# Bioremediation of soils polluted by petroleum hydrocarbons by *Pseudomonas putida*

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## ABSTRACT

In order to clean up soils contaminated with hydrocarbons, the bioremediation activity of *Pseudomonas putida* was studied. *Pseudomonas putida* is a bacterium that can withstand the harshest environmental conditions. It is able to metabolize a wide range of petroleum hydrocarbons which is used as a source of carbon and energy. Given the potential of this microorganism, an experiment wasconducted on this strain.

For the isolation of this microorganism, a sample ofsoil from the Vakinankaratra region in the urban commune of Antsirabe II, Madagascar was microbiologically analysed. The bacterial identification was based on a study of the morphological, physicochemical and sequential analysis of the 16S rDNA gene.

The isolated strain was then inoculated into soil polluted by diesel engine oil from the garage at Universityof Saint Joseph Antsirabe, Madagascar. The kinetics ofbacterial growth showed that the biomass increased from $4.10^7$ to $3.10^{13}$ CFU/g at the end of the experimentation. A growth rate of $0,32h^{-1}$ and a generation time of 2,16 hours were noted. The quantification of the residual hydrocarbons according to the EPA method (Environmental Protection Agency) 3540C have made it possible to deduce the capacity of degradation of the bacterial strain which is 0,2 mg of hydrocarbon per gram of soil per day. After 3 months of biological treatment, the concentration of the residual petroleum hydrocarbons had been reduced by 30%(from 18000 mg/kg to 5000 mg/kg). Thus, the ability of *Pseudomonas putida* to decontaminate polluted soils with hydrocarbons has been observed.

***Key words**: petroleum hydrocarbons, bioremediation, Pseudomonas putida, soil, oil.*

## I. INTRODUCTION

Petroleum hydrocarbons are potentially toxic organic compounds. Their presence in the soil has negative impacts on both environmental quality and human health (Robert, 1996). Several technics have been proposed to reduce the amountof petroleum hydrocarbons in the soil. Among these technics is bioremediationwhichis to the most ecological and the cheapest.Itconsists to use living organisms or microorganisms to clean up contaminated sites (Ballerini and Vandecasteele, 1999). Bacteria are the most used in bioremediation, however, depolluting activities have been seenwith algae (*Selenastrumcapricornutum*) and fungi (*Aspergillusniger*). Moreover, Madagascar has a broader microbial diversity. There are pathogens microorganisms as *Salmonella, Escherichia coli, ….*And there are beneficial microorganisms, it is even profitable for food, and environments because it allows to protect them, improve their qualities (Tsirinirindravo and *al, 2016 ;*Mananjara and *al, 2016*).

This is why a study was conducted on the bioremediation the soils polluted with hydrocarbons by *Pseudomonas putida* in order to develop an efficient bioremediation system. *P. putida* is an ubiquitous bacterium, mostly found in therhizosphere zone of*Pinusradiata* (Mukerji*et al.,* 2006). The bacterium is classified as a chemo-organotroph because of its capability to metabolize a wide range of carbon compounds including petroleum hydrocarbons. Different manipulations were carried out at the microbiological and physico-chemical laboratories of the University of Saint Joseph Antsirabe, Madagascar.

## II. MATERIALS AND METHODS

### Biological material

The sampling of the soils to analyze was taken from the soil inVakinankaratra region in the urban commune of Antsirabe II, Madagascar. *Pseudomonas putida* is a ubiquitous bacterium found in large quantity in the root zone of *Pinusradiata* (Bowen *et al.,* 1976). The samples were taken from three different rhizosphere zones of *Pinusradiata* at 15 cm deep.

### Bacterial isolation

To make the soil suspension, 10 g of the sample of soil sample are mixed with 90 ml of Buffered Peptone Water. 1 ml of this suspension is plated oncetrimide agar plates and then incubated at 30°C for 48 hours. The fluorescent bacteria colonies at 254 nm are purified in view of being identified (Maksimova*et al.,* 1994).

### Bacterial identification

The purified bacterial colonies are identified by a succession of biochemical tests (Garrity*et al*., 2002) and 16S rDNA sequence analysis.

### Phenotypic study

The identification of the pure strains is firstly based on a macroscopic observation to determine cultural characteristics. A microscopic observation ata fresh state and after Gram staining made it possible to characterize the bacterial morphology. The respiratory type as well as the respiratory enzymes are both determined. The biochemical characterization is carried out through various tests, namely: test on medium HAJNA-KLIGLER, test on medium SIMMONS CITRATE, test on medium MANNITOL-MOBILITY-NITRATE, test on medium LYSINE-IRON, gelatintest (Nelson, 2002) and the carbon auxanogram (Latour*et al.,* 1997). In addition, an analysis of the 16S rDNA sequence of the presumtived strainof*P. putida* is necessary in order to confirm the strain identity.

## III. Molecular study

### Extraction of genomic DNA

The "universal" extraction procedure using SDS and proteinase K has been adopted with some modifications (Daniel *et al.,* 1995). In order to realizethe extraction of the genomic DNA, the strain to be analyzed is firstly cultivated in nutrient broth for 24 hours at 30 °C. Two ml of the bacterial suspension are poured in sterilized Eppendorf tubes and centrifuged at 12000 rpm for 10 minutes. In the obtained pellet are poured 467 µl of the TE buffer solution, 3 µl of 20 mg / ml of proteinase K and 30 µl of 10% SDS. After incubation at 37 °C for 12 hours, the released proteins are precipitated with 400 µl of phenol / chloroform (50:50). The DNA suspended in the aqueous phase is then collected by centrifugation at 12000 rpm for 10 minutes. To ensure that the DNA samples are free of contaminants, 4µl of RNase (100mg/ml) are added then incubated for 2 minutes at room temperature. Finally, the DNA is precipitated by addition of sodium acetate and isopropanol, then collected by centrifugation at 13000 rpm for 10 min and dissolved in TE buffer. The DNA solutions are finally stored at -20 °C for amplification (Maloy, 1990).

### Qualitative analysis of DNA on agarose gel

To prepare the agarose gel, 30ml of TBE buffer are mixed with 0,3g of agarose. The obtained mixture is boiled until complete dissolution of the agarose. After having put in place the electrophoresis cell

and the comb, the gel is poured ina manner as to obtain a thickness of 3 to 5 mm. After the solidification of the latter, the comb is removed and the gel is immersedin TBEbuffer as migration buffer. The first well is loaded with a size marker (FERMENTAS, Lambda DNA / HindIII Marker). In the other well are poured: 8 µl of DNA sample, 2 µl of loading buffer (BIO LABS Gel Loading Dye). The manipulation is carried out in a horizontal electrophoresis apparatus (BIO RAD, Mini-Sub Cell GT) at 100 V for 15 minutes. After the migration of the DNA fragments, the gel is stained with ethidium bromide.

**Amplification and sequencing of the 16S rDNA region**

The target gene is amplified by conventional PCR in a programmable thermocycler(PTC-100). The chosenprimer pair limit the rDNA sequence to be replicated.The latter has been described by Weisburg in 1991:

27F (forward): (5 'AGAGTTTGATCMTGGCTCAG 3')

1492R (reverse): (5 'TACGGYTACCTTGTTACGACTT 3')

The PCR reaction is carried out in 25 µl reaction volume containing 5 µl of genomic DNA, 1 µl of each primer used, 0,125 µl of Taq polymerase, 2,5µl of PCR buffer, 2 µl of MgCl2, 0,5µl. OfdNTP. The thermocycler is programmed as follows: an initial DNA denaturation phase at 94 °C for 5 minutes and then 35 cycles of 1 minute at 94 ° C for DNA denaturation, 1 minute at 60 ° C for hybridization of primers and 1,5 minutes for elongation phase. The reaction is finished by increasing the temperature to 72 ° C for 10 minutes. The amplicons obtained are analyzed by agarose gel electrophoresis.

For sequencing the gene of interest, the PCR products are electrophoresed on an automated Applied Biosystems 3730xl DNA sequencing apparatus, using 50 cm capillary arrays and a POP-7 polymer. The results obtained are analyzed on PE-Biosystems version 3.7.

**Phylogenetic analysis of sequences**

The sequences are compared with those found in GenBank database the National Center for Biotechnology Information (NCBI) using the BLAST program. Genetic affiliation is evaluated using the Phylip 3.69 software. The results obtained are then represented in the form of a phylogenetic tree.

**Biological test**

The biological test sits on 3 months. It is realized in a sealed glass container of about 4500 $cm^3$to avoid contamination from outside. The container is covered by perforated aluminum foil to ensure aeration (Tarayre 2012, Bidaud 1998). The strain identified as *Pseudomonas putida* constitutes the inoculum in the experimental device. The setting of the device is done in three steps:

The preparation of the preculture consists in inoculating 3 handles of the strain in 100 ml of nutritive broth. The preparation is incubated at 30 ° C for 24 hours.

Then, 500 g of soil (dry weight) are autoclaved at 121 ° C at 1.5 bar for 2 hours. The soil is then dried at 103 ° C for 24 h and placed in a desiccator until it is cooled. 1.8% of diesel engine oil is added to the soil (El, 1998). Then, 150g of *Pinus* sawdust representing 30% of the weight of the soil is used as texturizer (Bidaud, 1998). Finally, 0.4% of yeast beer autoclaved and grinded which useas a source of nitrogen are also added in the soil (Tarayre, 2012).

The last step consists to inoculate 100ml of preculture in the previously prepared soil. The device is placed in the shade at room temperature (23 ° C). Every seven days, the soil is stirred and 20 ml of distilled water are poured on.

**Monitoring bacterial growth**

The evolution of the bacterial population is followed by microbiological analysis. To do this, 10 g of the soil sample are mixed with 90 ml of EPT solution in order to make the soil suspension. A succession of dilutions till $10^{-3}$ is done from the main suspension. Then, 1 ml of each dilution is plated oncetrimide agar plates. After incubation for 24 hours at 30°C, the bacterial count was done according to the ISO7218 standard.

**Determination of Residual Hydrocarbons (Method 3540C)**

The residual hydrocarbon concentration in the soil is also evaluated during the process. The 3540C method was adopted with some modifications. To do this, the soil is prepared in advance before the extraction of the hydrocarbon. 10 g of soil to be analysed are dried at 103 ° C for 24 h then placed in a desiccator until cooling. 5 g of dried soil are placed in an extraction cartridge then immersed with 150 ml of hexane. The extraction lasts 16 hours and the evaporation of the product is collected in a flask of 250 ml capacity containing pumice stones. The flask is then placed in a rotary evaporator to evaporate the hexane. The solvent and water residues in the flask are removed at 103 °C for 15 minutes, followed by a cooling in a desiccator. Finally, the volume of the extracted product is determined. In addition, the concentration of residual hydrocarbon present in the soil expressed in mg / kg is specified by the following formula (Center of Expertise in Environmental Analysis of Quebec, 2016):

$$C = \frac{AXV}{Q}$$

Thus :

- C : concentration of hydrocarbon in theanalysed sample (mg/kg)
- A : concentration of hydrocarbon in the injected extract (ng/µl)
- V : final volume of the analysed extract (ml)
- Q : dry weight of the analysedsample (g)

## IV. RESULTS

**Bacterial phenotypic identification**

The results of the cultural, morphological, physiological and biochemical studies permit to characterize the isolated bacterial strains.

Six strains were isolated. They are Gram negative, aerobic, mobile, oxidase positive. Fluorescent pigment production at 254 nm was observed on all strains (fig.1). Two strains produce floral notes. The biochemical identification of the strains confirmed the identity of the 6 isolated strains. Of which, 2 were identified as *Pseudomonasputida*, 2 *Pseudomonas aeruginosa*, 1 *Pseudomonas fluorescens* and 1 *Pseudomonas chlororaphis*. Table 1 gives the biochemical characteristics of each isolated bacterial strain.

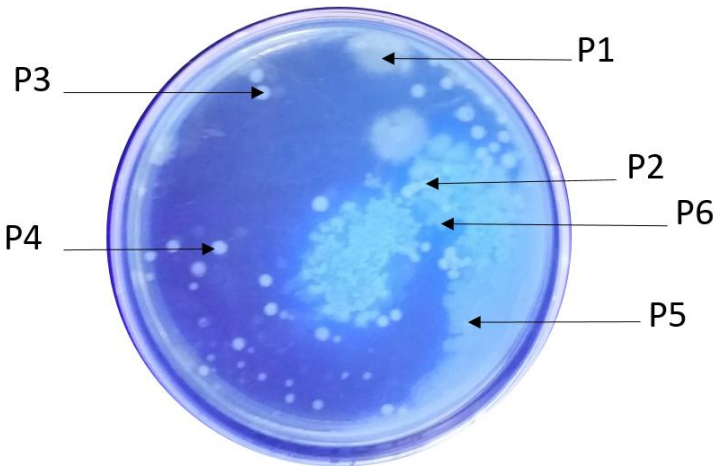


Figure 1 : Bacterial colonies observed at 254 nm after incubation on cetrimide agar

Table 1: Biochemical Characteristics of Isolated Bacterial Strains

| Strains | HAJNA-KLIGLER | | | | MANNITOL-MOTILITY | | LYSINE-IRON | | SIMMONS CITRATE | NITRATE REDUCTASE | GELATIN TEST | CARBON AUXANOGRAM | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Glu | Lac | $H_2S$ | $CO_2$ | MANNITOL | MOTILITY | LDC | LDA | | | | SACCHAROSE | XYLOSE | TREHALOSE |
| ***Pseudomonas aeruginosa*** | - | - | - | - | - | + | - | + | + | + | + | + | + | - |
| ***Pseudomonas chlororaphis*** | - | - | - | - | - | + | - | + | + | - | - | + | + | + |
| ***Pseudomonas fluorescens*** | - | - | - | + | - | + | - | + | + | - | + | + | + | - |
| ***Pseudomonas putida*** | - | - | - | - | - | + | - | + | + | - | - | + | + | - |

LDC : Lysine DeCarboxylase LDA : Lysine DeAminase Source : author, 2017

**Molecular identification**

Agarose gel electrophoresis of the extracted DNA fragments revealed unique and intact bands. The extract can be used for the amplification step of the 16S rDNA region. Electrophoresis is shown in Figure 2.

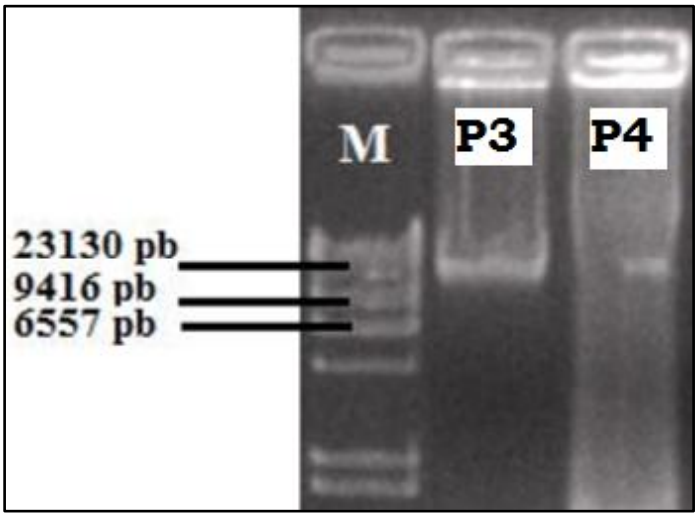


Figure 2 : Agarose gel electrophoresis of the genomic DNA of strains presumed to be *Pseudomonas putida* P3 and P4 (the first band represents the size marker)

Electrophoresis of the amplified 16S rDNA fragments showed that the size of the fragments was around 1500bp. Figure 3 shows Electrophoresis of amplified DNA fragments.

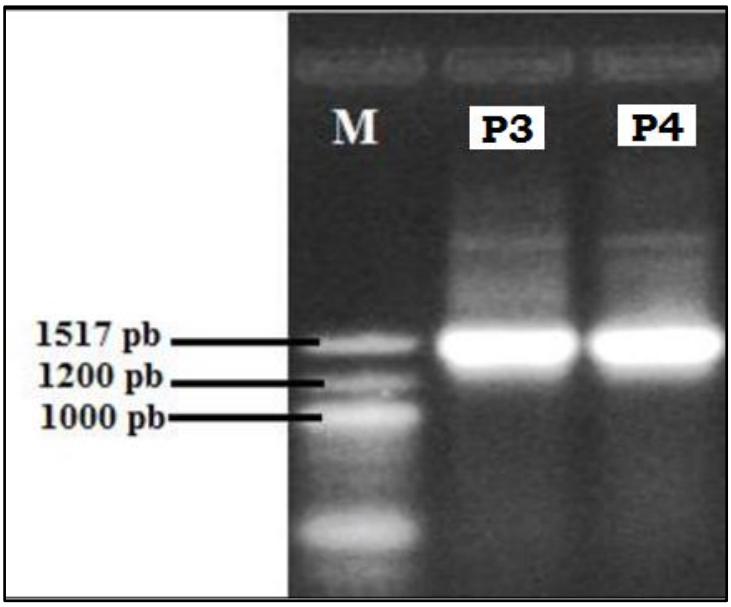


Figure 3 : Agarose gel electrophoresis of the amplified 16S rDNA fragments of the presumptive strain *Pseudomonas putida* P3 and P4 (the first band represents the size marker)

**The phylogenetic tree**

The assessment in BLAST of the 16S rDNA sequences of the P3 and P4 strains showed respectively 97.8% and 98% sequence similarity with *Pseudomonas putida*. This similarity is represented by a phylogenetic tree in Figures 4 and 5.

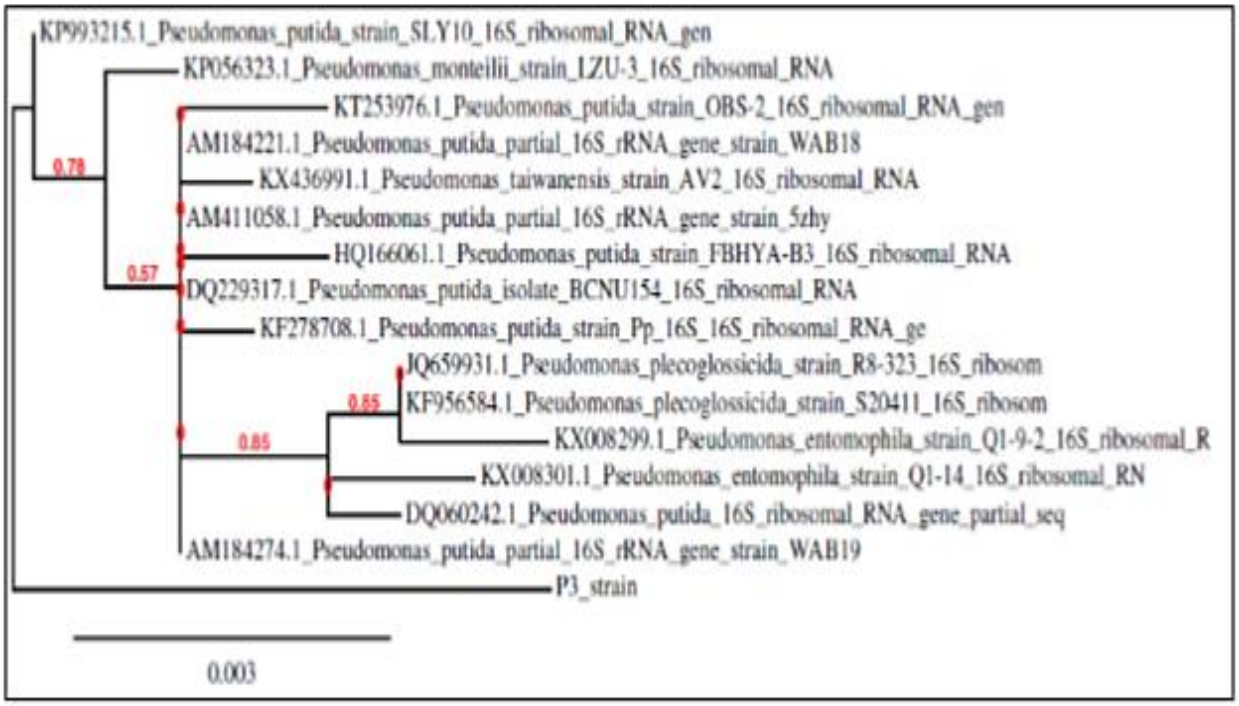


Figure 4: Phylogenetic tree based on the 16S rDNA sequences of Pseudomonasputida P3 strain and related species of BLAST database

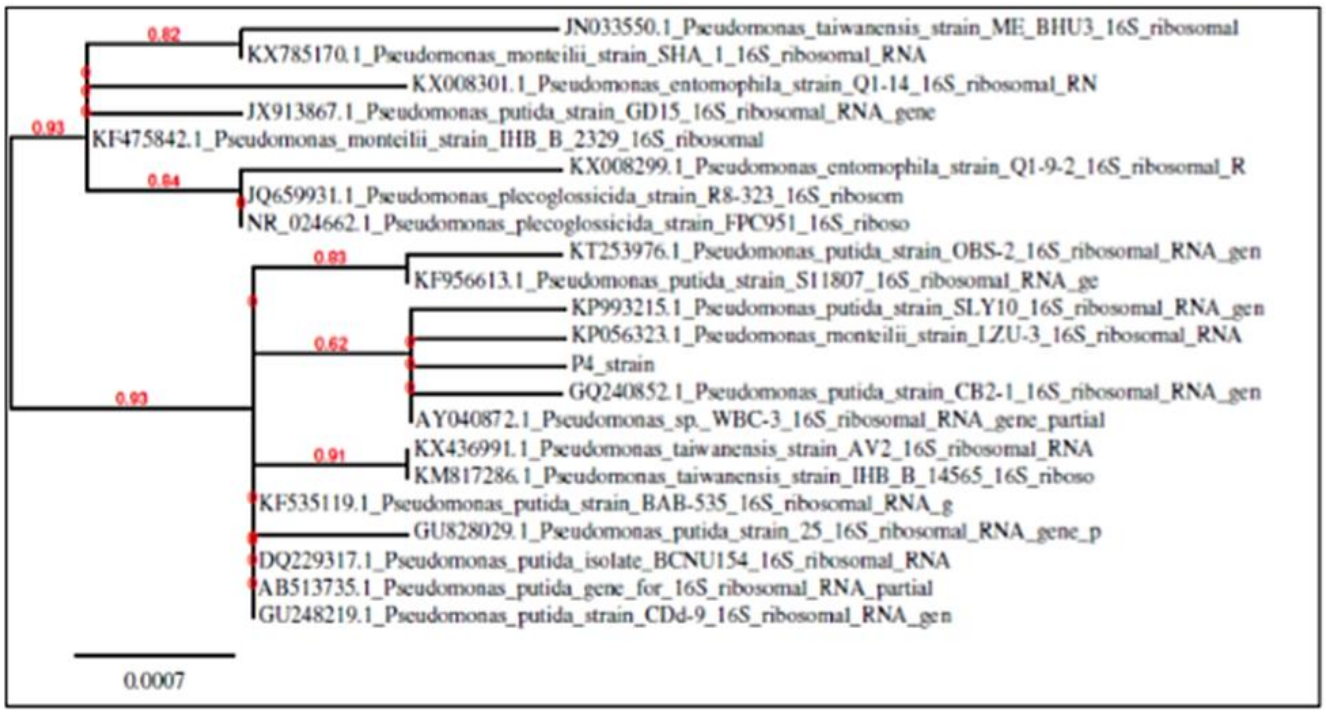


Figure 5: Phylogenetic tree based on 16S rDNA sequences of Pseudomonas*putida* P4 strain and related species of BLAST database

**Abundance of microorganisms in the rhizosphere zone of Pinusradiata**

Fluorescents *Pseudomonas* are the most common bacterial species found in the rhizosphere zones (Garcia *et al*., 2001). *P. aeruginosa, P. putida, P. fluorescens, P. clhororaphis*belong to this kind of bacteria andthey are called PGPR (Plant Growth Promoting Rhizobacteria). Figure 6 shows the abundance of *Pseudomonas* species in the root zone of *Pinusradiata*.

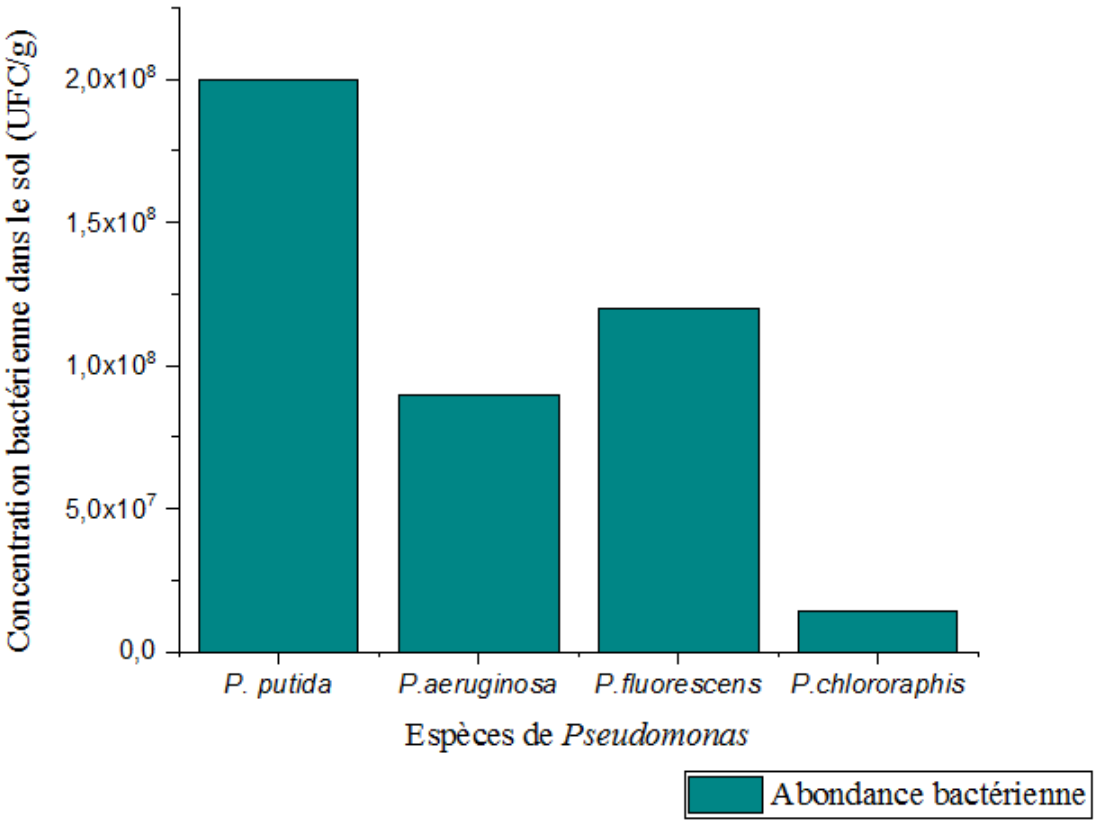


Figure 6: Bacterial distribution of *Pseudomonas* species in the root zone of *Pinusradiata*

**Biological test**

The results show a reduction of hydrocarbon concentration from 18000 mg/kg to 5000 mg/kg in 3 months. The degradation rates is around 30%. Figure 7 shows the evolution of the degradation rate of the hydrocarbon by *Pseudomonas putida*.

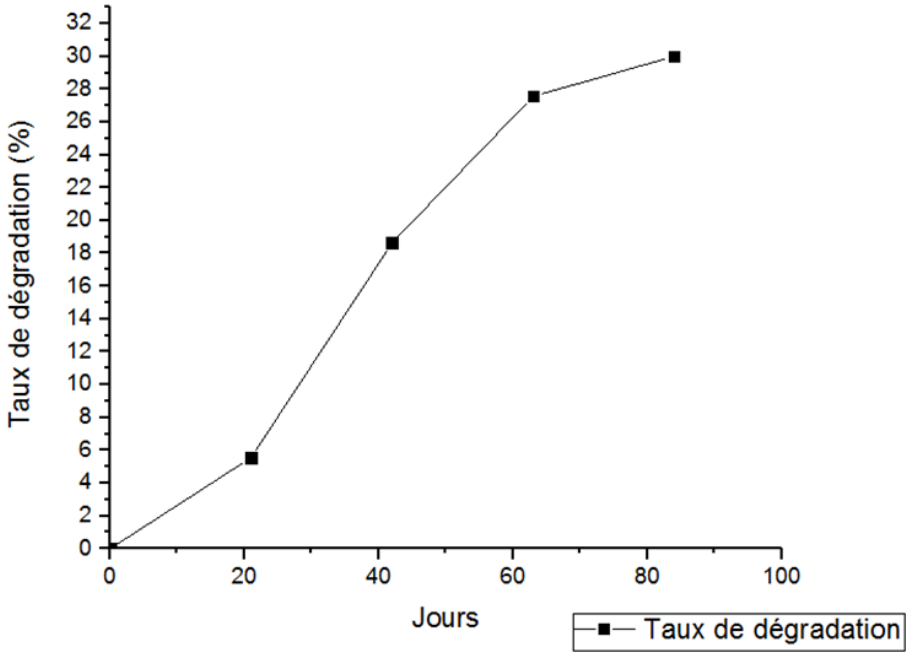


Figure 7: Degradation rate of hydrocarbon on 3 months

The bacterial population increases considerably from $4.10^7$ CFU/g to $3.10^{13}$ CFU/g and the generation time is 2,16h. During the first weeks, bacteria acclimatizes in the new culture medium. It follows an acceleration phase and exponential phase of growth. The bacterial growth is inversely proportional to the degradation of the hydrocarbon (Figure 8).

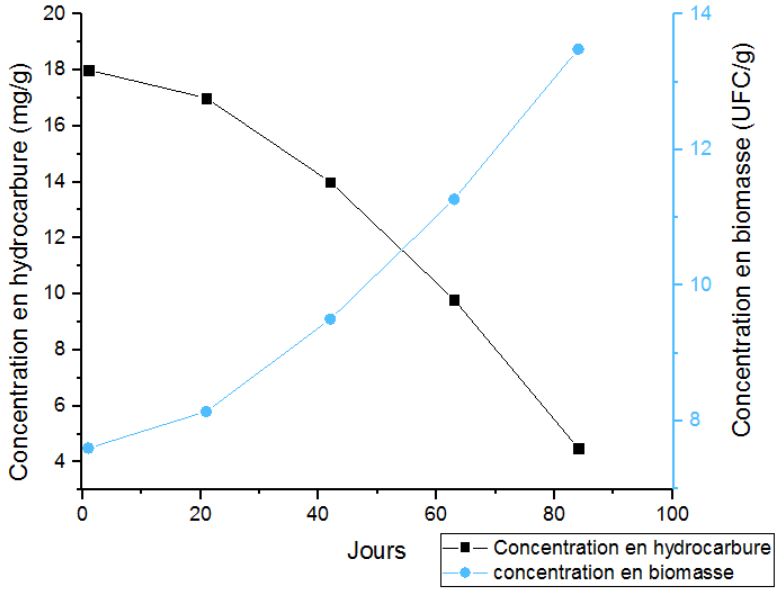


Figure 8: Evolution of the biomass and the hydrocarbon concentrations during the bioremediation of polluted soil using *Pseudomonas putida.*

At the end of the experiment, a production of $1,45.10^{12}$ CFU/g of soil per day was noted. This increased growth correspond to a degradation capacity of $2,52.10^{-4}$g of hydrocarbon per gram of soil per day.

## V. DISCUSSION

The bacterial isolation of a soil sample from the region of Vakinankaratra made it possible to deduce the presence of 4 different strains of *Pseudomonas* in the rhizosphere zone of *Pinusradiata*. These strains are*Pseudomonas aeruginosa, Pseudomonas fluorescens, Pseudomonas chlororaphis*and *Peudomonasputida*.

Based on the phenotypic characters, it was noted that all these strains belong to the genus *Pseudomonas* (Public Health England, 2015). The comparison of the identification results obtained with those of Bossis*al* in 2000 makes it possible to identify the strains P3 and P4 as *Pseudomonas putida*. However, phenotypic studies are not sufficient to confirm the identity of the strains. Thus, a molecular characterization has been carried out. The comparison in NCBI of the 16S rDNA of strains P3 and P4 respectively displays a similarity of 97,8% and 98% with *Pseudomonas putida*. Results of the obtained 16S rDNA sequences show that the P3 and P4 encoded strains belong to the species *Pseudomonas putida*.

About the experiment carried out on*Pseudomonas putida*, a bioremediation activity was observed. Indeed, the decrease in hydrocarbon concentration means that the strain is able to use petroleum hydrocarbon as the unique carbon and energy source (Jirasripongpun, 2002). This variation of the hydrocarbon concentration corresponds to a degradation capacity of 0,25 mg of hydrocarbon per day, which is not insignificant. The work carried out by Vinothini and its collaborators in 2015 on *Pseudomonas putida* showed a degradation rate of 98,8%, compared to the values obtained during the experiment, the latter is not yet optimized. Especially since during the first weeks of experimentation, the rate of degradation was not considerable (5,5%), a preculture in a medium containing hydrocarbon is therefore required.

There are several parameters to consider for effective bioremediation. On the one hand, the use of a consortium shows to be more efficient because of the diversity of catabolic enzymes that each microorganism used (Ghazali*et al*., 2004). Physicochemical characters also affect bioremediation. Specifically, the addition of texturizer such as sawdust promotes the supply of oxygen as an electron acceptor during metabolic reactions (Bidaud, 1996). The addition of bio-surfactant, temperature, pH, and moisture also contribute to the increased ability of the bacteria to degrade (Thwaites*et al*., 2007).

## VI. CONCLUSION

This experiment shows the presence in soil of a strain able to clean up soil contaminated by petroleum hydrocarbons. The results obtained reflect the ability of *Pseudomonas putida* to decrease to 30% the hydrocarbons concentration in 3 months. Despite the complexity of the chemical composition of the engine oil, the strain is found to be able to degrade this pollutant and to resist its toxic activity. The effectiveness of bioremediation depends not only on the biological agent used, but also on the physicochemical characteristics of the soil to be treated. Nitrogen content, moisture and aeration of the soil significantly affect this bacterial activity. The biological system used, the bioaugmentation and biostimulation has been appropriate.

However, several factors such as oxygen supply, pH, and humidity must be optimized to promote the bioremediation activity of the strain.

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