PREDICTING TARGET RELEASE PROFILE OF ANTIMICROBIALS FROM CONTROLLED RELEASE PACKAGING

by

AISHWARYA BALASUBRAMANIAN

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ABSTRACT OF THE DISSERTATION

Predicting Target Release Profile of Antimicrobials from

Controlled Release Packaging

By AISHWARYA BALASUBRAMANIAN

Dissertation Director:

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Controlled release packaging (CRP) is an innovative technology that uses the package to deliver active compounds, such as antimicrobials in a controlled manner to enhance food safety and quality. There is an optimum range of release profiles of antimicrobial from CRP, called "Target release profile" that depends on food composition, packaging material, shelf life and temperature, to produce an effective inhibition of microbial growth for the desired shelf life.

The objective of the research is to develop mathematical model to predict target release profile of antimicrobials from CRP. Target release profile is the missing link for advancing research and development in CRP. Quantifying it helps polymer scientists design packages, tailor-made for the food and shelf life requirements for effective inhibition of microorganisms under different stress conditions.

This objective is achieved by quantifying the critical parameters influencing target release profile through a model system based on two hypotheses. The first hypothesis was developed to express target release profile in form of a quantifiable parameter such as diffusivity. The hypothesis was tested by generating release profile based on literature

data for potassium sorbate (bacteriostatic antimicrobial) diffusivities and evaluating their effect on the growth of *Escherichia coli* DH5α. The results show that not all release profiles were effective in inhibiting the growth of the organisms. There was an optimum range of release profiles, thereby an optimum range of diffusivities suitable to extend the lag period of *E.coli* DH5α for the 24 hours period tested. Diffusivity between 7.5 $x10^{-12}$ m^2/s and $2.60x10^{-13}$ m^2/s was needed to provide complete inhibition of the microorganism for 24 hours when 0.2 g (1 mg/mL) was added to the polymer. Increasing the amount of antimicrobial in polymer to 0.4 g and 0.6 g increased the effective range.

The second hypothesis was developed to quantify the optimum diffusivity based on minimum inhibitory concentration (MIC) of the antimicrobial and its effect on microbial lag period. The hypothesis states that the release rate of antimicrobials from the package during the inherent lag period of the organism must be equal or more than their MIC to produce an effective inhibition of the organism over the desired shelf life. The results supported the hypothesis that a minimum of 0.5 mg/mL has to be delivered during the inherent lag period of *E.coli* DH5α.

The results from the hypotheses were used to develop the target release rate model. The model is simple and takes into account the antimicrobial efficacy (MIC), microbial growth kinetics (lag period) and correlates them with the release kinetics of antimicrobial from polymer (diffusivity). The target release model was validated by evaluating the effect of nisin (bactericidal antimicrobial) release profile on the growth of *Micrococcus luteus*. The results validated the model and also showed that the predicted release profile was highly effective considering controlled release may use only 15% nisin to achieve complete inhibition of *M. luetus* rather than instant addition of 100% nisin.

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I strongly believe that we are not born but made by the people around us. I place on record the unstinted support and encouragement of my parents, sister, in-laws, and friends. I would like to specially express my deepest love and appreciation to my husband Prabhu for believing in me and son, Ashwin for his patience. I thank everyone around me who contributed in making my dream a reality.

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1. INTRODUCTION

Controlled release packaging (CRP) is an innovative technology that uses packaging as a delivery system to release active compounds, such as antioxidants and antimicrobials, at a targeted rate to slow down reaction kinetics of food deterioration, thereby enhancing quality and safety of foods during extended storage. The uniqueness of CRP is the ability to control or manipulate the release rate of active compounds based on the expected deterioration kinetics in food. To achieve control, it is essential to understand the deterioration kinetics in food based on its composition and environmental factors and design package suitable to deliver the active compounds at a targeted rate that would slow down the deterioration kinetics. Therefore, the objective of this study is to understand and model the "target release profile" or the critical release profile of antimicrobials from CRP films, based on microbial growth kinetics in food, to provide the required microbial inhibition in food for the desired shelf life of the food product.

The release rate of antimicrobials from a polymeric film to a food involves three steps: (1) diffusion within the polymer toward the interface between polymer and food, (2) mass transfer across the interface, and (3) dispersion into the bulk food. The amount of antimicrobial released over time is not constant and the release trend can be profiled as an initial fast release rate, followed by slower rates. Usually the release is controlled by the diffusion step due to the high diffusion resistance in the polymer. Thus, to develop a CRP system containing antimicrobial for microbial inhibition, it is important to quantify the rate of antimicrobial diffusion since the release profile of antimicrobials from CRP film is a critical factor determining the microbial growth kinetics.

Microbial growth has an inherent short lag period and the amount of antimicrobial added during that period is vital. Very slow release (low diffusivity) may result in lower concentrations of antimicrobial delivered to food, thereby resulting in insufficient amounts to inhibit growth while faster release (high diffusivity) may cause large concentration of antimicrobial delivered to food leading to resistance development and mutation in the organism. Thus, there is an optimum range of release profiles of antimicrobials from CRP system, called “target release profiles”, that depends on packaging materials, food composition and temperature to produces an effective inhibition of microbial growth and maintain their concentration at a safe level over the desired shelf life. Target release profile is the missing link for advancing research and development of CRP technology. It helps integrate packaging research with food research by quantifying effective release of antimicrobials from polymers into food under different environmental conditions to reduce microbial population to a safe level for the targeted shelf life. The need for target release profile of antimicrobials from CRP system will be explained below in a sequential manner.

Microbial contamination is considered one of the major deterioration modes in food. High incidence of food borne infections in the United States, with an estimate of around 76 million illnesses annually and an annual loss of around $6.9 billion, makes microbial contamination a serious concern and challenge to food scientists [1]. The increase in FSIS recalls of process foods and fresh produce in recent times has increased concerns among consumers. Moreover, the increase in the resistance of organisms to antimicrobials and development of mutant strains [2] drives the need to find new processing techniques that helps to enhance safety and maintain nutritional value of food.

Unlike lipid oxidation, microbial growth has short lag period or lag phase. Lag phase is the initial growth phase of the bacteria where the cells number remains constant prior to their exponential growth. In this thesis lag phase or lag period is defined as the period when there is an ***effective reduction of microbial cell number (cfu/mL) below the FDA/USDA approved level and maintaining the concentration at that level over the desired shelf life***. Consequently, to enhance safety and extend shelf life of food, we need to extend this lag period of microbial growth.

Traditionally, active compounds such as antimicrobials were "instantly released" into food by mixing them directly into initial food formulations. Though instant addition results in immediate inhibition of microorganisms (should it occur in the product), once the antimicrobials are consumed in the reaction, either due to complex interactions with the food matrix or by natural degradation over time, protection ceases and food quality degradation increases rapidly. Increasing levels of additives in the formulation may not always be feasible as the levels in foods are highly regulated by FDA. Moreover, higher concentrations could develop resistance or mutant strains in microbes [3]. To overcome these disadvantages CRP systems were developed that continuously replenish antimicrobials thereby regulating their concentrations in food at a targeted level that is effective in slowing down microbial growth kinetics and rendering it safe for human consumption.

Previous research in our laboratory aided in validating the concept of slow or timed release. Simulated slow addition of nisin, an antimicrobial, using a syringe pump to inhibit the growth of *Listeria monocytogenes* showed that slow addition of nisin was an effective mode of delivery to inhibit microbial growth. Similar effects were seen in timed

release of antioxidant such as tocopherol to inhibit lipid oxidation. Simulated slow addition of tocopherol into linoleic acid using a syringe pump showed that the induction period is significantly extended using slow or timed release of 300 ppm tocopherol at the rate of 50 ppm/day compared to instant addition of 300 ppm [4]. Our results provide the motivation for developing slow release systems of antimicrobials to enhance food safety and quality. Two immediate challenges for developing these systems are (1) delivering antimicrobials in a practical manner and (2) filling the knowledge gap to help design these systems.

Slow or timed release can occur in an uncontrolled manner if we fail to understand the effect of release rates on reaction kinetics of food deterioration reactions. For example, it cannot be guessed whether manipulating release rates of 300 ppm tocopherol to 25 ppm/day or 100 ppm/day will show the same effect as 50 ppm/day. Thus, understanding and quantifying target release profiles is imperative to provide a "controlled release rate" suitable to inhibit deterioration in food.

The concept of target release is complex because release of antimicrobials depends on packaging variables such as polymer type, food variables such as amount of water or fat in food and temperature, while the growth kinetics of microbes depend on food variables, and temperature. A systematic approach was taken to develop a mathematical model to predict target release profile by identifying critical parameters and testing hypotheses that would help quantify the effect of the parameters on antimicrobial release profiles.

Quantification of target release profile would help polymer scientists design food packages tailor-made for the food and shelf life requirements. For example, if diffusivity or diffusion coefficient (D) is identified as the major packaging parameter influencing

release, then the target release profile is quantified as a function of D and shelf life. The model developed would help predict D, if given the required shelf life (block arrow in Figure 1). Similarly the model would predict target release profile from the D of a polymer and consequently the shelf life (regular arrow in Figure 1).

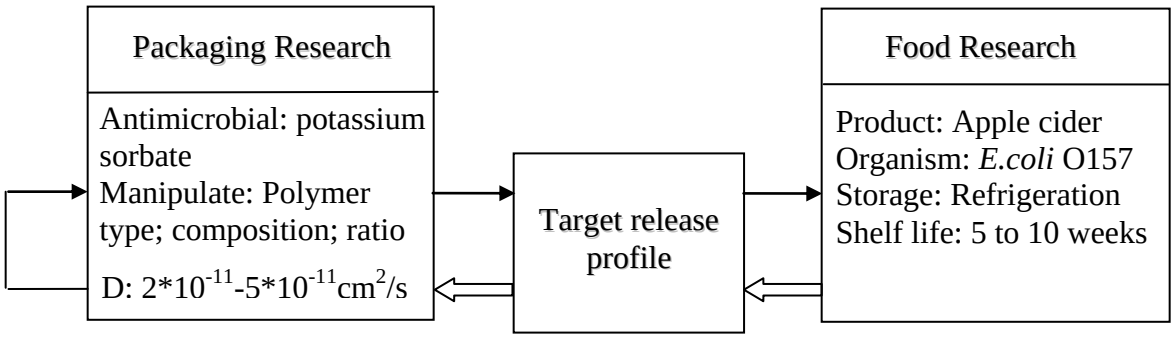

Figure 1: The application of target release profile in real-life systems

2. LITERATURE REVIEW

2.1. Controlled Release Packaging (CRP)

2.1.1. Concept of controlled release packaging

CRP is an innovative technology that use packaging as a delivery system to release of active compounds at a required rate that will slow down the reaction kinetics of food deterioration, thus enhancing the quality and safety of foods during extended storage [3, 5].

CRP is generally used as the food-contact layer or active layer in a multilayer film or a coating on a film or container consisting of one or more other supporting/barrier layers such as a gas barrier layer. Figure 2 helps to illustrate the concept of CRP, wherein the active compounds are added to the active layer and the barrier layer acts as the supporting layer. The active layer of the film is designed to control or manipulate the release of these compounds into food. These layers along with the active compounds form the major components of the controlled release packaging system. The compounds are slowly released over a period of time into food (Time t_1; Figure 2) and at the same time they are consumed by the food (Time, t_2; Figure 2) to slow down the kinetics of food deterioration reactions. This can be further explained through the graphs in Figure 2. The active compounds diffuse from the package slowly at a variable rate which is manipulated by the design of active layer, namely varying thickness or using different types and combination of polymers, based on the type of food and the target reaction kinetics, namely lipid oxidation or microbial growth rate. This differentiable release helps to maintain the deterioration index of the food at a low rate and thus extend the quality and shelf life of the food.

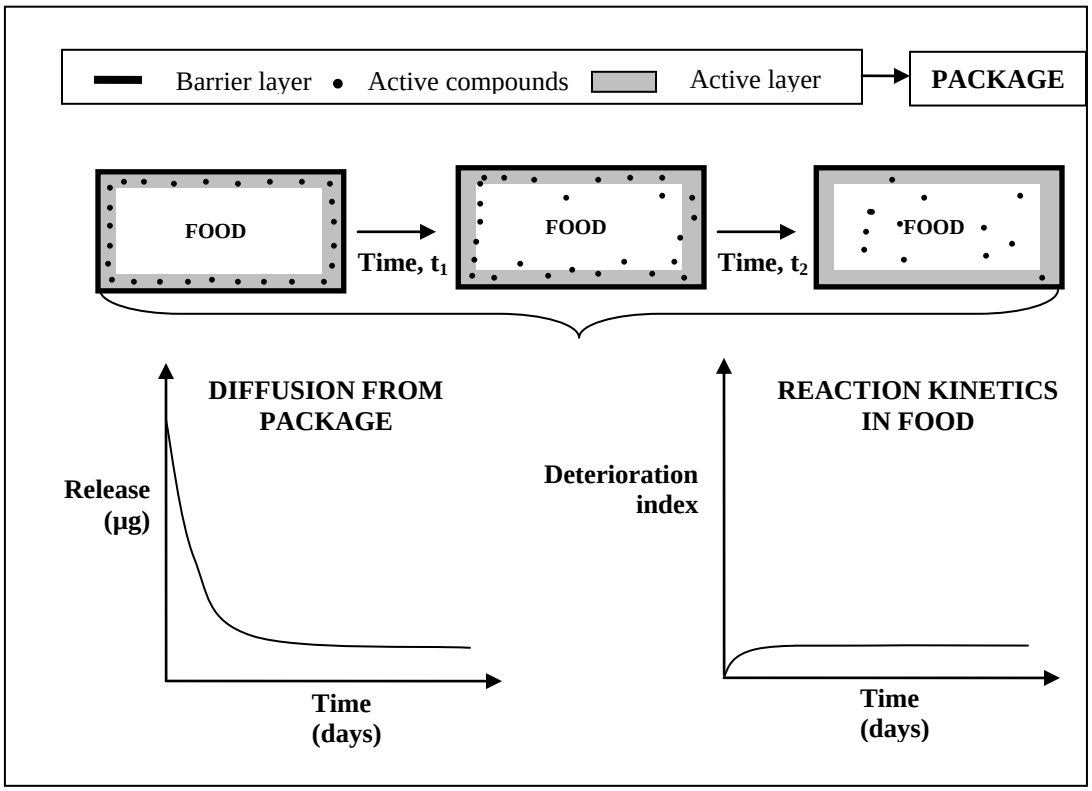

Figure 2: Mechanism of controlled release from CRP system

2.1.2. Uniqueness of CRP as active packaging

The four major functions of packages include containment, communication, convenience and protection. Traditionally, food packaging offers protection from the external environmental factors such as temperature, humidity and human handling. In recent times new generations of packaging materials have been developed to offer "active" protection from internal factors such as oxygen/carbon dioxide production, ethylene production, moisture, lipid oxidation, browning and microbial activity. These technologies are collectively known as "Active packaging" (Figure 3).

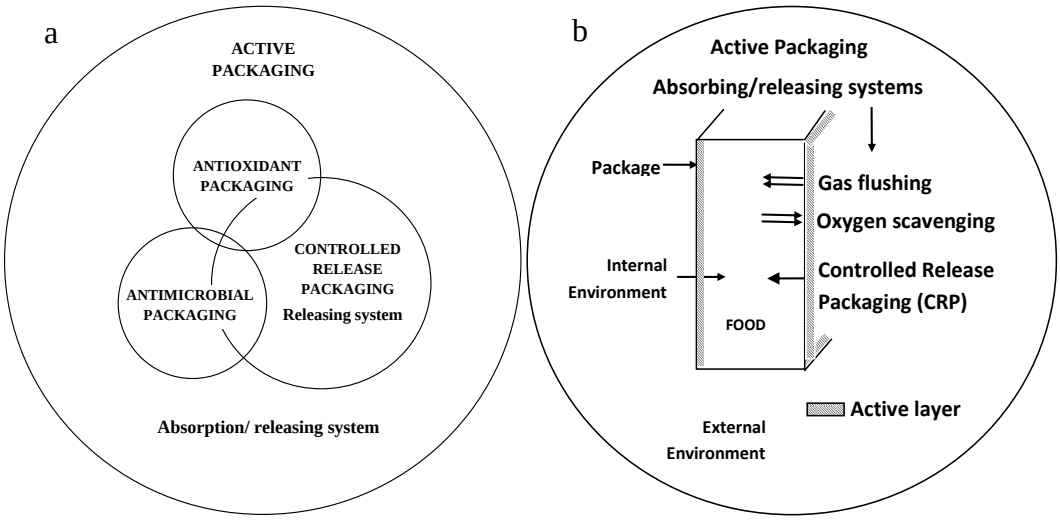

Figure 3: (a) Types of active packaging; (b) Example of an active packaging system including releasing and absorbing materials in the active layer

Active packaging is an innovative concept developed to actively modify the internal environment by continuously interacting with the food over the stipulated shelf-life (Figure 3). It is defined as an intelligent system that modifies the environment inside the package thus altering the state of the packaged food system or headspace to improve food quality through extension of shelf-life, maintenance of microbial safety or enhancement of sensory qualities [6-9]. Active packaging has gained much popularity due to the increased desire for high-quality, natural, safe and fresh products by consumers [10, 11].

Active packaging is a broad area that includes all absorption systems (e.g., O_2/CO_2 scavengers, ethylene and moisture) and releasing systems (e.g., ethanol/CO_2 emitters and antioxidant/antimicrobial) [8, 11, 12]. Thus antimicrobial packaging, antioxidant packaging and controlled release packaging are all forms of active packaging (Figure 3).

The uniqueness of CRP lies in the ability of the package to control the release of the antioxidant or antimicrobial into the food system, thereby modifying the internal

environment of the package over an extended period of time. Thus, the emphasis in CRP is “controlled” or “manipulated”, which not only fulfills the functions of an active packaging system but also deliberately regulates the release of the active compounds based on the targeted need (deterioration reactions).

The other aspect of CRP that sets it apart as an active packaging is its mechanism of release. “Slow release”, which was found to be effective, release active compounds over a longer period of time [13]. Packaging could be used as a delivery system to slowly release active compounds. Slow release often occurs in an uncontrolled manner and may result in wastage of active compound. For example, lipid oxidation reactions have an inherent induction period where the primary oxidation product remains constant. Dumping antioxidants during this period is not necessary and may also lead to pro-oxidation [14]. In contrast, the lag period in microbial growth kinetics is very short and slow addition of antimicrobials could result in no inhibition. CRP helps to overcome the limitation by releasing active compounds in a controlled manner by releasing higher amounts of antimicrobial during the lag period of the organism curbing growth and lesser amount during inherent induction period of lipid oxidation (without overloading the food system) and continuing the slow release for the desired. The “controlled release” based on the understanding of the deterioration kinetics helps maintain food quality and safety for the desired shelf life.

2.2. Antimicrobial releasing CRP system

2.2.1. Advantages of slow release compared to instant addition

Instant addition of antimicrobials via formulation often results in instant inhibition of microorganisms. However, the survivors will continue to grow, especially when antimicrobials added by formulation get depleted. Depletion of antimicrobial may also be due to complex interactions with the food matrix or by natural degradation over time, which is expected with instant addition [15, 16]. Simulated slow addition of nisin, an antimicrobial, using a syringe pump to mimic a packaging film, showed inhibition of *Listeria monocytogenes* [2]. The results clearly showed that slow addition (1000 IU/mL of nisin over a period of 100 hours) was more effective in inhibiting microbial growth than instant addition. Also a combination of instant addition (200 IU/mL) and slow release (200 IU/mL; 500 IU/mL over 100 hours) proved more effective than any single delivery mode. Instant addition of 200 IU/mL and slow release of 1000 IU/mL both resulted in development of antimicrobial-resistant mutants, but a combination resulted both in reduced cell counts and lack of mutation; instead, the cells adapted and regained their sensitivity to nisin following one passage through nisin-free medium.

2.2.2. Antimicrobial release from synthetic controlled release films/coating

In the case of food products, standardized films have far more potential than the simple addition of antimicrobials due to the ability to vary release rates by varying the chemical structure and type of polymer. Also, synthetic polymers are cheaper and more readily available than biopolymers. Lysozyme, a natural antimicrobial known to inhibit lactic acid bacteria causing wine malolactic fermentation, was incorporated in polyvinyl

alcohol (PVOH) films. The varied degree of cross linking of the films helps to control or vary release rates of the antimicrobial to provide effective inhibition [11]. Antibacterial and antimycotic effects of potassium sorbate added to high density polyethylene (HDPE) and low density polyethylene (LDPE) films on American cheeses has been studied. Sorbate released from HDPE films was found to be effective and able to stabilize the cheese for 5 months at room temperature[17]. This may be due to the higher diffusivity of HDPE compared to LDPE[18]. LDPE films containing 1000 mg/kg imazalil showed complete inhibition of *A. toxicarius* and *Penicillium sp.* in cheddar cheese [19]. LDPE films containing 0.5 and 1.0% w/w triclosan exhibited antimicrobial activity in an agar diffusion assay against *Staphylococcus aureus, Listeria. monocytogenes, E. coli O157:H7, Salmonella enteritidis* and *Brocothrix thermosphacta* [20]. LDPE films incorporated with natural antimicrobial such as propolis and clove extracts showed positive inhibition of *L. plantarum* and *F. oxysporum* [21]. Preliminarily studies by Suppakul and others (2002) showed that linear low density polyethylene (LLDPE) films incorporated with 0.05% linalool or methyl chavicol limited growth of *E.coli* [22].

Solution coating of antimicrobials using carriers on a synthetic polymer has continuously shown potential as CRP system, due to the ability to manipulate the antimicrobials release easily by manipulating the carrier. Moreover, large antimicrobial compounds can be incorporated into synthetics matrices without hassle using coating techniques. Nisin containing carriers such as methylcellulose (MC)/ hydroxypropyl methylcellulose (HPMC) coatings on LDPE showed effectively inhibited *S. aureus* and *L. monocytogenes* [23]. Packaging paper coated with a styrene-acrylate copolymer emulsion containing 100 ppm propyl paraben exhibited a slow but continuous inhibition of *Saccharomyces*

cerevisiae for about 60 hours (lag period) compared to control (3 hours lag period with no paraban) [24]. Similar studies on coating made with styrene-acrylate copolymer containing triclosan (87±9 mg triclosan/cm^3coating) inhibited *Enterococcus faecalis* [25].

2.2.3. Antimicrobial release from controlled release biopolymer films/coating

Biopolymer based antimicrobial packaging for food is being explored widely due to increasing in environmental concerns and need for natural materials. Biopolymer films includes both edible films and coatings and can be classified into several categories: carbohydrate-based (also known as hydrocolloids), protein-based, lipid-based, and composites [10]. Beef muscle was packed with milk protein based film containing 1.0 % (w/v) oregano and stored at 4 ºC [26]. The samples at the end of storage showed a 0.95 log reduction of *Pseudomonas sp.* level and 1.12 log reduction of *Escherichia coli* O157: H7 level compared to samples without film.

Biopolymer films have a huge potential as antimicrobials due to the swelling properties of most water-based biopolymers, and ease of manipulation of release rates by altering cross-linking. Cross-linked starches have been used in food applications for years, and have properties that extend well into controlled-release applications. The shelf life of strawberries coated with starch-based coatings containing potassium sorbate was effectively increased from 14 days to 28 days [27]. Derivatives of cellulose, such as methylcellulose and hydroxypropyl methylcellulose combined with fatty acids such as lauric, palmitic, stearic, and arachidic acid reduced their swelling property, thereby slowing down release of potassium sorbate [28].

Addition of calcium and sodium ions as cross linkers, greatly improved the antimicrobial release properties of alginates. Calcium alginate treatment with nisin on poultry skin contaminated with *Salmonella Typhimurium*^{Nar} population after 72 to 96 h of exposure at 4ºC showed reductions from1.8 to 4.6 log cycles [29]. Cha et al. (2002) found that Na-alginate film containing a cocktail of nisin, lysozyme, ethylene diamine tetraacetic acid (EDTA), and grape fruit seed extract (GFSE) reduced populations of both gram-positive and gram-negative bacteria [10].

2.3. Antimicrobials tested in the study

Potassium sorbate and nisin were the two antimicrobials used in this study due to their entirely different mechanism of action on the microorganisms. Potassium sorbate is one of the most common antimicrobials that is commercially used and is bacteriostatic in nature. It inhibits growth without actually killing the organisms. In contrast, nisin is bactericidal and one of the most effective antibacterial peptide, specific for gram positive organisms. The antimicrobial activity and mechanism of action of both antimicrobial is discussed below.

2.3.1. Antimicrobial activity of potassium sorbate

Potassium sorbate (CH_3-CH=CH-CH=CH-COOK) is the potassium salt of sorbic acid. Highly water soluble, it is used extensively in the food industry for its ability to inhibit or delay microbial growth in foods. The carboxyl group and the conjugated double bonds of the sorbic acid (Figure 4) are highly reactive, thus making it effective as an antimicrobial [30].

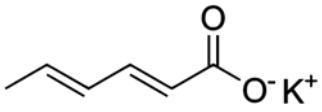

Figure 4: Structure of Potassium sorbate

Potassium sorbate releases sorbic acid when dissolved in water. They are effective up to pH 6.5 but the effectiveness increases as the pH decreases. The increased activity at low pH, approaching pKa 4.76, is attributed to the increased amount of undissociated acid. The undissociated form is the most effective antimicrobial form [31, 32]. 0.3% potassium sorbate shows effective inhibition of variety of molds, yeast and bacteria and is considered in most cases the maximum allowable limit in food.

Table 1: Microorganisms inhibited by potassium sorbate (adapted from "Antimicrobials in Food "[33])

Mold	***Alternaria, Ascochyta, Ascosphaera, Aspergillus, Botrytis, Cephalosporium, Chaetomium, Cladosporium, Colletotrichum, Cunninghamella, Curvularia, Fusarium, Geotrichum, Gliocladium, Helminthosporium, Heterosporium, Humicola, Monilia, Mucor, Penicillium, Phoma, Pepularia, Pestalotiopsis, Pullularia, Rhizoctonia, Rhizopus, Rosellinia, Sporotrichum, Trichoderma, Truncatella, Ulocladium***
Yeast	***Brettanomyces, Candida, Cryptococccus, Debaryomyces, Endomycopsis, Hansenula, Kloeckera, Pichia, Rhodotorula, Saccharomyces, Sporobolomyces, Torulaspora, Torulopsis, Zygosaccharomyces***
Bacteria	***Acetobacter, Achromobacter, Acinetobacter, Enterobacter, Aeromonas, Alcaligenes, Alteromonas, Arthrobacter, Bacillus, Campylobacter, Clostridium, Escherichia, Klebsiella, Lactobacillus, Micrococcus, Moraxella, Mycobacterium, Pediococcus, Proteus, Pseudomonas, Salmonella, Serratia, Staphylococcus, Vibrio, Yersinia***

Product	Concentration	Microorganism inhibited

Cheese	**1000µg/mL**	***Penicillium verrucosum var. cyclopium***
	<=6%	***Penicillium roqueforti ; Mucor miehi***
	<=6%	***Streptococcus salivarius var. thermophilus; Lactobacillus delbrueckii var.Bulgaricus***
	1%	***L. monocytogenes***
	0.3%	***E. coli O157:H7***
Meat products	**<0.3%**	***Escherichia coli, S. aureus, Clostridium perfringens, Yersinia enterocolitica, Brochothrix thermosphacta, Serratia liquefaciens, Lactobacillus, Clostridium sporogenes, Bacillus cereus, Bacillus licheniformis,***

2.3.2. Mechanism of action by potassium sorbate

The mechanism of microbial inhibition by potassium sorbate is not yet clear. The mechanism of action depends on factors such as microbial type, substrate type and composition, environmental factors, and concentration of sorbate [34-37]. Sorbate inactivates microbial cells either by inhibiting enzyme activity or nutrient uptake by the cells. Sorbate inhibits a variety of enzymes including sulfhydryl enzymes and yeast alcohol dehydrogenase [38]. Sorbate also inhibits oxygen uptake of the microbial cells by interfering with acetyl coenzyme A formation [39]. Sorbate may also cause cell starvation by inhibiting nutrients such as glucose and amino acids [40]. Sorbate causes damage to the outer cell membrane thereby inhibiting cell growth [41]. Sorbic acid was also found to be a weak-acid stressor causing expression of high amount of protein [42]. ATP depletion by sorbate was often found to be the cause for inhibition [43].

2.3.3. Antimicrobial activity of nisin

Nisin, an antibacterial peptide produced by *Lactococcus lactis*, is the only GRAS status bacteriocin used to inhibit growth of *Listeria monocytogenes* and *Clostridium botulinum* in foods [44]. Nisin inactivates gram positive bacteria, including heat-resistant types, making it one of the most sought out natural antimicrobial. Nisin, commercially available as Nisaplin® (Danisco), contains 2.5% active ingredient (nisin) in a base of lactose and milk solids and is extremely stable when stored under refrigerated conditions [45]

Nisin has a flexible, three dimensional structure with 34 amino acids and a molecular weight of 3150 Dalton. The flexibility of nisin is determined by its internal thioether ring. The stability and solubility of nisin depends on both temperature and pH of the system it is added to. In both low acid and high acid foods increasing temperature to 250 °F destroyed nisin activity by 25-50% [46, 47]. Nisin solubility decreased with increasing pH, but this behavior is not considered a major issue due to its lower level of usage in food.

Nisin not only inactivates vegetative cells but also inhibits outgrowth of spores formed by bacteria such as *Bacillus cereus* and *Bacillus sporothermodurans* [45]. Nisin is particularly toxic to the lactic acid bacteria, responsible for spoilage of low pH foods such as salad and alcoholic products, that are not generally heat processed, Nisin is not as effective against gram negative bacteria because it cannot penetrate the lipopolysaccharide layer of the cell wall.

2.3.4. Mechanism of action by nisin

The bacterial action of nisin results primarily from disruption of bacterial cytoplasmic membranes. The "Wedge model" defined by Driessen et al. (1995) proposes that nisin forms transient pores in the cell by inserting into the membrane without losing contact with the surface [48]. Another study suggested that nisin adheres to a peptidoglycan precursor for subsequent pore formation [49, 50]. NMR data shows that the cationic amino acids of nisin interact with negatively charged phospholipids in the membrane and nisin hydrophobic group immerse in the membrane core, while the other amino acids orient themselves perpendicular to the membrane. This bends the phospholipid bilayer causing the pore to open. Some studies also show that bacteria develop resistance to nisin with continued exposure to the peptide [51] due to modification of the membrane either in cell wall content or in the hydrophobicity and thickness of cell wall [52].

2.4. Mechanism of antimicrobial release from CRP films

The antimicrobial release from packaging into food is a 3-step process (Figure 5) [53].

- Diffusion of antimicrobial within the polymer matrix
- Partitioning of antimicrobial at the polymer/food interface
- Solubilizing into the food matrix

Very often the diffusion of antimicrobial to the interface is the rate determining step since the packaging layers are relatively thin compared to the food. Thus partitioning across the interface is negligible, especially with high affinity of antimicrobials to the food system. The diffusion of antimicrobials within the polymer matrix could be a time dependent

factor or a time independent factor depending on the type of polymer and the food (depends on affinity of the active compound to food).

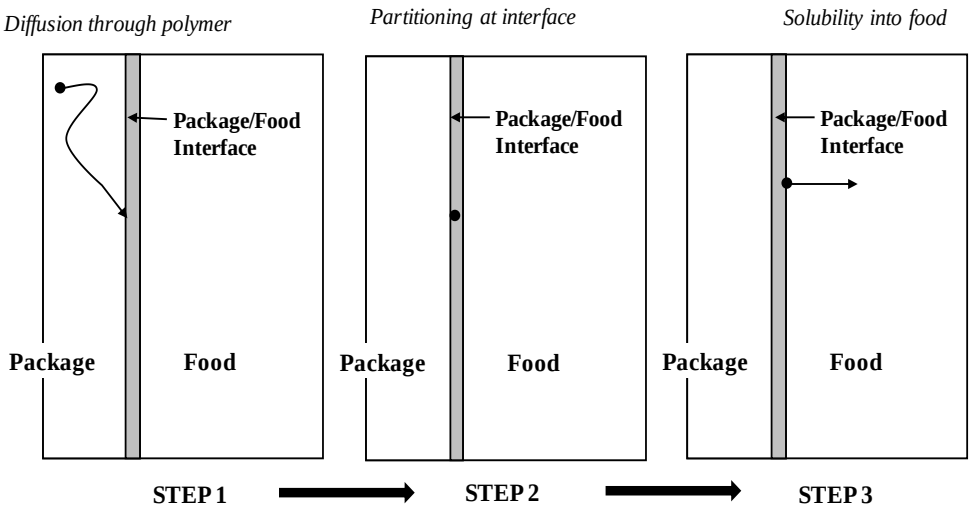

Figure 5: Mechanism of antimicrobial release from package into food

2.4.1. Diffusion of antimicrobial based on Fickian diffusion

The transport of antimicrobials through polymer membranes occurs due to random movement of molecules [54]. The diffusion within the polymer matrix is governed by the concentration gradient within the matrix. The transport of antimicrobials from polymer to the food occurs due to the concentration difference and can be described by Fick's first law of diffusion in which the flux, J in the direction of flow is proportional to the concentration gradient (dc/dx).

$$J = -D\left(\frac{\partial c}{\partial x}\right)$$

where, J (mol cm^{-2} s^{-1}) is diffusion flux, D (cm^2 s^{-1}) is the diffusion coefficient or diffusivity, c (mol cm^{-3}) is antimicrobial concentration, and x (cm) is distance in the flow direction. The first law is suitable only for steady state situations where the concentration of the active compound in the polymer does not vary with time. This is not true in real life conditions where the diffusion process is unsteady within the polymer. Thus the second law of Fickian diffusion is given by rate of change of antimicrobial concentration within the membrane and is obtained through first law of diffusion and mass balance [53].

$$\frac{\partial c}{\partial t} = D\left(\frac{\partial^2 c}{\partial x^2}\right)$$

where, D (cm^2 s^{-1}) is the diffusion coefficient or diffusivity, c (mol cm^{-3}) is antimicrobial concentration, and x (cm) is distance in the flow direction and t (s) is time. Fick's second law is suitable only for isotropic systems where the antimicrobials are uniformly distributed within the polymer. Thus, the diffusivity is independent of position (x), concentration (c), and time (t), and therefore is a material property. Diffusivity is a widely exploited parameter, useful in quantifying as how fast or slow the antimicrobial would be released from a particular material.

Depending on the boundary conditions the second law can be solved to provide different solutions. Generally for CRP films the solutions are obtained for plain sheets based on Crank's diffusion models for different boundary conditions.

2.4.2. Diffusion models for Fickian diffusion through planar sheet

2.4.2.1. Model 1: Simple diffusion model for unsteady state, short time, <60% diffusion

The controlled release film is considered as a planar sheet of thickness, L, and the antimicrobials are uniformly distributed in the film. There are two simple models that are generally used to estimate diffusivity within polymers when the release of antimicrobials is unidirectional, the time is short, and the ratio of amount of antimicrobial released to the food and the amount of antimicrobial in the polymer after time t is less than 2/3 [55]:

$$\frac{M_{f,t}}{M_{p,0}} = \frac{2}{L}\left(\frac{Dt}{\pi}\right)^{0.5}$$

and

$$\frac{M_{f,t}}{M_{f,\infty}} = \frac{2}{L}\left(\frac{Dt}{\pi}\right)^{0.5}$$

where $M_{f,t}$ is the amount of antimicrobial in food at time t; $M_{p,0}$ is the initial amount of antimicrobial in the packaging film; $M_{f,\infty}$ is the amount of antimicrobial in food after at equilibrium; D is the diffusivity of the antimicrobial in the polymer (cm^2/s) and t is time (s). The diffusivity is calculated by plotting the ratio of $M_{f,t}/M_{p,0}$ versus $t^{0.5}$.

The above models are popular and widely used to estimate diffusivity within polymers. The model does not take into account antimicrobial partitioning at the package/food interface. The model is also used for swellable biopolymers, if the amount and time taken for swelling is negligible compared to the release rate of antimicrobials. To estimate diffusivity of active compounds from films the general experimental design is to immerse

the film containing the active ingredient in a solvent and quantify release over time. This would modify the model slightly to accommodate for the bi-directional release and the diffusivity is calculated as below

$$\frac{M_{f,t}}{M_{f,\infty}} = \frac{4}{L}\left(\frac{Dt}{\pi}\right)^{0.5}$$

The diffusivities calculated using the models are listed in Table 1. The effect of temperature on diffusivities can be clearly seen. Increase in temperature causes increase in diffusivity and the relationship can be obtained using Arrhenius equation, which will be discussed in Section 2.5.6.

Table 2: Summary of diffusivity of antimicrobials based on Model 1

Polymer	Antimicrobial	Diffusivity (m^2/s)
Chitosan [16]	Acetic acid	$1.19x10^{-12}$ at 4°C $1.49x10^{-12}$ at 10°C $2.59x10^{-12}$ at 24°C
Chitosan [16]	Propionic acid	$0.91x10^{-12}$ at 4°C $1.27x10^{-12}$ at 10°C $1.87x10^{-12}$ at 24°C
Whey Protein Isolate: Glycerol (1:1) [56]	lyzozyme	$9.87x10^{-14}$ at 4°C $1.29x10^{-13}$ at 10°C

		$2.92x10^{-13}$ at 22°C
Whey Protein Isolate: Glycerol (2:1) [56]	lyzozyme	$4.76x10^{-15}$ at 4°C $2.16x10^{-14}$ at 10°C $3.68x10^{-14}$ at 22°C
Whey Protein Isolate: Glycerol (3:1) [56]	lyzozyme	$6.26x10^{-16}$ at 4°C $6.11x10^{-15}$ at 10°C $2.90x10^{-14}$ at 22°C
k-carageenan [57]	Potassium sorbate	$1.29x10^{-13}$ at 5°C $2.98x10^{-13}$ at 25°C $4.24x10^{-13}$ at 40°C
Alginate film with 3% $CaCl_2$ [58]	Potassium sorbate	$3.94x10^{-11}$ at 25°C
Gluten [59]	Potassium sorbate	$3.1x10^{-12}$ at 4°C $4.1x10^{-12}$ at 10°C $7.5x10^{-12}$ at 20°C
Gluten-beeswax [59]	Potassium sorbate	$2.2x10^{-12}$ at 4°C $3.0x10^{-12}$ at 10°C $5.6x10^{-12}$ at 20°C
Gluten-Acetylated monoglycerides [59]	Potassium sorbate	$1.6x10^{-12}$ at 4°C $2.2x10^{-12}$ at 10°C

		$3.2x10^{-12}$ at 20°C
Acetylated monoglycerides [59]	Potassium sorbate	$2.7x10^{-13}$ at 20°C
Beeswax [59]	Potassium sorbate	$2.4x10^{-16}$ at 20°C
Acrylic polymer coating [15]	nisin	$4.2x10^{-12}$ at 10°C
Vinyl acetate-ethylene co-polymer [15]	nisin	$9.3x10^{-12}$ at 10°C
Cast corn-zein films [60]	nisin	$6.5x10^{-16}$ at 5°C $7.7x10^{-15}$ at 25°C $3.1x10^{-14}$ at 35°C $6.4x10^{-14}$ at 45°C
Heat pressed corn-zein films [60]	nisin	$3.4x10^{-15}$ at 5°C $2.9x10^{-14}$ at 25°C $9.2x10^{-14}$ at 35°C $1.1x10^{-13}$ at 45°C
Cast wheat-gluten film [60]	nisin	$5.1x10^{-15}$ at 5°C $3.5x10^{-14}$ at 25°C $7.5x10^{-14}$ at 35°C $1.3x10^{-13}$ at 45°C
Heat pressed wheat-gluten films [60]	nisin	$3.6x10^{-15}$ at 5°C $3.7x10^{-14}$ at 25°C

		$5.9x10^{-14}$ at 35°C $7.7x10^{-14}$ at 45°C

2.4.2.2. Model 2: Diffusion model for unsteady state, large time, finite package, infinite food

The controlled release film is considered as a planar sheet of thickness, L, and the antimicrobials are uniformly distributed in the film. The release of antimicrobials is assumed to be bi-directional since the films containing active ingredients are immersed in a model solvent and the release of compound in solvent is quantified over time. This model is especially useful to calculate diffusivity of an antimicrobial over a long time assuming that the food is of infinite volume so no antimicrobial remains at the interface as. If sufficient time is provided for diffusion then all the antimicrobials will move from package to food. This model is especially useful for release studies in model food systems where an infinite amount of food simulant is added compared to the polymer and the antimicrobial has a high affinity for the simulant. Thus most antimicrobials will be released when the system is mixed well for a large period of time [55]. This model is expressed in the equation,

$$\frac{\boldsymbol{M_{f,t}}}{\boldsymbol{M_{p,0}}} = \mathbf{1} - \sum_{\boldsymbol{n=0}}^{\infty} \frac{\mathbf{8}}{\boldsymbol{(2n+1)^2\pi^2}} \boldsymbol{exp}\left\{\frac{\boldsymbol{-D(2n+1)^2\pi^2 t}}{\boldsymbol{L^2}}\right\}$$

where, where $M_{f,t}$ is the amount of antimicrobial in food at time t; $M_{p,0}$ is the initial amount of antimicrobial in the packaging film; $M_{f,\infty}$ is the amount of antimicrobial in food after at equilibrium; D is the diffusivity of the antimicrobial in the polymer (cm^2/s)

and t is time (s). This model provides more accurate quantification of D compared to Model 1as it includes the partitioning effect, which is common in real life condition. To simplify the situation, model food is used to eliminate portioning effect and thus model 2 is rarely used. The WPI studies with lyzozyme compared model 1 and 2 and found that with 1:1 ratio of WPI and glycerol there was no significant difference between the diffusivities obtained from the two models, but when the ratio increased (WPI>glycerol) the diffusivities obtained from model 2 were significantly smaller than model 1 especially at 4 and 10°C (Table 3) [56] . Similar variations in diffusivities occurred between the two models largely in heat pressed wheat gluten films, especially at high temperatures [60]. The models did not show any significant difference in diffusivities for potassium sorbate release from alginate films made with 3% calcium chloride (Table 3) [58].

Table 3: Summary of diffusivity of antimicrobials based on Model 2

Polymer	Antimicrobial	Diffusivity (m^2/s)
Alginate film with 3% $CaCl_2$ [58]	Potassium sorbate	$3.18x10^{-11}$ at 25°C
Whey Protein Isolate: Glycerol (1:1) [56]	lyzozyme	$1.1x10^{-13}$ at 4°C $1.17x10^{-13}$ at 10°C $2.82x10^{-13}$ at 22°C
Whey Protein Isolate: Glycerol (2:1) [56]	lyzozyme	$3.25x10^{-15}$ at 4°C $1.65x10^{-14}$ at 10°C $2.97x10^{-14}$ at 22°C

Whey Protein Isolate: Glycerol (3:1) [56]	lyzozyme	$3.13x10^{-16}$ at 4°C $7.83x10^{-15}$ at 10°C $2.18x10^{-14}$ at 22°C
Coating on paper with Vinyl acetate-ethylene co-polymer binder [61]	nisin	$1.13x10^{-11}$ at 10°C
Cast corn-zein films [60]	nisin	$6.8x10^{-16}$ at 5°C $8.1x10^{-15}$ at 25°C $3.3x10^{-14}$ at 35°C $6.2x10^{-14}$ at 45°C
Heat pressed corn-zein films [60]	nisin	$3.8x10^{-15}$ at 5°C $3.0x10^{-14}$ at 25°C $9.8x10^{-14}$ at 35°C $1.2x10^{-13}$ at 45°C
Cast wheat-gluten films [60]	nisin	$5.5x10^{-15}$ at 5°C $2.8x10^{-14}$ at 25°C $4.9x10^{-14}$ at 35°C $7.2x10^{-13}$ at 45°C
Heat pressed wheat-gluten films [60]	nisin	$3.6x10^{-15}$ at 5°C $2.8x10^{-14}$ at 25°C

		$2.8x10^{-14}$ at 35°C $6.8x10^{-14}$ at 45°C

2.4.2.3. Model 3: Diffusion model for unsteady state, finite package, finite food

The controlled release film is considered as a planar sheet of thickness, L, and the antimicrobials are uniformly distributed in the film. The release of antimicrobials is designed to be unidirectional. This model is generally used to calculate diffusivity for systems where the food is of finite or limited volume. This will result in significant partitioning of the antimicrobial at the package/food interface, which needs to be taken into consideration to calculate diffusivity [55].

$$\frac{M_{f,t}}{M_{f,\infty}} = 1 - \sum_{n=1}^{\infty} \frac{2 \propto (1+\propto)}{1+\propto +\propto^2 q_n{}^2} exp\left\{\frac{-Dq_n{}^2 t}{L^2}\right\}$$

where, q_n is the non-positive roots of $\tan q_n = -\alpha q_n$ and the roots could be obtained from Table 4.1 in Crank; $M_{f,t}$ is the mass of active compounds transferred to food at time t; $M_{f,\infty}$ is the mass of active compound in the food at equilibrium. The partition coefficient, given by K_p, and it is the ratio of concentration of food to concentration of polymer at equilibrium,

$$K_p = \frac{C_{f,\infty}}{C_{p,\infty}} = \frac{M_{f,\infty}}{M_{p,\infty}}\left(\frac{V_p}{V_f}\right) = \frac{M_{f,\infty}}{M_{p,0} - M_{f,\infty}}\left(\frac{V_p}{V_f}\right)$$

where, $M_{p,0}$ is the initial mass of active compound in the film; $C_{f,\infty}$ is the concentration of active compound in food at equilibrium; $C_{p,\infty}$ is the concentration of active compound in

polymer at equilibrium; V_f is the volume of food; V_p is the volume of polymer. Therefore,

$$\propto = \frac{M_{f,\infty}}{M_{p,0} - M_{f,\infty}} = K_p \left(\frac{V_f}{V_p}\right)$$

The above model is generally used when the partitioning was not anticipated in the experiment planned. It is expensive and tedious to change conditions and run experiments and thus the model is an alternate way to calculate diffusivity [62]. The model is also effective in experiments using real food systems. Examples of diffusivities calculated from Model 3 are listed in Table 4.

Table 4: Summary of diffusivity of antimicrobials based on Model 3

Polymer	Antimicrobial	Partition coefficient	Diffusivity (m^2/s)
Styrene-acrylate copolymer coating [24]	Propyl paraban	4.65×10^{-3} at 30°C	2.01×10^{-14} at 30°C
Carboset coating [63]	Propyl paraban	1.72×10^{-3} at 30°C	9.78×10^{-15} at 30°C

2.5. Diffusion of antimicrobial based on non-Fickian diffusion

Non-Fickian diffusion usually refers to films that are highly swellable so they are not driven by concentration gradient. The diffusivity is not a material property and it varies with time. Non-Fickian diffusion is significant in highly hydrophilic polymers or hydrophobic polymers in contact with hydrophobic food. The diffusion of food solvents

into the polymer causes morphological changes in the polymer matrix resulting in variation in diffusivity over time.

Very few mathematical models have been developed to describe their solvent uptake and antimicrobial release for highly swellable films. As in Fickian diffusion the release of antimicrobial from these swellable films are explained by a 3 step process [64]:

- Diffusion of solvent into the polymer
- Relaxation kinetics of polymer matrix
- Diffusion of antimicrobial through the swollen polymer network

The models have been developed based on the above 3 conditions, incorporating initial and final boundary conditions to obtain suitable solutions. Models developed by Buonocore [65] and others on highly swellable films were successfully able to quantify diffusivity of the solvent, antimicrobial and the partition coefficient at the package/food interface (Table 5). Factors influencing the release of these antimicrobials from the polymers can be clearly seen by the variations in their diffusivity and will be explained in the next section.

Table 5: Summary of diffusivity of antimicrobials from highly swellable films

Polymer	Antimicrobial	Diffusivity of water into film (m^2/s)	Diffusivity of antimicrobial (m^2/s)	Partition coefficient
7.7% w/w PVOH films crosslinked with glyoxal [65]	Lysozyme	5.41×10^{-12}	3.83×10^{-15}	431.37
	Nisin		3.01×10^{-14}	152.8
	Sodium benzoate		1.25×10^{-12}	55.82

2.0% w/w PVOH films crosslinked with glyoxal [65]	Lysozyme	$1.12x10^{-11}$	$2.45x10^{-14}$	45.62
	Nisin		$3.16x10^{-13}$	67.58
	Sodium benzoate		$4.2x10^{-12}$	60.01
0.77% w/w PVOH films crosslinked with glyoxal [65]	Lysozyme	$1.80x10^{-11}$	$2.10x10^{-13}$	15.06
	Nisin		$6.24x10^{-13}$	34.57
	Sodium benzoate		$2.55x10^{-12}$	25.96
0.077% w/w PVOH films crosslinked with glyoxal [65]	Lysozyme	$3.86x10^{-11}$	$9.98\ x10^{-13}$	6.33
	Nisin		$8.61x10^{-13}$	26.73
	Sodium benzoate		$2.54x10^{-12}$	21.39

2.6. Factors influencing antimicrobial release from polymers

2.6.1. Size of active compound

The molecular weight of active compounds influences their ability to be entrapped within the polymer matrix. Thus, the mobility of antimicrobials within the matrix depends on their size, unless the antimicrobial has chemical interactions with the polymer. Small molecular weight compounds entrapped in a matrix release faster than larger molecular weight compounds. For example, sodium benzoate releases from PVOH films faster than nisin and is evident from their diffusivities [65].

2.6.2. Compatibility of antimicrobial with polymer films

Compatibility of the antimicrobial with the polymer mediated by molecular associations controls release of the antimicrobial from the films. Very high compatibility (binding) of antimicrobial with polymer results in no release, while incompatibility causes instant

release. Controlled release of antimicrobials is feasible only if the antimicrobials are physically entrapped within the polymer matrix rather than being chemically bound. The polymer network can then be manipulated to vary release. A wide variety of approaches have been used to modify films and their release properties. Exchanging acid for salt forms, hydrogen bonding for charges, is one used frequently. Han and Flores (1997) showed that 1.0% potassium sorbate incorporated in LDPE films and showed their effectiveness in inhibiting yeast and mold growth [66], while Weng and Hotchkiss (1993) found no inhibition when 1.0% sorbic acid was added to LDPE [67]. The difference was due to binding of the acid to the polymer [68].

Anhydrides, on the other hand, are incompatible with PE due to their higher molecular weight and low polarity [67]. Benzoic anhydride incorporated in LDPE films showed complete inhibition of *Rhizopus stolonifer*, *Penicillium sp*. and *Aspergillus toxicarius* on potato dextrose agar (PDA). The films also showed effect against mold growth on surfaces on packaged cheese and toasted bread stored at 6 ºC. Similarly PE films containing 20 mg/g of benzoic anhydride was effective in suppressing microbial growth in tilapia fillets stored at 4 ºC for a period of 14 days [67].

Hydrophilic nisin incorporated into hydrophobic linear low-density polyethylene (LLDPE) caused repulsion of the film and coalescence of nisin treatment solution droplets. This may lead to non-uniform mixing of nisin and eventually localized antimicrobial activity in the film [69, 70]. Similarly when nisin was added to acrylic polymer, the hydrophilicity of the polymer caused adsorption and retention of nisin compared to vinyl acetate-ethylene copolymer [71].

2.6.3. Polymer matrix

The polymer matrix is the internal polymer network with free space in which the antimicrobials are entrapped. The distribution and compactness of the polymer molecules determines the movement of the antimicrobials within the matrix. Polymer type is a contributing factor for manipulating release. For example, it was reported that release rate of the natural antioxidant, tocopherol, from polypropylene (PP) into 95% aqueous ethanol was the slowest followed by its release from high density polyethylene (HDPE) and low density polyethylene (LDPE), respectively [18]. This could be due to methyl groups on PP orienting on outside, forcing chain into α-helix. The neighboring helices associate by Van-Der-Waal forces packing into tight rigid structures, thereby increasing crystallinity. The active compounds could get trapped in the helices, restricting movement, causing slower release rate.

Crosslinking of polymers is another approach manipulating polymer networks varying release mode [72]. Diffusivity of antimicrobial can be increased by limiting the degree of crosslinking. Zactiti et al. studied the effect of degree of calcium crosslinking in alginate films on the release of potassium sorbate and found that increasing concentration of crosslinker decreased the diffusivity of potassium sorbate [73]. Similarly, increasing crosslinking in pectin films using calcium chloride also depressed the release of nisin [74].

Plasticizers added for film formation increase the free space by forming gels which are more open and have elastic networks, thereby facilitating release. Whey protein isolate films formed with glycerol, a plasticizer, shows a variation in the release of lysozyme. Films formed with equal ratios of WPI and glycerol containing lysozyme shows high

diffusivity ($2.92x10^{-9}$ at 22°C) of the antimicrobial compared to films formed with 3:1 ratio of WPI and glycerol ($2.90x10^{-10}$ at 22°C) [56].

Addition of hydrophobic materials to hydrophilic polymers reduces swellability due to water absorption, thereby slowing down the antimicrobial release. The addition of lipids causes pore constriction and increased blind porosity of the hydrophilic network, thereby blocking antimicrobial mobility through the network [75]. 1.0% Lauric acid or 0.5% cinnamaldehyde or eugenol added to Chitosan films significantly decreased diffusivity of acetic acid [16]. Also, addition of beeswax or acetylated monoglycerides decreases the diffusivity of potassium sorbate by 20% to 50% [59].

2.6.4. Processing method

The effect of processing methods on release rate of antimicrobials is primarily due to the processing conditions. The stability of the antimicrobial depends on the temperature, pH and other chemicals used for making the films during processing. Processing conditions could vary the chemical properties of antimicrobials, thus resulting in loss of antimicrobials or inactivation of antimicrobial activity. The antimicrobial effectiveness of 1% Grape fruit seed extract (GFSE) on ground beef was evaluated by incorporating them in multilayered PE films by co-extrusion and solution coating [76]. It was found that coating with the aid of a polyamide binder enhanced antimicrobial activity than co-extrusion. Solution coating of GFSE showed activity against *M. flavus, E. coli, S. aureus,* and *Bacillus subtilis,* while co-extruded films with GFSE showed inhibition of *M. flavus* only.

The processing method also influences the release rate of antimicrobials. This may be due to the modifications in polymer matrix due to the processing conditions, leading to a compact network or a looser network. The diffusivity of nisin incorporated in cast corn zein films and heat pressed corn zein films was compared. It was found that the diffusivity significantly reduced in cast films compared to heat pressed films [77].

2.6.5. Food composition

The components in food significantly affect the release of antimicrobials. The release rate of hydrophilic or hydrophobic antimicrobials depends on the water activity and fat levels of food. Thus the release can also be manipulated by varying the composition of food instead of manipulating the polymer. The diffusivity of propyl paraban, a hydrophobic antimicrobial, from carboset coating into 10% ethanol, 50% ethanol and n-heptane was evaluated. It was found that propyl paraban released instantaneously into 50% ethanol and n-heptane, while slow release was observed with 10% ethanol [63].

The physiochemical characteristics of food also influence the antimicrobial activity. For example the antimicrobial activity of potassium sorbate is due to the acid dissociation and it occurs only at low pH. When the effect of food pH on the diffusivity of potassium sorbate was evaluated, it was found that there was no significant difference in the diffusivity of sorbate from k-carageenan film at pH 3.8, 5.2 and 7.0 [57].

2.6.6. Storage conditions

Temperature is the major factor influencing diffusivity of antimicrobials from polymers. Increase in temperature increases diffusivity and this is clearly seen in tables 1 and 2. The

temperature effect on diffusivity is generally explained by solubility of antimicrobials in films, nature of adhesive forces at the package/food interface and on molecular mobility [53]. This effect is explained through Arrhenius equation showing that it is thermodynamic in nature [60]. The effect of temperature on diffusion is controlled by the activation energy and not due to morphological changes in the polymer. The Arrhenius equation for temperature effect on diffusivity is given by,

$$\boldsymbol{D = D_0\, exp\left(\frac{-E_a}{RT}\right)}$$

Where, D is the diffusion coefficient (cm^2/s), D0 is a constant, E_a is activation energy for the diffusion process (J/mol), R is universal gas constant (J/mol K), and T is absolute temperature (K). High activation energy indicates an increased sensitivity of diffusivity to temperature. The temperature effect on diffusivity can be exploited to our advantage as the microbial activity also increases with temperature.

2.7. Conceptual framework of CRP

Development of a controlled release package requires fundamental understanding of the packaging system, factors governing the release of active compounds from the packaging films, the food system, and functional relationships between all these components. Not all this information is currently available, and certainly not in coordinated and integrated form. Therefore a road map in form of conceptual framework was developed distilled from the knowledge gained from the research by Dr. Yam and Dr. Schaich and team at Rutgers University.

The conceptual framework is divided into two parts -- packaging research and food research.

The packaging research includes process, structure and property variables. The process variables are variables that could be directly controlled to manipulate the mobility of the active compounds incorporated inside the polymer matrix. They include the active compounds, polymer composition and processing methods. The effect of process variables on release has been discussed in section 2.4.

The structure variable is the variations in the polymer structure and morphology causing control in mobility of the antimicrobials. This is influenced by composition of the polymer and processing method. For example, smart blending (based on the science of chaotic advection) of LDPE and PP results in variations in polymer morphology leading to significant difference in diffusivity [78].

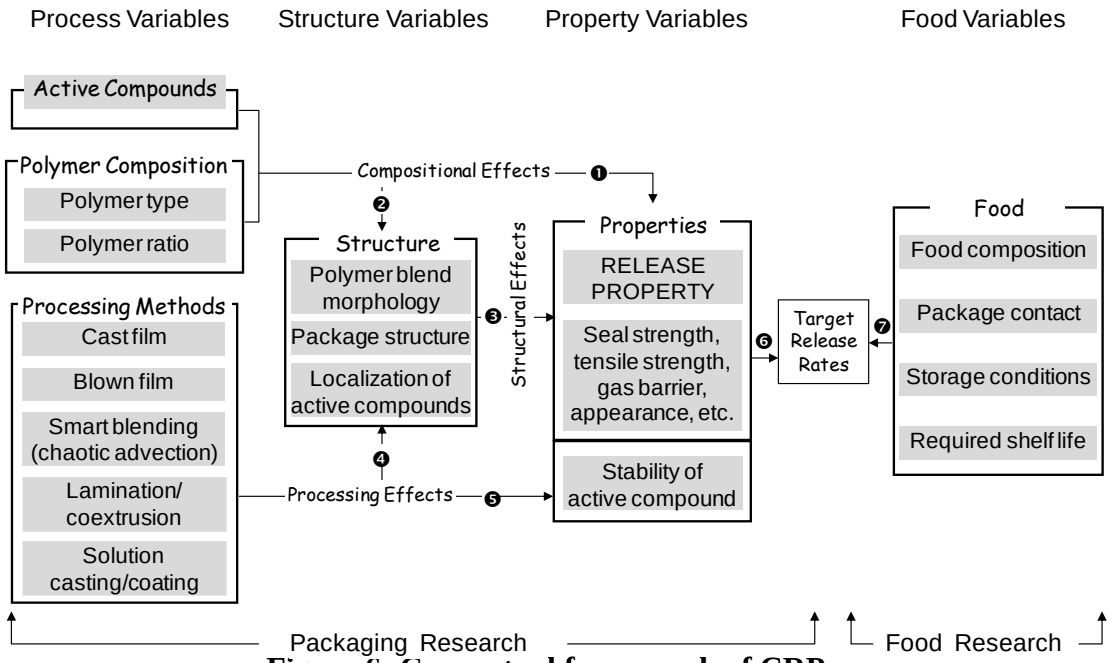

Figure 6: Conceptual framework of CRP

All the variations to the package in the form of process and structure variables not only influence the variations in tensile strength, moisture and gas barriers but also modify its ability to release active compounds

The food research on the other hand includes the food variables required for efficient design and delivery of CRP technology like food composition; consistency and contact with package; storage conditions; shelf life requirements. The packaging research and food research is linked by the concept called target release profile which is essential to take the CRP technology to the next level.

2.7.1. Target release profile of antioxidants from CRP

Previous work on target release profile of antioxidants from CRP by Zhu (2008) defines it as the optimum rate at which an antioxidant must be released from the food package to

replenish that consumed in oxidation reactions of food to provide maximum induction period [4].

He showed that target release profile depends on temperature and that the optimum release rate to produce maximum induction period in lipid oxidation increased with increasing temperature. The effect of 300ppm of tocopherol released at varying rates (20ppm/day-150ppm/day) using a syringe pump on linoleic acid was evaluated. It was found that at 30°C, the target release profile was about 40 ppm; at 40°C, it was about 75ppm/day and at 50°C, the target release profile was about 150 ppm. That is releasing 300ppm of tocopherol at the rate of 40ppm/day for 7.5 days at 30°C provided the maximum induction period (160 days) compared to instant addition of 300ppm (40 hours). However increasing the rate above 40ppm/day causes self reaction of tocopherol and thereby decreased the induction period.

The above results clearly showed that there is an optimum release rate of antioxidants that is suitable to produce a maximum induction period of lipid oxidation. Release rates faster than the optimum rate could cause pro-oxidation, while very slow release rates may not be sufficient for inhibition, thereby showing a small induction period. Target release profile depends on temperature and increasing temperature increases the target release profile of antioxidants.

3. CONCEPT OF TARGET RELEASE PROFILE

3.1. The need for target release profile

Based on previous studies and conceptual framework proposed in this research group, we can see that without understanding the extent of release and quantifying the effect of different parameters on release rates, CRP would be a technology that is effective only in lab. Current research on antimicrobial CRP pertains either to developing new polymers that can vary release of active compounds; new antimicrobials that have the ability to inhibit a wide range of microbial strains and/or different food materials inoculated with organisms that shows a positive effect with CRP. All the aforementioned steps are needed to understand the factors influencing release rates and contributing to controlled release. However now that we have sufficient understanding it is important to quantify these parameters and connect them to take CRP technology from “test tube” to real life.

Extending the idea of target release profile from CRP films, we can say that release of antimicrobials from the polymers is not always able to inhibit microbial growth. An antimicrobial may inhibit more than one strain of microbes in culture but not show the same activity against all the strains after releasing from CRP, especially if the release rate does not match the growth rate of the organism. Sorbic anhydride released from polyethylene films shows a better inhibition of slow-growing *Penicillium sp.* compared to fast growing *Aspergillus niger* [79]. Although the antimicrobial shows bacteriostatic on both strains in a test tube, if the organisms grow at a different rate than the antimicrobial is released, CRP may inhibit one and not the other. Thus it is imperative we quantify the release rate of antimicrobials from various CRP films.

3.2. Framework to understand target release profile

The complexity of target release profiles arises from the influence of multiple factors such as packaging, food and environmental variables on the release rate of antimicrobials from films. Thus a framework was developed (Figure 7) to show the interrelationship between these factors and the way target release profile may be used to connect the release rate of antimicrobials to the growth rate of microorganisms.

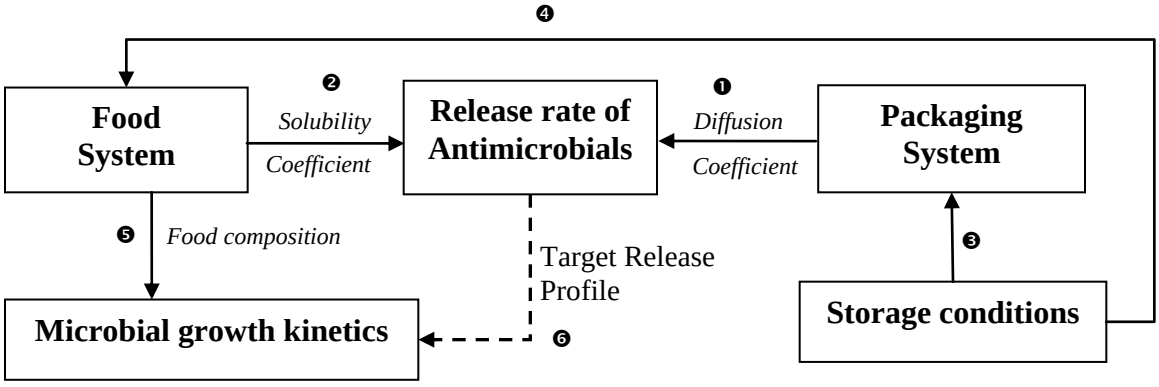

Figure 7: Framework for target release profile

The effectiveness of antimicrobials depends on the release rate from polymers. In real-life situations the release rate of antimicrobials from a particular package depends on their diffusion inside the package❶, i.e., how fast or how slow the compounds move inside the package and solubility of antimicrobials in food❷ , which in turn depends on percentage of water and fat (food composition).

Storage conditions like temperature could influence the diffusion coefficient of a package, thereby altering the release rate of antimicrobials from a package❸. At the same

time, the food reaction kinetics❹, in this case, the microbial growth kinetics are also influenced by the storage conditions such as temperature and RH, as well as by food composition❺ namely, pH and water activity. Thus the release profile of antimicrobials from a package must correspond to the microbial growth rate in such a way that there is a quick reduction (faster release rate) in the viable microbial count during the lag period /induction period of microbial growth and continue to maintain (slow release rate) and extend this lag phase over the desired period of storage.

The effective or the target release profile for a particular shelf life depends on all the above mentioned variables, thus making the system complex. It is critical to not only develop the concept of target release profile but also quantify it, so that once the food technologist determines the target release profile of a food item based on the functional relationships mentioned above, the packaging engineer can then design a controlled release packaging to match this target release profile.

The framework helps organize the factors influencing target release profile in a systematic manner. The functional relationships can be simplified and written as,

- Antimicrobial release rate = f {diffusivity, food composition, temperature}
- Microbial lag period = f {food composition, temperature, antimicrobial}
- Shelf life = f {antimicrobial release rate, microbial lag period, temperature}
- Target release profile = f {diffusivity, microbial lag period, temperature, shelf life}

3.3. Research gaps

3.3.1. The concept of target release profile for antimicrobial release from CRP

The slow release of antioxidants and antimicrobials from packages shows inhibits lipid oxidation and microbial growth respectively. Further work on integrating the antioxidant release from package with that of lipid oxidation kinetics in food using the concept of target release profile showed that there is an optimum release rate that provides maximum inhibition of lipid oxidation without accelerating lipid breakdown. Slow release of antioxidants, is not sufficient to inhibit radical chains of oxidation while very fast release causes pro-oxidation in lipids. Similarly microorganisms are known to develop resistance if exposed to large amount of antimicrobials. This similarity between the microbial growth and lipid oxidation reaction leads to the question, "Is there an optimum release profile for antimicrobial release from CRP?"

3.3.2. Limitation of previous work using syringe pump

The previous work on slow release of active compounds from CRP used syringe pump to mimic the release from polymers. The syringe pump was used to deliver a constant release rate of antimicrobials (200IU/mL, 500IU/mL, and 1000IU/mL for 100 hours) and antioxidants (300 ppm of tocopherol at 75 ppm/day for 4 days) from package into food.

Though the constant release rate is suitable to prove the concept of slow release using CRP and target release profile they do not help in quantifying target release profile as the release from package is highly variable. The release from the packaging polymer is usually at a fast rate during the initial period followed by a period of slow rate and finally a constant release rate (Figure 8). Thus mimicking this variable release rate is needed to

understand the effect of release rate on reaction rate. Both the above mentioned gaps and the solutions are elaborately analyzed and discussed in the next section.

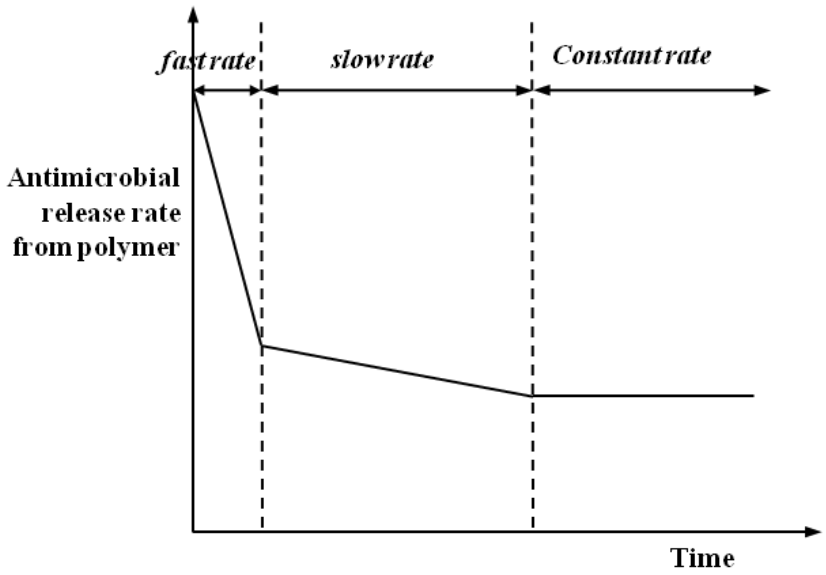

Figure 8: General trend of antimicrobial release rate from package into food

Previous work on slow release of active compounds from CRP a modeled release from polymers using syringe pump was used to deliver a constant release rate of antimicrobials (200IU/mL, 500IU/mL, and 1000IU/mL for 100 hours) and antioxidants (300 ppm of tocopherol at 75 ppm/day for 4 days) from package into food.

Though constant delivery rate is suitable to prove the concept of slow release using CRP and target release profile it does not quantify the variable target release profile from an actual package where release occurs usually at a fast rate during the initial period followed by a period of slow rate and finally a constant release rate (Figure 8). Thus mimicking this variable release rate is needed to understand how release rate controls

reaction rate. Both the above mentioned gaps and the solutions are analyzed and discussed in more detail in the next section.

4. PROBLEM FORMULATION

The overall objective of this research is to predict the target release profiles of antimicrobial release from CRP system. To achieve the objective, two hypotheses were proposed based on the previous studies.

4.1. Evidence leading to hypothesis

4.1.1. Hypothesis 1

There is an optimum range of diffusivities suitable to extend microbial lag period over the desired shelf life.

The microbial growth kinetics depends on the release rate of antimicrobials. Slow release rate of nisin at the rate of 200 IU/mL (IU of antimicrobial/ml of microbial culture media) or 500 IU/mL over a period of 100 hours does not inhibit *L.monocytogenes* while increasing the rate to 1000 IU/mL showed inhibition of the organism by extended the lag period to over 100 hours [2]. On the other hand, faster release rate (10 IU/mL/hour) caused the organism to develop resistance when exposed to nisin immediately after collecting them from nisin treatment and also exposed to nisin after an overnight recovery in the absence of nisin. In other words, high release induced both short-term and long-term resistance development in the organisms, which is of serious concern.

Similar results were seen with rapid antioxidant release and lipid oxidation. Slow release of tocopherol (300 ppm at 20 ppm/day) into linoleic acid at 30ºC gave an induction

period of 6 days and increasing the release rate to 75 ppm/day did not increase the induction period proportionately to 22 days but only to 10 days. Most importantly induction period did not increase proportionately but reached a peak and then decreased. The maximum induction period (14 days) was produced with a release rate of 40 ppm/day. This rate producing the maximum determined the optimum release rate and was defined as target release profile [4].

From previous studies, we can understand that the response of microbial growth kinetics to antimicrobial release rates from CRP system will be similar to the response of lipid oxidation reactions to antioxidant release rates. Therefore, there should be an optimum range of release rates suitable to inhibit microbial growth for the desired shelf life. But target release profile is merely concept and to quantify it, it must be expressed into a quantifiable parameter. Based on theoretical understanding and the framework of target release profile (Section 3.2), the release rate can be quantified from diffusivity from the polymer. Thus it is hypothesized that there is an optimum range of diffusivities suitable to extend microbial lag period over the desired shelf life.

Outcome if tests support hypothesis: Target release profile expressed in form of diffusivities

4.1.2. Hypothesis 2

The release rate of antimicrobials from the package during the inherent lag period of the organism must be equal or more than their MIC to produce an effective inhibition of the organism over the desired shelf life.

This hypothesis was based on the evidence that microbial lag period is short i.e., the inherent lag period or period taken by the organisms to adapt themselves to their environment is very short. Thus maximum need of antimicrobial is during this period. The organism grows exponentially during the log period and addition of any amount of antimicrobial during that period may not be sufficient to inhibit growth.

MIC is the minimum concentration of antimicrobial that shows maximum inhibition or microorganisms. Thus, the release rate of antimicrobial from the package during the inherent lag period of the organism must be fast and at least equal to the MIC.

Outcome if tests support hypothesis: Quantify optimum diffusivity in terms of MIC and microbial lag period

4.2. Objective achieved through hypothesis

The hypotheses will be tested and if the hypotheses are supported, the data will help determine the variable influencing target release profiles. Determining such variables would help achieve the objective to quantify or predict target release profile by integrating package and food and developing mathematical models that predict target release profile of antimicrobials from CRP (Figure 9).

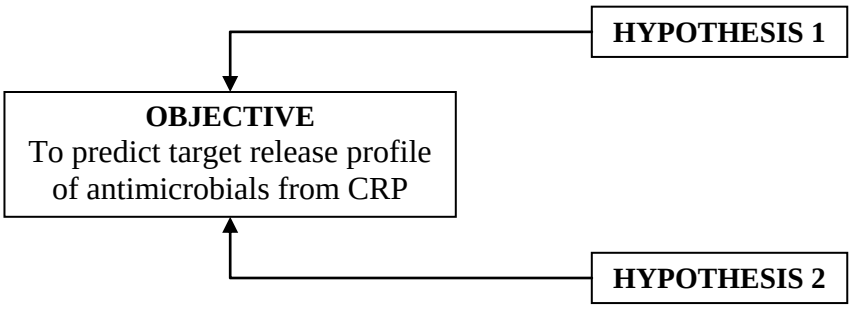

Figure 9: Hypothesis leading to objective

5. EXPERIMENTAL DESIGN

5.1. Parameters identified for the study

5.1.1. Diffusivity

Diffusivity or diffusion coefficient has been identified as the major packaging parameter that directly influences the release rate of antimicrobials into food. Diffusivity is a material property and a polymer parameter that can be quantified. When the target release rate is quantified based on diffusivity, it can easily be translated to the type packaging material which can then be manufactured by film manufacturers. Diffusivity is measurable and is a parameter estimated in most research (see section 2.4) to compare the release rate of antimicrobials from different polymers. Diffusivity can also be directly manipulated by manipulating process and structure variables of the package (see section 2.6). Polymers that follow Fick's law or Fickian diffusion have been selected for the study.

5.1.2. Microbial lag period

The lag period of the growth kinetics is a critical parameter as the objective of adding antimicrobials is to extend the lag period of an organism. This lag period can directly be translated to shelf life since once the microbes enter the log period the food is either considered spoiled, leading to loss in quality or could cause safety concerns.

The lag period depends highly on the food system and the internal environment of the package. The lag phase is shorter when the conditions are favorable for the micro-organisms and longer as the conditions become unfavorable. The release rate of the

antimicrobials, or the target release rate, should correspond to the variations in lag period and thus quantifying lag period will help quantify target release rate.

5.1.3. Minimum Inhibitory Concentration (MIC)

MIC, the minimum concentration of antimicrobial that shows maximum inhibition of microorganisms, depends on the initial microbial count. MIC is considered as an experimental parameter since maximum stress must be imposed on the organism during the lag period when the microorganism is acclimating to the environment and sustained stress is needed to prevent further growth. Delivering MIC during the lag period will ensure enough stress to either kill or injure the cells. Slow delivery of the rest will provide sustained stress.

This idea has some limitations since not all microorganisms have a lag period. Usually food composition and storage conditions are designed to inhibit growth rate of microbes and thus it is reasonable to expect the microorganisms to have a lag period based on the food type and storage requirements.

5.2. Experimental plan

The experimental plan was made based on understanding the factors influencing release profiles and identifying the major parameters influencing the target release profile. Since Hypothesis 1 helps test whether the concept of target release profile can be expressed in form of quantifiable parameter such as diffusivity, experiments were designed to evaluate the effect of diffusivities on microbial growth kinetics. Hypothesis 2 was designed to evaluate the effect of MIC and diffusivity on microbial growth kinetics. Testing 1 and 2

would help develop mathematical model for predicting target release profile which would then be validated using a bactericidal system (Figure 10).

Hypothesis 1 was tested by evaluating the effect of potassium sorbate diffusivities on *E.coli* DH5α. Measures of potassium sorbate diffusivity from polymers were obtained from the scientific literature. These values were then used to predict release profiles [59].

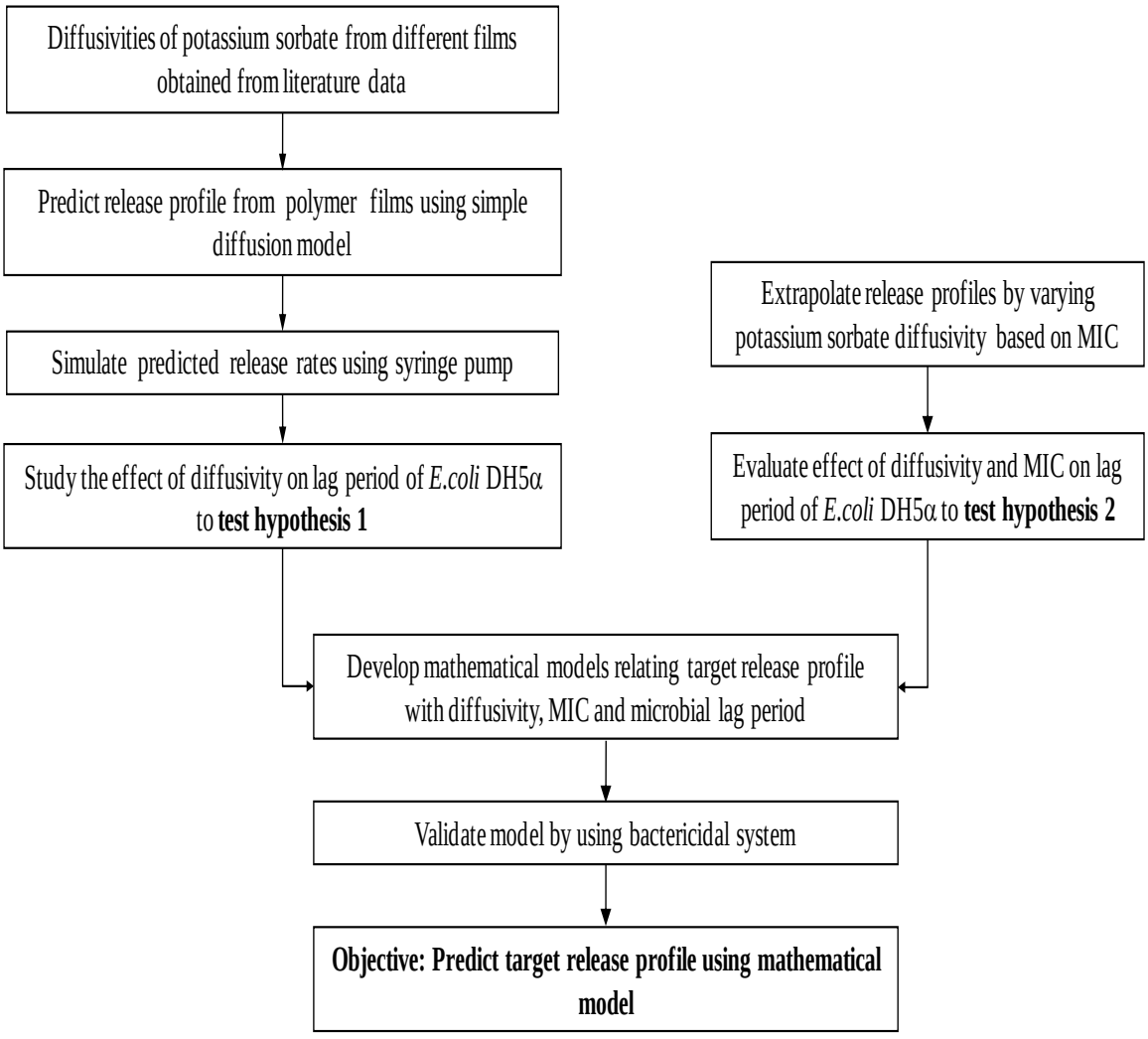

Figure 10: Experimental plan to predict antimicrobial target release profiles

Hypothesis 2 was then tested by extrapolating diffusivity based on amount of antimicrobial released during the inherent lag period of the organism and evaluating the

microbial growth kinetics. Testing the hypothesis helps determine the position of the parameters in the model. Thus the objective is achieved by developing mathematical models integrating diffusivity, MIC and microbial lag period to predict target release profile. The model was then validated using nisin/ *Micrococcus luteus* system, where nisin was bactericidal. The experimental plan helps integrate package and food research by developing a simple model to understand and quantify target release profile.

5.3. Materials for testing hypothesis 1 and hypothesis 2

5.3.1. Potassium sorbate

Potassium salt of sorbic acid (2, 4-Hexadienoic acid, potassium salt, 99% pure) was obtained from Acros organics. Three different concentrations (1 mg/mL, 2 mg/mL and 3 mg/mL) of potassium sorbate in culture media were used for the study, with 3 mg/mL (0.3% of food wt/wt) being the maximum allowable limit in food. Potassium sorbate is either dissolved directly in the growth media for instant addition or dissolved in water for controlled release studies based on the concentrations used for the study.

5.3.2. Tryptic soy broth (TSB)

Tryptic soy broth containing dextrose and yeast extract powder were obtained from MP Biomedicals. 30 g of the TSB dry powder and 6 g of yeast extract were mixed with 1000 mL of water to prepare the growth media for the bacteria. The pH of the media was adjusted to 5.0 using 3M concentrated hydrochloric acid. The Tryptic soy agar plates were made by adding 15 g granulated agar to the above mixture, autoclaving it at 121ºC and pouring it into sterile petridishes for solidification.

5.3.3. E.coli DH5α

Working cultures of *E.coli* DH5α was maintained on tryptic soy broth (TSB, Difco), pH 5.0, containing glucose and yeast extract, at 4 °C. The pH of the media is lowered to 5.0, to ensure high activity of potassium sorbate, using 3M concentrated hydrochloric acid. The cells were sub-cultured weekly and grown aerobically with agitation in tryptic soy broth, pH 5.0, at 30 °C. Periodic enumeration of the cells was done by serially diluting the cultures in phosphate buffered saline (PBS) and growing on tryptic soy agar.

5.4. Modes of delivery of potassium sorbate

The antimicrobial was delivered both by instant addition, which is the direct addition of antimicrobial to the formulation, and controlled release based on the model prediction of release kinetics from polymers.

5.4.1. Instant addition

Different concentrations of potassium sorbate (0.25 mg/mL to 3 mg/mL) were added to 200 mL of the growth media (TSB) at pH 5.0 and autoclaved. 2 mL (100X dilution) of *E.coli* DH5α, grown in TSB without antimicrobials for 15 hours at 30ºC, was added to the above mixture. The contents were shaken at 150 rpm and stored at 30ºC in the incubator. The growth of the organisms was evaluated by plate count method (see section 5.7) over a period of 24 hours and the inhibitory effect of potassium sorbate was determined by plotting the growth curve of log (CFU/mL) versus time.

5.4.2. Simulation of controlled release using syringe pump

5.4.2.1. Syringe pump description

A syringe pump is a small programmable mechanical pump that can deliver liquid materials through a syringe at a specified rate through its sophisticated flow control mechanism. A NE-1000 single syringe pump was used for the (Figure 11), obtained from New Era Pump Systems Inc. This was a bench top set-up 5 3/4" x 8 3/4" in area and holds up to 60 mL in the syringe. The pump rates can be increased from 0.73 µL/hr to 2120 mL/hr. Since rates are largely dependent on the inner area of the syringe (not just displacement rates), must be matched to desired release rates.

Figure 11: Syringe pump

The syringe pump can both infuse and withdraw and is controlled by a microcontroller system that drives a step motor. The syringe containing the antimicrobial solution was placed between the syringe holding block and the pusher block (Figure 12) and held in place by a spring mechanism.

The plate adjustment knob helps to keep the pusher block plate exactly touching the syringe plunger flange to ensure there is no time lapse during release. The syringe was driven by a drive screw-drive nut mechanism. The pump has a keypad interface that controls start/stop, varies the rates and volume by changing phase and also monitors flow.

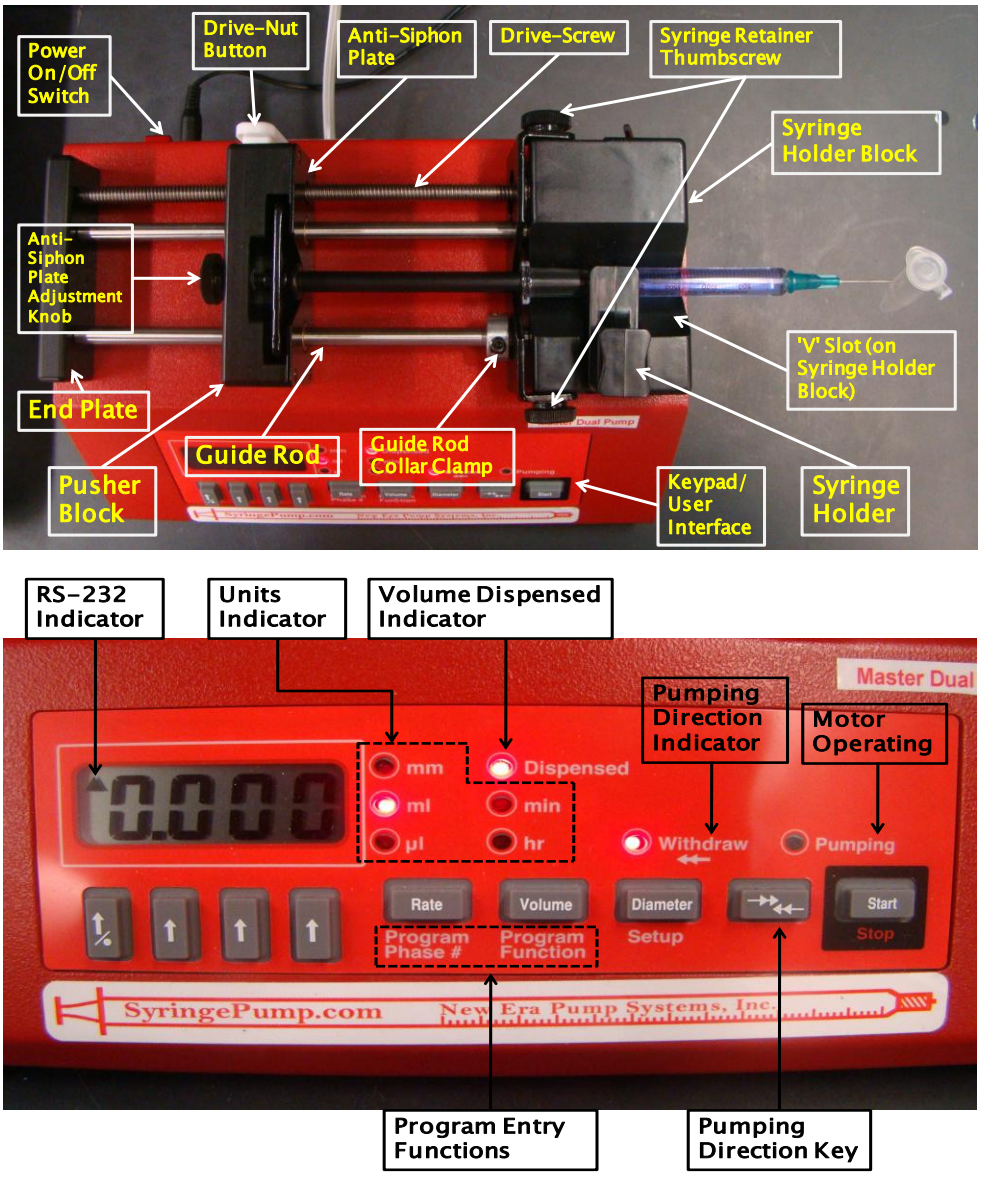

Figure 12: Parts of a syringe pump

The pump was connected to the computer using the RS232 communications port of the syringe pump. Automation with the computer simplifies the process and helps running the pump for days without moving the pump. WinPump control, a Windows™ program, is used to control all functionality of the pump when connected to the computer.

5.4.2.2. Precision and accuracy of syringe pump

The precision and accuracy of the syringe pump system was evaluated to avoid variations in the release rates from the model specification (Figure 13). The precision and accuracy of the syringe pump was evaluated by varying the release rate of colored water from the 2.5 mL Hamilton gas-tight syringe from 4 µL/hr to 1000 µL/hr using different size needles (20G1, 23G1 and 25G5/8). Delivery was 96-99% reproducible and accurate for high concentrations (100-1000µL/hr), and 94-98% for low concentrations (4-100 µL/hr).

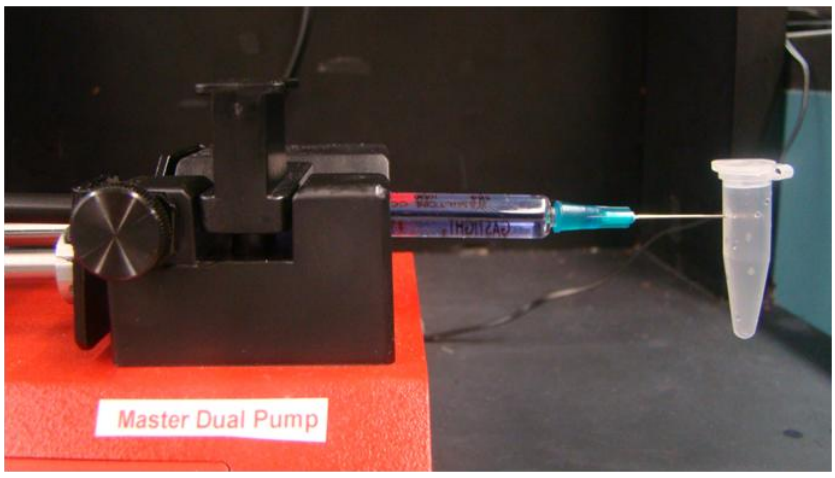

Figure 13: Syringe pump set-up to evaluate precision and accuracy

5.4.2.3. Syringe pump set-up

The syringe pump experiments were set-up as shown in the Figure 14. The syringe used for the experiments was a 2.5 mL Hamilton gas-tight syringe with an inner diameter of 7.28 mm. The release rates for these syringes can be varied from a minimum of 3.67 µL/hr to a maximum of 480.91 mL/hr. Potassium sorbate (0.6 g) was mixed in 2.5 mL water for 0.3% concentration and 0.2 g in 2.5 mL water for 0.1% concentration is taken in the syringe to be released at a desired rate. BD 25G5/8 precision glide needles were directly inserted into the autoclavable 1litre Nalgene PP bottles through the septum caps. This set-up ensures that there is no gap between the needle and the cap and thus prevents contamination. The syringe pump with the bottle was placed on a shaker and stirred at 150 rpm for 24 hours to ensure uniform mixing of organisms in the growth media and accelerate cell growth. The entire set-up is then placed in environmental chamber at 30ºC for the whole period of the study.

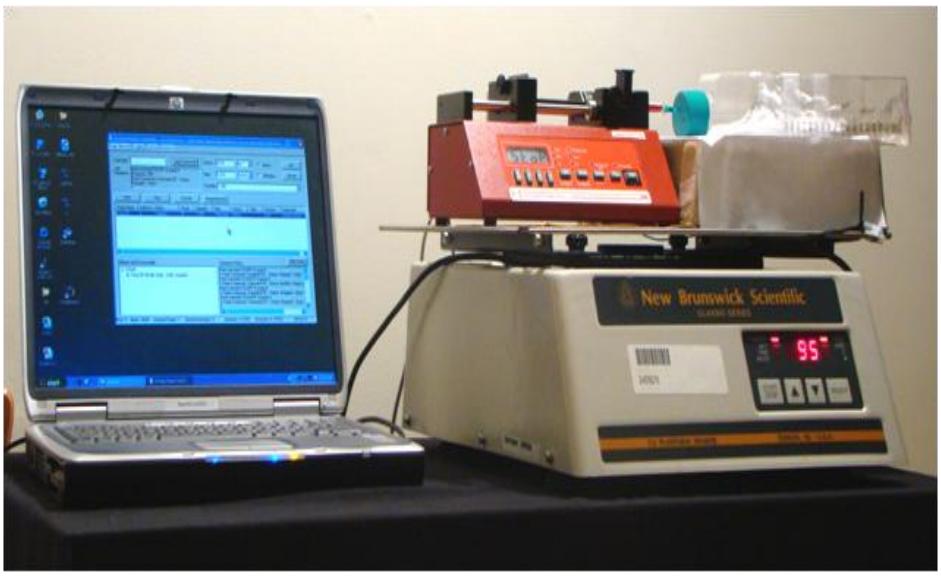

Figure 14: Syringe pump set-up

The release rates specified by model prediction were simulated by the syringe pump using the WinPump software (section 2.3.2). In this way the syringe pump helps to mimic release rates from different polymers without actually making those polymers and can facilitate quantification of target release profiles.

5.5. Diffusivity of potassium sorbate from literature to test hypothesis 1

Data on diffusivity of potassium sorbate from different polymer films that follow Fick's law or Fickian diffusion was selected from the literature. The diffusivities around 30°C were selected to ensure a wide range of release rates varying from very slow to very fast release rates. Potassium sorbate released from gluten films has the fastest release rate due to the high diffusivity (7.8×10^{-12} m^2/s) and beeswax had the slowest release rate (2.4×10^{-16} m^2/s). Table 6 lists the diffusivities of potassium sorbate obtained from the literature and used in the computer model for this study.

Table 6: Diffusivities of potassium sorbate obtained from polymers based on literature data

Polymer	**D, m^2/s**
k-carageenan	**$2.6x10^{-13}$**
Gluten	**$7.5\ x10^{-12}$**
Gluten-beeswax	**$5.6\ x10^{-12}$**
Gluten-Acetylated monoglycerides	**$3.2\ x10^{-12}$**
Beeswax	**$2.4\ x10^{-16}$**

5.6. Generation of diffusivities and release profile of potassium sorbate using simple diffusion model to test hypothesis 2

To realistically test the effect of potassium sorbate diffusivities on *E.coli* DH5α, it is critical to know the feasible range (the lowest diffusivity and highest diffusivity) that could be produced using CRP films. This range was determined from previous research on sorbic acid and potassium sorbate diffusion shown in Table 6 from polymers [59]. The high diffusivity was taken as $7.5\ x10^{-12}\ m^2/s$, which is based on sorbic acid diffusion from gluten films and low diffusivity as $2.4\ x10^{-16}\ m^2/s$, based on sorbic acid diffusion from beeswax films. Diffusivities within this range were generated based on diffusion model for short time. The following equation (Crank's diffusion model) describes the release of antimicrobial from a polymeric film based on the assumptions that release rate is controlled by Fickian diffusion, antimicrobial in the polymer is initially distributed

uniformly, the total amount of antimicrobial in the film is released to the food at equilibrium, antimicrobial is released from one side of the film, contact time is short, and the ratio of $M_{f,t}/M_{p,0} \leq 0.67$.

$$\frac{\mathbf{M_{f,t}}}{\mathbf{M_{p,0}}} = \frac{\mathbf{2}}{\mathbf{L}}\sqrt{\frac{\mathbf{D \times t}}{\boldsymbol{\pi}}} \quad \textbf{(1)}$$

where, $M_{f,t}$ is amount of antimicrobial in the food at time t (µg), $M_{p,0}$ is initial amount of antimicrobial in the film (µg), L is film thickness (m), D is diffusion coefficient or diffusivity of antimicrobial in the polymer (m^2/s), and t is time (s).

Model Assumptions

- Diffusion of potassium sorbate from the polymers follows Fickian diffusion
- D is a constant and depends only on temperature
- Potassium sorbate is dispersed uniformly within the polymer and the surface concentrations are uniform
- The release of potassium sorbate is unidirectional from x=0 to L
- The ratio of surface area to thickness is very large and thus the edge effect is negligible
- Potassium sorbate has high affinity to the solvent used for release study
- Solubility factor is negligible compared to diffusivity
- The solvent phase is stirred vigorously and no concentration gradients exist within the solvent
- Resistance to mass transfer negligible

- The initial concentration of potassium sorbate in the solvent is zero
- Large or infinite solvent is used compared to the polymer, therefore the partition coefficient is negligible

Diffusivities were generated by assuming reasonable values of L, $M_{p,0}$, $M_{f,t}$ and t in Eq. 1. The release profiles ($M_{f,t}/M_{p,0}$) were then generated using the calculated D and substituting back in Eq. 1.

5.6.1. Evaluation of minimum inhibitory concentration (MIC) using plate count method to test hypothesis 2

The minimum inhibitory concentration (MIC) is defined as the lowest concentration of antimicrobial required to produce maximum inhibition. The MIC of potassium sorbate required for inhibition of *E.coli* DH5α was found using plate count method. Five different concentration of potassium sorbate (0.25 mg/mL, 0.5 mg/mL, 0.75 mg/mL, 1 mg/mL, 2 mg/mL and 3 mg/mL) was evaluated on the growth of ~10^7 cfu/mL cells of *E.coli* DH5α. The MIC obtained is then compared with release rates to quantify target release profile.

5.6.2. Predicting lag period of *E.coli* DH5α using a logistic model

Logistic or primary model are useful in predicting the microbial lag period. Bacterial growth plotted against logarithm of the organisms as a function of time generates a sigmoidal curve, where the lag phase is just after time zero, followed by exponential phase with maximum growth and finally a stationary phase [80]. This growth may be described by the equations,

$$\log N(t) = A + \frac{D}{1 + e^{-B(t-M)}}$$

$$\boldsymbol{t_{lag} = M - \frac{2}{B}\left(1 - \frac{2}{(1 + e^{BM})}\right)} \quad \textbf{(2)}$$

Where, M = time at which half the maximum cell concentration is reached

B = maximum specific growth rate, μ_{max}

A = value of lower asymptote

D= difference between the lower and upper cell density

The lag period (t_{lag}) of *E.coli* DH5α in the absence of potassium sorbate (control) was obtained from the curve as time at the intersection of the horizontal line of initial count and tangent line of exponential growth plotted from the logistic or primary model shown above.

5.7. Determination of Growth Kinetics of *E.coli* DH5α for instant addition and controlled release of potassium sorbate

The ability of *E.coli* DH5α to grow in the absence and presence of potassium sorbate was determined using plate count method at 30 °C. The effect of instant addition or direct addition of potassium sorbate into growth media at zero time on the growth kinetics of *E.coli* DH5α were determined using the plate count method at 30 °C. The cells were cultured in TSB to an OD of 0.8-1.0 at 600 nm (SmartSpec™ 3000, Bio-Rad Laboratories, Hercules, CA, USA). The cells were then diluted 100 times into sterile PET bottles (Fisher Scientific, Pittsburgh, PA, USA) containing 200 mL TSB to obtain an initial concentration of ~10^7 CFU/mL. Samples from the PET bottles were taken periodically for up to 24 h and diluted serially as needed, using PBS. One hundred micro

liters of the diluted sample was uniformly spread on the plates and the cell growth was enumerated over time by spread plate method. The number of organisms grown was counted, plates were used in the model calculations only if the number of cells grown was in the range of 30-300. Two replicates of each dilution are plated on 4 plates and thus a total of 8 plates were evaluated for each time period. The inhibitory effect of potassium sorbate was observed by plotting the growth curve of log (CFU/mL) versus time. The enumeration studies for controlled release of potassium sorbate were carried out the same way as described for instant addition experiments.

5.8. Quantification of target release profile through mathematical models

Once growth kinetics,for various release rates of the antimicrobial were obtained mathematical models were developed based on the functional relationships mentioned in section 3.2. The experiments were designed with a limited number of variables, as the effect of one on the other can be quantified more clearly. Once a basic model was developed, additions of other variables influencing target release profile were added to it.

5.9. Validating model using nisin/ *Micrococcus luteus* system

The developed model was validated by evaluating the effect of nisin, a bactericidal antimicrobial, on the growth of *Micrococcus luteus*. The experimental design consisted of determining the growth kinetics of *M. luteus* for (1) instant addition of nisin and (2) controlled release of nisin, where the controlled release was simulated to mimic the release profile of nisin from packaging films.

5.9.1. Bacterial strains, culture conditions, and nisin solution

A working culture of *M. luteus* ATCC 10420 was maintained on tryptic soy agar plates (TSA, Difco) supplemented with 1% glucose and 0.6% yeast extract at 4°C. The cells were sub-cultured weekly and grown aerobically with agitation in tryptic soy broth at 30°C. Periodic enumeration of the cells was done on tryptic soy agar by serially diluting the cultures in phosphate buffered saline (PBS).

Nisin powder (2.5%, balance NaCl and milk solids) with a minimum potency of 10^6 IU/g was obtained from Sigma (St Louis, MO, USA). A nisin stock solution (7.45×10^{-3} µmol/mL) was prepared by dissolving the appropriate amounts of powder in 0.02 N HCl (pH 1.7). The stock solution was then autoclaved at 121°C for 15 min and stored at 4°C.

The nisin concentration for instant addition was varied between 1.49×10^{-4} µmol/mL and 7.45×10^{-3} µmol/mL. Final concentrations obtained by adding no more than 2 mL's of appropriately diluted nisin stock solution to 200 mL TSB. The nisin solution for controlled release was prepared by diluting the stock solution with nisin (0.02 N HCl, pH 1.7) to reach a final concentration of 0.596 µmol nisin/mL, again adding no more than 2 ml nisin to minimize cell dilution in the culture. To minimize cell dilution due to addition of nisin solution, the total volume of solution added to the culture was kept under 1% (v/v).

5.9.2. Determination of growth kinetics for instant addition experiment

The effect of instant addition or direct addition of nisin into growth media at zero time on the growth kinetics of *M. luteus* was determined using the plate count method at 30°C. The cells were cultured in TSB to an OD_{600} of 0.8-1.0 (SmartSpec™ 3000, Bio-Rad

Laboratories, Hercules, CA, USA). The cells were then diluted 100 times into sterile PET bottles (Fisher Scientific, Pittsburgh, PA, USA) containing 200 mL TSB to obtain an initial concentration of ~10^6-10^7 CFU/mL. Samples were taken from the PET bottles periodically for 48 hours and serial dilution was carried out, as needed, using PBS. One hundred µL of the diluted sample was uniformly spread on the plates and the cell growth was enumerated over time by spread plate method. The inhibitory effect of nisin was observed by plotting the growth curve of log (CFU/mL) vs. time. The lag period of *M. luteus* in the absence of nisin (control) was obtained from the curve as time at the intersection of the horizontal line of initial count and tangent line of exponential growth.

5.9.3. Prediction of target release profile of nisin

Optimal diffusivity was quantified from the developed model, was then substituted in eq. 1 to obtain the target release profile. The target release profile was then simulated using a syringe pump to evaluate the growth kinetics of *M. luteus* in growth media.

5.9.4. Generation of Release Profiles of Nisin using Crank's Diffusion Model

Release profiles bracketing the optimum rates were evaluated to further validate the model. The procedures for generating the release profiles ($M_{f,t}$ versus t) were as follows: (1) reasonable values of L, $M_{p,0}$, $M_{f,t}$, and t were assumed to calculate D values using Eq. 1, and (2) release profiles were then generated using the calculated D values and Eq. 1. Nisin, the antimicrobial, L was assumed to be 3 mil (76.2 µm), and $M_{p,0}$ was taken as 1.49 µmol ($7.45x10^{-3}$ µmol/mL of growth media) which was the highest amount used in the instant addition experiment. Values of $M_{f,t}$ and t for calculating D values were

selected based on the results of instant addition experiment. The release profiles were generated using the D values and Eq. 1 for a time period based on the results of instant addition experiment.

5.9.5. Simulation of Controlled Release Profile of Nisin using Syringe Pump System

The release profile of the nisin solution was simulated by a syringe pump system which consisted of a NE-1000 single syringe pump (New Era Pump Systems Inc., Wantagh, NY, USA) controlled by a Windows-based computer using WinPump Control software. The rates of release were delivered according to Crank's model (Eq. 1) to mimic the release of nisin from CRP films. Appropriate amounts of nisin solutions from the sterile gas-tight syringe (Hamilton, Reno, NV, USA) mounted on the syringe pump were injected into a sterile PET bottle (Fisher Scientific, Pittsburg, PA, USA) containing 200 mL TSB inoculated with *M. luteus* (~$5x10^6$ CFU/mL). The syringe pump with the bottle was placed in an incubator at 30ºC and stirred at 150 rpm to ensure uniform mixing. The setup ensures immediate mixing of nisin into the broth and also uniform mixing of *M. luteus* within the broth.

5.9.6. Determination of Growth Kinetics for Controlled Release Experiments

The ability of *M. luteus* to grow in the presence and absence of nisin was evaluated using the plate count method at 30°C. The enumeration studies were carried out the same way as described in Section 5.7.

5.9.7. Data Reproducibility

To test reproducibility of results, each experiment was performed twice in quadruplicates.

6. RESULTS AND DISCUSSION

6.1. Testing hypothesis 1

The theory behind Hypothesis 1 is that for the same amount of antimicrobial added to the CRP film, not all diffusivities will have the same inhibitory effect on a microorganism. In practical terms, this means that for the same amount of antimicrobial in a packaging film, different polymers and blends will show different levels of inhibitory effect on the targeted microorganism because they will release the antimicrobials at different rates. It is logical to expect then, that there is an optimum range of diffusivities suitable to suitable to extend microbial lag period over the desired shelf life.

The release profile for potassium sorbate diffusivities generated from literature data (see section 5.5) is shown in Figure 15. No addition or control without potassium sorbate is diffusivity 0 and instant addition of 0.3% potassium sorbate is of diffusivity ∞. The effect of release rates designed to match varying diffusivities on the growth kinetics of *E.coli* DH5α was evaluated; diffusivity of potassium sorbate plotted as time vs. colony forming units per mL (CFU/mL) is shown in Figure 16.

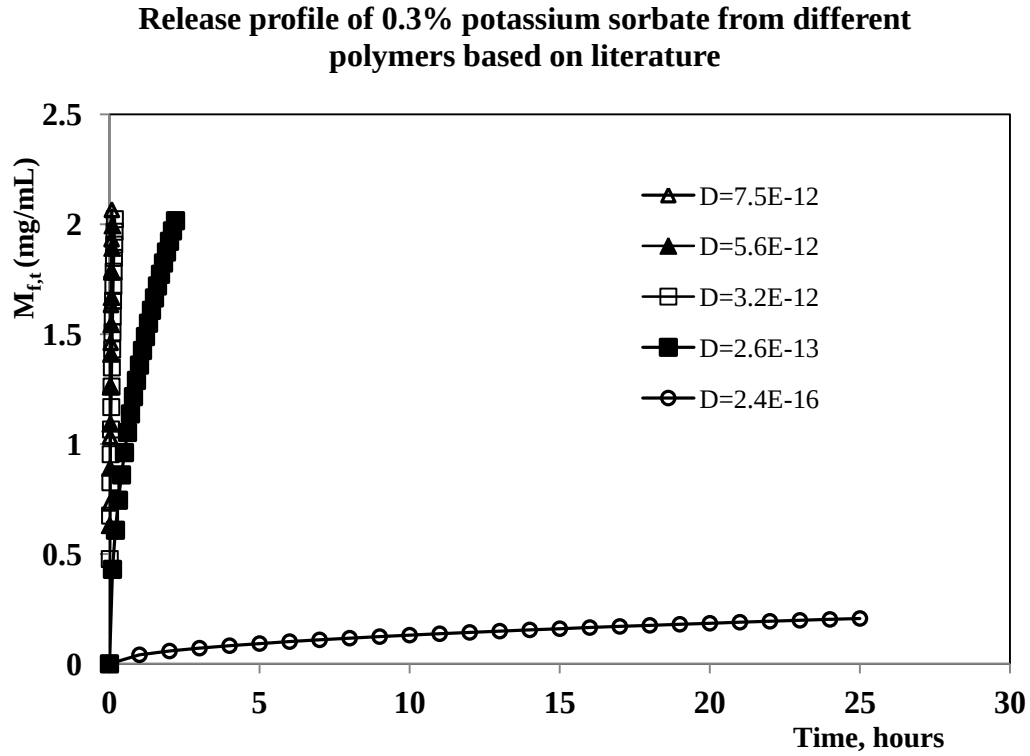

Figure 15: Release rate of 0.3% potassium sorbate by varying diffusivities

Results show that as the diffusivity increases growth of the microorganism slows. Instant addition of 0.3% potassium sorbate showed complete inhibition of *E.coli* DH5α for the required shelf life of 24 hours. Slow release rate of potassium sorbate from beeswax films ($D=2.4x10^{-16}$ m^2/s) was insufficient to inhibit *E.coli* DH5α (Figures 16). The growth kinetics resembled the control without addition of antimicrobial. However as the diffusivity increased moving to gluten ($7.5x10^{-12}$ m^2/s) and k-carageenan film models ($2.6x10^{-13}$ m^2/s) the lag period correspondingly increased and produced complete inhibition of the organism for the period of 24 hours.

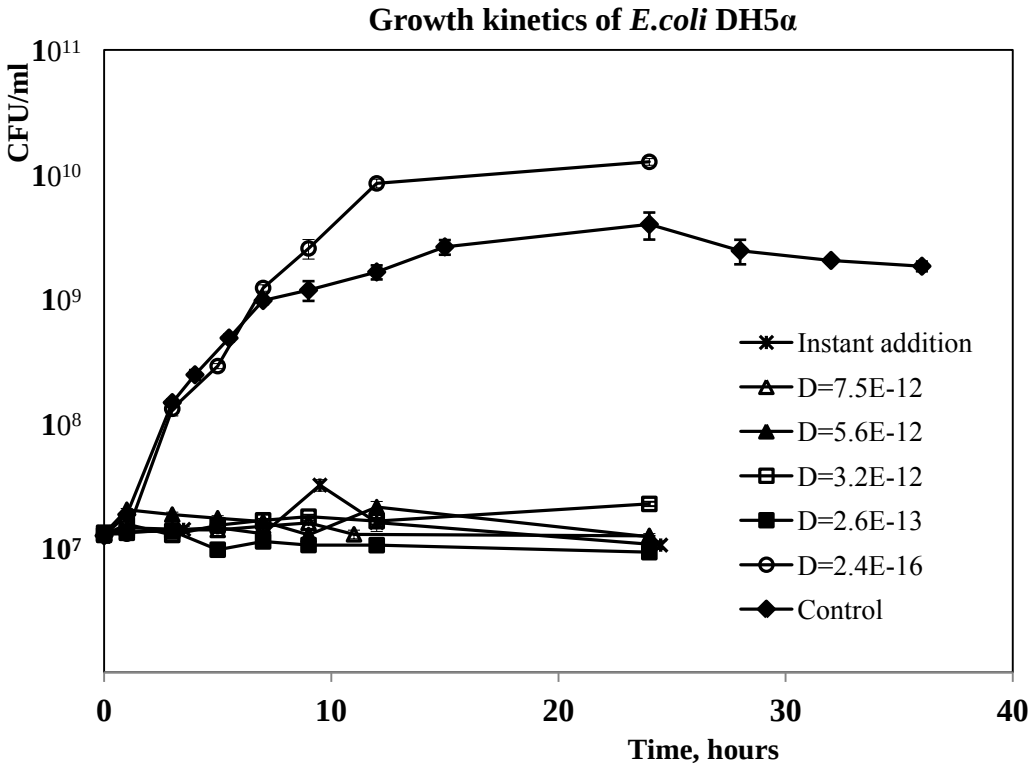

Figure 16: Growth kinetics of *E.coli* DH5α by varying diffusivities of 0.3% potassium sorbate

Although the concentration of potassium sorbate was 3 mg/ml in all cases, about 67% of the antimicrobial was released within 24 hours when diffusivities were between 7.5×10^{-12} m^2/s to 2.6×10^{-13} m^2/s, while only 10% was released from beeswax film model (2.4×10^{-16} m^2/s). In terms of the hypothesis the gluten and carageenan rates provide antimicrobial doses above the MIC while the besswax dose was considerably below this level. From the results it can be extrapolated that decreasing diffusivities from 2.6×10^{-13} m^2/s to 2.4×10^{-16} m^2/s would decrease the concentration of potassium sorbate released in 24 hours, thus narrowing the range of effective diffusivities that would cause complete inhibition of the organisms.

Although these results do not cover the entire range of diffusivities, the observation that for the same amount of antimicrobial in CRP films not all diffusivities had the same inhibitory effect on the growth of the microorganism **supports Hypothesis 1 that there is an optimum range of diffusivities for antimicrobial release from CRP system to inhibit microbial growth, thereby extending the lag period and maintaining the product at a safe level over the desired shelf life.**

Further knowing that the release is controlled by the diffusion step (see Section 2.4), the antimicrobial release from CRP films referred as release profile was quantified using diffusivity or diffusion coefficient (D, m^2/s), a packaging parameter that shows how fast or slow the antimicrobial moves within the polymer. Results demonstrate a selective range of diffusivities suitable to inhibit microbial growth and thus target release profile can be expressed in form of diffusivity for quantification.

6.2. Testing hypothesis 2

Hypothesis 2 was designed to quantify the minimum diffusivity required for maximum or complete inhibition of microbial growth in food. Based on the rationale that bacterial cells adapt during the lag period to recover and adjust to the new environment before initiating exponential growth [81, 82], the hypothesis assumed that the amount of antimicrobial added during the inherent lag period of the microorganism determines its effectiveness for inhibiting subsequent growth and proliferation. To provide a maximum stress for microorganisms the amount of antimicrobial given during the microbial lag period must be equal or greater than the MIC, the minimum concentration required for complete inhibition of the organism.

The hypothesis was tested by,

- Measuring the MIC of potassium sorbate by instant addition of 0.25 mg/mL, 0.5 mg/mL, 0.75 mg/mL, 1 mg/mL, 2 mg/mL and 3 mg/mL to determine the concentration required to completely inhibit of *E.coli* Dh5α
- Determining the lag period of *E.coli* Dh5α in the absence of potassium sorbate (section 5.6.2)
- Determining growth kinetics of *E.coli* Dh5α as a function of release profile and concentration of potassium sorbate added (above and below MIC) during the inherent lag period of the organism (predicted from step 2) (Section 5.6)
- Repeating step 3 for three different concentrations of potassium sorbate in polymers ($M_{p,0}$ varied between 1 mg/mL to 3 mg/mL)

6.2.1. Instant addition of potassium sorbate to obtain MIC

Growth kinetics of *E.coli* DH5α exposed to instant addition of 0.25 mg/mL, 0.5 mg/mL, 0.75 mg/mL, 1 mg/mL, 2 mg/mL and 3 mg/mL are shown in Figure 17. Results showed that a minimum of 0.5 mg/mL is needed for an initial count of ~10^7 cells to completely inhibit the organism for 24 hours. Concentration below 0.5 mg/mL does not show inhibition and the organisms continue to grow. Figure 17 also shows that potassium sorbate is bacteriostatic in nature and it does not kill *E.coli* Dh5α but maintains the cells in the lag period.

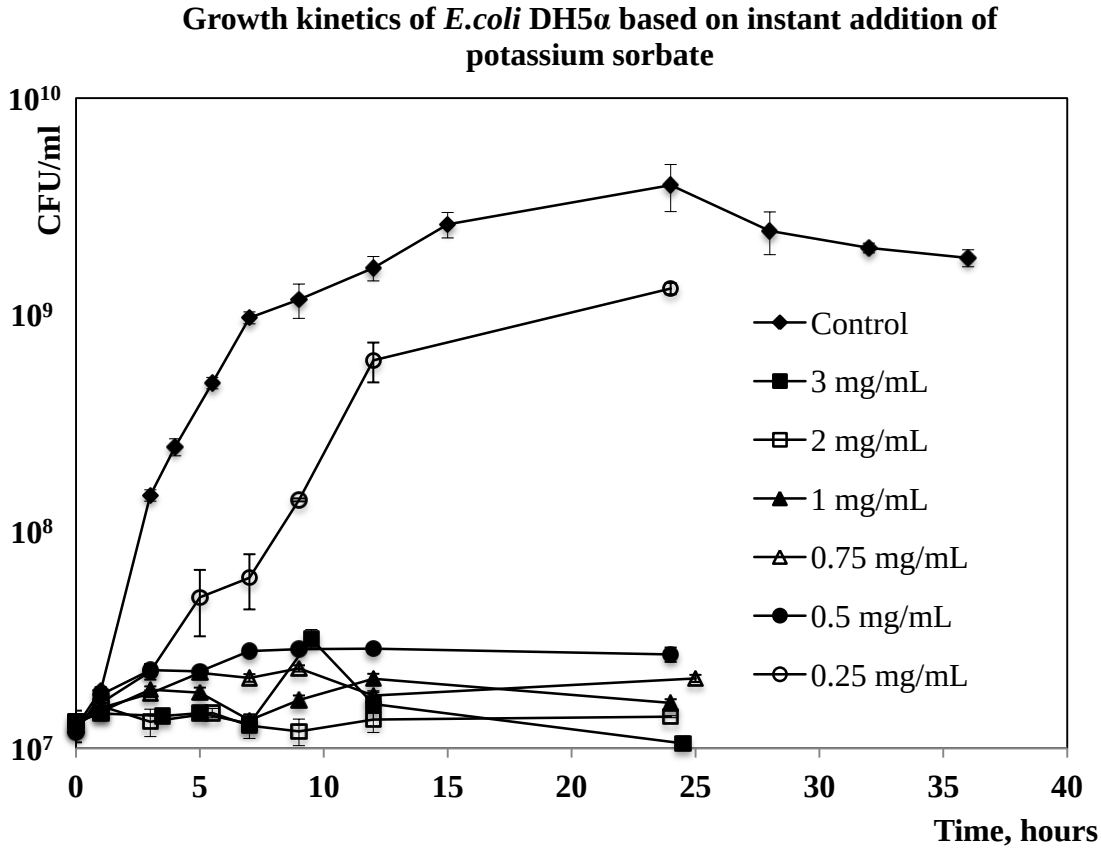

Figure 17: Quantifying MIC based on instant addition of different concentrations of potassium sorbate

6.2.2. Quantifying lag period of *E.coli* Dh5α

The lag period of *E.coli* Dh5α was quantified using logistic model (Section 5.6.2). In plotting of N(t) vs time (Figure 18) the slope BD/4 was found as 0.3598. The difference between lower and upper asymptote (D) was calculated as 9.6, therefore B was calculated as 1.5×10^{-1}. The time at which the growth was maximal (M) was found as 9. Substituting B and M in Equation 2 show that the lag period of *E.coli* Dh5α was 1.16 hours.

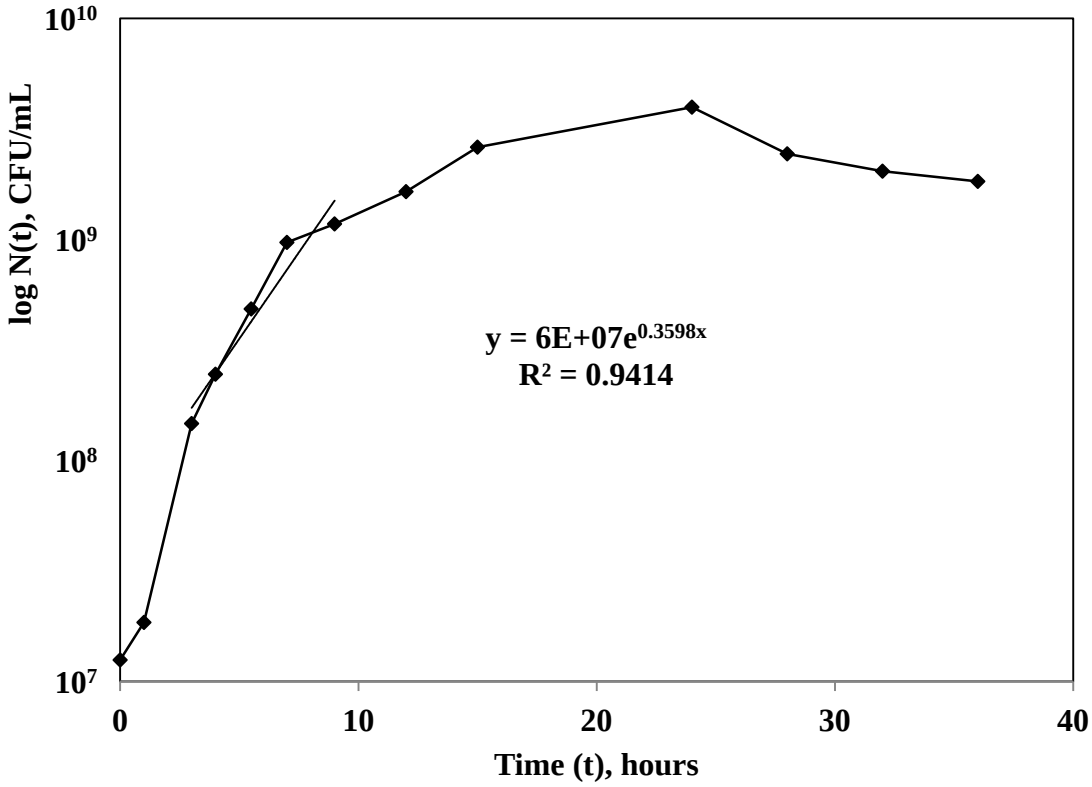

Figure 18: Lag period of *E.coli* DH5α quantified using logistic model

6.2.3. Evaluating growth kinetics of *E.coli* DH5α by releasing three concentration of potassium sorbate from polymer ($M_{p,0}$) at the different diffusivities

To evaluate the effect of potassium sorbate diffusivities on *E.coli* DH5α, it is critical to know the feasible range (the lowest diffusivity and highest diffusivity) that could be produced using CRP films. This range was obtained from previous research on sorbic acid and potassium sorbate diffusion from polymers [59]. The high diffusivity was taken as 7.5 $x10^{-12}$ m^2/s, based on sorbic acid diffusion from gluten films, and low diffusivity as 2.4 $x10^{-16}$ m^2/s, based on sorbic acid diffusion from beeswax films. Diffusivities within this range were generated from the diffusion model for short time as described in section 5.6 by assuming, thickness, L as 3 mil (76.2 μm), initial concentration of potassium

sorbate in polymer, $M_{p,0}$ was varied from 1 mg/mL to 3 mg/ml and time, t as 1.16 h (lag period of *E.coli* DH5α). The concentration of antimicrobial released over time, $M_{f,t}$ was varied between 0.15 mg/ml to 0.75 mg/ml and the diffusivities were calculated by substituting these values in Equation 1 (Table 7).

Table 7: Potassium sorbate diffusivities generated from Crank's diffusion model

	$M_{p,0}$, mg/ml	**1**	**2**	**3**
t, h	**$M_{f,t}$, mg/ml**	**D, m^2/s**		
1.16	0.15±0.01	$2.61x10^{-14}$	$6.53x10^{-15}$	$2.90x10^{-15}$
	0.25±0.01	$6.82x10^{-14}$	$1.71x10^{-14}$	$7.58x10^{-15}$
	0.31±0.01	$1.08x10^{-13}$	$2.64x10^{-14}$	$1.20x10^{-14}$
	0.50±0.01	$2.60x10^{-13}$	$6.82x10^{-14}$	$3.03x10^{-14}$
	0.75±0.01	$6.14x10^{-13}$	$1.53x10^{-13}$	$6.82x10^{-14}$

The release profile predicted by substituting diffusivities in Crank's model (Eq.1) was plotted as time vs. concentration of potassium sorbate released (Figure 19, 21, 23). The growth kinetics of the organism based on the diffusivity of potassium sorbate is plotted as time vs. colony forming units per mL (CFU/mL) (Figure 20, 22, 24).

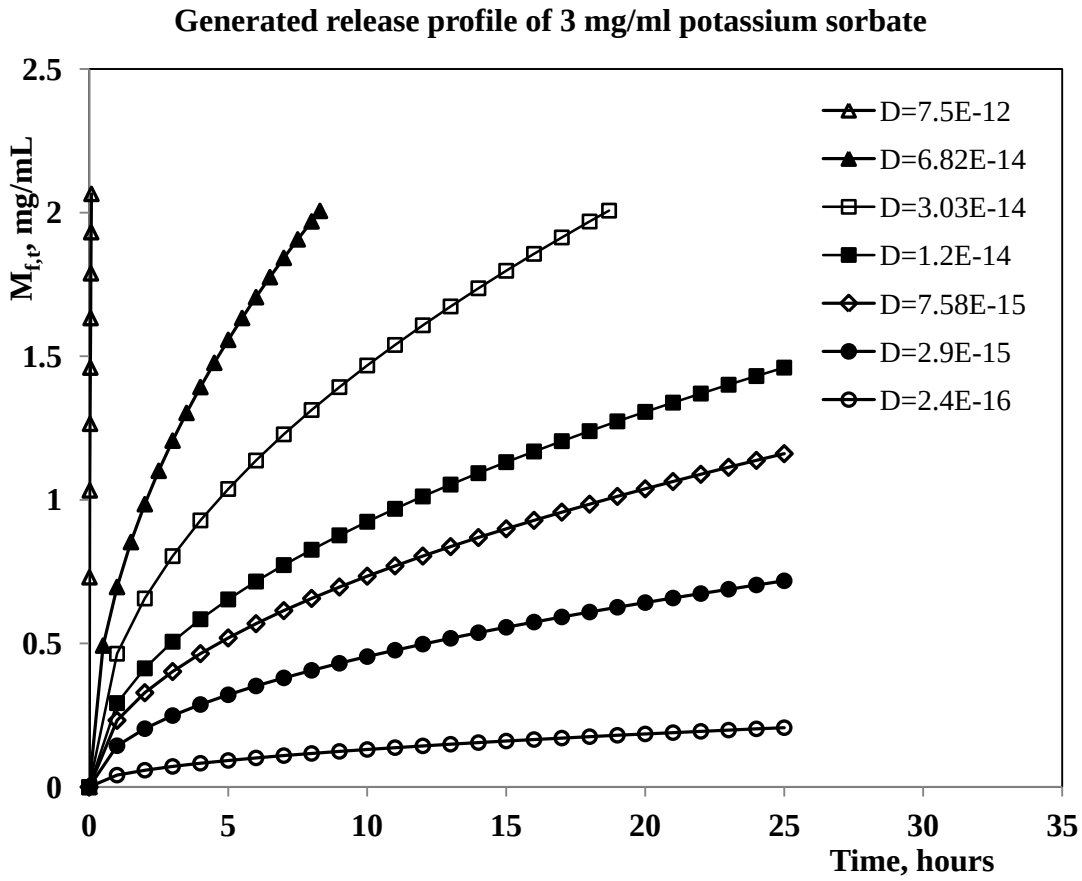

Figure 19: Effect of diffusivities on release profiles of 3 mg/ mL potassium sorbate, based on Crank's diffusion model

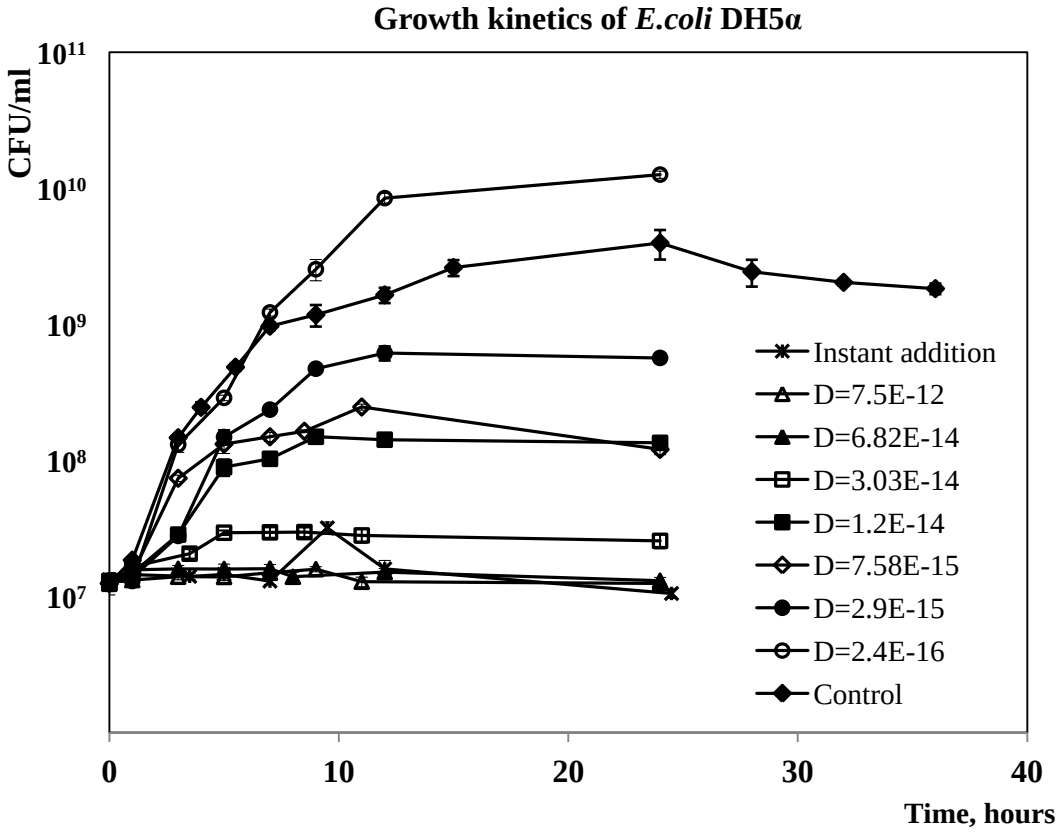

Figure 20: Effects of 3 mg/mL potassium sorbate diffusivity (release profiles) on growth kinetics of *E.coli* Dh5α

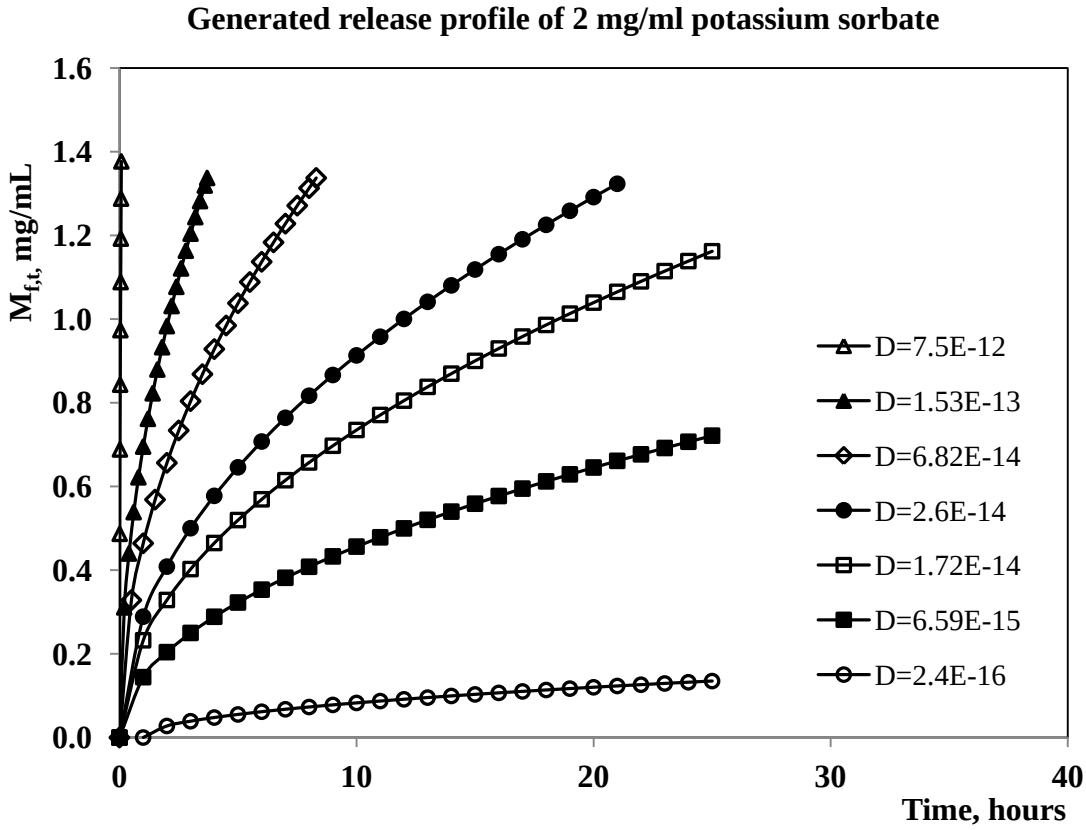

Figure 21: Effect of diffusivities on release profiles of 2 mg/ mL potassium sorbate based on Crank's diffusion model

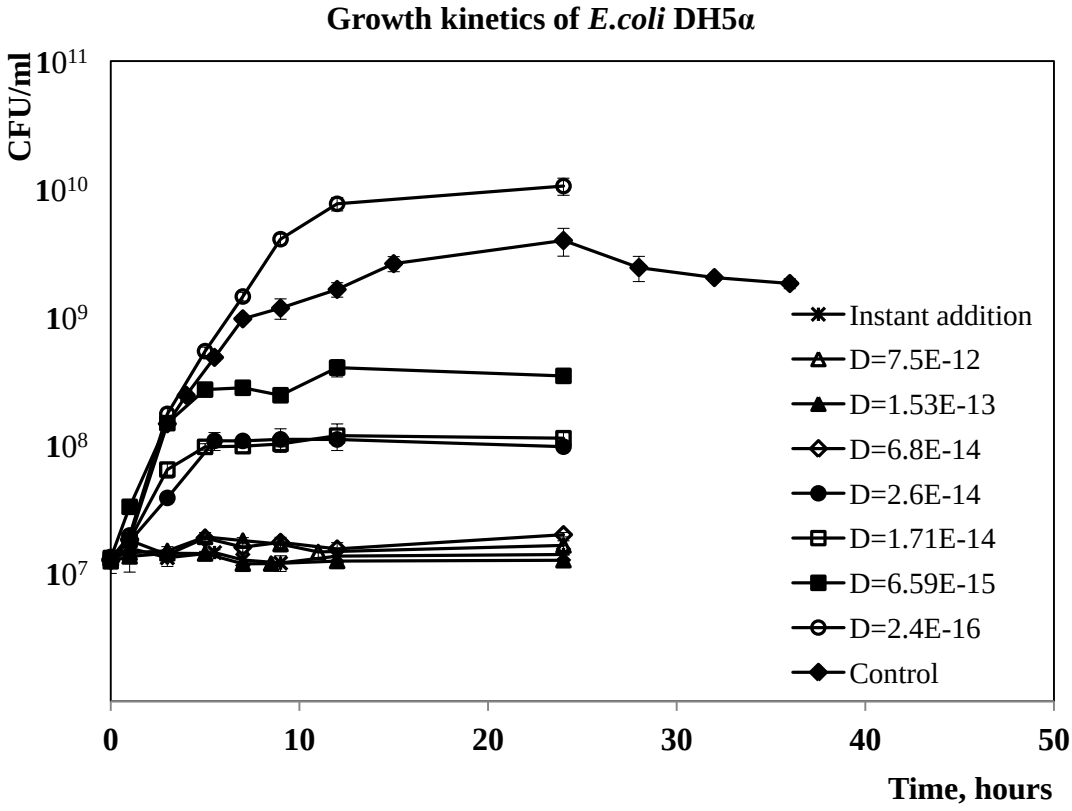

Figure 22: Effects of 2 mg/mL potassium sorbate diffusivity (release profiles) growth kinetics of *E.coli* Dh5α

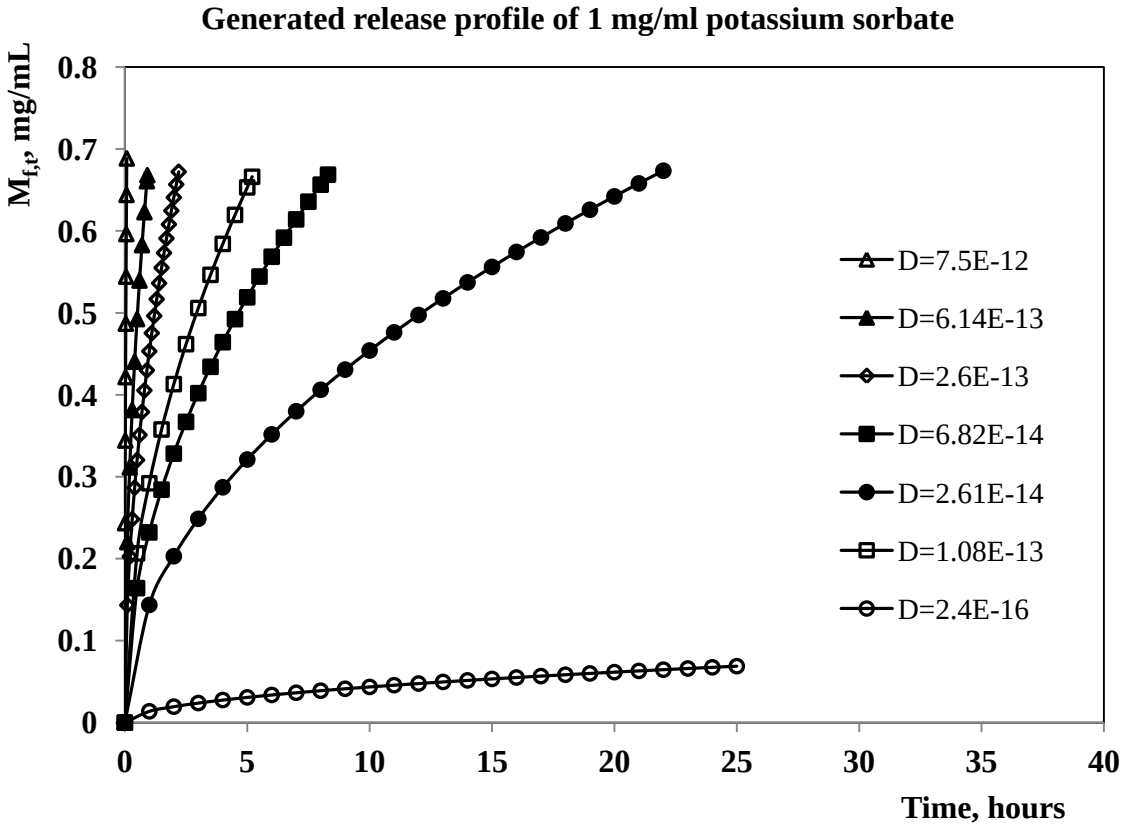

Figure 23: Effect of diffusivities on release profile of 1 mg/ mL potassium sorbate based on Crank's diffusion model

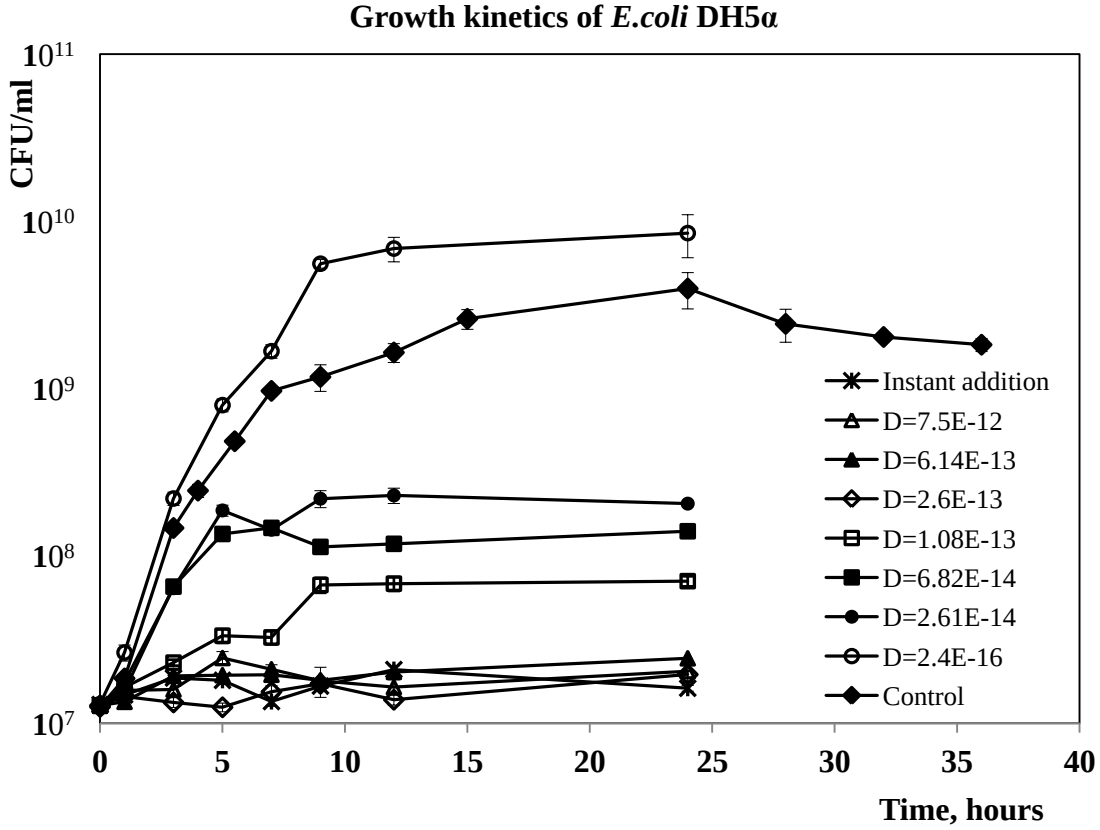

Figure 24: Effects of 1 mg/mL potassium sorbate diffusivity (release profiles) growth kinetics of *E.coli* Dh5α

Results clearly show that not all diffusivities, for the same amount of potassium sorbate in the model polymer, show the same level of growth inhibition in *E.coli* DH5α. For $M_{p,0}$ of 0.2 g (1 mg/mL), a minimum diffusivity of 2.60×10^{-13} m^2/s was needed to provide complete inhibition of the microorganism for 24 hours (Figure 24). Potassium sorbate diffusivity less than 2.60×10^{-13} m^2/s does not show the required inhibition of *E.coli* DH5α comparable to instant addition. Similar results were observed when $M_{p,0}$ was increased to 0.4 g (Figure 22) and 0.6 g (Figure 20). A minimum diffusivity of 6.82×10^{-14} m^2/s ($M_{p,0}$ = 0.4 g) and 3.03×10^{-14} m^2/s ($M_{p,0}$ = 0.6 g) was needed for complete inhibition of *E.coli* DH5α for 24 hours at 30 °C. Moreover increasing the amount of potassium sorbate in the model polymer increased minimum diffusivity. These results further confirmed

Hypothesis 1 that there is an optimum range of diffusivities suitable to provide the required microbial inhibition in food.

The results also showed that a minimum of ~0.5 mg/ml of potassium sorbate released during the inherent lag period of *E.coli* DH5α (1.16 h) showed complete inhibition of the organism (Figure 25). Concentrations lower than that did not have the same level of inhibition. We know from the instant addition studies that the MIC of potassium sorbate for *E.coli* DH5α is 0.5 mg/mL.

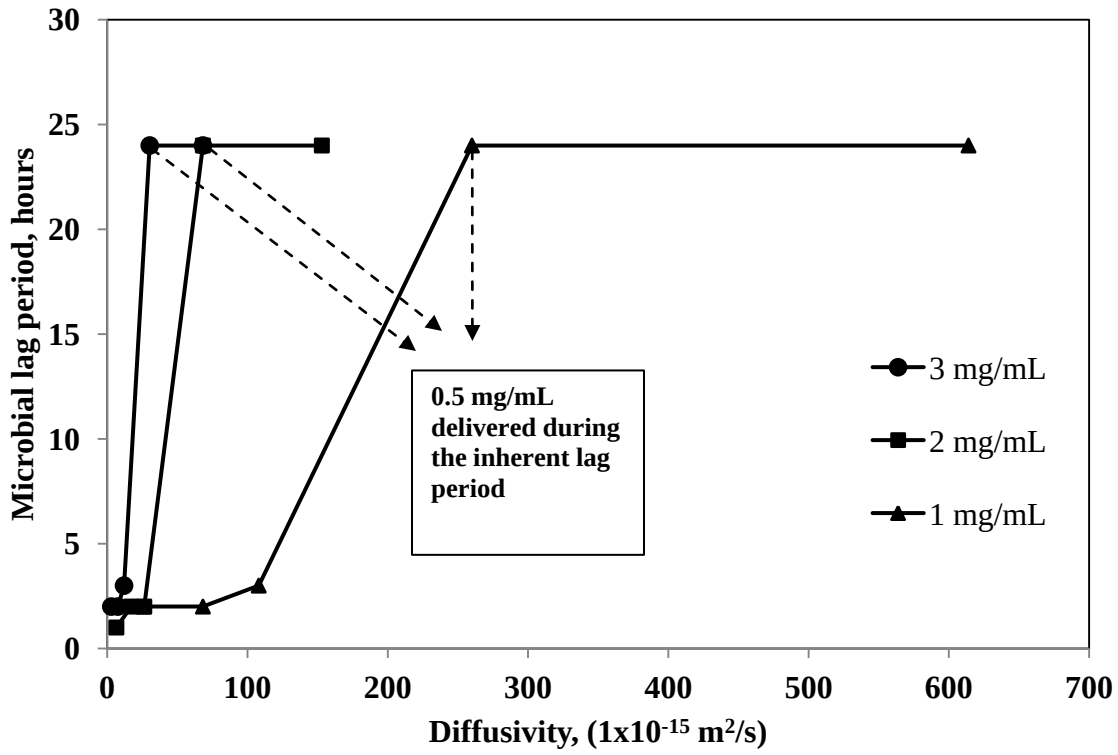

Figure 25: Microbial lag period as a function of diffusivity and antimicrobial concentration in polymer

Thus, **results support hypothesis 2** and it can be **concluded that the concentration of antimicrobial added during the inherent lag period of the microorganism must be**

equal to or greater than its MIC. These results further suggests that lethal or maximum antimicrobial stress is needed during the lag phase of a microorganism to injure cells and inhibit growth, while maintaining stress will help prevent further growth. The addition of MIC and microbial lag period in the model would help quantify the minimum diffusivity that is needed to provide the maximum inhibition of the microorganisms in the system.

6.2.4. The reason behind effectiveness of Hypothesis 2

Most theories about the release kinetics of antimicrobial from packaging expects that the concentration of antimicrobial must be maintained above the critical inhibitory concentration of the contaminating microorganisms [7, 83]. Though in theory it is ideal, putting it into effect or quantifying it requires further understanding of the microbial growth kinetics.

Addition of an antimicrobial to a system containing microorganisms either weakens the organism (scenario A: bacteriostatic antimicrobials) or injures/kills the cells (scenario B: bactericidal antimicrobials) (Figure 26). In scenario A (Figure 27) where the microorganisms are weakened increase in time from 0 to 1 does not decrease cell number but the cells at t =1 (N1) are weakened and therefore may not need as much stress as the cells at time 0 (N0). Lowering concentration from C0 to C1 would help maintain the stress, thereby preventing the microorganisms from growing back. In scenario B (Figure 25) the cell count is lowered from N0 to N1 due to the antimicrobial over time t=0 to t=1. Thus lowering concentration from C0 to C1 will continue to inhibit cell growth. In scenario B the idea of MIC comes to mind. MIC is highly relative to the cell number.

Lowering cell number from N0 to N1 basically lowers MIC, therefore lower concentrations are sufficient to provide the stress and injure cells.

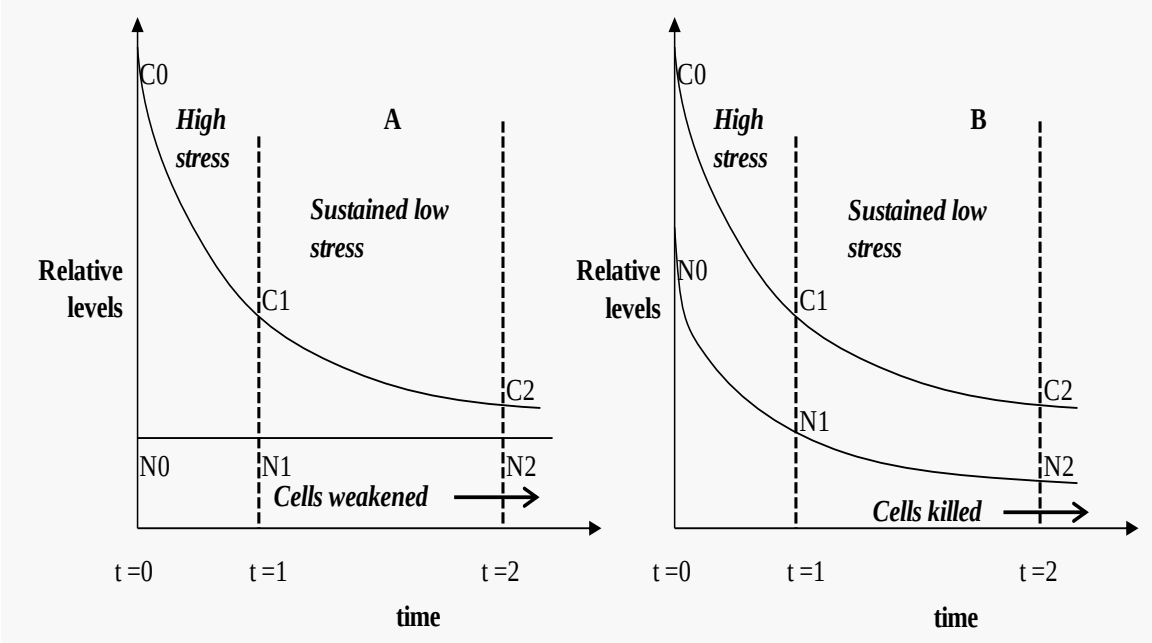

Figure 26: Hypothetical graph explaining microbial cell count and antimicrobial concentration in a system

In both cases it is critical to give high stress during the time when the microbes are at their weakest stage trying to adapt themselves to the environment. Controlling organisms in log or exponential phase would require high concentration of antimicrobial and may not be feasible. Sustained stress through continues delivery of antimicrobial would prevent further growth.

Thus, it was hypothesized that the quantifiable or effective concentration that can provide the maximum stress during the inherent lag period of the organisms must be equal or greater than MIC and the results support the hypothesis. Sustained stress is provided through controlled release technology (assuming early migration is not excessive) and is determined by the amount of antimicrobial added to the polymer. In instant addition, all

the antimicrobials are delivered at time 0, so the antimicrobials would provide enough stress to kill cells but since the antimicrobials can be depleted in the process, extended protection may not possible.

6.3. Effect of antimicrobial concentration on diffusivity

Figure 25 showed that the effective range of diffusivities required for complete microbial inhibition decreased with decrease in antimicrobial concentration. Figure 27 further showed that for the same diffusivity of 6.82×10^{-14} m^2/s, 3 and 2 mg/mL potassium sorbate in polymer shows complete inhibition but 1 mg/mL is not sufficient to provide the required inhibition of *E.coli* DH5α. In practical terms, the effective range of polymers suitable to inhibit microbial growth decreases with decrease in antimicrobial concentration. Hypothetically, if LDPE containing 3 mg/mL (of food) potassium sorbate is suitable to inhibit growth of *E.coli* DH5α, reducing concentration to 1 mg/mL may not be effective under the same conditions, and may require, instead a polymer with higher diffusivity.

Case in point the minimum diffusivity to obtain maximum microbial inhibition increases as antimicrobial concentration decreases, i.e., when the antimicrobial concentration in polymers is decreased then faster release profile would be required to completely inhibit the microorganisms. For example, as the concentration of potassium sorbate decreased from 3 mg/mL to 1 mg/mL, the minimum diffusivity that produced an effective inhibition of the organisms increased from 3.03×10^{-14} to 2.6×10^{-13} m^2/s.

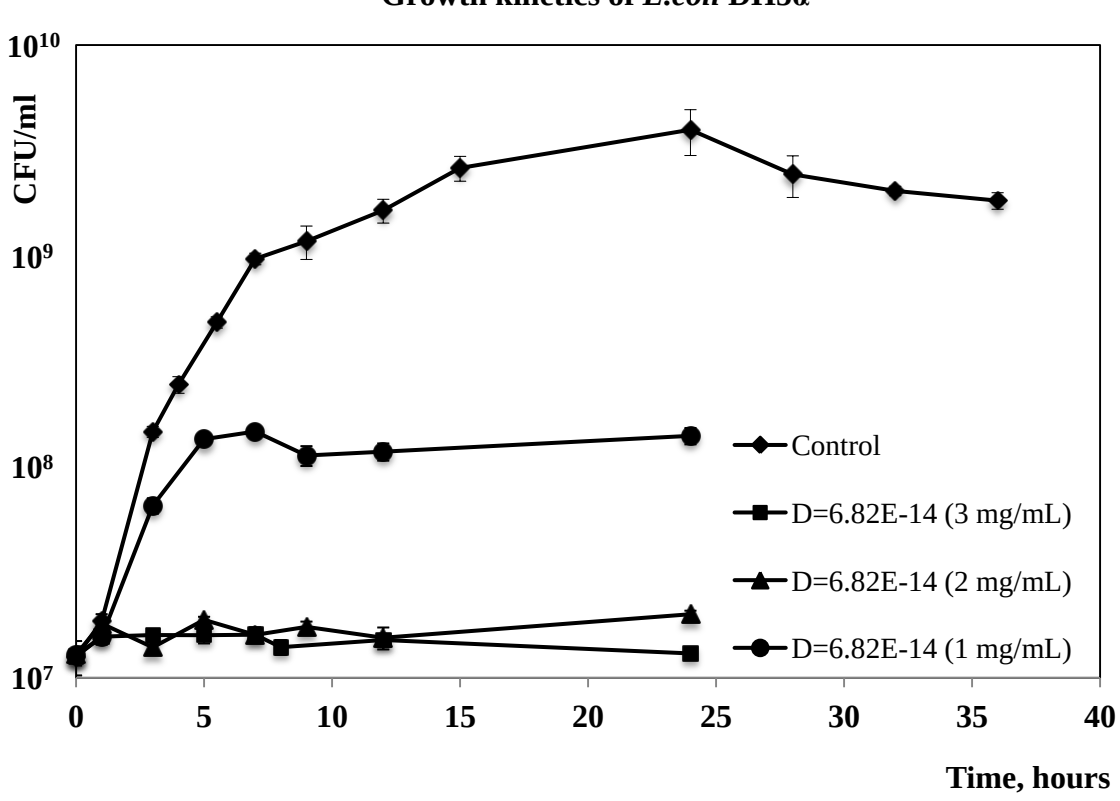

Figure 27: Effect of diffusivity and antimicrobial concentration in a model polymer on the growth of *E.coli* DH5α

6.4. Effect of critical parameters identified on target release profile based on experimental results

The framework of target release profile (Section 3.2) was used to develop a functional relationship to integrate packaging, food and environmental parameters. The relationship was given as,

Target release profile = f {diffusivity, microbial lag period, temperature, shelf life}

6.4.1. Target release profile as a function of diffusivity and microbial lag period

As the diffusivity increases the microbial lag period increases. There is an optimum range of diffusivities that provided complete inhibition of the organisms for the required shelf life.

Experimental results show that diffusivity between $2.6x10^{-14}$ and $7.5x10^{-12}$ m^2/s for 3 mg/mL potassium sorbate in polymer (Figure 18); $6.82x10^{-14}$ and $7.5x10^{-12}$ m^2/s for 2 mg/mL potassium sorbate in polymer (Figure 20); $2.6x10^{-13}$ and $7.5x10^{-12}$ m^2/s of 0.1% (Figure 23) potassium sorbate produced an effective inhibition for the required shelf life of 24 hours.

6.4.2. Target release profile as a function of diffusivity, concentration of antimicrobial and microbial lag period

As the concentration decreases the effective range of diffusivities that is suitable to produce complete inhibition of the organism decreases.

Experimental results show that as the concentration of potassium sorbate decreased from 3 mg/mL >2 mg/mL >1 mg/mL, the minimum diffusivity that produced an effective inhibition of the organisms decreased from $2.6x10^{-13}$ $>6.82x10^{-14}$ $>3.03x10^{-14}$ m^2/s (Figures 20, 22 and 24).

Antimicrobial effectiveness depends on the diffusivity and in turn their release rates from the package more than the concentration of the antimicrobial in the package.

Experimental results show that for a constant diffusivity at $6.14x10^{-14}$ m^2/s, potassium sorbate concentration from 2 mg/mL to 3 mg/mL completely inhibits of *E.coli* DH5α while at 1 mg/mL concentration the cells continued to grow (Figure 27).

6.4.3. Target release profile as a function of MIC and microbial lag period

To produce an effective inhibition of the organisms, the initial amount of antimicrobial released from the package during the inherent lag period of the organism should be equal or more than the MIC of antimicrobial (Figure 25).

Experimental results show that release of a minimum of 0.5 mg/mL of potassium sorbate during the inherent lag period of *E.coli* DH5α (1.16 h) showed complete inhibition of the organisms. A minimum diffusivity of $3.03x10^{-14}$ m^2/s for 3 mg/mL potassium sorbate, $6.82\ x10^{-14}$ m^2/s for 2 mg/mL and $2.6x10^{-13}$ cm^2/s for 1 mg/mL potassium sorbate is required to produce an effective inhibition for the 24 hour shelf life.

Figure 28 illustrates the reasoning behind the model. The release rate of antimicrobials from polymer is not constant. The antimicrobial releases faster during the initial period of time due to the high concentration gradient and then the rate starts to slow down finally tapering off to almost constant rate. Similarly the microbial growth curve starts with a lag period where the bacterial cells adapt themselves to their external environment, followed by the exponential phase where the cells double and finally the stationary and the death phase where the growth rate is almost constant. To develop effective antimicrobial CRP films the challenge lies in correlating the release profile of the antimicrobial from polymers with the growth kinetics of the organism.

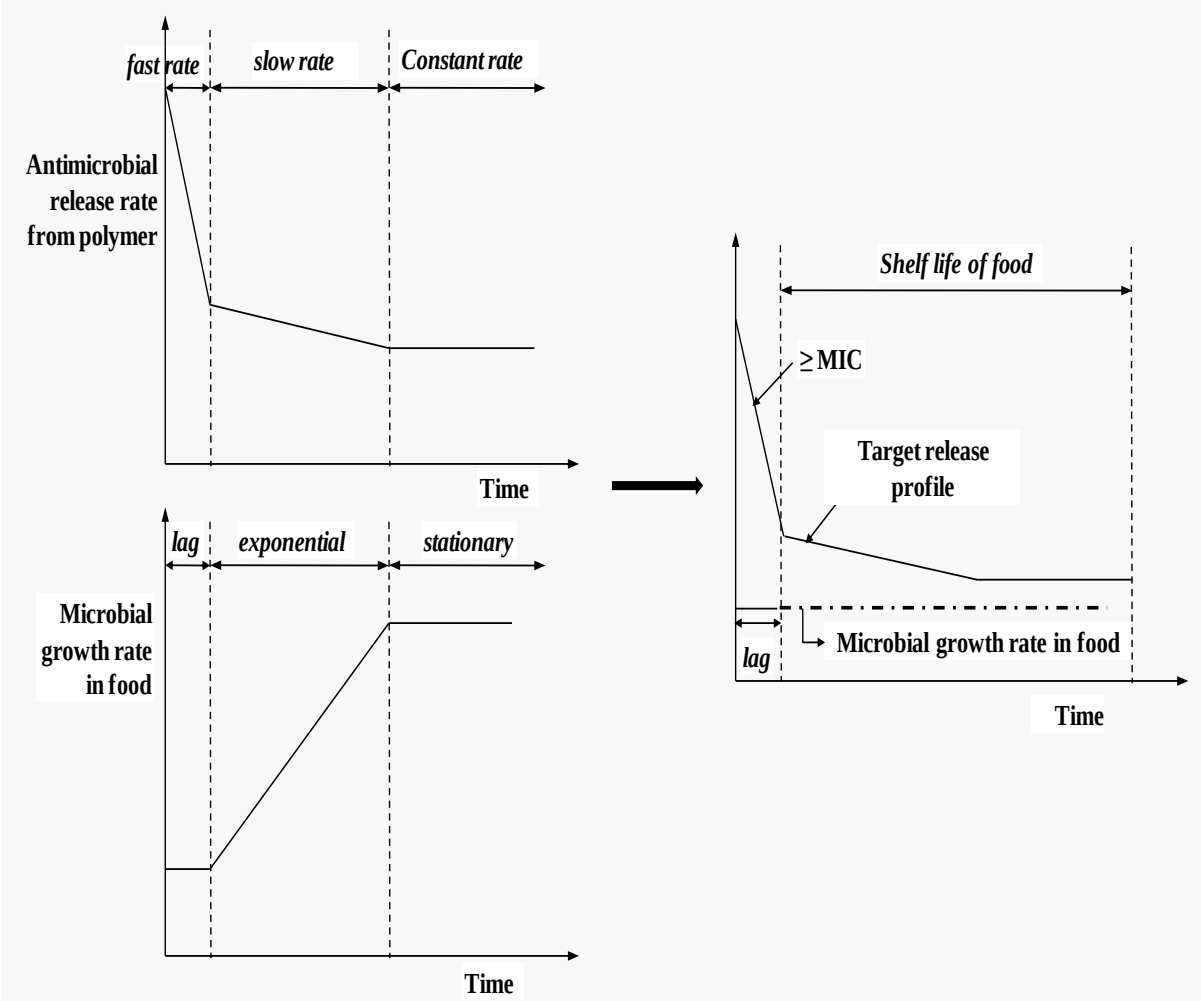

Figure 28: Illustration to show target release profile

The correlation can be brought out by determining the amount of antimicrobial that needs to be delivered during a particular growth phase. We understand that the microorganism may be at their weakest state during the lag period when they are trying to adapt themselves to their environment. Once the lag phase crosses the organisms enters the exponential phase where controlling growth involves high amounts of antimicrobial and nearly impossible to eliminate all cells. Thus we can assume maximum stress is needed during the initial lag period of the organism to injure cells. This is of help as the release

profile shows the initial fast rate and correlating the time of initial fast rate with microbial lag period would help deliver the needed stress.

The second question arises as how much antimicrobial is needed to cause the injury during the lag period was answered by understanding the MIC of antimicrobial needed for a particular microorganism under the given conditions. Initial fast release must deliver antimicrobials equal or greater than the MIC for maximum stress of the organisms during their lag period and slow release of the remaining amount would continually maintain the stress preventing them from growing back. The shelf life parameter in the target release profile determines the amount of antimicrobial in packaging and the time required for the slow delivery.

6.5. Mathematical model to quantify target release profile

The experimental results support the hypothesis and thus it was clearly shown (Section 6.8) that target release profile depends on diffusivity, concentration, microbial lag period and MIC of the antimicrobial. Based on the functional relationship the Crank's model for antimicrobial diffusion was modified to quantify the minimum diffusivity that is effective to produce complete inhibition of the organism. Simple diffusion model to quantify release rates is given as,

$$\frac{\boldsymbol{M_{f,t}}}{\boldsymbol{M_{p,0}}} = \frac{\mathbf{2}}{\boldsymbol{L}}\left(\frac{\boldsymbol{Dt}}{\boldsymbol{\pi}}\right)^{\mathbf{0.5}}$$

where,

$M_{f,t}$ = amount of antimicrobial in food at time t (µg)

$M_{p,0}$ = initial amount of antimicrobial in the packaging film (µg)

D = diffusivity of the antimicrobial in the polymer (cm^2/s)

L = thickness of the film (cm)

t = time (s)

Since the diffusivity (D) is a material property, the model was evaluated by replacing time with the inherent lag period of the organism (time taken for the organism to increase by 1 log) and M_t as the minimum inhibitory concentration based on the initial microbial load.

$$\frac{\boldsymbol{MIC_{IL} * V_f}}{\boldsymbol{M_{p,0}}} = \frac{\boldsymbol{2 * A}}{\boldsymbol{V_p}} \sqrt{\frac{\boldsymbol{D * t_{lag}}}{\boldsymbol{\pi}}} \quad (3)$$

MIC_{IL} = Minimum inhibitory concentration for a initial microbial load (µg/mL)

$M_{p,0}$ = initial amount of antimicrobial in the packaging film (µg)

t_{lag} = Time taken for the organism to increase by 1 log

V_f = Volume of food (mL)

V_p = Volume of the polymer (m^3)

A = Surface area of the polymer (m^2)

D = Diffusivity, m^2/s

The model follows the same assumptions as the Crank's diffusion model and also added to that the amount of antimicrobial in the polymer is assumed to the maximum permissible level that can be added to food. It is predicted that the diffusivity and

ultimately the release rate obtained through this model will be the minimum release rate required for an effective or complete inhibition of the organisms. Though this is a simple model, it should provide a meaningful insight into parameters that govern target release profile. The model helps packaging scientists develop CRP systems based on minimum diffusivity values.

6.5.1. Limitations of the model

The model assumes diffusivity as the major factor determining release. This may be true for most liquid foods but in some case the release from the package to food is governed by the food composition and antimicrobial compatibility with the food. The model does not account for antimicrobial migration in food but would be useful for surface treatment of solid foods. The model is envisioned to be effective for liquid foods where the antimicrobial can migrate into food and distribute uniformly. Throughout, as well as, for systems where the package is in close contact with the food and only surface treatment of the product is required. The model does not include the shelf life parameter. Including the shelf life parameter would help determine the concentration of antimicrobial required in the polymer.

6.6. Validation of target release model

6.6.1. Instant addition of nisin to obtain MIC

Micrococcus luteus is highly sensitive to nisin and is considered an indicator organism to evaluate nisin activity. Thus, very low concentrations are sufficient to cause inhibition.

The MIC of nisin for *M. luteus* was determined by evaluating the effect of instant addition of nisin (six concentrations) of nisin on the growth of the organisms (Table 8).

Table 8: Effective nisin concentration used for instant addition

Concentration of 2.5% nisin (µg/mL)	**Effective nisin concentration (µmol/mL)**
0.5	$1.49x10^{-4}$
1	$2.98x10^{-4}$
2.5	$7.45x10^{-4}$
5	$1.49x10^{-3}$
10	$2.98x10^{-3}$
25	$7.45x10^{-3}$

The organism was inhibited by nisin at all concentrations compared to control (absence of nisin) (Figure 29). Adding as low as $1.49x10^{-4}$ µmol/mL nisin showed 2 log reduction but the organisms continued to grow after 9 hours. Doubling the amount ($2.98x10^{-4}$ µmol/mL) reduced cell count by ~ 4 logs but the inhibition was effective only for 9 hour. Increasing levels almost 50 times ($7.45x10^{-3}$ µmol/mL, the highest concentration tested) also shows same effect. Though the cell count was reduced by almost 6 logs, the organisms started to grow back after 12 hours.

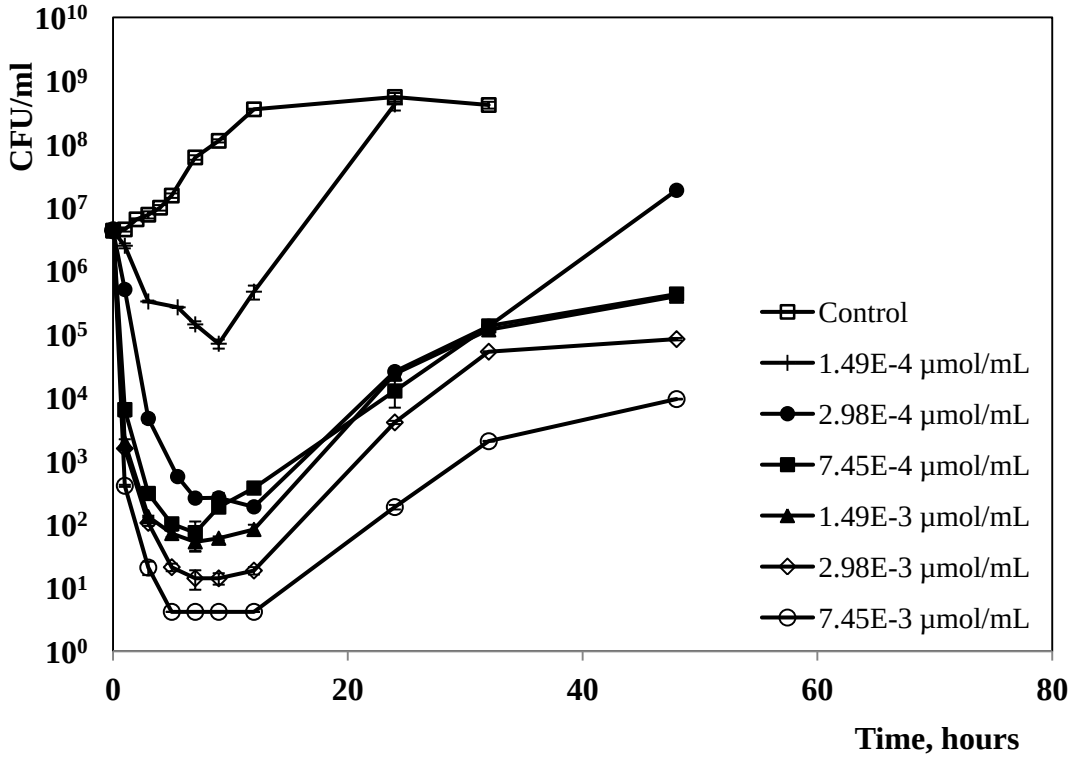

Figure 29: Instant addition of nisin to quantify MIC

The growth of *M. luteus* may be either due to the depletion of nisin in the system or due to the sustained exposure of the organisms to nisin resulting in resistance development [2]. Since the highest concentration of 7.45×10^{-3} µmol/mL was most effective among the instant addition concentrations, it is used as the concentration of nisin in the polymer for controlled release experiments. This concentration corresponds to a $M_{p,0}$ of 1.49 µmol.

6.6.2. Quantifying lag period of *M. luteus*

The lag period of *M. luteus* was quantified using a logistic model (section 5.6.2). In a plot of (Figure 30) log N(t) vs time, the slope BD/4 was 0.3951. The difference between lower and upper asymptote (D) was calculated as 8.54, therefore B was calculated as 1.85×10^{-1}.

The time at which the growth was maximal (M) was found as 12. Substituting these values of B and M in Eq. 2 shows that the lag period of *M. luteus* was 3.3 hours.

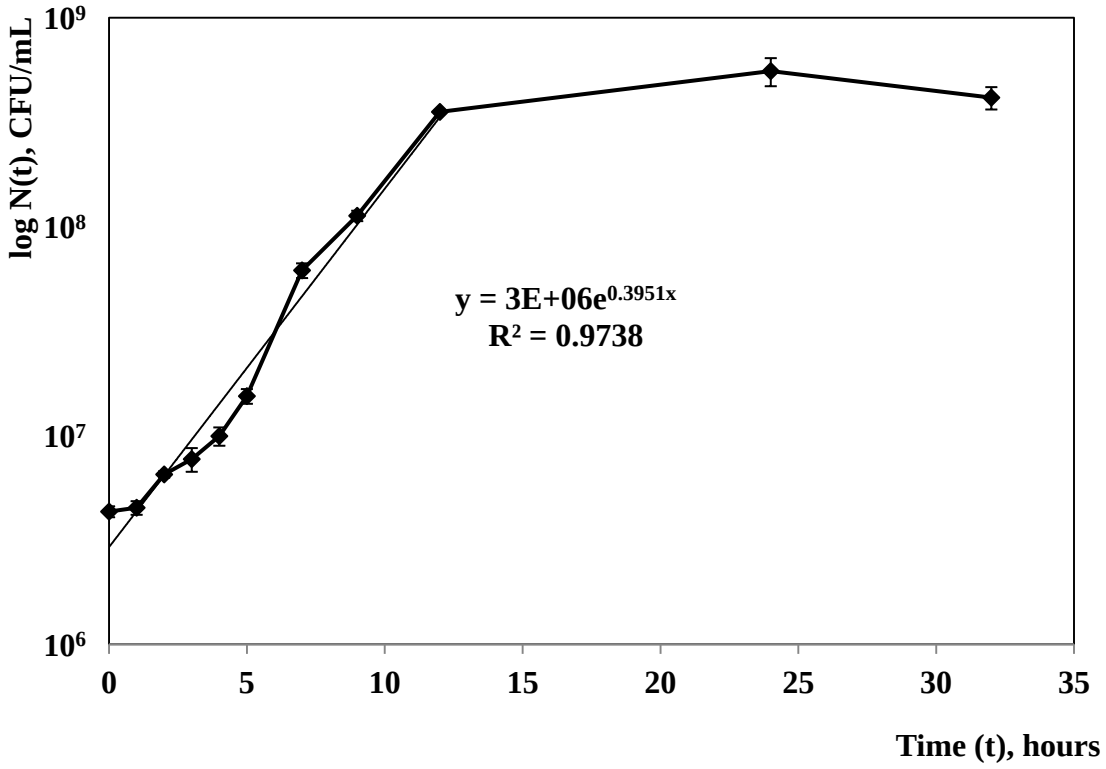

Figure 30: Lag period of *M. luteus* quantified using logistic model

6.6.3. Predicting minimum diffusivity of nisin from target release model

Minimum diffusivity was obtained by substituting MIC, t_{lag}, L and $M_{p,0}$ in Eq. 3 (Table 9). Figure 29, the MIC was taken as 2.98×10^{-4} µmol/mL. Although the cells grew back after 9 hours, 2.98×10^{-4} µmol/mL was the minimum concentration that produced 4 log reduction in cells. The minimum diffusivity was calculated as 6.13×10^{-16} m^2/s from Eq.3. This minimum diffusivity was then substituted in eq. 1 to obtain the target release profile.

Table 9: Prediction of minimum nisin diffusivity from target release model

Prediction from target release model	
Mp,0	1.49 µmol
L	76.2 µm
MIC	2.98×10^{-4} µmol/mL
t_{lag}	3.3 hours
D	6.13×10^{-16} m2/s

`

6.6.4. Validating target release profile on the growth of *M. luteus*

To validate the target release profile other profiles were generated by varying the concentration of antimicrobial released during inherent lag period of *M. luteus* above and below the MIC values. As shown in Table 10, four values of $M_{f,t}$ corresponding to nisin concentrations within the range of 1.49×10^{-4} and 1.49×10^{-3} µmol/mL were chosen, which represented low concentrations in the range (1.49×10^{-4} and 7.45×10^{-3} µmol/mL) used in the instant addition experiment. The lag time of 3.3 hours obtained from the control sample (without antimicrobial) was used as t to obtain D values ranging between 1.53×10^{-16} m^2/s and 1.53×10^{-14} m^2/s as shown in Table 10. The choice of this lag time for t was based on the assumption that the amount of nisin added to the growth medium during the inherent lag period of the organism was highly effective at inhibiting subsequent growth and proliferation.

Table 10: Target release profile predicted from the target release model

Lag period	Concentration of nisin released during lag period (based on instant addition)	D, m^2/s
3.3 hours	$1.49x10^{-4}$ µmol/mL	$1.53x10^{-16}$
	$2.98x10^{-4}$ µmol/mL	$6.13x10^{-16}$
	$7.45x10^{-4}$ µmol/mL	$3.83x10^{-15}$
	$1.49x10^{-3}$ µmol/mL	$1.53x10^{-14}$

The D values in Table 10 and Eq. 1 were used to the generate release profiles ($M_{f,t}$ versus t) with fixed values of $M_{p,0}$ (1.49 µmol) and L (76.2 µm) (Figure 31). Higher D values corresponded to faster release profiles. As in the instant addition experiment, time was simulated up to 48 hours.

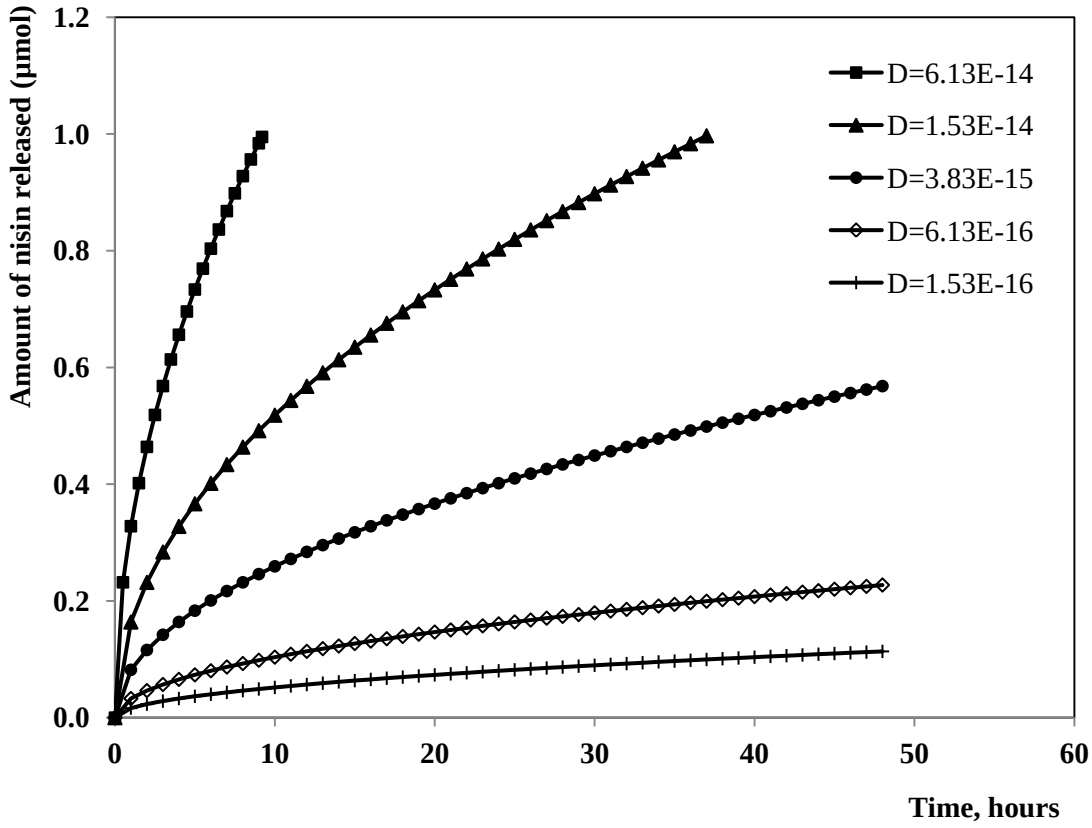

Figure 31: Target release profile and other generated release profiles

Based on Crank's diffusion model, the maximum amount that can be released into food in a short time is 67% of the total antimicrobial in the polymer ($M_{f,t}/M_{p,0} \leq 2/3$). For highest diffusivity of $6.13x10^{-14}$ m^2/s, 67% of nisin (0.994 µmol) was released within 9 hours, while for the lowest diffusivity $1.53x10^{-16}$ m^2/s only 8% nisin (0.113 µmol) was released in 48 hours. For a diffusivity of $1.53x10^{16}$ m^2/s, 67% of nisin was released in 37 hours and the amounts of nisin added for diffusivities of $6.13x10^{-16}$, and $3.83x10^{-15}$ m^2/s after 48 hours were 0.227, and 0.568 µmol, respectively. Compared to the highest $M_{p,0}$ of 1.49 µmol used in instant addition, these amounts correspond to 15, and 38%, respectively.

Figure 32 validates prediction that a minimum diffusivity of $6.13x10^{-16}$ m^2/s produces the target release profile suitable for complete inhibition of *M. luteus*. Thus, minimum D value required to effectively inhibit microbial growth is about $6.13x10^{-16}$ m^2/s which corresponds to the total amount of nisin released (0.227 µmol) or final concentration in the media ($1.14\ x10^{-3}$ µmol/mL) after 48 hours. 0.227 µmol is equal to 15% of the amount used for the best result (1.49 µmol) obtained from instant addition, which is equal to the MIC selected. For growth curves of faster release profiles (D = $6.12\ x10^{-16}$ m^2/s or above), complete inhibition of *M. luteus* was observed for at least 48 hours at diffusivities below the minimum value, microbial growth was growing similar to control. The growth curve of the slowest release profile (D = $1.53x10^{-16}$ m^2/s) did not cause a decrease in cell number.

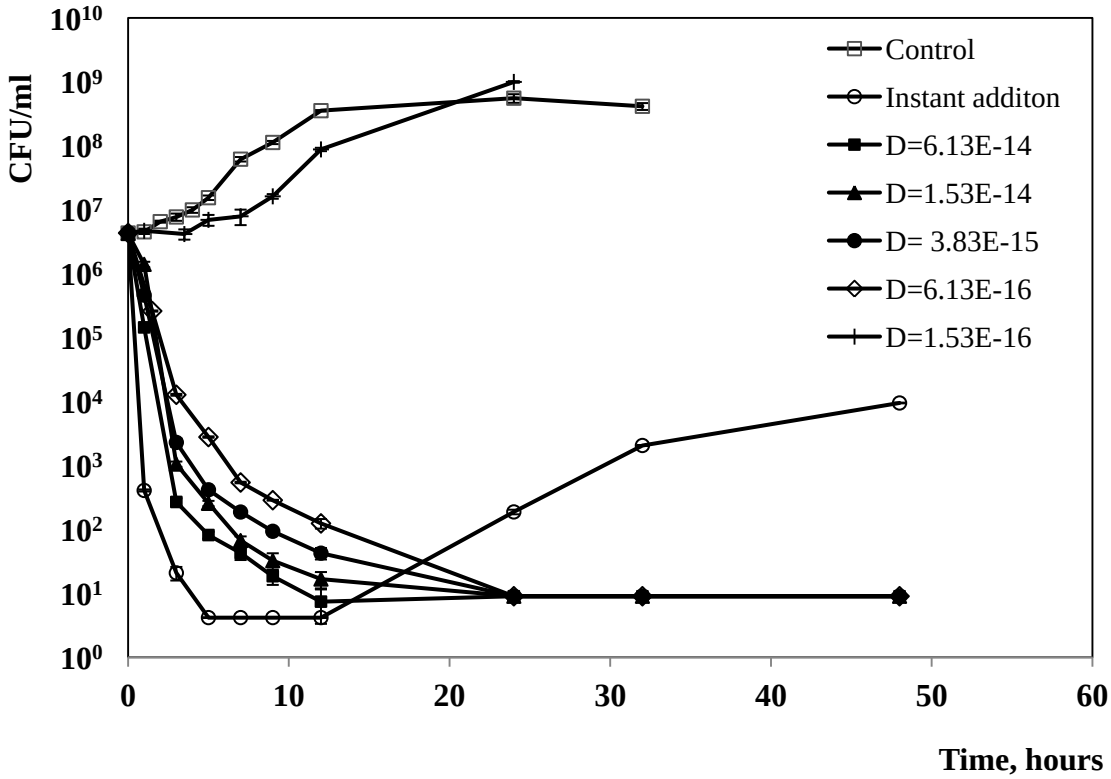

Figure 32: Inhibition of *M. luteus* by controlled release of nisin

Since the growth curve of instant addition shows that inhibition was not sustained after 12 hours, the delivery of antimicrobial was more effective using controlled release than instant addition.

The results show that the predicted target release profile was highly effective considering controlled release may use only 15% nisin to achieve better results than instant addition of 100% nisin. The fast initial rates of these profiles are necessary to provide lethal stress to kill or injure the cells, while the subsequent slower rates with persistent release of small amounts of nisin are sufficient to suppress recovery of the injured surviving cells [84]. Thus, the combination of initial fast rate and subsequent slower rate provides good overall microbial inhibition.

6.6.5. Model limitation evaluated based on nisin concentration in polymer on growth of *M. luteus*

The target release rate model assumes the amount of antimicrobial added in the polymer should be the highest permissible amount or the highest amount of antimicrobial that could be added into the polymer. Therefore the predicted diffusivity based on the lower the concentration of antimicrobial in the polymer ($M_{p,0}$) would not provide the desired inhibition. This is especially critical in antimicrobials whose activity is not reversible as nisin. Though nisin is highly effective against *M. luteus* once nisin is utilized to disrupt the cells their activity is lost and cannot be used to kill another cell.

Also from the instant addition studies it can be theorized that there may be deterioration in nisin activity over time. Increasing concentration of nisin 50 times (1.49×10^{-4} µmol/mL

to 7.45×10^{-3} µmol/mL) was effective in killing the cells but the cells continued to grow after 12 hours (Figure 29). This may be due to deterioration of nisin or complete utilization of nisin by 12 hours but for the same initial number of organisms in the broth the increase in the amount of antimicrobial did not linearly decrease the cell number suggesting that there may be some loss of nisin activity over time.

To test the limitation of the model lower initial concentration of nisin in polymer (1.49×10^{-1} µmol/mL) was released by varying the amount of nisin released during the lag period of the organism (3.3 h) (Table 11).

Table 11: Generated release profile of nisin by lowering nisin concentration in polymer

Nisin amount in polymer ($M_{p,0}$)	Concentration of nisin released during lag period (based on instant addition)	D, m^2/s
1.49×10^{-1} µmol	1.49×10^{-4} µmol/mL	1.53×10^{-14}
	2.98×10^{-4} µmol/mL	6.13×10^{-14}
	5.96×10^{-4} µmol/mL	2.45×10^{-13}

The concentration of 2.98×10^{-4} µmol/mL released during the lag period of *M. luteus* was the MIC taken for the validation studies (see section 6.6.3). The D values in Table 11 and Eq. 1 were used to generate release profiles ($M_{f,t}$ versus t) in Figure 33 with fixed values of $M_{p,0}$ (0.149 µmol) and L (76.2 µm). Higher D values corresponded to faster release profiles. As in the instant addition experiment, time was simulated up to 48 hours.

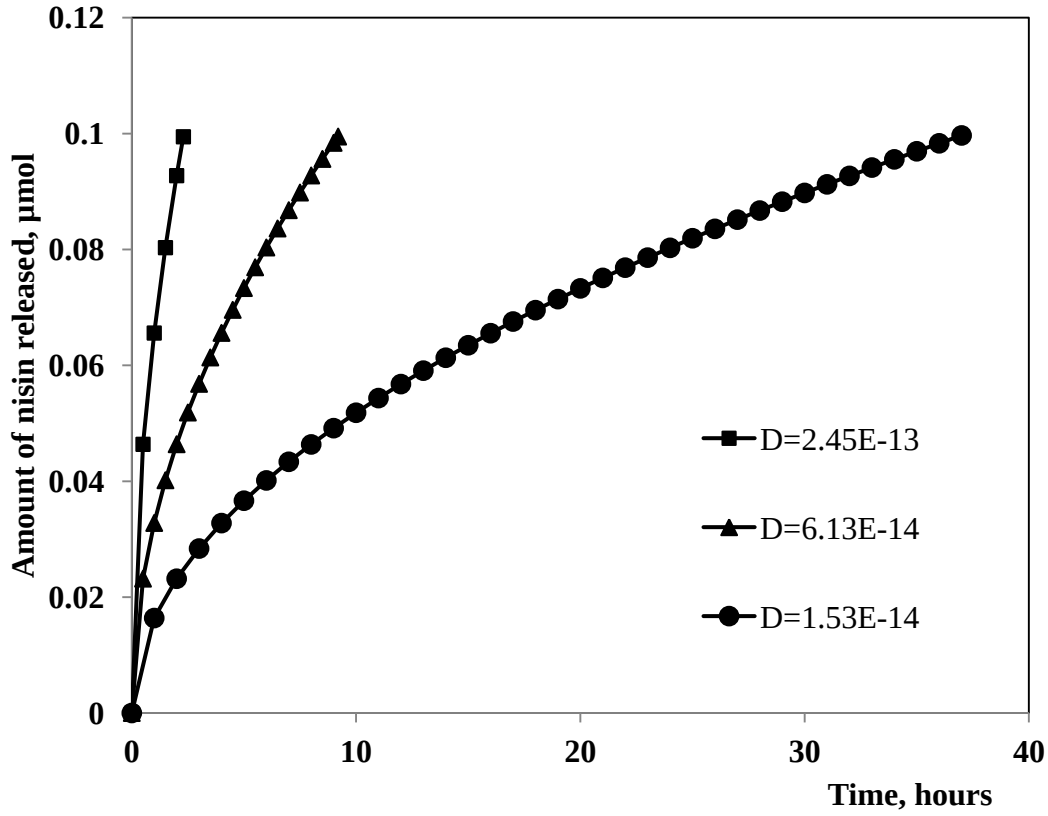

Figure 33: Generated release profile based on 0.149 µmol nisin in polymer

The release profile was simulated using a syringe pump and the results (Figure 34) show that above the optimum diffusivity ($6.13x10^{-14}$ m^2/s), calculated based on MIC, there is a decline in cell number but in both cases the cells grow back after 12 hours. The results though validate hypothesis 2 that a minimum of MIC must be delivered during the inherent lag period of the organism, it also confirms the limitation of the model.

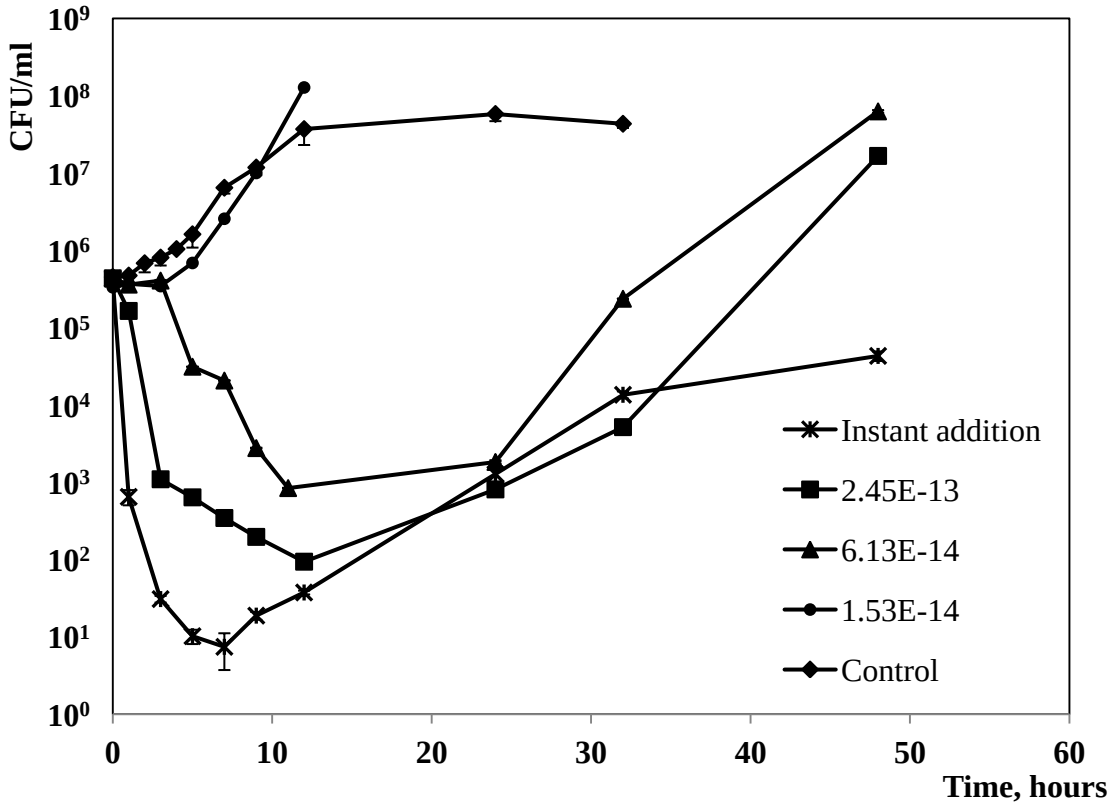

Figure 34: growth kinetics of *M. luteus* based on the generated release profile of 0.149 µmol nisin in polymer

6.6.6. Effect of amount of antimicrobial released (Scenario 1) and time of release (Scenario 2) on the growth of microorganism

Two scenarios were analyzed to further understand the implications of diffusivity, amount released over time and amount of antimicrobial in polymer.

6.6.6.1. Scenario 1: Diffusivity was maintained at 6.14x10-14 m2/s; nisin amount in polymer was varied at 1.49 µmol and 0.149 µmol

Variation in the concentration of nisin in polymer would vary the amount of nisin released over time for the constant diffusivity. From Figure 35 it can be seen that if the

concentration in polymer was decreased 10 times, the amount of nisin released in around 9 hours is decreased 10 times. Though in both cases the amount of nisin released during inherent lag period of *M. luteus* is equal (when nisin concentration in polymer is 0.149 µmol) or greater (when nisin concentration in polymer is 1.49 µmol) than MIC, the total amount of nisin released over time is vastly different and it depends on concentration of nisin in polymer.

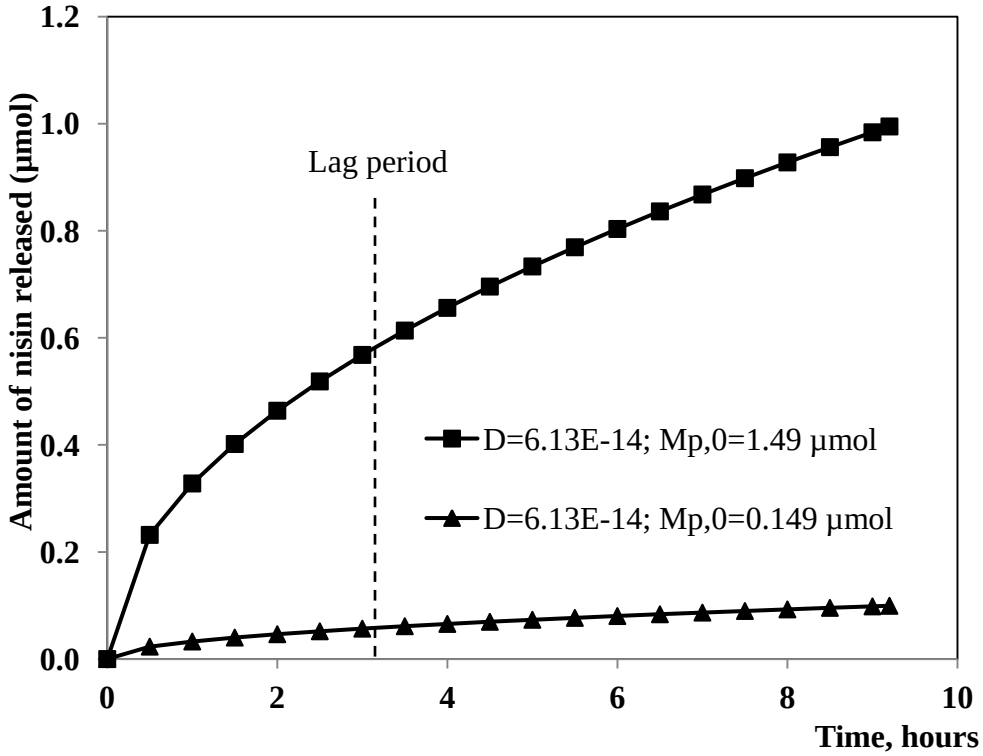

Figure 35: Release profile of nisin keeping diffusivity constant and varying amount of nisin in polymer

The growth kinetics of *M. luteus* (Figure 36) for the corresponding release profile shows that there is an optimum amount of nisin in polymer required to provide complete inhibition of *M. luteus* for 48 hours keeping the diffusivity constant at 6.14×10^{-14} m^2/s. . So it can be said that for the same diffusivity of polymer not all concentrations of

antimicrobial in the polymer may be suitable to inhibit microbial growth. There is an optimum concentration of antimicrobial in polymer that would be effective in inhibiting the microorganism.

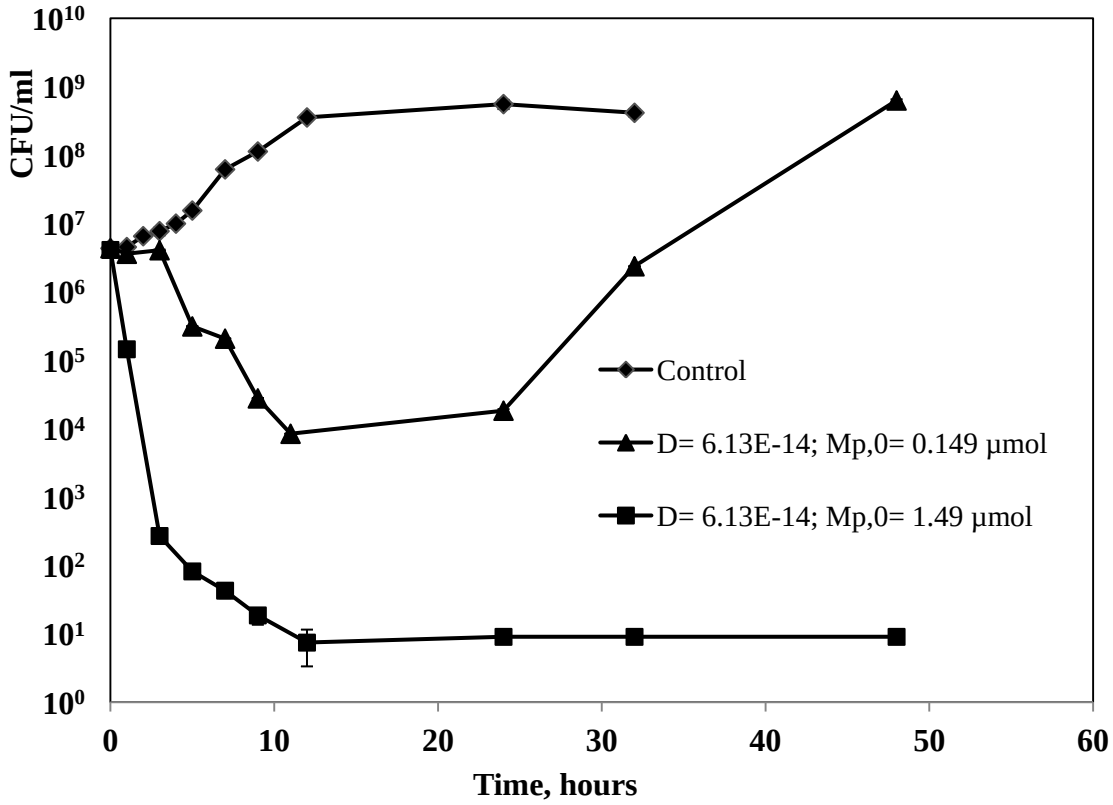

Figure 36: Effect on nisin amount in polymer on the growth of *M. luteus*

6.6.6.2. Scenario 2: Amount of antimicrobial released over time is maintained constant; nisin amount in polymer was varied at 1.49 µmol and 0.596 µmol

In this scenario the amount of nisin released over time is maintained constant even when the amount of nisin in polymer was increased by 2.5 times. This would imply two different diffusivities (Figure 37) where the diffusivity with 0.596 µmol in polymer would be 9.58 $x10^{-14}$ m^2/s and with 1.49 µmol in polymer would be 1.53 $x10^{-14}$ m^2/s. In

this case though the amount of nisin released over time is equal in both cases, the time taken for release is decreased with decrease in nisin amount in polymer. Lowering nisin amount from 1.49 to 0.596 µmol lowered the time taken for release by 31 hours.

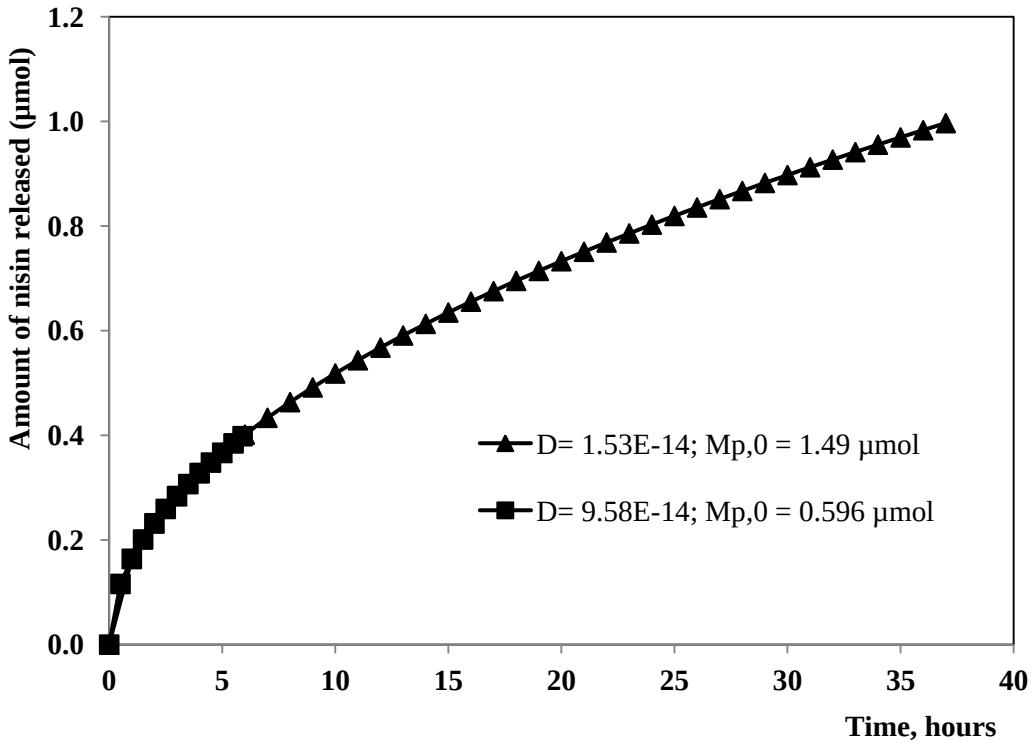

Figure 37: Similar release profile based on varying diffusivity and amount of antimicrobial in polymer

Though the amount of nisin released over time is similar since the amount of nisin in polymer is lowered, the time taken for nisin release is lowered. Even in this scenario the concentration of nisin released during the inherent lag period of the organism is greater than MIC. The growth of *M. luteus* (Figure 38) based on the current scenario shows complete inhibition of the organism when the nisin is released for 37 hours (1.53×10^{-14} m^2/s) but when the release time is lowered (9.58×10^{-14} m^2/s) it is observed that the organisms starts to grow back after 24 hours and the level of inhibition is not same.

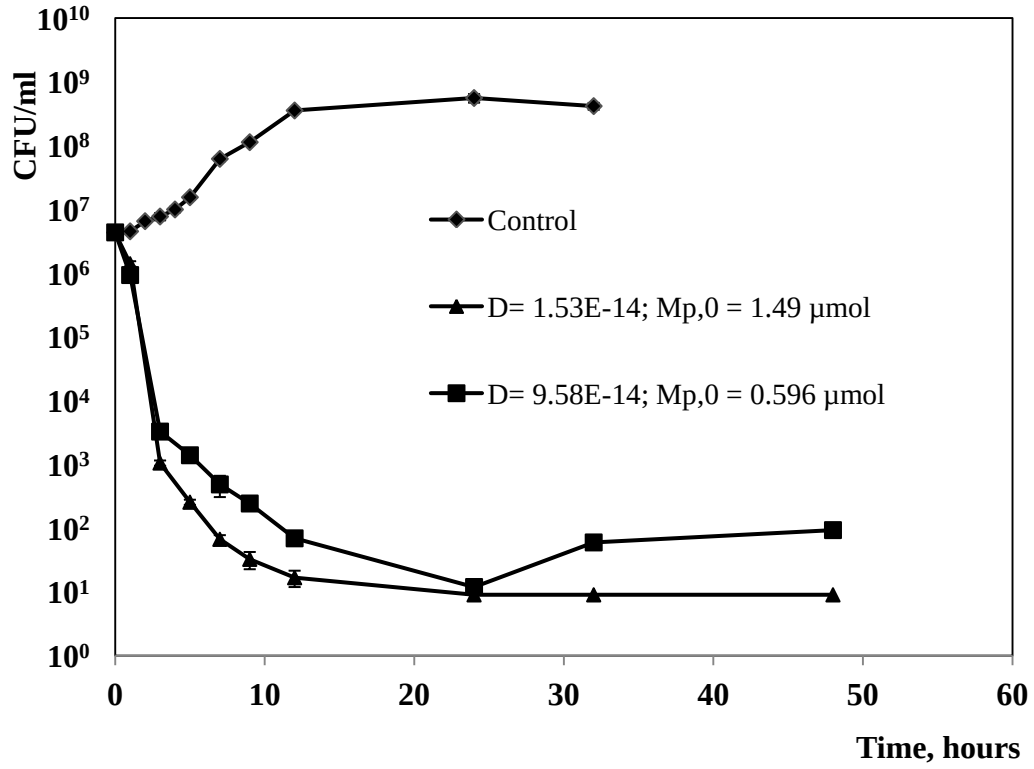

Figure 38: Effect of time of release on growth kinetics of *M. luteus*

In the validation studies the desired period was taken as 48 hours. Thus it can be hypothetically taken that the shelf life requirement is 48 hours. Controlled release of nisin for complete 48 hours shelf life requires a minimum amount of nisin in polymer. It can be understood from the results in scenario 2 that the effectiveness of controlled release not only depends on the concentration of antimicrobial released during the initial fast phase of release but also how long would the remaining antimicrobial from polymer be released during the slow release phase. The time taken for slow release is dependent on the desired shelf life of the food under study. Thus it is critical to include the shelf life parameter in the model to obtain the minimum amount of antimicrobial required in the polymer to obtain an effective inhibition of the microorganism, which would essentially determine the time taken for release.

The target release model may not be a solution for every problem and as any model has limitations. Yet the model is simple and takes into account the antimicrobial efficacy (MIC), microbial growth kinetics (lag period) and correlates them with the release kinetics of antimicrobial from polymer (diffusivity).

7. CONCLUSIONS

The following conclusions are made from the study.

- The ratio of amount of antimicrobial in food to the microbial load at any time t is critical to obtain complete inhibition. Ratio <MIC implies that the antimicrobial concentration in food is not sufficient to cause inhibition. On the other hand if the ratio >>MIC, then the concentration of the antimicrobial is very high and this may result in either deterioration of the antimicrobial or resistance development in the organism. Therefore a ratio = MIC would be ideal to inhibit microbial load and keep them at a safe level for the desired time

- The target release profile is thus defined as an optimum range of release of antimicrobials from CRP films suitable to extend microbial lag period and maintain it at a safe level for the required shelf life. Target release profile is dependent on packaging, food and environmental factors and functional relationship is given as,

- Target release profile = f {diffusivity, microbial lag period, temperature, shelf life}

- Amount of antimicrobial released over time is not constant and the release tend can be profiled as an initial fast rate, followed by slow rate, finally tapering down to constant rate. This release profile is usually governed by high diffusion resistance from the packaging film. Thus there is an optimum diffusivity of antimicrobial from packaging that is suitable to inhibit microbial growth.

- Microbial lag period increases with increasing diffusivity. This is evident as the inherent lag period or period taken by the organisms to adapt themselves to their environment is very short. Thus maximum need of antimicrobial is during this period. Increasing diffusivity increases the initial release thus providing instant and lethal stress which killed or injures cells, followed by sustained stress from slow release which suppressed the recovery or resistance development of injured cells.

- The target release profile is obtained by quantifying diffusivity based on releasing antimicrobial concentrations equal or greater than the minimum inhibitory concentration during the microbial lag period.

- The mathematical model to predict target release profile is based on the diffusion model. The model is simple and takes into account the antimicrobial efficacy (MIC), microbial growth kinetics (lag period) and correlates them with the release kinetics of antimicrobial from polymer (diffusivity) ***(see section 6.5***).

- The target release model was found effective with both bacteriostatic (Potassium sobate/ E.coli DH5α) and bactericidal systems (Nisin/ *M. luteus*).

- Release profile generated based on target release model lowered nisin concentration to as little as 15% compared to 100% with instant addition to provide complete microbial inhibition for longer period times.

- The decrease in the antimicrobial concentration used in the package decreases the range of diffusivities suitable to produce an effective inhibition of microbial growth. The implication of this conclusion is that if the concentration of

antimicrobial is reduced then the range of polymers that are suitable to inhibit microbial growth for the required shelf life decreases.

- The target release model is highly effective only if maximum permissible amount of antimicrobial is added to the packaging film. This is critical for high shelf life requirements and also for irreversible antimicrobials, where the antimicrobial depletes over time.

- For the same packaging film, varying antimicrobial amounts in polymers would vary the amount released over time. On the other hand, for the same amount of antimicrobial added to different packaging film the diffusivity changes thereby varying the amount released over time. In both cases there is a probability of shifting the ratio of antimicrobial amount to microbial load << or >> than MIC. Thus optimizing both amount of antimicrobial in polymer and the diffusivity is critical to obtain the target release profile. This conclusion is also important for making cost effective controlled release packages. The packaging manufactures, based on the cost of antimicrobial or the polymer, can decide on the polymer type and antimicrobial concentration to be used.

- The increase in shelf life requirements of the food would further increase the amount of antimicrobial required in the polymer. The increase in antimicrobial in polymer would help ensure sustained stress over the required shelf life to prevent injured cells recuperating. For irreversible antimicrobial like nisin this is especially important as either deterioration or depletion of antimicrobial over time would cause cells to grow back.

8. FUTURE WORK

8.1. Effect of temperature on target release profile

The increase in temperature increases diffusivity and in most cases the temperature increase (to a certain extent) also increases the microbial growth rate. If the rate of increase in both cases is proportional, then the target release model would be effective. For example from the nisin/*M. luteus* it is known that a minimum of 2.98×10^{-4} µmol/mL concentration of nisin is required to inhibit ~10^6 cells with a lag period of 3.3 hours at 30 °C. If the temperature of the system is decreased from 30 °C to 10 °C, then the diffusivity would also decrease (based on the Arrhenius equation). This would imply that the amount of nisin released over time would be reduced.

$$\boldsymbol{D = D_0\, exp\left(\frac{-E_a}{RT}\right)}$$

On the other hand decrease in temperature would decrease the growth rate of *M. luteus*, thereby increasing the lag period. Hypothetically if the lag period increases three times (9.9 hours) then the concentration of nisin released during this time should be atleast 2.98×10^{-4} µmol/mL. Thus for the current target release model to be effective the diffusivity should decrease (low temperature) or increase (high temperature) in proportion to the increase (low temperature) or decrease (high temperature) of microbial lag period.

To test this theory nisin release from cast corn-zein films [60] at three temperatures (5 °C, 25 °C and 35 °C) based on diffusivity values provided in literature was [60] evaluated. The release profile (Figure 39) was generated based on diffusion model for short time as

described in section 5.6 by assuming, thickness, L as 3 mil (76.2 µm), initial concentration of nisin in polymer, $M_{p,0}$ was assumed as 1.49 µmol and D obtained from literature at three different temperature.

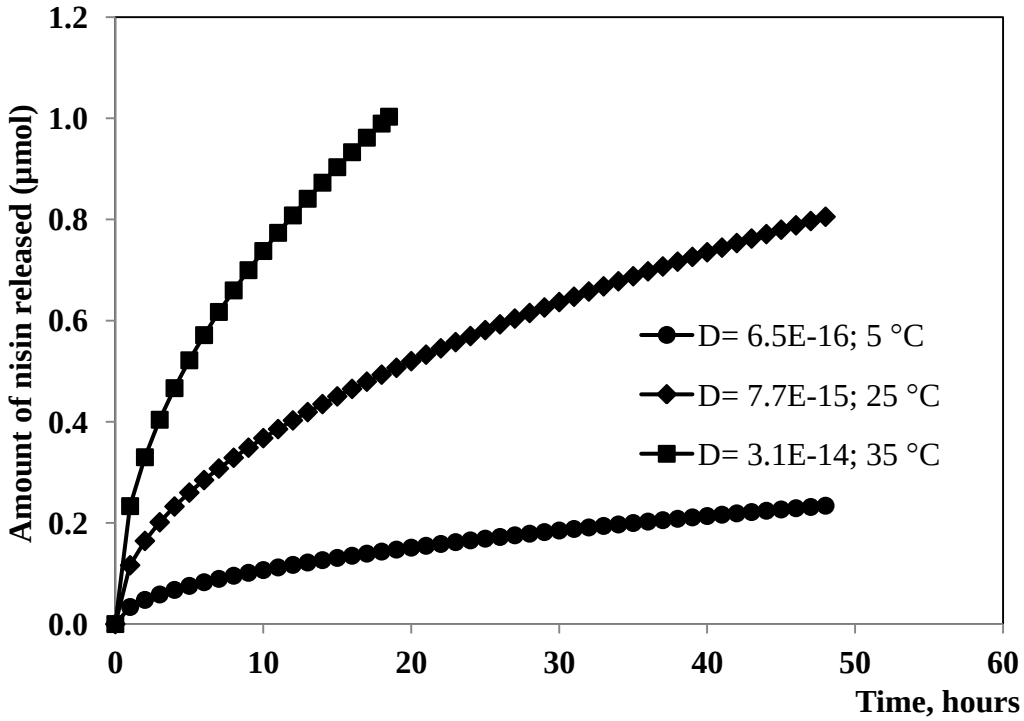

Figure 39: Generated release profile of nisin based on effect of temperature on diffusivity

The release profile curves show that as temperature increases the diffusivity increases. The total time required for the release at different temperatures was plotted to see the trend (Figure 40). It can be seen that the release time decreases almost 12 times from 875 hours to 75 hours when temperature increased from 5 °C to 25 °C.

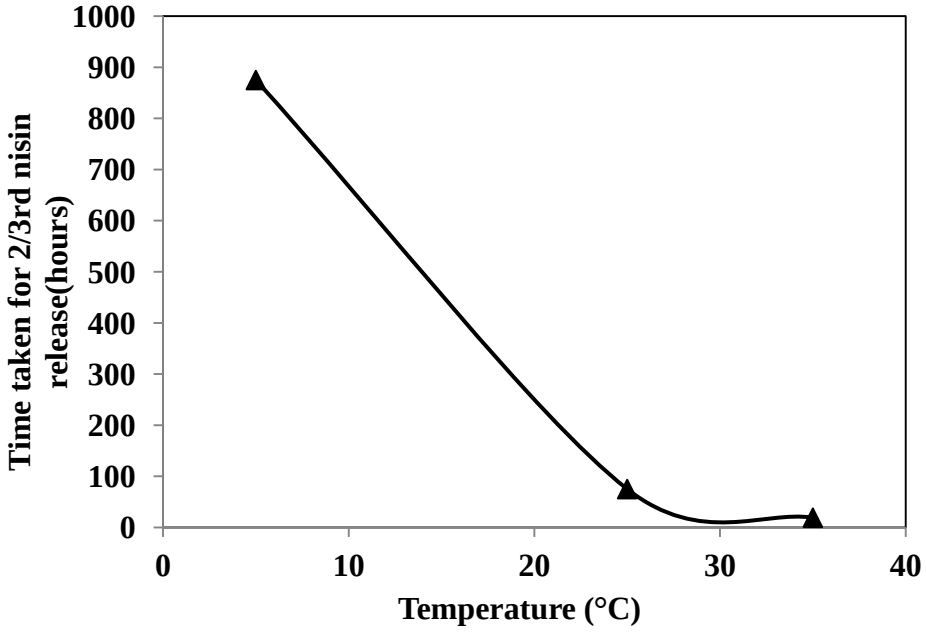

Figure 40: Effect of temperature on time taken for nisin release from corn-zein films

The lag period of *listeria monocytogenes* was quantified for the same three temperatures (5 °C, 25 °C and 35 °C) using PMP predictive microbial modeling [85]. The simulation was done by assuming 5.9 log (CFU/mL) *L. monocytogenes* cells were growing in broth at pH 6 and with 0.5% [g/dL] sodium chloride. The lag period was plotted with respect to temperature (Figure 41). It can be seen that the lag period decreases almost 15 times from 66.5 hours to 4.5 hours when temperature increased from 5 °C to 25 °C. This trend is very similar to that of the release trend (Figure 40).

The amount of nisin released during the lag period was evaluated and plotted (Figure 42). It was found that the amount of nisin released at three temperatures during the lag period of *L. monocytogenes* was consistent and remained between 0.26 to 0.33 µmol. In this case the increase in temperature increased diffusivity and decreased microbial lag period proportionately. Therefore quantifying target release profile at one temperature would be effective with increase in temperature. This theory needs to be validated with real system.

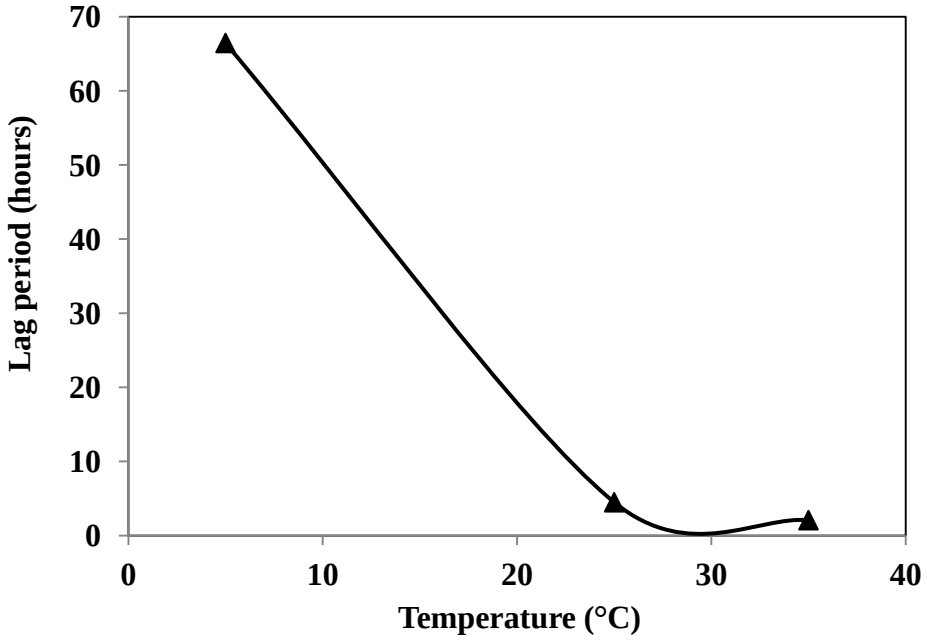

Figure 41: Generated lag period of *L. monocytogenes* based on increase in temperature

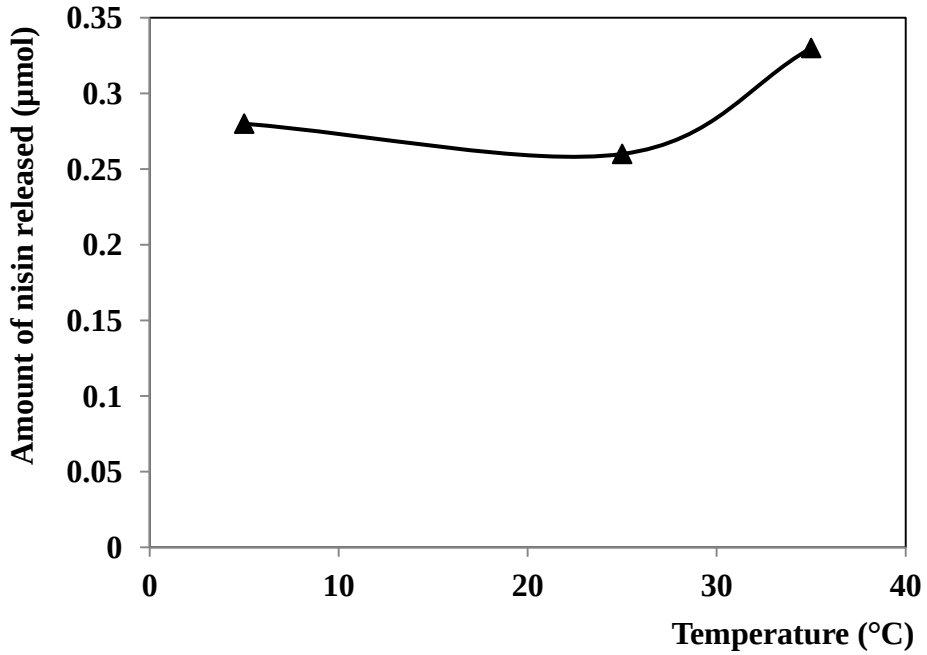

Figure 42: Amount of nisin released during the lag period of *L. monocytogenes* with increase in temperature

8.2. Target release model validation with real system

The model needs to be validated with real system. This includes both the packaging film and the food system. Migration of antimicrobial from packaging to food plays a vital role in deciding the success of the model. As described in section 2.4, the diffusion of antimicrobial into food is a 3-step process (Figure 4) [53].

- Diffusion of antimicrobial within the polymer matrix
- Partitioning of antimicrobial in the polymer/food interface
- Solubility into the food matrix

The target release model developed assumes the resistance offered by the polymer is dominant over the other two steps, resulting in diffusivity being the primary factor governing release. This may be true in liquid system where the orange juice or milk is in close contact to the package and the migration of antimicrobial would be facilitated or in solid food like meat where the product is vacuum packed. Also the antimicrobial selected for the study should be of high efficacy against the target microorganism so that lower concentrations can be added to polymer.

To test the model with real system the following steps needs to be carried out,

1. The lag period of the microorganism in the real food system must be quantified based on the desired storage conditions of the food system.
2. The MIC of the antimicrobial for the organism must be quantified based on high initial count of the target microorganism. This takes into account the worst case

scenario and MIC of any antimicrobial is relative to the number of microorganisms in the system.

3. Optimum diffusivity is obtained by substituting values from steps 1 and 2 into target release model.
4. Polymer type/blend/processing methods needs to be manipulated to obtain a film with diffusivity similar or higher than the optimum diffusivity obtained (see section 2.6).
5. The release profile of the antimicrobial from the developed CRP film should be tested with the food system without the microorganisms.
6. The Food system containing known concentration of microorganism is then is packed with the developed CRP films containing antimicrobial and the growth kinetics of the microorganism is quantified over time.

The model needs to be validated with different types of foods and then parameters needs to be added to the model based on the obtained results. This would help expand the boundary conditions of the model thereby making it suitable to more than one system.

8.3. Extrapolation of target release rate model to other type of CRP systems

The target release model developed in this research is based on antimicrobial release from packaging films. There are multiple ways to add and release antimicrobial from polymers and some of them are shown in Figure 43. The antimicrobials instead of being embedded in the packaging material can be applied as coating and also immobilized in

the package. In the latter case release of antimicrobial is not a possibility and may not be suitable. On the other hand antimicrobials can also be volatile and they can be released to the package head space and then condensed to food. This is particularly important where there is no contact between package and food. Multi-layered system containing antimicrobial in one layer and migration/permeation through other layers (without antimicrobial) would further control release.

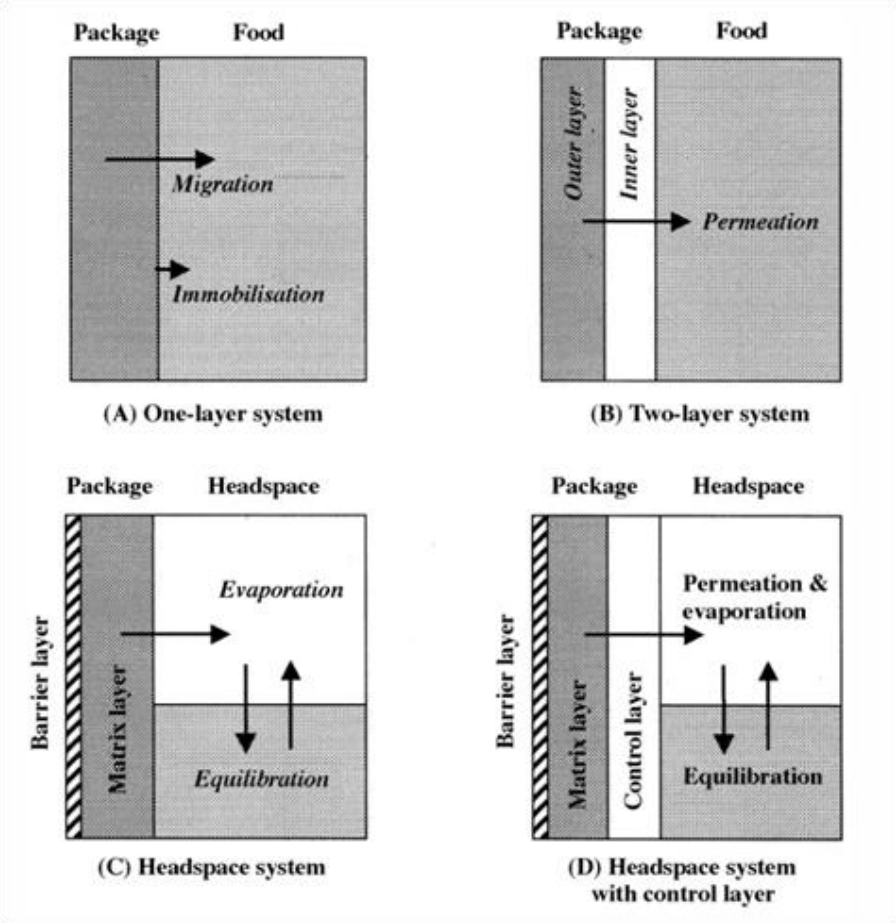

Figure 43: Migration of antimicrobials from packaging systems [83]

For all the above mentioned systems, target release profile can be quantified and modeled based on the understanding of the mechanics of antimicrobial release from polymers and microbial growth kinetics.

8.4. Integrated decision support system for CRP application

An integrated decision support system is essential for the application of the antimicrobial CRP system. The decision support system which would be a collaborative effort of academicians and food industries would be beneficial for enhancing food security. Figure 44 is a schematic to explain the framework of the decision support system.

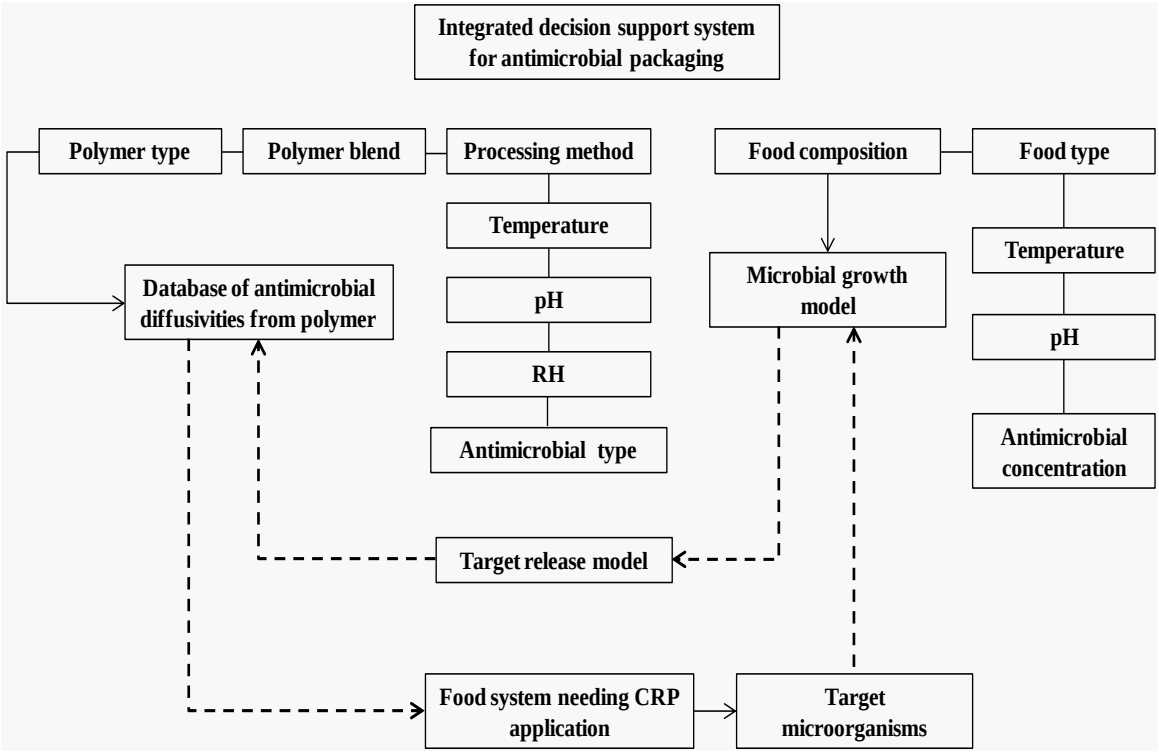

Figure 44: Integrated decision support system for antimicrobial packaging

The decision support system would include packaging database and microbial data base. The data base are envisioned like that of Genbank (http://www.ncbi.nlm.nih.gov/genbank/), where the researchers gets the opportunity to upload information on the antimicrobial diffusivity from packaging based on polymer properties, processing conditions and the external experimental conditions.

Similarly a microbial database would be a growth model platform to predict microbial growth based on the database information about food composition, type (real food vs broth), storage conditions and stress due to antimicrobial concentration. Existing programs like PMP (pathogen modeling program) by USDA (http://pmp.arserrc.gov/ PMPOnline.aspx) could be integrated and expanded.

Having the database would then be utilized for food application. If an end user wants to select an antimicrobial package for their food application, the following steps needs to be carried out,

1. Input the target microorganism that needs to be inhibited into the microbial modeling program
2. Select antimicrobial based on options and input other information pertaining to food and the storage conditions

Once the information is given, the integrated modeling program would take the input conditions and based on the microbial model would quantify the microbial lag period of the target organism, MIC of the antimicrobial for the target organism and feed it to the target release model which would quantify the optimum diffusivity. This would loop into

the diffusivity database to select optimum packaging parameters. The end user would now get information about the antimicrobial concentration, the type of polymer that may match their criteria and other available information pertaining to the polymer and antimicrobial, if any. Constantly updating and maintaining the database would prevent redundancy and also make information available to the end users.

Executing the idea of an integrated system may not be as easy, it is not far-fetched. There are currently databases available in medical and health fields. Moreover any supply chain management thrives on the ability to have integrated modeling system. A database with information pertaining to package and food would help people in developing countries where the knowledge flow is limited due to the limitations in available resources. Therefore in this era of technology and with the increasing need for food security an integrated system needs to be more than just a vision.

9. REFERENCES

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