Potency of Chitosan as a Bioactive Edible Coating for Preservation of Meat of Common Shrimps (*Crangon crangon*)

Dissertation

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Dr. rer. nat.

Tri Erny Dyahningtyas

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Untersuchungen zur Wirksamkeit von Chitosan als bioaktive essbare Beschichtung zur Konservierung von Fleisch von Nordseegarnelen (*Crangon crangon*)

Dissertation

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Tri Erny Dyahningtyas

aus Yogyakarta, Indonesien

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List of abbreviations

ACS	American Chemical Society
a_w	Water activity
BAnz.	(ger.: *Bundesanzeiger*) Federal Gazette
BMBF	(ger.: *Bundesministerium für Bildung und Forschung*) Federal Ministry of Education and Research
BMJ	(ger.: *Bundesministeriums der Justiz*) Federal Ministry of Justice
BVL	(ger.: *Bundesamt für Verbraucherschutz und Lebensmittelsicherheit*) Federal Office of Consumer Protection and Food Safety
CFU	Colony forming units
CPD	Critical point dryer
cP	centi-Poise
Da	Dalton (unit of molecular weight)
DCl	Deuterium chloride
DDA	Degree of deacetylation
D_2O	Deuterium oxide
ED	European Directive
EFFoST	European Federation of Food Science and Technology
e.g.	(lat.: *exempli gratia*) for example
EN	(ger.: *Europäische Normen*) European Standards
etc.	(lat.: *et cetera*) and other things
et al.	(lat.: *et alii*) and others
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
GRAS	Generally recognized as safe
HPLC	High Performance Liquid Chromatography
ICFMH	International Conference of Food Microbiology and Hygiene
ICMSF	International Commission on Microbiological Specifications for Foods

ISO	International Organization for Standardization
kDa	kilo Dalton (unit of molecular weight)
LD_{50}	Median lethal dose
LMH	(ger.: *Lebensmittelmikrobiologie und Hygiene*) Food Microbiology and Hygiene
M	Molar concentration (molarity)
MAP	Modified atmosphere packaging
Mc	Moisture content
MHB	Mueller Hinton broth
MIC	Minimum inhibitory concentration
mPas	milliPascal seconds
M_w	Molecular weight
M_η	Molecular mass (viscosimetric molecular weight)
NMR	Nuclear magnetic resonance
OPA	Ortho-Phthaldialdehyde
PCA	Plate count agar
RP	Reverse-phase (a type of HPLC column)
SCD	Sputter Coater Dryer
SEC	Size exclusion chromatography
SEM	Scanning electron microscope
TMSP-d	Trimethylsilyl-3-propionate, sodium salt D4
TPC	Total plate count
TS	Technical specification
TVBN	Total volatile basic nitrogen
TVC	Total viable count
U.K.	The United Kingdom
U.S.	The United States
w/v	Weight per volume
XLD	Xylose lysine deoxycholate
v/v	Volume per volume

List of figures

List of tables

Table of contents

Table of contents

Table of contents

1 Introduction

1.1 Chitosan

1.1.1 Sources and structure

Chitosan is a polysaccharide obtained by deacetylation of chitin, which is the major constituent of the exoskeleton of *Crustacea* (No et al. 2002). Chitosan was first discovered by Rouget in 1859 during boiling of chitin in a concentrated potassium hydroxide solution, which resulted in deacetylation of chitin (Muzzarelli 1977). Chitosan is a naturally regenerating resource that can be further enhanced by artificial culturing. It was also reported that chitosan and chitin are contained in cell walls of fungi (Sudarshan et al. 1992). At the present time, most chitosan in practical and commercial use comes from the production of deacetylated chitin originating from the shells of crab, crawfish, and shrimp, which are the most readily available sources of chitosan (Knorr 1994; Shahidi et al. 1999; No et al. 2007). Recent advances in fermentation technology suggest that the cultivation of fungi (*Aspergillus niger*) can provide an alternative source of chitosan (Teng et al. 2001; Rabea et al. 2003).

Chitosan is a polycationic polymer with specific structure and properties and contains more than 5000 glucosamine units. According to its chemical structure, chitosan is composed of 2-amino-2-deoxy-D-glucose (glucosamine) monomers, which are linked β-1-4-glycosidically, whereas chitin is composed of *N*-acetyl-glucosamine monomers, as presented in Figure 1.1 (Rabea et al. 2003).

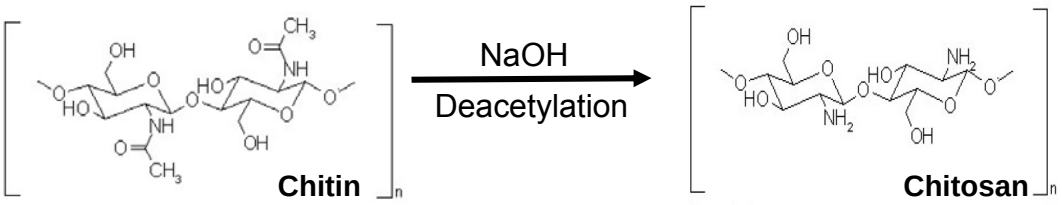


Figure 1.1: Preparation of chitosan by deacetylation of chitin (Rabea et al. 2003)

1.1.2 Physicochemical properties

The most important physicochemical characteristics of chitosan are the degree of deacetylation (DDA) and the molecular weight (M_w), since these parameters play a major role for the quality of chitosan in its various applications (Kumar 2000; Tharanathan and Kittur 2003; Kumar et al. 2004).

The difference between chitin and chitosan lies in the DDA (Muzzarelli 1977). The DDA determines the contents of free amino groups in the polysaccharide and means the ratio of *N*-acetyl-D-glucosamine to D-glucosamine structural units. When the DDA is higher, the number of free amino groups of chitosan is also higher (Allan et al. 1984; Draget 1996). The DDA controls the degree of crystallinity and hydrophobicity of chitosan due to variations in the hydrophobic interactions which control the loading and release characteristics of chitosan matrices. The DDA also controls the degree of cross-linking of chitosan in the presence of any suitable cross-linker (Draget 1996). The DDA of chitosan usually ranges from 70–95 %, where in foods and food products, it ranges from 75–80 % and in pharmaceuticals from 90–95 %. Most publications use the term chitosan when the DDA is higher than 70 % (Tsai et al. 2002; Kumar et al. 2004). The DDA value of chitosan depends on the preparation procedure (Schatz et al. 2003). Methods to determine the DDA of chitosan are infrared spectroscopy, ultraviolet spectrophotometry, titration, gas chromatography,

thermal analysis, and dye adsorption (Maghami and Roberts 1988; Kumar et al. 2004).

Another important physicochemical characteristic of chitosan is the molecular weight (M_w), although details on the underlying chemical and physical effects of chitosan for this parameter are still unknown. However, considerable evidence has been gathered indicating that most of the physiological activities and functional properties of chitosan depend on their molecular weight (Rabea et al. 2003).

The M_w of chitosan is a key parameter in the preparation of chitosan complexes, particularly in the industry, since the transfection efficiency strongly correlates with M_w. The commercial molecular weights of chitosan are in the range of 2000 to 2050 kDa (Illum 1998).

Chitosan with high M_w renders very stable complexes, but due to its bulk molecules, the interacted efficiency is very low (Zhang et al. 2004). A scanning electron microscopy (SEM) image of chitosan with a high M_w of 640 kDa is shown in Figure 1.2.

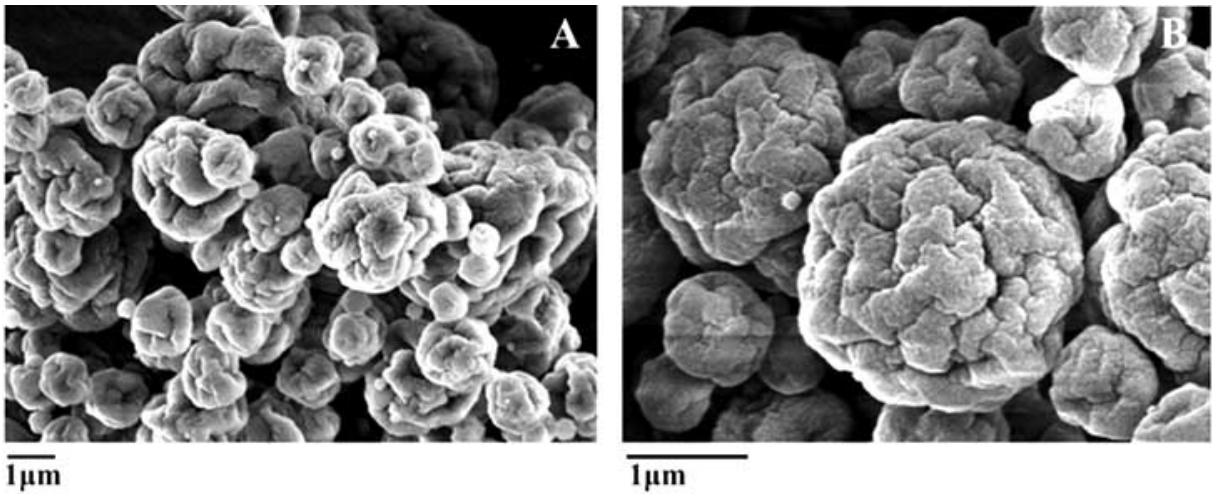


Figure 1.2: SEM image of high-molecular-weight chitosan added with **(A)** 0.2 % cross-linker and **(B)** without addition of any cross-linker (Heras et al. 2009)

Recently, chitosan with low M_w has become the main concern to be used alternatively to improve the efficiency of transfection. Chitosan with low M_w possesses a higher activation energy and can effectively interact with other negatively charged compounds (Beysseriat et al. 2006).

The molecular weight of chitosan can be determined by several methods, such as light scattering spectrophotometry, gel permeation chromatography, and viscometry. Among these methods, viscometry is the simplest, most rapid, and widely applied method for determination of molecular weight of chitosan (Bough et al. 1978; Kumar 2000).

The main difference between chitin and chitosan lies in their solubility. Through deacetylation of chitin to obtain chitosan, the insoluble chitin is transformed into the acid-soluble chitosan. For instance, chitosan is soluble in dilute aqueous acids such as acetic acid at a concentration of 0.1 M (Hirano 1996; Chen and Tsaih 1998). Chitosan is soluble under acidic conditions due to the free protonable amino groups present in the D-glucosamine units (Heras et al. 2009).

Chitosan is insoluble in water, alkali, and organic solvents, but soluble in most solutions of organic acids when the pH of the solution is less than 6.0. The pH-dependent solubility of chitosan is attributed to its amino groups (—NH_2), which become protonated upon dissolution at a pH of less than 6.0 to form cationic amine groups (—NH_3^+), increasing intermolecular electric repulsion and resulting in a polycationic soluble polysaccharide (Singla and Chawla 2001; Rafaat and Sahl 2009). The most widely used acids for dissolving chitosan are acetic acid and formic acid (Muzzarelli 1977). Some diluted inorganic acids, such as nitric acid, hydrochloric acid, perchloric acid, and phosphoric acid can also be used to prepare a chitosan solution but only after prolonged stirring and warming (Cho et al. 2000).

One of the most characteristic properties of many polymers, including chitosan, is their ability to form viscous solutions; therefore, chitosan may function as thickeners, stabilizers, or suspending agents and its solutions are able to show pseudoplastic and viscoelastic properties (Cho et al. 2000). Moreover, the viscosity of chitosan is affected by degree of deacetylation, molecular weight, concentration, types of solvents, pH value of the prevailing solution and ionic strength, as well as temperature (Kumar 2000). The viscosity of 1 % (w/v) commercial chitosan in 1 % (v/v) acetic acid at 25 °C is in the range of 10 to 1000 mPas (Cheng et al. 2005).

1.1.3 Biological properties

Much of the recent commercial interest in chitosan arises due to its several favourable biological properties, which are natural, biodegradable, biocompatible, in bland taste, and non-toxic (Muzzarelli 1996). Moreover, other biological properties such as analgesic, antitumoregenic, hemostatic, hypocholesterolemic, and antioxidant properties have also been reported (Kumar 2000; Tharanathan and Kittur 2003). These biological properties make chitosan, on the one hand, as an excellent choice for a natural food additive component and a valuable material for pharmaceutical, biomedical as well as industrial applications (Shahidi et al. 2002; Rafaat and Sahl 2009). On the other hand, chitosan is economically inexpensive because it is a natural compound obtained by deacetylation of chitin, which is produced from shrimp, crab, and crawfish shells waste (Knorr 1994).

Chitosan is susceptible to enzymatic degradation by enzymes from a variety of sources (Muzzarelli 1997), including non-specific enzymes, such as lysozyme presents in tears, saliva, blood, and milk (Rhoades and Roller 2000), chitinases (Sikorski et al. 2005), cellulases or hemicellulases, proteases such as papain and

pronase (Kumar et al. 2005), lipases, ß-1,3-1,4-glucanases, and chitosanases (Kimoto et al. 2002). Chitosanases have been generally recognized as enzymes that attack chitosan, catalyzing the endohydrolysis of ß-(1→4)-glycosidic linkages between D-glucosamine residues in partly acetylated chitosan (Kim et al. 2003).

One of the most important biological properties of chitosan is its biocompatibility, where it should not be affected by the host and at the same time should not elicit any undesirable local or systemic effects (Tharanathan and Kittur 2003). Chitosan is well tolerated by living tissues, including the skin, the ocular membranes, and the nasal epithelium, and has thus been proven valuable for a wide range of biomedical applications (Kumar et al. 2004).

The toxicity of chitosan compared with other natural polysaccharides is low, thus, chitosan has attracted much attention with regard to food applications (Shahidi et al. 2002; No et al. 2007). It has been reported that the purity of chitosan influences its toxicological profile. The safety of chitosan in terms of inertness and low or no toxicity has been demonstrated by *in vivo* toxicity studies. The oral LD_{50} (median lethal dose) of chitosan in mice was found to be in excess of 16 g/kg of body weight per day, which is higher than that of sucrose (Singla and Chawla 2001; Bowman and Leong 2006).

1.1.4 Antimicrobial activity and mode of action

Chitosan possesses a specific structure with reactive amino groups (Figure 1.3). Due to its reactive amino groups, chitosan shows antimicrobial properties, thus, it becomes a bioactive compound with antimicrobial function (Rabea et al. 2003; Kumar et al. 2004).

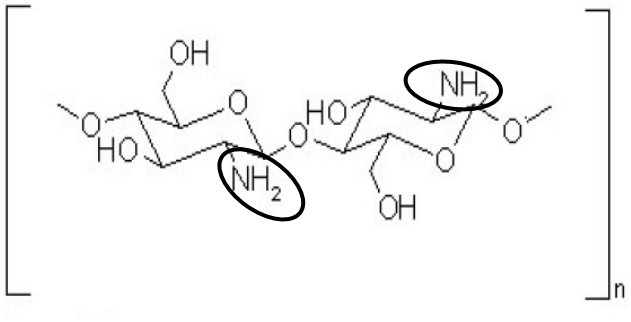


Figure 1.3: Chitosan structure with its reactive amino groups

Due to its antimicrobial activity, chitosan can inhibit the growth of a wide range of microorganisms such as bacteria, fungi, and yeast (Sudarshan et al. 1992; Sagoo et al. 2002). Chitosan generally has a stronger antimicrobial activity against bacteria than against fungi (Tsai et al. 2002). However, chitosan shows its antibacterial activity only in an acidic medium because of its poor solubility above pH 6.5 (Tsai and Su 1999; No et al. 2002).

Because of the positive charge on the C-2 position of the glucosamine monomer below pH 6, chitosan is more soluble and has a better antimicrobial activity than chitin (Rabea et al. 2003). The exact mechanism of the antimicrobial action of chitosan has so far not been fully elucidated, but several hypothetical mechanisms have been proposed (Sudarshan 1992; Rabea et al. 2003; No et al. 2007).

The first mechanism which is the most feasible hypothesis is a change in cell permeability due to interactions between positive charges on the C-2 of the glucosamine monomer of chitosan and negatively charged microbial cell membranes. This interaction leads to the leakage of proteinaceous and other intracellular constituents in the cell (Leuba and Stössel 1986; Papineau et al. 1991; Sudarshan 1992; Rabea et al. 2003).

The other mechanism involves the binding of chitosan to DNA to inhibit mRNA synthesis (Hadwiger et al. 1986). It has been proposed that when chitosan is

liberated from the cell wall of fungal pathogens by plant host hydrolytic enzymes, chitosan penetrates the nuclei of the fungus and interferes with mRNA and protein synthesis. Thus, the organism may be impaired by both its own chitosan and the host phytoalexin induced by the liberated chitosan (Hadwiger and Loschke 1981).

Other antimicrobial mechanisms of chitosan are the chelation of metals, spore elements, and essential nutrients. However, no further information has been gathered regarding these mechanisms (Cuero et al. 1991).

Recent studies on antibacterial activity of chitosan revealed that chitosan is more effective in inhibiting growth of gram-positive bacteria than that of gram-negative bacteria (No et al. 2002). The inhibitory activity of chitosan towards gram-positive and gram-negative bacteria should be considered in terms of its chemical and structural properties (Rabea et al. 2003). As a polymeric cationic macromolecule with positively charged amino groups, chitosan is able to penetrate the cell wall of gram-positive bacteria that consists chiefly of peptidoglycan and lacks an outer membrane. However, as a polymeric cationic macromolecule, chitosan may have less capability to interact with both bacterial cell membranes of gram-negative bacteria, mainly to pass the outer membrane, since this membrane functions as an efficient outer permeability barrier against chitosan (Je and Kim 2006). On the contrary, chitosan can easily disrupt the cell wall of gram-positive bacteria because the major constituents of the cell wall are composed of peptidoglycan and very little protein (Sudarshan et al. 1992; Helander et al. 2001; Rabea et al. 2003; Je and Kim 2006). The penetration of the cell wall of bacteria leads to the leakage of proteins and other important intracellular constituents of bacteria so that they can not grow any further (Helander et al. 2001).

Furthermore, chitosan has attracted attention in various fields of application due to its antimicrobial activity (Sagoo et al. 2002; Shahidi et al. 2002; No et al. 2007), which

greatly depends on its degree of deacetylation and molecular weight (Uchida et al. 1989; Jeon et al. 2001).

1.1.5 Applications and regulatory status

The main driving force in the development of new applications for chitosan lies in the facts that the polysaccharide is not only naturally abundant, but it is also non-toxic and biodegradable (Muzzarelli 1977). Beside that, chitosan is economically inexpensive compared to other synthetic polymers (Rabea et al. 2003). Chitosan has attracted attention in various fields of application due to its antimicrobial activity against a wide range of microorganisms (Sagoo et al. 2002), due to its better solubility than chitin, and due to its physicochemical properties, namely DDA and M_η (Kumar 2000; Tharanathan and Kittur 2003; Kumar et al. 2004).

Moreover, due to its protonated amino groups with the positive charge, chitosan has ability to interact with many negatively charged compounds, such as polyanions, dyes, proteins, and DNA (Kumar et al. 2004). Similarly, chitosan is able to act as a flocculating agent due to its ability to form an insoluble chelate-complex with heavy-metal ions. This property has been particularly used in Japan for waste water treatment since 1975 (Hirano 1996).

Other applications of chitosan in various fields are such as a potential elicitor of plant defense responses (Cote et al. 2000; Kim et al. 2005), as an additive in the food industry (Rhoades and Roller 2000; Roller 2003), as a hydrating agent in cosmetics (Kumar et al. 2004; Kofuji et al. 2004), and more recently as a pharmaceutical agent in biomedicine (Carlson et al. 2008; He et al. 2008).

Chitosan has received regulatory approval as functional food ingredients in some Asian countries such as Japan and Korea during the last decade. Moreover, Japan

produces dietary cookies and noodles enriched with chitosan (Hirano 1996). The inclusion of chitosan was considered in 2003 by the Codex Alimentarius Commission (Paul and Sharma 2000). In the field of pharmaceutical application, chitosan has been included in the European Pharmacopoeia since 2002 (Ph. Eur. 2002).

The percentages of utilization of chitosan in various fields of application are presented in Figure 1.4.

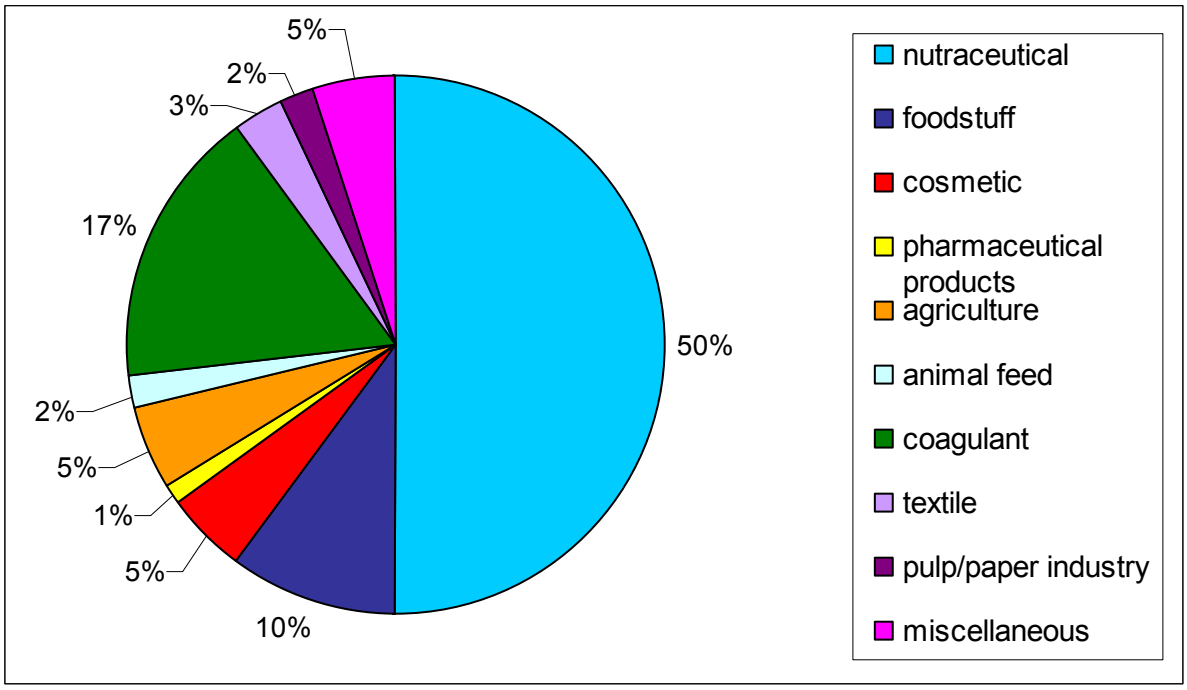


Figure 1.4: Percentages of utilization of chitosan in various fields of application (Heras et al. 2009)

1.2 Shrimps

1.2.1 Nature, sources, and economical importance

Shrimps are the most important group of crustaceans (*Crustacea*), and belong to the phylum of Arthropoda and the order of Decapoda. They are distributed all over the world and live mainly in aquatic environments (Boyd and Clay 1998). Of the nearly

2500 known species of shrimps, 344 species are suitable for human consumption (Schminke 1996).

North Sea shrimps such as a species *Crangon crangon* grows slowly and is small in size. Adults are typically 30-50 mm long, although individuals up to 90 mm have been recorded (Figure 1.5). The shrimps have cryptic coloration, which can change to brown-sand color, to adapt to the environment. They live in shallow water, which can also be slightly brackish, and feed nocturnally. During the day, they remain buried in the sand to escape predatory birds and fishes, with only their antennae protruding (FAO 2005-2006).

Figure 1.5: Shrimp species *Crangon crangon*

C. crangon is the commercially important species of shrimps in Germany. This species is also found in the Irish Sea, the Baltic Sea, the Mediterranean Sea, and the Black Sea, as well as in Scandinavia and in parts of the Atlantic coast of Morocco. It has various names such as common shrimp, brown shrimp, sand shrimp, and prawn in British English. The commercial catch of *C. crangon* takes place in summer and autumn (Schminke 1996). Over 38.811 tons of *C. crangon* were caught in 2007, with 80 % of this total attributed to Germany and the Netherlands (FAO 2006-2007).

The world production of shrimps, both captured and farmed, is about 6 million tons, of which about 60 % enters the global market. Globally, about 60 % of the shrimps production comes from fishing, while 40 % is from farming. The recent world shrimps catch has been about 3.4 million tonnes per year, with Asia being the most noteworthy area for shrimps fishing, accounting for 55 % of the world catch. The main producers of shrimps in the world are Indonesia, India, China, the USA, and Thailand (Sikorski 1990). However, the international shrimps world markets are only concentrated in the USA, Japan, and Europe (Gillett 2008).

Due to high commercial value of shrimps, their processing and trading are highly specialized. Shrimps are sold alive, fresh, frozen, canned, dried, and in a number of different forms, such as whole, headless, shell-on, and peeled (Boyd and Clay 1998). Shrimps are considered on the world market as high-value commodities and are one of the most important internationally traded fishery products, with a value of U.S. $10 billion, consisting 16 % of global fishery exports. Shrimps fisheries generate substantial economic benefits, especially for many developing countries (Gillett 2008; FAO 2009).

1.2.2 Spoilage of shrimp meat and its preservation

Seafood spoilage can be considered as any change which renders seafood products unacceptable for human consumption (Smith et al. 1996). Shrimp is a highly perishable food due to its high water activity value within the range of 1.00-0.95 (Fontana 2000). The shelf life and quality of shrimp during storage is greatly influenced by enzymatic changes and microbiological changes, mainly due to the growth and activity of gram-negative aerobic bacteria (Quattara et al. 2001; Baixas-Nogueras et al. 2002). Shrimps spoil more rapidly than fish due to their chemical

composition which contains a lot of non-protein nitrogenous compounds that encourage accelerated spoilage (Smith et al. 1996; Ólafsdóttir et al. 2005).

During the last several years, reliable methods have been developed to extend the shelf life of shrimp and to avoid health hazards for consumers (Al-Dagal and Bazaraa 1999). However, these methods have some limitations for shrimp preservation. In addition, the most common preservation method used so far to extend the shelf life of shrimp during storage is freezing, mainly in the shrimp industry (Huidobro et al. 2002). Beside that, in the fishery industry, most of the shrimps are iced on board of the fishing boat after catching and are processed in factories nearby the fishing area within 5–7 days from the time of catching (Valdimarsson et al. 1998). Several methods of shrimp preservation applied so far are summarized in Table 1.1.

Table 1.1: Methods applied for shrimp preservation and their limitations

Preservation methods	Limitations	References
Cold storage in ice	Poor texture and shape, loss of colour, high energy cost	Shamshad et al. (1990), Lakshamanan et al. (2002), Rogério et al. (2001)
Modified ice storage	Poor texture and loss of colour	Harrison and Heinz (1989)
Cook-chill process	Poor texture and shape	Venugopal (1993)
Super-chilled storage at 0–4 ^{o}C	Poor texture, loss of colour, high energy cost, absence of 'cold chains'	Fatima et al. (1988)
Liquid ice	Loss of colour, high energy cost	Ólafsdóttir et al. (2005)
Modified atmospheres packaging storage in ice	Poor flavour, cost of the packaging	Baka et al. (1999), Lopez-Caballero et al. (2002)
Gamma-radiation	High energy cost, poor shape	Yeh and Hau (1988)
Treatment with organic acids and their salts	Flavor changes, poor texture, loss of colour	Benner et al. (1994), Mosffer et al. (1999)
Combined cook-irradiation effect	High energy cost, poor texture and shape	Quattara et al. (2001)

However, under chilled storage, the shelf life of shrimp is limited by enzymatic and microbiological spoilage. Even under freezing storage conditions, quality deterioration of shrimp is not always completely suppressed; likewise, reactions leading to oxidative and enzymatic changes and protein degradation may still proceed (Smith et al. 1996; Simpson et al. 1997; Shahidi et al. 2002). Beside that, freezing at a temperature of -20 °C alters the physical structure of foods. Moreover, storage at -20 °C for weeks or months is possible, but microorganisms can still grow in pockets of liquid water trapped within the frozen mass. For long-term storage, temperatures of -80 °C, which are also called the temperatures of “dry ice”, are necessary. However, maintenance of such low temperatures is very expensive and consequently it is not used for routine foods storage (Madigan et al. 2009).

With increasing consumer demands for fresh seafood products with extended shelf life and advantageous energy cost, it is therefore necessary to develop an alternative preservation method to maintain the quality and freshness of shrimp meat and at the same time, to economize on energy cost. Moreover, recently, food quality and safety has become major concerns in the food industry as consumers prefer fresher and minimally processed products. In particular, bacterial contamination of ready-to-eat products is an issue with regard to human health (Quattara et al. 2000; Pranoto et al. 2005). So far, the use of preservatives in shrimp meat by direct application such as spraying or dipping has been done to overcome bacterial contaminations (Quattara et al. 2000). However, direct surface application of preservatives onto foods has some limitations because the active substances could be neutralized, evaporated or may diffused inadequately into the bulk of the foods (Torres et al. 1985; Siragusa and Dickson 1992).

Currently, a new concept is being developed in which preservatives as antimicrobial compound can be incorporated into coatings or films in order to maintain high

concentrations of preservatives on the surface of foods for longer storage time (Guilbert 2000). Edible coatings or films have been investigated for their abilities to retard moisture, oxygen, aromas, and solute transports (Quattara et al. 2000). Moreover, edible coatings or films constitute one of the most effective methods to maintain food quality (Guilbert 2000; Quattara et al. 2002; Pranoto et al. 2005).

Due to environmental concerns, edible coatings prepared from biopolymers such as proteins, polysaccharides, and lipids are generally used as carriers for various antimicrobials (Quattara et al. 2001). Chitosan as a natural polymer has been proved to qualify as a major material for edible coatings or film due to its non-toxicity, biodegradability, biofunctionality, biocompatibility, and antimicrobial properties (Wang 1992; Muzzarelli 1996). Therefore, chitosan is suitable for use as edible coating or film for shrimp meat preservation. Another antimicrobial agent acting as a “secondary preservative” such as plant extracts may be added into chitosan coating solution to strengthen the antimicrobial efficacy (Dutta et al. 2009).

At the present time, little is known about the efficacy of such technology for the control of microbial growth and the extension of shelf life of shrimp meat.

1.2.3 Assessment of shrimp meat quality

Shrimps, like other kinds of seafood, are generally assessed by sensory methods based on changes in their appearance, odor, color, flavor, and texture. Speed, simplicity, and low costs are the main advantages of these methods. However, sensory analyses are inherently subjective, even when panel members have received extensive training (Koutsoumanis et al. 1999; Baixas-Nogueras et al. 2002). For this reason, recently, biochemical methods have been developed to measure the amounts of degradation products derived from either bacterial or endogenous enzymatic activity. In particular, biochemical parameters, such as volatile and non

volatile amine levels, have been used to assess shrimp freshness (Thorarinsdóttir and Ólafsdóttir 2003).

Besides that, the quality of shrimp meat during storage is influenced by activity of microorganisms, thus, the quality assessment of shrimp meat during storage depends on changes in microbiological parameters (Huis in't Veld 1996). Therefore, the evaluation of quality and shelf life of shrimp meat during storage is based on changes in microbiological parameters and biochemical parameters throughout the storage period. The microbiological parameters are such as total cell count of aerobic mesophilic bacteria, growth of tested gram-positive bacteria, and growth of tested gram-negative bacteria. The biochemical parameters consist of pH value, water activity, content of total volatile basic nitrogen, and content of biogenic amines (Huis in't Veld 1996; Smith et al. 1996; Ólafsdóttir et al. 2005).

1.2.3.1 Assessment of microbiological parameters

Recently, much attention has been paid to the occurrence of pathogenic microorganisms in consumed seafood. Due to growing demands of consumer for safer and better quality of seafood, quality and safety aspects of seafood in trade have become important, since fresh seafood is prone to certain microbiological contaminations (FAO 2009).

During harvesting, processing, and handling, shrimps may become contaminated with a wide range of microorganisms and common foodborne pathogens. These foodborne pathogens are usually called non-indigenous pathogenic bacteria. These bacteria are normally associated with humans or warm-blooded animals and their faeces, and are not naturally present on shrimps. Subsequently, during distribution and storage, contamination due to microorganisms can rapidly develop and cause serious spoilage of shrimps. After harvesting, shrimps die immediately and may have

high bacterial counts by the time they are deposited at the processing plant on shore (Ólafsdóttir et al. 2005; Huss et al. 2000).

North Sea shrimps such as *C. crangon* are traditionally boiled on board of the fishing boat, thus, eliminating much of the contamination flora. However, they are recontaminated during subsequent handling and cooling in seawater, particularly by psychrotrophic bacteria. Since shrimps are rich in free amino acids, vitamins, and minerals, the cooking water in the vessel provides a good medium for bacterial growth (Huis in't Veld 1996; Smith et al. 1996; Huss et al. 2000). In addition, pH of shrimp meat is nearly neutral, at about 7.0. Thus, shrimp meat is a suitable living and proliferation space for bacteria (Thorarinsdóttir and Ólafsdóttir 2003). Moreover, under unhygienic storage conditions, microorganisms rapidly spoil the shrimp meat. This may be harmful to human health by causing infections and intoxications (Jeong et al. 1991). It is worthwhile to note that, when the shrimps die, their immune system collapses and bacteria are allowed to proliferate freely. On the shrimp shell, the bacteria to a large extent colonize the scale pockets. During storage, they invade the flesh by moving between the muscle fibres (Thorarinsdóttir and Ólafsdóttir 2003).

Furthermore, like other semi-ready-to-eat foods, cooked shrimps are considered as a serious health risk food for consumer due to their capability to associate with spoilage bacteria, which play an important role for consumer health (Huss 1997; Gillett 2008). The spoilage bacteria, such as *Salmonella* sp., *Listeria* sp., *Escherichia coli*, and *Staphylococcus aureus* possibly occur on cooked shrimps caused by handling, processing, and storage conditions (Huss 1997; Huss et al. 2000).

Among these bacteria, *Staphylococcus aureus* has a great importance because this strain can produce heat-stable toxins causing food poisoning (Madigan et al. 2009). These toxins continue to persist on shrimps during cooking, and thus cooked shrimps may create a great risk (Loir et al. 2003). *S. aureus* is a facultatively anaerobic, gram-

positive coccus, catalase positive, and able to convert hydrogen peroxide (H_2O_2) to water and oxygen, which makes the catalase test useful to distinguish staphylococci from enterococci and streptococci. *S. aureus* is the most common cause of staphylococcal infections (Madigan et al. 2009; Dykes 2010).

Besides that, cooked shrimps are often contaminated by *Escherichia coli*, which belong to the family *Enterobacteriaceae*. *E. coli* is a gram-negative, non-spore-forming, straight rod arranged in pairs or singly, facultatively anaerobic bacterium with an optimum growth temperature of 37 °C. Its further characteristics are oxidase-negative, catalase-positive, fermentative (glucose, lactose, D-mannitol, D-sorbitol, arabinose, maltose), reduces nitrate, and β-galactosidase-positive. Approximately 95 % of the strains are indole and methyl red positive (Fratamico and Smith 2006). All strains of *E. coli* are negative in the Voges-Proskauer test. Most strains do not hydrolyze urea or produce H_2S in triple sugar iron (TSI) medium and are unable to use citrate as a sole carbon source. In addition, a strain of *E. coli* which caused foodborne disease, ranging from mild enteritis to serious illness and death is *E. coli* O157:H7 (Wilshaw 2000). There have been several studies on the presence of coliforms in seafood because of concern about the health of seafood consumers, mainly in fish. In contrast, there have been relatively few studies about the presence of coliforms notably *E. coli* in shrimp (Greenwood et al. 1985; Sikorski 1990; Hansen et al. 2008).

Furthermore, cooked shrimps often act as a source of foodborne infection such as *Listeria monocytogenes,* which causes listeriosis. *L. monocytogenes* is a non-spore forming gram-positive and catalase positive rod shaped bacterium. It can grow under anaerobic or microaerophilic conditions and under a wide temperatures range (0–45 °C) with an optimum range of 30–37 °C (Huss et al. 2000; Madigan et al. 2009).

Because *L. monocytogenes* can grow at low temperatures, thus, it is considered as psychrotrophic bacterium that can easily adapt and grow under the conditions of most foods. Its capacity to grow at refrigerated temperatures (at 4–7 °C) can be one of the most important factors supporting its presence at the end of the shelf life of non-sterile refrigerated products (Dykes 2010). Thus, ready-to-eat (RTE) shrimp meat is a potential source of the occurrence of listeriosis due to long storage period at refrigerated temperature, at which *L. monocytogenes* is able to further proliferate (Hatha et al. 2003; Ahmed and Anwar 2007). It must be taken into account that the presence of *L. monocytogenes* in foods has become a concern in recent years (Conner et al. 1986; Shahidi et al. 2002).

Lastly, a genus of bacteria that may potentially contaminate shrimp is *Salmonella* (Dalgaard et al. 1995; Bhaskar et al. 1995). *Salmonella* belongs to the family of *Enterobacteriaceae*. They are gram-negative, non-spore-forming rods bacteria which are facultatively anaerobic, catalase-positive, oxidase-negative, and generally motile with peritrichous flagella. The genus *Salmonella* consists of over 2500 serovars, as determined by its somatic (O) and flagellar (H) antigens (Cai et al. 2005). The serotypes are closely related, many of which are potentially pathogenic for humans and animals (Yan et al. 2003). *Salmonella* can cause salmonellosis, a widely distributed foodborne disease. It constitutes a major public health burden and represents a significant cost in many countries (WHO 2005). The clinical characteristics of human salmonellosis are usually characterized by abdominal pain, diarrhoea, nausea, and sometimes vomiting. It is generally agreed that the food chain is the major source of *Salmonella* infection for humans (WHO 2007). Many factors such as inadequate supplies of clean water, inadequate sanitary measures, lack of food hygiene, and food safety measures have been responsible for increased incidences of foodborne salmonellosis (Miko et al. 2005). The occurrence of

salmonellosis in humans is mainly caused by *S. typhimurium* through food poisoning (Madigan et al. 2009).

The microbiological criteria for cooked shrimps used so far are recommended by European Union guideline 93/51/EWG (1994*)*. According to these criteria, *L. monocytogenes* and *S. typhimurium* must be not detectable (N.D.) in 25 g of shrimp meat sample, whereas *E. coli* and *S. aureus* have the certain upper limiting and recommended values, as presented in Table 1.2.

Table 1.2: Recommendations of microbiological criteria for cooked shrimps (EU guideline 93/51/EWG 1994)

Microorganisms	Upper limiting value	Recommended value
Escherichia coli	100/g	10/g
Staphylococcus aureus	1000/g	100/g
Salmonella typhimurium	N.D. in 25 g	N.D. in 25 g
Listeria monocytogenes	N.D. in 25 g	N.D. in 25 g
Aerobic mesophilic bacteria	10^6/g	10^5/g

The total numbers of organisms allowed to grow in seafood vary enormously such reported by several investigators. The total viable count (TVC) of aerobic mesophilic bacteria was proposed at a value of 10^6 CFU/g, when sensory spoilage was detected in seafood (Fieger and Novak 1961; Gill 1986; Huis in't Veld 1996). In addition, International Commission on Microbiological Specifications for Foods of the International Union of Microbiological Societies also proposed the acceptability limit of 10^6 CFU/g for fresh fish (ICMSF 1986; Shahidi et al. 2002). Eventually, the TVC of 10^6 CFU/g has been proposed as a guideline for shrimp meat freshness (Ólafsdóttir et al.1997).

1.2.3.2 Assessment of biochemical parameters

Shrimp meat freshness is generally assessed based on changes in biochemical parameters, such as pH value, content of total volatile basic nitrogen (TVBN), water activity value (a_w), and content of biogenic amines during storage. In particular, biochemical parameters are used to significantly assess the quality of shrimp freshness (Quattara et al. 2002).

pH value is an indicator for shrimp meat spoilage due to its effects on activities of microorganisms and enzyme during storage. Usually pH decreases during anaerobic formation of lactic acid by microbial activity during the first hours after the death of shrimp. However, microbial metabolism may also lead to an increase in pH during storage time (Haard 1992; Smith et al. 1996). Krishnakumar et al. (1985) showed reduction of total nitrogen in shrimp stored in ice because some compounds containing nitrogen were leached out. Changes in pH value showed a good correlation with microbiological results. This is also reflected by TVBN accumulation which indicated the spoilage progress (Cobb et al. 1977; Chan et al. 2006).

The occurrence of TVBN is one of the characteristic features attributed to changes in biochemical parameters occurring in marine fish muscle during spoilage. This parameter is widely considered to be a useful index of seafood freshness (Ólafsdóttir et al. 2006).

In fact, the European Union regulation considers a TVBN value of 30 mg/100 g as the limit above which shrimp is not acceptable for human consumption (Baixas-Nogueras et al. 2002). Increases in the TVBN contents of shrimp meat during storage may be attributed to several enzymatic processes, namely, deamination of free amino acids, degradation of nucleotides, and oxidation of amines, among others (Simpson et al. 1997).

The biochemical parameter for notably assessing the hygienic quality of seafood is biogenic amines content due to their potential toxic effects. The biogenic amines are non volatile amines and are found at very low levels in fresh shrimp. However, they can be accumulated in association with bacterial spoilage in shrimp meat during storage. It is highly recommended to reduce and even to avoid the consumption of food containing biogenic amines, even at low concentrations (Xue et al. 2007).

Biogenic amines are generated by microbial decarboxylation of amino acids in food products. Their formation in foods depends on availability of free amino acids, the presence of decarboxylase positive microorganisms and favorable conditions, which allow growth of microorganisms and subsequent decarboxylase activity (Taylor et al. 1995). The most significant biogenic amines occurring in shrimp are putrescine, cadaverine, tyramine, agmatine, histamine, and spermidine. The importance of estimating the concentration of biogenic amines in seafood and its products is related to their impact on human health and food quality (Benner et al. 2003). However, in fact, only histamine has been extensively studied up to now and for instance, histamine is the only biogenic amine which has a legally established regulation level according to the European Union that has set the maximum average content at 100 mg/kg for raw seafood (Karovicova and Kohajdova 2005). The Food and Drug Administration (FDA) lowered the toxicity level of histamine from 100 to 50 mg/kg, recommending that not only histamine but also the contents of other biogenic amines have to be taken into account (Baixas-Nogueras et al. 2002). Moreover, some studies have reported that the accumulation of biogenic amines in raw fish increased progressively during storage. Thus, biogenic amines have been proposed as potential markers for evaluating of seafood freshness (Gill 2005).

Another parameter for shrimp meat freshness assessment is water activity (a_w). The concept of water activity is an important property that is used to predict the quality

and safety of food with respect to microbial growth, rates of deteriorative reactions, chemical and physical properties. Controlling water activity is an important way to prevent spoilage and to maintain foods quality (Gibbs and Gekas 1998; Fontana 2000).

Water activity is the ratio of the water vapor pressure (p) over a food to the water vapor pressure of pure water (p_o) at a given temperature, as expressed by the formula:

$$a_w = \frac{p}{p_o}$$

Thus, pure water has a water activity value of exactly 1.00.

The water activity of a food describes the availability of free water in the food, and hence its availability to act as a solvent and participate in chemical or biochemical reactions and growth of microorganisms of the food system. Water activity influences not only microbial spoilage but also chemical and enzymatic reactivity as well as the storage stability of foods, since some deteriorative processes in foods are mediated by water (Beuchat and Rockland 1987; Rockland and Stewart 1998).

Water activity has a direct implication for microbiological safety of foods. Microorganisms generally grow best within a range of a_w values of 0.95-0.98, while most of them cease growing at $a_w < 0.90$. Microorganisms causing spoilage in seafood are generally inhibited in certain range of a_w. Table 1.3 shows the minimal water activity values for growth of bacteria causing spoilage in shrimp meat.

Table 1.3: Minimal a_w values for growth of bacteria causing spoilage in shrimp meat (Beuchat 1983; Gibbs and Gekas 1998; Fontana 2000)

Strain Species	Group of Bacteria	Minimal a_w value
Escherichia coli	gram-negative	0.95
Salmonella typhimurium	gram-negative	0.91
Listeria monocytogenes	gram-positive	0.92
Staphylococcus aureus	gram-positive	0.80

Water activity also strongly influences enzymatic reactions, spontaneous autocatalytic lipid oxidation reactions, and physical properties such as textural properties and appearance of seafood. Finally, a_w plays a significant role in shelf life of foods. The relationship between water activity and physical properties of foods can be explained with an example namely that foods with high a_w have textural properties that are described as moist, tender, juicy, and chewy. If the water activity value of foods is lowered, undesirable textural properties such as hard, dry, stale, and tough occur (Labuza 1987; Fontana 2000).

Throughout history man has controlled the water activity of foods by traditional methods of preservation such as drying, sugaring, salting, and freezing. These methods were applied to reduce the a_w values of foods, because most microorganisms can not further grow well at low a_w values. Thus, the shelf life of foods could be extended (Bourne 1987; Board and Gould 1991; Chirife 1993).

Nowadays, the shelf life of foods is described with regards to the microbial, chemical or biochemical, and physical quality of foods (Gibbs and Gekas 1998; Fontana 2000). Shrimp meat as highly perishable food with high a_w value of 0.95-1.00 needs to be treated with a right method using a right preservative agent, therewith the desired a_w values in shrimp meat during storage can be maintained. Thus, in one side, their quality and shelf life indicated by chemical or biochemical and physical quality can be extended. On the other side, growth of microorganisms causing spoilage in shrimp meat can be inhibited simultaneously.

1.3 Bioactive edible coatings

1.3.1 Definition and regulatory status

Edible coatings may be defined as a thin layer of material that covers the surface of the food and can be eaten as part of the whole product (Guilbert et al. 1996).

The major benefit of the edible coatings is that they can be consumed along with the food, can provide additional nutrients, may enhance sensory characteristics, and may include quality-enhancing antimicrobials. Beside that, the edible coatings act as a barrier to the external elements such as moisture, oil, vapour, and thus prevent dehydration and protection of foods as well as extend their shelf-life (Guilbert 2000). Because they may be consumed, the composition of edible films or coatings must be conform to the regulations that apply to the food product concerned (Guilbert et al. 2002).

According to the European Directive 1995 and 1998 (Vargas et al. 2008) and the USA Code of Federal Regulations (FDA 2006) edible coatings are those coatings that are formulated using food-grade biological materials that qualify as generally recognized as safe (GRAS). The amount of edible coating components used should be not higher than that is necessary to accomplish the intended effect, and the components have to be qualified as GRAS (FDA 2006). Moreover, the cost of the technology and raw materials from which coatings are produced has to be relatively low (Vargas et al. 2008).

1.3.2 Functionality and composition

Edible coatings have to fulfil some specific functional requirements, which depend on the type of material used and their properties in human metabolism, such as sensory properties and barrier properties. The sensory properties of edible coatings are

described as transparent, tasteless, and odourless, whereas the barrier properties require that coatings must have an adequate water vapour and solute permeability and selective permeability to gases and volatile compounds (Guilbert et al. 1996; Vargas et al. 2008). Furthermore, edible coatings are used commercially to reduce moisture loss, prevent physical damage, enhance product appearance, and carry food ingredients. By incorporating antimicrobials, the functionality of edible coatings can be expanded to protect foods from microbial spoilage and extend shelf life. Furthermore, edible antimicrobial coatings have the potential to enhance the safety of foods (Vargas et al. 2008; Kilincceker et al. 2009).

There are several mechanisms involved in extending shelf life of food by coatings. These include controlled moisture transfer between food and surrounding environment, controlled release of chemical agents like antimicrobial substances, antioxidants, reduction of oxygen partial pressure in the package that results in a decreased rate of metabolism, controlled rate of respiration, high impermeability to certain substances like fats and oils, temperature control, structural reinforcement of food and coat flavor compounds and leavening agents in the form of microcapsules (Kittur et al. 1998; Vargas et al. 2008).

Recently, the development of new edible coatings with improved functionality and performance for fresh and minimally processed foods has become one of the challenges and recent investigations in food industry. In the past few years, research efforts have focused on the design of new eco-friendly coatings based on biodegradable polymers, which not only reduce the requirements of packaging but also lead to the conversion of by-products of the food industry into value-added film-forming components. The new generation of edible coatings is especially designed to allow the incorporation and/or controlled release of antioxidants, vitamins,

nutraceuticals, and natural antimicrobials (Vargas et al. 2008; Dutta et al. 2009; Chiu and Lai 2010).

The major components used in formation of edible coatings are biological materials such as polysaccharides, proteins, and lipids as well as their derivatives. The minor components such as plasticizers and functional ingredients may additionally be incorporated to enhance or improve the functional properties of edible coatings (Guilbert et al. 2002; Suyatma et al. 2005). The composition of materials used in formation of incorporated edible coatings is summarized in Table 1.4.

Table 1.4: Composition of materials used in formation of edible coatings

Major components	Minor components
Polysaccharides and their derivatives: Chitosan, cellulose, starch, alginate, gums, pectin, carrageenan, etc.	**Plasticizers:** Glycol, glycerol, phospholipids, etc. Function: Maintaining the flexibility and extensibility of edible coatings during storage
Protein and their derivatives: Gelatin, corn zein, casein, gluten, whey protein, egg white protein, keratin, etc.	**Acid or base:** Acetic acid, lactic acid, formic acid, etc. Function: To regulate pH of edible coatings
Lipids and their derivatives: Waxes, glycerides, fatty acids, cocoa butter, shellac, milkfat fraction, etc.	**Functional ingredients:** Antimicrobial agents: spices, plant extract, nisin, lysozyme, etc. Function: Improvement of efficacy and quality of edible coatings

Furthermore, in food preservation, the edible coatings improved by incorporating another antimicrobial agent are intended to obtain optimally functions of edible coatings against a wide range of foodborne microorganisms in foods. This incorporated antimicrobial agent acts as a “secondary preservative” in edible coatings (Pranoto et al. 2005; Holley and Patel 2005). Thus, the edible coating may

optimally protect foods from microbial spoilage and extend their shelf life (Vargas et al. 2008; Dutta et al. 2009; Chiu and Lai 2010).

There is a very wide range of compounds that can be used in the formulation of edible coatings and their choice depends mainly on the target application. The selection of appropriate materials for coating composition is greatly influenced by intrinsic food properties (pH, water activity, and composition) and extrinsic factors such as temperature, relative humidity during processing, and storage (Baldwin 1999; Guilbert et al. 2002; Vargas et al. 2008).

Furthermore, due to growing environmental concerns and regulations to develop environmentally friendly packaging materials by food industry, natural biopolymers, such as chitosan have the advantage over synthetic biopolymers because they are biodegradable and renewable raw materials. Thus, they can be used effectively to make biodegradable coatings and packagings to replace short shelf life plastics (Cutter 2006; Dainelli et al. 2008).

1.3.2.1 Edible coatings of chitosan enhanced with plasticizer

Plasticizers are additives used to increase the flexibility or plasticity of polymers, and occasionally they are used only to facilitate the polymer processing (Suyatma et al. 2005). Generally, plasticizers are defined by two purposes, namely to aid in processing and to modify the properties of the final product. For films and coatings, there are different definitions of plasticizers depending on the purpose of the polymer-plasticizer system. In this case, plasticizers can be defined as small low molecular weight, non volatile compounds added to polymers to reduce brittleness, impart flexibility, and enhance toughness of films. Furthermore, as a specific definition for coatings, plasticizers reduce flaking and cracking by improving coating flexibility and toughness. In general, plasticizers reduce intermolecular forces along

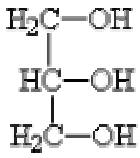
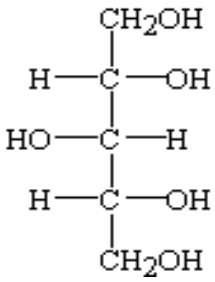
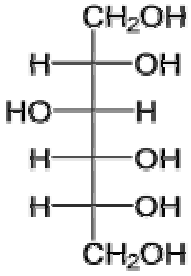
the polymer chains, thus, increasing film toughness and flexibility (Krochta and Sothornvit 2005).

Chitosan edible coatings or films are quite brittle due to extensive intermolecular forces such as hydrogen bonding, electrostatic forces, and hydrophobic bonding. Plasticizers are required to interrupt intermolecular forces resulting in greater flexibility and toughness. However, there are disadvantages of using plasticizers in edible coatings or films. For instance, at low concentration of plasticizers, antiplasticization in films and coatings can occur, in which it attributed to several mechanisms, such as reduction of polymer free volume, interaction between the polymer and plasticizer, and film or coating rigidity (Guilbert et al. 1996; Seow et al. 1999). Moreover, adding a low concentration of plasticizer can lead to an increase in polymer crystallinity, due to lowering the energy barrier for a change of polymer state (Lourdin et al. 1997). Antiplasticization increases the brittleness of chitosan edible films and coatings, which is the opposite function to plasticization. Therefore, plasticizers must be used in the correct amount to obtain the advantage of enhancing the films and coatings properties. Plasticizers are generally required at approximately 10 % to 60 % on dry basis, depending on the stiffness of the polymer (Chang et al. 2000; Guilbert et al. 2002).

Several theories have been proposed to explain the mechanisms of plasticization action. The earliest is the free volume theory, which involves the intermolecular spaces in polymer. The lubricant theory postulates that plasticizers, by interspersing themselves, act as internal lubricants by reducing frictional forces between polymer chains. The gel theory states that the rigidity of polymer comes from three-dimensional structures, and plasticizers take effect by breaking polymer-polymer interaction. For edible films and coatings, the most useful concepts are the gel theory and the free volume theory (Suyatma et al. 2005; Krochta and Sothornvit 2005).

The permanence of plasticizers in polymers depends on the size of the plasticizer molecule and on the rate of diffusion in polymers. Larger plasticizer molecules possess lower volatility, resulting in greater permanence. Moreover, polarity and hydrogen bond capability will influence the volatility of plasticizers (Park et al. 2002). Commonly used plasticizers in coating and film systems are monosaccharides, disaccharides or oligosaccharides, lipids and their derivates, which are phospholipids, fatty acids, surfactant, and polyols (glycerol, sorbitol, xylitol, and polyethylene glycol), (Baker et al. 1994; Baldwin 1999; Krochta and Sothornvit 2005). The chemical structure and molecular weight (M_w) of glycerol, xylitol, and sorbitol are presented in Table 1.5.

Table 1.5: Chemical structures and molecular weights of glycerol, xylitol, and sorbitol (Krochta and Sothornvit 2005)

Plasticizer	Chemical structure	Molecular weight (M_w)
Glycerol		92
Xylitol		152
Sorbitol		182

Ideal plasticizers are miscible and compatible in all proportions with plastic components, and they may be added to polymers in solution (dispersion technique) or after solvents have been removed (absorption technique). Commonly used plasticizers in coating and film systems are monosaccharides, disaccharides or oligosaccharides, polyols (glycerol, sorbitol, xylitol, and polyethylene glycol), lipids and their derivates, which are phospholipids, fatty acids, and surfactants (Baker et al. 1994; Baldwin 1999; Krochta and Sothornvit 2005).

In addition, plasticizers should have low volatility, as well as being non-toxic and aromas free (Suyatma et al. 2005).

The selection of plasticizers requires consideration of three basic criteria, namely compatibility, efficiency, and permanence. It is necessary to use plasticizer that is compatible with the intended polymer, in this case, chitosan. Compatibility depends on polarity, structural configuration (shape), and molecular weight (size) of plasticizer. Good compatibility results from the plasticizer and polymer having a similar chemical structure. Therefore, different polymers require different plasticizers. Generally, good plasticizers provide high plasticization at low concentration and exhibit rapid polymer diffusion and interaction. The plasticizer efficiency is defined as the quantity of plasticizer required to produce the desired film or coating mechanical properties. One method to define the efficiency is the lowering of the glass transition temperature at a given amount or volume fraction of plasticizer. There is no exact number to indicate the efficiency of each plasticizer, because it depends on the polymer properties (Krochta and Sothornvit 2005).

1.3.2.2 Edible coatings of chitosan enforced with garlic extract

Recently, due to the increasing demand for natural food additives, more extensive efforts are being made in research for alternative traditional and natural antimicrobial

agents such as spice extracts (Holley and Patel 2005; Hwang et al. 2009). In the field of edible coatings and films, incorporation of “secondary preservatives” such as spice extracts into coating solution or film matrix is one of the most challenging technologies for food preservatives industry and pharmaceutical industry (Fazilah et al. 2008; Dutta et al. 2009).

Allium is the largest and most important representative genus of the *Alliaceae* family and comprises 450 species, widely distributed in the northern hemisphere. Garlic (*Allium sativum*) is mainly composed of sulfur-containing compound such as allicin, diallyl disulfide, and diallyl trisulfide that possesses better antimicrobial activity than the corresponding basic form (Nychas 1995). The biological effects of garlic are related to its thiosulfinates and volatile sulfur compounds, which are also responsible of their characteristic pungent aroma and taste. Moreover, garlic possesses antioxidant properties (Nychas 1995; Miron et al. 2000).

The use of garlic in various forms of products such as powder, oil, and aqueous extracts is gaining attention worldwide, however garlic extracts have been found effective against a wide range of foodborne microorganisms (Oparaeke et al. 2007). Furthermore, in food applications, the use of garlic with both antioxidants and antimicrobial activities may be useful to maintain the quality, extend shelf life and prevent economic loss of foods (Yin and Cheng 2003; Hwang et al. 2009).

The mode of the antimicrobial action of garlic extract to strongly inhibit the growth of bacteria is due to its main active agent, allicin, which is able to penetrate cell membranes. This is caused possibly by the feature of the bacteria cell envelope that may influence the access to periplasmic and cytoplasmic enzymes (Miron et al. 2000; Douglas and Bakri 2005). In addition, antimicrobial activity of garlic is stronger against gram-negative bacteria, which are mainly derived from the family

Enterobacteriaceae, due to a greater sensitivity of species of *Enterobacteriaceae* to allicin (Lanzotti 2006; Corzo-Martínez et al. 2007).

Due to its antimicrobial activity, garlic can synergistically strengthen the antimicrobial activity of chitosan coating to inhibit the growth of spoilage microorganisms in foods. Thus, chitosan with its positively charged amino group can more easily interact with negatively charged microbial cell membranes, leading to the leakage of proteinaceous and other intracellular constituents of the microorganisms, mainly gram-negative bacteria (Shahidi et al. 1999).

The biosynthetic pathway of thiosulfinates in garlic is presented in Figure 1.6, in which alliin is converted to allicin, so that garlic shows an antimicrobial effect.

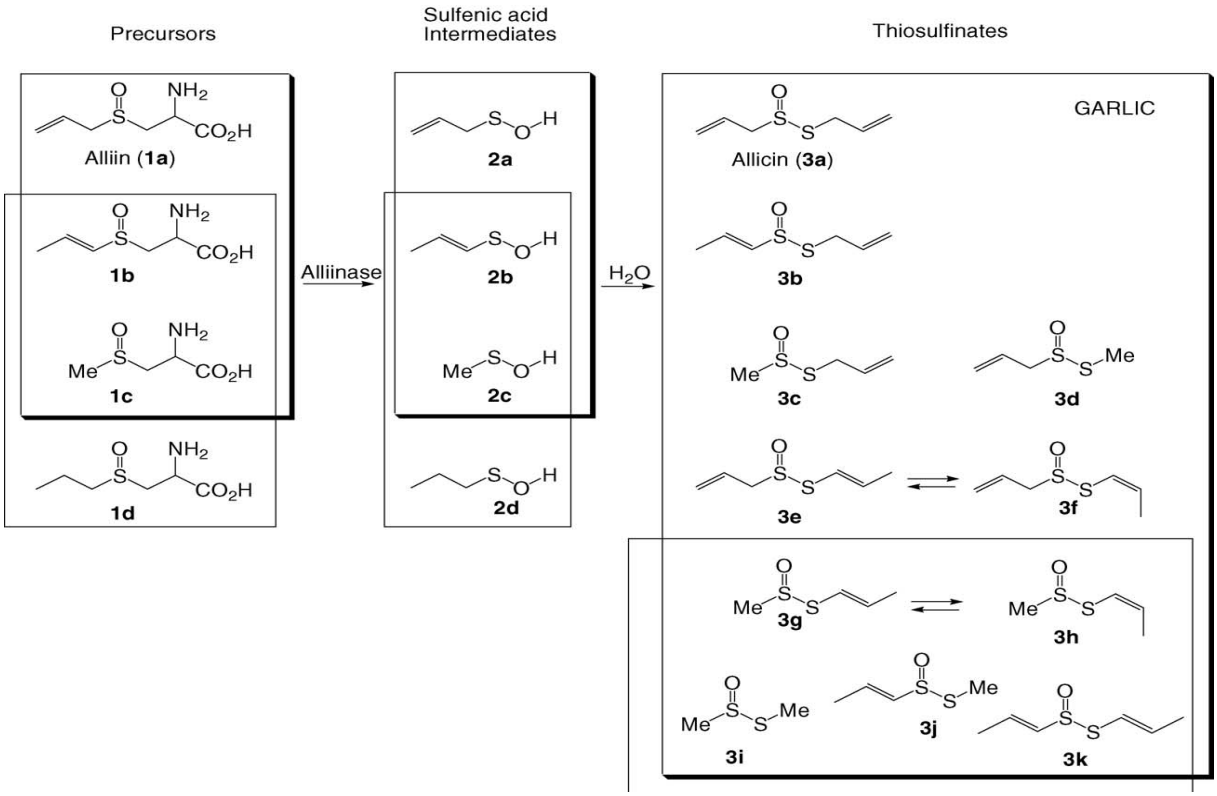


Figure 1.6: Biosynthetic pathway of thiosulfinates in garlic (Lanzotti 2006)

1.3.3 Edible coatings of chitosan for food preservation

Chitosan is one of the most common biopolymers that can be used in the formation of coatings or films and offers a wide range of unique applications in the food industry, including preservation of foods from microbial deterioration and extension of their shelf life (Guilbert et al. 1996; Vargas et al. 2008).

The use of edible coatings of chitosan to extend shelf life and improve the quality of both fresh and frozen foods has been examined during the past few years (Kester and Fennema 1986; Labuza and Breene 1989; Durango et al. 2006) due to their non-toxicity, eco-friendly, and biodegradable nature (Hoagland and Parris 1996; Kittur et al. 1998).

The mechanisms of chitosan edible coatings in extending shelf life of foods are influenced by the properties of chitosan. These include controlled moisture transfer between food and surrounding environment, controlled release of chemical agents like antimicrobial substances, antioxidants, reduction of oxygen partial pressure in the package that results in a decreased rate of metabolism, controlled rate of respiration, high impermeability to certain substances like fats and oils, temperature control, structural reinforcement of food and coat flavor compounds and leavening agents in the form of microcapsules (Sebti and Coma 2002; Vargas et al. 2008).

Furthermore, in detail, the outer layers of chitosan edible coatings provide supplementary essential properties for controlling physiological, morphological and physicochemical changes in foods (Kittur et al. 1998; Vargas et al. 2008). For example, it can protect against food borne microorganisms, thus, the shelf life of fresh foods can be extended. Besides that, edible coatings of chitosan possess moderate water permeability which is useful for retarding of moisture loss in foods so that the freshness of foods could be maintained (Guilbert et al. 1996). Moreover, due to the ability to reduce lipid oxidation and discoloration, edible coatings of chitosan

can retain shelf life of fresh foods with high water activity during storage at low temperature. In addition, appearance of food products in retail packages can be enhanced by edible coatings of chitosan. Similarly, during frying of breaded food products, edible coatings of chitosan can reduce oil uptake; thus, the appearance of fried breaded food products may be enhanced (Guilbert 2000; Guilbert et al. 2002).

1.4 Aim of the work

Recently, the use of natural preservatives in food application becomes very popular and is greatly promising, particularly for food industry due to growing consumer awareness regarding to synthetic preservatives. Chitosan as a natural biopolymer is qualified as a potential food preservative due to its antimicrobial activity against a wide range of foodborne pathogenic bacteria and spoilage microorganisms (Sagoo et al. 2002), where the antibacterial activity of chitosan depends on its physicochemical properties namely degree of deacetylation and molecular mass (No et al. 2002). Chitosan generally possesses a stronger antimicrobial activity against gram-positive bacteria than against gram-negative bacteria (Sudarshan et al. 1992). To strengthen the antimicrobial activity of chitosan against gram-negative bacteria, another antimicrobial agent as a “secondary preservative” such as plant extracts may be added into chitosan as a coating solution (Dutta et al. 2009). Currently, increasing attention has been paid to develop and test edible coatings or films with antimicrobial properties in order to improve quality and shelf life of foods (Pranoto et al. 2005). However, at the present time, little is known about the efficacy of the incorporating method into coatings or films based on chitosan for controlling of microbial growth and extension of shelf life of shrimp that is very easy to spoil during storage.

The first purpose of the present work is to study the potency of chitosan to inhibit seafood spoilage bacteria namely gram-positive bacteria (*Staphylococcus aureus,*

Listeria monocytogenes) and gram-negative bacteria (*Escherichia coli, Salmonella typhimurium*). Beside that, the influence of the physical characteristics of chitosan such as molecular mass (M_η) and degree of deacetylation (DDA) is observed.

The second purpose is to check the efficacy of chitosan as a potential coating for shrimp meat preservation. In order to determine the potency of chitosan coating to improve quality and extend shelf life of shrimp meat during storage, changes in the microbiological parameters (growth of total aerobic mesophilic bacteria, growth of gram-positive bacteria as well as growth of gram-negative bacteria) and changes in the biochemical parameters (pH value, content of total volatile basic nitrogen, water activity, and content of biogenic amines) are monitored. Furthermore, chitosan is enforced with garlic extract in order to achieve an improved function of chitosan edible coatings for shrimp meat preservation.

2 Materials and Methods

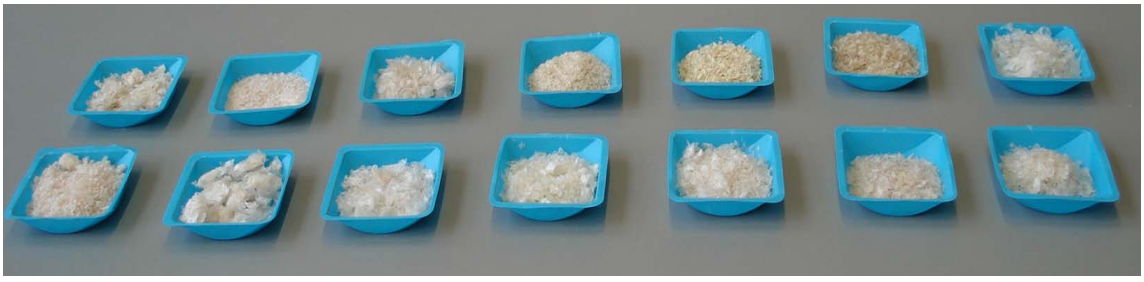
2.1 Preparation of testing materials

2.1.1 Chitosan charges

2.1.1.1 Production of chitosan charges

Chitosan charges of different chain lengths were delivered by Seehof Laboratorium GmbH (SeeLab, Wesselburen, Germany) and Cognis GmbH (Düsseldorf, Germany). The chitosan charges of SeeLab GmbH are produced from shells of the shrimp species *Crangon crangon* (Figure 2.1 A), whereas the chitosan charges of Cognis GmbH are manufactured from the species *Pandalus borealis* shells (Figure 2.1 B).

A)



B)

Figure 2.1: Chitosan charges of **(A)** SeeLab GmbH and **(B)** Cognis GmbH

2.1.1.2 Treatment and storage of chitosan charges

Each chitosan charge was packed in sealed transparent plastic bags. In order to identify the charges of chitosan precisely, internal codifications such as SN for the charges of SeeLab and CN for the charges of Cognis were used. Each plastic bag containing a chitosan charge was labelled with an internal code followed by a serial number. Afterwards, each chitosan charge in a plastic bag was stored at refrigerator temperature of 4–6 °C before used. The tested chitosan charges are listed in Table 2.1.

Table 2.1: Chitosan charges with different chain lengths

Producer	Internal codification	Various charges
SeeLab GmbH	SN	SN 8
		SN 10
		SN 12
		SN 14
		SN 17
		SN 18
		SN 19
		SN 20
		SN 21
		SN 22
		SN 25
		SN 26
		SN 27
Cognis GmbH	CN	CN 2
		CN 4
		CN L
		CN S (old charge)
		CN S (new charge)

2.1.1.3 Preparation of chitosan stock solutions

To obtain the stock solution of chitosan charge at a concentration of 1 % (w/v), 10 g of each chitosan charge were dissolved in 1000 mL of 1 % (v/v) acetic acid (Merck, Darmstadt, Germany) and mixed by stirring at 40 °C for 2 hours. Finally, the chitosan stock solution was filtered through a sterilized 0.45 µm microporous membrane

(Sartorius, Goettingen, Germany) by using a vacuum pump (KnF Neuberger, Otto Steiner, Hamburg, Germany) to remove any undissolved particles.

2.1.2 Garlic extract

2.1.2.1 Purchase and storage of garlic samples

The most common spice for seafood preservative in Indonesia is garlic (*Allium sativum*). For the present study, it was purchased from the Indonesian grocery (Steindamm, Hamburg, Germany).

After arriving in the laboratory, fresh garlic was immediately used to obtain its optimal efficacy. The rest thereof was stored at room temperature for the next using.

2.1.2.2 Preparation of garlic extract stock solution

1 g of husked garlic was strongly crushed with a pestle and mortar, and then allowed to stand for 10 min to produce allicin that possesses antimicrobial activity, enzymatically out of alliin. Then, 99 mL of sterile 0.9 % (w/v) sodium chloride solution (Merck, Darmstadt, Germany) were added and the mixture was blended to a homogenous state using a laboratory stomacher (type BA 7021, Seward Medical, London, U.K.) for 2 min. A second homogenization step was performed using an Ultra-Turrax (type 25, Janke & Kunkel, IKA-Labortechnik, Staufen, Germany) at speed 4000 rpm for 1-2 min. The homogenate was afterwards filtered through a 0.45 µm sterile microporous membrane (Sartorius, Goettingen, Germany) by using a vacuum pump (KnF Neuberger, Otto Steiner, Hamburg, Germany) to get the extract. The stock solution of garlic extract with a concentration of 1 % (w/v) was then kept in a sterilized flask and stored at temperature of 2-6 °C.

2.1.3 Shrimp meat samples

2.1.3.1 Purchase and handling of shrimps (*C. crangon*)

Shrimps (*C. crangon*) were purchased from the local fish market (Altona, Hamburg, Germany) and delivered by using a cold box to the laboratory of Division of Food Microbiology and Biotechnology of University of Hamburg (Hamburg, Germany).

2.1.3.2 Treatment and preparation of shrimp meat samples

In the laboratory, shrimps were peeled, their heads were removed, and subsequently they were washed with distilled water, then immediately drained to remove all the water upon them. Shrimp meat was promptly soaked in the chitosan coating solution for research needs.

2.2 Assays for antimicrobial activity

2.2.1 Microorganisms

Four strains of bacteria causing spoilage in seafood were tested for antibacterial activity of each chitosan charge. These consist of two gram-negative bacteria (*Escherichia coli* LMH 1N and *Salmonella typhimurium* LMH 2N) and two gram-positive bacteria (*Listeria monocytogenes* LMH 34P 10587 and *Staphylococcus aureus* LMH 5P). The four strains (Table 2.2) were taken from the laboratory culture collections of Division of Food Microbiology and Biotechnology (Institute of Food Chemistry, Department of Chemistry, University of Hamburg, Germany).

Table 2.2: Internal codification of tested strains

Strain	Group of bacteria	Internal codification
Escherichia coli	Gram-negative	LMH 1N
Salmonella typhimurium	Gram-negative	LMH 2N
Listeria monocytogenes	Gram-positive	LMH 34P
Staphylococcus aureus	Gram-positive	LMH 5P

2.2.2 Culture conditions

2.2.2.1 Cultivation of strains on Standard 1 agar

The strains of *E. coli, S. typhimurium*, *L*. *monocytogenes,* and *S. aureus* were primarily stored on Standard 1 agar slant (Merck, Darmstadt, Germany) and stored at 4 °C before using.

2.2.2.2 Inoculation of cultures in Mueller-Hinton broth

Fresh subculture was monthly carried out to maintain bacterial viability. For the preparation of seeding cultures for antimicrobial activity tests of chitosan charges as well as garlic extract, a loopful of each bacterial inoculum from Standard 1 agar slant (Merck, Darmstadt, Germany) was taken and inoculated into 10 mL of Mueller Hinton broth (MHB, Merck, Darmstadt, Germany). The suspension was then incubated at 37 °C for 24 h. After reaching a cell titre of 10^7 CFU/mL, it was used as inoculum for antimicrobial activity assay.

2.2.2.3 Determination of viable bacterial count on plate count agar

0.1 mL of incubated MHB suspension of each strain was diluted 10-fold serially up to 10^{-5} in 0.9 % (w/v) sterile sodium chloride solutions (Merck, Darmstadt, Germany). Viable cells of each strain were enumerated by spread plating of 0.1 mL aliquot of the dilution series onto the surface of PCA (Merck, Darmstadt, Germany). The plates

were triplicated and incubated at 37 °C for 24–48 h in an incubator (Type B 5060 E, Heraeus, Hanau, Germany). The grown colonies of each strain were afterwards counted and presented as log CFU/mL.

2.2.3 Preparation of antimicrobial solutions and inoculation of strains in the solutions

2.2.3.1 Preparation of chitosan solution in MHB

An appropriate volume of each chitosan charge stock solution at a concentration of 1.0 % (w/v) was aseptically diluted in sterilized MHB (Merck, Darmstadt, Germany) to get final concentrations of 0.002–0.20 % (w/v), followed by stirring to homogenize.

2.2.3.2 Preparation of garlic extract in MHB

An appropriate volume of 1.0 % (w/v) garlic extract stock solution was aseptically diluted in sterilized MHB (Merck, Darmstadt, Germany) to get final concentrations of 0.002–0.20 % (w/v), followed by stirring to homogenize garlic extract-MHB mixture. The garlic extract-MHB solutions could be used within seven days of storage at refrigerator temperature (4–6 °C).

2.2.3.3 Preparation of chitosan-garlic extract solution in MHB

1 mL garlic extract at a concentration of 0.1 % (w/v) was added into sterile chitosan SN 22 solution to obtain chitosan-garlic extract (Ch-G) stock solution at a concentration of 0.1 % (w/v). The mixture was then stirred at 40–60 °C for 2 h until completely dispersed. The chitosan-garlic extract solution was finally filtered through a sterile 0.45 µm microporous membrane (Sartorius, Goettingen, Germany) to remove any undissolved particles by using a vacuum pump (KnF Neuberger, Otto

Steiner, Hamburg, Germany) at room temperature. This stock solution was placed in a sterile flask and could be used within seven days at storage temperature of 2–6 °C. The chitosan-garlic extract solution in MHB was prepared as follows, an appropriate volume of 1.0 % (v/v) chitosan-garlic extract stock solution was aseptically diluted in sterilized MHB (Merck, Darmstadt, Germany) to get final concentrations of 0.002–0.20 % (w/v), followed by vigorously stirring to homogenize each chitosan-garlic extract-MHB solution.

2.2.3.4 Inoculation of strains in the chitosan-MHB solutions

1.0 mL of each strain with a cell titre of 10^7 colony forming unit (CFU/mL) was inoculated into 10 mL of chitosan-MHB solution at chitosan-concentrations of 0.002–0.20 %. The pH of each solution was adjusted at various values of 4.5–6.5 by using 1.0 N HCl or NaOH (Merck, Darmstadt, Germany) to study the effect of pH on antimicrobial activity. Then each strain suspension was incubated at 37 °C for 24–48 h with shaking at 100 rpm by rotary shaker (Gyrotory Model G 76, New Brunswick Scientific, Edison, USA). These inoculated strains in chitosan-MHB solution were used to measure the antimicrobial activity and minimum inhibitory concentration (MIC) of each chitosan charge.

2.2.3.5 Inoculation of strains in the chitosan-garlic extract-MHB solutions

1.0 mL of each strain at cell titre of 10^7 CFU/mL was inoculated into 10 mL of each sterilized garlic extract-chitosan-MHB solution in concentration range of 0.002–0.20 % (w/v) and the pH of each suspension was adjusted to 5.5. Each strain suspension was incubated at 37 °C for 24–48 h with shaking at 100 rpm using a rotary shaker (Gyrotory Model G 76, New Brunswick Scientific, Edison, USA). These inoculated strains in the garlic extract-chitosan-MHB solution were used to measure

the antimicrobial activity and the MIC of each combined chitosan-garlic extract solution.

2.2.4 Study the influence of pH on antimicrobial activity

1.0 mL of cell suspension containing 10^7 CFU/mL of each strain was inoculated into 10 mL of chitosan-MHB solution at chitosan-concentrations of 0.002–0.20 %. The pH of each solution was adjusted using 1.0 N hydrochloric acid solution (Merck, Darmstadt, Germany) at various values which were 4.5–6.5 using pH meter TA 10 plus (Schott, Mainz, Germany). In the following, each cell suspension was incubated at 37 °C for 24–48 h with shaking at 100 rpm by rotary shaker (Gyrotory Model G 76, New Brunswick Scientific, Edison, USA). Then the dilution series (10^{-1}–10^{-5}) of each cell suspension were prepared in 0.9 % (w/v) sterile sodium chloride solution (Merck, Darmstadt, Germany). The cell count of each strain at each pH value was then determined by spread plating of 0.1 mL aliquot of the appropriate dilutions series onto the surface of agar plates (PCA, Merck, Darmstadt, Germany). The plates were triplicated and incubated at 37 °C for 48 h at incubator (Type B 5060 E, Heraeus, Hanau, Germany). The influence of pH on antimicrobial activity of each chitosan charge was indicated by differences of the cell count of control and tested strain at each pH value.

2.2.5 Determination of antimicrobial activity

2.2.5.1 Determination of antimicrobial activity of chitosan

Each inoculated strain in the chitosan-MHB solution that has been incubated at 37 °C for 24–48 h was diluted 10-fold serially to 10^{-5} in 0.9 % (w/v) sterile sodium chloride solution (Merck, Darmstadt, Germany). Viable cells of each strain were enumerated

by spread plating of 1 mL of the dilutions series of the chitosan-MHB solution over the surface of agar plates (PCA, Merck, Darmstadt, Germany) and followed by incubation at 37 °C for 48 h in the incubator (type B 5060 E, Heraeus, Hanau, Germany). The grown colonies of each tested strain were then counted and compared with the control group (the strain inoculated in MHB without chitosan) and the acetic acid group, in which each chitosan charge in MHB was replaced with 1.0 % (v/v) sterile acetic acid (Merck, Darmstadt, Germany). The antimicrobial activity of each chitosan charge is indicated by the amount of viable cells (CFU/mL) of the test strains, which could still survive in the presence of the chitosan charge tested. Triplicate estimations of the enumeration of viable count were carried out to ensure the reproducibility of the results.

2.2.5.2 Determination of antimicrobial activity of garlic extract

Antimicrobial activity of garlic extract was determined by the viable cell count method. Each inoculated strain in the garlic extract-MHB solution that has been incubated at 37 °C for 24–48 h was diluted 10-fold serially to 10^{-5} in 0.9 % (w/v) sterile sodium chloride solutions (Merck, Darmstadt, Germany). Viable cells of each strain were determined by spreading of 0.1 mL aliquot of the appropriate dilution series onto the surface of agar plates (PCA, Merck, Darmstadt, Germany). The plates were triplicated and incubated at 37 °C for 48 h in the incubator (Type B 5060 E, Heraeus, Hanau, Germany). The grown colonies were then counted and compared with that of the control in which garlic extract was replaced with 0.9 % (w/v) sterile sodium chloride solution (Merck, Darmstadt, Germany). The antimicrobial activity of garlic extract solution was determined by the differences of the cell count (CFU/mL) of the controls and the garlic extract samples after incubation. Triplicate estimations of the

enumeration of viable count were carried out to ensure the reproducibility of the results.

2.2.5.3 Determination of antimicrobial activity of chitosan-garlic extract solution

Antimicrobial activity of each chitosan-garlic extract (Ch-G) solution with different concentrations of 0.002–0.20 % (w/v) was determined by the viable cell count method. Each inoculated strain in the chitosan-garlic extract-MHB solution that has been incubated at 37 °C for 24-48 h was diluted 10-fold serially to 10^{-5} in 0.9 % (w/v) sterile sodium chloride solutions (Merck, Darmstadt, Germany). Viable cells of each strain were enumerated by spread plating of 0.1 mL aliquot of the appropriate dilution series onto the surface of agar plates (PCA, Merck, Darmstadt, Germany). The plates were triplicated and incubated at 37 °C for 48 h in the incubator (type B 5060 E, Heraeus, Hanau, Germany). The grown colonies on PCA of Ch-G samples were then counted and compared with the cell count of the control, in which Ch-G solution was replaced with 0.9 % (w/v) sterile sodium chloride solution (Merck, Darmstadt, Germany). The antimicrobial activity of each Ch-G solution is indicated by the differences of the amount of viable cells of controls and the Ch-G samples after incubation. Triplicate estimations of the enumeration of viable count were carried out to ensure the reproducibility of the results.

2.2.6 Determination of the minimum inhibitory concentration (MIC)

2.2.6.1 Determination of the MIC of chitosan

Each chitosan charge solution was added directly into sterile liquid agar (PCA, Merck, Darmstadt, Germany) to get final concentrations of 0.002–0.20 % (w/v) and its pH

was adjusted to 5.5. Each chitosan charge-PCA suspension was poured into each Petri dish and afterwards allowed to stand overnight until all the plates became solid. Viable cells of each strain were enumerated by spread plating of 0.1 mL aliquot of cell suspension dilution series (10^{-1}–10^{-5}) onto the surface of chitosan-agar (PCA, Merck, Darmstadt, Germany). The plates were triplicated and incubated at 37 °C for 72 h in the incubator (type B 5060 E, Heraeus, Hanau, Germany). The grown colonies were then counted and compared with the control. The MIC of each chitosan charge was determined as the lowest concentration of each chitosan charge solution required to inhibit bacterial growth after incubation at 37 °C for 72 h completely (Andrews 2001).

2.2.6.2 Determination of the MIC of chitosan-garlic extract

The MIC of chitosan solution enhanced with garlic extract (Ch-G) was determined by the viable count method. Each concentration of (Ch-G) solution was added directly into sterile liquid plate count agar (PCA, Merck, Darmstadt, Germany) for final concentrations of 0.002–0.20 % (w/v) and its pH was adjusted to 5.5. This (Ch-G)-agar suspension was poured into each Petri dish and afterwards allowed to stand overnight until all the plates became solid. Viable cells of each strain were enumerated by spread plating of 0.1 mL aliquot of the appropriate dilution series (10^{-2}–10^{-5}) onto the surface of garlic extract-chitosan-agar (PCA, Merck, Darmstadt, Germany). The plates were triplicated and incubated at 37 °C for 72 h in the incubator (Type B 5060 E, Heraeus, Hanau, Germany). The grown colonies were then counted and compared with the control. The MIC of each (Ch-G) solution was determined as the lowest concentration of each (Ch-G) solution required to inhibit bacterial growth after incubation at 37 °C for 72 h completely (Andrews 2001).

2.3 Study the potency of chitosan for shrimp meat preservation

2.3.1 Treatment of shrimp meat samples with chitosan SN 22

The potency of chitosan SN 22 solution was tested for shrimp meat preservation. Previously, chitosan solution had to be plasticized in order to prevent the brittleness, so that chitosan solution can optimally coat the shrimp meat samples during storage. For this purpose, polyol plasticizers such as xylitol, sorbitol, and glycerol were examined to enhance chitosan coating solution.

2.3.1.1 Testing several plasticizers to enhance chitosan coating solution

100 mL of 0.1 % (w/v) chitosan SN 22 solution was added with 1 mL of 10 % (w/v) food grade xylitol, sorbitol, and glycerol (Merck, Darmstadt, Germany) and then mixed by vigorously stirring at 40–50 °C for 1 h until each plasticizer was completely dispersed. The plasticized suspension was then degassed using ultrasonic bath (Sonorex super RK 102 H, Bandelin Electronic, Berlin, Germany) for 15 min. The solution was finally filtered through a 0.45 µm sterile microporous membrane (Sartorius, Goettingen, Germany) to remove any undissolved particles. Thereafter, the plasticized film formed out of chitosan SN 22 was poured into sterile glass plates (diameter 9 cm, Karl-Roth, Karlsruhe, Germany) at a thickness of about 1 mm. The plates were then dried at 60–80 °C in the oven (Type B 5060 E Heraeus, Hanau, Germany) and then conditioned in a forced air oven (Cytoperm Heraeus 8080, Kendro Laboratory Product, Germany) at 50 % relative humidity (RH) and 23 °C for 3–5 weeks before using. The obtained films were carefully peeled from the plates and used to determine the moisture content of chitosan film (see section 2.5.5.1).

2.3.1.2 Coating of shrimp meat samples with chitosan SN 22 solution enhanced with plasticizer

The best plasticizer used to enhance chitosan SN 22 solution was chosen after getting the results of the tests described in section 2.3.1.1. Each 10 g of shrimp meat sample was soaked in the 0.1 % (w/v) plasticized chitosan SN 22 solution for 1 h and then allowed to drip dry for 2 min. Shrimp meat of the control group was left untreated, except that shrimp meat was soaked in 1 % (v/v) acetic acid solution to which chitosan had not been added. The coated shrimp meat samples were then placed in Petri dishes and dried at 40 °C for 2 h in a forced air oven (Cytoperm Heraeus 8080, Kendro Laboratory Product, Germany) in order to form the chitosan coatings. The same protocol was used for the control. The Petri dishes containing coated shrimp meat samples were covered with Parafilm and then stored at refrigerator temperature (at 4–6 °C) and room temperature (22–23 °C) for up to 14 days for subsequent quality assessments, namely microbiological and biochemical analysis which were performed every 24 h (see section 2.5.3 and 2.5.4).

2.3.2 Treatment of shrimp meat samples with chitosan-garlic extract solution enhanced with plasticizer

Each 25 g of shrimp meat was soaked in the 0.1 % (v/v) plasticized chitosan-garlic extract (Ch-G) solution for 1 h in a cold room (at 4–6 °C). Shrimp meat samples of the control group were soaked in 1.0 % (w/v) acetic acid solution without chitosan. Both the control and coated shrimp meat samples were dried at 40 °C for 2 h in a forced air oven (Cytoperm Heraeus 8080, Kendro Laboratory Product, Germany) in order to form the edible coatings. Coated shrimp meat samples were then stored at refrigerator temperature (at 4–6 °C) and room temperature (22–23 °C) for up to 30

days for subsequent quality assessments, namely microbiological and biochemical analyses (see section 2.5.3 and 2.5.4).

2.4 Study the influence of plasticizer on edible film of chitosan-garlic extract

2.4.1 Preparation of chitosan-garlic extract film enhanced with plasticizer

100 mL of 0.1 % (v/v) chitosan-garlic extract (Ch-G) solution was enhanced with 1 mL of 10 % (w/w) food-grade glycerol (Merck, Darmstadt, Germany) and then mixed by vigorously stirring at 40–50 °C for 1 h until the glycerol was completely dispersed. The plasticized (Ch-G) solution was then degassed using ultrasonic bath (Sonorex super RK 102 H, Bandelin Electronic, Berlin, Germany) for 15 min. The solution was finally filtered through a 0.45 µm sterile microporous membrane (Sartorius, Goettingen, Germany) to remove any undissolved particles. The plasticized film forming (Ch-G) solution was casted onto sterile glass plates (diameter 9 cm, Karl-Roth, Karlsruhe, Germany) at a thickness of about 1 mm. The plates were then dried at 60–80 °C in the oven (Type B 5060 E Heraeus, Hanau, Germany) and then conditioned in a forced air oven (Cytoperm Heraeus 8080, Kendro Laboratory Product, Germany) at 50 % relative humidity (RH) and 23 °C for 3–5 weeks before using. The obtained films were carefully peeled from the plates and used for determination of the plasticizer effect on the microstructure of (Ch-G) film using scanning electron microscopy (see section 2.5.5.2).

2.5 Analytic Assays

2.5.1 Physical analysis of chitosan

2.5.1.1 Determination of intrinsic viscosity

The measurement was performed by means of an Ubbelohde capillary viscometer (type Ic, Schott, Hofheim, Germany) with a shear rate ranging from 500 to 1500 s^{-1} at a temperature of 25 °C. 0.1 % (w/v) stock solutions of the chitosan charges to be investigated were prepared by 12 h shaking and subsequent filtration using a nylon filter with a pore size of 20 µm. From stock solutions, the desired concentration series were prepared by dilution with the corresponding solvents (0.5 M acetic acid/0.2 M sodium acetate buffer).

2.5.1.2 Determination of molecular mass

Light scattering measurements were performed using a coupled system of various apparatuses consisting of size exclusion chromatography (SEC), a multi-angle laser light scattering photometer (MALLS, Dawn DSP, Wyatt Technology Corp., Santa Barbara, CA, USA; λ=633 nm) and a refraction detector (dRI, Shodex RI-71, Showa Denko, Tokyo, Japan) at 25 °C (Kulicke and Böse 1984; Kulicke et al. 1993; Klein et al. 1998, Kulicke et al. 1999). Chitosan samples were dissolved in an eluent (0.1 % (v/v) CF_3COOH + 0.1 mol/L NaCl) by shaking for 12 h and 1 mg/mL polymer solutions were filtered using a 0.45 µm membrane filter (Sartorius, Goettingen, Germany). The mixed eluent of 0.1 % (v/v) CF_3COOH and 0.1 mol/L NaCl was selected in such a way that a high signal to noise ratio was obtained. Separation was carried out at a flow rate of 0.5 mL/min with an injected sample volume of 75 µL via three columns (type NOVEMA 3000 Å/1000 Å/300 Å, particle size 12 µm, Polymer

Standards Service, Mainz, Germany). Recording and evaluation of light scattering data was conducted by means of the Astra 5 software (Wyatt Technology Corp, Santa Barbara, CA, USA) using a refractive index increment of dn/dc = 0.185 mL/g (Schatz et al. 2003).

2.5.2 Determination of degree of deacetylation (DDA)

2.5.2.1 ^{1}H NMR spectroscopy

10 mg of each non-degraded chitosan charge was dissolved in 0.8 mL of D_2O (in reference: TMSP-d_4 = 3-trymethylsilyl 3,3,2,2 tetradeuteropropionic acid, Na salt) and one drop of DCl overnight. The solution was transferred to an NMR test tube (d = 5 mm).

2.5.2.2 ^{13}C NMR spectroscopy

200 mg of each degraded chitosan charge was dissolved in 5 mL of D_2O (in TMSP-d_4) and two drops of DCl overnight. The solution was transferred to an NMR test tube (d = 10 mm). In order to be able to take ^{13}C NMR spectra with a high enough resolution for determination, chitosan solutions had to be degraded by ultrasonic prior to this for approximately 4 h (Schittenhelm and Kulicke 2000). For this purpose, 1 % (w/v) acetic acid was used as a solvent. Ultrasonic degradation is the only method that allows a selective degradation of polymer molecules in the centre of chains without chemical side reactions, elimination of side groups or formation of monomers and oligomers (Kulicke et al. 1993). All NMR measurements were carried out at a measuring temperature of 70 °C with a relaxation time of 5 seconds, the number of scans being 512 (^{1}H NMR) and 10240 (^{13}C NMR), respectively.

2.5.3 Microbiological analysis of shrimp meat samples

2.5.3.1 Determination of total viable count of aerobic mesophilic bacteria

10 g of the shrimp meat samples coated with chitosan SN 22 were put aseptically into sterile plastic bags (Seward Medical Stomacher, London, U.K.) and homogenized in 90 mL sterile 0.9 % (w/v) NaCl solution (Merck, Darmstadt, Germany). The homogenization of the samples was performed using a laboratory stomacher (type BA 7021, Seward Medical, London, U.K.) for 2 min. A second homogenization step was performed using an Ultra-Turrax (type 25, Janke & Kunkel, IKA-Labortechnik, Staufen, Germany) at speed 4000 rpm for 1-2 min. To determine the total cell count in the shrimp meat samples coated with (Ch-G) solution, 25 g of these samples were homogenized in 225 mL sterile 0.9 % (w/v) NaCl solution (Merck, Darmstadt, Germany). The same procedure was followed as mentioned above.

Total cell count in the shrimp meat samples was enumerated by spread plating of 0.1 mL of dilution series (10^{-1}–10^{-5}) of inoculum onto surface of sterile plate agar (PCA, Merck, Darmstadt, Germany). The plates were triplicated and incubated at 37 °C for 24–72 h. The colonies grown on the plates were counted using the colony counter, and total cell count of aerobic mesophilic bacteria were indicated as colony forming unit per gram of shrimp meat (CFU/g).

2.5.3.2 Determination of growth of gram-positive and gram-negative bacteria

Prior the determinations of growth of the test gram-negative and gram-positive strains by enumerating of their grown colonies, several analytic tests for identification and characterization of the colony of each tested strain were conducted, according to the scheme in Figure 2.2.

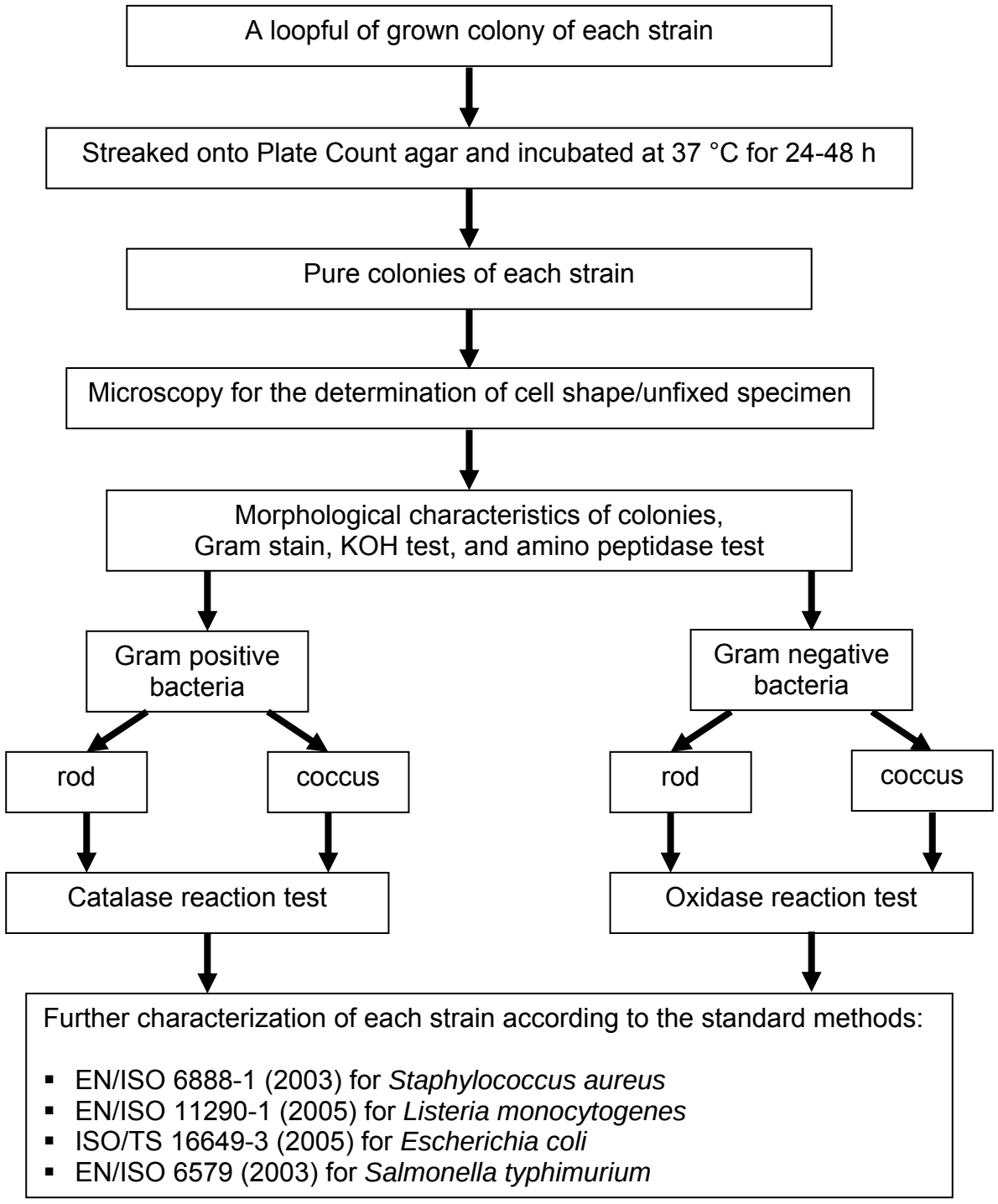


Figure 2.2: Scheme for identification and characterization of bacteria

2.5.3.2.1 Determination of growth of *Staphylococcus aureus*

A 0.1 mL aliquot of *S. aureus* LMH 5P at a cell titre of 10^2 CFU/mL was supplemented into every 10 g of coated shrimp meat samples before storage at refrigerator temperature and room temperature. The finished stored coated shrimp

meat samples were homogenized in 90 mL sterile alkaline saline peptone water using a laboratory stomacher (type BA 7021, Seward Medical London, U.K.) for 2 min and continued by using an Ultra-Turrax (type 25, Janke & Kunkel, Type 18/10, IKA-Labortechnik, Staufen, Germany) at speed 4000 rpm for 1-2 min. The growth of *S. aureus* LMH 5P was monitored by spread plating of 0.1 mL of dilutions series (10^{-1}–10^{-3}) of the homogenate onto selective Baird-Parker agar (BPA, Merck, Darmstadt, Germany) which was prior enriched with sterile Egg Yolk Tellurite Emulsion 20 % (Merck, Darmstadt, Germany). The plates were triplicated and incubated at 37 °C for 24–48 h.

The typical grown colonies of *S. aureus* LMH 5P were prior identified and characterized according to scheme in Figure 2.2. Further identifications of this strain were conducted according to the standard method of EN/ISO 6888-1 (2003), namely the verification of the colonies of this strain, such as the typical morphology, gram-positivity, and catalase positivity verification, as well as the verification of the *S. aureus* colonies by coagulase test using Staphyslide test (bioMérieux sa, Marcy I`Etoile, France).

The growth of *S. aureus* LMH 5P was monitored by enumerating of the grown colonies (CFU/g) every 24 h over a 14 d storage period and for period storage after 14 d up to 30 d, every 48 h.

2.5.3.2.2 Determination of growth of *Listeria monocytogenes*

A 0.1 mL aliquot of *L. monocytogenes* LMH 34P at a cell titre of 10^2 CFU/mL was supplemented into every 10 g of coated shrimp meat samples before storage at refrigerator temperature and room temperature. The finished stored coated shrimp meat samples were prior homogenized in 90 mL L-PALCAM-Listeria selective enrichment broth (according to van Netten et al., Merck, Darmstadt, Germany) using

a laboratory stomacher (type BA 7021, Seward Medical London, U.K.) for 2 min and followed by using an Ultra-Turrax (type 25, Janke & Kunkel, Type 18/10, IKA-Labortechnik, Staufen, Germany) at speed 4000 rpm for 1-2 min. The homogenate was then incubated at 30 °C for 48 h. The growth of *L. monocytogenes* on shrimp-meat samples was determined by spread plating of 0.1 mL of dilution series (10^{-1}–10^{-3}) of the homogenate onto PALCAM-Listeria selective agar (Merck, Darmstadt, Germany) which was prior enriched with PALCAM-Listeria selective supplement (according to van Netten et al., Merck, Darmstadt, Germany). The plates were triplicated and incubated at 30 °C for 48 h.

The typical grown colonies of *L. monocytogenes* LMH 34P were prior identified and characterized according to scheme in Figure 2.2. Further identifications of this strain were conducted according to the standard method of EN/ISO 11290-1 (2005), namely the verification of the suspicious colonies of this strain onto Trypton Soya Yeast Extract agar (TSYEA, Merck, Darmstadt, Germany), such as the typical morphology, gram-positivity, and biochemical verification of the colonies using API *Listeria* test (bioMérieux sa, Marcy l`Etoile, France).

The growth of *L. monocytogenes* LMH 34P was monitored by enumerating of the grown colonies (CFU/g) every 24 h over a 14 d storage period and for period storage after 14 d up to 30 d, every 48 h.

2.5.3.3 Determination of growth of gram-negative bacteria

2.5.3.3.1 Determination of growth of *Escherichia coli*

Each 10 g of coated shrimp meat sample was prior supplemented with 0.1 mL aliquot of *E. coli* LMH 1N at a cell titre of 10^2 CFU/mL before storage at refrigerator temperature and room temperature. The finished stored coated shrimp meat samples

were afterwards homogenized in 90 mL warmed (at 37 °C) buffered peptone water solution using a laboratory stomacher (type BA 7021, Seward Medical London, U.K.) for 2 min and followed by using an Ultra-Turrax (type 25, Janke & Kunkel, IKA-Labortechnik, Staufen, Germany) at speed 4000 rpm for 1–2 min. The growth of this strain was monitored by spread plating of 0.1 mL of the homogenate dilutions series (10^{-1}–10^{-4}) on selective MacCONKEY agar (Merck, Darmstadt, Germany). The plates were triplicated and incubated at 37 °C for 24–48 h.

The typical grown colonies of *E. coli* LMH 1N were prior identified and characterized according to scheme in Figure 2.2. Further identifications of this strain were conducted according to the standard method of ISO TS 16649-3 (2005), namely the verification of the suspicious colonies onto Endo agar (Merck, Darmstadt, Germany) according to the typical morphology, gram-negativity, lactose-positivity, and oxidase-negativity of the colonies.

The growth of *E. coli* LMH 1N was monitored by enumerating of the grown colonies (CFU/g) every 24 h over a 14 d storage period and for period storage after 14 d up to 30 d, every 48 h.

2.5.3.3.2 Determination of growth of *Salmonella typhimurium*

Each 10 g of coated shrimp meat sample was prior supplemented with 0.1 mL aliquot of *S. typhimurium* LMH 2N at a cell titre of 10^2 CFU/mL before storage at refrigerator temperature and room temperature. The finished stored coated shrimp meat samples were homogenized in 90 mL warmed (37 °C) buffered peptone water solution using a laboratory stomacher (type BA 7021, Seward Medical London, U.K.) for 2 min and continued by using an Ultra-Turrax (type 25, Janke & Kunkel, Type 18/10, IKA-Labortechnik, Staufen, Germany) at speed 4000 rpm. After incubating the homogenate for 18 h at 37 °C, a 0.1 mL aliquot thereof was pipetted into 10 mL of

Salmonella enrichment broth (according to Rappaport and Vassiliadis, Merck, Darmstadt, Germany), and followed by incubating at 43 °C for 24 h. The growth of this strain was monitored by spread plating 0.1 mL of the homogenate dilutions series (10^{-1}–10^{-3}) onto xylose lysine deoxycholate (XLD) agar (Merck, Darmstadt, Germany). The plates were triplicated and incubated at 37 °C for 24–48 h.

The typical grown colonies of *S. typhimurium* LMH 2N were prior identified and characterized according to the scheme in Figure 2.2. Further identifications of this strain were conducted according to the standard method of EN/ISO 6579 (2003), namely the verification of the suspicious colonies onto XLD agar (Merck, Darmstadt, Germany) according to the typical morphology, gram-negativity, oxidase negativity and lactose-negativity of the colonies.

The growth of *S. typhimurium* LMH 2N was monitored by enumerating of the grown colonies (CFU/g) every 24 h over a 14 d storage period and for period storage after 14 d up to 30 d, every 48 h.

2.5.4 Biochemical analysis of shrimp meat samples

2.5.4.1 Determination of pH value

10 g of coated shrimp meat samples after storage at refrigerator and room temperature were homogenized in 50 mL sterile 0.9 % (w/v) sodium chloride (Merck, Darmstadt, Germany) using a laboratory stomacher (type BA 7021, Seward Medical London, U.K.) for 2 min. A second homogenization step was performed using an Ultra-Turrax (type 25, Janke & Kunkel, IKA-Labortechnik, Staufen, Germany) at speed of 4000 rpm for 1–2 min. The pH of the shrimp meat homogenate was measured using pH meter (TA 10 plus, Schott AG, Mainz, Germany) every 24 h over a period of 14 d and for period storage after 14 d up to 30 d, every 48 h.

2.5.4.2 Determination of water activity value

The water activity (a_w) value of coated shrimp meat samples after storage at refrigerator and room temperature were measured by Hygromess Labo (type 4701, Hygrocontrol GmbH, Hanau, Germany) every 24 h over a 14 d storage period and for period storage after 14 d up to 30 d, every 48 h.

2.5.4.3 Determination of total volatile basic nitrogen content

Determination of total volatile basic nitrogen (TVBN) content in this research was performed according to the official method which is taken from “Collection of Official Methods” of Food, Commodities, and Feed Code section 64, issued by Federal Ministry of Justice (BMJ 2005).

2.5.4.3.1 Preparation of sample

10 g of finished stored coated shrimp meat was carefully crushed using a kitchen mixer (type HR 2870, Philips GmbH, Hamburg, Germany) for 3 min. Then 90 mL of 0.6 N perchloric acid solution (Sigma-Aldrich Chemical, Steinheim, Germany) was added to it and the mixture was blended to a homogenous state using a laboratory stomacher (type BA 7021, Seward Medical, London, U.K.) for 2 min. A second homogenization step was performed using an Ultra-Turrax (type 25, Janke & Kunkel, IKA-Labortechnik, Staufen, Germany) at speed 4000 rpm for 1–2 min. The homogenate was afterwards extracted through fluted filter paper 150 mm (Sartorius, Goettingen, Germany). The extract of shrimp-meat could be used within seven days at storage temperature of 2–6 °C.

2.5.4.3.2 Water-steam-distillation process

50 mL of coated shrimp meat extract was placed in a flask for steam-distillation, added by some drops of phenolphthalein solution (Sigma-Aldrich Chemical, Steinheim, Germany), silicon antifoam emulsion (Wacker-Chemie GmbH, Burghausen, Germany) and 6.5 mL of 20 % (w/v) potassium hydroxide solution (Sigma-Aldrich Chemical, Steinheim, Germany), and followed by starting of steam-distillation unit (Büchi K-314, Labortechnik AG, Flawil, Switzerland) immediately. At the same time, the distillation discharge pipe was set to be dipped into 100 mL of 0.3 % (w/v) boric acid (Sigma-Aldrich Chemical, Steinheim, Germany) in a 250 mL Erlenmeyer flask containing some drops of Tashiro-indicator (Merck, Darmstadt, Germany). The distillation was proceeded about 10 min until 100 mL volume of the distillate was obtained.

2.5.4.3.3 Titration of distillate

100 mL of collected distillate (green colour) was then titrated by 0.01 N hydrochloric acid standard solution (Merck, Darmstadt, Germany) using a titration unit (Titroline alpha plus, Schott AG, Mainz, Germany) until the neutralization point was reached (by changing of distillate colour from green to grey). As control or blank samples 50 mL of 0.6 N perchloric acid (Sigma-Aldrich Chemical, Steinheim, Germany) were used. The same procedure was followed for the treatment of the controls. The content of TVBN was calculated as:

$$\text{TVBN [mg/100 mg]: } \frac{(V_1 - V_o) * c * F * 2 * 100 * 14}{m}$$

V_1 = volume of 0.01 N hydrochloric acid standard solution which was consumed by the titration of samples

V_o = volume of 0.01 N hydrochloric acid standard solution which was consumed by the titration of blank or control

c = concentration of hydrochloric acid standard solution

F = factor of hydrochloric acid standard solution

m = weight of shrimp meat sample (g)

2.5.4.4 Determination of the content of biogenic amines

2.5.4.4.1 Chemicals

All reagents were HPLC grade quality from Merck (Darmstadt, Germany), Karl-Roth (Karlsruhe, Germany), and Sigma-Aldrich Chemical (Steinheim, Germany). All solutions were prepared with ultra pure water that was obtained from a Milli Q-System (Millipore, Berford, France).

2.5.4.4.2 Equipment

Chromatographic experiments were performed using JASCO HPLC 2000 Series (Tokyo, Japan). The HPLC system consisted of a FP-2020 Plus fluorescence detector, CO-2060 column oven, PU-20800 pump, and AS-2055 auto sampler. The chromatographic separations were carried out using a column RP-C 18 (250 mm x 4 mm x 5 µm, Merck, Darmstadt, Germany). Afterwards, the chromatographic data were collected and recorded by the Chromeleon© Dionex 1996-2001 software Version 6.70 Build 1820.

2.5.4.4.3 Preparation of eluents and derivatization solution

2.5.4.4.3.1 Preparation of eluent A

8.03 g of sodium acetate anhydrous (Merck, Darmstadt, Germany) was dissolved in 800 mL of ultra pure water (Millipore, Berford, France) and then its pH was adjusted to 4.5 with 96 % (w/v) acetic acid (Sigma-Aldrich Chemical, Steinheim, Germany). Afterwards, 2.16 g of sodium octanesulfonate (Sigma-Aldrich Chemical, Steinheim, Germany) was added into the solution that was prior placed in a 1000 mL volumetric flask and filled with ultra pure water (Millipore, Berford, France) up to the mark. This solution was imperishable in sealed storage vessel.

2.5.4.4.3.2 Preparation of eluent B

12.73 g of sodium acetate anhydrous (Merck, Darmstadt, Germany) was dissolved in 600 mL of ultra pure water (Millipore, Berford, France) and its pH was adjusted to 4.5 with 96 % (w/v) acetic acid (Sigma-Aldrich Chemical, Steinheim, Germany). Afterwards, 2.16 g of sodium octanesulfonate (Sigma-Aldrich Chemical, Steinheim, Germany) and 230 mL acetonitrile (Merck, Darmstadt, Germany) were added into the solution that was prior placed in a 1000 mL volumetric flask and filled with ultra pure water (Millipore, Berford, France) up to the mark. This solution was imperishable in sealed storage vessel.

2.5.4.4.3.3 Preparation of borate buffer

61.8 g of boric acid (Karl-Roth, Karlsruhe, Germany) and 40 g of potassium hydroxide (Merck, Darmstadt, Germany) were first dissolved in ultra pure water and then carefully transferred into a 1000 mL volumetric flask. Afterwards, it was filled)

up to the mark with ultra pure water (Millipore, Berford, France. This solution was imperishable in sealed storage vessel.

2.5.4.4.3.4 Preparation of derivatization solution

1.5 g of polyethylene lauryl ether (BRIJ® 35, Merck, Darmstadt, Germany) was dissolved in 5 mL of methanol (Sigma-Aldrich Chemical, Steinheim, Germany) and followed by adding 500 mg of o-Phthaldialdehyde (OPA, Merck, Darmstadt, Germany) and 500 mL of borate buffer (Sigma-Aldrich Chemical, Steinheim, Germany) into the solution respectively. After addition of 1.5 mL of mercaptoethanol (Merck, Darmstadt, Germany), the solution had to be degassed using ultrasonic bath (Sonorex super RK 102 H, Bandelin Electronic, Berlin, Germany) for at least 30 min. Afterwards, the solution was ready for use and had to be kept in the dark or brown flask, stored at cool temperature, and used within a day.

2.5.4.4.4 Preparation of biogenic amines standard solution

2.5.4.4.4.1 Preparation of biogenic amines stock solution

Histamine hydrochloride, putrescine dihydrochloride, cadaverine dihydrochloride, agmatine sulphate, and tyramine hydrochloride (Sigma-Aldrich Chemical, Steinheim, Germany) had to be stored under exsiccator over concentrated 98 % sulphuric acid (Sigma-Aldrich Chemical, Steinheim, Germany) at least for 24 h before using, to prevent their hygroscopic activity. The stock solution of each amine was prepared in 0.6 N perchloric acid (Sigma-Aldrich Chemical, Steinheim, Germany) with a concentration of 1000 mg/L.

2.5.4.4.4.2 Preparation of calibration solution

0.05 mL, 0.10 mL, 0.2 mL, 0.5 mL, 1.0 mL, 2.5 mL, and 5.0 mL of each stock solution were transferred into a 100 mL volumetric flask and afterwards were filled up to the mark with 0.6 N perchloric acid (Sigma-Aldrich Chemical, Steinheim, Germany). These solutions correspond to biogenic amines standard contents of 5 mg, 10 mg, 20 mg, 50 mg, 100 mg, 250 mg, and 500 mg amine/kg of substrate.

2.5.4.4.4.3 Preparation of internal standard solution

1.6-Diaminohexane (Sigma-Aldrich Chemical, Steinheim, Germany) was stored under exsiccator over concentrated 98 % sulphuric acid (Sigma-Aldrich Chemical, Steinheim, Germany) at least for 24 h before using. 160 mg thereof was dissolved with 0.6 N perchloric acid (Sigma-Aldrich Chemical, Steinheim, Germany), and then carefully transferred into a 100 mL volumetric flask. Afterwards, it was filled up to the mark with 0.6 N perchloric acid.

2.5.4.4.5 Preparation of shrimp meat samples

10 g of coated shrimp meat was carefully crushed using a kitchen mixer (type HR 2870, Philips GmbH, Hamburg, Germany) for 3 min. Then 45 mL of 0.6 N perchloric acid (Sigma-Aldrich Chemical, Steinheim, Germany) was added to it and the mixture was blended to a homogenous state using a laboratory stomacher (type BA 7021, Seward Medical London, U.K.) for 1-2 min. A second homogenization step was performed at 4000 rpm for 1-2 min by Ultra-Turrax (type 25, Janke & Kunkel, IKA-Labortechnik, Staufen, Germany). The suspension solution was centrifuged at 5000 rpm for 15 min using a laboratory centrifuge (Biofuge, Heraeus, Kendro Laboratory Product, Germany). The shrimp meat extract was obtained by filtering the supernatant through a fluted filter paper 125 mm (Roth, Karlsruhe, Germany).

The extract of shrimp meat could be used within seven days at storage temperature of 2-6 °C. The shrimp meat extract was prior filtered through membrane filter 0.20 µm (Sartorius, Goettingen, Germany) before injecting to HPLC equipment system.

2.5.4.4.6 Chromatographic conditions

During chromatographic separation of the biogenic amines using a reversed phase chromatographic column, a gradient system consisting of eluent A and B with the following composition was used, as shown in Table 2.3.

Table 2.3: Composition of optimized gradient for column elution

Time (min)	Eluent A (%) (described in section 2.5.4.4.3.1)	Eluent B (%) (described in section 2.5.4.4.3.2)
0	20	80
5	50	50
10	40	60
15	50	50
20	40	60
25	80	20

The gradient elution program was controlled by a system controller at the flow rate of 1.0 mL/min, and the flow rate of the derivatization solution was 0.7 mL/min. The derivatization process was performed online through a T-branch connector (JASCO, Tokyo, Japan) which was connected to the third pump (PU-20800, JASCO, Tokyo, Japan). The elution of derivatization solution was detected by monitoring the fluorescence detector at excitation 366 nm and emission 450 nm wavelengths. The analytical chromatographic column was set at 40 °C throughout the experiment. The volume of biogenic amines standards and the shrimp meat samples injected into HPLC system was 20 µL.

2.5.4.4.7 Chromatographic identification and quantitative determination

The right peak of each biogenic amine was identified by comparing of the respective retention time between samples and standard substances. The internal standard was used for accurate identification of each peak. The concentration of each biogenic amine was calculated directly by interpolation of the ratio in the corresponding linear calibration curve standard between 5 and 500 mg/kg. Afterwards, the biogenic amine content was quantitatively calculated by comparison of the peak area between samples and standard and was indicated as mg amine/kg of substrate.

2.5.5 Physical analysis of plasticized chitosan-garlic extract film

2.5.5.1 Determination of moisture content of the film

The chitosan thin film samples were stored for at least 3 weeks at 23 °C in a forced air oven (Cytoperm Heraeus 8080, Kendro Laboratory Product, Germany). After weighing, they were conditioned at 20-25 °C in a dessicator (Büchi, Labortechnik AG, Flawil, Switzerland) containing cupric sulfate pentahydrate saturated solution (Merck, Darmstadt, Germany). During storage for up to 96 h, the film samples were removed and immediately weighed every 24 h. This procedure was repeated at least in triplicate until an equilibrium weight was reached. The moisture content of the chitosan film samples was calculated using the equation (Anglès and Dufrense 2000):

$$Mc = \frac{(W_t - W_0)}{W_0} * 100$$

Mc = moisture content (%)
W_t = weights (g) of samples after certain time (t)
W_0 = the initial weight (g) of film samples

2.5.5.2 Scanning electronic microscopic recording of chitosan-garlic extract film enhanced with glycerol

The cut wet films had to be dried using freeze dryer (Alpha-1-4, Martin Christ GmbH, Osterode, Germany) for 24 h, then film sample was immediately put in desiccator (Büchi, Labortechnik AG, Flawil, Switzerland) for at least 24 h. Afterward film samples were further dried according to critical point drying method (Gerstenberger and Leins 1978) using Critical Point Dryer (CPD 030, BAL-TEC GmbH, Schalksmuehle, Germany). Then, the dried film was placed again in desiccator (Büchi, Labortechnik AG, Flawil, Switzerland) for at least 24 h. Furthermore, the film samples were taken out of desiccator (Büchi, Labortechnik AG, Flawil, Switzerland) and sputtered with gold using sputter coater (SCD 050, BAL-TEC GmbH, Schalksmuehle, Germany). The gold coated cut films were ready to be taken of their microstructure of surface and cross section films using scanning electron microscope (XL-200, Phillips, California, USA).

3 Results

For shrimp meat preservation, charges of chitosan with different chain lengths (Table 2.1) had to be tested for their antimicrobial activity against four microorganisms causing spoilage in shrimp meat, namely *E. coli*, *S. typhimurium, L. monocytogenes*, and *S. aureus* (Table 2.2). During this testing, the concentrations and pH values of chitosan charges had to be optimized simultaneously.

Beside that, the physical characteristics of chitosan, such as molecular weight (M_η) and degree of deacetylation (DDA), had to be determined. Furthermore, the influence of the physical characteristics on the antimicrobial activity of chitosan was tested. The charge of chitosan with the best antimicrobial activity was used for the experiments to preserve shrimp meat.

To test potency of chitosan to preserve shrimp meat, the experiments were conducted at refrigerator temperature and room temperature. Each sample was assessed every 24 h and compared with control (samples untreated with chitosan). Assessment consisted of monitoring changes in microbiological parameters and biochemical parameters. The microbiological parameters were total cell count of aerobic mesophilic bacteria and growth of gram-positive and gram-negative bacteria, whereas the biochemical parameters consisted of pH value, water activity value, content of total volatile basic nitrogen, and content of biogenic amines. Afterwards, the optimum conditions for the preservation of shrimp meat with chitosan obtained from the preliminary study were used to treat shrimp meat in the full-scale study. The procedures in this step followed the procedures of the preliminary study (see Figures 3.1 and 3.2).

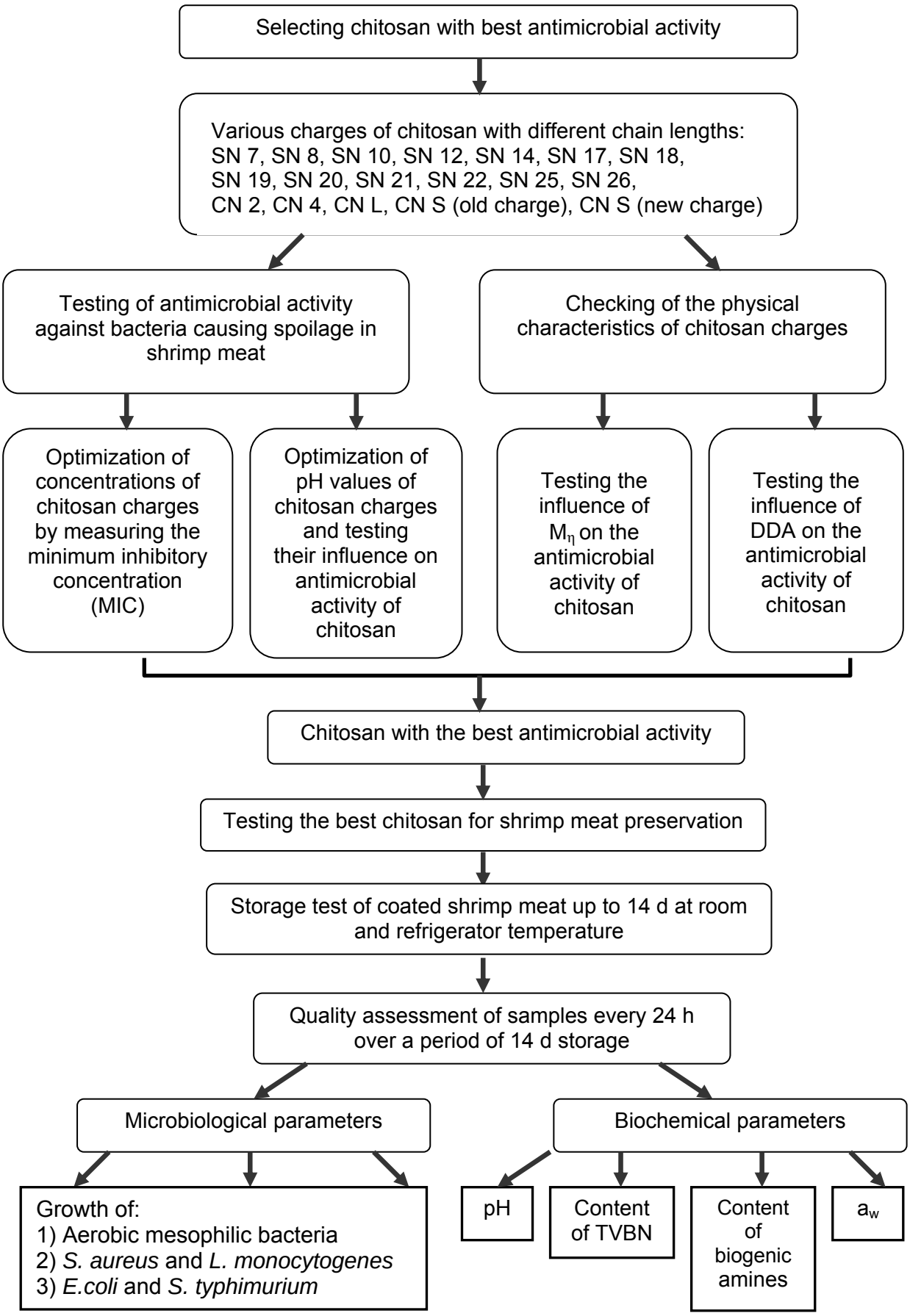


Figure 3.1: Scheme of the preliminary study for the optimization of shrimp meat preservation conditions

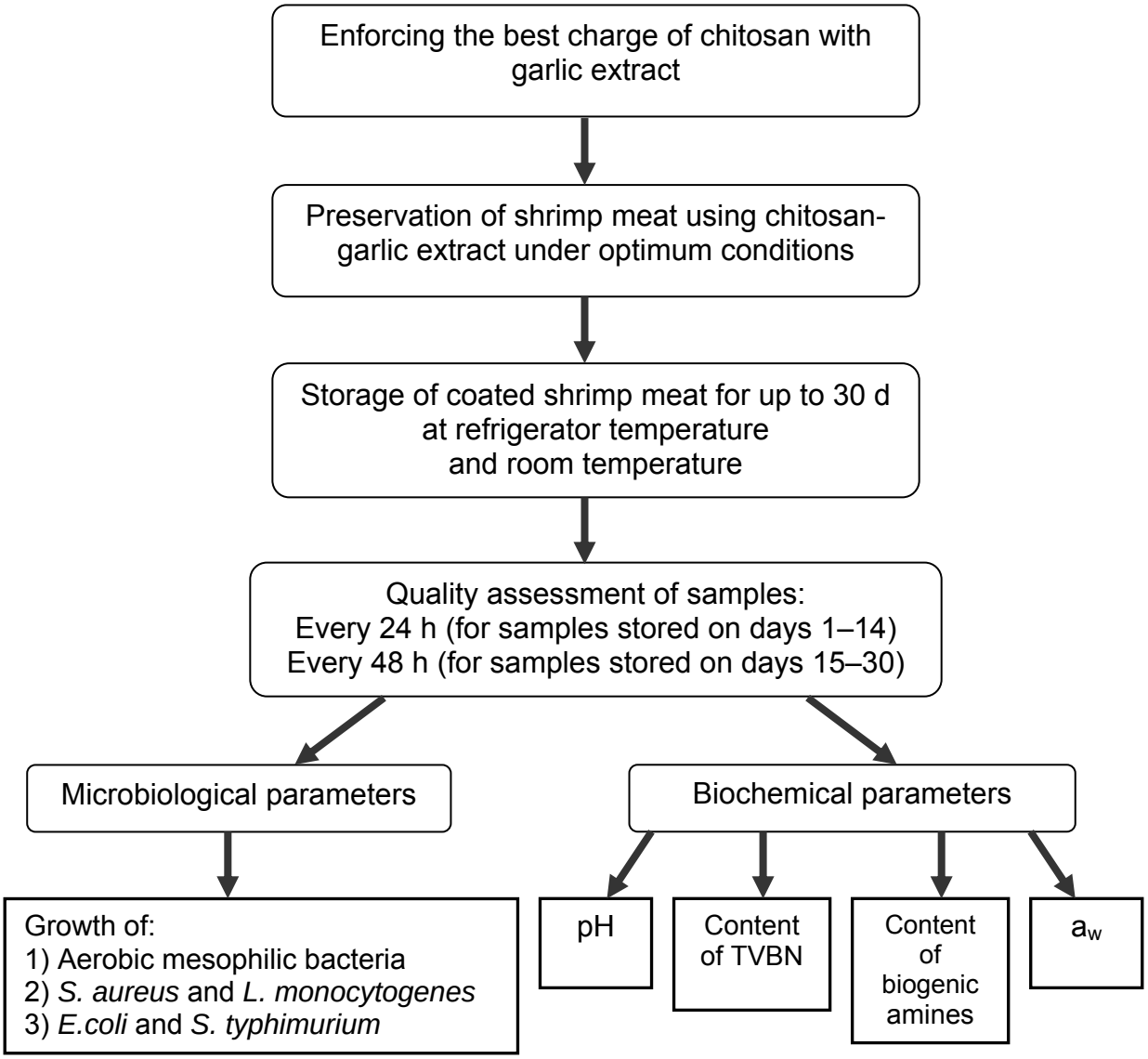


Figure 3.2: Scheme of the full-scale study for shrimp meat preservation under optimum conditions

3.1 Molecular mass and degree of deacetylation of chitosan

To assess the influence of degree of deacetylation (DDA) and molecular mass (M_η) of chitosan charges on their antimicrobial activity, both physical characteristics of the tested chitosan had to be determined. The M_η and the DDA of chitosan charges are presented in Table 3.1.

Table 3.1: The M_η and DDA data of the tested chitosan charges

No.	Charge of chitosan	Degree of deacetylation (DDA) [%]	Molecular mass (M_η) [g/mol]	Viscosity (η) [mL/g]
1.	SN 7	90	5.1×10^5	967
2.	SN 8	80	5.3×10^5	994
3.	SN 10	74	5.9×10^5	1227
4.	SN 12	76	3.9×10^5	797
5.	SN 14	97	1.5×10^5	337
6.	SN 17	81	2.4×10^5	547
7.	SN 18	87	9.3×10^5	1516
8.	SN 19	85	2.9×10^5	643
9.	SN 20	80	5.7×10^5	1051
10.	SN 21	88	1.3×10^6	1890
11.	SN 22	80	1.5×10^5	397
12.	SN 25	87	3.9×10^5	792
13.	SN 26	98	1.9×10^5	466
14.	SN 27	97	1.7×10^5	424
15.	CN 2	89	6.7×10^5	1193
16.	CN 4	87	2.0×10^5	494
17.	CN L	84	1.1×10^6	1730
18.	CN S (old charge)	80	3.3×10^5	708
19.	CN S (new charge)	81	3.2×10^5	681

As shown in Table 3.1, the chitosan charges tested show various DDA values which can be categorized as high (85–98 %), medium (80–84 %), and low (< 80 %). The DDA values range from 98–74 %, in which charge SN 26 with a DDA of 98 % showed the highest value and charge SN 10 a DDA of 74 % showed the lowest value. The high DDA values range from 85–98 %, namely for charges SN 19 (85 %), SN 18, SN 25, and CN 4 (87 %), CN 2 (89 %), SN 21 (88 %), CN 2 (89 %), SN 7 (90 %), SN 27, SN 14 (97 %), and SN 26 (98 %). The medium DDA values range from 80–84 %, referring to charges SN 8, SN 20, SN 22, and CN S (old charge) with a DDA of 80 %, SN 17 and CN S (81 %), and CN L (84 %). The low DDA values amount to below 80 %.

In case of the M_η values, the tested chitosan charges showed quite similar values ranging from 1.5×10^5–1.3×10^6 g/mol. Most charges possess M_η values ranging in the order of 10^5, with the lowest value being 1.5×10^5 g/mol (charges SN 14 and SN 22). There seems to be a correlation between the molecular weight of chitosan and its viscosity. For example, charge SN 21 with the highest M_η value of 1.3×10^6 g/mol also possesses the highest viscosity of 1890 mL/g. It was also observed that other charges with relatively high M_η values (> 5.7×10^5 g/mol) also have high viscosities (> 1050 mL/g); these are SN 10, SN 18, SN 20, CN 2, and CN L. It is worthwhile to note, although the charges SN 14 and SN 22 have the same M_η (1.5×10^5 g/mol); however, they possess slightly different viscosities of 337 mL/g and 397 mL/g, respectively (see Table 3.1).

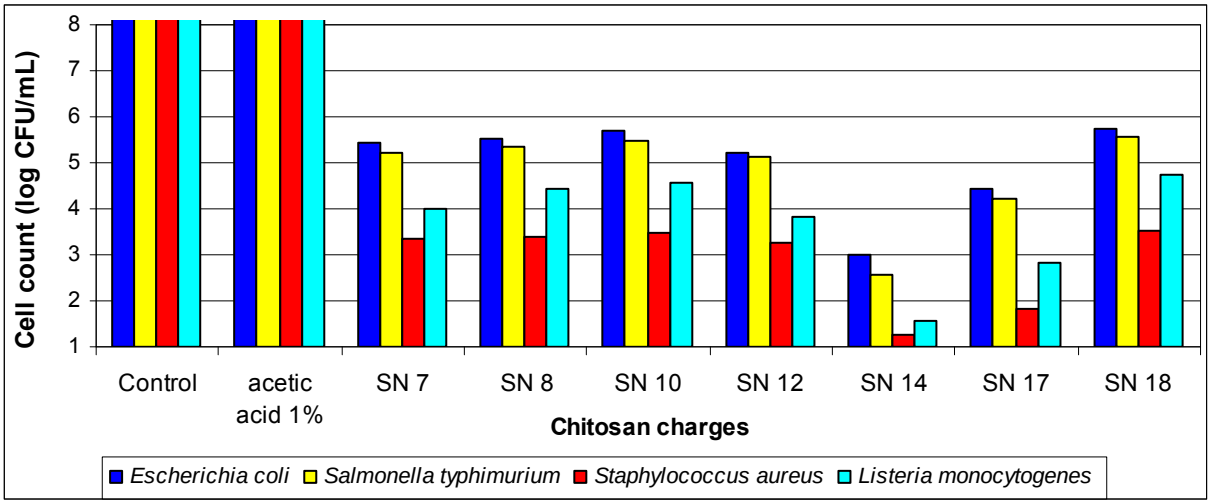
In this research, the influence of the M_η and DDA of chitosan charges on their antimicrobial activity against the tested bacterial strains causing spoilage in shrimp meat (Table 2.2) was studied.

3.2 Antimicrobial activity of chitosan

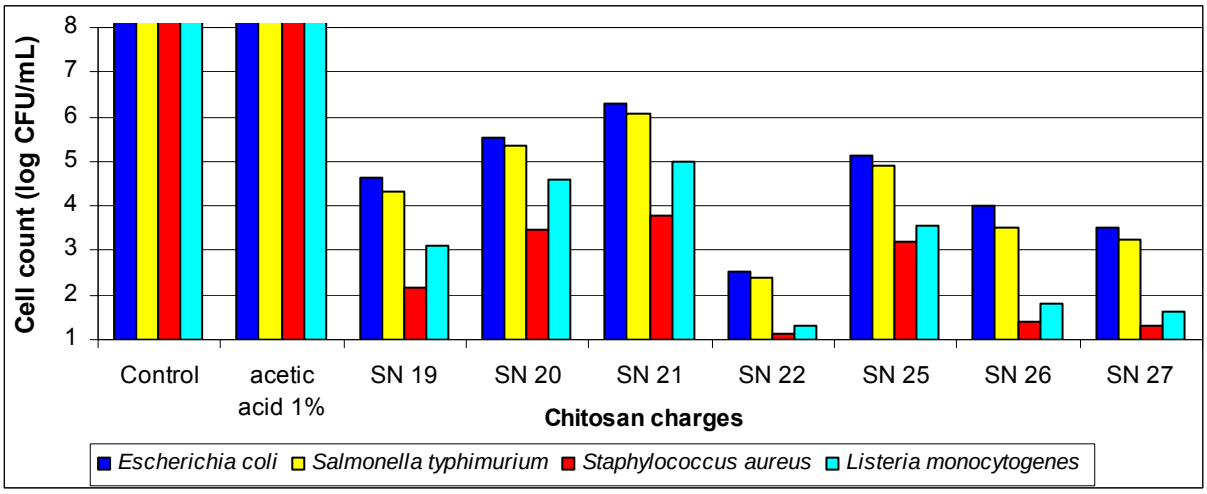
In this study, the antibacterial activity of each chitosan charge was assessed against four strains of bacteria causing spoilage in shrimp meat. These consist of two gram-negative bacteria and two gram-positive bacteria namely *E. coli* LMH 1N, *S. typhimurium* LMH 2N, *L. monocytogenes* LMH 34P, and *S. aureus* LMH 5P (see Table 2.2). Chitosan charges of different chain lengths were delivered by Seehof Laboratorium (SeeLab) GmbH and Cognis GmbH (see Table 2.1).

All tested charges of chitosan markedly inhibited the growth of the test bacteria at a concentration of 0.1 % (w/v) and generally showed a stronger antimicrobial effect against gram-positive bacteria than against gram-negative bacteria (see Figures 3.3 A.1–2 and B).

A.1)



A.2)



B)

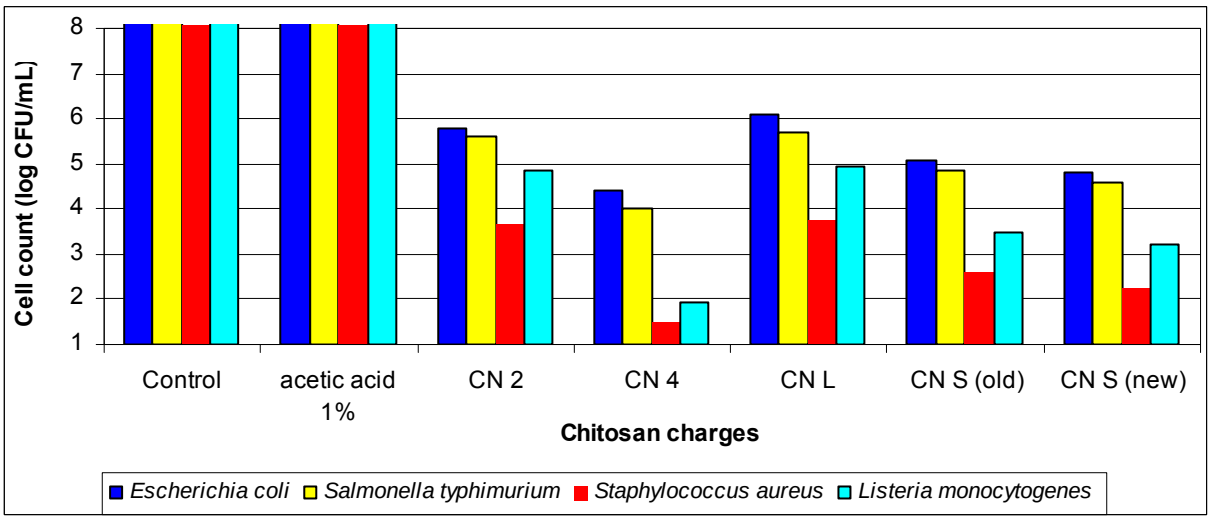


Figure 3.3: Antimicrobial activity of the test chitosan charges of SeeLab GmbH **(A.1, A.2)** and Cognis GmbH **(B)** compared to untreated samples (control) and samples treated with acetic acid 1 % (v/v)

Chitosan charges SN 22, SN 27, CN 4, SN 26, SN 17, and SN 19 with quite low molecular mass (M_η) values of 1.5 × 10^5–2.9 × 10^5 g/mol (see Table 3.1) showed stronger antimicrobial activity against the four tested strains, mainly against the tested gram-positive bacteria. Especially, growth of *S. aureus* was almost completely suppressed by all tested chitosan charges (Figures 3.3 A.1–2 and 3.3 B).

The chitosan charges with high M_η values of 3.2 × 10^5–1.1 × 10^6 g/mol possessed weak antibacterial activity against the four tested strains. The chitosan charges with high molecular mass are CN S (new charge), CN S (old charge), SN 25, SN 7, SN 8, SN 20, SN10, SN 18, CN 2, CN L, and SN 21 (see Table 3.1).

The same tendency was displayed for the gram-negative strains tested. Growth of *E. coli* and *S. typhimurium* was inhibited more effectively by chitosan charges with low M_η values (1.5 × 10^5–2.9 × 10^5 g/mol), although inhibition of both gram-negative strains was weaker than that of the tested gram-positive strains.

The chitosan charges SN 8, SN 20, SN 22, and CN S (old charge) with the same DDA value of 80 % showed varying antimicrobial activities, which tend to be stronger with decreasing molecular mass (M_η). One of them even showed the best antimicrobial activity, namely charge SN 22 with the lowest M_η of 1.5 × 10^5 g/mol.

3.2.1 Optimization of pH for antimicrobial activity of chitosan

The effect of pH on the antimicrobial activity of chitosan against the test bacteria was evaluated for all charges at pH values of 4.5, 5.0, 5.5, 6.0, and 6.5 of chitosan solution at a concentration of 0.1 % (w/v). The lowest pH value was set as 4.5 because growth of *E. coli* is inhibited in an environment with a pH value of < 4.5. The upper pH value was limited to 6.5 because chitosan is insoluble in an environment with a pH value of > 6.5. Evaluation of the effect of the pH value on the antimicrobial

activity of chitosan was performed simultaneously for all chitosan charges at the given pH values, against each test strain.

In order to clearly observe the results of the pH effect on the antimicrobial activity of chitosan, only the effects of pH on the antimicrobial activity of the three chitosan charges SN 22, SN 14, and SN 27 that showed the best antimicrobial activities are presented (Figures 3.4 A–D), although the evaluation was performed on each charge of chitosan (see Table 3.1).

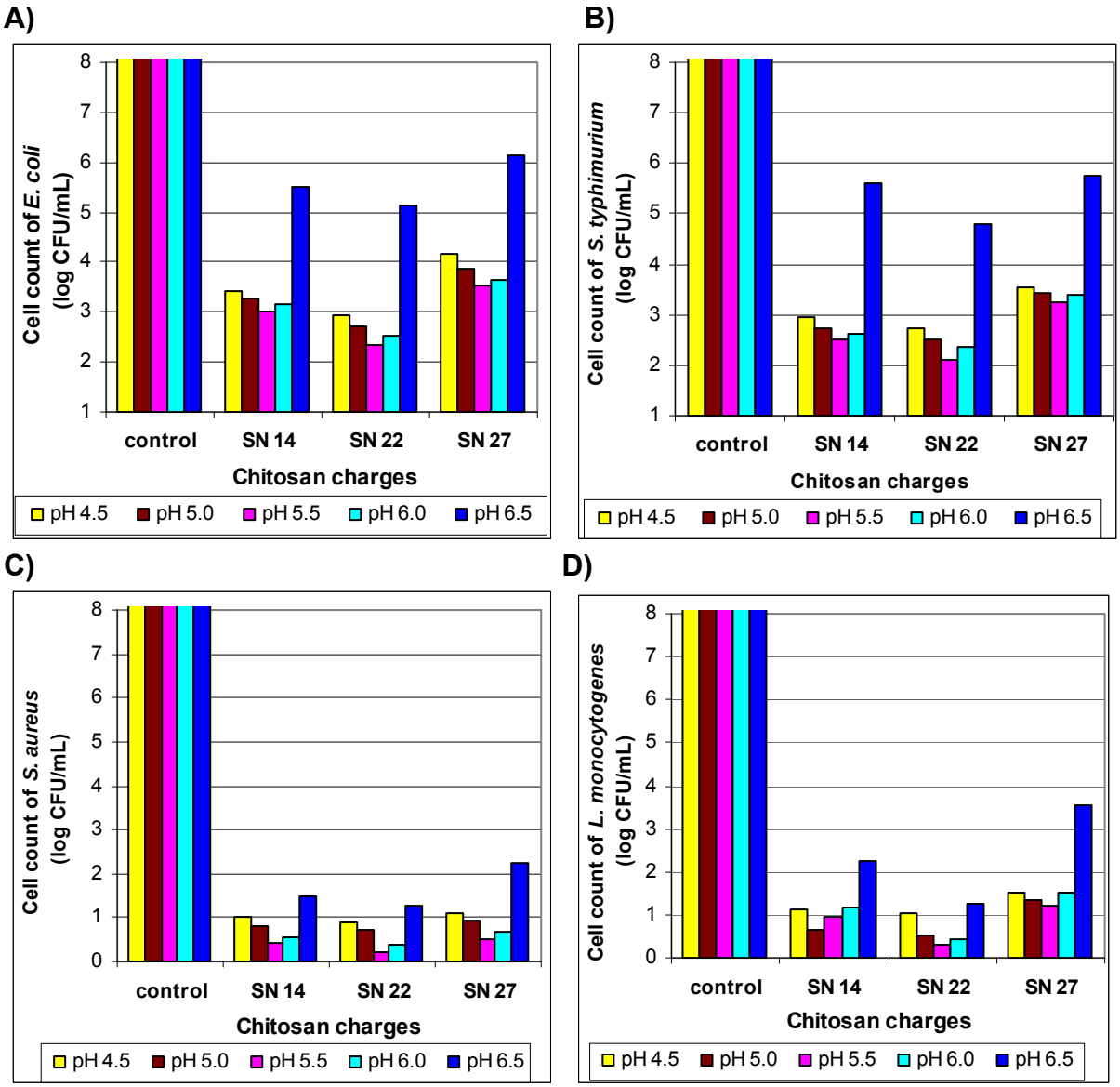


Figure 3.4: Influence of pH on the antimicrobial activity of chitosan charges SN 14, SN 22, and SN 27 against **(A)** *E. coli*, **(B)** *S. typhimurium,* **(C)** *S. aureus*, and **(D)** *L. monocytogenes* compared to control

As shown in these Figures, the antimicrobial activity of chitosan against the four test bacteria is influenced by pH value, with the strongest activity being found at a pH value of 5.5, followed by the pH value of 6.0 with the second best activity. Furthermore, it can be obviously seen that the antimicrobial activities of chitosan decreased drastically, when the pH values are higher than 6.0.

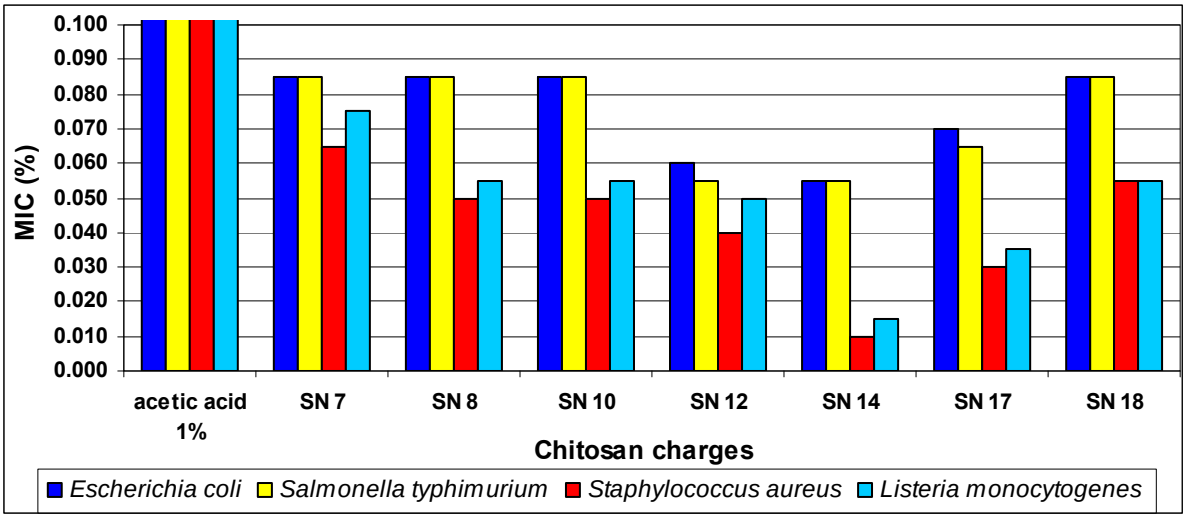
In general, at the pH values ranging from 4.5–6.5, all chitosan charges in this research showed a stronger antimicrobial activity against the gram-positive test strains than against the gram-negative test strains. For example, *S. aureus* at pH 5.5 showed the lowest number of viable cells at value of 1.5×10^1 CFU/mL, 3.5×10^1 CFU/mL, and 5.2×10^1 CFU/mL after treatment with chitosan SN 22, SN 14, and SN 27, respectively.

3.2.2 Minimum inhibitory concentration of chitosan

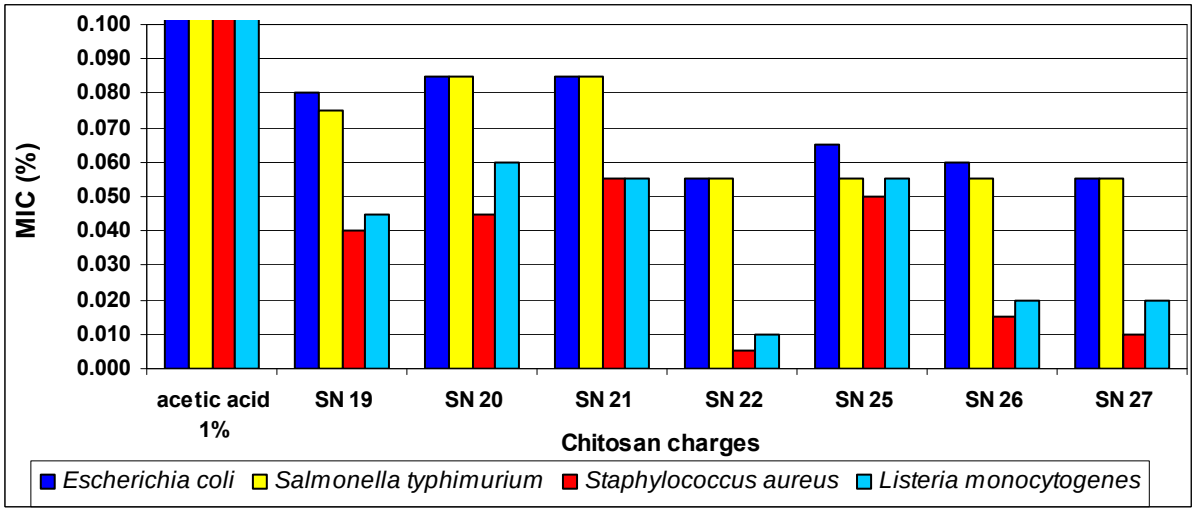
To obtain the minimum inhibitory concentration (MIC) of the chitosan charges to optimally inhibit the four tested bacteria incubated at 37 °C for 72 h at a pH value of 5.5, the antimicrobial activities of chitosan charges at concentrations of 0.002–0.20 % (w/v) were examined and compared to the antimicrobial activity of acetic acid at a concentration of 1 % (v/v).

The result of MIC values ranged from 0.005–0.1 % (w/v), as shown in Figures 3.5 A.1, A.2, and B. All tested chitosan charges showed stronger antibacterial activity against the test gram-positive bacteria, with MIC values ranging from 0.005–0.050 %. The MIC of chitosan charges against the test gram-negative strains ranged from 0.055–0.085 %. The lowest MIC value of 0.005 % was exhibited by charges SN 22 for inhibiting *S. aureus*.

A.1)



A.2)



B)

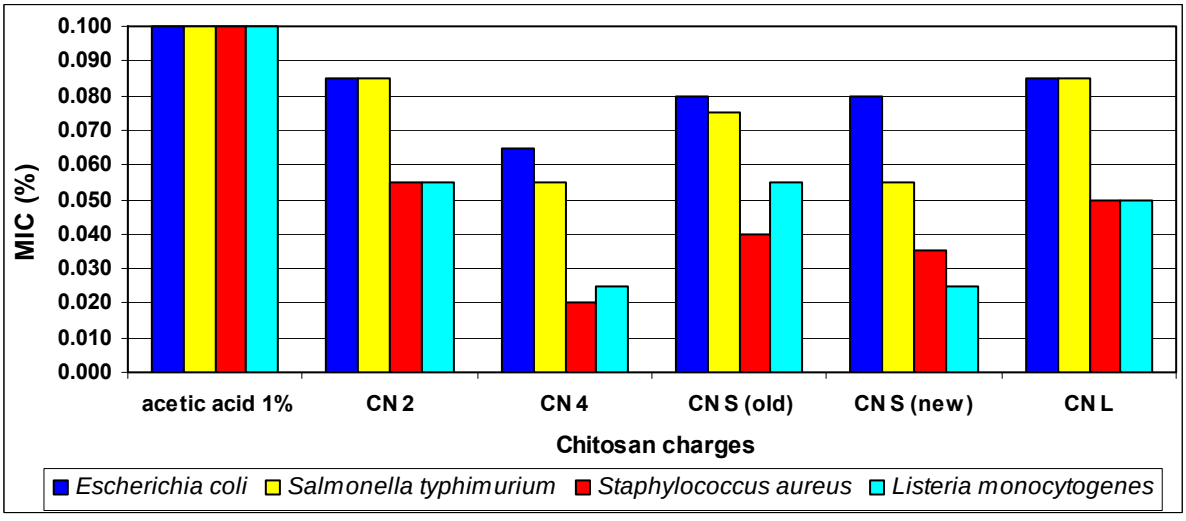


Figure 3.5: Minimum inhibitory concentrations of chitosan charges of **(A.1, A.2)** SeeLab GmbH and **(B)** Cognis GmbH compared to acetic acid 1 % (v/v)

The chitosan charges SN 22, SN 27, CN 4, SN 26, SN 17, and SN 19 with quite low M_η values in the range of 1.5×10^5–2.9×10^5 g/mol (see Table 3.1) were more effective in inhibiting all the tested strains than those with higher M_η values. Acetic acid at a concentration of 1 % (v/v) showed no antimicrobial activity against the gram-positive and gram-negative test strains (Figure 3.5 A.1, A.2, and B).

Regarding the influence of the DDA on the MIC value of chitosan, there is a distinct tendency that chitosan charges with medium DDA values ranging from 80–84 % showed a stronger effect on their MIC values. These were charges SN 8, SN 20, SN 22, and CN S (old charge). Charge SN 22 showed the strongest inhibitory effect at the lowest MIC of 0.005 % against the four test strains.

Comparing the MIC values shown in Figures 3.5 A.1–2 and 3.5 B, it was also exhibited that the growth of *S. aureus* LMH 5P could be easily inhibited by all the charges of chitosan tested, whereas *E. coli* LMH 1N was lowest inhibited.

3.3 Testing the potency of chitosan for shrimp meat preservation

The results of the antimicrobial activity assay of chitosan show that charge SN 22 exhibited the strongest antimicrobial activity against the four tested strains. In the following, this charge was used for experiments to preserve shrimp meat. This test was aimed to elucidate the effect of chitosan charge SN 22 in maintaining the quality of shrimp meat during storage.

3.3.1 Enhancing the chitosan solution with plasticizer for shrimp meat coating

Chitosan SN 22 solution was used to coat shrimp meat samples, as described in section 2.3.1. In order to form a good coating solution to preserve shrimp meat during storage, chitosan as a polymer needs to be more flexible and ductile.

For this reason, food-grade plasticizers were added to chitosan SN 22 solution to enhance its coating properties. Moreover, in order to determine the plasticizer that can optimally enhance the chitosan coating, polyol plasticizers such as glycerol, xylitol, and sorbitol were tested.

3.3.1.1 Moisture content of plasticized chitosan thin films

The effects of glycerol, xylitol, and sorbitol on the moisture contents of chitosan SN 22 thin films are presented in Figure 3.6. It can be clearly seen that glycerol leads to the highest moisture content in chitosan thin films, in comparison with xylitol and sorbitol. The plasticizing effects of glycerol, xylitol, and sorbitol on the moisture contents of chitosan films decreased during storage.

Glycerol showed the best plasticization ability and stability in maintaining the moisture content of chitosan thin films throughout the storage. At the beginning of storage, chitosan films plasticized with glycerol reached an initial moisture content of 62 %. During a 96-h storage period, these moisture contents could be significantly maintained. At the end of 96 h of storage, a slight decrease in the moisture content of chitosan films was found and reached a level of 61 % (Figure 3.6).

On the contrary, the moisture contents of chitosan films plasticized with xylitol and sorbitol decreased from the first 24 h up to the end of storage. For example, the moisture content of chitosan films plasticized with sorbitol decreased to a level of 29 % from the initial moisture content of 33 % within 24 h of storage. Similarly, xylitol could not maintain the initial moisture content in the plasticized chitosan films within 24 h of storage, and the moisture content decreased from 33 % to 27 %. After 24 h of storage, the decreases in the moisture contents in chitosan films plasticized with sorbitol and xylitol were greater, even at 72 h of storage; the moisture content reached a low level of < 20 %.

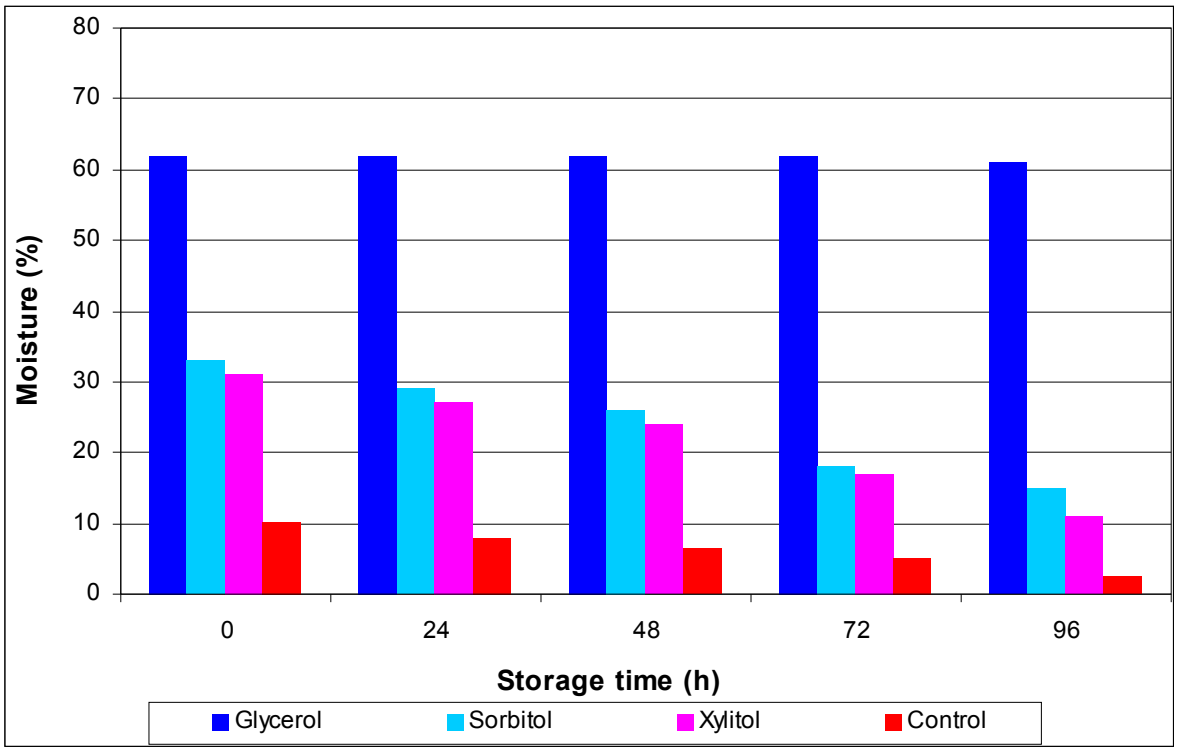


Figure 3.6: Moisture contents of chitosan SN 22 thin films enhanced with various plasticizers at a concentration of 10 % (w/v) compared to control (unplasticized films)

3.3.1.2 Optimization of plasticizer concentration

To obtain the optimum concentration of glycerol necessary to enhance the chitosan coating solution for shrimp meat preservation, several concentrations of glycerol in the range of 10–40 % (w/v) were tested. The moisture contents in the chitosan films plasticized with glycerol at various concentrations was determined (see section 2.3.1.1).

The effects of varying concentrations of glycerol on the moisture content in chitosan thin films are presented in Figure 3.7. At a concentration of 20 % (w/v), glycerol showed the highest plasticization activity, with the moisture contents of the chitosan films reaching 72 %. Indeed, the concentration of 20 % (w/v) was the optimum level of glycerol to enhance the chitosan films. Comparing the concentrations of 10 %, 30 % and 40 % (v/v), it is observed that the plasticizer activity decreased, with the

moisture content reaching the levels of 62 %, 65 %, and 58 %, respectively. As observed during the research, at a glycerol concentration of 10 %, the chitosan films became brittle and easy to tear. On the other hand, at higher concentrations of 30 % and 40 % (w/v), the chitosan films became rigid and inelastic because the density of glycerol was too high, and thus, the resulting moisture contents in chitosan films were lowered.

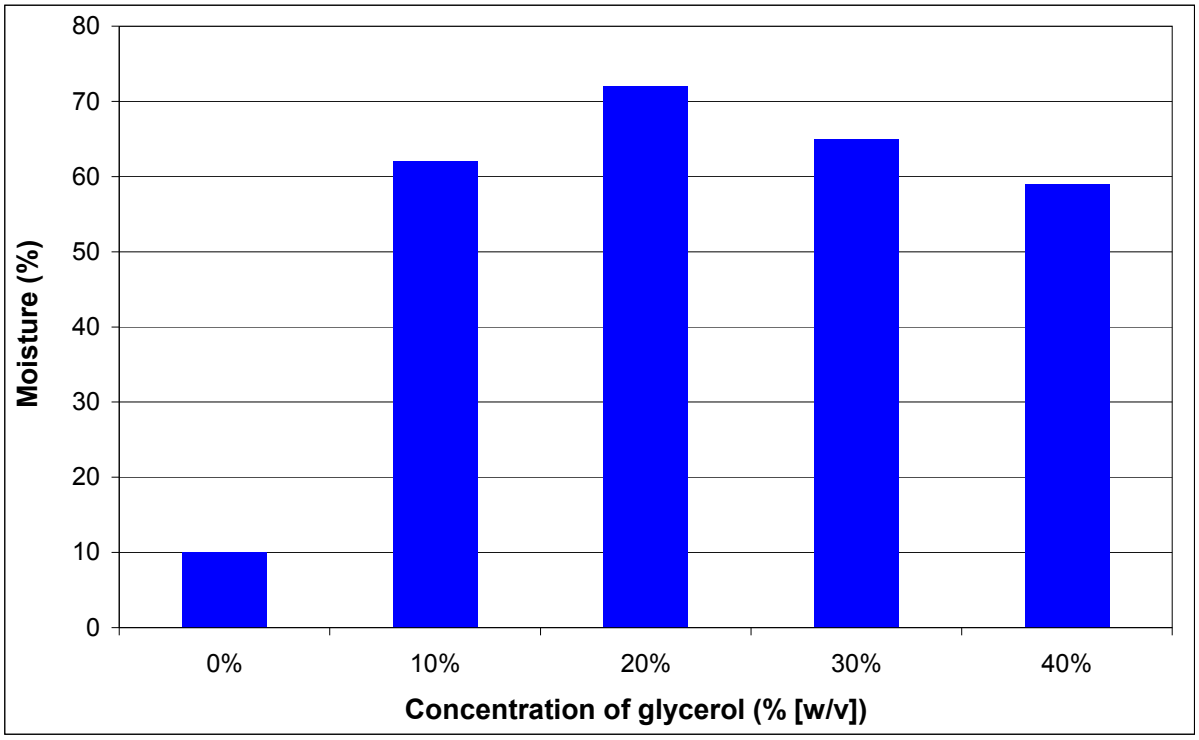


Figure 3.7: Moisture contents of chitosan SN 22 thin films enhanced with various concentrations of glycerol

The moisture content in chitosan films plasticized with glycerol at a concentration of 30 % (w/v) reached a level of 65 %, and those films plasticized at a concentration of 40 % (w/v) reached a level of 58 %. In general, at 20 % (w/v) glycerol concentration, the chitosan films were tough, long-lasting, flexible, and easy to be peeled from the plates, compared to those plasticized with 10 %, 30 %, and 40 % (w/v) glycerol, as shown in Figures 3.8 A–D.

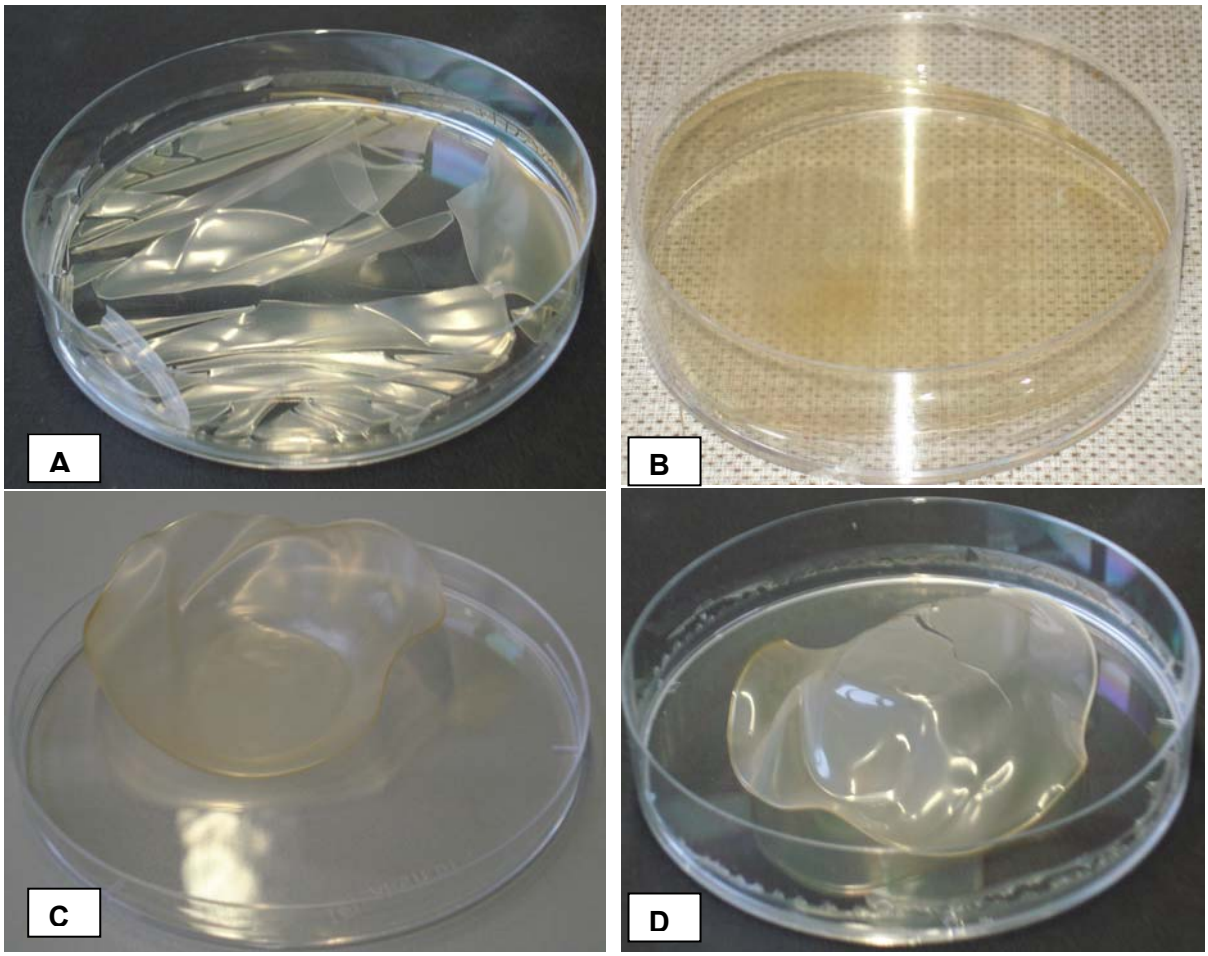


Figure 3.8: Chitosan SN 22 films plasticized with various concentrations of glycerol **(A)** 10 % (w/v), **(B)** 20 % (w/v), **(C)** 30 % (w/v), and **(D)** 40 % (w/v)

Afterwards, the chitosan SN 22 solution plasticized with 20 % (w/v) glycerol was used for shrimp meat coating. The storage test of the coated shrimp meat samples was performed at refrigerator temperature (at 4–6 °C) and room temperature (22–23 °C) for up to 14 days for subsequent quality assessments, namely microbiological and biochemical analyses which were performed every 24 h (see section 2.3.1).

3.3.2 Microbial quality assessment of shrimp meat samples

To evaluate the effect of chitosan SN 22 on the microbiological parameters of shrimp meat, three microbiological analyses were conducted, namely changes in total viable

cell count (TVC) of aerobic mesophilic bacteria, changes in growth of *S. aureus*, *L. monocytogenes*, *E. coli*, and *S. typhimurium*.

3.3.2.1 Total viable count of aerobic mesophilic bacteria

The viable count (TVC) of aerobic mesophilic bacteria of shrimp meat samples were indicated by the colonies grown on PCA that expressed as CFU/g of shrimp meat (see section 2.5.3.1). Changes in TVC of aerobic mesophilic bacteria in the shrimp meat samples during storage are shown in Figure 3.9.

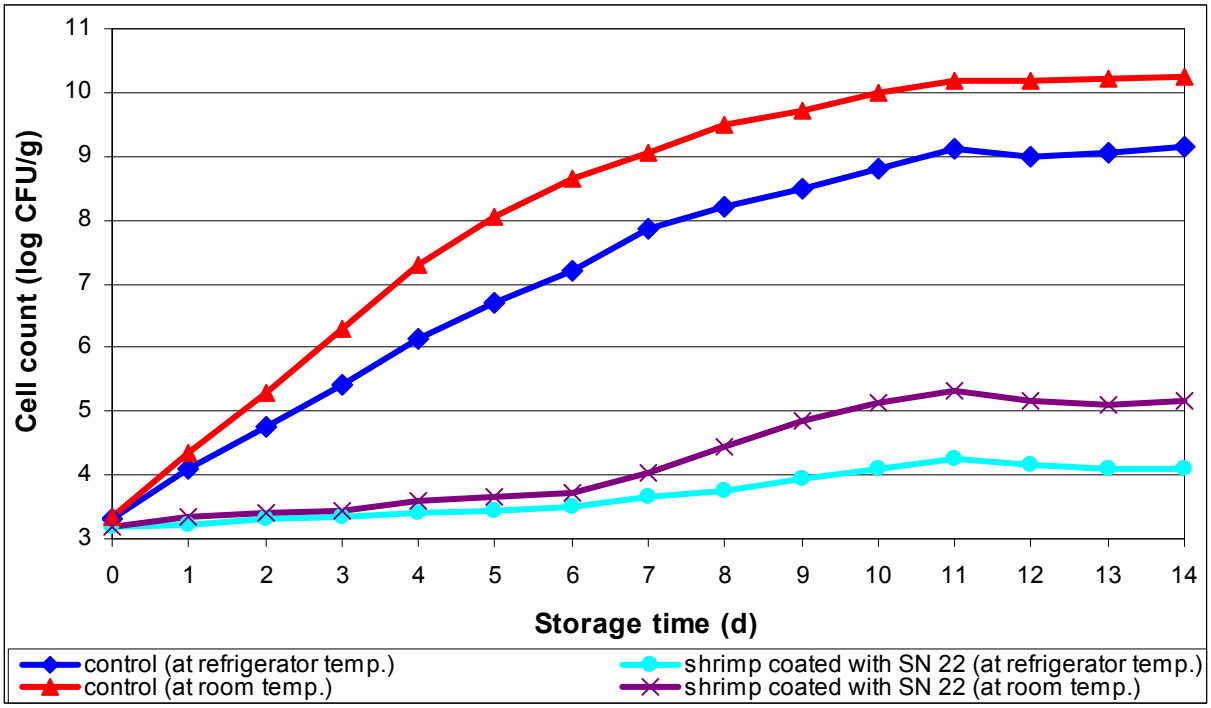


Figure 3.9: Total viable count of aerobic mesophilic bacteria on shrimp meat coated with chitosan SN 22 compared to control during storage at refrigerator and room temperature

The initial cell count of aerobic mesophilic bacteria of the shrimp meat was 1.5×10^3 CFU/g for both refrigerator and room temperature storage. The coated shrimp meat samples stored at refrigerator and room temperature showed a long lag phase up to day 6 of storage and reached total aerobic cell counts of 5.5×10^3 CFU/g and 6.5 ×

10^3 CFU/g respectively. After 6 days of storage, a slight growth became apparent for the coated samples stored at both temperatures. In this growth phase, the highest total aerobic cell count of 2.1 × 10^4 CFU/g of shrimp meat for refrigerated storage and 2.5 × 10^5 CFU/g for room temperature storage were reached. In addition, the growth phase of aerobic cells in the coated samples stored at room temperature increased more rapidly than that in the coated shrimp meat samples stored at refrigerator temperature. After 11 days of storage, the populations reached the stationary phase with the cell count of 1.5 × 10^4 CFU/g, which lasted to the end of storage. Furthermore, during the entire storage period of 14 days, the TVC of the coated shrimp meat samples stored at both temperatures never exceeded the level of 10^6 CFU/g of shrimp meat.

Unlike the coated samples with a long lag phase at the beginning of the storage period, there was no lag phase apparent for the uncoated shrimp meat (control) stored at both temperatures. Growth of the aerobic cell populations on the shrimp control exponentially increased from day 1 of storage. The exponential growth further steadily increased and reached the TVC at a value of 3.5 × 10^6 CFU/g on day 3 of room temperature storage, whereas at refrigerator temperature the value of 1.1 × 10^6 CFU/g was reached on day 4 of storage. The highest TVC values were reached on day 11 of storage with values of 1.1 × 10^9 CFU/g and 1.8 × 10^{10} CFU/g of shrimp meat for refrigerator and room temperature storage, respectively. After 11 days of storage, the populations reached the stationary phase with the cell count of 2.2 × 10^5 CFU/g, lasting until the end of storage (see Figure 3.9).

In general, the rate of microbial proliferation of the chitosan-coated shrimp meat was slower than that of the control samples. The growth of aerobic mesophilic bacteria on coated shrimp meat samples was effectively inhibited by chitosan charge SN 22 within the first 6 days of storage. In addition, the slowest bacterial growth was found

in the shrimp meat samples coated with chitosan charge SN 22 at refrigerator storage (see Figure 3.9).

3.3.2.2 Growth of gram-positive bacteria

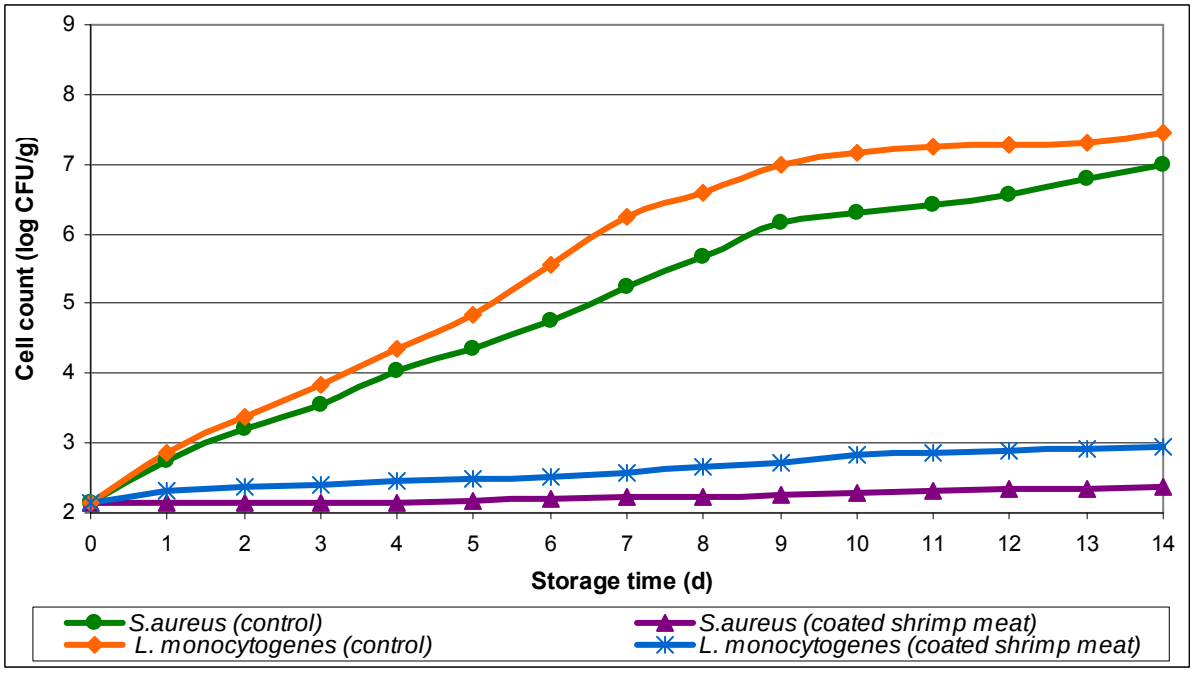
To evaluate the effect of chitosan charge SN 22 on growth of gram-positive bacteria on shrimp meat samples, the strains of *S. aureus* LMH 5P and *L. monocytogenes* LMH 34P were tested. To assess the growth of *S. aureus*, Baird Parker selective agar was used, whereas PALCAM-Listeria selective agar was used to determine the growth of *L. monocytogenes*. The coated shrimp meat samples were first suplemented with 0.1 mL aliquot of each gram-positive strain at a cell titre of 10^2 CFU/mL before storage at refrigerator and room temperature (see sections 2.5.3.2.1 and 2.5.3.2.2).

The effects of chitosan charge SN 22 on the growth of *S. aureus* and *L. monocytogenes* are presented in Figures 3.10 A and 3.10 B for refrigerator storage and room temperature storage, respectively.

The initial value of each tested strain through cell supplementation was 1.5×10^2 CFU/g for both storage temperatures. The rate of microbial proliferation of the coated shrimp meat samples was generally slower than that of the control samples, which showed an exponential phase immediately after the first day of storage, especially for the control samples stored at room temperature (Figure 3.10 B).

The growth of *S. aureus* and *L. monocytogenes* on shrimp meat samples coated with chitosan SN 22 at refrigerator and room temperature storage did not begin immediately, but a lag phase became apparent lasting up to day 6 of storage. After 7 days, the slight growth phase became apparent for the test strains at both storage temperatures.

A)



B)

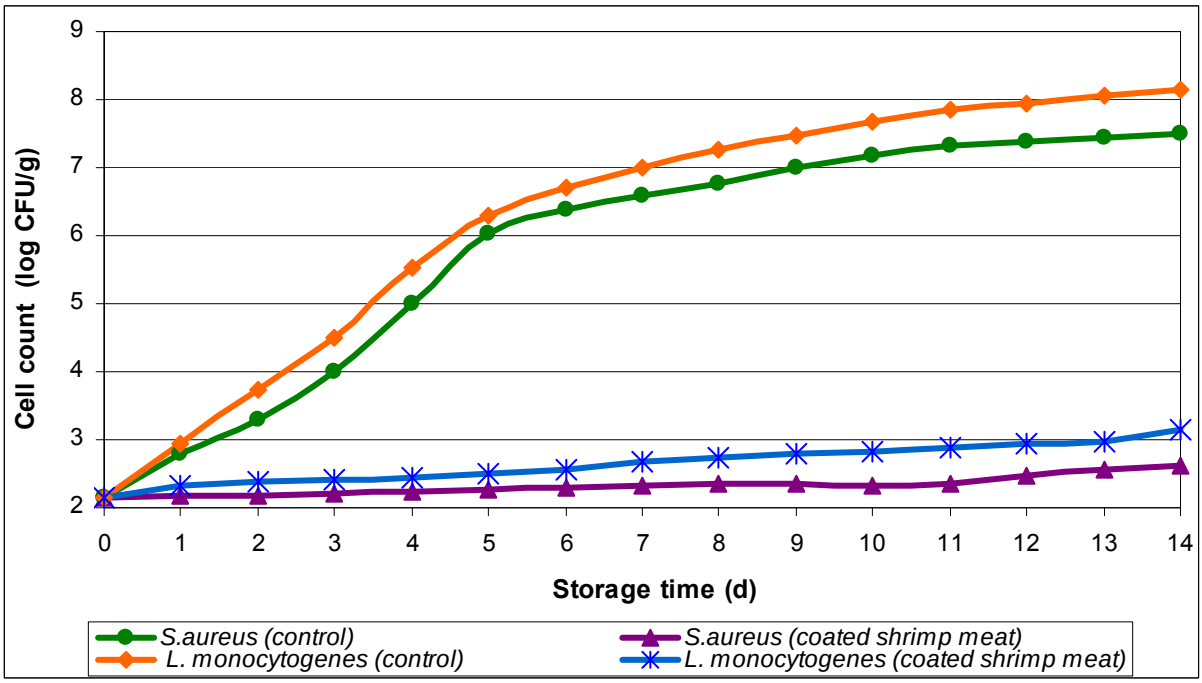


Figure 3.10: Growth of *S. aureus* and *L. monocytogenes* on shrimp meat coated with chitosan SN 22 compared to control during storage at **(A)** refrigerator temperature and **(B)** room temperature

During refrigerator storage, the growth of both test strains on the coated shrimp meat samples started to increase slightly just after 6 days. Starting on day 7 of storage, a slight growth of both test strains became apparent, in which the growth of *S. aureus*

reached a value of 2.1 × 10^2 CFU/g of shrimp meat and *L. monocytogenes* reached a value of 7.1 × 10^2 CFU/g of shrimp meat (Figure 3.10 A). The growth of these strains further on increased slightly up to day 12 of storage, when the slight growth phase ended. After 12 days of storage, the populations of both tested strains reached the stationary phase, with cell counts of 4.0 × 10^2 CFU/g and 9.7 × 10^2 CFU/g of shrimp meat for *S. aureus* and *L. monocytogenes*, respectively. This stationary phase lasted until the end of storage. The results show that the growth rate of *L. monocytogenes* LMH 34P was distinctly higher than that of *S. aureus* LMH 5P during refrigerator storage (Figure 3.10 A).

The growth of both test strains on the coated shrimp meat samples stored at room temperature showed a similar trend to those stored at refrigerator temperature. However, the growth rates of these strains were higher on coated shrimp meat samples stored at room temperature. For example, after 7 days of room temperature storage, the growth of *S. aureus* reached a value of 3.2 × 10^2 CFU/g and the growth of *L. monocytogenes* increased to 8.0 × 10^2 CFU/g of shrimp meat (Figure 3.10 B). After that, the populations of these strains increased until the end of storage, with the final values of 6.1 × 10^2 CFU/g and 1.5 × 10^3 CFU/g for *S. aureus* and *L. monocytogenes*, respectively. The growth rate of *L. monocytogenes* LMH 34P was higher than that of *S. aureus* LMH 5P during room temperature storage (Figure 3.10 B).

In general, the growth rates of both test strains on chitosan-coated shrimp meat samples were much lower than those of the control samples at both storage temperatures. The growth rates of both test strains on the control samples exponentially increased from the first day of storage. This exponential growth further on increased quickly and reached a level of 10^6 CFU/g of shrimp meat after 4 days of room temperature storage (Figure 3.10 B). After 6 days of refrigerated storage, a

level of 10^6 CFU/g of shrimp meat for the growth of *L. monocytogenes* was reached, whereas *S. aureus* reached this level after 8 days. The exponential phase of *L. monocytogenes* ended on day 11 of storage, after that the stationary phase began with the cell count of 4.5×10^7 CFU/g of shrimp meat, which lasted up to the end of storage (Figure 3.10 A).

3.3.2.3 Growth of gram-negative bacteria

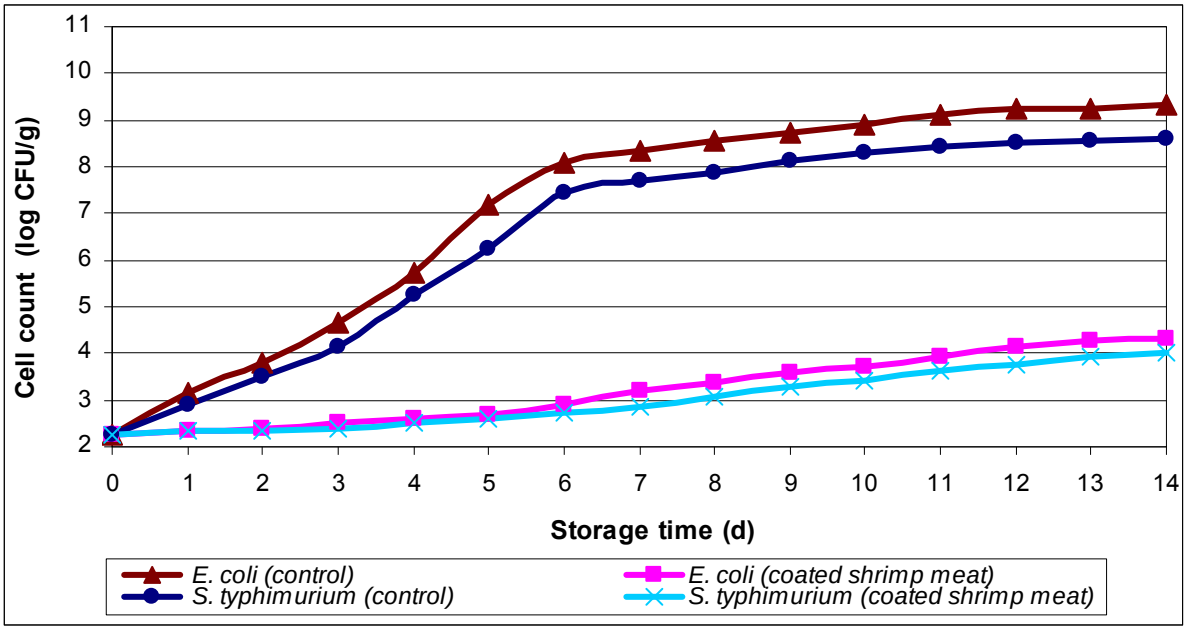
To assess the effects of chitosan charge SN 22 on growth of gram-negative bacteria on shrimp meat samples coated with chitosan SN 22, the test strains of *E. coli* LMH 1N and *S. typhimurium* LMH 2N were used. The growth of *E. coli* LMH 1N was determined using selective MacCONKEY agar, while growth of *S. typhimurium* LMH 2N was evaluated using xylose lysine deoxycholate (XLD) agar (see sections 2.5.3.3.1 and 2.5.3.3.2).

The effects of chitosan charge SN 22 on growth of *E. coli* and *S. typhimurium* on shrimp meat are presented in Figures 3.11 A and 3.11 B for samples stored at refrigerator temperature and room temperature, respectively.

The initial value of each test strain through cell supplementation was 1.5×10^2 CFU/g for both storage temperatures. The growth rate of the tested gram-negative strains on coated shrimp meat samples was generally higher than those of the tested gram-positive strains.

During refrigerated storage, chitosan SN 22 effectively inhibited both gram-negative strains tested on the shrimp meat samples, thereby a lag phase of the test strains became apparent lasting up to day 3 of storage (Figure 3.11 A). However, the growth of both test strains slightly increased after 3 days, especially for *E. coli*, which reached a value of 5.5×10^2 CFU/g of shrimp meat. After 7 days of refrigerated storage, the growth of both strains clearly started to increase (Figure 3.11 A).

A)



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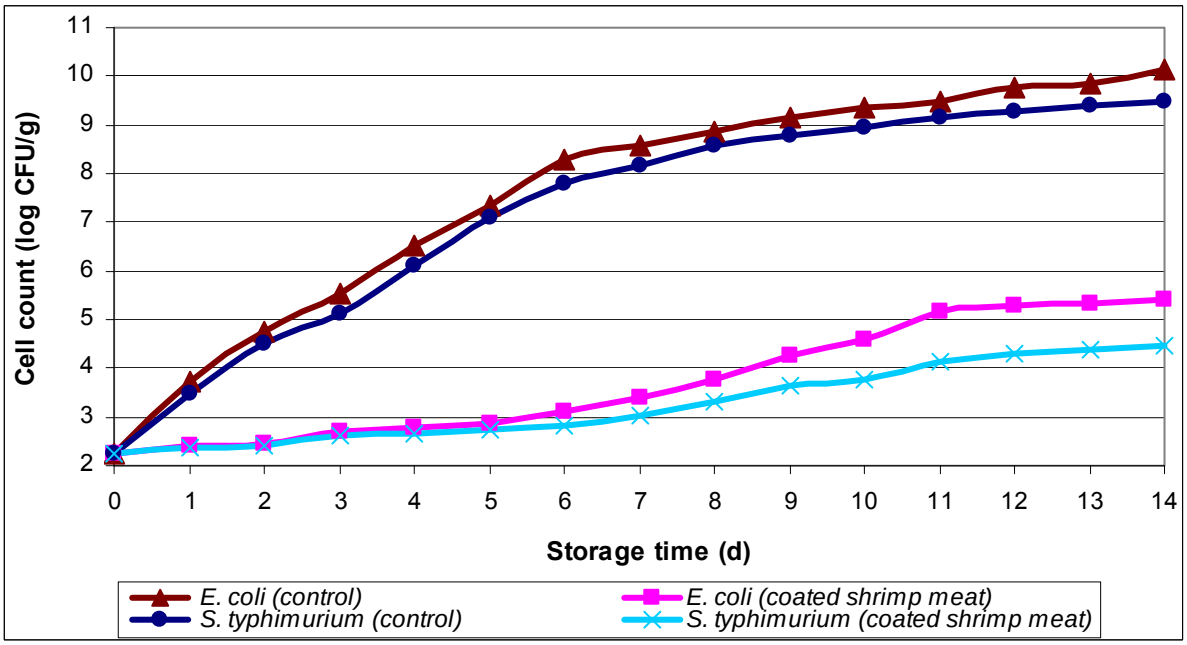


Figure 3.11: Growth of *E. coli* and *S. typhimurium* on shrimp meat coated with chitosan SN 22 compared to control during storage at **(A)** refrigerator temperature and **(B)** at room temperature

During the growth phase at refrigerator temperature, distinct changes in growth of both strains appeared, in which the growth of *E. coli* was generally higher than *S. typhimurium*. For example, as shown in Figure 3.11 A, on day 7 of storage, the

growth of *E. coli* reached a value of 1.2 × 10^3 CFU/g of shrimp meat, whereas the growth of *S. typhimurium* reached a lower value of 8.2 × 10^2 CFU/g of shrimp meat. After 7 days of storage, both strains on coated shrimp meat samples further grew until day 12 and reached a value of 7.3 × 10^3 CFU/g of shrimp meat for *S. typhimurium*, whereas *E. coli* grew up to a value of 1.1 × 10^4 CFU/g of shrimp meat. On day 12 of refrigerated storage, the growth of both strains on coated samples stopped. Afterwards, both strains entered the stationary phase, in which *S. typhimurium* reached a value of 9.9 × 10^3 CFU/g of shrimp meat and *E. coli* could slowly grow up to a value of 2.2 × 10^4 CFU/g of shrimp meat at the end of storage (Figure 3.11 A).

As presented in Figure 3.11 B, a similar trend was shown on growth of both test gram-negative strains on shrimp meat coated with SN 22 during storage at room temperature. In the beginning of storage, a lag phase of both tested strains was apparent up to day 2. However, changes in growth of these strains were already found on day 3, at which the growth of *S. typhimurium* slightly increased to 6.1 × 10^2 CFU/g of shrimp meat, whereas *E. coli* reached a value of 7.2 × 10^2 CFU/g of shrimp meat. These strains on the coated samples entered the growth phase simultaneously on day 6 of storage. Starting on day 7 of room temperature storage, the growth rate between *S. typhimurium* and *E. coli* were more distinct than those at refrigerator temperature. In general, during room temperature storage, the growth rate of both test strains was higher than that stored at refrigerator temperature (see Figures 3.11 A and 3.11 B).

As shown in Figure 3.11 B, the growth of *E. coli* steadily increased starting on day 7 up to day 11 of storage, at which *E. coli* reached a value of 1.5 × 10^5 CFU/g of shrimp meat. In comparison to that, growth of *S. typhimurium* was much lower until day 11 of storage, at which this strain reached a value of 1.1 × 10^4 CFU/g of shrimp

meat. The stationary phase began on day 12 until the end of storage, in which the growth of the test strains further slowly increased up to a value of 4.5×10^4 CFU/g and 5.5×10^4 CFU/g for *S. typhimurium* LMH 2N and *E. coli* LMH 1N, respectively.

Unlike to growth of the test strains on coated shrimp meat samples which possessed a long lag phase during the first 2–3 days of storage at both temperatures, the test strains on control samples entered the exponential growth phase immediately on the first day of storage at both temperatures. Starting on day 5 of refrigerated storage, the growth of both strains reached the values of 10^6 and 10^7 CFU/g of shrimp meat for *E. coli* and *S. typhimurium*, respectively (see Figures 3.11 A). After 11 days of storage, the populations of both strains entered the stationary phase, reaching the cell counts of 2.2×10^9 CFU/g and 8.1×10^8 CFU/g of shrimp meat for *E. coli* and *S. typhimurium*, respectively. This stationary phase lasted until the end of storage (Figure 3.11 A).

Similar growth trends were found for both strains on control samples stored at room temperature (Figure 3.11 B), although their growth rate were higher than those at refrigerator temperature (Figure 3.11 A). As shown in Figure 3.11 B, the exponential growth of *E. coli* reached a value of 2.5×10^7 CFU/g of shrimp meat on day 5 of storage, whereas *S. typhimurium* reached a value of 1.1×10^7 CFU/g of shrimp meat This exponential growth lasted up to day 11 of storage, and then the populations of both test strains entered the stationary phase until the end of storage. However, it seemed that slow growth of both strains may still occur during this phase, especially for *E. coli* LMH 1N on control samples stored at room temperature with increasing of the population counts less than 1 log unit (Figure 3.11 B).

3.3.3 Biochemical quality assessment of shrimp meat samples

3.3.3.1 pH value

To determine the effect of chitosan SN 22 on changes in pH value of shrimp meat during storage, a homogenate of coated shrimp meat samples in 0.9 % (w/v) sodium chloride was used (see section 2.5.4.1).

The initial pH values of the shrimp meat homogenate were in the range of 7.0–7.1. During storage at refrigerator and room temperature, the pH values increased to a pH range of pH 7.2–8.3. Changes in pH values of shrimp meat samples during 14 days of storage are presented in Figure 3.12.

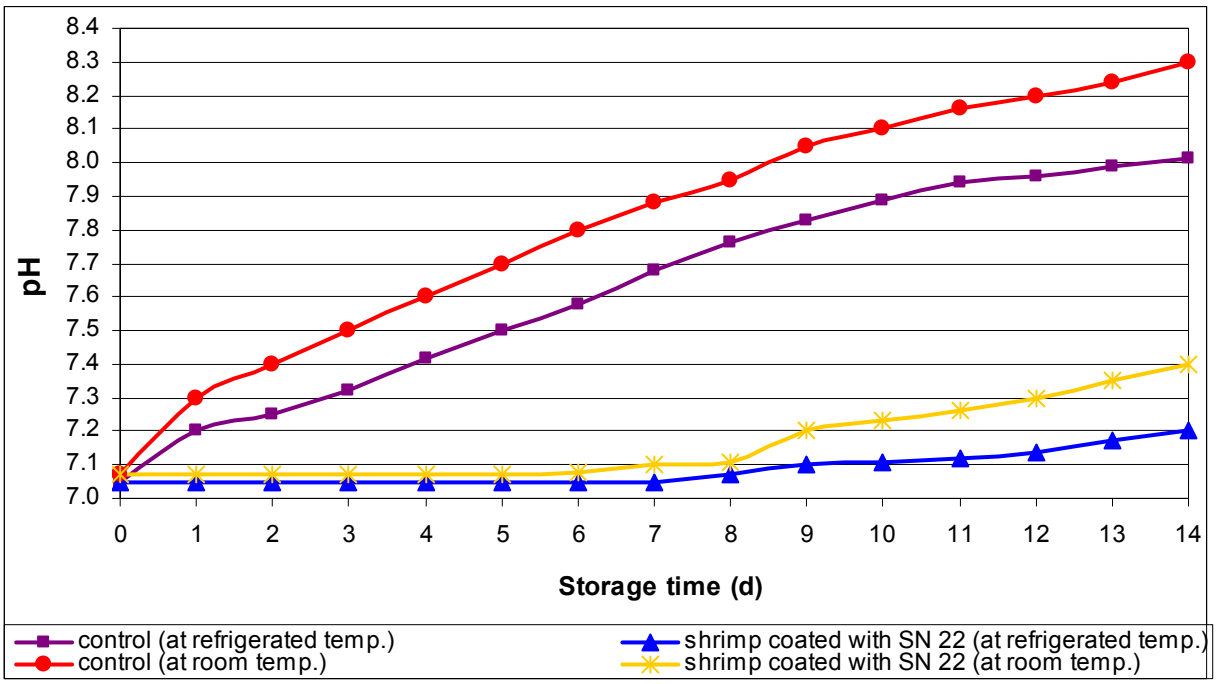


Figure 3.12: Changes in pH value of shrimp meat coated with chitosan SN 22 compared to control during storage at refrigerator and room temperature

The pH values of coated shrimp meat samples stored at both temperatures were shown to be quite similar until day 6 of storage (pH ≤ 7.1). However, starting from day

7, pH values of these samples started to increase slightly until day 9 of storage. Significant differences between pH values of these samples were found after 9 days. The differences continuously increased up to the end of storage time with pH value of 7.2 for fridge-stored samples and 7.4 for samples stored at room temperature.

In general, the pH values of control shrimp meat were higher than those of coated samples stored at both temperatures. For example, pH value of coated shrimp meat samples stored at refrigerator temperature started to increase significantly just after 8 days of storage with pH value of 7.1, whereas the pH value of the control samples at the same temperature already increased starting from day 1 of storage, when it reached a pH value of 7.2. A similar trend was found for control shrimp meat stored at room temperature, at which a very sharp increase was already found on day 1 of storage, when it reached a pH value of 7.3 (Figure 3.12).

The pH value of the control samples stored at refrigerator temperature increased steadily from day 1 of storage and reached pH 7.7 on day 7 of storage. This steady increase lasted up to the end of storage and reached the final pH value of 8.0. During room temperature storage, increase in pH value of control shrimp meat was much higher and reached pH 7.7 on day 5 of storage. This increase lasted up to the end of storage, when it reached a value of 8.3.

3.3.3.2 Water activity value

Determination of a_w value in this work was performed to study the effect of chitosan SN 22 on changes in freshness of shrimp meat during storage. Changes in water activity of shrimp meat coated with chitosan SN 22 compared to uncoated samples (control) during storage at refrigerator and room temperature are presented in Figure 3.13.

The initial a_w values of the shrimp meat samples in the present study were of 0.99. During refrigerated storage, the initial a_w values of coated shrimp meat samples could be maintained by chitosan SN 22 until day 13 of storage. A slight decrease was found at the end of the refrigerated storage, when a_w reached a value of 0.98.

During room temperature storage, chitosan SN 22 could keep the initial a_w values (0.99) until day 3 of storage. Water activity (a_w) of the coated samples started to decrease slightly down to 0.98 on day 4, whereupon this a_w value could be maintained up to day 11 of storage. On day 12 of storage, a_w further decreased down to 0.97, reaching a value of 0.96 at the end of room temperature storage.

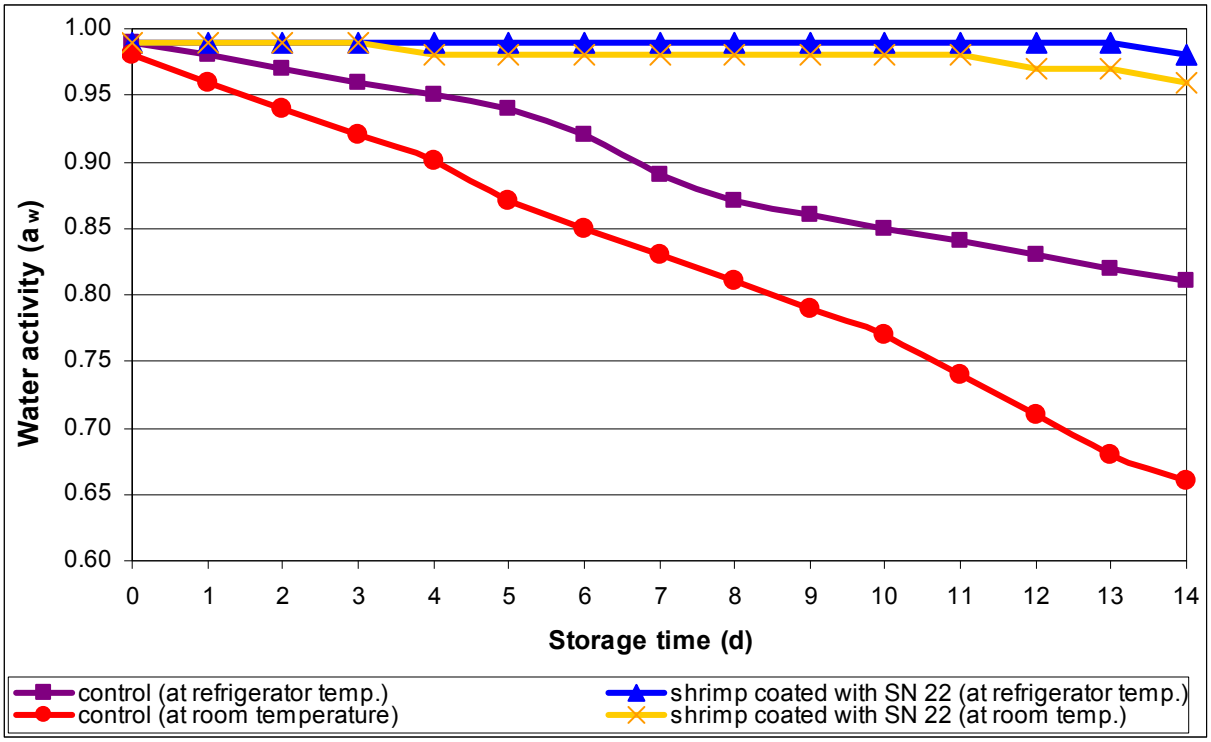


Figure 3.13: Changes in a_w value of shrimp meat coated with chitosan SN 22 compared to control during storage at refrigerator and room temperature

For uncoated shrimp meat samples (control), the initial a_w of 0.99 began slightly to decrease down to 0.98 on day 1 of storage, and later further decreased until the end

of refrigerated storage down to 0.81. For the control samples stored at room temperature, the first decrease of the initial a_w of 0.99 to 0.96 occurred from day 1 of storage, followed by a steady decrease until the end of storage down to 0.67. Both the final a_w values of 0.81 and 0.67 of the control samples exceeded the lower limit of acceptable a_w value for shrimp meat freshness (0.95), according to Fontana (2000).

3.3.3.3 Content of TVBN

In this work, the determination of TVBN content consists of 3 steps, namely preparation of samples, water-steam-distillation process, and titration of distillate. The preparation of samples was performed by extracting shrimp meat samples using 0.6 N perchloric acid (see section 2.5.4.3).

Changes in TVBN content of shrimp meat samples are presented in Figure 3.14. The initial TVBN contents were close to 10 mg/100 g of shrimp meat at both storage temperatures.

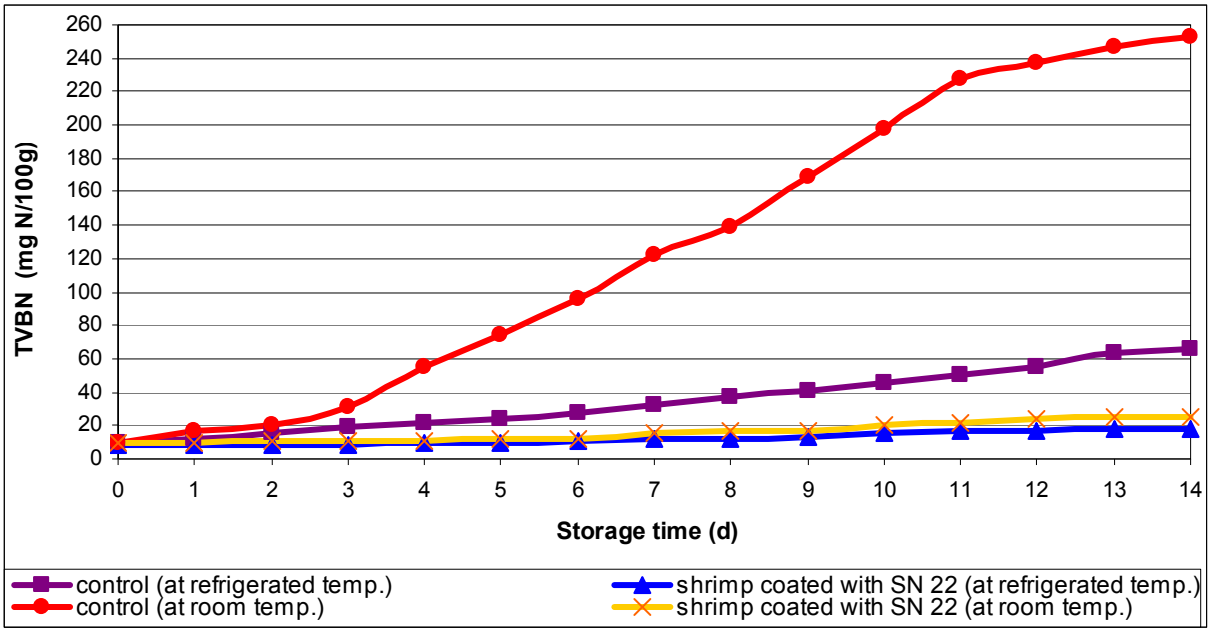


Figure 3.14: Contents of TVBN of shrimp meat coated with chitosan SN 22 compared to control during storage at refrigerator and room temperature

In general, TVBN content increased with time of storage at refrigerator and room temperature. A latency period was observed in TVBN accumulation of coated shrimp meat samples stored at refrigerator temperature and room temperature. Significant differences between the TVBN contents of coated shrimp meat samples and their controls were found on day 7 during refrigerated storage, whereas during storage at room temperature they were already found on day 3.

In general, the TVBN contents of control samples were higher than those of the coated samples. The TVBN content of shrimp meat samples stored at refrigerator temperature started to increase slightly just after 9 days of storage, whereas the TVBN content of the control increased rapidly after 3 days of storage. The maximum TVBN content of the coated samples at the end of storage was 18.45 mg/100 g of shrimp meat. In contrast, the increase in TVBN content of the control samples reached a maximum level of 66.15 mg/100 g of shrimp meat at the end of storage. During room temperature storage, TVBN content of coated shrimp meat increased to 15.06 mg/100 g of shrimp meat after 7 days, reaching a level of 25.33 mg/100 g at the end of storage.

TVBN content of the control shrimp meat reached a level of 37.30 mg/100 g of shrimp meat on day 3 of room temperature storage. This increase lasted up to the end of storage at room temperature, when it reached a level of 258.52 mg/100 g of shrimp meat (Figure 3.14).

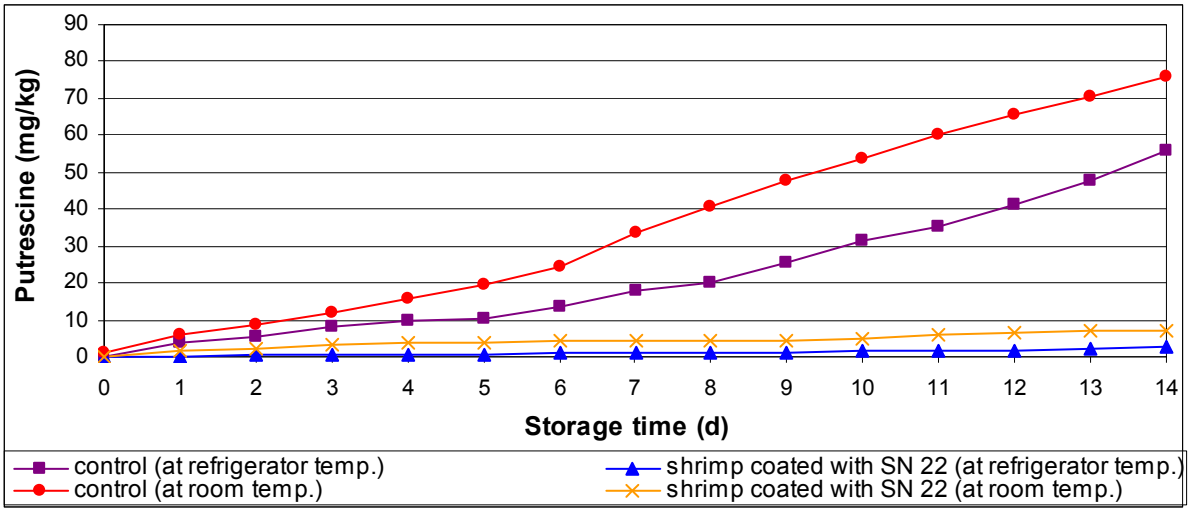
3.3.3.4 Content of biogenic amines

The quantitative contents of biogenic amines were determined by HPLC with a fluorescence detector after derivatization performed by *o*-Phthaldialdehyde (see section 2.5.4.4). The quantitative changes in biogenic amines content of shrimp meat

coated with chitosan SN 22 during a 14-day storage period at refrigerator and room temperature are presented in Figures 3.15 A–E.

During refrigerated storage, the only biogenic amine found on day 5 of was putrescine, at a concentration of < 1 mg/kg (Figure 3.15 A). Another biogenic amine found on coated shrimp meat later was cadaverine (Figure 3.15 B).

A)



B)

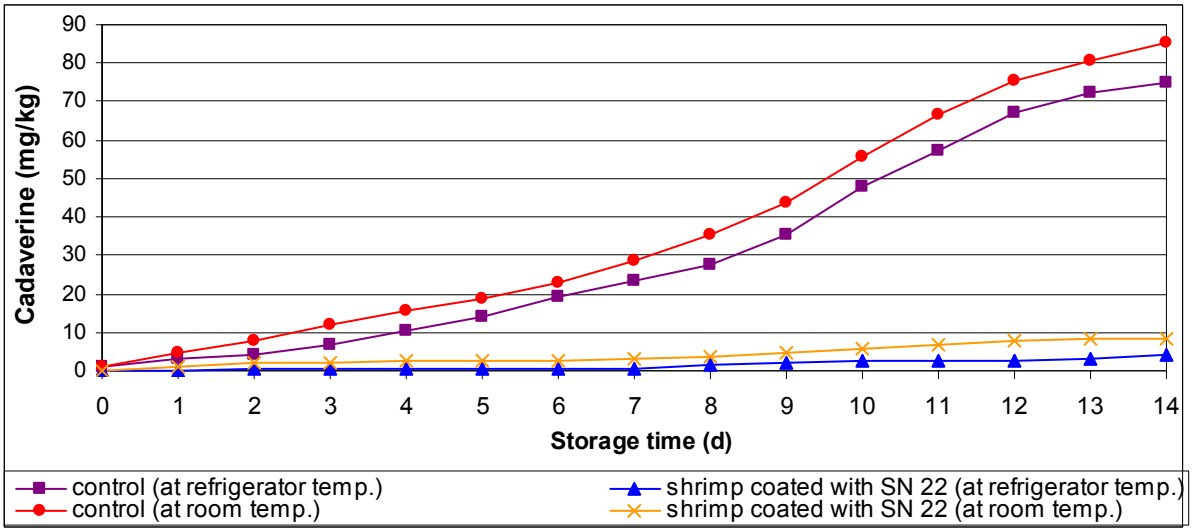


Figure 3.15 A, B: Changes in the content of **(A)** putrescine and **(B)** cadaverine of the shrimp meat coated with chitosan SN 22 compared to control during storage at refrigerator and room temperature

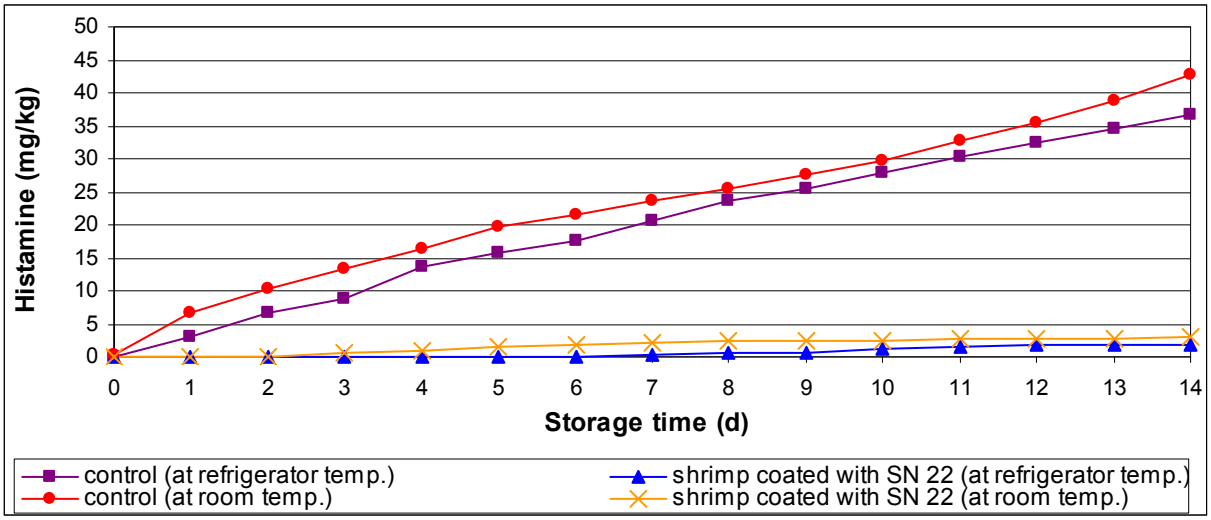
The concentration of putrescine and cadaverine started to increase slightly on day 7 of refrigerated storage. These biogenic amines further increased in concentration up

to the end of storage, at which the concentration of cadaverine (4.5 mg/kg of shrimp meat) was higher than the concentration of putrescine (2.5 mg/kg of shrimp meat), as shown in Figures 3.15 A–B. In contrast to other biogenic amines, putrescine and cadaverine appeared initially on coated shrimp meat when stored at room temperature. The two biogenic amines in coated shrimp meat increased gradually in their concentration up to the end of storage. It is worthwhile to note that the content of putrescine became lower than cadaverine content after day 9 until the end of storage, at which the concentration of putrescine and cadaverine reached a value of 7.3 mg/kg and 8.5 mg/kg of shrimp meat, respectively. Regarding the control of both biogenic amines, their content reached levels of > 50 mg/kg after 14 days of storage at both temperatures (Figures 3.15 A–B).

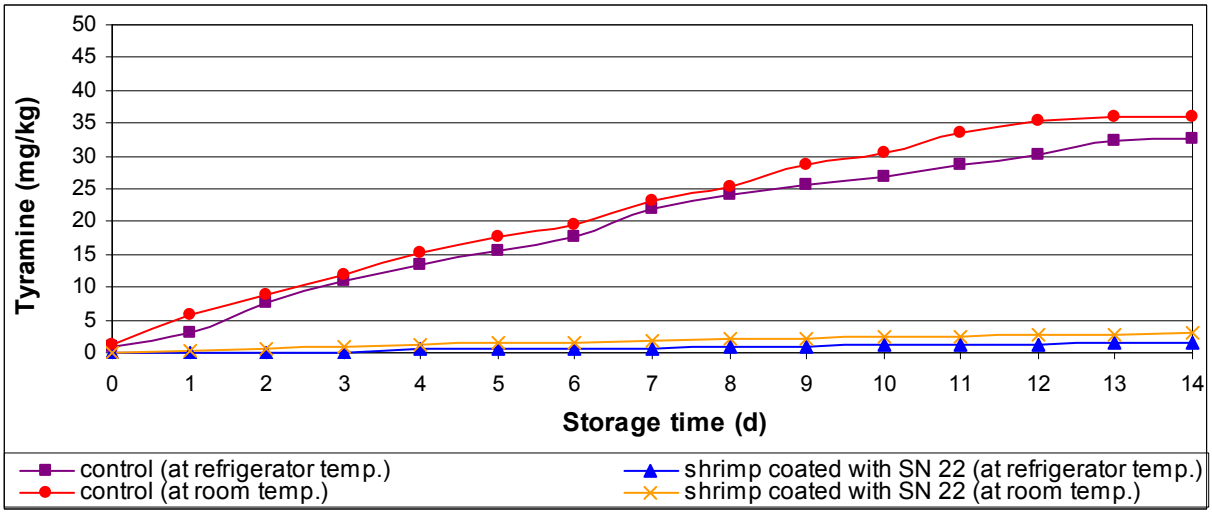
Histamine, tyramine, and agmatine in coated shrimp meat samples were detectable to some extent, particularly after 7 days of room temperature storage (Figures 3.15 C–E). Histamine slightly increased at a level of < 1 mg/kg on day 3 until day 8 of room temperature storage, but no further distinct changes were seen after that up to the end of storage. Histamine reached the final content at a level of 2 mg/kg and 3 mg/kg at the end of storage at refrigerator and room temperature, respectively. In contrast to the coated samples, the content of histamine in the control samples reached levels of more than 35 mg/kg of shrimp meat at the end of storage at both temperatures (Figure 3.15 C).

Tyramine and agmatine in coated samples simultaneously appeared slightly on the first day and remained detectable up to day 11 of room temperature storage. Later, agmatine increased to a level of 4 mg/kg, which was slightly higher than the content of tyramine (3 mg/kg), at the end of room temperature storage. Both tyramine and agmatine in coated samples reached the same concentration of 2 mg/kg at the end of refrigerated storage (Figures 3.15 D–E).

C)



D)



E)

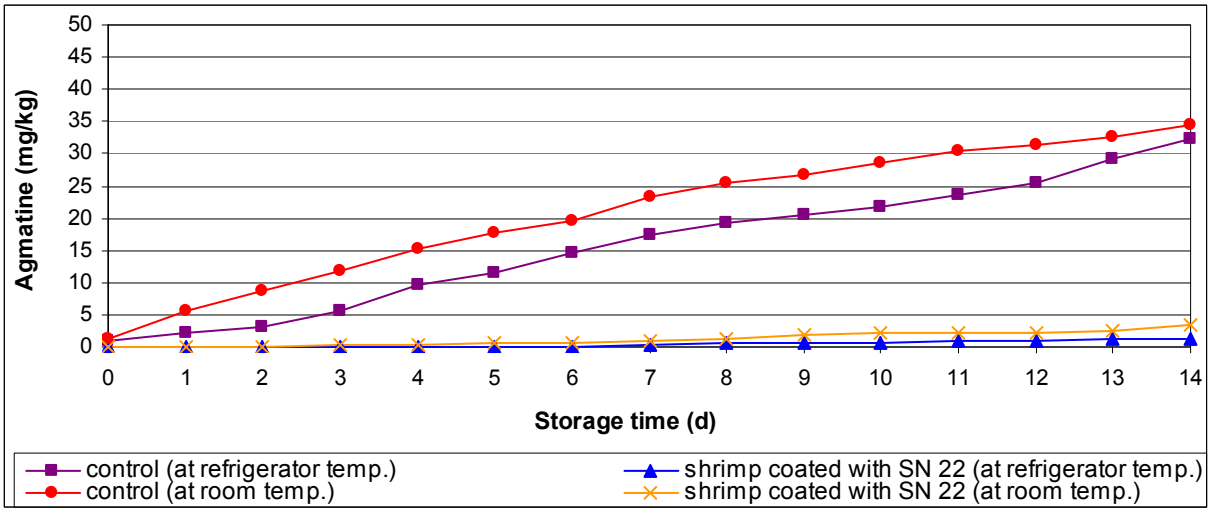


Figure 3.15 C, D, E: Changes in content of **(C)** histamine, **(D)** tyramine, and **(E)** agmatine of shrimp meat coated with chitosan SN 22 compared to control during storage at refrigerator and room temperature

Unlike to the coated samples, the content of tyramine and agmatine in the control reached the final levels of more than 30 mg/kg at the end of storage at both temperatures (Figures 3.15 D–E).

In general, no significant changes in the concentration of histamine, tyramine, and agmatine were found in the coated samples during storage at both temperatures, compared to their control. At the end of storage, the concentrations of these biogenic amines in coated samples were < 5 mg/kg (Figures 3.15 C–E).

3.4 Testing the potency of chitosan enhanced with garlic extract for shrimp meat preservation

Chitosan charge SN 22 showed a potency to control changes in microbiological parameters and the biochemical parameters during a 14-day storage period (see Figure 3.1). Furthermore, the optimum conditions obtained from experiments without garlic enforcement were used to treat 25 g shrimp meat in order to evaluate changes in microbiological parameters and biochemical parameters during storage in the full-scale step. In this case, the storage period of samples was prolonged to 30 days in order to ensure an efficacy of chitosan SN 22 for shrimp meat preservation. In addition, the weight of the test samples in the full-scale step was 25 g of shrimp meat, according to the recommendation of the microbiological assay for cooked shrimps (EU guideline 1994). The procedures in the preliminary study were also performed for the full-scale study.

3.4.1 Enhancing antimicrobial activity of chitosan by incorporating garlic extract

Based on the results of antimicrobial activity and minimum inhibitory (MIC) of chitosan SN 22, it is evident that chitosan SN 22 markedly inhibited growth of the test

strains at a concentration of 0.1 % (w/v). However, chitosan SN 22 mainly showed stronger antimicrobial effect against the gram-positive strains than against gram-negative strains tested. Therefore, it is necessary to improve the antimicrobial efficacy of chitosan SN 22 against the tested gram-negative strains, so that their growth on preserved shrimp meat samples can be reduced more effectively. For this purpose, chitosan SN 22 coating solution was enforced with garlic extract.

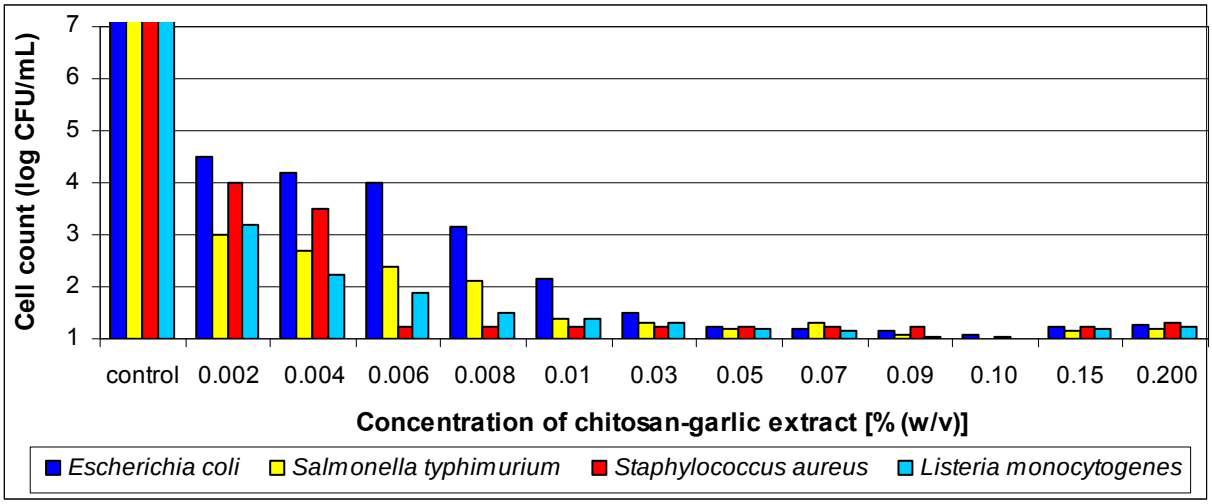
3.4.1.1 Antimicrobial activity of chitosan-garlic extract solution

Antimicrobial activity of chitosan enforced with garlic extract (Ch-G) solution in 1 % (v/v) acetic acid with different concentrations of 0.002–0.20 % (w/v) was determined by the viable cell count method. For that purpose, the four strains causing spoilage on shrimp meat were tested (see Table 2.2). The antimicrobial activity of Ch-G solution at each concentration is indicated by the differences of the amount of viable cells of controls and the test strains, which could further grow in the presence of each Ch-G solution during incubation (see section 2.2.5.3).

The inhibitory effects of Ch-G solution were compared to the inhibitory effects of garlic extract at the same concentrations of 0.002–0.20 % (w/v) (see section 2.2.5.2). The inhibitory activity of Ch-G and the inhibitory activity of garlic extract against the test strains are presented in Figures 3.16 A–B.

The optimum concentration of Ch-G solution was seen at a concentration of 0.1 % (w/v) (Figure 3.16 A). The Ch-G solution showed a strong antibacterial activity against the four bacterial strains tested, particularly against *L. monocytogenes* and *S. typhimurium,* whereby these strains were completely suppressed at a concentration of 0.1 % (w/v). The growth of *E. coli* and *S. typhimurium* were almost completely inhibited at this optimum concentration, reaching the cell counts < 10 CFU/mL (Figure 3.16 A).

A)



B)

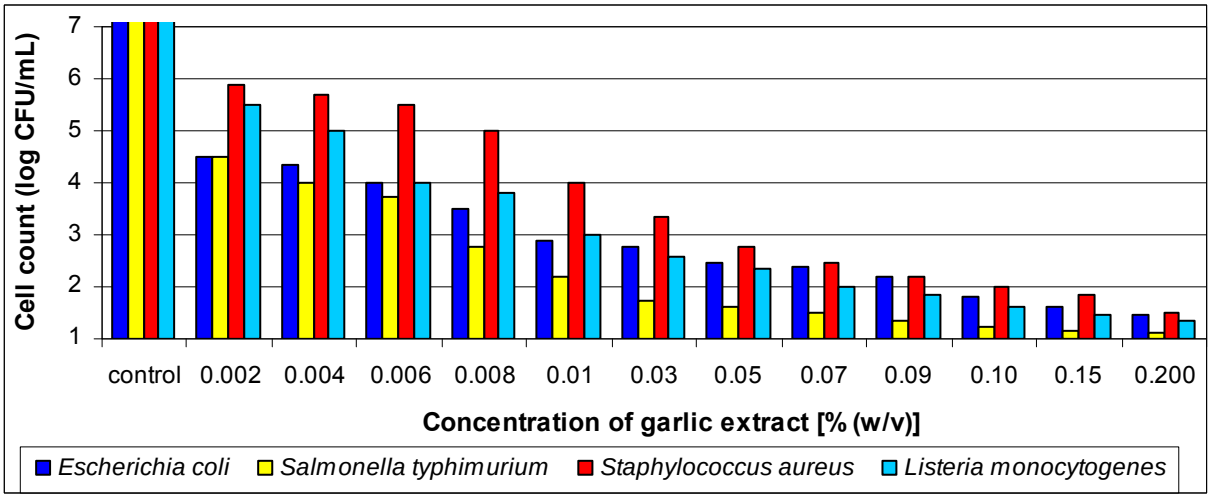


Figure 3.16: The inhibitory activity of various concentrations of **(A)** chitosan-garlic extract and **(B)** garlic extract on the tested strains compared to control

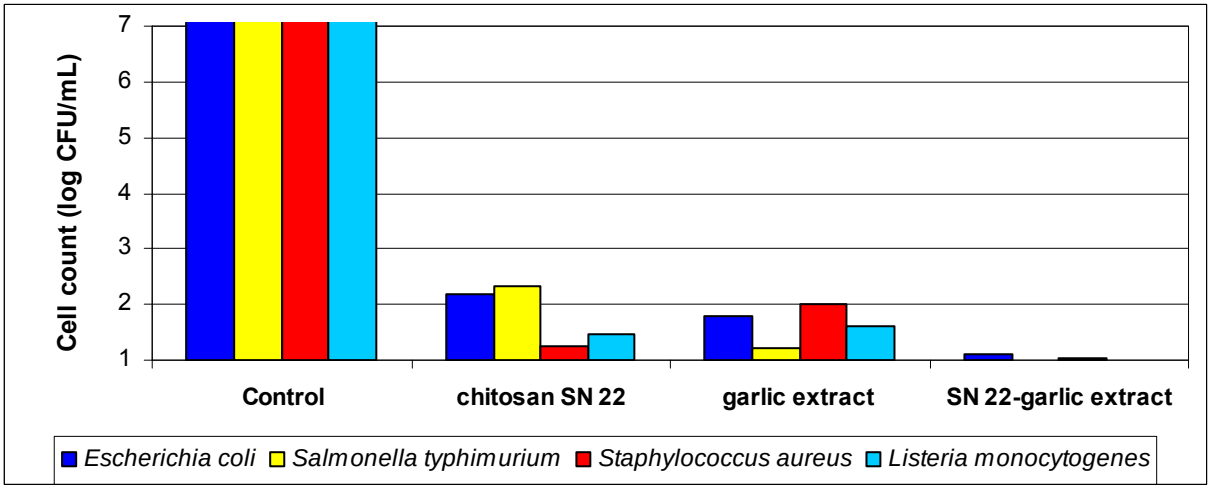


Figure 3.17: Comparison of inhibitory activity of chitosan SN 22 and chitosan-garlic extract at the optimum concentration of 0.1 % (w/v)

Garlic extract markedly inhibited the growth of the four test strains and showed a stronger antimicrobial effect against the test gram-negative strains than against the test gram-positive strains (Figure 3.16 B). At the optimum concentration of 0.1 % (w/v), *E. coli* and *S. typhimurium* were more effectively inhibited, thereby its initial cell count of 10^7 CFU/mL could be reduced to 8.5×10^1 CFU/mL and 2.1×10^1 CFU/mL, respectively (Figure 3.16 B).

In Figure 3.17, the differences between inhibitory activity of chitosan SN 22 and chitosan-garlic extract at the optimum concentration of 0.1 % (w/v), compared to control are shown. At the same concentration of 0.1 % (w/v), chitosan SN 22 showed stronger inhibitory activity against the tested gram-positive strains than against the test gram-negative strains, especially against *S. aureus,* where its initial cell count of 10^7 CFU/mL can be reduced to 10^1 CFU/mL by chitosan SN 22.

On the contrary, garlic extract showed stronger inhibitory effect against the gram-negative strains and showed a weaker antimicrobial activity concerning *S. aureus*. Based on to the antimicrobial characteristics of chitosan and garlic extract, it is worthwhile to combine both of them to obtain the optimum antimicrobial activity against the four bacterial strains tested in this present work (Figure 3.17).

3.4.2 Microbiological quality assessment of shrimp meat samples coated with chitosan-garlic extract

3.4.2.1 Total viable count of aerobic mesophilic bacteria

The initial cell count of aerobic mesophilic bacteria of shrimp meat coated with chitosan-garlic extract were 1.5×10^3 CFU/g for refrigerator and room temperature storage. As presented in Figure 3.18, shrimp meat samples coated with chitosan-garlic extract showed a long lag phase up to day 14 of storage at both temperatures.

After that, a slight growth phase was seen up to day 24 of storage, in which the growth of aerobic mesophilic bacteria of the coated shrimp meat reached total cell counts of 1.2×10^4 CFU/g of shrimp meat during refrigerator storage. The growth of aerobic mesophilic bacteria of the coated samples stored at room temperature was more pronounced and reached total cell counts of 7.5×10^4 CFU/g of shrimp meat at the end of storage (Figure 3.18).

In contrast to the coated samples that showed a long lag phase in the beginning of storage, there was no lag phase apparent for control samples stored at both temperatures. The exponential phase of aerobic cell populations of control samples began on day 1 of storage. This exponential phase further increased and reached the highest value of total aerobic cell counts on day 16 of storage with values of 5.1×10^9 CFU/g and 2.5×10^{10} CFU/g of shrimp meat for refrigerator and room temperature storage, respectively.

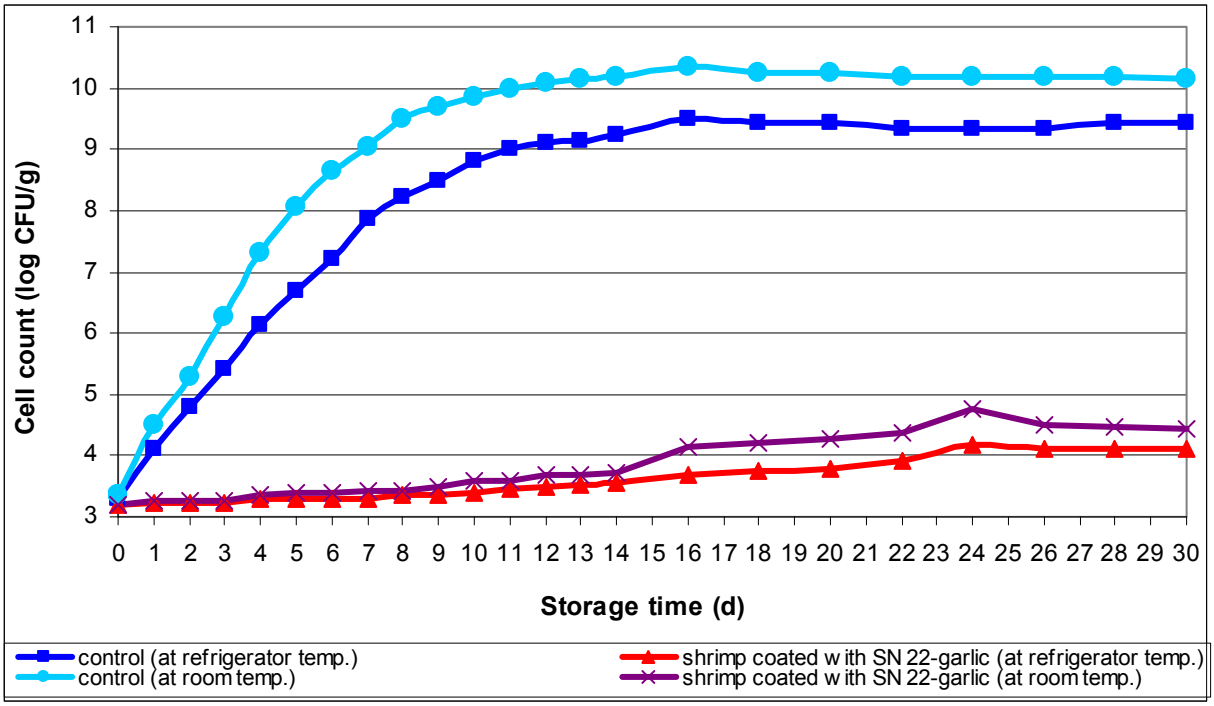


Figure 3.18: Total viable count of aerobic mesophilic bacteria of shrimp meat coated with chitosan-garlic extract compared to control during storage at refrigerator and room temperature

The aerobic cell populations of the control samples entered the stationary phase after 16 days of storage, reaching the cell count of 4.5×10^9 CFU/g and 1.5×10^{10} CFU/g for refrigerated and room temperature storage, respectively. This stationary phase lasted until the end of storage (Figure 3.18).

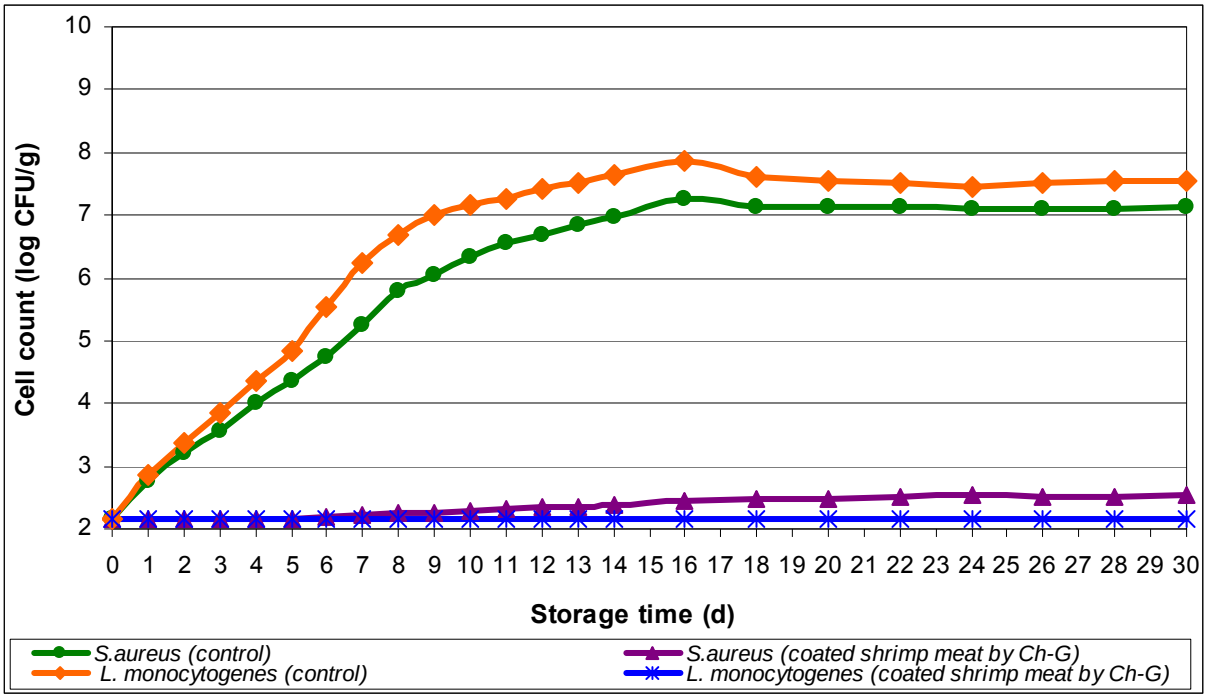
In general, the rate of microbial proliferation of the chitosan-coated shrimp meat was slower than that of the control. The growth of total aerobic cell count on coated shrimp meat samples was effectively inhibited by chitosan SN 22 enforced with garlic extract during 30 days of storage. In addition, the slowest bacterial growth was found on shrimp meat samples coated with chitosan-garlic extract at refrigerator storage (Figure 3.18).

3.4.2.2 Growth of gram-positive bacteria

To evaluate the effect of chitosan charge SN 22 on changes in growth of *S. aureus* LMH 5P and *L. monocytogenes* LMH 34P on shrimp meat coated with chitosan-garlic extract, 25 g of coated shrimp meat samples were inoculated with 0.1 mL aliquot of each test strain at a cell titre of 10^2 CFU/mL prior to storage at refrigerator and room temperature. The same protocol was performed for the control (uncoated shrimp meat).

The effect of chitosan SN 22 enforced with garlic extract on growth of gram-positive bacteria is shown in Figures 3.19 A and B. The initial value of each test strain through cell supplementation was 1.5×10^2 CFU/g for refrigerator and room temperature storage. The rate of microbial proliferation on coated shrimp meat samples was generally slower than on controls which show exponentially growth immediately after the first day of storage, especially on the control samples stored at room temperature.

A)



B)

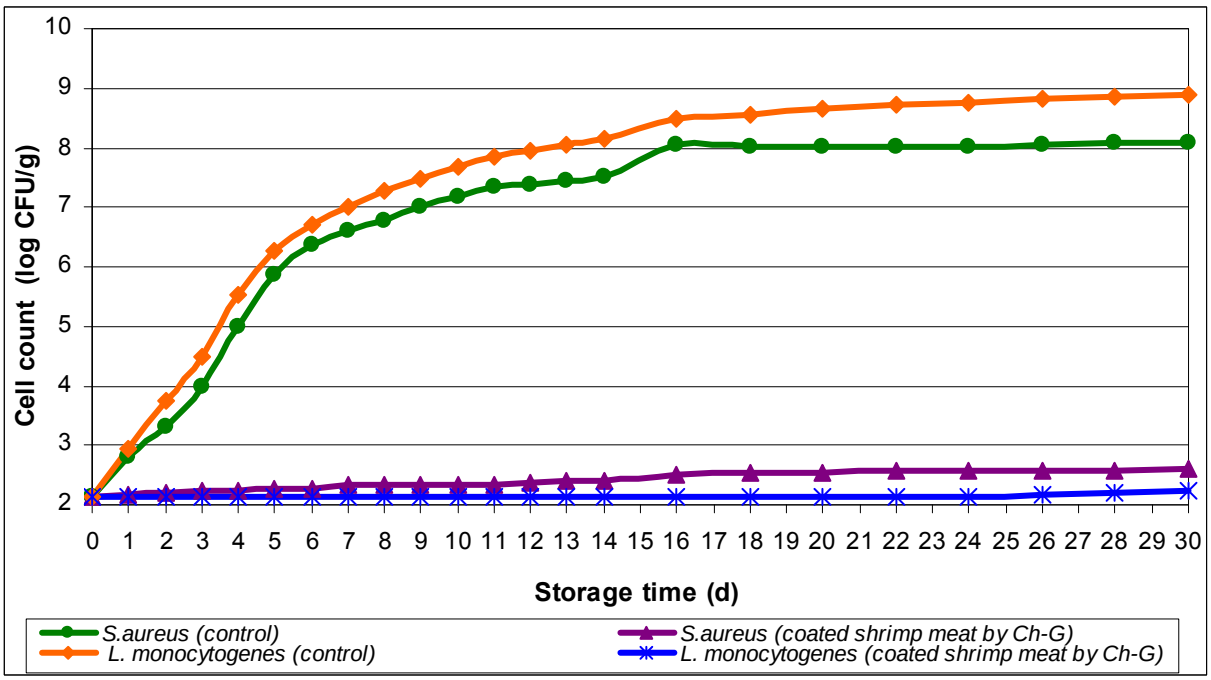


Figure 3.19: Changes in growth of *S. aureus* and *L. monocytogenes* on shrimp meat coated with chitosan-garlic extract compared to control during storage at **(A)** refrigerator temperature and **(B)** room temperature

The growth of gram-positive bacteria on shrimp meat samples coated by chitosan-garlic extract at both storage temperatures did not start immediately, but a long lag

phase became apparent up to day 21 of storage for *S. aureus.* The growth of *S. aureus* LMH 5P on coated shrimp meat samples started to increase slightly just on day 22 of storage both temperatures and reached a value of 4.2×10^2 CFU/g of shrimp meat.

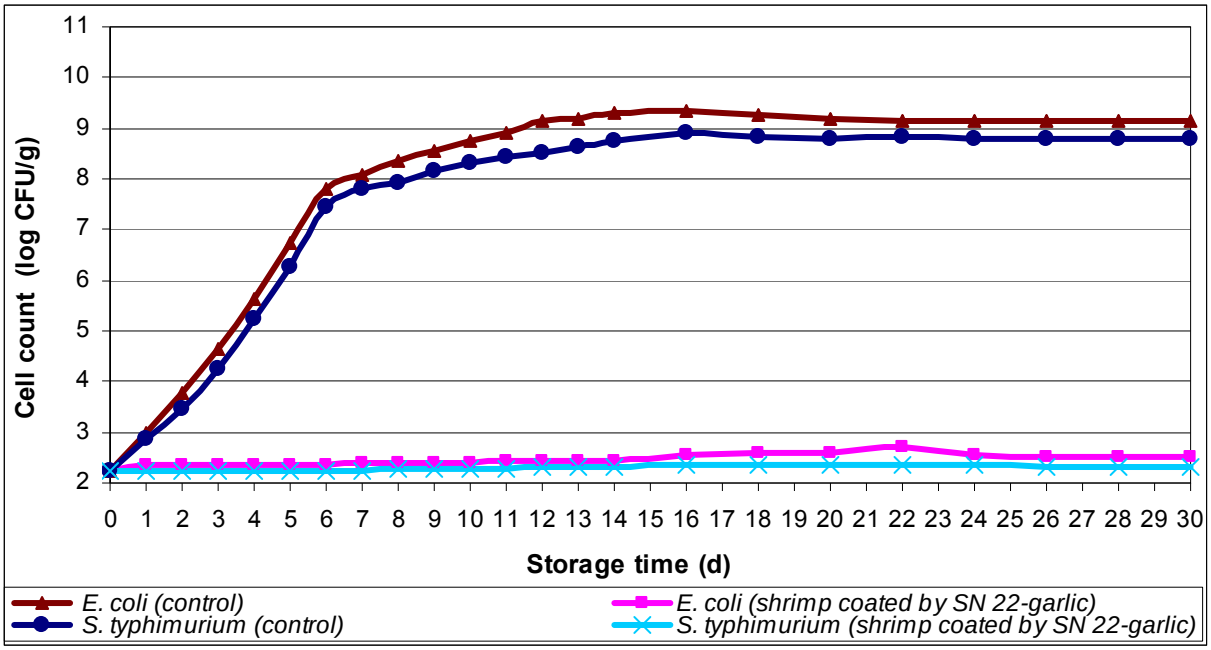
No growth of *L. monocytogenes* was found on coated shrimp meat during 30 days of refrigerated storage. Similarly, there was almost no growth of *L. monocytogenes* on coated shrimp meat during room temperature storage. Its initial count of 1.5×10^2 CFU/g increased only to 1.7×10^2 CFU/g of shrimp meat during 30 days of room temperature storage. This means, the growth of *L. monocytogenes* on shrimp meat coated with Ch-G was less than 1 logarithmic unit, and thus it was negligible.

3.4.2.3 Growth of gram-negative bacteria

To evaluate the effect of chitosan charge SN 22 on changes in growth of *E. coli* LMH 1N and *S. typhimurium* LMH 2N on shrimp meat coated with chitosan-garlic extract, 25 g of coated shrimp meat samples were inoculated with 0.1 mL aliquot of each strain at a cell titre of 10^2 CFU/mL prior to storage at refrigerator and room temperature. The same protocol was performed for the control (uncoated shrimp meat).

The effect of chitosan SN 22 enforced with garlic extract on growth of the test gram-negative bacteria is presented in Figures 3.19 A and 3.19 B. The initial value of each test strain through cell supplementation was 1.5×10^2 CFU/g for refrigerator and room temperature storage. In general, the rate of microbial proliferation of coated shrimp meat samples was slower than on control, which showed exponentially growth immediately after the first day of storage, especially for the control samples stored at room temperature.

A)



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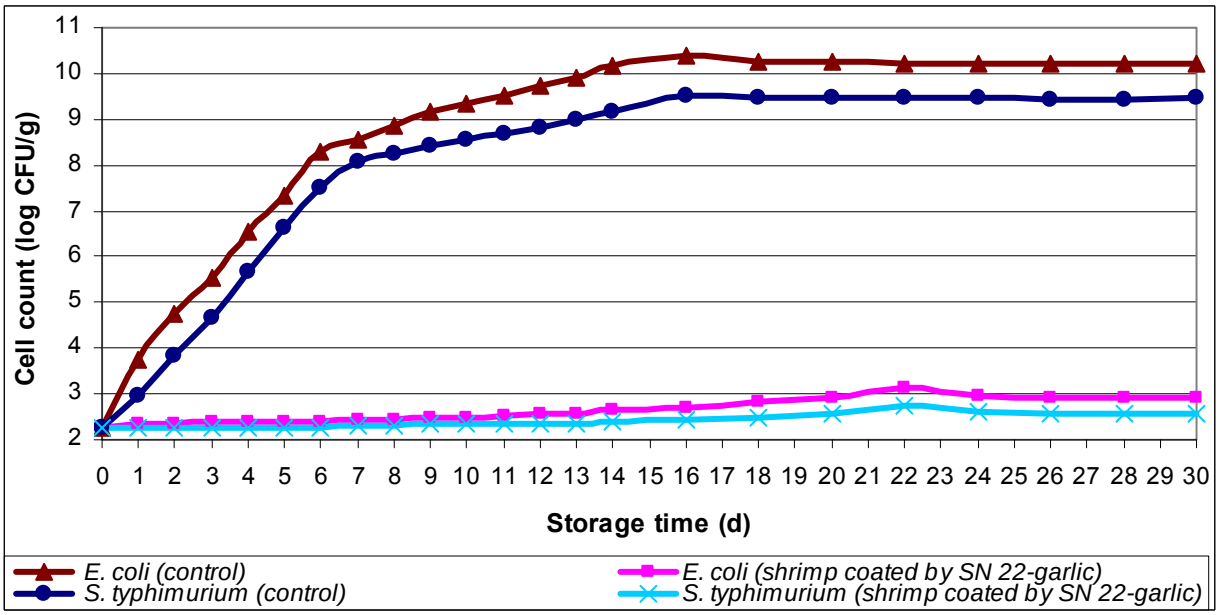


Figure 3.20: Changes in growth of *E. coli* and *S. typhimurium* on shrimp meat coated with chitosan-garlic extract compared to control during storage at **(A)** refrigerator temperature and **(B)** room temperature

The growth of the test gram-negative strains began with a long lag phase lasting up to day 20 and 15 of storage at refrigerator and room temperature, respectively. During storage at refrigerator temperature, the growth of *E. coli* on coated shrimp

meat samples started to increase slightly just on day 22 of storage and after that entered the stationary phase, reaching a value of 6.5 × 10^2 CFU/g of shrimp meat. During refrigerated storage, no changes in growth of *S. typhimurium* were found.

The same pattern was seen on the growth of both test strains on coated samples stored at room temperature. A slight growth of *E. coli* began on day 15 lasting to day 22, then the populations entered the stationary phase, when it reached a value of 9.7 × 10^2 CFU/g of shrimp meat. A similar trend was seen on growth of *S. typhimurium*, although, the growth rate of this strains was lower than that of *E. coli*. No distinct increase in the growth of *S. typhimurium* was found until day 22 of storage. After that, the stationary phase became apparent, when the populations of this strain reached the cell count of 5.1 × 10^2 CFU/g of shrimp meat, which lasted to the end of storage (Figure 3.20 B).

3.4.3 Biochemical quality assessment of shrimp meat samples coated with chitosan-garlic extract

3.4.3.1 pH value

The initial pH values of shrimp meat homogenate were in the range of 7.0–7.1. During 30 days of storage at refrigerator and room temperature, pH value increased to a pH range of 7.3–8.7. Changes in pH values of shrimp meat coated with chitosan-garlic extract (Ch-G) during refrigerated and room temperature storage are presented in Figure 3.21.

The pH values of coated shrimp meat stored at both temperatures were found to be quite similar until day 13 (pH ≤ 7.1). During refrigerated storage, no changes in the pH values were found until day 16 of storage. After that, the pH values of these samples increased up to the end of storage and reached a final value of 7.3.

Similarly, during room temperature storage, the initial pH value of the coated samples could be maintained by the Ch-G solution until day 13 of storage. Starting on day 14, the pH increased slightly up to the end of storage and reached a final value of 7.5.

In contrast, pH values of control samples immediately increased from the beginning of storage. The increase in pH value of the control samples were rapid and lasted until the end of storage, reaching pH 8.4 and 8.7, for refrigerated and room temperature storage, respectively.

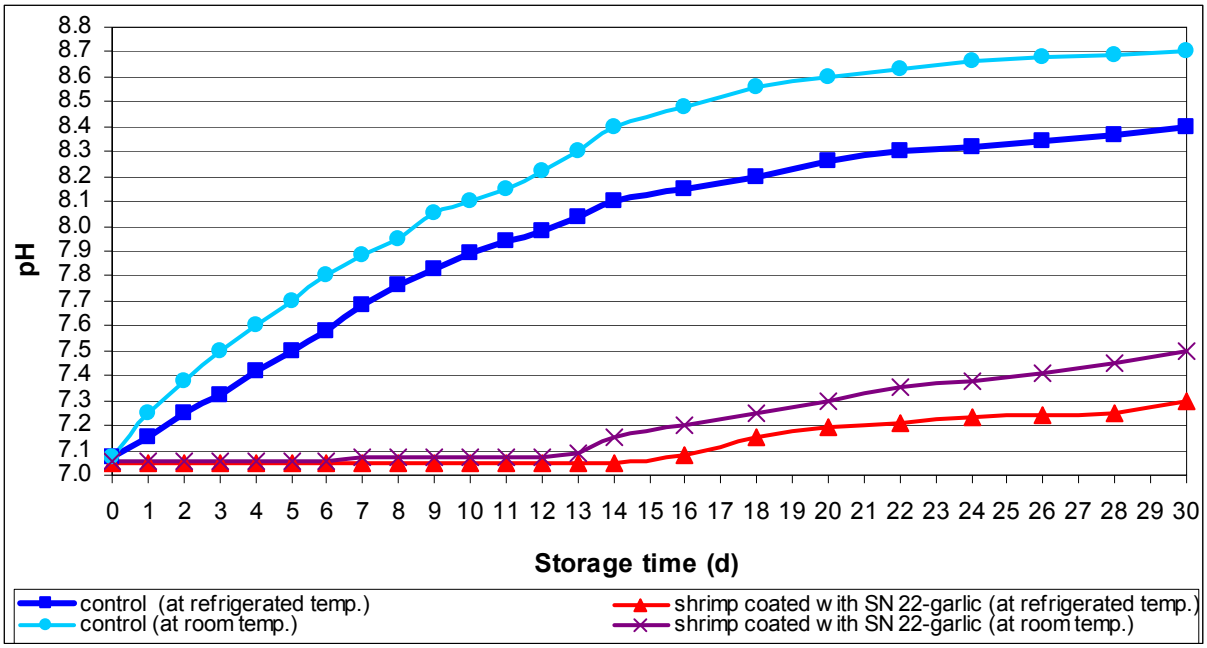


Figure 3.21: Changes in pH value of shrimp meat coated with chitosan-garlic extract compared to control during storage at refrigerator and room temperature

3.4.3.2 Water activity

The changes in water activity (a_w) of shrimp meat coated with chitosan-garlic extract during storage at refrigerator and room temperature are presented in Figure 3.22. The initial a_w of the shrimp meat samples tested in this work was 0.99. During refrigerated storage, the initial a_w (0.99) of the coated samples could be maintained up to day 14, whereas during room temperature storage, it could only be maintained

up to 7 days. A slight decrease in the a_w value of the coated samples stored at refrigerator temperature was found on day 16 up to day 24 of storage, in which the a_w value decreased to 0.98. During room temperature storage, a slight decrease down to 0.98 was found on day 8 and kept constant up to day 14. The a_w of the coated samples slightly decreased to 0.97 on day 16 until day 22. Then, a decrease down to 0.95 was found on day 24 up to day 26. Afterwards, the a_w values further decreased and ended at 0.94 on day 30 of storage at room temperature.

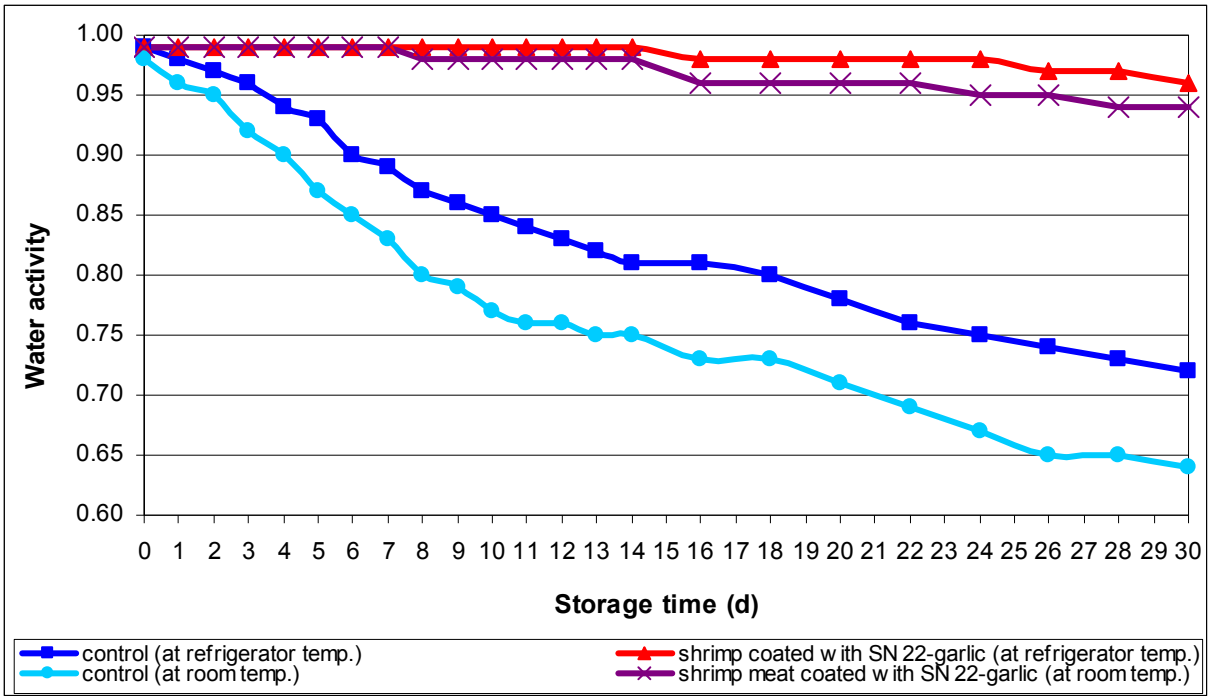


Figure 3.22: Changes in water activity value of shrimp meat coated with chitosan-garlic extract compared to control during storage at refrigerator and room temperature

On the contrary, a_w values of the control samples immediately decreased from the beginning of storage. The decrease in a_w value of the control samples was rapid and lasted until the end of storage, especially at room temperature, reaching the final values of 0.73 and 0.64 for refrigerated and room temperature storage, respectively.

3.4.3.3 Content of total volatile basic nitrogen (TVBN)

Changes in TVBN content of shrimp meat coated with chitosan-garlic extract during storage are presented in Figure 3.23. TVBN content increased during the time of storage at refrigerator and room temperature. The TVBN content of shrimp meat coated with chitosan-garlic extract started to increase only after 16 days of refrigerated storage.

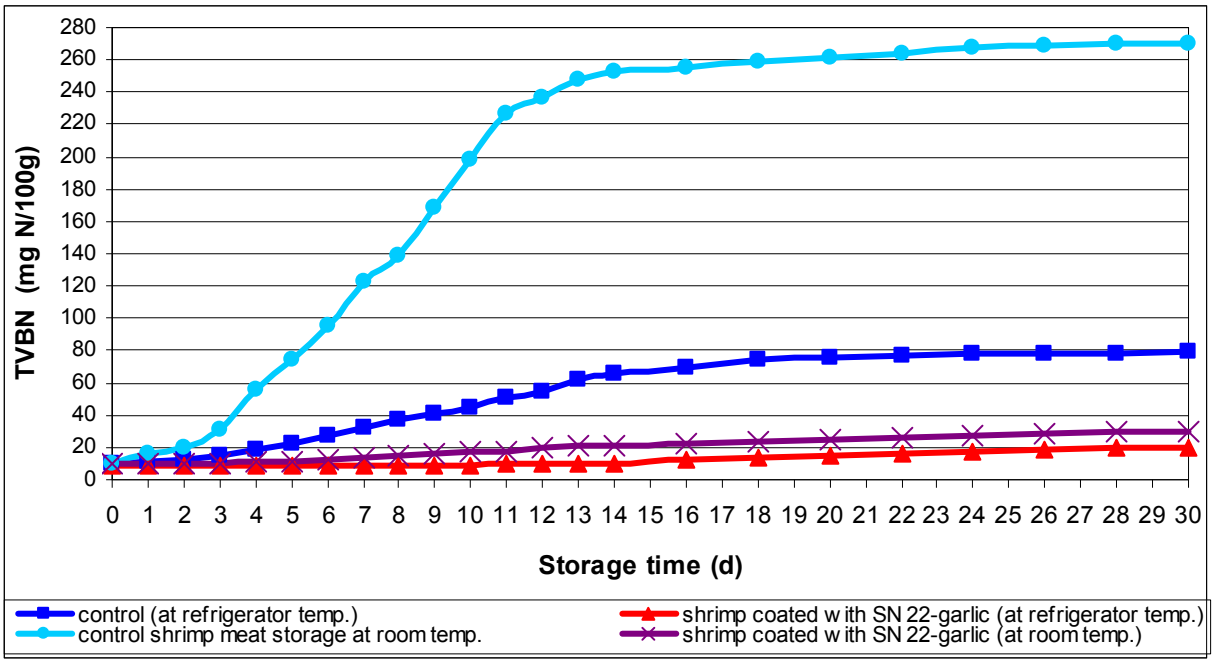


Figure 3.23: Changes in TVBN value of shrimp meat coated with chitosan-garlic extract compared to control during storage at refrigerator and room temperature

The maximum TVBN content of the coated samples after 30 days of refrigerated storage was 19.83 mg/100 g of shrimp meat, whereas TVBN content of the control increased steadily up to 79.51 mg/100 g of shrimp meat at the end of refrigerated storage.

During room temperature storage, TVBN content of the coated samples increased to 14.16 mg/100 g of shrimp meat after 7 days, and reached a maximum content of 30.50 mg/100 g after 30 days of storage.

The TVBN content of the control samples stored at room temperature reached 31.26 mg/100 g of shrimp meat after 2 days, which exceeded the acceptable level of 30.00 mg/100 g of shrimp meat intended for human consumption according to EU regulation (Baixas-Nogueras 2002). After 2 days of room temperature storage, the TVBN content of the control steadily increased up to 270.52 mg/100 g of shrimp meat on day 30.

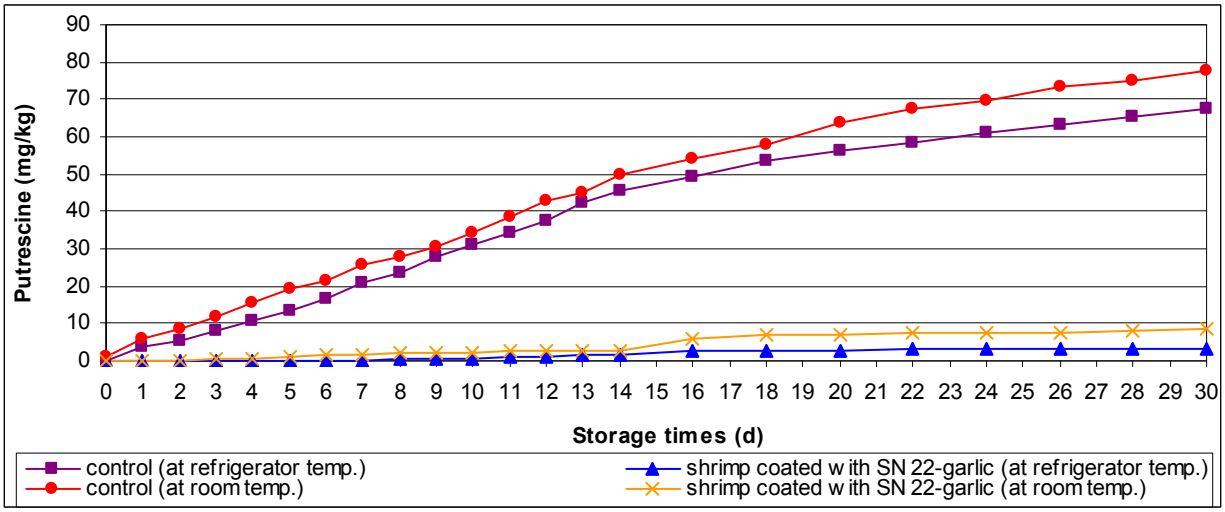
3.4.3.4 Content of biogenic amines

The quantitative changes in content of biogenic amines in shrimp meat samples coated with chitosan SN 22 enforced with garlic extract (Ch-G) are presented in Figures 3.24 A–E. The quantitative contents of biogenic amines were determined by HPLC with a fluorescence detector after derivatization performed by *o*-Phthaldialdehyde (see section 2.5.4.4).

There were no significant changes in content of biogenic amines in coated shrimp meat up to 14 days of refrigerated storage. The only biogenic amines found on day 14 of refrigerated storage were putrescine and cadaverine at values less than 1.0 mg/kg of shrimp meat (Figures 3.24 A–B). Close to the end of storage, the contents of putrescine were lower than that of cadaverine, whose contents dominated up to the end of storage with a final value of 7.5 mg/kg of shrimp meat, whereas the contents of putrescine reached a level of 2.0 mg/kg of shrimp meat (Figures 3.24 A and B).

During room temperature storage, there were more distinct changes in concentration of putrescine until day 26. Afterwards, the content of cadaverine increased more rapidly than that of putrescine and ended at 10.0 mg/kg of shrimp meat on day 30 of storage. At the same time, the contents of putrescine reached a level of 9.0 mg/kg of shrimp meat (Figures 3.24 A and B).

A)



B)

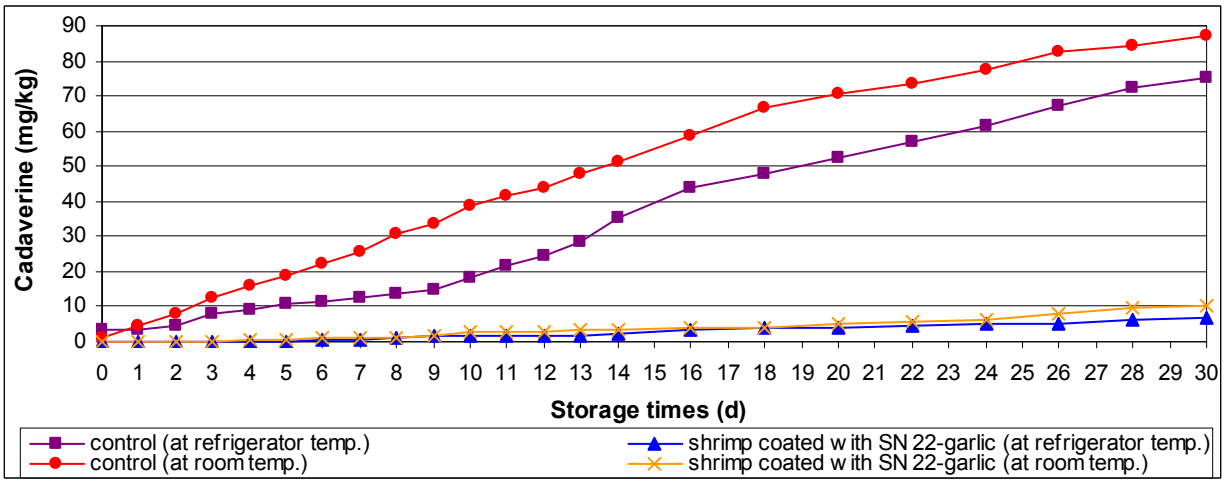


Figure 3.24 A, B: Changes in content of **(A)** putrescine and **(B)** cadaverine of shrimp meat coated with chitosan-garlic extract compared to control during storage at refrigerator and room temperature

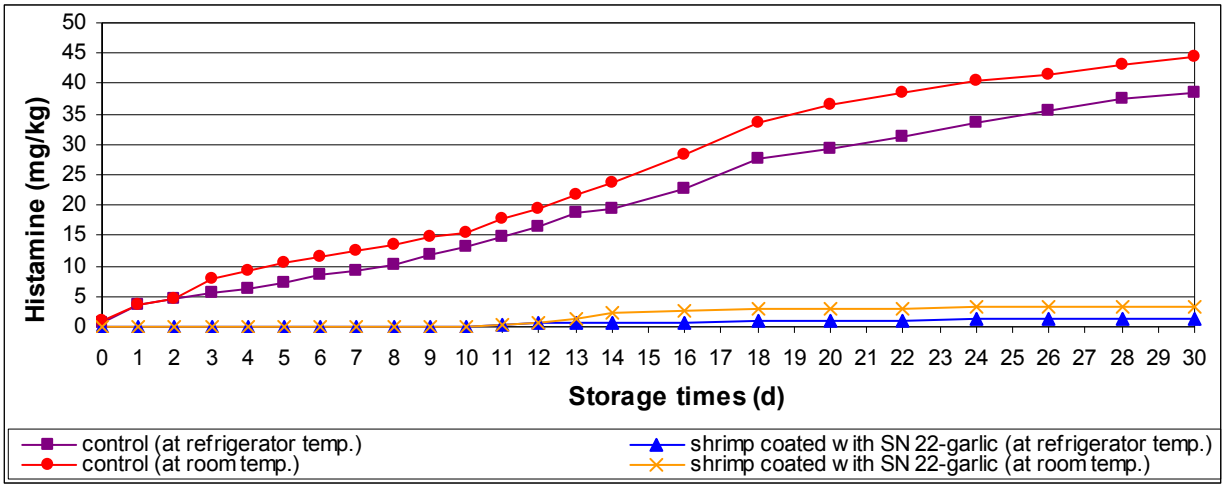
No distinct changes in concentration of histamine, tyramine, and agmatine in coated shrimp meat samples during refrigerated storage were found. During room temperature storage, a slight increase in the content of histamine lasting up to the end of storage was seen (Figures 3.24 C–E).

Histamine, tyramine, and agmatine in coated shrimp meat samples were detectable to some extent after 14 days of storage, particularly at room temperature (Figures 3.24 C–E). Starting from day 16 of refrigerated storage, a slight increase in

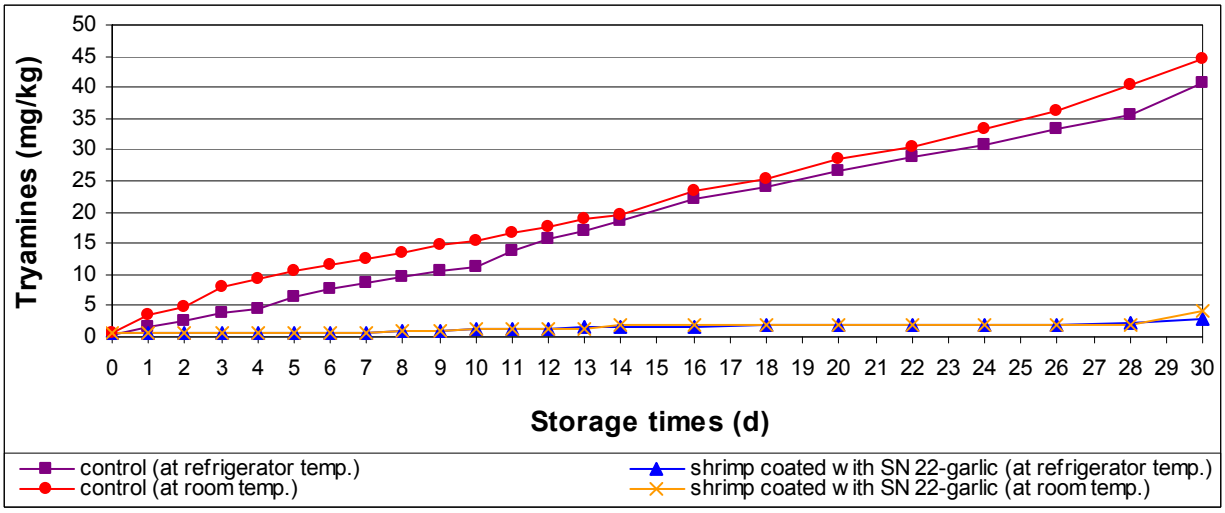
concentration of these biogenic amines at a level of < 1 mg/kg was seen. No further distinct changes in concentration of these biogenic amines were found until the end of storage. Histamine reached final contents of 2 mg/kg and 4 mg/kg after 30 days of storage at refrigerator and room temperature, respectively (Figures 3.24 C). Tyramine and agmatine in the coated samples showed the same pattern of changes in their concentrations during storage at both temperatures, in which no distinct changes in contents of both biogenic amines were found. These biogenic amines appeared slightly after 14 days of storage and their concentration remained constant up to day 28 of both storage temperatures. Later, tyramine reached final concentrations of 3 mg/kg and 4 mg/kg of shrimp meat, whereas agmatine increased to 2 mg/kg and 3 mg/kg of shrimp meat after 30 days of storage at refrigerator and room temperature, respectively (Figures 3.24 D–E).

In general, no significant changes in the concentration of histamine, tyramine, and agmatine were found in the coated shrimp meat samples during storage at both temperatures, compared to their control, whose contents increased from the beginning until the end of storage. After 30 days of storage at both temperatures, the concentrations of histamine, tyramine, and agmatine in the samples coated with Ch-G were less than 5 mg/kg of shrimp meat, whereas their concentration in the control samples were more than 35 mg/kg of shrimp meat (Figures 3.24 C–E).

C)



D)



E)

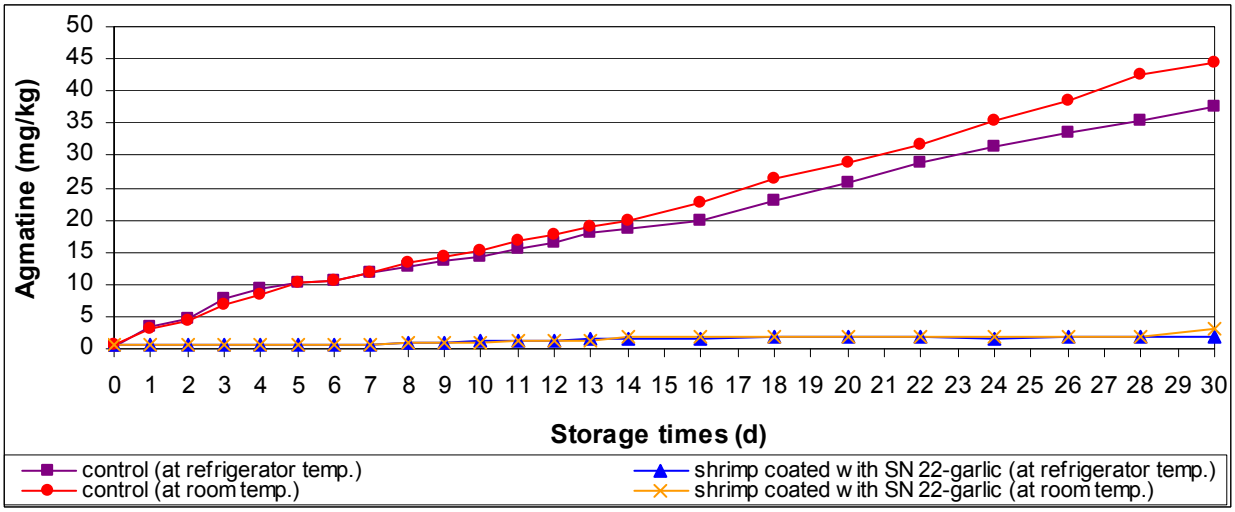


Figure 3.24 C, D, E: Changes in content of **(C)** histamine, **(D)** tyramine, and **(E)** agmatine of shrimp meat coated with chitosan-garlic extract compared to control during storage at refrigerator and room temperature

3.4.4 Scanning electronic microscopy of microstructure of chitosan films

In general, the chitosan SN 22 films plasticized with 20 % (w/v) glycerol presented good flexibility, rigidity, and high tear resistance (see Figure 3.8 B).

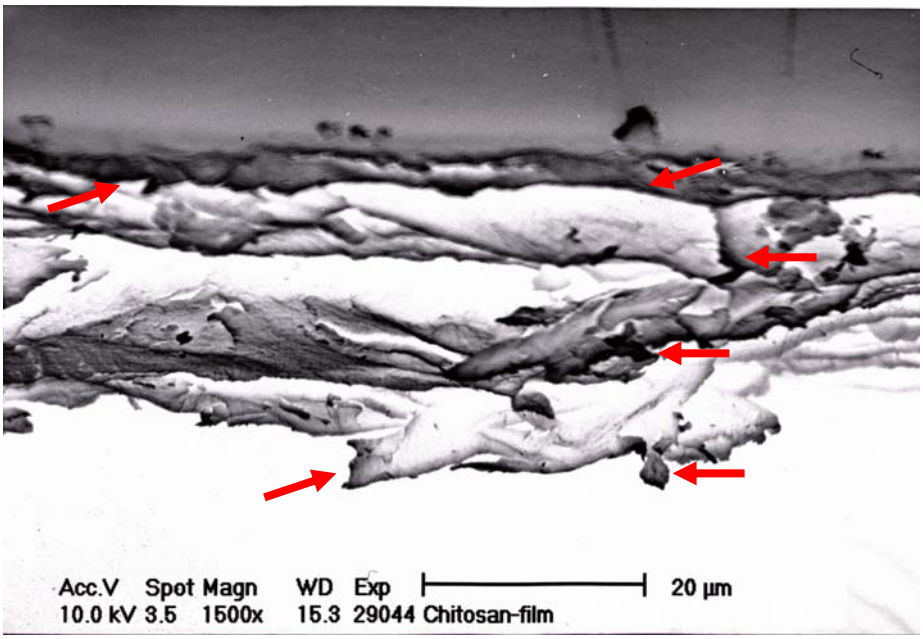
Later, the plasticized chitosan SN 22 films were enforced with garlic extract. The manufactured chitosan-garlic extract Ch-G films plasticized with 20 % (w/v) glycerol were expected to have desired characteristics of films such as flexible, rigid, and high tear resistance.

To know the influence of glycerol on the characteristics of Ch-G films, the microscopic view of their surface and cross section were taken by scanning electron microscopy (SEM).

The microscopic views of cross section of Ch-G films without plasticizer and with plasticizer are presented in Figures 3.25 A and 3.25 B, respectively. The SEM view of surface of Ch-G film without plasticizer is shown in Figure 3.26 A, whereas Figure 3.26 B shows the SEM view of surface Ch-G film with plasticizer.

The microstructures of cross section of Ch-G films without plasticizer are full of folds and cracked, which are indicated by arrows (Figures 3.25 A). In contrast to this, the SEM view of cross section of Ch-G film with plasticizer shows that microstructures of Ch-G films are smooth, homogeneous porous, and unfolded (Figure 3.25 B).

A)



B)

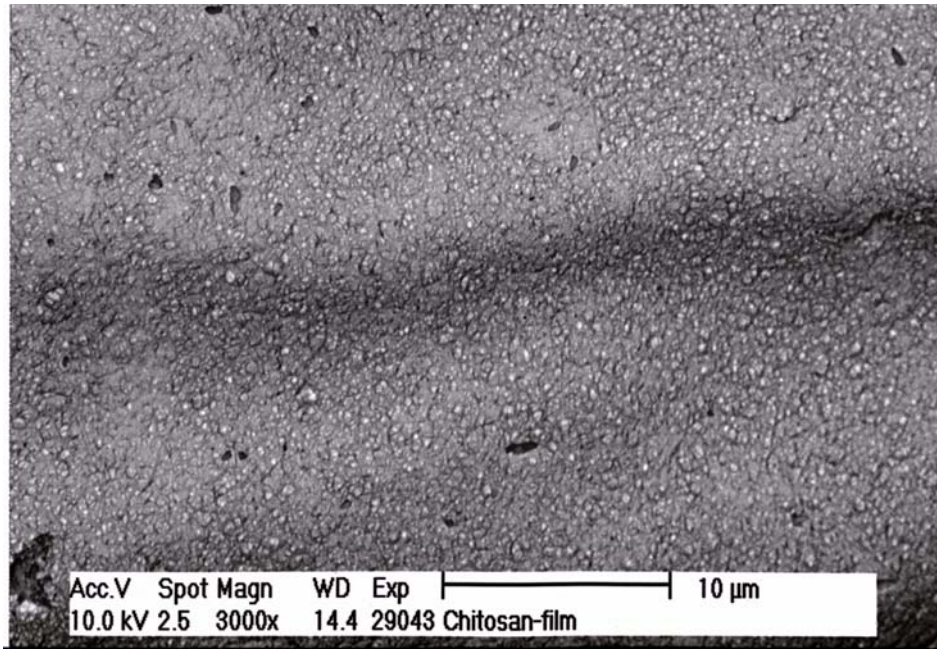
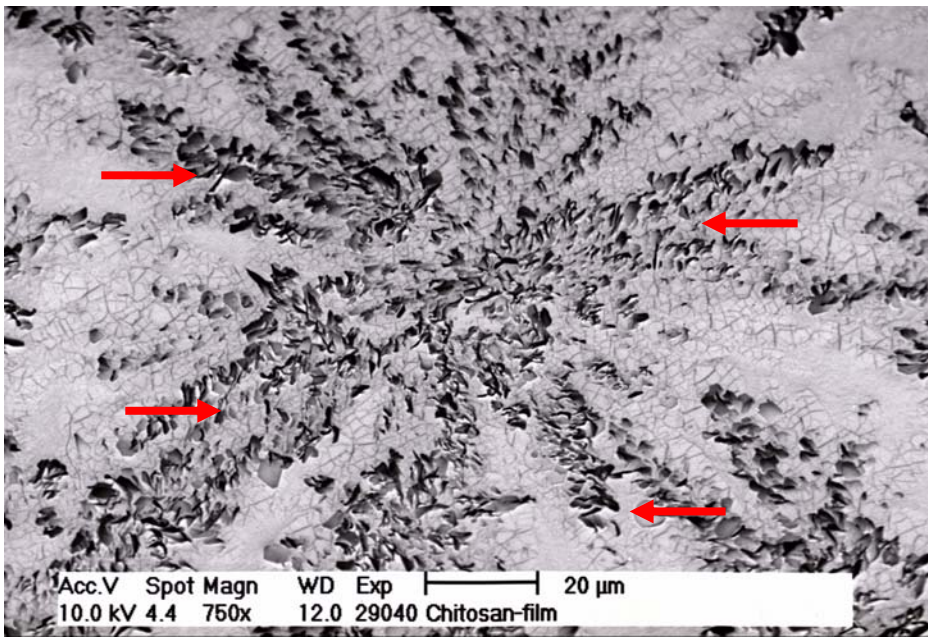


Figure 3.25: SEM view of cross section of chitosan-garlic extract film **(A)** without plasticizer with arrows indicating folds and cracks and **(B)** with plasticizer

A Similar pattern was found on SEM view of surface of Ch-G film with plasticizer and without plasticizer, as shown in Figure 3.26 A and 3.26 B.

A)



B)

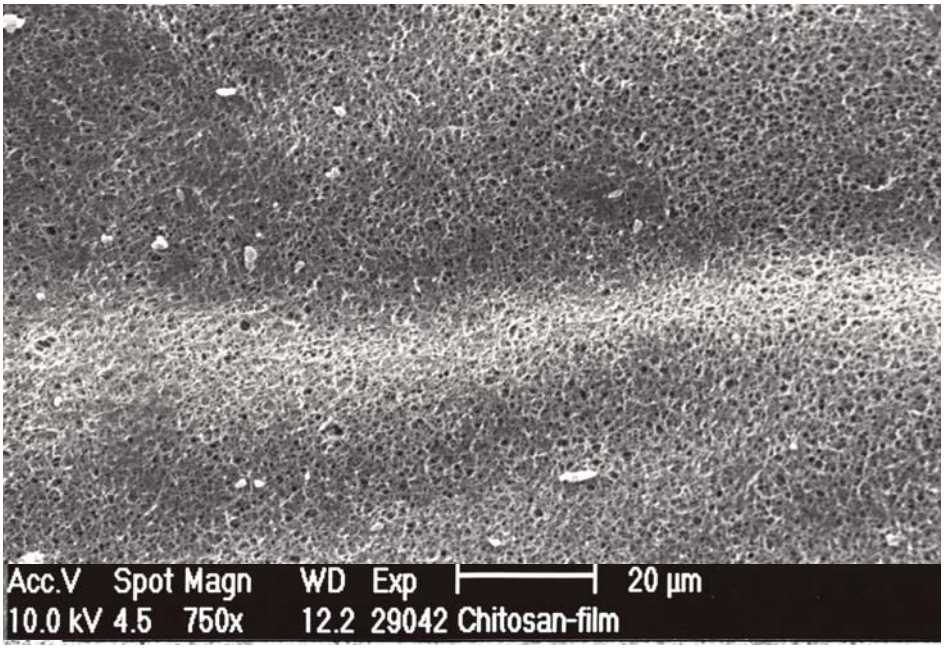


Figure 3.26: SEM view of surface of chitosan-garlic extract film **(A)** without plasticizer (arrows indicating inhomogeneous pores) and **(B)** with plasticizer

As shown in Figure 3.26 A, the film Ch-G without plasticizer shows a very perforated surface area microstructure caused by inhomogeneous pores, which are spread irregularly over the surface of Ch-G film (indicated by the arrows). Evidently, the expected characteristics of surface Ch-G film without plasticizer were not achieved.

On the contrary, SEM view of surface of Ch-G films with plasticizer shows a smooth and unfolded microstructure with homogeneously spread pores (Figure 3.26 B). Only using plasticizer, the expected characteristics of surface Ch-G film can be obtained.

4 Discussion

4.1 Potency of chitosan as antimicrobial substance

In the present study, all chitosan charges markedly inhibited the growth of the gram-positive bacteria tested (*S. aureus* LMH 5P and *L. monocytogenes* LMH 34P) and gram-negative bacteria (*E. coli* LMH 1N and *S. typhimurium* LMH 2N) at a concentration of 0.1 % (w/v), as seen in Figures 3.3 A.1–2 and 3.3 B. These results are in accordance with the literature mentioning that, at lower concentration, the polycationic chitosan probably binds to the negatively charged bacterial surface, thus causing agglutination, disturbing the cell membrane, and causing cell death due to leakage of intracellular components. At higher concentrations, the larger number of positive charges of chitosan may impart a net positive charge to the bacterial surface to keep them in suspension and prevent agglutination (Sudarshan et al. 1992). Similarly, Young et al. (1982) found that the electrophoretic mobility of bacteria changed after treatment with high concentrations of poly-α-amino acids, but only at lower concentration did agglutination occur and thus the growth of bacteria was inhibited.

Several investigations were conducted to prove these theoretical statements. Wang (1992) found that 0.5 % (w/v) chitosan was ineffective in inhibiting *S. typhimurium*. Beside that, Allan et al. (1984) reported that *S. aureus* was negligibly inhibited at a chitosan concentration of 0.1 % (w/v), and *E. coli* was only slightly affected at a level as high as 1.0 % (w/v). In this work, the growth of *S. aureus* was almost completely suppressed (< 10 CFU/mL), whereas the growth of *E. coli* with the initial cell count of 10^7 was inhibited to 10^2 CFU/mL by chitosan SN 22 at a concentration of 0.1 % (w/v).

All the gram-positive bacteria tested were sensitive to the antimicrobial activity of chitosan, with MIC values ranging from 0.005–0.050 %. On the other hand, the gram-negative strains tested were less sensitive to the antimicrobial activity of chitosan, with MIC values ranging from 0.055–0.085 % (see Figures 3.5 A.1, A.2, and B). These results are consistent with previous studies reporting a stronger bactericidal effect of chitosan on gram-positive bacteria (No et al. 2002).

The inhibitory activity of chitosan towards gram-positive and gram-negative bacteria should be considered in terms of its chemical and structural properties. As a polymeric cationic macromolecule, chitosan is able to penetrate the cell wall of gram-positive bacteria, which consists chiefly of peptidoglycan and lacks the outer membrane (Sudarshan et al. 1992; Helander et al. 2001; Rabea et al. 2003). However, as a polymeric cationic macromolecule, chitosan might have less potency to damage the cell wall of gram-negative bacteria, whose cell wall has an outer membrane which constitutes the outer surface of the cell wall. Thus, chitosan may not easily pass the outer membrane of gram-negative bacteria, since this membrane functions as an efficient outer permeability barrier against chitosan (Je and Kim 2006). Chitosan with its positively charged amino groups may interact with the negatively charged microbial cell wall leading to the leakage of proteinaceous and other important intracellular constituents of bacteria so that they cannot grow any further (Helander et al. 2001; Pranoto et al. 2005).

With regard to the application of chitosan for shrimp meat preservation, it is worthwhile to note that the presence of *L. monocytogenes* in foods has become a concern in recent years (Conner et al. 1986; Ahamad and Marth 1989; Shahidi et al. 2002). Besides that, the microbiological criteria for cooked shrimps used so far are recommended by European Union (1994*)*. According to this guideline, *L.*

monocytogenes and *S. typhimurium* must not be detectable (N.D.) in 25 g of sample (see Table 1.2).

However, in the preliminary study, all chitosan charges could not optimally inhibit the growth of *L. monocytogenes* to reach the above-mentioned recommended value. The reason for this is probably that the antimicrobial activity of chitosan was not yet in the optimum condition.

Therefore, in order to obtain the optimum antimicrobial effectiveness of chitosan in this present study, several factors influencing the antimicrobial activity of chitosan, such as pH and physical characteristics of chitosan had to be optimized. Furthermore, chitosan was used for shrimp preservation under improved conditions, so that a significant health hazard by consumption of seafood contaminated with bacteria could possibly be reduced or prevented by proper chitosan treatment.

4.1.1 Influence of molecular mass and degree of deacetylation of chitosan on their antimicrobial activity

Antimicrobial activities of chitosan are greatly dependent on its physical characteristics, most notably molecular weight and degree of deacetylation (DDA). Moreover, in the various fields of chitosan application, both DDA and molecular weight have a great impact.

In the present study, the molecular weight of chitosan charges was determined by viscometry, therefore, it is called as the viscometric molecular weight (M_η) or molecular mass (Kumar 2000; Kulicke et al. 1999). The considerable evidence has been gathered indicating that most of the physiological activities and functional properties of chitosan depend on its molecular weight, although the chemical and physical processes underlying some of the applications of chitosan and its derivatives are still unknown in detail (Rabea et al. 2003).

Several studies revealed that the antimicrobial activity of chitosan depends on its DDA, whereby the antimicrobial activity of chitosan generally increases with increasing DDA (Franz et al. 2002; Tsai et al. 2002). Therefore, the screening of the most effective charge of chitosan providing the strongest antimicrobial activity is related to both physical characteristics (Sano et al. 2002; Rafaat and Sahl 2009).

As seen in Figures 3.3 A.1–2 and 3.3 B, chitosan charges with relative lower M_η (1.5×10^5–3.9×10^5 g/mol) exhibited stronger antimicrobial activity against the four strains tested. Especially for *S. aureus,* its growth was almost completely suppressed by all chitosan charges used in this research. The charges with higher M_η (5.1×10^5 to 1.3×10^6 g/mol) were weaker in inhibiting of the strains tested. Similarly, No et al. (2002) reported that growth of both *E. coli* and *Bacillus cereus* is inhibited more effectively by chitosan charges with M_η of 470 g/mol and 746 g/mol than by chitosan with M_η of 1106 g/mol and 1671 g/mol. Jeon et al. (2001) observed that chitosan charges with M_w of 224 and 1106 kDa possess weak or no antibacterial activity against *S. typhimurium*, compared with a chitosan charge with a M_w of 28 kDa. In addition, with regard to viscosity, Cho et al. (1998) reported that the antibacterial activity of chitosan for *E. coli* and *Bacillus* sp. increased with decreasing viscosity from 1000–10 cP.

Beside that, it is also worthwhile to perceive the relationships between the molecular weight and the antimicrobial activity of chitosan oligomers which have also been reported by various investigators. Jeon et al. (2001) reported that chitosan oligomers with M_w of 1–10 kDa was critical for microorganism inhibition and their efficacy increased with M_w. Sekiguchi et al. (1994) investigated the antibacterial activities of chitosan oligomers with M_w ranging from 2350–21.600 Da for various bacteria. Growth of *B. cereus* on agar culture was suppressed by 0.2–0.3 % (w/v) chitosan oligomer with a M_w of 11.000 Da. Furthermore, Uchida et al. (1989) reported that

chitosan oligomer II (a mixture of triose and tetraose) failed to display antibacterial activity against *E. coli* at a concentration of 0.5 %, while chitosan oligomers I (a mixture of tetraose and heptaose) possessed antibacterial activity. No et al. (2002) reported that the antibacterial activity of chitosan oligomers varied depending on their molecular weight average (M_η) and the particular bacterium. However, from this it could be concluded that chitosan oligomers with low M_η of 1 kDa showed relatively higher antimicrobial activity against gram-negative bacteria, while chitosan oligomers with M_η of 4 kDa and 2 kDa exhibited effective antimicrobial activity against gram-positive bacteria compared to those with higher M_η of 7, 10, and 22 kDa.

The correlation between the molecular weight and the DDA on the antimicrobial activity of chitosan depends on the positive charges number of protonated chitosan and the number of the negative charges on the microbial surface (Leuba and Stössel 1986; Tsai and Su 1999; Tsai et al. 2002). If the chitosan charges possess a relatively low DDA, they are not sufficiently able to bind the negative charges on bacterial surface by polycationic action. On the other hand, when the chitosan charges have a relatively high DDA, they form linearly chained structures due to intermolecular electrostatic repulsion. Thus, the inhibition activity of chitosan might be retarded by less entrapment of bacteria due to the steric hindrance (Sano et al. 2002). Therefore, in this research, chitosan charge SN 22 with the relatively low molecular mass (M_η) of 1.5×10^5 g/mol and the moderately high DDA of 80 % evidently has the strongest antimicrobial activity at a chitosan concentration of 0.1 % (w/v).

The results are similar to those investigated by Wu et al. (2006). They reported that the antibacterial activity of chitosan with a molecular mass of 440 kDa (within M_η range of 439–805 kDa) and a DDA of 75.3 % (within DDA range of 68.4–93.7 %) exhibited the strongest antimicrobial activity against the growth of *E. coli* and *B. cereus*. Sano et al. (2002) observed that chitosan with a molecular mass of 3 kDa

(M_η ranging from 0.8–6 kDa) and a DDA of 60 % (DDA ranging from 10–90 %) was the most effective in inhibiting the growth of *Streptococcus sobrinus* and *Streptococcus mutans.*

The mechanism of interaction between chitosan and cell surface of bacteria is based on electrostatic attractive force. This means that polymer chains of chitosan with their positively charged groups attach to the negatively charged bacterial cell surface. If the molecular mass (M_η) of chitosan is low, its polymer chains have greater flexibility to bind more than one cell. Thus, bridges between bacterial cells and polymer chains of chitosan are quickly formed, so that the bacteria are immediately inactivated (Sano et al. 2002; No et al. 2007; Wu et al. 2006).

Regarding the influence of the DDA on the antimicrobial activity of chitosan charge SN 22, it can be described that a DDA value of 80 % expresses an adequate positive charge of chitosan to bind the negative charge on the microbial surface. This interaction leads to the leakage of proteinaceous and other intracellular constituents of bacteria cells, so that they can not grow any further (Sudarshan et al. 1992; Helander et al. 2001; Rabea et al. 2003; Je and Kim 2006).

4.1.2 Influence of pH on the antimicrobial activity of chitosan

The effect of the pH value on the antimicrobial activity of chitosan against four spoilage bacteria on shrimp meat (*E. coli*, *S. typhimurium, S. aureus*, and *L. monocytogenes*) was evaluated. The pH value of each chitosan charge solution was set using 1.0 N hydrochloric acid solution. The lowest pH was set as 4.5 because the growth of *E. coli* is inhibited in an environment with a pH value of < 4.5. The upper pH value was limited to 6.5 because chitosan is insoluble in an environment with a pH value of > 6.5 (No et al. 2002). In addition, Sudarshan et al. (1992) reported that no

antibacterial activity was evident at pH 7.0 because the amino groups of chitosan at pH 7.0 were no longer significantly charged, thereby bacteria cells could not be clumped. Thus, the chitosan charges were poorly soluble at pH 7.0 with regard to their acid dissociation constant (pK_a) of approximately 6.3.

Some background theories support these present results in relation to the importance of the influence of pH on the antimicrobial activity of chitosan. Since the pK_a of chitosan is approximately 6.3, the amino groups on the chitosan carry positive charges when the pH value is below 6.0 (Wicken and Knox 1983; Chung et al. 2003). Furthermore, Lyubina et al. (1983) and Chung et al. (2003) underlined that chitosan optimally reacts with negatively charged bacteria and further inhibits bacterial growth, when chitosan is present in a properly acidic environment. Beside that, chitosan has almost no antimicrobial activity in a neutral environment (at pH value of approximately 7.0). In order to optimize the antimicrobial activity of chitosan, controlling the pH value by using a compound or substance which can increase the acidity in the environment of chitosan must be carried out.

Several investigators have reported results comparable to those found in the present study. No et al. (2002) observed that the antibacterial activity of chitosan was affected by pH, with greater activity being found at lower pH values. For example, at pH 4.5, *E. coli* showed 10^5–10^6 CFU/mL viable cells, whereas at pH 5.9, it showed 10^6–10^7 CFU/mL viable cells. At pH ≤ 5.5, chitosan at a concentration of 0.03 % (w/v) could completely suppress the growth of *L. monocytogenes*. Tsai and Su (1999) reported that a low pH value increased the bactericidal effect of chitosan against *E. coli*. Yun et al. (1999) noted that the MIC of chitosan was 1.4–1.7 times lower at pH 3.6–3.8 than at pH 5.9–6.0. Wang (1992) found that the antibacterial activity of chitosan against five species of foodborne pathogens (*Yersinia enterocolitica, S. aureus, E. coli, L. monocytogenes, S. typhimurium*) was stronger at pH 5.5 than at

pH 6.5. These results clearly indicate that chitosan shows its antimicrobial activity only in an acidic medium due to its degree of protonation. Besides that, it is evident that the application of chitosan to acidic foods will enhance its effectiveness as a natural preservative.

4.2 Enforcing the antimicrobial activity of chitosan with garlic extract

The results in the preliminary work revealed that chitosan SN 22 showed a stronger antimicrobial effect against the gram-positive test strains than against the gram-negative test strains. A possible reason for this is that chitosan with its positively charged amino group can not damage the cell wall of gram-negative bacteria in order to interact with the negatively charged microbial cell membrane. This interaction leads to the leakage of proteinaceous and other intracellular constituents of bacterial cells so that they can not continue to grow (Sudarshan et al. 1992; Helander et al. 2001; Rabea et al. 2003; Je and Kim 2006). Chitosan may damage the cell wall of gram-positive bacteria because the cell wall is mainly composed of peptidoglycan, teichoic acid, and very little protein. On the contrary, the cell wall of gram-negative bacteria is more complex and contains various lipopolysaccharides, proteins, and lipids beside peptidoglycan (Shahidi et al. 1999; Pranoto et al. 2005).

Therefore, to obtain a stronger antimicrobial efficacy of chitosan SN 22 against the gram-negative strains tested causing spoilage in shrimp meat, the antimicrobial efficacy of chitosan SN 22 needs to be improved. For this purpose, chitosan SN 22 coating solution was enforced with garlic extract.

The reason to choose garlic extract in this work was due to its highly active organosulfur compounds which possess antimicrobial activity. These active compounds are such as allicin, diallyl disulfide, and diallyl trisulfide (Nychas 1995; Pranoto et al. 2005; Corzo-Martinez et al. 2007). The main antimicrobial constituent

of garlic has been identified as the oxygenated sulfur compound, thio-2-propene-1-sulfinic acid *S*-allyl, which is usually referred to as allicin (Douglas and Bakri 2005; Rose et al. 2005). Moreover, recently, the use of natural plant extracts has become desirable for the development of new food products and nutraceuticals as well as new packaging systems, including bioactive coatings and films (Fazilah et al. 2008). Furthermore, bioactive edible coatings and films are a very important issue for food industry due to environmental concerns and regulations to develop environmentally friendly packaging materials (Guilbert et al. 1996; Park and Zhao 2004; Dainelli et al. 2008).

According to this statement, in the present study, enforcing the antimicrobial activity of chitosan SN 22 coating solution by incorporating garlic extract is a way to prepare bioactive edible coatings for seafood preservation, whereby chitosan and garlic are natural or biological products.

As presented in Figure 3.17, garlic extract alone (without chitosan) showed stronger antimicrobial activity against gram-negative bacteria than against gram-positive bacteria. The mode of the antimicrobial action of garlic extract to strongly inhibit the growth of gram-negative bacteria is due to its main active agent, allicin, which is able to penetrate cell membranes (Miron et al. 2000; Douglas and Bakri 2005). This is caused possibly by a feature of the bacteria cell wall that may enable access to periplasmic and cytoplasmic enzymes. The differential effect of garlic extract on inhibiting the test gram-positive and gram-negative strains can be seen in Figure 3.16 A–B and 3.17).

In comparison to the antimicrobial activity of chitosan without garlic extract in this work, it is obvious that incorporation with garlic resulted in stronger antimicrobial activity of chitosan, mainly against gram-negative bacteria. On the one hand, chitosan with its positively charged amino groups possesses stronger antimicrobial

activity against gram-positive bacteria. On the other hand, garlic extract with its main active agent, allicin, showed to be more effective against gram-negative bacteria tested. This is in accordance with Miron et al. (2000), who reported that garlic inhibits potential harmful *Enterobacteriaceae* such as *Salmonella* sp. and coliforms more effectively, probably due to greater sensitivity of *Enterobacteriaceae* to allicin.

As observed in this work, chitosan and garlic extract act synergistically and can optimally inhibit the growth of the gram-positive and gram-negative bacteria tested. Therefore, by evaluation of microbiological and biochemical parameters of shrimp meat during storage, it is evident that the chitosan SN 22 solution enhanced with garlic extract used for shrimp meat coating can optimally maintain the quality of shrimp meat during storage.

Studies on the extension of the shelf life of foodstuff by chitosan coating have so far been limited to fruits and vegetables. In this case, chitosan has been claimed as an excellent shelf life extender (Shahidi et al. 2002; Park and Zhao 2004). There are a number of studies regarding application of chitosan coating for fresh fruits and vegetables. For examples, El-Ghaouth and Arul (1991) investigated chitosan for coating of fresh strawberries. Du et al. (1997) reported about the effects of chitosan coating on the storage of peaches, pears, and kiwis. Jiang and Li (2001) studied the effects of chitosan coating on the post-harvest life and quality of logan fruit. Recently, Vargas et al. (2006) studied the quality of strawberries coated with chitosan-oleic acid during refrigerated storage.

On the contrary, only one study on the extension of the shelf life of seafood by chitosan coatings or films has so far been published, namely by Shahidi et al. (2002), who reported on chitosan as an edible invisible film for herring and Atlantic cod.

Moreover, it must be underlined that no work on the extension of the shelf life of shrimp meat by chitosan coatings and films has been presented up to now.

Furthermore, there is no study on using chitosan coatings and films incorporated with another natural antimicrobial agent such as plant extract to extend the shelf life of shrimp meat (Baker et al. 1994; Hotchkiss and Appendini 2002; Cagri et al. 2004).

4.3 Potency of chitosan and chitosan-garlic extract coating solution for shrimp meat preservation

4.3.1 Potency of chitosan and chitosan-garlic extract coating solution to prevent microbial spoilage of shrimp meat

Spoilage is most rapid and evident in proteinaceous foods such as seafood, which possesses a high moisture content and a neutral or slightly acidic pH. Therefore, a wide range of microorganisms can easily grow on seafood (Smith et al. 1996). Shrimp may become contaminated during subsequent handling and processing due to coliforms, staphylococci, and other mesophilic organisms. Therefore, this work was focused on microorganisms involved in the spoilage of shrimp meat and on studying the potency of chitosan as a preservative agent to reduce microbial proliferation in shrimp meat.

4.3.1.1 Changes in total viable count of aerobic mesophilic bacteria

The quality of shrimp meat during storage is influenced by changes in microbiological parameters. During storage, the bacteria on shrimp meat steadily grow and cause spoilage.

Chitosan SN 22 at a concentration of 0.1 % (w/v) was able to control growth of aerobic mesophilic bacteria, which was indicated by limitation of total viable count (TVC) on shrimp meat to 10^4 CFU/g at refrigerator temperature and 10^5 CFU/g at room temperature, over a period of 14 d storage. These values are much lower than

the TVC values of uncoated shrimp meat (control), which reached the values up to 10^9–10^{10} CFU/g of shrimp meat (see Figure 3.9).

Furthermore, chitosan enforced with garlic extract at a concentration of 0.1 % (w/v) was able to control the TVC of aerobic mesophilic bacteria on shrimp meat samples down to a value of 10^4 CFU/g of shrimp meat, at the end of a 30-day refrigerated and room temperature storage, respectively. These final values are much lower than the final TVC values of control, which reached the values up to 10^9–10^{10} CFU/g of shrimp meat during storage at both temperatures (Figure 3.18).

By comparing the above results, it is evident that chitosan-garlic extract solution strongly retarded the growth of aerobic mesophilic bacteria on the shrimp meat samples. For example, chitosan without garlic extract limited the growth of these bacteria on coated shrimp meat to 10^4–10^5 CFU/g after 14 days of storage at refrigerator and room temperature, respectively. On samples coated with chitosan-garlic extract, after 14 days, the growth of these bacteria increased only to 10^3 CFU/g of shrimp meat stored at both temperatures. Furthermore, chitosan-garlic extract limited the growth of aerobic mesophilic bacteria on the coated samples to 10^4 CFU/g of shrimp meat after 30 days of storage at both temperatures.

The present results are obviously better concerning the effect of chitosan on microbial spoilage than those presented by Simpson et al. (1997) who observed that after 8 days of refrigerated storage (4–7 °C), the TVC of shrimp (*Pandalus borealis*) samples treated with 1 % (w/v) chitosan increased to 10^6 CFU/g from the initial cell count of 2.5×10^3 CFU/g. For shrimp samples treated with chitosan at a concentration of 2 % (w/v), the TVC increased to 10^5 CFU/g, after 8 days of storage. Ólafsdóttir et al. (2005) reported the TVC changes of shrimp (*P. borealis*) samples stored at a freezing temperature of -1.5°C. During 7 days of storage, the TVC on shrimp samples increased to 3.0×10^8 CFU/g from the initial cell counts of 2.4 ×

10^5 CFU/g. Similarly, Wang et al. (2008) investigated the TVC increase of fresh cod (*Gadus morhua*) treated by the modified atmosphere packaging (MAP) method combined with superchilled storage. The TCV of MAP-treated cod samples increased from the initial cell count of 5.3×10^4 CFU/g to the final cell count of 2.6×10^7 CFU/g after 21 days chilled storage (-1.0 °C).

Several investigators reported total cell counts of aerobic bacteria at values of 10^6–10^8 CFU/g, when sensory spoilage was detected in foods (Fieger and Novak 1961; Gill 1986; Kraft 1992). Similar trends were observed in seafood, such as reported by Stenstrom (1985) who observed total aerobic bacteria count of 10^6 CFU/g on cod fillets after 6 days of storage at 2 °C. Shahidi et al. (2002) reported that cod and herring fillets coated with chitosan reached total aerobic bacteria counts of 10^6 CFU/g during the entire storage period of 12 days, whereas uncoated samples and samples treated with 1 % (w/v) acetic acid exceeded the level of 10^6 CFU/g fish after 6 and 10 days, respectively. The International Commission on Microbiological Specifications for Foods (ICMSF 1986) proposed the acceptability limit of 10^6 CFU/g for fresh fish. In addition, the EU (1994) recommended the upper limiting value of 10^6 CFU/g for cooked shrimps. In this work, shrimp meat samples were also considered as unwholesome when the total cell counts were higher than 10^6 CFU/g of shrimp meat.

The mechanisms of chitosan in inhibition of growth of spoilage bacteria have been reported by several investigators. Papineau et al. (1991) and Tsai et al. (1998) reported that chitosan is believed to chelate certain ions from the lipopolysaccharide (LPS) layer of the outer membrane of bacteria. Thus, it has been suggested that alterations in the LPS layer may cause the outer cell surface to become more permeable, thereby releasing intracellular components of bacteria. Furthermore, the chitosan coating acts as a barrier against oxygen transfer and leads to the inhibition of growth of aerobic bacteria. Tsai and Su (1999) noted that chitosan caused leakage

of glucose and lactate dehydrogenase from *E. coli* cells. Furthermore, the antibacterial mechanism of chitosan involves a cross-linkage between the polycations of chitosan and the anions on the bacterial surface, which changes the membrane permeability. Simpson et al. (1997) observed that chitosan could extend the lag phase of growth for all the microorganisms tested such as *E. coli*, *S. aureus*, *S. typhimurium*, *Pseudomonas fluorescens*, *Pseudomonas vulgaris,* and *Bacillus cereus*. At higher concentration of ≥ 0.01 % (w/v), chitosan was bactericidal with the TPC showing little or no colony growth except for *P. fluorescens*. In this case, the rate of microbial proliferation in samples treated with chitosan was generally slower than that of the controls in the logarithmic phase. *B. cereus* required chitosan at concentrations of ≥ 0.02 % (w/v) for a bacteriocidal effect, while *E. coli* and *P. vulgaris* showed minimal growth at concentrations of 0.005 % (w/v), but were completely inhibited at concentrations of ≥ 0.0075 % (w/v).

The results in this work prove that the presence of garlic extract enforced antimicrobial activity of chitosan SN 22 against microbial spoilage on shrimp meat. As observed in this work, chitosan with its antimicrobial constituent amino groups (Sagoo et al. 2002) and garlic extract with its antimicrobial component allicin (Nychas 1995) act synergistically and effectively inhibit the growth of the total aerobic mesophilic bacteria.

The growth of aerobic mesophilic bacteria on shrimp meat coated with chitosan-garlic extract was lower due to its higher antimicrobial activity caused by enforcement of garlic extract into chitosan coatings solution. Thus, in this work, the spoilage on shrimp meat samples caused by aerobic mesophilic bacteria was significantly reduced.

4.3.1.2 Changes in growth of gram-positive bacteria

During harvesting, processing, and handling, shrimps may become contaminated with a wide range of microorganisms and common foodborne pathogens. Furthermore, during subsequent distribution and storage, contamination due to microorganisms can rapidly develop and cause serious spoilage of shrimps, especially due to listeria and staphylococci (Dykes et al. 2010).

Recently, the presence of *L. monocytogenes* in foods has become a big concern (No et al. 2002; Mejlholm et al. 2008). The growth of *Listeria* on shrimps may be influenced by several factors such as temperature, pH, competitive microflora, and the presence of food additives (Mejlholm et al. 2008). The growth rate of *L. monocytogenes* on cooked crustaceans, including shrimps is higher than that of other ready-to-eat (RTE) foods such as smoked seafood and soft cheeses (Hatha et al. 2003; Ahmed and Anwar 2007).

Regarding staphylococci, they may occur on shrimps during handling and processing, because staphylococci are normal members of the local flora of the skin and upper respiratory tract of nearly all humans and are often opportunistic pathogens (Madigan et al. 2009). Beside that, staphylococci are common spoilage bacteria, particularly in aerobically stored foods with high water content and a neutral pH value, such as seafood (Gram and Huss 1996; Huis in't Veld 1996). Among the microorganisms associated with cooked foods, *Staphylococcus aureus* has a great importance because this strain can produce several heat-stable toxins causing food poisoning (Madigan et al. 2009). These toxins continue to persist in foods during cooking, thus, cooked shrimps may create a great risk by *S. aureus* (Loir et al. 2003; Ahmed and Anwar 2007).

In this work, chitosan SN 22 at a concentration of 0.1 % (w/v) was able to reduce cell count of the tested gram-positive strains, namely *L. monocytogenes* and *S. aureus*

during storage. With the initial cell count of 1.5 × 10^2 CFU/g, the growth of *S. aureus* was limited to 4.0 × 10^2 CFU/g and the growth of *L. monocytogenes* was inhibited to 9.8 × 10^2 CFU/g, at the end of a 14-day refrigerated storage (see Figure 3.10 A).

In case of quality determination of shrimps concerning growth of gram-positive bacteria in this work, the results obtained were compared with the available standards of microbiological criteria for cooked shrimps, as recommended by EU (1994). According to this guideline, the cell count of *S. aureus* is limited to 10^3 CFU/g of shrimp meat, whereas the recommended value of this strain amount to 10^2 CFU/g of shrimp meat. *L. monocytogenes* must be not detectable (N.D) in 25 g of shrimp meat (see Table 1.2).

The growth of *S. aureus* with cell count of 2.5 × 10^2 CFU/g on coated shrimp meat samples met the recommended value of 10^2 CFU/g of shrimp meat, whereby the initial cell count of *S. aureus* through supplementation (1.5 × 10^2 CFU/g) increased to 4.0 × 10^2 CFU/g, at the end of a 30-day of storage. This cell count (2.5 × 10^2 CFU/g) was even much lower than the upper limiting value (10^3 CFU/g) of *S. aureus*. However, *L. monocytogenes* with the initial cell count of 1.5 × 10^2 CFU/g grew to 8.2 × 10^2 CFU/g, which did not meet the microbiological criteria for cooked shrimps (EU 1994).

In general, chitosan SN 22 showed a strong effect on retarding the growth of *S. aureus* on coated shrimp meat samples during refrigerated storage. This result may be elucidated by several theories. Chitosan generally has a stronger antimicrobial activity against gram-positive bacteria than against gram-negative bacteria (Tsai et al. 2002; Sagoo et al. 2002), especially against *S. aureus* (No et al. 2002). This is probably due to a lack of an outer membrane of the cell wall of *S. aureus*, like other gram-positive bacteria (Sudarshan et al. 1992; Je and Kim 2006). Thus, chitosan may easily damage the cell wall and leading to leakage of protein and other

important constituents of bacterial cells (Helander et al. 2001), thereby the cells of *S. aureus* can not grow further on. In addition, the ability of *S. aureus* to grow depends on extrinsic factors such as storage temperature. During refrigerated storage, *S. aureus* as a mesophilic strain can not grow well, because its optimum temperature for growth is at 37 °C (Farrel and Upton 2007).

Unlike *S. aureus* as a mesophilic strain, *L. monocytogenes* is a psychrotolerant strain, which is able to proliferate at refrigerator temperatures (at 4–7 °C). This ability is one of the most important factors for the presence of *L. monocytogenes* at the end of storage period of refrigerated products (Madigan et al. 2009; Dykes et al. 2010). Therefore, the cell count of *L. monocytogenes* on the coated shrimp meat samples was relatively higher at the end of a 14-day refrigerated storage period, compared to that of *S. aureus*.

Furthermore, during a 30-day refrigerated storage, chitosan-garlic extract (Ch-G) limited the cell count of *S. aureus* to a value of 2.5×10^2 CFU/g of shrimp meat at the end of storage, whereas no growth was seen for *L. monocytogenes* during this storage (see Figure 3.19 A). However, during room temperature storage, a very slight growth of *S. aureus* was found on day 22 until the end of storage, with a final value of 5.1×10^2 CFU/g. This means, chitosan-garlic extract could effectively suppress the growth of *S. aureus* up to day 22 of room temperature storage. No growth was found for *L. monocytogenes* during this storage (see Figure 3.19 B).

During refrigerated storage, the cell count of *S. aureus* on coated shrimp meat met the recommended value of 10^2 CFU/g (EU 1994), whereby the cell count of this strain increased from the initial cell count of 1.5×10^2 CFU/g to the final value of 2.5×10^2 CFU/g. During room temperature storage, Ch-G solution limited the cell counts of *S. aureus* to 3.6×10^2 CFU/g of shrimp meat, which met the upper limiting value of *S. aureus* for shrimps intended for human consumption (EU 1994).

Increase in cell count of *L. monocytogenes* was not detectable on coated shrimp meat during storage at both temperatures. At the end of 30 days, the final cell count of *L. monocytogenes* at both temperatures were 1.5×10^2 CFU/g, which were the same with the initial cell count 1.5×10^2 CFU/g (see Figure 3.19 A). This means, chitosan-garlic extract completely suppressed the growth of *L. monocytogenes* during a 30-day storage period, thus, no growth of this strain was found. These results are in accordance with the recommendation of microbiological criteria for cooked shrimps (EU 1994).

Specific studies on growth and survival of *S. aureus* on shrimps are rarely found. Many investigators preferred to observe the prevalence of *S. aureus* on other kind of foods, such as fish fillet and pork slices. Likewise, investigations on growth and survival of *L. monocytogenes* on shrimps and their products are also limited. Most studies were so far focused on preventing prevalence of *L. monocytogenes* on shrimps and their products.

Paranjpye et al. (2008) used a steam pasteurization method to eliminate naturally contaminating *L. monocytogenes* in cooked-peeled shrimp. They exposed that the viable cells of *Listeria* were limited to 16 CFU/25 g of shrimp after steam-cooking for 45, 60, and 90 s. However, the product suffered a minor loss in flavour, was slightly tougher and weighed up to 25 % less. Mejlholm et al. (2008) observed the growth of *L. monocytogenes* in brined shrimp (*Pandalus borealis*) combined with modified atmosphere packaging (MAP) method during a 40-day chilled storage (at 7–8 °C). No clear effect of MAP method combined with chilled storage against growth of *L. monocytogenes* was observed in their investigation. Similarly, Chiu and Lai (2010) reported the reduction of cell count of *L. monocytogenes* and *S. aureus* on pork slices coated with Hsian-tsao (*Mesona procumbens*) leaf gum matrices combined with green tea (*Camellia sinensis*) extracts. During an 8-day refrigerated storage, the

cell count of *L. monocytogenes* and *S. aureus* were limited to 10^3–10^4 CFU/g of pork slices, whereby the initial cell count of both strains were 10^9 CFU/g.

In this work, growth of *L. monocytogenes* and *S. aureus* on shrimp meat coated with chitosan-garlic extract was evidently lower, especially the growth of *L. monocytogenes*. This is due to the higher inhibitory activity of chitosan-garlic extract, whereby chitosan SN 22 acts synergistically with garlic extract to inhibit the growth of gram-positive strains tested. In addition, the relative low pH (5.5) of the chitosan-garlic extract solution used to coat shrimp meat may also contribute to the reduction of CFU of *L. monocytogenes.* This is in agreement with Hatha et al. (2003) and Dykes et al. (2010) who stated that *L. monocytogenes* can not grow well in an acidic environment.

4.3.1.3 Changes in the growth of gram-negative bacteria

Much attention has been paid to the occurrence of pathogenic microorganisms in seafood intended for consumption. For example, there have been several studies on the presence of coliform bacteria on seafood, mainly on fish, because of concerns regarding the health of seafood consumers. In contrast, relatively few studies have been performed on the presence of *E. coli* on shrimps (Greenwood et al. 1985; Sikorski 1990; Hansen et al. 2008).

The occurrence of spoilage bacteria on shrimps may be found since their catching time. North Sea shrimps, such as *C. crangon* are traditionally boiled on board of the shipping boat and cooled by fresh water directly after boiling, thereby eliminating much of the contamination flora. However, shrimps may be recontaminated during later handling. Since crustaceans are rich in free amino acids, the water in the cooking vessels provides a good medium for bacterial growth (Mejlholm 2008).

Besides that, during subsequent handling and processing, shrimps may become contaminated with spoilage bacteria such as salmonellae, coliforms, and other pathogenic bacteria. Furthermore, distribution and storage can possibly bring about a change in the cell number, proliferation rate, and composition of the spoilage bacteria of shrimps, mainly by *Enterobacteriaceae* such as *Salmonella* sp. and coliforms. *Salmonella* sp. and *E. coli* belong to *Enterobacteriaceae*. Among *Salmonella* strains, *S. typhimurium* is responsible for most common outbreaks of foodborne salmonellosis in humans. Most strains of *E. coli* are not pathogenic in humans, but a few strains are potential foodborne pathogens (Tindall et al. 2005; Madigan et al. 2009).

S. typhimurium and *E. coli* are common spoilage bacteria, particularly in aerobically stored foods with a high water content and neutral pH such as seafood (Gram and Huss 1996; Huis in't Veld 1996). Moreover, *S. typhimurium* and *E. coli*, like most of the other gram-negative rod-shapes bacteria, are commonly occurring in the environment and may contaminate foods from many sources, and they are able to utilize a wide range of materials as growth substrates (Dainty and Mackey 1996; Dykes et al. 2010). High numbers of *S. typhimurium* and *E. coli* may also be found in water. It has been shown that *E. coli* and *Salmonella* can survive for very long periods in water. Besides that, due to their mesophilic characteristics, salmonellae and coliforms may become responsible for spoilage at temperatures above of 15 °C (Huss 1995; Huis in't Veld 1996).

In general, increases in cell count (CFU) of the gram-negative strains tested in coated shrimp meat samples was higher than those of the gram-positive strains tested. This may be because chitosan SN 22 as a polymeric cationic macromolecule has a lower potential to pass the outer membrane, since this membrane functions as an efficient outer permeability barrier against chitosan (Je and Kim 2006).

In this work, chitosan SN 22 limited the growth of *S. typhimurium* and *E. coli* to a level of 10^4 CFU/g, at the end of a 14-day refrigerated storage (see Figure 3.11 A). A similar trend for both gram-negative strains tested was seen on coated shrimp meat samples stored at room temperature, whereby at the end of storage, chitosan SN 22 limited the growth of *S. typhimurium* and *E. coli* to levels of 10^4 CFU/g and 10^5 CFU/g, respectively (see Figure 3.11 B).

According to the microbiological criteria for cooked shrimps (EU 1994), the final cell count of *E. coli* on shrimp meat coated with chitosan SN 22 did not meet the upper limiting value of 10^2 CFU/g, whereas *S. typhimurium* must be not detectable (N.D.) in 25 g of shrimp meat (see Table 1.2).

Regarding chitosan-garlic extract for shrimp meat preservation, the effectiveness of retarding growth of the gram-negative strains on the coated shrimp meat was much stronger than that of chitosan SN 22 alone (without garlic extract). At refrigerator temperature, no growth of *S. typhimurium* was found up to the end of 30 days of storage. Similarly, *E. coli* showed a long lag phase lasting until day 20 of storage (Figure 3.20 A). The same pattern was found in the growth of the two strains during room temperature storage, at which after day 14, the cell count of *S. typhimurium* slightly increased and reached a final value of 5.1×10^2 CFU/g on day 30. A slight growth of *E. coli* was found and reached a final value of 9.5×10^2 CFU/g (Figure 3.20 B).

The above results demonstrate that chitosan-garlic extract strongly delayed the growth of the gram-negative strains tested on the coated shrimp meat samples during a 30-day refrigerated storage, especially *S. typhimurium*. This is due to the higher inhibitory activity of chitosan-garlic extract, whereby chitosan SN 22 and garlic extract synergistically inhibited the gram-negative strains tested.

So far, specific studies on the growth rate or survival of *Salmonella* on shrimps and shrimp products are limited, mainly there are studies on the prevalence of *Salmonella* sp. on treated or preserved fish. Iyer and Shrivastava (1989) investigated the viability of *Salmonella* in cooked shrimp homogenates at freezing temperatures (-20 °C and -40 °C). They found that all strains of *Salmonella* were resistant to freezing (-40 °C), thus growth of these strains could not be retarded. Hatha et al. (2003) and Heinitz et al. (2000) observed growth and survival of *Salmonella* in cooked shrimp treated in a relatively high salting condition combined with drying. They observed that *Salmonella* was able to survive in a high salt condition, even treatment by the combination of salting and drying could not totally suppress this strain. In addition, Chiu and Lai (2010) studied the antimicrobial activity of edible coating based on tapioca starch matrices decolourized with Hsian-tsao (*Mesona procumbens*) leaf gum combined with green tea (*Camellia sinensis*) extracts against *E. coli* DH10beta and *S. enterica* BCRC 10747 on pork slices. During a 8-day storage, no effect of this edible coating was found against both strains tested.

The present results show that chitosan-garlic extract at a concentration of 0.1 % (w/v) proved to be able to limit the growth of *E. coli* up to day 22 of storage, and to suppress the growth of *S. typhimurium* on shrimp meat samples during a 30-day refrigerated storage entirely.

4.3.2 Potency to control changes in biochemical parameters of shrimp meat

4.3.2.1 Changes in pH value

The pH of shrimp meat gives some valuable information about its quality change. Changes in pH influence spoilage because of its effect on the microorganisms and on enzyme activity (Smith et al. 1996; Ólafsdóttir et al. 2006). Usually, pH value

decreases during anaerobic formation of lactic acid, as reported by Smith et al. (1996), who observed that the postmortem pH of seafood usually decreased to about 5.5–6.5 because of the lactic acid produced under anaerobic conditions. However, the microbial metabolism of proteolitic bacteria leads to an increase in pH during storage time. The pH changes showed good correlation with the amount of CFU. They also reflected TVBN accumulation and indicated spoilage progress, such as reported by Krishnakumar et al. (1985), who observed a reduction of total nitrogen on shrimps stored in ice because some compounds containing nitrogen were leached out.

The fact that pH of the coated shrimp meat samples increased slowly during storage at refrigerator temperature and room temperature was probably due to an effective inhibitory activity of chitosan and its enforcement with garlic extract on the growth of proteolitic bacteria causing decomposition of nitrogenous compounds. The present results show that chitosan SN 22 retarded the pH increase of coated shrimp meat to a value of 7.2 and 7.4 at the end of a 14-day of refrigerated and room temperature storage, respectively (see Figure 3.12). Furthermore, the pH increase on shrimp meat was retarded by chitosan-garlic extract to a value of 7.3 and 7.5 at the end of 30 days of refrigerated storage and room temperature storage, respectively (see Figure 3.21). These final pH values met the upper limit of an acceptable pH at a value of 7.6, as recommended by the European Commission for shrimps intended for human consumption (Abu Bakar et al. 2008). In contrast to this, the pH values of uncoated samples (control) increased steadily during storage at both temperatures and reached values of > 8.0, which exceeded the upper limiting pH value of 7.6 for shrimps (Abu Bakar et al. 2008). Thus, uncoated shrimp meat was unacceptable.

The present results are in accordance with previous observations conducted by Bilinski et al. (1983) and Haard (1992) who emphasized that the pH changes were

strongly influenced by the spoilage microorganisms. Huss (1995) and Jackson et al. (1997) reported that there was a continuous increase in pH for all sample groups, especially for untreated samples (control), probably due to the metabolism of microorganisms producing alkaline compounds like amines formed by the deamination of amino acids. Ólafsdóttir et al. (2006) stated that the microbial metabolism leads to an increase in pH on shrimp samples during storage time and that the pH changes showed a good correlation with microbial growth (CFU). In addition, Kilincceker et al. (2009) asserted that pH values increase due to the presence of proteolytic bacteria and autolytic enzymes in fish meat. During storage, decomposition of nitrogenous compounds leads to an increase in pH on fish meat, thereby affecting its quality.

In this work, chitosan-garlic extract proved to be effective in maintaining the initial pH value and retarding the pH increase on shrimp meat during storage, compared to those reported by previous investigators. Ólafsdóttir et al. (2005) observed that the pH of shrimp samples stored in liquid ice (-1.5 °C) increased during 7 days of storage, reaching a final value of 8.0 from the initial value of 7.4. Similarly, Kilincceker et al. (2009) reported a pH increase on fish meat coated with a mixture of wheat and corn enhanced with 1 % (w/w) garlic powder and 1 % (w/w) onion powder during storage at a temperature of -18 °C. On day 30 of storage, the pH increased to 6.4 from an initial value of 6.0, whereby the upper limit of the acceptable pH value for fish meat intended for human consumption amount to 6.5.

Chitosan-garlic extract (Ch-G) coating solution at a concentration of 0.1 % (w/v) was able to control the increase in pH of coated shrimp meat during a 30-d storage period at refrigerator and room temperature. Moreover, the present results show that the Ch-G solution maintained the pH values of coated shrimp meat samples close to the initial pH value (7.1) for a longer time and retarded the pH increase below the upper

limit of acceptable value for shrimps intended for human consumption. This is due to the higher inhibitory activity of chitosan-garlic extract, which synergistically inhibited the spoilage microorganisms causing pH increases in coated shrimp meat.

4.3.2.2 Changes in water activity

Shrimps are a highly perishable food with high water activity (a_w) values within the range of 1.00–0.95 (Fontana 2000). Due to its high a_w value, shrimp meat can easily undergo spoilage during storage because microorganisms causing spoilage in shrimp meat can generally grow well within a_w values range of 0.95–0.98 (Beuchat 1983; Gibbs and Gekas 1998).

Water activity influences not only microbial spoilage but also chemical and enzymatic reactivity as well as the storage stability of foods, since some deteriorative processes in foods are mediated by water (Beuchat and Rockland 1987; Rockland and Stewart 1998). In addition, there is a relationship between water activity and physical properties of foods. If the water activity value of foods is high, the desirable textural properties, such as moist, tender, juicy, and chewy occur on foods. On the contrary, undesirable textural properties, such as hard, dry, and stale appear, if the water activity value of foods is lowered (Labuza 1987; Fontana 2000).

It is not easy to control the water activity value in order to inhibit the microbial spoilage in foods, and at the same time to maintain their desirable physical properties. Several traditional methods of preservation such as drying, sugaring, and salting have been applied in order to prolong the shelf life of foods (Board and Gould 1991). These traditional methods were intended to reduce water activity of foods, so that microorganisms causing spoilage in foods can not grow any longer (Chirife 1993; Gibbs and Gekas 1998). However, there are disadvantages of decreasing the water activity by these traditional methods, namely loss of the physical quality properties,

leading to poor texture and impaired appearance of foods with regard to color, shape, size, and gloss (Fontana 2000; Malcoim 2002). Furthermore, the problem of discoloration on shrimp meat became one of the biggest serious concerns of the shrimp industry, as shrimps usually undergo rapid deterioration as a result of their unique biological and biochemical characteristics (Simpson et al. 1988; Yan et al. 1990).

In this work, the potency of chitosan SN 22 to control the water activity of shrimp meat during storage was tested. Due to its antimicrobial activity and ability to retard moisture loss (Durango et al. 2006), chitosan is qualified to be used as a preserving agent to maintain the quality of shrimp meat during storage, and at the same time, to get its desirable physical properties. Besides that, to optimize the preservation process of shrimp meat with chitosan during storage, the coating method was applied in the present study. Furthermore, to maintain the quality of shrimp meat optimally and to extend its shelf life, chitosan SN 22 was incorporated with garlic extract.

Changes in the a_w value of shrimp meat coated with chitosan SN 22 during storage at refrigerator and room temperature can be seen in Figure 3.13. The presented results show that chitosan SN 22 proves useful as a preserving agent for controlling the a_w value of shrimp meat during 14 d of storage. In general, the a_w values of shrimp meat coated with chitosan SN 22 during storage at both temperatures met the acceptable a_w values for shrimp meat freshness, which are in the range of 1.00–0.95 (Fontana 2000).

Regarding the changes in the a_w value of coated shrimp meat with chitosan-garlic extract during storage at both temperatures, it can be observed that the initial a_w value of shrimp meat (0.99) could be maintained up to day 14 of refrigerated storage (see Figure 3.22). Although the a_w value slightly decreased to a value of 0.98 at day

16 of storage, chitosan-garlic extract could maintain the a_w value of shrimp meat until the end of storage, with the final a_w value of 0.96.

A similar trend was found for changes in the a_w value of coated shrimp meat stored at room temperature, whereby chitosan-garlic extract could maintain the initial a_w value of shrimp meat (0.99) up to day 7 of storage. Afterwards, the a_w value decreased to a value of 0.98 up to day 14 of storage and reached the final value of 0.94 at day 30 of storage (see Figure 3.22).

The present results demonstrate that chitosan-garlic extract could clearly maintain the initial a_w value of 0.99 during 30 d of storage, so that the freshness and the shelf life of shrimp meat could be extended. On the other hand, due to their high antimicrobial activity, chitosan-garlic extract strongly inhibited growth of microorganisms causing spoilage in shrimp meat, thus delaying the microbial spoilage without reducing the a_w value.

These present results are in accordance with the theoretical review summarized by Abbas et al. (2009). They mentioned that the detection of spoilage on fish can be determined by controlling a_w and at the same time, the spoilage can be retarded by reducing a_w of fish by preservation methods such as drying and freezing. If a_w reduced to 0.6, the growth of bacteria and moulds can be prevented. In addition, Fontana (2000) reported that microorganisms such as the bacteria causing spoilage in shrimp meat, have a limiting a_w level below which they can not grow. *E. coli* does not grow at $a_w < 0.95$; *Salmonella* is inhibited at $a_w < 0.91$; *S. aureus* can generally not grow at $a_w < 0.80$, and *L. monocytogenes* does not further grow at $a_w < 0.92$.

Although water activity is an important property that is used to predict the quality and safety of foods, particularly for seafood, up to now no published scientific paper can be found that describes only a_w determination to assess seafood quality. Most investigators generally preferred to choose sensory evaluation for the assessment of

freshness and quality of seafood, especially for commercial purposes. Speed, simplicity, and low cost are the main advantages of this method (Baixas-Nogueras et al. 2002; Ólafsdóttir et al. 2005).

Shrimps, particularly in the shrimp industry, are also generally assessed by sensory methods based on changes in their appearance, rather than measuring changes in their water activity value. However, sensory analyses are inherently subjective, even when the panel members have received extensive training. Moreover, measurements of sensory parameters have not been documented up to now (Koutsoumanis et al. 1999; Ólafsdóttir et al. 2005).

4.3.2.3 Changes in content of TVBN

The occurrence of total volatile basic nitrogen (TVBN) is one of the characteristic features attributed to changes in biochemical parameters. This parameter is widely considered to be a useful index of seafood freshness (Ólafsdóttir et al. 2006).

In this work, the initial TVBN contents reached values of 10 mg/100 g of shrimp meat. The TVBN contents of shrimp meat coated with chitosan SN 22 did not immediately increase, but a lag phase was apparent during refrigerator as well as room temperature storage (see Figure 3.14). On the contrary, the TVBN contents of uncoated samples (control) already increased after day 3 of storage, especially during room temperature storage. Afterwards, a steady increase of the TVBN content occurred up to the end of storage (see Figure 3.14). The sharp increase in TVBN content was due to the increase in total plate count of aerobic mesophilic bacteria (Baixas-Nogueras et al. 2002; Shahidi et al. 2002).

The possible reason for the results is that chitosan may possess antimicrobial properties so that chitosan is able to inhibit the growth of the microorganisms, which are responsible for the production of volatile amines on shrimp meat (Ólafsdóttir et al.

2006). Besides that, the level of TVBN of shrimp meat samples depends on the temperature and time of storage. These two factors also influence the production of volatile amines through their effect on the growth of microorganisms (Smith et al. 1996; Baixas-Nogueras et al. 2001).

In this work, the TVBN content of coated shrimp meat stored at refrigerator temperature was lower than that of samples stored at room temperature, possibly due to the fact that the growth of microorganisms responsible for TVBN production is retarded at refrigerator temperature (Shahidi et al. 2002; Kilincceker et al. 2009). However, some psychrotolerant bacteria keep on growing at refrigerator temperature (No et al. 2002; Madigan et al. 2009). Thus, the TVBN content of coated shrimp meat stored at refrigerator temperature may continue to increase. Furthermore, the increase in the TVBN contents of shrimp meat during storage may also be attributed to several enzymatic processes, namely deamination of free amino acids, degradation of nucleotides, and oxidation of amines, among others. These enzymatic processes can also occur at refrigerator temperature (Simpson et al. 1997; Baixas-Nogueras et al. 2001; Shahidi et al. 2002).

Therefore, chitosan SN 22 was used for shrimp meat preservation in the present study because chitosan is able to limit the bacterial growth and to delay the enzymatic activities that are responsible for TVBN production in shrimp meat samples during storage. The fact that chitosan SN 22 generally showed stronger antimicrobial effects against gram-positive bacteria is in accordance with the investigations reported by Sudarshan et al. (1992), No et al. (2002), and Rabea et al. (2003). In order to improve the antimicrobial activity of chitosan against gram-negative bacteria, garlic extract was added to the chitosan SN 22 solution, so that all bacteria involved in the production of TVBN in shrimp meat could be optimally inhibited. The content of TVBN of the shrimp meat samples coated with chitosan

enhanced with garlic extract Ch-G solution was reduced down to a level of 19.83 mg/100 g of shrimp meat at the end of a 30-day refrigerated storage period. At room temperature storage, Ch-G solution reduced the TVBN content down to a level of 30.05 mg/100 g of shrimp meat (Figure 3.23).

The correlation between the TVBN content and the spoilage level in marine fish and crustaceans was explained by Baixas-Nogueras et al. (2002) and Shahidi et al. (2002). They mentioned that the spoilage level in marine fish and crustaceans during storage depends on their TVBN levels. If TVBN content is low, the spoilage level in marine fish and crustaceans is also low. In this work, the TVBN contents in shrimp meat coated with Ch-G solution during a 30-day storage at both temperatures were in accordance with the acceptable levels of 30–35 mg/100 g of shrimp meat intended for human consumption according to the EU regulation (Baixas-Nogueras et al. 2002). This means that, if the TVBN contents in shrimp meat are lower than 30–35 mg/100 g of shrimp meat, the shrimp meat is still acceptable.

It is obvious from these results that the Ch-G solution at a concentration of 0.1 % (w/v) extends the shelf life of shrimp meat and keeps the TVBN content in the shrimp meat samples at low level during storage. Moreover, the results in this work are evidently better compared to those reported by other investigators. Shahidi et al. (2002) observed the TVBN content of samples of cod (*Gadus morhua*) and herring (*Clupea harengus*) coated with chitosan during a 12-day storage period at 4 °C, whereby the TVBN content increased to a value of 21.94 mg/100 g and a value of 24.19 mg/100 g for cod samples and herring samples, respectively. Ólafsdóttir et al. (2005) reported that the TVBN content of shrimp (*Pandalus borealis*) samples increased to > 70 mg/100 g on day 4 of storage under different cooling conditions. Wang et al. (2008) investigated the TVBN content of cod (*G. morhua*) samples treated by the combined methods of modified atmosphere packaging (MAP) and

super-chilled storage, reaching the TVBN content at a value of 36.2 mg/100 g at the end of a 24-day storage period.

4.3.2.4 Changes in content of biogenic amines

Biogenic amines are generally present at low levels in fresh seafood and are the result of microbial amino acid decarboxylation. Their accumulation is associated with bacterial spoilage (Kalač and Krausová 2005; Hwang et al. 2009).

In accordance with the above theory, it is evident in this work that the contents of the biogenic amines in shrimp meat samples coated with chitosan and with chitosan-garlic extract (Ch-G) solution were low. The only biogenic amines found in shrimp meat samples coated with chitosan SN 22 in the beginning of storage were putrescine and cadaverine (Figures 3.15 A and 3.15 B). The two biogenic amines were also found in shrimp meat samples coated with Ch-G solution (Figure 3.24 A and 3.24 B).

The low content of biogenic amines in the shrimp meat samples were due to the treatment with chitosan SN 22 and with chitosan-garlic extract. These results are in agreement with those of Nishibori et al. (2001) and Xue et al. (2007) who reported that low levels of biogenic amines are typical of well-treated seafood products. Biogenic amines occur in foods when free amino acids and bacteria containing decarboxylases are present together at a suitable temperature and pH value. The low levels in polyamine content may be explained by the fact that microorganisms can use these compounds as nitrogen sources, although it could also be due to deamination reactions (Halasz et al. 1994; Bardocz 1995). Based on this theory, it can be elucidated that, when shrimp meat is coated with chitosan and chitosan-garlic extract, the decarboxylase producing bacteria do not grow well. Therefore, free amino acids are not converted to biogenic amines. Moreover, during refrigerated

storage, the activities of the decarboxylases are weaker (Mietz and Karmaz 1978; Mietz and Karmaz 1981; Kalač and Krausová 2005).

Putrescine appeared earlier compared to other biogenic amines in this work, this is in agreement with Veciana-Nogués et al. (1995), Baixas-Nogueras et al. (2001), and Rezaei et al. (2007). In addition, Baixas-Nogueras et al. (2002) and Rezaei et al. (2007) explained that putrescine is the physiological precursor of other biogenic amines formed during normal metabolic processes and naturally present in cells of living organisms. Thus, normally, putrescine appears earlier than other biogenic amines (Silla-Santos 1996; Elliasen et al. 2002; Kalač and Krausová 2005).

Moreover, putrescine and cadaverine have been suggested as freshness indicators for several fish species because these two biogenic amines are the most important amines found during the spoilage of fish. In fish species, cadaverine usually starts to increase later than putrescine. However, its level at the end of the storage is generally higher (Mackie and Fernandés-Salguero 1987; Dawood et al. 1988; Moral and Ruiz-Capillas 2001).

Similarly, in this work, cadaverine and putrescine appeared earlier than other biogenic amines contained in shrimp meat. In general, putrescine started to increase earlier than cadaverine during storage at both temperatures. However, shortly before the end of storage, cadaverine increased more rapidly than putrescine, and its level at the end of storage was higher than that of putrescine. This is in accordance with Moral and Ruiz-Capillas (2001) who reported that the low initial amounts of cadaverine was found in fresh fish. Due to bacterial lysine-decarboxylase activity, cadaverine is produced during storage of fish.

The accumulation of tyramine and histamine, which are aromatic biogenic amines, was only found shortly before the end of storage. This is in accordance with a theory mentioning that the occurrence of tyramine in seafood indicates an initial stage of

decomposition. In addition, tyramine levels are important from the toxicological point of view (Baixas-Nogueras et al. 2001). Therefore, tyramine and histamine are less suitable as freshness indicators in seafood compared to other biogenic amines (Baixas-Nogueras et al. 2001; Gill 2005; Rezaei et al. 2007). Furthermore, the occurrence of histamine is extremely variable and its production is a function of time, temperature, and the microflora present during seafood storage. The enzymes involved in the production of histamine during shrimp meat storage, require temperatures greater than 15 °C (Fletcher et al. 1995; Gill 2005). Thus, in this work, the contents of histamine in shrimp meat during room temperature storage are higher than when stored at refrigerated temperature.

The values of agmatine on coated shrimp meat were less than 1 mg/kg throughout the storage period, and no significant differences were observed between its values in coated shrimp meat stored at refrigerator temperature and at room temperature. However, agamatine showed a particular profile during storage of shrimp meat at both temperatures. This peak profile could be explained by the fact that, by some microorganisms, agmatine may be formed from arginine as an intermediate metabolite in the putrescine production pathway (Moral and Ruiz-Capillas 2001).

Recently, the contents of biogenic amines have been proposed to indicate seafood spoilage, particularly for fish, since their concentration increases progressively as the fish deteriorates (Antoine et al. 2002; Xue et al. 2007; Rezaei et al. 2007). According to the Food and Drug Administration (FDA), the regulatory limits focus on the content of histamine at a level of 50 mg/kg, while other biogenic amines have no regulatory limits. For instance, the guiding limit for tuna acceptance is 50 mg/kg, according to the FDA (Baixas-Nogueras et al. 2001).

So far, no exact regulation limit for the content of biogenic amines in shrimp meat has been published. There are only few published reports in literature on the role of

biogenic amines in determining the shelf life of shrimp. Mietz and Karmaz (1978) and Vasundhara et al. (1995) reported that in shellfish such as shrimp, putrescine was found to be the dominant amine, because of the high levels of arginine in the shellfish. Other biogenic amines have been reported to be not so dominant in shrimp. Similarly, in this work, the level of putrescine was higher than that of histamine, tyramine, and agmatine.

The shelf life of shrimp meat coated with chitosan SN 22 and chitosan-garlic extract stored at refrigerator temperature is longer than when stored at room temperature. These results are in accordance with Kalač and Krausová (2005) and Abu Bakar et al. (2008) who reported that during refrigerated storage, the activities of decarboxylases, which are responsible for the production of biogenic amines in foods, are weaker.

Moreover, chitosan-garlic extract strongly suppressed the production of biogenic amines, indicated by low levels of ≤ 10 mg/kg at the end of a 30-day storage period at both temperatures. This is due to inhibition of growth of bacteria containing decarboxylases by chitosan SN 22 as well as its enforcement with garlic extract, thereby suppressing the production of biogenic amines in shrimp meat.

Hwang et al. (2009) investigated the production of biogenic amines in samples of salted and fermented anchovy (*Engraulis japonicus*) treated with garlic extract at a concentration of 5 % (w/v). At the end of a 30-day fermentation period, the concentration of putrescine and cadaverine in the samples reached levels of 37 mg/kg and 95 mg/kg, respectively.

In this work, the biogenic amines contents in shrimp meat samples treated with chitosan-garlic extract at a concentration of 0.1 % (w/v) were strongly suppressed. At the end of a 30-day refrigerated storage period, putrescine and cadaverine were retarded to levels of 3 mg/kg and 8 mg/kg, respectively, whereas at the end of room

temperature storage, they were suppressed to levels of 9 mg/kg and 10 mg/kg, respectively.

The reason for this may be that the growth and the decarboxylase activities of the four test strains causing spoilage on shrimp meat samples may be suppressed due to a stronger antimicrobial activity of chitosan enforced with garlic extract. Both antimicrobial components of chitosan and garlic extract, which are low viscosity chitosan with free amino groups and allicin, act synergistically to inhibit effectively growth of decarboxylase-producing bacteria, thus suppressing the biogenic amines production on shrimp meat during storage.

4.4 The influence of plasticizer on chitosan coating solution for shrimp meat preservation

The formation of a chitosan coating on the surface of shrimp meat is dependent on two types of interaction, namely cohesion and adhesion. Cohesion is effected by attractive forces between the chitosan molecules themselves, whereas adhesion results from attractive forces between the chitosan film and the substrate. Cohesive forces in chitosan films can result in the undesirable property of brittleness (Krochta and Sothornvit 2005). To overcome this limitation, food-grade plasticizers were added to the chitosan film formulation to decrease the cohesive forces. By reducing the cohesive forces in chitosan films, the mobility of the polymer chains, the plasticization action, and the flexibility of the coatings or films are increased.

In order to determine which plasticizer can optimally improve chitosan SN 22 coating, food-grade polyols plasticizers such as glycerol, xylitol, and sorbitol were tested. Those plasticizers were selected due to their hydroxyl groups, which can form hydrogen bonds with the chitosan polymer and thus increase the ductility and the

flexibility of chitosan SN 22 coatings or films. Besides that, the selection of polyol plasticizers considers their compatibility with chitosan coatings or films, whereby the compatibility depends on the polarity, structural configuration, and molecular weight of the selected plasticizers (Krochta and Sothornvit 2005). The polarity of these polyols positively affects their hydrophilicity and water solubility, thus their plasticizing effect increases. Moreover, the plasticizing effect of polyols can be particularly attributed to their ability to intercalate between polymer molecules, to bind water, and to disrupt intermolecular polymer associations. The relative effectiveness of different polyols may be attributed to their varying ability to associate with water (Gontard et al. 1993; Shaw et al. 2002; Suyatma et al. 2005). In addition, polyols qualify as plasticizers since they have low volatility, as well as being non-toxic and aroma free (Krochta and Sothornvit 2005). Beside that, glycerol, xylitol, and sorbitol can be isolated from plants, thus, they are biodegradable and environmentally friendly compounds. These characteristics are very important for industry due to environmental concerns (Padgett et al. 1998).

Furthermore, with regard to the structural configuration and molecular weight of glycerol, xylitol, and sorbitol, these polyols possess the simple structural configurations without bulky branches, resulting in small size, indicated by their molecular weights, as presented in Table 1.5 (Krochta and Sothornvit 2005).

The plasticizing effect of glycerol, xylitol, and sorbitol was determined by measuring the moisture content in chitosan films. Glycerol showed the best plasticization activity and stability compared to the chitosan films plasticized with xylitol and sorbitol. The possible reason is due to the infinitely water solubility of glycerol at 25 °C (Griffin and Lynch 1988; Shaw et al. 2002). Moreover, due to its high water solubility, glycerol is able to prevent the evaporation of water and the loss of moisture from the coatings or film matrixes during storage (Suyatma et al. 2005). Cervera et al. (2004) observed

that the crystallinity of chitosan-amylose starch films tends to increase during storage, and the crystallinity development is well-known to increase the rigidity and brittleness of chitosan coatings or films.

The results in this work support the argument that the effectiveness of glycerol as plasticizer can be attributed to its higher water solubility and hydrophilicity relative to the other polyols tested in this work. In addition, with regard to structural configuration and molecular weight of the tested plasticizers, glycerol has the simplest structural configuration and the lowest molecular weight (M_w = 92) compared to xylitol (M_w = 152) and sorbitol (M_w = 182), as presented in Table 1.5 (Krochta and Sothornvit 2005).

The volatility of plasticizers must be taken into account for their correct choice, because it influences the stability of chitosan coatings or thin films during storage and application. In this case, the least volatile plasticizer, glycerol, is recommended for use (Mangavel et al. 2003; Cervera et al. 2004; Suyatma et al. 2005).

To obtain the appropriate composition of chitosan coatings and films in this work, glycerol as the best plasticizer had to be used in the correct concentration, in order to obtain the advantage of enhancing the properties of films and coatings. According to Chang et al. (2000) and Guilbert et al. (2002), plasticizers are generally required at approximately 10–60 % on dry basis, depending on the stiffness of the polymer. Based on this statement, in the present study, glycerol at concentrations of 10–40 % (w/v) was tested. The effects of various concentrations of glycerol on the moisture contents of the chitosan thin films are presented in Figure 3.7. Glycerol showed the highest plasticization activity at concentration of 20 % (w/v), at which the moisture content of the plasticized chitosan films reached 72 %. Regarding the plasticization activity of glycerol at a low concentration of 10 %, the moisture content in the plasticized chitosan films was lower (61 %). This is in accordance with the

observation conducted by Guilbert et al. (1996) and Seow et al. (1999) who reported that at low concentrations of plasticizers, antiplasticization can occur in films and coatings, which can be attributed to several mechanisms such as reduction of polymer free volume, interaction between the polymer and plasticizer, and film or coating rigidity. Moreover, Lourdin et al. (1997) found that adding a low concentration of plasticizer could lead to an increase in polymer crystallinity, due to a lowering of the energy barrier for a change of polymer state. Indeed, crystallinity development is well known to increase the rigidity and brittleness of chitosan coatings or films. In addition, several investigators such as Caner et al. (1998), Coma et al. (2002), and Suyatma et al. (2005) stated that at a high plasticizer content, the plasticization activity to enhance the coating properties and the coating process decreased.

Several similar studies on plasticization activity using polyols on whey protein isolate films revealed that glycerol and sorbitol increased the films permeability and extensibility and reduced the film strength (McHugh et al. 1994; Alexeev et al. 2000). Moreover, Butler et al. (1996) found that glycerol could maintain the water barrier and mechanical properties of plasticized chitosan films during storage. In addition, Gontard et al. (1993) proposed in their study on wheat gluten films that the mode of action of plasticizers involved a modification of the moisture uptake of films. However, little is known of the plasticizing effects of polyols such as xylitol and sorbitol in film and coating systems. It must be emphasized that with regard to coatings and films manufactured from chitosan, little or no work has been published about the effects of plasticizers on their moisture content during storage up to now.

4.5 Future prospects

Chitosan SN 22 as an edible natural biopolymer proved to be effective as a preservative coating for shrimp meat during storage. The coating method used to

maintain the quality of shrimp meat showed that a very good preservation could be achieved throughout a 30-day storage period. Moreover, incorporation of garlic extract into the chitosan coating solution revealed an excellent synergistic inhibitory effect against microorganisms causing spoilage in shrimp meat, thus, the shelf life of shrimp meat was greatly extended.

Due to the great effectiveness of chitosan and its enforcement with garlic extract for shrimp meat preservation, and with chitosan and garlic extract being natural materials, the use of synthetic preservatives and chemical additives that have so far been generally used to improve quality and to extend shelf life of seafood during storage, can be replaced. Recently, the use of natural preservatives in food applications has become very popular and has turned out to be very promising, particularly for the food industry.

Furthermore, due to the increasing demand for natural food additives, more extensive efforts are currently being made in research for alternative traditional and natural antimicrobial agents such as spice extracts (Holley and Patel 2005; Hwang et al. 2009). Recently, traditional and natural antimicrobial agents have been reviewed with regard to their potential value for use as “secondary preservatives” in foods (Douglas and Bakrie 2005; Rose et al. 2005) and the regulatory status of many of these in the USA was outlined lately (Holley and Patel 2005). In addition, incorporation of “secondary preservatives” into coating solutions or film matrices is one of the most challenging technologies in the field of edible coatings and films (Fazilah et al. 2008; Dutta et al. 2009).

Due to environmental concerns and regulations to develop environmentally friendly packaging materials, bioactive edible coatings or films constitute one of the most important issues for the food industry due to their biodegradability (Park and Zhao 2004; Lopez-Rubio et al. 2004; Cutter 2006; Dainelli et al. 2008).

In conclusion, chitosan and its enforcement with garlic extract as a preservative coating may be used as a promising alternative to replace the use of classical synthetic preservatives and conventional methods of preservation, due to growing demands of consumers for safer and better quality foods and food industry for environmentally friendly packaging materials.

Furthermore, regarding the environmental awareness, edible coatings of chitosan incorporated with garlic extract may offer a promising alternative as an environmentally friendly packaging material that can replace some non-degradable plastic packaging. Thus, the existing environmental problems caused by the disposal of plastic waste from consumers and industries may gradually be solved in the future. In addition, because chitosan edible coating can be consumed along with the food, the disposal of food packaging waste can be reduced.

This work may hold some future contributions for my home country, Indonesia, which is one of the main producers of shrimp in the world. Using chitosan obtained through deacetylation of chitin, which is biotechnologically produced from shrimp shell waste, is a promising ecological and an economical challenge. On the one hand, the problem of disposal of the large quantities of shrimp shell waste may thus be solved. On the other hand, due to the high potential of chitosan as a natural food preservative, and with respect to the growing consumer awareness regarding synthetic preservatives, the food industry sector may potentially be opened with new marketing opportunities.

5 Summary

The focus of this work was to study the potency of chitosan as edible coating to improve the quality and to extend shelf life of shrimp meat during storage. Chitosan as a natural biopolymer qualifies as a potential food preservative due to its antimicrobial activity. The potency of various charges of chitosan with different chain lengths was initially tested to inhibit seafood spoilage bacteria, namely gram-positive bacteria (*Staphylococcus aureus, Listeria monocytogenes)* and gram-negative bacteria (*Escherichia coli, Salmonella typhimurium)*. Simultaneously, the concentrations and pH values of chitosan charges had to be optimized. Beside that, the physical characteristics of chitosan, such as molecular mass (M_η) and degree of deacetylation (DDA) had to be determined in order to evaluate the influence of these physical characteristics on the antimicrobial activity of chitosan.

The chitosan charge SN 22 (M_η = 1.5 × 10^5 g/mol, DDA = 80 %) showed the best antimicrobial activity against the four test strains at a concentration of 0.1 % (w/v) and at an optimum pH value of 5.5. Chitosan SN 22 added with glycerol as a plasticizer was applied as coating solution to preserve shrimp meat samples, which were then stored for up to 14 days at room temperature and refrigerator temperature (at 4–7 °C). The changes in microbiological parameters (growth of total aerobic mesophilic bacteria, growth of gram-positive and gram-negative bacteria) and biochemical parameters (pH value, content of total volatile basic nitrogen, water activity, and content of biogenic amines) were monitored during storage. Chitosan SN 22 at a concentration of 0.1 % (w/v) can inhibit the growth of mesophilic bacteria and that of the four spoilage bacteria, and can extend the shelf life of shrimp meat.

To achieve an improved preservation function for shrimp meat, chitosan SN 22 coating solution was further enforced with garlic extract. Chitosan-garlic extract at a concentration of 0.1 % (w/v) was shown to partially or completely suppress the growth of the tested bacterial strains, particularly *S. typhimurium* LMH 2N strain and *L. monocytogenes* LMH 34P/NCTC 10587. This potency was further tested for preservation of shrimp meat samples and monitored during storage for up to 30 days at room temperature and refrigerator temperature (at 4–7 °C). Chitosan-garlic extract can reduce the growth of mesophilic bacteria down to 10^4 CFU/g of shrimp meat (the acceptable upper limit amounts to 10^6 CFU/g), whereas the growth of the four spoilage bacteria are almost completely or entirely suppressed. Monitoring of biochemical parameters showed that chitosan-garlic extract can maintain the pH value below to the acceptable upper limit of 7.8, reduce the TVBN content down to the acceptable upper limit of 30–35 mg N/100 g of shrimp meat, retard increase in the contents of biogenic amines, and keep the water activity value of shrimp meat near the initial value of 0.99, during a storage period of 30 days.

The present results indicate that chitosan coating solution enforced with garlic extract proved to be optimal for improving the quality and the shelf life of shrimp meat during the storage.

Zusammenfassung

Der Schwerpunkt der vorliegenden Arbeit besteht in der Testung der Wirksamkeit von Chitosan als essbare Beschichtung zur Verbesserung der Qualität und zur Verlängerung der Haltbarkeit von Garnelenfleisch während der Lagerung. Chitosan ist ein natürliches Biopolymer, das aufgrund seiner antimikrobiellen Wirkung als Konservierungsmittel für Lebensmittel geeignet ist. Die Wirksamkeit verschiedener Chitosan-Chargen mit unterschiedlichen Kettenlängen wurde zunächst an Bakterien

getestet, die am Verderb der Meeresfrüchte beteiligt sind. Hierzu gehören Gram-positive Bakterien (*Staphylococcus aureus, Listeria monocytogenes)* und Gram-negative Bakterien (*Escherichia coli, Salmonella typhimurium*). Gleichzeitig wurden die Konzentrationen und die pH-Werte der zum Einsatz kommenden Chitosan-Chargen optimiert. Die physikalischen Eigenschaften von Chitosan, wie molekulare Masse (M_η) und Deacetylierungsgrad (DDA) wurden ermittelt, um deren Einfluss auf die antimikrobielle Aktivität von Chitosan zu analysieren.

Die Chitosan-Charge SN 22 (M_η = 1,5 x 10^5 g/mol; DDA = 80 %) zeigt bei einer Konzentration von 0,1 % (w/v) und einem pH-Wert von 5,5 die beste antimikrobielle Aktivität gegen die vier untersuchten Stämme. Chitosan SN 22 wurde darüber hinaus als Beschichtungslösung unter Zusatz eines Weichmachers (Glycerin) zur Konservierung der Garnelenfleischproben benutzt, die dann bis zu 14 Tage bei Raum- und Kühlschranktemperatur (bei 4–7 °C) gelagert wurden. Die veränderten mikrobiologischen Parameter (Entwicklung der Gesamtkeimzahl der aeroben mesophilen Bakterien, Wachstum der Gram-positiven sowie der Gram-negativen Bakterien) und die veränderten biochemischen Parameter (pH-Wert, Gehalt an flüchtigem basischem Stickstoff, Wasseraktivitätswert und Gehalt an biogenen Aminen) wurden während der Lagerung beobachtet. Chitosan SN 22 kann bei einer Konzentration von 0,1 % (w/v) sowohl das Wachstum der mesophilen Bakterien als auch das der vier Bakterienstämme hemmen, die für den Verderb von Garnelenfleisch verantwortlich sind. Chitosan kann somit die Haltbarkeit des Garnelenfleisches verlängern.

Um eine verbesserte Konservierung von Garnelenfleisch zu erzielen, wurde der Chitosan-Beschichtungslösung ein Knoblauchextrakt beigefügt. Chitosan-Knoblauchextrakt mit einer Konzentration von 0,1 % (w/v) zeigt eine fast vollständige

bzw. vollständige Unterdrückung des Wachstums der getesteten Bakterienstämme, vor allem von *S. typhimurium* LMH 2N und *L. monocytogenes* LMH 34P/NCTC 10587. Diese Wirkung wurde zudem für die Konservierung der Garnelenfleischproben getestet und bei einer Lagerung von bis zu 30 Tagen bei Raum- und Kühlschranktemperatur (bei 4–7 °C) verfolgt. Der Chitosan-Knoblauchextrakt kann das Wachstum der mesophilen Bakterien auf bis zu 10^4 KBE/g Garnelenfleisch begrenzen (der annehmbare obere Grenzwert beträgt 10^6 KBE/g). Das Wachstum der vier untersuchten Bakterienstämme wurde hierbei fast vollständig bzw. vollständig unterdrückt. Die Ergebnisse der biochemischen Analysen zeigen, dass Chitosan-Knoblauchextrakt für einen Lagerungszeitraum von 30 Tagen den pH-Wert unter der zulässigen Obergrenze von 7,8 hält, den TVBN-Gehalt unterhalb der zulässigen Obergrenze von 30–35 mg N/100 g Fleisch hält, die Erhöhung des Gehalts an biogenen Aminen verzögert und die Wasseraktivität des Garnelenfleisches nahe dem Anfangswert von 0,99 stabilisiert. Es ist gelungen zu zeigen, dass die Beschichtungslösung aus Chitosan-Knoblauchextrakt beim Einsatz als Konservierungsmittel einen sehr guten Effekt auf die Qualität und die Haltbarkeit von Garnelenfleisch während der Lagerung hat.

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7 Appendix

7.1 Hazardous chemicals

7.1.1 List of hazardous chemicals

Chemicals	Hazard symbol	Risk phrase	Safety phrase
Acetic acid	C, F	R10, R35	(S1/2), S23, S26, S45
Acetonitrile	F, Xn	R11, R20/21/22, R36	(S1/2), S16, S36/37
Agmatine sulphate	F	R36/38, R23/24/25	S22
Boric acid	T	R60, R61	S53, S45
BRIJ® 35	N, Xn	R36/38, R52/53	S26, S37/39
Cadaverine dihydrochloride	Xi	R36/37/38	S26, S36
Calcium chloride dihydrate	Xi	R36	S22, S24
Cupric sulfate pentahydrate	N, Xn	R22, R36/38, R50/53	S22, S60, S61
Deuterium chloride	F, Xn	R12, R20/21/22, R36	(S1/2), S16, S36/37
Deuterium oxide	F, Xn	R12	S2, S9, S16, S33
1.6-Diaminohexane	Xi	R38	S3/7, S24
Histamine hydrochloride	T	R60, R61	S53, S45
Hydrochloric acid	C	R34, R37	S26, S45
Methanol	F, T	R11, R23/24/25, R39/23/24/25	S7, S16, S36/37, S45
Mercaptoethanol	T+	R22, R24, R34, R51/53	S26, S36/37/39, S45, S61
Perchloric acid	C, O	R5, R8, R35	(S1/2), S23, S26, S36, S45
Phenolphthalein	T	R45, R62, R68	S53, S45

Chemicals	Hazard symbol	Risk phrase	Safety phrase
o-Phthaldialdehyde	F, Xi	R36/37/38	S7, S15, S16, S24/25
Potassium hydroxide	C, Xn	R22, R35	(S1/2), S26, S36/37/39, S45
Polyethylene lauryl ether	Xn	R22, R38, R41	S26, S36/37/39
Putrescine dihydrochloride	F, Xi	R36/37/38	S7, S15, S16, S24/25
Silicon antifoam emulsion	Xi	R36/37/38	S26, S36
Sodium acetate anhydrous	Xi	R36/37/38	S26, S36
Sodium hydroxide	C	R35	S26, S37/39, S45
Sodium octane sulfonate	F, Xi	R36/37/38	S7, S15, S16, S24/25
Sulphuric acid	C	R35	S26, S30, S45
3-trymethylsilyl 3,3,2,2 tetra-deuteropropionic acid, Na salt	F, Xn	R12, R19, R22, R66, R67	S16
Trifluoroacetic acid	F+, Xn	R20, R35, R52/53	S9, S26, S27, S28 S45, S61
Trimethylsilyl-3-propionate, sodium salt D4	F, Xn	R12, R19, R22, R66, R67	S3/7, S8, S24/25
Tyramine hydrochloride	Xi	R36/38	S24/25

7.1.2 Abbreviation and description of hazard

Abbreviation	Hazard	Description of hazard
O	Oxidising	Chemicals that react exothermically with other chemicals.
F+	Extremely flammable	Chemicals that have an extremely low Flash low flash point and boiling point, and gases that catch fire in contact with air.

Abbreviation	Hazard	Description of hazard
F	Highly flammable	Chemicals that may catch fire in contact with air, only need brief contact with an ignition source, have a very low flash point or evolve highly flammable gases in contact with water.
T+	Very toxic	Chemicals that at very low levels cause damage to health.
T	Toxic	Chemicals that at low levels cause damage to health.
Xn	Harmful	Chemicals that may cause damage to health.
C	Corrosive	Chemicals that may destroy living tissue on contact.
Xi	Irritant	Chemicals that may cause inflammation to the skin or other mucous membranes.
N	Dangerous for the environment	Chemicals that may present an immediate or delayed danger to one or more components of the environment

7.1.3 Risk phrases and description of risk

Abbreviation	Description of risk
R5	Heating may cause an explosion
R8	Contact with combustible material may cause fire
R10	Flammable
R11	Highly flammable
R12	Extremely flammable
R19	May form explosive peroxides
R20	Harmful by inhalation
R20/21/22	Harmful by inhalation, in contact with skin and if swallowed

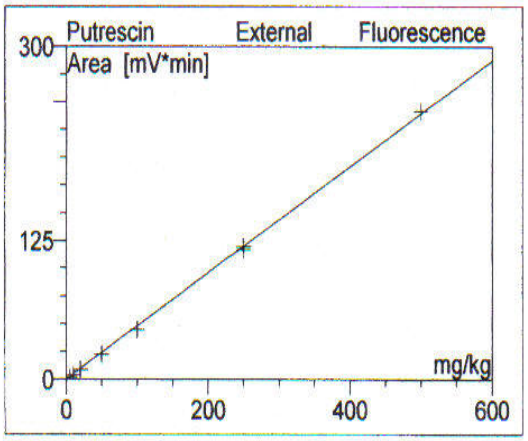
Abbreviation	Description of risk
R22	Harmful if swallowed
R23/24/25	Toxic by inhalation, in contact with skin and if swallowed
R23/24/25	Toxic by inhalation, in contact with skin and if swallowed
R24	Toxic in contact with skin
R34	Causes burns
R35	Causes severe burns
R36	Irritating to eyes
R37	Irritating to respiratory system
R38	Irritating to skin
R36/37/38	Irritating to eyes, respiratory system and skin
R36/38	Irritating to eyes and skin
R39/23/24/25	Toxic: danger of very serious irreversible effects through inhalation, in contact with skin and if swallowed
R41	Risk of serious damage to eyes
R45	May cause cancer
R50/53	Very toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment
R51/53	Toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment
R52/53	Harmful to aquatic organisms, may cause long-term adverse effects in the aquatic environment
R60	May impair fertility
R61	May cause harm to the unborn child
R62	Possible risk of impaired fertility
R66	Repeated exposure may cause skin dryness or cracking
R67	Vapours may cause drowsiness and dizziness
R68	Possible risk of irreversible effects

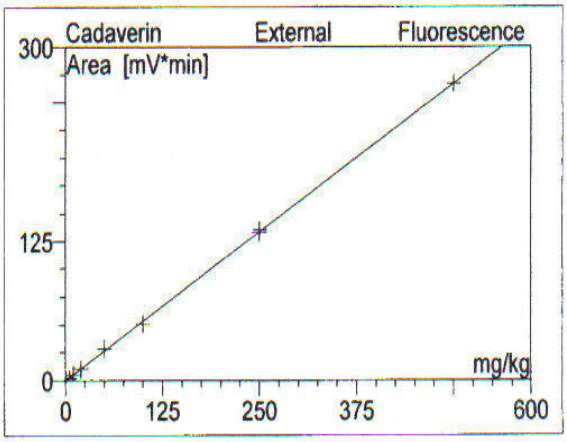
7.1.4 Safety phrases and description of safety

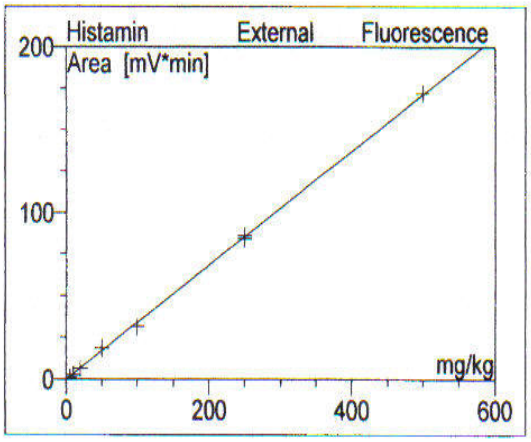
Abbreviation	Description of safety
(S1/2)	Keep locked up and out of the reach of children
S2	Keep out of the reach of children
S3/7	Keep container tightly closed in a cool place
S7	Keep container tightly closed
S8	Keep container dry
S9	Keep container in a well-ventilated place
S15	Keep away from heat
S16	Keep away from sources of ignition - No smoking
S22	Do not breathe dust
S23	Do not breathe gas/fumes/vapour/spray (appropriate wording to be specified by the manufacturer)
S24	Avoid contact with skin
S24/25	Avoid contact with skin and eyes
S26	In case of contact with eyes, rinse immediately with plenty of water and seek medical advice
S27	Take off immediately all contaminated clothing
S28	After contact with skin, wash immediately with plenty of (to be specified by the manufacturer)
S30	Never add water to this product
S33	Take precautionary measures against static discharges
S36	Wear suitable protective clothing
S36/37	Wear suitable protective clothing and gloves
S36/37/39	Wear suitable protective clothing, gloves and eye/face protection
S37/39	Wear suitable gloves and eye/face protection
S45	In case of accident or if you feel unwell seek medical advice immediately (show the label where possible)

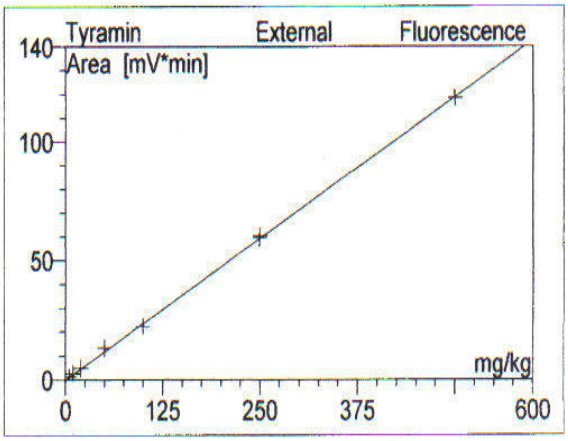
Abbreviation	Description of safety
S53	Avoid exposure - obtain special instructions before use
S60	This material and its container must be disposed of as hazardous waste
S61	Avoid release to the environment. Refer to special instructions/safety data sheet

7.2 Calculation of biogenic amines content from the standard curve







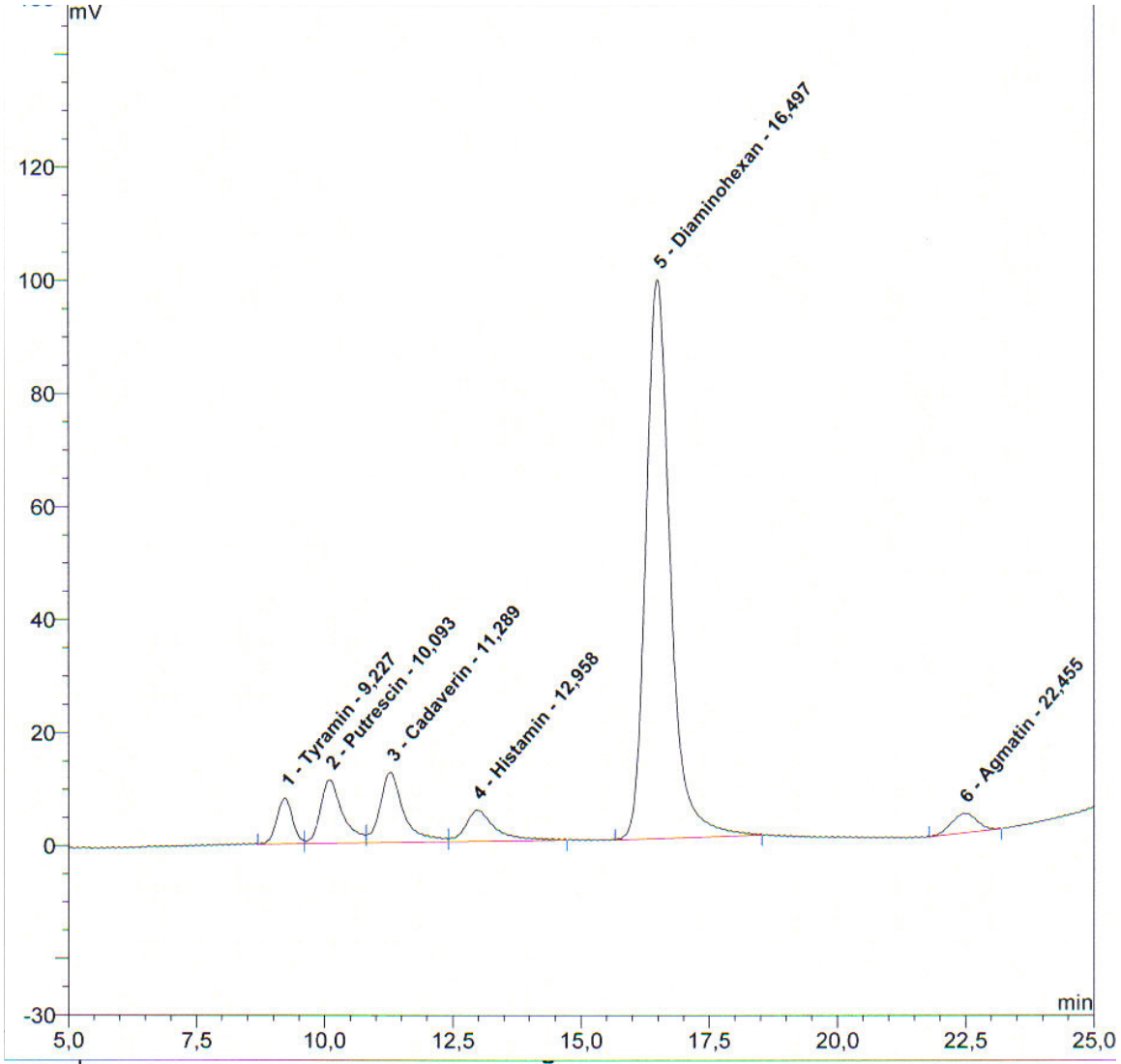


No.	Ret.Time min	Peak Name	Cal.Type	Points	Coeff.Det. %	Offset	Slope	Curve
1	6,15	Tyramin	Lin	8	99,9565	0,0000	0,2373	0,0000
2	6,67	Putrescin	Lin	8	99,9451	0,0000	0,4786	0,0000
3	7,36	Cadaverin	Lin	8	99,9782	0,0000	0,5339	0,0000
4	8,22	Histamin	Lin	8	99,9443	0,0000	0,3425	0,0000
5	10,40	Diaminohexan	Lin	8	96,8389	0,0000	0,1800	0,0000
6	14,00	Agmatin	Lin	8	99,9474	0,0000	0,2507	0,0000
Average:					99,4351	0,0000	0,3372	0,0000

7.3 List of HPLC chromatogram examples of biogenic amines

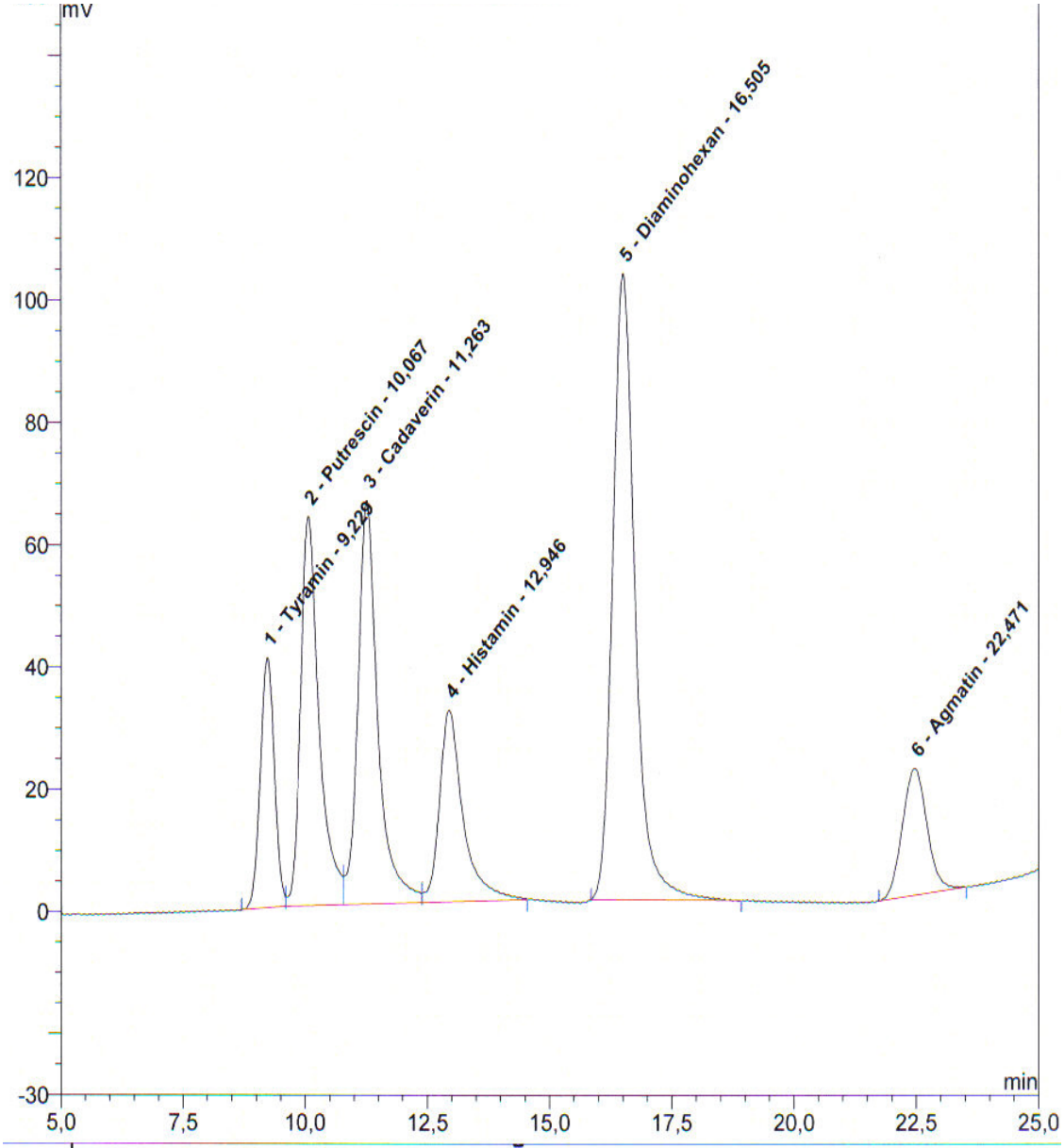
7.3.1 Chromatograms profile of biogenic amines standard

A) At a concentration of 10 mg/kg (with 5-diaminohexane as the internal standard)



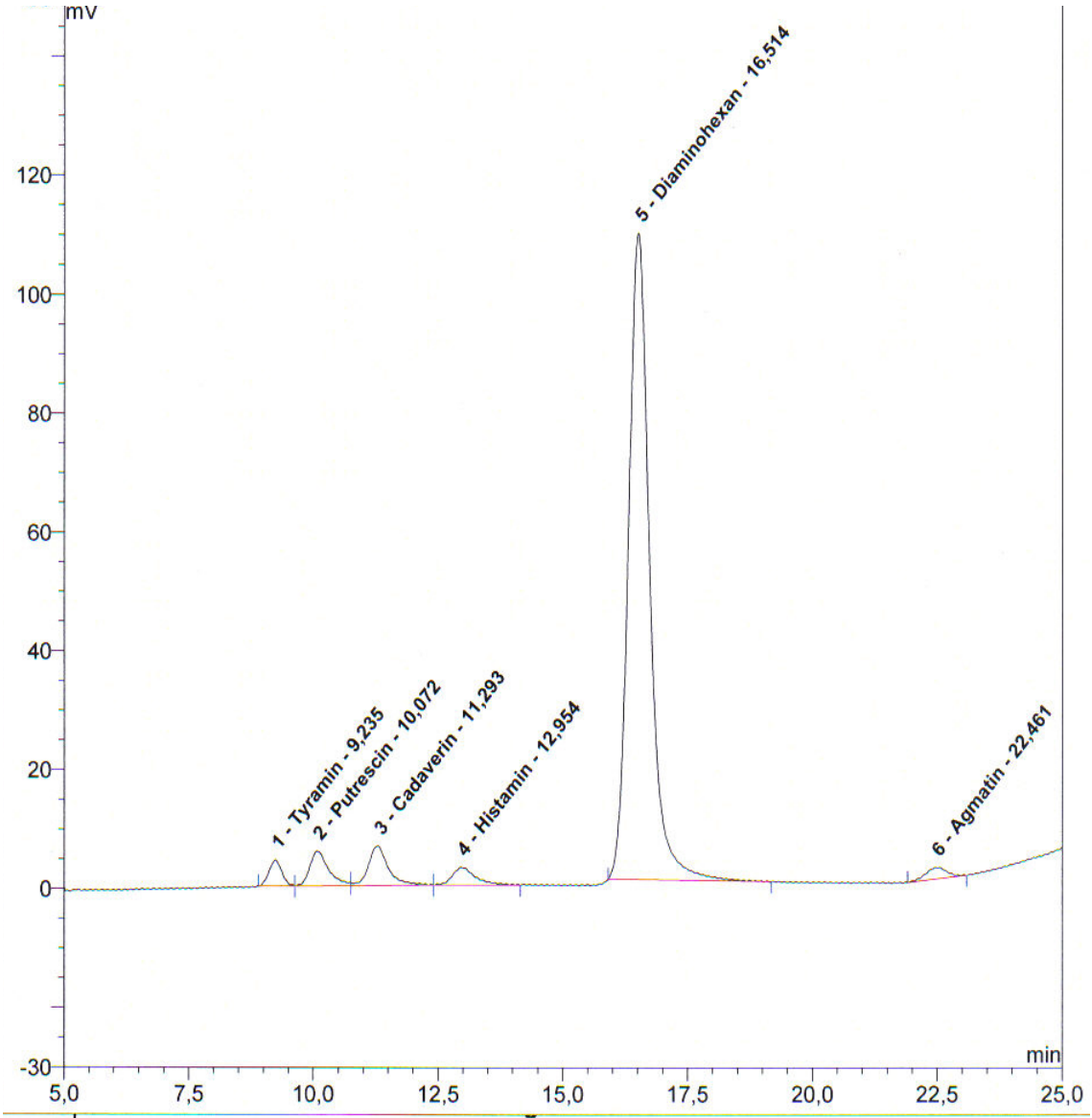
	min		mV	mV*min	%	mg/kg	mV
1	9,23	Tyramin	8,134	2,870	3,81	11,252	0,158
2	10,09	Putrescin	11,153	5,620	7,46	9,522	0,158
3	11,29	Cadaverin	12,397	6,602	8,76	11,040	0,158
4	12,96	Histamin	5,537	3,680	4,88	10,454	0,158
5	16,50	Diaminohexan	98,746	54,521	72,35	253,846	0,158
6	22,46	Agmatin	3,554	2,067	2,74	8,683	0,158
Total:			139,522	75,362	100,00	304,796	

B) At a concentration of 50 mg/kg (with 5-diaminohexane as the internal standard)



	min		mV	mV*min	%	mg/kg	mV
1	9,23	Tyramin	40,736	13,617	8,73	53,379	0,138
2	10,07	Putrescin	63,565	27,963	17,92	47,375	0,138
3	11,26	Cadaverin	65,966	30,992	19,86	51,821	0,138
4	12,95	Histamin	31,262	17,452	11,19	49,568	0,138
5	16,51	Diaminohexan	102,290	53,837	34,51	250,661	0,138
6	22,47	Agmatin	20,732	12,162	7,80	51,083	0,138
Total:			324,551	156,024	100,00	503,887	

7.3.2 Chromatogram profile of biogenic amines in the shrimp meat sample (with 5-diaminohexane as the internal standard)



	min		mV	mV*min	%	mg/kg	mV
1	9,23	Tyramin	4,333	1,256	2,00	4,924	0,124
2	10,07	Putrescin	5,782	2,492	3,98	4,223	0,124
3	11,29	Cadaverin	6,612	2,998	4,78	5,012	0,124
4	12,95	Histamin	2,969	1,585	2,53	4,502	0,124
5	16,51	Diaminohexan	108,703	53,384	85,14	248,549	0,124
6	22,46	Agmatin	1,991	0,983	1,57	4,130	0,124
Total:			130,390	62,698	100,00	271,341	

Curriculum Vitae

Personal data:

Name:	Tri Erny Dyahningtyas
Nationality:	Indonesian

Education background:

Doctoral Program	Division of Food Microbiology and Biotechnology, Institute of Food Chemistry, Department of Chemistry, The University of Hamburg, Germany
Master of Science	Department of Chemistry, Kosin University, Busan, South Korea
Bachelor of Science	Department of Chemistry, Gadjah Mada University, Yogyakarta, Indonesia

Scholarship:

For taking PhD degree	Awarded by German Protestant Church Development Service *(Evangelischer Entwicklungsdienst e.V.- EED)*, Bonn, Germany
For attending the German course	Awarded by German Protestant Church Development Service *(Evangelischer Entwicklungsdienst e.V.- EED)*, Bonn, Germany, for

	the preparation of doctoral program (elementary-advanced level),
For conducting Master degree	Awarded by Korean Church Development Service, Busan, South Korea
For accomplishing Bachelor degree	Awarded by Gloria Foundation, Ministry of Education and Research, Yogyakarta, Indonesia

Award:

Talented young researcher	Granted by Department of Chemistry, Kosin University, Busan, South Korea for conducting “foreign research stay” for 2.5 months at the Division of Bioinorganic chemistry, Department of Chemistry of The University of Tokyo, Japan
Best performance talented young student	Granted in the frame of “Best Performance Talented Young Student” competition, organized by Gloria Foundation, under supervision of Ministry of Education and Research, Yogyakarta, Indonesia

Work experience:

Education staff	At Department of Biology of Duta Wacana Christian University, Yogyakarta, Indonesia, responsible for the chemistry lessons

The head of the laboratory	At Biology Department of Duta Wacana Christian University, Yogyakarta Indonesia

Personal Skill:

Languages	Indonesian: mother tongue
	English: proficient in writing and speaking
	German: advanced level
	Korean: elementary level
Computer	MS Office (word, power-point, excel)
	Adobe Photoshop