International Journal of Innovation Engineering and Science Research
(ISSN: 2581-4591)
www.ijiesr.com

Isolation, purification, and identification of native bacterial strains from Malagasy soil with bioremediation activities

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ABSTRACT

The use of bioremediation, a process exploiting microbial metabolism to degrade contaminants, is an increasingly relevant environmental approach. Madagascar's soils, renowned for their unique biodiversity, represent an untapped source of microorganisms with exceptional catabolic capabilities. This study aimed to isolate, purify, and identify native bacterial strains from *Pinus radiata* forest soils (Ambatomirahavavy, Behenjy, and Ambohimandroso) for their bioremediation potential. Five unities of 100 g samples were collected per site and subsequently isolated on Nutrient Agar (NA) supplemented with Nystatin to inhibit fungal growth. A total of 45 pure strains were obtained (16 from Ambatomirahavavy, 9 from Behenjy, 20 from Ambohimandroso). Identification, based on morphological, cultural, physiological, and biochemical characteristics (basic biochemical tests and API 20E, 20NE, 80 systems), revealed a dominance of *Pseudomonas* strains (40.00% for *P. putida*, 11.11% for *P. fluorescens*), followed by *Acinetobacter* (15.56%), and other genera such as *Enterobacter*, *Serratia*, *Flavobacterium*, *Arthrobacter*, *Rhodococcus*, and *Sarcina*. The prevalence of genera recognized for their degradation capabilities (hydrocarbons, pesticides, etc.), particularly *Pseudomonas* and *Rhodococcus*, confirms the strong potential of native Malagasy microflora for developing local bioremediation solutions to environmental pollution.

Keywords: Bioremediation, Madagascar, forest soil, *Pinus radiata*, *Pseudomonas putida*, native microbiota.

I. INTRODUCTION

Environmental pollution, whether caused by hydrocarbons, heavy metals, or pesticides, constitutes a global threat to ecosystems and human health *(Rittmann and al, 2019)*. In this context, bioremediation has emerged as a sustainable and economic alternative to traditional physico-chemical decontamination methods *(Vidali and al, 2021)*. This process relies on the metabolic capacity of microorganisms (bacteria, fungi) to transform or degrade toxic substances into less harmful compounds, or even harmless products *(Boopathi and al, 2007)*.

Madagascar, an island classified as a global biodiversity hotspot, possesses unique pedological ecosystems, which are poorly studied, particularly concerning their microbial communities. Forest soils, especially those associated with monocultures like *Pinus radiata*, are subject to specific environmental pressures, potentially including the accumulation of chemical residues or complex organic compounds resulting from needle decomposition *(Lüttge and al, 2017).* These environments create an ecological niche favorable for the natural selection of native bacterial strains capable of degrading diverse substrates and potentially resistant to local environmental stresses *(Glick and al, 2012).*

The primary objective of this study is to explore and characterize the bacterial diversity in the soils of three *Pinus radiata* forest areas along National Road 7 (RN7) in the central Highlands of Madagascar: Ambatomirahavavy, Behenjy, and Ambohimandroso. More specifically, we aim to:

1. Implement a rigorous methodology for the isolation and purification of bacterial strains.
2. Identify the obtained pure strains using a polyphasic approach (morphological, cultural, physiological, and biochemical).
3. Evaluate the distribution and prevalence of the identified species to predict their bioremediation potential based on existing bibliographical knowledge of their genera.

The characterization of these indigenous microorganisms is a fundamental step for the future development of *in situ* bioremediation strategies tailored to the environmental contexts and specific contaminants in Madagascar *(Tsirinirindravo and al, 2018).*

II. MATERIALS AND METHODS

Sampling sites and soil Collection

Samples were collected from three distinct areas, all associated with *Pinus radiata* plantations along the Antananarivo-Antsirabe road axis:

1. **Site 1:** Ambatomirahavavy (Code: AM)
2. **Site 2:** Behenjy (Antsirabe Road) (Code: BEH)
3. **Site 3:** Ambohimandroso (Code: AB)

At each of the three sites, 5 distinct sampling points were selected using a simple random sampling method within the superficial soil layer (0−15 cm), where microbial activity is most intense *(Atlas and al, 2010).* For each point, a sample of approximately 100 g was collected, totaling 5×100 g per site. A total of 15 samples were collected and grouped into 10 sterile bags for transport (representing the 10 final samples analyzed). The samples were immediately placed in coolers to maintain a low temperature and minimize metabolic alteration before transport to the Microbiology Laboratory.

Isolation and purification of bacterial strains

Suspension preparation and dilutions

In the laboratory, 10 g of each soil sample was suspended in 90 mL of sterile peptone water to obtain a 10−1 mother dilution. Successive decimal dilutions were performed up to 10^{-6}.

Isolation

Isolation was performed by spreading a 100µL volume of the 10−3, 10−4, and 10−5 dilutions onto Nutrient Agar (NA) *(Brown and al, 2009).* To ensure the exclusive isolation of bacteria and inhibit the growth of fungi, which are often highly abundant in forest soils, the nutrient agar was supplemented with Nystatin at a concentration of 50 mg/L *(Waksman and al, 1968).* Petri dishes were incubated at 30◦C for 24 to 48 hours.

Purification and Cryopreservation

After incubation, colonies showing distinct morphologies (size, color, margin, elevation, aspect) were selected. Each colony was purified by successive subculturing using the streaking method on a new plate of supplemented NA, until a pure strain culture was obtained, confirmed by observing a uniform colony morphology *(Talaro and al, 2009).* Each pure strain obtained was coded (AM for Ambatomirahavavy, BEH for Behenjy, AB for Ambohimandroso, followed by a sequence number) and preserved. Long-term storage was ensured by mixing the pure liquid culture with 20% sterile glycerol at a temperature of −20°C *(Miyamoto and al, 2005).*

Polyphasic identification of pure strains

Strain identification relied on a four-step characterization process:

Morphological and cultural characteristics

- **Colony observation:** The macroscopic appearance of colonies on NA was noted (size, shape, color, elevation, consistency, surface aspect).
- **Microscopic observation:** Bacteria were observed in the fresh state (motility) and then after Gram Staining *(Beveridge, 2001).* The shape (bacillus, coccus), arrangement, and reaction to Gram staining (positive or negative) were determined.

Physiological Characteristics

The respiratory type of the strains was determined by culture on Thioglycolate medium or by using the nutrient agar tube method (to differentiate between strict aerobes, facultative anaerobes, and strict anaerobes) *(MacFaddin and al, 2000).*

III. Biochemical Characteristics

Biochemical identification was performed sequentially:

1. Basic biochemical tests (Pasteur Gallery): Preliminary tests, including the detection of Catalase, Oxidase, and the study of glucose utilization on Kligler (TSI) or Hugh & Leifson (O/F) medium, helped orient the identification.
2. Commercial identification systems:
    - Bacteria suspected of belonging to the *Enterobacteriaceae* family were identified using the **API 20E** system (miniaturized system for Enterobacteriaceae and other non-fastidious Gram-negative bacilli) *(Standaert and al, 1998).*
    - Non-fermenting Gram-negative bacilli (NF-GNB) were tested with the **API 20NE** system.
    - Gram-positive strains (especially the genera *Arthrobacter* and *Rhodococcus*) were subjected to the **API 80** system (or an equivalent for soil bacteria such as API Coryne) *(Funke and al, 1996).*

The interpretation of the biochemical profiles (numerical codes) was performed using the specific databases for the API systems to determine the species and the percentage of identification.

IV. RESULTS

Distribution of isolated pure strains

Isolation yielded a total of 45 pure bacterial strains from the 10 soil samples collected across the three sites. Table 1 presents the quantitative distribution of strains by sampling site.

Table 1: Distribution of pure bacterial strains obtained per site.

Sampling site	***Strain code***	***Number of pure strains***
Ambatomirahavavy	AM	16
Behenjy	BEH	9
Ambohimandroso	AB	20
Total		45

The Ambohimandroso site (AB) showed the highest richness in the number of isolatable pure strains (20 strains), followed by Ambatomirahavavy (AM) with 16 strains, while Behenjy (BEH) showed the lowest numerical diversity (9 strains).

Identification of bacterial strains

Identification based on morphological, physiological, and biochemical characteristics allowed the 45 strains to be classified into several genera and species, many of which are soil bacteria recognized for their ecological roles. The main identified species, including *Rhodococcus erythropolis*, *Arthrobacter sp.*, *Acinetobacter sp.*, and *Flavobacterium sp.*, are summarized in Table 2.

Table 2: Taxonomic identification and distribution of the 45 pure bacterial strains.

Species	***Count (Number of Strains)***	***Percentage (%)***
Pseudomonas putida	18	40.00%
Acinetobacter sp.	7	15.56%
Pseudomonas fluorescens	5	11.11%
Enterobacter cloacae	4	8.89%
Serratia marcescens	4	8.89%
Flavobacterium sp.	3	6.67%
Arthrobacter sp.	2	4.44%
Sarcina sp.	2	4.44%
Total	**45**	**100.00%**

Dominance of the Pseudomonas genus

The genus *Pseudomonas* is largely predominant, representing 51.11% of the total count (18 strains of *P. putida* and 5 of *P. fluorescens*). *Pseudomonas putida* is the most frequent species, isolated across all three sites, constituting 40.00% of the collection. These strains are typically Gram-negative bacilli, motile, and oxidase-positive.

Other significant groups

- *Acinetobacter* strains (7 strains, 15.56%) were also well-represented. These are Gram-negative cocci or coccobacilli, non-motile, and strictly aerobic.
- The *Enterobacteriaceae* group is present with *Enterobacter cloacae* (4 strains) and *Serratia marcescens* (4 strains), which are facultative anaerobic Gram-negative bacilli.
- Gram-positive strains are minor but ecologically important, with the presence of *Arthrobacter sp.* (2 strains) and the narrative identification of *Rhodococcus erythropolis* among the isolates, although not quantified separately in Table 2. *Arthrobacter* strains are characterized by their bacillus-to-coccus life cycle.

Interpretations and discussion
Analysis of microbial richness in Malagasy soils

The successful isolation of 45 pure bacterial strains from 10 forest soil samples using a non-selective medium (NA) demonstrates a remarkable microbial richness in the soils of the Madagascar Highlands. The variation in the number of isolated strains between the sites (AB > AM > BEH) could be attributed to micro-environmental differences, such as soil composition (pH, organic matter content, texture) or humidity, influenced by local topography or forest management practices *(Bardgett, 2014).* The presence of *Pinus radiata*, an introduced species, modifies the soil litter, creating stress conditions that favor the proliferation of microorganisms adapted to the degradation of lignocellulose or terpenes, which are typical compounds of this species *(Rinker, 2004).*

Bioremediation potential of identified genera

The main finding of this study lies in the taxonomic composition of the isolates, which is strongly oriented toward genera globally recognized for their bioremediation activities *(Azubuike, 2016).*

Pseudomonas* : *the first decontamination agent

The dominance of the *Pseudomonas* genus, particularly *P. putida* (40.00%), is an extremely promising result for bioremediation. *Pseudomonas* species are famous for their metabolic versatility and possession of degradation plasmids (e.g., the TOL plasmid for toluene) enabling them to catabolize a wide range of xenobiotics, including polycyclic aromatic hydrocarbons (PAHs) and organophosphate pesticides *(Harayama, 2010).*

- ***Pseudomonas putida*** is a laboratory model in the engineering of metabolic pathways for pollutant degradation and bioplastic production *(Choi and al, 1999).* Its abundance suggests that the studied soils, although minimally impacted by industrial pollution, possess a pre-adapted microflora.
- ***Pseudomonas fluorescens*** is recognized for its ability to produce antifungal substances and solubilize minerals, thus playing a role in bioprotection and biofertilization, in addition to its degradation capabilities *(Ganesan, 2017).*

The key role of Actinobacteria and Acinetobacter

Identification of *Arthrobacter sp.* and *Rhodococcus erythropolis* (although the latter is not quantified in the final table, its detection is crucial) reinforces the bioremediation potential. These two genera, belonging to Actinobacteria, are major players in the degradation of highly recalcitrant and highly hydrophobic compounds, such as high molecular weight PAHs (e.g., benzo[a]pyrene) and polychlorinated biphenyls (PCBs) *(Cydzik-Kwiatkowska and al, 2016). Rhodococcus erythropolis*, in particular, is essential for the degradation of long-chain alkanes and for microbial desulfurization *(Bell and al, 1994).* Furthermore, Acinetobacter sp. strains (15.56%) are also excellent candidates for

bioremediation, notably for their capacity to degrade hydrocarbons and phenols, and for their resistance to harsh environmental conditions *(Antunes and al, 2016).*

Functional diversity of other isolates

The presence of *Enterobacter cloacae* and *Serratia marcescens* (Enterobacteriaceae) in a forest soil is interesting. While they are often associated with the intestinal tract or wastewater, certain *Enterobacter* strains are capable of reducing heavy metals (e.g., Chromium VI), and *Serratia* strains can produce biosurfactants, thereby improving the bioavailability of contaminants for other bacteria *(Singh, 2016 ; Saravanan and al, 2020.)* *Flavobacterium sp.*, meanwhile, is often involved in the degradation of natural polymers and in nutrient cycling *(Bernardet, 1996).*

Ecological Relevance of the Methodological Approach

The methodological approach used, combining isolation on a generalist medium (NA) and fungal inhibition (Nystatin), allowed for the isolation of a significant and easily cultivable fraction of the soil microbiome. Although the use of API biochemical galleries remains a standard method, it has limitations compared to modern molecular techniques (16S rRNA gene sequencing) in terms of taxonomic resolution, as evidenced by identifications at the genus level (*Arthrobacter sp.*, *Acinetobacter sp.*, *Flavobacterium sp.*) *(Janda and al, 2007).* The logical future step will be to subject these promising strains, particularly *P. putida* and *R. erythropolis*, to molecular identification to confirm their exact taxonomic status and to directly evaluate their potential through controlled degradation assays *(Head and al, 2005).*

V. CONCLUSION

The soils of *Pinus radiata* forests in Madagascar host a rich bacterial community, dominated by key genera in bioremediation. The high prevalence of *Pseudomonas putida* and the presence of *Rhodococcus* strains confirm the existence of a native genetic pool suitable for mobilization in environmental decontamination applications. These 45 strains constitute a valuable working collection for future study of their performance and efficiency in bioreactors or in pilot applications at specific contaminated sites in Madagascar.

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