Cellulolytic Bacteria Associated with Sloughing Spoilage of California Ripe Olives

ISHWARLAL B. PATEL and REESE H. VAUGHN

Department of Food Science and Technology, University of California, Davis, California 95616

Received for publication 22 December 1971

Sloughing spoilage of California ripe olives during processing is characterized by severe softening, skin rupture, and flesh sloughing. It was assumed that cellulolytic activity was responsible for skin rupture and sloughing of flesh, and so a deliberate search was made for cellulolytic bacteria from olives undergoing sloughing spoilage. A bacterium identified as Cellulomonas flavigena was highly cellulolytic, attacking filter paper, carboxymethyl cellulose (CMC) gel, and olive tissue. Other bacteria attacking CMC, but not filter paper, enhanced the activity of the Cellulomonas strain when grown in mixed culture, although they did not, in pure culture, have any effect on filter paper. These latter cultures (all degraded olive tissue) represented the genera Xanthomonas, Aerobacter, and Escherichia. Other noncellulolytic bacteria belonging to the genera Alcaligenes, Kurthia, and Micrococcus also were used for study of mixed culture fermentation of cellulose by C. flavigena. Cellobiose accumulation at levels of 1.0% (w/v) and above suppressed growth of C. flavigena.

In a previous study (11), gram-negative pectinolytic bacteria were reported to be associated with the sloughing spoilage of California ripe olives during processing. However, in spite of rapid softening, rupture of the skin and sloughing of the flesh were not observed. Because skin rupture and flesh sloughing as well as generalized tissue softening are typical symptoms of this spoilage, it was postulated that free-living, cellulase-producing microbes might also be associated with the deterioration. Therefore a deliberate search was made for cellulolytic bacteria from olives undergoing sloughing. This report describes the results of that search and clearly associates cellulolytic bacteria with sloughing spoilage of olives.

MATERIALS AND METHODS

Isolation and culture media. The basal medium used for growth and isolation of cellulose-decomposing organisms was the one described by Han and Srinivasan (4). The cellulotic substrates were Whatman no. 1 filter paper and carboxymethyl cellulose (CMC) (type 7HF, Hercules Inc.). The latter was used at a concentration of 2.0% as recommended by Goto and Okabe (3).

Enrichment, isolation, and purification. The sloughed olives investigated were of commercial origin and represented the Mission, Manzanilla, and Sevillano varieties. The covering liquid was a dilute aqueous solution of lye, or salt, or both. About 1 ml of this solution from the sloughed olives was inoculated into a test tube containing approximately 10 ml of the basal medium and a strip of filter paper so placed that its top portion projected about ¾ inch (ca. 1.9 cm) above the surface of the liquid. After 5 to 7 days at 30 C on a Rollor drum machine (model TC-5, New Brunswick Scientific Co.), if cellulolytic bacteria were present, a patch of yellow-pigmented material appeared at the air-liquid interface of the paper. Then a small piece of the paper showing slight disintegration at the air-liquid interface was transferred aseptically with sterile forceps to a new tube of the same medium. This process was repeated seven or eight times to enrich the cellulolytic organisms. Then the paper from the last enrichment was removed, macerated in a small amount of sterile physiological saline solution, and streaked onto plates containing nutrient agar, CMC agar (0.5% CMC in basal medium) plus 1.5% agar, and filter paper agar (a plate of nutrient agar covered with a filter paper disc), respectively.

Representative colonies which developed on each of these agar media were picked and inoculated into fresh filter paper medium and into CMC gel medium. Cellulolytic cultures were further purified by alternately plating to test purity and enriching in the filter paper medium and CMC gel until pure cultures were obtained (3).

Two filter paper decomposing cultures were obtained. On the basis of morphology and colony characteristics they appeared to be identical, so only one was retained for further study. Four cultures which attacked CMC gel but not filter paper were...
also retained for further study. Three noncellulolytic cultures were isolated for mixed-culture fermentation studies.

Identification of the bacteria. The cultures retained for further study were identified by conventional methods used for generic and species allocation. General references included Breed et al. (1), Society of American Bacteriologists (9), and Skerman (8). When necessary the original literature was consulted.

Preparation of crude cell-free cellulolytic enzyme solutions. The one filter paper decomposing isolate was grown in the basal medium containing 0.25% filter paper (w/v) or 1% CMC (w/v). After incubation for 5 days at 30°C with continuous shaking, cell-free preparations were obtained by centrifugation in a Sorvall, Super Speed R.C.-2, automatic refrigerated centrifuge at 9,500 rev/min for 15 min at 3 to 4°C. The clear supernatant fluid which contained the cellulolytic enzyme(s) was collected. A 1-ml amount of 1% Merthiolate (thimerosal powder, N.F.) was added per 100 ml of solution. The solutions were stored in a refrigerator until used.

The four cellulolytic cultures unable to macerate filter paper were grown in the basal medium containing 1% CMC (type 7MF, Hercules Inc.). After incubation for 10 days at 30°C with continuous shaking, cell-free solutions were prepared by centrifugation at 9,500 rev/min for 20 min at 3 to 4°C. The clear supernatant fluid was collected and preserved as described above.

Gravimetric determination of cellulolytic activity. The growing 18- to 24-hr cultures were inoculated into 250-ml Erlenmeyer flasks containing 100 ml of basal medium and a limited amount of filter paper (110 to 120 mg per flask). After 5 days of incubation at 30°C on a continuous shaker, the flasks were removed, and their contents were filtered, washed, and dried in a Gooch crucibles of 30-ml capacity and medium porosity according to the procedure of Lemberg and Colmer (5).

Viscosimetric determination of cellulolytic enzyme activity. The activity of the crude enzyme preparations was determined by measurement of the changes in viscosity of the CMC (type 7MF) substrates that were induced by the enzymes with an Ostwald viscosimeter as described by Nortje and Vaughn (7).

The volume of crude enzyme solution was always 10 ml. The volume of 1% CMC (T7MF) was always 20 ml. The reaction time was always 30 min. The effect of pH was determined at 30°C. The effect of temperature was studied at pH 6.0 with the enzyme produced in the presence of filter paper and at pH 6.5 with CMC as the substrate.

Softening of olives by crude enzyme preparations. The possibility exists that cellulolytic degradation of olive tissue can occur during their storage in salt brine as well as during the final stages of processing when leaching with water is used to remove the lye used to destroy the bitter glucoside oleuropin. It was not possible in the laboratory to exactly duplicate the conditions existing in industry. However, the in vitro tests were designed to duplicate all but the physical pressure produced by 3- to 4-ft depths of olives.

Ability of the crude enzyme preparations to soften olive tissue was done with Manzanilla ripe process fruit and with Sevillano variety olives from salt brine storage with preparations adjusted to various pH values (range 4.5 to 8.0) with McIlvaine (6) buffer and preserved with Merthiolate. Controls, regardless of the variety, consisted of olives, buffer solution of the desired pH, and Merthiolate to preserve the mixture. Both of the varieties were submerged in the enzyme solutions for 5 days at 30°C. The ripe process olives were observed daily for signs of softening, skin rupture, and flesh sloughing by sight and feel. After 5 days the Sevillano olives were put through the ripe processes (see Vaughn [10] and Cruess [2] for details). After the lye had penetrated to the pit, the olives were leached with three to four changes of water each day for 5 days. Signs of disintegration were observed daily during the 15 days of processing.

The Manzanilla ripe olives were desalted so they would simulate olives during the washing period used to leach the residual lye from the fruits during the final stages of processing prior to canning (see above). It is during the 4- to 5-day washing period that sloughing spoilage becomes apparent.

The brine storage (7.0% salt, 0.35% total acidity as lactic acid, and pH 4.85) Sevillano fruits during the 15 days required for processing would lose all of the salt and total acidity because of leaching, and the final pH would be in the range of 7.0 to 8.0 during the final washing period.

RESULTS

Solely on the basis of the tests shown in Tables 1 and 2, the eight isolates described appeared to be in seven different genera and eight species of bacteria. The salient features of each follow.

Cellulomonas flavigena. The C. flavigena culture (DS) was the only strongly cellulolytic bacterium isolated. It disintegrated filter paper and CMC readily and caused skin rupture and flesh sloughing of olives. The characteristics are identical to the description of C. flavigena found in Bergey's Manual, 7th ed. (1).

The genus Xanthomonas. The two cultures representing the genus Xanthomonas caused liquefaction of CMC gel and disintegrated olive tissue, but did not attack filter paper. The culture DB has been allocated to X. stewartii, although plant pathogenicity was not tested. The other culture (98C) was not allocated to a specific species, although it resembled the descriptions of X. pruni and X. maculifoliigardeniae found in Bergey's Manual (1), the only difference being that culture 98C did not digest milk.

The coliform bacteria. The coliform bacteria culture 98A was able to liquefy CMC gel and degrade olive tissue but not filter paper. It had all of the characteristics of Aerobacter
Table 1. Morphological and physiological characteristics of the isolates

| Taxonomic allocation | Culture no. | Form | Cell size (µm) | Motility (flagella) | Nutrient broth* | Gram stain | Colony pigment* | Gelatin liquefaction | Nitrate reduction | Starch hydrolysis | Urea decomposition | Litmus milk† | Optimal temperature (°C) |
|----------------------|-------------|------|----------------|---------------------|-----------------|------------|-----------------|---------------------|-------------------|-----------------|----------------------|-------------|-------------------------|
| *C. flavigena*       | DS          | Rods | 0.5–0.8 × 1.5–2.2 | Nonmotile           | T                | Variable   | Greyish yellow | +                   | +                 | –               | –                    | AR          | 25–35                  |
| *X. steavartii*       | DB          | Rods | 0.5–0.8 × 1.5–2.2 | Nonmotile           | RST              | –          | Light yellow   | –                   | –                 | –               | –                    | RCD         | 25–35                  |
| *X. sp.*             | 98C         | Rods | 0.5–0.8 × 1.5–2.2 | Polar               | PRST             | –          | Yellow         | +                   | +                 | –               | –                    | AR          | 25–35                  |
| *Aerobacter cloacae*  | 98A         | Rods | 0.5–1.0 × 1.0–2.0 | Peritrichous        | SRT              | –          | White          | +                   | +                 | –               | –                    | ACDG        | 30–37                  |
| *Escherichia intermedia* | 4A        | Rods | 0.5–1.0 × 1.0–2.0 | Peritrichous        | ST               | –          | Greyish white  | –                   | +                 | –               | –                    | AR          | 25–35                  |
| *Kurthia bessonii*    | CA          | Rods | 1.0–1.5 × 2.0–3.5 | Peritrichous        | ST               | Variable   | Greyish white  | +                   | –                 | +               | +                    | NP          | 30–37                  |
| *Micrococcus sp.*     | CW          | Spherical | 1.0–1.3 diam  | Nonmotile           | ST               | +          | White          | –                   | +                 | –               | –                    | A           | 30–37                  |
| *Alcaligenes faecalis* | AW         | Rods | 0.5–0.7 × 1.0–2.0 | Peritrichous        | T                | –          | Greyish white  | –                   | +                 | –               | –                    | Alk         | 30–37                  |

*P, pellicle; R, ring; S, sediment; T, turbidity.

*Pigment of cultures grown on nutrient agar.

†A, acid; Alk, alkaline; C, curd; D, digested; G, gas; N, neutral; P, peptonized; R, reduced.
| Taxonomic allocation | Culture no. | Indole production | Methyl red test | V.-P* reaction | Citrate utilization | H₂S production | Filter paper in peptone broth | Carboxymethyl cellulose-gel liquefication* | Glucose* | Lactose | Maltose | Sucrose | Mannitol | Glycerol | Starch | Dextrin | Salicin | Cello-biose |
|----------------------|-------------|-------------------|----------------|----------------|--------------------|----------------|-----------------------------|------------------------------------------|----------|---------|---------|---------|----------|---------|-------|--------|--------|------------|
| *Cellulomonas flavigena* | DS          | -                 | ±              | -              | -                  | -              | +*                         | 3-4                                     | A        | A       | A       | A       | A        | A       | A     | A      | A      | A          |
| Xanthomonas *stewartii* | DB          | -                 | -              | +              | -                  | -              | -                          | 5-6                                     | A        | A       | A       | -       | -        | A       | A     | A      | A      | A          |
| Xanthomonas sp.       | 98C         | -                 | -              | -              | +                  | -              | 8-9                         | A                                       | A        | A       | A       | A       | A        | A       | A     | A      | A      | A          |
| *Aerobacter cloacae* | 98A         | -                 | -              | +              | +                  | -              | 15-16                       | AG                                      | AG       | AG      | AG      | AG      | A        | AG      | AG    | AG     | AG     | AG         |
| Escherichia *intermedia* | 4A          | +                 | +              | +              | +                  | -              | 20-21                       | AG                                      | A        | AG      | AG      | AG      | AG       | AG      | -     | AG     | AG     | AG         |
| *Kurthia bessonii* | CA          | -                 | -              | +              | -                  | -              | A                                                  | A                                      | A        | A       | A       | A       | A        | A       | A     | -      | -      | -          |
| *Micrococcus* sp.  | CW          | -                 | ±              | -              | -                  | -              | -                          | -                                       | -        | -       | -       | -       | -        | -       | -     | -      | -      | -          |
| *Alcaligenes faecalis* | AW          | -                 | -              | +              | -                  | -              | -                          | -                                       | -        | -       | -       | -       | -        | -       | -     | -      | -      | -          |

* Voges-Proskauer reaction + or - for production of acetoin plus diacetyl from glucose.
* Days required to liquefy carboxymethyl cellulose-gel completely.
* A, acid; G, gas.
* Fibers separated upon slight agitation after 1 week of incubation at 30 C.
* Citrate utilized after 7 days of incubation at 30 C.
cloacae and was so allocated. The other culture (4A) also was cellulolytic to a degree, but was the slowest of the five cellulolytic cultures studied. Even though culture 4A utilized citrate slowly and formed only acid from lactose, it was allocated to the Escherichia intermedia group. An alternative would be to call it Paracolobactrum intermedium on the basis of its anomalous lactose fermentation.

Kurthia bessonii. Culture CA was not cellulolytic, but was the only pectolytic organism found in this study. This culture differs only slightly from the descriptions found in Bergey's Manual (1) and given by Skerman (8) for K. bessonii. The main differences are the variability of the Gram stain and the slight amount of acid produced from carbohydrates.

The Micrococcus. Culture CW was placed in the genus Micrococcus solely on morphological grounds, but it could not be identified satisfactorily even as to genus because of many variations in morphological and physiological characteristics. These characteristics, however, best match those of the genera Micrococcus or Gaffkya as found in Bergey's Manual (1) or Skerman (8).

Alcaligenes faecalis. The other noncellulolytic culture (AW) had all of the characteristics of A. faecalis as found in Bergey's Manual (1) and was so named. It is felt that neither A. faecalis nor the unidentified micrococcus play any important role in mixed fermentation acceleration of cellulolytic activity as will be shown below.

All of the isolates involved in this study could grow in the presence of 8% sodium chloride (w/v) in nutrient glucose, tryptone, and yeast extract broth.

Cellulolytic activity. The results given in Table 3 compare the attack of C. flavigena on filter paper in pure culture and in mixed culture with the other isolates. The degree of digestion (percent of solubilized cellulose) was 32.35% when C. flavigena was grown alone. Digestion increased about 25 to 50% when the bacterium was grown in mixed culture with the isolates able to use CMC. The noncellulolytic A. faecalis and Micrococcus sp. had little effect on increasing filter paper digestion.

Five of the eight cultures involved in this study grew well on CMC-gel and liquefied it at varying rates. However, because of the slowness of their reaction rates, C. flavigena was used to produce crude enzyme to study the effect of pH and temperature on cellulolytic activity as measured by viscosimetry. Data on the effect of pH are shown in Fig. 1; those on the effect of temperature are shown in Fig. 2.

| Cultures          | Wt of filter paper (mg) | Final wt of residues (mg) | Degree of digestion (% solubilized cellulose)* |
|-------------------|-------------------------|---------------------------|-----------------------------------------------|
| C. flavigena      | 115.3                   | 78.0                      | 32.35                                         |
| C. flavigena plus X. stewartii | 118.3                   | 21.5                      | 81.82                                         |
| C. flavigena plus Xanthomonas sp. | 119.7                   | 22.1                      | 81.54                                         |
| C. flavigena plus A. cloacae | 113.2                   | 24.8                      | 78.09                                         |
| C. flavigena plus E. intermedia | 117.5                   | 50.9                      | 56.68                                         |
| C. flavigena plus K. bessonii | 116.9                   | 42.0                      | 64.07                                         |
| C. flavigena plus Micrococcus sp. | 115.6                   | 70.6                      | 38.92                                         |
| C. flavigena plus A. faecalis | 114.8                   | 73.4                      | 36.06                                         |

*Degree of digestion of cellulose was measured gravimetrically after the organisms were inoculated into 100 ml of media containing filter paper (Whatman no. 1) as a sole source of carbon. Inoculum was 1.0 ml of cell suspension for single-culture and 0.5 ml of each for mixed-culture fermentation. Incubation was for 5 days at 30 C on a continuous shaker.

The cell-free crude enzyme filter paper preparation was most active at about pH 6.0 and had good activity in the range of pH 5.0 to 7.0, whereas the preparation obtained with CMC as the substrate was most active at about pH 6.5 and had good activity in the range of pH 5.0 to 7.5. As shown, the optimum temperature for activity of the crude enzymes was 50 C regardless of the substrate.

Degradation of olive tissue. Crude, cell-free enzymes prepared from the cellulolytic bacteria all softened desalted Manzanilla ripe-olive tissue as shown in Table 4. In the range of pH 7.0 to 8.0 the softening was pronounced, and there was skin rupture and flesh sloughing typical of the spoilage as observed under commercial conditions.

Nearly identical results were obtained with brine storage Sevillano olives that were treated with crude, cell-free enzymes and then were put through the ripe-pickling process. Figure 3 shows in vitro sloughing spoilage.

Softening and skin rupture of olives subjected to the crude enzymes was consistent and involved all of the olives tested to a greater or lesser degree. In contrast, the control olives showed no evidence of softening or sloughing.

Cellulolytic activity of pectolytic bacteria previously studied. Seventeen of the 19 cul-
TABLE 4. Softening of California ripe olives by crude bacterial enzyme preparations

| pH of enzyme preparation | Crude enzyme produced by: |
|--------------------------|---------------------------|
|                          | C. flavigena | X. ste- | Xantho- | A. clo- | E. inter- |
|                          |              | wartii  | monas   | acae   | med- |
| A*                      | B*           | A       | A       | A      | A |
| 4.5                     | -            | -       | -       | -      | - |
| 5.0                     | -            | -       | -       | -      | - |
| 5.5                     | +            | ±       | ±       | ±      | - |
| 6.0                     | +            | ±       | +       | ±      | ± |
| 6.5                     | +            | +       | +       | +      | + |
| 7.0                     | + +          | +       | ++      | +      | + |
| 7.5                     | + + +        | +       | ++      | +      | + |
| 8.0                     | + + + +      | +       | ++      | +      | + |

*Enzymes produced in the basal medium with 1% CMC (type 7MF).

Fig. 2. The effect of temperature on the activity of the cellulolytic enzymes produced by C. flavigena. O, Filter paper substrate; Δ, carboxymethyl cellulose substrate.

Fig. 1. The effect of pH on the activity of the cellulolytic enzymes produced by C. flavigena. O, Filter paper substrate; Δ, carboxymethyl cellulose substrate.

Properties of a number of pure cultures described in 1969 (11) were still available in the laboratory culture collection. These cultures were examined for their ability to attack CMC gel, degrade cellobiose, and disintegrate filter paper. None of the cultures could disintegrate filter paper but, surprisingly, 11 of the cultures could completely liquefy CMC gel, and 14 decomposed cellobiose with acid or acid and gas production, depending on the genus. The Achromobacter species culture 178, previously shown to be extremely pectolytic, caused complete liquefaction of CMC gel in 3 days, the same time required by C. flavigena.

Five of the cultures including Aerobacter aerogenes 43, Aeromonas liquefaciens 46, Achromobacter sp. 178, E. intermedia 177 and Paracolobactrum aerogenoides 38 were grown in mixed culture with C. flavigena in the basal medium with filter paper strips for 5 days at 30 C on the Rollor drum agitator. The mixed cultures had the same pronounced effect in increasing solubilization of filter paper cellulose over that produced by C. flavigena in pure culture as was observed with the cultures isolated in this study.

DISCUSSION

Association of cellulolytic bacteria with sloughing spoilage of olives confirms the speculation by Vaughn et al. (11) that cellulolytic...
microbes might cause the skin rupture and flesh sloughing so characteristic of this spoilage. Some of the pectolytic bacteria previously associated with softening, but not sloughing, were also found to be cellulolytic and also increased cellulose solubilization when grown in mixed culture with C. flavigena. The cultures, pectolytic or not, that degraded CMC all decomposed cellubiose. A concentration of 1.0% cellubiose (w/v) or more was found to inhibit C. flavigena. It is possible that the other bacteria favor the activity of C. flavigena by keeping the cellubiose at a low level. However, noncellulolytic, cellubiose-degrading yeasts grown with C. flavigena did not increase solubilization of the filter paper so this explanation is suspect. Another possibility is that the bacteria unable to decompose filter paper cellulose supply micronutrients to C. flavigena. This latter speculation is suspect, because the two noncellulolytic, cellubiose-negative bacteria used in this study did not materially increase cellulose degradation when grown in mixed culture with C. flavigena. It is also possible that, once C. flavigena has degraded the filter paper to a certain degree of polimerization, the CMC-degrading bacteria can attack the lesser degree of polimerization cellulose and thus increase the total solubilization of the filter paper cellulose. A concise explanation obviously is dependent on further research. In any event, both cellulolytic and pectolytic enzymes produced by various bacteria are involved in sloughing spoilage of olives.

C. flavigena and the other cellulolytic cultures also caused marked tissue destruction when grown in sterile olives in brine.

ACKNOWLEDGMENTS

This investigation was supported by the Olive Administrative Committee administering Federal Olive Order no. 932.

We thank H. J. Phaff of this Department for cellubiose-degrading yeast cultures.

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