PECTOLYTIC ENZYMES OF GARLIC

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PECTOLYTIC ENZYMES OF GARLIC

By

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INTRODUCTION

Fabian and Wenzel (1945) investigated the influence of garlic on the softening of genuine Kosher dill pickles. In their work they used fresh, fresh moldy, fresh chopped, dehydrated, and sterilized garlic. Garlic vinegar was also used. The garlic used was added in the normal amount and in from 5 to 10 times the normal amount of garlic. Their results showed that the lots of pickles which contained no garlic, sterilized garlic, or dehydrated garlic kept the best and showed the least amount of softening. They found the highest percentage of spoilage and the greatest rate of softening in the lots of pickles to which 10 times the normal amount of fresh garlic cloves had been added, and also in those lots which contained garlic known to be heavily contaminated with molds and bacteria.

It was significant in their investigation that those lots of pickles which contained garlic and toluene to prevent microbial growth became slippery after 6 weeks, while another lot that contained toluene but no garlic showed no softening at all.

PRESENT INVESTIGATION

Due to the fact that garlic in above normal amounts in Kosher dills and also garlic in pickles free from microbial growth caused softening, it was decided to investigate the possibility that:

Normal sound garlic may contain pectolytic enzymes *per se*, and to study the role of molds found on garlic in softening Kosher dill pickles.

REVIEW OF LITERATURE

The commercial preparation of overnight genuine Kosher dill pickles has been described by Schucart (1942) in the following manner: "Cucumbers, usually not exceeding 1,000 size, are washed and packed in barrels with several layers of dill weed, as in packing genuine dill pickles. One to 1½ pounds of garlic and mixed spices are added; and the stock covered with a salt brine of 22 degree to 25 degree salometer. In some cases a quart of 100 grain vinegar is added. The barrels are closed and sent to cold storage where despite the cold temperature, 36 to 38 degrees Fahrenheit, curing at a slow rate continues, and if kept too long (6 to 9 months) spoilage may be heavy. After the intake season is past, barrels are withdrawn from storage and delivered to the trade."

Greater losses due to softening occur in genuine dill pickles than in salt stock. Fabian and Johnson (1938) attribute this to the lower salt concentration of the genuine dills. In some years as high as 50 percent of the genuine dill pickles packed by certain salters have spoiled. Spoilage not only occurs in the fermented cucumbers but also occurs in the finished product after it has been bottled and marketed.

The spoilage of pickles is progressive (Fabian and

Johnson (1938) and Fabian, Bryan, and Etchells (1932). The first indication of spoilage appears on the skin of the pickles and is known as a "slip". Because of spoilage, the skin of the pickle is slippery and easily removed. Progressively, the deeper cells of the pickle become involved causing the whole pickle to become soft. Cucumbers in which spoilage has progressed to this stage are known as "mushy" cucumbers.

Fabian, Bryan, and Etchells (1932) studied the morphological changes from normally cured pickles to slippery and very soft pickles. A microscopic study showed that normally cured pickles showed decided plasmolysis but the cells were intact and appeared normal. Slippery pickles when examined showed that the pectic material in the lamella of the epidermal and parenchymatous cells was no longer evident. Pickles in the advanced stage of decomposition (mushy pickles) showed marked morphological differences. Practically all the cell walls of the epidermal and parenchymatous cells had disappeared.

Fabian and Johnson (1932) isolated an organism, Bacillus mesentericus fuscus which was capable of causing "slips" in 6 to 12 hours and "mushy" pickles in 12 to 24 hours. By using a stain, ruthenium red, the complete absence of the pectic materials was noted. Sections of pickles that had been extracted with ammonium oxalate to remove the pectic substances also showed absence of pectic materials. As

their chemical analysis showed the same amount of pectic substances present in normal and spoiled pickles, they conclude that the bacterial enzyme produced by Bacillus mesentericus fuscus is a protopectinase and possibly a pectase, but not a pectinase. They add, however, that this does not preclude the possibility of a pectinase being formed if given sufficient time.

They found the bacterial enzymes dissolving pectic substances in pickles was inhibited by 2 percent salt and 1.1 percent acetic acid or 0.7 percent lactic acid. It was also found that acids and heat were found to increase the susceptibility of the pickles to softening, either immediately as by cooking, or over a long period of time with a weak acid.

Fabian and Faville (1949) isolated and identified a mold, Oospora lactis which was the cause of two cases of pickle spoilage. In one case the pickles had been allowed to freshen too long allowing the organism to initiate growth, while in the other case the mold was isolated from dirty barrels which had been used to pack processed dills. For enzyme production the molds were grown in dextrose broth for 7 days. Freshened salt stock was then added to the broth and layered with toluene to prevent microbial growth. The molds isolated from both samples were able to produce slippery pickles in 6 to 8 hours and "cheesy" pickles in 10 to 12 hours.

Jones (1909) studied the enzyme pectinase produced by

Bacillus carotovorus and certain other soft rot organisms. Various experiments were undertaken by him to determine the cultural conditions necessary for optimum enzyme formation having in mind the conclusions of previous investigators that enzyme production in certain cases is a starvation phenomenon. On the contrary, he noted that there seems to be a perfect correlation between the rate and the vigor of the growth of the organism and the amount of enzyme developed. He also studied the effect of heat, acids, alkalies, and long storing of the enzyme. The enzyme produced by B. carotovorus was capable of softening carrots very quickly by dissolving the middle lamella leaving the cells free.

Pittman and Cruess (1929) studied the hydrolysis of pectin by various microorganisms. Their method consisted of growing the different organisms in apple juice to which one percent of commercial powdered apple pectin was added. The bottles were stored for nearly four months and at the end of such time were tested for pectin content, viscosity, and jellying power. One phase of the experiment was designed to determine the protective effect of dextrose on hydrolysis of pectin in solution by Penicillium glaucum. Their results indicate that as long as such a sugar is present, P. glaucum will not attack the pectins as rapidly as it will in the absence of sugar; and that when the supply of sugar is exhausted, the rate of pectin hydrolysis materially increased. Of the microorganisms tested, two molds, P. glaucum and a Pythium sp. exerted the greatest hydrolytic

action. The former gave a more rapid hydrolysis of pectin in media of pH 6 and pH 5 than in pH 3.

Pectin is described as the viscous colloidal substance extracted from plant tissue. It belongs to the group of substances known as hemi-celluloses found throughout the cell walls of plant life. Botanically, the pectins are identified largely with the middle lamella of the cell walls. Softening action of pickles has been considered to be due to the result of action by pectin splitting enzymes on the pectin composing the middle lamella of cucumber tissue.

Phaff and Joslyn (1947) list two principle types of pectic enzymes: pectinesterase (syn. pectase, pectin-methoxylase, pectin methylesterase), which catalyzes the de-esterification of pectin by removal of the methoxyl groups, and polygalacturonase (syn. polygalacturonidase, pectinase, pectolase, pectin polygalacturonase), which catalyzes the glycosidic hydrolysis of pectin or pectic acid.

In regards to protopectinase which Davison and Willaman (1927) included in their three categories of pectic enzymes, which include the two just mentioned, viz. pectinase and pectase, Phaff and Joslyn state: "Pectin has never been prepared as a product of the hydrolysis of protopectin by protopectinase, probably owing to the presence of PG (polygalacturonase) and PE (pectinesterase), which decompose soluble pectin as formed. More critical work is needed, however, to prove with certainty the existence or non-

existence of such an enzyme."

Protopectinase as defined by Davison and Willaman (1927) is that enzyme which converts protopectin of the cell wall into soluble pectin and also attacks the intercellular substance with resultant maceration of the tissue.

Recently Kertesz (1951) states that the complex macromolecules of pectic substances would seem to offer a number of possibilities for enzyme action, and that in spite of this there are only two enzymes which are definitely known; pectin methylesterase, and pectin polygalacturonase. He further adds that a protopectinase might also exist, but that so far this enzyme has been demonstrated only by its action on plant tissues and a chemical definition of the reaction is impossible until the nature of protopectin itself is clarified. As to the recently discovered pectic acid depolymerase in tomatoes, Kertesz (1948) is in doubt that it is an enzyme in the active sense of the word; but it appears now that the loss of pectinic substances in processed tomato products occurs as a result of the cooperative action of tomato pectinesterase and the pectic acid depolymerase.

Jansen and MacDonnel (1945) studied the influence of the methoxyl content of pectic substances on the action of polygalacturonase. In their work they freed the glycosidase from pectinesterase by treatment with acid. The action of the glycosidase was then compared with such substrates as

pectin, alkali-prepared and enzyme prepared pectinic and pectic acids, and methyl glycoside of polygalacturonic methyl ester. They found that de-esterification must occur before polygalacturonase can act on pectic substances.

As Etchells and Bell (1951) state in their paper, "considerable importance is then placed on the part pectinesterase may play in the destruction of the pectin of cucumbers in relation to salt stock softening. The presence of the esterase alone would not necessarily be a causative agent, but together with the glycosidase enzyme system, softening of salt stock could readily take place."

Calesnick, Hills, and Willaman (1950) studied the properties of a commercial fungal pectase preparation. They found the fungal pectase differs from the higher plant pectases in pH relationship, in its response to mono- and divalent cations, and in its thermal behavior. The fungal pectase had activity from pH 2 to 6.5, whereas the pectases of tomatoes, alfalfa, orange and tobacco are inactive below pH 4. Optimum activity was reported to be at pH 5. Optimum salt concentration varied with pH, with the pectase being more sensitive to calcium than to sodium ions. The fungal pectase is also more sensitive to temperature than tomato pectase with complete inactivation at 62° C, whereas, tomato pectase is only 50 percent inactivated at 62° C.

Pectinesterase occurs in the roots, leaves, and fruits of higher plants and is also produced by a number of micro-

organisms. In higher plants it is more or less free of polygalacturonase according to Phaff and Joslyn (1947).

Kertesz (1951) states that it is doubtful that polygalacturonase occurs in higher plants along with pectinesterase. The enzyme pectinesterase occurs in such diverse plants as alfalfa, lilac leaves, molds, malt, tobacco stems and leaves, and the fruits of cherry, tomato, and eggplant. It is not abundant in most plants, however. Some of the better sources are alfalfa, the citrus fruits flavedo and albedo, tobacco stems and leaves, tomatoes, white clover, and pea vines.

Garlic, to our knowledge, has not been mentioned in the literature as a source of pectolytic enzymes.

EXPERIMENTAL METHODS

Preparation of Material

There are several methods used for pectinesterase determination. Kertesz (1951) lists the following: 1) determining the change in ester content, 2) determining the increase in free carboxyl groups, and 3) estimating the amount of methanol liberated.

Our determination was based on the increase in free carboxyl groups, by titrating with 0.1 or 0.5 N NaOH. The procedure followed closely the work of Hills and Mottern (1947) and Bell and Etchells (1951).

Figure 1 shows the apparatus used in determining the pectinesterase in garlic. The reaction was carried out in a water bath at a constant 30° C temperature. The water was heated with two heating elements thermostatically controlled and kept in agitation by means of a stirrer attached to an air driven motor. The reaction mixture proper, contained in a 600 ml beaker, was also agitated. The temperature in the beaker was also maintained at 30° ± 0.5° C. The pH of the reaction mixture was taken with a Beckman pH meter equipped with extension leads. A 10 ml microburette was used to add 0.1 or 0.5 N NaOH as needed.

The enzyme extract was prepared in the following

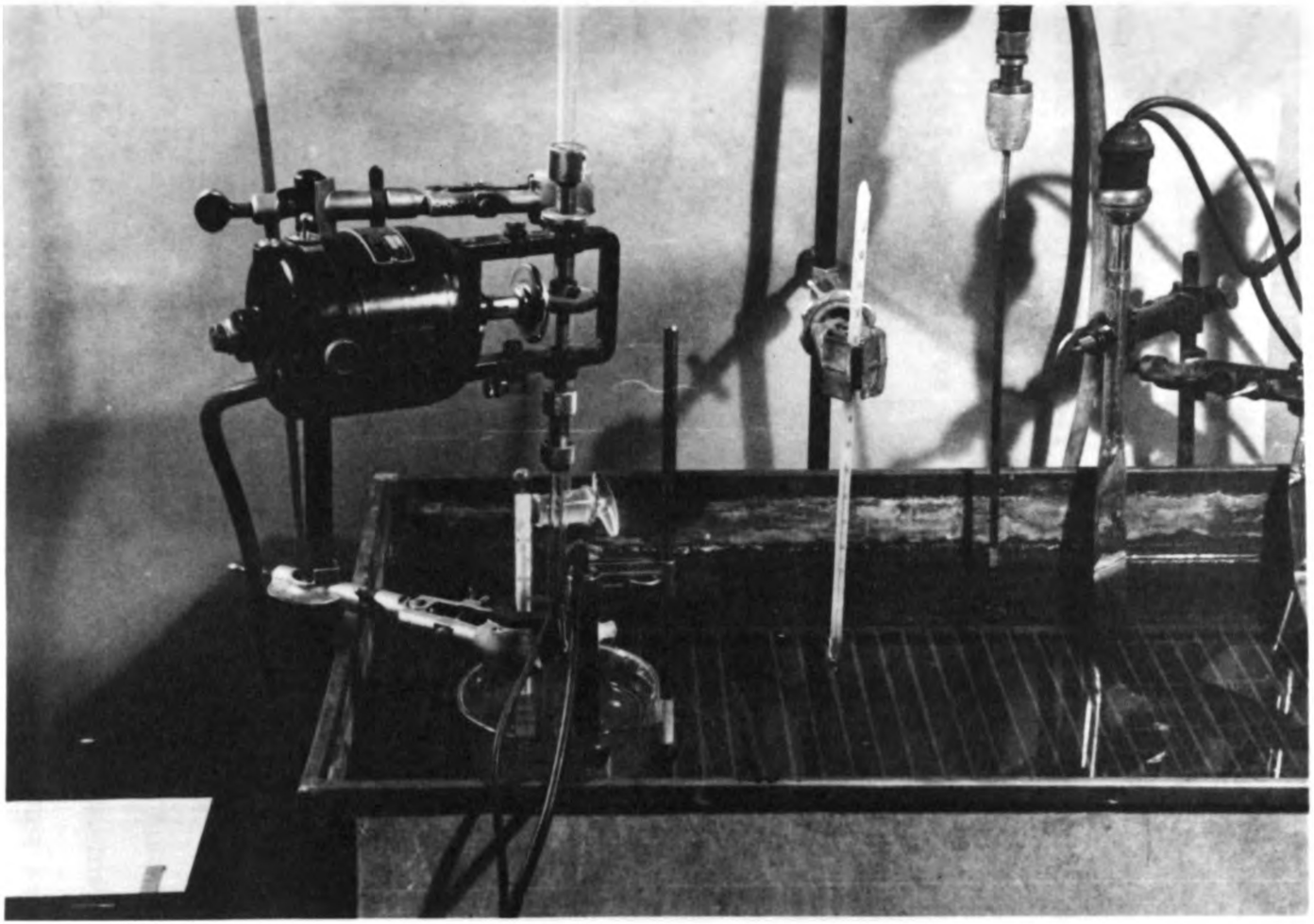

Figure 1. Apparatus for the quantitative determination of garlic pectinesterase.

manner: Clusters of garlic were broken into individual cloves which were examined very carefully for any signs of microbial decomposition. Only those cloves that were sound and normal in appearance were used. They were placed in a quart mason jar and held in a deep freeze unit at a temperature of -20° C. When ready to use, the cloves were allowed to partially thaw. Because of the low moisture content of garlic (64-66 percent) enough distilled water was added to the cloves to secure a consistency necessary for proper maceration of the garlic tissue. Approximately 500 g of garlic cloves and 270 ml of distilled water were blended in a Waring blendor for 20 minutes. Enough NaCl was added to give a 2.4 percent concentration to the macerate. The pH of the garlic-distilled water macerate was 5.6.

According to Kertesz (1951) pectinesterase can be easily desorbed from water insoluble cellular tissues by the use of fairly strong salt solutions; by raising the pH of the tissue macerate above 5; or by using a combination of these two conditions.

After thoroughly blending the macerate was squeezed by hand through several layers of cheesecloth and then filtered through coarse filter paper using a Buchner filter flask. The extract was layered with toluene and stored at 7° C until used.

Qualitative Test for Pectinesterase

The following qualitative test for pectinesterase in garlic based on the observations of Etchells et al (1949) was run as follows. Thirty ml of a 3 percent pectin* solution was placed into each of two 50 ml Erlenmeyer flasks. The pectin was buffered at pH 4 with NaOH and potassium acid phthalate buffer. To flask No. 1 was added 5 ml of the above garlic extract, and to flask No. 2 was added 5 ml of garlic extract that had been inactivated by heating to 80° C for 15 minutes. Five drops of toluene was placed in each flask; they were tightly stoppered, and placed in a 30° C water bath. Table I shows the results.

TABLE I

QUALITATIVE TEST FOR PECTINESTERASE IN GARLIC

Flask number	Time in hours			
	12	24	36	48
1	+	+	+	++
2	-	-	-	-

- = absence of gel; + = gel; ++ = solid gel

The results in Table I show that in flask No. 1 a gel was observed which, indicated that the enzyme was inactivated. In flask No. 2 no gel occurred which indicated that the enzyme was inactivated. Therefore, pectinesterase is present in garlic.

*Pectin obtained from California Fruit Growers Exchange. Labeled was "Special Pectin for Enzyme Testing" No. 447-U-7.

Quantitative Test for Pectinesterase

The quantitative test for pectinesterase in garlic was run according to the following procedure: Two hundred ml of a 1 percent pectin solution, 5 ml of 0.2 M sodium oxalate solution and sufficient 2 M NaCl to give a final concentration of 0.15 M in the reaction mixture were added to a 600 ml beaker. The above solutions were made up to a final volume of 500 ml by the addition of distilled water and 25, 40, and 50 ml quantities of the garlic extract respectively. The garlic extract was not added to the mixture, however, until all the other solutions had been heated to 30° C in a 600 ml beaker. The beaker was then placed in a water bath at 30° C and 25, 40, and 50 ml of the garlic extract respectively added. The pH was then adjusted to 7.5 with 0.1 N NaOH for the 25 and 40 ml quantities of garlic extract and with 0.5 N NaOH for the 50 ml sample. They were maintained at this pH and temperature until the reaction was completed.

Tables II, III, and IV show the results of using 25, 40 and 50 ml of the garlic extract in a quantitative determination of the pectinesterase of garlic.

Figure 2 shows the de-esterification curves obtained for the different quantities of extract used.

"The number of milliequivalents of ester bonds hydrolyzed per minute per unit volume or weight of enzyme material at pH 7.5, 30° C, and with 0.15 M sodium chloride when acting on a 0.4 percent pectin solution is designated as a

TABLE II

TIME AND AMOUNT OF 0.1 N NaOH REQUIRED TO COMPLETE REACTION WHEN 25 ML OF THE GARLIC PECTINESTERASE EXTRACT WAS USED

Minutes	Ml of 0.1 N NaOH used	Minutes	Ml of 0.1 N NaOH used
0	0.00	86	0.95
2	0.40	88	1.00
4	0.75	90	0.50
6	0.60	92	1.10
8	0.67	94	0.75
10	1.18	96	0.60
12	0.67	98	0.57
14	1.04	100	0.65
16	0.39	102	0.60
18	0.90	104	0.70
20	0.50	106	0.76
22	0.90	108	0.66
24	0.75	110	0.63
26	0.70	112	0.60
28	1.18	114	0.75
30	0.53	116	0.65
32	1.16	118	0.65
34	0.58	120	0.53
36	0.80	122	0.64
38	0.87	124	0.73
40	1.03	126	0.55
42	0.60	128	0.55
44	0.90	130	0.67
46	0.60	132	0.58
48	1.05	134	0.58
50	0.30	136	0.57
52	0.87	138	0
54	0.68	140	0.80
56	0.73	142	0.35
58	0.77	144	0.41
60	0.80	146	0.35
62	1.05	148	0.46
64	0.60	150	0.40
66	0.60	0	0
68	0.79	156	1.05
70	0.60	0	0
72	0.40	160	0.48
74	1.16	0	0
76	0.82	167	0.95
78	0.68	0	0
80	0.75	173	0.40
82	0.75	0	0
84	0.85	177	0.39

TABLE II CONTINUED

Minutes	Ml of 0.1 N NaOH used	Minutes	Ml of 0.1 N NaOH used
0	0	199	0.07
184	0.45	0	0
0	0	204	0.05
190	0.11	0	0
0	0	209	0.01
195	0.22	0	0
0	0	212	0.00
		Total ml of 0.1 N NaOH used	56.36

0 = no readings taken

TABLE III

TIME AND AMOUNT OF 0.1 N NaOH REQUIRED TO COMPLETE REACTION WHEN 40 ML OF THE GARLIC PECTINESTERASE EXTRACT WAS USED

Results of Two Trials

Minutes	Ml of 0.1 N NaOH used		Minutes	Ml of 0.1 N NaOH used	
	Trial 1	Trial 2		Trial 1	Trial 2
0	0.00	0.00	87	0.87	0.96
3	1.15	1.10	90	1.49	1.17
6	1.75	1.75	93	1.29	1.29
9	1.65	1.42	96	1.05	1.15
12	1.36	1.63	99	1.18	1.15
15	1.48	1.45	102	0.84	1.10
18	1.56	1.37	105	0.87	1.00
21	1.77	1.85	108	0.94	0.75
24	1.82	1.35	111	1.02	0.90
27	1.29	1.55	114	0.73	0.65
30	1.27	1.55	117	0.37	0.77
33	1.65	1.42	120	0.40	0.63
36	1.70	1.63	123	0.47	0.35
39	1.60	1.56	126	0.33	0.60
42	1.28	1.49	129	0.30	0.35
45	1.46	1.58	132	0.38	0.34
48	1.63	1.47	135	0.47	0.21
51	1.69	1.38	138	0.23	0.35
54	1.30	1.35	141	0.20	0.25
57	1.59	0	144	0.08	0.15
60	1.59	3.17	147	0.06	0.15
63	1.32	1.55	150	0.12	0.10
66	1.58	1.05	153	0.20	0.15
69	1.97	1.37	156	0.13	0.10
72	0.45	1.38	159	0.09	0.05
75	1.45	1.90	162	0.04	0.02
78	1.55	1.60	165	0.05	0.00
81	1.37	1.12	168	0.00	
84	1.48	1.58			
			Total ml of 0.1 N NaOH used	56.80	56.29

0 = no readings taken

TABLE IV

TIME AND AMOUNT OF 0.5 N NaOH REQUIRED TO COMPLETE REACTION WHEN 50 ML OF THE GARLIC PECTINESTERASE EXTRACT WAS USED

Results of Two Trials

Minutes	Ml of 0.5 N NaOH used		Minutes	Ml of 0.5 N NaOH used	
	Trial 1	Trial 2		Trial 1	Trial 2
0	0.00	0.00	72	0.35	0.27
3	0.45	0.49	75	0.26	0.29
6	0.27	0.27	78	0.30	0.25
9	0.38	0.37	81	0.24	0.22
12	0.33	0.28	84	0.22	0.39
15	0.33	0.39	87	0.30	0.19
18	0.41	0.30	90	0.25	0.26
21	0.28	0.34	93	0.22	0.24
24	0.33	0.38	96	0.24	0.22
27	0.32	0.35	99	0.17	0.19
30	0.34	0.30	102	0.19	0.15
33	0.43	0.30	105	0.15	0.18
36	0.31	0.36	108	0.07	0.14
39	0.35	0.31	111	0.13	0.13
42	0.28	0.33	114	0.04	0.12
45	0.39	0.33	117	0.07	0.16
48	0.35	0.35	120	0.08	0.08
51	0.25	0.27	123	0.04	0.04
54	0.39	0.34	126	0	0.07
57	0.30	0.31	129	0	0.00
60	0.26	0.27	132	0.40	0.04
63	0.35	0.35	135	0.00	0.04
66	0.35	0.35	138	0.00	0.00
69	0.30	0.26			
			Total ml of 0.5 N NaOH used	11.49	11.35

0 = no readings taken

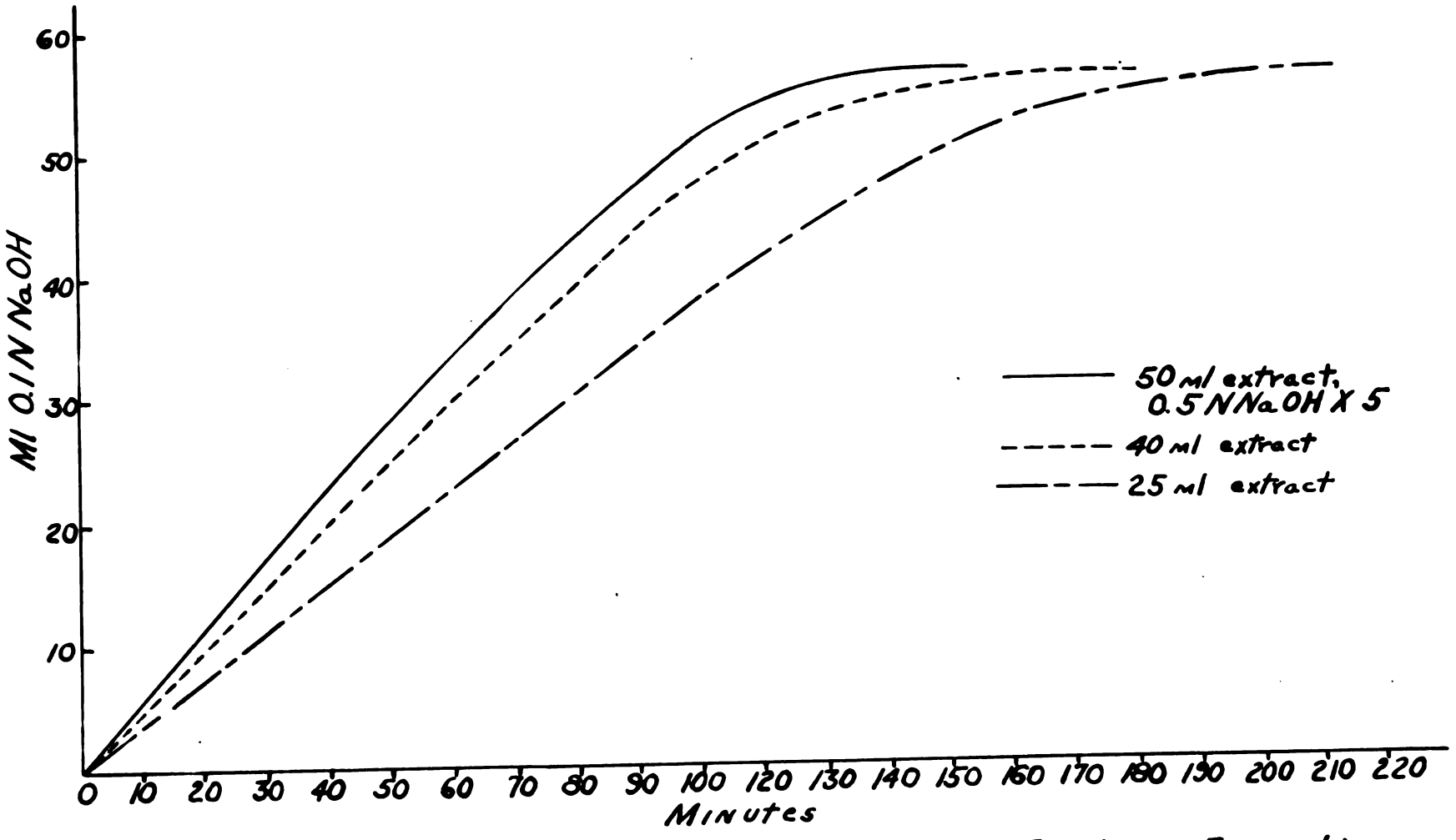

Figure 2. Time curves for the de-esterification of pectin by 25, 40 and 50 ml quantities of garlic extract.

unit of pectinesterase" by Bell et al (1950). They explain that this unit is very similar to that described by Lineweaver and Ballou (1945) with symbol PE, and Hills and Mottern (1947) with symbol K.

Table V gives the calculations of pectinesterase units for the different quantities of enzyme extract used.

TABLE V

CALCULATION OF PE UNITS

Amount of extract	Minutes of reaction	No. of milliequivalents of hydrolyzed ester bonds	PE units x 100
25	40	1.56	0.156
40	39	2.00	0.128
40	39	1.96	0.126
50	39	2.26	0.116
50	39	2.22	0.114

Effect of Various Concentrations of Salt on the Activity of Garlic Pectinesterase

The effect of NaCl on garlic pectinesterase activity was determined at concentrations of 0.15 M (0.88 percent), 0.50 M (3.0 percent), and 0.63 M (3.7 percent), using 200 ml of the reaction mixture. The NaCl added was dissolved in the distilled water that was added to the reaction mixture to bring the volume to 200 ml. In calculating the salt used the 2.4 percent that was originally in the garlic extract was taken into consideration. The reaction was carried out

for a 30 minute period. The total amount of 0.1 N NaOH consumed in the 30 minute period was used to determine the activity of the enzyme. The percentages of salt used is typical of the range of salt used in Kosher dills. Table VI shows the activity found at the different salt levels.

TABLE VI

ACTIVITY OF GARLIC PECTINESTERASE AT VARIOUS SALT CONCENTRATIONS

Exp. No.	Salt concentration	Ml of 0.1 N NaOH used	Percent relative activity
1	0.15 M (0.88%)	6.14	100
2	0.50 M (3.0%)	4.41	72
3	0.63 M (3.7%)	3.45	56

This indicates that pectinesterase present in the garlic would still have an activity of 56 to 72 percent in the range of the salt concentrations found in Kosher dill pickles.

Effect of pH on Garlic Pectinesterase Activity

The effect of pH on garlic pectinesterase activity was determined at pH levels of 7.5, 6.0, 5.0 and 4.0. One-tenth N HCl was used in adjusting the pH. Two hundred ml of the reaction mixture was used. Sodium chloride concentration was maintained at 0.15 M, and the reaction was carried out for a 30 minute period. The total amount of 0.1 N NaOH consumed in the 30 minute period was used to

to determine the activity of the enzyme. Table VII shows the activity found at the various pH levels.

TABLE VII

ACTIVITY OF GARLIC PECTINESTERASE AT DIFFERENT pH LEVELS

Exp. No.	pH	Ml of 0.1 N NaOH used	Percent relative activity
1	7.5	9.89	100
2	6.0	7.22	73
3	5.0	3.47	35
4	4.0	0.36	3.6

As the pH is decreased from 7.5, the activity of the garlic pectinesterase was diminished.

Heat Inactivation of Garlic Pectinesterase

The effect of heat on garlic pectinesterase was studied because of the wide use of fresh garlic cloves in making certain types of pickles such as genuine, processed, and fresh pasteurized dill pickles.

The temperature used to inactivate the enzyme was 72° C which is equivalent to 165° F, the temperature used to pasteurize genuine, processed, and fresh dill pickles. Genuine and processed dill pickles may or may not be pasteurized depending upon the equipment available and the manufacturer but of course it is necessary to pasteurize

the fresh dills; otherwise they would spoil immediately.

The method was similar to that used by Kertesz (1938) in his studies on heat inactivation of tomato pectin-methoxylase (pectinesterase). The test is as follows: A 1 percent pectin solution was made to which was added 15 drops of methyl red indicator to every 100 ml of pectin solution. The pH of the pectin solution was adjusted to 6.2. with 0.1 N NaOH. Twenty-five ml of the 1 percent pectin solution and 5 drops of toluene were placed into 125 ml flasks. Five ml quantities of the garlic pectinesterase extract was placed in 20 ml pyrex test tubes. The test tubes were placed in a rack and immersed in a 72° C water bath to inactivate the enzyme. The tubes were allowed to remain in the bath for various lengths of time (Table VIII) after which the contents of each tube were emptied in the flasks containing the 1 percent pectin solution, mixed thoroughly and incubated at 30° C for 24 hours. At the end of this time the pH was taken. If the enzyme was inactivated, the pH would not be lowered and the indicator would still have its yellow appearance. If the enzyme was not inactivated the pH would be lowered with the formation of a red color due to action of the enzyme on the pectin. The results of this experiment are given in Table VIII.

Since 5 ml of the garlic pectinesterase was used, it was not possible to heat this quantity to 72° C immediately. A thermometer suspended a short distance from the bottom of the tubes gave the temperature of the extract in the tubes.

TABLE VIII

EFFECT OF HEAT ON GARLIC PECTINESTERASE

Time in water bath	Temperature of tubes in bath, °C	Activity	pH of pectin after 24 hrs.
30 sec.	45	+	3.95
45 sec.	54	+	3.97
1 min.	60	+	4.00
2 min.	70	+	4.55
3 min.	72	+	5.72
4 min.	72	+	5.62
5 min.	72	-	6.31
7 min.	72	-	6.32
10 min.	72	-	6.40
12 min.	72	-	6.40

+ = red color; - = yellow color

The enzyme was not inactivated until a temperature of 72° C had been reached at the end of 5 minutes.

Pectinesterase Activity of Dehydrated Powdered Garlic and Dehydrated Garlic Flakes

Commercial powdered garlic and garlic flakes were tested for the presence of pectinesterase. The powdered garlic was packed in 1950 and the garlic flakes in 1951.

Sixty-gram quantities of each product, 240 ml of distilled water and 2 ml of toluene were placed in flasks which were tightly stoppered and allowed to stand overnight in the refrigerator at 7° C. This mixture was then macerated

in the Waring blendor with 2 percent NaCl and filtered through cheesecloth and coarse filter paper. The activity was quantitatively tested by the method previously described except 20 ml quantities of the garlic extract were used. The reaction mixture contained 200 ml and 0.1 N NaOH was used in the titrations. Inactivated controls of the powdered garlic and dehydrated garlic flakes required 0.41 and 0.43 ml respectively of 0.1 N NaOH for titration. These values were taken into consideration when calculating the PE units in Table IX. The reactions were allowed to proceed for 30 minutes. Results are given in Table IX.

TABLE IX

PECTINESTERASE ACTIVITY OF POWDERED DEHYDRATED GARLIC AND GARLIC FLAKES

Extract	Amount of extract (ml)	Minutes of reaction	No. of milli-equivalents of hydrolyzed ester bonds	PE units x 100
Powdered garlic	20	30	0.202	0.033
Garlic flakes	20	30	0.237	0.039

Since the work was done with fresh garlic and dehydrated garlic powder and flakes, it was necessary to use different amounts of water to prepare the extract. This makes comparison of the pectinesterase activity of the respective extracts difficult. In the preparation of the fresh garlic extract 500 g of the cloves was macerated in 270 ml of distilled water. When 25 ml of the extract was incubated

for 30 minutes with the 3 percent pectin solution, a PE unit of 0.156 was obtained, Table V. In the case of the dehydrated powder and chips 240 ml of distilled water was used to 60 g of the powder and flakes respectively. When 20 ml of these extracts was incubated with the 3 percent pectin solution for 30 minutes, PE units of 0.033 and 0.039 respectively were obtained, Table IX. The label of the dehydrated products used stated that 1 lb of the dehydrated product was equivalent to 4 lb of the fresh product. A very rough calculation indicates that fresh garlic was approximately four times more active than the dehydrated garlic products tested. Many factors can influence this difference in activity such as age, purity and method of preparation of the dehydrated products.

Test for a Polygalacturonase-like Enzyme in Garlic

Recently, Bell (1951) reported the presence of a pectolytic enzyme similar to polygalacturonase in various parts of the cucumber fruit and plant. Bell states that the glycosidic hydrolysis of pectin or pectic acid to galacturonic acid would not necessarily have to be complete for salt stock to become soft, and refers to the work of Fabian and Johnson (1938) where it was found that both mushy and firm salt stock had the same pectin content when measured as calcium pectate.

Bell et al (1949) reported on the softening of cucumber salt stock in relation to polygalacturonase activity. They

found that the polygalacturonase-like enzyme reacted similarly to commercial polygalacturonase (pectinol) in respect to temperature, pH, salt and time of incubation.

Kertesz and McColloch (1949) reported an enzyme in ripe tomatoes that was capable of the depolymerization of pectic acid. They refer to it as pectic acid-depolymerase.

With the findings of the above workers in mind, it was decided to test for the presence of other pectolytic enzymes in garlic with special reference to polygalacturonase.

Experimental procedure. The method used was similar to that of Bell (1951), and Bell, Etchells and Jones (1949) and will be described later in the paper. Briefly, it consists of measuring the loss in viscosity of a 3 percent pectin solution caused by the action of pectolytic enzymes or more specifically by a polygalacturonase-like enzyme.

In order to prevent gel formation in the garlic extracts, the pectinesterase was destroyed by adjusting the pH of the extracts to a value of 3 and incubating at approximately 40° C for 24 hours in a water bath. Extracts were prepared as previously described from the fresh garlic, powdered garlic and garlic flakes respectively. These extracts were tested by adding 5 ml of the extract and 5 drops of toluene to 30 ml of a 3 percent pectin solution buffered at pH 4 to 50 ml flasks. The flasks were tightly stoppered and incubated at 30° C for 7 days. Viscosity readings were taken at the end of one and 7 days by means

of a 25 ml volumetric pipette. The dropping time was measured with a stopwatch. A loss in viscosity of the pectin solution would indicate the presence of a polygalacturonase-like enzyme in the garlic extract.

The formation of a gel showed that in the fresh garlic extract the pectinesterase was not inactivated by this method so that it was impossible to test for the polygalacturonase-like enzyme. On the other hand the dehydrated garlic powder and flakes showed no loss in viscosity and no gel formation which indicated the presence of little or no pectinesterase and the absence of a polygalacturonase-like enzyme.

To check the action of the garlic extract on pickles, several freshened salt stock pickles were kept in the garlic extract for a month and showed no sign of softening.

The gel formation by fresh garlic in the above experiment may be explained by Kertesz (1951), who states that the procedure for inactivating pectinesterase must be established for each kind of enzyme preparation.

Decomposed Garlic as a Source of a Polygalacturonase-like Enzyme

Since dehydrated garlic did not show any evidence of a polygalacturonase-like enzyme, it was decided to test for this enzyme in decomposed garlic. Furthermore, decomposed garlic has already been implicated as a cause of softening in genuine Kosher dill pickles by Wenzel and Fabian (1945). Therefore, it was thought that a study of

several of the molds found on decomposed garlic would be of interest.

Wenzel and Fabian (1945) found Aspergilli and Penicillium molds on garlic and attributed the formation of pectolytic enzymes by these fungi as the cause of spoilage in genuine Kosher dill pickles.

The literature cites many fungi capable of producing pectolytic enzymes, and certain of the genera such as Penicillium and Aspergilli, are commercially used to produce pectolytic enzymes for clarifying of fruit juices. Penicillium glaucum is used to produce pectolytic enzymes sold under the trade name of "Pectinol".

Experimental procedure. Several clusters of garlic were placed in mason jars to which a small amount of water was added to hasten the development of mold growth. After one week there was considerable growth. Molds present on the garlic clusters were identified as Penicillium canescens and Fusarium oxysporum. These were cultured on Sabouraud dextrose agar.

The molds isolated were first grown on a pectin medium developed by Manchester and Baier (1945) to determine their ability to liquefy pectin. Both molds grew very well on this medium. P. canescens liquefied the pectin readily, while F. oxysporum liquefied it slowly and only partially.

As production of pectolytic enzymes may be more or less profuse according to the substrate used, these molds were

grown in pure culture on sterile garlic cloves. Sound, fresh garlic cloves with the layer of skin removed were immersed in a solution of 1:1250 Roccal for 5 minutes. The cloves were then removed and rinsed twice in sterile water. The cloves were aseptically placed into two sterile mason jars and inoculated with the two molds. Several of the cloves were placed in dextrose broth and in Sabouraud dextrose agar to check on the sterility of the cloves. One lot of cloves was run as a control without any inoculum. The mason jars were placed in a dark room to keep the garlic cloves from sprouting. After three weeks of incubation, the inoculated cloves were completely covered with growth and very badly decomposed, while the uninoculated control showed no change.

Fifteen g of each of the two lots of moldy garlic were macerated in the Waring blendor in 45 ml of distilled water. The uninoculated control and two clusters of garlic that had been allowed to decompose naturally were treated in the same manner. The extract from each lot was filtered through cheesecloth and then through filter paper to remove any particles that would interfere with the viscosity measurements. After 5 drops of toluene was added, the flasks were stoppered and placed in the refrigerator at 7° C.

The methods used to test for the presence of pectolytic enzymes in the extract was similar to that of Etchells et al (1949). The method used in these experiments was as follows: A 3 percent pectin solution was buffered at a pH

of 4 with potassium acid phthalate and NaOH. It was then filtered through cotton, layered with toluene, stoppered tightly, and stored in the refrigerator until used.

As a known source of pectolytic enzymes a commercial product, "Pectinol M"*, was used. One portion was inactivated by heating for 15 minutes at 80° C and then tested for its activity.

Thirty ml of the 3 percent pectin solution was added to 50 ml Erlenmeyer flasks. To each flask were added 5 ml of the prepared extracts and 5 drops of toluene after which the flasks were stoppered and placed in the 30° C water bath. Viscosity readings were made at the end of 1, 2, 4, and 6 days.

The dropping time of the solutions was measured with a 25 ml volumetric pipette. The pectin solution was drawn into the pipette with a rubber suction bulb to a mark on the pipette which was calibrated to deliver 25 ml. The end of the pipette was cut off to give a faster delivery rate. The time required to empty the pipette was taken with a stopwatch.

Since all readings were taken with the same pipette, it was necessary to clean the pipette thoroughly after each reading. The pipette was cleaned by flushing it with boiling water, followed by an alcohol, and then a boiling water rinse, and finally in an alcohol and acetone rinse.

The activity of the enzyme was measured by the loss in viscosity of the pectin solution. The loss in viscosity

*"Pectinol M" obtained from Rohm and Haas Co., Philadelphia, Pa.

was calculated by comparing the loss in viscosity of the active enzyme solution to the inactive control which should show no loss in viscosity. The results obtained are given in Table X.

TABLE X

PECTOLYTIC ACTIVITY OF THE VARIOUS EXTRACTS EXPRESSED AS PERCENT LOSS IN VISCOSITY

Source of enzyme	Ave. dropping time in seconds at the end of days				Percent loss in viscosity
	1	2	4	6	
Extract from naturally decomposed garlic	15.0	10.4	9.1	8.9	81.6
Inactivated 0.1% Pectinol M	48.5	48.4	47.6	47.4	2.2
0.1% Pectinol M	12.5	10.5	9.4	8.5	82.0
F. oxysporum garlic extract	54.6	59.6	72.3	83.5	0
P. canescens garlic extract	9.3	8.8	8.5	8.5	82.4
Control -- uninoculated garlic extract	57.5	58.0	58.5	59.1	0
Control -- inactivated garlic extract	51.1	48.7	49.9	48.5	5.0

The results in Table X show that the extract from the decomposed P. canescens garlic showed a loss in viscosity comparable to the 0.1 percent Pectinol M, as did also the naturally decomposed garlic extract. The decomposed F. oxysporum garlic extract and the uninoculated garlic

extract showed no loss but a gain in viscosity and after two weeks both of these extracts showed a gel formation.

DISCUSSION

In the quantitative determination of the garlic pectinesterase, values of 0.156×10^{-2} to 0.144×10^{-2} PE units per ml were obtained when using 25 to 50 ml of the garlic extract respectively. The difference in values may be due to the concentration of enzyme, the concentration of substrate, or due to storage of the garlic extract. The amount of 0.1 N NaOH or 0.5 N NaOH used in titrating when various quantities of the garlic extract were used gave comparable results as to the total amount of milliequivalents of hydrolyzed ester bonds. In the case of the 25 ml quantity of extract used 56.36 ml of 0.1 N NaOH was used, while in the case of the 40 and 50 ml quantities, 56.54 and 11.42 ml of 0.1 N and 0.5 N NaOH respectively were used. This would give values of 5.63, 5.65, and 5.63 milliequivalents of hydrolyzed ester bonds for the 25, 40, and 50 ml quantities of the extracts respectively. Although different PE values were obtained for the different quantities of extract used, the total amount of hydrolyzed ester bonds indicates the completeness of the reaction in all cases.

It was found that the normal salt range of the various kinds of Kosher dill pickles decreased the activity of garlic pectinesterase from 56 to 72 percent. However, at the beginning of a genuine Kosher dill fermentation the salt

concentration is generally greater than the values tested for in these experiments, viz. 3.0 and 3.7 percent. Therefore, the concentration of salt at the beginning of a genuine Kosher dill fermentation would be unfavorable for the activity of garlic pectinesterase.

The activity of the garlic pectinesterase diminished as the pH decreased from 7.5 to 4.0. Thus, in genuine Kosher cucumber fermentation the activity of the garlic pectinesterase would be at its greatest before the fermentation actually began. Therefore, in actual conditions, the pH at the beginning of the fermentation would be favorable for the activity of the enzyme while the salt concentration would not.

Since pectinesterase has been found in garlic, it may be significant in paving the way for softening of genuine Kosher dill pickles. Jansen and MacDonnel (1945) have demonstrated that de-esterification must occur before polygalacturonase can act on pectic substances. Therefore, garlic could be responsible for the de-esterification of pectin in genuine Kosher dill pickles.

It has been shown in this paper that moldy garlic could be a source of polygalacturonase. The extract from the garlic decomposed by P. canescens gave an activity comparable to commercial Pectinol M, as did the extract from the garlic that was allowed to decompose naturally. The extract inoculated with the F. oxysporum showed a gain in viscosity and a gel formation at the end of two weeks.

This could be attributed to formation of pectinesterase by the F. oxysporum mold or to the extraction of more of the garlic pectinesterase due to the decomposition of the garlic.

The heat inactivation tests showed that the garlic pectinesterase was inactivated at the end of 5 minutes under the conditions of the experiment. Since fresh pasteurized dills are processed at approximately 165° F for a period of 15 to 20 minutes, the garlic pectinesterase would doubtless be inactivated. The same would be true if genuine and processed dills were pasteurized at this temperature and time. Most processors of such pickles use chopped garlic so that the heat penetration into the garlic would be faster in such cases.

SUMMARY

1. Garlic was found to contain the enzyme pectinesterase in amounts of 0.156×10^{-2} to 0.114×10^{-2} PE units per ml depending on the amount of extract used.
2. Garlic pectinesterase showed decreased activity as the pH and salt concentration approached those found in different kinds of Kosher pickles.
3. Pectinesterase was found in dehydrated garlic products. The powdered garlic and garlic flakes showed similar activity but showed approximately one-fourth the activity of the fresh garlic extract.
4. Polygalacturonase was not demonstrated in the dehydrated garlic products.
5. Naturally decomposed garlic and garlic decomposed by the mold, *Penicillium canescens*, showed pectolytic activity comparable to commercial Pectinol M, while garlic decomposed by *Fusarium oxysporum* showed a strong gel formation.

BIBLIOGRAPHY

Bell, T. A. (1951). Pectolytic enzyme activity in various parts of the cucumber plant and fruit. Bot. Gaz., 113 (2), Dec.

Bell, T. A., Etchells, J. L., and Jones, I. D. (1951). Pectinesterase in the cucumber. Arch. Biochem., 31 (3).

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

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

Davison, F. R., and Willaman, J. J. (1927). Biochemistry of plant diseases. IX. Pectic enzymes. Bot. Gaz., 83, 329-361.

Fabian, F. W., and Bryan, C. S., and Etchells, J. L. (1932). Mich. Agr. Exp. Sta. Tech. Bull. 125.

Fabian, F. W., and Faville, L. W. (1949). Isolation and identification of a mold, Oospora lactis, as the causative agent of two cases of pickle spoilage. Fruit Products Jour., 28, 297-298.

Fabian, F. W., and Johnson, E. A. (1938). Experimental work on cucumber fermentation. Mich. Agr. Exp. Sta. Tech. Bull. 157.

Hills, C. H., and Mottern, H. H. (1947). Properties of tomato pectase. Jour. Biol. Chem., 168, 651-663.

Jansen, E. F., and MacDonnel, L. R. (1945). Influence of methoxyl content of pectic substances on the action of polygalacturonase. Arch. Biochem., 8 (1), Oct., 97-111.

Jones, L. R. (1909). Pectinase, the cytolytic enzyme produced by Bacillus carotovorus and certain other soft rot organisms. N.Y. Agr. Exp. Sta. (Geneva) Tech. Bull. 11.

Kertesz, Z. I. (1939). Pectic Enzymes. III. Heat inactivation of tomato pectin-methoxylase (pectase). Food Research, 4 (2), 113-116.

Kertesz, Z.I. (1951). "The pectic substances". Interscience Publisher, New York.

Lineweaver, H., and Ballou, G. A. (1945). The effect of cations on the activity of alfalfa pectinesterase (pectase). Arch. Biochem., 6, 373-387.

McColloch, R. J., and Kertesz, Z, I. (1949). Recent developments of practical significance in the field of pectic enzymes. Food Tech., 3, March, 94-96.

Phaff, H. J., and Joslyn, M. A. (1947). The newer knowledge of pectic enzymes. Wallerstein Lab. Comm., 10, 133-148.

Pittman, G. A., and Cruess, W. V. (1929). Hydrolysis of pectin by various microorganisms. Ind. and Eng. Chem., 21, 1292-1295.

Schucart, H. S. (1942). Practical observations on the manufacture of Kosher style dill pickles. Fruit Products Jour., 21, 206-212.

Wenzel, F. W., and Fabian, F. W. (1945). Experimental work on cucumber fermentation. Mich. Agr. Exp. Sta. Tech. Bull. 199.

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