CHARACTERIZATION AND GROWTH PARAMETERS OPTIMIZATION OF PHENOL DEGRADING BACTERIA FROM BATU TANNERY WASTEWATER

Elroi Tarekegn Legesse

A Thesis Submitted to the Department of Applied Biology, School of Applied Natural Science Presented in Partial Fulfillment of the Requirements for the Degree of Masters in Applied Biology Specialization in Biotechnology

Office of Graduate Studies

Adama Science and Technology University

May, 2024

Adama, Ethiopia

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DECLARATION

I hereby declare that this Master Thesis entitled **Characterization and Growth Parameters Optimization of Phenol Degrading Bacteria From Batu Tannery Wastewater** is my work and has not been submitted to any university for a similar purpose. The references used in this thesis are duly recognized by proper citations.

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RECOMMENDATION OF ADVISOR

We, the advisors of this thesis, hereby certify that we have read the revised version of the thesis entitled **"Characterization and Growth Parameters Optimization of Phenol Degrading Bacteria From Batu Tannery Wastewater"** prepared under my guidance by **Elroi Tarekegn** submitted in partial fulfillment of the requirement for the degree of Masters of Science in Biotechnology. Therefore, we recommend the submission of revised version of the thesis to the department following the applicable procedures.

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APPROVAL PAGE

We the advisors of the thesis entitled "**Characterization and Growth Parameters Optimization of Phenol Degrading Bacteria From Batu Tannery Wastewater"** by Elroi Tarekegn. Hereby certify that the recommendation and suggestion made by the board of examiners are appropriately incorporated into the final version of the thesis.

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Co-advisor/ Co-supervisor	Signature	Date

We, the undersigned, members of the Board of Examiners of the thesis by Elroi Tarekegn have read and evaluated the thesis entitled **"Characterization and Growth Parameters Optimization of Phenol Degrading Bacteria From Batu Tannery Wastewater"** and examined the candidate during open defense. Therefore, to certify that the thesis is accepted for partial fulfillment of the requirement of the degree of Master of Science in Biotechnology.

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Table of Contents

Contents **Page**

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF APPENDICES

Appendices **Page**

LIST OF ACRONYMS AND ABBREVIATIONS

ABIS _____ Advanced Bacterial Identification Software

BBP _____ benzyl butyl phthalate

BTI _____ Batu tannery industry

DEHP _____ di-ethyl hexyl phthalate

DO _____ dissolved oxygen

EDCs _____ endocrine-disrupting chemicals

eDT _____ extended direct transfer

FDRE _____ Federal Democratic Republic of Ethiopia

HCCA _____ α-cyano-4-hydroxycinnamic aci

LIS _____ The leather industry

LSV _____ Logarithmic Score Value

MALDI-TOF _____ Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectroscopy

MCL _____ maximum contamination level

MSM _____ minimal salt media

PDBI _____ phenol-degrading bacteria isolates

US EPA _____ United States Environmental Protection Agency

WHO _____ World Health Organization

ABSTRACT

Phenolic compounds pose a threat to human and animal health, and the accumulation of phenol introduces toxicity to both flora and fauna, potentially leading to fatal consequences upon high exposure. Despite the availability of various methods for phenolic removal, biodegradation using microorganisms stands out as an environmentally friendly approach. This study aimed at screening, characterizing, and assessing the phenolic removal efficiency of bacteria isolated from tannery wastewater in Addis Ababa, along with the optimization of growth parameters of the phenol degrading isolates. Wastewater samples (500 mL) were collected from Batu Tannery industry using sterile bottles. The screening of phenolic-degrading bacteria isolates (PDBI) was carried out on minimal salt media supplemented with 200-2000 ppm phenol crystal and nitro-phenol. The cultures were identified using biochemical and morphological characterization. Optimization and phenolic degradation assay were performed for PDBI. Data were analyzed using Microsoft Excel office (2008), and Origin version 2019b. The results revealed that out of 49 isolates, 8 (PDBI-7, PDBI-9, PDBI-11, PDBI-12, PDBI-15, PDBI-21, PDBI-23, and PDBI-49) isolates were found to be effective in phenols (Phenol crystal and nitrophenol) biodegradation at the concentration of 600-2000 $_{ppm}$. These isolates were identified as Bacillus subtilis PDBI-9 and Klebsiella pneumoniae PDBI-49. Based on optimization, the maximum Cell Dry Weight (g/l) and $OD_{600\ nm}$ recorded for B. subtilis PDBI-9 and K. pneumoniae PDBI-49 at pH 7and 30°C. Glucose gave the maximum Cell Dry Weight (CDW)(g/L) for PDBI-49, whereas mannitol gave the minimum Cell Dry Weight (CDW)(g/L) for PDBI-9. The maximum (0.00867± 0.00031) and minimum (0.00113 ± 0.00021) CDW(g/L) obtained when peptone and KNO_3 utilized by PDBI-49 and PDBI-9, respectively. Study showed that K. pneumoniae (PDBI-49) (86.24%) was more effective in degrading phenolic compounds (Phenol crystal and nitrophenol) than B. subtilis (PDBI-9) (76%). In conclusion, B. subtilis PDBI-9 and K. pneumoniae PDBI-4) employed for the phenol biodegradation.

Keywords: *Biodegradation, efficiency, isolates, leather, optimization, phenol, and Tannery*

1. INTRODUCTION

The leather industry (LIS) has a significant contribution to the economies of several developing nations such as India, China, Turkey, Brazil, Ethiopia, Pakistan, and Bangladesh. In Ethiopia, there are over 20 leather processing factories, and the majority of these factories, which account for over 90% of the total, dispose of their wastewater directly into nearby water bodies and open land without any treatment. Nevertheless, by the environmental policy of the Ethiopian federal government, effluent laws, and guidelines have recently been created (Leta *et al*., 2004). All development-related laws, policies, and results of the Federal Democratic Republic of Ethiopia (FDRE) are based on the country's Constitution. Ethiopia's air pollution, workplace safety and health, and the impact of climate change on health are all covered under the Constitution. For example, everyone has the constitutional right to a clean and healthy environment. To make sure of this, the government has an obligation. In accordance with the Constitution, development programs and projects must not harm or destroy the environment. The FDRE Health Policy offers recommendations and directions for preventing pollution of the environment caused by hazardous chemical wastes (Mitike *et al*., 2016).

One of the world's most important environmental problems is waste management. Different industries create a variety of wastewater pollutants; which are difficult and costly to treat. Wastewater characteristics and levels of pollutants vary significantly from industry to industry (Anupama *et al*., 2013). Over the past few decades, a variety of foreign substances have been introduced into the environment as a consequence of industrial advancements. The buildup of these substances has led to environmental pollution and has been a contributing factor to numerous harmful impacts on living organisms. Addressing these contaminations has driven the creation of novel technologies with varying degrees of efficacy and cost. Conventionally, wastewater was managed through physicochemical techniques, but more recently, biodegradation has gained significant attention and is being employed as a cost-effective alternative that offers the potential for the complete breakdown of organic compounds (Mailin & Firdausi, 2006).

Phenols are common chemicals that are widely used in many industries, including polymeric-resin-producing companies and petroleum refining, petrochemical, tannery industries, and pharmaceutical plants. They are, therefore, major pollutants found in many industrial wastewater

(Geng *et al*., 2006). The aromatic chemical phenol and its higher homologs have a hydroxyl group linked to the benzene ring. Phenolic compounds come from a variety of commercial and natural sources, and they are widely dispersed in the environment (Arutchelvan & Atun, 2019). Phenol pollutants, which can dissolve in water relatively easily and build up in the soil, lead to widespread contamination of surface water, and soil due to their highly toxic nature. Phenol is also known as hydroxyl benzene or phenic acid. It can exist as a colorless solid or pink substance with a low melting point. However, its boiling point is high due to the presence of hydrogen bonds. The harmful and environmentally unacceptable pollution impacts of phenolic effluent have been documented on a global scale (April *et al*., 2014; Ruiz-Ordaz *et al*., 2001).

Effluent from tanneries is regarded as one of the most dangerous industrial pollutants. The primary concerns stem from wastewater that contains heavy metals, harmful chemicals, chloride, and lime, along with elevated levels of dissolved and suspended salts and other contaminants. These pollutants have the potential to significantly hinder the growth of various organisms, including microorganisms (Moyo *et al*., 2001). The compound phenol, which is present in concentrations ranging from 5 to 2000 parts per million, poses a significant threat to all forms of life due to its high toxicity. As a result of this characteristic, it is classified as a priority pollutant (Salem *et al*., 2021). Due to these adverse health effects of phenolics, as per the rules of the World Health Organization (WHO), the maximum permissible level for phenol in the environment is 0. 01 mg/L (Saravanan *et al*., 2008).

Phenol is a key component in the production of bisphenol A, commonly used in plastic manufacturing, as well as caprolactam for the production of synthetic fibers and nylon. In the medical field, phenol plays a role in the formulation of various products such as general antiseptics, disinfectants, lotions, ointments, mouthwashes, and salves. It also has applications in the production of paints, tanning dyes, lacquers, perfumes, and inks (Ahmad, 2014). Phenol is one of the most often utilized organic compounds in use, and serves as the fundamental structural component of many synthetic organic compounds, including insecticides and agricultural chemicals. Natural sources of phenol include coal and other forms of dead organic materials that are decaying, such as rotting vegetables (Basha *et al*., 2010). The release of these substances into the environment without treatment carries significant concerns for the health of people, animals,

and aquatic systems (Villegas *et al*., 2016). It has been demonstrated that phenolic chemicals are poisonous to people, animals, and plants (Lin & Wu, 2011).

It is well recognized that acute phenol exposure can result in headaches, gastrointestinal discomfort, and skin irritation. Phenol is easily absorbed through skin and mucosa yet is harmful to the nervous system, heart, kidneys, and liver (Wang *et al*., 2011). Since it quickly penetrates the skin, it may be lethal if consumed, inhaled, or absorbed via the skin. It may also cause serious eye and respiratory system irritation. At quantities of 5 to 25 mg/l, it is thought to be potentially carcinogenic to humans and could be fatal to fish. The photosynthesis of diatoms and blue-green algae is inhibited by phenol at concentrations of 0.1 g/ml and higher. Phenol inhibits the nitrification process at 1000 mg/l and above (Rajani, 2015). Phenol can lead to eye irritation, swelling, and ultimately, vision loss. Exposure to high concentrations of phenol can result in chronic health issues such as liver damage, vomiting, and nervous disorders. When phenol is in solution form, it readily penetrates the skin, and it undergoes metabolism primarily in the liver, although metabolism can also occur in the lung and kidney. It's important to note that even relatively low concentrations of 5 mg/l are highly lethal to fish, while a concentration of 20 mg/l can be fatal for humans (Kafilzadeh *et al*., 2010; Ravikumar *et al*., 2011)

Several techniques for eliminating phenol, such as coagulation, incineration, chemical oxidation, liquid membrane permeation, adsorption, and other non-biological approaches, encounter certain limitations, like the generation of hazardous byproducts and elevated expenses. Furthermore, a few of these methods may necessitate energy consumption, such as radiation and ozone, in addition to using chemical reagents like catalysts and oxidizers (Moghadam *et al*., 2016a). The main disadvantage noted about physicochemical approaches is overcome by biological treatments of phenol, which are extremely appealing and effective procedures. Biological methods are efficient and use pure and mixed microbial strains to break down phenol and its derivatives into harmless chemicals (Wang *et al*., 2007). The most significant environmentally friendly agents for the detoxification and degradation of industrial contaminants during the biological treatment of industrial wastewaters are microbes (bacteria and fungi). Bacterial cultures (Pseudomonas, Bacillus) or various yeast and fungus species (Penicillium, Aspergillus, Candida, Trichosporon) are the microorganisms used (Bonfá *et al*., 2013).

The aim of this study is to evaluate how well microbes can degrade phenolic compounds found in tannery wastewater, as opposed to costly non-biological methods such as chemical oxidation, adsorption, liquid membrane permeation, coagulation, and incineration. Microbial removal of phenol is considered a cost-effective solution worldwide. This research focuses on screening, characterizing, and studying the phenolic removal efficiency of bacteria isolated from tannery wastewaters in Batu Tannery Industry (BTI) Addis Ababa, and optimizing growth parameters.

1.1 Statement of the problem

People who consume phenol could experience abnormal blood pressure, as well as damage to their liver and kidneys. There have also been reports of cardiac toxicity, which manifests as a weakening pulse, lowered blood pressure, and cardiac depression. It is important to note that a single gram of phenol has been linked to a deadly consequence in humans. Furthermore, worries about the possible carcinogenic effects of drinking water with high phenolic component concentrations have surfaced. The World Health Organization (WHO) set a limit of 1 mg/l to control the concentration of phenol in drinking water in response to these harmful effects on human health (Nuhoglu & Yalcin, 2005).

Despite its significant contribution to income and job creation, the tannery industry is currently grappling with a serious challenge surrounding the disposal of untreated wastewater, and poses a significant health risk to people. The effluent from tanneries contains a range of harmful pollutants, including phenolic compounds that can cause various health problems due to hazardous discharge on both land and water. Although phenol compounds have multiple uses, such as in the production of plastics, pharmaceuticals, disinfectants, and dyes, in the tannery industry, phenol is primarily used as a tanning agent to produce high-quality leather. Phenol is also used as a preservative to prevent bacterial and fungal growth in raw hides and skins, a disinfectant to sanitize tanning equipment, tools, and surfaces in the tannery, and a coagulating agent for protein-based materials in the tanning process.

Overall, phenol plays an important role in the tanning industry and is used to improve the quality and durability of leather products. They also have antimicrobial properties and are used as preservatives in some products. However, exposure to phenol compounds can be harmful to humans and the environment. Phenolic compounds are a cause of major concern due to their

serious health and environmental implications. Phenol, in particular, has potentially harmful long-term and acute health impacts, with lethal amounts of exposure leading to comas, respiratory arrest, muscle weakness, tremors, and erratic breathing. Furthermore, constant exposure to phenol can cause skin, eye, and mucous membrane irritation, anorexia, weight loss, diarrhea, vertigo, salivation, and dark urine color. Chronic exposure to phenols in animals can lead to irritation in the digestive and neurological systems and damage the cardiovascular, kidneys, and liver tissues.Due to its eco-friendliness, affordability, and potential for complete mineralization of the phenol substrate, biological approaches that use microorganisms to remove phenol are favored to.

Due to its eco-friendliness, affordability, and potential for complete mineralization of the phenol substrate, biological approaches that use microorganisms to remove phenol are favored to physicochemical methods (Prpich & Daugulis, 2005). Although there have been many studies on the degradation of phenols by microorganisms, the majority of these have focused on microbes isolated from contaminated sites of industrial effluents and wastewater across the world. However, little attention has been paid to these toxic compounds in Ethiopia. Therefore, this thesis focuses on the isolation and characterization of bacteria from tannery wastewaters in Addis Ababa, and their ability to remove phenolic compounds. Additionally, the study seeks to optimize growth parameters for these bacteria to improve their phenolic removal efficiency. Overall, this research aims to address the serious health risks posed by untreated wastewater from tannery industries in Ethiopia. This research study presents valuable information about the isolation and potential development of bacteria that can resist phenol, thus providing important insights into the field of wastewater treatment and management.

1.2 Significance of the study

This study provided information about the harmful effects of phenol. It has acute and long-term health impacts, and excessive exposure can cause comas, respiretory arrest, muscle weakness, tremors, and erratic breathing. Exposure to phenol can also result in skin, eye, and mucous membrane irritation, anorexia, weight loss, diarrhea, salivation, and dark urine color. Additionally, chronic exposure of animals to phenol can harm their digestive, neurological, liver, kidney, and cardiovascular systems, making it a toxic chemical for humans via oral intake. Efficient cleaning methods and procedures that can eliminate phenol and its compounds from water are essential since they are harmful and difficult to degrade. Microorganisms, including aerobes and anaerobes, are commonly used to degrade phenols,

which were isolated and identified through biochemical tests, MALDI TOF, and growth parameters. The findings of this study contribute to enriching the knowledge base on biodegradation removal methods for phenol. The use of biological methods of removing phenolic compounds from tannery wastewater is a cost-effective and efficient technology that can benefit both developed and developing nations,especially African countries.

1.3 Objectives of the Study

1.3.1 General objective

- The general purpose of this study is to characterize phenolic degrading microbes isolated from industrial wastewater in Addis Ababa in order to promote ecofriendly pollution removal strategies.

1.3.2 Specific objectives

- The specific objectives of the current project were
- To Isolate and screen phenol degrading bacterial isolates from industrial wastewaters
- To characterize phenolic degrading bacterial isolates using biochemical tests, and MALDI-TOF system
- To optimize conditions (pH, Temperature, carbon and N sources) for phenol degrading potent isolates in order to evaluate their phenol removal efficiency

1.4 Scope of the Study

This study concentrated only on the isolation, screening, biochemical and MALD TOF MS identification of potential phenol degrading bacteria from the in-let wastewater site, denitrification site of the wastewater treatment process, primary treatment site, secondary treatment site and out-let wastewater. This research didn‘t test with enhancement of phenol degradation using phenol degrading bacteria. Nor was this research aimed to quantitatively assess the enzymatic activities of the isolated phenol degrading bacteria but focused only on the identification and phenol removal efficiency.

2.LITERATURE REVIEW

2.1 Tannery industry process and their economic importance

The emergence of modern tanning in Ethiopia dates back to 1918 and 1927 with the establishment of the then ASCO (currently Addis Tannery) and Darmar/ Awash (currently ELICO) tanneries, respectively. Between 1954 and 1976, Dire, Modjo, and Kombolcha tanneries were established (Darge, 1995). The leather industry sector is one of the growing economic sectors in Ethiopia. However, the sector is constrained by different issues like external parasites, inappropriate management of animals, faults during slaughtering, and improper handling of skin before reaching the tannery, due to which the sector is losing a large amount of money due to the decline in quality and the fall in export price. Currently, 27 tanneries in Ethiopia produce all forms of hides and skins and finished leather for the domestic and export markets. These tanneries have an average daily soaking capacity of 107,850 pieces of sheep skin, 51,550 pieces of goatskin, and 9,800 pieces of hide. The annual capacity reaches approximately 48 million (32.4 million sheep and 15.5 million goat) skins and 2.9 million hides (Adem, 2019).

According to the United States Agency for International Development [USAID] (2013) report, the existing 27 tanneries in Ethiopia produce all forms of hides and skins and finished leather for the domestic and export markets with average daily soaking capacity of 107,850 pieces of sheep skin, 51,550 pieces of goatskin, and 9,800 pieces of hide. Meanwhile, the annual capacity reaches approximately 48 million (32.4 million sheep and 15.5 million goat) skins and 2.9 million hides. However, the capacity to process hides and skins greatly exceeds domestic supply, particularly for raw sheep and goatskins (USAID, 2013).

In modern society, waste is created by several sectors, including consumers, industry, agriculture, mining, energy, and transportation. Depending on the processes involved, different amounts and levels of hazardous waste are released from industrial activity. Of all the industrial wastes, tanning effluents are thought to be the most dangerous (Shen, 1995). Animal rawhide and skin, especially that of the cattle hide, undergo tanning to produce the highly tenacious and adaptable leather. The material has been in use since ancient times and continues to be popular today (Kesarwani *et al*., 2015). The leather industry is regarded as one of the most environmentally polluting sectors, raising concerns about its potential negative impact on the environment. India is estimated to manufacture around 2 billion square meters of processed leather, and the industry has set a goal to eventually double this production quantity. Even

though the tanning process mostly uses waste from the livestock industry, a variety of chemicals are used to change basic materials into the finished product. As a result, the leather sector uses up resources and produces pollutants that affect the environment (Dumitra *et al*., 2022, 2023).

One of the oldest industries in the world is tanning, and it generates a significant amount of waste, both liquid and solid. Chemicals and other potentially hazardous materials are used in the process of turning raw hides and skins into leather. Because of this, a lot of tanneries have put in place waste management systems to lessen the environmental effect of their operations (Kanagaraj *et al*., 2006). Water is necessary for several stages of the leather manufacturing process as it allows for reactions between added chemicals and the hide. The huge amount of water required for this process leads to the production of around 145 billion gallons of wastewater annually. However, only around 65-75% of the chemicals used in the process are consumed, resulting in wastewater that is highly polluting (Sathish *et al*., 2015).

During the tanning process (Table 1), a lot of chemicals are used to turn the partially soluble protein called "collagen" in animal hides and skins into durable leather goods. These chemicals include acids, alkalis, auxiliaries, biocide, chromium salts, dyes, phenolics, sulfates, sulphonated oils, surfactants, and tannins.

Unfortunately, some of the chemicals used end up in wastewater rather than being fully adhered to the hide or skins (Lofrano *et al*., 2008). It was found that the most closely linked substances to any tannery waste were chlorinated phenols and chromium. That's right it has been found that waste-exposed organisms suffer greatly from the presence of chlorinated phenols, such 3, 5-dichlorophenol, which function as an organic pollutant associated with the tanning business. Protein and carbohydrate breakdown in organic matter dissolved oxygen (DO) in stream waters is reduced by microbial breakdown (Mwinyihija *et al*., 2006). During the post-tanning and finishing phases of leather processing, various types of phenolic compounds, including monohydric, dihydric, and trihydric phenolics, are released into the atmosphere. In water, the acceptable limit for these phenolic compounds, represented as phenol (C_6H_5OH), is 10.2 mg/l. However, in drinking water, the concentration should not exceed 0.3 mg/l. It has been documented that the presence of sulfide, ammonia, phenol, and chromium in tannery wastewater can be harmful to freshwater fish (Kanagaraj *et al*., 2006).

Table 1. The process of tanning leather by incorporating both chrome and vegetable tanned leather (Kesarwani et al., 2015).

Processing of leather				
PREPARATORY STAGE Ware Housing Beam House Soaking Liming De-Fleshing De-Liming Bating Pickiling		TANNING Chrome Tanning Vegetable Tanning Aldehyde Tanning Synthetic Tanning		POST-TANNING Operation Samming Splitting Neutralizing Greasing Drying Finishing

Syntants are a class of chemicals designed for re-tanning or tanning leather. They have an intricate chemical structure made up of many substances such as acrylic resins, phenol, naphthalene, formaldehyde, and syntans based on melamine. These elements are still present in

the effluent after being partially bound to the skins in standard chemical and biological wastewater treatment methods (Lofrano *et al*., 2007). An important technical and environmental concern that requires immediate attention is the high concentration of non-biodegradable contaminants found in tannery wastewater (Moreira *et al*., 2009).

2.2 Phenolic Compounds and its use in the tannery industry

The aromatic molecules phenol and its derivatives have hydroxyl (OH-), methyl (CH_3), amide (NH_2), or sulphonic groups connected to the benzene ring structure (Figure 1). Phenols are widely distributed in the environment and naturally appear as plant building components (Africa & Africa, 2010). The amount, types, and locations of substituted groups on the aromatic ring(s) determine the phenolic compounds' toxicity and effects on the environment. Certain phenolic compounds have the potential to be hazardous to humans and other animals at high doses. One class of organic pollution that is frequently created by industrial activities, including the production of leather goods, is phenolic compounds. Because these substances can be hazardous

and persistent in the environment, they may have negative consequences on both human and environmental health (Nicell *et al*., 1993).

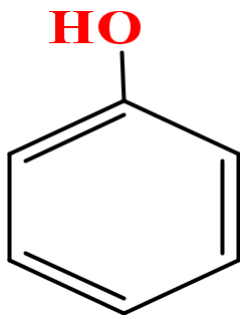

Figure 1. Chemical structures of phenol (Basha *et al*., 2010).

Phenol is a polar molecule and has some solubility in water. When a substance is soluble in water, it can dissolve in it and become distributed in the water cycle much more easily. This implies that it can travel through rivers, lakes, and watersheds before being absorbed by living things. The behavior and fate of a chemical in the environment are significantly influenced by its solubility in water (Oller *et al*., 2011). According to the US EPA, phenol is a major pollutant that is regulated by inspection and limits on the amount of phenol in drinking water. This is due to the fact that phenol is a poisonous compound that, in large enough doses, can be fatal to people. To safeguard the public's health, the US EPA has set a maximum contamination level (MCL) for phenol in public drinking water systems at one milligram per liter (mg/L) (Michałowicz & Duda, 2007). The EPA stated in 2004 that phenolic chemicals constitute a substantial source of pollution for waterways. They cause aquatic mortality, stunt growth, weaken resistance to disease, and encourage the growth of undesirable aquatic vegetation, among other negative consequences. Serious ecological issues may arise if phenolic contamination gets into subsurface water supplies. For this reason, it is essential that the maximum amount of phenol allowed in industrial effluents is 0.5 mg per liter. Because of these worries, it is crucial to remove phenol from the environment, especially from water and water resources (Kafilzadeh *et al*., 2010).

To convert raw leather into forms that may be used for a variety of products, the leather industry uses a wide range of chemicals. These substances are essential to the process of preparing leather. Describe the many kinds of chemicals that are utilized in the leather business and the toxicity that goes along with them (Sivaram & Barik, 2019). Aromatic compounds like phenol and its derivatives have a hydroxyl group attached to the benzene ring. Phenolic chemicals are

widely dispersed throughout the environment and can be found in a wide range of industry and natural sources. The main application of chlorinated phenols is the protection of wood against insects and fungi. Nevertheless, they also come from other processes like the bleaching of pulp, the burning of organic waste and municipal solid waste, the partial conversion of phenoxy pesticides like 2,4-dichlorophenoxyacetic acid and 2,4,6-trichlorophenoxyacetic acid, and the preservation of leather in the tannery sector (Shah & Thakur, 2002).

The leather industry has been subject to limitations on the use of specific chemicals by regulatory bodies. Plasticizers like di-ethyl hexyl phthalate (DEHP), benzyl butyl phthalate (BBP), and di-butyl phthalate (DBP) are used in the microporous artificial leather covering. But the European Union has made it mandatory for items containing more than 0.5% of these phthalates to be labeled as such because of their possible harm to reproduction. Additionally, according to the recommended rules, the amount of nonyl phenol—a substance used in leather finishing—in completed items should not exceed 0.1% (Sivaram & Barik, 2019). The growing amount of water treated in wastewater treatment facilities indicates the expansion of industries and progress in society. While modern wastewater treatment methods are capable of producing high-quality effluents, the presence of certain inhibitory substances, such as phenols, can still have detrimental effects on the treatment process. The main antimicrobial substances present in wastewater are phenols, which are frequently produced by the agro-processing food (including wineries and olive mills), petroleum, textile, and steel industries (Esteban *et al*., 2022).

2.3 Environmental concerns of phenolic compounds and on living things

The continuous and enduring environmental problem of pollution is causing great concern to the world society. As a xenobiotic, phenol is frequently found in contaminated habitats as a result of industrial processes, mining, and agricultural activities, among other natural or man-made activities. High concentrations of phenol build up as a result in our ecology. Consequently, increasing production of hazardous compounds or environmental leakage might have a negative impact on the quality of soil and groundwater (Ahmad, 2014). Widely present in aquatic environments, phenol (C_6H_5OH) is acknowledged as one of the most prevalent organic contaminants. The fact that it is soluble in water, has a stable chemical composition, and may spread across the ecosystem makes it a ubiquitous contamination. Oil refineries, chemical factories, and companies that produce explosives, paper, wood, resin, dye, and pulp are the main sources of phenolic compounds found in water bodies (Kottuparambil *et al*., 2014).

There are serious risks to human health and ecosystems when industrial wastewater containing phenolic compounds is released. The environment may be impacted by the use of phenol and its derivatives in a variety of industrial sectors. The particular industrial processes at play have a major impact on the concentration of phenolic compounds in surface water, which can have unfavorable impacts on local ecosystems and human populations (Raza *et al*., 2018). The pKa value, hydrophobicity, and reactivity of organic compounds are some of the qualities that might modify their environmental toxicity. Phenolic compounds are known to cause acute health problems, such as skin-related problems and serious organ failures, such as those affecting the stomach, lungs, and kidneys, which can be fatal. The carcinogenic properties of phenolic chemicals, with an emphasis on chlorophenols in particular (Czaplicka, 2004).

An enormous number of chemicals produced by industrial processes contaminate water supplies and have harmful, carcinogenic, and mutagenic qualities that endanger the environment's ecological balance (Busca *et al*., 2008). Sewage effluents and agricultural chemical runoff are the primary sources of endocrine-disrupting chemicals (EDCs) in rivers and lakes in North America and Europe. Elevated levels of EDCs in aquatic settings are largely caused by uncontrolled discharge from houses and industries into rivers in developing nations such as Africa and Asia. It has been found through experimental research that endocrine-disrupting qualities are exhibited by both artificial substances and naturally occurring estrogens and secondary metabolites from plants (Falconer *et al*., 2006).

2.4 Effects of phenolic compounds on Human Health

The widespread presence of aromatic organic pollutants, such phenol and its derivatives, in soil, surface water, and subsurface water has become a major reason for concern. It is commonly known that phenols are extremely harmful to humans, other living things, including aquatic life. They are considered to be among the most hazardous toxins, posing significant challenges in terms of their elimination (Al-Khalid & El-Naas, 2012). Phenols demonstrate toxic effects on both fish and humans, disrupting various metabolic processes (Monteiro *et al*., 2000).

Human health has been shown to be negatively impacted by phenolic substances such as aminophenols, chlorophenols, nitrophenols, methylphenols, and chlorophenols (Mun *et al*., 2005). Moreover, it is commonly known that phenolic compounds have genotoxic effects and can interfere with hormone balance. Phenolic compounds are common examples of endocrine-disrupting chemicals (EDCs), and they include chlorophenols, nonylphenols, 4-tert-octylphenol,

and BPAs. These chemicals possess the ability to disrupt regular hormonal functions, mimic endogenous hormones, and occupy their receptors in many organisms, resulting in notable hormonal dysregulation and related health hazards. For example, BPA causes disruptions to microtubule organization and centrosome activity, exhibiting a variety of cancer-promoting effects (Panigrahy *et al*., 2022). Research has shown that bisphenol A and certain alkylphenols can impair an animal's development of the mammary gland, which can have an endocrine disruptive effect on humans. The tendency of bisphenol A to postpone the onset of puberty in girls has also been revealed by similar research. Sipping on liquids with high phenol concentrations, such as drinking water, causes gastrointestinal distress and tremors in the muscles that make walking difficult (Anku *et al*., 2017).

People who consume phenol could experience abnormal blood pressure, as well as damage to their liver and kidneys. There have also been reports of cardiac toxicity, which manifests as a weakening pulse, lowered blood pressure, and cardiac depression. It is important to note that a single gram of phenol has been linked to a deadly consequence in humans. Furthermore, worries about the possible carcinogenic effects of drinking water with high phenolic component concentrations have surfaced. The World Health Organization (WHO) set a limit of 1 mg/l to control the concentration of phenol in drinking water in response to these harmful effects on human health (Nuhoglu & Yalcin, 2005). Phenol has detrimental effects on various organs and tissues, including the kidneys, liver, muscles, and eyes. It is known to cause skin irritation and necrosis. The skin's coagulation occurs due to the reaction between phenol and amino acids in the keratin of the epidermis and the collagen in the deeper layers of the skin, leading to skin damage. While a dose of 1 gram of phenol can be fatal to an adult man, tolerance to the substance varies significantly. Some studies suggest that individuals have survived even after being exposed to as much as 30 g (equivalent to 60 ml of a 50% solution, w/v) of phenolic mixtures. The rapid absorption of phenol through the skin, ranging from 60 to 90% (w/v), indicates that even a small amount of contact with the forehead or palm could be fatal (Michałowicz & Duda, 2007; Owicz & Duda, 2016).

Because phenol interacts hydrophobically with lipid bilayers, it can damage cellular membranes, which is why it is poisonous. Its capacity to provide free electrons to oxidized substrates, resulting in oxidative stress, is connected to phenol's reactivity with biomolecules. The production of free radicals and reactive oxygen species, such as superoxide radicals or hydrogen peroxide, is what causes this oxidative stress. Because phenolic compounds are very reactive,

they can easily participate in radical processes that lead to the lipid peroxidation of cellular membranes. Additionally, phenols are able to enter intracellular regions and harm a variety of organelles, such as the nucleus, mitochondria, and endoplasmic reticulum, as well as the enzymes and nucleic acids that come with them (Kottuparambil *et al.*, 2014). When phenol is consumed over an extended period of time at concentrations between 10 and 240 mg/L, it can cause a number of symptoms, including mouth sores, diarrhea, black urine excretion, and eye impairment. It is estimated that blood levels of phenol can range from 4.7 to 130 mg/100 mL, which is lethal. The nervous system and vital organs, including the spleen, pancreas, and kidneys, are adversely affected by phenol (Baker *et al*., 1978; Manahan, 2022).

2.5 Treatment methods for removal of phenolic compounds

2.5.1. Ion Exchange

The removal of an ion from an aqueous solution by replacing another ionic species isthe basic principle of Ion exchange method of xenobiote removal. Natural and syntheticmaterials which are specially designed to enable ion exchange operations at high levels are used to perform this ion exchange for removal of organic and inorganic pollutants for purification and decontamination of industrial effluent. The main features of the ionic resins include properties such as like adsorption capacity, porosity, density etc (Zorpas et al., 2010).

2.5.2. Adsorption

Adsorption is a widely used wastewater treatment method for colour, heavy metals and other inorganic and organic impurities present in the industrial effluents. Adsorption of phenol onto activated carbon is a widely studied treatment method because of the affinity of phenols for the active surface of carbon. Mechanisms involved in the adsorption process mainly focus on the selection of the adsorbent material like their particle size surface area and porosity etc. pore size distribution and surface area of the activated carbon are two important factors that affect the adsorption of phenol. Because of the high cost involved in the usage of activated carbon, materials basically obtained from low-cost agricultural wastes; activated carbon prepared from various raw materials such as sawdust, nut shells, coconut shells etc were been used as adsorbent (Zawani et al.,2009). These adsorbents are basically used for the effective removal and recovery of phenolic pollutants from wastewater streams (Basso et al., 2002; Park et al., 2006).

2.5.3. chemical oxidation

Chemical treatment involves the use of chemical agents to completely destroy or convert the contaminants to harmless or less toxic compounds, or intermediates that can be further degraded by microorganism. Chemical oxidation of organic pollutants especially phenol is a promising alternative when wastewater contains nonbiodegradable and/or toxic contaminants and also when the contaminant concentration is high. The chemical agents used are normally strong oxidants. The most commonly used oxidants that initiate the oxidation reactions include hydrogen peroxide which is widely used for this purpose (Dias-Machado et al., 2006 ; Ksibi, 2006). Before wastewater is discharged into water bodies, phenols, which are frequently present in many wastewater types, must be removed. This is required because phenols are highly poisonous and can endanger both human and animal life even at low amounts.

Phenols have been eliminated using a number of techniques, including ion exchange, reverse osmosis, membrane filtering, photocatalytic degradation, adsorption, electrochemical oxidation, ozone/hydrogen peroxide oxidation, biological techniques, and Fenton's reagent. Certain methods, either applied singly or in combination, have shown promise in the successful removal of phenols. These methods, however expensive and complex, have proven to be effective in eliminating phenols from wastewater. When considering phenol therapies, biological approaches are far more effective and attractive than physicochemical ones because they minimize a number of major disadvantages with the latter (Jain *et al*., 2004). By using particular microorganisms or a mixture of microbial strains, biological techniques can convert phenol and its derivatives into non-toxic compounds. These biological methods are a good choice for wastewater treatment since they are not only economical but also efficient at eliminating phenol (Wang *et al*., 2007).

The use of physicochemical methods has several disadvantages, such as high energy consumption, high implementation costs, and the production of toxic byproducts, low efficiency, and restricted applicability outside of a certain concentration range. For the elimination of phenol, biological treatments are more advantageous than physicochemical approaches due to these disadvantages. A different suggestion is to investigate biological processes as a more appropriate strategy in light of the difficulties in removing phenol using conventional treatment methods like chemical coagulants, hydrogen peroxide, and filtration, particularly the less biodegradable lignin-like polymer (Galanakis, 2014). The capacity to transform phenol into non-toxic chemicals, reduced energy requirements, cost-effectiveness, and a broader variety of application are some benefits of using biological processes. When it comes to phenol

elimination, biological therapies are generally a more appealing and effective choice than physicochemical ones (Banerjee & Ghoshal, 2011).

2.5.4 Biological methods of phenolic compounds degradation

Modernization and economic expansion have changed several industrial sectors with the goal of improving human well-being. On the other hand, these modifications have had detrimental effects on our ecosystem that will take time to resolve. Extensive industrialization contributes to significant climate changes and has direct effects on ecosystems. Numerous dangerous chemicals are released into the air, water, and land by industrial activities. This dangerous waste is extremely dangerous to human health and may even contribute to the development of cancer in some cases (Sellami *et al*., 2021). Because it's easy to handle, inexpensive, and has positive effects on the environment, biodegradation is the method of choice for getting rid of phenol (Tay & Moy, 2005). The two primary types of biological methods used to remove phenolic chemicals from wastewater are enzymatic and microbial methods. By using bacteria, yeast, and fungi, the microbial method breaks down phenolic chemicals into innocuous byproducts like carbon dioxide and water. Phenolics are successfully changed by this procedure into materials that are safe for ingestion by humans. This method is thought to be workable since some microorganisms, such as phenolics, depend on aromatic compounds for carbon or nourishment, which makes them useful phenolic removal agents (Anku *et al*., 2017).

Many environments, including hot springs, dry regions, frozen landscapes, brackish lakes, and seas, are home to microorganisms. Polluted places include heavy metal-contaminated sites, landfills, oil spills, pesticide-ridden farmlands, and wastewater treatment plants are common sources of microbes with the capacity to degrade contaminants. Under both aerobic and anaerobic circumstances, these microorganisms use the hazardous pollutants as a source of carbon and energy. Because of their metabolic activity, the microbes are also able to break down or change the harmful pollutants into less harmful molecules (Garima, T. & Singh, 2014). It is usual to isolate microorganisms from habitats contaminated by tannery wastes, and these organisms have demonstrated a high level of resistance to typical contaminants prevalent in these locations, including phenolic compounds and chromium (Shah & Thakur, 2002). Remediation of contaminated regions, detoxification of wastewater, and removal of organic pollutants can all be accomplished with success by using microbial degradation in practice. Many different kinds of bacteria, both anaerobic and aerobic, can use phenol as their only energy and carbon source.

Given the extensive distribution of phenol in the environment, this capability is very noteworthy. These bacteria can be useful in microbial degradation processes as demonstrated by the way they use phenol, which shows that they can help remove phenolic chemicals and restore contaminated environments (Pradeep *et al.*, 2015).

The breakdown of phenols is caused by the metabolic processes of a variety of microorganisms, including actinomycetes, bacteria, and fungi. Bacterial strains such as *Bacillus sp, Pseudomonas sp*, *Acinetobacter* sp, *Achromobacter* sp, and others are involved in this process. Fungal groups such as *Corious versicolor*, *Fusarium* sp, *Phanerocheate chrysosporium*, *Ralstonia* sp, and *Streptomyces sp* have also been demonstrated to be effective in breaking down phenol. Nonetheless, when exposed to higher concentrations of phenol, these microorganisms experience substrate inhibition, which hinders their growth (Nair *et al*., 2008). Several bacterial species have been identified and classified as phenol-degrading microorganisms due to the hypothesis that only a limited number of microorganisms are capable of utilizing phenol as their sole carbon and energy source. Notable examples of these bacterial species belonged are including such as *Acinetobacter,.Arthrobacter citreus, Alcaligenes faecalis, Bacillus subtilis, Bacillus brevis, Pseudomonas putida, Rhodococcus erythropolis, Sphingomonas,* and *Serratia marcescens,* These bacterial species have been identified for their unique ability to degrade phenol, signifying their importance in the natural breakdown of phenolic compounds in diverse environments(Liu *et al.*, 2016).

Investigating the biodegradation of phenol is mostly dependent on bacterial isolates. Breaking down phenolic chemicals is a common use of *Pseudomonas,* one of the bacterial genera that has been investigated extensively. Their remarkable capacity to flourish on chemical substances has made these bacteria well-known. Researchers have discovered putative pathways and identified the enzymes that aid in the process by studying phenol biodegradation using a variety of bacterial species (Nair *et al*., 2008b). *Pseudomonas* bacteria have been utilized as models for typical phenol-degrading microorganisms. While *P. cepacia* isolated from industrial wastewater can degrade 2.5 g/L phenol in 144 h (17.36 mg/L per hour), *P. putida* can degrade 1 g/L phenol in 162 h (6.17 mg/L per hour). In 48 hours (15.27 mg/L per hour), *Acinetobacter calcoaceticus* can break down 91.6% of 0.8 g/L of phenol. A fresh strain of *Rhodococcus aetherivorans* degrades 0.5 g/L of phenol at a rate of 35.7 mg/L per hour. The mutant M1 of *Rhodococcus ruber* SD3 can immobilize cells to digest 98% of 2 g/L of phenol (27.2 mg/L per hour) in 72 hours (Xu *et al*., 2021).

2.5.5 Aerobic biodegradation of phenol

In wastewater treatment, the aerobic biodegradation of phenol, which involves the complete breakdown of phenol into its mineral components, is typically the preferred method. Phenol is known to act as an inhibitory substance, even at low concentrations (100 mg/L), and is often used as a convenient model for studying the degradation kinetics of aromatic compounds (Christen *et al*., 2012). The aerobic degradation of phenol begins with the introduction of oxygen. Under aerobic conditions, a monooxygenase enzyme called phenol hydroxylase facilitates the monohydroxylation of the aromatic ring at an ortho position relative to the existing hydroxyl group. This process leads to the formation of catechol, which serves as the primary intermediate in the microbial breakdown of phenol across different strains. Depending on the specific microbial strain involved, catechol is further metabolized through two different pathways. In the ortho-cleavage pathway, catechol is oxidized by catechol 1,2-dioxygenase, resulting in the production of succinyl Co-A and acetyl Co-A. Alternatively, in the meta-pathway, catechol is oxidized by catechol 2,3-dioxygenase, leading to the formation of pyruvate and acetaldehyde (Mishra & Kumar, 2017).

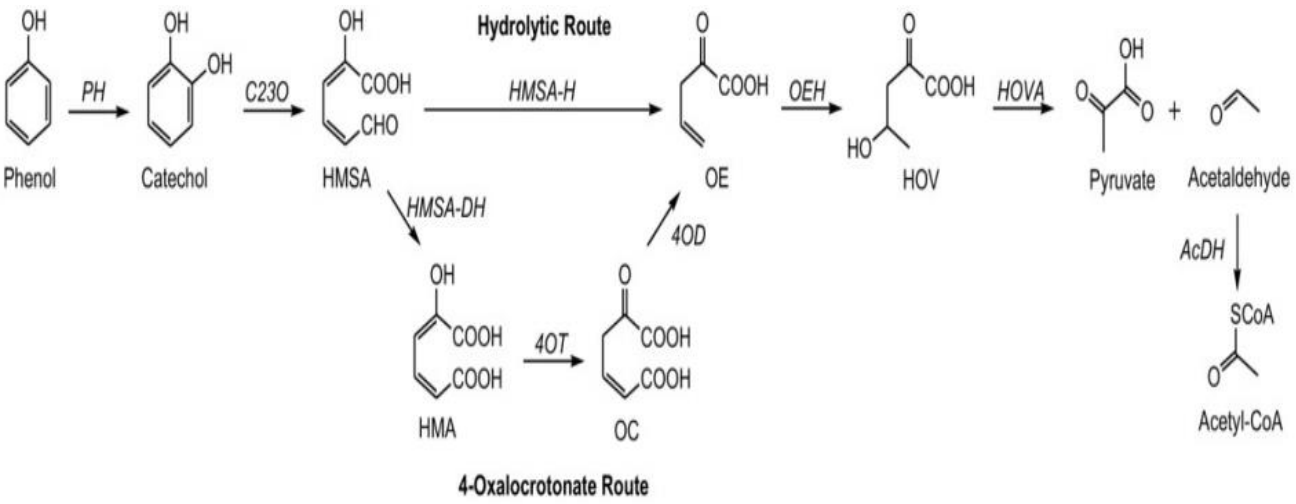

Figure 2. Aerobic biodegradation Pathway

2.5.6 Anaerobic biodegradation of Phenol

The degradation of phenol in an environment lacking oxygen is possible. However, compared to the aerobic process, our understanding of this anaerobic process is relatively limited. It is only recently that the process of anaerobic phenol degradation has been elucidated and comprehended, despite the availability of several anaerobic microorganisms capable of

degrading phenol in pure cultures. The initial studies on phenol decomposition in the absence of oxygen primarily focused on its breakdown under methanogenic conditions (Van scnte & Young, 2000). Due to its aromatic structure, phenol exhibits resistance to natural biodegradation, and phenolic compounds are known for their high stability, making it difficult to break down the benzene ring. However, certain microorganisms have the ability to tolerate phenol and utilize it as a source of carbon and energy. These microorganisms possess specific enzymes that are capable of cleaving the benzene ring, enabling the process of biological degradation to occur. Through the action of these enzymes, the microorganisms can initiate the breakdown of phenol and ultimately metabolize it (Koçberber, 2009).

2.6 Enzymes Responsible For The Biodegradation

2.6.1 Oxygenases

It is the enzyme that changes the hydrophobic organic compound to more watersoluble and thus it can be broken down by a larger number of other microorganisms. Two major classes of oxygenases are known. They are monooxygenase and dioxygenase. These enzymes participate in the oxidative metabolism of a wide variety of chemicals of pharmaceutical, agricultural and environmental significance. Some of the most widely recognized substrates for this class of enzymes are the aliphatic and aromatic hydrocarbons of both endobiotic and xenobiotic sources.

2.6.2 Hydroxylase

Phenol hydroxylase catalyses the degradation of phenol via two different pathways initiated either by ortho or meta cleavage. There are many reports on phenol hydroxylase and catechol 2, 3 dioxygenase involved in the biodegradation of phenol. Phenol-degrading aerobic bacteria are able to convert phenol into nontoxic intermediates of the tricarboxylic acid cycle via an ortho or meta pathway. The monooxygenation of the aromatic ring constitutes the first step in the biodegradation of many phenolic compounds. This process is carried out by flavoprotein monooxygenases, which use electrons of NAD(P)H to activate and cleave a molecule of oxygen through the formation of an intermediate flavin hydroperoxide and enable the incorporation of an oxygen atom into the substrate (Moonen et al., 2002). These reactions can be catalyzed by a single polypeptide chain or by multicomponent enzymes (Berkel et al., 2006). It has been reported as a class of monooxygenases, consisting of a small reductase component that uses NAD(P)H to reduce a flavin that diffuses to a large oxygenase component that catalyzes the hydroxylation of aromatic substrate (Berkel et al., 2006).

2.6.3 Monoxygenases

This class of enzymes inserts one atom of the oxygen molecule into the substrate, and the other atom of oxygen becomes reduced to water, i.e. two reluctant (substrates) are needed. They are also more complex in action, and can catalyze several different types of O insertion reactions. Since monoxygenases oxidize 2 substrates, they are also called mixed function oxidizes. Also since one of the main substrates becomes hydroxylated, they are also called hydroxylases.

2.6.4 Ecology of phenol degrading bacteria

Several bacterial species have been identified and characterized as microorganisms capable of utilizing phenol as the sole source of carbon and energy. These bacterial species are *Acinetobacter,.Arthrobacter citreus, Alcaligenes faecalis, Bacillus subtilis, Bacillus brevis, Pseudomonas putida, Rhodococcus erythropolis, Sphingomonas,* and *Serratia marcescens,* It has been observed that native microbial species have a higher adaptability and competitive advantage over non-indigenous microorganisms in remediating environments contaminated with phenol. Phenolic wastewater samples were gathered from the effluent of an oil refinery located in South China (Liu *et al*., 2016).

Bacteria, yeast, and fungi have the ability to utilize phenolic compounds. While phenol is known for its toxic and substrate inhibitory properties, it can paradoxically serve as a carbon and energy source for various bacterial strains. These strains are classified within the genera of *Bacilli, Aureobasidium. Klebsiella, Ochrobactrum, Pseudomonas,* and *Rhodococcus.* Despite its potential adverse effects, phenol is metabolized and utilized by these bacterial species as a nutrient for their growth and energy production. Samples were obtained from various locations within the effluent treatment plant of a phenol-formaldehyde resin manufacturing industry (Veeraraghavan & Choudhury, 2019). *Paenibacillus mucilaginosus* was isolated sewage wastewater samples of Assiut hospitals area of Assiut University, Assiut (Salah *et al*., 2020). *Rhodococcus sp* reported from sediment and effluent samples that discharge from a point of a tannery, in Argentina, Elena, and Córdoba Province, (Paisio *et al*., 2012). *A. calcoaceticus* has demonstrated its efficiency in treating phenolic wastewater. The bacterium could effectively reduce 91.6% of a phenol concentration of 0.8 g/L within a two-day period and exhibited tolerance towards phenol at a concentration of 1.7 g/L. These findings highlight the effectiveness of *A. calcoaceticus* in the treatment of phenolic wastewater (Liu *et al*., 2016).

2.7 Determination of Phenol degradation efficiency

Numerous liquid- or gas-chromatographic techniques and electrochemical techniques have been developed to enable selective and sensitive analysis of phenol in aqueous media, across various

sample types (Brega *et al*., 1990). VIS (visible) molecular absorption spectrometry is considered to be a suitable technique for developing a simple, rapid, and cost-effective analytical method for the quantitative determination of phenol in aqueous media. VIS spectrometry utilizes the absorption of light in the visible range (typically between 400 and 700 nm) by the analyte of interest. Phenol exhibits characteristic absorption peaks in the visible region, allowing for its quantification through spectrophotometric measurements. The technique involves straightforward instrumentation and measurement procedures, making it accessible to a wide range of users. VIS spectrometry provides fast results, allowing for high sample throughput and efficient analysis, and it is generally more affordable compared to other spectroscopic instruments, making this method cost-effective for routine phenol analysis. VIS spectrometry can be applied to various aqueous samples containing phenol, including wastewater, environmental samples, and industrial effluents (González *et al*., 2003).

Because phenol forms a colored compound that absorbs light at a particular wavelength, spectrophotometric methods are the preferred approach for quantifying phenol in aqueous media. The underlying idea behind these techniques is that phenol can compound or react chemically with a reagent to generate a colored species. The amount of phenol contained in the sample is exactly proportionate to the color's intensity. One way to quantify the concentration of phenol is to measure the absorption of light at the right wavelength, which is normally established through prior calibration. A number of variables, including the sample matrix's composition and the necessary sensitivity and selectivity, influence the choice of particular reagent and wavelength for analysis. In a number of aqueous mediums, accurate and precise measurement of phenol can be achieved by utilizing suitable spectrophotometric techniques and optimizing the reaction conditions. (Tanasă et al., 2022).

2.7.1 4-Aminoantipyrine as Analytical reagent

Emerson suggested using 4-aminoantipyrine as a colorimetric reagent in 1943 to determine the presence of phenolic compounds. Consequently, 4-aminoantipyrine was dubbed Emerson's reagent. The phenolic substance, 4-aminoantipyrine, and an alkaline oxidant were combined in a high pH solution for the suggested reaction. Preventing the production of a quinonoid substitution product and guaranteeing precise measurement were the goals of keeping the pH high. For this reaction, potassium ferricyanide was used as the oxidant. The amount of phenol present was directly correlated with the intensity of the resultant colour, known as antipyrine red.

This technique made use of the 4-aminoantipyrine colorimetric assay to quantitatively analyse phenolic chemicals. The use of stable reagents, quick findings, and ease of manipulation are just a few benefits of Emerson's approach. It is a flexible and trustworthy method for phenol measurement since it works with a broad range of phenolic material concentrations. (Emerson, 1943).

2.7.2 $FeCl_3$ as Analytical reagent

The determination of phenol is based on the reaction between phenol and ferric chloride in aqueous media, which leads to the formation of a soluble purple reaction product. This reaction is specific to phenol and allows for its selective detection and quantification. When phenol is mixed with ferric chloride in an aqueous solution, a chemical reaction occurs, resulting in the formation of a colored complex. The exact mechanism of this reaction involves the coordination of phenol molecules with ferric ions, leading to the formation of a purple-colored species. By measuring the intensity of the color produced, typically using spectrophotometric techniques, the concentration of phenol in the sample can be determined. The color intensity is directly proportional to the amount of phenol present, enabling quantitative analysis. This phenol-ferric chloride reaction is widely used in analytical methods for phenol determination due to its specificity and the easily measurable color change it produces. The purple reaction product allows for sensitive and reliable detection of phenol in aqueous media. To determine the qualitative characteristics of the purple complex formed between phenol and FeCl3, VIS (visible) absorption spectra were recorded in the wavelength range of 470 to 700 nm, with distilled water serving as the reference (Apostica *et al.*, 2018).

2.8 Factors affecting Biodegradation of phenolic compounds from tannery wastewaters

The chemical composition and structure of phenolic compounds have an important role on the biodegradation performance. Taking Chlorophenol as an example, those with more halogen substituents are more susceptible to biodegradation. At the same time, the position of substituent has a great effect on biodegradation, such as the degradation sequence of chlorophenol is as follows: Adjacent position > Separated position > Opposing positions. Natural microorganisms often have poor degradation ability of phenolic compounds, but the microbial degradation ability of phenolic compounds can be greatly improved by mutagenesis. The changes of environmental conditions such as temperature, pH value, dissolved oxygen, nutrients and toxic substances,

affect the metabolic activity of microorganisms and the physicochemical properties of phenols, thus affecting the biodegradation performance of phenolic compounds (Zhao *et al*., 2018).

3. MATERIALS AND METHODS

3.1 Description of the study area

Addis Ababa seat both for the Federal Democratic Republic of Ethiopia (FDRE) and the Oromia National Regional State Government is a diplomatic capital and headquarters for different organizations. Geographically, The latitude of Addis Ababa, Ethiopia is 9.005401, and the longitude is 38.763611. Addis Ababa, Ethiopia is located at Ethiopia country in the Cities place category with the GPS coordinates of 9° 0' 19.4436" N and 38° 45' 48.9996" E. km^2. There are 11 sub-cities (Kifle ketema) and about 99 Kebeles with a population of about 2.1 million with an eight percent annual growth rate, a density of 5936.2/km2 (Bogale, 2012).

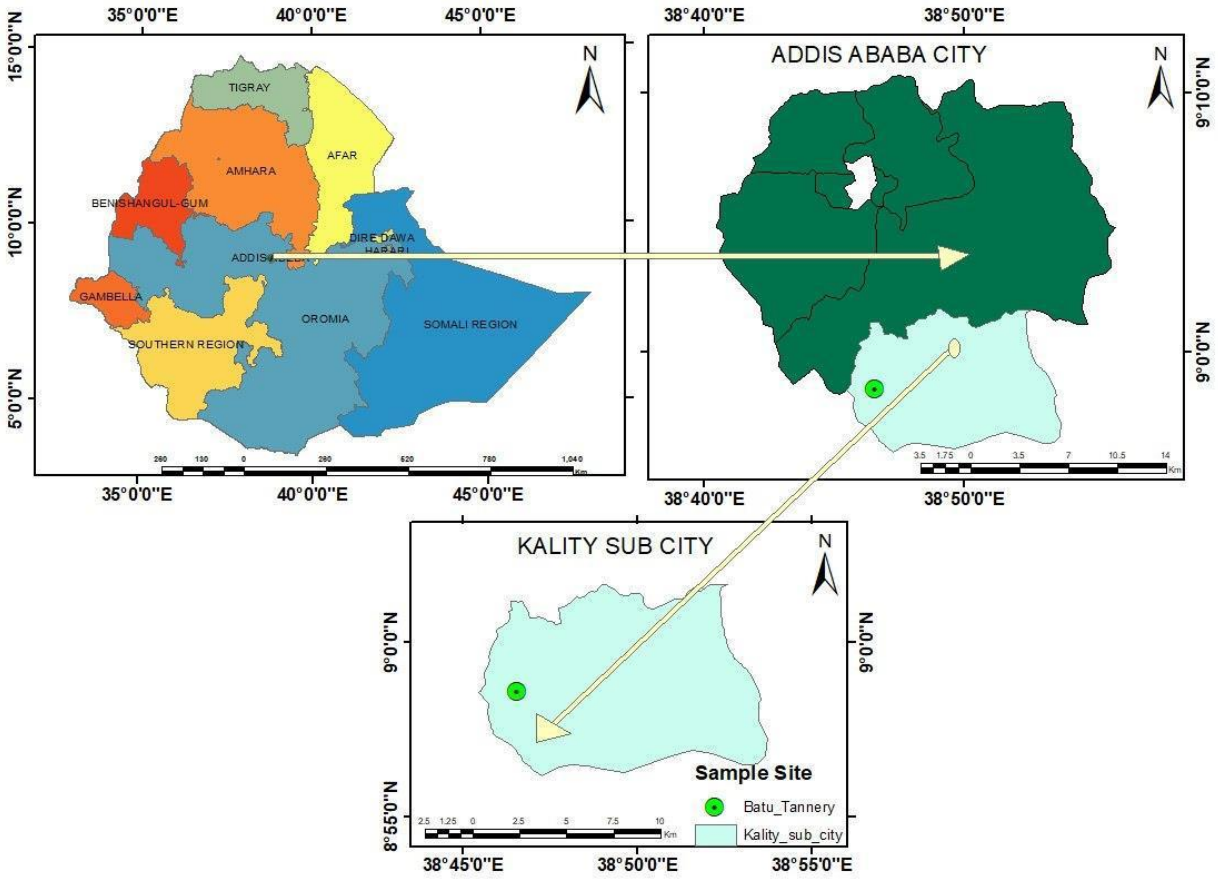

Figure 3. Map of Addis Ababa town and sites of wastewater samples collected from kality sub

3.2 Sample Collection

Using a sterile plastic container, a total of 10 (ten) wastewater samples (500 ml) were collected from chosen Batu Tannery during dry seasen wastewater (effluent) from various places. Batu tannery was selected because it found in urban areas. A sterile plastic container was used to collect the samples, and an ice box was used to transfer the samples to Applied Biology laboratory, ASTU. The samples were kept at 4°C for 24 h until the isolation and screening procedures started. In the experiments, phenol with a purity of 99.0% was used (Salem et al., 2021).

Figure 4. Site samples were collected

3.3 Isolation and Screening of possible phenol-degrading bacteria

To screen isolate cultures, wastewater samples were obtained from the BTI and inoculated from wastewater into flasks with minimal salt medium (MSM) (g/L) (KH_2PO_4 0.5, K_2HPO_4 0.5, $CaCl_2$ 0.1, NaCl 0.2, $MgSO_4 \cdot 7H_2O$ 0.5, $MnSO_4 \cdot 7H_2O$ 0.01 g, $FeSO_4 \cdot 7H_2O$ 0.01 g and NH_4NO_3 1.0). The media were supplemented with phenol as the only carbon source, at various concentrations (200, 500, 800, 1000, 1200, and 2000 ppm), and incubated for 7 d on a rotary shaker at 120 rpm and 30°C. After one week of incubation, 10 ml of the culture broth was transferred to a new shaking flask with 90 ml of fresh MSM medium . The final culture broth was serially diluted and disseminated on LB-agar (tryptone 10 g, yeast extract 5 g, and NaCl 5 g per liter) and MSM agar plates enriched with phenol (500 mg/L). This procedure was carried out three times. Colonies that emerged on the agar plates after 3-4 d of incubation at 30°C were selected and sub-cultured, to obtain pure cultures. Microscopy was used to check purity, especially optical microscopes, allow for the observation of the size and shape of microorganisms in a sample. If microorganisms exhibit uniform shapes, it indicates purity; otherwise, variations in shape

suggest impurities. Impurities or contaminants may introduce microorganisms of different sizes and shapes compared to the pure substance (Salem *et al*., 2021).

3.4 Identification of the Selected Bacteria

The isolated colonies were examined using an optical microscope to determine their morphological characteristics. Additionally, the typical physiological and biochemical traits of bacterial strains that possess the ability to degrade phenol, such as motility and Gram staining, were observed.

3.4.1 Morphological Characterization

Bacterial strains isolate was examined for colony morphology; cell shape and size of the isolated bacterium were studied using the microscope.

3.4.2 Gram Staining

To determine whether the strains were gram-positive or gram-negative bacteria, a process of microscopic inspection using slide preparations and gram-staining techniques was utilized. A single, purified colony was extracted using a loop from the plate and placed in the center of a clean slide, which was then smudged. To conduct the gram staining of the isolate, a smear was prepared from an overnight viable culture. The slide was heat-fixed and crystal violet was applied for 1 min. The slide was gently washed with tap water and covered with gram's iodine for 1 min before being washed off by tilting the slide. The smear was decolorized with 95% ethyl alcohol for 15 sec and then rinsed with tap water. The smear was then covered with safranin for one minute and washed with tap water. Finally, the smear was blotted and left to dry in the air before being examined under the oil immersion objective (Wilmotte & Herdman, 2001).

3.5 Biochemical Tests

Various biochemical tests were performed to distinguish between the different phenol-degrading bacterial isolates. These tests included the catalase test, citrate utilization test, H_2S production test, indole production test, motility test, urease test, gelatin hydrolysis test, and starch hydrolysis test. Based on the results of these tests, the bacteria could be classified into different genera to which they belong. These biochemical tests help in identifying and characterizing the bacterial isolates based on their metabolic capabilities and enzymatic activities.

3.5.1 Catalase test

This test that was used to test for the presence of the enzyme catalase. A loop full of the isolated bacteria was smeared on a clean microscope slide and 2-3 drops of 3% (v/v) H_2O_2 was added

directly on the smear, a positive test resulted in immediate bubbling (O_2 formed), while a negative test showed no bubbling (Taylor & Achanzar, 1972).

3.5.2 H_2S production, motility and indole production (SIM) test

To assess the isolated organism's sulfur reduction, indole production, and motility, SIM (Sulfide Indole Motility) medium (g/L) (Beef extract, 3; $Na_2S_2O_3$ 0.025; Peptic digest of animal tissue, 30; Peptonized iron, 0.2 and agar 3) was employed. For this experiment, SIM medium (Peptone from casein 20.0; peptone from meat 6.6; ammonium iron(II)citrate 0.2; sodium thiosulfate 0.2; agar-agar 3.0) was prepared, and poured into test tubes and allowed to solidify. The test tubes were then inoculated by inserting the inoculating needle into the center to a depth of half an inch. Subsequently, the test tubes were incubated at 30 °C for 24 h. After incubation, the test tubes were examined for the presence of a black precipitate (indicating H_2S production), a fuzzy area around the stab line (indicating motility), and the addition of 2-3 drops of Kovac's indole reagent. The formation of a red band following the addition of the reagent was observed to determine the production of indole (Vashist *et al*., 2013).

3.5.3 Citrate test

For the citrate test, Simmons Citrate agar (SCA) was prepared using the following ingredients: (g/L) (Bromo thymol blue, 0.08; $MgSO_4$, 0.2; (NH_4) $2HPO_4$, 1 and agar 15). The agar medium contained citrate as the sole carbon source and ammonium salts as the sole nitrogen source. Fresh, pure phenol degrading bacterial colonies that were 18-24 h old were streaked on the slants, and the test tubes were incubated at 30°C for 2-4 d. If the bacteria were capable of metabolizing citrate, a color change would occur in the SCA, transitioning from green to deep blue (MacWilliams, 2009).

3.5.4 Urease test

The urease test was conducted by preparing urea agar slants using a urea agar base. The urea agar base contained (g/L) (Dextrose 1; Disodium phosphate, 1.2; Monopotassium phosphate, 0.8; NaCl, 5; Peptone, 1; Phenol red, 0.012 and Agar, 15). After sterilization and cooling, 40% (w/v) urea solution was added to the agar base. Pure phenol-degrading bacterial colonies, aged 18-24 h, were streaked on the slants and then incubated at 30 °C for up to 5 d. Bacterial colonies capable of producing urease and converting urea into carbon dioxide (CO_2), water (H_2O), and ammonium (NH_4) caused a color change on the slants from yellow to bright pink (Brink, 2016).

3.5.5 Starch hydrolysis test

The starch hydrolysis test assessed the ability of phenol-degrading bacteria to produce the enzyme amylase, which breaks down starch into smaller sugar molecules. Starch agar (g/L) (Beef extract, 3; starch, 10; agar 12) is also used in differentiating members of various genera which have both amylase-positive and amylase-negative species. Heat the medium to dissolve the starch and sterilize it by autoclaving at 121°C for 15 min. Using a sterile inoculating loop or needle, streak the surface of the starch agar plate with the phenol-degrading bacterial isolate being tested. Incubate the inoculated starch agar plate at 30°C for 24-48 h is suitable. After the incubation period, flood the surface of the plate with iodine solution. The iodine solution reacts with starch to form a dark blue or black color. Observe the plate for any zone of clearing around the bacterial growth (Archana Lal, 2016).

3.5.6 Gelatin hydrolysis test

In this method, a heavy inoculum of 18- to 24-h-old bacterial culture is stab-inoculated into tubes containing nutrient gelatin (g/L) (Peptone, 5.0; Beef extract, 3.0; Gelatin, 120.0; Final pH: 6.8 ± 0.2 at 30°C). The inoculated tubes and an uninoculated control tube are incubated at 25°C, or at the test bacterium‘s optimal growth temperature, for up to 1 week, and checked every day for gelatin liquefaction. Gelatin normally liquefies at 28°C and above, so to confirm that liquefaction was due to gelatinase activity, the tubes are immersed in an ice bath for 15 to 30 min. Then, tubes are tilted to observe if gelatin has been hydrolyzed. Hydrolyzed gelatin will result in a liquid medium even after exposure to cold temperature (ice bath), while the uninoculated control medium will remain solid. For weak positive results, incubate the inoculated nutrient gelatin tube longer until complete liquefaction is observed. The hydrolysis of gelatin indicates the secretion of gelatinase by the test organism into the medium (Clarke, 1953).

3.5.7 Triple Sugar Iron Agar Test

To facilitate the observation of carbohydrate utilization patterns, TSI Agar (g/L) (Peptone, 20; Yeast extract: 3; Beef extract, 3, Lactose: 10; Sucrose, 10; Glucose, 10; Sodium chloride, 5; Ferrous sulphate: 0.2; Sodium thiosulphate, 0.3; agar, 12; distilled water; Phenol red, 0.024) contains three fermentative sugars, lactose and sucrose in 1% concentrations and glucose in 0.1% concentration. Due to the production of acid during fermentation, the pH falls. The acid base indicator Phenol red is incorporated for detecting carbohydrate fermentation that is indicated by the change in color of the carbohydrate medium from orange red to yellow in the presence of acids. In case of oxidative decarboxylation of peptone, alkaline products are produced and the pH rises. This is indicated by the change in color of the medium from orange red to deep red.

Adjust pH to 7. Then add phenol red indicator. Sterilize by autoclaving at 121°C for 20 min. Allow the medium to set in slopped form with a butt about 1 inch long. The medium is used in the form a butt and slant. Using sterile technique, the test organism was inoculated into the medium by means of stab and streak inoculation. An uninoculated tube was kept as control both tubes were incubated at 30°C for 24 h and the reaction was observed (Welsh, 2019).

3.5.8 Identification of phenol degrading bacteria species using MALDI-TOF MS

The phenol-degrading bacterial isolates were subjected to analysis using Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS), a technique that identifies microorganisms based on their protein or peptide profiles. The analysis was conducted at Wudase diagnostic center located in Addis Ababa. Prior to analysis, the bacteria were cultured overnight. The extended direct transfer (eDT) method, following the manufacturer's instructions, was employed for sample analysis (Xiao et al., 2012). This method involved using 70% formic acid to disrupt the cell membranes of the bacteria and facilitate the extraction of microbial proteins or peptides for characterization. To prepare the target plate, which contained 94 wells, it was thoroughly cleaned and dried. A distinct colony from the overnight bacterial culture was directly applied to the wells, creating a thin film that was allowed to dry. The dried spot was then overlaid with 1µl of 70% formic acid, followed by drying at room temperature. Subsequently, 1µl of α-cyano-4-hydroxycinnamic acid (HCCA) solution was applied and allowed to dry. Once the samples were completely dried, the target plate was placed inside the MALDI Zybio for analysis. The samples are to be said adequately identified to the species level when the logarithmic score value (LSV) is greater than or equal to 1.9 (Tsirogianni *et al*., 2005).

3.6 Optimization of growth conditions for isolated phenol-degrading bacteria

In order to determine the physiological growth requirements and optimal growth conditions of the isolated bacteria two specific isolates, namely PDBI-9, and PDBI-49, were selected for analysis. The two isolates, PDBI-9 and PDBI-12, were chosen for their phenol tolerance and ability to degrade phenol. Various growth factors were investigated, including incubation temperature, pH range, carbon source, and nitrogen source. The analysis of optimal growth conditions followed a systematic approach. Each growth factor was examined individually using a one-factor-at-a-time completely randomized method, with triplicate measurements taken for each condition. This method allowed for a comprehensive assessment of the impact of each growth factor on the bacterial growth. Different incubation temperatures were tested to identify the temperature at which the bacteria exhibited the highest growth rate. Similarly, various pH

ranges were investigated to determine the pH value that favored the optimal growth of the bacteria. In addition, different carbon sources and nitrogen sources were examined to identify the most suitable sources for promoting bacterial growth. The triplicate measurements ensured reliability and consistency in the results, providing a more accurate assessment of the optimal growth conditions for the isolated bacteria.

3.6.1 Effect of temperature on the growth and phenol degradation

To find out the optimum growth temperature of the isolated phenol-degrading bacteria the isolates were all cultured on the aforementioned medium, with the same volume of media and for the same amount of time. The bacterial isolate was grown in Minimal salt medium (MSM) (g/l) (KH_2PO_4 0.5 g, K_2HPO_4 0.5, $CaCl_2$ 0.1, NaCl 0.2, $MgSO_4 \cdot 7H_2O$ 0.5, $MnSO_4 \cdot 7H_2O$ 0.01, $FeSO_4 \cdot 7H_2O$ 0.01 and NH_4NO_3 1.0) with 1000 mg/l of phenol at different temperature values (25, 30, 35,37 and 40°C) at pH 7 using 250 ml flasks. The cultures were placed on a shaker (120 rpm) at the above-mentioned temperatures. The growth was measured at 48 h and phenol degrading bacteria growth was measured using UV light (Salem *et al*., 2021). After 48 h, the optical density and biomass of the cultured medium were taken in triplicates and recorded. The generated data were analysed using the Microsoft office Excel Software.

3.6.2 Effect of pH on the growth and phenol degradation

To find out the optimum pH range of the isolated phenol degrading bacteria the isolates were all cultured on the same medium, with the same volume of medium and for the same duration of time. The bacterial isolate was grown in MS – medium. The effect of pH (6-8) on growth and phenol degradation was tested. The broth cultures were grown using mechanical shaker at 30°C using MS medium, which supplemented with 1000 mg/l phenol and 5 % (w/v) inoculum size in 250 mL flask. The biomasses were measured at 48 h. and phenol degrading bacteria growth was measured by UV light (Salem *et al*., 2021). After 48 h, the optical density and biomass of the cultured medium were taken in triplicates and recorded. The generated data was analyzed using the Microsoft office Excel Software.

3.6.3 Effect of nitrogen sources on the growth and phenol degradation

Effect of different nitrogen sources on growth and phenol degradation was determined. Each culture was grown as shake cultures at 35°C in MSM (g/L) (KH_2PO_4 0.5 g, K_2HPO_4 0.5, $CaCl_2$ 0.1, NaCl 0.2, $MgSO_4 \cdot 7H_2O$ 0.5, $MnSO_4 \cdot 7H_2O$ 0.01, $FeSO_4 \cdot 7H_2O$ 0.01 and NH_4NO_3 1.0) supplement with 1000 mg/L phenol and in the source of nitrogen was replaced with peptone,

yeast extract, KNO_3 and NH_4SO_4 at concentration of 2 g/L. The growth was measured at 48 h and phenol degrading bacteria growths were measured using UV light (Salem *et al*., 2021). After 48 h, the optical density and bio mass of the cultured medium were taken in triplicates and recorded. The generated data were analyzed using the Microsoft office Excel Software.

3.6.4 Effect of carbon source on the growth and phenol degradation

To find out the most suitable carbon source for the growth of the phenol degrading bacterial isolates, four carbon containing organic compounds were used. MSM (g/L) (KH_2PO_4 0.5, K_2HPO_4 0.5, $CaCl_2$ 0.1, NaCl 0.2 g, $MgSO_4 \cdot 7H_2O$ 0.5, $MnSO_4 \cdot 7H_2O$ 0.01, $FeSO_4 \cdot 7H_2O$ 0.01; and NH_4NO_3 1.0) was prepared. The carbon sources (2%, w/v) glucose, fructose, mannitol and sucrose were added to each prepared minimal salt medium. After autoclaving, 250 ml medium was poured into 500 ml Erlenmeyer flasks and 200 µl of 24 h old fresh inoculum of the isolated bacteria was inoculated into the medium. Then the flasks were incubated for 30°C for 48 h under agitation at 120 rpm. After 48 h, the optical density and bio mass of the cultured medium were taken in triplicates and recorded (Salem *et al*., 2021). The generated data were analyzed using the Microsoft office excel software.

3.7 Phenol degradation efficiency of phenol degrading bacteria isolates

Preparing a culture of phenol-degrading bacteria and adjusting it to an optical density at 600 nm ($OD_{600\ nm}$) of 1.0. A final concentration of 2% (v/v) of the bacterial inoculum was then added to flasks containing minimal salt media (MSM) with phenol 1000 mg/l as the sole carbon source. The range of phenol concentrations in the flasks was 1000 mg/L. The flasks were incubated at 30°C with shaking speed of 120 rpm for a duration of 4 d. Samples were collected periodically to measure both biomass and optical density of phenol degradation. The optical density content was monitored spectrophotometrically by measuring the absorbance at a wavelength of 540 nm. To determine the concentration of phenol in the samples, 2 g ferric chloride was used as a color reagent. The reaction between phenol and ferric chloride resulted in the formation of a colored product, which could be quantified using spectrophotometry. By comparing the absorbance of the samples with a calibration curve prepared with known concentrations of phenol, the concentration of phenol in the samples could be determined (Apostica *et al*., 2018).

3.8 Data analysis

Data were computed into Microsoft excel to calculate mean, standard deviation, and standard error for isolate's biomass (CDW, g/ml) against different carbon source, physical parameter such

as pH and temperatures. IMB SPSS 24 and OriginPro8.5 statistical software were used 30 for data analysis. All data were mean of experiments performed in triplicate. Descriptive statistics were used to summarizing quantitative data.

4. RESULTS AND DISCUSSION

4.1 Isolation and screening of phenol-degrading bacteria from tannery wastewater

In this study, a total of 49 isolates were screened from the wastewater originated from the BTI (Table 3/Figure 5,6). These isolates were found to degrade phenol . As indicated in Figure 3a, these PDBIs exhibited various colony colors when grew on Luria-Bertani (LB) medium. It has been recognized that 600 to 2000 $_{ppm}$ (99%, w/v) of phenol crystal and nitrophenol served as a specific carbon source for the growth of these PDBIs. It was observed that over time and through acclimation, bacteria exhibited increase in biomasses when phenolic compounds supplied as the sole carbon sources, indicating a gradual adaptation to the compound. Similarly, it has been reported (Salem *et al*., 2021) that numerous bacterial and fungal species can tolerate environments which were contaminated with phenolic compounds.

Xu *et al*., (2021)) employed a consistent methodology to ensure the efficiency of the medium and to selectively isolate bacterial strains which are effective for phenol degradation from samples of tannery wastewater. By utilizing the characteristics of these isolated microorganisms, solutions that are efficient and economically feasible are being developed, offering a workable and affordable way to reduce environmental impact. Notably, phenol served as the primary carbon and energy source for all colonies thriving on the solid medium containing phenol. The isolates exhibited significantly accelerated growth rates in medium containing up to 2 g/L of phenol, as detailed in Table 2. This standardized approach ensures the effective isolation, cultivation and identification of phenol-degrading bacteria, laying the groundwork for potential applications in environmentally sustainable technologies.

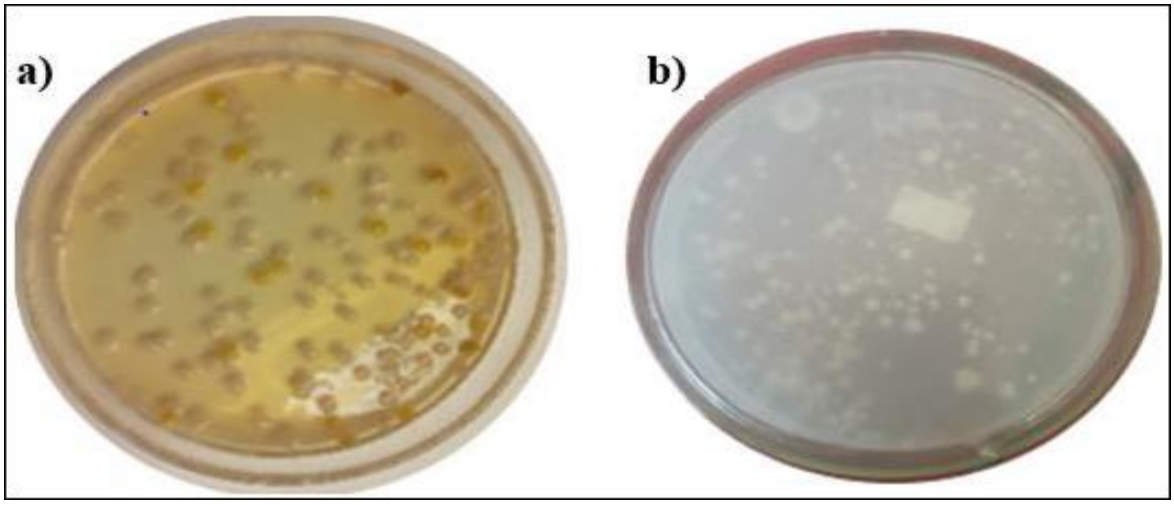

Figure 5. a) indicates phenol degrading bacteria growth on LB agar, b) The presence of phenol-degrading bacteria growth is indicated on MSM agar.

The recent PDBIs exhibited resistance to phenol compounds was accomplished through a meticulous selection process. Out of 49 isolates, PDBI-7, PDBI-9, PDBI-11, PDBI-12, PDBI-15, PDBI-21, PDBI-23, and PDBI-49 were recognized as a potential isolate to grow on MSM that supplied with various concentration of phenolic compound as displayed in the Table 2. These isolates could utilize phenolic compound as the main carbon sources. Subsequent treatment with various concentration of phenolic compound ensured the selection of these PDBIs, which capable to withstanding this compound.

Notably, isolates PDBI-9 and PDBI-12 demonstrated resilience even at higher phenol (crystal and nitrophenol) concentrations, specifically at 2000 $_{ppm}$. Furthermore, isolates PDBI-49 and PDBI-21 exhibited the capability to withstand elevated phenolic concentrations, reaching up to 2000 ppm (Table 2). In line with this study, (Paisio *et al*., 2012) reported that microorganisms living in environments contaminated with tannery wastes show tolerance to chromium and phenolic compounds.

Table 2: The growth of phenol-degrading isolates was observed as they utilized phenol as a carbon source.

S.N.	Phenol concentration (Phenol crystal and nitrophenol) (ppm)							
	Isolates	**200**	**400**	**600**	**800**	**1200**	**2000**	**3000**
1	PDBI-7	+	+	+	+	-	-	-
2	PDBI-9	+	+	+	+	+	-	-
3	PDBI-11	+	+	+	+	-	-	-
4	PDBI-12	+	+	+	+	+	+	-
5	PDBI-15	+	+	+	+	-	-	-
6	PDBI-21	+	+	+	+	+	-	-
7	PDBI-23	+	+	+	+	-	-	-
8	PDBI-49	+	+	+	+	+	+	-

Legend : the use of "+" indicates the growth capacity of phenol-degrading bacteria, while "-" indicates the absence of growth in phenol-degrading bacteria.

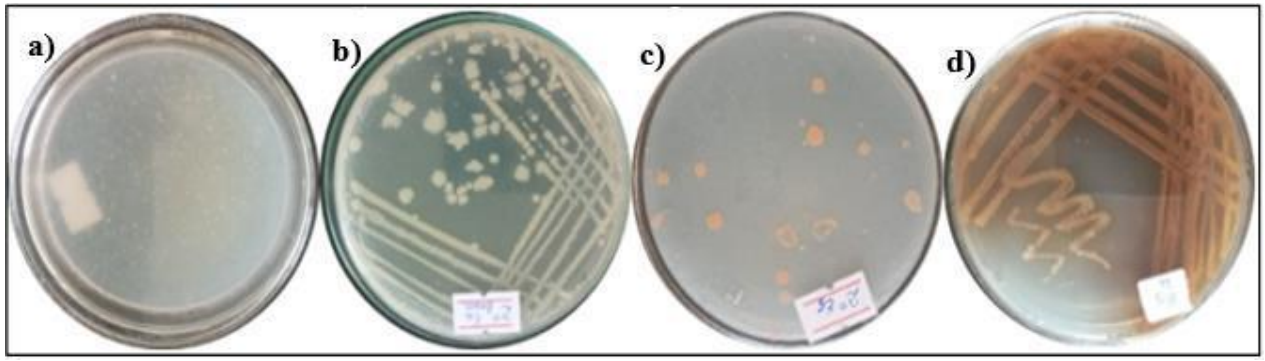

Figure 6: Process of isolating, and screening these bacterial isolates which were capable of degrading phenol from tannery wastewater. The bacteria were grown on Mineral Salt Medium (MSM) and Luria-Bertani (LB) agar. a) PDBI-7 growth on MSM (Minimal Salt Medium). b) PDBI-7 growth on LB (Luria-Bertani) medium. c) PDBI-49 growth on MSM (Minimal Salt Medium). d) PDBI-49 also shows growth on LB (Luria-Bertani) medium.

4.1.1 Gram staining and morphological characterization for PDBIs

Distinct morphological and biochemical characteristics were observed in the pure cultures of phenol (phenol crystal and nitrophenol) degrading bacterial isolates . Forty-nine (49) isolates were found to grow on an MSM broth from them eight isolates were resist 600 $_{ppm}$ phenol as the sole carbon source and energy (Table 2**)**.

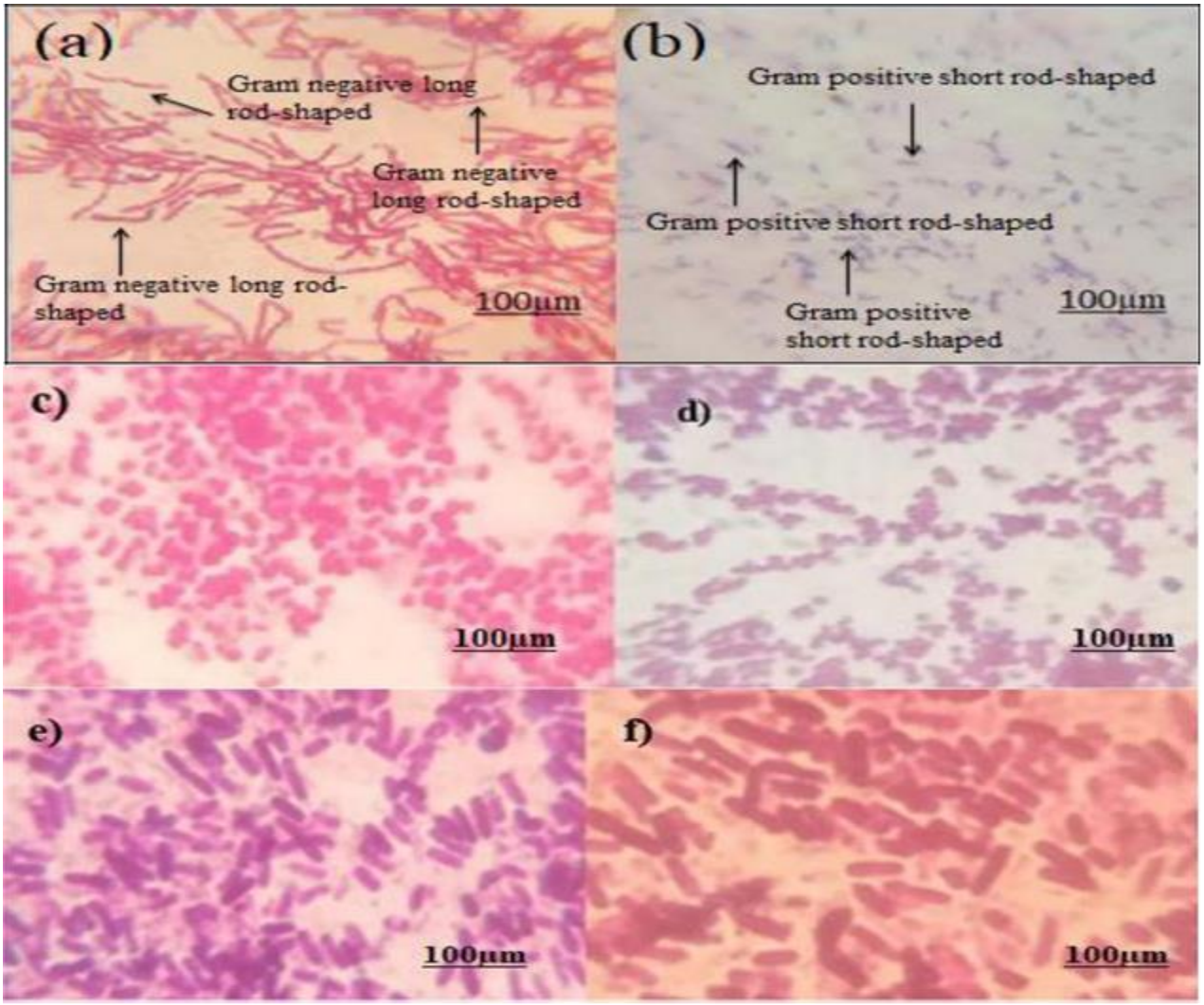

Figure 7. Gram staining for PDBIs, which obtained from BTI. a) PDBI-49 is a long-rod-shaped Gram-negative, which can degrade phenolic compounds. b) PDBI-9 indicated a short rod-shaped Gram-positive isolate which can degrade phenolic compounds. c) PDBI-46 is gram negative cocci, which can degrade phenolic compounds d) PDBI-1 is gram positive cocci, which can degrade phenolics compounds e) PDBI-29 a short rod gram positive isolate, which can degrade phenolics compounds f) PDBI-37 a short-shaped gram negative isolate, which can degrade phenolics compounds

Interestingly, eight isolates were found to resist 600 $_{ppm}$ of phenol (phenol crystal and nitrophenol), while four of the isolates were able to resist even higher concentrations 1200 $_{ppm}$ and two of them were able to resist even higher concentrations, specifically 2000 $_{ppm}$ (Table 3). PDBI-9 and PDBI-12 were Gram-positive rod-shaped isolates and PDBI-21 and PDBI-49 were Gram negative rod shaped. Upon observation of their Gram staining images, isolate (a) appeared as a Gram-negative short rod, while isolate (b) appeared as a Gram-positive short rod (Figure 5).

4.1.2 Biochemical tests for PDBI

Out of the 8 isolates 5 were found to be *Bacillus*, 3 isolates were Enterobacter genus were also isolated from tannery wastewaters (Table 3).

Table 3. Identification of the bacteria isolated from the tannery wastewater sample grown on mineral salt agar medium

S.N.	Biochemical tests																		
	Bacterial Isolate Code	Gram staining		Catalase test	Citrate test	Gelatinhydrolysis Test		Indole test	Motility	Protease	Starch hydrolysis	Urease test	Fermentation						Mald tof identification
													Gas production		Mannitol	Fructose	Sucrose		
1	PDBI-7	+	R	+	+	+	+	-	+	+	+	-	F	-	F	F	F	F	*B. licheniformis*
2	PDBI-9	+	R	+	+	+	+	-	-	+	+	-	F	+	F	F	F	F	*B. subtilis*
3	PDBI-11	+	R	-	+	+	+	-	-	+	+	-	F	+	F	F	F	F	*B. licheniformis*
4	PDBI-12	+	R	+	+	+	+	-	+	+	+	+	F	+	F	F	F	F	*B. subtilis*
5	PDBI-15	-	R	+	-	-	+	-	+	+	-	-	F	+	F	F	F	F	*P. merabilis*
6	PDBI-21	-	R	+	+	+	-	-	-	+	+	+	F	-	F	F	F	F	*K.pneumoniae*
7	PDBI-23	+	R	+	+	+	-	-	-	+	+	+	F	-	F	F	F	F	*Bacillus cereus*
8	PDBI-49	-	R	+	+	+	-	-	-	+	-	+	F	-	F	F	F	F	*K.pneumoniae*

Legend : PDBI indicates phenol degrading bacteria isolates, + = Positive, - N= Negative, F = Fermented, NF = No Fermentation, R= rod

4.2 Identification of phenol degrading bacteria isolates by MALDI-TOF MS

In this study, various species of phenol-degrading bacterial isolates were identified as *B. cereus* (PDBI-23), *B. subitilis* (PDBI-9, PDBI-12), *B. licheniformis* (PDBI-7), *Bacillus* sp. (PDBI-11), *Klebsialla* sp. and *P. merabilis* (PDBI-15) from tannery wastewater. The results of this investigation offer evidence in favour of the existence of such bacterial species in the specified situation *Bacillus* spp (P. Taylor & Yusuf, 2013), *K. pneumoniae*, *P.subtili* (Ashkan *et al*., 2023). *B. licheniformis* PDBI-7 was obtained from the in-let wastewater site, while *B. subtilis* PDBI-9, *Bacillus sp* PDBI-11, and *B. subitilis* PDBI-12 were isolated from the denitrification site of the wastewater treatment process. *P.merabilis* PDBI-15 was obtained from the primary treatment site, and *Klebsialla* sp. PDBI-21 and *B. cereus* PDBI-23 were isolated from the secondary treatment site. Lastly, *K. pneumoniae* PDBI-49 was obtained from the out-let wastewater (Table 4). In line this study, various phenol degrading bacterial species were identified using MALDI-TOF MS, which is highly precise for identification of bacterial isolates, which collected from Batu Tannery industry. The experts further clarified that the occasional inability of MALDI to identify certain bacteria may be attributed to the absence of those organisms in the existing databases, rather than being a result of a procedural error (Bizzini & Greub, 2010).

Table 4: MALDI-TOF MS analysis for isolated phenol degrading bacterial species these obtained from BTI

S.N	Sample code	Species	LSV
1	PDBI-7	*B. licheniformis*	2.18
2	PDBI-9	*B. subtilis*	2.44
3	PDBI-11	*Bacillus* sp.	1.66
4	PDBI-12	*B. subtilis*	2.18
5	PDBI-15	*Proteas merabilis*	2.40
6	PDBI-21	*Klebsialla* sp.	1.91
7	PDBI-23	*B. cereus*	2.24
8	PDBI-49	*K. pneumoniae*	2.45

Legend: PDBI indicates phenol degrading bactria isolates LSV stands for Logarithmic Score Value

4.3 Physiological characterization and growth condition of phenol degrading bacteria

4.3.1 Effect of carbon sources on phenol degrading bacteria

Carbon sources (2%) and phenolic compounds (1000 $_{ppm}$) were employed for the growth of certain PDBIs and considerable amounts of CDW (g/L) and $OD_{600\ nm}$ (Figure 6) were recorded for *B. subtilis* PDBI-9 and *K. pneumoniae* PDBI-49 (Table 4). Both isolates exhibited maximum growth CDW (g/l) and OD $_{600\ nm}$ production when glucose was supplied as the sole carbon source (Figure 5). As indicated in Figure 4, the least CDW (g/l) and OD $_{600\ nm}$ exhibited for sucrose. The highest CDW (g/l) and OD $_{600\ nm}$ were recorded when glucose used for *B. subtilis* PDBI-9 (0.005 ± 0.0002 g/L, 1.093 ± 0.003785939) and *K. pneumoniae* PDBI-49 (0.005 ± 0.0003gms, 1.071 ± 0.002) isolates (Figure 6), respectively. In this respect, it was reported that the addition of non-toxic substances such as glucose activates cell viability and degradation process (Topp and Hanson, 1988).

In this study, variations were observed in terms of the measured CDW (g/l) and OD $_{600\ nm}$ for the isolates at a 95% confidence interval ($p<0.05$). As illustrated in (Figure 6a), there were no statistically significant variations observed in CDW g/l among glucose and fructose ($p=0.450$), fructose and sucrose ($p=0.130$), fructose and mannitol ($p=0.206$) as well as sucrose and mannitol ($p=0.795$). However, there was significant variations recorded for glucose and sucrose ($p=0.009$), glucose and mannitol ($p=0.043$), and glucose and sucrose ($p=0.001$), for isolate PDBI-9. For the isolate PDBI-49 there were no statistically significant variations observed in CDW g/ml among glucose and fructose ($p=0.377$) which may be attributed to their similar nature of being simple sugars (Figure 6c). In addition, glucose supports cell densities and microbial growth that increased its degradation activity (Loh & Wang, 1997). Sachan *et al*., (2019) reported that the best carbon source for phenol-degrading bacterial isolates, which sourced from petrochemical wastes that could support the growth of the isolates and reduce the concentration of phenol present in the medium to 1% glucose (w/v). The same authors stated that the same isolates were failed to grow in the absence of glucose.

Mane et al. (2014) found the suitability of glucose for various phenol degrading bacterial isolates, these collected from industrial sewages However, recently Patil et al. (2023) have stated that the addition of carbon sources showed a negative effect on phenol degradation for

these isolates, which showed maximum growth and phenol degradation when phenol was supplemented as a maincarbonsource.

The precarious nature of this occurrence may be due to the isolated bacterial strains' capacity to evolve and choose phenol as their sole source of carbon and energy, increasing the density of the microorganisms. It has been reported that, phenol-degrading bacteria that have been isolated from phenol-contaminated environments have the capacity to withstand the extreme conditions of phenol contamination while also reproducing, expanding their cell size, and assimilating the phenol mass (Patil et al., 2023).Growth of diversities of bacterial cells can be determined by specific carbon sources like glucose. However, some of these isolates highly utilize carbon source like glucose due to its simple structure, monomer. These isolates may use this specific carbon source for the formation of their cellular structures (Beisel & Afroz, 2015). As illustrated in Figure 5, significant variation observed for CDW (g/L) between glucose & mannitol (*p=0.048*), and glucose & sucrose (*p=0.027*) for *B. subtilis* PDBI-9 (Table 2). Highly significant variation was noticed among all carbon sources for *B. subtilis* PDBI-9 with $OD_{600\ nm}$ (*p=0.000*) (Figure 6). No variation detected among fructose, mannitol and sucrose for CDW (g/L) with *B. subtilis* PDBI-9 (*p>0.05*) (Table 2).

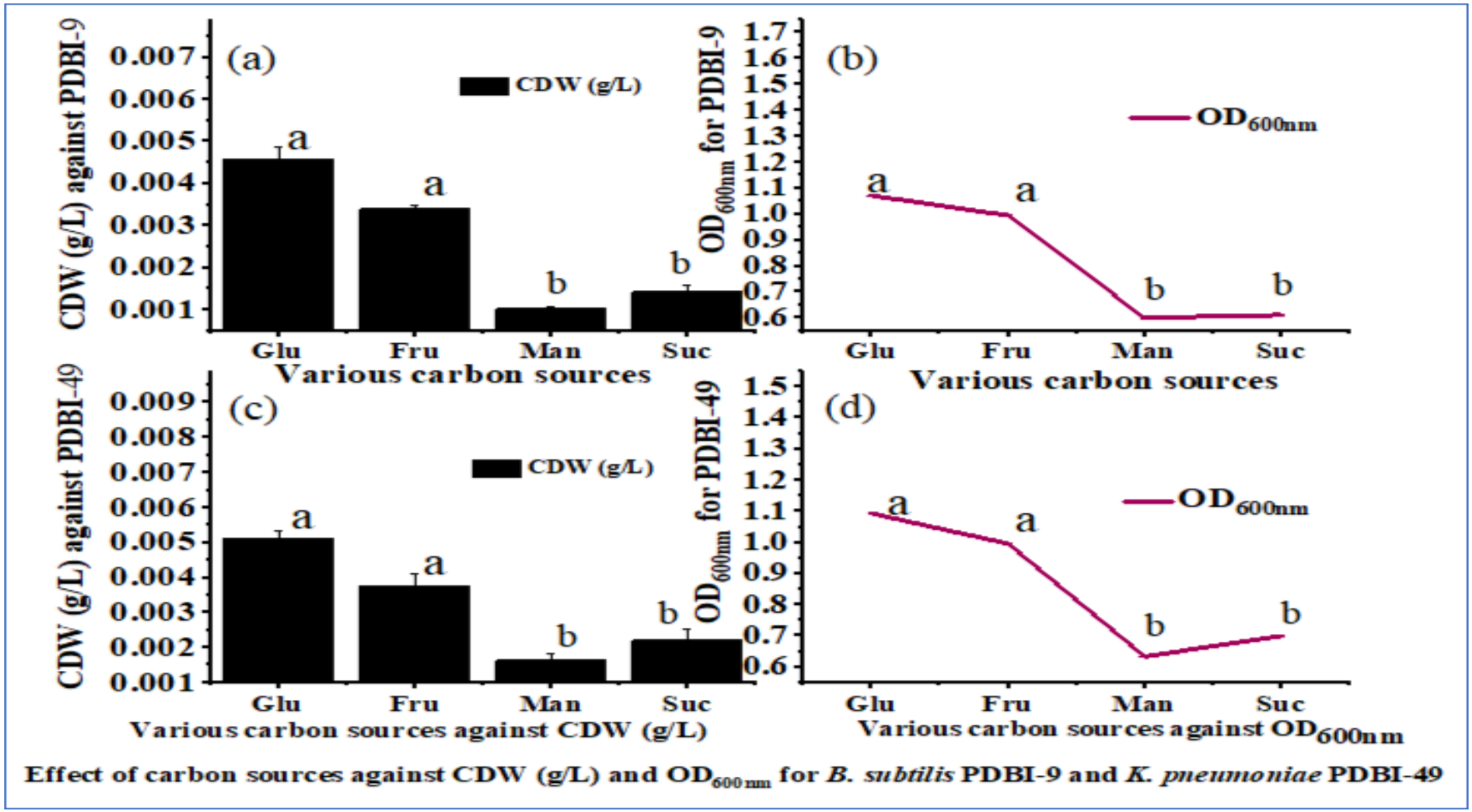

Figure 8: Effect of carbon source against *B. subtilis* PDBI-9 and *K. pneumoniae* PDBI-49 in terms of CDW (g/L) and OD_{600nm}. (a) CDW (g/L) against *B. subtilis* PDBI-9 due to various carbon sources. (b) OD600nm against *B. subtilis* PDBI-9 due to fructose, glucose, mannitol and sucrose. (c) CDW (g/L) against *K. pneumoniae* PDBI-49 due to various carbon sources. (d) OD_{600nm} with significant variation among all carbon sources *(p=0.000*) against *K. pneumoniae* PDBI-49 at 30°C. In this study, data represented the mean ± SD of three replicates (n=3). One-way Anova exhibited that significant variations were observed for phenol degrading bacterial isolates having different letter on their respective bars and line graphs against CDW (g/L) and OD_{600nm}, respectively.

Recent study validated that highly significant variations were also observed for CDW (g/L) between glucose & mannitol (*p=0.021*), and glucose & sucrose (*p=0.028*) against *K. pneumoniae* PDBI-49(Ashkan et al. (2023)). Whereas, there was no variation detected between fructose & glucose (*p=0.377*), fructose & mannitol (*p=0.312*), and fructose & sucrose (*p=0.171*) against *K. pneumoniae* PDBI-49 for CDW (g/L) (Table 2). It was also confirmed that very high variations were noticed in terms of $OD_{600\ nm}$ among glucose, fructose, mannitol and sucrose (p=0.000) for these *B. subtilis* PDBI-9 and *K. pneumoniae* PDBI-49 , phenol crystal and nitrophenol degrading isolates, respectively (Figure 6). Similarly, previous study showed that variation were detected between fructose & molasses, fructose & ribose, glucose & molasses, glucose & ribose, and Molasses & ribose (*p<0.05*) against *Bacillus* sp LPPI-18 with PHA producing isolate at 37°C in terms of CDW (g/L) (Mohammed and Ray, 2022) . The same authors stated that no variation detected between fructose and glucose for *Bacillus* sp LPPI-18 strain, isolate obtained from Loktak Lake soil sediment. Effect of various biomasses (g/L) (*p>0.05*) were noticed between *P. aeruginosa* PDI-1 & *P. balearica* PDI-17, *P. aeruginosa* PDI-1 & PDI-21 (not identified), and *P. balearica* PDI-17 & PDI-21 when Polyethylene supplied as a main carbon source at its 0.5 % (w/v) concentration (Nedi *et al*., 2024). Contrary to this study, no variations (*p>0.05*) had been noticed among *P. aeruginosa* PDI-1, *P. balearica* PDI-17, PDI-21 (not identified), phenol degradation isolates in terms of biomasses (g/l) (Nedi et al., 2024) at 0.05% (w/v) concentration of Polyethylene that supplemented as a carbon source for these strains.

4.3.2 Effect of nitrogen source on phenol degrading bacteria

Peptone was the preferred source of nitrogen for growth against *B. subtilis* PDBI-9 and *K. pneumoniae* PDBI-49 isolates with highest CDW (g/l) (0.00740.0004 and 0.0087± 0.00031and OD_{600nm} (1.086±0.004 and 1.106±0.005), respectively (Figure 7). It was found that *B. subtilis* PDBI-9 (0.0056±0.00031g/L &1.055± 0.005) and *K. pneumoniae* PDBI-49 (0.0076±0.0002 g/L &1.075±0.0012) were also preferred yeast extract as a suitable nitrogen source for their growth against CDW (g/L) and $OD_{600\ nm}$, respectively. In this test, significant variations were observed between the organic nitrogen source peptone and the inorganic nitrogen sources in the growth of PDBI-9 (*p=0.011*) and (*p=0.007*) as well as PDBI-49 (*p=0.008*) and (*p=0.004*) for (NH4)2SO4 and KNO3, respectively (Figure 9b). However, no significant variation was detected for both PDBI-9 (*p=0.165*) and PDBI-49 (*p=0.165*) isolates when using peptone and yeast extract as the sole nitrogen sources.

Numerous prior investigations substantiate the observation that bacteria exhibit a preference for organic nitrogen sources over inorganic ones (Ashkan et al. 2023). A study by Salem *et al*. (2021) have revealed that for the phenol-degrading *Bacillus* sp. (C2), yeast extract supplementation as the sole nitrogen source resulted in the most significant growth, followed by peptone as the second most favourable option. Furthermore, Ashkan *et al*. (2023) Have demonstrated that *K. pneumoniae* and *K. variicola* strains, which specialized in peptone degradation were isolated from petrochemical-contaminated soil, and exhibited maximal growth when supplied with organic nitrogen sources, specifically peptone and yeast extract, respectively. Notably, although these isolated phenol-degrading bacteria exhibited a preference for organic nitrogen sources, inorganic nitrogen sources were still capable of sustaining bacterial growth. In this study $(NH_4)_2SO_4$ was more supportive for the growth of *B. subtilis* PDBI-9 and *K. pneumoniae* PDBI-49 isolates compared to KNO_3. Additionally, Ahmad *et al*., (2011) have reported that *Acinetobacter* sp. AQ5NOL 1, which isolated from industrial effluent, $(NH_4)_2SO_4$ is the most suitable nitrogen source for growth and phenol degradation compared to sodium nitrate, ammonium chloride, cysteine, and histidine.

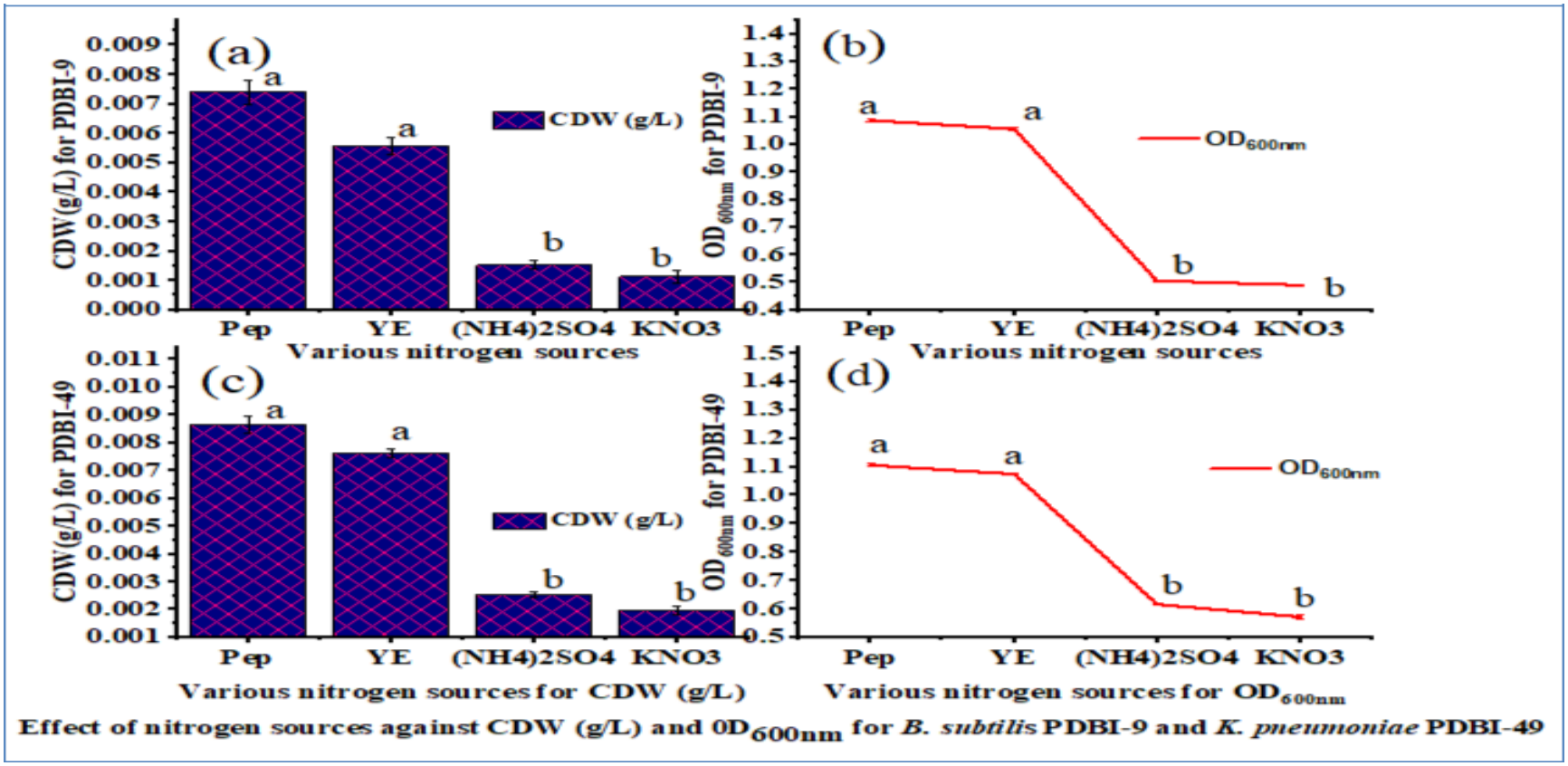

Figure 9: Effect of nitrogen source against *B. subtilis* PDBI-9 and *K. pneumonia* PDBI-49 interms of CDW(g/l) and OD 600nm. a) CDW (g/l) *B.subtilis* PDBI-9 due to various nitrogen sources. b) OD 600nm against B. subtilis PDBI-9 due to pepton, yeast extract, (NH4)2SO4, KNO3. c) CDW (g/l) *K. pneumonia* PDBI-49 due to various nitrogen sources. d) OD600nm with significant variation among all carbon sources (p=0.) against *K. pneumonia* PDBI-49. In this study, data represented the mean ± SD of three replicates (n=3). One-way Anova exhibited that Signiant variations were observed for phenol degrading bacterial isolates having different letter on their respective bars and line graphs against CDW (g/L) and OD_{600nm}, respectively.

4.3.3 Effect of temperature on phenol degrading isolated bacteria

After 48 h measurements, it was found that 30°C was the optimal growth temperature for *B. subtilis* PDBI-9 and *K. pneumoniae* PDBI-49 bacterial isolates (Figure). In terms of OD_{600nm} and CDW(g/l) multiple comparison of One-way ANOVA at LSD Post Hoc Test indicated that statistically significant variations detected among 25, 30 , 35 , 37 and 40°C ($p<0.05$) for both isolates. Specifically, great significant variations observed between 25 & 30, 35 & 30, 37 & 30 and 40 & 30°C ($p=0.00$) at optimum pH (Figure 9a and c). The maximum CDW (g/l) and OD_{600nm} noticed for *B. subtilis* PDBI-9 (0.0086 ± 0.00015, and 0.65 ± 0.0064) and *K. pneumoniae* PDBI- 49 (0.009 ± 0.00031, & 0.623 ± 0.005) isolates, respectively. The minimum growth of the bacterial isolates obtained for CDW (g/l) and OD_{600nm} at 40°C. However, at optimum pH value, no statistically significant variations were detected in terms of CDW (g/ml) when PDBI-9 isolates were incubated at 35 and 37°C ($p=0.249$) as well as at 25 and 40°C ($p=0.621$). In the case of CDW (g/ml) when PDBI-49 was incubated at optimum pH, there were no significant variations recorded between the optimal temperature of 30 and 35°C ($p=0.531$) as well as 30 and 37°C ($p=0.107$) which shows a relative range of temperature tolerance by the isolates. The growth of these PDBIs might be retarded due to elevated temperatures. In agreement with this study, it was reported that a phosphate solubilizing bacterial isolate, which was obtained from root samples of ryegrass (*l*olium *perenne L*.) did not grow at 40°C (Er & Ogut, 2010).

Liu *et al*., (2016) have reported that for phenol-degrading bacterial isolate, the biomass and phenol degradation reached the maximal values at the temperature 30°C, which corresponded precisely with our isolates' optimum growth temperature. Notably, these isolates' growth and phenol-degrading capacity significantly declined when exposed to temperatures of 40°C and beyond. It was documented by Ashkan *et al*. (2023) that S3, S10 and S18 were found to be suitable phenol-degrading bacteria.

The variation in the ideal growth temperature for phenol-degrading bacteria can be attributed to both species' differences and the prevailing climatic conditions in the regions where the bacterial samples were obtained. For instance, *A. calcoaceticus* strain PA, as reported by Liu *et al*., (2016), and our current isolates, *B. subtilis* PDBI-9 and *K. pneumoniae* PDBI-49, were isolated from areas with an average temperature of approximately 28±2°C. In contrast, the three phenol-degrading bacteria isolated by Ashkan *et al*. (2023), (S3, S10, and S18), which were identified as

P. aeruginosa, *K. pneumoniae*, and *K. variicola*, respectively, originated from a region with an average temperature of 33±2°C, occasionally reaching up to 40°C. This distinction underscores that microorganisms have evolved to adapt to the temperature conditions of their natural habitats. Their enzymes and other essential proteins are finely tuned to operate optimally at these specific temperatures. However, it's important to note that microorganisms are not limited to growing only within their ideal temperature ranges; they can still thrive outside these ranges. The optimal temperature signifies the conditions under which their cellular machinery operates at peak efficiently.

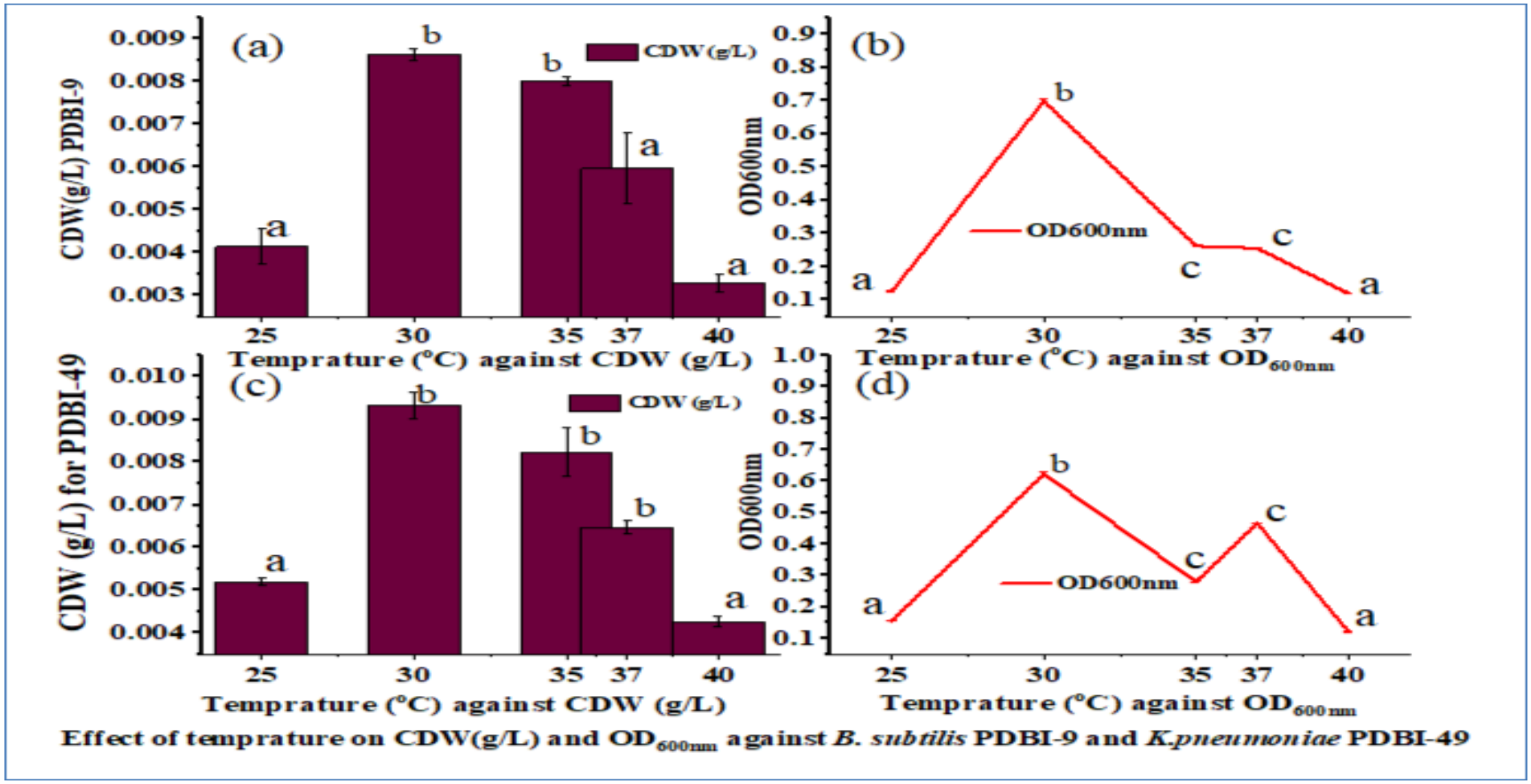

Figure 10: Effect of temperature source against *B. subtilis* PDBI-9 and *K. pneumoniae* PDBI-49 in terms of CDW (g/L) and OD_{600nm}. (a) CDW (g/L) against *B. subtilis* PDBI-9 due to various temperature. (b) OD600nm against *B. subtilis* PDBI-9 25, 30, 35, 37, and 40 ^{0}C. (c) CDW (g/L) against *K. pneumoniae* PDBI-49 due to various temperatures. (d) OD_{600nm} with signifcant variation among all temperatures *(p=0.)* against *K. pneumoniae* PDBI-49 at 37°C. In this study, data represented the mean ± SD of three replicates (n=3). One-way Anova exhibited that signicant variations were observed for phenol degrading bacterial isolates having different letter on their respective bars and line graphs against CDW (g/L) and OD_{600nm}, respectively.

4.3.4 Effect of pH on Phenol Degrading Isolated Bacteria

Various CDW (g/l) and OD_{600nm} were recorded at 6, 6.5, 7, and 8 pH for *B. subtilis* PDBI-9 and *K. pneumoniae* PDBI-49 isolates (Figure 10a). Among the tested pH values, pH 7.0 , a neutral range, gave better CDW (g/l) and OD_{600nm} and exhibited to be a suitable pH range for the growth of these PHBIs. As indicated in Figure 10a, a considerable amount of CDW (g/L) (0.003 ± 0.0003, 0.005 ± 0.0002) and OD_{600nm} (1.097±0.002, 1.114 ± 0.003) were documented for *B. subtilis* PDBI-9 and *K. pneumoniae* PDBI-49 isolates, respectively. As indicated in (Figure 9 a and c) there is a significant variation ($p<0.005$) observed between pH6, pH6.5, pH7.5, pH8 and pH7 in terms of OD_{600nm} and CDW (g/l) at optimum temperature 30℃ for both isolates. Indeed, this study identified a significant variation in pH tolerance of the isolates PDBI-9 and PDBI-49 there is statistically significant variation between pH7 & pH6, pH7 & pH6.5, pH7 & ph8 with ($p=0.000$) (Figure 9 b and d) at OD_{600nm} and CDW (g/l). It was observed that the pH value had an impact on microbial growth. The maximum growth of PDBI-9 and PDBI-49 occurred at the optimum pH value of 7.0, indicating that these PDBI-9 and PDBI-49 exhibited enhanced metabolic activities at this pH. Conversely, minimal growth of *B. subtilis* PDBI-9s observed at pH 6, pH 6.5 and pH 8.

Salem *et al*. (2021) reported that the phenol-degrading isolate such as *Bacillus* sp. *C2* and *Pseudomonas* sp. P4, these obtained from an effluent of ceramic factories and the effluent of a petrochemical company, respectively, showed maximum growth and phenol degradation at pH 7 which coincides to the optimum growth and phenol degradation pH of our isolates. Contrary to the recent study, it is reported that (Moghadam *et al*., 2016b) most of these phenol-degrading bacterial isolates prefer to grow at pH 7-9, and the optimum growth exhibit at pH 8 for phenol-degradation. On the other hand, it was described that there is a variation of optimum pH for the growth and degradation of phenol due to phenol degrading bacterial isolates (Khleifat, 2006). For example, *K. oxytoca* exhibited optimal growth and phenol degradation at pH 6.8 a similar result to *K. pneumoniae* PDBI-49. When evaluating the growth of the isolated bacteria, it's worth noting that at pH8, the second-highest bacterial growth demonstrated by *B. subtilis* PDBI-9 and *K. pneumoniae* PDBI-49. Specifically, their growth recorded at 0.985±0.003 and 0.003±0.0002 for B. subtilis PDBI-9, and 0.997±0.002 and 0.003 ± 0.0002 for K. pneumoniae PDBI-49 with OD600nm and CDW(g/l), respectively. Previous studies have shown that various strains of B. subtilis are pH tolerant in the range of 5-9 although most have optimum growth at pH 7. Some other strains slightly prefer the alkaline range, at pH8. A previous (Sidorova *et al*., 2020) study

confirmed that when determining the optimum pH level for *B. subtilis* BZR , a high growth is recorded at pH 8.0. For the other *B. subtilis* BZR 517 strain, it was reported that the optimal growth was detected in the range of pH 6.0-8.0 with CFU/ml and OD600nm.

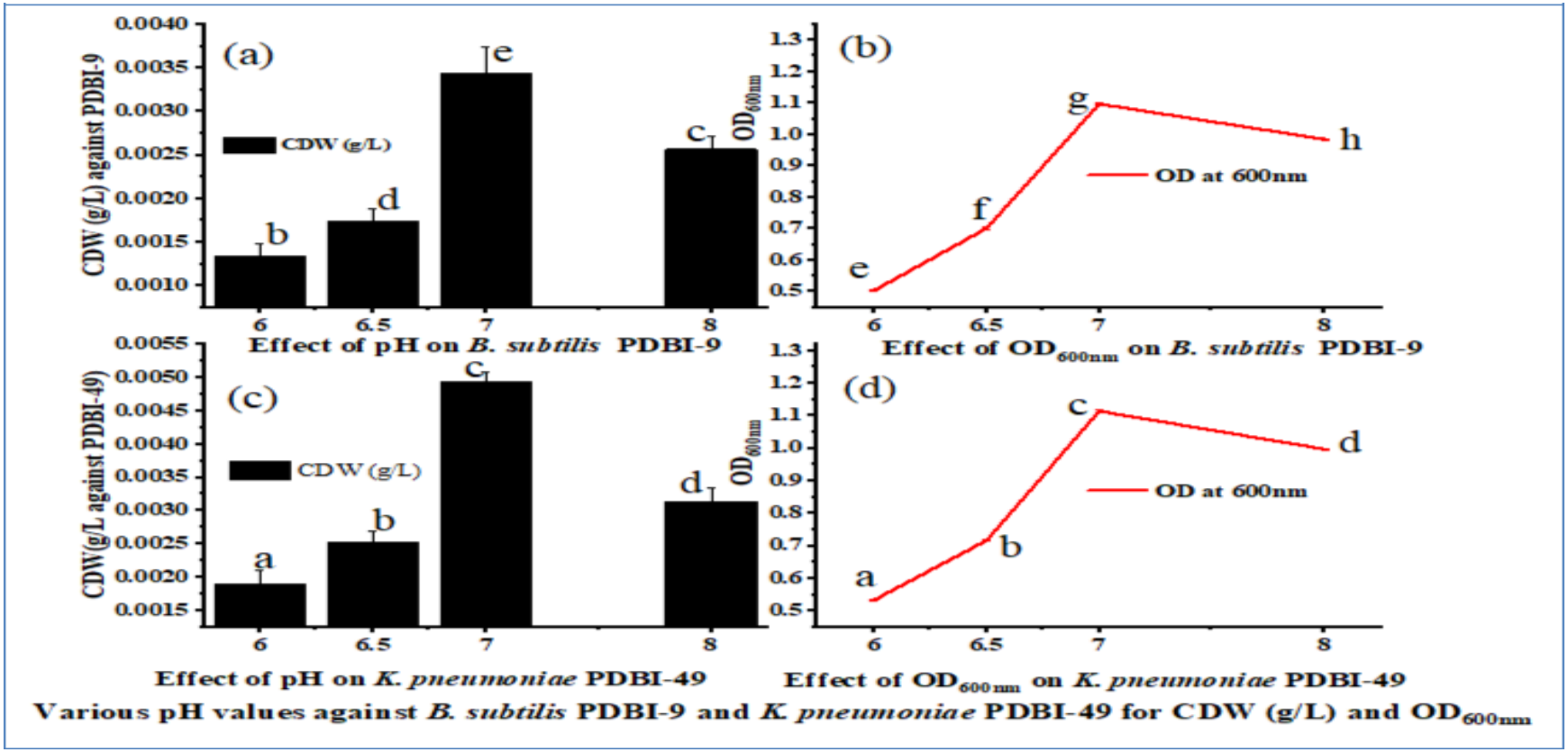

Figure 11: Effect of carbon source against *B. subtilis* PDBI-9 and *K. pneumoniae* PDBI-49 in terms of CDW (g/L) and OD_{600nm}. (a) CDW (g/L) against *B. subtilis* PDBI-9 due to various pH. (b) OD_{600nm} against *B. subtilis* PDBI-9 due to 6, 6.5, 7, and 8. (c) CDW (g/L) against *K. pneumoniae* PDBI-49 due to various pH. (d) OD_{600nm} with signifcant variation among all pH sources (p<0.05) against *K. pneumoniae* PDBI-49 at 30ºC. In this study, data represented the mean ± SD of three replicates (n=3). One-way Anova exhibited that signicant variations were observed for phenol degrading bacterial isolates having different letter on their respective bars and line graphs against CDW (g/L) and OD_{600nm}, respectively.

4.4 Phenol Degrading Bacteria Efficiency Analysis

The results of this study confirmed the phenol degradation capabilities of the tested strain of phenol-degrading bacteria (*B. subtilis* PDBI-9 and *K. pneumoniae* PDBI-49; (Table5). The bacterial culture exhibited efficient growth and utilization of phenol as the sole carbon source. According Apostică *et al.,* (2018), the colorimetric assay using ferric chloride proved to be a suitable method for quantifying phenol concentration in the samples. The absorbance values at OD_{540nm} were used to determine the phenol concentration, while the calibration curve prepared with known concentrations of phenol allowed for the quantification of phenol concentration in the samples.

Table 5. Phenol degrading bacteria efficiency analysis

Time Interval (hours)	Phenol Degradation (%) against *B. subtilis* PDBI-9	Phenol Degradation (%) against *K. pneumoniae* PDBI-49
0-8	3.7	6.6
8-16	6.5	12.7
16-24	7.9	18.8
24-32	10.6	32.97
32-40	15.7	29.7
40-48	18.4	38
48-56	21.7	43
56-64	26.44	52.84
64-72	44.7	59.4
72-80	57.54	66.64
80-88	70	79.2
88-96	76	86.24

Table 5. analysis degradation percentages of phenol against *B. subtilis* (PDBI-9) and *K. pneumoniae* (PDBI-49) over time course.

The application of visible (VIS) molecular absorption spectrometry emerges as the most suitable methodology for developing an uncomplicated, swift, and cost-effective analytical protocol for quantifying phenol in aqueous matrices (Fiamegos *et al*., 2000). Often, spectrophotometric

analysis relies on the phenol-4-aminoantipyrine (4-AAP) dye complex for phenol detection in aqueous solutions.

In our investigation, due to the unavailability of 4-aminoantipyrine (4-AAP) dye, we employed the phenol-ferric chloride ($FeCl_3$) dye complex to ascertain phenol concentrations in treated samples. The reaction between phenol and $FeCl_3$ occurs rapidly, within 1-2 min, obviating the necessity for pH adjustment. The resulting purple complex exhibits a maximum visible spectrum at 540 nm compared to distilled water, and its absorbance remains stable for at least 10 h . This method facilitates the determination of phenol in aqueous media across a relatively broad concentration range (0.09-2.30 mg·mL-1) with satisfactory detection limits (Apostica *et al.*, 2018). The results of the study revealed that within a 96 h period, bacteria were able to reduce the concentration of phenol at 1000 ppm. The measurements were taken at 8-hour intervals. During the initial 8-hour period, *K. pneumoniae* PDBI-49 was observed to degrade 6.6% of phenol, while *B. subtilis* (PDBI-9) degraded 3.7% of the phenolic compound.

The results demonstrate that *K. pneumoniae*-PDBI-49 was more effective in degrading phenol when compared to *B. subtilis*-PDBI-9, achieving a degradation rate of 86.24% versus 76% within a 96-hour time course. According to a study conducted by (Carlos *et al*., 2011) *K. pneumoniae*, *P. aeruginosa*, and *E. coli* exhibited significant resistance to a phenol concentration of 1000 mg L-1. (Sandhibigraha *et al*., 2020) reported that the metabolic adaptability of *B subtilis* exihibited against various phenolic compounds. Study reveals that this particular isolates effectively degraded significant proportions of different phenolic compounds within a 48 h. Specifically, it achieved degradation rates of 94.79% for phenol, 84.96% for 2-chlorophenol, 90.90% for 4-CP, 92.82% for 2,4-dichlorophenol, 75.53% for 2,4,6-trichlorophenol, 89.52% for 4-nitrophenol, and 90.02% for pentachlorophenol. The metabolic adaptability of *B. subtilis* makes them effective microorganisms for the purpose of bioremediating industrial effluent contaminated with a wide range of phenolic compounds.

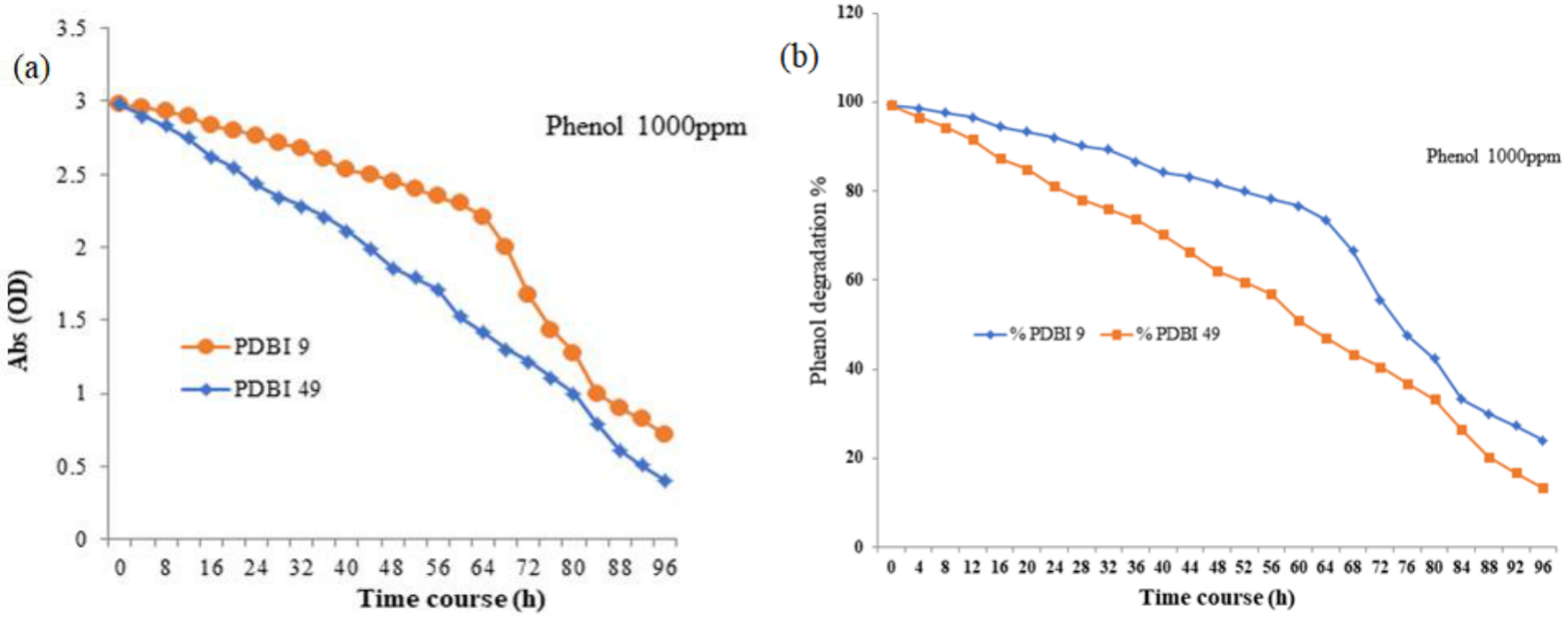

Figure 12: (a) Phenol degrading efficiency bacteria absorbance and Time course (b) phenol degradation efficiency in percentage and Time course

5. CONCLUSION AND RECOMMENDATION

5.1 Conclusion and Recommendations

Evaluation of growth conditions for the two isolates showed that temperature of 30^{o}C with pH range 5-8 and Glucose and Peptone as sole sources of carbon and nitrogen, respectively, were optimal growth conditions the isolates. The results demonstrate that *K. pneumoniae*-PDBI-49 was more effective in degrading phenol compared to *B. subtilis*-PDBI-9 This study provides valuable insights into the potential use of these phenol-degrading bacteria for bioremediation purposes in industrial wastewater treatment. Based on the research findings, Further research can focus on the characterization of the phenol degradation pathways and the optimization of other growth parameters to enhance the efficiency of phenol degradation by these isolates. Additionally, identification of organisms at the species level using 16S rRNA can be a valuable focus for further study. This information suggests that these two strains *B. subtilis* PDBI-9 and *K. pneumoniae* PDBI-49, have demonstrated the strongest capacities and may hold significant promise for future research and investigations into their unique attributes. It is proposed to employ gene manipulation and other advanced technologies for the enhancement of phenol degradation genes while concurrently eliminating their pathogenic elements.

6. REFERENCES

Adem, M. (2019). Production of hide and skin in Ethiopia; marketing opportunities and constraints: a review paper. Cogent Food & Agriculture, 5(1), 1565078.

Africa, S., & Africa, S. (2010). *Endocrine disrupting chemicals (phenol and phthalates) in the South African environment: a need for more monitoring*. *36*(5), 671–682.

Ahmad, S. A. (2014). Phenol and its toxicity. *Environmental Microbiology and Toxicolog*, *2*(January), 11–24.

Ahmad, S. A., Syed, M. A., Arif, N. M., Shukor, M. Y. A., & Shamaan, N. A. (2011). Isolation, identification and characterization of elevated phenol degrading acinetobacter sp. strain AQ5NOL 1. *Australian Journal of Basic and Applied Sciences*, *5*(8), 1035–1045.

Al-Khalid, T., & El-Naas, M. H. (2012). Aerobic biodegradation of phenols: A comprehensive review. *Critical Reviews in Environmental Science and Technology*, *42*(16), 1631–1690. https://doi.org/10.1080/10643389.2011.569872

Anku, W. W., Mamo, M. A., & Govender, P. P. (2017). Phenolic Compounds in Water: Sources, Reactivity, Toxicity and Treatment Methods. *Phenolic Compounds - Natural Sources, Importance and Applications*. https://doi.org/10.5772/66927

Anupama, S., Pradeep, N. V., & Hampannavar, U. S. (2013). Anaerobic Followed by Aerobic Treatment Approaches for Spentwash Using MFC and RBC. *Sugar Tech*, *15*(2), 197–202. https://doi.org/10.1007/s12355-013-0205-1

Apostica, A. G., Ichim, T., Radu, V. M., & Bulgariu, L. (2018). Simple and rapid spectrophotometric method for phenol determination in aqueous media. *Bulletin of the Polytechnic Institute of Jassy*, *64*(3), 9–18. https://www.researchgate.net/publication/330598807

April, V. I., Trimbake, P. D., Ambawade, M. S., & Mane, A. V. (2014). International Journal of Research in Chemistry and Environment Isolation of Phenol Degrading Microorganisms from Sewage and Degradation under Optimized Conditions. *International Journal of Research in Chemistry and Environment*, *4*(2), 18–30.

Archana Lal, N. C. (2016). Starch Agar Protocol. *Starch Agar Protocol*, *November 2012*, http://www.microbelibrary.org/library/laboratory-t. http://www.microbelibrary.org/library/laboratory-test/3780-starch-agar-protocol

Arutchelvan, V., & Atun, R. C. (2019). Degradation of phenol, an innovative biological approach. *Adv. Biotech. Micro*, *12*, 555-835.

Ashkan, M. F., Al-quwaie, D. A., Motwali, E. A., Alqahtani, F. S., & El-fattah, H. I. A. (2023). Pseudomonas aeruginosa and Klebsiella variicola Strains Seeds Germination. *Molecules*, *28*(1203), 1–19. https://doi.org/https://doi.org/10.3390/ molecules28031203

Baker, E. L., Landrigan, P. J., & Bertozzi, P. E. (1978). Phenol poisoning due to contaminated drinking water. *Archives of Environmental Health*, *33*(2), 89–94. https://doi.org/10.1080/00039896.1978.10667314

Banerjee, A., & Ghoshal, A. K. (2011). International Biodeterioration & Biodegradation Phenol degradation performance by isolated Bacillus cereus immobilized in alginate. *International Biodeterioration & Biodegradation*, *65*(7), 1052–1060. https://doi.org/10.1016/j.ibiod.2011.04.011

Basha, K. M., Rajendran, A., & Thangavelu, V. (2010). Recent advances in the Biodegradation of Phenol : A review. *Asian J. Exp. Biol. Sci.*, *1*(2), 219–234.

Beisel, C. L., & Afroz, T. (2015). Rethinking the hierarchy of sugar utilization in bacteria. *Journal of Bacteriology*, *198*(3), 374–376. https://doi.org/10.1128/JB.00890-15

Bizzini, A., & Greub, G. (2010). Matrix-assisted laser desorption ionization time-of-flight mass spectrometry, a revolution in clinical microbial identification. *16*, *11*, 1614–1619.

Bonfá, M. R. L., Grossman, M. J., Piubeli, F., Mellado, E., & Durrant, L. R. (2013). Phenol degradation by halophilic bacteria isolated from hypersaline environments. *Biodegradation*, *24*(5), 699–709. https://doi.org/10.1007/s10532-012-9617-y

Bogale, Y. A. (2012). Evaluating transport network structure: Case study in Addis Ababa, Ethiopia (Master's thesis, University of Twente).

Brega, A., Prandini, P., Amaglio, C., & Pafumi, E. (1990). Determination of phenol , m- , o- and pcresol , paminophenol and pnitrophenol in urine by high-performance liquid chromatography. *Journal of Chromatography A*, *535*, 311–316.

Brink, B. (2016). Urease Test Protocol. American Society for Microbiology. *American Society for Microbiology*, *November 2010*, 1–7.

Busca, G., Berardinelli, S., Resini, C., & Arrighi, L. (2008). Technologies for the removal of phenol from fluid streams : A short review of recent developments. *Journal of Hazardous Materials*, *160*, 265–288. https://doi.org/10.1016/j.jhazmat.2008.03.045

Carlos, J. C. Z. as, Martha, A. S. rez H., Maria, T. G. G. lez, Moacute nica, N. S. nchez G. lez, & Ulrico, J. L. pez C. (2011). Biosorption of metals by phenol-resistant bacteria isolated from contaminated industrial effluents. *African Journal of Microbiology Research*, *5*(18), 2627–2631. https://doi.org/10.5897/ajmr11.211

Christen, P., Vega, A., Casalot, L., Simon, G., & Auria, R. (2012). Kinetics of aerobic phenol biodegradation by the acidophilic and hyperthermophilic archaeon Sulfolobus solfataricus 98/2. *Biochemical Engineering Journal*, *62*, 56–61. https://doi.org/10.1016/j.bej.2011.12.012

CLARKE, S. K. (1953). A simplified plate method for detecting gelatine-liquefying bacteria. *Journal of Clinical Pathology*, *6*(3), 246–248. https://doi.org/10.1136/jcp.6.3.246

Czaplicka, M. (2004). Sources and transformations of chlorophenols in the natural environment. *Science of the Total Environment*, *322*(1–3), 21–39. https://doi.org/10.1016/j.scitotenv.2003.09.015

Dumitra, C. M., Alexandrescu, L., Pantazibajenaru, M., Deselnicu, D. C., & Semenescu, A. (2022). Leather Industry in Romania-an Overview. *ICAMS Proceedings of the International Conference on Advanced Materials and Systems*, *313*, 407–412. https://doi.org/10.24264/icams-2022.IV.4

Dumitra, C. M., Deselnicu, D. C., & Semenescu, A. (2023). *WASTE FROM THE LEATHER INDUSTRY – A RESEARCH IN CURRENT CONTEXT*. *March*.

Emerson, E. (1943). The condensation of aminoantipyrine. II. A new color test for phenolic compounds. *Organic Chemistry*, *8*, 417–428.

Esteban, M., Azerrad, S., Menashe, O., & Kurzbaum, E. (2022). Journal of Water Process Engineering Efficient biodegradation of phenol at high concentrations by Acinetobacter

biofilm at extremely short hydraulic retention times. *Journal of Water Process Engineering*, *47*(April), 102781. https://doi.org/10.1016/j.jwpe.2022.102781

Falconer, I. R., Chapman, H. F., Moore, M. R., & Ranmuthugala, G. (2006). Endocrine-Disrupting Compounds: A Review of Their Challenge to Sustainable and Safe Water Supply and Water Reuse. *Environmental Toxicology*, 181–191. https://doi.org/10.1002/tox.20172

Fiamegos, Y. C., Stalikas, C. D., Pilidis, G. A., & Karayannis, M. I. (2000). Synthesis and analytical applications of 4-aminopyrazolone derivatives as chromogenic agents for the spectrophotometric determination of phenols. *Analytica Chimica Acta*, *403*(1–2), 315– 323. https://doi.org/10.1016/S0003-2670(99)00644-3

Galanakis, C. M. (2014). Recovery and Removal of Phenolic Compounds from Olive Mill Wastewater. *Journal of the American Oil Chemists' Society*, 1–18. https://doi.org/10.1007/s11746-013-2350-9

Garima, T. & Singh, S. (2014). Application of bioremediation on solid waste management. *J Bioremed Biodegr*, *5*, 24.

Geng, A., Soh, A. E. W., Lim, C. J., & Loke, L. C. T. (2006). Isolation and characterization of a phenol-degrading bacterium from an industrial activated sludge. *Applied Microbiology and Biotechnology*, *71*(5), 728–735. https://doi.org/10.1007/s00253-005-0199-z

González, M., Guzmán, B., Rudyk, R., Romano, E., & Molina, M. A. (2003). Spectrophotometric determination of phenolic compounds in propolis. *Acta Farmaceutica Bonaerense*, *22*, 243–248. doi

Heilbuth, N. M., Linardi, V. R., Monteiro, A. S., da Rocha, R. A., Mimim, L. A., & Santos, V. L. (2015). Estimation of kinetic parameters of phenol degradation by bacteria isolated from activated sludge using a genetic algorithm. *Journal of Chemical Technology and Biotechnology*, *90*(11), 2066–2075. https://doi.org/10.1002/jctb.4518

Jain, A. K., Gupta, V. K., & Jain, S. (2004). Removal of Chlorophenols Using Industrial Wastes. *ENVIRONMENTAL SCIENCE & TECHNOLOGY*, *38*, 1195–1200.

Kafilzadeh, F., Farhangdoost, M. S., & Tahery, Y. (2010). Isolation and identification of phenol degrading bacteria from lake parishan and their growth kinetic assay. *African Journal of Biotechnology*, *9*(40), 6721–6726. https://doi.org/10.5897/AJB10.665

Kanagaraj, J., Velappan, K. C., Babu, N. K. C., & Sadulla, S. (2006). *Solid wastes generation in . 65*(July), *the leather industry and its utilization for cleaner environment A review* 541–548.

Kesarwani, P., Jahan, S., & Kesarwani, K. (2015). A review on leather processing. *International Journal of Applied Research*, *1*(9), 977–982. www.allresearchjournal.com

Khleifat, K. M. (2006). Biodegradation of phenol by Ewingella americana: Effect of carbon starvation and some growth conditions. *Process Biochemistry*, *41*(9), 2010–2016. https://doi.org/10.1016/j.procbio.2006.04.015

Koçberber, N. (2009). International Biodeterioration & Biodegradation Enhancement of phenol biodegradation by Ochrobactrum sp. isolated from industrial wastewaters. *International Biodeterioration & Biodegradation*, *63*, 778–781. https://doi.org/10.1016/j.ibiod.2009.06.006

Kottuparambil, S., Kim, Y. J., Choi, H., Kim, M. S., Park, A., Park, J., Shin, W., & Han, T. (2014). A rapid phenol toxicity test based on photosynthesis and movement of the freshwater flagellate, Euglena agilis Carter. *Aquatic Toxicology*, *155*, 9–14. https://doi.org/10.1016/j.aquatox.2014.05.014

Leta, S., Assefa, F., Gumaelius, L., & Dalhammar, G. (2004). Biological nitrogen and organic matter removal from tannery wastewater in pilot plant operations in Ethiopia. *Applied Microbiology and Biotechnology*, *66*(3), 333–339. https://doi.org/10.1007/s00253-004-1715-2

Lin, Y. H., & Wu, C. L. (2011). Sensitivity Analysis of Phenol Degradation with Sulfate Reduction Under Anaerobic Conditions. *Environmental Modeling and Assessment*, *16*(2), 213–225. https://doi.org/10.1007/s10666-010-9243-1

Liu, Z., Xie, W., Li, D., Peng, Y., Li, Z., & Liu, S. (2016). Biodegradation of phenol by bacteria strain Acinetobacter Calcoaceticus PA isolated from phenolic wastewater. *International Journal of Environmental Research and Public Health*, *13*(3). https://doi.org/10.3390/ijerph13030300

Lofrano, G., Aydin, E., & Russo, F. (2008). *Characterization , Fluxes and Toxicity of Leather Tanning Bath Chemicals in a Large Tanning District Area (IT)*. 529–542. https://doi.org/10.1007/s11267-008-9177-7

Lofrano, G., Meriç, S., Belgiorno, V., & Napoli, R. M. A. (2007). *Fenton ' s oxidation of various-based tanning materials*. *211*, 10–21. https://doi.org/10.1016/j.desal.2006.03.589

Loh, K. C., & Wang, S. J. (1997). Enhancement of biodegradation of phenol and a nongrowth substrate 4-chlorophenol by medium augmentation with conventional carbon sources. *Biodegradation*, *8*(5), 329–338. https://doi.org/10.1023/A:1008267607634

MacWilliams, M. P. (2009). Citrate Test. *American Society for Microbiology*, *December 2009*, 1– 7. http://www.asmscience.org/

Mailin, M., & Firdausi, R. (2006). High Performance Phenol Degrading Microorganisms Isolated from Wastewater and Oil-Contaminated Soil. *Malaysian Journal of Microbiology*, *2*(3), 32–36. https://doi.org/10.21161/mjm.220606

Manahan, S. E. (2022). (2022). *Environmental chemistry. CRC press.* (11th Editi). https://doi.org/https://doi.org/10.1201/9781003096238

Mane, A. V, Trimbake, P. D., & Ambawade, M. S. (2014). Isolation of Phenol Degrading Microorganisms from Sewage and Degradation under Optimized Conditions. *International Journal of Research in Chemistry and Environment*, *4*(2), 18–30. https://doi.org/ISSN 2248-9649

Michałowicz, J., & Duda, W. (2007). Phenols – Sources and Toxicity. *Polish J. of Environ. Stud*, *16*(3), 347–362.

Mishra, V. K., & Kumar, N. (2017). Microbial Degradation of Phenol: A Review. *Water Pollution & Purification Research*, *4*(1), 17–22.

Mohammed, S., & Ray, L. (2022). Polyhydroxyalkanoate recovery from newly screened Bacillus sp. LPPI-18 using various methods of extraction from Loktak Lake sediment sample. Journal of Genetic Engineering and Biotechnology, 20(1), 115 https://doi.org/10.1186/s43141-022-00392-7

Moghadam, M. S., Safaei, N., & Ebrahimipour, G. H. (2016). Optimization of phenol biodegradation by efficient bacteria isolated from petrochemical effluents. *Global Journal of Environmental Science and Management*, *2*(3), 249–256. https://doi.org/10.7508/gjesm.2016.03.004

Monteiro, Á. A. M. G., Boaventura, R. A. R., & Rodrigues, E. (2000). Phenol biodegradation by Pseudomonas putida DSM 548 in a batch reactor. *Biochemical Engineering Journal*, *6*, 45–49.

Moreira, R. F. P. M., Schro, H. F., Schrank, S. G., Bieling, U., & Jose, H. J. (2009). *Generation of endocrine disruptor compounds during ozone treatment of tannery wastewater confirmed by biological effect analysis and substance specific analysis*. 31–38. https://doi.org/10.2166/wst.2009.762

Moyo, B., Phiri, O., Mumba, P., & Kadewa, W. (2001). Assessment of the impact of industrial effluents on water quality in Patancheru and environs. *India. Hydrogeology*, *9*(3), 297– 312.

Mun, M., Markey, C. M., Wadia, P. R., Luque, E. H., Rubin, B. S., Sonnenschein, C., & Soto, A. M. (2005). Perinatal Exposure to Bisphenol-A Alters Peripubertal Mammary Gland Development in Mice. *Endocrine Society*, *146*(9), 4138–4147. https://doi.org/10.1210/en.2005-0340

Mwinyihija, M., Meharg, A., Dawson, J., Strachan, N. J. C., & Killham, K. (2006). *An Ecotoxicological Approach to Assessing the Impact of Tanning Industry Effluent on River Health*. *324*, 316–324. https://doi.org/10.1007/s00244-005-1049-9

Nair, C. I., Jayachandran, K., & Shashidhar, S. (2008). Biodegradation of phenol. *African Journal of Biotechnology*, *7*(25), 4951–4958. https://doi.org/10.4018/978-1-5225-8903-7.ch045

Nicell, J. A., Bewtra, J. K., Biswas, N., & Taylor, E. (1993). *REACTOR DEVELOPMENT FOR PEROXIDASE CATALYZED POLYMERIZATION A N D PRECIPITATION OF PHENOLS FROM WASTEWATER*. *27*, 1629–1639.

Nuhoglu, A., & Yalcin, B. (2005). Modelling of phenol removal in a batch reactor. *Process Biochemistry*, *40*(January 2004), 1233–1239. https://doi.org/10.1016/j.procbio.2004.04.003

Oller, I., Malato, S., & Sánchez-pérez, J. A. (2011). Science of the Total Environment Combination of Advanced Oxidation Processes and biological treatments for wastewater decontamination — A review. *Science of the Total Environment, The*, *409*(20), 4141– 4166. https://doi.org/10.1016/j.scitotenv.2010.08.061

Owicz, J. M. Ł., & Duda, W. (2016). PHENOLS TRANSFORMATIONS IN THE ENVIRONMENT AND LIVING PHENOLS TRANSFORMATIONS IN THE ENVIRONMENT AND LIVING ORGANISMS. *Current Topics in Biophysics*, *May*.

Paisio, C. E., Talano, M. A., González, P. S., Busto, V. D., Talou, J. R., & Agostini, E. (2012). Isolation and characterization of a Rhodococcus strain with phenol-degrading ability and its potential use for tannery effluent biotreatment. *Environmental Science and Pollution Research*, *19*(8), 3430–3439. https://doi.org/10.1007/s11356-012-0870-8

Panigrahy, N., Priyadarshini, A., Sahoo, M. M., Verma, A. K., Daverey, A., & Sahoo, N. K. (2022). A comprehensive review on eco-toxicity and biodegradation of phenolics: Recent progress and future outlook. *Environmental Technology and Innovation*, *27*, 102423. https://doi.org/10.1016/j.eti.2022.102423

Patil, A. H., Mishra, R. M., Pendse, A. S., & Kundar, R. R. (2023). *STUDY OF PHENOL DEGRADING BACTERIUM ISOLATED FROM A PETROCHEMICAL CONTAMINATED SITE*. *17*(2), 306–319. https://doi.org/10.5281/zenodo.8018938

Pradeep, N. V., Anupama, S., Navya, K., Shalini, H. N., Idris, M., & Hampannavar, U. S. (2015). Biological removal of phenol from wastewaters: a mini review. *Applied Water Science*, *5*(2), 105–112. https://doi.org/10.1007/s13201-014-0176-8

Prpich, G. P., & Daugulis, A. J. (2005). Enhanced biodegradation of phenol by a microbial consortium in a solid-liquid two phase partitioning bioreactor. *Biodegradation*, *16*(4), 329–339. https://doi.org/10.1007/s10532-004-2036-y

Rajani, V. (2015). *Microbial Degradation of Phenol- A Review*. *2*(February), 46–54.

Ravikumar, S., Parimala, P. S., & & Gokulakrishnan, R. (2011). Biodegradation of phenolic compounds by using halotolerant microbes. *Plant, Animal and Environmental Sciences, 1*, 38-45.

Raza, A. W., Lee, J., & Raza, N. (2018). Removal of phenolic compounds from industrial waste water based on membrane-based technologies. *Journal of Industrial and Engineering Chemistry*. https://doi.org/10.1016/j.jiec.2018.11.024

Ruiz-Ordaz, N., Ruiz-Lagunez, J. C., Castañón-González, J. H., Hernández-Manzano, E., Cristiani-Urbina, E., & Galíndez-Mayer, J. (2001). Phenol biodegradation using a repeated batch culture of Candida tropicalis in a multistage bubble column. *Revista Latinoamericana de Microbiologia*, *43*(1), 19–25.

Sachan, P., Madan, S., & Hussain, A. (2019). Isolation and screening of phenol-degrading bacteria from pulp and paper mill effluent. *Applied Water Science*, *9*(4), 1–6. https://doi.org/10.1007/s13201-019-0994-9

Salah, A., Abdel-wahab, M., & El-latif, A. (2020). Isolation and Genetic Identification of Phenol Degrading Bacterium from Wastewater of Assiut University Hospitals. *Egyptian Sugar Journal*, *15*, 13–27.

Salem, S., Abd El-Fattah, H., Abdelbasit, H., & Mahgoub, S. (2021). Isolation and Characterization of Phenol Degrading Bacteria From Industrial Wastewater and Sewage Water. *Zagazig Journal of Agricultural Research*, *48*(2), 443–457. https://doi.org/10.21608/zjar.2021.175276

Sandhibigraha, S., Chakraborty, S., Bandyopadhyay, T., & Bhunia, B. (2020). A kinetic study of 4-chlorophenol biodegradation by the novel isolated bacillus subtilis in batch shake flask. *Environmental Engineering Research*, *25*(1), 62–70. https://doi.org/10.4491/eer.2018.416

Saravanan, P., Pakshirajan, K., & Saha, P. (2008). Growth kinetics of an indigenous mixed microbial consortium during phenol degradation in a batch reactor. *Bioresource Technology*, *99*(1), 205–209. https://doi.org/10.1016/j.biortech.2006.11.045

Sathish, M., Madhan, B., Sreeram, K. J., Rao, J. R., & Nair, B. U. (2015). AC SC. *Journal of Cleaner Production*. https://doi.org/10.1016/j.jclepro.2015.06.118

Sellami, K., Couvert, A., Nasrallah, N., Maachi, R., Tandjaoui, N., Abouseoud, M., & Amrane, (2021). Bio-based and cost-effective method for phenolic compounds removal using cross-linked enzyme aggregates. *Journal of Hazardous Materials*, *403*(June 2020), 124021. https://doi.org/10.1016/j.jhazmat.2020.124021

Shah, S., & Thakur, I. S. (2002). *Enrichment and characterization of a microbial community from tannery effluent for degradation of pentachlorophenol*. 693–698.

Shen, T. T. (1995). Industrial Pollution Prevention. In: Industrial Pollution Prevention. Environmental Engineering. In *Springer, Berlin, Heidelberg*. https://doi.org/https://doi.org/10.1007/978-3-662-03110-0_2

Sidorova, T. M., Asaturova, A. M., Homyak, A. I., Zhevnova, N. A., Shternshis, M. V., & Tomashevich, N. S. (2020). Optimization of laboratory cultivation conditions for the synthesis of antifungal metabolites by bacillus subtilis strains. *Saudi Journal of Biological Sciences*, *27*(7), 1879–1885. https://doi.org/10.1016/j.sjbs.2020.05.002

Sivaram, N. M., & Barik, D. (2019). Toxic Waste From Leather Industries. In *Energy from Toxic Organic Waste for Heat and Power Generation*. Elsevier Ltd. https://doi.org/10.1016/B978-0-08-102528-4.00005-5

Tanasă, I. D., Bulgariu, A. E., Siriţeanu, C. Ă. T. Ă. L. I. N. A., & Bulgariu, L. A. U. R. A. (2022). Spectrophotometric determination of rifampicin in aqueous solution. *Bul. Inst. Politeh. Iaşi*, 25–34.

Tay, S. T., & Moy, B. Y. (2005). Comparing activated sludge and aerobic granules as microbial inocula for phenol biodegradation. *Applied Microbiology and Biotechnology*, *67*, 708– 713. https://doi.org/10.1007/s00253-004-1858-1

Taylor, P., & Yusuf, R. O. (2013). Desalination and Water Treatment A comparison of the efficacy of two strains of Bacillus subtilis and Pseudomonas fragii in the treatment of tannery wastewater. *51*, *16–18*(October 2014), 37–41. https://doi.org/10.1080/19443994.2012.751155

Taylor, W. I., & Achanzar, D. (1972). Catalase test as an aid to the identification of Enterobacteriaceae. *Applied Microbiology*, *24*, 58-61.

Tsirogianni, E., Aivaliotis, M., Karas, M., & Tsiotis, G. (2005). Detection and characterisation of catechol 2,3-dioxygenase in an indigenous soil Pseudomonad by MALDI-TOF MS using a column separation. *Biodegradation*, *16*(2), 181–186. https://doi.org/10.1007/s10532004-4885-9

United States Agency for International Development (2013). Value chain analysis for Ethiopia: Meat and live animals, hides, skins and leather and dairy.

Van scnte, P. M., & Young, L. Y. (2000). Biodegradation of phenol: Mechanisms and applications. *Bioremediation Journal*, *4*(1), 1–18.https://doi.org/10.1080/10588330008951128

Vashist, H., Sharma, D., & Gupta, A. (2013). A review on commonly used biochemical test for bacteria. *Journal of Life Science*, *1*, 1-7.

Veeraraghavan, A., & Choudhury, A. R. (2019). Degradation of Phenol , an Innovative Biological Approach. *Innovative Biological Approach*, *January*.

Vijayaraghavan, P., Gnana, S., & Vincent, P. (2013). A simple method for the detection of protease activity on agar plates using bromocresolgreen dye. *Journal of Biochemical …*, *4*, 628–630. http://jbiochemtech.com/index.php/jbt/article/viewArticle/jbt419

Villegas, L. G. C., Mashhadi, N., Chen, M., Mukherjee, D., Taylor, K. E., & Biswas, N. (2016). A Short Review of Techniques for Phenol Removal from Wastewater. *Current Pollution Reports*, *2*(3), 157–167. https://doi.org/10.1007/s40726-016-0035-3

Wang, Y., Song, J., Zhao, W., He, X., Chen, J., & Xiao, M. (2011). In situ degradation of phenol and promotion of plant growth in contaminated environments by a single Pseudomonas aeruginosa strain. *Journal of Hazardous Materials*, *192*(1), 354–360. https://doi.org/10.1016/j.jhazmat.2011.05.031

WANG, Y., TIAN, Y., HAN, B., ZHAO, H. bing, BI, J. nan, & CAI, B. li. (2007). Biodegradation of phenol by free and immobilized Acinetobacter sp. strain PD12. *Journal of Environmental Sciences*, *19*(2), 222–225. https://doi.org/10.1016/S1001-0742(07)60036-9

Welsh, C. T. (2019). *Microbiology: a laboratory manual. Pearson.*

Wilmotte, A., & Herdman, M. (2001). The Archaea and the Deeply Branching and Phototrophic Bacteria. In *Bergey's Manual of Systematic Bacteriology. Volume One*.

Xiao, D., Zhao, F., Lv, M., Zhang, H., Zhang, Y., Huang, H., Su, P., Zhang, Z., & Zhang, J. (2012). Rapid identification of microorganisms isolated from throat swab specimens of community-acquired pneumonia patients by two MALDI-TOF MS systems. *Diagnostic Microbiology and Infectious Disease*, *73*(4), 301–307. https://doi.org/10.1016/j.diagmicrobio.2012.04.004

Xu, N., Qiu, C., Yang, Q., Zhang, Y., Wang, M., Ye, C., & Guo, M. (2021). Analysis of Phenol Biodegradation in Antibiotic and Heavy Metal Resistant Acinetobacter lwoffii NL1. *Frontiers in Microbiology*, *12*(September), 1–12. https://doi.org/10.3389/fmicb.2021.725755

Zhao, L., Wu, Q., & Ma, A. (2018). Biodegradation of phenolic contaminants: Current status and perspectives. In IOP conference series: Earth and environmental science (Vol. 111, No. 1, p. 012024). IOP Publishing.

7. APPENDICES

Appendix 1: Supplementary Figure S_1: Illustrative pictures during site of sample collection, isolation (screening) and isolation of PDBIs

Figure S1: Isolation of PDBIs and their Biochemical tests

Appendix 2: Mean, SD, minimum and maximum value for CDW (g/L) against B. subtilis PDBI-9 and *K. pneumoniae* PDBI-49 at various Carbon source, nitrogen source, pH, and temperature values using SPSS

Table 1. Mean, SD, minimum and maximum value for CDW (g/L) against *B. subtilis* PDBI-9 and *K. pneumoniae* PDBI-49 at various carbon sources using SPSS

Descriptive								
CDW (g/L) and OD600nm for PDBI-9 and PDBI-49								
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
CDW (g/L) of Glucose against B. subtilis PDBI-9	3	.0045667	.00030551	.00017638	.0038078	.0053256	.00430	.00490
CDW (g/L) of Fructose against B. subtilis PDBI-9	3	.0034000	.00010000	.00005774	.0031516	.0036484	.00330	.00350
CDW (g/L) of Sucrose against B. subtilis PDBI-9	3	.0010333	.00005774	.00003333	.0008899	.0011768	.00100	.00110
CDW (g/L) of Manito against B. subtilis PDBI-9	3	.0014333	.00015275	.00008819	.0010539	.0018128	.00130	.00160
CDW (g/L) of Glucose against K. pneumoniae PDBI-49	3	.0051333	.00020817	.00012019	.0046162	.0056504	.00490	.00530
CDW (g/L) of Fructose against K. pneumoniae PDBI-49	3	.0037667	.00035119	.00020276	.0028943	.0046391	.00340	.00410
CDW (g/L) of Sucrose against K. pneumoniae PDBI-49	3	.0016333	.00020817	.00012019	.0011162	.0021504	.00140	.00180
CDW (g/L) of Manito against K. pneumoniae PDBI-49	3	.0022000	.00034641	.00020000	.0013395	.0030605	.00180	.00240

OD600nm of Glucose against B. subtilis PDBI-9	3	1.0713333	.0020 8167	.00120185	1.0661622	1.0765045	1.06900	1.07300
OD600nm of Fructose against B. subtilis PDBI-9	3	.9943333	.0020 8167	.00120185	.9891622	.9995045	.99200	.99600
OD600nm of Sucrose against B. subtilis PDBI-9	3	.6010000	.0030 0000	.00173205	.5935476	.6084524	.59800	.60400
OD600nm of Manito against B. subtilis PDBI-9	3	.6113333	.0015 2753	.00088192	.6075388	.6151279	.61000	.61300
OD600nm of Glucose against K. pneumoniae PDBI-49	3	1.0933333	.0037 8594	.00218581	1.0839285	1.1027381	1.08900	1.09600
OD600nm of Fructose against K. pneumoniae PDBI-49	3	.9956667	.0041 6333	.00240370	.9853244	1.0060090	.99100	.99900
OD600nm of Sucrose against K. pneumoniae PDBI-49	3	.6343333	.0011 5470	.00066667	.6314649	.6372018	.63300	.63500
OD600nm of Manito against K. pneumoniae PDBI-49	3	.6993333	.0015 2753	.00088192	.6955388	.7031279	.69800	.70100
Total	48	.4202396	.4465 5125	.06445412	.2905746	.5499046	.00100	1.09600

Table 2. Mean, SD, minimum and maximum value for OD_{600nm} against *B. subtilis* PDBI-9 and *K. pneumoniae* PDBI-49 at various pH values using SPSS

Descriptive								
OD600nm (g/L) against PDBI-9 and 49								
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
OD_{600nm} against pH6 for B. subtilis PDBI-9	3	.5036667	.00251661	.00145297	.4974151	.5099183	.50100	.50600
OD600nm against pH6.5 for B. subtilis PDBI-9	3	.7020000	.00400000	.00230940	.6920634	.7119366	.69800	.70600
OD600nm against pH7 for B. subtilis PDBI-9	3	1.0973333	.00152753	.00088192	1.0935388	1.1011279	1.09600	1.09900
OD600nm against pH8 for B. subtilis PDBI-9	3	.9850000	.00264575	.00152753	.9784276	.9915724	.98200	.98700
OD600nm against pH6 for K. pneumoniae PDBI-49	3	.5333333	.00251661	.00145297	.5270817	.5395849	.53100	.53600
OD600nm against pH6.5 for K. pneumoniae PDBI-49	3	.7166667	.00152753	.00088192	.7128721	.7204612	.71500	.71800
OD600nm against pH7 for K. pneumoniae PDBI-49	3	1.1143333	.00251661	.00145297	1.1080817	1.1205849	1.11200	1.11700
OD600nm against pH8 for K. pneumoniae PDBI-49	3	.9970000	.00200000	.00115470	.9920317	1.0019683	.99500	.99900
Total	24	.8311667	.23627060	.04822853	.7313983	.9309350	.50100	1.11700

Table 3. Mean, SD, minimum and maximum value for CDW (g/L) against *B. subtilis* PDBI-9 and *K. pneumoniae* PDBI-49 at various temperatures values using SPSS

Descriptive								
Temperatures against CDW (g/L) and OD600nm for PDBI-9 and 49								
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
CDW (g/L) against 25oC for B. subtilis PDBI-9	3	.004133	.0004163	.0002404	.003099	.005168	.0038	.0046
CDW (g/L) against 30oC for B. subtilis PDBI-9	3	.008633	.0001528	.0000882	.008254	.009013	.0085	.0088
CDW (g/L) against-35oC for B. subtilis PDBI-9	3	.008000	.0001000	.0000577	.007752	.008248	.0079	.0081
CDW (g/L) against-37oC for B. subtilis PDBI-9	3	.005967	.0008327	.0004807	.003898	.008035	.0053	.0069
CDW (g/L) against 40 oC for B. subtilis PDBI-9	3	.003267	.0002082	.0001202	.002750	.003784	.0031	.0035
CDW (g/L) against 25oC for K. pneumoniae PDBI-49	3	.005200	.0001000	.0000577	.004952	.005448	.0051	.0053
CDW (g/L) against 30oC for K. pneumoniae PDBI-49	3	.009333	.0003055	.0001764	.008574	.010092	.0090	.0096
CDW (g/L) against 35oC for K. pneumoniae PDBI-49	3	.008233	.0000577	.0000333	.008090	.008377	.0082	.0083
CDW (g/L) against 37oC for K. pneumoniae PDBI-49	3	.006467	.0001528	.0000882	.006087	.006846	.0063	.0066

CDW (g/L) against 40oC for K. pneumoniae PDBI-49	3	.004267	.0001155	.0000667	.003980	.004554	.0042	.0044
OD600nm against 25oC for B. subtilis PDBI-9	3	.125667	.0025166	.0014530	.119415	.131918	.1230	.1280
OD600nm against 30oC for B. subtilis PDBI-9	3	.652333	.0064291	.0037118	.636363	.668304	.6450	.6570
OD600nm against 35oC for B. subtilis PDBI-9	3	.262333	.0015275	.0008819	.258539	.266128	.2610	.2640
OD600nm against 37oC for B. subtilis PDBI-9	3	.256333	.0005774	.0003333	.254899	.257768	.2560	.2570
OD600nm against 40oC for B. subtilis PDBI-9	3	.120000	.0010000	.0005774	.117516	.122484	.1190	.1210
OD 600nm against 25oC for K. pneumoniae PDBI-49	3	.131667	.0015275	.0008819	.127872	.135461	.1300	.1330
OD600nm against 30oC for K. pneumoniae PDBI-49	3	.622667	.0050332	.0029059	.610163	.635170	.6180	.6280
OD600nm against 35oC for K. pneumoniae PDBI-49	3	.280000	.0020000	.0011547	.275032	.284968	.2780	.2820
OD600nm against 37oC for K. pneumoniae PDBI-49	3	.256667	.0015275	.0008819	.252872	.260461	.2550	.2580
OD600nm against 40oC for K. pneumoniae PDBI-49	3	.122667	.0020817	.0012019	.117496	.127838	.1210	.1250
Total	60	.144692	.1935076	.0249817	.094703	.194680	.0031	.6570