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Microbial Degradation of Natural Rubber Latex by a novel Species of *Bacillus* sp. SBS[25] isolated from Soil

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ABSTRACT: *Bacillus* sp. SBS25, a gram positive bacteria isolated from soil was found to be effective in the biodegradation of natural rubber latex. The products of latex biodegradation were separated from the ether extract by thin layer chromatography. Gas chromatography and mass spectrometry showed the biodegradation of the major compound into three simpler fractions with low molecular weights. This was further confirmed by Fourier transform infrared spectroscopic analysis. A single band in the control at 2856/cm got split into four subunits such as 2805.45, 2774.12, 2692.40 and 2602.50/cm possibly representing C-H- stretching, the formation of carboxylic acids and hydroxyl stretching respectively. Decrease in the total organic carbon of the medium, accumulation of lipids in the bacterial cell and formation of acids in the extract were also observed during the biodegradation of the natural rubber latex.

Key words: Natural rubber latex, Microbial degradation, *Bacillus* sp. SBS25, cis 1, 4 – polyisoprene, Oxidative cleavage, cellular lipids

INTRODUCTION

Accumulation of rubber waste is becoming a worldwide problem. It is worth trying to develop a microbial process for natural rubber waste disposal. The main constituent of natural rubber is cis 1, 4 – polyisoprene with an average molecular mass about 10^6 Da. It is relatively resistant to microbial decomposition compared with many other natural polymers. However, there were many reports published on the application of microorganisms on the treatment of natural rubber latex centrifugation effluent (Jayachandran *et al.*, 1994). The rate and extent of microbial degradation is influenced by the rubber formulations, the microorganism present and their interaction with the environment. The wastage of valuable rubber and the disposal of waste tires have become two major problems of rubber industry leading to the environmental pollution.

According to previous reports, natural rubber degrading bacteria mostly belongs to the group of Actinomycetes (Heisey and Papadatos, 1995; Jendrossek, 1997; Linos *et al.*, 1999; Rifat and Yosery, 2004; Banh *et al.*, 2005). Recently, certain thermophilic bacteria were also reported to be rubber degrading (Ibrahim *et al.*, 2006). Degradation of natural rubber latex by two gram negative bacteria viz. *Xanthomonas* sps. (Tsuchi and Takeda, 1990) and *Pseudomonas aeruginosa* (Linos *et al.*, 2000) were reported in previous works. But there were no reports on gram positive bacteria other than the Actinomycetes. Biodegradation of natural rubber latex is a rare event and in this study we report a novel species of *Bacillus* as an efficient system for the degradation of natural rubber latex.

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MATERIALS & METHODS

Microorganisms were isolated from the latex contaminated soil, which was collected from the premises of rubber plantations. The enrichment culture was prepared by the addition of 0.5mL latex to 50 mL soil soup at regular intervals. Aliquots of 100mL mineral salt latex medium were

prepared with KNO_3 (0.1g), K_2HPO_4 (0.1g) and $MgSO_4.7H_2O$ (0.05g) in which latex was added as the sole source of carbon (Low *et al.*, 1992). Latex was added at different dilutions of 1:5000, 1:10000, 1:15000 and 1:20000 and was inoculated with the selected organism. Organism was pre-cultivated in nutrient broth overnight. Nutrient broth was centrifuged at 10000 rpm for 20 minutes. Pellets were washed in saline, re-suspended in 4mL saline and was used as the inoculums at 3% v/v concentration. Each of these 100mL aliquots was inoculated with the above inoculums and was incubated on a rotary shaker at 150 rpm for 10 days. Similarly mineral salts latex agar plates were also inoculated with the organism and was incubated at 37^0C for 10 days. The organism that was able to grow in the medium containing maximum concentration of latex was selected for the further studies. Identification of the selected strain was done by carrying out morphological and biochemical analysis of the strain. The organism was identified based on Bergey's Manual of Systematic Bacteriology (Sneath, 1986). De-proteinisation of latex was done by treating latex with equal amount of sodium dodecyl sulphate (SDS) (15mg/5mL). It was centrifuged at 10000rpm for 20 minutes. The lower aqueous layer was discarded and the upper creamy portion was again treated with SDS. The same procedure was repeated twice (Tangboriboonrat *et al.*, 1993).

The amount of CO_2 liberated was determined during the cultivation of cells in the mineral salts latex medium in which latex was the sole source of carbon provided (Berekaa *et al.*, 2000). The released CO_2 was trapped in a solution of 1N NaOH and was quantified by titrating of the remaining NaOH with 0.1 N HCl (Alvarez,2003). *Bacillus* sp. SBS25 was inoculated into the medium containing de-proteinised latex and was kept in shaker at 150rpm at room temperature for 21 days. The medium was centrifuged and the supernatant was collected. The supernatant was used for the degradation analysis and the pellet was used for lipid analysis.

The supernatant of the de-proteinised latex medium after biodegradation was subjected to the analysis of Total organic carbon (TOC-5000A, Shimadzu, Japan). Total organic and inorganic carbon was analyzed at seven-day intervals up to twenty-one days. An un-inoculated medium was also kept along each trial as control.

The supernatant was extracted twice with double amount of diethyl ether. The extract was allowed to evaporate in a water bath at 50^0C. Then it was subjected to Thin Layer Chromatography, Gas chromatography/Mass spectrometry (Quadrupol mass spectrometer, Varian 1200L) and Fourier Transform Infrared Spectrometry (ThermoNicolet, AVATAR 370 DTGS). GC/MS and FTIR analysis were done at STIC, Cochin University, Kerala, India.

Analysis of biodegradation products by TLC was performed on silica gel plates using the following solvent system: glacial acetic acid-chloroform-methanol (2:65:15, v/v) and the spots were detected by spraying conc. H_2SO_4. The portion where spots obtained was scraped out and was dissolved in ether. It was centrifuged at 4000 rpm for 10mins to remove the silica gel. Supernatant was subjected to volumetric analysis to check the presence of acids.

In order to analyze the presence of acids about1mL of the above supernatant was added to 2mL of the saturated solution of sodium bicarbonate solution taken in the test tube and was checked for the liberation of CO_2.

Lipids were extracted from the whole cells with a mixture of methanol: chloroform (2:1, v/v) (Alvarez *et al.*, 1996). TLC was performed using the solvent system: hexane-diethyl ether- acetic acid (80:20:1, v/v/v) (Alvarez, 2003). The spots of lipids were visualized after brief exposure to iodine vapor. Palmitic acid was used as the reference substance. The portion of silica gel containing the spot was scraped out and dissolved in diethyl ether for the qualitative analysis of lipids as follows:

In order to carry out the emulsification about0.5mL of the above supernatant solution was added to 2mL water in a test tube and it was shaken well to check the formation of an emulsion. The above test was repeated with a solution of bile acid instead of water. For the saponification test about 10mL of 10% alcoholic KOH solution was added to 2mL of the above supernatant solution in a test tube. The mixture was boiled for 10-15 minutes or until the saponification was complete.

To the hot solution, conc. HCl was added slowly until the mixture is acidic and the formation of fatty acids was checked.

RESULTS & DISCUSSION

50 strains isolated from the soil by enrichment culture with latex were screened for the latex degrading ability. The strain that could grow on both latex agar medium and liquid medium with higher concentration of latex was selected as the most efficient strain for the degradation of natural rubber. The organism was later identified as *Bacillus* sp. SBS25 based on its morphological and biochemical properties as per Bergey's Manual of Systematic Bacteriology. The identity was confirmed by sending culture for identification at Institute for Microbial Technology, Chandigarh, India.

Natural rubber latex contains proteins as the major impurity. So it was deproteinised (Low *et al.*, 1992; Heisey and Papadatos, 1995). De-proteinised latex was used for the degradation studies. The organism could grow at a pH range of 5 –9 and at a temperature of 15^0C – 42^0C. Analysis of the biodegraded latex medium after the removal of biomass by centrifugation was done by TOC, TLC, GCMS and FTIR analysis.

The CO_2 evolution was estimated by alkalinity method to confirm the growth of the organism. The amount of CO_2 liberated gradually increased and became 35.2mg/l on the fourth week while that of un-inoculated sample remained nil (Fig.1)

Total organic carbon of the water soluble fraction was observed at 7 day intervals for 21 days. There was a gradual decrease in the water soluble organic carbon of the inoculated sample and finally it became nil. A slight decrease of the organic carbon was obtained in the un-inoculated sample (Fig. 2).

In TLC, three bands with Rf value 0.98, 0.74 and 0.36 were obtained in the degraded latex medium while only a single band with Rf value 0.99 was obtained in the control indicating the possible biodegradation of latex into two products. One of the band portion when subjected to volumetric analysis with sodium bicarbonate showed brisk effervescence with liberation of CO_2 indicating the presence of acid in the biodegraded natural rubber latex.

Presence of cellular lipids was observed from the TLC of the cellular extract. It was identified from the brown colored band obtained on the silica gel plate on exposure to iodine vapors. It was further confirmed by the qualitative analysis of lipids such as emulsification and saponification. In the emulsification test, when supernatant was added to water, it formed a temporary emulsion. It was separated and rose to the top, up on standing. When the above test was repeated with a solution of bile acid instead of water, a stable emulsion was formed. In the saponification test, on the addition of conc. HCl to the boiled mixture containing the supernatant and KOH, free fatty acids were

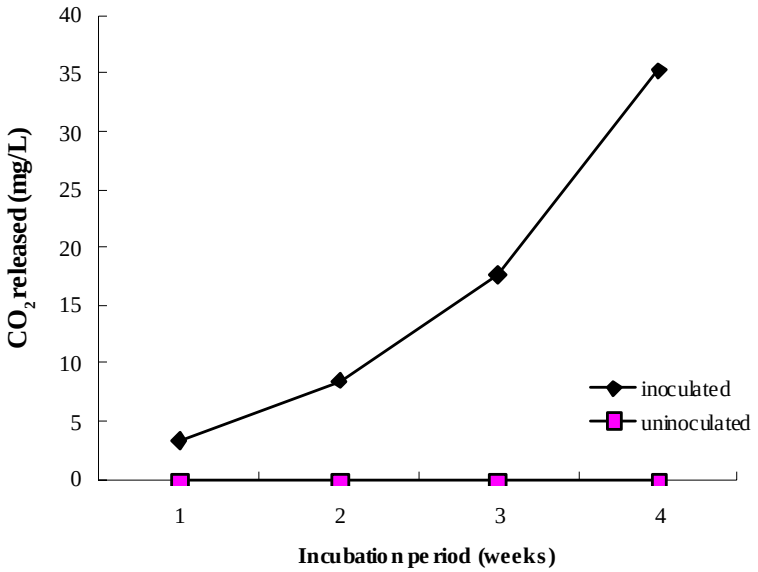

Fig. 1. CO_2 released in the inoculated and uninoculated medium at 7 days interval

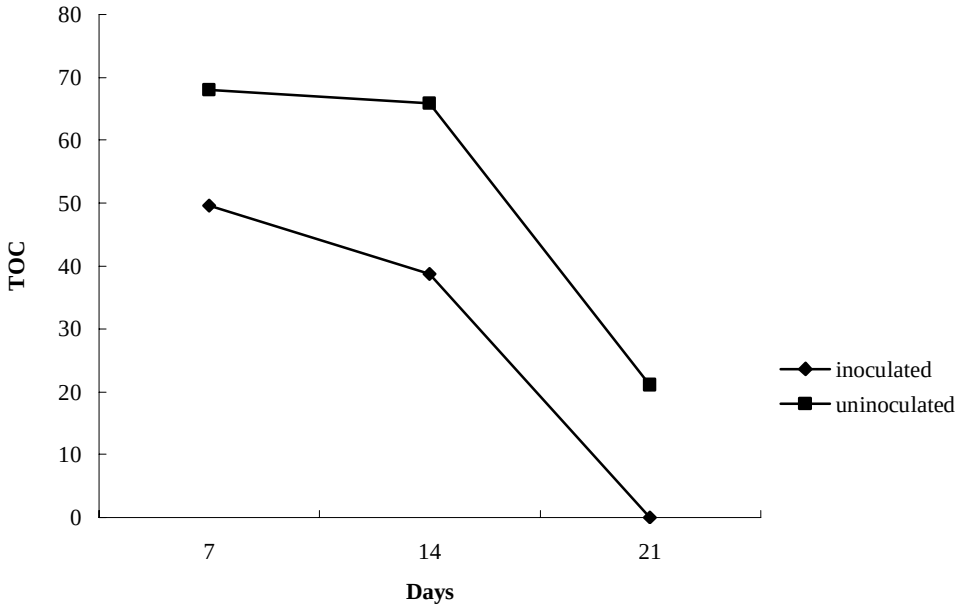

Fig. 2. Total organic carbon of the medium inoculated with *Bacillus* sp. SBS25 (A) and uninoculated control (B) at 7 days intervals

formed and aroused to the top as a clear oily layer. When the solution was allowed to cool, the fatty acids get solidified and formed a cake.

GC/MS of the control showed a peak at retention time of 10.77mins. with a parental molecular ion m/e 221(Table-1). In the biodegraded latex there were peaks at 11.045minutes, 11.346 minutes and 11.808 minutes having molecular ion at m/e 137,154 and 152 respectively. Of these peeks, the peek with m/e 154 was a minor peak. Another peak obtained in GC of control at retention time 16.703 minutes, with the parental molecular ion at m/e 303 was shifted to retention time17.627 with the parental molecular ion at m/e 220 (Table-1).FTIR analysis of the medium after degradation showed significant changes in the structure of the polymer. The broad absorption band at 3388.44/cm showed the polymeric association in control. But it was narrowed and shifted to 3483.74/cm in the treated sample (Table 1).

A reduction in the region at 881/cm, (900-800/cm) in the FTIR of the treated sample might be indicating the reduction in double bond by oxidative cleavage. Broadening of the band at 1637/cm (1680-1600/cm), indicated the formation of aldehyde in lower frequency region (Linos, *2000a*). The splitting of the band at 1462/cm into 1490, 1445 and 1415/cm (1500-1400 /cm) showed the CH_3 deformation vibrations. The band at 2856/cm in untreated sample was shifted to 2862 /cm in the treated sample. There is a sharp decrease at 2933/cm (3000-2800 /cm) which are the region of CH_3 stretching vibrations.

The increase in the CO_2 liberation provided a clear evidence of the degradation and mineralization of the rubber substrate. Similar results were also observed by Berekaa *et al.* (2000). In previous studies, mineralization of the rubber substrate was reported where it was determined by the percentage of CO_2 evolved (Berekaa et al., 2000, Alvarez, 2003 and Ibrahim *et al.*, 2006). In the present work, it was also confirmed by the analysis of TOC of the inoculated medium.

Even though there was a clump forming tendency, both in the inoculated and un-inoculated latex sample, the clump formation was very less in the inoculated medium. It might be due to the increased solubility of latex due to the microbial action. In the control, the soluble latex was slowly undergoing partial coagulation forming clumps thereby resulting in the decrease of the soluble latex. This might be the reason behind the decrease of total organic carbon in the un-inoculated sample. However, in the treated sample, the TOC was decreasing at a much faster rate even in the absence of large clump formation. Finally, the TOC of the treated sample decreased

Table 1. Results obtained from GC and FTIR of the extract of the uninoculated and inoculated mineral salts latex medium

Sample	GC analysis		FTIR analysis	
	No.of peaks	Time (minutes)	Absorption band (cm^{-1})	Differences in the absorption band.
Uninoculated	2	10.77	3388	Broad
		16.703	2856	
			1462	
Inoculated	4	11.045	3483	Narrowed
		11.346	2862	Shifted
		11.808	1637	Broadened
		17.627	1490	New
			1445	New
			1415	New
			881	Reduced

to zero showing the complete mineralization of the soluble latex where the TOC of the treated un-inoculated sample remained at 21.06.

In TLC, there were two more spots in the inoculated sample in comparison with the un-inoculated sample and one of them showed the presence of acids. Presence of acids among the degradation products of poly cis-1, 4-isoprene was reported in earlier works also (Bode *et al.*, 2000; Bode *et al.*, 2001).According to Alvarez *et al.* (2003), the actinomycetes, which could degrade hydrocarbons, were reported to produce cellular lipids from oxidation products. Storage lipids were accumulated in the form of insoluble inclusions in the cellular cytoplasm. So the presence of lipids in the bacterial cells that cultivated in the mineral salts latex medium made it clear that the bacteria was able to consume the hydrocarbon and could deposit it as cellular lipids.

Tsuchii and Takeda, (1990), explained the degradation of rubber as a two step reaction. They found that the crude enzyme isolated from *Xanthomonas* sps. could degrade the isoprene chain mainly into two fractions. In the first step, the original polymer with very high molecular weight was degraded into polymers with medium molecular weights. In the second step, the polymers with medium molecular weight were again degraded to form polymers with low molecular weight. So the wide molecular weight distribution of the degraded fraction in the MS suggested the random scissions of the original polymer in endwise form (Tsuchii and Takeda, 1990).

In the FTIR analysis, the shifting of this band to 3483.74/cm in the treated sample supported the de-polymerization of latex. According to the previous reports by Linos et al., 2000a, there was a splitting of band at 2853/cm into two bands at 2855 and 2841/cm indicating the formation of two different bonding environments. But here, the single band in the control at 2856/cm got split into four subunits such as 2805.45, 2774.12, 2692.40 and 2602.50/cm possibly representing C-H-stretching, the formation of carboxylic acids and hydroxyl stretching respectively.

The mechanism involved in the degradation of natural rubber may be the oxidative cleavage which is very well reflected in the reduction of double bond character and in the presence of aldehydes. The formation of acids was also clear from the results of TLC which was also supported by FTIR data. This also supported the oxidative cleavage of natural rubber latex during biodegradation with the selected *Bacillus* sps.

Previous studies on natural rubber biodegradation with various microorganisms indicated that during rubber degradation, oxidative cleavage of the double bond in the poly cis1, 4 isoprene backbone occurred as the first step (Rose and Steinbuchel, 2005). Presence of aldehydes and ketones were reported among the degradation products in several studies (Linos *et al.*, 2000a, Linos *et al.*, 2000b; Berekaa *et al.*, 2000; Rose *et al.*, 2004). Carboxylic acids were usually formed after the formation of alcohols and aldehydes according to the degradation pathway of n-alkanes (Wyatt, 1984).

CONCLUSION

Bacillus sp. SBS25 was able to degrade natural rubber effectively. It used the hydrocarbon of natural rubber as the sole source of carbon and energy. It was able to produce degradation products with low molecular weights. The results of TLC, TOC, GCMS and FTIR strongly confirmed the degradation of natural rubber by *Bacillus* sp. SBS25.

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