The Employment of Endophytic Bacteria for Phytodegradation of Pyridine

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Abstract. Pyridine is considered a heterocyclic aromatic chemical that is poisonous and carcinogenic to a variety of living species. The use of plant and endophytic bacteria to improve the efficiency of pollutants extraction is considered a viable technique since the endophytic bacteria help in the adaptation of the plant itself in various ecosystems and have significant ecological importance because they improve the soil fertility and quality. This research aims to stimulate the pyridine phytodegradation by Phragmites australis plants using the endophytic bacterial strain, Acinetobacter by inoculation these bacterial cells to the plants to see if it might increase plant growth and pyridine phytodegradation. In the present study, the system of pyridine phytodegradation basins with the vertical subsurface flow (VSSF) was adopted, since this system has better ventilation. In addition, the retention time is several hours due to the penetration of water molecules to the layers of packing materials of the basin, which have a relatively high hydraulic conductivity. After conducting the experiments, samples were collected and tests were done to find out the optimum conditions. The results were recorded as 40 plants of P. australis/m² of VSSF systems; bacterial cells concentration, 250 mg/L; pyridine concentration, 400 mg/L; temperature, 35 °C and pH, 8±2 for 10 hrs incubation duration. As a result, endophytic bacteria can break down toxic organic substances in combination with certain plants. When the endophytic bacterium, Acinetobacter was not used to enhance the role of Phragmites australis plants in the pyridine-phytodegradation process, the rate of phytodegradation was reduced to less than 30% at a pyridine concentration of 700 mg/L, indicating the importance of this endophytic bacterium in the pyridine phytodegradation process.

1. Introduction
Heterocyclic nitrogen bases, including pyridine and its derivatives, are one of the most significant substances containing N as a heteroatom [1]. It is utilized as an industrial solvent, particularly in the dehydrochlorination process, and has prospective uses in the production of colors, explosives, insecticides, and medicines [2]. The USEPA has classified pyridine as a priority pollutant (the United States Environmental Protection Agency) [3]. Pyridine is particularly toxic pollution because it would be poisonous to organisms even at low concentrations [4]. As a result, industrial wastewaters containing pyridine must be treated before being discharged into the environment to avoid harming natural ecosystems [5]. Different physical and chemical methods are available for the treatment of wastewaters containing pyridine and its derivatives [1], i.e., ultrafiltration [6], adsorption [7], ion exchange [8], ozonation [9], Fenton oxidation [10], and are employed to remove pyridine from wastewater. Moreover, a number of engineering and biology solutions have been created to help restore polluted ecosystems. Specifically, phytoremediation, which is recognized as one of the most practical, environmentally acceptable, and cost-effective options for the remediation of organic contaminants that are hazardous to the environment and human health. It is described as the use of plants to remove contaminants from the environment or render them harmless [11]. Phytoremediation of organic substances can occur by phytostimulation, which is the stimulation of microbial biodegradation in the rhizosphere, or the region...
around plant roots, or via phytotransformation, the plant's absorption and destruction of organic pollutants [12]. This kind of biodegradation is affected by a number of variables, including temperature, amount of acclimatization, and the types and features of the microbe population [13]. The employment of plants and microbes in tandem is a promising technique for the treatment of industrial and residential wastewater [14]. Plants sustain microbial populations through plant-endophytes relationships, and microorganisms promote plant development and pollutant degradation in exchange [15]. Endophytes are microorganisms (fungi and bacteria) that infiltrate the live, interior tissues of plants without inflicting any immediate harm and they may be separated from weeds, fruit plants, and essential crops' roots, stems, leaves, and inflorescences [16]. Endophytic bacteria are pathogen-controlling and processing facility bacteria that can be found in almost all plant species. The interaction of plants and endophytes can play an important role in the breakdown of hazardous pollutants in the rhizosphere [17]. Many researchers have utilized various endophytic bacterial species to remediate organic and inorganic contaminants. Germaine et al. [18] documented inoculating the pea (Pisum sativum) with a genetically engineered bacterial endophytic species that spontaneously degraded 2, 4-dichlorophenoxyacetic acid. Chen et al. [19] described heavy metal bioremediation using endophytic bacteria L14 (EB L14) isolated from the cadmium hyper-accumulator Solanum nigrum L. Oliveira et al. [20] identified three strains from cerrado plants capable of degrading various components of petroleum, diesel oil, and gasoline. The effects of toxic concentrations of essential and non-essential ions, as well as organic compounds, in the soil on plant-sensitive enzymes, which can result in plant growth inhibition and death, as well as toxicity accumulative symptoms in the plant must be considered when selecting the plant for phytodegradation approach [21]. Whiting et al. [22] investigated the impact of rhizosphere bacteria on the uptake of Zn by a hyperaccumulator (Thlaspi caerulescens) and a non-accumulator (Thlaspi arvense). Their findings revealed that the hyperaccumulator's biomass production and Zn absorption were stimulated by the rhizosphere microflora, while T. arvense was unaffected. Assisted phytoremediation by microorganisms' benefits both parties since plants provide nutrients to microorganisms, which grow and acquire improved plant ability for degradation ability, lowering the phytotoxicity of polluted soil; yet, there is a scarcity of scientific evidence on this plant-microorganism synergism [17, 23]. Endophytic bacteria influence plant development and growth directly through biological nitrogen fixation, digestive acceleration, and phytohormone synthesis, which confers resistance to biotic factors [24]. Endophytic bacteria and plants have a beneficial relationship that results in considerable improvements in plant growth, biomass, root length, dry matter production, and grain yield. The goal of this research is to illustrate the stimulation of pyridine phytodegradation by the synergistic use of plant-endophytic bacterium, Acinetobacter at the optimum environmental and operational conditions; temperature, pH, and pyridine concentration.

2. Materials and Methods

2.1. Chemicals, microorganisms, plant and culture conditions
All the chemicals utilized in the studies were either analytical as well as laboratory quality. Pyridine (C5H5N) was procured from VWR Chemicals BDH® (figure 1). The pH of the medium was initially adjusted to 7.0 ± 0.2 using weak hydrochloric acid (0.1 N).
The roots are the major entrance for microorganisms, and endophytic bacteria are most common in this section of the plant as shown in figure 2 [24]. The endophytic bacterium, Acinetobacter was screened from the root of Phragmites australis. The strain was purified using the streaking inoculation technique, and one clone was injected in 50 mL of LB liquid medium. After leaving the culture overnight at 35 °C, the cells were centrifuged and washed three times with sterile water [25].

**Figure 1.** Chemical structure of pyridine.

**Figure 2.** Representation of occurrence of endophytic microorganisms in the plant originating in the rhizosphere.

Phragmites australis, A major aquatic plant on the banks of the Tigris River in Salah Al-Din Governorate, Iraq, was chosen as the reed plant in this study. The plants were cleaned in water to remove suspended debris and clay from their roots before being placed in plastic bags until the planting procedure in the specified planting basins was achieved. 40 plants per square meter were extensively grown in the experimental ponds. This criterion was chosen based on previous research [26]. Before beginning the tests, the plants were grown and acclimated for two weeks.

2.2. Wastewater samples
The synthetic wastewater samples simulated pyridine-polluted wastewater. Purified pyridine solutions were added to the mineral salt medium (MSM) as carbon and nitrogen sources for bacterial growth. This mineral salt medium (MSM) comprises of many organic and inorganic compounds which represented synthetic wastewater as illustrated in Table 1. In synthetic wastewater samples, the organic compound peptone has been introduced as a source of biological matter.
Table 1. Mineral salt medium (MSM).

| Compound               | Chemical formula | Added amount (mg/L) |
|------------------------|------------------|---------------------|
| **a. Mineral compounds** |                  |                     |
| Potassium phosphate    | K$_2$HPO$_4$     | 15                  |
| Calcium chloride       | CaCl$_2$         | 20                  |
| Magnesium sulfate      | 7H$_2$O.MgSO$_4$ | 20                  |
| Zinc sulfate           | ZnSO$_4$         | 1                   |
| Ferric chloride        | FeCl$_3$         | 2                   |
| Sodium bicarbonate     | NaHCO$_3$        | 50                  |
| Ammonium chloride      | NH$_4$Cl         | 10                  |
| **b. Organic compound** |                  |                     |
| Peptone                | -                | 50                  |

2.3. Setup of Experiments

The glass basins used in the tests have dimensions of (60 cm × 26 cm) and a height of (30 cm). The basin base was covered in three layers: 3 cm of 10-mm aggregates in the first layer, 3 cm of sand in the second layer, and 4 cm of agro-sand in the third layer as shown in figure 3. In this study, three basins have been used for batch-mode procedure with a single exposure to pyridine polluted-water influent and bacterial inoculation (250 mL). Vertical subsurface flow (VSSF) was chosen as the regime for the phytoremediation system in this study because it provides greater ventilation. Furthermore, the retention duration is many hours due to water molecules penetrating the basin's layers of packing materials, which have a rather high hydraulic conductivity. one as a reference and the other to conduct the experiments of synthetic wastewater biotreatment. The wastewater samples were supplied using a vertical subsurface flow regime (VSSF). The experiments were conducted at operational modes of the hydraulic loading rate (HLR = 0.02 m/d) for 60 days. The treated effluent obtained from each basin was collected through a pipe subjected at the end of each basin. Following the establishment of the plants, the basins were divided into three duplicates for two experiments of phytodegradation:

- E1: Planted basin with pyridine-polluted water.
- E2: Planted basin irrigated with pyridine-contaminated water and inoculated with bacterial consortia.

Figure 3. Sketch of Vertical subsurface flow basin used in the experiments.
2.4. Plant growth
The plant growth was estimated at the end of the experiment by selecting three out of 10 plants in the same experimental unit randomly according to the method mentioned by [27]. Then the root and fresh weight were identified. Plant samples were dried at 70 °C degrees in constant weight. The dry root biomass and above-water parts (summation of shoots and leaves) are reported in terms of grams per square meter (g/m²). The relative growth rate (RGR) was based on total dry biomass and was estimated using equation (1):

$$RGR = \frac{\ln W_2 - \ln W_1}{T} \quad \text{eq. (1)}$$

Where:
RGR: relative growth rate of a plant, (g/d).
W₁ and W₂: the initial and final dry weights of used plants, respectively, of a whole plant sample, (g).
T: experiment time, (d).

2.5. Physical and chemical investigations
The analyses were conducted in accordance with the analytical methods described in Standard Methods [28]. The values of pH of the samples before and after phytoremediation experiments were estimated by pH-meter (HANA, HI.8424). Turbidity was determined by turbidity meter (HANNA, HI 93703-11). During the simulated phytodegradation tests, the development of cultures was measured spectrophotometrically (UNICO UV-2800, USA). The calibration curve was used to determine the cell dry weight (CDW, g/L) based on OD₆₀₀. The concentration of pyridine was determined using a high-performance liquid chromatography (HPLC) system (Shimadzu LC-20AT, Japan) outfitted with a Prominence SPD-20A UV–Vis Detector (detected at 254 nm). Prior to injection, samples were filtered via 0.2 μm syringe filters [29, 31].

2.6. Calculating the percentage of removal
The removal of each pollutant indicator as percentage was estimated after application of phytoremediation experiments, using equation (2):

$$R = \frac{C_1 - C_2}{C_1} \times 100 \quad \text{eq. (2)}$$

Where:
R: the percentage of pollutant removal (%).
C₁: Initial concentration of pollutant before phytoremediation experiments, (mg/L).
C₂: Final concentration of pollutant after phytoremediation experiments, (mg/L).

3. Results and Discussion
3.1. Phytodegradation of pyridine without inoculated bacterial cells
To investigate the synergistic use of the plant-endophytic bacterium, duplicate experiments in batch mode using the same operational and environmental conditions for the plant-bacterium cells ones but without inoculation of bacterial cells. The results of these experiments were shown in figure 4. The best percentage of pyridine-phytodegradation by P. australis was 80 % for 400 mg/L pyridine-concentration. When the concentration of pyridine was above 700 mg/L, the plants were shocked and phytodegradation decreased to less than 30 %.
3.2. Effect of bacterial concentration on pyridine-phytodegradation

For bacterial inoculation; after the samples were centrifuged, *Acinetobacter* cells were suspended in 0.9 percent sterile NaCl solution to produce a bacterial consortium. Different initial concentrations of biomass cells are used; from 50 to 300 mg/L of aqueous solution to investigate the effect of bacterial concentration on pyridine-phytodegradation as shown in figure 5. The optimum bacterial cells concentration was 250 mg/L, which simulated the pyridine phytodegradation by *P. australis* to 92%. This could be attributed to the ability of bacterial cells that were injected colonized the rhizosphere of *P. australis* and may be directly or indirectly engaged in the removal of pyridine from wastewater by stimulating plant growth and/or detoxifying this pollutant [30]. Colonization governs the relationship between the host plants and the endophytic species, which is regulated by growth stage, physiological status, type of plant tissue, agricultural operations, and environmental variables such as temperature, water supply, and nutrients [24].

3.3. Effect of temperature on pyridine-phytodegradation

Pyridine phytodegradation took place at temperatures ranging from 25°C to 38°C (figure 6). The highest rate of pyridine degradation was discovered at 35°C, whereas temperatures over 38°C or below 25°C were unfavourable for pyridine degradation by of *P. australis*. This is in agreement with the results of previous studies by Deng et al. and Kvesitadze et al. [29,30].
3.4. Effect of pH on pyridine-phytodegradation
As shown in figure 7, the optimum pH for pyridine phytodegradation activity of the endophytic bacteria Acinetobacter was 8±2, and it declined outside of this range. Furthermore, pyridine-phytodegradation appeared to be stable enough when the pH was equal to 8. Pyridine phytodegradation was favoured under alkaline (pH 8) environments [29].

4. Conclusions
The interaction of plants, soil, and microbes is critical for phytoremediation approaches. Phragmites australis, often known as common reed in English, was chosen for this study because of its various benefits, including its ability to produce oxygen gas at a rate of 5-12 mg of O₂/m².d. In order to simulate the pyridine-phytodegradation by P. australis; the endophytic bacterium, Acinetobacter was inoculated to the plants. The results illustrated that the optimum conditions of the pyridine phytodegradation in the presence endophytic bacterium cells for 10 hrs. incubation duration, were as follows: 40 plants of P. australis/m²; bacterial cells concentration, 250 mg/L; pyridine concentration, 400 mg/L; temperature, 35°C and pH, 8±2. When the endophytic bacterium, Acinetobacter was not used to enhance the role of Phragmites australis plants in the pyridine-phytodegradation process, the rate of phytodegradation was reduced to less than 30% at a pyridine concentration of 700 mg/L, indicating the importance of this endophytic bacterium in the pyridine phytodegradation process. As a result, endophytic bacteria can breakdown toxic organic substances in combination with certain plants.

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