DISCUSSION
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Isolation and purification of bacterial strains

The waste rubber accumulation is a major environmental hazard and hence the management of worn out rubbers is emerging as a significant problem even in the developed countries. In this context, biodegradation is a significant possible remedy as it is more economical and environmental friendly process. India is one of the largest producers of rubber in the world market. In India, the south east region is found to be suitable for the cultivation and processing of rubber of which Kerala constitutes the major land area for the rubber plantations. In Kerala, Kottayam district is considered as the largest producer of rubber.

In the present study, the soil sample was collected for the isolation of the organisms, from Devalokam which comes under Kottayam district. During the harvesting of latex, latex that spill on the soil get accumulated beneath the rubber trees and forms the earth scrap. It constitutes a mixture of latex with soil which may comprise a group of bacteria that occur naturally in the soil. The soil bacteria that found in such scraps may be adapted to live on that substrate and may be able to consume the same to a certain extent. So such a mixture was selected as the source to isolate rubber degrading bacteria.

In previous studies the rubber degrading organisms were isolated from deteriorated rubbers (Tsuchii et al., 1985), Hevea tree bark, cuplump rubber and rubber sheets that buried under soil for more than four months (Low et al., 1992). Organisms were also isolated from tar contaminated soils (Kummer et al., 1999) and tyres (Linos et al., 2000, Roy et al., 2005).
Enrichment media is prepared inorder to make the organism adapted to the specified substrate. During the enrichment culture those organisms that fail to survive in the unpleasant environment disappear from the culture. Enrichment techniques with solid rubber was reported to be successful in the isolation of several rubber degrading bacteria (Heisey and Papadatos, 1995; Linos et al., 1999; Linos et al., 2000). Many of them had high rubber degrading activity but did not necessarily form clearing zones.

Unlike the previous studies, in the present study, latex was given as the sole source of carbon in the MSLM supplied to the organisms. Usually a small amount of glucose (0.5%) was also added as carbon source in the medium to initiate the growth of microorganisms (Low et al., 1992). According to Jendrossek et al. (1997), even though the addition of glucose supported the growth of the organism it was found to repress the synthesis of natural rubber degrading enzymes in most strains. But here, the organism could grow with out any additional carbon source and thus proved that it could be able to consume latex. The growth of the organism in the medium, resulted in the gradual reduction of the latex content in MSLM when compared to the uninoculated control.

In the present study, the several organisms were isolated which included *Pseudomonas, Alcaligenes, Acinetobacter, Kurthia, Arthrobacter, Agromyces* and *Micromonospora*. Of these, *Arthrobacter, Agromyces* and *Micromonospora* belonged to actinomycete group (Table-1).

Actinomycetes are the major group of organism that was reported to possess the rubber degrading ability. Of the several organisms isolated by Jendrossek et al., (1997) and Rifaat and Yosery (2004), those organism which showed the rubber degrading potential belonged to the group of actinomycetes, and the majority of the organisms were identified
as *Streptomyces*. The other organisms included *Micromonospora, Actinoplanes, Gordona, Nocardia, Dactylosporangia, Actinomadura, Amycolatopsis* (Haeisey and Papadatos, 1995) and *Mycobacterium furtuitum* (Linos et al., 2000a; Berekaa et al., 2000). Fungal organisms isolated were *Fusarium* spp. and *Metarrhizium anisopliae* (Low et al., 1992). Chuphal et al., (2005) have isolated rubber degrading *Paecilomyces* sp. and *Pseudomonas syringae* from the sediment core of drainage of the pulp and paper mill industry.

Screening of the organism was not possible by a biochemical assay, as there was no method for the quantitative determination of the reduction in rubber latex. So certain qualitative parameters were selected to screen the organisms as reported in the earlier studies (Spence and van Niel, 1936; Jendrossek et al., 1997; Linos et al., 2000a). Spence and van Niel (1936) developed a sensitive clear zone technique by emulsifying natural rubber latex in mineral agar resulting in an opaque medium. Growth on many rubber degrading organism resulted in visible clear zone formation around the colonies. This method has been accepted in many of the later studies (Jendrossek et al., 1997; Linos et al., 2000a; Rifaat and Yosery, 2004).

In the present study, the isolated organisms were individually cultivated on the latex medium and their efficacy to degrade rubber latex was evaluated. The major criteria used to select the potent organisms were the size of the bacterial colonies and the appearance of clear zones (Jendrossek et al., 1997; Rifaat and Yosery, 2004) along with the ability to reduce the latex content in the MSLM (Low et al., 1992).

Though there was clear zone producing strains among the isolated organism, their ability to reduce the latex content in MSLM was
less compared to that of *B. pantothenticus*. This strain could also form extensive growth on rubber latex agar medium (Table-2).

According to Linos *et al.* (2000a), there were two types of organisms- those which produced clear zones and those which did not form clear zones. Most of the actinomycetes were reported to be capable of producing clear zones (Jendrossek *et al.*, 1997). But in several studies, it was observed that those organisms which did not produce clear zone were the potent degraders of rubber hydrocarbon (Linos *et al.*, 1999; Linos *et al.*, 2000a; Linos *et al.*, 2000b). According to Arenskotter *et al.* (2004), the bacteria that did not form translucent halos were able to grow on the surface of rubber particles in liquid culture and they represent the most potent rubber- degrading bacterial strains.

The isolated organism, *Bacillus pantothenticus*, exhibited an interesting branching growth pattern with which it was able to spread their colonies up to the edge of the agar plates starting from the centre. The bacteria that exhibit tip-splitting (branching) growth were referred as *T* morphotype. Ben- Jacob *et al.*, (1994) have derived *T* morphotype from *Bacillus subtilis* 168 grown on low nutrient substrate. The bacterial colonies adopt various shapes as the growth conditions were varied, from compact at high nutrient levels to fractal and dense-branching patterns at low nutrient levels (Ben- Jacob *et al.*, 1995).

Swarming in *B. subtilis* was reported to require the presence of flagella (Ohgiwari *et al.*, 1992), although flagella independent swarming has also been described recently (Kinsinger *et al.*, 2003). According to the earlier studies, the swarming requires the extraction of water from the agar or reduction of surface friction, processes in which surfactants presumably played a role (Bees *et al.*, 2000). *B. subtilis* was reported to
be able to grow with a branched swarming pattern (Julkowska et al., 2004).

*B. subtilis* (Matsushita, 1997) and *Proteus mirabilis* (Watanabe et al., 2002) were found to be able to swarm on the agar plate and showed various colony patterns on the agar medium depending on the environmental conditions. Komoto *et al.* (2003), had reported the knotted branching pattern of *Bacillus circulans* by swarming on a hard agar medium.

In the present study, *Bacillus pantothenticus* showed normal growth on the nutrient agar without any branching. It showed branching when cultivated on the MSLAM. The medium lacked sufficient nutrients or nutrients were not in the absorbable form (Plate 8).

**Process of biodegradation**

Most heterotrophic bacteria favour a pH near neutrality, where fungi were more tolerant of acidic conditions (Atlas, 1988). The hydrocarbon concentration and pH of the samples were found to have direct effect on the microbial population size and species diversity of the soil samples.

To confirm the degradation, the change in different parameters such as latex content, protein content and pH were observed during the process. The supernatant of the medium after centrifugation showed a reduction in the OD at 238nm (absorption maxima of latex) which can be assumed due to the reduction in the amount of latex. Of the different concentration of the latex tested, maximum reduction of the latex content was observed in the concentration, 1:5000 on the completion of 20 days (Fig.1). This may be due to the failure of microorganism to grow on limited nutrients. When protein content was estimated, there was a slight
increase initially and then showed a gradual decrease (Fig.2). Low et al. (1992) also observed a reduction in the rubber, nitrogen and protein content. The slight increase in the protein content that occurred initially may be due to the increase in bacterial cells due to the growth of the organisms. Reduction in the protein content indicated the degradation of latex. pH was found to be increasing during the process of biodegradation of rubber which was similar to the previous reports by Low et al. (1992) (Fig.3).

When coagulated rubber was treated with Bacillus pantothenticus, there was a weight loss of 5% with in 2 weeks, while there was no apparent weight loss in the uninoculated control (Table-3). This indicated that weight loss in the inoculated flask was due to biological process rather than non biological process such as oxidation. There was a reduction in the DRC by 2%. The weight loss might be due to consumption of latex by bacteria attaching to its surface. As the biomass increased, the cells formed might have been deposited on the rubber surface making it thick. It was difficult to remove the bacteria that was attached to the rubber surface and hence the dry weight was taken for analysis. That may be the reason why the weight of rubber remained constant after two weeks.

A weight reduction of purified natural rubber by 5-10% due to biodegradation in 10 months was reported by Low et al. (1992). Weight reduction > 10% in vulcanized natural rubber and 27% in the unvulcanized natural rubber was observed when 0.5 cm strips in latex medium were subjected to biodegradation (Heisey and Papadatos, 1995). They obtained nearly 100% weight loss of natural rubber when 60mg was treated in 100ml medium after 8 weeks. Tsuchii and Tokiwa (2001) found that the rate of degradation increased with shaking of the
culture medium and they obtained 36% reduction of tyre rubber particles after 8 weeks of treatment.

Initially the biodegradation studies were carried out in the natural rubber latex without affecting any pretreatment. The natural rubber latex was composed of 25-35% rubber hydrocarbon, 1-1.8% protein, 0.4% amino acids and amides, etc. Shaposhnikov et al. (1952) reported that in the case of microorganisms that had grown at the expense of impurities such as proteins, resins and carbohydrates present in the natural rubber, growth did not occur on rubber if the impurities were removed. Hence the mere presence of the organisms on the rubber cannot prove that it was utilizing the rubber hydrocarbon as it can grow on the non rubber contents in the latex. Organic impurities in the rubber could also support the microbial growth (Cundell and Mulcock, 1973) without affecting the biodegradation of the rubber hydrocarbon. The microorganism could also deteriorate rubber as a result of cometabolism using impurities as their carbon and energy sources. (Cundell and Mulcock, 1973). So it was important to check whether the organism could grow on the purified rubber. For that, the latex was subjected to deproteinisation. The deproteinised latex was used in all the further studies.

In the previous studies, latex was purified by centrifugation and resuspension of the cream in water was made to get a 5% rubber latex. (Jendrossek et al., 1997). Low et al., (1992) have purified natural rubber to remove non rubbers by ultra centrifugation and the rubber cream obtained was deproteinised by treating it with 10% sodium dodecyl sulphate. It was also purified by repeated centrifugation and washing with 0.002% Tween 80. Top layer of each centrifugation step was used for the next centrifugation (Jendrossek and Reinhadrt, 2003). Same
procedure was followed in the present study but, instead of Tween 80, SDS was used.

So in all the subsequent studies, the selected organism was inoculated into the mineral salts medium which was prepared with the deproteinised latex.

Natural rubber latex is only partially soluble in water. Natural rubber latex is a polymer of 8,000-30,000 isoprene-units and is usually very difficult to monitor the minute structural changes which may takes place during the microbial action. FTIR analysis sharply focuses on the structure and types of bond in an organic compound and hence can be accepted as a fingerprint of that compound. Any minute change taking place in a compound at structural level will be reflected in the FTIR spectrum. Hence in the present study, FTIR analysis was taken as the criteria for the optimization of the various factors affecting the biodegradation of natural rubber latex.

Many progressive structural changes taking place during the biodegradation of natural rubber latex were very evident in the FTIR spectra taken at different incubation time (Fig.5 to Fig.9). The major progressive change was associated with the band at 3200-3400 cm\(^{-1}\). This range is a characteristic feature of polymeric association. There was a gradual reduction of absorption in this range right from 7 days to 35 days. However from the 3\(^{rd}\) week onwards the band at 3200-3400 cm\(^{-1}\) range was partially nil. This could be accepted as a solid proof for the depolymerisation of natural rubber latex. Another major change was associated with the band at 1581 cm\(^{-1}\). This region was a characteristic feature of C-C multiple bond stretching in dienes.

There was a shift of the band from 1581 cm\(^{-1}\) to 1596 cm\(^{-1}\) during the 3\(^{rd}\) week of treatment. As the minimum time for maximum stable
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A structural deviation could be an ideal feature for the biodegradation, 21 days incubation was selected as the optimum incubation period.

The degradation of polymers may also depend on the amount of carbon substrate available in the medium. In the present study, deproteinised latex was the only carbon source provided in the medium (Fig.10 to Fig.14). Of the different concentration provided, the medium with 1.5% latex showed maximum reduction in the band at 3200-3400 cm\(^{-1}\) range. There was the appearance of two small bands at 1600 and 1577 which were not present in the other latex concentrations.

3200-3400 cm\(^{-1}\) is the characteristic region for polymeric association. The maximum reduction of the band at this region could be taken for most efficient conditions for degradation. The presence of two bands in the 1600 range indicated the presence of C=O under different structural environments. This unique representation was clearly visible in the FTIR spectrum of the sample at 1.5%.

At 0.5% there was slight reduction in the polymeric association. 0.5% concentration of the substrate was comparatively less, as this was the only carbon source provided to the organism which might be insufficient for their growth. At 1%, there was significant reduction in the band at 3200-3400 cm\(^{-1}\). It represented the reduction in the region of polymeric association.

In the concentration 0.5%, there was the band at the region 3200-3400 cm\(^{-1}\) remained prominent as there was no significant change occurred in the polymeric structure. However, the band at 1724 cm\(^{-1}\) was shifted to 1639 cm\(^{-1}\). In the concentration 1%, the band at 3200-3400 cm\(^{-1}\) was reduced and there was a small peak appeared at 1600 cm\(^{-1}\). In the other two concentrations of latex- 2% and 2.5%, there were not much differences from the control sample.
Thus the medium with 1.5 % latex was found to be optimum for the biodegradation by *Bacillus pantothenticus*. It may be difficult for the organism to exist in the high concentration of latex while very low concentration may limit the growth of the organism and thus reduce the degradation rate also.

Biodegradation of the polymer may also depend upon the pH of the medium. The influence of pH on the degradation was analysed by conducting the process at different pH and it was found that it increases as the degradation proceeds. It was similar to the observations of Low *et al.*, (1992). At alkaline pH of 8, there was significant changes in the FTIR spectrum of the sample (Fig.18). At the pH 8, the band at 3200-3400 cm\(^{-1}\) was highly reduced and there was the appearance of two more bands at 1685 cm\(^{-1}\) and 1654 cm\(^{-1}\) along with another at 1600 cm\(^{-1}\), that obtained in the other optimum conditions. In the case of analysis conducted at two other pH, there was only a slight reduction in the band at 3200-3400 cm\(^{-1}\). So pH 8 was found to be the optimum pH.

Temperature of incubation is an important parameter as far as the degradation of any polymer is concerned. Increase or decrease in temperature may itself contribute to the depolymerisation of various compounds (Li *et al.*, 1998). Hence to find out the optimum temperature for the microbial action on latex, separate controls were kept during each trial (Fig.20 to fig.25). At 25\(^{\circ}\)C, not much changes were reflected in the FTIR spectrum. How ever in the FTIR spectrum of the control and treated samples kept at 30\(^{\circ}\)C incubation showed some variations. There was a sharp characteristic decrease in the band corresponding to the range 3200-3400cm\(^{-1}\), which was characteristic feature of polymeric association. However at 35\(^{\circ}\)C, the FTIR of both the control and treated
sample were almost similar. It followed that organism could use the natural latex effectively at ambient condition of temperature 30°C.

Thus the optimum conditions for the degradation of the natural rubber latex was found to be the medium with pH 8 and with 1.5% latex content. The optimum incubation conditions involved a temperature of 30°C and incubation period of 21 days.

The growth of the organism in MSLM were compared with that in nutrient broth. It was found that the growth was slow in the stress condition. The organism could complete all phases of growth in 96 h when it was grown in nutrient broth, where as it took 144 h to complete the growth in MSLM. In MSLM the organism exhibited an extended log phase upto 72 h. This clearly indicated the growth of the organism under limited nutrients.

**Analysis of biodegradation**

In many previous studies, mineralisation of the rubber substrate was reported and was monitored by estimating the percentage of CO₂ evolved. (Berekaa *et al.*, 2000; Alvarez, 2003; Ibrahim *et al.*, 2006). The increase in the CO₂ liberation, provided a clear evidence of the degradation and mineralisation of the rubber substrate (Fig. 28). Dibble and Bartha (1979) reported increase in CO₂ evolution over the range of 1.25-5% hydrocarbon concentration. The methodology to estimate the liberation of CO₂ was explained by Berekaa *et al.* (2000), where the liberated CO₂ was trapped in a solution of Ba(OH)₂. It was later titrated with HCl. CO₂ release was >30% from the untreated raw natural rubber as well as from purified natural rubber (Berekaa *et al.*, 2000) while it was 35.2mg/l in the present study. CO₂ was assumed to be formed in the final stage of the degradation process and was indicative of the complete
mineralisation. In the present work, the biodegradation was also confirmed by the analysis of TOC of the inoculated medium (Fig. 29).

Even though there was a clump forming tendency, both in the inoculated and uninoculated 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 uninoculated 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 almost to zero showing the complete mineralisation of the soluble latex where the TOC of the uninoculated sample remained at 21.06.

In TLC, there was two more spots in the inoculated sample in comparison with the uninoculated sample. It indicated the formation of two compounds as the degradation products and one of them showed the presence of acids (Table-4). 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).

Formation of phospholipids in the bacterial cells when they grow at the sole expense of long chain hydrocarbons was reported by Makula and Finnerty (1970). Phospholipids of Micrococcus cerificans when they were grown at the expense of long chain alkanes were studied by Makula and Finnerty (1970) and found that alkane grown cells contained almost 50% more phospholipids than acetate grown cells.

According to Alvarez (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.

In the GCMS analysis, the appearance of two smaller peaks instead of a large peak that formed in the control (Fig.30, Fig.37), indicated the conversion of a high molecular weight compound into two low molecular weight compounds which supports the results of TLC.

In the GC analysis of the control there was a peak at 9.512 min. In the treated sample there was a corresponding peak at 9.516 min. However the mass spectroscopy showed that the mass distribution in both the cases were different. This indicated that these two components were not same but different. It followed that the biodegradation had resulted in the shifting of the peak from 9.512 to 9.516 min as a result of the slight structural modification (Fig. 31 and Fig. 38).

In the GC of the treated sample there was three new peaks nearer to 11 min. i.e. at 11.045 min, 11.346 min and 11.808 min. These three peaks on mass spectroscopic analysis gave entirely different molecular ions(Fig. 39-41). This indicated the formation of three different components with structural similarity as a result of biodegradation on natural rubber latex. Similarly, the peaks in the control at 10.77 min (Fig. 32), 14.055 (Fig. 33), 14.345 (Fig. 34), 16.703 min (Fig. 35) and at 21.481 min. (Fig. 36) were totally absent in the treated sample.

GC analysis of the treated sample also gave many new peaks at 14.949 min, 15.132 min and also at 17.627 min. The mass distribution of all these peaks were different (Fig. 43-45) and were unique to the biodegraded sample.
FTIR analysis showed the structural variations that occurred in the polymer during the process of biodegradation (Fig. 45). A reduction in the region at 881 cm\(^{-1}\), (900-800 cm\(^{-1}\)) 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\(^{-1}\) (1680-1600 cm\(^{-1}\)), indicated the formation of aldehyde in lower frequency region (Linos et al., 2000\(^a\)). The splitting of the band at 1462 cm\(^{-1}\) into 1490, 1445 and 1415 cm\(^{-1}\) (1500-1400 cm\(^{-1}\)) showed the CH\(_3\) deformation vibrations. The band at 2856 cm\(^{-1}\) in untreated sample was shifted to 2862 cm\(^{-1}\) in the treated sample. There was a sharp decrease at 2933 cm\(^{-1}\) (3000-2800 cm\(^{-1}\)) which was the region of CH\(_3\) stretching vibrations.

According to Low et al., 1992 though the degradation of natural rubber was observed, there was not any significant chemical modification occurred in the polymer during the treatment with fungi and actinomycetes. But Linos et al. (2000\(^a\)) found that, there was decrease in cis 1,4 double bonds, the formation of carbonyl groups and the change of the overall chemical environment. In the present study, the FTIR analysis indicated the oxidative attack at the double bond which might be the first metabolic step of the rubber latex biodegradation process.

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 weight. 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) (Fig. 38-42).

In the FTIR analysis, the shifting of the band to 3483.74 cm\(^{-1}\) in the treated sample supported the depolymerisation of latex. According to the previous reports by Linos et al. (2000\(^a\)), there was a splitting of band at 2853 cm\(^{-1}\) into two bands at 2855 and 2841 cm\(^{-1}\) indicating the formation of two different bonding environments. But here, the single band in the control at 2856 cm\(^{-1}\) got split into four subunits such as 2805.45, 2774.12, 2692.40 and 2602.50 cm\(^{-1}\) 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. Formation of aldehydes during the degradation process were stated in several reports where it was identified by staining with schiff's reagent (Linos et al., 2000\(^b\); Ibrahim et al., 2006). The formation of acids was also clear from the results of TLC which was also supported by FTIR data. All these evidences supported the oxidative cleavage of natural rubber latex during biodegradation with the selected organism.

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., 2000\(^a\), Linos et al., 2000\(^b\); Berekaa et al., 2000; Rose et al., 2005). Carboxylic acids, were usually formed after the
formation of alcohols and aldehydes according to the degradation pathway of n-alkanes (Wyatt, 1984).

Scanning electron microscopy which showed the colonization of bacteria on the rubber surface gave a clear evidence for the adhesive growth behavior of the strain. Major alteration and deterioration of the rubber surface due to the bacterial growth was evident on the surface compared to the control (Plate 10-12) Roughening of the rubber surface, development of a granular appearance on the fractured edges and increased porosity due to the enzymatic digestion were reported to be the characters of the detrioration (Heisey and Papadatos, 1995). These features could be clearly observed in the present study also. Colonization behavior and biofilm formation of *P. auruginosa* AL98 on NR latex glove material was investigated by SEM. (Linos *et al.*, 2000b). Microbial colonies on the rubber surface was also observed with the help of SEM by (Roy *et al.*, 2006), after the treatment with organism for 45 days.

*Bacillus pantothenticus* 8063 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, FTIR and SEM strongly confirmed the degradation of natural rubber by *Bacillus pantothenticus*.

**Enzymes in rubber latex degradation**

Since natural rubber was a high molecular mass compound and was too large to be taken up by the bacteria, the polymer has to be cleaved extracellularly as the first step (Jendrossek *et al.*,1997). So the bacteria capable of producing extracellular enzyme will be more suitable for cleaving the hydrocarbon chain. Natural rubber degrading actinomycetes apparently have other highly substrate specific
polyisoprenoid oxygenases that are responsible for the first oxidation step of natural rubber (Jendrossek et al., 1997).

Even though there were several reports on the rubber degrading enzymes, there were no reports on the enzyme responsible for the degradation process till the isolation of the enzyme RoxA by Braaz et al., (2004) recently.

Tsuchii and Takeda (1990), isolated a rubber degrading Xanthomonas strain which was able to produce an extracellular enzyme. They observed the rubber degrading activity of the crude enzyme isolated from Xanthomonas. Two fractions, one with high molecular weight and the other with low molecular weight were obtained as degradation products. The latter composed mainly of 1 component which was identified as 12-oxo-4,8-dimethyl trideca-4,8-diene 1-al (acetonyl diprenyl acetoaldehyde, $[\text{ALP}_2\text{A}_1]$).

Later, a gene of Xanthomonas sp. whose gene product could be involved in the rubber degradation was cloned (Jendrossek and Reinhardt, 2003). Ros et al. (2004) found lcp (latex clearing protein) in Streptomyces sp. K30 to be essential for rubber degradation.

Recently, Xanthomonas strain was found to secrete a protein having an apparent molecular mass of 65 KDa during the growth on latex (Braaz et al., 2004) which was referred to as RoxA. RoxA of Xanthomonas sp. strain 35Y did not exhibit any homology with lcp of Streptomyces sp. strain K30. The purified RoxA protein contained about 2 mol heme per mol RoxA protein and had strong absorption at 406 nm (Braaz et al., 2004). This is a characteristic of heme-containing proteins. Incubation of the purified RoxA protein with latex and oligo(cis-1,4-isoprene) resulted in accumulation of the same product (12-oxo-4,8-dimethyltrideca-4,8-diene-1-al), that obtained by Tsuchii and Takeda
(1995), as a major degradation product with a molecular weight of 236-Da (Braaz et al., 2004, Braaz et al., 2005).

In the present study also, an extracellular rubber degrading enzyme was isolated from *Bacillus pantothenicus* 8063 which when supplied alone without the organism showed degradation of latex in the medium. The enzyme preparation also showed absorption at 406nm, the characteristic absorption maxima of heme containing RoxA. The partially purified enzyme was subjected to carbonyl content and RoxA assay (Braaz et al., 2004). The conditions for the enzyme reaction was also optimized and found that the enzyme activity was high when 0.2% substrate (concentration) was given at a pH of 7 at 50°C and at an incubation time of 16 hours (Fig 45 to 49).

Natural rubber latex is a polymer of hydrocarbon and hence solubility is very less. This has been identified as a major bottle neck in the biodegradation studies of natural rubber. There were many attempts to increase the solubility of natural rubber latex using many types of detergents. But in most of the cases, detergents could be inhibitory to the enzyme action. This might be denaturing the enzyme resulting in the complete inhibition of the enzyme activity.

Effect of surfactant treated latex on the enzyme reaction were also observed by providing different kinds of surfactants such as Triton X100, biosurfactant and SDS (Fig.50 and Fig.51). They were provided in various concentrations such as 0.5%, 1%, 1.5%, 2% and 2.5%. In the case of triton X100, all concentrations provided were found to have inhibitory effect on the enzyme reaction which was similar to the previous reports (Braaz et al., 2004). SDS which was also reported to be inhibiting, when supplied to the reaction mixture, was found to inhibit the enzyme reaction. This was due to the denaturing of enzymes with SDS. So an
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An attempt was made to remove the SDS from the reaction mixture before adding the enzyme. Being an anionic surfactant, its removal was possible through dialysis. When the SDS was removed from the reaction mixture through dialysis, before the addition of enzyme, there was an increase in the enzyme activity up to the SDS concentration of 2%.

There was 100% enzyme inhibition in both triton X100 and SDS treated samples. However, when the SDS was dialysed out after solubilising the latex, the enzyme showed better activity. SDS might have resulted in the increased solubility of hydrocarbon resulting in the formation of more amount of solubilised substrate for enzymatic action.

There are still controversy regarding the effects surfactants on biodegradation. It was reported that soaps being highly surface active were known to displace proteins from latex particles (Chin et al., 1974). There were reports on the inhibitory effects of surfactants (Zhang et al., 1997) as well as on the enhancing effects of surfactants (Barkay et al., 1999).

Of the different concentrations of biosurfactant provided, a slight enhancement on the enzyme reaction was observed on the supplement of biosurfactant only at a very low concentration (0.5%). Higher concentrations were found to have no effect. Application of biosurfactant in the biodegradation of natural rubber like polymer was not reported yet.

Surfactants are surface-active compounds capable of reducing surface and interfacial tension between liquids, solids, and gases (Desai and Banat, 1997). Most of the surfactants currently in use are chemically synthesized. However, increasing environmental awareness has led to serious consideration of biological surfactants as possible alternatives to existing products (Kim et al., 1999). Biosurfactants are diverse group of surface-active compounds that are produced by a wide variety of
microorganisms (Banat, 1995). The increasing interest in the potential application of microbial surface active compounds is based on their broad range of functional properties. Biosurfactants offer several advantages over their chemical counterparts such as their ecological acceptance, biodegradability and production from renewable substrates (Fiechter, 1992). With increasing environmental awareness and emphasis on a sustainable society in harmony with the global environment, natural surfactants are becoming much more important (Holmberg, 2001). Microorganisms can produce a range of biosurfactants providing new possibilities for industrial applications (Parra, 1989).

The main classes of biosurfactants are glycolipids, lipoamino acids and lipopeptides (Lang and Wullbrandt, 1999). Among the wide type of biosurfactants, the best-studied microbial surfactants are rhamnolipids produced by *Pseudomonas aeruginosa* (Maier and Soberon-Chavez, 2000). They are the most effective surfactants known today. *Pseudomonas oleovorans* produces significant amounts of surfactants when grown on n-alkanes (Schmid *et al.*, 1998). They were also found to be effective in the biodegradation of hydrocarbons. The rhamnolipid biosurfactant was reported to have significant positive effects on the bioremediation of gasoline-spilled soil (Rahman *et al.*, 2002).

The production of biosurfactant is regulated by microbial growth substrate and the process. Usually the biosurfactants are produced from pure substrates. As it is necessary to find a more economical production process, studies were undertaken for the production of biosurfactants through biotechnological routes employing inexpensive, renewable substrates (Mercade, *et al.*, 1993). The success of biosurfactant production depends on the development of cheaper processes and the use of low cost raw materials, which account for the 10-30% of the
The use of economic substrates such as hydrophobic wastes meets one of the requirements of a competitive process for biosurfactant production. *Pseudomonas aeruginosa* was reported to produce rhamnolipids from waste frying oils (Haba et al., 2000). It has shown that waste cooking oils are suitable substrates for biosurfactant production.

In the present study, a biosurfactant developed in our lab was used for the increased solubilisation of latex for enhancing the enzyme action. It is a rhamnolipid produced by a *Pseudomonas aeruginosa* strain using used coconut oil as the substrate for the microbial growth. Reusing of used frying oil is hazardous for human health. So it is highly appreciatable if it can be provided as a high-energy source for microbial growth and transformation into high value products. Hence the present attempt to utilize the biosurfactant of the rhamnolipid type to enhance the enzymatic action on latex is a novel idea that opens up wide area for future research.

The enzyme treated latex was subjected to FTIR analysis and the spectrum was compared with that of the control. The enzyme treatment could facilitate many changes in the structure of latex. The FTIR spectrum showed reduction in the polymeric region along with many new bands from 1750 cm$^{-1}$ to 1000 cm$^{-1}$. Most of the band formed on the FTIR analysis of the biodegradation of the deproteinised latex under optimum condition (Fig.45) were also indicated in the FTIR of the enzyme treated sample (Fig.52).

**Microbial treatment of latex centrifugation effluent**

The selected rubber degrading strain was applied in the treatment of the effluent from the rubber processing factories. The effluent was strongly acidic in nature (Madhu et al., 1991). This might be due to the presence of formic acid or sulphuric acid added for the coagulation of latex (Jayachandran et al., 1998). TSS, TDS and MLSS were found high
in the effluent. TSS is a combination of organic particles, silt and sand that either floats on the surface, or is in suspension in water or wastewater. The effluent was composed of large amount of tiny rubber particles and this might have resulted in high value of suspended particles. TSS clouds the water. Toxic contaminants adhere to solids that eventually settle to the bottom, contaminating bottom sediments and smothering bottom-dwelling organisms.

Often, pollution in surface waters is not measured in terms of concentrations of the individual contaminants, but it is measured in terms of their aggregate potential in bringing oxygen depletion. Both BOD and COD were found high in the effluent. High values of COD and BOD indicated the organic nature of the pollutants (Table-5).

The latex effluent was inoculated with high concentration of young cultures of *Bacillus pantothenticus* 8063 in order to evaluate the efficiency of organism in bringing down the organic load in latex effluent during specific period of incubation. It was able to produce a considerable reduction in the COD (Fig. 53).

An activated sludge system was also developed for the effective treatment of latex centrifugation effluent. Activated sludge treatment is a process where waste water was treated aerobically by a microbial consortium dominated by heterotrophic bacteria. They were flocculated in the mixed liquor under a supply of excessive oxygen which is necessary to form discrete clumps of micro organisms. The latex effluent with the inoculated sludge was subjected to bacterial analysis and several strains were isolated (Table-6). It was reported that high sulphate levels due to excessive usage of sulphuric acid in the coagulation of skim latex and the presence of high ammoniacal nitrogen might have caused adverse effects on the bacterial viability and function (Ponniah *et al.*, 1976). In the
Discussion

Present study, sludge from a rubber processing unit was used as the inoculum. Here, in addition to the sludge inoculum, a heavy inoculum of *Bacillus pantothenticus* 8063 was also added to the system. The sludge biomass or floc is the basic operational unit in the mixed liquor. The slime forming bacteria grow and form flocs. These flocs form a substratum to which protozoa and sometimes filamentous bacteria and fungi attach. The organism in the presence of sludge could reduce the COD as well as BOD to a less hazardous level.

In the present study, the activated sludge system was developed by fill and draw mechanism. Both the COD and BOD were monitored during the acclimatization process. The trends in the deviation of COD and BOD were given in Fig. 54 and Fig. 55 respectively.

Fig. 54 clearly indicated decrease in COD during the acclimatization process. There was a gradual decrease up to 15 days. However, after that system almost entered into equilibrium. From 18<sup>th</sup> day onwards whatever organic load being added was completely removed within 4 days of treatment. A similar trend was observed in BOD analysis also. The analysis of MLSS showed a gradual decrease (Fig. 56) while SVI decreased gradually up to 12<sup>th</sup> day and then remained stable up to 21<sup>st</sup> day (Fig. 57).

The effluent from latex centrifugation unit possesses high values of COD and BOD and it cannot be released to the water bodies without proper treatment. Microorganisms are in general more effective compared to multicellular organism in the effluent treatment. Exploitation of the degradative capability of microorganisms is the fundamental basis of organic pollutant bioremediation. Biological methods are considered superior to physicochemical methods in the treatment of effluent since the later may cause secondary pollution. So, it is possible that existing
microbial resources can be applied for the reduction of pollution parameters in the effluent. In this context, the activated sludge system proposed in the study offers a promising choice for the effective treatment of the natural rubber latex centrifugation effluent.

Hence the present research attempt to exploit the wide possibilities of biodegradation in natural rubber latex opens up many new ideas. The novel bacteria selected in the study, *Bacillus pantothenticus* 8063 could carry out the biodegradation of natural rubber latex. All the DRC, TLC, CO$_2$ liberation, lipid deposition, TOC, GC/MS and FTIR analysis supported that the organism could effectively degrade natural rubber latex. The enzyme RoxA involved in the natural rubber degradation could be isolated and could be partially purified. The project also reveals various possibilities in the treatment of natural rubber latex centrifugation effluent. Further studies in this area may bring many other novel informations with which the biodegradation of natural rubber latex and the treatment of latex centrifugation effluent can be scaled up to tackle many of the current environmental problems.