AEROBIC DECOMPOSITION OF GUAYULE SHRUB (*PARTHENIUM ARGENTATUM* GRAY)[1]

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INTRODUCTION

Aerobic microbial decomposition of guayule shrub (*Parthenium argentatum* Gray) by natural flora has recently been investigated as part of the research program dealing with natural rubber carried out at this Laboratory. In the course of this work, we obtained data of general interest on the broad problem of the aerobic decomposition of woody tissue by natural flora and the biochemical changes thus produced. Only those changes taking place in the first two or three weeks were investigated, because such periods appear to be as long as would be industrially feasible in rubber recovery. The data obtained show that under proper conditions a marked disintegration of the tissue can be brought about in one to two weeks. Aspects of the problem which bear upon the technology of guayule rubber will be presented elsewhere at a later date.

Decomposition of plant materials by microorganisms has been studied extensively in relation to soil fertility, peat formation, and manures. The voluminous literature on this subject has been reviewed by Waksman (1932, 1940). With few exceptions emphasis has been placed primarily on the changes in the material undergoing decomposition and secondarily upon the agents bringing them about.

Guayule is a small, profusely branching shrub which is native to the plateau of northern Mexico and the southwestern part of the United States. The ecology and anatomy of the plant were extensively investigated by Lloyd (1911). Certain aspects of the anatomy of the plant have been further investigated recently by Artschwager (1943).

The aerobic decomposition of whole and defoliated guayule was employed by Spence (1933) to improve the quality of rubber recovered in the industrial milling process. The agents responsible for the changes were not investigated, but from the nature of the environment, such as high moisture, and from the resulting effects (thermogenesis, ammonia production, etc.) it can be assumed that they were microbial. He called this process "retting." Although the term cannot be used here in its strict sense as applied to flax, it does signify that the changes are microbiological and is so used in this paper.

[1] Natural Rubber from Domestic Sources. Paper No. 3.

[2] This is one of four Regional Research Laboratories operated by the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture.

METHODS

Preparation of shrub

The air-dry, two-year-old shrub was mechanically defoliated and cut in 1- to 2-inch lengths in an ensilage cutter. It was then passed through a cane mill in which pieces larger in diameter than one-quarter inch were crushed to about this thickness and many of the smaller pieces were cracked. The shrub treated in this way had a density of about 16 pounds per cubic foot on a dry-weight basis, or 46 pounds per cubic foot at 65 per cent moisture. The resultant air space in the mass at 65 per cent moisture, assuming a density of 1 for the wet shrub, was about 25 per cent of the volume occupied by the shrub.

Sampling and analysis

Chemical. The prepared shrub was riffled to a 1- to 2-per cent sample, which was cut fine, mixed, and used for chemical analysis. The retting shrub was sampled by removing the entire charge and mixing it thoroughly, then removing about 70 pounds (wet weight), which was cut fine, mixed, and grab-sampled for analysis. All chemical analyses except moisture are expressed on a dry-weight basis.

The chemical constituents were determined by standard methods of the Association of Official Agricultural Chemists (1940) for analysis of plants. The pH was determined by a glass electrode meter upon a sample prepared as follows: Five grams of finely macerated shrub were covered with 15 ml of distilled water; after 5 minutes the pH of the supernatant was measured.

Microbiological. Two 40- to 50-g (wet weight) samples were removed aseptically each day from the retting mass to sterile petri dishes. One sample was usually taken from 3 to 4 inches below the surface and one at the center of the mass. A representative 10-g portion of each sample was cut up with sterile pruning shears, placed in a sterile 90-ml water blank and held for 30 minutes, with occasional shaking. It was then shaken vigorously for 3 minutes. Appropriate dilutions for the assay were made in sterile water blanks. Preliminary investigations showed beef-extract peptone agar, adjusted to pH 6.8, to be a suitable substrate for making plate counts of the bacteria present. Media containing various concentrations of guayule leaf infusion did not allow the maximum number of organisms to develop. Addition of glucose to the nutrient agar failed to improve the substrate. Resin emulsion agar (Allen, Naghski, and Hoover, 1944) was also unsatisfactory for total counts because the colonies developed more slowly, although it proved useful for detection of resin-digesting organisms. All microbiological data are expressed on a wet-weight basis.

Appropriate dilutions were made for the determination of fungi in poured plates of glucose-peptone-acid agar, adjusted to pH 4.5 with H_2SO_4.

As the temperatures at which the experiments were conducted were at the upper limit for the mesophilic microorganisms and the lower limit for the thermophilic forms, plates for bacteriological analysis were poured and incubated at the average temperature of the retting shrub. In experiment U24S5 parallel plates

were also incubated at 37 C. Incubation time varied with temperature; a period of 24 hours was found sufficient for 42 C and higher, and 2 days for the lower temperatures. All acid-agar plates were incubated at 32 C, and readings were made on the third and fifth days.

To detect anaerobic spore-formers, 1 g of the sample was cut fine, transferred aseptically to 9 ml of sodium-thioglycolate meat-infusion broth (Brewer, 1940), shocked at 80 C for 10 minutes, and cooled. Serial dilutions were made in tubes of the same medium and incubated at 32 C.

The medium of Dubos (1928) was used for the detection of mesophilic cellulose fermenters and that of Viljoen, Fred, and Peterson (1926) was used for the thermophilic ones.

The usual laboratory methods were employed in the study of pure cultures isolated in the course of the investigation. The isolates were classified according to Bergey (1939) and Henrici (1930).

TABLE 1

Biochemical classification of cultures isolated from microflora of decomposing guayule

MEDIUM	SACCHAROLYTIC	NEUTRAL	PROTEOLYTIC
Litmus milk	ACR	AR, R, or Alk. R	Alk R or Alk Pep
Gelatin liquefaction	–	–	+
Glucose broth	A	A or sl A	Alk or sl A
Lactose broth	A	sl A – or Alk	– or Alk

A = acid; C = curd; R = reduction; Alk = alkaline; Pep = peptonization; sl = slight; – = negative or no change; + = positive.

Classification of the microflora. About 15 representative cultures were isolated from countable plates of each sampling and studied culturally, morphologically and biochemically. On the basis of gram reaction and morphology, the organisms present were divided into the following three groups: (1) gram-negative rods, (2) spore-forming gram-positive rods, and (3) actinomycetes. Occasional cultures of cocci, non-sporulating gram-positive rods, spore-forming gram-negative rods, and yeasts were found; all of these were classed as "others."

All cultures were classified on the basis of reaction after incubation for two weeks in litmus milk, nutrient gelatin, glucose broth and lactose broth into the following: (1) Saccharolytic, (2) Neutral, (3) Proteolytic. The neutral group comprises those organisms which are not definitely saccharolytic or proteolytic. The characteristics of these groups are given in table 1. It is obvious that the conditions chosen are rather arbitrary and a somewhat different distribution might be arrived at if other criteria were employed. Reid, McKinstry, and

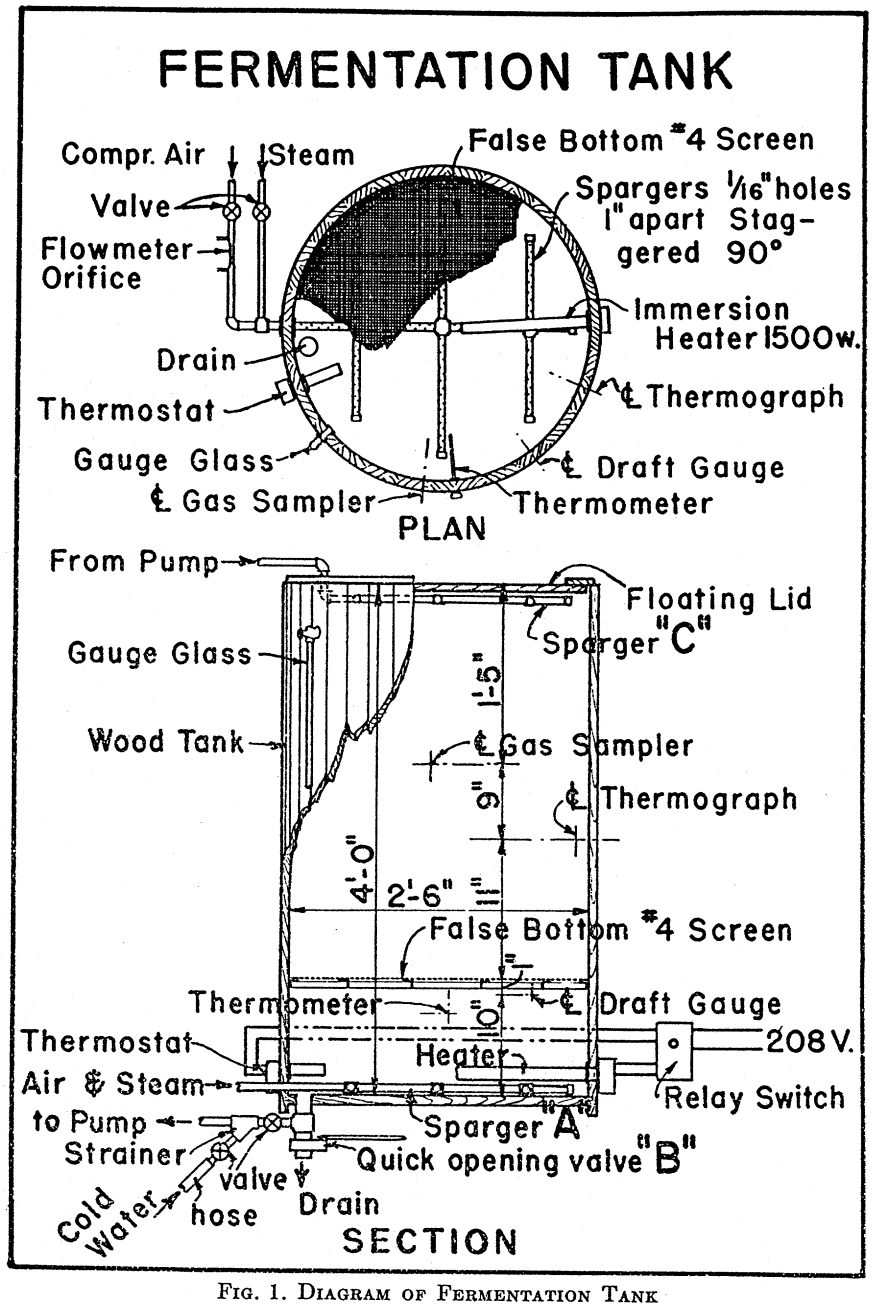

FIG. 1. DIAGRAM OF FERMENTATION TANK

Haley (1938) have discussed the effect of time of incubation upon physiological classification and the effect of adaptation of the organism to the cultural conditions upon its physiological response.

Equipment and procedure for retting

Retting was carried out in a 150-gallon wooden tank (figure 1) equipped as shown. The shrub was held on a false bottom 12 inches above the bottom of the tank. Thirty-five kg of dry shrub, prepared as described previously, filled the tank to a depth of 12 inches. Conditions in the retting mass were controlled as follows:

Preboiling. The shrub was covered with tap water and submerged by a perforated false top held down by stones. Steam was passed into the water through sparger A (figure 1) upon the bottom of the tank. The water was brought to a boil in 30 to 40 minutes and then boiled for 30 minutes, after which the froth of resinous material upon the surface was skimmed off, and the water drained through gate valve B. The shrub was cooled in about 10 minutes by spraying it with cold water. In addition to removing some of the resinous matter, preboiling softened the surface tissues by hydration.

Inoculation. Because of the difficulties inherent in the application of pure culture techniques to the microbiological decomposition of large tonnages of plant material, inoculation was not attempted. The prolific natural flora of the shrub was not destroyed by the preboiling treatment. Its response to controlled physical conditions was studied and is presented here.

Temperature control. Observation of the temperature was made by means of a thermograph, the heat-sensitive portion of which was buried in the middle of the mass. About 24 gallons of water in the bottom of the tank were maintained at the desired temperature, ± 1 C, by means of an electric heater controlled by a thermostat. The water was removed and replaced with fresh water daily. When thermogenesis raised the temperature of the shrub above that desired, this water was pumped up and sprayed on the shrub through the sparger C until the temperature of the mass approximated that of the water. Evaporation of the water spray cooled the circulating water to a temperature slightly below that maintained beneath.

Aeration. A constant flow of humidified and warmed air through the mass was obtained by passage of compressed air through a needle valve, a calibrated flowmeter, the sparger A, and the electrically heated water in the bottom of the tank.

EXPERIMENTAL RESULTS

Effect of temperature on decomposition of boiled shrub

A series of three experiments was carried out in which preboiled shrub was retted at three temperature levels. The conditions of these experiments are summarized in table 2.

The results of experiment U24S3 are of especial interest, in that the temperature was held in a range above that considered normal for mesophilic organisms and below the thermophilic range of 50 to 60 C. The temperature changes, taken from the thermograph charts, are shown in figure 2. These data can be more satisfactorily interpreted in relation to the microflora and the chemical

TABLE 2

Retting conditions in temperature series

EXPERIMENT NO.	TEMPERATURE OF RETTING	INITIAL DRY WEIGHT	LOSS IN WEIGHT ON BOILING	TIME OF RETTING	MOISTURE CONTENT ON FOURTH DAY	AIR FLOW
	C	*kg*	%	*days*	%	*Ft³/hr/ft² shrub*
U24S4.....	35–38	23.2	13.3	7.5	67.91	2.5
U24S3.....	42–48	52.8	8.93	12.6	61.05	1.4
U24S5.....	50–54	28.4	—	14	61.07 (fifth day)	2.1

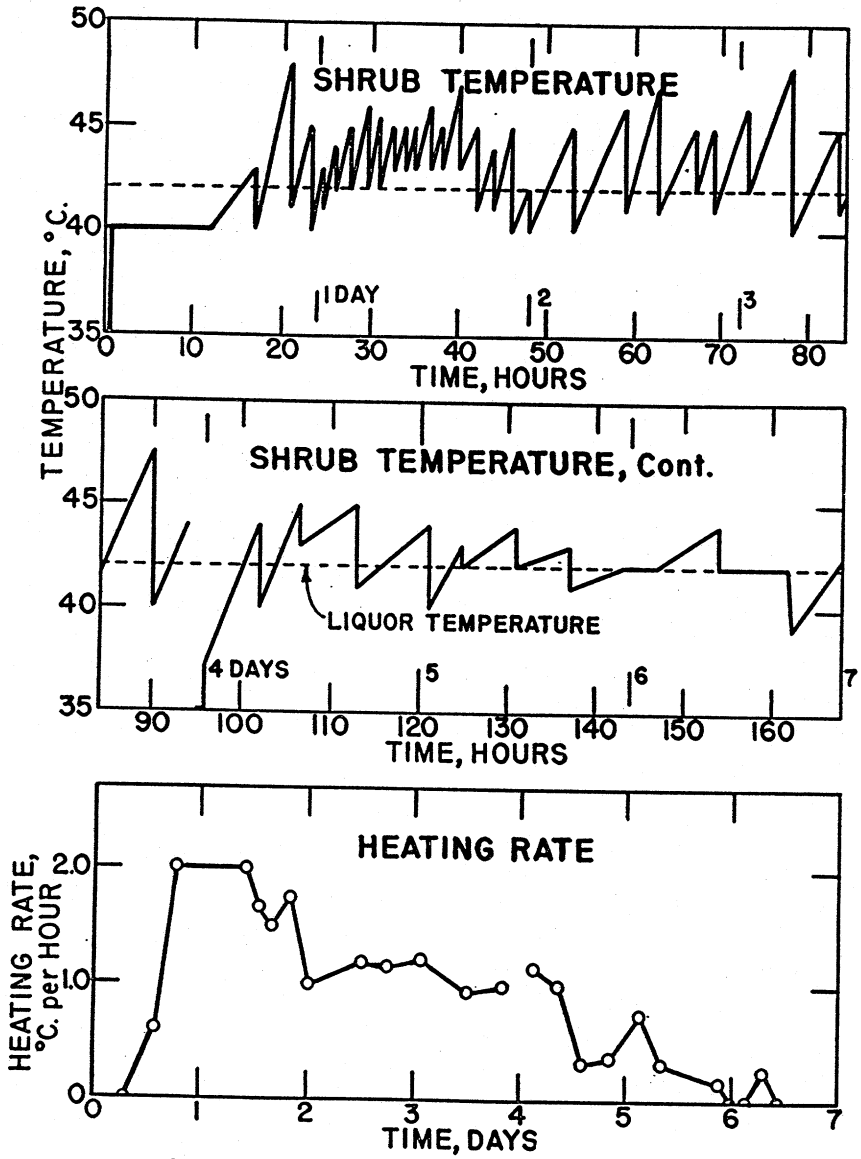

FIG. 2. HEATING OF SHRUB IN EXPERIMENT U24S3

changes if the rate of heating is plotted against time, as in the lower part of figure 2.

(Measurement of the rate of heat production in the uninsulated system used cannot give true results for the caloric output of the fermentation, as various heat losses and secondary effects, such as frequency of cooling, affect the data. Thus, comparable data for experiment U24S4 could not be obtained because the maintenance of a temperature below 38 C required frequent circulation of cooling water. In experiment U24S5 the heat losses to the exterior were so great that they largely masked thermogenesis. In all experiments carried out in the range 40 to 52 C (U24S3, -6, -7, -8), thermogenesis was measured.)

The total numbers of bacteria plus actinomycetes and of fungi appearing in the three experiments were strikingly similar (figure 3). The initial rate of development of the microflora after the shrub was boiled for 30 minutes is noteworthy. Counts of a billion per gram were reached on the first or second day. The only significant difference in the total counts was the smaller number of fungi in the retting shrub held above 50 C.

(It is apparent that some error in determination of total numbers of bacteria plus actinomycetes occurred in the ninth-day sampling of experiment U24S5. This error, of course, lowers the number of organisms in the various biochemical classes.)

The biochemical classification of the bacteria plus actinomycetes in the three experiments is presented in figure 4. Consideration of these data is facilitated by comparison with the changes in chemical composition and pH (figure 5). Losses in dry weight, water-soluble carbohydrates, and nitrogen due to boiling the shrub are shown in the initial portions of the graphs. Measurement of pH was made after the shrub was boiled. Other experiments confirm the major conclusions to be drawn from these data; namely, that the water-soluble carbohydrates were completely removed in a week, whereas the nitrogen content fell slightly (to about 1 per cent) and remained at that level. The correlation of the decreasing numbers of saccharolytic organisms and the end of the thermogenic phase with the removal of available carbohydrates is shown by figures 2, 4, and 5.

A further criterion of microbiological attack upon woody tissue is the softening and disintegration of the substrate due to digestion of the hemicellulose, pectin, and other structural constituents. In the absence of any satisfactory objective method for measuring this effect, the softening was judged by the feel of the tissue.

Maintenance of a temperature of 35 to 38 C in experiment U24S4 necessitated spraying the shrub at hourly intervals. Certain differences observed in this experiment are attributed to the consequent greater moisture content of the shrub (table 3). The natural redox indicator in the shrub was in the reduced (yellow) state, in contrast to the brownish-black color characteristic of its oxidized form. The pH was significantly lower throughout. In general the shrub seemed to be somewhat waterlogged, and, as is shown in table 3, it disintegrated more rapidly than in the other experiments. The appearance of large numbers

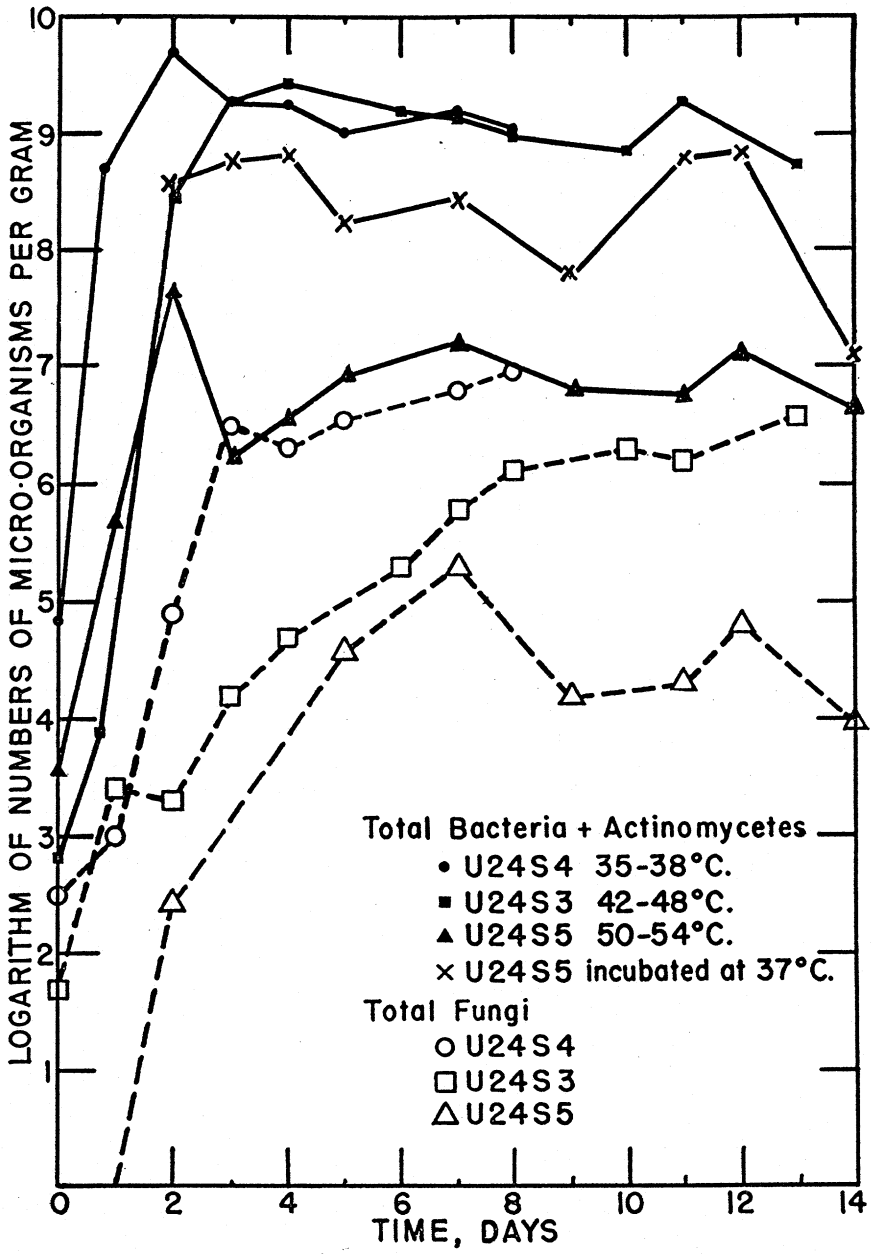

FIG. 3. EFFECT OF TEMPERATURE ON TOTAL NUMBERS OF MICROORGANISMS ON RETTING GUAYULE

of yeast-like fungi was consistent with the observation that the shrub was waterlogged. These results are analogous to those observed by Lambert and Davis (1934) in aerobic and anaerobic portions of mushroom compost heaps.

Retting of unboiled shrub

In experiment U24S6 a similar lot of 38.4 kg was prepared and retted in the same manner except for omission of the preboiling. In this case, the shrub was

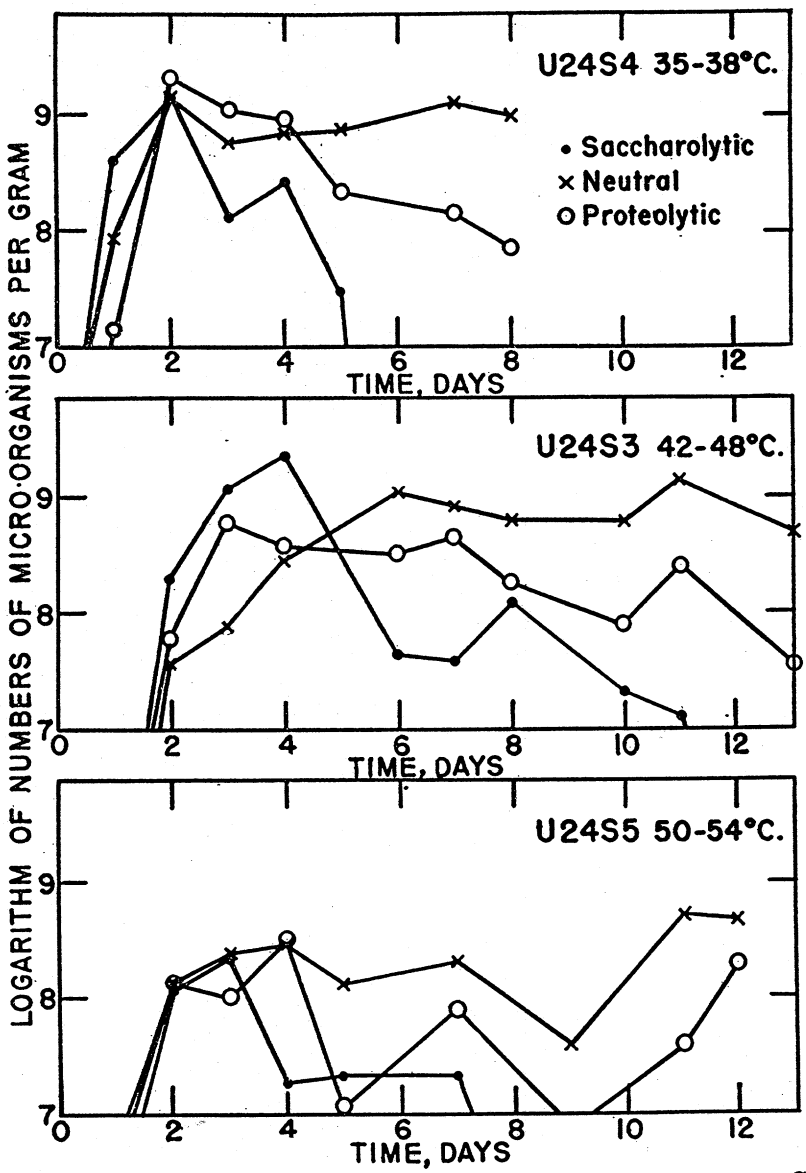

FIG. 4. EFFECT OF TEMPERATURE OF RETTING GUAYULE ON THE PHYSIOLOGICAL CLASSIFICATION OF THE MICROORGANISMS

hydrated by circulating water, kept at 44 C, twelve times the first day and less frequently thereafter. As the previous series had shown the distribution of types to be relatively insensitive to temperature variation, the temperature was

allowed to range between 42 and 50 C. The total numbers of bacteria plus actinomycetes and of fungi, and the biochemical classification of the bacteria plus actinomycetes, are plotted in figure 6. Comparison of these data with those

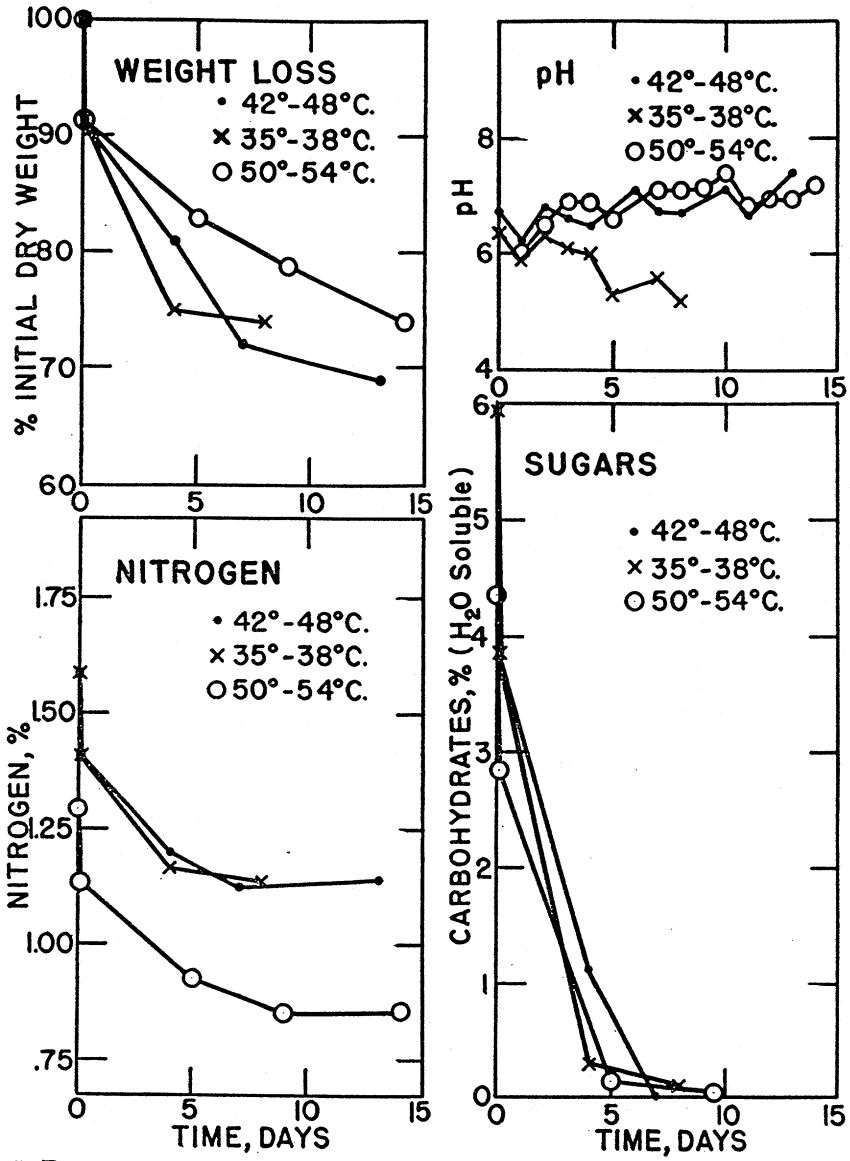

FIG. 5. EFFECT OF TEMPERATURE ON CHEMICAL CHANGES OCCURRING IN RETTING GUAYULE

obtained in experiment U24S3 shows no significant differences in the floras. The changes in chemical composition (table 4) were also similar to those observed in the comparable experiment. Thermogenesis was no longer apparent after

4 days. This phase is here again correlated with the presence of a saccharolytic flora and available carbohydrates. Maceration of the tissue appeared to take place somewhat more slowly than in experiment U24S3.

Effect of bulk on retting

Inasmuch as the process under investigation was aerobic, the effect of bulk was studied next, in experiments U24S7 and U24S8. A greater bulk necessarily increases the difficulty of removing the heat of thermogenesis and maintaining adequate aerobiosis. In each case, 130 kg of shrub (unboiled) was solidly packed into the retting tank and pressed down. The resultant density was 54 pounds per cubic foot at a moisture content of 65 per cent. Microbiological analysis was handicapped by the necessity of sampling only near the top of the bulk. The results therefore were not so consistent as in the previous experiments but did show the same general trends. Thermogenesis was essentially complete after a week, and analysis showed that the available carbohydrates were exhausted at that time. Each experiment was continued for a total of 21 days. At the end of this time a loss in dry weight of about 29 per

TABLE 4

Chemical changes in retting of unboiled shrub in experiment U24S6

TIME	H_2O	N	WATER-SOLUBLE CARBOHYDRATES	LOSS IN WEIGHT	pH
days	%	%	%	%	
0	18.00			0	6.5
4	61.06	1.06	<0.15	14.0	7.9
8	63.60	1.01	0.00	16.6	7.7

cent (29.4 and 28.4 per cent) had been obtained. Maceration of the tissue paralleled that observed in experiment U24S6. A + + effect was present in 14 days and a + + + effect in 21 days. The latter indicates extensive but not complete disintegration of the tissue.

The possibility of anaerobiosis was investigated by analyzing for CO_2 (in the gas phase) at the center of the mass throughout the thermogenic period. A sampler of rubber pressure tubing was buried with one end in the center of the decomposing material and daily analyses were made. Because of the large bulk the air flow was set at 6.5 cubic feet per cubic foot of shrub per hour. The degree of packing was judged by the rate of drainage of water through the shrub and the development of back pressure in the air chamber beneath the false bottom. Passage of air or water through a heterogeneous mass such as this is affected by stratification of the finer material, with consequent channeling. Therefore no general interpretation of the data in table 5 can be made. They do show, however, that under these particular conditions the microflora can produce CO_2 tensions approaching anaerobiosis. Heating and the pH values were concurrently lowered. After the fifth-day sampling, the upper portion of the mass was loosened as much as possible without removing it. The effect

of this loosening is apparent in the stimulation of the rate of heating and the sharp increase in pH.

Invasion of intact guayule twigs by microorganisms

The previously discussed experiments were carried out upon shrub which had been crushed by passage through a cane mill. If the organisms could invade intact woody tissue rapidly enough, however, the crushing operation could be dispensed with. Accordingly, 400 gm of intact dry twigs from one-quarter to one-half inch in diameter were cut into 3- to 4-inch lengths, hydrated by immer-

TABLE 5

Changes in temperature, pH, CO_2 content of the gas phase, and packing, in bulk retting
(Aeration at 6.5 $ft^3/ft^3/hr$)

TIME	INCREASE IN TEMPERATURE	pH	CO_2	PACKING*
days	*C/hr*		%	
1	1.1	5.4	2.9	0
2	2.3	7.2	6.5	0
3	1.25	6.9	6.5	+
4	0.8	—	10.6	++
5	0.7	5.4	14.9	+++
6	1.6	7.5	6.3	+†
7	0.8	7.6	11.6	++

*0 = free flow of air and water; + to ++++ = decreasing rate of drainage of water and ncreasing back pressure in the air chamber beneath the shrub.

† The top 18 inches of shrub was loosened with a pitchfork following the fifth-day observations.

sion in tepid water, and incubated at 42 C. At 2-day intervals the shrub was plated out according to this scheme:

1. Untreated control
2. Scrubbed with a brush, rinsed with sterile distilled water, dried with a towel, dipped momentarily into alcohol, and flamed
   - A. Whole pieces of twigs
   - B. Twigs subdivided aseptically
     - (1) Bark
     - (2) Wood

As a check upon the experimental procedure, certain samples were plated immediately after scrubbing (without flaming), and others were scrubbed and then passed several times through the flame of a bunsen burner. These samples (table 6) gave counts which differed only slightly from the corresponding samples which had been treated with alcohol and flamed, demonstrating that the alcohol treatment was only slightly toxic to the organisms within the twig.

The major portions of the bacteria and fungi were removed by scrubbing and rinsing with sterile water, and therefore had been upon the exterior of the twigs. The results obtained upon the separated bark and wood showed that the remain-

ing organisms had been present primarily in the bark of the intact twigs. Moreover, the mycelial fungi did not invade the wood more readily than did the bacteria. It was concluded on the basis of these data that a preliminary crushing of the tissue is essential for the rapid aerobic decomposition of the wood.

It should be noted that the total count was far less than that observed in the larger-scale experiments (figures 3 and 6). This can be, at least partly, attributed to the difficulty in hydrating the small amount of selected twigs used and in maintaining hydration. If greater hydration could have been attained, it is probable that greater invasion of the wood would have resulted.

TABLE 6

Invasion of uncrushed guayule twigs by microorganisms

DAYS	CONTROL		Scrubbed, then flamed with ethanol					
	Whole		Whole		Bark		Wood	
	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi
	millions per gm	*thousands per gm*	*millions per gm*	*thousands per gm*	*millions per gm*	*thousands per gm*	*millions per gm*	*thousands per gm*
2	620	2,370	170*	180*				
4			5.2	0.3				
6	470	50	16.3	0.6	28.6	2.0	0.16	0.1
8	347	60	45.0*	3.0*	60.0*	2.1	0.04*	<0.01*
			26.4	<0.01	3.3	<0.01	12.2	<0.01
10	340	9	100†	4†				
			26‡	0.2‡				

* Scrubbed only.
† Washed without scrubbing, not flamed.
‡ Flamed without ethanol.

Identity of microorganisms

Although our primary aim was the study of the biochemical activities of the natural flora, some of the 1108 cultures isolated were further studied and classified. Those organisms capable of attacking the "resin" of guayule rubber and of the guayule plant are discussed by Allen, Naghski, and Hoover (1944).

Bacteria and actinomycetes. The saccharolytic forms were a distinct group, readily recognized by their fermentative powers (table 1). Ninety-one per cent of these were gram-negative short rods, which comprised 20 per cent of the microflora. Growth on agar slant is white, filiform, and abundant; agar colonies are white and convex. These organisms do not liquefy gelatin. Growth occurs at 50 C but not at 55 C. They were classified into three species of *Achromobacter* (table 7), on the basis of motility, reduction of nitrates, and indole production, according to Bergey's manual. This classification is also correlated with ability to decompose resins.

A few other saccharolytic forms were encountered, including gram-positive spore-forming rods, gram-positive non-spore-forming rods, gram-positive cocci, and actinomycetes, none of which were identified.

The neutral forms were more difficult to classify, since the reactions they produced in litmus milk and in carbohydrate media were slight and variable. Furthermore, prolonged maintenance in stock culture resulted in a diminished attack on carbohydrates, with the result that a previous slightly acid reaction became neutral or even alkaline. This group represented large numbers of gram-negative non-spore-forming rods, gram-negative spore-forming rods, and actinomycetes, and a small number of miscellaneous forms, including cocci, gram-positive non-spore-forming rods, and yeast-like forms. The gram-negative non-spore-forming rods comprised 37 per cent of the total flora and 63 per cent of the neutral organisms. They are very similar in their cultural and biochemical characteristics. Growth on agar slant is white, filiform, and abundant, turning slightly yellowish by transmitted light. Gelatin is not liquefied; litmus milk is unchanged or becomes slightly alkaline; glucose broth becomes slightly acid, then slightly alkaline; lactose broth is unchanged or becomes slightly alkaline; starch is not hydrolyzed; indole is not produced; reduction of nitrates

TABLE 7

Characteristics of saccharolytic Achromobacter sp.

ORGANISM	FLAGELLA	REACTION OF NITRATES*	INDOLE PRODUCTION*	RESIN DECOMPOSITION*
A. lacticum (Kramer) Bergey *et al.*	None	−	−	+
A. ubiquitum (Jordan) Bergey *et al.*	None	+	−	−
A. reticularum (?) (Jordan) Bergey *et al.*	Peritrichous	+	+†	−

* − = negative; + = positive.

† Described by Bergey as not producing indole.

to nitrites is variable. Growth occurs at 50 C but not at 55. On the basis of flagellation these organisms are subdivided into four species:

	Flagella
(a) *Pseudomonas incognita* Chester	Monotrichous.
(b) *Pseudomonas putida* Migula	Lophotrichous
(c) *Achromobacter alcaliaromaticum* (Berlin) Bergey *et al.*	Peritrichous
(d) *Achromobacter candicans* (Frankland and Frankland) Bergey *et al.*	absent—non-motile

The spore-forming rods appeared to be a homogeneous group of organisms. They represented only 3 per cent of the organisms isolated and only 6 per cent of the neutral forms. About 90 per cent of these were gram negative. Vegetative cells are 0.4 to 0.6 x 2.2 to 3.1 microns; terminal ellipsoid endospores are 0.9 to 1.2 x 1.0 to 1.3 microns. Sporangia are generally swollen terminally. The rods are motile by means of peritrichous flagella. Growth on agar slants is scant, white to transparent; agar colonies are small (0.5–1 mm); gelatin is not liquefied; litmus milk, glucose, and lactose broth become slightly acid, then slightly alkaline. Most strains grow at 55 C. They undoubtedly belong to the *Bacillus circulans* group. Considering the close resemblance between the

numerous members of this group, no attempt was made to determine the species.

Ninety-three per cent of the *Actinomyces* isolated belonged to the neutral group of organisms, and comprised 27 per cent of the neutral organisms isolated. They were important in the later stages of retting (7 to 21 days), when they comprised over half the flora. Since we were primarily interested in the earlier stages, however, no attempt was made to determine the species represented, although it appeared from certain preliminary studies that two species predominated, one producing slight acidity in carbohydrate media, the other an alkaline reaction.

Twenty per cent of the cultures isolated from retting guayule were the proteolytic forms. Of these, 20 per cent were gram-negative rods. Cells are 0.5 to 0.6 x 1.0 to 2.0 microns and are motile by means of a single polar flagellum. Growth on nutrient agar is white, filiform, and abundant, the medium rapidly becoming a deep blue-green; gelatin is completely liquefied, becoming deep blue-green; nutrient broth becomes turbid and similarly pigmented, the presence of pyocyanin being readily demonstrated; litmus milk is completely peptonized, having an alkaline reaction within 2 days; glucose broth becomes slightly acid; lactose broth turns alkaline; starch is not hydrolyzed; nitrates are reduced to nitrites; indole is not produced. Growth occurs at 45 C but not at 50 C. These forms are classified as *Pseudomonas aeruginosa* (Schroeter) Migula (Reid, Naghski, Farrell, and Haley, 1942; Seleen and Stark, 1943). They were found in largest numbers in experiment U24S4, fairly often in U24S3, but only occasionally in U24S7 and U24S8. None were detected in U24S5 or U24S6. They were not found when the temperature of the retting shrub exceeded 45 C.

The other species is characterized as gram-negative non-motile rods, 0.5 to 0.7 x 1.0 to 2.1 microns. Agar colonies are yellow, convex, entire; growth on agar slant is yellow, filiform, and abundant; gelatin is rapidly liquefied; litmus milk is completely proteolyzed, with alkaline reaction; dextrose broth is made slightly acid, turning alkaline; lactose broth slowly turns alkaline; starch is hydrolyzed; indole is produced; nitrates are not reduced to nitrites. Growth occurs at 45 C but not at 50 C. This description is in agreement with the limited description of *Flavobacterium fecale* Bergey *et al.* These forms made up 20 per cent of the proteolytic organisms. They were observed only in experiment U24S4, which was held at 35 to 38 C throughout.

The other proteolytic gram-negative rods were not classified, as they occurred only occasionally and varied considerably in their cultural and biochemical reactions. They probably represented other species of *Pseudomonas* and *Achromobacter*.

The gram-positive spore-forming rods made up 37 per cent of the proteolytic organisms and without exception were *Bacillus subtilis* Cohn.

Only a few (5 per cent) of the actinomycetes were placed in this group of organisms. They appeared in small numbers in all the experiments except U24S6.

Fungi. The fungi present in guayule shrub represented many forms, including

species of *Alternaria*, *Mucor*, *Rhizopus*, and *Aspergillus*. Selection took place during retting, however, and after 3 to 4 days *Aspergillus fumigatus*[3] was the predominating mold. It appears highly significant that this organism should also be capable of attacking guayule resin (Allen, Naghski, and Hoover, 1944). It was present in counts of 10^5 to 10^6 per gram in all experiments. In experiment U24S4, extremely large numbers of yeast-like forms were also found; they made up over 90 per cent of the total fungal flora in this experiment.

Others. The large numbers of facultative organisms made it difficult to obtain an estimate of the number of anaerobes in the shrub, either by deep-tube or plate methods. It was possible, however, to detect their presence in the spore state by heat shocking the material at 80 C for 10 minutes and making serial dilutions in sodium thioglycolate infusion broth. Occasionally numbers as high as 10^5 per gram were observed. In stained sections of these samples, plectridia were readily demonstrated, existing between the cells in the pith.

The presence of cellulose fermenters could not be demonstrated conclusively. On several occasions they were detected on shrub that had undergone fermenta- for 18 to 21 days. Subcultures from the initial tubes failed to digest filter paper. This group was not investigated further.

Microscopic examination of shrub that had been retting 14 to 21 days revealed basidiomycete mycelium characterized by clamp cells. When these samples were permitted to remain in petri dishes at room temperature for about 10 days, typical sporophores were produced. No attempt was made to identify these forms further.

After 2 days of retting, the shrub in experiment U24S4 was covered with specks of white mucoid material. Microscopic examination showed these to be composed of masses of motile, short, rod-shaped bacteria, tangled masses of fungal mycelium, yeast-like cells, and numerous protozoa. This was the only experiment in which protozoa were detected.

DISCUSSION

Carlyle and Norman (1941) have called attention to the fact that heat is always evolved in biological decomposition and therefore under adiabatic conditions, where heat losses are prevented, the oxidation of any plant constituent is a thermogenic process. Thermogenesis in the natural heating of hay, composts, and the like therefore depends on the production of heat at a rate substantially greater than the rate of heat loss. Under the conditions of these experiments the oxidation of water-soluble carbohydrates by the saccharolytic organisms in the flora was clearly correlated with the phase of active heating. This phase was complete in 4 to 7 days.

A mixed flora of neutral and proteolytic organisms, primarily actinomycetes, pseudomonads, and *Achromobacter*, predominated in the second and third weeks. The major criterion for the separation of these groups was gelatin liquefaction. Although neutral organisms were present in greatest number, a very heavy

[3] We are indebted to Dr. K. B. Raper, Fermentation Division, Northern Regional Research Laboratory, for the identification of these organisms.

growth of proteolytic forms was also present at this time. The total counts obtained were in the billions per gram, calculated on a wet weight basis. The biochemical activities of a type of flora which is present to the extent of 10^8 organisms per gram cannot be neglected even if other organisms are present in numbers of 10^9. Therefore the decomposition observed in the latter phase can best be attributed to a mixed non-saccharolytic bacterial flora and fungi, and not to any one group of organisms.

The fungi, primarily *Aspergillus fumigatus*, were found in large numbers (*ca.* 10^6 g) throughout the experiments. Various workers, however, have called attention to the fact that vegetative mycelium may have a biochemical activity far greater than is indicated by dilution plating methods (Reid, McKinstry, and Haley, 1938) and on the other hand sporulation may produce large counts without much resultant biochemical activity. Fungi are generally considered to be of prime importance in the decomposition of forest litter, and their ability to attack woody tissue has been well established (Waksman, 1932). In view of these facts, we do not feel that the relative biochemical importance of the fungi and bacteria can be estimated upon the basis of these data.

The differences in the floras which developed on boiled and unboiled shrub do not appear to be of any significance. Although boiling the shrub for 30 minutes would be expected to eliminate all but spore-forming organisms, a profuse mixed flora developed with great rapidity. No effort was made to determine the source of the gram-negative organisms which appeared. Organisms of the same biochemical characteristics and essentially the same species predominated in the boiled and unboiled shrub. Therefore it appears that the composition of the substrate and the environment determine the succession of types of organisms.

Although the physiological grouping of the organisms was quite similar at the three temperature ranges investigated, characterization of the isolates brings out differences in the floras. At 35 to 38 C large numbers of forms growing at low temperature (*P. aeruginosa* and *F. fecale*) were found. Actinomycetes developed slowly, but after the fifth day exceeded the bacterial population. Large numbers of yeast-like fungi and the presence of protozoa were characteristic of this temperature.

At 42 to 48 C bacteria were the first to develop and remained dominant throughout the retting period. Yeast-like fungi and protozoa were not observed and only small numbers of the low-temperature organisms were present. Actinomycetes were slow in developing and equalled the number of bacteria only after two weeks of retting. *A. fumigatus* was the dominant fungus.

At 50 to 54 C the development of actinomycetes was greatly limited, and *A. fumigatus* also appeared to be inhibited by the higher temperature. Spore-forming bacteria in counts of 10^7 per gram were demonstrated by culturing at 52 C. By culturing at 37 C, however, bacteria common to the other temperature ranges were detected in counts of 10^9, showing that on decomposing plant material these mesophilic forms were active above 50 C, although they did not appear on agar plates incubated at 52 C.

These results are consistent with those of Waksman, Cordon, and Hulpoi

(1939) on the influence of temperature upon the flora in the composting of stable manures. Carlyle and Norman (1941) in their study of thermogenesis in the decomposition of straw showed that the mesophilic flora was active even above 50 C.

The rapid aerobic disintegration observed is further confirmation of the results of Stoller, Smith, and Brown (1937) and of Stoller (1943) upon rapid composting of plant residues. Their results with herbaceous plant materials are comparable with the extensive softening and disintegration of woody tissue obtained in these experiments.

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SUMMARY

Aerobic decomposition of crushed guayule shrub was carried out at 36, 44, and 52 C for one to three weeks. The profuse microflora was tested for its biochemical reactions, and the predominating organisms were identified.

A 20 to 30 per cent loss of dry weight, with marked softening and disintegration of the woody tissue, was observed in 5 to 14 days.

The disappearance of water-soluble carbohydrates and the existence of a saccharolytic flora were correlated with the markedly thermogenic phase occurring during the first week.

A mixed neutral and proteolytic flora followed during the second and third weeks.

Fungi, primarily *Aspergillus fumigatus*, occurred in numbers of 10^5 to 10^6 per gram throughout the whole period.

The organisms of major importance in the three physiological groups were:

Saccharolytic: *Achromobacter lacticum*, *Achromobacter ubiquitum*, *Achromobacter reticularum* (?).

Neutral: *Pseudomonas incognita*, *Pseudomonas putida*, *Achromobacter alcaliaromaticum*, *Achromobacter candicans*, *Actinomyces* spp.

Proteolytic: *Pseudomonas aeruginosa*, *Flavobacterium fecale*, *Bacillus subtilis*.

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