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Investigation of Indole-3-Acetic Acid Production and Bioremediation Activity of *Pseudarthrobacter sp* UMK-PNF5

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DECLARATION

I declare that this thesis entitled “Investigation of IAA Production and Bioremediation Activity by *Pseudarthrobacter* sp UMK-PNF5” is the results of my own research except as cited in the references.

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KELANTAN

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ABSTRAK

Pertanian adalah penting untuk pengeluaran makanan global dan kesihatan alam sekitar, ia merupakan aspek yang berkait rapat untuk kesejahteraan manusia dan kelestarian ekosistem. *Pseudarthrobacter sp.* dan kepentingan IAA (asid indol-3-acetic) berkait rapat dalam konteks interaksi antara tumbuhan dan mikrob. IAA, sebagai hormon tumbuhan memainkan peranan penting dalam pelbagai proses fisiologi tumbuhan, termasuk pembahagian sel, pemanjangan, dan perbezaan. *Pseudarthrobacter sp.*, yang mampu menghasilkan IAA berpotensi untuk mempengaruhi pertumbuhan dan perkembangan tumbuhan secara positif. Melalui pembebasan IAA, *Pseudarthrobacter sp.* dapat meningkatkan pertumbuhan akar, meningkatkan kecekapan penyerapan nutrien, dan memberikan ketahanan terhadap tekanan biotik dan abiotik dalam tumbuhan. Oleh itu, bakteria ini merupakan bakteria yang meningkatkan pertumbuhan tumbuhan (PGPB) dan komponen berharga dalam amalan pertanian yang bertujuan untuk meningkatkan produktiviti tanaman dan ketahanannya. Walau bagaimanapun, tahap pengeluaran semasa asid indol-3-acetic (IAA) oleh *Pseudarthrobacter sp.* masih kurang dan tidak mencukupi untuk kegunaan praktikal. Kaedah konvensional untuk menangani cabaran alam sekitar dari aktiviti industri adalah mahal dan menghasilkan produk sampingan yang toksik. Sebaliknya, bioremediasi adalah teknik mesra alam yang menunjukkan potensi untuk menyingkirkan pencemaran dengan berkesan. *Pseudarthrobacter sp.* telah membuktikan keupayaannya untuk menguraikan pelbagai bahan toksik, menjadikannya calon yang ideal untuk menilai potensi penambahbaikan *Pseudarthrobacter sp.* UMK-PNF5. Kajian ini bertujuan untuk menyiasat pengeluaran IAA oleh *Pseudarthrobacter sp.* UMK-PNF5 di bawah kepekatan L-tryptophan yang berbeza, khususnya 1000, 3000, 6000, dan 9000 µg/mL. Selain itu, kajian ini mengkaji pengeluaran IAA *Pseudarthrobacter sp.* UMK-PNF5 dengan menggunakan sumber karbon dan nitrogen yang berbeza, termasuk molasses dan fruktosa sebagai sumber karbon, dan beef extract dan tryptone sebagai sumber nitrogen, dengan penambahan 1000 µg/mL kepekatan L-triptofan. Ia menunjukkan penyerapan yang lebih tinggi menghasilkan lebih banyak IAA. Selanjutnya, penyelidikan mengeksplorasi kapasiti bioremediasi *Pseudarthrobacter sp.* UMK-PNF5 dalam memecahkan fenol, menyediakan pandangan tentang syarat pertumbuhan yang optimum dan penentuan sisa fenol. Penemuan ini menunjukkan bahawa *Pseudarthrobacter sp* UMK-PNF5 tidak boleh menguraikan fenol. Penemuan ini akan meningkatkan pemahaman kita tentang keupayaan *Pseudarthrobacter sp.* UMK-PNF5, menawarkan aplikasi berpotensi dalam pertanian mampan dan penambahbaikan alam sekitar.

Kata Kunci: Pengeluaran Asid Indol Asetik (IAA), Bioremediasi, Sumber Karbon, Sumber Nitrogen, *Pseudarthrobacter sp.* UMK-PNF5

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ABSTRACT

Agriculture is vital for global food production and environmental health, which are interconnected aspects crucial for human well-being and ecosystem vitality. *Pseudarthrobacter sp*. and the importance of IAA (indole-3-acetic acid) are closely intertwined in the context of plant-microbe interactions. IAA, as a plant hormone, plays a crucial role in various physiological processes of plants, including cell division, elongation, and differentiation. *Pseudarthrobacter sp*., being capable of producing IAA, can potentially influence plant growth and development positively. Through the secretion of IAA, *Pseudarthrobacter sp.* can enhance root growth, improve nutrient uptake efficiency, and confer resistance to biotic and abiotic stresses in plants. Consequently, this bacterium holds promise as a plant growth-promoting bacterium (PGPB) and a valuable component of sustainable agricultural practices aimed at enhancing crop productivity and resilience. However, current production levels of Indole-3-acetic acid (IAA) by *Pseudarthrobacter sp*. fall short of practical use. Conventional methods for addressing environmental challenges from industrial activities are both expensive and generate toxic byproducts. In contrast, bioremediation is an eco-friendly technique that shows promise for effectively eliminating pollutants. *Pseudarthrobacter sp.* has demonstrated its ability to degrade various toxic substances, making it an ideal candidate for evaluating the remediation potential of *Pseudarthrobacter sp.* UMK-PNF5. This study aims to investigate the production of IAA by Pseudarthrobacter sp. UMK-PNF5 under different concentrations of L-tryptophan, specifically 1000, 3000, 6000, and 9000 µg/mL. Additionally, the study examines the IAA production of *Pseudarthrobacter sp.* UMK-PNF5 using different carbon and nitrogen sources, including molasses and fructose as carbon sources, and beef extract and tryptone as nitrogen sources, with the addition of 1000 µg/mL concentration of L-tryptophan. It shows higher absorbance produces higher IAA. Furthermore, the research explores the bioremediation capacity of *Pseudarthrobacter sp.* UMK-PNF5 in breaking down phenol, providing insights into optimal growth conditions and residual phenol determination. These findings show *Pseudarthrobacter sp* UMK-PNF5 cannot degrade phenol. These findings enhance our understanding of the capabilities of *Pseudarthrobacter sp.* UMK-PNF5 offers potential applications in both sustainable agriculture and environmental remediation.

Keywords: Indole Acetic Acid (IAA) Production, Bioremediation, Carbon sources, Nitrogen sources, *Pseudarthrobacter sp.* UMK-PNF5

UNIVERSITI MALAYSIA KELANTAN

FYP FBKT

TABLE OF CONTENT

UNIVERSITI MALAYSIA KELANTAN

FYP FBKT
UNIVERSITI MALAYSIA KELANTAN

FYP FBKT

UNIVERSITI MALAYSIA KELANTAN

FYP FBKT

LIST OF TABLES

UNIVERSITI
MALAYSIA
KELANTAN

LIST OF FIGURES

FYP FBKT

UNIVERSITI MALAYSIA KELANTAN

LIST OF ABBREVIATIONS

IAA	Indole-3-Acetic Acid	1
PGPB	Plant Growth Promoting Bacteria	3
Ph	Potential of hydrogen	5
-OH	Hydroxyl groups	15
LB	Luria Bertani	17
L	Litre	17
MM-M9	Minimal Medium M9	18
O.D	Optical Density	20
nm	Nanometre	20
mg/mL	milligrams per milliliter	23
CFU	Colony Forming Unit	24
mL	Milliliter	24
PGPR	Plant Growth Promoting Rhizobacteria	27
NADH/NADPH	Nicotinamide adenine dinucleotide	36
FADH2	Flavin adenine dinucleotide	36

FYP FBKT
UNIVERSITI MALAYSIA KELANTAN

LIST OF SYMBOLS

µg/mL	micrograms per milliliter	16
°C	Degree Celcius	17
±	Plus-minus	21
%	Percentage	21
µm	micrometer	22

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

The relationship between Indole-3-acetic acid (IAA) and bioremediation lies in the potential of certain bacteria, such as those belonging to the genus *Pseudarthrobacter sp*, to play a role in both processes. *Pseudarthrobacter sp*, being Gram-positive bacteria with the ability to grow on various carbon sources, including amino acids, are known for their versatility in different environments, including those associated with animals, soil, water, and other environmental materials (Rachid & Güngör, 2023).

One aspect of interest is the production of IAA by these bacteria. Indole-3-acetic acid is a plant hormone that influences various aspects of plant growth and development, particularly in controlling cell elongation and differentiation (Etesami & Glick, 2024). The ability of *Pseudarthrobacter sp* to produce IAA suggests a potential link between these bacteria and plant growth.

In the context of bioremediation, *Pseudarthrobacter sp* and similar bacteria can contribute to the degradation of pollutants and contaminants in the environment (Huang et al., 2021). Bioremediation involves the use of biological organisms to break down complex organic compounds into simpler and less harmful forms (Huang et al., 2021). The enzymes produced by microorganisms, including bacteria like *Pseudarthrobacter sp*, play a crucial role in catalyzing these transformation processes.

This study aims to explore whether *Pseudarthrobacter sp* has the potential for bioremediation processes and whether it can release or enhance the production of Indole-3-acetic acid (IAA) as part of its metabolic activities. This dual function, involving environmental cleanup through bioremediation and potentially influencing plant growth through IAA production.

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1.2 Problem Statement

Agriculture and environmental health are the most essential responsibilities we have to concerns. Agriculture is essential for food production while environmental health is essential for human health as well as for the health of flora and fauna, which are exposed to environmental hazards via air and water (Kumar & Kumar, 2019). Agriculture can provide the required resources for producing crops and raising livestock to support the world's population. IAA is a vital plant growth regulator that is frequently utilized to improve crop yield and quality (Solano et al., 2023). However, the current levels of IAA production by *Pseudarthrobacter sp.* are frequently insufficient for practical applications, and it is necessary to identify the optimal fermentation conditions to increase the IAA yield (Tshishonga, 2020). The reduction in the usage of chemical fertilizers and pesticides that can result from optimizing fermentation conditions for IAA generation by *Pseudarthrobacter sp.* will have far-reaching consequences for sustainable agriculture.

Pollutant found in industrial wastewater and oil accidents can have significant environmental and health effects. The conventional methods for removing these pollutants, such as physical and chemical remedies are frequently expensive and can generate toxic byproducts. In recent years, bioremediation has emerged as a promising technique for removing organic pollutants, such as phenol and oil. *Pseudarthrobacter sp.* were reported to degrade toxic substances such as polycyclic aromatic hydrocarbons, pesticides herbicides, heavy metals, cyanide compound and aromatic compound (Kumar et al., 2020). In this study, the ability of *Pseudarthrobacter sp.* UMK-PNF5 would be evaluated.

FYP FBKT
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1.3 Objectives

1.To determine IAA productions by *Pseudarthrobacter sp* UMK-PNF5 under different L-Tryptophan concentrations.

2.To determine the IAA production of *Pseudarthrobacter sp* UMK-PNF5 by using different carbon and nitrogen sources.

3.To assess the degradation of phenol by *Pseudarthrobacter sp* UMK-PNF5.

1.4 Scope of Study

Pseudarthrobacter sp. UMK-PNF5 was isolated from the paddy rhizosphere in a previous study. This bacterium demonstrated the ability to produce indole-3-acetic acid (IAA), suggesting its potential role as a plant growth-promoting bacterium (PGPB) (Kamaruzaman et al., 2022). The bacterium could establish beneficial interactions with plant roots, contributing to the overall health and growth of plants in the rhizosphere. In this study, the optimization of IAA production was carried out using different concentrations of L-tryptophan, carbon sources, and nitrogen sources. The concentrations of L-tryptophan used were 0, 1000, 3000, 6000, and 9000 µg/mL. Molasses and fructose were utilized as carbon sources, while beef extract and tryptone were employed to optimize various nitrogen sources. The phenol degradation study involved a investigation into the capacity of *Pseudarthrobacter sp.* UMK-PNF5 to degrade phenol, a significant environmental pollutant. The research delved into understanding the efficiency of *Pseudarthrobacter sp.* UMK-PNF5 in degrading phenol, elucidating the underlying mechanisms involved in the degradation process.

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1.5 Significances of Study

The use of microorganisms for bioremediation may help in mitigating the environmental impact of these pollutants and restore affected ecosystems. Bioremediation utilizing microorganisms is a sustainable method for decontaminating polluted sites because it is a natural process that does not rely on harmful chemicals or extensive energy consumption. As a plant growth hormone that can enhance crop yields and plant health, the production of Indole-3-acetic acid (IAA) is significant for agriculture. Additionally, it can aid in reducing the need for chemical fertilizers and promoting sustainable agricultural practices. Bioremediation of phenol and oil spills and the production of IAA can contribute to an understanding of microbial ecology, biogeochemistry, and the interactions of microbes with the environment.

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CHAPTER 2

LITERATURE REVIEW

2.1 *Pseudarthrobacter sp*

Pseudarthrobacter sp. is a genus of bacteria that belongs to the family *Micrococcaceae*. The genus includes various species of Gram-positive, aerobic, and non-motile bacteria (Xu et al., 2017). *Pseudarthrobacter* bacteria are commonly found in soil, water, and other environmental habitats. These bacteria are known for their ability to adapt to diverse environments and tolerate harsh conditions such as extreme temperatures, pH levels, and salinity (Chen et al., 2021). *Pseudarthrobacter sp.* can produce antimicrobial compounds that inhibit the growth and proliferation of pathogenic organisms (Etesami & Glick, 2024). These antimicrobial substances act directly on pathogens, disrupting their cellular processes and hindering their ability to cause disease (Amaresan et al., 2020). Additionally, *Pseudarthrobacter sp.* engages in competition for resources within the rhizosphere environment, were plant roots release exudates rich in nutrients (Yang et al., 2023). They have also been isolated from contaminated sites and have shown the capability to degrade a wide range of organic compounds, including pollutants. *Pseudarthrobacter sp*. has been studied for its potential applications in bioremediation, as they possess enzymes and metabolic pathways that can break down pollutants and contribute to the cleanup of contaminated sites (Chen et al., 2021). Additionally, they have been investigated for their production of extracellular enzymes, antimicrobial compounds, and biosurfactants (Benaud et al., 2021).

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2.2 Indole-3-Acetic Acid production

IAA refers to indole-3-acetic acid, a naturally occurring plant hormone also known as auxin. IAA is essential for modulating numerous aspects of plant growth and development, including cell elongation, root formation, tropisms (responses to stimuli), and fruit development(Gomes & Scortecci, 2021). IAA biosynthesis is the synthesis and secretion of indole-3-acetic acid by certain organisms, such as plants and bacteria(Duca & Glick, 2020). IAA is involved in numerous growth responses and is produced by numerous plants as part of their normal physiology(Ma et al., 2022). Some bacterial species can generate IAA via enzymatic pathways. These bacteria can convert the amino acid tryptophan into IAA using enzymes such as tryptophan-2-monooxygenase (IAA monooxygenase) and tryptophan-5-monooxygenase (IAA oxidase) (Kurosawa et al., 2009). IAA production by bacteria, such as those found in the rhizosphere can promote plant growth and development(Das et al., 2019). When microorganisms produce IAA in the rhizosphere, it can affect root development, improve nutrient uptake, increase plant stress tolerance, and stimulate plant growth(Etesami & Adl, 2020).

2.3 Roles of IAA in plant growth

The production of Indole-3-Acetic Acid (IAA) plays a significant function in a variety of biological processes and has several significant ramifications. IAA is a vital plant hormone involved in growth and development regulation. It influences cell elongation, root development, vascular tissue differentiation, apical dominance, and fruit development. IAA helps stimulate root growth, promote the formation of lateral roots, and modulate tropic responses such as phototropism and gravitropism. Additionally, increased IAA levels stimulate the development of lateral roots, which increases the root surface area for nutrient assimilation. IAA can also increase root hair production, which improves nutrient and water absorption by enhancing the root-soil interface. IAA production may be affected occupancy, soil degradation, and a wide range of environmental factors, such as flooding, drought, salinity, temperature (extreme heat and cold), and heavy metal contamination. (Ojuederie et al., 2019).

Certain microbes that produce IAA can contribute to the suppression of plant diseases. As part of their antagonistic activity against plant pathogens, certain bacteria produce IAA, which inhibits their growth or disrupts their virulence mechanisms. In addition, IAA can enhance the plant's own defense mechanisms, such as the production of antimicrobial compounds and the activation of acquired systemic resistance. Plant pathogens have developed numerous strategies for evading or suppressing fundamental host defenses. (Dou & Zhou, 2012).

2.4 Factors influence IAA production

The production of Indole-3-Acetic Acid (IAA) in plants and microorganisms is influenced by numerous factors. IAA production is significantly influenced by environmental factors. Light, temperature, humidity, nutrient availability, and water availability are environmental factors that can influence IAA synthesis (Lopes et al., 2021). The availability of nutrients, specifically nitrogen and carbon sources, can influence IAA production. For optimal IAA synthesis, microorganisms and plants need adequate nutrient levels. Nutrient deficiencies or imbalances can contribute to altered IAA production (Etesami & Maheshwari, 2018). IAA production may be modulated by interactions between IAA and other plant hormones. Hormones including cytokinins, gibberellins, abscisic acid, and ethylene can affect the synthesis, transport, and metabolism of IAA(Anfang & Shani, 2021). The equilibrium and interplay between these hormones contribute to the overall regulation of IAA production. Genes and genetic pathways control the production of IAA. Expression and activity of enzymes involved in IAA biosynthesis are genetically controllable. IAA production can be influenced or regulated by genetic factors, including mutations and genetic variations (Duca & Glick, 2020).

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2.5 Environmental Factors Influencing Indole-3-Acetic Acid Production in Microorganisms

The production of indole-3-acetic acid (IAA) in microorganisms is intricately governed by environmental factors that significantly impact the metabolic pathways involved. The composition of the growth medium, encompassing carbon, nitrogen, and phosphorus sources, plays a pivotal role in modulating IAA synthesis (Etesami & Glick, 2024). Specific carbon and nitrogen sources influence microbial metabolism, affecting the availability of precursors essential for IAA production (Widawati, 2020). Additionally, the pH of the medium is a critical determinant, with various microorganisms displaying distinct optimal pH ranges for IAA synthesis. Factors such as temperature and oxygen levels further contribute to the efficiency of IAA biosynthesis, as optimal conditions for microbial growth and enzyme activity are imperative (George et al., 2020). Essential cofactors and minerals, including iron and magnesium, intricately modulate the enzymatic reactions crucial for IAA production (Borah et al., 2023). Genetic factors and microbial strain diversity contribute to variations in IAA synthesis capacities among different microorganisms. In the case of *Pseudarthrobacter sp.*, a fact worth noting is its ability to thrive in diverse environments, including extreme conditions, showcasing its potential resilience and adaptability as an IAA-producing microorganism (Redondo-Gómez et al., 2021). The comprehensive understanding of these environmental factors not only sheds light on the intricate mechanisms governing microbial IAA synthesis but also provides a foundation for potential agricultural applications, where the targeted use of specific microorganisms, like *Pseudarthrobacter sp.*, could enhance plant growth and stress tolerance.

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2.6 Genetic Regulation of IAA Production

The genetic regulation of Indole-3-Acetic Acid (IAA) production serves as a molecular blueprint guiding the intricate synthesis of this crucial plant hormone within microorganisms, exemplified by *Pseudarthrobacter sp* (Kumawat et al., 2023). This process initiates with the biosynthesis of tryptophan, a pivotal precursor for IAA production, wherein specific genes orchestrate the synthesis of this amino acid (Yoshida & Fernie, 2023). Dedicated enzymes, encoded by these genes, play a fundamental role in ensuring an adequate supply of tryptophan (Keswani et al., 2020). Subsequently, the involvement of IAA biosynthetic genes becomes prominent, directing the transformation of tryptophan into IAA through enzymes such as tryptophan-2-monooxygenase and tryptophan-5-monooxygenase (Pavlova et al., 2017).

The regulatory control of IAA biosynthesis extends to the transcriptional level, where intricate networks of transcription factors modulate the expression of genes involved in the pathway (Yoon et al., 2020). This transcriptional regulation allows microorganisms to respond to environmental cues and optimize IAA production based on external conditions. Moreover, feedback inhibition mechanisms add a layer of sophistication to the process, ensuring that as IAA levels accumulate, the enzymes responsible for its synthesis are regulated, preventing excessive production (Dong & Lin, 2021).

The profound understanding of these genetic mechanisms not only enriches our comprehension of microbial physiology but also holds significant biotechnological promise. Applications such as genetic engineering and synthetic biology approaches can leverage this knowledge to tailor and enhance IAA production in microorganisms like *Pseudarthrobacter sp* (Etesami & Glick, 2024). These biotechnological strategies open avenues for optimizing IAA yields for applications in agriculture, where enhanced plant growth and stress tolerance can be achieved, and environmental remediation, where microorganisms can be tailored to mitigate the impact of pollutants through efficient IAA synthesis (Sudheer et al., 2020). The synergy between genetic regulation and biotechnological tools underscores the potential for innovative and sustainable solutions in agriculture and environmental management.

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2.7 Optimization of Fermentation Conditions for Enhanced IAA Yield

The optimization of fermentation conditions for enhanced Indole-3-Acetic Acid (IAA) yield is a critical aspect of harnessing the plant growth-promoting potential of microorganisms, particularly exemplified by *Pseudarthrobacter sp* (Benadjila et al., 2022*)*. This process involves the systematic adjustment of various parameters to create an environment that maximizes the production of IAA. One key consideration is the choice and concentration of carbon sources, such as molasses and fructose, which serve as essential substrates for microbial metabolism (Ammar & Philippidis, 2021). The availability of nitrogen sources, represented by components like beef extract and tryptone, also plays a pivotal role in influencing IAA production (Elsoud et al., 2023). The concentration of the precursor L-tryptophan further becomes a focal point for optimization, with variations in its levels impacting the efficiency of IAA biosynthesis (Castiglione et al., 2021). The fermentation conditions encompass environmental factors such as temperature, pH, and oxygen levels, which need meticulous calibration to create an environment conducive to the enzymatic reactions involved in IAA synthesis (Dhere & Ghavri, 2023).

Moreover, the duration of the fermentation process is a critical factor, as prolonged exposure to optimized conditions may lead to increased IAA yields (Bunsangiam et al., 2021). The optimal conditions for IAA production is not only vital for maximizing yields but also holds significance for the potential biotechnological applications of *Pseudarthrobacter sp*. in sustainable agriculture and environmental remediation (Alotaibi et al., 2022).

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2.8 Bioremediation

Bioremediation is the use of living organisms, such as bacteria, fungi, plants, or enzymes, to remove, degrade, or transform pollutants from polluted environments (S. Bala et al., 2022). It is a cost-effective and environmentally favorable method for cleaning up contaminated sites and mitigating pollution. Bioremediation involves the introduction or stimulation of microorganisms or plants native to the environment or specifically selected for their ability to degrade pollutants at a contaminated site (Sharma, 2020). These organisms use pollutants as a source of energy or nutrients, converting them into less hazardous substances or non-toxic forms (Saravanan et al., 2021). Depending on the character of the contaminants and the site's conditions, various bioremediation techniques are employed. This strategy involves treating the contaminants directly at the contaminated site. To degrade pollutants in place, microorganisms or plants are introduced or stimulated in contaminated soil, groundwater, or sediment. Bioremediation is applicable to a wide variety of contaminants, such as petroleum hydrocarbons (e.g., crude oil, gasoline), chlorinated solvents, pesticides, heavy metals, and other organic or inorganic contaminants (Neethu et al., 2019). It is used in industrial sites, landfills, oil accidents, and groundwater contamination, among other contaminated environments.

The success of bioremediation depends on the selection or introduction of organisms with the metabolic capabilities necessary to target specific pollutants (Saroj Bala et al., 2022). These organisms can either occur naturally in a polluted environment or be selected for their ability to degrade pollutants. Utilizing existing biodiversity or introducing suitable organisms, bioremediation facilitates the degradation of pollutants into less hazardous substances or their transformation into non-toxic forms.

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2.9 Effectiveness of bioremediation

Bioremediation's efficacy can vary depending on several factors, such as the nature and extent of contamination, site-specific conditions, remediation techniques chosen, and the characteristics of the contaminants themselves (Xia et al., 2019). Some contaminants are biodegradable more readily than others. The efficacy of bioremediation is contingent on whether the selected microorganisms or plants have the metabolic capacity to degrade the specific contaminants present (Enerijiofi, 2021). Certain contaminants may be more difficult to eliminate and necessitate the use of specialized microbial strains or additional treatment methods. Recent research indicates that the use of multiple living organisms will undoubtedly result in more efficient and effective bioremediation, and will pave the way for the exploration of greater microbial diversity (Kour, Kaur, Devi, Yadav, Singh, Joshi, Singh, Suyal, Kumar, Rajput, et al., 2021). Environmental factors such as temperature, pH, moisture content, oxygen availability, and nutrient concentrations significantly affect the activity and growth of bioremediation microorganisms (Adams et al., 2015). It is necessary to sustain optimal environmental conditions so that microbial populations can flourish and effectively degrade contaminants. The availability and equilibrium of these nutrients in a contaminated environment can influence microbial activity and the rate of contaminant degradation (Kour, Kaur, Devi, Yadav, Singh, Joshi, Singh, Suyal, Kumar, & Rajput, 2021). To maximize microbial growth and improve biodegradation rates, nutrient supplementation may be required (Varjani & Upasani, 2019). Regular monitoring of contaminant concentrations, microbial activity, and environmental parameters is essential for determining bioremediation's progress and efficacy.

In designing and implementing bioremediation strategies, several parameters are crucial. These parameters optimize the process and ensure that contaminants are effectively degraded. In order to accelerate microbial growth and decomposition, environmental parameters must frequently be altered during bioremediation. (Ren et al., 2018). Understanding the nature and concentration of contaminants is essential for selecting appropriate bioremediation approaches and determining the practicability of the process, which are two crucial bioremediation parameters. Maintaining optimal conditions for the growth and metabolic activity of degrading organisms is essential. Important are the presence and abundance of microorganisms capable of degrading the contaminants (Mbachu et al., 2020). Numerous bioremediation procedures rely on

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aerobic degradation, which necessitates a sufficient oxygen supply (Nagda et al., 2022). In an anaerobic environment, many microorganisms can ferment organic carbon and make hydrogen gas (Alvarez et al., 2017) .To support their growth and pollutant degradation, microorganisms require essential nutrients such as nitrogen, phosphorus, and trace elements (Mbachu et al., 2020).

2.10 Role of microorganisms in pollutant degradation

Bioremediation relies heavily on microorganisms to degrade pollutants. They possess a variety of metabolic capabilities that allow them to use pollutants as sources of energy and nutrients, ultimately degrading them into less hazardous or non-toxic forms (Doukani et al., 2022). Numerous microorganisms have evolved the ability to generate enzymes that degrade specific pollutants. These enzymes facilitate the transformation of complex organic compounds into simpler, more manageable forms that microorganisms can use as carbon and energy sources (Doukani et al., 2022). Microorganisms can target a vast array of contaminants, including hydrocarbons, chlorinated solvents, pesticides, and aromatic compounds, among others(Manian, 2023). Environmental samples such as soil, detritus, and water contain diverse microbial communities. Different microorganisms within these communities possess distinct metabolic capabilities, enabling the concurrent degradation of multiple pollutants (Saroj Bala et al., 2022).

Communities of microorganisms frequently exhibit synergistic interactions, in which the metabolic activities of one microorganism support or enhance the activities of others (Fouad et al., 2022).This cooperative behavior improves the overall efficacy of decomposition and ensures the complete destruction of pollutants (Zhou et al., 2023). Microorganisms can adapt to fluctuating environmental conditions and exposure to pollutants. Over time, microbial communities can evolve and select for microbes with enhanced degradation capacities. This adaptation enables microorganisms to efficiently degrade pollutants that were previously inaccessible or toxic to them, thereby enhancing bioremediation capability (Alori et al., 2022). Microorganisms not only contribute to the degradation of pollutants, but also to their detoxification and mineralization (Aragaw, 2021). Through metabolic processes, microorganisms can convert noxious pollutants into less dangerous compounds or inert forms. This detoxification

FYP FBKT
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lessens the environmental impact of pollutants and facilitates the restoration of polluted ecosystems (Nisa et al., 2022).

Frequently, microorganisms form biofilms, which are cell communities encased in a matrix of extracellular polymeric substances (Mahto et al., 2022). Biofilms provide a protective environment for microorganisms, enabling them to endure severe conditions and persist in contaminated environments (Mishra et al., 2022). Biofilms also enhance pollutant degradation by facilitating the proximity of various microbial species, fostering metabolic interactions, and bolstering the overall resilience of the microbial community (Wu et al., 2023). Involved microorganisms in biodegradation processes not only degrade pollutants, but also contribute to nutrient cycling (S. Mishra et al., 2021). As microorganisms degrade pollutants, they release essential nutrients back into the environment, thereby promoting ecosystem health and fostering the development of other organisms (Timmis & Ramos, 2021).

2.11 Enzymes involved in bioremediation activities of *Pseudarthrobacter sp*

Pseudarthrobacter sp. is a genus of bacteria renowned for its diverse metabolic capabilities and potential for degrading pollutants. While the specific enzymes involved in biodegradation can vary depending on the particular strain of *Pseudarthrobacter sp.* and the targeted pollutants, one common group of enzymes associated with *Pseudarthrobacter sp.* is dioxygenases (Asimakoula et al., 2022). Dioxygenase enzymes play a crucial role in the initial stages of aromatic compound degradation. *Pseudarthrobacter* strains may possess dioxygenases that initiate the breakdown of pollutants such as phenols, catechols, and other aromatic compounds by catalyzing the introduction of molecular oxygen into the aromatic ring (Vanhoutte, 2021). The presence of dioxygenases in *Pseudarthrobacter sp.* is highly significant due to their pivotal role in the breakdown of aromatic compounds. Dioxygenases catalyze the crucial initial steps of aromatic compound degradation, making them essential for pollutant removal and bioremediation processes (Medić & Karadžić, 2022). Dioxygenases function by facilitating ring hydroxylation or aromatic ring cleavage, wherein they insert two atoms of oxygen (O2) into the aromatic ring. This process results in the formation of a dihydroxylated

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intermediate, marked by the incorporation of hydroxyl groups (-OH) into the aromatic ring structure (Rahaman et al., 2016).

The addition of hydroxyl groups by dioxygenases significantly alters the electronic and chemical properties of the aromatic ring, rendering it more reactive and amenable to subsequent degradation steps (Maier, 2019). This enhanced reactivity is vital for breaking down recalcitrant aromatic compounds that would otherwise persist in the environment. The dihydroxylated intermediate produced through dioxygenase activity serves as a starting point for further metabolism within *Pseudarthrobacter* cells. Other enzymes and metabolic pathways act upon this intermediate, leading to its continued breakdown and eventual mineralization. *Pseudarthrobacter sp.* exhibit a remarkable capability to target and degrade a wide range of aromatic pollutants such as phenols. Phenols are organic compounds characterized by the presence of a hydroxyl group (-OH) directly attached to an aromatic ring (Abbas et al., 2017).

Pseudarthrobacter strains produce specific dioxygenases tailored to hydroxylate these aromatic pollutants. The hydroxylation process initiated by these dioxygenases is instrumental in the breakdown and degradation of phenols and similar aromatic compounds commonly found in polluted environments. The diverse metabolic capabilities of *Pseudarthrobacter sp.* , coupled with their possession of specific dioxygenases, underline their potential as effective bioremediators (Asimakoula et al., 2022). By harnessing these enzymatic activities, *Pseudarthrobacter* strains can actively participate in the removal of aromatic pollutants from contaminated sites, thereby contributing to environmental cleanup efforts. It's worth noting that while the general principles of dioxygenase-mediated degradation apply to *Pseudarthrobacter sp*., the specific enzymes involved can vary among strains and their environmental niches. This adaptability allows *Pseudarthrobacter sp* to address a broad spectrum of aromatic pollutants encountered in different polluted habitats.

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CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Bacterial Strain

Pseudarthrobacter sp UMK-PNF5 is a plant-growth promoting rhizobacteria. The bacterium was isolated from paddy field by a previous study. This bacterium demonstrated the ability to produce indole-3-acetic acid (IAA), suggesting its potential role as a plant growth-promoting bacterium (PGPB) (Kamaruzaman et al., 2022).

3.1.2 Materials and Apparatus

Apparatus that were used in this research include orbital Incubator shaker, petri dish, conical flask, centrifuge, spectrophotometer, rotary shaker, laminar flow chamber, autoclave machine, Bunsen burner, electronic balance, micropipette, tips, inoculating loop, sterile falcon tube, media bottles, beakers, measuring cylinder, aluminium foils, parafilm, gauze, cotton, cuvette, syringe filter 0.22µm, and syringe.

3.1.3 Chemical and reagent

The materials that were used in this research are L-tryptophan, molasses, fructose, beef extract, tryptone, Phenol, glucose, 4-aminoantipyrine solution, potassium ferricyanide solution, 2N ammonium hydroxide, acetone, sodium hydroxide, potassium dihydrogen, sodium chloride, ammonium chloride, indole-3-acid, nutrient agar, LB broth and LB agar.

3.2 Methods

3.2.1 Preparation of media

a) Luria-Bertani Medium

The Luria-Bertani (LB) medium was prepared by dissolving 25 grams of Luria-Bertani (LB) agar powder in 1 L of distilled water. The mixture was thoroughly stirred to ensure the complete dissolution of the agar powder. Following this, the medium was subjected to sterilization through autoclaving at 121°C for 15 minutes. After autoclaving, the medium was allowed to cool to a temperature suitable for handling. Subsequently, the sterilized LB agar was poured into a sterile Petri dish and left to solidify, forming a solid medium suitable for bacterial cultivation. To make Luria-Bertani (LB) broth, a conical flask was employed. Sterile gauze and cotton were used to cover the flask's opening, creating a barrier against external contaminants while allowing for aeration.

b) Nutrient Agar Medium

The nutrient medium was prepared by combining 1 L of distilled water with 13 grams of nutrient agar powder. The mixture was thoroughly stirred to ensure the complete dissolution of the agar powder. Following this, the medium was subjected to sterilization through autoclaving at 121°C for 15 minutes. After autoclaving, the medium was allowed to cool to a temperature suitable for handling. Subsequently, the sterilized nutrient agar was poured into a sterile Petri dish and left to solidify, forming a solid medium suitable for bacterial cultivation. To make nutrient broth, a conical flask was employed. Sterile gauze and cotton were used to cover the flask's opening, creating a barrier against external contaminants while allowing for aeration.

c) Minimal Medium M9 (MM-M9)

The minimal medium M9 (MM-M9) was prepared by weighing and dissolving 3g of KH_2PO_4 (potassium dihydrogen phosphate), 0.5g of NaCl (sodium chloride), and 1g of NH_4Cl (ammonium chloride) in 100ml of distilled water. The resulting solution was sterilized by autoclaving at 121°C for 15 minutes to eliminate potential contaminants. After cooling to room temperature, 2g of glucose was added as the carbon source as showed in Table 3.1.

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Table 3.1: Composition of Minimal Medium M9 (Soma et al., 2023)

Composition of Minimal Medium M9 (MM-M9)	Concentration (g/mL)
Potassium Dihydrogen Phospate (KH2PO4)	3
Sodium Chloride (NaC1)	0.5
Ammonium Chloride (NH4C1)	1
Glucose (C6H12O6)	2

d) L-Tryptophan Stock Preparation

The L-tryptophan stock solution was prepared by adding 0.5 grams of L-tryptophan powder to a sterile Falcon tube to achieve a concentration of 50000 µg/mL. Subsequently, 15 ml of distilled water was carefully added to the sterile falcon tube, and the contents were mixed thoroughly to dissolve the L-tryptophan powder. To adjust the pH and facilitate solubility, 5 ml of a 0.2g/mL solution of 1 molar NaOH was slowly added to the mixture. The reaction resulted in the formation of a soluble salt of L-tryptophan. The solution was then drawn into a sterile syringe, and to ensure sterility, it was passed through a sterile 0.22 µm syringe filter into another sterile falcon tube. This step effectively removed any particulate matter or microbial contaminants.

Table 3.2: Composition of L-Tryptophan Stock (Lazzari et al., 2019)

Composition of L-Tryptophan Stock	Concentration (g/mL)
L-Tryptophan (C11H12N2O2)	0.5
Sodium Hydroxide (NaOH)	0.2
Distilled Water (dH2O)	15

3.2.2 Evaluation of Indole-3-Acetic Acid Production

a) Preparation of Salkowski Reagent

The preparation of the Salkowski reagent took place in the fume hood to ensure safe handling of chemicals. In this process, 1 ml of 0.5M iron (III) chloride, 50 ml of distilled water, and 30 ml of concentrated sulfuric acid were added to sterile falcon tube. The procedure began with the careful measurement of 1 ml of 0.5M iron (III) chloride, followed by the addition of 50 ml of distilled water. Subsequently, 30 ml of concentrated sulfuric acid was introduced to the mixture.

Table 3.3: Composition of Salkowski Reagent ((Ham et al., 2021))

Composition of Salkowski Reagent	Concentration (g/mL)
Iron (III) Chloride (FeCl3)	1
Sulfuric Acid (H2SO4)	30
Distilled Water (dH2O)	50

b) Preparation of Standard Curve of Indole-3-Acetic Acid

10 mg of IAA powder was weighed out and mixed with 10 ml of acetone. IAA standard solutions were prepared, each containing IAA at concentrations of 0, 2, 4, 5, 10, 20, 30, 60, 100, 120, 150, and 200 µg/ml. Subsequently, 1 ml of IAA Standard Solution was mixed with 2 ml of Salkowski reagent and kept in the dark at room temperature for 25 minutes. A blank was generated by mixing 2 ml of Salkowski reagent with 1 ml of distilled water. The optical density (OD) of the mixtures was measured at 530_{nm}, and a graph was constructed to illustrate the relationship between the quantity of IAA and the corresponding absorbance values.

c) Indole-3-Acetic Acid Production Assay

The *Pseudarthrobacter sp* UMK-PNF 5 strain was inoculated into Luria-Bertani (LB) broth and incubated at 36°C until they reached OD ±0.4. Then, different concentrations of tryptophan which are 100, 500, 1000, 3000, 6000 and 9000 µg/ml added to the culture respectively. After being incubated at 37°C with agitation at 121 rpm for 7 days, the bacterial culture was subsequently centrifuged at 9000 rpm for 15 minutes. The supernatant was filtered using a 0.22-µm syringe filter and mixed with 2ml of Salkowski reagent and incubated in the dark at room temperature for 25 minutes. Absorbance was measured at $O.D_{530}$.

3.2.3 Evaluation of Indole-3-Acetic Acid Production by using different Nitrogen and Carbon sources

a) Indole-3-Acetic Acid Production Assay by using different Nitrogen and Carbon Sources with addition of L-Tryptophan

i. Nitrogen Sources

The *Pseudarthrobacter sp* UMK-PNF 5 strain was cultured by inoculating it into Luria-Bertani (LB) broth, and the culture was allowed to grow at 36°C until reaching an optical density (OD) of approximately ±0.4. Subsequently, 1% of different nitrogen sources, specifically beef extract and tryptone, were separately added to Luria-Bertani (LB) media supplemented with 250µg/mL of L-tryptophan. Following a 4-day incubation period, the bacterial culture underwent centrifugation at 9000 rpm for 15 minutes. The resulting supernatant was then subjected to filtration using a 0.22-µm syringe filter to eliminate any particulate matter. The filtered supernatant was mixed with 2 ml of Salkowski reagent and incubated in the dark at room temperature for 25 minutes. This incubation allowed for the development of a colorimetric reaction indicative of the presence of indole compounds, such as indole-3-acetic acid (IAA) produced by the bacteria. After 25-minute incubation, the absorbance of the mixture was measured at 530_{nm} using a spectrophotometer.

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ii. Carbon Sources

The *Pseudarthrobacter sp* UMK-PNF 5 strain was cultured by inoculating it into Luria-Bertani (LB) broth, and the culture was allowed to incubate at 36°C until reaching an optical density (OD) of approximately ±0.4. Subsequently, 1% of different carbon sources, specifically molasses and fructose, were separately added to Luria-Bertani (LB) media supplemented with 250µg/mL of L-tryptophan. Following a 4-day incubation period, the bacterial culture underwent centrifugation at 9000 rpm for 15 minutes. The resulting supernatant was then subjected to filtration using a 0.22-µm syringe filter to eliminate any particulate matter. The filtered supernatant was mixed with 2 ml of Salkowski reagent and incubated in the dark at room temperature for 25 minutes. This incubation allowed for the development of a colorimetric reaction indicative of the presence of indole compounds, such as indole-3-acetic acid (IAA) produced by the bacteria. After 25-minute incubation, the absorbance of the mixture was measured at 530_{nm} using a spectrophotometer.

3.2.4 Phenol Bioremediation by using *Pseudarthrobacter sp* UMK-PNF5

a) Bacterial strain and growth conditions

Pseudarthrobacter sp. UMK-PNF5 was cultivated in Luria-Bertani broth (LB) medium, employing 300 mg/ml of phenol and 400 mg/L of glucose as the carbon and energy sources. The bacterial culture was then subjected to agitation in a rotary shaker at 180 rpm at 30°C, allowing for optimal growth conditions.

b) 4-aminoantipyrine Colometric Preparation

The 4-aminoantipyrine colorimetric method was initiated by preparing a 2% solution of 4- aminoantipyrine was created by dissolving the compound in water and ensuring complete dissolution. Simultaneously, an 8% potassium ferricyanide solution was prepared by dissolving the compound in an appropriate solvent. Concurrently, a 2 N ammonium hydroxide solution was made by diluting ammonium hydroxide with water. Once these solutions were ready, they were combined in a clean container in the following proportions: equal parts of the 2% 4- amino

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antipyrine solution and the 8% potassium ferricyanide solution, along with two parts of the 2 N ammonium hydroxide solution. To achieve the optimal conditions for the reaction, the pH of the mixture was carefully adjusted to 6.9 ± 0.1 using a pH meter. This was done by adding small amounts of either hydrochloric acid or sodium hydroxide as needed. The final step involved thorough mixing of the combined solutions to ensure a homogeneous reaction mixture.

Table 3.4: Composition of 4-aminoantipyrine reagent (Asimakoula et al., 2023)

Composition of 4-aminoantipyrine reagent	Concentration (g/mL)
4-aminoantipyrine	1
2 N ammonium hydroxide	30
Potassium ferricyanide	50

c) Assessment of growth and determination of residual phenol in culture medium

The culture was initially inoculated with an optical density of 0.15 at 600 $_{nm}$, and the growth of bacteria was monitored by measuring optical density at 600 nm at different time intervals. Subsequently, the culture was inoculated with boil-dead cells to establish an abiotic control. Viable cell counts were determined by counting colony-forming units (CFU) on spread plates. After 48 hours of incubation at 30°C, 100 µL of *Pseudarthrobacter sp* UMK-PNF5 was inoculated onto agar plates, and the viable count was determined. 1 mL samples were taken, centrifuged, and filtered using 0.22 µm syringe filters. The concentration of phenol was determined using the 4-aminoantipyrine colorimetric method. The resulting mixture was diluted with 1 mL of distilled water and allowed to react for 15 minutes at room temperature. Following incubation, a spectrophotometer was used to measure the absorbance at 510 nm, and a calibrated standard curve was employed to determine the phenol concentration.

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CHAPTER 4

RESULTS AND DISCUSSION

4.1 Evaluation of Indole-3-Acetic Acid production under different L-Tryptophan concentrations

To assess the production of Indole-3-Acetic Acid (IAA) in *Pseudarthrobacter sp* UMK-PNF5, the strain was cultivated as an inoculum in Luria-Bertani (LB) broth until an optical density of approximately 0.5 at 530 $_{nm}$ was attained. Subsequently, the culture was transferred to 50 mL of Luria-Bertani (LB) broth supplemented with varying concentrations of L-tryptophan (0, 1000, 3000, 6000, and 9000 µg/ml) to induce IAA production. The quantification of IAA was performed utilizing a previously prepared standard curve. The IAA content was determined based on the IAA Standard Curve show in Figure 4.1.

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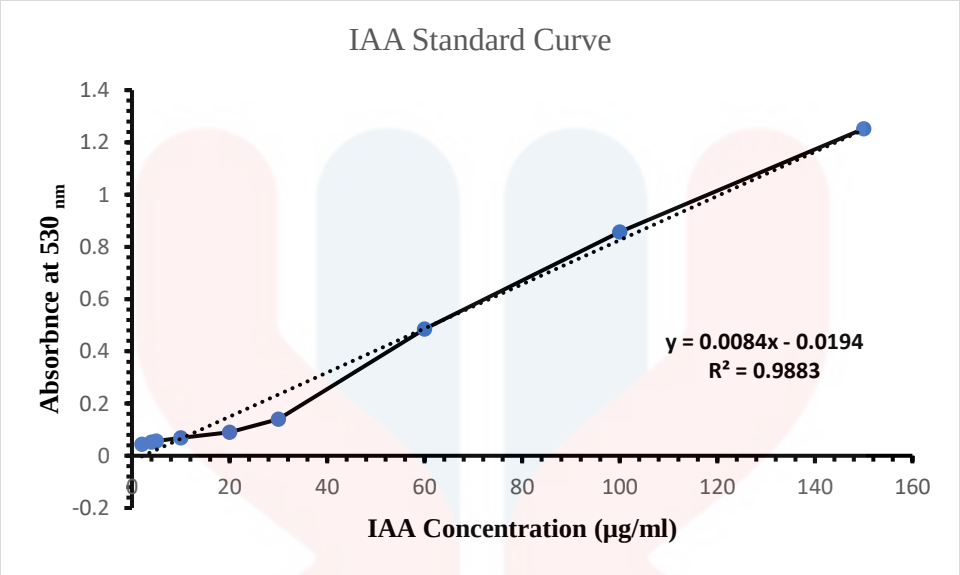

Figure 4.1: IAA Standard Curve

The data presented illustrates the relationship between indole-3-acetic acid (IAA) production in micrograms per milliliter (µg/ml) and the corresponding absorbance at 530_{nm}. As IAA production increases from 2 µg/ml to 150 µg/ml, there is a noticeable upward trend in absorbance at 530_{nm}. This pattern is clearly visualized in a scatter plot of the data points, where the absorbance values progressively rise with higher concentrations of IAA. The scatter plot suggests a positive correlation between IAA production and absorbance at 530_{nm}, indicating that an increase in IAA concentration is associated with an elevation in absorbance levels. The correlation coefficient, a measure of the strength and direction of this relationship, would likely confirm the observed positive correlation. This outcome suggests that the assay or experiment used to measure IAA production is detecting higher concentrations of IAA through increased absorbance values, highlighting a potential quantitative relationship between the two variables. Further statistical analysis, such as calculating the correlation coefficient and performing regression analysis, could provide additional insights into the nature and strength of this correlation. These findings contribute valuable information for studying plant hormone production and its quantification, emphasizing the importance of accurately measuring IAA levels for a comprehensive understanding of plant physiological processes.

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4.2 Evaluation of Indole-3-Acetic Acid Production with Different L-Tryptophan Concentration

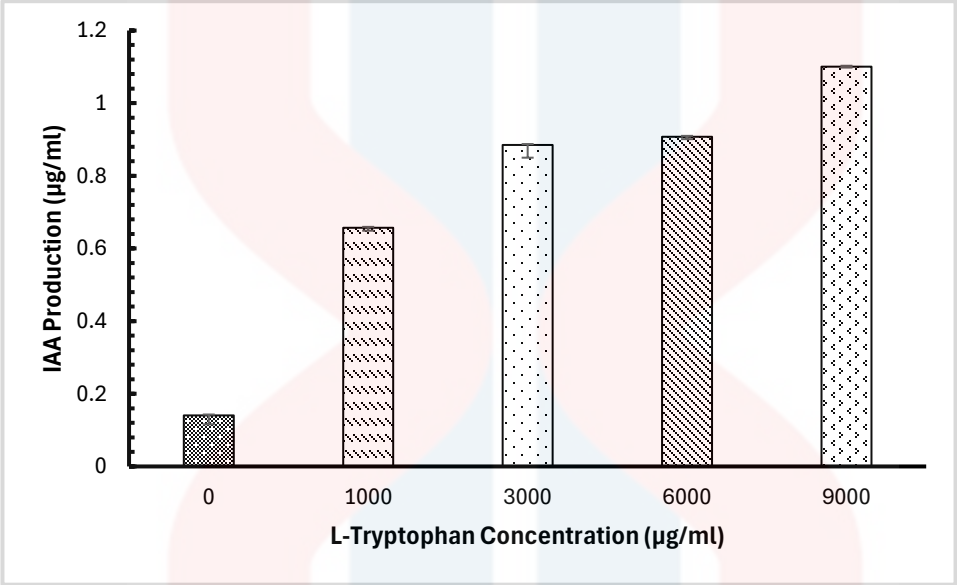

Figure 4.2: IAA Production by *Pseudarthrobacter sp* UMK-PNF5

Based on previous study, the study discussed the optimization of fermentation conditions to enhance the production of indole acetic acid (IAA) by *Pseudarthrobacter sp.* NIBRBAC000502770, a bacterium isolated from shooting range soil in South Korea. IAA is a vital compound recognized for its role in promoting plant growth and is typically synthesized by plant growth-promoting rhizobacteria (PGPR). The findings underscored the potential of *Pseudarthrobacter sp.* NIBRBAC000502770 as a valuable biological agent for enhancing plant growth. Moreover, optimizing the fermentation medium could pave the way for cost-effective large-scale production of this microorganism for agricultural applications (Asimakoula et al., 2023).

In Figure 4.2, the scatter plot provided a comprehensive depiction of the intricate relationship between L-tryptophan concentration (µg/ml) and indole-3-acetic acid (IAA) production (µg/ml) based on the experimental data. The figure revealed a pronounced and

nuanced pattern, highlighting a clear positive correlation between L-tryptophan levels and subsequent IAA production. At the lowest L-tryptophan concentration (0 µg/ml), the corresponding IAA production was minimal, registering at 0.141 µg/ml. This initial observation is in accordance with well-established biochemical knowledge. L-tryptophan serves as a crucial precursor for IAA biosynthesis in plants. The absence or low concentration of L-tryptophan restricts the availability of the substrate required for enzymatic conversion to IAA, resulting in lower overall IAA production (Keswani et al., 2020).

As L-tryptophan concentrations increased, there was a discernible and consistent rise in IAA production. This observation aligns seamlessly with the biochemical understanding that L-tryptophan is a pivotal precursor in the pathway leading to IAA synthesis. The positive correlation observed in figure 4.2 emphasizes the direct influence of L-tryptophan availability on the production of IAA, a key plant hormone involved in various growth and developmental processes (Noor et al., 2023).

Notably, at higher L-tryptophan concentrations (6000 µg/ml and 9000 µg/ml), there was a further escalation in IAA production. This could suggest a dose-dependent response, where elevated concentrations of the precursor molecule lead to increased enzymatic activity, consequently resulting in higher levels of IAA synthesis (Keswani et al., 2020). This concept is grounded in enzymatic kinetics, where the rate of product formation is influenced by the availability of the substrate.

However, it is imperative to acknowledge the potential existence of a saturation point in this relationship. Beyond a certain threshold concentration of L-tryptophan, further increases may not proportionally enhance IAA production. This saturation point could be attributed to factors such as enzyme saturation or other regulatory mechanisms within the biosynthetic pathway, emphasizing the complexity of the biological processes involved.

In the context of the observed positive correlation between L-tryptophan concentration and indole-3-acetic acid (IAA) production, the potential existence of a saturation point suggests intricate molecular and regulatory mechanisms at play (Moliszewska & Nabrdalik, 2020). However, beyond a certain threshold, a saturation point is acknowledged, and this phenomenon may be governed by several factors. Enzyme saturation is a plausible mechanism, where the catalytic activity of key enzymes becomes saturated at high substrate concentrations, limiting

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further increases in IAA production (López-Gómez et al., 2024). Additionally, regulatory mechanisms such as feedback inhibition may come into play, wherein elevated IAA levels negatively regulate the activity of essential enzymes in the biosynthetic pathway, preventing uncontrolled escalation (Yadav, 2020).

The results presented in Figure 4.2 not only enhance our comprehension of how L-tryptophan influences IAA production but also carry significant practical implications. This understanding is particularly valuable in agricultural settings, as it informs strategies for manipulating plant hormone levels to improve crop growth and yield. The biosynthetic pathway leading to the production of indole-3-acetic acid (IAA), a crucial plant hormone, is intricately linked to the concentration of L-tryptophan (Zhang et al., 2022). Tryptophan-dependent IAA biosynthesis primarily involves the activity of several enzymes. The initial step is catalyzed by tryptophan synthase, which converts chorismate to tryptophan (Suárez Pérez, 2020). Subsequently, the enzyme tryptophan aminotransferase facilitates the conversion of L-tryptophan to indole-3-pyruvic acid (IPA). The rate-limiting step in IAA biosynthesis is often attributed to the enzyme indole-3-pyruvate decarboxylase, which converts IPA to indole-3-acetaldehyde (Yue et al., 2021). Finally, indole-3-acetaldehyde is further transformed into IAA through the action of aldehyde dehydrogenases (Shah et al., 2021). The regulation of these enzymatic steps is finely tuned by the concentration of L-tryptophan, acting as a key precursor. Elevated levels of L-tryptophan can enhance the activity of these enzymes, thereby promoting increased IAA production. This intricate interplay underscores the significance of L-tryptophan in modulating the biosynthesis of IAA, a pivotal regulator of plant growth and development (Etesami & Glick, 2024).

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4.3 Determination of IAA production of *Pseudarthrobacter sp* UMK-PNF5 by using different carbon and nitrogen sources

To evaluate the synthesis of Indole-3-Acetic Acid (IAA) by *Pseudarthrobacter sp* UMK-PNF5 under varying carbon and nitrogen sources, the bacterial strain underwent initial cultivation in Luria-Bertani (LB) broth until reaching an optical density of approximately 0.5 at 530 nm. Following this, the culture was transferred to 50 mL of Luria-Bertani (LB) broth supplemented with diverse carbon sources, specifically molasses and fructose, along with beef extract and tryptone as nitrogen sources. This manipulation aimed to induce IAA production, with the addition of 250µg/ml of L-tryptophan to the medium. The quantification of IAA was accomplished by employing a previously established standard curve. The IAA content was determined based on the IAA Standard Curve show in Figure 4.1.

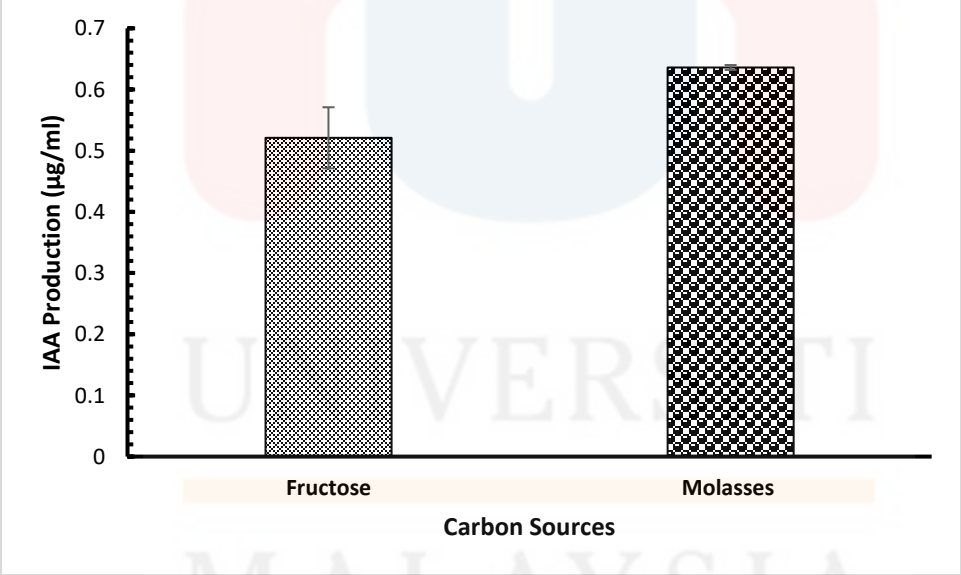

Figure 4.3: Effect of fructose and molasses as a carbon source on IAA production by *Pseudarthrobacter sp* UMK-PNF5

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In Figure 4.3, the absorbance values at 530_{nm}, representing the impact of different carbon sources (fructose and molasses) on a biological system, provided a detailed and insightful exploration of their effects. The experimental data indicated that fructose resulted in an absorbance of 0.521, whereas molasses lead to a slightly higher absorbance of 0.635.

The discernible difference in absorbance values suggested distinct effects of these carbon sources on the growth or metabolic activity of the investigated organism, which could have been a microbial culture or a plant cell culture. Absorbance at 530_{nm} was frequently utilized as a surrogate for cellular density or metabolic activity, and in this context, the higher absorbance associated with molasses implied its potential as a more favorable carbon source. This observation aligned with the understanding that molasses, being a complex mixture, provided a diverse array of sugars, minerals, and other nutrients that could robustly support microbial or cellular growth (Luo et al., 2021).

The specific nutrient compositions of molasses play a pivotal role in supporting cellular growth and Indole-3-Acetic Acid (IAA) production in microbial cultures, offering valuable insights for designing optimized culture media in biotechnological applications (Blanco Parte et al., 2020). Molasses, derived from the sugar extraction process, encompasses a diverse array of sugars, including sucrose, glucose, and fructose (Mangwanda et al., 2021). These sugars serve as essential carbon sources, providing energy for microbial metabolism and cellular growth (Bajic & Sanchez, 2020). Additionally, molasses contains trace elements, vitamins, and minerals derived from the original plant material, enriching the medium with crucial nutrients that support overall cellular health and function (Mourad et al., 2018).

In the context of IAA production, the diverse composition of molasses becomes particularly significant. Tryptophan, an amino acid precursor for IAA biosynthesis, is often present in molasses, contributing to the efficiency of the IAA production pathway (Rocha et al., 2024). The availability of a spectrum of carbon sources and essential nutrients in molasses influences microbial metabolic pathways, potentially enhancing the yield of IAA (Rocha et al., 2024).

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As a primary carbon source, these sugars serve as essential substrates for microbial metabolism, providing energy for cellular growth and the synthesis of vital cellular components (Jeckelmann & Erni, 2020). This knowledge informs the design of optimized culture media for biotechnological applications by enabling the formulation of media with balanced carbon sources and additional nutrients to support enhanced cellular growth and, consequently, heightened IAA production. The strategic incorporation of molasses into culture media serves as a fundamental step in creating environments conducive to microbial activities, laying the foundation for sustainable applications in agriculture and environmental management (Eyegheleme, 2023).

The lower absorbance reading observed for fructose in comparison to molasses can be attributed to the inherent compositional differences between these carbon sources and their impact on microbial growth. Fructose, being a simple monosaccharide, serves as a singular and straightforward carbon source for microbial metabolism (Sánchez-Martínez et al., 2020). As a standalone sugar, fructose may offer a more limited spectrum of nutrients compared to the complex mixture found in molasses. Consequently, microorganisms exposed to fructose might encounter a narrower range of substrates, potentially resulting in a less pronounced metabolic response and, consequently, a lower absorbance at 530 nm. This lower absorbance is indicative of reduced indole-3-acetic acid (IAA) production. Conversely, molasses with its diverse array of components, provides a richer and more varied set of nutrients for microbial utilization (Zhang et al., 2021). This can lead to a more robust metabolic response, reflected in higher absorbance readings at 530 nm and, consequently, increased IAA production.

Furthermore, fructose is directly assimilated by microorganisms through various metabolic pathways. Its rapid utilization might result in a shorter duration of active growth compared to the sustained microbial activity that can be sustained with the diverse sugars present in molasses (Kampen, 2014). The intricacies of fructose metabolism, including potential regulatory mechanisms and the absence of additional co-substrates, may contribute to the observed lower absorbance (Liu et al., 2020).

In contrast, molasses encompasses a mixture of sugars, including sucrose, glucose, and fructose, offering a richer and more varied nutrient profile. Microorganisms exposed to molasses are likely to encounter a more diverse set of carbon substrates, promoting prolonged and more extensive metabolic activities (Khatun, 2022). The complex composition of molasses provides a

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broader spectrum of nutrients, supporting microbial growth and potentially leading to a higher absorbance reading at 530 nm.

The detailed analysis of these findings also extends to the potential optimization of culture conditions according to specific research objectives. If the objective was to achieve higher biomass or metabolic activity, molasses might have been a more suitable choice due to its capacity to support robust growth. On the other hand, if the focus was on specific metabolic pathways or product synthesis, tailored carbon sources might have warranted consideration.

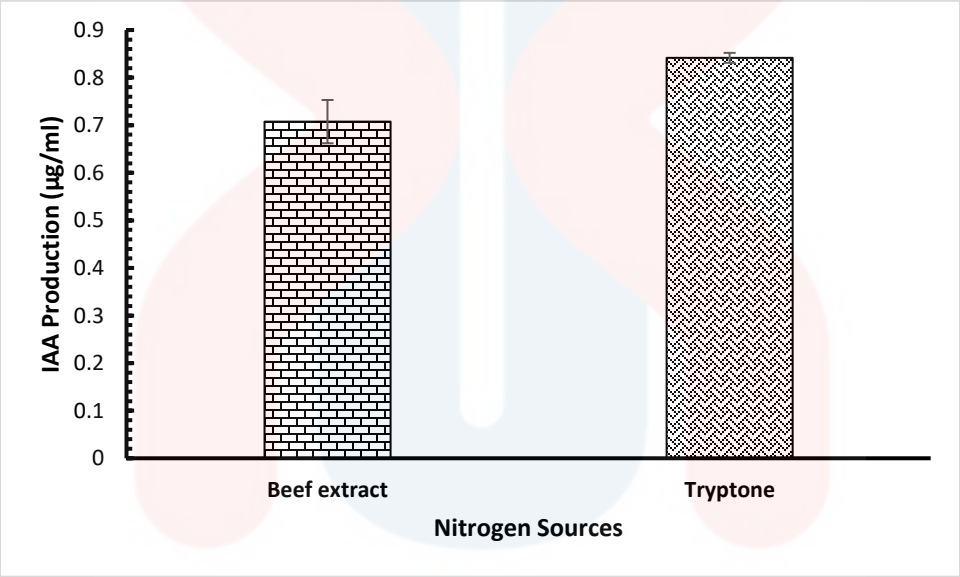

Figure 4.4: Effect of beef extract and tryptone as a nitrogen source on IAA production by *Pseudarthrobacter sp* UMK-PNF5)

In Figure 4.4, the IAA production in µg/ml resulting from different nitrogen sources, specifically beef extract and tryptone, was explored, providing a detailed understanding of their respective impacts on the biological system. The experimental data revealed that beef extract led to an IAA production of 0.658, while tryptone resulted in a higher IAA production of 0.842. The observed disparity in IAA production values suggests distinct effects of these nitrogen sources on the physiological responses of the investigated organism, likely a microbial or plant cell culture. Indole-3-acetic acid (IAA) is a key plant hormone known for its role in various growth and

developmental processes, and its production serves as an indicator of the organism's response to different nitrogen sources (Etesami & Glick, 2024).

The higher IAA production associated with tryptone, compared to beef extract, indicates that tryptone might have provided a more conducive nitrogen environment to produce IAA. Tryptone is a complex mixture of peptides and amino acids, and its ability to support higher IAA production aligns with the fact that IAA biosynthesis often involves precursor molecules derived from amino acids (Hernández-Fernández et al., 2022).

The higher Indole-3-Acetic Acid (IAA) production observed when using tryptone as a nitrogen source prompts an exploration into the specific amino acids or peptides within tryptone that could potentially serve as precursors for IAA biosynthesis. Tryptone, a complex mixture of peptides and amino acids derived from the enzymatic digestion of casein, contains a diverse array of organic compounds (Amaresan et al., 2020). In the context of IAA biosynthesis, certain components of tryptone are particularly noteworthy.

Tryptophan, an essential amino acid, is a primary precursor for IAA biosynthesis in many microorganisms. Within tryptone, the presence of tryptophan becomes a key factor contributing to enhanced IAA production. Tryptophan serves as the substrate for enzymes involved in the conversion of tryptophan to IAA, such as tryptophan-2-monooxygenase and tryptophan-5-monooxygenase (Zhou et al., 2021).

Peptides in tryptone, comprising various combinations of amino acids, may also play a role in providing additional precursors for IAA biosynthesis. Proteolytic breakdown of peptides within tryptone could release amino acids, including tryptophan, and other compounds that participate in the IAA synthesis pathway (Sypka et al., 2021).

Comparatively, beef extract, another nitrogen source commonly used in microbial cultures, may lack the abundance of tryptophan present in tryptone. This difference in tryptophan content could explain the observed variations in IAA production. Tryptone's richer composition in tryptophan may offer a more favorable substrate pool for the enzymatic reactions leading to increased IAA synthesis. Additionally, investigating the expression levels of genes associated with IAA biosynthesis under different nitrogen sources may provide insights into the molecular mechanisms underlying the observed differences. Overall, the exploration of tryptone's

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composition and its influence on IAA production offers a pathway for understanding the intricacies of microbial metabolism and optimizing conditions for enhanced IAA yields (Chen, 2021).

Conversely, the inverse relationship between lower absorbance and diminished indole-3-acetic acid (IAA) production, as well as higher absorbance correlating with elevated IAA production, suggests that beef extract, under the specified experimental conditions, may not have been as proficient in fostering IAA synthesis. This disparity in IAA production values emphasizes the critical importance of thoughtfully choosing nitrogen sources in experimental setups, given their substantial influence on the measured biological responses.

4.4 Phenol bioremediation by using *Pseudarthrobacter sp* UMK-PNF5

Pseudarthrobacter phenanthrenivorans Sphe3 efficiently catabolizes phenol as its sole source of carbon and energy, primarily through the catechol ortho-cleavage pathway. The main degradation pathway was determined by analyzing the transcription of genes involved in phenol degradation, assessing the activity of the corresponding enzymes, and detecting the metabolic products generated during phenol degradation. This comprehensive approach allowed for the elucidation of the specific biochemical pathways utilized by Sphe3 for the breakdown of phenol (Asimakoula et al., 2022).

In this study, the *Pseudarthrobacter sp* UMK-PNF5 culture was inoculated at 30°C with 125 rpm for 48 hours using boiled dead cells. Subsequently, the culture was transferred to an LB agar plate to assess colony-forming units (CFU). Unfortunately, after inoculating for 48 hours, the CFU results revealed that no single cell was able to grow on the plate. This unexpected outcome raises several questions, prompting a deeper exploration into the factors that might have influenced the bioremediation potential of *Pseudarthrobacter sp.* UMK-PNF5 under the given conditions.

One plausible explanation for the lack of observed growth could be related to the strain's adaptation to the MM-M9 medium. Microbial strains often exhibit varying degrees of adaptability to different nutritional environments, and the failure of *Pseudarthrobacter sp.* UMK-

PNF5 to thrive in the provided medium could be attributed to a lack of compatibility (Amaya-Gómez et al., 2020). Microbial growth depends on the availability and assimilation of specific nutrients, and the selected medium may not have provided the necessary elements essential for the strain's metabolic processes (Di Caprio, 2021).

Moreover, the inhibitory effect of phenol on the growth of *Pseudarthrobacter sp.* UMK-PNF5 is a critical consideration. Phenol, as a target compound for bioremediation, may have been present in concentrations that exceeded the strain's tolerance threshold. High phenol concentrations can exert toxic effects on bacteria, leading to growth inhibition and a subsequent failure in initiating the degradation process (Panigrahy et al., 2022).

The phenol degradation pathway typically requires essential nutrients and cofactors to facilitate enzymatic reactions. These may include nitrogen sources such as amino acids or nitrogenous compounds, phosphorus-containing compounds like phosphate ions, sulfur-containing compounds such as sulfate ions, and trace elements like iron, magnesium, and manganese. Additionally, specific cofactors such as NADH/NADPH (nicotinamide adenine dinucleotide), FADH2 (flavin adenine dinucleotide), and various coenzymes may be essential for enzymatic activity within the phenol degradation pathway. The absence or deficiency of any of these nutrients or cofactors can significantly impact the efficiency of the phenol degradation process. Therefore, ensuring the presence of these essential components is crucial for effective phenol biodegradation. Bioremediation processes are intricate and involve specific enzymatic pathways. The lack of certain co-factors could have hampered the bacterium's ability to efficiently initiate and complete the phenol degradation process (Marghade et al., 2021). Identifying and supplementing these essential elements in subsequent experiments could potentially enhance the biodegradation efficiency of *Pseudarthrobacter sp.* UMK-PNF5.

The incapacity of *Pseudarthrobacter sp*. UMK-PNF5 to thrive in the presence of phenol can be attributed to a combination of factors rooted in the intrinsic properties of phenol and the strain's limitations in adapting to and mitigating its toxic effects. Phenol, known for its cytotoxicity, imposes severe stress on microbial cells by disrupting cell membranes and critical cellular processes (Jagaba et al., 2022). *Pseudarthrobacter sp*. UMK-PNF5 may lack efficient mechanisms to counteract these deleterious effects, leading to compromised cell integrity and eventual death in the phenol-rich environment. Furthermore, phenol degradation pathways often

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demand substantial energy expenditure and specific enzymatic activities (Panigrahy et al., 2022). If the strain struggles to allocate energy effectively or lacks the requisite enzymatic machinery for phenol metabolism, it would hinder its ability to detoxify and survive in the presence of phenol (Said et al., 2021). The absence or inefficiency of adaptive responses to phenol, such as the absence of detoxification enzymes or transporters, could further contribute to the observed inability of *Pseudarthrobacter sp*. UMK-PNF5 to endure in phenol-contaminated environments (Patel et al., 2022). The influence of environmental conditions on microbial activity cannot be understated. Variations in pH, temperature, and oxygen levels can significantly impact the performance of bacteria in bioremediation processes (M. Mishra et al., 2021). Fine-tuning these environmental parameters based on the strain's preferences and optimizing the conditions for phenol degradation might be crucial for achieving positive results in future experiments.

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CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

In conclusion, this study investigates various aspects of *Pseudarthrobacter sp.* UMK-PNF5, including its ability to produce indole-3-acetic acid (IAA) under different L-tryptophan concentrations, the influence of different carbon and nitrogen sources on IAA production, and its capacity for phenol degradation. The findings shed light on the metabolic capabilities of *Pseudarthrobacter sp.* UMK-PNF5 and its potential applications in agriculture and environmental remediation. Through meticulous experimentation, it was determined that the bacterium exhibited varying levels of IAA production depending on the concentration of L-tryptophan and the types of carbon and nitrogen sources provided. Additionally, the study revealed that *Pseudarthrobacter sp.* UMK-PNF5 does not possess the capability to degrade phenol, a significant environmental pollutant, indicating limitations in its potential for bioremediation. These insights contribute to a deeper understanding of the bacterium's capabilities and pave the way for further research into harnessing its biotechnological potential for sustainable agricultural practices and environmental protection efforts.

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5.2 Recommendations

Based on the comprehensive analysis of the experimental results, it is advisable to explore alternative strategies for optimizing the production of indole-3-acetic acid (IAA) by *Pseudarthrobacter sp.* UMK-PNF5. This exploration could entail investigating additional factors beyond L-tryptophan concentrations, such as variations in growth conditions, pH levels, or co-cultivation with other microorganisms known to enhance IAA production. Additionally, considering the significant influence of carbon and nitrogen sources on IAA production, conducting a more extensive screening of different substrates and their combinations could offer valuable insights for maximizing IAA yields. Furthermore, since the study has demonstrated that *Pseudarthrobacter sp.* UMK-PNF5 lacks the capability to degrade phenol, it is recommended to explore alternative bacterial strains or bioremediation approaches to address phenol contamination in environmental settings. Additionally, conducting detailed genomic and proteomic analyses of *Pseudarthrobacter sp.* UMK-PNF5 may provide valuable insights into the underlying genetic and biochemical mechanisms governing its metabolic activities, thereby guiding targeted strategies for improving its bioremediation potential. Pursuing these avenues of research can enrich our understanding of the potential applications of *Pseudarthrobacter sp.* UMK-PNF5 and contribute to the development of more efficient and sustainable agricultural and environmental practices.

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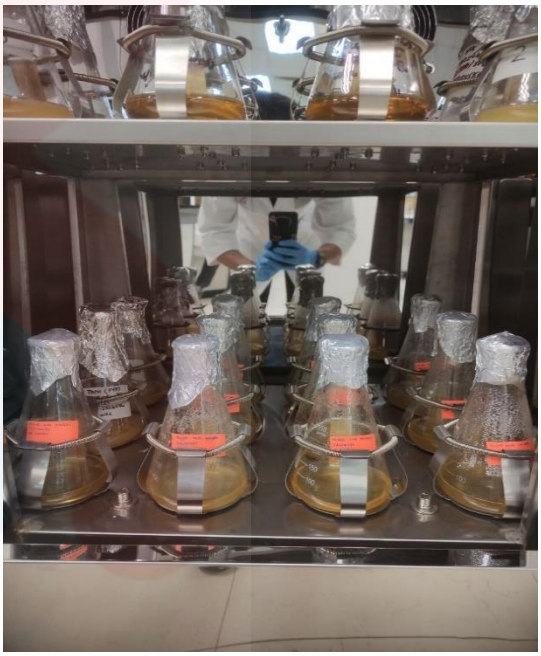
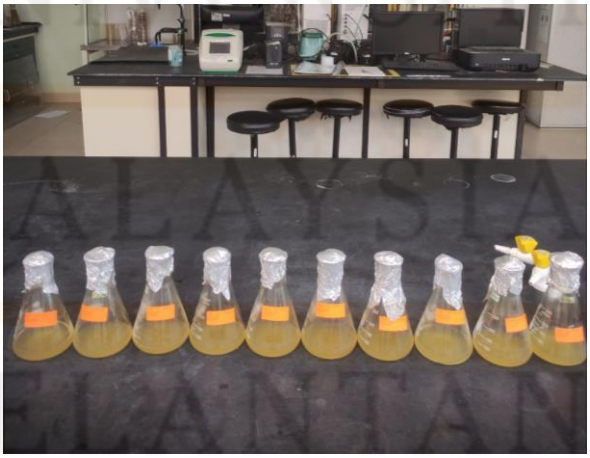
APPENDIX A

Incubation of *Pseudarthrobacter sp* UMK-PNF5 in LB Broth





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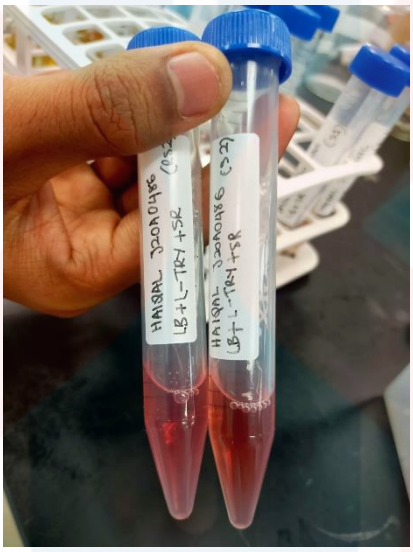
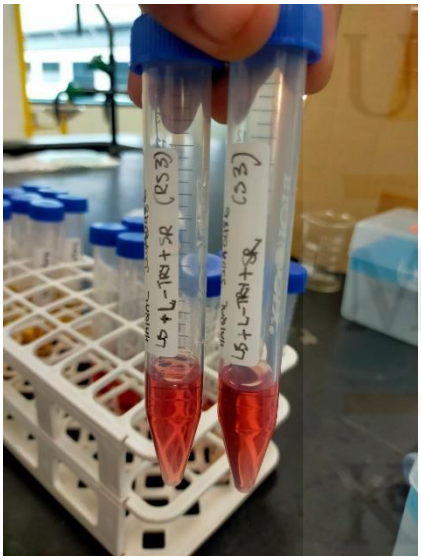
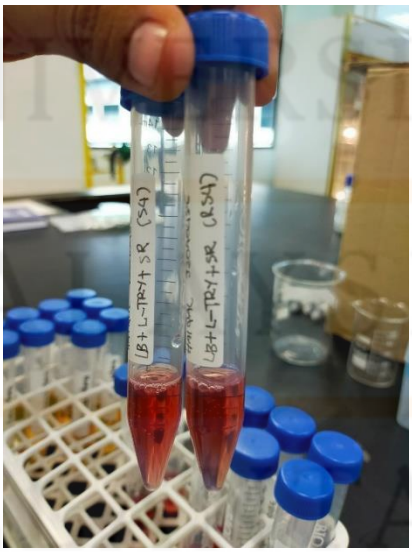
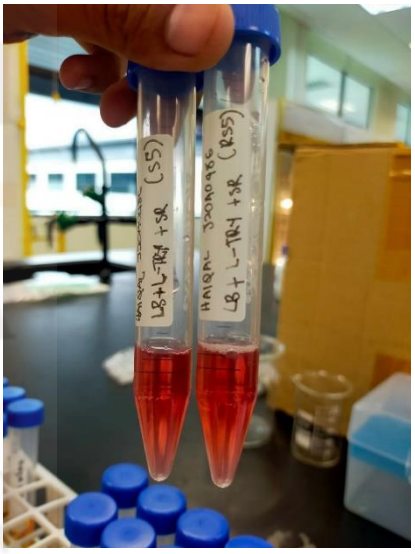
APPENDIX B

The color of the *Pseudarthrobacter sp.* UMK-PNF5 sample changed after adding the Salkowski reagent.



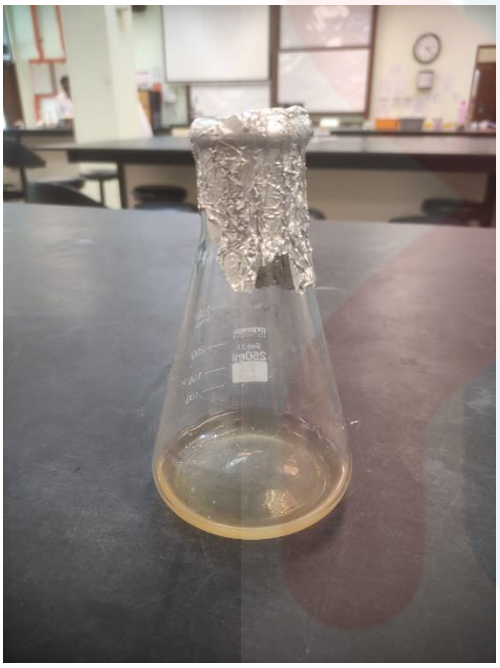


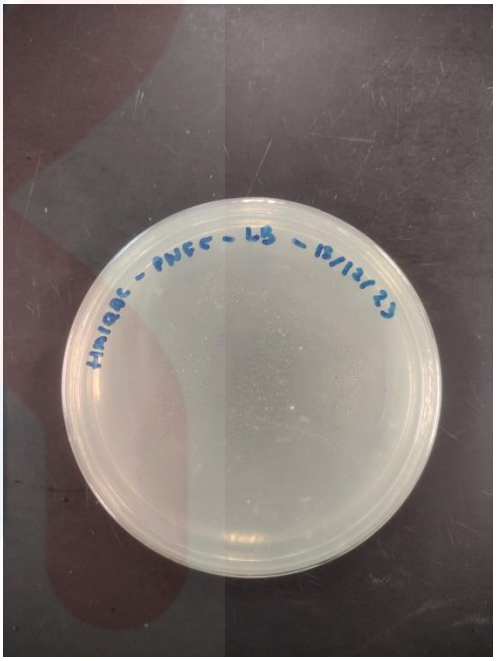




APPENDIX C

The appearance of Pseudarthrobacter sp. UMK-PNF5 changed after phenol was added and after incubation.





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APPENDIX D

The raw data of IAA productions by Pseudarthrobacter sp. UMK-PNF5 under different L-tryptophan concentrations and the IAA production of Pseudarthrobacter sp. UMK-PNF5 by using different carbon and nitrogen sources.

IAA Production (µg/ml)	Absorbance at 530 nm
2	0.044
4	0.052
5	0.056
10	0.069
20	0.09
30	0.14
60	0.485
100	0.857
150	1.252

L-Tryptophan Concentration (µg/ml)	Replicate 1	Replicate 2	Average	Stdeviation
0	0.116	0.165	0.1405	0.0245
1000	0.665	0.649	0.657	0.008
3000	0.85	0.92	0.885	0.035
6000	0.914	0.901	0.9075	0.0065
9000	1.098	1.103	1.1005	0.0025

Carbon sources	Replicate 1	Replicate 2	Average	Stdeviation
Fructose	0.571	0.471	0.521	0.05
Molasses	0.632	0.64	0.636	0.004

Nitrogen sources	Replicate 1	Replicate 2	Average	Stdeviation
Beef extract	0.753	0.662	0.7075	0.0455
Tryptone	0.831	0.852	0.8415	0.0105