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Isolation and screening of Chitinolytic bacteria and their potential in biocontrol of phytopathogenic microorganisms

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Dedication

I dedicate this work

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List of abreviations

PBS : Phosphate-buffered saline

ANOVA : Analysis of variance

PCA : Plate count agar

HCl : Hydrochloric acid

$K_2H_2PO_4$: Dipotassium phosphate

KH_2PO_4 : Potassium dihydrogen phosphate

$MgSO_47H_2O$: Magnesium sulfate heptahydrate

NaCl : Sodium chloride

KCL : Potassium Chloride

Na_2HPO_4 : Sodium phosphate dibasic

NH_4Cl : Ammonium chloride

CMC : CarboxyMethylCellulose

CaCl $2H_2O$: Calcium chloride dehydrate

KNO_3 : Potassium nitrate

$MgSO_4$: Magnesium sulfate

$CaCl_2$: Calcium chloride

$FeCl_3$: Iron trichloride

KI : Potassium iodide

NH_3 : Ammonia

HCN : Hydrogen cyanide

PDA : Potato dextrose agar

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Introduction

It is expected that by 2050, there will be approximately 9.1 billion people on Earth. Thus, an approximate 70% increase in agricultural food production is necessary to meet the demands of the world's expanding population (Grethe et al., 2011).

Plants are currently facing numerous biotic and abiotic stress factors caused by global warming and environmental pollution, leading to a significant reduction in their yield. Biotic stress factors involve fungi, bacteria, virus, nematodes weeds, and insects (Moustafa-Farag et al., 2019). Among them, fungal pathogens are the most severe limiting factor for crop production worldwide. Chemical fungicides continue to be used extensively as the primary strategy for disease control. However, their widespread use not only incurs high costs but also leads to the accumulation of hazardous pollutants in both environment and humans (Raju et al., 2003).

Due to the negative impacts of chemical fungicides on non-target organisms (Köhl et al., 2019), biological control techniques have emerged as significant tools for disease management. Various studies provide evidence that certain microorganisms effectively inhibit the growth of pathogenic species (Panth et al., 2020).

Among these organisms, chitinolytic bacteria have been identified as being widespread in different environments, such as soil, water and living organs (Krithika and Chellaram, 2016). They have distinct characteristics and properties depending on their location (Herdyastuti et al., 2012). These bacteria have recently been developed for use as a biocontrol agent against pathogenic fungi (Rathore and Gupta, 2015; Veliz et al., 2017) and to improve soil fertility (Sharma et al., 2010**;** Herdyastuti et al., 2012) by substituting chemical pesticides that cause damage to the ecosystem (Veliz et al., 2017)**.**

These bacteria generate enzymes called chitinases, their role is to break down chitin and use it as a source of energy (Downing and Thomson, 2000)**.** chitin is the structural component found in many organisms: molluscs, crustaceans, algae, fungi and marine (Patel and Goyal, 2017). It is the second most common polymeric polysaccharide, exhibiting excellent biocompatibility and degradability. This polysaccharide is regarded as a biological source for biofuels and other high-value functional compounds, potentially replacing chemical energy sources (Yadav et al., 2019).

Chitinases have been reported to actively inhibit the growth of various phytopathogenic fungi by degrading their chitin cell wall (Downing & Thomson, 2000; Brzezinska et al., 2013). In addition to their use in the biological control of pathogenic fungi and insects, it has also been demonstrated that these enzymes can be used for marine waste management (Poria et al., 2021; Dhole et al., 2021).

In this study, our focus is on investigating the chitinase enzymes by rhizospheric bacterial isolates obtained from Fava bean, potato, Onion and Turnip rhizosphere. Additionally, we aim to assess the potential of these isolates to control phytopathogenic fungi *in vitro* and *in vivo.*

The manuscript is divided into three parts:

- The first part is devoted to a literature review, including an introduction to biological control, the various biocontrol agents, chitinase-producing bacteria and their impact on pathogenic microorganisms.
- The second part describes the methodology used in this study,
- The third part is reserved to results and discussion.

Finally, a general conclusion summarizes the main findings of this study.

1. Biological Control

The controversies over the use of chemical pesticides in agriculture continue to heighten public awareness of the current effects these practices have on the environment and human health (Heimpel & Mills, 2017). Several studies suggest biological control approaches as an alternative that could prove to be a more potent and environmentally-friendly solution.

1.1. Definition

Biological control refers to the direct or indirect suppression of pest and weed populations that have an effect on the environmental, human health and food safety, through microorganisms (Heimpel & Mills, 2017). It is also defined as the use of an organism to reduce the population density of another organism and thus includes the control of animals, weeds and diseases (Bale et al., 2008).

In entomology, it refers to using living predatory insects or entomopathogenic nematodes to suppress pest populations, in plant pathology, it involves employing microorganisms to inhibit diseases and control pathogenic weeds. The living organism used in this approach is termed a "biological control agent" (Pal and Gardener, 2006).

The biocontrol agents use diver mechanisms such as predation, parasitism, pathogenicity or competition to reduce the populations of invasive and harmful species (Boivin, 2001; Heimpel & Mills, 2017).

1.2. History and development

Biological control has a long history, dating back to the domestication of the cat to control rodents that threatened stored food supplies. The use of beneficial insects, such as ladybirds, to manage aphid infestations has been known to good gardeners for a long time. Likewise, the protection traditionally afforded to certain insectivorous birds, such as the active installation of nest boxes, is part of what is now known as the "promotion" of natural enemies (Jourdheuil et al., 1991).

In the 19th century, pigs were used to destroy the overwintering forms of insects that ravage forests and poultry in fields and the end of this century saw the use of auxiliary insects against harmful insects and weeds (Jourdheuil et al., 1991).

The first scientific study to trigger modern biological control was carried out by the American entomologist Charles Valentine Riley. The *Icerya purchasi* scale insect was introduced from Australia to California, causing havoc in citrus orchards. In response, Riley was sent in a mission to Australia to find natural enemies of the scale insect, and brought back the ladybird *Novius cardinalis*. In less than two years, as a result of the introduction of this ladybird predator, mealybug populations were reduced to an economically viable level (Sforza et al., 2008).

Since then, biological control has been widely adopted worldwide as an ecological and sustainable alternative to chemical pesticides. While challenges persist, biological control continues to be an important component of integrated pest management programs globally.

1.3. Biocontrol agents

1.3.1. Predatory insects

Different species of arthropodes are thought to be natural predators of ravagers, using a variety of predatory strategies to manage ravageur populations (DeBach et al., 1991). In a variety of cultures, species such as *Harmonia axyridis* have been successful in combating aphids (Koch, 2003), while other species such as *Chrysoperla carnea* have achieved significant commercial success as control agents, particularly for aphids (Tauber et al., 2000; Turquet et al., 2009). The larvae of syrphes (*Diptera: Syrphidae*) have also been identified as natural biological control agents (Dunn et al., 2020).

1.3.2. Predatory mites

Mites are predatory arthropods found in soil, plants and compost heaps, mainly used in gardens and greenhouses. They attack all stages of phytophagous mites, eggs and immature insects. With 8 legs and rapid reproductive and development cycles, they are able to follow fluctuations in pest populations, making them highly effective biocontrol agents (Ellis and Atthowe, 1996; Flint et al., 1998). Species such as *Amblyseius swirskii*, *Phytoseiulus*

persimilis and *Neoseiulus cucumeris* are the most widely used, accounting for two-thirds of the total market for arthropod biocontrol agents (Van Lenteren, 2012).

1.3.4. Entomopathogenic nematodes

Entomopathogenic nematodes are effective biocontrol agents against a multitude of anthropoid pests, such as leafminers *(Liriomyza spp.)*, carpenter beetle *(Prionoxystus robiniae)* and hummingbird bugler *(Hemaris thysbe)*, in contrast to plant pathogenic nematodes which can be considered as pests (Helder et al., 2015)

In most cases, these nematodes can cause death on their own, but they generally have a mutualistic relationship with entomopathogenic bacteria (Dillman and Sternberg, 2012). The most extensively studied species are *Steinernema* with *Xenorhabdus* and *Heterorhabditis* with *Photorhabdus* (Hazir et al., 2004). At the third larval stage, known as the infective juvenile, these parasites enter their hosts and release a bacterial load which, in turn, generates a toxic mixture of secondary metabolites that kill the insect pest (DeBach et al., 1991; Dillman and Sternberg, 2012).

1.3.4. Bacteria

Genera such as *Agrobacterium, Alcaligenes, Arthrobacter, Bacillus, Enterobacter, Erwinia, Pseudomonas* and *Rhizobium* have all demonstrated the ability to protect plant against pathogenic bacteria and fungi (Schaechter, 2009). Numerous studies have demonstrated the efficacy of these microorganisms in biological control, highlighting their significant role in plant protection and their potential to enhance agricultural yields (Niranjan et al., 2005). These genera use different mechanisms to suppress plant diseases, fluorescent *Pseudomonas* which are efficient colonizers of the plant surface and the endosphere can restrict plant pathogens by siderophores or by nutritional competition (O'Sullivan and O'Gara, 1992; Ellis et al., 1999; Lugtenberg et al., 2001).

Bacillus species are one of the most widely used bacteria as biopesticides because of their availability in soil and on plant surfaces, their ability to form endospores and, mainly their capacity to produce bacteriocins, cyclic lipopeptides and hydrolytic enzymes such as chitinase and protease (McSpadden Gardener, 2004; Ongena and Jacques, 2008; Guleria et al., 2016).

1.3.5. Fungi

The use of entomopathological fungi instead of chemical pesticides has proved to be a new alternative for pest control (Waqar Islam et al., 2021). These fungi are parasitic micro-organisms that can infect arthropods through the cuticle, unlike bacteria and viruses. They are classified into different genera such as *Beauveria, Cordyceps* (Isaria), *Hirsutella, Metarhizium* and *Nomuraea* (Leger and Wang, 2010; Humber, 2012).

Beauveria bassiana is an entomopathogenic fungus naturally occurring in soil (Rohrlich et al., 2018). It has been shown to effectively control a variety of pests, including termites, malaria-transmitting mosquitoes, whiteflies, and more recently, pecan aphids (Lovett and Leger, 2018; Ramakuwela et al., 2020). Other species such as *Cordyceps fumosorosea* have demonstrated a powerful antagonistic effect against whiteflies (Sandhu et al., 2012).

1.3.6. Viruses

In the quest for new alternatives to chemical pesticides, viruses have proven effective in suppressing a wide range of pests, particularly anthropod pests. Both RNA and DNA viruses have demonstrated this capability.

Baculoviruses have been isolated from over 700 species of arthropods, these viruses are able to provoke large epizootics among these species especially lepidopteran and sawfly pests, thus being used as biological agents mostly applied using spray application technology (Erlandson, 2008).

1.4. Biological control of phytopathogenic fungi

1.4.1. Phytopathogenic fungi

70 to 80% of plant diseases are caused by phytopathogenic fungi. They have adverse effects on crop growth, yield and productivity. However, some diseases are not caused by a single pathogen, but rather by the synergy of several pathogens (Shang et al., 2016).

Fungi have developed numerous biochemical and mechanical strategies to colonize plants and obtain nutrients. These interactions lead to a wide range of outcomes, ranging from beneficial symbiotic relationships to host death facilitated by the production of secondary

metabolites or enzymes that degrade polymers, acting as virulence factors (Salvatore and Andolfi, 2021).

It's important to know that phytopathogenic fungi can be categorized into two main groups: biotrophic pathogens, which establish intimate interactions with plants and can survive by using living tissue; and necrotrophic pathogens, which destroy tissue to extract nutrients. Additionally, there are hemibiotrophic pathogens, which initially behave as biotrophs before transitioning to necrotrophy (Doehlemann et al., 2017).

Phytopathogenic fungi produce toxins that can play a key role in the development of plant diseases, adversely affecting host plants. These phytopathogenic toxins are mainly low molecular weight secondary metabolites, that can produce specific symptoms such as wilting, growth inhibition, chlorosis, necrosis and leaf spots (Shang et al., 2016). They act mainly on the cell membrane, mitochondria and chloroplasts of host plants, destroying the plant or interfering with its metabolism (Shang et al., 2016). Among the most common phytopathogenic fungi are *Alternaria*, *Fusarium*, *Botrytis*, *Geotrichum*, *Penicillium* and *Sclerotinia* (Azeem et al., 2022).

1.4.2. Microbial Agents Against Phytopathogens fingi

In recent years, the use of actinomycetes as biocontrol agents against phytopathogenic fungi has provided an alternative to the application of synthetic fungicides. Actinomycetes are Gram-positive bacteria and ubiquitous, it has been isolated from different environments, including terrestrial, marine and hypersaline environments, wetlands and plant (Torres-Rodriguez et al., 2022). The main antagonistic mechanisms used by actinomycetes to control phytopathogenic fungi are competition for space and nutrients, production of antibiotics, siderophores, lytic enzymes, volatile organic compounds (VOCs) and induction of host resistance. Actinomycetes also promote plant growth and development through the synthesis of phytohormones (Torres-Rodriguez et al., 2022).

Another biocontrol agent is bacteria, which have been reported as antagonistic microorganisms, in particular strains of the genus *Bacillus*, *Pseudomonas* and *Agrobacterium* (Nowocień and Sokołowska, 2020). Most interactions between antagonistic bacteria and phytopathogens involve inhibition through antimicrobial substances (Raaijmakers et al., 2002), competition for nutrients and/or space (Antoun and Prévost, 2005), inactivation of pathogen germination factors, degradation of pathogenicity factors such as toxins, and some

bacteria have the ability to parasitize and degrade pathogen spores (Whipps, 1997). Plant growth-promoting bacteria (PGPB), are a promising group of bactéria capable of enhancing plant yields and limiting plant pathogens, they could be used as an alternative to limit the use of chemical fungicides (Azeem et al., 2022).

Several fungal species have been employed as biocontrol agents against phytopathogenic fungi, demonstrating their potential in sustainable disease management strategies. Antagonists belonging to the genus *Trichoderma* are among the most commonly isolated soil fungi. They are extensively researched and commercially utilized as biopesticides, biofertilizers, and soil amendments due to their capacity to protect plants and manage pathogen populations across diverse soil environments. Additionally, Trichoderma spp. are known producers of various biologically active substances, such as cell wall-degrading enzymes and secondary metabolites (Vinale et al., 2008).

In addition, Endophytic fungi are micro-organisms that live in a mutualistic relationship within plant tissue without causing pathological symptoms. Fungi obtain protection and nutrients from host plants and, in return, can contribute to their growth and nutrient uptake. Endophytic fungi prevent damage to plants through their ability to release enzymes, antibiotics, hydrogen cyanide and volatile compounds that inhibit the activities of pathogens and induce systemic resistance. Several studies have demonstrated the antagonistic activity of endophytic fungi against phytopathogenic fungi (Nuaimy and Hawar, 2024).

Still in the fungi family, another endophytic fungi, named *Ceriporia lacerate,* is reported recently to be a biological control agent. This white rot fungus grows on living and dead trees in some boreal forests. It uses lignin, cellulose and proteins as sources of carbon and nutrients through the production of extracellular enzymes, such as manganese peroxidase, laccase, lignin peroxidase, cellulase, protease and phosphatase. *C. lacerata* (HG2011) is a newly discovered species isolated from the Jinyun National Forest Park in south-west China. it is mentioned that this fungus has the ability to produce extracellular hydrolases and siderophores, thereby enhancing nutrient uptake by plants. Studies on the antimicrobial potential of *C. lacerata* HG2011 against six pathogenic fungi and two oomycetes revealed that soluble and volatile metabolites produced by this fungus suppressed the growth of all pathogens and induced morphological distortions in mycelia (Yin et al., 2023).

1.5. Mechanisms of biological control

1.5.1. Antibiotic production

Antibiosis is the most effective mechanism used by biocontrol agents to fight against pathogens. It corresponds to the production of antimicrobial compounds to inhibit the growth of pathogens (Johansson, 2003). Actinomycetes produce a class of antibiotics known macrolides, which inhibit the synthesis of fungal proteins. Amphotericin B changes permeability and induces cell lysis by binding specifically to ergosterol in the fungal cell membrane (Torres-Rodriguez, 2022). Weller (2007) has classified several bacterial strains whose production of compounds such as phenazines and DAPG (2,4-diacetylphloroglucinol) is directly linked to the inhibition of pathogen growth. Other microorganisms also produce volatile antibiotics such as hydrogen cyanide, ammonia, 2,3-butanediol, and acetoin (Ahmed et al., 2008).

1.5.2. Competition for space and nutrients

Competition is defined as the consumption or control of access to nutrients, space, or any other factor whose availability is limited. Competition between two or more micro-organisms starts over the same carbon; azote; micro and micro elements, or the same space required for their growth. Competition is an effective biocontrol mechanism when the antagonist is present in sufficient volume and assimilates nutrients more quickly and in greater quantities than the pathogen (Lahlali et al., 2022).

A particular case of competition for nutrients is based on competition for iron. This element is often present in the soil in an insoluble form (ferric iron (Fe^{3+}). Biocontrol agents secrete iron-binding ligands (siderophores), these chelators have a high affinity for sequestering iron from microenvironments, thus depriving phytopathogenic agents of one of their growth factors (Pal and Gardener, 2006; Torres-Rodriguez et al., 2022).

1.5.3. Production of lytic enzymes

The production of lytic enzymes is another mechanism of biological control. The various biological control agents produce lytic enzymes such as chitinase, β-1,3-glucanase and protease, which break down the fungal cell wall, causing a loss of membrane integrity and release intracellular material and lead to cell death (Torres-Rodriguez et al., 2022).

2. Chitinase: An overview of properties and applications

2.1. Chitin

The second most abundant polysaccharide in nature which at least 10 gigatons are being synthesized and degraded each year in the biosphere, chitin is a long-chain polymer of covalent β-(1→4)-linkages forming units of N-acetylglucosamine (it's also referred to as (1-4)-2-acetamido-2-deoxy-ß-D-glucan). There are mainly two forms found in nature: α-chitin (the N-acetylglucosamine chains are aligned in an antiparallel manner) constituent of the nematode and rotifers eggshells, fungi cell walls, hydrozoa calyces, mollusks radulae and the cuticles of arthropods; The β-chitin (the chains are parallelly aligned) found in the peritrophic membranes, cocoons and exoskeletons of insects, cuttlefish bone and the shells of brachiopods and mollusks. γ-chitin, although less common in nature, features a three-chain unit cell arrangement with two chains oriented 'up' and one 'down (Muzzarelli, 1999).

Chitin is not soluble in water and it is only hydrolyzed by acids like HCl to produce colloidal chitin used in the study of chitinases, furthermore chitin is hydrolyzed by chitinase and lysozymes (Blackwell, 1988; Muzzarelli, 1999; Beier and Bertilsson, 2013).

2.2. Chitinase

2.2.1. Overview

Chitinases are glycoside hydrolases that break the ß-l ,4-glycosidic bonds between the N-acetylglucosamine units in chitin. They are found in fungi, bacteria, archaea, rotifers, algae, carnivorous plants and in the digestive tracts of higher animals, the operational mode of chitinase typically follows these steps : (1) cleaving the polymer into water-soluble oligomers; (2) splitting these oligomers into dimers; (3) cleavage the dimers into monomers (Henrissat, 1999; Beier et bertilsson 2013).

2.2.2. Classification

Chitinases can be classified based on amino acid sequence analysis of their catalytic and non-catalytic domains, gene sequence analysis, and their structural characteristics.

2.2.2.1. Amino acid sequence analysis

a. Catalytic domain

Comparisons of chitinases amino acid sequences showed that their catalytic domains can be grouped in two families 18 and 19 (Henrissat, 1999).

Family 18: Containing 180 members mostly found in viruses, eukaryotes and prokaryotes, they all operate through the retention of the anomeric configuration at the cleavage point implying a double displacement mechanism.

Family 19: Containing more than 130 members mainly found in plants, they operate by inverting the anomeric configuration at the site of cleavage.

b. Non-catalytic domains

Similarly, amino acid sequence analysis groups the non-catalytic domains of chitinases (chitin-binding domains) into three families. The first two families contain modules exclusive to chitinases, while the third family includes modules found in cellulases and serine proteases (Henrissat, 1999).

2.2.2.2. Gene sequence analysis

With gene sequencing, chitinases can be classified into 6 classes based on characteristics such as N-terminal sequence, enzyme localization, isoelectric pH, signal peptide presence, and inducer specificity (Patil et al., 2000; Shakhbazau and Kartel', 2008).

Class I: Found strictly in plants, they have leucine or valine- rich signal peptide with vacuolar localization and a cysteine-rich N-terminal.

Class II: Found in plants, fungi and bacteria, they are pathogen-induced chitinases that share sequence similarity with class I but lack the cysteine-rich N-terminal motif.

Class III: They don't share any sequence similarities with class I and II

Class IV: Shares characteristics with class I especially the immunological properties but are considerably smaller in size.

Class V and VI: Each of them has only one representative and is still not well-characterized

2.2.2.3. Structure

a. Endochitinase

Produces soluble, low-molecular-weight multimers of N-acétylglucosamine (GlcNAc) such as chitotetraose, cleavage occurs randomly over the entire length of chitin microfibril (Sahai and Manocha, 1993).

b. Exochitinase

Catalyzes the progressive release of diacetylchitobiose and no monosaccharides or oligosaccharides are formed, cleavage only occurs at the non-reducing end of the chitin microfibril (Sahai and Manocha, 1993). Exochitinases are also subcategorized into two main types:

Chitibiosidases: release the di-acetylchitobiose dimer one by one from the chitin microfibril, resulting in the non-formation of monosaccharide or oligosaccharides.

β-1,4- N-acetylglucosaminidases : split the multimer products of the chitobiosidases and endochitinases into monomers of N-acetylglucoseamine.

2.2.3. Role of chitinase

In nature, chitinases play vatious roles: in algae, higher plants and vertebrates, they serve a defensive role; in microorganisms, they digest chitin or partially hydrolyze the chitinous cell wall to facilitate cell proliferation; they also play a role in parasitism.

In insects and crustaceans, chitinases degrade the chitin in the exoskeleton during the molting (ecdysis) process (Koga et al., 1999). Chitinases also play a significant role in morphogenesis in fungi and contribute to maintaining the balance between carbon and nitrogen in genera (Patil et al., 2000; Thakur et al., 2023).

2.2.4. Applications of chitinase

2.2.4.1. Industrial applications

Production of chitooligosaccharides

Chitooligosaccharides exhibit antibacterial, antioxidant, anti-inflammatory, and prebiotic properties. They also contribute to the formation of root nodules, and serve as elicitors of plant defense. These compounds are produced from chitin by using chitinase; for example,

chitobiose, which is widely used in the food, cosmetic, and pharmaceutical industries, is one of the most common product. Chitinases from *Streptomyces griseus* and *Bacillus* are used for the production of chitooligomers. Aadditionally, Chitinases from *Vibrio alginolyticus* are employed to convert colloidal chitin into chitopentose and chitotriose (Tran et al., 2019; Rani et al., 2020; Poria et al., 2021).

Single cell protein production

Single-cell protein (SCP), is a protein food additive used as as a sustainable alternative to conventional protein sources. Chitinase from *S. marcescens*, *Pichia kudriavzevii*, *Candida tropicalis*, *Saccharomyces cerevisiae*, *Hansenula polymorpha* and *Myrothecium verrucaria* degrades chitin found in marine waste, therby producing unicellular proteins (Revah-Moiseev and Carroad, 1981; S Patil, 2014; Poria et al., 2021).

Protoplast isolation

Protoplasts are valuable tools in the understanding and study of fungi, they are primarily used for cell free extracts and fungal organelles preparation, the main fungi used in the preparation of fungal protoplasts are *T. reesei*, *P. florida*, *A. niger*, and *A. bisporus* (Dahiya et al., 2005; Waghmare et al., 2011; Poria et al., 2021).

2.4.2. Biomedical applications

Chitinases can play a role in the regulation of cellular and humoral immune responses. Helminths and allergens induce the production of acidic mammalian chitinase (AMCase), which in turn initiates protective immune responses against intestinal nematodes. Additionally, bacterial chitinases, such as those from *Lactobacillus rhamnosus*, have been found to exhibit inhibitory properties against the opportunistic pathogen *Candida albicans*. (Vannella et al., 2016; Allonsius et al., 2019; Poria et al., 2021).

2.4.3. Waste management application

Approximately 6 to 8 million tons of marine chitinous waste is generated annually, bacterial and fungal chitinases present a more cost-effective and eco-friendly alternative to the physical or chemical waste management (Patil and Jadhav, 2015; Saini et al., 2020; Poria et al., 2021).

2.4.4. Biocontrol application

Several phytopathogenic insects and fungi possess chitinous exoskeletons or cell walls. Chitinases have been identified for their biocontrol activities against these organisms. For instance, chitinases from *Penicillium ochrochloron* have been shown to effectively control populations of *Helicoverpa armigera* by increasing larval and pupal mortality (Poria et al., 2021).

Application of Chitinolytic microorganisms in biocontrol

While pesticides are essential for protecting monocultures from insects and diseases, they also pose significant environmental and public health risks. This unsustainable agricultural practice cannot persist if it continues to destroy ecosystems in the name of safeguarding our crops

Safer and more environmentally sound, chitinolytic microorganisms are used as an alternative to pesticides as biocontrol agents (Herrera-Estrella and Chet, 1999) for different phytopathogens, mainly by degrading their chitinous cell walls or exoskeletons (Poria et al., 2021). Indeed, many plant chitinases extracted from wheat, barley, and maize exhibit antagonistic activity against fungi by acting as endochitinases (Roberts and Selitrennikoff, 1988). Similarly, *Serratia marcescens*, one of the most studied chitinase-producing bacteria, has been shown to inhibit *Sclerotium rolfsii* (Ordentlich et al., 1988)

Moreover, chitinase producing fungi like *Beauveria bassiana* or *Metarhizium anisopliae*, considered as entomopathogenic, are used to control insect pests. They achieve this by producing chitinolytic and proteolytic enzymes that enable them to penetrate the peritrophic membrane and exoskeletons of insects, which are primarily composed of chitin. (Herrera-Estrella and Chet, 1999; Patil et al., 2000 ; Dukariya and Kumar, 2020).

1. Soil sampling

Four soil samples were collected in March 2024, from different agricultural fields located in Bejaia: Ait Idris Taskriout (36,5665540, 5, 2785297); Akbou (36, 4530316, 4, 5348299); Tizi Ahmed (36, 6548767, 5, 163762) and Adkar (36, 7471292, 4, 7108861). Sampling was conducted in the root zones (rhizospheres) of the following crops, respectively: fava bean, onion, potato, and turnip. The soil collected was placed in sterile vials and transported to the laboratory (Figure 1).

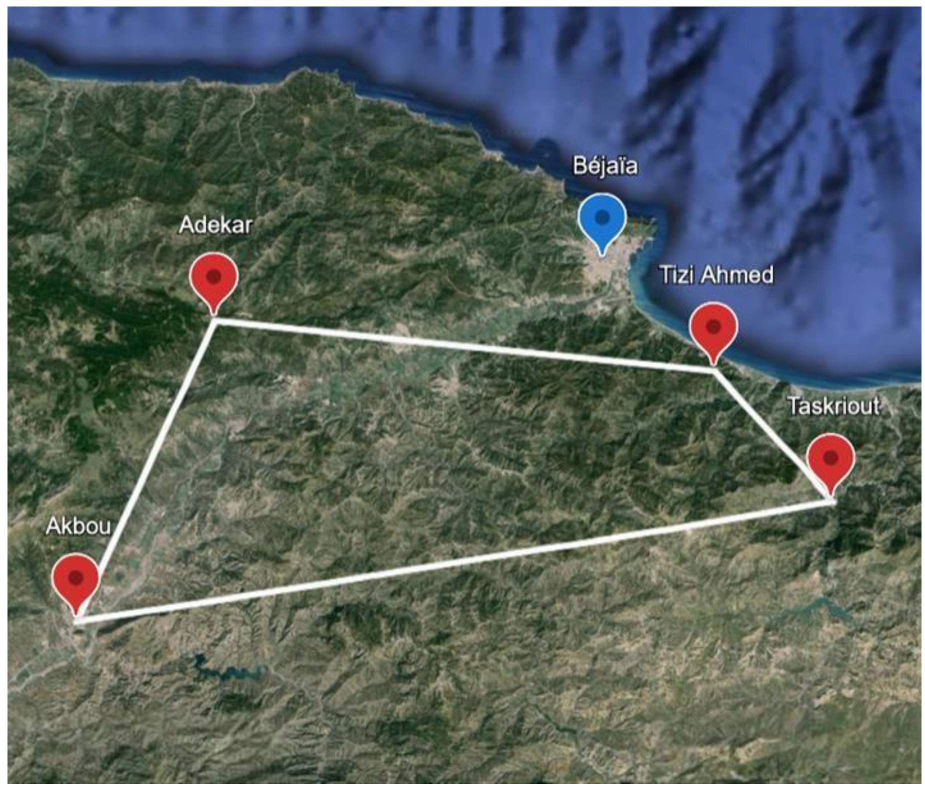

Figure 1 : Sampling locations

2. Isolation and purification of soil bacteria

One gram of each soil sample was suspended in 10 ml PBS (Phosphate-buffered Saline) (Annex I) and 1 ml of each soil solution was serially diluted from 10^{-1} to 10^{-7} in the same broth medium. 1 ml of each dilution was spread on Plat Count Agar (PCA) (Annex II) in duplicate, using the "flooding technique", and then incubated for 24h at 30°C. Finally, successive subcultures of all colonies are performed until getting pure colonies (Figure 2).

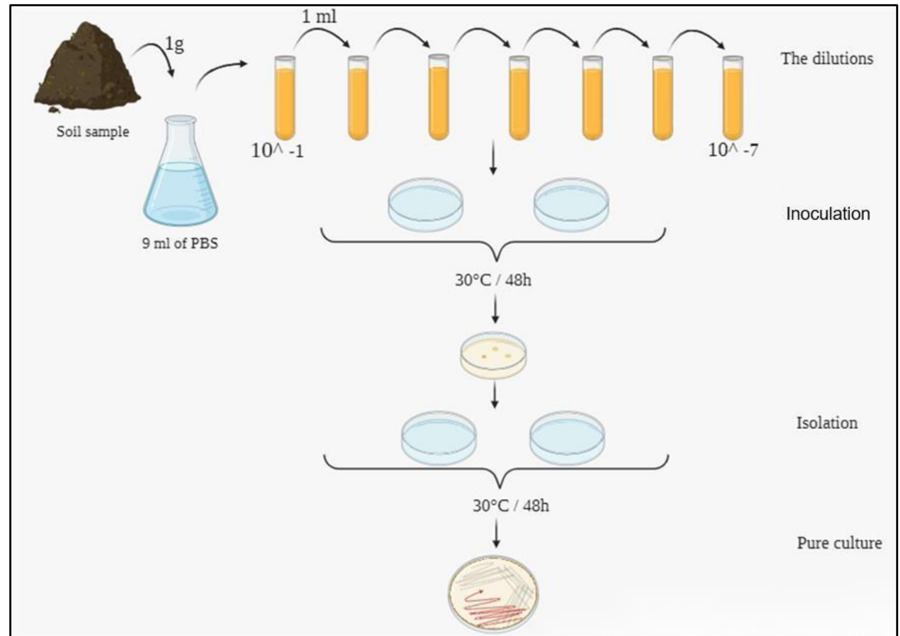

Figure 2 : Isolation and purification steps

3. Chitinase activity assay

3.1. Preparation of chitin

The chitin used in our study was extracted from shrimp shells following the Hisham et al. (2021) method, firstly the shrimp shells was washed and dried in the oven for four days, then ground into powder. Subsequently, the shrimp shell powder underwent deproteinization by a treatment with 1M of sodium hydroxide at a solid to solvent ratio of 1:5 (w/v) for 20h at ambient temperature. Following this, demineralization was carried out by treatment with 2.5% HCl at a solid to solvent ratio of 1:5 (w/v) for 16 hours at room temperature. The resulting residue was centrifuged, soaked, and washed with distilled water until reaching a neutral pH (Figure 3). This final residue is referred to as chitin.

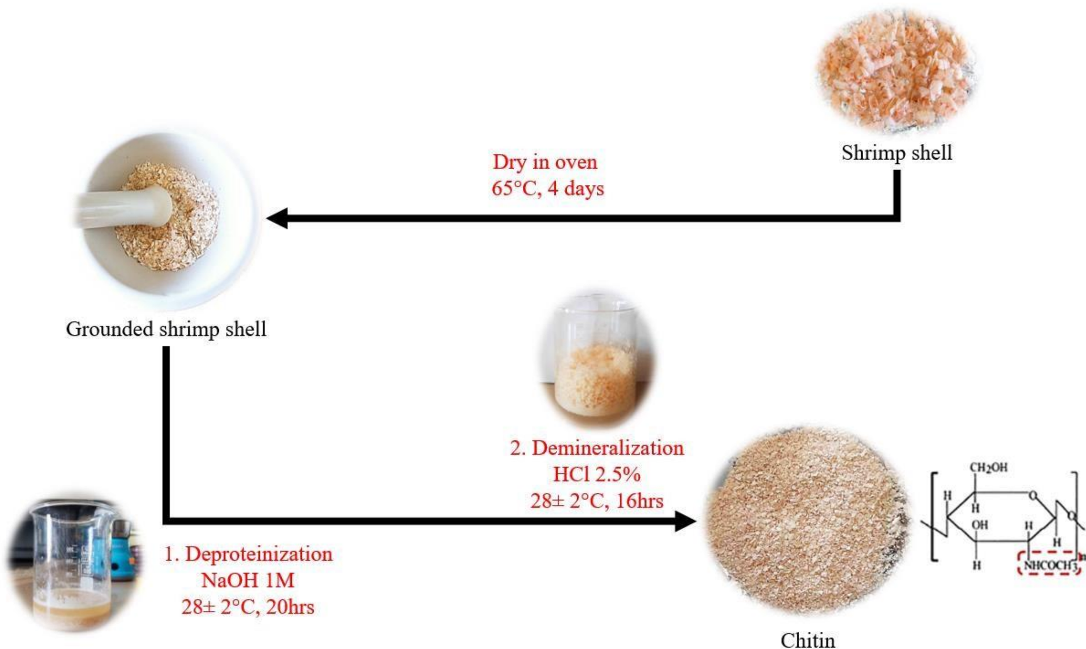

Figure 3 : Isolation and purification steps of chitin

3.2. Preparation of colloidal chitin

Colloidal chitin was prepared according to the method of Berger & Reynold (1958) with slight modification. 10g of chitin was placed into a 1L flask with 200ml of HCL. After stirring the mixture for two hours, 800ml of distilled water were added. The mixture was then left overnight to enable separation of the pellet and supernatant.

The mixture was filtered through a funnel containing two layers of filter paper. The collected colloidal chitin on the filter was subsequently washed with distilled water until it achieves a neutral pH. It was then dried for thirty minutes in a drying oven before being ground using a mortar and pestle.

3.3. Culture medium preparation

The medium used was prepared by combining the following elements (amounts given in grams per litre): colloidal chitin (0.8); $K_2H_2PO_4$ (2.7); KH_2PO_4 (0.3); $MgSO_47H_2O$ (0.7); NaCl (0.5); KCL (0.5); yeast extract (0.13), agar (15). The pH of the medium was adjusted to seven. Subsequently, it was poured into flasks and autoclaved (Kopečný et al., 1996).

3.4. Chitinase activity

24h grown cultures of bacterial isolates were inoculated onto the previously prepared medium using the spot method. After incubation for 3 days at 30°C, isolates displaying a clear halo around their colonies were chosen for further tests (Kopečný et al., 1996).

4. Biochemical identification

The selected isolates were identified through a few biochemical tests only, specifically Gram staining, catalase test, and oxidase test. This represents a partial identification rather than a comprehensive one.

4.1. Gram staining

Gram staining was performed for the selected isolates using the following standard method:

The technique begins with heat-fixing a bacterial smear on a glass slide, followed by staining with crystal violet for one minute, then fixed with a solution of iodine (Lugol's solution) for another minute. Decolorization is then achieved by briefly washing with alcohol for 30 seconds, and then rinsed with water. Counterstaining with fuchsine was applied for one minute, followed by a final rinse. The slides were dried and a drop of immersion oil was applied. The slides were then observed under a light microscope with a 100X objective.

4.2. Catalase test

A small amount of bacterial colony from each isolates was mixed with a drop of hydrogen peroxide on a glass slide. Immediate effervescence indicates a positive catalase reaction, confirming the presence of catalase enzyme

4.3. Oxidase test

Add a disc of oxidase to a tube containing the bacterial suspension. A development of purple coloration indicates a positive oxidase reaction.

5. Factors influencing chitinase production

For chitinase production, the culture medium was inoculated with 5µl of 24h grown cultures (OD 0.08-0.1) of selected isolates, K3, K4, K5 and K6. Four different factors, namely

temperature, pH, concentration of $MgSO_4$ and incubation time, were considered for the study to verify the influence of these parameters on chitinase activity.

5.1. Effect of the temperature of incubation

Temperature is known as an important parameter for chitinase production since the growth of bacteria is affected by low or high temperatures. Thus, chitinase production was quantified at 25, 30, 35, 40 and 45 °C for 4 days.

5.2. Effect of pH

One of the crucial physicochemical factors influencing the overall production of chitinase is pH. To determine the influence of pH in chitinase production by the selected isolates, chitinase assay was conducted in chitin medium adjusted to different pH: 4, 5.5, 7, 8.5 and 10. The media were inoculated and incubated at 30°C for 4 days.

5.3. Effect of magnesium sulfate

Magnesium sulfate is important elements that effect the production of chitinase by bacteria. Its effect was studied using chitin medium supplemented with Magnesium sulfate at final concentrations of 0.04; 0.05; 0.06; 0.07%. Media were inoculated and incubated at 30°C for 4 days.

5.4. Effect of incubation time

Selected bacteria were inoculated into chitin medium, and incubated at 30°C. Chitinase production was estimated at regular time intervals of 24 h for six days.

6. Enzymatic tests

In order to verify the ability of the selected isolates to produce other enzymes, various enzymatic activities were examined using the agar diffusion method (Figure 4). For each activity, a 5ul of each selected isolate was inoculated onto the appropriate medium, then the plates were subsequently incubated at 30 ± 2°C. All tests were conducted in duplicate.

6.1. Cellulase activity (Carder, 1986)

The selected isolates were inoculated in CMC (Carboxy Methyl Cellulose) agar, containing (g/l): Na_2HPO_4 (6), KH_2PO_4 (3), NaCl (0.5), NH_4Cl (1), yeast extract (3), CMC (7), agar-agar (15) and incubated at 30°C for 7 days. After the incubation, to visualize the

hydrolysis zone, the agar medium was flooded with 1% red Congo solution for 20 min then washed with 1 M NaCl and kept overnight (Jaradat et al., 2008). Clear zones around the colony indicated the production of extracellular cellulase (Figure 4) Carder (1986).

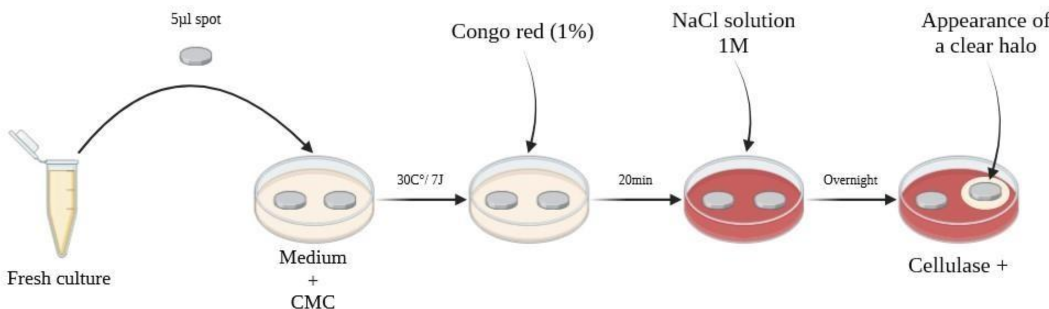

Figure 4: Cellulolytic activity

6.2. Esterase and lipase activity

These activities were tested as described by (Sierra, 1957), on a medium containing in g/l : peptone (10), NaCl (5), $CaCl_2$ $2H_2O$ (0.1), agar (18), and 1% of sterilized tween 80 for esterase activity and tween 20 for lipolytic activity, the pH was adjusted to 7.4. After inoculation, Petri dishes were incubated for 48h at 30°C. Activity is shown by an opaque zone around the colonies (Figure 5)(Carrim et al., 2006).

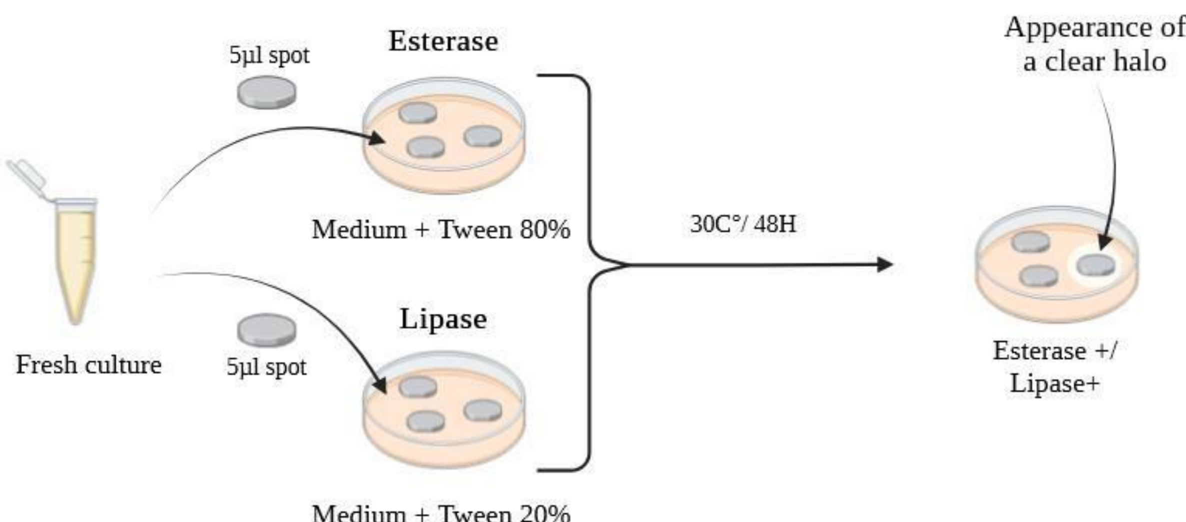

Figure 5: Esterase and lipase activity

6.3. Proteolytic activity

This test was made on a medium containing in g/l : pancreatic casein (5) ; yeast extract (2,5) ; Glucose (1) and Agar (15). The medium was adjusted to pH 7. In parallel, 100ml of sterile 10% skimmed milk solution were added to the medium. After inoculation, Petri dishes were incubated for 48h at 30°C. Proteolytic activity is revealed by a clear halo around the colonies (Figure 6) (Bach and Munch, 2000).

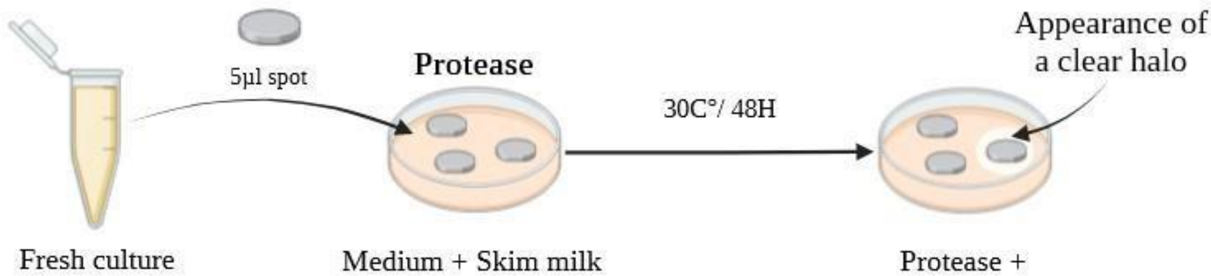

Figure 6: Proteolytic activity

6.4. Urease activity

The medium was prepared by adding in g/950 ml: peptone (1) ; glucose (1) ; NaCl (5) ; Na_2HPO_4 (1,2) ; KH_2PO_4 (0,8) ; red phenol (0,012) ; Agar (15). The PH was adjusted to 6.8. After autoclaving, 50ml of a 40% urea solution pre-sterilized by filtration (porosity 0.22 µm), were added to the medium. The activity is shown by a pink halo around the colonies (Figure 7) (Christensen, 1946).

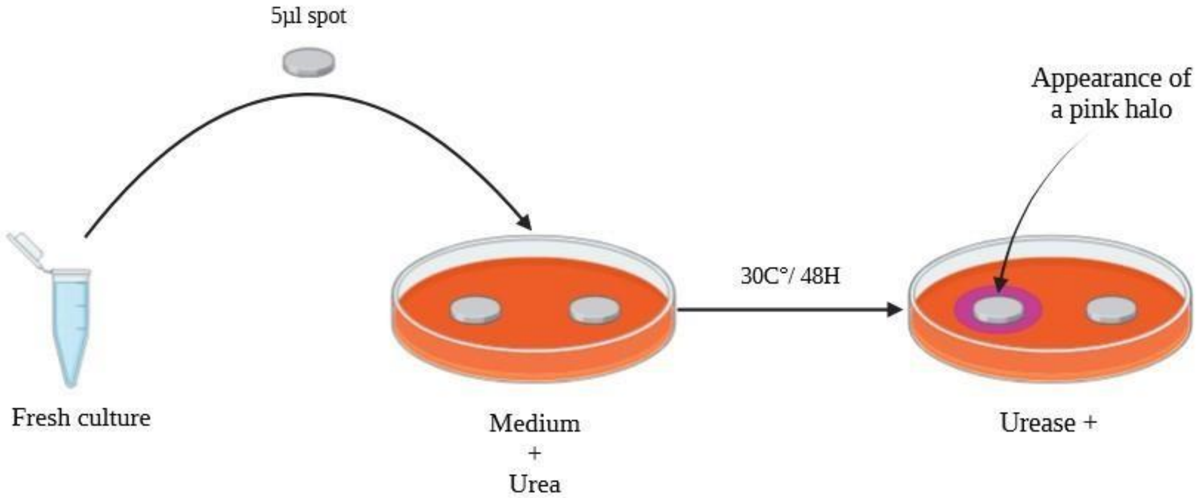

Figure 7 : Ureolytic activity

6.5. Amylolytic activity

The ability of isolates to hydrolyze soluble starch was verified in agar starch medium, which contains in (g/l): KNO_3 (0.5); K_2HPO_4 (1); $MgSO_4$ (0.2); $CaCl_2$ (0.1); $FeCl_3$ (0.001); soluble starch (10); agar (15). The pH was adjusted to 7.2. The isolates were inoculated and incubated at 30°C for 72 hours. After incubation, Lugol´s solution (1 g iodine, 2 g of KI and 300ml of distilled water) was poured onto medium surface. After a few minutes, the excess solution was removed, and the dishes were rinsed with distilled water. The activity was indicated by a clear zone around the colonies. (Figure 8) (Raj et al., 2009).

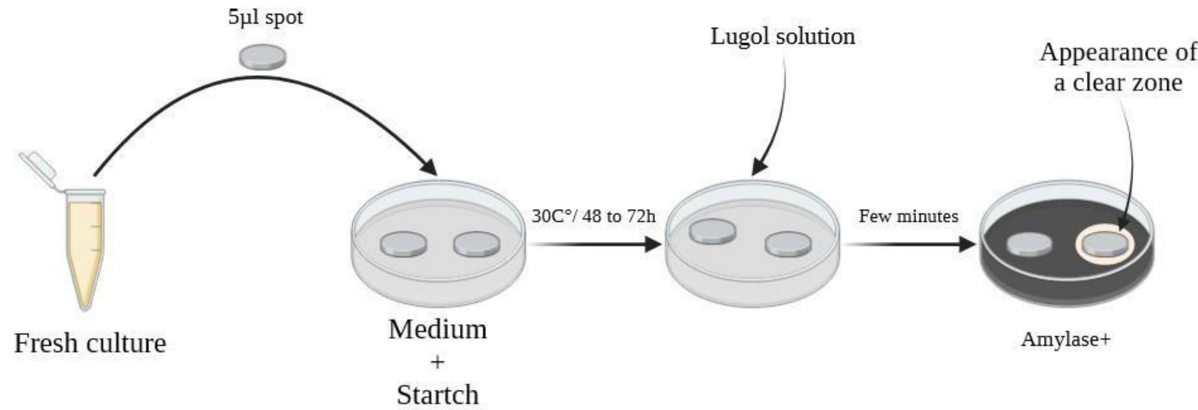

Figure 8 : Amylolytic activity

7. Bioactive compounds with Antifungal properties

7.1. Ammonia NH_3 production

This qualitative test followed the method outlined by Cappuccino and Sherman (1992). It involved inoculating 100 µl of the bacterial suspension into 10 ml of peptone water. After incubation at 30°C for 96 hours, 500 µl of Nessler's reagent was added to each tube. The emergence of a yellow or orange color indicate the production of ammonia (NH_3). (Figure 9).

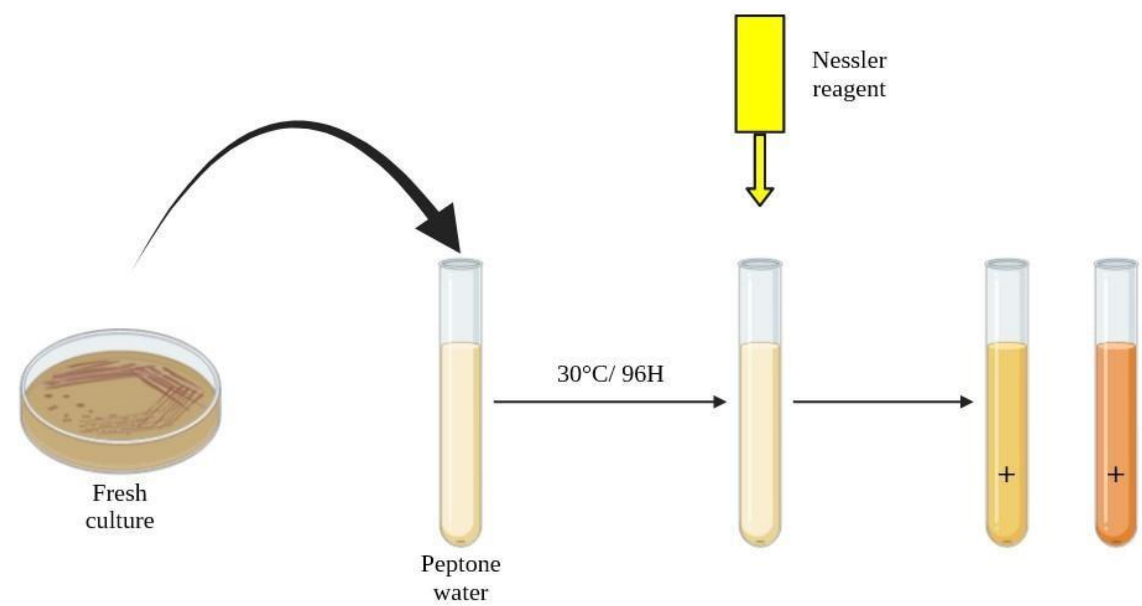

Figure 9 : Ammonia production

7.2. Hydrogen cyanide production (HCN) (Lorck, 1948)

The production of HCN was performed according to the method of Lorck (1948). The bacterial culture was cultured on Petri dishes containing nutrient agar medium (Annex II) supplemented with glycine (4.4 g/l). A disc of Whatman paper (N°42, 8 cm diameter) saturated with sodium picrate solution (5% of picric acid and 2% of anhydrous sodium carbonate) was placed inside the lids of the plates. The Plates were then sealed with Parafilm and incubated at 30°C/96 h. A color change of the Whatman paper from yellow to orange or brown indicated production HCN (Figure 10).

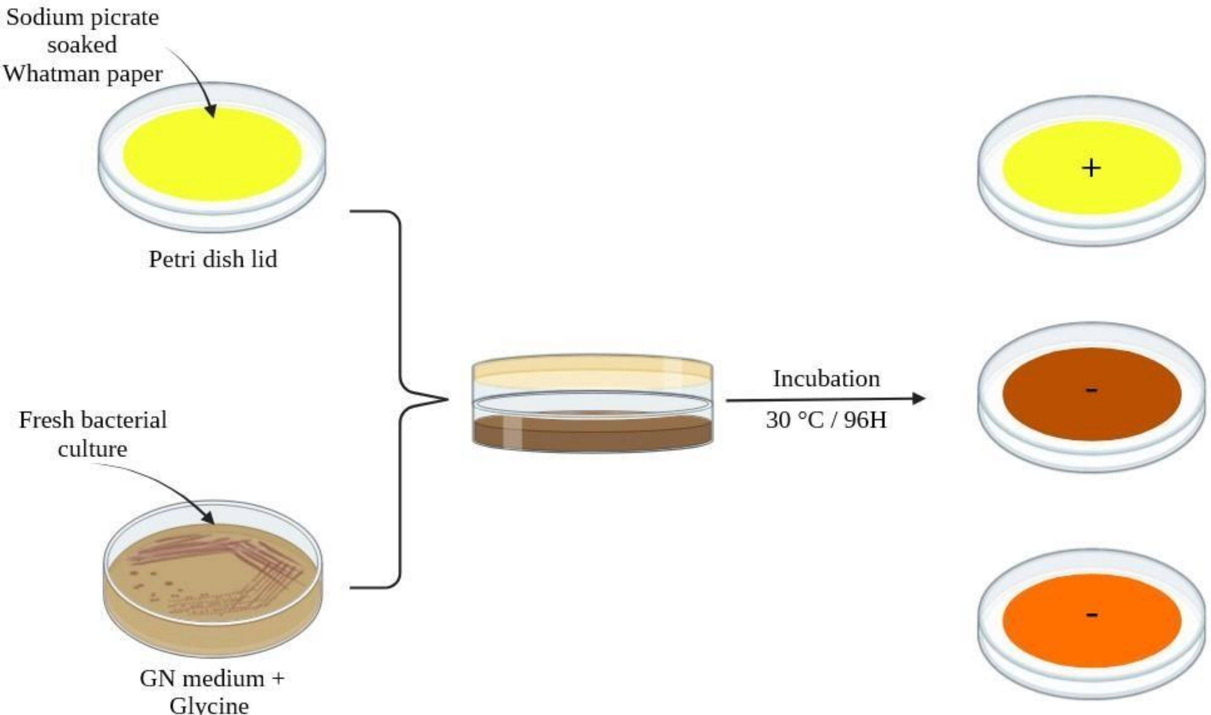

Figure 10 : HCN production

8. Antifungal test

This test was used to assess the efficacy of our strains against pathogenic fungi *in vitro* and *ex vivo* (apple fruits).

8.1. *In vitro* test

Four phytopathogenic fungi were tested, *Fusarium* sp., *Aspergillus niger*, *Penicillium* sp. And *Alternaria* sp. The fungal species were grown in Potato Dextrose Agar (PDA) (Annex II) (Figure 11).

Figure 11 : Fungi used in the test

The selected isolates were tested for their antifungal activities against the four fungal species, following the method described by Sagahon et al. (2011). Fungal plugs measuring 5 mm in diameter were inoculated in the center of the petri plates, and two spots of each bacterial isolate were inoculated 2.5 cm away from the fungal inoculum (two isolate per plates). Plates without potential bacterial antagonists served as negative controls. The plates were then incubated at 25 ± 2°C for 5 to 7 days and inspected daily (Figure 12). The percentage of growth inhibition (PGI) of the fungus was recorded and calculated using the formula described by Siddiki (2000):

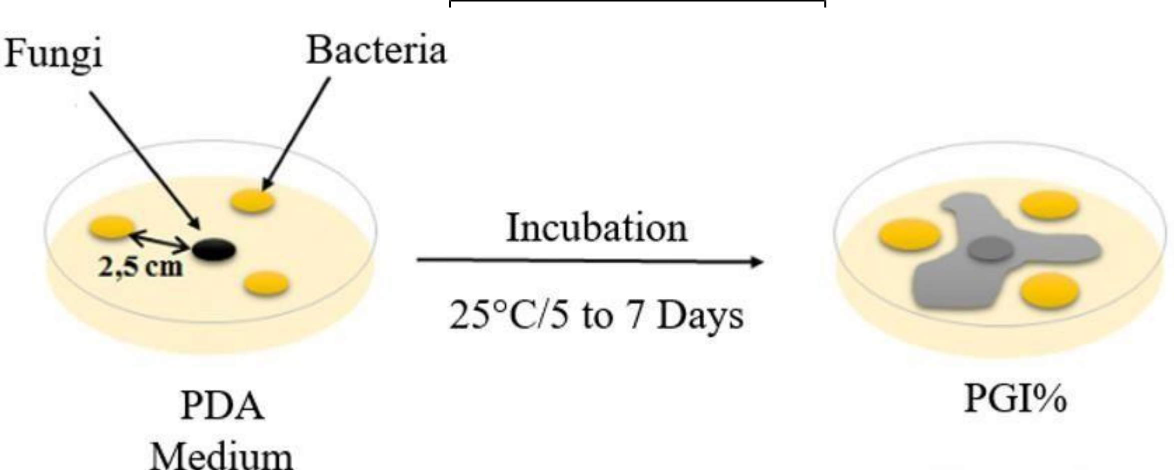

Figure 12 : Antifungal test

8.2. *In vivo* test

K5 and K6 bacterial isolates were tested for their capacity to control the growth of *Penicillium* sp. and *Alternaria* sp. on apple fruits. These steps were followed to realize this test (Figure 12):

Select fruits that are free from injury and rot, and of uniform size and maturity stage;

Disinfect the fruit surface by immersing in 2% hypochlorite solution for 1 to 2 minutes, followed by rinsing twice with sterilized water;

Allow the fruits to air dry at room temperature under sterile conditions;

Create wounds in the equatorial zone of each apple fruit with 2 wells (3 mm wide × 3 mm deep), adjusted to the fruit size.

Inoculate each well with 30 µl of bacterial strains (1×10^8 cells/ml). For the control, replace the bacterial strain with 30 µl of sterilized distilled water. Allow the fruits to sit for 2 hours at room temperature under sterile conditions.

Add 15 µl of *Penicillium* sp. or *Alternaria* sp. spore suspension to each well, excluding the control.

Incubate the fruits in disinfected boxes at 20°C with 95% to 98% relative humidity for 4 to 6 days (Tabli, 2017)

Rotting is evaluated by measuring the area of lysis.

To highlight the protective effect of the isolates, the apples are photographed and the lysis surfaces are measured using Image J software.

Figure 13 : Steps of the in vivo antifungal test on apples

1-Humidification of boxes; **2-** Preparation of the wells; **3-** Treatment; **4-**Transfer of the apples into boxes; **5-** Incubation

9. Statistical analysis

Each experiment was performed in four replicates, and data were statistically analyzed using the tow way ANOVA test (analysis of variance).

1. Isolation and screening of rhizobacteria

Macroscopic observation of the morphological appearance of colonies on PCA medium revealed a wide diversity in size, shape, color and surface characteristics. Therefore, 61 different colonies were isolated from each soil sample crop: 9 (Fava bean), 21 (potato), 13 (Onion) and 18 (Turnip).

Agricultural soils, particularly rhizospheric soils harbor an exceptionally high microbial biomass and species diversity. Indeed, just 1 gram of rhizosphere soil contain between 10^8 and 10^{11} cultivable cells. Due to its significant genetic, ecological, functional, and taxonomic diversity (Saleem et al., 2015; Fierer, 2017), the soil microbiome serves as a crucial reservoir of microbial traits that could benefit plant growth and health (Saleem et al.,2019).

2. Chitinase activity assay

The following graph illustrates the chitinolytic activity of the isolates obtained by the measurement of the diameter of the clear zones.

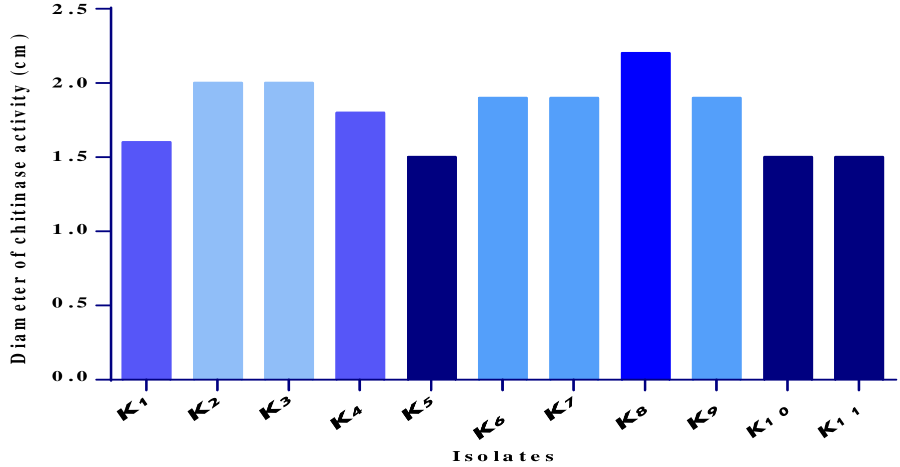

Figure 14: Diameters of chitinase activity for all initial isolates

The obtained results indicate that only 11 isolates exhibit Chitinolytic activity, as shown in the figure 13 : K1: 1.6 cm, K2: 2 cm, K3: 2 cm, K4: 1.8 cm, K5: 1.5 cm, K6: 1.9 cm, K7: 1.9 cm, K8: 2.2 cm, K9: 1.9 cm, K10: 1.5 cm, and K11: 1.5 cm. However, for subsequent tests, we selected only four isolates (K3, K4, K5, K6).

Chitinolytic bacteria are prevalent in various soil types, particularly in agricultural lands (surface soil, macropores, and rhizosphere) (Bundt et al., 2001; Fierer et al., 2007; Kurniawan et al., 2018; Nayak et al., 2020). Someya et al. (2011) highlighted their diversity by screening 100 isolates from different plant rhizospheres. Additionally, De Boer et al. (1998) and Tran et al. (2018) demonstrated the presence of chitinolytic bacteria in non-agricultural environments by isolating them from dune soil and freshwater.

3. Biochemical identification

The results of the chemical tests for the selected isolates are shown in the following table and figures.

Table I: Results of Gram staining

Strains	Gram	Shape
K3	-	Bacille
K4	-	Bacille
K5	-	Bacille
K6	+	Bacille

Table II: Biochemical identification test

Chemical test / Strain	K3	K4	K5	K6
Catalase test	+	+	+	+
Oxidase test	+	+	+	+

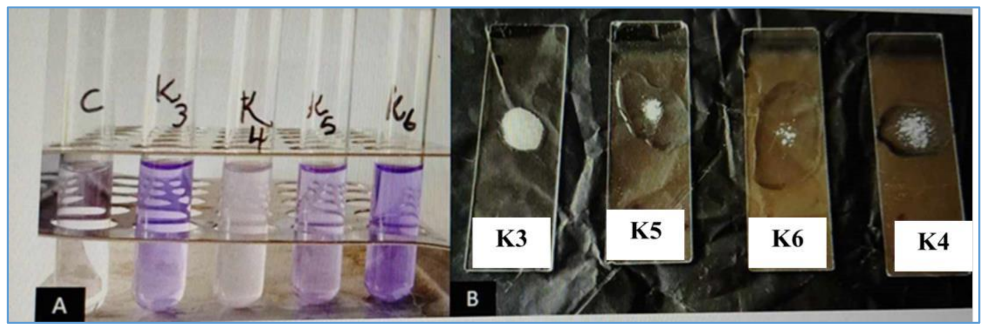

Figure 15: Biochemical test: A) Catalase test; B) oxidase test

Almost all isolates are Gram-negative. Similar results were obtained by Grobelak and al (2015) who showed that the rhizosphere is colonized mainly by a Gram-negative microbial potato community.

A bacterium that is Gram-negative, bacillus-shaped, oxidase-positive, and catalase-positive (K3, K4 and K5) could belong to the genus *Pseudomonas*. *Pseudomonas* bacteria are typically Gram-negative, rod-shaped, and often test positive for oxidase and catalase. These characteristics align with numerous species within this genus, such as *Pseudomonas protegens*, *Pseudomonas fluerescens*, and other *Pseudomonas* species commonly found in soil and other natural environments.

Pseudomonas sp. are known as plant growth-promoting rhizobacteria (PGPR), they are widely used in agriculture as natural biocontrol agents (Kumar et al., 2014). The genetically best-characterized biocontrol agents belong to the genus *Pseudomonas* (Bloemberg and Lugtenberg, 2001). The complex of *Pseudomonas fluorescens* includes several species described as plant growth-promoting rhizobacteria (PGPR) potentially active in biocontrol and biofertilization (Garrido-Sanz et al., 2016).

A bacterium that is Gram-positive, bacillus-shaped, oxidase-positive, and catalase-positive (K6) could belong to the genus *Bacillus*. Bacteria of the genus *Bacillus* are one of the main groups of rhizobacteria known for their application in the biocontrol of several phytopathogens. They produce several bioactive compounds effective against bacteria, fungi, protozoa, and viruses (Bottone and Peluso, 2003).

4. Factors influencing chitinase production

Multiple investigations indicates that bacterial chitinase production is affected by temperature, pH, incubation time, carbon source, nitrogen source, metal ions, etc (Singh et al., 2009; Jholapara et al.,2013; Aliabadi et al., 2016 ; Natsir et al., 2017; Poria et al., 2021).

4.1. Effect of incubation temperature

The following graphs illustrate the effect of various temperatures on the chitinolytic activity of the selected isolates.

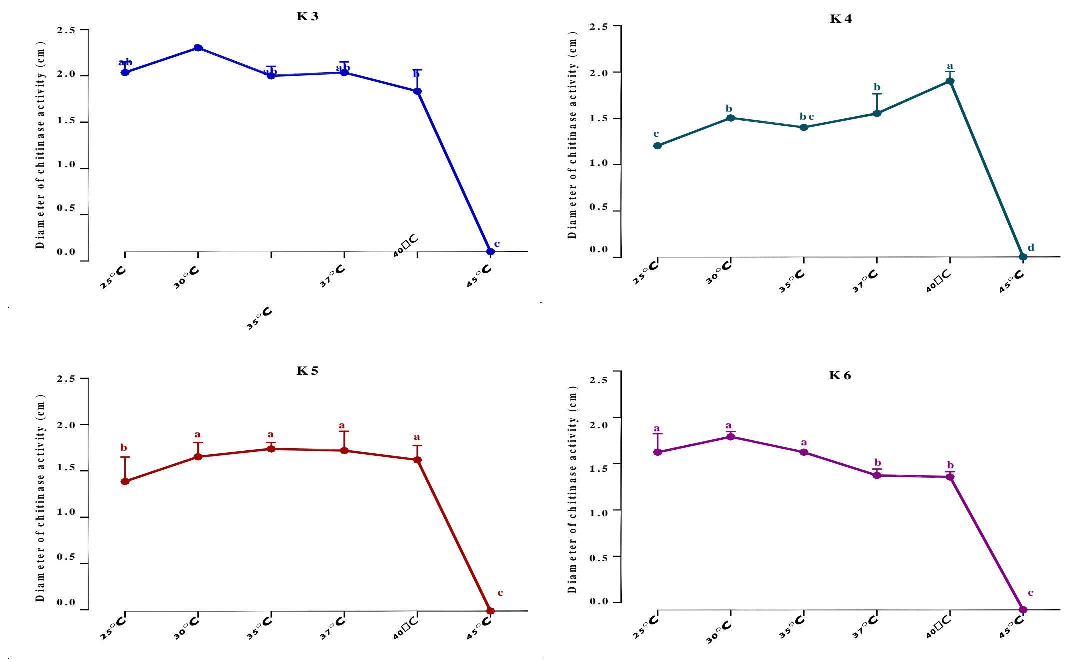

Figure 16: The effect of incubation temperature on production of chitinase

Error bars represent standard deviations. Values followed by the same letter do not represent significant difference. Values followed by different letter represent significant difference ($p \leq 0.05$)

We observe that isolates K3 and K6 share the same temperature optimum of 30°C, where we recorded a significant chitinolytic activity ($p \leq 0.05$). This finding aligns with the results reported by Natsir et al. (2017) who showed that a gram negative *Aerobacter* was the optimal temperature and Singh et al. (2010) showed that 30°C was the optimal for *Paenibacillus sp. D1*. Isolate K5 exhibited its highest and significant activity at 35°C. Karunya et al. (2010) reported that *Bacillus subtilis* produced the most chitinase at 35°C.

Isolate K4 demonstrated superior and significant production of chitinase at 40°C, which corresponds to the results obtained by Sudha (2020) *Bacillus thuringiensis strain LS1* had maximum chitinase production at 40C°. Although our strains exhibited activity across a wide temperature range from 25°C to 40°C, no activity was detected at 45°C, contrary to the findings of Singh (2009), where chitinase activity didn't decrease at elevated temperatures up to 45°C.

4.2. Effect of pH

The following graphs illustrate the effect of various pH on the chitinolytic activity of the selected isolates.

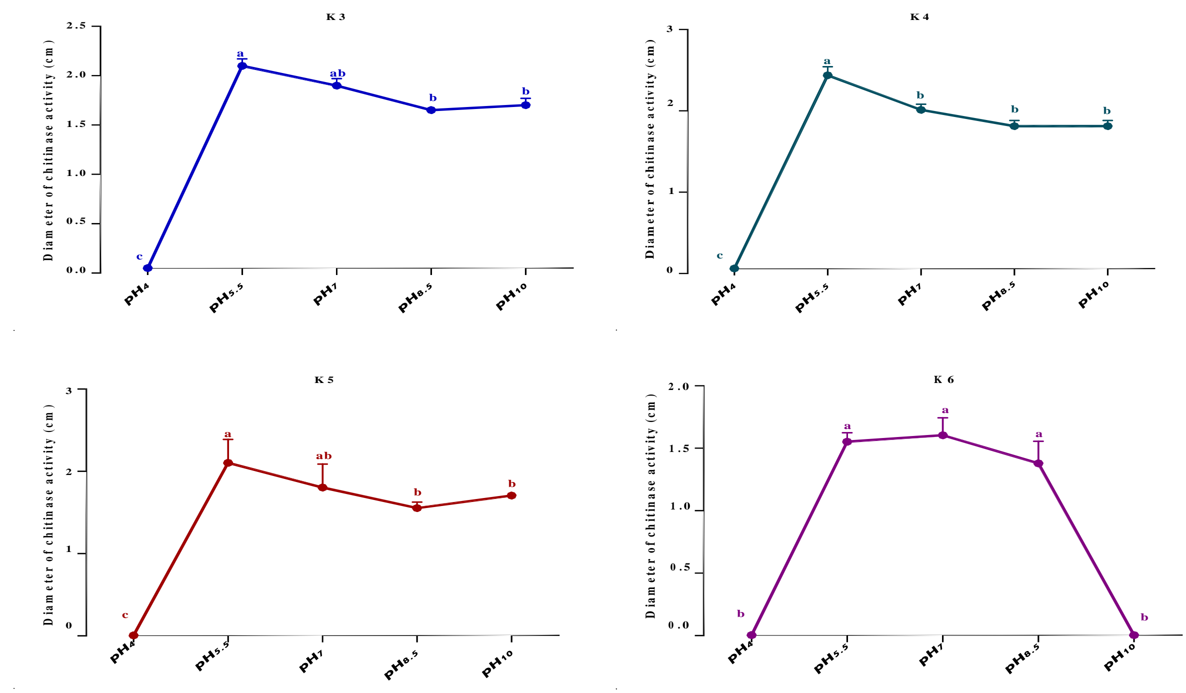

Figure 17: The effect of pH on the production of chitinase by the selected isolates

Error bars represent standard deviations. Values followed by the same letter do not represent significant difference. Values followed by different letter represent significant difference ($p \leq 0.05$)

As reported by multiple investigators (Rousk et al., 2009; Singh et al., 2009;Jholapara et al., 2013; Sudha, 2020), pH levels not only influence chitinolytic activity but also bacterial growth. Additionally, Tripathi et al. (2018) suggest that soil pH can favor specific bacterial taxa based on their pH preferences. In our study, none of the isolates prosuce chitinase at pH 4. Rousk et al. (2009), reported that soil bacterial growth rate and biomass decreased dramatically below pH 4.

However, all isolates demonstrated optimal and significant activity at pH 5.5, except for the K6 isolate, which has its optimum at pH 7. This aligns with the investigations of Mathivanan et al. (1998) and Jholapara et al. (2013), indicating that chitinolytic activity is most pronounced between pH 5 and 7. Furthermore, our results regarding the absence of

activity at pH 10 for the K6 isolate align with Jabeen et al. (2018), who reported minimal chitinolytic activity for *Stenotrophomonas maltophilia* at pH 11.

4.3. Effect of Incubation time

The following graphs illustrate the effect of various time incubation on the chitinolytic activity of the selected isolates.

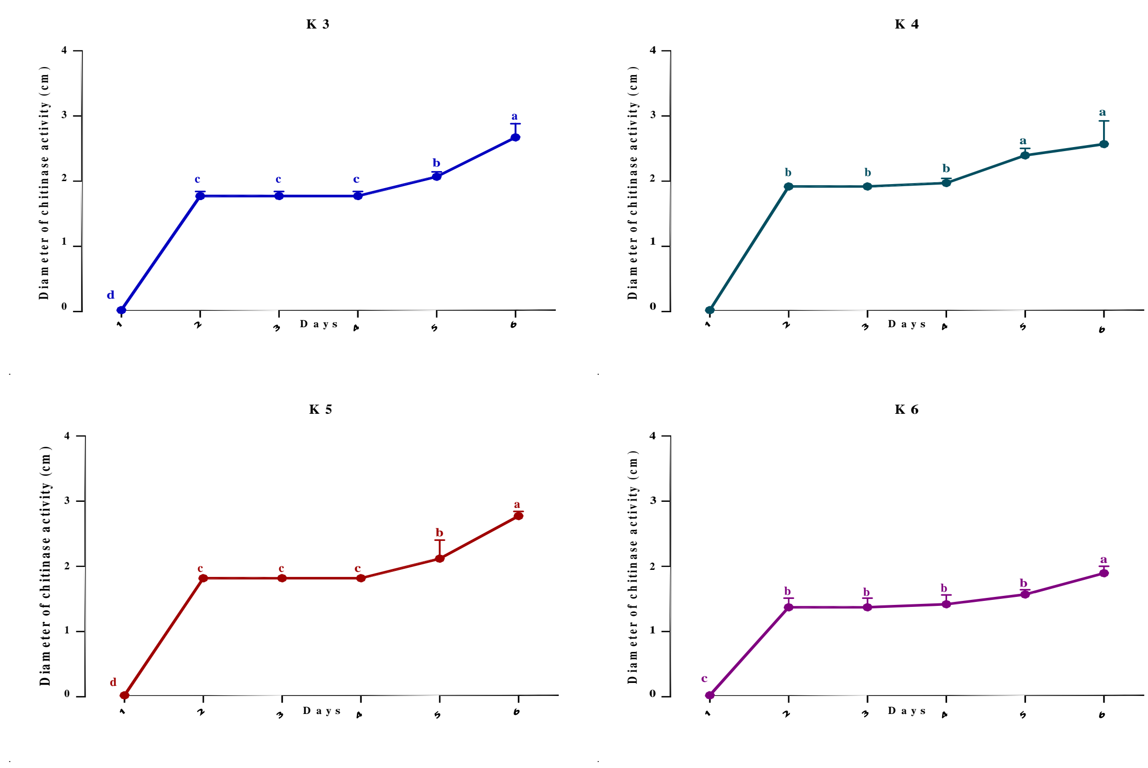

Figure 18: The effect of incubation time on the production of chitinase by isolates

Error bars represent standard deviations. Values followed by the same letter do not represent significant difference. Values followed by different letter represent significant difference ($p \leq 0.05$)

The chitinolytic activity of our isolates showed a consistent and logical increase over the span of six days, aligning with Singh's findings in 2010 where *Paenibacillus sp D1* had similar activity to our isolates. Additionally, investigation by Fathalla (2020) and Karunya et al. (2011) indicates that the chitinolytic activity of *S. liquefaciens* and *B. subtilis* had an optimum production at the 4th day of incubation. Moreover, Chakrabortty et al. (2012) observed that *Serratia marcescens* exhibited its highest chitinase production on the third day.

4.4. MgSO4 concentration

The following graphs illustrate the effect of various concentration of MgSO4 on the chitinolytic activity of the selected isolates.

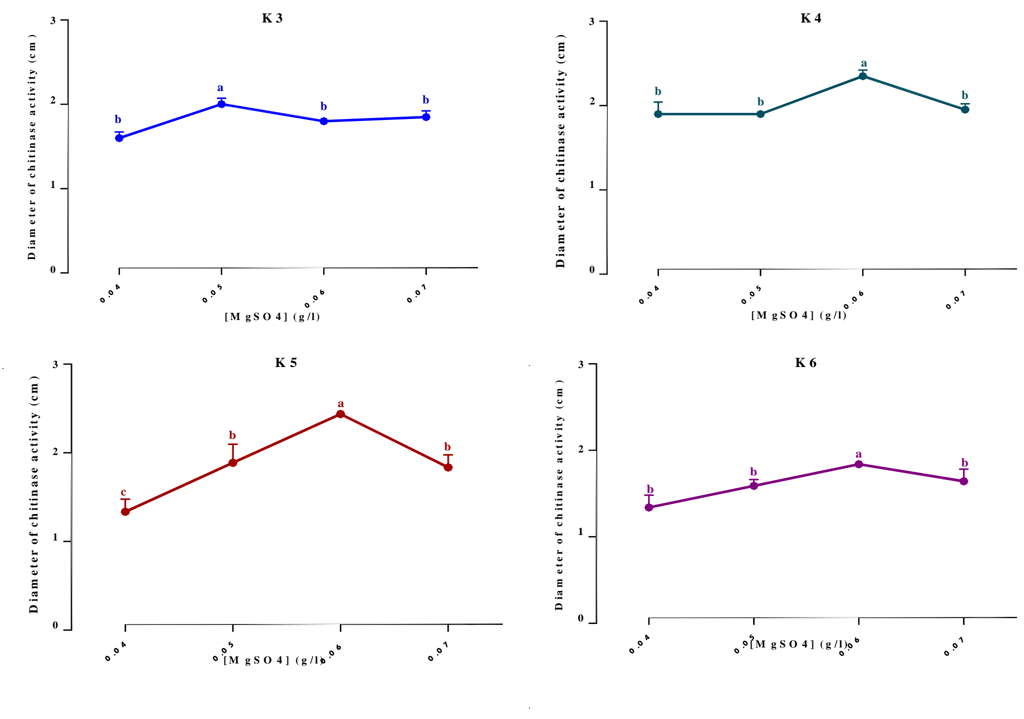

Figure 19: The effect of MgSO4 concentration on the production of chitinase by the selected isolates.

Error bars represent standard deviations. Values followed by the same letter do not represent significant difference. Values followed by different letter represent significant difference ($p \leq 0.05$)

In our current study, MgSO4 demonstrated an effect at concentrations lower than those used in the original chitinase assay. Specifically, isolates K4, K5, and K6 had their optimum at 0.06 g/l while isolate K5 had it’s optimum at 0.05 g/l.

Jholapara et al. (2013), found that *Bacillus cereus* strain had its optimum of chitinase activity at 0.06 g/l of MgSO4. Additionally, Han et al. (2008) demonstrated that a decrease in MgSO4 concentration had a positive impact on chitinase production by *Streptomyces sp. Da11*.

5. Enzymatic tests

In addition to chitinase, which was previously highlighted for its role in biological control, the bacterial lytic enzymes studied in this research play crucial roles in biocontrol, plant growth promotion, and biofertilization. Our results indicate that all isolates produced the studied enzymes, except for K6, which did not produce esterase. (Table III)

Table III: Results of enzymatic activities

Isolates	Cellulase	Lipase	Protease	Esterase	Amylase	Urease
K3	+++	+	++	+	+	++
K4	+++	+	++	+	++	++
K5	+++	+	++	+	++	++
K6	+++	++	++	-	+	++

+: diameter between 7 and 10 mm, ++: diameter between 10 and 20 mm and +++ > 20mm

5.1. Cellulase activity

According to Malik et al. (2023) and Thapa et al. (2020), cellulose stands out as the most abundant polymer in nature, making it a primary renewable agricultural waste globally. Cellulase enzymes play a crucial role in catalyzing cellulose into various compounds, thereby enhancing agricultural soils.

All of our bacteria have exhibited cellulolytic activity, a trait commonly found among soil bacteria. As reported by Sethi et al. (2013), various bacterial species such as *Pseudomonas fluorescens, Bacillus subtilis, Escherichia coli*, and *Serratia marcescens* isolated from soil have been shown to produce cellulase enzymes. These enzymes exhibit biocontrol activities by degrading the cell walls of different types of phytopathogenic fungi, as demonstrated by Jadhav et al. (2017). Furthermore, endophytic cellulase-producing bacteria contribute to maintaining the health of their host plants by providing tolerance and resistance against both abiotic and biotic challenges, as well as by promoting plant growth. This beneficial role of endophytic cellulase-producing bacteria in plant health has been highlighted by studies such as those conducted by Lata et al. (2018) and Oukala et al. (2021).

5.2. Lipase activity

Lipase-producing bacteria, including *Bacillus, Pseudomonas*, and *Burkholderia*, are widely distributed and can be found in various environments such as contaminated soil, lake water (Ilesanmi et al., 2020), and agro-industrial waste (Maldonado et al., 2014). As reported by Gupta et al. (2004) lipase enzymes, produced by all of our isolates, play a critical role in

the bioremediation of polluted soil by breaking down oils and fatty substances, as demonstrated by Jia Fu Lin et al. (2012).

Many authors such as Golani et al. (2016); Habibollahi & Salehzadeh (2018); Ilesanmi et al. (2020) have isolated different lipolytic bacteria from oil contaminated soils. Moreover, lypolytic bacteria could play a role in shrimp waste management according to Cornejo et al (2021).

5.3. Protease activity

Protease enzymes produced by all our isolates play diverse roles in soil. They are crucial in biocontrolling nematodes, as evidenced by Darwesh et al. (2019) where they have extracted protease from 14 actinomycetes isolates, similarly, Siddiqui et al. (2005) showed that populations of *meloidogyne incognita,* nematode responsible of root-knot disease was suppressed by an extracellular protease of *Pseudomonas fluorescens.*

Antifungal proteases and serine alkaline proteases of bacterial origin can regulate the population of phytopathogenic fungi and insects (Morton et al., 2003; Chang et al.,2007; Tian et al., 2007; Vranova et al., 2013). Caballero et al. (2020) demonstrated that proteases from Bacillus spp. improve soil fertility by inducing key metabolic enzymes such as dehydrogenase and favoring Plant Growth Promoting Rhizobacteria (PGPRs).

5.4. Esterase activity

Microbial esterase produced by K3, K4, and K5 plays a key role in soil health and fertility by hydrolyzing ester bonds in organic compounds. Recently Yamamoto-Tamura et al. (2015) showed that soil fungal esterase can contribute to the biodegradation of aliphatic polyester agricultural mulch film in cultivated soils.

Bhatt et al. (2021) showed that, esterases have a major role in bioremediation of soils by degrading organophosphate, carbamate, and pyrethroid pesticides.

5.5. Amylase activity

Amylases produced by all of our isolates are common in soil. For instance, Yassin et al. (2021) isolated amylolytic bacteria from soil in extreme environments. Bacteria or fungi can produce amylase; their main role is to hydrolyze complex polysaccharides like starch to glucose, thereby enriching soils with nutrients (Joshi et al, 1993; Singaram and kamalakumari, 2000; Naga Raju et al., 2017).

Recently Elamary and Salem (2020) highlighted the biomedical role of soil bacteria amylase in the inhibition of clinical biofilm-forming bacteria.

5.6. Urease activity

In this study, we found that all of our bacteria produced urease. Urease-producing bacteria play a crucial role in enriching agricultural soil by hydrolyzing urea fertilizers into NH3 and CO2. These bacteria play a key role in regulating nitrogen supply to plants following the application of urea fertilizers (Rotini, 1935; Andrews et al., 1989; Byrnes and amberger, 1989; Das and Varma, 2010). According to Li et al. (2013), four urease-producing isolates from garden soil exhibited high removal rates of heavy metals, ranging from 88% to 99%, after 48 hours of incubation. This highlights the bioremediation capabilities of these bacteria.

6. Bioactive compounds with Antifungal properties

6.1. Ammonia (NH_3) and Hydrogen cyanide (HCN) production

Table IV: Results of ammonia and HCN production

Isolates	NH3	
K3	++	+
K4	++	++
K5	+	++
K6	++	+
Isolates	**HCN**	
K3	+	
K4	+	
K5	+	
K6	+	

All of our isolates produced ammonia and HCN reflecting the results obtained by (Goswami et al., 2015) where *Pseudomonas aeruginosa BG* had biocontrol and PGPR properties by producing HCN and Ammonia.

Hydrogen cyanide and ammonia are volatile metabolite widely produced by soil bacteria. HCN is commonly categorized as a biological agent, as shown by Qessaoui et al. (2018), where HCN and chitinase producing *Pseudomonas* were able to control efficiently *tetranychus urticae,* a phytophatogenic pest. Similarly, Halimursyadah et al. (2023) suggested that HCN and chitinase rhizobacteria isolates from Patchouli rhizosphere could be used to

protect plants form pathogen attacks. However, Rijavec and Lapanje (2016) suggest that HCN-producing bacteria do not fulfil the role of biocontrol agents but instead play a role in regulating the availability of phosphate in the soil, akin to ammonia-producing bacteria, thereby contributing to soil richness and fertility as PGPR (Joseph et al., 2007).

Researchers also showed that ammonia production is a mechanism used to control pathogenic fungi (Kavitha et al., 2013), it also plays a key role in signalization during the interaction between rhizobacteria and plants (Becker et al., 2002).

7. Antifungal test

7.1. *In vitro* test

Chitin, being the main component of fungal cell walls, makes chitinolytic soil bacteria well-known as antifungal agents due to their ability to degrade these walls. (Medina et al., 2016). However, they are not the only type of bacteria that exhibit an antagonistic effect on fungi. Cellulase and protease-producing bacteria also play a role in the biocontrol of phytopathogenic fungi (Jadhav et al., 2017).

The results of the antifungal activity are showed in the graph below.

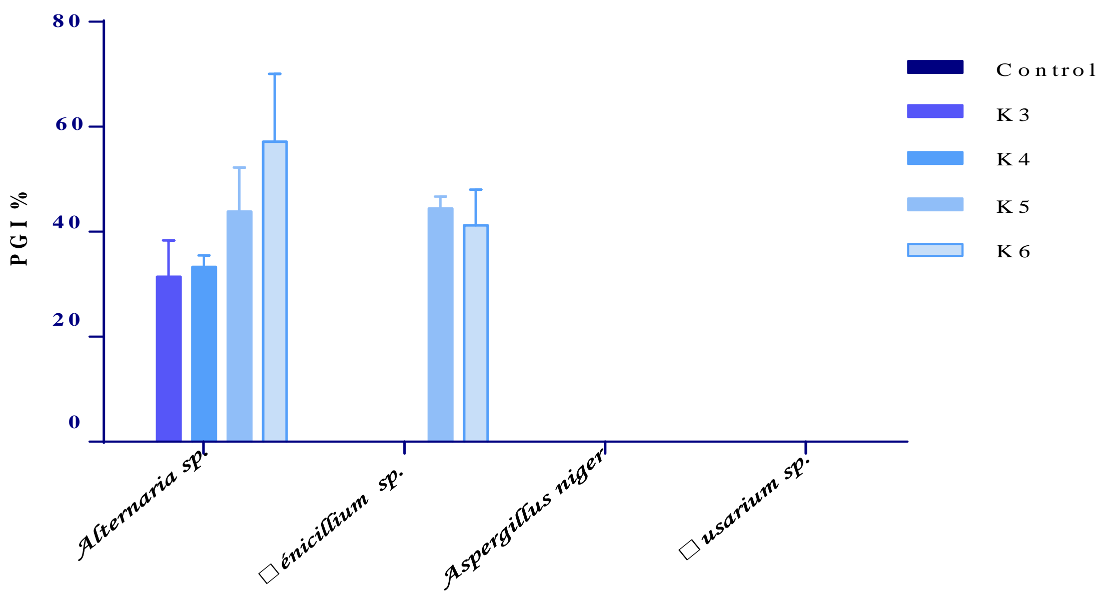

Figure 20: Percentage of fungal growth inhibition

Our isolates exhibited antifungal activity against *Alternaria* sp. with the following inhibition rates: K3: 31.42%, K4: 33.32%, K5: 34.80%, and K6: 57.13%. Against *Penicillium*

sp., the inhibition rates were K5: 44.44% and K6: 41.26%. Isolates K5 and K6 demonstrated the highest activity against *Penicillium* sp. and *Alternaria* sp.. These findings align with the results of Medina et al. (2016), who reported that chitinolytic strains of *Bacterium sp., Burkholderia cepacia, Burkholderia gladioli*, and *Paenibacillus sp.* were effective in degrading the mycelium of both *Alternaria* and *Penicillium.*

Similarly Tozlu et al. (2018) showed that strains of *B. subtilis, B. pumilus* and *B. megaterium* isolated from the rhizosphere and phyllosphere of wild and traditionally cultivated plants were effective in the control of *Alternaria alternata.*

7.2. *In vivo* test

The figures below represent the results of the antifungal effect of the isolates K5 and K6 on apple fruits

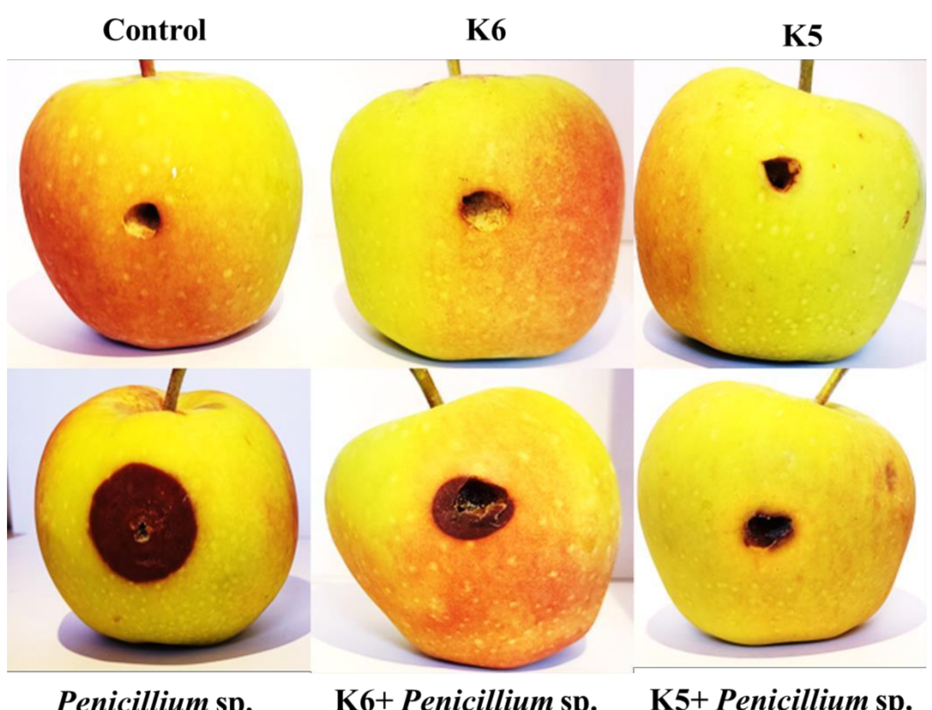

Figure 21: Appearance of the lysis surfaces obtained on apples infected by *Penicillium* sp. and treated with the isolates K5 and K6

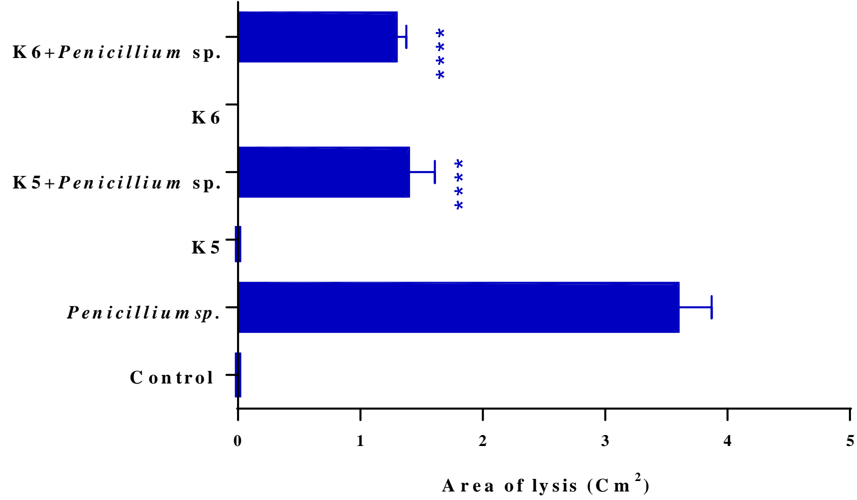

Figure 22: The values of the lysis areas obtained on apples infected by *Penicillium* sp. and treated with the isolates K5 and K6

ns : Non significative (p≥0,05) ; **** : p≤0.001

In our study, isolates K6 and K5 showed no lysis area compared to the control, suggesting that these isolates do not adversely affect apple fruits. Furthermore, when these isolates were inoculated with *Penicillium* sp., there was a significant decrease in the lysis area compared to the positive control (*Penicillium* sp.). This indicates that the K6 and K5 isolates possess biocontrol properties against *Penicillium* sp.

Antifungal activity can be attributed to the isolates' capacity to produce various lytic enzymes such as chitinase, protease, and cellulase, along with volatile antifungal compounds like HCN and NH3, all known for their antifungal properties (Kavitha et al., 2013; Vranova et al., 2013; Jadhav et al., 2017; Halimursyadah et al., 2023).

Our results were similar to those reported by Wang et al. (2016), where *Bacillus subtilis* effectively inhibited *Penicillium expansum* (pathogen of apples) both *in vitro* and *in vivo* on apples fruits. Similarly, Wallace et al. (2017) demonstrated that *Penicillium expansum* mold on McIntosh and Spartan apples could be inhibited by protease-producing *Pseudomonas fluorescens* isolated from the rhizosphere of pulse crops, they suggested that the biocontrol properties of their isolates were related to their ability to compete for nutrients and space, to produce inhibitory metabolites, and to the biofilm formation targeting conidial germination and mycelial growth.

Apples are not only targeted by *Penicillium* sp. For example, Jamalizadeh et al. (2010) demonstrated that *Botrytis mali* poses a threat to apple fruits. In the same study, they showed the biocontrol properties of *Bacillus* sp. isolated from the rhizosphere of wheat against this fungus. Similarly, Calvo et al. (2007) demonstrated the ability of *Rahnella aquatilis* to inhibit both *Penicillium expansum* and *Botrytis cinerea* on infected apple fruits.

Conclusion

Chitinolytic bacteria play a crucial role in the degradation of chitin, helping to recycle nutrients and suppress pathogens

In Present investigation, four soil samples were collected from different agricultural fields located in Bejaia. After isolation and purification 61 bacterial isolates were obtained and tested for their ability to produce chitinase enzyme.

Four isolates were selected and underwent preliminary biochemical identification (Gram staining, catalase, and oxidase tests). The obtained results lead us to suggest that the K3, K4 and K5 isolates could belong to the genus *Pseudomonas* and K6 to the genus *Bacillus.*

The results indicate that the variations in physicochemical parameters such as pH, Temperature, Incubation time and $MgSO_4$ concentration influence chitinase production by the selected isolates.

The optimal conditions for chitinase production were observed at different temperatures: 30°C for K3 and K6, 35°C for K5 and 40°C for K4. The pH conditions favoring production were pH 5.5 for all isolates except for K6 which had its optimum at pH 7. and the optimal $MgSO_4$ concentrations was 0.05% for K3 and 0.06 for K4, K5 and K6. The optimum production was observed on the 6th day for all isolates.

Our results indicate that all isolates produced HCH, Ammonia and all hydrolytic enzymes, except for K6, which did not produce esterase.

All isolates exhibit antifungal activity against *Alternaria* sp. with the following inhibition rates: K3: 31.42%, K4: 33.32%, K5: 34.80%, and K6: 57.13%. Against *Penicillium* sp., the inhibition rates were K5: 44.44% and K6: 41.26%.

There was a significant decrease in the lysis area compared to the positive control (*Penicillium* sp.) in the *in vivo* test.

From this study we conclude that our isolates could be used in the biological control of pathogenic microorganisms, thereby paving the way for practical applications in the biocontrol and management of plant diseases.

Perspectives

At the end of this study, several perspectives appear necessary for better utilization of these isolates:

Optimization of chitinase production

Testing the effect of isolates on the biocontrol of other plants pathogens

Verification of isolates' ability to produce other agriculturally relevant molecules

Molecular identification of the isolates

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Annex 1: *Phosphate-buffered saline*

phosphate-buffered saline

NaCl……………………………………………………………………………8g

KCl…………………………………………………………………….…. 0.2g

KH_2PO_4……………………………………………………………………..0.24g

Na2HPO4……………………………………………………………...1.44g

PH…………………………………………………….………..7.0±0.2

Annex 2: *Composition of the culture media used (for 1 litre of medium)*

Luria Bertani (LB)

NaCl……………………………………………………………………………5g

Tryptone………………………………………………………………………10g

Yeast extract……………………………………………………………….....5g

PH…………………………………………………………………………7.2±0.2

Plat count agar (PCA)

glucose……………………………………………………………………..1g

Tryptone…………………………………………………………………....5g

Yeast extract…………………………………………………………………2.5g

Agar………………………………………………………………………..12g

PH…………………………………………………………………...7.2±0.2

Nutrient agar

Peptone………………………………………………………………...5g

Meat extract…………………………………………………………….1g

Yeast extract………………………………………………………….....2g

NaCl…………………………………………………………………….....5g

Agar……………………………………………………………………...7.5g

PH……………………………………………………………………7.2±0.2

Potato Dextrose Agar (PDA)

Potato……………………………………………………………………………...200g

Glucose……………………………………………………………………......20g

Agar………………………………………………………………………….....15g

PH………………………………………………………………………......5.4±0.2

Abstract

Chitinolytic bacteria play a crucial role in the degradation of chitin, helping to recycle nutrients and suppress pathogens. The current study focuses on the isolation of Chitinase-producing rhizobacteria and the verification of their potential to control phytopathgenic fungi. Four soil samples were collected from different agricultural fields located in Bejaia. 61 isolates were obtaine and from 11 chitinase-producing bacteria, four isolates were selected. The influence of temperature, pH, incubation time and concentration of $MgSO_4$ on chitinase production were verified. The selected isolates were also tested for their ability to control *Alternaria* sp., *Penicillium* sp., *Fusarium* sp. and *Aspergillus niger in vitro,* and *Penicillium* sp. *in vivo* on apple fruits. Enzymatic activities (protease, amylase, lipase, urease, esterase), ammonia and HCN production were also carried out. The optimal conditions for chitinase production were observed at different temperatures: 30°C for K3 and K6, 35°C for K5 and 40°C for K4. The pH conditions favoring production were pH 5.5 for all isolates except for K6 which had its optimum at pH 7 and the optimal $MgSO_4$ concentrations was 0.05% for K3 and 0.06 for K4, K5 and K6. The highest chitinase production was observed on the 6th day for all isolates. All isolates produced HCH, Ammonia and all hydrolytic enzymes, except for K6, which did not produce esterase. All isolates exhibit antifungal activity against *Alternaria* sp. with the following inhibition rates: K3: 31.42%, K4: 33.32%, K5: 34.80%, and K6: 57.13%. Against *Penicillium* sp., the inhibition rates were K5: 44.44% and K6: 41.26%. In vivo test, there was a significant decrease in the lysis area compared to the positive control (*Penicillium* sp.).

Key words: Biocontrol, Chytinolytic Bacteria, Antifungal Activity, *Penicillium* Sp., Chitin

Résumé

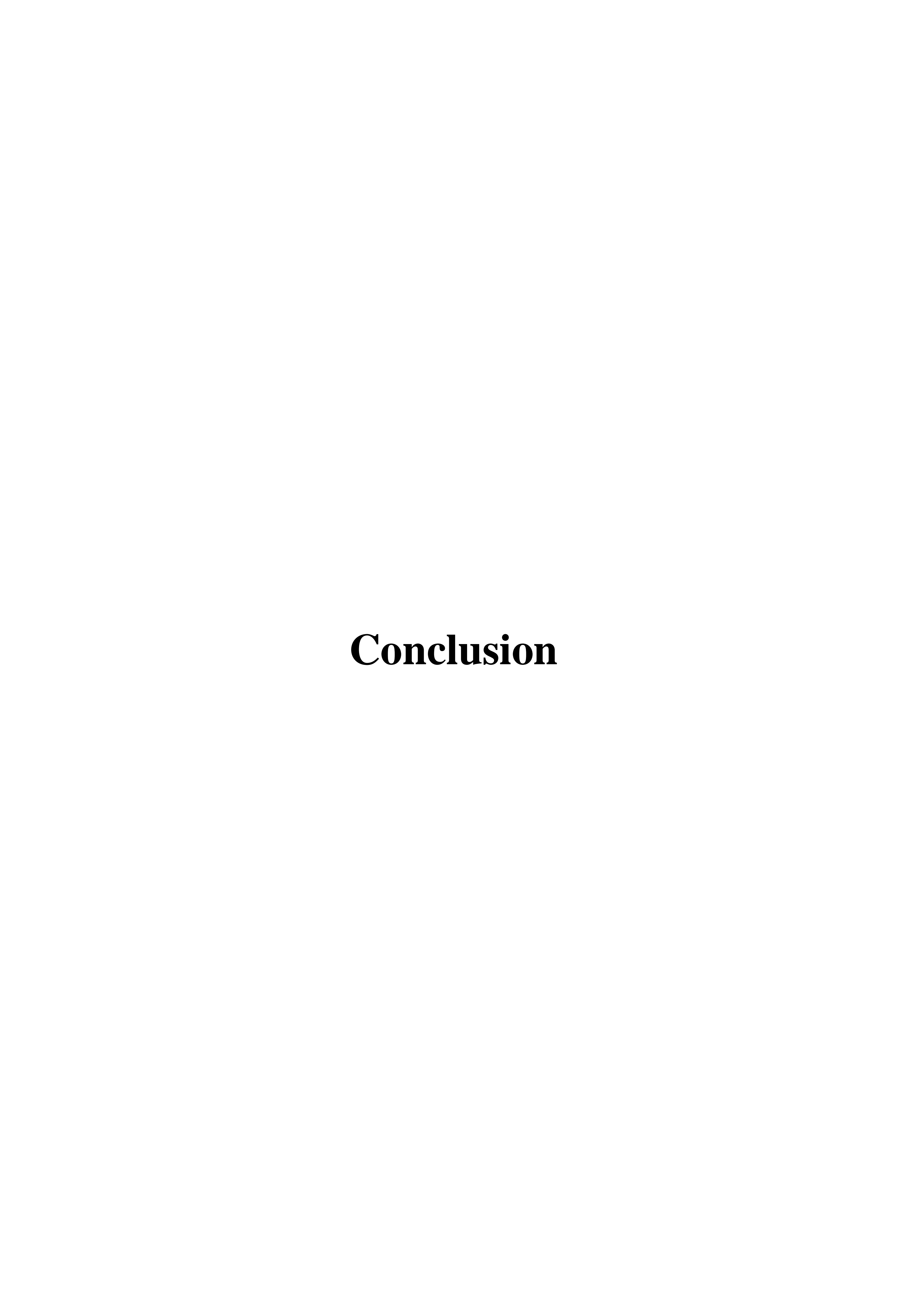
Les bactéries chitinolytiques jouent un rôle crucial dans la dégradation de la chitine, en participant à recycler les nutriments et à contrôler les agents pathogènes. La présente étude se concentre sur l'isolement des rhizobactéries productrices de chitinase et la vérification de leur potentiel dans le contrôle de champignons phytopathogènes. Quatre échantillons de sol ont été collectés dans différents champs agricoles situés à Bejaia. 61 isolats ont été obtenus et parmi 11 isolats producteurs de chitinase, quatre isolats ont été sélectionnés. L'influence de la température, du pH, du temps d'incubation et de la concentration de MgSO4 sur la production de chitinase a été vérifiée. Les isolats sélectionnés ont également été testés pour leur capacité à contrôler *in vitro* les champignons phytopathogènes *Alternaria* sp., *Penicillium* sp., *Fusarium* sp. et *Aspergillus niger*, et *in vivo,* sur des pommes, *Penicillium* sp. Des tests de recherche d´activités enzymatiques (protéase, cellulase, amylase, lipase, uréase et estérase), de production d'ammoniac et de HCN ont également été réalisées. Les conditions optimales pour la production de chitinase ont été observées à différentes températures : 30°C pour K3 et K6, 35°C pour K5 et 40°C pour K4. Les conditions de pH favorisant la production étaient de 5,5 pour tous les isolats à l'exception du K6 qui avait son optimum à pH 7. Les concentrations optimales de MgSO4 étaient de 0,05 % pour K3 et de 0,06 pour K4, K5 et K6. La meileur production de chitinase a été observée au 6ème jour pour tous les isolats. Tous les isolats produisaient du HCN, du l'ammoniac et toutes les enzymes hydrolytiques, à l'exception du K6, qui ne produisait pas d'estérase. Tous les isolats présentent une activité antifongique contre *Alternaria* sp. avec des taux d'inhibition suivants : K3 : 31,42 %, K4 : 33,32 %, K5 : 34,80 %, et K6 : 57,13 %. contre *Penicillium* sp., les taux d'inhibition étaient K5 : 44,44 % et K6 : 41,26 %. Le test *in vivo*, il y a eu une diminution significative de la zone de lyse par rapport au contrôle positif (*Penicillium* sp.).
Mots-clés : Biocontrole, Bacteries Chitinolytiques, Activité antifongique, *Penicillium* Sp., Chitine

Results and Discussion

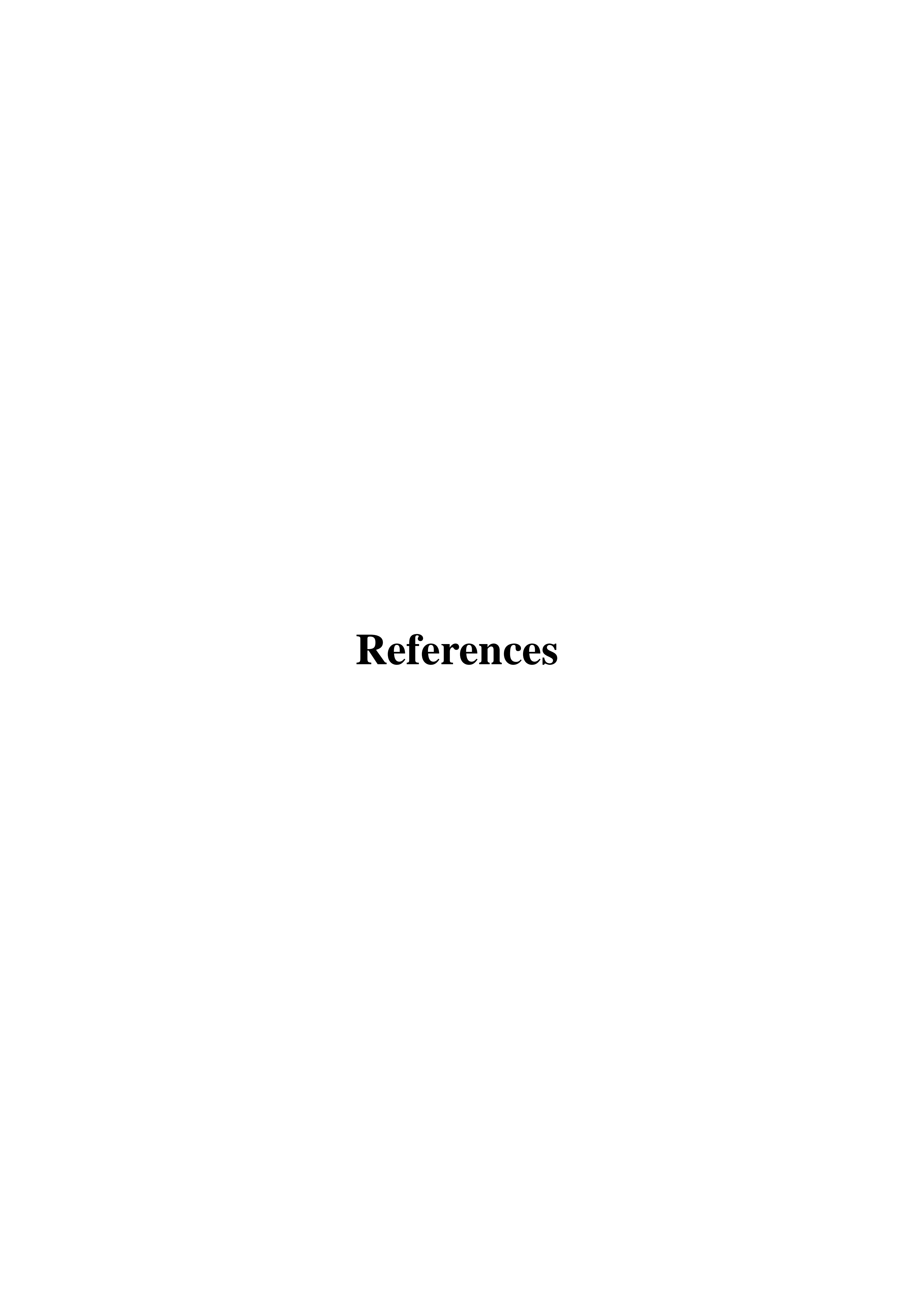
Material and Methods

Review

Introduction



Conclusion



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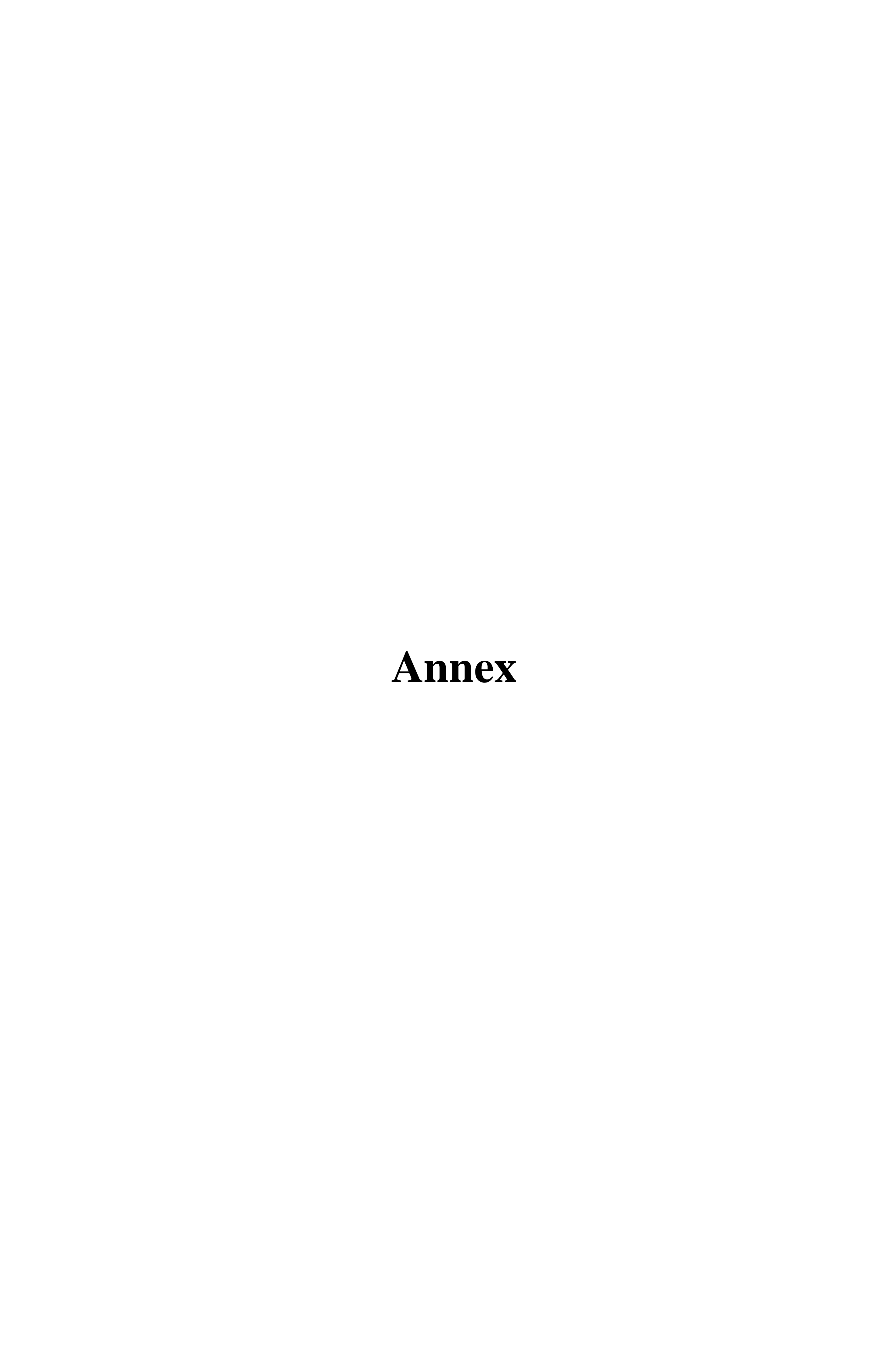

Annex