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Some Pink Yeasts Associated with Softening of Olives[1]

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Pink yeasts identified as *Rhodotorula glutinis* var. *glutinis*, *R. minuta* var. *minuta*, and *R. rubra* produce polygalacturonases which cause a slow softening of olive tissue. Both pectin methyl esterase and polygalacturonase are produced when cultures are grown in appropriate media. Crude, cell-free dialyzed enzyme preparations measured viscosimetrically exhibited optimal activity on sodium polygalacturonate at *p*H 6.0 and 40 C, and were active in the range of *p*H 4.0 to 9.0 and 10 to 50 C. Cultures grown in sterilized olives and brine at *p*H 4.0 with sterile glucose added aseptically caused a slow softening of tissue as measured with a Christel texturometer. Similar results were obtained when crude, cell-free enzyme preparations were added to olives in buffer solution at *p*H 6.0 with Merthiolate. Commercial control of these yeasts is easy if anaerobic conditions can be provided. Otherwise, the industry has to resort to manual removal of the film from the brine surface, either by skimming or by flagellation.

A softening of fermented Spanish-type green olives in barrels opened for grading and repacking in glass jars was first called to the attention of the senior author late in 1956. The softening was sporadic and quite variable in degree. Some lots were only soft at the stem end. The majority were progressively softer and represented a type of softening known in the industry as "stem-end shrivel" [*see* Nortje and Vaughn (8)]. On examination, the brines invariably were devoid of pectinolytic microorganisms which could be associated with the softening. A puzzling observation was that the softening was confined to the Sevillano variety fruit.

During the fall and winter of 1958–1959, intensive study of the problem was initiated, and weekly or biweekly visits to three plants in the San Joaquin valley were made. In this period, it became apparent that pink yeasts might possibly be the cause of the spoilage.

In the San Joaquin valley, it had been the practice since the start of commercial production of Sevillano variety Spanish-type green olives in the 1930's to ferment the fruit with the barrels upright and the head, fitted with two small bung openings (1.9 cm), flooded to the top of the chime. This was done so that salt could be added periodically to avoid salt shrivel, to which this variety is very susceptible. In contrast, the Manzanilla, Mission, and other varieties had always been fermented in barrels placed on their sides from the beginning and fitted with a cellar bung of the type used in the wine industry [*see* Vaughn et al. (13)].

It was observed that the exposed brines in the chimes on the heads of some of the barrels, although clear during October and about half of November, slowly began to develop tiny, very thin, pinkish islands composed of yeasts. As the days shortened, the yeast growth developed into a thin, veil-like, more or less transparent film covering the entire surface of the brine. Later, during the middle of the winter, when the sun was lowest and there was much fog, the films became thick pellicles, and the heads of the barrels accumulated an abundant pinkish sediment of yeast cells. Even though the yeast films frequently were washed off by the flooding of the heads of the barrels during rains, because of the initial contamination they soon reappeared and continued to flourish until the brines had reached the desired salt concentration and the barrels were placed on their sides and fitted with cellar bungs. By the end of the salt stabilization period, all of the barrels still remaining upright were supporting the growth of the pink yeasts on the exposed brines to a greater or lesser degree.

Since the commercial production under ob-

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servation was coded as to the day, month, and year of barreling for fermentation, study of the records showed that the Sevillano olives stored from late October on until the end of the harvest were most likely to be more or less softened.

During the period of observation just described, it also was noted that similar yeast films developed on the surface of brines covering olives destined for processing as California canned ripe olives that were stored in tanks in the open. Samples of the infected brines from both barrels and storage tanks were streaked on malt powder-pectate gel at *p*H 4.2 (12). Surprisingly enough, most of the pink colonies developing on the gel were pectinolytic and caused a slow softening under and around the periphery of each colony.

This circumstantial evidence linking the pink yeasts with the softening of the Spanish-type green olives soon was verified, for an isolated and purified culture caused a slow stem-end softening when inoculated into sterile Spanish-type green olives in brine in vitro. With this causal proof, the solution for commercial control was obviously to create anaerobic conditions, for the pink yeasts are strict aerobes.

During the 1959–1960 season, additional experiments were done to determine whether the conclusions reached the previous year were valid. Pectinolytic pink yeast cultures taken from Spanish-type and storage brines were isolated and purified for study. The following is a description of the 41 pectinolytic yeasts, including their taxonomy, pectinolytic activity, and other characteristics of importance.

MATERIALS AND METHODS

Identification of the yeasts. The purified isolates were differentiated as to genus and species by use of the procedures described by Lodder and Kreger-van Rij (4) and Wickerham (14). 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. Growth at various temperatures was determined by measuring the diameter of the giant colony after 7 days of incubation of cultures grown on malt-agar plates. Salt tolerance was determined in Difco YNB broth with 0.5% (w/v) glucose adjusted with McIlvaine's disodium phosphate-citric acid buffer to *p*H 4.6. The basal salt medium was sterilized in an autoclave and, after sterile filtration, the glucose was added under aseptic conditions.

The effect of the initial *p*H of the medium on growth also was determined in YNB broth with 0.5% (w/v) glucose. Each *p*H increment was adjusted with McIlvaine's buffer. The basal medium was sterilized in an autoclave; then, after sterile filtration, the glucose solution was added aseptically as before.

Two different substrates were used to determine lipolytic activity. The first, described by Sierra (9), contained polyoxyethylene sorbitan monooleate (Tween 80). The second contained an olive oil emulsion in place of cotton seed oil in the medium described by Starr (10).

Proteolytic activity also was determined in two different media: malt powder-gelatin (4) and casein-agar (1).

Preparation of enzyme solutions. Crude enzyme preparations were obtained by growing the cultures in a basal standard nutrient broth containing 1.0% glucose, 0.25% pectin (NF), or 0.25% sodium polypectate (w/v), as the best of several different media tested. The *p*H of all three media was adjusted to 6.0 ± 0.1 after sterilization. The cultures were grown in the various media for different times, and then the cells were removed by centrifugation. The supernatant fluid was dialyzed against distilled water for 24 hr at 5 C and then concentrated in a dialysis bag by evaporation in the cold to one-tenth of the original volume or (since 1966) lyophilized and then brought to the desired volume with distilled water. With some cultures, it was necessary to use a 25 times or more concentration.

Determination of pectolytic activity. The primary objective of this study was the association of the pink yeasts with the softening of olives in brine. Therefore, qualitative methods were used to study the activity of the enzymes these yeasts produce rather than attempting a more elaborate quantitative study with emphasis on the softening enzymes.

Pectin methyl esterase production was demonstrated by the cup-plate method with the use of the reagents described by McComb and McCready (6). Polygalacturonase production was detected by the modified cup-plate assay described by Nagel and Vaughn (7). No attempt was made to quantitate polygalacturonase production by this method, because it was felt that positive demonstration of degradation of pectinous substances and the softening of olives by the crude enzymes was sufficient. Instead, the effects of temperature, *p*H, and other factors on the activity of the crude enzyme preparations were determined by measuring changes in the viscosity of pectinous substances induced by the enzymes. These measurements were made with an Ostwald viscosimeter under carefully controlled conditions (8). The end products of degradation also were determined by qualitative chromatographic means with *n*-butyl alcohol-water-acetic acid (4:3:2) as the solvent system, since it was determined that none of the yeasts produced pectin or pectic acid *trans*-elimase enzymes. The products of degradation were determined on descending chromatograms with Whatman no. 4 paper developed for 18 to 24 hr at room temperature. End-product spots on the chromatograms were located by spraying with a silver nitrate reagent (11) or by the method of Demain and Phaff (2).

In vitro softening of olives. To reproduce the softening, Manzanilla variety "small" (also called "standard" and "select") size olives from commercial salt storage were used. For the first test, the olives were placed in 400-ml E flasks, covered with commercial brine of typical composition (8.25% sodium chloride, 0.4% total acid as lactic, traces of hexose, and a *p*H of 4.0), and then plugged with cotton and steamed for

1 hr on 3 successive days with a temperature in the center of the brine of 65 to 70 C. After cooling, six flasks of sterile olives were inoculated with strain 1 of *Rhodotorula glutinis* var. *glutinis* and six served as uninoculated controls.

For the second experiment, the chemical composition of the covering brine was changed so that the salt concentration was 6.5% and the total acidity was 0.4% as lactic acid; the *p*H was still 4.0, and 0.5% sterile glucose was added after the olives had been heated for 1 hr on 3 successive days as before. Six flasks were inoculated with strain 1 of *R. glutinis* var. *glutinis* and six uninoculated flasks were used as controls.

After various intervals of incubation at 20 C, the olives were pitted and cut in half. Each sample (40 g) was tested for decreased firmness with the Christel texturometer.

Other in vitro softening experiments were done by use of centrifuged, dialyzed, concentrated (25 times) crude culture preparations and the same lot of olives as above. In this latter test, the olives were treated as described by Nortje and Vaughn (8).

RESULTS

All of the cultures were strictly aerobic (oxidative), asporogenous yeasts classified as members of the genus *Rhodotorula* because of pigment production on solid media, lack of fermentative activity, and oxidative assimilation patterns characteristic of that genus.

Species allocation. Most of the cultures (39) had the characteristics of *R. glutinis* var. *glutinis*. One culture was identified as *R. minuta* var. *minuta* and another as *R. rubra*. The primary characteristics which separate these three species are shown in Table 1. It is important to note that none of the cultures used *i*-inositol as a substrate in YNB agar. Pigmented yeasts of the genus *Cryptococcus* are known to use *i*-inositol as a source of energy in YNB agar or broth.

Components of the olives and brines as substrates. Compounds known to be present in olives and brines that might serve as substrates for the growth of the yeasts included the hexoses fructose and glucose, ethyl alcohol, mannitol, acetic, lactic, succinic, and citric acids, the alkaline hydrolysis products of the bitter glucoside, oleuropein, including glucose, arabinose, and various polyphenolic compounds, olive oil, and pectin. The phenolic components were not used as possible substrates because known samples were not available in the quantities needed to test all cultures. However, most of the other compounds except the degradation products of pectin supported the growth of at least some of the yeasts if the temperature and *p*H were favorable.

Fructose and glucose were readily assimilated by all cultures, as were ethyl alcohol and mannitol. Utilization of the D and L isomers of arabinose differed considerably: all cultures used the D isomer but only five strains attacked L-arabinose.

There also was some difference in the utilization of the organic acids. Only 10 isolates used acetic acid in YNB broth at *p*H 4.5. However, if the YNB broth had an initial *p*H of 7.0 to 7.2, all 41 strains readily assimilated the acetate. In contrast, all cultures used citric, lactic, and succinic acids in the basal medium at *p*H 4.5.

Lipolytic activity of the *Rhodotorula* cultures was considerable. All 41 strains caused a rapid, abundant development of lipase on the medium containing polyoxyethylene sorbitan monooleate (Tween 80), and 37 of the 41 cultures were able to degrade olive oil in the spirit blue medium. It was noted that growth was not retarded on the Tween 80 medium, whereas giant colony development on the spirit blue-olive oil-agar was quite slow in comparison. It may be that the indicator dye has a deleterious effect on the growth of the yeasts or that the nutrients in the medium were not nearly optimal for the cultures. These possibilities were not investigated.

Proteolytic activity was not great, as only 18 cultures of *R. glutinis* var. *glutinis* caused weak liquefaction of gelatin. Casein was not attacked.

The ability to use pectin and its degradation products as a sole source of carbon in YNB broth or agar was nil even though all of the cultures produced pectinolytic enzymes in standard nutrient broth containing either glucose or pectin,

TABLE 1. *Identification of the species of Rhodotorula*

Species allocation	Compound utilized[a]						
	KNO_3	Maltose	Sucrose	Melezitose	Raffinose	Lactose	*i*-Inositol
R. glutinis var. *glutinis* (39 cultures)	+	+	+	+	+	−	−
R. minuta var. *minuta* (1 culture)	−	−	+	+	−	−	−
R. rubra (1 culture)	−	+	+	+	+	−	−

[a] Yeast carbon base agar was used to test NO_3 utilization; yeast nitrogen base agar and broth were used to determine carbohydrate assimilation.

TABLE 2. *Salt tolerance of the pink yeasts*

Species	NaCl (w/v)					
	10%	12%	14%	16%	18%	20%
R. glutinis var. *glutinis*	39[a]	39	39	10	1	1
R. minuta var. *minuta*	1	0	0	0	0	0
R. rubra	1	1	1	1	1	1

[a] Number of cultures growing.

pectic acid, sodium polygalacturonate, or polygalacturonic acid.

Unlike many of the pectinolytic bacteria and molds already studied, the species of *Rhodotorula*, although producing lower oligouronides from pectin, are unable to further degrade them. This fact already has been reported by Luh and Phaff (5) with another yeast, *Saccharomyces fragilis*.

Salt tolerance. In the California olive industry, a brine with a maximum of about 8% (w/v) salt is commonly used for fermentation of the green, Spanish-type olives and other olives stored in brine for future processing as California canned ripe olives. The latter may be covered with a brine containing 10 to 12% (w/v) salt if stored into the summer or carried over to the next season.

The data shown in Table 2 indicate that all of the pink yeasts readily tolerate 10% (w/v) salt, and some of them grow at salt concentrations well above those normally used in olive storage throughout the world (10 to 12%, w/v). It may be concluded that the salt concentrations used in the industry will deter only a few of these yeasts if other conditions for their growth are provided.

Effect of temperature. Although some of the cultures grew over a temperature range of 5 to 30 C, none grew appreciably at 37 C after 1 week of incubation. At 5 C, the culture representative of *R. rubra* and 21 isolates of *R. glutinis* var. *glutinis* were able to grow slowly. At 12 C, an additional 11 isolates of the latter species grew, and the diameter of the giant colonies increased somewhat. All 41 isolates grew at 20 C and, based on the giant colony diameters, which ranged from 6.0 to 11.0 mm with an average of 8.8 mm, this temperature was considered to be nearly optimal. A temperature of 30 C was already strongly inhibitory to 17 of the cultures, including the strain of *R. rubra* and 16 isolates of *R. glutinis* var. *glutinis*, for they failed to develop visible colonies. It is interesting to record that the culture of *R. minuta* var. *minuta* grew best between 20 and 30 C and the isolate of *R. rubra*, between 5 and 20 C, whereas representatives of *R. glutinis* var. *glutinis* grew between 5 and 30 C, the majority quite well; 21 and 23 cultures, respectively, of a total of 39.

Effect of pH on growth. There is considerable variation between the *p*H of the brines of Spanish-type green olives and that of the brines of olives stored for further processing. On initial brining, the former may range from 8.5 to 11.0, whereas the storage olives not treated with lye will be in the range of 5.5 to 6.9. The *p*H values of these brines drop, and at the end of the fermentations the Spanish-type brines are controlled to 3.8 to 4.0 and the storage olives drop naturally to a range of 4.2 to 4.5.

The yeasts all grew over a *p*H range of 3.0 to 8.0, and the majority tolerated a *p*H of 8.5. They grew especially well in the acid range.

Pectolytic enzyme activity. All of the cultures

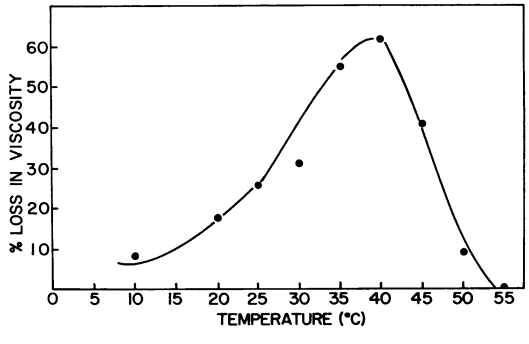

FIG. 1. *Effect of temperature on polygalacturonase activity of R. glutinis var. glutinis 1. Reaction time, 30 min.*

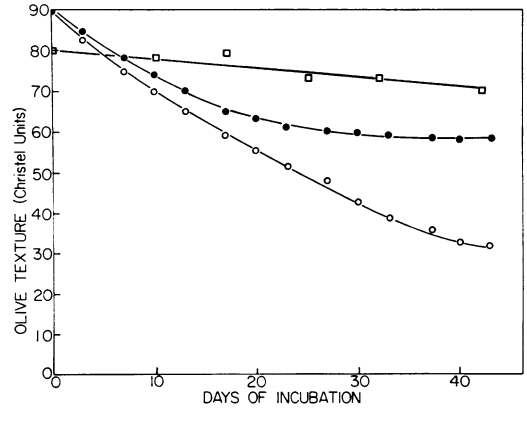

FIG. 2. *In vitro softening of olives by R. glutinis var. glutinis 1. Symbols: □, uninoculated control, 8% salt, pH 4; ●, inoculated, 8% salt, pH 4, no glucose; ○, inoculated, 6.5% salt, pH 4, 0.5% glucose.*

produced both pectin methyl esterase and polygalacturonase. Although all of the isolates were constitutive in this respect, a few cultures would not produce detectable polygalacturonase unless incubated for 18 days. These included the one isolate of *R. minuta* var. *minuta* and two strains of *R. glutinis* var. *glutinis*.

It is noteworthy that if the cultures could be grown at 30 C, 10 C above their optimum for maximal growth, the crude enzyme preparations had considerably more activity. Hsu and Vaughn (3) have already reported that production of polygalacturonic acid *trans*-eliminase was greatly stimulated under conditions of restricted growth of *Aeromonas liquefaciens* accomplished by substrate restriction in a continuous-feeding culture or by restricting divalent cations in a batch culture. It would appear that temperature of incubation as well as nutrition has a role in the increase or decrease in pectinolytic enzyme production.

The end products of polygalacturonic acid degradation were all saturated oligouronides. The mono, di, tri, tetra, penta, and hexa polymers were identified. The crude enzyme preparations varied considerably in their activity. The stronger ones regularly degraded polypectate to mono-, di-, and trigalacturonic acid when the original medium contained both glucose and polypectate. Yet, if the same culture had been grown in the medium containing only glucose, from four to six oligouronides were identified.

The crude enzyme was active over the range of temperatures normally associated with brined olives and, as shown in Fig. 1, exhibited greatest activity at 40 C.

Softening of olives. The growth of *R. glutinis* var. *glutinis* (strain 1) on the surface of brined sterile Manzanilla olives caused a slow yet measurable loss in olive texture (Fig. 2). The amount was related to the composition of the brine. More softening occurred when the salt was reduced to 6% and 0.5% glucose was added.

Experiments with crude enzyme preparations paralleled the growth tests. Unless the preparations were concentrated, the olives were softened slowly over a comparable period of time.

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