THE BACTERIAL FLORA OF AUSTRALIAN FLAX RETTING

By G. W. LANIGAN*

[*Manuscript received April* 12, 1951]

Summary

An account is given of investigations of both the aerobic and anaerobic bacterial flora associated with Australian flax retting.

Of three media tested, nutrient agar enriched with glucose and yeast extract proved most suitable for aerobic plate counts. While this count varied considerably from ret to ret, its maximum was consistently attained near the end of the first day and at the conclusion of a ret it was at times as little as one-fiftieth of the maximum. Predominant among the aerobes were lactic acid types of *Streptococcus* and species of *Paracolobactrum*. Species of *Flavobacterium*, *Achromobacter*, and the coliform group were present in smaller numbers. Other genera represented in some rets were *Leuconostoc*, *Lactobacillus*, and *Microbacterium*. Maximal presumptive coliform counts of retting liquor varied between 10^2 and 10^8 per ml., 55 per cent. of samples giving counts between 10^4 and 10^6 per ml.

Direct plating of heated suspensions of retted flax on glucose yeast extract agar was the most effective of three methods employed for isolation of the retting bacteria. Four distinct types of retting bacteria (clostridia) were found. Types I and II of these were evidently the predominant retting agents of Australian flax, while types III and IV appeared to play a minor part. Type III retting clostridium was identified as *Clostridium felsineum*, but the other three differ significantly from currently recognized species. Type I is microaerophilic; type II is related to the butylic group as exemplified by *Clostridium acetobutylicum*; and type IV is characterized by the formation of a non-diffusible, canary-yellow pigment. Of the non-retting clostridia isolated, 87 per cent. were varieties of *Clostridium butyricum*.

I. INTRODUCTION

The mixed bacterial flora that develops when flax straw is immersed in water at a suitable temperature is derived largely from the soil in which the crop has been grown. In some instances, no doubt, where water from lakes or streams is used, this water may contribute to the bacterial population of the retting tank; but only in exceptional cases would this contribution be appreciable. In the field, bacteria and spores are transferred to the roots by direct contact and to the stems through such agencies as wind and rain. Thus it may be assumed that the composition of the flora is fixed before the straw enters the retting tanks. Doubtless, however, some types of bacteria find conditions in the retting liquor inimical to their development and are soon outgrown, while the proportions of those surviving and multiplying in this environment can be shown to vary with the stage of retting. As would be expected.

* Flax Research Laboratory, C.S.I.R.O., Highett, Vic.

there is present, in addition to the true retting species, a large, mixed, subsidiary flora. Previous investigators in several countries have established that the responsible agents in water retting are spore-forming, anaerobic, or microaerophilic bacteria; in modern systems of classification, members of the genus *Clostridium*. The subsidiary flora, on the other hand, comprises species with all degrees of atmospheric requirements (Beijerinck and Van Delden 1904; Störmer 1904; Carbone and Tombolato 1917; Ruschmann and Bavendam 1925; Orla-Jensen and Kluyver 1939; Markova 1940; Allen 1946; Enebo 1947).

The literature relating to the true retting bacteria has been reviewed recently by Enebo (1947) and in a publication by the D.S.I.R., Great Britain (1948), so that no useful purpose would be served here by a repetition of this. However, some of the earlier work will be discussed at a later stage in relation to the present author's findings. Suffice to say that the retting of flax in various localities has been attributed to an organism, or group of organisms, termed variously *Granulobacter* (*Plectridium, Clostridium*) *pectinovorum* or to the orange-pigmented *Clostridium felsineum* or to both. The exact characteristics of the former are still difficult to ascertain from the literature, owing possibly to the existence of several varieties or even distinct species with retting ability. Bergey *et al.* (1948) do not accord specific rank to this bacterium but list it as a probable variety of *Clostridium butyricum*. On the other hand, *Cl. felsineum* has been subjected to detailed study and its characteristics are well established (Ruschmann and Bavendam 1925; McCoy and McClung 1935; Bergey *et al.* 1948). The very marked retting activity of the latter organism has been reported by various authors. In 1946, Allen concluded that the bacteria most commonly active in the retting of English flax were strains of *Clostridium tertium*. This appears to be the first occasion on which this clostridium has been found associated with flax retting.

It has been demonstrated (Behrens 1903; Rossi 1904; Rossi and Carbone 1909) that some species of *Bacillus* can ret flax under strongly aerobic conditions, and Allen (1946) isolated an enzyme powder with retting activity from cells of a variety of *B. subtilis*. However, bacteria of this type are believed to play no part in quiescent water retting where anaerobic conditions are soon established.

Relatively little attention has been given to the subsidiary bacterial flora of flax retting. Störmer (1904) identified *Pseudomonas fluorescens*, a coliform bacterium, and yellow-pigmented bacteria, presumably species of *Flavobacterium*, as the principal non-retting types, with yeasts and *Oidium*-like fungi appearing towards the end of the ret. Stutzer (1927) found, *inter alia*, that coliform bacteria and *Streptococcus lactis* were present throughout the ret, while *Bacillus* spp. were detectable in the early stages only and, in 1946, Allen reported essentially similar findings in a study of English flax retting. Enebo (1947), who investigated the flora of Swedish flax rets, found that paracolon bacilli and species of *Flavobacterium* usually predominated, while a *Pseudomonas* species and two species of *Micrococcus* were frequently present in smaller numbers. Apparently Enebo did not encounter lactic streptococci.

In Australia, the large-scale water retting of flax is a comparatively new industry, which developed rapidly in the years following the outbreak of World War II. No previous bacteriological studies of the process in this country have been reported, although Jensen (1941) published an account of his investigations of the fungal flora associated with dew retting, which has been practised in some of our flax-growing districts until quite recently. It is the purpose of the present paper to record the results of studies in the bacteriology of local flax retting carried out by the author during the past four years. The subsidiary flora as well as the retting bacteria have been investigated. Investigation of the aerobic and facultative species was carried out and is described first; this approach appearing logical in view of the prior development of these bacteria in the actual retting process.

II. The Aerobic and Facultatively Anaerobic Flora

(*a*) *Materials and Methods*

(i) *Aerobic Plate Counts.*—Laboratory tank rets, employing the standard "Belgian" type of schedule, were the source of material for enumeration and isolation of the aerobic species of bacteria present in flax retting liquor. These rets utilized a variety of flax crops grown in different districts. Standard nutrient agar was found to be unsuitable for aerobic plate counts, as many of the colonies that developed on this medium were too small for convenient counting. Allen (1946) reported that nutrient agar gave lower counts than either his carrot extract agar or potato agar. Three media were investigated in the present study; namely, a glucose yeast extract agar (G.Y.A.), a potato extract agar (P.A.), and Allen's (personal communication) carrot extract agar (C.A.).

For the counts, samples of liquor were withdrawn aseptically from a number of laboratory rets at intervals of 24 hr. throughout the retting period. In each case, liquor was taken from three levels in the tank and a composite sample prepared by mixing in a shaking machine. Serial dilutions of these samples to 1 in 10^7 were prepared in sterile distilled water. Duplicate counts were made with each of the three media described above. Incubation was at 30°C.

(ii) *Isolation of Aerobic and Facultative Species.*—Several of each type of colony present were picked from the plate cultures used for the aerobic counts and replated twice to ensure purity of the strains. Stock cultures were preserved under sterile liquid paraffin on either nutrient agar or glucose yeast agar, according to individual requirements.

(iii) *The Coliform Flora.*—In addition to observations regarding these organisms made in the general study of aerobic species, a specific investigation of the coliform flora was carried out. Presumptive coliform counts, using MacConkey's neutral red, lactose, bile salt, peptone water, were made on samples of liquor from 18 laboratory and 11 mill rets. The mill samples were transported to the laboratory packed in ice. The cultures from the highest and second highest dilutions, showing acid and gas formation within 48 hr. at 37°C.,

were plated out on MacConkey agar. After incubation at 37°C. for 24 hr., representative colonies were picked off and replated to ensure purity. The pure cultures were submitted to the standard tests employed for identification and typing according to the scheme of Wilson *et al.* (1935).

TABLE 1
AEROBIC PLATE COUNTS OF BACTERIA IN FLAX RETTING LIQUORS

Ret No.	Retting Time (hr.)	Count (millions per ml.) G.Y.A.[1]	P.A.[2]	C.A.[3]
1	24	43.0	—	44.0
2	24	85.0	79.5	86.5
	48	9.4	9.3	9.7
	72	7.5	6.9	7.0
	96	5.2	5.3	—
3	24	370.0	347.0	348.0
	48	39.0	39.0	34.0
	72	35.7	29.8	32.0
	96	10.4	7.6	—
4	24	164.5	175.0	159.0
	48	6.6	2.7	7.0
5	24	27.6	22.8	19.0
	48	16.3	14.9	11.7
	72	11.2	10.7	5.1
	96	5.0	4.05	1.95
6	24	84.5	94.5	90.0
	48	34.0	33.0	38.5
	72	11.0	11.0	9.5
	96	1.8	2.0	2.2
7	24	61.0	55.0	51.0
	48	49.0	51.5	45.0
	72	13.0	8.0	6.5
	96	3.0	1.4	3.0

[1] G.Y.A., glucose yeast extract agar.
[2] P.A., potato extract agar.
[3] C.A., carrot extract agar.

(*b*) *Experimental and Results*

(i) *Enumeration of Aerobic Flora.*—Table 1 summarizes the results of aerobic plate counts on samples of liquor from several laboratory rets. The figures given are means of duplicate determinations. Statistical analyses of these results were carried out and the significance of deviations was determined by use of the χ^2 test, with appropriate corrections for continuity. In the examination of variation between duplicate results, the values of χ^2 were found to be in good agreement with expectation, thereby indicating that the method was of satisfactory accuracy. The effect of medium on the count was significant at

the 1 per cent. level in approximately one-half of the tests. The media, in descending order of count were, generally, G.Y.A., P.A., and C.A., the counts being evenly spaced at 24 and 48 hours, but at 72 and 96 hours G.Y.A. and P.A. were close together. Highly significant interactions of medium and dilution occurred in two instances but in neither were the above conclusions affected.

Although G.Y.A. usually gave slightly higher counts than did P.A., it was observed that colonies were frequently better developed and any pigmentation was more marked on the latter medium than the former. It would seem therefore that P.A. may be better suited to some of the bacterial types present in retting liquor.

At first, colony counts were made after incubation of plates for 2, 3, 4, 5, and 6 days. These early tests indicated that 3-4 days was the most suitable incubation period. At this stage, colonies were of a convenient size for counting and did not increase in numbers on further incubation. Moreover, with more prolonged incubation, colonies of micro-aerophilic species usually appeared and rendered counting uncertain, since some of these approximated in size the smaller aerobic colonies. Towards the end of a ret, aerobic bacterial plate counts were frequently unreliable, owing to the development of large numbers of yeast colonies.

By reference to Table 1, it will be seen that, after 24 hours retting, the aerobic plate count is higher than at any subsequent stage, and it therefore appears that this count reaches its maximum at or about the end of the first day of retting. In the rets studied here, it will be seen that the maximal aerobic plate count varies considerably; viz. from 27.6 to 370×10^6 per ml., and at the end of the retting period the count may be as low as one-fiftieth of its maximum, the rate and extent of this decline varying from ret to ret also.

(ii) *Composition of the Aerobic Flora.*—Detailed study of 183 cultures showed that they comprised the following genera, the percentage of the total belonging to each genus being shown in parentheses: *Streptococcus* (35.0), *Paracolobactrum* (34.0), *Flavobacterium* (9.8), *Achromobacter* (6.6), *Lactobacillus* (4.9), *Aerobacter* (4.4), *Leuconostoc* (3.3), *Microbacterium* (1.0), *Escherichia* (0.5), and *Bacillus* (0.5).

In the following paragraphs, details of the morphological and cultural characteristics of the various bacterial groups encountered are given. In most cases, specific identification has been achieved but in others this has not been possible. For the purpose of placing them on record, those organisms not conforming in their properties to recognized species are described in some detail.

Streptococcus.—Morphologically, the cultures classified as members of this genus were Gram-positive, spherical to ovoid diplococci, with little tendency to form chains of more than four to six cells, even in liquid media. They were identified as *Streptococcus lactis* (Lister) Löhnis, being differentiated from *Strep. faecalis* on the one hand and *Strep. cremoris* on the other by the following properties: growth at 40° but not at 45°C., and growth in the presence of 4 per cent. but not 6.5 per cent. sodium chloride.

Leuconostoc.—The six cultures tentatively allotted to this genus differed from the other streptococci in that they produced little or no change in litmus milk medium. In this respect and in view of the marked stimulation of their growth by yeast extract, they certainly resemble described species of *Leuconostoc* (Hucker and Pederson 1930; Bergey *et al.* 1948). On the other hand, none of the strains isolated showed evidence of polysaccharide gum formation in sugar media. Moreover, they did not correspond in their reactions to any of the species listed by Bergey *et al.* (1948). In view of the small numbers found, however, these cultures have not been investigated further.

Paracolobactrum.—Cultures classified as members of this genus had the general characteristics of the coliform group of bacteria but exhibited a delayed or incomplete fermentation of lactose. In lactose peptone water, acid formation was generally evident in 2-5 days and was usually accompanied by a small bubble of gas in the Durham tube, which did not increase appreciably on prolonged incubation. In litmus milk medium, acid only or acid with slow coagulation occurred. Apart from their slow or incomplete fermentation of lactose, 57 of the cultures of this type had the characteristic properties of the *aerogenes-cloacae* group of bacteria. Most strains liquefied gelatin within 2 weeks at 22°C. Indole production was variable. Sixteen strains formed a golden pigment of varying intensity on nutrient agar. Consequently, it was thought at first that they may be related to the plant pathogens, *Erwinia carotovora* and *E. erivanensis;* however, they did not exhibit any digestive action on sterile, raw carrot or potato. Thus these strains must be regarded as pigmented varieties of paracolons. The observed characteristics of the cultures in this group identify them as *Paracolobactrum aerogenoides* Borman, Stuart, and Wheeler (1944).

The remaining five cultures of paracolon bacilli, while utilizing citrates as a sole source of carbon, were positive in the methyl red test, did not form acetyl methyl carbinol, and did not liquefy gelatin. Accordingly, they were identified as *Paracolobactrum intermedium* Borman, Stuart, and Wheeler (1944).

Flavobacterium.—Eighteen cultures were identified as members of the genus *Flavobacterium.* Three of these were *Flavobacterium estero-aromaticum* (Omelianski) Bergey *et al.* (1948). The remaining cultures represented six types, but none could be definitely identified with the species described by Bergey *et al.* (1948). The principal features of these unidentified strains are given in Table 2.

Achromobacter.—None of the cultures classified as members of this genus could be identified with species described by Bergey *et al.* (1948). The properties of the 12 strains studied are recorded in Table 2.

Aerobacter.—Of the eight cultures isolated that belonged to this genus, six were *Aerobacter aerogenes* (Kruse) Beijerinck and two were *Aerobacter cloacae* (Jordan) Bergey *et al.* (1948). On the basis of Wilson's (1935) classification, four of the *A. aerogenes* strains were type I and two were type II.

TABLE 2

CHARACTERISTICS OF ORGANISMS CLASSIFIED AS SPECIES OF *ACHROMOBACTER* AND *FLAVOBACTERIUM*

Genus	No. of Cultures	Pigment on Agar	Motility	Gelatin Liquefaction	Litmus Milk	Nitrate Reduction	Glucose	Maltose	Sucrose	Lactose	Mannitol
Achromobacter	11	None	+	Not liquefied	Rennett *C*, slowly peptonized	Reduced to nitrite	*A*	*O*	*A* slow	*O*	*O*
	1	None	+	Stratiform; slow	Rennet *C*, slow. Not peptonized	Not reduced	*A*	*O*	*A*	*O*	*A*
Flavobacterium	9	Pale golden	+	Saccate; 3-4 weeks	*A*, *C* slow	Reduced to nitrite	*A*	*A*	*O*	*A* slow	*A*
	2	Pale golden	+	Infundib.; 5-12 days	*A*, *C* slow	Reduced to nitrite	*A*	*A*	*A*	*A*	*A*
	1	Pale golden	+	Infundib.; 5-12 days	Rennet *C*, peptonized	Reduced to nitrite	*A*	*A*	*A*	*O*	*A*
	1	Pale golden	—	Not liquefied	*A*, slight	Reduced to nitrite	*A*	*A*	*O*	*O*	*A*
	1	Pale yellow	—	Stratiform; slow	*A*, slight	Reduced to nitrite	*A*	*A*	*A*	*A*	*O*
	1	Yellow	+	Stratiform; slow	*A*	Reduced to nitrite	*O*	*O*	*O*	*O*	*O*

Gelatin incubated at 22°C., all other cultures at 30°C. *A* = acid; *C* = coagulation; *O* = no change.
None of the cultures produced indole.

Escherichia.—The sole representative of this genus isolated was identified as *Escherichia intermedium* (Werkman and Gillen) Vaugh and Levine. It belonged to Wilson's type 1.

Lactobacillus and *Microbacterium.*—Table 3 summarizes the characteristics of the 11 cultures identified as members of these genera. Apart from generic allocation, it has not been possible to identify any of them with described species. Their infrequent occurrence in flax retting liquor has not warranted further investigation.

TABLE 3

CULTURAL CHARACTERISTICS OF THE GRAM-POSITIVE, NON-MOTILE, NON-SPORING BACILLI ISOLATED

No. of Cultures	Litmus Milk	Catalase	Nitrate Reduction	Glucose	Maltose	Sucrose	Lactose	Mannitol	Generic Classification
5	*O* or sl. *A*	—	Not reduced	*A*	*A*	*A*	*O*	*O*	*Lactobacillus*
4	*A, C*	—	Not reduced	*A*	*A*	*O*	*A*	*O*	*Lactobacillus*
2	*A* with slow *C*	+	Reduced to nitrite	*A*	*O*	*O*	*A*	*O*	*Microbacterium*

Other features common to the three groups were aerobic and facultatively anaerobic growth; absence of motility; and no liquefaction of gelatin. *A* = acid; *C* = coagulation; *O* = no change.

Bacillus.—Only one culture belonging to this genus was isolated. Its characteristics indicated that it was a variety of *Bacillus megatherium* De Bary.

(iii) *The Coliform Flora.*—In the investigation of the aerobic flora of flax retting liquor described above, only nine cultures belonging to the coliform group of bacteria were isolated. Thus their proportion of the total cultures studied; viz. 5 per cent., would not indicate that they constituted a very significant part of the aerobic species present. However, in view of the importance attached to the presence of coliform bacteria when assessing the pollutional effect of retting effluent on streams etc., it was decided to study a wider range of retting liquors, using more specific methods for the detection of these bacteria. Accordingly, presumptive coliform counts were made on a further 29 samples of retting liquor; 18 from laboratory rets and 11 from mill rets. Figure 1 shows the percentage distribution of these counts. All counts were made after 20-24 hr. retting, i.e. when the aerobic plate count was at or near its maximum.

It will be seen from Figure 1 that the 24-hr. presumptive coliform count is subject to wide variations from ret to ret. Fifty-five per cent. of the counts were found to lie between 10^4 and 10^6 per ml., while the remainder were fairly evenly distributed above and below these limits, the total range of the counts being from 10^2 to 10^8 per ml. The fact that only 24 per cent. of the liquor

samples gave counts higher than 10^6 per ml. probably explains why so few coliform bacteria were detected in the study of the aerobic flora, based on cultures used for aerobic plate counts, which ranged from 30 to 300×10^6 per ml.

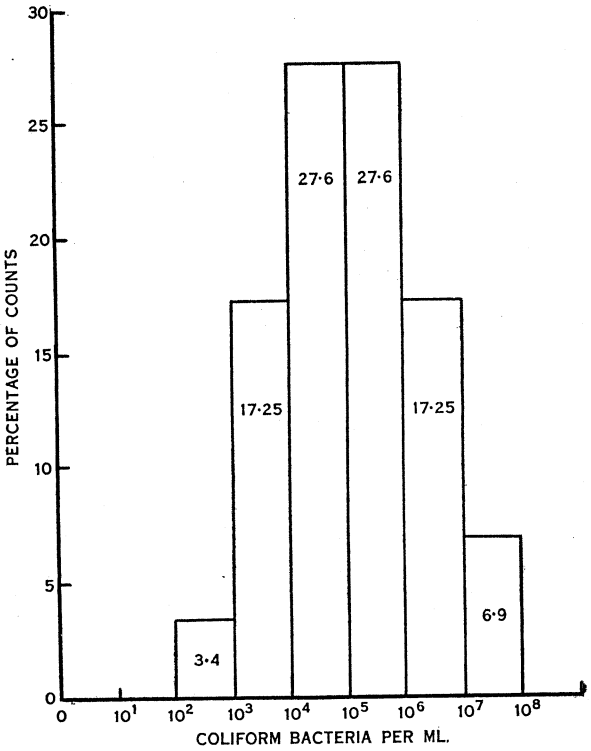


Fig. 1.—Percentage distribution of presumptive coliform counts.

By plating and selection of discrete colonies, 110 bacterial strains were isolated from the presumptive coliform count tubes. As would be expected, however, it was found that, in a number of instances, duplicate cultures of the same coliform type had been recovered from the one ret. For this reason 21 cultures were discarded. One other culture proved to be a species of *Proteus* and was also discarded. Ultimately, therefore, 88 cultures remained to be classified and, of these, 26 were shown to be paracolon types by their delayed or incomplete fermentation of lactose. The coliform and paracolon types found and the percentage of the total belonging to each type are shown in Table 4.

Identification of the various coliform types was based on the classification of Wilson *et al.* (1935) and that of the paracolon species was in accordance with descriptions given in Bergey's Manual (1948).

III. The Anaerobic and Micro-Aerophilic Flora

(*a*) *Materials and Methods*

In order that the investigation of the anaerobic flora of Australian flax retting, particularly the retting species, might be reasonably comprehensive, 41 flax samples were obtained from all of the mills employing water retting. Moreover, where possible, crops were selected that had been grown in localities representative of the total supply of flax straw to the particular mill. Samples were obtained of six crops from each of the six Victorian mills, viz. Ballarat, Colac, Drouin, Lake Bolac, Myrtleford, and Strathkellar; of two crops from the Hagley mill in Tasmania; and of three crops from the Boyup Brook mill in Western Australia. All samples were retted in the laboratory, using the Belgian type of anaerobic retting schedule in current use in Australia. At the completion of retting, lengths of straw were withdrawn aseptically and at random from each sample and allowed to drain for a short time in a sterile tube. After being cut into lengths of approximately ¼ in., 5 g. of the retted straw samples were shaken mechanically with glass beads in 95 ml. of sterile distilled water, to obtain an even distribution of the bacteria present. Serial dilutions of these primary suspensions were prepared and used for the isolation of anaerobic bacteria as described below.

Table 4
ISOLATIONS FROM COLIFORM COUNT TUBES

Type	Percentage
Esch. coli type I	11.4
Esch. intermedium type I	17.1
Esch. intermedium type II	5.7
Aerobacter aerogenes type I	17.1
Aerobacter aerogenes type II	1.1
Aerobacter cloacae	8.0
Irregular type II	3.4
Irregular type IV	2.3
Irregular type VI	4.6
Paracol. coliforme	1.1
Paracol. intermedium	3.4
Paracol. aerogenoides	25.0

As this study was to be limited to the spore-forming anaerobic species present, all suspensions of retted straw were heated in a water-bath for 20 min. at 80°C. to destroy vegetative cells, in particular those of the facultative anaerobes. Three different methods were used for isolation of clostridial species from the heated suspensions of retted straw; namely:

(*A*) Incubation at 37°C. in a glucose potato mash medium* for 7 days after fermentation commenced, followed by heating for 20 min. at 80°C., then plating out on G.Y.A.;

* As described in "Manual of methods for pure culture study of bacteria." Leaflet No. 2, p. 22. Biotech. Publications, Geneva, N.Y., 1944.

(*B*) Incubation at 37°C. in the raw potato broth of Allen (1946) for 7-10 days, followed by heating to destroy vegetative cells and plating out of the tubes from the highest dilutions producing softening of the potato;

(*C*) Direct plating of the retted straw suspension on G.Y.A.

In all three methods, representative colonies were picked from the plates into glucose potato mash, and after a short period of incubation, i.e. when fermentation was obvious, the cultures were replated. Usually, three platings were needed to ensure purity. Each strain recovered was tested for freedom from aerobic contaminants. Unless otherwise stated, all cultures of anaerobic organisms were incubated in specially made jars, in an atmosphere of hydrogen and nitrogen plus 5 per cent. CO_2, the oxygen being removed by means of a palladium-asbestos catalyst — Wright's (1943) cold capsule as described by Hayward (1945).

Allen's (1946) raw potato medium was modified by substituting yeast-peptone water for the nutrient broth used by him, as better growth of most cultures isolated was thereby obtained. Growth of these anaerobes in the raw potato medium from small inocula, such as high dilutions of retted straw, was rendered much more certain by simultaneous inoculation with a non-retting aerobic organism, such as *Aerobacter aerogenes,* or one of the paracolon species described in the section relating to the aerobic flora. This technique obviated the problem of dissolved oxygen, which could not be removed by the usual process of heating and, in addition, it permitted incubation without resort to special anaerobic jars. The cells of the aerobe were, of course, readily destroyed by subsequent "pasteurization."

Methods (*A*) and (*B*) were used for straw samples from Colac, Hagley, Lake Bolac, and Strathkellar; methods (*B*) and (*C*) were used for the Ballarat, Drouin, and Western Australian samples; and method (*C*) only was employed in the study of the samples from the Myrtleford district.

The ability, or otherwise, of the various anaerobic cultures to ret flax was determined by the method of Allen (1946). This method employed a medium prepared by sterilizing air-dry flax straw in tubes at 15 lb. steam pressure for 20 min. and subsequently covering the straw with sterile water. It was found, however, that yeast water gave better results than tap water and this has therefore been used throughout this investigation.

After a suitable period of incubation to test sterility, the sterile flax medium was usually inoculated with 1 ml. of an actively growing potato mash culture of the various strains of clostridia. Some of the organisms, however, did not grow well in potato mash and, in such cases, glucose yeast peptone water cultures were used as inoculum. The cultures were incubated at 37°C. until the straw was retted, or for a maximum of 10 days. As with the raw potato broth cultures, simultaneous inoculation with a non-retting facultative anaerobe, such as *A. aerogenes,* and "aerobic" incubation was usually superior to incubation in an anaerobic jar. It was realized that objection to this practice could be raised, on the grounds of symbiotic effects resulting in a retting action which might not be evident in pure cultures. Therefore, all strains were

tested both in pure culture and in association with the facultative anaerobe. None of the organisms studied showed evidence of retting in the latter cultures without it being shown in the former also.

However, almost invariably, retting proceeded more rapidly in the mixed cultures. As Allen (1946) has pointed out, the use of heat-sterilized flax for determining the retting ability of a particular organism is open to the objection that sterilization produces some physical and possibly chemical changes in the straw, which could lead to false positive results. Experience has shown, however, that this test does give a clear-cut differentiation of retting and non-retting types of bacteria. While there were obvious differences in the rate and completeness of retting by different strains, the changes produced in the flax by retting types were profoundly different from those occurring in the presence of non-retting organisms, such as members of the aerobic flora. A very few anaerobic organisms were isolated, however, which did not conform to any of the main retting groups encountered, and which appeared capable of bringing about partial retting of the sterilized flax. It has not yet been possible to determine whether these few strains would have a similar effect on unheated flax and, in any event, it seems most unlikely that they play a part of any great importance in normal retting.

(*b*) *Experimental and Results*

(i) *Isolation.*—Initially, 535 cultures of spore-forming anaerobic bacteria were isolated and purified by the methods outlined above. These were submitted to a preliminary screening based on morphology, colonial characteristics, and action on sterilized flax. As a result of this screening, approximately half of the cultures isolated from each ret were discarded as obvious replications, while, wherever possible, duplicate or triplicate strains of each type found were retained. Ultimately, 293 cultures were submitted to detailed investigation.

Table 5 shows the proportions of retting and non-retting members of the genus *Clostridium* isolated by each of the three methods employed. Unfortunately, because of overlapping of work with flax from different sources, the complete unsuitability of method *A* did not become apparent until cultures from a considerable number of rets had been studied in some detail. This method was then immediately discarded. With regard to methods *B* and *C*, it will be seen from Table 5 that the latter yields twice the proportion of retting organisms obtainable by the former and is therefore the method of choice. It is of interest to note that, even with the direct plating of method *C*, equal numbers of retting and non-retting types of sporing anaerobes were isolated.

(ii) *The Retting Flora.*—The observed characteristics of the retting strains of sporing anaerobes isolated showed that they constituted four distinct, homogeneous groups labelled for convenience types I-IV. The members of three of these groups (I, II, and IV) had properties that clearly differentiated them from any of the species described by Bergey *et al.* (1948), but group III was readily identified as *Clostridium felsineum* (Carbone and Tombolato) Bergey *et al.* (1948). Retting clostridium type I, which was micro-aerophilic, appeared

similar to the *Plectridium pectinovorum* of Störmer (1904) and the plectridial types described by Weizmann and Hellinger (1940). Its relationship to these organisms and significant points of difference will be discussed later.

TABLE 5

EFFECT OF ISOLATION METHOD ON PROPORTION OF RETTING AND NON-RETTING CLOSTRIDIA

Origin of Flax	Method *A*		Method *B*		Method *C*	
	Retting	Non-Retting	Retting	Non-Retting	Retting	Non-Retting
Colac	6	39	11	24	—	—
Hagley	0	9	2	4	—	—
Lake Bolac	0	46	14	41	—	—
Strathkellar	0	32	2	26	—	—
Ballarat	—	—	8	26	24	16
Drouin	—	—	7	27	36	35
Boyup Brook	—	—	9	12	14	11
Myrtleford	—	—	—	—	22	33
Totals	6	126	53	160	95	95
	(4.5%)	(95.5%)	(24.8%)	(75.2%)	(50.0%)	(50.0%)

The retting activity of clostridium type I was less marked than that of the the other three types. Generally, retting was incomplete and a few strains did little more than soften and loosen the cortical and epidermal tissues. Morphological features of retting clostridium type I are shown in Plate 1, Figures 9 and 10, and typical colonies are illustrated in Plate 1, Figures 1-3. All strains of *Cl. felsineum* isolated proved to be very active retting agents; in fact, by far the most active of the four types encountered. None of the author's strains of *Cl. felsineum* fermented inulin and none could be shown to reduce nitrates, even at 10 p.p.m. KNO_3, although all reduced 1000 p.p.m. of nitrite to ammonia. Thus, in the first two properties they differed from the description of Bergey *et al.* (1948). It is perhaps worthy of note that one of Carbone's original strains, possessed by the author, could only reduce 10 p.p.m. of KNO_3 and also failed to ferment inulin. Consequently, it would appear that nitrate reduction and inulin fermentation are variable properties of *Cl. felsineum.* Photomicrographs of a local strain of this organism are shown in Plate 1, Figures 13 and 14, for comparison with the other types.

The characteristics of the Australian retting clostridia types II and IV, which do not conform to descriptions of recognized clostridial species, are given in detail in the following paragraphs. All cultures were grown anaerobically at 37°C. and morphological descriptions are based on Gram-stained smears from glucose yeast agar stroke cultures.

(iii) *Retting Clostridium Type II.*—Twenty strains of this type were studied.

Morphology and staining, medium size rods with markedly incomplete fission at 24 hours; but at 48 hr. the chains and filaments have largely disappeared. Rods are 0.6-0.8 μ diam. and mostly 2-7 μ long; axis straight to slightly curved, ends rounded, sides parallel; motile; Gram-positive when young.

Sporulation occurs early and freely. The body of the sporangium is only slightly greater in diameter than the vegetative cells but it is distended by an elliptical spore, which arises subterminally, although in the majority of mature sporangia the spore appears terminal. Sporangial cells are usually slightly curved, some appreciably so. Their length varies between 5 and 12 μ, with the majority 6-9 μ. Free spores are elliptical in outline, their dimensions varying between 1.6 and 2.4 μ in length and 1.0 and 1.2 μ in width; most are 1.8-2.0 μ × 1.1 μ (Plate 1, Figs. 11 and 12).

Glucose gelatin, liquefied within 7-21 days.

Nutrient agar slope, little or no growth.

Nutrient broth, at most, slight deposit.

Glucose yeast agar surface colonies, characteristic colony irregular in outline, with a "woolly" or myceloid margin. In 3 days on dry plates, they are 2-3 mm. diam., but on even slightly moist media there is a marked tendency to spread, forming effuse, amoeboid projections. The colony is differentiated into a circular, low-convex, opaque, greyish white, central knob, and an effuse, translucent marginal zone. The centre is smooth, glistening, and butyrous, while the effuse portion is largely embedded in the agar. This penetration of the medium is myceloid in appearance. The relative sizes of the convex centre and the effuse margin vary from strain to strain. Occasionally, the central, convex zone is unusually well developed and slightly viscid in consistency, while the effuse, myceloid margin is quite narrow or may be almost non-existent. It is considered that this type of colony may represent a mucoid phase (Plate 1, Figs. 4-6).

Glucose yeast agar deep colonies, in 3 days, approximately spherical, "woolly" balls, 1 mm. diam.; medium shattered by gas.

Glucose yeast agar stroke culture, abundant growth, filiform on dry slopes but spreading with finger-like projections if medium is moist; slightly raised, smooth, glistening, butyrous centre; effuse, matt margin with myceloid penetration of the agar; "woolly" periphery.

Glucose yeast peptone water, heavy turbidity and heavy amorphous deposit; much gas.

Litmus milk, acid, reduction, gas, and clot; frequently a stormy coagulation; no digestion of the curd.

Potato mash, active fermentation with "head"; complete diastatic action.

Cooked brain medium, some gas; no blackening or digestion.

Coagulated egg albumin, no visible effect but, after several days, slight softening is usually detected by probing.

Indole, not formed.

Hydrogen sulphide, small to moderate amounts produced on glucose yeast agar in 3-7 days.

Nitrates, not reduced.

Nitrites, reduced, presumably to ammonia, in 2-3 days.

Carbohydrates and related compounds, acid and gas produced from glucose, galactose, maltose, sucrose, lactose, starch, and pectin. Inulin, mannitol, glycerol, and calcium lactate not fermented. Cultures in glucose yeast peptone water have a distinct odour of butanol.

Sterilized flax in yeast water, active retting by all strains.

Raw potato in yeast water, rapid disintegration.

Optimum temperature, not determined; grows well at 30° and 37°C.

Atmospheric requirements, obligate anaerobe.

Distinctive features, colony on glucose yeast agar; requires fermentable carbohydrate for growth; ability to ret flax.

Habitat, presumably soil.

(iv) *Retting Clostridium Type IV.*—Three strains of this type were studied.

Morphology, slender rods, 0.4 μ diam. × 2-7 μ long, most 3-4 μ; occurring singly, in pairs end to end and in small clusters with some palisade formation, some short filaments; axis straight to slightly curved, ends rounded, sides parallel; motile; Gram-positive when young. Sporulation occurs early and freely. The cell is distended at sporulation by an elongated, subterminal spore with a pronounced terminal "cap." The spore frequently occupies half the length of the sporangium, which is usually curved and often shows a definite kink corresponding in position to the junction of the spore and the cytoplasm. Sporangial length is fairly uniform, most cells being 6-7 μ long with a maximum range of variation from 5 to 8 μ. The free spores are long and narrow, elliptical or bean-shaped in outline, 2.2-3.0 μ long × 0.7-0.9 μ wide, most being 2.5-2.7 μ × 0.8 μ (Plate 1, Figs. 15 and 16).

Glucose gelatin, liquefied in 7-11 days.

Nutrient agar, no growth.

Nutrient broth, no growth.

Glucose yeast agar surface colonies, in 3 days, circular, 1-1.5 mm., entire, low-convex or umbilicate, smooth and glistening, opaque, canary-yellow by reflected light, viscid consistency (Plate 1, Figs. 7 and 8).

Glucose yeast agar deep colonies, biconvex discs, 1 mm. diam. in 3 days, canary-yellow colour, medium disrupted by gas.

Glucose yeast agar stroke culture, good, filiform growth; smooth and glistening surface; canary-yellow, non-diffusible pigment; soft butyrous to viscid consistency.

Glucose yeast peptone water, heavy uniform turbidity with much gas, pale yellow viscid deposit.

Litmus milk, acid, gas, reduction, coagulation; usually a stormy clot within 2-4 days; no digestion of curd.

Potato mash, active fermentation with "head," pale canary-yellow pigment, complete diastatic action.

Cooked brain medium, slight gas, no blackening or digestion.

Coagulated egg albumin, no visible change but after several days softening can be detected by probing.

Indole, not formed.

Hydrogen sulphide, traces formed on glucose yeast agar.

Nitrates, not reduced.

Nitrites, 0.1 per cent. KNO_2 reduced, presumably to ammonia.

Carbohydrates and related compounds, acid and gas produced from glucose, galactose, maltose, sucrose, lactose, starch, and pectin. Inulin, mannitol, glycerol, and calcium lactate not fermented. Cultures in glucose yeast peptone water have marked odour of butanol.

Sterilized flax straw in yeast water, active retting; this organism appears to possess retting ability intermediate between that of *Cl. felsineum* and type II.

Raw potato in yeast water, rapid disintegration.

Optimum temperature, not determined; grows well at 37°C. and well but more slowly at 30°C.

Atmospheric requirements, strictly anaerobic.

Distinctive features, formation of a non-diffusible canary-yellow pigment; morphology of sporangia; active retting of flax; no growth in the absence of a fermentable carbohydrate.

Habitat, probably soil.

(v) *Relative Importance of the Four Retting Clostridia.*—In view of the observed differences in retting activity of the organisms encountered in this investigation, it was obviously desirable to establish, if possible, the relative frequencies with which each type occurred in Australian flax retting. Retting clostridia types I, II, III, and IV, respectively, were found in approximately ¾, ½, 1/3, and 1/10 of the rets studied. However, it was shown in Table 5

that marked differences existed in the numbers of retting organisms recovered by the three methods employed and, consequently, these overall figures do not give a true picture of the respective frequencies with which the four types of retting clostridia occur. In view of this deficiency, the numbers and percentages of the four types isolated by each method have been determined and these figures are presented in Table 6.

TABLE 6
EFFECT OF ISOLATION METHOD ON RECOVERY OF THE FOUR TYPES OF RETTING CLOSTRIDIA

Method of Isolation	No. of Rets	No. of Rets from which Isolated			
		Type I	Type II	Type III	Type IV
A	18	3 16.7%	0	0	0
B	33	16 48.5%	4 12.1%	1 3.0%	2 6.1%
C	21	15 71.5%	14 66.7%	10 47.6%	1 4.8%

It is evident from Table 6 that only the results obtained by method *C* are of real value in determining the relative importance of the various types of retting organisms in Australian flax retting. From these findings it appears that clostridial types I and II are equally prominent in our retting, while type III is less commonly present. So few cultures of type IV were isolated that it is apparent that this organism plays little part in flax retting in this country. The dominant role of types I and II and the minor activity of type III become even more evident from a consideration of the following three observations. In all rets from which retting bacteria were isolated, either type I or type II was found. In 43 per cent. of the rets for which method *C* was used, both of these organisms were present in similar numbers. On the other hand, retting clostridium type III was never found alone and, in fact, only an odd colony of this organism was usually found on the plates. It might be argued, of course, that the method of isolation was not well suited to type III clostridium and that this was responsible for the relative paucity of its colonies. That this is not so is shown by the fact that, with some Belgian flax examined and in some rets inoculated with this organism, its colonies were well developed and predominant on the plates. It is therefore evident that clostridial types I and II are the agents most commonly responsible for flax retting in Australia and that, in about half of the rets, both types are present in approximately equal numbers. The significance of these findings will be discussed in a later section of this paper.

(vi) *The Non-retting Anaerobic Flora.*—Of 381 cultures of non-retting clostridia initially isolated, 174 were studied in detail, the remainder being discarded as obvious replications after the preliminary screening. On the basis

of morphological and cultural characteristics, these non-retting clostridia were separable into six groups.

Groups I, II, and III, comprising 87 per cent. of the cultures studied, had morphological and cultural properties that identified them with the *Clostridium butyricum* group (Bergey *et al.* 1948). Group I differed from group II in failing to reduce nitrates, although both reduced nitrites to ammonia. Thus these groups were identified as varieties of *Cl. butyricum* Prazmowski and *Cl. multifermentans* Bergey *et al.*, respectively. It is noteworthy, however, that both groups of organisms showed a wider range of fermentative activity than the type species, in their consistent fermentation of mannitol, glycerol, and calcium lactate. On the other hand, none of the strains produced the typical "stormy" fermentation of milk. The single culture of group III did not ferment starch, inulin, mannitol, glycerol, or lactate and failed to reduce nitrates. Thus it was identified as *Cl. beijerinckii* Donker.

The colonial morphology of these butyric clostridia was subject to appreciable variation from strain to strain. As a considerable number of strains have been studied here and the colony types found may therefore be deemed representative of those likely to be encountered, descriptions and percentages of the various forms found in groups I and II are shown in Table 7.

TABLE 7
PERCENTAGES OF VARIOUS COLONY FORMS IN *BUTYRICUM*-TYPE CLOSTRIDIA

Description of Colony	Percentage of Cultures	
	Group I	Group II
A. Circular, entire, smooth, glistening, low-convex, opaque to translucent, creamy white; typical colony	47	59
B. Body of colony as for *A* but with translucent peripheral buds or effuse, ragged outgrowths	5	33
C. Slightly irregular, slightly umbonate, opaque centre, translucent spangled margin	32	8
D. Circular, pulvinate, collapsing later, greyish white, viscid, semi-fluid; mucoid variant	13	0
E. As for *A*, but pale mauve pigment formed when first isolated	3	0

It will be seen from Table 7 that approximately half of the strains in both groups formed the typical colony of *Cl. butyricum* (colony type *A*). The several variant colonies, however, tended to be more common in one or other of the two groups. Thus, colony type *B* was found predominantly in group II, while types *C*, *D*, and *E* were most commonly formed by group I, types *D* and *E*, in fact, being confined to the latter group.

None of the other non-retting clostridia, groups IV-VI, could be identified with currently recognized species and, as relatively few cultures of each were isolated, indicating that they were adventitious types of no significance to the retting process, they have not been studied further.

IV. Discussion and Conclusions

(*a*) *The Aerobic and Facultatively Anaerobic Flora*

The aerobic plate count of liquor from anaerobic rets with Australian flax has been found to reach its maximum at or about the end of the first day. From then on, the count steadily declines and, towards the end of the ret, may fall to as little as one-fiftieth of its maximum. The maximal count varied appreciably from ret to ret. In the rets studied here, the total range of variation was from 26 to 370 $\times$ 10^6 per ml. The results obtained in the present study are in good agreement with those of Allen (1946) in England, who reported counts of 20 to 60 $\times$ 10^6 per ml. at 20 hr., falling to about 5 $\times$ 10^5 per ml. at 65 hr., in anaerobic rets, while the liquor from aerated rets gave much higher counts which remained high throughout the ret. Allen also found that counts per gram of moist straw were somewhat higher than in the liquor.

All of the aerobic bacteria isolated from retting liquor in this investigation are representative of types commonly found in soil and water and on plants. Thus, their presence in flax rets might almost have been predicted. As far as is known at present, none of the aerobic species encountered is able to ret flax. None of the strains tested had any apparent effect on flax sterilized in the autoclave. On the other hand, the lactic streptococci, the paracolons, and the coliforms, which constitute the majority of the aerobic flora, are active fermenters of the simpler carbohydrates and related substances. Consequently, by their action on the soluble constituents of the flax stem, they may be expected to contribute appreciably to the total acidity of retting liquor. Thus, the importance of giving the flax straw a preliminary rinse would be based, in part, on the removal of soluble carbohydrates, thereby limiting the early fall in pH of the liquor caused by the activities of the aerobes. Too rapid and marked a fall in pH during the very early stages of retting might well retard germination of the spores of the retting bacteria. This hypothesis is in accord with earlier views of Ruschmann (1923) and Allen (1946), but is disputed, in part at least, by Enebo (1947). The latter investigator maintains that the aerobic flora are of no great importance to the appearance of volatile acids in the first phase of retting. This contention is based on determinations of the acetic : butyric acid ratio at different stages of retting. As this ratio was the same at 20 hr. as at the conclusion of Swedish rets, Enebo concluded that the true retting bacteria, which produced butyric acid, were active much earlier than had been believed formerly and that the division of the retting period into "preliminary phase" and "principal phase" by Ruschmann (1923) was purely artificial.

The views of Störmer (1904) and Ruschmann (1923), that the development of aerobic species during the preliminary state of retting, with consequent removal of oxygen from solution, paves the way for the subsequent activities of the anaerobic retting bacteria, have not been disputed by later workers. Indeed, there can be no doubt that the aerobic flora, by lowering the redox potential of the liquor to a level consonant with the germination

of the anaerobic spores, makes possible the comparatively simple industrial application of the anaerobic fermentation process known as retting, without resort to the special equipment and precautions needed to provide an anaerobic environment.

The composition of the aerobic flora of Australian retting liquors has proved to be broadly similar to that found by previous investigators in other countries. Streptococci of the types found here were also reported by Stutzer (1927) and Allen (1946). Paracolon bacteria, which were reported by Enebo (1947) in his study of flax retting in Sweden, were also much more common in Australian retting than the coliform types found by such workers as Störmer (1904), Stutzer (1927), and Allen (1946). The occurrence of large numbers of paracolons in flax retting liquors is of interest since, although this group has received a good deal of attention in relation to gastro-intestinal disorders in recent years, little other work appears to have been carried out with them. In this paper, members of this group have been classified for convenience according to Bergey *et al.* (1948) in the genus *Paracolobactrum* Borman, Stuart, and Wheeler (1944). It is considered, however, that this generic separation is an artificial one and that these organisms are probably variants that have lost, in varying degrees, their activity towards lactose, since this sugar is rarely encountered in their natural environment. Some evidence for this contention is given in a paper by Mushin (1949) who found that some of her cultures regained the ability to ferment lactose rapidly, after repeated subculture in media containing this sugar. A few tests made by the present author have yielded similar results in the adaptation of strains from retting liquor.

Other types of aerobic bacteria isolated from Australian flax retting, namely species of *Flavobacterium, Achromobacter, Lactobacillus,* and *Microbacterium,* are widely distributed on natural products and their presence is probably adventitious. The isolation of only one species of *Bacillus* confirms the conclusion of several previous investigators that bacteria of this type play no part in anaerobic flax retting. The appearance of large numbers of yeasts in the liquor towards the end of the ret, i.e. when the pH has fallen to between 4 and 5, is in keeping with the usual sequence of events where vegetable material is undergoing microbial decomposition.

It was observed in the present investigation that the lactic streptococci were consistently relatively more numerous in the later stages of retting than the other aerobic bacteria, such as the paracolons and coliforms. This is in accord with the earlier findings of Stutzer (1927) and Allen (1946). Obviously, the streptococci tolerate the acidic conditions prevailing towards the end of a ret (pH 4.2-4.4) better than do the other types. The predominance of streptococci in the later stages of retting probably accounts, in part, for their constituting such a high proportion of the total aerobic organisms isolated here, since approximately equal numbers of colonies were picked from the pour plate cultures of liquor samples taken at each retting stage.

(*b*) *The Coliform Flora*

Although members of this bacterial group were not recovered in appreciable numbers from the aerobic plate count cultures, the use of more specific methods demonstrated that they are well represented in flax retting liquor. Their numbers and types, however, vary considerably from ret to ret, the range of maximal counts being from 10^2 to 10^8 per ml. With the exception of *Escherichia coli* type II, all of Wilson's (1935) major types and three of the irregular types were found from time to time. It is of interest to note that, even with this isolation method based on acid and gas production in MacConkey's broth, almost 30 per cent. of the cultures recovered were paracolon types.

Of the coliform bacteria isolated from the cultures used for presumptive counts, 11.4 per cent. were *Esch. coli* type I. To this figure may be added the 3.4 per cent. of irregular type II, making a total proportion of 14.8 per cent. of faecal types. This proportion is only slightly lower than that reported by Allen (1946), who found 16.7 per cent. of faecal strains in 150 cultures.

A finding of some considerable interest in the present study was that all but one of the faecal strains was isolated from mill retting liquor. That is to say, while faecal coliform bacteria were present in only one of 18 laboratory rets, they were found in 10 of 11 mill rets, nine of the latter yielding *Esch. coli* type I. The reason for the more frequent occurrence of faecal types in mill retting liquor is not clear at present. However, as thorough cleaning of mill retting tanks between rets is not possible, owing to the rough, pitted nature of the walls and other structural features, it is thought that some form of carry-over from ret to ret may be responsible. Whether this transfer is of bacterial cells, or of some substance favourable to the multiplication of the faecal strains is not evident at this juncture. There is no apparent reason why any one of the various types present should gain the ascendency by transfer from ret to ret, unless the environment favoured it more than the others but, with such rapidly multiplying bacteria as the coliform group, it seems reasonable to assume that, once introduced, a particular type could persist in a tank and, given a favourable medium, thereby constitute an appreciable proportion of the flora in successive rets. On the other hand, the possibility of transfer of some growth-promoting substance, which is particularly stimulatory to the faecal coliform types, cannot be ruled out. The laboratory retting tanks, which are constructed of stainless steel with a highly polished inner surface, can be much more thoroughly cleaned between rets and the amount of material carried from ret to ret must be very small. Thus, in laboratory rets, it appears unlikely that the flora on the straw itself would be augmented by seeding from the tank walls. Moreover, the presence of faecal coliform strains in only one of 18 laboratory rets studied is in keeping with the fact that these bacteria are not encountered in material free from contamination by animal excreta.

Recalculation shows that, of the coliform bacteria isolated from mill rets, 28.1 per cent. were *Esch. coli* type I and 9.4 per cent. were irregular type II,

a total of 37.5 per cent. of faecal types. Consequently, mill retting effluents may be expected to have a definite effect on the faecal coliform count of any body of water into which they may be discharged. The magnitude of this effect would, of course, vary according to the volume of retting effluent discharged in a given time and the capacity of the lake or flow rate of the river in question, and could only be determined by periodical analyses.

(*c*) *The Retting Flora*

The method of isolation employed has been shown to have a marked influence on the proportions of retting and non-retting clostridia recovered from heated suspensions of retted flax. Even with the most suitable method found to date, however, namely direct plating on glucose yeast extract agar, approximately equal numbers of both groups are usually recovered. It would now appear that the true retting organisms constitute a relatively small part of the total bacterial flora of retting flax and consequently, it might well be anticipated that their activity would be influenced, to some extent at least, by variations in the composition of the subsidiary flora. Thus, the interrelationships of various non-retting organisms and the retting species may prove a fruitful field for future investigations.

Four distinct types of retting clostridia have been identified in this study of Australian flax retting. Two of these, viz. types I and II, are evidently the agents predominantly responsible for retting in this country, while types III and IV are less frequently present and then only in relatively small numbers, so that their role must be a minor one. Indeed, type IV has only been isolated from three of the large number of flax samples examined. Tests on sterilized flax straw in yeast water, using a number of strains of each type, have revealed noteworthy differences in the respective retting abilities of the four organisms. Type III, *Clostridium felsineum,* proved outstanding with regard to both rate and effectiveness of retting. This observation agrees with earlier findings in this field (Carbone and Tobler 1922; Markova 1940; Ruschmann and Bartram 1943; Enebo 1947). Clostridial types II and IV were less efficient retting agents than *Cl. felsineum,* although apparently effective by qualitative tests in a longer time, while type I, the organism most commonly encountered here, was by far the least active of the four. In fact, many strains of type I left the flax incompletely retted after 7-10 days incubation at 37°C.

The fact that *Cl. felsineum* is not commonly present in large numbers, and that less active and frequently inefficient agents predominate, in Australian flax retting probably bears a significant relationship to the slowness of retting in this country and the frequently under-retted nature of our fibre. This hypothesis and experiments relating thereto were discussed in an earlier publication (Lanigan 1950). However, in the light of the evidence now available, some modification of views expressed then would appear necessary. Initially, it was thought that the active retting agent, *Cl. felsineum,* was sparsely distributed in the soil of our flax-growing districts and, consequently, only appeared in an occasional ret; but Table 6 shows that the direct plating

method led to the detection of this organism in almost half the rets examined in this way. The isolation of this clostridium from such a high proportion of these rets suggests that it may actually be present in most or all of them, but in numbers too small to permit detection, in the absence of a suitable enrichment technique. Thus, it would now appear that the lack of prominence of *Cl. felsineum* in Australian flax retting may be due, not to its infrequent presence on the straw, but rather to environmental factors during retting. There are, of course, many ways in which the environment could prove unsuitable for a particular species and these may be either peculiar to some flax crops or common to all. If the foregoing assumption is correct, then it would be anticipated that inoculation of rets with pure cultures of *Cl. felsineum* would be ineffective, unless substances carried over in the inoculum obviated environmental deficiencies. The author's experiments on rets inoculated with *Cl. felsineum* are incomplete at present and no definite conclusions can yet be drawn from them regarding the value of such inoculation. The following observations are, however, pertinent to this discussion. While the retting of some flax samples is greatly improved by the inoculation, the effect with others is only slight. Moreover, in the relatively few cases investigated to date, it has only been possible to recover *Cl. felsineum* from some of the straw samples at the conclusion of retting. Thus, there is evidence that this clostridium fails to survive, or does not multiply appreciably, following inoculation of some Australian flax rets, and this lends support to the suggestion made above that scarcity of *Cl. felsineum* in our retting may be attributable to environmental factors. As it has been demonstrated that inoculation with this organism leads to marked acceleration and improvement of the retting of some Australian flax straw, it is obviously of great importance that an attempt should be made to establish the underlying causes of its failure to survive and multiply in other rets.

Of the various retting clostridia reported in the literature, only *Cl. felsineum* is accorded specific rank in the current edition of Bergey's Manual (1948). Most of the others are listed as probable varieties of *Cl. butyricum.* There is no doubt, however, that retting clostridia generally, and the plectridial species in particular, exhibit quite different morphological and biochemical characteristics from those of *Cl. butyricum.* It is evident, therefore, that a detailed comparative study of existing cultures of retting bacteria should be undertaken, in order to establish their taxonomic relationships. Three of the four retting agents encountered in the present study differ significantly from currently recognized species of *Clostridium* but it is not the author's intention to name them as new species at this stage, since this would only aggravate an already confused situation. A few comments on clostridial types I, II, and IV may, nevertheless, be justified.

The characteristics of the Australian retting clostridium type I agree well with those of retting organisms described by Weizmann and Hellinger (1940), who considered that their organisms should be classified with Störmer's (1904) plectridial forms as *Clostridium pectinovorum* Störmer, except for the fact that

the Australian strains were micro-aerophilic, as were Störmer's strains (Bergey *et al.* 1948). It is possible, of course, that varieties of *Cl. pectinovorum* differ in the degree of their oxygen tolerance. *Cl. tertium* (Henry) Bergey *et al.* (1948) is the only saprophytic, micro-aerophilic clostridium given specific status by Bergey *et al.* (1948) but, while this organism also forms terminal, elliptical spores, Weizmann and Hellinger's and the author's strains of *Cl. pectinovorum* differ from *Cl. tertium* in liquefying gelatin, actively fermenting potato mash, and in not reducing nitrates. Accordingly, there appear to be good reasons for the recognition of *Clostridium pectinovorum* Störmer as a (micro-aerophilic) species of the genus *Clostridium.*

Type II retting clostridium, an obligate anaerobe, has the general characteristics of the butylic group of clostridia, but does not correspond to any of the species described by Bergey *et al.* (1948). For example, it resembles, in some respects, *Cl. acetobutylicum* but differs significantly from this organism in its morphology, colonial form, fermentation of pectin, and non-fermentation of mannitol and inulin. It appears likely therefore that the Australian retting clostridium type II is a new species.

The yellow-pigmented, retting clostridium type IV exhibits similar biochemical properties to those of *Cl. felsineum.* However, in addition to obvious differences in pigmentation, these two clostridia show marked differences in the morphology of their sporangia and spores and in their colonial form. Apart from two cellulose-digesting clostridia, which are obviously unrelated to clostridium type IV, no yellow-pigmented clostridia are described by Bergey *et al.* (1948). However, de Graaf (1930), mentioned by McClung (1943), described a yellow-pigmented clostridium which he called *Cl. xanthogenum,* and McClung (1943) reported that he had isolated three or four similar types. Unfortunately, descriptions of these organisms are not yet available to the author, so that no comparison of them with local strains is possible. There seems little doubt, however, that at least one species of yellow-pigmented clostridium, other than the cellulose digesters, exists.

(*d*) *The Non-Retting Anaerobic Flora*

The fact that some 87 per cent. of the non-retting clostridia, isolated in this study of Australian flax retting, proved to be varieties of *Clostridium butyricum* is, perhaps, not unexpected, since such organisms are known to be common in cultivated soils, on plants, and on other natural products. Conditions during retting are obviously favourable for their development, as they are usually present in numbers equalling those of the retting bacteria. It is tempting to speculate whether or not these butyric anaerobes are of assistance to the retting species, for example, by virtue of the known ability of some varieties to fix atmospheric nitrogen. As far as is known, however, this possibility has not been investigated.

All but one of the 151 strains of *Cl. butyricum*-like anaerobes studied by the author failed to correspond in their properties with the varieties listed by Bergey *et al.* (1948). Thus, although they were separable into *butyricum* and

multifermentans types, they consistently fermented mannitol, glycerol, and lactate, thereby showing overlapping properties of these two starch-fermenting varieties. Varieties of *Cl. butyricum* with similar properties to those described here were reported by Tabachnick and Vaughn (1948) to be capable of fermenting tartrates. Thus the biochemical properties of these butyric anaerobics from retted flax, together with the occurrence of five distinct colonial types, emphasize the variability existing in this group of clostridia.

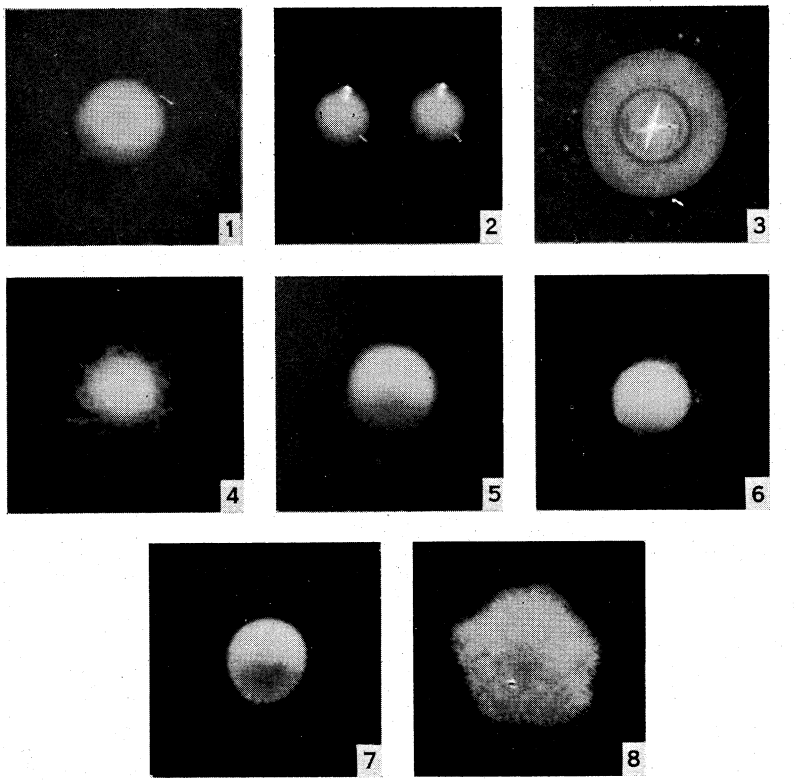
V. Acknowledgments

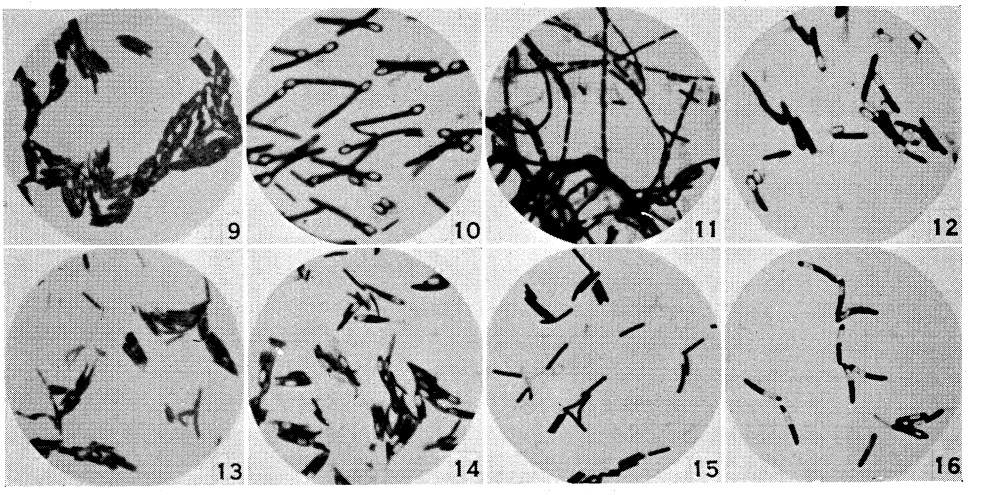
The author desires to express his appreciation of the constructive criticism offered by Dr. Nancy Hayward, Senior Lecturer, Bacteriology School, University of Melbourne, and his indebtedness to Mr. E. J. Williams, Section of Mathematical Statistics, C.S.I.R.O., for statistical analysis of the aerobic plate counts. Valuable technical assistance by Miss A. M. Norton is also gratefully acknowledged.

VI. References

Allen, L. A. (1946).—*J. Soc. Chem. Ind.* **65**: 365, 373.
Behrens, J. (1903).—*Zbl. Bakt.* II **10**: 524.
Beijerinck, M. W., and Van Delden, A. (1904).—*Arch. Néerl. Sci.* (2) **9**: 418.
Bergey, D. H., *et al.* (1948).—"Manual of Determinative Bacteriology." 6th Ed. (Williams and Wilkins Co.: Baltimore.)
Borman, E. K., Stuart, C. A., and Wheeler, K. M. (1944).—*J. Bact.* **48**: 351.
Carbone, D., and Tobler, F. (1922).—*Faserforschung* **2**: 163.
Carbone, D., and Tombolato, A. (1917).—*Staz. Sper. Agr. Ital.* **50**: 563.
D.S.I.R., Great Britain (1948).—Flax retting with aeration. Water Pollution Res. Lab. Tech. Pap. No. 10: 10-2.
Enebo, L. (1947).—*Acta Agric. Suecana* **2** (3): 319.
de Graaf, J. (1930).—*Ned. Tijdschr. Hyg.* **4**: 219.
Hayward, Nancy J. (1945).—*Proc. Ass. Clin. Path.* **1**: 5.
Hucker, G. H., and Pederson, C. S. (1930).—New York Agric. Exp. Sta. Tech. Bull. No. 167: 66.
Jensen, H. L. (1941).—*Proc. Linn. Soc. N.S.W.* **66**: 276.
Lanigan, G. W. (1950).—*Nature* **165**: 516.
Markova, Z. S. (1940).—*Mikrobiol.* (*U.S.S.R.*) **9**: 824.
McClung, L. S. (1943).—*J. Bact.* **46**: 507.
McCoy, Elizabeth, and McClung, L. S. (1935).—*Arch. Mikrobiol.* **6**: 230.
Mushin, Rose (1949).—*Aust. J. Exp. Biol. Med. Sci.* **27**: 543.
Orla-Jensen, A., and Kluyver, A. J. (1939).—*Zbl. Bakt.* II **101**: 257.
Rossi, G. (1904).—*Annu. Scu. Agr. Portici* **5**.
Rossi, G., and Carbone, D. (1909).—*Annu. Scu. Agr. Portici* **9**.
Ruschmann, G. (1923).—Grundlagen der Röste, Leipzig.
Ruschmann, G., and Bartram, H. (1943).—*Zbl. Bakt.* II **105**: 326, 433.
Ruschmann, G., and Bavendam, W. (1925).—*Zbl. Bakt.* II **64**: 340.
Störmer, K. (1904).—*Zbl. Bakt.* II **13**: 171, 306.
Stutzer, M. J. (1927).—*Zbl. Bakt.* II **69**: 164.
Tabachnick, J., and Vaughn, R. H. (1948).—*J. Bact.* **56**: 435.
Weizmann, C. H., and Hellinger, Esther (1940).—*J. Bact.* **40**: 665.
Wilson, G. S., *et al.* (1935).—The bacteriological grading of milk. Med. Res. Coun. Spec. Rep. No. 206. (H.M.S.O.: London)
Wright, B. M. (1943).—Army Pathology Service, Great Britain. Current Notes, No. 9.

BACTERIAL FLORA OF AUSTRALIAN FLAX RETTING





Explanation of Plate 1

Figs. 1-8.—The colonies illustrated were grown on glucose yeast extract agar, at 37°C. x10.
Figs. 1-3.—Retting clostridium type I.
Fig. 1.—Low convex, butyrous colony; 3 days.
Fig. 2.—Pulvinate, rubbery colony; 2 days.
Fig. 3.—Collapsed form of colony in Figure 2. The inner ring is the original outline; 3 days.
Figs. 4-6.—Retting clostridium type II.
Fig. 4.—Typical colony with myceloid margin; 3 days.
Fig. 5.—Smooth, regular, viscid colony; 3 days.
Fig. 6.—Colony type intermediate between those in Figures 4 and 5; 3 days.
Figs. 7-8.—Retting clostridium type IV.
Fig. 7.—Typical colony; 3 days.
Fig. 8.—Larger, irregular colony on sparsely seeded plate; 3 days.

Figs. 9-16.—Photomicrographs are of Gram-strained preparations from cultures on glucose yeast extract agar, at 37°C. x900.
Fig. 9.—Vegetative cells of retting clostridium type I.
Fig. 10.—Sporangia and spores of retting clostridium type I.
Fig. 11.—Vegetative cells of retting clostridium type II.
Fig. 12.—Sporangia and spores of retting clostridium type II.
Fig. 13.—Vegetative cells of *Cl. felsineum.*
Fig. 14.—Sporangia and spores of *Cl. felsineum.*
Fig. 15.—Vegetative cells of retting clostridium type IV.
Fig. 16.—Sporangia of retting clostridium type IV.