Detection of Potential Eco-friendly Microbes to Clean-Up the Polluted Water in Sri Lanka

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ABSTRACT

Microbial biodegradation of pollutants has intensified in recent years as mankind strives to find sustainable ways to clean up contaminated environments. These biological processes play a major role in the removal of contaminants in the polluted environment. Utilization of catabolic versatility of naturally occurring microorganisms in biodegrading processes is an essential process to degrade or convert such compounds. Recent developments in molecular microbial ecology offer new tools that facilitate molecular analyses of microbial population at contaminated sites. Both conventional and the molecular methods were used in this study to identify the bacteria from different polluted environments. Bacteria were isolated from the rubber latex contaminated water, municipal waste, petroleum waste and agricultural waste. Then, microbial DNA was isolated and amplified with Pseudomonas aeruginosa specific primers. The amplification of 162 bp specific region of catabolic gene of Pseudomonas aeruginosa confirmed the presence of this organism in the contaminated water collected from different climatic regions in Sri Lanka.

KEYWORDS: *Biodegradation, Polluted water, Pseudomonas aeruginosa*

Introduction

Environmental pollution is increased as a consequence of industrialization in all over the world. Thousands of hazardous waste sites have been generated worldwide resulting from the accumulation of xenobiotics in soil and water over the years. Increment of this pollution caused many hazards for all organisms, even for humans such as carcinogenicity and toxicity. The elimination of wide range of pollutants and waste from the environment is an absolute requirement to promote a sustainable development of our society with low environmental impact.

Bioremediation provides an alternative to chemical treatments.Bioremediation uses naturally occurring microorganisms to degrade various types of wastes. Contaminants are often potential energy sources for microorganisms. Microorganisms survive in contaminated habitat because they are metabolically capable of utilizing its resources (Lovley, 2003). Like all living creatures, microbes need nutrients, carbon, and energy to survive and multiply. Such organisms are capable of breaking down chemicals to obtain food and energy, typically degrading them into harmless substances such as carbon dioxide, water, salts, and other innocuous products.

The aim of this work is to isolate the bacteria from contaminated sites to assess their potential for bioremediation to develop a byproduct useful for bioremediation purposes.Traditional culture dependent methods are insufficient to isolate many microorganisms from the ecological niche (Hugenholtz *et al*., 1998; Martin Laurent *et al*., 2001; Allan *et al*., 2005). Therefore, it is necessary to explore bioremediation agents using modern molecular approach to overcome the limitation factors involved in poor cultivability of microbes (Phoebe *et al*., 2001).

Materials and Methods

Bacteria and Culture

Bacteria were isolated from the rubber latexcontaminated water which was collected from the area of industrial zone. The water sample were streaked in nutrient agar medium and incubated at 37^{o}C for 24 hr. Bacterial isolates were selected based on the colour, shape and the morphology of the colonies. To test for the utilization of rubber latex particles by these bacteria, the selected bacterial isolates were grown in enrichment medium containing latex.

Enrichment for Bacterial Isolates

The enrichment culture was prepared by the addition of rubber latex to the nutrient agar broth medium. Selected bacterial isolates were pre cultivated in nutrient broth for overnight. Nutrient agar broth was centrifuged at high speed for 10 min. Pellet was washed in saline water (3 %), re-suspended in 4 ml saline and was used as the inoculum at 3 % v/v concentration. Nutrient broth medium containing 3% latex was used as the sole source of carbonto be used as diluents. Latex dilutions (1:1000, 1:500, 1:200, and 1:100) were used. In addition inoculum (0.3 ml) from each of these selected isolate was added to the nutrient broth and incubated separately on a rotary shaker at 120 rpm for 12 days. The bacterial isolates which grew in the latex dilution were selected. The selected strains were then grown in 5 ml of 100 % latex for 48 hr in the shaker at 120 rpm.

Bacterial Identification

Microbial properties of the isolates were determined by gram staining and catalase test. Bacteria smear was stained with crystal violet for 2 min followed by gram's iodine for 1 min and thensafranin for 1 min. Catalase activity of the bacterial isolates were tested with 3% H_2O_2. Identification of isolates present in different sources of wastewater was also performed by DNA analysis.

Bacterial Identification by DNA Based Methods

Bacterial cultures isolated from industrial effluent and wastewater collected from contaminated water withmunicipal, petroleum, industrial and agricultural wastefrom two differentclimatic zones, especially the wet zone (Colombo) and the intermediate zone (Kurunagala) were used for the DNA extraction. For each DNA extraction, two different protocols were used. In the first protocol, DNA was extracted from the bacterial culture obtained from the wastewater contaminated with the rubber latex. The second protocol was performed based on the method described for DNA extraction directly from wastewater (Chaudhuri *et al*., 2006).

Genomic DNA Extraction from Bacteria

In the first protocol, the lysis step was performed by adding 10 µl of 1 % SDS to 800 µl of bacterial suspension and a gentle rotation was done for approximately 5 min. Then the bacterial suspension was kept in a water bath at 65ºC for 30 min and left to cool to room temperature.Stirring rod was placed into the lysed bacterial suspension and ice cold 100 % ethanol was added slowly down the stirring rod and rod was rotated for 5 min. Rod with bacterial DNA was immersed in 70 % ethanol for 2 min. Then DNA was dissolved in 300 µl of TE buffer. Equal volume of chloroform was added to the DNA sample and mixed well. Mixture was centrifuged at high speed for 1 min at room temperature. Aqueous phase was transferredinto a fresh tube. This procedure was repeated twice to separate the aqueous phase. DNA was precipitated by addition of 100 % ethanol and centrifugation. Ethanol was removed and pellet was air-dried. DNA was dissolved in 20 µl of TE buffer and was stored at -20ºC**.**

The second protocol was performed based on the method described for DNA extraction directly from wastewater (Chaudhuri *et al*., 2006) (see below). For visualizing the DNA extracts, each extract was electrophoresed on 0.8% agarose gel in 1xTBE buffer, stained with ethidium bromide and examined under ultraviolet (UV) light.

Microbial DNA Extraction from Wastewater

In the second protocol, wastewater obtained from industrial, municipal, petroleum and agricultural waste was enriched with glucose (10 g/L) by keeping overnight in

the shaker and then DNA was directly extracted by the modified direct extraction method.

The wastewater sample was centrifuged to pellet down the cells at high speed for 15 min. This was performed three times to pool the pellet. The pellet was washed twice with wash buffer (50 mMTrisHCl, pH 8.00, 5mM EDTA, pH 8.00) before lysis. The pellet was dissolved in 500 µl lysis buffer (100 mMTrisHCl, pH 8.00, 100 mM EDTA, pH 8.00, 1.5 M NaCl) and centrifuged at high speed for 15 min. Supernatant was separated and 75 µl NaOAC and 500 µl of ice cold isopropanol were added and centrifuged at high speed for 15 min. Pellet was washed with 70 % ethanol. Ethanol was removed and pellet was air-dried until ethanol was evaporated. Then the pellet was resuspended in 25 µl of deionized water. Extracted DNA was electrophoresed on 0.8 % agarose gel containing ethidium bromide and visualized under UV light.

Test Organism

To screen for the presence of the bioremediation agent in contaminated water in Sri Lanka, specific primerswere designed and used to identify the bacteria *Pseudomonas aeruginosa*as it is acommon bacteria having biodegrading ability, present in all types of waste in all part of the world (Ekanayake*et al*., 2010).

PCR Amplification

A region 162 bp from the catabolic gene of dimethylglycinewas amplified using the forward primer and F 5'GAACGTGCTGGTCTACGACA3' and the reverse primerR5'GGGATACATGCTGCGGTAGT3'. Each 20 µl PCR mixture contained 40 ngDNA, 200 µM dNTPs, 0.7 µM each of two opposing primers, 1X PCR buffer, 2.5 mM $MgCl_2$ and 0.8 units of Taq polymerase. The amplification cycle consisted of an initial denaturation step of 5 min at 94^{o}C followed by 30 cycles of 1 min at 94^{o}C, 1 min at 58^{o}C, 1 min at 72^{o}C and the final extension step of 10 min at 72^{o}C was included. Final holding temperature was 4^{o}C. Template DNA was omitted from the reaction mixture for the negative control. Amplified PCR products were electrophoresed on 1 % agarose gel containing ethidium bromide and visualized under ultraviolet light.

Results and Discussion

Isolation and Characterization of Bacteria from Industrial Effluent

Bacteriaisolated from the industrial effluent by enrichment culture with rubber latex were screened for the latex degrading ability. The isolates that could grow on liquid medium with higher concentration of latex (100%) were selected as the most efficient isolates for the degradation of natural rubber latex (data not shown).

Most of these bacterial isolates screened for this study, were white in colour and appeared bluish in colour under the direct light and negative for gram staining and

positive for the catalase test. Most aerobic organism makes catalase. Based on its morphological and biochemical properties, these isolates were identified as gram negative and aerobic. They found to be characteristics of *Pseudomonas aeruginosa*. In order to identify this bioremediation organism in contaminated sites from different climatic zones and from different types of wastewater, the microbial populations in wastewater were screened by DNA based techniques. Therefore, genomic DNA was first isolated and then subjected to PCR amplification using species specific primers.

Extraction of Microbial DNA

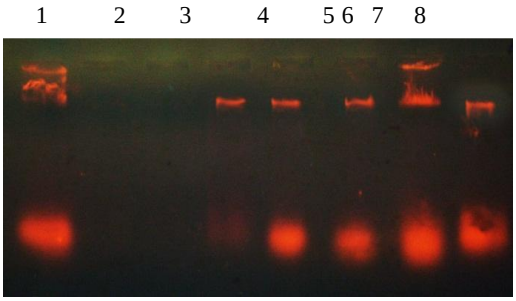

Figure 1: Extraction of Genomic DNA from Bacteria using Protocol 1
Lane 1-8: DNA isolated from different Bacterial colonies

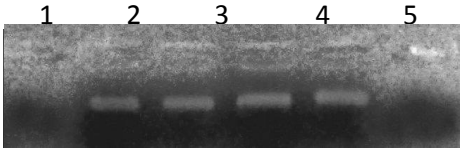

Figure 2: Extraction of Microbial DNA from Wastewater

Lane 1: DNA directly isolated from wastewater. Lane 2 – 5: DNA isolated from optimized wastewater samples (Lane 2: Municipal waste, Lane 3: Petroleum waste, Lane 4: Industrial waste and Lane 5: Agricultural waste). Lane 6: Negative control.

DNA extracted from bacterial culture bythe first protocols provided a good yield of DNA which could be used in PCR amplification (Figure 1).Direct lysis for DNA extraction by optimization with glucose proved to be very efficient in providing large amounts of DNA from environmental samples (Figure 2) while DNA extraction without optimization was not efficient. All extraction products obtained by different protocols could be amplified with the primers specific for *Pseudomonasaeruginosa*. Satisfactory amplified products were obtained indicating that the DNA was good in quality in all the DNA preparations and the DNA samples were free of PCR inhibitors.

PCR Amplification of Catabolic Gene Fragment

PCR amplification was performed to detect the 162 bp region of catabolic gene in *Pseudomonas aeruginosa*using specific primers. The amplified band was observed between 564 bp and 125 bp region of the lambda ladder, confirming the presence of expected PCR product (Figure 3). The absence of band innegative control explained the reliability of the PCR reaction. However further confirmation is recommended. This could be possible by sequencing the amplified product.

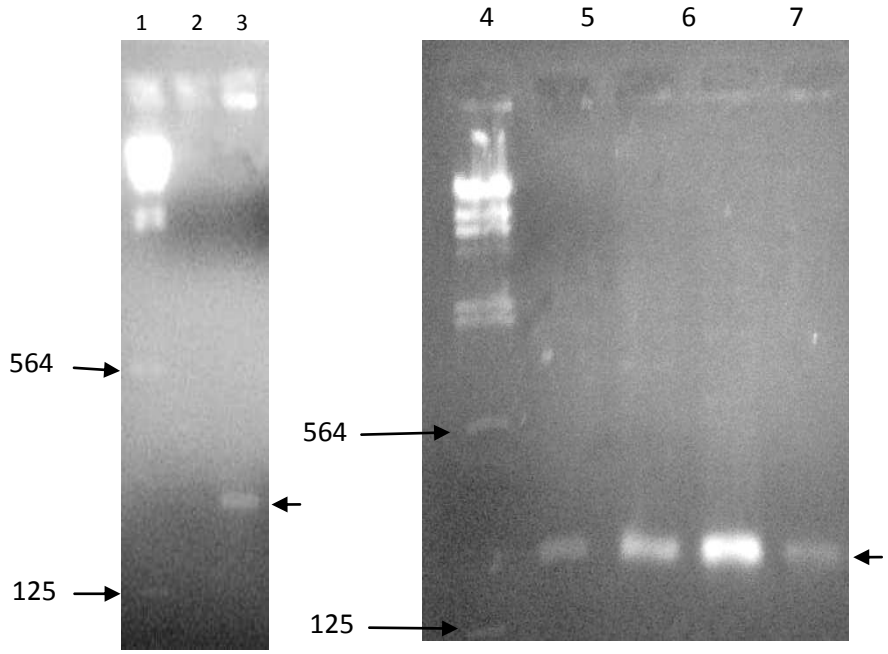

Figure 3: PCR Amplification of Catabolic Gene Fragment from Environmental Samples

Lane 1 & 4: Lambda DNA ladder, Lane 2: Negative control, Lane 3 & 7: Industrial waste sample (Makandura), Lane 5: Municipal waste (Maharagama), Lane 6: Petroleum waste (Maharagama), Lane 8: Agricultural waste(Makandura).

Conclusions

Bacteria isolated from industrial effluent were able to grow and degrade natural rubber latex effectively. These bacterial isolates were identified as gram negative and aerobic. DNA extracted from both bacterial culture and directly from wastewater was good in quality and suitable for PCR amplification. However, DNA extracted directly from wastewater gave high yield of DNA when wastewater was enriched with glucose. The bacterium *Pseudomonas aeruginosa*was identified from various typesof contaminated sites in different parts of the island. Further confirmation is required for the detection of this organism. This study should be extended to identify the potential bioremediation organism from other regions of the country.

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