ANTIMICROBIAL EFFECTS OF ALLYL ISOTHIOCYANATE AND MODIFIED ATMOSPHERE ON PSEUDOMONAS AERUGINOSA IN FRESH CATFISH FILLET UNDER ABUSE TEMPERATURES

by

YU-HSIN PANG

A Thesis submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

in partial fulfillment of the requirements

for the degree of

Master of Science

Graduate Program in Food Science

written under the direction of

Kit L. Yam

and approved by

_____

_____

_____

_____

New Brunswick, New Jersey

Oct, 2012

ABSTRACT OF THE THESIS

Antimicrobial Effects of Allyl Isothiocyanate and Modified Atmosphere on *Pseduomonas Aeruginosa* in Fresh Catfish Fillet under Abuse Temperatures

By YU-HSIN PANG

Thesis Director:

Kit L. Yam

Pseudomonas aeruginosa, a common spoilage microorganism on fresh catfish products, can grow rapidly at temperatures above 4°C during storage and transportation. To protect against temperature abuse and extend the shelf life of catfish, an antimicrobial packaging system examined in this study. Allyl isothiocyanate (AIT), an extract of horseradish oil, is a well-known antimicrobial agent, which has been proved to have an excellent antimicrobial capability on various microorganisms. The packaging system consists of combing AIT with modified atmosphere (MA), a common antimicrobial method, to inhibit the growth of *P. aeruginosa.*

The objectives of this research were: 1) to investigate the antimicrobial effects of AIT in vapor phase and MA on *P. aeruginosa* in fresh catfish fillet; 2) to develop models to predict the lag phase and shelf life of fresh catfish products as a function of AIT and MA. Three concentrations (0, 18 and 36 μg/L) of AIT in vapor phase were used to inhibit the growth of *P. aeruginosa* cocktail (3-strains) at 8, 15 and 20°C. In addition, the

antimicrobial capability of MA (49% CO_2, 0.5% O_2 and 50.5% N_2) and MA combined with AIT were also investigated.

Our results showed that temperature has the most significant impact on the growth rate of *P. aeruginosa.* Lowing temperature could decrease the maximum growth rate of *P. aeruginosa* and extend its lag phase and shelf life. Increasing gaseous AIT concentration could elongate lag phase and reduce growth rate of *P. aeruginosa* effectively. In addition, AIT alone reduced *P. aeruginosa* counts within the initial several hours. The application of MA had a similar antimicrobial effect as AIT under low storage temperature. Moreover, to attain a longer shelf life of fresh catfish fillet, the combination of AIT and MA was included in this study. The synergistic effect of AIT and MA could extend the shelf life of fresh catfish from 4 days (control) to 23 days at 8°C.

Models developed from this study may be applied to estimate the lag phase of *P. aeruginosa* and shelf life of catfish under abuse temperature conditions. The results could assist food industry to predict the shelf life of catfish products, which may be further optimized with appropriate AIT concentration in a MA packaging.

ACKNOWLEDGEMENT

First and foremost, I would like to express my deepest thanks to my advisor, Dr. Kit L. Yam. I am grateful to him for providing me the opportunity to work in his lab and for his invaluable guidance, advices, and support. I learnt not only science knowledge but also life philosophy from him.

I also want to express my deeply gratitude to Dr. Shiowshuh Sheen in USDA/ARS/ERRC, Wyndmoor, PA for technically and mentally supporting me in this project. The experiments were conducted mostly in ERRC under his guidance. He was always available when I had questions and concerns.

I am grateful to Dr. Linshu Liu for giving me the great opportunity to work in USDA/ARS/ERRC and agreeing to serve on my committee. I greatly appreciated his inputs, suggestions and friendly encouragement.

I wish to thank Dr. Hotchkiss for kindly agreeing to be one of my committee. His inputs, suggestions and comments are appreciated.

I would like to thank Dr. Dongsun Lee in South Korea for giving kindly help to my research. He was always listening and answering my questions patiently. His knowledgeable but humble personality was impressive.

I want to thank the secretaries of Department of Food Science, Paulette Arico, Karen Ratzan, Debbie Koch, Karin Conover, Miriam Gonzalez, Laura Amador and Irene Weston for assisting me in administrative work. In addition, I would like to thank Yakov Uchitel and David Petrenka for their help and support.

I would like to express my sincere appreciation to my labmate Siyuan Zhou for providing his valuable opinion to my project. In addition, I would like to thank my labmates,

Saifanassour Arabi, Aishwarya Balasubramanian, Peng Yuan, Luni Shen, Xi Chen, Carol Saade, Soumi Ray, Spurti Ravi, Malathi Vakkalanka, Vara Prodduk, Saikiran Chaluvadi, Mansi Trivedi, Tulsi Patel for creating a fun and friendly environment in which to discuss and learn.

Special thanks to my boy friend, Li Zhang for his love, tolerant, great support, and encouragement. He was so kind to read my thesis and paper patiently, make helpful comments and challenge me with all kinds of questions. In addition, he gave me a big help on statistic analysis and model explanation in this research.

Finally and most importantly, this work would not have been accomplished without enormous love and continuous support from my family. Therefore, I would like to thank my father Chen-I Pang, my mother Mei-Shueh Lin and my sister Wen-Hsin Pang for their unconditional love and support. To them I dedicate this thesis.

TABLE OF CONTENT

LIST OF TABLES

LIST OF ILLUSTRATIONS

1. INTRODUCTION

Catfish is an important economic contributor in the United States. Over 420 million pounds of processed catfish were produced in the US in 2011[1]. Compared to other agricultural commodities, catfish has relatively higher commercial value in the market. However, catfish is also highly perishable that limits its distribution feasibility and then reduces its economic value. During transportation, storage temperature fluctuation declines the quality of catfish. Therefore, proper processing method and packaging system should be applied to maintain the quality and safety of fresh catfish.

Three primary deterioration factors may affect the quality and safety of catfish, which are autolysis, chemical oxidation and microbial growth[2]. Among these deterioration factors, microbial growth is the major cause of fish spoilage, which could produce amines, alcohols, aldehydes, sulfides, organic acids and ketones with unpleasant and unacceptable off-flavor, odor and color[3]. Since the storage environment of fresh catfish is aerobic and under refrigerate temperature, aerobic and psychrotrophic bacteria are highly concerned with the safety and quality of catfish. *Pseudomonas aeruginosa*, one of the specific spoilage organisms in seafood, conforms to the characteristic of spoilage organism, so it is selected as target microorganism in this study.

Allyl isothiocyanate (AIT) is considered to be a promising candidate as natural antimicrobial agent, which is well known for its effective inhibiting capability of microbial growth in foods. AIT is a colorless and volatile essential oil, which shows excellent antimicrobial effect in its vapor phase. To prevent the loss of gaseous AIT and maintain its antimicrobial ability during storage time, AIT can be incorporated into food packaging to create an antimicrobial environment. This antimicrobial packaging system

can be used to eliminate or inhibit the growth of *P. aeruginosa* or other microorganisms. However, the pungent odor of AIT limits its applied concentration, thus reducing the antimicrobial effect of food packaging. Therefore, modified atmosphere (MA) is combined with AIT to achieve complementary effect for this antimicrobial packaging system.

MA is an extensively used technology for perishable seafood product. The concept of MA is controlling the level of CO_2 and O_2 in the food packaging system to extend the shelf life of food products by suppressing the growth of both aerobic spoilage and pathogenic organisms. Since CO_2 is slightly soluble in water, high percentage of CO_2 can form carbonic acid with water to create an acidic environment to inhibit the growth of aerobic microorganisms. However, the water-soluble property of CO_2 may also bring some negative impact to the antimicrobial packaging system. While CO_2 gradually dissolves into water or the moist surface of catfish, the internal pressure decreases and then causes packaging collapsing. In addition, carbonic acid, the forming compound of CO_2 and water, may affect the texture of catfish, because it can lower the water holding capability of catfish by reducing the pH of flesh[4, 5]. Therefore, the application of CO_2 in high percentage should be avoided in packaging systems of seafood to prevent the above consequences.

Since both AIT and MA have their limitations when applied in packaging system, but could be complementary to each other, the combination of these two antimicrobial methods could be a potential candidate as an antimicrobial packaging system to obtain a sufficient antimicrobial effectiveness thus attaining a longer shelf life of fresh catfish. Due to the shelf life of fresh catfish has been proven to be strongly depended on storage

temperatures[6], the antimicrobial effect of AIT in vapor phase and MA on *P. aeruginosa* in catfish fillet held in different abuse temperatures was investigated in this study. Three parameters (lag phase, maximum growth rate and shelf life) were utilized to analyze the antimicrobial effect of this antimicrobial packaging system.

Predictive models were established base on the result of antimicrobial effect of AIT, MA and their combinations. The models developed in this study could be used to estimate the lag phase of *P. aeruginosa* and shelf life of catfish at different storage conditions. The function of the models is to assist food industry in predicting the shelf life of catfish products, which may be further optimized with appropriate AIT concentration in a MA packaging system.

2. REVIEW OF LITERATURE

2.1. Catfish

Catfish belongs to the order-*Siluridae*, which is usually found in slow-flowing fresh water such as large rivers and lakes. However, catfish have good adaptation of environment, so they also can live in strong current of rivers[7]. Catfish are easy to rear in warm climates and reach the marketable size in a short period of time[8]. The size range of catfish is quiet broad. Some of the smallest species of catfish are only 1 cm and the largest species can grow up to 2 m[9]. The larger species of catfish can be farmed for food and some particular smaller species such as the genus *Cordoras* are being popular in many aquariums[10]. Therefore, catfish has a huge commercial importance in the market.

Catfish have been farm-raised in the US for several decades, and more than half of all the species of catfish live in Americas. Mississippi, Alabama, Arkansas and Texas are the top four states to produce catfish and supply 95% of total sales in the United States[1]. Because of the year-round availability, consistent quality and healthy attributes of catfish, the consumption of catfish products has grown rapidly in the recent years[11]. According to the USDA report, over 423 million dollars of catfish were sold in the United States during 2011 and increased 5% from 403 million dollars in previous year[1]. In recent years, the unit price of catfish is also rising gradually, as shown in Table 1.

Table 1: Farm-raised catfish prices received by producers by month in United States: 1997-2011 (adopted from USDA report[12])

[Quantity processed by major processors and the prices received for fish delivered to the processing plant's door. Price includes charges for any services provided by the processing plant, such as seining and hauling, but does not include adjustments based on year-end settlements]

Year	January	February	March	April	May	June	
	(dollars per pound)	(dollars per pound)	(dollars per pound)	(dollars per pound)	(dollars per pound)	(dollars per pound)	
1997	0.730	0.730	0.730	0.730	0.730	0.720	
1998	0.690	0.730	0.780	0.790	0.790	0.780	
1999	0.703	0.714	0.732	0.756	0.777	0.775	
2000	0.744	0.788	0.789	0.789	0.785	0.786	
2001	0.693	0.696	0.697	0.694	0.687	0.669	
2002	0.549	0.555	0.565	0.561	0.574	0.588	
2003	0.529	0.544	0.585	0.630	0.618	0.586	
2004	0.668	0.703	0.723	0.728	0.720	0.689	
2005	0.725	0.731	0.733	0.725	0.722	0.721	
2006	0.727	0.729	0.745	0.785	0.796	0.807	
2007	0.837	0.838	0.838	0.841	0.840	0.817	
2008	0.658	0.688	0.743	0.757	0.776	0.794	
2009	0.810	0.770	0.773	0.763	0.762	0.763	
2010	0.764	0.765	0.785	0.804	0.796	0.786	
2011	0.931	1.003	1.075	1.141	1.169	1.231	

Year	July	August	September	October	November	December	Average
	(dollars per pound)	(dollars per pound)	(dollars per pound)	(dollars per pound)	(dollars per pound)	(dollars per pound)	(dollars per pound)
1997	0.710	0.700	0.690	0.690	0.690	0.690	0.712
1998	0.760	0.740	0.730	0.710	0.700	0.700	0.743
1999	0.768	0.743	0.728	0.716	0.713	0.716	0.737
2000	0.760	0.741	0.727	0.710	0.696	0.682	0.751
2001	0.656	0.624	0.610	0.596	0.566	0.554	0.647
2002	0.590	0.582	0.576	0.568	0.560	0.544	0.568
2003	0.564	0.552	0.560	0.567	0.610	0.629	0.581
2004	0.682	0.683	0.683	0.695	0.689	0.690	0.697
2005	0.723	0.724	0.724	0.724	0.724	0.726	0.725
2006	0.812	0.811	0.832	0.836	0.837	0.838	0.795
2007	0.762	0.731	0.697	0.682	0.666	0.650	0.767
2008	0.818	0.827	0.827	0.825	0.823	0.821	0.776
2009	0.771	0.769	0.772	0.768	0.765	0.763	0.771
2010	0.788	0.790	0.816	0.832	0.841	0.861	0.801
2011	1.252	1.277	1.275	1.262	1.257	1.251	1.172

Since catfish is tropical fish, its production in winter is relatively less than in summer, so most of them are imported to the United States during wintertime. Cambodia, China, Thailand and some other Asian countries are the major sources of imported catfish for America. In 2010, up to 14.6 million pounds of catfish were imported from the above countries[13].

Figure 1 showed the change of transportation amount of catfish around the entire year for 2010 and 2011, from which similar trend could be observed for both years. Because large amount of catfish were produced in summer time, the transportation amount of catfish were also correspondingly increased. However, transportation during summer had some potential hazards for fresh catfish, due to high environmental temperature. The hot weather would cause the abuse temperature conditions more frequently, which could accelerate chemical oxidation and also create a suitable growth environment for both spoilage and pathogenic bacteria.

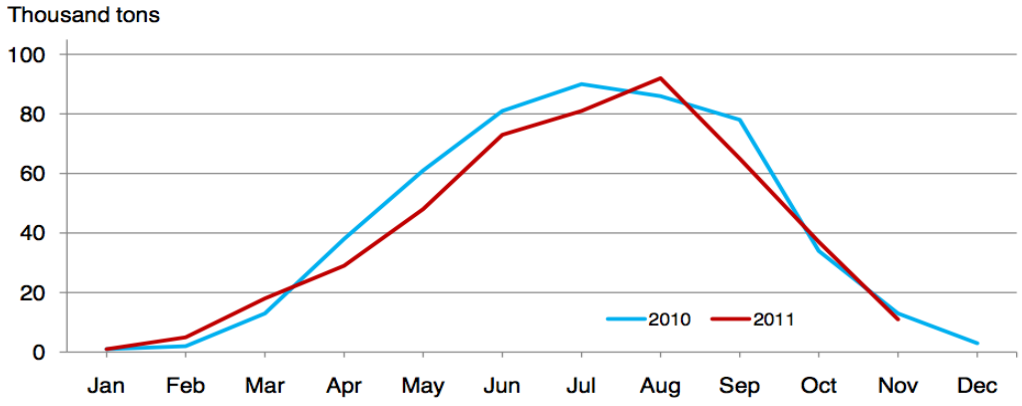

Figure 1: Foodsize catfish feed delivered in United States (adopted from USDA report[14])

2.2. Quality and shelf life of fish

The quality of catfish is decided by the integration of sensory characteristic. Appearance, odor, flavor, and texture are the critical characteristics to evaluate the quality level of fish.

Since the change of colors, flavors and odors are tangible spoilage phenomenon for consumers, these sensory characteristics are immediate concerns for fresh fish products. Various intrinsic and extrinsic factors affect the rate of spoilage in fresh fish such as storage temperature, availability of oxygen, initial population of microorganisms, pH of muscle, activity of intracellular enzymes, nutrients and lipid content[15]. The spoilage of fresh fish can be divided to two main pathways: microbiological and biochemical deterioration. The biochemical pathways are typically including autolysis and chemical oxidation[3].

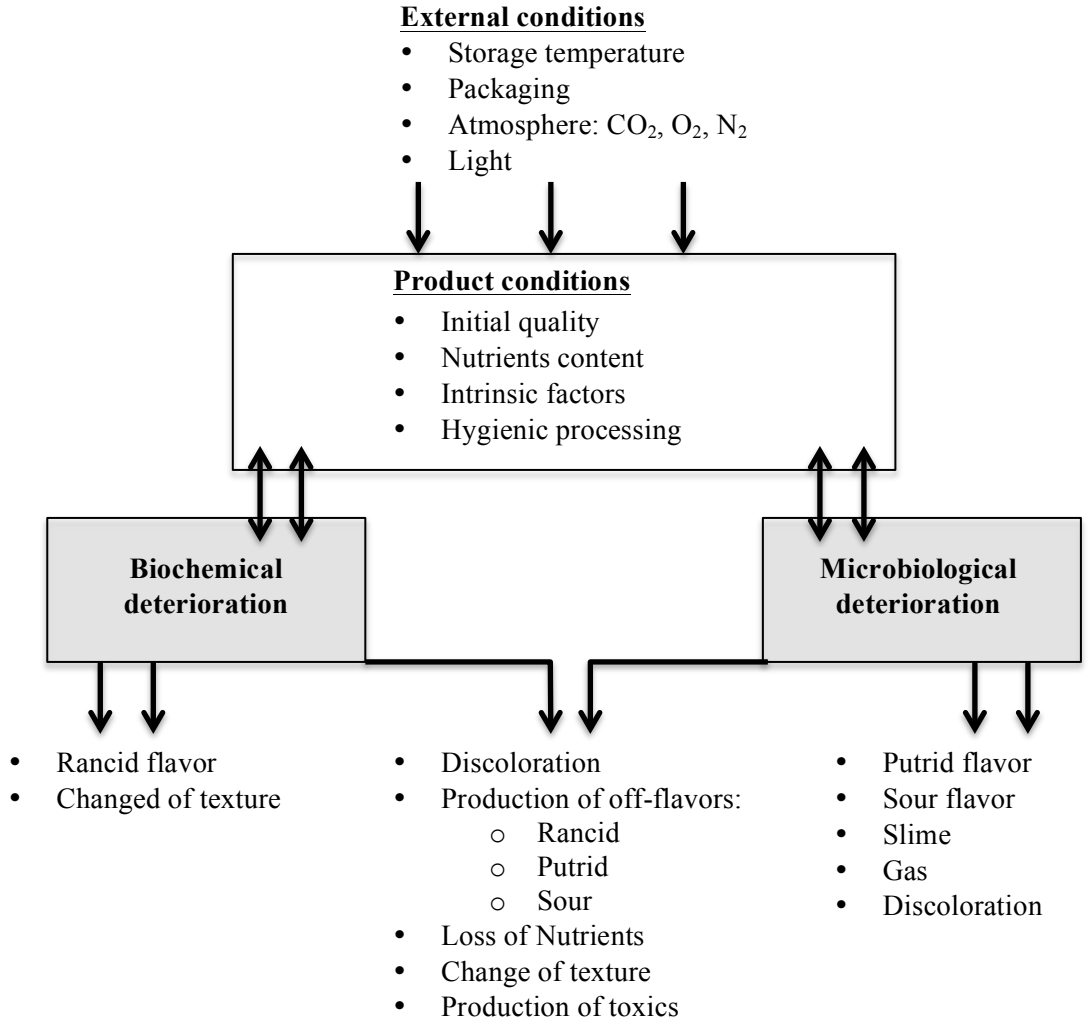

Figure 2: Quality deterioration during storage of fish (modified from Jos and Huis, 1996[16])

Since the microbiological and biochemical deterioration of fish have different mechanisms, these two deterioration modes start at different periods of storage. Figure 3 is a spoilage model of fish products during chill storage temperature. Generally, the microbial spoilage happens earlier than biochemical spoilage, and the initial population of specific spoilage organisms (SSO) is around 3 - 3.5 $\log_{10}$ cfu g^{-1} in fish. For the biochemical deterioration, the induction period of chemical spoilage may depend on the

fat content and fatty acid profile of fish species, and the range of lipid content in catfish muscle is 2.5% - 3.8%.

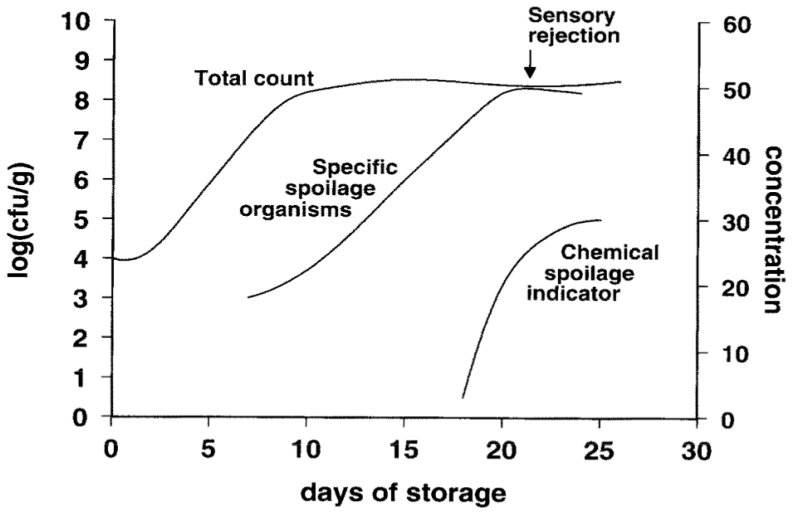

Figure 3: Model of changes in total count (TVC), specific spoilage organisms (SSO) and chemical spoilage indices during chill storage of a fish product (adopted from Gram and Huss[6]).

2.2.1. Microbial growth

Microbial spoilage is a rising concern for food industry, since it has been estimated that up to 25% of agricultural commodities including fishery product are lost due to microbial activities every year[17]. In addition, among the deterioration modes of fresh fish, microbial growth has been recognized the most crucial factor to cause fish spoilage[4], and the spoilage model of fish in Figure 3 also showed that microbial spoilage begin at the initial stage during the storage.

Microorganisms can produce amines, alcohols, aldehydes, sulfides, organic acids and ketones with unpleasant and unacceptable flavors and odors[18]. Discoloration and slime formation may also be detected during microbial activities[4]. Microbiological activities

influence the sensory characteristics of fresh fish in different ways and the sensory manifestation by different microbiological activities is shown in Table 2.

Table 2: Microbiological spoilage of fish (adopted from Gram and Huss[6])

Microbiological activity	Sensory manifestation
Breakdown of food components	Slime formation, off-flavors
Production of extracellular polysaccharide material	Large visible pigmented or non-pigmented colonies
Growth of molds, bacteria, yeasts	Production of gas
Production of CO_2	Discoloration, softening texture

All of fish commodities have their own distinctive microbial flora, and the total amount of microorganisms on fresh fish ranges from 2 to 7 $\log_{10}$ cfu g^{-1}[19]. Different composition of microbial flora in fresh fish depends on its origin, species, season and fishing ground[15]. Several important intrinsic factors influence the growth of microorganism and the spoilage of fish[6].

1. The poikilotherm nature of fish: The poikilothermal fish provide bacteria a broad temperature range to grow. Generally, psychrotrophic Gram-negative bacteria such as *Pseudomonas, Vibrionaceae* and *Shewanella* are dominating in the temperate water fish. Microbial flora in the tropical fish are similar to the temperate water fish, but with slightly higher load of Gram-positive bacteria[19].
2. High post-mortem pH (> 6.0) in the fish flesh: The amount of carbohydrate in the muscle tissue of fish are usually lower than 0.05%, so only little amounts of lactic acid can be produced after death. The relatively high pH environment allows the growth of pH-sensitive spoilage bacteria such as *Shewanella*[6].

3. The presence of trimethylamineoxide (TMAO): TMAO is the major source of umami for fish and also thought to have osmoregulatory function in live fish[20]. Some spoilage bacteria can decompose TMAO to trimethylamine (TMA), the formation of TMA is responsible for off-odors and off-flavors of fish[21].

On the other hand, the major extrinsic factors for fish spoilage are inappropriate storage temperature and irregular processing procedure. Temperature abuse during transportation, processing and storage of fresh fish without any antimicrobial method may create a suitable environment for both spoilage and pathogenic bacteria to grow[22]. Unhygienic processing such as inappropriate slaughter manner may also increase the risk of microbial contamination. Aerobic and chill storage environment is very common in daily life, because consumers usually store fresh fish in the refrigerators.

The spoilage microorganisms developing in aerobically stored fish typically consist of Gram-negative psychrotrophic bacteria. Therefore, under aerobic refrigerator temperature, the flora is composed almost exclusively by *Pseudomonas aeruginosa* and *Shewanella putrefaciens*[6].

Table 3: Specific spoilage bacteria of fresh and packed fish stored at chilled environment (adopted from Gram and Huss[6])

Atmosphere	Specific spoilage organisms of fresh, chill fish			
	Temperate waters		Tropical waters	
	Marine	Fresh	Marine	Fresh
Aerobic	*S. putrefaciens* *Pseudomonas spp.*	*Pseudomonas spp.*	*S. putrefaciens* *Pseudomonas spp.*	*Pseudomonas spp.*
Vacuum	*S. putrefaciens*	Gram-positive bacteria	Lactic acid bacteria	Lactic acid bacteria
	P. phosphoreum	Lactic acid bacteria		
CO_2	*P. phosphoreum*	Lactic acid bacteria	Lactic acid bacteria / TMAO reducing bacteria	Lactic acid bacteria / TMAO reducing bacteria

2.2.2. Autolysis

Autolysis is defined as the degradation of muscle and skin constitution by endogenous enzymes[23]. The impact of which is on textural quality including losing the elasticity of flesh and draining out of the blood water. However, autolysis does not responsible to off-odors and off-flavors in the early stage[18].

Live fish contain numerous enzyme systems, and these intracellular and extracellular enzymes are distributed throughout the fish muscle. Once a fish is dead, all of the anabolic and biosynthetic processes are terminated owing to the absence of blood circulation. Without blood circulation, no oxygen and nutrients is available to supply the process of anabolic and biosynthetic. As a result, only the catabolic or the degenerative and degrading reactions are active which lead to the accumulation of catabolic products. Table 4 shows the possible impact of autolysis by enzymes on fish flesh[23].

Table 4: Summary of autolytic changes in chilled fish (adopted from FAO[24])

Enzyme(s)	Substrate	Changes Encountered	Prevention
Glycolytic enzymes	Glycogen	Production of lactic acid, pH of tissue drops, Loss of water-holding capacity in muscle	Fish should be allowed to pass through rigor at temperatures as close to 0°C Pre-rigor stress must be avoided
Autolytic enzymes involved in nucleotide breakdown	ATP, ADP, AMP, IMP	Gradual production of bitterness loss of fresh fish flavor	Avoid pre-rigor stress and improve handling.
Cathepsins	Proteins, peptides	Softening of tissue	Avoid rough handling during storage
Chymotrypsin, trypsin, carboxy-peptidases	Proteins, peptides	Autolysis of visceral cavity in pelagic (Belly-bursting)	Avoid freezing/ thawing or long-term chill storage
Calpain	Myofibrillar proteins	Softening of tissue	Remove calcium
Collagenases	Connective tissue	Softening and gaping of tissue	Short time and chilled temperature storage
Trimethylamine Oxide (TMAO) demethylase	TMAO	Formaldehyde	≤ -30°C storage temperature, avoid freeze/thawing

It is difficult to prevent the autolysis of fresh fish as the enzymes are dispersed throughout the whole flesh of fish. However, controlling the storage temperature and pH can be effective to delay the autolysis. Since low temperature reduces the activities of all enzymes in fish flesh, decreasing storage temperature can effectively slow down the rate of autolysis[23, 25]. In addition, keeping the pH of muscle around 7 with the treatment of suitable buffer systems can also prevent the activities of some enzymes and minimize the autolysis in fish[23].

2.2.3. Chemical oxidation

Chemical oxidation of fish is mainly because of the oxidative deterioration of unsaturated fatty acids[26], which may be induced by light, metal ions or excessive heat during processing or storage. Lipid oxidation influences the quality of fish by producing off-flavor, off-odor and off-color compounds. Moreover, some of the byproducts may also affect the nutritional quality and change the texture of fish.

Due to the existence of high degree unsaturated fatty acids, chemical oxidation is one of the major deterioration modes for fish species with high lipid content[18]. Several factors influence the lipid content and fatty acid composition in fish commodities, which are size, species, tissue, diet, season and living environmental conditions. The lipid content in the muscle of catfish is around 2.5% to 3.8% (w/w), and the viscera usually contains higher lipid, ranging from 4.5 - 5.9%. Besides, winter catfish contain slightly higher lipid content than summer samples[27].

Chemical oxidation can be initiated either by enzymatic or nonenzymatic reactions. Lipolysis is a process of enzymatic hydrolysis of lipids. In this process, lipases can degrade glyceride and form free fatty acid, which is responsible for declining quality of fat and bring rancidity for fish. Lipases can be found in food products or derived from psychrotrophic microorganisms[16]. Three major enzymes involved in fish lipid hydrolysis are triacyl lipase, phospholipase A2 and phospholipase B[28].

Non-enzymatic oxidation happens due to the catalysis of heme proteins, which could produce lipid hydroperoxides[18]. Heme proteins including hemoglobin and myoglobin are the major catalysts of lipid oxidation in the muscle of fish. The transition metals such as iron (Fe), which can be found in heme proteins, are known to catalyze lipid oxidation

and the iron amount of heme can also decide the pro-oxidative power of hemoglobin and myoglobin[15]. Autoxidation begins when iron in the ferrous form (Fe^{2+}) is converted to the ferric met form (Fe^{3+}). This oxidized met form can break down lipid hydroperoxides to initiate and propagate lipid oxidation[29]. In addition, low pH environment may also accelerate lipid oxidation, because heme proteins are partially unfolded under acidic environment and provide more access to participate in oxidation[15, 18]. Therefore, stabilizing hemoglobin and myoglobin in the reduced ferrous form and controlling the environmental pH are expected to retard lipid oxidation in fish muscle[15].

2.3. Antimicrobial agent - Allyl isothiocyanate (AIT)

With the increasing demands by consumers, good preservation techniques become necessary in order to minimize deteriorations and maintain the quality and nutrition values of catfish[18]. Since consumers are becoming aware of healthy concept, natural ingredients having antimicrobial properties such as essential oils are preferred instead of chemical preservatives[30, 31]. Many researches have also proved that essential oil can be incorporated into food packaging systems which can effectively maintain the antimicrobial activity of the internal environment during storage[32].

Nedorostova and others[33] studied the effect of twenty-seven essential oils on five types of microorganisms and observed that only two essential oils (garlic and horseradish oil) were capable to inhibit the growth of all tested microorganisms, including *Pseudomonas aeruginosa, Listeria monocytogenes, Staphylococcus aureus, Eschericia coli, Salmonella entertidis.* Moreover, horseradish oil exhibited the highest antimicrobial potential compared to the other essential oils. The major compound in horseradish oil is allyl isothiocyanate (AIT)[34], which is responsible for its antimicrobial effect[35, 36].

AIT can be extracted from mustard (*Brassica nigra* and *Brassica juncea*) seed or horseradish root. It is released upon injury or mechanical disruption tissue of plants to protect plants from the attack by herbivores[36]. However, it is also harmful to the plant itself, so glucosinolate is a harmless form to store AIT in the plants. When an animal chews the plant, glucosinolate can be hydrolyzed by myrosinase and form AIT to repel the animals[37].

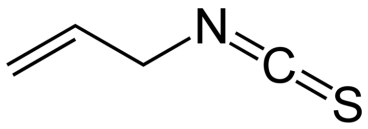

Figure 4: Molecular structure of AIT

AIT is a colorless and volatile compound with organosulfur group (CH_2CHCH_2NCS) (Figure 4). Most of organosulfur compounds bring strong and distinctive odor for plants such as mustard, horseradish and wasabi[36, 38]. AIT is slightly soluble in water but well soluble in most of organic solvents[39]. AIT in liquid phase results in weak antimicrobial ability, however vapor phase AIT with low concentration is unexpectedly considered as an effective antimicrobial agent to inhibit most of typical food spoilage microorganisms[35].

The antifungal and antibacterial capability of AIT is proved by many studies. AIT is recognized as a sulfhydryl inhibitor, which can inhibit the growth of microorganism by causing oxidative cleavage of disulfide bond to inactive the intracellular enzymatic reactions. For antifungal effect, higher concentration of AIT can inhibit the oxygen uptake for yeast[35, 36].

However, the practical application concentration of AIT is limited due to the pungency flavor which can affect the acceptability of a food product[40]. Therefore, other

preservation technologies should be applied in order to enhance the antimicrobial effect in a packaging system.

2.4. Modified atmosphere packaging (MAP)

MAP is one of the most extensively reviewed technologies for perishable seafood products[41]. The concept of MAP is replacing the air in the packaging with a different gas mixture[42]. Generally, high percentage of CO_2 and low percentage of O_2 are used in modified atmosphere packaging. CO_2 with high percentage (≥40%) can effectively suppress the development of aerobic organisms by inhibiting various bacterial enzymatic and biochemical pathways[6, 43, 44]. The antimicrobial capability may increase with the increasing percentage of CO_2 in the atmosphere.

The antimicrobial effect of CO_2 is complicated and four possible activity mechanisms of CO_2 on microorganisms are identified by previous researches[42], which are:

1. Interfere nutrients uptake and absorption of microorganism by altering the cell membrane function.
2. Inhibit or decrease the rate of intracellular enzymatic reactions of microorganism.
3. Reduce the intracellular pH by penetrating of bacterial membranes.
4. Denaturalize the proteins of microorganism by changing its physical and chemical properties.

Since CO_2 is slightly soluble in water, high percentage of CO_2 can form carbonic acid (as shown in Figure 5) with water to create an acidic environment, thus inhibiting the growth of aerobic microorganisms. However, the water-soluble property of CO_2 can also bring some negative impact to the antimicrobial packaging system. While CO_2 gradually dissolves into water or the moist surface of catfish, the internal pressure also decreases,

leading to the collapse of package. In addition, carbonic acid, the forming compound of CO_2 and water, may affect the texture of catfish, because it can lower the water holding capability of catfish by reducing the pH of flesh[5, 6]. Therefore, the application of CO_2 in high percentage should be avoided in the packaging systems of fish to prevent the above consequences.

Gas dissolution

$$CO_2\ (g) \leftrightarrow CO_2\ (aq)$$

Carbonic acid formation

$$CO_2\ (aq) + H_2O \leftrightarrow H_2CO_3\ (aq)$$

Carbonic acid equilibrium

$$H_2CO_3\ (aq) \leftrightarrow H^+\ (aq) + HCO_3^-\ (aq)$$

$$HCO_3^-\ (aq) \leftrightarrow H^+\ (aq) + CO_3^{2-}\ (aq)$$

Figure 5: The chemical equilibriums of carbonic acid forming

Since both AIT and MA have their applied limitations in food packaging, but could be complimentary to each other, these two antimicrobial methods could be combined in one packaging system to obtain their complementary antimicrobial effect thus extending the shelf life of fresh catfish.

2.5. Predictive model

Fresh fish is one of the perishable food commodities. The spoilage of fish usually changes the sensory manifestation by the production of off-flavor and off-odor compounds. Therefore, monitoring and controlling the quality of fish is one of the major goals in the fish industry[45].

Predictive models can be used to estimate the shelf life of fish in a production and distribution chain. Depending on the intrinsic and extrinsic factors, each fish product has its own specific spoilage organism. In order to obtain an accurate prediction of shelf life for each fish product under different storage conditions, it is important to select and apply a microbial model based on the spoilage process of fish product[46]. Two main factors of fish spoilage should be collected for shelf life predictions[47].

1. Specific spoilage organisms—base on the different storage or living conditions, each fish product has its specific spoilage organism.
2. Spoilage level—the fish spoilage may be detected, when the population of specific spoilage organism reaches the spoilage level.

The shelf life of fish can be estimated through the predictive models base on the defined specific spoilage organism and its population level. In the past, most of predictive models were developed by conducting experiments in liquid media such as broth instead of actual food products, because it is much easier to control different factors in broth than in real food item. Recently, many researches have observed that the growth behaviors of microorganism in broth and in catfish are different, because significant factors such as food matrix structure and microbial interactions are significantly different in both

systems[46]. To achieve better accuracy of the predictive models, commercial catfish products were used for model establishment in this study.

3. OBJECTIVES

Two major objectives are included in this research.

1. Investigate the antimicrobial effect of vapor phase AIT (0, 18 and 36 μg/L) and MA (49% CO_2, 0.5% O_2 and 50.5% N_2) on *P. aeruginosa* in fresh catfish fillet held at different abuse temperatures (8, 15 and 20°C).
2. Establish predictive models to estimate the lag time of *P. aeruginosa* and the shelf life of fresh catfish under various storage conditions.

The first objective can be divided into three tasks: to compare the growth behaviors of *P. aeruginosa* in broth and in catfish; to investigate the antimicrobial effect of 18 and 36 μg/L AIT in vapor phase alone and MA alone (49% CO_2, 0.5% O_2 and 50.5% N_2) at 8, 15 and 20°C; and to evaluate the combination effect of AIT in vapor phase and MA at 8, 15 and 20°C.

The impacts and uniqueness of this research are:

(1) Compare the growth behaviors of *P. aeruginosa* in broth and in catfish. Broth is recognized as an optimum matrix for the growth of microorganisms, so much research has been done in broth as food simulant. This research demonstrated that the growth behavior of *P. aeruginosa* in broth and actual catfish were different.

(2) Inhibit the growth of *P. aeruginosa* in catfish fillet by gaseous AIT and MA combination. The synergistic effect of AIT and MA may significantly extend the shelf life of fresh catfish fillet at abuse temperature conditions.

(3) Provide information for food industry to predict the shelf life of fresh catfish products. The system may be further optimized with appropriate AIT concentration in a MA package.

4. MATERIALS AND METHODS

4.1. Overview of experimental procedures

The experiment was designed based on research objectives mentioned in the previous section.

Figure 6 shows the flow chart of the experimental design, which was divided into three parts:

- Growth behavior study. It included observing the growth behavior of *P. aeruginosa* in broth at 8 and 15°C and the growth behavior of *P. aeruginosa* in fresh catfish fillets at 8, 15 and 20°C as the controls for antimicrobial study.
- Antimicrobial study of AIT and MA. It included defining the range of AIT concentrations which were applied in this study; determining the antimicrobial effect of gaseous AIT (18 and 36 μg/L) on *P. aeruginosa* in fresh catfish fillets at 8, 15 and 20°C; determining the antimicrobial effect of MA (49% CO_2, 0.5% O_2 and 50.5% N_2) on fresh catfish fillets at 8, 15 and 20°C, and investigating the combination effect of AIT and MA combination at 8, 15 and 20°C.
- Predictive model development and validation. It includes analyzing the data from antimicrobial study using Baranyi model to obtain lag phase, maximum growth rate of *P. aeruginosa* and shelf life of fresh catfish; establishing predictive models to estimate the lag phase of *P. aeruginosa* and shelf life of fresh catfish; and validating the predictive models by additional independent storage experiments.

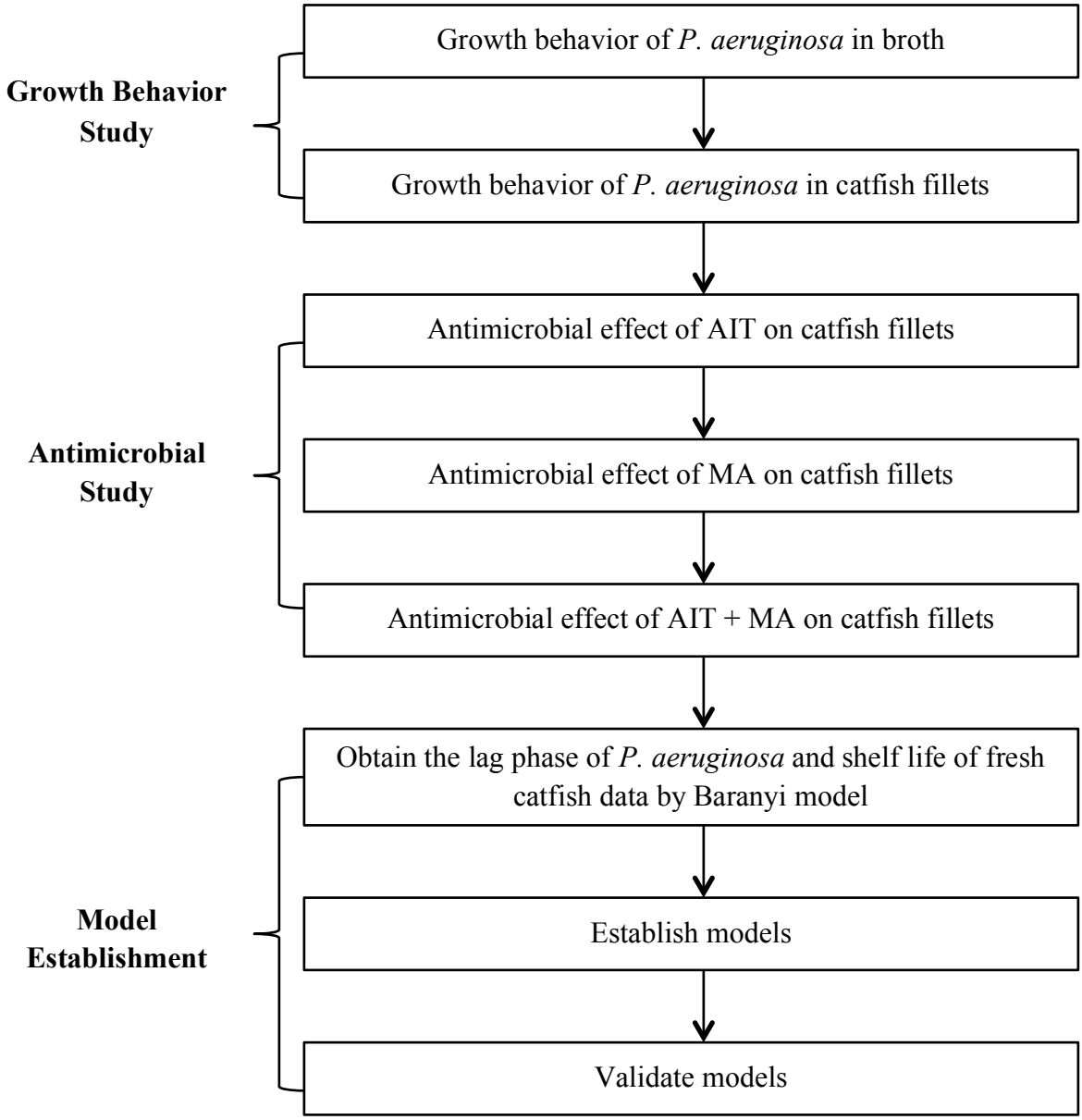

Figure 6: Experimental design flow chart

4.2. *P. aeruginosa* strains and cocktail preparation

Three strains of *P. aeruginosa* (ATCC #15442, #10145 and #27853) obtained from American Type Culture Collection (Manassas, VA) were used in this study. All of the strains are commonly found in fresh fish.

Each strain of *P. aeruginosa* was kept at -80°C as stock cultures separately and transferred monthly to maintain their viability. For preparing *P. aeruginosa* cocktail suspensions, each strain of *P. aeruginosa* stock cultures was taken by using a loopful culture and transferred to a 10 ml Brain Heart Infusion (BHI, Difco, Sparks, MD). Each *P. aeruginosa* suspension was incubated at 37°C for 6 hours and then a second transfer was applied where 100 µl of *P. aeruginosa* was taken from the suspension by micropipetter and added into a new 10 ml BHI for a second incubation at 37°C for 24 hours. 1.0 ml of each strain was taken, mixed together and further diluted using sterile 0.1% peptone water (PW) to attain approximately 5 $\log_{10}$ cfu g^{-1} of *P. aeruginosa* cocktail.

4.3. Growth behavior of *P. aeruginosa* in broth

Appropriate amount of *P. aeruginosa* cocktail suspension was added into two 10 ml BHI cultures to obtain 3.5 $\log_{10}$ cfu g^{-1} as initial concentration, which were incubated at 8 and 15°C respectively and further microbiological tests were conducted over time. For microbiological test, appropriate serial dilutions were performed for microbiological enumeration. *P. aeruginosa* cells were counted by duplicate spread plate on Pseudomonas Isolation Agar (PIA, Difco, Sparks, MD) after incubation at 30°C for at least 24 hours. The colonies number was counted and reported as $\log_{10}$ cfu g^{-1} for every sample.

4.4. Fresh catfish fillets preparation and inoculation

Fresh catfish fillets were purchased from local market. To avoid the interference of background bacteria on catfish, the fillets were frozen for irradiation. The irradiation process was performed in USDA-ARS-ERRC (United States Department of Agriculture-Agriculture Research Service, Eastern Regional Research Center, Wyndmoor, PA).

Before each experiment, catfish fillets were thawed at room temperature (25°C) for around 2 hours, cut into 15 g pieces (3 × 3 × 1.5 cm), and placed in a sanitized 250 ml Mason jar (one piece per jar) (Ace hardware corporation, Shouthampton, PA). This preparation process was performed in a laminar flow biosafety hood. The Mason jar was served as a model storage system and the volume of headspace was constant in this study. *P. aeruginosa* cocktail suspension was diluted using sterile 0.1% PW to achieve target inoculation level, and 100 µl of the diluted suspension was evenly applied to the surface of each catfish piece to yield approximately 3.5 $\log_{10}$ cfu g^{-1} of *P. aeruginosa*, which was to simulate the common situation of fresh catfish[48]. Once the population level of *P. aeruginosa* exceeded 5 $\log_{10}$ cfu g^{-1}, the shelf life of catfish fillet was determined to be terminated, because above this level *P. aeruginosa* might change the color of flesh and cause unacceptable flavors and odors of catfish as shown in Figure 7[49].

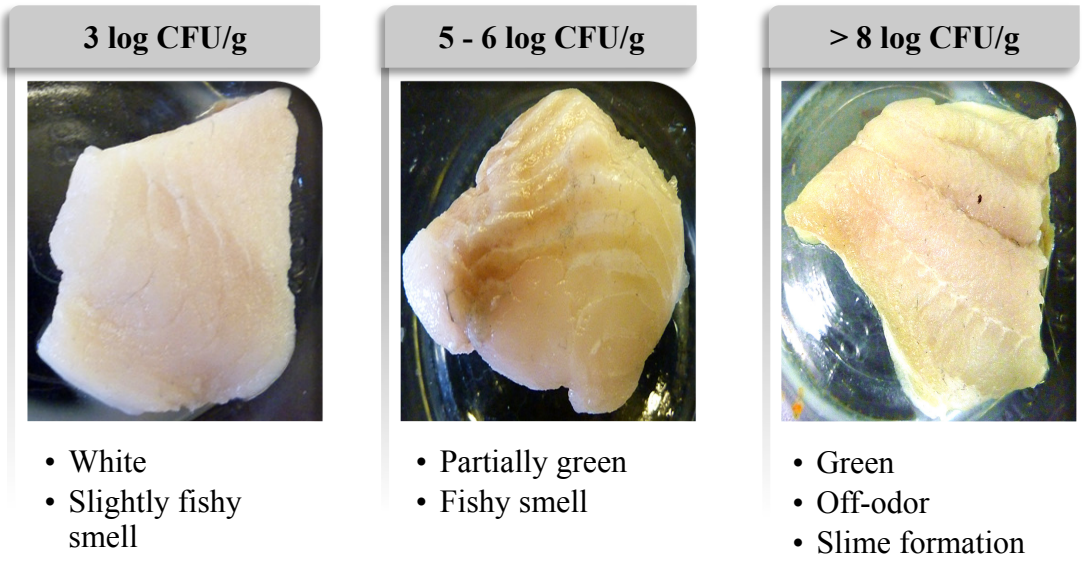

Figure 7: Sensory manifestation with the growth of *P. aeruginosa*

4.5. Screening test of the antimicrobial capability of AIT

A screening test was conducted to confirm that AIT in vapor phase has the antimicrobial effectiveness to inhibit the growth of *P. aeruginosa.* 100 µl of *P. aeruginosa* suspension was spread on the Pseudomonas Isolation Agar, and then 0.005, 0.01, 0.03 and 0.05 mg AIT in liquid phase was added into individual filter papers which were placed on the covers of petri dish in advance. The plates were inoculated at 37°C for 24 hours.

4.6. Gas phase AIT preparation

To study the effect of AIT (purity: ≥93%, Sigma-Aldrich Co., Saint Louis, MO), two amounts (0.005 and 0.01 mg) were added onto the 1 cm × 2 cm filter papers which were placed in each Mason jar in advance as an AIT carrier and then the jar was sealed immediately. Catfish fillet samples without the addition of AIT were used as controls. These samples were stored in the incubators set at 8, 15 and 20°C, and taken out at different intervals for microbiological evaluation in order to obtain the growth curves.

The equilibrium concentrations of gaseous AIT in Mason jar with 0.005 and 0.01 mg AIT filter papers were calculated as 18 and 36 μg/L ($\frac{weight\ of\ liquid\ AIT \times purity\ of\ AIT(\%)}{volume\ of\ jar}$) respectively, assuming the AIT in the filter paper vaporized completely into the headspace. The headspace AIT concentrations in Mason jar with filter paper and the Mason jar with filter paper plus fresh catfish fillet were confirmed to be the same using gas chromatography (HP Series 5890A, Hewlett Packard, New York, NY).

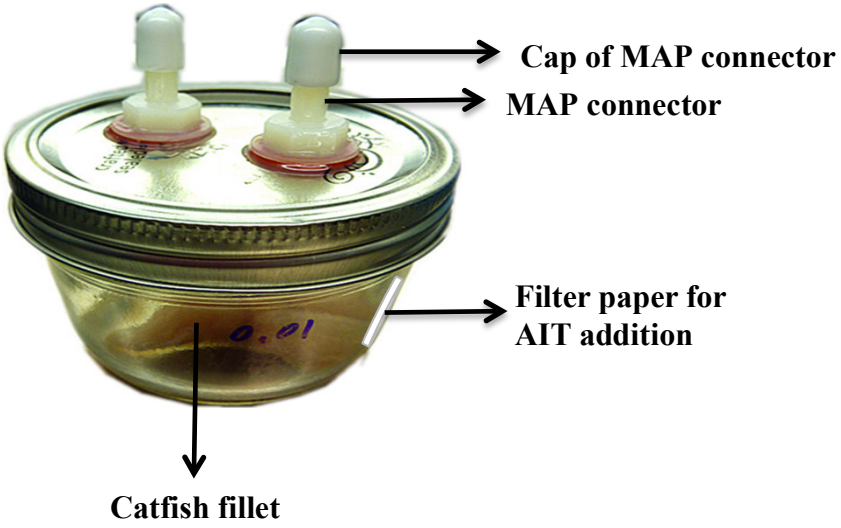

Figure 8: The device of sample

4.7. MA procedure

In order to evaluate the antimicrobial effect of modified atmosphere (MA) on *P. aeruginosa*, the lids of Mason jars were modified to incorporate two connectors (McMaster-Carr Supply Company, Dayton, NJ) for gas flushing. Inoculated catfish fillet samples were placed in the Mason jars and gas mixture was flushed into the jars, as shown in Figure 9. For treatment with MA alone, 50% CO_2, 50% N_2 gas mixture (Airgas, Cheshire, CT) was flushed through the connectors into Mason jar at 50 kpsi for 1 minute and then the connectors were covered by the caps immediately. For AIT and MA

combination, different volumes of AIT (0.005 and 0.01 mg) were added quickly onto the filter paper through the connectors after gas flushing and then the connectors were sealed immediately. These samples were stored at 8, 15 and 20°C for further microbiological analysis. CO_2 and O_2 percentages inside Mason jar were monitored in triplicate at 0, 2, 4, 17 and 23 days throughout the storage periods using O_2 / CO_2 analyzer (Model 902D Dual Trak, Quantek Instruments, Grafton, MA). The percentage of N_2 was calculated by subtracting the percentage of CO_2 and O_2 from 100%.

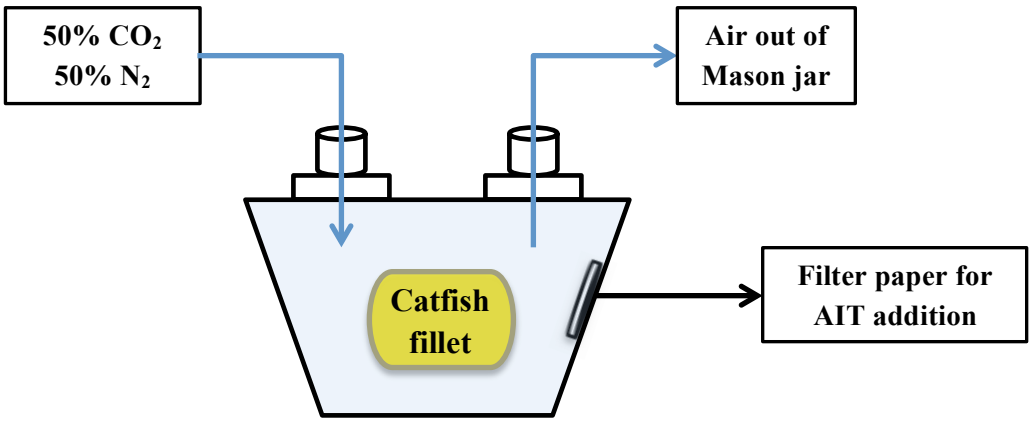

Figure 9: The demonstration of gas flushing

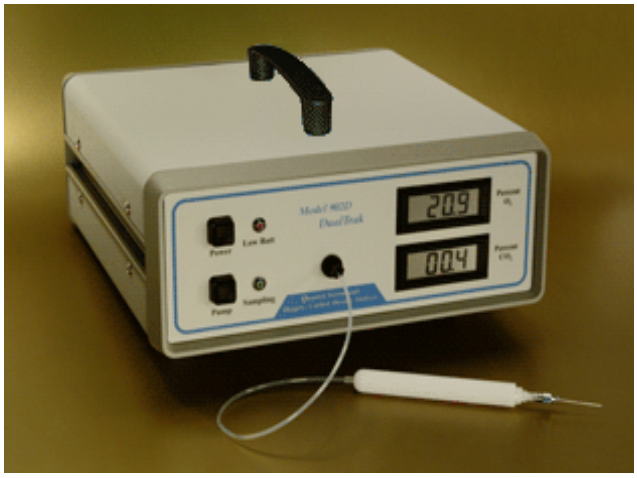

Figure 10: O_2 / CO_2 Analyzer (Model 902D DualTrak)

4.8. Microbiological analysis

After a certain period of storage under different conditions, each catfish fillet sample was placed in a sterile filter bag (80 ml, Fisherbrand, Pittsburgh, PA) with addition of 15 ml 0.1% sterile PW to make a 1:1 dilution (W/V), then homogenized using a Stomacher Lab Blender 400 (Tekmar, Cincinnati, OH) at 300 rpm for 2 minutes. Proper serial dilutions were performed for microbiological enumeration. *P. aeruginosa* cells were counted by duplicate spread plate on Pseudomonas Isolation Agar after incubation at 30°C for at least 24 hours. The colonies number was counted and reported as $\log_{10}$ cfu g^{-1} for every sample.

4.9. Predictive model

A graphical procedure for predictive model development is shown in Figure 11. Stages 1 to 4 were the microbiological experiments, which had been described in the previous sections and stages 5 and 6 were for building and validation model.

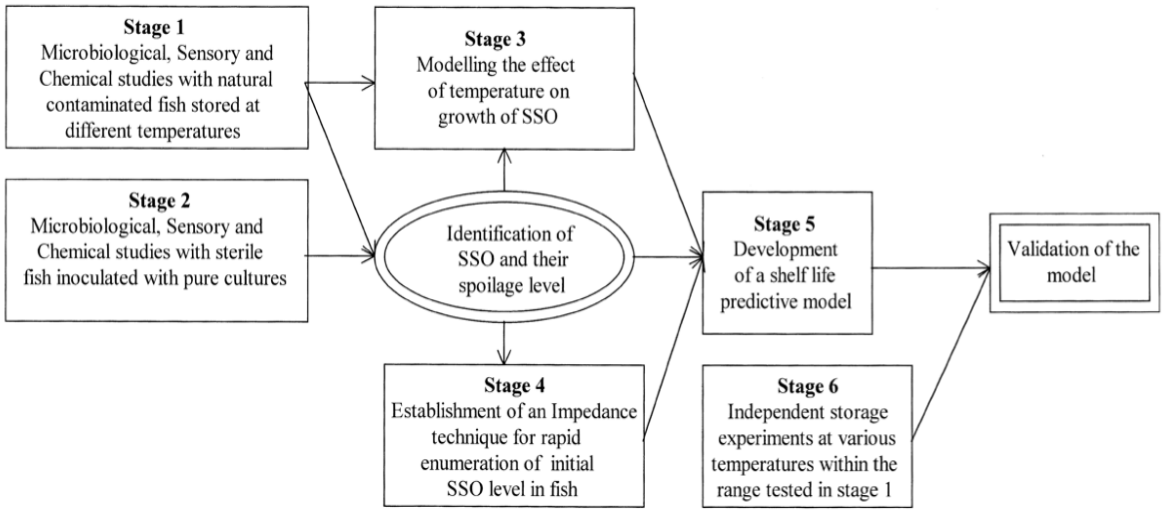

Figure 11: Experimental procedure of developing a microbial model for rapid predictions of shelf life of fresh catfish. (adopted from Koutsoumanis and Nychas[46])

4.9.1. Model establishment

The growth data gathered from the enumeration of *P. aeruginosa* on fresh catfish fillet under different storage conditions were analyzed using DMFit software (Figure 12) on Combase website (www.combase.cc).

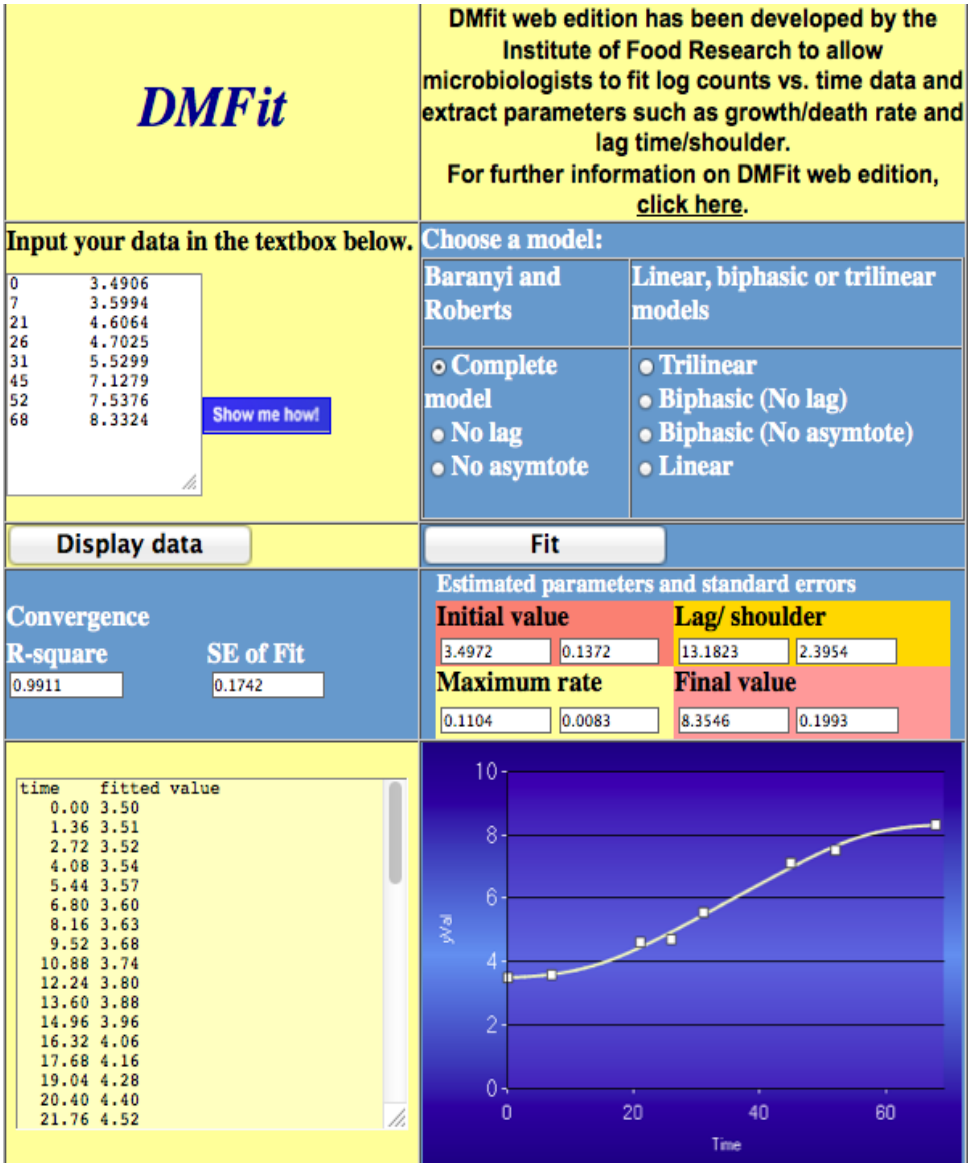

Figure 12: DMFit software on Combase website (www.combase.cc)

In the DMFit software, Baranyi model[52] was applied for data fitting to obtain lag time and maximum growth rate of *P. aeruginosa* and shelf life of catfish at each experimental conditions. The explicit form of the model is the following:

$$y(\mathrm{t}) = y_0 + \mu_{max} t + \frac{1}{\mu_{max}} \ln\left(e^{-\nu \cdot t} + e^{-h_0} - e^{-\nu \cdot t - h_0} \right) - \frac{1}{m} \ln\left[1 + \frac{e^{m\mu_{max} t} + \frac{1}{\mu_{max}} \ln\left(e^{-\nu \cdot t} + e^{-h_0} - e^{-\nu \cdot t - h_0} \right) - 1}{e^{m\,(\,y_{max} - y_0\,)}} \right]$$

where $y(\mathrm{t}) = \ln\big(x(t)\big)$ with $x(t)$ the cell concentration $\left(\frac{cfu}{ml}\right)$.

$y_0 = \ln(x_0)$, $y_{max} = \ln(x_{max})$.

x_0 and x_{max} are the initial and the asymptotic cell concentration, respectively.

μ_{max} is the maximum specific growth rate $\left(\frac{1}{h}\right)$.

m is a curvature parameter to characterize the transition from the exponential phase.

ν is a curvature parameter to characterize the transition to the exponential phase.

h_0 is a dimensionless parameter quantifying the initial physiological state of the cells.

The lag time λ (h) can be calculated as $\frac{h_0}{\mu_{max}}$.

The shelf life was estimated by the period of time during which *P. aeruginosa* grow from initial concentration to 5 $\log_{10}$ cfu g^{-1} (termination of shelf life). The linear regression procedures as well as a three-way ANOVA were performed using SAS (SAS, v9.1) and significant factors were also determined ($P < 0.05$).

4.9.2. Model validation

To validate the model developed in this study, additional data from independent experiments were needed to evaluate how well the model could predict lag phase and

shelf life by using simple linear regression. Fresh catfish samples treated with AIT alone at the concentration of 9 μg/L at 12°C and MA alone at 12°C were selected to validate our predictive models. The experiments were conducted using the same procedure as previous experiments in this study. For the AIT treatment sample, 0.0025 mg AIT (9 μg/L) was added onto the 1 cm × 2 cm filter papers and then the jar was sealed immediately. For the MA treatment sample, 50% CO_2, 50% N_2 gas mixture was flushed through the connectors into Mason jar at 50 kpsi for 1 minute, and then the connectors were covered by the caps immediately. Both samples were stored at 12°C and taken out at pre-determined times for microbiological evaluation to obtain growth curves of *P. aeruginosa*. The microbiological evaluation was analyzed by the same procedure as mentioned previously.

5. RESULTS AND DISCUSSIONS

5.1. Effect of growth mediums

Growth behaviors of *P. aeruginosa* in broth and catfish were observed to be different in this study as shown in Figure 13. The growth rate of *P. aeruginosa* was temperature dependent which increased with increasing storage temperature. From Figure 13, it can be obviously seen that *P. aeruginosa* grew faster in catfish than in broth. At 8°C, it took about 325 hours for *P. aeruginosa* to obtain 5 $\log_{10}$ cfu g^{-1} in broth, while it only took 90 hours in catfish. In other words, the growth rate of *P. aeruginosa* in catfish was about 3.6 times faster than in broth.

Although broth is a simple matrix that different factors can be controlled easily, it still cannot completely replace the actual food system in microbiological study. Many factors such as structure, microbial interaction and nutrients of food system can also affect the growth behavior of *P. aeruginosa*[46].

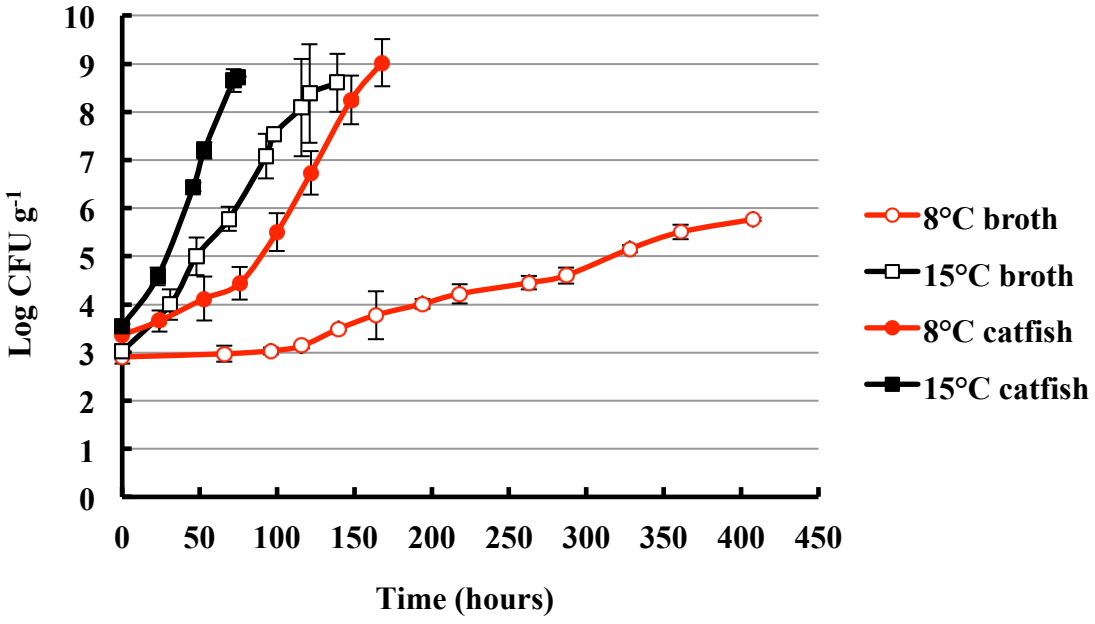

Figure 13: Growth behavior of *P. aeruginosa* in broth and in fresh catfish

5.2. Influence of gas phase AIT

5.2.1. Screening test of AIT

The result of screening test proved that AIT effectively inhibited the growth of *P. aeruginosa* as shown in Figure 14. After incubated at 37°C for 24 hour, all of the samples showed a clear inhibition zone on the plate and the diameter of inhibition zone increased with the increasing amount of AIT added.

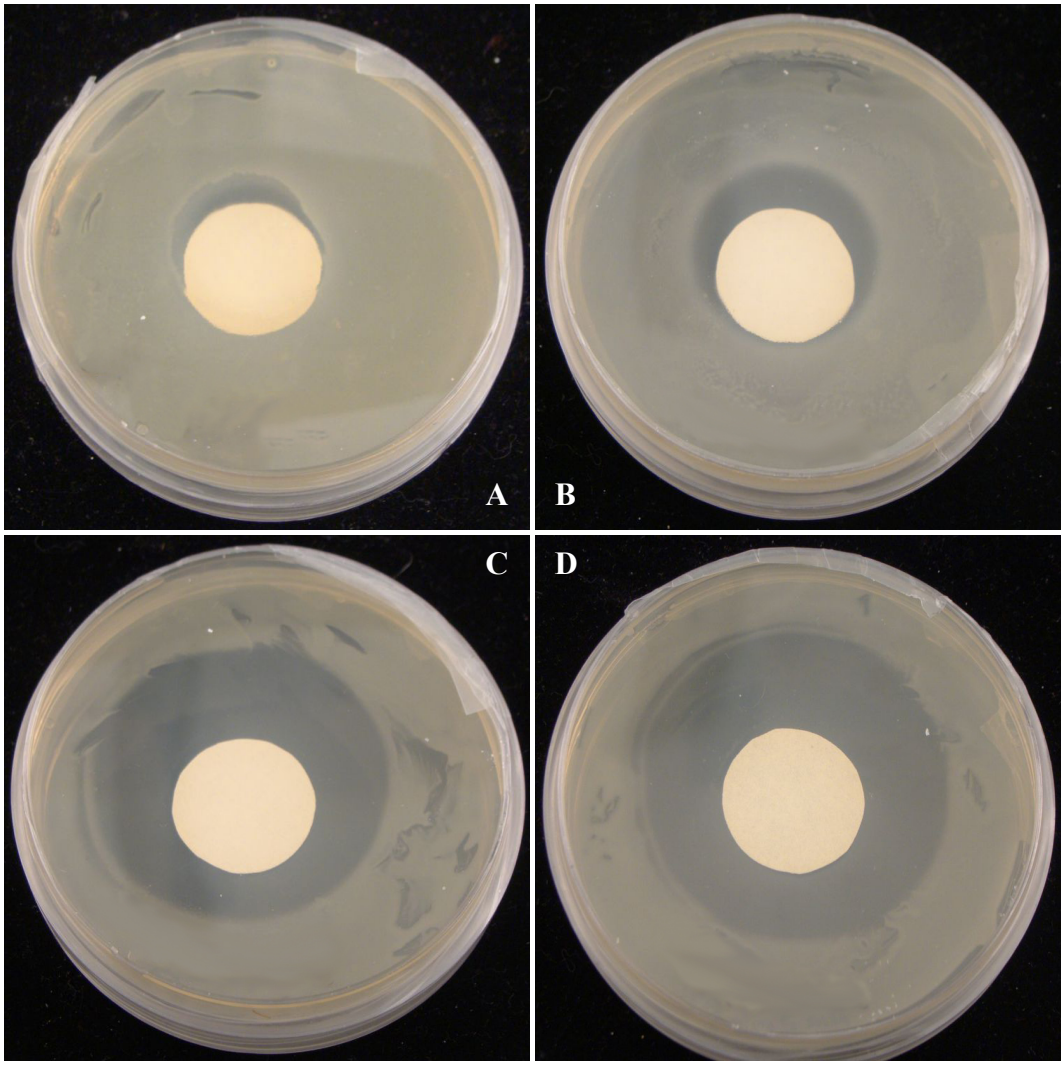

Figure 14: Screen test: Antimicrobial effect of AIT with different concentrations (A) 0.005 mg AIT (B) 0.01 mg AIT (C) 0.03 mg AIT (D) 0.05 mg AIT

5.2.2. Antimicrobial effect of AIT

The effect of AIT on *P. aeruginosa* in catfish fillets at different storage temperature was summarized in Table 5, 6 and 7. Temperature was the major factor to affect the growth of *P. aeruginosa* in this study. Lag phase increased from 5.3 hours to 59.2 hours as temperature decreased from 20 to 8°C without addition of AIT, while the maximum growth rates decreased from 0.20 to 0.05 $\log_{10}$ cfu h^{-1} were observed. Among three temperature conditions, catfish fillet stored at 8°C showed the longest shelf life, which was approximately three times longer than that at 15°C and seven times longer than at 20°C.

Three concentration levels of AIT (0, 18 and 36 μg/L, 0 as the control) were selected to apply on catfish fillets. AIT with concentrations more than 36 μg/L level might be rejected by consumers in the sensory test.

Table 5: Lag phase of *P. aeruginosa* under different storage conditions

	Storage temperature (°C)		
	8	15	20
Lag phase (hours)			
Control	59.2 ± 5.5	12.9 ± 2.7	5.3 ± 0.8
18 μg/L AIT	62.1 ± 6.5	23.4 ± 1.4	16.3 ± 2.5
36 μg/L AIT	232.7 ± 27.5	31.5 ± 5.3	22.6 ± 5.9
MA	214.4 ± 8.9	36.9 ± 1.1	13.1 ±1.1
MA + 18 μg/L AIT	≥ 550	50.2 ± 1.0	42.4 ± 4.4
MA + 36 μg/L AIT	≥ 550	56.2 ± 1.2	51.5 ± 5.0

Mean values ± standard errors

Table 6: Maximum growth rate of *P. aeruginosa* under different storage conditions

	Storage temperature (°C)		
	8	15	20
Maximum growth rate (log cfu/hour)			
Control	0.050 ± 0.004	0.089 ± 0.008	0.201 ± 0.012
18 μg/L AIT	0.001 ± 0.000	0.084 ± 0.003	0.159 ± 0.026
36 μg/L AIT	0.066 ± 0.051	0.095 ± 0.011	0.115 ± 0.053
MA	0.026 ± 0.001	0.103 ± 0.000	0.110 ± 0.003
MA + 18 μg/L AIT	-	0.147 ± 0.012	0.231 ± 0.043
MA + 36 μg/L AIT	-	0.074 ± 0.025	0.205 ± 0.115

Mean values ± standard errors
-, not determined

Table 7: Shelf life of catfish under different storage conditions

	Storage temperature (°C)		
	8	15	20
Shelf life (hours)			
Control	84 ± 3	28.7 ± 1.7	12.2 ± 0.2
18 μg/L AIT	132 ± 14	42.4 ± 1.2	27.5 ± 1.7
36 μg/L AIT	267 ± 23	49.2 ± 3.2	41.1 ± 2.4
MA	284 ± 3	50.5 ± 0.5	26.5 ± 1.8
MA + 18 μg/L AIT	≥ 550	61.9 ± 1.2	50.7 ± 4.3
MA + 36 μg/L AIT	≥ 550	75.5 ± 7.0	62.5 ± 5.2

Mean values ± standard errors

The growth curves of *P. aeruginosa* with different concentrations of AIT are showed in Figure 15, 16 and 17, which indicated the bacteria load was slightly reduced in the initial several hours. For example, 36 μg/L AIT treatment reduced about 0.8 $\log_{10}$ cfu g^{-1} of *P. aeruginosa* at 8°C in the first 25 hours and similar effect was also observed in the rest of the samples stored at different temperatures.

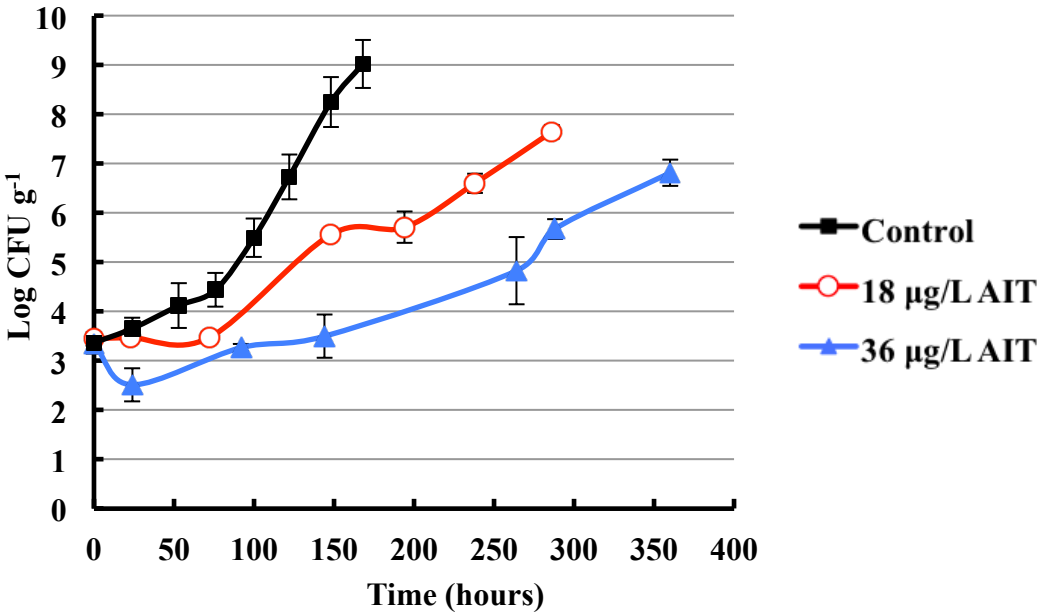

Figure 15: Growth curves of *P. aeruginosa* cocktail in fresh catfish fillet with different concentrations of AIT at 8°C.

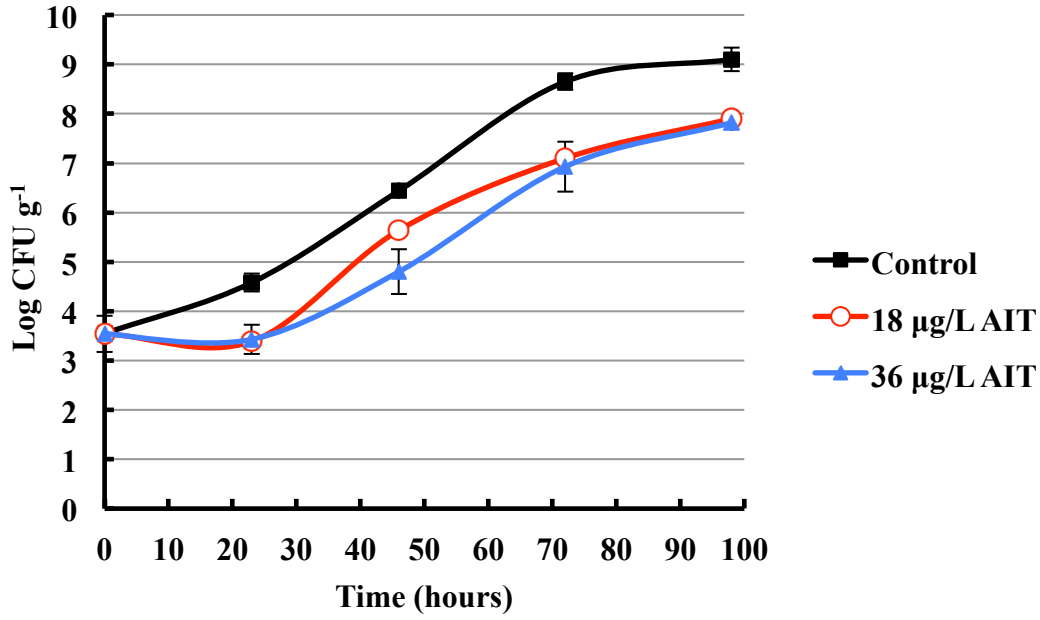

Figure 16: Growth curves of *P. aeruginosa* cocktail in fresh catfish fillet with different concentrations of AIT at 15°C.

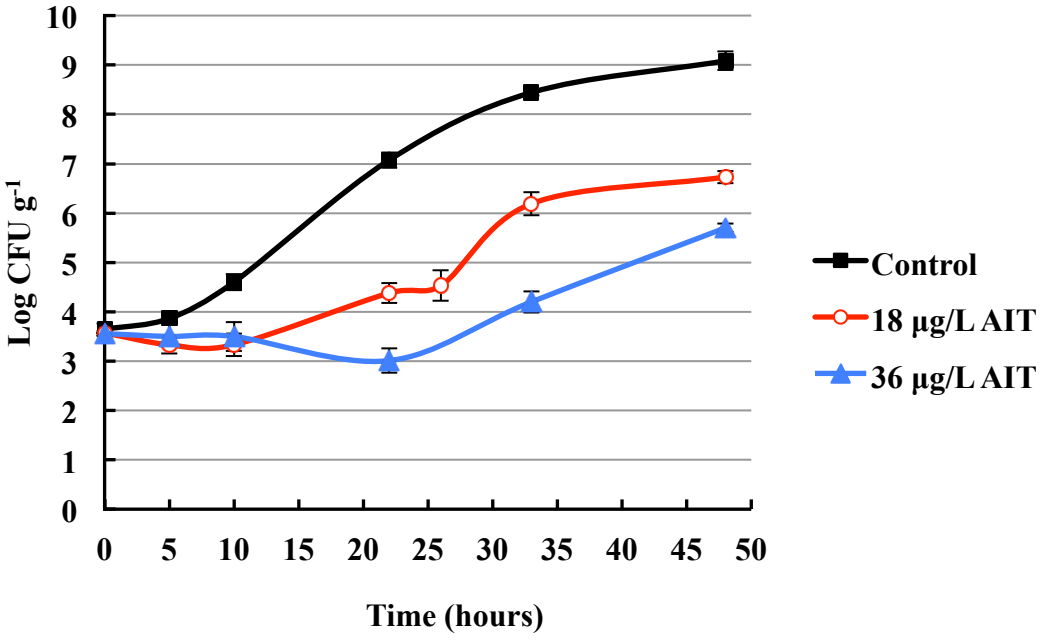

Figure 17: Growth curves of *P. aeruginosa* cocktail in fresh catfish fillet with different concentrations of AIT at 20°C.

The antimicrobial effect of AIT was illustrated by three parameters, lag phase, maximum growth rate and shelf life in Tables 5 – 7, respectively. Compared to the controls, 36 µg/L AIT treatment provided about three times longer lag phase at all three storage temperatures and the shelf life was also extended based on *P. aeruginosa* growth threshold of 5 $\log_{10}$ cfu g^{-1}. This study demonstrated that AIT was an effective antimicrobial agent to extend the lag phase of *P. aeruginosa* and to prolong the shelf life of catfish fillets. However, no positive relationship between maximum growth rate and concentrations of gaseous AIT was observed. In other words, higher AIT concentration did not provide lower maximum growth rate of *P. aeruginosa*. According to the antimicrobial mechanisms presented in the literature[35], AIT might only partially reduce some intracellular enzymatic reactions in *P. aeruginosa* to keep them stay in the lag

phase, but once the survival cells adapted to the imposed stresses, they could recover and then behave similar to the normal cells.

5.3. Influence of MA

5.3.1. Fluctuation of gas compositions

The change of gas composition in the headspace of Mason jar was determined at room temperature and the results were shown in Table 8. It could be found that the percentage of CO_2 decreased while the present of O_2 increased during storage. The possible explanation for this observation might be partially due to the dissolution of CO_2. Since CO_2 was slightly soluble in water, part of CO_2 could dissolve into the moist surface of catfish fillet and the percentage of dissolved CO_2 increased with the decreasing storage temperature conditions.

Table 8: The change of gas composition in headspaces during storage of catfish fillets

Days of storage	Gas composition % 50% CO_2 / 50% N_2	
	CO_2 (%)	O_2 (%)
0	49 ± 0.8	0.5 ± 0.3
2	43.5 ± 1.1	2 ± 0.8
4	36.7 ± 3.5	2.3 ± 1.2
17	30.7 ± 2.9	4.6 ± 0.9
23	26.8 ± 5.4	7.5 ± 3.6

5.3.2. Antimicrobial effect of MA

The growth curves of *P. aeruginosa* in catfish fillet with/without MA stored at different temperatures were shown in Figure 17, 18 and 19. According to the results, MA showed a strong antimicrobial potential on *P. aeruginosa* growth, which was in agreement with

other published reports demonstrated that major aerobic spoilage microorganisms such as *P. aeruginosa* could be inhibited by higher level (> than 40%) of CO_2[55, 56]. All of the MA samples showed around three times longer lag phase than those without MA treatment. In addition, a longer shelf life could also be obtained by the MA samples at three different temperature conditions.

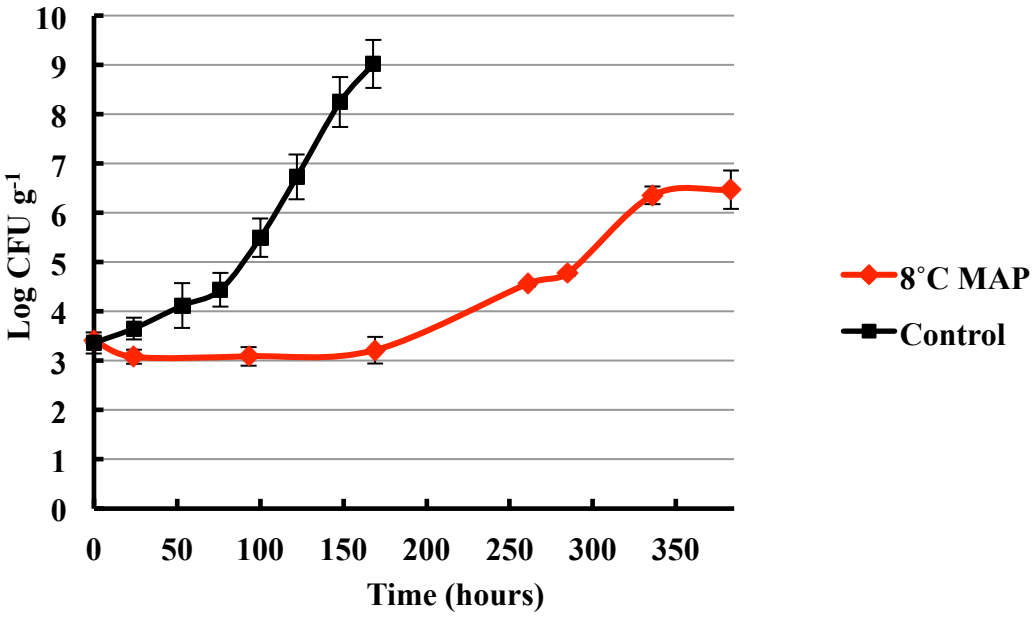

Figure 18: Growth curves of *P. aeruginosa* cocktail in fresh catfish fillet with/without MA (49% CO_2, 0.5% O_2 and 50.5% N_2) at 8°C.

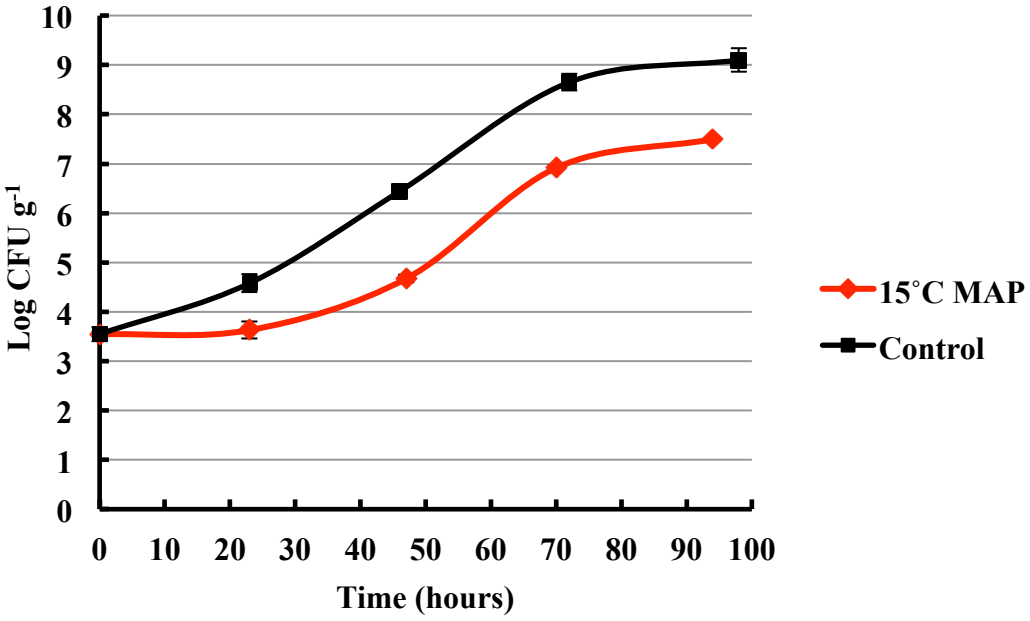

Figure 19: Growth curves of *P. aeruginosa* cocktail in fresh catfish fillet with/without MA (49% CO_2, 0.5% O_2 and 50.5% N_2) at 15°C.

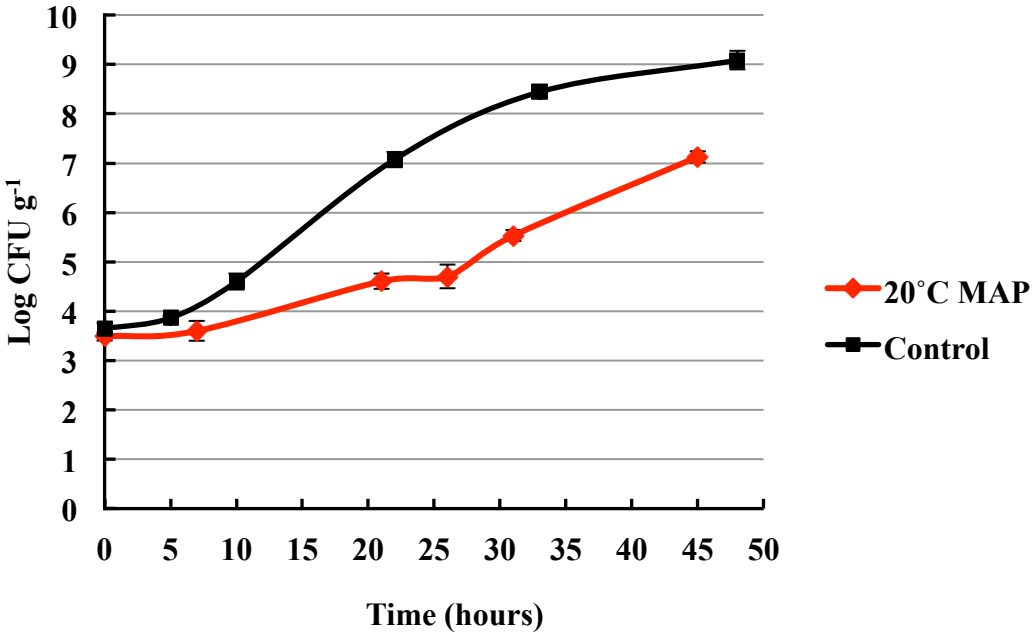

Figure 20: Growth curves of *P. aeruginosa* cocktail on fresh catfish fillet with/without MA (49% CO_2, 0.5% O_2 and 50.5% N_2) at 20°C.

The comparison between AIT and MA treated samples was showed in Tables 5 - 7. MA exhibited similar antimicrobial capability as 36 μg/L AIT treatment at lower storage temperature conditions such as 8 and 15°C, but its antimicrobial effect at higher temperature was less effective than 36 μg/L AIT treatment in this study. The relatively weak solubility of CO_2 at 20°C might account for the decreasing antimicrobial effectiveness[33]. CO_2 was slightly soluble in water and the major factor to affect its solubility was environmental temperature. The solubility of CO_2 at 0°C is around 3.38 g CO_2/kg H_2O which reduces to 1.73 g CO_2/kg H_2O at 20°C[53]. The dissolved CO_2 might lower the pH at catfish fillet surface by creating carbonic acid and this acidic environment could inhibit the growth of aerobic spoilage organisms[43, 54].

5.4. Influence of AIT and MA combination on catfish

To extend the lag phase and shelf life of fresh catfish fillet, AIT combined with MA was applied and the synergistic effects were evaluated. AIT and MA combination can reduce more bacteria load in the initial several hours than AIT alone treatment as shown in Figure 21, 22 and 23. At 8°C, 36 μg/L AIT alone treatment can reduce 0.5 $\log_{10}$ cfu g^{-1} of *P. aeruginosa* in the first 53 hours, but about 1.57 $\log_{10}$ cfu g^{-1} of *P. aeruginosa* can be reduced under 36 μg/L AIT and MA combination treatment in the same condition.

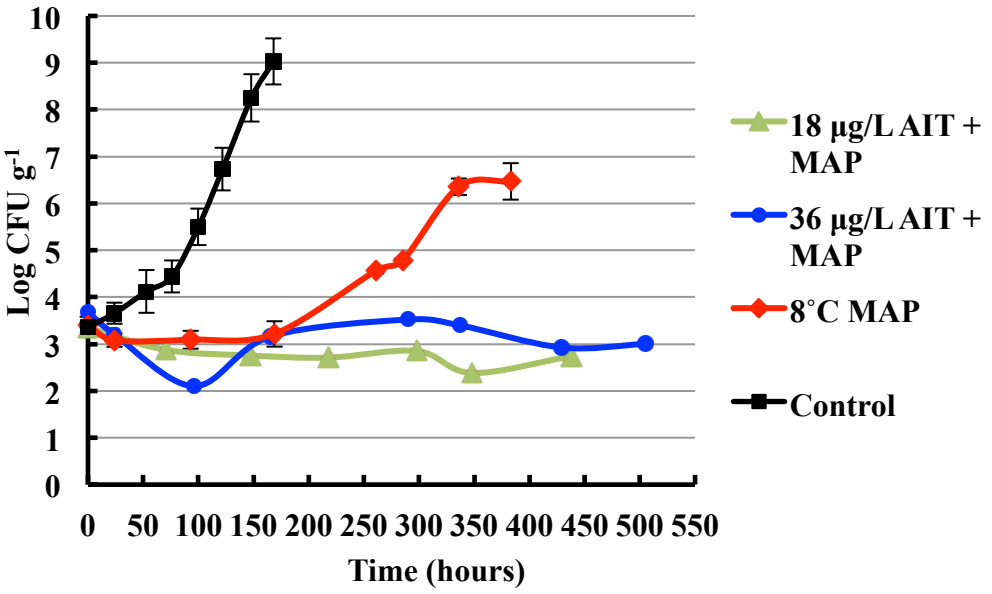

Figure 21: Growth curves of *P. aeruginosa* cocktail in fresh catfish fillet under different storage conditions at 8°C.

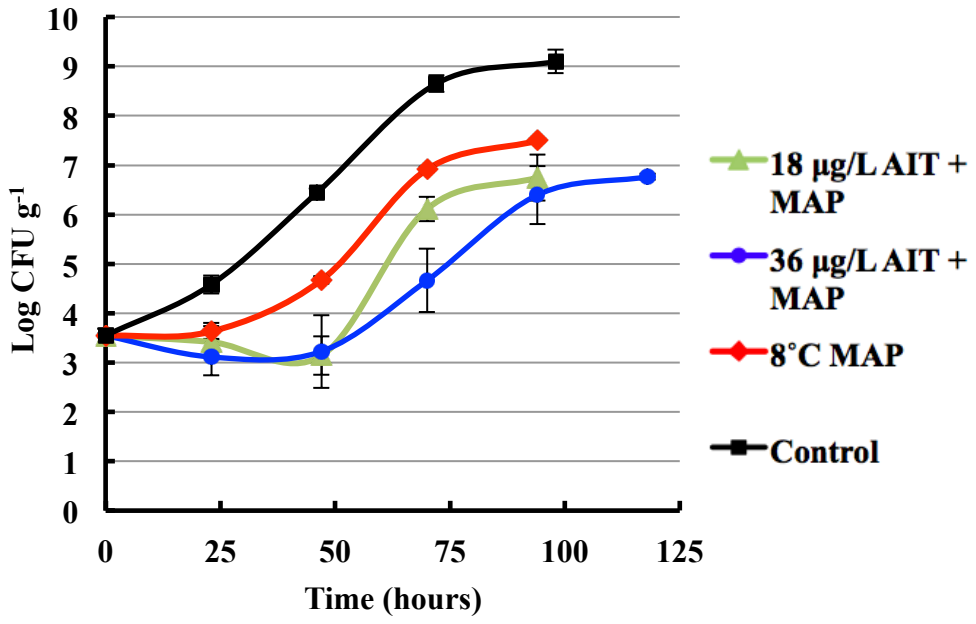

Figure 22: Growth curves of *P. aeruginosa* cocktail in fresh catfish fillet under different storage conditions at 15°C.

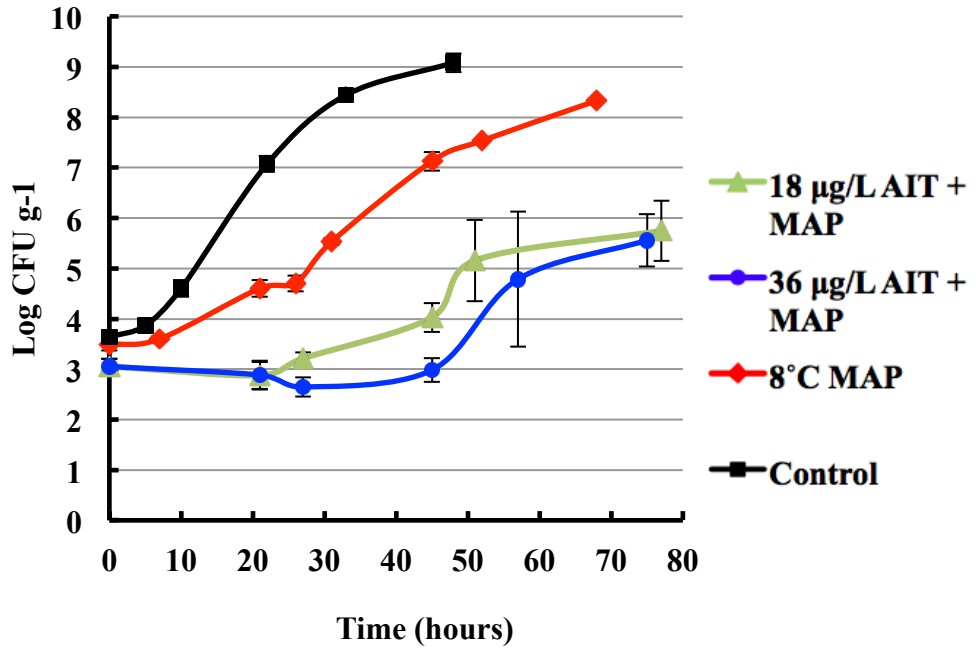

Figure 23: Growth curves of *P. aeruginosa* cocktail in fresh catfish fillet under different storage conditions at 20°C.

In Table 5 -7, the synergistic effect was observed when AIT and MA combination applied to catfish samples. 18 µg/L AIT alone sample showed 132 hours shelf life and MA alone sample showed 285 hours shelf life at 8°C as shown in Table 5. However, the AIT and MA combination showed more than 550 hours shelf life, which was 32% longer than the sum of shelf life of AIT alone and MA alone. In Table 5 and 7, the combination of AIT and MA prolonged the lag phase of *P. aeruginosa* for around 1.5 - 4 times longer, and resulted in at least 1.2 times longer shelf life than those of AIT or MA alone at 15 and 20°C. Compared to the controls, shelf life of catfish can be extended from 4 days to 23 days (550 hours) at 8°C. Although the synergistic effect is not obtained at higher temperature such as 15 and 20°C, this combination may still extend the shelf life of catfish fillet for more than 2.6 times (hours vs. hours control).

No further experiments were conducted beyond 550 hours in this study, because after that time *P. aeruginosa* spoilage might no longer be the critical issue due to other molds growth. In this case, biochemical deteriorations including lipid oxidation or autolysis should also be considered for determining the shelf life of fresh catfish fillet. Since the study was terminated at 550 hours, the shelf life at 8°C was assigned to be 550 hours for both AIT concentrations combined with MA. In addition, *P. aeruginosa* was not able to grow under the treatment of AIT combined with MA within 550 hours at 8°C, so no maximum growth rate data were presented in Table 6.

According to ANOVA as shown in Tables 9, 10 and 11, all factors (temperature, AIT and MA) showed significant effect on prolonging lag phase of *P. aeruginosa* and the shelf life of fresh catfish fillet. The ANOVA results also indicated that the synergistic effect of AIT and MA was very significant, which suggested the combination of AIT and MA could be used as an effective antimicrobial system to resist temperature abuse and extend the shelf life for fresh catfish fillet. However, for the maximum growth rate of *P. aeruginosa*, only temperature showed significant impact, which decreased along with the decreasing storage temperatures, AIT and MA alone did not affect the growth rates of *P. aeruginosa* after the cells adapted to the stresses. Although the interaction of AIT and MA appeared to be statistically significant according to ANOVA (Table 11), it did not necessarily mean their interaction had actual effect on maximum growth rate, because when the major factors (AIT and MA) were insignificant, it was highly likely that their interaction could be inaccurately determined to be significant by using ANOVA[57].

Table 9: Summary of the lag phase results obtained by ANOVA.

Factor	d.f.	Sum Sq	Mean Sq	F	P
Temperature	2	116773	58387	1163.988	< 0.001
AIT	2	14513	7257	144.665	< 0.001
MA	1	16906	16906	337.045	< 0.001
Temperature * AIT	4	34474	8619	171.818	< 0.001
AIT * MA	2	3784	1892	37.715	< 0.001
Temperature * MA	2	19532	9766	194.694	< 0.001
Residuals	34	1705	50		

Table 10: Summary of the shelf life results obtained by ANOVA.

Factor	d.f.	Sum Sq	Mean Sq	F	P
Temperature	2	878169	439084	790.598	< 0.001
AIT	2	79242	39621	71.340	< 0.001
MA	1	168374	168374	303.168	< 0.001
Temperature * AIT	4	85278	21320	8.387	< 0.001
AIT * MA	2	11462	5731	10.319	< 0.001
Temperature * MA	2	231557	115778	208.466	< 0.001
Residuals	40	22215	555		

Table 11: Summary of the maximum growth rate results obtained by ANOVA.

Factor	d.f.	Sum Sq	Mean Sq	F	P
Temperature	2	0.14167	0.07083	66.539	< 0.001
AIT	2	0.00298	0.00149	1.402	0.259
MA	1	0.00410	0.00410	3.853	0.057
Temperature * AIT	4	0.00569	0.00142	1.337	0.276
AIT * MA	2	0.02180	0.01090	10.239	< 0.001
Temperature * MA	2	0.00057	0.00029	0.267	0.766
Residuals	34	0.03619	0.00107		

5.5. Predictive models

Polynomial models, a common type of model for empirical modeling, were selected to predict the lag phase of *P. aeruginosa* and the shelf life of fresh catfish in this study. However, the limitation of empirical model was that the predictive ranges were confined to the selected parameters with experimental design[58].

5.5.1. Lag phase model

The lag time obtained from three levels of AIT (0, 18 and 36 μg/L) treatment with/without MA were applied to develop predictive models using the general linear regression procedures (SAS, v9.1). Models developed for the lag time without and with MA treatment were shown below in Equation (1) and (2), respectively.

$$\text{Lag phase} = 225.30 - 30.11T + 4.36AIT - 0.38AIT \times T + 1.01T^2 + 0.08AIT^2 \quad (1)$$

$$\text{Lag phase} = 634.99 - 66.96T + 0.71AIT - 0.06AIT \times T + 1.80T^2 + 0.03AIT^2 \quad (2)$$

where lag phase was the lag time in hours, T is temperature in °C, and AIT is concentration of allyl isothiocyanate in μg/L. The R^2 was 0.87 for Equation (1) and 0.99 for Equation (2).

In Equation (2), two terms, AIT and its interaction with temperature, were not significant in the linear regression analysis ($P > 0.05$). However, the statistical insignificance of AIT did not necessarily mean that AIT had no actual effect on lag phase or shelf life. The existence of MA might abate the significance of AIT in the linear regression analysis, but the effect of AIT could be observed from experimental result. In Table 5, the lag time of catfish sample was 214 hours at 8°C under MA treatment, however the sample treated with AIT and MA combination exhibited a lag time of more than 550 hour. Therefore, the terms related to the effect of AIT should still be kept in the linear regression model.

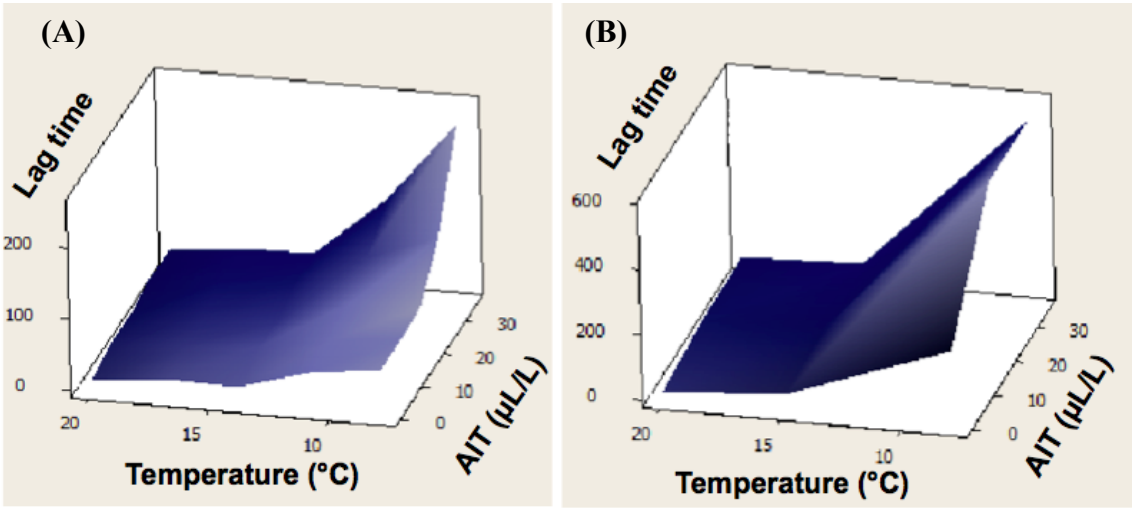

Figure 24: Surface plot of lag time models: (A) lag time plot without MA, (B) lag time plot with MA.

Figure 24 was the surface plot of lag time models, which showed that higher concentration of AIT with lower storage temperature had more effective antimicrobial effect by providing a longer lag phase of *P. aeruginosa*. Compared Figure 24 (A) and (B), the sharp slope in (B) demonstrated that the participation of MA in AIT packaging system could obviously extend the lag phase at lower storage temperature.

Figures 25 and 26 were the comparison of experimental data and predicted values from the models. In Figure 25, when the experimental data were close to the predicted value, the corresponding point on the graph should be close to the solid line. Once the observed value equaled to the predicted value, the point would be exactly on the solid line (slope =1.0). When the predicted values were over or under estimated, the point would be above or below the solid line, respectively. The dotted line represents 95% confident range for the predictive models. Figure 26 (with MA treatment) showed narrower 95% confident range than Figure 25 (without MA treatment), which indicated that the linear regression model with MA treatment could predict lag time closer to the experimental ones[58].

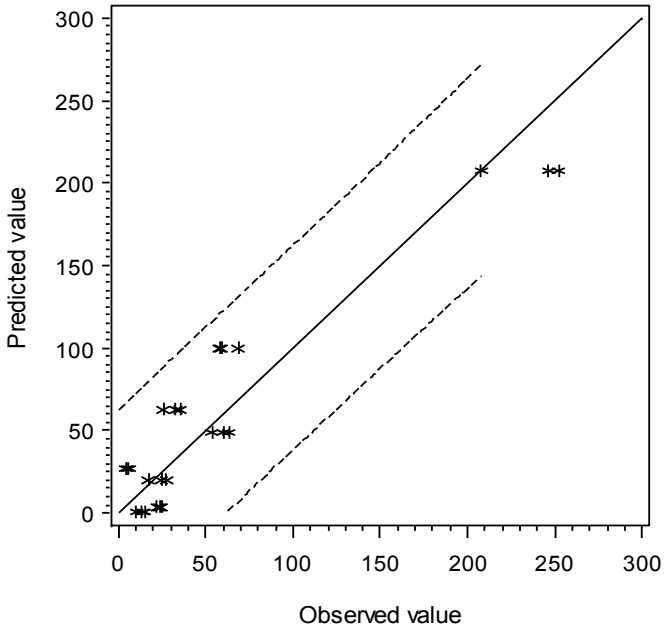

Figure 25: The observed vs. predicted values of lag phase without MA using Eq. (1)

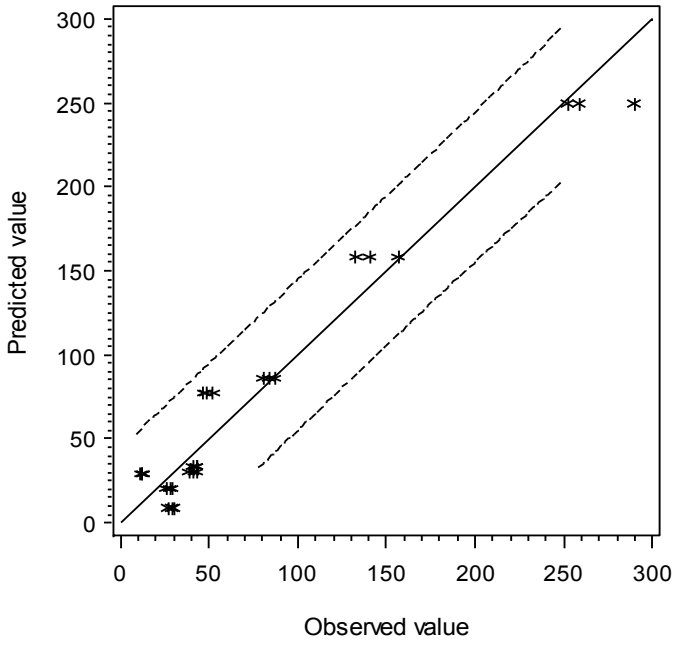

Figure 26: The observed vs. predictive values of lag phase with MA Eq. (2)

5.5.2. Shelf life model

Equation (3) was developed to predict the shelf life of catfish fillet without MA treatment, and Equation (4) was for the treatment with MA.

$$\text{Shelf life} = 326.75 - 40.17T + 6.50AIT - 0.37AIT \times T + 1.27T^2 + 0.03AIT^2 \quad (3)$$

$$\text{Shelf life} = 854.10 - 91.07T + 1.38AIT - 0.01AIT \times T + 2.49T^2 - 0.02AIT^2 \quad (4)$$

where shelf life was in hours. The R^2 was 0.95 for Equation (3) and was 0.99 for Equation (4).

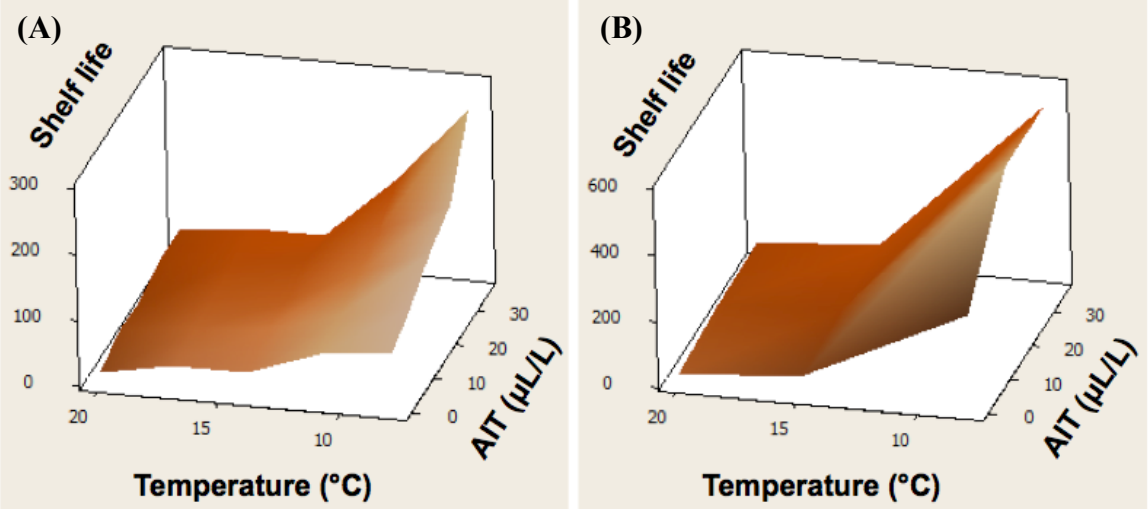

Figure 27: Surface plot of shelf life models: (A) shelf life plot without MA, (B) shelf time plot with MA.

Figure 27 was the surface plot of shelf life models, which is similar to the surface plot of lag time models. MA and AIT combination at low storage temperature increased the shelf life dramatically, and a longer shelf life was obtained at higher concentration AIT with lower storage temperature.

According to Figure 28 and 29 both predictive models for shelf life showed narrow 95% confident ranges, which indicated that the linear regression models could predict shelf life fairly close to the experimental data.

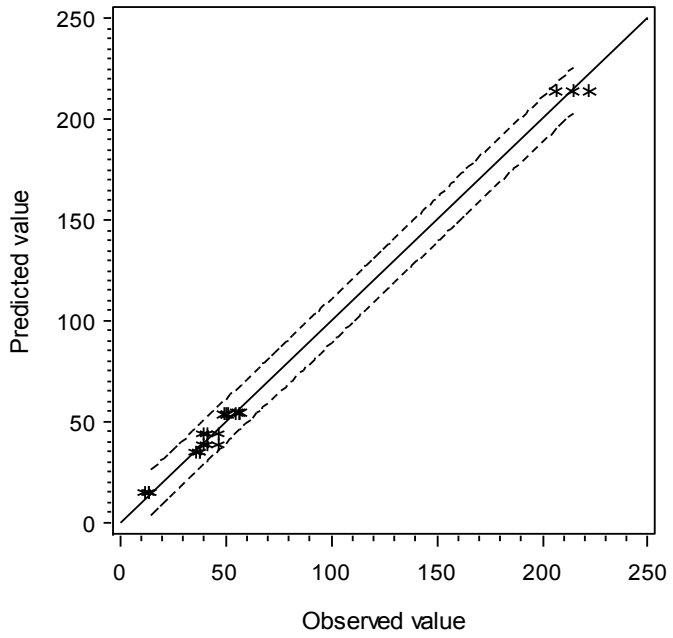

Figure 28: The observed vs. predicted values of shelf life without MA using Eq (3).

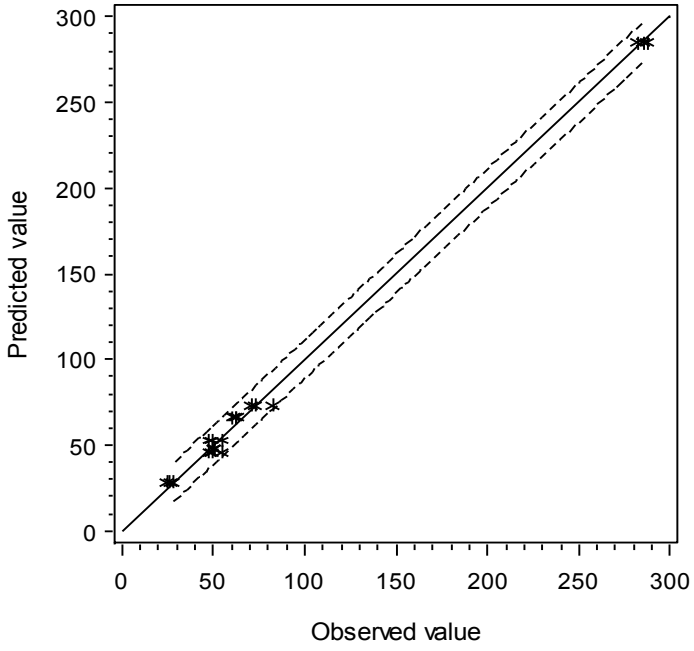

Figure 29: The observed vs. predicted values of shelf life with MA using Eq. (4)

5.5.3. Maximum growth rate model

Since neither AIT nor MA showed significant effect ($P > 0.05$) on maximum growth rate (Table 11), predictive models were not established for maximum growth rate in this study.

5.5.4. Model validation

Linear regression models (with/without MA) needed to be validated to ensure their accuracy. 9 μg/L AIT treatment at 12°C was selected to validate the linear regression models without MA treatment (Equation (1) and (3)), and MA (49 % CO_2, 0.5% O_2 and 50.5% N_2) treatment at 12°C was applied to validate the models with MA treatment (Equation (2) and (4)). Lag time and shelf life were estimated from these two independent experiments and the predicted values were obtained from their corresponding linear regression models.

The comparison of validation data and predicted values from linear regression models for treatment without MA was shown in Figure 30. According to the linear regression model, the predicted lag time of *P. aeruginosa* under 9 μg/L AIT treatment at 12°C should be 27.45 hours, but the actual lag time from the experiment was 22.06 hours. The difference between experimental and predicted values was 24%. The shelf life of fresh catfish fillet determined from the experiment was 46.62 hours, and its predicted value was 47.23 hours. The difference between them was 1%. Both of validation data presented shorter lag phase than predicted results.

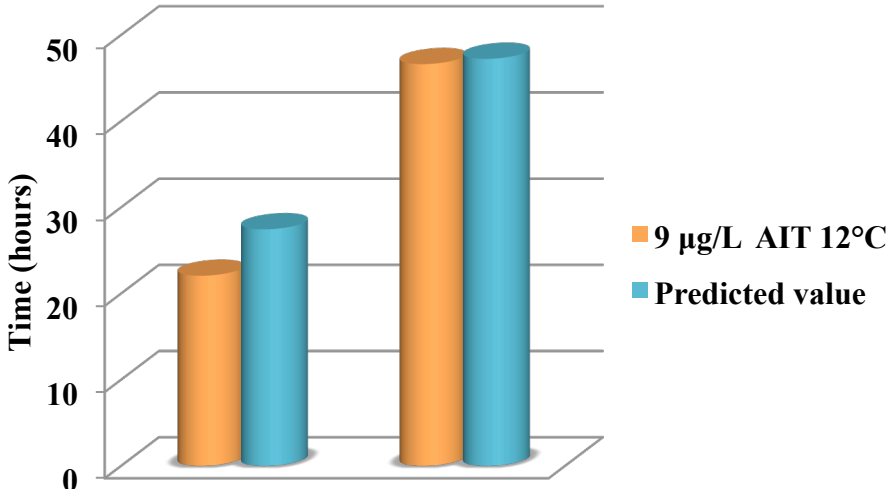

Figure 30: Comparison of experimental data and predicted values for 9 µg/L AIT sample at 12°C

Figure 31 showed the experiment data and the predicted values from linear regression models for treatment with MA. The lag time of *P. aeruginosa* under MA condition at 12°C from the experiments was 82.40 hours, and the predicted value was 90.36 hours. The difference of lag time between validation data and predicted result was 10%. The shelf life of fresh catfish fillet from the experiment and the predictive model were 114.67 and 119.82 hours, respectively. The difference between them was 4%.

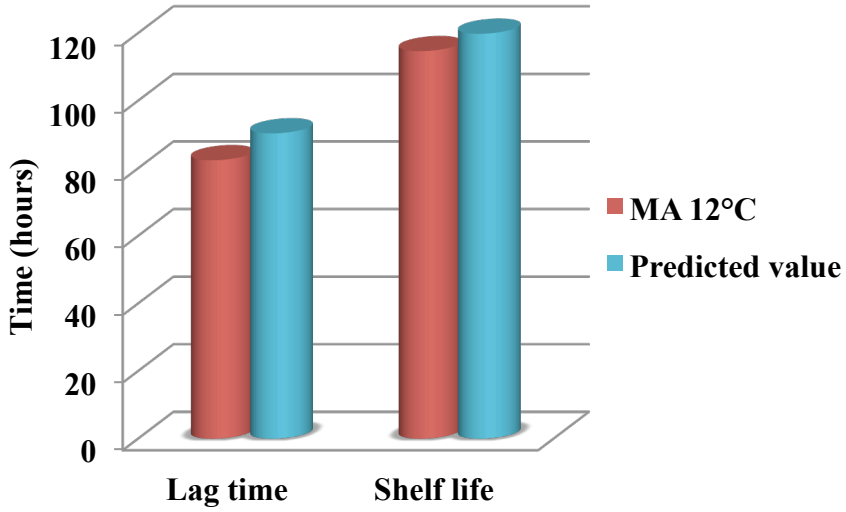

Figure 31: Model validation for lag time and shelf life with MA alone at 12°C

Most of our models showed very good correlation coefficient with R-squared (R^2) value close to 1, except the lag phase predictive model without MA treatment (R^2 = 0.87). However, the R^2 value of 0.87 was still acceptable for a linear regression analysis. Since the predictive models developed in this study were empirical, the high R^2 values could imply that our models were able to fairly well predict lag time and shelf life within the designed parameter ranges. The comparison of predicted and experimental values in Figure 25, 26, 28, 29 showed narrow 95% confident zones, which also indicated that the predicted values were quite close to the corresponding experimental values.

In addition, the validation experiments were conducted to confirm the reliability of our linear regression models. Two validation samples were treated within designed parameter ranges, 9 μL/L AIT alone and MA alone treatment at 12° C. The biases between experimental and predicted values were within 10%, excluding the lag phase predictive model without MA treatment. The high percent error of lag phase predictive model

without MA treatment, with a value of 24%, could be caused by the relatively low fitness of linear regression model ($R^2 = 0.87$), so further investigation might be necessary.

6. CONCLUSIONS

6.1. Conclusion on antimicrobial effect of gas phase AIT

This study suggested that AIT was an effective antimicrobial agent to significantly inhibit the growth of *P. aeruginosa* because it provided 1.5 - 3 times longer shelf life of fresh catfish fillet than the controls. The study also demonstrated that the lag phase of *P. aeruginosa* and shelf life of fresh catfish was extended with the increasing gaseous AIT concentration. In other words, higher gaseous AIT concentration showed more effective antimicrobial effect on *P. aeruginosa*.

6.2. Conclusion on antimicrobial effect of MA

MA showed similar antimicrobial capability as 36 μg/L AIT treatment at lower storage temperature (8 and 15°C), but its antimicrobial effect at higher temperature (> 20°C) was less effective in this study. The more effective antimicrobial effect of MA at low temperature condition is due to more CO_2 can dissolve into moist surface of fresh catfish fillet at low temperature condition which can created an acidic environment to inhibit the growth of microorganism.

6.3. Conclusion on antimicrobial effect of AIT and MA combination

The combination of gaseous AIT and MA (49% CO_2, 0.5% O_2 and 50.5% N_2) created positive combination effect on *P. aeruginosa* and further extended the shelf life of fresh catfish fillets. At 8°C, 18 μg/L AIT with the aid of MA showed 32% longer shelf life than the sum of shelf life of AIT and MA alone which also extended the shelf life of catfish fillets from 4 days to more than 23 days (550 hours). At 15 and 20°C, although the

combination effect was not effective as low storage temperature, the combinations still extended at least 2.6 times longer shelf life of fresh catfish fillet than the controls.

Therefore, AIT combination with MA can be a potential candidate as an antimicrobial packaging system for seafood industry to extend the shelf life of their fresh catfish products.

6.4. Conclusion on predictive models

In this study, polynomial models were developed to predict the lag phase of *P. aeruginosa* and the shelf life of fresh catfish under different storage conditions. The surface plot of predictive models supported the previous result in this study that higher gaseous AIT concentration with low storage temperature provided better antimicrobial effect, and AIT combined with MA obviously extend the lag phase at lower storage temperature.

Our models reasonably well predict the lag phase of *P. aeruginosa* and the shelf life of fresh catfish fillet with AIT and MA treatment under abuse temperature conditions. The result can help food industry predict the growth behavior of *P. aeruginosa* and further optimize the usage of AIT in a MA packaging system.

7. FUTURE WORK

The antimicrobial effect of gaseous AIT on *P. aeruginosa* has been proved in this study. To expand the application of gaseous AIT, the antimicrobial effectiveness of AIT on other spoilage or pathogenic microorganisms including molds or *Clostridium botulinum* etc. should be investigated in the future. In addition, incorporating AIT with other preservation technologies such as controlled release packaging can also broaden its application. Controlled release packaging can prolong the effect of AIT in a packaging system thus improving antimicrobial effect. Although the antimicrobial effect of AIT improved with its increasing concentration, the pungent odor of AIT might not be accepted by every consumer, so sensory test needs to be conducted to optimize the range of acceptable AIT concentration.

Only one gas mixture was used in this study for MA treatment. However, different gas mixtures need to be tested, because microorganisms have different susceptibility of oxygen; different microorganism should also be tested under MA systems to define the optimum gas mixture.

Since the AIT and MA combination treatment could effectively extend the shelf life of fresh catfish fillet to 23 days, chemical oxidation might become a major concern after such a long period of storage. Therefore, the addition of antioxidant such as tocopherol or ascorbic acid should be investigated for improving the quality of fresh catfish fillet.

REFERENCES

1. National Agricultural Statistic Service N and Agricultural Statistic Board USDoA, USDA, Catfish production. 2012.
2. Tryfinopoulou P, Tsakalidou E, and Nychas G-JE. Characterization of Pseudomonas spp. Associated with Spoilage of Gilt-Head Sea Bream Stored under Various Conditions. *Applied and Environmental Microbiology*, 2002; **68**: 65-72.
3. Gram L and Dalgaard P. Fish spoilage bacteria-problems and solutions. *Current Opinion in Biotechnology*, 2002; **13**(3): 262-266.
4. Davis HK, **Fish and Shelfish** in Principles and Applications of Modified Atmosphere Packaging of Foods. 2 ed. 1998, *Blackie Academic*: London, UK.
5. Floros JD and Matsos KI, **Introduction to modified atmosphere packaging**, in Innovations in Food Packaging. Han JH, Editor. 2005, *Elsevier*. p. 159-172.
6. Gram L and Huss HH. Microbiological spoilage. of fish and fish products. *International Journal of Food Microbiology*, 1996; **33**(1): 121-137.
7. Bruton MN. Alternative life-history strategies of catfishes. *Aquatic Living Resources*, 1996; **9**: 35-41.
8. With a view to fisheries- catfish, the basics. Available from: http://www.fisheriesmanagement.co.uk/catfish/catfish_introduction.htm.
9. Nelson JS. Fishes of the world. 2006: *John wiley & sons. Inc*.
10. Catfish-Wikipedia. Available from: http://en.wikipedia.org/wiki/Catfish.
11. Hoke ME, et al. Stability of Washed Frozen Mince from Channel Catfish Frames. *Journal of food science*, 2000.
12. National Agricultural Statistic Service N and Agricultural Statistic Board USDoA, USDA, Catfish processing. 2012.
13. National Agricultural Statistic Service N and Agricultural Statistic Board USDoA, USDA. Catfish processing. 2011.
14. National Agricultural Statistic Service N and Agricultural Statistic Board USDoA, USDA. Catfish feed deliveries. 2011.
15. Garner KS. Effect of carbon monoxide muscle quality of spanish mackerel.2004,University of Florida:
16. Jos H.J and Veld Hit. Microbial and biochemical spoilage of foods: an overview. *International Journal of Food Microbiology*, 1996; **33**(1): 1-18.
17. Baird-Parker TC. The Production of Microbiologically Safety and Quality of Foods. The Production of Microbiologically Safe and Satble Foods. 2000, Gaithersburg, MD: *Aspen Publishers Inc*. 3-18.
18. Ghaly AE, et al. Fish Spoilage Mechanisms and Preservation Techniques: Review. *American Journal of Applied Sciences*, 2010; **7**: 846-864.
19. Liston J. Microbiology in Fishery Science. Advances in Fishery Science and Technology, ed. Connell JJ. 1980, Farnham. 138-157.
20. Parry RT. Principles and Applications of Modified Atmosphere Packaging of Food. 1993, London: *Blackie Academic & Profesional*.
21. Dalgaard P, Gram L, and Huss HH. Spoilage and shelf-life of cod fillets packed in vacuum or modified atmospheres. *International Journal of Food Microbiology*, 1993; **19**(4): 283-294.

22. Kim CR, et al. Extending Shelf Life of Refrigerated Catfish Fillets Using Sodium Acetate and Monopotassium Phosphate. *Journal of Food Protection*, 1995; **58**(6): 644-647.
23. Mukundan MK, Antony PD, and Nair MR. A review on autolysis in fish. *Fisheries Research*, 1986; **4**(3-4): 259-269.
24. FAO. Post-harvesr changes in fish. FAO Fisheries and aquculture department 2005; Available from: http://www.fao.org/fishery/topic/12320/en.
25. Callow EH, The autolysis of the muscle of the cod fish. 1924: Camberidge.
26. Ramanathan L and Das NP. Studies on the control of lipid oxidation in ground fish by some polyphenolic natural products. *Journal of Agricultural and Food Chemistry*, 1992; **40**(1): 17-21.
27. Hwang KT, et al. Fatty acid composition and oxidation of lipis i korean catfish. *Journal of the American Oil Chemists Society*, 2003; **81**(2): 123-127.
28. Audley MA, Shetty KJ, and Kinsella JE. Isolation and properties of phosphilase A from pollock muscle. *Journal of Food Science*, 1978; **43**: 1771-1775.
29. Everse J and Hsia N. The toxicities of natice and modified hemoglobins. *Free Radical Biology & Medicine*, 1997; **22**(6): 1075-1099.
30. Nychas GJE, **Natural antimicrobials form plants.**, in New Methods of Food Preservation. 1995, *Blanckie Academic and Professional* London. p. 58-89.
31. Lopez P, et al. Solid- and Vapor-Phase Antimicrobial Activities of Six Essential Oils: Susceptibility of Selected Foodborne Bacterial and Fungal Strains. *Journal of Agricultural and Food Chemistry*, 2005; **53**(17): 6939-6946.
32. Nguyen VT, Gidley MJ, and Dykes GA. Potential of a nisin-containing bacterial cellulose film to inhibit Listeria monocytogenes on processed meats. *Food Microbiology*, 2008; **25**(3): 471-478.
33. Nedorostova L, et al. Antimicrobial properties of selected essential oils in vapour phase against foodborne bacteria. *Food Control*, 2009; **20**(2): 157-160.
34. Matan N, et al. Antimicrobial activity of cinnamon and clove oils under modified atmosphere conditions. *International Journal of Food Microbiology*, 2006; **107**(2): 180-185.
35. Delaquis PJ and Mazza G. Antimicrobial Properties of Isothiocyanates in Food Preservation. *Food Technology*, 1995; **49**: 73-84.
36. Fenwick GR, et al. Glucosinolates and their breakdown products in food and food plants. *Critical Reviews in Food Science and Nutrition*, 1982; **18**(2): 123-201.
37. Delaquis PJ and Sholberg PL. Antimicrobial Activity of Gaseous Allyl Isothiocyanate. *Journal of Food Protection*, 1997; **60**: 943-947.
38. Allyl isothiocyanate. Available from: http://en.wikipedia.org/wiki/Allyl_isothiocyanate - cite_note-Ullmann-3.
39. Romanowski F and Klenk H, **Thiocyanates and Isothiocyanates, Organic**, in Ullmann's Encyclopedia of Industrial Chemistry. 2000, *Wiley-VCH Verlag GmbH & Co. KGaA*.
40. Sara B. Essential oils: their antibacterial properties and potential applications in foods- review. *International Journal of Food Microbiology*, 2004; **94**(3): 223-253.
41. Corbo MR, et al. Thymol and modified atmosphere packaging to control microbiological spoilage in packed fresh cod hamburgers. *International Journal of Food Science & Technology*, 2009; **44**(8): 1553-1560.

42. Sivertsvik M, Jeksrud WK, and Rosnes JT. A review of modified atmosphere packaging of fish and fishery products – significance of microbial growth, activities and safety. *International Journal of Food Science & Technology*, 2002; **37**(2): 107-127.
43. Daniels JA, Krishnamurthi R, and Rizvi SSH. A Review of Effect of Carbon Dioxide on Microbial Growth and Food Quality. *Journal of Food Protection*, 1984; **48**(6): 532-537.
44. Lampila LE, **Modified atmosphere-packaging** in Microbiology of marine food products. Ward DR and Hackney C, Editors. 1991, *Van Nostrand Reinhold* New York. p. 373-393.
45. Koutsoumanis K. Predictive Modeling of the Shelf Life of Fish under Nonisothermal Conditions *Applied and Environmental Microbiology*, 2001; **67**(4): 1821-1829.
46. Koutsoumanis K and Nychas G-JE. Application of a systematic experimental procedure to develop a microbial model for rapid fish shelf life predictions. *International Journal of Food Microbiology*, 2000; **60**(2,Äì3): 171-184.
47. Dalgaard P. Modelling of microbial activity and prediction of shelf life for packed fresh fish. *International Journal of Food Microbiology*, 1995; **26**(3): 305-317.
48. Laycock RA and Regier LW. Pseudomonads and achromobacters in the spoilage of irradiated haddock of different preirradiation quality. *Applied Microbiology*, 1970; **20**(3): 333-341.
49. Boziaris IS, Kordila A, and Neofitou C. Microbial spoilage analysis and its effect on chemical changes and shelf-life of Norway lobster (Nephrops norvegicus) stored in air at various temperatures. *International Journal of Food Science & Technology*, 2011; **46**(4): 887-895.
50. FDA. Agency Response Letter GRAS Notice No. GRN 000180. 2006.
51. Korb KA and Chism GW. A Rapid Method for Determining Allylisothiocyanate in Horseradish-Containing Products. *Journal of Food Science*, 1989; **54**(3): 778-779.
52. Baranyi J and Roberts TA. A dynamic approach to predicting bacterial growth in food. *International Journal of Food Microbiology*, 1994; **23**: 277-294.
53. Knoche W, **Chemical reactions of CO^2 in water.** , in Biophysics and physiology of carbon dioxide. C. Bauer GGHB, Editor. 1980, *Springer-Verlag*: Berlin. p. 3-11.
54. Silva JL and White TD. Bacteriological and Color Changes in Modified Atmosphere-Packaged Refrigerated Channel Catfish. *Journal of Food Protection*, 1994; **57**: 715-719.
55. Jiménez SM, et al. Spoilage microflora in fresh chicken breast stored at 4 °C : influence of packaging methods. *Journal of Applied Microbiology*, 1997; **83**(5): 613-618.
56. Tsigarida E, Skandamis P, and Nychas GJE. Behaviour of Listeria monocytogenes and autochthonous flora on meat stored under aerobic, vacuum and modified atmosphere packaging conditions with or without the presence of oregano essential oil at 5 °C. *Journal of Applied Microbiology*, 2000; **89**(6): 901-909.

57. Ryan TP, **Modern experimental design**, in Design with more than two levels. 2007, *Wiley*. p. 248-290.
58. Sheen S, Hwang C-A, and Juneja VK. Modeling the impact of chlorine on the behavior of Listeria monocytogenes on ready-to-eat meats. *Food Microbiology*, 2011; **28**(5): 1095-1100.