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Colonization and degradation of rubber pieces by *Nocardia* sp.

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Abstract

The growth of a *Nocardia* sp. occurs essentially on the insoluble rubber substrate and the cells are tightly bound to the rubber in the initial stage of the growth in spite of vigorous stirring of the cultures. The colonization of rubber pieces was followed by staining with Schiff reagent, and it was revealed that not only the thickness of rubber pieces, but also their length and width greatly influenced microbial colonization and degradation of natural rubber products. Among rubber pieces of various shapes, long strips were most rapidly covered by many microbial colonies and experienced the highest rate of rubber degradation. The rate of degradation (expressed by % weight loss) of the long strips of rubber was a linear function of surface area per unit weight of rubber. Thin and wide films of rubber were also rapidly colonized and degraded, while the colonization and degradation of short and narrow pieces were substantially slower and less extensive.

Introduction

Rubber baloons and plastic bags are now considered to be a danger to wild animals when disposed of in the natural environment. It is important to know the potential for their microbial degradation from both the viewpoint of prolongation of usage and of waste disposal problems. There have been a number of reports on microbial deterioration of rubber products and the effect of curing conditions and rubber formulations were examined (Simpson 1988; Williams 1986). The methods employed in these degradation tests, however, were usually time consuming and inadequate for comparison of many samples (Zyska 1988).

As we reported previously, a strain of *Nocardia* that we isolated, utilizes various kinds of vulcanized natural rubber products as a carbon substrate (Tsuchii et al. 1985) and the soft type products like gloves were rapidly degraded. The pure culture of the organism was used to examine microbial degradation of a series of the vulcanizates and we reported that the resistance of the vulcanizates was in good correlation with the cross-link density, and that addition of carbon black as a filler made the vulcanizates markedly more resistant to microbial attack (Tsuchii et al. 1990).

In contrast to growth in common media where carbon substrate is dissolved, the growth of microorganisms upon natural rubber products is very slow and the effect of culture conditions upon microbial growth on an insoluble solid substrate like rubber has not been well characterized. In comparing the degradation rates of various kinds of rubber products, consideration should be given not only to the effect of curing conditions and rubber formulations but also to the effect of thickness and length. The purpose of the present study was to examine the culture characteristics of the rubber-degrading microorganism in vitro, and in particular, the effect of shapes and surface area on microbial colonization and degradation of rubber pieces in stirred flasks was precisely examined.

Materials and methods

Rubber products

Rubber pieces varying in length and width were cut from three kinds of soft type natural rubber products of different thickness. Latex glove A (phoenix brand, 0.1 mm in thickness) was obtained from Sunrex Japan Co., Ltd., Surgical glove B (New Doctor hand, 0.2 mm thickness) was from Fujilatex Co., Ltd. and Rubber band C (O-band, 1.0 mm in thickness and width) was purchased from Kyowa Co., Ltd. Rubber band D (0.5 mm thickness and 1.0 mm width) and Rubber band E (0.5 mm in thickness and 0.5 mm in width) were prepared by cutting band C and were also used as growth substrate. These products contain about 3% of low molecular weight organic materials (chloroform or acetone soluble) and about 3% of inorganic constituents (hot-toluene insoluble). Rubber pieces were extracted with chloroform before use in order to remove chemicals with microbiocidal activities.

Some of the rubber pieces were used for benzene swelling test. The test specimens were preserved in benzene for 2 days at 30° C, and the volume fraction of the rubber network (Vr) in the swollen gel was estimated. Vr of these rubber products were 0.19–0.22, indicating that all products were susceptible to microbial attack (Tsuchii et al. 1990).

Microorganism and preculture

Nocardia sp. 835A, which was the strongest decomposer of solid rubber, was used throughout this study (Tsuchii et al. 1985).

A preculture of a strain 835A which had been grown on glove A for 19 days was used as the inoculum for the degradation test. The test flasks were inoculated with 5 ml of the preculture containing 1.0 to 2.0 $\times$ 10^7 viable cells per ml of the medium. The viable cells in the preculture were counted by plating 0.1 ml aliquots of the appropriate dilution onto Yeast extract-malt extract agar (Pridham et al. 1956–57). The conditions for the preculture are the same as that for the degradation test and a long strip of glove A was used as a sole carbon substrate.

Methods of the degradation tests in stirred flasks

To examine the microbial degradation of various kinds of rubber pieces, growth experiments in which rubber was used as the sole carbon substrate for the growth were carried out.

Rubber pieces were added to 100 ml of mineral salt medium in 300 ml erlenmeyer flasks, and each of the flasks was aerated by stirring with a Teflon coated magnetic bar (50 mm in length and 7 mm in diameter) at 600 rpm and 30° C. Initial dry weight of rubber added to each flask was 70 to 80 mg. The composition of the mineral salt medium was as follows: $(NH_4)_2SO_4$, 10.0 g; KH_2PO_4, 2.0 g; K_2HPO_4, 16.0 g; $MgSO_47H_2O$, 0.2 g; NaCl, 0.1 g; $CaCl_2$, 0.02 g; $FeSO_4$, 0.01 g; $Na_2MoO_42H_2O$, 0.5 mg; $Na_2WO_42H_2O$, 0.5 mg; MnSO, 0.5 mg in 1 liter distilled water, pH 7.5.

After a 14 days' incubation, the residual rubber was collected by filtration with gauze and very small particles passed through gauze were considered as degraded completely. The mixture of rubber and cells was extracted by boiling in 1N NaOH. The fragments of rubber were then thoroughly washed with water and weighed after being dried *in vacuo*. The rubber weight loss (%) was calculated according to the following equation, 100 $\times$ (Initial weight of rubber - Residual rubber after alkali treatment)/Initial weight.

The cells suspended in the medium were collected by centrifugation and weighed after being dried *in vacuo*. Protein content of the alkali extract and the suspended cells was about 30% of the dried cell weight and that of the cells of the organism grown on a glucose medium was 32%. The protein was estimated by the modified method of Lowry et al. (Herbert et al. 1971).

Methods of the cultures in shaken flasks

Rubber pieces were added to 100 ml of the salt medium in 500 ml erlenmeyer flasks or Sakaguchi flasks (apple-shape flasks). The erlenmeyer flasks were shaken with a rotary shaker (120 rpm) and the Sakaguchi flasks with a reciprocal shaker (120 rpm).

Staining of rubber-degrading colonies

The actively growing colonies of strain 835A on the rubber surface were visualized clearly by staining with Schiff reagent (Ehrlich et al. 1948). The purple color produced by the reagent was evidence that isoprene oligomers containing aldehyde group were produced and accumulated during the microbial degradation of rubber (Tsuchii et al. 1985).

Table 1. The effect of culture methods on rubber degradation.

Culture method	Weight loss (%) of glove A[a]	Weight loss (%) of glove B	Weight loss (%) of band D
Magnetic stirrer	89 ± 11[b]	59 ± 4	35 ± 2
Rotary shaker	78 ± 14	40 ± 1	28 ± 1
Reciprocal shaker	52 ± 9	10 ± 5	22 ± 7

a) After 14 days cultivation, weight losses of long strips (120 mm) were calculated by the formula: 100 × (initial weight of rubber – residual rubber after alkali treatment)/initial weight.
b) Mean ± deviations in more than duplicate experiments.

Results

The effect of the culture methods upon rubber degradation

The weight losses of the long strips (120 mm) of rubber after 14 days cultivation period are shown in Table 1. It was apparent that the all three kinds of rubber products were most rapidly degraded in stirred flasks and that the degradation rate was slower in shaken flasks. In the rest part of this study, the stirred flask experiments were carried out.

Time course of the degradation of a long strip of glove A

The typical time course of the growth of the Nocardia sp. on a long strip of glove A in stirred flask is shown in Fig. 1. The cell growth started after a lag period of 3 days. Many but small colonies can be seen on rubber surface on day 3 (about 30/mm^2 on average, and less than 0.1 mm in diameter). The collony diameter reached as large as 0.2 mm on day 5, and more than half of the surface was then covered with the growth. In the early stage of the growth, the mycelium of the organism attached tightly to the rubber and the weight loss of the mixture of rubber and the cells was negligible in spite of the considerable amount of the growth. Only after alkali treatment of the mixture, the consumption of rubber by the organism was estimated from the weight loss.

Although the rubber surface was completely covered with dense growth of rubber-degrading organism after 7 days cultivation, there was little or no suspended cells in the culture medium. After 9 to 12 days, the cells were released from the rubber surface into the medium, and the strip was broken up to many small fragments on day 14.

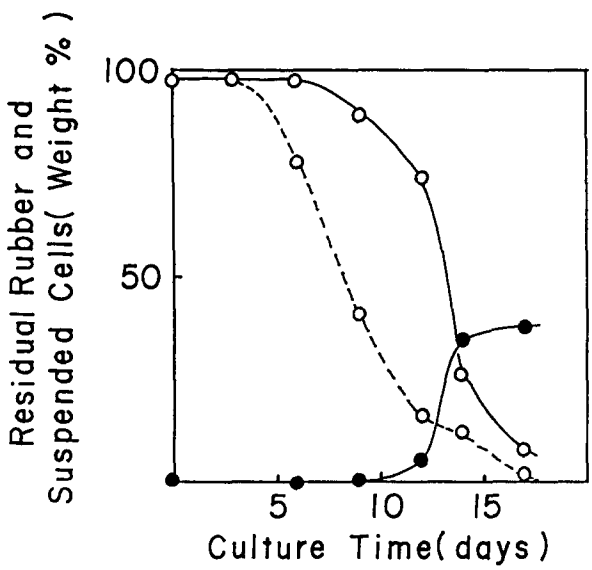

Fig. 1. Time course of rubber degradation. A long strip of glove A in a stirred flask was degraded by strain 835A. Symbols: ○ — ○, weight of the rubber strip before alkali treatment; ○ - - - ○, weight of the rubber strip after alkali treatment; ● — ●, weight of the cells suspended in the medium.

The effect of the length upon colonization and degradation

The effect of the length of rubber pieces upon degradation rate is summarized in Fig. 2.

In the case of very thin films from glove A (0.1 mm thickness and 5 mm in width), the surface of the short strips, as well as that of long strips, were uniformly covered with many colonies after 7 days' cultivation

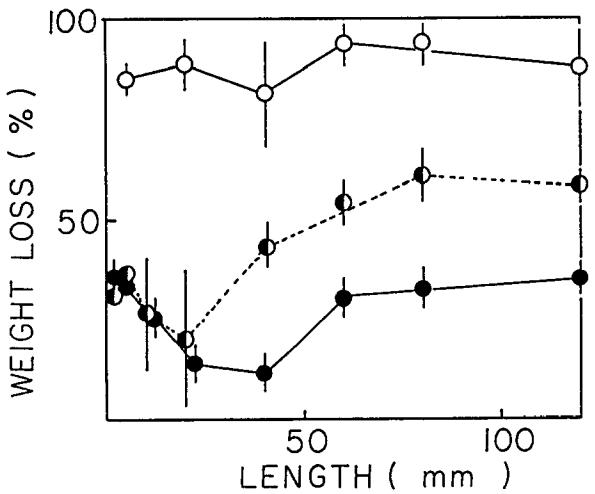

Fig. 2. The effect of length upon microbial degradation of rubber pieces. After 14 days cultivation, weight losses were estimated. Symbols: ○ — ○, glove A (0.1 mm in thickness and 5 mm in width); ◐ — ◐, glove B (0.2 mm in thickness and 3 mm in width); ● — ●, band D (0.5 mm in thickness and 1.0 mm in width). The vertical lines in the figure indicate the deviation of values in more than duplicate experiments.

and the degradation rate was not significantly influenced by the length of the rubber strips.

In the case of glove B (0.2 mm thickness and 3 mm in width) and band D (0.5 mm thickness and 1.0 mm width), the length of the rubber strips greatly influenced the colonization and the degradation rate. Both the long strips of glove B and band D were uniformly covered with growth of the organism after 14 days' cultivation (Fig. 3). On the contrary, not all the surface of the short pieces of band D was covered with microbial growth, and the growth was sparse and not uniformly distributed on the short pieces of glove B. The individual colonies can be separately observed on the surface of band D short pieces (Fig. 4) and the number of colonies was about 50/mm^2 on average.

In the case of glove B and band D, longer strips measuring 60 mm or more in length were rapidly degraded, while a considerable depression of microbial degradation was observed in 20 mm and 10 mm strips. The very short and small strips (1.5 to 5.0 mm in length) were not uniformly colonized, but degraded rather rapidly.

The effect of width upon microbial degradation

The effect of width is summarized in Fig. 5.

In the case of glove A (20 mm in length), thin films of rubber with widths of 3 to 20 mm were rapidly

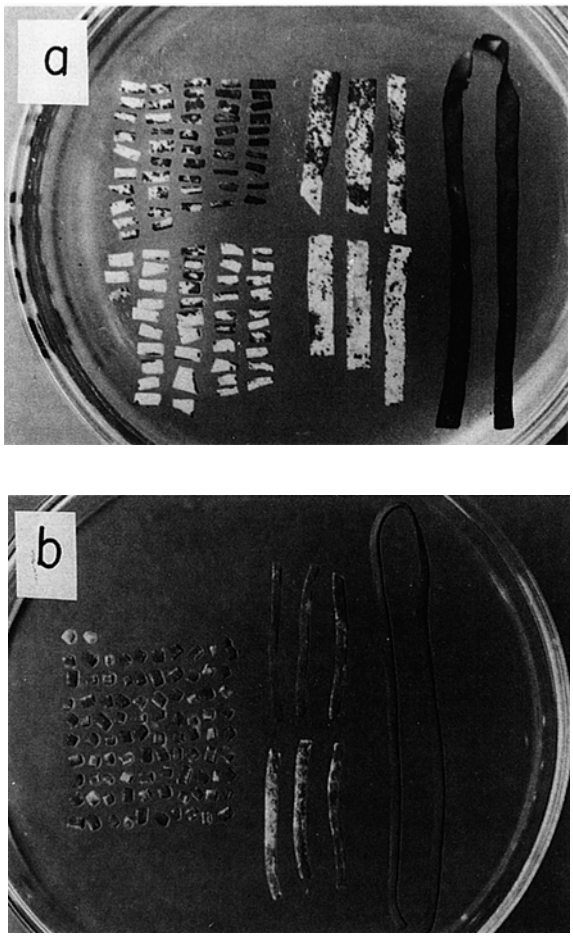

Fig. 3. The effect of length upon microbial growth. After 14 days cultivation, the growth of strain 835A was visualized by staining rubber pieces with Schiff reagent. (a): the microbial growth on glove B, left (very short pieces with length of 1.5 mm); center (short strips with length of 20 mm); right (a long strip with length of 120 mm). (b): band D, left (1.5 mm in length); center (20 mm); right (120 mm).

colonized and degraded. On the contrary, the growth had not covered all the surface of the narrowest film of the glove A (1.5 mm width) even after 14 days and the microbial degradation was substantially depressed.

In the case of glove B (0.2 mm in thickness and 20 mm in length), a depression of microbial colonization and degradation with decreasing width of the rubber film was apparently observed. More than 40/mm^2 of colonies can be seen on the surface of the 6 mm width rubber films of glove B after 14 days cultivation, while the number of colonies on the surface of the film measuring 3 mm in width was 15/mm^2 on average. Very few colonies (2/mm^2 on average) were observed on the narrowest films.

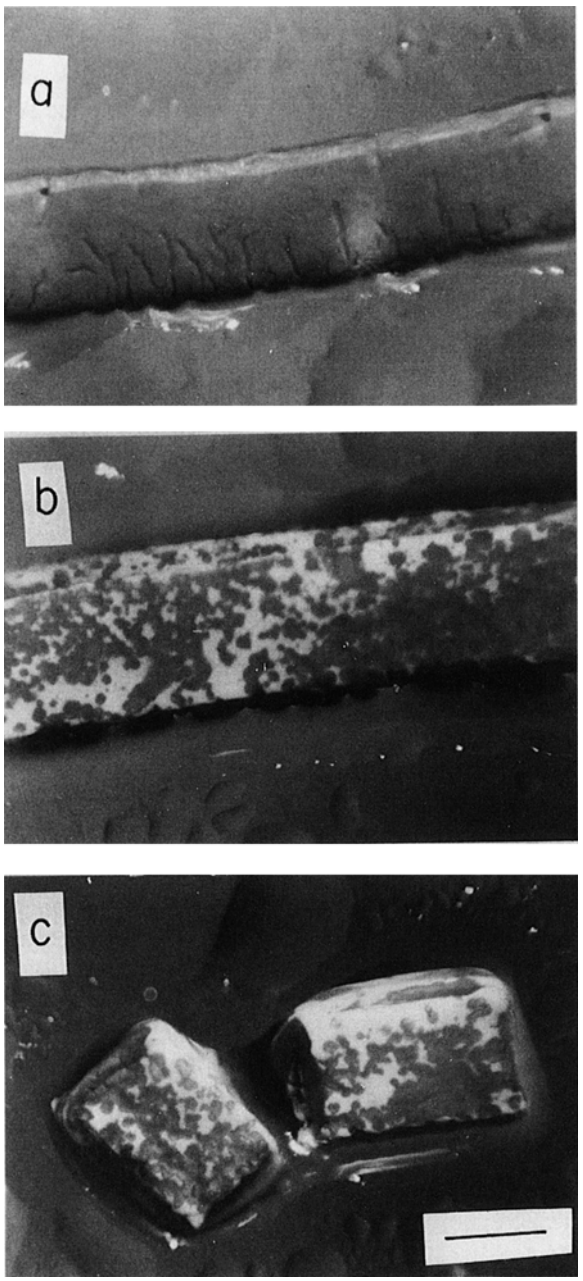

Fig. 4. The effect of length upon colonization of band D. Colonization of rubber pieces after 14 days was followed by staining with Schiff reagent. (a), a long strip of band D (120 mm in length); (b), a short strip (20 mm); (c), very small pieces (1.5 mm). The bar represents 1.0 mm.

The effect of thickness and surface area upon degradation rate

The effect of thickness upon microbial degradation was apparently observed in Fig. 2. The data regarding long

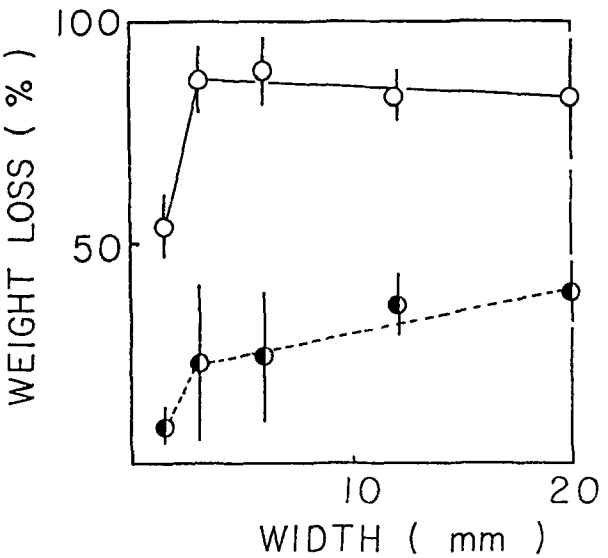

Fig. 5. The effect of width upon microbial degradation. Symbols: ○ — ○, glove A (0.1 mm in thickness and 20 mm in length); ◐ — ◐, glove B (0.2 mm in thickness and 20 mm in length). The vertical lines indicate the deviations of values.

(120 mm) and short (20 mm) strips of rubber are rearranged in Fig. 6 according to the surface area. With the long strips, the rate of degradation (expressed by weight loss %) was a linear function of surface area per unit weight of rubber, regardless of thickness and width. The weight losses of short strips were also in good correlation with the surface area, but the degradation rate of glove A, which had the largest surface area, was exceptionally high. Besides, deviation of the data for short strips was larger than that for long strips.

Discussions

The adherence of microorganisms to various insoluble target substrates is considered to be a key factor in the further utilization of these substances (Minato & Suto 1979; Iham & Gould 1990; Ohmura et al. 1993). It has been observed that cells of *Clostridia* are tightly bound to the insoluble cellulose substrate in the initial stages of fermentation despite vigorous stirring of the cultures (Bayer et al. 1983). The growth of *C. cellulolyticum* on crystalline cellulose occurs when bacteria are attached to the substrate and it was postulated that cellulose colonization occurs according to the following process: adhesion, colonization, and release (Gelhaye et al. 1993a, 1993b). It is supposed that attach-

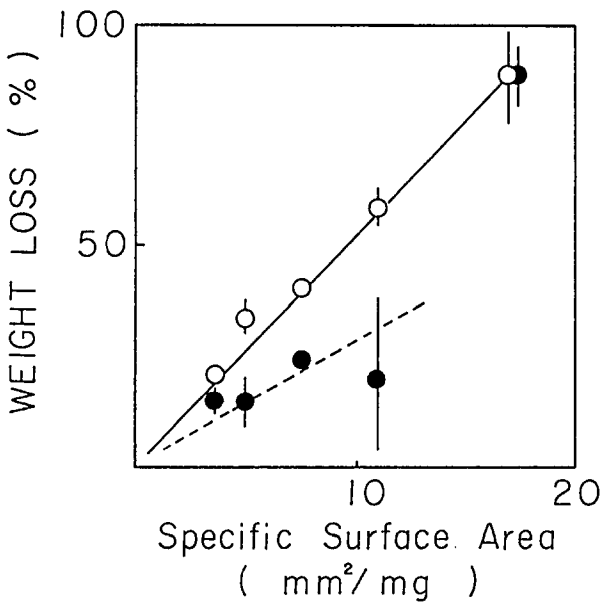

Fig. 6. The effect of surface area. Symbols: ○ — ○, long strips of rubber (120 mm in length); ● - - - ●, short strips (20 mm). From left to right, the circles represented band C, band D, band E, glove B and glove A.

ment and colonization of ruminal bacteria is necessary for the digestion of highly ordered crystalline cellulose (Kudo et al. 1987). It was suggested that the surface of poly(hydroxybutyrate) was important as a growing field for the degrading-bacterium and spherical holes were observed as the results of the colonization (Nishida & Tokiwa 1992).

The growth of *Nocardia* sp. also occurs essentially on the insoluble rubber substrate and the cells are tightly bound to the substrate in the initial stage of the growth. Although the considerable amount of the growth was observed on the surface of rubber pieces, the suspended cells in the medium was negligible in spite of vigorous stirring. In the early growth stages, the cells of the organism consists of branched filaments, and each one of the colonies can be separately observed on rubber surface. After 12 days cultivation, when most of the films of glove A was degraded, the filamentous cells fragment to shorter rods or cocoid units and released into the medium.

It was demonstrated that a reduction in substrate particle size and, thus, an increase in surface area per unit mass results in an increase both in leaching rate of zinc sulfide (Torma et al. 1972) and in the rate of sulfur oxidation by *Thiobacillus* sp. (Laishley et al. 1986). On the while, the rate of copper leaching from chalcopyrite minerals by *T. ferrooxidans* increased with increasing particle size from 0.06 to 1.4 mm, in spite of decreasing surface area. It was found that the cell attachment efficiency in a shake flask was higher for larger particles, and the higher rate of leaching with increasing particle size is supposed to be attributable to the attachment efficiency (Kumar et al. 1991).

In this communication, the effect of rubber shapes and surface area on the rate of rubber degradation by *Nocardia* sp. was studied. Among rubber pieces of various shapes, the long strips (120 mm) were rapidly and uniformly colonized and degraded most rapidly in stirred flasks. With these long strips, the rate of microbial degradation (expressed by weight loss %) was a linear function of surface area per unit weight of rubber. Thin and wide films of rubber were also rapidly colonized and degraded, while the colonization and degradation of short and narrow pieces, which have essentially identical surface area with long strips, were substantially slower and less extensive. The fact that the rate of degradation was always in good correlation with the number of colonies per unit area of the rubber surface suggest that the efficiency of colonization plays an important role and that the colonization could be a key factor affecting the degradation rate.

The movement of rubber pieces in stirred flasks differed considerably depending on their length and width, and hence rubber pieces of different shapes have different flow field around them. In order for colonization to take place, microbial cells must collide with the rubber surface and attach to it. Hence, the rate of colonization will be proportional to the number of collisions and the attachment efficiency. From this point of view, the relative velocity of water flows (and suspended cells) against the rubber surface is an important factors affecting the colonization efficiency. Consequently, the effect of rubber shapes upon colonization and degradation could be attributable to the effect of the shapes upon water flows around the rubber piece and collisions of cells suspended in water with the rubber surface. In order to estimate the rate of degradation, the effect of water flows around the rubber piece upon oxygen supply and cell proliferation also should be taken into consideration.

The long strips tended to get entangled around the bar (Fig. 7a) and strongly push the water away and forward. As a result, the number of collisions between cells and the rubber surface can be supposed to be great. In contrast, the short and narrow strips of glove B and band D floated in the medium and were carried

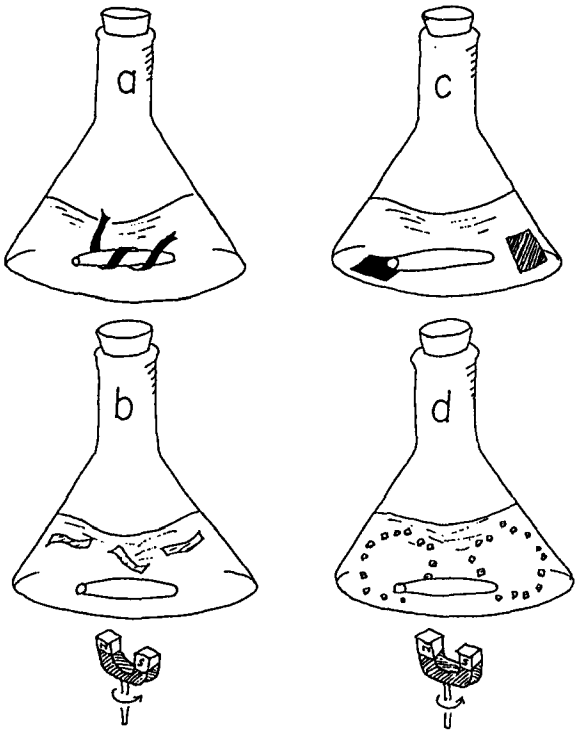

Fig. 7. Schematic diagrams of the movement of rubber pieces in stirred flasks. (a), a long strip of rubber; (b), short and narrow pieces of rubber; (c), wide and thin films; (d), very small pieces.

by the water flow in the flasks (Fig. 7b) and the relative velocity of cells against the rubber surface may be small. Consequently, the frequency and relative velocity of collisions between cells and rubber may be small in spite of vigorous stirring. In the case of glove A, the thin films readily stuck to each other, and the adhering film aggregate tended to attach to the magnetic bars. This observation may explain the exceptional high rate of colonization and degradation of the short pieces of glove A (Fig. 9). Since the rubber films with width of 20 and 12 mm tightly adhered to the glass wall at the bottom of the flask (Fig. 7c) and did not readily separate from the wall at the initial stage of growth, the relative velocity of water flows (and suspended cells) against the rubber surface may be large. Still shorter and very small pieces having lengths of 5 to 1.5 mm followed the stirring of magnetic bars and turned vertically in the medium (Fig. 7d). Although the number of colonies on the surface were not so many, they were degraded rather rapidly probably because that their specific surface area was relatively high.

Jar-fermenter experiments for microbial degradation of a commercial surgical glove revealed that rapid and uniform colonization of the rubber films is the most important rate-determining step (Kajikawa et al. 1991). The present study also demonstrated that the colonization efficiency is very important for rapid degradation of rubber pieces. The experimental conditions of degradation test described in the text gives a result with sufficient reproductivity in a relatively short time period and the method was considered suitable for comparison of various kinds of rubber products in laboratory conditions. The degradation of long strips in stirred flasks, however, would be inadequate for the treatment of a large amount of waste products. Further study will be required to improve the culture conditions for colonization and degradation of short and narrow pieces of rubber, particularly in the case of hard type rubber products like tire.

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