EXTRACTION OF PECTIN FROM

WATERMELON RIND

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INTRODUCTION

1.1 Problem Statement

A significant percentage of the total U.S. watermelon crop, which was 146,000 total planted acres in 2005 (USDA NASS, 2005), is wasted each year due to the inability to sell the total yields within the peak growing season. This waste is a result of second class melons being left in the field and many producers harvesting their melon crop at most twice in a growing season. Also a large number of seeded pollinators are left in the field due to the increasing U.S. consumer demand for seedless watermelons. All of these factors contribute to the nearly 30% of the watermelon crop that goes unharvested each year. This wasted crop represents a significant potential for the development of value-added products from watermelon. If all watermelons were harvested, a watermelon biorefinery could be developed in which a number of value-added products could be produced utilizing the entire watermelon biomass.

A biorefinery is a concept in which the total biomass of a biological product would be used to produce an array of value-added products resulting in minimal or no waste of the biomass. The basic principles of a biorefinery are that the feedstock is processed using chemical, thermal, physical, or biological processes to produce fuels,

chemicals, commodities, and other materials (Kamm et al., 2004). The biorefinery concept has been successfully integrated into crops such as corn and sugarbeet (Ohara, 2003).

A watermelon biorefinery would be possible if all or nearly all components of the watermelon biomass could be utilized as value-added products in an economical fashion. The longterm goal of this project is to look into the possibility of applying the general biorefinery concept to the watermelon crop. Since watermelon rind constitutes nearly a third of the watermelon weight, value-added products from the rind would be a critical part of the watermelon biorefinery and the rind will be the focus of this project. The initial goal is to explore the extraction of pectin from the watermelon rind.

Because of the success found in the citrus industry for extraction of pectin from peel, it is of interest to explore pectin extraction from watermelon rind. The citrus industry discovered the need for utilizing some of its wastes as value-added products decades ago and has incorporated production of some of these smaller products into the main production of citrus products such as juice. The extraction of orange juice yields 55% juice with 45% wet mass residues left over, resulting in a large amount of waste material for disposal (Braddock, 2004). Pectin is extracted from the citrus waste residue along with some other value-added products including essential oils, flavenoids and liminoids, and the production of dried cattle feed pellets. The basic pectin extraction procedure for citrus peels and other procedures that have been successfully applied to pectin extraction from a number of other plant materials will be applied to watermelon rind.

1.2 Research Objectives

The main objectives of this research project were to determine the feasibility of pectin extraction from watermelon rind through optimization of pectin extraction methods. The methods chosen for optimization were acid extraction, the standard method utilized in commercial pectin extraction, and enzymatic extraction, a method that has shown considerable promise in laboratory extractions and some commercial extractions over the past couple of decades. The specific objectives were to:

1. Investigate acid extraction procedures to produce the highest obtainable yield from watermelon rind
2. Investigate enzymatic extraction procedures for highest obtainable yield from watermelon rind
3. Compare acid and enzyme extraction methods in terms of yield and quality.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Watermelon Background

Watermelon is a warm-season crop from the cucurbit family, which also contains other melons such as cantaloupe and gourds such as squash and pumpkin (Robinson et al., 1997). There are three recognized species of watermelon; Citrullus *lanatus*, Citrullus *ecirrhosus* Cogn., and Citrullus *colocynthis* (Robinson et al., 1997). The domesticated varieties belong to the species C. *lanatus* which should not be confused with the wild populations designated as C. *lanatus* var. *citroides* (Robinson et al., 1997). With over 1200 varieties available for a number of different growing conditions, watermelon is obtainable to be enjoyed throughout the world.

The 50 main varieties common to the United States can be sorted into four general categories. Allsweet watermelons are 20-25 pounds, Ice Box watermelons are 5-15 pounds, Seedless watermelons are 10-15 pounds and have little to no seeds, and Yellow Flesh watermelons are 10-30 pounds with yellow or orange flesh (All About, 2004). The popularity of the seedless variety has resulted in its increased production and consumption in recent years.

The seedless cultivars were developed through crosses of tetraploid and diploid cultivars. These triploid cultivars contain less viable pollen and as a result are always planted with diploid cultivars in a 1:3 or 1:4 diploid to triploid ratio to increase pollination (Robinson et al., 1997).

Harvesting is done at full maturity, starting one month after full bloom, and continuing for several weeks. Watermelon is generally picked by hand due to the fragility of the rind. Watermelons are cut from the vine and carried to a straw-packed truck for field loading. The melons are then transported to packing sheds for grading and bin and carton loading (Hurst, 2002). The melons are checked for size and maturity and the melons that do not qualify are discarded. The packed melons are cooled to about 15˚C and shipped generally without refrigeration (Hurst, 2002).

Traditionally in the United States watermelon was enjoyed mainly as a summertime treat, and it still is with 40% of annual sales occurring in June and July (Mizelle, 2002). Today watermelon can be eaten year-round with availability from U.S. growers from April through November and imported availability from October through June (Fields, 2004). The annual volume sales of watermelon after the month of August increased 10% to over 20% from 1986 to 1996, indicating that it is being utilized more throughout the year (Mizelle, 2002). Watermelon is available for purchase as whole melons or quarter sections in the produce section of grocery stores and as a minimally processed component of fruit salads available from grocery stores and many food service retailers.

In 2000-02 watermelon was reported to be the leading U.S. melon crop based on production, per capita consumption, and planted area (Lucier et al., 2001). The per capita

consumption per person in 2001 was 13.2 pounds, which was down from a peak of 16.8 in 1996 but relatively steady in comparison to recent years (Lucier et al., 2001). The total acreage planted in the United States with watermelon in 2005 was 146,000 and the total acreage harvested was approximately 136,400 (USDA NASS, 2005). The estimated production for 2005 was approximately 37,896,000 cwt and the total monetary value in 2005 for the United States watermelon production was approximately $410,281,000 (USDA NASS, 2005). Based on these statistics, 9600 acres of watermelon crop were not harvested and were left as unutilized waste in the field. This indicates a significant untapped resource for the production of value-added products.

According to the USDA-ARS, in 1998 approximately 10% of watermelon production was sold as a minimally processed product (Perkins-Veazie et al.). In 2003 the fresh cut fruit market increased 40% with watermelon being at least 14% of the total market (Information, 2003). This signifies a rapidly increasing market for minimally processed watermelon and therefore an increased waste stream coming from the processors.

2.2 Watermelon Composition

Watermelon biomass can be categorized as three main components which are the flesh, seed, and rind. As shown in Figure 2.1, the flesh constitutes approximately 68% of the total weight, the rind approximately 30%, and the seeds approximately 2% (Kumar, 1985).

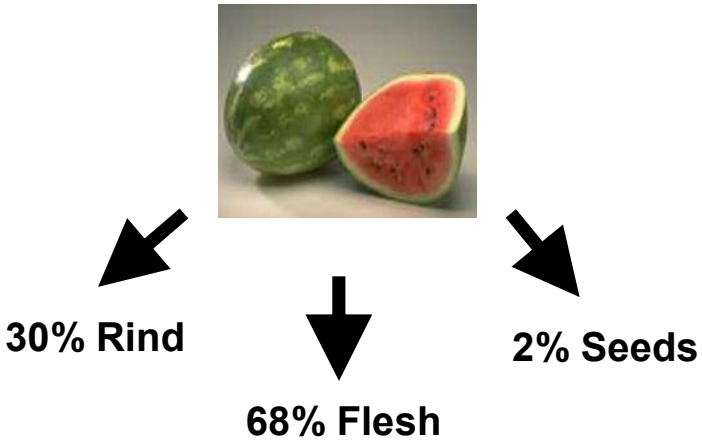

Figure 2.1. Breakdown of watermelon biomass.

The composition of the flesh, seed, and rind vary considerably. One hundred grams of watermelon flesh was analyzed and found to contain 92.6g water, 0.5 g protein, 0.2 g fat, 6.4 g total carbohydrate, 0.3 g fiber, 0.3 g ash, and a number of vitamins and minerals including 0.7 mg calcium, 590 international units (IU) vitamin A, 0.03 mg thiamine, 0.03 mg riboflavin, 0.2 mg niacin, and 7 mg ascorbic acid (Huor, 1979). The seed is approximately 42% kernel and 58% hull (Ramakrishna, 1985). Watermelon seed was found to be 8.32% moisture and 91.7% dry matter (Olaofe, 1994). The composition of the watermelon seed kernel was determined to be 35.7% crude protein, 50.1% crude oil, 4.83% crude fiber, 3.60% total ash, and 5.81% nitrogen free extract (El-Adawy et al., 2001). Approximately 4.36% of the rind is peel and the other is the inside whitish portion (Kumar, 1985). One study states that the rind is 93.8% moisture, 0.49% ash, 0.1% nitrogen, and 2.1% sugars (Bawa et al., 1977). Singh et al. (1975) determined the skin of fully ripened watermelon to contain approximately 20% cellulose, 23% hemicellulose, 10% lignin, 13% pectin, 7 mg/g silica, and 12% silica free minerals. According to research conducted by the ARS laboratory the rind contains 2-20 mg/g dry weight of the amino acid citrulline (Perkins, 2004). The rind is higher in percent fresh

weight, dietary fiber, and potassium but lower in total sugar than the flesh (Perkins-Veazie, 2002).

2.3 Watermelon Biorefinery

In order for the biorefinery concept to work for the watermelon crop it is necessary to consider value-added products for all of the components. This section will detail the array of possible value-added products from the watermelon crop, as outlined in Figure 2.2.

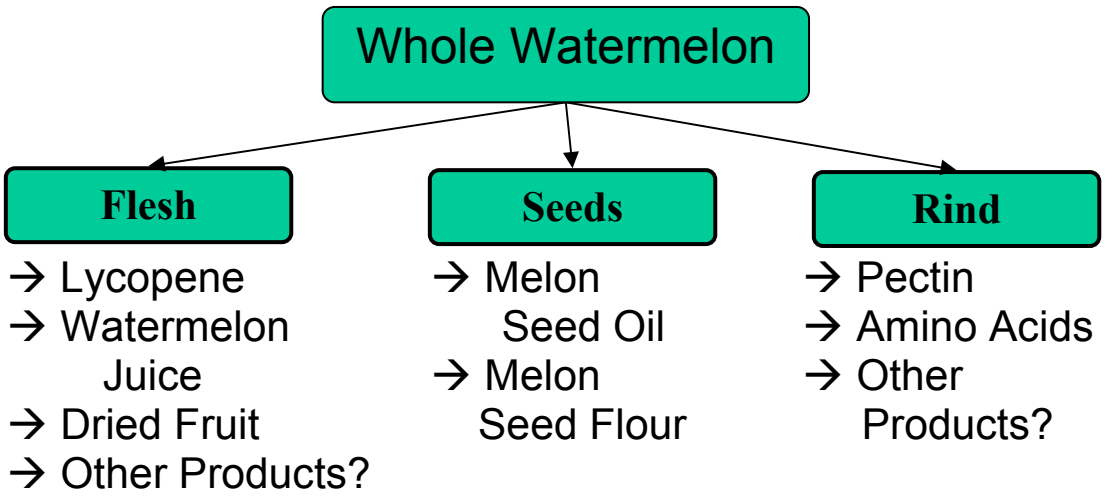

Figure 2.2. Overview of watermelon biorefinery concept.

A number of value-added products could be produced from the watermelon flesh. Watermelon flesh contains 39-78 μg/g lycopene, which is up to 60% more than the average tomato content (Perkins-Veazie, 2001). Lycopene is a carotenoid with antioxidant properties and is currently being researched due to its potential health benefits. A recent major use for watermelon flesh is for the extraction of lycopene as a

nutritional supplement (Arnold, 2002, Davis et al., 2003, Perkins-Veazie, et al. 2001). As discussed previously, minimally processed watermelon has become an increasing market for use of watermelon flesh. Another use of the watermelon flesh is for juice extraction. Many studies have been conducted to evaluate the possibility of pasteurizing and commercializing watermelon juice (Silva et al., 1991, Huor et al., 1980). A recent patent details a process for making a commercial packaged watermelon juice drink by juicing the whole watermelon with the exception of the seeds (Marks et al., 2003). A watermelon puree is also being produced commercially. In 1971 a French patent was filed for a soft candied watermelon fruit (Richaud, 1975). Additional research was done at Oklahoma State University to determine the feasibility of producing a dried watermelon fruit.

Seeded watermelons are still a large part of the total market and therefore the seed portion of the watermelons should be considered. Several studies have already been conducted on the feasibility of the use of watermelon seeds including flour production for protein supplementation and oil extraction as described below. These processes could be applied within the biorefinery concept to utilize the seed component of the seeded type of watermelons. Seeds of certain watermelon varieties are used widely in other countries on a small-scale as a source of oil or protein (El-Adawy et al., 2001, Kamel et al., 1985). Sharma et al. (1986) concluded that watermelon kernel proteins would be a valuable supplement to most protein sources other than cereals. Another study concluded that watermelon oilseed flour has good protein solubility and would be a suitable protein for food formulation and stabilizing of colloidal food systems (Olaofe, 1994). Akpapunam et al. (1981) found that watermelon seed proteins could be used to nutritionally supplement

the proteins of cowpeas, a staple legume in many tropical regions which is poor in sulfur-containing amino acids. The results of this study were a protein digestibility of 80% for a mixture of cowpea and watermelon proteins and a lysine availability of 93% for watermelon flour.

Several possibilities exist for the use of watermelon rind to produce value-added products. It is possible to juice just the rind but no commercial uses for this juice have been noted (McGregor, 2004). The USDA ARS is currently processing a patent to utilize extracted rind citrulline, an amino acid that helps to remove nitrogen from the blood for conversion to urine (Perkins, 2004, Pons, 2003). Other research has been conducted on the utilization of the rind as an ingredient in products including pickle, candy, vadiyam, and cheese (Madhuri et al., 2003, Kumar, 1985, Simonne et al., 2002). A patent from 1976 listed watermelon rind as a nonassimilable product for use in the production of a low calorie pasta product (Blake et al., 1975). Huor (1979) reported that high-grade pectin was extracted from watermelon rind. Another method for pectin extraction from watermelon rind was discussed by Crandall et al. (1981). A process was patented in 1989 listing watermelon rind as a possible source for the liberation of pectin from the tissue using Bacillus microorganisms (Sakai, 1989).

2.4 Pectin Background

Pectin is defined as complex mixtures of polysaccharides that make up approximately one third of the cell-wall dry substance of most types of plants (Van Buren, 1991). The function of pectin in plants is to contribute structural integrity to the

cell wall and adhesion between cells. Pectin is primarily made up of D-galacturonic acid joined by α-(1-4) glycosidic linkages (Van Buren, 1991). As a part of the plant structure, pectin is a complex mixture of blocks of homogalacturonic acid called 'smooth regions' mixed with blocks of homogalaturonic acid containing many neutral sugars including rhamnose, galactose, arabinose, xylose, and glucose called 'hairy regions' (IPPA, 2001). A percentage of the galacturonic acid residues are generally esterified with methanol.

The pectins of a plant can be water-soluble, chelator soluble, or protopectins (Van Buren, 1991). The methods of extraction will vary based on the actual makeup for each particular plant type. For example, protopectins are brought into solution by hot dilute acids. The general makeup of the pectin content varies with ripening of the plant and it is fairly easily brought into solution depending on the plant type (Van Buren, 1991). After extraction pectin consists of smooth galacturonic acid regions with a few neutral sugars still attached (IPPA, 2001). Commercial pectin extraction is mainly from citrus peel and apple pomace, but several other sources exist such as sugar beets and sunflower heads.

Extracted pectin can be categorized into two major categories depending on the percentage of galacturonic acid residues that are esterified with methanol. A degree of methoxylation (DM) greater than 50% is considered high methoxyl pectin and a DM below 50% is considered low methoxyl pectin (Braddock, 1999). A subset of low methoxyl pectin exists called amidated pectin that is produced through de-esterification of high methoxyl pectin with ammonia (Braddock, 1999). These types of pectin can be used for a wide range of end uses as their structures will yield a variety of gelling and texturizing abilities under differing conditions.

Pectin is capable of forming gels with sugar and acid. Because of this gelling ability one of the well-known uses of pectin is in high sugar jams and confectionery jellies, dating back to at least the 18th century (IPPA, 2001). Because it is a natural additive for foods, pectin is being considered for a number of applications beyond the traditional jams and jellies. Pectins are now used as thickeners, water binders, and stabilizers. It is used in yogurts and pastry glazes and as a stabilizer in drinkable yogurts and blends of milk and fruit juices (May, 1990). Pectin is also being used as a texturizing fat replacer to mimic the mouth-feel of lipids in low-calorie foods and shorter chain galacturonic acids have been considered as clarification agents in fruit juices (Braddock, 1999). Pectin has also been investigated for its usefulness in the pharmaceutical industry. Among other uses it has been considered in the class of dietary fibers known to have a positive affect on digestive processes and to help lower cholesterol (Braddock, 1999). It also is utilized to stabilize liquid pharmaceutical emulsions and suspensions.

2.5 Pectin Extraction Methods

Pectin is produced commercially from citrus peel and apple pomace. The extraction conditions vary from facility to facility and are dependent on the pectin source. Extraction most commonly occurs using a dilute mineral acid, usually hydrochloric, sulfuric, or nitric acids.

Commercial pectin extraction as detailed by the International Pectin Producers Association is described as the following (IPPA, 2001). A factory receives previously washed and dried apple pomace or citrus peel from a number of sources. The material is

added to hot water and a dilute mineral acid is added for extraction. Sufficient time elapses to allow extraction to occur and then the solids are separated from the pectin containing liquid through filtration or centrifugation. The remaining solution is concentrated and mixed with an alcohol for pectin precipitation. The precipitated pectin is separated and washed with alcohol to remove impurities. The pectin is dried, ground to a powder, and blended with other additives, if necessary.

Laboratory scale extractions have been conducted to determine optimal pectin extraction conditions and the feasibility of pectin extraction from a number of different plant materials. The effects of temperature, time, and pH on pectin yield for orange pectin using nitric acid extraction was investigated by Aravantinos-Zafiris et al. (1991). Optimal extraction conditions of pH 1.6, 84˚C, and 64 min resulted in yields up to nearly 26% of the dried peel weight. Galacturonic acid content, methoxyl content, and ash were reported to be independent of the extraction variables. Optimal extraction conditions found through varying extraction time, pH, and temperature for pectin extraction from sugar beet pulp were reported as the use of hydrochloric acid to adjust pH to 1.5 extracted for 4 hours at 80˚C (Phatak et al., 1988). The resulting pectin yield was 19.53% dry basis at these extraction conditions. Extraction pH, time, and liquid to solid ratio were optimized in a study on pectin extraction from tropical fruits (Simpson et al., 1984). Optimal conditions for extraction of pectin from grapefruit rinds at room temperature using ethanol for precipitation were pH 2.0, 24 hours, and a liquid to solid ratio of 5:1, which resulted in 4.10 g dry pectin per g fresh fruit. The extraction conditions were applied to various tropical fruits and some of the resulting yields were 2.74, 2.14, 3.02,

and 3.16 g dry pectin per g fresh fruit for guava, mango, passion fruit, and bread fruit, respectively.

Many studies have been conducted on the feasibility of utilizing enzymes for pectin extraction. Based on the success of these methods on other pectin sources, it would be beneficial to look into enzymatic pectin extraction for watermelon peel. One study used *Trichoderma viride* cellulase, *Aspergillus niger* hemicellulase, and a crude glycosidase complex from Xanthomonaas campestris to extract pectin from pumpkin pulp (Shkodina et al., 1998). The extraction conditions were a 3:50 dry solid to liquid ratio, 30˚C, 20 hours, and 250 mg of hemicellulase, 50 mg of cellulase, or culture fluid of *X. campestris*. The data showed that there was a considerable increase in yield, from approximately 5% using acid extraction up to 22% using cellulase extraction, with cellulase producing the highest yield. The enzymatically extracted samples had low molecular weights with more difficulty in gelation. Another study by Donaghy et al. (1994) successfully extracted pectin from citrus peel and apple pomace but not from sugar beet pulp using polygalacturonase from *Kluveromyces fragilis*. Optimal extraction of pectin from citrus peel at yields of 16 to 20% of the dry matter was reported for conditions of a solid to liquid ratio of 1:12, 24 hours, 37˚C, and 1.2 U enzyme activity. Ghanem et al. (1991) reported that solid to liquid ratio, incubation period, age and size of microbial inoculum, and pH all influenced microbial extraction of pectin from beet. Solid to liquid ratio, extraction time, and extraction temperature were optimized in a study using *Trichosporon penicillatum,* which produces a protopectinase activity for microbial pectin extraction from citrus peel (Sakai et al., 1980). Optimal conditions were

reported as a 1:2 solid to liquid ratio, 15 to 20 hours, and 30˚C resulting in 2.5 g pectin per 100 g of peel.

These studies indicate that a considerable increase in yield can be obtained by using enzymes for pectin extraction. These procedures and others could be tested on watermelon waste to determine if the use of enzymes to extract pectin would be more beneficial than the use of acids.

2.6 Watermelon Rind Pectin Extraction

With the previously specified pectin content of 13% the extraction of pectin from watermelon rind could be a viable utilization of this portion of the watermelon biomass. One of the methods for extraction of watermelon rind resulted in a yield of 4.65 kg per ton (approximately 5.1 g/kg) of watermelon, which is 150-300 kg pectin per hectare of watermelon (Huor, 1979). The method of Crandall et al. (1981) resulted in a 150 grade pectin yield of 20% on a dry weight basis. This was reported to be approximately one third of the expected yield for lime or lemon peel. Crandall (1981) followed a referenced method for citrus peel pectin extraction. The referenced method uses a 45-minute extraction with 1.0 M nitric acid at pH 1.6 and 90˚C, precipitation with 2 volumes of isopropyl alcohol, and a series of alcohol washes.

2.7 Pectin Analysis

A number of different factors are often analyzed to determine the quality and purity of pectin. The accepted measure of pectin purity is by the determination of the anhydrogalacturonic acid (AGA) content. A percentage above 65% is considered to be the typical minimum level for pectins used for various applications according to information available from the International Pectin Producers Association (IPPA, 2001). The purest citrus pectins contain 85-90% AGA (Braddock, 1999). Another factor considered for pectin analysis is the degree of methoxylation. This measure will determine the usage of the pectin and whether it should be classified as high methoxyl (above 50%) or low methoxyl (below 50%). High methoxyl citrus pectin is generally at 70-80% DM (Braddock, 1999). It is also of interest to determine the ash content, molecular weight, and degree of amidation for a pectin sample. Molecular weight is often determined as an indication of the gelling quality of the pectin. Amidation is sometimes desired in low methoxyl pectins because it can increase gel formation.

Pectin is standardized according to the IFT Pectin Standardization Method of 1959 (IFT Committee, 1959). This procedure utilizes the SAG method of standardization to measure the sugar holding capacity of a test gel. A standard 65˚ Brix pectin gel is made, poured into a jelly glass, and left to dry for 20-24 hours. The jelly glass is inverted and the amount of sag is measured with a Ridgelimeter. The jelly grade is determined from this measurement and the pectin is then standardized to 150 jelly grade by diluting with sugar. 150 jelly grade means that 1 kg of standardized pectin will turn 150 kg of sugar into a standard gel.

CHAPTER 3

EXPERIMENTAL MATERIALS AND METHODS

3.1 Overview of Methodology

The overall goals of this project were to optimize the variables within each extraction method in order to produce the highest yield of pectin from watermelon rind. All procedures were variations of methods outlined in published papers for extraction of pectin from various plant materials. The acid extractions followed the procedure outlined in Crandall et al. (1978a), which was referenced in Crandall et al. (1981) for use in watermelon rind pectin extraction. The enzymatic extractions followed the procedure outlined by Shkodina et al. (1998). All experiments were performed within the Biosystems and Agricultural Engineering laboratories in the Food and Agricultural Products Center and the Advanced Technology Research Center at Oklahoma State University.

3.2 Preparation of Watermelon Rind

Watermelons were purchased from local retailers for use in experiments. Because of seasonal changes in varieties available for purchase, an effort was made to buy

watermelons in larger numbers so that watermelon variety would be the same within all trial sets of a tested extraction variable. Seedless watermelons were preferred for testing and were purchased when available. With the exception of the initial acid extraction trials and the first trial set of the acid extraction solid to liquid ratio experiment, a single watermelon was utilized as the peel source for a set of trials within a variable. The acid extraction solid to liquid ratio experiment required a larger amount of peel so the individual trials were conducted using different watermelons of the same variety and similar maturity level.

The rind including the skin was separated from the flesh and the flesh was discarded. The rind was ground to uniform size using a food processor at high speed. The ground peel was then leached with tap water to remove soluble solids. The leaching process was performed by adding approximately 2.5 times the peel weight of 30˚C water to the ground peel and stirring for 10 min. The mixture was strained with a flat metal screen and the procedure was repeated. The strained peel was pressed to uniform dryness. Sampling of the ground peel was conducted before and after the leaching process to determine the total solids content of the sample. The samples were weighed and dried at least 24 hours in a forced air oven held at 80˚C. The amount of soluble solids removed in the leaching process was determined by the difference in sample weights. The pressed peel, as shown in Figure 3.1, was placed in a sealed bag and held at refrigerated temperature unless the extraction procedure was performed immediately after the peel preparation.

Figure 3.1. Watermelon peel after completion of preparation process.

3.3 Acid Extraction Methodology

A Thermolyne Cimarec 2 magnetic stirring hot plate manually held within range of the desired temperature was utilized for the initial studies and a Precision (cat model 66302-26) shaking waterbath was utilized for all of the final studies. Extraction solution pH was measured using a Thermoelectron pH probe attached to an Orion portable meter that has a calibration point at pH 1.68, near the pH of the extractions. The pH probe, which was rated for temperatures up to 100˚C, was always used in conjunction with an Orion automatic temperature compensation probe.

3.3.1 Acid Extraction Procedure

Seven hundred and fifty mL of deionized (DI) water was measured into a 2000 mL Erlenmeyer flask and maintained at the desired temperature using the stirring hot plate or the shaking waterbath which are both shown in Figure 3.2.

(A) (B)

Figure 3.2. Setup for pectin extraction using the stirring hot plate (A) and the shaking waterbath (B).

A weighed portion of pressed peel was added to the water. Measured amounts of acid were added to the peel-water mixture until the desired pH was obtained. The mixture was agitated at a constant temperature until the desired extraction time had elapsed. The pH and temperature were recorded and the mixture was allowed to cool in an ice waterbath until it reached 55˚C. The mixture was centrifuged at 5050 rpm for 10 min. The filtrate was vacuum filtered as shown in Figure 3.3 using Whatman #4 filter paper and the solids were resuspended in 400 mL of 60˚C DI water for 5 min.

(A) (B)

Figure 3.3. Filtration setup (A) and filtered watermelon peel mass (B).

The centrifugation and filtration steps were repeated. The filtered solutions were combined and approximately twice the volume of alcohol was added for overnight precipitation, which is shown in Figure 3.4.

Figure 3.4. Acid extracted watermelon pectin after overnight precipitation.

The pectin was separated from the alcohol solution using a double layer of cheesecloth and the samples were washed three times with 70% alcohol and once with undiluted alcohol to remove any impurities. The resulting pectin was dried under vacuum at 50˚C

in aluminum sample dishes until all moisture was removed. Samples were cooled, weighed and ground using a mortar and pestle. Ground samples were stored in small plastic sample bags.

3.3.2 Preliminary Experimentation Methods

Initial extraction conditions were chosen as a solid to liquid ratio of 0.133 g/mL, pH 1.65 using 1 N nitric acid, time of 45 min, temperature of 90˚C, and precipitation with isopropanol using seedless watermelon as the peel source.

Experiments were conducted to compare centrifugation and cheesecloth as retrieval methods for the precipitated pectin. Extraction conditions were a solid to liquid ratio of 0.133 g/mL in 750 mL deionized water extracted for 45 min at 90˚C at pH 1.60. In the centrifugation method, all precipitation solution was centrifuged at 5050 rpm for 15 min. After centrifugation, liquid was drained off and separated pectin was retrieved. This process was repeated after precipitation and after each washing step. In the cheesecloth method, all precipitation solution was strained using a double layer of cheesecloth to remove pectin from the solution. This process was repeated after precipitation and after each washing step.

An experiment was conducted extracting the same peel twice to determine if additional pectin could be removed from the peel mass. The peel was extracted under the original extraction conditions of a peel to liquid content of 0.133 g/mL, 45 min, 90˚C, and pH 1.60. After extraction the solution was centrifuged and filtered. The remaining solids were reextracted utilizing the same extraction conditions. This second filtrate was

kept separate from the first and both were precipitated with 2 volumes of isopropanol, washed with alcohol, and dried under vacuum at 50˚C.

The method of Rouse et al. (1976) was followed for extraction of pectin using Valencia oranges. The extraction procedure was the same as the method outlined in a previous section and used a solid to liquid ratio of 0.258 g/mL in 1100 mL deionized water extracted for 45 min at 90˚C. Solids were washed with 400 mL of 40˚C deionized water. Precipitated pectin was collected through a combination of cheesecloth filtering and centrifugation of the remaining precipitation solution.

Solid to liquid ratios of 0.133 g/mL and 0.258 g/mL were tested using 100 g and 193.2 g of pressed peel in 750 mL of DI water. The extraction conditions were pH 1.65 adjusted using 1 N nitric acid for 45 min at 90˚C.

An experiment was conducted using 1 N hydrochloric acid to adjust pH and ethanol for precipitation and was tested at both solid to liquid ratios of 0.133 and 0.258 g/mL in 750 mL water for 45 min at 90˚C and pH 1.65. Separate experiments were conducted using 1 N nitric acid and 1 N hydrochloric acid in combinations with isopropanol and ethanol on different watermelon sources using 45 minute extraction periods at pH 1.65, 95˚C, and a solid to liquid ratio of 0.258 g/mL using 750 mL DI water.

3.3.3 Methods for Testing of Extraction Parameters

Upon completion of initial experiments, procedures were set up to determine the optimal extraction solid to liquid ratio, time, pH, and temperature. All extractions were

conducted using 1 N nitric acid to adjust pH and 2 volumes of isopropanol for pectin precipitation.

Solid to liquid ratio was tested using measured amounts of pressed peel in 750 mL of DI water, which were 150, 175, 180, 193.2, 210, and 225 g of peel with corresponding solid to liquid ratios of 0.133, 0.233, , 0.258, and 0.280 g/mL, respectively. The procedure outlined in the previous section was followed using the hot plate as the heating source and seedless watermelon rind. The specific extraction conditions were 90˚C for 45 min at pH 1.65.

Solid to liquid ratio was reexamined later in the project to verify the obtained results. Extraction was conducted using the stirring hot plate and a scaled down extraction with 375 mL deionized water so that all extractions could be conducted using a single watermelon peel source. The solid to liquid ratio was tested at values of 0.233, 0.247, 0.258, and 0.28 g/mL and the extraction conditions were 90˚C for 45 min at pH 1.65 using seedless watermelon peel. Washing of extracted solids was scaled down to 200 mL of deionized water. Experiments were conducted using two different types of seedless watermelons to compare solid to liquid ratios of 0.133 and 0.258 g/mL using the same extraction conditions and the shaking waterbath.

Extraction time was tested at 30, 45, 60, 75, and 90 min on the stirring hot plate using extraction conditions of 90˚C at pH 1.65 with a solid to liquid ratio of 0.258 g/mL using 750 mL deionized water and seedless watermelon rind.

Extraction temperature was tested at 80, 90, and ~100˚C using the stirring hot plate and at 85, 90, and 95˚C using the shaking waterbath. Extraction parameters for both

experimental setups were 45 min at pH 1.65 with a solid to liquid ratio of 0.258 g/mL in 750 mL deionized water using seedless watermelon rind.

Extraction pH was tested at values near 1.55, 1.65, and 1.75 using measured amounts of 1 N nitric acid to adjust the pH to the desired value. Extraction was performed using the stirring hot plate with seeded watermelon rind as the peel source and using the shaking waterbath with seedless watermelon rind as the peel source. Extraction parameters were 45 min at 95˚C with a solid to liquid ratio of 0.258 g/mL in 750 mL deionized water for both the hot plate and the waterbath.

Studies were conducted utilizing the optimal extraction conditions to determine the influence of watermelon maturity and variety on pectin yield. Watermelons were harvested from the OSU Department of Horticulture Vegetable Research Station in Bixby, Oklahoma. The seedless varieties Bobbie and 5144 and the seeded variety Lantha were chosen for this study. Bobbie and Lantha were obtained from Rupp Seeds and 5144 was obtained from Hazara. All watermelons utilized in this study were started as transplants. Watermelons were picked at varying degrees of ripeness. Initial determination of ripeness was based on the apparent maturity due to rind coloration on the watermelon belly, vine tendril condition nearest the watermelon, and sound resulting from thumping of the watermelon. This was conducted with the expertise of Dr. Niels Maness, OSU postharvest horticulture specialist. These classifications were confirmed according to flesh characteristics and taste. Ripe was classified as a melon with smooth, crisp flesh and a good flavor. Slightly overripe was classified as a melon with slightly cracked flesh and a slightly off flavor. Overripe was classified as a melon with very cracked flesh and off flavor. Very overripe was classified based on extremely cracked,

mushy flesh and an off odor. The maturity study was conducted using all three watermelon varieties. The experiments utilized watermelon rind from watermelons considered to be ripe and at varying stages of overripe as described. The variety study was conducted by comparing only the ripe watermelons from all three varieties. The acid extraction procedure detailed previously was utilized for both sets of experiments using the optimal extraction conditions of a time of 45 min, temperature near 95˚C using the stirring hot plate as the heating source, peel to liquid ratio of 0.258 g/mL, and pH 1.65 using 1 N nitric acid.

3.4 Enzymatic Extraction Methodology

3.4.1 Enzymatic Extraction Procedure

A stock buffer solution was prepared and 100 mL allotments of properly diluted solution were measured into 250 mL flasks with 0.01% sodium azide as an antibacterial agent. A measured amount of peel was added to each flask. All flasks were acclimated to 50˚C in a New Brunswick Scientific shaking waterbath and a determined amount of enzyme was added to each flask. The peel enzyme mixtures were agitated at constant temperature as shown in Figure 3.5 for a set amount of time.

Figure 3.5. Extraction setup for enzymatic pectin extraction of watermelon rind.

The mixtures were vacuum filtered through Whatman #4 filter paper and the solids were resuspended in 60 mL of room temperature DI water. The filtration process was repeated and the filtered solutions were combined and approximately twice the volume of alcohol was added for overnight precipitation as shown in Figure 3.6.

Figure 3.6. Enzyme extracted watermelon pectin after overnight precipitation.

The pectin was separated from the alcohol solution using a double layer of cheesecloth and the samples were washed three times with 70% alcohol and once with undiluted alcohol to remove any impurities. The resulting pectin was dried under vacuum at 50˚C in aluminum sample dishes until all moisture was removed. Samples were cooled,

weighed and ground using a mortar and pestle. Ground samples were stored in small plastic sample bags.

3.4.2 Preliminary Experimentation Methods

3.4.2.1 *Trichoderma viride* cellulase. *Trichoderma viride* cellulase was purchased from Fisher for use in enzymatic pectin extraction trials. The method detailed in the previous section was followed by extracting watermelon peel for 20 hours at 30˚C using 50 mg enzyme in 50 mL 50 mM pH 5.0 citrate buffer and isopropanol for precipitation. The extraction procedures were repeated with solid to liquid ratio of 0.20 and 0.50 g/mL. The solid to liquid ratio was tested again at 0.20 and 0.50 g/mL using ethanol instead of isopropanol as the precipitation alcohol. Using dried peel the solid to liquid ratio was tested at the equivalent of wet peel amounts of 0.129, 0.257, and 0.386 g/mL using the same extraction conditions and ethanol for precipitation. The trial was repeated using wet peel at solid to liquid ratios of 0.083, 0.257, and 0.386 g/mL.

In the next set of experiments enzyme loading was varied at 50 mg, 75 mg, and 100 mg of enzyme. Extraction conditions were 100 mL of 50 mM citrate buffer at pH 3.65 with a solid to liquid ratio of 0.25 g/mL held at 55˚C for 24 hours. The enzyme loadings were increased to 100, 175, and 257 mg and trials were conducted under the previous conditions with a new buffer solution at pH 4.45.

Buffer solutions were tested using 100 mL solutions of 50 mM citrate buffer, 100 mM acetate buffer, and 100 mM citrate buffer. Extraction conditions were a solid to liquid ratio of 0.25 g/mL and enzyme loading of 100 mg held at 55˚C for 22 hours.

3.4.2.2 Enzyme Screening. The enzymes Multifect GC, Multifect XL, and Multifect CL were obtained from Genecor, CelluPract was obtained from Biopract, and Fibrilase was obtained from Iogen. These enzymes were tested using 75 mM sodium acetate buffer pH 4.5 and 50 mM citrate buffer pH 4.4. All extractions used ethanol for precipitation. Extraction conditions were 100 mL of buffer with a solid to liquid ratio of 0.25 g/mL at 50˚C for 24 hours. Enzymes tested using acetate buffer were 100 mg of T. viride cellulase, 0.5 mL of Fibrilase, 0.5 mL of Multifect CL, and 0.5 mL of Multifect GC. Enzymes tested using citrate buffer were 0.5 mL of Multifect CL, 0.5 mL of Multifect GC, 0.5 mL of Fibrilase, 0.5 mL of Multifect XL, and 0.5 mL of CelluPract AL. Extractions were repeated for Multifect GC, Fibrilase, and CelluPract using the same extraction conditions but with a shorter extraction time of 12 hours for CelluPract.

3.4.2.3 Multifect XL. Solid to liquid ratio, enzyme loading, extraction time and buffer pH were adjusted to determine their effect on pectin yield using Multifect XL. Extraction parameters for all experiments were 50˚C for approximately 24 hours with a solid to liquid ratio of 0.25 g/mL in 50 mM citrate buffer pH~4.45 using an enzyme loading of 1.4 FPU/g. The parameter of interest was varied while keeping the others as stated. Solid to liquid ratio was tested at 0.12, 0.25, and 0.50 g/mL and enzyme loading was tested at 0.7, 1.4, 2.1, and 2.8 FPU/g. Time was tested at 20 and 24 hours. Buffer pH was adjusted at intervals between 4.4 and 5.0.

3.4.2.4 Fibrilase. Solid to liquid ratio, enzyme loading, extraction time, and buffer pH were adjusted to determine their effect on pectin yield using Fibrilase. Extraction parameters for all experiments were 50˚C for approximately 24 hours with a solid to liquid ratio of 0.25 g/mL in 50 mM citrate buffer pH~4.3 using an enzyme loading of 1.3 FPU/g. The parameter of interest was varied while keeping the others as stated. Solid to liquid ratio was tested at 0.12, 0.25, and 0.50 g/mL and enzyme loading was tested at 0.7, 1.3, 2.0, and 2.7 FPU/g. Extraction time was tested at intervals between 16, 20, 24, and 28 hours. Buffer pH was varied at intervals between 4.0 and 4.8.

3.4.2.5 CelluPract. Enzyme loading, extraction time, and buffer pH were adjusted to determine their effect on pectin yield using CelluPract. All extractions were conducted using 50 mM citrate buffer pH~4.4 with a solid to liquid ratio of 0.25 g/mL at 50˚C for 2 hours with an enzyme loading of 1.4 FPU/g while varying only the parameter of interest. Extraction times of 6 and 8 hours were tested followed by a separate experiment with extraction times of 2, 4, 6, and 8 hours. Extractions at 1, 2, 3, and 4 hours were conducted next. Enzyme loading was tested at 0.7, 1.4, 2.1, and 2.8 FPU/g. Buffer pH was varied at intervals between 3.5 and 5.0.

3.4.3 Methods for Testing of Extraction Parameters

Upon completion of initial experiments, procedures were set up to determine the optimal buffer concentration and pH, solid to liquid ratio, enzyme loading, and extraction time for CelluPract, Fibrilase, and Multifect XL. All experiments followed the extraction

procedure outlined previously using seedless watermelon rind, 100 mL of citrate buffer, an extraction temperature of 50˚C, and 2 volumes of ethanol for pectin extraction.

Buffer concentration was tested at 25, 50, and 100 mM for citrate buffer. The extraction conditions for CelluPract were 2 hours, enzyme loading of 2.1 FPU/g, solid to liquid ratio of 0.25 g/mL, and buffer pH 4.25. The extraction conditions for Fibrilase were 20 hours, enzyme loading of 2.0 FPU/g, solid to liquid ratio of 0.25 g/mL, and buffer pH 4.35. The extraction conditions for Multifect XL were 2 hours, enzyme loading of 2.1 FPU/g, solid to liquid ratio of 0.25 g/mL, and buffer pH 4.65.

Buffer pH was tested in 50 mM citrate buffer within the optimal pH usage ranges listed by the manufacturer for each enzyme. The pH values tested were 3.25, 3.5, 3.75, 4.0, 4.25, and 4.5 for CelluPract, 4.0, 4.3, 4.6, and 4.9 for Fibrilase, and 4.25, 4.5, 4.75, and 5.0 for Multifect XL. The extraction conditions for CelluPract were 2 hours, enzyme loading of 2.1 FPU/g, and solid to liquid ratio of 0.25 g/mL. The extraction conditions for Fibrilase were 20 hours, enzyme loading of 2.0 FPU/g, and solid to liquid ratio of 0.25 g/mL. The extraction conditions for Multifect XL were 20 hours, enzyme loading of 2.1 FPU/g, and solid to liquid ratio of 0.25 g/mL.

Solid to liquid ratio was tested using peel contents of 11, 18, 25, 32, and 39 g of peel in 100 mL of buffer solution with resulting ratios of 0.11, 0.18, 0.25, 0.32, and 0.39 g/mL, respectively. Extraction parameters for CelluPract were 50 mM citrate buffer pH 4.3 with an enzyme loading of 2.1 FPU/g for 2 hours. Extraction parameters for Fibrilase were 50 mM citrate buffer pH 4.3 with an enzyme loading of 2.0 FPU/g for 20 hours. Extraction parameters for Multifect XL were 50 mM citrate buffer pH 4.6 with an enzyme loading of 2.1 FPU/g for 20 hours.

Solid to liquid ratio was reexamined after completion of the enzyme loading and time trials. Solid to liquid ratios of 0.18 and 0.25 g/mL were examined at enzyme to peel ratios of 4.6 and 3.0 FPU/g for Fibrilase and Multifect, respectively. Extraction conditions were 50˚C for 15 hours using 100 mL of 50 mM citrate buffer at pH 4.5 for Fibrilase and 4.7 for Multifect XL. Solid to liquid ratio trials were also repeated for CelluPract at 0.18 and 0.25 g/mL with enzyme loadings of 7.8 and 9.7 FPU/g for 0.18 g/mL solid to liquid ratio and 5.6 and 7.0 FPU/g for 0.25 g/mL solid to liquid ratio. Extraction conditions were a 50 mM citrate buffer pH 4.0 for 2 hours.

Enzyme loadings were tested for CelluPract at intervals ranging from 1.4 to 7.0 FPU/g. Using a solid to liquid ratio of 0.25 g/mL, enzyme loadings of 1.4, 2.1, 2.8, 3.5, 4.2, 5.6, and 7.0 FPU/g were tested. Extraction for CelluPract occurred using 50 mM citrate buffer pH 4.0 for 2 hours. Enzyme loadings were tested for Fibrilase at intervals ranging from 1.3 to 6.5 FPU/g. Using a solid to liquid ratio of 0.25 g/mL, enzyme loadings of 1.3, 2.0, 2.7, and 3.4 FPU were tested. Using a solid to liquid ratio of 0.18 g/mL, enzyme loadings of 3.7, 4.7, 5.6, 6.5, and 7.4 FPU/g were tested. Extraction for Fibrilase occurred using 50 mM citrate buffer pH 4.5 for 20 hours. Enzyme loadings were tested for Multifect XL at intervals ranging from 1.2 to 5.8 FPU/g. Using a solid to liquid ratio of 0.25 g/mL, enzyme loadings of 1.2, 1.8, 2.4, 3.0, and 4.2 FPU were tested. Using a solid to liquid ratio of 0.18 g/mL, enzyme loadings of 2.5, 3.3, 4.2, 5.0, and 5.8 FPU/g were tested. Extraction for Multifect XL occurred using 50 mM citrate buffer pH 4.7 for 20 hours.

Extraction time was tested at 1, 1.5, 2, 2.5, and 3hours for CelluPract and 8, 12, 16, 20, and 24hours for Fibrilase and Multifect XL. The extraction conditions for

CelluPract were 50 mM citrate buffer pH 4.0 with a solid to liquid ratio of 0.25 g/mL at an enzyme loading of 5.6 FPU/g. The extraction conditions for Fibrilase were 50 mM citrate buffer pH 4.5 with a solid to liquid ratio of 0.18 g/mL at an enzyme loading of 5.5 FPU/g. The extraction conditions for Multifect XL were 50 mM citrate buffer pH 4.7 with a solid to liquid ratio of 0.18 g/mL at an enzyme loading of 3.3 FPU/g.

An experiment was conducted to compare pectin yield resulting from extraction under similar conditions for CelluPract, Fibrilase, and Multifect XL. Extraction occurred at 50˚C for 2 hours with a solid to liquid ratio of 0.18 g/mL and an enzyme loading of 3.9 FPU/g using 50 mM citrate buffer at pH 4.0, 4.5, and 4.7 for CelluPract, Fibrilase, and Multifect XL, respectively.

An experimental trial was conducted using combinations of Fibrilase and Multifect XL. Extraction occurred at 50˚C for 2 hours with a solid to liquid ratio of 0.18 g/mL using 50 mM citrate buffer pH 4.5. Enzyme loadings were 3.7 FPU/g for Fibrilase and 3.0 FPU/g for Multifect XL. Additional tests were conducted using a constant 4.7 FPU/g for Fibrilase and varying enzyme loading at 3.0, 4.2, and 5.8 FPU/g for Multifect XL.

3.5 Methods for Comparison of Extraction Conditions

Two varieties of seedless watermelons were tested utilizing the chosen extraction conditions. Conditions for the acid extraction were 95˚C using the waterbath to maintain temperature for 45 min using 1 N nitric acid to adjust the pH to 1.65 at a solid to liquid ratio of 0.258 g/mL. CelluPract extraction was conducted at 50˚C for 2 hours using 50

mM citrate buffer pH 4.0 with a solid to liquid ratio of 0.18 g/mL and at enzyme to peel ratios of 7.8 and 9.7 FPU/g. Conditions for Fibrilase extraction were a 15 hour extraction at 50˚C using 50 mM citrate buffer pH 4.5 with a solid to liquid ratio of 0.18 g/mL and an enzyme to peel ratio of 4.6 FPU/g. Multifect XL extraction was conducted at 50˚C for 15 hours using 50 mM citrate buffer pH 4.7 with a solid to liquid ratio of 0.18 g/mL and an enzyme to peel ratio of 3.0 FPU/g.

3.6 Commercial Enzyme Screening

The enzymes were screened using the filter paper standard assay procedure for cellulase analysis of the International Union of Pure and Applied Chemistry (Ghose, 1987). The method was followed as detailed below. Whatman No. 1 filter paper was cut into 1.0 x 6.0 cm strips weighing 50 ± 5 mg. Dinitrosalicylic Acid (DNS) reagent was prepared and stored at room temperature. Stock solutions of 50 mM citrate buffer, pH 4.8, and 10 mg/mL anhydrous glucose were prepared and stored at refrigerated temperature. Solutions of CelluPract, Fibrilase, and Multifect XL enzymes were prepared at dilutions between 1:150 and 1:225 using citrate buffer. One ml of citrate buffer was added to 25 mL test tubes for all samples, blanks, and standards. Glucose standards were made at concentrations of 6.7, 5.0, 3.3, and 2.0 mg/mL using dilutions of the 10 mg/mL glucose solution in citrate buffer. 0.5 mL of the appropriate standard was added to a test tube. 0.5 mL of enzyme was added to the appropriate test tube. At least two dilutions of each enzyme were used so that the target reading of 2.0 mg glucose could be obtained from the reaction conditions. An enzyme blank was created for each

enzyme dilution. A spectro zero sample was made by adding 0.5 mL of citrate buffer to a test tube. All enzyme samples were acclimated in a 50˚C waterbath and a filter paper strip was added to each enzyme sample and mixed. All samples were incubated exactly 60 min in the waterbath. Three mL of DNS reagent was added to each sample and mixed using a vortex mixer. The same was done to all standards, enzyme blanks, and the spectro zero blank. All samples were boiled together for exactly 5 min and immediately placed in an ice waterbath. 20 mL of DI water was added to each sample and mixed several times by inversion. Samples were held for 20 min at room temperature and then allotments were transferred to 4 mL disposable cuvettes and read at 540 nm wavelength using a Varian Cary 50 BIO uv/visible spectrophotometer. Resulting enzyme activity was calculated and reported in Filter Paper Units (FPU) per milliliter.

3.7 Pectin Analysis

3.7.1 Percent Yield

The percent yield of the pectin was determined as the dry pectin weight divided by the dry weight of the pressed peel.

3.7.2 Galacturonic Acid

Two procedures were jointly conducted to determine the galacturonic acid content of selected pectin samples. Pectin samples were prepared as detailed below according to

the method of Wilkins et al. (2005). Pectin samples were prepared by addition to 50 mM sodium acetate buffer, pH 4.7, in Erlenmeyer flasks at 1% w/v. Sodium azide was added at 0.01% to all flasks as an antimicrobial agent. The samples were acclimated in the New Brunswick Scientific waterbath at 45˚C and 500 uL of a 10% pectinase solution was added to each flask. Hydrolysis occurred for 24 hours with agitation. Samples were heated to approximately 60˚C in an oven to inactivate the pectinase enzyme.

The first method was an adapted HPLC method from Wilkins et al. (2005) which was based on the method of Clarke et al. (1991). Sample dilutions at 1:500 were analyzed for galacturonic acid content. The following procedure is repeated from the referenced method using minor adjustments. A CarboPac PA1 column was used on a Dionex HPLC, regulated at room temperature. A mobile phase of aqueous 16 mM NaOH ran for 25 min followed by a linear gradient to aqueous 100 mM NaOH and 150 mM sodium acetate buffer over 30 min. A step gradient to aqueous 200 mM NaOH for 15 min was used as a column wash and followed by an aqueous 16 MM NaOH 15 minute re-equilibration step. The mobile phase was maintained at 1 mL/min. Detection was conducted with an electrochemical detector with pulsed-amperometric detection. External standards were used to calibrate the detector response.

The second method followed the colorimetric analysis of Kintner et al. (1982) which is based on the commonly accepted analysis method of Blumenkrantz et al. (1973). The method relies on the appearance of a chromagen when a solution of m-hydroxydiphenyl reacts to the uronic acid content of a sample heated in concentrated sulfuric acid/ sodium tetraborate solution. The Kintner method corrects for carbohydrate interference that may occur in the original method. Dilutions at 1:250 of the pectinase

hydrolyzed pectin solutions were analyzed for galacturonic acid content following the method detailed below.

A stock solution of 1 mg/mL galacturonic acid monohydrate was prepared and stored at refrigerated temperature. A 0.0125 M solution of sodium tetraborate decahydrate in concentrated sulfuric acid was prepared by stirring overnight for complete dissolution of the sodium tetraborate and was stored at room temperature. Solutions of 0.15% m-hydroxydiphenyl in 0.5% sodium hydroxide and 0.5% sodium hydroxide were prepared and stored at refrigerated temperature. One mL galacturonic acid standards were prepared at concentrations of 5, 15, 30, 45, 60, and 75 ug/mL using the 1 mg/mL stock solution of galacturonic acid and were added to 25 mL test tubes. Next 1:250 dilutions of the 0.01 g/mL pectinase treated pectin samples were made and 1 mL of each sample was added to 25 mL test tubes. A 1 mL sample blank was made for each sample tested. A reagent blank was also made using 1 mL DI water. All samples were cooled in an ice waterbath and 6 mL of sodium tetraborate/ sulfuric acid solution was added to each. Each sample was vortexed several times to ensure complete mixing and all samples were then heated in a 100˚C waterbath for exactly 5 min and cooled immediately in an ice waterbath. 0.1 mL of the m-hydroxydiphenyl/ sodium hydroxide solution was added to all of the standards and samples. 0.1 mL of sodium hydroxide solution was added to the sample blanks and the reagent blank. All samples were vortexed thoroughly and portions of each were poured into 4 mL disposable cuvettes. After 20 min each sample was read at 520 nm wavelength using a Varian Cary 50 BIO uv/visible spectrophotometer.

Samples chosen for galacturonic acid analysis were from the optimal extraction conditions for acid, CelluPract, Fibrilase, and Multifect XL extractions. Samples were analyzed from three separate experimental runs to obtain an average content from a range of samples for the colorimetric method. Only one set of samples was analyzed using the HPLC method. Colorimetric analysis was also conducted on acid extracted pectin which was obtained under extraction temperature conditions of 85, 90, and 100˚C to determine the impact of extraction temperature on galacturonic acid content. All three samples were from the same trial run that used a common watermelon peel source. CelluPract extracted samples from extraction times of 1, 2, and 4 hours were analyzed using the colorimetric method to determine the impact of extraction time on galacturonic acid content. All three samples were from the same trial run that used a common watermelon peel source.

3.7.3 Degree of Methoxylation

Samples were prepared for analysis using the procedure of Iglesias et al. (2004). Pectinase hydrolyzed pectin solution was properly diluted to yield 12 mL of 8.33 mg/mL solution. The solution was treated with 1 mL of 1 N NaOH for 30 min to hydrolyze the methyl esters. The solution was diluted to yield 20 g. The gas chromatography procedure outlined in the referenced material was followed with adjustments for analysis of methanol in the sample. A 2mx2mm ID Porapak N 80/100 mesh column was used in an Agilent 6890 gas chromatograph. Oven temperature was fixed at 115˚C, injector temperature was 210˚C, and detector temperature was 220˚C. Helium was used as the

carrier gas at a flow rate of 40 mL/min for sample volumes of 1 μL. Dilutions of methanol in deionized water were used to produce a standard curve. Degree of methoxylation (DM) was calculated as the molar ratio of methanol to galacturonic acid. The galacturonic acid values used for this calculation were obtained using the m-Hydroxydiphenyl method.

3.8 Statistical Analysis

All data sets for testing of extraction parameters were performed at least in duplicate. Values presented in tables and figures represent the average of the individual data points collected for a particular variable. A completely randomized design was utilized in this study. All statistical analysis was done on MiniTab 6.0 statistical software with analysis of variance based on t-tests using a 95% confidence level.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Explanation of Material Usage and Experimental Setup

Fresh pressed peel was chosen for use in all extractions instead of dried peel. This decision was based on the loss of yield reported in use of dried peel in pectin extraction. Crandall et al. (1978b) discovered a reduction in both pectin yield and pectin grade due to drying lime and lemon peel in direct fired rotary driers. Because the goal of this project was to optimize pectin yield, it was decided that fresh pressed peel would be used so that drying conditions would not cause a loss in pectin yield. A separate study could be conducted in the future to determine the optimal drying characteristics for watermelon peel.

Factorial experiments were not conducted for either extraction method due to the limited amount of peel available from a single watermelon source, the length of time necessary to conduct each individual trial, and the limited availability of experimental instrumentation and supplies that were necessary to conduct each trial. To conduct a factorial experiment with these limitations, fewer extraction parameters and levels within each parameter could be investigated. Instead, optimization was performed for each parameter individually and the best results were carried over into optimization of the next

parameter. A single watermelon source was utilized for each optimization trial so that variation in pectin content from watermelon to watermelon would not be a factor within each set of results.

4.2 Acid Extraction Experiments

Acid extractions were started utilizing a hot plate with magnetic stirring capability. All tests of extraction parameters and watermelon maturity and variety were conducted using the hot plate. The Precision shaking waterbath with heating capability up to 100˚C was desired for use due to its ability to hold extraction temperatures within a tighter range. However, because of lengthy maintenance requirements the waterbath was not available for use until much later in the project. Once the waterbath was working, several of the optimization experiments were repeated utilizing the shaking waterbath to determine if any variations would exist due to its use. The final comparison of acid extraction and enzymatic extraction was conducted utilizing the waterbath.

4.2.1 Preliminary Experiments

Initial yields averaged less than 10% when following the Crandall referenced extraction method using a solid to liquid ratio of 0.133 g/mL in 750 mL deionized water extracted for 45 min at 90˚C. Experimental trials were conducted to determine if certain steps had an impact on improving pectin yield. Other pectin extraction methods were

examined to determine which process variations existed and what process points could be of interest.

4.2.1.1 Pectin Retrieval. It was possible that pectin loss was occurring during the precipitation step and the alcohol washes. There was a large cloudy mass within the liquid that was possibly pectin that was not being recovered and retained in the following separation and washing steps. Experiments were set up to test the method of retrieving the pectin from the alcohol after precipitation. Experiments were conducted under standard extraction conditions comparing centrifugation and cheesecloth as retrieval methods for the precipitated pectin. The pectin yields for the centrifugation and cheesecloth methods were 14.3% and 10.6%, respectively. These results indicated that more pectin was retained using the centrifugation method.

4.2.1.2 Multiple Extractions. The first process point to be examined was the number of extractions performed on the peel mass. Several of the referenced studies performed a series of extractions on the peel mass to produce a higher volume of solubilized pectin. Two extractions at 80˚C, the first for 60 min and a reextraction of the peel mass for 10 min, were reported for peach pectin extraction (Chang et al., 1973). A similar process was applied to the watermelon peel extraction by performing two extractions on the same peel mass and separating the resulting filtrates to determine pectin yield. A very small amount of pectin precipitated from the second extraction. Very little of the pectin remained at the end of the alcohol washings and the pectin yield was considered to be negligible. It appeared that all solubilization of the watermelon

pectin occurred during the first extraction period and a second extraction was not necessary.

4.2.1.3 Citrus Peel Extraction. Because no major increases in pectin yield had occurred from the changes made so far, it was decided that it would be beneficial to repeat a citrus pectin extraction utilizing oranges for comparison. The method of Rouse et al. (1976) was followed for pectin extraction. Valencia oranges were used to conduct the study in the same manner as in the referenced study. The resulting pectin yield was considerably higher than any of the watermelon rind pectin extractions at 32.7% of the pressed peel dry weight. The precipitated pectin behaved completely different from the watermelon pectin that was precipitated utilizing the original method. The precipitated citrus pectin congealed and clumped together and was very easy to separate from the precipitation alcohol using cheesecloth and then wash with alcohol to remove impurities.

4.2.1.4 Solid to Liquid Ratio. The Rouse et al. (1976) method was examined for potential differences between it and the current method that might influence pectin yield. The solid to liquid ratio was noticed to be considerably different from the original method and was tested on watermelon peel to determine whether it was a factor in the pectin yield. The solid to liquid ratio was changed to 0.258 g/mL using 1 N nitric acid to adjust pH to 1.65 at 90˚C. The resulting yield was 27.0% of the dried pressed peel weight, which was considerably higher than was observed for the lower solid to liquid ratio. Several positive differences were observed in the experimentation process. In the centrifugation step following the extraction, the solids were noticed to pellet more than

with the lower solid to liquid ratio, making it easier to filter the solution. The precipitated pectin behaved similar to the orange peel pectin and was easy to separate from the alcohol and wash in the alcohol washings.

The procedure was repeated using different peel sources at the same solid to liquid ratio. Experiments were conducted at the same amounts of peel and water and a half scale version. The resulting pectin yields were 18.0% and 23.4% of the dried pressed peel weight, respectively. The process was determined to be repeatable and an experiment was conducted to determine the difference in yield between the old and new solid to liquid ratios utilizing the same peel source. Keeping the water volume constant at 750 mL, pressed peel contents of 100 g (old method) and 193.2 g (new method) were utilized resulting in pectin yields of 11.95% and 19.33%, respectively. The precipitation alcohol was observed to be much clearer for the higher solids content, indicating that more pectin was precipitating out of solution for that sample. The precipitation solution was centrifuged after recovery of the congealed pectin using cheesecloth to determine how much pectin was lost in the cheesecloth retrieval process. A very small amount of pectin was collected from the higher solids sample after centrifugation and was determined to be negligible in comparison to the amount collected through cheesecloth straining. Thus, utilizing the new solid to liquid ratio, it was no longer necessary to centrifuge the pectin alcohol mixture to retrieve the remaining pectin.

4.2.1.5 Effect of Acid Type. Because a number of different mineral acids are reported in extraction conditions for different pectin sources (Braddock, 1999), it was considered worthwhile to examine the impact of acid type on watermelon pectin yield.

The combination of 1 N HCl and ethanol was tested at solid to liquid ratios of 100 g and 193.2 g in 750 mL water using 45 minute extraction periods at pH 1.65 and 95˚C. The resulting pectin yields were 16.0% and 10.8% for solid to liquid ratios of 100 and 193.2 g, respectively. Although the yield was higher than average for the 100 g sample, for the 193.2 g peel sample it was lower than using nitric acid and isopropanol. Experimentation was then conducted to determine if different extraction acid and precipitation alcohol combinations would have an effect on pectin yield. 1 N nitric acid and 1 N hydrochloric acid were used in combination with isopropanol and ethanol on different seedless watermelons using 45 minute extraction periods at pH 1.65, 95˚C, and a solid to liquid ratio of 0.258 g/mL using 750 mL DI water. As shown in Table 4.1, the nitric acid and isopropanol appeared to be slightly better than the hydrochloric acid and isopropanol combination and was utilized in all of the remaining acid extraction experiments. Ethanol was found to promote less congealing in the watermelon pectin samples than isopropanol and the yields produced for both acid types in combination with ethanol were lower as a result.

Table 4.1. Comparison of pectin yield (dry weight basis) utilizing different extraction acid and precipitation alcohol combinations. Extraction conditions were a solid to liquid ratio of 0.258 g/mL, pH 1.65, 95˚C, and 45 min.

		Alcohol Type	
		Isopropanol	**Ethanol**
Acid Type	**1 N Nitric Acid**	20.7%[a]	15.0%[a]
	1 N Hydrochloric Acid	19.0%[a]	13.3%

[a]Values are averages of duplicate samples

4.2.2 Effect of Solid to Liquid Ratio

Because of large differences in both pectin yield and precipitation behavior that were observed with the adjustment to the solid to liquid content of the extraction procedure, it was deemed necessary to test a broader range of solid to liquid ratio to determine the best extraction value. Peel content was tested at 0.133, 0.20, 0.233, 0.247, 0.258, 0.28, and 0.30 g/mL in 750 mL DI water on the stirring hot plate and the resulting pectin yields are shown in Figure 4.1. Due to the large number of peel to liquid contents being tested and the limited amount of peel available from a single watermelon, not all trials could be completed from the same watermelon peel sample. Peel was utilized from different seedless watermelons all purchased from the same bin. A second scaled down experiment was conducted a year later to verify that the trend still occurred. This experiment utilized the same extraction conditions as before for peel contents of 0.133, 0.23, 0.258, and 0.28 g/mL in 375 mL DI water. These extractions used peel from a single watermelon source at half the water content of the first series of extractions. The experiments were scaled down so that all of the experiments could be completed using a single watermelon source. There was not a difference in pectin yield due to solid to liquid ratio for any of the samples ($p=0.201$). Based on data trends the 0.258 g/mL content appeared to produce a slightly higher pectin yield, as shown in Figure 4.1.

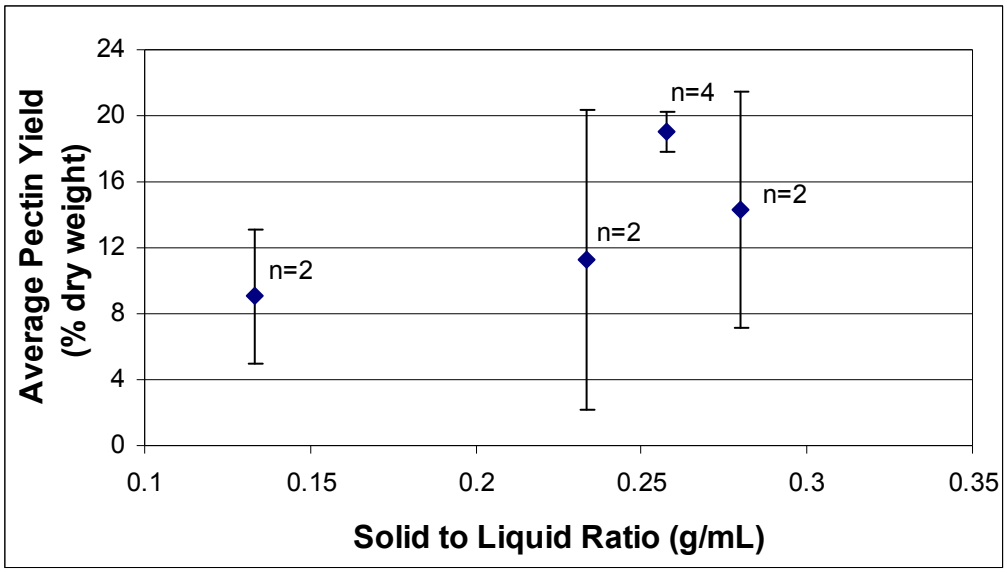

Figure 4.1. Effect of peel content on pectin yield at extraction conditions of 45 min, 90˚C, and pH 1.65 with 1 N nitric acid using the stirring hot plate. Sample size and standard deviation are indicated for each average value.

The solid to liquid ratio trials were repeated using the shaking waterbath. Experiments were scaled down to 375 mL DI water so that all trials could be from the same watermelon source. Extraction conditions were kept the same as the hot plate experiments to test peel contents of 0.233, 0.247, 0.258, and 0.28 g/mL. Trials were repeated on two different seedless watermelon varieties at solid to liquid ratios of 0.133 and 0.258 g/mL. These trials were repeated to determine if the same jump in pectin yield would occur as was observed during the initial experimentation trials that triggered the examination of solid to liquid ratio. All trials produced similar amounts of pectin at the tested solid to liquid ratios and did not exhibit the original trends. A solid to liquid ratio of 0.258 g/mL was used in further extractions.

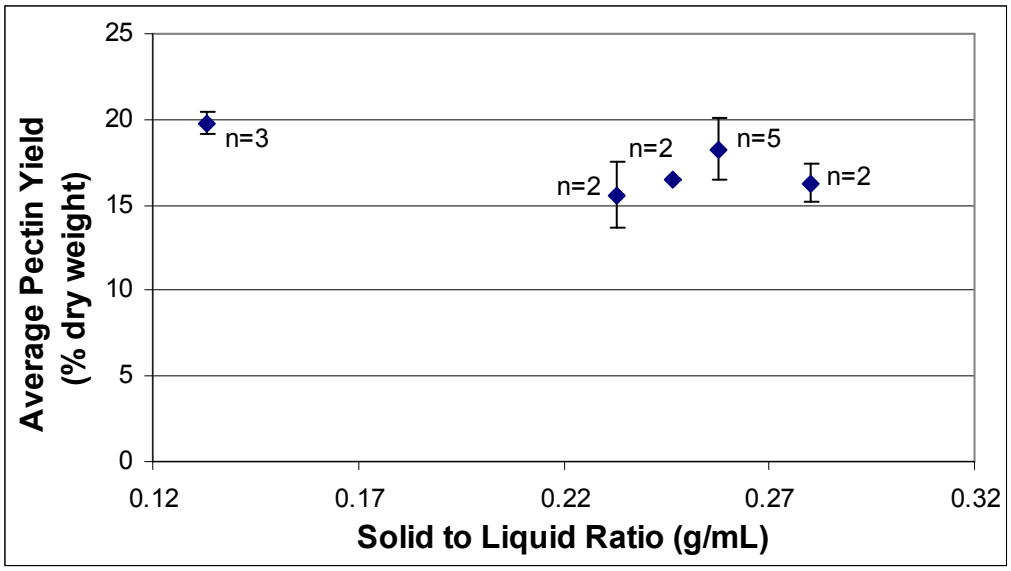

Figure 4.2. Effect of peel content on pectin yield at extraction conditions of 45 min, 90˚C, and pH 1.65 with 1 N nitric acid using the shaking waterbath. Sample size and standard deviation are indicated for each average value.

These results indicate that there was a difference in pectin yield between solid to liquid ratios of 0.133 and 0.233 g/mL (p=0.046), as shown in Table 4.2.

Table 4.2. Average pectin yields for acid extraction using the waterbath at varying solid to liquid ratios. Values with the same superscript are not significantly different.

Solid to liquid ratio (g/mL)	Average Pectin Yield (% dry weight)
0.133	19.8 ±0.7[b]
0.233	15.6 ±1.9[a]
0.247	16.5 ±0.01[a,b]
0.258	18.3 ±1.8[a,b]
0.280	16.3 ±1.1[a,b]

4.2.3 Effect of Temperature

The effect of temperature on pectin yield was examined next. Extraction temperatures of 80, 90, and near 100˚C were examined. There was no significant

difference in yield due to temperature (p=0.097). The results shown in Figure 4.3 show a data trend of an increase in pectin yield with an increase in extraction temperature.

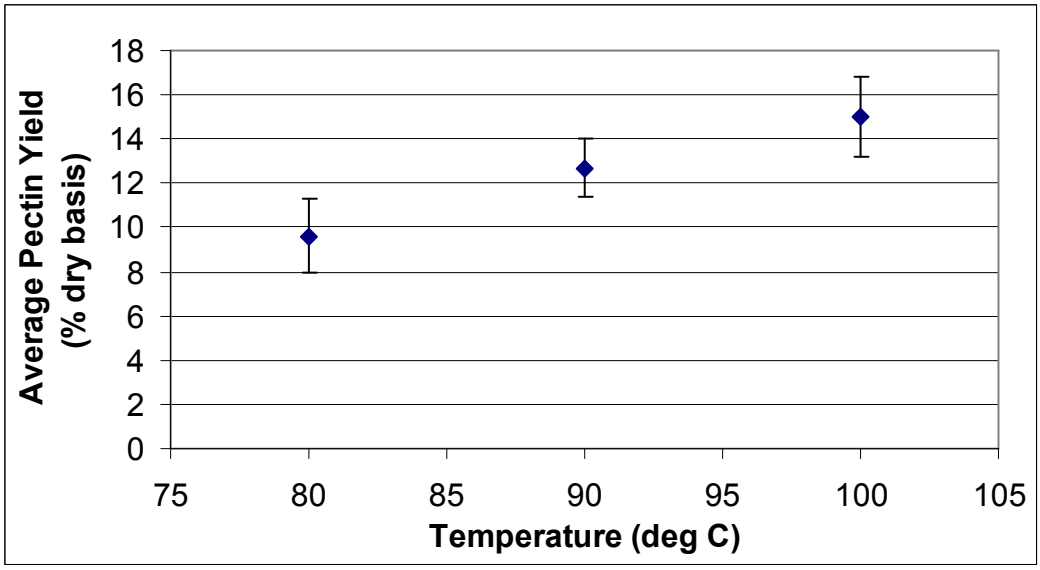

Figure 4.3. Effect of temperature on acid extracted pectin yield at extraction conditions of 45 min, pH 1.65, and solid to liquid ratio of 0.258 g/mL using a hot plate. All values are averages of duplicate samples with sample standard deviation indicated by error bars.

The process was repeated utilizing the shaking waterbath, which could more accurately hold temperature at a desired value. A tighter temperature range of 85, 90, and 95˚C was tested to determine if there were any significant changes. Due to heat loss with removal of the waterbath cover when the extraction is started, it was decided that 95˚C would be the highest temperature tested. The same extraction parameters were used as in the hot plate trials, which were pH 1.65, 45 min, and a solid to liquid ratio of 0.258 g/mL. There was not a difference between the yields resulting from temperature (p=0.051). The same data trend of increase in pectin yield with an increase in temperature was found using the waterbath, as shown in Figure 4.4. Based on this trend, an extraction temperature of 95˚C was chosen and used in all further extractions.

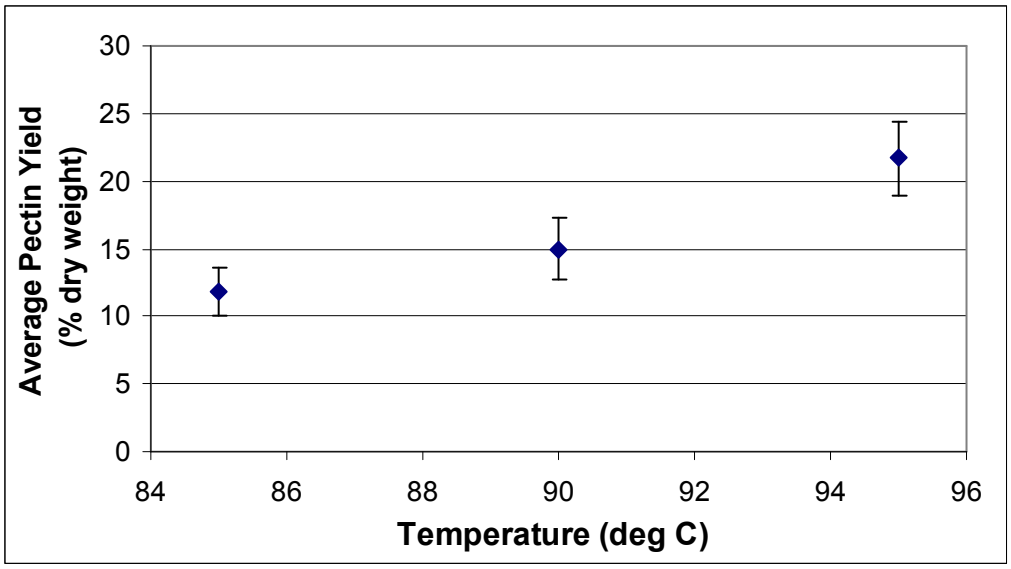

Figure 4.4. Effect of temperature on acid extracted pectin yield at extraction conditions of 45 min, pH 1.65, and solid to liquid ratio of 0.258 g/mL using a waterbath. All values are averages of duplicate samples with sample standard deviation indicated by error bars.

4.2.4 Effect of Time

The effect of time on pectin yield was examined to determine if increasing extraction time would increase pectin yield. There was no difference in pectin yield between any of the extraction times ($p=0.729$). No noticeable trend occurred with increasing extraction time from 45 min to 90 min, as shown in Figure 4.5. Because no increase in yield was observed with increasing time, the extraction time was left at 45 min, which was the extraction time used in the referenced method (Crandall et al., 1978a).

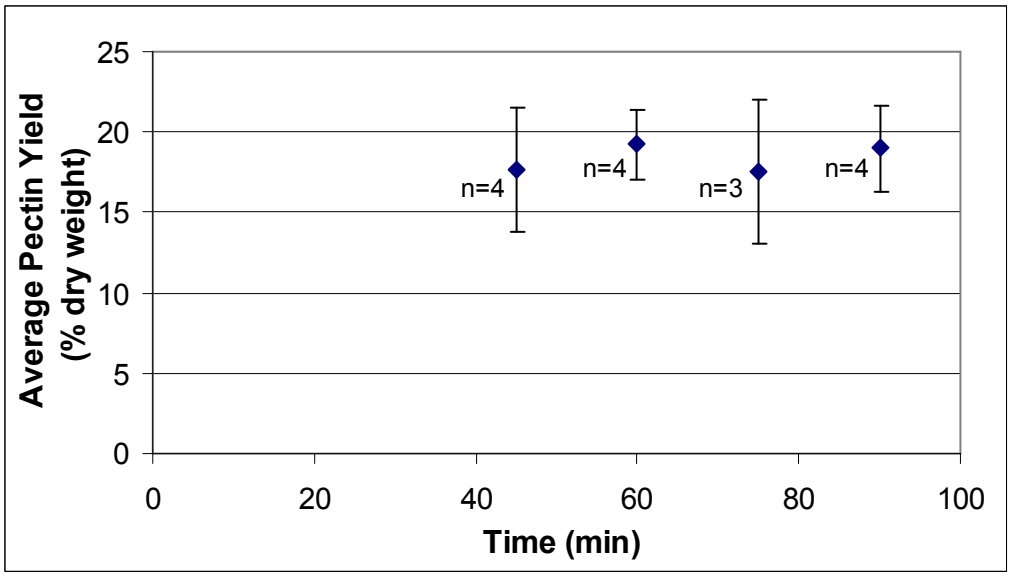

Figure 4.5. Effect of time on acid extracted pectin yield at extraction conditions of 95˚C, pH 1.65 using 1 N nitric acid, and solid to liquid ratio of 0.258 g/mL using a hot plate. Sample size and standard deviation are indicated for each average value.

4.2.5 Effect of pH

The effect of pH on pectin yield was determined using increasing amounts of 1 N nitric acid to lower the pH to the desired values of approximately 1.55, 1.65, and 1.75. Because of slight changes in solution pH throughout the extraction, it was difficult to repeatedly reach an exact pH so approximately 2% error was allowed when measuring pH. The first trial was performed using seeded watermelon rind with the hot plate and the second trial was performed using seedless watermelon with the waterbath. A seeded watermelon was used for the first trial because it was the only watermelon that was available during that time period. The pH trial was conducted the second time with a seedless watermelon to verify that the same trends would exist with a seedless watermelon. Both trials showed that pectin yield was greater at pH 1.65 ($p=0.007$) than for values above and below 1.65 as shown in Figure 4.6. Pectin yields due to pH 1.55

and 1.75 were statistically the same. pH 1.65 was considered optimal and used for all remaining extractions.

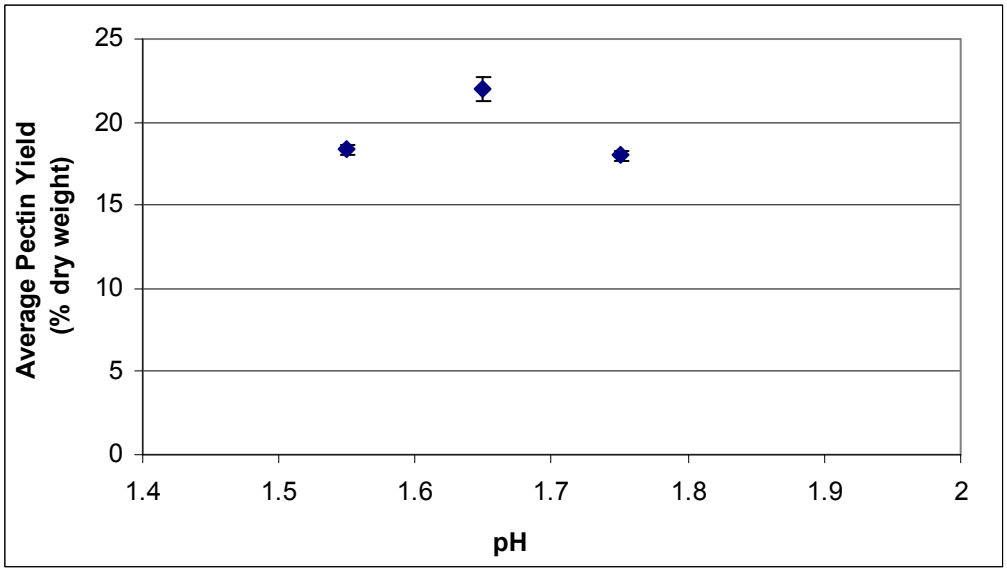

Figure 4.6. Effect of pH using 1 N nitric acid on acid extracted pectin yield at extraction conditions of 45 min, 95˚C, and solid to liquid ratio of 0.258 g/mL. All values are averages of duplicate samples with sample standard deviation indicated by error bars.

4.2.6 Effect of Watermelon Maturity

A large portion of watermelon waste that could be utilized for pectin extraction comes from unharvested watermelons. Because there is likely to be a large variation in the maturity levels of the unharvested melons, it was of interest to determine what differences might exist in pectin yield due to maturity. Three watermelon varieties were picked for use in this study, which were the seedless varieties Bobbie and 5144 and the seeded variety Lantha. Melons were classified as ripe, slightly overripe, overripe, and very overripe. These classifications were determined according to flesh characteristics and taste. Ripe was classified as a melon with smooth, crisp flesh and a good flavor. Slightly overripe was classified as a melon with slightly cracked flesh and a slightly off

flavor. Overripe was classified as a melon with very cracked flesh and off flavor. Very overripe was classified based on extremely cracked, mushy flesh and an off odor. As shown in Figure 4.7, there appear to be no definite trends in pectin yield due to maturity level for the three varieties tested. There was no statistical difference in pectin yield due to maturity for either Bobby or Lantha. For 5144 the very overripe and ripe pectin yields were statistically the same and were both significantly higher than the slightly overripe and overripe pectin yields which were statistically the same, as shown in Figure 4.7.

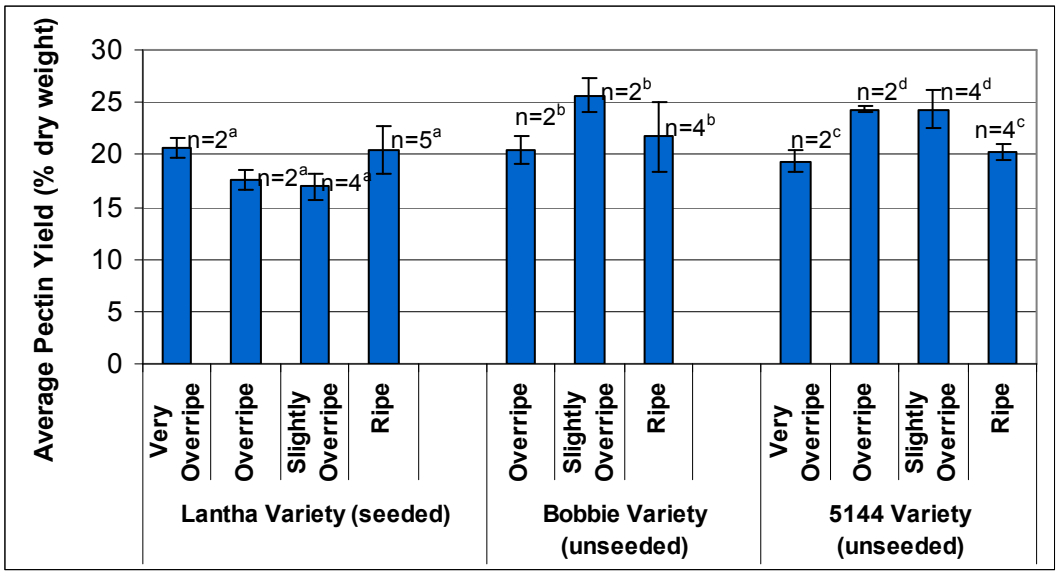

Figure 4.7. Effect of watermelon harvest maturity on pectin yield for varieties Lantha, Bobbie, and 5144 using 1 N nitric acid to adjust pH to 1.65 at extraction conditions of 45 min, 95˚C, and solid to liquid ratio of 0.258 g/mL. Sample size and standard deviation are indicated for each average value. Statistical significance was determined within each variety and values with the same superscript are not significantly different.

4.2.7 Effect of Watermelon Variety

It was also of interest to determine whether watermelon variety had an effect on pectin yield. The ripe watermelons from the maturity studies for seedless varieties

Bobbie and 5144 and the seeded variety Lantha were used for this analysis. As shown in Figure 4.8, no difference in pectin yield was found due to watermelon variety (p=0.637).

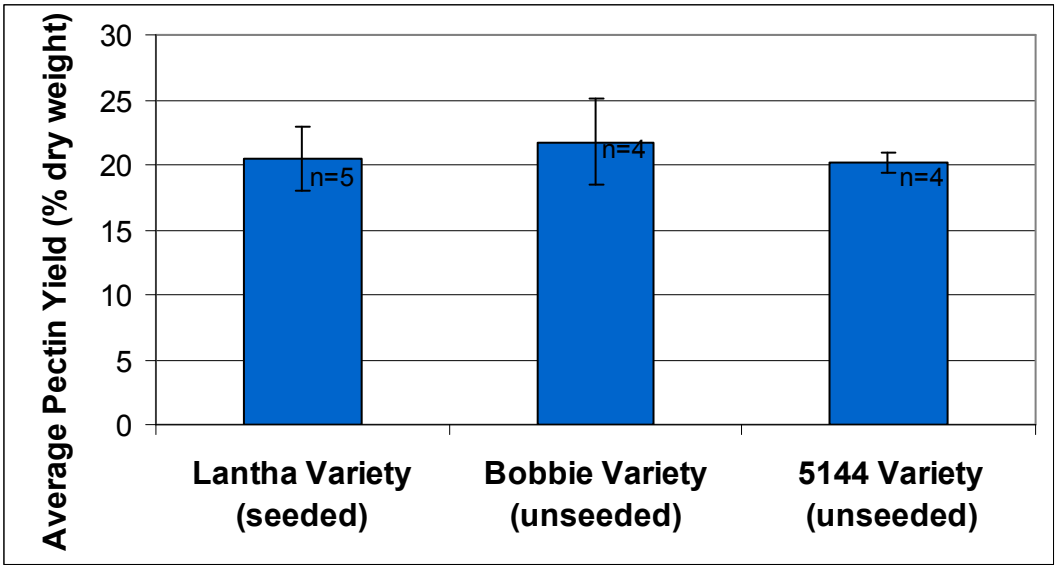

Figure 4.8. Effect of watermelon variety on pectin yield for Lantha, Bobbie, and 5144 varieties using 1 N nitric acid to adjust pH to 1.65 at extraction conditions of 45 min, 95˚C, and solid to liquid ratio of 0.258 g/mL. Sample size and standard deviation are indicated for each average value.

4.3 Enzymatic Extraction Experiments

Enzymatic extractions were conducted to determine whether this could be a suitable method for extraction of pectin from watermelon rind. In studies conducted by other researchers, enzymatic extractions yielded higher amounts of pectin when compared to the traditional acid extractions (Shkodina et al., 1998, Sakamoto et al., 1995). Enzymatic extractions could also prove to be more cost effective when used in the watermelon biorefinery.

In order to efficiently run the large number of experiments required to optimize all of the parameters for the enzymes chosen, the extraction temperature was held at 50˚C

for all optimization experiments. This temperature was within the manufacturer's range for all enzymes tested and allowed for testing of all enzymes at once for a given allotment of watermelon peel.

Based on the reported factors influencing pectin yield through enzymatic extractions and bioextractions, buffer pH, solid to liquid ratio, enzyme loading, and extraction time were chosen as the parameters to optimize for enzymatic watermelon pectin extraction yield. Buffer concentration was also investigated to ensure that the best concentration had been chosen for all enzymes tested.

4.3.1 Preliminary Experiments

4.3.1.1 *Trichoderma viride* cellulase

The first enzymatic pectin extraction trials were conducted using *Trichoderma viride* cellulase. This enzyme was chosen for initial extraction experiments because it had resulted in the highest yield and quality of pectin from pumpkin, which is also within the Cucurbit family (Shkodina et al., 1998). The watermelon rind pectin obtained using *T. viride* enzyme was less than 1% of the dry weight. Also, the pectin did not congeal well and produced a sort of grainy precipitate. Adjustments were made to the extraction parameters to determine if a better yield could be obtained with this enzyme.

Solid to liquid ratio was changed to 0.20 and 0.50 g/mL resulting in negligible pectin yields for 0.2 g/mL and 0.25% of the dry weight for 0.5 g/mL. These initial trials utilized isopropanol for precipitation due to its success in precipitating watermelon rind pectin in acid extractions. Because of the low pectin yields, it was of interest to try

ethanol for precipitation. Ethanol was listed in a number of other studies as the desired alcohol for enzyme extracted pectin precipitation (Sakai et al., 1980, Shkodina et al, 1998). The solid to liquid ratio was tested again using ethanol as the precipitation alcohol. Using dried peel the solid to liquid ratio was tested at the equivalent of wet peel amounts of 0.129, 0.257, and 0.386 g/mL resulting in 0.9, 3.8, and 2.4% pectin, respectively. Dried peel was used because no whole watermelons were available at that time for purchase. In addition to the increase in pectin yield an improvement was observed in the precipitation conditions of the pectin. With ethanol precipitation the pectin congealed and was easy to separate from the precipitation alcohol. The trial was repeated using wet peel at solid to liquid ratios of 0.083 g/mL, 0.257 g/mL, and 0.386 g/mL. The solid to liquid ratios of 0.257 g/mL and 0.386 g/mL resulted in 2.7% and 0.9% pectin, respectively. There appeared to be a difference in the pectin yield due to solid to liquid ratio using both dried and fresh pressed peel. A solid to liquid ratio of 0.25 g/mL was chosen based on this observation.

The next parameter to be varied was enzyme loading. Three samples were tested using 50, 75, and 100 mg of enzyme. The temperature was increased to 55˚C from the referenced 30˚C based on the ideal range provided in the manufacturer's specifications for the enzyme. The resulting pectin yields were 0.59, 0.83, and 2.7% for enzyme loadings of 50, 75, and 100 mg, respectively. The enzyme loadings were increased to 100, 175, and 257 mg and trials were conducted under the previous conditions with a new buffer solution at pH 4.45. The resulting pectin yields were 2.1, 3.0, and 4.0%, respectively.

Buffer solutions and concentrations were adjusted next to determine their effect

on pectin yield. 100 mL solutions of 50 mM sodium citrate buffer, 100 mM sodium acetate buffer, and 100 mM citrate buffer were used for extraction. The resulting pectin yields were 1.2, 0.4, and 1.5%, respectively.

The yields for this enzyme were all consistently low regardless of the adjustments in extraction parameters. Because it did not appear to be possible to increase yields to reasonable amounts, this enzyme was determined not to be useful for pectin extraction.

4.3.1.2 Enzyme Screening. Several additional enzymes were tested to determine their resulting pectin yields. The enzymes were screened using two different buffer solutions: a 75 mM sodium acetate buffer and a 50 mM sodium citrate buffer. When using the acetate buffer, negligible amounts of pectin precipitated in the ethanol and it was determined that acetate buffer would not be compatible with the extraction process. Extractions were repeated using 50 mM citrate buffer at pH 4.4 for enzymes Multifect CL, Multifect GC, Fibrilase, Multifect XL, and CelluPract. As shown in Table 4.2, pectin yields of 9.7% and 15.7% were obtained for Multifect CL and Multifect XL, respectively. Pectin yields of 11.5% and 15.8% were obtained for Multifect GC and Fibrilase, respectively. No pectin congealed in the ethanol for the CelluPract enzyme but the solution was very cloudy, an indication that pectin had possibly been solubilized by the enzyme but had not precipitated. This cloudiness had been observed in initial acid extractions and precipitated pectin resulted after other factors were adjusted. CelluPract was chosen for repeat extractions. CelluPract was extracted under the same conditions but with a shorter extraction time of 12 hours. As shown in Table 4.3, the resulting pectin yield was 11.5% for CelluPract. Multifect XL and Fibrilase were chosen for

further testing based on the yields produced in these initial studies. Due to the fact that the precipitation alcohol solution was still very cloudy and the pectin yield had increased significantly with the reduction in extraction time, CelluPract was chosen for additional time studies to determine if the yield would increase as extraction time was decreased.

Table 4.3. Pectin yields for enzymes using 50 mM citrate buffer and a solid to liquid ratio of 0.25 g/mL extracted at 50˚C for approximately 24 hours.

Enzyme	Pectin Yield (% dry basis)
Multifect GC	11.5
Multifect XL	15.7
Fibrilase	15.8
Multifect CL	9.7
CelluPract	11.5[a]

[a]12 hour extraction time instead of 24 hours

4.3.1.3 Multifect XL. Solid to liquid ratio, enzyme loading, extraction time and buffer pH were adjusted to determine their effect on pectin yield using Multifect XL. Solid to liquid ratio was tested at 0.12, 0.25, and 0.50 g/mL. As shown in Table 4.4, solid to liquid ratio appeared to have an impact on pectin yield and was greatest with a solid to liquid ratio of 0.25 g/mL. It would be beneficial to test solid to liquid ratios within the tested range to determine at which value extraction should occur.

Table 4.4. Initial pectin yields for extraction using Multifect enzyme at solid to liquid ratios of 0.12, 0.25, and 0.50 g peel/mL using 50 mM citrate buffer pH~4.45 with an enzyme loading of 1.4 FPU/g at 50˚C for approximately 24 hours.

		Pectin Yield (% dry weight)	
Solid to liquid ratio (g/mL)	**0.12**	5.0	11.2
	0.25	13.1	17.7
	0.50	10.4	6.2

Enzyme loading was tested at intervals of 0.7, 1.4, 2.1, and 2.8 FPU/g. As shown in Table 4.5, enzyme loading caused an increase and then decline in yield with increasing amount for one trial and a continuous increase in yield for the other trial. Additional data

should be obtained in further experiments to determine which trend is correct.

Table 4.5. Initial pectin yields for extraction using Multifect at enzyme loadings of 0.7, 1.4, 2.1, and 2.8 FPU/g using 0.25 g peel/mL in 50 mM citrate buffer pH~4.45 at 50˚C for approximately 24 hours.

		Pectin Yield (% dry weight)			
		Seedless Watermelon 1	Seedless Watermelon 2	Seedless Watermelon 3	Seedless Watermelon 4
Enzyme Loading (FPU/g)	0.7	11.6	14.2	-	-
	1.4	13.3	17.7	17.3	22.2
	2.1	10.3	20.1	18.7	25.4
	2.8	-	-	24.5	24.6

Time was tested at 20 and 24 hours to determine if time impacted yield. As shown in Table 4.6, no apparent trend existed for the times tested but the differing results indicate that further extractions should be conducted to determine the influence of time.

Table 4.6. Initial pectin yields for extraction using Multifect enzyme at times of 20 and 24 hours using 0.25 g peel/mL in 50 mM citrate buffer pH~4.45 with an enzyme loading of 1.4 FPU/g at 50˚C.

		Pectin Yield (% dry weight)	
		Seedless Watermelon 1	Seedless Watermelon 2
Time (hours)	20	22.7	18.7
	24	21.9	25.4

Buffer pH was adjusted at intervals between 4.4 and 5.0 and, as shown in Table 4.7, the results indicate that the lower pH produced a higher yield and a broader range of pH values should be tested to determine which should be chosen for extraction.

Table 4.7. Initial pectin yields for extraction using Multifect enzyme at buffer pH ranging from 4.4 to 5.0 using 0.25 g peel/mL in 50 mM citrate buffer with an enzyme loading of 1.4 FPU/g at 50˚C for approximately 24 hours.

		Pectin Yield (% dry weight)		
		Seedless Watermelon 1	Seedless Watermelon 2	Seedless Watermelon 3
pH	4.4	-	-	12.4
	4.5	11.2	13.8	-
	4.6	-	-	11.4
	4.75	7.9	7.4	-
	4.8	-	-	11.2
	5.0	6	5.3	5.9

Fibrilase. Solid to liquid ratio, enzyme loading, extraction time and buffer pH were adjusted to determine their effect on pectin yield using Fibrilase. Solid to liquid ratio was tested at 0.12, 0.25, and 0.50 g/mL. Based on this first set of results shown in Table 4.8 and the data from the Multifect XL trials it was determined that a solid to liquid ratio of 0.25 g/mL would be used for further initial studies using Fibrilase.

Table 4.8. Initial pectin yields for extraction using Fibrilase enzyme at solid to liquid ratios of 0.12, 0.25, and 0.50 g peel/mL using 50 mM citrate buffer pH~4.3 with an enzyme loading of 1.3 FPU/mL at 50˚C for 24 hours.

		Pectin Yield (% dry weight)
Solid to liquid ratio (g/mL)	**0.12**	8.6
	0.25	9.5
	0.50	3.0

Enzyme loading was tested at 0.7, 1.4, 2.0, and 2.7 FPU/g. As shown in Table 4.9, yield increased with increasing enzyme loading and should be tested at higher values to determine the enzyme loading for extraction.

Table 4.9. Initial pectin yields for extraction using Fibrilase enzyme at enzyme loadings of 0.7, 1.4, 2.0, and 2.7 FPU/g using 0.25 g peel/mL in 50 mM citrate buffer pH~4.3 at 50˚C for 24 hours.

		Pectin Yield (% dry weight)		
		Seedless Watermelon 1	**Seedless Watermelon 2**	**Seedless Watermelon 3**
Enzyme Loading (FPU)	**0.7**	0.6	-	-
	1.3	9.5	15.7	13.1
	2.0	18.2	17.1	16.0
	2.7	-	-	18.0

Extraction time was tested at intervals between 16 and 28 hours. As shown in Table 4.10, pectin yield was higher in most trials at extraction times of 16 and 24 hours and a broader range should be tested to determine extraction time.

Table 4.10. Initial pectin yields for extraction using Fibrilase enzyme at extraction times of 16, 20, 24, and 28 hours using 0.25 g peel/mL in 50 mM citrate buffer pH~4.3 with an enzyme loading of 1.3 FPU/g at 50˚C.

		Pectin Yield (% dry weight)		
		Seedless Watermelon 1	Seedless Watermelon 2	Seedless Watermelon 3
Time (hours)	16	13.1	-	24.1
	20	-	16.8	20.2
	24	15.7	16.0	19.8
	28	-	13.0	-

Buffer pH was varied at intervals between 4.0 and 4.8 resulting in no apparent trend in yield due to pH as shown in Table 4.11.

Table 4.11. Initial pectin yields for extraction using Fibrilase enzyme with buffer pH ranging from 4.0 to 4.8 using 0.25 g peel/mL in 50 mM citrate buffer with an enzyme loading of 1.3 FPU/g at 50˚C for 24 hours.

		Pectin Yield (% dry weight)		
		Seedless Watermelon 1	Seedless Watermelon 2	Seedless Watermelon 3
pH	4.0	8.2	7.3	9.8
	4.2	-	-	12.0
	4.3	12.3	10.8	-
	4.4	-	-	8.0
	4.6	14.6	10.5	9.4
	4.8	-	-	12.0

4.3.1.4 CelluPract. The CelluPract precipitation solution was still very cloudy after the first 12 hour extraction and it was of interest to test shorter extraction times to see if a higher yield would result. Solid to liquid ratio had proven to be optimal at 0.25 g/mL for both Multifect XL and Fibrilase and was chosen for use in CelluPract initial trials. Extraction times of 6 and 8 hours were tested resulting in yields of 13.4 and 9.8%, respectively. A trial with duplicate samples was conducted with extraction times shortened to 2, 4, 6, and 8 hours resulting in higher yields at 2 and 4 hours as shown in Table 4.12. Extractions at 1, 2, 3, and 4 hours were conducted resulting in the highest yields at 2 and 3 hours, as shown in Table 4.12. Based on these results an extraction time

of 2 hours was used for the remaining initial trials.

Table 4.12. Initial pectin yields for extraction using CelluPract enzyme varying extraction time between 1 and 8 hours using 0.25 g peel/mL in 50 mM citrate buffer pH~4.4 with an enzyme loading of 1.4 FPU/g at 50˚C.

		Pectin Yield (% dry weight)				
		Seedless Watermelon 1	Seedless Watermelon 2		Seedless Watermelon 3	Seedless Watermelon 4
Time (hours)	1	-	-	-	16.1	16.7
	2	-	18.0	17.2	17.9	16.7
	3	-	-	-	14.5	17
	4	-	15.6	21.9	9.5	7.9
	6	13.4	12.3	12.4	-	-
	8	9.8	5.0	-	-	-

Enzyme loading was tested at 0.7, 1.4, 2.1, and 2.8 FPU/g and yield increased with an increase in enzyme loading, as shown in Table 4.13.

Table 4.13. Initial pectin yields for extraction using CelluPract enzyme for enzyme loadings of 0.7, 1.4, 2.1, and 2.8 FPU/g using 0.25 g peel/mL in 50 mM citrate buffer pH~4.4 at 50˚C for 2 hours.

		Pectin Yield (% dry weight)	
		Seedless Watermelon 1	Seedless Watermelon 2
Enzyme Loading (FPU/g)	0.7	10.6	11.0
	1.4	16.0	-
	2.1	19.4	-
	2.8	17.8	17.6

Buffer pH was varied at intervals between 3.5 and 5.0 and as shown in Table 4.14.

Lower pH produced higher yields.

Table 4.14. Initial pectin yields for extraction using CelluPract enzyme varying pH between 3.5 and 5.0 using 0.25 g peel/mL in 50 mM citrate buffer with an enzyme loading of 1.4 FPU/mL at 50˚C for 2 hours.

		Pectin Yield (% dry weight)			
		Seedless Watermelon 1	**Seedless Watermelon 2**	**Seedless Watermelon 3**	**Seedless Watermelon 4**
pH	**3.5**	-	-	-	20.3
	3.8	-	-	19.3	20.8
	4.0	21.2	16.3	16.8	18.6
	4.2	-	-	13.8	19.8
	4.4	-	-	15.1	-
	4.5	17.0	15.2	-	16.0
	4.6	-	-	16.2	-
	4.8	-	-	15.5	-
	5.0	12.8	10.8	12.4	-

4.3.2 Enzyme Selection

Based on pectin yields in the preliminary studies, Multifect XL, Fibrilase, and Cellupract were all chosen for further analysis and comparison. It was of interest to compare pectin yield and quality due to enzyme type. All three enzymes contained different enzyme combinations according to the manufacturer specifications. CelluPract was reported to be a cellulase with beta-glucanase and xylanase activities. Multifect XL was reported to contain cellulase and endoxylanase activity. Fibrilase did not specify enzyme activity other than cellulase.

4.3.3 Commercial Enzyme Activity

The three enzymes that were chosen for exploration of enzymatic pectin yield were analyzed to determine cellulase activity. Repeated trials were conducted to minimize variation and the resulting activity levels and averages are shown in Table 4.15. The average cellulase activities for CelluPract, Fibrilase, and Multifect XL were found to

be 70, 67, and 60 FPU/mL, respectively. Two different samples of Multifect XL were analyzed due to the use of both in the enzymatic extraction experiments. The old sample was expected to last throughout the entire experimentation process and, due to unexpected additional trials, it was necessary to introduce a new enzyme sample. The old sample had an activity level that was 9 FPU/mL greater than the replacement new sample. Because the new Multifect XL enzyme was used in replacement of the old before the enzyme loading trials were conducted, no adjustment was made for the difference in activity levels.

Table 4.15. Cellulase enzyme activities for CelluPract, Fibrilase, and Multifect XL enzymes.

Enzyme	Average Enzyme Activity (FPU/mL)
CelluPract	**70 ±1.1**[a]
Fibrilase	**67 ±1.2**[a]
Multifect XL $_{new}$	**60 ±1.8**[a]
Multifect XL $_{old}$	**69 ±0.6**[b]

[a]Values are for sample sets of 4
[b]Values are for duplicate samples

4.3.4 Effect of Buffer Concentration

The effect of buffer concentration on pectin yield was tested for CelluPract, Fibrilase, and Multifect XL. Citrate buffer solutions were made at concentrations of 25, 50, 100, and 125 mM. As shown in Figure 4.9, buffer concentration had no impact on pectin yield for CelluPract ($p=0.423$). No increase in yield was observed beyond a buffer concentration of 50 mM so it was chosen as the buffer concentration for further extractions.

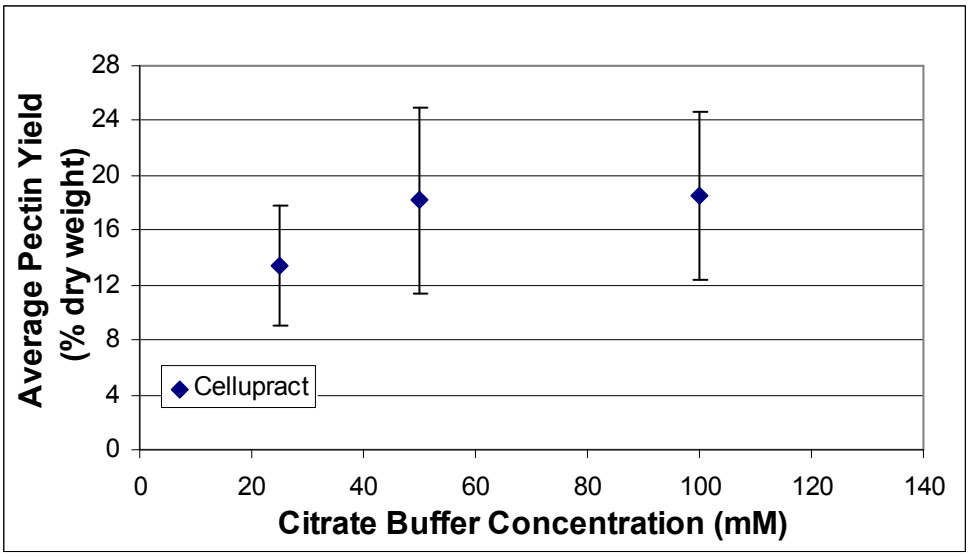

Figure 4.9. Effect of citrate buffer concentration on pectin yield using CelluPract enzyme with extraction conditions of pH 4.25, 50˚C, 2 hours, enzyme loading of 2.1 FPU/g, and solid to liquid ratio of 0.25 g/mL. All values are averages of triplicate samples with sample standard deviation indicated by error bars.

As shown in Figure 4.10, buffer concentration had no impact on pectin yield for Fibrilase ($p=0.238$). No increase in yield was observed beyond a concentration of 50 mM so it was chosen as the buffer concentration for further extractions.

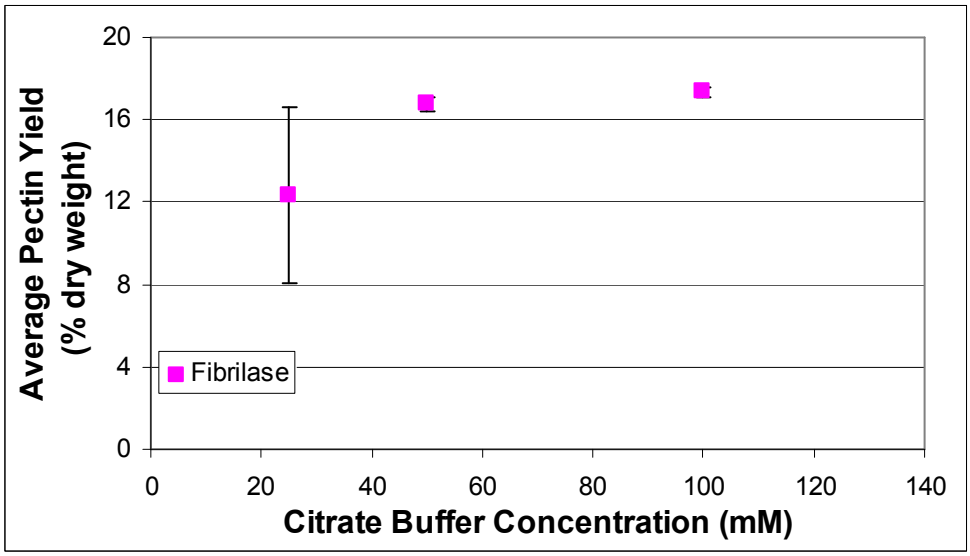

Figure 4.10. Effect of citrate buffer concentration on pectin yield using Fibrilase enzyme with extraction conditions of pH 4.35, 50˚C, 20 hours, enzyme loading of 2.0 FPU/g, and solid to liquid ratio of 0.25 g/mL. All values are averages of triplicate samples with sample standard deviation indicated by error bars.

As shown in Figure 4.11, buffer concentration had no significant impact on pectin yield for Multifect XL (p=0.076). Based on data trends 50 mM buffer concentration was chosen for further experiments. Statistically there was no difference in pectin yield due to the changes in buffer concentration.

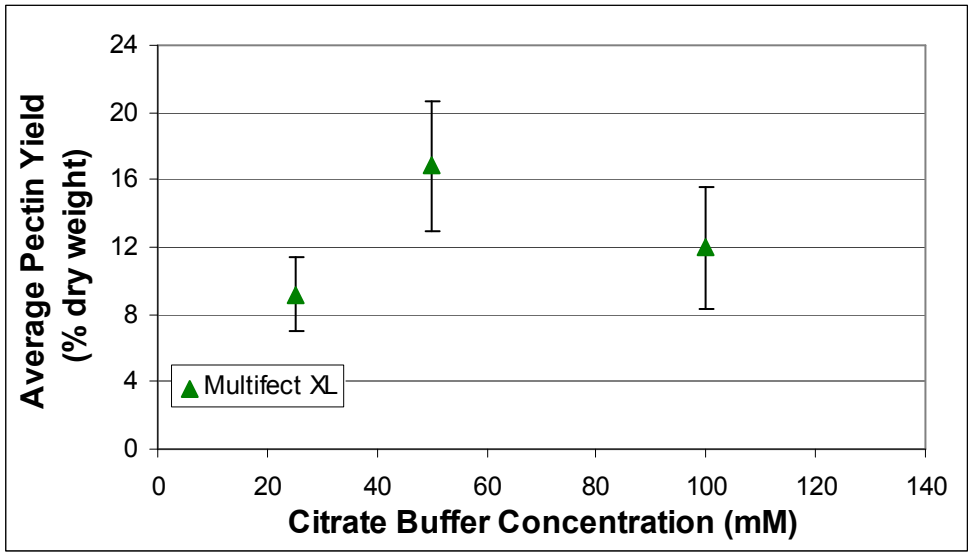

Figure 4.11. Effect of citrate buffer concentration on pectin yield using Multifect XL enzyme with extraction conditions of pH 4.65, 50˚C, 20 hours, enzyme loading of 2.1 FPU/g, and solid to liquid ratio of 0.25 g/mL. All values are averages of triplicate samples with sample standard deviation indicated by error bars.

4.3.5 Effect of Buffer pH

The effect of buffer pH on pectin yield was tested for CelluPract, Fibrilase, and Multifect XL. 50 mM citrate buffer solutions were made at pH values spanning the recommended pH usage range given by the manufacturer of each enzyme. For CelluPract, pH was tested at 3.25, 3.5, 3.75, 4.0, 4.25, and 4.5. As shown in Figure 4.12, pectin yield did not have an effect on pectin yield (p=0.381). Based on slight data trends a buffer pH of 4.0 was chosen for further extractions.

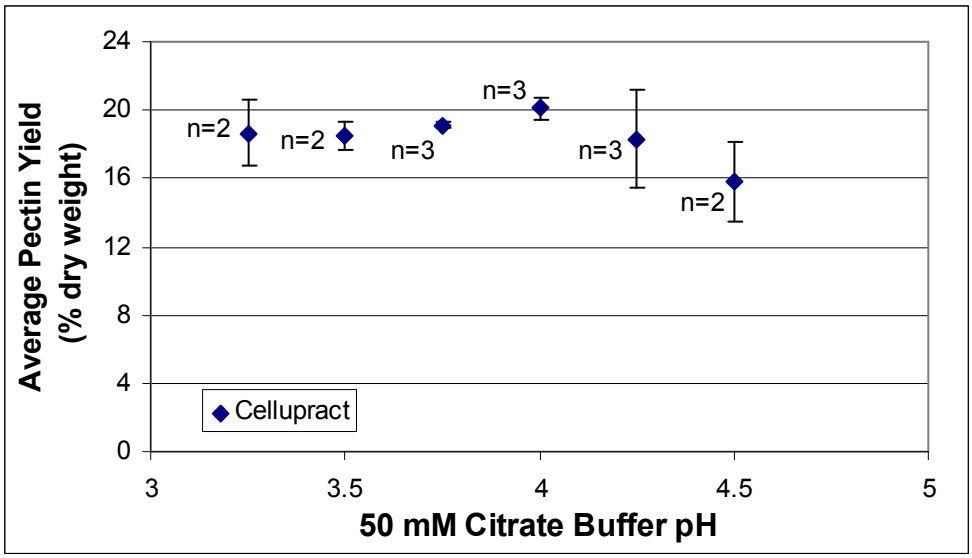

Figure 4.12. Effect of citrate buffer pH on pectin yield using CelluPract enzyme with extraction conditions of 50 mM buffer, 50˚C, 2 hours, enzyme loading of 2.1 FPU/g, and solid to liquid ratio of 0.25 g/mL. Sample size and standard deviation are indicated for each average value.

For Fibrilase, pH was tested at 4.0, 4.3, 4.6, and 4.9. As shown in Figure 4.13, no change in pectin yield occurred due to buffer pH (p=0.261). Based on data trends, pH 4.5 was chosen for the remaining trials.

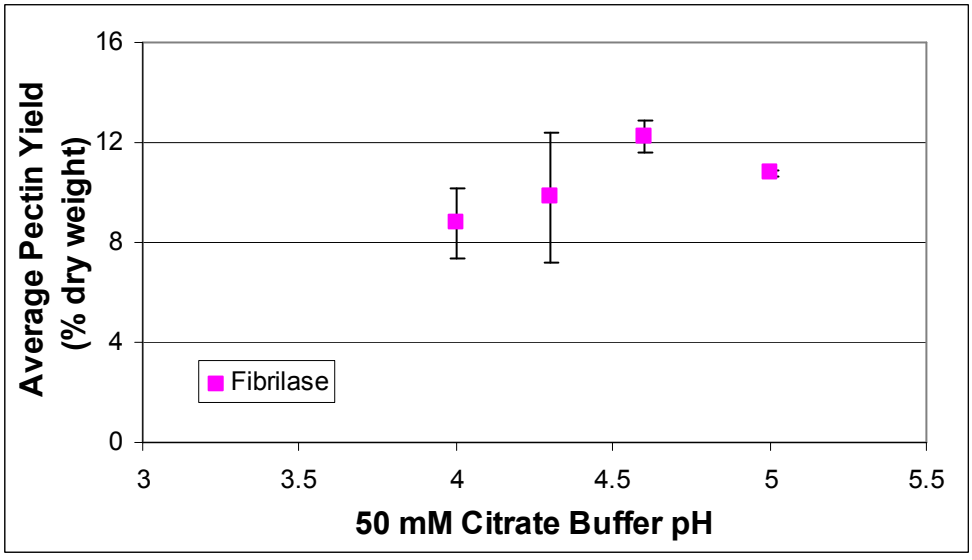

Figure 4.13. Effect of citrate buffer pH on pectin yield using Fibrilase enzyme with extraction conditions of 50 mM buffer, 50˚C, 20 hours, enzyme loading of 2.0 FPU/g, and solid to liquid ratio of 0.25 g/mL. All values are averages of triplicate samples with sample standard deviation indicated by error bars.

For Multifect XL, pH was tested at 4.25, 4.5, 4.75, and 5.0. As shown in Figure 4.14, pectin yield was not influenced by buffer pH (p=0.86). Based on data trends, buffer pH 4.7 was chosen for the remaining experiments.

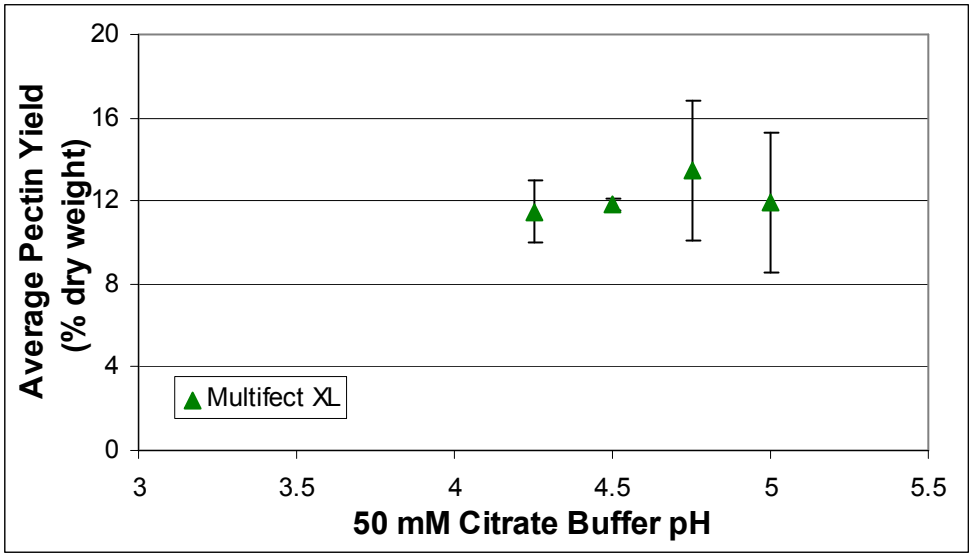

Figure 4.14. Effect of citrate buffer pH on pectin yield using Multifect XL enzyme with extraction conditions of 50 mM buffer, 50˚C, 20 hours, enzyme loading of 2.1 FPU/g, and solid to liquid ratio of 0.25 g/mL. All values are averages of triplicate samples with sample standard deviation indicated by error bars.

4.3.6 Effect of Solid to Liquid Ratio

Solid to liquid ratio, enzyme loading, and time were all considered to be interrelated based on the possibility that adjustment of one parameter could have a significant impact on the outcome of the other parameters. Solid to liquid ratio was chosen for optimization first out of the three parameters based on the large difference in yield between the middle value of 0.25 g/mL and the other values of 0.12 and 0.50 g/mL in the initial extraction trials for Fibrilase and Multifect XL. This large difference made it appear necessary to optimize first. Solid to liquid ratio was tested at values of 0.11,

0.18, 0.25, 0.32, and 0.39 g/mL for all three enzymes. As shown in Figure 4.15, pectin yield for CelluPract extraction was not affected by solid to liquid ratio (p=0.15). Based on data trends a solid to liquid ratio of 0.25 g/mL was chosen for remaining experiments.

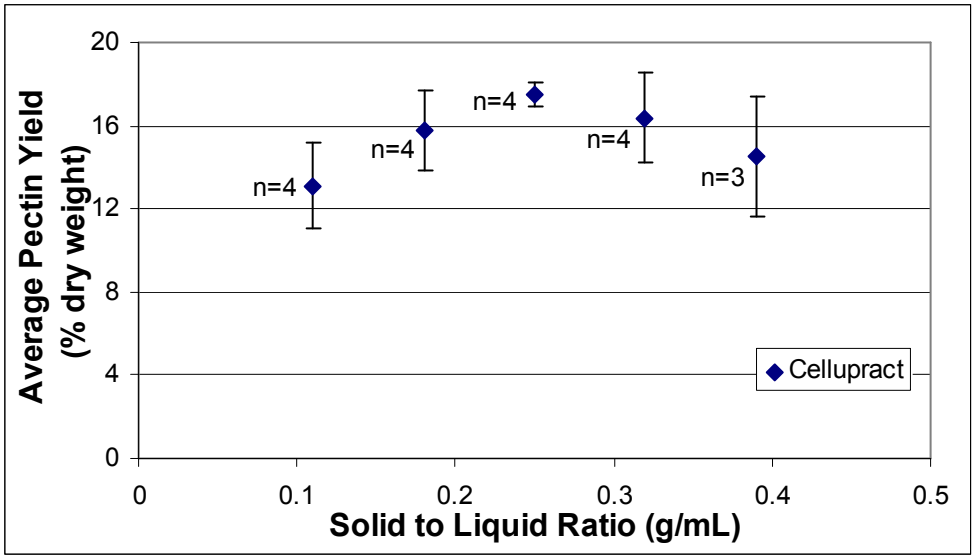

Figure 4.15. Effect of solid to liquid ratio on pectin yield using CelluPract enzyme with extraction conditions of 50 mM citrate buffer pH 4.3, 50˚C, 2 hours, and enzyme loading of 2.1 FPU/g. Sample size and standard deviation are indicated for each average value.

As shown in Figure 4.16, no difference in pectin yield occurred due to solid to liquid ratio for Fibrilase (p=0.201). A solid to liquid ratio of 0.18 g/mL was chosen for the remaining experiments.

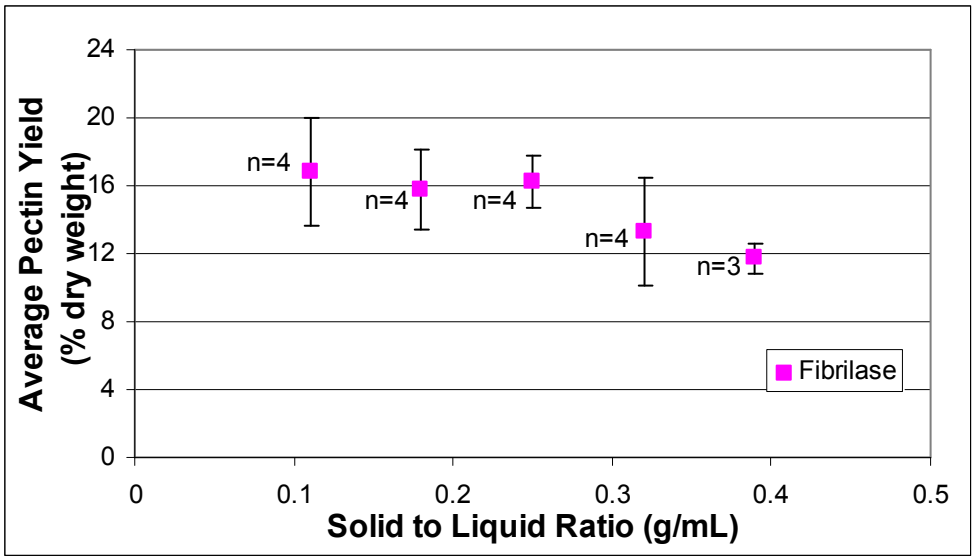

Figure 4.16. Effect of solid to liquid ratio on pectin yield using Fibrilase enzyme with extraction conditions of 50 mM citrate buffer pH 4.3, 50˚C, 20 hours, and enzyme loading of 2.0 FPU/g. Sample size and standard deviation are indicated for each average value.

As shown in Figure 4.17, pectin yield for Multifect XL was not affected by solid to liquid ratio (p=0.325). Based on data trends a solid to liquid ratio of 0.18 g/mL was chosen for the remaining experiments.

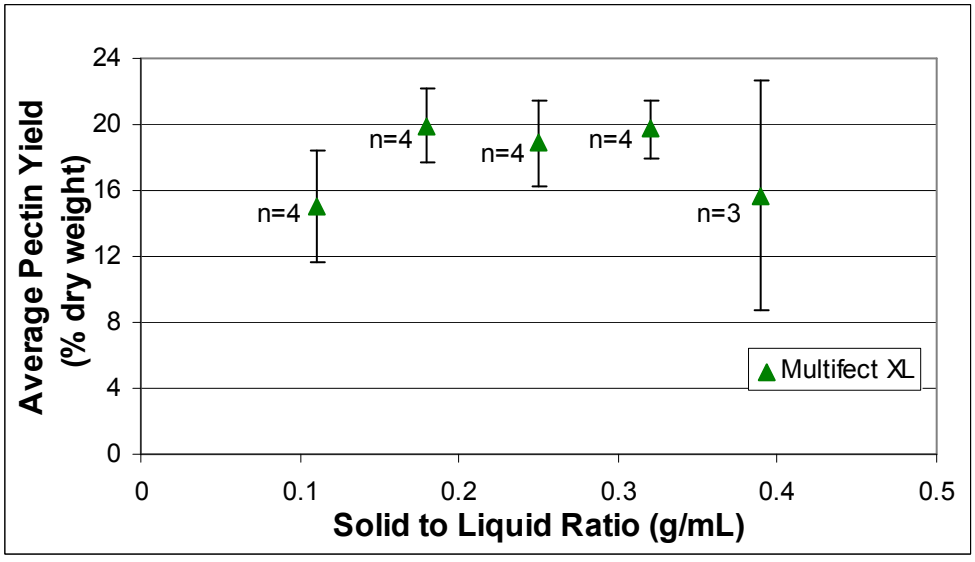

Figure 4.17. Effect of solid to liquid ratio on pectin yield using Multifect XL enzyme with extraction conditions of 50 mM citrate buffer pH 4.6, 50˚C, 20 hours, and enzyme loading of 2.1 FPU/g. Sample size and standard deviation are indicated for each average value.

4.3.7 Effect of Enzyme Loading

Enzyme loading, which was expressed as the enzyme to peel ratio, was the next parameter to test. Enzyme loadings were tested at periodic intervals ranging from 1.4 to 7.0 FPU/g for CelluPract. There was no significant difference between enzyme loadings ($p=0.085$). As shown in Figure 4.18, data trends increased until a certain point when the peel appeared to be saturated with enzyme. Above a ratio of approximately 5.6 FPU/g the resulting pectin yield was relatively stable. Based on this observation a value of 5.6 FPU/g was chosen as the optimal enzyme loading.

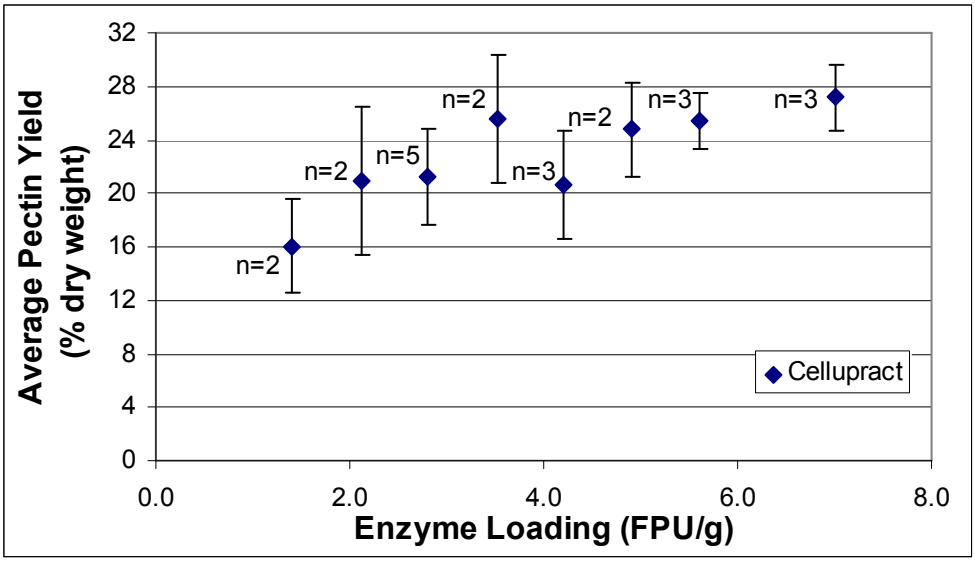

Figure 4.18. Effect of enzyme to peel ratio (FPU/g) on pectin yield using CelluPract enzyme with extraction conditions of 100 mL of 50 mM citrate buffer pH 4.0, 50˚C, and 2 hours with a solid to liquid ratio of 0.25 g/mL. Sample size and standard deviation are indicated for each average value.

Enzyme loadings were tested for Fibrialse at periodic intervals ranging from 1.3 to 7.4 FPU/g. As shown in Figure 4.19, pectin yield increased until a ratio of about 4.6 FPU/g after which the yield started to decline. This pattern occurred consistently for

several watermelon peel samples and as a result the critical point of 4.6 FPU/g was chosen for Fibrilase pectin extraction.

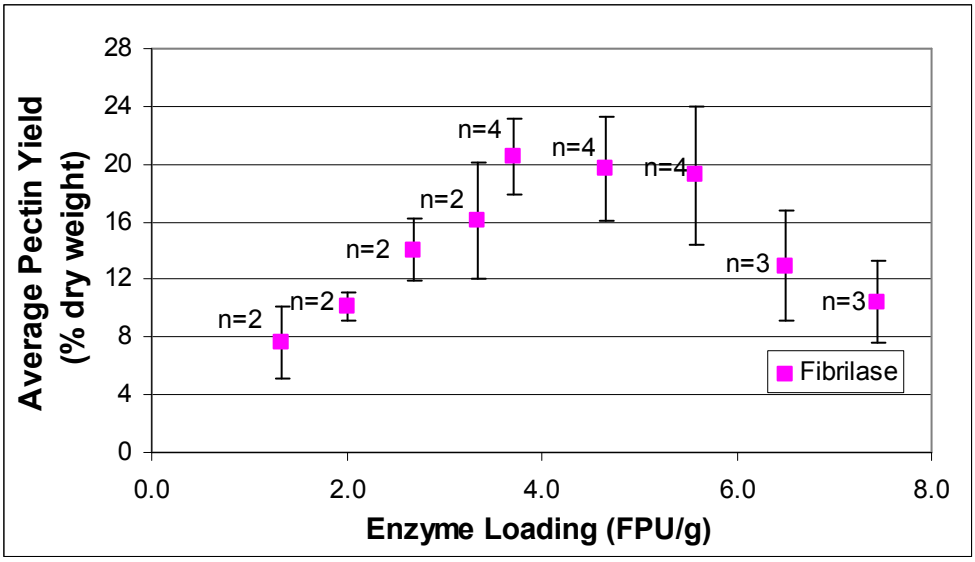

Figure 4.19. Effect of enzyme to peel ratio (FPU/g) on pectin yield using Fibrilase enzyme with extraction conditions of 100 mL of 50 mM citrate buffer pH 4.5, 50˚C, and 20 hours with a solid to liquid ratio of 0.18 g/mL. Sample size and standard deviation are indicated for each average value.

The enzyme loadings and resulting average yields and their statistical significance are duplicated in Table 4.16. The enzyme loadings of 3.7, 4.7, and 5.6 FPU/g resulted in significantly higher pectin yields than both the lower and higher enzyme loadings (p=0.002).

Table 4.16. Average pectin yields for Fibrilase enzyme loadings. Values with the same superscript are not significantly different.

Enzyme Loading (FPU/g)	Average Pectin Yield (% dry weight)
1.3	7.6[a]
2.0	10.1[a]
2.7	14.1[a,b]
3.4	16.1[a,b]
3.7	15.3[b]
4.7	14.5[b]
5.6	15.5[b]
6.5	9.9[a,b]
7.4	8.0[a]

Enzyme loadings were tested at periodic intervals ranging from 1.2 to 5.8 FPU/g for Multifect XL. As shown in Figure 4.20, pectin yield increased until a ratio of about 3.0 FPU/g after which the yield started to rapidly decline. This pattern occurred consistently for several watermelon peel samples and as a result the point of 3.0 FPU/g was chosen for Multifect XL pectin extraction.

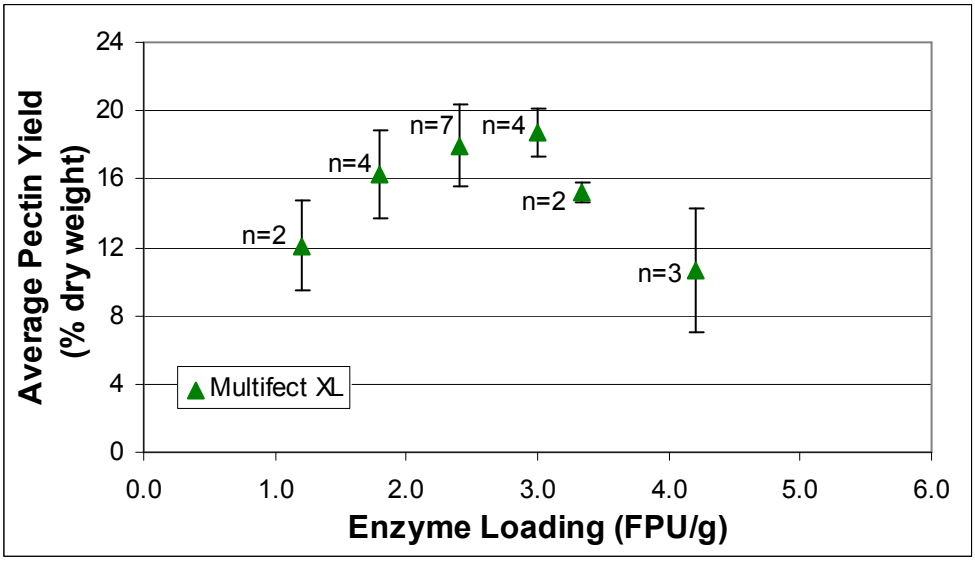

Figure 4.20. Effect of enzyme to peel ratio (FPU/g) on pectin yield using Multifect XL enzyme with extraction conditions of 100 mL of 50 mM citrate buffer pH 4.7, 50˚C, and 20 hours with a solid to liquid ratio of 0.18 g/mL. Sample size and standard deviation are indicated for each average value.

Several of the resulting yields were significantly different from each other. The enzyme loadings and average yields are duplicated in Table 4.17. The enzyme loadings of 2.4 and 3.0 FPU/g resulted in significantly higher pectin yields than both the lower and higher enzyme loadings (p=0.003).

Table 4.17. Average pectin yields for Multifect XL enzyme loadings. Values with the same superscript are not significantly different.

Enzyme Loading (FPU/g)	Average Pectin Yield (% dry weight)
1.2	12.1[a]
1.8	16.3[a,b]
2.4	15.7[b]
3.0	18.7[b]
3.3	7.5[a,b]
4.2	8.5[a]

Both Fibrilase and Multifect XL exhibited a parabolic trend in pectin content with increasing enzyme to peel content. Donaghy et al. (1994) also found that lower enzyme activity was optimal for extraction of pectin from citrus peel using a polygalacturonase that had been produced by *Kluveromyces fragilis* on whey.

4.3.8 Effect of Time

The effect of time was examined to determine its impact on enzymatic pectin yield. Extraction times of 1, 1.5, 2, 2.5, and 3 hours were tested for CelluPract. As shown in Figure 4.21, there was no difference in pectin yield due to extraction time ($p=0.107$). Based on these results an extraction time of 2 hours was chosen. No statistical difference in pectin yield was observed due to the changes in extraction time.

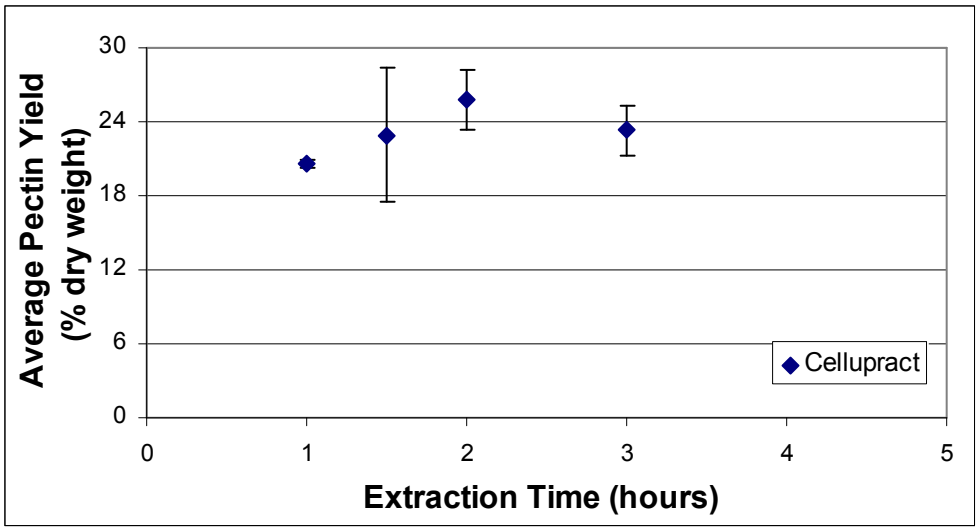

Figure 4.21. Effect of time on pectin yield using CelluPract enzyme with extraction conditions of 50 mM citrate buffer pH 4.0, 50˚C, enzyme loading of 5.6 FPU/g, and solid to liquid ratio of 0.25 g/mL. All values are averages of duplicate samples with sample standard deviation indicated by error bars.

Extraction times of 12, 16, 20, and 24, hours were tested for Fibrilase. As shown in Figure 4.22, there was no difference in pectin yield as a result of extraction time ($p=0.179$). An extraction time of 15 hours was chosen.

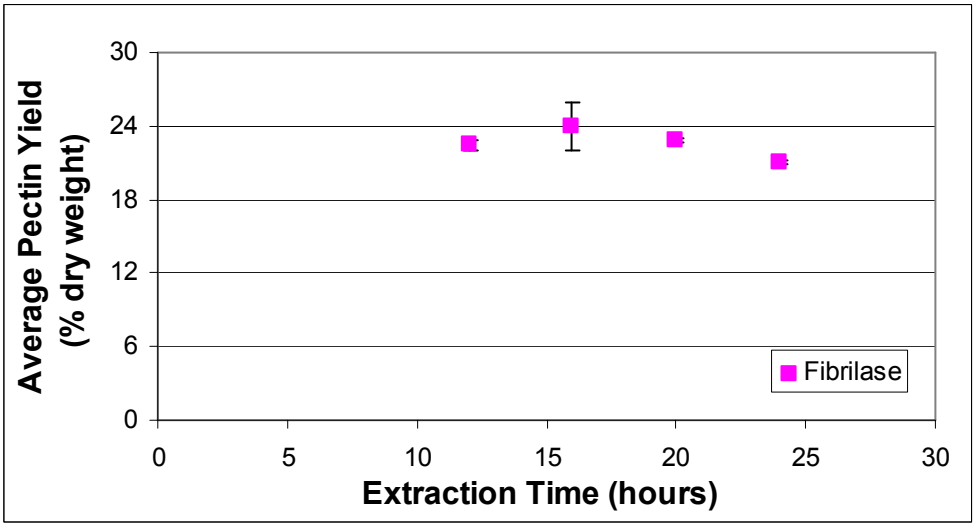

Figure 4.22. Effect of time on pectin yield using Fibrilase enzyme with extraction conditions of 50 mM citrate buffer pH 4.5, 50˚C, enzyme loading of 5.6 FPU/g, and solid to liquid ratio of 0.18 g/mL. All values are averages of duplicate samples with sample standard deviation indicated by error bars.

Extraction times of 12, 16, 20, and 24 hours were tested for Multifect XL. As shown in Figure 4.23, no definite pattern in pectin yield resulted from the extraction time trials for Multifect XL (p=0.761). An extraction time of 15 hours was chosen.

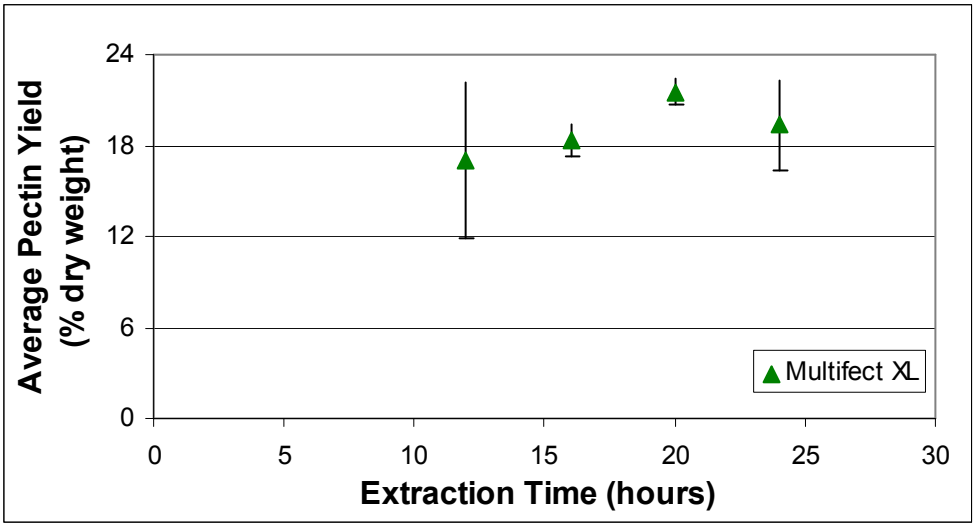

Figure 4.23. Effect of time on pectin yield using Multifect XL enzyme with extraction conditions of 50 mM citrate buffer pH 4.7, 50˚C, enzyme loading of 3.3 FPU/g, and solid to liquid ratio of 0.18 g/mL. All values are averages of duplicate samples with sample standard deviation indicated by error bars.

Time trials were also conducted on Multifect XL utilizing the same extraction conditions except with a solid to liquid ratio of 0.25 g/mL. The results for these trials are shown in Figure 4.24. There was no difference in pectin yield due to extraction time (p=0.794). Based on data trends an extraction time of 15 hours was chosen for remaining experiments.

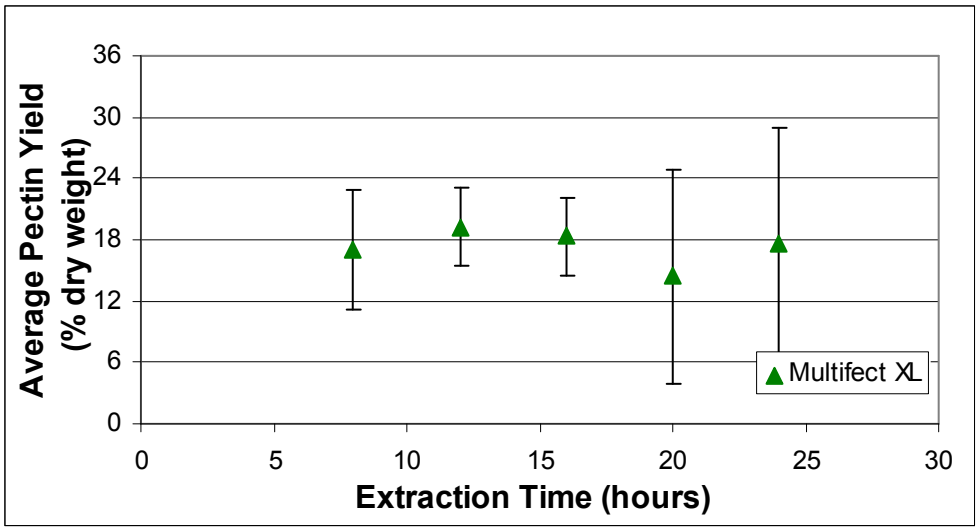

Figure 4.24. Effect of time on pectin yield using Multifect XL enzyme with extraction conditions of 50 mM citrate buffer pH 4.7, 50˚C, enzyme loading of 2.4 FPU/g, and solid to liquid ratio of 0.25 g/mL. All values are averages of triplicate samples with sample standard deviation indicated by error bars.

4.3.9 Comparison of Enzymes

It was of interest to compare the three enzymes utilizing similar extraction parameters. Extraction occurred at 50˚C for 2 hours at a solid to liquid ratio of 0.18 g/mL and an enzyme loading of 3.9 FPU/g using 50 mM citrate buffer at pH 4.0, 4.5, and 4.7 for CelluPract, Fibrilase, and Multifect XL, respectively. An enzyme loading of 3.9 FPU/g was chosen because, although it was well below the saturation enzyme loading for CelluPract, it was near the peak enzyme loading for both Fibrilase and Multifect XL and was before the drastic decline in yield for each of these enzymes. An extraction time of 2 hours was chosen because it was within the timeframe at which yield was still high for CelluPract. As shown in Figure 4.25, a significantly larger yield was obtained using CelluPract followed by Multifect XL and then Fibrilase. CelluPract pectin yield was

found to be different from the pectin yields of both Fibrilase and Multifect XL which were the same (p=0.001).

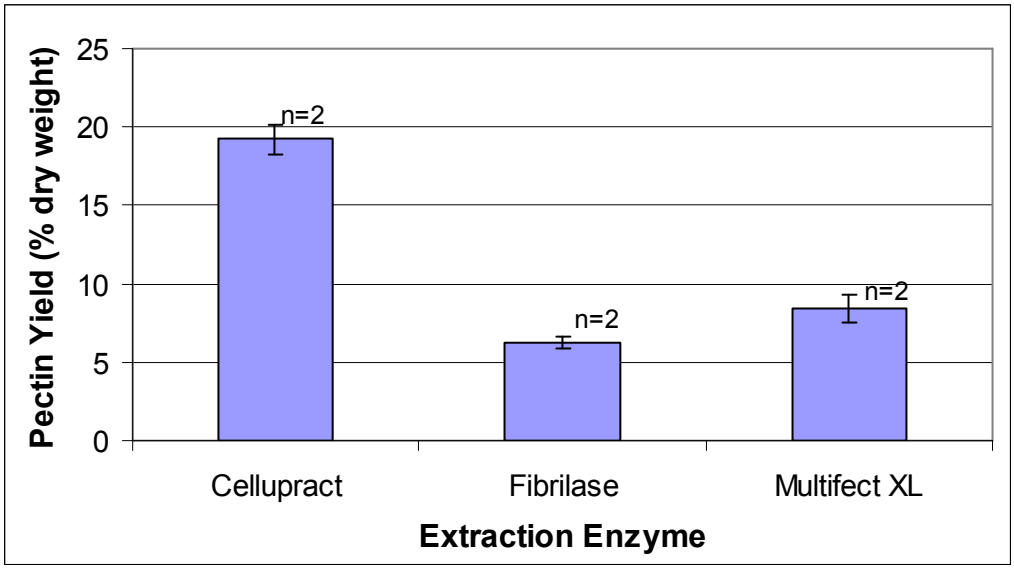

Figure 4.25. Comparison of pectin yield using CelluPract, Fibrilase, and Multifect XL enzymes for pectin extraction at common extraction conditions of 100 mL of 50 mM citrate buffer at 50˚C for 2 hours at a solid to liquid ratio of 0.18 g/mL and an enzyme loading of 3.9 FPU/g. Sample size and standard deviation are indicated for each average value.

4.3.10 Reexamination of Solid to liquid ratio

Because of the apparent impact of enzyme to peel ratio for both Fibrilase and Multifect XL, a smaller experiment was conducted to determine if a different solid to liquid ratio would be more desirable utilizing the optimal enzyme to peel ratio. Solid to liquid ratios of 0.18 and 0.25 g/mL were examined at enzyme to peel ratios of 4.6 and 3.0 FPU/g for Fibrilase and Multifect, respectively. As shown in Table 4.18, no noticeable difference resulted in pectin yield due to solid to liquid ratio for both enzymes. Based on this lack of difference in yield between the two solid to liquid ratios, a solid to liquid ratio of 0.18 g/mL was chosen for both enzymes.

Table 4.18. Comparison of average pectin yield for Fibrilase and Multifect XL at solid to liquid ratios of 0.18 and 0.25 g/mL using enzyme loadings of 4.6 and 3.0 FPU/g, respectively, at 50˚C for 15 hours using 50 mM citrate buffer. All values are for duplicate samples.

		Average Pectin Yield (% dry weight)
Fibrilase	**0.18 g/mL**	20.0 ±0.8
	0.25 g/mL	20.3 ±0.4
Multifect XL	**0.18 g/mL**	18.2 ±0.4
	0.25 g/mL	17.1 ±1.1

Solid to liquid ratio trials were repeated for CelluPract even though it did not exhibit the same enzyme to peel characteristics seen for Fibrilase and Multifect XL. Solid to liquid ratio was tested at 0.18 and 0.25 g/mL with enzyme loadings of 7.8 and 9.7 FPU/g for 0.18 g/mL solid to liquid ratio and 5.6 and 7.0 FPU/g for 0.25 g/mL solid to liquid ratio. The results are shown in Table 4.19. Pectin yield was slightly higher at a solid to liquid ratio of 0.18 g/mL for all trials. Also, although the enzyme loading trials indicated a saturation point for the enzyme, these trials show that a higher pectin yield could be obtained using an enzyme loading past the initial point that was originally chosen.

Table 4.19. Pectin yields for CelluPract enzyme using combinations of a solid to liquid ratio of 0.18 g/mL at enzyme loadings of 7.8 and 9.7 FPU/g and a solid to liquid ratio of 0.25 g/mL at enzyme loadings of 5.6 and 7.0 FPU/g. Extraction conditions were 100 mL 50 mM citrate buffer extracted for 2 hours at 50˚C.

		Pectin Yield (% dry weight)	
		Enzyme Loading	
		140 FPU	174 FPU
Solid Content	**0.18 g/mL**	27.6 ±1.0[b]	32.5 ±0.8[a]
	0.25 g/mL	23.8 ±2.2[b]	25 ±0.6[a]

[a]Values are for duplicate samples
[b]Values are for sample sets of 4

4.3.11 Extraction Using Enzyme Combinations

An experimental trial was conducted using combinations of Fibrilase and Multifect XL to extract pectin from watermelon rind. A selection of combinations of these enzymes was utilized to determine if an increase in yield would result from any of the enzyme groupings. As seen in Table 4.20, all combinations resulted in higher yields than the average yields obtained by the enzymes individually. The best combination out of those tested was Multifect XL at 4.2 FPU/g and Fibrilase at 4.7 FPU/g which resulted in an average pectin yield of 31.8%.

Table 4.20. Comparison of pectin yield using different combinations of Fibrilase and Multifect XL enzymes for pectin extraction.

		Pectin Yield (% dry weight)		
		Multifect XL Enzyme Loading		
		3.0 FPU/g	4.2 FPU/g	5.8 FPU/g
Fibrilase Enzyme Loading	3.7 FPU/g	20.7		
	4.7 FPU/g	23.3	31.8[a]	26

[a]Average of duplicate samples

4.4 Comparison of Extraction Methods

Two varieties of seedless watermelons were separately tested utilizing the chosen extraction conditions for each method. As shown in Figure 4.26, CelluPract produced the highest pectin yields, averaging approximately 34.1% followed by the combination of Multifect XL and Fibrilase at 31.7%. Acid extraction produced an average 20.2% and Fibrilase and Multifect XL produced average pectin yields of 18.7 and 18.0%, respectively. There was no difference between pectin yield of acid extracted, Fibrilase

extracted, and Multifect XL extracted pectin. CelluPract and Multifect XL/Fibrilase combination pectin yields were significantly higher than all of these (p=0.000). There was no difference between CelluPract and Multifect XL/Fibrilase combination pectin yields. Based on yield alone CelluPract and the Multifect XL/Fibrilase combination are the optimal choices for watermelon rind pectin extraction.

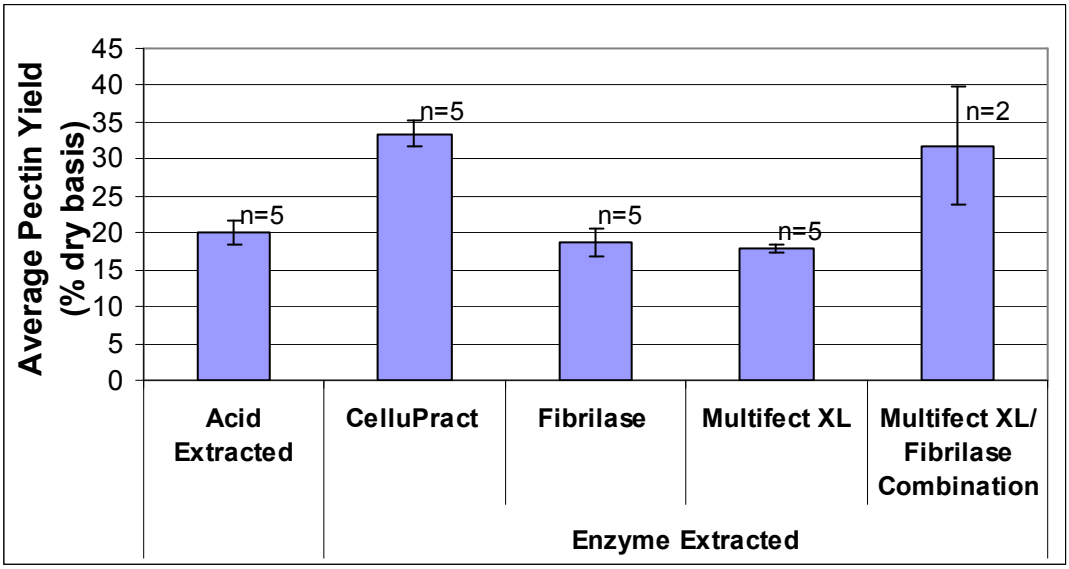

Figure 4.26. Comparison of average pectin yield using chosen extraction methods for acid extraction and enzymatic extraction using CelluPract, Fibrilase, and Multifect XL enzymes. Sample size and standard deviation are indicated for each average value.

4.5 Pectin Analysis

4.5.1 Galacturonic Acid Content

It was necessary to establish a method for dissolving the watermelon pectin samples for analysis. Pectin samples were dried using a vacuum oven until all moisture was removed so that pectin yields could accurately be reported for the experiments. Drying the samples completely resulted in a thin sheet of pectin layering the bottom of

the weighing dish used for sample drying. The samples were removed and ground using a mortar and pestle, but it was not possible to produce a fine powder that could more easily be brought into solution for pectin analysis. No other method of producing a pectin powder proved successful due to the small samples sizes of less than 3 g for acid extracted pectin and less than 0.5 g for enzyme extracted pectin. The pectinase treatments of the samples proved to be the most successful method of dissolving the watermelon pectin samples. The process was conducted on a pure citrus pectin sample obtained from Sigma with a galacturonic acid content of 82.7% to verify that the treatment did not affect the analysis. Galacturonic acid analysis of the citrus pectin using the m-hydroxydiphenyl method resulted in an average galacturonic acid content of 81.8%. Compared to the actual content of 82.7%, this was felt to support the pectinase method of dissolving the watermelon pectin samples.

Due to the time requirement for preparation and analysis of the pectin samples for galacturonic acid content, only selected samples were analyzed. Samples were chosen from three different extraction trials comparing the optimal extraction conditions for acid extraction and the enzymes CelluPract, Fibrilase, and Multifect XL. As shown in Table 4.21, the highest galacturonic acid content resulted from the acid extraction method with an average value of 68.6%. The enzyme extracted samples were all lower in galacturonic acid content with Multifect XL containing the highest percentage at 60.2%, followed by Fibrilase at 56.2% and CelluPract at 47.0%. Galacturonic acid content ranged from an average of 52.0% to 57.3% depending on the combinations of Fibrilase and Multifect XL. Lower galacturonic acid content in enzyme extracted samples was also reported by Shkodina et al. (1998) for pumpkin pectin extraction. The galacturonic acid content for

acid extracted pumpkin pectin was approximately 60%. Galacturonic acid contents of approximately 38, 32, and 36% were obtained by using *Trichoderma veride* cellulase, *Aspergillus niger* hemicellulase, and a crude *Xanthomonas campestris* glycosidase, respectively (Shkodina et al., 1998).

Table 4.21. Comparison of galacturonic acid content using the m-Hydroxydiphenyl method for a pure citrus pectin sample, acid extracted watermelon rind pectin, and enzyme extracted watermelon rind pectin using CelluPract, Fibrilase, and Multifect XL enzymes. Results are divided into segments based on watermelon rind source. Citrus results are for duplicate samples and all watermelon results are for sample sets of 7.

	Pectin Type				
			Watermelon-Enzyme Extracted		
	Citrus	Watermelon-Acid Extracted	CelluPract	Fibrilase	Multifect XL
Average % Galacturonic Acid	81.8 ±2.4	68.6 ±7.1	47.0 ±3.8	56.2 ±7.4	60.2 ±4.8

Galacturonic acid analysis using the HPLC method also showed the highest galacturonic acid content for acid extracted pectin at an average value of 71.7%, as shown in Table 4.22. Enzyme extracted galacturonic acid contents were all lower with Fibrilase having the highest content at 46.3% followed by Multifect XL at 43.8% and CelluPract at 43.1%. The galacturonic acid contents for all of the enzyme extracted samples were lower than for the m-Hydroxydiphenyl method. It would be of interest to reexamine galacturonic acid content of another set of samples using the HPLC method to determine if the enzymatic results would continue to be lower for this method than for the m-Hydroxydiphenyl method. This was not possible during experimentation due to limited availability of the HPLC.

Table 4.22. Comparison of galacturonic acid content using the HPLC analysis method for a pure citrus pectin sample, acid extracted watermelon rind pectin, and enzyme extracted watermelon rind pectin using CelluPract, Fibrilase, and Multifect XL enzymes. Results are divided into segments based on watermelon rind source. All values are for duplicate samples.

	Pectin Type				
			Watermelon-Enzyme Extracted		
	Citrus	Watermelon-Acid Extracted	CelluPract	Fibrilase	Multifect XL
Average % Galacturonic Acid	82.1 ±4.3	71.7 ±9.0	43.1 ±3.2	46.3 ±7.1	43.8 ±5.2

The high temperatures required for acid extraction could cause degradation of the pectin and have an impact on the resulting quality of the samples. It was of interest to analyze galacturonic acid content to determine the impact of increasing extraction temperatures. Samples were analyzed from an experimental trial testing extraction temperatures of 85, 90, and 95˚C. As shown in Table 4.23, there was no apparent difference in galacturonic acid content due to increasing the extraction temperature. Aravantinos-Zafiris et al. (1991) also found that galacturonic acid content was independent of extraction conditions in nitric acid extraction of orange pectin. It was beneficial to discover that there was no difference in galacturonic acid content due to extraction temperature because the pectin yields were significantly higher at extraction temperatures of 95˚C than at both 85 and 90˚C.

Table 4.23. Galacturonic acid content determined by m-Hydroxydiphenyl analysis for watermelon rind pectin produced using acid extraction at temperatures of 85, 90, and 95˚C. Extraction conditions included addition of 1 N nitric acid to pH 1.65, 45 min, and a solid to liquid ratio of 0.258 g/mL. All values are for duplicate samples.

	Acid Extracted Pectin Temperature		
	85C	90C	95C
Average % Galacturonic Acid	71.0 ±2.5	71.8 ±4.2	70.5 ±0.4

Extraction time had a large impact on pectin yield for CelluPract pectin extraction. Because there was such a difference in resulting pectin yield over a 24 hour

span, it was of interest to determine if a loss of quality was occurring with increasing extraction time. Samples were analyzed from an experimental trial testing extraction times of 1, 2, and 4 hours. As shown in Table 4.24, here was no apparent difference in galacturonic acid content due to increasing the extraction time.

Table 4.24. Galacturonic acid content determined by m-Hydroxydiphenyl analysis for watermelon rind pectin produced through CelluPract enzyme extraction at times of 1, 2, and 4 hours. Extraction conditions included 50 mM citrate buffer pH 4.0, 50˚C, enzyme loading of 140 FPU, and a solid to liquid ratio of 0.25 g/mL. All values are for duplicate samples.

	CelluPract Extracted Pectin Time		
	1 hour	2 hours	4 hours
Average % Galacturonic Acid	50.8 ±1.2	50.4 ±2.5	50.6 ±1.8

Thibault et al. (1988) reports use of a method for purification of pectins using copper acetate. Application of this method to enzymatically extracted citrus pectin resulted in a reported increase in galacturonic acid content from values of 36.8 and 50.0% to 56.7 and 59.3%, respectively. Application of this purification method to enzymatically extracted watermelon pectins could result in more desirable galacturonic acid contents.

4.5.2 Degree of Methoxylation

The degree of methoxylation was determined for selected samples that had been analyzed for galacturonic acid content. A standard citrus sample was treated using the same method used on the watermelon pectin. The resulting average degree of methoxylation of 51.8% for the citrus pectin was lower than the known value of 66.5%. Because the experimental degree of methoxylation differed considerably from the known value, it could be best to take the watermelon pectin degree of methoxylation values as

general ranges and not exact values. As shown in Table 4.25, the acid extracted watermelon pectin degree of methoxylation was around 46%. The CelluPract, Fibrilase, and Multifect XL extracted pectin degree of methoxylation values were around 38, 38, and 40%, respectively. These numbers indicate that all watermelon pectin samples can be classified as low methoxyl pectins.

Table 4.25. Comparison of degree of methoxylation for a pure citrus pectin sample, acid extracted watermelon rind pectin, and enzyme extracted watermelon rind pectin using CelluPract, Fibrilase, and Multifect XL enzymes. All values are for duplicate samples.

	Pectin Type				
	Citrus	Watermelon-Acid Extracted	Watermelon-Enzyme Extracted		
			CelluPract	Fibrilase	Multifect XL
Average Degree of Methoxylation (%)	51.8 ±0.6	45.6 ±1.5	38.3 ±3.5	38.0 ±2.4	40.1 ±0.4

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Pectin was successfully extracted from watermelon rind. Pectin yields for all extraction methods were increased through the optimization of extraction parameters. Conclusions for the extraction procedures are as follows:

Acid Extractions

1. Extraction using nitric acid and precipitation using isopropanol appeared to be the best acid/ alcohol combination.
2. Further experimentation indicated that there was not a difference in pectin yield due to solid to liquid ratio.
3. No significant difference in pectin yield occurred with increasing temperature, although the trend showed higher yields with increased temperature. An extraction temperature of 95˚C was chosen for further extractions.
4. No difference in pectin yield occurred with increasing extraction time from 45 min to 90 min.

5. Extraction at pH 1.65 was significantly different from other tested values based on pectin yields and was chosen for use in further extractions.
6. No trend in pectin yield appeared to exist due to watermelon maturity for the tested varieties of Lantha, Bobbie, and 5144. No significant differences in pectin yield due to maturity existed for Lantha or Bobbie. Very overripe and ripe maturity levels were significantly different from overripe and slightly overripe maturity levels for 5144.
7. No difference in pectin yield occurred due to watermelon variety in comparison of ripe watermelons for Lantha, Bobbie, and 5144 varieties.

Enzymatic Extraction

1. The use of *Trichoderma viride* cellulase for watermelon rind pectin extraction resulted in a minimal amount of pectin.
2. Extraction with citrate buffer and precipitation with ethanol appeared to be the best combination of buffer and precipitation media.
3. Precipitation with isopropanol resulted in negligible pectin yields.
4. Citrate buffer concentration did not result in a significant difference in pectin yield. A citrate buffer concentration of 50 mM was chosen for CelluPract, Fibrilase, and Multifect XL extractions based on data trends.
5. Buffer pH did not result in a significant difference in pectin yield. Citrate buffer pH 4.0, 4.5, and 4.7 were chosen for CelluPract, Fibrilase, and Multifect XL extractions, respectively based on data trends.

6. Solid to liquid ratio did not result in a significant difference in pectin yield. A solid to liquid ratio of 0.18 g/mL was chosen for CelluPract, Fibrilase, and Multifect XL extractions, respectively based on data trends.
7. Enzyme loading did not result in a significant difference in pectin yield for Cellupract. Enzyme loading resulted in significantly higher pectin yields at 3.7, 4.7, and 5.6 FPU/g for Fibrilase and 2.4 and 3.0 FPU/g for Multifect XL than at higher or lower enzyme loadings. CelluPract pectin yields appeared to plateau with increasing enzyme loadings while Fibrilase and Multifect XL pectin yields decreased beyond the optimal enzyme loadings. Enzyme loadings of 9.7, 4.6, and 3.0 FPU/g were chosen for CelluPract, Fibrilase, and Multifect XL extractions, respectively based on data trends.
8. An extraction time of 2 hours was chosen for CelluPract extraction and an extraction time of 15 hours was chosen for Fibrilase and Multifect XL extractions based on data trends.
9. CelluPract extraction resulted in significantly higher pectin yields in comparison to Fibrilase and Multifect XL extractions under similar extraction conditions.
10. Extractions using an enzyme combination of Fibrilase and Multifect resulted in higher pectin yields than extraction using either of the enzymes individually.

Comparison of Extraction Methods

1. Extraction conditions for acid extraction were chosen as 95˚C using the waterbath to maintain temperature for 45 min and using 1 N nitric acid to adjust the pH to 1.65 at a solid to liquid ratio of 0.258 g/mL.

2. Extraction conditions for CelluPract were 50˚C for 2 hours using 50 mM citrate buffer pH 4.0 with a solid to liquid ratio of 0.18 g/mL and at enzyme loading of 9.7 FPU/g.
3. Extraction conditions for Fibrilase were 50˚C for 15 hours using 50 mM citrate buffer 4.5 with a solid to liquid ratio of 0.18 g/mL and at an enzyme to peel ratio of 4.6 FPU/g.
4. Extraction conditions for Multifect XL were 50˚C for 15 hours using 50 mM citrate buffer pH 4.7 with a solid to liquid ratio of 0.18 g/mL and at an enzyme to peel ratio of 3.0 FPU/g.
5. CelluPract extraction resulted in significantly higher pectin yields than acid extraction, Fibrilase extraction, and Multifect XL extraction.

Pectin Analysis

1. Acid extraction resulted in the highest galacturonic acid content at an average value of 68.6% based on the colorimetric method. Galacturonic acid contents for CelluPract, Fibrilase, and Multifect XL were 47.0, 56.2, and 60.2%, respectively.
2. All watermelon pectin extractions resulted in low methoxyl pectins.

5.2 Recommendations for Further Research

It would be beneficial to explore other enzymes for extraction of pectin from watermelon rind. Protopectinases, glycosidases, and polygalacturonases could all be tested to determine their optimal conditions for extraction of pectin from watermelon

rind. It could also be of interest to further investigate the combination of Fibrilase and Multifect XL for pectin extraction or other combinations of enzymes.

It could be beneficial to determine the gelling characteristics of watermelon pectin to determine which uses it would be most beneficial for if commercial production were achieved.

An economic evaluation of the processes could also help in determining the feasibility of watermelon pectin extraction as part of the watermelon biorefinery.

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VITA

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Scope and Method of Study: Watermelon rind pectin extraction was investigated using acid and enzymatic extractions. The objectives were to optimize acid and enzyme extraction procedures for highest obtainable yield and quality.

Findings and Conclusions: Acid extraction was conducted using nitric acid and precipitated with isopropanol. Extraction conditions of 45 minutes, pH 1.65, 95˚C, and 0.258 g/mL solid to liquid ratio resulted in a pectin yield of 20.2%.

Enzymatic extractions were conducted using CelluPract, Fibrilase, and Multifect XL enzymes at extraction conditions of 50˚C, 0.18 g peel/mL, 50 mM citrate buffer. Extraction time and pH were 2 hours and 4.0, 15 hours and 4.5, and 15 hours and 4.7 for CelluPract, Fibrilase, and Multifect XL, respectively. Pectin yields were 34.1, 18.7, and 18.0% for CelluPract, Fibrilase, and Multifect XL, respectively.

Galacturonic acid content was 69, 47, 56, and 60% for acid, CelluPract, Fibrilase, and Multifect XL extracted pectin. All extractions resulted in low methoxyl pectins.

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