

**APPLICATION OF LACTIC ACID, NISIN AND
CHITOSAN ON QUALITY OF PRAWN
(*FENNEROPENAEUS MERGUIENSIS*)**

ALIREZA SHIRAZINEJAD

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**APPLICATION OF LACTIC ACID, NISIN AND
CHITOSAN ON QUALITY OF PRAWN
(*FENNEROPENAEUS MERGUIENSIS*)**

by

ALIREZA SHIRAZINEJAD

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PENGGUNAAN ASID LAKTIK, NISIN DAN KITOSAN TERHADAP KUALITI UDANG (*FENNEROPENAEUS MERGUIENSIS*)

ABSTRAK

Kesan asid-asid organik terpilih (asid laktik, askorbik, sitrik dan malik) untuk memperlahankan kerosakan bakterial udang yang disejukkan telah dikaji. Asid laktik, apabila digunakan pada paras 2% (v/v) dalam nisbah 1:2 (udang:larutan asid lactic; w/v), tidak memberi kesan buruk pada sifat-sifat organoleptik udang tetapi dapat menghalang pertumbuhan mikroorganisma yang tidak diingini. Asid laktik (2% v/v) dalam kombinasi dengan nisin (0.04 g/L atau 1600 IU/mL) mempamerkan kesan perencatan ketara pada *Pseudomonas* spp dan bakteria menghasilkan H₂S yang dianggap sebagai mikroorganisma perosak utama dalam udang sejuk tersimpan. Kesan “permeabilizing” asid laktik (yang mengakibatkan pembebasan lipopolisakarida daripada membran luar bakteria) pada *Shewanella putrefaciens* (dikenalpasti sebagai bakterium perosak utama dalam udang) telah dipertingkatkan oleh kehadiran nisin.

Rawatan sel *Shewanella putrefaciens* dengan gabungan asid laktik (0.04% v/v) dan nisin (1600 IU/mL) mengakibatkan pembebasan tertinggi bahan menyerap UV (OD_{260nm}) ke dalam supernatan sel-bebas, peningkatan yang ketara dalam pengambilan NPN, dan kesan terbesar ke atas “permeabilization” daripada sel *S. putrefaciens* ($P < 0.05$) yang juga dilihat melalui mikroskop pendarfluor. Kajian perubahan permukaan sel *S. putrefaciens* menunjukkan kejatuhan ketara dengan tekstur yang sangat kasar pada struktur permukaan sel apabila sel-sel telah dirawat dengan asid laktik dalam kombinasi dengan nisin. Perubahan morfologi sel berikutan pendedahan kepada rawatan yang sama menunjukkan struktur vesikular pada membran luar. Asid laktik dalam kombinasi dengan nisin amat meningkat

pembentukan “vesicle” yang mengakibatkan perubahan ketara pada membran luar dan integritinya. Ini telah digambarkan oleh visualisasi ultrastruktur sel-sel *S. putrefaciens* dengan menggunakan mikroskop elektron penghantaran.

Manfaat daripada menggunakan agen-agen pengawet, secara kombinasi bukannya individu, dalam lanjutan hayat penstoran udang sejuk turut disahkan apabila asid laktik digunakan dalam kombinasi dengan nisin dan kitosan (LA-N-Chit). Gabungan agen-agen antimikrobial seperti ini tidak menjejaskan sifat-sifat organoleptik udang dirawat tetapi terus memperpanjangkan hayat penstoran udang sejuk (berdasarkan atas parameter mikrobiologi dan biokimia) ke 14 hari. Rawatan LA-N-Chit mampu mengurangkan populasi *Vibrio parahaemolyticus* dalam udang disejuk dan disuntikkan ke tahap di bawah had maksimum yang dibenarkan bagi patogen ini dalam makanan laut ($3 \log_{10}$ CFU/g) selepas 7-11 hari.

Pendekatan pengawetan gabungan untuk mencapai lanjutan hayat penstoran produk terus disiasat dalam udang dibungkus-vakum yang disimpan pada 4°C. Rawatan udang dengan kitosan atau LA-N-Chit, sebelum pembungkusan vakum dan penyimpanan sejuk pada 4°C, mampu untuk melanjutkan hayat penstoran produk kepada 21 hari berbanding 11 hari yang dipamerkan oleh sampel yang tidak dirawat. Gabungan LA-N-CHIT terbukti menjadi rawatan yang paling berkesan terhadap pertumbuhan *Listeria* dalam udang dibungkus-vakum.

Kesimpulannya, keberkesanan penghalangan mikrobial dan penyelenggaraan kualiti biopengawet berbeza yang digunakan dalam kajian ini didapati mengikut perintah ($P < 0.05$): LA-N-CHIT \geq CHIT > LA-N > LA > N \geq Kawalan, sama ada udang dibungkus-udara atau dibungkus-vakum. Pembungkusan vakum difahami memanjangkan hayat penstoran kedua-dua udang kawalan dan udang dirawat lebih lagi, sebanyak 4-7 hari, apabila dibandingkan dengan sampel dibungkus-udara.

APPLICATION OF LACTIC ACID, NISIN AND CHITOSAN ON QUALITY OF PRAWN (*FENNEROPENAEUS MERGUIENSIS*)

ABSTRACT

The effects of selected organic acids (lactic, ascorbic, citric and malic acids) on retarding bacterial spoilage of refrigerated prawns were studied. Lactic acid, applied at a level of 2% (v/v) in the ratio of 1:2 (prawn:lactic acid solution; w/v), had no adverse effects on the organoleptic properties of prawn but inhibited the growth of undesirable microorganisms. Lactic acid (2% v/v) in combination with nisin (0.04 g/L or 1600 IU/mL) exhibited pronounced inhibitory effects on *Pseudomonas* spp and H₂S-producing bacteria which were the main spoilage microorganisms in refrigerated stored prawn. The permeabilizing effect of lactic acid (leading to liberation of lipopolysaccharides from bacterial outer membranes) on *Shewanella putrefaciens* (identified as a major spoilage bacterium in prawn) was enhanced by the presence of nisin.

Treatment of *Shewanella putrefaciens* cells with a combination of lactic acid (0.04% v/v) and nisin (1600 IU/mL) resulted in the highest release of UV-absorbing material (OD_{260nm}) to the cell-free supernatant, a significant increase in the uptake of NPN, and the greatest effect on permeabilization of *S. putrefaciens* cells ($P<0.05$) which was also visualized through fluorescent microscopy. Studies of the surface changes of *S. putrefaciens* cells showed pronounced collapse with greatly roughened texture on the cell surface structure when the cells were treated with lactic acid in combination with nisin. Morphological changes of the cells following exposure to the corresponding treatments indicated vesicular structures on the outer membranes. Lactic acid in combination with nisin remarkably increased vesicle formation leading to pronounced changes on the outer membrane and its integrity. This was illustrated by ultrastructure visualization of *S. putrefaciens* cells using transmission electron

microscopy.

The benefit of using preservative agents, in combination rather than individually, in shelf-life extension of refrigerated prawn was further confirmed when 2% lactic acid was used in combination with 1600 IU/mL nisin and 1% chitosan (LA-N-CHIT). Such a combination of antimicrobial agents did not adversely affect the organoleptic properties of the treated prawns but further extended refrigerated prawn shelf-life (based on microbiological and biochemical parameters) to 14 days. LA-N-CHIT treatment was able to reduce the population of *Vibrio parahaemolyticus* in inoculated refrigerated prawns to a level below the maximum permissible limit for this pathogen in seafoods ($3 \log_{10}$ CFU/g) after 7-11 days.

The combined preservation approach to attain product shelf-life extension was further investigated in vacuum-packed prawns stored at 4°C. Treatment of prawn with chitosan or LA-N-CHIT, prior to vacuum packaging and refrigerated storage at 4°C, was able to extend the shelf-life of the product to 21 days as opposed to the 11 days exhibited by untreated samples. The combination of LA-N-CHIT proved to be the most effective treatment against growth of *Listeria* in vacuum-packed prawn.

In conclusion, the microbial inhibitory effectiveness and quality maintenance of different biopreservatives used in the present study followed the order ($P < 0.05$): LA-N-CHIT \geq CHIT > LA-N > LA > N \geq Control, whether the prawn was air-packed or vacuum-packed. Vacuum packaging understandably prolonged the shelf-life of both control and treated prawns even further, by as much as 4-7 days, when compared to the corresponding air-packed samples.

CHAPTER ONE

INTRODUCTION

1.1 Research Background

Seafood consumption has been raised steadily and this is because of larger variety of seafoods available compared to other meat products, consumer's demand for alternative types of healthy foodstuff and more affordable pricing. Thus, market demand for prawn, one of the most popular seafoods in the world, continues to grow rapidly. According to FAO (2012), Oceania is of relatively marginal importance in global aquaculture production. Production from this region consists mainly of marine molluscs (63.5 percent) and finfishes (31.9 percent), while crustaceans (3.7 percent, mostly marine shrimps) and other species (0.9 percent) constitute less than 5 percent of its total production. The global consumption of marine shrimps is 27.2% in comparison with marine fishies which is 18.8% (FAO, 2012). Hence, prawn can be considered as a product with the highest commercial value in the seafood trade.

On the other hand, post-harvest losses of seafood and seafood products occur in various forms. The physical loss of material is caused by, for example, poor handling and preservation. Economic losses happen when spoilage of fresh product causes a value-decrease or when there is a requirement to reprocess cured fish, increasing the cost of the final product. Additionally, improper handling and processing techniques can decrease nutrients, leading to nutritional loss.

Fish products losses caused by spoilage are estimated at 10 to 12 million tonnes per year, accounting for around 10% of the total production from capture fisheries and aquaculture (FAO, 2012). Therefore, reducing post-harvest losses requires reducing spoilage and reducing spoilage requires improved handling on

board, processing, preservation, and transportation, all of which are particularly deficient in small-scale fishery industries.

In addition, regarding to safety issues, fish and shellfish products can easily transfer foodborne pathogens. They can be contaminated with pathogenic and spoilage bacteria during harvesting, production, and distribution due to improper handling and storage condition. Eleven percent of foodborne outbreaks could be traced to seafood products in the United States, and bacterial pathogens are involved in about 25% of the disease outbreaks linked to seafood (Bean and Griffin, 1990). Among the pathogenic bacteria is marine vibrios, such as *V. parahaemolyticus* found in high numbers in shellfish and in shellfish-eating fish from tropical waters (Nilsson et al., 2002).

Prawn is categorized as a highly perishable food. Its shelf-life during storage at refrigerated temperatures and shipping is influenced by enzymatic and microbiological changes. Microbiological activity is responsible for spoilage or deterioration and thereby establishes shelf-life of the products. It has been indicated that microbial contamination can lower the quality of prawn, and reduce shelf-life leading to economic loss (Kanduri and Eckhardt, 2002; Wan Norhana, 2010a).

Due to the high perishability potency of such products, several preservation techniques for fresh prawn have been applied to prolong the shelf-life and to reduce the risk of associated health hazards. Such techniques include chilled storage (Rogerio et al., 2001, Lakshmanan et al., 2002), application of liquid ice (Huidobro et al., 2002), high-pressure and vacuum-packaging (Lopez-Caballero et al., 2000), modified atmosphere packaging (Baka et al., 1999; Lopez-Caballero et al., 2002; Mejlholm et al., 2008), gamma irradiation (Ito et al., 1993), ozonated water (Lu,

2009), and the use of chemical preservatives such as chlorine dioxide and sodium metabisulfite (Januario and Dykes, 2005).

Application of chemical preservatives such as chlorine and chlorine dioxide as decontaminating agent has been widely used in the food and seafood industry. For example, chlorine dioxide (ClO₂) as has been utilized in the food industry including; vegetable processing (Reina et al., 1995), fish processing, meat processing (Cutter and Dorsa, 1995) and, poultry processing (Tsai et al., 1995). It has been applied as an antimicrobial agent against *Vibrio parahaemolyticus* (Puente et al., 1992). Also it has been shown its effectiveness in reducing microorganisms in water used for washing and handling fish fillets, whole fish, and scallops (Kim et al., 1999), reducing bacterial loads, enhancing freshness, extending shelf-life and improving safety of prawn (Lin et al., 1996; Andrews et al., 2002). However, chlorine and chlorine dioxide are not considered generally recognized as safe (GRAS) due to the health problems. Therefore, the aim of the present study is to use lactic acid as natural and food grade preservative in combination with other biopreservatives, as alternative chemical preservatives to maintain quality and to enhance safety of prawn.

Lactic acid, which has been widely investigated in the decontamination of meat (Zhou et al., 2010) and poultry products (Loretz et al., 2010), might also have the potential to be an effective alternative decontamination treatment for preservation of fish products (Ghaly et al., 2010), although very few studies have been carried out on shellfish and, in particular, prawn decontamination. On the other hand, other natural preservatives such as nisin (a bacteriocin produced by *Lactococcus lactis*) have been used to control meat-borne pathogens and/or spoilage bacteria. Nisin inhibits many Gram-positive food-borne pathogens such as *Listeria*

and *Clostridium*, but could not be used alone for shellfish products because their initial spoilage flora primarily consists of Gram-negative bacteria (Chen and Hoover, 2003). However, several studies have indicated an effect of nisin on Gram-negative bacteria when it is combined with other compounds such as organic acids, ethylenediaminetetraacetic acid (EDTA) and ethyl alcohol by increasing the rate of nisin penetration through Gram-negative cell walls (Gogus et al., 2006). Furthermore, in prawn processing, one of the main waste materials during processing is the removed head and shells, which are rich sources of chitin and chitosan. The applications of chitosan are extensive and range from food wrapping films to wound healing applications. The antimicrobial activity of chitosan targets a wide range of organisms and draws attention to its potential as a natural food preservative. It has been demonstrated that chitosan showed antimicrobial effects against different types of microorganisms such as bacteria, fungi and yeasts (Kong et al., 2010).

Therefore, the main objective of the present study was to evaluate the application of lactic acid and/or its combination with other biopreservatives such as nisin and chitosan, in the decontamination of banana prawns (*Fenneropenaeus merguensis*), in an attempt to maintain quality and enhance safety of banana prawns during storage at refrigerated temperature.

1.2 Research Objectives

In order to achieve the main objective, experiments were conducted into the following specific objectives:

- To study the preservative effects of lactic acid on bacterial spoilage of refrigerated prawn in comparison with other organic acids.
- To study the enhancing preservative effects of lactic acid in combination with

nisin on bacterial spoilage of prawn during refrigerated storage.

- To investigate mode of action of lactic acid in combination with biopreservatives; nisin or chitosan against prawn spoilage bacteria.
- To evaluate the effects of lactic acid in combination with nisin and chitosan on storage quality of refrigerated prawn.
- To determine the effects of lactic acid in combination with nisin and chitosan on quality and safety of vacuum-packed prawn.

CHAPTER TWO

LITERATURE REVIEW

2.1 Prawn (*Fenneropenaeus merguensis*)

Fenneropenaeus merguensis is commonly known as banana prawn or white prawn. In Malaysia, its commercial name is *udang kaki merah* or *udang pasir*; in Thailand, *kung chaebauy*; in Japan, *tenjikeubi* or *bananaebi*. This marine species lives in shallow water between 10 to 45 meters on muddy bottoms. Juveniles are commonly found in estuarine and adults mostly survive in marine environments. The life cycle of prawn is shown in Figure 2.1.

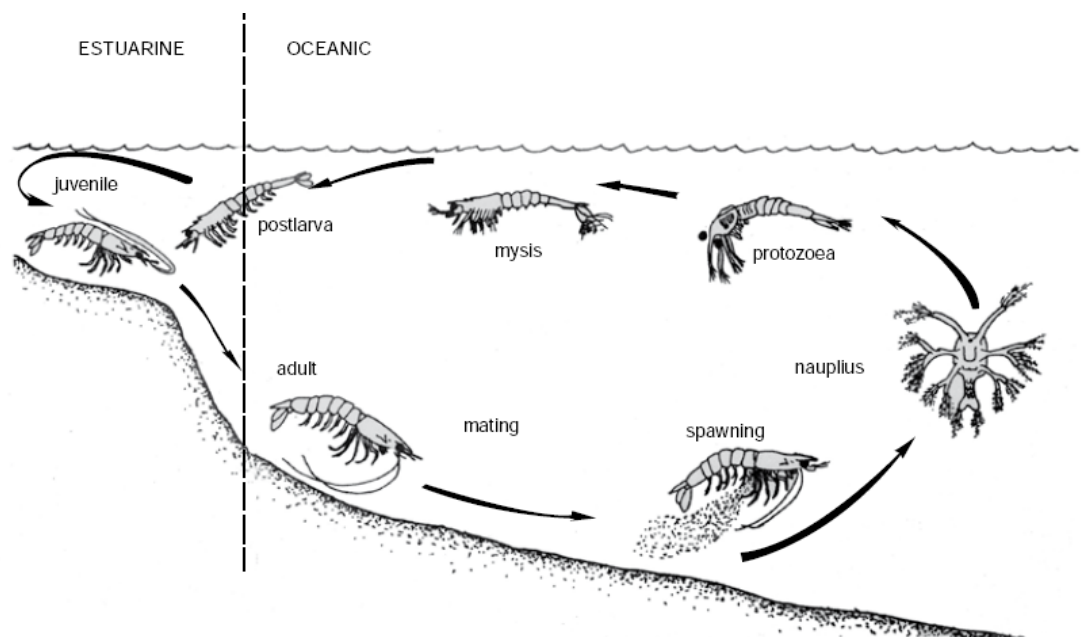


Figure 2.1 Life cycles of prawns
(Source: Montgomery, 2010)

This species has its natural habitats ranging from the Persian Gulf and Pakistan through the Malay Archipelago and South China Sea to Australia. It is one of the indigenous species in South East Asia with a high natural population. The scientific classification of *Fenneropenaeus merguensis* is shown in Table 2.1. The

morphology and anatomy of this species is given in Appendix 1. It should be mentioned that at the end of week 12, males and females are distinguishable by the visible thelycum plates in the females (Hoang et al., 2002). The ventral surfaces of reproductive organs are presented in Appendix 2.

Table 2.1 Scientific classification of *Fenneropenaeus merguensis*

Kingdom	Animalia
Phylum	Arthropoda
Subphylum	Crustacea
Class	Malacostraca
Order	Decapoda
Family	Penaeidae
Genus	<i>Fenneropenaeus</i>
Species	<i>Fenneropenaeus merguensis</i> (De Man, 1888)
Synonym(s)	<i>Penaeus indicus merguensis</i> (De Man, 1888) <i>Penaeus merguensis</i> (De Man, 1888)
Common name(s)	Banana prawn, Banana shrimp

Source: Perez Farfante and Kensley (1997)

Fenneropenaeus merguensis is one of the main species cultured in ponds in Thailand and is also an important catch in Malaysia and the Philippines (Phongdara et al., 1999). *Fenneropenaeus merguensis* is highly nutritious, being rich in vitamins A, B₁, B₂, B₃, B₆, B₁₂, D and Omega 3 (Holland et al., 1993). It is also a good source of selenium and tryptophan (Akiyama et al., 1992). It is categorized as a low acid food with a pH value ranging from 6.8-7.2. The United States is the world's largest shrimp market. According to the annual report of exported and imported shrimp between the U.S. and Malaysia in 2010, the recorded export and import values were USD262,656 and USD106,260,169, respectively (U.S. Foreign Trade, 2010).

2.2 Quality Properties of Prawn

2.2.1 Prawn as highly perishable food

Prawn is a highly perishable food because of its pH (6.8 - 7.2) and water activity of 0.99-1.00 which are conducive for microbial growth (Forsythe, 2000). Postmortem changes are known to occur more rapidly in prawn than in fish. The high content of free amino acids and other soluble non-protein nitrogenous substances contribute to the desirable, delicate and sweet taste of prawn (Zeng et al., 2005). However, those compounds facilitate rapid bacterial spoilage which is accompanied by production of large quantities of volatile base nitrogen and, consequently, the pH rises (Jay, 2005). It has been known that both bacterial and enzymatic changes are responsible for quality deterioration of seafoods. Enzymatic reactions, arising from the actions of naturally occurring enzymes in muscle tissue, are responsible for loss of freshness. However, the spoilage of raw seafood is ultimately due to bacterial activity. Furthermore, seafood spoilage is dynamic with spoilage mechanisms varying with different microbial spoilage groups and being dependent upon the type and composition of the product, as well as its origin and the conditions under which it is stored (Gram, 2010).

In general, microbial spoilage in seafood occurs as a result of the growth of bacteria that have colonized muscle surface. Thus, post-harvest, decomposition begins and involves prawn-surface bacteria which originate from the marine environment or from contamination during handling and washing. Quality deterioration of raw seafood, which is highly temperature-dependent, can be reduced by the use of low storage temperatures.

2.2.2 Pathogenic bacteria in prawn

Seafood contains various potential pathogens which are of public health concern. Aquatic pathogens found in such products include *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Listeria monocytogenes*, *Clostridium perfringens* and *Clostridium botulinum*. The activity of these pathogens is dependent on the storage, handling and processing conditions of the raw material (Fraser and Sumar, 1998).

Vibrio parahaemolyticus is a Gram-negative, halophilic and facultatively anaerobic bacterium found naturally in marine waters. Studies have indicated that *V. parahaemolyticus* is associated with raw or improperly cooked fish, shellfish and mussel samples (Daniels et al., 2000; Terzi and Gucukoglu, 2010). Infection with this bacterium can cause diarrhea, vomiting, abdominal cramps and fever. Such food poisoning symptoms, which are generally mild, usually surface within 4 – 96 h after ingestion of the pathogen (Wong et al., 2000). *V. parahaemolyticus* is normally mesophilic but can apparently grow at refrigerated temperatures down to 5°C (Twedt, 1999). Contaminated raw, improperly cooked, and cooked re-contaminated fish and shellfish have been implicated in cases of gastroenteritis. The level of contamination of *V. parahaemolyticus* in seawater and in harvested seafood varies with the season, being reportedly higher during the warmer months (DePaola et al., 1990).

Listeria monocytogenes, a Gram-positive bacterium, is a foodborne pathogen that is widely distributed in the environment and occurs naturally in many raw foods (Farber and Peterkin, 2000). It has been isolated from both domestic and imported, fresh, frozen, and processed seafood products, including crustaceans, molluscan shellfish, and finfish (Jinneman et al., 1999; Elliot and Kvenberg, 2000). It is psychrotrophic, halotolerant, and can grow in the temperature range from -0.4 to 50°C

and NaCl range from 0 to 10% (Junttila et al., 1998; Jay et al., 2005). Infection by *L. monocytogenes* causes fever, headache, diarrhea, meningitis, septicemia, spontaneous abortion, and vomiting (Farber and Petrkin, 1991). *L. monocytogenes* is considerably more resistant to heat than *Vibrio* or enteric pathogens (Dorsa et al., 1993) and also more halotolerant, being able to withstand elevated sodium chloride levels better than enterics. Vacuum-packed seafoods are a well-recognized source of *L. monocytogenes* (Gonzalez-Fandos et al., 2009).

2.3 Factors Influencing Bacterial Spoilage in Prawn

Generally, the factors influencing growth of spoilage bacteria during storage of food products may be grouped as follows:

(1) Intrinsic factors (properties of product) such as moisture content, pH and acidity, nutrient content, biological structure, redox potential (rH), naturally occurring and/or added antimicrobials, and competitive microflora. In prawns, the major intrinsic factor that promotes the growth of pH-sensitive spoilage bacteria such as *Shewanella putrifaciens* is apparently the high postmortem pH (>7.0), a consequence of the high content of non-protein nitrogen and the low content of carbohydrates that can produce only a small amount of lactic acid postmortem (Gram and Huss, 1996);

(2) Extrinsic factors (properties of the environment surrounding the product) such as storage temperature, relative humidity, and gas composition;

(3) Processing factors, and

(4) Implicit factors (the interactions between microorganisms) which can lead to suppression of some groups or species by antagonism or competition for nutrients.

A more complete list of intrinsic, extrinsic, processing and implicit factors is shown in Table 2.2. These factors will be discussed in some detail in the following section. It is hoped that this will impart a better understanding of the roles of these factors in influencing microbial growth and survival, and consequently spoilage activities in perishable food products such as prawn.

Table 2.2 Major factors affecting microbial growth and survival in foods

Intrinsic Factors		Processing		
Chemical	Physical	factors	Extrinsic factors	Implicit factors
Nutrients	Water activity (a_w)	Change in food composition	RH during storage	Microbial growth rate
pH & buffering capacity	Ice & freeze concentration	Changes in types of microorganisms	Time & temperature during storage	Synergistic effects (food components, microorganisms)
Oxidation-reduction potential (redox potential)	Changes in structure	Changes in numbers of microorganisms (heat treatments, irradiation)	Types of packaging/atmospheres: oxygen level, presence of other gases (CO ₂ specifically)	Antagonistic effects (food components, microorganisms)
Antimicrobial substances (naturally occurring & added antimicrobials)	Changes in microstructure (emulsification) & biological structure		Storage/holding condition	Symbiosis (cooperative growth between microorganisms)

Source: Adapted from Gould (2000)

2.3.1 Composition of prawn as an intrinsic factor on bacterial spoilage

The chemical composition of prawn consists of 78.47% moisture, 19.12% protein, 1.35% non-protein dry matter or ash, 1.06% fat and 0% Carbohydrate (Nurnadia et al., 2011). The energy value of a gram of prawn is 4.94 KJ/g. The zero percent of

carbohydrate (or $< 0.5\%$ according to other values reported in the literature) in prawn does not account for glycogen accumulating in the muscles (because of the poikilothermic behavior of fishery products). This may have important consequences for the microbiology of such products as the postmortem pH is > 6.0 in most fresh seafoods.

The most important chemical compounds in relation to the microbiology of seafood products are the non-protein nitrogen (NPN) fractions which consist of low-molecular-weight (vs. high-molecular-weight fractions as proteins), water-soluble, nitrogen-containing compounds including amino acids, nucleotides, and trimethylamine oxide. The NPN is the major substrate for bacterial spoilage. Prawn contains a high content of NPN in the region of 602 mM solute concentration (Willmer et al., 2004). More information about composition of boiled and processed prawn is presented in supplementary table of shellfish composition in Appendix 3.

2.3.2 Storage temperature and spoilage bacteria in prawn during refrigerated storage

Storage temperature plays an important role (as an environmental factor) in influencing growth rate and type of spoilage microorganisms in highly perishable food products such as prawn. Storage at refrigerated temperatures reduces microbial proliferation that causes spoilage, thereby extending product shelf-life. Storage at temperatures below the levels required for optimal growth of microorganisms leads to extended doubling (generation) time and, consequently, a prolongation of the lag-phase. A storage temperature below the minimal temperature required to support growth of a particular microorganism would result in continued extension of the lag-phase until cell multiplication ends (Doyle et al., 1997). Refrigeration will obviously

restrict the growth of mesophiles, which constitute a major proportion of the initial population of microflora, and allow psychrotrophic microorganisms to grow and eventually dominate the microflora (Ashie et al., 1996). Therefore, in the case of raw prawns, as microbial growth occurs during chilled storage, the composition of the microflora is altered or dominated by a few or often a single microbial species, usually of the genus *Pseudomonas* and/or *Shewanella* (Chinivasagam et al., 1996). Although these bacteria often comprise only a small proportion of the initial microflora, the types of bacteria that ultimately predominate during chilled storage are reflective of these genera. These specific spoilage microorganisms are the ones that cause the chemical changes and production of off-odors that lead to unacceptability of the product. Consequently, the shelf-life of the product is dependent upon the growth of these specific spoilage microflora.

The shelf-life of prawn equates to the storage time until it reaches the point of unacceptability through spoilage. The endpoint of spoilage may be defined by a certain maximum acceptable bacterial level (ca. \log_{10} CFU/g), or an acceptable off-odor/off-flavor or appearance. The numbers and types of microorganisms, mainly bacteria, initially present and their subsequent growth are the predominant factors determining the shelf-life of the product (Lalitha and Surendran, 2006). The most commonly encountered or predominant bacteria associated with spoilage of refrigerated seafood (including shellfish) products are *Pseudomonas* spp., *Shewanella putrefaciens*, and *Shewanella putrefaciens*-like bacteria (Chinivasagam et al., 1998). Furthermore, *Pseudomonas* spp., *Shewanella putrefaciens*, and members of Vibrionaceae were the three main types of surface microflora identified in tropical water shrimps (*Penaeus*) after 16 days of storage at chilling temperatures (Gram and Huss, 2000).

2.3.2.1 *Shewanella putrefaciens*

S. putrefaciens is an aquatic Gram-negative bacterium belonging to the family Vibrionaceae (MacDonell and Colwell, 1985). *S. putrefaciens*-like bacteria have also been isolated consistently from spoiled chilled products. As such, *S. putrefaciens* probably deserve the epithet “fish spoilage bacterium”. The number of *S. putrefaciens* (as H₂S-producing bacteria) on iced marine fish is linearly correlated with the remaining shelf-life of the product. This bacterium reduces trimethylamine oxide to trimethylamine in anaerobic respiration and is capable of producing H₂S and other sulfides. It should be noted that a higher pH may allow *S. putrefaciens*, which does not grow at pH < 6.0, to contribute to the spoilage microflora (Gram and Huss, 1996).

Prawns are generally considered spoiled when the total aerobic plate count increases to approximately 7 log₁₀ CFU/g and/or the hydrogen sulphide producing bacterial count reaches about 6 log₁₀ CFU/g (Lalitha and Surendran, 2006). Furthermore, according to Olafsdottir et al. (1997), total viable counts of 7-8 log₁₀ CFU/g appear to be typical for fish products at the point of sensory unacceptability.

2.3.2.2 *Pseudomonas* spp.

Pseudomonas spp. are usually able to compete successfully in aerobically packed refrigerated products (Kraft, 1992). The genus *Pseudomonas* is characterized by a competitive growth rate, even at refrigerated temperatures. These important spoilage bacteria can metabolize a wide variety of carbohydrates, proteins and lipids in foods (Ray, 2003). In addition, pseudomonads are able to grow within the pH range of 5.5 to 7.0, while many bacteria species are less capable of competing under refrigerated temperatures at lower pH in this range (Jackson et al., 1997). As pseudomonads are

greatly oxidative, they are able to utilize low-molecular weight nitrogen compounds as a source of energy. This is a clear competitive advantage for pseudomonads since prawn contains relatively very low (or even zero) levels of simple sugars, and more complex energy sources such as protein and fat do not serve as significant substrates for growth until later in spoilage when high bacterial populations are attained.

Low temperature storage is mostly used in the seafood industry as well as in the retail market (e.g. storing fresh seafoods in ice, which has always been the consumer's primary choice). This temperature control method can inhibit microbial growth as well as slow down autolytic reactions. However, the use of chilling temperatures alone can only slightly delay microbial spoilage. In order to further extend product shelf-life, other strategies such as method of packaging, type of preservatives or their combination, involving the application of multiple barrier preservation techniques, would have to be adopted. Such strategies would be especially useful during processing of shellfish products (such as prawns) and other high-value seafoods.

2.3.3 Packing condition and spoilage of prawn during storage

The shelf-life of fresh seafood products is limited due to the growth and biochemical activities of Gram-negative, psychrotrophic bacteria in the presence of atmospheric oxygen (Erkan et al., 2006). Seafood stored under aerobic condition is spoiled due to the growth and activity of one bacterial group (so-called specific spoilage microorganisms such as *S. putrefaciens* and *Pseudomonas* spp.) causing the important chemical changes (Hozbor et al., 2006). Murcia et al. (2003) indicated that conventional air-packed had higher microbiological counts (which include aerobic

mesophilic and psychrotrophic bacteria) compared to samples that were kept in vacuum or modified atmosphere packaging.

Under vacuum-packaging conditions, air is evacuated from gas-impermeable pouches followed by sealing. Upon storage of a vacuum-packaged food product, an increase in CO₂ occurs as a result of both tissue and microbial respiration where O₂ is consumed and CO₂ is released in equal volumes (Jay et al., 2005). Vacuum-packaging reduces the level of the aerobic respiratory bacteria such as *Pseudomonas* spp. However, *Shewanella* spp. develops easily under vacuum conditions due to their ability to utilize trimethylamine oxide as an electron acceptor (anaerobic respiration). The reduction of trimethylamine oxide into trimethylamine leads to specific fishy off-odors (Farn et al., 1996). Vacuum-packaging favors the growth of lactic acid bacteria such as *Lactobacillus* spp., *Carnobacterium* spp., and *Leuconostoc* spp. which become the predominant flora. They are mixed with typical Gram-negative spoilage bacteria (e.g. *Enterobacteriaceae*), and are involved in spoilage processes that result in the production of off-odors and biogenic amines (Gram and Dalgaard, 2002).

It should be mentioned that packaging condition affects on bacterial ecology of products during storage at controlled temperature. For instance, under anaerobic conditions, lactic acid bacteria (LAB) can grow to large numbers and emerge as the predominant microorganisms. Although LAB do not originate from aquatic environments, some species have been found in freshwater fish and their surrounding environment and they have been implicated in the spoilage of certain fish products (Francoise, 2010). Previous research has indicated the dominance of lactobacilli such as *Lactobacillus curvatus*, *Lactobacillus sakei*, *Lactobacillus plantarum*, *Leuconostoc* spp. and *Carnobacteria* spp. in vacuum-packed products

during chilled storage (Truelstrup Hansen and Huss, 1998). Dalgaard et al. (2003) identified LAB strains found in cooked and brine shrimp stored under a modified atmosphere over the temperature range from 0 to 25°C. Joffraud et al. (2001) showed that *Lactobacillus* spp. can release large amounts of volatile compounds and, therefore, are possibly responsible for the off-odors perceived on spoiled vacuum-packed cold-smoked salmon. In contrast, according to Francoise (2010), the role of LAB in spoilage of high carbohydrate-containing food products is more important than in products with low carbohydrate contents.

Lactic acid bacteria are Gram-positive bacteria that produce lactic acid either as a sole product of metabolism (homolactic fermentation) or as a major end product (heterolactic fermentation) during fermentation of carbohydrates. The antimicrobial compounds produced by lactic acid bacteria are organic acids, bacteriocins, H₂O₂, diacetyl, etc. (Nilsson and Gram, 2002). They are able to grow at refrigerated temperatures and they can usually tolerate gas atmosphere packaging, low pH, high salt concentration and the presence of different additives (Calo-Mata et al., 2008). They are responsible for food fermentation processes and are also commonly found in non-fermented food, including dairy products, meat products, fish products, fruits, vegetables and cereals.

In refrigerated fish products, studies have shown the existence of a variable microflora comprising different proportions of lactic acid bacteria and *Enterobacteriaceae*, at levels of 6-7 log₁₀ CFU/g, at the end of shelf-life (Leroi et al., 2001). While members of the *Enterobacteriaceae* appear to be consistently represented in the microflora of fresh prawn, their numbers would decrease during chilled storage under aerobic conditions (Gram and Huss, 1996; Leitao and Rios,

2000). The activity of *Enterobacter* strains has been reported to be minimal at low temperatures (Gennari et al., 1999; Leitao and Rios, 2000).

Enterobacteriaceae are Gram-negative bacteria. The important genera in the family of *Enterobacteriaceae* include *Citrobacter*, *Enterobacter*, *Escherichia*, and *Klebsiella*. Most members of *Enterobacteriaceae* are mesophilic, but there are also psychrotrophic taxa (e.g. *Serratia* spp.) that can multiply in refrigerated foods such as meat, fish and milk (Lindberg et al., 1998). *Enterobacteriaceae* have been isolated from spoiled vacuum-packed meat. In meat products, a change in atmosphere e.g. by vacuum-packaging, will inhibit the respiratory pseudomonads and cause a shift in the dominant microflora to lactic acid bacteria, *Enterobacteriaceae* and sometimes *Brochothrix thermosphacta* (Dainty and Mackey, 1992).

2.4 Quality Characteristics of Seafood Products

2.4.1 Microbiological activity

Microbial activity is the most important factor affecting the shelf-life of raw seafoods. The total viable count is usually used as an index for acceptability to establish standards, guidelines and specifications (Olafsdottir et al., 1997). However, as mentioned previously, only a small fraction of the microorganisms (mainly bacteria) present on seafood is actually of importance where product spoilage is concerned. Consequently, total viable counts in seafood correlate poorly with the degree of freshness or remaining shelf-life as shown in Figure 2.2.

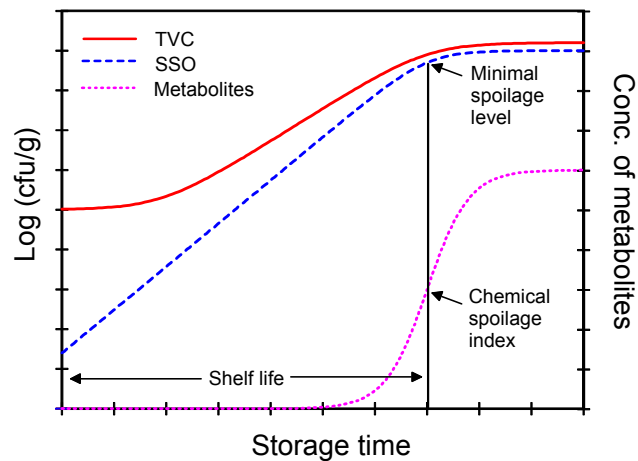


Figure 2.2 Specific spoilage organism (SSO) concept. Typical changes in total viable counts, SSOs and metabolites produced by SSOs during storage of fresh seafood. (Source: Dalgaard, 2006)

During storage of seafood under particular conditions of temperature, atmosphere, salt concentration, water activity (a_w), and preservatives, specific spoilage organisms (SSOs) grow faster than the remaining microflora, and eventually produce the metabolites responsible for off-flavors and product rejection (Figure 2.2). Consequently, the numbers of SSOs can be used as objective quality indices for shelf-life determination in seafoods.

2.4.2 Biochemical quality

In seafoods, total volatile basic nitrogen (TVB-N) primarily includes trimethylamine (TMA), dimethylamine and ammonia. The European Commission (Commission Decision 95/149/EC, 1995) has specified that TVB-N is to be used if sensory evaluation indicates doubt about the freshness of different fish products (Limbo et al., 2009). Critical limits of 25, 30 and 35 mg/100 g have been established for different groups of fish species (Sukran et al., 2006). TMA is a microbial metabolite and it can only be used as an index of spoilage and not freshness. Development of TMA in seafood depends primarily on the content of the substrate, trimethylamine-

oxide, in the raw material. It is also noteworthy that the TMA concentration at the time of product rejection depends on the storage conditions. Furthermore, some spoilage bacteria in seafoods produce one or more of the biogenic amines which are heat-stable and, as such, appropriate for the evaluation of freshness of the raw material used in canned products. However, production of biogenic amines in seafood depends on concentrations of the free amino acid substrates and is, therefore, strongly species dependent (Dalgaard, 2000).

2.4.3 Sensory evaluation

Sensory evaluation is a method for the assessment of freshness and quality of products. It is commonly used in the fish sector and fish inspection services. Sensory evaluation can be applied to all species of fish and fish products. The evaluation is fast and non-destructive, with the results often reflecting the criteria the consumer uses in evaluating acceptability (Connell, 1995). The rapid method, used by consumers and inspectors in the market, is the appearance of the fish products, particularly the color and luster of the prawn. The disadvantages are that the evaluations of inspectors might be non-standardized and the results obtained might be subject to the personal whims and biases of the assessors. However, most trade is based on sensory assessments, although measurements are not always objective and documented (Alasalvar and Taylor, 2002).

One of the most used methods to quantify consumer acceptance is the 9-point hedonic scale, which has been used in both academic and industrial consumer research in western cultures, especially in America and Europe (Yeh et al., 1998). The word "hedonic" is of Greek origin and relates to the degree or magnitude of "like" or "dislike". This test is used to quantify the degree of preference for a

product and measures how well products are liked or which products are preferred. This method provides a balanced 9-point scale for “liking” which is arranged based on the following order: like extremely, like very much, like moderately, like slightly, neither like nor dislike, dislike slightly, dislike moderately, dislike very much, and dislike extremely (Harry and Hildegard, 1998).

Descriptive sensory test is another method of sensory evaluation. It is involved detection (discrimination) and description of both the qualitative and quantitative sensory components of the food products by trained panelists (Lawless and Haymann, 1998). The qualitative aspects of a product include aroma, color, flavor, texture of a product, which distinguish it from others. Sensory judges then quantify these product aspects in order to facilitate description of the perceived product attributes. Descriptive sensory analyses are used for quality control, comparison of product prototypes to understand consumer responses in relation to products’ sensory attributes, and for sensory mapping and product matching (Gacula, 1993).

2.5 Preservation Techniques to Control Spoilage and Pathogenic Bacteria in Fish and Shellfish Products

The major food preservation methods, as summarized in Table 2.3, can be categorized as methods to: (a) delay or prevent microbial growth, (b) inactivate microorganisms, and (c) restrict access of microorganisms to products. Due to the high perishability potency of seafood products, several preservation techniques for fresh prawn have been applied to prolong their shelf-life and to reduce the risk of health hazards. Such techniques include chilled storage (Rogerio et al., 2001; Lakshmanan et al., 2002), application of liquid ice (Huidobro et al., 2002), high-pressure processing and vacuum-packaging (Lopez-Caballero et al., 2000), modified

atmosphere packaging (Baka et al., 1999; Lopez-Caballero et al., 2002; Mejlholm et al., 2005), gamma irradiation (Ito et al., 1993), treatment with ozonated water (Lu, 2009), and the use of chemical preservatives such as chlorine dioxide (Andrews et al., 2002) and sodium metabisulfite (Januario and Dykes, 2005).

Table 2.3 Major methods of food preservation

Methods to delay or prevent microbial growth
<ul style="list-style-type: none">• Reducing temperature<ul style="list-style-type: none">- chilled storage, frozen storage• Reducing water activity<ul style="list-style-type: none">- curing with salt, drying, conserving with sugar• Reducing pH<ul style="list-style-type: none">- acidification (e.g, using of organic acid), fermentation• Removing oxygen<ul style="list-style-type: none">- vacuum or modified atmosphere packaging• Modified atmosphere packaging<ul style="list-style-type: none">- replacing air with CO₂, O₂, N₂ combinations• Using preservatives<ul style="list-style-type: none">- inorganic (e.g, sulfite, nitrite)- organic (e.g, benzoate, propionate, sorbate)- bacteriocin (e.g, nisin)- antimycotic (e.g, natamycin)• Surface coating
Methods to inactivate microorganisms
<ul style="list-style-type: none">• Heating<ul style="list-style-type: none">- pasteurization- sterilization• Radiation• High pressure processing
Methods to restrict access of microorganisms to products
<ul style="list-style-type: none">• Packaging• Cleaning• Aseptic processing

Source: Adapted from Gould (1996); Prokopov and Tanchev (2007)

Although some of these methods such as chilled storage may be effective to some extent in controlling bacterial growth and spoilage activity in fish products, refrigerated temperatures between 4°C and 8°C are not sufficient to ensure

complete cessation of growth of psychrotrophic spoilage and pathogenic bacteria. Therefore, additional preservation methods are necessary to maintain quality and to ensure the safety of chilled products (Huss, 1997). The preferred preservation techniques are those based on a combination of different factors (“hurdles”) to delay, prevent or inhibit the growth of undesirable microorganisms in order to enhance the safety, stability and sensory quality of the products (Gould, 2000). The potential hurdles used for food preservation are temperature (high or low), pH (low or high), water activity (low or high), modified atmosphere, pressure (high or low), ultrasound (high), radiation (UV, microwaves, ionizing radiation), preservatives, etc. (Leistner, 1994). In addition, such new preservation techniques are a direct response to increased consumer demand for tasty, nutritious, natural and easy-to-handle food products. Table 2.4 shows the nexus between consumer requirements and food industry reactions aimed at developing appropriate preservation techniques for food products.

The new and emerging preservation technologies such as using bacteriocins like nisin, herbs and spices, and enzymes (see the examples in Appendix 4), adopt more natural approaches. The application of combinations of preservatives has the advantage of minimizing problems with bacterial adaptation to stresses, known as resistance, because of the relatively lower concentrations of each preservative used and potential synergistic effects. Knowledge of the synergistic actions of preservation technologies when used in combination would greatly assist the development of effective mild preservation strategies. The main hurdle preservation technique for fish and fish products involves the use of low storage temperature, different preservatives, and vacuum or modified atmosphere packaging.

Table 2.4 Consumer requirements and food industry reactions

Trends in consumer requirements

- Improved convenience
 - in preparation, storage, shelf-life
- Higher quality
 - in flavor, texture, appearance
- Fresher
- More natural
 - with less additives
- Nutritionally healthier
- Minimally packaged
- Safer

Food industry reactions

- Milder processing
 - minimal overheating
 - less intensive heating
 - nonthermal alternatives to heat
- Fewer additives
 - less “chemical” preservatives
- Use of “hurdle” technology or “combination preservation” systems
- Evaluation of natural antimicrobial systems as food preservatives
- Less use of salt, saturated fats, sugar; more low-calorie foods

Reduced, environmentally friendly

- Packaging
 - Elimination of food poisoning microorganisms
-

Source: Adapted from Gould (1996)

2.5.1 Cell wall structure of Gram-negative bacteria as target spoilage bacteria in seafoods

Christian Gram developed the Gram stain in 1884 and it became evident that bacteria could be divided into two major groups; Gram-positive and Gram-negative, based on their response to the Gram-stain procedure. The Gram-positive cell wall consists of a single 20 to 80 nm thick homogeneous peptidoglycan or murein layer lying outside the plasma membrane (Figure 2.3). In contrast, the Gram-negative cell wall is quite complex. It has a 2 to 7 nm peptidoglycan layer surrounded by a 7 to 8 nm thick outer membrane (Prescott, 2002).