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Contribution to the selection of spore-forming bacteria capable of producing bioplastics

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Abbreviation list

PHA Polyhydroxyalkanoate

PET Hugh density polyethylene

PVC Polyvinyl chloride

LDPE Low density polyethylene

PP Polypropylene

Ps Polystyrene

NaOH sodium hydroxide

HCL Hydrogen chloride

pH Hydrogen potential

(NH4)2SO4 Diammonium sulfate

Na2HPO4·7H2O Sodium phosphate dibasic heptahydrate

KH2PO4 Monopotassium phosphate

MgSO4 Magnesium sulfate

CaCl2·7H2O Calcium chloride heptahydrate

MnCl2·4H2O Manganese(II) chloride tetrahydrate

CoSO4·7H2O Cobalt(II) sulfate heptahydrate

CuCl2·2H2O Copper(II) chloride dihydrate,

ZnSO4·7H2O Zinc sulfate heptahydrate

FeSO4·7H2O Iron(II) sulfate heptahydrate

FeCl3·H2O Iron(III) chloride monohydrate

H3BO3 Boric acid

CoCl2·6H2O Cobalt(II) chloride hexahydrate

MnCl2·4H2O Manganese(II) chloride tetrahydrate

Na2MoO4·2H2O Sodium molybdate dihydrate

NiCl2·6H2O Nickel(II) chloride hexahydrate

CuSO4·5H2O Copper(II) sulfate pentahydrate

GM Growth medium

AM PHA accumulation medium

LB Luria-Bertani

LBB Luria-Bertani

MM Minimal medium

PBS Phosphate Buffered Saline

UV Ultra violet

FTIR Fourier Transform Infrared

“ Look deep into nature, and then you will understand everything better “

Albert Einstein

Introduction

The Earth is facing a grave environmental crisis due to the rampant pollution caused by human activities, with the overproduction and indiscriminate use of synthetic plastics leading to a global plastic pollution pandemic, with millions of tons of plastic waste accumulating in our oceans, landfills, and natural ecosystems every year **(Geyer et *al*., 2017)**. This plastic pollution not only harms delicate ecosystems and marine life but also poses a significant threat to human health and well-being **(Barnes et *al*., 2009; Thompson, Moore, et *al*., 2009)**.

In the face of this daunting challenge, we must look to innovative and sustainable solutions that can help mitigate the impact of plastic pollution, and one such solution lies in the remarkable capabilities of the microbial world, where microorganisms, such as bacteria and fungi, have evolved remarkable metabolic pathways and the ability to produce a wide range of useful compounds, including bioplastics **(Vijay & Tarika, 2019)**.

Bioplastics, particularly those derived from polyhydroxyalkanoates (PHAs), offer a promising alternative to traditional petroleum-based plastics, as they are produced by bacteria through fermentation processes and possess similar properties to conventional plastics while being biodegradable and environmentally friendly **(Anjum et *al*., 2016).** The study of PHA-based bioplastics presents a unique opportunity to explore sustainable solutions to the plastic pollution crisis, and replacing traditional plastics with bioplastics derived from PHA-producing bacteria can offer numerous benefits for the environment and human well-being, including biodegradability **(Koller, 2018)**, a reduced carbon footprint **(Sriyapai et *al*., 2022),** contribution to a circular economy, environmental protection **(Arora et *al*., 2021)**, and alignment with sustainable development goals .

The aim of this research is to explore the potential of *Bacillus* genus for polyhydroxyalkanoates (PHAs) production, focusing on three main chapters.

The literature will examine polyhydroxyalkanoates (PHAs) and their production by various bacteria, particularly those within the *Bacillus* genus. It will discuss the characteristics of *Bacillus*, including its role, physiology, morphology, metabolism, and taxonomy, before evaluating its growth ability and PHA production capacity using different carbon sources.

The materials and methods chapter will detail the protocols used to select the producing bacteria and produce PHAs.

While the results and discussion chapter will present and analyze the experimental findings, comparing them with existing literature and exploring the implications.

The manuscript will conclude by summarizing the key findings and suggesting future research directions for advancing PHA production from *Bacillus sp.*

LITERATURRE REVIW

1- Literature review

1.1- Nature of plastic

1.1.1- Plastic

1.1.1.1- Definition

Plastic is a broad term that refers to a wide range of synthetic or semi-synthetic organic polymeric materials, typically derived from petrochemicals or natural substances such as cellulose, coal, natural gas, or crude oil **(Andrady & Neal, 2009; Geyer et *al*., 2017)**. These materials are characterized by their ability to be molded into various shapes and forms through the application of heat and/or pressure, and they exhibit properties such as durability, elasticity, flexibility, and resistance to heat, water, organic solvents, oxidation, and ionizing radiation **(Hopewell et *al*., 2009; Thompson, Swan, et *al*., 2009) .**

Plastics are generally composed of long chains of repeating molecular units called monomers, which are linked together through a process called polymerization **(Hakkarainen & Albertsson, 2004).**

The specific properties of a plastic material are determined by the type and arrangement of the monomers, as well as the degree of cross-linking between the polymer chains .

1.1.1.2- History

The history of plastics can be traced back to the use of naturally occurring polymeric materials, such as gums and shellac, which were utilized by various civilizations as early as 1600 BC **(Williams & Rangel-Buitrago, 2022)**. The development of plastics then evolved from the chemical modification of natural materials, like rubber and cellulose, to the invention of the world's first fully synthetic plastic, Bakelite, in 1907 by Leo Baekeland in New York. Bakelite was a revolutionary material that was hard, water-resistant, and a poor conductor of electricity, making it suitable for a wide range of applications **(Geyer, 2020).**

The 20th century saw a rapid expansion of the plastics industry, driven by the demand for synthetic materials during World War II, and the growing consumer demand for the adaptable and versatile properties of plastics **(Gilbert, 2017)**. However, the increasing environmental concerns about plastic pollution in the 1960s led to a shift in public perception, highlighting the need to

address the challenges of plastic waste management while maintaining the benefits of these materials **(Williams & Rangel-Buitrago, 2022).**

1.1.1.3- Types of plastic

- **The most common forms of plastic**

The most common types of plastic include Polyethylene Terephthalate (PET), High-Density Polyethylene (HDPE), Polyvinyl Chloride (PVC), Low-Density Polyethylene (LDPE), Polypropylene (PP), and Polystyrene (PS) **(Geyer et *al*., 2017; Hopewell et *al*., 2009)**.

- **PET** is a thermoplastic polymer widely used for food and drink packaging due to its ability to prevent oxygen from entering. However, PET can contain antimony trioxide, a carcinogen, and is generally recommended for single-use and disposal.
- **HDPE** is a more stable and safer plastic option, commonly used for grocery bags, juice containers, and shampoo bottles. HDPE has a high strength-to-density ratio and is often recycled. Some studies have shown HDPE can leach estrogen-mimicking additives, so caution is advised .
- **PVC** is considered the most toxic and hazardous plastic, as it can lead to the leaching of chemicals such as BPA, phthalates, lead, dioxins, mercury, and cadmium. PVC is the second most used resin and the hardest to recycle due to its unique chemical characteristics.
- **LDPE** is the cheapest and the easiest to make plastic, used for grocery bags, plastic wraps, and wire or cable coverings. It is considered safe for use, but it is very difficult to recycle .
- **PP** is a durable and heat-resistant plastic used for reusable water bottles, hot food containers, and disposable diapers. It is a relatively safe option for food and drink applications, but it is also difficult to recycle.
- **PS** is extremely hard to recycle and it is recommended to be avoided, as it may leach styrene which is a known toxicant that can affect the brain, nervous system, lungs, liver, and immune system.

- **Processes used for the production of plastic**

The plastic manufacturing process generally involves the following steps **(Yang et *al*., 2008)**:

Material Preparation: The raw materials used in plastic manufacturing, such as petrochemicals like ethylene and propylene, are cleaned and processed to remove impurities.

Polymerization: The raw materials undergo polymerization, a process where small molecules (monomers) are chemically linked together to form long-chain polymers. Different polymerization techniques are used, such as addition polymerization, condensation polymerization, and ring-opening polymerization, depending on the type of plastic being produced.

Additives: After polymerization, various additives like colorants, stabilizers, plasticizers and other materials are mixed with the polymer resin to modify the physical and chemical properties of the plastic.

Molding: The plastic is then shaped into the desired form through various molding techniques, such as:

- ✓ Injection molding: Molten plastic is injected into a mold and cooled to solidify.
- ✓ Extrusion: Molten plastic is forced through a die to create continuous shapes like pipes or sheets.
- ✓ Blow molding: Molten plastic is blown into a mold to create hollow shapes like bottles.
- ✓ Thermoforming: Plastic sheets are heated and formed over a mold.
- ✓ Compression molding: Heated plastic is placed in a mold and compressed to shape.

Finishing: The final stage involves removing any excess material from the molded product and adding any necessary finishing touches, such as labeling or packaging.

1.1.1.4 The effects of plastic toxicity on the environment

The toxicity of plastic can have significant impact on the environment through various mechanisms. According to scientific researches plastic pollution can lead to environmental toxicity through several pathways

Microplastic and macroplastic contamination can have distinct impacts on the environment, with macroplastics directly harming larger animals through entanglement or ingestion, while microplastics infiltrate ecosystems and food webs, adsorbing and transporting harmful chemicals, and requiring tailored mitigation strategies **(Laskar & Kumar, 2019)**

- **Migration of Chemicals into Food**

Chemical additives in plastics can migrate into food and water, posing a threat to human health and the environment. Examples of toxic chemicals that can migrate from plastics include trichloroethane, acetone, methylene chloride, methyl ethyl ketone, styrene, toluene, benzene, and 1,1,1 trichloroethane **(Kao, 2020)**.

- **Biological Effects**

Microorganisms can transform complex organic matter into simple compounds, but the presence of microplastics can disrupt this process, leading to ecological instability. Microplastics can also act as vectors for pathogens increasing the spread of diseases **(Sohail et *al.*, 2023)**.

- **Air and Water Pollution**

The production and disposal of plastics can generate significant air and water pollution, contributing to environmental toxicity **(Kasavan et *al.*, 2021)**.

- **Human Health Impacts**

Exposure to plastic particles and chemicals can cause a range of health issues, including cancer, birth defects, and reproductive problems **(Koch & Calafat, 2009; Proshad et *al.*, 2018)**.

- **Physical degradation**

Plastic particles can be broken down into smaller pieces through abrasion, thermal degradation, and biological processes leading to the formation of microplastics and nanoplastics.

- **Chemical degradation**

Chemical additives in plastics, such as heavy metals, can leach into the environment causing toxicity and bioaccumulation in organisms **(Choi et *al.*, 2021)**. Heavy metals like Antimony (Sb), Arsenic (As), Barium (Ba), Beryllium (Be), Cadmium (Cd), Chromium (Cr), Cobalt (Co), Copper (Cu), Iron (Fe), Lead (Pb), Manganese (Mn), Nickel (Ni), Selenium (Se), Vanadium (V), and Zinc (Zn) are commonly used as additives in plastics for stabilizing actions. These metals can be toxic to organisms and have been linked to various health issues, including cancer, birth defects, and genetic changes **(Hahladakis et *al.*, 2018)**.

1.1.1.5- Plastic biodegradation

Plastic degradation by microbes is a sustainable approach to managing plastic waste and mitigating its environmental impact. Microorganisms, such as bacteria and fungi, can degrade various types of plastics through different mechanisms, including biodegradation, photodegradation and thermo-oxidative degradation **(Zeenat et *al.*, 2021)**.

Biodegradation is the most common mechanism of plastic degradation by microbes. In this process, microorganisms break down plastic polymers into smaller molecules, such as carbon dioxide, water and humus. This process requires the formation of a biofilm on the surface of the plastic particles, which allows colonization by microorganisms **(Choi et *al.*, 2023)**.

Several studies have demonstrated the biodegradation of various types of plastics by microbes. For example, bacteria such as *Alcanivorax borkumensis* and *Bacillus* genus. have been shown to degrade polyethylene and polypropylene, respectively, fungi such as *Penicillium simplicissimum* have also been found to degrade polyethylene **(Tania & Anand, 2023)**.

Microbial degradation of plastics typically occurs at relatively slow rates, often taking weeks, months, or even years to achieve significant degradation (See table 1) **(Gewert et *al.*, 2015)**.

Table 1 : Polyethylene (PE), Low-Density Polyethylene (LDPE), and High-Density Polyethylene (HDPE) Degradation Potential of Organisms Reported in Literature **(Zeenat et *al.*, 2021)**

Organisms	**Plastic types**	**Degradation time (Days)**	**Biodegradation efficiency (%)**	**Reference**
Pseudomonas fluorescens	PE	270	18	**Thomas et *al.* (2015)**
***Bacillus vallismortis* bt-dsce01**	LDPE	120	75	**Skariyachan et *al.* (2017)**
Klebsiella pneumoniae CH001	HDPE	60	18,4	**Awasthi et *al.* (2017)**
Aspergillus oryzae strain A5	LDPE	112	36,4	**Muhonja et *al.* (2018)**
Bacillus cereus strain A5	LDPE	112	35,72	**Muhonja et *al.* (2018)**
Trichoderma viride RH03	LDPE	45	5,13	**Munir et *al.* (2018)**
Aspergillus nomius RH06	LDPE	45	6,63	**Munir et *al.* (2018)**
Bacillus sp.* & *Paenibacillus sp	PE	60	14,7	**Parkand Kim (2019)**
Aspergillus flavus	HDPE	100	5,5	**Taghavi et *al.* (2021)**
Bacillus siamensis	LDPE	90	8,46	**Maroof et *al.* (2021)**

The complex structure and recalcitrant nature of many plastics poses challenges for a complete biodegradation. Furthermore, the conditions required for optimal microbial degradation, such as specific temperatures, pH levels and nutrient availability, are difficult to replicate on a large scale.

1.1.2- Bioplastic (Polyhydroxyalkanoate)

While microorganisms have demonstrated the ability to degrade various types of plastics, the process is often slow and incomplete with plastic residues that still remaining even after microbial treatment.

As a result, plastic pollution remains a significant environmental concern with millions of tons of plastic waste accumulating in landfills and oceans each year **(Williams & Rangel-Buitrago, 2022)**. Given the limitations of microbial degradation in fully, addressing the plastic pollution crisis, alternative solutions are urgently needed.

One promising approach is the development of bioplastics which are plastics derived from renewable and biodegradable sources such as plant biomass, agricultural waste or microbial fermentation. Bioplastics offer the potential for a complete biodegradation under natural conditions, without leaving behind harmful residues. Furthermore, the production of bioplastics can be designed to be more energy-efficient and environmentally friendly compared to traditional petroleum-based plastics. By transitioning towards bioplastics and other sustainable materials to mitigate the long-term impact of plastic pollution and create a more circular economy**(Arora et *al*., 2021).**

1.1.2.1- Generalities

Bioplastics are a class of polymers derived from renewable biomass sources, such as vegetable fats and oils, corn starch or microbiota, rather than petroleum. They are designed to be biodegradable and compostable, making them more environmentally friendly comparing to the traditional petroleum-based plastics. Bioplastics can be produced from various feedstocks including agricultural waste, lignocellulosic biomass and even municipal solid waste. The production process typically involves microbial fermentation, enzymatic catalysis or chemical synthesis **(Medeiros Garcia Alcântara et *al*., 2020)**.

One of the most promising types of bioplastics is Polyhydroxyalkanoates (PHAs). PHAs are linear polyesters produced by bacterial fermentation of sugar or lipids. They have similar

properties as like as conventional plastics, such as thermoplasticity and elasticity, making them suitable for a wide range of applications**(Raza et *al.*, 2018)**.

Bioplastics offer several advantages over traditional plastics including biodegradability, reduce carbon footprint and circular economy potentiality. However, they also face challenges such as higher production costs, limited availability and sometimes inferior mechanical properties compared to conventional plastics **(Sarkingobir & Lawal, 2021)**.

Despite these challenges, the demand for bioplastics is expected to grow significantly in the coming years, driven by increasing environmental awareness, government regulations and consumer preferences for sustainable products.

1.1.2.2- Discovery

The discovery of bioplastics dates back to the early 19th century, with Alexander Parkes creating the first man-made plastic, Parkesine, from cellulose in 1862. However, the development of bioplastics took a significant turn in 1926 when Maurice Lemoigne discovered polyhydroxy butyrate (PHB) which has been considered as the first known bioplastic made from bacteria. Polyhydroxyalkanoates (PHAs), a family of biopolymers, have been evolved beyond their origins as mere bioplastics with various microorganisms such as bacteria and archaea, naturally producing them as a mean of storing carbon and energy. PHAs are composed of hydroxyalkanoic acid monomers and can be synthesized as homopolymers or copolymers. One of the key advantages of PHAs is their biodegradability, as they can be broken down by microorganisms into carbon dioxide, water and biomass, making them an attractive alternative for reducing plastic pollution and promoting sustainability **(Behera et *al.*, 2022)**.

1.1.2.3- Structure

Polyhydroxyalkanoates (PHAs) are a family of biopolyesters composed of hydroxyalkanoic acid monomers. The general structure of PHA can be represented as follows **(Sharma et *al.*, 2021)**:

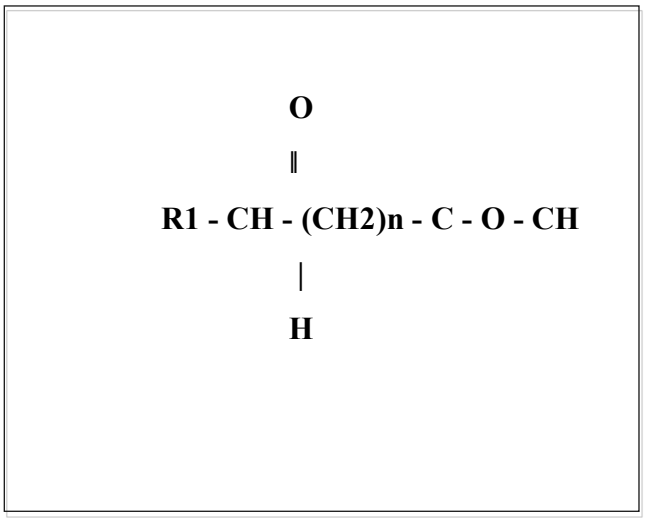

Figure 1 : Basic structure of PHA **(Samrot et *al.*, 2021)**.

With:

- R1 can be a hydrogen atom (H) or an alkyl group (CnH2n+1);
- n is an integer representing the number of methylene (-CH2-) groups.

The most common PHA monomers include:

- 3-hydroxybutyrate (3HB);
- 3-hydroxyvalerate (3HV);
- 3-hydroxyhexanoate (3HHx);
- 4-hydroxybutyrate (4HB).

PHAs can be classified into three main groups based on the number of carbon atoms in the monomers:

- Short-chain-length PHAs (scl-PHAs): 3-5 carbon atoms per monomer unit (e.g., PHB, PHBV).
- Medium-chain-length PHAs (mcl-PHAs): 6-14 carbon atoms per monomer unit (e.g., mcl-PHA containing 3-hydroxyhexanoate, 3-hydroxyoctanoate, and 3-hydroxydecanoate).
- Long-chain-length PHAs (lcl-PHAs): more than 14 carbon atoms per monomer unit.

The specific properties of PHAs depend on the monomer composition and can be tailored for various application.

1.1.2.4- PHA's characteristics

Polyhydroxyalkanoates (PHAs) are biodegradable polymers that exhibit a range of properties, making them a promising alternative to traditional plastics. The main characteristics of PHA include **(Hu et *al.*, 2011)**:

- **Biodegradability**: PHAs are biodegradable, meaning they can be broken down by microorganisms into carbon dioxide, water and biomass.
- **Thermoplasticity**: PHAs are thermoplastic, meaning they can be melted and reformed multiple times without undergoing any significant chemical change.
- **Elastomeric behavior**: PHAs exhibit elastomeric behavior, allowing them to stretch and recover their original shape.
- **Simple tunability**: PHAs can be produced with different physical properties by varying the production process and materials used.
- **Immunotolerance**: PHAs are non-toxic and biocompatible, making them suitable for biomedical applications.
- **High degree of polymerization**: PHAs have a high degree of polymerization, resulting in high molecular mass and polymer characteristics similar to conventional plastics.
- **High crystallinity**: PHAs are highly crystalline, which contributes to their mechanical properties and biodegradability.
- **Optical activity**: PHAs are optically active, meaning they exhibit stereospecificity in their repeating units.
- **Piezoelectric properties**: PHAs have piezoelectric properties, which can be useful in biomedical applications such as bone growth stimulation.
- **Solubility**: PHAs are soluble in halogenated solvents such as chloroform, dichloromethane or dichloroethane but insoluble in water.

1.1.2.5- The essential genes and enzymes for PHA biosynthesis

The key genes and enzymes involved in the formation of these biopolymers, specifically Polyhydroxyalkanoates (PHA), are a crucial set of molecular components that play a vital role in the biosynthetic pathway including the essential genes and enzymes such as PHA synthase (PhaC), β-ketothiolase (PhaA), Acetoacetyl-CoA reductase (PhaB), Phasin (PhaP) and Regulator proteins (PhaR), which collectively facilitate the conversion of carbon sources into PHA monomers,

ultimately leading to the formation of these biodegradable and biocompatible biopolymers**(Hu et *al.*, 2011)**.

- **PHA synthase (PhaC):** This is the key enzyme responsible for the polymerization of hydroxyalkanoic acid monomers into PHA.
- **β-ketothiolase (PhaA):** This enzyme catalyzes the condensation of two acetyl-CoA molecules to form acetoacetyl-CoA, which is a precursor for PHA biosynthesis.
- **Acetoacetyl-CoA reductase (PhaB):** This enzyme reduces acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA, which is the monomer for the most common PHA, polyhydroxybutyrate (PHB).
- **Phasin (PhaP):** Phasins are proteins that bind to the surface of PHA granules and regulate their size and number.
- **Regulator proteins (PhaR):** These proteins regulate the expression of PHA biosynthesis genes and phasin genes.

1.1.2.6- Biological synthesis of Polyhydroxyalkanoates (PHA) via microbial fermentation

Biological synthesis of Polyhydroxyalkanoates (PHA) through microbial fermentation is a well-studied process with significant implications for sustainable bioplastic production. PHAs, being biodegradable polyesters produced by various microorganisms offer a promising alternative to traditional plastics. The microbial fermentation process involves the conversion of carbon sources into PHA monomers, which are then polymerized to form PHA biopolymers. Research has delved into optimizing microbial strains **(Ali et *al.*, 2017)**, fermentation conditions, **(Dañez et *al.*, 2020)** and genetic engineering approaches to enhance PHA production efficiency and tailor the properties of these bioplastics for diverse applications.

The fermentation process for producing PHA by microorganisms involves several steps and conditions that are crucial for optimal production :

Inoculation: The fermentation process begins with the inoculation of a microorganism, such as *Pseudomonas putida*, into a nutrient-rich medium. This step ensures the growth of the microorganism and the initiation of PHA production **(Law et *al.*, 2001)**.

Growth Phase: The microorganism grows and multiplies in the medium, consuming the nutrients and producing PHA as a byproduct. This phase is characterized by rapid cell growth and PHA accumulation.

PHA Accumulation Phase: As the microorganism continues to grow, it begins to accumulate PHA within its cells. This phase is characterized by the production of PHA granules, which are composed of hydroxyalkanoic acid monomers**(Ratnaningrum et *al*., 2019)**.

Harvesting: The fermentation process is terminated by harvesting the microorganism, which is typically done by centrifugation or filtration. The resulting biomass is then processed to extract the PHA **(Akdoğan & Çelik, 2018)**.

1.1.2.6- PHA producing microorganisms

Microorganisms have emerged as a promising source for the production of bioplastics, offering a sustainable alternative to traditional plastics. Through fermentation processes, certain bacteria and fungi can convert simple sugars into complex polymers, such as polyhydroxyalkanoates (PHA) and polyhydroxybutyrates (PHB) (See table 2).

Table 2: Production of polyhydroxyalkanoates (PHAs) by various microorganisms using variety of carbon sources

Microorganisms	Carbon source	Dry cell weight (DCW) (g/L)	PHAs yield (g/L)	PHAs accumulation (%)	References
Burkholderia cepacian ATCC 17,759	Glycerol	5.8	4.8	82.76	**(Zhu et *al.*, 2010)**
Bacillus sp. NA10	Cardboard industry effluent	7.8	5.202	66.70	**(Bhuwal et *al.*, 2014)**
Bacillus thuringiensis IAM 12,077	Starch	3.6	2.6	72.3	**(Gowda & Shivakumar, 2014)**
Cupriavidus necator	Fructose	11.6	7.48	64.48	**(Aramvash et *al.*, 2015)**
Pseudomonas aeruginosa	Mustard oil	–	9.01	20.1	**(Javed & Jamil, 2015)**
Bacillus sp.	Sugarcane bagasse	9.0	5.0	55.55	**(Getachew & Woldesenbet, 2016)**
Pseudomonas putida	Waste frying oil	4.90	2.80	57	**(Putida et *al.*, 2018)**
Bacillus siamensis PD-A10	Orange peel	2.66	2.16	81.2	**(Vijay & Tarika, 2019)**
Bacillus megaterium	Molasses	–	19.52	60.02	**(Mascarenhas, 2019)**
Bacillus cereus NDRMN001	Rice bran	36.26	33.19	91.54	**(Narayanan, Kandasamy, et *al.*, 2020)**

1.1.2.7- Methods for Detecting PHA-Producing Bacteria

PHA-producing bacteria can be detected using various methods including staining techniques, spectrophotometric methods, infrared and FTIR spectroscopy, HPLC and polymerase chain reaction (PCR) amplification of PHA synthesis genes.

Non-Molecular Techniques for Detecting PHA-Producing Bacteria

- ✓ **Staining Techniques**

Sudan Black B Staining**:** Used as a presumptive test for the presence of PHB granules in bacterial cells **(Pandolfi, n.d.).**

Nile Blue A Staining**:** Staining method to detect PHA granules in bacterial cells **(Oshiki et *al.*, 2011)**.

Phase Contrast Microscopy: Utilized for screening large numbers of isolates by observing intracellular refractive granules suggesting the presence of PHB **(Godbole, 2016)**.

- ✓ **Spectrophotometric Methods**

UV-Vis Spectroscopy: Measures the absorbance of PHA at specific wavelengths for detection.

Infrared and FTIR Spectroscopy**:** FTIR Spectroscopy: Analyzes the infrared absorption spectra of PHA for identification **(Oshiki et *al.*, 2011)**.Molecular Techniques for Detecting PHA-Producing Bacteria

Polymerase Chain Reaction (PCR)**:** PCR Amplification of PHA Synthesis Genes used for amplifying genes involved in PHA synthesis to detect PHA-producing bacteria.

NMR Techniques**:** NMR Analysis coupled with staining techniques for identification of polyhydroxyalkanoates in specific bacterial species.

Electron Microscopy**:** Electron Microscopy used in combination with staining techniques for visualizing PHA granules in bacterial cells **(Berlanga et *al.*, 2006)**.

1.1.2.8- Effect of operational parameters on PHA production by bacteria

Polyhydroxyalkanoates (PHA) production is influenced by various operational parameters including substrate concentration, pH, temperature and nutrient availability. These parameters can significantly impact the efficiency and yield of PHA production, making it crucial to optimize them for optimal results **(Cabrera et *al.*, 2019)**.

Substrate concentration

Substrate concentration plays a critical role in PHA production: high substrate concentrations can lead to inhibition and reduced PHA productivity, while low concentrations can result in reduced biomass growth and PHA accumulation.

pH

PH is another critical parameter that affects PHA production. Optimal pH ranges for PHA production vary depending on the microorganism used but generally, pH 6.5-7.5 is considered optimal.

Temperature

Temperature also influences PHA production: optimal temperatures for PHA production vary depending on the microorganism used but generally, temperatures between 25-37°C are considered optimal.

Nutrient availability

Nutrient availability, particularly nitrogen and phosphorus, is essential for PHA production. Insufficient nutrient availability can lead to reduced biomass growth and PHA accumulation.

1.1.2.8- PHA's extracting and recovery

Various methods have been developed for the extraction and recovery of Polyhydroxyalkanoates (PHA) from bacterial cells. These methods aim to efficiently extract PHA while minimizing degradation and maintaining its properties.

❖ **Extraction methods**

- **Solvent Extraction**

Solvent extraction is one of the most widely used methods for PHA recovery. It involves the use of organic solvents to dissolve and extract PHA from the bacterial cells. The PHA is then recovered by precipitation or evaporation of the solvent.

Some commonly used solvents include:

- Chloroform;
- Dichloromethane;
- Dimethyl carbonate (DMC).

Solvent extraction can achieve high PHA purity and recovery yields, but it requires the use of large quantities of organic solvents, which can be costly and environmentally harmful.

- **Chemical Digestion**

Chemical digestion methods involve the use of chemicals to selectively degrade or dissolve the non-PHA cellular materials (NPCM), leaving behind the PHA.

The most commonly used chemicals are:

- Sodium hypochlorite (NaOCl);
- Sodium hydroxide (NaOH);
- Acids (e.g., sulfuric acid, hydrochloric acid).

Chemical digestion methods are relatively simple and cost-effective, but they can cause degradation of the PHA and require careful optimization of the chemical concentrations and reaction conditions.

- **Enzymatic Digestion**

Enzymatic digestion methods use enzymes to selectively degrade the NPCM, leaving behind the PHA.

The enzymes used include:

- Proteases (e.g., pronase, proteinase K);
- Nucleases (e.g., DNase, RNase);
- Lysozyme.

Enzymatic digestion methods are gentle and can minimize PHA degradation, but they can be more expensive and time-consuming compared to chemical digestion methods.

- **Mechanical Disruption**

Mechanical disruption methods use physical forces to break down the bacterial cells and release the PHA.

Some commonly used methods include:

- Bead milling;
- High-pressure homogenization;
- Ultrasonication.

Mechanical disruption methods can be effective in extracting PHA without using chemicals, but they can be energy-intensive and may require additional purification steps.

- **Supercritical Fluid Extraction**

Supercritical fluid extraction uses supercritical fluids, such as carbon dioxide (CO2), to extract PHA from bacterial cells. The supercritical fluid acts as a solvent and can be easily separated from

the extracted PHA by adjusting the pressure and temperature.

Supercritical fluid extraction is a promising method for PHA recovery as it is environmentally friendly and can minimize PHA degradation, but it requires specialized equipment and optimization of the extraction conditions.

❖ **Purification of PHA after Extraction**

After the extraction of PHA from bacterial cells, the extracted PHA needs to be purified to remove impurities and enhance its quality **(Zribi-Maaloul et *al*., 2013)**.

- **Solvent Removal**

The first step in the purification process is to remove the solvent used for extraction. This can be done by evaporation, filtration or centrifugation.

- **Precipitation**

The PHA solution is then cooled to a temperature that allows the PHA to precipitate out of the solution. This step helps to separate the PHA from the solvent and other impurities.

- **Solid-Liquid Separation**

The precipitated PHA is then separated from the remaining solvent and impurities through solid-liquid separation techniques such as filtration or centrifugation.

- **Decolorization**

To enhance the purity of the PHA, a decolorization step may be necessary to remove any color bodies or impurities. This can be achieved through adsorption using activated carbon or other decolorizing agents.

- **Drying**

The purified PHA is then dried to remove any remaining solvent content. This can be done by increasing the temperature of the PHA to evaporate the solvent, resulting in dry PHA ready for further processing or application.

1.1.2.9- Scaling up PHA production from Lab to Industry

Scaling up PHA production from lab to industry involves several key steps (G.-Q. Chen, 2010):

Step 1: Strain Development

Strain Selection: Select a microorganism capable of producing PHA.

Strain Improvement: Use genetic engineering techniques to enhance PHA production by introducing genes that improve PHA biosynthesis, such as the phaC gene.

Step 2: Fermentation Optimization

Fermentation Conditions: Optimize fermentation conditions such as temperature, pH and oxygen levels to maximize PHA production.

Fermentation Scale-Up: Scale up fermentation from shake flasks to laboratory and pilot fermenters to increase PHA production.

Step 3: Cell Harvesting and Separation

Cell Harvesting: Harvest cells using centrifugation or filtration to separate them from the fermentation broth.

Cell Disruption: Disrupt cells using methods such as sonication or enzymatic digestion to release PHA.

Step 4: PHA Extraction and Purification

Solvent Extraction: Use organic solvents such as chloroform or dichloromethane to extract PHA from the cell disruption mixture.

Precipitation: Precipitate PHA from the solvent using methods such as cooling or addition of a precipitant.

Filtration: Filter the PHA precipitate to remove impurities and achieve high purity.

1.1.2.10- Biodegradability of Polyhydroxyalkanoates (PHA)

The biodegradability of Polyhydroxyalkanoates (PHA) has been extensively studied and proven **(Zhou, 2023)**.

PHA can be degraded entirely in nature under anaerobic conditions, such as in sediments and landfills, into carbon dioxide, methane and water. Under aerobic conditions, such as in soil and marine environments, PHA can be degraded into carbon dioxide and water.

During PHA degradation, microbial exoenzymes play an essential role in breaking down the completely non-soluble PHA into smaller water-soluble substances. Afterward, microbes can use these water-soluble substances as carbon and energy sources to further degrade them into carbon dioxide, methane and water, creating a carbon cycle for PHA production and degradation. Compared to other biodegradable plastics, PHA has better biodegradability in the natural environment.

The predicted pathway of PHA degradation involves microbial exoenzymes breaking down PHA into smaller water-soluble substances, which are then further degraded by microbes into carbon dioxide, methane and water.

PHA can be fully degraded to water and carbon dioxide under aerobic conditions or methane under anaerobic conditions in various environments, such as seawater, sewage and soil.

- **Biodegradation pathway**

The biodegradability of PHA materials is attributed to the action of extracellular PHA depolymerases secreted by microorganisms in various natural environments. The biodegradation process involves the colonization of PHA surfaces by microorganisms, which secrete enzymes that degrade PHA into HA monomers. These monomers are then utilized by the cells as a carbon source for biomass growth **(Boey et *al*., 2021)**.

Microbial Exoenzymes

Microbial exoenzymes play an essential role in breaking down the completely non-soluble PHA into smaller water-soluble substances.

Water-Soluble Substances

The water-soluble substances are then further degraded by microbes into carbon dioxide, methane, and water

Carbon Cycle

The carbon cycle for PHA production and degradation involves microbes using the water-soluble substances as carbon and energy sources to further degrade them into carbon dioxide, methane, and water

1.1.2.11- Fields of application

Polyhydroxyalkanoates (PHA) have been extensively studied for various applications due to their unique properties, such as mechanical strength, biocompatibility and biodegradability **(Pradhan et *al*., 2020)**.

- **Environmental Applications**

As a biodegradable Packaging, bioplastics can be used to create compostable packaging materials that can break down naturally in the environment, reducing plastic waste and pollution.

- **Bioplastics for Consumer Electronics:**

Bioplastics can be used to create eco-friendly electronics, such as biodegradable circuit boards and casings, reducing electronic waste and pollution.

- **Food Service**

Bioplastics can be used to create biodegradable cutlery and plates, reducing the environmental impact of disposable tableware.

- **Agricultural Applications:**

PHA can be used to fabricate mulch films, reducing nutrient loss and soil erosion.

Slow-release fertilizers : PHA can be used to fabricate slow-release fertilizers, improving plant growth.

- **Textile Applications:**

PHA can be used to fabricate biodegradable fibers, reducing the amount of textile waste.

- **Medical Applications:**

Bioplastics can be used to create biodegradable medical implants, such as sutures and bone prostheses, reducing the environmental impact of medical waste.

- **Aerospace and Automotive Applications:**

Bioplastics can be used to create biodegradable materials for aerospace and automotive application.

1.2- Bacteria of *Bacillus* genus

The genus *Bacillus* is a group of Gram-positive and rod-shaped bacteria that are widely distributed in various environments, including soil, water and air. Characterized by its ability to form endospores which are highly resistant to environmental stressors such as heat, radiation and chemicals. *Bacillus* spp. exhibit a vegetative form with a rod-shaped morphology, typically motile with a flagellum at one or both ends, and capable of growing in a variety of environments **(D'Agostino & Cook, 2016)**.

The sporulated form of *Bacillus* genus is characterized by the formation of endospores, which are highly resistant to environmental stressors, formed within the vegetative cell through sporogenesis, involving the formation of a spore coat and the dehydration of the cell **(Olarte & Bratcher, 2023)**.

1.2.1- History

The genus *Bacillus*, named by Christian Gottfried Ehrenberg in 1835, is a group of Gram-positive rod-shaped bacteria that have been extensively studied in various fields of microbiology. The term "*Bacillus*" is derived from the Latin word "bacillus," meaning "stick," which refers to the rod-shaped morphology of these bacteria.**(Turnbull, 1996)**.

Early studies on *Bacillus* genus focused on their ability to form endospores which are highly resistant to environmental stressors. Ferdinand Cohn later amended the genus to include spore-forming, Gram-positive and aerobic or facultatively anaerobic bacteria **(Nicholson et *al.*, 2000)**.

1.2.2- Habitat

Bacillus genus bacteria are commonly found in soil, water and decaying organic matter. They are also found in the gastrointestinal tract of humans and animals, where they play a role in the breakdown of complex nutrients. *Bacillus* is able to thrive in a wide range of environments due to its ability to form endospores, which provide it with protection against environmental stressors **(Jenson, 2014)**.

1.2.3- Bacteriological Characteristics

Bacillus genus is a Gram-positive, aerobic, spore-forming bacteria with the following key characteristics (See table 3) **(Aksoy et *al.*, 2018) (Aminian-Dehkordi et *al.*, 2019)**.

Table 3 : Key characteristics of *Bacillus* genus.

Cell Morphology	They are a large, rod-shaped bacteria with a cell length up to 100 µm and a diameter of 0.1 µm, which is quite large for bacteria. The cells often occur in pairs and chains.
Gram Staining	Gram-positive
Spore Formation	They are an endospore-forming bacteria, capable of producing highly resistant spores under unfavorable conditions.
Motility	motile
Oxygen Requirement	They are an aerobic bacterium, requiring oxygen for growth.
Catalase Production	catalase-positive
Carbohydrate Metabolism	They can utilize various carbohydrates as carbon and energy sources, such as L-arabinose, D-glucose, and D-mannitol.
Protein Production	known for their ability to produce a wide range of proteins and enzymes, including penicillin amidase, amylases, glucose dehydrogenase, and amino acid dehydrogenases, making it a valuable organism in biotechnology.
Sporulation	The bacteria undergo a complex process of sporulation, forming highly resistant endospores in response to nutrient depletion or other environmental stresses.
Bacteriophage Susceptibility	*Bacillus* genus Cells are susceptible to various bacteriophages, which has been exploited in phage typing and genetic studies.

1.2.4- Ecological Role of *Bacillus* genus

Bacillus plays a significant role in various ecological processes including decomposition and nutrient cycling, plant growth promotion, bioremediation, symbiotic relationships, adaptation to diverse habitats, pathogenesis **(Padgham & Sikora, 2007)** and food web **(Schultz et *al.*, 2017)**. It is involved in the decomposition of organic matter, breaking down complex molecules into simpler compounds that can be used by other organisms, and contributes to nutrient cycling in the environment. Additionally, it possesses plant growth-promoting activity, including biocontrol ability against plant's pathogens, making it beneficial for agriculture **(Poveda & González-Andrés, 2021)**. *Bacillus* genus has been used in bioremediation processes to clean up pollutants and contaminants from the environment and to form symbiotic relationships with other organisms, such as plants, providing them with essential nutrients and services. **(Vary et *al.*, 2007)**

1.2.5- Physiology

- **Temperature**

Bacillus cells grow at temperatures ranging from 3 to 45 °C, with the optimal growth temperature around 30 °C. Some isolates from an Antarctic geothermal lake have been found to grow at temperatures up to 63 °C

The permissive temperature for sporulation of *Bacillus* genus is up to 42 °C, which is 4–5 °C lower than the temperature required for growth **(Strnadová et *al.*, 1991)**.

- **pH**

The optimal pH for growth of *Bacillus* genus is around pH 7.0 **(Poveda & González-Andrés, 2021)**.

- **Trophic Type**

Bacillus genus is a group of an heterotrophic bacteria, meaning they obtain their energy by breaking down and utilizing organic compounds. They are chemoorganotrophic, using organic carbon compounds as their source of energy and carbo **(Hur et *al.*, 2024)**.

- **Respiratory Type**

Bacillus genus contain aerobic bacteria that require oxygen to carry out their metabolic processes including respiration. they use oxidative phosphorylation to generate energy from the breakdown of glucose and other organic compounds, and also employ glycolysis to break down glucose into pyruvate, which is then converted into lactate or other compounds. Although primarily

aerobic *Bacillus* species can also grow under anaerobic conditions when necessary, using alternative metabolic pathways such as fermentation. This versatility in respiratory modes allows them to thrive in a wide range of environments, from soil to industrial settings **(Goswami et *al.*, 2018)**.

1.2.6- Morphology

- ✓ **Macroscopic Description of *Bacillus* genus**

Bacillus genus form concave and smooth colonies in appearance with a milk-white color (See table 4).

Table 4 Macroscopic Description of *Bacillus* genus.

Characteristics	Description
Colony Morphology	Concave and smooth.
Color	Milk-white.
Macro-Capsules	Present, with micro-capsules dispersed throughout the colony.

- ✓ **Microscopic observation**

Bacillus genus appear on a microscope as Gram-positive rod-shaped bacteria with endospores (Se table 5).

Table 5 : Microscopic observation of *Bacillus* genus.

Characteristics	Description
Cell Morphology	Rod-shaped, up to 100 µm in length, 0.1 µm in diameter.
Cell Arrangement	Often in pairs and chains, joined by polysaccharides on cell walls.
Cell Wall Composition	Contains polysaccharides facilitating cell arrangement.

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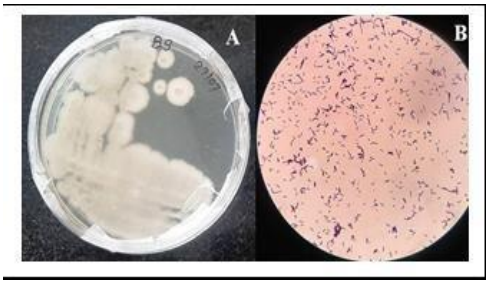
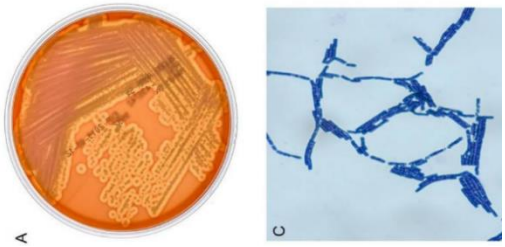
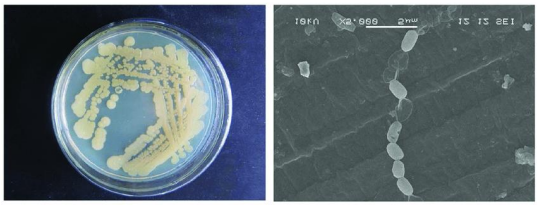
✓ **Macroscopic and Microscopic Observations of the most known *Bacillus* Species**

Table 6 : Macroscopic and Microscopic Observations of the most known *Bacillus* Species

Species	Macroscopic Observation	Microscopic Observation	References
 **Figure 2 :** *Bacillus subtilis*	Off-white, round, entire, opaque colonies (2 mm).	Gram-positive, motile, and spore-forming rods.	**(Errington & van der Aart, 2020)**
 **Figure 3 :** *Bacillus cereus*	White color, irregular, entire, opaque colonies (8-9 mm)	Gram-positive, motile, and spore-forming rods	**(Tirloni et *al.*, 2022)**
 **Figure 4 :** *Bacillus megaterium*	Concave, smooth, and milk white	Rod-shaped morphology	**(Liu et *al.*, 2014)**

1.2.7- Spores formation

Bacillus genus spores are highly resistant to environmental stressors such as heat, radiation and chemicals due to their unique structure and composition. Each spore consists of a thick proteinaceous coat called the exosporium, which is covered by a thin layer of peptidoglycan and lipoteichoic acid.

The spore also contains a central core composed of DNA, RNA and proteins, which are protected by a thick layer of calcium dipicolinate **(Goswami et *al.*, 2018)**.

- **Characteristics of Spores**

The spores of *Bacillus* genus are highly resistant to heat, with a thermal resistance of up to 121°C for 30 minutes. They are also resistant to radiation, with a dose of up to 10 kGy. Additionally, the spores are resistant to chemicals such as disinfectants and antibiotics **(Setlow et *al.*, 2009)**

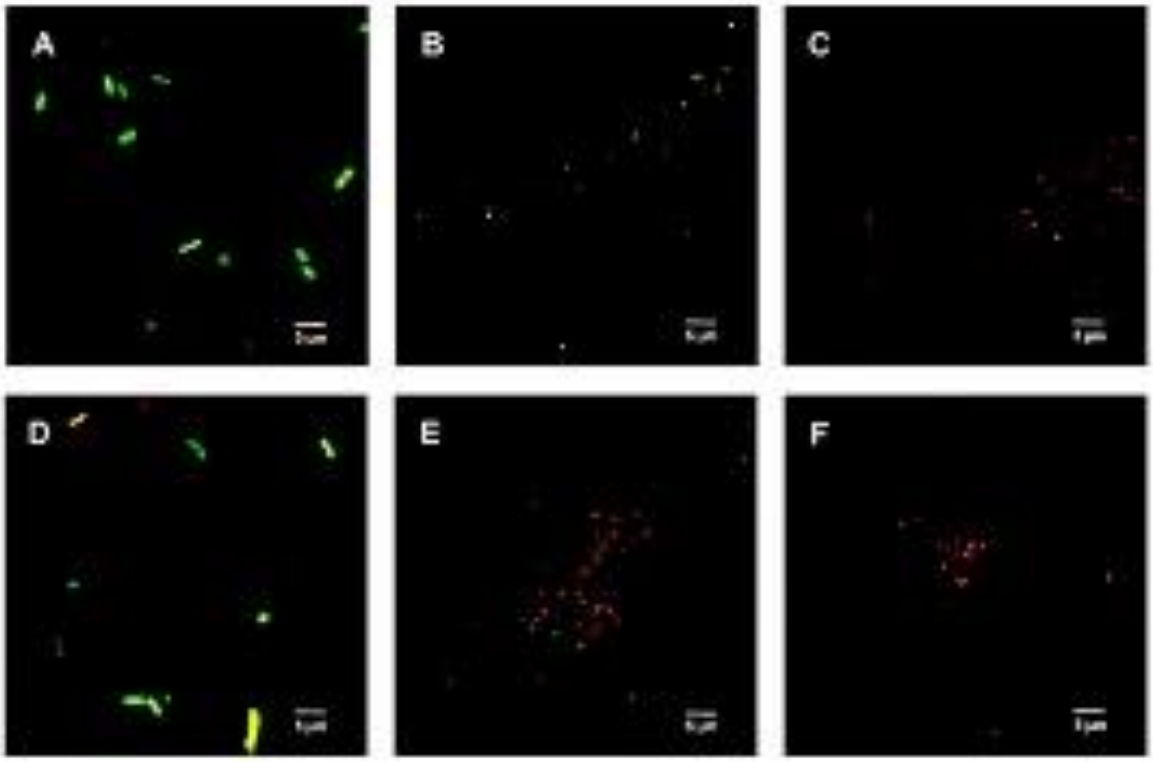


Figure 5: Live/Dead assay of *Bacillus spp.* spores with TFDG at 60-min incubation. The green indicates viable spores, while the red indicates non-viable spores. (A) *B. cereus* spores control; (B*) B.cereus* spores with 312.5 µg/mL TFDG; (C) *B.cereus spores* with 625 µg/mL TFDG; (D) *B. subtilis* spores control; (E) *B.subtilis* spores with 312.5 µg/mL TFDG; and (F) *B.subtilis* spores with 625 µg/mL TFDG **(Yussof et *al.*, 2022)**.

1.2.8- Primary and secondary metabolites of *Bacillus* bacteria

Bacillus genus is known to produce a diverse array of primary and secondary metabolites. Primary metabolites are essential compounds directly involved in growth and development, while secondary metabolites often have specialized functions such as defense and communication. Researches on *Bacillus* species has revealed the production of primary metabolites like amino acids, lipids and sugars, crucial for basic cellular functions **(Hur et *al.*, 2024)**. Additionally, the bacteria synthesize secondary metabolites such as butanediol, cyclic dipeptides and fatty acids **(Foster et *al.*, n.d.)**, which may play roles in defense and signaling pathways.

Understanding the metabolic profile of *Bacillus* genus sheds light on its physiological capabilities and potential applications in various fields.

1.2.9- Taxonomy

Taxonomy is the scientific discipline of classifying and naming organisms based on their similarities and evolutionary relationships. It provides a hierarchical system for organizing living things into groups and subgroups, allowing the identification and study of species.

The taxonomy of *Bacillus* genus is important for understanding its evolutionary relationships, identifying it accurately, and studying its characteristics and potential applications.

their taxonomy is as follows:

Kingdom: *Bacteria*

Phylum: *Firmicutes*

Class*:* *Bacilli*

Order: *Bacillales*

Family*:* *Bacillaceae*

Genus: *Bacillus*

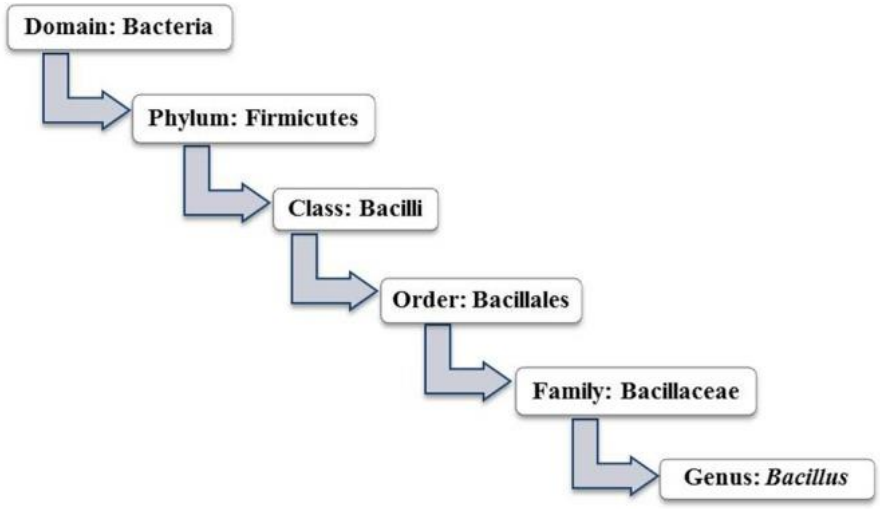


Figure 6 : *Bacillus* genus's taxonomy **(Bergey's Manual of Systematic Bacteriology, n.d.; Elshaghabee et *al.*, 2017)**

Materials and Methods

2- Materials and Methods

Polyhydroxyalkanoates (PHAs) are biodegradable polymers produced by various microorganisms, including *Bacillus spp*., as a carbon and energy storage mechanism. This study aimed to detect PHAs from a *Bacillus spp.* isolate obtained from a soil sample.

The *Bacillus spp.* isolate was cultured in a suitable medium to enhance PHA production.

Different detection methods were used to identify the presence of PHAs in the bacterial cells, including techniques such as Fourier Transform Infrared (FTIR) spectroscopy , UV Lampe and fluorescence microscopy using Nile blue dye.

2.1- Sampling

Soil samples were aseptically collected from the rocky sites located within the premises of the ***Enterprise Nationale des Granules (ENG)***, a state-owned enterprise specializing in the production and processing of various granular materials. The soil samples were transported to the laboratory for further processing and analysis.

This soil sample was chosen due to its favorable environmental conditions, which are known to support the growth and survival of the genus *Bacillus*. Specifically, the soil's pH and nutrient profiles are conducive to the optimal growth of *Bacillus* species also the presence of calcium carbonate (CACO3) in the soil also contributes to the unique conditions that favor the growth of *Bacillus* species, while the endospores of *Bacillus* provide resistance to the harsh conditions found in the rocky sites, such as high temperatures and low water availability, which are known to inhibit the growth of other genera. Other genera can hardly thrive in these conditions, making it a unique environment for studying *Bacillus* species.

2.2- Isolation and purification

1g of the soil sample was diluted with 9 mL of physiological water and a Serial dilutions of $10^{-1}$to 10^{-3} was prepared .The mixture was subjected to a thermal treatment at 80°C for 15 minutes in a water bath. The treated mixture was then plated onto Peter dishes containing LB agar and incubated at 30°C for 24 hours. The resulting colonies were purified by repeated streaking on LB agar plates.

2.3- Preliminary identification's Tests

Several identification tests were performed on the purified Bacterium :

- **Gram Staining**

A small aliquot of the bacterial culture was aseptically transferred to a clean microscope slide to be mixed with a drop of sterile physiological saline solution. The sample was then fixed to the slide using a Bunsen burner flame to prevent cell movement or changes during staining.
Crystal violet, a primary stain, was then applied to bind to the peptidoglycan layer in the cell wall of Gram-positive bacteria. A mordant, Lugol solution, was used to enhance the binding of the dye. After decolorization with 95% ethanol, the cultures were counterstained with fuchsine,

The stained cultures were observed under an optical microscope with objective (X40) then with immersion (X100) to determine the Gram reaction, with Gram-positive bacteria appearing purple and Gram-negative bacteria appearing pink.

- **Catalase test**

A few drops of hydrogen peroxide were added to the cultures, and the production of oxygen bubbles was observed. The formation of bubbles, corresponding to the release of oxygen gas, indicates that the bacterium is catalase-positive. The absence of bubbles signifies that it is catalase-negative.

- **respiratory type**

The respiratory type of the bacterial culture is determined using various biochemical tests, including the meat liver agar medium, which is a deep tube medium. The medium is inoculated with the bacterial culture ensuring the inoculum is evenly distributed throughout the medium, and incubated at 37°C for 24 hours.

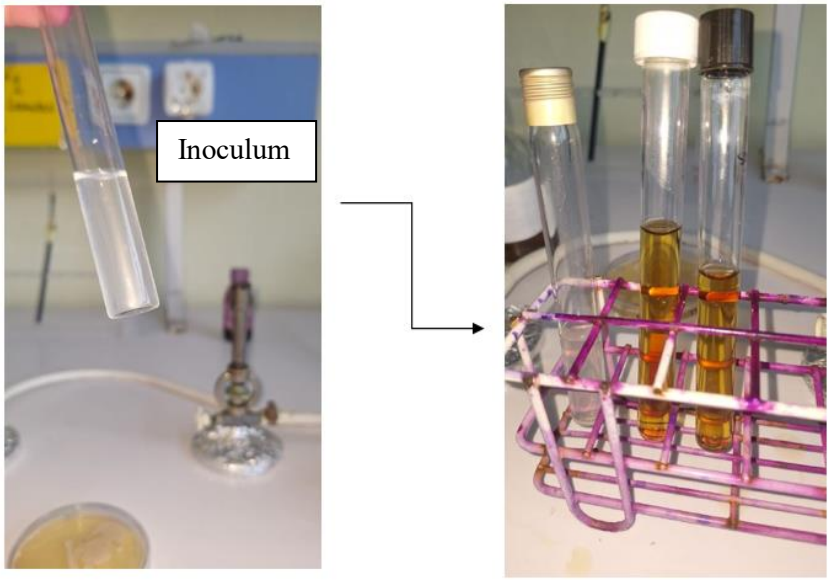


Figure 7: Respiratory test.

The type of respiration exhibited by the microorganism can be determined by observing the growth of the microorganism in the medium, with different types of growth indicating different types of respiration. For example, **aerobic growth** is characterized by the presence of colonies at the top of the tube, while **anaerobic growth** is characterized by the presence of colonies at the bottom of tube. **Facultative anaerobes** growth is characterized by the presence of colonies in both aerobic and anaerobic zones **(Camille, 2014)**.

2.4- Production of Polyhydroxyalkanoates by *Bacillus* isolate

Three distinct protocols were employed in this research, each designed to optimize the analysis of polyhydroxyalkanoates (PHA) from *Bacillus spp*.

2.4.1 First protocol

- **Media used**

Preparation of growth medium (Nile Blue Agar Screening Medium)

- Glucose: 10 g/L
- Meat extract: 3 g/L
- Peptone: 5 g/L
- Sodium chloride: 8 g/L
- Agar: 15 g/L

After mixing all the ingredients together the pH of the medium was adjusted to 7.0 using 0.1N NaOH , the medium was then sterilized at 121°C for 20 min **(Ratnaningrum et *al.*, 2019)**.

This carbon-rich nutrient agar medium, containing Nile blue (See apendix), was used for qualitative screening of PHA-producing organisms.

Preparation of Minimal Medium for PHA Accumulation

- Glucose: 10 g/L;
- (NH4)2SO4: 1 g/L;
- Na2HPO4·7H2O: 6.7 g/L ;
- KH2PO4: 0.1 g/L ;
- MgSO4·7H2O: 0.2 g/L ;
- Ferrous ammonium citrate: 60 mg/L ;
- CaCl2·7H2O: 10 mg/L ;

- Trace element solution: 1 mL/L.

The trace element solution contained the following components per liter :

- $MnCl_2 \cdot 4H_2O$: 1.98 g ;
- $CoSO_4 \cdot 7H_2O$: 2.81 g ;
- $CuCl_2 \cdot 2H_2O$: 0.17 g ;
- $ZnSO_4 \cdot 7H_2O$: 0.29 g ;
- $FeSO_4 \cdot 7H_2O$: 2.78 g.

After mixing all the ingredients together the pH of the medium was adjusted to 7.0 using 0.1N NaOH , the medium was then sterilized at 121°C for 20 min **(Ratnaningrum et al., 2019)**.

This minimal medium is used to induce and maximize PHA accumulation in the bacterial isolates. This medium was formulated based on previous studies and contained a suitable carbon source, limited nitrogen and essential nutrients required for growth and PHA synthesis. The specific composition of this optimized medium was designed to promote the highest possible PHA yields in the target bacterial species.

- **Detecting PHA-Accumulating Bacteria: A Qualitative Approach using UV light**

The qualitative determination of PHA accumulation in the bacterial isolates was performed using Nile blue staining. The isolates were grown by streaking onto Nile Blue Agar Screening Medium **(Martinez & Henary, 2016)** and incubated at 30°C for 24 hours.

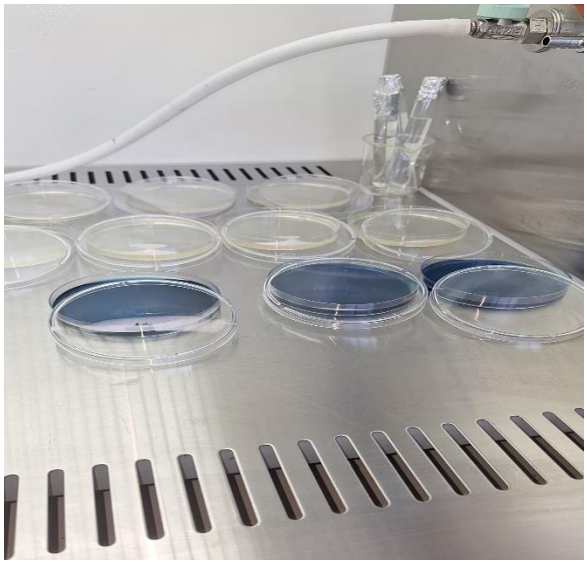


Figure 8: Nile Blue Agar Screening Medium poured into petri dishes.

The colonies were then observed under UV light at a short wavelength of 235 nm, and positive colonies exhibited fluorescence. To confirm the presence of PHA-accumulating bacteria, the positive colonies were further grown in nutrient broth medium and incubated at 30°C for 24 hours. The nutrient broth culture was then inoculated into minimal medium broth containing 0.2% Nile blue solution and incubated at 30°C for 24 hours. A 10 µL sample of the culture was then observed under a fluorescence microscope with a magnification of 400, and positive cells exhibited pink color fluorescence **(Ratnaningrum et *al.*, 2019)**.

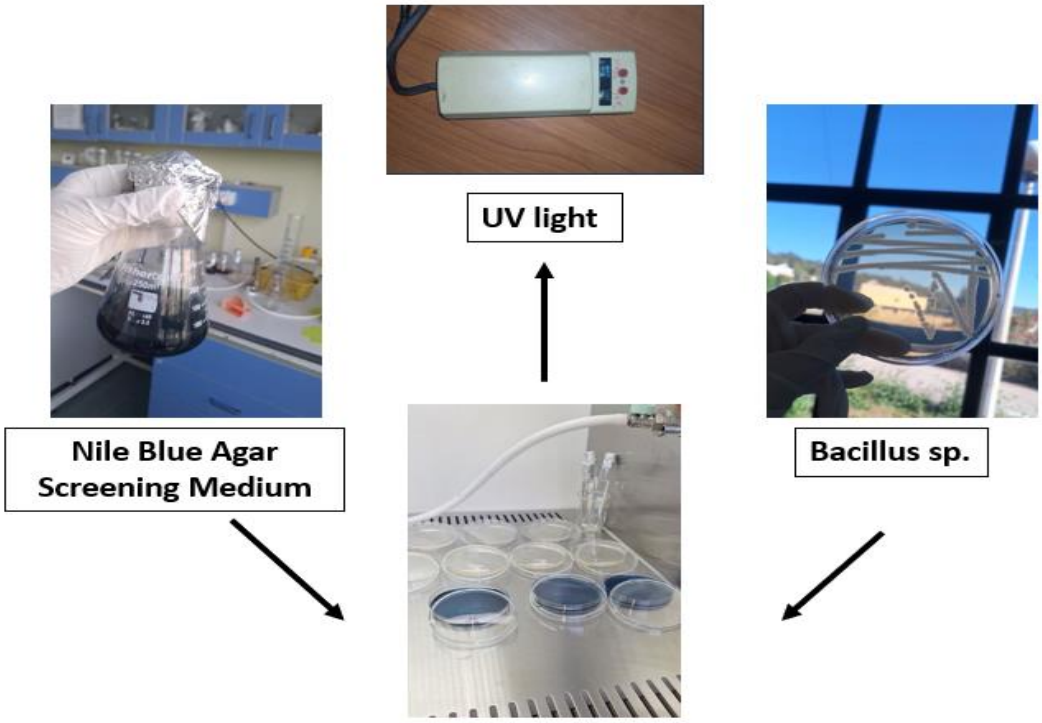


Figure 9 Diagram of the first protocol.

2.4.2- Second protocol

- **Media used and inoculation**

Growth medium (LBB)

containing:

- 10 g/L tryptone;
- 5 g/L yeast extract;
- 10 g/L sodium chloride.

Minimal medium (MM)

The minimal medium (pH 7.0) contained per liter:

- Glucose: 10 g;
- (NH4)2SO4: 1 g;
- MgSO4·7H2O: 0.2 g;
- KH2PO4: 3.6 g;
- Na2HPO4·2H2O: 7.2 g;
- Trace element solution: 1 mL;

→ After mixing all the ingredients together the pH of the medium was adjusted to 7.0 using 0.1N NaOH , the medium was then sterilized at 121℃ for 20 min **(Akdoğan & Çelik, 2018)**.

- **Cell Culture Conditions**

The bacterial culture was grown overnight on Luria-Bertani (LB) broth at 30°C on a rotary shaker with an agitation rate of 200 rpm. The incubated cells were then harvested by centrifugation, and the pelleted cells were resuspended in 50 mL minimal medium. The inoculated flasks were subsequently incubated at 30°C with agitation at 200 rpm on a rotary shaker for a period of 66 hours **(Akdoğan & Çelik, 2018)**.

- **Harvesting and washing Cells**

Harvesting: The cells from the incubated culture were harvested by centrifugation at 2650xg for 10 minutes.

Initial Resuspension: The pelleted cells were resuspended in PBS buffer (See apendix).

First Centrifugation and Supernatant Discard: The resuspended cells were centrifuged, and the supernatant was discarded.

Second Resuspension: The pelleted cells were again resuspended in PBS buffer.

Second Centrifugation and Supernatant Discard: The resuspended cells were centrifuged, and the supernatant was discarded.

- **Detecting PHA-Accumulating Bacteria: A Qualitative Approach using fluorescence microscopy**

To detect the presence of intracellular polyhydroxyalkanoate (PHA) granules, bacterial isolates were prepared on a glass slide, heat-fixed and stained with Nile blue A**(Pei et *al.*, 2023)**. The staining process involved the following steps:

Preparation of the Slide: A small aliquot of the bacterial culture was aseptically transferred to a clean microscope slide.

Heat Fixation: The slide was heat-fixed to ensure the cells were firmly attached to the slide.

Staining with Nile Blue A: A 1% aqueous solution of Nile blue A (see apendix) was applied to the slide for 10 minutes. This allowed the dye to bind to the PHA granules.

Washing and Drying: The slide was washed with water and then 8% aqueous acetic acid for 1 minute to remove excess stain. The slide was then dried with filter paper to remove any remaining moisture.

Covering with a Glass Cover Slip: A glass cover slip was placed over the stained cells to protect them from immersion oil during microscopy.

Microscopy: The stained slide was examined using a fluorescence microscope with an excitation wavelength of approximately 460 nm. PHA granules stained with Nile blue A were visible within cells, with individual granules often visible under the microscope.

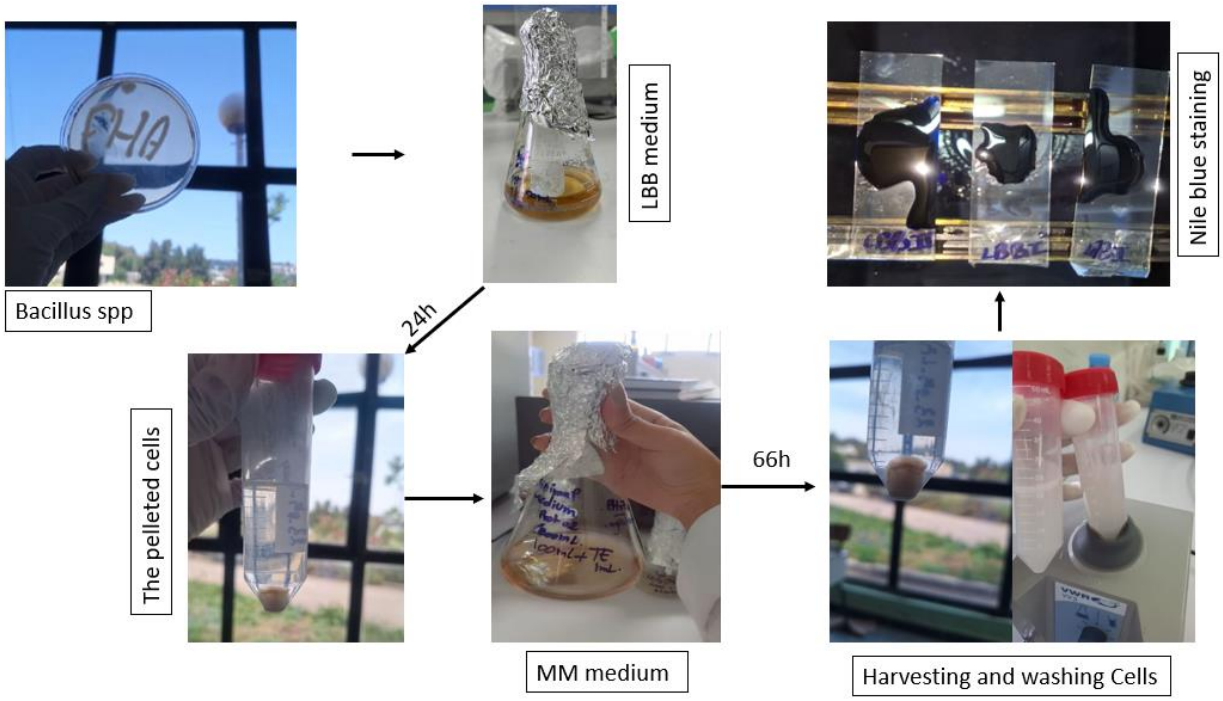


Figure 10 Diagram of the second protocol.

2.4.3- Third protocol

- **Media used**

Growth medium GM

The growth medium used in this protocol consisted of a nutrient-rich medium, LB broth, which contained 10 g/L of tryptone, 5 g/L of yeast extract, and 10 g/L of NaCl. Additionally, 10 g/L of glucose was added to the medium to provide a carbon source for bacterial growth.

After mixing all the ingredients together the pH of the medium was adjusted to 7.0 using 0.1N NaOH, the medium was then sterilized at 121°C for 20 min.

PHA accumulation medium AM

The PHA accumulation medium was designed to promote the accumulation of PHA in bacterial cells. The medium contained the following components:

- 3.57 g/L of Na2HPO4;
- 0.25 g/L of (NH4)2SO4;
- g/L of KH2PO4;
- 0.20 g/L of MgSO4·7H2O;
- 20 g/L of glucose;
- 1 ml trace elements.

The trace elements solution was prepared by dissolving the following components in 100 mL of distilled water:

- 0.60 g of FeCl3·H2O;
- 0.002g of CaCl2·2H2O;
- 0.03 g of H3BO3;
- 0.002 g of CoCl2·6H2O;
- 0.010 g of ZnSO4·7H2O;
- 0.003 g of MnCl2·4H2O;
- 0.003 g of Na2MoO4·2H2O;
- 0.0024 g of NiCl2·6H2O;
- 0.001g of CuSO4·5H2O;

After mixing all the ingredients together the pH of the medium was adjusted to 7.0 using 0.1N NaOH, the medium was then sterilized at 121°C for 20 min.

- **Inoculation and Incubation**

A colony of *Bacillus sp.* was inoculated with 100 mL of sterile GM medium and incubated for 18h at 37°C with 200 rpm shaking to enrich the cell population. The culture from GM medium (25 mL) was centrifuged at 2610g for 10 min. The supernatant was discarded, and the pellet was resuspended in 100 mL of AM medium. The culture was incubated at 37°C for 72 h with 200 rpm shaking.

- **Serial Dilutions and Streaking**

Serial dilutions of $10^{-1}$to 10^{-7} of the cell culture from MG medium were prepared by diluting the culture with 0.9% NaCl solution. The culture from each dilution was streaked on production agar plates (AM medium with 20 g/L of glucose, 1 mL/L of trace elements solution and 1.5% agar) and incubated for 16 h at 37°C.

- **Detecting PHA-Accumulating Bacteria: A Qualitative Approach using FTIR**

The cells from each single colony of the plates incubated previously, were carefully isolated and then subjected to Fourier transform infrared (FTIR) analysis (See apendix), a non-destructive spectroscopic technique that provides detailed information about the molecular structure and chemical composition of the cells, allowing for the identification and characterization of the polyhydroxyalkanoates (PHA) produced by the bacterial isolates **(Manish Kumara et *al.*, 2016)**.

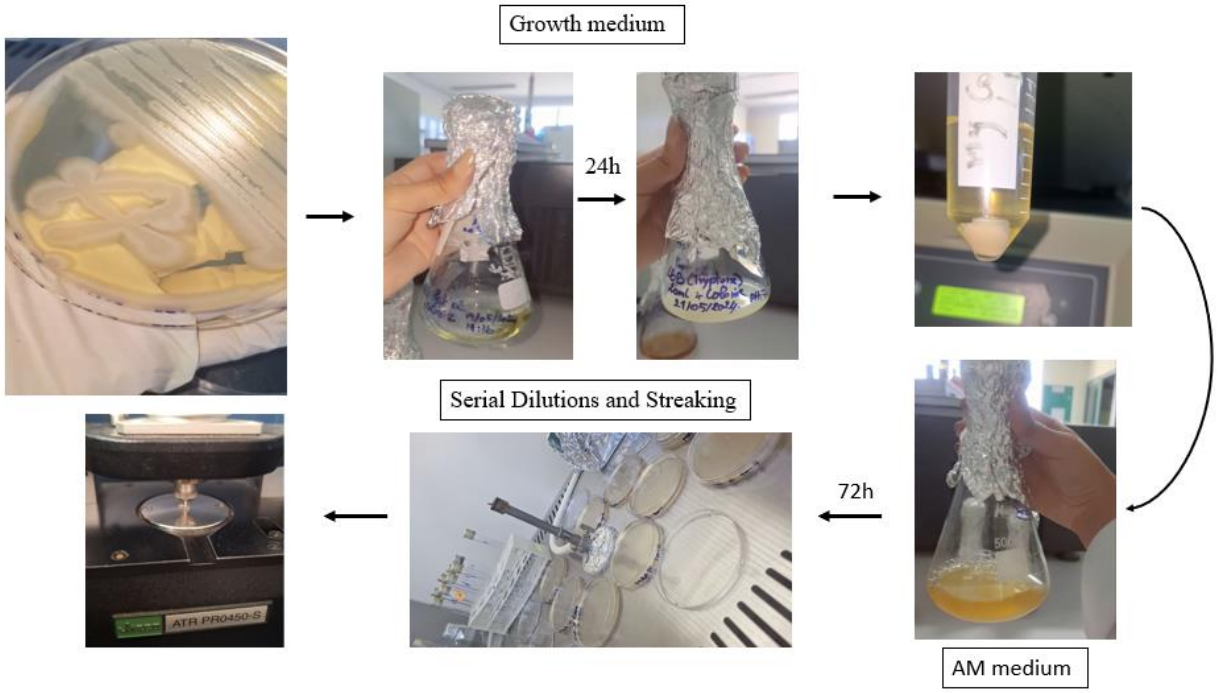


Figure 11: Diagram of the third protocol.

RESULTS

AND

DISCUSSION

3- Results and discussion

This study investigated the production of polyhydroxyalkanoates (PHA) by *Bacillus spp.* using a combination of molecular and biochemical techniques. The results provide an overview of the PHA production capabilities of the bacterial isolates.

3.1- Isolation and purification of *Bacillus* genus

Soil samples were subjected to thermal treatment to selectively isolate *Bacillus species*, which are known to form heat-resistant endospores, after that thermal treatment, a single strain was isolated. To confirm the purity of the isolate, additional purification steps were performed to ensure the homogeneity and absence of contaminants. The purified strain is in principle a *Bacillus sp.* due to its heat resistant.

3.2- Preliminary Identification of *Bacillus* genus

- **Gram staining**

Gram staining was performed to determine the cell wall characteristics of the isolated *Bacillus sp.* The positive result (See table 6) confirmed that they are Gram-positive bacteria, which is consistent with the known characteristics of *Bacillus* species.

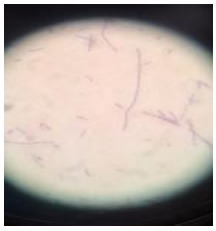
- **Catalase test**

A positive catalase test result was observed, indicated by the immediate formation of bubbles upon the addition of hydrogen peroxide (See table 6). The bubbles were produced due to the rapid breakdown of hydrogen peroxide into water and oxygen gas by the catalase enzyme present in the *Bacillus* species.

- **respiratory type**

The respiratory test revealed that the isolated *Bacillus sp.* is aerobic, meaning they require oxygen for growth and metabolism (See table 6). This is consistent with the known characteristics of *Bacillus* species, which are typically aerobic and require oxygen for growth.

Table 7 : Identification results of isolated bacterium from a soil sample of rocky sites.

Identification test	Result	Figure
Isolation and purification	Well isolated	 **Figure 12:** *Bacillus sp.* Isolated from a soil sample of rocky sites
Gram staining	Gram +	 **Figure 13:** Gram positive *Bacillus sp.*
Catalase	Catalase +	 **Figure 14:** Bubbles observed during the catalase test
Preliminary identification	The results of these tests suggest that the bacterium belongs to the *Bacillus* genus and classified as *Bacillus sp.*	

3- Detecting PHA-Accumulating Bacteria

3-1 First protocol

The qualitative determination of PHA accumulation in the bacterial isolates using Nile blue staining did not yield any positive results. Despite the isolates being grown on carbon-rich nutrient agar media containing Nile blue and incubated at 30°C for 24 hours.

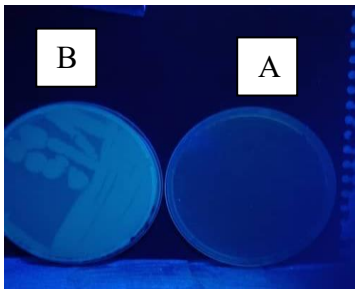


Figure 15 : Observation of the isolated *Bacillus sp* streaking onto Nile bleu screening medium under UV light (A) comparing to staking of the same the isolated colonies of *Bacillus sp.* streaking onto LB medium (B)

no fluorescence was observed under UV light at a short wavelength of 235 nm. This suggests that the bacterial isolates may not be capable of accumulating PHA.

A comparative analysis was conducted between the results obtained in this study and those reported in an earlier investigation that utilized the same experimental approach **(James & Umesh, 2024; Oshiki et *al.*, 2011)**. *Bacillus spp.* are commonly used to produce polyhydroxyalkanoates (PHAs), and the presence of PHA can be detected using Nile blue A dye under UV light with fluorescence (See figure 16)

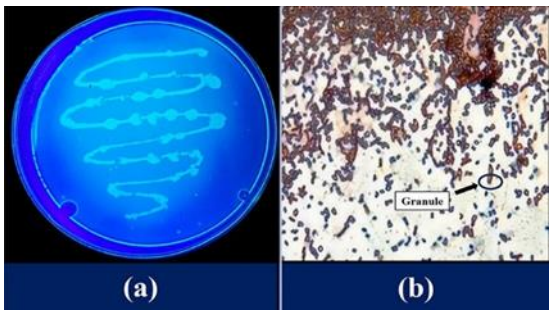


Figure 16 : a Nile blue staining on plate b Sudan black stain showing black PHA granules (inset) in red vegetative cells.

In contrast, our experiment failed to detect PHA, this negative result may be due to several factors, including the high concentration of Nile blue solution in the PHA accumulation medium that inhibited the grow or the absence of PHA-producing genes in the bacterial isolates. Further studies are needed to identify the underlying causes and to optimize the conditions for PHA production.

3-3 Second protocol

In this study, a reliable protocol was developed to detect the presence of intracellular PHA granules in bacterial isolates using Nile blue A staining. The results of this protocol were highly positive, with PHA granules clearly visible within the cells under the microscope.

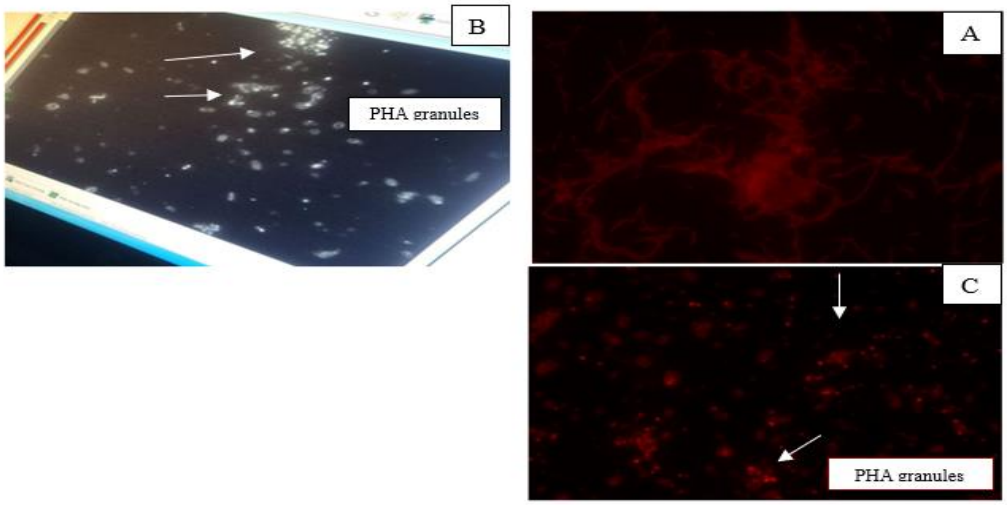


Figure 17 : fluorescent microscope observation of A- *Bacillus sp*. isolated from a soil sample of rocky sites cultured on LB B,C- *Bacillus sp.* isolated from a soil sample of rocky sites harvested from MM after 66h and stained with Nile blue.

The detection of polyhydroxyalkanoates (PHA) in *Bacillus sp.* cultures was achieved using a protocol involving centrifugation **(Akdoğan & Çelik, 2018)** and Nile Blue A staining, followed by observation under a fluorescent microscope. Strong fluorescence was observed, indicating the presence of PHA in the cultures.

This positive result is consistent with a recent study by **(Martínez-Gutiérrez et al., 2018)** that also employed the same protocol and found strong fluorescence, confirming the presence of PHA (See figure 18).

The successful detection of PHA in both studies suggests that the protocol is reliable and effective in detecting PHA in *Bacillus spp.* cultures, with centrifugation ensuring the separation of the bacteria from the medium and Nile blue A staining specifically binding to the PHA, allowing for its detection under the fluorescent microscope.

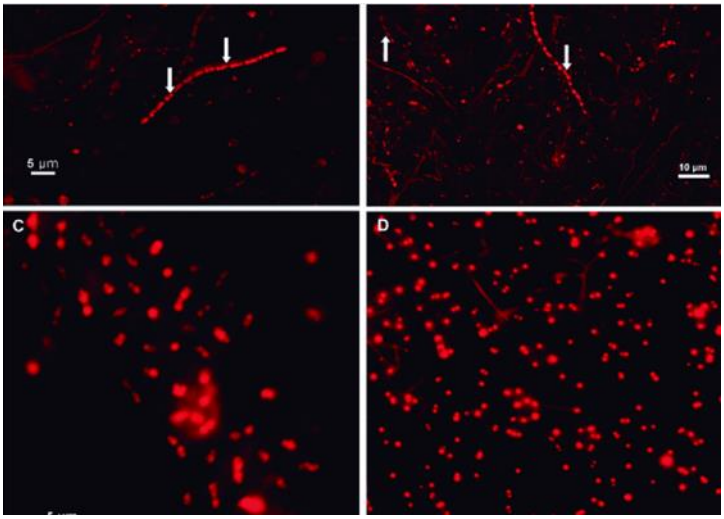


Figure 18: Micrographs of environmental samples and strains showing PHA granules. Nile Red stains of ESSA A1 and ESSA A4 mats (A and B, respectively); inset in (B) displays an additional long filamentous morphotype observed in the respective sample; a representative of the three Halomonas strains (C) and of Paracoccus strain (D). Brightly refractile cytoplasmic inclusions of the representative Halomonas (E), Paracoccus (F) and Staphylococcus (G) strains. Sudan Black stain of Planomicrobium strain (H). Arrows indicate presumptive PHA granules. Full-size DOI: 10.7717/peerj.4780/fig-1.

The Nile blue A dye effectively bound to the PHA granules, allowing for their detection with high sensitivity and specificity. The protocol was able to accurately identify PHA-producing bacteria, which is crucial for various applications such as biotechnology and environmental monitoring.

The Nile blue A staining protocol used in this study has several advantages, including its high sensitivity and specificity for detecting PHA granules. It is also relatively simple and rapid, making it a practical choice for routine screening of bacterial isolates. However, it does require the use of specialized equipment, such as an epifluorescence microscope.

3-2 Third protocol

The results of this study demonstrate the successful production of polyhydroxyalkanoates (PHAs) by *Bacillus sp.* using a fermentation medium. The fermentation process was optimized to achieve a maximum PHA production after 72 hours. The produced PHA was characterized using Fourier Transform Infrared (FTIR) spectroscopy, which revealed a strong absorption peak at 1633 cm^{-1}, indicating the presence of ester functional groups characteristic of PHAs. due to its ability to detect specific absorption peaks characteristic of PHA molecules **(Manish Kumara et al., 2016)**.

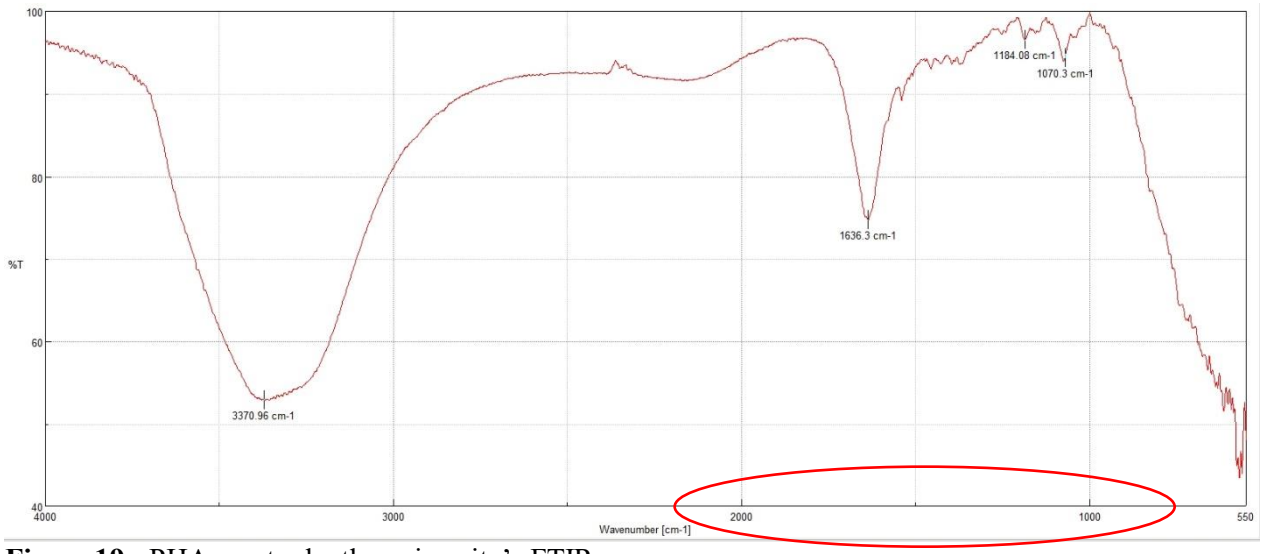


Figure 19 : PHA spectra by the university's FTIR.

These findings are consistent with previous research on PHA production by *Bacillus sp.* using similar fermentation conditions (See figure 20).

The FTIR spectrum of the produced PHA shows a strong absorption peak between 2000 and 1500 cm^{-1}which is typical of PHAs and confirms the successful production of these bioplastics.

The results also demonstrate the effectiveness of the fermentation medium in supporting the growth and PHA production by *Bacillus sp.*

The results of this study are significant because they demonstrate the potential of *Bacillus sp.* as a microbial source for PHA production.

The use of FTIR spectroscopy to detect PHA production is a novel approach that offers several advantages over traditional methods, including increased sensitivity and specificity. The results of this study also highlight the importance of optimizing fermentation conditions to achieve maximum PHA production.

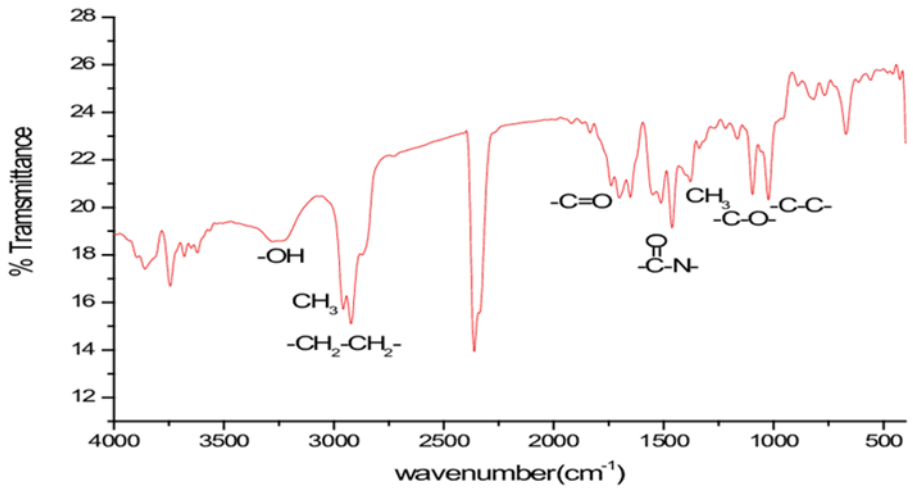


Figure 20*:* FT-IR spectra of PHA synthesized by *Bacillus sp.* ISTC1. Confirmatory Analysis of PHA Production GC-MS analysis of PHA and lipids: The in situ transesterified samples of *Bacillus sp.* ISTC1, in the GC-MS profile depicted the presence of PHA, 3-hydroxyvalerate (RT 16.35) and Fatty Acid Methyl Esters (FAMES) such as Hexadecanoic acid, methyl ester, Pentanoic acid,Cyclopentaneundecanoic acid, methyl ester, 13-Docosenoic acid, methyl ester, Eicosanoic acid, methyl ester within the cells. The results also confirm that bacterial production of 3-hydroxyvalerate, a monomeric unit of poly (3-hydroxyvalerate) (PHV) which is a non-PHB homopolymer of medium chain length (mcl-PHA). There have several reports of production of PHB from bacteria. While, 3-hydroxybutanoic acid provides stiffness, 3-hydroxypentanoic acid promotes flexibility to the back bone of the polymer, which is made up of carbon and oxygen atoms.

CONCLUSION

4- Conclusion and perspectives

In conclusion, the present study aimed to develop and evaluate three protocols for the qualitative determination of polyhydroxyalkanoate (PHA) accumulation in bacterial isolates preliminary identified as *Bacillus sp.* The first protocol, utilizing Nile blue staining, failed to detect PHA accumulation in the isolates, suggesting that the isolates may not be capable of accumulating PHA or that the conditions for PHA production were not optimal. The second protocol, employing Nile blue A staining, successfully detected PHA granules within the cells and to confirm that production with a more specific approach, the third protocol, involved the use of FTIR spectroscopy. The FTIR analysis revealed a strong absorption peak at 1633 cm^{-1} indicating the presence of ester functional groups characteristic of PHAs.

These results demonstrate the successful production of polyhydroxyalkanoates (PHAs) by *Bacillus sp.* using a fermentation medium, highlight the effectiveness of Fourier Transform Infrared (FTIR) spectroscopy and fluorescence microscopy in detecting and identifying PHA-producing bacteria, providing a reliable and accurate method for monitoring PHA production. In contrast, the use of UV light to detect PHA production was found to be unreliable and did not provide a consistent or accurate result. Therefore, FTIR spectroscopy and fluorescence microscopy are recommended as a more reliable and effective methods for detecting PHA production

The production of bioplastics by microorganisms holds immense promise for transforming the plastics industry and mitigating its environmental impact, with numerous prospectives for further research and development in this rapidly evolving field.

The molecular identification of the isolated strain is a crucial step in understanding the taxonomic classification of the isolate, which can be achieved through techniques such as 16S rRNA gene sequencing. Moreover, understanding the PHA production mechanism involves elucidating the PHA metabolic pathway. Additionally, scaling up PHA production by the isolate to enhance PHA yields and reduce production costs. Furthermore, the use of biomass waste as a source of carbon instead of glucose can significantly reduce the cost and environmental impact of PHA production.

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Abstract

The world is facing a severe plastic pollution crisis, with devastating effects on the environment and human health. Bioplastics, produced from certain bacteria, offer a promising solution to this problem. This study aimed to investigate the production of PHA by sporulating bacteria, *Bacillus sp.*, isolated from soil of rocky sites in the region of Ouled Rahmoun, Constantine.. PHA production is typically favored by nutrient-limiting conditions, such as low levels of nitrogen and phosphorus, and high levels of carbon sources like glucose or fructose. This is because PHA synthesis occurs intracellularly, and the bacteria need to store energy and carbon in the form of PHA granules when they are unable to grow due to limited nutrients. The soil sample from rocky sites yielded after a thermal treatment at 80°C a pure culture expected to be a *Bacillus sp.* In addition to that a preliminary identification tests were conducted on the isolated culture, revealing a strain with a white color, rod-shaped morphology, and Gram-positive staining and a catalase activity, consistent with its classification as a member of the *Bacillus* genus. To produce and detect PHA, specific protocols were employed. Initially, the *Bacillus sp.* was inoculated onto Nile blue agar screening medium and incubated at 30°C for 24 hours. However, this protocol failed to detect PHA using UV light to observe fluorescence in petri dishes. Consequently, a second protocol was implemented, involving fluorescence microscopy to visualize granules within cells after centrifugation and harvesting from minimal medium following 66 hours of incubation at 200 rpm. This protocol yielded positive results. To further confirm the presence of PHA, a third protocol was performed, where the isolated culture was incubated in PHA accumulation broth medium for 72 hours at 200 rpm, then streaked onto PHA accumulation agar medium and analyzed using Fourier Transform Infrared (FTIR) spectroscopy. The FTIR spectrum exhibited a wavelength of 1633 cm-1, characteristic of the ester functional groups present in PHA. The results of this study demonstrate the potential of *Bacillus sp.* to produce PHA, a biodegradable and renewable plastic. The study highlights the importance of developing robust and reliable methods for screening and characterizing PHA-accumulating *Bacillus sp.* with potential applications in bioplastics production. Further research is needed to develop more efficient and scalable methods for PHA production, as well as to explore the potential applications of PHA in various industries.

Key words : PHA , Polyhydroxyalkanoate , *Bacillus.*, bacteria , bioplastic , plastic pollution.

Résumé

Le monde est confronté à une grave crise de la pollution plastique, qui a des effets dévastateurs sur l'environnement et la santé humaine. Les bioplastiques, produits à partir de certaines bactéries, offrent une solution prometteuse à ce problème. Cette étude a pour but d'étudier la production de PHA par des bactéries sporulantes, *Bacillus sp.,* isolées du sol de sites rocheux dans la région d'Ouled Rahmoun, Constantine. La production de PHA est généralement favorisée par des conditions limitant les nutriments, telles que de faibles niveaux d'azote et de phosphore, et des niveaux élevés de sources de carbone comme le glucose ou le fructose. En effet, la synthèse des PHA se fait au niveau intracellulaire et les bactéries ont besoin de stocker de l'énergie et du carbone sous forme de granules de PHA lorsqu'elles sont incapables de se développer en raison d'un manque de nutriments. L'échantillon de sol provenant de sites rocheux a produit, après un traitement thermique à 80°C, une culture pure censée être un *Bacillus sp.* En outre, des tests d'identification préliminaires ont été effectués sur la culture isolée, révélant une souche de couleur blanche, une morphologie en forme de bâtonnet, une coloration Gram positive et une activité catalase, compatible avec sa classification en tant que membre du genre *Bacillus*. Des protocoles spécifiques ont été utilisés pour produire et détecter le PHA. Dans un premier temps, le *Bacillus sp.* a été inoculé sur un milieu de criblage gélosé au bleu de Nil et incubé à 30°C pendant 24 heures. Cependant, ce protocole n'a pas permis de détecter la PHA en utilisant la lumière UV pour observer la fluorescence dans les boîtes de Petri. Par conséquent, un deuxième protocole a été mis en œuvre, impliquant la microscopie à fluorescence pour visualiser les granules à l'intérieur des cellules après centrifugation et récolte du milieu minimal après 66 heures d'incubation à 200 tours par minute. Ce protocole a donné des résultats positifs. Pour confirmer la présence de PHA, un troisième protocole a été mis en œuvre : la culture isolée a été incubée dans un bouillon d'accumulation de PHA pendant 72 heures à 200 tours/minute, puis étalée sur un milieu gélosé d'accumulation de PHA et analysée par spectroscopie infrarouge à transformée de Fourier (FTIR). Le spectre FTIR présentait une longueur d'onde de 1633 cm-1, caractéristique des groupes fonctionnels ester présents dans la PHA.Les résultats de cette étude démontrent le potentiel de *Bacillus sp.* à produire du PHA, un plastique biodégradable et renouvelable. L'étude souligne l'importance de développer des méthodes robustes et fiables pour cribler et caractériser les *Bacillus sp.* accumulant le PHA et ayant des applications potentielles dans la production de bioplastiques. Des recherches supplémentaires sont nécessaires pour développer des méthodes plus efficaces et évolutives pour la production de PHA, ainsi que pour explorer les applications potentielles du PHA dans diverses industries.

Mots clés : PHA, Polyhydroxyalkanoate, *Bacillus*, bactéries, bioplastique, pollution plastique.

ملخص

يواجه العالم أزمة تلوث بلاستيكية حادة، لها آثار مدمرة على البيئة وصحة الإنسان. تقدم اللدائن الحيوية المنتجة من بكتيريا معينة حلاً واعداً لهذه المشكلة. هدفت هذه الدراسة إلى التحقيق في إنتاج "PHA" بواسطة بكتيريا "*Bacillus sp.*" المعزولة من تربة المواقع الصخرية في منطقة أولاد رحمون بقسنطينة. عادةً ما يكون إنتاج PHA مفضلًا في الظروف التي تحد من المغذيات، مثل انخفاض مستويات النيتروجين والفوسفور، ومستويات عالية من مصادر الكربون مثل الجلوكوز أو الفركتوز. ويرجع ذلك إلى أن تخليق PHA يحدث داخل الخلايا، وتحتاج البكتيريا إلى تخزين الطاقة والكربون في شكل حبيبات PHA عندما تكون غير قادرة على النمو بسبب محدودية العناصر الغذائية. وقد أسفرت عينة التربة المأخوذة من المواقع الصخرية بعد المعالجة الحرارية عند 80 درجة مئوية عن مستنبت نقي يتوقع أن يكون من بكتيريا العصيات العصوية. بالإضافة إلى ذلك، أجريت اختبارات تعريف أولية على البكتيريا المعزولة كشفت عن سلالة ذات لون أبيض ومورفولوجيا على شكل عصا ، وصبغة إيجابية الجرام ونشاط ايجابي للكاتلاز، بما يتفق مع تصنيفها كعضو في جنس العصيات. لإنتاج وتحفي PHA، تم استخدام بروتوكولات محددة. في البداية، تم تلقيح بكتيريا *Bacillus sp.* في وسط فحص Nile blue agar وحضنت في درجة حرارة 30 درجة مئوية لمدة 24 ساعة. ومع ذلك، فشل هذا البروتوكول في الكشف عن PHA باستخدام الأشعة فوق البنفسجية لمراقبة الفلورة في أطباق بتري. وبناءً على ذلك، تم تنفيذ بروتوكول ثانٍ يتضمن الفحص المجهري الفلوري لتصور الحبيبات داخل الخلايا بعد الطرد المركزي والحصاد من Minimal Medium بعد 66 ساعة من الحضانة عند 200 دورة في الدقيقة. أسفر هذا البروتوكول عن نتائج إيجابية. ولزيادة تأكيد وجود PHA، تم تنفيذ بروتوكول ثالث، حيث تم تحضين البكتيريا المعزولة في وسط محفز لانتاج PHA لمدة 72 ساعة عند 200 دورة في الدقيقة، ثم تم نثرها على الوسط المحفز الصلب وتحليلها باستخدام التحليل الطيفي بالأشعة تحت الحمراءFTIR). أظهر طيف الأشعة تحت الحمراء التحويلية (FTIR) طولًا موجيًا يبلغ 1633 سم-1، وهو ما يميز المجموعات الوظيفية للإستر الموجودة في PHA. تُظهر نتائج هذه الدراسة قدرة البكتيريا *Bacillus sp.* على إنتاج PHA، وهو بلاستيك قابل للتحلل الحيوي ومتجدد. وتسلط هذه الدراسة الضوء على أهمية تطوير طرق قوية وموثوقة لفحص وتوصيف PHA المتراكم من البكتيريا *Bacillus sp.* مع التطبيقات المحتملة في إنتاج البلاستيك الحيوي. هناك حاجة إلى إجراء المزيد من البحوث لتطوير طرق أكثر كفاءة وقابلة للتطوير للإنتاج، وكذلك لاستكشاف التطبيقات المحتملة له في مختلف الصناعات.

Appendix

LB medium

Luria-Bertani (LB) medium was used for the growth and maintenance of bacterial isolates. The composition of LB medium per liter is as follows **(Bertani, 1951)**:

- Tryptone: 10 g
- Yeast extract: 5 g
- Sodium chloride (NaCl): 10 g

The components were dissolved in distilled water and the pH was adjusted to 7.0 using 1 M sodium hydroxide (NaOH) or 1 M hydrochloric acid (HCl). The medium was then sterilized by autoclaving at 121℃ for 15 minutes.

1% Nile blue solution

A 1% Nile blue solution was prepared by dissolving 1 g of Nile blue A in 100 mL of distilled water. The solution was mixed thoroughly until the dye was completely dissolved. The prepared solution was stored in a dark bottle at room temperature and protected from light.

Figure21: 1% Nile blue solution

Preparation of PHOSPHATE BUFFER SALINE (PBS)

- 8 grams of sodium chloride (NaCl)
- 0.2 grams of potassium phosphate monobasic (KH2PO4)
- 1.44 grams of sodium phosphate dibasic (Na2HPO4)
- 1 liter of distilled water

The solution is passed through a 0.22 µm filter to remove any impurities and is stored at room temperature or in the refrigerator for up to 1 week.

FTIR Analysis

Fourier Transform Infrared (FTIR) spectroscopy was used to characterize the chemical composition and functional groups of the bacterial isolates. The FTIR analysis was performed using a Thermo Scientific Nicolet iS10 FTIR spectrometer equipped with a Smart iTR attenuated total reflectance (ATR) accessory. The samples were prepared by placing a small amount of the bacterial cells directly on the ATR crystal. The FTIR spectra were collected in the range of 4000-400 cm-1 with a resolution of 4 cm-1 and 32 scans per sample.

The obtained FTIR spectra were analyzed using the OMNIC software (Thermo Scientific). The characteristic peaks were identified and compared with reference spectra to determine the presence of PHA and other functional groups in the bacterial cells.

Figure 22 : FTIR spectroscope at the university of *Frères Mentouri Constantine1*

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Recherche topic : Contribution to the selection of spore-forming bacteria capable of producing bioplastics

Abstract :

The world is facing a severe plastic pollution crisis, with devastating effects on the environment and human health. Bioplastics, produced from renewable resources, offer a promising solution to this problem. One of the most promising bioplastics is polyhydroxyalkanoates (PHA), produced by certain bacteria. This study aimed to investigate the production of PHA by sporulating bacteria *Bacillus sp.* isolated from soil of rocky sites in the region of Ouled Rahmoun, Constantine. *Bacillus sp.* are known for their ability to produce PHA, a biodegradable and renewable plastic. PHA production is typically favored by nutrient-limiting conditions, such as low levels of nitrogen and phosphorus, and high levels of carbon sources like glucose or fructose. This is because PHA synthesis occurs intracellularly, and the bacteria need to store energy and carbon in the form of PHA granules when they are unable to grow due to limited nutrients. The study used three protocols to detect PHA within the cells: the first used Nile blue A as a dye in the production medium itself, but failed due to Nile blue A high concentration; the second did not use any dye and the produced PHA was characterized using Fourier Transform Infrared (FTIR) spectroscopy; and the third used centrifugation after 66h of incubation in the production medium and then a fluorescence microscopy using Nile blue A, which showed clearly the PHA granules within the cells. The results of this study demonstrate the potential of *Bacillus sp.* to produce PHA, a biodegradable and renewable plastic. The study highlights the importance of developing robust and reliable methods for screening and characterizing PHA-accumulating *Bacillus sp.* with potential applications in bioplastics production. Further research is needed to develop more efficient and scalable methods for PHA production, as well as to explore the potential applications of PHA in various industries.

Key words : PHA , Polyhydroxyalkanoate , *Bacillus ,* bacteria , bioplastic , plastic pollution.

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