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Microbial Degradation of Natural Rubber

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Until recently, most reports of biodegradation of natural rubber (NR) had been on vulcanised rubber and rubber products. This paper describes the microbial degradation of raw and purified rubber from Hevea brasiliensis *Muell Arg. by several micro-organisms. The degradation was accompanied by an increase in pH of the medium and a decrease in the rubber, nitrogen and protein contents. A reduction in the molecular weight of the polymer was observed. From IR spectroscopy, no significant modification of NR occurred during the biodegradation. Rubbers obtained from inoculated latex did not differ significantly in their Wallace Plasticity (P_o) values from those of controls, whereas their change in accelerated storage hardening test (ASHT) conditions was significantly lower than that of controls. Screening of micro-organisms capable of utilising NR for growth was carried out by incubating each test organism in (1:10 000) diluted sterile latex, followed by assessment on the ability of the organism to clear the milky rubber solution.*

The microbial deterioration of natural rubber (NR) has been a subject of active research for many years. Sohngen and Fol[1] reported that two *Actinomyces* species were capable of using NR as the sole carbon source. Later, Spence and Van Niel[2] isolated four more strains of *Actinomyces*, and Kalinenko[3] a strain of *Streptomyces*, which were capable of decomposing rubber. Most of the studies on microbiological deterioration of rubber, however, had been concentrated on the microbial degradation of vulcanised rubber and rubber products like underground pipe-joint rings[4–7], cable insulators[8–13] and rubber gloves[14]. The degraded and leaky nature of these rubber products after long usage was attributed partly to microbial attack. Evidence for the degradation of vulcanised rubber by micro-organisms has not been unequivocal, since the degradation of the rubber polymer is not always distinguished from the degradation of other constituents and fillers in the vulcanisate. While the microbial degradation of rubber pipe-joint rings and underground cable insulators was shown to have disadvantages in the long term, this same microbial degradation was used to advantage in the elimination of worn-out rubber. Faber[15] reported that microbial degradation was a feasible method in the disposal of disused rubber tyres. The microbial breakdown of uncompounded natural rubber was also observed[16,17].

This paper describes the microbial degradation of raw and purified rubber from *Hevea brasiliensis* by several micro-organisms.

MATERIALS AND METHODS

Methods of Screening for Micro-organisms

Isolation of micro-organisms (fungi and actinomycetes) was carried out by plating pieces of *Hevea* tree bark, rubber from cup-lumps and rubber sheets buried under soil for more than four months on selective media. The isolation from soil under rubber was by the soil dilution plate method. The media used were: 2% malt extract – rose bengal – streptomycin, a modified selection medium for fungi[18] and starch-casein for actinomycetes[19]. These were subsequently maintained on 2% malt extract agar. A rapid method of screening for the ability of

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micro-organisms to utilise natural rubber was devised in the laboratory. This was carried out by incubating each isolate in a (1:10 000) diluted sterile latex, obtained from sterile tapping[20] and supplemented with sterilised nutrient salts[2] consisting of 0.1% KNO_3, 0.1% K_2HPO_4 and 0.05% $MgSO_4 . 7H_2O$. Micro-organisms which could clear the latex suspension within one week of incubation were subjected to a second test in a higher concentration of latex (1:5000), containing nutrient salts as described above and these were selected for further studies.

Incubation with Latex

Selected micro-organisms were inoculated into latex which was diluted (1:4) with nutrient solution, buffered with sodium hydrogen maleate, pH 7.0 and pasteurised at 0.35 kgf/cm^2 pressure for 30 min. This was then incubated at 29°C.

During the incubation period, samples were removed at regular intervals (usually ten days) and their pH determined with a pH meter. These were subsequently coagulated with heat. The rubber coagulums were then washed with water, dried at 60°C overnight and rolled to a uniformly thin (0.32–0.36 cm) sheet, after six passes through the mill. The milled rubber was soaked with water for at least 3 h for further extraction of non-rubbers. The washed and milled rubber was dried to a constant weight in an oven (60°C), before samples were removed for further analysis.

Incubation with Purified Rubber

Purified rubber *i.e.* rubber devoid of non-rubbers, was prepared from rubber cream obtained after ultracentrifugation of fresh latex by the method of Subramaniam[21]. Rubber cream was deproteinised by dispersion in 10% sodium dodecyl sulphate. After three washes with water, the rubber was coagulated in ethanol. The coagulated rubber was then subjected to soxhlet extraction with hot ethanol. The purified rubber thus obtained was milled six times and dried to a constant weight.

All inoculation experiments were performed in replicates of four. Pieces of purified rubber, (each weighing approximately 10 g and measuring 7 cm^2 × 0.32 – 0.36 cm thick) were sterilised with propane oxide. These were then incubated at 29°C in a solution of nutrient salts (0.1% KNO_3, 0.1% K_2HPO_4, 0.05% $MgSO_4.7H_2O$ and 0.5% glucose), modified after Spence and Van Niel[2]. Test samples were inoculated with fungal isolates, *Fusarium* spp. and *Metarrhizium anisopliae*, while controls remained uninoculated.

At the end of the incubation period of ten months, the rubber pieces were washed with distilled water, dried and dissolved in chloroform. After filtration to remove mycelia and cell debris, the dissolved rubber was spread on glass plates and evaporated to dryness at room temperature, in air, under a fan. The weight and other properties of these rubber pieces were then determined.

Estimation of Non-rubber Content

Incubation mixtures[22] containing buffered latex and inocula were centrifuged at 21 000 r.p.m. for 1 h in a Beckman Spinco L2-65B ultracentrifuge, using rotor 21. The clear sera thus obtained were either used directly, or concentrated further before analysis. Protein concentration in the sera was determined by the Lowry method[23]. The amino acid, sugar and lipid contents of the sera were estimated qualitatively by thin layer and paper chromatography, coupled with appropriate staining procedures.

Polyacrylamide Gel Electrophoresis

The sera obtained after ultracentrifugation of the incubation mixtures (described above) were dialysed against distilled water. The dialysed sera were then lyophilised and reconstituted in a minimal amount (2 ml) of water. Aliquots (50 µl) of these were then applied on polyacrylamide gels for electrophoresis. Polyacrylamide gel electrophoresis

at pH 8.3 was carried out according to the procedures of Ornstein[24] and Davies[25] Protein bands were visualised after staining in Coomassie brilliant blue.

Measurement of Molecular Weight and Raw Rubber Properties

Molecular weight of rubber was determined by gel permeation chromatography as described by Subramaniam[26]. Nitrogen content was determined by a semi-micro Kjeldahl procedure[27]. Wallace Plasticity (P_o) and plasticity change under accelerated storage hardening test (ASHT) conditions ($\triangle P$), were measured by established methods[27].

RESULTS AND DISCUSSION

Screening of Microbes

Using the method devised in our laboratory, micro-organisms capable of utilising NR were identified by their ability to clear a latex suspension, presumably by consuming the rubber *(Figure 1)*. To-date, more than 200 micro-organisms, belonging mainly to actinomycete and genera *Fusarium, Trichoderma, Penicillium, Paecilomyces* and *Aspergillus* had been screened for their ability to utilise rubber for growth. Micro-organisms which had shown promise in the biodegradation of raw and purified NR are *Fusarium solani* (from buried rubber), *Metarrhizium anisopliae* (obtained from the Commonwealth Mycological Institute, United Kingdom) and a few species of actinomycete from buried rubber and soil.

Electron Microscopy of Microbial Growth in Latex

Electron microscopy (EM) examination of one of these microbial cultures *(Metarrhizium anisopliae)* grown in diluted latex revealed the presence of osmiophilic rubber-like particles inside the hypha *(Figure 2)*.

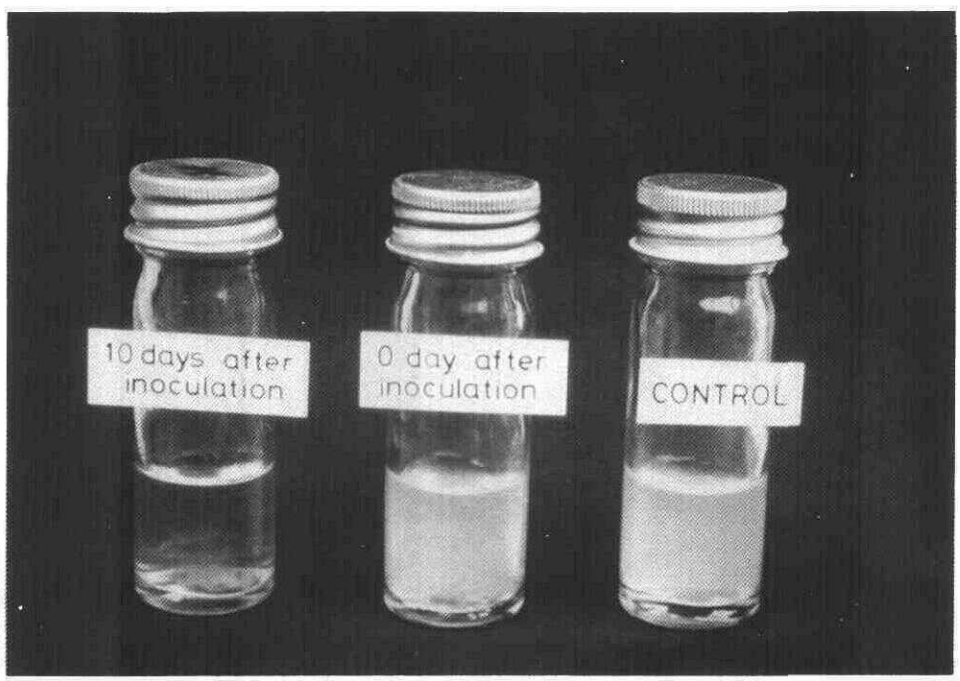
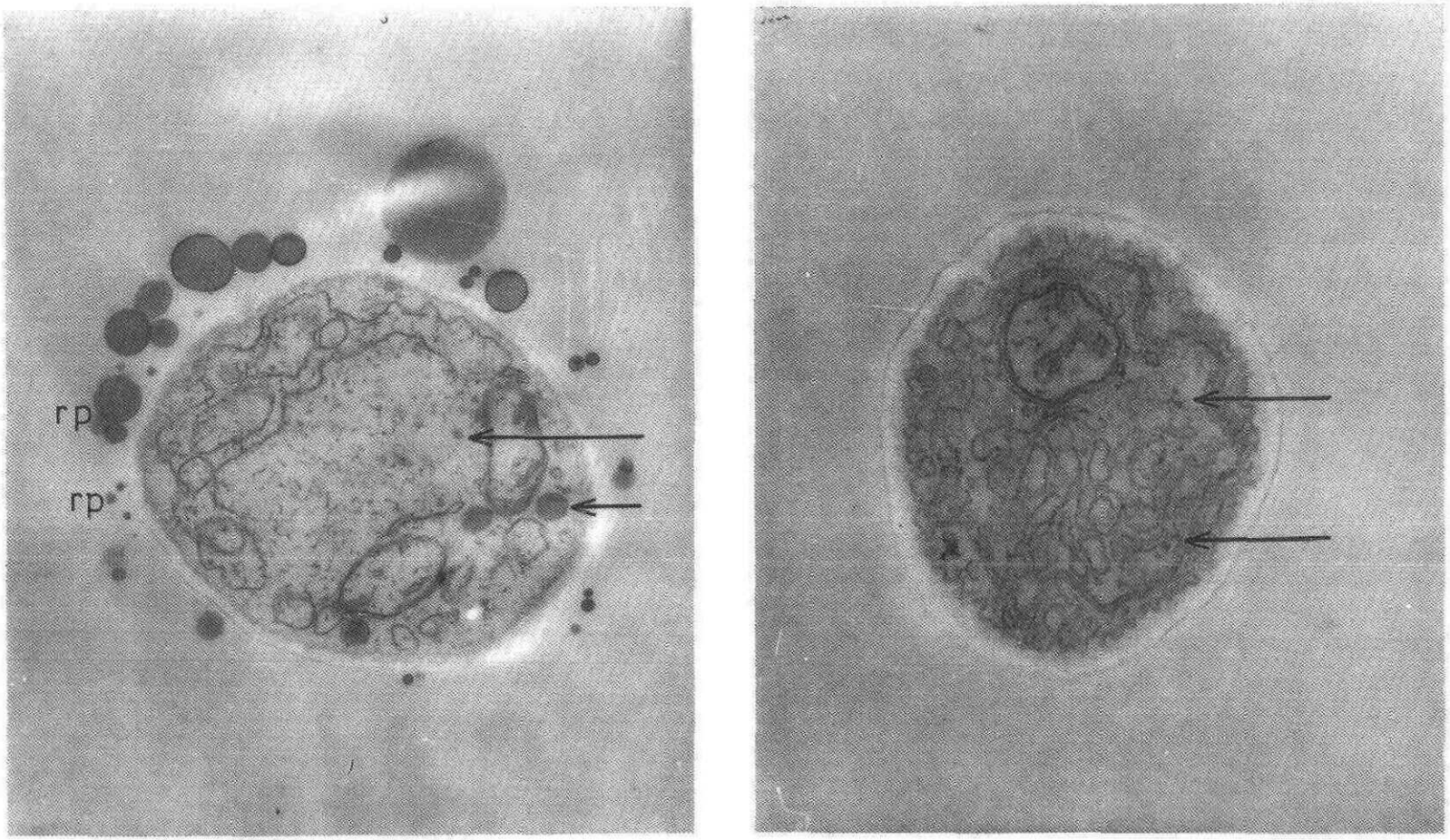


Figure 1. A rapid method of screening for the ability of micro-organisms capable of clearing a sterile, diluted latex suspension.



Cultured in latex (test)

Cultured in growth medium (control)

Figure 2. Electron microscopy of a hypha of Metarrhizium anisopliae *cultured in latex (test) and growth medium (control) showing osmiophilic particles resembling rubber-like particles (arrows) and rubber particles (rp). Magnification 30 000 ×.*

This suggested that the microbe was capable of 'comsuming' rubber particles with the probability of degrading the rubber. On the other hand, similar EM examination of a hypha of *M. anisopliae* in growth medium (control), showed that although osmiophilic particles were present in the hypha, these were different in structure and did not resemble rubber particles in their ultrastructure *(Figure 2)*.

Incubation with Latex

Incubation with diluted pasteurised latex with these selected micro-organisms resulted in an increase in pH of the incubation medium from neutral to alkaline *(Figure 3)*. Simultaneous with the increase in pH of the incubation medium, a distinct lowering of the nitrogen content of latex was also demonstrated *(Figure 4)*. These changes in pH and nitrogen content of rubber were shown to be more rapid in the initial three weeks of incubation, than the subsequent period of incubation. This change in pH from neutral to alkaline was distinctly different from that reported for assisted biological coagulation[28], where incubation of latex with bacteria resulted in a change of pH of the latex from neutral to acidic. This suggested that the growth of the above micro-organisms in latex was not *via* the metabolic conversion of carbohydrates to fatty acids as observed in assisted biological coagulation, but rather, through some other undetermined process. The rapid decline in nitrogen content of latex as incubation progressed implied that growth was probably supported by the breakdown of nitrogenous non-rubbers in latex.

As incubation progressed, a decrease in protein content in the inoculated latex serum compared to controls was recorded *(Figure 5)*. Polyacrylamide gel electrophoresis of serum from the incubation mixtures also revealed fewer protein bands in the inoculated samples compared to control uninoculated samples. Comparisons of liquid chromatographic separations between sera from control (uninoculated latex) with that from inoculated latex demonstrated that there were fewer amino acids, sugars and lipids in the inoculated latex sera than in the control. The depletion of proteins in the latex as microbial growth developed supports the suggestion that growth of these micro-organisms was primarily at the expense of non-rubbers like proteins in the latex.

Rubbers obtained from inoculated latex were not significantly different in their Wallace Plasticity (P_o) values from that of the control (uninoculated latex) *(Figure 6)*. On the other hand, their change in hardening ($\triangle P$) under accelerated storage hardening test (ASHT) conditions was shown to be distinctly lower than that of the control *(Figure 7)*. The depressed $\triangle P$ values of rubbers from inoculated latex, compared to control rubbers implied that there was decreased cross-linkage between the NR molecules due to the accelerated storage hardening process. This decreased cross-linkage was probably a result of a decreased non-rubber content in the rubbers, since non-rubbers and aldehyde groups are essential for cross-linkages[29]. The decreased non-rubber content in rubbers from inoculated latex had already been confirmed colorimetrically for proteins and chromatographically for lipids, carbohydrates and amino acids.

Reductions of 17% and 24% were recorded in the weight average molecular weight of rubbers from inoculated latex compared to uninoculated controls *(Table 1)*.

The discrepancy in differences in molecular weight between rubbers from inoculated latex and controls, by the two methods of measurement *i.e.* Wallace Plasticity and gel permeation chromatography was probably due to the presence of microgel[30]. While P_o is a measurement of the molecular weight of the entire dry rubber sample, *i.e.* the linear as well as cross-linked rubber molecules, the GPC method measures only the *soluble,* linear rubber molecules, the cross-linked rubber molecules being filtered off as microgel in the process.

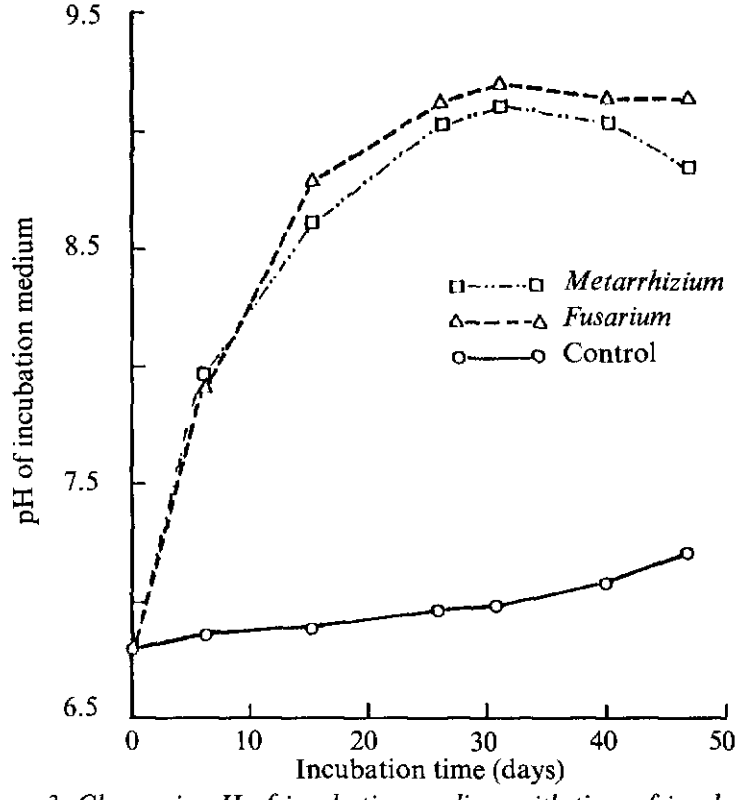


Figure 3. Change in pH of incubation medium with time of incubation.

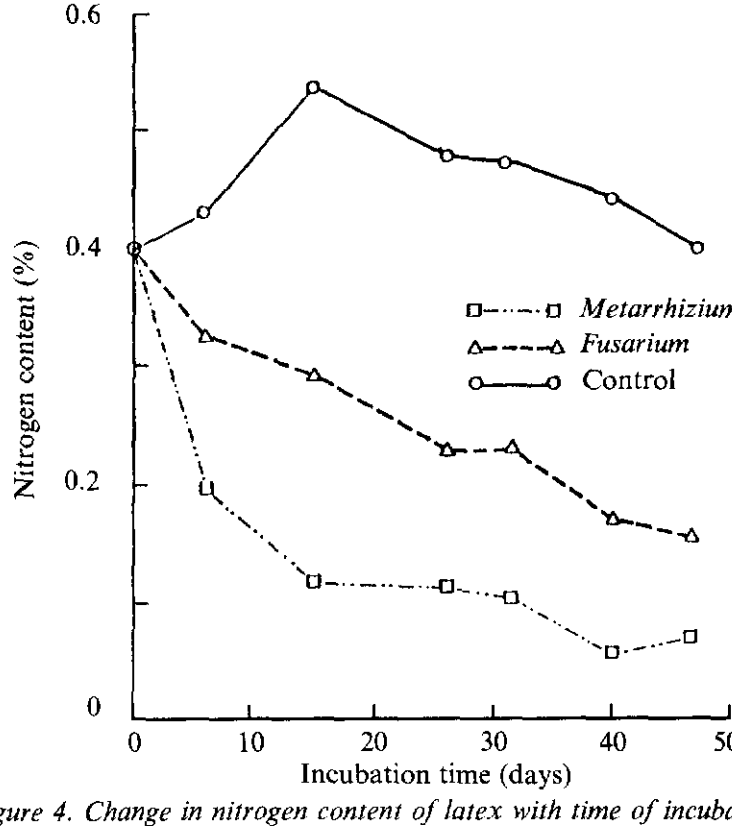


Figure 4. Change in nitrogen content of latex with time of incubation.

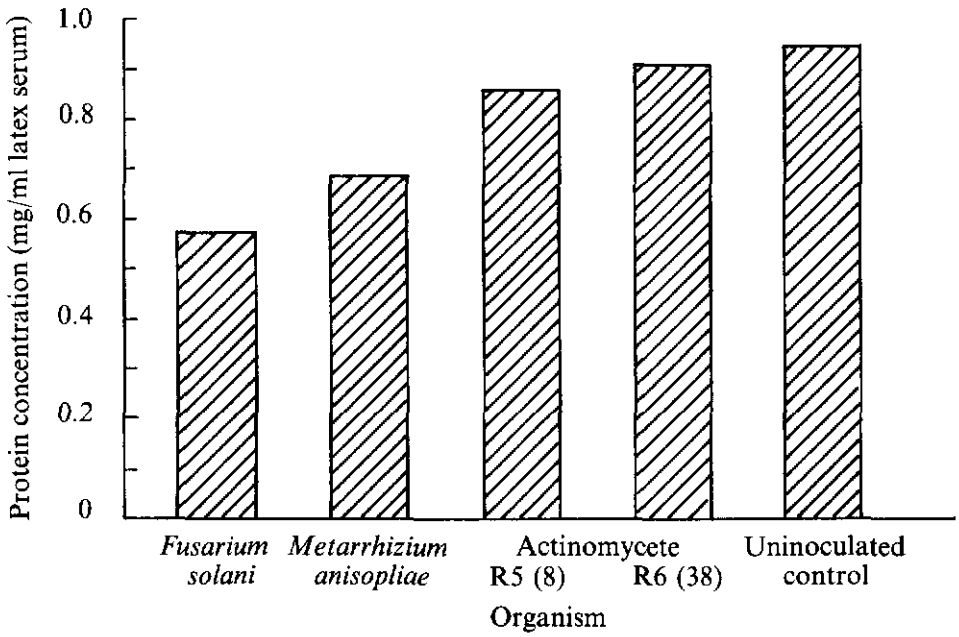


Figure 5. Determination of protein concentration in the serum of inoculated latex.

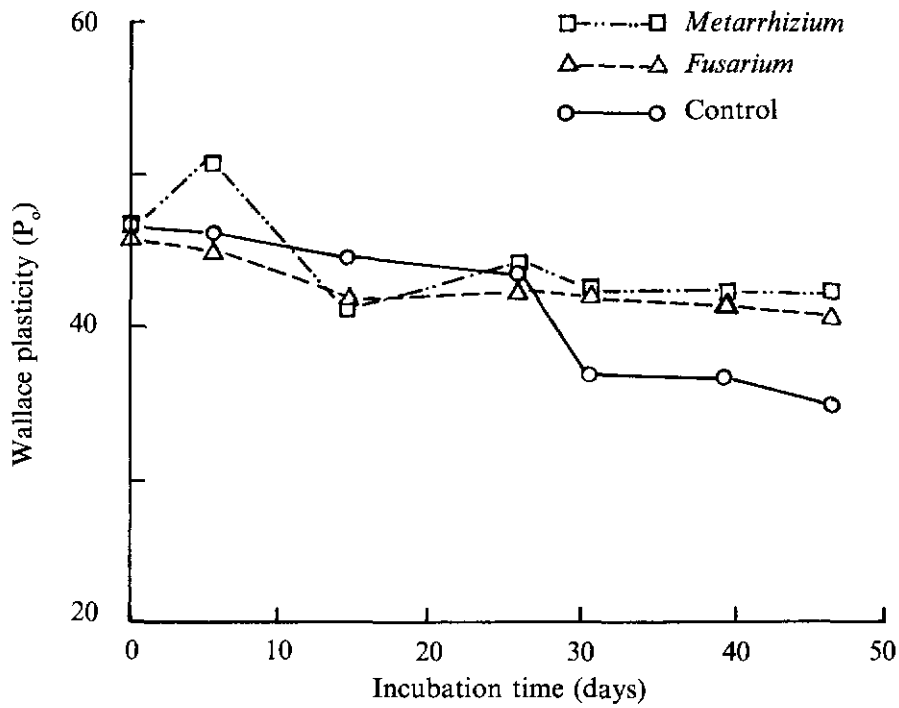


Figure 6. Change in Wallace plasticity of rubber with time of incubation.

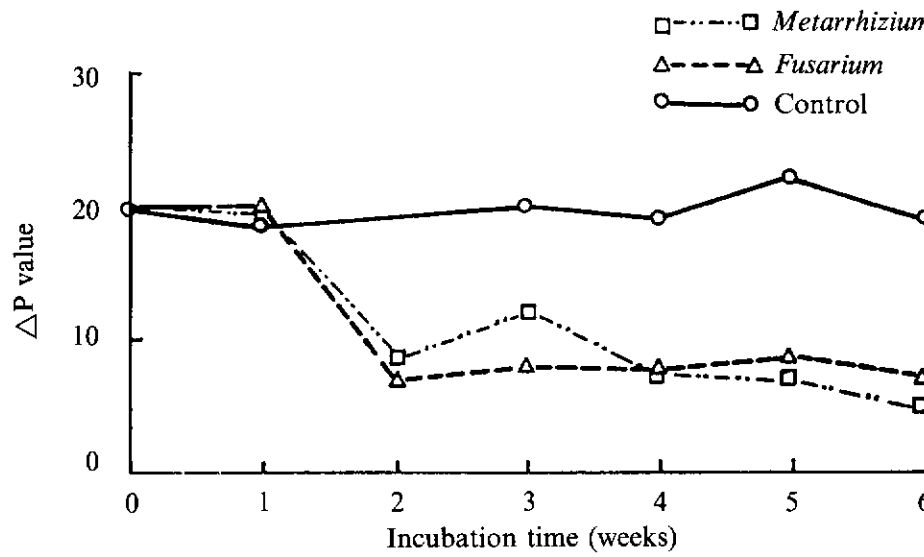


Figure 7. Change in hardening under accelerated storage hardening test conditions of rubber with time of incubation.

TABLE 1. MOLECULAR WEIGHT DETERMINATION (M_w) BY GPC OF RUBBERS FROM COAGULUM OF INOCULATED LATEX

Organism	$M_w \times 10^{-5}$	% M_w of treated control	% reduction in M_w
Rubber from control uninoculated latex	10.01239	100.0	–
+ *Fusarium solani*	8.32875	83.2	16.8
+ Actinomycete R6 (38)	7.57863	75.7	24.3

Incubation with Purified Rubber

When pieces of purified rubber, each of approximately 10 g and measuring 7 cm^2 × 0.32 – 0.36 cm thick, were incubated with selected micro-organisms for long periods (ten months), a reduction of 5%–10% in weight of purified rubber was observed *(Figure 8)*. Solubility tests on these rubbers suggested that rubbers which were incubated with selected micro-organisms were less soluble in toluene than control uninoculated rubbers *(Figure 9)*. Besides solubility, differences in colour and elasticity were noted between inoculated and uninoculated rubbers. In general, controls were dark-brown in colour and were very sticky. The inoculated rubbers, on the other hand, remained fairly light in colour with tinges of pink or white, depending on the nature of the micro-organisms used for the experiment. The inoculated rubbers were more elastic than the controls. The observed differences in properties between the inoculated and control samples were probably attributed to the presence of microbial growth. Over a ten-month incubation period, oxidation of purified rubber would

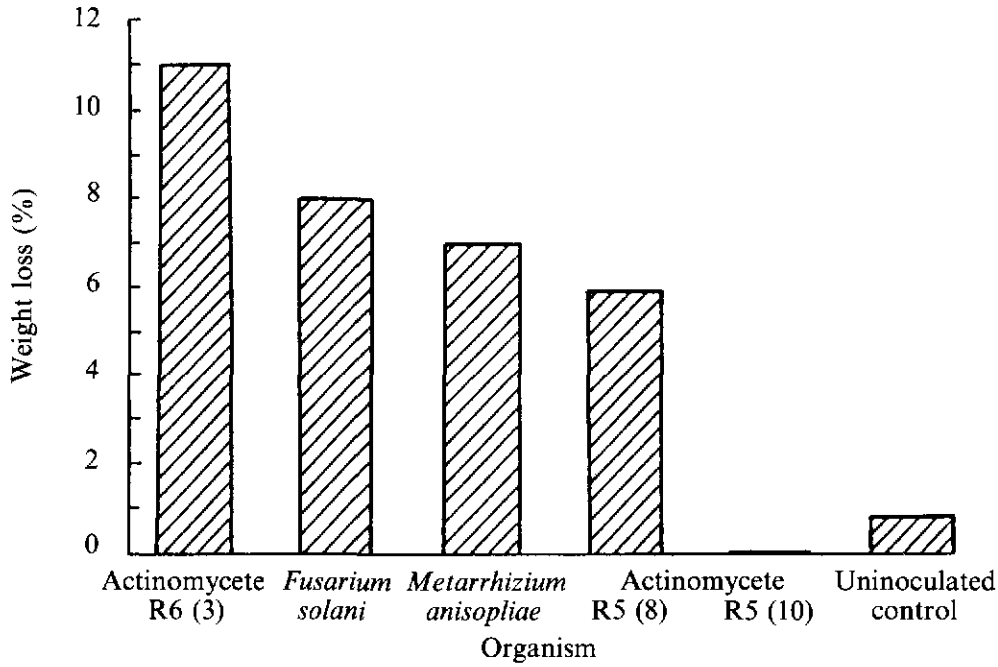


Figure 8. Weight loss of purified rubber after ten months incubation with selected micro-organisms.

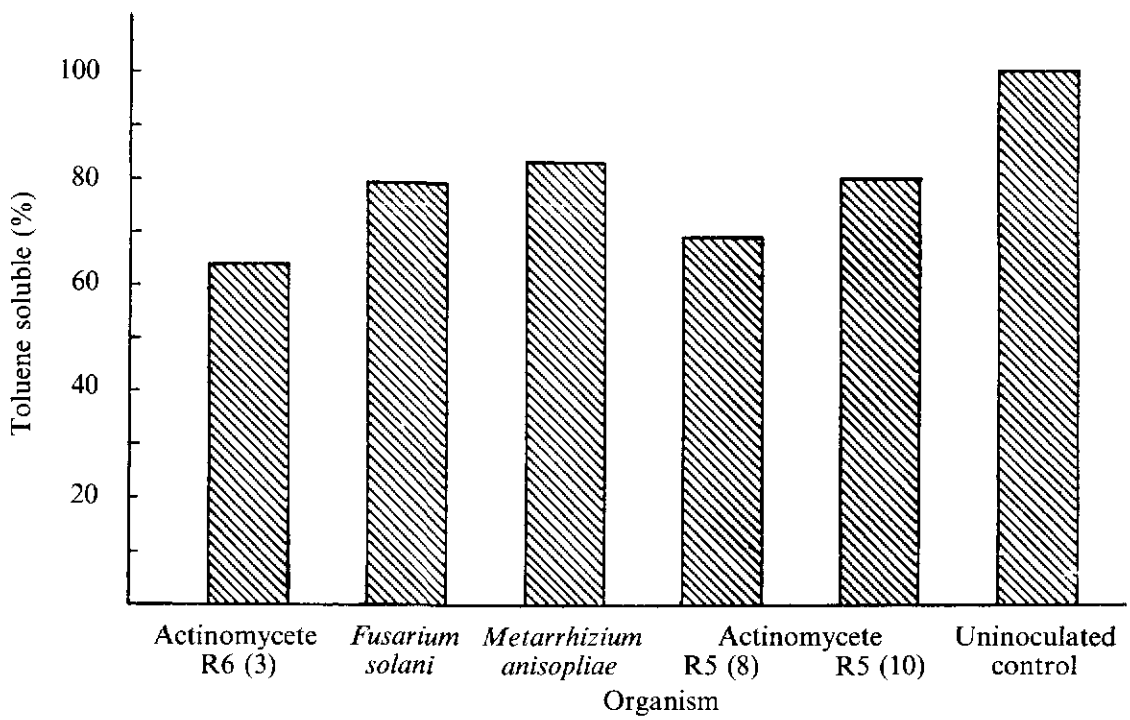


Figure 9. Solubility of purified rubber after ten months' incubation with selected micro-organisms in toluene.

have occurred, with the resultant stickiness in the control uninoculated samples. The inoculated samples, on the other hand, might not have been subjected to the same extent of oxidation, because of the protective barrier of microbial growth.

Infra-red (IR) spectroscopy of rubbers obtained from inoculated latex as well as inoculated purified rubbers had so far suggested that no significant chemical modifications of the NR polymer had occurred. Only a slight reduction of molecular weight of 5%–13% was recorded.

Shaposhnikov[31] reported that micro-organisms grew at the expense of non-rubbers such as proteins, resins and carbohydrates present in natural rubber, and growth did not occur on rubber when these non-rubbers were removed. However, as selected micro-organisms were shown to grow on purified rubber *i.e.* rubber devoid of non-rubbers, this is direct evidence that micro-organisms can grow at the expense of the polyisoprene chain. Although a small amount of carbon source (0.5% glucose) was present to initiate the growth of micro-organisms, subsequent growth was probably supported by carbon source derived from purified rubber. The 5% – 10% decrease in weight of inoculated purified rubbers, together with the molecular weight reduction data on the inoculated purified rubbers confirmed that selected micro-organisms were capable of degrading the NR polymer. Since only purified rubber was used, the above results reflected changes in the NR polymer and not in the non-rubber content (as was the case of raw rubber) and the compounding materials (as in the case of vulcanised rubber and rubber products[32]). However, as both control and inoculated rubbers did not differ significantly in their IR spectra, and the polymer molecular weight was only slightly reduced, it was concluded that the selected micro-organisms attacked the NR polymer from the chain ends. This is similar to that observed by Williams[17] who found that biodeterioration of *Penicillium variabile* on natural rubber smoked sheet and pale crepe resulted in a 15% reduction in the molecular weight of the NR polymer without significant changes in the IR spectra between the degraded and undegraded rubbers.

CONCLUSIONS

Micro-organisms (fungi and actinomycetes) capable of degrading purified rubber, without significant chemical modification to the polymer were demonstrated. The observed microbial degradation appeared to be from the *ends* of the polymer, rather than by internal cleavage of the polymer. This form of microbial degradation was shown to be different from assisted biological coagulation, which was mainly bacterial. The lowering of protein and nitrogen contents which resulted from this microbial activity could be promising for the recovery of rubber from skim latex. However, more work would be necessary before this could be applied.

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