ABSTRACT

KINETICS OF THERMAL INACTIVATION OF POLYGALACTURONASE IN SPENT CHERRY BRINES

By

Panagiotis Evagelos Athanasopoulos

Sweet cherries, used for manufacturing into maraschino, cocktail, or glacé fruit, are stored in calcium bisulfite brine. Spent cherry brine is a strong pollutant and creates serious disposal problems. If the spent brine could be reclaimed and reused, the disposal problem would be reduced in magnitude. The polygalacturonase enzyme may be present in reclaimed brine and results in enzymatic softening of the new cherry lot. The inactivation of the enzyme by heat would provide the required safety to prevent losses of cherries due to enzymatic softening.

Commercial sweet cherry brines were used to investigate the heat resistance of purified polygalacturonase which was diluted to a concentration of 1 mg enzyme per ml of brine. One ml of the enzyme dilution was heated in test tubes using a two constant-temperature bath technique followed by cooling in an ice-water bath. The plate and cup assay was used to estimate the enzyme activity during

inactivation determinations. Least-squares statistical analysis was used to evaluate experimental results and determine appropriate constants.

The inactivation of the polygalacturonase enzyme in cherry brines was described by first-order functions. The rate constant (k) at 70 °C was 4.93×10^{-2} sec^{-1} and the decimal reduction time (D) at the same temperature was found to be 46.8 sec. The Z value for thermal resistance was calculated from the thermal resistance curve and found to be 8.4 °C. The activation energy was computed from the Arrhenius plot and had a value of 64.7 Kcal mol^{-1}. The effect of pH on the enzyme inactivation rate was studied and the results indicate that the polygalacturonase enzyme in cherry brines exhibits higher resistance in the pH 2.8 - 3.5 range. Sugar concentrations higher than 12 percent found to have a significant protecting effect on the enzyme. Enzyme dilution in brine with higher SO_2 and higher pigment content had higher resistance to inactivation by heat. Partially inactivated enzyme in brine was tested for regeneration capability at room temperature and under refrigeration conditions. Results indicated that the inactivation of that enzyme is irreversible.

KINETICS OF THERMAL INACTIVATION OF POLYGALACTURONASE IN SPENT CHERRY BRINES

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Panagiotis Evagelos Athanasopoulos

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To my dear wife

Gina

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

In the United States, the cherry brining industry has increased since 1930 (Steele and Yang, 1960). Between 40 and 45 percent of the total sweet cherry production is brined. In Michigan, a 75 percent of the production is brine processed (Woodruff, 1975). In the manufacture of maraschino and glacé cherries, the fruit is held in calcium bisulfite brine. First, cherries are bleached to a bright light straw color before processing.

Spent brine is normally discharged to municipal sewage system or to local streams. Waste water from cherry brining industry is characterized by high sulfur dioxide content, high chemical oxygen demand, low pH, and color. Reclamation of spent brine for re-use has been proposed of reducing the pollution problems created from the disposed brine. The problems in recycling of spent brine are: (a) The brine has reduced bleaching power which will usually prevent the production of a bright yellow color in the cherries during brine reuse and (b) the polygalacturonase enzyme may be present in the spent brine and result in enzymatic softening of the fruit.

A process for used brine reclamation has been developed at Oregon State University, (Soderquist, 1971), which consists of the following units: (a) sand filters for suspended solids removal, (b) activated carbon contactors for anthocyanin and polyphenol adsorption, (c) pasteurization unit for polygalacturonase enzyme inactivation, and (d) chemical reactor for adjustment of SO_2 concentration and pH.

The kinetics of thermal inactivation of polygalacturonase in spent cherry brine has not been investigated and data required for design of the pasteurization unit of the reclamation system are not available in current literature. This study has been conducted to evaluate the appropriate design parameters.

The specific objectives of this research are:

1. To study the inactivation rates of the polygalacturonase enzyme as a function of (a) temperature, (b) pH, (c) dissolved sugars, and (d) cherry variety.
2. To investigate the regeneration capability of the partially inactivated enzyme in brine under different conditions.
3. To conduct mathematical analysis of experimental data and evaluate the appropriate design parameters.

LITERATURE REVIEW

A. Structure of the Industry

The chief cherry varieties for brining in Michigan are Napoleon, Windsor, Schmidt, and Emperor Francis. Cherries are hand picked or mechanically harvested. Very little damage occurs during mechanical harvest depending on the variety and maturity (Levin et al., 1969). As maturity advances, the machine harvested fruit decreases in quality much more rapidly than hand picked fruit (Thienes et al., 1969). A considerable percentage of mechanically harvested cherries has attached stems which could create problems if briners wished to have fruits free of stems. Thienes (1969), reported that a reduction in cherry grade may occur by delaying the brining for even two hours after harvesting.

To minimize the adverse effect of bruising on quality, cherries are brined in a matter of seconds after harvesting by placing the cherries in barrels which are half-full of brine. These barrels are transported to the briner plant where they are sold. Since cherry weight is difficult to be obtained after cherries have been placed in brine, they are sold by volume (Whitenberger, 1969). Inside the processing plant, the cherries are kept in large tanks,

in brine, for at least 5-6 weeks for their color to be removed.

Two general types of brine are commercially used. Type one uses the method of gas sulfur dioxide introduced into a suspension of calcium carbonate or calcium hydroxide. There is no effect on texture due to the use of different calcium salts, (Vibbert, 1976). In type two method, the bleaching agent is sodium bisulfite ($NaHSO_3$). Sodium bisulfite and calcium chloride are dissolved in water and pH is adjusted with commercial hydrochloric acid. Brined cherries are shipped to finisher plant in plastic lined tote bins and tank trucks. Years ago, and sometimes today, cherries were transported in brine. Currently, considerable quantities of brined cherries are shipped in water with citric acid added as a preservative.

Cherries with high pigment content are not completely bleached by sulfur dioxide brines. In addition, dark spots (sunburn, bruising) are partially bleached. Cherries with these dark blemishes are unacceptable for finishing because the final product has lower market value. A secondary bleaching is necessary to remove these discolorations. A number of bleaching agents have been tested with different degrees of success. Sodium chlorite ($NaClO_2$) has been reported (Beavers, 1968, and Payne, 1969) as a successful agent leading to snow white product of firm texture and free of off-flavors. Brined cherries are leached in cold water to remove SO_2, and they are bleached

in an acidified solution of sodium chlorite. After bleaching, sodium chloride is removed (leached in water) and they are returned to calcium bisulfite brine.

The next step is steaming and pitting. Accordingly, prepared cherries are soaked in running water to remove most of the sulfur dioxide. Then, they are boiled in water which is changed several times until the desired tenderness is obtained and the sulfur dioxide content is below 100 ppm (for maraschino cherries) or below 20 ppm (for fruit salad).

Bleached cherries are ready for dyeing. They are covered with the 30 percent sugar syrup containing dye. Twenty-four hours later, the syrup concentration is increased (by evaporating water in a vacuum pan) gradually, to a final strength of 72-74 percent sugar. When the dye has penetrated sufficiently, the syrup is acidified to pH 3.6-3.9 using citric acid for color fixation. Dyed cherries are drained out of the syrup and packed.

The general steps of the brining cherries industry are illustrated in the following flow sheet, shown in Fig. 1.

B. Brine Reclamation

The sweet cherry industry is a source of pollution. Solid wastes are produced during pitting and stemming processes, while waste water is the most important pollutant of this industry (Beavers et al., 1970). Sources of waste

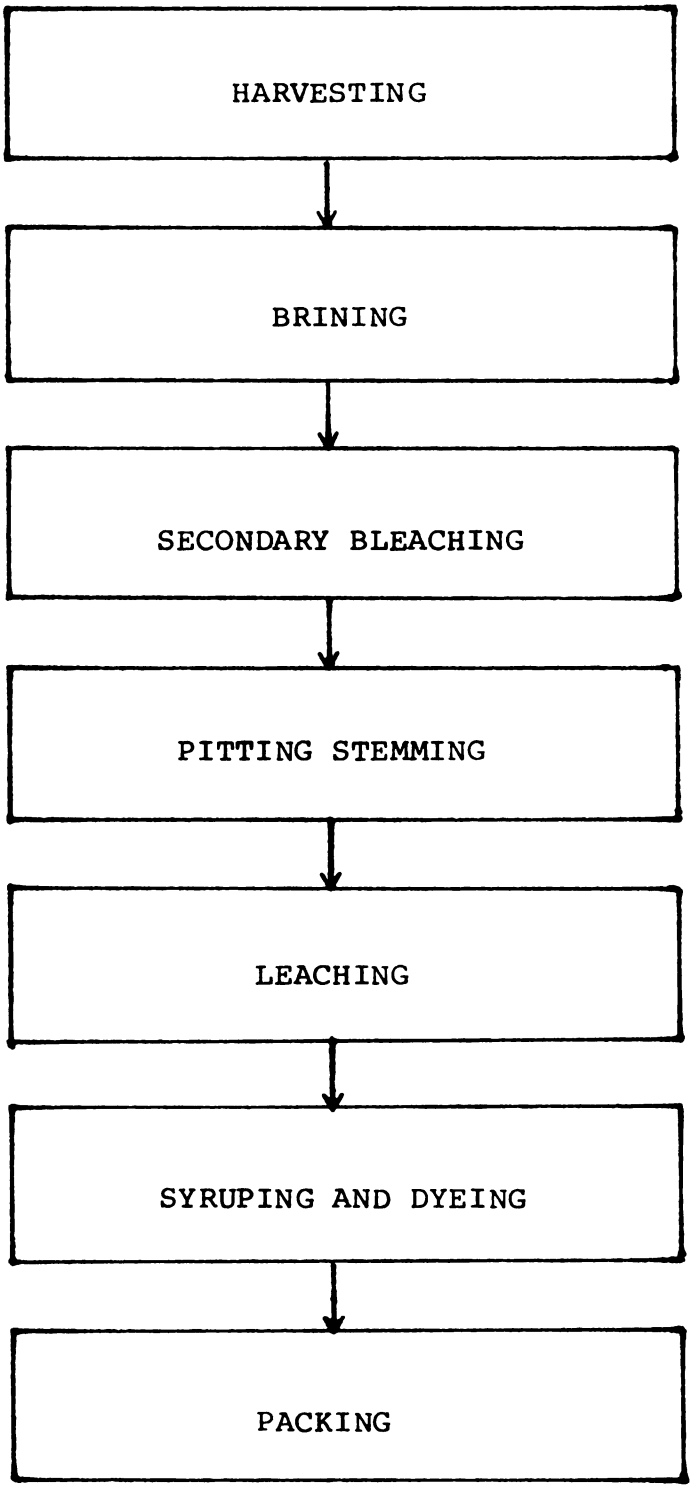

Fig. 1. Major steps in maraschino cherry industry.

water are: brining, secondary bleaching, leaching, and syruping and dyeing.

The most important pollutants of this industry (Soderquist, 1971) are soluble solids, which consist principally of dextrose, acids, anthocyanins, polyphenols, free sulfur dioxide, SO_2, combined SO_2, sulfate (SO_4) and calcium (Ca^{++}). In addition, spent brine is characterized by low pH; effluent from dyeing consists principally of sucrose, color, and high temperature.

Spent brine makes up the largest volume of the effluent and it is characterized by high strength. Payne et al. (1969) reported that spent undiluted brine may contain 4,000 mg/l free SO_2, 1,000 mg/l sulfite, 4,000 mg/l calcium, and a pH of 3.5. Chemical oxygen demand (COD) of 150,000 mg/l is common. Soderquist (1971) reported that spent cherry brine is approximately 500 times stronger than domestic wastewater.

The high sulfur content is directly related to very high chemical oxygen demand (COD). The dissolved oxygen in the water is depleted by microbia growth, stimulated by the high levels of organics present, and it creates anaerobic conditions. This anaerobiosis is conducive to the growth of organisms, such as Desulfovibrio sp. (McKinney, 1962). These microflora generate hydrogen sulfide and other malodorous reduced sulfur compounds resulting in serious odor problems.

Chemical and physical methods for brine treatment have been described by Sapers (1975). Application of hydrogen peroxide for odor control of sweet cherry brining waste is under investigation (Gerrish, 1975). The wastewaters from the brined cherry pitting process were found to be highly treatable in an aerobic biological treatment process (Mauldin et al., 1975).

By recycling the commercially wasted spent brine, the volume of the waste water released from a cherry process is significantly reduced. The spent brine contains dissolved pigment and has a little bleaching capacity (Beavers et al., 1970). Pigment must be removed before re-use so that the brine is adequate to bleach successfully a new load of cherries. Experiments have shown that dissolved anthocyanins and polyphenols, present in spent brine, can be removed by treatment with active carbon (Beavers et al., 1970, Soderquist, 1971, Sapers, 1975). A reclamation system for spent brine should include a sand filter to remove suspended solids, which may escape from screens, a series of active carbon contactors to adsorb most of the pigments present in the brine, and an adjusting unit for full strength brine preparation (Soderquist, 1971).

Reclaiming spent brine may not be an economical process for finishers located at a distance from cherry brining areas. Finishers brine may be used as a syrup basis, after removing Ca^{--}, SO_2, and concentrating it 1.78 fold by evaporation. The level of pesticides in brine have

to be taken under consideration, as well, for using spent brine for syrup production (Sapers, 1975).

One important problem in re-using reconditioned brine is the enzyme polygalacturonase (PG). If any portion of the brine is contaminated with the enzyme, the entire bulk stock of brine could become contaminated during the reclamation process (Beavers et al., 1970). Re-using brine contaminated with polygalacturonase could lead to enzymatic hydrolysis of the pectic substances of the cherries resulting in a soft unacceptable product. A safe inactivation method of the enzyme is pasteurization (Watter, 1961; Soderquist, 1971; Sapers, 1975).

Brine reclamation and re-use would reduce sewerage charges and lower costs of chemicals for new brine production. Economic analysis by Soderquist (1971) has shown that the net annual savings is a function of the plant size. A processor handling 1,000 tons of Black Republican cherries per year would have negative results ($574 per year or $0.06 per ton). A larger plant handling 5,000 tons per year would have a profit of $10,109 per year or $2.02 per ton, while production on a level of 10,000 tons per year would result in a net annual cost savings of $27,300 per year or $3.06 per ton. The above results have been derived under economic conditions of the year 1970. By incorporating a pasteurization unit in the reclamation system, the cost will be somewhat higher.

Preliminary work for syrup preparation from used cherry brine, which contains about 8 percent sugars, showed that 100 gal. brine produces 56 gal. of 15-17 percent soluble solids syrup base. By adding 3.8-4.0 lb of sugar per gal. of concentrate, about 80 gal. of 40 Brix syrup is produced. The cost for that process has been estimated to be $11.32 per 100 gal. of brine (Sapers, 1975).

C. Pectic Substances and Pectic Enzymes

The pectic substances, which are carbohydrate derivatives, are widely distributed in plant tissues where they fill the intercellular spaces (middle lamella). Pectins are composed mostly of hydrogalacturonic acid residues. Other carbohydrates (Joslyn and Denel, 1963), such as arabinose, galactose, sorbose, rhamnose, are attached to the chains of unhydrogalacturonic acid units. The carboxyl groups in pectic polygalacturonides are either free or partially esterified with methyl alcohol or may form salts with various cations.

The characteristics and composition of pectic substances depend on many factors (Kertesz, 1951). These factors include the origin of the sample, the methods used in the preparation of the tissue, the methods of extraction and separation, the type and extent of purification, and the analytical procedures used in assay and characterization.

Highly esterified pectins in 65 percent sugar solutions yield firm gels. The term PECTIN comes from that

property. It has been derived from the Greek "πηκτός" meaning to congeal or solidify. Partial hydrolysis of the methyl esters leads to low methoxyl pectins, which form gels with calcium ions at lower sugar concentrations. Complete hydrolysis of the methyl esters leads to the pectic acids in which there is one free carboxyl group per unit of galacturonic acid. The jelly grade of pectin is significantly affected by the molecular weight. Pectins with high molecular weight, such as 180,000 yield firm gels with a large amount of sugar (1g pectin with 220-300 g sugar). On the other hand, pectins with molecular weight 30,000 fail to form jelly.

Several workers have estimated the amount of pectic substances in cherries. Kertesz (1951) summarizes: (a) for an unripe sample of cherries, 11.4 percent pectic substances on a dry matter basis; (b) for a ripe sample 4.3 percent; (c) for stone-free cherries, 0.35 percent as pectic acid on a fresh weight basis; (d) for Morella cherries, 0.16 percent as calcium pectate; (e) for red cherries 0.28 percent; and (f) for white cherries 0.31 percent.

Montmorency cherries have been studied (Al-Delaimy, 1963) under different storage conditions and varying degrees of maturity. The total pectins found to be in the range 0.304 to 0.466 percent on a fresh fruit weight basis are expressed as unhydrouronic acid.

The chemistry of pectic substances has been extensively reviewed by Kertesz (1951), Dewel and Stutz (1958),

and Joslyn and Dewel (1963). The enzymes which catalyze the degradation of pectic substances are called "pectic enzymes." Enzymes, catalyzing the formation of pectin from its water insoluble precursor protopectin, are called protopectinases, but they have never been isolated (Reed, 1966).

At the present time, it is almost impossible to obtain a clear picture of the relationship between the different kinds of pectic enzymes which have been isolated from various micro-organisms. Kertesz (1951) discusses two enzymes pectin-methylesterase, and pectin-polygalacturonase. Reed (1966) classifies the pectic enzymes into two groups. The enzymes which split the ester linkage between methyl esters of the galacturonic acid molecules, called methylesterase (ME), and that which split the glycosidic linkage between adjoining galacturonic acid molecules, called polygalacturonases (PG).

The group of polygalacturonases includes enzymes able to act within the polygalacturonic acid molecules on α-1,4- linkages (endopolygalacturonases) and enzymes catalyzing the step-wise splitting off of galacturonic acid residues from the non-reducing end of the chain (Exopolygalacturonases).

Some enzymes act principally on methylated substances (pectins) and are called polymethylgalacturonases (endo- and exo-) while others act on substrates containing free carboxylic acid groups (pectic acids) and are called

polygalacturonases (endo- and exo-). The latter completes the degradation of the pectins on which methylesterases have acted.

The complexity of classification is greater than described in the above paragraph. It has been recognized that a nonhydrolytic type of cleavage of the α-1,4 linkage occurs, called trans-elimination (Reed, 1966). The reaction is enzymatically catalyzed, and the enzymes have been called transeliminases. These enzymes are not included in the general classification proposed by Demain and Phaff (1957) to help clear up the complexity problem. The most recent classification and nomenclature is that recommended by the International Union of Biochemistry (1973).

The activity of an enzyme can be measured by determining the substrate loss, by estimating some new compounds or groups formed by the enzyme action, or by measuring the changes in physical properties (viscosity). All of these methods have been used for polygalacturonase determination. The techniques and the limitations of each procedure are well described by Kertesz (1951). The interferometric method has been proposed by Rukhlyadeva and Korchagina (1975) for commercial pectic enzyme preparations. It is a rapid (less than two hours) and accurate method ($\pm$2%). Interferometer readings (m) are obtained using several dosages of a given preparation and the enzyme activity A is calculated in arbitrary units from the equation: $A = (.6425xm + 19.62) \div (n \times 1000)$.

Recently, the solid media technique has been introduced in the quantitative determination of enzyme activity. This technique, developed for microorganism cultivation, has been used for detection of enzyme production by microorganisms (Hankin and Anagnostakis, 1975). The agar "cup-plate" diffusion technique has been applied to the quantitative determination of polygalacturonase (Dingle et al., 1953). This test has been used by Steele and Yang (1960) and by Beavers et al. (1970) for polygalacturonase activity determination in cherry brines.

D. Polygalacturonase in Brined Cherries

The polygalacturonases have been detected in higher plants such as tomatoes (Patel et al., 1960), avocados and pears (McCready et al., 1954). This enzyme is produced by a number of microorganisms including bacteria yeast and mold, such as Saccharomyces fragilis (Patel et al., 1959; Phaff et al., 1956), Aspergillus niger (Mill et al., 1961; Tuttobello and Mill, 1961), Aspergillus sp. (McClendon et al., 1963).

Softening of brined cherries was reported for the first time in 1954 (McCready et al., 1954). The damage to cherries was slight, and an in-depth investigation was not initiated until 1957 when extensive softening damage occurred in Oregon and Washington. The cherry softening which occurred seemed to be identical to that reported in 1954. The problem was investigated and it was established

that the enzyme polygalacturonase was responsible for the softening (Steele et al., 1960).

The enzyme polygalacturonase has not been detected in cherries. Investigations on Montmorency cherries (Al-Delaimy et al., 1966) indicated the enzyme was not present. Severan varieties of fresh cherries tested by Steele (1960) were free of polygalacturonase. Probably, the sources of the enzyme are microorganisms which gain access in cherries after damage during the ripening period, or it enters the brines with stems and plant particles.

The activity of the enzyme in calcium bisulfite brines does not follow a definite pattern. It has been reported that the enzyme remained active in the brine for a period of only 13 to 25 days (Yang et al., 1960). On the other hand, commercial samples of brined cherries showed enzyme activity three months after brining, but it stopped after six months in brine (Beavers et al., 1970). Brekke et al. (1966) found active polygalacturonase in brine from softened commercially brined cherries for over one year. Cherries brined the second year in this brine produced soft fruit.

Current results indicate that the degree of activity and the period of activity of polygalacturonase in cherry brine is not predictable. Any investigation on reclamation of brine must include tests for the detection of polygalacturonase which should be conducted on each lot of brine before it is released for reuse. No extensive study

of cherry polygalacturonase has been conducted, and the inactivation of the enzyme in different brines has remained a problem. Methods suggested for inhibition of polygalacturonase in cherry brines are: (a) Using alkyl aryl sulfate (Steele et al., 1960), (b) adjusting pH in the range from 1.0 to 1.6 (Yang et al., 1960), and (c) increasing the concentration of calcium chloride in the brine (Brekke et al., 1966). None of the mentioned methods found commercial application, however, and the inactivation by heat has been proposed as the only approach (Soderquist, 1971; Watters et al., 1961; Sapers, 1975).

E. Enzyme Kinetics

The magnitude of enzymatic activity is usually proportional to the concentration of the enzyme. This proportionality is exhibited at least during the earliest stages of the reaction. The linear relationship between enzyme concentration and rate of reactionn has been detected in hydrolysis of milk fat and olive oil by purified milk lipase (Chandan and Shahani, 1963).

In the earliest stages of an enzymatic reaction and for very short time intervals, the substrate concentration can be assumed constant. This is particularly true in the case when the substrate is present in excess, and the amount of product formed is proportional to time,

$$dP / dt = Ko \qquad (1)$$

where dP/dt is the rate of product formation, and Ko is a constant. In other words the enzymatic reaction follows zero-order kinetics. Beyond the initial phase, the reaction slows down with time. Most enzymatic reactions follow the kinetics of a first-order reaction described by the equation,

$$dP / dt = K (S-P) \qquad (2)$$

where (S-P) is the concentration of substrate remaining at any given time, t, and K is the first order reaction constant.

The enzymes are proteins in nature, and they have highly specialized biological activity. Most protein molecules retain their biological capacity to function only in a very narrow range of temperature and pH. Exposure of proteins to extremes of temperature or pH results in denaturation of the molecule. When the enzymes are heated, they lose their ability to catalyze a chemical reaction. The polypeptide backbone of the protein molecule is not broken, but the polypeptide chain is unfoled and the protein molecule loses its folded structure (Lehninger, 1970).

As the temperature is increased, two simultaneous reactions occur (Messing, 1975): (a) The enzyme activity increases, as observed in most chemical reactions, and (b) The enzyme stability decreases due to thermal denaturation. The net result of these two reverse effects is a bell-shaped curve.

Inactivation of enzymes by heat is a very common procedure in the food processing industry. Vegetables and some fruits are heat treated before canning or freezing. Cloudy juices, such as orange and tomato juice retain their cloudiness by inactivating the pectic enzymes by heat.

Lipoxidase, inactivated by heat and by ionizing energy, exhibit first order kinetics (Farkas and Golblith, 1962). Kinematic procedures employing D values were used by Yamamoto et al. (1962) for kinetic studies on heat inactivation of peroxidase in sweet corn. A first order reaction was observed for the initial 95 to 98% reduction of the enzyme activity.

The inactivation of an enzyme can be described by first order kinetics,

$$\frac{dC}{dt} = - kC \tag{3}$$

where dC/dt is change in activity with time, k is the reaction constant and C the activity at any time. After intergration, equation (3),

$$\int_{Co}^{C} \frac{dC}{C} = -\int_{0}^{t} kdt \tag{4}$$

the following equation is obtained,

$$ln \frac{C}{Co} = - kt \tag{5}$$

where Co is the initial enzyme activity and t is the heating time. The natural log can be changed to log with base 10 and the equation (5) can be written,

$$\log \frac{C}{Co} = \frac{-kt}{2.303} \quad \text{or} \quad \log C = \log Co - \frac{kt}{2.303} \qquad (6)$$

Equation (6) describes a straight line on log C versus t coordinates. The intercept is log Co and the slope is k/2.303. Measuring the enzyme activity C, at different times t, both the initial activity Co, and the rate constant k, can be computed. The constant k is highly pH depended. Plot of the log k versus pH resulted in a "boat shaped" curve (Farkas and Golblith, 1962), with high k at pHs 4 and 9 and low k at pHs 5 and 7.

Yamamoto et al. (1962) employed techniques used in thermobacteriology to study kinetics of enzyme inactivation by heat. Decimal reduction time, D, is the time required to reduce enzyme activity by 90 percent at a given temperature T. This time can be computed from equation (6) for C = 10 and Co = 100 (reduction of activity by 90 percent) as following:

$$\log \frac{C}{Co} = \log \frac{10}{100} = - \frac{k}{2.303} t' \quad \text{or}$$

$$1 = \frac{k}{2.303} t' \quad \text{and} \quad t' = D = \frac{2.303}{k} \qquad (7)$$

where t' is the time required for reduction of activity by 90 percent. Substituting k in equation (6) with 2.303 / D, from (7),

$$\log \frac{C}{Co} = -\frac{t}{D} \quad \text{and} \quad \frac{C}{Co} = 10^{-t/D} \tag{8}$$

By plotting log D values against temperature, a straight line is obtained. This is the "thermal resistance curve." The temperature required to reduce D value by 90 percent is called "factor Z" which is characteristic for each enzyme. The equation of the thermal resistance curve is,

$$D_{T_1} = D_{T_2} \; 10^{\frac{T_2 - T_1}{Z}} \tag{9}$$

If the Z factor is known and the D value is known at certain temperature T_1, the D value can be computed at any given temperature T_2.

As heating time t increases, the enzyme activity C decreases. This activity can be very low, but it never is zero (eq. 5). The time required to reduce the enzyme activity to a certain desired degree at a given temperature is called "thermal inactivation time." In establishing the thermal death time for a certain enzyme, the following should be taken into consideration:

(a) The curve log C versus t sometimes is not a straight line, but a broken one. If the degree of inactivation beyond the break point is desired, two "D" values should be evaluated, one for each portion of the broken curve.

(b) The regeneration phenomenon has been observed in some enzymes. Peroxidase was regenerated in samples of peas that had been blanched just sufficiently to destroy the original peroxidase activity (Pinsent, 1962). The

regeneration occurred after a few months in peas stored at -18 °C, or within a few hours in thawed peas held at room temperature. The maximum regeneration observed was 4 percent.

F. Heating and Cooling Methods

A number of methods have been developed for bacteria spores destruction studies. Stumbo (1965) describes the most common methods. Techniques, employed for bacteria spores studies, may be used for studying other biological products in their original form or after proper modification. A thermoresistometer, for example, designed for bacteria spores study has been properly modified for studying the destruction of vitamins by heat (Mulley et al., 1975). Techniques for heating can be devided into two categories. These employing temperatures higher than the boiling point of the water (oil baths, steam heating) and those using a constant temperature water bath, which are more common in enzymes studies.

The constant temperature water bath technique can be described as follows: A small amount of liquid (.5-2 ml), in which the substance to be studied has been dissolved, is put in small diameter glass tubes. A number of test tubes are placed into the constant temperature bath, and samples are removed at scheduled time intervals, and then cooled in ice-water.

The constant temperature bath gives good results when the heat resistance of the substance under study is

relatively high. In such cases, the heating time is relatively long allowing the liquid inside the tube to reach the bath temperature. Agitation is sometimes employed to assure as much uniform heating as possible. When short times have to be employed for heat treatment, two problems arise. First, the temperature of the liquid increases exponentially and, as the heating time is short, it never reaches the bath temperature. Samples removed from the bath at different times have been heated to different temperatures which are different from that of the constant temperature bath. In addition, the pH may change with temperature and this change might have an effect on the heat resistance of the substance under study. This is usually corrected by using buffer solutions.

METHODS AND PROCEDURES

A. Heating and Cooling of the Samples

To determine the inactivation effect of heat on polygalacturonase in cherry brine some alternative heating methods required evaluation. To select a method for heat treatment of the samples some preliminary tests were run. It was established that time less than 60 seconds was required to inactivate the enzyme by more than 90 percent at 70 °C. Since the temperatures required for studying the kinetics of inactivation of the enzyme polygalacturonase in cherry brines were well below the boiling point of water, the constant temperature bath technique was selected. To overcome the limitations of the water bath, described previously, the method was modified and two constant temperature water baths were employed, one having a higher temperature than the other. The higher temperature bath will be called "heating bath" while the other, having a lower temperature, will be referred to as "holding bath."

The temperature history of the liquid in the test tube was studied in preliminary heating and cooling tests. Test tubes, 8 mm in diameter and 20 mm in length, were used. A thermocouple was placed in a tube, containing

1 ml brine, at a distance of about 1/3 of the brine height from the bottom (slowest heating point). The sample was heated in the heating bath at different temperatures. After conducting an appropriate number of tests, the heating curves were constructed (Fig. 2). Using these curves, the time required for the heated sample to reach a desirable temperature could be estimated as follows:

a. The heating and holding bath temperatures are measured.
b. The temperature difference between the two baths is calculated.
c. The temperature difference line is drawn (dotted lines in Fig. 2).
d. The intercept of dotted and solid line is projected on the time axis, and the heating time is directly read.

Example for heating time estimation. Measured temperatures: heating bath 75 °C, holding bath 69 °C. The temperature difference is T = 75 - 69 = 6 °C. The intercept of 6 °C (dotted line) and 75 °C (solid line) is projected on time axis (Fig. 2) and the heating time is estimated 34.5 seconds.

The sample in the test tubes was heated in the heating bath for a predetermined time, and then it was transferred from the heating bath to the holding bath. A thermocouple temperature recorder was used to check whether the estimated heating time was the correct one. As was

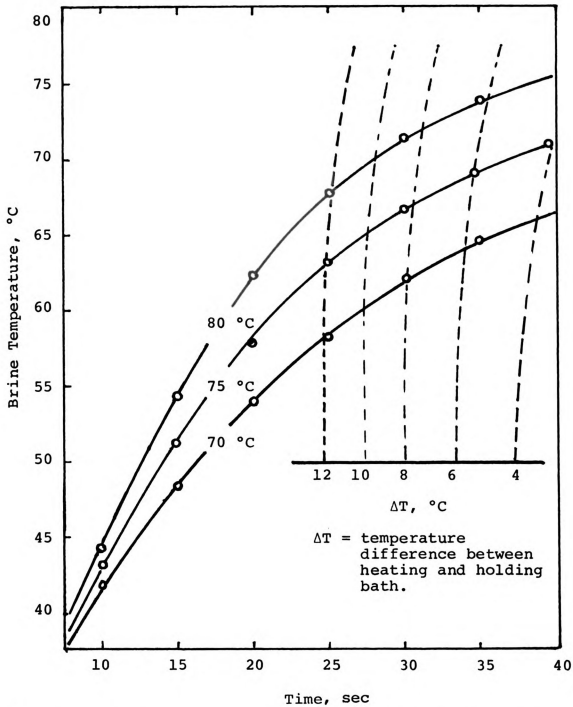

Fig. 2. Temperature history of 1 ml brine in test tube, at the slowest heating point. Curves show heating from room temperature, 24 °C (70, 75, and 80 °C are heating bath temperatures).

indicated previously, the temperature was measured at the slowest heating point. Tubes were removed at 10 or 15 seconds intervals and placed in an ice-water bath for cooling. Since the heating and cooling were not instantaneous, both contributed to the enzyme lethality.

A number of tests were run to determine the cooling rates of the sample. To study the temperature history during the cooling process, the thermocouples were placed near the top of the liquid at the location of the slowest cooling point. Fig. 3 illustrates the shape of the cooling curves from different temperatures to 0 °C (32 °F).

B. Enzyme Source and Concentration

Purified commercial polygalacturonase (Pectinase),[1] from Aspergillus niger, was used in this investigation. Concentration of 1 mg/ml of brine was selected. Concentration of 1 mg/ml of polygalacturonase may soften the cherries extensively within 12 days (Steele and Yang, 1960). Beavers et al. (1970) reported failure of pure polygalacturonase (1 mg/ml) to completely break down the texture of the brined cherries. The failure was not due to the enzyme concentration but to the absence of pectinesterases which promote the polygalacturonase activity. Pectinesterases have been detected in cherries (Al-Delaimy, 1963). The proper amount of the purified enzyme was dissolved in

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[1]Pectinase is the trade name for polygalacturonase supplied by SIGMA Chemical Company, St. Louis, MO.

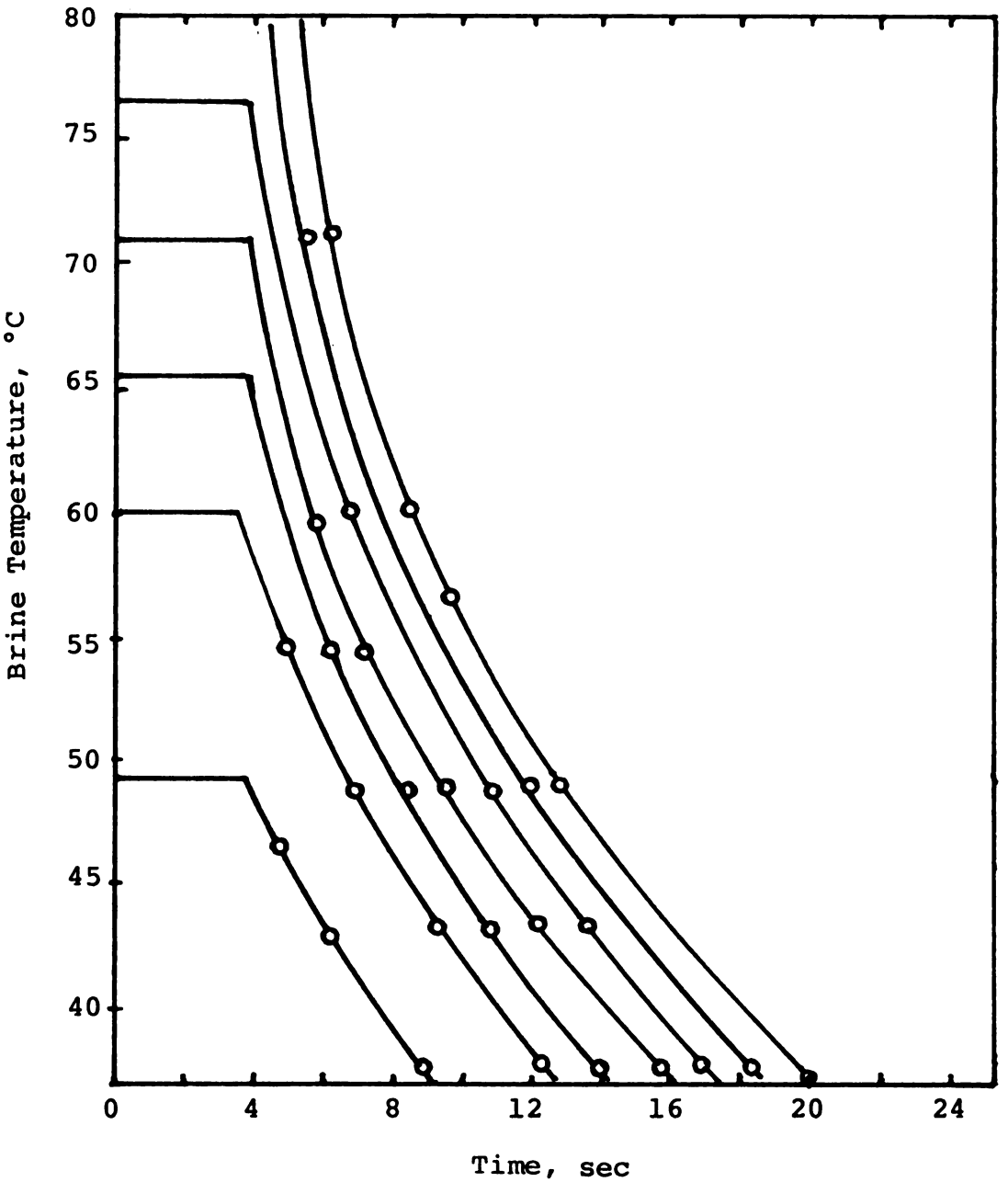

Fig. 3. Cooling curves for 1 ml of brine in test tubes from various temperatures to 0 °C.

commercial brine so as to obtain an enzyme concentration of 1 mg enzyme per ml of brine. One ml of that solution was placed into each test tube, and heat treated as previously described.

C. Measurement of Polygalacturonase Activity

The "cup-plate" technique developed by Dingle et al. (1953) was used. The exact assay for the enzyme activity is described in the Appendix B. Dingle et al. (1953) studied the effect of pH of the gel on the diameter of zones produced by polygalacturonase. They suggested that the pH be adjusted to 4 $\pm$ 0.1 by using 0.1 N sodium hydroxide. The substrate used in this experiment was found to have the appropriate pH, and no adjustment was necessary.

The ingredients necessary for the gel formation were blended and the mixture was transferred to 100 ml Erlenmeyer flasks. The flasks with the substrate were sterilized in an autoclave at 15 psig. (250 °F). Sterilization time of 20 minutes at 15 psig. has been suggested by Steele et al. (1960). It was found that sterilization for 20 minutes resulted to a soft gel, difficult to be handled for cup formation and for development of the clear zone. The probable reason for the soft gel was the partial hydrolysing of the agar. Preliminary tests were run to establish the proper sterilization time. It was found that sterilization for 5 minutes resulted in desirable firm gel formation. That sterilization time was employed for all the tests.

The next step in the procedure involved 15 ml of the liquid substrate that were transferred to 15x85 mm Petri dishes and allowed to solidify. The amount of the liquid in each dish has a significant effect on the diameter of the clear zone. The effect of the gel thickness in the plate on the zone diameter is illustrated in Fig. 4. Very small thickness (5 ml per dish) resulted in a non clear zone, while, very high thickness resulted in smaller diameter. A thickness of 2.1 mm (or 15 ml per dish) was found to give well defined and large diameter clear zone. It must be noted, however, the larger the diameter the greater the accuracy.

Small plastic collars (9 mm diameter) were inbedded in the media to provide cups into which brine, containing enzyme, was placed. Using a device, similar to eye dropper, two drops of the sample were placed in each cup, each drop was approximately 0.065 ml and then, the plate was incubated for 20 hours at 36 °C (96.8 °F). The enzyme action on the substrate in the incubated Petri dishes was not visible. The clear zone was developed by flooding with 2 N hydrochloric acid for about 5 minutes. The development was completed by rinsing with distilled water. The activity of the enzyme was indicated by the formation of a clear zone. The diameter of the formed zone is a function of the enzyme concentration. Dingle et al. (1953) showed that the plot of the enzyme concentration (on log scale) vs the zone diameter is a straight line. Some other factors, such as

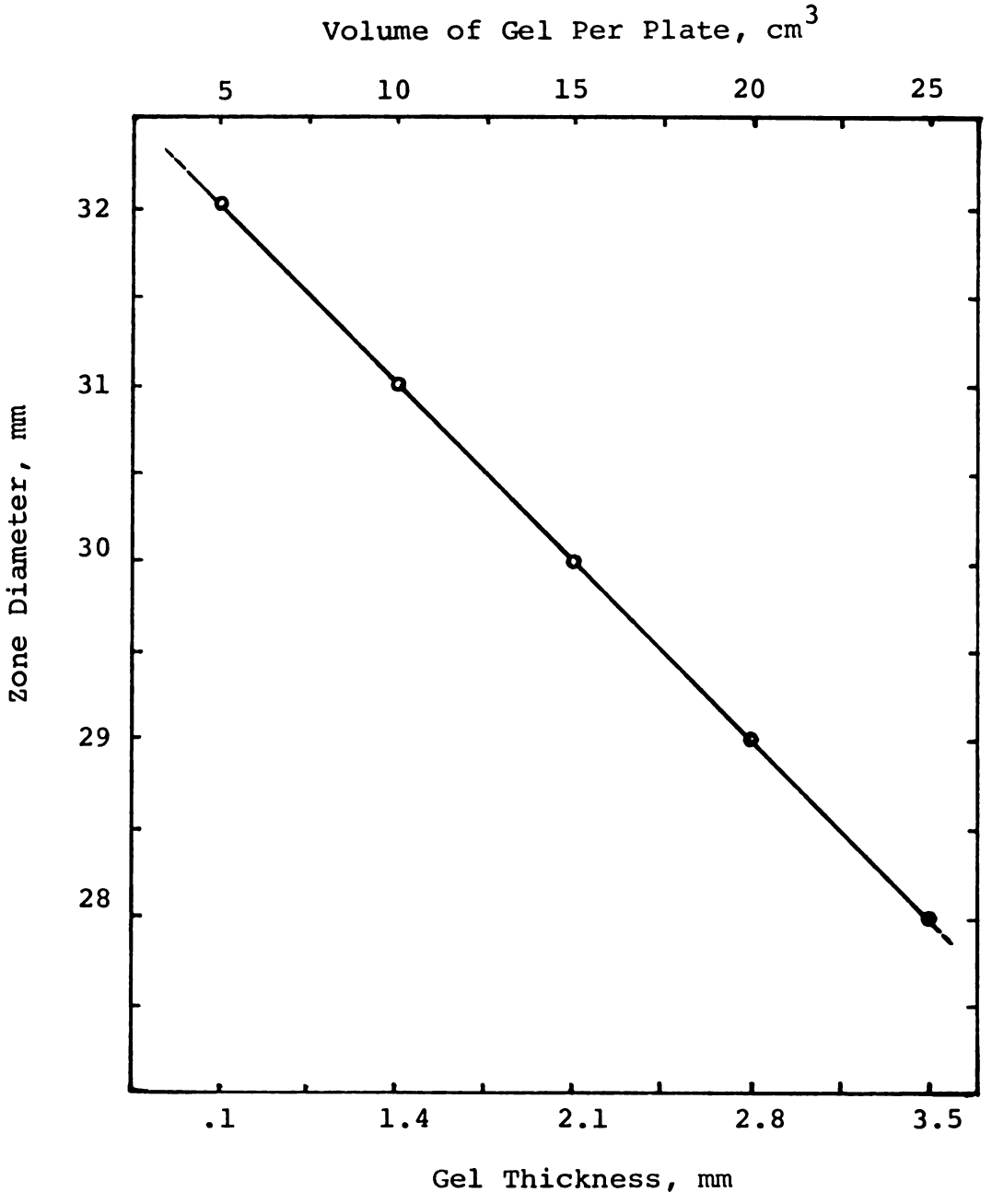

Fig. 4. Effect of gel thickness on zone diameter of 1 mg/l ml enzyme concentration and brine pH = 3.

the gel firmness and the incubation temperature and time, may have an effect on the zone diameter.

A number of enzyme dilutions in brine, ranging from 10 to .001 mg/ml, were prepared and six dishes were inoculated with each dilution. After incubation and development, the diameter of the clear zone was measured and the average of the six diameters were plotted against the concentrations of the enzyme on semi log paper. The plot (Fig. 5) was used as a reference curve to convert the zone diameters obtained from the tests to enzyme concentration.

It was noted that the same enzyme dilution, incubated in Petri dishes containing solid media from different batches, might result in clear zones with a different diameter. This is probably caused by some unidentified factors which affected the gel firmness, and in turn, the diffusion rate of the enzyme. For the results to be comparable, the following procedure was followed:

a. The diameter of the clear zone (average of three plates) of the control sample was determined for each substrate batch.

b. From the intercept of the observed diameter line and the 100% activity line, a curve was drawn parallel to the standard curve (example curve in Fig. 5).

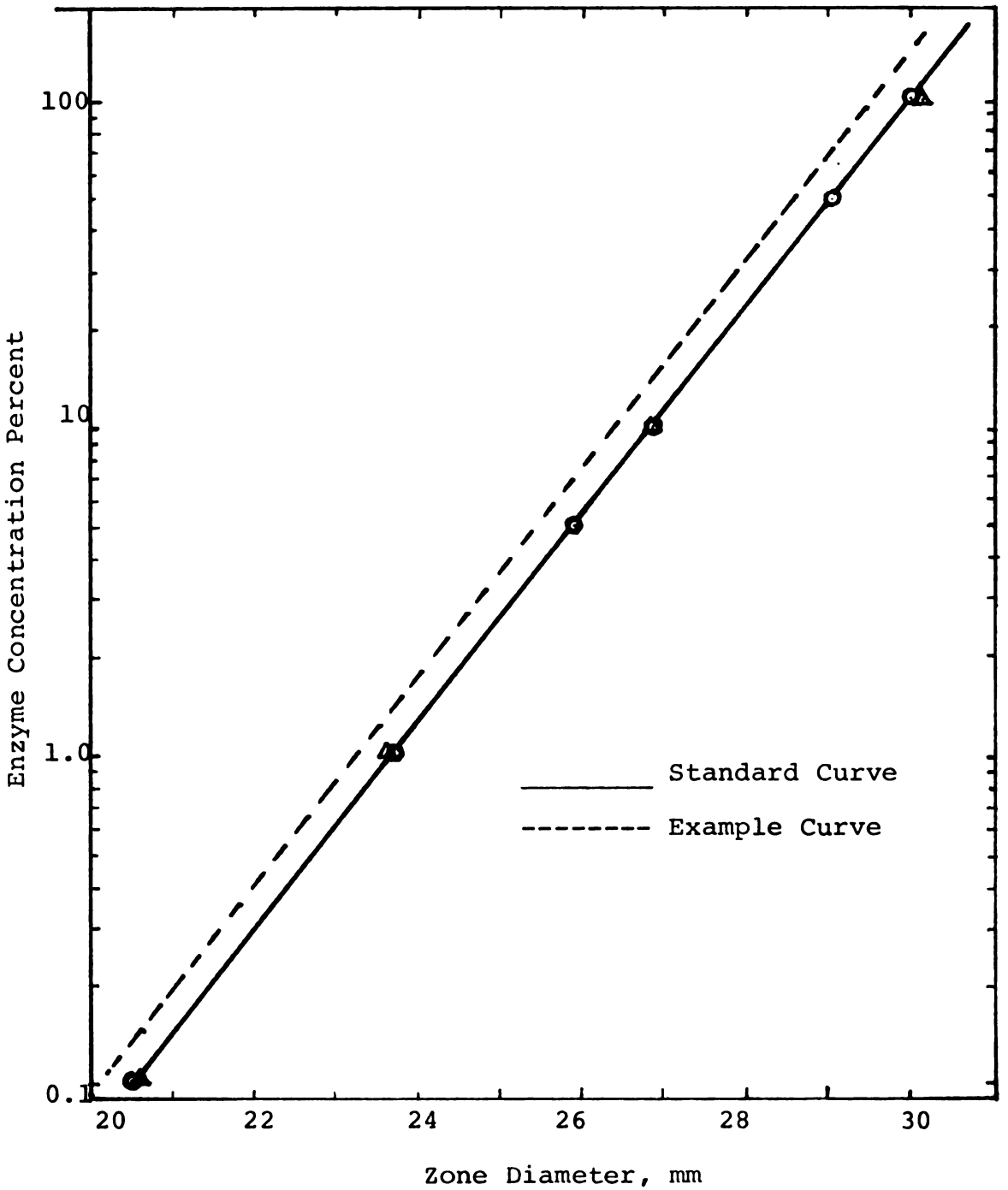

Fig. 5. Standard curve for enzyme activity estimation.

c. The dotted curve was considered as a standard curve for all the tests for which the same substrate was used.

D. pH Adjustment

The pH of the commercial brines at the time of preparation is about 2.8. The pH increases during the storage of the brines reaching about 3.8 by the end of the period. The resistance of the enzyme to inactivation by heat was determined at four different pH levels to represent the normal commercial pH range. The pH of the commercial Napoleon brine tested was 3.0. Samples were adjusted to pH = 2.5 by using hydrochloric acid, and to pH = 3.5, and 4.0 by using sodium hydroxide.

E. Dissolved Solids Level

After the cherries have been cured and equilibrated, the brine will contain soluble solids of 6 to 12 percent (Beavers et al., 1970). The soluble solids consist principally of sugars, pigments, acids, and other soluble constituents diffused from cherries. Napoleon brine was found to have a dissolved solids concentration 9 percent (measured with ABBE refractometer). Corn syrup was used to increase the dissolved solids concentration to 12 and 14 percent. Spent brine contains the same as corn syrup sugars, namely fructose and glucose (Sapers, 1975).

Dissolved solids concentration of 14 percent is the highest concentration which might be expected in the brine after several years of re-use.

F. Cherry Variety

Commercial brines from two cherry varieties were studied. Windsor brine is characterized by high SO_2 and high pigment concentration. Napoleon brine contains less pigment and about one-half as much SO_2 as Windsor brine.

G. Regeneration Capability of the Enzyme

Commerical Napolean brine which had been heat treated to inactivate the enzyme was stored under room conditions and in refrigerator at 4 °C. The enzyme activity was determined at selected time intervals for 82 days.

RESULTS AND DISCUSSION

A. Effect of Temperature

1. Order of the Reaction

To determine the order of enzyme inactivation reaction, the measured percent activities were plotted versus time in Fig. 6. Under the experimental conditions, data fit a straight line plot on semi-log coordinates and were described by a first order function. Fig. 6 illustrates the effect of the temperature on the rate of enzyme inactivation. The straight lines can be described by equation (6). It must be noted that the higher the temperature the higher the rate constant which results in steeper curves. The time scale in Fig. 6 corresponds to the heating time in the holding bath. The intercept of the curves with the percent activity indicates the enzyme activity at the time when the sample was transferred from the heating bath to the holding bath. As initial activity Co, in equation (6), is considered the activity described by the intercept. Thus any factor affecting the value of the intercept does not have any effect on the slope of the inactivation curves. The activity value determined

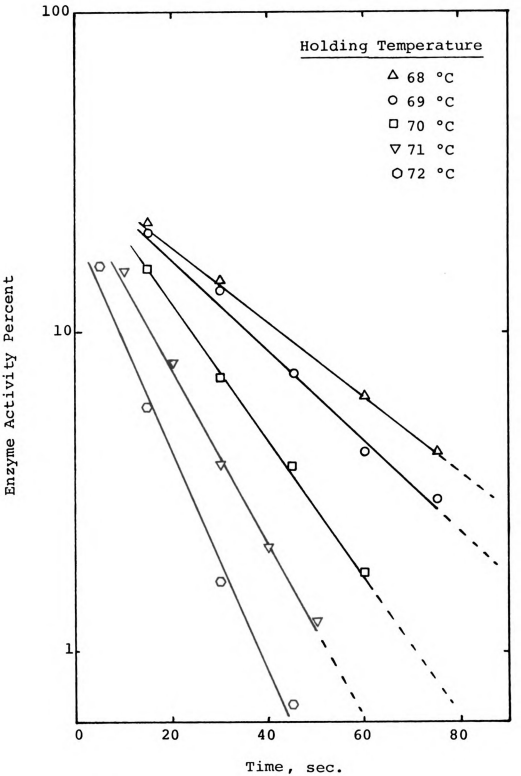

Fig. 6. Effect of temperature on the inactivation rate for the polygalacturonase enzyme in Napolean brine (pH = 3.0).

by the intercept is always less than the initial activity due to the partial inactivation occurring during heating and cooling. The value of the intercept is affected by the following:

a. The effect of the heat on the enzyme activity, described by the curves, corresponds to the heating in the holding bath. Some inactivation occurs during heating in the heating bath and during cooling. The contribution of heating and cooling to the total lethality is not indicated in Fig. 6.

b. The temperature of the sample was measured at the slowest heating point and since the sample was not agitated, all the other points are at higher temperatures. The thin layer of the brine in contact with the tube walls would be heated more than all the other points. This nonuniform temperature distribution contributes to depletion of the intercept.

c. The other factor which may have a significant effect on the intercept value is the method used for the enzyme activity measurement. As it has been described above, a standard curve was used to convert the zone diameter to enzyme activity. In each test, a line parallel to the standard was drawn based on the zone diameter average of three control dishes, as described in the previous chapter. A small error in the diameter measurement

results in significant movement of the intercept. By changing the control diameter by one milimeter (from 29.5 to 30.5) the activity indicated by the intercept decreases from 33 to about 18 percent.

Factor (b) may be controlled closely. By employing agitation, more uniform heating of the samples should be expected, and the temperature measured will be very close to the average sample temperature. The factor (c) was difficult to control and could be a deficiency of the method.

2. Rate Constants

Equation (5) can be rewritten as follows:

$$C = Co\ e^{-kt} \qquad (10)$$

The concentration indicated by the intercept may be considered as initial concentration Co. The inactivation rate constants were computed from equation (10) by using the exponential regression program in Wang Computer. The enzyme activities percent at different times and temperatures are summarized in Table A3. Fig. 7 illustrates the normalized inactivation curves for three temperatures. Table 1 shows the rate constants as they were evaluated by using Wang Computer (2200 series). Computed values of the rate constant are similar to that found in thermal inactivation of polygalacturonase in cucumber brine. The

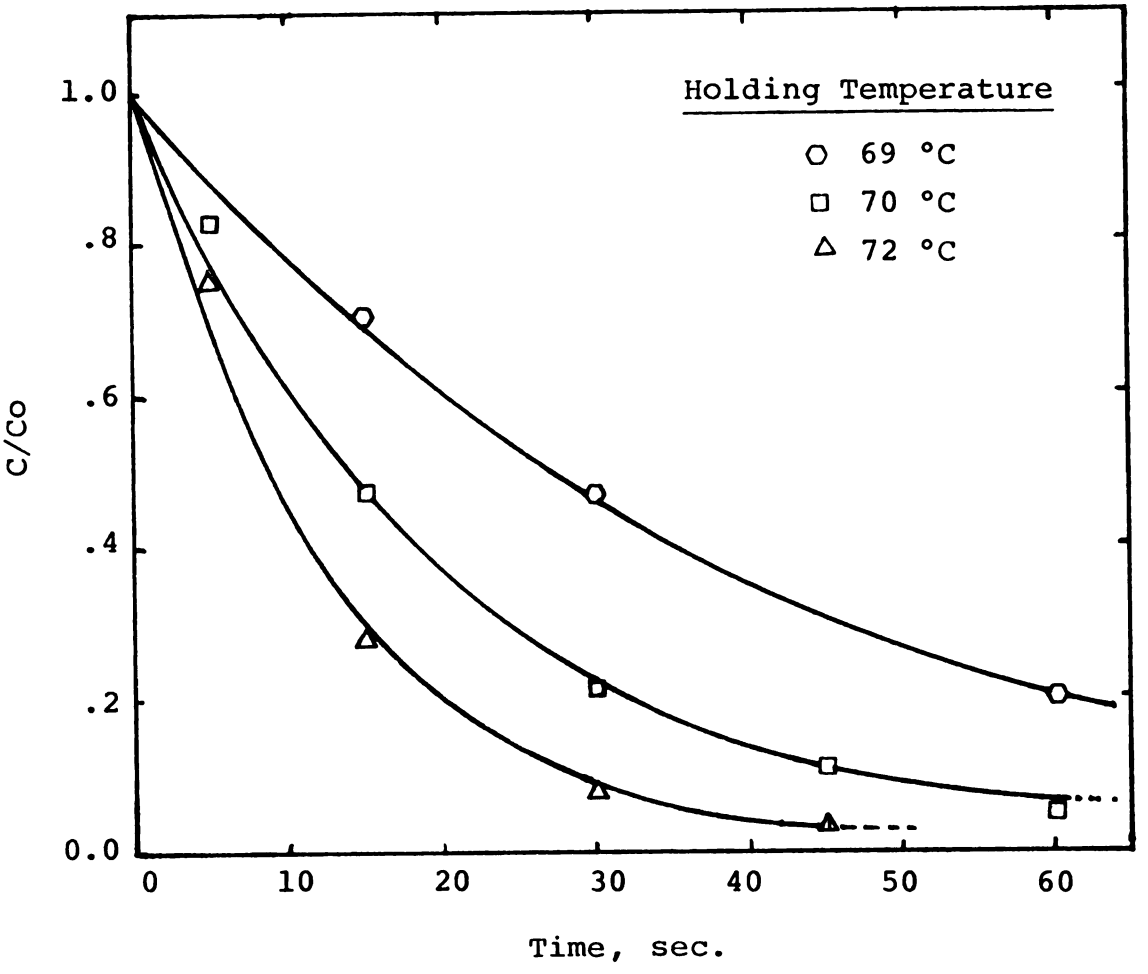

Fig. 7. Normalized inactivation rate curves for polygalacturonase enzyme in Napoleon brine (pH = 3.0).

Table 1.--Rate constants for inactivation of polygalacturonase in Napoleon brine (pH = 3.0).

Temperature (°C)	Correl.[1] Coeff.	D (sec)	k x 10^2 (sec^{-1})
68	.9995	84.9	2.712
69	.9969	67.59	3.407
70	.9994	46.76	4.925
71	.9988	36.71	6.274
72	.9934	29.26	7.870

[1]For C/Co versus time (Table A3).

rate constant in the latter case found to be equal to 7.7×10^{-2} sec at 79.5 °C (Chavana, 1976).

Fig. 7 indicates that the experimental points fit well to the ideal curves which describe the inactivation process. The curves illustrated in Fig. 7 have been obtained by using least squares fit-power curve. Thus the inactivation process follows first order kinetics. This may be supported by the high correlation coefficients shown in Table 1. The D values in Table 1 computed from k values (eq. 7).

The D values were plotted versus temperature on semilog coordinates. The straight line obtained and shown in Fig. 8 is called the "thermal resistance curve." Using Fig. 8, the decimal reduction time can be estimated at different holding bath temperatures. As the temperature increases, the D becomes smaller and smaller. The

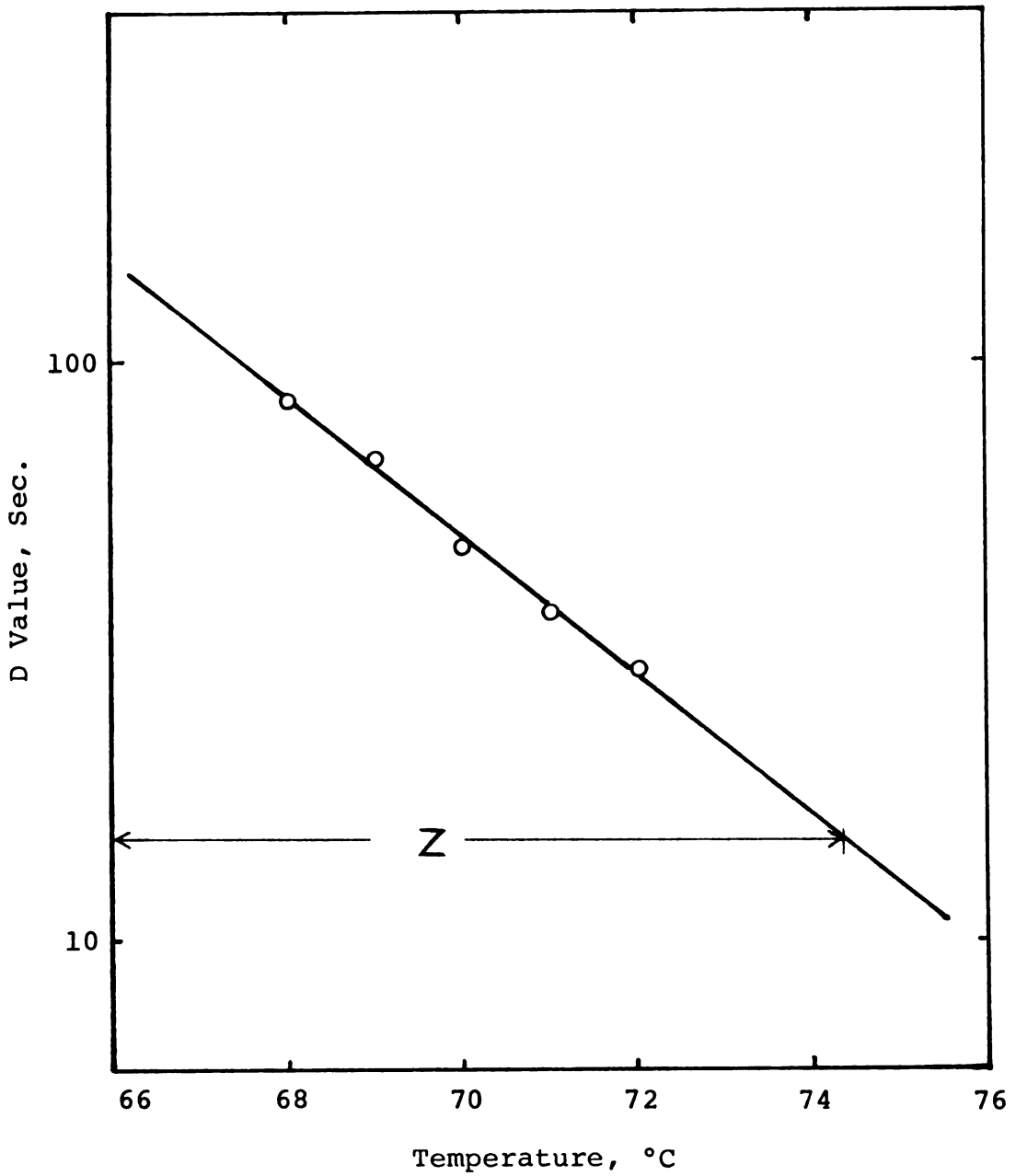

Fig. 8. Thermal resistance curve for polygalacturonase in Napoleon brine (pH = 3.0).

Table 2.--Data for evaluation of the activation energy for heat inactivation of polygalacturonase in Napoleon brine (pH = 3.0).

$k \times 10^2$ (sec^{-1})	T (°C)	Tab (°k)	$(1/Tab) \times 10^3$ ($°k^{-1}$)
2.712	68	341	2.932
3.407	69	342	2.924
4.925	70	343	2.915
6.274	71	344	2.907
7.870	72	345	2.899

temperature change required for changing D by 90 percent is called "Z" value and it was found to be 8.4 °C (15.12 °F).

The Z value is characteristic for each micro-organism, nutrient or enzyme and the Z value can be used to compute the decimal reduction time (D) at any desired temperature. The thermal resistance curve (Fig. 8) can be described by the equation:

$$\log D_1 = \log D_2 - \frac{T_2 - T_1}{Z} \qquad (11)$$

or

$$D_1 = D_2 \, 10^{\frac{T_2 - T_1}{Z}} \qquad (12)$$

if Z has been evaluated and D is known at a certain temperature, the decimal reduction time can be computed at any temperature.

3. Activation Energy for Inactivation

Another way to express the influence of temperature on rate constant, and in turns on decimal reducation time, is the well-known Arrhenius equation:

$$k = A\, e^{(-Ea/RTab)} \qquad (13)$$

where k is reaction rate constant, A is a constant, Ea is the activation energy, Tab is the absolute temperature, and R is the gas constant. Equation (13) can be written as follows:

$$\log k = \log A - Ea/(2.303)RTab \qquad (14)$$

Equation (14) describes a straight line known as the "Arrhenius plot." The Arrhenius plot for polygalacturonase inactivation in Napoleon cherry brine at pH = 3.0 is illustrated in Fig. 9. The constant A is evaluated from the intercept, while the activation energy is computed from the slope of the curve. The slope is equal to Ea/(2.303)R.

The rates of chemical reactions generally increase with increasing temperature. Increasing in temperature results in increasing of the collision frequency and, for a certain reactant concentration, an increasing of reaction rate. It must be noted that only a small proportion of the collisions occur with the required energy to promote the reaction. This minimum energy required for a successful reaction is called activation energy. Equation (13)

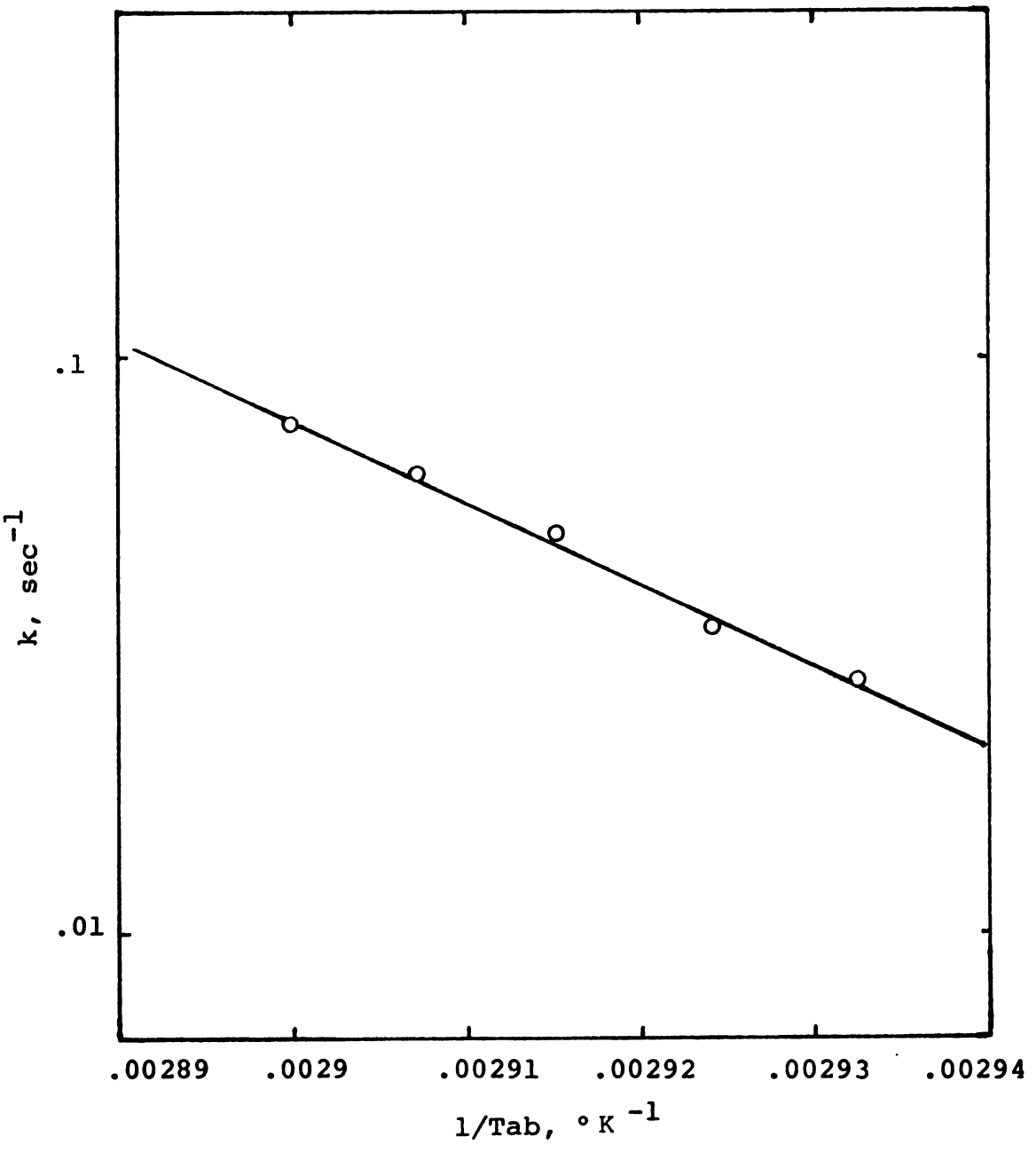

Fig. 9. Arrhenius plot for polygalacturanase inactivation in Napoleon brine CpH = 3.0).

formulated empirically by Arrhenius is generally used to correlate the rate constant, the temperature, and the activation energy. Enzyme catalyzed reactions exhibit behavior similar to chemical reactions up to a point (Segel, 1975), and equation (13) can be used to describe the effect of temperature on the reaction rate.

To explain the magnitude of the activation energy for inactivation of an enzyme, the structure of the enzyme must be taken into consideration. Enzymes are complicated protein molecules and their catalytic properties are associated to their highly ordered tertiary structure. The tertiary structure of the enzymes is maintained primarily by noncovalent bonds which generally are weak bonds. Hirschelder (Gould, 1959) suggested that the energy of activation is proportional to the strength of the bond that must be broken during the rate determining step of a reaction.

Fig. 9 illustrates the Arrhenius plot for the enzyme polygalacturonase in commercial cherry brine, from Napoleon cherries, having pH equal to 3.0. The slope of the curve, calculated with Wang Computer, was found:

$$\text{slope} = Ea / (2.303)\ R = 14{,}181.8 \qquad (15)$$

and the activation energy:

$$Ea = 64{,}668.2\ \text{Cal} \times \text{mol}^{-1} \qquad (16)$$

The activation energy for inactivation found for the polygalacturonase enzyme falls in the range generally accepted for inactivation of enzymes by heat. This range (Messing, 1975) is from 50,000 to 150,000 cal mol^{-1}. The relatively small Ea indicates that the enzyme molecule has a very delicate and fragile structure (Segel, 1975).

B. Effect of pH on Inactivation Rate

The inactivation rate was studied at pH values of 2.5, 3.0, 3.5, and 4.0 and at the temperature of 69 °C. Commercial brine from Napoleon cherries was used in that experiment.

Data obtained from the tests are shown in Table A4. The inactivation rate curves are illustrated in Fig. 10. Using the intercept of these curves as initial concentration, Co, the normalized curves have been constructed for two pHs (Fig. 11). The rate constants were calculated by using Wang Computer. Results of the calculation are summarized in Table 3. The rate constant of the inactivation reaction is highly pH dependent.

The effect of pH on the decimal reduction time is not linear, as it is indicated in Fig. 12. The resistance of the enzyme to inactivation by heat is higher at pH values between 2.8 to 3.5 than at pH values of 2.5 and 4.0. As it is illustrated in Fig. 12, the enzyme exhibits more stability at pH values usually employed in commerical brines.

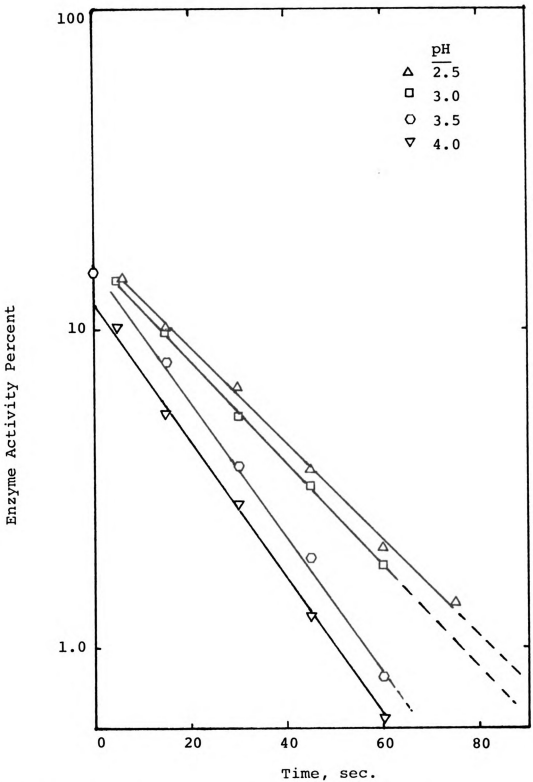

Fig. 10. Inactivation rate curves for polygalacturonase enzyme in Napoleon brine at 69 °C and different pH values.

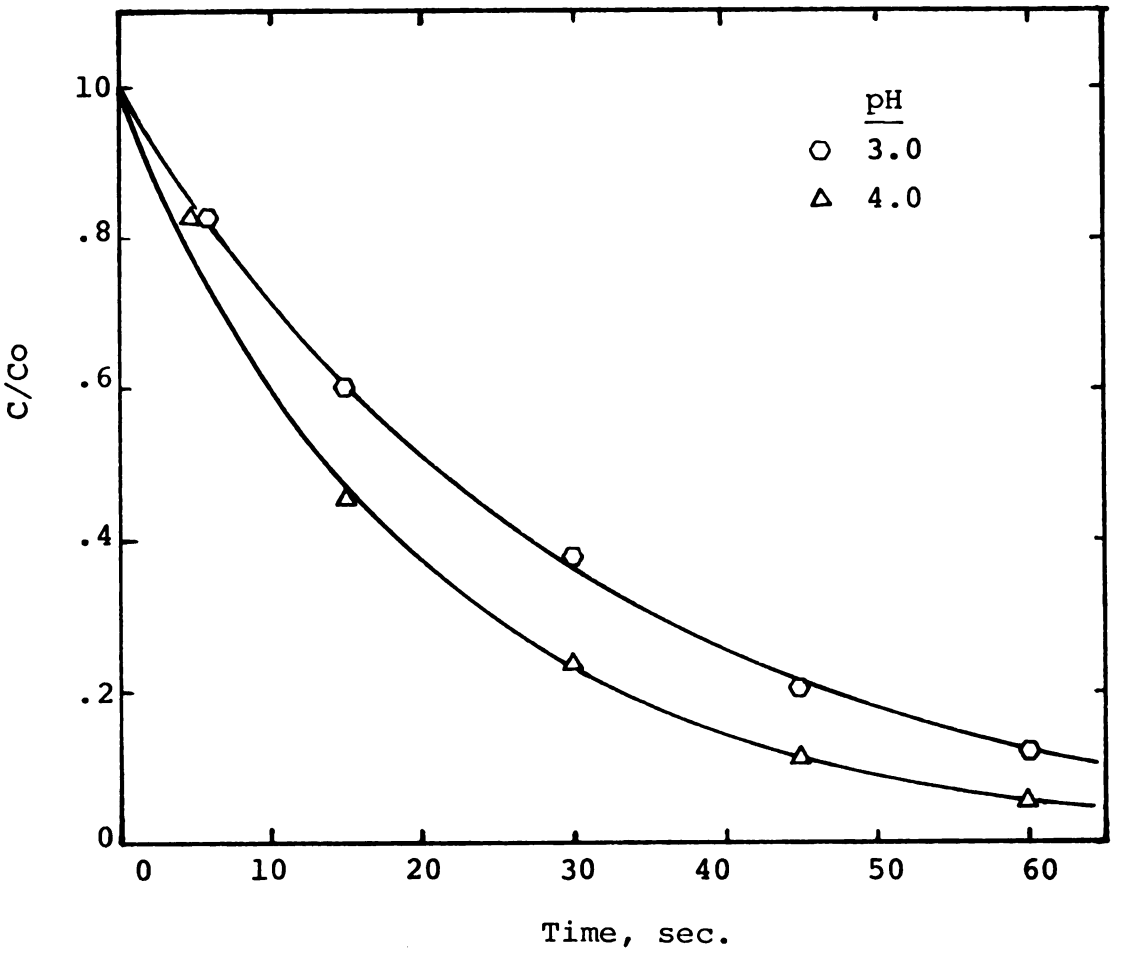

Fig. 11. Effect of pH on inactivation rate of polygalacturonase in Napoleon brine at 69 °C.

Table 3.--Rate constants of polygalacturonase inactivation in Napoleon brine at 69 °C and different pH values.

pH	Correlation[1] Coefficient	$k \times 10^2$ (sec^{-1})	D (sec)
2.5	.9988	4.848	47.5
3.0	.9984	3.464	66.54
3.5	.9976	3.812	60.41
4.0	.999	4.947	46.55

[1]For C/Co versus time (Table A4).

Table 4.--Rate constants of polygalacturonase inactivation in Napoleon brine at different sugar concentrations.

Sugar (%)	Correlation[1] Coefficient	$k \times 10^2$ (sec^{-1})	D (sec)
9	.998	3.462	67
12	.997	3.299	70
14	.994	2.574	92.5

[1]For C/Co versus time (Table A5).

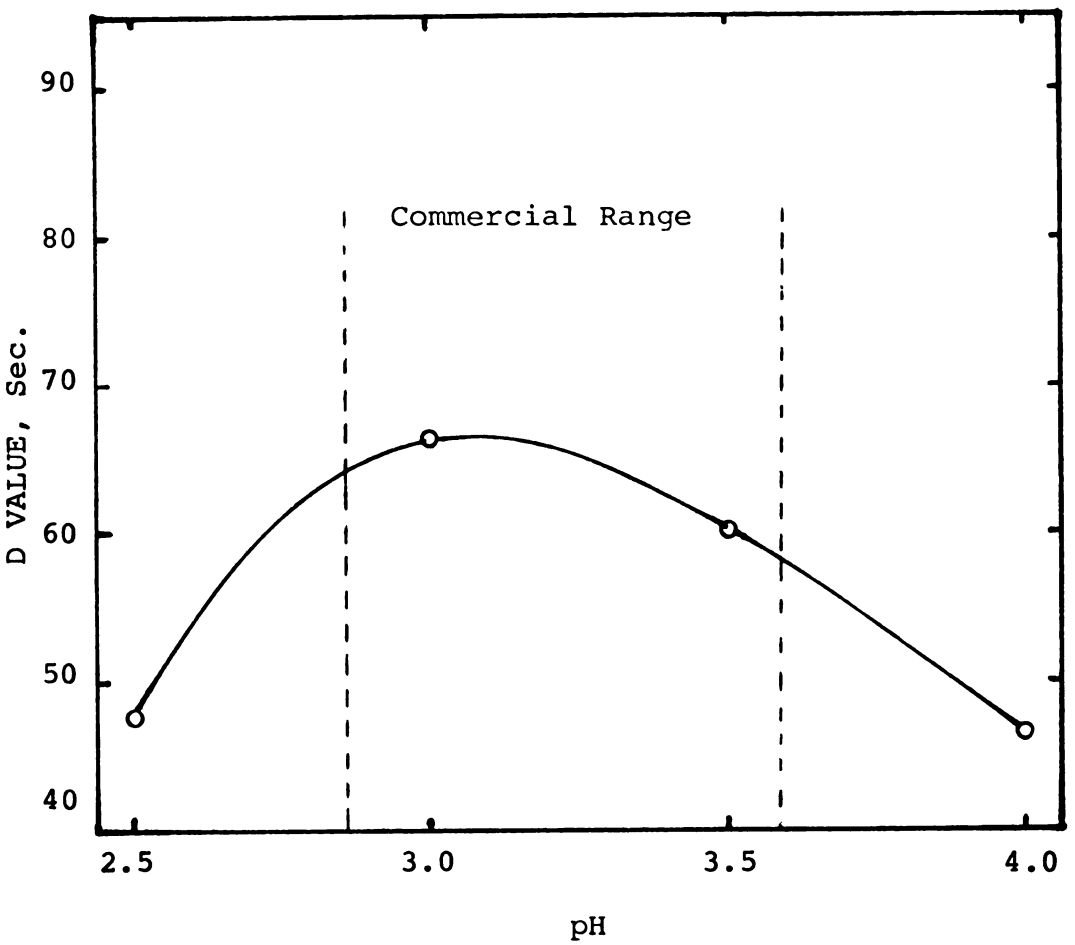

Fig. 12. Effect of pH on the decimal reduction time at 69 °C.

The "t" test was used to determine the significance level of the effect of pH on decimal reduction time. The calculated values were compared with "t-statistics" from tables and found the difference between pH 2.5 and 3.0, and 3.5 and 4.0 to be significant, while the difference of D at pHs 3.0 and 3.5 is not significant with probability of 99 percent.

Results, similar to that obtained in the lab for polygalacturonase, have been reported for other enzymes. Farkas and Goldblith (1962) found that the inactivation rate of the enzyme lipoxidase is faster at pH values 4.0 and 9.0 than at pH values between 4 and 7. The stability to inactivation by heat of 1.0 mg/ml solutions of purified spinach catalase was higher at pH values of 5.0 or 7.0 than at pH 9.0 at 55 °C (Sapers and Nickerson, 1962).

The effect of pH on the enzymes activity has been studied more extensively than the effect of pH on the thermal inactivation rate. Probably, the mechanism involved in the effect of pH on activity should be different than that in inactivation by heat. Lehninger (1970) describes the possible parameters which influence the pH activity profiles of the enzymes. The effect of pH on the inactivation rate may be more complicated and is not well understood.

To explain the effect of pH on the inactivation rates, the nature of the enzymes must be recalled. The enzymes are the largest class of proteins. As proteins, the enzymes exhibit an acid-base behavior in solution which

is largely determined by the relatively large number of ionizable R groups of the various aminoacids. The contribution of the terminal amino and carboxyl groups in that behavior is very little. Due to different types of ionizing groups, the enzyme molecule has positive or negative charge which is pH dependent. There is always a pH at which the enzyme molecule has no charge, and this is called isoelectic point. At pH values other than the isoelectic point, the molecule is charged and the electrical potential of the molecule is determined by its nature (by the pk of the ionizable groups) and by the pH.

The enzyme molecules in solution exhibit colloidal behavior. Proteins, and in turn enzymes, have been characterized as thermodynamically stable, or reversible, colloidal systems (Kruyt, 1952). Colloidal particles carry an electric primary charge the sign of which can be either positive or negative. The magnitude and the sign of the primary charge are frequently affected by the pH. The net electrical charge of a colloidal dispersion is zero. This is due to accumulation of opposite charge ions (counter-ions) near the surface of the particle resulting in the formation of double layer at the interface between a solid and water. The counter-ions are attracted by electrostatic forces generated by the primary charge of the particle the magnitude of which is affected by the pH. This attraction results in a concentration gradient of the ions, and a thermal energy is required to diffuse the ions away from

the surface of the particle. This attraction of the counter-ions may be considered as an activation energy barrier for inactivation which is pH affected and which must be over-come for inactivation to occur. As a result, the activation energy is expected to be affected by pH.

C. Effect of Sugar Concentration

Samples of commercial brine from Napoleon variety were treated as it is described by Sapers (1975) for syrup production from brine. The pH of that brine, which is essentially free of calcium bisulfite, was adjusted to 3.0 by using a small amount of sodium hydroxide. The sugars content of the brine was measured by the ABBE refractometer and found 9 percent. High fructose corn syrup was used to adjust the sugars concentration in two samples to 12 and 14 percent. The heat inactivation rates were studied in all three sugar levels, namely 9, 12, and 14 percent.

The results of the test are summarized in Table A5. The rate constant was calculated by using Wang Computer and the decimal reduction time computed from equation (7). Values of both k and D are shown in Table 4, in which the correlation coefficient of C/Co versus time is included. Fig. 11 indicates that the experimental points fit well to the curve described by equation (10). The inactivation rates of the enzyme polygalacturonase in brines with different sugar contain is illustrated in Fig. 13. The effect of the sugar concentration on the decimal reduction time is described by the curve shown in Fig. 14.

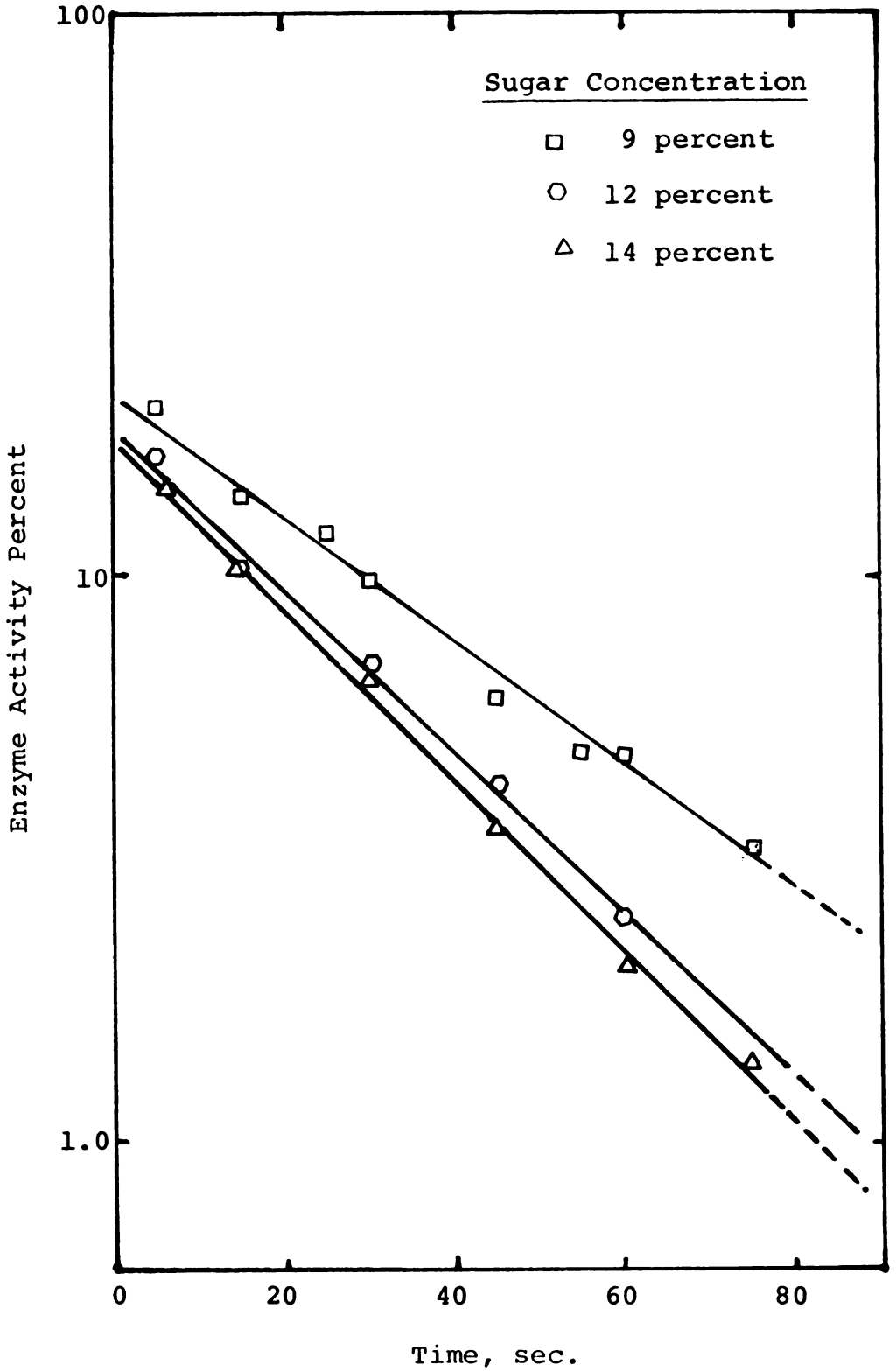

Fig. 13 Effect of dissolved sugar concentration on inactivation rate of polygalacturonase in Napoleon brine at 69 °C and pH = 3.0.

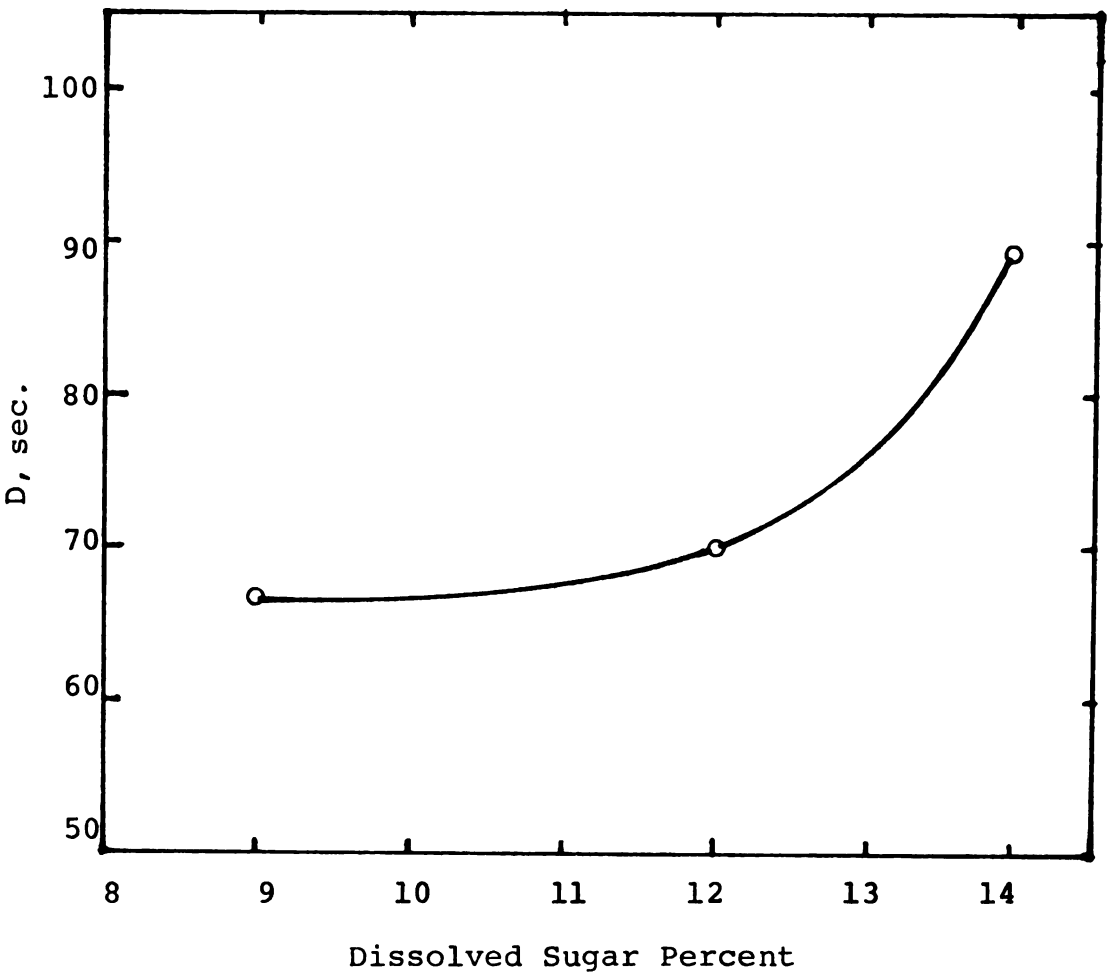

Fig. 14. Effect of dissolved sugars on D value at 69 °C and pH = 3.0

At lower sugar concentrations the inactivation rate is not significantly affected by the sugar content. As is illustrated by the curve (Fig. 13), the decimal reduction time is increased when the sugar concentration is higher than 12 percent. This effect is significant, as it was determined by the "t" test, and has to be taken into consideration when pasteurization units are designed for brines used more than one year because the concentration of the sugar in such brines will be higher and higher from year to year. An observation in Fig. 8 and 14 indicates that the pasteurization temperature must be increased by about 1.5 °C to overcome the effect of the higher sugar concentration at pH = 3.0.

The effect of the sugar concentration on the resistance of the enzyme was expected. Similar observations have been reported in thermobacteriology. It has been reported (Stumbo, 1965) that high sugar concentrations tend to increase the heat resistance of microorganisms. The sugars concentration is expected to affect the activation energy, as well. The mechanism of the protective effect of sugar on heat inactivation of the enzyme is not known. A possible explanation may be that the rich in hydroxyl groups sugars form hydrogen bonds with uncharged polar R groups of the aminoacid residues existing in the enzyme molecule. Additional heat energy is required to break down these hydrogen bonds which may tend to increase the enzyme resistance to inactivation by heat.

D. Effect of Cherry Variety

Some briners change proportions of ingredients in brine formulations for different varieties in attempts to get the desired product (Brekke et al., 1966). For varieties with higher pigments content, higher SO_2 concentrations are used.

Two different commercial brines were tested to determine the effect on the decimal reduction time. One brine was from Napoleon and the other from Windsor variety. The main differences of the two brines were: (a) The SO_2 concentration in Napoleon brine was about 7,000 ppm, while in Windsor brine it was twice as much. (b) The $CaCl_2$ concentration in Windsor brine was higher than that in Napoleon. (c) The concentration of the pigments was higher in Windsor brine which had red color. The pH of both brines was adjusted to 3.0 and enzyme at a concentration of 1 mg/ml was used. The inactivation rate of the enzyme was tested at 69 °C.

Table A6 summarizes data obtained in the laboratory. The rate constant found to be lower in Windsor brine having higher concentration in SO_2, Ca, and pigments. The calculated "t" values were compared with "t-statistics" from tables, and it was found that the effect of the variety on the D value was significant with probability 99 percent. The inactivation rate curves are shown in Fig. 15.

The effect of the various brines on the decimal reduction time is not easy to explain because of the

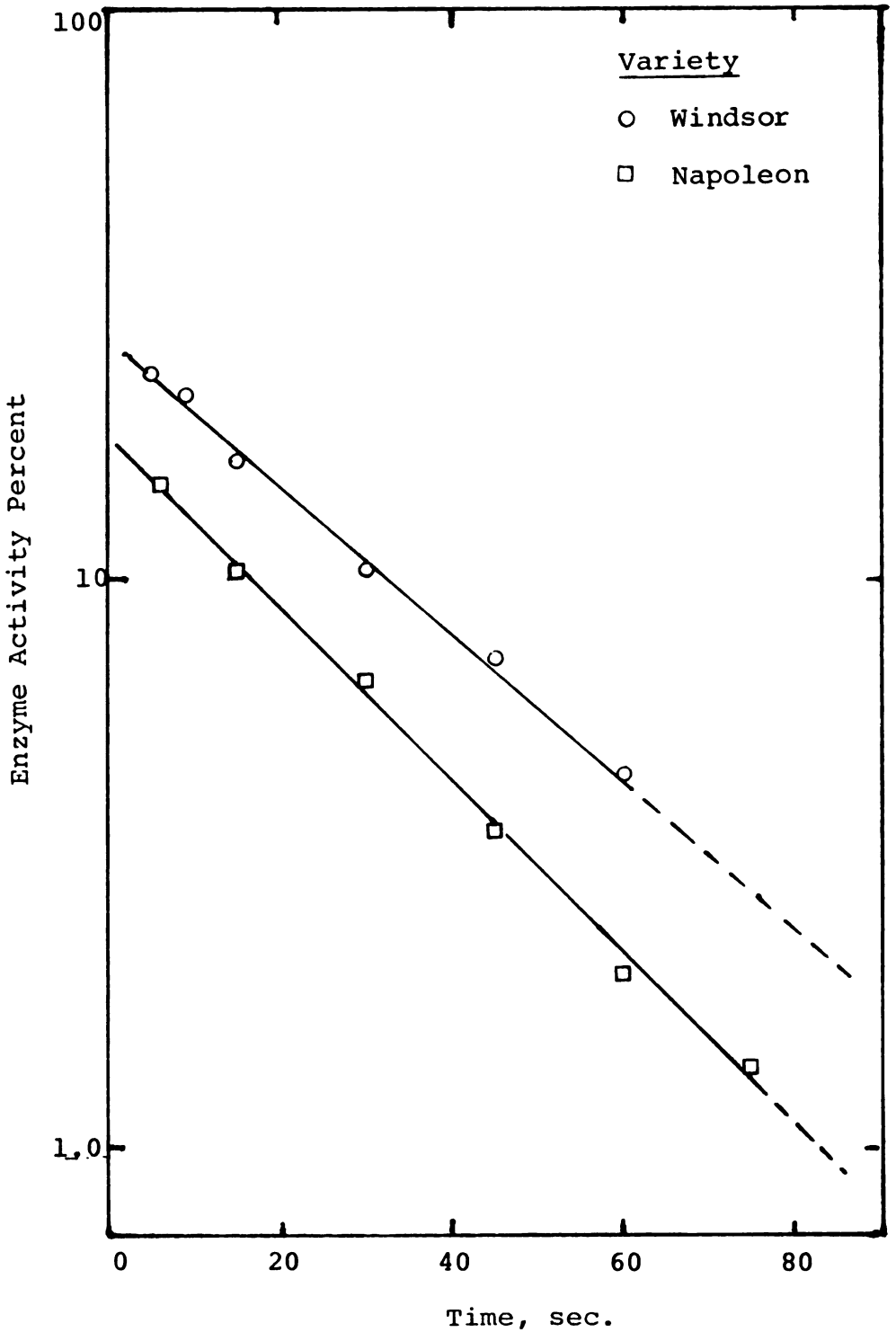

Fig. 15. Effect of cherry variety on inactivation rate of polygalacturonase in brine at 69 °C and pH = 3.0.

complexity of the system. The possible explanation may be the difference in ionic strength of the brines. Ionic strength is defined by the equation:

$$I = \frac{1}{2} MiZi^2 \qquad (17)$$

Where Mi is the molarity of the ion and Zi is the charge of the ion (regardless of sign). An enzyme may be more stable in one buffer of a certain pH than in another buffer with the same pH and different ionic strength (Segel, 1975). Dyson et al. (1973) showed that not the kind of the cations (monovalent or divalent) but the ionic strength alone affected the enzyme activity.

When brines of different formulations have to be pasteurized, the effect of the ionic strength on the inactivation rates of the enzyme should be taken into account in designing pasteurization units.

E. Regeneration Capability of the Enzyme

Napoleon brine was adjusted to pH = 3.0 and purified polygalacturonase was dissolved in it so as to obtain enzyme concentration 0.1 percent expressed as weight by volume. The brine was heated so as the enzyme activity was reduced to .88 percent of the initial activity. The heat treated brine was divided into two lots one of which was stored at room temperature and the other under refrigeration conditions at 4 °C. The lots were tested for activity at time intervals.

No regeneration was observed for storage time equal to 82 days, which indicates that the enzyme inactivation is irreversible (Table 5). In the sample stored at room temperature a precipitate, which probably consisted from crystals of calcium sulfate, was formed. This precipitation was not found to have any effect on the enzyme activity.

F. Applications

The parameters evaluated by this work cannot be used for design purposes before evaluating the thermal death time (F). This parameter is the time required to reduce the enzyme activity to a level at which it should be unable to result in softening of the cherries packed in that brine.

The thermal death time is difficult to evaluate from data in current literature because different types of enzyme preparations and different substrates have been used by the researchers. Brekke et al. (1966) used Pectinol A and found that it softened cherries when 0.05 to 0.20 percent of the weight of the fruit was added. This enzyme concentration is roughly equivalent to 0.5 to 2 mg of the enzyme per ml of brine. Steele and Yang (1960) found that polygalacturonase, in "cats claw" infected fruit, had activity equivalent to 1.5 to 0.5 mg/ml of Pectinol M solutions. Softening and disintegration of these cherries was excessive and their appearance was similar to that reported by McCready and McComb (1954). Purified polygalacturonase enzyme was added by Beavers et al. (1970) in

Table 5.--Activity of polygalacturonase inactivated in Napoleon brine by 99.12 percent.

Storage Time (Days)	Room Temperature		Refrigerator	
	Mean Diam. (mm)	Enzyme Activity (%)	Mean Diam. (mm)	Enzyme[1] Activity (%)
0	23.5[2]	0.88[1]	23.5[1]	0.88
16	23.5[2]	0.88	23.5[2]	0.88
26	23.0[3]	0.90	23.0[3]	0.90
34	24.5[4]	0.88	24.4[4]	0.82
38	23.5[2]	0.88	23.5[2]	0.88
65	23.0[3]	0.90	23.0[3]	0.90
74	23.5[2]	0.88	23.4[2]	0.82
82	23.0[3]	0.90	23.0[3]	0.88

[1]Activity at time zero = 0.88 percent of the initial.

[2]Control Zone diameter = 3.0 mm.

[3]Control Zone diameter = 29.5 mm.

[4]Control Zone diameter = 31.0 mm.

cherry brine in a concentration of 1 mg/ml and cherries were packed in the brine on an one weight by weight basis. They found that a decrease in texture accompanies the polygalacturonase activity.

The concentration of the enzyme of the type used in this study, which is capable to produce soft fruit, should be determined experimentally. Data obtained from such a study will allow for determination of the degree of inactivation of the enzyme for safe re-use of brine. Preliminary studies indicated that inactivation of the enzyme up to 99.3 percent follows the described kinetic mechanism (Fig. A1). As long as the desirable degree of inactivation has been established, the F value can be evaluated for a certain temperature. If F value is known at one temperature T, it can be computed at any temperature T', using the equation:

$$F_T = F_{T'} \times 10^{\frac{T'-T}{Z}} \qquad (18)$$

where Z has already been computed from the thermal resistance curve (Fig. 18) and found 8.4 °C.

In a continuous pasteurization process three segments are well defined: (a) heating, (b) holding, and (c) cooling. In such a process, the heating and cooling of the pasteurized liquid can be considered uniform. The contribution of the heating and cooling to the total lethality can be calculated. The following example illustrates the procedure to be used for the equivalent

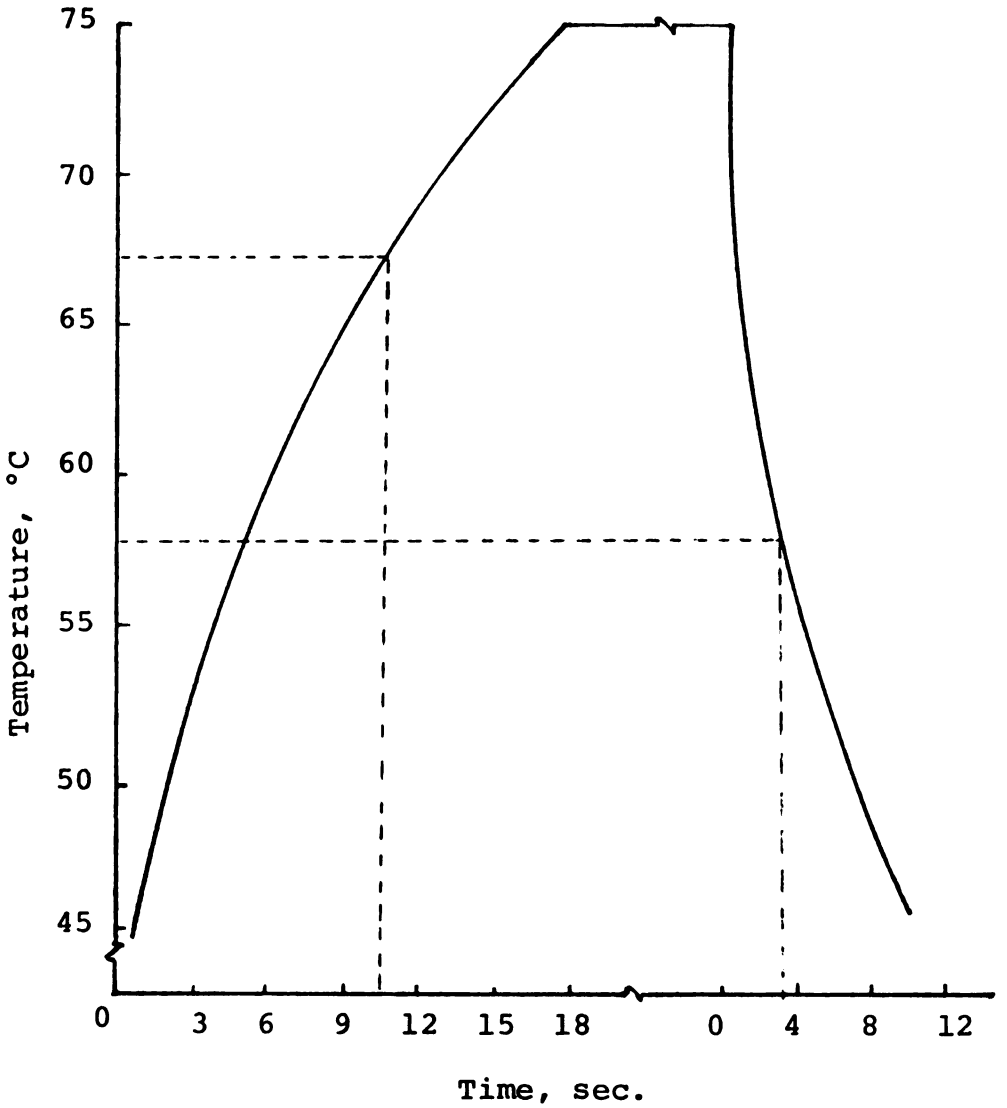

Fig. 16. Heating and cooling profiles for lethality calculation.

process time determination and for sizing the holding tube of the heat exchanger.

Problem: Tubular heat exchanger is to be used for cherry brine determination. The flow rate is 10 gal per min, the holding tube inside diameter is 1 inch, the thermal death time for the enzyme is F_{75} = 10 sec, and the Z value is 8.4 °C. Assuming that the heating and cooling process is described[1] by Fig. 16, calculate the minimum holding time at 75 °C and the length of the holding tube.

Solution:

1. The time required for heating and cooling is divided in appropriate intervals (3 sec. for heating, 2 sec. for cooling) as it is shown in Table 6.
2. The midpoint temperature is evaluated from Fig. 16 (column 2 in Table 6).
3. The lethality for each time interval is computed by multiplying the time intervals with the lethality computed (column 3). Time intervals for heating selected 3 sec. and for cooling 2 sec.
4. The total lethality effect of heating and cooling is the sum of column 4. This is expressed as equivalent process time at 75 °C and it is 3.824 seconds.

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[1]The heating and cooling profiles may be obtained by running a pilot plant or from theoretical calculations (Welty, 1974).

Table 6.--Contribution of heating and cooling to the total lethality of the enzyme in brine.

Midpoint Time (sec)	Midpoint Temp. T (°C)	Midpoint[1] Lethality	Time Interval[2] Lethality
		Heating	
1.5	47.25	.0005	.0015
4.5	56.50	.0063	.0188
7.5	62.50	.0395	.0975
10.5	67.25	.1195	.3851
13.5	71.0	.334	1.0021
16.5	73.75	.70989	2.1297
		Subtotal	3.635
		Cooling	
1.0	65.9	.0825	.16508
3.0	57.8	.00896	.0179
5.0	53.25	.00257	.0051
7.0	47.25	.0005	.0010
		Subtotal	.18908
		Total lethality	3.824

[1]Midpoint lethality = $10^{\frac{T-75}{Z}}$, where Z = 8.4 °C.

[2]Time interval lethality = Midpoint lethality x time interval.

5. The minimum holding time of the brine at 75 °C is: F_{75} - 3.824 = 10 - 3.824 = 6.175 seconds. Time of 6.5 sec. may be used for holding tube sizing.
6. The brine flow rate is 10 gal per min or 10 ÷ 7.5 = 1.34 ft^3 per min.
7. The velocity of the brine in the holding tube is: V = UA where A is the cross area of the tube, so U = V ÷ A = (1.34 ÷ πD^2)/4 = (1.34) ÷ (3.14 x $(1/12)^2$/4 = 245.8 ft/min = 4.1 ft/sec.
8. The length of the holding tube should be: L = Ut = 4.1 ft/sec 6.5 sec = 26.65 ft or 27 ft.

The holding tube may have coil shape and if the diameter of each turn is 1 ft, the total number of turns will be N = 26 ÷ πD = 8.49 or 9 turns.

SUMMARY AND CONCLUSIONS

Kinetics of inactivation of the polygalacturonase enzyme by heat in commercial cherry brines was studied. Commercial purified preparation of polygalacturonase was used at a concentration of 1 mg per ml of brine and tested at different temperatures, pH levels, at different dissolved sugar concentrations, and in brines from different cherry varieties. The regeneration capability of partial inactivated enzyme at room temperature (26 °C) and refrigerator temperatures (4 °C) was studied, as well. The agar "cup-plate" diffusion technique was used in this work for the quantitative determination of polygalacturonase.

Laboratory data were analyzed (Wang Computer, 2200 series) and the following were obtained.

1. The inactivation of the polygalacturonase enzyme by heat can be described by a first-order function.
2. The rate constant at 70 °C was 4.925×10^{-2} sec^{-1} and the decimal reduction time at the same temperature 46.8 sec.
3. The activation energy for enzyme inactivation in Napoleon cherry brine at pH = 3.0 computed from the Arrhenius plot was 64.2 $Kcal\text{-}mol^{-1}$.

4. The Z values for the temperatures range investigated was 8.4 °C.
5. A significant effect of pH on the decimal reduction time was found for the 2.5, 3.0, and 4.0 pH range while the difference between the effect of pH 3.0 and 3.5 was insignificant. The highest resistance of the enzyme to heat inactivation was at pH = 3.0.
6. Sugar concentrations higher than 12 percent seem to have a significant protective effect on the enzyme.
7. Enzyme preparation in brine from the Windsor cherry variety exhibited more resistance to inactivation by heat than enzyme in brine from Napoleon cherries.
8. No regeneration was observed within 82 days of storage for partially inactivated enzyme.

Additional investigation is necessary to establish the concentration of the enzyme needed to create softening of cherries, and in turn, the degree of inactivation required for safe re-use of pasteurized spent cherry brine. The most common cherry variety should be tested for softening in various brines containing different enzyme concentrations.

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APPENDICES

APPENDIX A

TABLES ILLUSTRATING RAW AND CALCULATED DATA

APPENDIX A

TABLES ILLUSTRATING RAW AND CALCULATED DATA

Table A1.--Data for standard curve construction. Polygalacturonase enzyme was diluted in commercial Napoleon brine at pH = 3.0.

Enz[2] Conc. (mg/ml)	Clear Zone Diameter, mm						Aver. Diam. (mm)	Stand. Deviat.
	I	II	III	IV	V	VI		
10.0	33.0	33.0	32.8	33.0	33.0	33.0	32.967	.069
5.0	32.5	32.5	31.8	32.5	32.0	32.0	32.22	.269
1.0	30.0	30.5	30.0	30.0	30.2	30.0	30.12	.1725
0.5	28.5	29.0	29.0	28.5	29.0	29.0	28.84	.2182
0.1	27.0	27.0	27.0	27.0	27.0	27.0	27.00	0.0
0.05	25.5	26.0	. .	26.0	25.9	26.0	25.8	.224
0.01	23.5	23.5	23.5	24.0	23.5	24.0	23.67	.218
0.001	20.5	20.5	20.5	20.5	20.5	20.5	20.417	.173

Table A2.--Effect of temperature on zone diameter for polygalacturonase in Napoleon brine (pH = 3.0).

Time sec	Clear Zone Diameter, mm						Mean Zone Diam. mm	Stand. Deviation
	1st Trial			2nd Trial				
	I	II	III	I	II	III		
				68 °C[1]				
0	28.0	28.0	27.5	28.0	28.0	28.0	27.92	.204
15	27.5	27.5	27.5	27.5	27.0	27.5	27.42	.204
30	27.0	27.0	26.5	27.0	26.5	27.0	26.83	.258
60	26.0	25.5	25.5	25.5	25.5	26.0	25.67	.258
75	25.0	25.0	25.5	25.0	25.0	25.5	25.17	.258
				69 °C[2]				
6	28.0	28.0	27.5	28.0	27.5	28.0	27.83	.258
15	27.5	27.5	27.0	27.0	27.5	27.5	27.34	.258
30	27.0	26.5	. .	27.0	26.5	. .	26.75	.289
45	26.0	26	25.5	26.0	26.0	26.0	25.92	.204
60	25.0	25.5	25.5	25.0	25.0	25.0	25.17	.258
75	25.0	24.5	24.5	24.5	24.5	25.0	24.67	.258
				70 °C[3]				
0	28.0	28.0	28.0	28.0	28.0	28.0	28.01	.00
5	27.5	28.0	27.5	28.0	28.0	27.5	27.75	.274
15	27.0	27.0	26.75	27.0	27.0	27.0	26.95	.167
30	26.0	26.0	25.75	26.0	25.75	25.75	25.86	.233
45	25.0	25.0	25.0	25.0	25.0	25.0	25.0	.00
60	23.75	24.0	24.0	24.0	24.0	23.75	23.91	.202

Table A2.--(cont'd.).

Time sec	Clear Zone Diameter, mm						Mean Zone Diam. mm	Stand. Deviation
	1st Trial			2nd Trial				
	I	II	III	I	II	III		
				71 °C[4]				
0	27.5	28.0	27.5	27.5	28.0	27.5	27.67	.258
10	27.0	26.5	27.0	27.0	27.0	27.0	26.92	.204
20	26.0	26.0	26.0	26.0	26.0	26.0	26.0	.00
30	25.0	25.0	25.0	25.0	25.0	25.0	25.0	.00
40	24.0	24.5	24.5	24.0	24.0	24.0	24.2	.258
50	23.5	23.5	23.0	23.5	23.5	23.5	23.42	.204
				72 °C[5]				
5	26.0	26.0	26.0	26.0	26.0	26.0	26.0	.00
15	24.5	24.5	24.5	24.5	25.0	24.5	24.6	.224
30	23	23	. .	22.5	23.0	23.0	22.9	.224
45	21.5	21.5	22.0	22.0	21.5	21.5	21.67	.258
60	21.0	21.0	20.5	20.5	21.0	21.0	20.84	.258

[1]Control Zone diameter = 29.5 mm.

[2]Control Zone diameter = 29.5 mm.

[3]Control Zone diameter = 29.5 mm.

[4]Control Zone diameter = 29.5 mm.

[5]Control Zone diameter = 28.5 mm.

Table A3.--Effect of temperature on polygalacturonase inactivation in Napoleon brine (pH = 3.0).

Temp. °C	Time sec	% Enzyme Activity	C/Co[1]
	0	31.0	.968
	15	22.0	.687
68	30	14.5	.453
	60	6.2	.194
	75	4.2	.131
	6	30.0	.908
	15	20.5	.620
69	30	13.8	.417
	45	7.4	.224
	60	4.2	.127
	75	3.0	.091
	0	33.5	.993
	5	27.8	.824
70	15	15.8	.468
	30	7.2	.213
	45	3.8	.113
	60	1.78	.053
	0	26	.960
	10	15.5	.572
71	20	8.0	.295
	30	3.85	.142
	40	2.12	.078
	50	1.23	.045
	5	16.00	.9976
72	15	5.80	.2795
	30	1.65	.0795
	45	0.68	.328

[1]Co is: 68 °C = 32.033, 69 °C = 33.05, 70 °C = 33.73
71 °C = 27.087, and 72 °C = 20.75.

Table A4.--Effect of pH on inactivation rate of polygalacturonase in Napoleon brine at 69 °C.

pH	Time sec	Mean Diam. mm	Standard Deviation	% Enzyme Activity,C	C/Co[1]
	0	26.917	.1725	15.0	.956
	15	25.917	.1725	7.8	.497
2.5	30	25.084	.1725	3.7	.236
	45	24.084	.1725	1.9	.121
	60	23.167	.218	0.8	.05100
	6	27.834	.258	14.5	.824
	15	27.34	.258	10.2	.579
3.0	30	26.75	.289	16.6	.375
	45	25.92	.204	3.6	.204
	60	25.17	.258	2.05	.116
	75	24.67	.258	1.39	.079
	5	27.67	.218	14.0	.841
	15	27.25	.231	9.5	.571
3.5	30	26.40	.183	5.38	.323
	45	25.834	.218	3.4	.204
	60	24.917	.1725	1.8	.108
	5	26.37	.194	10.0	.8299
	15	25.5	.00	5.4	.448
4.0	30	25.0	.00	2.8	.232
	45	23.5	.00	1.25	.104
	60	22.5	.00	0.6	.0498
	75	21.625	.1936	0.31	.026

[1] pH : 2.5 3.0 3.5 4.0

Co : 15.686 17.60 16.6418 12.049

Table A5.--Effect of sugar concentration on polygalacturonase inactivation in Napoleon brine at 69 °C (pH = 3.0).

Brix (°)	Time (sec)	Mean Diam. (mm)	Standard Deviation	Enz. Act. (%)	C/Co[1]
	6	27.83	.258	14.5	.8239
	15	27.33	.258	10.2	.5795
9	30	26.75	.289	6.6	.3750
	45	25.92	.204	3.6	.2045
	60	25.17	.258	2.05	.11648
	75	24.61	.258	1.39	.0789
	5	28	.0	16.5	.8967
	15	27.34	.218	10.2	.5543
12	30	26.84	.218	7.1	.3859
	45	26.17	.218	4.3	.2337
	60	25.42	.173	2.5	.1359
	5	27.79	.237	20	.9257
	15	27.29	.237	14	.6480
	25	21.08	.172	12	.5554
14	30	26.75	.235	9.5	.4397
	45	26.17	.218	6.1	.2823
	55	25.84	.218	4.9	.2268
	60	25.83	.218	4.8	.2222
	75	25.34	.218	3.3	.1527

[1]Co is: for 9° = 17.59, 12° = 18.4, 14° = 21.605.

Table A6.--Effect of cherry variety on inactivation rate of polygalacturonase at 69 °C (pH = 3.0).

Variety	Time (sec)	Enzyme Activity,C (%)	Standard Deviation	C/Co[1]	Notes
W	4	24.00	.00	.9123	kx10[2]
I	5	23.00	.1725	.8743	2.9068
N	9	21.00	.204	.7983	
D	15	16.00	.218	.6082	CORR.COEFF.
S	30	10.0	.224	.3820	.99697
O	45	7.2	.224	.7737	
R	60	4.55	.188	.1796	
N	6	14.5	.258	.8239	kx10[2]
A	15	10.2	.258	.5795	3.46II
P	30	6.6	.289	.3750	
O	45	3.6	.204	.2045	CORR.COEFF.
L	60	2.05	.258	.11648	.9984
E	75	1.39	.258	.17898	
O					
N					

[1]Co is: Windsor = 26.307, Napoleon = 17.60

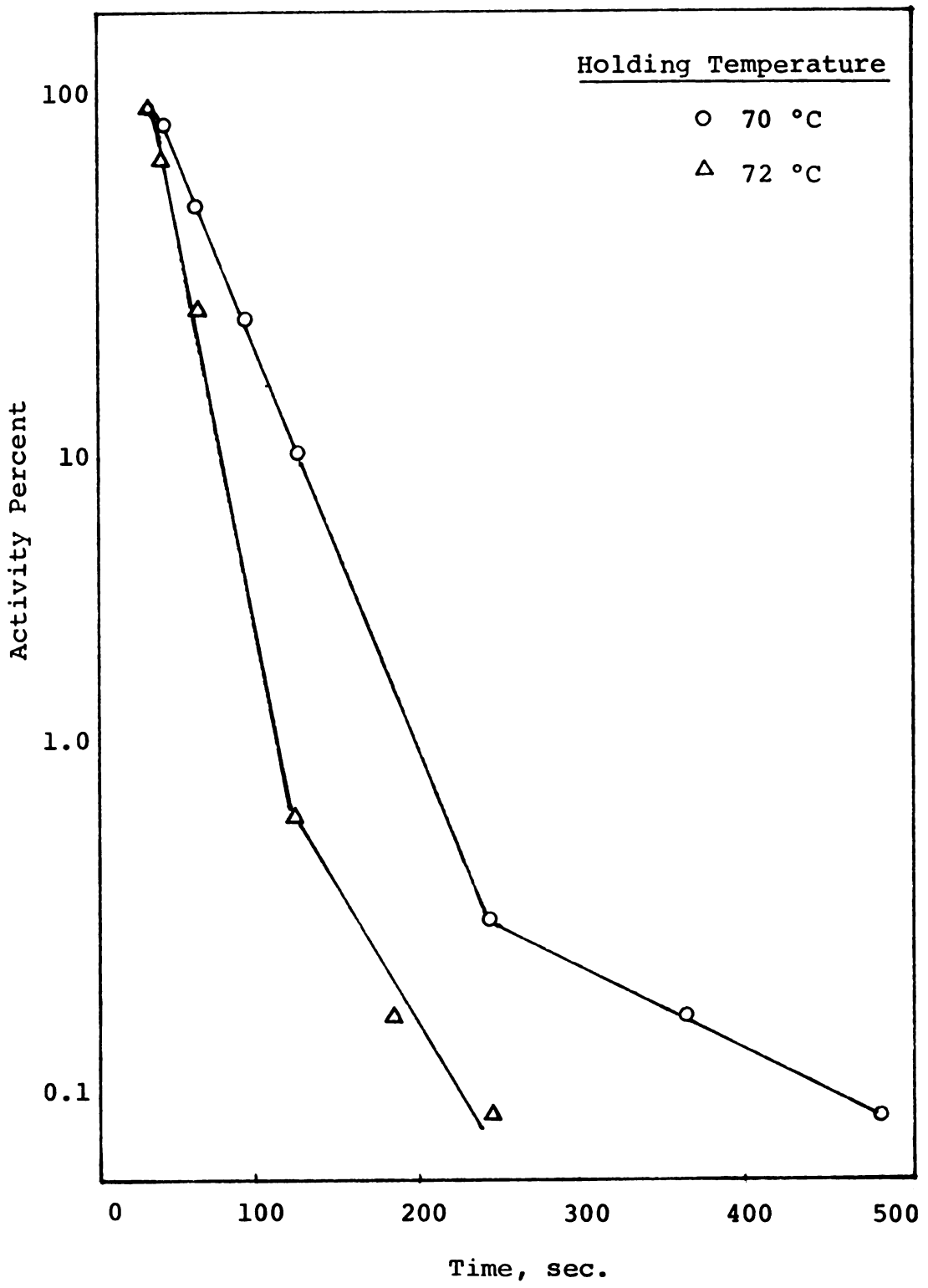

Fig. A1. Long time inactivation profile for polygalacturonase in Napoleon brine at pH = 3.0.

APPENDIX B

CUP PLATE METHOD OF ANALYAIS FOR POLYGALACTURONASE ENZYMES

APPENDIX B

CUP PLATE METHOD OF ANALYSIS FOR POLYGALACTURONASE ENZYMES

The following is the cup plate method for polygalacturonase enzyme assay according to the method of Dingle and others (1953) as given by Beavers et al. (1970).

One thousand milliliters of medium is prepared as follows:

1. To 500 ml distilled water in a one-quart blender jar, add 2.500 g of polygalacturonic acid.
2. While blending add 75 ml of 0.1 N sodium hydroxide. (Check pH, which should be 4.0 $\pm$ 0.1.)
3. Add 2.5 g ammonium oxalate and 10.0 g potassium acid phthalate. Continue to blend until completely dissolved.
4. Transfer to a 1-liter Erlenmeyer.
5. Blend 20.0 g Bacto Agar into 425 ml of distilled water and add 0.25 g thymol.
6. Transfer to a second 1-liter Erlenmeyer.
7. Steam both flasks about 30 minutes or until dissolved. (Agar will become clear above 97 °C.)

8. Cool contents of both flasks to 50 °C; mix in a 2-liter flask.
9. Dispense to flat bottomed petri dishes, 25 ml per dish.

Small stainless steel collars (8 mm dia.) may be imbedded in the media to provide cups into which a few drops of brine can be placed. The collars (1/4 inch long) can be cut from stainless steel tubing.

With an eye dropper or similar device, dispense four or five drops of test sample into a cup. Incubate overnight at 35 °C. At the end of the incubation period, pour 6 N hydrochloric acid over the media in the plates. Allow to stand one hour and observe. If polygalacturonase is present, a clear zone will appear around the cup; the diameter will vary depended on the concentration of the enzyme.

Polygalacturonase activity is evaluated by measuring the diameter of the clear zone, including the diameter of the cup. Control samples of solutions of pectin enzymes in water or brine should be included in any series of tests. Solutions containing 1, 0.1, and 0.1 percent of pectin enzyme will give clear zones of different diameters around the cup. By comparing the sample of cherry brine under test with the enzyme solutions, one can estimate the degree of polygalacturonase activity in turns of concentration of the pectic enzyme used.