EXOCELLULAR PECTOLYTIC ENZYMES PRODUCED BY MOLDS ISOLATED FROM BLACK RASPBERRIES

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EXOCELLULAR PECTOLYTIC ENZYMES PRODUCED BY MOLDS ISOLATED FROM BLACK RASPBERRIES

By

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INTRODUCTION

In some instances, arbitrary mold count tolerances by Governmental agencies make little or no allowance for the types of produce, weather conditions, or the genera of molds which may be present.

In effect, while the Howard method doubtless has shown considerable merit when applied to tomatoes, its widespread use for other food products is difficult to justify unless certain basic fundamentals are considered. Too frequently, the term "mold" is deemed synonymous with "rot" without any evidence to show that the product or products concerned are structurally or chemically degraded.

It is alleged that the Howard mold count standard for black raspberries has been set unofficially by the Government at 40-percent positive fields. This is the same as that allowed for tomato paste, catsup and puree. Black raspberries are essentially different from tomatoes and it is by no means possible to predict from one fruit the behavior of another, even under given conditions. Producers and packers of black raspberries have sustained serious economic losses occasioned by seizure and rerouting of produce especially when humid conditions have prevailed during the growing season. This has occurred in Michigan which supplies about 80-percent of the U. S. black raspberry canned pack and a substantial proportion of the frozen pack. No evidence has been offered thus far to show that the berries are damaged in any way when minute

amounts of molds are present.

The present study was undertaken to determine the extent of available information concerning the degradation of black raspberries after picking and to investigate experimentally, the differences, if any, which might exist in the pectolytic properties of several important molds isolated from black raspberries.

REVIEW OF LITERATURE

It has long been believed that pectin occurs as calcium pectate in the intercellular layers of plant tissues, and that this calcium pectate serves as a cement between the adjacent cell walls. Loconti and Kertesz (22) presented evidence that the firmer texture which is given to tomatoes by treating them with calcium chloride was due to the formation of calcium pectate. The mechanics of firming apple slices with calcium has similarly been explained by Baker (1).

Kertesz (19) presented an extensive review of the literature relative to the chemical and physical properties of pectic substances. He stated that although pectic substances may serve a wide variety of unknown purposes in plants, the contribution of the insoluble pectic constituents in the middle lamella of plants to rigidity, is one of the most important known functions and that softening and maceration of the tissues occur when the pectic substances are transformed. Pectin materials in a state of transition can be found in cell sap and Kertesz suggested that this solubility may be governed at least partly by the degree of demethylation. This hypothesis was substantiated by the work of Leinbach, Seegmiller and Wilbur (21) which showed that dissolved pectinic substances of

a high degree of methylation were present in the cell sap of strawberries and raspberries.

When overripening of fruit or vegetables, excessive heating, fungal or bacterial rotting occurs, it may be shown that cementing pectic material between adjacent cell walls is removed. During the ripening of apples, Carre (8) showed that the amount of pectic substances decreased and that the parenchymous cells became loosened or completely detached from one another. Personius and Sharp (25) demonstrated that during the cooking of potatoes, there is an extensive decrease in cell wall adhesion by a weakening of the intercellular substances. Fabian and Johnson (11) demonstrated the development of enzymatic activity by Bacillus mesentericus fuscus which produced decomposition of pectic materials in the tissues of cucumbers. Kertesz (19) stated that the pectolytic enzymes produced by microorganisms are responsible for most plant diseases related to the destruction of pectic constituents. This same author also suggested (18) based on the work of Robertson et al. (30) that depolymerization and hydrolysis of pectin as well as other saccharides may be caused by hydrogen peroxide in the presence of ascorbic acid. However, Phaff (27) indicated that hydrogen peroxide has not been conclusively demonstrated in plant tissues.

In general, the transformations which pectin substances undergo, aside from those noted during heating, have been attributed to the activity of pectic enzymes.

Despite the complex macromolecules of pectin substances which are thought to be chainlike combinations of anhydrogalacturonic acid units possessing carboxyl groups distinguishing them from the poly-

saccharides, there are only two known pectic enzymes, pectin-methylesterase (P. M., pectinesterase, pectase) and pectin-polygalacturonase (P. G., pectinase, pectolase) (19).

Pectin-methylesterase has been characterized as the enzyme responsible for destruction of pectinic acid by hydrolysis of the methyl ester groups in pectinic acid and pectin. Pectin-methylesterase is commonly found in the root, stems, leaves and ruit of many higher plants and without making any claims to completeness Kertesz (19) listed eighteen sources of this enzyme. In plant tissues, pectin-methylesterase, according to Phaff (27), occurs more or less free from pectin-polygalacturonase.

Kertesz (19) made a general statement that the activity of pectin-methylesterase of plant origin increases as the pH is elevated from 4.0 to 7.0. Above pH 7.0, however, the situation is confused and the addition of salt causes a drift in the pH optimum. McColloch (24) showed that heat inactivation of pectin-methylesterase of plant origin occurred at 30° C.

Pectin-methylesterase, according to Phaff (27) always appeared present in fungal or bacterial preparations when pectin-polygalacturonase was noted and Jansen and MacDonnel (16) found that little glycosidic hydrolysis occurs without demethylation of the pectin in commercial fungal pectinases (a group designation for an enzyme complex composed of at least one or more pectic enzymes in addition to pectin-polygalacturonase). Fungal pectin-methylesterase from a commercial pectinase was active in a salt free medium at pH 4.0 and had a well defined optimum at about 4.5 - 5.0. With salts, the activity of the enzyme was increased, especially near the pH optimum

but there was not an increase in the pH value at which the action occurred.

Calesnick, Hills and Willaman (6) reported that complete inactivation of fungal pectase occurred at pH 3.5 when extracts were heated for 30 minutes at 62° C. Calesnick et al. (6) showed that fungal pectase with which they had experimented, was quite stable and that there was no loss in the activity of the enzyme at pH 6.25 when it was stored for 2 weeks at 23° C.

Pectin-polygalacturonase has been considered responsible for the destruction of pectinic acid by hydrolysis of the 1,4 glycosidic linkages of the polygalacturonic acid skeleton of pectic acid or pectinic acids with the formation of polygalacturonic acids of smaller molecular size and of (mono) galacturonic acid. Kertesz (19) listed germinating barley as the only proven source of this enzyme in plants. However, many microorganisms have been reported to produce pectin-polygalacturonase and commercial preparations which include this enzyme are widely used. Fish and Dustman (14) found that fungal pectin-polygalacturonase had a pH optimum in the neighborhood of 3.5 but other workers (17) have found it in the pH range of 3-7. Among the reasons suggested by Kertesz (19) to explain this wide variation, is that many workers used unpurified pectinase preparations and that simultaneous action of the two enzymes pectin-methylesterase and pectin-polygalacturonase must have been common. In addition, he explained that the deesterifying enzyme changing pectinic acid to pectic acid might also have had a profound effect on the pH optimum reported. Furthermore, the same author stated (19) that the pH optimum for fungal pectin-polygalacturonase under most

conditions is in the range of pH 3.5 - 4.0. Phaff (26) in studies with Penicillium chrysogenum proved that complete inactivation of the pectin-polygalacturonase occurred at 80° C in ten minutes.

Undoubtedly, one of the most important considerations in the production of fungal pectic enzymes is the medium. Menon (23) presented data which indicates that the nutrients present influence to a considerable extent the precise behavior of the enzymes of any particular fungus. He suggested that the enzyme is the same in all instances but that certain of its properties are greatly influenced by the absorption of the substances from the medium. Fernando (13) showed that the optimum activity of Botrytis cinerea on potato decoction media was at pH 6.5 but that substances which caused a drift to the alkaline side, such as peptone, yielded very active enzyme solutions while those which caused an acid pH drift (ammonium chloride, nitrate and sulfate) produced good growth but low enzymatic activity.

The literature concerning fresh raspberries is particularly meager. Rendle (29) was concerned with the development of mushiness of raspberries during their transportation to canning factories. He felt that raspberries are, perhaps, the most difficult of all fruits to keep intact and to transport or handle. He proved that changes which took place following ripening occurred even faster after the fruit had been picked. Although Rendle did not report the extraction of an enzyme in this work, he showed that changes in pectic constituents were arrested when the berries were heated. He concluded that the character and degree of enzymatic activity in raspberries is considerably more marked than in other fruits. When he employed the

Carre and Haynes method (7) for an estimation of pectin as calcium pectate, he showed that the pectin content of berries decreased from 0.92 to 0.34-percent when storage was for six days at 18 to 24° C. In this study he also reported the pH value of canned raspberries as 3.3.

Kertesz (19) noted that it is surprising that the percentage of pectic constituents in strawberries does not decrease when this fruit becomes overripe and he concluded that the pectic constituents of strawberries are more stable than those of raspberries.

Lee (20) presented the following data showing that the black raspberry, Rubus occidentalis L. respires at a consistently higher rate than the red raspberry or the purple variety.

	Milligrams CO_2 evolved per kg fresh weight per hour at		
Variety	60°F	40°F	32°F
Cumberland (black)	100.7	38.5	19.9
U. S. D. A 322 (purple)	87.5	36.9	25.1
Latham (red)	82.2	30.7	17.5

He also reported that the approximate composition of red and black raspberries was as follows:

	Water %	Protein %	Fat %	Ash %	Total Carbo-hydrate %	Fibre %	Sugars %	Acid as citric %
Red	83.4	1.1	0.6	0.47	14.4	2.3	7.2	1.34
Black	80.7	1.5	1.6	1.65	15.6	3.5	7.9	1.16

In general, berry fruits such as raspberries, blackberries and dewberries are subject to attack by the same molds (15). These include

Botrytis sp., Rhizopus sp., Penicillium sp., Phyllostocinia sp., Carpogena sp., Elsinoe veneta sp., and Cladosporium sp. Probably, the most common and widely spread rot of strawberries is Botrytis and it has been a major concern of raspberry growers as well. It develops and spreads throughout the whole fruit and causes the berry to dry up and become tough and leathery. It was further stated that Botrytis is a good example of a mold which may be overlooked in the early stages on raspberries. When the aerial mycelium of a berry is moistened, the mycelium seems to disappear. In referring to Botrytis as the cause of spoilage of stone fruit, it was stated that the organism gains entrance to the fruit through breaks in the skin but occasionally it has been found to penetrate sound epidermis.

Dodge and Wilcox (9) reported that any delay beyond a few hours which increases the time interval between picking and sale to a consumer, affords an opportunity for the development of fruit rots and molds affecting raspberries. These authors also added that raspberries which are soft from being overripe or from rainy weather are quickly attacked by certain mold fungi which cause decay and that in hot or wet weather, picking may be required at least every other day.

Fabian et al. (12) investigated the factors responsible for high mold counts which were found to occur on black raspberries which had been grown in the State of Michigan. These workers found that no tissue breakdown of the berries was noted, even when the mold count, as determined by the Howard method, was 80-percent. Visible mold was rarely found and when present it indicated that the berries had

not been freshly picked. Despite prompt picking and delivery to the canning factories, mold counts by the Howard method during a humid season sometimes reached 40-percent. As a result packers had to drastically reduce the season's output of canned and frozen berries.

In the same study, it was also reported that the molds most commonly found in black raspberries were, in decreasing order of incidence, Alternaria humicola, Cladosporium sp., Pullularia sp., Fusarium sp., Botrytis cinerea and Penicillium sp.

Organoleptic tests on berries which showed a high mold count failed to indicate that the quality of the fruit had been affected in any way. Beneke (4) calculated on the basis of the 56-percent positive Howard mold count that if 100-percent positive fields had been obtained, the volume of mold present would have amounted to only 0.00162-percent of the total puree volume.

Fabian et al. (12) suggested that the present Howard mold count standard for black raspberries is unjustly severe and particularly so when berries are grown under hot and humid conditions.

STUDY OF MATERIALS

The work reported in this investigation was conducted during the season when fresh fruit was unavailable. Therefore, it was important that the berries resemble as much as possible their original condition and especially contain pectin which would serve as a carbon source for microorganisms.

Initially, it was believed that frozen berries would be satisfactory; but tests on berries which had been stored since the previous season's pack showed that the pectin content of these berries,

as determined by their ability to form a gel, was very small or negligible.

Canned black raspberries which were available on the local market were packed in sugar sirup; and although tests showed that they were capable of showing good gelation, Pitman and Cruess (28) observed in a series of experiments with Penicillium glaucum that when sugar was present in the medium, it was invariably utilized before the pectin was attacked. Accordingly, it was decided not to use berries packed in this manner.

The black raspberries which were finally chosen for the studies were obtained from a Michigan processor and had been packed in water for use in bakeries and restaurants. All cans had the same code number and the berries appeared sound.

Sample portions of the berries from several cans were crushed and tested using a Beckman glass electrode pH meter. A pH value of 3.85 was noted for all samples. Alcohol precipitation tests showed that a substantial amount of pectin was present.

STUDY OF METHODS

Phaff and Joslyn (27) list five methods for the measurement of polygalacturonase activity: increase in reducing substances measured as aldoses; decrease in alcohol precipitate; decrease in optical rotation; drop in viscosity of the pectin solution; and decrease in calcium pectate. For the purpose of this investigation all of these methods had decided limitations, many of which were similar to those noted by Bell et al. (2) during a study of methods for work on cucumbers. All methods with the exception of the drop in viscosity of pectin solution depend on the development of a

strong glycosidic hydrolysis which might require several hours and the use of a large amount of substrate.

Measurement of changes in optical rotation was employed by Ehrlich (10) in a study of enzymatic activity which occurred in pectin solutions. It was stated (19) that the specific rotation of pectinic and pectic acid solutions is constant in the pH range of 3 to 6.5 and that the optical rotation decreases during enzymic hydrolysis.

Trial experiments were conducted using a Schmidt and Hench polarimeter and a sodium light source, but it was observed that this method had several disadvantages and was unsuitable for the present study. It was necessary to clarify the samples to be tested and readings, when obtained, were of insufficient accuracy to show correlation. Probably, this was due to the concentration of the pectin solutions, which approximated 1-percent, used in this work. Another obvious difficulty with this method, had it been used, would have been the influence of arabans on observed readings (19). The arabans which often accompanies pectic substances in plants is strongly levorotatory while the pectinic substances are dextrorotatory and even the presence of minute amounts of the arabans has a considerable effect on the optical rotation of pectic substances.

METHODS EMPLOYED

The molds which had been most prevalent on black raspberries during the 1950 season were obtained. These were Alternaria humicola, Cladosporium sp., Pullularia sp., Fusarium sp., Botrytis cinerea and Penicillium sp. Subcultures were made periodically during the study. Potato dextrose agar (Difco), pH 5.6, was used as a solid

medium unless otherwise specified.

As required for inoculations, the molds were grown on large slants prepared by the addition of 200 ml of agar to one-liter dilution bottles. After 2 weeks incubation at 22° to 25° C, 50 ml of sterile distilled water was added to each bottle and spore suspensions prepared by gently rubbing the agar surfaces with an inoculating needle. The suspensions were removed, placed in screw capped bottles and thoroughly shaken.

A 1-percent solution of special pectin* in a sodium citrate-citric acid buffer solution at pH 4.0 was prepared for demonstrating enzymatic activity by the loss in viscosity method. The buffer solution was prepared according to the method given by Bell et al. (2) and consisted of weighing out 2 gm each of sodium citrate and citric acid and dissolving in 100 ml of distilled water. The solution was heated over a Bunsen burner until the temperature reached 60°C and then placed in a Waring blendor. The pectin then was added slowly in a sprinkling manner to the liquid which was simultaneously agitated with a glass rod. To insure uniformity, the suspension was stirred in the blendor for a period of 1 minute and then was passed through two thicknesses of cheesecloth. The solution was allowed to stand for several hours before use to offset any increase in apparent viscosity (19).

Changes in the viscosity of test pectin solutions have been

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* Designated 447-U-7. Obtained from the California Fruit Growers Exchange. Manufacturer's specifications showed the following percentage analysis: moisture, 7.7; ash 1.2; methoxyl 10.6; galacturonic acid 87.3.

widely used to measure pectolytic enzyme activity. It was reported by Jansen and MacDonnell (16) that a 50-percent change in the viscosity of a 1-percent solution occurred with only a 2-percent breakage of the glycosidic bonds. Phaff (26) used this method in studies with Penicillium chrysogenum, Bell et al. (2) in determining softening of pickles and Bell (3) in studying cucumbers. Kertesz (19) pointed out that the decrease in viscosity is a very sensitive indicator of even traces of pectin-polygalacturonase action. This author further stated that it is unlikely that at the pH of the reaction mixtures, demethoxylation would have any significant effect on the viscosity as compared to the viscosity drop produced by pectin-polygalacturonase.

It is interesting to note that a 1-percent hydrolysis of the polygalacturonic chains causes approximately a 33-percent reduction in the viscosity, while a 1-percent demethoxylation causes a slight change. Therefore, viscosity is not governed by the methylester content but rather by the average molecular size of the pectinic acid.

Bell et al. (2) added heated and unheated extracts to 3-percent test pectin solutions and incubated the mixtures at 30°C for 6 days. Relative dropping times were then determined by allowing the solutions to flow through a 20 ml pipette.

Phaff (26) added heated and unheated extracts to 1.5-percent test pectin solutions and then incubated the mixtures at 25°C for 30 minutes. Dropping times were measured employing Ostwald viscometers.

The procedure used in the present investigation to measure the loss in viscosity was essentially a combination of the methods employed by Phaff and by Bell. In each instance, to determine pectolytic activity 2 ml of the unheated extract was mixed with 20 ml of

the test pectin solution. This mixture was incubated at 30°C in a thermostatically controlled water bath for 45 minutes. At the end of this time, 5 ml amounts were placed in each of 2 Ostwald viscometers. These were then placed in a water bath and dropping times measured. Each time a determination was made, the initial viscosity of the pectin solutions and a heated extract from the same source served as a contro. To obtain a heated extract, a sample was placed in a water bath and kept at 80°C for 10 minutes. The inactivated extract was then added to the pectin solution and the same procedure followed as above. Decrease in viscosity was expressed as a percentage of the viscosity of the test pectin solution and the heated extract according to the formula $\frac{A - B}{A} \times 100$. A represents the viscosity of the heated enzyme mixture and B the viscosity of unheated enzyme mixture.

The incubation period was longer than the 30 minutes employed by Phaff (26) to permit time for adequate cleaning of the Ostwald viscometer. Acetone, alcohol and hot water rinses as were used by Bell *et al.* (2) did not prove satisfactory for cleaning the Ostwald viscometers and gels usually formed which could not be removed from the small capillaries. A satisfactory procedure, developed during the early part of this work, consisted of flushing hot chromate-sulfuric acid solution through the Ostwald viscometers, followed by four hot water rinses and finally by a distilled water rinse. The viscometers were then dried by filtered air under pressure. The same two Ostwald viscometers were used in all tests reported.

EXPERIMENTS WITH CITRUS AND APPLE PECTIN

Samples of high grade citrus* and apple** pectin were obtained. Two media were prepared by the addition of these pectins in a 1-percent concentration to a mineral base described by Phaff (26). This substrate consisted of tap water, 0.1-percent ammonium nitrate, 0.1-percent dihydrogen phosphate, and 0.05-percent magnesium sulfate. The ingredients appeared likely to support the growth of the molds with pectin as the sole source of carbon since Phaff obtained luxuriant growth of P. chrysogenum in this medium. Each preparation was thoroughly mixed and filtered through cheesecloth to obtain even distribution of the pectin.

The pH value of the medium containing apple pectin was 5.4.

The pH value of the medium containing citrus pectin was 6.3.

The media was dispersed in 1000 ml amounts into 2000 ml Erlenmeyer flasks which were then plugged with cotton and sterilized. Mold suspensions of Alternaria humicola, Cladosporium sp., Pullularia sp., Fusarium sp., Botrytis cinerea and Penicillium sp. were prepared as described under Methods. Five ml of spores representing each genus was inoculated into an individual flask containing apple pectin medium. A duplicate set of flasks containing citrus pectin were also inoculated. Incubation was at 22° to 25° C for 7 days. The flasks were then carefully tilted and samples of the medium in each instance were removed aseptically for testing.

The molds showed a pronounced difference in their ability to produce exocellular pectolytic enzymes (Tables 1 and 2).

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

** Apple pectin - 170 grade, uncut, Speas and Co., Philadelphia, Pa.

Fusarium sp. and Alternaria humicola showed the greatest production of pectolytic enzymes. Exocellular enzymatic activity produced by Penicilium sp. was appreciable in the medium containing citrus but not in apple pectin. Botrytis cinerea, in the medium containing citrus pectin, demonstrated pectolytic activity similar to that of polygalacturonase while in the medium containing apple pectin, an increase in the viscosity of test pectin solutions was noted. This increase in viscosity was probably due to methylesterase production.

The variation noted in the development of the enzymes in the two media could probably be attributed to the differences in the pH values of the media.

EXPERIMENTS WITH BLACK RASPBERRY JUICE

Juice was expressed from the black raspberries with a screw type fruit press. The juice obtained was thoroughly mixed and 1000 ml quantities were distributed into 3000 ml Erlenmeyer flasks. Large flasks were chosen in order to provide a broad surface area for growth of the molds. The flasks were plugged with cotton and sterilized. Mold suspensions of Alternaria humicola, Cladosporium sp., Pullularia sp., Fusarium sp., Botrytis cinerea and Penicillium sp. were prepared as already described.

Five ml of mold suspension prepared from each genus was inoculated into an individual flask of juice. Incubation was at 22° to 25° C for 7 days. Unless otherwise specified, determinations for the degree of pectolytic activity were conducted following 3 and 6 day incubation periods of all cultures of molds with the exception

TABLE 1: Pectolytic activity produced by molds in citrus pectin medium (pH 6.3) after 7 days incubation at 22° to 25° C

Mold	Dropping time of fluid (minutes and seconds)				Activity of fluid (percent)		
	Viscometer 1		Viscometer 2		Viscosity drop		
	Heated	Unheated	Heated	Unheated	Viscometer 1	Viscometer 2	Average
Alternaria humicola	9'57"	7'38"	8'39"	6'32"	23.3	24.5	23.9
Cladosporium sp.	10'05"	9'07"	8'47"	7'46"	9.6	11.6	10.6
Pullularia sp.	10'08"	8'40"	8'42"	7'23"	14.5	15.1	14.8
Fusarium sp.	9'57"	6'25"	8'32"	5'40"	35.5	33.6	34.5
Botrytis cinerea	9'46"	8'51"	8'31"	7'35"	9.4	10.9	10.1
Penicillium sp.	9'32"	8'52"	8'32"	8'06"	7.0	5.1	12.1

TABLE 2: Pectolytic activity produced by molds in apple pectin medium (pH 5.4) after 7 days incubation at 22° to 25° C

Mold	Dropping time of fluid (minutes and seconds)				Activity of fluid (percent)		
	Viscometer 1		Viscometer 2		Viscosity drop		
	Heated	Unheated	Heated	Unheated	Viscometer 1	Viscometer 2	Average
Alternaria humicola	9'49"	8'25"	8'20"	7'24"	14.3	11.2	12.7
Cladosporium sp.	10'11"	9'42"	8'41"	8'25"	4.7	3.1	3.9
Pullularia sp.	10'09"	9'12"	8'48"	8'05"	9.4	8.1	8.7
Fusarium sp.	9'54"	7'22"	8'37"	6'29"	25.8	24.8	25.3
Botrytis cinerea	9'44"	9'48"	8'24"	8'30"	*	*	*
Penicillium sp.	9'55"	9'54"	8'31"	8'29"	--	--	--

* denotes increase.
-- denotes change less than 3-percent.

of Penicillium sp. More tests were made on this species in view of the interesting results obtained by Phaff (26) for P. chrysogenum.

Tables 3, 4 and 5 illustrate the pectolytic activity of the molds grown on the black raspberry juice. A comparison of these data shows that the greates pectolytic activity was produced by Penicillium sp. followed by Pullularia sp. and Fusarium sp. A progressive decrease in the viscosity of test pectin solutions was produced by Penicillium sp. (Table 5). While a rather pronounced variation was noted between some readings, it is likely that this were caused by the use of a new solution of test pectin near the end of the experiment. Botrytis cinerea produced an enzyme which consistently increased the viscosity of test pectin solutions.

One of the most interesting observations noted was the lack of production of pectolytic enzymes by Alternaria humicola and Cladosporium sp.

Alternaria humicola did not develop visible growth on the surface of the black raspberry juice during these tests. Observations made after 10 days also failed to show surface growth. A precipitate in the juice was noted, however, and the development of sub-surface growth was confirmed by microscopic examination.

The degree of enzymatic activity noted for the molds in black raspberry juice was not related to the amount of visible mold which grew on the surface of the medium.

In some instances, readings of dropping times as determined by viscometer 1 did not correlate with viscometer 2 as well as had been noted in previous tests. In cases of doubtful readings, tests were repeated. Errors were undoubtedly due to small particles of the

TABLE 3: Pectolytic activity produced by molds in black raspberry juice after 3 days incubation at 22° to 25° C

Mold	Dropping time of fluid (minutes and seconds)				Activity of fluid (percent)		
	Viscometer 1		Viscometer 2		Viscosity drop		
	Heated	Unheated	Heated	Unheated	Viscometer 1	Viscometer 2	Average
Alternaria humicola	10'47"	10'59"	9'40"	9'39"	--	--	--
Cladosporium sp.	11'52"	11'23"	9'17"	9'36"	--	--	--
Pullularia sp.	10'08"	8'50"	9'03"	7'40"	12.8	15.3	14.0
Fusarium sp.	x	x	x	x	x	x	x
Botrytis cinerea	9'53"	10'33"	8'49"	9'36"	6.7	*	*

x denotes results not determined.
* denotes increase in viscosity.
-- denotes change less than 3-percent.

TABLE 4: Pectolytic activity produced by molds in black raspberry juice after 7 days incubation at 22° to 25° C

Mold	Dropping time of fluid (minutes and seconds)				Activity of fluid (percent)		
	Viscometer 1		Viscometer 2		Viscosity drop		
	Heated	Unheated	Heated	Unheated	Viscometer 1	Viscometer 2	Average
Alternaria humicola	10'30"	10'15"	9'05"	9'10"	--	--	--
Cladosporium sp.	10'34"	10'12"	9'03"	9'03"	--	--	--
Pullularia sp.	10'18"	6'59"	8'57"	6'12"	32.2	30.7	31.4
Fusarium sp.	10'15"	8'32"	8'47"	7'18"	16.7	16.8	16.7
Botrytis cinerea	9'29"	10'16"	8'36"	8'45"	*	*	*

-- denotes change less than 3-percent.
* denotes increase in viscosity.

TABLE 5: Pectolytic activity produced by *Penicillium* sp. in black raspberry juice

Time of growth (days)	Dropping time of fluid (minutes and seconds)				Activity of fluid (percent)		
	Viscometer 1		Viscometer 2		Viscosity drop		
	Heated	Unheated	Heated	Unheated	Viscometer 1	Viscometer 2	Average
0	10'36"	10'55"	9'00"	9'10"	--	--	--
1	8'40"	7'42"	9'30"	8'35"	11.2	11.0	11.1
2	8'25"	7'19"	7'40"	6'40"	12.0	13.0	12.5
3	8'07"	6'10"	7'17"	5'11"	24.0	28.8	26.4
5	9'55"	7'49"	8'23"	6'48"	21.2	18.9	20.0
7	9'52"	5'50"	8'13"	5'14"	40.9	36.3	38.6

-- denotes change less than 3-percent.

raspberries which remained in suspension and interfered with the viscosity readings.

DEVELOPMENT OF MOLD ON WHOLE BLACK RASPBERRIES

It seemed desirable to provide at least some indication of the relative ability of molds to develop quickly on the surface of black raspberries, although it could not be assumed that mold behavior on canned berries would be the same as on fresh berries.

Thirty gm amounts of the water pack berries were aseptically removed from the cans and placed in sterile 200 ml Erlenmeyer flasks. Two ml of the spore suspension prepared from each of the six mold cultures was dropped onto the surface of the berries contained in the six flasks respectively. The berries were then incubated at 22° to 25° C and observed intermittently for the development of visible mold growth.

In 18 hours, moderate growth of Penicillium sp. was noted and barely visible growth of Botrytis cinerea and Cladosporium. At the end of 24 hours, Fusarium, Alternaris and Pullularia were faintly visible. By 36 hours all molds had overgrown the exposed surfaces of the berries.

DISCUSSION

Alternaria humicola and Cladosporium sp. under the conditions of the present investigation showed a lack of ability to produce pectolytic enzymes in black raspberry juice. This is particularly interesting in view of the findings of Beneke (5) that these two molds had accounted for approximately 70-percent of all molds detected in black raspberries during the 1950 growing season. By contrast,

Pullularia sp., Fusarium sp., Botrytis cinerea and Penicillium sp. all showed active pectolytic enzyme production in black raspberry juice. When Alternaria humicola and Cladosporium sp. were cultured in mineral media, both secreted pectolytic enzymes. Of the 6 molds investigated, the degree of activity exhibited by Alternaria humicola in mineral medium with pectin as the sole source of carbon was exceeded only by Fusarium sp.

Botrytis cinerea, which is known to have a pronounced degenerative effect on some fruits, showed evidence of a pectin-methylesterase type of action when grown in mineral medium containing citrus pectin. In black raspberry juice and in mineral medium containing apple pectin, a pectin-polygalacturonase type of enzymatic action was noted for the same organism.

This investigation has shown that production of pectolytic enzymes by molds is not related to the amount of visible mold. It seems obvious that in the grading of a food product, the effect of fungal activity on the food itself should be a primary consideration rather than the mere demonstration that fungi are present. The Howard method makes no allowance for the degradative properties of fungi as a means of evaluating the soundness and sanitary condition of a food product. The limitations of the Howard method become even more apparent when it is realized how little volume actually is occupied by molds in a foodstuff, even when up to a 100-percent mold count is observed.

While it is recognized that any fruit or vegetable benefits from expeditious handling, it is unreasonable to expect that growers of black raspberries, for example, should be put in the economically

disadvantageous position whereby they must harvest within an extremely restricted period of hours, in the hope that their produce will meet the present Howard mold count standard.

SUMMARY

1. Alternaria humicola, Cladosporium sp., Pullularia sp., Fusarium sp., Botrytis cinerea and Penicillium sp., which had been isolated from black raspberries, differed markedly in their ability to produce pectolytic enzymes when grown in black raspberry juice under given conditions.

2. Alternaria humicola and Cladosporium sp. showed a lack of ability to produce pectolytic enzymes when grown in black raspberry juice.

3. Enzyme production was most evident in black raspberry juice by Penicillium sp., Pullularia sp. and Fusarium sp.

4. Botrytis cinerea when grown in black raspberry juice secreted an enzyme which increased the viscosity of test pectin solutions.

5. All of the molds studied except Alternaria humicola produced visible growth on the surface of black raspberry juice under the conditions of the test.

6. Fusarium sp., and Alternaria humicola showed the greatest production of pectolytic activity in apple and citrus pectin media.

7. On the surface of whole, water-packed berries, Penicillium sp. developed macroscopic growth most quickly, followed in order by Botrytis cinerea, Cladosporium sp., Fusarium sp., Alternaria humicola and Pullularia sp.

8. Factors relating to Howard mold count standards were reviewed

and evidence was presented to show that the present mold count standard for black raspberries is unreasonable.

LITERATURE CITED

1. Baker, G. L. Explanation of the role of calcium salts in the firming of apple slices. Fruit Products J. and Am. Food Mfg., 3, 197-198. 1947.

2. Bell, T. A., Etchells, J. L. and Jones, I. D. Softening of commercial cucumber salt-stock in relation to polygalacturonase activity. Food Tech., 4 (4), 157-163. 1950.

3. Bell, T. A. Pectolytic enzyme activity in various parts of the cucumber plant and fruit. Botanical Gaz. 113 (2), 216-221. 1951.

4. Beneke, E. S. The volume occupied by fungus hyphae in raspberry fruits. Mich. Agr. Exp. Sta. Quat. Bull., 33 (2), 124-126. 1950.

5. Beneke, E. S. Unpublished manuscript. 1950.

6. Calesnick, E. J., Hills, C. H. and Willaman, J. J. Properties of a commercial fungal pectase preparation. Arch. Biochem. 29, (2), 432-440. 1950.

7. Carre, M. H.,and Haynes, D. The estimation of pectin as calcium pectate and the application of this method to the determination of the soluble pectin in apples. Biochem J.,16, 60-69. 1922.

8. Carre, M. H. An investigation of the changes which occur in the pectic constituents of stored fruit. Biochem J., 16, 705-712. 1922.

9. Dodge, B. O., and Wilcox, R. B. Diseases of raspberries and blackberries, U. S. D. A., Washington F. Bull. 1488. 1941.

10. Ehrlich, F. Pectolase. Enzymologia 3, 185-99. 1937. (Obtained from C. A. 8565)

11. Fabian, F. W., and Johnson, E. A. Experimental work on cucumber fermentation X Zymological studies of the cause of soft cucumbers. Tech. Bul., 9-16, Mich. Agr. Exp. Sta. 1938.

12. Fabian, F. W., Beneke, E. S., Erickson, F. J., and Floate R. What is a reasonable mold count for black raspberries in the great lakes region. Canner, May 5, 1951.

13. Fernando, M., Ann. Botany (N. S.) 1-727. 1937.

14. Fish, V. B., and Dustman, R. B. The enzyme activities of Pectinol A on pectin and other substances. J. Am. Chem. Soc., 67, 1155-1045. 1945.

15. Food and Drug Administration, Microanalysis of food and drug products. U. S. Government Printing Office, Washington. 171 pp. 1951.

16. Jansen, E. F., and MacDonnel, L. R. Influence of methoxyl content of pectic substances on the action of polygalacturonase. Arch. Biochem., 8, 1, 97-99. 1945.

17. Joslyn, M. A., and Phaff, H. J. Recent advances in the chemistry of pectic substance. Wallerstein Lab. Comm. X, (29), 39-56. 1947.

18. Kertesz, Z. I. A possible non-enzymatic mechanism of changes occurring in the pectic substances and other polysaccharides in living plants. Plant Phy. 18 (2), 308-309. 1943.

19. Kertesz, Z. I. The pectic substances, ed. 1, Interscience, New York, 1951, 628 pp.

20. Lee, F. E. in the Chemistry and Technology of Food Products, ed. by Jacobs, M. B., Interscience Publishers, New York, (2nd ed. 1951).

21. Leinbach, L. R., Seegmiller, C. G., and Wilbur, J. S. Composition of red raspberries, including pectin characterization. Food tech., 5 (2), 51-54. 1951.

22. Loconti, J. D., and Kertesz, Z. I. Identification of calcium

pectate as the tissue-firming compound formed by treatment of tomatoes with calcium chloride. Food. Res., 6 (5), 499-508. 1940.

23. Menon, K. P., V. Ann. Botany, 187, 134. 1948.

24. McColloch, R. J., and Kertesz, Z. I. Pectic enzymes VIII. A comparison of fungal pectin-methylesterase with that of higher plants, especially tomatoes. Arch. Biochem., 19 (2), 217-229. 1947.

25. Personius, C. J., and Sharp, P. F. Adhesion of potato-tissue cells as influenced by pectic solvents and precipitants. Food Res., 4, 229-306. 1939.

26. Phaff, H. J. The production of exocellular pectic enzymes by Penicillium chrysogenum 1. On the formation and adaptive nature of polygalacturonase and pectinesterase. Arch. Biochem., 13 (1), 67-81. 1946.

27. Phaff, H. J., and Joslyn, M. A. A newer knowledge of pectic enzymes. Wallerstein Lab. Com. X (30), 133-148. 1947.

28. Pitman, G. A., and Cruess, W. V. The hydrolysis of pectin by various microorganisms. Ind. Eng. Chem., 21, 1292-95. 1929.

29. Rendle, T. Observations on changes in raspberries after picking. Analyst, 58, 73. 1933.

30. Robertson, W. van B., Ropes, M. W., and Bauer, W. Degradations of mucins and polysaccharides by ascorbic acid and hydrogen peroxide. Biochem. J., 35, 903-908. 1941.