

ABSTRACT

Maruvada, Rashmi. **Evaluation of the importance of enzymatic and non-enzymatic softening in low salt cucumber fermentations** (Under the direction of Roger F. McFeeters.)

Retention of a firm, crisp fruit texture is a major consideration for pickled vegetables including pickles made from fermented cucumbers. Cucumbers fermented in low salt (3% or less NaCl) without added calcium soften rapidly. However, it is not clear if softening of cucumbers in low salt occurs as a result of enzymatic or non-enzymatic mechanisms. The objective of this research was to assess the relative importance of non-enzymatic and enzymatic reactions in cucumbers in the softening that occurs when cucumbers are fermented and stored at a low salt concentration.

Fermentation of cucumbers in 6.0% NaCl plus 18 mM calcium maintained firmness better than any treatment with reduced salt. Heat treatment of cucumbers sufficient to inactivate cell wall degrading enzymes before fermentation helped reduce firmness loss during fermentation and storage. However, heat treatment of cucumbers after fermentation did not prevent firmness loss during storage at 30°C. This suggested that reactions that resulted in softening of the cucumber tissue during storage had occurred during the fermentation period and that heating after fermentation could not prevent those changes.

Polygalacturonase activity could not be detected in the cucumbers used in these experiments. α -D-galactopyranosidase and β -D-glucopyranosidase lost their activity completely in cucumbers blanched at 65°C. Other enzymes detected in the cucumbers that hydrolyzed p-nitrophenylglycosides lost ~60 to 80% of their activity at this blanch temperature. Pectinesterase retained over 70% of its activity in cucumbers blanched at 65°C. Pectinesterase along with all of the glycosidases detected in fresh cucumbers lost all detectable activity when cucumber slices were blanched at 85°C. At equal ionic strength, α -D-galactopyranosidase and β -D-galactopyranosidase were inhibited to a greater extent by calcium ions than sodium ions. However, for β -D-glucopyranosidase, the inhibition was dependent primarily on the ionic strength of the solution. During the first week of fermentation in 1.75% NaCl, there was a complete loss of activity of all the glycosidases assayed.

The fact that cucumbers heated sufficiently to inactivate all the enzymatic activities measured and fermented at a low salt concentration lost firmness compared to cucumbers fermented in 6% salt with 18 mM calcium chloride showed that non-enzymatic reactions were important in low salt softening. In three of four lots of cucumbers fermented there was additional softening if the cucumber tissue did not receive a heat treatment. This suggested that enzymatic reactions can also contribute to the softening that occurs during fermentation and storage of cucumbers at low salt levels. However, it was not possible to associate softening with specific enzymatic reactions.

**EVALUATION OF THE IMPORTANCE OF ENZYMATIC AND NON-
ENZYMATIC SOFTENING IN LOW SALT CUCUMBER FERMENTATIONS**

By

RASHMI MARUVADA

A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the Degree of
Master of Science

FOOD SCIENCE

Raleigh

2005

APPROVED BY:

Dr. Allen E. Foegeding

Dr. Van-Den Truong

Dr. Roger F. McFeeters

Chair of Advisory Committee

DEDICATION

To my husband for his motivation and support.

To my parents for their immense blessings.

BIOGRAPHY

Rashmi Maruvada was born on November 19, 1977 in a small town in India. She was raised in central India from where she completed her high school. She obtained her Bachelor of Science degree in Biology and Master of Science in Chemistry in India. She moved to United States after marriage in June 2001 and decided to obtain her Master of Science degree in Food Science at North Carolina State University, Raleigh, NC under the direction of Dr. Roger F. McFeeters in USDA Vegetable Fermentation Laboratory.

ACKNOWLEDGEMENTS

I would like to thank Dr. Roger F. McFeeters for giving me the opportunity to work under his direction while completing my Master of Science degree in Food Science. I am grateful to him for all the support, guidance and supervision that he has provided throughout my project. I admire his patience and owe him greatly for teaching me the skills and critical thinking that I have gained from this project. Thank you for answering all my questions and teaching me something new every time I visited your office.

I also would like to thank my committee members Dr. Allen E. Foegeding and Dr. Van-Den Truong for their suggestions and guidance. I also extend my gratitude to all my lab members especially Sabine Morrison, Suzanne Johanningsmeier, Roger Thompson and Janet Hayes for their support and technical assistance throughout my project.

Finally I would like to thank my parents, in-laws, sisters and especially my husband for their love, support, patience, guidance and blessings that each one has showered on me. All of them have helped me to accept any challenge in life and achieve my personal and professional goals.

TABLE OF CONTENTS

	Page
List of Tables	vi
List of Figures	vii
Chapter 1. Literature Review	1
1.1 The cucumber pickle industry	2
1.2 Structure of plant cell wall	5
1.3 Cucumber texture and non-enzymatic softening in acid brines	7
1.4 Calcium effects in other fruits and vegetables	9
1.5 Cucumber cell wall degrading enzymes	10
1.6 Methods used to measure enzyme activity in cucumbers	12
1.7 References	17
Chapter 2. Evaluation of the importance of enzymatic and non-enzymatic softening in low salt cucumber fermentation.	26
2.1 Abstract	27
2.2 Introduction	29
2.3 Materials and methods	32
2.4 Results and discussion	41
2.5 Conclusions	52
2.6 References	53

LIST OF TABLES

	Page
Table 2.1. Experimental design to test the effect of low salt, blanching and post fermentation heating on the firmness retention of size 2A fermented cucumbers. Cucumbers were packed and fermented whole. After fermentation half the jars were sliced and repacked.....	56
Table 2.2. Experimental design to test the effect of blanch treatments on firmness retention of cucumber slices fermented in 1.75% NaCl.	57
Table 2.3. Experimental design to test the effect of blanching and calcium ion addition on texture retention and enzyme activities in cucumber slices fermented in 1.75% NaCl	58
Table 2.4. p-nitrophenyl glycosides used to detect different glycosidase activities in 6% NaCl extracts from fresh cucumbers.....	59
Table 2.5. The structures present in plant cell wall polysaccharides.....	60
Table 2.6. Cell wall exo-O-glycosylhydrolases and the wall polymers that are their potential natural substrates	61
Table 2.7. Enzyme activities in fresh and blanched cucumber tissue	62

LIST OF FIGURES

	Page
Figure 1.1. Flow diagram for cucumber pickle processing.....	25
Figure 2.1. Firmness changes in whole cucumber mesocarp tissue after fermentation. Control contains 6.0% NaCl, 18 mM CaCl ₂ . Other treatments contain 1.75% NaCl without added calcium. Blanching was done at 75°C for 15 minutes. Post fermentation heating was done to a center jar temperature of 75°C for 15 minutes.....	63
Figure 2.2. Firmness changes in sliced cucumber mesocarp tissue after fermentation. Control contains 6.0% NaCl, 18 mM CaCl ₂ . Other treatments contain 1.75% NaCl without added calcium. Blanching was done at 75°C for 15 minutes. Post fermentation heating was done to a center jar temperature of 75°C for 15 minutes.....	64
Figure 2.3. Firmness changes in cucumber mesocarp tissue after fermentation. Control contains 6.0% NaCl, 18 mM CaCl ₂ . Other treatments contain 1.75% NaCl without added calcium. Blanching was done at 55°C, 65°C and 75°C for 15 min.....	65
Figure 2.4. Major polysaccharides in primary cell wall of plants.....	66
Figure 2.5A. Changes in the activity of α-D-galactopyranosidase from fresh cucumbers on increasing ionic strength. Ionic strength was changed by having different concentrations of NaCl and CaCl ₂ in the reaction mixture.....	67
Figure 2.5B. Changes in the activity of β-D-galactopyranosidase from fresh cucumbers on increasing ionic strength. Ionic strength was changed by having different concentrations of NaCl and CaCl ₂ in the reaction mixture.....	68
Figure 2.5C. Changes in the activity of β-D-glucopyranosidase from fresh cucumbers on increasing ionic strength. Ionic strength was changed by having different concentrations of NaCl and CaCl ₂ in the reaction mixture.....	69

Figure 2.6A. Firmness changes in two lots of cucumbers during and after fermentation. Control contains 6.0% NaCl, 18 mM CaCl ₂ . Other treatments contain 1.75% NaCl. 18 mM CaCl ₂ added to the calcium treatments.....	70
Figure 2.6B. Firmness changes in two lots of cucumbers during and after fermentation. Control contains 6.0% NaCl, 18 mM CaCl ₂ . Other treatments contain 1.75% NaCl. 18 mM CaCl ₂ added to the calcium treatments.....	71
Figure 2.7. Changes in pectinesterase activity during cucumber fermentation and storage. Panels A and B show the results from two lots of cucumbers.....	72
Figure 2.8. Changes in α-L-arabinopyranosidase activity during cucumber fermentation and storage. Panels A and B show the results from two lots of cucumbers.....	73
Figure 2.9. Changes in α-D-galactopyranosidase activity during cucumber fermentation and storage. Panels A and B show the results from two lots of cucumbers.....	74
Figure 2.10. Changes in β-D-galactopyranosidase activity during cucumber fermentation and storage. Panels A and B show the results from two lots of cucumbers.....	75
Figure 2.11. Changes in α-D-mannopyranosidase activity during cucumber fermentation and storage. Panels A and B show the results from two lots of cucumbers.....	76
Figure 2.12. Changes in β-D-xylopyranosidase activity during cucumber fermentation and storage. Panels A and B show the results from two lots of cucumbers.....	77

CHAPTER 1

Literature Review

1.1 The Cucumber Pickle Industry

1.1.1 Statistics

Cucumbers are the members of cucurbit family. They are the fourth most cultivated fresh vegetable crop in the world after tomatoes, brassicas (e.g., broccoli, spinach, cabbage), and onions (FAOSTAT, 2004). About four percent of the world's cucumbers are produced in the United States. U.S. production ranks fourth behind China, Turkey and Iran (Lucier et al., 2000; FAOSTAT, 2004).

Cucumis sativus is the commonly consumed species of cucumber in the U.S. It is consumed either raw or used for pickling. *Cucumis anguria*, found commonly in India and Africa, is mostly used to make pickles. Pickled foods are those to which salt and/or vinegar are added as a primary means of preservation (Fleming and Moore, 1983). Pickling cucumbers remain the third leading shelf stable (packed in cans/jars) vegetable in terms of consumption, after tomatoes and sweet corn (Lucier et al., 2000). Between 1994 and 2004 pickling cucumber production ranged from 1,127.4 million pounds in 1996 to 1,297 million pounds in 2003 (USDA, 2004).

About 60% of cucumber consumption is in the fresh form and 40% are pickled. Pickling cucumbers are commercially processed into three general groups of products: (1) pickles made from fermented brine-stock cucumbers (35%), (2) fresh-pack pickles (40%), and (3) refrigerated pickles (25%) (Fleming et al., 2002). Approximately 40% of pickled cucumbers are consumed away from home, mainly at fast food chains as U.S. consumers continue to increase consumption of food away-from-home (Lucier, 2003). Consumers

have been moving away from salty foods and the pickle industry has been changing to accommodate them. The average pickle's salt content today is lower than the 1970's and 1980's because fresh pack pickles are a larger share of the market and contain less sodium than fermented pickles. Although low-sodium pickle products have not gained wide acceptance, the demand for high quality, mild (low salt and low acid) refrigerated pickles has increased over past 10 years. Processors have responded with a wide range of products (Lucier et al., 2000).

1.1.2 Pickle Processing

Figure 1.1 shows a general process diagram for pickled cucumber products produced in the United States (Fleming, 1982). Refrigerated pickles are preserved without a thermal process by mild acidification ($\text{pH} < 4.6$) and refrigeration (1 to 4°C) and the use of preservatives, primarily sodium benzoate (Fleming et al., 1983). Refrigerated products generally retain more fresh cucumber flavor and crispness than other processing methods.

Fresh-pack cucumber pickles are prepared by packing fresh cucumbers in glass jars, covering with brine containing salt, vinegar, color, and flavorings, followed by pasteurization to kill vegetative microorganisms to make the products shelf stable. Fresh-pack pickles are the dominant pickle products in retail groceries and supermarkets.

The major pickle product consumed through the food service industry is hamburger dill chips. Hamburger dill chips along with small sweet pickles sold in groceries are manufactured from fermented salt stock pickles. Fermentation is done in large open top

tanks made from fiberglass or plastic materials, though a few wooden tanks continue to be used. Cucumbers are typically brined in 6% NaCl (Fleming et al., 1981) with 0.1 to 0.5% calcium chloride (Fleming et al., 1978; Buescher et al., 1979). The salt directs the course of fermentation because only homofermentative lactic acid bacteria, usually *Lactobacillus plantarum* is able to grow and produce acid at such high salt concentrations. The high salt concentration and calcium also prevent softening of cucumbers. The combination of salt, calcium, and the acid produced by lactic acid bacteria due to fermentation of sugars in the cucumbers preserves the cucumbers microbiologically and maintains a firm texture in the cucumbers during storage for a year or more (Fleming et al., 1981). After fermentation is completed, some processors may add additional salt, in some cases to give a total concentration up to 16%, to deal with dilution of brine due to rain or to reduce freezing in tanks during the winter in the Northern United States and Canada.

The salt concentrations used for fermentation and storage of cucumbers are much higher than is acceptable in products made from the cucumbers. Therefore, a desalting step must be used to lower the salt concentrations in fermented cucumbers before they are made into hamburger dill chips or other products (Humphries et al., 1989). This desalting step is called “processing” in the industry. The salt leached from the brine stock normally is discarded into water bodies. Sodium chloride disposal is a serious problem due to its contamination of fresh surface water and groundwater. The U.S. Environmental Protection Agency (EPA) limits chloride discharge in fresh waters to 230 ppm (EPA, 1987). Strict environmental regulations have resulted in some companies having to

abandon brining operations, and others having to pay sizeable expenses for discharge into municipal wastewater or install expensive lagoons and equipment for waste handling.

The concentrated salt brine remaining in the fermentation tanks after the cucumbers are removed is usually recycled and used in the brining fresh cucumbers (Fleming et al., 1996). When reusing fermentation brine, it is important to be sure the brine does not contain softening enzymes, that could soften the next batch of cucumbers (Fleming et al., 1983).

Research has been done to reduce salt by brining cucumbers at lower salt concentrations. This is achieved by addition of a starter culture to assure fermentation with a suitable homofermentative lactic acid bacterium, and fermentation in closed anaerobic tanks (Fleming et al., 1988) or plastic lined boxes (Fleming et al., 2002). Fermentation in anaerobic tanks is done to prevent dilution by rainwater, microbial contamination from the environment, and surface spoilage by aerobic yeasts. However, it has not been possible to consistently retain acceptable firmness for a year or more with reduced salt as can be done when cucumbers are fermented in 6% salt. Also, in reduced salt conditions cucumbers may be more susceptible to anaerobic microbial spoilage if the final pH is 3.6 or higher (Fleming et al., 1989).

1.2 Structure of plant cell wall

The texture of processed pickles is dependent on the cell wall structure of cucumbers during processing and storage. The plant cell wall is a highly complex organ. Carpita et

al. (2001) have estimated that up to 15% of the genes in Arabidopsis may be involved in cell wall synthesis and modification. The primary walls of young plant cells as well as the edible tissues of fruits, including cucumbers, are mainly composed of cellulose, pectin and matrix polysaccharides. Matrix polysaccharides include hemicelluloses that bind tightly, but noncovalently, to cellulose microfibrils cross-linking them into a complex network (Zablockis et al., 1995). Most matrix polysaccharides are branched molecules modified by various sugars (Robyn et al., 1999). For example xylan, the major hemicellulose polymer found in plants, consists of a β -1,4-linked D-xylose backbone and can be substituted by different side groups such as L-arabinose, D-galactose, acetyl, feruloyl, p-coumaroyl, and galacturonic acid residues (Wilkie and Woo, 1977). A second hemicellulose commonly found is (galacto-) glucomannan (Timell, 1967), which consists of a backbone of β -1,4-linked D-mannose and D-glucose residues with D-galactose side groups. Xyloglucans consists of a β -1,4-linked D-glucose backbone substituted by D-xylose. Xyloglucans interact with cellulose microfibrils by the formation of hydrogen bonds, thus contributing to the structural integrity of the cellulose network (Carpita and Gibeaut, 1993).

Cellulose consists of linear β -1,4-linked D-glucopyranose chains that are hydrogen bonded into crystalline structures called microfibrils (Kolpak and Blackwell, 1976). Cellulose has the function of giving rigidity and resistance to tearing, while the hemicelluloses and pectic substances confer plasticity and the ability to stretch (McFeeters 1985; Cosgrove, 2001; Imam et al., 2005).

Pectins form another group of heteropolysaccharides and consist of a backbone of α -1,4-linked D-galacturonic acid residues. It is located in the primary cell wall and constitutes the principal component of the middle lamella (Roberts, 1990). Pectins have homogalacturonan smooth and rhamnogalacturonan hairy regions (Schols et al., 1995). In native pectin, the galacturonic acid units are to a variable extent esterified with methanol (McFeeters and Armstrong, 1984), thereby forming methyl-D-galactopyranosyl residues. Pectin can be enzymatically deesterified, creating a blockwise distribution of deesterified homogalacturonan units (Willats et al., 2001). This reaction takes place during plant growth where highly methyl-esterified homogalacturonan deposited in cell walls is deesterified (Femenia et al., 1998). Deesterified blocks can be cross-linked with calcium, resulting in gel formation which contributes to intercellular adhesion. The pattern of methyl esters is likely to contribute distinct mechanical and porosity properties to the cell wall matrix. Methyl-D-galactopyranosyl residues of pectin are substrates for pectin methylesterase (PME).

1.3 Cucumber texture and non-enzymatic softening in acid brines

Cucumber mesocarp tissue, the fleshy layer of the cucumber between the peel (exocarp) and seed area (endocarp) of the fruit, contains about 1% cell wall on a fresh weight basis (McFeeters and Lovdal, 1987). The composition of monosaccharides in the cell wall is similar to other dicotyledons. Glucose, primarily present as cellulose, makes up 40% of the total monosaccharide residues. Galactose and galacturonic acid are each 21% of the sugars. The remaining 18% of the sugars include xylose 8%, mannose 4.5%, arabinose 4% with rhamnose and fucose each accounting for less than 1% of the monosaccharides.

The degree of methylation of the galacturonic acid residues is about 50% (McFeeters and Lovdal, 1987). The natural calcium content of cucumber tissue ranges from 2 mM to 8 mM, but the calcium concentration is usually toward the lower end to the range (McFeeters and Fleming, 1989).

Non-enzymatic softening of fruit and vegetable tissue at pH <4 has been attributed to acid hydrolysis of the polysaccharides in the cell wall (Doesburg, 1961). The results of Smidsrod et al. (1966) showed that pectic substances would be the cell wall polysaccharides most susceptible to acid hydrolysis in the pH range that occurs in fermented and acidified cucumbers. However, Krall and McFeeters (1998) found that acid hydrolysis of pectin would occur too slowly to be the cause of softening in the pH range of fermented cucumbers.

Non-enzymatic softening of cucumber tissue has been investigated by blanching at 80°C to inactivate pectinesterase, though it may not always preclude some recovery of activity during low pH storage (McFeeters et al., 1985). This temperature was sufficient to inactivate an endo-polygalacturonase that has been purified from ripe cucumber endocarp tissue (McFeeters et al., 1980). McFeeters and Fleming (1990) found that the entropy and enthalpy of activation for cucumber tissue softening in acid solutions was more similar to the thermodynamics of protein denaturation, which involves conformation changes of polymers, rather than hydrolysis of glycosidic bonds. Furthermore, calcium was found to inhibit non-enzymatic softening by causing a sufficiently large reduction in the entropy of activation for softening such that the free energy of activation increased as

calcium was added to cucumbers. This suggested that calcium inhibited softening by inhibiting conformational changes in cell wall polymers that led to softening of the tissue. Fleming et al. (1978) first observed that calcium inhibited softening in fermented cucumbers. McFeeters et al. 1995 found that both calcium concentration and temperature had a major effect upon the softening rate of cucumbers fermented in 2% NaCl. However, the kinetics of fermented cucumber tissue softening was more complex than occurred in blanched, non-fermented tissue. As a result it was not possible to carry out the same analysis of the thermodynamics of softening as was done for cucumber tissue which had been blanched to inactivate enzymes (McFeeters and Fleming, 1990).

1.4 Calcium effects in other fruits and vegetables

Calcium has been shown to inhibit softening in many processed fruits and vegetables. Some of these include brined jalapeno peppers (Saldana and Meyer, 1981; Howard et al., 1994), brined yellow banana peppers (Lee et al., 1999), pickled peppers (Fleming et al., 1993), refrigerated apple slices (Ponting et al., 1971) and cooked potatoes (Hughes et al., 1975). A high calcium concentration in the cell wall of living plant tissues inhibits autolysis by tissue enzymes, cell expansion, and fruit ripening (Ferguson, 1984; Burns and Pressey, 1987; Ma et al., 1999). In processed plant tissues, the mechanism by which calcium inhibits autolysis is not well understood, but it has been suggested that calcium inhibits the activity of pectin degrading enzymes such as endo-acting polygalacturonase (Konno et al., 1984; Pagel et al., 1990; Ma et al., 1999) directly, or by modifying the pectin substrate (Jauneau et al., 1994). Another possible explanation is that endo-

polygalacturonase becomes trapped in gelled pectin, thereby inhibiting movement of the enzyme (Virk et al., 1990; Jauneau et al., 1994).

1.5 Cucumber cell wall degrading enzymes

Softening of cucumbers in commercial fermentations has been an important problem for pickling industry. Investigation into this problem was started as early as 1950's (Bell et al., 1950). Since then, many attempts have been made to determine how softening may occur by both enzymatic and non-enzymatic mechanisms. The softening of small brined cucumbers under commercial conditions was demonstrated to be caused by pectinolytic and cellulolytic enzymes (Etchells et al., 1955). These enzymes were found to be fungal enzymes that are introduced into curing brines chiefly by the way of fungus-laden flowers that remain attached to cucumbers when they were brine, though some of the enzymes were likely on the fruit itself (Etchells et al., 1958).

Though they have not been directly related to softening, several enzymes that can degrade cell wall components have been reported in cucumber fruit. Bell (1951) reported that pickling cucumbers contain pectinolytic enzymes that are most active in fruits larger than those used for pickling as they approach maturity. Activity in cucumbers of pickling size was found to be very low or absent. Bell and Etchells (1951) were the first to demonstrate pectinesterase activity in cucumbers. They reported a pH optimum of 7.5 and found that the activity decreased as the pH was lowered to pH 3.5, which is in the range of the pH of fermented cucumbers. McFeeeters et al. (1985) determined the relationship between blanch temperature and pectinesterase inactivation in cucumber

slices. Heating at 81°C completely inactivated the enzyme, but some recovery of activity was observed during subsequent brining and storage of the cumber slices. Yemencioglu and Cemeroglu (1999) found that partially purified pectinesterase from cucumbers was heat stable below 60°C and showed activation at 55°C. Rapid inactivation of the enzyme was observed at 80 and 90°C. Optimum conditions for pectinesterase activity were 60°C and pH 9.0. Pressey and Avants (1975) reported an exo-polygalacturonase in cucumbers. Based upon the observations of Bell and Etchells (1951) that the highest pectolytic activity in cucumbers was in the endocarp tissue of mature cucumbers, McFeeters et al. (1980) purified and characterized an endo-polygalacturonase from ripe pickling cucumbers. Saltviet and McFeeters (1980) showed that this polygalacturonase activity could be induced in immature cucumbers exposed to ethylene. Endo-beta-1,4-glucanase, glucomannan-splitting enzymes, and some exo-glycosidases were detected in cucumbers based upon their ability to hydrolyze p-nitrophenyl glycosides (Meurer and Gierschner, 1992; Mujer and Miller, 1991) extracted and characterized β -xylosidase isozymes from cucumber seeds. The presence of several different glycosidases has been observed in pears (El-Rayah-Ahmed and Labavitch, 1980), olives (Heredia et al., 1993), and Semillon grapes (Takayanagi et al., 1997) based upon the ability of tissue extracts to hydrolyze p-nitrophenyl glycosides.

Despite the presence of enzymes in cucumbers that can degrade cell wall polysaccharides, the importance of these enzymes in causing softening of cucumber tissue during fermentation and storage is not clear. Pectinesterase has been found to demethylate pectin in brined non-fermented cucumber slices (McFeeters et al., 1985) and

fermented cucumbers (Hudson and Buescher, 1986; McFeeters et al., 1995). Miller et al. (1989) observed increases in the activities of enzymes in cucumbers that degrade cell wall polysaccharides after the cucumbers were mechanically damaged, but there was not a clear correlation between increases in activity and changes in cucumber tissue texture. The mechanisms and conditions of storage under which the fermented cucumber tissue softens as a result of the action of enzymes are not known. There are no data that show hydrolysis of glycosidic linkages in cell wall polysaccharides by cucumber enzymes during fermentation, nor data that demonstrate changes in texture as a result of enzymatic activities.

1.6 Methods used to measure enzyme activity in cucumbers

There are many ways to measure the activities of different enzymes. The type of assay employed depends on the type of enzyme being assayed as well as the matrix in which the enzyme is present. In order to determine the different enzymes qualitatively and/or quantitatively, specific substrates are needed.

1.6.1 Pectin methyl esterase

The action of pectin methyl esterase results in the release of methanol and consequently in the formation of carboxylic acid groups, and allows two ways to monitor the action of the enzyme. The release of the H⁺ can simply be monitored in a nonbuffered system using a pH indicator which changes color in the range of the optimal pH of the pectinesterase that is assayed (Versteeg, 1979; Pilnik and Voragen, 1991). A more quantitative assay to measure the action of the enzyme based upon formation of free carboxyl groups is to do an automated titration at the optimal pH (nonbuffered) where the

amount of NaOH necessary to maintain the initial pH is measured (Lee and MacMillan, 1968; Versteeg, 1979).

The release of methanol can be measured using gas-liquid chromatography (GLC) directly (McFeeters and Armstrong, 1984) or after conversion of the methanol to methyl nitrite (Litchman and Upton, 1972). Although this latter method is quite sensitive and reliable, it is also quite laborious (Pilnik and Voragen, 1991). Alternatively, methanol can also be determined by HPLC on an Aminex column 87H column (Voragen et al., 1986) although this method may not be sensitive enough for all purposes. Colorimetric methods have been developed in which methanol is oxidized to formaldehyde (Cameron et al., 1992; MacMillan et al., 1993), but they are not easy to perform (Bordenave, 1996).

1.6.2 Polygalacturonase

Polygalacturonases hydrolyze the α -galactopyranosyl linkages of pectin chains by random action (endopolygalacturonase) or by attack at the non-reducing terminal residues of the chains (exopolygalacturonase). This results in the liberation of a series of oligogalacturonates (mono-, di-, and trigalacturonates) (Rombouts and Pilnik, 1980), which can be measured spectrophotometrically with reagents that react with reducing sugars (Rexova-Benkova and Markova, 1978; Blais et al., 1992; Lorrungruang et al., 1993). The dinitrosalicylic acid reagent developed by Sumner and Sisler (1944) and modified by Miller (1959) provides a method for determining reducing sugars in a variety of biological fluids. The reaction is based on the reduction of sugar by dinitrosalicylate under alkaline conditions to yield 3-amino-5-nitrosalicylate (Miller, 1959). A method that is more sensitive than the dinitrosalicylate method for measuring release of reducing

groups from polypectate is Nelson's reducing sugar assay (1944). Cupric ions are reduced to cuprous ions, which give a blue color upon the addition of asenomolybdate reagent. Color is measured at 600 nm. The very sensitive and more simple to use bicinchoninic acid (BCA) reducing sugar assay (Waffenschmidt and Jaenicke, 1987) can be used to detect both endo and exo-carbohydrases. Use of this reagent has been adapted to microtiter plates so that large numbers of samples can be analyzed simultaneously for carbohydrase activities (Meeuwesen et al., 2000).

Substrate for reducing sugar assays for polygalacturonases are usually limited to the use of demethylated polypectate rather than pectin that has a substantial degree of methylation. The methods cited all require heating in alkaline conditions. Under these conditions, methoxylated pectins depolymerize by a β -elimination mechanism and thus give a high and variable background level of absorption when assaying for polygalacturonase activity. (Rexova-Benkova and Marcovic, 1976; Voragen et al., 1995).

Viscometric methods for assaying pectinase activity are free from this disadvantage. Bell et al. (1955) measured the activity of pectinases and cellulases in cucumbers and fermentation brines using viscometric methods. A percent loss in viscosity of a standard pectin solution is calculated over time. The relative viscosity of the standard pectin solution is influenced by pectin concentration, pH, temperature and addition of sodium chloride. All of these factors must be controlled for making quantitative comparisons. One advantage of this method is that it is capable of detecting enzyme activity in extremely low concentrations (Bell et al., 1950). A disadvantage is that it does not allow

measurement of the number of bonds hydrolyzed. Buescher and Burgin (1992) measured polygalacturonase activities in simulated and commercial cucumber pickle brines to evaluate using a diffusion plate assay. They found that the diffusion plate assay was a suitable and convenient alternative to the viscometric procedure for monitoring polygalacturonase activities in brines used for the bulk storage of pickles.

A method for the detection of polygalacturonase activity in fungi has been developed by McKay (1988) using ruthenium red staining of fungal colonies on polygalacturonate-agarose plates. Ruthenium red was shown to penetrate beneath the surface layers of the gel, in the regions surrounding a fungal colony where degradation of polygalacturonate had occurred. Without degradation of polygalacturonate ruthenium red did not penetrate the medium, was restricted to binding to the surface layers and was easily washed off. The medium containing undegraded polygalacturonate was a colorless clear background and areas of polygalacturonate degradation around the colonies were visualized as an intense purple-red halo. The method has been used to screen yeasts and filamentous fungi for polygalacturonase secretion.

1.6.3 Glycosidases

Glycosidases catalyze the selective hydrolysis of glycosidic bonds in oligosaccharides, polysaccharides, and their conjugates (Ma et al., 2002). A variety of assay methods for glycosidases are currently available, such as spectrophotometric assays (Lederberg, 1950; Gatt and Rapport, 1966; Dacremont and Kint, 1968; Van Hoof and Hers, 1968; Dangelmaier and Holmsen, 1980; Rauscher, 1984), fluorometric assays (Leaback and

Walker, 1961; Wong, 1970; Dangelmaier and Holmsen, 1980; Daniels and Glew, 1984), coupled enzyme assays (Barnett et al., 1967), detection of product sugars (Halliwell and Halliwell, 1984), and titrimetric monitoring of hydrogen fluoride liberated from glycosyl fluorides (Barnett, 1971). One of the most commonly used methods is the spectrophotometric assay using synthetic substrates such as p-nitrophenyl or o-nitrophenyl glycosides. Since the optimum pH of most plant glycosidases is in the acidic to neutral range (pH 4–7), the standard assay with these substrates usually involves the addition of alkaline solution after the enzymatic reaction to change the liberated p-nitrophenol or o-nitrophenol into its photometrically detectable anionic form, which is yellow in color (Li and Li; 1972; Dangelmaier and Holmsen, 1980). Fluorometric assays using 4-methylumbelliferyl glycosides is an alternative method with improved sensitivity for a few sugars. Development of fluorescence from the released aglycon also requires an alkaline solution (Dangelmaier and Holmsen, 1980; Daniels and Glew, 1984).

1.7 References

- Barnett, J.E.G., Jarvis, W.T.S., and Munday, K.A. 1967. Enzymic hydrolysis of the carbon-fluorine bond of -D-glucosyl fluoride by rat intestinal mucosa. *Biochem. J.* 103, 699–611.
- Barnett, J.E.G. 1971. The hydrolysis of glycosyl fluoride by glycosidases. *Biochem. J.* 123, 607–611.
- Bell, T.A., Etchells, J.L., and Jones, I.D. 1950. Softening of commercial cucumber salt-stock in relation to polygalacturonase activity. *Food Technol.* 4 (4), 157-163.
- Bell, T.A., Etchells, J.L., and Jones, I.D. 1951. Pectinesterase in the cucumber *Arch. Biochem. Biophys.* 31 (3), 431-441
- Bell, T.A., Etchells, J.L., and Jones, I.D. 1955. A method for testing cucumber salt-stock brine for softening activity. USDA- ARS Bulletin. 72-5, 15 pp.
- Blais, P., Peter, A.R., and Charest, P.M. 1992. Kinetics of the production of polygalacturonase and pectin lyase by two closely related formae speciales of *Fusarium oxysporum*. *Exp. Mycol.* 16, 1-7.
- Bordenave, M. 1996. Analysis of pectin methylesterase. In: H.F. Linskens, J.F. Jackson, eds. Modern methods of plant analysis. Vol. 17 – *Plant cell wall analysis*. Berlin: Springer-Verlag, pp 165-180.
- Buescher, R.W., Hudson, J.M., Adams, J.R. 1979. Inhibition of polygalacturonase softening of cucumber pickles by calcium chloride. *J. Food Sci.* 44(6), 1786-7.
- Buescher, R.W. and Burgin, C. 1992. Diffusion plate assay for measurement of polygalacturonase activities in pickle brines. *J. Food Biochem.* 16(1), 59-68.
- Burns, J.K., and Pressey, R. 1987. Ca²⁺ in cell walls of ripening tomato and peach. *J. Am. Soc. Hort. Sci.* 112, 783–787.
- Cameron, R.G., Bulsig, B.S., and Shaw, P.E. 1992. Adaptation of a spectrophotometric assay for pectin methylesterase to a kinetic microplate reader. *J. Food. Sci.* 47,1006-1008.
- Carpita, N., Tierney, M., and Campbell, M. 2001. Molecular biology of the plant cell wall: searching for the genes that define structure, architecture and dynamics. *Plant Molecular Biology*. 47, 1–5.

Carpita, N.C., and Gibeaut. D.M. 1993. Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *Plant J.* 3, 1–30.

Cosgrove, D.J. 2001. Wall structure and wall loosening. A look backwards and forwards. *Plant Physiol.* 125(1), 131-134.

Dacremont, G., and Kint, J.A. 1968. GM1-ganglioside accumulation and -galactosidase deficiency in a case of GM1-gangliosidosis (Landing disease). *Clin. Chim. Acta* 21, 421–425.

Dangelmaier, C.A., and Holmsen, H. 1980 Determination of acid hydrolases in human platelets. *Anal. Biochem.* 104, 182–191.

Daniels, L.B., and Glew, R.H. 1984. In *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Ed.), Vol. 4, pp. 217–226, VCH, Weinheim.

Doesburg, J.J. 1961. Retention between the behavior of pectic substances and changes in firmness of horticultural products during heating. *Qual. Plant. Et Mater. Veg.* VIII:115.

El-Rayah-Ahmed, A., and Labavitch, J.M. 1980. Cell wall metabolism in ripening fruit. II. Changes in carbohydrate-degrading enzymes in ripening 'Bartlett' pears. *Plant-Physiology.* 65(5), 1014-1016.

EPA. 1987. Water quality criteria; availability of document. Fed. Reg. 52: 37655-37656. *Environ. Prot. Agency.* Washington, D.C.

Etchells, J.L., Bell, T.A., and Jones, I.D. 1955. Studies on the origin of pectinolytic and cellulolytic enzymes in commercial cucumber fermentation. *Food Technol.* 9(3), 14, 16.

Etchells, J.L., Bell, T.A., Monroe, R.J., Masley, P.M., and Demain, A.L. 1958. Populations and softening enzyme activity of filamentous fungi on flowers, ovaries, and fruit of pickling cucumbers. *Appl. Microbiol.* 6 (6), 427-440.

FAOSTAT Data, 2004. *Agricultural Production.* Last updated February 2004 Food and Agriculture Organization of the United Nations

Femenia, A., Garosi, P., Roberts, K., Waldron, K.W., Selvendran, R.R., and Robertson, J.A. 1998. Tissue-related changes in methyl-esterification of pectic polysaccharides in cauliflower (*Brassica oleracea* var. *botrytis*) stems. *Planta.* 205(3), 438-444.

Ferguson, I.B. 1984. Calcium in plant senescence and fruit ripening. *Plant Cell Environ.* 7, 477–489.

- Fleming, H.P., Thompson, R.L., Bell, T.A., and Hontz, L.H. 1978. Controlled fermentation of sliced cucumbers. *J. Food Sci.* 43(3), 888-891.
- Fleming, H.P. and McFeeters, R.F. 1981. *Shelf life of fresh-pack cucumber pickles*. Advisory statement published and distributed by Pickle Packers International, Inc., St. Charles, IL.
- Fleming, H.P. 1982. Fermented vegetables. In *Economic Microbiology. Fermented Foods*. Vol. 7 (chapter 7), A. H. Rose, ed., Academic Press, Inc., New York, NY, pp. 227-258.
- Fleming, H.P. and Moore, W.R., Jr. 1983. *Pickling*. In Processing of Horticultural Crops in the United States by G. Fuller and G. G. Dull. In CRC Handbook of Processing and Utilization in Agriculture, Vol. II, Part 2: *Plant Products*. I. A. Wolff, ed., CRC Press, Inc., Boca Raton, FL, pp. 397-463.
- Fleming, H.P., McFeeters, R.F., Daeschel, M.A., Humphries, E.G., and Thompson, R.L. 1988. Fermentation of cucumbers in anaerobic tanks. *J. Food Sci.* 53 (1), 127-133.
- Fleming, H.P., Daeschel, M.A., McFeeters, R.F., and Pierson, M.D. 1989. Butyric acid spoilage of fermented cucumbers. *J. Food Sci.* 54(3), 636-639.
- Fleming, H.P., Thompson, R.L., and McFeeters, R.F. 1993. Firmness retention in pickled peppers as affected by calcium chloride, acetic acid, and pasteurization. *J. Food Sci.* 58(2), 325-330, 356.
- Fleming, H.P., Thompson, R.L., and McFeeters, R.F. 1996. Assuring microbial and textural stability of fermented cucumbers by pH adjustment and sodium benzoate addition. *J. Food Sci.* 61(4), 832-836.
- Fleming, H. P., Humphries, E. G., Fasina, O. O., McFeeters, R. F., Thompson, R. L., and Breidt, Jr., F. 2002. BAG-IN-BOX TECHNOLOGY: Pilot system for process-ready, fermented cucumbers. *Pickle Pak Sci.* VIII (1), 1-8.
- Gatt, S., and Rapport, M.M. 1966. Isolation of -galactosidase and -glucosidase from brain. *Biochim. Biophys. Acta*. 113, 567– 576.
- Halliwell, G., and Halliwell, N. 1984. In *Methods of Enzymatic Analysis*. (Bergmeyer, H. U., Ed.), Vol. 5, pp. 18–31, VCH, Weinheim.
- Heredia, A., Guillen, R., Jimenez, A., and Fernandez-Bolanos, J. 1993. Activity of glycosidases during development and ripening of olive fruit. *Zeitschrift-fuer-Lebensmittel-Untersuchung-und-Forschung*. 196(2), 147-151.

- Howard, L.R., Burma, P., and Wagner, A.B. 1994. Firmness and cell wall characteristics of pasteurized jalapeno pepper rings affected by calcium chloride and acetic acid. *J. Food Sci.* 59(6), 1184-1186.
- Hudson, J.M.; and Buescher, R.W. 1986. Relationship between degree of pectin methylation and tissue firmness of cucumber pickles. *J. Food Sci.* 51, 138-140, 149.
- Hughes, J.C., Grant, A., Faulks, R.M., 1975. Texture of cooked potatoes: The effect of ions and pH on the comprehensive strength of cooked potatoes. *J. Sci. Food Agric.* 26, 739.
- Humphries, E.G. and Fleming, H.P. 1989. Anaerobic tanks for cucumber fermentation and storage *J. Agric. Eng. Res.* 44, 133-140.
- Imam, S., Shey, J., Wood, D., Glenn, G., Chiou, Bor-Sen; Inglesby, M., Ludvik, C., Klamczynski, A., and Orts, W. 2005. Structure and function of complex carbohydrates in produce and their degradation process. *Produce Degradation*. 563-597.
- Jauneau, A., Cabin-Flaman, A., Verdus, M.C., Ripoll, C. Thellier, M. 1994. Involvement of calcium in the inhibition of endopolygalacturonases activity in epidermis cell wall of Linum usitatissimum. *Plant Physiol. Biochem.* 32, 839–846.
- Kolpak, F. J., and Blackwell, J. 1976. Determination of the structure of cellulose II. *Macromolecules*. 9, 273–278.
- Konno, H., Yamaya, T., Yamasaki, Y., and Matsumoto, H. 1984. Pectic polysaccharide breakdown of cell walls in cucumber (*Cucumis sativus*) roots grown with calcium starvation. *Plant Physiol.* 76, 633–637.
- Krall, S.M. and McFeeters, R.F. 1998. Pectin hydrolysis: Effect of temperature, degree of methylation, pH, and calcium on hydrolysis rates. *J. Agric. Food Chem.* 46 (4), 1311-1315.
- Leaback, D.H., and Walker, P.G. 1961. Studies on glucosaminidase. 4. The fluorimetric assay of N-acetyl-beta-glucosaminidase. *Biochem. J.* 78, 151–156.
- Lederberg, J. 1950. The beta-D-galactosidase of *Escherichia coli*, strain K-12. *J. Bacteriol.* 60, 381–392.
- Lee, M., and MacMillan, J.D. 1968. Mode of action of pectic enzymes. I. Purification and properties of tomato pectinesterase. *Biochemistry*. 7: 4005-4010.
- Lee, Y., and Howard, L.R. 1999. Firmness and phytochemical losses in pasteurized yellow banana peppers (*Capsicum annuum*) as affected by calcium chloride and storage. *J. Agric. Food Chem.* 47, 700-703.

Li, Y.T., and Li, S.C. 1972. In Methods in Enzymology (Ginsburg, V., Ed.), Vol. 28, pp. 702–713, Academic Press, New York.

Litchman, M.A., and Upton, R.P. 1972. Gas chromatographic determination of residual methanol in food additives. *Anal. Chem.* 44:1497-1497.

Lorrungruang, C., Elegado, F.B., and Fujio, Y. 1993. Some properties of polygalacturonase from *Rhizopus japonicus* IFO 5318. *J. Fac. Agric. Kyushu Univ.* 37:297-305.

Lucier, Gary, and Biing-Hwan Lin, 2000. *Americans Relish Cucumbers*. Agricultural Outlook, Economic Research Service, United States Department of Agriculture.

Lucier, Gary. 2003. *Vegetable Consumption Away from Home on the Rise*. Economic Research Service, United States Department of Agriculture.

Ma, J.F., Yamamoto, R., Nevins, D.J., Matsumoto, H., Brown, P.H. 1999. Al binding in the epidermis cell wall inhibits cell elongation of okra hypocotyls. *Plant Cell Physiol.* 40, 549–556.

Ma, Seung-Jin., Hiratake, J., Nakai, Y., Izumi, M., Fukase, K., Kusumoto, S., Sakata, K. 2002. A Direct Continuous Spectrophotometric Assay for Glycosidases with 3-Nitro-2-pyridyl Glycosides by Tautomerization of 2-Hydroxy-3-nitropyridine. *Analytical Biochemistry*. 302(2), 291-297.

MacMillan, G.P., Johnston, D.J., Morel, J.B., and Perombelon, M.C.M. 1993. A pH independent assay for pectin methyl esterase for use in column chromatography. *Anal. Biochem.* 209, 377-379.

McFeeters, R.F., Bell T.A., and Fleming, H.P. 1980. An endo-polygalacturonase in cucumber fruit. *J. Food Biochem.* 4 (1), 1-16.

McFeeters, R.F., and Armstrong, S.A. 1984. Measurement of pectin methylation in plant cell walls. *Anal. Biochem.* 139, 212-217.

McFeeters, R.F., Fleming, H.P., and Thompson, R.L. 1985. Pectinesterase activity, pectin methylation, and texture changes during storage of blanched cucumber slices. *J. Food Sci.* 50(1), 201-205, 219.

McFeeters, R.F. 1985. *Changes in pectin and cellulose during processing*. In Chemical Changes in Food During Processing, T. R. Richardson and J. W. Finley, eds., Avi Publishing Company, Westport, CT, pp. 347-372.

McFeeters, R. F. and Lovdal, J. A. 1987. Sugar composition of cucumber cell walls during fruit development. *J. Food Sci.* 52 (4), 996-1001.

McFeeters, R.F. and Fleming, H.P. 1989. Inhibition of cucumber tissue softening in acid brines by multivalent cations: inadequacy of the pectin "egg box" model to explain textural effects. *J. Agric. Food Chem.* 37(4), 1053-1059

McFeeters, R.F. and Fleming, H.P. 1990. Effect of calcium ions on the thermodynamics of cucumber tissue softening. *J. Food Sci.* 55(2), 446-449.

McFeeters, R.F., Brenes B. M., and Fleming, H. P. 1995. Softening rates of fermented cucumber tissue: effects of pH, calcium, and temperature. *J. Food Sci.* 60(4), 786-788, 793.

McKay. 1988. A plate assay method for the detection of fungal polygalacturonase secretion. *FEMS Microbiology Letters.* 56(3), 355-358.

Meeuwsen, P.J.A., Vincken, J.P., Beldman, G., and Voragen, A.G.J. 2000. A universal assay for screening expression libraries for carbohydrates. *J. Biosci. Bioengin.* 89, 107-109.

Meurer, P and Gierschner, K. 1992. Occurrence and effect of indigenous and eventual microbial enzymes in lactic acid fermented vegetables. *Acta-Alimentaria.* 21(2), 171-188.

Miller, G.L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* 31, 426-428.

Miller, A.R., Dalmasso, J.P. and Kretchman, D.W. 1989. Developmental variation of cell wall degrading enzymes from cucumber (*Cucumis sativus*) fruit tissues. *Can. J. Bot.* 67, 817-821.

Mujer, C.V., and Miller, A.R. 1991. Purification and properties of β -xylosidase isozymes from cucumber seeds. *Plant Physiol.* 82, 367-376.

Nelson, N. 1944. A photometric adaption of the Somogyi method for the determination of glucose. *J. Biol. Chem.* 153:375-380

Pagel, W., and Heitefuss, R. 1990. Enzyme activities in soft rot pathogenesis of potato tubers: Effect of calcium, pH, and degree of pectin esterification on the activities of polygalacturonase and pectate lyase. *Physiol. Mol. Plant Pathol.* 37, 9–26.

Pilnik, W., and Voragen, A.G.J. 1991. The significance of endogenous and exogenous pectic enzymes in fruit and vegetable processing. In PF Fox, ed. *Food Enzymology*, Vol. 1. London: Elsevier Applied Science, pp 303-336.

Ponting, J.D. Jackson R., Watters, G. 1971. Refrigerated apple slices: effects of pH, sulfites and calcium on texture. *J. Food Sci.* 36, 349-350.

- Pressey, R., and Avants, J.K. 1975. Cucumber polygalacturonase. *J. Food Sci.* 40, 937-939.
- Rauscher, E. 1984. In *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Ed.), Vol. 4, pp. 157-161, VCH, Weinheim.
- Rexova-Benkova, L. and Marcovic, O. 1976. *Pectic enzymes*. In advances in Carbohydrate Chemistry and Biochemistry, Vol. 33, (R.S. Tipson and D. Horton, Eds.) pp. 323-385, Academic Press, New York.
- Rexova-Benkova, L., and Markova, M. 1978. *Pectic enzymes*. In advances in Carbohydrate Chemistry and Biochemistry, Vol. 33, (R.S. Tipson and D. Horton, Eds.) pp. 323-385, Academic Press, New York.
- Roberts, K. 1990. Structures of the plant cell surface. *Current Opinion in Cell Biology*. 2(5), 920-928.
- Robyn M. Perrin, Amy E. DeRocher, Maor Bar-Peled, Weiqing Zeng, 1,4 Lorena Norambuena, Ariel Orellana, Natasha V. Raikhel, Kenneth Keegstra. 1999. Xyloglucan Fucosyltransferase, an Enzyme Involved in Plant Cell Wall Biosynthesis. *Science*. 284 (5422), 1976-1979.
- Rombouts, F. M., and Pilinik, W. 1980. Pectic enzymes. In: *Economic Microbiology*. Microbial Enzymes and Bioconversions. A. H. Rose, ed. Vol. 5. Academic Press, New York, pp 227-282
- Saldana, G., and Meyer, R. 1981. Effects of added calcium on texture and quality of canned jalapeno peppers. *J. Food Sci.* 46, 1518-1520.
- Saltveit, M.E., Jr. and McFeeters, R.F. 1980. Polygalacturonase activity and ethylene synthesis during cucumber fruit development and maturation. *Plant Physiol.* 66 (6), 1019-1023.
- Schols, H.A., Vierhuis, E., Bakx, E.J., Voragen, A.G.J. 1995. Different populations of pectic hairy regions occur in apple cell walls. *Carbohydrate Research*. 275(2), 343-60.
- Smidsrod, O., Haug, A., Larsen, B. 1966. The influence of pH on the rate of hydrolysis of acidic polysaccharides. *Acta Chem. Scand.* 20, 1026-1034.
- Sumner, J.B., and Sisler, E.B. 1944. A simple method for blood sugar. *Arch. Biochem.* 4, 333-336.
- Takayanagi, T., Okuda, T., Yokotsuka, K. 1997. Changes in glycosidase activity in grapes during development. *Journal of the Institute of Enology and Viticulture*. Yamanashi University, 32, 1-4.

- Timell, T.E. 1967. Recent progress in the chemistry of wood hemicelluloses. *Wood Sci. Technol.* 1, 45–70.
- USDA, National Agricultural Statistics Service, Vegetable Yearbook 2004. “U.S. cucumbers for pickles: Supply, utilization, and price, farm weight, 1979-2004.”
- Van Hoof, F., and Hers, H.G. 1968. The abnormalities of lysosomal enzymes in mucopolysaccharides. *Eur. J. Biochem.* 7, 34–44.
- Versteeg, C. 1979. Pectinesterases from the orange fruit – their purification, general characteristics and juice cloud destabilizing properties. PhD thesis, Wageningen University.
- Virk, S.S., Cleland, R.E. 1990. The role of wall calcium in the extension of cell walls of soybean hypocotyls. *Planta.* 182, 559–564.
- Voragen, A.G.J., Schols, H.A., and Pilnik, W. 1986. Determination of the degree of methylation and acetylation of pectins by HPLC. *Food Hydrocolloids.* 1, 65-70.
- Voragen, A.G. J., Pilnik, W., Thibault, J.F., Axelos, M.A.V., and Renard, M.G.C. 1995. In *Food Polysaccharides and Their Applications* (Stephen, A. M., ed.) Marcel Dekker, Inc., New York, pp. 287–339.
- Waffenschmidt, S. and Jaenicke, L. 1987. Assay for reducing sugars in the nanomole range with 2'2'-bicinchoninate. *Anal Biochem.* 165, 337-340.
- Wilkie, K.C.B., and Woo, S.L. 1977. A heteroxylan and hemicellulosic materials from bamboo leaves, and a reconsideration of the general nature of commonly occurring xylans and other hemicelluloses. *Carbohydr. Res.* 57:145–162.
- Willats, W.G., Orfila, C., Limberg, G., Buchholt, H.C., van Alebeek, G.J., Voragen, A.G.J., Marcus, S.E., Christensen, T.M., Mikkelsen, J.D., Murray, B.S., Knox, J.P. 2001. Modulation of the degree and pattern of methyl-esterification of pectic homogalacturonan in plant cell walls. Implications for pectin methyl esterase action, matrix properties, and cell adhesion. *J. Biol. Chem.* 276(22), 19404-19413.
- Wong, K.P. 1970. A new fluorometric assay of beta-glucuronidase in tissues and serum. *Anal. Biochem.* 35, 35–45.
- Yemencioglu, A and Cemeroglu, B. 2004. Some characteristics of pectin methylesterase from cucumbers (*Cucumis sativus*). *Gida.* 29(1), 51-55.
- Zablockis, E., Huang, J., Muller, B., Darvill, A.G., Albersheim, P. 1995. Characterization of the cell-wall polysaccharides of *Arabidopsis thaliana* leaves. *Plant Physiol.* 107(4), 1129-38.

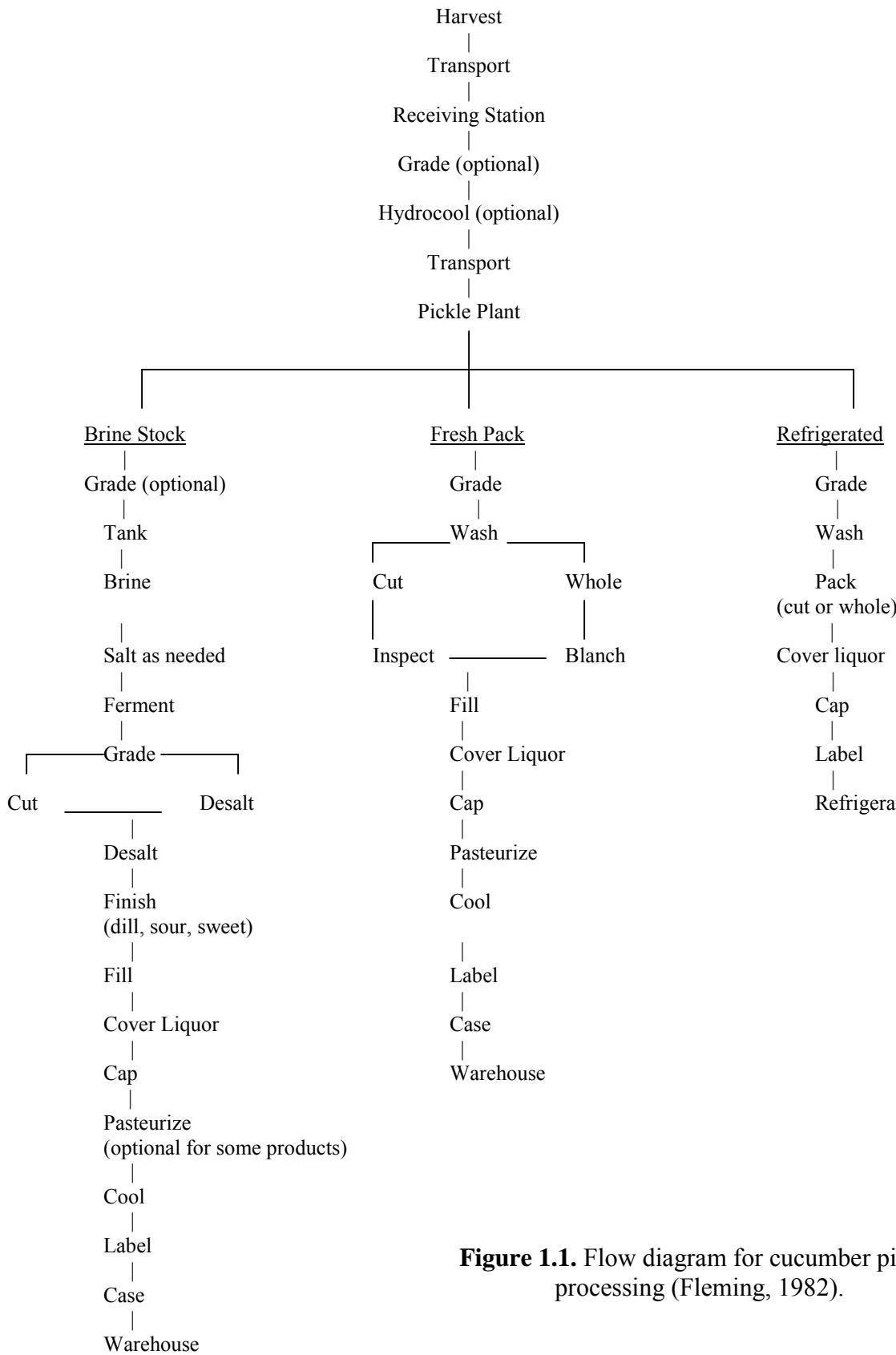


Figure 1.1. Flow diagram for cucumber pickle processing (Fleming, 1982).

CHAPTER 2

Evaluation of the importance of enzymatic and non-enzymatic softening in low salt cucumber fermentations

ABSTRACT

Retention of a firm, crisp fruit texture is a major consideration for pickled vegetables including pickles made from fermented cucumbers. Cucumbers fermented in low salt (3% or less NaCl) without added calcium soften rapidly. However, it is not clear if softening of cucumbers in low salt occurs as a result of enzymatic or non-enzymatic mechanisms. The objective of this research was to assess the relative importance of non-enzymatic and enzymatic reactions in cucumbers in the softening that occurs when cucumbers are fermented and stored at a low salt concentration.

Fermentation of cucumbers in 6.0% NaCl plus 18 mM calcium maintained firmness better than any treatment with reduced salt. Heat treatment of cucumbers sufficient to inactivate cell wall degrading enzymes before fermentation helped reduce firmness loss during fermentation and storage. However, heat treatment of cucumbers after fermentation did not prevent firmness loss during storage at 30°C. This suggested that reactions that resulted in softening of the cucumber tissue during storage had occurred during the fermentation period and that heating after fermentation could not prevent those changes.

Polygalacturonase activity could not be detected in the cucumbers used in these experiments. α -D-galactopyranosidase and β -D-glucopyranosidase lost their activity completely in cucumbers blanched at 65°C. Other enzymes detected in the cucumbers that hydrolyzed p-nitrophenylglycosides lost ~60 to 80% of their activity at this blanch temperature. Pectinesterase retained over 70% of its activity in cucumbers blanched at

65°C. Pectinesterase along with all of the glycosidases detected in fresh cucumbers lost all detectable activity when cucumber slices were blanched at 85°C. At equal ionic strength, α -D-galactopyranosidase and β -D-galactopyranosidase were inhibited to a greater extent by calcium ions than sodium ions. However, for β -D-glucopyranosidase, the inhibition was dependent primarily on the ionic strength of the solution. During the first week of fermentation in 1.75% NaCl, there was a complete loss of activity of all the glycosidases assayed.

The fact that cucumbers heated sufficiently to inactivate all the enzymatic activities measured and fermented at a low salt concentration lost firmness compared to cucumbers fermented in 6% salt with 18 mM calcium chloride showed that non-enzymatic reactions were important in low salt softening. In three of four lots of cucumbers fermented there was additional softening if the cucumber tissue did not receive a heat treatment. This suggested that enzymatic reactions can also contribute to the softening that occurs during fermentation and storage of cucumbers at low salt levels. However, it was not possible to associate softening with specific enzymatic reactions.

INTRODUCTION

Retention of firm crisp texture of cucumbers during storage and processing is important for pickle industry. Approximately 40% of the United States pickling cucumber crop is temporarily preserved by brine fermentation and storage. Traditionally cucumbers are fermented in 5-8% NaCl to permit rapid growth of lactic acid bacteria which convert fermentable sugars to lactic acid and other end products (Fleming et. al., 1987). After fermentation salt concentrations may be increased from 12 to 16% in order to avoid excessive dilution of brine from rainfall, to reduce the potential for microbial spoilage, and in cold climates to minimize freezing of the tanks in winter. Since finished pickle products contain only 2% to 4% salt, cucumbers are desalted after fermentation and storage. The salt leached out of fermented cucumbers is normally discarded, which creates a disposal problem. The U.S. Environmental Protection Agency (EPA) limits chloride discharge in fresh waters to 230 ppm (EPA, 1987). Thus it would be preferable to brine and store cucumbers at a sufficiently low concentration of salt to reduce or eliminate the necessity for desalting. However, it is not possible to assure firmness retention during storage when salt concentrations are sufficiently low so desalting is not required.

It has been shown that addition of calcium to fermenting cucumbers helps to improve the firmness retention and to make it unnecessary to increase salt levels during post fermentation storage (Buescher et al. 1979; Fleming et al., 1978, 1987; McFeeters and Fleming, 1991). Fleming et al. (1996) demonstrated that pH 3.5 was the optimum pH for assuring microbial stability and texture retention of brine-stock cucumbers at 4% salt.

However in lower salt, and at lower pH the firmness of cucumbers was decreased more rapidly. This type of softening as pH is lowered was also reported by McFeeters et al. (1995). The rate of softening was reduced by increasing calcium concentration.

The softening that occurs during fermentation and storage of cucumbers in low salt may be a result of enzymatic or non-enzymatic reactions that result in disruption of the cell walls of the fruit. For non-enzymatic softening, the evidence indicates non-covalent reactions, such as conformational changes in the cell wall polysaccharides, cause the softening. McFeeters and Fleming (1990) found both large entropy and enthalpies of softening for blanched and acidified cucumber tissue. Calcium ions slowed the rate of softening by reducing the entropy of activation. This is similar to the thermodynamics of protein denaturation, which occurs as a result of conformational changes that do not involve breaking of covalent bonds. In addition, the thermodynamic parameters for tissue softening were very different from those observed for acid hydrolysis of pectin (Krall and McFeeters, 1998), which is the primary polysaccharide for holding plant cell walls together. Among the major plant cell wall polysaccharides, pectin is the most susceptible to acid hydrolysis within the pH range of acidic foods (Smidsrod et al., 1966).

Cucumbers contain the enzymes that have been associated with cell wall changes and tissue softening during fruit ripening and in food processing. These include pectinesterase (Bell et al., 1951), exo-polygalacturonase (Pressey and Avants, 1975), endo-polygalacturonase (McFeeters et al., 1980), cellulase (Miller et al., 1989), endo-xylanase (Mujer et al., 1991), β -xylosidase (Mujer and Miller, 1991), and an endo-1,4- β -

glucanase (Omran et al., 1991). Endo-polygalacturonase activity was very low in immature cucumbers, but activity increased in the seed cavity during ripening or when immature cucumbers were exposed to ethylene (Saltveit and McFeeters, 1980). Enzymatic tissue softening has been shown to be responsible for economic losses in commercial cucumber fermentations, but this softening was found to be caused by fungal polygalacturonases (Bell et al., 1950; Etchells et al., 1958) rather than enzymes from the cucumber. Fasina et al., (2002) have attempted to improve firmness retention during reduced salt fermentation of cucumbers by using a blanch treatment to inactivate enzymes. However, it has not been shown that cucumber enzymes, which can degrade cell wall polysaccharides, have a significant role in cucumber tissue softening during fermentation and subsequent storage in low salt conditions.

The objective of this research was to assess the relative importance of non-enzymatic and enzymatic activities in cucumbers in the softening that occurs when cucumbers are fermented and stored with a low salt concentration.

MATERIALS AND METHODS

Fresh pickling cucumbers (size 2A; 25-32 mm diameter) of unidentified cultivar were obtained from a local processor. The fruit were in good condition, not notably desiccated, diseased, or mechanically damaged. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Fermentation procedure. Fermentations with whole cucumbers were done by packing size 2A cucumbers in 46 oz. jars with a 55:45 packout ratio (748 g cucumbers: 612 g brine). Equilibrated concentrations of NaCl, CaCl₂, and acetic acid in the brines are shown in table 2.1 along with blanch treatments. The jars were hermetically sealed by heating caps in boiling water and then closing by hand. A single rubber septum was put in each cap to allow removal of liquid samples during incubation. The day after packing, the jars were inoculated with *Lactobacillus plantarum* MOP3: M6 to get 10⁶ cfu/ml of the culture in each jar. Jars were stored at 30°C. Ten days after packing, brine samples from different treatments were taken to measure pH and the amount of lactic acid and acetic acid produced. Sampling was repeated after 14 days. If the acid concentrations at 10 and 14 day analysis were similar, fermentation was considered to be complete. However, if acid concentration increased between 10 and 14 days, the fermentation period was continued till 21 days. After fermentation was complete, the jars in one treatment with 1.75% NaCl were heated at 75°C in the center of the jar for 15 minutes. Half of the total number of jars from each treatment were sliced into 6 mm thick slices using a 7/32 cusinart plate and were repacked into 8 oz jars with the same fermentation brine with a

packout ratio of 55% cucumber slices: 45% brine. Texture analysis was done on cucumbers stored both as whole cucumbers and slices.

For experiments in which cucumbers were fermented as slices the cucumbers were first sliced, blanched, if required, and then packed into jars. Slices were cut to 6 mm thickness using 7/32 cusrinart plate. All slices in an experiment were randomly mixed prior to blanching and filling into 16 oz jars with a 55:45 packout ratio (264 g cucumbers: 216 g brine). As before, the jars were hermetically sealed by heating caps with a rubber septum in boiling water and then closed by hand. Jars were inoculated the day after filling with *Lactobacillus plantarum* MOP3: M6 to get 10^6 cfu/ml of the culture in each jar. Jars were stored at 30°C. Treatments for these experiments are shown in tables 2.2 and 2.3.

Culture preparation. *Lactobacillus plantarum* MOP3: M6 was obtained from the culture collection maintained by the Food Fermentation Laboratory, U.S. Department of Agriculture, Agriculture Research Service, Raleigh, North Carolina. Cultures were maintained at -80°C in MRS broth (Difco Laboratories, Detroit, MI) containing 16% glycerol. Cultures were streaked onto MRS agar plates and grown for 24 hrs at 30°C. Colonies (3-4) of the culture were transferred to 10 ml of MRS broth and grown overnight at 30°C. Cells were then centrifuged (5000 X g) and washed with 8.5 g/L NaCl (saline) and re-suspended in 10 ml saline. The culture was plated on MRS medium to determine actual cell numbers. Cells resuspended in saline solution were inoculated in to sealed jars.

Blanching. Whole cucumbers or cucumber slices were placed in a wire mesh basket and blanched in a steam kettle containing 120-150 liters of tap water. Whole cucumbers were blanched at 75°C for 15 min. Slices were blanched either at 55°C, 65°C or 75°C for 15 minutes or at 65°C or 85°C for 6 minutes followed by cooling under tap water (5-10°C) for 15 minutes or 6 minutes respectively. Cucumbers were then drained of excess water and packed. The blanch times were sufficient to raise the internal temperature of the cucumbers or slices to the target temperature (Fasina and Fleming, 2001).

Heating fermented cucumbers. For the heat treatment shown in table 2.1, whole fermented cucumbers packed in the fermentation jars were heated after fermentation to a center jar temperature of 75°C for 15 minutes. The time and temperature was similar to that of blanched whole cucumbers for that experiment.

Fermentation Treatments. The treatments included in the experiment are shown in tables 2.1, 2.2 and 2.3.

Texture measurement. Firmness measurements were made with a TA.TX2 Texture Analyzer (Texture Technologies Corp., Scarsdale, NY/Stable Micro Systems, Godalming, Surrey, UK) using a 3 mm diameter punch. Data were collected and analyzed using *Texture Expert* software (Texture Technologies Corp., Scarsdale, NY/Stable Micro Systems, Godalming, Surrey, UK). The force required to puncture the cucumber mesocarp was recorded and expressed in Newtons (N). Firmness measurements were done on 15 pieces of cucumber mesocarp from each of two jars from each treatment and

at each sampling time (Thompson et al., 1982). For the treatments shown in table 2.1, texture analysis on repacked slices was done at 2 days, 3, 7 and 10 weeks after repacking. Whole cucumbers from the same experiment were analyzed for texture at 4, 7, 10 and 13 weeks after packing. Firmness measurement on whole cucumbers was done on a 6 mm thick slice from the center of each cucumber. For the treatments shown in table 2.2, texture analysis was done at 2 days, 3, 6, 9 and 12 weeks after packing. Texture analysis was done on fresh cucumber samples at 2 days, 1, 2, 4, 8 and 12 weeks after packing for treatments listed in table 2.3.

Extraction of fresh and fermented cucumbers for enzyme activity measurements.

All cucumber slices packed in a jar were put in a Waring blender and blended for 15 minutes until a homogeneous slurry was obtained. No brine from the jars or additional water was added during blending. Sufficient dry NaCl was added while blending to obtain an equilibrated concentration of 6% NaCl in the slurry. The blended cucumber slurry was filtered through a single layer of Miracloth, a rayon polyester cloth with about a 0.25 mm pore size, (Calbiochem, San Diego, CA) and the filtrate centrifuged in a Sorvall RC 5B refrigerated centrifuge (Model 825 MP) at 13, 000 rpm (5000 x g) for 10 minutes. An aliquot of the supernatant cucumber juice (5-10 ml) was refrigerated and used to assay pectinesterase activity. The remainder of the supernatant juice was placed in 23 mm diameter x 30 cm dialysis bags (~ 25 to 30 ml in each bag). The volume of cucumber juice in the bags was first reduced about 2 to 5 fold by covering the bags with dry polyvinylpyrrolidone (PVP 360). Then PVP was washed off the bags using distilled water and the concentrated juice was dialyzed in 0.2 M, pH 5.5 sodium acetate buffer.

For 20 ml of concentrated cucumber juice 2 liters of 0.2 M, pH 5.5 sodium acetate buffer was used. The dialysis buffer was changed three times for each dialysis. After dialysis the juice was centrifuged again to get a clear liquid for doing enzyme assays.

Enzyme Assays. Glycosidase activities were measured by release of p-nitrophenol from the appropriate glycoside. All commercially available p-nitrophenyl glycosidases of sugars present in plant cell wall were tested to determine if they were enzymatically hydrolyzed by a dialyzed, pH 5.5, 6% NaCl extract from fresh cucumbers. Reaction mixtures (total volume 1 ml) contained dialyzed cucumber juice appropriately diluted with 0.2 M, pH 5.5 sodium acetate buffer. Reaction mixtures were equilibrated at 30°C in a water bath prior to addition of substrate. A 20 microliter aliquot of each substrate solution (table 2.4) was added to 1.0 ml of the diluted, centrifuged cucumber juice to initiate the reaction. Samples (200 microliters) were taken from a reaction mixture 1, 10, 20 and 30 minutes after substrate addition. Samples were transferred to a well in a Costar flat bottom, white 96 well plate (COS96fw) that contained 4 microliters of 3N NaOH to stop the enzymatic reaction and raise the pH to produce a yellow color due to ionization of p-nitrophenol released from the p-nitrophenyl glycoside substrate. The absorbance was measured at 399 nm with a Tecan Saphire plate reader (Tecan US, Research Triangle Park, NC). For each substrate control reactions were done with boiled cucumber extract in 0.2 M, pH 5.5 sodium acetate buffer and with sodium added. Reaction rates were calculated as nmoles of p-nitrophenyl glycoside released per minute per gram fresh weight of cucumber based upon an absorbance standard curve for p-nitrophenol under the

conditions of the assay. For each extract duplicate reactions were done with each substrate.

Extracts from fermenting cucumbers were prepared and assayed in the same way as fresh cucumber extracts. Adjustments were made in dilution of the enzyme extracts and in sampling times to take into account the loss of activity that occurred as a result of blanching cucumbers or during fermentation.

Pectinesterase activity was measured based upon the amount of NaOH required to maintain the pH of a pectin solution at pH 7.0 as pectinesterase hydrolyzed methoxyl groups from the pectin and released free carboxyl groups from galacturonic acid residues. Rapid set citrus pectin (Sigma Chemical Co., St. Louis, MO) was dissolved in 0.15 M NaCl to give a 0.5% solution. Twenty ml of the pectin substrate was put in a 50ml beaker with a magnetic stirring bar. Two ml of the cucumber juice prepared for pectinesterase assay was added to it. The pH was initially adjusted to 7.0 with 0.1 M NaOH. The pH was then maintained at 7.0 by addition of 0.05 N NaOH as the release of free carboxyl groups by pectinesterase proceeded. Measurement of pH was done with a Fischer Accumet pH meter using a corning semi-micro combo electrode. Control reactions were done with boiled cucumber extract and substrate in 0.2 M, pH 5.5 sodium acetate buffer with no cucumber extract added. Reactions were done in duplicates.

Polygalacturonase was assayed by the release of reducing groups at 30°C as a result of hydrolysis of sodium polypectate measured by the procedure of Nelson (Nelson, 1944).

Reagent solutions for the Nelson assay were copper reagent A, copper reagent B, ammonium molybdate reagent. Copper reagent A was made by mixing 25 g of anhydrous sodium carbonate, 20 g of sodium bicarbonate, 25 g of sodium potassium tartarate (Rochelle salt) and 200 g of sodium sulfate (anhydrous) in 1000 ml water. Copper reagent B was made by mixing 30 g of CuSO₄.5H₂O in 200 ml water containing 4 drops of conc. sulfuric acid. Equal amounts of copper reagent A and copper reagent B were mixed to make copper working reagent. Ammonium molybdate reagent was made by mixing two solutions; one containing 25 g of ammonium molybdate dissolved in 450 ml distilled water along with 21 ml conc. H₂SO₄ and the second one containing 3 g of sodium arsenate heptahydrate dissolved in 25 ml water. Both solutions were mixed and placed in an incubator at 37°C for 24 to 48 hrs. Galacturonic acid was used as the standard for this assay.

A mixture of 3 ml of 0.4 M, pH 5.5 Na acetate buffer and 3 ml of 0.333% Na polypectate was used as the substrate. Four milliliters of cucumber juice prepared for polygalacturonase assay was added to the 6 ml substrate and the test tubes were kept in 30°C water bath. One milliliter samples were taken at five time intervals (1 min, 2 hr, 4 hr, 6 hr, and 10 hr) and mixed with 1.0 ml of copper working reagent to stop the reaction. The samples were then boiled for 20 min immediately followed by cooling under tap water for 10 min. One milliliter of ammonium molybdate reagent was added to the samples. After color developed, the samples were centrifuged for 5 min at 12,000 x g for removal of precipitated substrate. Absorbance measurements were made at 600 nm using a Varian Cary 300 Bio UV-Visible spectrophotometer. Boiled cucumber extract and substrate in 0.2 M, pH 5.5 sodium acetate buffer with no cucumber extract added were

used as controls. Duplicate reactions were run. Reaction rates were calculated as nanomoles of galacturonic acid reducing groups released per minute per gram fresh weight of cucumber.

Effect of brine components on glycosidase activities. Fresh cucumbers were extracted with 6% NaCl and prepared for glycosidase activity assays as described above except the cucumber juice was not concentrated with PVP.

The three of the most active glycosides found in fresh cucumbers were used to evaluate the effect of NaCl and CaCl₂ concentration on inhibiting enzymatic activity. These were α-D-galactopyranosidase, β-D-galactopyranosidase and β-D-glucopyranosidase. The procedure as described above for sugar glycosidase assays was followed except that in addition to 0.2 M, pH 5.5 sodium acetate buffer, variable concentrations of sodium chloride and calcium chloride were added. Several equilibrated concentrations of NaCl (0%, 1%, 1.75%, 3.5% and 6%) were 18 mM calcium chloride or a combination of 0 mM, 18 mM, 36 mM, 100 mM, 330 mM calcium chloride. A series of calcium chloride concentrations (0, 18, 36, 100, 330 mM) was added to either 1.75% or 6% sodium chloride. These concentrations were achieved by making appropriate dilutions of NaCl and CaCl₂ stock solutions. A 10% NaCl stock solution was made in buffer to keep the pH constant and appropriate dilutions were made. 500 ml of this solution was made by dissolving 8.2g Na acetate and 50 g of NaCl. The pH was adjusted to 5.5 by 3N HCl. Similarly 500 ml of 0.5 M CaCl₂.2H₂O solution was made by dissolving 36.75 of CaCl₂.2H₂O and 8.2g Na acetate in water and adjusting the pH to 5.5 using 3N HCl.

Substrate solution (20 μ l) was added to the reaction mixture to give 3 mM of each substrate in the reactions. From the reaction mixture 200 μ l of samples were taken at 1, 10, 20 and 30 minutes after addition of substrate. Duplicate reactions were done for each substrate.

Statistical analysis. The ANOVA and GLM procedures of SAS[®] (Statistical Analysis Systems, Cary, NC) were used for all statistical computations and inferences.

RESULTS AND DISCUSSION

Effect of salt concentration and heat treatments on cucumber firmness. Fleming et al. (1987) showed that fermentation and storage of cucumbers in 1.0 M (5.8%) NaCl, 18 mM calcium and 53 mM acetic acid would result in fermented cucumbers with commercially acceptable firmness after one year or more of storage. To investigate the types of softening that occurs when cucumbers are fermented at a low salt concentration, the control cucumbers were brined and fermented with 6% NaCl, 18 mM calcium and 53 mM acetic at 30°C. Cucumbers, for experimental treatments, were fermented in only 0.3M (1.75%) NaCl without added calcium to assure that softening would occur relatively rapidly. Different heat treatments were initially investigated for their ability to affect firmness retention during storage.

Figure 2.1 shows changes in the firmness of mesocarp tissue during storage at 30°C after the active fermentation period. As expected whole cucumbers stored in 6% NaCl, 18 mM calcium did not show any substantial loss in firmness. Fermentation and storage in 0.3 M (1.75%) NaCl without added calcium resulted in a large loss in firmness. Cucumbers blanched at 75°C prior to fermentation retained firmness better than non-heated cucumbers. However, cucumbers heated at 75°C after the completion of fermentation softened similar to the low salt cucumbers that received no heat treatment. This result indicated that the changes that would result in more softening of the cucumber tissue during storage had occurred during the fermentation period. Heating after fermentation could not stop or reverse those changes.

Commercially cucumbers are fermented as whole fruit. However, experimentally it would be possible to apply heat more uniformly, use smaller amounts of sample, and do texture measurements and blend tissues more rapidly if experiments were done with sliced cucumbers. Therefore, after fermentation of the cucumbers in the experiment shown in figure 2.1, jars of all treatments were opened, sliced, repacked in the same fermentation brine. The sliced cucumbers were stored along with the jars of whole cucumbers. Figure 2.2 shows changes in firmness of the sliced, fermented cucumbers. The pattern of texture change was similar to that for whole cucumbers.

Figure 2.3 shows texture changes in another lot of cucumbers in which, with the exception of a low salt, no heat treatment, cucumbers were sliced prior to doing blanch treatments and fermentation. For the control cucumbers fermented with 6% NaCl and 18 mM calcium chloride there was about a 2N loss in firmness during fermentation with no further loss for 9 weeks. Just like the whole cucumber fermentations, the fermentation of slices in low salt resulted in softer slices. There was an initial firmness loss in the first two days after packing cucumbers and a slow reduction in firmness during 12 weeks of fermentation and storage. Firmness retention improved as the temperature of the blanch treatments increased from 55°C to 75°C. There was not an initial firmness loss when whole cucumbers were fermented. However, with only 0.3 M (1.75%) NaCl firmness declined more rapidly so that by 12 weeks cucumbers fermented whole were nearly as soft in texture as slices, where much of the firmness loss occurred in the first two days.

The effect of heating cucumbers prior to fermentation on firmness retention indicated that enzymatic activity had some role in softening cucumber tissue in low salt fermentation. However, the fact that cucumber tissue remained much more firm with 6% NaCl and added calcium clearly showed heating was not sufficient to overcome all the softening that resulted from reducing salt and calcium.

Enzymatic activity in fresh cucumbers. The next step was to determine which enzymatic activities that might be involved in cell wall degradation were present in cucumbers. Fresh cucumber extracts were analyzed for enzyme activities that might be involved in the degradation of cell wall polysaccharides (figure 2.4). Polygalacturonase and pectinesterase measurements were based upon release of reducing groups from polypectate and release of free carboxyl groups from high methoxyl pectin, respectively. Detection of all other enzyme activities was based upon release of p-nitrophenol from the p-nitrophenyl glycosides. All commercially available p-nitrophenyl glycosidases of sugars that occur in cucumber cell walls (McFeeters and Lovdal, 1987) were tested for their ability to be hydrolyzed by dialyzed extracts from fresh cucumbers even if the glycosidic bond was not one known to occur in plant cell walls (table 2.5).

The cucumber juice extracted using salt did not cause hydrolysis of those p-nitrophenyl glycosides with glycosidic linkages that are not known to occur in plant cell walls (table 2.6, Nos. 1-4). Five synthetic substrates with glycosidic linkages known to occur in cell wall were hydrolyzed by the cucumber extract (table 2.6, Nos. 5-9). In addition, p-nitrophenyl- α -D-mannoside was hydrolyzed (table 2.6, No. 10). This linkage is not

known to be present in plant cell walls, but has been found in the side chains of glycoproteins (Fry, 1995). Hydrolysis of p-nitrophenyl- β -D-mannoside and p-nitrophenyl- α -L-rhamnopyranoside did not occur to a detectable extent even though these glycoside linkages occur in cell walls (table 2.6, Nos. 11 & 12). This may be because these bonds only occur in the main chains of polysaccharides and not as end groups or side chains. However, α -D-xylopyranoside and α -L-arabinofuranoside glycosidic linkages occur as a side chain in cells wall polysaccharides, but no hydrolysis was seen (table 2.6, No. 13 & 14). The pattern of glycosidase activities observed suggests that several enzymes likely to hydrolyze sugar linkages in cell wall polysaccharides were present in these extracts. All of the p-nitrophenyl glycosidase activities detected here have previously been found in cucumbers (Mujer and Miller, 1991; Meurer and Gierschner, 1992), but no estimate of the amount of activity present in cucumbers was reported. Activities of all p-nitrophenyl glycosidase detected in fresh cucumbers plus a low level of α -glucosidase activity have also been found in ‘Bartlett’ pears (El-Rayah-Ahmed and Labavitch, 1980). Similar to cucumbers, ‘Bartlett’ pears did not show α -D-xylopyranosidase and α -L-arabinofuranosidase activities. They also are not known to contain α -L- and β -L-fucopyranosidase. All of these glycosidase activities except for β -glucosidase have also been detected in olives (Heredia et al., 1993). Activities of all six glycosidases found in cucumbers have also been reported in grapes (Takayanagi et al., 1997). These activities appear to be widely distributed in fruits and to have the ability to hydrolyze sugar residues from cell wall polysaccharides (Fry, 1995). However, the importance of these glycosidases in plant tissue softening is not clear (Tateishi et al., 2005).

In addition to glycosidases capable of hydrolysis of p-nitrophenyl glycosidases, pectinesterase and polygalacturonase activities were measured. Pectinesterase activity, much higher than the activities of any of the glycosidases in terms of the nmoles/min of bonds hydrolyzed per gram of cucumber tissue was detected (table 2.7). However, polygalacturonase activity could not be detected in the cucumbers used in these experiments even though the same assay method was used as in other investigations of cucumber polygalacturonase (McFeeters et al., 1980; Salveit and McFeeters, 1980).

Effect of ionic strength on activity of glycosidases: Calcium ions greatly reduce the rate of non-enzymatic softening of cucumber tissue (McFeeters and Fleming, 1990). This is presumed to be a result of binding to cell wall polysaccharides whether by cross-linking ionized carboxyl groups in pectin (Grant et al., 1973) or by some other binding mechanism (McFeeters and Fleming, 1990). Creation of cross-links among cell wall polysaccharide molecules could reduce the ability of enzymes to diffuse through the cell wall matrix and hydrolyze polysaccharides. In addition high concentration of ions could inhibit enzyme activities directly. It was thus of interest to investigate the combined effect of different levels of NaCl and CaCl₂ on the activity of enzymes. For this purpose, the three glycosidases that showed high activity in fresh cucumbers were selected. These were α -D-galactopyranosidase, β -D-galactopyranosidase and β -D-glucopyranosidase. The activity of these enzymes in cucumbers was analyzed in the presence of different

combinations of NaCl and CaCl₂ at pH 5.5, including the concentrations used for cucumber fermentations in this study.

The results in figures 2.5 A, B and C show that each of these enzymes was partially inhibited as sodium and calcium concentrations increased. For the 6% salt, 18 mM calcium concentration that effectively prevented cucumber softening during fermentation and storage, the inhibition was 88%, 56% and 49% for α -D-galactopyranosidase, β -D-galactopyranosidase and β -D-glucopyranosidase respectively. At equal ionic strength α -D-galactopyranosidase and β -D-galactopyranosidase were inhibited to a greater extent by calcium ions than sodium ions. However, for β -D-glucopyranosidase the inhibition was dependent primarily on the ionic strength of the solution.

These results show that some inhibition of glycosidases with specificity for glycosidic bonds in cell walls occurs when the ionic strength is high enough to maintain a high degree of tissue firmness. Thus, prevention of softening may in part be the consequence of partial inhibition of glycosidases, ion binding to cell walls that inhibit non-enzymatic softening reactions (McFeeters and Fleming, 1990), and, perhaps, physical restriction of the ability of enzymes to attack cell wall polysaccharides for which there currently exists no known measurement technique.

Heat inactivation of enzymatic activities in fresh cucumber slices. In the initial experiments, heating cucumber tissue improved firmness retention. Therefore it was of interest to determine the heat stability of the enzymes detected in fresh cucumbers. For

that reason, cucumbers were blanched at 65 and 85°C and the slurry obtained from the blanched cucumber slices was assayed for pectinesterase and those glycosidases that showed activity in fresh cucumber juice. It was observed that when cucumbers are blanched at 85°C all of the glycosidases detected and pectinesterase were inactivated. However, activity of some of the enzymes survived after heating slices for 6 min. at 65°C (table 2.7). Four of the six sugar glycosidases lost 60%-80% of their activity as compared to fresh cucumbers. α -D-galactosidase and β -D- glucosidase, lost all detectable activity after a 65°C blanch. Pectinesterase was the most heat resistant enzyme among those assayed in that it retained 71% of the activity. Heat inactivation of pectinesterase has also been reported by McFeeters et al. (1985). It was shown that pectinesterase is inactivated at 81°C, but not when cucumbers are blanched at 66°C or less. Meurer and Gierschner (1992) also found that pectinesterase was more heat stable than polygalacturonase because when cucumbers are heated at 65°C, pectinesterase retained 42% of its activity while polygalacturonase retained only 16% of its activity as compared to non heated slices. However, when cucumbers are heated at 79°C, both pectinesterase and polygalacturonase were inactivated completely. Unfortunately, for these experiments, the activity of polygalacturonase could not be detected even in fresh cucumbers and hence could not be compared to other enzymes.

Enzyme and texture changes during cucumber fermentation and storage.

After studying the effects of heat treatment and cucumber storage in 6% NaCl and 1.75% NaCl in the presence as well as in the absence of CaCl₂, and the behavior of cell wall polysaccharide degrading enzymes in fresh cucumbers, it was of interest to study the

significance of enzymatic activity on the texture retention of cucumbers during fermentation and storage. Sliced cucumbers were fermented and texture measurements were made during fermentation and storage. Pectinesterase and enzymes found in fresh cucumbers that hydrolyzed p-nitrophenyl sugars (table 2.6) were measured at each sampling point. Enzymes were extracted from all cucumber samples with 1M NaCl and then dialyzed in 0.2 M acetate buffer, pH 5.5. Therefore, activity differences reflect changes in the amount of active enzyme and not differences caused by variation of the amount of salt during extraction or the amount of NaCl or calcium ions in reaction mixtures.

With all treatments, enzyme activities declined rapidly after brining. Some residual pectinesterase could be detected in cucumbers during fermentation and storage. Reactivation of pectinesterase activity, such as was observed in brined, non-fermented cucumbers blanched at 81°C (McFeeters et al., 1985), was not observed during fermentation and storage of blanched cucumber slices. All of the other enzymes assayed had lost all detectable activity after one week with the exception of α -D mannopyranosidase. In the control treatment with 6% NaCl and 18 mM calcium some activity remained after one week in one of the lots of cucumbers (figure 2.11A). To the extent that six enzymes measured in these experiments are representative of the enzymes in cucumbers that degrade the cell wall polysaccharides, these results suggested that any enzymatic degradation of the cell wall that contributed to firmness loss would occur in the first few days after brining.

Loss in firmness was seen in non-heated cucumbers stored in 0.3M (1.75%) NaCl (figures 2.1, 2.3, 2.6A and 2.6B). For three of these lots of cucumbers, blanching cucumbers before fermentation resulted in some improvement in firmness retention. Since blanching at 75°/85°C would have inactivated all, or at least most of the enzymes that might degrade the cell wall, the difference in firmness between the high temperature blanch and the non-heated cucumbers may be firmness loss caused by enzymes in the cucumbers. The lack of difference in texture between blanched and non-blanced cucumbers in one experiment (Figure 2.6B) could be a result of a higher natural concentration of calcium in the tissue that reduced tissue softening.

The firmness loss in cucumbers blanched at 75°/85°C compared to the cucumbers fermented with 6% NaCl and 18 mM calcium ions must be a result of non-enzymatic softening reactions. The mechanism of non-enzymatic softening in acid conditions is not well understood. However, the results of Krall and McFeeters (1998) show that pectin hydrolysis in the pH range of fermented cucumbers is very slow. The data of Smidsrod (1966) indicated that pectic polysaccharides would be the most rapidly hydrolyzed polysaccharides present in cell walls in the pH range of acid foods. The results of those studies strongly indicate that non-enzymatic acid hydrolysis could not be responsible for softening. The thermodynamics of tissue softening is more consistent with non-enzymatic softening being a result of conformational changes in the cell wall components (McFeeters and Fleming (1990).

The control treatment with high salt (6% NaCl) and calcium (18 mM) always maintained a substantially firmer texture than any other treatment (figures 2.1, 2.2, 2.3, 2.6A and 2.6B). This combination of sodium and calcium chlorides therefore, must be able to inhibit softening, whether enzymatic or non-enzymatic. Calcium has been shown to inhibit non-enzymatic softening based upon its ability to lower the entropy of activation sufficiently that it raises the free energy of activation of the reaction (McFeeters and Fleming, 1990). However, in the fermented cucumbers, salt clearly must contribute to the prevention of non-enzymatic softening, since the difference between the 1.75% NaCl, 18 mM CaCl₂ and the control fermentations was only 4.25% NaCl (Figures 2.6A and 2.6B). There are three possible mechanisms by which 6% NaCl, 18 mM calcium inhibit softening related to enzymatic degradation. The salt and calcium may reduce the activities of enzymes that can degrade cell wall polysaccharides (Figure 2.5A-C) as a result of the high ionic strength partially inhibiting the activity of some enzymes. Cross linking of cell wall polysaccharides with calcium ions, whether by the ‘egg box’ mechanism (Grant et al., 1973) or by some other cross linking mechanism (McFeeters and Fleming, 1989), could maintain the structure of the wall despite some limited degradation of the polysaccharide. In addition, cross linking of polysaccharides by metal ions could reduce the ability of enzymes to diffuse in the wall matrix and gain access to polysaccharide glycosidic bonds and carry out hydrolysis reactions.

These results show some benefit for blanching cucumber tissue to reduce texture loss, but the improvement is not sufficient to maintain adequate firmness with low salt and calcium concentrations. Even if blanching were more effective, it is not practical in

commercial processing conditions to heat and cool many tons of cucumbers each day, while at the same time maintaining sufficient heating and cooling capacity to carryout the heating and cooling operations required to process fresh pack cucumbers.

The 6% NaCl, 18 mM calcium fermentations did provide conditions to consistently prevent softening, whether by enzymatic or non-enzymatic mechanisms. The ability of high concentrations of calcium to inhibit enzymatic activity equal to or better than NaCl at the same ionic strength (Figures 2.5A and 2.5B) as well as the ability of low calcium concentrations to reduce softening, raises the possibility that brining cucumbers with CaCl₂ only at lower concentrations than NaCl might provide a means to maintain cucumber firmness while substantially reducing waste generation. Even if the concentration of calcium required were so high that some removal of calcium from the fermented cucumbers were necessary, disposal of brines containing calcium chloride into soils would not be as detrimental as is disposal of NaCl. In areas where soils are deficient in calcium, such as the costal plain of North Carolina, addition of brines to soils containing waste calcium to soils might be environmentally beneficial.

CONCLUSIONS

Fermentation of cucumbers in 6.0% NaCl plus 18 mM calcium maintained firmness better than any treatment with reduced salt. Heat treatment of cucumbers sufficient to inactivate cell wall degrading enzymes before fermentation helped reduce firmness loss during fermentation and storage. However, heat treatment of cucumbers after fermentation did not prevent firmness loss during storage at 30°C. This suggested that reactions that resulted in softening of the cucumber tissue during storage had occurred during the fermentation period and that heating after fermentation could not prevent those changes. Glycosidases and pectinesterase rapidly lost activity at the beginning of the fermentation process. However, textural changes related to enzymatic mechanisms may not be fully evident until later in the storage period.

The effect of heating cucumbers prior to fermentation on firmness retention indicated that enzymatic activity had some role in softening cucumber tissue in low salt fermentation. During fermentation of cucumbers that are not blanched, activities of certain glycosidases like α -D-galactopyranosidase, β -D-galactopyranosidase and β -D-glucopyranosidase may be partially inhibited by high ionic strength in the fermentation brine caused by the presence of high NaCl and calcium. In addition to reducing the activity of cucumber enzymes early in the fermentation, cross-linking of cell wall polysaccharides by sodium and calcium ions might also restrict the ability of enzymes to attack cell wall polysaccharides and thereby prevent softening. Since calcium chloride may be more effective in preventing softening at lower concentrations than NaCl, fermentation of cucumbers in calcium chloride without the use of NaCl should be considered.

REFERENCES

- Bell, T.A., Etchells, J.L., and Jones, I.D. 1950. Softening of commercial cucumber salt-stock in relation to polygalacturonase activity. *Food Technol.* 4 (4), 157-163.
- Bell, T. A., Etchells, J. L., and Jones, I. D. 1951. Pectinesterase in the cucumber. *Arch. Biochem. Biophys.* 31 (3), 431-441.
- Buescher, R.W., Hudson, J.M., Adams, J.R. 1979. Inhibition of polygalacturonase softening of cucumber pickles by calcium chloride. *J. Food Sci.* 44(6), 1786-7.
- El-Rayah-Ahmed, A., and Labavitch, J.M. 1980. Cell wall metabolism in ripening fruit. II. Changes in carbohydrate-degrading enzymes in ripening 'Bartlett' pears. *Plant-Physiology.* 65(5), 1014-1016.
- EPA. 1987. Water quality criteria; availability of document. Fed. Reg. 52: 37655-37656. *Environ. Prot. Agency.* Washington, D.C.
- Etchells, J.L., Bell, T.A., Monroe, R.J., Masley, P.M., and Demain, A.L. 1958. Populations and softening enzyme activity of filamentous fungi on flowers, ovaries, and fruit of pickling cucumbers. *Appl. Microbiol.* 6 (6), 427-440.
- Fasina, O.O. and Fleming, H.P. 2001. Heat transfer characteristics of cucumbers during blanching. *J. Food Eng.* 47, 203-210.
- Fasina, O., Fleming, H., and Thompson, R. 2002. Mass transfer and solute diffusion in brined cucumbers. *J. Food Sci.* 67 (1), 181-187.
- Fleming, H. P., Thompson, R. L., Bell, T. A., and Hontz, L. H. 1978. Controlled fermentation of sliced cucumbers. *J. Food Sci.* 43 (3), 888-891.
- Fleming, H.P., McFeeters, R.F., and Thompson, R.L. 1987. Effects of sodium chloride concentration on firmness retention of cucumbers fermented and stored with calcium chloride. *J. Food Sci.* 52 (3), 653-657.
- Fleming, H.P., McDonald, L.C., McFeeters, R.F., Thompson, R.L., and Humphries, E. 1991. Fermentation of cucumbers without sodium chloride. *J. Food Sci.* 60(2), 312-315, 319.
- Fleming, H.P., Thompson, R.L., and McFeeters, R.F. 1996. Assuring microbial and textural stability of fermented cucumbers by pH adjustment and sodium benzoate addition. *J. Food Sci.* 61(4), 832-836.

Fry, S.C. 1995. Polysaccharide-Modifying Enzymes in the Plant Cell Wall. *Annual Review of Plant Physiology and Plant Molecular Biology*. 46, Page 497-520.

Grant, G.T., Morris, E.R., Rees, D.A., Smith, P.J.C., and Thom, D. 1973. Biological interactions between polysaccharides and divalent cations: The egg-box model. *FEBS Lett.* 32, 195-198.

Heredia, A., Guillen, R., Jimenez, A., and Fernandez-Bolanos, J. 1993. Activity of glycosidases during development and ripening of olive fruit. *Zeitschrift-fuer-Lebensmittel-Untersuchung-und-Forschung*. 196(2), 147-151.

Howard, L.R and Buescher,R.W. 1990. Cell wall characteristics and firmness of fresh pack cucumber pickles affected by pasteurization and calcium chloride. *Journal of Food Biochemistry*. 14(1), 31-43.

Krall, S.M. and McFeeters, R.F. 1998. Pectin hydrolysis: Effect of temperature, degree of methylation, pH, and calcium on hydrolysis rates. *J. Agric. Food Chem.* 46 (4), 1311-1315.

Kačuráková, M., Capek, P., Sasinková, V., Wellner, N. and Ebringerová, A. 2000. FT-IR study of plant cell wall model compounds: pectic polysaccharides and hemicelluloses. *Carbohydrate Polymers*. 43(2), 195-203.

McFeeters, R. F., Bell, T. A., and Fleming, H. P. 1980. An endo-polygalacturonase in cucumber fruit. *J. Food Biochem.* 4 (1), 1-16.

McFeeters, R.F., Fleming, H.P., and Thompson, R.L. 1985. Pectinesterase activity, pectin methylation, and texture changes during storage of blanched cucumber slices. *J. Food Sci.* 50(1), 201-205, 219.

McFeeters, R. F. and Lovdal, J. A. 1987. Sugar composition of cucumber cell walls during fruit development. *J. Food Sci.* 52 (4), 996-1001.

McFeeters, R.F. and Fleming, H.P. 1989. Inhibition of cucumber tissue softening in acid brines by multivalent cations: inadequacy of the pectin "egg box" model to explain textural effects. *J. Agric. Food Chem.* 37(4), 1053-1059

McFeeters, R.F. and Fleming, H.P. 1990. Effect of calcium ions on the thermodynamics of cucumber tissue softening. *J. Food Sci.* 55 (2), 446-449.

McFeeters, R.F. and Fleming, H.P. 1991. pH Effect on calcium inhibition of softening of cucumber mesocarp tissue. *J. Food Sci.* 56 (3), 730-732, 735.

McFeeters, R.F., Brenes B.M., and Fleming, H.P. 1995. Softening rates of fermented cucumber tissue: effects of pH, calcium, and temperature. *J. Food Sci.* 60(4), 786-788, 793.

Meurer P. and Gierschner K. 1992. Occurrence and effect of indigenous and eventual microbial enzymes in lactic acid fermented vegetables. *Acta-Alimentaria*. 21(2): 171-188.

Miller, A.R., Dalmasso, J.P and Kretchman,D.W. 1989. Developmental variation of cell wall degrading enzymes from cucumber (*Cucumis sativus*) fruit tissues. *Canadian-Journal-of-Botany*. 67(3), 817-821.

Mujer, C.V and Miller, A.R. 1991. Purification and properties of beta-xylosidase isozymes from cucumber seeds. *Physiologia-Plantarum*. 82(3), 367-376.

Mujer, C.V., Kretchman, D.W. and Miller, A.R. 1991. Partial purification and properties of an endo-xylanase from cucumber seeds. *Physiologia-Plantarum*. 81, 327-334.

Nelson, N. 1944. A photometric adaption of the Somogyi method for the determination of glucose. *J. Biol. Chem.* 153:375-380.

Omran, H., Buckenhueskes, H., Jaeckle, E., and Gierschner, K. 1991. Characteristics of pectinesterase, exo-polygalacturonase and an endo-beta-1,4-glucanase from pickled cucumbers. *Deutsche-Lebensmittel-Rundschau*. 87(5), 151-156.

Pressey, R., and Avants, J.K. 1975b. Cucumber polygalacturonase. *J. Food Sci.* 40, 937-939.

Saltveit, M.E., Jr. and McFeeters, R.F. 1980. Polygalacturonase activity and ethylene synthesis during cucumber fruit development and maturation. *Plant Physiol.* 66 (6), 1019-1023.

Smidsrod, O., Haug, A., Larsen, B. 1966. The influence of pH on the rate of hydrolysis of acidic polysaccharides. *Acta Chem. Scand.* 20, 1026-1034.

Takayanagi, T., Okuda, T., Yokotsuka, K. 1997. Changes in glycosidase activity in grapes during development. *Journal of the Institute of Enology and Viticulture*. Yamanashi University, 32, 1-4.

Tang, H.C.L. and McFeeters, R.F. 1983. Relationships among cell wall constituents, calcium and texture during cucumber fermentation and storage. *J. Food Sci.* 48(1), 66-70.

Tateishi, A., Mori, H., Watari, J., Nagashima, K., Yamaki, S., and Inoue, H. 2005. Isolation, characterization, and cloning of a-L-arabinofuranosidase expressed during fruit ripening of Japanese pear. *Plant Physiology*. 138, 1653-1664.

Thompson, R.L., Fleming, H.P., Hamann, D.D., and Monroe, R.J. 1982. Method for determination of firmness in cucumber slices. *J. Text. Studies*.13 (3), 311-324.

Table 2.1. Experimental design to test the effect of low salt, blanching and post fermentation heating on the firmness retention of size 2A fermented cucumbers. Cucumbers were packed and fermented whole. After fermentation half the jars were sliced and repacked.

Treatment No. ¹	Description	NaCl, % ²	Calcium, mM ²	Blanch temp., °C	Post fermentation temp., °C	Whole (W)/ Sliced (S)
1	High salt, Ca ²⁺	6	18	—	—	W
2	Low salt	1.75	0	—	—	W
3	Low salt, heated post-fermentation,	1.75	0	—	75	W
4	Low salt, blanch	1.75	0	75	—	W
5	High salt, Ca ²⁺	6	18	—	—	S
6	Low salt	1.75	0	—	—	S
7	Low salt, heated post-fermentation,	1.75	0	—	75	S
8	Low salt, blanch	1.75	0	75	—	S

¹ Acetic acid was added to equilibrate at 53 mM in each treatment.

² Equilibrated concentrations.

Table 2.2. Experimental design to test the effect of blanch treatments on firmness retention of cucumber slices fermented in 1.75% NaCl.

Treatment No. ¹	Description	NaCl, % ²	Calcium, mM ²	Blanch temp., °C
1	High salt, Ca ²⁺	6	18	—
2	55°C blanch	1.75	0	55
3	65°C blanch	1.75	0	65
4	75°C blanch	1.75	0	75
5	Low salt	1.75	0	—
6	Whole, low salt	1.75	0	—

¹ Acetic acid was added to equilibrate at 53 mM in each treatment.

² Equilibrated concentrations.

Table 2.3. Experimental design to test the effect of blanching and calcium ion addition on texture retention and enzyme activities in cucumber slices fermented in 1.75% NaCl.

Treatment No. ¹	Description	NaCl, % ²	Calcium, mM ²	Blanch temp., °C
1	High salt, Ca ²⁺	6	18	—
2	Low salt	1.75	0	—
3	Low salt, Ca ²⁺	1.75	18	—
4	65°C blanch	1.75	0	65
5	85°C blanch	1.75	0	85

¹ Acetic acid was added to equilibrate at 53 mM in each treatment.

² Equilibrated concentrations.

Table 2.4. p-nitrophenyl glycosides used to detect different glycosidase activities in 6% NaCl extracts from fresh cucumbers.

Substrate used to detect glycosidase activity	Sigma-Aldrich catalog number	Solvent	Stock solution (mg/ml of solvent)	Concentration (mM) in stock solution	Concentration (mM) in reaction mixture
4-nitrophenyl- α -L-arabinopyranoside	N 3512	50% acetone & water	40.7	150.0	3.0
4-nitrophenyl- α -D-galactopyranoside	N 0877	50% acetone & water	42.8	140.0	2.8
4-nitrophenyl- β -D-galactopyranoside	N 1252	50% acetone & water	45.2	150.0	3.0
4-nitrophenyl- β -D-glucopyranoside	N 7006	50% acetone & water	45.2	150.0	3.0
4-nitrophenyl- α -D-mannopyranoside	N 2127	50% acetone & water	45.2	150.0	3.0
4-nitrophenyl- β -D-xylopyranoside	N 2132	50% acetone & water	40.7	150.0	3.0
4-nitrophenyl- α -L-arabinofuranoside	N 3641	100% acetone	27.2	100.0	2.0
4-nitrophenyl- β -L-arabinopyranoside	N 0520	50% acetone & water	40.7	150.0	3.0
4-nitrophenyl- β -D-fucopyranoside	N 3378	50% acetone & water	40.7	145.0	2.9
4-nitrophenyl- β -D-galacturonide	N 8755	100% acetone	45.2	145.0	2.9
4-nitrophenyl- α -D-glucopyranoside	N 1377	50% acetone & water	47.3	155.0	3.1
4-nitrophenyl- β -D-mannopyranoside	N 1268	50% acetone & water	45.2	150.0	3.0
4-nitrophenyl- α -L-rhamnoside	N 3513	60% acetone & 40% water	42.8	150.0	3.0
4-nitrophenyl- α -D-xylopyranoside	N 1895	50% acetone & water	40.7	150.0	3.0

Table 2.5. Structures in plant cell wall polysaccharides (Kacurakova et al. (2000).

S. No.	Compound	Structure	
		Backbone	Side chain
1	Pectin	$\alpha\text{-}(1 \rightarrow 4)\text{-GalpA} +$ RhaGalUA-I	$\alpha\text{-Araf-(1 \rightarrow 4,5)\text{-Galp}_n,$ $\alpha\text{-}(1 \rightarrow 3,5)\text{-Araf}_n$
2	Rhamnogalacturonan	$\alpha\text{-}(1 \rightarrow 4)\text{-GalpA}$ branched at O-3 $\alpha\text{-}(1 \rightarrow 4)\text{-Rhap}$ branched at O-4	$\beta\text{-GlcA}$ $\beta\text{-}(1 \rightarrow 4)\text{-Galp}$
3	Galactan	$\beta\text{-}(1 \rightarrow 6)\text{-Galp}$	—
4	Arabinan	$\alpha\text{-}(1 \rightarrow 5)\text{-Araf}$ branched at O-2 or O-3	$\alpha\text{-Araf}$
5	Arabinogalactan	$\beta\text{-}(1 \rightarrow 6)\text{-Galp}$ branched at O-3	$\alpha\text{-Araf}$ $\alpha\text{-}(1 \rightarrow 5)\text{-Araf}_n$
6	Arabinogalactan	$\beta\text{-}(1 \rightarrow 3)\text{-Galp}$ branched at O-6	$\alpha\text{-Arap}, \alpha\text{-}(1 \rightarrow 3)\text{-Araf}_n$ $\beta\text{-Galp}, \beta\text{-}(1 \rightarrow 6)\text{-Galp}$
7	Arabinogalactan (Type II)	$\beta\text{-}(1 \rightarrow 3)\text{-Galp}$ branched at O-6	$\beta\text{-}(1 \rightarrow 6)\text{-Galp}, \alpha\text{-Araf},$ $\alpha\text{-}(1 \rightarrow 5)\text{-Araf}_n$
8	Arabinogalactan (Type II) + Glucomannan (9:1, (w/w))	$\beta\text{-}(1 \rightarrow 3)\text{-Galp}$ branched at O-6 + $\beta\text{-}(1 \rightarrow 4)\text{-GlcP},$ $\beta\text{-}(1 \rightarrow 4)\text{-ManP}$	$\beta\text{-}(1 \rightarrow 6)\text{-Galp}, \alpha\text{-Araf},$ $\alpha\text{-}(1 \rightarrow 5)\text{-Araf}_n$
9	Arabinogalacto- rhamnoglycan	$\beta\text{-}(1 \rightarrow 6)\text{-Galp}$ branched at O-3 $\alpha\text{-}(1 \rightarrow 4)\text{-Rhap}$	$\alpha\text{-Araf},$ $\alpha\text{-}(1 \rightarrow 5)\text{-Araf}$
10	Xyloglucan	$\beta\text{-}(1 \rightarrow 4)\text{-GlcP}$ branched at O-6	$\alpha\text{-Xylp}, \alpha\text{-}(1 \rightarrow 2)\text{-Xylp},$ Terminal $\beta\text{-}(1 \rightarrow 2)\text{-Galp},$ $\alpha\text{-FucP}$
11	Glucan	$\alpha\text{-}(1 \rightarrow 6)\text{-GlcP}$	—
12	Glucomannan	$\beta\text{-}(1 \rightarrow 4)\text{-ManP}$ $\beta\text{-}(1 \rightarrow 4)\text{-GlcP}$	—
13	Galactoglucomannan	$\beta\text{-}(1 \rightarrow 4)\text{-ManP}$ $\beta\text{-}(1 \rightarrow 4)\text{-GlcP}$	$\alpha\text{-Galp}$
14	Arabinoxylan + galactoglucomannan	$\beta\text{-}(1 \rightarrow 4)\text{-Xylp} +$ $\beta\text{-}(1 \rightarrow 4)\text{-GlcP} +$ $\beta\text{-}(1 \rightarrow 4)\text{-ManP}$	$\alpha\text{-Araf}, \alpha\text{-4MeGA}, \alpha\text{-Galp}$

Table 2.6. Cell wall exo-O-glycosylhydrolases and the wall polymers that are their potential natural substrates.

No.	Enzyme	Wall polymers that are possible substrates	Activity found (+)/not found (-)
1	β -L-arabinopyranosidase	none known	—
2	β -D-fucopyranosidase	none known	—
3	β -D-galacturonidase	none known	—
4	α -D-glucopyranosidase	none known	—
5	α -L-arabinopyranosidase	arabinogalactan	+
6	β -D-xylopyranosidase	xylans	+
7	β -D-glucopyranosidase	cellulose, xyloglucan, callose, MLG	+
8	α -D-galactopyranosidase	Mannans, extensins	+
9	β -D-galactopyranosidase	RG-I, xyloglucan	+
10	α -D-mannopyranosidase	Glycoprotein side chains (e.g. peroxidase)	+
11	β -D-mannopyranosidase	mannans	—
12	α -L-rhamnopyranosidase	RG-II	—
13	α -D-xylopyranosidase	Xyloglucans	—
14	α -L-arabinofuranosidase	RG-I, xylans, extensins	—

Table 2.7. Enzyme activities in fresh and blanched cucumber tissue.

Enzymes	Fresh (nmol/min/g cucumber)	65°C blanch (% activity remaining)
Pectinesterase	2870 (± 410)	70.9
α -L-arabinosidase	0.012 (± 0.001)	19.4
α -D-galactosidase	0.63 (± 0.10)	<1
β -D-galactosidase	0.82 (± 0.12)	38.9
β -D-glucosidase	0.078 (± 0.099)	0
α -D-mannosidase	0.60 (± 0.06)	30.7
β -D-xylosidase	0.029 (± 0.010)	32.9

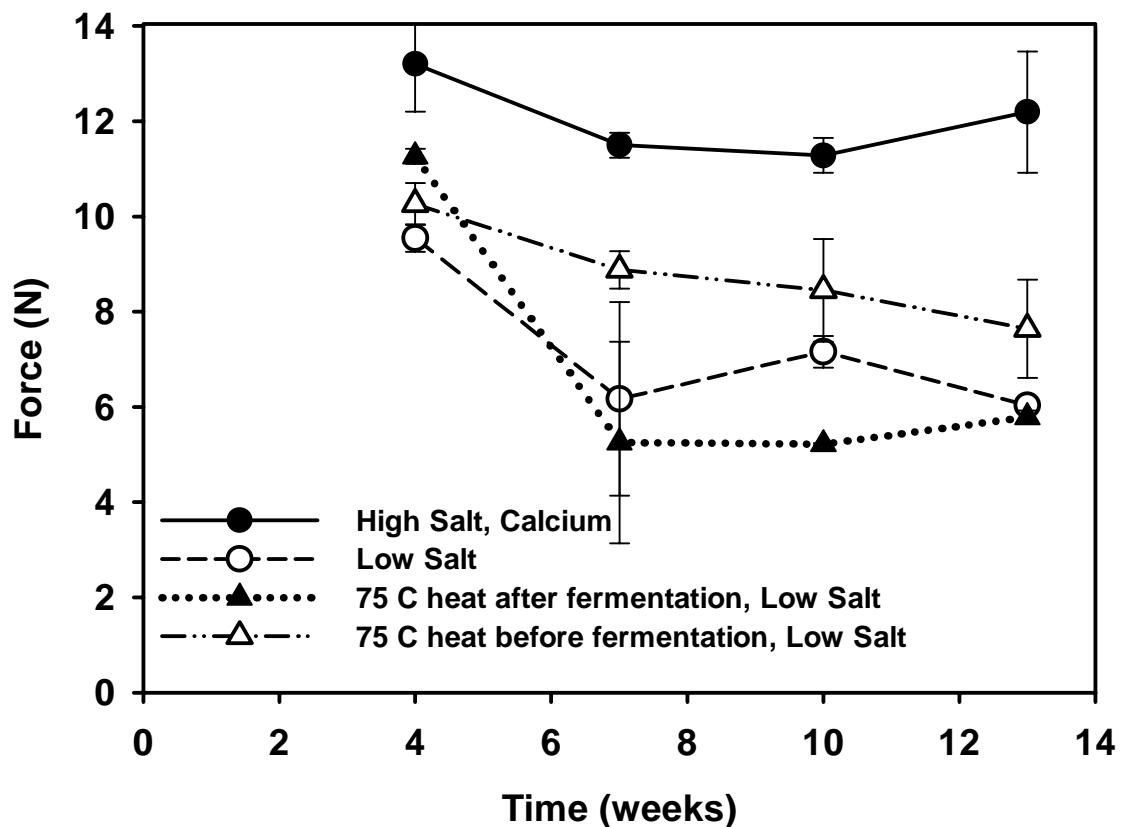


Figure 2.1. Firmness changes in whole cucumber mesocarp tissue after fermentation. Control contains 6.0% NaCl, 18 mM CaCl₂. Other treatments contain 1.75% NaCl without added calcium. Blanching was done at 75°C for 15 minutes. Post fermentation heating was done to a center jar temperature of 75°C for 15 minutes.

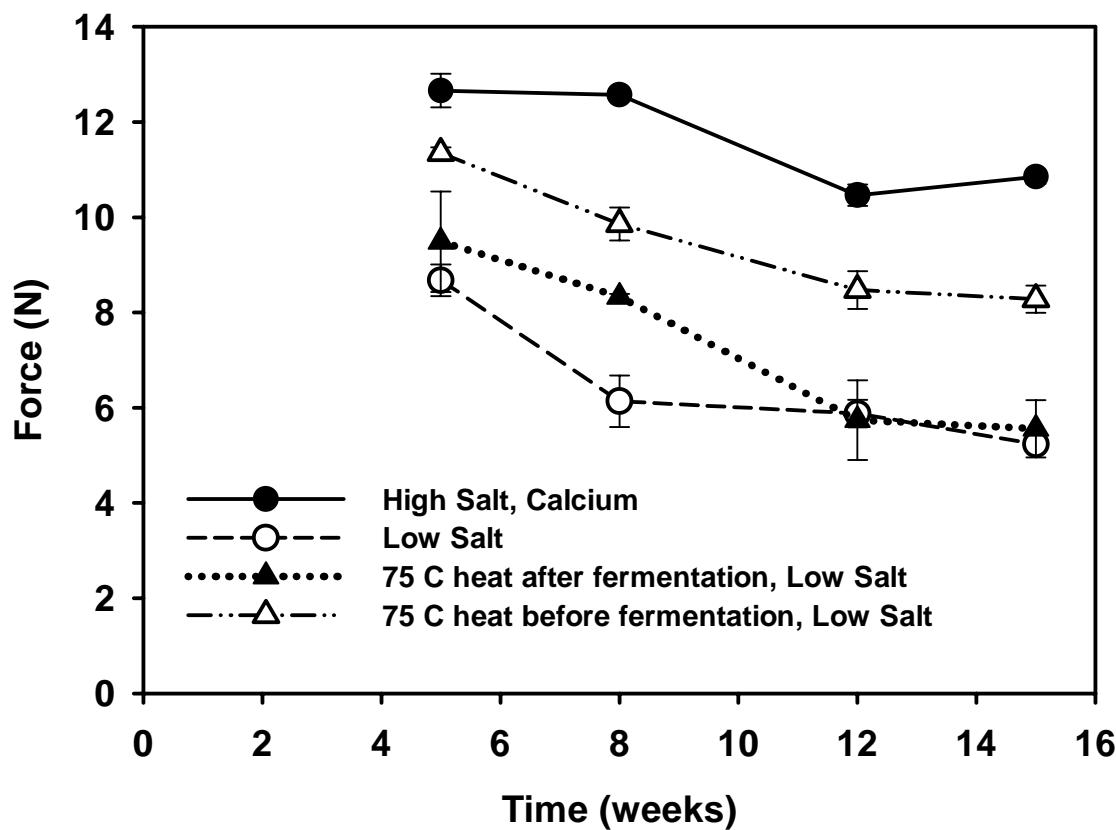


Figure 2.2. Firmness changes in sliced cucumber mesocarp tissue after fermentation. Control contains 6.0% NaCl, 18 mM CaCl₂. Other treatments contain 1.75% NaCl without added calcium. Blanching was done at 75°C for 15 minutes. Post fermentation heating was done to a center jar temperature of 75°C for 15 minutes.

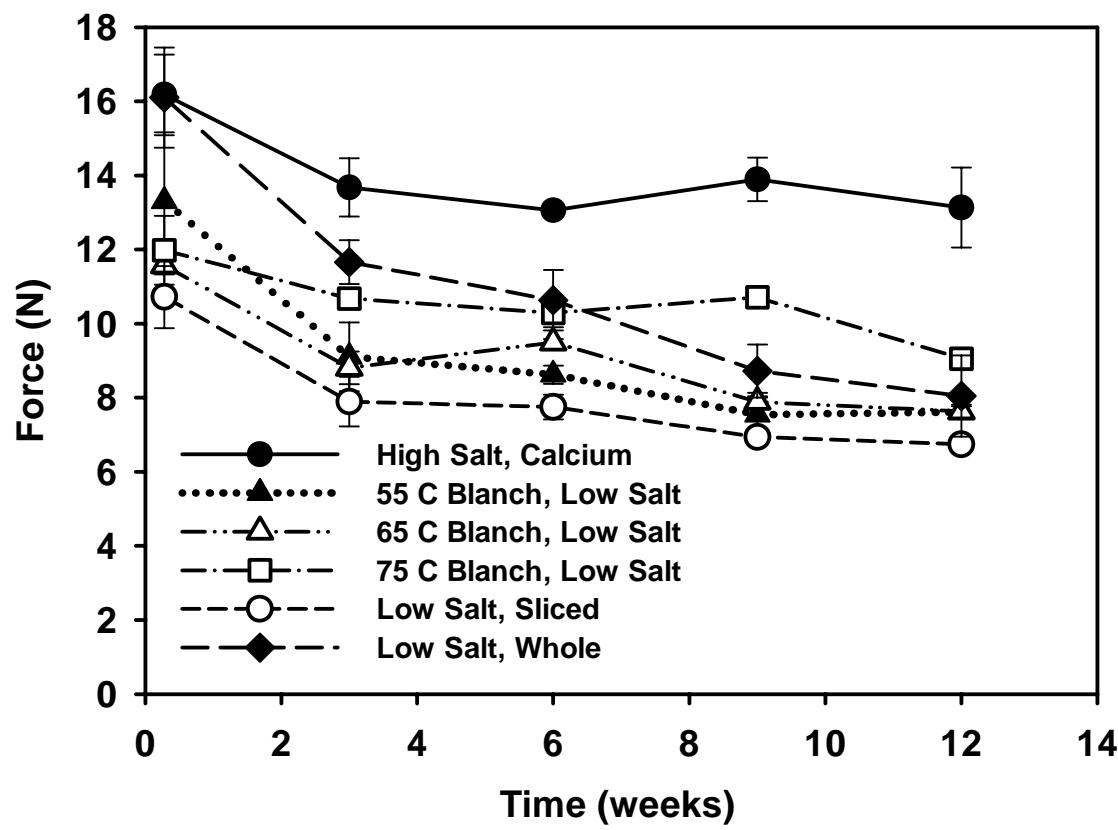
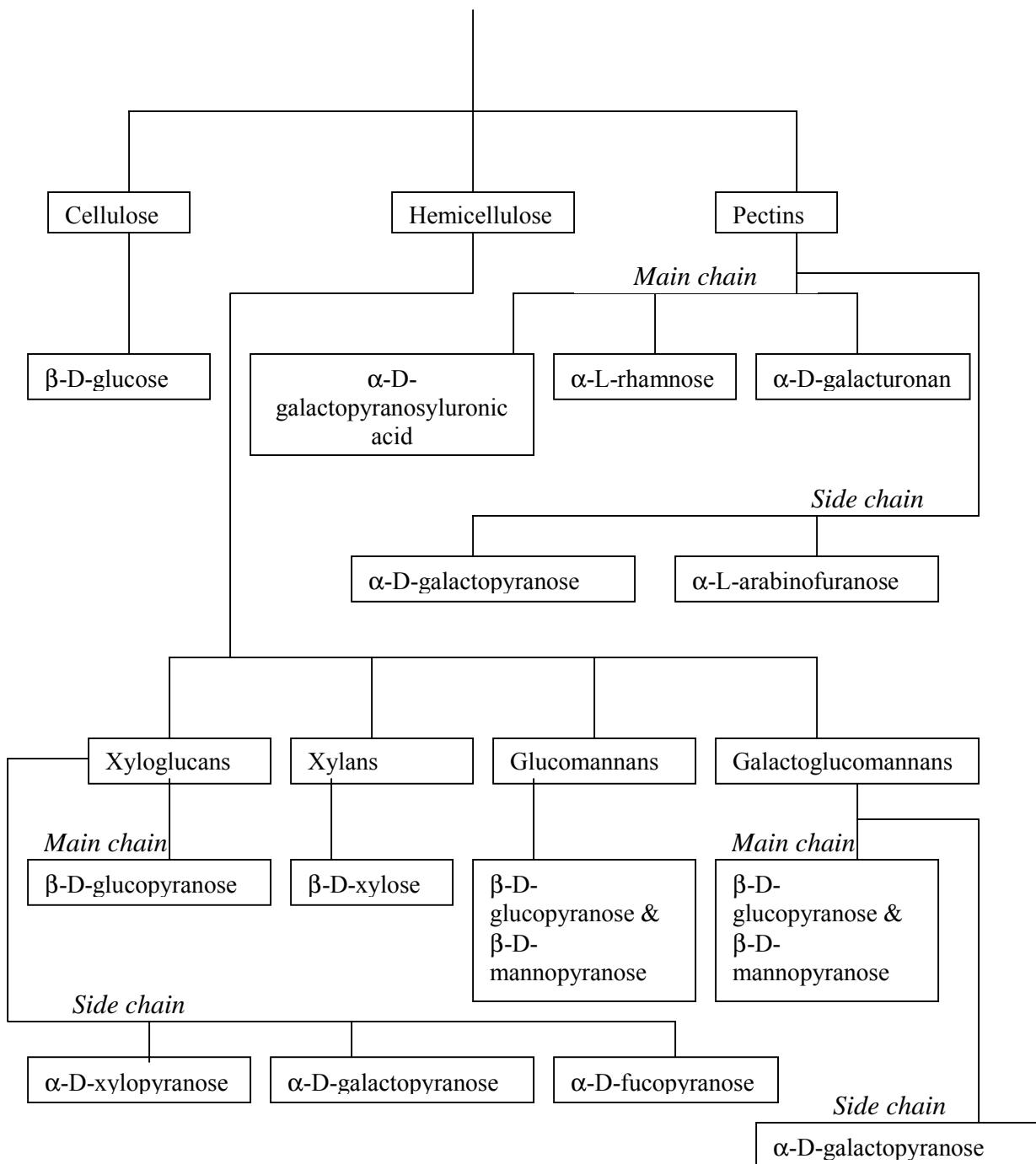


Figure 2.3. Firmness changes in cucumber mesocarp tissue after fermentation. Control contains 6.0% NaCl, 18 mM CaCl₂. Other treatments contain 1.75% NaCl without added calcium. Blanching was done at 55°C, 65°C and 75°C for 15 min.

Figure 2.4. Major polysaccharides in primary cell wall of plants



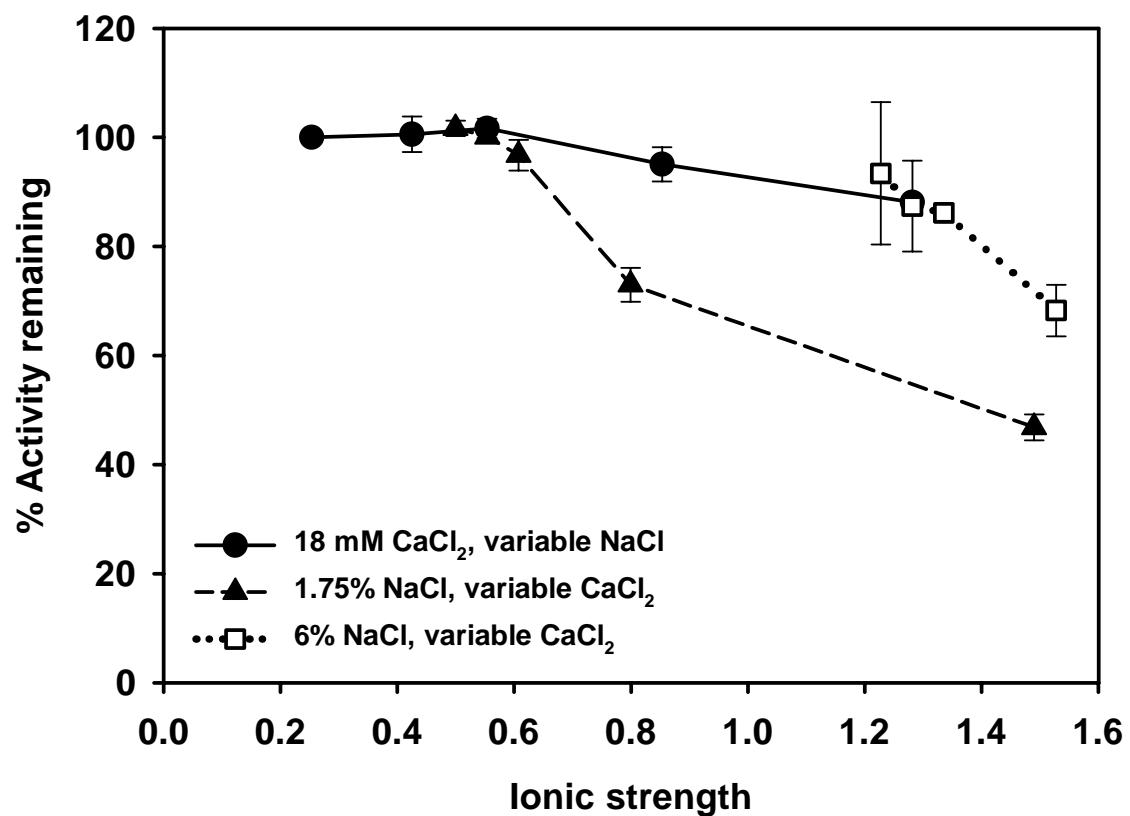


Figure 2.5A. Changes in the activity of α -D-galactopyranosidase from fresh cucumbers on increasing ionic strength. Ionic strength was changed by having different concentrations of NaCl and CaCl₂ in the reaction mixture.

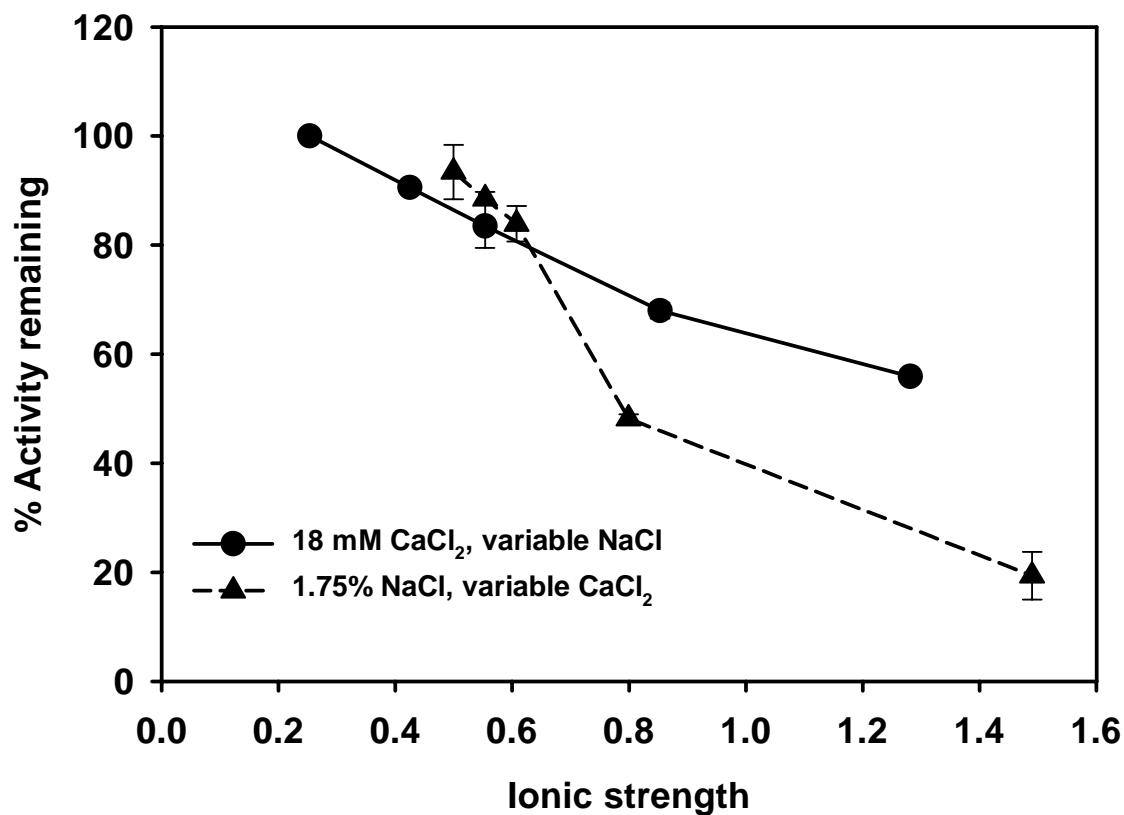


Figure 2.5B. Changes in the activity of β -D-galactopyranosidase from fresh cucumbers on increasing ionic strength. Ionic strength was changed by having different concentrations of NaCl and CaCl₂ in the reaction mixture.

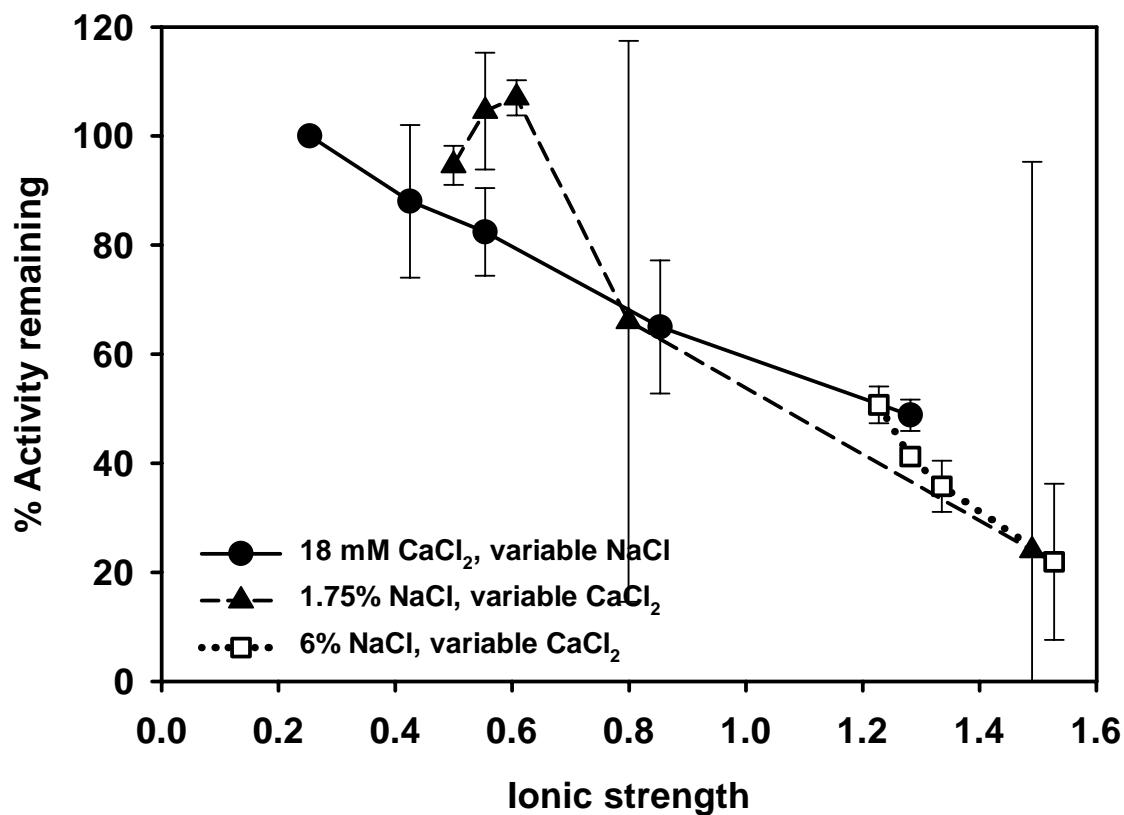


Figure 2.5C. Changes in the activity of β -D-glucopyranosidase from fresh cucumbers on increasing ionic strength. Ionic strength was changed by having different concentrations of NaCl and CaCl_2 in the reaction mixture.

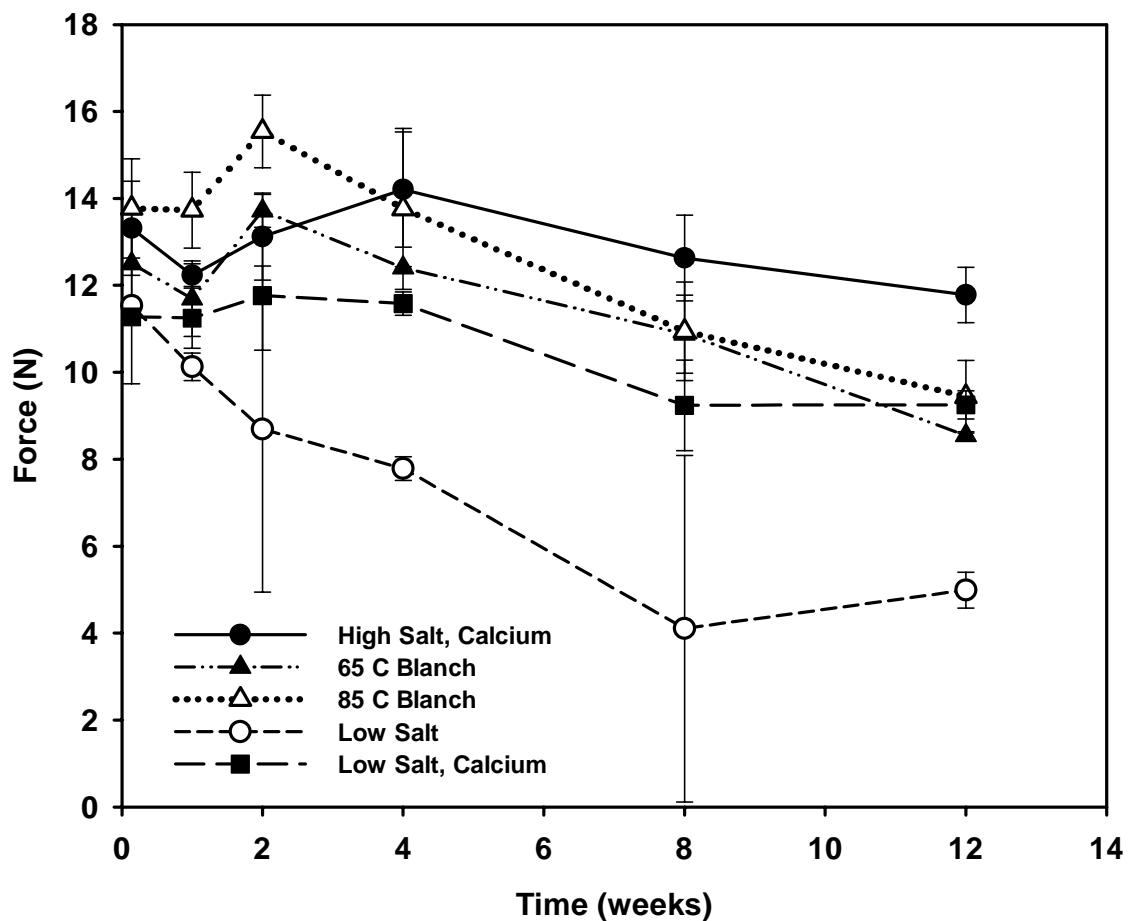


Figure 2.6A. Firmness changes in two lots of cucumbers during and after fermentation (Rep I). Control contains 6.0% NaCl, 18 mM CaCl₂. Other treatments contain 1.75% NaCl. 18 mM CaCl₂ added to the calcium treatments.

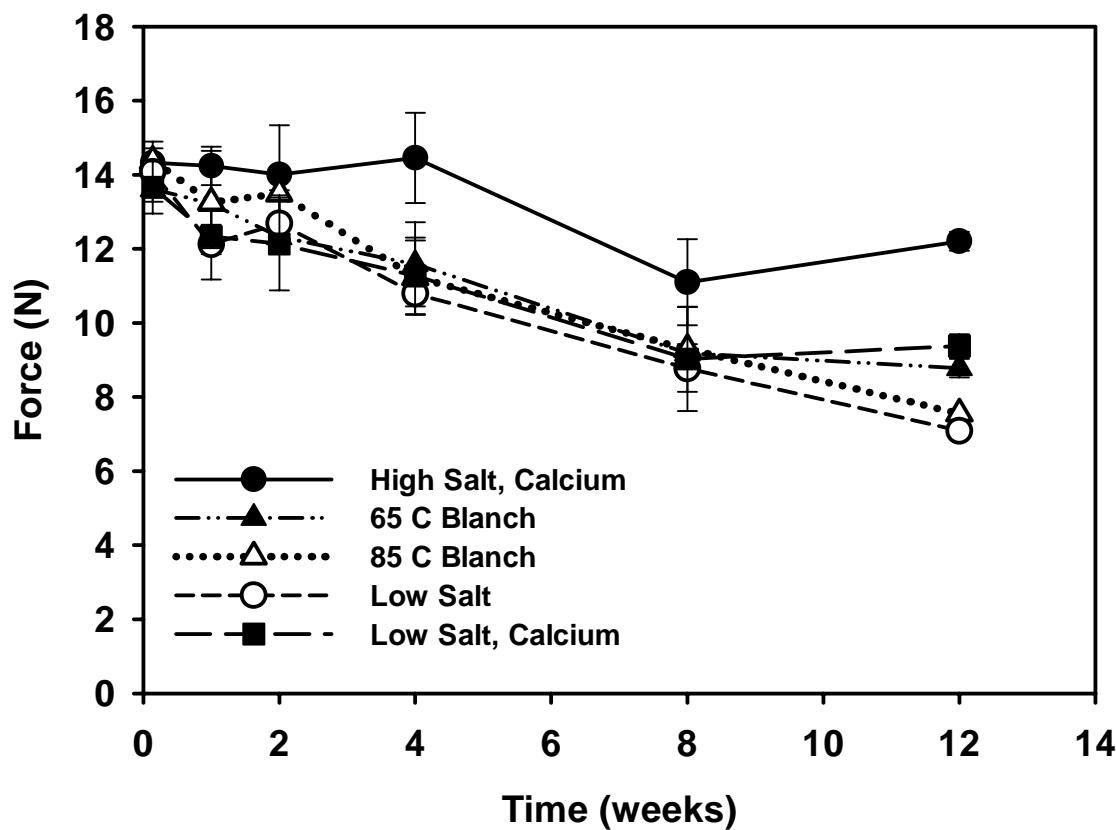


Figure 2.6B. Firmness changes in two lots of cucumbers during and after fermentation (Rep II). Control contains 6.0% NaCl, 18 mM CaCl₂. Other treatments contain 1.75% NaCl. 18 mM CaCl₂ added to the calcium treatments.

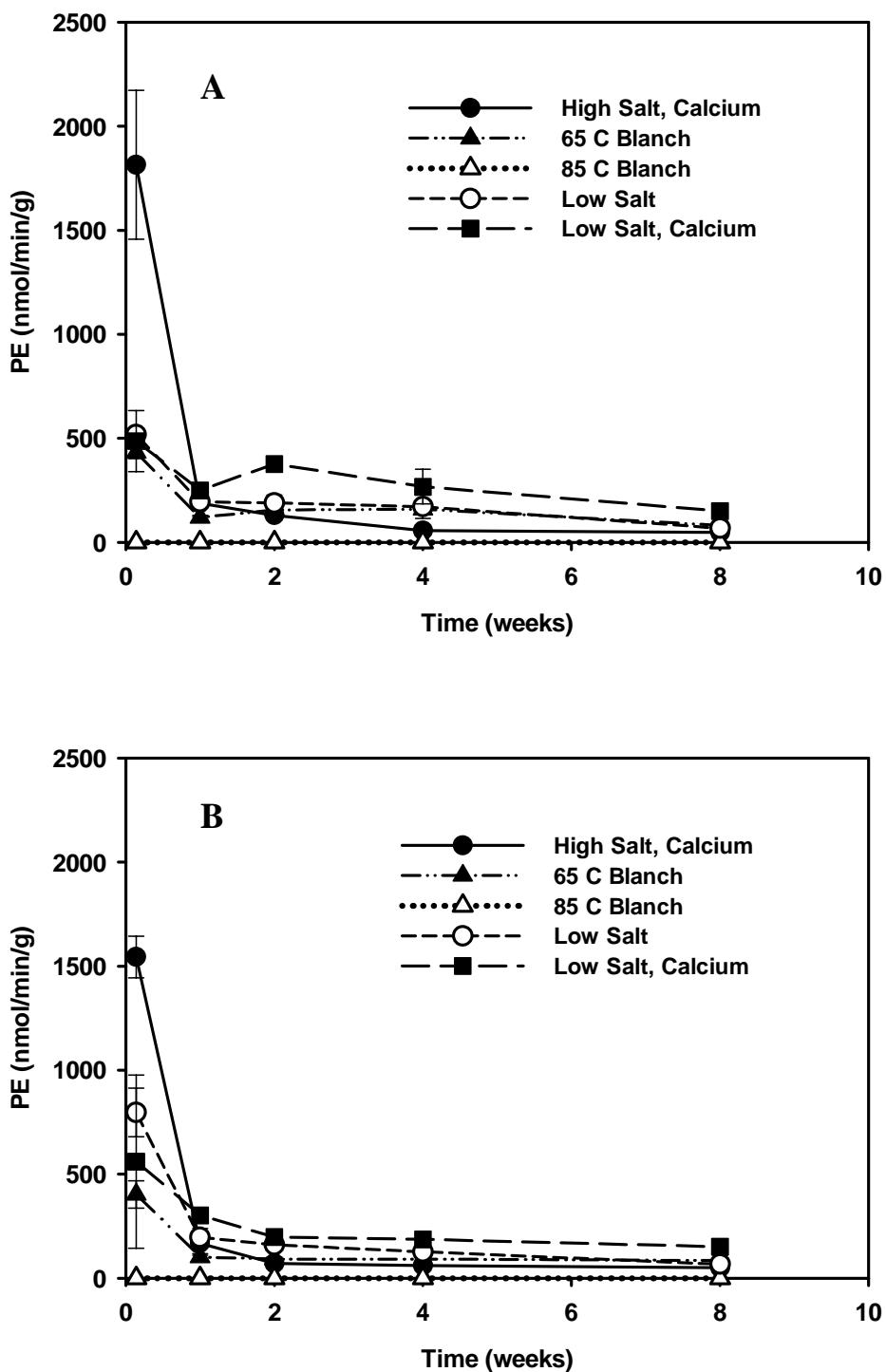


Figure 2.7. Changes in pectinesterase activity during cucumber fermentation and storage. Panels A and B show the results from two lots of cucumbers.

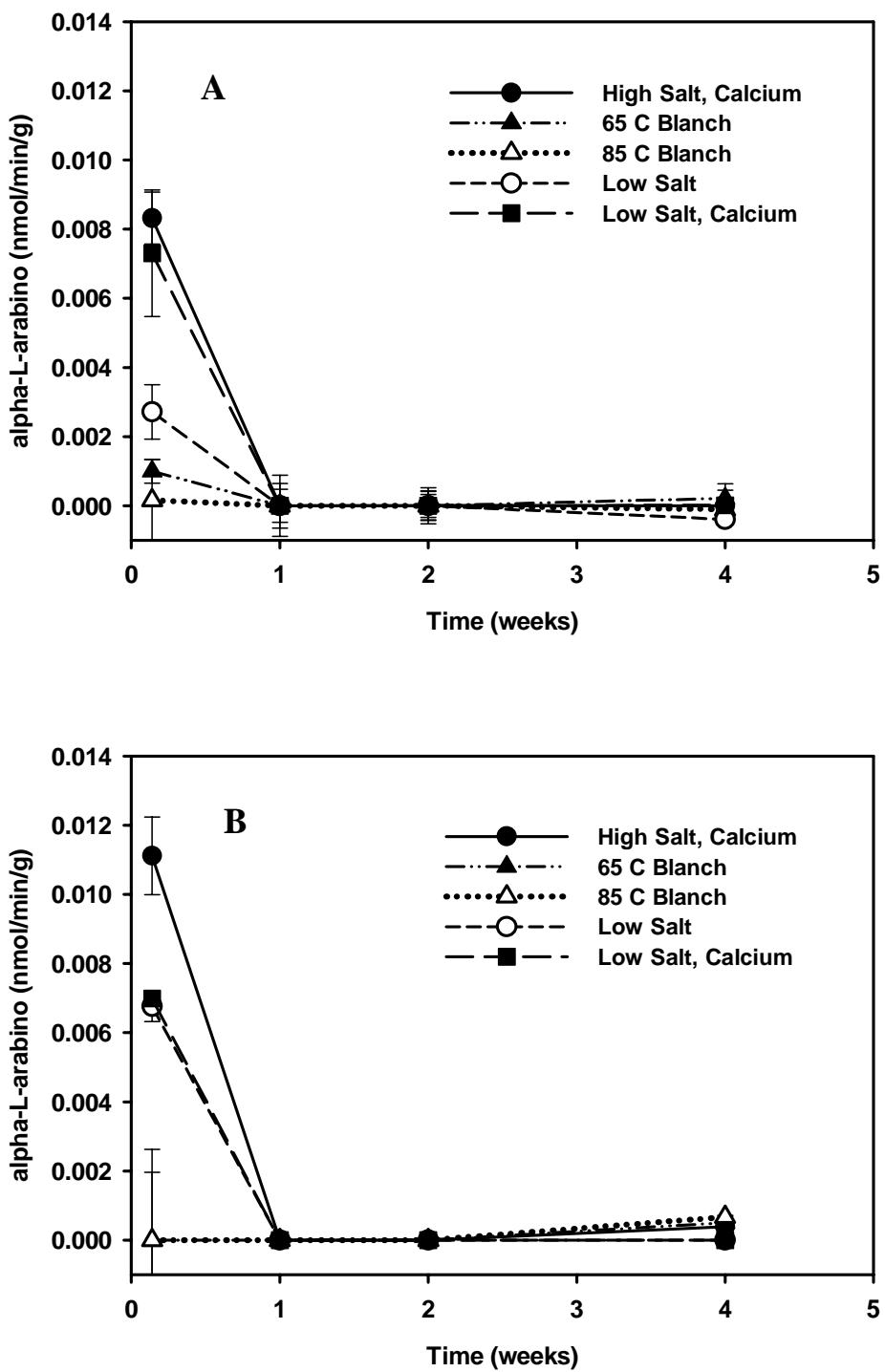


Figure 2.8. Changes in α -L-arabinopyranosidase activity during cucumber fermentation and storage. Panels A and B show the results from two lots of cucumbers.

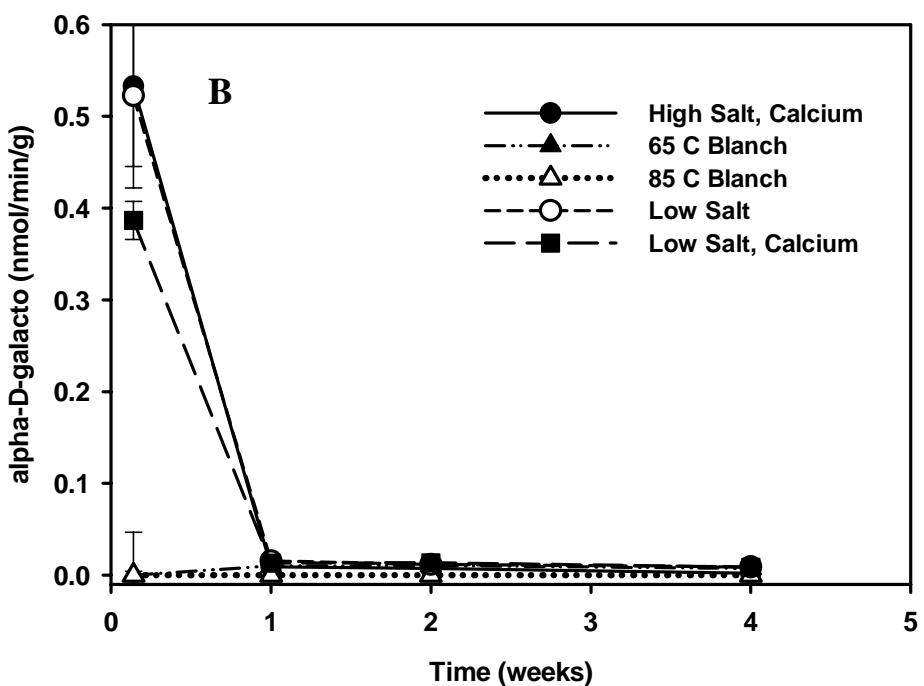
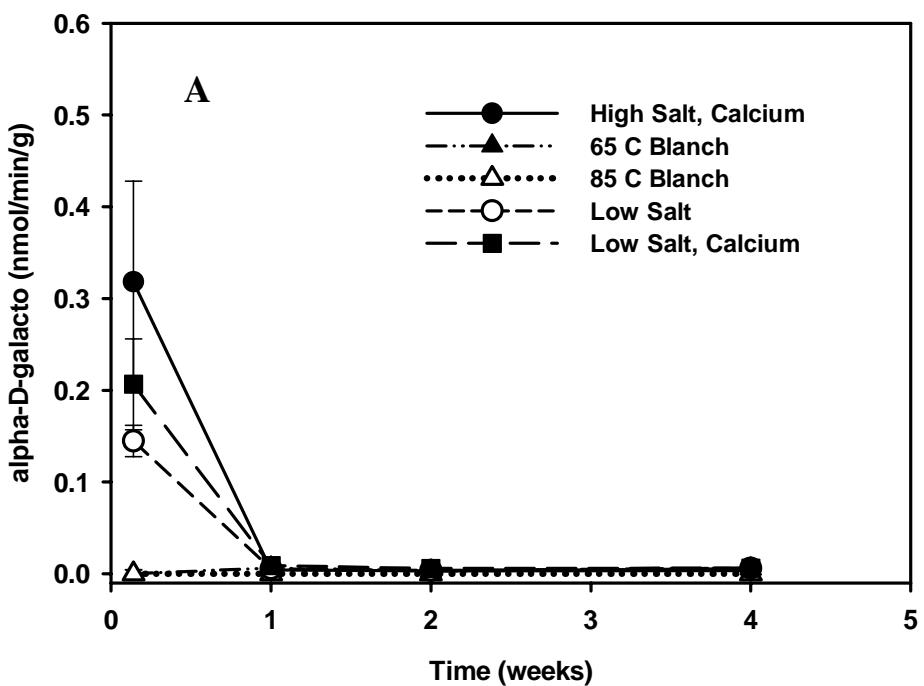


Figure 2.9. Changes in α -D-galactopyranosidase activity during cucumber fermentation and storage. Panels A and B show the results from two lots of cucumbers.

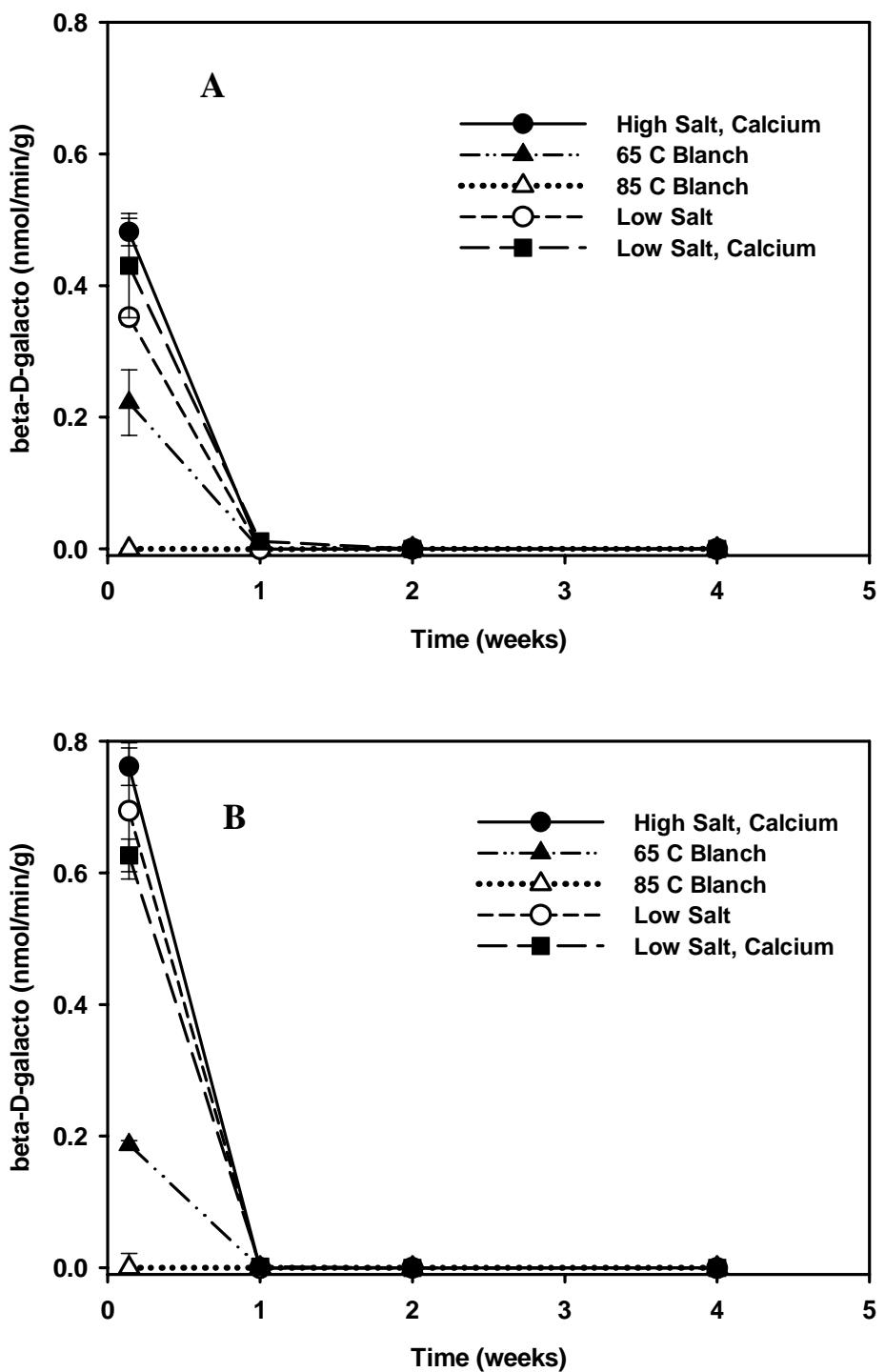


Figure 2.10. Changes in β -D-galactopyranosidase activity during cucumber fermentation and storage. Panels A and B show the results from two lots of cucumbers.

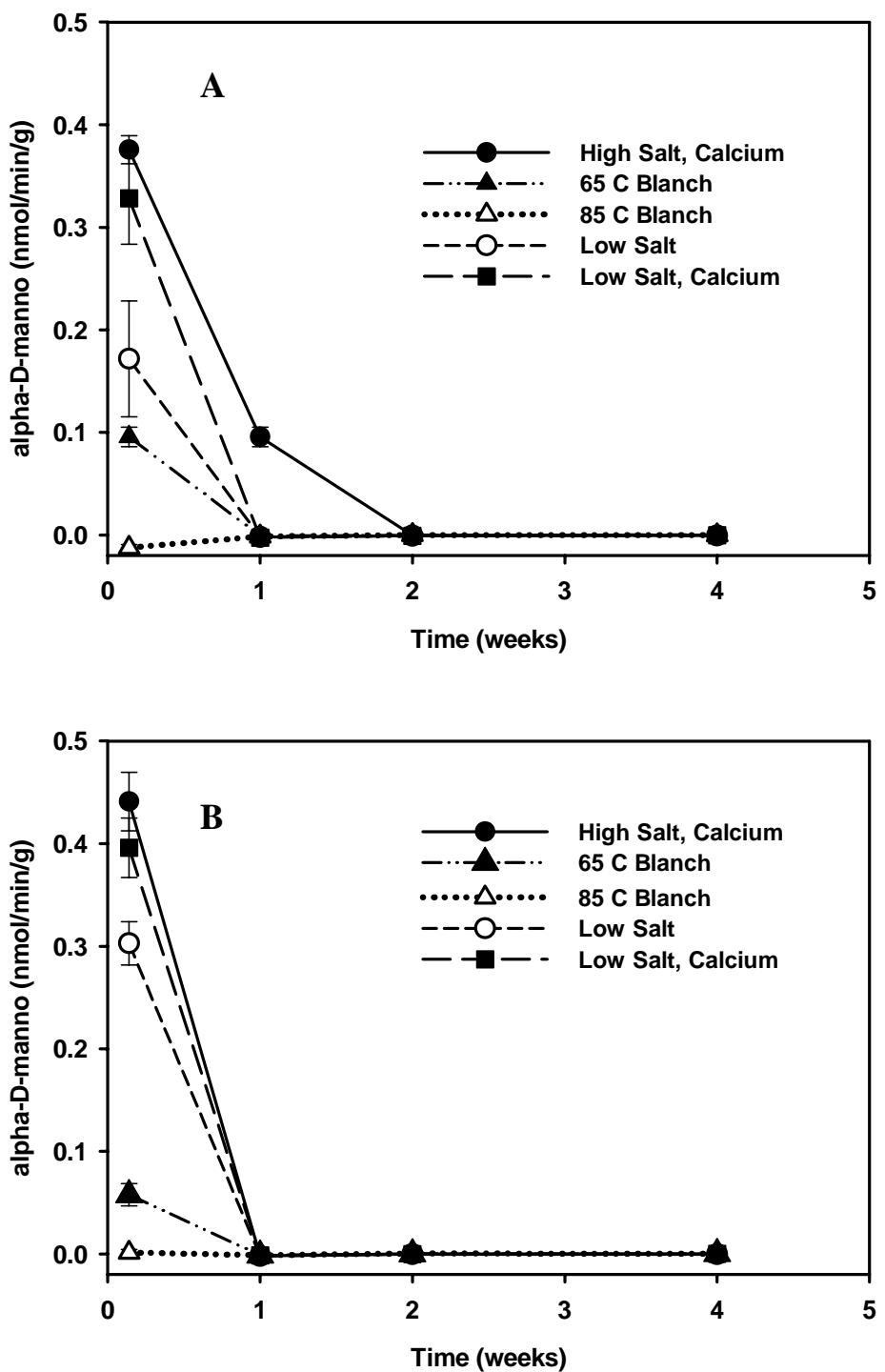


Figure 2.11. Changes in α -D-mannopyranosidase activity during cucumber fermentation and storage. Panels A and B show the results from two lots of cucumbers.

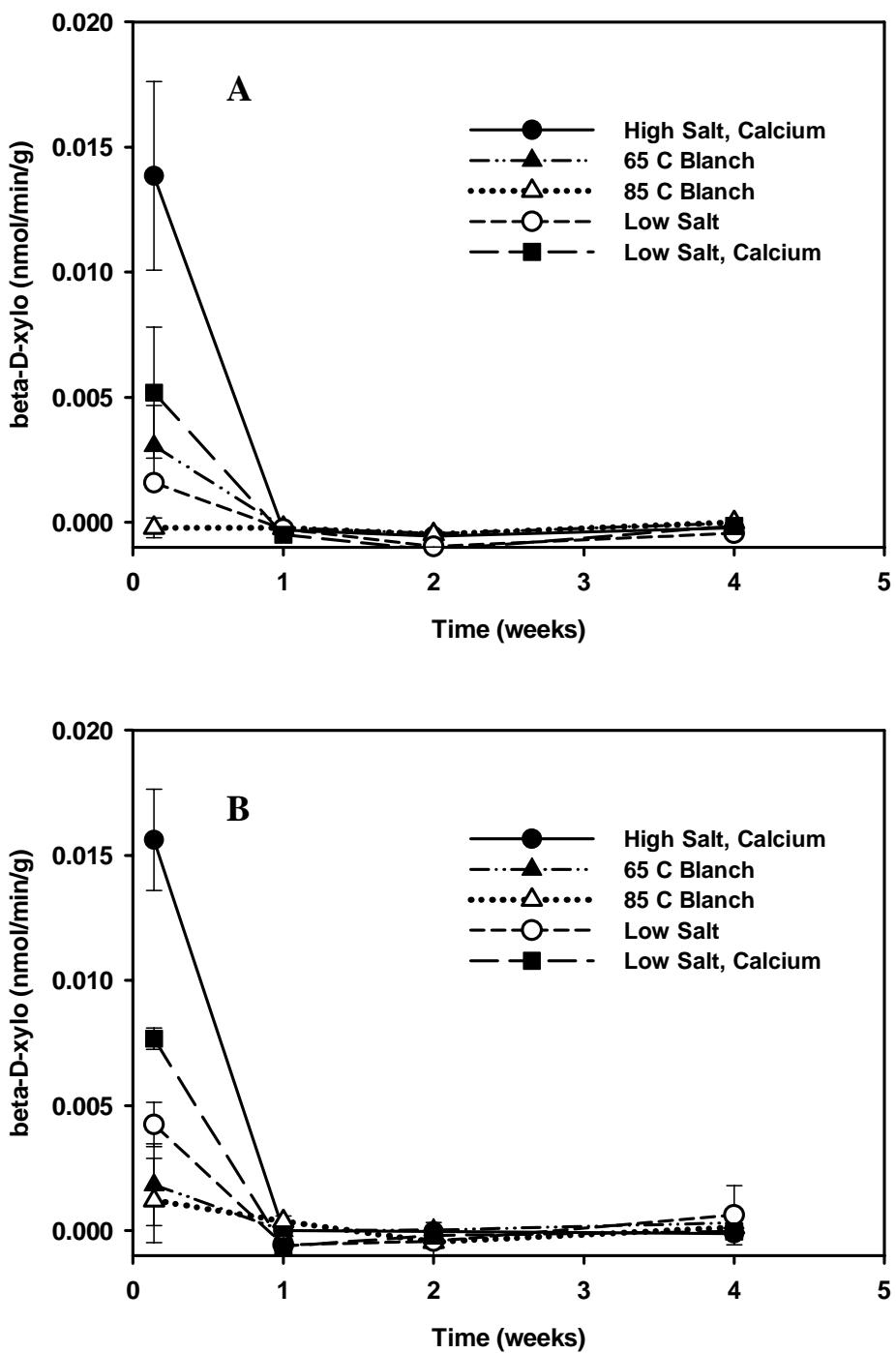


Figure 2.12. Changes in β -D-xylopyranosidase activity during cucumber fermentation and storage. Panels A and B show the results from two lots of cucumbers.