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Research Article

Artificial Rubber Mineralization by Co-Cultured Bacterial Strains Isolated from Rubber Plantation Area

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Abstract

Synthetic plastics are extensively used in packaging of products like food, pharmaceuticals, cosmetics, detergents and chemicals. Approximately 30% of the plastics are used worldwide for packaging applications. This utilization is still expanding at a high rate of 12% per annum. Hence, the removal of plastic from the environment has become a very important problem. The objective of the present study was Mineralization of artificial rubber by co-cultured Bacterial strains isolated from rubber plantation soil. Co-cultured bacterial strains had the capacity to mineralize plastic and Bioplastics. Mineralisation of the artificial rubber and plastics were confirmed by Spectrophotmetric and Fourier Transform Infra-Red (FTIR) studies. Artificial rubber, plastics and bioplastics degraded by the co-cultures were studied at different concentrations. Mineralization of artificial rubber was maximum (6.48×10^{-5}) on the 20th Day. The co-cultured bacterial strains were identified as *Bacillus cohnii* and *Brevundimonasnae jangsanensis*. Further the Co-cultured bacterial strains were applied for the treatment of plastic and bioplastics which was confirmed by SEM analysis. Hence such isolated co-cultures can be applied in the removal of artificial rubber, plastics and bioplastics present in the contaminated environment.

Keywords: *Bacillus cohnii*; *Brevundimonasnae jangsanensis*; Artificial rubber; Bioplastics; Mineralisation

Introduction

In recent years, the waste disposal problem has spurred mounting interest in the biodegradability of polymers, especially when the public is voicing greater concern about protecting human health and preserving the quality of our environment. Rubber and plastics, for instance, that became an integral part of contemporary life, already formed a significant part of wastes in municipal landfills. Concerns regarding the environmental impact of solid wastes, recycling and composting options are expected to increase as landfill capacity decreases. Managing waste is thus a challenge facing the global community.

Today, plastics are utilized in more applications and they have become essential to our modern economy. The plastics industry has benefited from 50 years of growth with a year on year expansion of 8.7% from 1950 to 2012. In the medical and safety area, plastics are enabling major breakthroughs. The latest medical techniques use plastics to unblock blood vessels, develop artificial corneas or hearing devices to name but a few. Plastics are indispensable for protection equipment such as helmets, firemen suits or bullet proof jackets. Plastics have made it possible for us to push the limits and go further, faster and safer than we have dared to go before [1].

Bioplastics are plastics derived from renewable biomass sources, such as vegetable fats and oils, corn starch, pea starch or microbiota. Bioplastics can be made from agricultural by products and also from used plastic bottles and other containers using microorganisms. Common plastics, such as fossil-fuel plastics (also called petrobased polymers), are derived from petroleum. Production of such plastics tends to require more fossil fuels and to produce more greenhouse gases than the production of biobased polymers (Bioplastics). Some, but not all, bioplastics are designed to biodegrade. Biodegradable bioplastics can break down in either anaerobic or aerobic environments, depending on how they are manufactured. Bioplastics can be composed of starches, cellulose, biopolymers, and a variety of other materials [1].

As these plastics and rubber are not biodegradable, dumping of these causes grave threat to human health and environmental pollution. Thus it is the need of the hour to work on the degradation aspects of these polymers. Synthetic plastics like polyester polyurethane, polyethylene with starch blend, are biodegradable, although most commodity plastics used now are either non-biodegradable or even take decades to degrade. This has raised growing concern about degradable polymers and promoted research activity world wide to either modify current products to promote degradability or to develop new alternatives that are degradable by any or all of the following mechanisms: biodegradation, photodegradation, environmental erosion and thermal degradation [2].

Due to similar material properties to conventional plastics [3,4] the biodegradable plastics (polyesters), namely Polyhydroxyalkanoates (PHA), polylactides, polycaprolactone, aliphatic polyesters, polysaccharides and copolymer or blend of these, and have been developed successfully over the last few years. The most important are poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate). Bioplastics (Biopolymers) obtained from growth of microorganisms or from plants which are genetically-engineered to produce such polymers are likely to replace currently used plastics at least in some of the fields [5].

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Microorganisms such as bacteria and fungi are involved in the degradation of both natural and synthetic plastics [6]. The biodegradation of plastics proceeds actively under different soil conditions according to their properties, because the microorganisms responsible for the degradation differ from each other and they have their own optimal growth conditions in the soil. Polymers especially plastics are potential substrates for heterotrophic microorganisms [7].

Hence, the present study focuses on the mineralization of artificial Rubber and bioplastics by co-cultured bacterial strains isolated from contaminated soil of rubber plantation area. The mineralization of artificial rubber and bioplastic material was evaluated by FTIR studies and Scanning electron microscopic observations. Such isolated Co-cultured bacterial strains shall be applied in the treatment of contaminated soild wastes sites harbouring artificial rubber and synthetic polymer.

Materials and Methods

Bacterial co-cultures and culture preparation

Bacterial co-cultures were isolated from contaminated soil of rubber plantation area was initially adapted and enriched with natural rubber and artificial Rubber (Latex Glove) as the sole carbon source. There were about two bacterial strains, which were enriched and isolated. These bacterial strains were identified by 16s RNA sequencing and the results showed that the bacterial strains belong to *Bacillus cohnii* and *Brevundimonasnae jangsanensis* [8].

Bacterial co-cultured strains was grown 150ml mineral salts medium prepared in conical flasks with the composition: Di-potassium hydrogen phosphate (K_2HPO_4) - 1g/L, Magnesium sulphate ($MgSO_4.7H_2O$) - 0.5g/L, Potassium nitrate (KNO_3) - 1g/L [9].

Cell morphology and the motility of cells in exponentially-growing liquid cultures were examined on freshly-prepared wet mounts by light microscopy. Plate counting (cfu/mL) was done on nutrient agar medium. The Bacterial co-cultures were studied for its growth on artificial rubber gloves, Plastic and bioplastic material as the sole carbon source. For the mineralisation study, mineral medium containing artificial rubber /Plastic/Bioplastic was inoculated with the bacterial co-cultures. Different conditions used for the degradation of phenol were (i) medium + Artificial rubber/ Bioplastic + Bacterial cocultures; (ii) medium + Artificial rubber/ Bioplastic and (iii) medium + bacteria co-cultures , with (ii) and (iii) serving as controls. The bacterial consortium was added to the medium at concentrations of 105 - 106 cfu/mL. The culture, in duplicate, was incubated at 37°C with shaking at 150 rpm and samples were withdrawn at 24 hours interval for 5-days. Then further sub culturing was done at 24 hours interval. The two bacterial strains, which were capable of degrading Natural rubber latex *Bacillus cohnii* and *Brevundimonasnae jangsanensis*, were used for the degradation of artificial rubber, Plastics and Bioplastics.

Mineralization of polymers by the bacterial cocultures

To study the mineralization of artificial rubber (Latex glove) was used as the substrate to study the mineralization. To 150 ml of Mineral Salts Medium 3mm of artificial rubber strips were added and logarithmic phase co-cultured bacterial isolates were inoculated and incubated at 37°C in Orbital shaker at 150 RPM. (Figure 1) shows the experimental set-up of the mineralization study. In the same way 3 mm strips of plastics and bioplastics were added in each of the conical flasks in duplicates and anlaysed for the mineralization. The polymer strips were examined for mineralization by viewing in Binocular light microscope, Dark field microscope and Scanning electron Microscopy for a period of 30 Days. Further mineralisation of the artificial rubber, Plastics and Bioplastics were confirmed by analysing the compounds released during the mineralization by performing FTIR spectroscopy and Scanning electron Microscopy.

Figure 1: Experimental set-up for mineralization of artificial rubber.

Rate of mineralization for the artificial rubber strips

The rate of mineralization was determined by quantitative analysis of $BaCO_3$. The mineral salts medium was sterilized and dispensed in bottles. Artificial rubber strips (3mm) were given as the sole carbon source. Then the co-cultured bacterial strains were inoculated. This was connected to the bottle containing 0.2M Ba $(OH)_2$ by using silicon pipes. The bottles were sealed properly to avoid the escape of carbondioxide as shown in (Figure 1). The set up was incubated at room temperature. Quantitative estimation of $BaCO_3$ was done by titrating it against 1N HCl [10] every 5th day for a period of 30 days.

Schiff's reagent test

Evidence for degradation and mineralization of cis-1,4-polyisoprene rubber hydrocarbon chain was obtained by staining treated artificial rubber strips with Schiff's reagent [11]. In a tightly stopper bottle, 10 ml of fuchsin reagent was added to a sample and kept for incubation for 10-30 minutes at room temperature. After

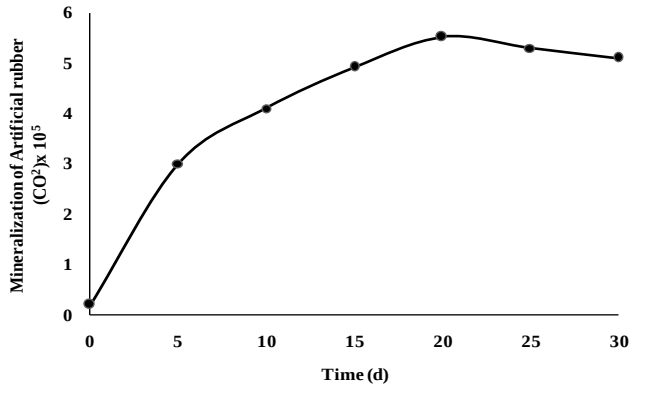

Figure 2: Mineralization of artificial rubber gloves.

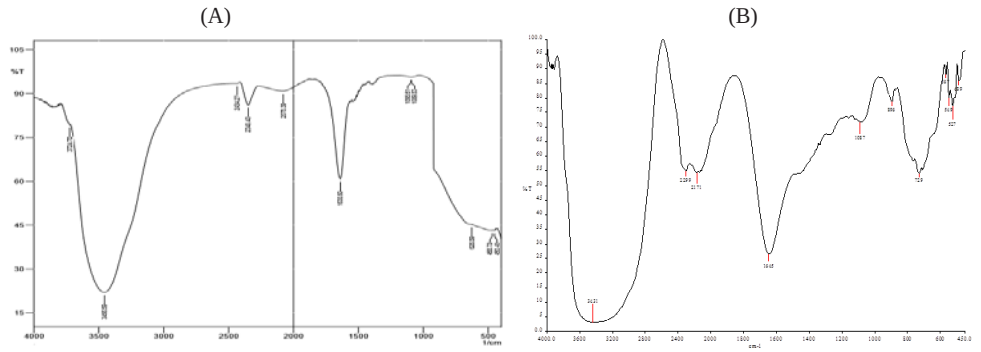

Figure 3: Confirmation of artificial rubber degradation by FTIR.

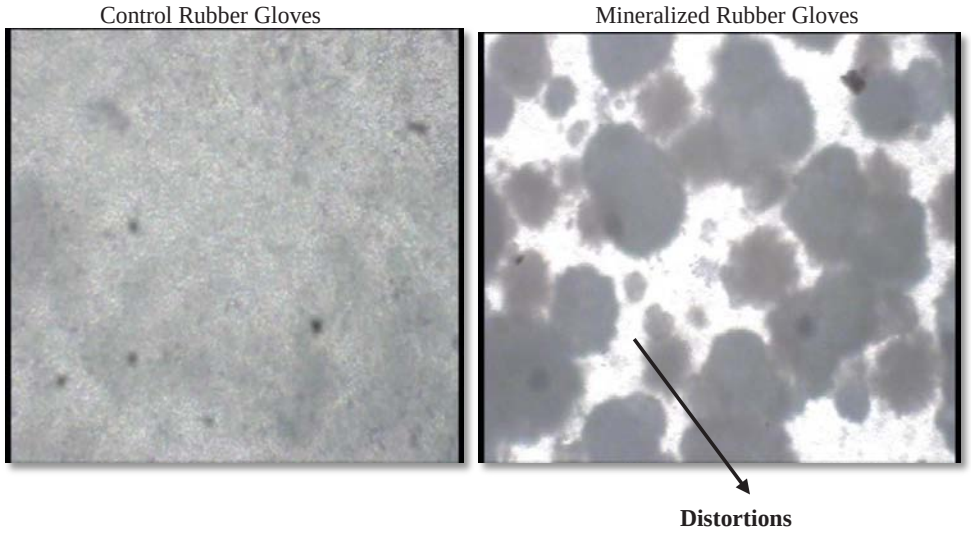

Figure 4: Confirmation of artificial rubber degradation by binocular microscope (100x).

10-30 minutes excess amount of the reagent was discarded and 10ml of the sulfite solution was added in order to suppress nonspecific reactions [12].

Products produced by mineralization of artificial rubber

Chemical changes that arose directly on the artificial rubber surface as a result of the mineralization were determined using FTIR Spectroscopy. It was performed in Perkin Elmer Spectrum from IIT Chennai. The samples were studied in transmittance spectra in IR range 4000 to 400 nm [13]. Further, the mineralizations of the samples were confirmed by analyzing it in Scanning electron Microscopy.

Results

There are many bacteria, which are able to hydrolyze starch; an ability to hydrolyze polymers of rubber and plastics, very few genera has been reported in the literature that could mineralize both [14-17]. Hence, this work was aimed in the isolation of bacterial co-cultures from rubber plantation area, which has the capability to hydrolyse polymer of higher molecular weight, that are naturally occurring like latex, plastics and bioplastics. To study the application of Natural Rubber Mineralization, artificial Rubber (Latex gloves), plastics and bioplastics were used as the carbon source by the isolated co-cultured bacterial strains. The co-cultured bacterial strains used for mineralization study belongs *Bacillus cohnii* and *Brevundimonasnae jangsanensis*, which were isolated from rubber plantation area that could mineralize natural rubber (latex). The co-cultured bacterial strains showed maximum growth on the 3rd Day and mineralization of (3.6 x 10^{-5}) on the 4th Day at the optimum concentration of 10 % of Latex [8].

Colonization of the artificial Rubber (Rubber gloves)

Latex gloves inoculated with the co-cultured bacterial strains were studied in the mineral salts medium for the breakdown of the polymer for the period of 30 Days. Mineralization of the artificial rubber was monitored for every 5 days interval by carbon-dioxide mineralization study. (Figure 2) represents the progression of CO_2 released during the mineralization of synthetic poly (cis-1,4-isoprene). From the figure it shows that mineralization of the artificial rubber by the isolated co-cultures bacterial strains were maximum (6.48 x 10-5) on the 20th Day of incubation. The results were further confirmed by performing Schiff's reagent test, FTIR analysis and Scanning electron Microscopy.

Schiff's reagent test

Rubber sheets, which were inoculated with co-cultured bacterial strains, turned to purple color and there was no color formation in

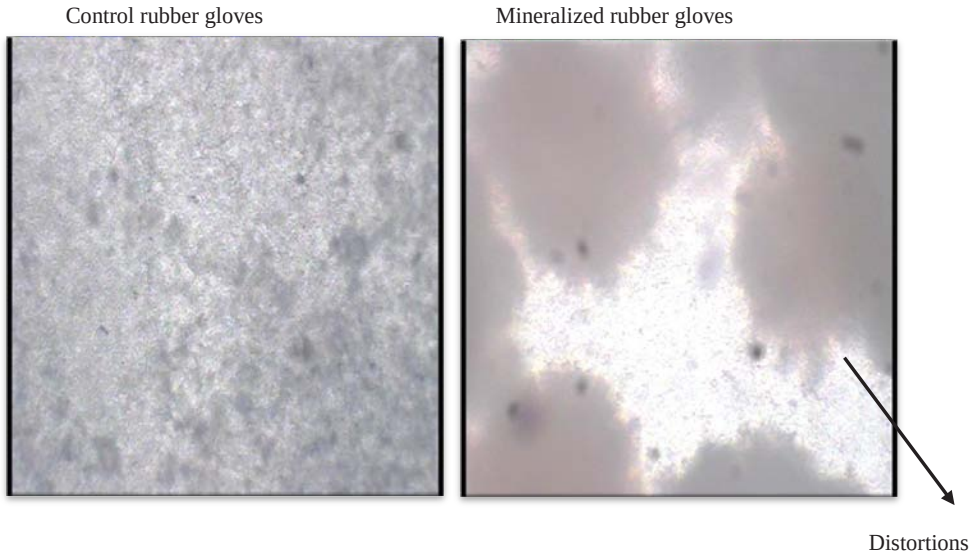

Figure 5: Confirmation of artificial rubber degradation by binocular microscope (400x).

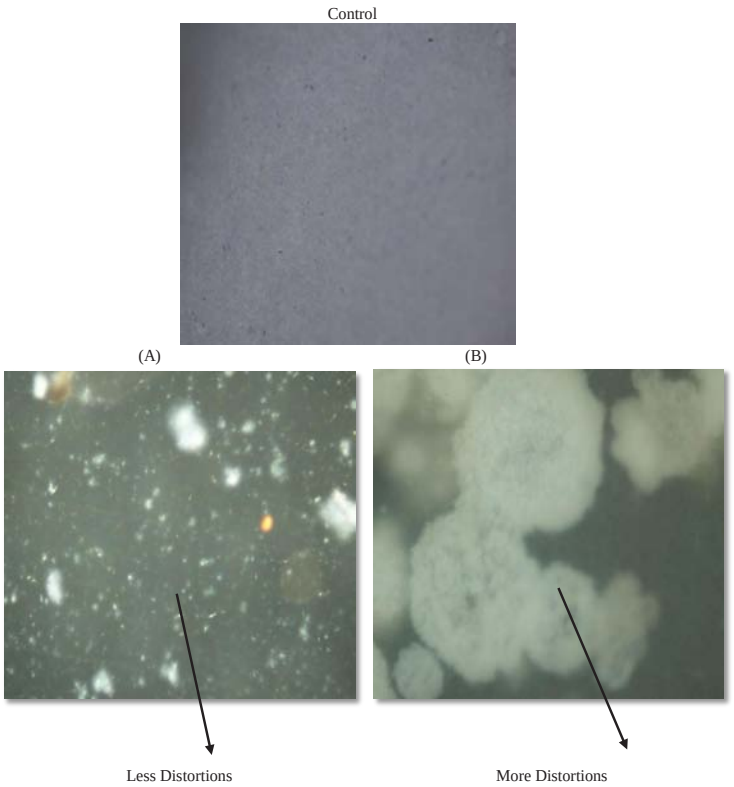

Figure 6: Confirmation of artificial rubber degradation by dark field microscopy. (A) 15 Days - Rubber Gloves (B) 30 Days - Rubber Gloves.

the control. Formation of purple color in the mineralized artificial rubber sample is due to the presence of aldehyde and ketone group, which were produced because of degradation of cis-1, 4-polyisoprene units.

FTIR analysis and microscopic observation

Artificial rubber, which was utilised by the bacterial co-cultures, was studied for their degradation products with FTIR analysis. (Figure 3) shows the FTIR spectrum of the artificial rubber on Day 5 (A) and Day 30 (B). FTIR studies showed the Peaks which were observed for 5^{th} and 30^{th} day at the wave length between 1638.60 cm^{-1} and 1645cm^{-1}, 1087 cm^{-1} respectively having H-C=O. C-H stretch and C=O stretch which indicates the presence of aldehydes and ketones, released as a result of artificial rubber degradation in the mineralised sample. Presence of these aldehyde and ketone group on the 30^{th} day further proved that the artificial rubber was mineralized by the co-cultured

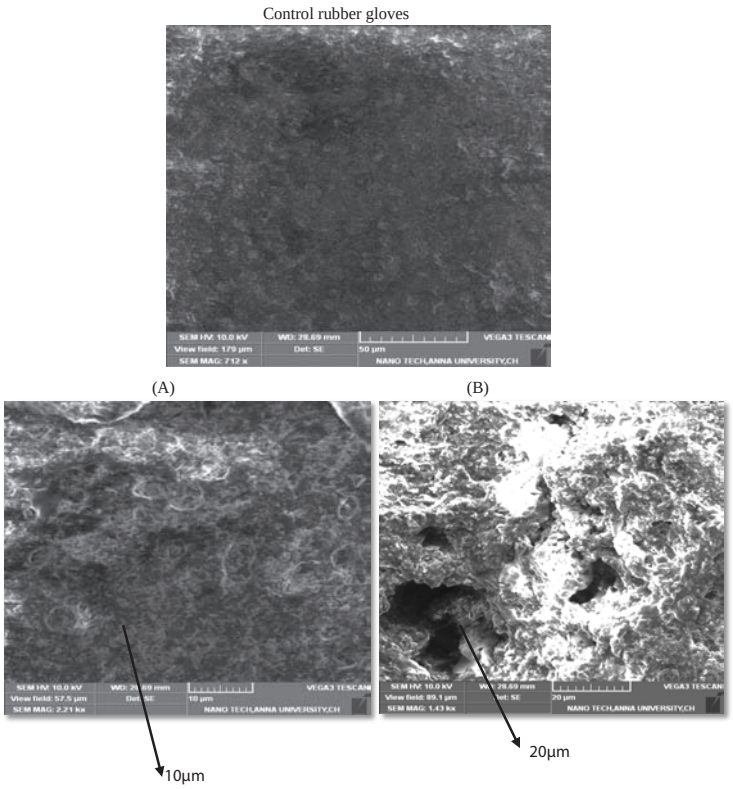

Figure 7: Confirmation of artificial rubber degradation by SEM. (A) 15 Days - Rubber Gloves (B) 30 Days - Rubber Gloves.

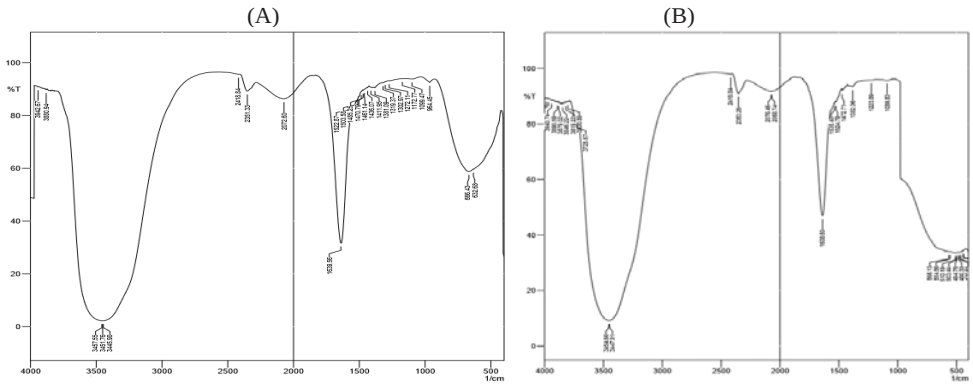

Figure 8: Confirmation of Plastic Degradation by FTIR (A) 5 Days - Plastic Pieces (B) 30 Days - Plastic Pieces.

bacterial strains [20]. Artificial rubber was utilized by the bacterial co-cultures was observed for distortion by observing under binocular microscopy. (Figure 4) shows the distortion of artificial Rubber under 100 times magnification. (Figure 5) shows the distortion of artificial rubber under 400 times magnification. Further Dark filed microscopic observation of the control sample, Day 15 sample (A), Day 30 sample (B) are shown in the (Figure 6). It was observed that 30th Day incubated artificial rubber was observed to have more Distortions than 15th Day Sample that confirms the mineralization of the artificial rubber by the isolated co-cultured bacterial strains.

SEM observation

The surface of the artificial rubber after mineralization with the cocultured bacterial strains was examined by Scanning electron Microscopy without the co-cultures which served as control. (Figure 7) shows the observation of control artificial rubber, Day 9 sample (A) and Day 30 sample (B) of mineralization of the artificial rubber. The uninoculated surface of the polymer was smoother than the surface of the rubber gloves, which was inoculated with the co-cultured bacterial strains. (Figure 7) shows the merging of the bacterial co-cultures along with the polymer and caused the disintegration with large holes appearing. This distortion started occurring only after 2 weeks of incubation. It was observed from the figure that 30th Day sample was observed to have more Distortions and disintegration than 15th Day Sample.

Table 1: Peaks obtained by FTIR and their functional groups confirming plastic degradation.

Day 5	Day 20
3000-3500 cm^{-1} - Amine	3000-3500 cm^{-1} - Amine
1639.56 cm^{-1} – Alkenes	1638.60 cm^{-1} – Alkenes
1400-1600 cm^{-1} – Carboxylic acids	1400-1600 cm^{-1} – Carboxylic acids

Table 2: Peaks obtained by FTIR and their functional groups confirming bioplastic degradation.

Day 5	Day 20
3000-3500 cm^{-1} - Amine	3000-3500 cm^{-1} - Amine
1639.56 cm^{-1} – Alkenes	1638.60 cm^{-1} – Alkenes
1000-1300 cm^{-1} – Esters	1000-1300 cm^{-1} – Esters
500-600- alkyl halides	500-600- alkyl halides

Degradation of plastics and bioplastics by the co-cultured bacterial strains

To study the application of co-cultured Bacterial strains, plastics and bioplastics were used as the substrate for further mineralization experiments. Plastics and Bioplastics, which are higher molecular weight polymer, were studied for the breakdown of the compounds for the duration of 20 Days.

Plastics which was utilised by the bacterial co-culture was studied for degradation products with FTIR analysis. (Figure 8) shows the FTIR spectrum of plastics used in the mineralization study, on Day 5 (A) and Day 30 (B) respectively. FTIR analysis of the plastic mineralized by the cocultures where studied for 5 days interval. The FTIR spectrum analysis of the peaks observed during the mineralization of the plastic strips on the 5th and 20th Day inoculated with the bacterial cocultures in the broth in figured in (Table 1). From the table it could be understood that the polystyrene material was degraded to amines, alkenes and carboxylic acids which confirms the mineralization of the plastic. Bioplastics material (Polyurethrene utilized by the cocultures of the bacteria is represented in (Table 2). Decomposition of urea units by release of ammonia contributes to the degradation of polyurethane. Sequentially the hydrolytic effects of microbial esterases could have broken the ester bonds of the urethane groups (H_2N-CO-OR). Polyurethrene breakdown products were analysed by FTIR and the bioplastic mineralised by the bacterial co-culture caused by the hydrolysis of ester bonds. (Table 2) shows the spectrum peaks obtained on the mineralization of bioplastics on the 5th day and 20th day interval. (Figure 9) shows the peaks obtained during the mineralization of the polyurthrene compound by FTIR analysis.

Discussion

In the present scenario where one side usage of rubber products has increased, the other side dumping of the used products has also increased. These wastes are degrading the environment and causing great threat to the environment. Physical and chemical techniques to solve the problem are causing even more threat instead of solving. Thus, biological techniques of using microbes to degrade these complex proteins are the only way to solve the issue in a healthy way.

Biodegradation is the process governed by different factors that include polymer characteristics, type of organism, and nature of pre-treatment. The polymer characteristics such as its mobility, tacticity, crystallinity, molecular weight, the type of functional groups and substituents present in its structure, and plasticizers or additives added to the polymer all play an important role in its degradation [20,21]. Microbial degradation is mainly carried out by various microorganisms such as bacteria and fungi. Plastics are biodegraded aerobically in wild nature, anaerobically in sediments and landfills and partly aerobically and partly anaerobically in composts and soil. Carbon dioxide and water are the products produced during aerobic biodegradation and carbon dioxide, water and methane are produced during anaerobic biodegradation [3]. Generally, the breakdown of large polymers to carbon dioxide (mineralization) requires several different organisms, with one breaking down the polymer into its constituent monomers, one able to use the monomers and excreting simpler waste compounds as by-products. Many of these polymeric substances are difficult to degrade because of their complex structure [1,9]. In the present study, an attempt was made to use the co-cultured bacterial strains, which were capable of mineralizing artificial rubber and other polymeric materials such as plastics and bioplastics.

Berekkaa *et al.* [17] have also used a technique to stain the artificial rubber strips with Schiff's reagent which indicated the presence of aldehydes in it. Processed artificial rubber was tested for mineralization and a few drops of Schiff's reagent were added to it. Appearance of purple color indicated the breakdown of double bonds of polyisoprene chains to form aldehyde thus confirming the test.

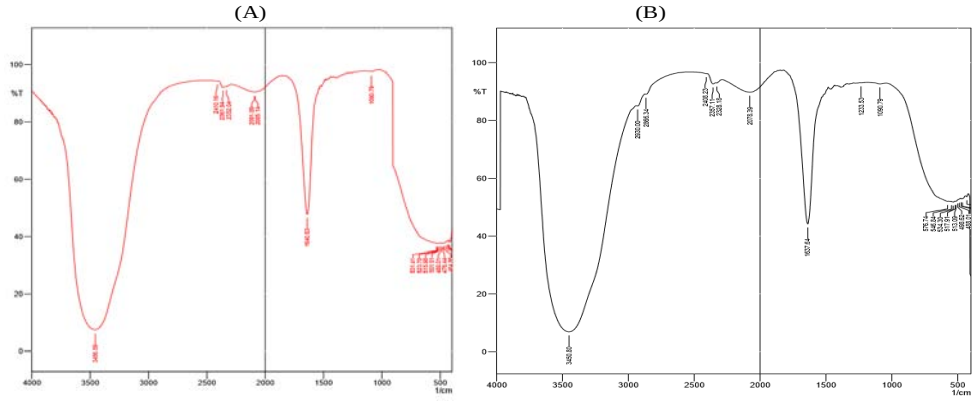

Figure 9: Confirmation of Bioplastic Degradation by FTIR (A) 15 Days - Bioplastic Pieces (B) 30 Days - Bioplastic Pieces.

In the present study the plastic strips used for mineralization changed to purple color which shows the presence of aldehydes with the isolated co-cultured bacterial strains, which proved the mineralization of artificial rubber strips.

In the present study, the co-cultured bacterial strains were used for the mineralization plastics and bioplastics as well. A number of aerobic and anaerobic microorganisms that degrade Polyhydroxyalkonate, particularly bacteria and fungi, have been isolated from various environments. *Aspergillus fumigatus*, Comamonas sp., Pseudomonas lemoignei and Variovorax paradoxus are among those found in soil, while in activated sludge *Alcaligenesfaecalis* and *Pseudomonas* have been isolated. Comamonas testosteroni has been found in seawater, *Ilyobacterdela fieldii* is present in the anaerobic sludge. PHA degradation by *Pseudomonas stutzeri* in lake water has also been observed [1].

Another bacterial strain *Bacillus megaterium* AF3, capable of degrading PHBV, was isolated from the soil and tested for degradation [22]. In the present study two bacterial strains which were isolated from rubber plantation area were identified to mineralize natural rubber (latex) and had the ability to show distortions with plastics and Bioplastics. The bacterial strains were identified by 16sr RNA sequencing as *Bacillus cohnii* and *Brevundimonasnae jangsanensis* [8].

Aamer Ali Shah *et al*. studied on the degradation of polyurthrene compounds by performing FTIR studies. Peaks were observed and were ranging around 2957 cm^{-1} (test) indicating the cleavage of C\H bonds and formation of C = C at the region of 1400-1600 cm^{-1}. It was also observed that the decomposition of urea units by release of ammonia contributes to the degradation of polyurethane. Sequentially the hydrolytic effects of microbial esterases could break the ester bonds of the urethane groups (H_2N-CO-OR) at 1715 cm^{-1}. In the present study FTIR analysis were performed on the degradation of plastics, which showed similar peaks at the region 1638.60 cm^{-1} of Alkenes, proving the degradation of plastics. Thus the current work proved the degradation of plastics and bioplastics by the isolated co-cultured bacterial strains.

Conclusion

Rubber products are widely used in our daily life. These products are made up of natural vulcanized rubber and other chemical additives. Due to vulcanization of the natural rubber these rubber are very resistant to high temperature and persist in environment for very long time. Rubber materials have been increasingly used now days in different area after usage its disposal is a very big solid waste problem. It cannot be easily recycled due to the sulphur cross linking formed during vulcanization. If they are burnt they release enormous amount of carbon-di-oxide and some other gases which cause environmental pollution and contribute to the global warming. Rubber products such as balloon which are disposed in the natural environment are considered to be dangerous to wild animals if they are consumed by animals. One of the alternative ways to solve these problems is to subject these products to biodegradation. In the present study the isolated cocultured bacterial strains were *Bacillus cohnii* and *Brevundimonasnae jangsanensis* could mineralize both artificial rubber, plastics and Bioplastics. Thus these strains can be used as an eco-friendly method for the mineralization of high molecular weight polymers. Future prospects of this study could be application of these co-cultured bacterial strains in the contaminated solid wastes containing rubber and plastic wastes.

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