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THEME

Screening identification and growth physiology of two *Trichoderma* sp strains of interest in the biotechnological field.

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Maria

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Abbreviation

° C: degree Celsius

µl: Microliter

ADD: Tryptophan Deaminase

ADSA-P : Axysymmetric Drop Shape Analysis by Profile

AmpC: Cephalosporinases

API 20^{E}: Analytical profile index 20^{E} (E = Enterobacteriaceae)

BGN: Gram negative bacilli

BN: Nutrient broth

C / N : carbon / nitrogen

CMC : Critical micellar concentration

CMC : Carboxy Methyl Cellulose

CMC: Carboxymethylcellulose

D : Diffusion coefficient

DHA: Arginine dihydrolase

DNS : 3,5-Dinitrosalicylic acid

Dyne / cm : Dyne Per Centimeter

E24 : Test of emulsification

ESBL: extended spectrum β-lactamase

GlcNAc : N-acetylglucosamine

GLU: Glucose

GN: Nutrient agar

H2O2: hydrogen peroxide

H2S: Hydrogen Sulphide

HCN : Hydrogen cyanide

He: Emulsion height

HLB : hydrophilic-lipophilic balance

Ht: Total height of the mixture

I: Intermediate

IAA : Indole Acetic Acid

IND: Indole

LDC: Lysine Decarboxylase

MH: Muller Hinton

NaCL: Sodium chloride

NaOH: Sodium hydroxide

NAPL : Non Aqueous Phase Liquids

NIT: Nitrate IV

O/W : oil-in-water

ODC: Ornithine Decarboxylase

PAH : aromatic hydrocarbons polycyclic

PDA : Potato Dextrose Agar

pH: Potential of Hydrogen

R: Resistance

rpm: Rotation per minute.

S: Sensitive

sp: species

spp: species plurimae

TLC : Thin layer chromatography

UFC: Colony Forming Unit

URE: Urea

UV : UltraViolet

VP: Reaction of Voges-Proskauer

W/O : water-in-oil

Summary

Introduction

Molds and yeasts, grouped together under the name "fungi", form a large group of microorganisms. Like bacteria, fungi are found everywhere, they are found in soil and decaying organic substances, in water, and in the air, which carries large amounts of spores (Boudih, 2011).

Over the past two decades, the development of microbiology has resulted in the production of a wide variety of biological materials, genetically modified or not, and capable of being used in processes for the production of vitamins, hormones, vaccines and drugs. Enzymes of microbial origin have various properties and specificities (Botton et al., 1999). There are countless ways of using enzymes. Indeed, fungal enzymes can be used in many industries.

The genus *Trichoderma* are known for their ability to produce high added value secondary metabolites. These metabolites may exhibit anticancer, antibacterial, anti-free radical, antioxidant and antifungal activities, etc (Carboué et al., 2017; De la Cruz Quiroz et al., 2015). Several *Trichoderma* species produced cell wall degrading enzymes such as proteases, glucanases and chitinases, these hydrolytic enzymes has significant effects on growth and development of the plant pathogens (Sivan et al. 1984; Coley-Smith et al. 1991; Lunge et al. 2012). Furthermore, *Trichoderma* species are able to produce antibiotics such as peptaibols, mycotoxins and more than 100 metabolites with antibiotic activities including polyketides, pyrones, terpenes, metabolites derived from amino acids and polypeptides (Sivasithamparam &Ghisalberti2002).

Les espèces de *Bacillus* sont également dotées de plusieurs caractéristiques qui font d‘eux bons candidats pour être utilisés comme producteurs de MAM à l‘échelle industrielle. En effet, les *Bacillus* sp. Produisentune diversité impressionnante de métabolites secondaires bioactifs y compris les MAM, biodégradables et compatibles avec l‘environnement (Singh et Cameotra., 2013). En plus, ils sont considérés par l‘OMS et la FAO comme des organismes généralement reconnus comme étant sans danger (GRAS : Generally Recognized As Safe). Enfin, leur particularité de former de spores qui leur confere l‘aptitude à résister aux conditions défavorables pendant la production industrielle en fermenteur et après leur séchage par lyophilisation en poudre qui se conserve longtemps. Depuis plus de 30 ans, la moitié des médicaments approuvée par la US Food and Drug Administration, incluant 74 % des anticancéreux et 73 % des antibiotiques, est issue des produits naturels ou leurs dérivés synthétiques (Demain., 2014 ; Newman et Cragg., 2016). Parmi les produits naturels mis sur

le marché, les NRP (lipopeptides) et les PAM ribosomaux (bactériocines) sont les métabolites spécialisés les plus représentés du fait de leur vaste gamme de bioactivités permettant de traiter de nombreuses pathologies infectieuses (Butler et al., 2014., 2017).

Among these many secondary metabolites is the synthesis of biomolecules called biosurfactants. Biosurfactants can be defined as the surfactant biomolecules produced by microorganisms with a wide range of applications. In recent years, due to their unique properties such as specificity, low toxicity and relative ease of preparation, these surfactant biomolecules have attracted great interest. Due to their unique functional properties, biosurfactants have been used in several industries (Volkering et al., 1998).

The worldwide production of surfactants amount increased to 17 million metric tons in 2000 (including soaps) expected future growth rates of 3-4% per year globally (Rahman and Gakpe ,2008). These chemically synthesized surfactants are mainly petroleum based and are usually non biodegradable thus remain toxic to the environment they find themselves. Also these compounds may bio-accumulate and their production processes and by-products can be environmentally hazardous, due to this increasing awareness on the need to protect the ecosystem, environmental scientist have been tightening environment regulations thus necessitating an increased interest in surfactants of microbial origin as possible alternatives to chemically synthesized ones (Benincasa, 2007).

This work aims to study the ability of strains of the *Trichoderma* genus and of the *Bacillus* genus to produce enzymes of biotechnological value and biosurfactants under favorable conditions to their growth. The realization of this modest study is structured in three sections: the first presents a bibliographic review, the second describes the material and methods used, and a third part devoted to the results and discussion which will be followed by a conclusion and perspectives.

Part I
Bibliographic Review

Chapter I Biosurfactants

1. General information

Biosurfactants are surfactant molecules produced by certain micro-organismes. Both their nature and their surfactant power are highly dependent on the type of the microorganism used (Bacteria, yeast, fungus), the strain tested as well as the nutrient substrate available for their cell development.

Among the various biosurfactants identified, we now find glycolipids, lipopeptides, phospholipids, neutral lipids, fatty acids or lypopolysaccharides. Like their synthetic chemical analogue, they can have emulsifying, foaming, wetting or dispersing properties. Some of these properties can, moreover, be preserved under extreme conditions such as pH acids, high temperature….(Herry and Bellon-Fontaine, 2001).

Given their potential and their harmlessness, they are still used in different fields of application such as environment, oil industry, agronomy or cosmetology and should quickly find their place in new application sectors such as the agro-food, pharmaceutical or even medical field (Herry and Bellon-Fontaine, 2001).

2. Definition

Surfactants (Surface Active Agents) are agents with surface activity (surfactants), chemically or biologically synthesized (biosurfactants) (Al-Arajil *et al.*, 2007).

The microbial surfactant or biosurfactant is a surfactant or surfactant having a hydrophilic pole and a hydrophobic pole, capable of being produced by a wide variety of microorganisms.

This synthesis is the result of a bioconversion carried out by a strain microbial from a hydrocarbon or carbohydrate substrate (Mimouni, 1995).

The interest in the use of biosurfactants comes from their interfacial properties which are a consequence of their molecular structure (hydrophilic and lipophilic pole) **(Fig. 01)** and which gives them an emulsifying power (Banat *et al.*, 1991).

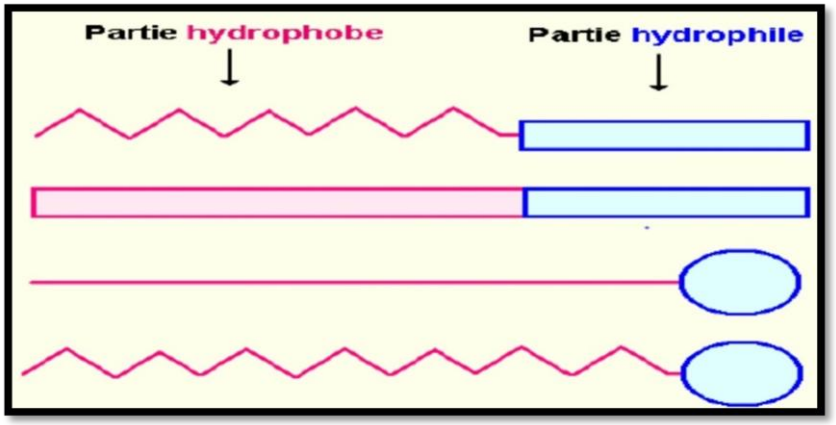


Figure 01: The most used representations to illustrate the two parts (hydrophilic and hydrophobic) amphiphilic molecules of surfactants (Larpent, 1995).

3. Classification of biosurfactants

Biosurfactants are classified according to their molecular weight and chemical structure (Rosenberg and Ron, 1999).

3.1 Classification according to molecular weight

According to Rosenberg and Ron (1999) there are two types of biosurfactants

- High molecular weight biosurfactants
  - Lipoproteins.
  - Complex fatty acids.
- Low molecular weight biosurfactants
  - Glycolipids.
  - Neutral lipids.
  - Lipoproteins.

3.2 Classification according to chemical structure

Based on the chemical structure, the following biosurfactants are distinguished, glycolipids, lipopolysaccharides, lipoamino acids, lipopeptides, proteincomplexes polysaccharides, phospholipids, fatty acids and neutral lipids (Georgiou *et al.*, 1992; Wagner and Lang, 1996).

According to Lang and Wullbrandt (1996) these different structures are arranged in four main classes of biosurfactants

-Glycolipids.

-Lipopeptides.

-Polymers: lipoproteins and lipopolysaccharides.

-Phospholipids, monoglycerides, diglycerides and fatty acids.

4. Physico-chemical properties of biosurfactants

According to Larpent (1995), biosurfactants are amphiphilic molecules with two functional parts

- A lipophilic, non-polarized part (soluble in non-polar solutions).
- A hydrophilic, polarized part (soluble in aqueous solutions).

This structure gives biosurfactant molecules a number of properties specific physico-chemical (Marcou, 1989; Larpent, 1995).

4.1 Physical characteristics of biosurfactants

4.1.1 Reduction in surface tension

Biosurfactants dramatically decrease the surface tension of the water itself in very dilute solutions. This appears in the following example: surface tension pure water is 72.80 mN / m (Laurila, 1985).

4.1.2 Critical micellar concentration (CMC)

The critical micellar concentration (CMC) is by definition the concentration of a surfactant (biosurfactant) above which some of the molecules dispersed within the aqueous solution come together as a micelle (Pore, 1992).

Micelles are formed when hydrophobic portions unable to form hydrogen bonds in the aqueous phase create a strong increase in energy free from the system.

One way to lower this energy is to isolate the hydrophobic part of water by adsorbing in to organic matrices or forming micelles (Haigh, 1996). In fact, in the micelles, the hydrophobic parts are grouped together towards the center and the hydrophilic portions remain in contact with water . **(Fig. 02)**

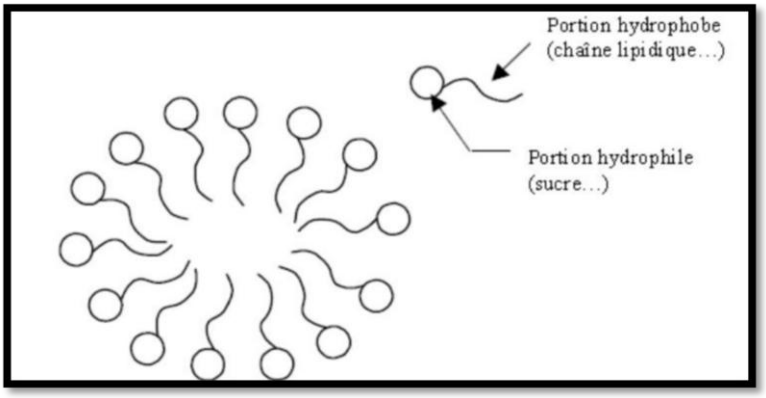


Figure 02: Schematic representation of a biosurfactant micelle (Gabet, 2004).

4.2 Modification of the structure of two-phase systems

An emulsion consists of the dispersion of one liquid in another in the form of microscopic droplets 0.1 to 100 nm in diameter. Generally speaking, the larger the diameter is smaller the more stable the emulsion is, depending on the types of dispersed phases, we distinguish the water-in-oil (W / O) and oil-in-water (O / W) emulsions, oil here refers to the non-phase aqueous (Zajic and Seffens, 1984).

4.2.1 Emulsification

The dispersion of one liquid in another is never completely stable. For prepare a stable emulsion, a third component can be added which will have properties distinct, first the formation of droplets, then once they have formed their stabilization (Zajic and Seffens, 1984).

4.2.2 Hydrophilic-lipophilic balance

The type of emulsion (water in oil or oil in water) that the emulsifier can improve, is indicated by its affinity for oil and water, quantified by a value called the hydrophilic-lipophilic balance or HLB. The latter is defined as the ratio between 20 times the molecular mass of the hydrophilic part and the molecular mass of the whole molecule. The most hydrophobic biosurfactants will have values below 7 (case of trehalose dimycolates), often between 3 and 6 (Zajic and Seffens, 1984), while the most hydrophilic such as rhamnolipids will have a coefficient greater than 7 (Haferburg *et al.,* 1986) most often between 8 and 10 (Zajic and Seffens, 1984).

5. Mode of action

Biosurfactants act in different ways, by mobilization and by solubilization.

5.1 Mobilization

By definition, mobilization depends on the ability of biosurfactants to reduce interfacial tensions between NAPL (Non Aqueous Phase Liquids) and water when the surfactant molecules share on the surface of the NAPL (Mc cray *et al.,* 2001).The mobilization is subdivided into two stages: a displacement stage and a dispersion.

The displacement corresponds to the release of the drops of NAPL from the porous medium thanks to the reduction of interfacial tensions.

Dispersion is the process in which NAPLs disperse in the aqueous phase in the form of fine emulsions (Bai *et al.,* 1997).

5.2 Solubilisation

Solubilization is the preferred mode of action for treating soils; it results from micelle formation. Solubilization is based on the ability of micellar solutions to increase the solubility of hydrophobic contaminants (Gabet, 2004). Generally, below the CMC, the monomers in solution have little or no effect on most hydrocarbons (Pennell *et al.,* 1993). Above the CMC, the phenomenon of incorporation of hydrocarbons into micelles appear, thus allowing an increase in their apparent solubility (Gabet, 2004). Thus, for the micelles to form it is necessary to add enough biosurfactant so that the aqueous phase concentration reaches the actual CMC. Sorption therefore depends on the concentration of biosurfactant in solution (Van Dyke *et al.,* 1993). Bai *et al.,* (1998) indicate that at high concentrations, solubilization is the primary mechanism.

6. Toxicity and biodegradability

Toxicity tests carried out by Haba *et al.,* (2003) found that rhamnolipids produced by a strain of *Pseudomonas* were classified as non-irritant and not toxic.

A study by Deschenes *et al.,* (1996) indicates that a biosurfactant produced by a strain of *Pseudomonas* did not affect overall microbial activity, even at high concentrations. In addition, these authors indicate that this biosurfactant was biodegradable.

According to Tabka (2015) biosurfactants are biodegradable (100%), non-toxic, biocompatible, hence the possibility of their use in pharmaceutical preparations and agro-food.

7. Potential of biosurfactants (advantages)

Biosurfactants can be as effective as and more beneficial than synthetic surfactants. They are highly specific, biodegradable, biocompatible with the environment (Mulligan, 2009), less sensitive to temperature, pH and extreme salinity (Suwansukho *et al.,* 2008), less toxic and can be synthesized into large amounts on expensive energy sources such as petroleum products (Sarubbo *et al.,* 2006), but also on renewable resources.

Another advantage of biosurfactants is the possibility of their modification by biotransformation to generate new products for specific needs (Samadi *et al.,* 2007). With the introduction of certain functional groups, biosurfactants provide new properties, thus surpassing chemical surfactants in many applications (Huang *et al.,* 2010).

These biomolecules exhibit a wide range of functional properties which allow their exploitation in various fields (Prommachan, 2002). However, it is not easy to rationalize the difference between their natural roles and our applications (Cameotra *et al.,* 2009; Hamme *et al.,* 2006; Marqués *et al.,* 2009).

Their ability to modify interfacial properties and their self-assembly into micelles or other nanostructures is crucial for several industrial processes such as the formation layers, diffusion, foaming, detergency, micellar catalysis, etc. (Xiaoyang *et al.,* 2009).

These biomolecules can also induce phase separation, solubilization, viscosity reduction (Ochsner *et al.,* 1995), flocculation, aggregation, desorption (Karanth *et al.,* 1999) as well as the stabilization and destabilization of emulsions (Huang *et al.,* 2010). Excellent technical characteristics such as emulsion formation (50-100%), lowering of the interfacial tension (~ 0.1 dyne / cm), lowering of the tension superficial (~ 27 dyne / cm), foaming power (stable form (15min)), wetting power, CMC (20-2000 mg / l) and antibiotic or fungicidal power (Tabka, 2015).

8. Physiological role of biosurfactants

Biosurfactants are produced by a variety of microorganisms, for example secretion as extracellular agents or attached to the cell membrane, mainly during their growth on water insoluble substrates. The physiological role of biosurfactants in producing microorganisms is not still well understood (Desai and Banat, 1997). The natural functions of biosurfactants; proposed or proven in the literature include: adhesion, emulsification, improvement bioavailability and desorption and defense strategies (Franzetti *et al.,* 2010).

8.1 Adhesion

Adhesion is a physiological mechanism for growth and survival microbial cells in natural media. A special case of membership is the growth of bacteria on water-insoluble

hydrocarbons, it is one of the main processes of bacterial transfer (Cameotra *et al.*, 2010). Given that the enzymes necessary for the oxidation of hydrocarbons are found on the cell membrane, the microorganism must therefore first come into contact with its substrate. In some situations, we see the formation of a biofilm due to secretion of the biosurfactant, which allows certain microorganisms to attach to some surfaces and inhibit others (Cameotra *et al.*, 2010). Another case is the modification of cellular hydrophobicity of microorganisms as needed. The microorganisms increase or decrease their cellular hydrophobicity by exposing respectively outside or inside the hydrophobic part of the cell membrane bound biosurfactants (Franzetti *et al.*, 2010).

8.2 Emulsification

Microorganisms hydrocarbonoclasts produce biosurfactants / bioemulsifiers allow it to grow on hydrophobic, water-insoluble substrates; by increasing the contact surface between the oil droplets and them.

8.3 Bioavailability and desorption

The major problem with high molecular weight hydrophobic compounds is their low solubility in water which increases their adsorption to surfaces, and which limits their availability to degrading microorganisms. Biosurfactants could improve this availability by desorbing them from surfaces and increasing their apparent solubility (Cameotra *et al.*, 2010). Biosurfactants that have a excellent surface activity are very effective in mobilizing compounds hydrophobic adsorbs on surfaces. An important property of surfactants is the formation of micelles beyond the critical micellar concentration (CMC). Micelles allow biosurfactants to increase the apparent solubility of hydrophobic compounds by incorporating them into their bodies, which in turn facilitates their microbial degradation (Eddouaouda, 2012). Desorption is thus advantageous for microorganisms in the event that conditions become unfavorable (for example accumulation of toxins or depletion of nutrients). At this point, the desorption allows microorganisms to expel from its habitat and seek other more favorable places (Cameotra *et al.*, 2010).

8.4 Defense strategies

Microorganisms secrete biosurfactants to survive in their natural habitats to protect against other microbes (Cameotra *et al.*, 2010).

9. Production of biosurfactants

The success of the use and production of biosurfactants requires a reduction production costs. This reduction can be achieved through the valuation of of low cost product growth substrates. For example, Makkar and Cameotra (2002) indicate that it is possible to use waste and agricultural products of a share to reduce costs, but also to reduce the quantities of waste to be treated various companies (used engine oils, etc.). A study carried out by Mercade and Manresa (1994) reports rhamnolipid production rates of 1.4 g / L for *Pseudomonas* grown on industrial by-products.

9.1 Producing micro-organisms

Biosurfactants are mainly produced by microorganisms growing aerobically in an aqueous medium containing one or more sources of carbon, such as carbohydrates, oils or hydrocarbons. Those microorganisms are generally yeasts, fungi or bacteria.

The main physiological role of the biosurfactant is to enable micro-organisms to grow on insoluble substrates by reducing stress interfacial between water and substrate, making the latter more easily accessible (Mata-Sandoval *et al.,* 2000).

The bacteria used to produce biosurfactants generally come from soils contaminated with hydrophobic molecules such as aromatic hydrocarbons polycyclic (PAH). They are therefore isolated from their natural environment and are cultivated in laboratory. This allows tests to be carried out to choose the best carbon source. And to optimize the culture media in order to obtain a maximum production rate. In all cases, the biosurfactant obtained is a mixture of several molecules. Through example, in the case of the biosurfactant produced by a strain of *Pseudomonas* aeruginosa UG2, a mixture of four rhamnolipids is obtained (Van Dyke *et al.,* 1993). Abalos *et al.,* (2001) indicate that seven homologues of rhamnolipids have been identified in cultures of *Pseudomonas aeruginosa* AT10.

Although many species produce biosurfactants, the regulation of their synthesis is still poorly understood, except for the strains of *Pseudomonas aeruginosa* and *Bacillus subtilis* which are the most studied bacteria (Banat, 2000). The biosynthesis of rhamnolipids by *Pseudomonas aeruginosa* strains is carried out during the exponential phase of growth and is due to a sequential transfer of glycosyl catalyzed by specific rhamnosyl transferases: there is donor intervention rhamnosyl, transferases. Two different transferases allow the formation of four different rhamnolipids (koch *et al.*, 1991). Biosurfactant molecules are associated with the membranes of bacteria and are also secreted into the medium (Thangamani and Shreve, 1994).

Bacteria produce low molecular weight molecules, which decrease effectively interfacial tensions, as well as polymers of high molecular weight, which bind strongly to surfaces (Ron and Rosenberg, 2002). Low mass biosurfactants molar are usually glycolipids, while those of high molar mass are consisting of polysaccharides, proteins, lipopolysaccharides or lipoproteins. The latter are less effective in reducing interfacial tensions, but more effective to surround the oil drops and prevent them from coalescing.

One of the techniques used to monitor the production of biosurfactants is ADSA-P ("Axysymmetric Drop Shape Analysis by Profile") which simultaneously determines the angle of contact and surface tension of liquid thanks to the profile of a drop remaining on a surface of a solid. The drops containing the producing microorganisms are placed on a fluorethylene-propylene surface and the profile of the drop is determined.

Other methods have been described as hemolysis of the blood, which is a known characteristic of certain biosurfactants and an emulsification index (E-24) obtained on a kerosine (Banat, 1995). The drop-collapsing test is used to see the colonies bacteria that

produce biosurfactants: drops containing cells in suspension are placed on an oil-covered surface; if the drop remains stable, this demonstrates the absence of the surfactant (Jain *et al.*, 1991).

9.2 Biosynthesis of biosurfactant

The production of biosurfactants is a phenomenon commonly observed during growth of a microorganism on water-insoluble substrates and reduction of surface tension of the medium as well as the formation of a stable emulsion indicates a efficient production (Pruthi *et al.*, 1995). The presence of surfactant is necessary for obtain a stable emulsion between two pure non-missible liquids (Krepsky *et al.*, 2007).

Cameotra (2009) explains this phenomenon as one of the behaviors of microorganisms to increase the bioavailability of several hydrophobic substrates, which are little used because of their insolubility in water.

Indeed, these bacteria synthesize biosurfactants which are either molecules intercellular, extracellular or localized on the cell surface (Prabhu *et al.*, 2003) to facilitate the diffusion of hydrocarbons or their derivatives inside the cell bacterial in order to degrade them (Al-Arajil *et al.*, 2007).

It is also interesting to know that the intermediate oxidation products of hydrocarbons do not persist in the soil like pollutants, but will be partially reabsorbed and metabolized by the same predominant microorganisms, as revealed The study of Dashti and it's collaborators in 2008.

However, biosurfactants can have other roles as important as emulsification, for example: adhesion to solid surfaces and the formation of biofilms (Acinobacter alasan), regulation of cellular energy level (sophorosis of T. bombicola), bactericidal activity (gramicidin, polymexin, surfactin), the pathogenicity of certain bacteria (*Pseudomonas rhamnolipids*), as well as the trapping of heavy metals (Vandecasteele, 2008).

9.2.1 Glycolipids

Glycolipids consist of carbohydrates in combination with a long chain of aliphatic acids or aliphatic hydroxy acids (Healy *et al.*, 1996; Ron *et al.*, 2002).

Many bacteria produce glycolipids, among these we distinguish: rhamnose-lipids (or rhamnolipids), glucose-lipids, trehalose lipids, pentasaccharide-lipids and varied mix.

According to Laurila (1985), bacterial glycolipids are grouped into two categories: glycosyl-diglycerides, and acylated sugar derivatives.

9.2.2 Lipopeptides

Lipopeptides are made up of a lipid attached to a polypeptide chain. Among Bacterial biosurfactants of lipopeptide nature, we distinguish: surfactin, lichenysin, lipoamino acids (ornithine), antibiotics (Arima *et al.*,1986; Grangermard *et al.*, 1999; Laurila, 1985; Zadjic and Mohamedy, 1984).

9.2.3 Phospholipids

Phospholipids are formed from alcohol and phosphate groups and chains lipid (Healy *et al.*, 1996). Bognolo (1999) indicates that although present in all microorganisms, there are few examples of extracellular production.

Various phospholipids are isolated from free cell culture of *Thiobacillus thiooxidans*. These phospholipids bind to the element sulfur which is necessary for growth cellular (Rosenberg and Ron, 1999).

According to the authors, the composition and type of major phospholipids in bacteria, yeasts and fungi, depend on the carbon source used and the conditions of growth culture, also the pH value of the culture medium.

9.3 Parameters influencing production

The type and amount of biosurfactants produced vary with the composition of the medium (source of carbon or other nutrients) and growing conditions (temperature, agitation, pH, ...).

9.3.1 Influence of the carbon source

The source of carbon is one of the parameters most influencing the production of biosurfactants, either by induction or by reducing the quantity produced. The sources of water soluble carbon (glycerol, glucose, mannitol or ethanol) are used to produce rhamnolipids. However, the yields appear to be lower than those obtained on insoluble substrates.

9.3.2 Influence of nitrogen

Numerous studies have shown that the synthesis of rhamnolipids occurs when there is had an excess of carbon in the medium or when the nitrogen was in limiting amount. Nitrogen can be supplied in different forms depending on the producing bacteria (Lang and Wullbrandt, 1999), however, in order to have optimum production yields, it is therefore necessary to have an ideal C / N ratio, and especially that nitrogen is a limiting factor (stress) for promote the production of biosurfactant (Gabet, 2004).

9.3.3 Influence of pH

The pH, which is a very important factor and which is more or less relative to the production, issaying a value higher or lower than the optimal one influences the production.

9.3.4. Influence of mineral salts

It would seem that a limiting concentration of magnesium, calcium, potassium sodium or trace elements induce an increase in production (Guerra Santos *et al.*, 1986).

9.3.5 Influence of oxygen

The availability of oxygen can also affect production through its effect on cell activity or growth (Gabet, 2004).

9.3.6 Influence of stirring speed

The culture media are agitated during the production of biosurfactant. For bacteria, an increase in the stirring speed induces an increase in the shearing speeds and therefore a lower yield. The opposite effect is observed when the producing organisms are yeasts (Desai and Banat, 1997).

10. General information on extraction methods and identification of biosurfactants

10.1 Methods of extraction of biosurfactants from the environment growth

To extract the biosurfactant from the growth medium, it is first necessary to separate the bacteria from this culture medium (by centrifugation for example).

The recovery of biotensiactants depends mainly on their ionic charge, their solubilization thus their localization (intracellular, extracellular or linked to cells).

The most used techniques are extractions by solvents: chloroforms / methanol, butanol, ethyl acetate, etc. (Desai and Banat, 1997) or techniques based on biosurfactant precipitation. These extractions can be carried out directly or after sedimentation of the producing cells.

It is possible to acidify the medium and then extract the surfactant with a mixture of solvents (chloroform / methanol) as done by Parra *et al.*, (1989). Generally, the acidification allows precipitation of the surfactant. Following this precipitation, a stage of Centrifugation followed by washing is necessary to recover and purify the surfactant.

There are continuous recovery techniques. Reiling *et al.*, (1986) carried out a adsorption on Amberlite XAD-2 followed by purification and lyophilization, giving yields varying from 60 to 90% in purity.

The ability of biosurfactants to aggregate to surfaces has also been used for retain on filtration membranes. For example an XM-50 membrane, in the threshold of cutoff is 5000 D, was used on 97% pure surfactin and recovery rate of 98% is obtained, while a membrane with a higher cut-off threshold (10,000 D) provides a recovery efficiency of 92% (Desai and Banat, 1997).

10.2 Biosurfactant identification methods

Once the products (biosurfactants) are purified, their structure must be determined. Among the various qualitative analyzes, the following are used most frequently:

Ultraviolet and visible light adsorption spectrometry (UV-VIS) which detects the presence of chromophore (Spoeckner *et al.*, 1999). Infrared spectrometry which determines the functional grouping (Peypoux *et al.*, 1999).

Mass spectrometry which gives molecular weight, indications on the structure and which has high resolution, provides elemental analysis of the molecule (Daniels *et al.*, 1999).

High-field nuclear magnetic resonance (proton and carbon 13 NMR) indicates the structure and conformation of the compounds to be analyzed (Daniels *et al.,* 1999).

11. Application and role of Biosurfactants

Biosurfactants have the ability of being biodegradable and non-toxic. In addition, some of them, secreted by extremophile organisms, remain functional despite drastic conditions of temperature, salinity or pH (Banat, 2000), this is why they can be used in many fields. The table below summarizes the applications and roles of biosurfactants in different areas:

Table 01: Different potential applications and roles of biosurfactants. (Santos *et al.,* 2016).

Industry	Application	Role of biosurfactants
Environment	Bioremediation; Spill clean-up operations hydrocarbons; Soil remediation and rinsing	Emulsification of oils, lowering of interfacial tension, dispersion of oils, solubilization of oils, wetting, spreading, detergency, foaming, corrosion inhibition in fuel oils and equipment, rinsing the soil.
Oil	Improved oil recovery; Demulsification	Emulsification of oils, lowering of interfacial tension, de-emulsification of oil emulsions, solubilization of oils, reduction of viscosity, dispersion of oils, wetting of solid surfaces, spreading, detergency, foaming, corrosion inhibition in fuel oils and equipment.
Operationmining	Heavy metal cleaning operations; Soil remediation; Flotation	Mooring and foaming, collectors and foams, metal removal ions from aqueous solutions, soils and sediments, heavy metals sequestering, spreading, corrosion inhibition in oils.
Food	Emulsification and demulsification; Functional ingredient	Solubilization of flavored oils, control of consistency, emulsification, wetting agent,

		spreading, detergency, foaming, thickener.
Medication	Microbiological; Pharmaceutical and therapeutic	Anti-stick, antifungal agents, antibacterial, antivirals, vaccines, gene therapy, molecules immunomodulatory.
Agriculture	Biocontrol; Fertilizers	Wetting, dispersion, suspension of pesticides powder and fertilizers, emulsification of pesticide solutions, facilitation of microbial biocontrol mechanisms, elimination of plant pathogens and increased bioavailability of nutrients for beneficial microbes associated with plants.
Beauty	Health and beauty products	Emulsification, foaming agents, solubilization, wetting agents, cleaning agents, antimicrobial agents, mediators of enzymatic action.
Cleaning	Laundry detergents	Detergents and disinfectants for laundry, wetting, diffusion, corrosion inhibition.
Textiles	Preparation of fibers; Dyeing and printing; Textile finishing	Wetting, penetration, solubilization, emulsification, detergency and dispersion, wetting and emulsification in finishing formulations, softening.
Nanotechnology	Synthesis of nanoparticles	Emulsification, stabilisation.

13. Biomedical application and therapeutic and antimicrobial activity Biosurfactants

According to Sneha (2012), several biosurfactants have shown antimicrobial action against bacteria, algae, fungi as well as viruses.

The iturine lipopeptide from *Bacillus subtilis* had shown antifungal activity (Besson *et al.*, 1976). Inactivation of the herpes viral envelope and retrovirus was observed with 80 mM of surfactin (Volkering *et al.*, 1997).

Rhamnolipids suppress the growth of harmful algae in flowering trees such as *Heterosigma akashivo* and *Protocentrum dentatum* at concentrations varying from 0.4 to 10.0 mg / l. A mixture of rhamnolipids obtained from *Pseudomonas aeruginosa* showed activity inhibitor against bacteria *Escherichia coli*, *Micrococcus luteus* and *Alcaligenes faecalis* (32 mg / ml), *Serratia marcescens* and *Mycobacterium phlei* (16mg / ml) as well as *Staphylococcus epidermidis* (8 mg / ml) in addition to excellent antifungal properties against *Aspergillus niger* (16 mg / ml), *Chaetonium globosum*, *Penicillium crysogenum*, *Aureobasidium pullulans* (32 mg / ml) and the phyto-pathogens *Botrytis cinerea* and *Rhizoctonia solani* (18mg / ml) (Kosaric *et al.,* 1993). Rhamnolipids and sophorolipids have been shown to be effective as antifungal agents against phyto-pathogens in seeds. A glycolipid surfactant from *Candida antartica*, has showed antimicrobial activity particularly against Grampositive bacteria (Kitamoto *et al.,* 1993).

CHAPTER II Fungi that produce biosurfactants

A) Introduction to the mushroom kingdom

1. Presentation of the fungal kingdom

The fungi, called Mycetes or Fungi, constitute a heterogeneous group. All are devoid of chlorophyll, which condemns them to a total heterotrophy vis-à-vis carbon (Bouchet *et al.*, 2005).

Some 1.5 million fungus grow on the surface of the globe and we know only 5 to 10% of this amount. Their number is estimated to date at around 100,000 species, but it is probably higher (Rubatzky *et al.*, 1997, ESIAB, 2021, Guy, 2015). The majority of fungi live as saprophytes in the soil, on dead or living plants, but only on their surface and without causing them damage. Many species are plant pests causing an economic problem. A few hundred are opportunistic and can become pathogenic for humans and animals. Finally, various species are symbiotic, either associated with *algae* in *lichens*, or associated with roots constituting mycorrhizae (Yuka and Hirokazu, 2014).

The basic cellular organization of fungi is the *thallus*, which makes up the *vegetative* system. This is characterized by a wide variety of structures, which range from a single-celled (yeast) most often to a filamentous form, which can show a considerable degree of differentiation. The set of filaments is called the mycelium. There are never real tissues like in higher plants or animals. They reproduce asexually and / or sexually through spores (Yuka and Hirokazu, 2014; Guy, 2015).

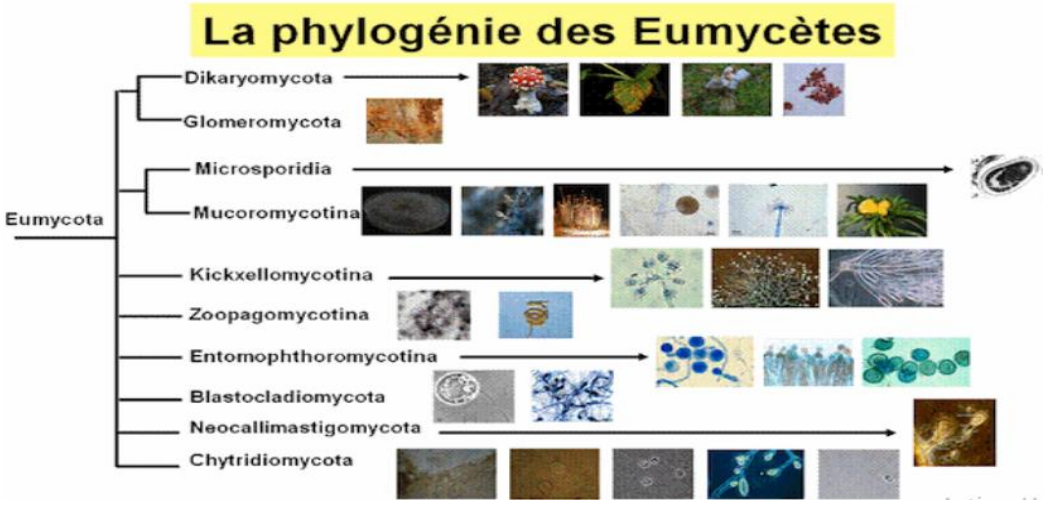


Figure 03: The phylogeny of *Eumycetes* (ESIAB, 2021).

B) The genus *Trichoderma*

1. General information about *Trichoderma*

Trichoderma species are ubiquitous imperfect saprophytic fungi belonging to the class of *Deuteromycetes*. The perfect form belongs to the class of *Ascomycetes* (*Hypocrea*), *aerobic* and *mesophilic*, durably versatile, which are commonly found in the soil. This genus includes approximately 20 cellulolytic species (http://mycota-crcc.mnhn.fr consulted on March 25, 2014).

They are usually crop contaminants and classically devoid of any pathogenicity. However, rare cases of *Trichoderma mycosis* (ear infections, pneumopathies and peritonitis in the *immunocompromised*) have been reported (Chabasse *et al.,* 2002; Schuster *et al.,* 2010) .

Various species produce antifungal metabolites such as trichoriazins and many mycotoxins (trichothecenes and cyclic peptides, gliotoxin, isocyanides, T-2 toxin, trichodermin) (Schuster *et al.,* 2010).

Trichoderma have an interest in the production of enzymes, certain species are used in biological control for the protection of trees and plant crops against the attack of phytopathogenic agents (Papavizas., 1985).

Some characteristics of the different *Trichoderma* species are shown in **(Fig. 04)** (Schuster *et al.,* 2010).

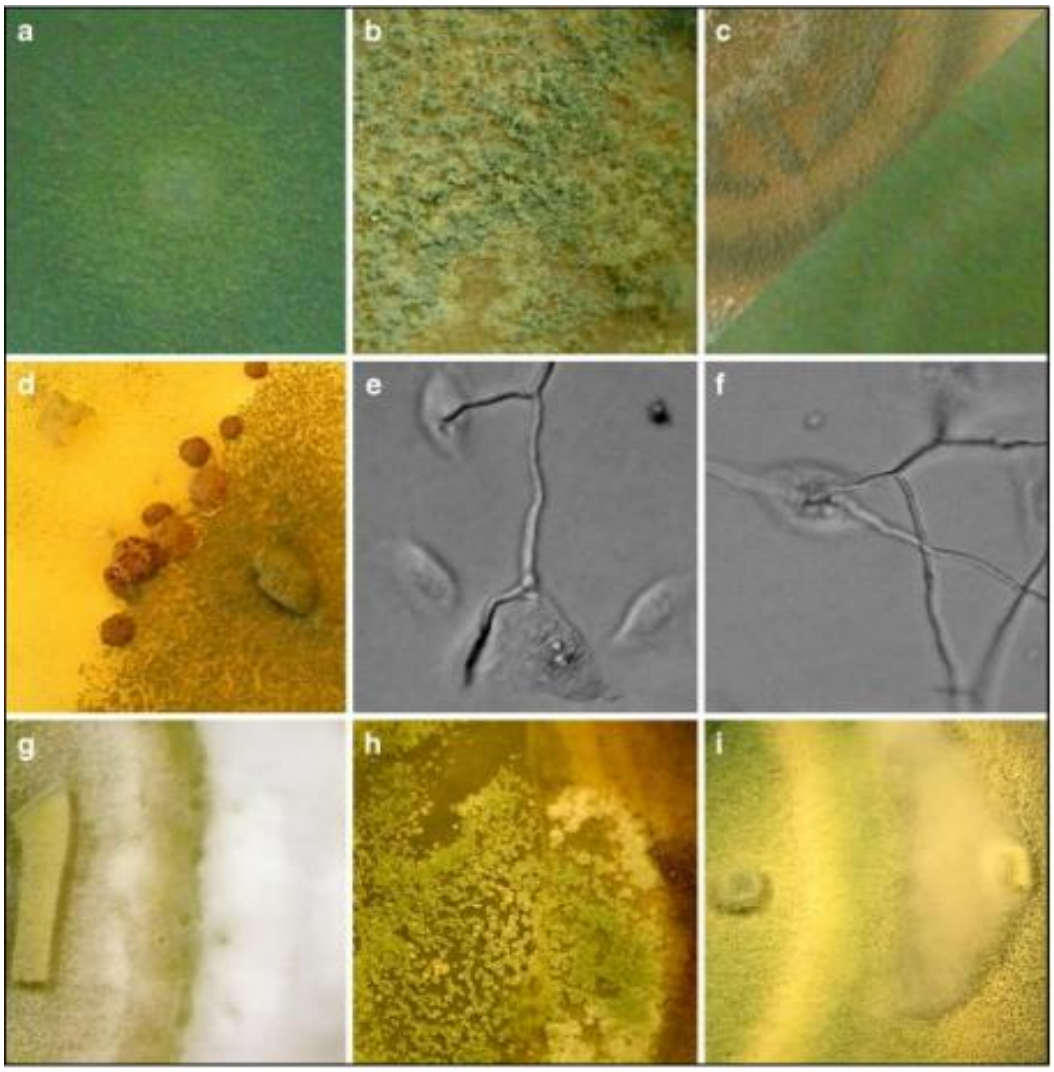


Figure 04: Characteristics of *Trichoderma* species; a: *T reesei* and b: *T. atroviride* growing on the plates c: *T. reesei* or *H. jecorina*. its increased growth more and more in daylight, formation of fruiting organ of *T. reesei* when crossed with a nature isolate of *H. jecorina*. (e, f) *T. longibrachiatum* which germinates and grows on human cells, g: *T. reesei* (left) during confrontation with Pythium ultimum h (right) *T. atroviride* (left) during confrontation with *R. solani* (right) (Schuster *et al.*, 2010).

2. The *Trichoderma* taxonomy

The division of the genus *Trichoderma* into species has been the subject of many studies and much controversy. In the living kingdom the limits of the "species" are based on the possibility of crossing between individuals. Or, *anamorphic* fungi of the genus *Trichoderma*, as such, have no known sexual reproduction, and therefore this trait cannot be used for their systematics. We then base ourselves on the cultural aspects and the morphology of the *sporogenous* apparatuses (Roquebert, 1996).

Modern fungal taxonomy has abolished the phylum *Deuteromycotina*, to which the genus *Trichoderma* belonged. The current taxonomic position of *Trichoderma* sp. Presented as follows (Bissett, 2004).

Phylum *Eumycetes*

Sub-branch *Ascomycotina*

Course *Sordariomycetes*

Orders *Hypocreal*

Family *Hypocraceae*

Genus *Trichoderma*

Table 02: Systematic sections of *Trichoderma* sp and some of the species belonging to it, according to Bisset (1991 a and b). * The aggregated species of Rifai (1969)

Trichoderma				
Section 1 Trichoderma	**Section 2 Pachybasium**	**Section 3 Hypocreanum**	**Section 4 Longibrachiatum**	**Section 5 Saturnisporum**
*T.viride**	*T.virens*	*T.lactea*	*T.longibrachiatum**	*T.satunisporum*
*T.koningii**	*T.hamatum**	*Comprend les formes anamorphiques d'hypocrea*	*T.pseudokoningii**	*T.ghanense*
*T.aureoviride**	*T.harzianum**		*T.citrinoviride*	
T.atroviride	*T.piluliferum**			
	*T.polysporum**			
	T.fasciculatum			
	T.flavofuscum			
	T.crasum			
	T.croseum			
	T.minutsiporum			
	T.tumentosum			
	T.fertile			
	T.longipilis			
	T.oblongisporum			
	T.strigosum			
	T.strictipillis			
	T.pubescens			
	T.spirale			

3. Cultural characters

These fungi have a very rapid and extensive growth on Sabouraud's medium at 25 ° C. (Chabasse *et al.,* 2002). They produce woolly colonies, initially white in color, appearing with age as greenish tufts isolated or arranged in concentric rings on the culture medium.

The woolly, fast growing colonies are white, yellow-green or green in color. The hyaline *conidiophores*, more or less compact in tuft, are septate, regularly and abundantly branched at right angles to the main axis. The *phialides*, ovoid to ellipsoidal, isolated or grouped in small numbers (2-3) are arranged on the branches, perpendicular to the axis.

The *unicellular conidia*, round or ellipsoidal, smooth or warty, are produced in mucilaginous masses forming glomeruli at the top of the phialides (Roquebert, 1996; Chabasse *et al.,* 2002).

4. Morphology of *Tricoderma* sp

The macroscopic appearance of *Trichoderma* sp is appreciated from cultures on suitable nutrient agars, distributed in Petri dishes. Fungal colonies may be slightly flaky or compacted in clumps. Between these two extremes, there are intermediate aspects. The colonies are colored according to the pigmentation of the phialides. Five days after its germination, the conidium gives rise to a mycelium which is initially white and sterile in the shape of a circle. Two days later, a green color is visible on the aerial parts of the mycelium, corresponding to conidiogenesis. Other regular concentric circles form thereafter, and between the 16th and the 20th day a thick felting is superimposed on the culture (Boutoumou and Boumaza, 2016)

Under an optical microscope one can observe a mycelium composed of yellow hyphae, *septes*, branched with smooth walls. The conidiophores **(Fig. 05)** have a conical or pyramidal shape. Very branched, they carry phialides in the form of flasks or keels. In turn, the phialides carry the spores (Cournut, 1984; Landreau, 2001; Kubicek *et al.,* 2003).

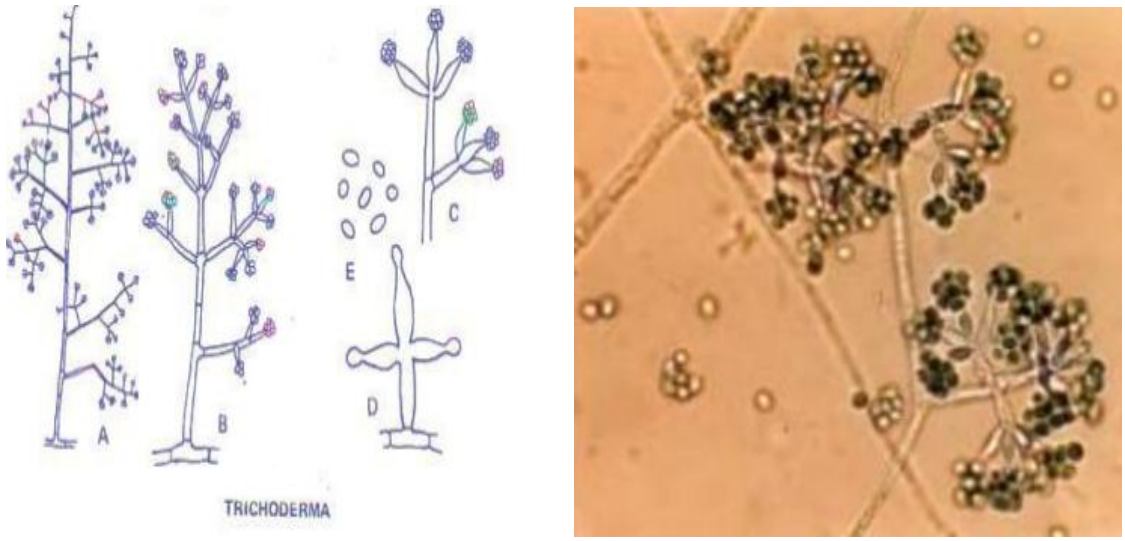


Figure 05: Morphology of *Trichoderma* sp. (A and B) Large conidiophores showing extensive branching. (C and D) Phialides showing the production of *conidia*. (E) *Conidia* (Barnett and Hunter, 1977) and microscopic appearance of *Trichoderma* sp (Rahman *et al.,* 2009).

5. Habitat of *Trichoderma*

Thanks to its great ability to adapt to different climatic conditions, the genus *Trichoderma* is very widespread in nature, both in terrestrial and marine environments (Roquebert, 1996; Esposito and Silva, 1998).

Indeed, *Trichoderma* sp. are notable for their rapid growth and ability to utilize different substrates and are, therefore, the major component in terrestrial and marine mycoflora (Widden and Abitrol, 1980; Kubicek *et al.,* 2003).

The *Trichoderma* sp. terrestrials develop in almost all soils (forest or cultivated) and on decaying plants. They frequently contaminate the compost of industrial cultivation of edible fungi, but are rarely parasites of living plants (Roquebert, 1996; Esposito and Silva, 1998)

The presence of *Trichoderma* sp in a terrestrial environment (6% of the total number of fungal species) seems comparable to that in a marine environment (6.4% to 10.4%) (Landreau, 2001). The abundance of *Trichoderma* sp in ecosystems is due to their ability to produce various bioactive substances and enzymes. They are therefore an important link in biological chains (Widden and Abitrol, 1980; Vining, 1990; Kubicek *et al.*, 2003).

6. Life Cycle *Trichoderma*

Five days after germination, the conidium gives rise to an initially white, sterile spit-shaped mycelium.

Two days later, a green color is visible on the aerial parts of the mycelium, corresponding to conidiogenesis **(Fig. 06).**

Other regular concentric carches form thereafter, and between the 16th and the 20th days a thick felting is superimposed on the culture (Corbaz, 1990).

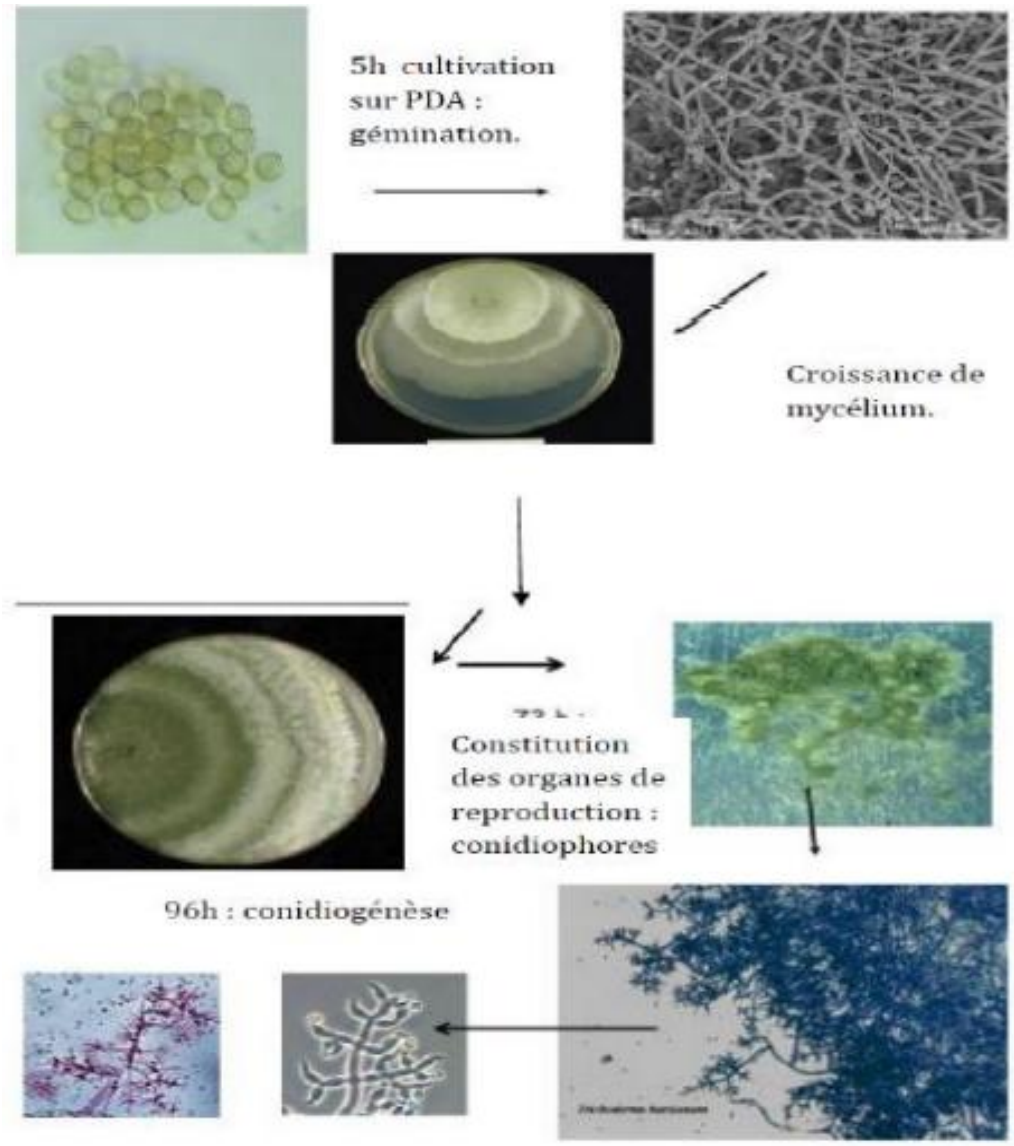


Figure 06: *Trichoderma* sp life cycle (Hamrouche and Kabouche, 2014).

7. Production of *Trichoderma*

Trichoderma typically grows in *PDA* (*Potatoes Dextrose Agar*) or *TSA (Tryptic Soy Agar*). It is known that *Trichoderma* grows best in *PDA* (Ubalua *et al.,* 2007; Verma 2007), other media like *TSA* are rarely used. *Trichoderma* has been produced in complex media such as municipal sewage and sludge and has shown excellent results (Verma 2007).

Trichoderma has also been shown to grow in barley cell wall preparations (Kanauchi *et al.,* 2001), with maximum growth at a temperature of 30 ° C. Growth at a temperature between 30 and 35 ° C is characterized by the high secretion of proteins which explains the strong synthesis of enzymes at this temperature. Davet and Comporota (1986) developed a technique for producing *Trichoderma* inoculum from a medium containing wheat and oatmeal. In order to select a substrate which could give *Trichoderma* greater competitive power against soil-borne *microorganisms,* five types of flour at various concentrations were tested on the initial inoculum of three *Trichoderma* strains. Davet *et al.,* (1981) demonstrated that the medium

based on wheat straw allows a longer conservation of the *Trichoderma* strains than the synthetic media or the soil devoid of nutrient support. Straw promotes the release by *Trichoderma* of volatile antibiotics (Davet 1983). **Table 03** shows the effect of different agro-industrial wastes on the density of *Trichoderma*.

Table 03: Effects of different agro-industrial wastes on the cell density of *Trichoderma Harzianum* (Singh *et al.,* 2007).

Déchets agroindustriels	UFC/g						
	Jours						
	30	60	90	120	150	180	210
Pseudotronc Banane	5.8×10^6	5.5×10^6	5.1×10^5	4.8×10^5	4.2×10^4	3.1×10^4	2.6×10^4
Compost	7.3×10^6	7.3×10^6	5.3×10^6	4.3×10^6	5.5×10^5	5.9×10^5	2.5×10^4
Maïs	5.4×10^6	5.4×10^6	3.8×10^6	3.4×10^6	5.3×10^5	7.9×10^4	2.4×10^4
Farine de maïs	3.3×10^7	3.3×10^7	3.0×10^7	5.8×10^6	6.7×10^5	6.0×10^5	8.3×10^4
Balle de Riz	6.4×10^7	6.4×10^7	6.2×10^7	5.9×10^7	1.2×10^6	7.8×10^5	5.2×10^4
Sciure	4.4×10^6	4.3×10^6	4.1×10^6	3.8×10^6	3.1×10^6	2.7×10^6	2.4×10^4
Grain de sorgho	2.1×10^7	2.1×10^7	1.9×10^7	1.6×10^7	1.5×10^7	3.3×10^6	2.1×10^4
Feuilles de thé usagées	8.0×10^8	8.0×10^8	8.0×10^7	7.8×10^7	7.5×10^7	6.5×10^7	6.1×10^4
Son de blé	2.4 x 107	2.1 x 107	2.0 x 107	2.0 x 107	9.8 x 106	6.4 x 106	5.7 x 104

8. Formulations based on *Trichoderma*

The antagonistic behavior of fungal species of the genus *Trichoderma* has been addressed in many research studies. It has been shown that if the defense activity of a biological agent is not related to the size of its population, the maintenance of any inoculum ensures prolonged action (Davet 1983).

The application of fungal agents like the genus *Trichoderma* to counter phytopathogens requires significant amounts of inocula with a formulation allowing good growth of the biocontrol agent against the pathogenic species. Davet *et al.,* (1981) have shown that *Trichoderma* inoculum, in the form of a simple spore powder, applied to non-sterile soil, is difficult to maintain and is not very active. To avoid this problem, it is absolutely necessary to introduce the *Trichoderma fungus* in a kind of substrate that allows it to escape the *microorganisms* in the soil. Several substrates were tested for the formulation of inocula based on *Trichoderma*. Thus, wheat bran has been widely used for the formulation of inoculae of several antagonists including *Trichoderma* (Rovira 1969; Chet *et al.,* 1979; Aziz *et al.,* 1997). A wheat bran formulation based on *Trichoderma harzianum* reduced damping-off caused by *Pythium* by 77% versus 32.4% by a simple sporal suspension of *Trichoderma* (Mukherjee *et al.,* 1989). This formulation was also very effective for the control against *Sclerotium rolfsii* (Chet 1987). The existing population of *Trichoderma* found in agricultural land does not exceed 102 CFU / g of soil (Chet 1987). Chet and Backer (1979) demonstrated that the minimum concentration required for soils should be around 106 CFU / g of soil.

9. Biochemical elicitors of disease resistance

Three classes of compounds secreted by *Trichoderma* inducing disease resistance are identified. They are proteins with enzymatic or other functions, homologous proteins encoded by virulence genes, oligosaccharides and, finally, other low molecular weight components that are released from cell walls (Harman *et al.,* 2004) . Prior to the discovery of resistance induction in plants by *Trichoderma*, there was the 22-k Da *xylanase* protein which is secreted by several species of *Trichoderma* (Yedidia *et al.,* 1999). This protein is able to trigger ethylene production and plant defense (Fuchs *et al.,* 1989; Anderson 1993). It is important to mention the mode of delivery of this protein. Indeed, this protein is translocated via the vascular system of tobacco after having been introduced to the petioles (Bailey *et al.,* 1991). It was therefore necessary that the translocation of this protein take place in the plant itself and not only at the level of the petioles.

10. Role (mode of action) of *Trichoderma*

Generally, *Trichoderma* inhibits or degrades pectinase and other enzymes which are essential for phytopathogens and in addition to its inhibitory effect on phytopathogens, *Trichoderma* is also able to induce localized and systematic resistance. Enhancement of plant growth by *Trichoderma* can take place either at the plant level (Lindsey *et al.,* 1967; Yedida *et al.,* 2001) or at the soil level (Chang *et al.,* 1986; Harman, 2000).

The induction of resistance in plants by *Trichoderma* has been studied and compared with the responses induced by *rhizobacteria*. *Trichoderma* is resistant to cyanides and produces two different enzymes which are capable of breaking down cyanides in the root zone (Ezzi and Lynch, 2002). Subsequently, this fungus can increase root growth, destroy toxic metabolites produced by *microflora*, and directly control root pathogens.

Microscopic observations on cultures of different fungi have shown that *Trichoderma* grows in parallel with *Rizoctonia Solani*. However, *Trichoderma* wraps around *Rizoctonia solani* and forms hooks thus preventing its development (Shalini *et al.,* 2007).

11. Importance of *Trichoderma* on biotechnological potential

Fungi of the genus *Trichoderma* are ubiquitous saprophytes in the soil. They sporulate abundantly, have few nutritional requirements, can grow rapidly and produce diverse ranges of secondary metabolites (ELAD, 2000; FREEMAN *et al.,* 2004; lAYALAKSHMI *et al.,* 2009). They have been indirectly promoted as biological control agents and plant growth stimulators (ELAD, 2000; FREEMAN *et al.,* 2004; BTISSAM *et al.,* 2007; lAYALAKSHMI *et al.,* 2009). *T harzianum* is the most widely used species for its effectiveness in biological control and in stimulating plant growth. There are formulations of *biopesticides* based on this species (CARON and LAMBERT, 2002).

Species of the genus *Trichoderma* influence plant growth by synthesizing *phytohormones* like *AIA* and improving the bioavailability of soil nutrients through the solubilization of phosphate and other nutrients like iron, copper, manganese and zinc (ALTOMARE *et al.,* 1999; RUDRESH *et al.,* 2005; GRAVEL *et al.,* 2007).

Trichoderma species generally utilize sources of nitrogen from ammonium compounds and proteins. The assimilation of nitrate is rare and depends on the species (MAHESH *et al.*, 2005). In addition, they have the ability to transform an extremely wide variety of natural organic materials promoting biofertilization in soils.

On the other hand, *Trichoderma* spp. are used commercially for the production of cellulases and other enzymes which degrade polysaccharide complexes (SCHUSTER and SCHMOLL, 2010), and frequently used in the food and textile industries (HARMAN, 2006).

12. Enzymes and secondary metabolites

Trichoderma are of capital importance in biotechnology for their biotransformation capacities. During the last decades the strains have been mainly used in the food industry to produce enzymes but also in the field of agro-industrial solid waste treatment to degrade the *lignocellulosic* compounds of bagasse in order to produce *bioethanol*. *Trichoderma* are the most widely used filamentous fungi for the production of cellulolytic enzymes "*cellulases*" which hydrolyze cellulose into simple sugars (glucose). There is not a single enzyme capable of degrading crude cellulose, it is rather an enzymatic complex consisting of *exo-cellulases*, *endo-cellulases* and *β-glucanases* that is used (Roussos 1985). Generally, the species *T. viride, T. reesei, T. longibrachiatum, T. harzianum* as well as *T. asperellum* are the most used to produce cellulases in solid media. The production of enzymes is variable from one strain to another. These are the hydrolytic enzymes such as *chitinases, cellulases, amylases, β-1,3-glucanases, proteases* and other *hydrolases* which degrade the cell wall of phytopathogenic fungi. They are among the most important enzymes involved in the defense mechanism exerted by *Trichoderma* (Howell 2003; Harman *et al.*, 2004).

Trichoderma are also capable of producing different bioactive molecules, among them the antifungal *6-pentyl-α-pyrone (6-PP)* which belongs to the group of volatile metabolites playing an antifungal role in the biological fight against phytopathogenic fungi such as *Botrytis cinerea, Rhizoctonia solani* and *Fusarium oxysporum* (Chen *et al.*, 2012; Huang *et al.*, 2011; De Marco *et al.*, 2002; Elad *et al.*, 1981).

6-PP is an unsaturated *ɣ-lactone* **(Fig. 07)** which has other names like *6-amyl-alpha-pyrone, 5-hydroxy-2,4-decadienoic acid ɣ-lactone*. It also has interesting aromatic potential; it is a molecule with a strong smell of coconut.

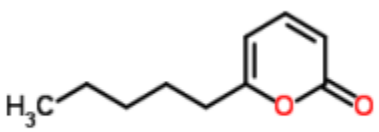


Figure 07: Chemical structure of 6-pentyl-α-pyrone.

CHAPTER III : The genus *Bacillus*

1. General characters and classification

The genus *Bacillus* encompasses Gram-positive bacteria, older cultures may appear Gram-negative. *Bacilli* are straight rods with a square or rounded end; of variable size from 0.5-2.5 x 1.2-10 µ. Their GC% is 32 to 69. They form endospores **(Fig. 08).** Most often mobile, with peritrichous flagella. They are aerobic, sometimes facultative, and catalase positive (Prescott *et al.,* 2003).

Several species of the genus *Bacillus* form capsules, sometimes including glutamic acid as in *B. megaterium*, and *B. subtilis*. The capsules of *B. pumilus*, *B. circulans*, *B. mycoides* and others contain the carbohydrate **(Table, 04).**

The genus *Bacillus* belongs to the class *Bacilli*, of the order *Bacillales*, and to the family of *Bacillaceae*. However, the classification currently used is based on the morphology and position of the spore. It subdivides the genus *Bacillus* into 03 groups:

Group I: made up of Gram-positive *bacilli* with a central or terminal, spherical or ovoid spore that does not deform the cell. This group is divided into 02 sub-groups; group IA consisting of *bacilli* greater than 1 µm in diameter and containing *B. anthracis, B. cereus, B. megaterium, B. mycoides, B. thringiensis, B. pseudomycoides* and *B. weihenstephanensis;* and group IB comprising *Bacilli* with a diameter of less than 1 µm: *B. coagulans, B. firmus, B. licheniformis, B. subtilis and B. pumilus.*

Group II: made up of Gram variable species presenting an ovoid, central or terminal, deforming spore: *B. circulans, B. stearothermophilus*….

Group III: made up of Gram variable *bacilli* and presenting a spherical, deforming, terminal or subterminal spore: *B. fusiformis, B. globisporus*….

2. Nutrition and growth

Most species of the genus *Bacillus* are chemoheterotophic, capable of respiration and use a variety of simple organic compounds (sugars, amino acids, organic acids). In some cases, they also ferment carbohydrates by a mixed reaction which typically produces glycerol and butanediol. Some species, such as *B. megaterium*, do not require any organic growth factor; others require amino acids, B vitamins, or both. The majority are mesophiles with optimum temperature between 30 and 45 C °, but the genus also contains a number of thermophilic species with optimum temperature above 65 C °.

In the laboratory, under optimal growth conditions, species of the genus *Bacillus* show generation times of about 25 minutes (Todar, 2003)

3. Ecology

Russian microbiologist *Winogradsky* considers *Bacillus* to be part of the natural flora of the soil. They become metabolically active when the appropriate substrates for their growth are

available, and form spores when nutrients are depleted. The latter phenomenon is accompanied by the production of antibiotics. Since most *Bacilli* can efficiently degrade a series of biopolymers (proteins, starch, pectin, etc.), it is assumed that they play a role in the biological cycles of carbon, and nitrogen (Todar, 2003).

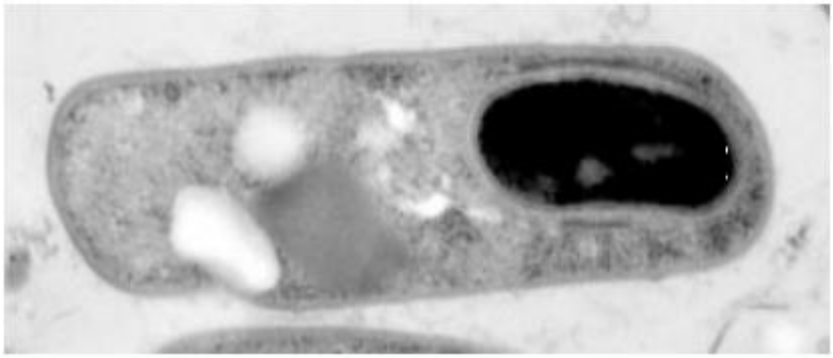


Figure 08: *Bacillus megaterium* with its endospore (Todar, 2003).

4. Ecophysiological groups

Members of species of the genus *Bacillus* are divided into various ecophysiological groups:

- Acidophiles: *B. acidocaldarius, B. coagulans*
- Alkalophiles: *B. alkalophilus*
- Halophiles: *B. pasteurii, B. pantothenticus*
- Psychrophiles: *B. insolitus, B. marinus, B. megaterium.*
- Thermophiles: *B. schlegelii, B .stearothermophilus*
- Denitrifiers: *B. azotoformans, B. cereus. B. licheniformis*
- Nitrogen fixers: *B. macerans, and B. polymyxa*
- Antibiotic producers: *B. brevis* (*gramicidin, tyrocidin*), *B. cereus* (cerexin), *B. pumilus* (*pumulin*), *B. subtilis* (*difficultiesidin, subtilin, mycobacillin*)

Antibiotic producing *Bacilli* share a full range of antimicrobial activity: bacitracin, pumilin, laterosporin etc., are effective against Gram positive bacteria; colistin and polymyxin are effective against Gram negative; difficultidin has a broad spectrum. *Mycobacillin* and zwittermicin are antifungals (Todar, 2003).

5. Pathogenicity

The genus *Bacillus* contains pathogenic insect species such as *B. larvae, B. popilliae, B. lentimorbis, B. thuringiensis.* Other animal pathogens; *B. cereus, B. circulans, B. anthracis*, have been isolated from human infections (Todar, 2003).

6. Sensitivity to antibiotics

Bacilli are generally sensitive to clavulanic acid, amoxicillin, gentamicin, amikacin, tetracycline. They are often resistant to lincomycin, colistin and frequently to fosfomycin (Todar, 2003).

7. Physiological characteristics

The general characteristics of the genus *Bacillus* are summarized in **Table 04**:

Table 04: characteristics of *Bacillus* (Guiraud, 1998).

	B.alvei	*B.circulans*	*B.macerans*	*B.polymyxa*	*B.anthracis*	*B.cereus*	*B.firmus*	*B.lentus*	*B.licheniformis*	*B.megaterium*	*B.panthothenicus*	*B.pumilus*	*B.subtilis*	*B.thuringiensis*	*B.brevis*	*B.pasteuri*	*B.sphaerisus*	*B.coagulans*	*B.stearothermophilus*
Groupe selon Priest	I	I	I	I	II	II	II	II	II	II	II	II	II	II	III	IV	IV	IV	IV
Morphologie sporale*	2	2	2	2	1	1	1	1	1	1	1/2	1	1	1	2	3	3	1/2	2
Position de la spore	C/T	C/T	T	C/T	C	C	C	C	C	C	T	C	C	C	C/T	T	T	C/T	T
Culture anaérobie	+	V	+	+	+	+	–	–	+	–	+	–	–	+	–	–	–	+	–
Acide sur glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	V	–	–	+	+
Gaz sur glucose	–	–	+	+	–	–	–	–	V	–	–	–	–	–	–	–	–	–	–
Mobilité	V	V	+	+	–	V	V	V	+	V	–	+	+	V	+	+	+	+	+
Retard sur glucose	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–
Acétoine	+	–	–	+	+	+	–	–	+	–	–	+	+	+	–	–	–	V	–
Lndole	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	V	–	–	–
Citrate	–	V	–	–	V	V	–	–	V	+	V	+	V	V	V	–	V	–	–
Amidon	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	–	+	+
Caséine	+	V	–	+	+	+	+	–	+	+	+	+	+	+	+	V	V	V	V
Gélatine	+	V	+	+	+	+	+	–	+	+	+	+	+	+	+	V	V	–	+
Lécithine	V	–	V	V	+	+	–	–	–	–	V	–	–	+	V	–	–	–	+
Uréase	–	V	–	–	V	V	–	+	–	V	–	–	–	V	–	+	V	–	V
Nitrate	–	V	+	+	+	+	+	–	+	V	V	–	+	+	V	V	–	V	V
Culture à 45 °C	V	+	+	V	–	V	V	–	+	V	+	+	+	+	+	–	V	+	V
Culture à 55 °C	–	–	–	–	–	–	–	–	+	–	–	–	V	–	V	–	–	+	+
Culture à 65 °C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+

* according to Bergey's Manual: group 1 = non-deforming oval spore; group 2 = oval deforming spore; group 3 = spherical deforming spore. V: variable; C: central spore; T: terminal

8. Isolation technique

Species of the genus *Bacillus* are easily isolated and easily developed in the laboratory. The simplest technique is to expose a diluted soil sample to 80 ° C for 15 minutes for aerobic sporulating forms, then spread it on nutrient agar and incubate it at 37 ° C for 24 hours or a few days. The first identifying characters contain catalase positive, Gram positive and spore formation (Todar, 2003)

For enumeration or isolation in a solid medium, the most widely used media are Bromocresol Purple Glucose Agar (BCP) (= DTA: Dextrose Triptone Agar) or milk agar.

The count in liquid medium is carried out on glucose broth with BCP (= DTB) or TSB broth (tryptone Soya broth). The enumeration of vegetative forms must use more selective or differential media.

The culture of isolated *Bacilli* is easily carried out on conventional culture media such as nutrient broth or ordinary nutrient agar (Guiraud, 1998).

9. Characteristics of some species of the genus *Bacillus*

- ***Bacillus coagulans***

Includes acidophilic strains. Spores are present in soils. Can multiply in acidic foods.

- ***Bacillus macerans***

Most of its strains fix N2 under anaerobic conditions. Degrades pectin polysaccharides. Some moderately thermophilic strains. Additionally was found in fruits stored at pH 3.8.

- ***Bacillus polymyxa***

Mycoid colonies, viscous. Synthesizes capsule, breaks down pectin and plant polysaccharides, fixes nitrogen under anaerobic conditions. Common spores. Produces the antibiotic polimyxyin.

- ***Bacillus cereus***

Thermophilic aero-anaerobic bacteria (certain strains can develop at 6 ° C), very widespread in the soil, on plants, in particular cereals, animal skin, etc. (Guiraud, 1998). B. cereus is used in the biological control of leek rust (Gourgaud et sanglier, 1992).

- ***Bacillus thuringiensis***

Distinguished from *Bacillus cereus* by pathogenicity to lepidopteran insects, and spore formation. It is the main biological insecticide marketed on a large scale (Gourgaud et sanglier, 1992).

- ***Bacillus megaterium***

Large cell, spore generator, aerobic. Grows in minimal medium without any additional growth factors. The spores are common in the soil.

- ***Bacillus pumilus***

The ubiquitous spores reproduce in the soil more frequently than those *Bacillus subtilis*.

- ***Bacillus subtilis***

With a genome containing 4.2 million base pairs, *B. subtilis* is not pathogenic for humans and does not produce endotoxins and can be easily cultivated in large-volume fermenters. The major problem with the use of *B. subtilis* is the low expression of genes. Only a few proteins could be produced at a rate greater than 1% of the total cellular proteins. *B. subtilis* degrades pectin and plant polysaccharides. Thrives in a minimal environment without growth factors. Common endospores. Much information on the biology, biochemistry and genetics of the Gram positive cell has been derived from the study of *B. subtilis* (Gougaud and Sanglier, 1992; Prescott *et al.*, 2003)

10. The Most Important Secondary Metabolites of *Bacillus* Species

10.1 Bacteriocins from *Bacillus* Species

Bacteriocins are bacterial ribosomally synthesized antimicrobial peptides lethal to bacteria other than the producing strain, usually against bacteria closely related to the producer (De Vuyst and Vandamme 1994; Riley and Wertz 2002). Compared to those produced by lactic acid bacteria, bacteriocins from the genus *Bacillus* have been relatively less recognized despite their broad antimicrobial spectra and high activity. Nonetheless, bacteriocins from *Bacillus* spp. may have great potential for application in food, agriculture, and pharmaceutical industries to prevent or control spoilage and pathogenic microorganisms (Lee and Kim 2010).

Several species of the *Bacillus* genus are bacteriocin-producers, such as *B. subtilis* which produces subtilin (Jansen and Hirschmann 1944) and subtilosin (Zheng and Slavik 1999), *B. coagulans*, *B. megaterium* and *B. thermoleovorans* producing respectively coagulin (Hyronimus *et al.,* 1998), megacin (Von Tersch and Carlton 1983) and thermoleovorin (Novotny and Perry 1992). Also, several bacteriocins associated with bacteria from the *Bacillus cereus* group have been studied and totally or partially characterized. These include bacteriocins produced by *B. thuringiensis* such as thuricin from strain HD2 (Favret and Yousten 1989), tochicin from strain HD868 (Paik *et al.,* 1997), thuricin 7 from strain BMG1.7 (Cherif *et al.,* 2001), entomocin 9 from strain subspecies entomocidus HD9 (Cherif *et al.,* 2003) and entomocin 110 from strain HD110 (Cherif *et al.,* 2008). Many bacteriocins have as well been partially characterized from *B. cereus* species such as cerein 7 from strain BC7 (Oscariz *et al.,* 1999), cerein 8A from strain 8A (Bizani *et al.,* 2005), and the bacteriocin-like inhibitory substance (BLIS) from the *B. cereus* type strain ATCC 14579T (Risøen *et al.,* 2004).

10.2 Siderophore

Plant growth and reproduction can be severely affected by various biotic and abiotic stresses. Among the abiotic stresses, iron (Fe) deficiency constitutes a major factor leading to a reduction in crop yield, especially in calcareous soils in which the solubility of Fe is extremely low (Kobayashi *et al.,* 2005).

One of the most widely utilized mechanisms of microbial iron acquisition is the production and excretion of siderophores, low molecular weight iron chelators that bind ferric iron with extremely high affinity and shuttle it into the cells (Zawadzka *et al.,* 2009). Thus the presence

of siderophore-producing micro-organisms in the rhizosphere contributes to plant health by complexing iron and making it less available to phytopathogenes that are generally not able to produce comparable Fe-transport systems (Chen *et al.,* 2009). It has been reported that under sterile soil system, plants show iron-deficiency symptoms and have fairly low iron level in roots which suggests the role of soil microbial activity in iron acquisition and plant growth (Raddadi *et al.,* 2007). Siderophore production was initially reported for Gram-negative bacteria and possibly implicated in their virulence potential. In *Bacilli*, several siderophores were detected, mainly schizokinen from *B. megaterium* and other from *B. subtilis, B. licheniformis* and the *B. cereus* group: *Bacillibactin* and *petrobactin* are produced by *B. cereus* (Park *et al.,* 2005), *B. anthracis* (Cendrowski *et al.,* 2004) and *B. thuringiensis* (Wilson *et al.,* 2006).

This character in the case of *B. thuringiensis* could be relevant for biocontrol of phytopathogenic fungi because of competition effects for iron, but also for providing the plant with iron (Raddadi *et al.,* 2007).

10.3 Polyketides

Polyketides are the other dominant family of secondary metabolites having relevant bioactivities. They are biosynthesized through decarboxylative condensation of malonyl-CoA derived extender units in a similar process to fatty acid synthesis (a Claisen condensation). Their biosynthetic pathway follows generally the same logic as in non ribosomally synthesized peptides and requires at least three domains. Essential domains of the modules harboured in bacterial type I polyketides synthase are acyl transferase (AT), ketosynthase and an acyl carrier protein (ACP) which needs to be activated by Ppant-transferse (Chen *et al.,* 2009). Polyketide represented by a wide range of active family molecules are currently used in human and animal health and in agriculture as antimicrobial (erythromycin, rifamycin), antifungal, immunosuppressant and antitumor agents. Polyketides are actually difficult to synthesise chemically which make their heterologous expression a promising alternative to the use of natural producers. Heterologous production systems can facilitate analysis of the catalytic properties of polyketide producing enzymes. Examples of heterologous hosts for polyketide protein production include bacteria such as *Escherichia coli, Bacillus subtilis*, and *Streptomyces coelicolor* (Rude and Khosla 2004).

Several polyketides were found to possess interesting activities. Bacillaene, produced by *B. subtilis* and *B. amyloliquifaciens,* inhibits prokaryotic protein synthesis by means of unknown mechanism, and exhibits high bacteriostatic activity against a wide spectrum of bacteria. Difficilin, another polyketides, was shown to be promising in its suppressive action against plant pathogen (Hamdache *et al.,* 2011).

10.4 AHL-Lactonases

In Gram-negative bacteria, the Quorum-Sensing (complex communication system) rely on the interaction of N-acylhomoserine lactones (AHLs), molecules that share identical homoserine lactone rings but vary in length and the substitution of the acyl side chain. AHL signals are involved in the regulation of a range of important biological functions, including

luminescence, antibiotic production, plasmid transfer, motility, virulence, biofilm formation and functional coordination among microbial communities (Whitehead *et al.*, 2001; Zhang 2003; Federle and Bassler 2003). The quorum-sensing pathways could be subverted by expressing "quorum quenching" enzymes that can hydrolyze AHL-signaling molecules. Dong *et al.*, (2002) studies have shown that *B. thuringiensis* strains, producing AHL-lactonase suppress the Quorum-Sensing dependent virulence of the plant bacterial pathogen E. carotovora through signal interference. These findings, illustrate the promising potential to explore the microbial antagonistic mechanisms such as signal interference, for the control and prevention of infectious diseases. Interfering in signaling pathways of pathogenic bacteria and suppressing their virulence potential, represent a serious alternative to the use of antibiotics and prevent from resistance appearance.

Part II
Materials And Methods

This work was carried out in the Scientific Police laboratory (Chevalley) at the level of the Bacteriology Department. This work was carried out during a 4-month internship period (beginning of March until end of June).

We started with the revivification and purification of our bacterial (2 strains of the genus *Bacillus*) and fungal (2 strains of the genus *Trichoderma*).

We have plotted as objectives:

- The re-identification and characterization of the four microbial strains of interest in the field of biotechnology by macroscopic, microscopic observations and physiological and biochemical tests.

- The search for enzymatic activities such as : Amylase; lecitinases; caseinase; lipases and gelatinases.

- The search for indol acetic acid (IAA).

- Evaluation of the production of siderophores.

- The production of biosurfactants.

Chapter I Fungal materials and methods

1. Biological materials

Two different fungal strains of *Trichoderma* spp (T_3 and T_{14}) were isolated from olive rhizosphere in the region of Bouira (Algeria), belonging to the Laboratory of Valorization and Conservation of Biological Resources (VALCORE) collection. Strains were supplied to us in Petri dishes grown on PDA medium **(Appendix 02)**. **(Fig. 09)**

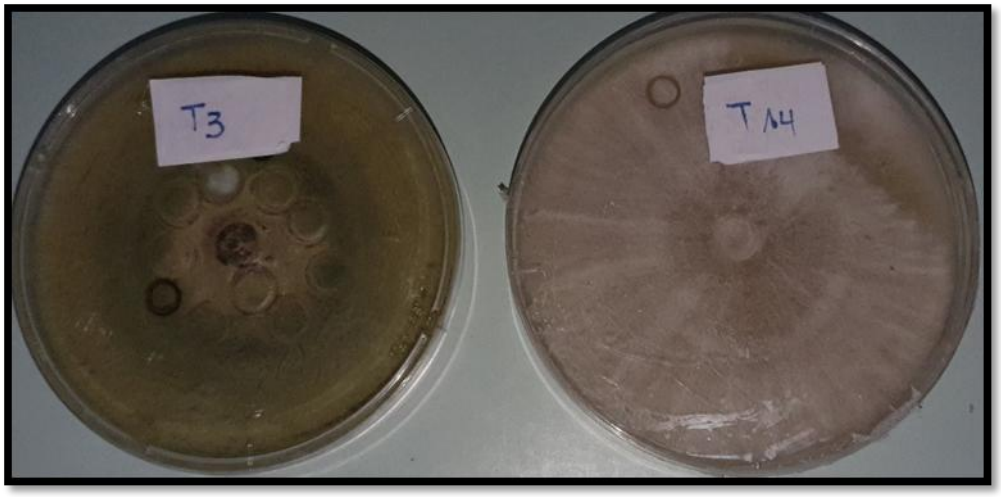


Figure 09: The two different fungal strains of *Trichoderma* species T_3 and T_{14}.

1.1 Purification and conservation of fungal strains

To ensure that the strain is from the same spore which confirms that the strain is genetically pure.The fungal colonies obtained were subjected to a monospore culture which is carried out in minimal medium **(Appendix 02)**. Once the culture is obtained, the strains were conserved, the method consists in transplanting the strains in tubes on slanted agar, the cultures are maintained for 7 days at 28°C, then stored at 4°C (Botton *et al.,* 1990).

1.2 Morphological study

The morphological identification is based on the morphological and cultural characteristics of isolated fungi in the pure state (Botton *et al.,* 1990).

1.2.1 Macroscopic study

Macroscopic observation is carried out on the face and the back of the Petri dish. It helps determine the shape, size, color, texture (velvety, woolly) and smell of the colony during its development. (Botton *et al.,* 1990). As well as the determination of the relief and consistency of colonies (Leslie and Summerell, 2006).

1.2.2 Microscopic study

It reveals the presence of the thallus, its nature: septate or not, as well as the characteristics of the fructifications, conidiophores and spores (Cahagnier and Ricard-Molard, 1998). And even the presence and absence of chlamydospores (Leslie and Summerell, 2006). Direct observation is made from 100 to 1000 magnification.

The microscopic characters are revealed following a scotch technique (or flag technique). This technique involves using a piece of tape to adhere a mycelia fraction from a young culture and stick it on a slide containing a few drops of methylene blue (Chabasse, 2002).

1.2.3 Monospore culture

The goal of monospore culture is to obtain genetically homogeneous fungal material (Booth, 1971). Initially, the strain is subcultured in a Petri dish containing PDA medium **(Appendix 02)** and allowed to develop for 05 to 06 days at 25 ° C. An explant is taken from the peripheral of the dish and introduced in to a tube containing 09 ml of sterile distilled water. The tube is shaken for a few minutes. After stirring, a spore suspension is obtained from which dilutions are made as follows:

1 ml of the spore suspension is taken and then introduced into a tube containing 9 ml of sterile distilled water (10^{-1}). This operation is repeated as many times until dilution (10^{-4}). From the last dilution (10^{-4}), 1 ml is taken and then filtered using filter paper. A drop of filtrate is taken and inoculated using a Pasteur pipette on minimum medium. After 24 hours of incubation, using a binocular magnifying glass, the location and delimitation of the germinating spores are carried out. These are removed and then placed in new Petri dishes containing PDA medium; then incubated at 28°C for 7 days (Rappily, 1968).

2. Screening for qualitative enzymatic activity of *Trichoderma* species

2.1 Protease assay

Protease activity was evaluated on solid medium Skim milk agar **(Appendix 02)** (Berg *et al.,* 2005). 6 mm agar disc cut from 5 day-old *Trichoderma* culture of each isolate was transferred to Petri dishes containing the selective medium. Clear zones appear around the transferred disc indicates a proteolytic activity.

2.2 Cellulase assay

The qualitative production of cellulases was screened according to the method described by Hankin and Anagnostakis (1975) on the Czapek-Mineral Salt Agar Medium **(Appendix 02)** supplemented with Carboxy Methyl Cellulose (CMC) 5.00g. The discs of 6 mm of *Trichoderma* cut from 5 days-old fungal culture of each isolate was inoculated in the center of the medium and incubated at 26 ± 2°C for 3 to 5 days. The plates were flooded with aqueous Congo red (2% w/v) solution **(Appendix 03)** for 15 min. Then, the agar surfaces were washed with distilled water and in the end plates were flooded with NaCl (1M) **(Appendix 03)** for 90 seconds. Formation of yellow-opaque area around the colonies indicates the production of cellulases.

2.3 Lipolytic activity

Lipolytic activity was evaluated on a solid medium **(Appendix 02)** according the methodology described by Howe and Ward (1976). *Trichoderma* isolates were inoculate and incubated for 5 days at the previously mentioned conditions. The presence of opaque precipitate around the colony indicated that *Trichoderma* spp. isolates were capable of hydrolyzing Tween 80.

2.4 Chitinase activity

Chitinase activity of the *Trichoderma* isolates was carried out on the Chitinase detection Medium **(Appendix 05)**, pH was adjusted to 4.7 (Agrawal and Kotasthane, 2012) The Medium containing Colloidal chitin as unique carbon source supplemented with Bromocresol purple. Method applied or adopted to prepare Colloidal chitin was there described by (Hsu, S. C., and J. L. Lockwood, 1975, and Seitzman, 2008) and it conserved at 4°C until further use. Culture plugs of the *Trichoderma* isolates were inoculated into the medium and incubated at 25 ± 2°C for 2–3 days.

Chitinase activity was evaluated by varying color from yellow to purple in the chitin hydrolysis region. Color intensity and diameter changing color of the Bromocresol purple around the colonies were used as the criteria to reveal the chitinase activity.

3. Screening for qualitative PGPF activity of *Trichoderma* species

3.1 Hydrogen cyanide (HCN) production

HCN production was screened qualitatively according to the method of Bakker and Schipper (1987). *Trichoderma* isolates were inoculated on solid medium containing Tryptic Soya Agar

(Appendix 02) supplemented with glycine. A Whatman filter paper impregnated by alkaline picric acid solution **(Appendix 03)** was placed under the top cover of each plate. The plates were incubated at 26±2°C for 7 days. **(Fig. 10)**

A change in color of the filter paper from yellow to light brown, brown or reddish brown was strong evidence for the production of HCN (Meera and Balabaskar, 2012).

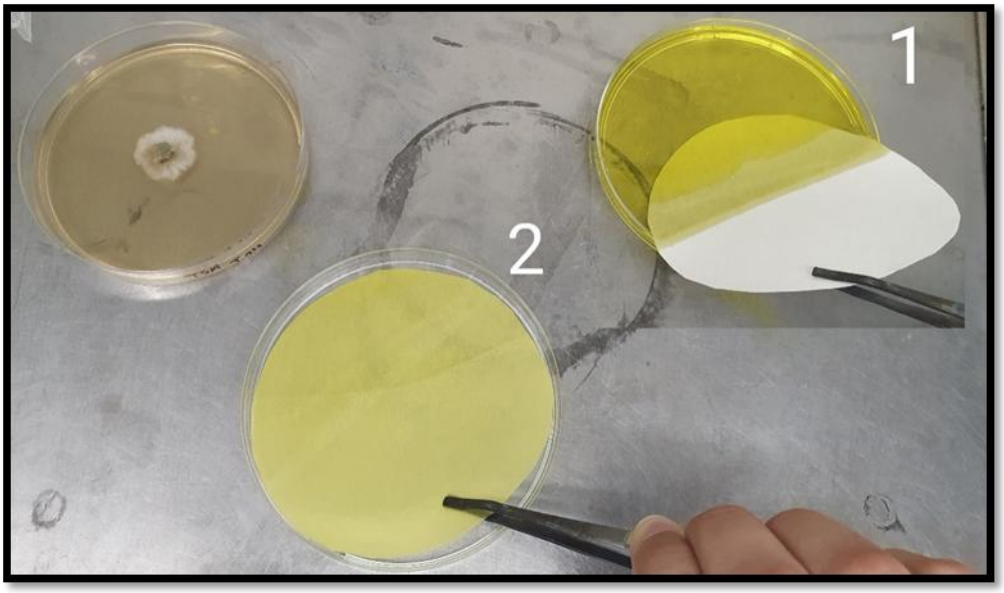


Figure 10: Method of impregnated Whatman filter paper by alkaline picric acid solution.

3.2 Nitrogen fixation activity

In order to evaluate nitrogen-fixing ability, medium lacking nitrogen **(Appendix 02)** was used, 6 mm diameter from pure culture of *Trichoderma* was inoculated in the center of a Petri dish containing nitrogen-fixing medium. After 3 days of inoculation, test of nitrogen-fixing ability was positive if the colony can grow normally on the selective medium (Zhang *et al.*, 2017).

3.3 NH3 production

Production of ammonia was tested in peptone water **(Appendix 02)** according the method of Bakker and Schipper (1987). Broth culture containing 10 ml peptone water were inoculated and incubated at 28°C for 72 h. 1 ml of Nessler's reagent **(Appendix 03)** was added to each tube. Development of color from yellow to brownish orange was recorded as a positive test for ammonia production.

3.4 Siderophore production

Siderophores production was evaluated according to the method of Hoyos-Carvajal *et al.*, (2009) on malt extract agar medium **(Appendix 02)** containing 8-hydroxyquinoline (50 mg/L) as chelator. Growth of strains tested on this medium after 5 days of incubation at 26±2°C was recorded as a positive result for siderophores production.

3.5 Dosage of Indole Acetic Acid (IAA)

Isolates of *Trichoderma* were screened for their efficiency of Indole Acetic Acid (IAA) production using L-tryptophan as precursor according to the method of Bric *et al.*, (1991). The essays were done in potato dextrose broth medium **(Appendix 02)**, which was supplemented with 1 g/l of L-tryptophan. The culture was incubated in a shaker with 170 rpm at 28 °C.

After 3 days of incubation. Production was qualitatively estimated by colorimetric assay.

The filtrates were centrifuged and then 2 ml of each supernatant was added to 2 ml of Salkowski reagent **(Appendix 03)**. The tube containing supernatants and Salkowski reagent was incubated for 10 min for color development.

The development of a pink or red color indicated IAA production.

3.6 Chitinase hydrolytic activity assay

Isolates of *Trichoderma* showed larger zone of chitin hydrolysis on solid medium were selected to evaluate their Chitinase hydrolytic activity. Method of Miller (1959) was adopted which consists in measuring the amount of N-acetylglucosamine released (GlcNAc) from the substrate colloidal (Legrand *et al.*, 1987).Two discs (6 mm diameter) from 5-day-old cultures of *Trichoderma* were inoculated into 50 ml of synthetic medium **(Appendix 02)**.

After incubation, broth was centrifuged at 8000×g for 5 min, then 1 ml of 1% colloidal chitin dissolved in 0.05M sodium acetate buffer (50 mM, pH 5,0) **(Appendix 03)** was added to 1 ml of supernatant (enzyme solution) and incubated in a water bath with constant agitation at 37°C for 1 h. After centrifugation at 5000 rpm for 15 min, 1 ml of supernatant was taken and added to 3 ml of reagent DNS, followed by boiling of the mixture for 5 min.

The absorbance was measured with spectrophotometer at 585 nm. One unit of chitinase activity was defined as the amount of enzyme that liberated 1 μmol of GlcNAc per min under the conditions of the study.

4. Screening and isolation of microbial strains that produce bio-surfactants

The search for microbial strains that produce biosurfactants is still a field of research of great interest to scientists, due to the diversity of molecules and the wide variety of uses of these molecules.

According to Viramontes-Ramos *et al.*, (2010), for the detection of microorganisms producing biosurfactants, hemolytic activity and emulsification were studied.

4.1 The E24 test of emulsification activity

This test was developed by Broderick and Cooney (1980), and modified by (Bodour *et al.*, 2004). It makes it possible to verify the ability of strains to emulsify a hydrophobic phase in a hydrophilic phase, as well as the detection of the emulsifying agent (biosurfactants) (Zajic *et al.*, 1984).

4.2 Preparation of the fermentation mash

Each microbial strain is inoculated into a 100 ml Erlen Meyer containing 10 ml of liquid M1 medium **(Appendix 02)**, the medium is added to 2% gasoil (hydrophobic phase), each Erlen

Meyer is blocked by the cotton to make sure the passage of oxygen. While avoiding contamination. Incubate at 30 ° C for 24 hours with stirring at 70 rpm. **(Fig. 11)**

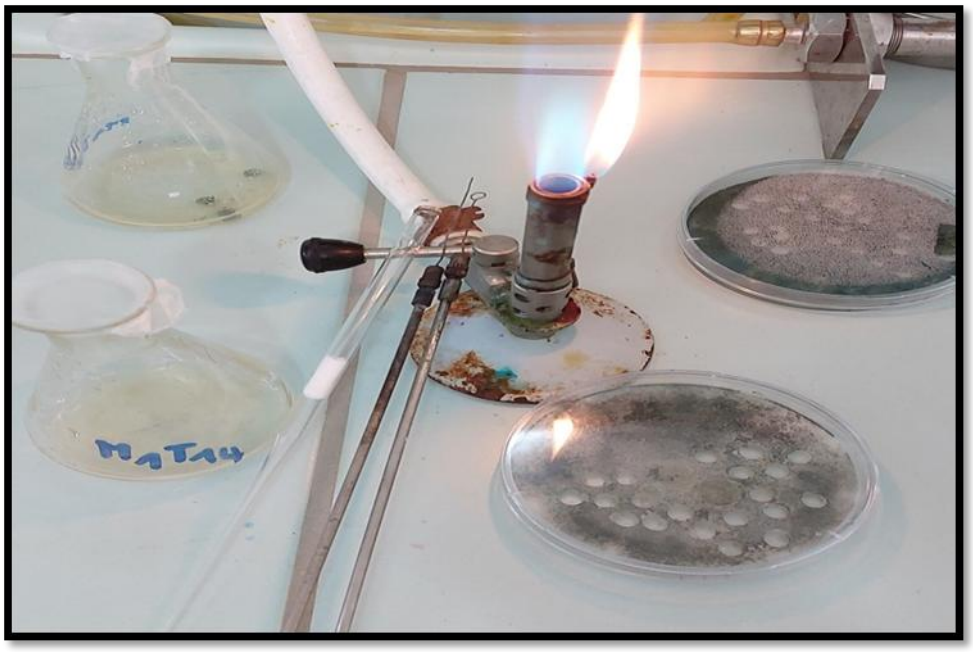


Figure 11: Preparation of the fermentation mash of the E24 test for fungal strain $T_{14.}$

4.3 Performance of the emulsification test

After 24 hours of incubation, 3 ml of fermentation must are mixed with an equal volume (3 ml) of gasoil in sterile tubes. The tubes are vortexed for 3 min, to assess the quality of the emulation with the naked eye after standing at room temperature for 24 hours, and this by comparison with the control or the fermentation wort and replace with the aseptic culture medium.

For the strains that emulsify the diesel, we obtain in the tube 3 phases:

- The 1st phase: the fermentation must at the bottom.
- The 2nd phase: the diesel emulsifies in the media.
- The 3rd phase: the fraction of the diesel that has not been emulsified on the surface (Ganesh, 2009).

4.4 Calculation of the E24 emulsion Index

The emulsification index (E24) was calculated by dividing the measured height of the emulsion layer by total mixture and multiplying times 100. **(Fig. 12)**

E24% = He / Ht ×100

Where: He: emulsion height, and Ht: total height of the mixture (VentySuryanti *et al.*, 2009)

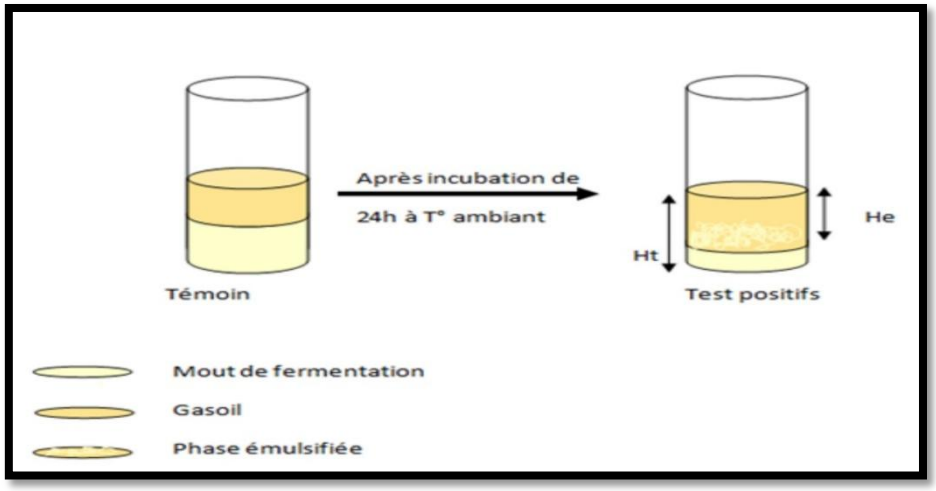


Figure 12: Emulsification test. (VentySuryanti *et al.*, 2009)

5. Extraction of bio-surfactants

5.1 Preparation of supernatant

From a 24-hour bacterial culture, the maximum colony is scraped and in the case of using a fungal culture, 12 discs of 6 mm in diameter are cut from pure culture. **(Fig. 13)**

Figure 13: Materials for preparation of fungal supernatant.

The biological material is placed in sterile flasks containing 160 ml of King B broth **(Appendix 02).** Homogeneity is ensured by a vortex, and the flasks are then incubated for 72 hours; (at 28°C for fungi and at 37°C for bacteria) this is the fermentation must.

The latter was centrifuged at 12,000 rpm / 20 min for the bacteria and at 15,000 rpm / 20 min for the fungi. In order to recover the supernatant which contains the biosurfactants separated from the cells.

5.2 The principle of extraction of bio-surfactants

- The recovered supernatant was mixed with an equal volume of chloroform: methanol (2: 1).
- In a separating funnel the mixture is subjected to stirring.

- Then has a decantation to allow the separation of the aqueous and organic phases.
- The solvent was evaporated off using a rotary evaporator.
- The biological material has been used as raw biosurfactants (Kumar *et al.,* 2006). **(Appendix 05)**

6. Extraction of antibiotics

6.1 Preparation of supernatant

The principle of preparation is the same as the preparation of the biosurfactant extraction supernatant, except that the centrifugation is done at 5,000rpm / 20min for bacteria and 10,000rpm / 20min for fungi. This operation allows good separation between the supernatant and the cell pellet.

6.2 Extraction of ATB from supernatant

- After centrifugation, the supernatant undergoes the following steps:
- The supernatant extraction is carried out by adding ethyl acetate solvent to a volume equal to the filtrate.
- Stir using a magnetic stirrer.
- Pour the contents into a separating funnel to allow the separation of the aqueous and organic phases.
- Recover the organic phase which constitutes the solvent.
- Evaporate under vacuum at 40°C using a rotary evaporator.
- Collect the dry residues in 0.6 ml of methanol. **(Appendix 05)**

7. Revelation of the extracts by TLC thin layer chromatography

- For the thin layer chromatography we used two solvent systems:

- Ethyl acetate: methanol = 100: 15 (V / V), often used for the separation of antibiotics.

- Chloroform: methanol: water = 65: 15: 2 (V / V / V), used as a developing solvent for biosurfactants.

- The tank used contains 100 ml of each solvent system **(Fig. 14)**, their atmosphere is saturated for 2 hours before the introduction of the silica gel plates.

- The silica gel plates are activated at 60 ° C for 15 min.

- The extracts are deposited in the form of 20 µl aliquots 3 cm from the lower edge of the plate and 2 cm from the side edges.

- The deposit is carried out in small fractions using a micropipette while drying as it goes under a current of cold air.

After migration, development is stopped when the solvent front reaches about 2 cm from the upper edge of the plates, they are removed from the tanks, the solvent front is then noted and the plates are dried at room temperature (Zitouni, 1995 and Ebadi *et al.*, 2018).

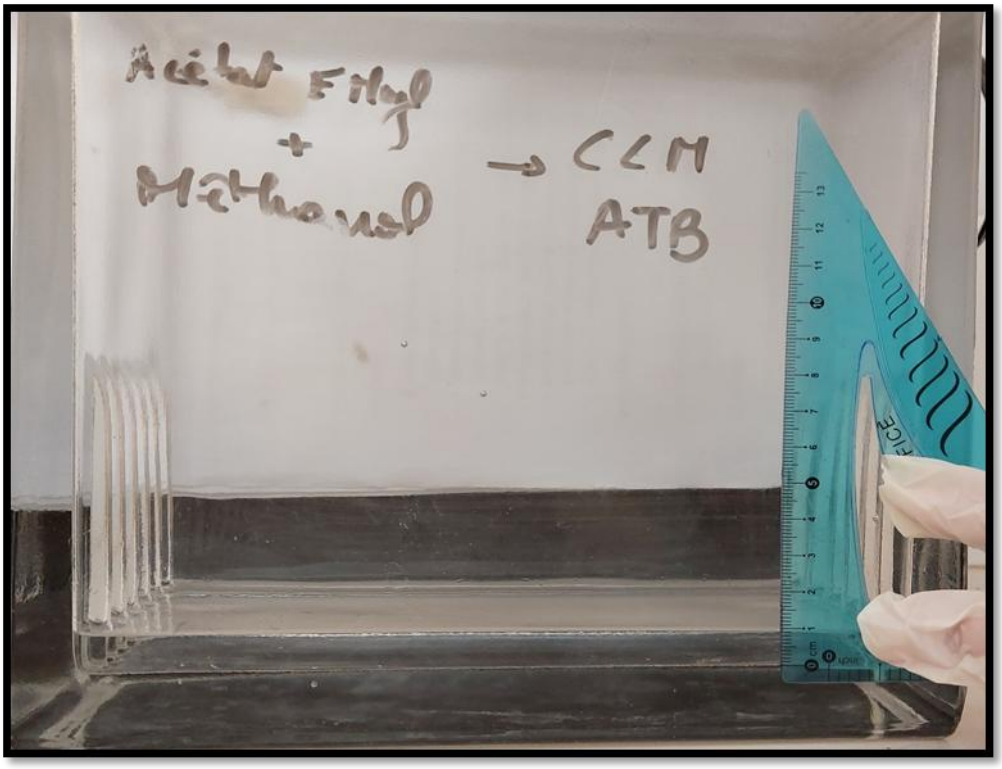


Figure 14: The tank used for the separation of antibiotics.

- The observations are made under UV at 350 nm.
- To identify the biosurfactants produced by the strains studied, two reagents were used:

Chromogenic reagent: spraying the chromogenic reagent (3 g of phenol and 5 ml of sulfuric acid dissolved in 95 ml of ethanol) forms brown spots on the plate indicating the presence of the lipid components.

Ninhydrin reagent: To detect lipopeptide biosurfactants they used a ninhydrin reagent (1 ml of centrifuged supernatant and 3 drops of 0.5% ninhydrin solution **(Appendix 03)** were placed in a 1.5 ml vial). Vials were then placed in a water bath for a few minutes to observe the color change of the reaction mixture.

CHAPTER II Bacteria

1. Biological materials

1.1 Bacterial strain

Two bacteriel strains were used in our study belong to the genus *Bacillus* sp. Bacterials starins were isolated from olive rhizosphere, belonging to the Laboratory of Valorization and Conservation of Biological Resources (VALCORE) collection.

1.2 Revivification of bacterial strain

This operation was carried out by streaking in Petri dishes containing the GN medium (nutrient agar), the latter were sealed with para film and labeled, then incubated at 37 ° C for 24 hours.

2. Test to verify the purity of the strain

In order to ensure the purity of our strains when carrying out our study, we carried out a series of basic tests

3. Macroscopic study

After incubation, the first identification criterion on which we base ourselves is that of the macroscopic appearance of the colonies seen with the naked eye, whether it be size, shape of the relief (convex, semi convex, flat), color, aspect (sticky, filamentous ...), transparency (opaque, translucent), appearance of the contours (regular, jagged), pigmentation, and appearance of the surface (smooth or rough) (solbi, 2013).

4. Microscopic study

4.1 Fresh examination

This is a quick method and consists of observing a bacterial suspension between slide and coverslip at the × 40 objective. The information obtained by this observation mainly concerns the mobility of bacteria (François *et al*., 2011).

- **Technical**

1) Place a small drop of sterile water on the slide.

2) Take a fraction of colonies on agar.

3) Make a homogeneous suspension in the drop of water by gradually incorporating the inoculum.

4) Cover with a coverslip, avoiding trapping air bubbles.

5) The liquid must not overflow (otherwise throw the slide into a disinfectant solution and start again).

6) Observe at the objective x40.

7) After observation, discard the fresh state in a container containing a disinfectant because the bacteria are alive.

Bacteria are considered mobile when very different paths are observed (moving in all directions). An immobile bacterium has normal agitating movements called Brownian movements, which should not be confused with mobility. Depending on the mobility observed, if it is present, it is possible to prejudge the type of ciliature of the bacterium (monotrich, peritrichium, etc.) which points to the isolated bacterium. (Denis, 2007).

4.2 Gram stain

A heat-fixed smear is stained for one minute with crystal violet; it is then rinsed quickly with distilled water, treated for one minute with a solution of lugol, and again rinsed quickly with distilled water. The stained smear is then subjected to a decoloration step by treating it with 95% ethanol for 15 to 30 seconds and then rinsed with distilled water. Then the smear is stained with fushin for 10 to 30 seconds and after a brief rinsing with distilled water, the smear is dried in a blotter or over the flame of a bunsen burner and examined with immersion objective (X 100). With this double staining, "Gram-positive" bacteria appear dark purple while "Gram-negative" bacteria are stained pink or red (Delarras, 2007).

4.3 The coloring of the spore

The staining of the spores was carried out according to the protocol described by (Dorner, 1926). Preparation of malachite green solution **(Appendix 3)**

- **Technical**

- Take a smear

- Cover the entire blade with 5% malachite green

- Heat the slide until the solution evaporates

- Let cool

- Rinse the blade with tap water

- Color the slide with 0.5% fushin (leave for 1 minute)

- Rinse the slide and dry

Staining with Malachite green, the spores appear dark green on a light green background (Delarras, 2007).

5. Biochemical study

5.1 Mannitol test - mobility

Mannitol is a D-mannose reduction product. It makes it possible to simultaneously research the fermentation of mannitol and mobility. The strains studied were inoculated into the medium by central puncture, and incubated at 30 ° C. ± 1 ° C. for 18 to 24 hours. The yellow turn of the medium indicates the fermentation of mannitol, a diffusion in the agar indicates the mobility of the bacteria (Marchal *et al*., 1991).

5.2 Catalase research

Catalase is an enzyme which is produced in abundance by bacteria with respiratory metabolism which can destroy H_2O_2 peroxides which has a lethal effect for the bacteria. This test is based on the decomposition of hydrogen peroxide (H_2O_2) into water and oxygen according to the following reaction (Rebiahi, 2012).

2 H2O2 ⟶ 2 H2O+ O2

- **Technical :**

- On a clean, dry slide, place a few drops of hydrogen peroxide at 10 volumes;
- Add the bacterial inoculum using a platinum loop or buttoned shepherd's pipette.
- Observe immediately with the naked eye.

- **Reading :**

The positive reaction is manifested by the appearance of air bubbles, the release of oxygen gas and the negative reaction manifests itself in the absence of air bubbles or effervescence.

5.3 Oxidase test

- **Principle**

This test demonstrates the production of the bacterium under study of the enzyme "oxidase". Bacteria with a complete respiratory chain are endowed with a cytochrome oxidase enzyme.

The oxidation of Tetramethyl-p-phenylenediamine indicates the presence of an oxidase in the bacterium oxidase (El Bouamri, 2017).

- **Technique**

On a clean glass slide, place a disc impregnated with oxidase solution fixed by sterile physiology water using a sterile flamed Pasteur pipette, place the colony to be studied on the disc and observe immediately

- **Reading**

A positive reaction results in a violet coloration within a few seconds, which highlights the colorless reduced form of methylated derivatives of paraphenylene diamine, in their purplish pink semiquinonic oxidized form (Lecour, 2017).

6. The AP20e biochemical gallery

It is a miniaturized and standardized version of conventional biochemical techniques for the identification of Gram-negative bacteria, including Enterobacteriaceae. It comprises 20 microtubes containing substrates in dehydrated form. These microtubes were inoculated with a bacterial suspension which reconstitutes the media. The reactions produced during the incubation period (18h-24h at 37C °) resulted in spontaneous color changes or revealed by the addition of reagents (Ayad, 2017).

- **Technical**

The bottom and lid of an incubation box are brought together with the distribution of approximately 5 ml of distilled water in the cells to create a humid atmosphere, without forgetting to write the reference of the strain on the side tab of the box. In fact, the gallery is removed from its individual packaging and placed in the incubation box, then the bacterial inoculum is prepared: a colony in 5 ml of physiological water, its opacity must be equivalent to 0.5 Mc Farland. . To inoculate the gallery, it is necessary to fill, using a Pasteur pipette, the tubes and wells of the CIT, VP and GEL tests with the bacterial suspension, and for the other tests; we will fill only the tubes (and not the cups) with the creation of an anaerobiosis in the tests: ADH, LDC, ODC, URE, H2S by filling their cup with sterile vaseline oil. Finally, incubation is carried out at 37 ° C ± 1C ° for 18-24 hours (Lagha, 2015).**(Fig. 15)**

Figure 15: The AP20e biochemical gallery after seeding of the strains.

- **Reading**

The API 20E galleries are read according to the supplier's instructions. After codification of the reactions in a digital profile, we refer to an analytical catalog where the identification is given with a percentage and an appreciation.

7. Study of the enzymatic ability of our bacterial strains

In order to determine the ability of isolates to produce enzymes of industrial interest, several enzymes were investigated, such as protease, cellulase, and phosohatase. After subculturing of the strains from the storage tubes, the screening was carried out by the spot method and in duplicate.

7.1 The solubilization of phosphates

The capacity of the isolated strains to dissolve tricalcium phosphate Ca3 (PO4) 2 was tested according to the method described by Nautiyal (1999).

The isolated bacteria are deposited in the form of spots (5 µl) on the NBRIP (National Botanical Research Institute's phosphate) solid medium **(Appendix 2)**.

After incubation at 28 ° C. / 10 days, the total diameter (diameter of the halo + diameter of the colony) is measured. The diameter of the solubilization halo for each of the strains is determined by subtracting the diameter of the colony from the total diameter.

7.2 The production of cellulases

The presence of cellulase is revealed by subculturing the isolates on the medium of Carder (1986) **(Appendix 2)**. The inoculated dishes were incubated for 8 days (Carrim *et al*., 2006).

After the end of the incubation, a previously prepared lugol solution **(Appendix 3)** was dispersed over the entire surface of the medium. After five minutes of contact, the excess was removed and the cans washed with distilled water. The presence of extracellular cellulase is manifested by the appearance of a clear halo around the colonies.

7.3 Proteolytic enzymes

The hydrolysis of casein is studied on a milk agar medium **(Appendix 2)**. The strains are seeded in a single streak and then incubated at 30C. The results are assessed daily for 72 hours. The appearance of a clear halo around the culture indicates degradation of the casein (Devos *et al*. 2009).

7.4 Chitinolytic activity

The demonstration of the chitinolytic activity consists in depositing 20 µl of each inoculum on the surface of the CMC medium **(Appendix 2)**. This test is carried out at an incubation temperature of 30 ° C.

The production of extracellular chitinase results in the appearance of clear areas around the colonies. The presence of clear halos around the colonies is observed after 7 days of incubation (Kim *et al*. 2003).

7.5 TSA medium (Tryptone Soya Agar)

The ability to produce hydrocyanic acid by strains of *Bacillus* is studied according to the method of Bakker and Schippers (1987). Each strain is inoculated, using a loop, on Trypticase soy medium (Fluka) **(Appendix 2)** supplemented with glycine (4.4 g / l).

Whatman 3MM paper (Ø = 5 cm) impregnated with a yellow solution **(Appendix 3)** is placed on the lid of each Petri dish in the inverted position.

These are sealed with Parafilm® paper and incubated at 28 ° C / 4 days. They are checked daily to identify the strains, HCN +, capable of turning the color of the paper, from yellow to orange (Abbas-Zadeh *et al*., 2010).**(Fig. 16)**

Figure 16: A Whatman 3MM paper (Ø = 5 cm) impregnated with a yellow solution **(Appendix 3)** placed on the lid of each Petri dish.

8. Performing the antibiogram

- The antibiogram is a microbiological laboratory technique designed to test the sensitivity of a bacterial strain to one or more antibiotics. It will therefore give indications on the in vitro efficacy of these antibiotics.
- To perform the antibiogram by the disc method, the bacterial culture is carried out on the surface of a Mueller Hinton agar **(Appendix 2)**, discs pre-impregnated with a known dose of antibiotic are deposited on the surface of the agar.
- The antibiotic diffuses from the disc by creating a concentration gradient from which we determine the diameter of the zone of inhibition which gives us an estimate of the minimum inhibitory concentration.
- The sensitivity or resistance characteristics of the bacterial strain is defined from a reference table published by the standardization of the Ministry of Health (Francois, 2011).

- **The technique**

- Pour 25ml of MH agar into a 90cm diameter Petri dish.
- Preparation of the suspension from a pure culture and dilute the suspected colonies: 1/100 in 10 ml of physiological water.
- Vortexing of the bacterial suspension to ensure its homogenization.
- Seed the suspension by swabbing; dip the swab in the suspension and wring it out on the edges, inoculate the dish by gently rubbing the swab on the agar

- Place the antibiotic discs using sterile forceps.
- Leave the dishes for 20 minutes at room temperature to allow pre-diffusion of the antibiotic, then incubate them for 18-24 hours at 37 ° C (Guy and Jean., 2006).**(Fig. 17)**

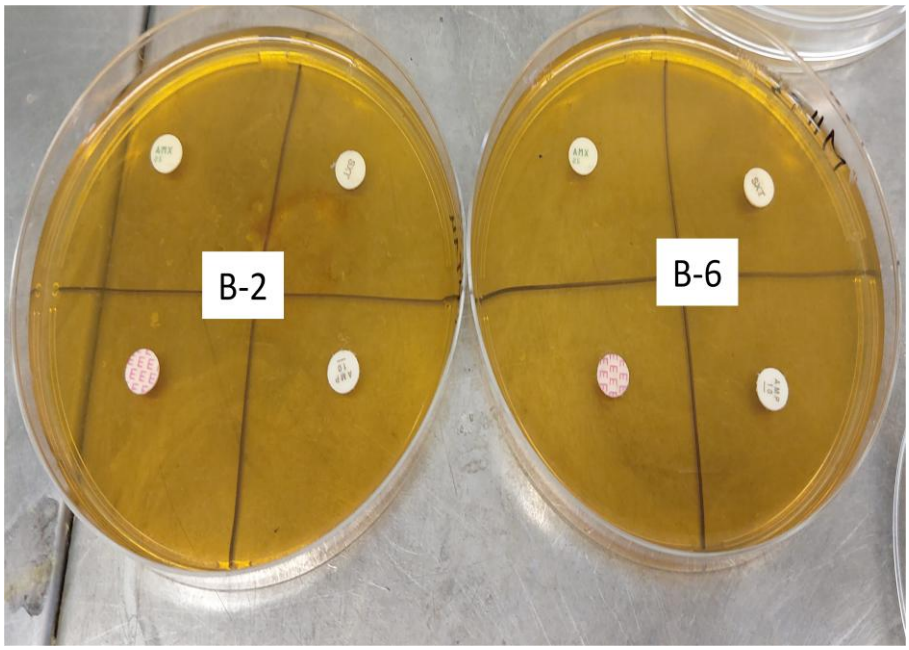


Figure 17: Location of antibiotic discs for our bacterial strains.

- **Reading and interpretation**

- A susceptible strain is one which can be reached by treatment at the usual dose by systemic route.
- An intermediate strain is a strain which can be reached by local treatment by increasing the doses systemically
- A resistant strain is one that is unlikely to react regardless of the dose and type of treatment (Avril *et al*., 2000).

Part III
Results And Discussion

Results and Discussion

Chapter I Fungal results and descriptions

1. Morphological identification of fungal isolates

1.1 Revivification of fungal strains

After 7 days of incubation at 25 ° C, the two fungal strains T_3 and T_{14} produce woolly colonies, initially white in color, and then appear in aging greenish tufts isolated or arranged in concentric rings on the centre of our culture; the back remains colorless.

The macroscopic appearance of fungal strains is demonstrated in the following figure: **(Fig. 18)**

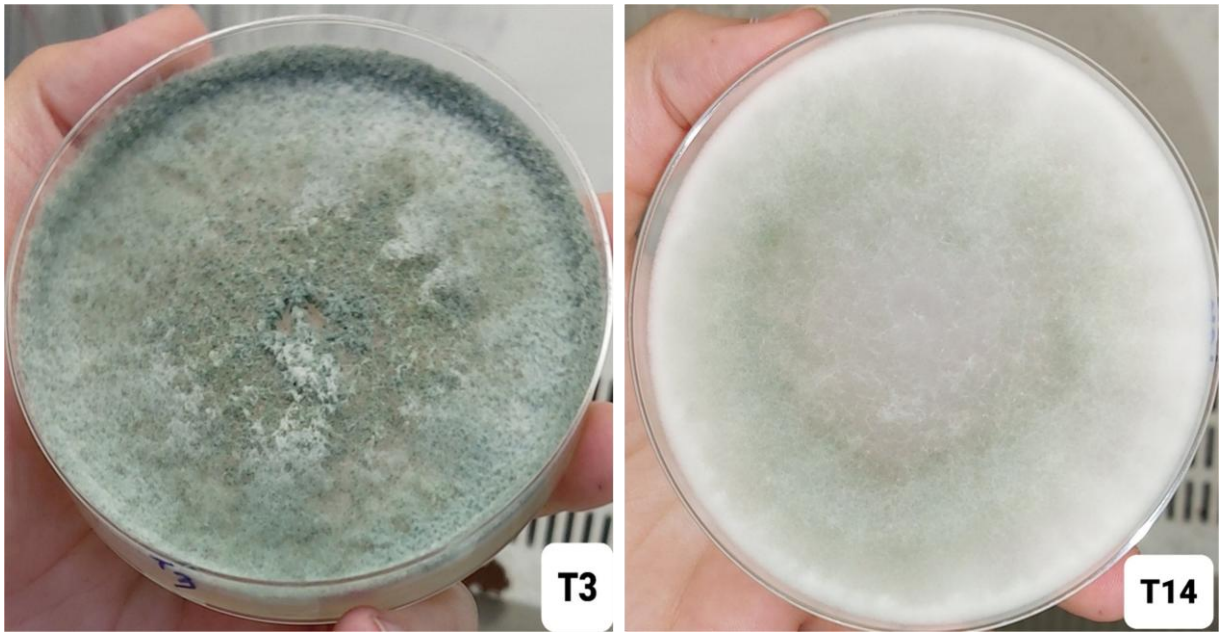


Figure 18: Macroscopic observation of the two strains after revivification.

1.2 Monospore culture

After 48 hours of incubation at 25 ° C, well isolated spores were obtained which are shown in the first two images: **(Fig. 19)**

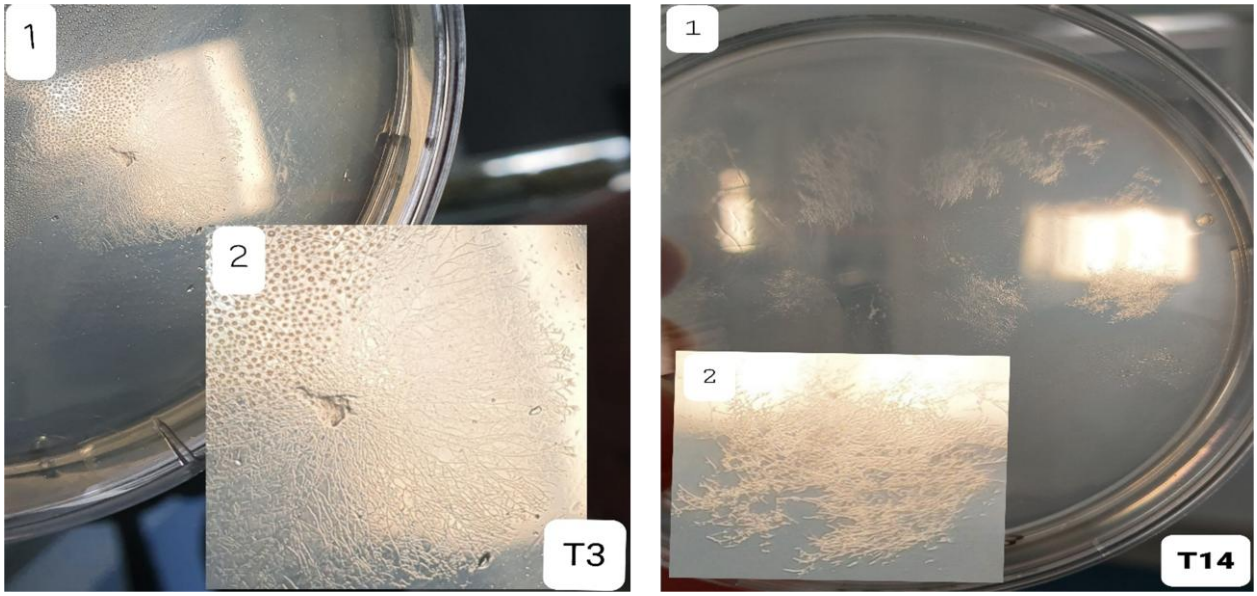


Figure 19: Results of monospore culture after 48h of incubation in minimal medium. Pictures taken without zoom (1). Pictures taken with zoom (2).

The spore identified is placed in culture in a new Petri dish containing the PDA medium. After six days of incubation at 25 ° C, we obtain pure mycelial colonies all from the same spore which allows us to ensure the purity of the strain and that they are 100% genetically identical. The result is shown in the following images: **(Fig. 20)**

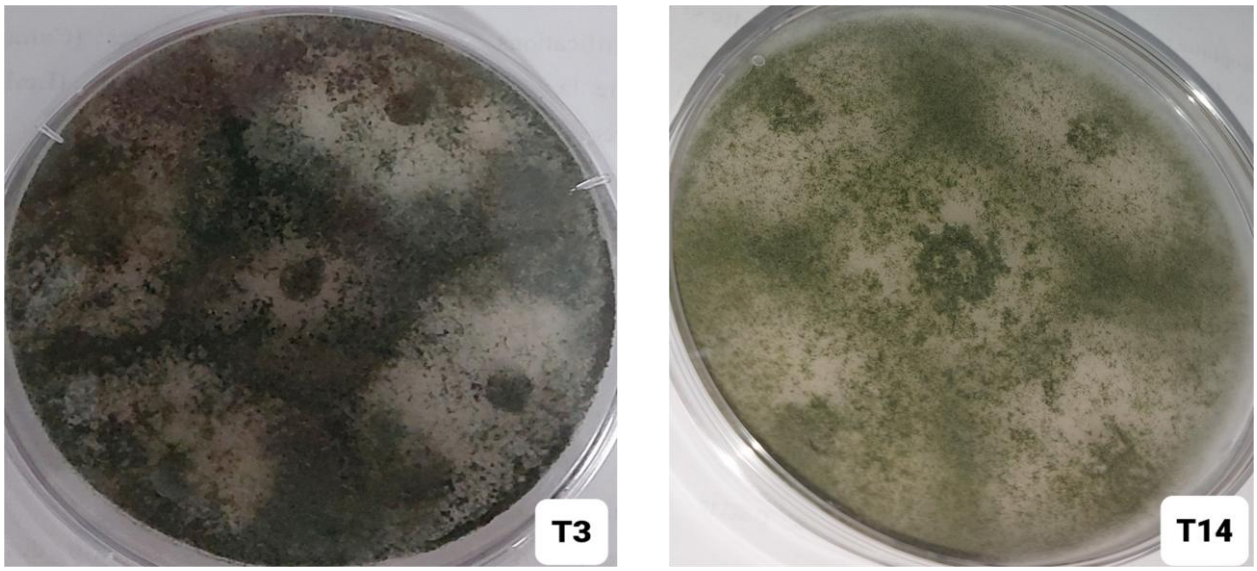
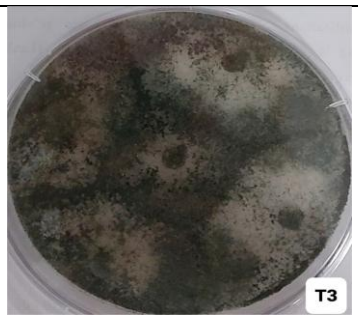
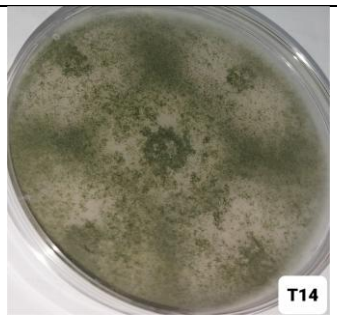


Figure 20: Results of monospore culture after 6 days of incubation in PDA medium.

1.3 Macroscopic study

The macroscopic characters of the various isolates selected were studied on the PDA medium most commonly used for this purpose, at an optimum temperature of 25 ° C (Botton, 1990) **(Fig. 20)**. The results obtained after 7 days of incubation are given in the following table.

Table 05: Table represents the macroscopic aspect of fungi.

	T_3	T_{14}
Figure	 **Figure 21:** Macroscopic observation of T_3.	 **Figure 22:** Macroscopic observation of T_{14}.
Number of colonies	Single colonies colonizing the entire of Petri dishe.	Single colonies colonizing the entire of Petri dishe.
Colony size	4 cm.	4 cm.
The obverse	White and pine green.	White and olive green.
The reverse	White to little bit green.	White to little bit green.
The aspect of strains	Cottony.	Cottony.

The macroscopic characters of the two fungal strains T_3 and T_{14} summarizes on **Table 05** correspond perfectly to the genus *Trichoderma* which was described by Botton (1990), Lyral and his collaborators (1998), also those of Chabasse and his collaborators (2002).

1.3 Microscopic study

Small, well-differentiated conidiophores appear on septate hyaline hyphae simple or branched. They bear phialides, also of small size, in the shape of keel. Swollen at their base, solitary or grouped by 3, the phialides are set at an angle right on the conidiophores.

The conidia, smooth or echinulate, globular, measure 2.5 to 3 µm in diameter. They gather in clusters at the top of the phialides, and thus form "false heads". (Chabasse *et al.*, 2002). **(Fig. 23)**

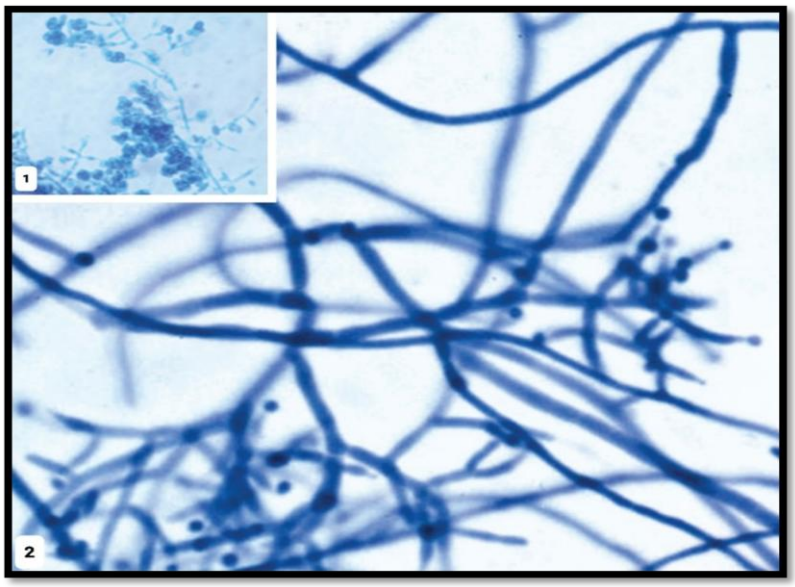


Figure 23: Microscopic observation of *Trichoderma* species. Clusters of conidia visualized at the top of the phialides at objective 10 (1). The phialides keel-shaped are arranged in whorls on angled branching conidiophores acute, the whole presenting a pyramidal aspect (2, objective 100).

2. Screening for qualitative enzymatic activity of *Trichoderma* species

To evaluate the production of extracellular enzymes was done on selective solid medium. Enzymatic activity was evaluated by measuring the halo of hydrolysis zone around the disc inoculated as follows: _ (no change): isolates showing no enzyme activity, + (halo zone less than 10 mm): isolates showing very low enzyme activity, ++ (halo zone of 10-30 mm): isolates showing low enzyme activity, +++ (halo zone of 40-60 mm):isolates showing high enzyme activity, ++++ (halo zone of 70 mm and above): isolates showing very high enzyme activity. The results are presented in the **Table 06**.

Table 06: Results of enzymatic activity of *Trichoderma* species.

Enzymatic activity of *Trichoderma* species	**T_3**	**T_{14}**
Protease assay	++++	+++
Cellulase assay	++++	+++
Lipolytic activity	+++	++
Chitinase activity	++++	++
Hydrogen cyanide (HCN) production	++++	++++
Nitrogen fixation activity	++++	++++
NH3 production	++++	++++
Siderophore production	–	–
Dosage of Indole acetic acid (IAA)	++	++

2.1 Protease assay

A clear zones appear around the transferred disc, that's indicates a proteolytic activity. **(Fig. 24)**. It has been suggested that the protease activity is involved in the degradation of pathogen cell walls, membranes and even proteins released by the lysis of the pathogen, thus making nutrients available for the mycoparasite (Goldman *et al.*, 1994).

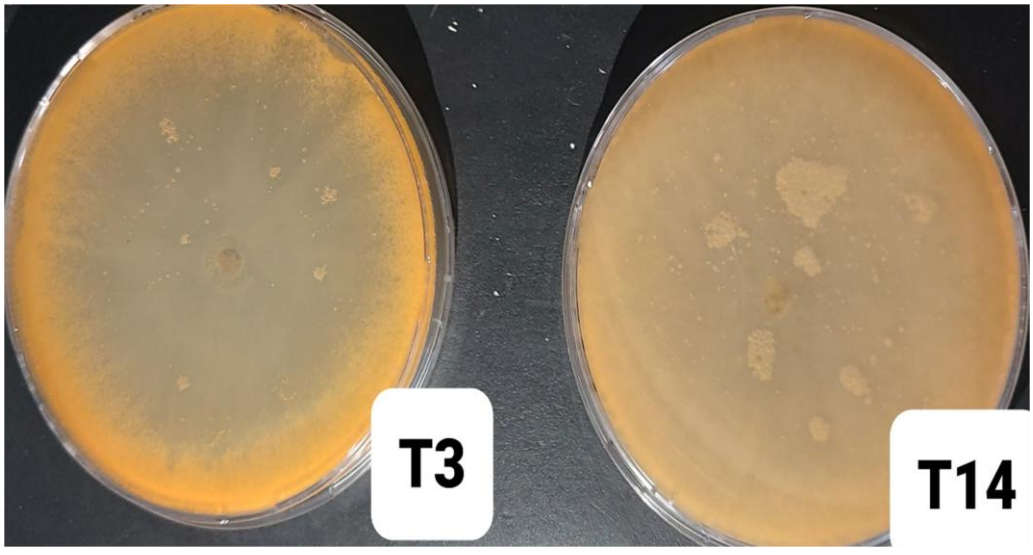


Figure 24: Results of protease assay.

2.2 Cellulase assay

Formation of yellow-opaque area around the colonies that indicates the production of cellulases. **(Fig. 25)**

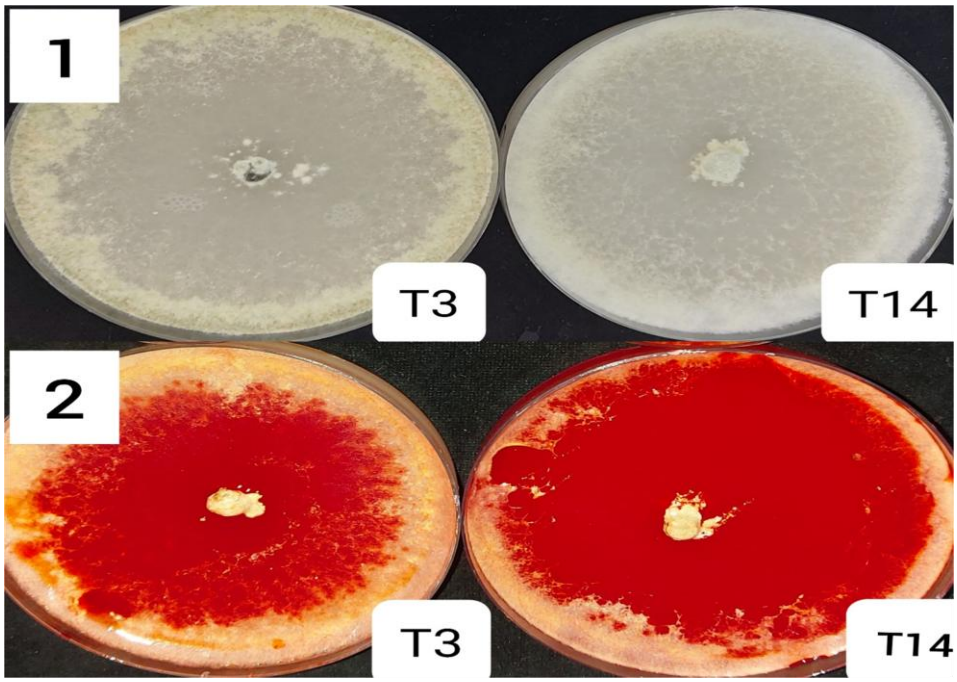


Figure 25: Results of cellulases assay. Pictures taken before addition of Congo red and NaCl solution (1). Pictures taken after addition of Congo red and NaCl solutions (2).

The fungi grown on the selective media supported the growth of the fungi by using cellulose as the carbon source (Khalid *et al.,* 2006). Cellulases are the enzymes responsible for the cleavage of the β–1, 4–glycosidic linkages in cellulose. The appearance of the clear zone around the colony after the addition of Congo red solution was strong evidence for the secretion of cellulase enzymes this results are in agreement with Kamala and Indira (2010). Cellulase and the -1, 3- glucanase are the two enzymes that play important role in the enzymatic degradation of cell walls of phytopathogenic fungi like *P. ultimum* during mycoparasitic interaction (Kamala and Indira, 2014).

2.3 Lipolytic activity

Lipolytic activity was evaluated according to the methodology described by Howe and Ward (1976), as we see in **Fig. 26** there's a presence of opaque precipitate around the colony indicated that *Trichoderma* spp isolates were capable of hydrolyzing Tween 80 according to Strakowska *et al.,* 2014

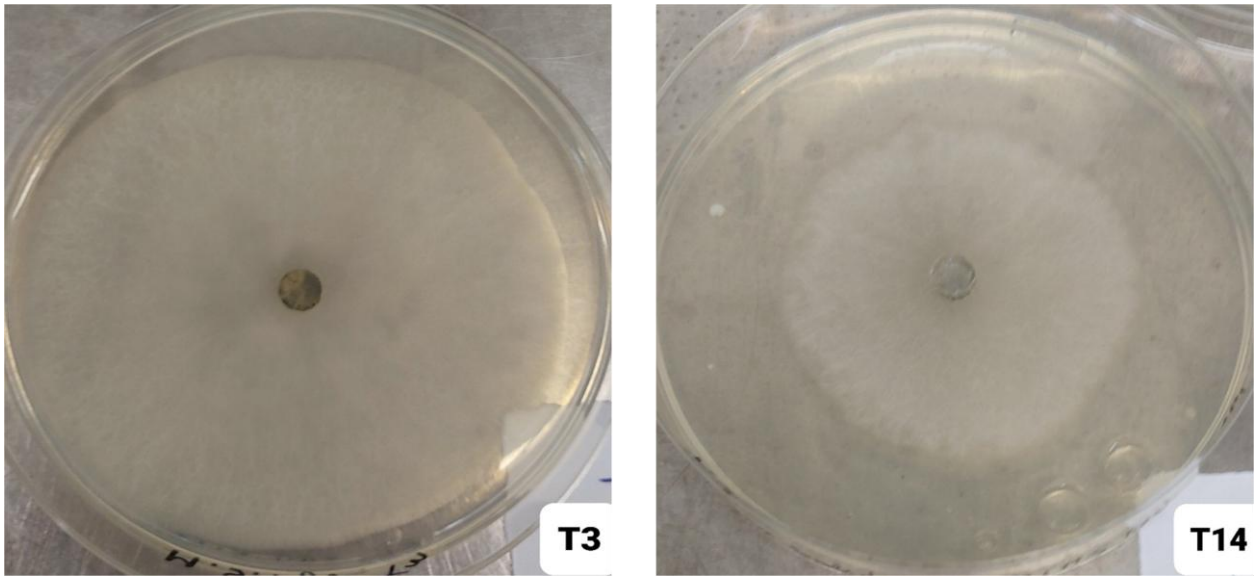


Figure 26: Results of lipolytic activity.

2.4 Chitinase activity

For chitinase activity, after 8 days of incubation we can see a varying of color from yellow to purple indicate a production of chitinase, this results are in agreements with those of Agrawal and Kotasthane in (2012) which observed the appearance of purple color in Petri dishes containing colloidal chitine as the alone resource of carbone and inoculated with *Trichoderma*. **(Fig. 27)**

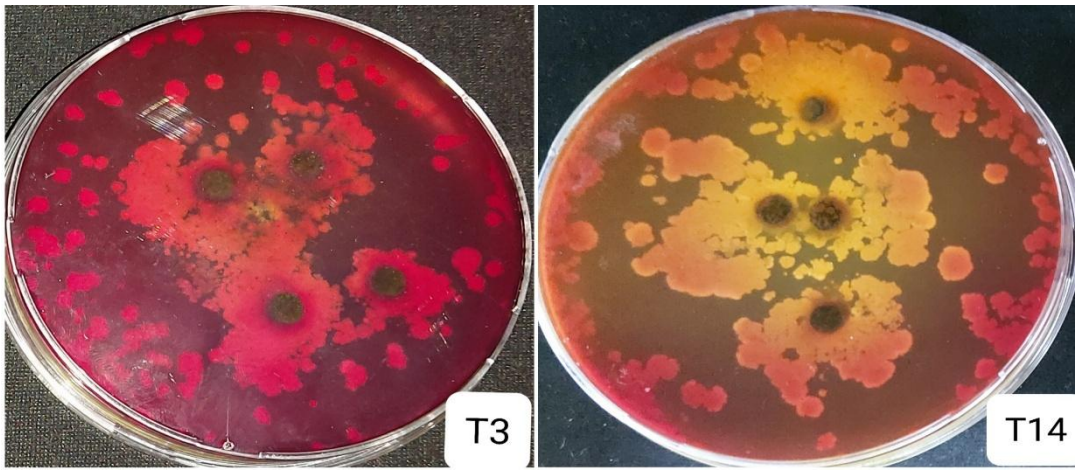


Figure 27: Results of chitinase activity.

3. Screening for qualitative PGPF activity of *Trichoderma* species

3.1 Hydrogen cyanide (HCN) production

HCN production was screened qualitatively; we observed a change in color of the filter paper from yellow to reddish brown that indicate the production of HCN. This results are agreed with the results of Meera and Balabaskar 2012. **(Fig. 28)**

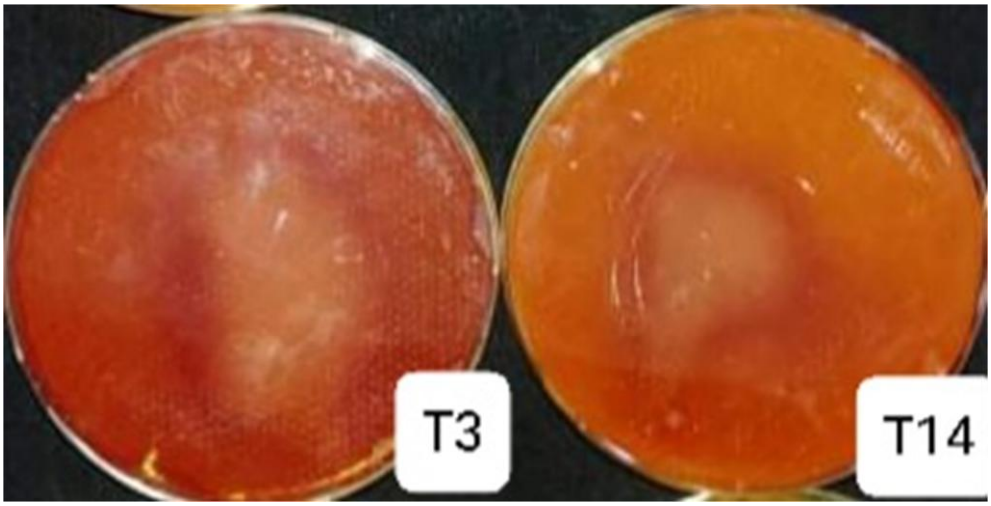


Figure 28: Results of hydrogen cyanide (HCN) production.

3.2 Nitrogen fixation activity

Nitrogen-fixing was screened according to Zhang *et al.,* 2017, after 3 days of inoculation, the test of nitrogen-fixing ability was positive because of the clearly growth of the colonies in media lacking nitrogen resources as shown in **Fig. 29**. This results are in agreement with Zhang *et al.,* 2017.

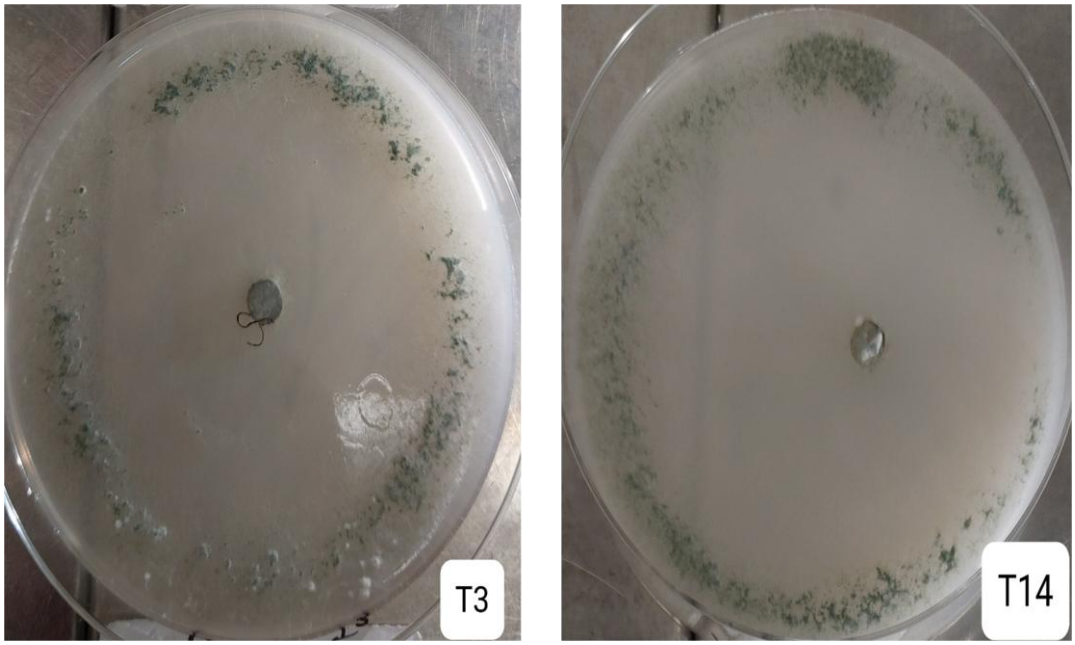


Figure 29: Results of nitrogen fixation activity.

3.3 NH3 production

Production of ammonia was tested according to Bakker and Schipper (1987), we can see in **Fig. 30** a development of color from yellow to brownish orange that's means a positive test for ammonia production according to Shoresh *et al.,* 2005.

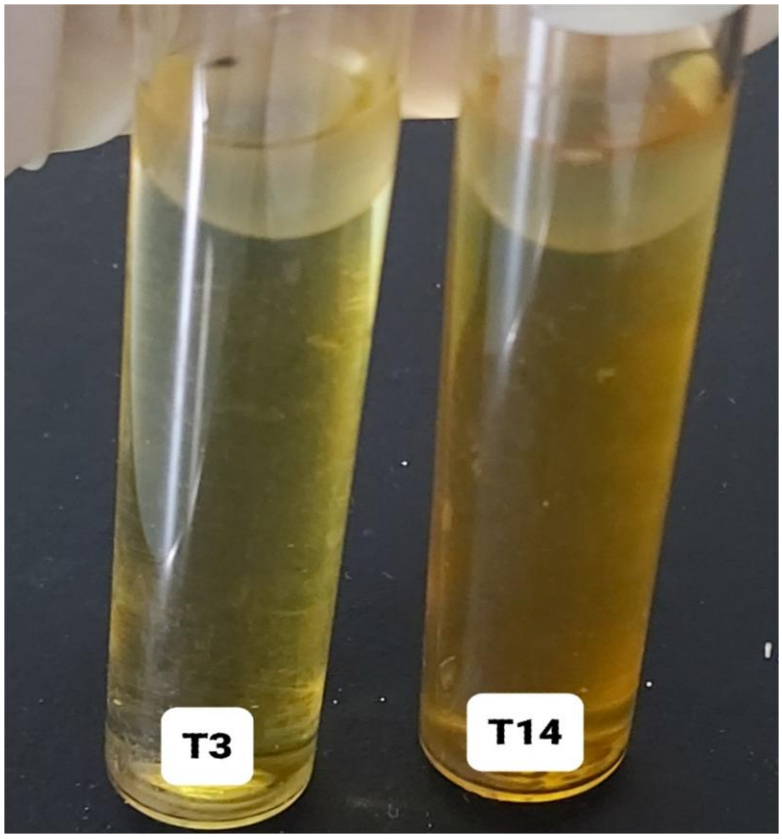


Figure 30: Results of ammonia production.

3.4 Siderophore production

As we observed in **Fig. 31** we don't have any growth of strains so the test of siderophores production considered in this case negative.

Our result is supported by López *et al.*, (2019) who reported that *Trichoderma atroviride* LBM 112 showed negative result for siderophores production.

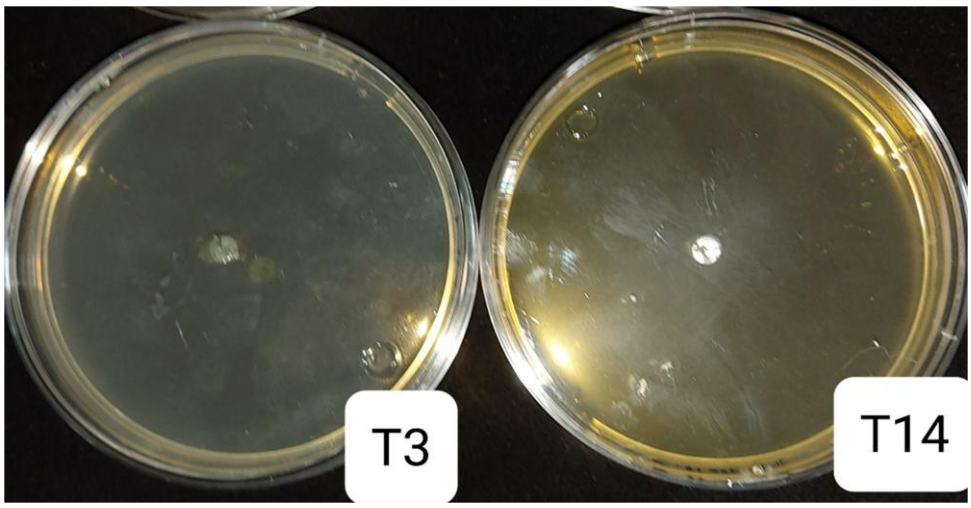


Figure 31: Results of siderophores production.

3.5 Dosage of Indole acetic acid (IAA)

We see in **(Fig. 32)** a development of color from yellow to pink and that's indicate IAA production and this results are in agreement with those observed by Gravel *et al.,* 2007.

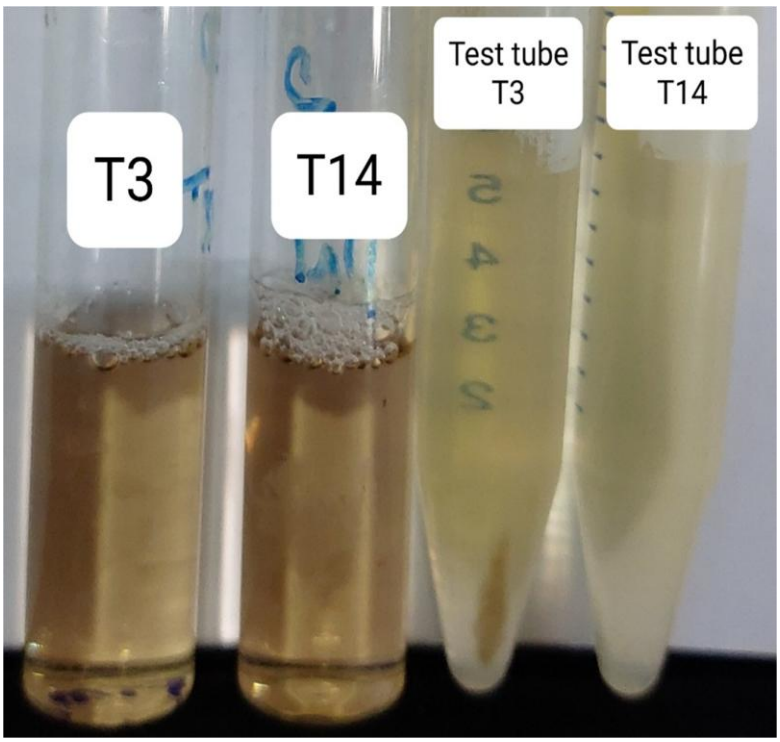


Figure 32: Results of dosage IAA.

3.6 Chitinase hydrolytic activity assay

Isolates of *Trichoderma* showed larger zone of chitin hydrolysis on solid medium were selected to evaluate their chitinase hydrolytic activity. The absorbance was measured with spectrophotometer at 585 nm. **(Fig. 33)**

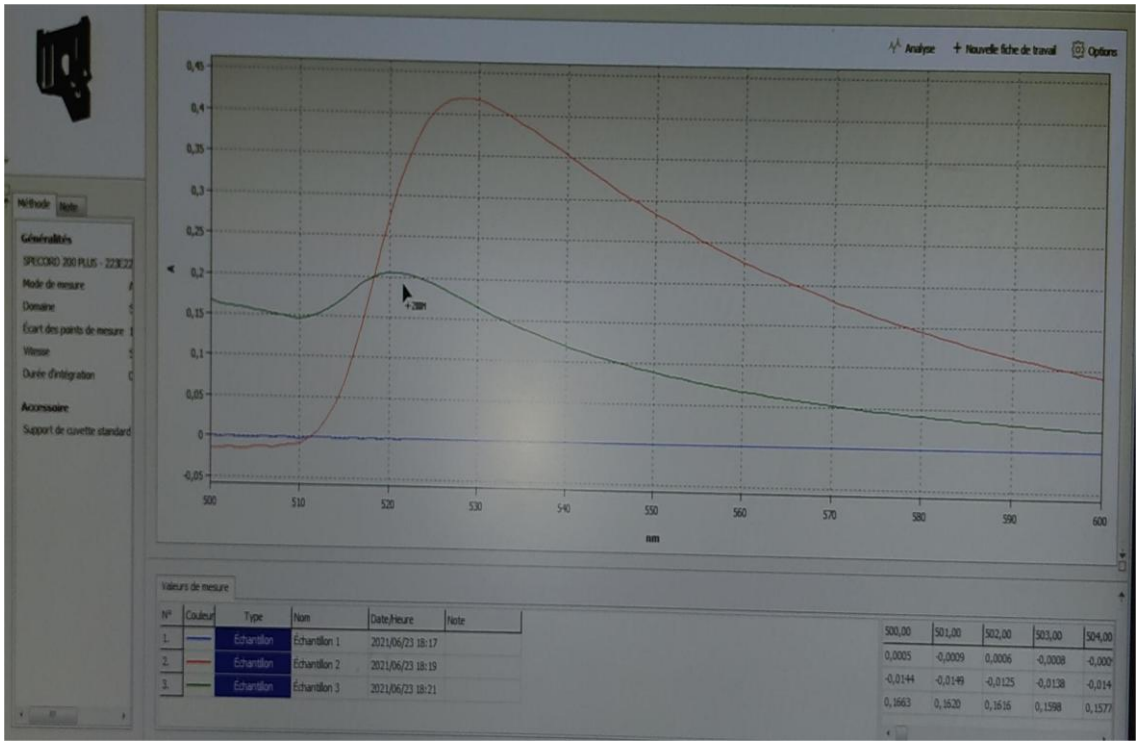


Figure 33: Absorbance diagram of chitinase hydrolytic activity assay.

The results of total chitinolytic activity assayed by measuring the amount of N-acetyl glucosamine released (reducing saccharides) from colloidal chitin and N-acetyl glucosaminidase (exochitinase) are positive in agreement with those of Agrawal and Kotasthane in (2012).

4. Screening and isolation of microbial strains that produce bio-surfactants

4.1 The E24 emulsification test

In order to confirm and estimate the production of the biosurfactant by the fungal strains tested, we performed the E24 emulsification test. The results obtained show that the two strains T_3 and T_{14} present a positive E24, this is confirmed by the appearance of an emulsion layer in the two tubes (The observation of the emulsion layer is done in relation to the control of the same strain.) these results are agreed with Silva *et al.*, (2019). **(Fig. 34)**

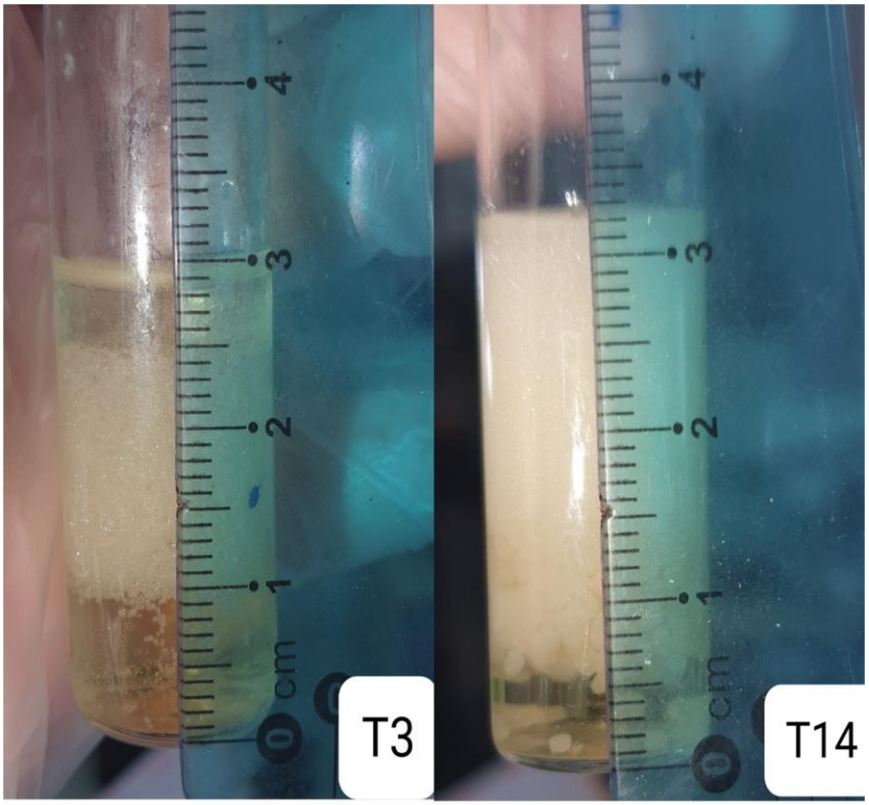


Figure 34: Results of emulsification test E24.

4.2 Calculation of the E24% index

The results of the E24% emulsification indices are presented in the following table:

Table 07: Table represents the percentages of E24.

Strains	**T_3**	**T_{14}**
He: emulsion height.	**2 cm.**	**2.7 cm.**
Ht: total height of the mixture.	**3 cm.**	**3.3 cm.**
E24%=He/Ht×100	**66.66 %.**	**81.81 %.**

From the results obtained, it appears that the two strains tested are capable to producing biosurfactants.

The percentages of the emulsification indices equal 66.66% for T_3 and 81.81% for T_{14}. The strain T_{14} have a percentage of E24 raised compared to that of the strain T_3. The ability to maintain at least 50% of the initial volume of the emulsion after 24 hours of its formation is considered as a criterion cited to confirm the production of biosurfactants (Nasr *et al.*, 2009).

Therefore, the fungi which grow on hydrocarbons generally produce emulsifiers which stimulate their growth and accelerate bioremediation (Ron and Rosenberg, 2002).

5. Extraction of bio-surfactants

The extracts of the biosurfactants obtained by the fungal cultures which are made by the method of Kumar *et al.,* 2006 with the mixture chloroform: ethanol (2: 1) as an extraction solvent are two transparent liquids. the biosurfactant obtained by the extraction of T_3 strains is more concentrated compared to that of T_{14}. **(Fig. 35)**

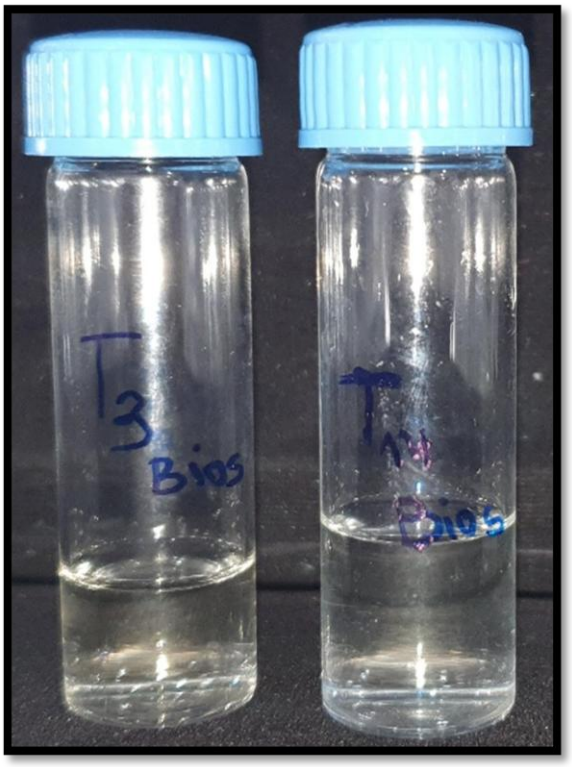


Figure 35: The extracts of biosurfactants obtained by T_3 and T_{14}.

6. Extraction of antibiotics

The extracts of antibiotics obtained by the fungal cultures which are made by ethyl acetate as an extraction solvent are two liquids with sand beige. The antibiotics extracts obtained by the extraction of T_3 strains is more concentrated compared to that of T_{14}. **(Fig. 36)**

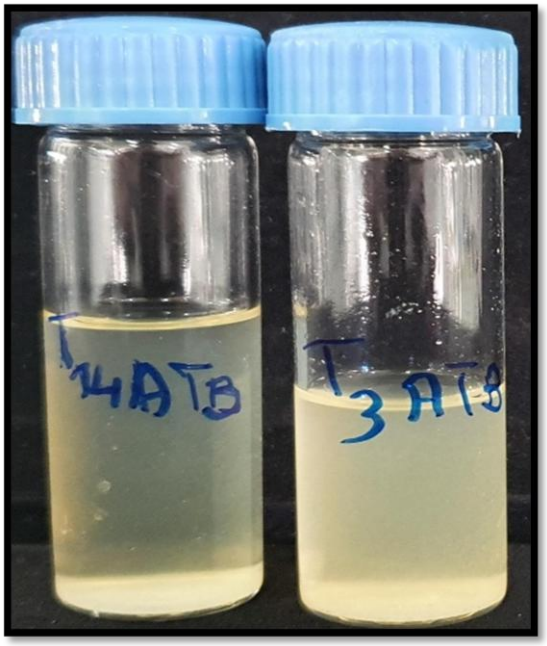


Figure 36: The extracts of ATB obtained by T_3 and T_{14}.

7. Revelation of the extracts by thin layer chromatography TLC

7.1 Revelation of biosurfactants extracts by TLC

After the revelation of the biosurfactants by thin layer chromatography with Chloroform: methanol: water = 65: 15: 2 (V / V / V) (Ebadi *et al.*, 2018) as a solvent, the solvent migration was rapid (54min) and the observation under UV at 350 nm shows migration plates **(Fig. 37)**. We calculated the frontal ratios in the following **Table 08**.

Table 08: Frontal report of biosurfactant's TLC.

Strains	**T_3**	**T_{14}**
Number of spots	1	2
Color of spots in white light	Transparent	Transparent
Color of under UV	Very radiant white	Very radiant white
Plates size	1.8 cm.	1.8 cm.
Distance traveled by the solvent (cm)	16.3	16.3
Distance traveled by the spot (cm)	15	15
frontal report	1.08	1.08

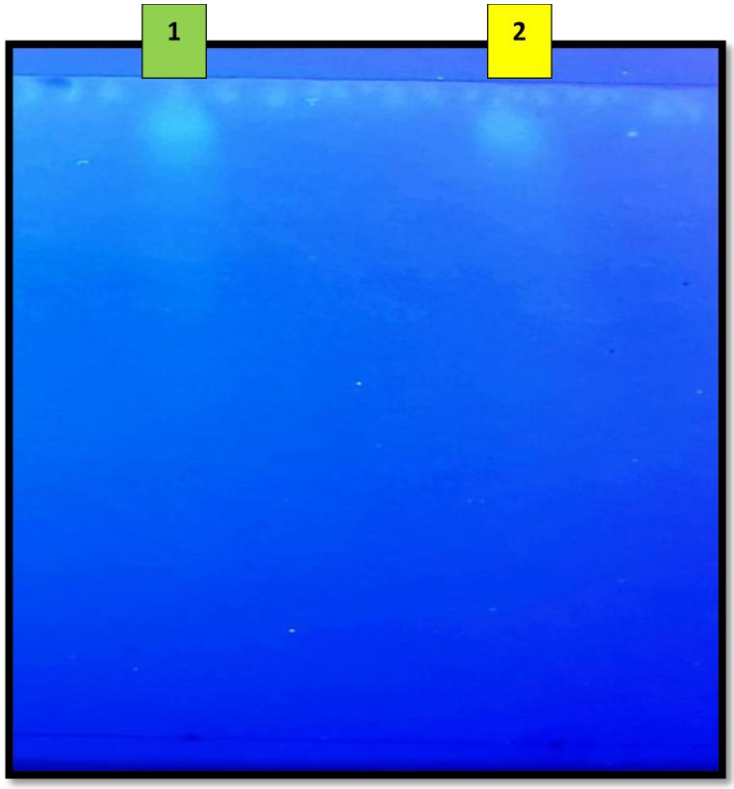


Figure 37: Observation of biosurfactant's TLC under UV at 350 nm.

The absence of pink spots after spraying the plate using a ninhydrin solution proves the absence of amino groups in the different biosurfactants studied. In addition, the absence of blue-green spots were also revealed after spraying with the plaque with a solution of phosphomolybdic acid, which shows the absence of fatty acid compounds in the two biosurfactants this results were very closed to those reported by Dorra, 2018.

7.2 Revelation of antibiotics extracts by TLC

After the revelation of the antibiotics by thin layer chromatography with Ethyl acetate: methanol = 100: 15 (V / V) (Zitouni, 1995) as a solvent, the solvent migration was rapid (50 min) and the observation under UV at 350 nm shows migration plates **(Fig. 38)**. We calculated the frontal ratios in the following **Table 09.**

Table 09: Frontal report of antibiotic's TLC.

Strains	**T_3**	**T_{14}**
Number of spots	3	4
Color of spots in white light	Transparent	Transparent
Color of spots under UV	Very radiant white	Radiant white
Plates size	2.3 cm.	2 cm.
Distance traveled by the solvent (cm)	16.3	16.3
Distance traveled by the spot (cm)	14.3	14.8
frontal report	1.13	1. 10

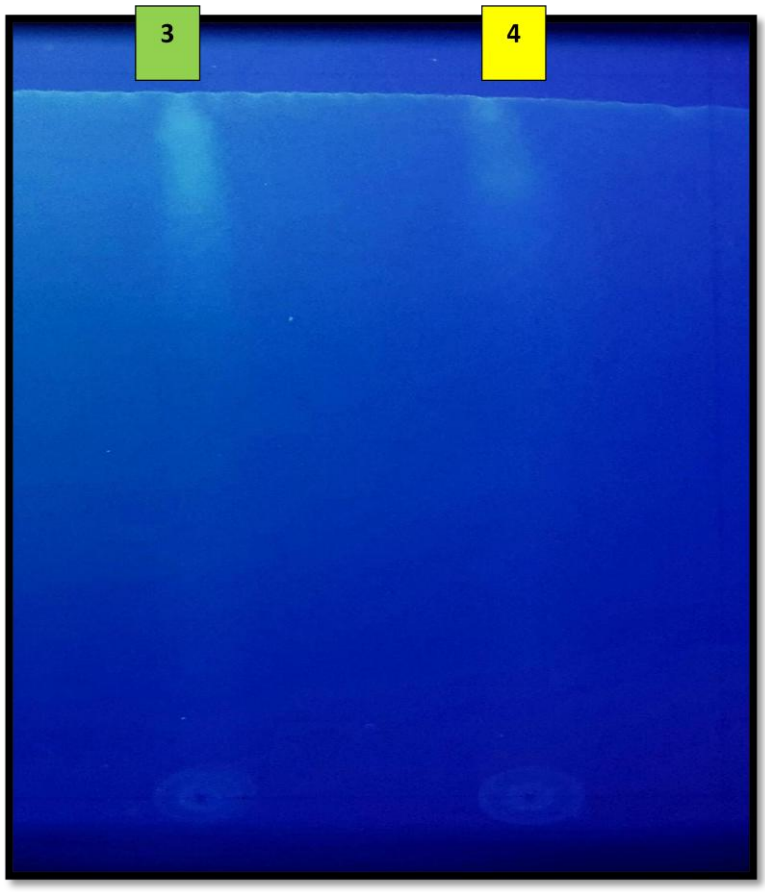


Figure 38: Observation of antibiotic's TLC under UV at 350 nm.

Chapter II Bacterial results and descriptions

I. Bacterial revivification

The determination of the morphology, the cellular arrangement, as well as the Gram of the strains were carried out on young strains cultivated at 37 °C on nutrient agar (NA) medium, on the other hand the formation of spore bodies was observed on old cultures.

II. The re-identification of bacterial strains

1. Macroscopic observation

The macroscopic appearance of the strains on NA made it possible to distinguish two different colonies **(Fig. 39), Table 10** shows the morphological appearance of the colonies on the NA medium.

Table 10: Morphological appearance of colonies obtained on NA.

The bacterial strain	Size (mm)	Form	Elevation	Aspect	contour	Consistency	Color	Odour
B1	3-4	Round colonies	Semi Domed	Smooth, opaque colonies	with regular edges	Creamy	Cream white	Presence of odora
B2	5	Round colonies	Semi Domed	Smooth, opaque colonies	with irregular edges	Creamy	Cream white	Presence of odora

The isolates observed were whitish in color, present a round shape colonies, smooth, opaque with regular edges (B1) and irregular (B2). Those isolates presented the morphological characteristics of the genus of *Bacillus.* According to Corbin (2004), *Bacillus* sp colonies have common characteristics has a whitish cream color with a round shape and irregular colonies .

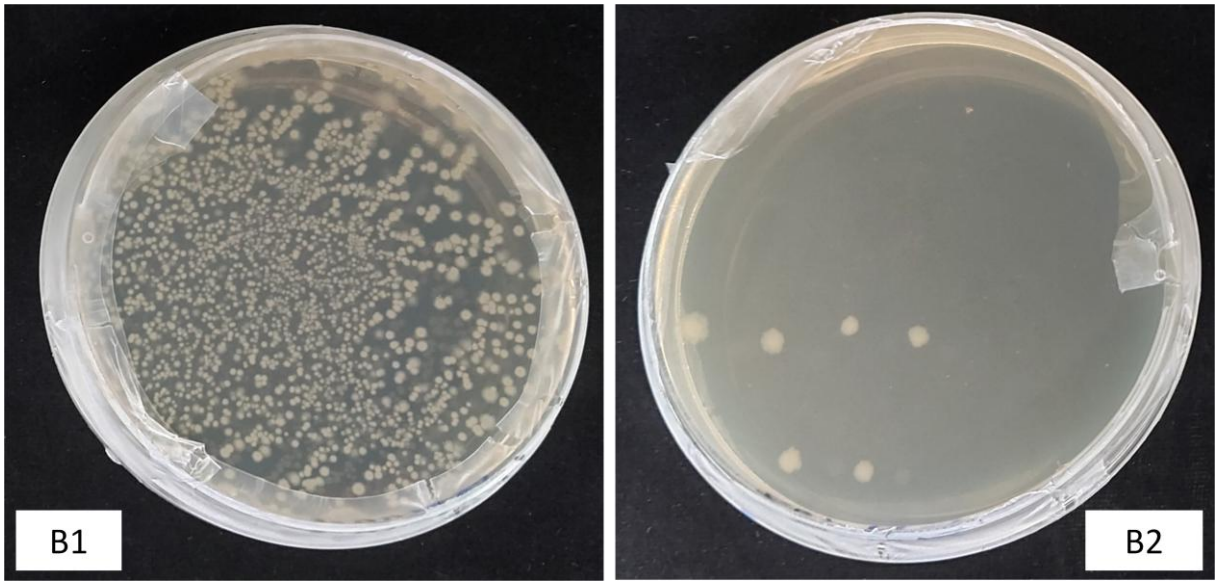


Figure 39: Photos show the morphological appearance of the colonies on the NA medium after 24h of incubation at 37°C.

2. Microscopic observation

2.1 Fresh examination

Observation of the cells in the fresh state indicates that both strains are immobile *Bacilli*. These results are compatible with that of (Fauchère and Avril, 2002).

2.2 Examination after Gram stain

The microscopic examinations which are carried out after Gram staining, showed that all the strains are *Bacilli*, Gram positive, appearing in isolated form or in pairs **(Fig. 40).**

This result shows similar microscopic characteristics with *Bacillus* sp by common, whose cells are rod-shaped and Gram-positive.

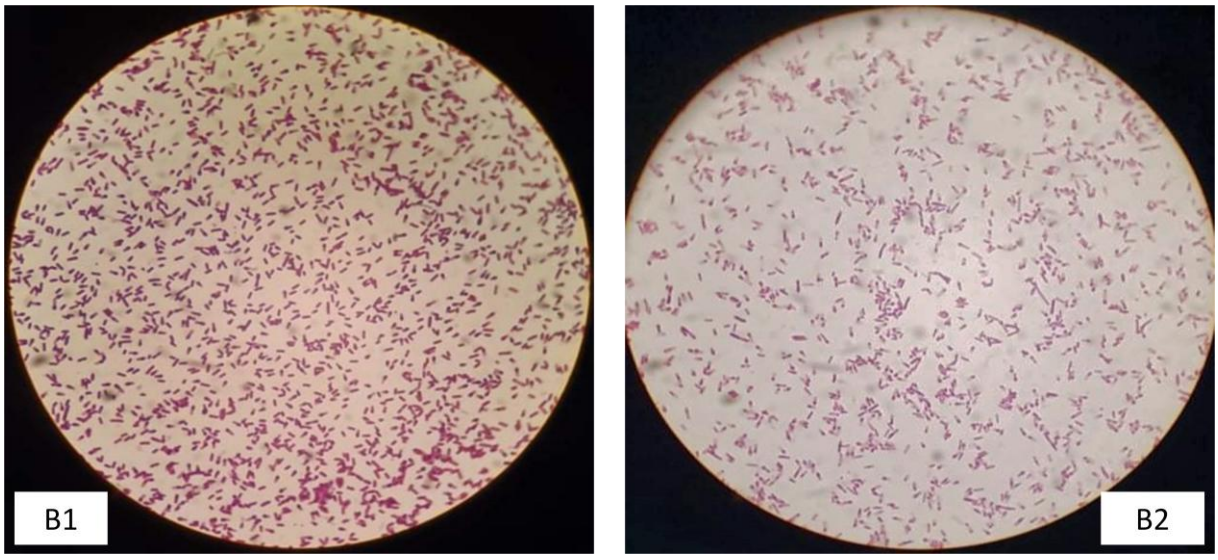


Figure 40: Optical microscopic observations of *Bacilli* after Gram staining.

2.3 The coloring of the spore

From the result shown in (**Fig. 41)** the two *Bacillus* strains formed spores that appear dark green on a light green background, these results are consistent with that of Curran (1957).

Spore formation was affected by many nutritional and other environmental factors and mineral salts were important (Kolodzie and Slepecky. 1962).

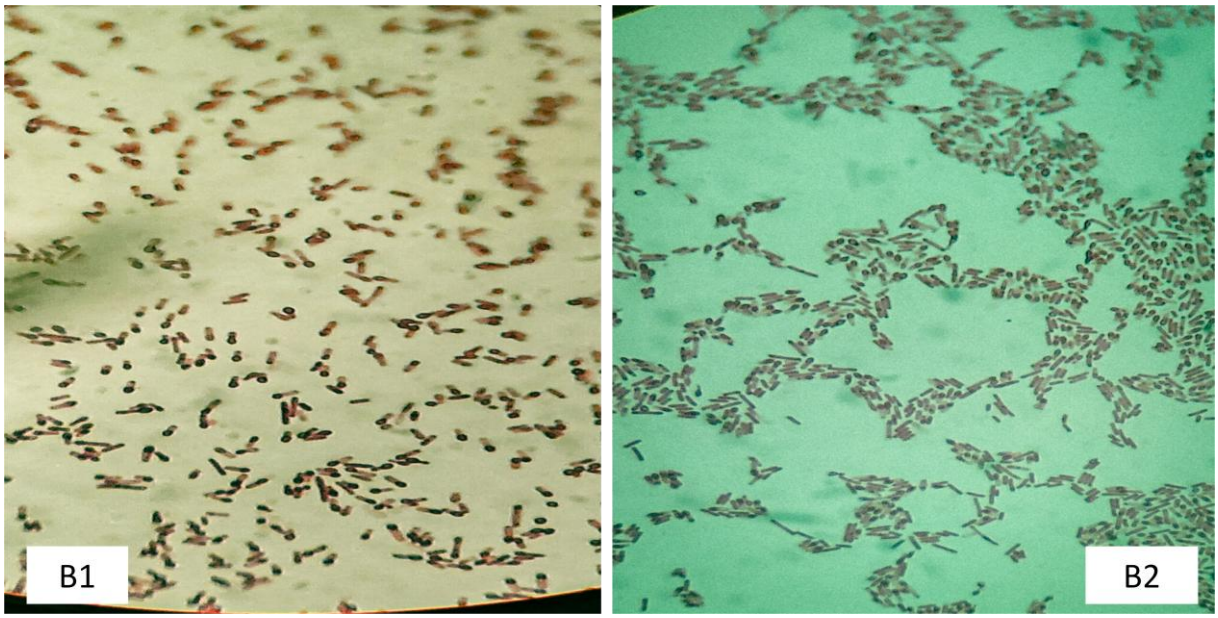


Figure 41: Microscopic observation of the spores after the malachite green staining of the two *Bacillus* strains.

3. Identification by orientation tests and biochemical identification

3.1 Mannitol test – mobility

From the result **(Fig. 42)** the two tubes indicate a negative result, the immobile bacteria persist along the central puncture and the medium keeps its initial color this indicates that the two bacterial strains have not acidified the medium (no yellow) therefore the strains are unable to ferment mannitol.

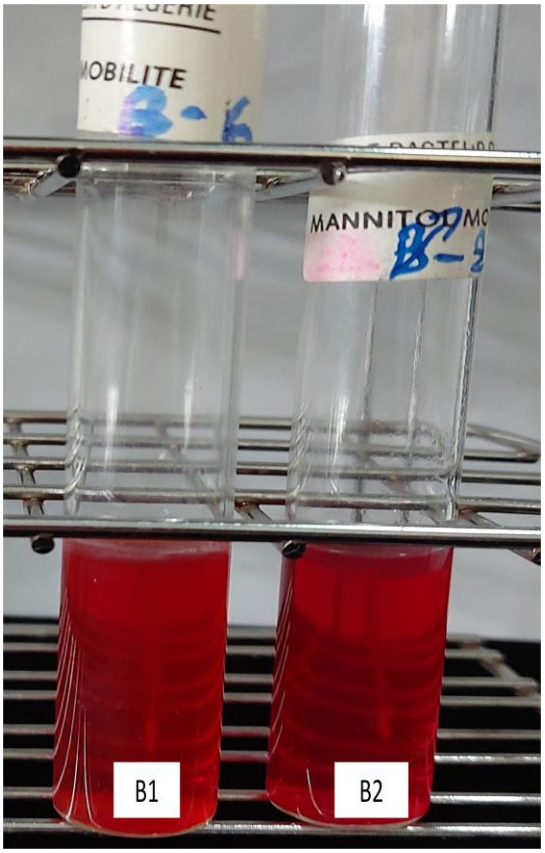


Figure 42: Mannitol test result –mobility of the two *Bacillus* strains.

3.2 Catalase test

The evolution of gaz bubbles means that there is production of the enzyme catalase and that the test is positive for both strains tested **(Fig. 43).** A positive catalase test indicates that *Bacillus* sp have the enzyme catalase. *Bacillus* sp is a genus of obligate aerobic or facultative aerobic bacteria, and positive for the enzyme catalase test. According to Barrow *et al.* (1993)

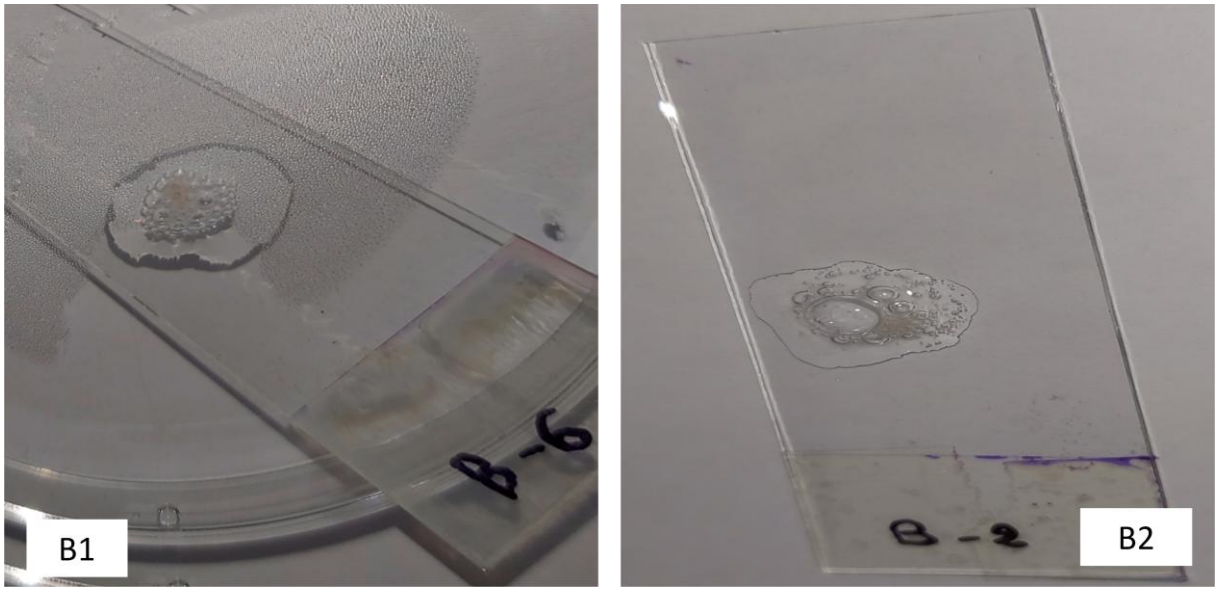


Figure 43: Catalase Test Results of Isolates.

3.3. Oxidase test

The purpose of the oxidase test is to look for a Cytochrome C system in bacteria (Oxidase positive). The results obtained after the oxidase test on the two strains studied show the presence of a purple color, which indicates that these strains were provided with oxidase (positive oxidase) **(Fig. 44).**

The oxidase enzymes of *Bacillus* sp play an important role in the transport of electrons during aerobic respiration. Enzyme oxidase produced by facultative aerobic bacteria such as *Bacillus* sp bacteria allows these bacteria to take advantage of available carbon sources (Priyani *et al*., 2006).

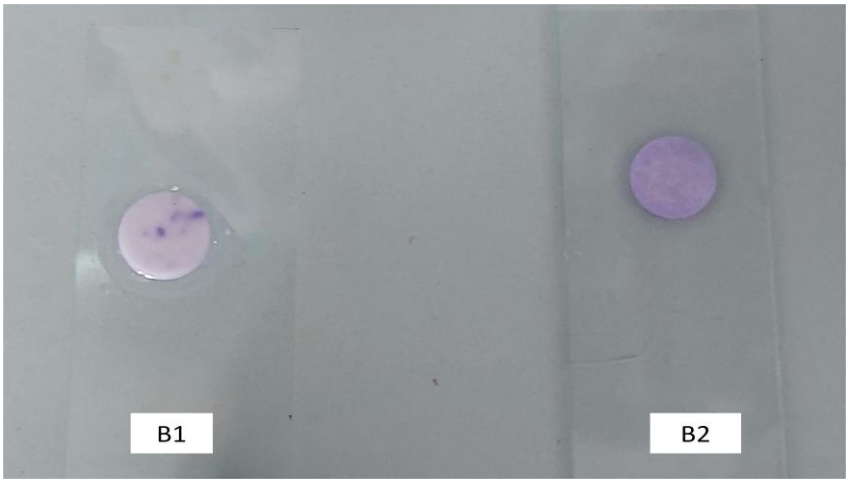


Figure 44: Isolate Oxidase Test Result.

Table 11: Results of physiological characters and biochemical tests of the two *Bacillus* strains.

Test / Strain	GRAM staining	Spore staining	Catalase	Mannitol-mobility		Oxidase
				Mannitol	mobility	
B1	+	+	+	-	-	+
B2	+	+	+	-	-	+

+: positive result / - : negative result

4. API 20^{E} Galleries

The identification of the two strains was determined by API20^{E} (identification system) using a free online tool (lab.upbm) **(Fig. 45)** and **(Fig. 46)** allowed to highlight the main biochemical characters linked to the two species studied.

The identified isolates are suspected of belonging to several different species according to the free online tool (lab.upbm).

Although phenotypic analyzes are important in microbial taxonomy, and are generally easy to study and very significant since the phenotypic criteria depend on the expression of genes which are often genetically stable, it is difficult to determine the species and bring them together in the genus *Bacillus*, nevertheless the results obtained are promising and prove that we are on the right track. Numerous studies suggest that the genus *Bacillus* constitutes a heterogeneous genus, phenotypically compared to most other bacterial genera (Claus and Berkeley, 1986) and that the species belonging to this genus are in continuous increase and are difficult to identify by traditional methods based on phenotypic characteristics (Woese, 1987).

Figure 45: Results of identification of the B1 strain by the API20E gallery and free online tool lab.upbm.

Table 12: Result of API 20^{E} gallery of strain B1.

Tests	**ONPG**	**ADH**	**LDC**	**ODC**	**CIT**	**H_2S**	**URE**	**TDA**	**IND**	**VP**	**GEL**
Results	+	+	-	-	+	-	+	+	-	+	+
Tests	**GLU**	**MAN**	**INO**	**SOR**	**RHA**	**SAC**	**MEL**	**AMY**	**ARA**	**OX**	**NO_2**
Results	-	-	-	-	-	-	-	-	-	+	+

+: positive result / - : negative result

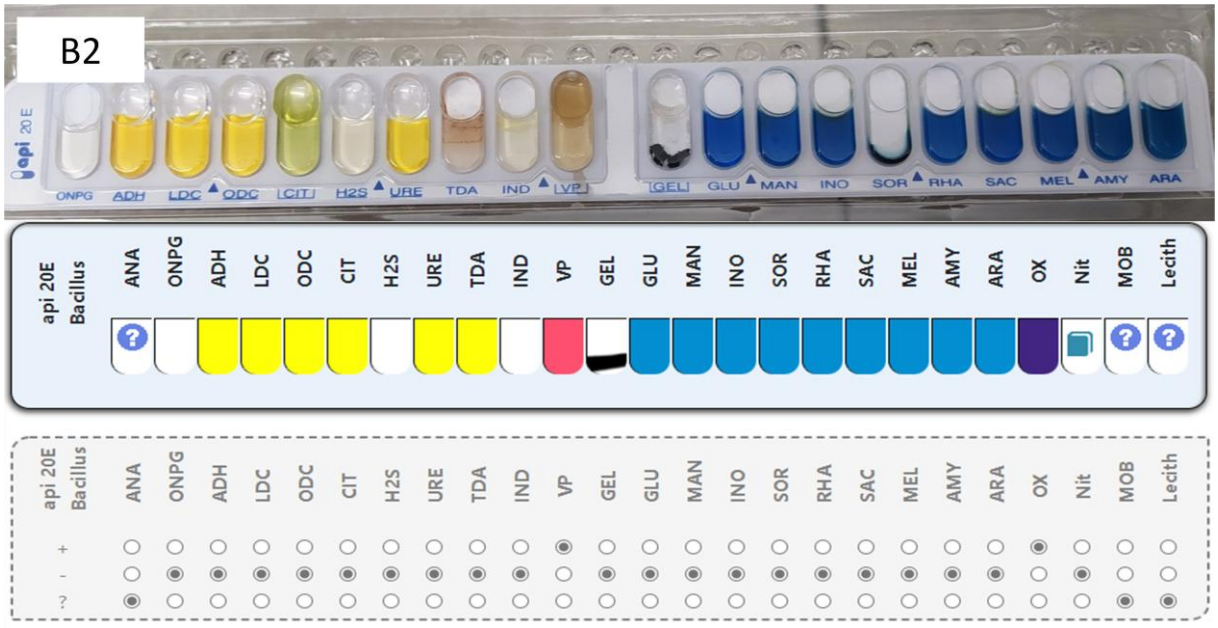


Figure 46: Results of identification of strain B2 by the API20E gallery and free online tool lab.upbm.

Table 13: Result of API 20E gallery of strain B2.

Tests	**ONPG**	**ADH**	**LDC**	**ODC**	**CIT**	**H_2S**	**URE**	**TDA**	**IND**	**VP**	**GEL**
Results	-	-	-	-	-	-	-	-	-	+	-
Tests	**GLU**	**MAN**	**INO**	**SOR**	**RHA**	**SAC**	**MEL**	**AMY**	**ARA**	**OX**	**NO_2**
Results	-	-	-	-	-	-	-	-	-	+	-

+: positive result / - : negative result

From these results obtained from our strains we can classify them according to the percentage of identification shown in **Table 14** and **Table 15.**

Table 14: Possible taxones for the *Bacillus* B1 strain.

The possible taxon to be	Crude probability
BreviBacillus choshinensis / centrosporus / brevis	<0.0001% and 1 exclusion
Bacillus sphaericus / fusiformis / badius	<0.0001% and 1 exclusion
Bacillus firmus	<0.0001% and 1 exclusion
Sporosarcina pasteurii	<0.0001% and 1 exclusion
Bacillus pumilus	<0.0001% and 1 exclusion

Table 15: Possible taxones for the *Bacillus* B2 strain.

The possible taxon to be	Crude probability
BreviBacillus choshinensis / centrosporus / brevis	raw probability of 0.7484% and 1 exclusion
Bacillus sphaericus / fusiformis / badius	0.6903% and 1 exclusion
Aneurinbacillus aneurinilyticus	0.1254% and 1 exclusion
Brevibacillus laterosporus	0.0056% and 1 exclusion
Bacillus firmus	0.0050% and 1 exclusion

5. Study of the enzymatic ability of our bacterial strains

5.1 The solubilization of phosphates

The two isolates of *Bacillus* showed a negative result reflected by an absence of the clear halos around the inoculation point **(Fig. 47).**

In our study, both bacterial strains were found to be poor solubilizers of inorganic phosphates.

Microorganisms release phosphate through the production of organic acids and / or through the secretion of H^+. Therefore, phosphate can be released by substitution of protons or its complexation with Ca^{2+} (Illmer and Schinner, 1995). Thus during solubilization, bacteria acidify the periplasmic space by direct oxidation of glucose. This process leads to the release of different organic acids (lactic, gluconic, isobutyric, acetic, glycolic, oxalic, malonic and succinic). However, these compounds are released with difficulty in the solid medium unlike the liquid medium (Nautiyal, 1999). The consequence of this phenomenon is manifested by the non-appearance of the transparency halo around the colonies of the strains.

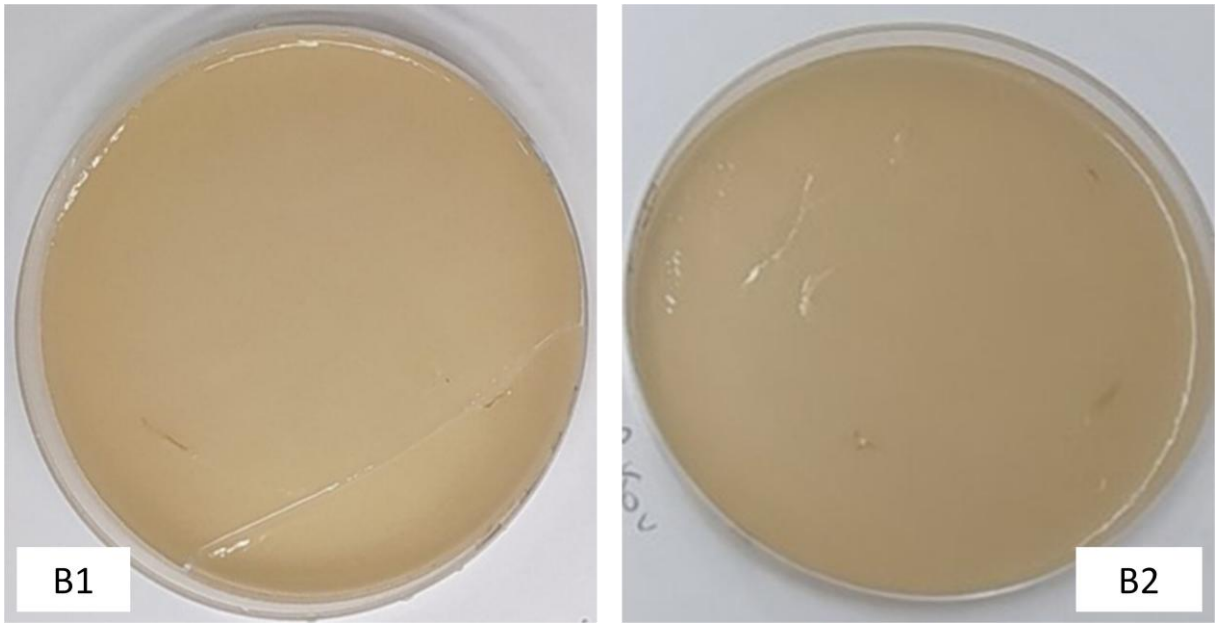


Figure 47: Negative results of phosphate solubilization by the two *Bacillus* strains.

5.2 Cellulase production

The results presented in (**Fig. 48)** show a clear degradation zone reflected by the secretion of the cellulase enzyme responsible for this degradation of the medium, the B2 strain exhibited a larger cellulase enzymatic activity with a clear zone diameter of 40mm followed by B1 with a clear area of 30mm.

Cellulase production with both *Bacillus* strains was significantly influenced by the type of carbon source in the basic medium. Carboxymethylcellulose (CMC) has been shown to be the most effective as the sole carbon source for the production of cellulase enzymes by the activity of *Bacillus* CMCase. These results are in agreement with those of Narasimha *et al*. (2006) and Niranjane *et al*. (2007) who found that carboxymethylcellulose was the best source of carbon, followed by cellulose for cellulase production.

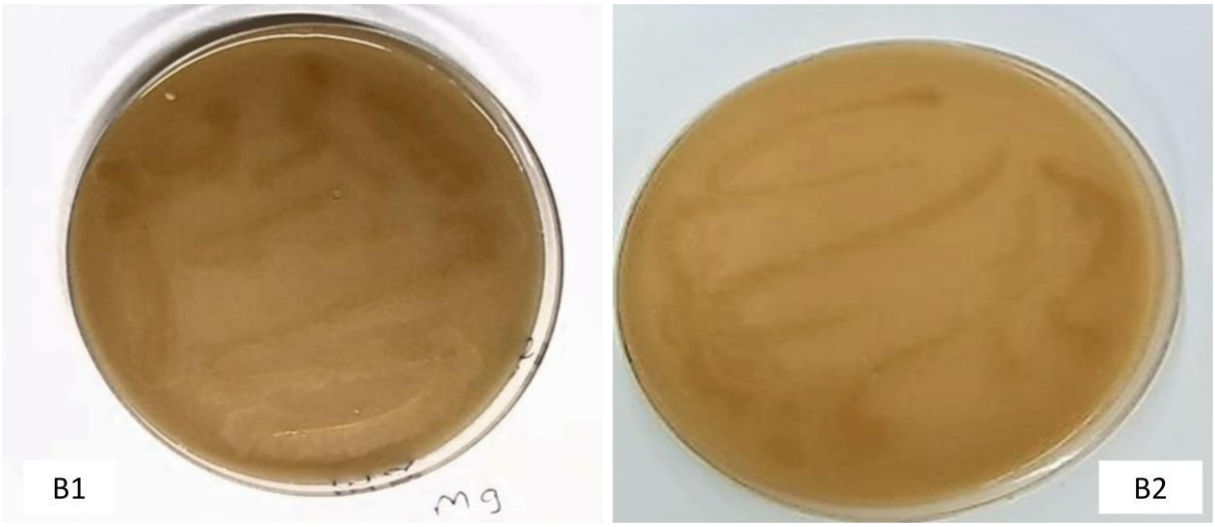


Figure 48: Results of cellulase production by the two *Bacillus* strains.

5.3 Proteolytic enzymes

The proteolytic activity was assayed using skimmed milk agar and expressed as the diameter of the clear zone in mm. Strain B2 exhibited the highest proteolytic activity with a clear zone diameter of 45mm followed by B1 with a clear zone of 35mm **(Fig. 49).** These *Bacillus* species produced a protease therefore were protease positive strains. Most of the predominantly neutral and alkaline commercial proteases are thought to have been produced from the genus *Bacillus* (Rao *et al*., 1998).

Figure 49: Results of the production of proteolytic enzymes by the two *Bacillus* strains.

5.4 Chitinolytic activity

Both strains of *Bacillus* showed positive chitinase activity exemplified by the formation of a clear zone produced on chitin agar plates. **(Fig. 50)**

Bacillus species have been shown to produce a high level of chitinolytic enzymes (Cody *et al.*, 1990; Champen *et al.*, 1999). A strong correlation between our results obtained.

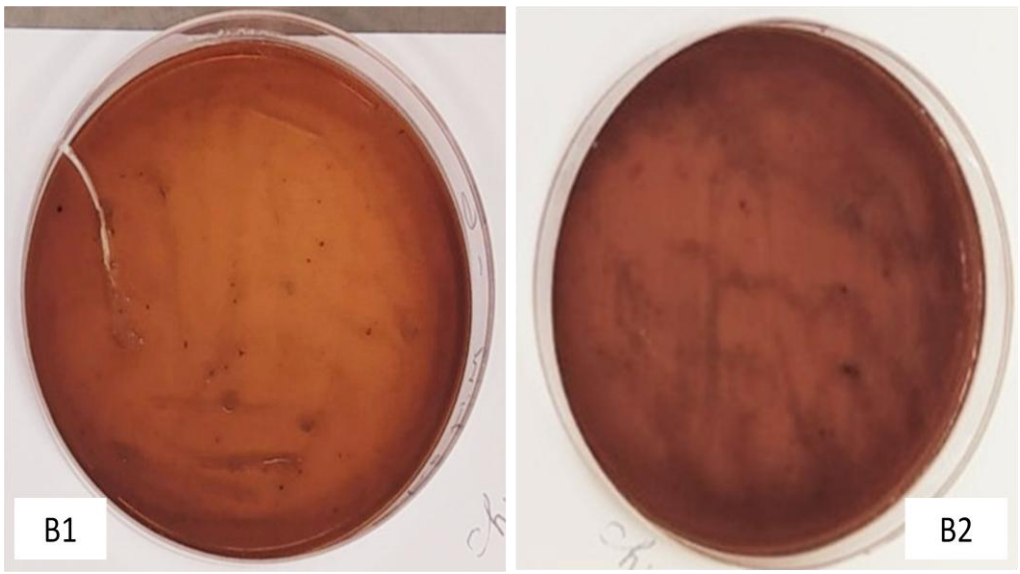


Figure 50: Results of chitinolytic activity by the two *Bacillus* strains.

5.5 Determination of HCN

Both *Bacillus* strains (B1, B2) produced HCN, as evidenced by the change in color of the filter paper from yellow to moderate brown and reddish. In the presence of glycine and picric acid, the dark brown color of the filter paper was observed, giving a clear indication of HCN production by bacterial strains **(Fig. 51)**. A similar result was also reported by Datta *et al*. (2011).

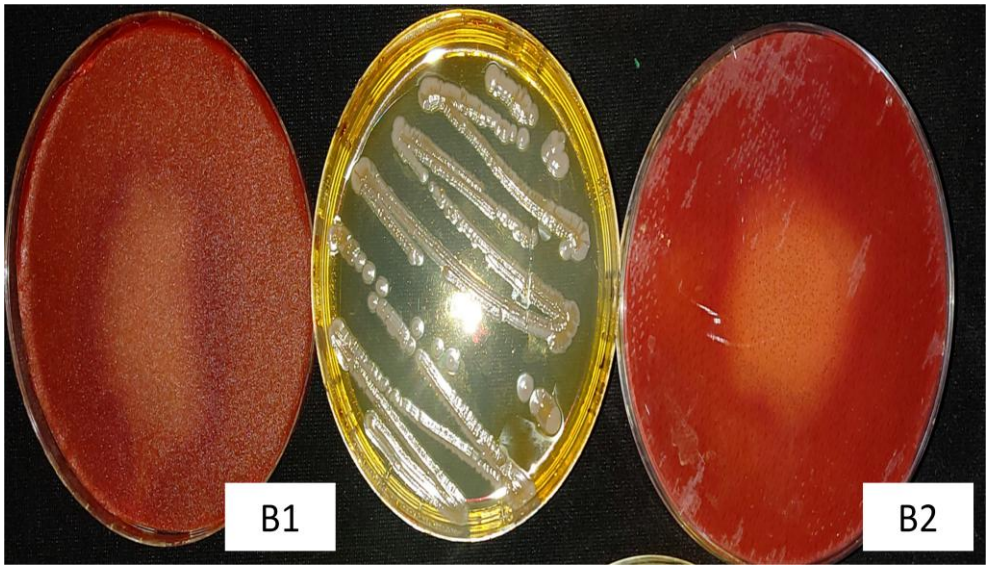


Figure 51: HCN production results by the two *Bacillus* strains.

Table 16: The results of the enzyme production for the two *Bacillus* strains.

Enzymatic activity	Phosphate solubilization	Cellulase	Proteolytic enzymes	Chitinases	Cyanogenesis
Strain B1	-	++	++	+	++
Strain B1	-	+++	+++	+	++

+ : medium / ++ : important / +++ : very important / - : no production

6. The antibiogram

The antibiogram allowed us to determine the result of the sensitivity or resistance of the strains studied to certain antibiotics using the solid-medium diffusion method. The inhibition diameters around the discs **(Fig. 52)** are measured **(Table 17)**, then they are compared to the critical diameters recommended by the Algerian committee (2016) **(Appendix 04).**

Table 17: Result of the antibiogram of the isolated strains.

ATBs / Strain		AMX25	SXT	AMP10	E
B1	**Spectrum**	**S**	**S**	**S**	**R**
	Diameter	**00 mm**	**26 mm**	**00 mm**	**17 mm**
B2	**Spectrum**	**S**	**S**	**S**	**R**
	Diameter	**00 mm**	**29 mm**	**00 mm**	**10 mm**

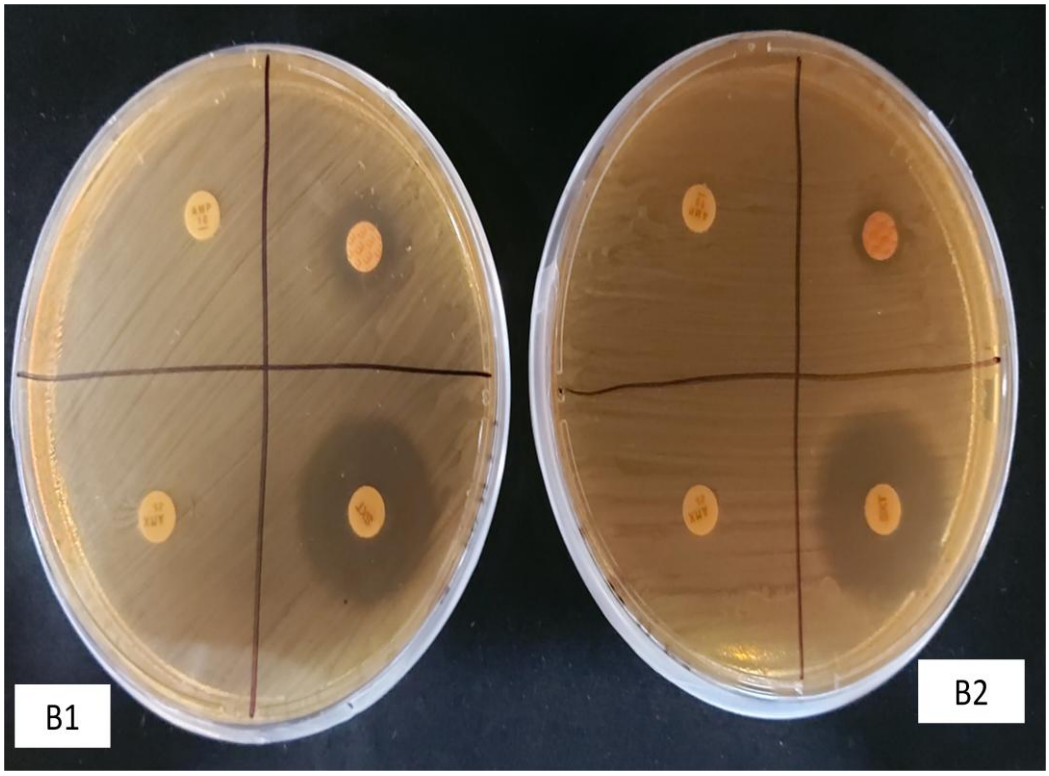


Figure 52: The results of the antibiogram of the two strains studied.

The result presented in **Table 17** indicates that the two strains are resistant to erythromycin which belongs to the family of macrolide antibiotics. Furthermore, both strains are sensitive to ampicillin and amoxicillin which belongs to the family of beta-lactam antibiotics and also sensitivity to trimethoprim which belongs to the family of diaminopyrimidine antibiotics.

The method we describe above is not considered an alternative to other biochemical and physiological tests used for conventional identification of *Bacilli*. However, some important additional information on taxonomic relationships between *Bacillus* species can be obtained by analyzing antibiotic sensitivity. Susceptibilities to certain antibiotics obviously reflect other forms of variation in *Bacilli* and adaptation to certain environmental conditions. These relationships are important for understanding the place occupied by these species in microbial associations (Reva *et al*., 1995).

7. Screening and isolation of microorganisms producing biosurfactants

7.1 Foaming activity

Both *Bacillus* strains showed a positive result in screening for the production of biosurfactants were inoculated into the production medium this positive result sums up on the presence of the foaming activity which was detected as the duration of stability of the foam **(Fig. 53).** A similar result has also been reported by (Priyadharshini and Latha, 2016).

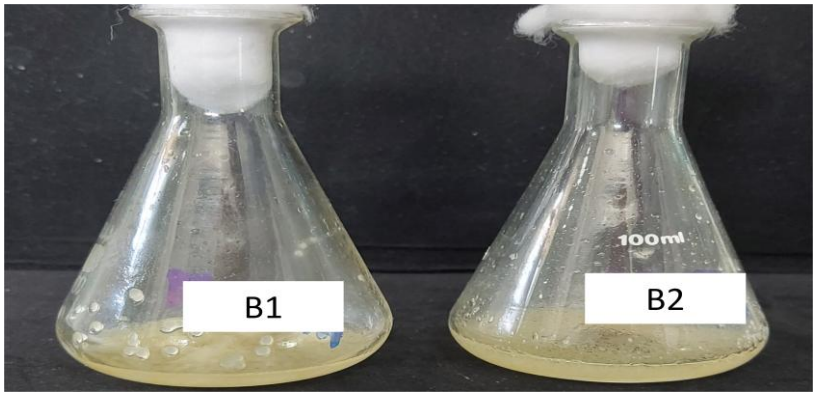


Figure 53: The foaming activity for the two strains of *Bacilli*.

7.2 Results of the E24 emulsification activity

The emulsification activity was verified with a positive result for the two *Bacillus* strains, after leaving the two tubes to stand for 24 h, the results of the emulsification index (E24) were calculated **(Table 18),** by measuring the emulsion layer, expressed as a percentage of the total height of the mixture in the tubes **(Fig. 54).** Similar results have also been reported by (Makkar and Cameotra. 1998).

The emulsification index (E24) of the supernatant of the two cultures against diesel reaches a maximum of 70% at 24 h.

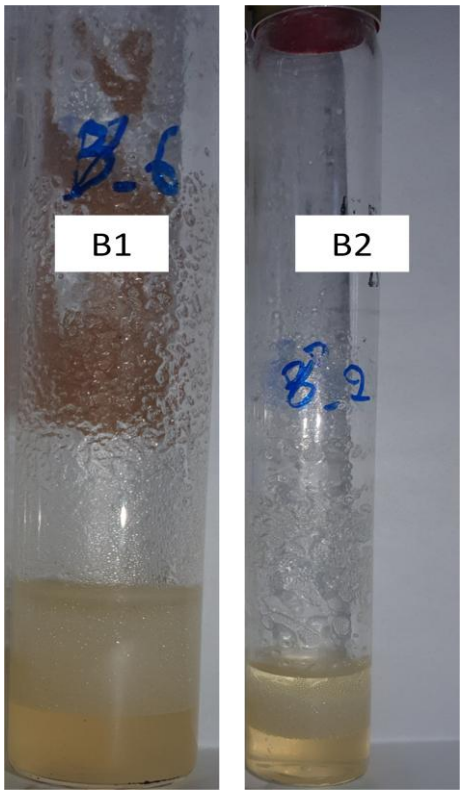


Figure 54: Emulsification index (E24) for the two *Bacillus* strains.

Table 18: The results of the emulsification index (E24) for the two *Bacillus* strains.

The strain	B1	B2
H_e (emulsion height)	**1.4 cm**	**1.4 cm**
H_t (total height of the mixture)	**2 cm**	**2 cm**
$E24\% = \frac{He}{Ht} \times 100$	**70%**	**70%**

7.3 Extraction of biosurfactants

The biosurfactants were extracted from the culture supernatant by the chloroform-methanol (2: 1) extraction method. The product was obtained as a transparent liquid with a creamy white material **(Fig. 55)** and the B1 culture showed more biosurfactant production, compared to the B2 culture. These results are similar to (Priyadharshini and Latha, 2016).

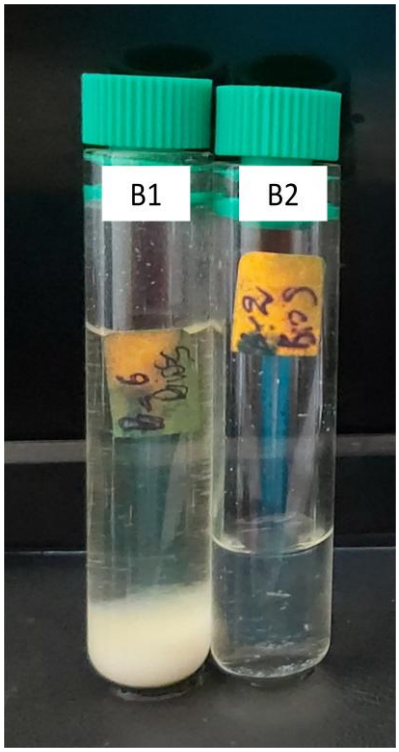


Figure 55: The result of extracting biosurfactants from the two *Bacillus* strains.

7.4 Extraction of antibiotics

The antibiotic extraction is done from the culture supernatant of the two *Bacillus* strains and only one organic solvent is used, ethyl acetate. The product was obtained as a transparent liquid with a white material on the bottom of the tubes **(Fig. 56).**

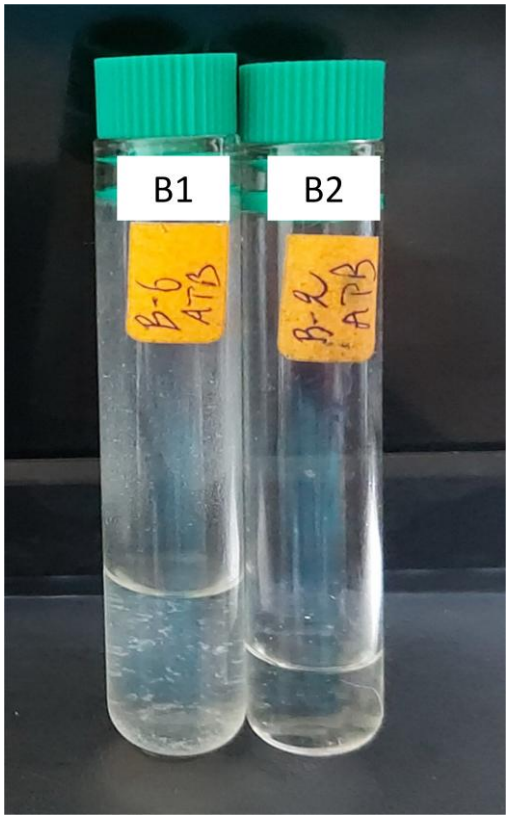


Figure 56: The result of the extraction of antibiotics by the two *Bacillus* strains.

7.5 The revelation of antibiotics and biosurfactants by TLC

7.5.1 The revelation of biosurfactants

The crude extract is analyzed by TLC. The eluent system used is a mixture of solvents: CHCl3 / CH 3OH / H 2 O (65: 15: 2, by vol). The results obtained from thin layer chromatography showed in **(Fig. 57).**

Spraying Molish's reagent revealed nothing. Also to check whether our extract contained lipopeptide molecules or not, we used another type of developer, Ninhydrin for the detection of lipopeptide biosurfactants.

Both of these tests were found to be negative. It can be concluded that this is probably a different class of biosurfactants than glycolipids (or those of other molecules containing a sweet part).

Table 19: The frontal ratios found on the TLC plate of biosurfactants.

The Strain	The extract of biosurfactants	Spot number	Distance traveled by the solvent (cm)	Distance covered by the spot (cm)	Frontal report
B1	Supernatant	Spot 02	16.3	15.4	1.05
B2	Supernatant	Spot 01	16.3	15.1	1.07

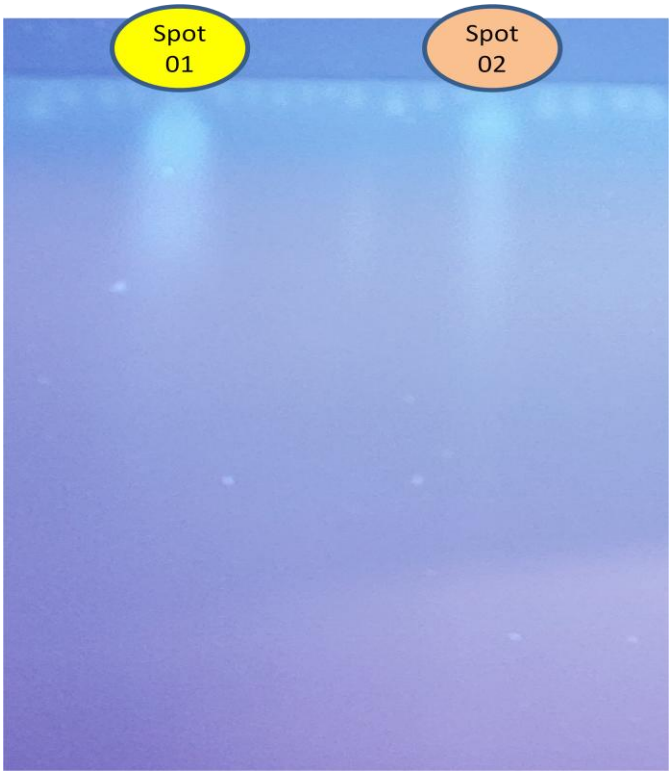


Figure 57: The results of the revelation of biosurfactants on thin layer chromatography observed under UV at 350 nm.

7.5.2 The revelation of antibiotics

A single system of migration solvents (ethyl acetate-methanol, 100/15) is used. The AM system was chosen for chemical revelations and purifications because it allows better separation of the spots which are localized under UV light and with the naked eye (Zitouni, 2005) **(Fig. 58).**

The FeCl3 revelation test (revealing phenols and hydroxamic acids) is negative. This assumes the presence of other antibiotic structures.

Table 20: The frontal ratios found on the TLC plate of the ATBs.

The Strain	The extract of biosurfactants	Spot number	Distance traveled by the solvent (cm)	Distance covered by the spot (cm)	Frontal report
B1	Supernatant	Spot 4	16.3	13	1.25
B2	Supernatant	Spot 3	16.3	13.6	1.19

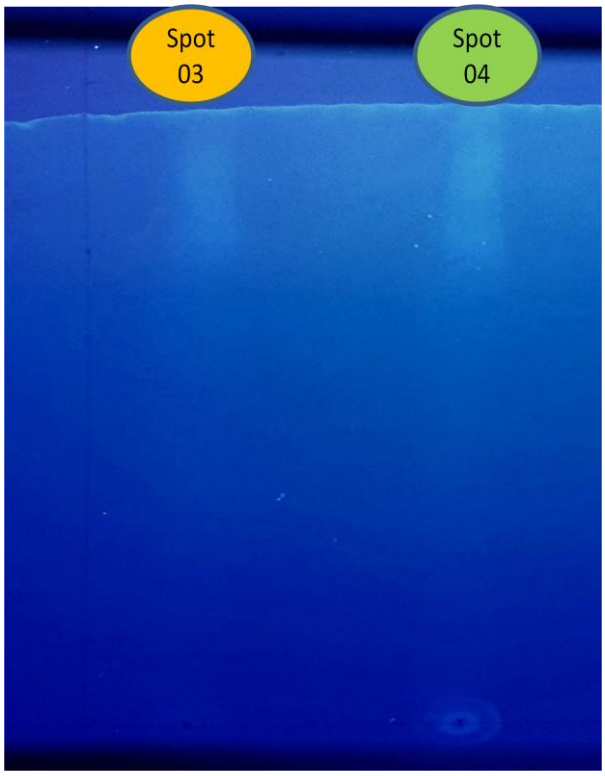


Figure 58: The results of the revelation of antibiotics on thin layer chromatography observed under UV at 350 nm.

Conclusion

The objectives of this study were to characterize the different aptitudes of strains related to the genus *trichoderma* and *Bacillus*, in particular the power of the synthesis of biotechnological enzymes and the capacity to produce biosurfactants.

The identification of our strains was carried out by macroscopic and microscopic observation, orientation tests (oxidase and catalase test) and biochemical analyzes by the various tests in the Api 20^{E} gallery for the two bacterial strains.

The production of extracellular enzymes examined by qualitative tests, allowed us to confirm the conclusion that the filamentous fungi of the genus *Trichoderma* and the two bacterial strains of *Bacilli* are microorganisms capable of adapting under various environmental conditions by their ability to secrete extracellular hydrolytic enzymes and biosurfactants.

Biosurfactants and microbial enzymes exhibit several properties that could be useful in many areas of the food industry; recently, their non-stick activity has gained attention as a new tool to inhibit and disrupt biofilms formed in food contact surfaces. The combination of particular characteristics such as emulsifying, anti-stick and antimicrobial activities exhibited by biosurfactants suggests potential application as versatile ingredients or additives.

In the light of the results obtained, it is desirable to complete this study with more in-depth approaches, namely:

- Identification of the strains studied and their taxonomic classification
- The study of the physicochemical properties of the biosurfactant produced to better understand its nature, its structure, etc.
- The production of the biosurfactant by testing other substrates (used oil, frying oil, etc.)
- Carrying out the antimicrobial activity test on pathogenic strains.

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Appendix

Appendix

Appendix 1: Materials Used

1. Equipment

- 37 ° C oven (BINDER)

- 105 ° C oven (memmert)

- Autoclave (Webeco)

- pH meter (inoLab)

- Stirrer

- Heating plate (VWR)

- Cold centrifuge (SIGMA)

- Photonic microscope (WETZLAR hund)

- Balance (KERN EMB 1200-1)

- Water bath (nüve bath)

- UV-visible spectrophotometer (BIOTECH ENGINEERING MANAGEMENT CO. LTD. (UK) VIS-7220G)

- Precision balance (KERN 770)

- Refrigerator (ENIEM)

- Vortex

- Bunsen burner

2. Glassware And Plastic Equipment

- Erlenmayers of 50ml, 100ml, 250ml, 500ml, 1000ml, 2000 ml

- Beakers of 10ml, 20ml, 50ml, 100ml, 250ml

- Plastic Petri dishes

- Graduated pipettes

- Pasteur pipettes

- Platinum handle

- Loop handle

- Wire handle

Appendix

- Test tubes

- blade

- Cover slat

- Metal clamps

- Micropipette

- Spectrophotometer cuvettes

- Sterile syringes

- Magnetic bars

- Sterile swabs

Appendix 2: Culture Media

- **Minimal medium**

- Glucose 2 g.
- Agar 18 g.
- Distilled water 1000 ml.
- Potato dextrose agar (PDA)
- Potato extract 200 g / L.
- Glucose 20 g.
- Agar 15g.
- pH 5.4

- **Skim milk agar**

- Skim milk 300 ml.
- Agar 20.00 g.
- Distilled water 1000 ml.

- **Czapek-Mineral Salt Agar supplemented with Carboxy Methyl Cellulose**

- Monopotassium phosphate 1.00 g.
- Magnesium sulfate heptahydrate 0.50 g.
- Sodium nitrate 2.00 g.
- Potassium chloride 0.50 g.
- Peptone 2.00 g.
- Agar 20.00g.
- Distilled water 1000 ml.

- **Solid medium**

- Sodium chloride 5 g.
- Calcium chloride 0.1 g.

- Peptone 10 g.
- Tween 80 10 g (polyoxyethylene sorbitan monooleate).
- Agar 20 g.
- Distilled water 1000 ml.

- **Chitinase detection Medium**

- Colloidal chitin 4.5 g.
- Magnesium sulfate heptahydrate 0.30 g.
- Diazanium sulfate 3.00 g.
- Monopotassium phosphate 2.00 g.
- Citric acid monohydrate 1.00 g.
- Bromocresol purple 0.15 g.
- Tween-80 0.20 ml.
- Agar 20 g.
- Distilled water 1000 ml.

- **Solid medium containing Tryptic Soya Agar (TSA)**

- Tryptic Soya Agar (TSA) 40 g.
- Glycine 4.4 g.
- Distilled water 1000 ml.

- **Medium lacking nitrogen**

- Monopotassium phosphate 0.2 g.
- Calcium carbonate 5.0 g.
- Magnesium sulfate heptahydrate 0.2 g.
- Sodium chloride 0.3 g.
- Mannitol 10.0 g.
- Calcium sulfate dihydrate 0.1 g.
- Agar 20.0 g.
- Distilled water 1000 ml.
- pH 7.2

- **Peptone water**

- Peptone 15 g.
- Distilled water 1000 ml.
- Malt extract agar medium
- Soja peptone 3 g.
- Malt extract 30 g.
- 8-hydroxyquinoline 50 mg.
- Agar 18 g.
- Distilled water 1000 ml.
- Potato dextrose broth medium (PDB) :

Appendix

- Put 200 grams of peeled and diced potatoes in a beaker and add 1000 ml of distilled water and bring to a boil.
- When the water decreases and thickens a little, remove it and squeeze it with gauze.
- The obtained juice supplemented with 1 g of L-tryptophan .

- **Synthetic medium (SM)**

- Bactopeptone 1 g.
- Urea 0.3 g.
- Monopotassium phosphate 2 g.
- Diazanium sulfate 1.4 g.
- Magnesium sulfate heptahydrate 0.3 g.
- Calcium Chloride hexahydrate 0.3 g.
- Solution of trace elements (Fe2+, Mn2+, Zn2+, and Co2+) 0.1 g.
- Glucose 0.2 g.
- Colloidal chitin 10 g.
- Distilled water 1000 ml.

- **Liquid M1 medium**

- Dipotassium phosphate 5 g.
- Monopotassium phosphate 5 g.
- Magnesium sulfate 2 g.
- Sodium nitrate 5 g.
- Yeast extract 3 g.
- Glucose 15 g.
- Distilled water 1000 ml.
- pH : 6.5

- **King B liquid medium**

- Casein peptone 20 g
- Magnesium sulfate 1.5 g
- Bi-potassium phosphate 1.5 g
- Glycerol 10 ml
- Distilled water 1000 ml
- pH : 7.2

- **Mueller Hinton agar**

- Meat extract 2 g.
- Acid hydrolyzate of casein 17.5 g.
- Starch 1.5 g.
- Agar 10 g.
- Distilled water 1000 ml
- pH : 7.4

Appendix

- **NBRIP medium (National Botanical Research Institute's phosphate) (Nautiyal, 1999)**

- Glucose 10 g
- Ca_3 (PO_4) 25g
- $MgCl_2$.6H2O 5g
- MgSO4.7H2O 0.25g
- KCl 0.2g
- (NH4) 2SO4 0.1g
- Agar agar 15g
- Distilled water q.s.p 1000 ml

- **Carder's Middle (1986)**

- Na2HPO4 6g
- KH2PO4 3g
- NaCl 0.5g
- NH4Cl 1g
- 3g yeast extract
- Cellulose 7g
- Agar 15g
- The pH was then adjusted to 7.4
- Distilled water q.s.p 1000 ml

- **GN medium containing 5% skimmed milk**

- Peptone 10g
- Meat extract 3g
- 3g yeast extract
- Sodium chloride 5g
- Agar 18g
- 5% topped milk
- Distilled water q.s.p 1000ml

- **CMC medium**

- Colloidal chitin 0.1 g
- KH 2 PO 4 0.7g
- K 2 HPO 4 0.3 g
- NaCl 4 grams
- MgSO 4 • 7H 2 O 0.5g
- FeSO 4 • 7H 2 O 1 mg
- ZnSO 4 • 7H 2 O 0.1 mg
- MnSO 4 • 7H 2 O 0.1 mg
- Agar 15 g
- Distilled water q.s.p 1000 ml

- **Soybean Trypticase Medium (Fluka)**

- Casein tryptic peptone 17g
- Papain soy peptone 5g
- NaCl 5g
- K2HPO4 2,5g
- Glucose 5g
- Agar 12g
- Distilled water q.s.p 1000ml

Appendix 3: Reagent and solutions

- **Nessler's reagent**

- Potassium iodide 70 g.
- Mercury iodide 100 g.
- Potash 100 g.
- Distilled water 1000 ml.

- **Salkowski reagent**

- 2 % of 0.5 M ferric chloride in 35 % perchloric acid.

- **DNS reagent**

- Sodium hydroxide 1 g.
- Potassium Sodium Tartare 18.2 g.
- 3,5-Dinitrosalicyclic Acid 1 g.
- Phenol 0.2 g.
- Sodium sulfite 0.05 g.
- Distilled water 100 ml.

- **Chromogenic reagent**

- Phenol 3 g.
- Sulfuric acid 5 ml.
- Ethanol 95 ml.

- **Ninhydrin solution**

- Ninhydrin 0.1 g.
- Chloroforme 100 ml.

- **Alkaline picric acid solution**

- Picric acid 2.5 g.
- Sodium carbonate 12.5 g.
- Distilled water 1000 ml.

- **Congo red (2% w/v)**

- Congo red powder 2 g.
- Distilled water 100 ml.

- **NaCl 1M**
- Sodium chloride 1 g.
- Distilled water 100 ml.

- **0.05 M sodium acetate buffer**
- Sodium acetate 4.10 g.
- Glacial acetic acid 2.85 mL.
- Distilled water 1000 mL
- pH : 5.0

- **Lugol**
- Iodine 5 g
- Potassium iodide 10 g
- Distilled water 100 ml

- **Alcohol**
- **Fuschines**
- **Gentian violet**
- **Immersion oil**
- **Distilled water**
- **NaOH solution**
- **Diesel fuel**
- **Methanol**
- **Hydrochloric acid**
- **Chloroform**
- **Acetat d'ethyle**

Appendix 04: List of antibiotics tested.

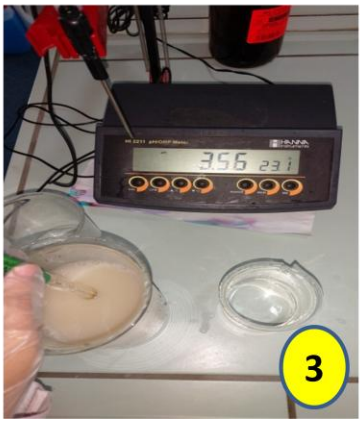
Family	Name of antibiotic	Coded	Disk load	Critical diameters (mm)		
				S	I	R
Beta lactams	Ampicillin	AMP10	10 µg	≥23		<16
	Amoxicillin	AMX25	25 µg			
Diaminopyrimidines	Trimethoprim	SXT	1.25 µg	≥40		<80
Macrolides	Erythromycin	E	15 µl	≥0.5		<08

Appendix

Appendix 05: Appendix 05 Protocol

Preparation of colloidal chitin

The preparation of the colloidal chitin was carried out according to mix between two modified protocol of Hsu, S. C., and J. L. Lockwood (1975) and Seitzman, B. (2008)



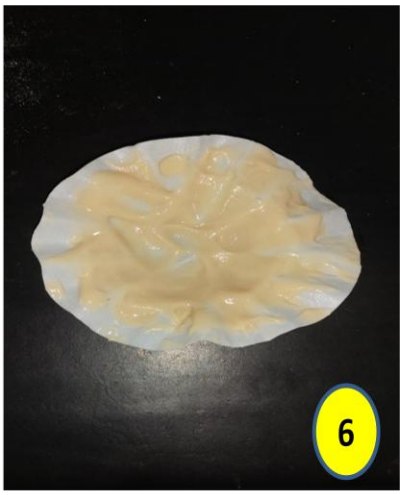
- Twenty grams of the chitin powder are treated with 150 mL of concentrated HCl (~ 12M) in a 1 L glass beaker. The hydrochloric acid is added slowly, with continuous stirring for 60 minutes under a chemical hood and at room temperature (25 ° C). The chitin-HCl mixture is then evaporated using a rotavapor. (1)

- The obtained (~ 100 ml) are then poured into two liters of cold distilled water to allow precipitation of the colloidal chitin; The whole is brought to 4 ° C under static conditions in order to facilitate the precipitation of the colloidal chitin. (2)

- After 12 hours, pH = 3.56 (3)

- The excess water is aspirated and the precipitate is placed in 50 ml centrifuge tubes, approximately 6 g in each tube and made up to 15 ml with distilled water, centrifugation at 5000 rpm for 15 min. after 15 min remove the supernatant and made up the tubes again to 14 ml with distilled water and centrifuging again under the same conditions, this action is repeated until neutralization of the pH (pH ~ 7). (4)

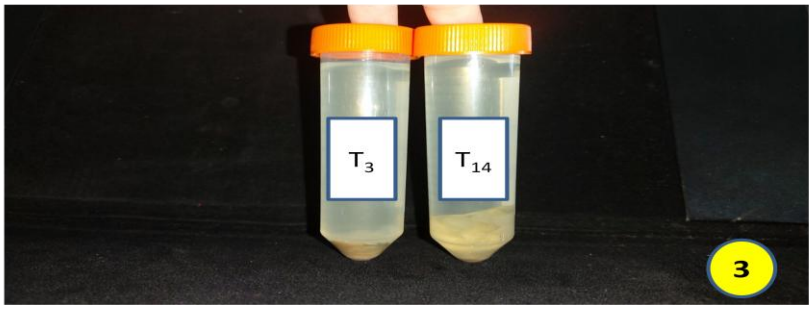
Appendix



- The resulting colloidal chitin is dried to remove as much moisture as possible, then sterilized and stored at 4 ° C. (5.6.7.8)

Appendix

Extraction protocol of bio-surfactants and antibiotic



- The fermentation must of fungal extrat after 72 hours. (1.2)
- Centrifugation at 15,000 rpm / 20 min. (3)

Appendix

- The recovered supernatant was mixed with an equal volume of chloroform: methanol (2: 1). (4)
- Decantation to allow the separation of the aqueous and organic phases. (5)
- The solvent was evaporated off using a rotary evaporator. (6)

Abstract:

The aim of this work is to study the ability of the group of microorganisms that play a significant role in biotechnology by producing many secondary metabolites with interest. The microorganisms studied belong to the genus *Trichoderma* with two species, namely T_3 and T_{14}, and two species of *Bacillus* (B_1 and B_2). *Trichoderma* and *Bacillus* isolates were investigated for the production of extracellular enzymes on solid medium, including: proteases, cellulases, lipases and chitinases, others enzymes and growth promoter molecules of plants (PGP) were also screened such as phosphatases, NH3, siderophore and production of indole acetic acid. All isolates tested shown proteolytic, cellulolytic and chitinolytic activity. Moreover, it's been able to produce phosphatases and Indol acetic acid with different degrees. We have observed that the tested stains lacking sedirophores production. But the more important results is the ability of all strains to produce an emulsifier, which is represented of biosurfactants, that can used in many fields such as the medical and petroleum fields, etc.. This is why, *Trichoderma* and *Bacillus* have the potential to be used for human needs to an even greater extent than before. Nevertheless, further studies are needed to increase the efficiency and safety of the application of these microorganisms.

Key words: *Trichoderma* species, enzymes, secondary metabolites, *Bacillus*, biosurfactants.

Résumé:

Le but de ce travail est d'étudier la capacité du groupe de micro-organismes qui jouent un rôle important en biotechnologie à produire de nombreux métabolites secondaires avec intérêt. Les microorganismes étudiés appartiennent au genre *Trichoderma* avec deux espèces, à savoir T_3 et T_{14}, et deux espèces de *Bacillus* (B_1 et B_2). Des isolats de *Trichoderma* et de *Bacillus* ont été étudiés pour la production d'enzymes extracellulaires sur milieu solide, notamment : les protéases, cellulases, lipases et chitinases, d'autres enzymes et molécules de promoteur de croissance des plantes (PGP) ont également été criblées telles que les phosphatases, NH3, sidérophores et production de acide indole acétique. Tous les isolats testés ont montré une activité protéolytique, cellulolytique et chitinolytique. De plus, il a été capable de produire des phosphatases et de l'acide acétique indol à différents degrés. Nous avons observé que les taches testées manquaient de production de sédirophores. Mais le résultat le plus important est la capacité de toutes les souches à produire un émulsifiant, qui est représenté par des biosurfactants, qui peut être utilisé dans de nombreux domaines tels que les domaines médicaux et pétroliers, etc. C'est pourquoi, *Trichoderma* et *Bacillus* ont le potentiel de être utilisé pour les besoins humains dans une mesure encore plus grande qu'auparavant. Néanmoins, d'autres études sont nécessaires pour augmenter l'efficacité et la sécurité de l'application de ces micro-organismes.

Mots clés : Espèce *Trichoderma*, enzymes, métabolites secondaires, *Bacillus*, biosurfactants.

الملخص:

الهدف من هذا العمل هو دراسة قدرة مجموعة الكائنات الحية الدقيقة التي تلعب دورًا مهمًا في التكنولوجيا الحيوية من خلال إنتاج العديد من المستقلبات الثانوية باهتمام. تنتمي الكائنات الحية الدقيقة المدروسة إلى جنس *التريكودارما* مع نوعين هما ت$_3$ و ت$_{14}$ ونوعين من *الباسيليس* ب$_1$ و ب$_2$. تم فحص عزلات *التريكودارما* و *الباسيليس* لإنتاج إنزيمات خارج الخلية على وسط صلب ، بما في ذلك: البروتياز، والسليولاز، والليباز، والكيتيناز، كما تم فحص الإنزيمات الأخرى وجزيئات محفز النمو في النباتات مثل الفوسفاتاز، السيديروفور، الامونيا وإنتاج اندول حامض الخليك. أظهرت جميع العزلات المختبرة نشاط حال للبروتين ومحلل للخلايا ومحلل للكيتين. علاوة على ذلك ، فقد تمكنت من إنتاج الفوسفاتاز وحمض الخليك إندول بدرجات مختلفة. لقد لاحظنا أن البقع المختبرة تفتقر إلى إنتاج السيديروفور. لكن النتائج الأكثر أهمية هي قدرة جميع السلالات على إنتاج مستحلب ، وهو عبارة عن مواد حيوية للتوتر السطحي ، يمكن استخدامها في العديد من المجالات مثل المجالات الطبية والبترولية ، إلخ. ولهذا السبب ، تمتلك *التريكودارما* و *الباسيليس* القدرة على تستخدم لتلبية احتياجات الإنسان إلى حد أكبر من ذي قبل. ومع ذلك ، هناك حاجة إلى مزيد من الدراسات لزيادة كفاءة وسلامة تطبيق هذه الكائنات الدقيقة.

الكلمات المفتاحية: أنواع *التريكودارما*، الإنزيمات، المستقلبات الثانوية، *الباسيليس*، العوامل الحيوية