



Research Article

BIOREMEDIATION OF PICOLINE BY ADAPTING *P. AERUGINOSA* ISOLATED FROM INDUSTRIAL EFFLUENT POLLUTED WATER

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ABSTRACT

Environmental pollution implies any alteration in the surroundings but it is restricted in use especially to mean any deterioration in the physical, chemical, and biological quality of the environment. All types of pollution, directly or indirectly, affect human health. Present scenario of pollution calls for immediate attention towards the remediation and detoxification of these hazardous agents in order to have a healthy living environment. The present study will deal with the use of naturally occurring microbes capable of bioremediating the picoline, which is a major environmental pollutant. The present work aimed at identifying the inhabitant bacteria in the highly polluted environments for their property to bioremediate picoline. The present study concludes that naturally developing species of *P. aeruginosa* can consume picoline and effectively eliminate it from its vicinity and this capacity of this organism is encoded by its plasmid.

Keywords: *P. aeruginosa*, picoline, plasmid, bioremediation and environmental pollution.

INTRODUCTION

The waste management technique that involves removal or neutralization of pollutants from a contaminated site using microorganisms is called bioremediation¹.

As day to day production of waste and pollutants being enhanced scientist's interest has grown in the microbial biodegradation procedures to find sustainable ways to clean up contaminated environments². Microbial xenobiotic metabolism is the soul basis behind these bioremediation and biotransformation methods where, this naturally occurring ability of microbes degrade, transform or accumulate a huge range of compounds including hydrocarbons (e.g. oil), polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), heterocyclic compounds (such as pyridine or quinoline), pharmaceutical substances, radionuclides and metals. Major methodological breakthroughs in recent years have deeply explored the microbial key biodegradative pathways and adaptation mechanisms to changing environmental conditions.

Unprecedented insights into genomic, metagenomic, proteomic, bioinformatic and other high-throughput analyses enabled scientists to survey into the details. To promote a sustainable development of our society, elimination of varied types of pollutants and wastes with low environmental impact is an absolute requirement. Microbial catabolic processes are versatile and by this virtue, degradation or conversion of contaminants takes place. Genome-based global studies open a new era in the field of Environmental microbiology, provided unprecedented *in silico* views of metabolic and regulatory networks and clues to the evolution of degradation pathways and to the molecular adaptation strategies to changing environmental conditions. Development of bioremediation technologies and biotransformation processes requires understanding of relative importance of different pathways and regulatory networks to carbon flux in particular environments and for particular compounds. Functional genomic and metagenomic

approaches are increasing our understanding and they will certainly accelerate this technology.

Aromatic compounds that are present in petroleum oil contain toxins and they contribute to both episodic and chronic pollution of the environment causing major ecological perturbations. Since oil spills of coastal regions and the open sea are poorly containable, marine environments are especially vulnerable and mitigation is difficult. It is estimated that both through human activities and natural seepage about 250 million liters of petroleum enter the marine environment every year³.

Picoline is a pyridine derivative and has three different methylpyridine isomers with a common chemical formula C₆H₇N. Picolines are more slowly degraded than their carboxylic acid counterparts and exhibit greater volatility. Volatilization is much extensive in water than in soil, owing to the adsorption of compounds to organic matter and soil clay⁴. Actinobacteria is the major bacterial isolates that mediate picoline degradation. Among the three isomers 3-Methylpyridine is the slowest degrading due to the impact of resonance in the heterocyclic ring. Degradation process of picoline generally excretes ammonium to the environment because picolines have nitrogen in excess to the required amount growth of microorganisms⁵.

The present work aims at identifying the inhabitant bacteria in the highly polluted environments for their property to bioremediate picoline.

MATERIALS & METHODS

Sample collection

Railway new colony is considered very near to the industrial area of Visakhapatnam and has effluent deposition point from various industries like cement, polythene, cosmetic and paint industries. This point is chosen as a potential point for the collection of water

sample. Sample is collected according to the standard WHO guidelines. The collected sample is brought to the lab in 60 min and was transferred to nutrient broth to assist the growth of bacteria.

Preparation and inoculation

The nutrient broth was purchased from HiMedia chemicals, which is in the powdered form. Dissolved 8 g of powder in 1000mL of distilled water and the conical flask was cotton plugged and wrapped with tin foil. This broth was then autoclaved for 15 minutes at 121°C. After the sterilization procedure the broth was taken in 5 different conical flasks each containing 200mL and in these 5 conical flasks 1mL of collected water sample was inoculated under sterile conditions and incubated at 37°C for 24 hrs.

Selective medium

Enzymatic Digest of Gelatin provides the nitrogen, vitamins, and carbon in Cetrimide Agar. Magnesium Chloride and Potassium Sulfate enhance the production of pyocyanin and fluorescein. Cetrimide (cetyltrimethylammonium bromide) is the selective agent. Cetrimide acts as a quaternary ammonium cationic detergent causing nitrogen and phosphorous to be released from bacterial cells other than *Pseudomonas aeruginosa*. Agar is the solidifying agent. Glycerol is supplemented as a source of carbon.

Acclimation to picoline

The procedure of gradual acclimatization to picoline was performed to make the isolated *Pseudomonas aeruginosa* adapted to the higher concentrations of picoline. This was done by first introducing the inoculums to the culture broth containing 1 part picoline and 9 parts glucose. After observing the growth in this broth, this would be further inoculated in to broth containing 2 parts picoline and 8 parts glucose and so on. This procedure would make the bacteria acclimatized, to gradually increasing picoline concentrations.

Plasmid curing

Add 50µl of SDS to 5ml of LB broth. Picoline resistant *P. aeruginosa* culture is inoculated to LB broth having different concentrations of SDS. Kept it for incubation (24 hrs) in a shaker incubator. After incubation, this culture is prepared for the plasmid isolation. Isolated plasmids are run on agarose gel electrophoresis.

Citrate utilization test

Inoculate Simmons Citrate Agar lightly on the slant by touching the tip of a needle to a colony that is 18 to 24 hours old. Incubate at 35°C to 37°C for 18 to 24 hours. Some organisms may require up to 7 days of incubation due to their limited rate of growth on citrate medium. Observe the development of blue color; denoting alkalization.

Catalase production test

Transfer a small amount of bacterial colony to a surface of clean, dry glass slide using a loop or sterile wooden stick. Place a drop of 3% H₂O₂ on to the slide and mix. A positive result is the rapid evolution of oxygen (within 5-10 sec.) as evidenced by bubbling. A negative result is no bubbles or only a few scattered bubbles. Dispose of your slide in the biohazard glass disposal container.

Oxidase production test

Take a filter paper soaked with the substrate tetramethyl-p-phenylenediamine dihydrochloride. Moisten the paper with sterile distilled water. Pick the colony to be tested with wooden or platinum loop and smear in the filter paper. Observe inoculated area

of paper for a color change to deep blue or purple within 10-30 seconds.

RESULTS AND DISCUSSION

The role of bacteria in the biodegradation and detoxification of the toxicants is well documented^{5, 6}. Matsumura et al. in 1976 first reported aerobic degradation of HCH, a persistent pesticide, by a *Pseudomonas* strain⁷. Later on, its degradation by a *Pseudomonas paucimobilis* was reported by Wada et al. in 1989⁸. The role of *Pseudomonas* species in the biodegradation of γ -HCH is also well established⁶. The bacteria involved in the metabolism of 2, 4-D and its other derivatives have been extensively studied by several investigators^{9, 10}. Biodegradations of carbamate pesticides by different bacteria were also demonstrated by several workers^{11, 12, 13}.

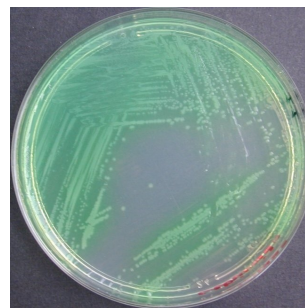


Figure 1: Growth of *P. aeruginosa* on selective medium, which is cetrimide agar medium

When the turbidity was observed in the inoculated broth, inoculum was prepared for the streak plate technique on selective cetrimide agar medium for *P. aeruginosa*. After inoculation the petriplate was incubated at 37°C for 48 hrs. Light green colonies were observed on the petridish shown in figure 1. There were single colonies observed, which were carefully transferred to the sterile nutrient medium for further studies.



Figure 2: Citrate utilization test. 1) Negative. 2) Positive.

Simmons citrate agar was prepared and sterilized. This was used for the preparation of agar slants to conduct citrate utilization test.

Citrate positive: Growth was visible on the slant surface and the medium was an intense Prussian blue. The alkaline carbonates and bicarbonates produced as by-products of citrate catabolism raised the pH of the medium to above 7.6, causing the bromothymol blue to change from the original green color to blue.

Citrate negative: Trace or no growth was visible. No color change occurred; the medium remained the deep forest green color of the uninoculated agar. Only bacteria that can utilize citrate as the sole carbon and energy source will be able to grow on the Simmons

citrate medium, thus a citrate-negative test culture will be virtually indistinguishable from an uninoculated slant

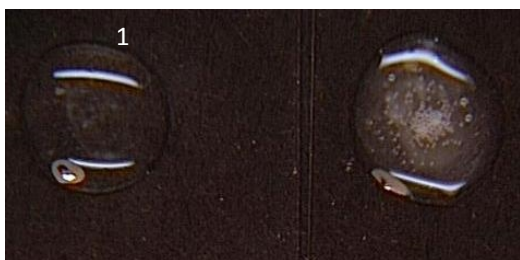


Figure 3: Catalase production test. 1) Negative. 2) Positive.

Catalase mediates the breakdown of hydrogen peroxide H_2O_2 into oxygen and water. To find out if a particular bacterial isolate is able to produce catalase enzyme, small inoculums of bacterial isolate is mixed into hydrogen peroxide solution (3%) and the rapid elaboration of oxygen bubbles occurs. The lack of catalase is evident by a lack of or weak bubble production. When compared to the control 3(1) the test has shown the positive result 3(2).



Figure 4: Oxidase production test. 1) Negative. 2) Positive.

The oxidase test is used to identify bacteria that produce cytochrome c oxidase, an enzyme of the bacterial electron transport chain. When present, the cytochrome c oxidase oxidizes the reagent (tetramethyl-p-phenylenediamine) to (indophenols) purple color end product. When the enzyme is not present, the reagent remains reduced and is colorless.

1. Positive: Development of dark purple color (indophenols) within 10 seconds
2. Negative: Absence of color

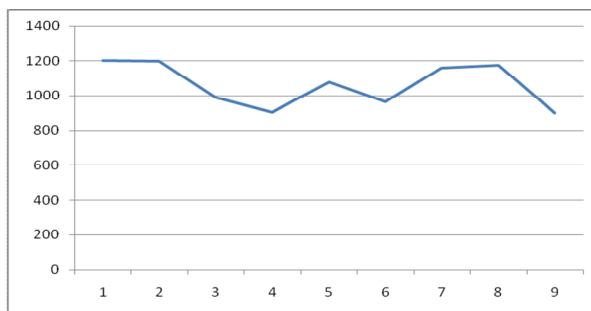


Figure 5: Graph showing the number of bacteria grown at different concentrations of picoline

Acclimation is the procedure that is followed to prepare the bacterial culture adapt to the new environment. The concentration category above represents the concentration enhancement of picoline i.e. as the concentration category increases, picoline concentration increases. The successful adaptation is manifested by the number of bacteria grown in the relevant broth. Figure 5 show that the given bacterial species have developed the function of thriving in the increasing concentrations of picoline, which is otherwise toxic. At highest concentration bacteria failed to thrive, hence number decreased.

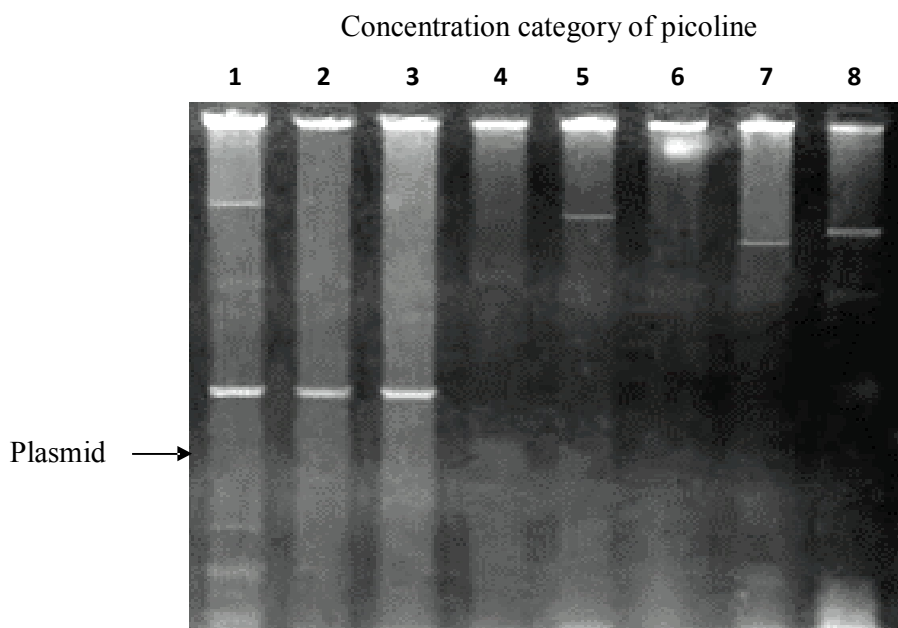


Figure 6: Agarose gel electrophoresis photograph showing bands corresponding to the plasmid isolated from the culture grown at different concentrations of SDS

Plasmid curing is the procedure to remove the plasmid present in the bacterium. *P. aeruginosa* which was grown at highest concentration of picoline, was cultured at different concentrations of SDS in the medium i.e. 0.1 (lane 2), 0.2 (lane 3), 0.3 (lane 4), 0.4 (lane 5), 0.5 (lane 6), 0.6 (lane 7) and 0.7 mg/ml (lane 8). After overnight incubation culture was used to isolate the plasmid and run on agarose gel electrophoresis. As per the figure 8 it is said that at 0.3 mg/ml concentration of SDS plasmid curing occurred, hence no plasmid observed at this point (lane 4). These plasmid cured bacteria was also unable to grow on picoline containing medium, confirming that picoline remediating activity is plasmid born.

CONCLUSION

As the picoline is considered as potential carcinogen, its removal from the environment has become the prime concern. The present study concludes that naturally developing species of *p. aeruginosa* can consume picoline and effectively eliminate it from its vicinity and this capacity of this organism is encode by its plasmid.

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