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DEGRADATION OF POLYISOPRENE RUBBER BY NEWLY ISOLATED *Bacillus* sp. AF-666 FROM SOIL

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Various microorganisms were screened for their ability to degrade polyisoprene rubber (natural rubber latex gloves). Strain AF-666, newly isolated from a soil sample, was selected as the best strain having the ability to grow on polyisoprene containing plates. The strain identified as *Bacillus* sp. AF-666, was found to degrade polyisoprene rubber, both on basal agar plates (latex overlay) as well as in liquid medium. Qualitative analysis of degradation was done through scanning electron microscopy (SEM) and Fourier transform infrared (FTIR) spectroscopy. SEM showed changes in surface morphology, like appearance of pits and cracks, and marked difference in transmittance spectra of test and control due to changes in the functional groups, was detected through FTIR. CO_2 evolution as a result of rubber degradation, was calculated gravimetrically by Sturm Test. About 4.43 g/l of CO_2 was produced in case of test, whereas, 1.57 g/l in case of control. The viable number of cells (CFU/ml) was also higher in test than in control. Present study may provide an opportunity for further studies on the applications of biotechnological processes as a tool for rubber waste management.

Rubbers can be used in thousands of ways; in the fabrics, mining, power generation, agriculture, transportation, paper industries, insulation of cables, wires and various equipment of livestock sheds [1]. Natural rubber (NR) consists of C_5H_8 units (isoprene), each containing one double bond in the *cis* configuration [2, 3] and used in manufacturing of polyisoprene and various copolymers. It is a major component of rubber tyres. Billions of discarded tyres are currently stockpiled around the world and there is continuous increase in its number every year. Those tyre stockpiles are hazardous for both health and environmental [4]. Isoprene and chloroprene are listed in the National Toxicology Program's Report on Carcinogens as potential human carcinogens [5]. Extensive study on different aspects of biodegradation of rubbers is required in order to solve the environmental problems due to rubber waste. It is important to understand the necessary requirements and the mechanisms involved in the biodegradation of natural and synthetic rubbers. For this purpose it is important to understand the interactions between materials and microorganisms and the biochemical changes that occur.

Microbial degradation of polyisoprene rubber has been studied by many scientists. It is a slow process, therefore, over weeks or even months of incubation is required to obtain its degradation products for analysis. *Streptomyces coelicolor* 1A formed clear zones of hydrolysis on NR latex overlay plates in 10–12 weeks of period [6]. The analytical data indicated that an oxidative attack at the double bonds of the polymeric chain is the first step in biodegradation of the cis-1,4-polyisoprene chain. The degradation products were analyzed as aldehyde and/or carbonyl groups, isolated from cultures of various rubber-degrading strains [2]. NR latex gloves and raw NR latex were treated with a *Nocardia* strain and a crude extracellular extract of a *Xanthomonas* strain respectively, resulted in the oligomers with molecular masses between 103 and 104 Da. The cleavage of the polymeric chain by oxygenative attack at the double bonds led to the apprearance of carbonyl groups for each oligomer at both ends, as detected by nuclear magnetic resonance and Infrared spectroscopy. Some new rubber-degrading strains belonging to the *Mycobacterium, Nocardia*, *Corynebacterium* group, such as *Gordonia polyisoprenivorans* strains VH2 and Y2K, *Gordonia westfalica* strain Kb1, and *Mycobacterium fortuitum* strain NF4, *Gordonia polyisoprenivorans* Kd2T (DSM 44302T) were isolated, showing enhanced degradation of NR, NR latex gloves, and synthetic *cis*-1,4-polyisoprene rubber (IR) [7–11]. Another important analytical method that can be used to test the biodegradation of polymers is CO_2 evolution test. Sturm test is used to measure the CO_2 evolved, which gives direct information on the bioconversion of the carbon backbone of the polymer to metabolic end products [12].

In the present study, we reported the isolation of a novel strain of bacteria from soil, showing the ability to degrade polyisoprene rubber. Results obtained from SEM, FTIR and Sturm test are discussed in this article. We have performed tests such as FTIR and Sturm test for the analysis of biodegradation of rubber using this strain which has not been reported previously.

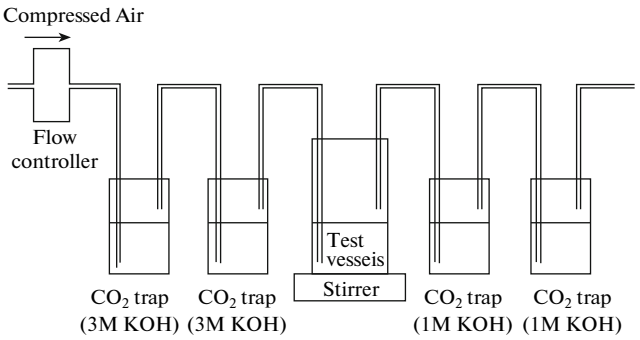

Fig. 1. Schematic diagram of Sturm test.

MATERIALS AND METHODS

Source of rubber. NR latex concentrate (1,4-polyisoprene rubber) was purchased from Sigma-Aldrich (GmbH, Germany). NR latex gloves (polyisoprene rubber) was purchased from BD Chemicals Ltd (UK). All the other chemicals/reagents used were purchased from Merck KGaA, Darmstadt, Germany.

Isolation of rubber degrading microorganisms. Soil samples were collected from solid waste disposal site in Islamabad, Pakistan. To isolate the rubber degrading microorganisms from soil, the NR latex glove pieces were taken in 100 ml of mineral salt medium in 250 ml Erlenmeyer flask and incubated at 37°C at 150 rpm. Mineral medium is composed of (g/l): K_2HPO_4 – 8.0, $(NH_4)_2SO_4$ – 0.5, $MgSO_4$ – 0.2, $CaCO_3$ – 0.5, KH_2PO_4 – 1.0, $MnSO_4$ – 0.0005, FeSO4 · 7H2O – 0.02, Na_2MoO_4 – 0.0005, $CuSO_4$ – 0.0005, NaCl – 0.1, $ZnSO_4$ – 0.0005, pH 7. After a week, 0.5 ml of culture broth along with latex glove pieces was transferred into the flask containing fresh mineral medium. This step was repeated five times. At the end of experiment, the culture was spread on NR latex overlay plates for single colony isolation. Isolate which showed best growth (AF-666) was stored in 200 g/l glycerol at –80°C.

Identification of rubber degrading microorganisms. The isolate was identified through 16S rRNA gene sequencing. For this purpose, the 16S rRNA gene was amplified from DNA using 27F' and 1494R' bacterial primers. The reaction mixtures for PCR consisted of sample DNA 1 μl, 10x PCR buffer 2 μl, deoxynucleoside triphosphate (dNTP) mix 2 μl, forward and reverse primer 2 μl each, Ex taq DNA polymerase (Takara Shuzo, Otsu) 0.5 μl, and distilled water 10.5 μl. At first, the reaction mixture was incubated at 96°C for 4 min. Then 30 cycles were performed as follows: 96°C for 1 min, 50°C for 1 min and 72°C for 1 min 30 s. Reaction was further incubated for 7 min at 72°C. The PCR product was purified with QiaQuick PCR purification kit (Qiagen, Hilden, Germany) and sequencing was performed using Big Dye terminator cycle sequencing kit (Applied BioSystems, Foster City, CA) and sequence was analyzed using ABI Prism 3100 DNA sequencer (PE Applied Biosystems, UK). The sequence was read using DNASIS and FinchTV. The complete sequence was compared with other 16S rRNA gene sequences using the BLASTN program in NCBI GenBank database. The sequence of strain AF-666 was deposited in GenBank under accession number FJ481932.

Analysis of biodegradation. *Plate assay.* In this case the degradation of NR latex concentrate by strain AF-666 was checked through plate assay. For this purpose, NR latex overlay plates were prepared by spreading 20 mg of latex concentrate solubilized in *n*-hexane, and then spread as thin film on mineral agar plates. The solvent was allowed to evaporate. The plates were inoculated with AF-666 and incubated at 37°C.

Growth conditions. NR latex glove pieces (1 g) were added to a 250 ml Erlenmeyer flask containing 100 ml of the mineral salt medium and inoculated with 5% (v/v) inoculum (Strain AF-666 was prepared in tryptic soya broth at 37°C and 150 rpm for 48 h). The flask was incubated at 37°C at 150 rpm for 3 weeks. The experiment was performed in triplicate. The inoculated mineral medium without latex glove pieces was used as control. The growth of the strain AF-666 was checked at the end of incubation, by measuring its OD_{600} through spectrophotometer.

Staining of latex gloves with Schiff's reagent. The NR latex glove pieces were removed from the flask and stained with Schiff's reagent to detect the formation of aldehyde groups on glove pieces, as the product of polyisoprene degradation. Staining was performed as described previously [13].

*Scanning electron microscopy (SEM).*The surface topology of the NR latex glove pieces was analyzed through scanning electron microscopy (LEO 440i, Leica, Bensheim, Germany) after culturing incubating in mineral salt medium with strain AF-666 for 3 weeks, to check for any structural changes. The washed pieces of NR latex glove were mounted on the aluminium stubs by silver coating under vacuum by evaporation in order to make the samples conducting. The images of the test (treated) and control (untreated) samples were compared.

Fourier transform infrared spectroscopy (FTIR). FTIR (FTX 3000 MX Bio Rad Ex-Clibur^{Tm} FTIR Series, USA) analysis was done to detect the degradation of NR latex glove after culturing in mineral salt medium, on the basis of changes in the functional groups. The correlation of absorption bands in the spectrum of an unknown compound with the known absorption frequencies was analyzed. The polymer pieces were mixed with KBr and made into a pellet, which was fixed to the FTIR sample plate. Spectra were taken in triplicate at 400 to 4000 wave-numbers cm^{-1} for each sample.

Biodegradation assay through Sturm test. CO_2 evolution as a result of biodegradation of polyisoprene rubber was determined by Sturm Test (Fig. 1). CO_2

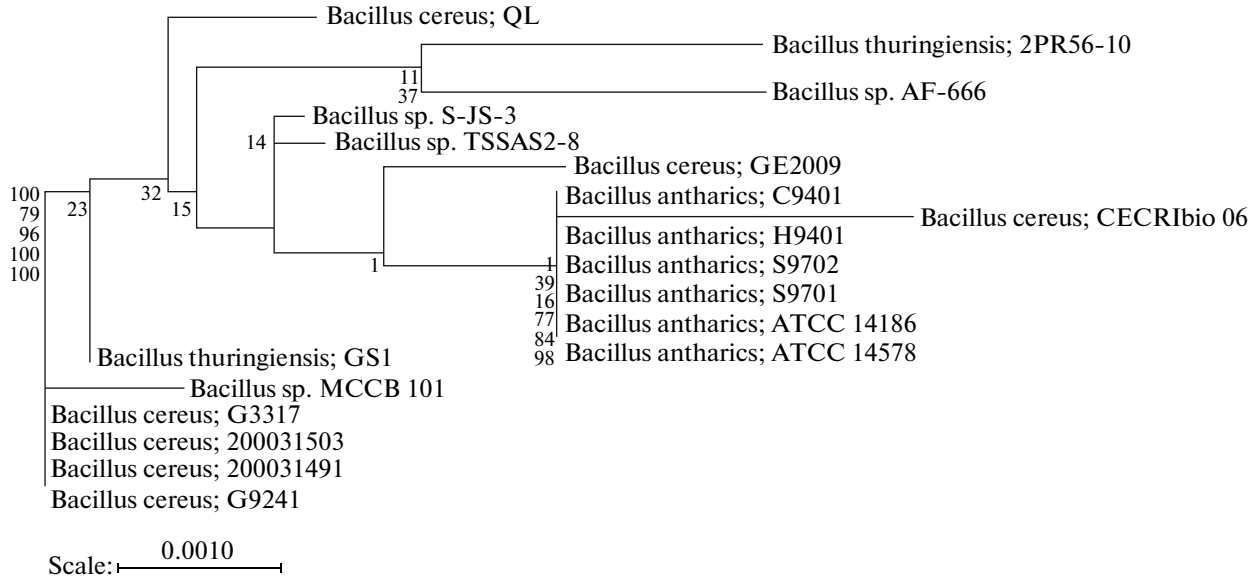

Fig. 2. Phylogenetic positions of strain AF-666 among the neighboring species. The phylogenetic tree was constructed by the method of neighbor joining, based on pair-wise comparisons of 16S rRNA sequences.

evolved as a result of degradation of polymeric chain was trapped in the absorption bottles containing KOH. Barium chloride solution was added to the CO_2 containing KOH bottles and as a result precipitates of barium carbonate (using CO_2 released by breakdown of polymer) were formed. CO_2 produced can be calculated gravimetrically by measuring amount (g/ml) of CO_2 precipitates evolved by addition of $BaCl_2$. Difference in the weights of precipitates both in test and control was observed [12].

RESULTS

Isolation of rubber degrading microorganisms. Ten bacterial strains isolated from soil were found capable of growing on mineral salt agar-NR latex overlay. Among them, AF-666 was chosen as the best strain, since this strain showed the maximum growth on latex agar plates.

Strain AF-666 was Gram-positive, motile and spore forming rod-shaped bacterium. The other physiological properties of strain AF-666 were as follows: casein, starch, lipid, and gelatin hydrolysis positive; urease-positive; nitrate reduction-positive; citrate positive; oxidase-negative; catalase-positive; indole production-negative. The strain could grow on glucose, lactose, fructose, mannose, and sorbitol but not on raffinose. Based on these characteristics, AF-666 was classified as a novel strain, closely resembling previously reported *Bacillus* sp. AF8 [14]. Strain AF-666 differed from *Bacillus* sp AF-8 in respect of oxidase and urease productivity and raffinose assimilation.

Identification of polyisoprene rubber degrading microorganisms. The sequencing result of strain AF-666 showed a total of 1.378 kb nucleotides of 16S rRNA. The resultant sequence was matched with those in the NCBI GeneBank and Ribosomal Database Project (RDP). The phylogenetic analysis of the 16S rRNA sequence showed that the strain AF-666 belongs to genus *Bacillus* having 99% similarities with *Bacillus* sp. TSSAS2-8 (Fig. 2).

Biodegradation studies. *Plate assay.* Clear zones around the strain AF-666 colonies on the opaque mineral agar- latex overlay plates indicated the degradation of latex concentrate. The size of zone increased with the increase in incubation period. Maximum zone of clearance was observed within 25 days of incubation, beyond this period no further increase was observed. The presence of a clear zone around the colony confirmed the presence of extracellular enzymes which degrade the latex concentrate.

Growth estimation. Bacillus sp. AF-666 showed best growth in the presence of NR latex glove pieces, the OD increased from 0.098 at the time of inoculation to 1.436 after 3 weeks of incubation.

Staining of latex gloves with Schiff's reagent. Colonies of AF-666 were visualized on the surfaces of NR latex glove pieces at the end of incubation. When stained with Schiff's reagent, the purple color around the colonies proved that isoprene oligomers containing aldehyde groups were produced and accumulated during the microbial degradation of latex glove.

Scanning electron microscopy. The Fig. 3a indicates the surface of control piece (uninnoculated) of NR latex glove, which is smooth with no pits. The glove pieces exposed to *Bacillus* sp. AF-666 for 2 weeks, showed a rough appearance unlike that of control. There were numerous irregular cracks, surface ero-

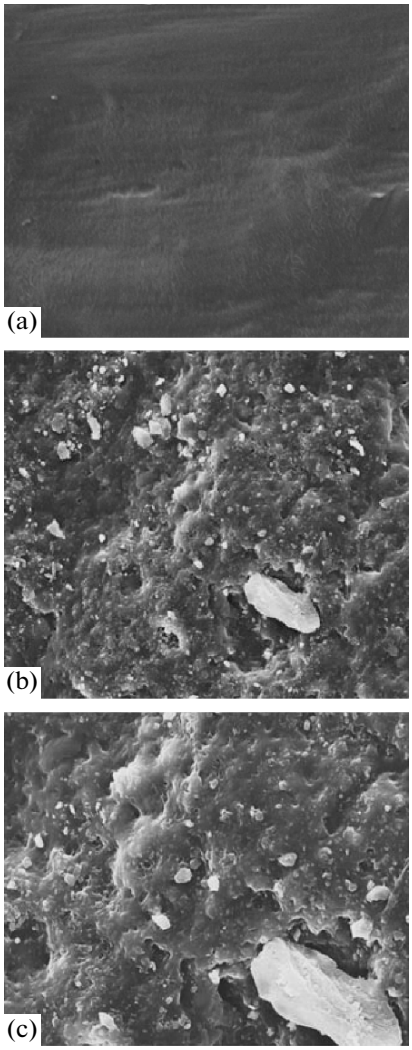

Fig. 3. Scanning electron micrographs of the polyisoprene rubber pieces after incubation in the presence of *Bacillus* sp. AF-666: (a) control (500×); (b) test sample (500×); (c) test sample (1000×).

sions, and pits of variable sizes on the surface of polymer film (Fig. 3b, c).

*Fourier transform infrared spectroscopy***.** FTIR analysis of the microbially treated pieces, showed few changes in the spectra as compared to the control (no exposure to microorganisms). There was decrease in peaks at the region 1200–1400 cm^{-1} in rubber pieces treated with bacterial culture as compared to control, indicating the breakdown of important functional groups like C=C, methyl and ester bonds and the formation of carbonyl functional group as a result of microbial degradation. A peak at 3034 cm^{-1}, present in control, disappeared after microbial treatment in case of test corresponds to CH_2 stretching, the peak at 835 cm^{-1} which corresponds to cis-1, 4 double bond decreased to 834 cm^{-1} after treatment with *Bacillus* sp. AF-666. Changes in the range of 1160 cm^{-1} indicates formation of aldehydes in case of test pieces as compared to control (Fig. 4).

Carbon dioxide evolution test (Sturm test). At the end of the experiment, gravimetric analysis of CO_2, evolved in case of test was higher (4.43 g/l), than control (1.57 g/l). The CFU/ml was also higher in case of test (80×10^7) than control (20×10^7), at the end of incubation.

DISCUSSION

The present research work was conducted to isolate the bacterial strains having the ability to degrade rubber. In the present study, bacterial colonies growing on latex overlay basal medium agar plates were selected for this study. Among all the isolates, *Bacillus* sp. AF-666 showed best growth on basal-latex overlay plates. Kalinenko [15] also used the latex overlay technique, and isolated several actinomycetes and fungi, for example, *Aspergillus oryzae* and *Penicillium* species, claiming that they all were able to consume NR obtained from *Taraxacum kok-saghyz* (rubber dandelion).

In the present study, the ability of *Bacillus* sp. AF-666 to degrade NR latex rubber was studied by Schiff's staining, SEM, FTIR and carbon dioxide evolution. Staining with Schiff's reagent revealed the presence of aldehyde groups. Clear purple color formation provided evidence for the occurrence of aldehyde containing degradation products. The isoprene oligomers, aldehyde and ketone groups formation after incubation of latex gloves with *G. polyisoprenivorans*, were detected by staining with Schiff's reagent, whereas the decrease in the number of double bonds in the polyisoprene chain was measured by FTIR-ATR spectroscopy [16].

Berekaa et al. [17] analysed latex glove material through FTIR after removing the *Gordonia* cells mechanically, as overgrown in the form of biofilm on the sample surface. The spectra revealed signals corresponding to those for cis-1,4-polyisoprene in *Hevea*-NR. These spectra exhibited the changes when compared with non-inoculated controls, such as; decrease in *cis*-1,4 double bonds, the appearance of ketone and aldehyde groups and the formation of two different bonding environments. These observations were finally interpreted as the consequence of an oxidative reduction of the polymer chain, in similar manner to that shown for the *Nocardia* sp. 835A. FTIR spectra showed the formation of aldehydes as well as CH_2-stretching and reduction in *cis*-1,4 double bonds. The degradation of NR latex gloves by *Gordonia* strains was analyzed through FTIR-ATR spectroscopy indicated a decrease in the number of cis-1, 4 double bonds in the polyisoprene chain, the appearance of ketone and aldehyde groups in the samples and the formation of two different bonding environments, which was a consequence from an oxidative reduction of the polymer chain length [13].

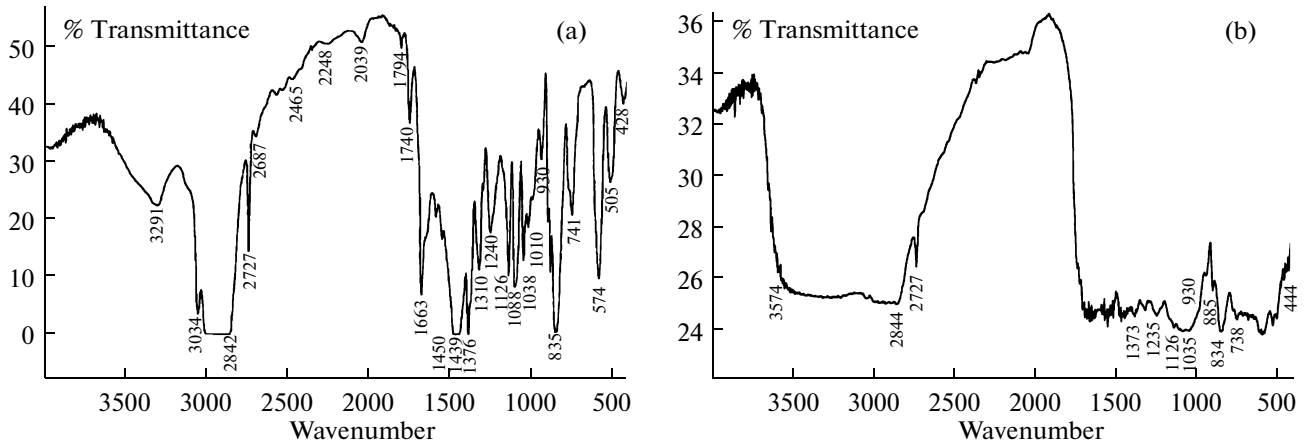

Fig. 4. FTIR spectrum of polyisoprene rubber pieces: (a) control; (b) after bacterial degradation.

In the present study the surface morphology of the polyisoprene rubber showed some changes on the surface, such as pits formation, erosions and roughness, when analyzed through SEM. Deterioration of samples of poly(cis-1,4-isoprene) and poly(trans-1,4-isoprene) by selected strains was verified by SEM after 5 weeks incubation. The surfaces of the Gutta percha (GP) films had almost totally deteriorated; cavities were confluent and separated cavities could hardly be distinguished. Cells of *G. polyisoprenivorans* VH2 caused remarkable deterioration as indicated by the appearance of large cavities in the rubber [18].

CO_2 evolution as a result of rubber biodegradation was determined by Sturm test. The microorganisms breakdown the polymers and the carbon is used for their metabolic activities and result in release of carbon dioxide as end product. The results showed the difference in amount of carbon dioxide produced by the test and the control. More amount of CO_2 was produced in case of test due to the utilization of rubber as a carbon source by microorganisms during their growth. The increased CFU/ml in case of test also proved the ability of microorganisms to utilize rubber and increase in number. Sturm test [19] was commonly employed for evaluation of the biodegradability of polymer materials [20]. Various modifications of this test have been used for the measurement of carbon dioxide evolution during degradation of biodegradable polymers [12], and the aliphatic and aromatic compounds [21]. Keeping in view the findings of these researchers we have employed the similar analytical method to determine the degradation of rubbers and were able to conclude that Sturm test can also be used to check degradation of rubbers.

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The phenomenon of biodegradation of rubber can be manipulated for rubber waste management which constitute major portion of solid wastes. Present study on biodegradation may provide an opportunity for further studies on the applications of biotechnological processes as a tool for rubber waste management. It can be concluded from the present study that *Bacillus* sp. AF-666 have a significant role in degradation of rubber and also that SEM, FTIR and Sturm test are good analytical tools that could provide the evidence of biodegradation. We have isolated a novel strain and performed tests such as FTIR and Sturm test for the analysis of biodegradation of rubber using this strain which has not been reported previously.

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