the concentration of the starch and the concentration of the amylase, respectively, were linearly related to the diameter of the zone of hydrolysis.

The method is rapid, convenient and highly accurate, the coefficient of variation being approximately one per cent, and is being used to develop a standard assay procedure for bacterial amylases.

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The Pectolytic Activity of Molds Isolated from Black Raspberries

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The use of the Howard mold count in the quality grading of food products had its inception in 1911 when it was first used in connection with tomato products to indicate the efficiency of trimming, sorting and manufacturing procedures (Howard, 1911). Needham and Fellers (1925) applied the Howard method to strawberries and blackberries and this method has since been applied to other berry fruits including raspberries. Mold counts are currently regarded by some workers as synonymous with the extent of decomposition and mushiness.

Rendle (1933) who was concerned with "mushiness" of raspberries during their transportation to canning factories, made no mention of molds. He showed that the pectic constituents cementing the cell walls of this fruit are subject to more rapid changes than occur in most fruits during ripening and that these changes take place even more rapidly after the fruit is picked.

Byssochlamys fulva was found responsible for the breakdown of pectinous material in raspberries and complete disintegration of the fruit (Olliver and Smith, 1933). The degree of softening was unrelated to the amount of visible mycelia.

Botrytis cinerea, which is known to attack berry fruits, required a pH of 6.5 on potato decoction medium for optimum pectolytic enzyme production (Fernando, 1937). When conditions were made acid little enzyme activity was noted despite good growth of the mold. It is concluded that relatively little is known as to the conditions under which fungi produce pectolytic enzymes (Phaff, 1946).

Recently, producers and processors of black raspberries in Michigan have been concerned, especially during humid seasons, with government seizure and rerouting of their produce executed on the basis of the Howard mold count. This matter was investigated by Fabian et al. (1951) who showed that no tissue breakdown of the black raspberries was noted even when the mold count as determined by the Howard method was 80 per cent. Beneke (1950a) isolated and identified the molds present in 1719 droplets of black raspberries. From this large number of droplets he isolated Alternaria sp. in 641 instances, Cladosporium sp. in 262 instances, Pullularia sp. 81 times, Fusarium sp. 66 times, Botrytis sp. 65 times, Penicillium sp. 59 times, Rhizopus sp. 25 times, Trichoderma sp. 21 times and eight other genera such as Aspergillus sp. and Oospora sp. in lesser numbers. The first two genera, Alternaria and Cladosporium, accounted for approximately 70 per cent of the total number of molds isolated from black raspberries. The present study was concerned with...
investigating the pectolytic activity of the principle molds.

**Materials and Methods**

**Cultures.** The mold isolates were grown on potato-dextrose agar (Difco) slants made in liter dilution bottles. After two weeks' incubation in darkness at 22 to 25 C, 50 ml aliquots of sterile distilled water were added to each culture and the surface growths gently removed. The suspensions were then placed in screw capped bottles and thoroughly shaken.

**Media.** The work reported in this investigation was conducted during the winter months when fresh fruit was unavailable; therefore canned water pack berries were employed. The berries showed sound structural characteristics and a substantial amount of pectin was present as demonstrated by alcohol precipitation tests. The berries were crushed and an extract obtained using a screw-type fruit press. The pH of this extract was 3.8. The extract was thoroughly mixed and 1000 ml quantities were dispensed into 3000 ml Erlenmeyer flasks.

A one per cent citrus pectin medium and a one per cent apple pectin medium, with pectin as the sole source of carbon were prepared using the mineral base described by Phaff (1946). These media were dispensed in the same manner as the black raspberry extract. The medium containing the apple pectin showed a pH of 5.4; and the pH of the medium containing the citrus pectin was 6.3.

**Preparation of Samples.** Each flask was inoculated with 5 ml of spore suspension and incubated at 22 to 25 C for seven days. Samples for testing were obtained by carefully tilting the flasks and aseptically removing portions of the culture media.

**Method of Enzyme Testing.** A 1 per cent pectin solution was prepared by adding special pectin to sodium citrate-citric acid buffer at pH 4.0 according to the method given by Bell et al. (1950). The pectin solution was allowed to stand for several hours before tests were conducted in order to offset any increase in apparent viscosity (Kertesz, 1951). Two ml of the culture fluid was added to 20 ml of the test pectin solution and the mixture was incubated in a thermostatically controlled water bath at 30 C for 45 min. Five ml of the mixture was then placed in each of two Ostwald viscosimeters and the dropping times at 30 C were noted. Control tests were run concurrently employing culture fluid which had been heated at 80 C for 10 min.

Decrease in viscosity was expressed as a percentage according to the formula:

\[
\frac{A - B}{A} \times 100 \text{ where } A = \text{viscosity test pectin solution + heated medium} \]

\[
B = \text{viscosity test pectin + unheated medium}
\]

The same two viscosimeters were used throughout the study.

**Effect of Pectic Enzymes on Black Raspberries.** One hundred ml amounts of 1 per cent solution of Pectinol M in distilled water were added to each of four 150 gm portions of the whole black raspberries. Heated Pectinol M solution was added to four other samples as controls. At the end of 5 hr the 2 lots of raspberries were compared macroscopically for disintegration. The samples were also placed on #40 mesh screens and drained weight readings were made after 2 min.

**Results**

The six molds tested grew well in the 3 media used. However, the behavior of the molds in producing pectolytic enzymes varied considerably.

*Supplied through the courtesy of Dr. E. S. Beneke, Department of Botany and Plant Pathology, Michigan State College, East Lansing, Michigan.

* Citrus pectin—178 grade, R.S. California Fruit Growers Exchange, Ontario, California.


* Designated 447-U-7, California Fruit Growers Exchange, Ontario, California. Manufacturer's specifications showed the following percentage analysis: moisture 7.7. ash 7.7, methoxyl 10.6, galacturonic acid 87.3.
The data presented in table 1 show the average changes in the viscosity of the test pectin solution obtained by sampling of the mold cultures following 7 days’ incubation. Tests made of the black raspberry extract after 3 days’ incubation showed the same relative pattern but pectolytic activity was less marked. *Alternaria humicola* and *Cladosporium* sp. showed little or no pectolytic enzyme activity in the black raspberry extract. This was in marked contrast to *Penicillium* sp., *Pullularia* sp., *Fusarium* sp., and *Botrytis cinerea*. *Botrytis cinerea* was the only mold which produced a metabolite, presumably an enzyme(s), in sufficient quantity to increase the viscosity of the test pectin solution. This behavior was noted when the black raspberry extract and the apple pectin medium were tested. When *Botrytis cinerea* was grown in citrus pectin medium, however, a decrease in viscosity of the test pectin solution occurred. *Penicillium* sp., in the black raspberry extract showed the most active pectolytic enzyme production. There-

**Fig. 2.** Disintegration of black raspberries by pectolytic enzymes. (a) Control, inactivated enzyme preparation. (b) One per cent Pectinol M.
fore, this was examined throughout the 7 day period. The average per cent loss in viscosity is given in figure 1.

The 1 per cent Pectinol M solution which was added to the whole black raspberries resulted in an almost complete disintegration of the berries within 5 hours to such an extent that their structural characteristics had disappeared (figure 2). After this time these samples showed an average loss of twelve percent in drained weight as compared to the weight of berries which had been covered with heat-treated Pectinol M solution.

**DISCUSSION**

While the limitations of the viscosity method of measuring pectolytic activity are well recognized (Kertesz, 1951), the application of the method in this study has served to emphasize the pronounced variations which may occur in production of pectolytic enzymes by molds. The most significant variation was the difference in pectolytic enzyme production by the various molds in the black raspberry extract.

The data presented here substantiate the work of Fabian et al. (1951) and explain how a high mold count may not necessarily be related to the pectolytic breakdown of black raspberries. Thus, if the molds present are primarily Alternaria humicola, Cladosporium sp., or other species producing little or no pectolytic activity within a short period of time, berries with a relatively high mold count may not show decomposition or mushiness.

It is appreciated that the production of pectolytic enzymes by molds depends on many factors. Individual strains of molds too, have been shown to vary greatly in their activity (Proskuriakov and Ossipov, 1939). In this study, the behavior of the molds in producing pectolytic enzymes was undoubtedly influenced by variations in the constituents of the media and the pH. While the literature shows the pH optimum for fungal pectin polygalacturonase to be in the neighborhood of 3.5, Kertesz (1951) cites several sources which have listed optima ranges up to pH 7.0. He suggests that such wide variations in these optimum ranges could be attributed to the combined action of pectin-methyl esterase and pectin-polygalacturonase acting simultaneously in the solutions tested. It would appear that pH alone was not the limiting factor in this study, as was noted by the appearance of optimum activity of Pullularia sp. and Penicillium sp. in black raspberry extract in contrast to Fusarium sp., which showed maximum pectolytic enzyme production in the citrus pectin medium.

Gel formation by Botrytis cinerea in the raspberry extract and in the apple pectin medium, both of which had low pH values, was likely due to the formation of a pectin-methylesterase like enzyme. Kertesz (1951) has pointed out that the activity of pectin-methylesterase of fungal origin, drops rapidly as the pH is raised above pH 5.0. This would presumably account for the lack of gel formation by Botrytis cinerea in the citrus pectin medium.

Unquestionably, factors other than the ability of molds to produce pectolytic enzymes enter into the breakdown of black raspberries. One consideration is the rapid pectic changes occurring during ripening and also following picking (Rendle, 1933). Ripe berries held for a prolonged period in the field or after picking would be expected to show a higher mold count than might be found in other classes of fruits. However, the presence of 100 per cent positive mold counts fields can be noted for this fruit when the mold filaments occupy as little as 0.00162 per cent of the total puree volume (Beneke, 1950b).

**ACKNOWLEDGMENT**

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**SUMMARY**

Representatives of the six genera of molds found most commonly in black raspberries during the 1950 season were tested for pectolytic enzyme production.

Under the conditions of these experiments Alternaria humicola and Cladosporium sp. produced little or no pectolytic activity when grown in black raspberry extract. This was in contrast to their behavior in other media and in contrast to Pullularia sp., Fusarium sp., Botrytis cinerea, and Penicillium sp.

In black raspberry extract Penicillium sp. and Pullularia sp. showed the greatest pectolytic activity.

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A Comparison of the Most Probable Numbers of Coliform Bacteria and Enterococci in Raw Sewage

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Because most of the species in the coliform group of bacteria are of nonfecal origin, and therefore of little value as indicators of pollution in waters used for swimming or for irrigation, it has long been felt that a test that employed the enterococci could serve as a supplement or a substitute for the above test. In recent years much work has been published suggesting the merits of such an enterococci test. Andrews (1906), Broadhurst (1915), Sherman (1937), Winter and Sandholzer (1946a), and Mallmann and Litsky (1951) have indicated that, although the enterococci are abundant in fecal matter, these organisms, unlike the coliform bacteria, are not found elsewhere in nature.

Past investigations have indicated relatively low numbers of enterococci in sewage or polluted waters (Winslow and Nibecker, 1903, U. S. Public Health Service, 1948, and Lattanzi and Mood, 1951); therefore, the use of these organisms as indicators of pollution has never been accepted in this country, although in England they are used in the routine analysis of water. Recently, Lattanzi and Mood (1951), employing the technique of Winter and Sandholzer (1946b) for the enumeration of enterococci, reported a ratio of 63 to 1 of Escherichia coli to enterococci in water samples from the harbor at New Haven, Connecticut. The United States Public Health Service, by the same technique, reported that the enterococcus determination was a less sensitive measure of bacterial densities of waters than the coliform determination (United States Public Health Service, 1948).

With the advent of azide dextrose broth (Difco) for the detection of streptococci, the problem of using enterococci as indicators of pollution had to be reinvestigated and reevaluated. Mallmann and Seligmann (1950), in a comparative study of media for the detection of streptococci in water and sewage, reported that azide dextrose broth was far superior to any of the other media employed for this purpose. Using this medium, Mallmann and Litsky (1951) reported that the numbers of coliform bacteria and enterococci were recovered in about the same numbers from soil freshly treated with sewage.

Because dextrose azide broth supported the growth of a few other nonstreptococcal forms, Litsky, Mallmann, and Fifield (1953) developed ethyl violet azide broth as a confirmatory medium for the enterococci. It was reported that, when using azide dextrose broth as a presumptive test and ethyl violet azide broth for the confirmation, 100 to 10,000 more enterococci were detected in polluted water than were detected by other methods. The effectiveness of this test suggested that a review of the coliform-enterococcus ratio in polluted water should be undertaken.

Methods

To test the validity of enterococci as test organisms for sewage pollution in bathing and irrigation waters, the relative numbers of these organisms in sewage must first be established. For this reason, samples of sewage from the settling tanks of the Amherst (Massachusetts) Treatment Plant were taken at various positions and depths with the aid of a depth-sampler to insure representative sampling. This experiment was carried out throughout the winter, spring, and summer months to insure the significance of the trends observed. Sewage at the Amherst Treatment Plant is diluted greatly with ground water during wet seasons and lower counts of microorganisms due to dilution are observed during

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1 Contribution No. 887 from the Massachusetts Agricultural Experiment Station, Amherst, Massachusetts.