

## **Folin-Ciocalteu Assay As A Rapid Colourimetric Screening Method For Evaluating PAH Degradation Abilities Of Heterotrophic Bacteria**

**Vasudha C. Bhatawadekar<sup>1§</sup>, Samir R. Damare<sup>1\*</sup>, Anita Garg<sup>2</sup>**

<sup>1</sup>Biological Oceanography Division, CSIR-National Institute of Oceanography,  
Dona Paula, Goa-403004, India.

<sup>2</sup>Analytical Services Division, CSIR-National Institute of Oceanography,  
Dona Paula, Goa-403004, India.

<sup>§</sup>Department of Microbiology, Goa University, Taleigao Plateau, Goa, India

\*Corresponding author: samir@nio.org

\*ORCID ID: 0000-0002-4201-4844

### **Abstract**

Bioremediation using microbes is an eco-friendly approach being explored for reclaiming PAH-contaminated areas. However, isolation and screening of potential bacteria to degrade PAHs is very laborious and cumbersome. To alleviate this issue, we describe a rapid method for screening the bacterial cultures for their ability to degrade PAHs using Folin-Ciocalteu (FC) assay. Six hundred bacterial isolates were tested for their ability to degrade PAH using FC assay. The cultures capable of degrading PAH show blue colouration, resulting from the reaction of FC reagent with phenolic intermediates generated during PAH degradation. Out of the 600 cultures screened, 64 showed an ability to degrade PAH. This study provides a very easy, rapid, less laborious, and sensitive method to screen a large number of bacterial cultures for their ability to degrade PAH.

**Keywords:** 1-naphthol; Bioremediation; Folin-Ciocalteu assay; Heterotrophic bacteria; Polycyclic aromatic hydrocarbons.

## Introduction

Polycyclic aromatic hydrocarbons (PAHs) are priority organic contaminants due to their toxic, mutagenic, and carcinogenic nature (Ghoshal et al. 2016). Incomplete combustion of carbon-containing material such as grass, wood, coal, petroleum and natural gas, forest fire, and volcanic eruption leads to the generation of PAHs (Ramzi et al. 2017). PAHs contain benzene rings fused in linear, angular, or cluster ways (Liu et al. 2017). Hydrophobic and intrinsic chemical stability leads to their persistence and ubiquitous occurrence in the environment (Paulik et al. 2016). They tend to adsorb on suspended particulate matter in the soil, air, water, and sediment. These features make them recalcitrant and less accessible for biological uptake and allow them to accumulate in the food chain leading to toxic, mutagenic, and carcinogenic effects on the living organisms of aquatic as well as terrestrial ecosystems (Masiol et al. 2012; Abdel-Shafy & Mansour 2016; Alegbeleye et al. 2017).

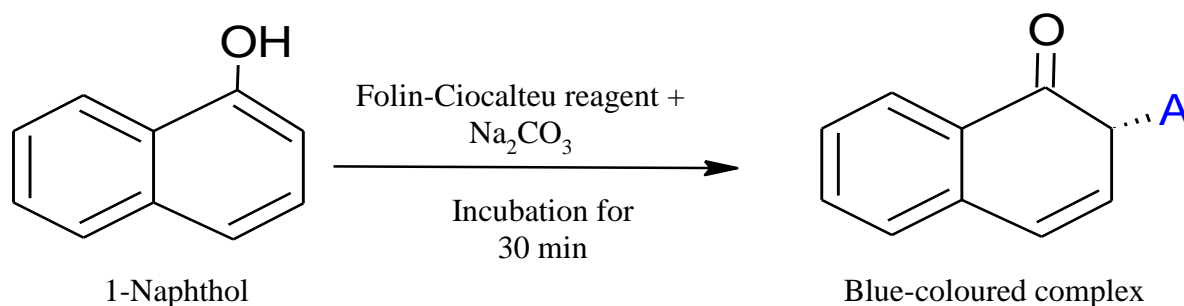
Different treatments have been practised to remove PAHs from the environment, including physical, chemical, and biological methods. Among these, biological treatment, also known as bioremediation, is an eco-friendly and cost-effective approach to treat these high-risk and toxic contaminants, as it does not exhibit a deteriorating effect on the environment (Aziz et al. 2018). Bioremediation involves using bacteria, fungi, and, or plants that convert toxic contaminants into a non-toxic or less toxic form (Bhatawadekar et al. 2021). Degradation of contaminants or pollutants using bacteria is an effective method due to their short generation time and quick adaptability (Ghoshal et al. 2016).

Characterisation of bacterial strains which can efficiently degrade pollutants is an essential step in studies involving the degradation of PAHs and their application at PAH-contaminated sites. Conventionally, the PAH-degrading bacteria were isolated using the enrichment and spray plate method (Thomas et al. 2016). Zhao et al. (2009) described a method in which cellulose acetate/nitrate filter paper was used for the adsorption of PAHs and, after 96 hours, checked for the growth of bacteria. Turbidity measurements, oxygen utilisation assessment, and carbon dioxide generation are also used to screen PAH-degrading bacterial strains (Habib et al. 2017). However, these methods are time-consuming, laborious, and uneconomical, and in the case of the spray plate method, equal distribution of PAHs on the agar plate is not possible.

The colourimetric method is a good choice as it is fast. One such method uses the colourimetric indicator 2, 6-dichlorophenolindophenol (DCPIP), a redox indicator. The general principle is that DCPIP, in its oxidised state, is a blue-coloured compound that accepts electrons and gets reduced to a colourless compound, DCPIPH<sub>2</sub>. This concept can be used to monitor the

utilisation of hydrocarbon by bacteria. DCPIP has been used for screening hydrocarbon-degrading bacteria in minimal salt medium (MSM) (Umar et al. 2017). The use of DCPIP for screening PAH-degrading bacteria has a disadvantage due to its non-specific nature and low colour stability in the solution (Jahn et al. 2020). Additionally, certain chemolithotrophs can use inorganic nutrients to grow and impart false-positive results as it is a redox reaction.

This study illustrates a method based on the Folin-Ciocalteu (FC) assay to resolve the problems associated with the methods mentioned above. It is routinely used for the determination of phenolic compounds. The aerobic bacterial degradation of PAHs leads to the production of stable phenolic intermediates like 9-phenanthrol, 1-hydroxy-2-naphthoic acid, 1, 2-dihydroxy-naphthalene, 1-hydroxypyrene, 1-hydroxy-6-methoxypyrene, 4, 5-dihydroxypyrene, 2,3-dihydroxybenzoate, 1-naphthol, etc. (Tiwari et al. 2010; Feng et al. 2012; Mangwani et al. 2014; Swati et al. 2019; Wu et al. 2019), which can react with FC reagent to produce blue-coloured complex.



Previously, a few researchers have used FC assay to monitor metabolite production during phenanthrene degradation (Samantha et al. 1999; Mangwani et al. 2014). However, no reports are available making use of the FC assay method to screen PAH-degrading bacteria rapidly are not explored. In this study, 600 bacteria were screened using FC reagent for their ability to degrade PAH, such as phenanthrene (Phe), fluoranthene (Flt), and pyrene (Pyr). Their metabolites were validated using GCMS analysis.

### Sample collection and isolation of bacteria

For isolating bacteria to be used for this study, sediment samples were collected using sediment grab, while surface water samples were collected using a Niskin water sampler from coastal regions of Goa (Divar mangrove, Zuari Estuary, Sal River, Mandovi Estuary, and Galgibaga) (Fig. 1) The sediment and water samples were collected in sterile sample collection bags and bottles respectively, maintained at 4°C, and immediately transported to the laboratory for further processing. Water samples and approximately 1 g of each sediment sample were serially diluted. The traditional spread-plate technique was used to obtain pure bacterial colonies on ZMA (Zobell marine agar) plates.

These plates were incubated at 28°C for 48 h. Morphologically distinct bacterial colonies were selected, purified, and maintained on ZMA plates for further study. 1000 bacterial colonies were obtained from different sampling locations after spread-plating on ZMA, and about 600 distinct colonies were purified. These were used to screen for their ability to degrade Phe, Flt, and Pyr.

### **Screening of PAH-degrading bacteria**

From the bacterial isolates obtained as detailed above, pure cultures were inoculated in 20 mL of sterile ZM (Zobell Marine) broth in 100 mL conical flasks and incubated overnight in a shaker at 28°C, 120 rpm. The overnight grown culture was reinoculated in 20 mL of fresh ZM broth in a 100 mL conical flask, and at an exponential stage of growth, the culture broth was centrifuged at 8000 g for 10 min to collect cell pellets. The cell pellet was washed twice with sterile MSM and resuspended in sterile MSM (Minimal Salt Medium) to remove the traces of the nutrient-rich organic medium, followed by the addition of MSM containing PAH (50 ppm Phe, 50 ppm Flt, and 50 ppm Pyr) (MPB – MSM plus PAH plus bacterial culture) as a sole carbon source, and incubated at 28°C, 120 rpm. Appropriate controls of MSM with PAHs without bacterial culture (MP - MSM plus PAH) and MSM without PAHs with bacterial suspension were maintained at similar conditions (MB - MSM plus bacterial culture). After 24 h incubation, 100 µL of the MPB, MP, and MB (each from control and test) were transferred into a 96-well microtitre plate containing 100 µL of Folin Ciocalteu (FC) reagent, followed by the addition of 80 µL of 10 % sodium bicarbonate to maintain an alkaline condition. This reaction mixture was incubated in the dark for 30 min and observed for colour change. Resorcinol solution (50 ppm) was used as a positive control. Amongst 600 bacterial isolates, a total of 63 strains showed blue colouration after adding FC reagent, indicating potential PAH degraders. The results for 12 bacterial isolates showing the ability to degrade one of the PAHs are shown in Fig. 2. The bacterial isolates (NIOSV1, NIOSV2, NIOSV3, NIOSV7, NIOSV8, NIOSV11, NIOSV12, NIOSV17, NIOSV18, NIOSV19, NIOSV20, and NIOSV22) able to degrade PAHs, developed a blue coloured complex in the presence of FC reagent. Negative controls (MSM with Phe, Flt, Pyr, and only MSM in E1, E2, E3, and E4 wells) showed no colour change. Another negative control set, MSM with bacterial inoculums without any PAHs in well numbers A4, A8, A12, B4, B8, B12, C4, C8, C12, D4, D8, and D12, also did not show any colour change. Resorcinol used as positive control showed prominent blue colouration (Fig. 2, Well E5). The potential of bacterial isolates to degrade various PAHs is shown in Fig. 3. Out of the total 600 bacterial isolates evaluated for their ability to degrade PAHs, 63 isolates were capable of degrading only Phe, 15 degraded only Pyr, 14 degraded only Flt, 9 degraded both Phe and Pyr, 7 degraded both Phe and Flt

4 degraded both Pyr and Flt while only 4 showed the ability to degrade all three PAH compounds evaluated in this study.

### **Identification of potential PAH-degrading bacteria**

The cell pellet was obtained from pure bacterial isolates grown in ZM broth by centrifugation at 12,000 rpm for 2 min. A DNA extraction kit (Nucleopore, Genetix, Biotech, India) was used to extract total genomic DNA as per the manufacturer's protocol. The universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492 R (5'-TACGGTTACCTTGTTACGACTT-3') were used to amplify 16S rDNA followed by PCR clean-up of amplified products using Promega Corporation, USA. Sequencing of nucleotide was performed using Genetic Analyzer 3730xl (ABI) based on the Big Dye terminator v 2.0 chain terminator chemistry. BLAST analysis was carried out using the NCBI database. Type strain sequences were downloaded from NCBI, and a phylogenetic tree was constructed using MEGA X (.....) (Fig 5).

### **Confirmation of PAH degradation using Gas Chromatography-Mass Spectrometry (GCMS) analysis**

The metabolites and PAH degradation by-products produced during the growth of the bacterial cultures were analysed using the GCMS. The metabolites and the degradation by-products from the culture broth were extracted with dichloromethane (DCM) and ethyl acetate. Briefly, the growing culture was initially acidified to pH 2.0 with 2M HCl, and to this, an equal volume of DCM was added and kept on a shaker at 160 rpm for 1 h, followed by vigorous shaking in a glass separating funnel and allowed to stand until two phases were separated. The extraction process was repeated thrice, and organic phases were pooled together and dried over anhydrous sodium sulphate. To the separated aqueous phase, an equal volume of ethyl acetate was added, and the same procedure was repeated thrice, as mentioned above. Then DCM extract was dried at 40°C while the ethyl acetate extract at 80°C using a rotary evaporator (Laborota 4000 Heidolph, Germany). Finally, this was resuspended in DCM and ethyl acetate and analysed using GC-MS (ThermoFisher Scientific 1300 GC coupled with Thermo TSQ8000 MS). DB-5 capillary column (60 m length, 0.25 mm internal diameter, 0.25 µm film thickness) was used for the analysis. The MS was operated in full scan mode with a temperature program of 70°C for 2 min hold, then raised the temperature by 30°C min<sup>-1</sup> up to 150°C, followed by increasing the temperature to 280°C by 4°C min<sup>-1</sup> rates and hold for 20 min. Pure helium (99.999%) was used as a carrier gas at a flow rate of 1.2 mL min<sup>-1</sup>. The GC-MS analysis identified the phenolic metabolites formed during the degradation of Phe, Flt, and Pyr by bacterial isolates. The mass spectrum with fragment ions at m/z 115 and 144 indicated that the compound is 1-

naphthol based on the m/z values and comparison with the mass spectra with the NIST library (Fig. 4).

The FC assay is a redox reaction between phenolic compounds and FC reagent at around pH 10. The FC reagent is composed of heteropoly-phosphotungstates/molybdates. The alkaline condition produces phenolate ions capable of reducing FC reagent and generating a blue-coloured complex (Sánchez-Rangel et al. 2013). This method utilises the production of phenolic metabolites during the degradation of PAHs (Steiber et al. 1994; Prabhu & Phale 2003), which are detected by Folin's reagent. Samanta et al. (1999) used the Folin reagent to quantify phenolic compounds generated during phenanthrene degradation by bacteria. Folin-Ciocalteu assay has also been used previously to confirm phenolic intermediates during bacterial degradation of Phe and Pyr (Mangwani et al. 2014). Production of phenolic metabolites has been reported earlier during the degradation of the three PAHs studied here, namely, Phe (Feng et al. 2012; Prabhu & Phale 2003), Flt (Cao et al. 2015; Reddy et al. 2018) and Pyr (Thomas et al. 2016; Tiwari et al. 2010). The method described here is based on the production of such phenolic metabolites, which react with FC reagent to produce a blue-coloured complex, considered a positive test.

The method described here has an advantage over other colourimetric assays like DCPIP, which is also a type of redox reaction. Bacteria able to utilise inorganic components of the medium can grow and mediate the electron transport chain, which leads to a change in colour, giving a false-positive result. Also, due to the environmental impact, there is a chance of oxidation of dye showing false positive results (Habib et al. 2017).

In this method, three negative control sets to eliminate false positive results were kept. The first negative control, MSM without PAH or bacterial culture, confirms that the components of the medium are not responsible for reducing the FC reagent. The second colourless negative control of MSM with PAHs confirms the stability of PAHs and no environmental oxidation of PAHs. The third colourless negative control of MSM with bacterial isolates without PAHs shows no interference of Chemolithotrophic bacteria using MSM components as a source of energy for their growth. The blue colouration in the positive control (resorcinol) represents the reaction between phenolic compounds with FC reagents. The method described here is highly specific to PAH degradation as phenolic intermediates are produced during the degradation of only PAH and no other hydrocarbons, such as alkanes or alkenes, as seen by the GCMS analysis.

## **Conclusion**

Screening and evaluation of PAH-degrading bacteria using conventional methods is laborious and time-consuming. This study describes a rapid and simple method, validated with GC-MS analysis, for screening many bacteria for their PAH degrading capabilities. The technique uses a simple colourimetric assay and doesn't require any particular instrument. The method is fast and accurate, with no false positive reports. This method will help researchers screen a large number of bacterial isolates for their PAH degradation ability, thereby boosting the possibility of using bioremediation to ameliorate PAH pollution.

## **Acknowledgements**

The authors are thankful to the Director CSIR-National Institute of Oceanography for providing all the facilities and infrastructure required to carry out this work. We acknowledge the funding received from DBT, Govt. of India, under the project GAP3297. The first author is thankful to CSIR for her Research Fellowship (18/12/2016(ii) EU-V). The work is part of the doctoral thesis of the first author to be submitted to Goa University. The authors appreciate the critical suggestions received from anonymous reviewers that helped in improving the manuscript. This is NIO contribution no.....

**Conflict of interest statement:** The authors declare that they have no conflict of interest in the publication.

**Author contribution statement:** The work was planned by the corresponding author. All the experimental work was carried out by the first author. The GCMS analysis was carried out by the first and last authors. All the authors contributed to writing the manuscript.

## **Compliance with Ethical Standards**

### **Conflicts of interest**

The authors declare that they have no conflict of interest in the publication.

### **Research involving Human Participants and/or Animals**

Not applicable to this manuscript.

### **Informed consent**

Not applicable to this manuscript.

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## Legend to Figures

**Figure 1:** Map of area showing sampling locations.

**Figure 2:** FC assay showing PAHs (phenanthrene (P), fluoranthene (F), and pyrene (Y)) degradation using different bacterial suspensions in a 96-well plate.

Well numbers A1, B1, C1, D1, A5, B5, C5, D5, A9, B9, C9, D9: Mineral salt medium with 50 ppm Phenanthrene with respective bacterial culture as mentioned below.

Well numbers A2, B2, C2, D2, A6, B6, C6, D6, A10, B10, C10, D10: Mineral salt medium with 50 ppm Fluoranthene with respective bacterial culture as mentioned below.

Well numbers A3, B3, C3, D3, A7, B7, C7, D7, A11, B11, C11, D11: Mineral salt medium with 50 ppm Pyrene with respective bacterial culture as mentioned below.

Well numbers A4, B4, C4, D4, A8, B8, C8, D8, A12, B12, C12, D12: Mineral salt medium without any PAH and with respective bacterial culture (Negative control) as mentioned below.

Well number E1: Minimal salt medium with Phenanthrene without bacterial culture.

Well number E2: Minimal salt medium with Fluoranthene without bacterial culture.

Well number E3: Minimal salt medium with Pyrene without bacterial culture.

Well number E4: Minimal salt medium without any PAH or bacterial culture.

Well numbers A1, A2, A3, A4: isolate NIOSV1;

Well numbers A5, A6, A7, A8: isolate NIOSV2;

Well numbers A9, A10, A11, A12: isolate NIOSV3;

Well numbers B1, B2, B3, B4: isolate NIOSV7;

Well numbers B5, B6, B7, B8: isolate NIOSV8;

Well numbers B9, B10, B11, B12: isolate NIOSV11;

Well numbers C1, C2, C3, C4: isolate NIOSV12;

Well numbers C5, C6, C7, C8: isolate NIOSV17;

Well numbers C9, C10, C11, C12: isolate NIOSV18;

Well numbers D1, D2, D3, D4: isolate NIOSV19;

Well numbers D5, D6, D7, D8: isolate NIOSV20;

Well numbers D9, D10, D11, D12: isolate NIOSV22;

Well number E5: Resorcinol 50 ppm (Positive control).

**Figure 3:** Degradation abilities of the bacterial isolates using FC assay method (T denotes the total number of bacterial isolates - 600; P denotes the number of bacterial isolates able to degrade Phenanthrene - 63; Y denotes the number of bacterial isolates able to degrade Pyrene - 15; F denotes the number of bacterial isolates able to degrade Fluoranthene - 14; PY denotes the number of bacterial isolates able to degrade both Phenanthrene and Pyrene - 9; PF denotes the number of bacterial isolates able to degrade both Phenanthrene and Fluoranthene - 7; FY denotes the number of bacterial isolates able to degrade Fluoranthene and Pyrene - 4; PFY denotes the number of bacterial isolates able to degrade all three compounds - 4).

**Figure 4:** GC-MS spectrum of 1-naphthol formed during Phenanthrene degradation by the bacterial culture.

**4A]** Chromatogram of (i) Control [MSM supplemented with Phenanthrene without bacterial isolate, RT- 20.39 (Phe)]; (ii) NIOSV8 (MSM supplemented with Phenanthrene and bacterial isolate, RT- 20.39 (Phe); 14.02 (1-naphthol)].

**4B]** Fragmentation pattern of 1-naphthol at 14.02 min.

**4C]** Structure of 1-naphthol formed during Phe degradation by potential bacterial strain based on the NIST 11 MS Library.

**Figure 5:** Phylogenetic tree constructed using Maximum Likelihood method and Tamura-Nei model for the identification of primary screened (FC assay) potential PAH degrading bacteria in MEGA X.

Figure 1.

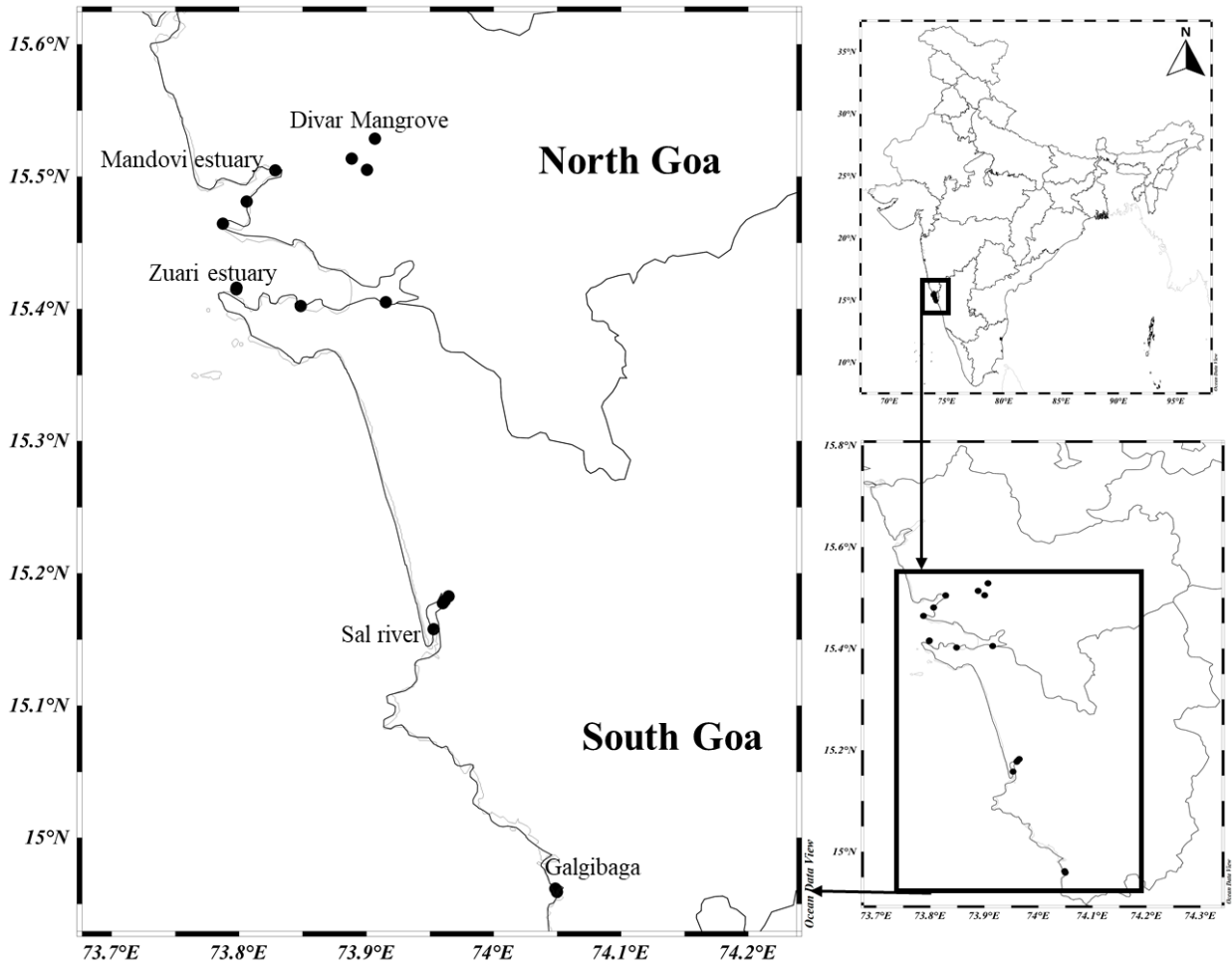


Figure 2.

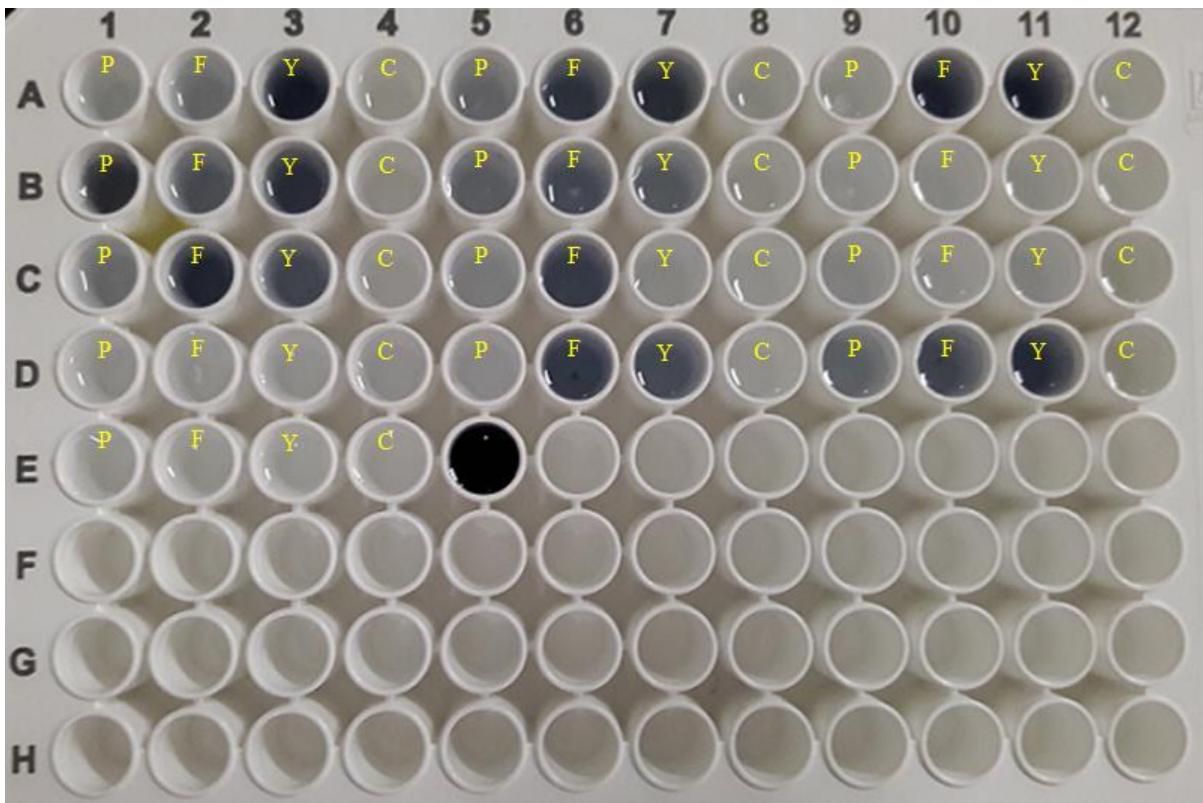


Figure 3.

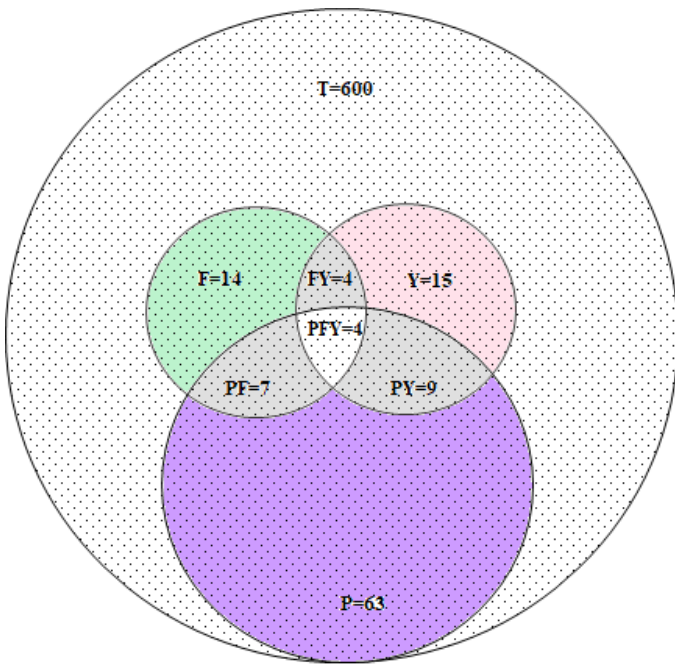
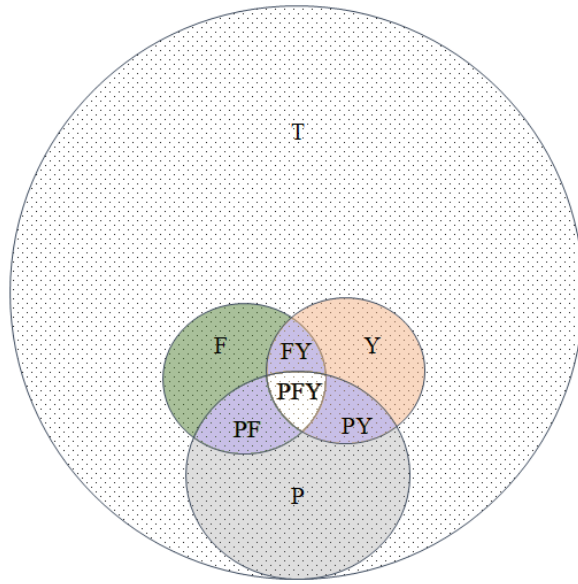
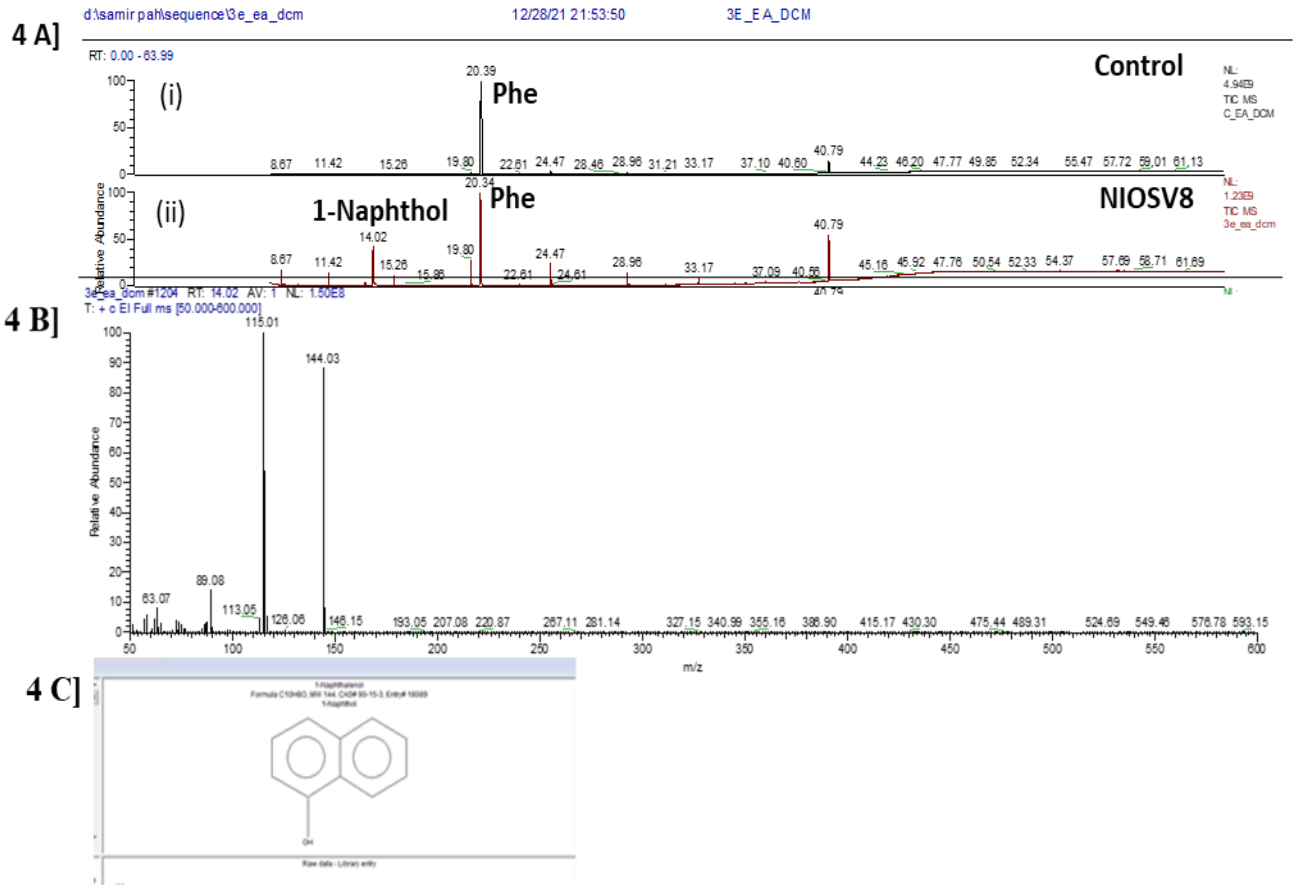
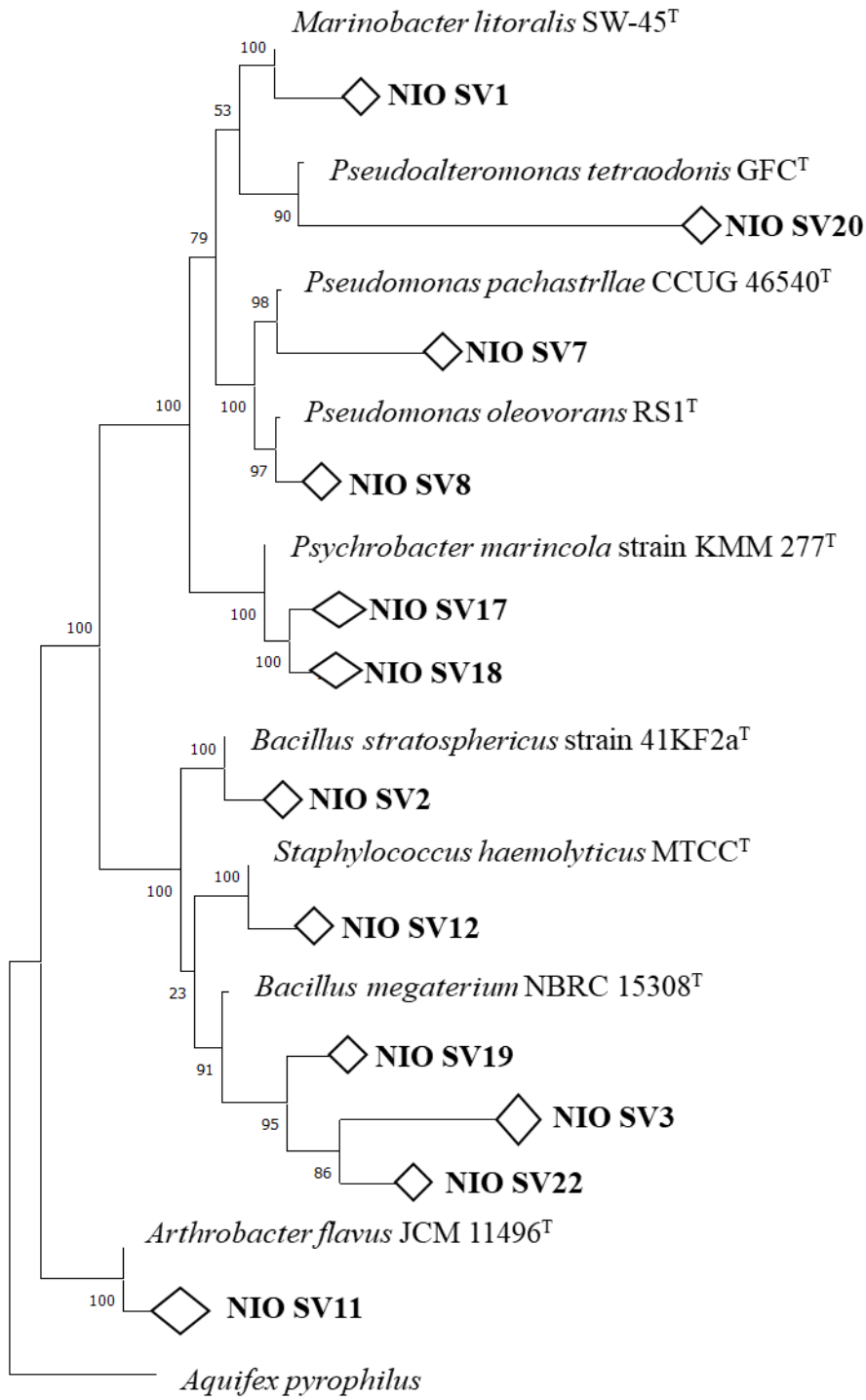


Figure 4.





0.10



**Supplementary Figure.** a) Isolated bacterial colonies on ZM agar plates b) Pure bacterial isolate

