Table V. Analysis of Variance for KCl Treatment and $CaCl_2$ or Tripolyphosphate Treatment of Lyophilized Meat

	df	KCl	$CaCl_2$ or tripoly-phos-phate	Mean pair difference	p
KCl–$CaCl_2$	6	1.6	1.1	0.5	<0.05
KCl–tripoly-phosphate	6	1.8	1.0	0.8	<0.01

fibril at the z lines, and Davey and Gilbert (1969) showed that EDTA suppressed the morphological changes in the myofibril during postmortem aging. As the amount of water-extractable Ca in meat increases during postmortem aging (Arnold *et al.*, 1956), this special effect of Ca ions is very interesting in the study of meat tenderization phenomenon. Studies are now in progress about the factors which affect the release of Ca ions from meat during postmortem aging.

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Pectinase Stabilization of Orange Juice Cloud

Robert A. Baker* and Joseph H. Bruemmer

The cloud of fresh orange juice was stabilized without heating by adding a commercial pectinase at 200 ppm. The level of stable cloud was proportional to pectinase concentration and to treatment temperature. Six other commercial pectinases were tested for stabilizing orange juice cloud. One pectinase accelerated clarification, but the other five stabilized with varying effectiveness. Pectin distribution analysis, pectinesterase assay of pectinase-treated juice, and pectin depolymerizing activity assays of the pectinases show the following. Pectinases degrade orange juice particulates and release bound pectinesterase into the juice. Pectinases stabilized orange juice cloud by depolymerizing pectic substances to soluble pectates instead of to insoluble pectates. And effectiveness of stabilization correlates with the ratio of depolymerizing activity on polygalacturonic acid to that on pectin. Selection of pectinases to stabilize natural orange juice cloud can be made on the basis of their depolymerizing activities.

Fresh orange juice contains finely divided particulates in suspension that give it a "cloudy" appearance. Analyses have shown this particulate material is composed almost exclusively of pectin, protein, and lipid (Baker and Bruemmer, 1969; Scott *et al.*, 1965). [Mizrahi and Berk (1970) found substantial quantities of hesperidin crystals in the juice cloud of Shamuti orange, but this characteristic appears to be peculiar to the Shamuti variety.] When this unstable colloidal system collapses, the juice clarifies. Once converted to an unattractive two-phase system of a flocculant sediment in a clear serum, the juice is no longer marketable. In addition, the cloud, which will not remain suspended, contains most of the characteristic orange flavor and color.

Heat is used commercially to stabilize orange juice against cloud loss. Because a temperature near 90° is required, processed orange juice sometimes acquires off-flavors from excessive heat (Bissett *et al.*, 1953; Kew and Veldhuis 1960, 1961). Heating stabilizes orange juice cloud by inactivating pectinesterase (PE), an enzyme that initiates a series of reactions leading to clarification. PE demethylates juice-soluble pectin, converting it to low-methoxyl pectin, which reacts with polyvalent cations to form insoluble pectates. Presumably, the precipitation of these pectates occludes the cloud particles and removes them from suspension (Dietz and Rouse, 1953). Until recently, juice-soluble pectin was presumed to form a colloidal matrix that supports the particulates and therefore was necessary for orange juice cloud stability (Rouse and Atkins, 1955).

We showed that soluble pectin was not necessary for cloud support, as a stable suspension of orange juice particulates could be made in water (Baker and Bruemmer, 1969). Because pectin is the source of the destabilizing low-methoxyl pectin, we proposed controlled pectin degradation as an alternative to heat denaturation of PE for stabilizing orange juice cloud. The proposal was supported by the stability of orange juice particulates in centrifugally prepared orange juice serum treated with a commercial pectinase (Baker and Bruemmer, 1969). As used throughout this paper, the term pectinase refers to commercial pectolytic enzyme preparations.

Demonstration that orange juice particulates formed a stable cloud in orange juice serum containing a commercial pectinase suggested that orange juice might be stabilized by adding a pectinase directly to whole juice. The present paper reports the first instance of stabilizing orange juice cloud by adding ppm levels of commercial pectinases to whole juice.

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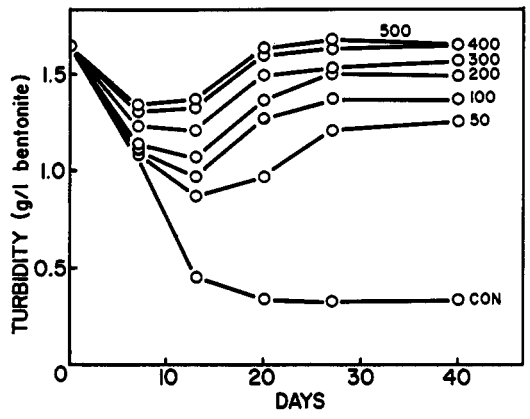

Figure 1. Effect of several concentrations (ppm) of Klerzyme on turbidity of orange juice at 4°

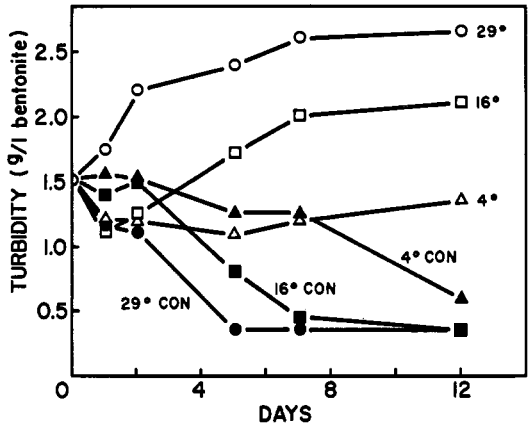

Figure 2. Effect of temperature on cloud stabilization with 200 ppm of Klerzyme

Effectiveness of a number of commercial pectinases in stabilizing cloud was shown to be related to their depolymerase action on pectic substances. An action mechanism of stabilization is proposed, based on this relationship.

METHODS

Cloud Stability. Juice from Valencia oranges was expressed by hand on an electric juice reamer and strained through a colander with 1.6-mm openings. After adding the pectinases at a concentration of 200 ppm, the juice samples were protected against spoilage with 0.1% sodium benzoate and stored in glass jars at 4°. The degree of clarification was determined by the procedure of Senn *et al.* (1955) with minor modifications. The samples were centrifuged for 10 min at 470 × *g*. Turbidity of the supernatant was measured as percent transmission through a 20-mm light path in a Lumetron colorimeter (Photovolt Corp., New York, N. Y.) equipped with a 650-mμ filter. Data were expressed as Bentonite units (Senn *et al.*, 1955).

Enzyme Activities. PE activity of the pectinases was determined by the method of Vas *et al.* (1967). Pectinases were dissolved in 15 ml of M/15 McIlvaine buffer of pH 3.8 containing 5% sodium chloride. After equilibrating at 30°, 50 ml of 1% citrus pectin was added to start the reaction. A Corning Model 10 pH controller (Corning Glass Works, Medfield, Mass.) was used to keep the pH at 3.8. This pH was selected because it was representative of the values frequently encountered in fresh juice. Data are expressed as pectinesterase units (PE_U) × 10^3 per gram of pectinase (Rouse and Atkins, 1955). PE activities of juice serum and peel extract were measured at pH 7.5 with 0.1 *M* phosphate buffer.

Depolymerase activities were determined by the procedure of Vas *et al.* (1967) with an Ostwald-type viscometer of 0.8 mm bore. Polygalacturonase activity (PG_{PGA}) was calculated from the time required for the pectinase to reduce the viscosity of 1% polygalacturonic acid by 25% corrected for the viscosity of water. One unit of PG_{PGA} is the activity required for this reduction to occur in 100 min. The combined activities of PG and polymethylgalacturonase (PMG) on pectin (PG_{PEC}) were calculated from the time required for the pectinase to reduce viscosity of 0.3% citrus pectin by 25%, corrected for the viscosity of water. One unit of PG_{PEC} is the activity required for this reduction to occur in 100 min.

Pectins Distribution. Pectinol 41-P and Pectozyme were added at 500 ppm to whole fresh orange juice to determine their effect on juice pectins. The samples were stored at 4° for 8 days with 0.1% sodium benzoate added as a preservative. Pectins were measured as water-soluble pectin, ammonium oxalate-soluble pectins (pectates), and NaOH-soluble pectin (protopectin) (Rouse and Atkins, 1955), and expressed as anhydrogalacturonic acid (AGA).

PE Fortified Juices. Orange peel PE was added to pectinase-treated juice samples to test stability against PE-initiated clarification. Two-hundred ppm of each of the pectinases was added to samples of fresh juice and stored for 3 days at 4°. Peel from the juiced fruit was dried for 2 days under vacuum at room temperature and then extracted for PE (MacDonnell *et al.*, 1945). The partially purified PE was assayed for activity, as was a juice serum sample obtained by centrifuging a portion of the control juice at 73,000 × *g* for 25 min. PE was added to the pectinase-treated samples to increase the soluble PE levels 5-, 10-, and 20-fold. Cloud densities of these PE-fortified samples and control samples were measured after 24 hr storage at 4°.

Materials. Polygalacturonic acid used in assays was obtained from Sunkist Growers, Inc., Corona, Calif. Pectin was Sigma grade II, derived from citrus (Sigma Chemical Co., St. Louis, Mo). Klerzyme 200 was received through the courtesy of Wallerstein Co., Morton Grove, Ill. Rohm & Haas Co., Philadelphia, Pa., generously provided samples of Pectinols 41-P, 42-E, 59-L, RA-5, and 5-B. Pectozyme was supplied by Fermco Laboratories, Chicago, Ill.

RESULTS AND DISCUSSION

Concentration. Klerzyme stabilized cloud when added to fresh orange juice at 4°. The data in Figure 1 show that stable cloud level is proportional to the concentration of Klerzyme between 50 and 400 ppm. The level is also proportional to the effectiveness in retarding loss of cloud in the first week when cloud is lost at all concentrations of Klerzyme. However, the loss is transient, and after 20 days cloud is reestablished at the approximate level of the initial juice. The increase in cloud during the third and fourth weeks probably reflects an increase in colloidal particles resulting from the degradation and fragmentation of juice particulates by Klerzyme.

Temperature. Figure 2 shows that the level of cloud stabilized by 200 ppm Klerzyme is proportional to storage temperature. Klerzyme stimulated clarification compared to controls during the first day of storage at 4° and 16° C. At 4° C clarification continued until day 5, whereas at 16° C the cloud level began to increase on day 2. The 29° C sample appeared to increase in turbidity immediately. However, other experiments showed that at 29° C cloud is lost during the first hour,

Table I. Bentonite Units of Orange Juice Cloud after Treatment for 34 Days at 4° with 200 ppm of Pectinases

Pectinase	Bentonite units, g/l.
Pectinol 41-P	1.97
Klerzyme 200	1.76
Pectinol 5-B	1.73
Pectinol 42-E	1.28
Pectinol 59-L	1.07
Pectinol RA-5	1.04
Pectozyme	0.35
None	0.40

Average of three experiments. Bentonite value for fresh juice was 2.18.

after which turbidity increases rapidly. These data indicate that Klerzyme has an initial clarifying action on orange juice and that the quicker this activity is controlled the higher the level of stable cloud.

Stabilization with Pectinases. Klerzyme is marketed as a clarifying agent for wine, apple juice, and other fruit juices. Its application as a stabilizer for orange juice cloud represents a novel use of this product. This unusual use prompted a survey of a number of commercial pectinases for similar activities. Table I shows that of the seven preparations examined, three (Pectinol 41-P, Klerzyme, and Pectinol 5-B) were very effective, three (Pectinols 42-E, 59-L, and RA-5) were moderately effective, and one (Pectozyme) was ineffective in stabilizing fresh orange juice cloud.

Those pectinases which were effective stabilizers showed a time pattern of change in cloud level similar to Klerzyme. The level of stable cloud was proportional to effectiveness in retarding loss of cloud during the initial period of storage. A representative experiment is shown in Figure 3 which gives the curves of two of these preparations, Pectinols 41-P and 5-B, as well as the stability curve for Pectozyme. The latter was the only pectinase that did not stabilize orange juice cloud to some extent.

Because PE is the natural orange juice-clarifying agent, and stable cloud levels are proportional to speed of suppressing clarification (Figures 1 and 2), soluble-PE activities of the Pectinol 41-P and Pectozyme-treated juices were assayed for a possible explanation of their differences in stabilizing effectiveness. Jansen *et al.* (1960) found that PG destroyed the ability of orange juice sacs to bind PE; therefore, PG in the commercial pectinases probably released PE from particulates in the treated juices. The Pectinol 41-P and Pectozyme-treated juices were examined for PE released into the serum after 2 and 5 days. Both the most and least effective stabilizers increased the serum PE three- to sixfold. While no correlation can be drawn between the amount of PE released and stabilizing effectiveness, these results suggest that the pectinase-treated juices can stabilize rapidly against clarification from PE release. Table II shows that adding clarifying levels of PE (20 × serum level) to treated juices stored for 3 days did not affect cloud stability, but markedly reduced turbidity of untreated control juice.

Change in Pectic Substances. When samples of Pectinol 41-P stabilized juice, Pectozyme-treated juice, and untreated control juice were examined for pectic substances after 8 days at 4°, the distribution shown in Table III was obtained. Both pectinases were equally effective in reducing the concentration of insoluble pectins. Soluble pectin concentration in the Pectinol 41-P-treated juice was slightly less than the control concentrations, while the concentration in the Pectozyme-

Table II. Effect of Added Orange PE on Cloud Stability (g/l. Bentonite) of Juice Containing 200 ppm of Pectinases for 3 Days at 4°

$PE_U \times 10^4$	0.65	3.90	7.15	13.65
Control	1.59	1.35	1.03	0.60
Pectinol 41-P	1.35	1.35	1.33	1.36
Klerzyme 200	1.29	1.26	1.35	1.36
Pectinol 5-B	1.20	1.20	1.22	1.20
Pectinol 42-E	1.13	1.20	1.19	1.19
Pectinol 59-L	0.73	0.73	0.72	0.72
Pectinol RA-5	0.32	0.30	0.32	0.33
Pectozyme	0.10	0.10	0.10	0.10

Table III. Effect of Pectinol 41-P and Pectozyme on Pectic Substances of Whole Juice. Juice Was Held for 8 Days at 4° After Addition of Pectinases at 500 ppm

Pectic substances	Control, mg/l. AGA	Pectinol 41-P		Pectozyme	
		mg/l. AGA	% of control	mg/l. AGA	% of control
Insoluble pectins	556	141	25	124	22
Soluble pectins	118	103	87	131	111
Insoluble pectates	185	95	51	401	217
Total	859	339	39	656	76
Oligogalacturonates[a]		520	61	203	24

[a] By difference (Control total minus treated total).

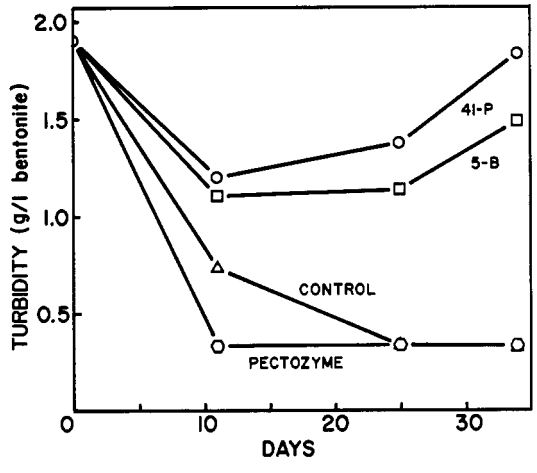

Figure 3. Effect of 200 ppm each of Pectinol 41-P, Pectinol 5-B, and Pectozyme on orange juice cloud stability at 4°

treated juice was slightly above control levels. Pectinol 41-P-treated juice had half as much and Pectozyme-treated juice had twice as much insoluble pectates as the control juice. Only 39% of the pectic substances in the control juice was recovered as such in the Pectinol 41-P-stabilized juice; the remaining 61% was degraded to oligogalacturonates. In contrast, only 24% of the pectic substances in the control juice was not recovered as such in the Pectozyme treated juice. Thus, the distribution of the pectic substances in the Pectinol 41-P and Pectozyme-treated juices suggests that pectinase stabilization involves degradation of insoluble pectins mainly to soluble pectates rather than to insoluble pectates as in unstable juice.

Enzymic Degradation of Pectin. Insoluble pectin can be degraded enzymically as shown schematically in Figure 4 (Demain and Phaff, 1957). Short-chain soluble pectin, low methoxyl pectin, and short-chain low methoxyl pectin constitute a series of soluble uronides, intermediate in chain length and degree of esterification between insoluble pectin and insoluble or soluble pectates. Three enzymes catalyze the degradation of pectin: PE, PG, and PMG. All three are

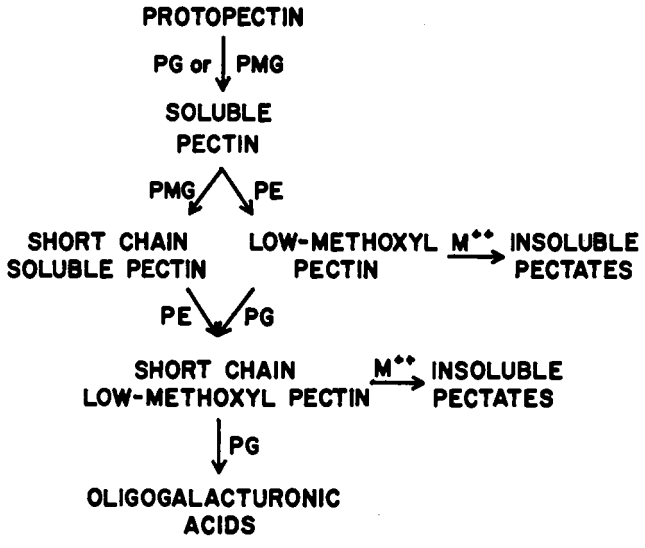

Figure 4. Possible pathways for enzymic breakdown of pectin by PE, PG, and PMG

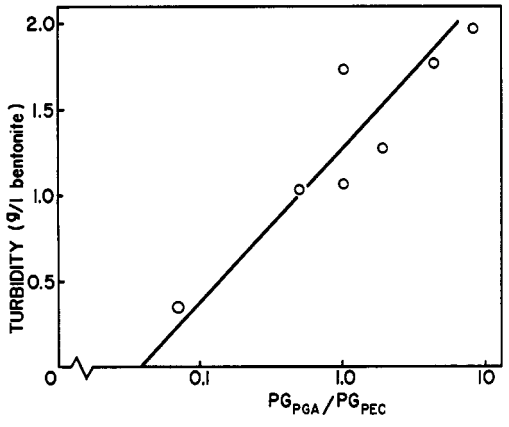

Figure 5. Regression of turbidity on ln of PG_{PGA}/PG_{PEC} ratio

involved in the formation of short-chain low methoxyl pectin; only PG controls its degradation. Pectin *trans*-eliminases (PTE) have not been included in this discussion. In a survey of eight commercial pectinases, Ishii and Yokotsuka (1971) found no or only a negligible amount of PTE activity.

The pectin distribution data in Table II can be interpreted by the enzyme reactions in Figure 4. Orange juice contains no detectable PG or PMG activity (Pilnik and Voragen, 1970), so that natural degradation in control juice occurs only through demethylation to low methoxyl pectin and precipitation as insoluble pectates. Stabilizing pectinases degrade pectin to oligogalacturonic acids which form soluble pectates. Nonstabilizing pectinases degrade pectin mainly to low methoxyl pectin stages from which insoluble pectates are formed. Thus, the data suggest that cloud-stabilizing effectiveness of pectinases depends on the relative ratio of formation and degradation of short chain low methoxyl pectin. Therefore, preparations with high PG activity and low PE and PMG activities would be the most effective stabilizers.

Pectin-Degrading Activities. Table IV ranks the pectinases in order of their effectiveness as cloud stabilizers with their PE, PG_{PGA}, and PG_{PEC} activities. Except for Pectinols 42-E and RA-5, PE activities of the pectinases are similar. The first four pectinases have the highest PG_{PGA} activities, but stability ranking does not correlate well with enzyme activity. PG_{PGA} is a measure of PG activity only, but PG_{PEC} is a measure of both PG and PMG activities because the native PE in

Table IV. PE, PG_{PGA}, and PG_{PEC} Activities of Commercial Pectinases

Pectinase	$PE_U \times 10^3$/g	PG_{PGA} U/g	PG_{PEC} U/g
Pectinol 41-P	29.7	1940	240
Klerzyme 200	31.2	100	23
Pectinol 5-B	14.9	79	80
Pectinol 42-E	304.	314	169
Pectinol 59-L	16.2	14	14
Pectinol RA-5	3.8	4	8.5
Pectozyme	25.3	12	179

juice would produce low methoxyl pectin substrate for PG. Thus, PG_{PEC} activity approximates the rate that the pectinase degrades orange juice-soluble pectin to short-chain low methoxyl pectin, while the rate of further degradation of short-chain low methoxyl pectin to oligogalacturonic acids in orange juice is approximated by PG_{PGA}.

Pectinol 41-P has much higher PG_{PGA} than PG_{PEC} activity, suggesting that pectin depolymerizing activity of this preparation s predominantly PG. Pectozyme, the clarifying pectinase, has much higher PG_{PEC} activity than PG_{PGA}, indicating that its pectin depolymerizing activity is primarily PMG.

Correlation of PG_{PGA}/PG_{PEC} with Stability. Pectinase activity data on pectin (PG_{PEC}) and on polygalacturonic acid (PG_{PCA}) provide a means of testing the interpretation of pectinase stabilization of cloud as an equilibrium between formation and degradation of short-chain low methoxyl pectin. Figure 5 shows the regression of turbidity on ln of the PG_{PGA}/PG_{PEC} ratio. The correlation coefficient for all seven preparations is 0.8549, indicating significance at the 99% level of probability. This significant correlation supports the pectin depolymerizing interpretation of pectinase stabilization of cloud. Pectinases with high PG and low PMG activities provide a means of degrading pectin to soluble products at rates surpassing those of formation of low-methoxyl pectin and insoluble pectates by pectinase-released PE. The pectinases are probably contaminated with other enzymes, some of which have been reported to destabilize juice cloud (Biggs and Pollard, 1970). Apparently, at the pectinase level of 200 ppm, these contaminants did not interfere with the PG/PMG cloud-stabilizing system.

In conclusion, the addition of certain pectinases to orange juice can substitute for heat in the stabilization of juice cloud. The most effective pectinases are those with high PG activity and low PMG activity. The effectiveness of a pectinase can be estimated from its ratio of depolymerase activities on polygalacturonic acid and pectin. Successful application of this method in the production of a cold pack juice would require a satisfactory cold sterilant and some means of preventing enzymic flavor deterioration as well. Modification of this method employing the minimal heat necessary to effect pasteurization has successfully produced juices with a dense, stable juice cloud (Baker and Bruemmer, 1971).

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Hydrolysis of Wood and Cellulose with Cellulytic Enzymes

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A procedure is described for determining the digestibility of cellulose, wood, modified cellulose, and modified wood with cellulytic enzymes. It is useful as a screening test to evaluate the effects of various physical and chemical treatments to improve the accessibility of cellulose carbohydrates to ruminants. Although not economical at the present time, fine grinding was the most effective treatment for increasing the digestibility of wood and cellulose. Treatment of aspen (*Populus tremuloides* Michx.) from pulpwood logs that contained mostly sapwood with dilute alkali increased the digestibility from 10% for the untreated wood to 50% for the treated wood. Treatment with liquid ammonia increased the digestibility to 36%. Reasons for the differences in digestibility found are discussed.

The carbohydrate content of wood varies between 70 and 80%. Considering the large quantities of wood available from low-grade forests, residues from logging operations, sawdust, shavings, and mill wastes, it is very tempting to develop means for treating wood so as to make the carbohydrate accessible to ruminants (Scott *et al.*, 1969). For such work, a screening test was needed to evaluate the effects of various treatments on the digestibility of wood. Such a procedure is described and is applied to substrates that have been treated by various physical and chemical means. It is based on measuring the hydrolysis of the polysaccharides by a cellulolytic enzyme.

MATERIALS AND METHODS

Preliminary work with several enzymes showed "Onazuka" SS (Kanematsu New York, Inc., New York, N. Y. 10004; 1500 μ/g of activity based on manufacturer's values for decomposition activity on filter paper) to be the most active of the commercially available cellulases tried. Therefore, it was selected for further work. This enzyme is derived from *Trichoderma viride* and contains appreciable quantities of hemicellulases. The powdered enzyme contains lactose, galactose, glucose, and arabinose amounting to 50% reducing sugar calculated as glucose. The principal sugar is lactose.

The enzyme solution was buffered at pH 4.6 with acetate buffer. It was prepared as follows.

Acetate Buffer. Dissolve 68 g of $NaC_2H_3O_2$–$3H_2O$ in about 500 ml of H_2O; add 29 ml of glacial acetic acid and dilute to 1 l.

Enzyme. Stir 25 g of powdered enzyme into a mixture of 900 ml of H_2O and 100 ml of acetate buffer for 1 hr. Filter off insoluble material.

The enzyme solution was stored in a refrigerator at 40°F. The solution was reasonably stable; a solution stored for 5 weeks had the same activity as a freshly prepared solution.

Substrates. The following substrates were used: a high-purity cotton linters pulp from Buckeye Cellulose Corp., equivalent to ICCA Pulp No. 1 of the International Committee for Cellulose Analysis; aspen (*Populus tremuloides*); a high α rayon grade softwood sulfite pulp designated ICCA Pulp No. 4; alfalfa (a reference feeding sample for ruminants, obtained from University of Wisconsin); cotton linters and aspen ground in a vibratory ball mill for 30 min (National Bureau of Standards, 1950; Pew and Weyna, 1962); cotton linters and aspen treated with liquid ammonia for 1 hr at 30° at approximately 150 psi (the samples were then air dried); cotton linters and aspen treated with dilute alkali for 1 hr at 30°. Five-gram samples were ground to pass a 40-mesh sieve and treated with 100 ml of 1% NaOH. The treated wood was then thoroughly washed and air dried.

All samples except those ground in the vibratory mill were ground in a Wiley mill to pass a No. 40 U.S. standard sieve. Since it was reported that high concentrations of sugars inhibit cellulolytic enzymes (Katz and Reese, 1968), 0.2-g samples were used in 6 ml of enzyme solution for the digestion.

An incubator was constructed from a forced draft oven whose temperature could be controlled within ±0.2° from 30 to 50°. Rotating racks were provided to hold vials of 8-ml capacity (17 mm diameter by 60 mm long) equipped with Teflon-lined screw caps. To provide agitation and to prevent packing or sticking of the ground sample during the digestion, the sample tubes were rotated end-over-end at 7 rpm.

The effectiveness of the enzyme system was evaluated by determining the loss in weight after digestion and by measuring the total sugars, as glucose, produced in the liquor.

The FPL method (American Society for Testing and Materials, 1970; Saeman *et al.*, 1954) for chromatographic analysis was used where the papers are irrigated with a butanol-

Forest Products Laboratory, Forest Service, U. S. Department of Agriculture, Madison, Wisconsin 53705.