APPLIED MICROBIOLOGY, Feb. 1972, p. 316-320
Vol. 23, No. 2

Fermenting Yeasts Associated with Softening and Gas-Pocket Formation in Olives

REESE H. VAUGHN, KENNETH E. STEVENSON,[1] BHALCHANDRA A. DAVÉ,[2] AND HYAN C. PARK[3]

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

Received for publication 8 March 1971

Fermenting, pectolytic yeasts were isolated from a massive commercial outbreak of softening and gas-pocket formation in olives that had been stored in acidified, low-salt brines in an attempt to reduce the problem of brine disposal. The suspected yeasts represented three different species: *Saccharomyces oleaginosus*, *S. kluyveri*, and *Hansenula anomala* var. *anomala*. All pectolytic cultures produced pectin esterase and polygalacturonase but no pectic acid *trans*-eliminase when grown in nutrient glucose broth. Crude, cell-free dialyzed enzyme preparations measured viscosimetrically exhibited optimal activity on sodium polygalacturonate at *p*H 6.0 and 45 C and were active in the range of *p*H 4.0 to 9.0 and 10 to 60 C.

For some time there has been increasing concern in California about the disposal of strong salt brines to prevent pollution of the soil and underground and surface waters. In an attempt to reduce the salt content in their olive storage brines, one company resorted to acidification with lactic acid to about *p*H 3.8 to 4.0 and used a brine of 3 to 4% instead of the customary 6 to 7% sodium chloride. Then, for some reason never revealed, the lactic acid was replaced by a mineral acid. (The low total titratable acidity values coupled with significant increases in chloride ions indicated that the unknown acidulant was hydrochloric acid.) Because the *p*H values dropped well below the level tolerated by the desirable lactic acid bacteria (as low as *p*H 2.6), the normal biological sequence of fermentation was disrupted. Yeasts, uninhibited by bacterial competition, predominated and spoiled the olives by a combination of gas-pocket formation and softening (Fig. 1).

This spoilage episode lead to the development by Vaughn et al. (14) of a method of salt-free storage for fruit to be used to make canned ripe olives. This method involved the use of a mixture of food-grade acetic and lactic acids plus sodium benzoate in water to cover the olives. It soon became evident that spoilage as described above would be a problem unless the olives in the salt-free solution were placed under anaerobic conditions throughout the period of storage. Otherwise, pectolytic yeasts and other acid-tolerant fungi thrived and caused deterioration of the olives (14).

It is anticipated that brine storage will, in time, be nearly completely replaced by the salt-free storage method, and the rest of the olives will be processed at harvest (direct cure method). Because of the difficulty of maintaining strictly anaerobic conditions, pectolytic fermenting yeasts still pose a hazard to olives in salt-free storage. Therefore, it is desirable to describe the yeasts isolated from the commercial outbreak, including their taxonomy, pectolytic activity, and other characteristics of importance.

MATERIALS AND METHODS

Isolation and identification of the yeasts. A total of 100 tanks of olives was involved. The spoilage ranged from 5% to approximately 97% based on the examination of 100 olives by sight for gas pockets and feel for softening. Samples of the acidified brines were collected for examination. Each one was tested for *p*H, total acidity as grams of lactic acid per 100 ml, and salt by hydrometry and chloride titration, and was examined under the microscope to determine the predominant microbial population. The samples were also plated on standard plate count agar to determine a viable count, on liver infusion-sorbic acid-agar to determine the pres-

[1] Present address: Department of Food Science and Human Nutrition, Michigan State University, East Lansing, Mich. 48823.

[2] Present address: Pennwalt Corporation, Decco Division Research Laboratory, Monrovia, Calif. 91016.

[3] Present address: C-17 Niemann, Norman, Okla. 73069.

FIG. 1. *Gassy spoilage of olives. This defect is commonly called "fish-eye" spoilage in the industry.*

ence of lactic acid bacteria, on Levine's eosine-methylene blue-agar to estimate the population of gram-negative bacteria, and on acidified malt-agar to obtain a "yeast" count. The standard plate count and the yeast count were approximately equal and agreed with the microscopic examination which suggested yeasts as the cause of spoilage. Since the bacterial counts were negligible, a search was made for pectolytic yeasts by using malt polypectate gel medium (13). Pectolytic yeasts (those producing depressions under and around the individual colonies) ranged from 5×10^6 to 5×10^7 per ml; nonpectolytic yeasts amounted to less than 10^6 per ml. Representative pectolytic yeast cultures were isolated and purified by appropriate restreaking on malt-agar (3).

The purified isolates were identified as to genus and species by the procedures of van der Walt (10, 11) and Wickerham (16). Difco yeast carbon base (YCB) and Difco yeast nitrogen base (YNB) were the basal media used for the study of nitrogen and carbon compound utilization. Salt tolerance and the effect of the initial *p*H of the medium on growth were determined as described by Vaughn et al. (15).

Preparation of enzyme solutions. Pectolytic enzyme production was tested in several different media. The three best media, basal nutrient broth containing 1% glucose, 0.25% pectin N. F., or 0.25% sodium polypectate (w/v) were chosen for crude, cell-free enzyme production. The *p*H value of all three media was adjusted so that it was 6.0 ± 0.1 after sterilization. The glucose medium was made by sterilization of the standard nutrient broth in the autoclave at 15 psi (approx. 121 C) and the glucose solution by filtration. Then the requisite amount of sterile glucose solution was added aseptically to the basal broth. The other two media were also sterilized at 15 psi but with the substrate present when heated. After growing different cultures in the media for various times, the cells were removed by centrifugation and the supernatant cell-free fluid was dialyzed against distilled water for 24 hr or more at 5 C. The dialyzed preparation was lyophilized and brought back to the desired volume with distilled water when used.

Determination of pectolytic activity. One of the primary objectives of this study was the association of the fermenting yeasts with the softening of olives in brine. Therefore, qualitative methods generally were used to determine the pectolytic activity of the enzymes the fermenting yeasts produced rather than attempting an extended quantitative study of them.

Pectin esterase and polygalacturonase production were demonstrated by the modified cup-plate assay described by Nagel and Vaughn (5). No attempt was made to quantitate pectolytic enzyme production by the cup-plate assay because it was believed that demonstration of degradation of pectic substrates and the softening of olives by the crude enzymes was sufficient. Instead, the effects of temperature and *p*H on the activity of the crude, cell-free enzyme preparations were determined by measuring changes in the viscosity of pectic substrates induced by the enzymes. These measurements were made with an Ostwald-Cannon-Fenske capillary viscosimeter under carefully controlled conditions (6). The end products of degradation also were determined by paper chromatography with *n*-butyl alcohol-water-acetic acid (4:3:2) as the solvent system. The degradation products were determined on descending chromatograms with Whatman no. 4 paper developed for 18 to 24 hr at room temperature. End products on the chromatograms were located by spraying with a silver nitrate reagent (9) or by the method of Demain and Phaff (2).

In vitro softening of olives. Small size Manzanilla variety olives from commercial storage were placed in 400-ml flasks and covered with a brine containing 6.5% NaCl and a mixture of lactic and acetic acids yielding a total acidity of 0.4% (w/v) calculated as lactic acid and a *p*H value of 4.0. The flasks were plugged with cotton and steamed for 1 hr on 3 successive days with a temperature in the center of the brine of at least 70 C. After cooling, a filter-sterilized glucose solution was added to the olives and brine to give a final concentration of 5% (w/v) glucose in the brine at time of addition of the sugar.

After at least 4 days of incubation at room temperature to check for sterility, the flasks were inoculated with selected yeasts and incubated at 20 C.

After various intervals up to 20 days, the olives were examined visually for gas-pocket formation and evidence of softening by feel. In some instances, the olives were tested for firmness with the Christel texturometer as described by Vaughn et al. (15).

RESULTS

All of the cultures investigated were fermentative, sporogenous yeasts classified as members of the genera *Hansenula* or *Saccharomyces* because of their individual characters concerning spore formation and fermentative and oxidative abilities.

Species allocation. Three species in the two genera were identified. Most of the cultures, 49 of 63, had the characteristics of *Saccharomyces oleaginosus* Santa Maria 1958. One culture was determined to be *S. kluyveri* Phaff, Miller et Shifrine 1956. The remaining 13 cultures were

found to be representative of *Hansenula anomala* (Hansen) H. et P. Sydow 1891 var. *anomala*. It is interesting that all cultures investigated conformed exactly to all of the key characteristics of the standard description of each of the three species identified (11, 16).

Components of the olives and brines as substrates. Compounds known to be present in olives and their brines which could serve as substrates for yeasts included fructose and glucose; ethanol and mannitol; acetic, lactic, succinic and citric acids; the alkaline hydrolysis products of the bitter glucoside oleuropein, including arabinose and glucose and different phenolic compounds; olive oil; and pectin. The phenolic products were not tested as possible substrates because known samples were not available in the quantities needed to test all cultures. Most of the other compounds listed except the lower oligouronides of pectin degradation supported growth of the yeasts if the *p*H and temperature were favorable.

The lower oligouronide breakdown products of pectin or polygalacturonic acid were not used as a sole source of carbon in YNB broth or agar even though growing cultures produced pectolytic enzymes in standard nutrient broth containing glucose, pectin N. F., or sodium polypectate. These observations further strengthen the idea that yeasts, although producing lower oligouronides from pectin, are unable to degrade them to the same extent as many of the pectolytic bacteria (4, 15).

Salt tolerance. Although the yeasts in this study were isolated from acidified, low-salt brines, it was thought desirable to determine their salt resistance to help increase our knowledge of their ability to grow in concentrations of NaCl commonly used with brines for olives and other produce. The results found in Table 1 show a significant difference in the salt tolerance of the three species. All of the cultures of *H. anomala* var. *anomala* grew in the presence of 10% NaCl, 11 of them grew in 12% salt (nearly the highest concentration commonly used in the world for storage of olives), 8 in 14% salt, and 6 in 16% NaCl. In contrast, the cultures of *S. kluyveri* and *S. oleaginosus* were much less resistant (Table 1).

pH tolerance. As might have been expected, all of the cultures, regardless of species, grew between *p*H values of 2.35 and 8.00 in McIlvanie's buffer with 0.5% glucose. All grew rapidly in the range of *p*H 3.0 to 6.5.

Pectolytic enzyme activity. All of the cultures comprising the three species identified in this study grew on malt or nutrient polypectate gel and produced depressions under and around the periphery of the individual colonies and gave positive qualitative tests for production of pectin esterase and polygalacturonase. However, when using the quantitative parameters for demonstration of pectin esterase and polygalacturonase activity, it was impossible to demonstrate pectolytic activity with any of the cultures of *H. anomala* var. *anomala*. In contrast, it was easy to quantitate pectolytic activity of the cultures of *S. kluyveri* and *S. oleaginosus* by use of the viscosimeter.

Crude, cell-free enzyme preparations of *S. kluyveri* culture no. 138 and *S. oleaginosus* culture no. 208 were found to cause greatest activity at 45 C with polygalacturonate or polypectate as substrates (Fig. 2). The crude enzyme preparations of these cultures were active over the range of temperatures normally associated with brined olives (10 to 50 C). Both species exhibited nearly the same polygalacturonase activity as measured by viscosimetric measurements. Use of all prior expertise failed to establish distinguishable polygalacturonase activity with any of the cultures of *H. anomala* var. *anomala*. It may be that the observed depression formation on the polypectate gel is a physical rather than a biological change. It also may be that the observed positive qualitative tests for pectin esterase and polygalacturonase for the cultures of *H. anomala* var. *anomala* were artifacts. In any event, one must conclude that there is no prime evidence that any of these latter cultures are pectolytic.

Softening of olives. The cultures of *S. kluyveri* and *S. oleaginosus* caused softening of olives under in vitro conditions, with or without

TABLE 1. *Salt tolerance of the fermenting yeasts*

Specie	No. of cultures[a] grown at NaCl concn (% w/v)								
	2	4	6	8	10	12	14	16	18
H. anomala var. *anomala* (13 cultures) ...	13[a]	13	13	13	13	11	8	6	0
S. kluyveri (1 culture)	1	1	1	0	0	0	0	0	0
S. oleaginosus (47[b] cultures)	47	45	22	21	1	0	0	0	0

[a] Number of cultures growing in 2 weeks at 30 C.

[b] Two cultures were lost at the time this study was made.

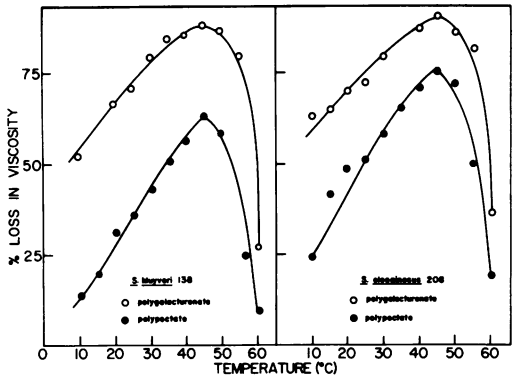

FIG. 2. *Effect of temperature on polygalacturonase activity of S. kluyveri and S. oleaginosus. Reaction time, 30 min.*

added glucose. This softening ability was inhibited in covering brines containing more than 6% NaCl or more than 0.1% actual acetic acid as part of the total acidity. (In normal storage of Spanish-type fermentation brines, the quantity of acetic acid may amount to as much as 50% of the total acidity.) The cultures of *H. anomala* var. *anomala* did not cause softening of olives under any of a variety of conditions tested. A more detailed study of Roby and Vaughn (*unpublished data*) confirmed this observation.

Gas-pocket formation was pronounced when any culture of the three species was grown in sterile olives covered with a brine containing 5% glucose.

DISCUSSION

The first reports of pectolytic activity by yeasts apparently were made by Cruess and Douglas (1) and Roelofsen (7) in 1936 (July and October, respectively). However, it was not firmly established until 1951 that yeasts sometimes did possess pectolytic activity. The work of Luh and Phaff (4) was reported and later confirmed by Roelofsen (8) in a reiteration of his 1936 publication, originally published in an obscure journal in the Dutch language.

Yeasts may be of considerable nuisance in the olive industry. Film-forming yeasts are especially notorious, for they rapidly oxidize the desirable acidity in the brines of storage Sicilian-style and Spanish-type olives if not controlled. Under conditions of abnormal growth, these same yeasts impart an undesirable yeasty taste to Sicilian- and Spanish-type green olives, especially when partially filled containers are refrigerated after opening. Quite frequently, fermenting yeasts cause a disruption of the normal lactic acid fermentation of Spanish-type green olives in California known as "stuck" fermentations. Although this phenomenon has been described by Vaughn et al. (12) and control measures are known, the identity of these yeasts still is lacking.

Only recently has it been demonstrated that, besides their nuisance value, yeasts can cause considerable spoilage with consequent economic loss through their pectolytic activity. It has been established that species of *Rhodotorula* cause stem-end softening of olives in brines stored for processing as California canned ripe olives or in Sicilian- and Spanish-type green olive fermentations (15). The present report describes three other species of yeasts isolated from spoiled olives. Two species, *S. oleaginosus* and *S. kluyveri*, are pectolytic and cause severe softening. Although not similar to commercial conditions, pronounced gas-pocket formation was produced by all three species in sterile olives covered with a brine containing 5% glucose. Since this defect produces unsalable fruit, cultures of *H. anomala* var. *anomala* may be of significance in the spoilage of olives even though they were shown not to cause softening. Perhaps other species are also involved, for 37 cultures of yeasts were lost in an accident early in the study.

ACKNOWLEDGMENTS

This investigation was supported by the Olive Advisory Board under authority of the Director of Agriculture of the State of California and by the Olive Administrative Committee administering Federal Olive Order no. 932.

M. W. Miller and H. J. Phaff of this department were most helpful with some matters of taxonomy.

LITERATURE CITED

1. Cruess, W. V., and H. C. Douglas. 1936. An interesting spoilage of Sicilian olives. J Fruit Producers **15**:334.
2. Demain, A. L., and H. J. Phaff. 1954. The preparation of tetragalacturonic acid. Arch. Biochem. **51**:114–121.
3. Lodder, J., and N. J. W. Kreger-van Rij. 1952. The yeasts. North-Holland Publishing Co., Amsterdam.
4. Luh, B. S., and H. J. Phaff. 1951. Studies on polygalacturonase of certain yeasts. Arch. Biochem. Biophys. **33**:212–227.
5. Nagel, C. W., and R. H. Vaughn. 1961. The characteristics of a polygalacturonase produced by *Bacillus polymyxa*. Arch. Biochem. Biophys. **93**:344–352.
6. Nortje, B. K., and R. H. Vaughn. 1952. The pectolytic activity of species of the genus *Bacillus:* qualitative studies with *Bacillus subtilis* and *Bacillus pumilus* in relation to the softening of olives and pickles. Food Res. **18**:57–69.
7. Roelofsen, P. A. 1936. Protopektinase vormende gisten. Verslag 16e vergadering Verenig. Proefstation Personeel Djember, October, 1936
8. Roelofsen, P. A. 1953. Polygalacturonase activity in yeast, *Neurospora* and tomato extract. Biochim. Biophys. Acta **10**:410–413.
9. Trevelyan, W. E., D. P. Proctor, and J. S. Harrison. 1950. Detection of sugars on paper chromatograms. Nature (London) **166**:444–445.

10. van der Walt, J. P. 1970. Criteria and methods used in classification, p. 34-113. *In* J. Lodder (ed.), The yeasts, a taxonomic study. North-Holland Publishing Co., Amsterdam.
11. van der Walt, J. P. 1970. Genus 16. *Saccharomyces* Meyen emend. Reess, p. 555-718. *In* Lodder (ed.), The yeasts, a taxonomic study. North-Holland Publishing Co., Amsterdam.
12. Vaughn, R. H., H. C. Douglas, and J. R. Gilliland. 1943. Production of Spanish-type green olives. Calif. Agr. Expt. Sta. Bull. **678**:1-82.
13. Vaughn, R. H., G. D. Balatsouras, G. K. York II, and C. W. Nagel. 1957. Media for detection of pectinolytic microorganisms associated with the softening of cucumbers, olives and other plant tissues. Food Res. **22**: 597-603.
14. Vaughn, R. H., M. H. Martin, K. E. Stevenson, M. G. Johnson, and V. M. Crampton. 1969. Salt-free storage of olives and other produce for future processing. Food Technol. **23**:124-126.
15. Vaughn, R. H., T. Jakubczyk, J. D. Macmillan, T. E. Higgins, B. A. Davé, and V. M. Crampton. 1969. Some pink yeasts associated with softening of olives. Appl. Microbiol. **18**:771-775.
16. Wickerham, L. J. 1970. Genus 7. *Hansenula* H. et P. Sydow, p. 276-315. *In* J. Lodder (ed.), The yeasts, a taxonomic study. North-Holland Publishing Co., Amsterdam.