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CONTROLLED RELEASE OF NISIN FROM A BIOPOLYMER BASED FILM FOR FOOD PACKAGING APPLICATIONS

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ABSTRACT

Nisin is a bacteriocin that is permitted to be used in food as a natural food preservative and is approved 'generally recognized as safe' (GRAS) by both Food & Drug Administration (FDA) and World Health Organization (WHO). Nisin is commercially available as Nisaplin®. Pure nisin has an activity of 40 x 10^6 IU/g and Nisaplin® has an activity of 1 x 10^6 IU/g. In some food systems, especially meat, it has been observed that nisin loses its activity over time because it binds to protein, fat etc. Several researchers have reported that the direct addition of antimicrobials to food leads to a loss of activity. However, this loss can be prevented to a great extent by controlled release of antimicrobials to food. This work was undertaken to develop a biopolymer-based film for the controlled release of nisin into food systems for inhibiting pathogenic organisms.

The first phase of the research identified potential biopolymers for the film matrix and evaluated their stability in an aqueous system. In the second phase, nisin was entrapped within seven biopolymer-based films and the antimicrobial activity of the films was evaluated by the agar diffusion method using *Micrococcus luteus*. The third phase of the research consisted of modeling the nisin release kinetics via a mechanistic diffusion model, as well as an empirical Weibull model. These models were validated by experiments in the final phase. In the final experiments with corn zein, nisin release was quantified using a high performance liquid chromatographic (HPLC) technique.

Using the agar diffusion method, it was found that xanthan films and locust bean films collapsed or dissolved on the agar surface without producing a distinguishable

inhibition zone. Kappa and iota carrageenan films did not collapse on the agar surface, but failed to inhibit *M. luteus*. It was hypothesized that nisin was not released from kappa and iota carrageenan due to the physical entrapment of nisin in the gel network. Blended films made with kappa and non-gelling lamda carrageenan exhibited inhibition against *M. luteus*. The amount of nisin released in the agar diffusion method increased as the concentration of lamda carrageenan in the film increased. Blended films made with kappa carrageenan and hydroxypropylmethyl cellulose (HPMC) also exhibited nisin release. Nisin release increased as the concentration of HPMC in the film increased.

Corn zein films containing Nisaplin® formed clear inhibition zones using the agar diffusion method. The nisin released from these films into citrate buffer was further quantified using a HPLC technique. The profile of released nisin demonstrated that as the corn zein concentration in the film increased, nisin release decreased. The diffusivity of nisin decreased from 38×10^{-11} to 8×10^{-11} cm^2/s (a fourfold decrease), as the corn zein concentration in the film increased from 4 to 10% (w/v). This observation may be attributed to increased tortuosity in the film. Other researchers also have reported that the release of active compounds from a matrix decreases as the biopolymer concentration increases.

Mathematical analysis of the early portion of the nisin release profile indicated that nisin release from corn zein films exhibits a pseudo-Fickian behavior. An empirical Weibull model was developed and tested. It demonstrated excellent prediction ($R^2 > 0.95$), power, and described the kinetics of nisin release well.

Corn zein films can be used to develop a matrix for controlled release of nisin in food system, thereby maintaining a constant microbial inhibitory effect. Also, release can

be controlled by varying the concentration of biopolymer in the matrix. Corn zein films can be used as coating within a package or as an edible coating for the product for controlled release of antimicrobials and other active compounds. Since corn zein is hydrophobic, it can also be successfully used for delivery of active compounds within aqueous food systems such as beverages, meat and other high moisture foods.

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Chapter 1

Introduction

The increase in food borne illness outbreaks and product recalls are major concerns that must be addressed by the food processing industry. Mead *et al*. (1999) reported that every year, food borne illnesses affect more than 76 million people, causing over 3,25000 hospitalizations and 5000 deaths in the U.S. alone. According to the Centre for Disease Control and Prevention (CDC) statistics on food borne outbreaks in 2006, there were a total of 1247 outbreaks, involving approximately 25,659 reported cases (Anon., 2006).

The addition of antimicrobials (active compounds that kill or prevent the growth of microorganisms) to foods during processing is an effective technique that is frequently employed to eliminate food borne pathogens. A bacteriocin is a proteinaceous substance that is produced by bacteria and it kills or inhibits the growth of other bacteria (Cleveland *et al*., 2001). The bacteriocin, nisin, was approved in Europe as a natural food preservative in 1969 (E 234) and since then, has found widespread application in foods from over 50 countries around the world. Nisin is used in a variety of foods, including dairy-based products, desserts, eggs, meat, fish, beer, beverages and cereal-based products (Schillinger *et al.*, 1996). Nisin is the only bacteriocin that is permitted to be used in food as a natural food preservative (Davies *et al.,* 1998 and Guerra *et al.*, 2005) and is approved as 'generally recognized as safe' (GRAS) by both Food & Drug Administration (FDA) and World Health Organization (WHO). Nisin is commercially

available as Nisaplin®. Pure nisin has an activity of 40 x 10^6 IU/mg and Nisaplin®, which contains 2.5 % nisin, has an activity of 10^6 IU/g (Zapico *et al.* 1999).

Nisin, with a molecular weight of 3500 Daltons, belongs to a group of bacteriocins called lantibiotics (Van de ven *et al.,* 1991). Lantibiotics are small polypeptides with molecular weights of 4000 Daltons or less (van Kraaij *et al*., 1999). Nisin ($C_{143}H_{230}N_{42}O_{37}S_7$) is a polypeptide with 34-amino-acids and is produced by *Lactococcus lactis* subsp. *lactis* during fermentation (Sebti *et al.,* 2004). Nisin attacks the target microorganism through pore formation in the plasma membrane. Since nisin is cationic, it binds to membranes containing a high concentration of anionic lipids. The anionic lipid content in Gram- positive bacteria is much higher than in Gram-negative bacteria (O'Leary *et al.,* 1988) and may be a key factor responsible for the higher activity of nisin against Gram-positive bacteria (Breukink *et al.,* 1997). Nisin shows lethal activity against food borne pathogens (*Listeria monocytogenes*, *Clostridium botulinum*) and spoilage organisms (*Lactobacillus helveticus, Brochothrix thermosphacta*) but has no effect on Gram-negative or fungi (Cha & Chinnan, 2003). Activity of nisin can be extended to Gram-negative bacteria by use of chelators to induce damage to the outer membrane (Cutter & Siragusa, 1995).

However, it has been observed that in some food systems, especially meat, nisin loses its activity over time. Published reports demonstrate that in a meat system, microorganisms are less sensitive to nisin as compared to *in vitro* studies. Davies *et al.* (1999) reported that the activity of nisin on meat surface depends on the meat system and the type of microorganisms present. Various factors such as storage temperature, storage time, pH, endogenous proteases, fat and protein in the food may be responsible for this

loss of activity. Loss of activity of nisin during storage or its degradation when in contact with food systems has not been well documented (Sanjurjo *et al.*, 2006).

Direct incorporation of antimicrobials to food may result in immediate destruction of the target microorganism, but may not be sufficient to prevent the recovery and growth of injured organisms (Hoffman *et al.* 2001). Cross-reaction with food components such as lipids and proteins also can cause antimicrobial activity loss (Han & Floros, 1997; Davies *et al.*, 1999). In many cases, microbial spoilage occurs primarily on the food surface. However, the inhibitory effect of antimicrobials introduced onto food surfaces may be limited, due to its diffusion into the bulk of the food (Vojdani and Torres, 1990; Torres *et al*., 1985).

The inhibitory activity can be extended by encapsulating the antimicrobial into packaging material to control the release rate of the antimicrobial into the food. Controlled release may be defined as a technique by which an active compound is made available to a target at a specific rate (Fan & Singh, 1989). Controlled release of an antimicrobial from a packaging material may ensure a constant supply that maintains a minimum inhibitory concentration (MIC) on the surface of the food.

This research was undertaken in an attempt to develop a biopolymer-based matrix for controlled release of nisin for food packaging applicable to an aqueous system. This would include beverages, meat and other high moisture foods. Controlled release would ensure that a minimum required amount of nisin concentration is maintained in the food. A set of preliminary experiments were conducted for the purpose of screening biopolymers. The main criterion was to select a matrix that would release nisin in a

controlled manner, would not completely bind nisin, nor would it collapse in the aqueous system, causing a burst effect (sudden release).

Several biopolymers, such as locust bean gum, xanthan gum, carrageenans (kappa, iota and lamda), hydroxypropylmethyl cellulose (HPMC) and corn zein were evaluated for their suitability to encapsulate and release nisin in a controlled manner in an aqueous system. Ripoche *et al.* (2006) evaluated nisin diffusion from a solution into a polysaccharide gel and found that as the gel concentration increased, the diffusion of nisin into it decreased. In the present study, after extensive screening experiments, we hypothesized that controlled release of nisin from a corn zein film into an aqueous system can be achieved by changing the concentration of corn zein in the film.

The overall goal of this research was to develop a biopolymer-based matrix for controlled release of nisin into a food system for inhibiting pathogenic organisms.

Chapter 2

Goals, Hypotheses and Objectives

Nisin is the **only** bacteriocin that is permitted to be used in food as a **natural** food preservative (Davies *et al.,* 1998 and Guerra *et al.,* 2005). However, in some foods such as meat, homogenized milk etc., nisin loses its activity over time. Various factors such as storage temperature, storage time, pH and binding of nisin to fat, protein and endogenous proteases (eg. Glutathione in meat) are reported to be responsible for this loss of activity. Another challenge is that direct incorporation of antimicrobial in foods causes it to diffuse into the bulk of the food product. As such there is a much lower concentration of antimicrobial on the food surface. Therefore, it is necessary to ensure that nisin is released into the food in a controlled manner, so that a minimum inhibitory concentration is always maintained on the food surface to inhibit pathogens.

2.1 Research questions

The overall goal of this project was to develop a biopolymer based matrix for the controlled release of nisin in an aqueous system. The following research questions were addressed.

- Is it possible to control the release of nisin from a blended film consisting of two biopolymers with different solubilities in water?

- Is it possible to control the release of nisin by controlling the dissolution of the film matrix?
- Is it possible to control the release of nisin from a film by varying the concentration of each biopolymer in the film?

2.2 Hypotheses & objectives

2.2.1 Hypothesis I

The nisin release of a blended film of two biopolymers can be controlled by varying the concentration of one of the polymers.

2.2.1.1 Objectives

1) To study the antimicrobial activity of films containing different proportions of kappa-carrageenan and hydroxypropylmethyl cellulose (HPMC) by agar diffusion method
2) To study the release kinetics of nisin from the above films into an aqueous system using HPLC technique

2.2.2 Hypothesis II

Increasing the concentration of corn zein in a film increases tortuosity of the film, thus reducing the diffusion of nisin through the film.

2.2.2.1 Objectives

3) To study the antimicrobial activity of corn zein film by agar diffusion method
4) To determine the effect of corn zein concentration (4, 6, 8 and 10% w/v) on the release kinetics of nisin into an aqueous system
5) To model the release of nisin through films of varying corn zein concentration

Chapter 3

Controlled Release of Nisin in Food: a Review

This chapter gives an overview of research conducted in the area of antimicrobial food packaging, emphasizing the necessity and techniques for controlled release of antimicrobials. The structure, stability and potential applications of the bacteriocin nisin are discussed from the perspective of food packaging. The loss of nisin activity in food systems is sought to be explained, which leads to our justification for undertaking the present research work on the controlled release of nisin from a biopolymer-based film. The chapter is divided into the following main sections:

- Nisin
- Quantification of nisin
- Loss of nisin activity
- Controlled release
- Matrices containing nisin
- Corn zein films containing nisin
- Factors affecting diffusion
- Mathematical modeling

3.1 Nisin

Bacteriocins are antibacterial proteins or peptides that inhibit the growth of other selected bacteria. Nisin is the only bacteriocin that is permitted to be used in food as a natural food preservative (Davies *et al.*, 1998 and Guerra *et al.*, 2005) and is GRAS by

both FDA and WHO. Nisin is commercially available as Nisaplin®. Pure nisin has an activity of 40 x 10^6 IU/g and Nisaplin® has an activity of 1 x 10^6 IU/g. The bacteriocin, nisin, was approved as a natural food preservative in 1969 (E 234) and since then, has found wide application in over 50 countries around the world.

3.1.1 Nisin structure

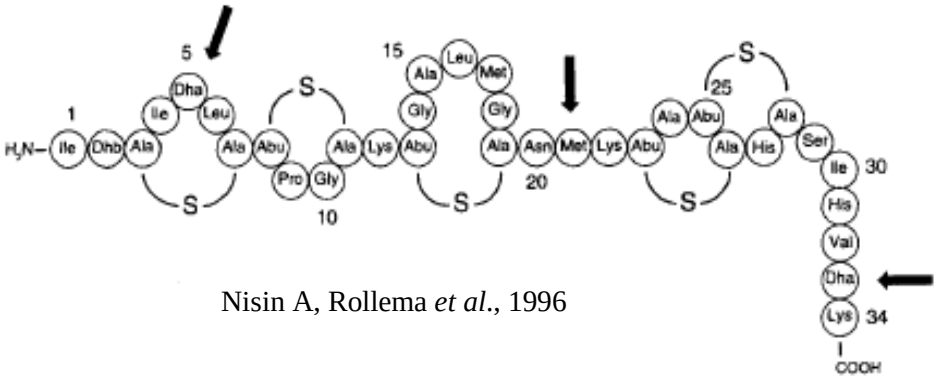
Nisin, a polypeptide with 34-amino-acids, is produced by *Lactococcus lactis* subsp. l*actis* during fermentation (Sebti *et al.*, 2004). Nisin (3500 Daltons) belongs to a group of bacteriocins called lantibiotics (small polypeptides < 5000 Daltons) and contains the uncommon amino acids lanthionine, β-methyl-lanthionine, dehydroalanine (Dha) and dehydrobutyrine (Dhb). Rollema *et al*. (1996) reported that unsaturated amino acids dehydroalanine (Dha) and dehydrobutyrine (Dhb) are formed from serine and threonine residues by a post-translational modification process. Additional reactions between cysteine residues and some of the unsaturated amino acids result in the formation of lanthionine and β-methyl-lanthionine residues. The thio-ether bridges of the lanthionines form intramolecular cross-links, resulting in five cyclic ring structures in the molecule. The presence of the lanthionine rings in nisin contributes to its high degree of hydrophobicity, rigid structure and thermal resistance.

Nisin ($C_{143}H_{230}N_{42}O_{37}S_7$) does not have any negative functional groups and has a net positive charge of +4, due to three lysine groups and one histidine group. Nisin has a dimension of 20 x 50 A°. The iso-electric point of nisin is slightly above 10.5 (Pfeiffer

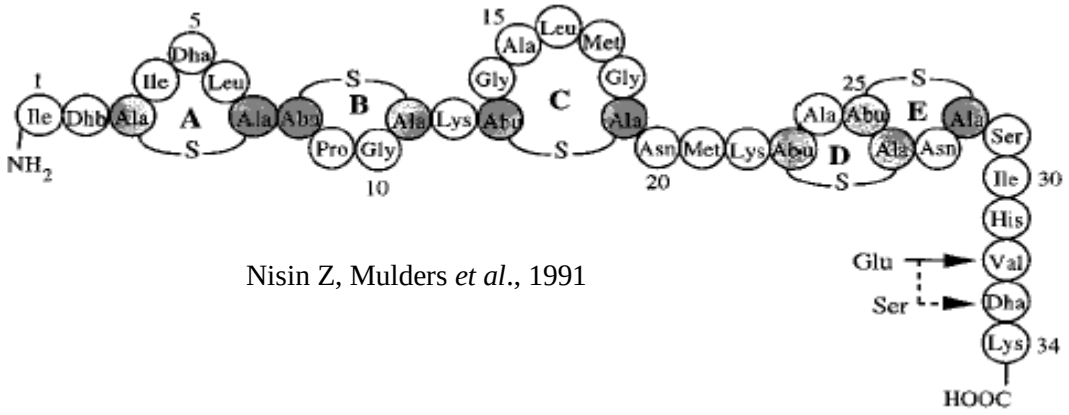
and Orben, 1997; Bani-Jaber *et al.,* 2000). Nisin also possesses amphipathic properties on account of its hydrophobic N terminal and hydrophilic C terminal.

The three known variants of nisin are nisin A, nisin Z and nisin Q (Fig. **3-1**). The structure of nisin A comprises of 34 amino acids, with 5 intramolecular sulfide rings. Nisin A and nisin Z have similar antimicrobial activities (Mulders *et* al., 1991). The only difference between them is that nisin A has a histidine at position 27 whereas nisin Z has an asparagine. Nisin Q, which was recently discovered, differs from nisin A in four amino acids and detailed characterization and structural analysis of nisin Q are yet to be studied (Zendo *et al.* 2003).

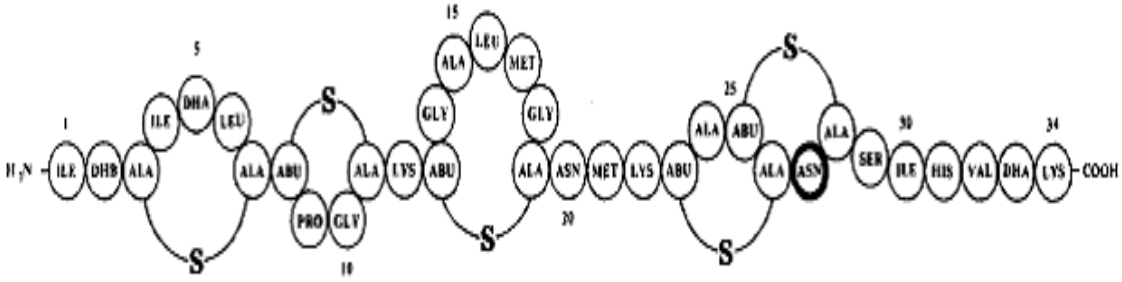
The thioether bonds give nisin two rigid ring systems, as shown in Fig. **3-2** (Rollema *et al.,* 1996). The ring systems, one at the N-terminal and the other at the C-terminal, are separated by a hinge region (amino acids 20-22) wherein considerable flexibility is observed. The ring systems give nisin a screw-like structure, possessing amphipathic properties. The amphipathic property of nisin is characterized by the N-terminal part being more hydrophobic than the C-terminal part and also the fact that the hydrophobic moieties are positioned opposite to the hydrophilic moieties throughout the length of the nisin molecule (Breukink and Kruijff, 1999). The hydrophilic C-terminal part contains positively charged side chains of lysine and histidine residues. This amphipathic character is imperative for its biological function (van de Ven *et al.*, 1991; van den Hooven *et al.*, 1996 a, b).



Nisin A, Rollema *et al*., 1996



Nisin Z, Mulders *et al*., 1991



Nisin Q, Zendo *et al*., 2003

Fig. **3-1** Structure of three natural nisin variants

3.1.2 Nisin stability and solubility

Nisin can be stored for up to two years ≤ 25°C, in a dry and dark environment (Delves-Broughton, 2005). Davies *et al.* (1998) studied the activity of nisin at two autoclave temperatures: 115°C for 20 min and 121°C for 15 min. They found that at pH 3, nisin demonstrated 5% and 15% activity loss, when autoclaved at 115 and 121°C, respectively. However, nisin solutions of pH 1 and pH 5, when autoclaved at 121°C, had an activity loss of more than 90% and 50%, respectively. This decrease does not prohibit its use in thermally processed, high pH foods, since the food can protect the nisin molecule (Heinemann *et al.*, 1965).

Nisin demonstrates increased solubility at low pH, but the solubility decreases as the pH is increased. Nevertheless, solubility is not an issue in foods, where low levels of nisin can be incorporated. The retention of nisin activity in a food system depends on the storage temperature, pH and period of storage (Delves-Broughton, 2005). Since the retention of nisin activity is better at low storage temperatures, a higher level of nisin needs to be incorporated if the food product is intended to be stored at a high temperature. Proteolytic enzymes in cold processed foods and common food additives, adsorption of nisin on titanium dioxide and sodium metabisulphite, also can cause loss of nisin activity in foods (Delves-Broughton, 2005).

During the process of nisin purification, the highest recovery of nisin was observed at a pH of 3-4. This was attributed to the fact that the net charge of nisin is more positive at a lower pH (Cheigh *et al.*, 2004; Hurst, 1981). As mentioned earlier, nisin does not have any negative functional groups and has a net positive charge of +4, on

account of three lysine groups and one histidine group. As the pH increases, histidine (pKa = 6.0) becomes protonated, thus losing its charge. However, lysine, by virtue of its high pKa (10.5), continues to be positive.

3.1.3 Mode of action of nisin

Nisin inhibits target microorganisms by pore formation in the plasma membrane. The action of nisin can be categorized into three steps: binding of nisin into target cell membrane followed by insertion into the lipid phase, which subsequently leads to pore formation (Breukink and Kruijff, 1999). Pore formation leads to cell death, due to the rapid efflux of essential cytoplasmic substances, such as amino acids, potassium ions and nucleotides, from the cytoplasm of a number of Gram-positive bacteria (Gao *et al*., 1991). Breukink *et al.* (1997) varied the anionic lipid content in membranes and found that nisin binding remarkably increased as lipid content increased above 40%. The anionic lipid content in Gram-positive bacteria is much higher than that in Gram-negative bacteria (O'Leary *et al.,* 1988) and may be responsible for the higher activity of nisin against Gram- positive bacteria (Breukink *et al.,* 1997).

Studies conducted by Breukink *et al*. (1997) and Kraaji *et al*. (1999) indicate that hydrophobic interactions are responsible for the insertion of the N-terminal part of the nisin into the cell membrane. Subsequent pore formation is due to the aggregation of nisin in the membrane. Though literature pertaining to pore formation of nisin is scanty, black-lipid membrane experiments indicate that the pore size is 1 nm (Benz *et al.,* 1991).

Nisin is reported to be less active against de-energized or starved cells (Ruhr & Sahl, 1985). In another study, Sahl *et al.* (1987) found that the activity of nisin against *Staphylococcus simulans* depended on the membrane potential. Hence, it was concluded that nisin needs an energized membrane for its activity and a threshold potential contributed by the anionic lipids was imperative for any nisin activity to be detected. This report was also confirmed by Kordel & Sahl (1986).

Development of bacterial resistance to antibiotics is a global problem (Kramer *et al*., 2004). An alternative is to use nisin which displays a broad spectrum of activity against Gram-positive bacteria (van Kraaji *et al*., 1999). Kramer *et al*. (2004) comment that no reports of resistance towards nisin has been reported, in spite of its prolonged use, as a preservative in the food industry and this could be because of the double mode of action of nisin. By binding to Lipid II, it inhibits cell wall synthesis and also forms pores in the cytoplasmic membrane. Kramer *et al*. (2004) further report that there is no role of Lipid II in nisin resistance. At the same time changes in the cell wall, deter nisin from gaining access to Lipid II, subsequently acquiring nisin resistance.

3.1.4 Applications of nisin in foods

Nisaplin® is the commercial grade, which contains 2.5% active nisin suspended in sodium chloride. Currently, 25g of Nisaplin® costs US$ 770.00 (Jozala *et al*., 2008). The nisin activity is expressed in International Units (IU).

1g pure nisin = 40×10^6 IU (1 μg pure nisin = 40 IU)

1g Nisaplin® = 10^6 IU (1 μg Nisaplin® = 1 IU)

Nisaplin® is widely used in food processing applications. It is effective over a range of pH (3.5-8.0) and is used in a variety of foods including processed cheese, sour cream; dairy and fat based desserts, yogurt, milk based beverages; fruit and vegetable based pulp and pasteurized juices; pasteurized liquid egg products, mayonnaise and salad dressings; pasteurized soups and sauces; canned vegetables; processed meat; beer etc (Nisaplin® Product description, PD 214210-1.OEN, Danisco; Schillinger *et al*., 1996; Delves-Broughton, 2005). The exact dosage of Nisaplin® added depends on the nature of the food material, but 25-500 mg/kg or liter of food is recommended. Nisaplin® is thoroughly dispersed in heat processed food, prior to heating. It can be added as a dry powder or as a suspension in water or milk (Nisaplin® Product description, PD 214210-1.OEN, Danisco). Nisin levels permitted to be used differ from country to country (Anon., 2002; Delves-Broughton, 2005).

3.2 Quantification of nisin

3.2.1 Agar diffusion method

This method involves the inoculation of the agar with an indicator organism sensitive to nisin. Wells of diameter approximately 7 mm were made on the agar plates with a cork borer. The nisin samples were dispensed into these wells and inhibition zones formed after 24 h were measured with the help of a vernier caliper.

3.2.1.1 Agar diffusion method: advantages

Agar diffusion method is a qualitative approach to test the activity of nisin. The method is not only cost effective and simple but it is also very sensitive, detecting nisin concentrations as low as 0.5 IU/ml or 0.0125 IU/μg. This procedure facilitates the detection of low levels of nisin activity, such as may be encountered in food applications.

3.2.1.2 Agar diffusion method: disadvantages

This technique is not recommended for quantitative analysis as it does not distinguish nisin from other interfering substances present in the food, thus causing false positive results (Tramer & Fowler, 1964). The agar diffusion method is time consuming, taking a minimum of two days and the inhibition zones produced are in logarithmic relation to the nisin quantity (Pfeiffer & Orben, 1997). Ripoche *et al.* (2006) reported that the inhibition zones in agar diffusion method were not proportional to the quantification technique they adopted. This observation may have been due to the lower sensitivity and poor reproducibility of this technique which depends on bacterial state, temperature, agar medium etc. This method would need to be refined before it could be employed for quantification.

3.2.2 Colorimetric method

Ripoche *et al.* (2006) used a colorimetric method bicinchoninic acid protein assay (BCA), as described by Wiechelman *et al.* (1988). Equal volumes of the sample and

QBCA reagent (Sigma) were mixed and heated at 60°C for 1 hour and the absorbance measured at 560 nm using a spectrophotometer.

3.2.3 HPLC technique

HPLC is an excellent quantitative approach to study the release kinetics of nisin from a matrix over a period of time. Rollema *et al.* (1995), Van Kraaij *et al.* (1997) and Buonocore *et al.* (2003 & 2004) adopted an HPLC protocol developed by Liu and Hansen (1990), with minimum modification, for the quantification of pure nisin. A reverse phase C-18 column of size 250 x 4 mm was used. The solvents used were water and acetonitrile (0.1% TFA), with a flow rate of 1 ml/min. Nisin was detected at a wavelength of 254 & 225 nm. The run time was 20 min, with nisin eluting after 10 min.

3.3 Loss of nisin activity

The loss of activity of nisin during storage or its degradation when in contact with food systems have not been well documented (Sanjurjo *et al.,* 2006). However, published reports indicate that in a meat system, microorganisms are less sensitive to nisin as compared to *in vitro* studies. Cutter & Miller (2004) developed antimicrobial films by soaking collagen films in Nisaplin® solution, followed by drying. Agar diffusion methods demonstrated that the collagen films retained nisin activity. Frankfurters, used as a model meat system, were wrapped in these films and subjected to temperature abuse, followed

by 14 days of refrigerated storage. It was found that both *Listeria monocytogenes* and *Brochothrix thermosphacta* were reduced to approximately 1 log CFU/g.

Davies *et al.* (1999) reported that the activity of nisin on meat surface depends on the meat systems and the type of microorganisms present. Scott & Taylor (1981) reported that *Clostridium botulinum* spore growth was prevented by 50 μg/ml nisin in a trypticase-peptone-yeast-glucose medium. However, nisin did not prove effective in a meat system, even at a concentration of 125 μg/ml. Salt, fat, pH, curing agents and food particle size are some of the factors that affect the activity of nisin in meat (Jung *et al.*, 1992). Ming *et al.* (1997) incorporated nisin in meat casings and found it to be ineffective against *Listeria monocytogenes* on the surfaces of beef and turkey. However, Guerra *et al*. (2005) developed a cellophane packaging material incorporating Nisaplin®, which was used to wrap fresh veal meat. The total plate count readings after 12 days of storage at 4°C was found to be approximately 1.5 log units lower than the initial microbial population.

Wiezerbicka *et al*. (1989) determined the amounts of sulfhydryl-containing enzymes, glutathione (GSH) in foods consumed by humans and found that concentration of GSH in beef chicken and pork was generally high (156 to 627 mmol/g wet weight) compared to that in plant and dairy based products. Rose *et al*. (1999) studied the inactivation of nisin in fresh meat. They found that nisin is bound to GSH and subsequently inactivated.

Zapico *et al*. (1998) studied the effect of homogenization of milk on nisin activity and on its bactericidal activity against *Listeria innocua*. They found that the bactericidal activity of nisin was considerably reduced by homogenization. The loss in activity was thought to be due to the adsorption of nisin on the fat-water interface, thus making it

unavailable for interaction with bacterial cells. In a similar study, Jung *et al*. (1992) observed that as the fat concentration was increased, nisin activity decreased.

Though nisin is stable at autoclaving temperatures, nisin films processed at higher temperatures showed activity loss. Hoffman *et al.* (1998) reported the loss of nisin activity in polyethylene films processed at 149°C, whereas no loss was observed in heat pressed corn zein films formed at 100°C.

3.3.1 Loss of nisin activity: mechanism

Rollema *et al.* (1996) reported that the Cα–Cβ double bonds in the nisin molecule are prone to chemical modification on account of its high reactivity, thus limiting the chemical stability of the compound (Fig. **3-2**).

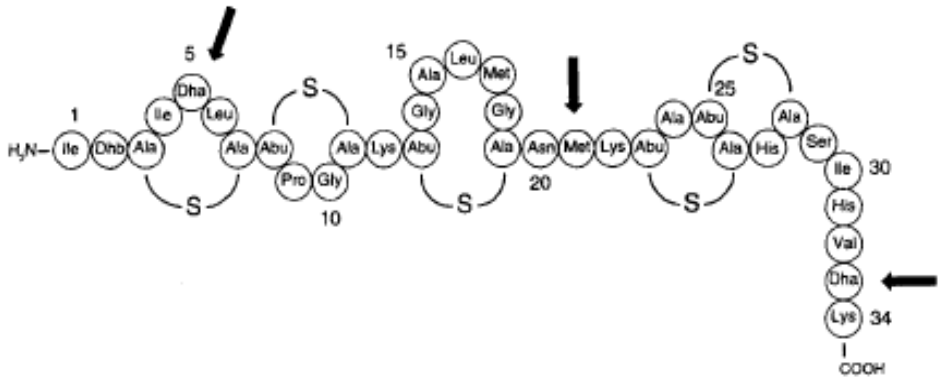


Fig. **3-2**: Primary structure of nisin A. Ala-S-Ala represents lanthionine, Abu-S-Ala β methyl-lanthionine, Dha dehydroalanine, and Dhb dehydrobutyrine. Arrows indicate the sites of modification. (Rollema *et al*., 1996)

Dehydroalanine (Dha) residues at position 5 (N-terminal) and position 33 (C-terminal) are susceptible to acid-catalyzed addition of water molecules to the double bond (Gross, 1967; Rollema *et al.* 1996). This bonding leads to the formation of 2-hydroxyalanine residue, which then splits into an N-terminal peptide amide and a C-terminal pyruvyl peptide, as shown in Fig. **3-3**. It was found that the removal of the C-terminal residues did not affect the biological activity. However, the modification of Dha5 at the N-terminal, which involved breaking open the first ring, significantly reduced the biological activity as compared to native nisin (Chan *et al.,* 1996; Rollema *et al.,* 1996). Thus, dehydroalanine (Dha) at position 5, or an intact first ring at the N-terminus is crucial for maintaining the functional properties of nisin. Chan *et al*. (1996) also report that activity is lost due to cleavage of Dha5 to yield [des-Dha5]-nisin. Prolonged storage of the nisin-incorporated food or heating in acid leads to the formation of [des-Dha5]-nisin which eventually causes loss of nisin activity (Chan *et al*., 1996).

Rollema *et al.* (1996) further reported that even the chemically modified nisin derivative contains an intact dehydrobutyrine (Dhb2) residue, which indicates that Dhb is more stable, as compared to Dha.

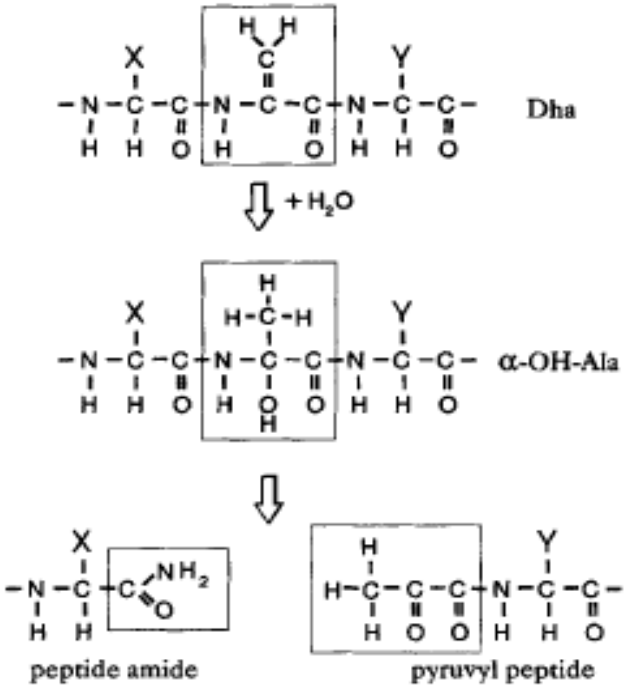


Fig. **3-3**: Acid-catalyzed hydrolysis of Dha causing cleavage of the polypeptide (Rollema *et al*., 1996)

3.4 Controlled release

Controlled release is a technique whereby an active compound is made available to a target, at a predetermined rate and duration, in order to produce a desired effect (Fan & Singh, 1989). Two main mechanisms involved in controlled release are dissolution of the matrix and diffusion ino the matrix (Fig. **3-4**). In the first case, solvent diffuses into the matrix causing the matrix to swell and dissolve thus releasing the active compound. In the second case, the solvent diffuses into the matrix and the soluble active compound diffuses out, wheras the matrix in itself is does not collapse.

According to Hoffman *et al.* (2001), incorporation of antimicrobials in the food package could reduce the post-process growth of food pathogens. Direct administration of antimicrobials in the food should result in the immediate destruction of the target microorganism, but would not prevent the recovery and growth of injured organisms. Microbial spoilage occurs primarily on the food surface. However, the inhibitory effect of the antimicrobials applied on the food surface is limited, on account of its diffusion into the bulk of the food system (Vojdani & Torres, 1990; Torres *et al*., 1985). The direct integration of antimicrobials in the food can also cause activity loss, due to its cross-reaction with food components such as lipids and proteins (Han & Floros, 1997; Davies *et al.,* 1999).

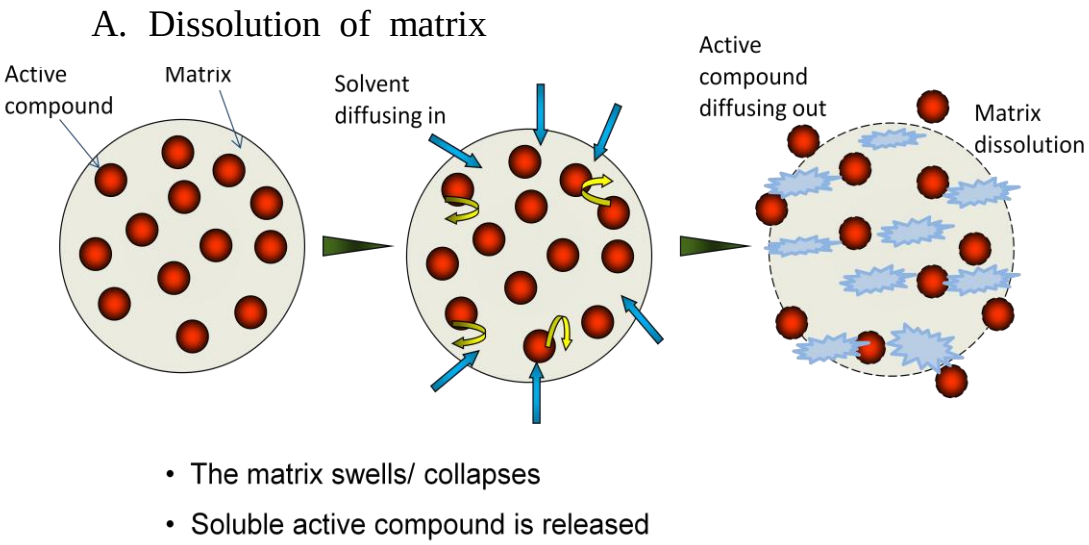
Antimicrobials incorporated in the film would serve to inhibit the microorganisms at the food surface. Thus, a minimum inhibitory concentration (MIC) is always maintained on the food surface and the antimicrobial retained in the film would prevent further contamination of food. Thus, the objective of antimicrobial food packaging is to transfer the active compound to the food, in order to maintain a predetermined concentration throughout the intended shelf life of the food (Buonocore *et al.,* 2003).

3.4.1 Control release techniques

Several innovative techniques have been employed to achieve controlled release, in the food and pharmaceutical industries. Tab. **3-1** lists a few potential techniques of controlled release and also describes the matrix used, the active compound incorporated and the release mechanism involved.

Table 3-1 Some controlled release techniques used in food and pharmaceutical industries

Matrix	Active compound	Release mechanism	Results	Ref.
Microspheres (30-50μ) Porous PLGA spheres	Growth hormone	Diffusion barriers created around the spheres with closed pores	Open pores - immediate release of hormone Closed pores – controlled release of hormone over a period of 30 days. .	Kim *et al*., 2006
Cross linked chitosan microspheres (60-120μ)	Vit B_3	Hydrolysis of ester in acidic media	Vit B_3 covalently bound to microsphere is released due to hydrolysis of the ester bonds. Release ↑ as pH↓	Szczubialka *et al*., 2006
Protein Microcapsules (35μ)	Anti-microbial compound	Break down of micro capsules by bacteria	Controlled release of antimicrobials depending on the rate at which capsules were degraded by the bacteria	Kromidas *et al*., 2006
Sodium alginate nanoparticles (235 nm)	3 anti-tubercular drugs	Cation induced controlled gelification of alginate	Inhalable nanoparticles could serve as carriers for controlled release of anti-tubercular drugs	Zahoor *et al*., 2005
X-linked hydrogel discs (PVP –PA amX)	Vit B_{12}	Drug release depends on the swelling behavior of the gels	High X-linking restricts the relaxation of the polymers and hence control the release of drug	Dengre *et al*., 2000
HME tablets containing Eudragit (RS PO & E PO)	Drug	Eudragit E PO (soluble) controls the release	↑ Eudragit RS PO (retardant): ↓ release ↑ Eudragit E PO: ↑ release	Fukuda *et al*., 2006
PLA-PEO-PLA triblock copolymer	Drug	PLA degrades & releases drug	Nearly zero order release. Crystalline PLA showed fast release compared to amorphous.	Agrawal *et al*., 2006
Calcium alginate microparticles	Nisaplin®	Dissolution of microparticles	Active against an indicator organism in milk	Wan *et al*., 1997



Fan & Singh, 1989

Fig. **3-4**: Controlled release mechanisms (A) Dissolution of the matrix (B) Diffusion through the matrix

3.5 Nisin incorporated matrices

Wan *et al*. (1997) incorporated nisin in calcium alginate microparticles of 150 μm. Nisaplin® (commercial grade containing 2.5% nisin) was incorporated in calcium alginate in the ratio 1:1, 1:4 and 1:9. The antimicrobial activity of the microparticles was tested in MRS broth and reconstituted skim milk. The nisin encapsulated in the alginate was found to be active against the target organism *Lactobacillus curvatus*.

Cutter *et al.* (2001) incorporated nisin in films made from polyethylene (PE) as well as in PE and polyethylene oxide blend films. The nisin activity of the film was determined by spot assay on TSBYE agar plates seeded with *Brochothrix thermosphacta*. They found that nisin incorporated films composed of polyethylene + polyethylene oxide were more effective than films made of polyethylene alone. The water solubility of polyetheylene oxide may account for the increased release and activity of nisin.

Cha *et al*. (2002) prepared 2% (w/w) kappa carrageenan films incorporated with nisin. The plasticizer used was a combination of glycerol and polyethylene glycol, in the ratio of 1:1. The antimicrobial activity of the films was tested by agar diffusion method. Kappa carrageenan films formed weak inhibition zones against *Micrococcus luteus* (Gram-positive). Kappa carrageenan fims, incorporated with nisin as well as ethylenediaminetetraacetic acid (EDTA), showed weak inhibition against *Listeria innocua* (Gram-positive).

In another study, Cha *et al*. (2003) investigated nisin incorporated films made of methyl cellulose (MC), hydroxypropylmethyl cellulose (HPMC), kappa carrageenan and chitosan. The films were made by heat pressing and solvent casting. As compared to the

heat pressed films, the cast films were observed to produce greater inhibitory zones in agar diffusion bioassay. Among the films studied, antimicrobial activity was found to be strongest in MC films. This observation was attributed to the non-ionic and water soluble nature of MC. The low release of nisin from 5 and 10% (w/w) kappa carrageenan films may have been due to ionic binding of nisin to kappa carrageenan.

Guerra *et al*. (2005) adsorbed nisin on cellophane and found the film to be antimicrobially active. Further experiments with chopped veal demostrated that the films were effective in controlling growth of *Enterococcus hirae*. Neetoo *et al*. (2007) studied the antimicrobial activity of five commercially available plastic films, that were coated with nisin. A cellulose based carrier solution was used for coating nisin on the films. The films retained more than 80% activity even after 12 weeks, when stored under refrigerated and room temperature conditions. Therefore, commercial packaging films can be coated with nisin to produce stable antimicrobial films.

Grower *et al*. (2004) coated low-density polyethylene (LDPE) films with methyl cellulose (MC) and hydroxypropylmethyl cellulose (HPMC) solutions containing different concentrations of nisin. They observed zones of inhibition after 8 days. However, the release was uncontrolled and did not produce consistent inhibition zones during the study.

3.6 Nisin incorporated corn zein films

Reiners *et al.* (1973) reported that four groups of proteins in corn, which could be distinguished on the basis of their solubility, are albumins (water soluble), globulins

(saline soluble), glutenin (dilute alkali soluble) and prolamine (soluble in 70% ethanol). Zein falls under a category of proteins called prolamines. Zein is a mixture of proteins and has an average molecular weight of 45,000 Da, in the native state. However, during extraction, some of its polypeptide chains break, yielding a product with a molecular weight of 25,000 -35,000 Da (Gennadios & Weller, 1990). Zein protein fraction α–zein is soluble in 95% ethanol while β–zein is soluble in 60% ethanol (Lasztity, 1984).

Some corn proteins extracted by reducing alcoholic solutions are also soluble in water in reducing conditions and are therefore called reducible soluble proteins (RSP) (Paulis *et al.*, 1977). The insoluble 50 kDa protein resistant to pancreatic digestion and heat, present in the RSP fraction of the corn flour act as an allergen in Immonoglobulin E (IgE) mediated food allergy to corn products (Pasini *et al*., 2002).

An abundance of hydrophobic and uncharged amino acids (ie. leucine, proline, alanine) compounded by a deficit of charged residues, accounts for the non-polar nature of corn zein (Paulis, 1982). The presence of 20-22% glutamic acid, which is mostly in the form of glutamine, contributes to the insolubility of zein in water through hydrogen bonding (Reiners *et al.*, 1973).

Zein is certified GRAS (Generally Recognized as Safe) by USFDA (United States Department of Food and Drug Administration). Zein is odorless, tasteless, water insoluble and edible, thus making it a suitable choice for food and pharmaceutical applications. It is now commonly used as a coating for candies, nuts, fruits etc. and hence, is often termed as ‘confectioner’s glaze’. Corn zein films are brittle and hydrophobic in nature. Corn zein films exhibit low oxygen permeability (Krochta, 1987). Presence hydrophobic and uncharged amino acids; leucine, proline and alanine make

corn zein films good moisture barriers (Padgett, 1996). Corn zein films have an off-white/ pale yellow color and are translucent. Corn zein films can form tough, glossy and grease proof coatings (Gennadios, 1990).

Hoffman *et al.* (2001) studied the antimicrobial activity of cast corn zein films, incorporated with nisin, lauric acid and EDTA. The films were found to be active against *Lactobacillus plantarum*. The film containing nisin reduced *Listeria monocytogenes* by 1 and 5.5 logs after 2 and 48 hours, respectively. Teerakarn *et al*. (2002) studied the nisin diffusivity through cast and heat-pressed corn zein films. They found that cast films had low nisin release and high retention, as compared to heat-pressed corn zein films. Nisin was incorporated in the cast film solution, but suspended as particles in the heat pressed films. This approach may have been the cause for the difference in release.

3.6.1 Plasticizers for corn zein films

Since corn zein is not miscible in water, cast corn zein films are prepared by dissolving it in organic solvents, such as ethyl alcohol and acetone. The alcohol solutions prevent the zein proteins from unfolding. Consequently, the bulky side groups keep the polypeptides from getting close to each other and arranging themselves in an ordered structure within the film matrix. This arrangement causes pure zein films to be brittle (Yang *et al.,* 1996). Hence, plasticizers are needed to make them more pliable. Plasticizers lower the glass-transition temperature (Tg) of the polymer and thus, reduce its modulus by separating the chains from each other and facilitating chain movement (Sperling, 1992)

When glycerol was used as plasticizer, it migrated to the surface of the corn zein films within a few hours after preparation, making the films brittle (Park *et al.*, 1992). Park *et al.*, 1994 and Parris & Coffin, 1997 reported that polyetheylene glycol (PEG) and polypropylene glycol (PPG) were better plasticizers, as compared to glycerol. Currently, zein films are prepared by using mixed plasticizers. Park *et al.* (1994) observed that glycerol plasticized films were very brittle and their elongation was improved by adding polyethylene glycol. In a similar study, Parris & Coffin (1997) studied the effect of different plasticizers on the tensile properties and water vapor permeability of corn zein films. They found that films plasticized with a combination of glycerol and PPG (Polypropylene glycol) in the ratio 1:3, exhibited elongation till break (ETB) values (a measure of flexibility) fifty times higher than glycerol plasticized films.

3.7 Factors affecting diffusion

3.7.1 Effect of molecular size and shape

Franssen *et al.* (2004) studied the diffusion of two antimicrobials, Natamycin® and pottasium sorbate in whey protein isolate (WPI) films. They found that the diffusion coefficient of Natamycin® was several orders lower than that of pottasium sorbate. This slow release of Natamycin® was attributed to its large molecular size and shape as against the small and linear sorbate.

3.7.2 Effect of plasticizer

Franssen *et al.* (2004) studied the effect of plasticizer on the diffusion of two preservatives Natamycin® and pottasium sorbate from WPI fims. Diffusion coefficients of Natamycin® and pottassium sorbate decreased as the glycerol content decreased. This observation may occur because as the glycerol content decreased, free volume available decreased, which in turn decreased the film flexibility. Thus slower release could be attained by adjusting the plasticizer content.

3.7.3 Effect of temperature and pH of release solution

Teerakarn *et al*. (2002) studied the nisin diffusivity through cast-corn zein films and heat-pressed corn zein films at different exposure temperatures (5, 25, 35 and 45°C). They found that kinetics of nisin diffusion through the films followed a Fickian diffusion model. Temperature dependence of nisin diffusion in the films demonstrated Arrhenius relationship. Similar reports were also made by Sebti *et al*. (2004). Mauriello *et al*. (2005) reported that lower pH of the release medium favored nisin diffusion from packaging film to water. This observation was probably due to higher solubility of nisin at a low pH. They also observed that at low temperatures, nisin release slowed down.

3.7.4 Effect of polymer concentration

Ripoche *et al.* (2006) and Sebti *et al.* (2004) studied nisin diffusion in agarose gels of varying concentration. They found that the nisin diffusivity coefficient decreased

with increase in agar concentration. This observation was attributed to an increase in pathlength of diffusion that may have been caused by the presence of a network. Giannkopoulos & Guilbert (1986) and Biquet & Guilbert (1986) reported similar observations with diffusion in agarose gels. They explained that the decrease in nisin diffusivity was probably because of gel rigidification as agar content increased. When agarose content increased, the pore size decreased, which probably decreased molecular diffusion in the gel. Ozdemir and Floros (2003) investigated potassium sorbate diffusion in whey protein films. They observed a decrease in sorbate diffusivity as the protein concentration in the whey protein film increased.

Zilberman and Sofer (2007) studied the controlled release of an enzyme, horseradish peroxidase (HRP) from composite fiber structures. They found that an increase in polymer concentration in the matrix resulted in smaller burst release and a more moderate release profile. They proposed that as the polymer concentration increased, the viscosity and polymer density also increased. This process in turn, lowered the solvent diffusion into the matrix and also reduced the free volume available for the diffusion of the active compound. In similar studies, Sun & Wang (1995), Sing & Nigam (1979) and Davis (1974) observed a reduction in active compound diffusivity as the polymer concentration in the matrix increased.

3.8 Mathematical modeling

The release of nisin from various matrices into the food systems may be predicted with the help of various modeling techniques. Several authors have reported that Fick's

second law satisfactorily explained nisin diffusion through various matrices (Teerakarn *et al*., 2002; Sebti *et al*., 2004; Ripoche *et al*., 2006).

3.8.1 Weibull model

The Weibull model has been used in several biological systems to describe diffusion, rehydration, matrix relaxation etc. (Marabi *et al*., 2003; Papadapoulou *et al*., 2006). This model describes the process as a sequence of probabilistic events.

$$\frac{M_t}{M_\infty} = 1 - \exp\left[-\left(\frac{t}{\alpha}\right)^{\beta}\right]$$

where, M_t = amount of nisin released at time t

M_∞ = amount of nisin released at equilibrium

α = scale parameter (s^{-1})

β = Weibull shape parameter

The scale parameter 'α' is proportional to the reciprocal of the process rate constant. The scale parameter defines the rate and represents the time needed to accomplish 63% of the process. The Weibull shape factor 'β' describes the shape of the curve and therefore, could be used to describe mechanisms like diffusion, relaxation and convection. When β = 1, the Weibull distribution describes a first order kinetics (Saguy *et al.*, 2005). Marabi *et al*. (2003) reported that the Weibull model could be used for describing mechanisms of water uptake. Papadapoulou *et al*. (2006) used a similar approach for describing drug release from tablets.

Chapter 4

Materials and Methods

This chapter describes the methodology adopted to complete the experiments outlined for this research. The first phase consisted of screening potential biopolymers for developing the film matrix, by evaluating its stability in an aqueous system. In the second phase, the active compound nisin was entrapped within the biopolymer-based films and the antimicrobial activity of the film was checked by agar diffusion method. The third phase consisted of modeling the nisin release kinetics with the help of mechanistic diffusion model, as well as an empirical Weibull model. These models were validated by experiments in the final phase. The nisin released from the films into an aqueous system was quantified using high performance liquid chromatographic (HPLC) technique.

4.1 Screening of biopolymers

Films were made with the one or more of the following biopolymers. Films developed with more than one biopolymer would hereafter be referred to as blended films.

- Locust bean gum (CPKelco, Lille Skensved, Denmark)
- Xanthan gum (CPKelco, Chicago, IL, USA)
- Kappa carrageenan (FMC BioPolymer, Philadelphia, PA, USA)
- Iota carrageenan (FMC BioPolymer, Philadelphia, PA, USA)
- Lamda carrageenan (FMC BioPolymer, Philadelphia, PA, USA)

- Hydroxypropyl methyl cellulose (Dow Chemicals, Midland, MI, USA)
- Corn zein (TCI America, Portland, OR, USA)

Nisaplin® (Danisco USA Inc., New Century, KS) was added during the film making process and the antimicrobial activity of the films was tested by agar diffusion method.

4.2 Films made with (A) locust bean gum and (B) xanthan gum

Two different films were developed using 1% (w/v) locust bean gum and xanthan gum respectively. The films were prepared by dissolving the bioploymers in distilled water using magnetic stirring. Polyethylene glycol (PEG 400, EMD Chemicals, Gibbstown, NJ, USA) (40% w/w, of the biopolymer) was used as a plasticizer and the mixture was heated to 70 °C. Heating caused the biopolymer to completely dissolve, thus forming a clear solution. Varying concentrations of Nisaplin® were then dissolved into the film solution. The films were cast in aluminum weighing boats (VWR International Inc., West Chester, PA, USA) and dried overnight in humidity and temperature controlled chamber (LAB-LINE® 680 APX, LAB-LINE Instruments, Melrose Park, IL, USA).

4.3 Films made with carrageenan

Films were made with 2% (w/v) of the different carrageenans kappa, iota and lamda according to the procedure outlined by Cha *et al*. (2003) and Su Cha *et al*. (2002). Blended films also were made with kappa-carrageenan and HPMC. The biopolymers

were dissolved in distilled water using magnetic stirring. Polyethylene glycol (PEG 400) (40% of the biopolymer) was used as a plasticizer and the mixture was heated to 70°C. Heating caused the biopolymer to completely dissolve, thus forming a clear solution. Nisaplin® (3000 mg) was dissolved in the film solution by magnetic stirring. The films were cast in aluminum weighing boats and dried 24 h in a temperature and humidity controlled chamber maintained at 50% RH and 25 °C. The flow chart for the preparation of carrageenan films is shown in Fig. **4.1** (Cha *et al*., 2003; Su Cha *et al*., 2002).

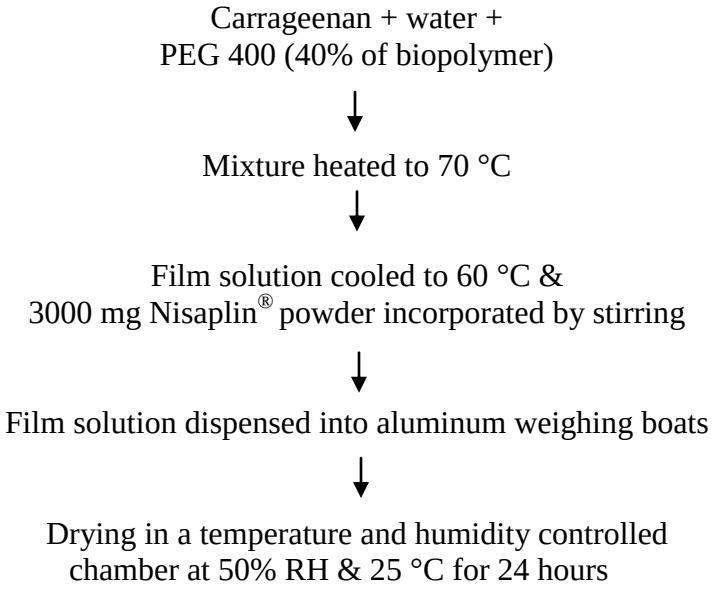


Fig. **4.1** Flowchart showing the preparation of carrageenan films

4.4 Films made with corn zein

Since corn zein is not miscible in water, cast corn zein films were prepared by dissolving varying concentrations of corn zein in 95% ethyl alcohol (190 Proof,

ACS/USP Grade, Brookefield, CT, USA). A simple experiment was conducted in order to identify the most suitable plasticizer combination for corn zein film (Appendix **A**). A plasticizer combination of PEG 400 (EMD Chemicals, Gibbstown, NJ, USA) and glycerol (EMD Chemicals, Gibbstown, NJ, USA) in the ratio 1:1 was found suitable for corn zein films and this is in agreement with published information (Park *et al.*, 1994).

Corn zein films are generally developed with a high concentration of corn zein (15-16% w/v) (Lawton, 2004; Dawson *et al*., 2003; Lai & Padua, 1997). In general, for controlled release purposes, the excipient should be developed preferably with low polymer concentrations. Therefore, nisin containing films were developed with low corn zein concentrations of 2, 3, 4, 5, 6, 8 & 12% (w/v). Preliminary experiments demonstrated that films with 2% concentration collapsed due to insufficient polymer, whereas those with 12% concentration did not show a quantifiable amount of nisin release. Based on these preliminary findings, corn zein films with concentrations 4, 6, 8 and 10% (w/v), were used for further studies.

Corn zein was dissolved in 95% ethanol. Plasticizers PEG 400 and glycerol were added to the film mix in the ratio 1:1. The film solution was heated to 70°C to completely dissolve corn zein and was held at that temperature for 5 minutes to remove the air bubbles. The solution was then cooled to 60 °C and 3000 mg Nisaplin® was incorporated in the solution. This experiment was replicated 4 times. The film composition is as shown in Table **4.1**.

Table **4.1** Composition of corn zein films

Corn zein concentration % (w/v)	Ethyl alcohol (ml)	Corn zein (mg)	Glycerol (µl)	PEG (µl)	Nisaplin® (mg)
4	50	2000	333	333	3000
6	50	3000	500	500	3000
8	50	4000	667	667	3000
10	50	5000	833	833	3000

The films were made in teflon casting plates that were specially fabricated with internal well dimensions of 17.5 x 7 x 1 cm (Fig. **4-2**). These dimensions were used to obtain 10 film pieces, 3.5 x 3.5 cm in size.

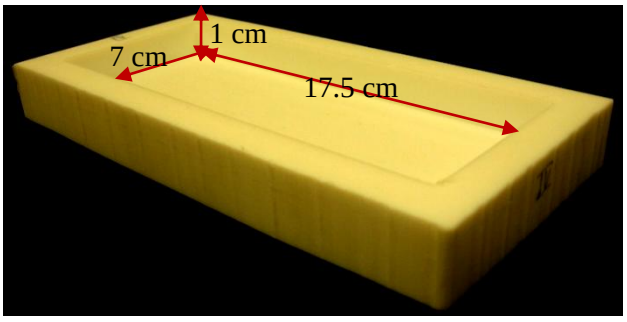


Fig. **4-2**: Teflon casting plate showing well dimensions

The plasticizer level was 40 % of the weight of biopolymer. For ease of measurement, glycerol and PEG were calculated on a volume basis (specific gravity of

glycerol and PEG is approximately 1.2). All the materials used viz. corn zein, ethyl alcohol and plasticizers are FDA approved. The procedure outlined by Lawton (2004), Dawson *et al*. (2003) and Lai & Padua (1997) was adopted for film preparation. Film preparation is shown below with the help of a process flow chart (Fig. **4-3 A & B**). The experiment was replicated four times.

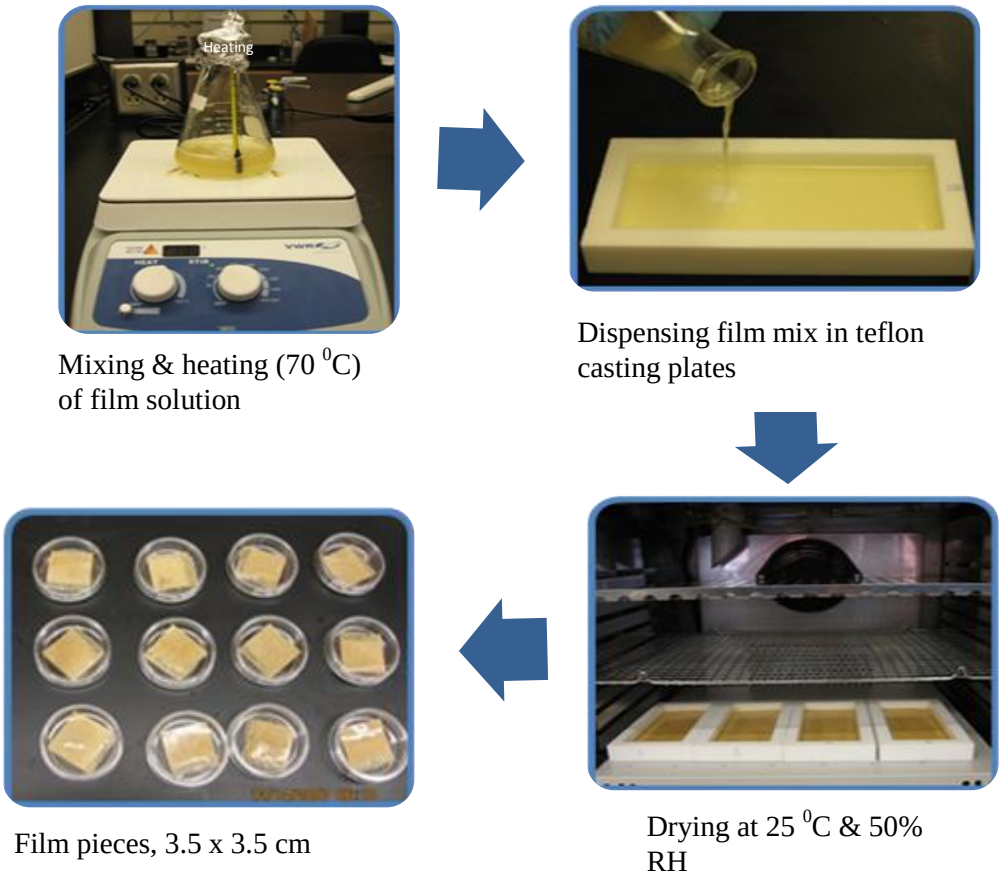


Fig. **4-3 A**: Pictorial chart showing the preparation of corn zein film

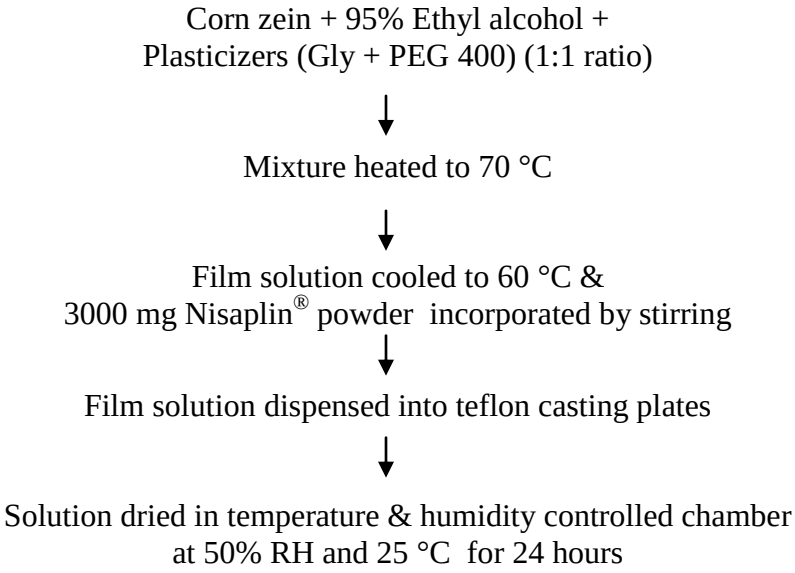


Fig. **4-3 B**: Flowchart showing the preparation of corn zein films

Control corn zein films (without Nisaplin®) were translucent and pale yellow in color and films with Nisaplin® were opaque. Corn zein films are generally brittle in nature and their quality improved on the addition of plasticizers.

4.4.1 Film thickness

The thickness of each film was measured at 20 different locations using a digital micrometer (IP65, Mitutoyo, Japan) to an accuracy of 0.001mm. The average thickness of each film was used later in diffusivity calculations.

4.5 Nisin release from films

The films were removed from the casting plates and cut into 10 pieces, 3.5 x 3.5 cm in size. Since 3000 mg Nisaplin® was added to the film solution, each piece contained 300 mg Nisaplin®, amounting to an activity of 300,000 IU. To ensure double sided diffusion from the films, petri dishes of size 60 x 15 mm were modified by sticking glass beads inside each of them (Fig. **4.4**). This process elevated the film and prevented it from resting on the petri dish bottom. This process also facilitated diffusion from both sides of the film. Citrate buffer (0.1 M, 3 pH) was dispensed on the film pieces in the dish to completely submerge the films.

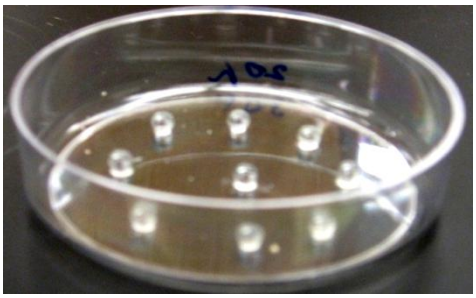


Fig. **4-4**: Petri dish modified to obtain double sided diffusion

The volume/surface area ratio for double sided diffusion experiments from polymeric films should be maintained between 0.31 to 155 ml/cm^2 (ASTM D 4754). A volume/surface area ratio of 0.82 ml/cm^2 was used for this study. As shown in Fig. **4.5**, nisin release was tested in a temperature & humidity chamber maintained at 25 °C and 50% RH. The samples were kept agitated at 50 rpm with the help of an orbital shaker (DS500E, VWR International Inc., West Chester, PA, USA) throughout the nisin release

study. Separate films were used for every reading. One ml of the release solution was withdrawn at predetermined intervals. The release solution was filtered with a 0.45 µm syringe filter (13 mm, 0.45 µm, Nylon, Restek, State College, PA, USA) and 50 µl of the release solution was injected in the HPLC for nisin quantification. The experiment was replicated four times.

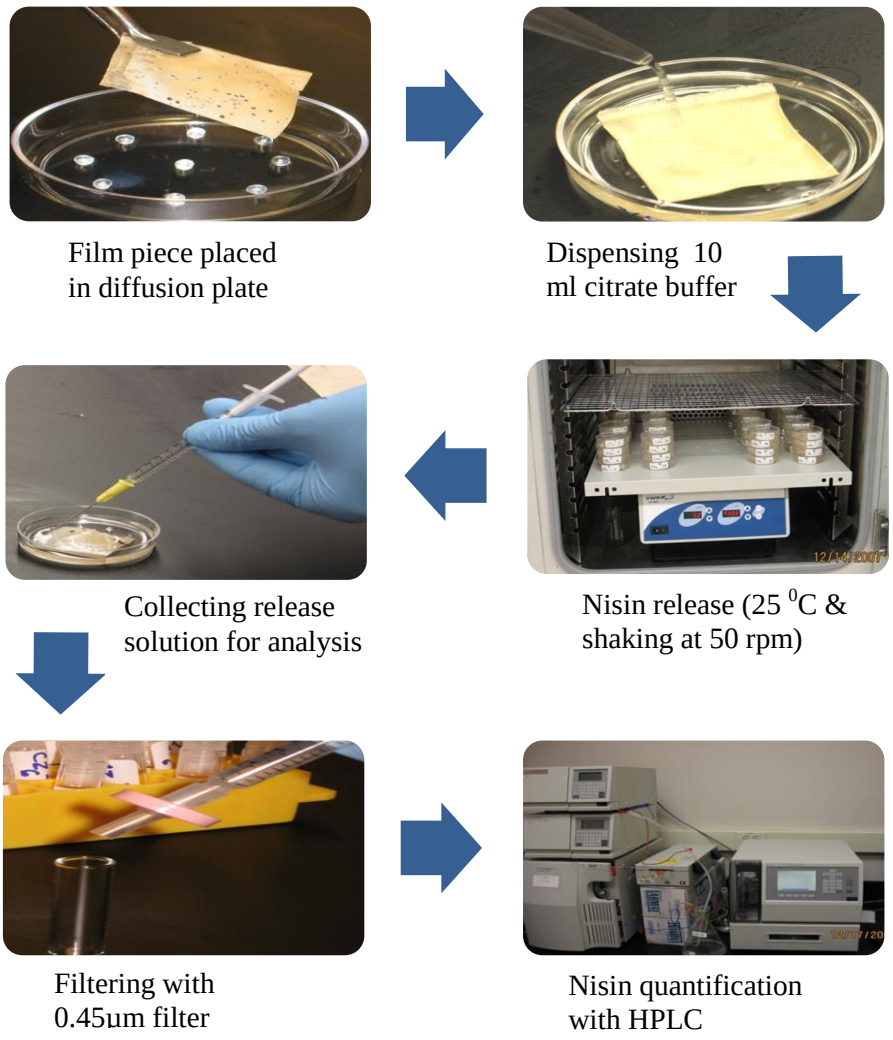


Fig. **4-5**: Pictorial chart showing nisin release studies

4.6 Antimicrobial activity of films

4.6.1 Agar diffusion method

The agar diffusion method was performed according to the procedure outlined by Pongtharangkul & Demirci (2004). The media consisted of nutrient broth, 0.75% agar (Difco laboratories, USA) and 1% Tween 20 (J.T. Baker, Phillipsburg, N.J., USA). Tween 20 makes the agar soft and aids the diffusion of nisin through the agar medium (Tramer & Fowler, 1964). Cha *et al*. (2003) used a full strength agar base which was overlaid with semisoft agar for making diffusion wells. However, in the present study, a 0.75% nutrient agar was directly used in order to avoid variability in diffusion of nisin into agars of different strength. Diffusivity of nisin decreases with an increase in agar concentration (Ripoche *et al.,* 2006 and Sebti *et al.,* 2004). After sterilizing the media, it was cooled to 40°C in a water bath and inoculated with 1% of an 18-hour culture of *Micrococcus luteus* (Source: Dr. Stephanie Doores, 420 Food Microbiology Lab, Food Science, Pennsylvania State University) an indicator organism that is sensitive to nisin (Pongtharangkul & Demirci, 2004). Twenty ml of the agar was dispensed into each petri dish (100 x 15 mm) and allowed to set for 30 minutes at room temperature (25 ^{0}C). Film discs of 7 mm diameter were cut with the help of a sterilized # 3 cork borer and placed on nutrient agar plates seeded with *Micrococcus luteus*. The inhibition zones formed after 24 hours (37 ^{0}C) were measured with digital vernier calipers (Catalogue # 62379-531, VWR, International Inc., West Chester, PA, USA) to the nearest 0.01 mm.

The nisin released from the films at different intervals were obtained as described in section 4.5. Since citrate buffer also can produce an inhibition zone in agar method, the nisin release from the films for testing antimicrobial activity was obtained in sterilized distilled water at 25°C with an agitation of 50 rpm. To measure the activity of the nisin release solutions, 4 to 5 wells of 7 mm diameter were made in the agar, using a # 3 cork borer. Fifty µl of the nisin solution was introduced into these wells. The inhibition zones formed were measured from the edge of the well (Fig. **4-6**) after 24 hours (37 ^{0}C) with digital vernier calipers. The assays were performed three times.

4.7 Nisin quantification

Nisin released from the films were quantified using standard nisin curves. For plotting standard curve, various concentrations of nisin solutions were prepared by serial dilutions of stock solution of Nisaplin®. A stock solution of 10,000 IU/ml was prepared by dissolving 100 mg Nisaplin® in 10 ml 0.02N HCl, followed by serial dilutions in distilled water. Standard curves were developed with agar diffusion method as well as using HPLC technique. The regression equation developed from the standard curve was used to calculate unknown concentrations of nisin released from the corn zein films.

4.7.1 Agar diffusion method

4.7.1.1 Nisin standard curve: agar diffusion method

Stock solution of Nisaplin® 10000 IU/ml was prepared in 0.02 N HCl (EMD Chemicals, Gibbstown, NJ, USA). Concentrations ranging from 0 to 10000 IU/ml were prepared, by serial dilutions in sterile distilled water. Media plates were prepared as described in section 4.6.1. To measure the activity of these solutions, 4 to 5 wells of 7 mm diameter were made in the agar, using a # 3 cork borer. Fifty µl of the above nisin solutions were dispensed into these wells.

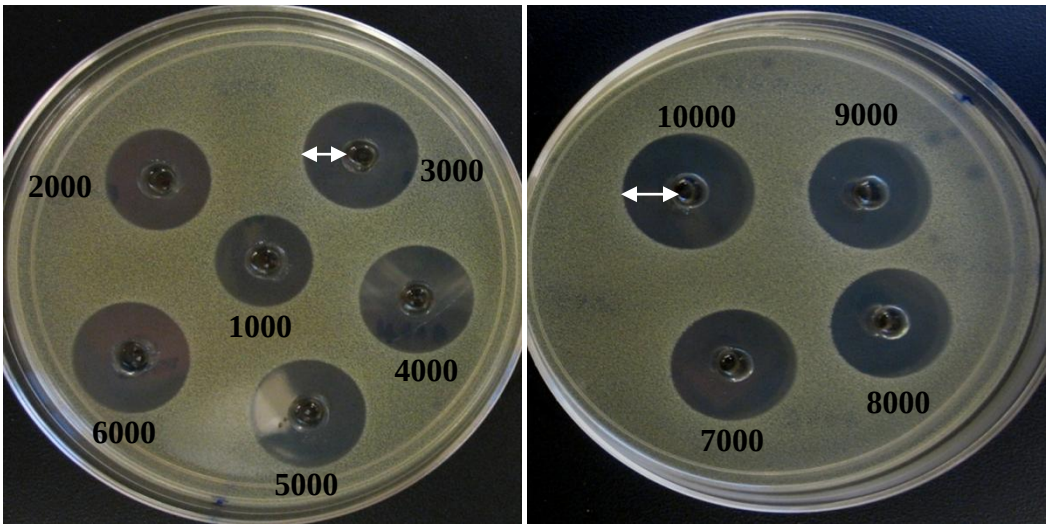


Fig. **4-6**: Inhibition zones made by solutions of known nisin concentrations (1000-10000 IU/ml). Inhibition zones were measured from the edge of the well.

The inhibition zones formed after 24 hours were measured (n=3), as explained earlier (section 4.6.1). The experiment was replicated thrice. Average of the inhibition zones (Fig. **4-6**) were plotted against known nisin concentrations to obtain the standard

curve, shown in Fig. **4-7**. It is evident from Fig. **4-6** & Fig. **4-7 A** that inhibition zones produced does not change significantly with increase in concentration. The trend line shown in Fig. **4-7 B** (developed after omitting the first two data points) indicates that as the nisin concentration increases there is only 0.05% increase in the inhibition zone. There is a weak dependence of inhibition zones on the nisin concentration. The slope 0.0005 is significantly different from slope zero ($p<0.05$). The relationship between inhibition zones and nisin concentration is non-linear. Probably the rate limiting factor of the inhibition zone is the quantity of nisin solution dispensed in the well. The solution starts diffusing into the surrounding agar and once the entire solution is diffused, the inhibition comes to a halt. On account of the inconsistency or inadequacy of the agar diffusion method, this standard curve (Fig. **4-7 B**) was not used for nisin quantification.

In a similar study, Pfeiffer and Orben (1997) reported that inhibition zones produced by agar diffusion method were a logarithmic function of the nisin quantity. Ripoche *et al.* (2006) attributed the lower sensitivity and poor reproducibility of this technique to bacteria state, temperature, agar medium etc. and stressed the need for it to be improved before it could be employed for quantification. Sanjurjo *et al.* (2006) reported that nisin evaluation by agar diffusion method was not satisfactory, due to the opposing effects of nisin diffusion and microbial growth. However, in a similar study, Teerakarn *et al.* (2002) investigated the effect of heat pressed and cast corn zein films on nisin diffusion and its activity. They developed a nisin standard curve by agar diffusion method by plotting inhibition zones on the Y axis and log nisin concentrations on the X axis and the standard curve was used for quantifying nisin release.

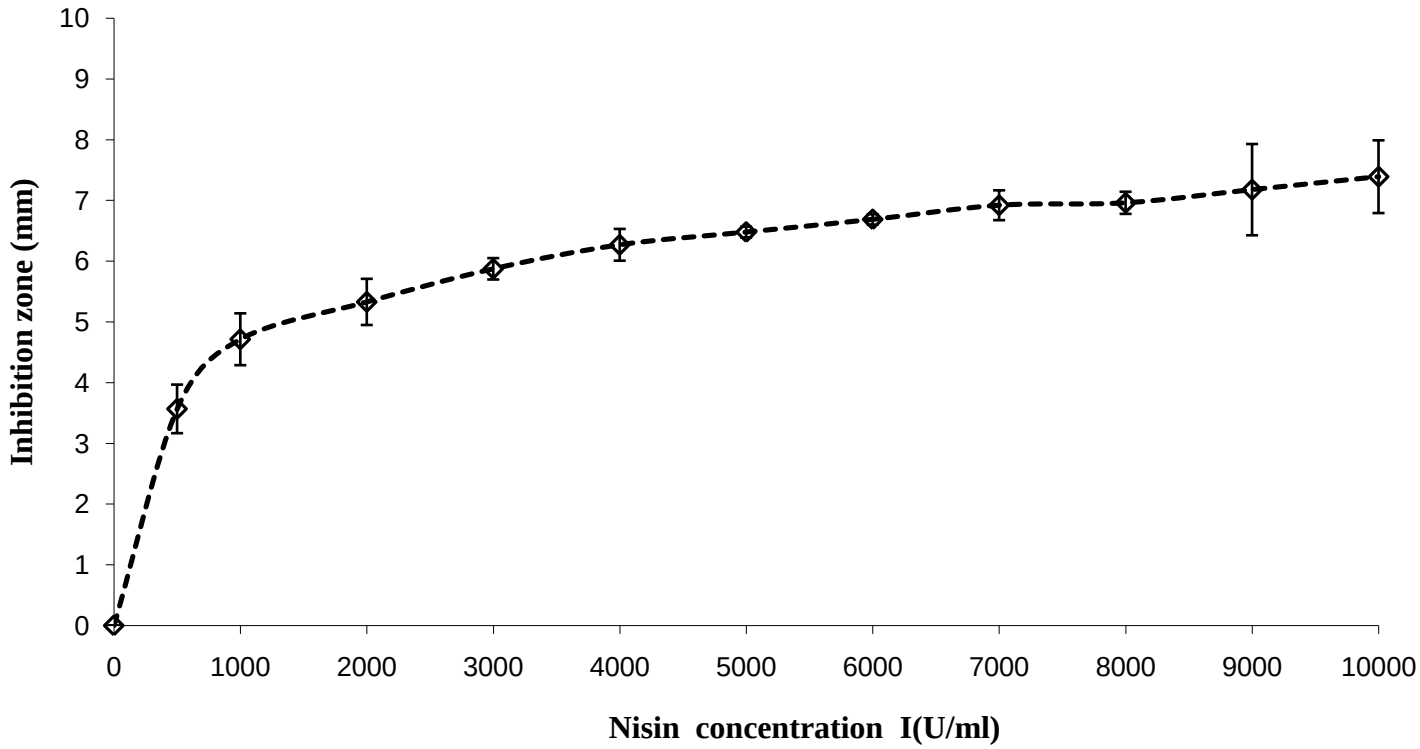


Fig. **4-7 A**: Nisin standard curve (0-10000 IU/ml) developed by agar diffusion method. Each data point is an average of 3 replications (Error bars: ±1 S.D). Symbol represents the experimental value and dotted line shows trend.

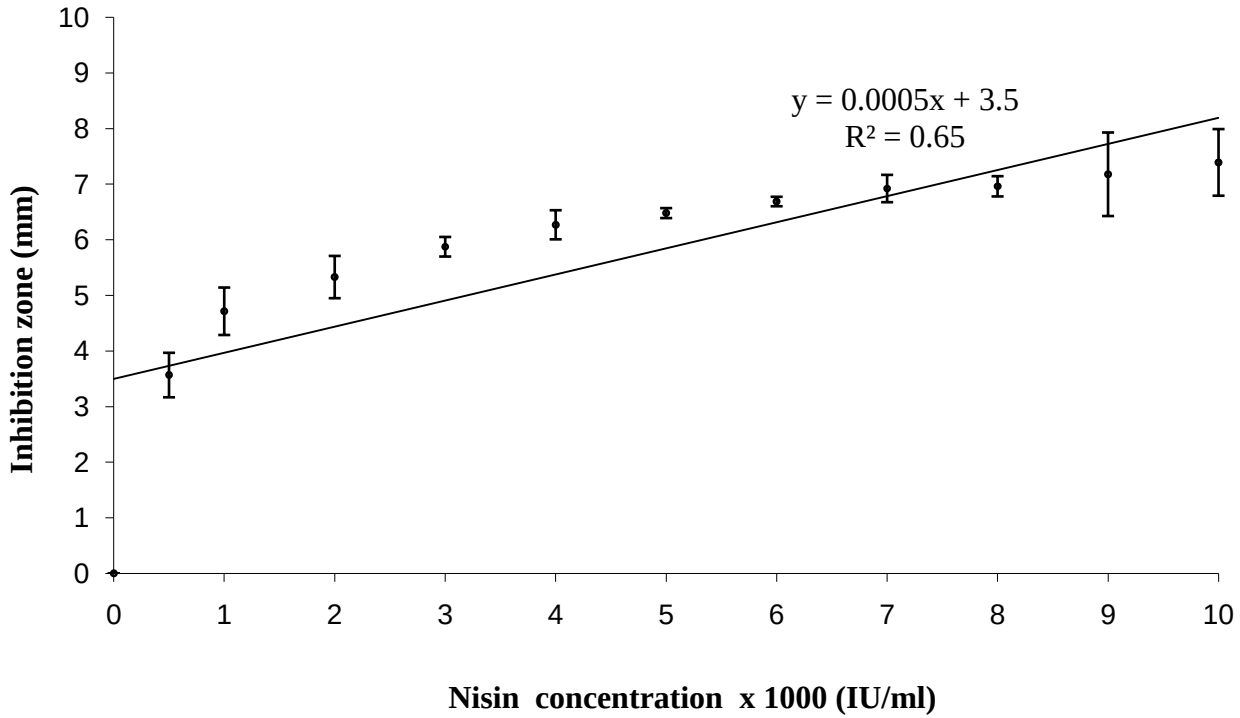


Fig. **4-7 B**: Nisin standard curve (0-10000 IU/ml) developed by agar diffusion method after omitting the first two data points. Each data point is an average of 3 replications (Error bars: ±1 S.D). Symbol represents the experimental value and solid line shows trend.

4.7.2 HPLC method

A C-18 reverse phase column (Ultra C18, Restek, State College, PA, USA) was used for quantifying nisin (Liu & Hansen, 1990 and Buonocore *et al.*, 2003). In reverse phase chromatography, the stationary phase (column packing material) is non-polar and the mobile phase (solvent) is polar, with respect to the sample. The stationary phase is comprised of silica (a reactive substance) bound with a hydrocarbon C18. A polar aqueous organic mixture acetonitrile + 0.1% TFA (Mallinckrodt Baker, Inc. Phillipsburg, NJ, USA) and water were used as mobile phase, with a gradient of 20-40%.

As the sample passes through the column, nisin, that is non-polar, was temporarily retained due to its attraction to the non-polar stationary phase. The polar components in the sample were eluted first and the non-polar nisin eluted subsequently. The run time was set at 20 minutes and the nisin peak was observed at 17-18 minutes. Nisin being hydrophobic, is initially retained on the hydrophobic stationary phase, but eluted later as the gradient increases.

4.7.2.1 HPLC operating parameters

A Waters HPLC system (Breeze software) was used with the following operating parameters:

Column: C 18 (Reverse phase), 250 mm x 4.6 mm

Packing: silica (particle size 5 µm & pore 100A°)

Injection volume: 50 µl

Run time: 20 minutes

Wavelength: 220 nm

Mobile phase: Acetonitrile + 0.1% Trifluroacetic acid (TFA) & DI water

Gradient: 20-40%

Solvent gradient and run time used by Liu & Hansen (1990) and Buonocore *et al.* (2003) were modified to get better peak resolution and work efficiency, and also to minimize the use of organic solvent. The solvent gradient used by Liu & Hansen (1990) and Buonocore *et al.* (2003) was 20-60%, for a run time of 60 and 20 minutes, respectively. This was modified to 20-40%, over a run time of 20 minutes.

4.7.2.2 Nisin standard curve: HPLC method

Pure nisin (40 x 10^6 IU/g), which was provided by Danisco (New Century, KS) was used for identifying the nisin peak (Fig. **4-8**). Stock solution of Nisaplin® 15000 IU/ml was prepared by dissolving 150 mg Nisaplin® (activity 10^6 IU/g) in 0.02N HCl (EMD Chemicals, Gibbstown, NJ, USA). Concentrations ranging from 0 to 15000 IU/ml were prepared, by serial dilutions in DI water (concentrations below 500 IU/ml could not be detected by HPLC). The solutions were filtered with a 0.45 µm syringe filter (Iso-disc, Nylon 13 mm x 0.45 µm, Restek, State College, PA, USA) and 50 µl of the samples were injected into the HPLC system. The nisin peak area in the chromatogram was integrated and plotted against known nisin concentrations to obtain the standard curve (Fig. **4-9**). The experiment was replicated thrice.

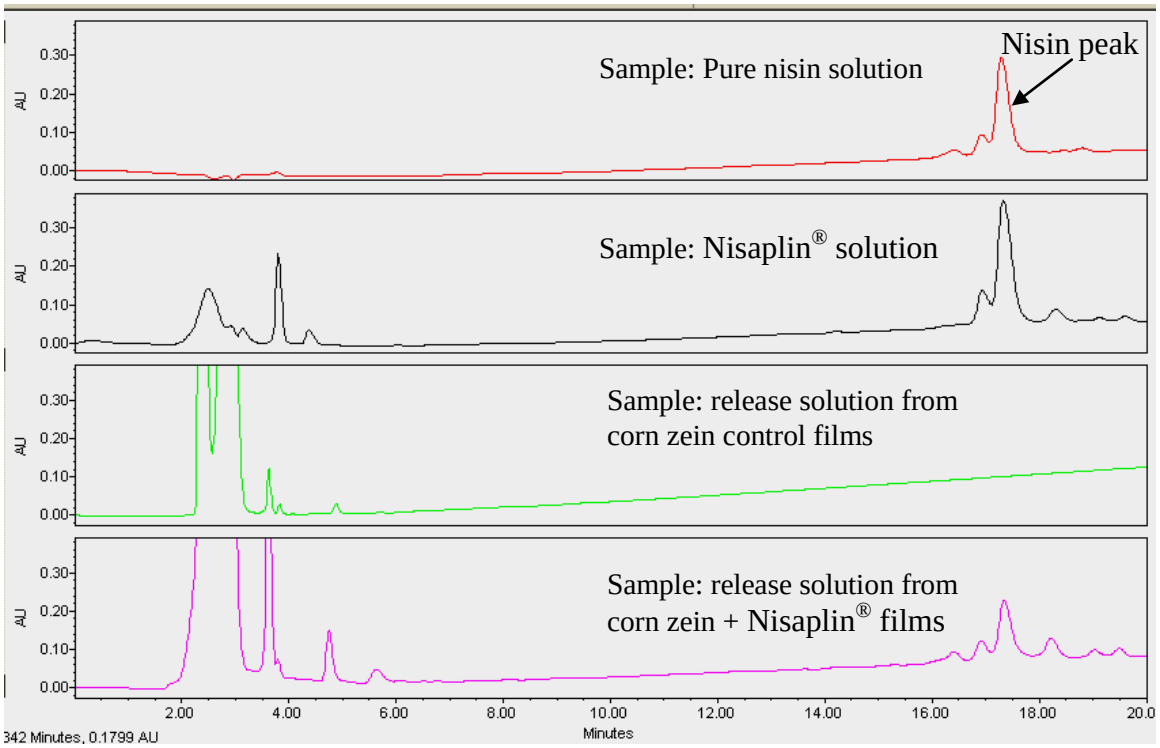


Fig. **4-8**: Chromatogram showing nisin peak in different samples

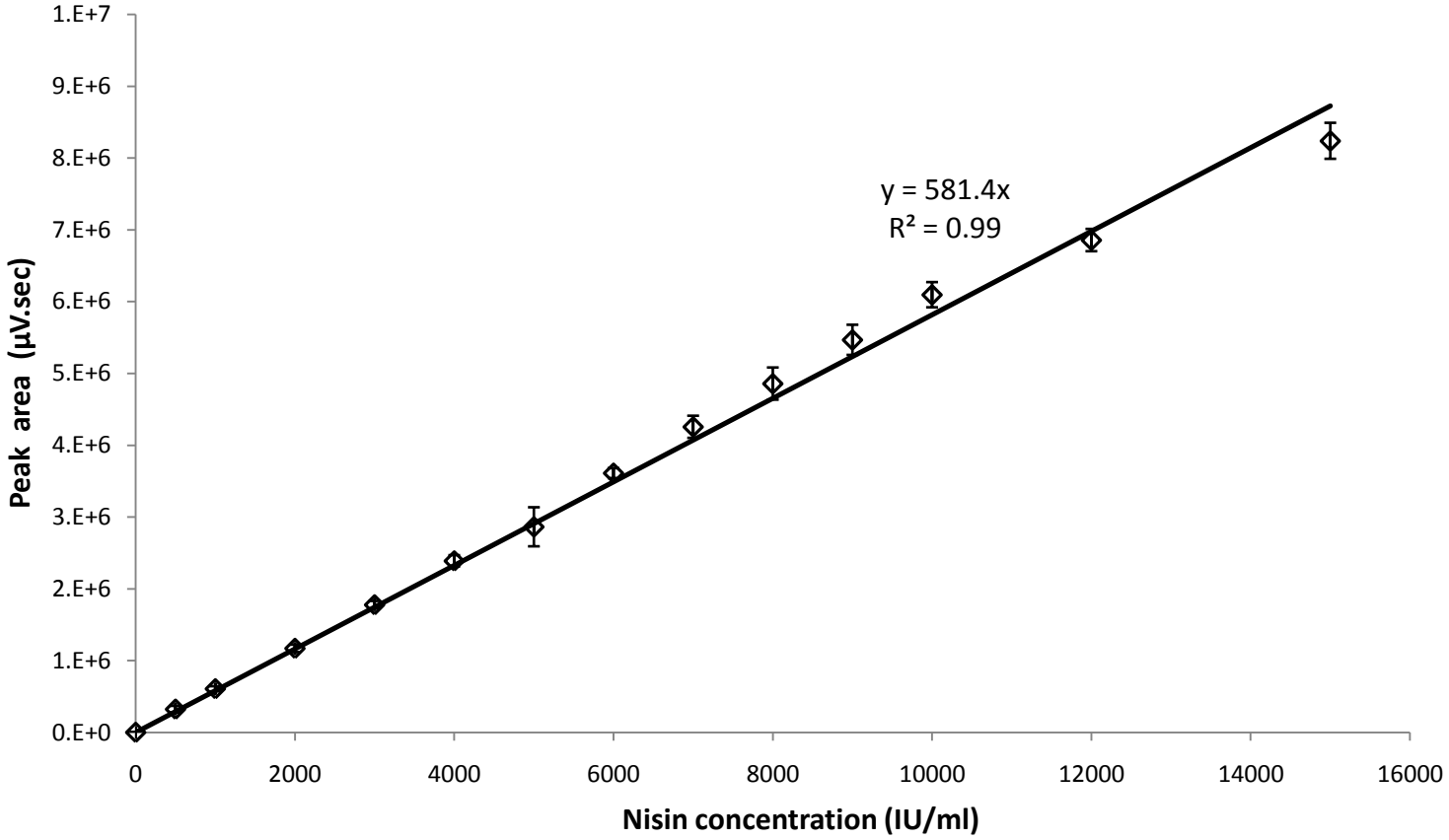


Fig. **4-9**: Nisin standard curve (0-15000 IU/ml) developed by HPLC method. Each data point is an average of 3 replications (Error bars: ±1 S.D). Symbol represents the experimental value and solid line shows trend.

The regression equation derived from the nisin standard curve (Fig. **4-9**) was used to calculate unknown nisin concentrations in the release solutions from corn zein films.

4.8 Nisin release: concentration dependent

To determine if the release of nisin was dependent upon the concentration of nisin, films with 4% corn zein concentration were made containing 100, 200 and 300 mg of Nisaplin®. Nisin release from these films was determined as described earlier (section **4.5**).

4.9 Microstructure of corn zein film

Differences in the morphology of corn zein films prepared by varying the polymer concentration were observed with a scanning electron microscope (SEM) (FEI Quanta 3100 ESEM, FEI Company, Hillsboro, OR). To observe the microstructure of the films and to see the distribution of Nisaplin® within the film matrix, cross-sectional views of the films were taken under low vacuum mode. Film samples were mounted directly on vertical sample holders with the help of double sided carbon stubs and scanning was done under low vacuum mode (0.68 Torr) with an accelerating voltage of 15 kV.

4.10 Glass transition temperature of corn zein films

A differential scanning calorimeter (DSC) (PerkinElmer, Pyris 1 DSC) was used to determine if there was a change in glass transition temperature (Tg) of corn zein films,

as the polymer concentration in the film changed. A procedure reported by Lai *et al.* (1997) was slightly modified for DSC studies. An empty pan was used as a reference and samples weighing 20-25 mg were pressure-sealed in aluminum pans. Samples were scanned from -10 to 140 °C at a rate of 5 °C/minute.

4.11 Statistical analysis

Statistical analysis was performed with the help of statistical software MINITAB (version 14). Effect of Nisaplin® concentration in the film and corn zein concentration in the film on nisin diffusivity was subjected to analysis of variance (ANOVA) and significance difference between the means was tested by Tukey's test.

Chapter 5

Mathematical Modeling

5.1 Diffusion mechanism

Diffusion is a process by which matter (molecules, ions or other small particles) are transported from a region of higher concentration to a region of lower concentration, by random molecular motion also known as Brownian motion (Cussler, 1997). Thus, the driving force for diffusion is a concentration (chemical potential) gradient.

Fick (1855) recognized that diffusion is analogous to Fourier (1822) heat conduction equation. Fick's first law relates to the concentration gradient at steady state. According to Fick's first law of diffusion,

$$J = -D\frac{\partial c}{\partial x} \qquad (\mathbf{5.1})$$

where, J = flux or the rate at which the matter permeates through a unit area (mol/m^2s)

D = diffusion coefficient (diffusivity) or the rate at which permeation takes place (m^2/s)

$\partial c/\partial x$ = concentration gradient (mol/m^3)

x = position (m) coordinate along the flow direction

The negative sign indicates that diffusion occurs in the direction opposite to that of increasing concentration. This equation is valid only for an isotropic medium, in which

the structure and diffusion properties in the neighborhood of any point are the same relative to all directions. D is numerically equal to the flux for unit concentration gradient. In dilute solutions, D is a constant, and in polymers, it depends on the concentration. D also depends on the nature of the medium, diffusing components and temperature (Islam, 2004). The value of diffusion coefficient in gases is around 2 x 10^{-5} m^2s^{-1}, in liquids it is 8 x 10^{-8} m^2s^{-1}, and solids it is around 2 x 10^{-11} m^2s^{-1} (Cussler 1997, Varzakas *et al*., 2005).

From the theory of mass balance (continuity equation), we can write:

$$\frac{\partial}{\partial x}(J) = -\frac{\partial c}{\partial t} \qquad (\mathbf{5.2})$$

Combining Fick's first law and the continuity equation, we have:

$$\frac{\partial}{\partial x}(J) = \frac{\partial}{\partial x}\left[-D\frac{\partial c}{\partial x}\right] = -\frac{\partial c}{\partial t} \qquad \text{or}$$

$$\frac{\partial c}{\partial t} = D\frac{\partial^2 c}{\partial x^2} \qquad (\mathbf{5.3})$$

Equation **5.3** is Fick's second law of diffusion which is limited to x direction and diffusivity D is independent of concentration. This model can be used for calculating concentration distribution at an unsteady state.

A standard procedure adopted, in order to investigate the diffusion mechanism involved in a thin sheet, is fitting the early portion of the curve (where the fractional release $M_t/M_\infty < 0.6$) to the power law model (Crank, 1975; Langer & Peppas, 1983; Ritger & Peppas, 1987; Ozdemir & Floros, 2001).

$$\frac{M_t}{M_\infty} = Kt^n \quad (5.4)$$

where, M_t is the amount of nisin released from the corn zein film at time t,

M_∞ is the nisin released at infinte time,

K describes the polymer solvent interaction and contact between two phases (K<1 for insufficient contact, K=1 for ideal system and K>1 for structural changes due to swelling).

n descibes the transport mechanism or diffusional exponent characteristic of release mechanism.

n = 0.5 (Case I transport) shows Fickian diffusion & release is proprtional to √t

n = 1.0 (Case II transport) shows non-Fickian mode & release is proportional to t

0.5 < n < 1.0 (Case III transport) shows anomalous or non-Fickian diffusion

n < 0.5 shows a pseudo-Fickian behavior, where the sorption curves resemble Fickian diffusion curves

5.1.1 Initial and boundary conditions

Nisin from the corn zein film diffused into a well stirred fluid (citrate buffer). The following assumptions were made:

- the corn zein matrix is isotropic and homogeneous (Scanning electron microscopy was performed to ensure homogenous distribution of Nisaplin® in corn zein films)

- diffusion takes place from both sides of the film
- diffusion is one dimensional
- diffusion is Fickian (Ripoche *et al*., 2006; Sebti *et al*., 2004)
- Surface concentrations at both the sides of the film are equal

If C is the concentration of nisin diffusing, the following initial and boundary conditions were set

Initial condition: $C = C_0$ at $t = 0$

Boundary conditions: $C = 0$, $x = -h/2$

$C = 0$, $x = h/2$

where, C_0 is the initial concentration of nisin uniformly distributed in the film and h is the film thickness.

Since citrate buffer is not saturated with nisin, we assume that nisin diffusion is into an infinite volume.

5.1.2 Analytical solutions

Nisin is released from the corn zein film into citrate buffer. The film matrix has uniform concentration of nisin C_0 and surface concentrations C_1. Considering the above boundary conditions, the solution for equation **5.3** is (Crank, 1975)

$$\frac{C}{Co} = \frac{4}{\pi}\sum_{n=0}^{\infty}\frac{(-1)^{\mathrm{n}}}{2\mathrm{n}+1}\exp\{-\mathrm{D}\,(2\mathrm{n}+1)^2\pi^2 t/h^2\}\left(\cos\frac{(2n+1)\pi x}{h}\right) \qquad (\mathbf{5.5})$$

Nisin retained in the corn zein film could be calculated by integrating Eq. **5.5** over space and time (Crank, 1975).

$$\frac{M_{t\prime}}{M_0} = \sum_{n=0}^{\infty} \frac{8}{(2n+1)^2\pi^2} \exp\{-D\,(2n+1)^2\pi^2 t/h^2\} \qquad (\mathbf{5.6})$$

where, $M_{t'}$ is the amount of nisin retained in the corn zein film at time 't' and

M_0 is the initial amount of nisin added in the film.

h is the film thickness (cm).

5.1.3 Diffusivity calculation

In one dimensional diffusion, diffusivity (diffusion coefficient) is defined as the rate of transfer of the diffusing substance across unit area of a section divided by the space gradient of concentration at that section (Crank, 1975).

A plot of $M_{t'}/M_0$ Vs t shows the desorption of nisin. If diffusion is Fickian with constant diffusivity D, plot of $M_{t'}/M_0$ Vs $\sqrt{t}$ will yield a straight line. The slope of this line, which is represented by k is used for diffusivity calculation.

For short times, when $M_{t'}/M_0 < 2/3$, the solution simplifies to (Teerakarn *et al.*, 2002)

$$\frac{Mt\prime}{M_0} = 1 - 4\left(\frac{Dt}{\pi h^2}\right)^{1/2} = 1 - kt^{1/2}$$

$$\therefore D = \left(\frac{kh}{4}\right)^2 \pi \qquad (\mathbf{5.7})$$

where, h = film thickness (cm) and k is the slope of nisin desorption curve.

5.2 Weibull model

The nisin release profile was also modeled using a Weibull equation (Eq. **5.8**). The Weibull model has been used in several biological systems to describe diffusion, rehydration, matrix relaxation etc. (Papadopoulou *et al*., 2006; Marabi *et al*., 2003). Papadopoulou *et al*. (2006) used this model to describe drug release from a matrix.

Equation **5.7** describes the process as a sequence of probabilistic events.

$$\frac{Mt}{Me} = 1 - \exp\left[-\left(\frac{t}{\alpha}\right)^{\beta}\right] \qquad (\mathbf{5.8})$$

where, M_t = amount of nisin released at time t

M_e = amount of nisin released at equilibrium

α = scale parameter (s)

β = Weibull shape parameter

The scale parameter ‘α’ is related to the reciprocal of the process rate constant. The scale parameter defines the rate and represents the time needed to accomplish 63% of the process. Weibull shape factor ‘β’ describes the shape of the curve and therefore could be used to describe mechanisms like diffusion, relaxation and convection. When $\beta = 1$, the Weibull distribution describes first order kinetics (Saguy *et al.,* 2005) and when $\beta > 1$, the model indicates that a complex process governs the nisin release (Mateus et al., 2007).

In the present research, Weibull model was used to describe the nisin release profiles for different corn zein concentrations and the predicted values were compared with that of experimental results. Weibull model was chosen to describe the profile of nisin released because, the Weibull parameters α and β would indicate the nisin release rate and mechanism of release respectively. Moreover, Weibull scale parameter α defines the rate and represents the time required to accomplish 63% of the release, which is comparable to the short time release ($M_t/M_\infty < 2/3$) in mechanistic diffusion model. Similarly, Weibull shape parameter β is comparable to the diffusion component n in the power law model. Thus a good comparison could be made between the empirical Weibull model and mechanistic diffusion model. The sum of least squares of the residuals (difference between experimental and predicted values) was minimized, subject to parameters α and β, using a 'Solver' function (Excel 2007).

Chapter 6

Results and Discussion

The overall goal of the project was to develop a biopolymer based matrix for controlled release of nisin for food packaging applications. Different sections of this chapter include:

- antimicrobial activity of the films
- validation experiments

6.1 Antimicrobial activity of various films

After various biopolymer films incorporated with Nisaplin® were developed, it was imperative to ensure that the films developed were antimicrobially active. Seven mm discs were cut from the films and placed on agar plates seeded with *Micrococcus luteus*, an indicator organism that is sensitive to nisin.

6.1.1 Antimicrobial activity of locust bean and xanthan films

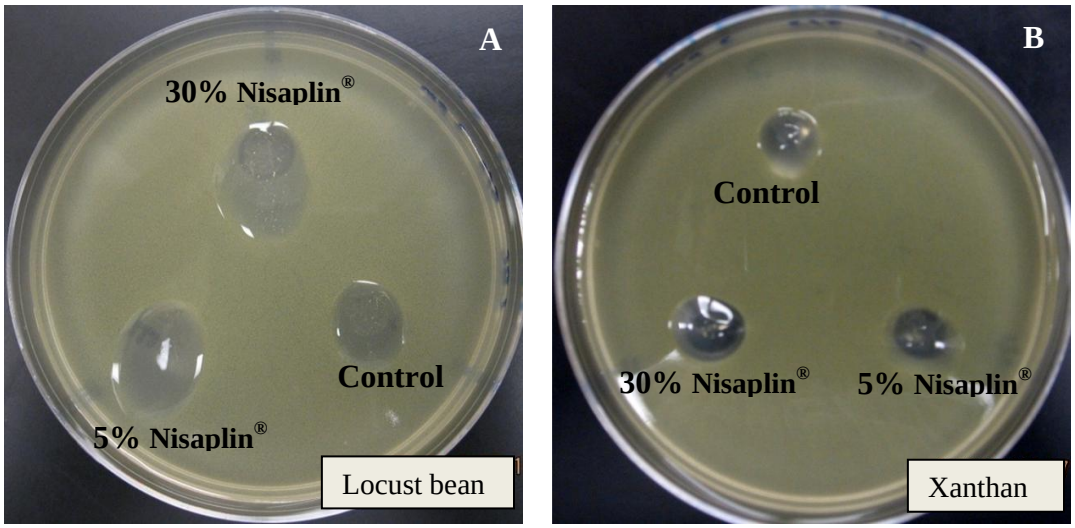


Fig. **6-1**: Antimicrobial activity of nisin incorporated (a) locust bean & (b) xanthan films (Nisaplin® was incorporated on percentage weight basis of the biopolymer)

The films made of locust bean and xanthan gums were transparent and flexible. As seen in Fig. **6-1**, these films did not form a clear zone but rather, collapsed due to its hydrophilic nature and formed a blob on the agar surface. Hence, these films were not found suitable for aqueous systems (beverages, meat and other high moisture foods). However, these films may be used as a nisin incorporated coating for intermediate moisture foods such as cake, cheese etc.

6.1.2 Antimicrobial activity of carrageenan films

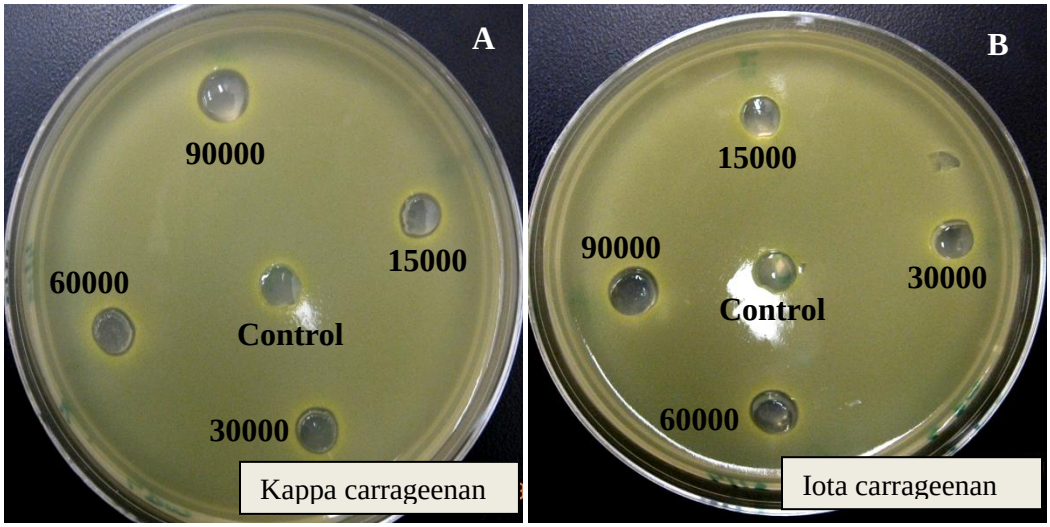


Fig. **6-2**: Antimicrobial activity of nisin incorporated (A) Kappa carrageenan & (B) Iota carrageenan films (Nisin activity expressed in IU).

Various amounts of Nisaplin® were mixed in 2% w/w kappa carrageenan and iota carrageenan films to give a nisin activity ranging from 15000 to 90000 IU. The carrageenan films were transparent and flexible. However, they did not release enough nisin to produce a distinguishable inhibition zone (Fig. **6-2**). The small tint seen around the discs may simply be a surface phenomenon (small amounts of nisin on the film surface). Cha *et al.* (2003) incorporated nisin in films made of different biopolymers including kappa carrageenan. It was observed from an agar diffusion test that nisin was not released from 2.5 and 5% (w/w) kappa carrageenan matrix. This observation was attributed to the ionic binding of cationic nisin by anionic (several $-OSO_3$ groups) kappa carrageenan. Similar reports of ionic binding of nisin to biopolymers are reported elsewhere (Guo *et al.,* 1998 and Hoffman *et al.,* 2001).

In another experiment, kappa carrageenan films of concentration 0.2, 0.5 and 1% (w/w) were mixed with 300 mg Nisaplin® (300,000 IU). However, reducing the carrageenan concentration and increasing the nisin content did not result in any significant amount of nisin activity in assays. In a similar study, Cha *et al.* (2002) mixed nisin in 1% (w/w) kappa carrageenan films and found that the nisin release from the film was very low (zone size less than 2 mm).

Further experiments demonstrated that kappa and iota (gelling carrageenans) did not show any release while non-gelling carrageenan lamda did exhibit nisin release (Fig. **6-3**). Therefore, nisin release from carrageenan is not solely prevented by ionic binding as inferred by Cha *et al.* (2003). Kappa, iota and lamda have 1, 2 and 3 ester sulfate (-OSO3) groups per disaccharide respectively, which contribute to their negative charge. There is a strong possibility that nisin was physically entrapped in the gel matrix formed by kappa or iota carrageenan while it diffused through the non-gelling lamda carrageenan. Due to hydration and polymer relaxation, the films swell (as evident in Fig. **6-2**) and therefore, may not release nisin. Baumgartner *et al.* (2005) reported that when a hydrophilic matrix comes in contact with water, it swells due to hydration and the gel layer constitutes a diffusional barrier that reduces further water uptake and curtails subsequent release of active compound.

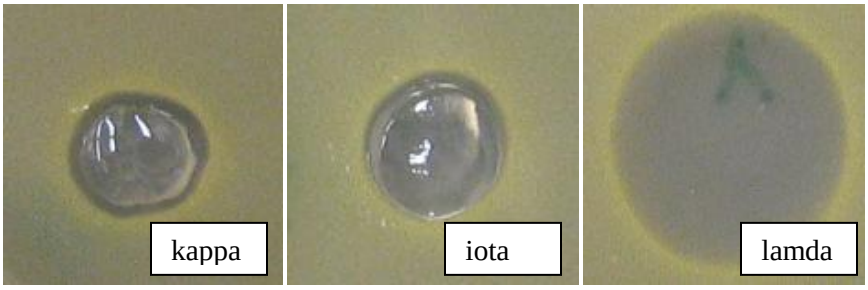


Fig. **6-3**: Antimicrobial activity of nisin incorporated films made of kappa, iota & lamda carrageenan (nisin activity of 90000 IU).

6.1.2.1 Antimicrobial activity of blended films made from kappa & lamda carrageenan and iota & lamda carrageenan films

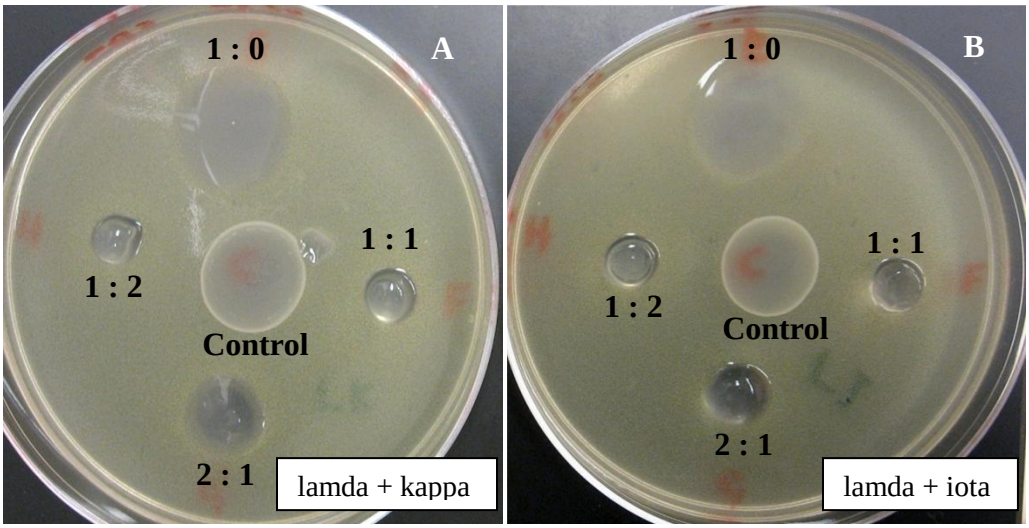


Fig. **6-4**: Antimicrobial activity of nisin incorporated blended films with different proportions of (A) lamda & kappa carrageenan (B) lamda & iota carrageenan.

Since lamda is a non-gelling carrageenan, nisin incorporated films made with pure lamda completely dissolved on the agar plates. However, these films did show some amount of nisin activity (Fig **6-3** & **6-4**). Blended films were made with varying proportions of lamda, incorporated with kappa and iota carrageenan respectively. From

Fig. **6-4**, it is evident that the nisin release increased, as the amount of lamda in the matrix increased. However, the films when submerged in an aqueous solution, formed a highly viscous solution. This viscosity made it difficult to filter prior to performing HPLC for nisin quantification.

After 48 hours encroachment of the *Micrococcus luteus* (yellow colored colonies) into the inhibition zones were observed. This indicates that at the periphery of the inhibition zones, nisin concentration is less and *M. Luteus* probably developed some resistance over a period of time.

6.1.2.2 Antimicrobial activity of blended films made from kappa carrageenan & hydroxypropylmethyl cellulose (HPMC)

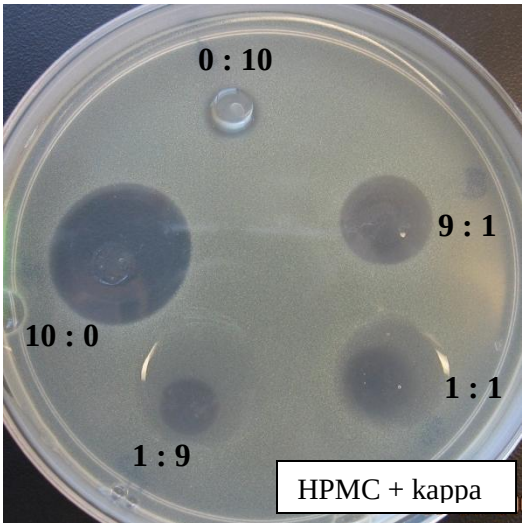


Fig. **6-5**: Antimicrobial activity of nisin incorporated blended films made of different proportions of HPMC & kappa carrageenan.

HPMC, a soluble biopolymer, formed transparent and flexible films which completely dissolved in agar plates. To improve the physical integrity and nisin release,

blended films of kappa carrageenan and non-ionic HPMC were made. As evident from Fig. **6-5**, nisin incorporated blended films made of kappa carrageenan and HPMC exhibited nisin release. Nisin release increased with an increase in HPMC content in the matrix. In a similar study, Cha *et al.* (2003) developed nisin incorporated blended films consisting of kappa carrageenan and methyl cellulose (MC 400 cps) in the ratio 1:1. The nisin activity observed in this matrix was attributed to the presence of non-ionic water soluble methyl cellulose. Grower *et al*. (2004) also reported that it is difficult to attain controlled and consistent release from a soluble matrix. Nevertheless, the blended films made by incorporating soluble components like lamda carrageenan or HPMC are antimicrobially active and could form an effective antimicrobial coating over intermediate moisture foods.

The blended films made of kappa carrageenan and HPMC, when submerged in an aqueous solution, formed a highly viscous solution. This viscosity made it difficult to filter the solution prior to performing HPLC for nisin quantification. The blended films made with lamda carrageenan also displayed similar behavior. The films made of HPMC & kappa carrageenan produced an unpleasant odor when immersed in water for more than 48 hours, the cause for which could not be ascertained.

6.1.3 Antimicrobial activity of corn zein films

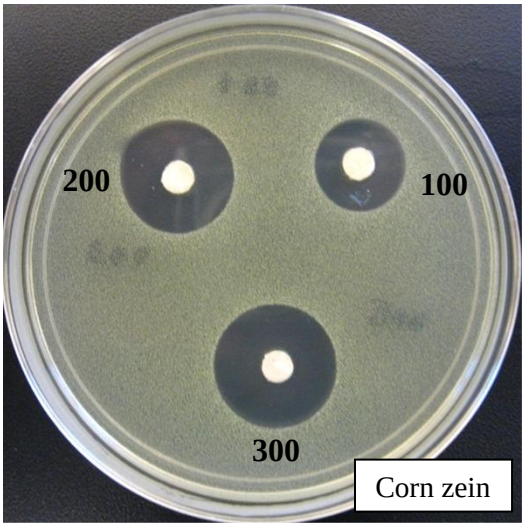


Fig. **6-6**: Antimicrobial activity of corn zein films incorporated with different amounts of Nisaplin® (mg).

Corn zein is immiscible in water and therefore, dissolved in 95% ethyl alcohol to cast films. Cast corn zein (CZ) films were slightly brittle compared to the previously discussed films made with locust bean, xanthan and carrageenan. Corn zein films had an off-white to pale yellow color. Differential scanning calorimetry showed that these films had a Tg of 18.4 °C. Discs cut out from corn zein films containing different amounts Nisaplin® were placed on agar plates. As seen in Fig. **6-6**, corn zein films exhibited nisin activity by producing distinct zones. The zone size also increased with an increase in Nisaplin® content.

It was hypothesized that by increasing the corn zein concentration in nisin incorporated films, the nisin release would decrease. Films made from 4, 8 and 12% (w/v) corn zein were prepared and were immersed in DI water to study nisin activity of

the released solution. Since corn zein is not miscible in water, the films remained stable. Wells of diameter 7 mm were made in the agar plates seeded with *Micrococcus luteus* and 50 µl of the release solutions were dispensed into the wells to study the nisin activity.

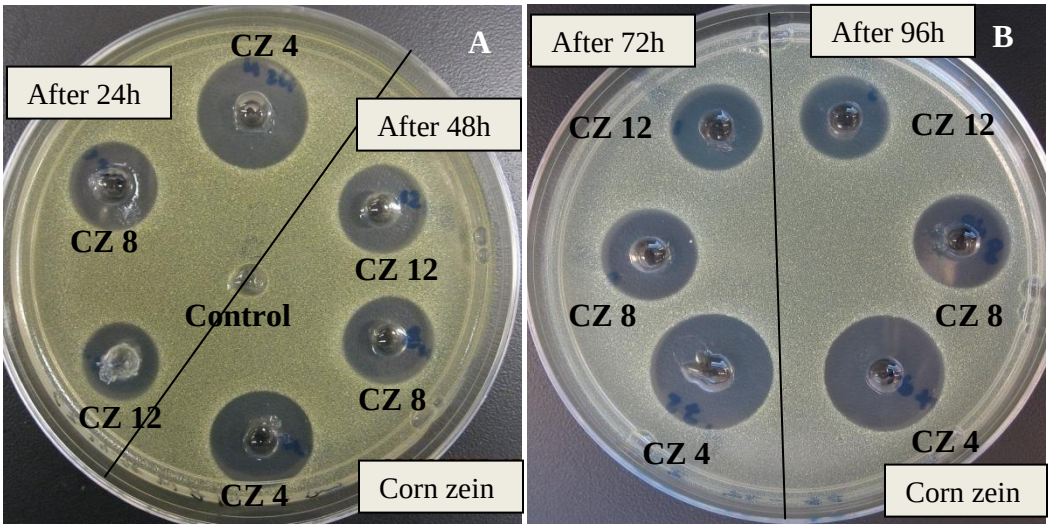


Fig. **6-7**: Antimicrobial activity of release solutions taken every 24 hours from corn zein films of different concentrations

It is clear from Fig. **6-7** that, nisin release decreased as the corn zein concentration in the film increased from 4-12% (w/v), which concurs with the hypothesis. At the same time, there was no significant change in zone diameters over time for a particular concentration. Possible reasons for this observation are discussed in section **4.7.1.1**.

Teerakarn *et al.* (2002) investigated the effect of heat-pressed and cast corn zein films on nisin diffusion and its activity. Using the agar diffusion method, it was determined that cast corn zein films were able to release nisin.

6.2 Validation experiments

6.2.1 Nisin release kinetics of corn zein films

On the basis of initial studies, corn zein films at concentrations of 4, 6, 8 and 10% (w/v) were made, containing 3000 mg of Nisaplin® and cut into 10 pieces (3.5 x 3.5 cm) such that each piece of film contained 300 mg Nisaplin® (nisin activity of 300,000 IU). Such a high concentration of Nisaplin® was chosen in order to ensure that the amount of nisin released would be sufficient to be detected by the HPLC method. Nisin released from the films was tested as explained earlier in section **4.5**.

6.2.2 Nisin standard curve

Standard curves were made with agar diffusion method as well as using the HPLC technique. However, due to the non-reliability and inconsistency associated with agar method (section **4.7.1.1**), the standard curve developed with this method was not used for nisin quantification. The nisin standard curve developed by the HPLC method (Fig. **4-9**) was used to calculate unknown concentrations of nisin released from the corn zein films.

6.2.3 Effect of amount of Nisaplin® on release kinetics

Corn zein films were incorporated with varying amounts of Nisaplin® in order to ascertain whether nisin release was concentration-dependent. The release profile (Fig. **6-8**) indicates that corn zein 4% (w/v) films, incorporated with varying amounts of Nisaplin®, exhibited a concentration-dependent release profile which increases with time. Nisin release increased with an increase in Nisaplin® concentration in the film.

A power law model (Eq. **5.4**) was used to determine if the nisin diffusion was Fickian or non-Fickian. The parameters in Eq. **5.4** were calculated from the plot of $\ln(M_t/M_\infty)$ vs ln(t) for early portion of nisin release (Fig. **6-9**). Values for n were derived from the slope of the curves in Fig. **6-9**. Value of n varies from 0.3 to 0.46. Since values of $n < 0.5$, it appears that the diffusion mechanism exhibits pseudo-Fickian behavior. Pseudo-Fickian behavior resembles a Fickian behavior and the diffusion is not solely due to concentration gradient. This observation is contrary to the assumption that nisin diffusion from the corn zein matrix is Fickian in nature. In pseudo-Fickian mechanism, sorption curves resemble Fickian curves, but approach to final equilibrium is very slow (Khan & Rousseau, 2006).

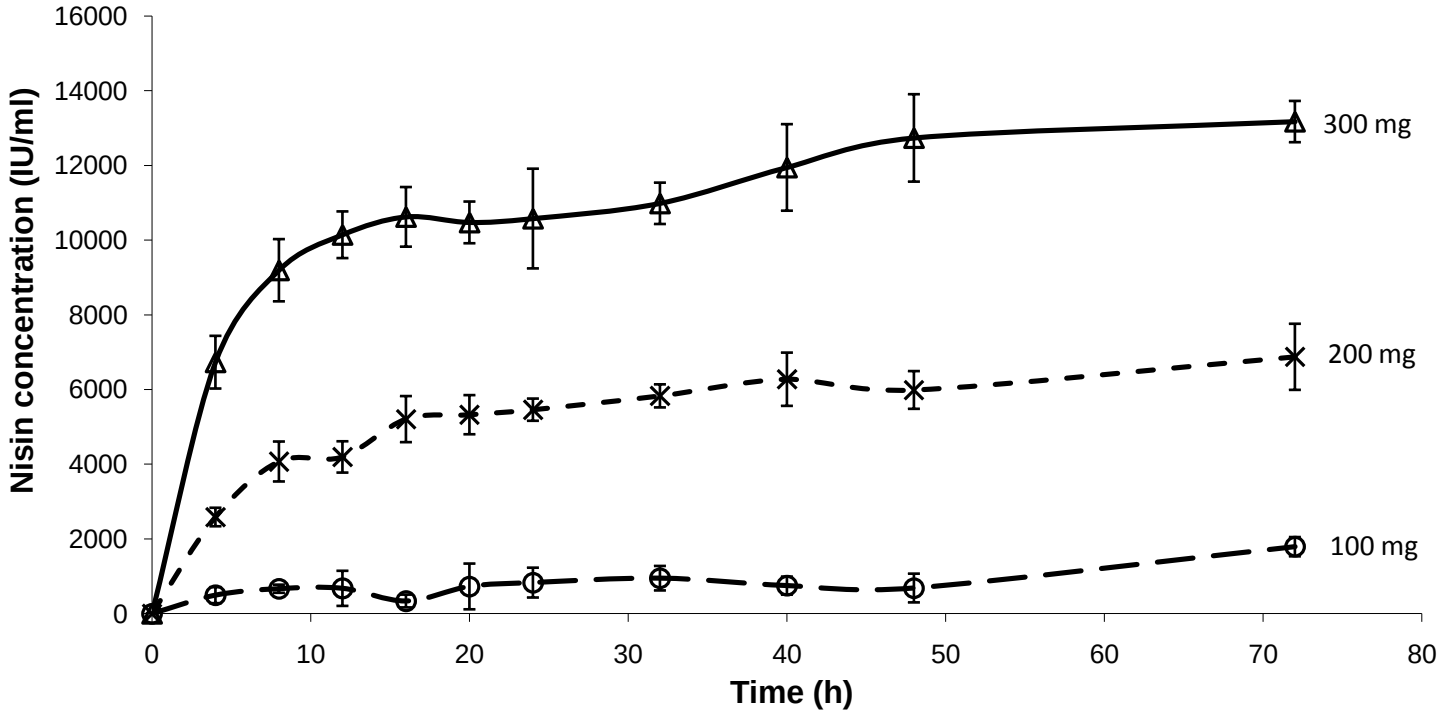


Fig. **6-8**: Nisin release after 72 h from corn zein 4% films containing different amounts of Nisaplin®. The films had an initial loading of 300, 200 and 100 mg Nisaplin® (1 mg Nisaplin® = 1000 IU). Each data point is an average of 4 replications (Error bars: ±1 S.D)

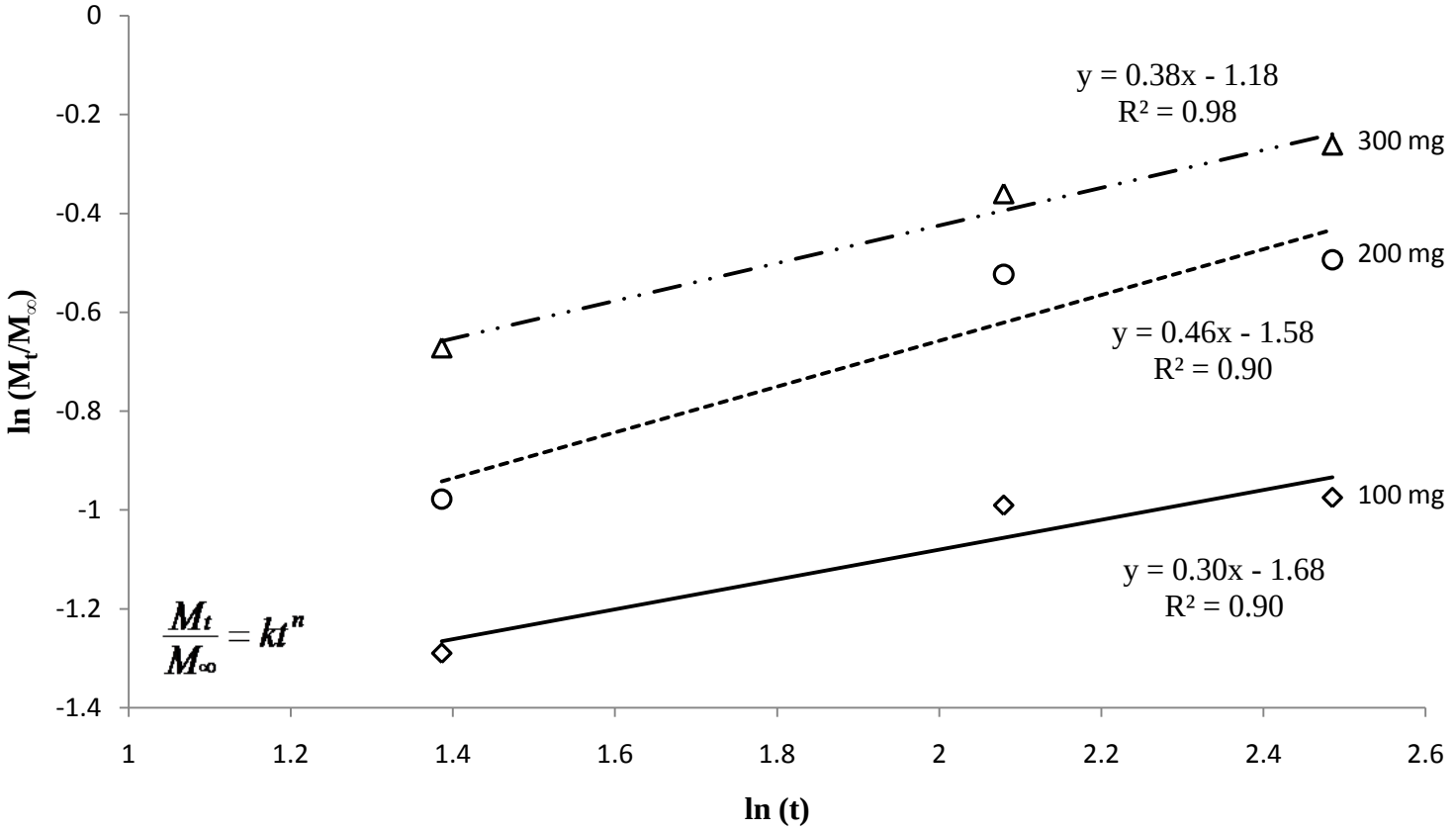


Fig. **6-9**: Plot of $\ln(M_t/M_\infty)$ vs ln(t) for early portion of nisin release from corn zein (4% w/v) films had an initial loading of 300, 200 & 100 mg Nisaplin®. Symbols represent the experimental data and lines show the trend. The slope of the curve represented by n (power law function shown in the inset) indicates the mechanism of release. M_t is the amount of nisin released in time t and M_∞ is the amount of nisin released at infinite time.

6.2.3.1 Nisin diffusivity (D) calculation

A plot of $M_{t'}/M_0$ vs t demonstrates desorption of nisin. If diffusion is Fickian with constant diffusivity D, plot of $M_{t'}/M_0$ vs $\sqrt{t}$ will yield a straight line with slope k, as shown in Fig. **6-10**. The data is forced into a linear relationship to get the slope k, which is used for calculating diffusivity D using Eq. **5.7**. The calculated diffusivity values are shown in Table **6-1**.

Table **6-1** Effect of Nisaplin® concentration in the film on nisin diffusivity

Amount of Nisaplin® (mg)	**Film thickness, h (cm)**	**Slope, k**	**Difffusion coefficient, D x 10^{-11} (cm^2/s)**
100	0.024	-0.006	2 ± 0.9^{a}
200	0.029	-0.040	17 ± 1.6^{b}
300	0.032	-0.085	38 ± 5.4^{c}

Diffusion coefficients with same superscripts are not significantly different at $p < 0.05$

Figure **6-11** demonstrates the effect of initial Nisaplin® concentration on nisin diffusivity. It is evident from Table **6-1** and Fig. **6-11** that nisin diffusivity through the film increases as amount of Nisaplin® in the film increases. In Table **6-1**, diffusivities with different superscripts are significantly different from each other. This shows that nisin release from corn zein film depends on the Nisaplin® concentration in the film ($p<0.05$). Nisaplin® contains almost 75% NaCl and 2.5% pure nisin, both of which are soluble in water. Nisaplin® incorporated in the film may have increased the hydrophilicity of the corn zein matrix, thus increasing its interaction with the solvent, which in turn, may have increased the diffusivity of nisin. Similar arguments were

presented by Ozdemir and Floros (2001) and Miltz and Rosen-Doody (1984) for potassium sorbate and styrene diffusion, respectively.

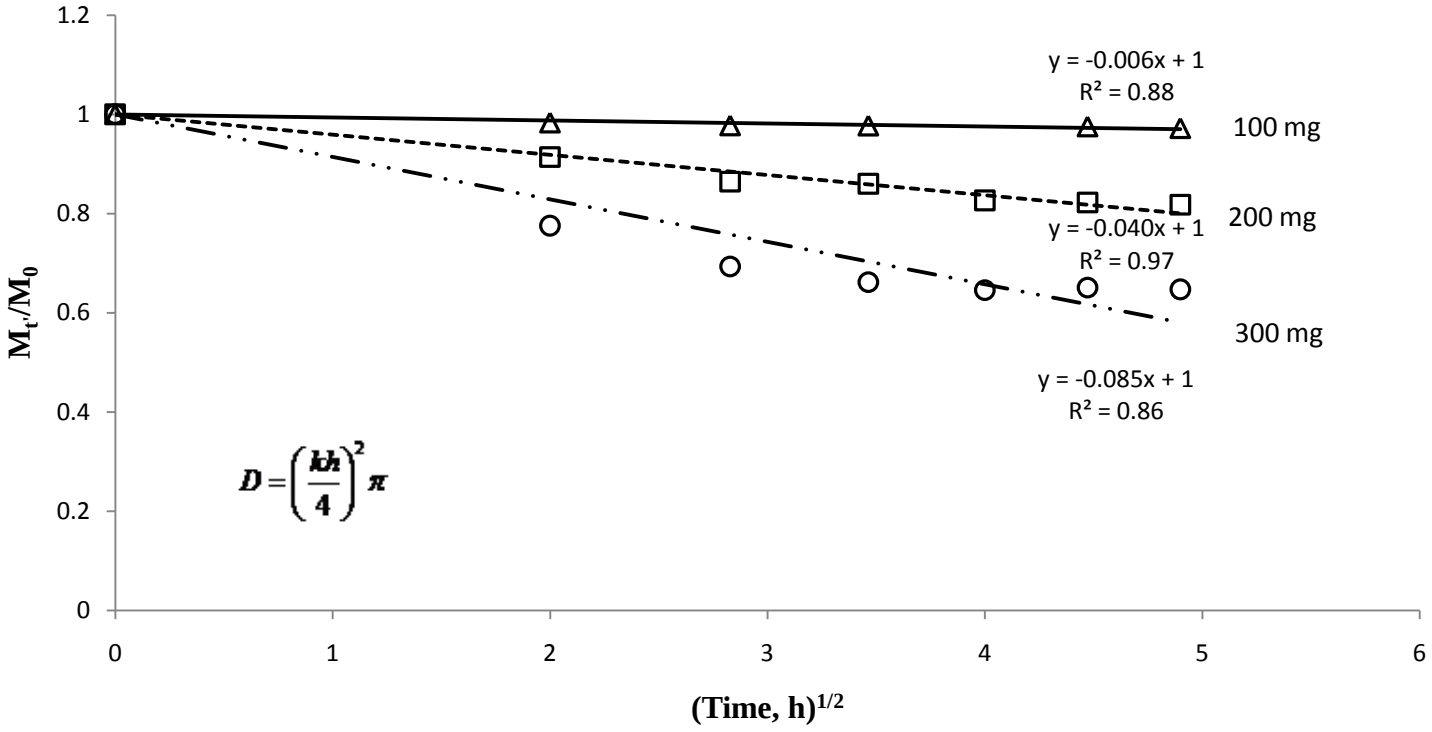


Fig. **6-10**: Nisin desorption after 24 h from films of corn zein concentration 4% (w/v). Symbols represent the experimental data and lines show the trend. The slope of the curve represented by k is used for calculating diffusivity D, using the formula shown in the inset. $M_{t'}$ is the amount of nisin retained in the film in time t and M_0 is the amount of nisin initially incorporated in the film.

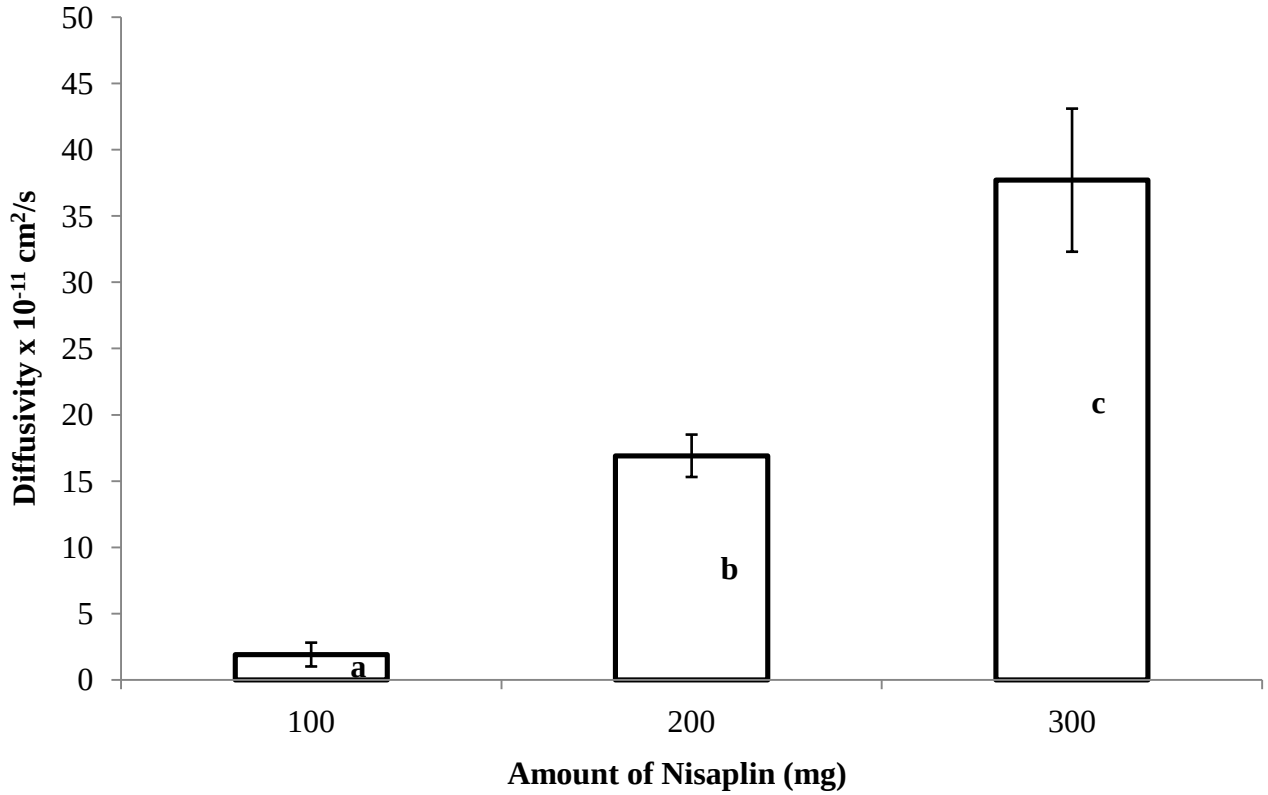


Fig. **6-11**: Effect of amount of Nisaplin® in the film on nisin diffusivity. Each data point is an average of 4 replications (Error bars: ±1 S.D). Diffusivities with same alphabets are not significantly different at $p < 0.05$

6.2.4 Effect of corn zein concentration on release kinetics

Figures **6-12** and **6-13** demonstrate the nisin release profile of corn zein films of concentration 4, 6, 8 and 10% (w/v) in 24 and 72 h, respectively. It is hypothesized that an increase in the concentration of corn zein in the film would result in decrease in the nisin release from the film. From Fig. **6-12** and **6-13**, it could be inferred that nisin release decreases as concentration of corn zein in the film matrix increases, which concurs with the hypothesis. Short time release from 4% films is comparatively higher. This may be due to increased solvent diffusion into the matrix and subsequent nisin release from the matrix at low polymer concentration. As the concentration of corn zein in the film increases, the polymer density increases. This phenomenon increases the tortuosity of the matrix, thus affecting the nisin diffusion.

Ripoche *et al*. (2006) investigated the mass transfer of pure nisin in agarose gels at concentrations of 3, 4 and 7% (w/w). They found that nisin diffusivity decreased with increase in agarose concentration in the gel. They further reported that, as the agarose content increased, the path length of diffusion increased, which in turn, limited nisin diffusion. In a similar study, Sebti *et al.* (2004) reported significant reduction in nisin diffusion as the agarose content increased from 3 to 8% (w/w) and attributed this observation to an increase in path length of diffusion. Giannakopoulos & Guilbert (1986) reported similar results for sorbic acid diffusion in agar gels and attributed the decrease in diffusivity to an increase in gel rigidity. Ozdemir and Floros (2003) investigated

potassium sorbate diffusion in whey protein films. They observed a decrease in sorbate diffusivity as the protein concentration in the film increased.

Zilberman & Sofer (2006) studied the controlled release of an enzyme, horseradish peroxidase (HRP) from composite fiber structures. They found that an increase in polymer concentration in the matrix resulted in smaller burst release and a more moderate release profile. They argued that as the polymer concentration increased, the viscosity and polymer density also increased. As a result the solvent diffusion into the matrix decreased and also reduced the free volume available for diffusion of the active compound. Davis (1974), Sun & Wang (1995) and Sing & Nigam (1979), in similar studies, observed a reduction in active compound diffusivity as the polymer concentration in the matrix increased.

It is evident from Fig. **6-13** that effect of corn zein concentration on nisin release is more evident at low corn zein concentrations. In films with corn zein concentration 8 and 10%, short time nisin release is different for both the concentrations. After about 40 h the release from 8 and 10% concentration films were almost similar. Probably once the polymer matrix is completely hydrated, polymer relaxation takes place and release from both this matrix is similar irrespective of the corn zein concentration in the two films.

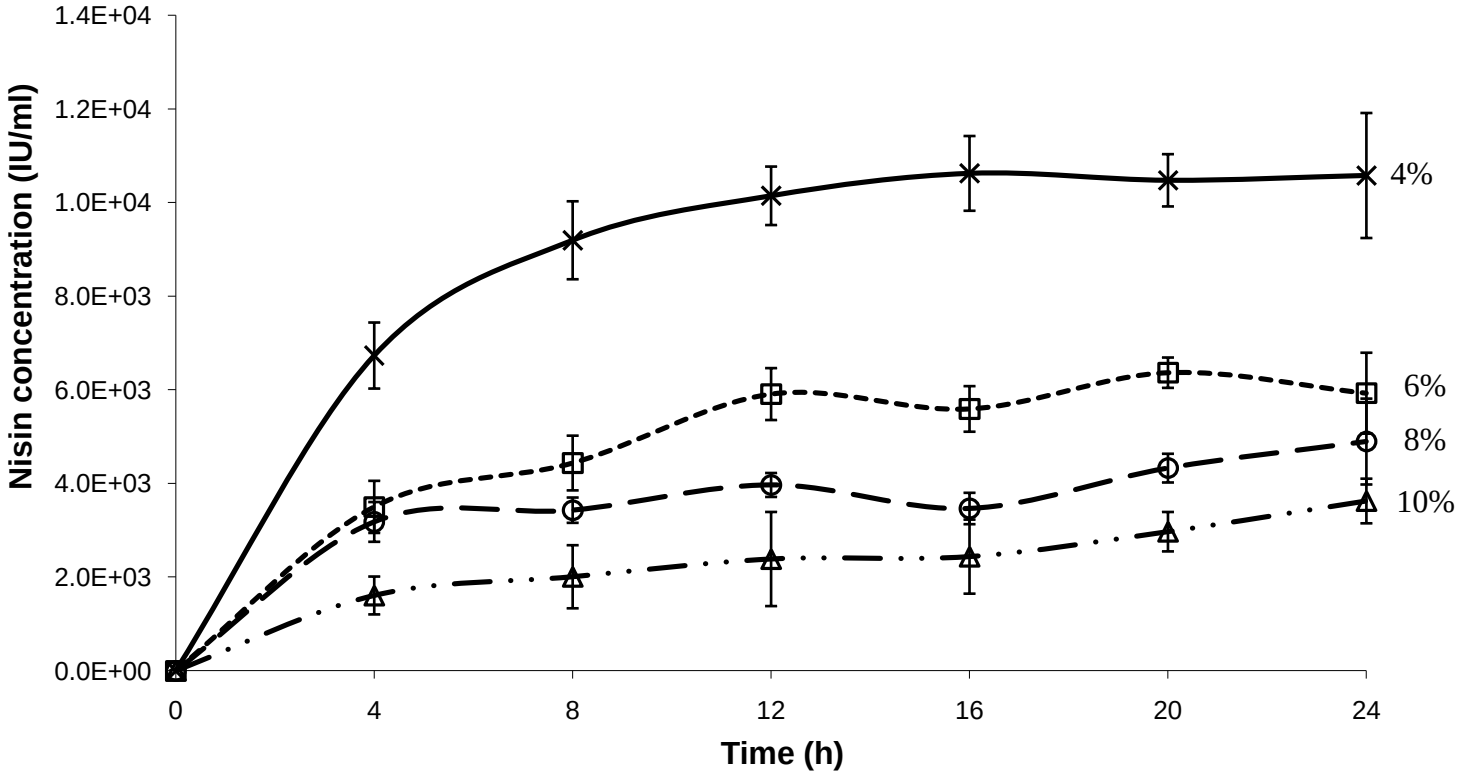


Fig. **6-12**: Nisin release in citrate buffer (3 pH, 25 ^{0}C) after 24 h from corn zein films of concentration 4, 6, 8 and 10% (w/v). Each piece of film is loaded with 300 mg Nisaplin®. Each data point is an average of 4 replications (Error bars: $\pm$1 S.D).

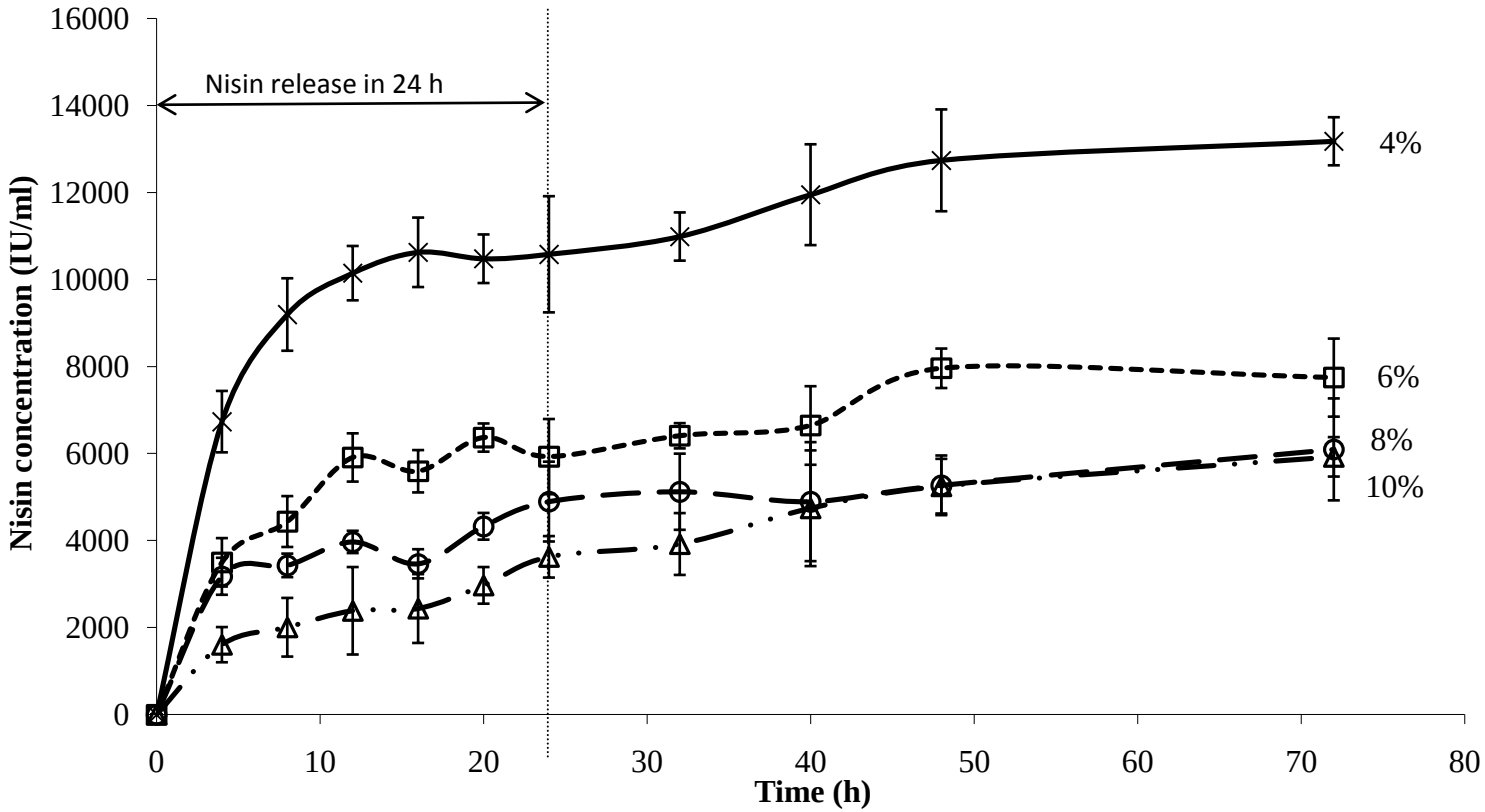


Fig. **6-13**: Nisin release in citrate buffer (3 pH, 25 ^{0}C) after 72 h from films of corn zein concentration 4, 6, 8 and 10% (w/v). Each piece of film contains 300 mg Nisaplin®. Each data point is an average of 4 replications (Error bars: $\pm$1 S.D).

A power law model (Eq. **5.4**) was used to determine if the nisin diffusion was Fickian or non-Fickian. The parameters in Eq. **5.4** were calculated from the plot of $\ln(M_t/M_\infty)$ vs ln(t) for early portion of nisin release (Fig. **6-14**). Values for n were derived from the slope of the curves in Fig. **6-14**. Value of n varies from 0.19 to 0.46. Since values of $n < 0.5$, it appears that the diffusion mechanism exhibits pseudo-Fickian behavior. This observation is contrary to the assumption that nisin diffusion from the film matrix is Fickian in nature. In pseudo-Fickian mechanism, sorption curves resemble Fickian curves, but approach to final equilibrium is very slow (Khan & Rousseau, 2006). Teerakarn *et al*. (2002) studied nisin diffusion in cast corn zein films and observed that the kinetics of nisin diffusion followed a Fickian diffusion model. Sebti *et al.* (2004) and Ripoche *et al.* (2006) studied nisin diffusion in agarose gel and observed that nisin diffusion shows a Fickian behaviour.

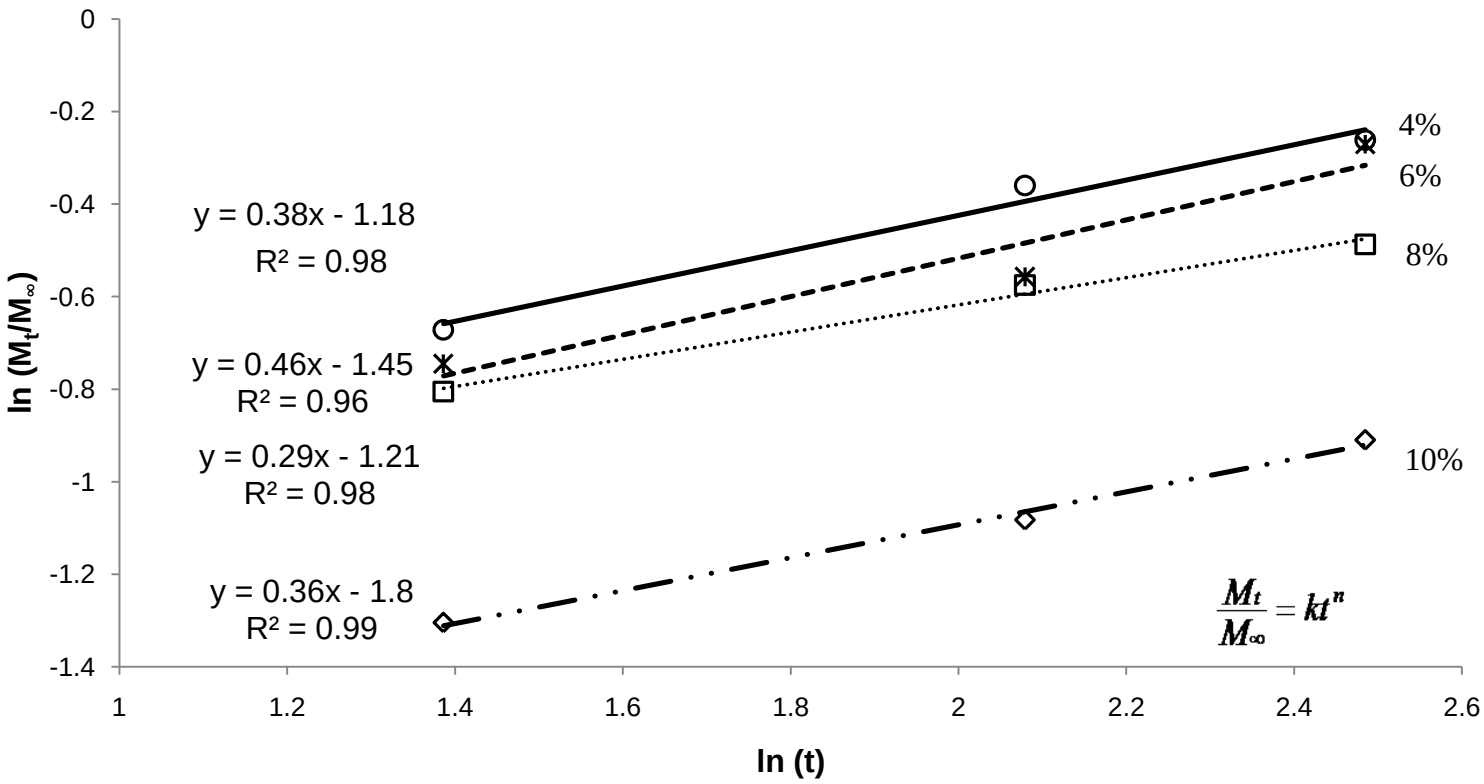


Fig. **6-14**: Plot of $\ln(M_t/M_\infty)$ vs $\ln(t)$ for early portion of nisin release from films of corn zein concentration 4, 6, 8 and 10% (w/v). Symbols represent experimental data and lines show the trend. The slope of the curve represented by n (power law function shown in the inset) indicates the mechanism of release. M_t is the amount of nisin released in time t and M_∞ is the amount of nisin released at infinite time.

6.2.4.1 Nisin diffusivity (D) calculation

A plot of M_t/M_0 vs t demonstrates desorption of nisin. If diffusion is Fickian with constant diffusivity D, plot of M_t/M_0 vs $\sqrt{t}$ will yield a straight line with slope k, as shown in Fig. **6-15**. The data is forced into a linear relationship to get the slope k, which is used for calculating diffusivity D using Eq. **5.7**. The calculated diffusivity values are shown in Table **6-2**.

Table **6-2** Effect of corn zein concentration in the film on nisin diffusivity

Corn zein conc. (% w/v)	**Film thickness, h (cm)**	**Slope, k**	**Difffusion coefficient, D x 10^{-11} (cm^2/s)**
4	0.0319	-0.085	38 ± 5.4^{a}
6	0.0384	-0.047	17 ± 3^{b}
8	0.0479	-0.034	15 ± 3^{bc}
10	0.0537	-0.023	8 ± 2^{cd}

Diffusion coefficients with same superscripts are not significantly different at $p < 0.05$

Figure **6-16** demonstrates the effect of corn zein concentration on nisin diffusivity. It is evident from Table **6-2** and Fig. **6-16** that nisin diffusivity through the film decreases as concentration of corn zein in the film increases. In Table **6-2**, diffusivities with different superscripts are significantly different. There is a fourfold decrease in diffusivity as corn zein concentration increases from 4 to 10% (w/v). This shows that nisin release from the film depends on the corn zein concentration in the film ($p < 0.05$).

The diffusivity values are comparable to the nisin diffusivity reported by Teerakarn *et al*. (2002) and Buonocore *et al.* (2003) who studied nisin release in water. Sebti *et al.* (2004) and Ripoche *et al.* (2006) studied nisin diffusivity in agarose gels and observed that nisin diffusivity decreases with an increase in agarose content in gel. Zilberman & Sofer, 2007 studied the release of an enzyme, horse radish peroxidase from composite fiber structures. They found that increase in polymer concentration in the matrix, increases the polymer density thus reducing the free volume. This in turn slows down the solvent diffusion into the matrix and subsequent release of the active compound from the matrix. Similar observations were also reported by Sun & Wang, 1995 and Sing & Nigam, 1979.

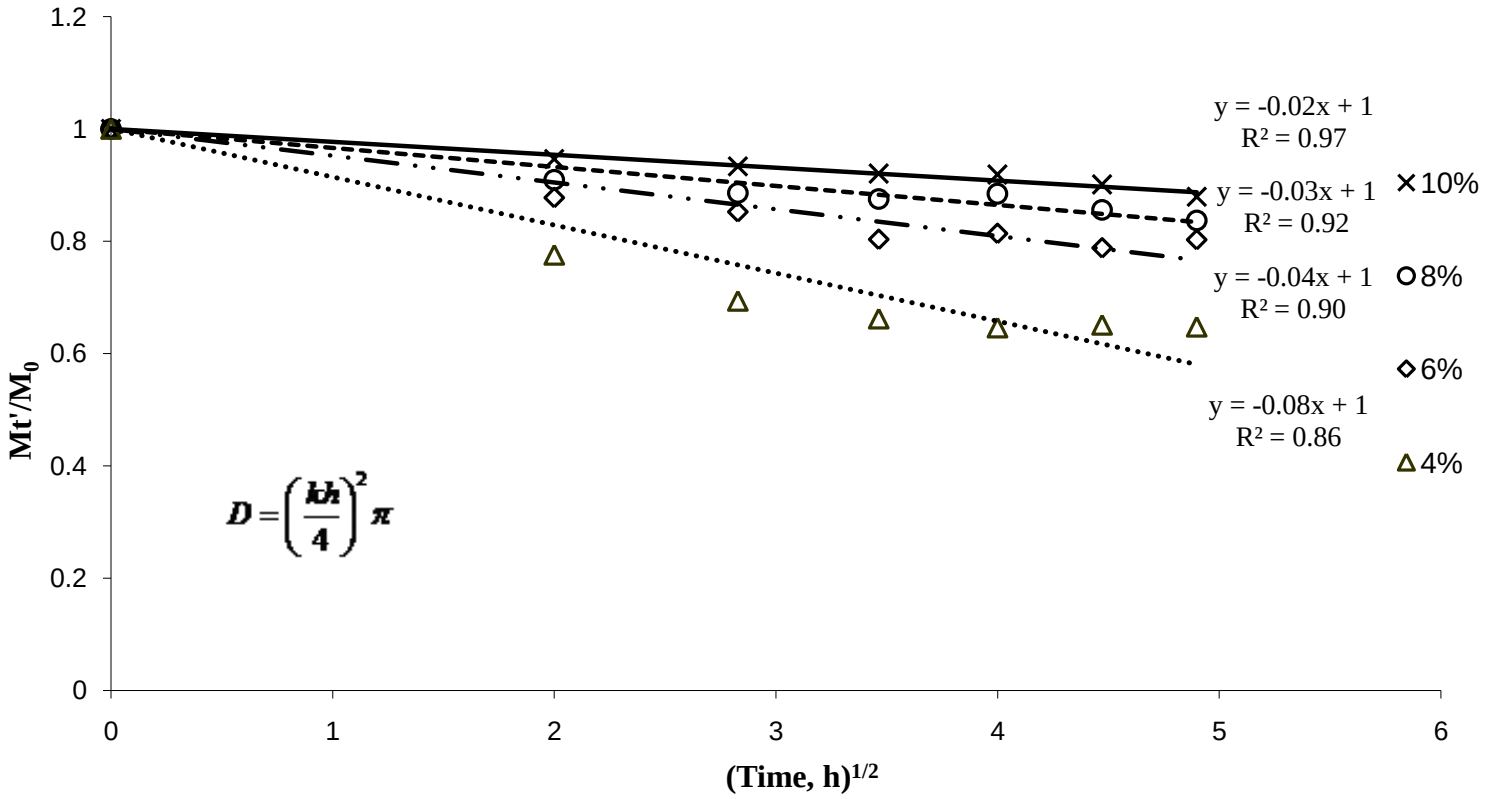


Fig. **6-15**: Nisin desorption after 24 h from films of corn zein concentration 4, 6, 8 and 10% (w/v). Symbols represent experimental data and lines show the trend. $M_{t'}$ is the amount of nisin retained in the film in time t and M_0 is the amount of nisin initially incorporated. The slope of the curve represented by k is used for calculating diffusivity D, using the formula shown in the inset.

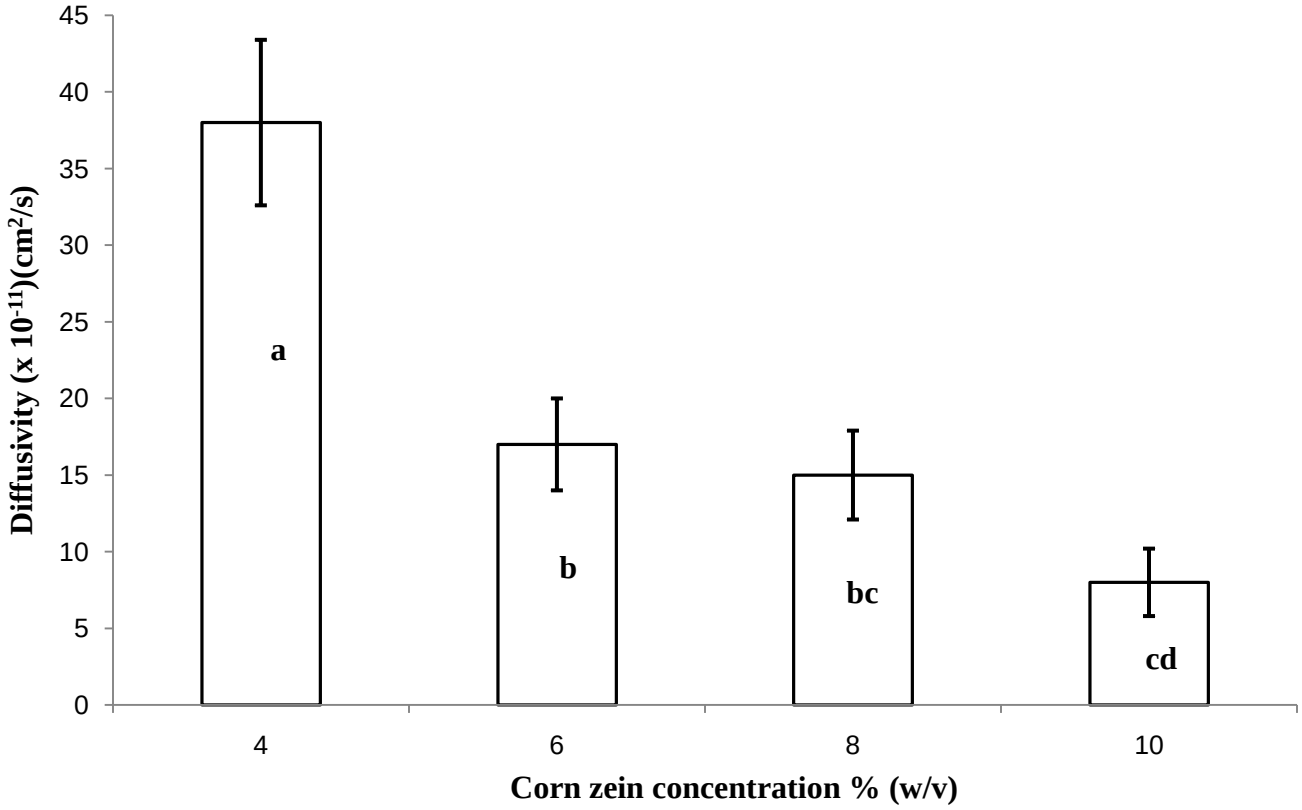


Fig. **6-16**: Effect of corn zein concentration in the film on nisin diffusivity. Each data point is an average of 4 replications (Error bars: $\pm$1 S.D). Diffusivities with same alphabets are not significantly different at $p < 0.05$

6.2.5 Nisin release: Weibull modeling

Fig. **6-17** demonstrates the experimental and predicted (Weibull model) nisin release from films of different corn zein concentration plotted as a function of time. The sum of least squares of the residuals (difference between experimental and predicted values) were minimized subject to parameters α and β using a 'Solver' function (Excel 2007). Weibull model gives an excellent prediction of experimental data with a coefficient of determination (R^2) 0.95 and above. The parameters α and β are shown in Table **6-3**.

Table **6-3** Parameters α and β calculated from Weibull model for films of corn zein concentration 4-10% (w/v)

Corn zein conc. (% w/v)	**Scale parameter (α)**	**Shape factor (β)**
4	6.73	0.49
6	9.37	0.59
8	11.28	0.48
10	25.21	0.90

A lower value of scale parameter α indicates that the process is faster at the beginning of the release (Mateus *et al.*, 2007). As seen in Table **6-2**, as corn zein concentration increases from 4 to 10% (w/v), there is also an increase in α from 6.73 to 25.21. This observation demonstrates that initial release is faster for lower corn zein concentrations as compared to that of higher corn zein concentrations. This finding is in agreement with the results (Fig. **6-16**). As corn zein concentration increases, the polymer

density in the matrix increases thus reducing the free volume. This in turn slows down the solvent diffusion into the matrix and subsequent release of nisin from the matrix. Similar observations were reported by Zilberman & Sofer (2007), Sun & Wang (1995), Sing & Nigam (1979) and Davis (1974).

Weibull factor β describes the shape of the release curve. Papadopoulou *et al*. (2006) report in a Weibull model,

$\beta < 0.75$ indicate Fickian diffusion and

$0.75 < \beta < 1$ indicates a combined mechanism (Fickian & case II transport)

$\beta = 1$ indicates first order kinetics

$\beta > 1$ indicates a complex mechanism

As evident from the β values (Tab. **6-3)**, corn zein films with concentrations 4, 6 and 8% (w/v) indicate a Fickian diffusion. However, unlike the 'n' component in 'Power law model', Weibull shape factor β does not differentiate between Fickian and pseudo-Fickian diffusion. Films with a corn zein concentration 10% (w/v) has a β value of 0.9, which indicates a combined mechanism of Fickian and case II transport (non-Fickian). At a corn zein concentration of 10% (w/v), probably release is governed by a nisin concentration gradient as wells as some other mechanism. After 40 h, release from films of 8 and 10% corn zein concentration are similar. Probably the effect of corn zein concentration in the film on nisin release is more evident at low concentrations.

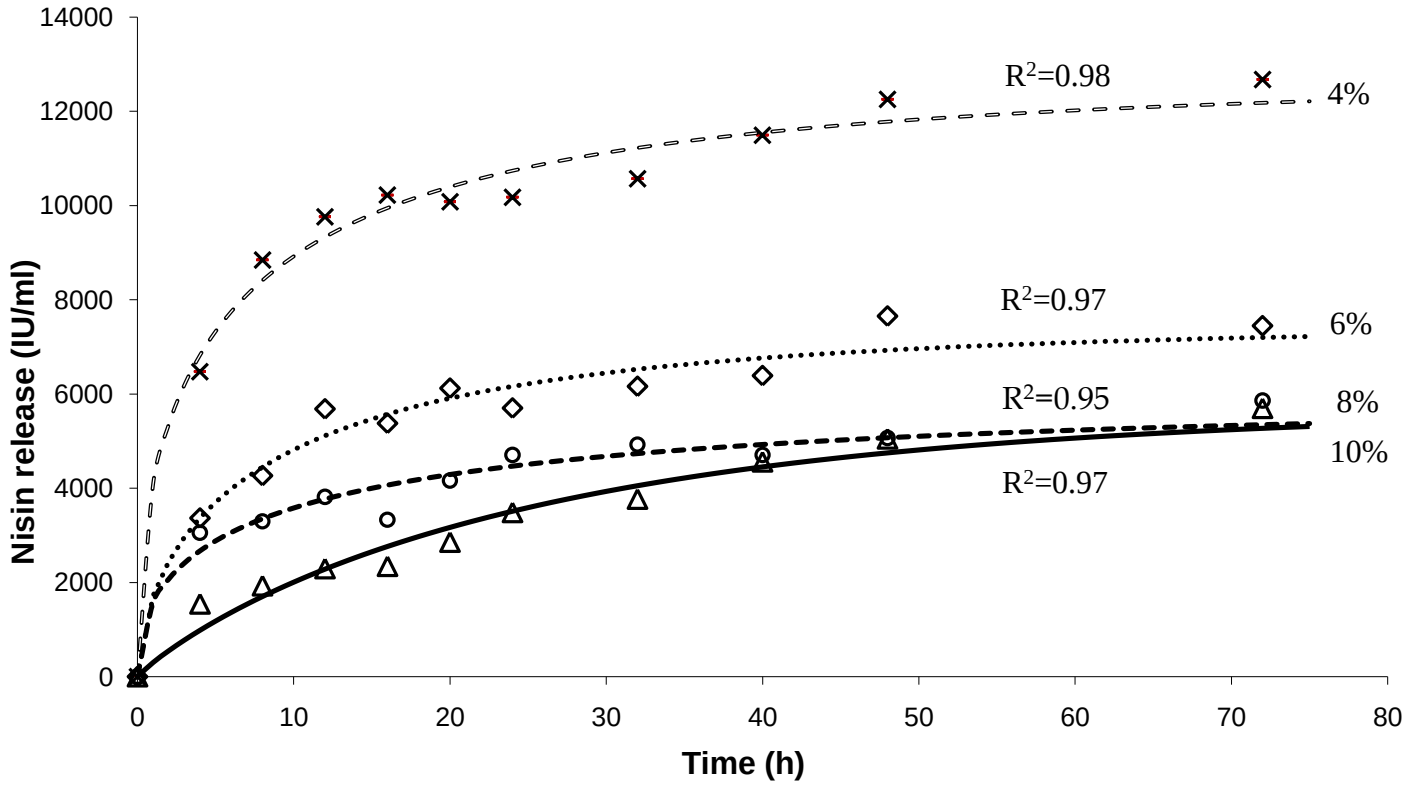


Fig. **6-17**: Experimental (symbols) and Weibull predicted (lines) nisin release from corn zein films in 72 h. CZ 4 to CZ 10 represent corn zein concentration 4, 6, 8 and 10% (w/v) in the film.

6.3 Microstructure of corn zein films

The microstructure of corn zein films of different concentration were studied with the help of a scanning electron microscope. A cross sectional view of corn zein (4%) film was done to study the distribution of Nisaplin® in the film before and after immersing in citrate buffer for 48 h, as demonstrated in Fig. **6-18 (A & B)** respectively. The images were captured at a magnification of 1200X. Fig. **6-18 A** demonstrates the uniform distribution of Nisaplin® in the corn zein film (the black arrows show NaCl crystals as Nisaplin® consists of 75% NaCl). Distribution of NaCl crystals show that Nisaplin® is uniformly distributed in the film. However, the crystals are not visible in Fig. **6-18 B** when films were submerged in citrate buffer, due to dissolution of the salt crystals.

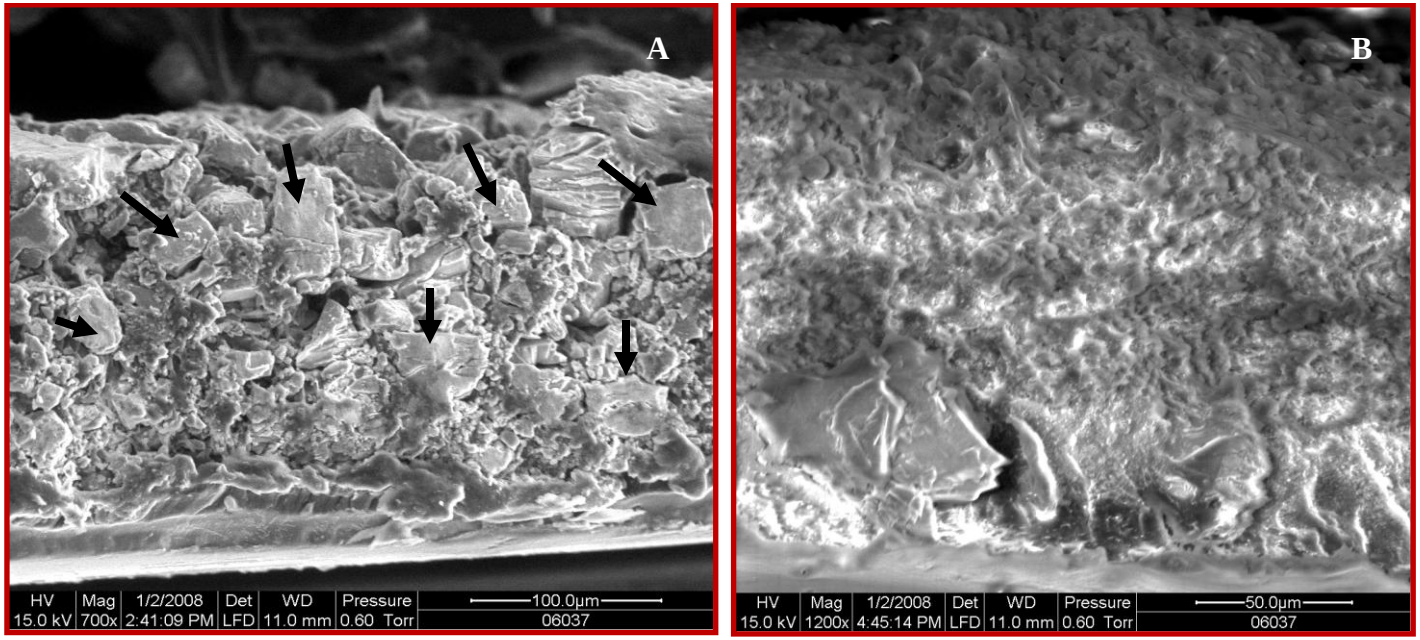


Fig. **6-18**: Cross-sectional SEM images of CZ 4 films (corn zein 4% w/v) (Magnification: 1200X)

(**A**) homogeneous distribution of Nisaplin® before immersing in citrate buffer. Arrows indicate uniform distribution of sodium chloride crystals which comprises of 75% of Nisaplin®

(**B**) film structure after immersing in citrate buffer for 48 h. Sodium chloride crystals are washed out in citrate buffer

6.4 Practical implications

Corn zein films can be used to develop a matrix for controlled release of nisin in food systems. Also, release can be controlled by varying the concentration of biopolymer in the matrix. Corn zein films can be used as coating within a package or as an edible coating for the product for controlled release of antimicrobials and other active compounds. Thin films of low corn zein concentration, 1 to 2% (w/v) could be prepared and used as a coating on fruits or vegetables. Dipping of the fruits or vegetables into film solutions would completely cover the product irrespective of its shape and uneven surface. Since corn zein is hydrophobic, it can also be successfully used for delivery of active compounds in beverages, meat and other high moisture foods.

If nisin diffusivity is known, the amount of nisin released from the matrix can be controlled to maintain a minimum required inhibitory concentration in the food. If the amount of nisin inactivated in the food or used up on account of bacterial interaction is determined, the amount of nisin required to be released into the food can be calculated with the help of a mass balance technique.

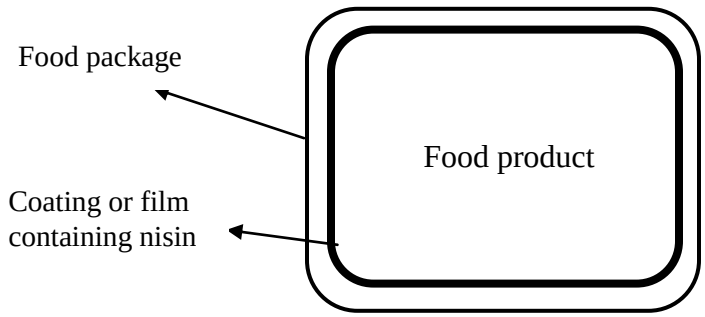


Fig. **6-19**: Schematic of food package with nisin coating

Chapter 7

Conclusions

Several biopolymer-based films were developed and their effectiveness in trapping and releasing nisin, as well as their stability in an aqueous food system was evaluated. Films were developed with one or more of the biopolymers xanthan gum, locust bean gum, carrageenans (kappa, iota & lamda), hydroxypropylmethylcellulose (HPMC) and corn zein.

Xanthan and locust bean films, being hydrophilic, collapsed or dissolved on the agar surface. The films did not produce any distinguishable inhibition zone, but instead, formed a blob on the agar plate.

Kappa and iota films did not collapse on the agar surface and also did not form any inhibition zone. Cha *et al.* (2003) observed from an agar diffusion test that nisin was not released from 2.5 and 5% (w/w) kappa carrageenan matrix. This observation was attributed to ionic binding, as nisin is a cation (+4 charge) and kappa carrageenan is anionic in nature, due to its several ester sulfate ($-OSO_3$) groups.

Two blended films were prepared using kappa & lamda carrageenan in different proportions and also iota & lamda carrageenan in different proportions. It was observed that the release from these films was directly proportional to the amount of lamda carrageenan incorporated in them. The fact that lamda films released nisin indicated that nisin release from the gelling carrageenans, kappa and iota, may have been prevented due to physical entrapment of nisin in the gel network. Hence, the lack of nisin release from

kappa and iota cannot be completely attributed to ionic binding, although that was the view put forth by Cha *et al.* (2003).

Films incorporated with nisin were prepared by blending different proportions of kappa carrageenan & HPMC, which is soluble in water. The inhibition zones formed in agar diffusion method verified that the films were antimicrobially active. Nisin release was found to increase with increase in HPMC content in the blended film. However, the HPMC films completely collapsed in water thus increasing the viscosity of the release solution, as a result of which the nisin released in this solution could not be quantified using HPLC. Similar challenges were faced with blended films made with lamda carrageenan also. It was concluded that in an aqueous system, it is difficult to control the nisin release from a soluble matrix, on account of it collapsing.

Agar diffusion demonstrated that corn zein films formed excellent inhibition zones. As amount of Nisaplin® in corn zein film (4% w/v) increased from 100 to 300 mg, nisin diffusivity increased almost twentyfold (2×10^{-11} to 38×10^{-11} cm^2/s), which indicates that nisin diffusivity through corn zein films is concentration dependent.

The diffusivity of nisin decreased from 38×10^{-11} to 8×10^{-11} cm^2/s, as the corn zein concentration in the film increased from 4 to 10% (w/v). Nisin diffusivity decreased fourfold as corn zein concentration in the film increased from 4 to 10% (w/v). This observation concurred with the hypothesis. This finding may have been on account of an increase in the tortuosity of the film, as the corn zein concentration in the film increased. Several researchers have reported that the release of an active compound from a matrix decreases as the biopolymer concentration in the matrix increases.

Mathematical analysis of the early portion of the release profile indicated that nisin release from corn zein films was pseudo-Fickian in nature. Deviation from Fickian behavior for polymers has been reported by several other researchers. The nisin release kinetics fitted with an empirical Weibull model showed excellent prediction ($R^2 > 0.95$).

Chapter 8

Suggestions for Future Research

Research has demonstrated that the bacteriocin nisin loses its activity when it comes in contact with food systems due to interaction with protein, fat and other constituents in the food. Several researchers have reported that while nisin activity was observed *in vitro*, activity was not always observed in model food systems. Hence, it is necessary that factors affecting activity loss of nisin in different food systems be identified, so that nisin can be used more effectively in foods.

In the present research, a corn zein based film incorporated with nisin was developed and its antimicrobial activity, as well as nisin release kinetics, was evaluated. The research can be further extended by comparing the activity of the nisin incorporated corn zein films with that of nisin directly incorporated in different food systems.

Although nisin is active against Gram-positive bacteria, several promising reports indicate that its activity can be extended to Gram-negative bacteria as well (Stevens *et al*., 1992; Cutter & Siragusa, 1995). Chelation of Mg^{++} and Ca^{++} ions in the lipopolysaccharide (LPS) layer of the outer membrane of the Gram-negative bacteria, results in destabilization of the LPS layer (Cutter & Siragusa, 1995). This destabilization increases cell permeability, which facilitates the penetration of nisin. Hence, further research could be carried out by encapsulating Nisaplin®, EDTA or Natamycin® in a corn zein matrix to address pathogenic microorganisms in the food.

In our study, blended films (kappa + lamda carrageenan; kappa carrageenan + HPMC) incorporated with nisin were found to be antimicrobially active, even though the nisin released into an aqueous system could not be quantified. The potential of these films as a food coating material for intermediate moisture foods, meat etc. should be explored further.

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Appendix A

Effect of plasticizers on film quality

Plasticizers	Film quality
No plasticizer	Very brittle film
Glycerol	Brittle film
Polyethylene glycol (PEG) 400	Films are flexible but very sticky
PEG 400 + Glycerol (ratio 1:1)	Good quality flexible films