

DEVELOPMENT OF CONTROLLED RELEASE ANTIMICROBIAL FILMS
FROM LOW METHOXYL PECTIN

By

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ABSTRACT OF THE THESIS

Development of Controlled Release Antimicrobial Films

from Low Methoxyl Pectin

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Biopolymer-based controlled release antimicrobial packaging is an innovative packaging that aids in controlled replenishment of antimicrobial compound at the food surface, where it is required the most. Various biopolymers have been evaluated for controlled release purposes; focus being mainly on their antimicrobial effectiveness. However, low methoxyl pectin, with the potential to control release of active compounds by forming different degrees of crosslinking, has not been exploited in antimicrobial packaging and there is a lack of understanding of different variables affecting the release properties of active compounds from pectin films.

The main objective of this thesis was to identify the key variables involved in the development of pectin-based antimicrobial films by evaluation of the effect of different variables such as composition variables (size of active compound and degree of calcium crosslinking), process variables (pH of pectin, method of crosslinking), and environmental variables on the release of antimicrobial compounds, especially nisin. A

secondary objective was to demonstrate the concept that release of active compounds could be altered by varying the degree of calcium crosslinking within the pectin matrix.

Antimicrobial compounds such as sodium benzoate, potassium sorbate and nisin were chosen based on their different physical and chemical properties. Coomassie blue dye was used as a model compound for proof of concept. Films were produced from LM pectin by varying the DE of pectin and calcium concentration using the solution casting method. Release of antimicrobial compounds into water as a food simulant was measured by UV/Visible spectrophotometry or HPLC or agar diffusion assay, depending on the antimicrobial compound being evaluated.

Degree of calcium crosslinking, pH of pectin slurry and method of calcium crosslinking were identified as the key variables controlling release of nisin from pectin films. Electrostatic attraction between pectin and nisin at pH above pKa of pectin was not sufficiently strong to prevent the release of nisin. Release studies with dye-containing pectin films demonstrated that variable release rates could be obtained by altering the degree of calcium crosslinking.

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1 INTRODUCTION

1.1 Controlled Release Packaging

1.1.1 Concept of Controlled Release Packaging (CRP)

Controlled Release Packaging, also referred to as CRP [1], is an innovative form of active packaging where active compounds such as antimicrobials and antioxidants are incorporated into a synthetic or natural polymer, and released from the polymer into food in a controlled manner.

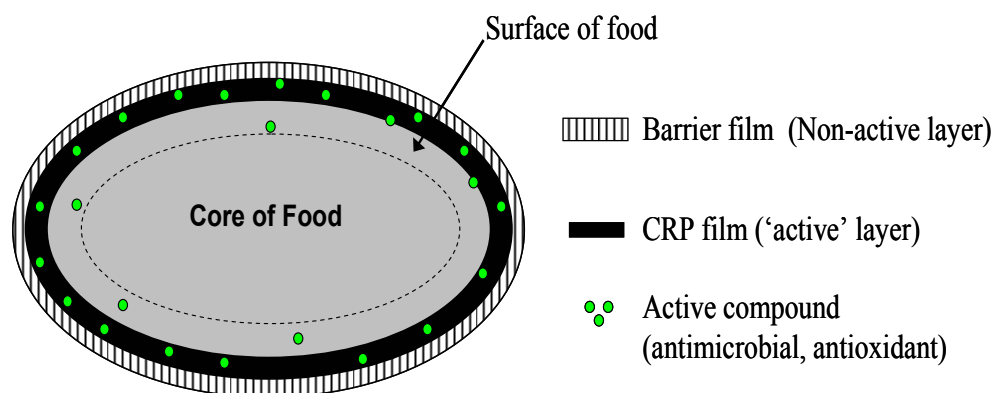


Figure 1: Concept of Controlled Release Packaging (CRP)

Illustrated in figure 1 is a typical CRP system, comprised of an inner active layer that is laminated with an outer barrier layer. The active compound may be incorporated into or coated onto the inner active layer and released continuously from the packaging material into food at a desired rate to improve food safety and quality.

1.1.2 Antimicrobial Packaging

Antimicrobial packaging, one of the early developments in the field of CRP, is a packaging system designed to release an antimicrobial compound on the food surface to delay microbial growth. The antimicrobial activity may be due to incorporation of an

antimicrobial compound into the packaging polymer or due to antimicrobial property of the polymer itself (e.g. chitosan) [2].

1.1.2.1 Motivation for Antimicrobial Packaging

Food companies all over the world are striving to minimize or delay food spoilage in order to develop safe and high quality food products with a longer shelf life. Post-process handling or moisture condensation on surface of refrigerated packaged foods are two reasons for microbial contamination and spoilage on the surface of packaged foods [3, 4]. Traditionally, antimicrobial compounds are added to entire food to inhibit or delay microbial spoilage. But, the main disadvantage of this traditional method is that the antimicrobial compound may not really be required in the interior part of food product, resulting in an excessive use of ‘preservatives’. Growing consumer demand for minimum or ‘zero’ use of preservatives has motivated researchers to develop alternate methods for extending shelf life of foods with minimum use of food additives. Use of antimicrobial sprays or dips [5] is one such option to deliver an antimicrobial compound at food surface, and at the same time it avoids excessive use of the antimicrobial compound. However, this method has some disadvantages such as – (i) it involves an additional manufacturing step, (ii) may be messy, and (iii) loss of effectiveness of dip at surface may occur due to diffusion of antimicrobial compound into food interior over time [6]. This is the main motivation for development of controlled release antimicrobial packaging; wherein the packaging can be designed to replenish the required amount of antimicrobial compound in a controlled manner at the food surface, where it is required the most!

1.1.2.2 Classification Based on Mode of Action

An antimicrobial packaging system may function in two ways [7-9]:

(i) Migration/release – In this case the antimicrobial compound inhibits microbial growth by migrating from packaging material onto the food surface. In case the released compound is volatile it will first migrate to the headspace and then condense onto the food surface (Figure 2). Non-volatile compounds diffuse directly from packaging material into food matrix (Figure 3). A volatile compound is more effective than a non-volatile compound for irregularly-shaped solid foods, where good contact between food and packaging material is not possible. Non-volatile antimicrobial compounds can be used effectively for semi-solid or liquid foods where excellent contact between food and packaging allows migration of the compound.

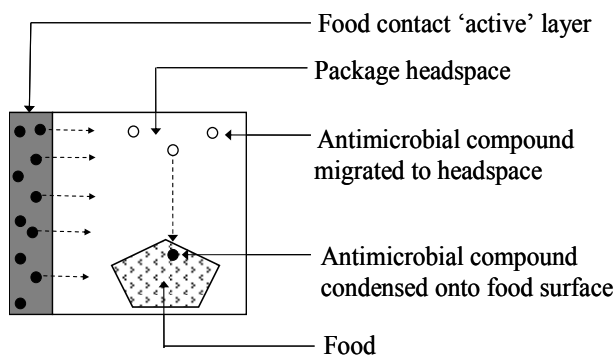


Figure 2: Mode of Action of a Volatile Antimicrobial Compound

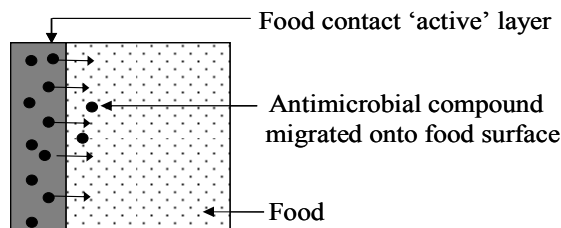


Figure 3: Mode of Action of a Non-Volatile Antimicrobial Compound

(ii) Immobilization – A non-food grade antimicrobial compound is immobilized on the food contact surface and inhibits microbial growth at contact surface without migrating from the packaging material (Figure 4). This mode requires very good contact between food and packaging material to effectively inhibit microbial spoilage.

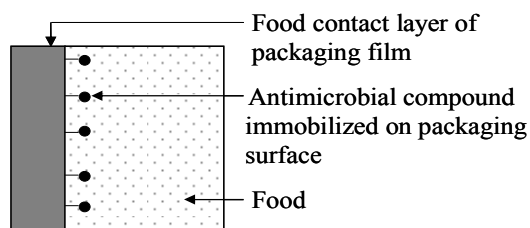


Figure 4: Immobilized Antimicrobial Packaging System

An indirect form of antimicrobial packaging is one in which the active compound inhibits microbial growth by absorbing factors that are favorable for microbial growth from the package; e.g. Oxygen or moisture absorbent sachets absorb oxygen or moisture, respectively, from package to prevent mould growth.

1.1.2.3 Significance of Antimicrobial Release Rate

For an antimicrobial packaging system to be effective, two critical factors must be considered while designing the system [8]. They are (i) rate of release of antimicrobial compound from packaging onto food surface, and (ii) rate of growth of microorganism to be inhibited. If the release rate of antimicrobial compound is slower than growth rate of target microorganism, microorganisms will grow before the antimicrobial compound is released. On the other hand, if the release rate of antimicrobial compound is faster than the microbial growth rate, the entire antimicrobial compound from package will be released too fast and the released antimicrobial agent will diffuse into food and will not be available at the surface to inhibit microbial growth at the desired time. In this case the

packaging system will be ineffective. An ideal antimicrobial packaging system should release the antimicrobial compound at a rate that matches the microbial growth rate. In addition, at any given time, the concentration of antimicrobial compound maintained at food surface should at least be equal to the minimum inhibitory concentration (m.i.c.) necessary to inhibit the target microorganism [10].

1.1.2.4 Factors Influencing Release of an Antimicrobial Compound from CRP

Some of the major factors which govern the release of active compound from packaging are [1, 8]:

- (i) Size of antimicrobial compound: Mobility of an antimicrobial compound within the polymer network depends on its size, provided there are no other chemical interactions involved between the antimicrobial compound and the polymer chain. A small sized compound will diffuse faster than a bigger compound through the same polymer matrix.
- (ii) Compatibility between antimicrobial compound and packaging polymer: If the antimicrobial compound binds with the packaging polymer i.e. they are compatible with each other, then the antimicrobial compound may remain inside the polymer matrix and this is not desirable for controlled release packaging. If the reverse is true, i.e. they are not compatible, then it may pose problems while incorporating the antimicrobial compound into the polymer matrix, or once incorporated, it may be released immediately, thereby defeating the purpose of controlled release. Ideally, the antimicrobial compound should be physically entrapped in the polymer matrix, to be released in a controlled manner by modifying the polymer matrix. Some of the factors that may influence the compatibility of antimicrobial compound and polymer matrix are polarity [11], pKa of biopolymers such as pectin and chitosan [12], pKa of antimicrobial compounds,

especially nisin. The effect of pKa of biopolymer and antimicrobial compound on film processing and controlled release properties is discussed in detail later in this chapter.

(iii) Extent of networking within polymer matrix: The effect of networking within a polymer on the release of active compound has been exploited successfully for designing controlled drug delivery applications [13-15] and is the focus of this thesis also. It is discussed in detail later in this chapter.

1.2 Edible/Biopolymer-Based Antimicrobial Packaging

1.2.1 Motivation for Biopolymer-Based Packaging

The past few decades have witnessed an alarming increase in use of petrochemical-based synthetic packaging materials because they are easily available in large quantities, are cheap and possess mechanical properties suitable for commercial food packaging applications. However, a major concern with use of synthetic polymers is their non-biodegradable character, which poses a serious threat to the environment. Marketing of eco-friendly packaging has increased consumer awareness about issues concerning environmental pollution [16, 17]. Therefore, the food and packaging industries are jointly focusing to develop alternate biodegradable packaging systems, including edible films and coatings for food.

Use of biopolymer-based packaging is not novel; edible films and coatings have been used for centuries [6, 18]. In China, a wax coating was used to delay moisture loss from citrus fruits during the twelfth century. During the fifteenth century, Yuba, an edible film made from soy milk was developed in Japan to help in food preservation [18]. Edible coatings to prevent shrinkage of meat were developed as early as the sixteenth

century. Further developments in use of edible coatings and films were seen in the nineteenth and early twentieth century when sugar and chocolate coating was used to prevent oxidation of nuts, oil-in water emulsion was coated on fruits and vegetables, gelatin casings were developed for sausages, etc.

1.2.2 Definition and Classification of Biopolymer-Based Packaging

Biopolymer-based packaging is defined as packaging made from renewable resources of agriculture or marine origin [17]. Edible packaging, as the name suggests, is made from edible natural biopolymers and can be used as an integral part of food. Biopolymers are categorized on the basis of their origin as:

- Natural – extracted from natural raw materials (e.g. starch, cellulose, protein, alginate, pectin);
- Chemically synthesized polymers from bioderived monomers (e.g. polylactic acid - PLA) [19];
- Microbial polymers (e.g. polyhydroxyalkanoates, polyhydroxybutyrates).

Though biopolymer-based packaging is environment friendly in comparison to synthetic polymers, there are some challenges associated with respect to its mechanical performance, commercial processing and cost [17].

In the past few decades, several films based on proteins, polysaccharides have been developed and characterized for application in food packaging.

1.2.3 Typical Components

Edible films are typically comprised of either a protein or a polysaccharide or any of the above in combination with lipids, a plasticizer and, if necessary, a cross-linking

agent. Proteins studied for edible packaging are whey protein, casein, corn zein, soy protein, wheat gluten, gelatin, and collagen [17, 20]. Polysaccharide based films have been developed using alginate, cellulose and cellulose derivatives, starch and starch derivatives, chitosan, carrageenan [21]. Coatings based on lipids such as vegetable oils, monoglycerides, acetylated glycerides, waxes such as paraffin wax, carnauba wax, beeswax, are used alone or in combination with proteins or polysaccharides for different purposes in food preservation [21]. Glycerol, sorbitol, polyethylene glycol (PEG) are some of the commonly used plasticizers that help to improve the flexibility of edible films [6]. Cross-linking agents such as calcium chloride (for alginate and low methoxyl pectin films) [4], glutaraldehyde (for gelatin films) [22] are used in the formation of films mentioned above to improve their mechanical strength, resistance to water, and other barrier properties.

1.2.4 Techniques for Manufacture of Edible Films

Technique for making an edible film depends on the material used for the film. Generally, films based on polysaccharides, except starch films, are formed by removal of solvent [6]. In this method, the hydrocolloid is dispersed in a solvent such as ethanol, water, to which plasticizer and crosslinking agent, if any, are added and the film-forming solution is cast on a flat surface and solvent is removed during drying step to form a thin film. In starch based films, film-making involves gelatinization of starch followed by casting of film [2, 23]. Extrusion is another technique for making biopolymer films [24, 25]. Protein films are made by different techniques such as solvent evaporation (wheat gluten, corn zein) or pH modification or thermal denaturation of protein (soy protein,

ovalbumin) or addition of crosslinking agent (whey protein) or gelation (gelatin), followed by casting [26].

1.3 Literature Review of Biopolymer-Based Antimicrobial Packaging

1.3.1 Biopolymers

Several biopolymers have been investigated for the purpose of antimicrobial films; selected references for the polysaccharide-based and protein-based films have been listed in table 1 and 2, respectively.

Table 1: Selected References for Polysaccharide-Based Antimicrobial Films

| Film | Antimicrobial Compound | Test Medium/ Food | Test Method | Reference |
|--------------------------------|--|------------------------------|--|------------------|
| Carrageenan | Potassium sorbate | Phosphate buffer | Diffusion cell | [27] |
| Carrageenan Sodium alginate | Nisin Lysozyme GFSE | Agar | Inhibition zone assay | [28] |
| Cellulose | Pediocin | Meat | Inhibition zone assay, effect on packed meat | [29] |
| Yam starch Chitosan | Chitosan | Culture medium | Shake flask method | [2] |
| Cellulose based paper | Nisin Lacticin | Agar, Cheese/ham | Inhibition zone assay | [30] |
| Alginate Apple puree | Plant essential oils (oregano, cinnamaldehyde, etc.) | Agar | Inhibition zone assay | [31] |
| Chitosan | Garlic oil, potassium sorbate, nisin | Agar | Inhibition zone assay | [32] |
| Konjac glucomannan Chitosan | Chitosan Nisin | Agar | Inhibition zone assay | [33, 34] |

| | | | | |
|--|--|-------------------------------------|--|------|
| Sodium alginate | Nisin | Beef | Spot assay | [35] |
| Chitosan | Acetic acid Propionic acid Lauric acid | Bologna Ham Pastrami | Bacterial enumeration of meat sample at timed intervals | [36] |
| Tapioca starch | Potassium sorbate | No study | Film characterization only | [23] |
| Yam starch Chitosan | Chitosan | Carrots | Bacterial enumeration of carrot samples at timed intervals | [37] |
| Alginate | Garlic oil | Agar | Inhibition zone assay | [38] |
| Alginate | Potassium sorbate | Potassium sorbate solution | Release study with diffusion cell | [4] |
| Tapioca starch | Nisin | Agar, Culture medium | Inhibition zone assay and release studies in liquid culture medium | [39] |
| HPMC* chitosan | Nisin | Agar | Inhibition zone assay | [40] |
| Chitosan coating on paperboard | Nisin | Distilled water, milk, orange juice | Release study with migration cell | [41] |
| Carrageenan Chitosan MC** HPMC* | Nisin | Agar | Inhibition zone assay | [42] |
| MC*-HPMC** coating | Nisin | Peptone broth | Release study | [43] |

| | | | | |
|-----------------------|-------------------|------------|--|------|
| Starch – potato, corn | Potassium sorbate | Strawberry | Effect of coating on different properties of fruit, including microbial inhibition, but not "controlled release" | [44] |
|-----------------------|-------------------|------------|--|------|

(* MC: Methyl cellulose; ** HPMC: Hydroxypropyl methyl cellulose)

Table 2: Review of Protein-Based Antimicrobial Films

| Film | Antimicrobial Compound | Test Medium/ Food | Test Method | Reference |
|--|--|------------------------------|--|------------------|
| Corn zein | Lysozyme/nisin | Agar | Inhibition zone assay | [3] |
| Soy protein isolate | Lysozyme/nisin | Agar | Inhibition zone assay | [3] |
| Whey protein isolate | Potassium sorbate | Water-glycerol | Release study | [45] |
| Whey protein isolate | p-amino benzoic acid, sorbic acid | Agar | Inhibition zone assay | [46] |
| Whey protein isolate | Oregano, rosemary, garlic essential oils | Agar | Inhibition zone assay | [47] |
| Corn zein Wheat gluten | Nisin | Water Agar | Release study Inhibition zone assay | [48] |
| Corn zein | Lysozyme, Albumin proteins, EDTA | Water | Release study | [49] |
| Soy protein isolate Wheat gluten Egg albumen Whey protein isolate | Nisin | Culture media | Release study | [50] |
| Corn zein | Lysozyme | Water | Release study | [51] |
| Whey protein isolate | p-amino benzoic acid, sorbic acid | Hot dogs | Surface inhibition | [52] |

| | | | | |
|----------------------|-----------------------------------|-------------------------|--------------------|------|
| Whey protein isolate | p-amino benzoic acid, sorbic acid | Bologna Summer sausages | Surface inhibition | [53] |
| Corn zein | Sorbic acid | Cooked sweet corn | Surface inhibition | [54] |

1.3.2 Antimicrobial Compounds

Nisin, potassium sorbate, lysozyme, essential oils such as oregano and garlic, and chitosan are some of the antimicrobial compounds researched for use in antimicrobial films; nisin being the most popular of them all.

1.3.2.1 Nisin and its Structure

Nisin is a small peptide produced by *Lactococcus lactis* subspecies *lactis*. It is considered GRAS and is the only bacteriocin approved for use in foods [55], the permitted limits varying across the globe. Commercially, nisin is available in the form of Nisaplin[®], a nisin ‘preparation’, which has only 2.5% nisin, the rest being lactose and other milk solids.

Nisin is composed of 34 amino acids and has a molecular weight of 3510 Daltons. The structure of nisin is shown in Figure 5 and was first reported by Gross and Morell in 1971 [56]. The presence of five internal thioether rings formed by lanthionine (Ala-S-Ala) and β -methyllanthionine (Abu-S-Ala) groups is responsible for the conformation of nisin. It is capable of forming dimers and oligomers [55]. It has a net positive charge due to lysine and histidine amino acid residues, pKa of their side chains being 10.2 and 7 respectively [57]. It possesses an amphipathic nature due to hydrophobic groups at the N-terminal and hydrophilic groups at the C-terminal. Solubility and thermal stability of nisin solution varies with pH. At pH 2, it survives autoclaving conditions of 121 °C for

15 minutes, but at higher pH of about 6, heating at 121 °C for 3 min is sufficient to destroy about 25-50% of the nisin [55]. Two major degradation products of nisin i.e. nisin¹⁻³² and (des-Δ Ala5)nisin¹⁻³² have been identified and antimicrobial activity has been characterized by Chan et al. [58]. In addition to loss of stability with increase in pH, solubility also drops drastically with increase in pH, but is not of much importance because of the low usage levels in food.

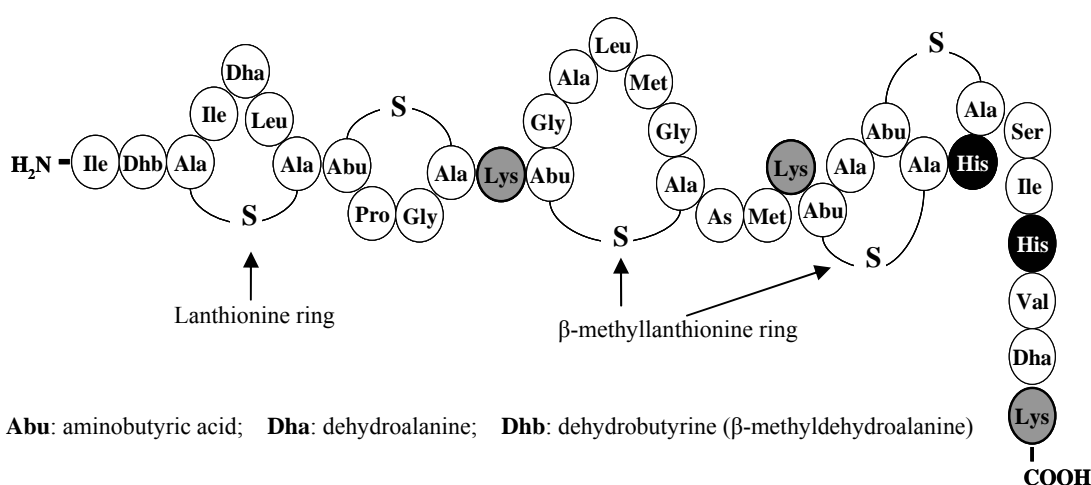


Figure 5: Structure of Nisin

1.3.2.2 Antimicrobial Activity

Nisin is a broad spectrum antimicrobial effective against Gram-positive bacteria, most importantly, those belonging to the *Bacillus* and *Clostridium* genera. Nisin is not only effective against vegetative cells, but it is also effective in inhibition of heat-resistant spores such as *Bacillus cereus*, *Clostridium botulinum*. This makes it a very effective antimicrobial for use in heat-treated foods where probability of spoilage due to surviving spores is high [55]. Penetration of the C-terminal of nisin into the cell membrane causes alteration of favorable pH conditions and consequently, rapid loss of cell metabolites from the cell. Presence of polyvalent cations such as Ca^{2+} , Mg^{2+} is thought to reduce the

antimicrobial effectiveness of nisin due to possible interaction of these ions with the negatively charged sites of the cell membrane and making these membrane sites unavailable for binding with nisin [59]. Nisin is effective against lactic acid bacteria, responsible for spoilage of low pH foods (e.g. salad dressings); *Listeria monocytogenes*, a pathogen; *Brocothrix thermosphacta*, responsible for spoilage in meat products. It is used mainly in the preservation of processed cheese and has potential for use in several products such as canned foods, juices, processed meat products, pasteurized liquid egg, and salad dressings.

1.3.2.3 Review of Nisin Usage in Biopolymer-Based Antimicrobial Films

Nisin has been studied extensively in antimicrobial packaging. Lee et al. [60] investigated the antimicrobial efficacy of a nisin-coated paper and found that even though only 9% of the total incorporated nisin could migrate to the food, it was still able to extend the shelf life of perishable foods such as milk cream and emulsions. Another group studied an antimicrobial edible coating of nisin on hydroxypropyl methyl cellulose [61]. Cha et al. [28] studied the effect of antimicrobial compounds such as nisin on the mechanical properties of sodium alginate and κ -carrageenan based films and reported that greater hydrophilic nature of sodium alginate films causes greater swelling than the carrageenan films, and as a result more nisin is released, thereby producing bigger zones of inhibition. Nisin immobilized on the surface of cellulose-based inserts and polyethylene/polyamide pouches was able to extend the shelf life of ham and sliced cheese when used in combination with modified atmosphere packaging [30]. Zhang et al. [62] conducted an important experiment to compare the antimicrobial effectiveness of different modes of delivery of nisin to a model system and found that a combination of

initial addition of nisin to the broth and slow release of nisin over a period of time was more effective than either of the delivery modes alone. Mechanical properties and/or antimicrobial activity of nisin-containing films made using biopolymers such as chitosan [32], konjac glucomannan [33, 34], sodium caseinate [63], poly(lactic acid) - pectin blends [64], hydroxypropyl methyl cellulose [40] have been evaluated. A study by Ko et al. [50] on the effect of pH of film-forming solution and surface hydrophobicity of protein films on antimicrobial activity of nisin showed that nisin was more effective when incorporated into hydrophobic protein films at a low pH. Immobilization of nisin in a calcium alginate gel was more effective than direct application of nisin on the surface of beef carcass [35, 65]. In a recent study, activated beads of alginate, modified to form covalent linkage with nisin, were not as effective as non-modified alginate-nisin solution coating on beef surface for inhibition of *Staphylococcus aureus* in beef [66]. Varying the molecular weight of hydroxypropyl methyl cellulose (HPMC) and methyl cellulose (MC) failed to control the release of nisin from HPMC/MC coated on LPDE films [43]. Cha et al. [42] compared the release of nisin from MC, HPMC, chitosan and carrageenan, either used as a coating on PE films or in the form of heat-pressed films made using above biopolymers in combination with PE pellets. It was found that in case of biopolymers coated onto PE, nisin was released at a varying rate, gradually increasing over 10 hours of the above mentioned study. However, in case of heat-pressed films, even after 5 days of release study, amount of nisin released was equal to that released in the first 2 hours of the release study.

1.3.2.4 Review of Potassium Sorbate in Biopolymer-Based Antimicrobial Films

Potassium sorbate is the second most studied antimicrobial compound in biopolymer-based packaging. Selected research work, relevant to controlled release applications, is mentioned in this section. The diffusivity of potassium sorbate in κ -carrageenan films was studied as a function of temperature and pH of the receiving solution and it was found that diffusivity was unaffected by pH of receiving solution and was a function of only temperature [27]. In another study, water-glycerol model system was used to study the diffusion of potassium sorbate from whey protein based films [45]. The proposed model describes the diffusion process as non-Fickian, where protein films swell due to diffusion of solvent (water) into the film matrix, followed by migration of potassium sorbate out of the swollen film matrix. The diffusion coefficients found using this liquid model system may not hold true in case of a film in contact with a semi-solid material. Zactiti et al. [4] studied the effect of degree of calcium crosslinking in alginate films on the release of potassium sorbate. This is an important experiment for developing controlled release films from pectin which is chemically similar to alginates and is discussed in detail later. Release kinetics of potassium sorbate incorporated into tapioca starch based edible films was studied in liquid media and semi-solid media and it was found that 30 minutes were required to release all the potassium sorbate from film into liquid media, whereas release into semi-solid media took slightly longer at 4 hours [67].

1.4 Pectin – A Promising Biopolymer for CRP

Pectin is a naturally occurring heterogeneous polysaccharide that has been used extensively in the food industry as a gelling agent. In the past decade, it has been pursued as a promising biopolymer for controlled drug delivery applications.

1.4.1 Structure of Pectin

Pectin is a methylated ester of polygalacturonic acid. The linear backbone of pectin, also known as homogalacturonan backbone, is made of a sequence of α -(1 \rightarrow 4) linked D-galactopyranosyluronic acid units [68, 69]. This backbone is periodically interrupted by α -(1 \rightarrow 2) linked L-rhamnose residues. Side chains of neutral sugars such as arabinose, xylose and galactose branch from the rhamnose portion of the chain. A schematic representation [70] for the pectin backbone is shown below in Figure 6.

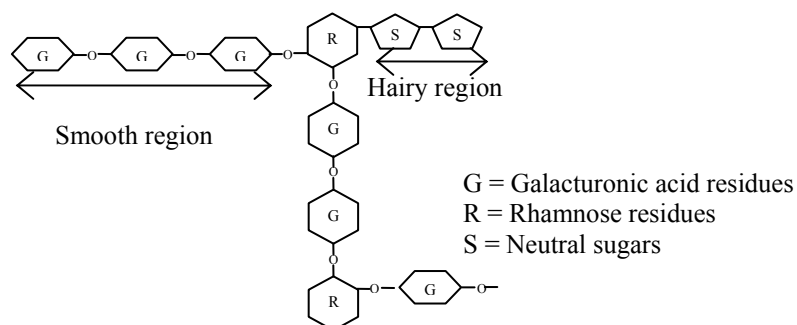


Figure 6: Schematic Diagram for Pectin Backbone

The galacturonic acid (GalA) residue may be partly esterified with methyl groups or exist as an amide or simply as free carboxyl groups (Figure 7) [70].

1.4.2 Degree of Esterification (DE)

The ratio of GalA groups present as esters to the total number of GalA groups present in a pectin chain is defined as the degree of esterification (DE). Another term,

degree of amidation (DA) is similarly defined as the ratio of GalA groups in the form of amides to the total GalA groups. The terms, DE and DA, together are known as degree of substitution (DS) of the given pectin. Natural pectin is generally highly esterified with a DE of 60-90%. Pectin with a specific DE can be produced by controlling the extent of demethylation.

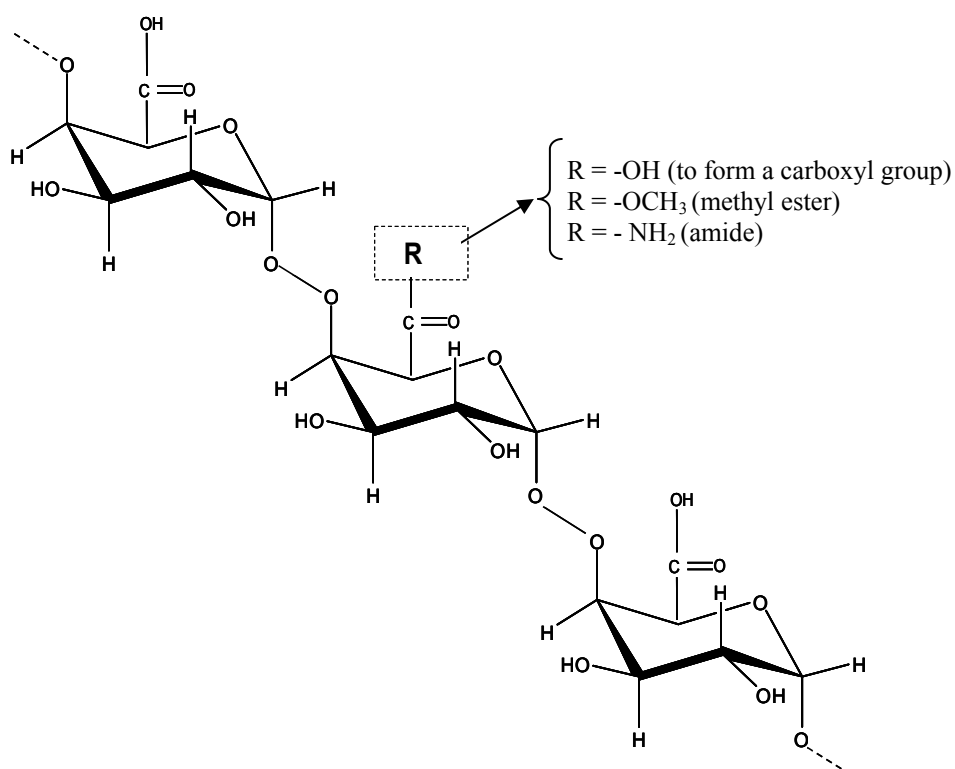


Figure 7: General Structure of Low Methoxyl Pectin Chain

1.4.3 Classification of Pectin

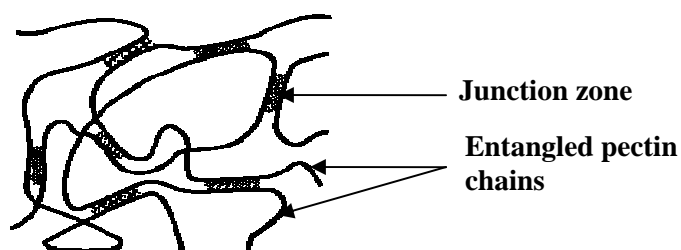
Pectin is classified on the basis of its degree of esterification (DE) as high methoxyl (HM) with a DE > 50% or low methoxyl (LM) with a DE < 50%. LM pectin is further classified as low methoxyl conventional (LMC), which has only ester and free carboxyl groups, and low methoxyl amidated (LMA), which has amide groups in addition to the

ester and carboxyl groups. In this thesis, pectin used is of the LMC type; hence only the DE term is used henceforth.

1.4.4 Gelling Mechanism

DE of pectin determines the gelling mechanism of pectin. HM pectin ($DE > 50\%$) gels only in the presence of sugar and specific pH conditions. LM pectin gels irrespective of presence of any specific pH or sugar. The only condition necessary for LM pectin gelation is the presence of divalent cations such as Ca^{2+} .

Pectin chains entangle to form junction zones over extended segments of the polymer (Figure 8). These junction zones are strengthened by number weak intermolecular interactions such as hydrogen bonding between free carboxyl groups and hydroxyl groups of adjacent pectin molecule, and hydrophobic interactions between the methyl ester groups.



<http://www.cpkelco.com/pectin/images/pectin03.gif>

Figure 8: Schematic Diagram for Gelation in HM pectin

Gelation of pectin depends on the charge of free carboxyl groups. When pH of solution is above pK_a , coulombic repulsion between the negatively charged carboxyl groups on pectin forces the pectin chains away from each other. This repulsion gives rise to well-hydrated individual pectin chains. Once the pH drops below pK_a , the carboxyl

groups lose their negative charge and pectin chains come closer due to decrease in repulsion, resulting in a gel. This is the general gelling mechanism for HM pectin.

In case of LM pectin, gelation mainly follows the “egg-box” model (Figure 9) (Grant et al., 1973, as cited in [68]). In this mechanism, two pectin chains, each in the form of a helix, undergo side-by-side association to form a dimer in which the carboxyl groups on adjacent pectin chains form ionic bonds with calcium ions. The ionic bonds formed are stable when there are at least seven carboxyl groups on each pectin chain in the dimer. Consecutive calcium crosslinks in a dimer result in the formation of a stable junction zone and aggregation of several layers of dimers results in gel formation. Presence of methyl esters in the pectin backbone interrupts the formation of junction zones. Therefore, in LM pectin with a lower DE, with less number of methyl ester groups, there is a better chance for formation junction zones with few calcium ions as compared to LM pectin with higher DE. Gelation depends on factors such as molecular weight, pH of pectin solution, presence of other groups such as acetyl and amide in the pectin chain, temperature.

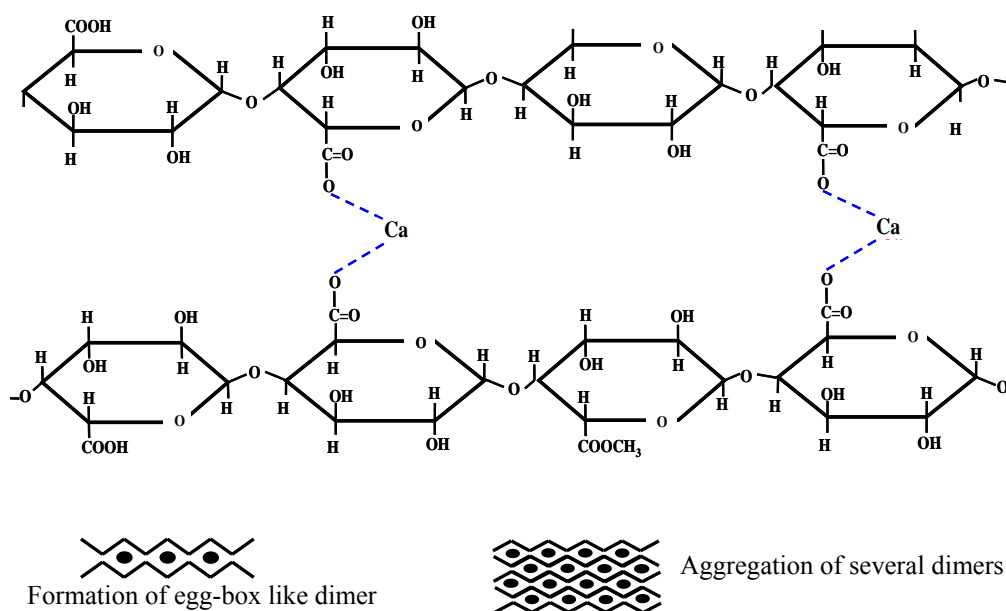


Figure 9: Schematic Diagram for Gelation in LM pectin

1.4.5 Review of Pectin Usage in Controlled Drug Delivery

Pectin is a hydrophilic polymer and this ability of pectin to form a hydrogel has made it a potential candidate for applications related to oral and gastrointestinal tract drug delivery. P. Srimornsak [70] has reviewed research work investigating the use of pectin for controlled drug delivery applications; some of them dating back to as early as 1981. Controlled drug delivery using pectin either as a tablet component or coating [71-73] based on the underlying principle of calcium cross-linking in the pectin matrix has been studied. Calcium crosslinking delays the swelling of pectin matrix (when pectin is used to coat the tablet) or erosion of tablet (when pectin is a component of the tablet), and these in turn delay the release of drug from the tablet. A schematic diagram showing the effect of crosslinking on swelling and hence release is shown later in section 1.4.7.2.

1.4.6 Review of Pectin in Antimicrobial Packaging

Extruded edible films from pectin-starch blends have been studied by Fishman et al. [24, 25], but without incorporation of antimicrobial compounds. Composite films of PLA-pectin blends containing nisin [64] have also been studied and antimicrobial activity of these films was evaluated. Overall, the above researchers have focused mainly on studying the processing aspects and physical characteristics of these composite films but not on controlling the release. Thus, there is plenty of scope for using pectin in controlled release of antimicrobials such as nisin by employing the concept of calcium crosslinking as used in controlled drug delivery.

1.4.7 Explanation of Terms and Concepts Related to Pectin and Controlled Release

1.4.7.1 Degree of Crosslinking

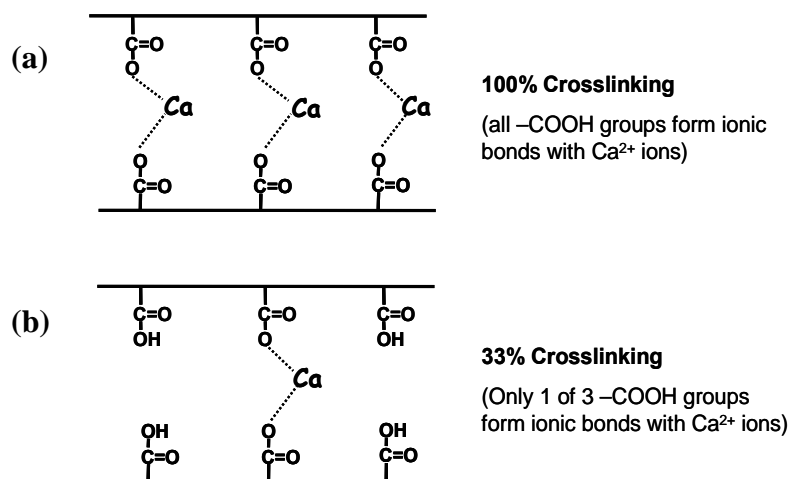


Figure 10: Schematic Diagram to Illustrate Different Degrees of Calcium Crosslinking

Degree of crosslinking in pectin films or coatings may be defined as the percent of free carboxyl groups in a pectin molecule that are involved in the formation of ionic bonds with calcium ions. The formation of these ionic bonds, also known as calcium

bridges or crosslinks, depends on the number of calcium ions made available to a pectin molecule of a particular DE. Illustrated in Figure 10 above is a hypothetical situation where two pectin chains, each with three free carboxyl groups, exist. If the amount of calcium ions provided is in excess, such that all three pairs of carboxyl groups are involved in the formation of crosslinks with calcium, then the degree of crosslinking obtained is 100% (Figure 10a). If the amount of calcium ions provided is limited, such that only one pair out of three pairs of carboxyl groups is involved in calcium crosslinking, then only 33% degree of crosslinking is achieved (Figure 10b). The number of free carboxyl groups depends on the DE of pectin used; lower the DE, higher the number of free carboxyl groups in a given pectin molecule and therefore, higher is the opportunity for the formation of calcium crosslinks.

Thus, degree of crosslinking can be manipulated by varying the degree of esterification (DE) of LM pectin used or by varying the amount of calcium ions, as shown in Figure 11. The amount of calcium ions will be referred to as calcium concentration and expressed as mg of Ca per g of pectin. One way to manipulate the degree of crosslinking (as explained earlier in figure 10a and 10b) is by varying the calcium concentration, for the same DE of pectin (DE 1, Figure 11). Another approach is to vary the DE of pectin used, keeping the calcium concentration constant. As mentioned earlier in gelling mechanism of LM pectin (section 1.4.4), as the DE decreases, the probability of calcium crosslinking increases than pectin with a higher DE and thus, different degrees of crosslinking can be achieved. The other logical way is to vary the number of pectin molecules by changing pectin concentration.

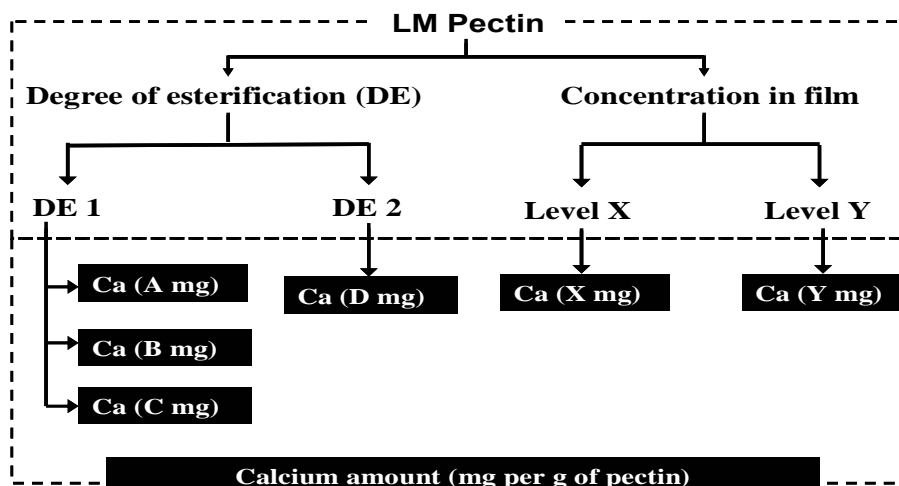
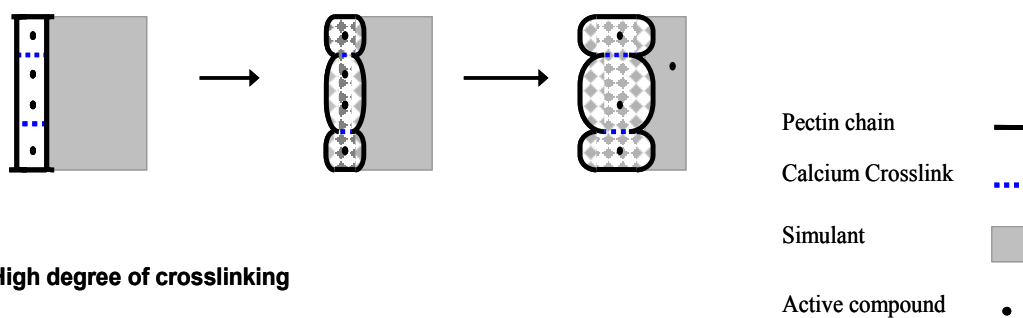


Figure 11: Different Ways to Manipulate Degree of Crosslinking

1.4.7.2 Degree of Swelling

Low degree of crosslinking



High degree of crosslinking

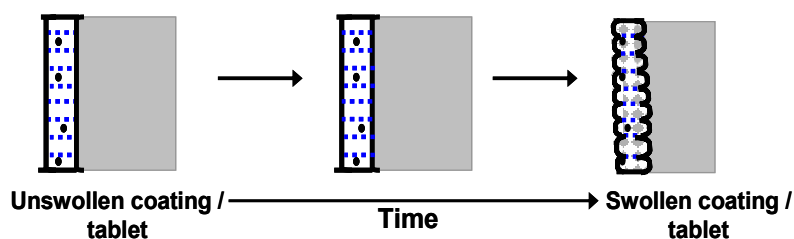


Figure 12: Effect of Calcium Crosslinking on Swelling of LM Pectin Film

Degree of swelling is typically defined as the % increase in weight or thickness of a crosslinked pectin film when suspended in a solvent like water. Swelling of film or tablet coating takes place when solvent (simulant) diffuses into the pectin matrix. As the films

swells, mobility of active compound inside the pectin matrix increases and it diffuses to the surface of film to be finally released into the simulant. It has been shown that extent of crosslinking has a direct effect on swelling of pectin coating in tablets; degree of swelling decreasing with increasing crosslinking. Illustrated above is a schematic representation of the effect of calcium crosslinking on release of active compound or drug from the pectin film or coating. The film/coating with more crosslinking takes longer to swell and hence release is delayed. Though not novel, this is an important concept that could be used to control the release of antimicrobial compounds from pectin films. The range of release rates that can be obtained from films with small variations in calcium crosslinking is yet to be determined.

1.5 Research Gaps and Opportunities

Based on the above literature review of antimicrobial edible films, some of the research gaps identified are as follows:

- Most of the studies conducted in this field have focused on evaluation of mechanical properties of antimicrobial films and their antimicrobial activity. However, except for some research work [4, 72], not many attempts have been made to control the release of antimicrobial compound by modifying the film composition or properties.
- Release kinetics of antimicrobial compound from edible film has not been investigated by many researchers; only antimicrobial efficacy of film, which is also important, has been studied.

- Many biopolymers possess charged groups and have the potential to form films with varying release rates; however, only a few of them such as alginate and pectin have been utilized for controlled release antimicrobial films.
- The potential of pectin, a biopolymer, extensively studied in drug delivery applications, has not been tapped in the area of antimicrobial edible films.
- Literature review of usage of nisin in biopolymers (section 1.3.2.3) indicates that very little research has been conducted with pectin-nisin films, probably due to the speculation that nisin, a positively charged peptide, might interact with negatively charged pectin, and may not be released from the pectin matrix. This presents an opportunity for study of release of nisin from pectin films.
- Nisin quantification is mostly done using the microbial assay. Since nisin forms multimers and degradation products that may still retain antimicrobial activity [58, 74], estimation of nisin (released from film) using the microbial assay may not represent the intact nisin, but it may be combined antimicrobial activity of nisin and/or its antimicrobially active derivatives. A correlation between chemical estimation of nisin (e.g. by HPLC) and estimation using the microbial assay (e.g. by agar diffusion assay) will provide information about stability of nisin when present in the film and after being released into the environment.

The objective of this thesis was to address the above research gaps.

1.6 Objectives

1.6.1 Overall Objective

An overall objective of our group was to develop edible/biopolymer based CRP films from low methoxyl (LM) pectin that can release an antimicrobial compound in a controlled manner to enhance safety and shelf life of food products with short storage periods. The biopolymer containing an active compound may be used as a single film in contact with food or in combination with an outer biopolymer layer or as a coating on another biopolymer. Fresh meats, cheese, fruits, packaged salads, etc. are the target food applications for this innovative antimicrobial packaging film.

1.6.2 Scope of Research

Previous research on use of pectin in tablets for controlled drug delivery [71, 72] and work done by Zactiti et al. [4] on effect of calcium crosslinking on potassium sorbate permeability through alginate films have demonstrated that degree of crosslinking is an important parameter governing the release characteristics of a tablet coating or alginate film. These experiments indicate that it is possible to develop controlled release packaging films from LM pectin with a wide range of release rates by simple variations in the degree of calcium cross-linking within the film. A database of film formulations possessing different release rates, generated from this study, will provide useful information to produce a CRP film from LM pectin with a desired release property simply by choosing the appropriate film formulation!

1.6.3 Challenges in Development of Pectin Films

Unlike synthetic polymers, pectin is a highly reactive biopolymer. Hence, in order to conclude that crosslinking is the only factor affecting the release of an antimicrobial compound from a LM pectin film, it is first necessary to develop an understanding about the different variables affecting pectin films, be it processing parameters or type of antimicrobial compound used or other environmental factors. As already mentioned, there is scope for development of nisin-containing pectin films. However, like pectin, nisin is also very reactive. Based on the understanding of chemical nature of pectin and nisin, some of the challenges foreseen in the development of pectin-nisin films are outlined below:

1.6.3.1 Effect of pH of Pectin Slurry on Pectin-Nisin Interaction

Nisin is positively charged at most pH conditions. Pectin has a pKa of about 3.5; pKa varies with DE of pectin. Therefore, depending on pH of pectin slurry, two scenarios (Figure 13) possible for pectin-nisin interaction [75] are stated below:

a) pH of pectin slurry is above the pKa of pectin: In this case, pectin in slurry will be negatively charged and therefore, positively charged nisin will interact with the negative sites ($-\text{COOH}$ groups) of pectin. Due to this interaction, nisin may bind to the pectin molecules and not be released from the film. Films containing nisin may still show antimicrobial activity due to immobilization of nisin on the film surface.

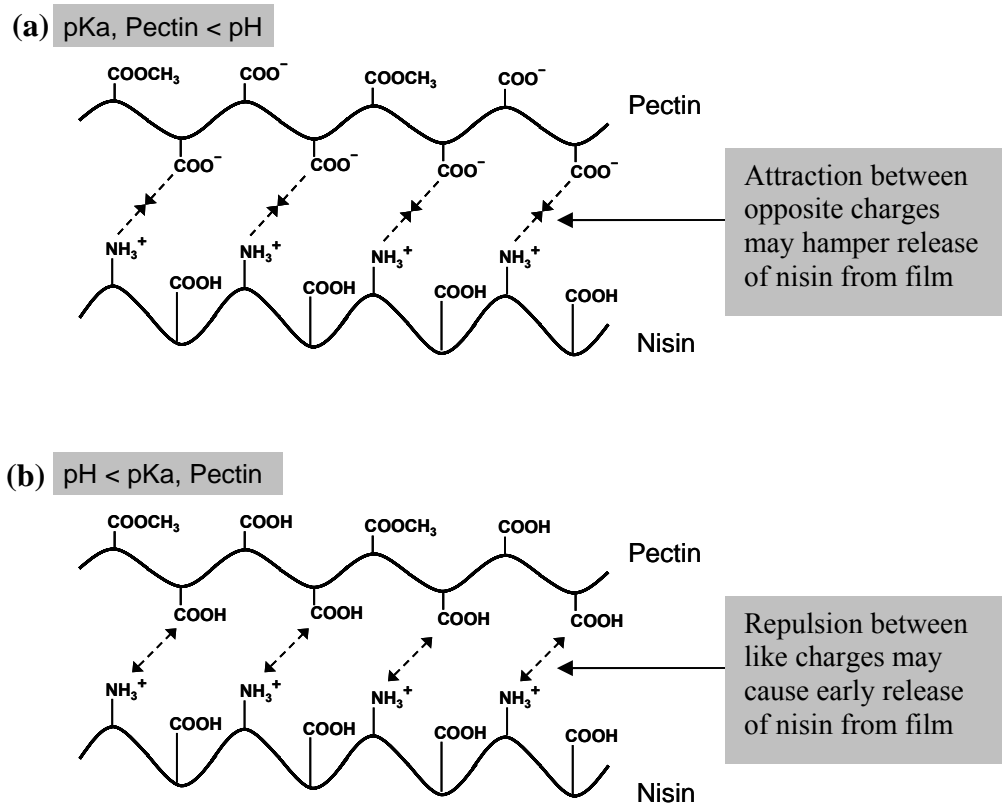


Figure 13: Schematic Diagram to Illustrate Pectin-Nisin Interactions

b) pH of pectin slurry is below the pK_a of pectin: In this case, the carboxyl groups of pectin will be in the protonated form, i.e. without any charge, resulting in a repulsion between pectin and nisin. As a result, nisin will not bind to the pectin molecules and can be released from the film. However, maintaining the pH of pectin slurry below its pK_a will affect the ability to form crosslinks with calcium ions.

1.6.3.2 Effect of pH of Pectin Slurry on Calcium Crosslinking

a) pH of pectin slurry is above the pK_a of pectin: In this case, pectin will be able to form crosslinks with calcium and different degrees of calcium crosslinking can be achieved.

b) pH of pectin slurry is below the pKa of pectin: In this case, pectin will not be able to form calcium crosslinks as effectively as in the case above. Entanglement of pectin chains will be responsible for gelation and film formation. Here, the advantage of being able to vary the release by varying the calcium crosslinking may not be realized to the maximum extent.

Thus, pH of pectin slurry is very important in designing controlled release pectin films containing nisin. The effect of pH of pectin on nisin interaction and calcium crosslinking may be summarized as shown in schematic diagram (Figure 14).

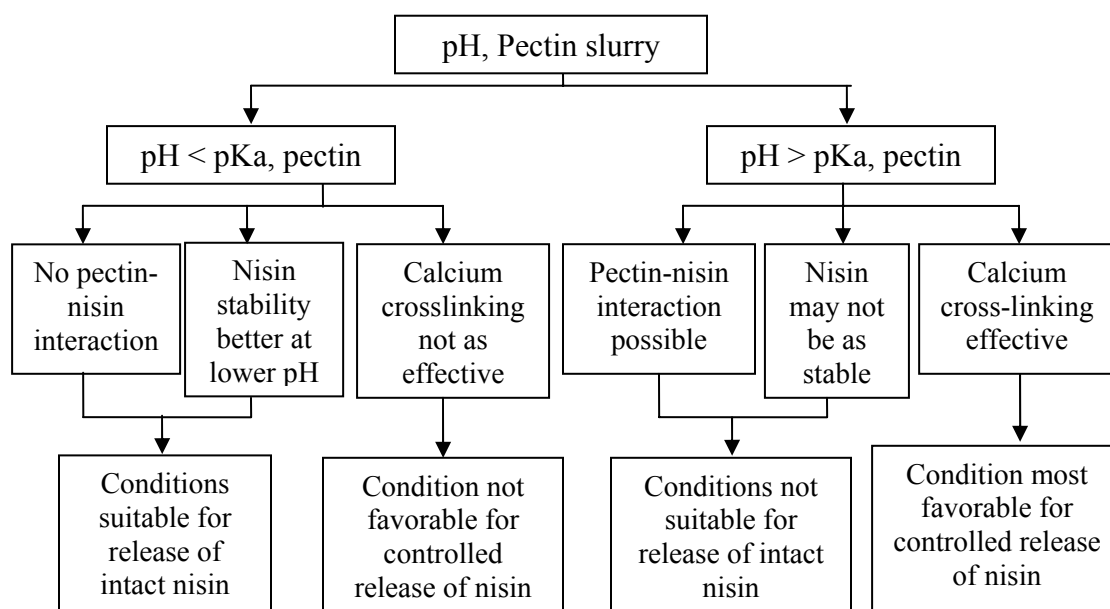


Figure 14: Overview of Impact of pH on Interactions of Pectin with Nisin and Calcium

1.6.4 Specific Objective

The specific objective of this research was to identify key variables by evaluating their effect on the release of antimicrobial compounds, especially nisin, from LM pectin films.

Based on the challenges outlined in section 1.6.3, the variables to be studied were categorized as follows:

1. Composition Variables:

- Size and chemical nature of active compounds: Here, three antimicrobial compounds - sodium benzoate (low molecular weight and aromatic ring compound), potassium sorbate (low molecular weight and straight chain compound), and nisin (highly reactive peptide with large molecular weight) – were chosen to evaluate the impact of their size on their release. Coomassie blue dye (medium molecular weight compound with several rings) was used as a model compound to prove the concept.
- Degree of calcium crosslinking: It is a product of two design variables - DE of pectin and calcium concentration. Here, the purpose was to understand the impact of the above design variables on release kinetics of the antimicrobial compounds mentioned above.

2. Process Variables:

- pH of pectin: The aim of studying this process variable was to evaluate its effect on the interaction of pectin with calcium and nisin and consequently, on the release kinetics of nisin.

- Sequence of nisin addition: Here, the aim was to determine whether pectin preferentially bound with calcium or nisin.
- Method of calcium crosslinking: Here, the purpose was to determine if release was affected by the method of calcium crosslinking – single-step or two-step crosslinking.

3. Environmental variable:

The main purpose of this experiment was to evaluate the effect of pH, both of pectin slurry and food simulant used in release study, on the recovery of nisin from pectin films. The other purpose was to compare the two methods used for nisin estimation - agar diffusion assay and HPLC.

Understanding from this study will help to optimize design variables for development of antimicrobial pectin films with variable release rates by simple variations in the degree of calcium crosslinking within the film.

1.7 Research Approach

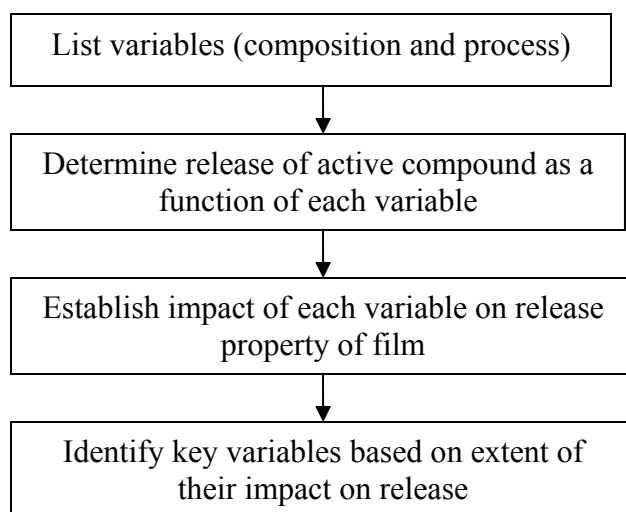


Figure 15: Research Approach to Identify Key Variables for Controlled Release LM Pectin Films

1.7.1 Proof of Concept

The research approach to attain the above goal is illustrated in the form of an experimental design (Figure 15). The first step was to establish a proof of concept using a model compound such as coomassie blue dye, the assumption being that its release was not affected by any sort of chemical interactions with pectin. Other advantages of using a dye for proof of concept were its fairly large size and ease of quantifying its release by a UV-Vis spectrophotometer. Moreover, release of dye from film could be easily observed visually.

Once proof of concept was demonstrated, the effect of key variables on release of different antimicrobial compounds would be evaluated.

1.7.2 Research Approach to Study Pectin-Nisin Interaction

Pectin-nisin interaction could be avoided by saturating the -COOH groups of pectin with calcium ions prior to addition of nisin. This may not be possible in films with lower levels of calcium concentration. In such conditions, some negative sites may still be available on pectin to bind with nisin and thus, either pectin-nisin interaction may take place or pectin-calcium interaction may take place. It is worth investigating to find out whether pectin preferably binds with any of the above. In order to seek this information, the sequence of addition of nisin and calcium can be changed, and the effect of the sequence of addition can be studied on the release of nisin.

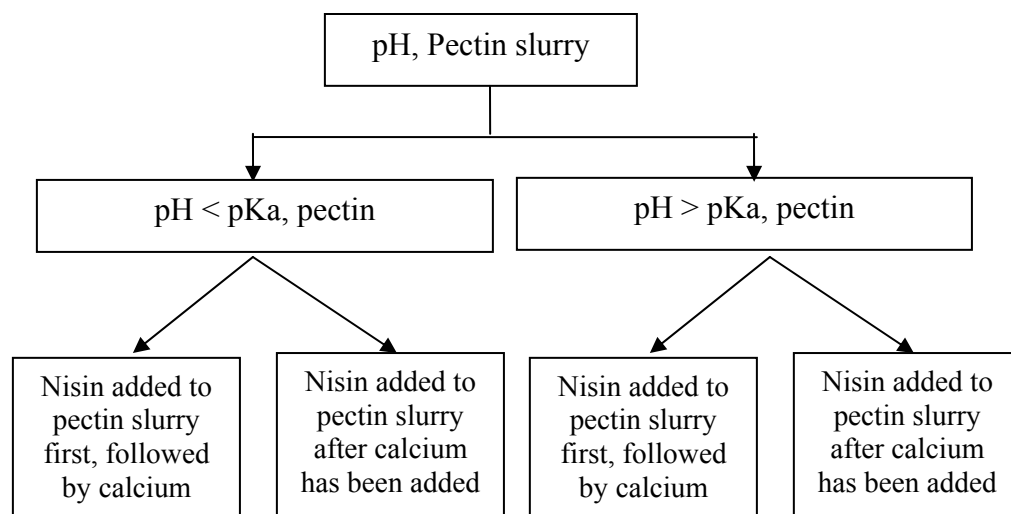


Figure 16: Research Approach to Investigate Preference for Pectin Interaction with Calcium and Nisin

2 EXPERIMENTAL

2.1 Materials

2.1.1 Pectin Selection

Commercial samples of low methoxyl pectin with varying DE were gifted by CP Kelco, San Diego, CA, USA. Their specifications are shown in table below. All pectin samples were used as is, without further purification.

Table 3: Specifications for LM Pectin from CP Kelco

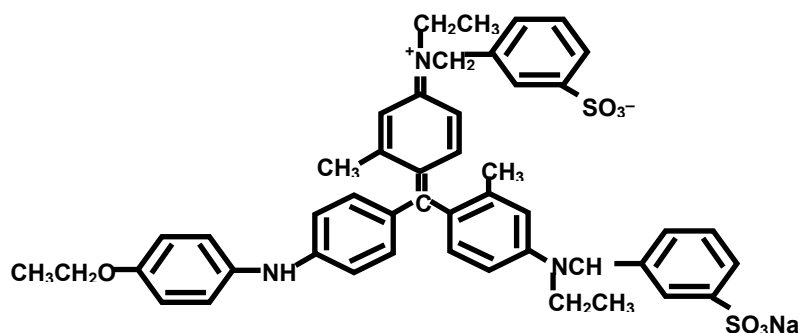
| Pectin Brand Name | Pectin Type | DE (Degree of Esterification) | DA (Degree of Amidation) | Suggested Calcium reactivity |
|--------------------------|------------------------------|--|---|---|
| GENU LM - 104 AS | Partly amidated low ester | 27 | 20 | Low |
| GENU LM - 101 AS | Partly amidated low ester | 35 | 15 | Low |
| GENU LM - 12 CG | Low ester | 35 | 0 | High |
| GENU LM - 18 CG | Low ester | 40 | 0 | Medium |
| GENU LM - 22 CG | Low ester | 50 | 0 | High sugar products |
| GENU LM - 5 CS | Low ester | 7 | 0 | High |

Pectin grades mainly used for this study were LM-12 (DE 35), LM-18 (DE 40) and LM-22 (DE 50). LM-5 was initially tried, but due to its extreme sensitivity to calcium, it was very difficult to incorporate even small amounts of calcium without causing any pregelation. The resultant films made using LM-5 pectin were very brittle and not suitable for release studies.

2.1.2 Active Compounds

2.1.2.1 Dye - for Proof of Concept

Coomassie Brilliant Blue G – 250 (research grade), sourced from Crescent Chemical Co., Inc., Hauppauge, New York, was used for proof of concept. This dye has a molecular weight of 854 and its chemical structure is as shown in Figure 17.



<http://www.serva.de/product/formulas/17524.gif>, accessed on 8 Sep 2007

Figure 17: Structure of Coomassie Brilliant Blue G – 250

2.1.2.2 Sodium Benzoate

Benzoic acid is one of the most commonly used GRAS preservatives in the food industry. It is naturally present in fruits, berries, cultured dairy products, black tea, etc. Due to the poor solubility of benzoic acid in water, its sodium salt is preferred for use in food preservation. The structures of benzoic acid and sodium benzoate are shown in Figure 18 [76].

Antimicrobial activity of benzoic acid is dependent mainly on pH and several other factors discussed in detail by Chipley [76]. It is effective in acidic foods at a pH below 4.5 and is typically used for products such as beverages, jams, sauces.



Figure 18: Structures of Benzoic Acid and Sodium Benzoate

The minimum inhibitory concentrations (MIC) for selected bacteria, yeasts and fungi have been listed by Chipley [76].

Sodium benzoate (>99% pure), in the form of a white powder, was purchased from Acros Organics.

2.1.2.3 Potassium Sorbate

Sorbic acid is another GRAS preservative commonly used in the food industry. Potassium salt is generally used in food due to the poor solubility of sorbic acid in water. The structures of sorbic acid and potassium sorbate are shown in Figure 19 [77].



Figure 19: Structures of Sorbic Acid and Potassium Sorbate

It is mainly effective against yeasts and molds and is used typically used for preservation of dairy products, baked goods, fruit and vegetable products, etc. [77].

Potassium sorbate (>99% pure), in the form of granules, was purchased from Sigma Aldrich.

2.1.2.4 Nisin

Pure nisin (40×10^6 IU per gram) was a gift from Chr. Hansen (Milwaukee, WI, USA). A stock solution (0.25% nisin) was prepared in acidic water (adjusted to pH 2 with conc. HCl) to obtain a final antimicrobial activity of 10^5 IU/ml and stored at 4 °C.

2.1.3 Plasticizer

Glycerol, a commonly used plasticizer in the manufacture of edible films, was purchased from Fisher Scientific.

2.1.4 Crosslinking Agent

Anhydrous calcium chloride, in the form of granules (4 mesh) was sourced from Sargent-Welch.

2.1.5 Media Preparation for Inhibition Zone Assay

The formulations used for preparing media for the inhibition zone assay is given in Table 4 below. All the ingredients were dispersed in deionized water and autoclaved at 121 °C for 15 minutes prior to use.

Table 4: Formula for Media Used in Inhibition Zone Assay

| Ingredients | TSBYE Nutrient Broth | Solid TSAYE Agar | Soft TSAYE Agar |
|--|-----------------------------|-------------------------|------------------------|
| Tryptic soy broth with dextrose (Bacto) | 30 g/L of water | 30 g/L of water | 30 g/L of water |
| Yeast extract powder (Bacto) | 6 g/L of water | 6 g/L of water | 6 g/L of water |
| Granulated agar (Difco Laboratories, Detroit, MI, USA) | - | 15 g/L of water | 7 g/L of water |

2.1.6 Bacterial Culture for Inhibition Zone Assay

An overnight culture of *Micrococcus luteus* ATCC 10420 was grown aerobically at 30 °C with agitation in TSBYE nutrient broth.

2.2 Methods

2.2.1 Preparation of Pectin Film

2.2.1.1 Single-Step Calcium Crosslinking

Aqueous slurry of pectin (4.56%, w/w) and glycerol (1.99%, w/w) was prepared by continuously mixing dry pectin powder in a mixture of deionized water and glycerol for almost an hour using a magnetic stirrer. Pectin slurry (35 g) was heated to 75 °C while being stirred continuously on a magnetic stirrer. To this continuously stirred hot pectin slurry, 3 ml of either coomassie blue solution (0.0395%) or 1% potassium sorbate solution or 1% sodium benzoate solution or 0.25% of pure nisin solution was added. Crosslinking agent i.e. calcium chloride solution (0.7%), heated to the same temperature as pectin slurry, was gradually added to the above slurry, being continuously stirred at 75 °C, to finally obtain the film-forming solution. The amount of calcium chloride solution added to the slurry varied with the desired degree of crosslinking and the values are given in Table 5. The hot film forming solution was immediately degassed and 25 g of film forming solution was poured into petri dishes (87mm diameter x 13 mm height) and allowed to dry overnight at 30 °C. The resultant film was peeled off from the petri dish and equilibrated at 50% RH, 25 °C for a day prior to using it for any tests.

Table 5: Requirement of Calcium Chloride for Films with Different Calcium Crosslinking.

| Desired Calcium Concentration in Film (mg of Ca per g of pectin) | Pectin Slurry (g) | Volume of Calcium Chloride Solution (0.7%) ml | Amount of water (ml) |
|---|--------------------------|--|-----------------------------|
| 0 | 35 | 0 | 12 |
| 9 | 35 | 6 | 6 |
| 13.5 | 35 | 9 | 3 |
| 18 | 35 | 12 | 0 |

2.2.1.2 Two-Step Calcium Crosslinking

For very high levels of calcium crosslinking or when very low DE pectin was to be used, it was difficult to make uniform films by the single-step crosslinking technique. Almost instantaneous gelling and solidification of the gel caused difficulty in pouring the gel to form uniform films. This imposed a practical limitation on the amount of calcium that could be added and so, a two-step crosslinking process was carried out.

Here, pectin slurry was prepared as described in above section. In the first step, low amount of calcium chloride was added to hot slurry, such that it was comfortable to pour the gel into petri dishes. The gel was allowed to dry overnight into a film and then this film was immersed in a known amount of calcium chloride solution and dried for 8-10 hours to obtain a higher degree of calcium crosslinking. The resultant film was rinsed 5 times, with 2 ml of deionized water each time; to remove any calcium or nisin adhered to the surface.

2.2.2 Release Studies

Approximately 0.8 g of film pieces were cut into 2 cm × 2 cm square pieces and immersed in 80 ml of deionized water in a 125 ml Erlenmeyer flask, which was shaken at

100 rpm and 23°C. Samples were withdrawn periodically and amount of active compound released was quantified by methods described in section 2.2.3.

Note: In these release studies, 100% water was used instead of the FDA recommended food simulants [78] like 10% ethanol because pectin precipitates in ethanol and methanol, thereby it might affect the swelling property of pectin film.

2.2.3 Quantification of Active Compounds

2.2.3.1 Quantification of Dye by UV-Visible Spectrophotometry

Coomassie blue dye released from film into water was quantified by UV/Visible spectrophotometry at 612 nm. A calibration standard curve for the quantification of dye was generated (Figure 20) at 612 nm using a Varian Cary 50 UV-Vis spectrophotometer (Palo Alto, CA, USA).

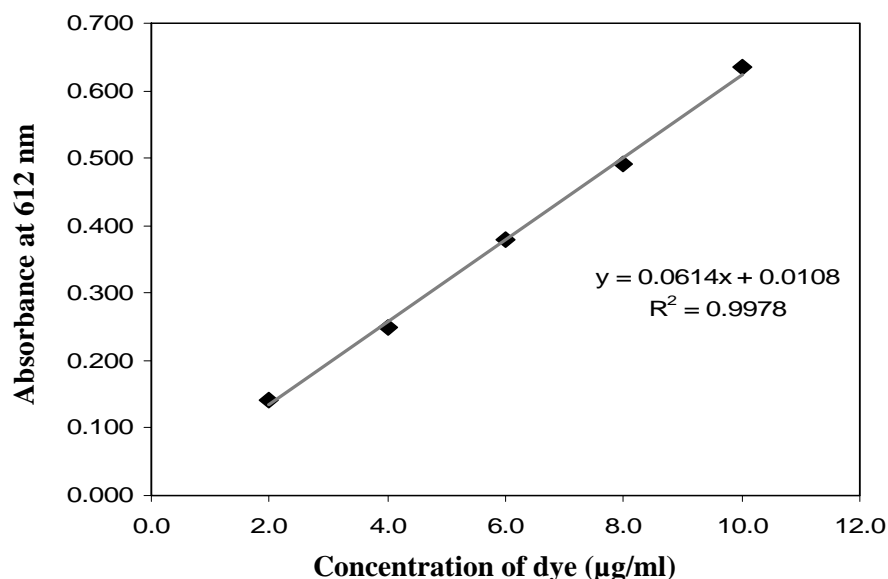


Figure 20: Calibration Curve for Coomassie Blue G – 250

2.2.3.2 Quantification of Sodium Benzoate and Potassium Sorbate by HPLC

Benzoic and sorbic acids and their respective salts were analyzed using RP-HPLC as per the standard protocol recommended by the USDA [79] and also used by Buonocore et al. [80]. Both these compounds were analyzed using the same method; they only differed in the wavelength of detection.

HPLC experiments were conducted on Shimadzu SCL-10Avp System (Kyoto, Japan) equipped with a LC10ADVP solvent delivery unit, FCV-10ALvp Low Pressure Gradient Unit, SIL-10Ai Autoinjector, and SPD-M10Avp Photodiode Array Detector. Data were processed using Class VP EZ Start Version 7.3 data acquisition software.

A silica based C18 column, Cosmosil – code no. 379-72 (Waters, MA, USA), with a particle diameter of 5 μm and dimensions of 250 mm \times 4.6 mm I.D. was used for the above quantification. 20 μl samples were injected.

The mobile phase for the above HPLC quantification was prepared by dissolving 0.68% (w/v) of potassium hydrogen phosphate in part of the water, followed by addition of 0.1% (v/v) of concentrated phosphoric acid and 25% (v/v) of HPLC grade acetonitrile. To this mixture, water was added to make up a mobile phase of water: acetonitrile in the ratio 75:25. The flow rate of mobile phase was maintained at 1 ml/min, at ambient temperature and wavelength of UV detection was set at 225 nm for benzoic acid and 260 nm for sorbic acid.

2.2.3.3 Microbial Assay for Nisin Activity

Antimicrobial activity of nisin released from film into water was assayed using the agar diffusion assay [81, 82]. Briefly, solid TSAYE agar plates were prepared by

dispensing 20 ml of sterile media in sterile petri dishes. Sterile soft TSAYE agar was melted in a microwave oven and tempered at about 45 °C. 4 ml of this agar was seeded with 40 µl of an overnight broth culture on *Micrococcus luteus* ATCC 10420 and dispensed over the solid agar mentioned above. Three wells of 6 mm diameter were bored into each agar plate with a sterile glass tube. 50 µl of liquid sample, either standard nisin solution or aliquot of samples collected at different time intervals during release study were pipetted into each well. The agar plates were then stored in refrigerator for 24 hours to allow diffusion of nisin, followed by incubation at 30 °C for 24 hours during which time clear inhibition zones were developed. Diameter of the inhibition zone developed was measured using a ruler. A calibration (figure) for standard nisin solutions ranging from 12.5µg/ml (equivalent to 2.5 IU/ml) to 100 µg/ml (equivalent to 4000 IU/ml) was constructed by plotting width of inhibition zone developed vs. log of corresponding nisin concentration. The amount of nisin in test solution was estimated by comparing the width of inhibition zone with that of standard nisin solutions.

In order to estimate amount of nisin released when film was placed in contact with soft agar, the previously described well diffusion assay was slightly modified. In this case, a bigger well with a diameter of 1.6 cm was bored into the seeded agar plate and the diameter of film sample placed on the seeded agar was also 1.6 cm. The agar plate with film was stored in refrigerator for different time intervals, followed by incubation at 30 °C for 24 hours.

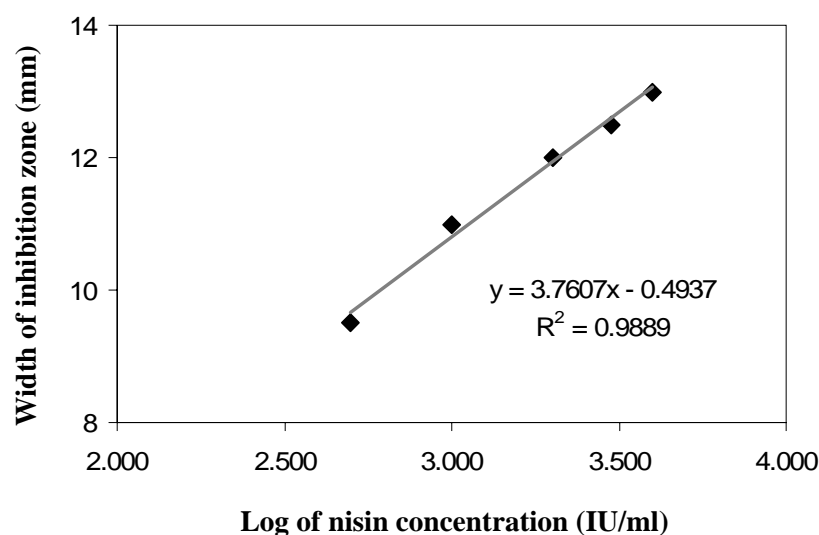


Figure 21: Calibration Curve for Nisin Estimation Using Agar Diffusion Assay

2.2.3.4 Quantification of Nisin by HPLC

Most common method for estimation of nisin is the inhibition zone assay mentioned in section 1.2.3.3. However, a HPLC method has been developed by Liu and Hansen [74].

Quantification of nisin released into water was made by adapting a modified method developed by Buonocore et al. [80]. HPLC experiments were conducted on Shimadzu SCL-10Avp System (Kyoto, Japan) mentioned in section 1.2.3.2.

A silica based C-18 reversed-phase column, ACE-5, with a particle diameter of 5 μm and dimensions of 150 mm \times 4.6 mm I.D. was used for the above quantification. Each column was preceded by a guard column. 50 μl samples were injected.

Mobile phase was comprised of water-acetonitrile, both containing 0.1% TFA. A gradient of 20-60% acetonitrile over 20 minutes at a flow rate of 1 ml/min was maintained. The wavelength of UV detection for nisin was set at 254 nm and nisin eluted

at 5.3 minutes. A calibration curve (Figure 22) was constructed for standard nisin solutions ranging from 5 to 125 $\mu\text{g/ml}$. Standard nisin solutions were prepared using the pH 2 water as a diluent in order to maintain nisin in the most stable form.

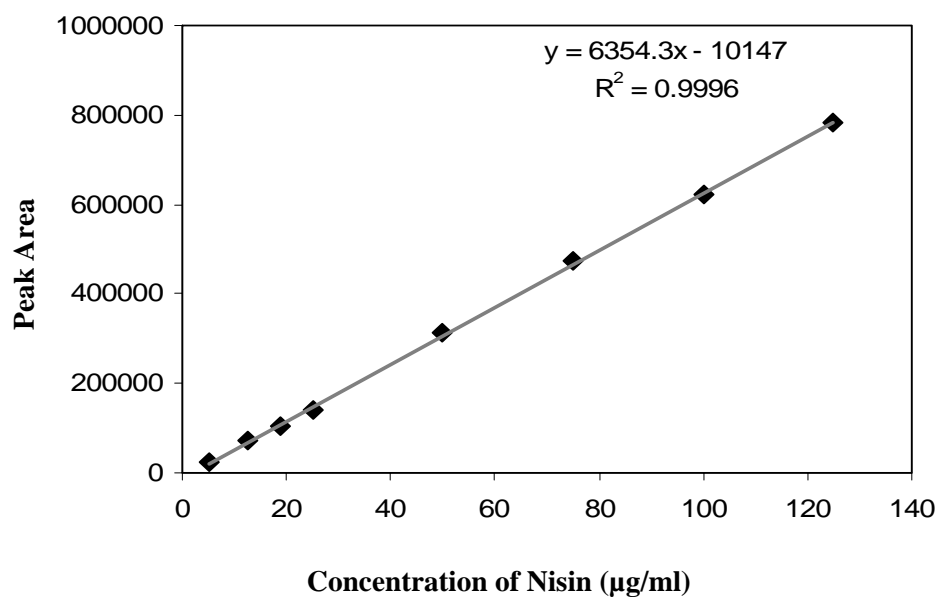


Figure 22: Calibration Curve for Nisin Estimation by HPLC

3 RESULTS AND DISCUSSION

3.1 Effect of Composition Variables on Release

As already mentioned, the main rationale to conduct an experiment for proof of concept was to get a sense of whether the degree of calcium crosslinking, one of the main composition variables was significantly effective in controlling the release of dye from pectin films, provided no other variables such as interactions with pectin or degradation of active compound were present. The dye chosen for proof of concept was stable, moderately large and easy to quantify. Results obtained from proof of concept were encouraging and provided a motive to study effect of other variables on release of active compounds.

3.1.1 Effect of Degree of Calcium Crosslinking

3.1.1.1 Effect of Calcium Concentration on Release of Dye from Pectin Film

Calcium concentration, one of the two main design variables affecting the degree of calcium crosslinking, was studied in this set of experiments. Pectin films containing dye were prepared as per method described in section 2.2.1. All films were made using LM-12 pectin (DE 35) and the amount of calcium in the films was varied to give a final calcium concentration, expressed as mg of Ca per g of pectin, in the dry films at 9, 13.6, 18, and 28. A control film was also made without any calcium. Release studies were conducted for each film and % of dye released was quantified for different time intervals, as shown below in Figure 23.

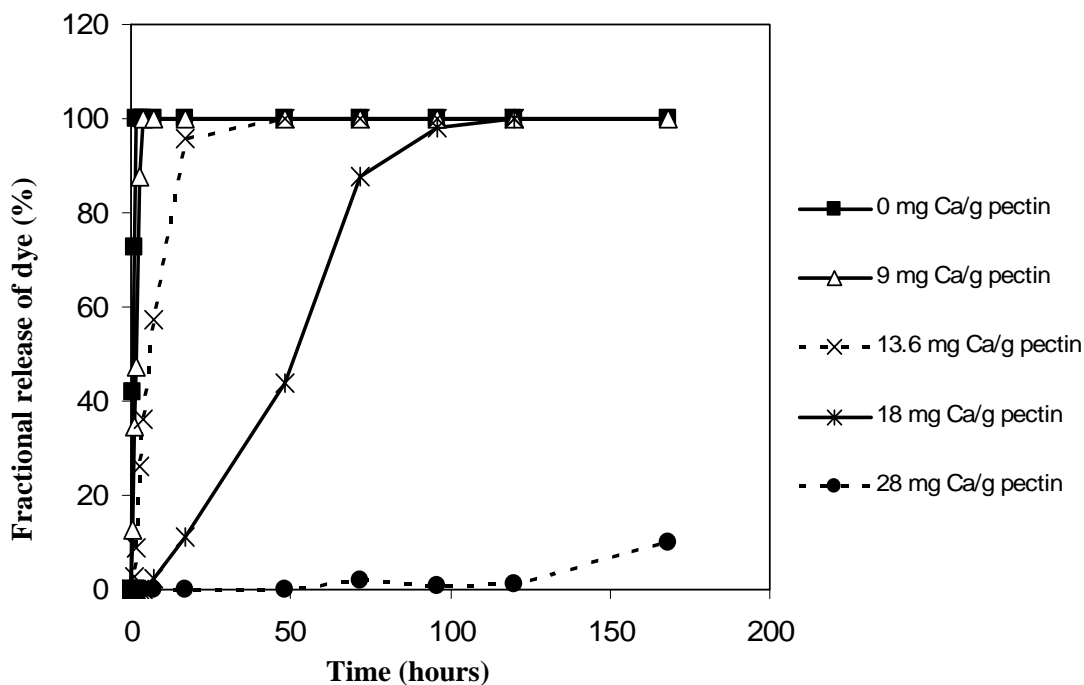


Figure 23: Effect of Calcium Concentration on Release of Dye from LM 12 (DE 35) Pectin Films

Time taken to release all dye from films with 0, 9, 13.6, 18 mg Ca /g pectin was 1.5 hours, 7 hours, 48 hours, 120 hours, respectively (Figure 23). For film with 28 mg Ca/g pectin, only 10% of dye was released after 7 days.

Similar release trend was observed in films made using LM-18 (DE 40) and LM-22 (DE 50) with different concentrations of calcium, as shown in Figure 24 and Figure 25, respectively.

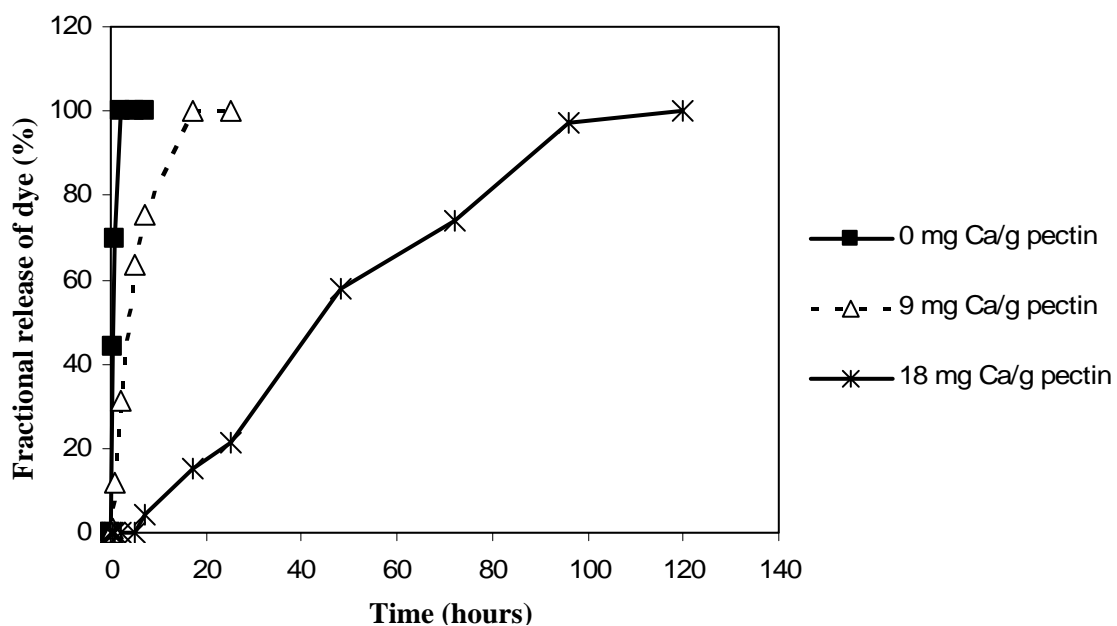


Figure 24: Effect of Calcium Concentration on Release of Dye from LM-18 (DE 40) Pectin Films

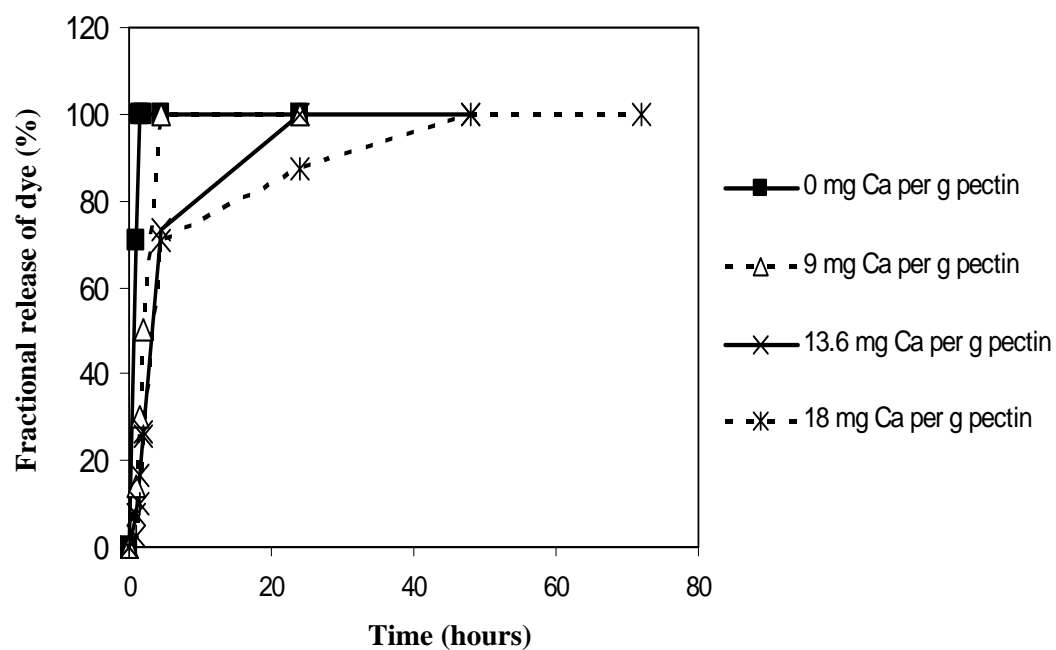


Figure 25: Effect of Calcium Concentration on Release of Dye from LM-22 (DE 50) Pectin Films

Results obtained from these release studies clearly demonstrated that the rate of release of dye from film decreased significantly with a small increase in calcium concentration.

3.1.1.2 Effect of DE of Pectin on Release of Dye from Pectin Film

Degree of esterification (DE) of pectin, the second design variable affecting the degree of calcium crosslinking in the pectin film, was studied in this set of experiments. Films were made using pectin with DE 35, 40 and 50 and for each DE calcium concentration were varied as shown in grid below.

Table 6: Effect of DE of Pectin and Calcium Concentration on Release of Dye from Film

| DE of Pectin | Calcium Concentration in Film (mg of Ca per g of pectin) | Time Taken for Release of All Dye (hours) |
|---------------|---|--|
| 35 (LM-12) | 0 | 1.5 |
| | 9 | 7 |
| | 18 | 120 |
| | 28 | 168 (~10% release) |
| 40 (LM-18) | 0 | 2 |
| | 9 | 17 |
| | 18 | 120 |
| 50 (LM-22) | 0 | 1.5 |
| | 9 | 4.5 |
| | 18 | 48 |

Table 6 gives a comparison of dye release time for films made with different DE pectins. For a film made with DE 50 pectin, at a calcium concentration of 18 mg Ca/g pectin, the dye release time was 48 hours, whereas for a film made with DE 35 pectin and same amount of calcium concentration, the total release time was 120 hours. This result showed that with a decrease in the DE of LM pectin from 50 to 35, the dye release time was extended from by 3 days. But, there was no significant difference in release rates for 35 DE and 40 DE films, indicating that in order to obtain a significant difference in release rates, a wide range of DE of pectin would be necessary.

Overall, the rate of release of dye was influenced by degree of calcium crosslinking within film, which was altered by varying the two design variables – calcium concentration or DE of pectin used (Figure 11). Degree of crosslinking within film was found to directly affect the rate of swelling and rate of dissolution of film in water, which in turn was thought to affect the release of an active compound from the swollen films. It is reasonable to say that the release of an active compound from such swellable films follows non-Fickian diffusion and depends on the degree of swelling of film and rate of dissolution of film in an aqueous food simulant. The sequence in which degree of crosslinking affects release of active compound may be summarized as shown in Figure 26.

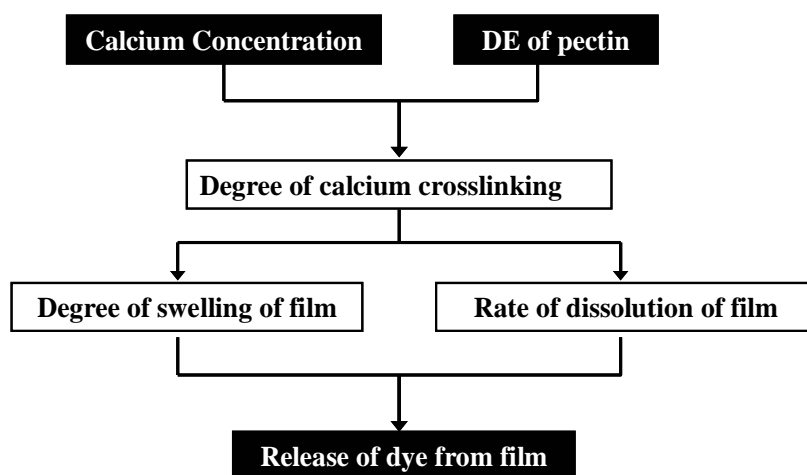


Figure 26: Summary of Effect of Crosslinking on Release of Active Compound from LM Pectin Film

Of the two design variables, it was clearly evident (Table 6) that a small change in calcium concentration produced reasonable difference in release rates. However, the same was not true in the case of DE of pectin, thereby indicating that calcium concentration could be a more dominant variable among the two. Since commercial LM

pectin grades are available within a narrow range of DE, use of this design variable is slightly restrictive for development of controlled release pectin films.

After proving the concept, the next step was to study the effect of other variables on release of active compounds.

3.1.2 Effect of Size of Active Compound

3.1.2.1 Sodium Benzoate

Pectin films containing approximately 1% of sodium benzoate (on dry film) were prepared and release tests were conducted as per method described in section 2.2.2 and amount of sodium benzoate released into food simulant was estimated using HPLC (section 2.2.3.2). The amount of benzoate that should be present in film theoretically was calculated on the basis of material balance of sodium benzoate added to film forming solution and weight of dry film obtained. Film was completely dissolved in water and total benzoate content in film was estimated to compare the theoretical % loading with the actual extractable amount (Table 7).

Table 7: Loading of Sodium Benzoate in Pectin Films - Theoretical vs. Total Extractable

| Sample | LM 22 (DE 50) | | LM 18 (DE 35) | |
|---------------|-----------------------------------|----------------------------|----------------------------|---|
| | mg of Ca per g of film | % Total (added) | % Total (added) | % Extractable of total added |
| 0.0 | | 1.30 | 11.22 | 0.930 |
| 9.0 | | 1.16 | 11.67 | 1.229 |
| 13.5 | | 1.18 | 12.43 | 1.177 |
| 18.0 | | 1.16 | 10.94 | 1.196 |

The total extractable benzoate was only about 8 -12% of the amount originally added. This meant that there was some kind of interaction between the rest of the

benzoate and pectin within the film during the release test with water as food simulant. A detailed study was conducted to find out the reason for such poor recovery of benzoate when films were dissolved in water.

3.1.2.1.1 Sodium Benzoate Entrapment in Pectin Films

Some of the probable reasons short listed were pH of film forming solution and release test food simulant, solubility of sodium benzoate in film forming solution or food simulant, thermal stability of sodium benzoate, interaction of sodium benzoate with calcium ions, interaction of sodium benzoate with pectin, etc. A logical decision tree method (Figure 27) was followed to rule out entrapment of benzoate in the different steps involved in development of film and subsequent release study.

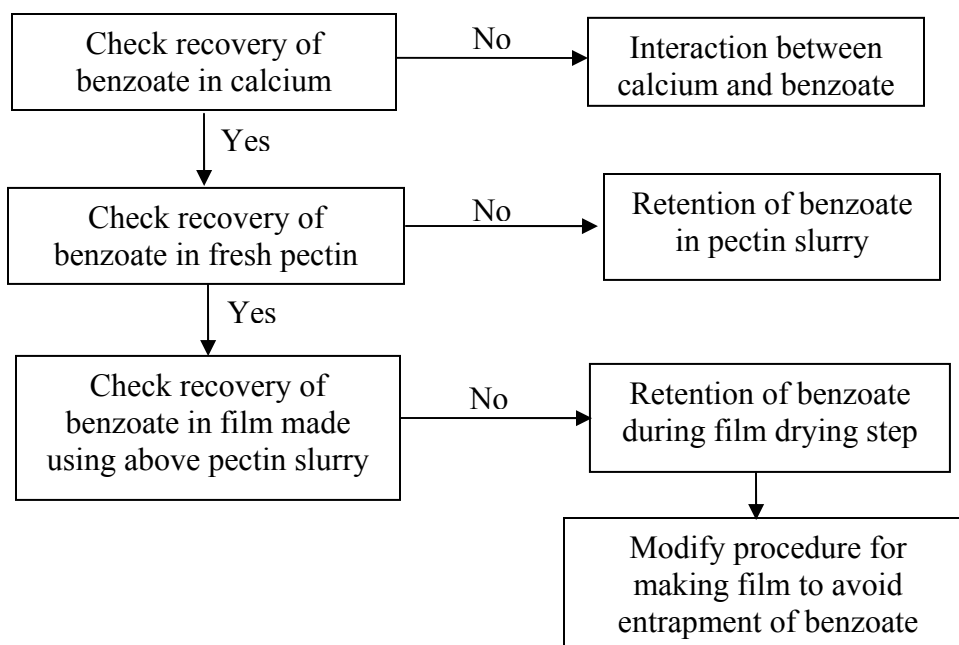


Figure 27: Decision Tree Diagram for Investigation of Benzoate Entrapment in Pectin Films

Table 8: Effect of Calcium on Recovery of Sodium Benzoate from Pectin Films

| Sample | Expected Benzoate Concentration ($\mu\text{g/ml}$) | Observed Benzoate Concentration ($\mu\text{g/ml}$) |
|--|--|--|
| Benzoate in deionized water | 118 | 121 123 116 |
| Benzoate and calcium chloride in deionized water | 118 | 121 |
| LM 18 pectin slurry without calcium | 24.6 | 18 |
| LM 18 pectin slurry with calcium | 24.6 | 20.5 |

The above results showed that benzoate could be recovered completely from deionized water, irrespective of presence of calcium ions. In case of benzoate recovery from pectin slurry, about 80% recovery was observed, irrespective of presence of calcium ions. Thus, the possibility of poor recovery due to interaction of sodium benzoate and calcium was ruled out. In fact, calcium dibenzoate is a known preservative approved for food use.

The next step was to check recovery of benzoate in fresh pectin slurry. Calcium was not added to the slurry in order to study effect of pectin only. pH of pectin slurry i.e. the film forming solution was found to be about 3 and hence recovery of benzoate from acidic pH solution was also checked to rule out any effect of pH. The results are shown in table below.

Table 9: Recovery of Sodium Benzoate from Fresh Pectin Slurry

| Sample | Expected Benzoate Concentration ($\mu\text{g/ml}$) | Observed Benzoate Concentration ($\mu\text{g/ml}$) | pH of Solution |
|--|--|--|----------------|
| Benzoate in deionized water | 30 | 34 | 6.9 |
| Benzoate in acidic water | 30 | 31.87 | 3.04 |
| 1% pectin solution (LM 18) in same solution as above (containing 30 $\mu\text{g/ml}$ benzoate) | 30 | 30.86 | 3.09 |

Full recovery of sodium benzoate from fresh pectin slurry (1% solution), at an acidic pH of 3, indicated that there was no interaction of benzoate with pectin till the step of pectin slurry preparation. So, two films were made with the top and bottom portion of the same pectin slurry used in the above estimation. These films were dissolved in water to form a 1% solution and the benzoate content was estimated. The results obtained are given in Table 10 below.

Table 10: Recovery of Sodium Benzoate from Pectin Film

| Sample | Expected Benzoate Concentration ($\mu\text{g/ml}$) | Observed Benzoate Concentration ($\mu\text{g/ml}$) | pH of Solution |
|---|--|--|----------------|
| Benzoate in water | 57.2 | 57 | 6.9 |
| Benzoate in acidic water (same concentration as in film forming solution) | 572 | 538 | 2.85 |
| 1% pectin solution (LM 18) containing 30 $\mu\text{g/ml}$ benzoate | 30 | 30.86 | 3.09 |
| Film made with top portion of above pectin slurry | 29 | 17.9 | 3.08 |
| Film made with bottom portion of above pectin slurry | 29 | 18.8 | 3.06 |

There was no problem with recovery of benzoate from fresh pectin slurry, but the films made with the same pectin slurry showed only 50-60% recovery. This was in confirmation with the first result (Table 7) in which only 10-12% of pectin could be recovered. This indicated that there was definitely some interaction occurring between pectin and sodium benzoate while the pectin slurry was being dried for 24 hours to form a film. Higher recovery in above study could be attributed to lesser interaction between benzoate and pectin due to difference in pectin content of films, 1% in above study (Table 10) as compared to 3.3% in the regular films used in the first study (Table 7).

Thermal stability of benzoate in acidic conditions was also tested by heating a solution of benzoate in deionized water, adjusted to a pH of 3, for same time-temperature conditions as in the preparation of film forming solution. Full recovery was seen and thus loss of benzoate due to heating was eliminated.

Some other tests were conducted, but the results obtained could not explain the poor recovery of benzoate from pectin films. Thus, sodium benzoate, though small in size, was not suitable for controlled release pectin films due to entrapment in pectin, resulting in very poor release.

3.1.2.2 Potassium Sorbate

It was speculated that the benzene ring in benzoic acid could be responsible for π -bonding, further leading to entrapment of benzoic acid in the pectin matrix. In order to prove this theory of π -bonding due to benzene ring, potassium sorbate was chosen. It has a pKa of 4.8, has similar molecular weight as sodium benzoate, but it is a straight chain compound. So, it was expected to be released completely from pectin films.

Release studies were conducted with pectin films containing 1% potassium sorbate (on a dry film). 0.8 g of film was suspended in 250 ml water and HPLC method was used to quantify amount of sorbate released from film into water. The results are shown in Figure 28 below.

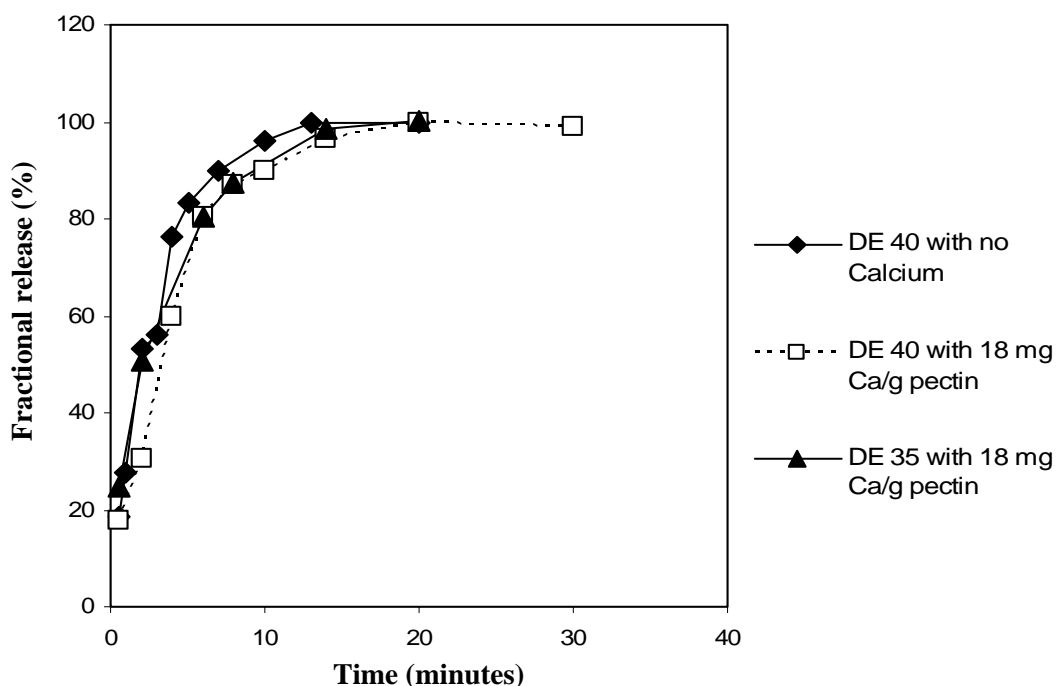


Figure 28: Effect of Calcium Concentration on Release of Potassium Sorbate from LM 12 (DE 35) and LM 18 (DE 40) Pectin Films

The observations from above release study were as follows:

- Only 45-50% of added potassium sorbate could be totally extracted, even after complete dissolution of the film in water. This observation was similar to that of pectin films containing benzoate, but in case of sorbate, the % total extractable was higher. Retention of potassium sorbate in pectin matrix has been reported by Guilbert et al. [83]

- Time taken to release the total extractable potassium sorbate (45-50% of added, as mentioned above) from a film crosslinked with 18 mg Ca/g pectin was only 10 minutes longer than that of a film without any calcium. Such fast release rates have also been reported for release studies with whey protein films and tapioca starch films.
- Film made using LM 12 (DE 35) pectin and containing 18 mg Ca/g pectin took more than 3 hours to begin swelling (observed visually) and started dissolving after 7-8 hours, but all free potassium sorbate was released in less than 30 minutes as shown in Figure 28 above. Thus, complete release of potassium sorbate even before the film began to swell could mean that effect of degree of swelling on release of active compounds was true only when the size of active compound was above a certain threshold. This was in confirmation with the proof of concept where same film compositions were used, but the active compound (dye) used was at least 7 times larger than potassium sorbate, big enough to be entrapped by the crosslinks within the film.

The above experiment with potassium sorbate confirmed the importance of choosing an active compound with an appropriate size such that it would not be released too quickly. Hence, nisin, an important antimicrobial compound, fairly big in size was considered for further development of antimicrobial pectin films. A preliminary study was conducted with nisin-containing films to test whether nisin could diffuse through pectin films; contrary to speculation that nisin would bind to pectin and hence could not be released from pectin films.

3.2 Preliminary Studies with Nisin Containing Pectin Films

It was thought that nisin may not be released from pectin films due its interaction with pectin. Hence, a preliminary study was conducted with nisin-containing films to find out if the above speculation was true or nisin could be released from pectin films. Films were made with LM 22 (DE 50) pectin using different protocols as given below:

- Control film – with 18 mg Ca/g pectin, without nisin
- Film 1 – In this case, slurry of pectin and calcium was made and known amount of nisin solution was added directly to the above slurry. The slurry was then allowed to dry overnight to form a film. Here, nisin (positively charged) may bind preferentially with pectin instead of calcium binding with pectin and hence may not release.
- Film 2 – In this case, slurry of only pectin and calcium was made, allowed to dry overnight to form a film. The film was then suspended in a known amount of nisin solution for 15 minutes to allow nisin to diffuse inside film. Here, the idea was to saturate some of the negative sites on pectin with calcium ions so that these negative sites would not be available to bind with nisin, thereby improving its chances of release as compared to film 1.
- Film 3 – In this case, the two-step calcium crosslinking method was used to saturate all the negative sites on pectin to form a film with maximum degree of calcium crosslinking. The film thus developed was then suspended in nisin solution for 15 minutes to allow nisin to diffuse inside film. Here, the assumption

was that all the negative sites on pectin would be saturated with calcium, leaving no site to bind with nisin.

The films made as per methods described above were placed on soft agar, inoculated with target microorganism *Micrococcus luteus* (100-fold dilution of an overnight culture), at 4°C overnight, followed by incubation at 30 °C for 24 hours. Zone of inhibition produced by nisin diffusion, as shown in Figure 29 was proof that nisin did not bind to pectin and was effective as an antimicrobial when incorporated in pectin films.

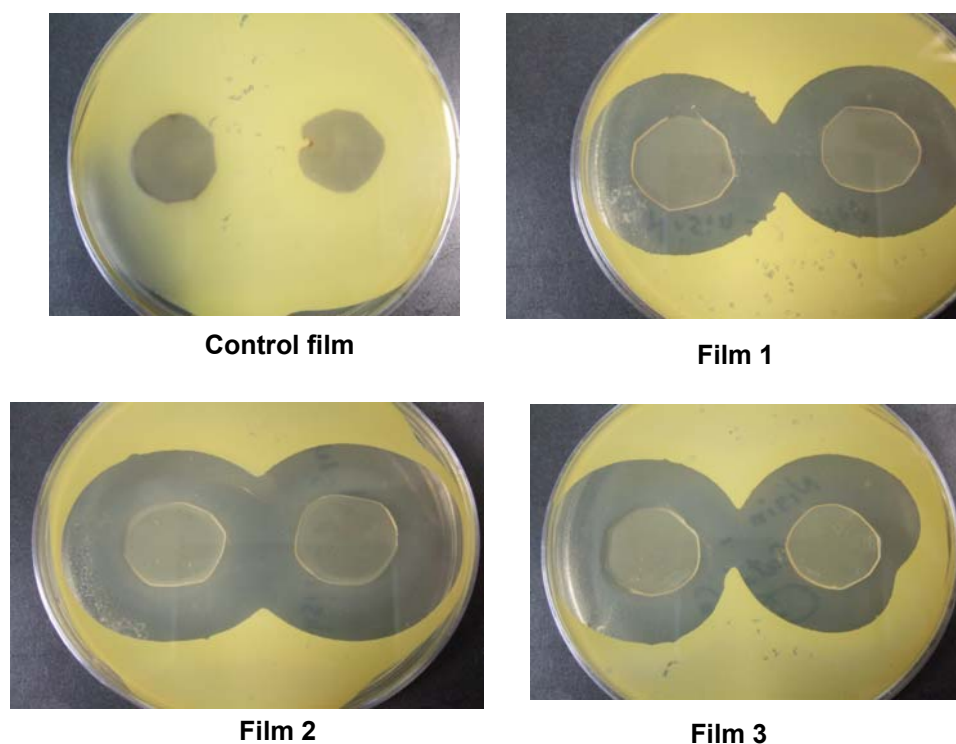


Figure 29: Antimicrobial activity of nisin-containing pectin films

No difference was observed in the zones developed by films made with different methods. The possible explanation for this was that the amount of nisin that diffused into the films 2 and 3 may not have been the same as the amount directly incorporated into film 1 and hence this experiment could not be used to compare the nisin released from

different films. However, this preliminary experiment gave an indication that nisin could indeed be release from pectin films and proved the speculation that nisin would bind to pectin wrong. It was however necessary to evaluate some of the processing variables to understand the pectin-nisin system better.

3.3 Effect of Processing Variables on Release of Nisin

3.3.1 Effect of Calcium Crosslinking Method

In order to study this processing variable, three films were made with LM 12 pectin (DE 35) at pH 3.2 as follows -

- Control film without calcium, with nisin (designated as '0' in the microbial assay).
- Single-step calcium crosslinked film containing 18 mg Ca per g of dry pectin, and with nisin. All calcium was added to the pectin slurry, prior to film formation (designated as '18' in the microbial assay).
- Two-step calcium crosslinked film containing a total of 100 mg of Ca per g of dry pectin, and with nisin (designated as '100' in the microbial assay). Here, 18 mg Ca per g of dry pectin was added to pectin slurry in the first step and the rest was added after film was formed.

Release studies were conducted with these films in water in a flask and nisin concentration of samples collected at different time intervals was estimated by well-diffusion assay. The results are shown in Figure 30 and Figure 31 .

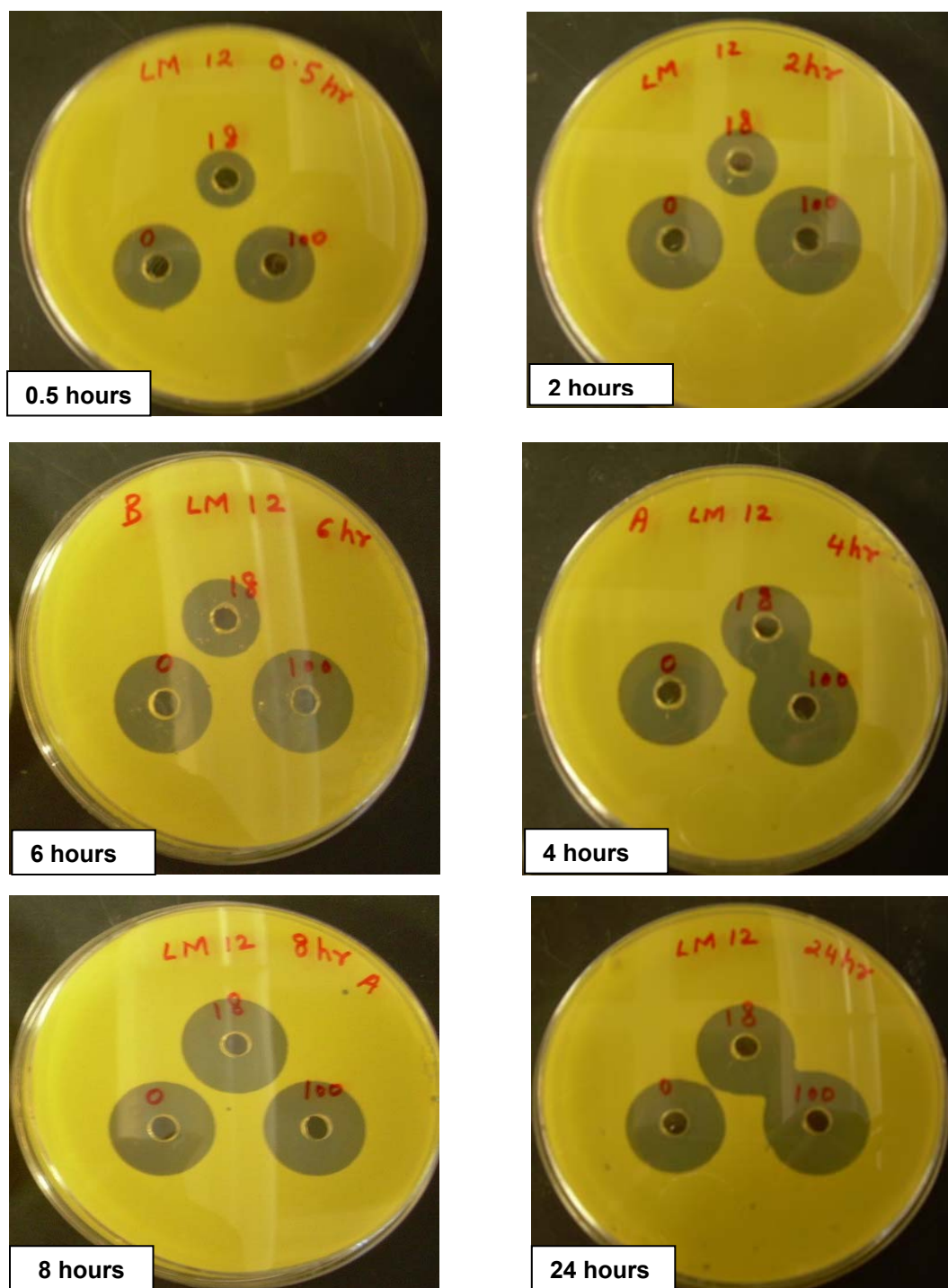


Figure 30: Effect of Calcium Crosslinking Method on Release of Nisin from Pectin Film (Inhibition Zone Assay)

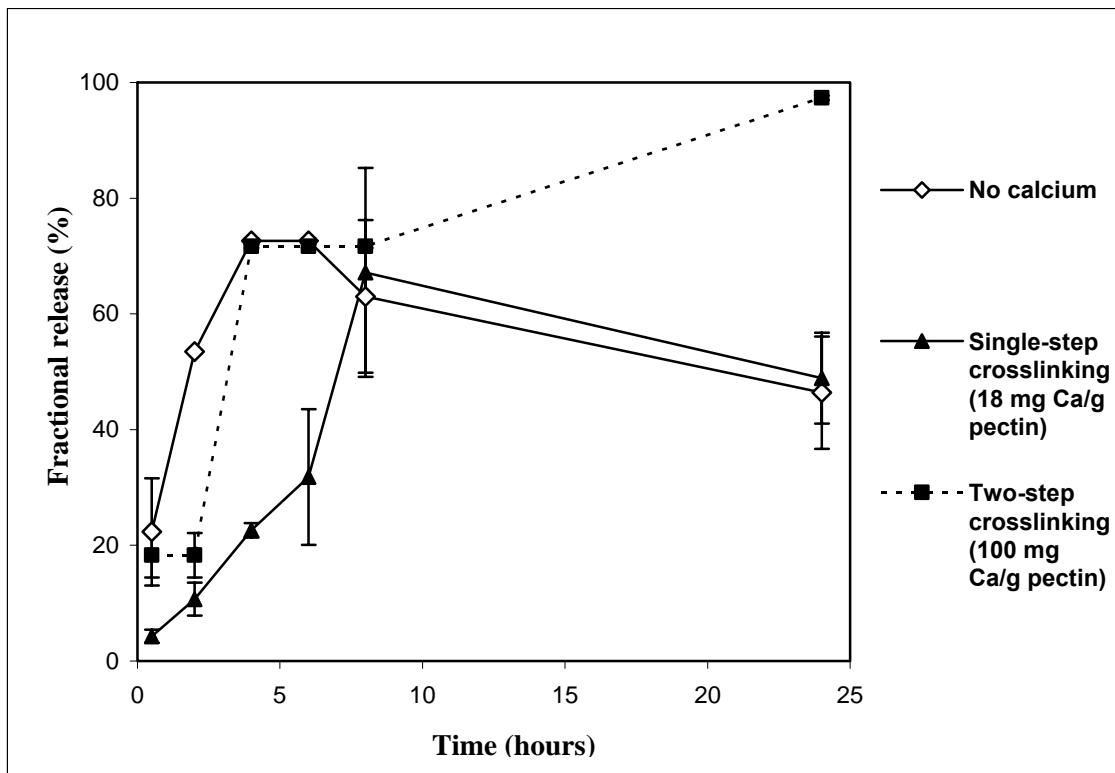


Figure 31: Effect of Calcium Crosslinking Method on Release of Nisin from Pectin Films

The film with 18 mg Ca/g pectin showed slower release of nisin as compared with film without calcium for the first 8 hours after which maximum nisin was released. In case of film containing 100 mg Ca/g pectin, made with the two-step crosslinking method, it should have ideally shown a release rate slower than 18 mg Ca/g pectin film. However, the opposite was observed, with the two-step film releasing maximum nisin in 3-4 hours, similar to control film without any calcium. A probable reason for this reverse trend could be attributed to the two-step process of crosslinking, which allowed enough time for entrapped nisin to migrate to the surface, resulting in its immediate release. Thus, the method of calcium crosslinking plays a role in the controlling the release of nisin.

The two-step method has been used in other studies and may not be the correct way to make controlled release films.

3.3.2 Effect of pH of Pectin and Sequence of Nisin Addition on Pectin-Nisin Interaction

As explained previously in the research approach, pectin-nisin interaction may be avoided by occupying the negative sites on pectin with calcium, so that few sites are available to bind with nisin. In order to establish a certain preference for interaction of pectin with either nisin or calcium, the sequence of addition of nisin was varied for two pH conditions of pectin slurry as follows:

- pH of pectin slurry < pKa of pectin: For this condition slurry was used as is because pH of pectin slurry for a 3.3% pectin concentration was already in the range 2.6 - 2.8.
- pH of pectin slurry < pKa of pectin: For this condition, pH of pectin slurry was adjusted to 3.85-3.95 using a 5% NaOH solution.
- For each pH condition, films were made with sequence of nisin addition as follows -
- Control film without calcium, with nisin (designated a '0' in the inhibition zone assay images).
- Nisin added to pectin slurry first, followed by calcium chloride solution. This film was designated as N1.
- Nisin added to pectin slurry after addition of calcium chloride solution. This film was designated as N2.

In N1 and N2, calcium concentration of 18 mg Ca/g dry pectin was maintained.

Release studies were conducted with these films suspended in water in a flask and nisin concentration of samples collected at different time intervals was estimated by well-diffusion assay. The results for $\text{pH} < \text{pKa}$ are shown in Figure 32 and Figure 33.

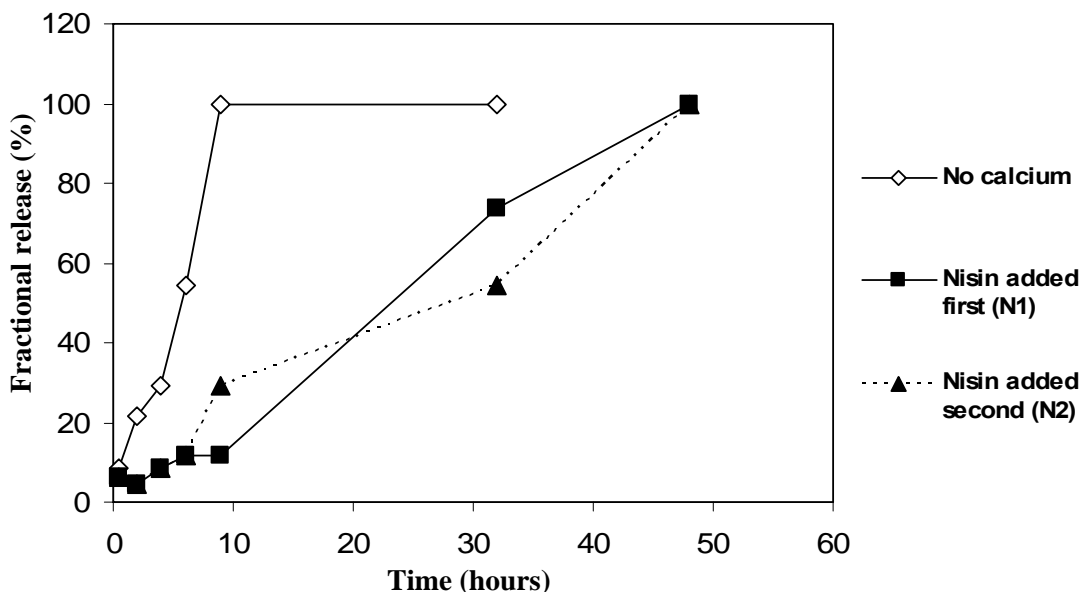


Figure 32: Effect of Sequence of Nisin Addition on Release of Nisin at $\text{pH} < \text{pKa}$ of Pectin

As seen in the images of inhibition zone assay (Figure 33), fractional release of nisin through films N1 and N2 followed the same trend indicating no significant impact of sequence of nisin addition on its release when pH of pectin slurry was below the pKa of pectin. At $\text{pH} < 3$, not much pectin-nisin interaction will occur, hence this result was expected. However, the fractional release curve shows more than 100% release, which is not possible. In order to find out a reason for higher release than expected, these same samples from above study were analyzed again for nisin concentration using HPLC. Surprisingly, no nisin could be detected in any of these samples. This could mean that derivatives of nisin may have formed, which were not detectable on HPLC, but had slightly better antimicrobial activity than nisin itself. It has been reported in literature that

nisin¹⁻³² is formed in mildly acidic conditions [58] and this has slightly better antimicrobial activity.

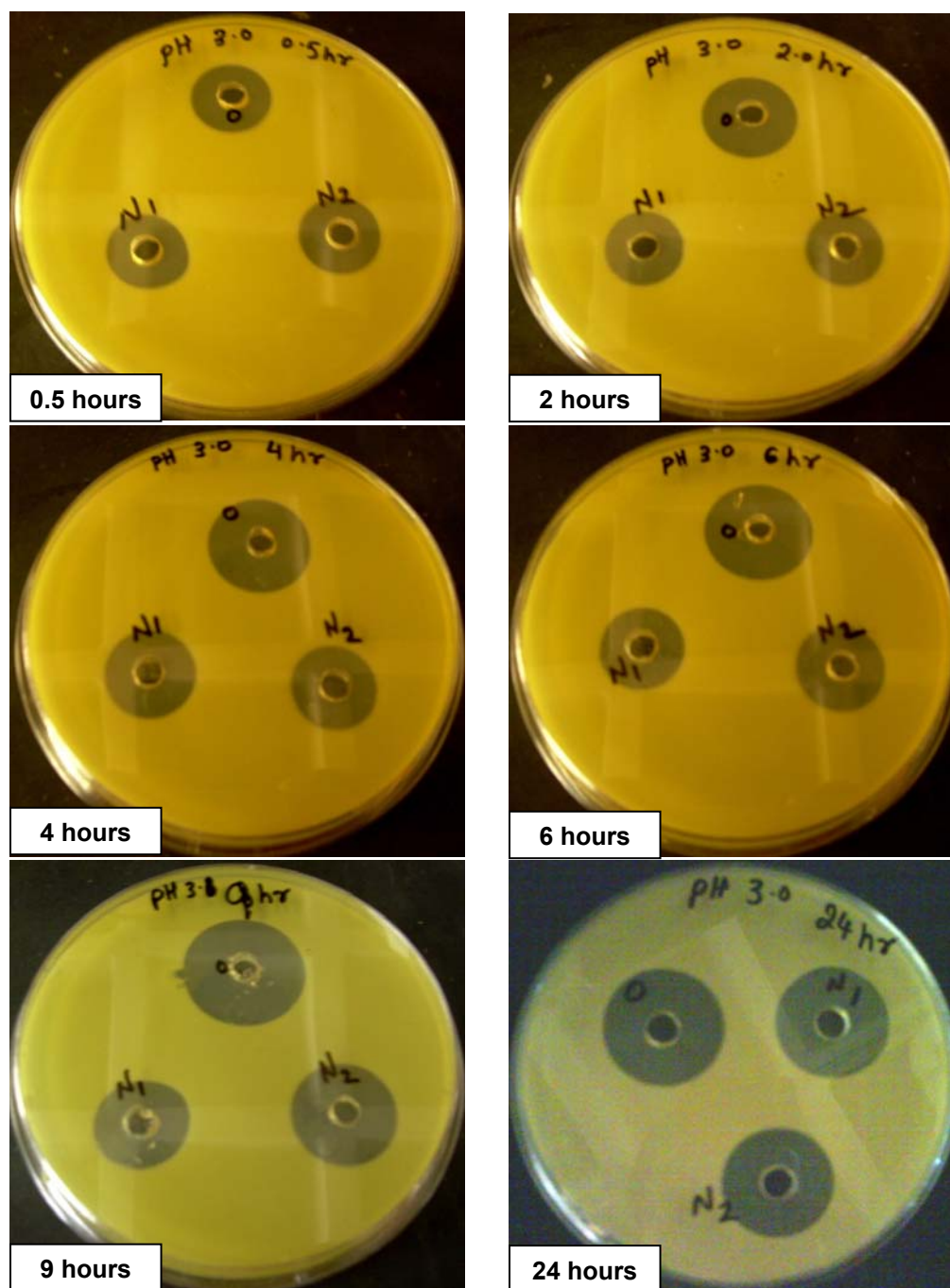


Figure 33: Effect of Sequence of Nisin Addition on Release of Nisin at pH < pKa of Pectin (Inhibition Zone Assay)

The results for $\text{pH} > \text{pKa}$ are shown in Figure 34 and Figure 35.

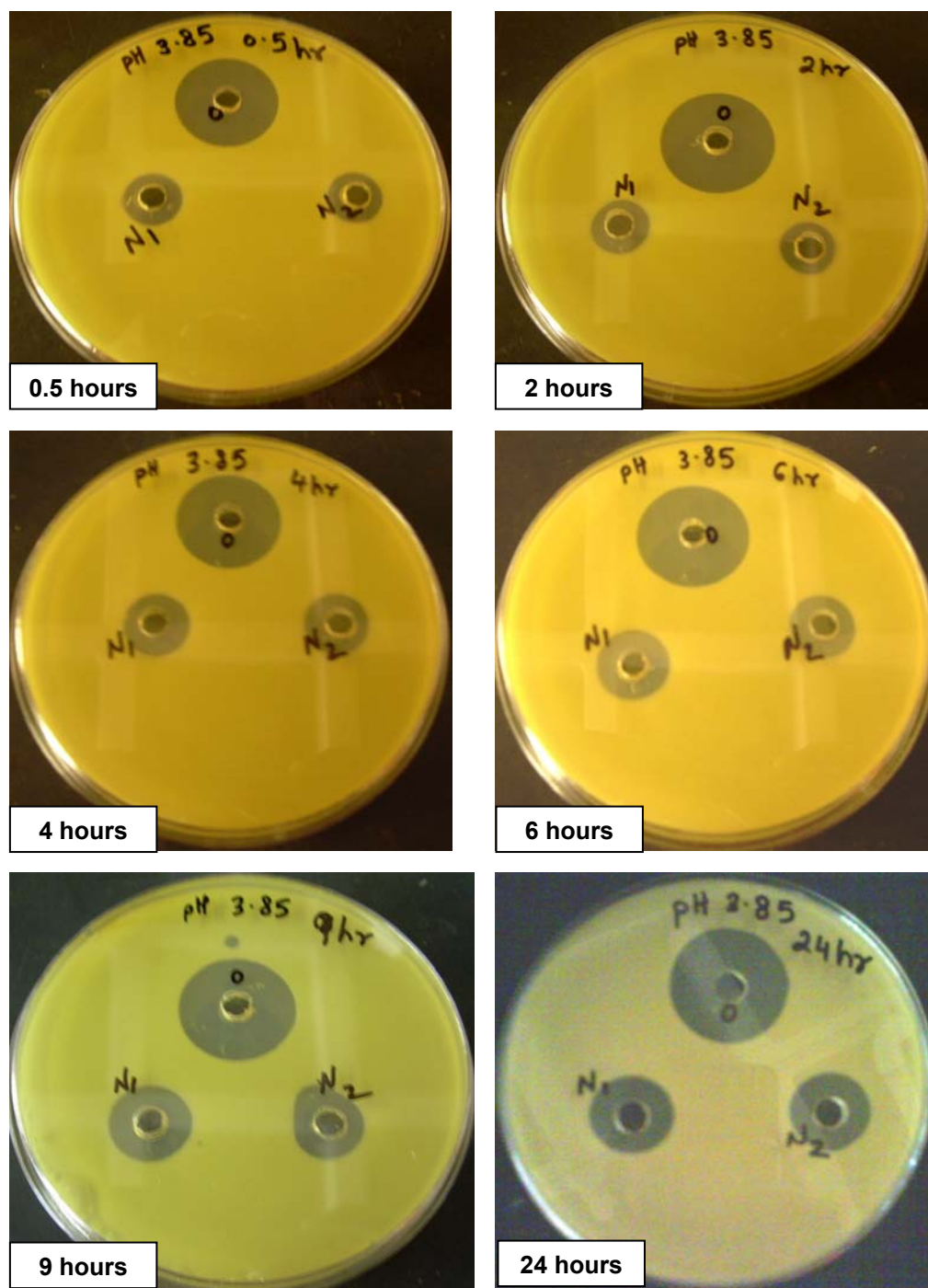


Figure 34: Effect of Sequence of Nisin Addition on Release of Nisin at $\text{pH} > \text{pKa}$ of Pectin (Inhibition Zone Assay)

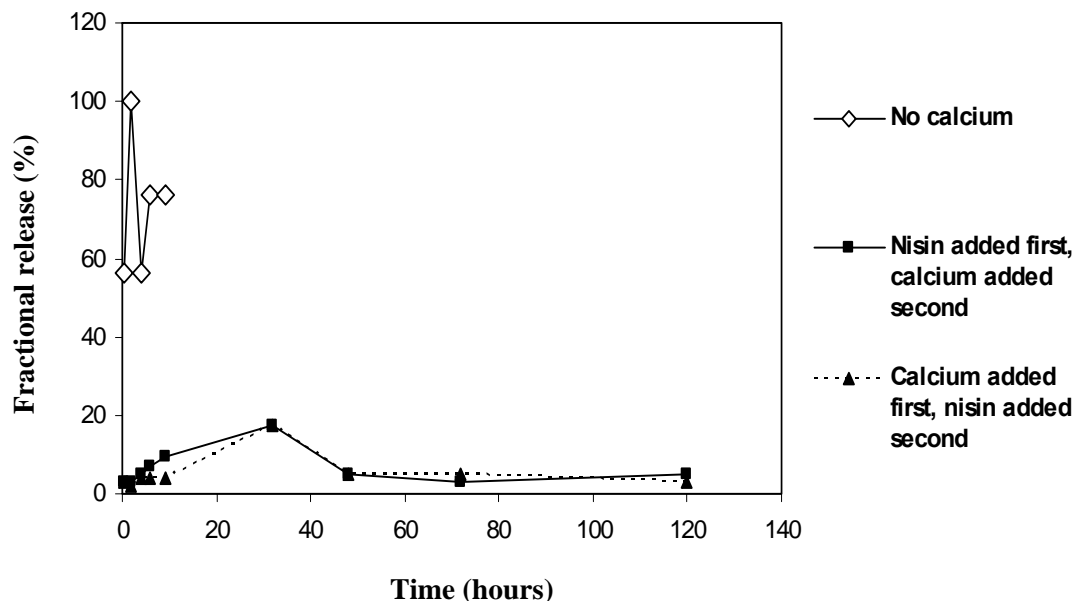


Figure 35: Effect of Sequence of Nisin Addition on Release of Nisin at pH > pKa of Pectin

In this case, pH was around 4 at which calcium crosslinking occurs. At this pH, films N1 and N2 did not dissolve for a week, whereas films N1 and N2 made at pH < pKa dissolved completely in two days further confirming that calcium crosslinking was more effective at this pH. Release of nisin was very low in films with 18 mg Ca/g pectin, irrespective of the sequence of addition of nisin, at pH of pectin slurry > pKa of pectin. The probable reasons for this low release could be:

- Nisin degraded in film forming step at given pH conditions, leaving only 20% of intact nisin, all of which was released in 24 hours from the two films N1 and N2;
- Nisin was released, but degraded over five days in the deionized water which was used as food simulant for the release study;
- More than 80% of nisin added to the films N1 and N2 was bound to the pectin, resulting in release of remaining 20% nisin;

- Calcium crosslinking was effective in controlling the release of nisin, resulting in a very slow release rate.

Between the control film and films N1 and N2, the processing conditions, pH, release study conditions were same, except for the presence of calcium crosslinking in films N1 and N2. So, the first 3 reasons should have been true for control film too. However, that was not observed and control film was able to release almost 80% of added nisin within a day. This means that calcium crosslinking was the main factor preventing all nisin from being released.

Thus, this experiment indicated the following:

- Sequence of nisin addition was not significant for controlled release.
- At $\text{pH} > \text{pKa}$ of pectin, calcium crosslinking was instrumental in preventing the release of nisin. However, it could not be concluded that at calcium concentration of 18 mg Ca/g pectin ‘controlled release’ was possible because the tight network of calcium crosslinks did not allow any further release of nisin. Release tests at lower levels of calcium concentration must be done to find out the extent of release of nisin.

3.4 Effect of Environmental Variables

In order to evaluate the effect of pH of pectin slurry and food simulant on the recovery of nisin, pectin films containing 18 mg Ca/g pectin were made as follows:

- pH of pectin slurry ($\text{pH}=2.7$) $<$ pKa of pectin: release study with deionized water at pH 2 and pH 5.

- pH of pectin slurry (pH=3.95) > pKa of pectin: release study with deionized water at pH 2 and pH 5.
- The film pieces (0.4 g) were powdered and completely dissolved into water at pH conditions mentioned and the solutions were analyzed for total nisin content using agar diffusion assay and HPLC. Results were obtained as shown in

Table 11: Comparison of nisin estimated by HPLC and Agar Diffusion Assay

| pH of Pectin Slurry | pH of Food Simulant | pH of Solution After Complete Dissolution of Film | % Recovery of Nisin as per HPLC | % Recovery of Nisin as per Agar Diffusion Assay |
|----------------------------|----------------------------|--|--|--|
| 2.7 | 2 | 1.95 | 83.6 ± 5.1 | 120.5 ± 25.8 |
| 2.7 | 5 | 2.8 | Not tested | 102.2 ± 0 |
| 3.95 | 2 | 2.07 | 76.9 | 107.8 ± 45.8 |
| 3.95 | 5 | 4.08 | 57.4 ± 3.6 | 61.5 ± 19.6 |

Preliminary results from HPLC and agar diffusion assays indicated that a combination of high pH of food simulant and high pH of pectin slurry was not favorable for nisin. 40% loss of nisin could have occurred at two stages – during film preparation step due to high pH of pectin slurry and after release to high pH water. This agreed with the previous result that showed 60-80% release of nisin from control film (without calcium) made with pH 3.85 pectin slurry (Figure 35). Time and high pH, in combination with calcium crosslinking, were probably responsible for low and delayed release of nisin from films N1 and N2 at pH 3.85.

4 CONCLUSIONS

The following were identified as key variables in the development of controlled release LM pectin films:

- **Degree of Calcium Crosslinking:** This composition variable was effective in controlling the release of an active compound, provided the size of active compound was above a certain threshold, and there was no binding of active compound to pectin molecules. Results obtained from proof of concept indicate that calcium concentration was the more dominant variable among the two design variables, DE of pectin and calcium concentration, responsible for different degrees of crosslinking. However, a systematic design of experiment will have to be conducted to further verify the above result.
- **pH of Pectin Slurry:** This process variable was as important as the degree of calcium crosslinking because it affected the calcium crosslinking ability of pectin, thereby affecting the release of active compound. Pectin slurry at a pH above the pKa of pectin was found to be effective. An important observation was that at this pH, electrostatic attraction between pectin and nisin was not strong enough to prevent the release of nisin from pectin films. Hence, nisin could be incorporated in controlled release pectin films.
- **Method of Calcium Crosslinking:** This was another important process variable for nisin-containing pectin films; the two-step method of crosslinking not being the preferred one as it allowed sufficient time for incorporated nisin to diffuse to the surface during film formation, resulting in faster release from film.

Other variables such as sequence of nisin addition, pH of food simulant did not affect the release properties of film to a great extent.

From the above results it can be concluded that for development of controlled release pectin films containing nisin pectin slurry should be maintained at a pH above the pKa of pectin and calcium crosslinking should be done by adding all calcium to the pectin slurry in a single step; the sequence of adding calcium and nisin not being important.

5 FUTURE WORK

- In this thesis, only one DE of pectin (DE 35) and one calcium concentration (18 mg Ca/g pectin) was used to study the effect of different variables on the release of nisin. A detailed study of release of nisin from films made using a range of calcium concentration and DE of pectin needs to be done to generate a database of film formulations with a range of release rates. The set of experiments for this study will be similar to the proof of concept study.
- Preliminary studies with nisin films placed on agar have shown that nisin is released when in contact with moist medium. Further work may be done to evaluate the performance of these films in a real food system. Nisin-containing pectin film may be used as an absorbent pad in the tray holding deli meat slices, the drip from the meat acting as a trigger for the films to swell and release nisin.
- Microscopic imaging could be another area of interest to find out the position of nisin inside the pectin matrix. Nisin can be made to fluoresce by tagging it with its antibody or by biotinylation and then imaged using Confocal Laser microscopy (personal communication with Dr. Peter Cooke of USDA, Wyndmoor, Pennsylvania).

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