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Plant Growth-Promoting *Rhizobium Nepotum* Phenol Utilization: Characterization and Kinetics

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Abstract: Phenol is a severe pollutant that harms the environment and, potentially, human health. This study aimed to investigate the biodegradability of phenol by the plant growth-promoting bacterium R. nepotum. That included studying the growth kinetics and the effects of growth conditions such as incubation temperature, pH, and the use of different substrate concentrations. As the primary substrate, six different starting concentrations of phenol were utilized. The ability of these cells to biodegrade phenol was greatly influenced by the culture conditions. After 36 and 96 hours of incubation at pH 7 and a temperature of 28 C, this organism grew the fastest and had the highest phenol biodegradation. The biodegradation rate is much higher at 700 mg/L, the highest of the six concentrations tried. In less than 96 hours of incubation, more than 90% of the phenol (700 mg/L) had been eliminated. The Haldane model has been the most accurate for determining the relationship between the initial concentration of phenol and growth rate. In contrast, the refined Gompertz model provided the most accurate depiction of phenol biodegradation over time. As predicted by the Haldane equation, the highest specific growth rate, half-saturation coefficient, and Haldane's growth kinetics inhibition coefficient are 0.7161 h1, 15.8 parts per million (ppm), and 292 parts per million (ppm), respectively. The equation of Haldane successfully fitted the experimental data by reducing the SSR (sum of squared errors) to 3.8x10 ^{3}. According to the results of the analysis by GC-MS for the bacterial culture sample, the hydroxylase enzyme was the first to convert the phenol molecule into catechol. The catechol was subsequently broken down into 2-hydroxymucconic semialdehyde by the 2,3-dioxygenase enzyme, which occurred through the meta-pathway. It is the first study showing that R. nepotum, a plant growth promoter, has high efficiency of phenol. In phenol-stressed conditions, this could help with rhizoremediation and crop yield preservation.

Keywords: *Rhizobium nepotum*, phenol, biodegradation, kinetics, plant growth-promoting bacteria.

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植物促生根瘤菌苯酚利用：表征和动力学

摘要：苯酚是一种严重的污染物，会危害环境，并可能危害人类健康。本研究旨在研究植物促生菌根瘤菌对苯酚的生物降解性。这包括研究生长动力学和生长条件的影响，例如孵化温度、酸碱度和不同底物浓度的使用。作为主要底物，使用了六种不同起始浓度的苯酚。这些细胞生物降解苯酚的能力很大程度上受培养条件的影响。在酸碱度7和28摄氏度的温度下孵育36和96小时后，该生物体生长最快，苯酚生物降解率最高。700毫克/升的生物降解率要高得多，是所尝试的六种浓度中的最高浓度。在不到96小时的孵育时间内，已消除了90%以上的苯酚(700毫克/升)。霍尔丹模型对于确定苯酚的初始浓度和生长速率之间的关系是最准确的。相比之下，改进的冈佩尔茨模型提供了随着时间的推移苯酚生物降解的最准确描述。正如霍尔丹方程预测的那样，最高的比生长率、半饱和系数和霍尔丹的生长动力学抑制系数分别为0.7161h1、15.8百万分之几和292百万分之几。霍尔丹方程成功地拟合了实验数据，将误差平方和降低到3.8x103。根据细菌培养样品的气相色谱-
质谱分析结果，羟化酶是第一个转化苯酚的酶。分子变成儿茶酚。儿茶酚随后被2,3-
双加氧酶分解为2-
羟基粘液酸半醛，该酶通过元通路发生。这是第一项表明植物生长促进剂根瘤菌具有高效苯酚的研究。在苯酚胁迫条件下，这有助于根际修复和作物产量保持。

关键词：根瘤菌, 苯酚, 生物降解, 动力学, 植物生长促进细菌。

1. Introduction

The utilization of chemical substances, particularly organic ones, like carbon or energy sources by living cells such as microbes, is a vital characteristic of all life forms. The most widely accepted narrative is that cells evolved to accept such polluting natural compounds. Many such compounds were caused by human intervention, resulting in environmental problems due to the resistance of mentioned microorganisms or their reluctance to mineralize [1, 2]. Even at relatively low amounts, phenol causes problems in the water supply, such as odor and taste. Furthermore, it is hazardous to various aquatic creatures at concentrations as low as mg/liter [3, 4]. As a result of their potential toxicity, phenol and its derivatives have been considered one of the priority pollutants maintained by the US Environmental Protection Agency (EPA) [5]. In addition to being carcinogenic, phenol can cause mortality when inhaled or absorbed through the skin.

Different strategies for phenol degradation, including physical or chemical methods, have significant disadvantages, including the requirement for considerable effort, high expense, and the development of unanticipated hazardous byproducts [3, 6]. Bacteria play a critical role in biodegradation via specialized enzymes called biocatalysts. Biocatalysts catalyze biological reactions without using chemically derived catalysts [7, 8]. These enzyme qualities facilitate their usage and modification for industrial uses, such as stabilizing enzymes across a temperature range, pH values, and various difficult reaction circumstances. Numerous enzymes have been addressed in various areas, including medicine, biology, and industrial applications such as livestock feed, biotransformation, textiles, food, and leather [9–17].

For decades, agricultural crop production has been resurrected using microorganisms promoting plant growth [18, 19]. Improved nutritional bioavailability and biosorption, decreased soil plant-bacteria via pathogenic factor control, the construction of materials that endorse plant growth, and the minimization of pollutants from the soil, such as dangerous compounds that can adversely impact plant growth benefits that these microbes provide [20]. Plant growth-promoting microorganisms, which can be employed instead of herbicides, and genetically altered plants are examples of such bacteria. Organic and inorganic fertilizers and pesticides for genetically altered plants and pests are produced by plant growth-promoting bacteria (PGPB). In addition, PGPB could help soil organisms cope with the effects of severe environmental stresses.

Desiccation and high concentrations of salts and heavy metals are examples of these adverse conditions. As a result, PGPB likely acts as a stimulant for regenerating agricultural fields that were previously unfit for fodder or nutrition farming.

The Al-Ghweiler Agricultural Station in Karak, Jordan, was used to isolate *R. nepotum*. It was detected using 16S rRNA techniques, and JOR18 MN083292 was allocated as the nucleotide entry number in Genbank. A motile gram-negative rod does not form spores [21]. It was discovered to be capable of phosphate solubility and nitrogen fixation. For the first time, this study looked at *R. nepotum's* capacity to degrade phenol under various growth conditions, including temperature, pH, and substrate concentration. The kinetics of *R. nepotum* growth on phenol biodegradation were also investigated using mathematical modeling.

2. Materials and Methods

2.1. Bacterial Strain

The Al-Ghweiler Agricultural Station in Al-Karak, Jordan, provided the *R. nepotum* strain used in this study. The identification of *R. nepotum* was made using 16S rRNA techniques (SUPREME laboratories, ENEA-Casaccia, Rome, Italy) and a nucleotide accession number in the Genbank given as (JOR18 MN083292). The strain was deposited in the MIRRI-It ENEA microbial collection in Italy.

2.2. Growth Culture Media

A phenol-free mineral medium was created. Three different solutions were first prepared. The first step was to make a 25,000 ppm phenol solution in deionized water filtering sterilized. The mineral medium was made by combining 1 gram K2HPO4, 1 gram NH4NO3, 0.5 gram (NH4)2SO4, 0.5 gram MgSO4, 0.5 gram KH2PO4, 0.5 gram NaCl, 0.02 gram CaCl2, and 0.02 gram FeSO4 in 1 liter deionized water. Before autoclaving, the correct volume of phenol was transferred to 125 mL flasks to be changed later. The third solution was Wolfe's mineral solution, which mixed 1.5 g of nitrilotriacetic acid with 500 mL of deionized water. The following ingredients were added after adjusting the pH to 6.5 using KOH: 100 mg $ZnSO_4$.7H2O, 0.01 g, 10 mg H3BO3, 10 mg $CuSO_4$.5H2O, 100 mg $ZnSO_4$.7H2O, 10 mg AlK $(SO_4)_2$. 100 mg $CoCl_2$.6H2O, 100 mg CaCl2, 10 mg $CuSO_4$.5H2O, 10 mg AlK $(SO_4)_2$.12H_2O, and 10 mg $FeSO_4$.7H2O. Then 500 mL of deionized water was added, bringing the total amount to 1 liter. The prepared Wolfe's mineral solution was sterilized using filtration to avoid thermal deterioration. 0.5 mL of Wolfe's solution was mixed with various amounts of phenol to make the mineral media with phenol. For example, to make 200 ppm of phenol, combine 0.4 mL phenol solution with 0.5 mL Wolfe's mineral solution, then add this mixture (0.9 mL) to 49.1 mL mineral medium. As a result, mineral media containing phenol concentrations of 200, 400, 700, 800, 1000, and 1200 ppm were created.

2.3. Plant Growth-Promoting Characteristics

Salkowski's reagent was used to indicate the ability of *R. nepotum* to convert L-tryptophan to indole-3-acetic acid (IAA), which can be detected by the formation of a pink or red color [22]. The product, IAA, is a carboxylic acid member of the auxin family. Auxins are well-known to promote branching and root growth in plants. Phosphate solubilization was determined by cultivating *R. nepotum* in Pikovskaya (PKO) medium at 30 ° C for seven days and monitoring it regularly for 7 days. A transparent halo enveloping the colonies clearly shows their ability to solubilize phosphate. Chrome azurol S (CAS) agar plates were used to detect the ability of *R. nepotum* to produce siderophore, which was indicated by the change in color from blue to orange [23]. Finally, the NFb medium was used to test *R. nepotum*'s ability to fix nitrogen (semi-solid New Fabian broth). *R. nepotum* was grown for 24 hours in Trypticase Soy Broth (TSB), washed twice, and resuspended in phosphate-buffered saline (PBS) to achieve a 600 nm absorbance of 0.5. The prepared culture was then inoculated into 4 mL of NFb medium and incubated at 28°C for 72 hours [24]. The sign of growth as a sub-surface pellicle on NFb medium indicates the ability of *R. nepotum* to fix nitrogen.

2.4. *R. Nepotum* Growth and the Biodegradation of Phenol

Mineral media containing 700 ppm of phenol were used to evaluate *R. nepotum*'s ability to utilize phenol as a single carbon source. First, *R. nepotum* grown to the middle of the log phase was centrifuged (4000 rpm for 15 min), washed, and resuspended in mineral media to reach an absorbance of 0.2 at 600 nm. Then the cultured bacteria were inoculated in a mineral medium containing 700 ppm phenol. The growth of the phenol-treated *R. nepotum* was monitored every 12 h for 96 h using a spectrophotometer at 600 nm. In parallel, uninoculated mineral media containing 700 ppm was processed as a control.

2.5. Phenol Assay Procedure

The phenol concentration in the treated phenol media cultured with *R. nepotum* was determined using the 4-amino-antipyrine colorimetric method [25]. Ammonium hydroxide (0.5 N), 2% w/v 4-amino-antipyrine, and 8% w/v potassium ferricyanide were used to make the reaction mixture. After 15 min of

incubation at room temperature, the OD of the mixture was measured using a spectrophotometer at 510 nm. The linear equation of the phenol standard curve was used to calculate the phenol concentration. Because many cells either stopped or showed no further degradation during this time or replicated the elapsed time during which all experiments were conducted, the concentration of residual phenol converted during the first 24 hours was used.

Calculating the average biodegradation of phenol, as proposed by Loh and Wang [26], avoids inaccuracies caused by widely differing lag stage lengths while determining the critical time required for complete biodegradation or when biodegradation is paused [27].

2.6. Effect of Temperature and pH on the Biodegradation of Phenol

R. nepotum grown in a mineral medium containing 700 ppm phenol was used in these tests. The pH effect on the phenol degradation was monitored at pH values of 5.5, 7, 8, and 9. The effect of the temperature was evaluated at temperatures of 25, 28, 33, and 37C.

2.7. GC-MS Analysis

Phenol and phenol derivatives concentrations in the cultured media were characterized using GCMS. An amount of 30 ml of each cultured media after 96h was collected and mixed with a stabilizer (4.5 ml of 10% CuSO4). After pH adjustment, the samples were extracted using dichloromethane. A rotary evaporator was used to remove the solvent, and anhydrous Na2SO4 was used to over-dry the sample. The crude extract was suspended in 5 mL methanol and subjected to GCMS analysis using a Varian Chrompack CP-3800 GC-MS-200 (Saturn) with a DP-5 column (30 m 0.25 mm i.d., 0.25 m film thicknesses). The mobile phase was helium gas at a flow rate of 1mL per minute. The MS source temperature was set to 180 °C, and the ionization voltage was 70 eV. After being held at 60 °C for 1 minute, the column temperature was gradually increased to 270 °C at a rate of 3 °C/min (isothermal). Under similar conditions, hydrocarbon standards (C8-C20) were evaluated.

Prior publications [28, 29] were used to identify the GCMS spectra, which were identified by matching their retention time and mass spectra to those in the National Institute of Standards and Technology (NIST)/Wiley collection, or by deducing the RTs using legitimate standards.

2.8. HPLC Analysis

An HPLC/UV-Vis detector (Shimadzu, LC-10A, Tokyo, Japan) and a Luna C18 column were used in this experiment (4.6 250 mm, 5 m, 100Ao). In a temperature-controlled column compartment, the phenol compound concentration was tested at 30°C. The mobile phase was made up of 0.1 percent acetic acid (solvent A) and 1:1 v/v percent (acetonitrile/methanol) (solvent B), with the following settings: 0.1-1 minute, 95 percent A; 1-6 minutes, 50 percent A; 6-10 minutes, 5 percent A. A UV detector with a volume of 10 liters and a flow rate of 1.0 mL/min was used (280 nm). Samples of culture media containing 700 ppm phenol were examined at various time intervals to validate the biodegradation of phenol (0, 12, 24, 48, 72, and 96 hr).

2.9. Analytical Quality Assurance and Validation Method

Four injections of phenol compound at five different concentrations (50, 250, 500, 1000, and 1500 mg/L) were used to determine linearity. The R2 value of 0.996 for phenol demonstrates good linearity.

The limits of detection (LOD) and quantitation were determined by diluting the standard solution until the signal-to-noise ratio (S/N) equaled three and ten, respectively (LOQ). The LOD and LOQ levels were 2.45 and 8.17 mg/L, respectively. A relative standard deviation (RSD) of 2.7 indicates that a good level of precision was obtained. Next, blank samples were spiked with varying quantities of standard phenol solution (50, 500, and 1500 mg/L) for the recovery test. All phenol compound recovery rates ranged between 83 and 106 percent at various spiking levels. These results are within the acceptable range of 70–120 percent [30].

2.10. Mathematical Modeling

The logistic equation is commonly used to describe the exponential and stationary stages of biomass growth in a batch system. The logistic equation is written in differential form as follows:

$$\frac{dX}{dt} = \mu_m X\left(1 - \frac{X}{X_m}\right)$$

where μ_m is the most significant specific growth rate (hr-1) feasible in a particular environment, and X_m is the higher cell concentration achievable in that environment (OD600).

The following formula for cell concentration is obtained by integrating the logistic equation:

$$X = \frac{X_o e^{\mu_m t}}{1 - \left(\frac{X_o}{X_m}\right)(1 - e^{\mu_m t})}$$

where X_o is expressed as OD600 (starting inoculum). Numerous kinetics models have been established to define the connection between substrate concentration S and specific growth rate μ .

When the substrate is available in high quantities and growth is controlled by inhibitor concentration, the Haldane equation has been the most commonly used

inhibitory expression:

$$\mu = \frac{\mu_{\max} S}{K_s + S + \frac{S^2}{K_I}}$$

where μ_{maxS} is the highest rate of Haldane's specific growth (hr^{-1}), K_I is the inhibition coefficient of Haldane (ppm).

The growth patterns of investigational biomass at varying primary concentrations of phenol were suited to the logistic equation utilizing the approach of non-linear regression. The used Solver add-in of Microsoft Excel 2007 was applied to establish the fitted model for parameters via lessening the SSR (sum of squared error).

The logistic model parameters and experimental values for varied initial phenol concentrations are shown in Fig. 1.

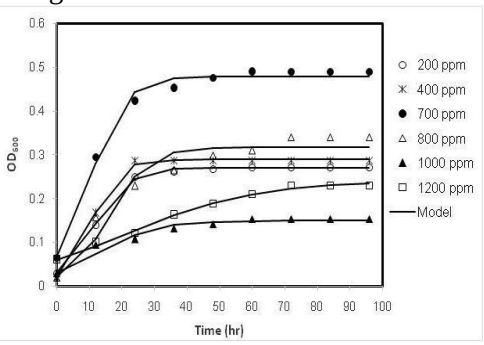

Fig. 1 The effect of different concentrations of phenol on the growth of *R. nepotum*

3. Results and Discussions

3.1. PGPB Characterization

R. nepotum was examined for its capability to solubilize phosphate, synthesize indole-acetic acid, fix nitrogen, and produce siderophore. As shown in table 1, *R. nepotum* is a nitrogen fixative bacteria and an indole-acetic acid producer. However, it is neither a phosphate solubilizer nor a siderophore producer.

The enzyme-nitrogenase complex, the formation of which is dependent on the nif gene, regulates the nitrogen fixation process. Bacterial growth was measured in NFCC-medium with a 0.5 percent bromothymol blue indicator [31]. Positive reactions, with the indicator turning pink or red, are used to identify isolates able to generate indole-3-acetic acid (IAA). Salkowski's reagent produces a pink compound that reacts with IAA, demonstrating the bacterial ability to convert L-tryptophan to IAA or other derivatives [22].

3.2. Growth Rates of *R. Nepotum* and Biodegradation of Phenol

In the current investigation, the phenol-degrading bacterium *R. nepotum* was used to biodegrade phenol. A 16S rDNA analysis was used to identify this bacterium.

Table 1 Nitrogen fixation, Siderophore production, Phosphate solubilization, and Auxin production of *R. nepotum*

Phylogenetic affiliation with similarity (16S rDNA sequencing)	Genbank Accession number	Plant Growth Promoting traits			
		Nitrogen fixation	Siderophore production	Phosphate solubilization	Auxin production
Rhizobium nepotum 99%	JOR18 MN083292	+	–	–	++

A minimum medium containing phenol was used as a carbon, and an energy source was used to verify that phenol was used. If organisms grow and biomass is formed, it is almost certainly a result of these substrates being consumed [3, 4, 26]. To determine the biodegradability of phenol, uninoculated phenol-containing minimal media broth (MMB) and inoculated heat-killed phenol-containing culture broth were used as negative controls. There was no evidence of phenol biodegradation, indicating that the presence of R. nepotum caused the biodegradation of phenol. Clearly, *R. nepotum* can utilize high phenol concentrations as a significant carbon and energy source (Table 2).

Table 2 *R. nepotum* biodegradation rate in different concentrations of phenol

Concentration (ppm)	Biodegradation rate (ppm/h)
200	4.15
400	6.25
700	8.33
800	8.25
1000	8.3
1200	8.2

3.3. Phenol Biodegradation Growth Kinetics of an *R. Nepotum*

As the primary substrate, six different amounts of phenol were utilized (Fig. 1).

With the non-linear regression technique, the *R. nepotum* growth biomass at various phenol concentrations was adapted to the logistic equation. For determining the model fitting parameters, the Solver add-in in Microsoft Excel 2007 was used to minimize the sum of squared error (SSR). Fig. 1 depicts the logistic regression profiles and experimental results at various phenol starting concentrations. The *R. nepotum* growth biomass reached its maximum stationary population size at a starting phenol concentration of 700 ppm. At an initial phenol level of 400 ppm, the biomass, on the other hand, grew at its fastest pace. Slow growth rates and small biomass size were reported with a more significant phenol dose (1200 ppm) (Table 3).

Table 3 Logistic growth kinetic parameters for phenol biodegradation by *R. nepotum*

Phenol Concentration (ppm)	μ_m μ_m (hr^{-1})	X_m X_m (OD_{600})	SSR
200	0.182	0.270	8.51×10^{-5}
400	0.244	0.289	8.46×10^{-5}
700	0.182	0.480	1.68×10^{-3}
800	0.168	0.316	6.75×10^{-3}
1000	0.111	0.150	6.78×10^{-4}
1200	0.051	0.240	3.00×10^{-5}

By graphing the biomass increase throughout the exponential phase, the specific growth rate (μ) for R. nepotum treated with different phenol concentrations was estimated. The particular growth rate is represented by the slope of the plot versus time.

$\frac{\ln X}{X_0}$ $\frac{\ln X}{X_0}$

The Haldane equation provided a compelling depiction of the relationship between the phenol concentration and the growth rate (Fig. 2).

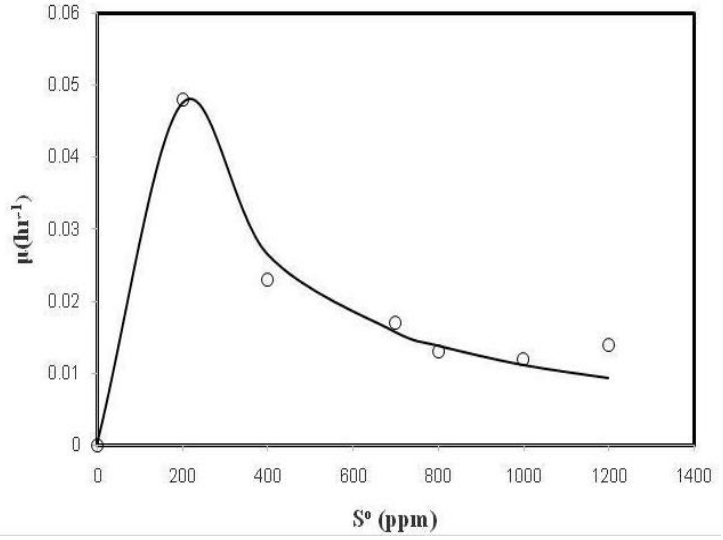

Fig. 2 Fitting of *R. nepotum* growth in the batch culture using the Haldane model

A non-linear regression technique based on SSR reduction was necessary to estimate Haldane's parameters. According to the Haldane equation, Haldane's growth kinetics inhibitory coefficient, half-saturation coefficient, and the highest specific growth rate were 292 ppm, 15.8 ppm, and 0.716 h1, respectively (Table 4).

These experimental data fit the Haldane equation with an SSR of 3.8 x10-3. Fig. 2 depicts a typical growth kinetics trend for an inhibiting substrate.

The *R. nepotum* growth rate increases linearly with increasing phenol concentration up to approximately 200 ppm, at which point it declines as the phenol concentration increases. The maximal specific growth rate recorded by Haldane in this investigation is close to values available in the published reports for phenol microbial degradation in various bacterial mediums.

The half-saturation coefficient (11 ppm) of the Haldane model says that phenol concentrations must constantly stay low to achieve a specific growth rate equal to half the maximum specific growth rate.

Table 4 Haldane's model parameters for the biodegradation of phenol by different bacteria

Microbial Strain	S_o S_o (ppm)	μ_{max} μ_{max} (hr-1)	K_s K_s (ppm)	K_I K_I (ppm)	Reference
Rhizobium nepotum	200-1200	0.716	15.8	292	This study
Pseudomonas putida	300-1000	0.031	63.9	450	[21]
Pseudomonas sp.	100-800	0.464	113.5	376.7	[32]
Mixed	0-800	0.26	25.4	173	[33]
Acinetobacter calcoaceticus	60-500	0.542	36.2	145	[34]
Pseudomonas WUST-C1	0-1600	2.5	48.7	100.6	[35]
Pseudomonas putida	25-800	0.900	6.93	284.3	[26]

The biomass response to inhibitory substances is measured by the inhibition coefficient of Haldane's growth kinetics. The value of the inhibitory constant (121 ppm) shows that phenol effectively suppresses biomass growth. The biodegradation of phenol was simulated using the updated Gombertz model [14].

$$S = S_o\left\{1 - exp\left\{-exp\left[\frac{R_m}{S_o}e(\lambda - t) + 1\right]\right\}\right\}$$

The modified Gombertz model [36] fits phenol biodegradation profiles reasonably well. The model's fitting parameters are listed in Table 5.

Table 5 Parameters using the Gompertz model on the different concentrations of phenol substrate

Phenol Concentration (ppm)	R_m R_m (mg/L.hr)	λ λ (hr)
200	6.5	7.2
400	6.3	7.1
700	10.3	5.9
800	7.7	11.4
1000	8.9	14.6
1200	8.3	3.8

At lower initial phenol concentrations, the phenol degradation rate increases as the concentration increases. The highest phenol biodegradation rate (10.30 ppm/h) was achieved at an initial phenol concentration of 700 ppm. The rate of phenol biodegradation, on the other hand, tends to be stable at more significant initial phenol concentrations. As a result of the growth profile and percent of phenol removal, the 700 ppm was chosen for further testing (Fig. 1 and 3).

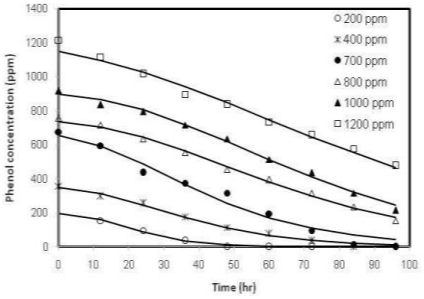

Fig. 3 modified Gompertz model fitted to the growth of *R. nepotum* at different phenol concentrations (mg/mL)

3.4. Effect of Temperature

Numerous physiological factors can occasionally affect bacteria's ability to biodegrade phenol. These parameters include phenol toxicity, pH, temperature, carbon, energy sources, and concentrations of microelements and macroelements. *R. nepotum* utilizes phenol as a sole carbon and energy source in this work. The factors to optimize are the incubation temperature and medium pH.

The effects of incubation temperatures on phenol degradation were investigated in this study. That was done at four different temperatures: 25, 28, 33, and 37 degrees Celsius. When incubation temperatures were altered, the results revealed a considerable difference in degradation rate (Table 6).

Table 6 Effect of growth conditions on the biodegradation rate of phenol by *R. nepotum*

Condition	Value	Biodegradation rate (ppm/h)
Incubate temperature (c)	25	7.5
	28	8.33
	33	7.3
	37	7
pH	5.5	4
	7	8.33
	8	7.8
	9	2

At 700 ppm phenol, any increase in temperature beyond 33°C resulted in a decline in growth once the temperature became less than 28°C. The temperature of incubation had a significant effect on cell mass formation and the rate of phenol degradation. Thus, the degradation of phenol occurs physiologically at room temperature, at which 28°C becomes the optimal temperature for *R. nepotum* cells. The temperature has been shown to have a physiologically significant impact on phenol fate [37]. Because it was discovered that 28°C is the best environmental factor for phenol degradation, or possibly because temperature influences the activities of the enzyme(s) involved [38, 39]. In the breakdown of organic pollutants, including phenol, the temperature has been as significant (or even more) than nutrition availability [40].

3.5. Effect of pH

The influence of pH on phenol concentration was tested on an uninoculated culture when studying pH settings to see if the phenol reduction was related to a chemical process or anything else. As shown in table 6, the biodegradation of phenol and the biomass growth rates were optimum at a pH of 7.0. It can be indicated that the pH of the medium has a critical effect on microorganism growth and, consequently, on phenol biodegradation [41]. The enzymes undoubtedly play a role in this catabolism pathway, and their maximal activity is, therefore, at pH 7.0. The appropriate pH plays a vital role in the biodegradation of these compounds, as previously noted. However, depending on the type of bacteria, the ideal pH varies. For example, Arthrobacter's optimal pH for the degradation of 4-CBA was 6.8 [42], which was similar to *Klebsiella oxytoca*'s optimum pH for the degradation of phenol [17, 28]. At the same time, *Halomonas campisalis* degraded phenol and catechol at pH ranging from 8 to 11 [43].

R. nepotum has the same ability to decompose phenol at pH 7 as Rhodococcus UKMP-5M [44], *Pseudomonas aeruginosa* PDM [29], and Pseudomonas sp. BZD-33 [45]. A range of aromatic compounds is biodegradable by oxygenase enzymes and the microorganisms that contain them [41, 46]. Several studies have shown that the concentration of yeast extract, which serves as carbon and nitrogen sources, is critical for maximizing the rate of phenol or other organic compound biodegradation.

Because phenol is the only carbon source, the high rate of phenol breakdown by *R. nepotum* cells could be explained by the availability of enough phenol-degrading enzymes. Furthermore, because the bacteria consume this substrate quickly, the usual reduction in phenol toxicity is further improved [3, 4, 47, 48].

3.6. GC-MS Analysis

In general, bacteria degrade phenol in two phases. The first is the hydroxylase enzyme's conversion of phenol to catechol in the presence of oxygen. Then, the catechol is degraded through either the ortho-pathway or the meta-pathway in the second phase. For example, catechol is degraded to cis-mucconic acid in the ortho-pathway by 1,2-dioxygenase. Then, it is converted to 2-hydroxymucconic semialdehyde in the meta-pathway by the 2,3-dioxygenase enzyme.

The biodegradation of phenol by *R. nepotum* was investigated using GCMS. As a control, phenol, and catechol were used. Fig. 4 shows that phenol (m/z = 94.0) washed out after 5.099 minutes, while catechol (m/z = 110.3) was stripped away after 8.194 minutes.

In comparison, *R. nepotum* degraded phenol to catechol and catechol to 2-hydroxymucconic semialdehyde using the GCMS analysis of the recovered products from the culture media sample (fig. 5), demonstrating that *R. nepotum* is employing a meta-pathway [41].

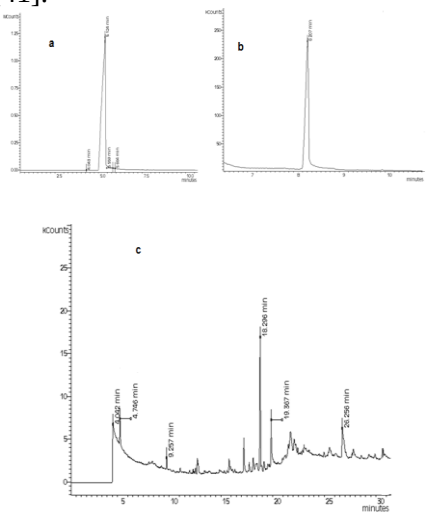

Fig. 4 The GC-MS chromatograms for the standards phenol (a), catechol (b), and the tested sample (c)

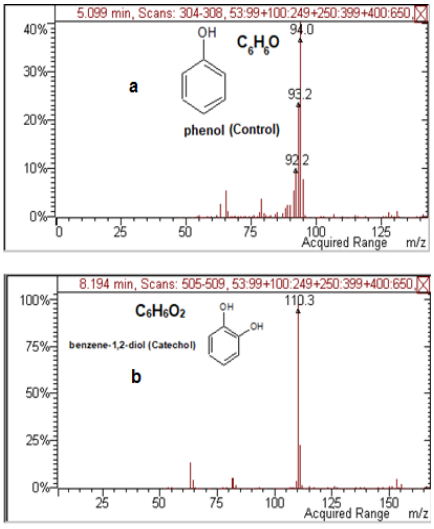

Fig. 5 MS fragmentation pattern of phenol (a) and catechol (b)

Furthermore, all intermediate molecules created throughout the degradation pathway underwent multiple oxidation stages, resulting in the meta pathway's synthesis of acetaldehyde and pyruvate, which eventually degraded into carbon dioxide and water.

However, due to the bacteria's efficient metabolism of these chemicals, it was logical to find them in our analysis. According to Fig. 6, catechol, acetaldehyde, and propanal can be generated from the phenol substrate, resulting in reverse Diels-Alder, coupling, or condensation processes between those fragments and phenol or catechol, as well as other fragments detected by GS-MS. The GC-MS chromatograms for the tested sample under investigation showed more than 15 peaks with various retention times, as shown in Fig. 7.

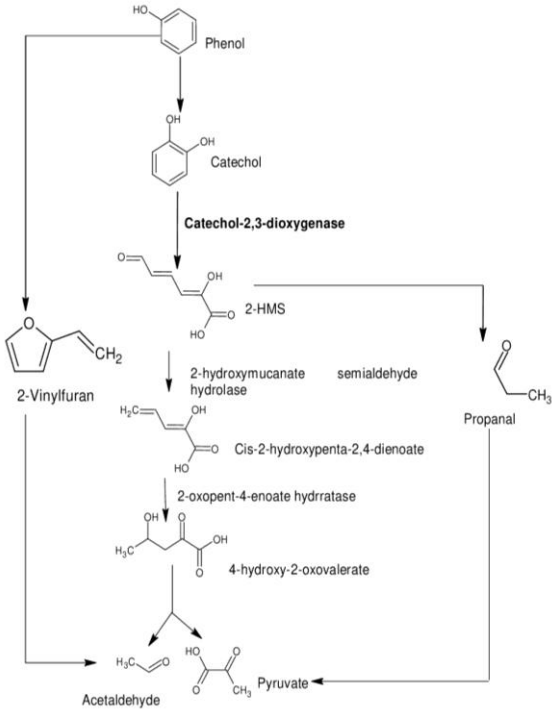

Fig. 6 The proposed Catechol-2,3-dioxygenase mechanism of phenol biodegradation by R. nepotum

The catechol or phenol degradation and condensation products were identified utilizing the NIST mass spectral database after GC-MS investigation of those significant peaks: Fig. 7 shows that the degradation of phenol happened via a reverse Diels-Alder reaction when 2-vinylfuran (1) was produced at (RT = 4.75 minutes and m/z =94.3). As illustrated in Fig. 7, the reaction of phenol and catechol produced [1,1'-biphenyl]-2,2',3,3'-tetrol (3), [1,1'-biphenyl]-2,3-diol (2), 4-phenoxy-phenol (5) and [11,21:24,31 terphenyl]-13,14,34-triol (4). Condensation of acetaldehyde with phenol and/or catechol, on the other hand, yielded a3-(1-hydroxyethyl) benzene-1,2-diol (6) (Fig. 7).

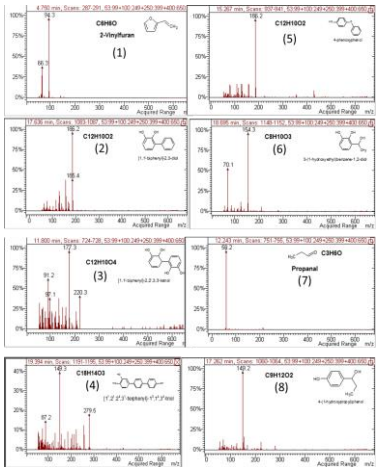

Fig. 7 MS fragmentation pattern of compounds (1-8)

The degradation sample produced a propanal (7) and the condensation between propanal and phenol and/or catechol generated a 4-(1-hydroxypropyl) phenol (8), 3,3'-(1-hydroxypropane-1,1-diyl)di(benzene-1,2-diol) (9), as shown in Fig. 4 and 5. Other condensation and elimination products such as, (*2Z,4Z*)-2,6-dihydroxy-6,6-bis(2-hydroxyphenyl) hexa-2,4-dienoic acid (10), 3-(3,4-dihydroxyphenyl)-2-hydroxypropanoic acid (11), (*3E*)-4-(3,4-dihydroxyphenyl)-2-oxopent-3-enoic acid (12), 3-(4-hydroxyphenyl)-2-oxopropanoic acid (13), 1,1'-[(*1E*)-but-1-ene-1,3-diyl] dibenzene (14), 4-(5-hydroxy-4-methylhexan-3-yl)phenol (15), as shown in Fig. 8, All of these degradation and condensation products demonstrate that catechol degradation occurs via the meta-pathway mechanism (catechol-2,3-dioxygenase).

3.7. HPLC Analysis

As indicated previously, gas chromatographic investigation (Fig. 9a-c) revealed that the degraded sample lacked phenol. Thus, the disappearance of a spectrum equivalent to phenol at 7 minutes and the presence of catechol as a function of time provide additional support for phenol degradation (Fig. 10).

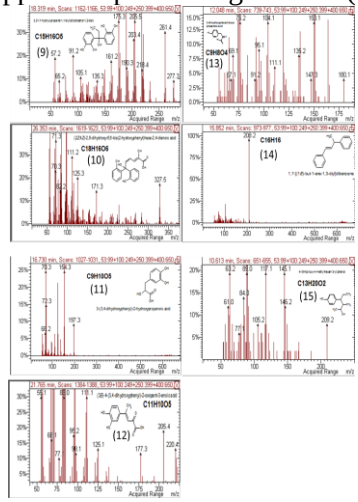

Fig. 8 MS fragmentation pattern of compounds (9-15)

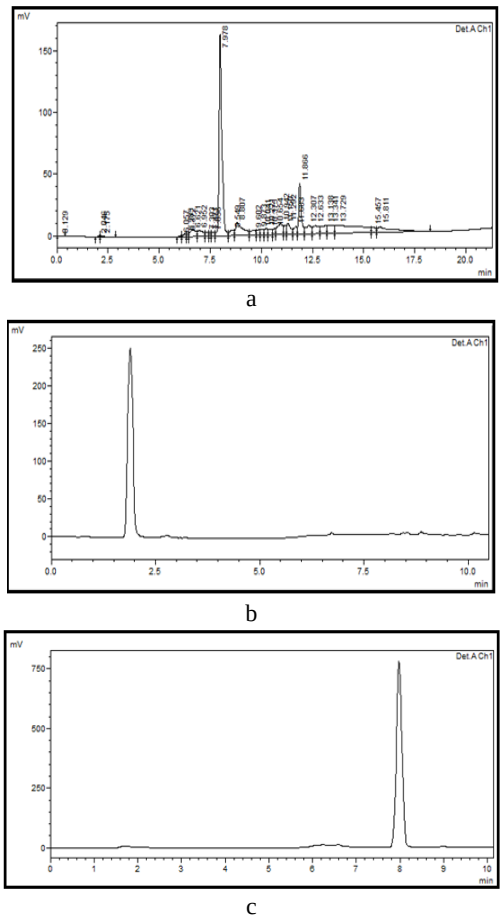

Fig. 9 HPLC chromatograms for phenol (a), catechol (b), and tested sample (c) at 0 times

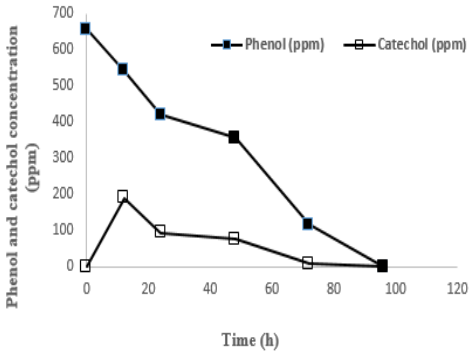

Fig. 10 Phenol and catechol concentrations of medium contain 700 ppm initial phenol concentration

4. Conclusion

Biological treatment has proven to be the most promising and cost-effective method for phenol removal. The ability of R. nepotum to biodegrade phenol and promote synchronous plant growth was revealed in this study. In phenol-stressed environments, this could help with rhizoremediation and crop yield preservation. This isolated soil bacterium, *R. nepotum*, is a powerful phenol degrader that can withstand phenol concentrations as high as 1200. When grown under optimal conditions, R. nepotum was capable of completely degrading 700 ppm of phenol in 96 hours.

Using the Haldane inhibition model, the maximum specific growth rate (max), half-saturation coefficient (Ks), and Haldane's growth kinetics inhibition coefficient (Ki) for phenol-dependent growth kinetics were estimated to be 0.716 (h^{-1}), 15.8 (mgL^{-1}) and 292 (mgL^{-1}) for phenol-dependent growth kinetics, respectively. Due to the volatile nature of this compound during aeration and intense mixing, however, these batch cultures have some limitations, including poor oxygen transfer. As a result, the impact of oxygen was neglected. It was supposed that the isolated *R. nepotum* growth and the rate of phenol degradation were inhibited exclusively by substrate concentration at certain conditions, including initial pH and temperature.

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