Pectin Decomposition by Species of Pseudomonas and Their Role in the Retting of Malvaceous Plants

S. M. Betrabet and J. V. Bhat

1958. Pectin Decomposition by Species of Pseudomonas and Their Role in the Retting of Malvaceous Plants. Appl. Environ. Microbiol. 6(2):89-93.

Pectin Decomposition by Species of *Pseudomonas* and Their Role in the Retting of Malvaceous Plants

S. M. Betrabet and J. V. Bhat

Fermentation Technology Laboratory, Indian Institute of Science, Bangalore, India

Received for publication July 8, 1957

Retting, which constitutes a vital step in the production of fibers like hemp, jute, and flax, is essentially a microbial decomposition process and depends upon the property of microorganisms to produce pectic enzymes that decompose pectic substances binding together the fibers. Although considerable information is available regarding the nature and activity of microorganisms involved in retting, (Ruschmann and Bavendamm, 1925a, b; Weizmann and Hellinger, 1940; Ruschmann and Bartram, 1943; Allen, 1944, 1946a, b; Debsarma, 1946; Hellinger, 1953) conspicuously no role, as yet, has been attributed to species of *Pseudomonas* in this process. The purpose of the present study is to report the dominance of *Pseudomonas* species in the retting of certain fiber yielding malvaceous plants and to present chemical evidence in support of the ability of these species to decompose pectin.

In his review on the utilization of pectic substances by microorganisms, Kertesz (1951) has rightly emphasized the need to add to our knowledge concerning the pectin fermenting bacteria which at present is limited and fragmentary. Of the 35 strains of plant pathogenic and fluorescent pseudomonads screened by Oxford (1944), for instance, only 6 strains could degrade pectic acid but the method adopted by him for detecting pectic acid decomposition was crude and qualitative in nature. Barinova's (1946) study, on the other hand, was confined to an analysis for the amount of pectin fermented by *Clostridium felsineum* and *Bacillus acetoethylicus* (*Bacillus macerans*). A complicating factor in the evaluation of earlier work in this direction was the impurity (nonpectic substances) contained in the pectic materials used in such work. In many reports, there is no mention of the kind of pectin used. Potter and McCoy (1952, 1955), however, had investigated in detail the fermentation of citrus pectin and pectic acid by *C. felsineum* and *Bacillus polymyxa*, but their report was limited to an investigation of these two bacteria. In the present investigation, we have put to test several isolates of *Pseudomonas* for their ability to ferment pectin *in vitro*. These isolations were made by enrichment culture method from the retted liquors of malvaceous plants.

Experimental Methods and Results

Enrichment and isolation of pectin decomposing bacteria. Malachra capitata, a good substitute for jute (Betrabet and Navalkar, 1956), and *Hibiscus cannabinus* were used for retting. The rettings were carried out both at room temperature (24 to 26 C) and at 37 C.

A medium of the following composition in 100 ml distilled water was used: Citrus pectin,[1] 1 g; Na_2HPO_4,

[1] Distillation Products Industries, Eastman Organic Chemicals Dept., Rochester, New York.

0.08 g; KH_2PO_4, 0.02 g; NaCl, 0.005 g; $MgSO_4 \cdot 7H_2O$, 0.05 g; $FeSO_4 \cdot 7H_2O$, 0.001 g; $CaSO_4 \cdot 2H_2O$, 5 ml (sat. sol); $(NH_4)_2SO_4$, 0.05 g; micronutrient solution,[2] 1 ml. This medium adjusted respectively at pH 7.0 and 4.8 was employed for the isolation of bacteria and molds and/or yeasts.

On the third day of retting, 1 ml of retted liquor was inoculated into the enrichment medium and incubated aerobically at room temperature and 37 C.

In every case, vigorous fermentation detected by gas formation was observed within 24 hr; after two successive transfers, inoculations were made on solid medium of the same composition. This medium proved to be satisfactory for both the isolation and purification purposes. In all, 40 pure cultures were isolated in this way.

For the isolation of pectin decomposing anaerobes, 0.05 per cent of sodium thioglycolate was incorporated in medium 1, at pH 7.4. The enrichments were set up with inoculum made in the following ways. On the third day of retting, the effluent in each flask was shaken thoroughly and used for inoculum. Separately inoculum was developed by using a few pieces of retted straw which were triturated in sterile distilled water under aseptic conditions. These served as inocula for the media in sterile, glass-stoppered bottles. Isolation from these enrichments were carried out by the shake culture method of Burri (1902) using a solid medium containing 1.5 per cent agar. By the repeated use of the shake culture method, six anaerobic strains were isolated in pure culture for further study. The anaerobe could ferment pectin and, as such, was identified as *Clostridium butyricum* var. *pectinovorum* in contradistinction to typical *C. butyricum* which does not ferment pectin.

Identification of microflora isolated from decomposing pectin. *The Manual of Methods for Pure Culture Study of Bacteria* (SAB, 1946) was used as a guide in the study of morphological and cultural characteristics of the bacterial isolates; and *Bergey's Manual of Determinative Bacteriology* (Breed *et al.*, 1948) was consulted for their identification. Results typical of these experiments are presented in table 1.

Aerobic microflora and volatile organic acids of typical laboratory rets. In the preliminary experiments, nutrient agar (NA), glucose agar (GA), and glucose yeast extract agar (GYA) were tried simultaneously for enumerating the total bacteria in the rets. Although GA and GYA gave slightly higher counts than NA, they suffered from the disadvantage that colonies grown

[2] Per 100 ml distilled water: $ZnSO_4 \cdot 7H_2O$, 1.1 g; $MnSO_4 \cdot H_2O$, 0.5 g; $CoSO_4$, 0.005 g; H_3BO_3, 0.005 g; Na_2MoO_4, 0.2 g; $CuSO_4 \cdot 5H_2O$, 0.0007 g.

TABLE 1

Identification of species isolated from decomposing pectin

Family	Genera and Species	No. of Strains	Incubation Temp
			C
Pseudomonadaceae	*Pseudomonas* spp.	17	24–26
Enterobacteriaceae	*Aerobacter aerogenes*	4	37
	Aerobacter aerogenes	2	24–26
	Aerobacter cloacae	2	37
	Escherichia freundii	1	37
	Escherichia spp.	2	24–26
Achromcbacteriaceae	*Alcaligenes bookeri*	2	37
	Flavobacterium aquatile	1	37
Bacillaceae	*Bacillus cereus*	2	37
	Bacillus spp.	2	37
	Clostridium butyricum var. *pectinovorum*	6	37

Note: Four molds belonging to the genus *Aspergillus* and ne yeast were isolated from the medium at acid pH.

TABLE 2

Dominant organisms and volatile acids of typical laboratory rets of Hibiscus cannabinus

Incubation Temp	Age of Retted Liquors	pH	Volatile Acids*			Total Counts	Dominant Organism
			F	Ac	B		
C	hr		g/L			millions/ml	
37	24	5.0	0.025	0.040	0.039	71.2	*Aerobacter cloacae*
	48	5.0	—	0.478	0.082	20	*Alcaligenes* spp.
	72	4.8	—	0.599	0.083	34	*Aspergillus* spp. and *Bacillus* spp.
	96	4.7	—	0.976	0.098	16	*Bacillus* spp.
24–26	24	5.0	0.026	0.066	0.042	428	*Pseudomonas* spp.
	48	4.5	—	0.624	0.092	880	*Pseudomonas* spp.
	72	4.4	—	0.758	0.073	160	*Pseudomonas* spp. and *Aerobacter aerogenes*
	96	4.6	—	1.252	0.097	47	*Aerobacter aerogenes*
24–26†	24	6.9	<0.005	<0.005	<0.005	1,580	*Pseudomonas* spp.
	48	7.0	"	"	"	38,808	*Pseudomonas* spp.
	72	7.2	"	"	"	230,000	*Pseudomonas* spp.
	96	7.5	"	"	"	Innumerable	*Aerobacter aerogenes*

* F = formic; Ac = acetic; and B = butyric.

† With constant aeration.

thereon were larger and of a spreading nature making accurate counts difficult. Colonies on NA were small, slow growing, and discreet. NA was therefore adopted for the enumeration of aerobic flora. The incubation period in general was 5 days and the temperature of incubation was the same as that under which the rettings were carried out.

To study the coliform and the pectin decomposing flora, MacConkey's agar and pectin agar were also used initially along with NA. All the types of organisms growing on pectin agar were, however, found to be similar to those growing on NA. MacConkey's agar did not prove to be of any special advantage for the appearance of coliforms. Hence NA alone was utilized for plate counts on all subsequent occasions.

Representative samples of the effluents were withdrawn aseptically from the respective laboratory rets at intervals of 24 hr throughout the retting period. Serial dilutions of these samples up to the 10^{-7} and 10^{-8} were prepared in sterile distilled water and plated in duplicate on NA; higher dilutions were made in the case of aerated effluents. (Aeration was effected by diffusing air through the rets.) The counts were made and the dominant organisms were isolated, purified and identified. Simultaneously, 1 to 1.5 L of the effluents were set aside for the estimations of volatile acids. The effluents were adjusted to pH 7.8 and the volumes were reduced to 10 ml each by evaporating on a boiling water bath. These concentrated effluents were adjusted to pH 2 and steam distilled. The partition chromatography technique recommended by Neish (1952) was employed for the estimations of the individual volatile organic acids. Table 2 brings out clearly the dominance of *Pseudomonas* in the liquors of the typical rets of *H. cannabinus*. The course followed by *M. capitata* retting and the microfloral picture presented by it was much the same as the other plant material studied (table 2).

Decomposition of pectin by Pseudomonas spp. To demonstrate the decomposition of pure pectin by the pseudomonads, the liver pectin medium used by Potter and McCoy (1952) was employed. This medium was dispensed in 20-ml quantities in 50-ml Erlenmeyer flasks and autoclaved for 25 min at 15 lb steam pressure. The final adjustment to pH 7 before inoculation was made with 1 N NaOH. A loopful of cells from the solid medium of each of the several strains of *Pseudomonas* was inoculated into culture tubes, each containing 5 ml of liver pectin medium, and incubated at room temperature. After 24 hr, the culture tubes were shaken well to obtain uniform suspensions and 0.05 ml was used as inocula for the flasks containing this medium. All the flasks were incubated at room temperature.

At intervals of 24 hr and on 4 consecutive days, one flask of each fermentation was subjected to the following analyses. The pH was determined and the titrable acidity estimated on 5 ml aliquots adopting the method followed by Potter and McCoy (1952). Pectin estimations were made by the method recommended by Kertesz (1951) because Hinton's (1940) method modified by Potter and McCoy (1952) proved unsuitable for the determination of pectic substances in the form of calcium pectate. The drawbacks of this method are obvious in the results reported by Potter and McCoy (1955). The values reported by them for calcium pectate (see tables 2 and 4) at various stages show an increase in the fermentable substrate over the initial amount when a decrease is to be expected as a result of bacterial activity; what is more, sudden rise and fall in the reported values are shown at different intervals. Similar results were recorded by us during the investigation with this method. At the initial stages of decomposition, a drop in the calcium pectate content was the feature. However, on prolonged incubation these values tended to exceed the initial. Resort to the Carré and Haynes' (1922) original method for the determination of pectic substances as calcium pectate was met with the same difficulties. Perhaps in all these methods, certain insoluble calcium salts of the by-products of pectin decomposition are precipitated along with the calcium pectate and tend to vitiate the results owing to their insolubility in acetic acid. Finally, however, a careful examination of the method recommended by Kertesz (1951) was not only found to be more accurate and reliable but the results were reproducible. The present results are based on this method.

TABLE 3

Decomposition of pectin by species of Pseudomonas

Pseudomonas spp.	Age in hr	pH	Acidity, ml 0.1 N NaOH per 5 ml	Calcium Pectate g per 100 ml
Strain 1	Initial	7	0.17	1.216
	24	4.8	0.57	0.683
	48	4.6	0.65	0.659
	72	4.5	0.79	0.347
	96	4.6	0.80	0.241
Strain 3	Initial	7	0.17	1.216
	24	4.7	0.51	1.050
	48	4.6	0.92	0.650
	72	4.6	1.03	0.300
	96	4.6	1.08	0.233

The results of two typical experiments of pectin decomposition by the *Pseudomonas* strains 1 and 3 are given in table 3.

Discussion

The results of the laboratory experiments indicate that the species belonging to the genus *Pseudomonas* are particularly important not only in the retting of *Malachra capitata* and *Hibiscus cannabinus* under controlled conditions but in the decomposition of pectin in

general. Among the aerobic microflora that developed in the aerated and unaerated rets, these species clearly dominated in both the series. Aeration during retting, however, facilitated to a greater extent the development of these species almost to the exclusion of the other forms of life during the first 72 hr and under the laboratory conditions. Studies with pure cultures of *Pseudomonas* species showed that they rapidly decomposed pure pectin. These observations would suggest that the species of *Pseudomonas* have an outstanding role in the process of retting, particularly under aerobic conditions, and that the greater part of the pectin decomposition observed during the process could be explained by the activities of *Pseudomonas*.

Another point of considerable interest is that *Pseudomonas* species seem to dwindle in the pectin enrichments and the laboratory rets incubated at 37 C, as at this elevated temperature *Aerobacter*, *Alcaligenes*, and the *Bacillus* form the dominant flora. Laboratory studies with the pure cultures also indicated that, for the development of *Pseudomonas*, temperatures above 30 C were less favorable than those below this level.

Pseudomonas species are known to be aerobic forms. As they developed at a rapid rate in the aerated rets, the systems did not show volatile acids and the pH increased (table 2). On the other hand, in the unaerated rets in the laboratory, the volatile acids tended to increase notably on longer incubation, a period during which both the pH and the total aerobic flora (including the pseudomonads) began to decrease, thus pointing out other species as being responsible for the formation of volatile acids. The analysis of decomposed pectin by the pure cultures of *Pseudomonas* also showed only a negligible amount of volatile acids despite the fact that their fermentative action resulted in the lowering of pH and the formation of a considerable amount of titrable acidity (table 3). This observation is in agreement with the information available on the metabolism of *Pseudomonas* species. The formation of butyric acid, however, is attributable to the concomitant activity of *Clostridium butyricum* var. *pectinovorum* active in those rets. It may be pointed out that, although butyric acid was not detected in the liquors derived from the aerated rets, the presence of the clostridia was demonstrable by the cultural methods, thus confirming Allen's report (1946a) with regard to the capability of anaerobes to remain dormant (in the sporulated state) under the adverse effect of aeration. It is also pertinent to note that, although Enebo *et al.* (1947) have reported the formation of appreciable quantities of acetic, propionic, and butyric acids along with traces of formic and valeric in the warm water retting of flax, the presence of propionic acid was at no time detected in the fermentation of *Malachra* and *Hibiscus*.

Retting presents, like many other processes in nature, a synergistic phenomenon. That it is so is well supported from several other experiments carried out in this laboratory (Unpublished Data). However, it is well to emphasize here that the pseudomonads seem to play a dominant part in the process as studied in the laboratory inasmuch as heavy inoculations of the species into the rets at the initial stages have at all times resulted in the completion of the process within 3 to 4 days in contrast to 9 to 12 days ordinarily required for the purpose. What is more, the rets inoculated with the clostridium alone or in combination with pseudomonads in equal proportions did not prove to be as effective as those inoculated with the pseudomonads alone despite the fact that the rets run were unaerated. From all of this, it would appear that species of *Pseudomonas* have an important role in the retting of *M. capitata* and *H. cannabinus*. It now remains to be established if the strains of *Pseudomonas* associated in the above rettings can be referred to the nearest species described in *Bergey's Manual* and also to ascertain whether they are equally significant in the industrial rets. This would represent the subject of another communication.

Acknowledgments

We are indebted to Dr. H. A. Barker of the University of California for his helpful suggestions and critical comments on the manuscript; to the Government of India for a senior scholarship to one of us (S. M. B.), and to the Director of this Institute for his keen interest.

Summary

Qualitative and quantitative studies of the microflora associated with the retting of *Malachra capitata* and *Hibiscus cannabinus* at 24 to 26 and 37 C with and without aeration have been reported. It has been shown that at the lower temperature the retting process is mostly brought about by the species belonging to the genus *Pseudomonas*. By chemical analysis the ability of the pseudomonads to decompose citrus pectin has been demonstrated.

REFERENCES

Allen, L. A. 1944 Spore-forming bacteria causing soft rot of potato and retting of flax. Nature, **153**, 224–225.

Allen, L. A. 1946a Recent developments in microbiology of retting. I. The microbial flora in different systems of retting. J. Soc. Chem. Ind. (London), **65**, 365–370.

Allen, L. A. 1946b Recent developments in microbiology of retting. II. The part played by bacteria in retting the flax and in purifying the liquor. J. Soc. Chem. Ind. (London), **65**, 373–376.

Barinova, S. A. 1946 On the decomposition of pectin by microbial ferments. Mikrobiologiya (U.S.S.R.), **15**, 313–318.

Betrabet, S. M. and Navalkar, B. S. 1956 Fibre yielding weeds of Bombay. *Malachra capitata* Lin. Indian Textile J., **66**, 682–683.

BREED, R. S., MURRAY, E. G. D., AND HITCHENS, A. P. 1948 *Bergey's manual of determinative bacteriology*, Ed. 6. The Williams & Wilkins Co., Baltimore, Maryland.

BURRI, R. 1902 Zur Isolierung der Anaeroben. Zentr. Bakteriol. Parasitenk., Abt. II, **8,** 534–537.

CARRÉ, M. H. AND HAYNES, D. 1922 The estimation of pectin as calcium pectate and the application of this method to the determination of the soluble apple pectin. Biochem. J., **16,** 60–69.

DEBSARMA, G. D. 1946 Biochemical investigation on jute retting. I. Isolation of micro-organisms from a jute-retting pit, their characterisation and their action on jute stems. Indian J. Agr. Sci., **16,** 453–458.

ENEBO, L., CARLSON, C. G., AND LUNDIN, H. 1947 On the formation of fatty acids during the retting of flax. Arch. Biochem., **12,** 339–347.

HELLINGER, E. 1953 Sporulating anaerobes on English flax. Nature, **171,** 1119.

HINTON, C. L. 1940 *Fruit pectins*. Chemical Publishing Co., Inc., New York, New York.

KERTESZ, Z. I. 1951 *Pectic substances*. Interscience Publishers, Inc., New York, New York.

NEISH, A. C. 1952 Analytical methods for bacterial fermentations. Natl. Research Council of Can. Ann. Rept. No. 46-8-3, 2nd revision.

OXFORD, A. E. 1944 Production of a soluble pectinase in a simple medium by certain plant pathogenic bacteria belonging to the genus *Pseudomonas*. Nature, **154,** 271.

POTTER, L. F. AND MCCOY, E. 1952 The fermentation of pectin and pectic acid by *Clostridium felsineum*. J. Bacteriol., **64,** 701–708.

POTTER, L. F. AND MCCOY, E. 1955 The fermentation of pectin and pectic acid by *Bacillus polymyxa*. J. Bacteriol., **70,** 656–662.

RUSCHMANN, G. AND BARTARM, H. 1943 *Bacillus felsineus* Carbone und Seine Bedeutung fur die Flachsrotte. Zentr. Bakteriol. Parasitenk., Abr. II, **105,** 326–351.

RUSCHMANN, G. AND BAVENDAMM, W. 1925a Zur Kenntnis der Rösterreger *Bacillus felsineus* Carbone und *Plectridium pectinovorum* (*Bac. amylobacter* A. M. et Bredemann). Zentr. Bakteriol. Parasitenk., Abt. II, **64,** 340–394.

RUSCHMANN, G. AND BAVENDAMM, W. 1925b Die Flaschsröste mit *Plectridium pectinovorum* (*Bac. amylobacter* A. M. et Bredemann) und *Bacillus felsineus* Carbone. Zentr. Bakteriol. Parasitenk., Abt. II, **65,** 43–58.

Society of American Bacteriologists 1946 *Manual of methods for pure culture study of bacteria*. Biotech. Publications, Geneva, N. Y.

WEIZMANN, C. AND HELLINGER, E. 1940 Studies on some strains of butyric acid producing pectridia isolated from hemp, jute and flax. J. Bacteriol., **40,** 665–682.