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Characterization of the Phenol-degrading *Pseudomonas* sp. strain Neni-4

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ABSTRACT

This research presents the characterization of *Pseudomonas* sp. strain Neni-4 , which was selected because it can metabolize phenol as its primary carbon source. The strain breaks down phenol through the meta-cleavage pathway , which is typical for many efficient phenol-degrading bacteria. The strain achieved its highest growth rate at temperatures ranging from 25 to 30°C and pH values between 7.0 and 7.5 without significant differences ($p > 0.05$) in these ranges. The bacterial growth reached its peak when phenol concentrations ranged from 500 to 700 mg/L. The best growth results occurred when using 1% (w/v) ammonium sulfate as the nitrogen source. The research investigated the impact of heavy metals on the phenol breakdown process. Cd(II) showed no inhibitory effect on the process , and similarly, Pb(II) and Ni(II) showed more than 90% activity was observed. The growth rate decreased by 27% when exposed to Cu(II), but Ag(I) and Hg(II) caused extreme inhibition, which reduced activity to 12% and 5% respectively. The degradation profile indicated a rapid decrease of phenol concentration from 500 mg/L to near-zero levels during the first five days, while the cell density increased, indicating active biodegradation. *Pseudomonas* sp. strain Neni-4 demonstrates potential for bioremediation of phenol-contaminated sites when operated under neutral pH and mesophilic temperature conditions.

INTRODUCTION

Phenol (C_6H_5OH) functions as an aromatic compound that exhibits both volatile characteristics and water-solubility and has become an increasingly dangerous environmental contaminant because it is commonly used in industrial applications and poses severe risks to human populations and ecosystems. The major sources of phenol pollution across the world include industrial emissions and improper waste management, along with agricultural waste, which results in major contamination of water bodies and soil throughout industrial areas in developing countries with inadequate environmental oversight [1]. Industrial uses of phenol are in petrochemicals and pharmaceuticals, as well as plastics, textiles, dyes , pesticides, and detergents. The manufacturing of bisphenol A (for polycarbonates), caprolactam (for nylon), and nonionic surfactants begins with phenol as a precursor material. The chemical serves as a solvent and plays a dual role in the production of resins and adhesives. Industries that generate phenolic wastewater include oil refineries, coal processing facilities known as coking plants, pulp and paper facilities, and chemical manufacturing operations. The improper treatment of coal conversion facilities can result in phenol emissions that reach levels up to several thousand parts per million [2–7].

The environmental impact , together with its health consequences, creates substantial problems. Low concentrations of phenol cause severe harm to aquatic species, which impacts both fish reproduction and enzyme activity. Exposure to phenol through ingestion or inhalation, or dermal contact causes human beings to develop symptoms which range from skin burns to liver and kidney damage. The combination of prolonged exposure to phenol has established links with toxic effects on body systems, neurological problems, and possible cancer-causing potential. Phenol enters groundwater because of its high solubility, which creates more dangers for people using untreated water sources or supplies that lack proper regulation [8,9]. Research has intensified on phenol site remediation due to its long-term environmental impact and health hazards. Physicochemical techniques, including adsorption with activated carbon and solvent extraction, and advanced oxidation processes with Fenton's reagent and ozone, together with membrane

filtration , work effectively yet require substantial cost and energy consumption and produce additional waste products. The environmental community now prefers bioremediation as its method of choice because it offers both cost-effective solutions and environmentally friendly practices [10].

Phenol degradation through microbial methods presents itself as a leading biological solution , which uses *Pseudomonas* , *Acinetobacter* and *Bacillus* bacteria to convert phenol into catechol before producing water and carbon dioxide. Bacteria use phenol hydroxylase and catechol dioxygenases as specific enzyme systems to break down phenol. The approaches of biostimulation, which add nutrients, and bioaugmentation , which add specific bacterial strains, work together to boost microbial activity in polluted areas. Environmental conditions, including pH levels, temperature, phenol concentration, and nitrogen source availability, determine the success rates of these degradation methods [11–14].

Microbial strain capabilities for phenol degradation have improved significantly through recent advances in genetic engineering and synthetic biology , which enable faster and more efficient degradation processes. The combination of monitoring tools that include biosensors and molecular techniques (qPCR, metagenomics) with these technologies creates promising prospects for enhanced site-specific remediation. Its biodegradation has been studied primarily with Pseudomonas species [15–22]. Other microorganisms that have been reported to degrade phenol include fungal species such as *Phanerochaete chrysosporium* [23], *Hormodendrum bergeri*, *Fusarium oxysporum,* and *Aspergillus flavus* var. coulmnaris [6], *Penicillium chrysogenum* [24], yeast species such as *Candida tropicalis* [25], *Aureobasidium pullulans* FE13 [26], bacterial species such as *Bacillus brevis* [27], *Alcaligenes* sp. [28], *Ochrobactrum* sp. [29], *Rhodococcus* species [30–34], and algae species [35]. It appears that phenol and phenolic compound contamination is widespread in Indonesia, as many groundwater wells have phenolic levels over the WHO limit, especially near petroleum mining and processing activities [36]. To address this, it is crucial to identify bacteria that can break down phenol and utilize them in bioremediation. In this study, we characterize the growth of *Pseudomonas* sp. strain Neni-4, a previously isolated molybdenum-reducing bacterium on phenol.

MATERIALS AND METHODS

Growth of *Pseudomonas* sp. strain Neni-4

The bacterium was previously isolated as a molybdenum-reducing bacterium [37]. The bacterium was subcultured in mineral salt medium (MSM) containing g/L): K_2HPO_4, 0.4; KH_2PO_4, 0.2; $MgSO_4$, 0.1; NaCl, 0.1; $MnSO_4.H_2O$, 0.01; $NaMoO_4.2H_2O$, 0.01; $Fe_2(SO_4).H_2O$,0.01; $(NH_4)2SO_4$, 0.4 in a 250 ml conical flask supplemented with 0.5 g/L or 500 mg/L phenol [34]. The phenol was filter-sterilized before being added to the medium. The incubation temperature was set at room temperature, and the shaking speed was set at 150 rpm.

Elucidation of Phenol Biodegradation Pathway

The ortho and meta pathways are the two main mechanisms by which phenols are biodegraded. The metabolite 2-hydroxymuconate semialdehyde can be identified in the supernatant using colorimetry due to its strong absorption at 375 nm, which is characteristic of the meta-ring-cleavage pathway [38].

The strain was cultured for 24 hours on an orbital shaker set at 150 rpm at room temperature in the aforementioned 100 mL MS medium supplemented with 500 mg/L phenol. After that, the culture was harvested at 10,000×g for 10 minutes. The resulting supernatant was then scanned in a quartz cuvette from 200 to 500 nm, using newly made MSM as the reference standard.

Growth characterization

Effect of pH

The MSM solution received different pH adjustments from 5.0 to 9.0 through 1 M HCl or 1 M NaOH before autoclaving. The solution received phenol (0.5 g L^{-1}) after sterilization. The bacterium received a 1% v/v inoculum into each flask before the flasks were placed at 150 rpm and 30°C for 5 days. The 4-aminoantipyrine colorimetric method was used to analyze phenol degradation in daily samples.

Effect of Temperature

The bacterial cultures were grown on phenol (0.5 g L^{-1}) in MSM at five different temperatures ranging from 20 to 40 °C. All other conditions were maintained constant. The experiment monitored phenol breakdown and bacterial growth over a 5-day period.

Effect of Phenol Concentration

The study evaluated phenol tolerance and degradation capacity through MSM solutions containing phenol concentrations of 0.1, 0.3, 0.5, 0.7, and 1.0 g L^{-1}. The bacterial cultures was incubated at 30°C and pH 7.0 and 150 rpm on an orbital shaker. Growth of the bacterium was monitored as before.

Effect of Nitrogen Source

The research investigated how different nitrogen sources affect phenol breakdown through the replacement of $(NH_4)_2SO_4$ with $NaNO_3$, KNO_3, urea and peptone at 0.4 g L^{-1} final concentration in MSM. The inoculated media underwent the same incubation process.

Analytical Methods

The 4-aminoantipyrine method measured phenol concentrations in culture supernatants by spectrophotometry at 510 nm. A UV-Vis spectrophotometer measured bacterial growth through optical density readings at 600 nm.

Statistical analysis

Comparison between groups was performed using a one-way analysis of variance with post hoc analysis by Tukey's test. $P < 0.05$ was considered statistically significant.

RESULTS

The optimum temperature and pH for bacterial growth

The ideal temperature for bacterial growth was determined by incubating *Pseudomonas* sp. strain Neni-4 with 500 mg/L phenol at 20 to 45 ºC. The optimal growth temperature range for *Pseudomonas* sp. strain Neni-4 on phenol was found span from 25 to 30 °C, with ANOVA analysis indicated no significant difference between these two values ($p>0.05$) (**Fig. 1**). The ideal pH was found by using a 20 mM inorganic phosphate buffer spanning the buffering capacity of phosphate from 5.8 to 7.8. *Pseudomonas* sp. strain Neni-4 thrived in environments with a pH range of between 7.0 and 7.5 (**Fig. 2**), with ANOVA analysis indicating no significant difference between these two values ($p > 0.05$).

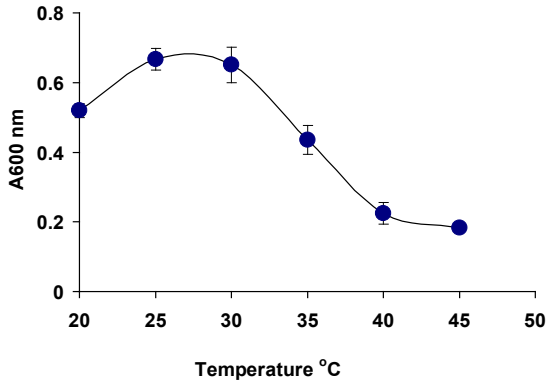

Fig. 1. *Pseudomonas* sp. strain Neni-4 bacterial growth as a function of temperature. Error bars represent mean ± standard deviation (n=3).

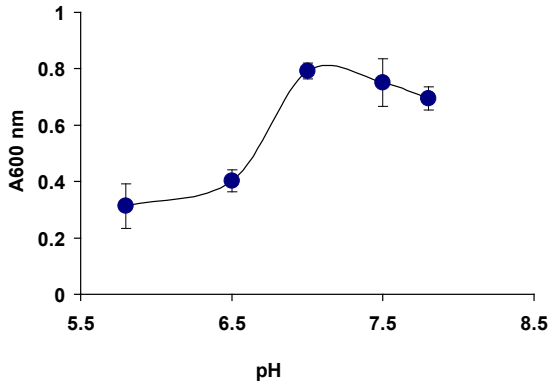

Fig. 2. *Pseudomonas* sp. strain Neni-4 bacterial growth as a function of pH. Error bars represent mean ± standard deviation (n=3).

The effect of carbon and nitrogen sources on growth

Fig. 3 shows that *Pseudomonas* sp. strain Neni-4 exhibits moderate phenol tolerance and degradation capability, as it was inhibited by 1000 mg/L of phenol. A concentration of between 500 and 700 mg/L of phenol was shown to be optimal for growth support as supported by ANOVA analysis. Ammonium sulfate was the best nitrogen source (**Fig. 4**) at 1% (w/v).

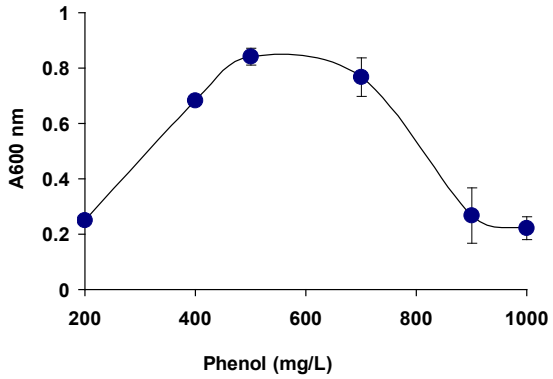

Fig. 3. *Pseudomonas* sp. strain Neni-4 bacterial growth as a function of phenol concentrations. Error bars represent mean ± standard deviation (n=3).

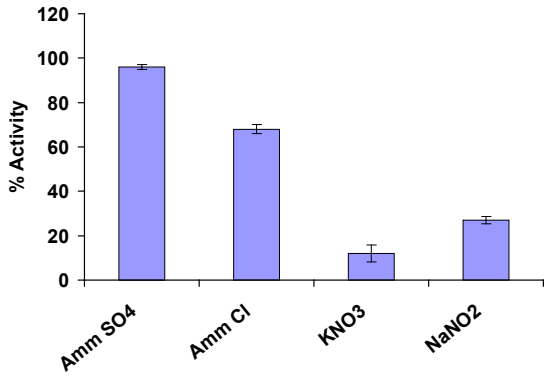

Fig. 4. *Pseudomonas* sp. strain Neni-4 bacterial growth as a function of nitrogen sources. Error bars represent mean ± standard deviation (n=3).

The effect of heavy metals on phenol degradation

The effect of various heavy metals on the growth of *Pseudomonas* sp. strain Neni-4 on phenol was assessed and expressed as a percentage relative to the control. The tested metals showed that Cd(II) had no inhibitory effect and maintained 100% activity, Pb(II) and Ni(II) retained high levels of activity at 97.2% and 91.7% respectively. Cu(II) moderately inhibited the growth, reducing activity to 27%, while Ag(I) and Hg(II) caused severe inhibition, with activities dropping to 12% and 5% respectively. These results indicate that the growth is highly sensitive to Hg(II) and Ag(I), whereas Cd(II), Pb(II), and Ni(II) have relatively minimal inhibitory effects (**Fig. 5**).

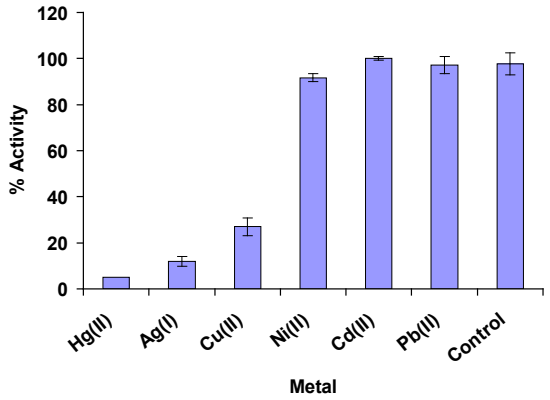

Fig 5. Effect of heavy metals at 2 mg/L final concentration on phenol degradation by *Pseudomonas* sp. strain Neni-4. Error bars represent mean ± standard deviation (n=3).

Growth and degradation profile of *Pseudomonas* sp. strain Neni-4 on phenol

The concentration of phenol decreased rapidly from 500 mg/L to nearly undetectable levels by day 5 (**Fig. 6**), while the bacterial cell count increased steadily, indicating active biodegradation. The control, without bacterial inoculation, showed no significant change in phenol concentration.

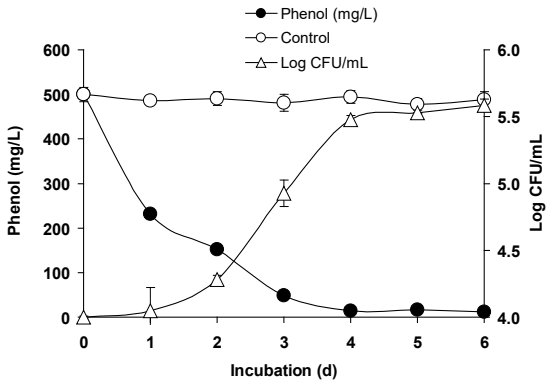

Fig. 6. Phenol degradation and bacterial growth over a 6-day incubation period. Error bars represent mean ± standard deviation (n=3).

DISCUSSION

There is limited information on phenol-degrading bacteria isolated from Indonesian soils, with only a few publications to date [37, 39–42]. This work contributes to the arsenal for future remediation strategies. Soil microbes have been found to break down phenol. The existence of 2-hydroxymuconate semialdehyde, a result of the meta-degradation of phenol, was shown by a high absorption at 375 nm in the UV spectrum of the culture supernatant when grown on phenol as the only carbon source (Data not shown). Many *Pseudomonas* phenol-degrading bacteria exhibit a meta cleavage pathway [39–43]. Research into the breakdown of phenol by different phenol-degrading bacterial species has shown either a combination of ortho- and meta-fissions, as in *Rhodococcus* sp. DGUM [44] and *Rhodococcus* sp. strain DK17 [45], or purely ortho-fission, as in *Acinetobacter* strains W-17 [46].

The strain exhibited broad optimum temperatures for growth on phenol, which is beneficial for commercial applications that require the microbe to adapt to a wide range of temperatures. Several phenol-degraders listed in the literature [9, 12–14, 51–60] can be most effectively grown at a relatively low temperature range. On the other hand, thermophilic *Bacillus stearothermophiles* can degrade phenol at temperatures as high as 50 °C [57], while psychrophilic *Pseudomonas* putida and a yeast strain may accomplish so at temperatures as low as 10 °C [58,59].

Over the past five years, scientists have discovered several strains that can withstand and mineralize phenol at starting concentrations exceeding 1000 mg/L. Bacteria that break down phenol showed very little growth at doses higher than 1500 mg/L, as phenol toxicity becomes apparent [60]. At phenol concentrations below 500 mg/L, several phenol-degrading bacteria exhibited lag phases [12–14,18,43,51,58,61–65]. Since ammonium sulphate was determined to be the most effective nitrogen source for *Pseudomonas* sp. strain Neni-4, we have chosen it for our subsequent optimization experiments. In addition to being cost-effective for use in future bioremediation projects, ammonium sulfate is readily available in large quantities. Most phenol-degraders prefer ammonium sulfate as a nitrogen source 18, 43, 51, 58, 61–65]. Although the specific growth rate of this strain was significantly reduced at high phenol concentrations, the degradation rate profile indicates that degradation is still occurring, suggesting that the strain prioritizes detoxification of the phenols over growth.

Pseudomonas sp. strain Neni-4, a previously isolated molybdenum-reducing bacterium, demonstrates effective phenol degradation via the meta-cleavage pathway and thrives under neutral pH (7.0–7.5) and mesophilic temperature conditions (25–30 °C). Its ability to degrade phenol at concentrations up to 700 mg/L, along with its tolerance to common environmental metal contaminants such as Cd(II), Pb(II), and Ni(II), underscores its potential as a robust candidate for bioremediation applications. However, growth inhibition by Ag(I) and Hg(II) suggests sensitivity to certain toxic heavy metals, which should be considered in future remediation works. The strain's rapid degradation kinetics and high cell proliferation during phenol removal further validate its use in treating phenol-contaminated wastewater or industrial effluents.

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