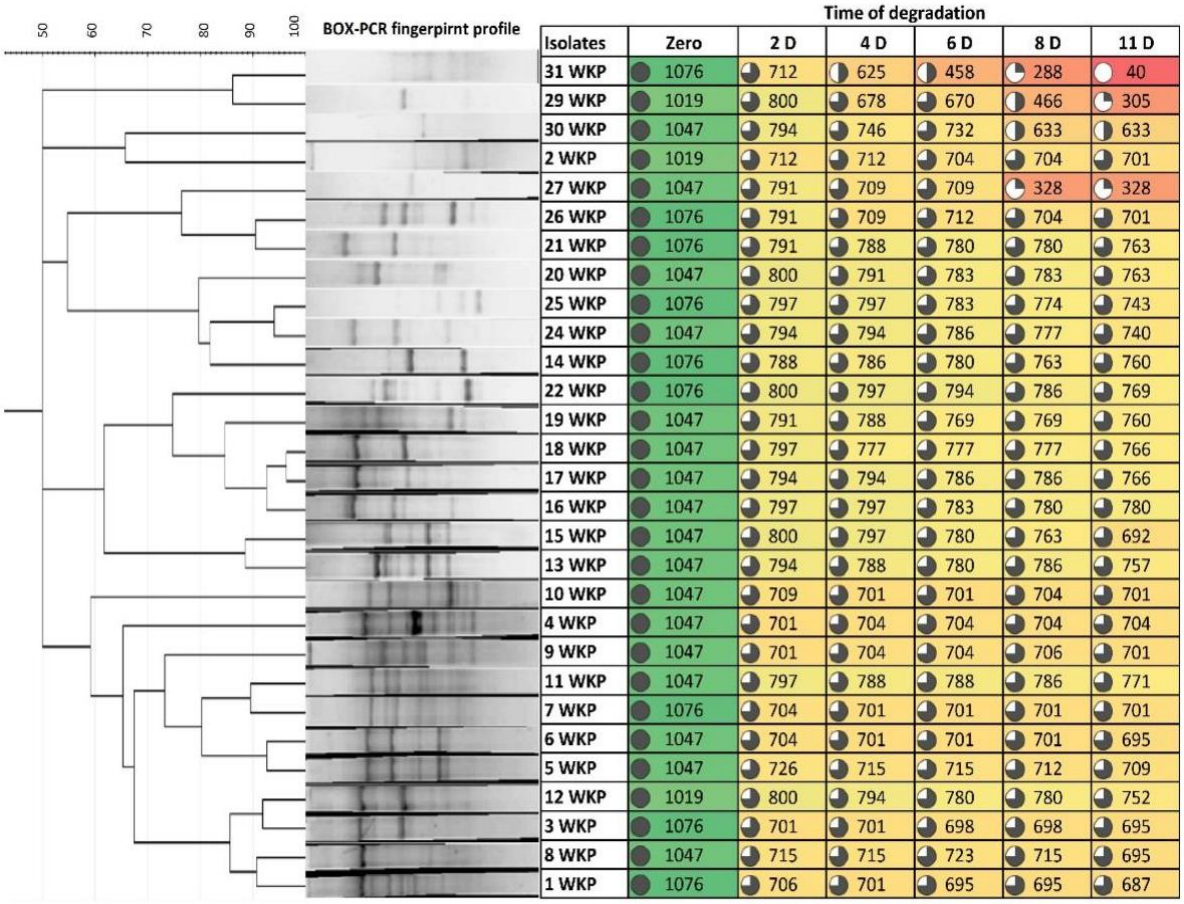


Fig. 5 Phenol degradation by *Rhodococcus phenolicus* 31 WKP, Cell Density at OD_{600} and viable cell count.

Characterization of the most potent bacterial isolate by 16S rRNA and phylogenetic analysis.

The 16S rRNA gene sequence analysis was performed on isolate 31 WKP, which possessed the highest phenol degradation ability. The 16S rRNA gene sequence showed 99.87% similarity to the *Rhodococcus phenolicus* (figure 7). Sequence was deposited in the NCBI GenBank database under the accession number PP819386.



Isolates	Time of degradation					
	Zero	2 D	4 D	6 D	8 D	11 D
31 WKP	1076	712	625	458	288	40
29 WKP	1019	800	678	670	466	305
30 WKP	1047	794	746	732	633	633
2 WKP	1019	712	712	704	704	701
27 WKP	1047	791	709	709	328	328
26 WKP	1076	791	709	712	704	701
21 WKP	1076	791	788	780	780	763
20 WKP	1047	800	791	783	783	763
25 WKP	1076	797	797	783	774	743
24 WKP	1047	794	794	786	777	740
14 WKP	1076	788	786	780	763	760
22 WKP	1076	800	797	794	786	769
19 WKP	1047	791	788	769	769	760
18 WKP	1047	797	777	777	777	766
17 WKP	1047	794	794	786	786	766
16 WKP	1047	797	797	783	780	780
15 WKP	1047	800	797	780	763	692
13 WKP	1047	794	788	780	786	757
10 WKP	1047	709	701	701	704	701
4 WKP	1047	701	704	704	704	704
9 WKP	1047	701	704	704	706	701
11 WKP	1047	797	788	788	786	771
7 WKP	1076	704	701	701	701	701
6 WKP	1047	704	701	701	701	695
5 WKP	1047	726	715	715	712	709
12 WKP	1019	800	794	780	780	752
3 WKP	1076	701	701	698	698	695
8 WKP	1047	715	715	723	715	695
1 WKP	1076	706	701	695	695	687

Fig 6. BOX-PCR fingerprints for the phenol-degrading bacterial isolates from Kitchener drainage wastewater.

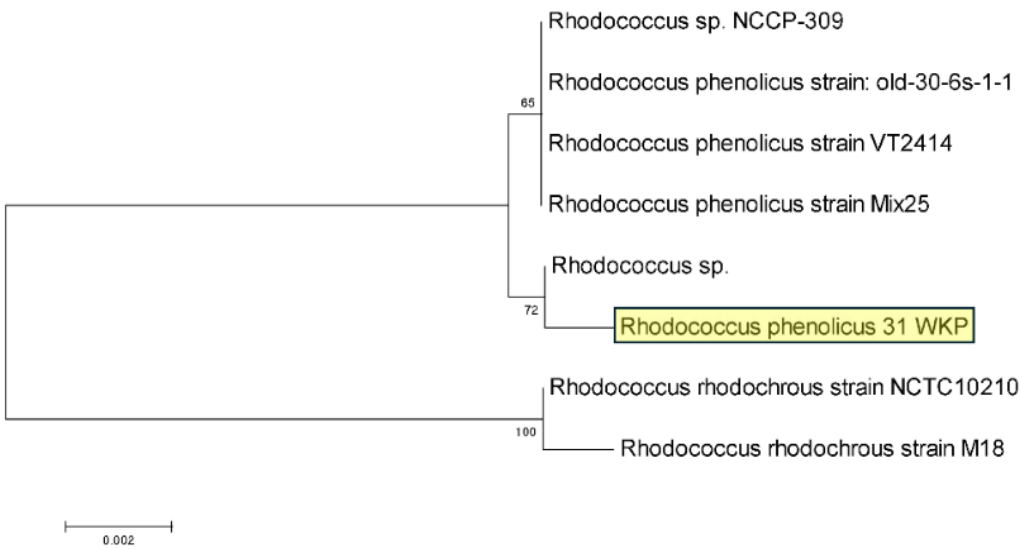


Fig 7. A neighbor-joining phylogenetic tree based on 16S rRNA gene sequences. Yellow highlight represents bacterial isolate obtained in this study, and bootstrap values are indicated at each node.

Conclusion and future perspectives

In conclusion, this study successfully isolated and identified a highly efficient phenol-degrading bacterium, Rhodococcus phenolicus, from the Kitchener wastewater drain in Kafr El-Sheikh, Egypt. The isolate demonstrated remarkable phenol degradation efficiency, achieving 96% removal of phenol at a concentration of 1000 mg/L over 11 days. This highlights its potential as a promising candidate for bioremediation of phenol-contaminated wastewater, particularly in industrial settings. The use of indigenous microorganisms for phenol degradation offers an eco-friendly and sustainable approach to mitigating water pollution caused by phenolic compounds. Furthermore, the high genetic diversity observed among the bacterial isolates underscores the potential for discovering additional strains with unique degradation capabilities.

Looking ahead, future research should focus on optimizing the conditions for large-scale application of Rhodococcus phenolicus in wastewater treatment systems, including factors such as pH, temperature, and nutrient availability. Additionally, exploring the synergistic effects of microbial consortia, combining Rhodococcus phenolicus with other phenol-degrading microorganisms, could enhance degradation efficiency and broaden the range of treatable pollutants. Genetic engineering and metabolic pathway studies could further improve the strain's degradation capacity and stability under varying environmental conditions. Finally, integrating this bioremediation approach with advanced treatment technologies, such as membrane filtration or photocatalytic degradation, could provide a comprehensive solution for addressing complex wastewater contaminants. By advancing these strategies, the findings of this study can contribute significantly to global efforts in combating water pollution and promoting environmental sustainability.

Conflict of interest

The authors declare that they have no conflict of interest.

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