Synergistic activity of Atlantic cod (*Gadus morhua* L.) piscidin with other antibiotic agents

Pongsaton Juntarut

Thesis for the degree of

Master of Science in Aquaculture

Faculty of Biosciences and Aquaculture

Bodø University College

May 2010

Acknowledgements

I am thankful to Dr. Jorge Fernandes, the best main supervisor, for all the help and kindness he has extended in the conduct of this research. I would also like to thank the members of my master's thesis committee; Ms Jareeporn Ruangsri, Dr. Kiron Viswanath, Dr. Monica Brinchmann for all the helpful advices in the improvement of my research. I would like to thank Jack-Asgard Bruun (University of Tromsø, Norway) for his help on the mass spectrometry analysis. I am also acknowledging the help of Ingvild Berg and Renate Karlsen (Bodø University College, Norway) in purchasing my chemicals and for arranging the needed equipment. I am also thankful to all the staff at the Faculty of Biosciences and Aquaculture, especially to the members of the Aquatic Animal Health and Welfare Group (Bodø University College, Norway) for helping me in one way or the other. I am also acknowledging the MUCO-COD Project for the financial support of this study. Lastly, I am extending my appreciation to the Norwegian Government for this master's scholarship.

Faculty of Biosciences and Aquaculture,

Bodø University College

18 May 2010

Pongsaton Juntarut

Abstract

Disease management is crucial in the aquaculture industry and to date it involves the usage of antibiotics to treat and prevent disease, especially bacterial infections. However, there is a growing concern over the problems caused by conventional antibiotics in aquatic and terrestrial environments (eg, bacteria resistant to multiple antibiotics). Therefore, it is crucial to reduce the usage of conventional antibiotics or find alternative novel antibacterial agents to manage bacterial diseases and antibiotic-resistant bacteria. Antimicrobial peptides are natural antibiotics produced from multicellular organisms and have the ability to kill bacteria and to synergise with other antibiotics. In this study I have analysed the *in-vitro* antibacterial activity of synthetic Atlantic cod piscidin and its ability to permeabilize the bacterial membrane. Moreover, I have investigated the antimicrobial properties of synthetic cod piscidin in combination with several conventional antibiotics, including oxolinic acid, oxytetracycline hydrochloride, and sulfadiazine/trimethoprim, which are amongst the most important groups of antibiotics for prevention of bacterial diseases in aquaculture. Five test bacteria, including two Gram-positive bacteria (*Micrococcus lysodeikticus* and *Planococcus citreus*) and three Gram-negative bacteria (*Yersinia ruckeri* VI 3629, *Vibrio anguillarum* VI-F-258-3 and *Aeromonas salmonicida* NCIMB 1102) were tested for synergistic activity of peptide-antibiotic combination. Antibacterial activity results showed moderate synergism i) between oxolinic acid and synthetic cod piscidin against *V. anguillarum* VI-F-258-3; ii) combined oxytetracycline hydrochloride and synthetic cod piscidin against *V. anguillarum* VI-F-258-3 and *A. salmonicida* NCIMB 1102; iii) and combined sulfadiazine/trimethoprim and synthetic cod piscidin against *M. lysodeikticus*, *A. salmonicida* NCIMB 1102, *Y. ruckeri* VI 3629. In contrast, no synergistic activity of either test antibiotics with synthetic cod piscidin was found against *P. citreus*. The data show that synthetic cod piscidin can reduce the concentration of conventional antibiotics required to inhibit bacterial growth of fish pathogenic bacteria, namely *Y. ruckeri* VI 3629, *V. anguillarum* VI-F-258-3 and *A. salmonicida* NCIMB 1102. Flow cytometry analysis revealed that this peptide could form stable pores in the bacterial membrane, which might be its main mechanism of action. These properties of synthetic cod piscidin highlight its potential an novel antibacterial agent that in a not so distant future may be used in disease control management in commercial aquaculture systems.

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Chapter 1. Introduction

1.1. Status of cod farming

1.1.1. Aquaculture industry

Atlantic cod (Fig. 1) is a marine cold-water fish species, which is demersal and distributed in the North Atlantic from inshore regions to deeper waters. This fish is omnivorous and while young fish feed on plankton and small invertebrates (such as crustaceans, polychaetes, and echinoderms), adults feed on invertebrates, fish and occasionally seaweeds (Cohen et al., 1990).

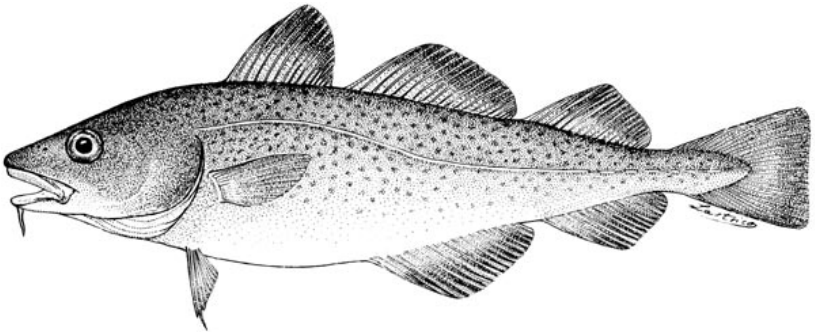


Figure 1. Atlantic cod, *Gadus morhua* Linnaeus 1758 (FAO, 2010)**.**

Nowadays, Atlantic cod is important for the aquaculture industry, especially in Norway, where it is a relatively new commercially farmed species (Grave et al., 2008). According to FAO, the global Atlantic cod capture has decreased steadily since 1968 until 2007 while its global aquaculture production has increased from 2001 until 2007 (Fig. 2) (FAO, 2010). Moreover, Norwegian aquaculture production of Atlantic cod has increased during the period 2000-2005 along with the usage of antimicrobial agents used in Norwegian aquaculture of Atlantic cod (Grave et al., 2008).

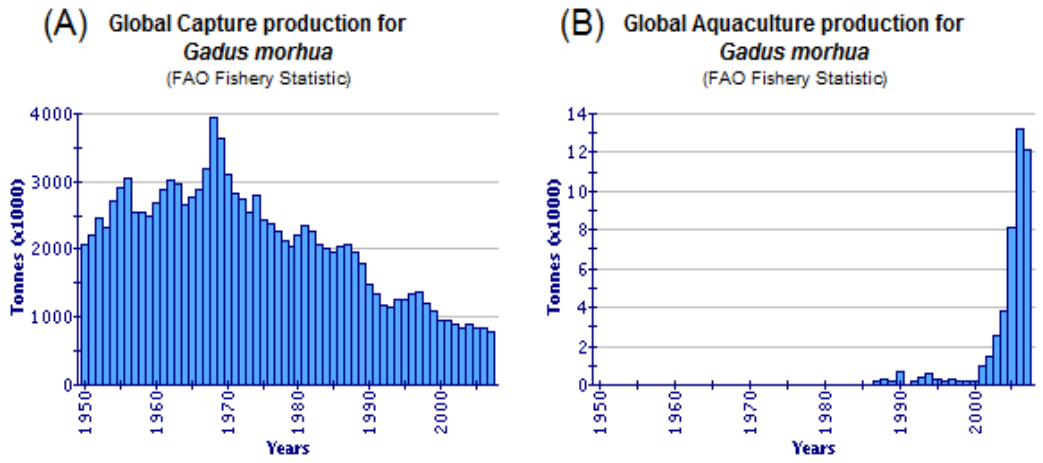


Figure 2. The global capture (A) and aquaculture production (B) for Atlantic cod (*Gadus morhua*) (FAO, 2010)**.**

1.1.2. Atlantic cod diseases

Atlantic cod farming has traditionally been affected by bacterial diseases caused by two dominant pathogens, including *Vibrio* sp. and *Aromonas* spp. (Samuelsen et al., 2006). Vibriosis is the most serious diseases that caused by *Vibrio* sp. (eg. *Vibrio anguillarum*). Head and eyes are the main target organs of infection and this disease has clinical signs such as fin erosion and haemorrhages. *Aeromonas salmonicida,* the causative agent of furunculosis in Atlantic salmon, also infects Atlantic cod. The clinical signs include haemorrhages and necrosis of several organs, such as gills and skin (Samuelsen et al., 2006). Importantly, francisellosis is a new serious bacterial disease affecting Atlantic cod in Norway (Nylund et al., 2006). This disease is caused by *Fracisella sp*. and is characterized by haemorrahagic nodules in the skin and swollen kidney and spleen (Olsen et al., 2006). Therefore, antimicrobial agents (e.g., antibiotics) are necessary to manage the pathogenic bacteria invading Atlantic cod in aquaculture and preventing disease outbreaks that cause huge economic losses.

1.2. Antibiotics

The most common compounds used in health management in aquaculture are antibiotics. An antibiotic is a biologically or synthetically produced substance (Graslund and Bengtsson, 2001) that has the ability to destroy bacteria or inhibit the bacterial growth (Sapkota et al., 2008). There are many different common groups of antibiotics including tetracyclines, quinolones, sulphonamides, aminopyrimidines, and amphenicols that are applied in aquaculture worldwide (Grave et al., 2008; Samuelsen et al., 2006; Schmidt et al., 2000). In particular, oxytetracycline, oxolinic acid, and combination of sulfadiazine with trimethoprim are the most commonly used antibiotics in Norwegian aquaculture (Sapkota et al., 2008). These antibiotic agents can protect cultured fish from bacterial infections to some extent but nowadays there are growing concerns with antibiotic-resistant bacteria. This issue has intensified to search for novel antimicrobial agents that can be used as alternative to antibiotics to treat and prevent bacterial diseases, whilst having less or no negative effect to other organisms in the environments surrounding aquaculture system.

1.2.1. Main groups of antibiotics used in aquaculture and their mechanisms

1.2.1.1. Quinolones are the group of antibiotics most commonly used in aquaculture. In particular, oxolinic acid is one antibiotic in this group that is widely applied to treat and prevent bacterial diseases in cultured commercial fish. It is a weak acid soluble in alkaline solution (Samuelsen et al., 1992) that is of synthetic origin and mainly used against Gram-negative bacteria, which it destroys by inhibiting their DNA-metabolism (Graslund and Bengtsson, 2001). These antibiotics binding to complexes between DNA and gyrase or topoisomerase IV, leading to changes in the enzyme conformation.

The resulting quinolone-gyrase-DNA complex then inhibits DNA replication (Hawkey, 2003).

1.2.1.2. Sulfonamides belong to an antibiotic group that is usually used in combination with trimethoprim against both Gram-negative and Gram-positive bacteria by a two step mechanism. In the first step, they impair tetrahydrofolate synthesis by inhibiting p-aminobenzoic acid, which leads to a decrease in nucleotide levels and in the second step they inhibit dihydrofol acid reductase, ultimately resulting in inhibition of bacterial growth (Graslund and Bengtsson, 2001; Vitale et al., 2003).

1.2.1.3. Tetracyclines are normally used in aquaculture to control bacterial diseases in salmonids, namely furunculosis. Oxytetracycline is usually used in aquaculture, since it has broad-spectrum activity against a wide range of Gram-negative or Gram-positive bacteria. These antibiotics inhibit protein synthesis (Graslund and Bengtsson, 2001) by protecting the aminoacyl tRNA and the ribosomes of bacteria (Chopra and Roberts, 2001).

1.2.2. The negative effect of antibiotics

1.2.2.1.Antibiotic-resistant bacteria

Normally antibiotic-resistant bacteria arise by DNA mutations as well as by horizontal gene transfer mechanisms such as conjugation, conjugative transposition (Cabello, 2006) and transduction with other bacteria (Sapkota et al., 2008). For example, oxolinic acid works by constraining the DNA-metabolism of bacteria but unfortunately oxolinic acid-resistant bacteria are able to repair the damaged DNA due to resistance mutations (Graslund and Bengtsson, 2001). Plasmid-mediated and plasmid-transmitted resistance (Graslund and Bengtsson, 2001) are common for sulfonamides and tetracycline, and trimethoprim resistances genes are found in plasmids of both Gram-negative and Gram-positive bacteria (Sorum and L'Abee-Lund,

2002). Moreover, it has been reported that oxytetracycline-resistance plasmids can be transferred from fish pathogenic bacteria (*Aeromonas*) to human *Escherichia coli* (Rhodes et al., 2000). This is a very clear example of how resistance genes from antibiotics resistant bacteria in aquatic environments can be transferred to terrestrial bacterial, which might lead to antibiotic resistant bacteria in the terrestrial environment.

1.2.2.2. The antibiotics residues accumulation

The leaching of antibiotics from uneaten feed, excretion of actives metabolites, and excessive usage of antibiotics can be deposited in sediments on the bottom of the pond, river or sea bed and lead to accumulation of antibiotics residues for long periods of the time. As the antibiotics residues diffuse into the sediment, some of them can be digested by aquatic organisms but the remaining antibiotic residues can induce antibiotic-resistant bacteria amongst the normal microflora in the sediment (Cabello, 2006). Antibiotic residues accumulate not only in the sediment but also distribute throughout the water column. These residues are in fact rich nutrients for growing plankton. The rapid growing of plankton due to high nutrient uptake leads to eutrophication, inducing changes in the ecological equilibrium (eg, producing an anoxic environment that has a negative impact to aquatic and terrestrial organisms (Chopra and Roberts, 2001)). In another study of antibiotic residues conducted in Norway, oxolinic acid residues have been detected in liver, plasma, and muscle of wild fish and crab; the source of this antibiotic residues was the medication administered in fish farms (Samuelsen et al., 1992). From these supporting reports, antibiotics residues from aquaculture activity in water column and sediment have negative impact directly to wild fauna and flora in aquatic environment. Therefore, it is necessary to find alternative antibiotics to use in the aquaculture industry.

1.3. Antimicrobial peptides and their mode of action

Antimicrobial peptides are natural antibiotics produced by multicellular organisms, including plants, insects, amphibians, teleosts and mammals (McCafferty et al., 1999) (Fig. 3). They are low molecular weight compounds that consist of less than 100 amino acids (Smith and Fernandes, 2009) and they have frequently been proposed as alternative anti-infective agents (Hancock, 1997). Antimicrobial peptides have been isolated from different cells, tissues and secretions from vertebrates, e.g., skin of rainbow trout (Fernandes and Smith, 2002), human urine (Park et al., 2001), epidermal mucus of Atlantic cod (Bergsson et al., 2005) and mast cells of hybrid striped bass (Silphaduang and Noga, 2001). Antimicrobial peptides are crucial components in innate defense against microbes (Boman, 1998), which is an important host defense mechanism for most living organisms both in terrestrial and aquatic environments but especially for aquatic organisms, which inhabit a complex environment rich in potential pathogens. Fish possess a diverse suite of antimicrobial peptides and these may play a more substantial role in the innate immune system of fish when compared to terrestrial mammals, which have a more highly evolved adaptive immune system (Douglas et al., 2003). Fish antimicrobial peptides have been isolated from different species (Table 1) and can be grouped in five families, based on their homology, secondary structure and genomic organization, including: the cathelicidins, defensins, LEAPs, piscidins and histone-dirived peptides (Smith and Fernandes, 2009).

Table 1. Antimicrobial peptides (AMPs) isolated from different species of fishes.

AMPs	Species of fish	References
CATH-1, -2	Rainbow trout (*Oncorhynchus mykiss*)	Chang et al. (2005)
CATH-1, -2	Atlantic salmon (*Salmo salar*)	Chang et al. (2006)
Histone H1	Coho salmon (*Oncorhynchus kisutch*)	Patrzykat et al. (2001)
Histone H2A	Rainbow trout (*Oncorhynchus mykiss*)	Fernandes et al. (2002)
Histone H2B	Atlantic cod (*Gadus morhua*)	Bergsson et al. (2005)
Piscidins	Hybrid striped bass (*Morone chrysops* x *Morone saxatilis*)	Silphaduang and Noga, (2001)
Pleurocidins	Atlantic halibut (*Hippoglossus hippoglossus*)	Patrzykat et al.(2003)
Moronecidins	Striped bass (*Morone saxatilis*)	Lauth et al.(2002)
Ribosomal	Atlantic cod (*Gagus morhua*)	Bergsson et al.(2005)
Hepcidins	Winter flounder (*Pleuronectes americanus*)	Douglas et al.(2003)
Hepcidin	Hybrid striped bass (*Morone chrysops* x *Morone saxatilis*)	Lauth et al.(2005)
Parasin I	Amur catfish (*Parasilurus asotus*)	Park et al.(1998b)

Antimicrobial peptides kill microbes through different mechanisms, namely by depolarizing and permeabilizing bacterial cell membrane, disrupting cell metabolism or interfering with DNA synthesis (Smith and Fernandes, 2009). The most common mode of action of antimicrobial peptides is explained by their ability to disrupt outer membrane of Gram-positive and Gram-negative bacteria by forming channels in the cytoplasmic membrane (Fig. 4). Typically, an antimicrobial peptide containing positively charged residues has the ability to interact with the negatively charged phospholipid heads on the bacterial membranes, their hydrophobic faces directed towards the bacterial membrane interior and their hydrophilic faces pointing inwards to form a channel that leads to release of intracellular components and causing bacterial cell dead (Hancock, 1997; Hancock and Chapple, 1999; Hancock and Scott, 2000).

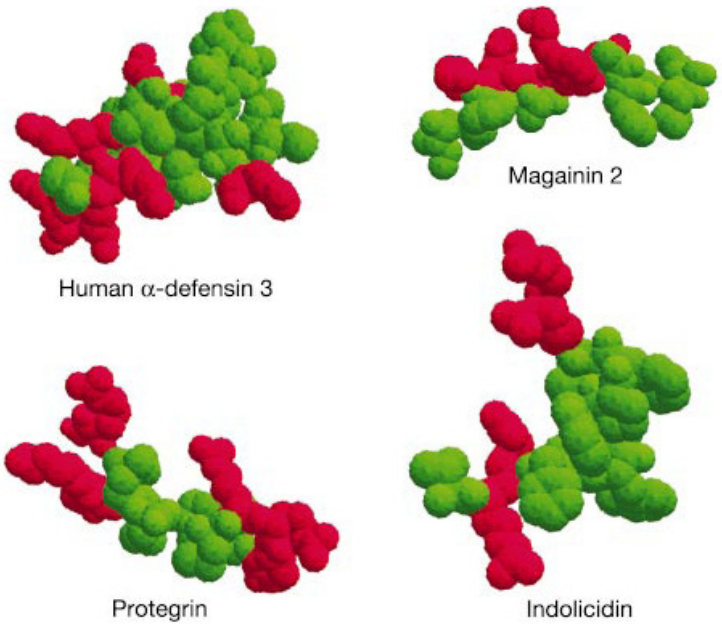


Figure 3. Several cationic antimicrobial peptides.

Red is the Basic amino acids (positively charged) are coded red, whereas hydrophobic amino acids are shown in green (Zasloff, 2002).

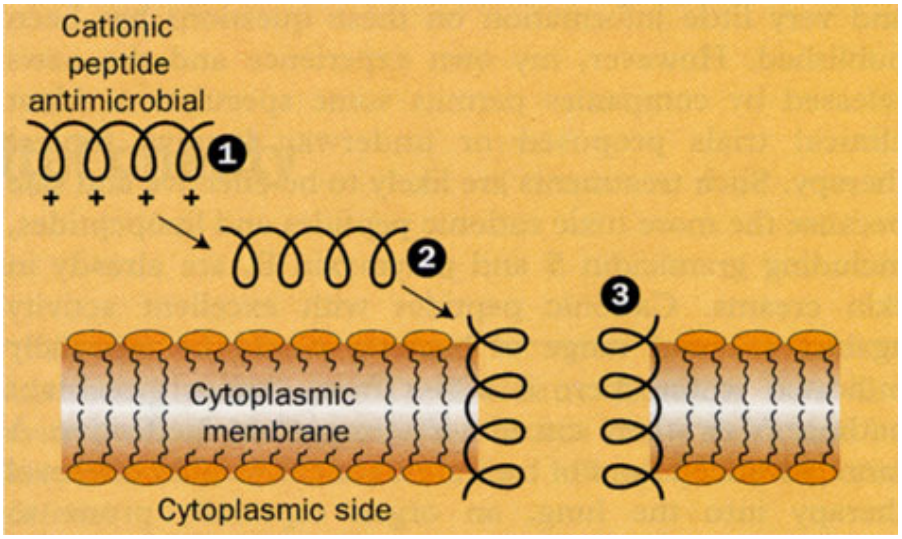


Figure 4. Mechanism of action of cationic peptides through pore formation .

Positively charged antimicrobial peptides bind the negatively charged phospholipids head groups in the bacterial cytoplasmic membrane. This interaction results in the formation of pores on the bacterial membrane, which then leads to leakage of ions and the intracellular contents of the bacterial cell (Hancock, 1997).

1.3.1. Piscidin antimicrobial peptide.

Piscidins are antimicrobial peptides composed of approximately 22 amino acid residues that have been found just only fish. They are characterized a by conserved amino-terminus rich in histidine and phenylalanine and the mature peptides have a high positive net charge at physiological pH. (Chekmenev et al., 2006; Fernandes et al., 2010; Silphaduang and Noga, 2001; Sung et al., 2008). Sun et al. (2007) reported that the mandarin fish piscidin contained four exons and three introns that coded for a prepropeptide consisting of a signal peptide 22 amino acid, 22 amino acids of a mature peptide (contained high positively charge) and 35 amino acids of a C-terminal prodomain that is similar to the moronecidin gene from hybrid striped bass (Lauth et al., 2002) (Fig. 5). There are many peptide members in this family, including pleurocidin, piscidins, chrysophsins, epinecidin-1 and dicentracin, which are grouped

as piscidins based on their similarities in genomic organization, amino acid sequence, secondary structure and phylogeny. Piscidin displays broad-spectrum activity against bacteria, fungus, protozoan parasites and even some enveloped virus (Smith and Fernandes, 2009). The piscidins are amphipathic cationic peptides that contain highly positively charge and also hydrophobic side chains in a linear α-helical structure (Fig. 6) that can be interact with the bacterial membrane through a similar mechanism to that of most antimicrobial peptides, leading to pore formation in the bacterial membrane followed by release of the intracellular contents (Fernandes et al., 2010; Noga et al., 2009). Piscidins have been found in the skin, intestine and gills (Cole et al., 2000; Cole et al., 1997; Lauth et al., 2002; Mulero et al., 2008), which are the mucosal tissues that exposed with pathogens in aquatic environment all the time. Therefore, piscidins are likely to play an important role in the first defence barrier against pathogen invasion in fish.

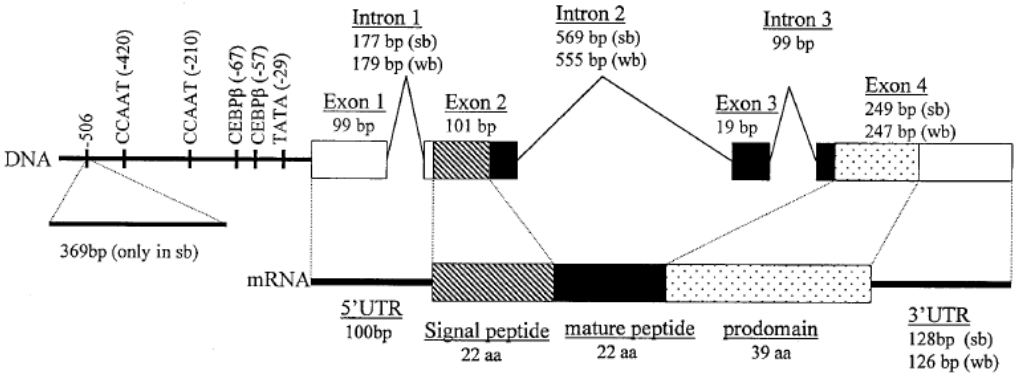


Figure 5. Bass moronecidin (piscidin) gene organization (Lauth et al., 2002).

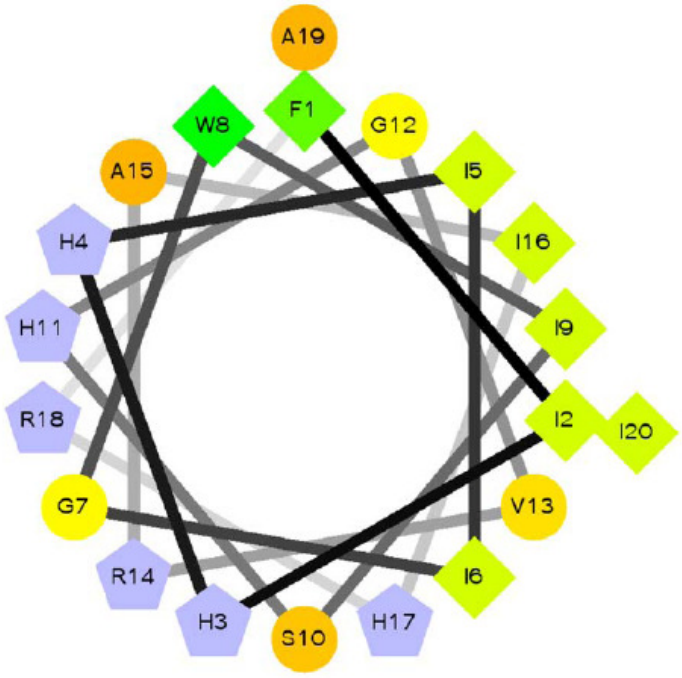


Figure 6. Amphipathic α-helical structure of piscidin from Atlantic cod (*Gadus morhua*). The hydrophilic residues are gray pentagons and hydrophobic residues are green diamonds. While the circles are neutral or polar amino acids (Fernandes et al., 2010).

1.4. Synergistic activity

Synergistic activity is defined as the combined positive interaction of two or more antimicrobial agents towards a single target molecule (McCafferty et al., 1999). The advantages of antimicrobial peptides over conventional antibiotics for therapeutic treatment are that they have broad spectrum for antibacterial, fungal and viral infection (Giacometti et al., 2000b) and they can synergise with other antibiotics (Cirioni et al., 2006; Giacometti et al., 2000b; Park et al., 2006) and antimicrobial peptides (Lauth et al., 2005; Patrzykat et al., 2001) to enhancing their antimicrobial activity . to kill multiple-resistant strains of bacteria (Marr et al., 2006). The synergistic activity is evaluated by using fractional inhibitory concentration (FIC) index,

$$FIC = FIC_A + FIC_B = [A]/ MIC_A + [B]/ MIC_B$$

Where [A] and [B] are the minimal inhibitory concentration (MIC) of drug A and drug B in the combination, MIC_A and MIC_B are the MIC of drug A and drug B alone, and FIC_A and FIC_B are the FIC of combined drug A and drug B.

The FIC index is interpreted as follow: ≤ 0.5, good synergism; 1.0, additive; and >4.0, antagonism (Yan and Hancock, 2001). Synergism is a positive interaction, inasmuch as antimicrobial activity of combined antimicrobial agents show significantly greater than their individual activity when tested separately. Additively is the result that shows antimicrobial activity of combined agents show no significant interaction when compared with individual activity when tested separately. On the other hand, antagonism is a negative interaction that antimicrobial activity of combined antimicrobial agents shows significantly less than their individual activity when tested separately. (Amsterdam, 2005).

This study focused on the synergistic potential of combining synthetic cod piscidin and conventional antibiotics to enhance their antibacterial activity *in vitro*

1.5. Main objectives

1. To purify and characterize synthetic cod piscidin.

2. To determine its spectrum of activity.

3. To investigate the kinetics of killing of synthetic cod piscidin.

4. To determine its haemolytic properties against cod erythrocytes.

5. To investigate if synthetic cod piscidin can disrupt the bacterial membranes through pore formation.

6. To ascertain if synthetic cod piscidins can potentiate the activity of other antibacterial compounds.

Chapter 2. Synthetic piscidin from Atlantic Cod (*Gadus morhua* L.) and its potential

2.1. Materials and methods

2.1.1. Sequence analysis

The protein sequences of teleosts piscidins were obtained from Gene bank of National Center for Biotecnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/) and then aligned with the ClustalW (http://align.genome.jp/) and performed the multiple sequence alignment by using BioEdit. Nine protein sequences of piscidins from difference teleost species (Table 2) including Atlantic cod were constructed the phylogenetic tree by Maximum likelihood using ATGC: Montpellier bioinformatics platform (PhyML, http://www.atgc-montpellier.fr/phyml/) and performed the phylogenetic tree by MEGA 4.

Table 2. The Gene bank of protein sequences of piscidin from teleost fishes that were obtained from NCBI database.

Teleost species	Gene	Gene bank
Gadus morhua	Piscidin	ACS91329.1
Morone chrysops x *Morone saxatilis*	Piscidin 3	P0C006.1
Epinephelus fuscoguttatus	Piscidin-like peptide	ADE06665.1
Epinephelus coioides	Piscidin-like peptide	ACE78291.1
Epinephelus akaara	Piscidin-like peptide	ACE78290.1
Larimichthys crocea	Piscidin-like peptide	ACE78289.1
Siniperca chuatsi	Moronecidin	AAV65044.1
Morone chrysops	Moronecidin	AAL40409.1
Morone saxatilis	Moronecidin	Q8UUG0.1

2.1.2. Peptide synthesis

The piscidin sequence, FIHHIIGWISHGVRAIHRAIHG was characterized from Atlantic cod by Ms Jareeporn Ruangsri, a doctoral student in Aquatic Animal Health and Welfare, Faculty of Biosciences and Aquaculture, Bodø University College (Ruangsri et al. unpublished). The synthetic cod piscidin was synthesized by Gen Script (The Biology CRO, USA) by using the mentioned sequence. The theoretical molecular weight was 2527 Da.

2.1.3. Peptide purification

2.1.3.1. High performance liquid chromatography

The synthesized peptide was chromatographed by C_{18} reversed-phase HPLC using 0.1% trifluoroacetic acid (TFA) in distilled water (solution A) and 0.1% TFA in acetonitrite (solution B). The synthesized peptide was dissolved in 0.1% TFA in distilled water and filtered with 0.2 μm pore filter (Whatman International Ltd, England) before purified by HPLC. One hundred microliter of sample was injected onto the column and eluted with solution A and solution B at a flow rate of 1 ml/min. The column was washed and equilibrated with both buffer solutions until no peptides remained in the column that was detected by absorbance at 280 nm. After HPLC, the interested fractions were lyophilized by freeze dryer until the solution was removed and then dry material was resuspended in 0.01% acetic acid in distilled sterile water and kept in -80°C until used for sodium dodecylsulfate polyacrylamind gel electrophoresis (SDS-PAGE) and antibacterial activity assays.

2.1.4. Sodium dodecylsulfate polyacrylamind gel electrophoresis (SDS-PAGE)

The synthetic cod piscidin purification was modified from Fernandes and Smith (2002) base on Schagger and Vonjagow (1987) by using SDS-PAGE with the Tris-Tricine system, 16% separating gel, 14% spacer gel, and 5% stacking gel (the gel preparation is followed Table 3). The three gel layers were separated following separating gel high approximately 4.5 cm, spacer gel 1.5 cm, and stacking gel 1 cm.

The purified peptide each interested fraction was prepared by mixing with protein loading buffer (0.61 % (w/v) Tris, 4% (w/v) SDS, 12% (w/v) glycerol, 0.01% (w/v) bromophenol blue, 2% (v/v) β-mercaptoethanol, pH 6.8), purified peptide : protein loading buffer = 1 : 1 and then denatured by heating at 95 °C for 5 min. The low molecular weight protein marker (New England BioLab, USA) in range 3.4-212 kDa was used. Anode (2.42 % (w/v) Tris, pH 8.90) and cathode (1.21 % (w/v) Tris, 1.79% (w/v) Tricine (BDH), 0.1% (w/v) SDS, pH 8.25) buffer were put in the electrophoresis chambers (BioRad, USA). Six microliter of marker was added into the first well and followed by the 20 µl of complex sample in stacking gel. The gels were run in a Mini-PROTEIN® Tetra cell electrophoresis (BioRad, USA) approximately for 2.30 h. (until the space between sample and end of separating gel was 1 cm) at constant current of 100 volt. The gels were stained with the coomassie staining (0.25 % (w/v) Coomassie brilliant blue R250, 45 % (v/v) methanol and 10 % (v/v) glacial acetic acid) for 30 min and following by immersed in destaining solution (25 % (w/v) methanol and 7.5 % (w/v) acetic acid) until show clearly blue band of protein marker in the gel.

Table 3. The details of polyacrylamind gel preparation.

Compositions	Separating gel	Spacer gel	Stacking gel
40 % Acrylamind:Bis (29:1), Accugel (ml)	3.1	0.77	0.62
Gel buffer (ml) (36.33% (w/v) Tris, 0.3 % (w/v) SDS, pH 8.45)	2.5	1.0	1.55
Water (ml)	0.65	1.23	4.08
80% (w/v) Glycerol (ml)	1.25	-	-
10% (w/v) APS (µl)	75	30	100
TEMED (µl)	7.5	3.0	10.0
Approximately total volume	7.5	3.0	6.25

This gel preparation for 16% separating gel, 14% spacer gel and 5% stacking gel that enough for 2 gels (separating gel 4.5 cm, spacer gel 1.5 cm, and stacking gel 1 cm) (Fernandes, 2002).

2.1.5. MALDI-TOF MS

The purified peptide fractions were collected based on antibacterial activity assay (two layer radial diffusion) of active fractions and approximately molecular mass by SDS-PAGE polyacrylamind gel electrophoresis (the theoretical molecular weight was 2527 Da). The molecular mass of collected fractions were determined by MALDI Micro MX (Waters/Micromass) using a nitrogen laser of 337 nm at the University of Tromsø, Norway. Each sample was mixed 1:1 with α-cyano-4-hydroxycinnamic acid. Glufibrinopeptide B was used as internal standard for mass adjustment (peptide mass, 1570.67 Da).

2.1.6. Protein quantification

The purified synthetic cod piscidin was measured the concentration by using a Quant-iT^{TM} protein assay kit (Invitrogen, U.S.A) following the manufacturer's protocol. Briefly, the working solution was prepared by diluting the Quant-iT^{TM} reagent 1:200 in Quant-iT^{TM} buffer. Three standards solution were used for calibrations; 10 µl of each standard solution was mixed with 190 µl of working solution in 0.5 ml PCR tube. Ten microliters of sample was added in 0.5 ml PCR tube and followed by adding 190 µl of working solution. The standards and samples were incubated for 15 min at room temperature and then the standards were equilibrated and followed by determining of protein concentration of the samples by Qubit^{TM} fluorometer (Invitrogen, U.S.A).

2.1.7. Antibacterial activity

2.1.7.1. Test bacteria

2.1.7.1.1. Culture condition

The various 12 bacterial strains including 4 Gram-positive bacteria and 8 Gram-negative bacteria (Table 5) were kindly given by Ms Jareeporn Ruangsri, a Ph.D. student in Aquatic Animal Health and Welfare at Bodø university college, Norway. All test bacterial strains were cultured under appropriate condition based on Fernandes et al. (2002) and Ruangsri et al. (In press). All bacterial strains were cultured in trypticase soy broth (TSB) (Merck KGaA, Darmstadt, Germany) or trypticase soy agar (TSA) (Fluka Analytical, Switzerland), supplemented with NaCl (Merck KGaA, Darmstadt, Germany) as appropriate that were prepared following the manufacturer's protocol (Table 4) and incubated at appropriate temperature which is showed in Table 5.

Table 4. The preparation of trypticase soy broth and agar.

Ingredients	Trypticase Soy Broth		Trypticase Soy Agar	
	Supplemented with 0.85% NaCl	Supplemented with 1.5% NaCl	Supplemented with 0.85% NaCl	Supplemented with 1.5% NaCl
Distilled water (ml)	1,000	1,000	1,000	1,000
Trypticase Soy Broth (g)	30	30	30	30
NaCl (g)	8.5	15	8.5	15
Agar (g)	-	-	15	15

Directions: All ingredients are suspended in glass bottle and sterilized at 121 °C for 15 minutes by autoclaving, cool to 40-45 °C and poured in sterile Petri dishes for trypticase soy agar. The broth and agar media were kept in 4°C until use.

Table 5. List of the bacteria, identification code, and their culture conditions.

Bacteria	Gram	Culture media+%NaCl/temperature condition
Micrococcus lysodeikticus	+	Trypticase soy agar or broth+0.85% NaCl / 37^{o}C
Planococcus citreus	+	Trypticase soy agar or broth +1.5% NaCl / 20^{o}C
Staphylococcus aureus ATCC 9144	+	Trypticase soy agar or broth+0.85% NaCl / 37^{o}C
Corynebacterium glutamicum ATCC 13032	+	Trypticase soy agar or broth+0.85% NaCl / 25^{o}C
Escherichia coli ATCC 25922	-	Trypticase soy agar or broth+0.85% NaCl / 37^{o}C
Vibrio anguillarum NCIMB 2133	-	Trypticase soy agar or broth +1.5% NaCl / 20^{o}C
Vibrio anguillarum VI-F-258-3	-	Trypticase soy agar or broth +1.5% NaCl / 20^{o}C
Vibrio anguillarum	-	Trypticase soy agar or broth +1.5% NaCl / 20^{o}C
Aeromonas salmonicida NCIMB 1102	-	Trypticase soy agar or broth +1.5% NaCl / 20^{o}C
Yersinia ruckeri NCIMB 2196	-	Trypticase soy agar or broth +1.5% NaCl / 20^{o}C
Yersinia ruckeri VI 3629	-	Trypticase soy agar or broth +1.5% NaCl / 20^{o}C
Psychrobacter immobilis	-	Trypticase soy agar or broth +1.5% NaCl / 20^{o}C

2.1.7.1.2. Growth curve determination

The bacterial strains in the Table 5 were sub-cultured onto TSA (suplemented NaCl as appropriate) under aseptic technique and incubated overnight at optimal temperature. Then several freshly colonies were picked and inoculated in the Erlenmeyer flasks (100 ml) containing 50 ml of TSB (supplemented NaCl as appropriate). The flasks were incubated at appropriate temperature condition (Table 5) with constant shaking for 200 rpm. The suspended bacterial cells in TSB were determined the optical density at 600 nm (Perni et al., 2005) by using spectrophotometer each indicated times after incubation until the growth curve of each bacteria reach to stationary phase. The optimal density each indicated times were performed in triplicate and then the average values were plotted with particular times to show the growth curve of each bacterial strain.

2.1.7.1.3. Cell density determination

In this experiment, all 12 bacterial stains (Table 5) were determined density (CFU/ml) by plating on TSA (supplemented NaCl as appropriate) at different concentration of bacterial suspension based on optical density at 600 nm. Drop plate method was modified from Herigstad et al. (2001) and applied to determine the bacterial cell density in this experiment. The bacteria on TSA from a freshly overnight culture were inoculated into the Erlenmeyer flasks (100 ml) that contained 50 ml of TSB and incubated at appropriate temperature (Table 5) with constant shaking for 200 rpm until reach to exponential phase. Then, the bacterial suspension in TSB was varied concentrations by diluting in the same medium and followed by measuring optimal density at 600 nm. Ten-fold serial dilution was made from each concentration of bacteria suspension with TSB. Thirty microliters of diluted bacterial suspension was plated on the TSA (supplemented NaCl as appropriate) for triplicate, overnight

incubated at optimal temperature. The bacterial colonies on agar plate in range 30-300 colonies were considered as standard number for counting under a phase contrast stereo microscope. The counted bacterial cells were calculated as the number of colony forming unit (CFU) per milliliter of culture media after correcting for dilutions. The bacterial cell density (CFU/ml) at particular optical density at 600 nm was plotted to show the relationship and the linear regression equation was obtained. Finally the bacterial density at 10^8 CFU/ml at optical density 600 nm was calculated by using linear regression equation which is the relationship of bacterial density (CFU/ml) and optical density at 600 nm.

2.1.7.2. Antimicrobial assay by two layer radial diffusion for selecting active fraction of synthetic cod piscidin after purification by HPLC

The two-layer radial diffusion method was modified from Smith et al. (2000) used for first screen of antibacterial activity of purified synthetic cod piscidin of each interested fractions after HPLC purification. The Gram-positive bacterium *P. citreus* was used for testing antibacterial activity of purified peptide in this assay. Briefly, 144 cm^2 square petri-dish was added 14 ml bacterial underlayer, consisted 1.5% NaCl, 1% bactotryptone, 0.5% yeast extract and 1% agarose in the distilled water and 2×10^6 CFU/ml of washed bacterium cells (log phase), and the underlayer was holed for 3 mm diameter by sterile pipette. Three microliters of interested fraction of the purified peptide was added into each well for duplicates and the control was 0.01 % acetic acid in sterile distilled water which is the diluent of sample. The plate was incubated at 4°C for 3 hours and then covered with 14 ml upperlayer, contained 6% TSB, 1.5% NaCl and 1% agarose and then incubated at 20 °C for 12-24 hours. The diameter of clear zone area (mm^2) in the underlayer agar was indicated antibacterial activity of each well.

2.1.7.3. Antibacterial activity by microtitre broth dilution assay for determination of minimal inhibitory concentrations (MICs) and plating for minimal bactericidal concentration (MBC) of purified synthetic cod piscidin

2.1.7.3.1. Preparation of bacterial cells for antibacterial activity assays

Twelve bacterial strains in Table 5 were prepared for the antibacterial activity assays of purified antibacterial peptide. Briefly, the freshly overnight bacterial colonies on TSA were inoculated in 50 ml of TSB, contained 1.5% or 0.85% NaCl as appropriate and incubated at optimal temperature (Table 5) with constant shaking 200 rpm until reached to exponential phase. The bacterial suspension was transferred into 50 ml sterile centrifuge tube and centrifuged (Heraeus Labofuge 400R centrifuge, USA) at 4,500 rpm for 10 min and washed by sterile saline water (approximately 1.5% or 0.85% NaCl depend on bacteria). The bacterial pellet was cleaned and resuspended in sterile saline water and centrifuged at 4,500 rpm for 10 min again. After the last centrifuge, the supernatant was drained and then bacterial pellet was resuspended in Mueller-Hinton broth (MHB) (Merck KGaA, Darmstadt, Germany) (the preparation is showed in Table 6 following manufacturer's protocol) that contained NaCl as appropriate. The resuspended bacterial cells in Mueller-Hinton broth was determined the optical density at 600 nm that corresponding to the bacterial density at 10^8 CFU/ml by using the spectrophotometer. Then bacterial density at 10^8 CFU/ml was diluted by Mueller-Hinton broth (supplemented NaCl as appropriate) for 10^5 CFU/ml that ready to use for MIC and MBC determination.

Table 6. The preparation of Mueller-Hinton broth.

Ingredients	Mueller-Hinton Broth	
	Supplemented 0.85% NaCl	Supplemented 1.5% NaCl
Distilled water (ml)	1000	1000
Mueller-Hinton broth (g)	21	21
NaCl (g)	8.5	15

Directions: All ingredients are suspended in glass bottle and sterilized by autoclaving at 121 °C for 10 minutes, cool to 40-45 °C and stored in 4 °C and protected from direct light.

2.1.7.3.2. Preparation of purified synthetic cod piscidin solution

The preparation of synthetic cod piscidin for determination of minimal inhibitory and bactericidal concentration was modified from Noga et al. (2009) Briefly the dry material of synthetic cod piscidin after freeze drying was resuspended in 0.01% acetic acid and determined the concentration by using a Quant-iT^{TM} protein assay kit (Invitrogen, U.S.A). Finally synthetic cod piscidin was diluted to desired concentration in 0.2% (w/v) bovine serum albumin/0.01% (v/v) acetic acid and stored in -80 °C until use.

2.1.7.3.3. Determination of minimal inhibitory concentrations (MICs) of purified synthetic cod piscidin

Determination of minimal inhibitory concentrations (MICs) of synthetic cod piscidin various 12 bacteria was performed by using a microtitre broth dilution assay in 96 microtitre plate (Becton Dickinson, France) that was modified from Fernandes et al. (2002). In this experiment the MIC of synthetic cod piscidin was defined as the lowest synthetic cod piscidin concentration that inhibits bacterial growth by 50% compared to the positive control (bacterial cells without peptide). Bacteria suspension in logarithm

phase of growth was prepared following 2.1.7.3.1. Ninety microliter of suspended bacterial cells in Mueller-Hinton broth (MHB), contained 10^5 CFU/ml was added into each well of sterile 96 well-microtitre plate for triplicate and followed by adding with 10 µl of a two-fold serial dilution of synthetic cod piscidin, diluted in 0.2% (w/v) bovine serum albumin/0.01% (v/v) acetic acid (Noga et al., 2009). The plate was incubated at the appropriate temperature (Table 5) until the optical density at 540 nm (Microplate reader, Fluostar optima, BMG Labtech GmbH, Offenburg, Germany) reached 0.2 in the positive control well, contained 90 µl of suspended bacteria and 10 µl of synthetic cod piscidin diluent. In addition, three negative controls were included: i) 90 µl of MHB and 10 µl of synthetic cod piscidin diluent, ii) 90 µl of MHB and 10 µl of synthetic cod piscidin solution, and iii) 100 µl of MHB only.

2.1.7.3.4. Determination of minimal bactericidal concentration (MBC) of purified synthetic cod piscidin

All 12 bacteria in Table 5 were determined the minimal bactericidal concentration (MBC), performed as described by Fernandes and Smith (2002). Briefly, 90 µl of washed bacteria containing approximately 10^5 CFU/ml in Mueller-Hinton broth (MHB) (supplemented 0.85 % or 1.5 % NaCl as appropriate) were added to each well in a 96-well microtitre plate and then 10 µl of two-fold serial dilution of synthetic cod piscidin was added. The control well contained 90 µl of suspended bacterial cells in MHB and 10 µl of synthetic cod piscidin diluents. Three negative controls were performed: i) 90 µl of MHB and 10 µl of synthetic cod piscidin diluent, ii) 90 µl of MHB and 10 µl of synthetic cod piscidin solution, and iii) 100 µl of MHB. All sample and controls were performed for triplicate. The microtitre plates were incubated in optimal temperature (Table 5) for 24 h and then the samples were plated on TSA (supplemented 0.85% or 1.5% NaCl as appropriate) and incubated at the appropriate

temperature (Table 5). The MBC was considered as the lowest concentration of synthetic cod piscidin that prevented colony forming on trypticase soy agar plates after incubation for 24 h.

2.1.8. Haemolytic activity

In the haemolytic activity of peptides was followed modification from Fernandes et al. (2002). Freshly blood (syringes were coated with peparin 150 IU/ml of blood) from Atlantic cod was washed with 10 mM phosphate buffered saline (PBS; 0.9 % (w/v) NaCl, pH 7.4) in the sterile centrifuge tube by centrifugation to remove the leucocytes (buffy coat) and plasma (supernatant). The cleaned erythrocytes are deposited in bottom of centrifuge tube was resuspended in PBS and packed by centrifugation at 800 g for 10 min at 4 °C, and then 2 % (v/v) packed cell volume of cod erythrocytes were diluted in PBS that ready to test with peptide. Three antimicrobial peptides; synthetic cod piscidin, cecropin P1, and melittin were diluted to give a range of concentrations from 0.2-1.4 µM. Eleven microliters of each concentration of each peptide was added to 100 µl of a 2 % packed cod erythrocytes in PBS and incubated at 37 °C for 30 min. The controls were performed: i) negative controls, 100 µl of 2 % packed cod erythrocytes in PBS and 11 µl of PBS, ii) positive controls, 100 µl of 2 % packed cod erythrocytes and 11 µl of 0.2 % (v/v) Triton X-100. All the samples and controls were centrifuged at 1000 g for 5 min at room temperature. One hundred microliters of the supernatant from each sample was diluted with 800 µl of PBS and measured the absorbance at 540 nm (Microplate reader, Fluostar optima, BMG Labtech GmbH, Offenburg, Germany). The percentage of haemolytic activity of peptide was calculated from the ratio of absorbance of sample and positive control, (Absorbance$_{540\ nm}$ of sample/Absorbacne$_{540\ nm}$ of positive control)×100.

2.1.9. Cytoplasmic membrane permeabilization assay

The membrane permeability assay was modified from Park et al. (2006) and Bunthof et al. (2001). A commercial viability and counting kit, the LIVE/DEAD *Bac*Light kit was used (Invitrogen, USA). Two molecular DNA stains SYTO 9 (green fluorescence) can pass intact cell membranes and propidium iodide (PI) (red fluorescence) can only enter through permeabilized cell membranes. The bacterial cells stained by the fluorescent dyes were analysed by flow cytometry (Cell counter, Beckman Coulter, USA) to show the fluorescence intensity of untreated and synthetic cod piscidin treated bacterium cells. Gram-positive bacterium *P. citreus* was selected to test in this assay. Briefly, bacterium colonies were picked from freshly overnight culture on TSA plates and inoculated in flasks, contained 50 ml of TSB (supplemented 1.5% NaCl), and incubated at 20 °C until reaching to exponential phase. Bacterium cells were harvested by centrifugation, washed with sterile saline (supplemented 1.5% NaCl) and resuspended in MHB (supplemented 1.5% NaCl). Ninety microliter of bacterial suspension containing 1×10^6 CFU/ml in MHB were mixed with 10 µl of different concentrations of synthetic cod piscidin, while 10 µl of MHB was added in 90 µl of bacterial suspension was considerate as control. All samples and control were incubated at 20 °C for 1 h, followed by adding 100 µl of working solution (1.5 µl of SYTO 9, 1.5 µl of PI, and 497 µl of sterile saline 1.5% NaCl) of LIVE/DEAD *Bac*Light bacterial viability and counting kit and incubated at room temperature for 15 min. Then the fluorescence intensity of samples and control was determined by a flow cytometer (Cell Lab Quanta, Beckman Coulter, USA).

2.1.10. Kinetic assay

The kinetic study, inhibiting rate of bacterium cell growth after incubation with synthetic cod piscidin was performed by fluorescence labeling using the commercial LIVE/DEAD *Bac*Light bacterial viability and counting kit (Invitrogen, USA) following method 2.1.9. analysed by flow cytometry (Cell lab Quanta, Beckman Coulter, USA). Briefly, *P. citreus* cells at exponential phase were washed with sterile saline 1.5 % NaCl and resuspended in MHB (supplemented 1.5% NaCl) to 10^6 CFU/ml. 90 µl of bacterium suspension were mixed with 10 µl of synthetic cod piscidin at 0.08 µM to against *P. citreus*, while the control was 90 µl of bacterium suspension and 10 µl of MHB. All samples and control were incubated at 20°C until the indicated times and then incubated with an equal volume of working solution (2.1.9.) of LIVE/DEAD *Bac*Light bacterial viability and counting kit before analyzing the fluorescence intensity by flow cytometric analysis.

2.2. Results

2.2.1. Peptide analysis

Cod piscidin protein sequence and piscidin family member were aligned, the results showed higher conserved amino acids found at the signal peptide region amongst modern fish species than mature and prodomain regions. In contradiction, less conserved amino acids can be identified along entire sequence of cod piscidin compared to piscidin from higher teleost (Fig. 7). The phylogenetic relationships of protein sequences of teleost piscidin orthologues was reconstructed. The result showed piscidin from Atlantic cod (*Gadus morhua*) is clustered together with large yellow croaker (*Larimichthys crocea*), while they are separated from other teleosts in Acanthopterygii superorder. In addition piscidin from all three grouper species are grouped together that more relate with mandarin fish (*Siniperca chuatsi*) than bass (*Morone chrysops*, *Morone saxatilis*, and *Morone chrysops* × *Morone saxatilis*,) (Fig. 8). This result suggest that piscidin from Atlantic cod, belong to Paracanthopterygii superorder has the same ancestor with teleosts piscidins from Acanthopterygii superorder, and it showed to have high relatively with the large yellow croaker (Fig. 9).

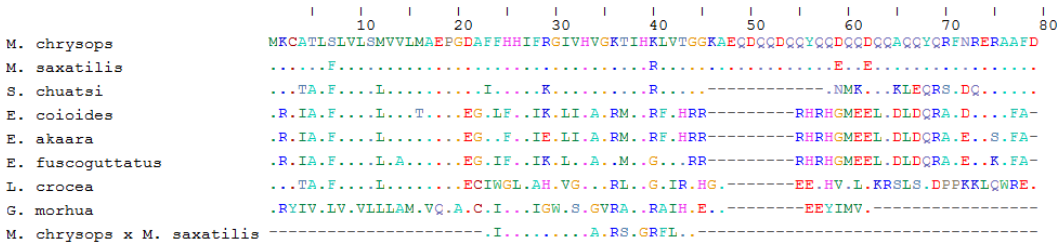


Figure 7. The ClustalW multiple sequence alignment of piscidin protein sequences from teleost. The identities are represented by dots, while dashes denote the gaps indicated to maximize alignments.

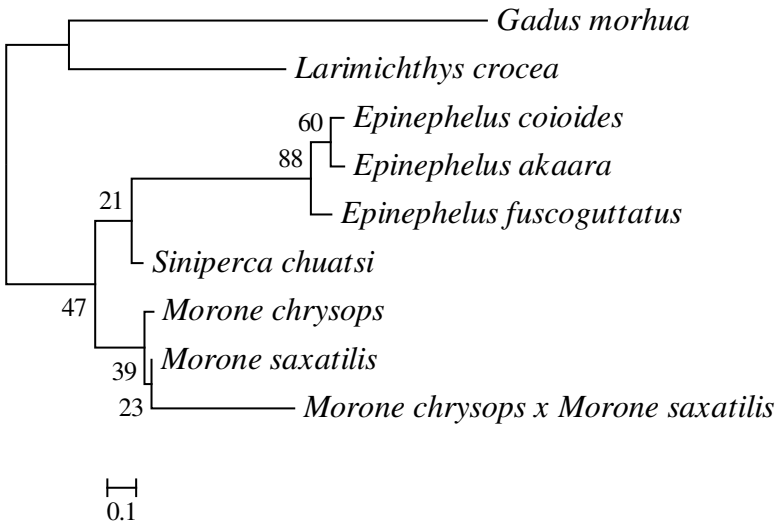


Figure 8. The phylogenetic relationship between teleost piscidins.

The phylogenetic relationship between teleost piscidins were analysed by Maximum likelihood of piscidin protein sequences using ATGC: Montpellier bioinformatics platform (PhyML) and performed the tree by MEGA 4. The number of each node indicated the percentage of bootstrapping after 100 replications and the aligned piscidin proteins sequences were showed in Fig. 7.

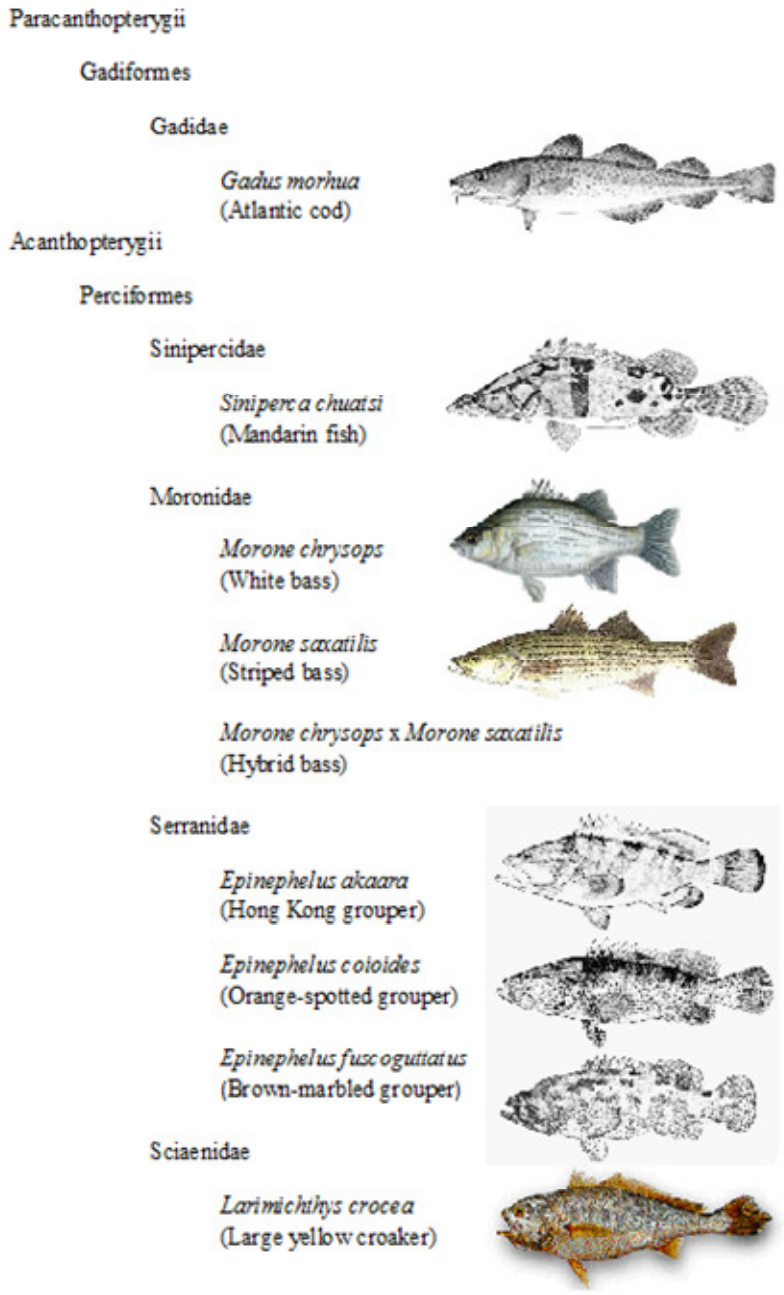


Figure 9. The taxonomic relationships of teleosts, were obtained protein sequences of piscidins. All fish pictures were obtained from FishBase; http://www.fishbase.org/ and FAO; http://www.fao.org/fishery/en).

2.2.2. Peptide purification

The synthetic peptide was purified by C_{18} reversed phase HPLC with water/acetonitrile gradient (Fig. 10, top panel), the interested fractions were collected from retention time of 35 to 50 min which are highly peaks. The fractions 35-50 were freeze dried by lyophilizing and resuspended in 0.01% acetic acid for antibacterial activity assay and SDS-PAGE. The antibacterial activity of fractions 35-50 against *P. citreus* using two layers radial diffusion assay (Fig. 10, bottom panel) was found that highly inhibition clear zone areas from retention time of 44 and 45 min. Moreover, SDS-PAGE result was performed a single band in retention time of 44 and 45 min which are indicated molecular mass below 3.4 kDa (Fig. 11) after coomassie staining. Fractions 44 and 45 of purified synthetic cod piscidin were collected to analyze molecular mass by the matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF MS) mass spectrum that showed a signal to molecular ion approximately at 2527 Da (Fig. 12) that similar to theoretical molecular weight (2527 Da) of obtained sequence, FIHHIIGWISHGVRAIHRAIHG. Finally yield of pure synthetic cod piscidin of fractions 44 and 45 were polled and quantified the concentration before determining the antibacterial activity assays.

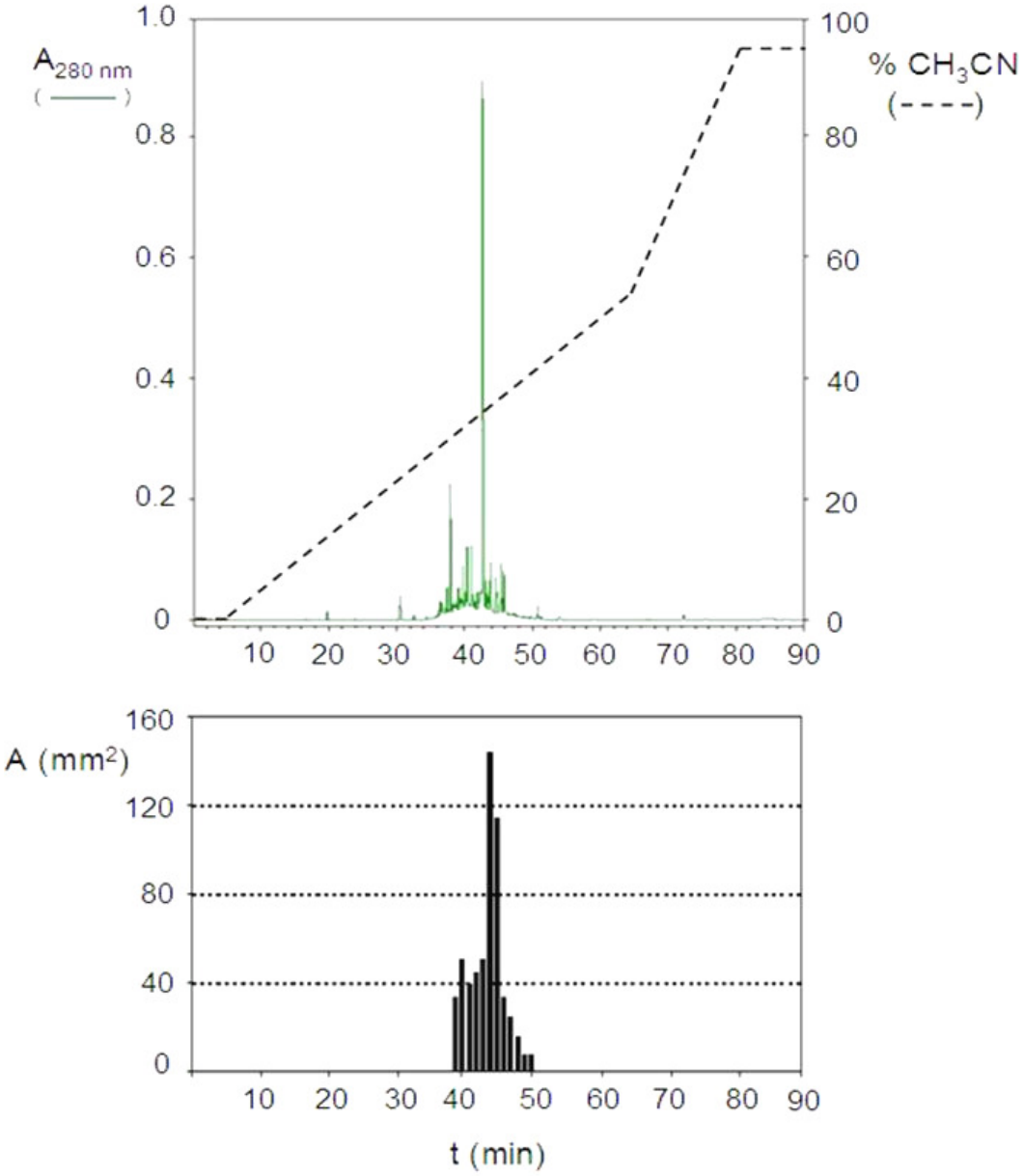


Figure 10. The purification of synthetic cod piscidin.

The purification was used C_{18} reversed-phase column for HPLC using 0.1% trifluoroacetic acid in acetonitrite and 0.1% trifluoroacetic acid in distilled water as gradient, as indicated by dotted line, and the solid line represents the absorbance at 280 nm (top panel). The bottom panel shows the antibacterial activity of the various fractions, as determined by two-layer radial diffusion assay against the Gram-positive bacterium *P. citreus*. The peak eluting at 44 and 45 min (chromatogram) had the highest antibacterial activity (histogram).

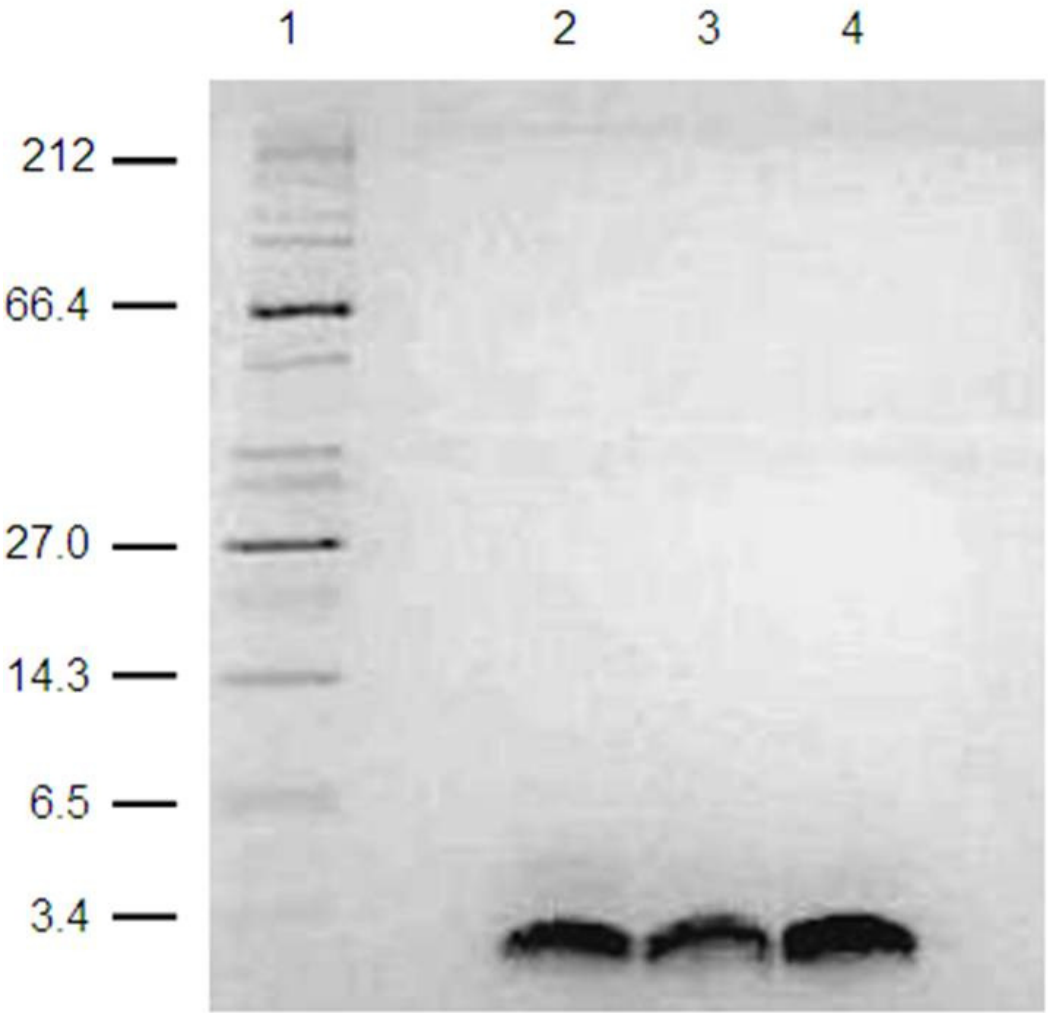


Figure 11. SDS-PAGE analysis of the active fractions of purified synthetic cod piscidin.

The active fractions were tested antibacterial activity against *P. citreus*. Lane 1: low-molecular weight protein markers; lane 2: non purified synthetic cod piscidin; lane 3: fraction 44 of purified synthetic cod piscidin by HPLC; lane 4: fraction 45 of purified synthetic cod piscidin by HPLC. Each lane contains 2.5 µl of sample, except the markers (7 µl). The numbers left side showed the molecular mass of the markers in kilodaltons (kDa).

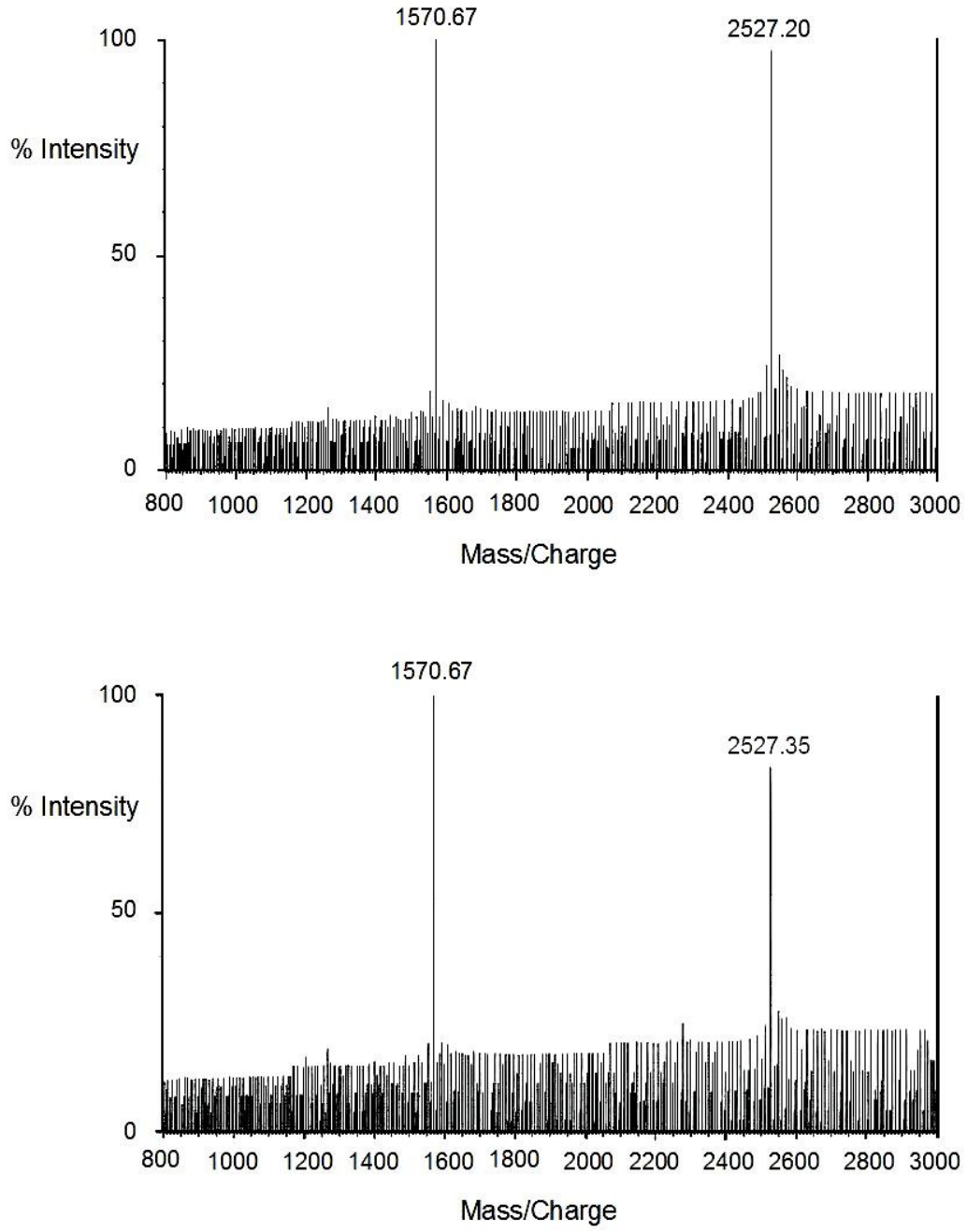


Figure 12. MALDI-TOF MS analysis of purified synthetic cod piscidin.

The fraction 44 and 45 from purified synthetic cod piscidin that are showed by monoisotopic mass result. A top picture is represented molecular mass of fraction 44 (2527.20 Da) and a bottom picture is represented molecular mass of fraction 45 (2527.35 Da). While peak at 1570.67 Da is corresponded to internal standard of mass adjustment.

2.2.3. Bacterial growth curve and their density

The preliminary study of bacterial growth determination and the determination of their density were used for antibacterial activity of antibacterial agents in this research. The investigation of bacterial growth was taken by optical density at 600 nm at indicated times (Appendex 1) during lag phase, log phase, and stationary phase of various Gram-positive and Gram-negative bacteria after incubation. The result showed the time during exponential phase of growth curve of *M. lysodeikticus*, *S. aureus* ATCC 9144, and *E. coli* ATCC 25922, incubated at 37 °C that appear short lag and log phase, the duration of their exponential phase of all three bacteria were selected at 3 hours after inoculation. While, *P. citreus* and *P. immobilis* were incubated at 20 °C showed the slow growth rate that were selected the duration of their exponential phase at 14 hours after inoculation. Whereas, The growth curve of *C. glutamicum* ATCC 13032, *V. anguillarum* NCIMB 2133, *V. anguillarum* VI-F-258-3, *V. anguillarum, A. salmonicida* NCIMB 1102, *Y. ruckeri* NCIMB 2196, and *Y. ruckeri* VI 3629 were performed the duration of their exponential phase at 8 hours after inoculation (Table 7).

Moreover, the relationship of optical density at 600 nm and bacterial density was calculated from linear regression equation, shown in Appendix 2. The results showed the optical density at 600 nm corresponding to 10^8 CFU/ml of each bacterium was provided in Table 7. Four bacteria; *M. lysodeikticus*, *P. citreus*, *C. glutamicum* ATCC 13032, and *V. anguillarum* shows optical density at 600 nm were 0.550, 1.022, 0.500, and 0.500, respectively that corresponding to 10^8 CFU/ml. For other Gram-positive and Gram-negative bacteria displayed optical density at 600 nm between rang 0.100-0.275 that corresponding to 10^8 CFU/ml.

Table 7. Bacterial strains and the duration of their exponential phase after inoculation and the optical density at 600 nm corresponding to 10^8 CFU/ml of bacterial density.

Bacterial strains	Exponential phase (h)	OD_{600} corresponding to 10^8 CFU/ml
M. lysodeikticus	3	0.550
P. citreus	14	1.022
S. aureus ATCC 9144	3	0.150
C. glutamicum ATCC 13032	8	0.500
E. coli ATCC 25922	3	0.156
V. anguillarum NCIMB 2133	8	0.200
V. anguillarum VI-F-258-3	8	0.100
V. anguillarum	8	0.500
A. salmonicida NCIMB 1102	8	0.156
Y. ruckeri NCIMB 2196	8	0.121
Y. ruckeri VI 3629	8	0.160
P. immobilis	14	0.275

The values represented as average of three replications for each sample. The growth and cell density curves were showed in Appendix 1 and 2, respectively.

2.2.4. Antibacterial activity of synthetic cod piscidin against various bacteria and its haemolytic activity against cod erythrocytes

The minimal inhibitory concentrations (MICs) and minimal bactericidal concentration (MBC) of synthetic cod piscidin against all 12 test bacterial strains were showed in Table 8. The result showed synthetic cod piscidin has more active against Gram-positive bacteria than Gram-negative bacteria with exception of *V. anguillarum* and *P. immobilis*. In addition, the MIC values of ranged between 0.04-5 μM of synthetic cod piscidin were performed against all four Gram-positive bacteria i.e. *M. lysodeikticus*, *P. citreus*, *S. aureus* ATCC 9144, *C. glutamicum* ATCC 13032 and two Gram-negative; *V. anguillarum*, *P. immobilis*. While the MBC were 4 times higher than MICs for *P. citreus* and 2 times higher than MICs for *C. glutamicum* ATCC 13032 and *P. immobilis*, while MBC of other tested bacteria were more than 5 μM.

The haemolytic activity of synthetic cod piscidin and cecropin P1 were showed percentage of haemolysis constantly around 30% from the lowest concentrations of 0.2 μM until highest concentrations of 1.4 μM. While, percentage haemolysis of melittin against cod erythrocytes was increased following the increasing of its concentrations, over 0.8 μM of melittin performed completely lytic for cod erythrocytes (Fig. 13). Therefore synthetic cod piscidin has less haemolytic effect than melittin which has a strong haemolystic activity against cod erythrocytes.

Table 8. The minimal inhibitory and bactericidal concentrations of synthetic cod piscidin against twelve test bacteria.

Bacterial strains	Gram	MICs (μM)[a]	MBC (μM)[b]
M. lysodeikticus	+	1.25-2.5	>5
P. citreus	+	0.04-0.08	1.25
S. aureus ATCC 9144	+	2.5-5	>5
C. glutamicum ATCC 13032	+	0.63-1.25	2.5
E. coli ATCC 25922	-	>5	>5
V. anguillarum NCIMB 2133	-	>5	>5
V. anguillarum VI-F-258-3	-	>5	>5
V. anguillarum	-	2.5-5	>5
A. salmonicida NCIMB 1102	-	>5	>5
Y. ruckeri NCIMB 2196	-	>5	>5
Y. ruckeri VI 3629	-	>5	>5
P. immobilis	-	0.63-1.25	2.5

The values were represented as average of triplicate of each sample.

[a] MIC, was defined as the lowest synthetic cod piscidin concentration that inhibits bacterial growth by 50% compared to the positive control (bacteria without peptide). MIC curves of synthetic cod piscidin against various bacteria were showed in Appendix 3.

[b] MBC, was considered as the lowest concentration of synthetic cod piscidin that prevented colony forming on TSA plates after incubation for 24 h.

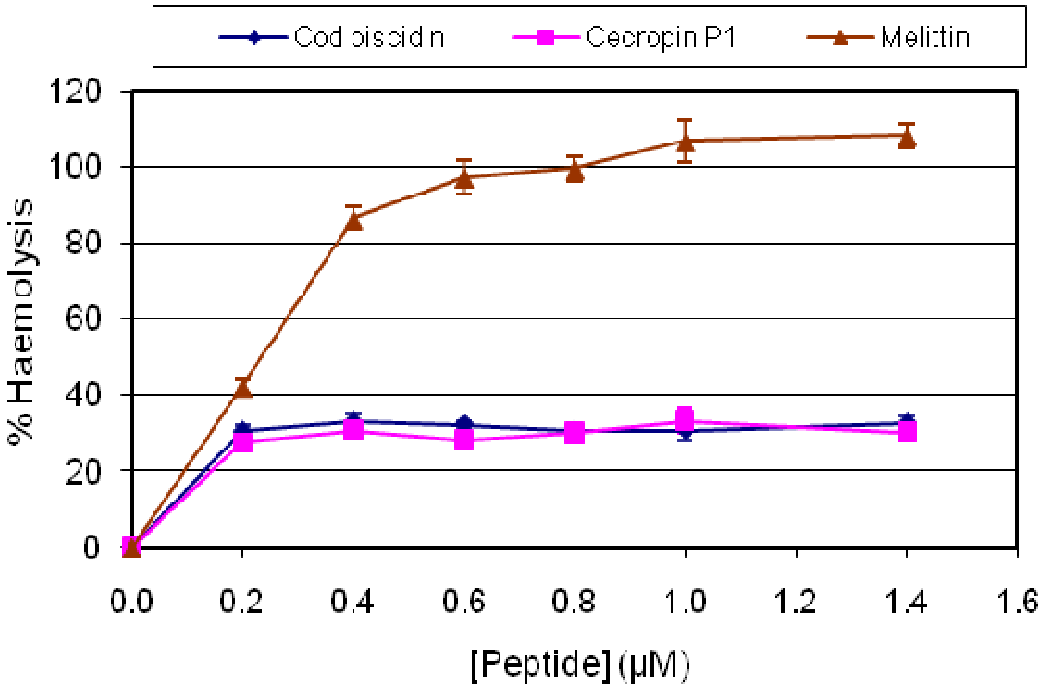


Figure 13. The haemolytic activity of peptides against erythrocytes of Atlantic cod.

The tested peptides were diluted to desired concentrations and incubated with a 2% (v/v) of cod erythrocytes for 30 min at 37 °C and performed for triplicate. Values were represented as means±standard error (vertical bars), n=3.

2.2.5. Permeabilization of bacterium cells treated by synthetic cod piscidin

Gram-positive bacterium *P. citreus* cells were treated with synthetic cod piscidin with two difference concentrations of 0.08 μM (upper value of MICs interval) and 1.25 μM (MBC). The result of treated bacterial cells with synthetic cod piscidin (0.08 μM and 1.25 μM) was observed fluorescence intensity of internalized PI (florescent red) in bacterial cells after analyzed by flow cytometric analysis. The percentages of permeable cells were increased with concentration of synthetic cod piscidin from 0.08 μM (44.45% permeabilised cells) to 1.25 μM (87.33% permeabilised cells) after incubation for 1 h. Therefore synthetic cod piscidin able to

form the pore in bacterium membranes that allowed small molecule of PI enter the bacterium cells. While, the control (bacteria without synthetic cod piscidin) was less observed PI fluorescence (1.83% permeabilised cells) in dead bacterium cells but showed higher percentage of live cells (89.19% intact cells) labeled with fluorescent green of SYTO 9 after incubation for 1 h (Fig. 14 and Fig. 15).

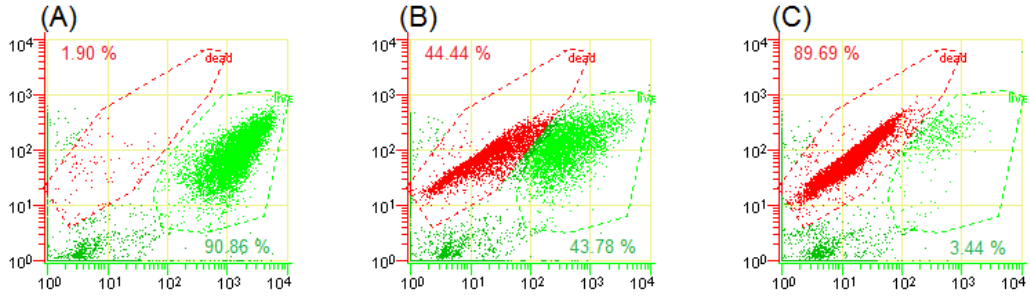


Figure 14. Permeabilization assay of synthetic cod piscidin against *P. citreus*.

This assay was observed by PI (fluorescence red labeled permeabilized cells) and SYTO 9 (fluorescence green labeled intact cells) labeling and analyzed by using flow cytometric analysis. A, control was *P. citreus* cells suspension were incubated without synthetic cod piscidin; B, *P. citreus* cells suspension were incubated with synthetic cod piscidin at 0.08 µM (upper value of MIC interval); and C, *P. citreus* cells suspension were incubated with synthetic cod piscidin at 1.25 µM (MBC). While, the particles outside the defined regions were considered as unknown.

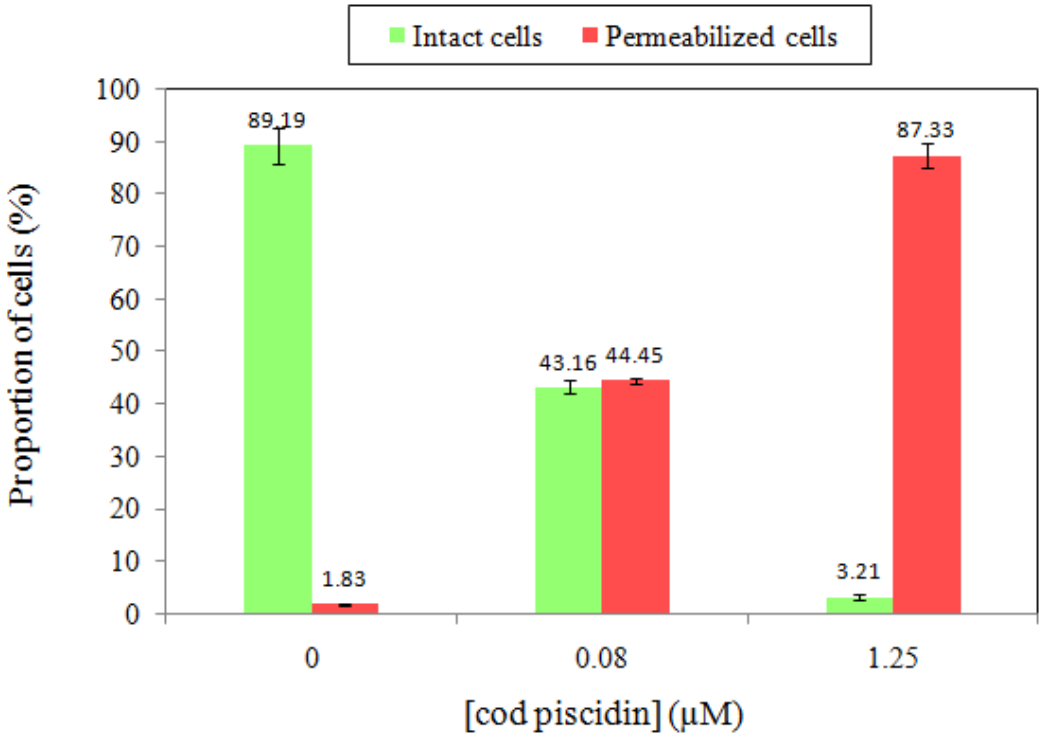
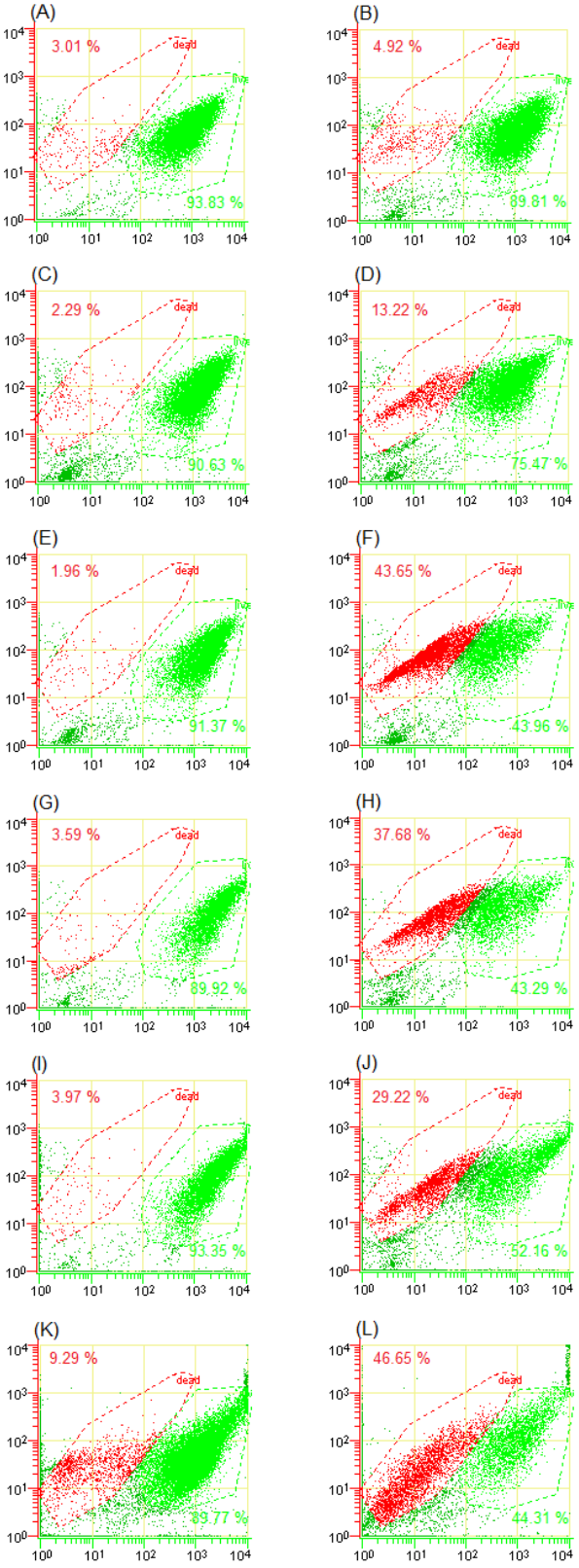


Figure 15. The average percentage of intact and permeabilized *P. citreus* cells.

The intact cells were labeled by SYTO 9 (fluorescence green), whereas permeabilized *P. citreus* cells were indicated by PI internalization (fluorescence red) after incubated at 20 °C for 1 h with two concentration of synthetic cod piscidin (0.08 and 1.25 µM). While the control was performed as test bacteria without synthetic cod piscidin (0 µM of synthetic cod piscidin) and analyzed by using flow cytometric analysis. Each sample and control was performed for triplicate, values are represented as means±standard error (vertical bars); n=3.

2.2.6. Kinetic study

The kinetic study of synthetic cod piscidin against Gram-positive bacterium *P. citreus* cells which is the highly sensitive bacterium to synthetic cod piscidin were investigated inhibiting growth rate after treated with synthetic cod piscidin at 0.08 μM (upper value of MIC interval) with increasing of incubation times (Fig. 16) by flow cytometric analysis. The results found at 0 h after incubation with synthetic cod piscidin was found percentage of live bacterium cells was around 90 % and the live bacterium cells were decreased with increasing time. After 1 h of incubation, treated bacterium cells with synthetic cod piscidin were showed constant percentage of live bacterium cells of 43.96 %, 43.29 %, 52.16 % and 44.31 % of 1, 3, 6, 24 h, respectively incubation times. While the control, untreated bacterium cells with peptide showed approximately 90 % of live bacterium cells every indicated times (0-24 h) after incubation at 20 °C.



The kinetic was analyzed by flow cytometric analysis. The pictures A, C, E, G, I, and K were untreated bacterium cells (control) at 0, 0.5, 1, 3, 6, and 24 h, respectively after incubation. While B, D, F, H, J, and L were treated bacterium cells with 0.08 µM (upper value of MIC interval) at 0, 0.5, 1, 3, 6, and 24 h, respectively after incubation. The particles outside the defined regions were considered as unknown.

Figure 16. The kinetic of synthetic cod piscidin against *P. citreus*.

2.3. Discussion

2.3.1. Sequence analysis

In this study found the piscidin from *G. morhua*, Paracanthopterygii superorder has relationships with teleost piscidins from Acanthopterygii superorder. Most of protein sequences of piscidin are taxonomic relationship within Acanthopterygii superorder with exceptions of *L. crocea* sequence that is high relatively with *G. morhua*. This result similar with Fernandes et al. (2010) that showed separation of piscidin sequences of *G. morhua* and *L. crocea* from the other teleosts. The reason that cod piscidin is different from the other teleost piscidins from Acanthopterygii superorder might be caused by longer evolutionary distance than those teleosts. However, piscidins were found in many teleost species, especially the members in Acanthopterygii superorder. In addition, nine species of teleosts from the 36 species tested were showed immunopositive with anti-piscidin 1 antibody that found in families Moronidae (*M. saxatilis*, *M. chrysops*, *M. saxatilis* × *M. chrysops*, *Dicentrarchu labrax*), Serranidae (*Epinephelus niveatus*), Sciaenidae (*Leiostomus xanthurus*, *Micropogonias undulates*), Siganidae (*Siganus rivulatus*), Belontidae (*Trichogaster leeri*), and Cichlidae (*Oreochromis niloticus*) which are the members in order Perciformes (Silphaduang et al., 2006). Moreover, Corrales et al. (2010) detected strong piscidin 4-positive cells in tissues (gill, intestine, stomach) of importance commercially fish including hybrid striped bass (*M. saxatilis* × *M. chrysops*), white bass (*M. chrysops*), striped bass (*M. saxatilis*), european seabass (*D. labrax*), and giltheaad seabream (*Sparus aurata*) by immunohistochemistry. The most of piscidins are found expression in order Perciformes of Acanthopterygii superorder, and in this study showed piscidin also was found in *G. morhua* that belong to family Gadidae of Paracanthopterygii superorder. Piscidins are present in many commercially importance

fish in aquaculture that included Atlantic cod, a new important cultured commercial fish species.

2.3.2. Peptide purification and identification

The synthetic cod piscidin was synthesized by using a sequence, FIHHIIGWISHGVRAIHRAIHG that was characterized from Atlantic cod and performed a molecular mass 2527 Da by MALDI-TOF MS analysis. The synthetic cod piscidin sequence contained 22 amino acid and showed highly histidin and isoleucine rich in its sequence that similar with 22 amino acid piscidin 1, 2, 3 were isolated from mast cells of hybrid striped bass and highly conserved histidine-rich and phenylalanine-rich (Silphaduang and Noga, 2001). Moreover, Lauth et al. (2002) discovered two isoforms of 22 amino acid moronecidin from skin and gills of hybrid striped bass that performed molecular mass 2543 and 2571 Da and highly histidine content in their sequences. While, piscidin 4 from gill of hybrid striped bass is 44 residues peptide with molecular mass 5329 Da that has highly homologous at its N-terminus to piscidin 1, 2, and 3 (Noga et al., 2009).

2.2.3. Bacterial growth curve and their density

In this study, all 12 bacteria were determined the growth curve which is the relationship between optical density at 600 nm with indicated times to select the appropriate incubation time during their exponential phage that used for antibacterial activity assays. Three bacteria including *M. lysodeikticus*, *S. aureus* ATCC 9144 and *E. coli* ATCC 25922 were incubated at 37 °C that growth faster than other bacterial which were cultured at 20 °C, while *P. citreus* and *P. immobilis* grew quite slow (Appendix 1).

The drop plate technique was applied in this study to determine the cell density, antibacterial activity of minimal bactericidal concentration and kinetic profile of

synergism. Duo to Chen et al. (2003) and Herigstad et al. (2001) performed the advantages of drop plate technique for bacterial density estimation; 1) saving the time for plating and colony counting process, 2) more accurately by counting under stereo-microscope, 3) less material cost because in one agar plate could be investigated for triplicate of each dilution, and 4) more space in incubator for the plates incubation. However, the error from drop plate technique might be caused by colonies overlapping that result to incorrect colony counting (Herigstad et al., 2001), and the pipetting during serial dilution process and dropping on agar plate also was considered as the weakness of this method.

2.2.4. Antimicrobial activity of synthetic cod piscidin

The synthetic cod piscidin has ability to inhibit the bacterial growth by i*n vitro* study using MIC and MBC estimation. The results performed synthetic cod piscidin seem to be more potential against Gram-positive bacteria (*M. lysodeikticus*, *P. citreus*, *S. aureus* ATCC 9144, and *C. glutamicum* ATCC 13032) than Gram-negative bacteria at micromolar concentration level. However, two Gram-negative bacteria including *V. anguillarum* and *P. immobilis* were inhibited *in vitro* growth by synthetic cod piscidin. Six Gram-negative bacteria including *E. coli* ATCC 25922, *V. anguillarum* NCIMB 2133, *V. anguillarum* VI-F-258-3, *A. salmonicida* NCIMB 1102, *Y. ruckeri* NCIMB 2196, and *Y. ruckeri* VI 3629 showed MIC and MBC values more than 5 µM, however if the synthetic cod piscidin is increased the test concentration then bacterial growth might be inhibited at higher than 5 µM. In this study was demonstrated the antibacterial activity of synthetic cod piscidin high potentiate against some bacteria, however there were many researches have been investigated ability of piscidins from fishes that resulted broad spectrum against bacteria, parasite, fungal and event virus.

Silphaduang and Noga (2001) isolated piscidin 1,2,3 from mast cell of hybrid striped bass that showed killing both Gram-positive and Gram-negative bacteria which are fish pathogens (e.g., *S. iniae*, *A. salmonicida*, and *V. alginolyticus*) and human pathogens (e.g., *S. aureus*, *E. coli*, *Streptococcus faecalis*, and *Pseudomonas aeruginosa*) that showed MIC in range 0.8-12.5 µg/ml, while MBC in range 1.6-25 µM. Moreover, piscidin 4 was isolated from healthy hybrid striped bass that have ability to against both fish and human pathogens e.g., *S. iniae* (MIC and MBC 12.5-25 µg/ml), *Listonella agnuillarum* (MIC and MBC 6.3 µg/ml), *S. aureus* (MIC and MBC 6.3-12.5 µg/ml) and *E. coli* (MIC and MBC 50 µg/ml) (Noga et al., 2009). Moreover Lauth et al. (2002) performed antibacterial activity of synthetic amidated moronecidin against both Gram-positive bacteria (e.g., *M. luteus* and methicilin-resistant *S. aureus* showed MIC 10-20 and 1.25-2.5 µM, respectively) and Gram-negative bacteria (e.g., *E. coli*, *A. hydrophila*, *V. cholera*, *Yersinia enterocolitica* showed MIC 5-10, >20, 2.5-5, 2.5-5 µM, respectively) that similar to antibacterial activity of bass moronecidin also showed by inhibiting bacterial growth, *S. iniae*, *E. coli*, *Y. enterocoliticus*, and *Serratia sonnei* at MIC in range 2.5-5 µM (Lauth et al., 2005). These published papers were confirmed that piscidins as a potent broad spectrum against bacteria growth by *in vitro* study. Unfutually, in this study determined the MIC and MBC of synthetic cod piscidin at maximum concentration of 5 µM that were not enough to inhibit i*n vitro* growth of most of Gram-negative bactria (*E. coli* ATCC 25922, *V. anguillarum* NCIMB 2133, *V. anguillarum* VI-F-258-3, *A. salmonicida* NCIMB 1102, *Y. ruckeri* NCIMB 2196, and *Y. ruckeri* VI 3629). This result similar to Sun et al. (2007) study that reported the antibacterial activity of synthetic amidated piscidin from mandarin fish (*S. chuatsi*) able to against many Gram-negative bacteria at more than 5 µM of piscidin concentration including *Aeromonas* (MICs 10->160 µM and MBCs 20->160 µM), *V.*

anguillarum (MICs 20-80 μM and MBCs 40-160 μM), *Y. ruckeri* (MICs 20-40 μM and MBCs 40-80 μM), and *E. coli* (MICs 5-10 μM and MBCs 10-20 μM). However in this study was found sensitive Gram-negative bacterium *P. immpbilis* was inhibited growth at MICs 0.63-1.25 and MBC 2.5 μM that can be suggested that this bacterium might have difference in cellular membranes and pathogenicity from other tested Gram-negative bacteria that performed MIC and MBC more than 5 μM.

Interestingly, piscidin was confirmed its ability to kill parasites by Coloni et al. (2008) that reported the antiparasitic activity of piscidin 2 from mast cells of hybrid striped bass able to against marine fish ectoparasites (*Cryptocaryon irritans*, *Trichodina sp*., and *Amyloodinium ocellatum*) and freshwater fish ectoparasites (*Ichthyophthirius multifiliis*), the protozoacidal concentration (PC_{min}, lowest concentration where at least one parasite died) showed against *Trichodina*, *Cryptocaryon* theront, *Amyloodinium* dinospore, and *Ichthyophthirius* theront of peptide concentration 12.5-100, 12.5, 6.3-12.5, and 6.3 μg/ml, respectively.

In addition piscidin also showed potential against fungal that was reported by Sung et al. (2008), piscidin 2 inhibit growth of human pathogenic fungal including *Malassezia furfur*, *Trichosporon beigelii*, and *Candida albicans* at MICs of 6.25, 1.56, and 6.25 μM, respectively. Moreover, moronecidin from bass also performed against filamentous fungi including *Neurospora crassa* (MICs 1.56-3.12 μM), *Aspergillus fumigates* (MICs 50-100 μM), *Fusarium oxysporum* (MICs 0.78-1.56 μM), *Fusarium culmorum* (MICs 0.39-0.78 μM), and yeast, *C. albicans*, *C. glabrata*, *C. lusitania*, and *C. tropicalis* at MICs 10-20 μM (Lauth et al., 2002).

Piscindins performed antibacterial, antifungal, antiparasitic activity, and surprisingly also showed antiviral activity property that piscidin-1N, 1H, 2, and 3 from hybrid striped bass were reported ability to reduce the infectivity of channel catfish

virus and frog virus3 at I_{50} (the concentration of piscidin that reduce viral infectivity by 50%) of 4-11 µM and 13-16 µM, respectively (Chinchar et al., 2004).

In this study demonstrated the ability of synthetic cod piscidin for antibacterial activity. Moreover, many reports were supported that piscidin has antimicrobial properties that able to against bacteria, fungal, parasite, and event virus. Therefore piscidin is appropriate to be a novel antimicrobial agent that might be applied for medication in aquaculture industry.

2.3.5. Haemolytic activity against cod erythrocytes

In this study was found synthetic cod piscidin has less haemolytic activity than mellitin, but still the same with cecropin P1. The piscidin 1, 2, and 3 from mast cells of hybrid striped bass were showed more haemolytic than magainin 2 but still less than melittin, approximately 10 µg/ml of piscidin 1, piscidin 2 and 100 µg/ml of piscidin 3 started lytic human erythrocytes (Silphaduang and Noga, 2001). While, four concentrations of bass hepcidin including 5.5, 11, 22, and 44 µM were incubated with hybrid striped bass erythrocytes at 37 °C showed no haemolysis was observed at 5.5 and 11 µM after incubation for 4 h, while 0.4-2.5 % haemolysis was showed after 3 h of incubation with 22 and 44 µM of bass hepcidin (Lauth et al., 2005). Moreover, moronecidin from hybrid striped bass has no haemolytic effect against human and sheep red blood cells below concentrations of 2.5 µM, while the concentrations over 5 µM were observed haemolysis until reach to 20-80 µM which were found 100% lytic human red blood cells, and over 10 µM also was observed haemolysis of sheep red blood cells (Lauth et al., 2002). In addition amidated and non-amidated fish piscidin 1 and 3 at concentration of 100 µg/ml was observed haemolytic effect against human red blood cells (Chekmenev et al., 2006).

2.3.6. Permeability of synthetic cod piscidin

In this study used commercial LIVE/DEAD *Bac*Light bacterial viability and counting kit to observe proportion of live and dead bacterium cells that were labeled by SYTO9 and PI and analyzed by using flow cytometric analysis. PI is a small molecule that can enters permeabilized cells and bound with double stranded nucleic acid and result red fluorescence, while intact bacterium cells were labeled fluorescence green of SYTO 9. In this study was found internal PI fluorescence in permeabilized *P. citreus* cells after incubated with 0.08 and 1.25 μM of synthetic cod piscidin that confirmed the ability of this peptide could disrupt bacteria membrane and allowed PI molecule enters inside the cells. However, some population of test bacterium cells could not be detected fluorescent labeled cells that identified as intermediate cells which might be caused by peptide still disrupting and forming the pore on bacterium cells membrane. Piscidin was demonstrated that its amphiphatic α-helical structure that contained high positively charge have potential to permeabilise bacterial membrane (Cotten et al., 2009). Cod piscidin was used in this study also has positive net charge and can be adopted the same structure (Rungsri et al. unpublished). Moreover piscidin 2 damaged fugal, *C. albicans* plasma membrane potential that was found accumulation of $DiBAC_4(3)$ (binds to lipid-rich intracellular components) in depolarized cells and also found pore forming in fungal membrane by detection fluorescent 1,6-diphenyl-1,3,5-hexatriene (associate with phospholipids within cytoplasmic membrane), lead to the releasing of intracellular cellular component and ions from the fungal cells (Sung et al., 2008). From these data were confirmed that main target of synthetic cod piscidin is the cytoplasmic membranes by pore formation which is the common mechanism of cationic antimicrobial peptides. For example, Park et al. (2006) investicated higher internalized PI fluorescence intensity of cationic antimicrobial peptide P5 treated *S.*

aureus cells than untreated and antibiotic chloramphenicol (act on inhibiting translation) treated cells by flow cytometric analysis. However, some antimicrobial peptides have no effect to cytoplasmic membrane, Buforin II could bind to DNA and RNA that lead to inhibiting cellular functions (Park et al., 1998a) and indolicidin that showed inhibiting DNA and protein synthesis (Subbalakshmi and Sitaram, 1998). Interestingly, in this study found synthetic cod piscidin at 0.08-1.25 µM could permeabilised *P. citreus* membrane that lead to bacterium growth inhibition around 44-87 % by using flow cytometric analysis. Moreover, the percentage of haemolytic activity of this peptide showed less haemolysis around 30 % against cod erythrocytes by peptide concentration in range 0.2-1.4 µM which covered the concentrations of 0.08-1.25 µM that can lysis *P. ctreus* cells for around 44-87 %.

2.3.7. Kinetic study

In this study, the bacterium growth was inhibited by synthetic cod piscidin at 0.08 µM (upper value of MIC interval) that was found the percentage of live cells were decreased from 0 h (89.81 %) to 30 min (75.47 %) and until 1 h (43.96 %) after incubation with peptide. In addition from 3, 6, and 24 h after incubation, treated bacterium cells with synthetic cod piscidin were showed constant percentage of live bacterium cells of 43.29 %, 52.16 % and 44.31, respectively. Lauth et al. (2002) observed kinetic of synthetic amidated white bass moronecidin against *S. aureus* and found 90 % of bacterium cells were killed within 1 and 10 min after incubated with 6 µM (2 times MIC) and 3 µM, respectively at 37 °C, however lower incubation temperature at 30 °C showed reducing of killing rate than at 37 °C either at 3 or 6 µM of peptide concentrations. Therefore the kinetic of antimicrobial peptides are dependent on time, peptide concentration, incubation temperature, and tested bacterial strain.

Chapter 3. Synergistic activity of synthetic piscidin from Atlantic Cod (*Gadus morhua* L.) with other antibiotic agents

3.1. Materials and Methods

3.1.1. Test bacteria

Three Gram-negative bacteria including *V. anguillarum* VI-F-258-3, *A. salmonicida* NCIMB 1102, *Y. ruckeri* VI 3629 and two Gram-positive bacteria including *M. lysodeikticus*, and *P. citreus* were selected study the synergistic activity of combined synthetic cod piscidin and antibiotic agents. All five bacteria were cultured under the appropriate conditions (Table 5) until reached to exponential phase. Suspended bacterial cells in media were washed and prepared following 2.1.7.3.1. Cleaned bacterial cells of 10^5 CFU/ml in saline Mueller-Hinton broth (supplemented NaCl as appropriate) were used for the determination of the MIC and MBC of antibacterial agents alone and in combination.

3.1.2. Preparation of antibacterial agents for antibacterial assays

Four antibiotic agents that are common use in aquaculture industry were used for synergistic activity study including: oxolinic acid, oxytetracycline hydrochloride, sulfadiazine/trimethoprim combination (combined as 5 parts of sulfadiazine to 1 part of trimethoprim) and all antibiotic agents are of commercial origin from Sigma-Aldrich. The antibiotic stock solution was prepared by using a modification from Bruun et al. (2000) based on the National Committee for Clinical Laboratory Standard (NCCLS). Briefly, the antibiotic solutions were prepared by weighting for desired

amount and dissolving into specific diluent of each antibiotic, and then mixed for homogenous solution by vortex mixer. The diluent was sterile water for oxytetracycline hydrochloride, 0.1 M NaOH for oxolinic acid and sulfadiazine, and 0.05 M HCl for trimethoprim. In addition oxolinic acid and sulfadiazine/trimethoprim solutions were adjusted the pH in range between 6-8 with 1 M NaOH and 1 M HCl. Then the final concentration of NaOH and HCl in antibiotic solutions including 0.0006 M NaOH and 0.004 M HCl for oxolinic acid, while 0.0004 M NaOH and 0.003 M HCl for sulfadiazine/trimethoprim. All antibiotic solutions were stored in 1.5 ml sterile micro tube in -80 °C.

The preparation of synthetic cod piscidin was following 2.1.7.3.2 base on Noga et al. (2009) method, briefly the synthetic cod piscidin was resuspended in 0.2% (w/v) bovine serum albumin/0.01% (v/v) acetic acid and kept in -80 °C until use for antibacterial activity test.

3.1.3. The determination of minimal inhibitory and bactericidal concentration of antibacterial agents

Determination of minimal inhibitory concentrations (MICs) and minimal inhibitory bactericidal concentration (MBC) of antibiotic agents and synthetic cod piscidin against test bacteria was followed 2.1.7.3.3 and 2.1.7.3.4 that was modified from Fernandes et al.(2002). Briefly, test bacterium cells 10^5 CFU/ml in Mueller-Hinton broth (supplemented NaCl as appropriate) was added into each well of 96 well-microtitre plates in volume 90 µl and mixed with 10 µl of two-fold serial dilutions of antibacterial agent for triplication. The positive control well, contained 90 µl of suspended bacteria and 10 µl of antibacterial agent diluent. Moreover, three negative controls were included: i) 90 µl of MHB and 10 µl of antibacterial agent diluent, ii) 90 µl of MHB and 10 µl of antibacterial agent solution, and iii) 100 µl of MHB. The

plates were incubated under appropriate condition of each test bacteria (Table 5) until the optical density at 540 nm reached to 0.2 in the positive control well. MIC was determined as the lowest antibacterial agent concentration that inhibits bacterial growth by 50% compared with the positive control.

In addition the MBC was observed by plating out from each well of 96 well-microtitre plates after incubation with antibacterial agents at appropriate temperature for 24 h on trypticase soy agar plate, contained 0.85% or 1.5% NaCl as appropriate. MBC was considered as the lowest concentration of antibacterial agent that prevented colony forming on trypticase soy agar after incubation for 24 h in appropriate temperature.

3.1.4. Synergistic activity of peptide-antibiotic combination

Synergistic study of combined synthetic cod piscidin with conventional antibiotics was determined following the modification from Park et al. (2006). The method involves determining the individual MIC of antibiotic and synthetic cod piscidin alone as well as the MIC of antibiotic and synthetic cod piscidin in the combination that was tested in 96 well plates. The initial concentration of the combined antibiotic and synthetic cod piscidin was based on the individual MIC of antibiotic and synthetic cod piscidin. Briefly, 90 µl of suspended bacterial cells 10^5 CFU/ml in MHB (supplemented NaCl as appropriate) was added in each well of 96 well plate and followed by adding of two-fold serial dilutions of combined antibacterial agents. The positive control well was contained 90 µl of bacterial cells 10^5 CFU/ml in MHB and 10 µl of antibacterial agent diluent. Three negative controls were performed: i) 90 µl of MHB and 10 µl of antibacterial agent diluent, ii) 90 µl of MHB and 10 µl of combined antibacterial agent solution, and iii) 100 µl of MHB. Each test sample and control were performed in triplicate, the plate was incubated at

appropriate temperature until the optical density at 540 nm of positive control well reached to 0.2. The MIC was determined as the lowest antibacterial agent concentration that inhibits bacterial growth by 50% compared with the positive control (Fernandes et al., 2002). The synergistic activity was assessed by determining the fractional inhibitory concentration (FIC) index. The FIC index is used as an indicator of synergistic activity of two antibacterial agents combination and calculated according to the equation:

FIC index= $FIC_A+FIC_B= [A]/MIC_A+[B]/MIC_B$

Where [A] and [B] are the MIC of drug A and drug B in the combination, MIC_A and MIC_B are the MIC of drug A and drug B alone, and FIC_A and FIC_B are the FIC of drug A and drug B. The FIC indexes are interpreted as follows: ≤0.5, good synergism; 1.0, additive; and >4.0, antagonism (Yan and Hancock, 2001).

3.1.5. Kinetics of synergism

Determination of kinetic of combined oxolinic acid and synthetic cod piscidin which showed synergistic activity against *V. anguillarum* VI-F-258-3 was evaluated base on Fernandes et al. (2002) by using the viable count of drop plate technique (Herigstad et al., 2001). Briefly, 90 µl of suspended bacteria approximately 10^5 CFU/ml in MHB (supplemented 1.5% NaCl) was mixed with 10 µl of combined antibacterial agents solution contained 0.031 µM of oxolinic acid and 5 µM of synthetic cod piscidin (based on MIC of individual oxolinic and synthetic cod piscidin against *V. anguillarum* VI-F-258-3) in 96 well plate and then incubated at 20 °C for indicated times. The kinetic study was monitored at 0 min, 30 min, 1h, 2h, 3h, 6h, 12h, and 24h after combined antibacterial agents addition. As controls including positive control which is the mixing between 90 µl of suspended bacteria approximately 10^5 CFU/ml in MHB and 10 µl of antibacterial agent diluents, while three negative

controls including: i) 90 µl of MHB and 10 µl of antibacterial agent diluent, ii) 90 µl of MHB and 10 µl of antibacterial agent solution, and iii) 100 µl of MHB. The test samples and controls were performed in triplicate. The sample each indicated time after incubation was diluted in media base on 10 fold serial dilution and plating by using drop plate technique (Herigstad et al., 2001) on TSA that contained 1.5% NaCl and the agar plates were incubated at 20 °C. The bacterial colonies were counted under stereo microscope following the bacterium colony density standard should be between in rang 30-300 colonies on agar plate. Finally the bacterial density (CFU/ml) was plotted with indicated time after incubation.

3.2. Results

3.2.1. Antibacterial activity against test bacteria

The minimal inhibitory concentrations (MICs) of synthetic cod piscidin showed more active to Gram-positive bacteria in range of MICs 0.04-2.5 µM than Gram-negative bacteria that showed MIC >5 µM. The MBC of synthetic cod piscidin against all tested bacteria were higher than 5 µM (maximal tested concentration) with exception of *P. citreus* was showed MBC of 1.25 µM (Table 9).

The MICs and MBC of antibiotic agents against Gram-positive bacteria (*M. lysodeikticus* and *P. citreus*) and Gram-negative bacteria (*V. anguillarum* VI-F-258-3, *A. salmonicida* NCIMB 1102, and *Y. ruckeri VI* 3629) were provided in Table 10. The result was found that MIC of sulfadiazine/trimethoprim against *M. lysodeikticus* was performed highest concentration (MICs 32-64 µM), while showed more active against *P. citreus* (MICs 0.031-0.063 µM). However, the MBC result of sulfadiazine/trimethoprim was not active against all five tested bacteria. The antibacterial activity of oxolinic acid and oxytetracycline hydrochloride was performed in range 0.016-0.5 µM against all five tested bacteria. The MBC of oxolinic acid also showed ineffective against *P. citreus* (MBC >64 µM), whereas very active against *A. salmonicida* NCIMB 1102 (MBC 0.063 µM). Moreover, MBC of oxytetracycline hydrochloride performed more active *A. salmonicida* NCIMB 1102 (MBC 1 µM) than other tested bacteria.

Table 9. The minimal inhibitory and bactericidal concentrations of synthetic cod piscidin against five test bacteria.

Bacteria	MIC (μM)[a]	MBC (μM)[b]
M. lysodeikticus	1.25-2.5	>5
P. citreus	0.04-0.08	1.25
V. anguillarum VI-F-258-3	>5	>5
A. salmonicida NCIMB 1102	>5	>5
Y. ruckeri VI 3629	>5	>5

The values were represented as average of triplicate of each sample.

[a] MIC, was defined as the lowest synthetic cod piscidin concentration that inhibits bacterial growth by 50% compared to the positive control (bacteria without peptide).

[b] MBC, was considered as the lowest concentration of synthetic cod piscidin that prevented colony forming on TSA plates after incubation for 24 h.

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Table 10. The minimal inhibitory and bactericidal concentrations of antibiotic agents against various bacteria.

Bacteria	Oxolinic acid		Oxytetracycline hydrochloride		Sulfadiazine/trimethoprim	
	MIC (μM)[a]	MBC (μM)[b]	MIC (μM)[a]	MBC (μM)[b]	MIC (μM)[a]	MBC (μM)[b]
M. lysodeikticus	0.125-0.25	1	0.063-0.125	8	32-64	>64
P. citreus	0.25-0.5	>64	0.031-0.063	16	0.031-0.063	>64
V. anguillarum VI-F-258-3	0.016-0.031	1	0.016-0.031	8	0.5-1	>64
A. salmonicida NCIMB 1102	0.016-0.031	0.063	0.031-0.063	1	2-4	32
Y. ruckeri VI 3629	0.031-0.063	16	0.125-0.25	32	0.5-1	>64

The values were represented as average of triplicate of each sample.

[a] MIC, The MIC was defined as the lowest antibiotic concentration that inhibits bacterial growth by 50% compared to the positive control (bacteria without peptide). The MIC curve was showed in Appendix 4.

[b] MBC, The MBC was considered as the lowest concentration of antibiotics that prevented colony forming on TSA plates after incubation for 24 h.

3.2.2. Synergistic activity

Five strains of bacteria including two Gram-positive bacteria; *M. lysodeikticus*, *P. citreus* and three Gram-negative bacteria; *Y. ruckeri* VI 3629, *V. anguillarum* VI-F-258-3, *A. salmonicida* NCIMB 1102 were tested synergistic activity of antibiotic-peptide combination. The synergistic activity results were showed in Table 11, 12, and 13 that reported moderate synergism for combined oxolinic acid and synthetic cod piscidin against *V. anguillarum* VI-F-258-3; combined oxytetracycline hydrochloride and synthetic cod piscidin against *V. anguillarum* VI-F-258-3, *A. salmonicida* NCIMB 1102; and combined sulfadiazine/trimethoprim and synthetic cod piscidin against *M. lysodeikticus*, *A. salmonicida* NCIMB 1102, *Y. ruckeri* VI 3629. In contrast, no synergistic activity of all three test antibioticss with synthetic cod piscidin was found against *P. citreus*. However, additive activity of combined oxolinic acid and synthetic cod piscidin against *P. citreus* showed one-fold decreasing in the MICs of individual antibacterial agent in combination.

Table 11. The FIC index of combined oxolinic acid and synthetic cod piscidin against test bacteria.

Bacteria	MIC (μM)		MIC of combination (μM)	FIC index	Interpretation
	oxolinic acid	piscidin	oxolinic acid, piscidin		
M. lysodeikticus	0.125-0.25	1.25-2.5	0.125-0.25, 1.25-2.5	2.0	Additive
P. citreus	0.25-0.5	0.04-0.08	0.125-0.25, 0.02-0.04	1.0	Additive
V. anguillarum VI-F-258-3	0.016-0.031	>5	0.008-0.016, 1.25-2.5	<0.7	Moderate synergism
A. salmonicida NCIMB 1102	0.016-0.031	>5	>0.031, >5	>3.0	Antagonism
Y. ruckeri VI 3629	0.031-0.063	>5	0.031-0.063, 2.5-5	<1.5	Additive

FIC index= FIC_A+ FIC_B = [A]/ MIC_A+[B]/ MIC_B , where [A] and [B] are the MICs of drug A and drug B in the combination, MIC_A and MIC_B are the MICs of drug A and drug B alone, and FIC_A and FIC_B are the FICs of drug A and drug B. FIC index are interpreted as follow: ≤0.5,good synergism; 1.0,additive; and >4.0,antagonism.

Table 12. The FIC index of combined oxytetracycline hydrochloride and synthetic cod piscidin against test bacteria.

Bacteria	MIC (μM)		MIC of combination (μM)	FIC index	Interpretation
	oxytetracycline hydrochloride	piscidin	oxytetracycline hydrochloride, piscidin		
M. lysodeikticus	0.063-0.125	1.25-2.5	0.031-0.063, 0.63-1.25	1.0	Additive
P. citreus	0.031-0.063	0.04-0.08	0.031-0.063, 0.04-0.08	2.0	Additive
V. anguillarum VI-F-258-3	0.016-0.031	>5	0.008-0.016, 1.25-2.5	<0.7	Moderate synergism
A. salmonicida NCIMB 1102	0.031-0.063	>5	0.016-0.031, 1.25-2.5	<0.7	Moderate synergism
Y. ruckeri VI 3629	0.125-0.25	>5	>0.25, >5	>3.0	Antagonism

FIC index= FIC_A+ FIC_B = [A]/ MIC_A+[B]/ MIC_B , where [A] and [B] are the MICs of drug A and drug B in the combination, MIC_A and MIC_B are the MICs of drug A and drug B alone, and FIC_A and FIC_B are the FICs of drug A and drug B. FIC index are interpreted as follow: ≤0.5,good synergism; 1.0,additive; and >4.0,antagonism.

Table 13. The FIC index of sulfadiazine/trimethoprim and synthetic cod piscidin against test bacteria.

Bacteria	MIC (μM)		MIC of combination (μM)	FIC index	Interpretation
	Sulfadiazine /trimethoprim	piscidin	sulfadiazine/trimethoprim, piscidin		
M. lysodeikticus	32-64	1.25-2.5	8-16, 0.63-1.25	0.7	Moderate synergism
P. citreus	0.031-0.063	0.04-0.08	>0.063, >0.08	>4	Antagonism
V. anguillarum VI-F-258-3	0.5-1	>5	0.5-1, 2.5-5	<1.5	Additive
A. salmonicida NCIMB 1102	2-4	>5	1-2, 1.25-2.5	<0.7	Moderate synergism
Y. ruckeri VI 3629	0.5-1	>5	0.25-0.5, 1.25-2.5	<0.7	Moderate synergism

FIC index= FIC_A+ FIC_B = [A]/ MIC_A+[B]/ MIC_B , where [A] and [B] are the MICs of drug A and drug B in the combination, MIC_A and MIC_B are the MICs of drug A and drug B alone, and FIC_A and FIC_B are the FICs of drug A and drug B. FIC index are interpreted as follow: ≤0.5,good synergism; 1.0,additive; and >4.0,antagonism.

3.2.3. The kinetic of oxolinic acid and synthetic cod piscidin in combination against *V. anguillarum* VI-F-258-3

In this study, the combination of oxolinic acid and synthetic cod piscidin showed moderate synergistic activity against *V. anguillarum* VI-F-258-3 that was determined kinetic profile at indicated times after incubation (Fig. 17). The concentration of combined oxolinic acid and synthetic cod piscidin were considered base on the combination between MIC of individual agent, then in combination contained oxolinic acid 0.031 μM (the upper interval MIC value) and 5 μM of synthetic cod piscidin (MIC > 5 μM). The result showed bacterium cells density were increased a little bit from 0 h to 2 h after incubated with combined oxolinic acid and synthetic cod piscidin at 20 °C. And then bacterium cells density were started decreasing after 2 h until 24 h after incubation. While a control was incubated with absence of combined oxolinic acid and synthetic cod piscidin that found bacterium cells density were increased from 0 h until 24 h after incubation at 20 °C.

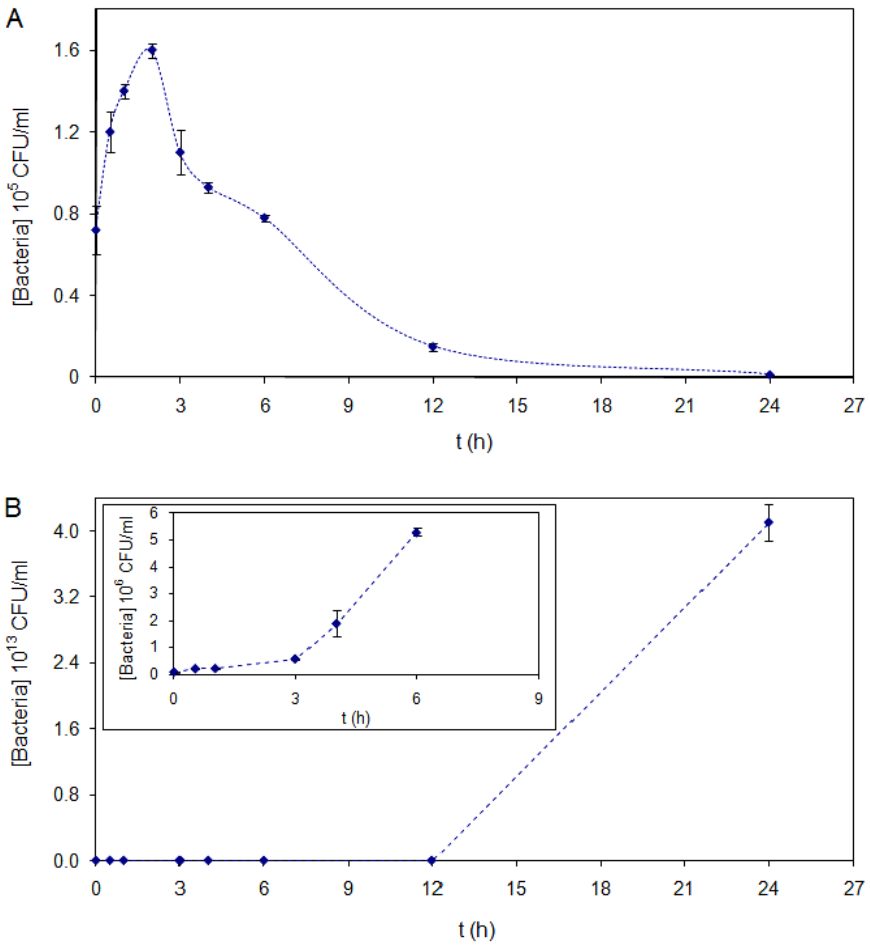

Figure 17. The kinetic of combined oxolinic acid and synthetic cod piscidin against *V. anguillarum* VI-F-258-3.

A, suspended *V. anguillarum* VI-F-258-3 approximately 10^5 CFU/ml in MHB were incubated with combined oxolinic acid (0.031 µM) and synthetic cod piscidin (5 µM) and plated on TSA for indicated times after incubation at 20°C. B, the control was contained *V. anguillarum* VI-F-258-3 suspension approximately 10^5 CFU/ml in MHB and incubated without combined antibacterial agents. Values are represented as means±standard error (vertical bars); n=3.

3.3. Discussion

3.3.1. Minimal inhibitory and bactericidal concentration of antibacterial agents

In this study found the individual MIC of synthetic cod piscidin were observed by MIC in rang 0.04-2.5 μM against 2 Gram-positive bacteria (*M. lysodeikticus* and *P. citreus*), while MIC of synthetic cod piscidin against all 3 strains of Gram-negative bacteria (*V. anguillarum* VI-F-258-3, *A. salmonicida* NCIMB 1102, and *Y. ruckeri* VI 3629) were more than 5 μM. In addition, only MBC of synthetic cod piscidin against *P. citreus* was investigated of 1.25 μM of peptide concentration, whereas the MBC of other test bacteria performed more than 5 μM of peptide concentration. However, the antibacterial activity of synthetic cod piscidin against different bacterial stains was discussed in the Chapter 2.

The antibacterial activity of conventional antibiotics; oxolinic acid, oxytetracycline hydrochloride, and sulfadiazine/trimethoprim were investigated MIC and MBC against various bacteria. In this study; ineffective antibacterial activity was found for sulfadiazine/trimethoprim combination against all five tested bacteria under consideration of MBC value. Moreover MICs of sulfadiazine/trimethoprim showed high concentration against *M. lysodeikticus* (MICs 32-64 μM), however the MICs of sulfadiazine/trimethoprim were 0.031-0.063 μM against *P. citreus*. Oxolinic acid showed very active antibacterial activity against *A. salmonicida* NCIMB 1102 (MBC 0.063 μM), whereas ineffective antibacterial against *P. citreu*s (MBC >64 μM). There are many reports for antibacterial activity of antibiotics used in aquaculture was investigated. Schmidt et al. (2000) observed the MICs of some antibiotics used in Danish aquaculture; oxolinic acid, sulfadiazine/trimethoprime combination 5:1, and oxytetracycline against *Y. ruckeri* (MICs of 2-8, 0.05-1, and 2-8 μg/ml, respectively),

and *Aromonas* resistant strains (MICs of 4-16, 205->1,024, and 32-256 µg/ml, respectively). Moreover, Bruun et al. (2000) reported the MICs of some antibiotics used in aquaculture; oxolinic acid, potentiated sulfadiazine (five parts sulfadiazine to one part trimethoprim), and oxytetracycline hydrochloride against *F. psychrophilum* (MICs of 0.13-0.25, 16-32, and 0.063-0.13 mg/ml, respectively), E. *coli* ATCC 25922 (MICs of 0.032-0.063, 2-4, and 1-2 mg/ml, respectively), S. *aureus* ATCC 29213 (MICs of 0.5-1, 1-2, and 0.25-1 mg/ml, respectively), and *Pseudomonas aeruginosa* ATCC 27853 (MICs of 16-32, 256-512, and 8-16 mg/ml, respectively). In this study, sulfadiazine/trimethoprim combination showed ineffective antibacterial activity against all five tested bacteria that similar with report of Bruun et al. (2000), showed less antibacterial effect of potentiated sulfadiazine against *Pseudomonas aeruginosa* ATCC 27853 (MICs 256-512 µg/ml) and *F. psychrophilum* NCIMB 1947 (MICs 16-32 µg/ml). Moreover, Myhr et al. (1991) have been tested MIC_{90} of antibiotics consisting enrofloxacin, flumequine, oxolinic acid, nitrofurazolidone, oxytetracycline, and combined trimethoprim-sulfadiazine against *V. anguillarum* serovar O1 and O2 strains by using drug microdilution method, the result showed MIC of nitrofurazolidone (MICs of 1-4 µg/ml) higher than MIC of combined trimethoprim-sulfadiazine (MIC of 0.5 µg/ml) and another antibiotics (oxytetracycline, oxolinic acid, enrofloxacin, and flumequine, MIC <0.5 µg/ml) against both V. *anguillarum* serovar. However, in the present was found antibiotic resistant bacteria that reported by Akinbowale et al. (2006) investigated antibiotics (e.g., oxolinic acid, trimethoprime potentiated sulfonamide, and oxytetracycline) resistance in isolated bacteria from aquaculture (e.g., fish and crustaceans farming) and environment sources in Australia was found antibiotic resistance is common in isolated bacteria, for examples; *Vibrio* spp. resistance to oxytetracycline (while, no resistance to trimethoprime potentiated

sulfamethoxazole and oxolinic acid), *Aeromonas* spp. resistance to oxolinic acid, oxytetracycline, and trimethoprime potentiated sulfamethoxazole, *Edwardsiella tarda* resistance to oxytetracycline (susceptible to oxolinic acid and trimethoprime potentiated sulfamethoxazole), and *Staphylococcu*s spp., *Micrococcus* spp. resistannce to oxolinic acid. From these supporting reports indicate that the antibiotics resistance bacteria were found in aquatic environment, caused by antibiotic used in aquaculture activity. It is possible that the antibiotic resistant gene from aquatic microorganisms might be transferred to terrestrial pathogens that might be caused of difficult to treat and prevent the diseases invading in terrestrial animals.

3.3.2. Synergistic study of synthetic cod piscidin and antibiotics combination

In this study was investigated the synergistic activity *in vitro* of synthetic cod piscidin from Atlantic cod with the conventional antibiotics, including oxolinic acid, oxytetracycline hydrochloride, and sulfadiazine/trimethoprim, which are amongst the most important antibiotic group for treatment and prevention of bacterial diseases in aquaculture. Interestingly, moderate synergy was found in this studies that showed FIC index <0.7 for combined synthetic cod piscidin with oxolinic acid, oxytetracycline hydrochloride, and sulfadiazine/trimethoprim against various strains of fish pathogenic bacteria including *V. anguillarum* VI-F-258-3, *A. salmonicida* NCIMB 1102, and *Y. ruckeri* VI 3629. The combination of antibacterial agents in this study showed synthetic cod piscidin can reduce the concentration of conventional antibiotics required to inhibit bacterial growth of most of test bacteria after combination. Many publications have been reported that combination of two antibacterial agents including combined antimicrobial peptide with antimicrobial peptide and also combined

antimicrobial peptide and antibiotic could enhanced their antimicrobial activity against various strains of bacteria than using antimicrobial peptide or antibiotic alone.

The synergistic interaction between the conventional antibiotic agent chloramphenicol and peptide A3 (sequenced from the N-terminus of *Helicobacter pylori* ribosomal protein L1) was found that their antibacterial activity *in vitro* against both Gram-negative and Gram-positive bacteria (*S. aureus*, *P. aeruginosa*, and *E. coli*) increased 2-8 fold more than using peptide A3 or antibiotic chloramphenicol alone (Park et al., 2004). Moreover Park et al. (2006) showed highest *S. aureus* membrane permeability of combined cationic antimicrobial peptide P5 with chloramphenical when compared with untreated and antibiotic treated bacterium cells by detection internalized PI fluorescence intensity using flow cytometric analysis. Another research was reported that synergy between pleurocidin and the lactic acid bacterial antimicrobial peptides destroyed outer membranes of bacterial (Luders et al., 2003). From these reports were confirmed that the combination of antimicrobial peptide and antibiotic could enhance their antibacterial activity and membrane permeability when compared with individual activity. Importantly the combination of antimicrobial peptide and antibiotic may solve the problem of antibiotic resistant bacteria that was supported by *in vitro* study of synergistic activity of combined antibacterial peptides nisin (produced by *Lactococcus lactis*) and ranalexin (isolated from bullfrog skin) with several antibiotics e.g., amoxycillin, and amoxicillin-clavulanate. The result was showed the killing activity was enhanced by against methicillin-resistant *Staphylococcus aureus* (MRSA) which is the resistance antibacterial treatment (Giacometti et al., 2000a). The similarly by Cirioni et al. (2006) showed the strong synergistic activity between α-helical antimicrobial peptides, magainin II and cecropin A with vancomycin that were investigated FIC indexes in range 0.312-0.458 against *S.*

ureus ATCC 25923, and *S. aureus* with intermediate resistance to vancomycin. In this study was observed moderate synergism of synthetic cod piscidin in combination with clinically used antibiotics in aquaculture. Unfortunately, antagonisms were found for combined synthetic cod piscidin with oxolinic acid, oxytetracycline hydrochloride, and sulfadiazine/trimethoprim against *A. salmonicida* NCIMB 1102, *Y. ruckeri* VI 3629, and *P. citreus*, respectively. This antagonism result was similar to Ulvatne et al. (2001) that reported no synergy between antibacterial peptides (P18, P15, P12, P9, and P6) and antibiotics (ampicillin, vancomycin, erythromycin, gentamicin, tetracycline, ciprofloxacin, and rifampicin) with exception of synergistic combination of antibacterial peptide P9 with ampicillin against *S. aureus* ATCC 25923, whereas, strong synergism of all antibacterial peptides (P18, P15, P12, P9, and P6) and erythromycin was observed against *E. coli* ATCC 25922. In addition, two synergistic interactions were investigated between enrofloxacin with antimicrobial peptides alamethicin and surfactin (FIC indexes were 0.75 and 0.56, respectively), whereas two indifference interactions were found between enrofloxacin with antimicrobial peptides globomycin and gramicidin S (FIC indexes were 1 for both) against *Mycoplasma pulmonis* (Fehri et al., 2007). In this study not only antagonism were found but additive activity also were investigated. The antagonism and additive activity might be caused by competition or alteration of biding site between combined antibacterial agents and particular binding site on bacterial cell. Moreover, Mueller-Hinton medium contained high ionic strength (Yan and Hancock, 2001) that might disrupt the combination of antibacterial agents and inhibit their antibacterial activity that might lead to additive and antagonistic activity of peptide-antibiotic combination. In addition, Fernandes et al., (2010) have mentioned that glycine residue at position 7 (Fig. 6) in amphipathic structure of cod piscidin might disrupt its function, which might related to

reducing of its antibacterial activity or ability to combine with antibiotics. Similar with Silphaduang and Noga (2001) report that a glycine substituted for histidin at position 17 of piscidin 3 might disrupt the its amphipathic α-helix structure and lead to reducing of its haemolytic and antibacterial activity as well.

Not only combination between antimicrobial peptide and antibiotic that can enhance their antibacterial activity but the combination between antibacterial peptides also can increase antibacterial activity when compared with individual activity. Lauth et al. (2005) showed synergism antibacterial activity of combined hepcidin and moronecidin, purified from the gills of hybrid striped bass against *S. iniae*, *E. coli*, *Y. enterocolitica*, and *S. sonnei*, the FIC indices were between 0.5-0.75 that were indicated good to moderate synergy. Moreover Luders et al. (2003) investigated the strong synergy between pleurocidin with antimicrobial peptides (produced by lactic acid bacteria); curvacin A, pediocin PA-1, and sakacin P that showed FIC indexes 0.19-0.5 against Gram-positive *Listeria ivanovii* Li4 and Gram-negative *E. coli* ATCC 14763 with exception of combined pleurocidin with sakacin P, showed FIC index 1.

Thus, the interaction between antimicrobial peptide with antibiotic is potentially enhancing antimicrobial activity and can supporting useful for antimicrobial therapy. Moreover, the combination of them could reduce the antibiotic concentration and increased antibacterial activity against antibiotic resistant bacteria by *in vitro* study. Therefore the combination of antimicrobial peptide and antibiotic might be used to manage antibiotic resistant bacteria problem and control bacterial diseases in aquaculture in the future.

Chapter 4. Conclusions

Disease management is crucial in the aquaculture industry, and to date it involves the usage of antibiotics to treat and prevent diseases, especially bacterial infections. However, there is a growing concern over the problems caused by conventional antibiotics in environment (e.g., bacteria resistant to multiple antibiotics). Therefore, it is crucial to reduce the usage of conventional antibiotics or find alternative novel antibacterial agents to manage bacterial diseases and antibiotic-resistant bacteria. Antimicrobial peptides are produced from multicellular organisms and have the ability to kill and inhibit bacteria via different mechanisms, namely by permeabilizing the bacterial cell membrane or disrupt cell metabolism. Therefore the antimicrobial peptides are considered as an attractive new alternative to conventional antibiotics. One significant advantage of antimicrobial peptides is that they have a broad spectrum of activity and can synergise with other antibiotics. In this thesis, I have investigated the antibacterial activity *in-vitro* of synthetic cod piscidin from Atlantic cod against different Gram-positive and Gram-negative bacteria and found synthetic cod piscidin to be more active against Gram-positive bacteria than Gram-negative bacteria with exception of *V. anguillarum* and *P. immobilis*. Interestingly, the permeabilization assay of synthetic cod piscidin against *P. citreus* revealed that this peptide permeabilized the bacterial membrane at its MICs and MBC values. Synthetic cod piscidin showed similar haemolytic activity to cecropin 1 and was less haemolytic than melittin against cod erythrocytes. Moreover, the synergistic activity *in-vitro* of synthetic cod piscidin was investigated with the conventional antibiotics, including oxolinic acid, oxytetracycline hydrochloride, and sulfadiazine/trimethoprim combination, which are amongst the most important antibiotics used for prevention of bacterial diseases in aquaculture. The peptide-antibiotic combination were tested

against two Gram-positive bacteria; *M. lysodeikticus*, *P. citreus* and three Gram-negative bacteria; *Y. ruckeri* VI 3629, *V. anguillarum* VI-F-258-3, *A. salmonicida* NCIMB 1102 which are common fish pathogens. The synergistic activity results showed moderate synergism for combined oxolinic acid and synthetic cod piscidin against *V. anguillarum* VI-F-258-3; combined oxytetracycline hydrochloride and synthetic cod piscidin against *V. anguillarum* VI-F-258-3, *A. salmonicida* NCIMB 1102; and combined sulfadiazine/trimethoprim and synthetic cod piscidin against *M. lysodeikticus*, *A. salmonicida* NCIMB 1102, *Y. ruckeri* VI 3629. In contrast, no synergistic activity of all three test antibiotics with synthetic cod piscidin was found against *P. citreus*. The data show that synthetic cod piscidin can reduce the concentration of conventional antibiotics required to inhibit bacterial growth of fish pathogenic bacteria (*Y. ruckeri* VI 3629, *V. anguillarum* VI-F-258-3, and *A. salmonicida* NCIMB 1102). These results provide a better insight into the mode of action of synthetic cod piscidin and suggest that it could be explored as an alternative antibiotic, which may be used in disease control management in commercial aquaculture systems in the future.

Appendix 1

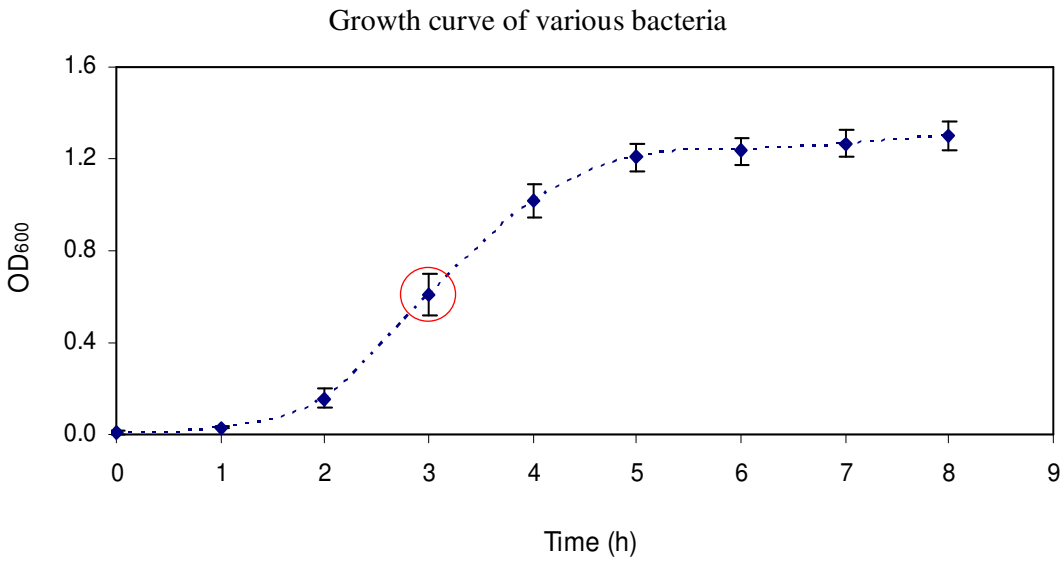

Figure A1.1. Growth curve of *M. lysodeikticus* that showed the relationship between optical densities at 600 nm and indicated times after incubation with appropriate culture conditions. The error bars were represented the standard error values for the average of triplicate. Red circle is indicated time during exponential phase.

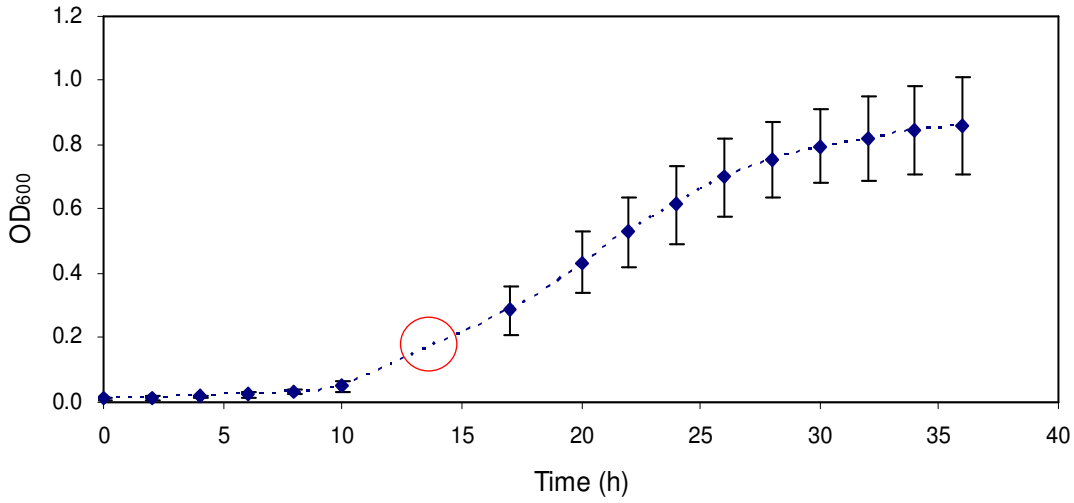

Figure A1.2. Growth curve of *P. citreus* that showed the relationship between optical densities at 600 nm and indicated times after incubation with appropriate culture conditions. The error bars were represented the standard error values for the average of triplicate. Red circle is indicated time during exponential phase.

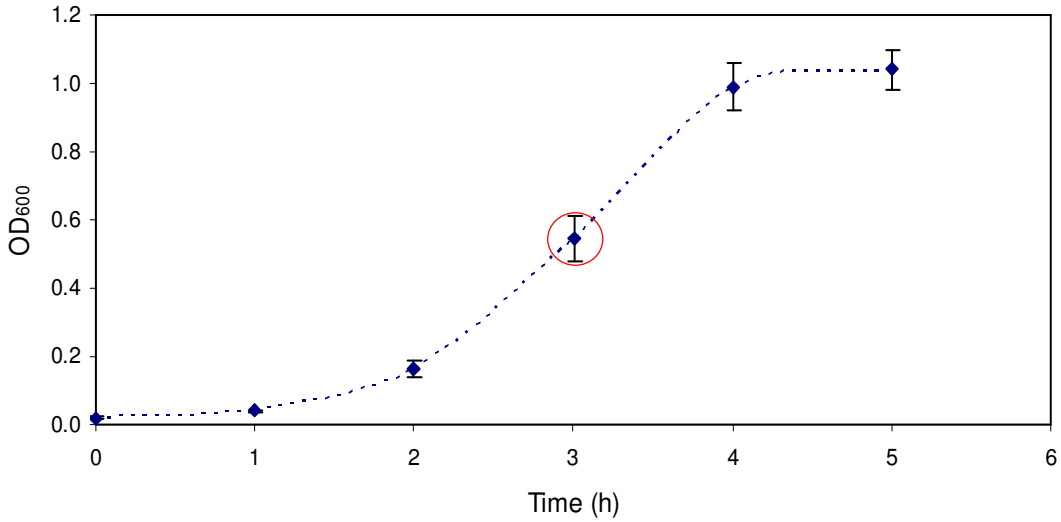

Figure A1.3. Growth curve of *S. aureus* ATCC 9144 that showed the relationship between optical densities at 600 nm and indicated times after incubation with appropriate culture conditions. The error bars were represented the standard error values for the average of triplicate. Red circle is indicated time during exponential phase.

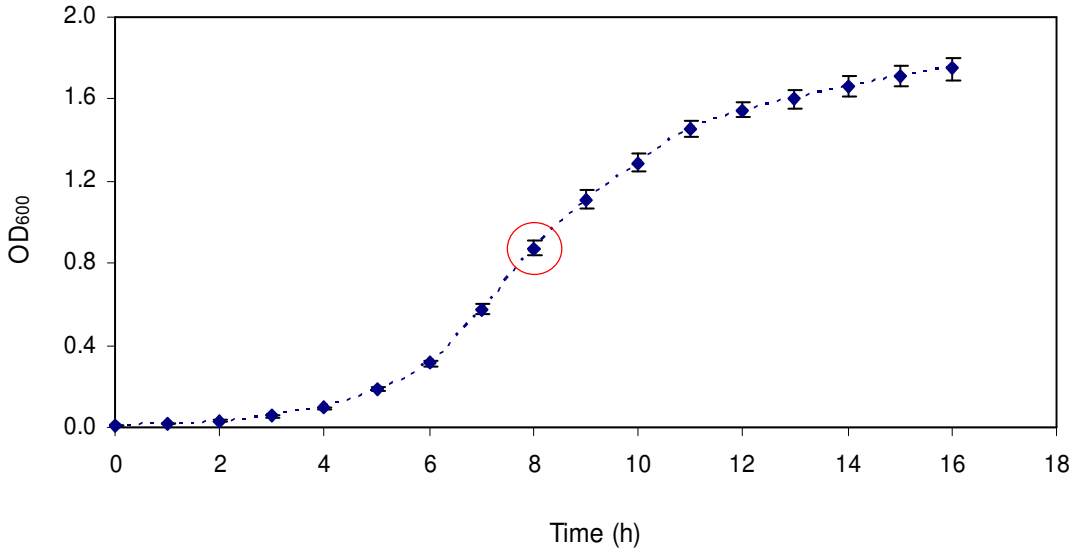

Figure A1.4. Growth curve of *C. glutamicum* ATCC 13032 that showed the relationship between optical densities at 600 nm and indicated times after incubation with appropriate culture conditions. The error bars were represented the standard error values for the average of triplicate. Red circle is indicated time during exponential phase.

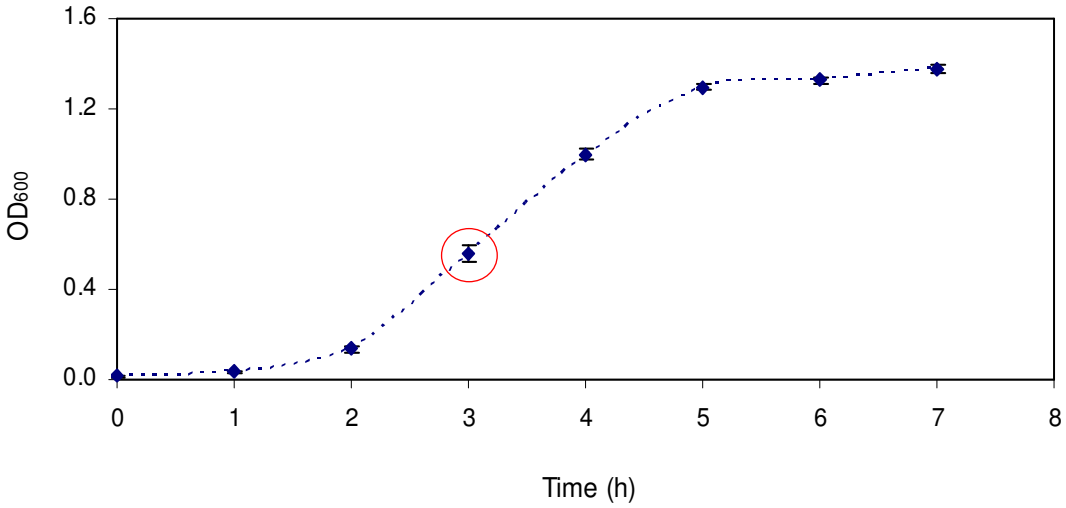

Figure A1.5. Growth curve of *E. coli* ATCC 25922 that showed the relationship between optical densities at 600 nm and indicated times after incubation with appropriate culture conditions. The error bars were represented the standard error values for the average of triplicate. Red circle is indicated time during exponential phase.

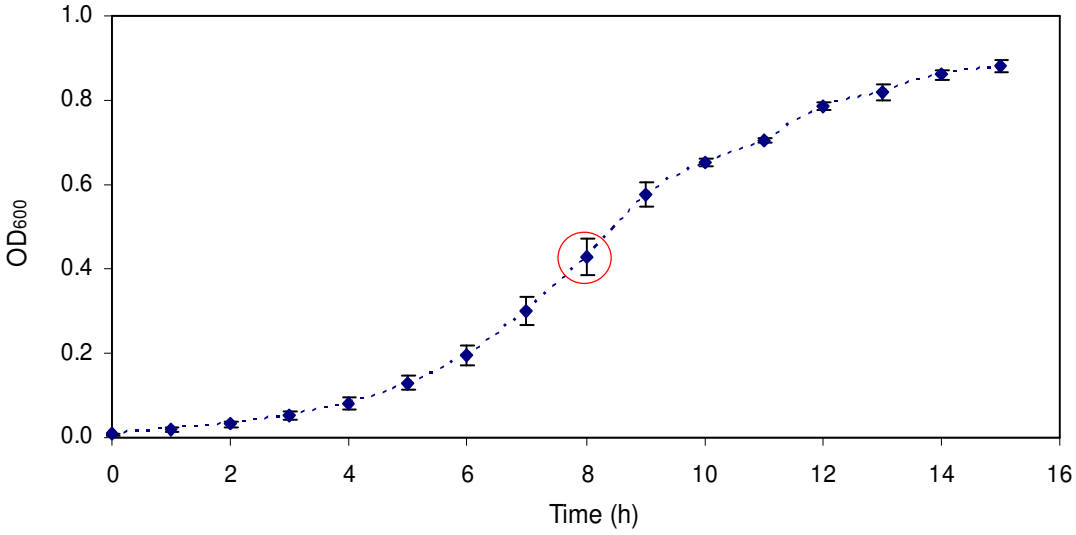

Figure A1.6. Growth curve of *V. anguillarum* NCIMB 2133 that showed the relationship between optical densities at 600 nm and indicated times after incubation with appropriate culture conditions. The error bars were represented the standard error values for the average of triplicate. Red circle is indicated time during exponential phase.

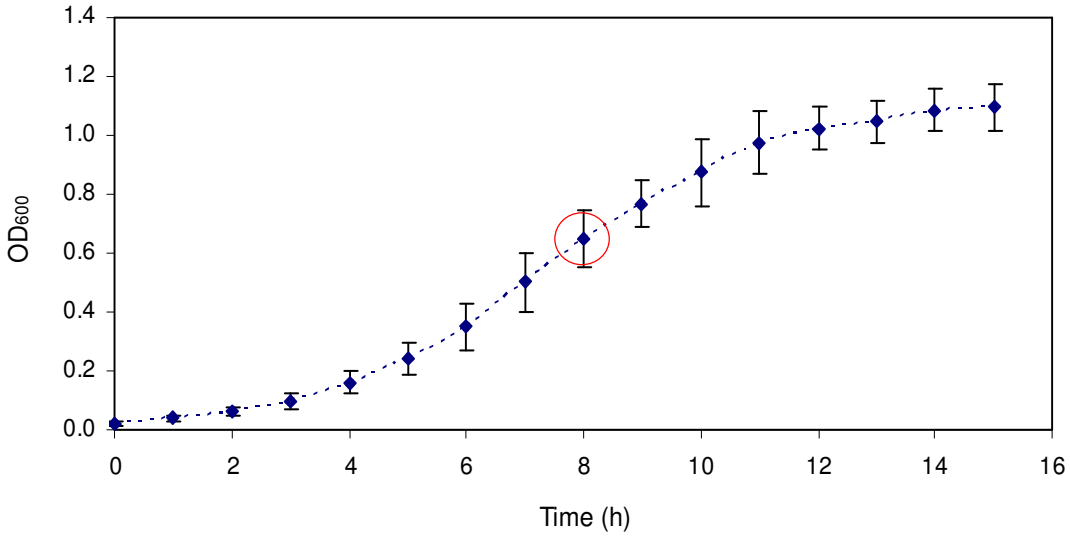

Figure A1.7. Growth curve of *V. anguillarum* VI-F-258-3 that showed the relationship between optical densities at 600 nm and indicated times after incubation with appropriate culture conditions. The error bars were represented the standard error values for the average of triplicate. Red circle is indicated time during exponential phase.

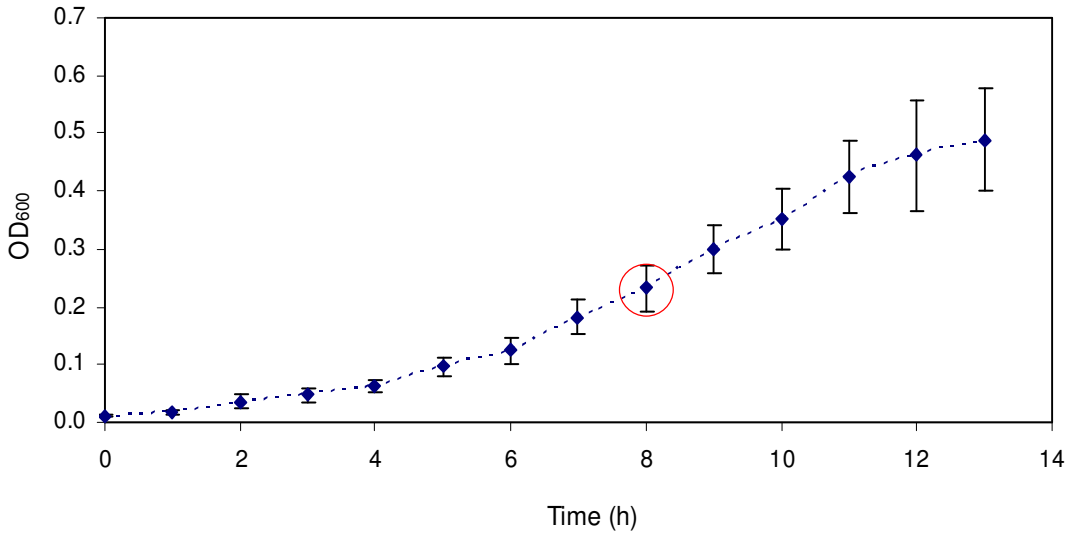

Figure A1.8. Growth curve of *V. anguillarum* that showed the relationship between optical densities at 600 nm and indicated times after incubation with appropriate culture conditions. The error bars were represented the standard error values for the average of triplicate. Red circle is indicated time during exponential phase.

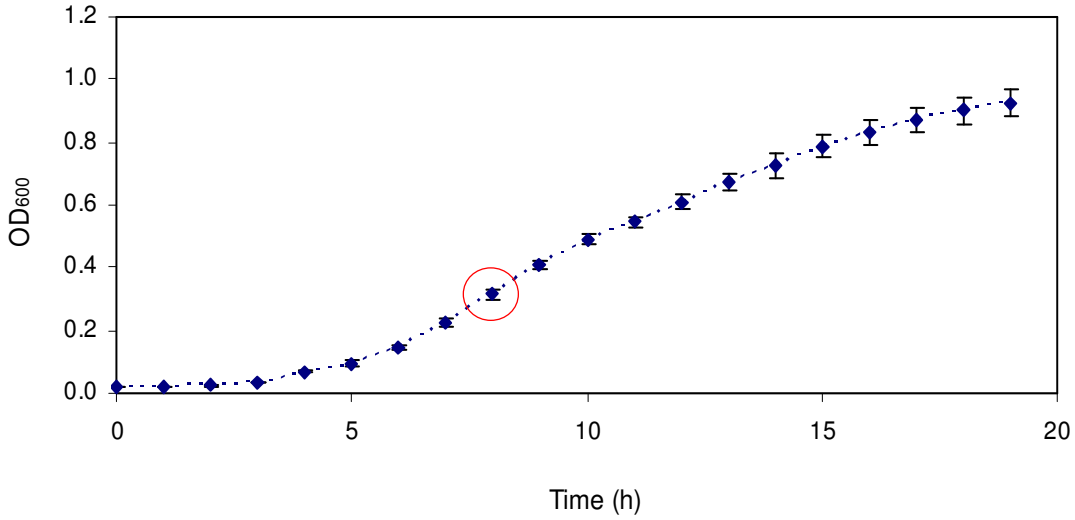

Figure A1.9. Growth curve of *A. salmonicida* NCIMB 1102 that showed the relationship between optical densities at 600 nm and indicated times after incubation with appropriate culture conditions. The error bars were represented the standard error values for the average of triplicate. Red circle is indicated time during exponential phase.

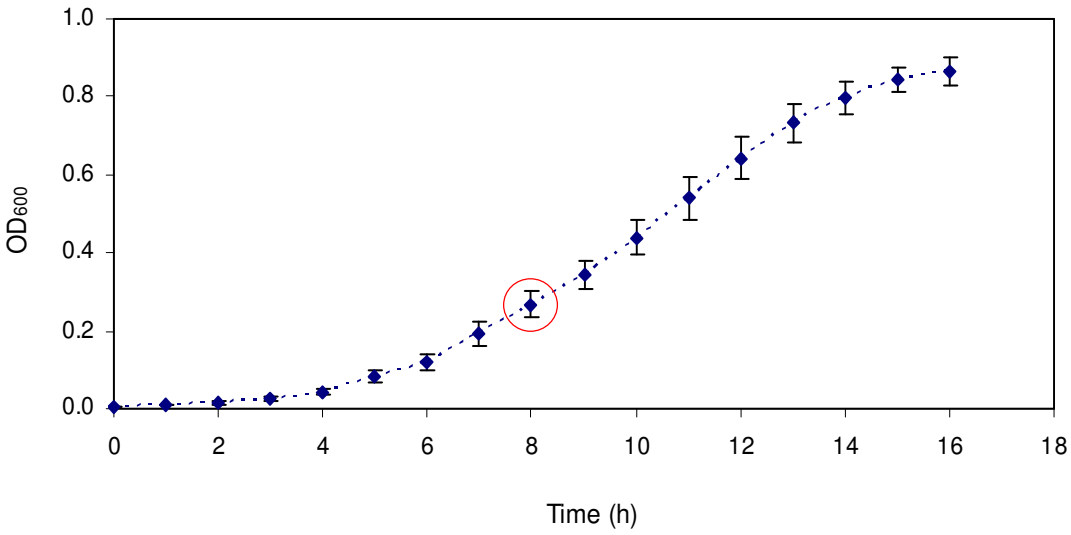

Figure A1.10. Growth curve of *Y. ruckeri* NCIMB 2196 that showed the relationship between optical densities at 600 nm and indicated times after incubation with appropriate culture conditions. The error bars were represented the standard error values for the average of triplicate. Red circle is indicated time during exponential phase.

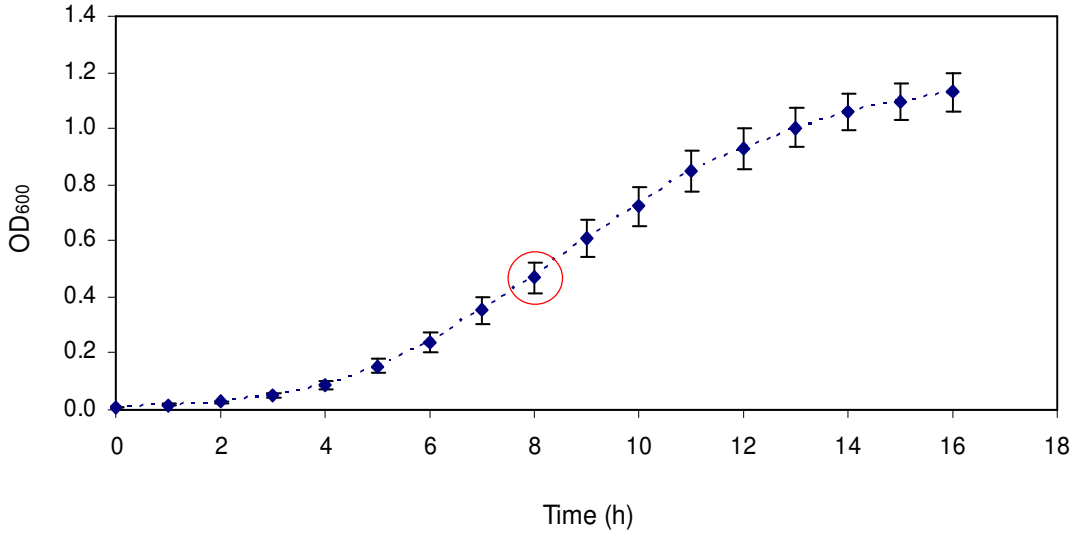

Figure A1.11. Growth curve of *Y. ruckeri* VI 3629 that showed the relationship between optical densities at 600 nm and indicated times after incubation with appropriate culture conditions. The error bars were represented the standard error values for the average of triplicate. Red circle is indicated time during exponential phase.

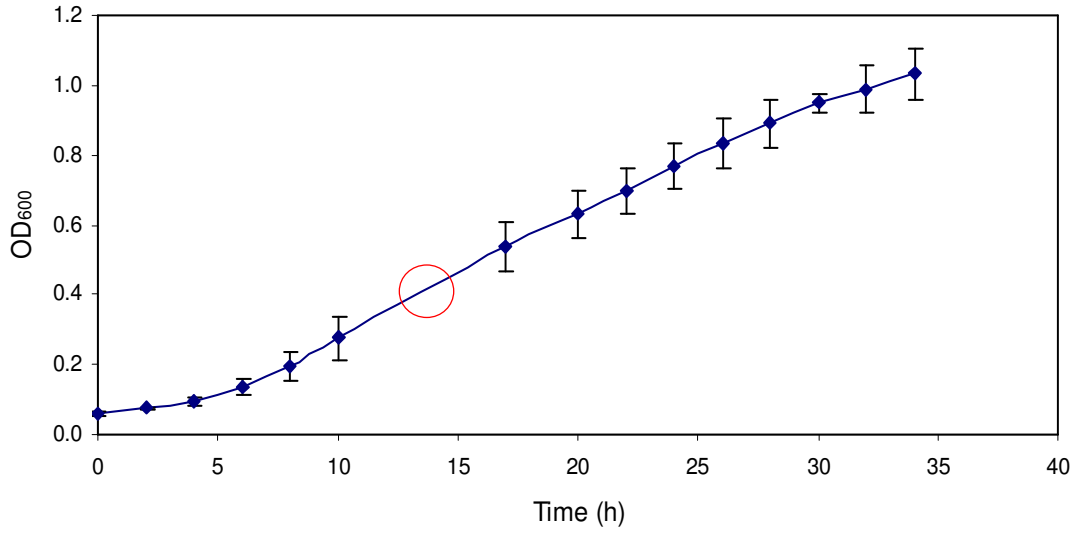

Figure A1.12. Growth curve of *P. immobilis* that showed the relationship between optical densities at 600 nm and indicated times after incubation with appropriate culture conditions. The error bars were represented the standard error values for the average of triplicate. Red circle is indicated time during exponential phase.

Appendix 2

The correlation ship between bacterial densities and optical density at 600 nm

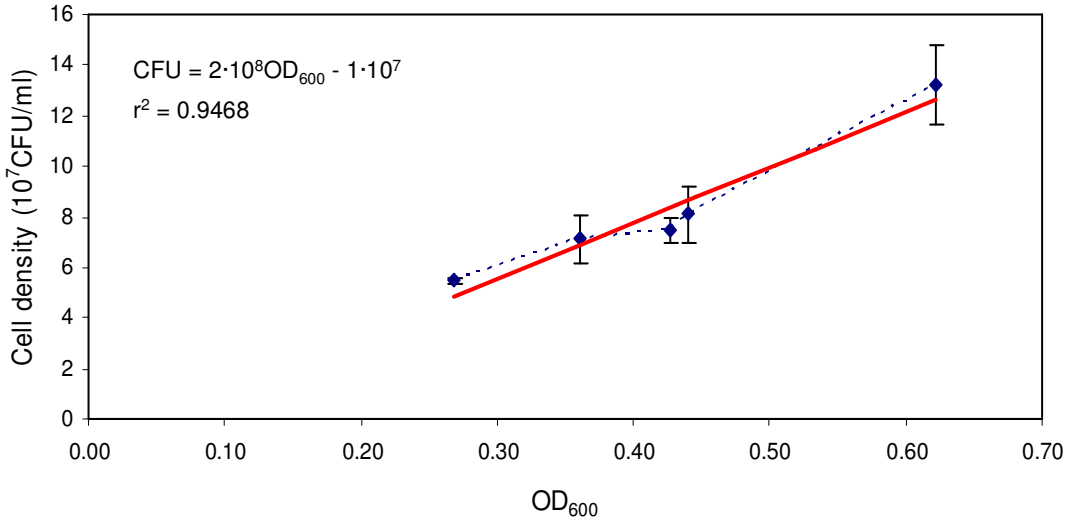

Figure A2.1. The relationship between cell density and optical density at 600 nm of *M. lysodeikticus*. Values are represented as means±standard error (vertical bars); n=3. The linear regression equation was used to calculate the bacterium cell density at particular optical density at 600 nm.

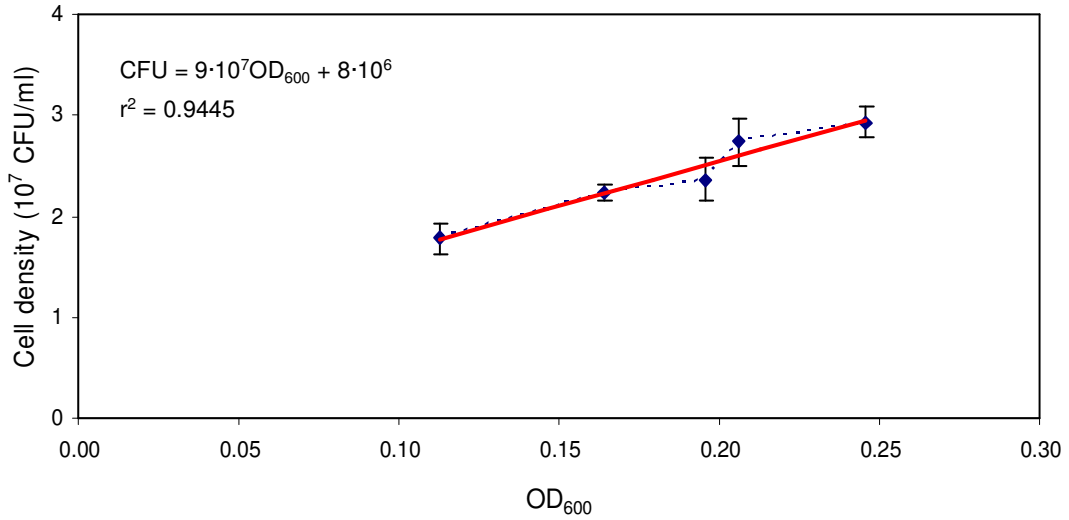

Figure A2.2. The relationship between cell density and optical density at 600 nm of *P. citreus* Values are represented as means±standard error (vertical bars); n=3. The linear regression equation was used to calculate the bacterium cell density at particular optical density at 600 nm.

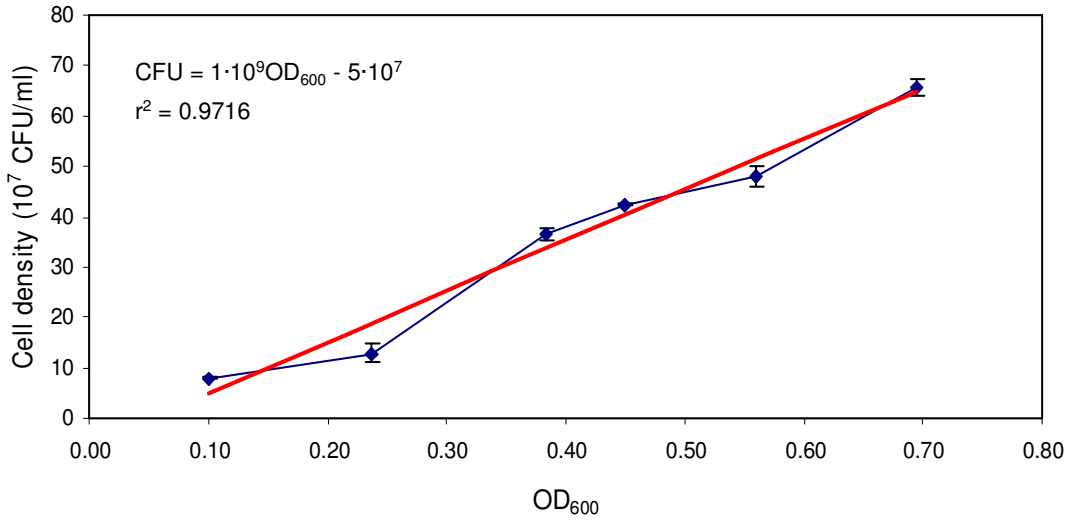

Figure A2.3. The relationship between cell density and optical density at 600 nm of *S. aureus* ATCC 9144. Values are represented as means±standard error (vertical bars); n=3. The linear regression equation was used to calculate the bacterium cell density at particular optical density at 600 nm.

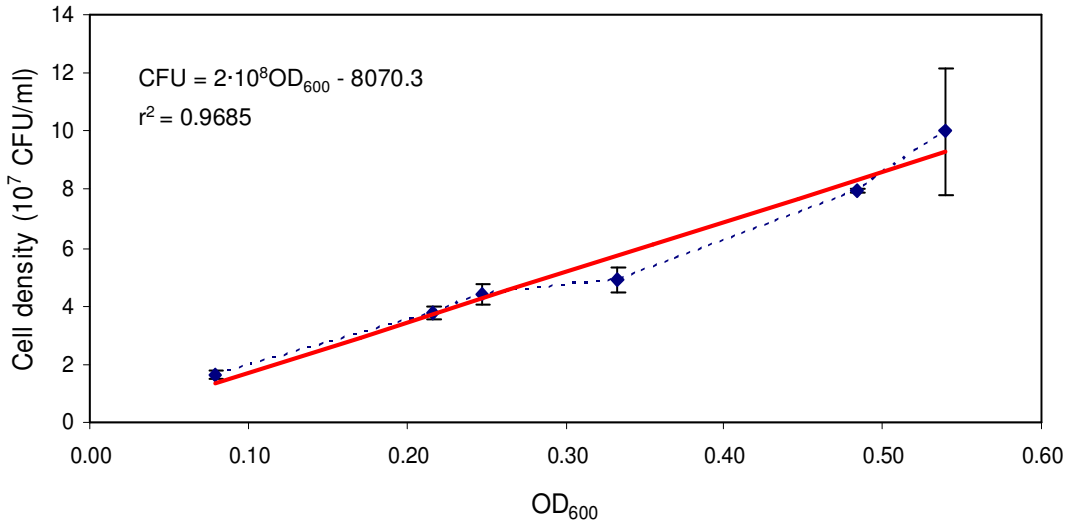

Figure A2.4. The relationship between cell density and optical density at 600 nm of *C. glutamicum* ATCC 13032. Values are represented as means±standard error (vertical bars); n=3. The linear regression equation was used to calculate the bacterium cell density at particular optical density at 600 nm.

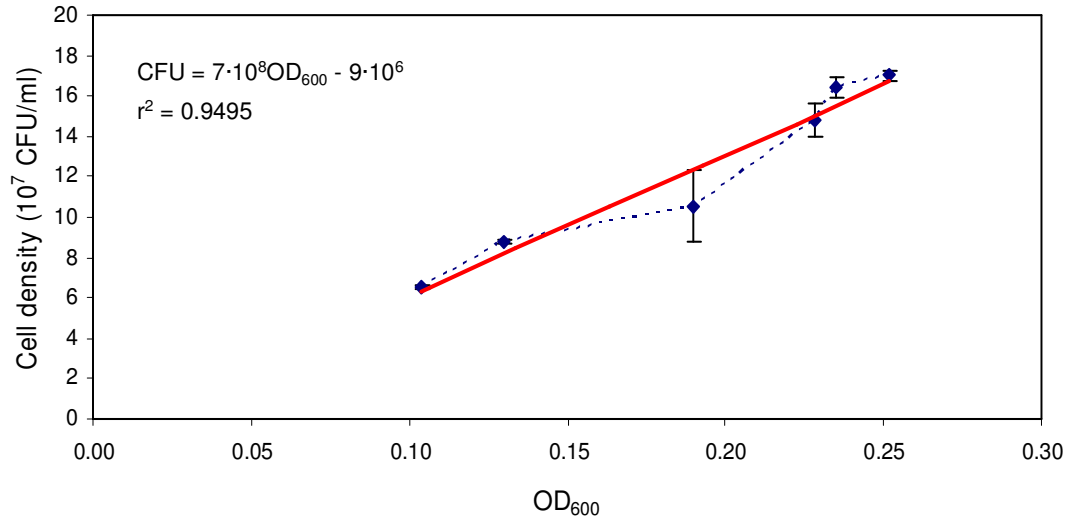

Figure A2.5. The relationship between cell density and optical density at 600 nm of *E. coli* ATCC 25922. Values are represented as means±standard error (vertical bars); n=3. The linear regression equation was used to calculate the bacterium cell density at particular optical density at 600 nm.

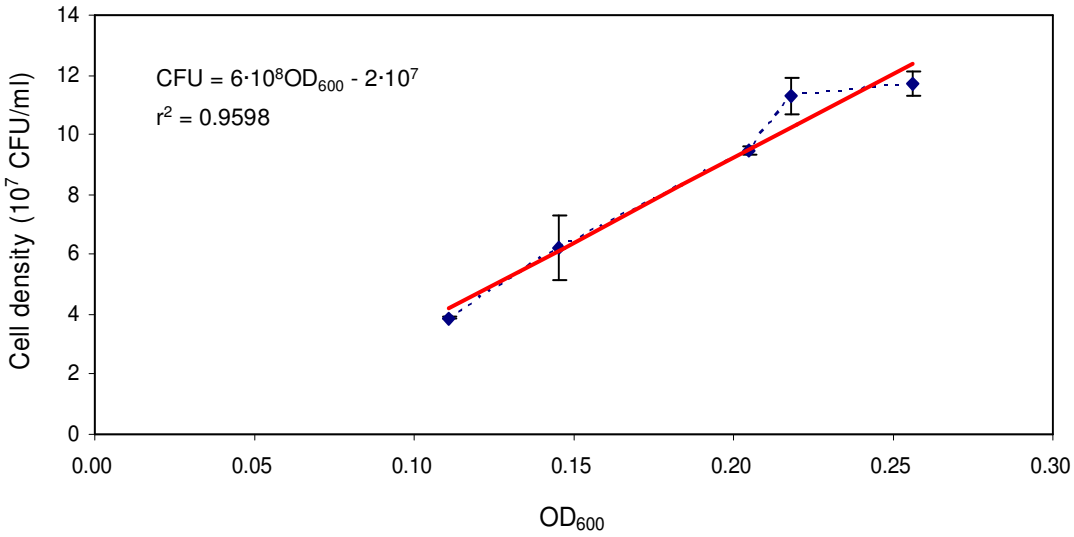

Figure A2.6. The relationship between cell density and optical density at 600 nm of *V. anguillarum* NCIMB 2133. Values are represented as means±standard error (vertical bars); n=3. The linear regression equation was used to calculate the bacterium cell density at particular optical density at 600 nm.

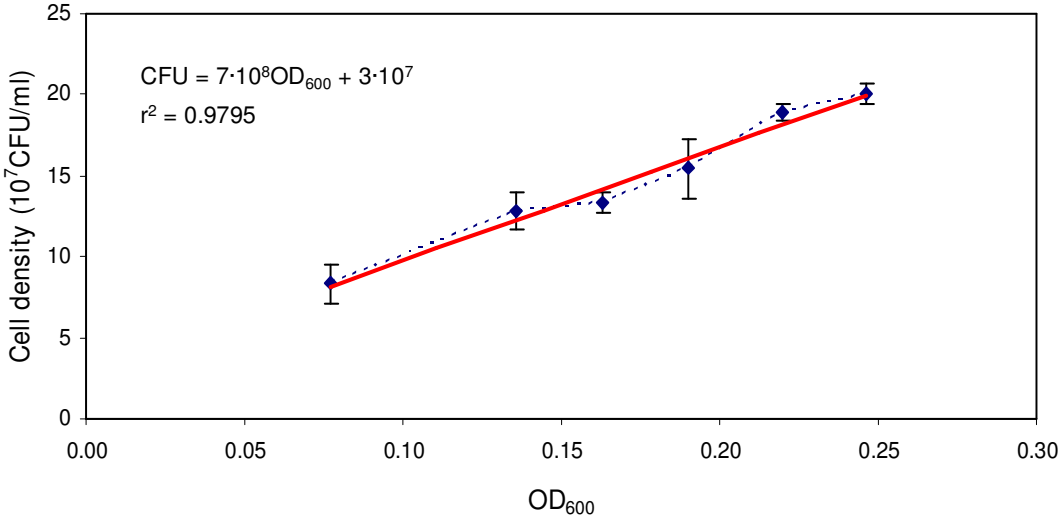

Figure A2.7. The relationship between cell density and optical density at 600 nm of *V. anguillarum* VI-F-258-3. Values are represented as means±standard error (vertical bars); n=3. The linear regression equation was used to calculate the bacterium cell density at particular optical density at 600 nm.

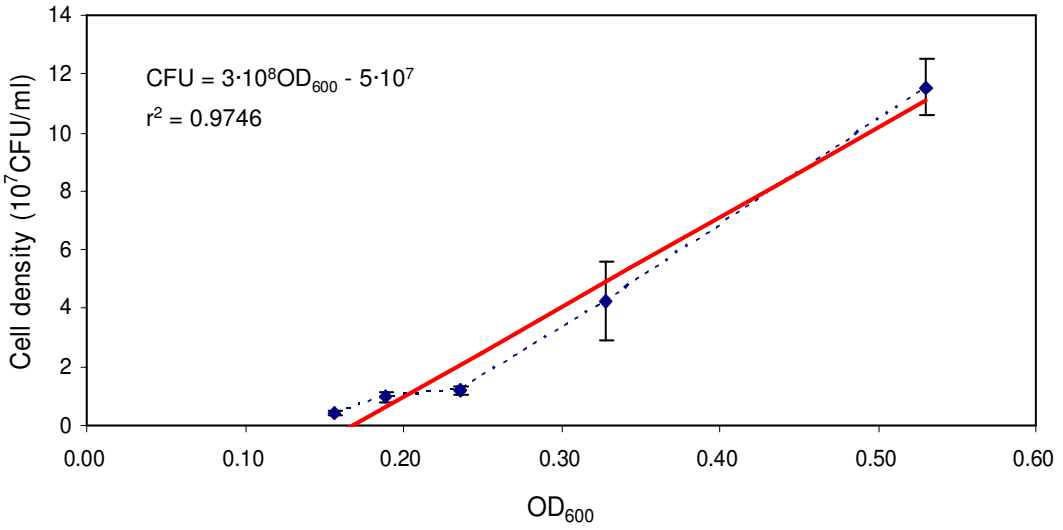

Figure A2.8. The relationship between cell density and optical density at 600 nm of *V. anguillarum*. Values are represented as means±standard error (vertical bars); n=3. The linear regression equation was used to calculate the bacterium cell density at particular optical density at 600 nm.

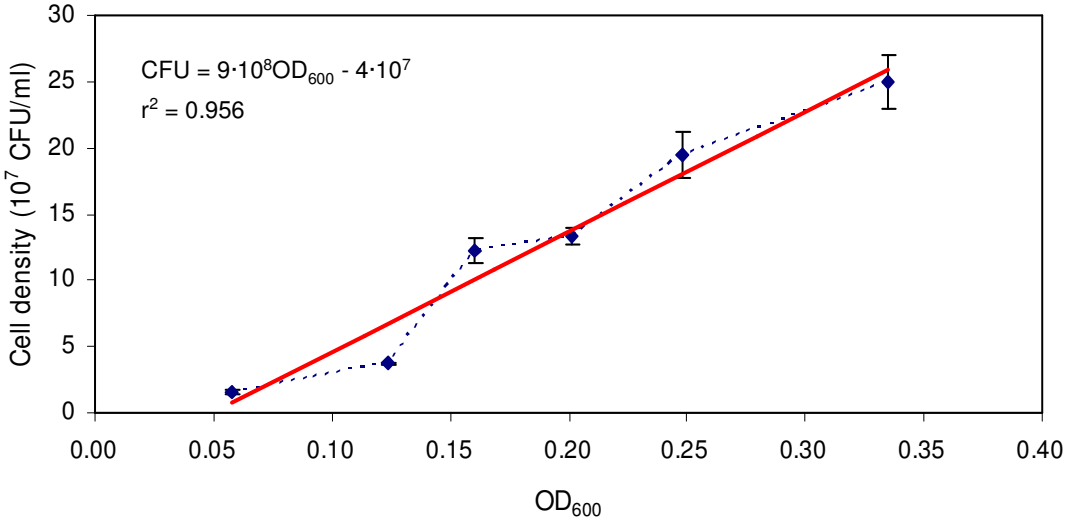

Figure A2.9. The relationship between cell density and optical density at 600 nm of *A. salmonicida* NCIMB 1102. Values are represented as means±standard error (vertical bars); n=3. The linear regression equation was used to calculate the bacterium cell density at particular optical density at 600 nm.

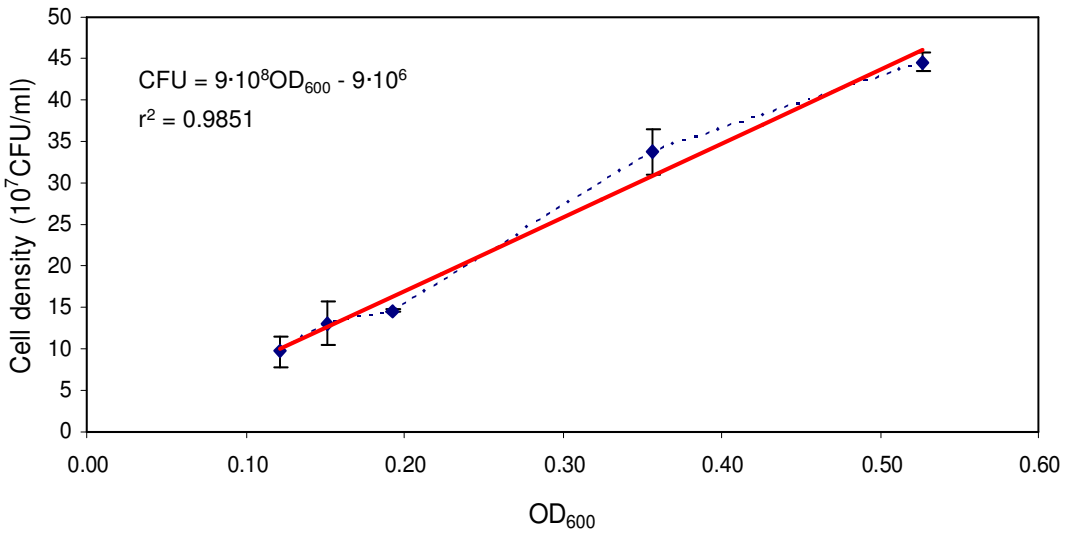

Figure A2.10. The relationship between cell density and optical density at 600 nm of *Y. ruckeri* NCIMB 2196. Values are represented as means±standard error (vertical bars); n=3. The linear regression equation was used to calculate the bacterium cell density at particular optical density at 600 nm.

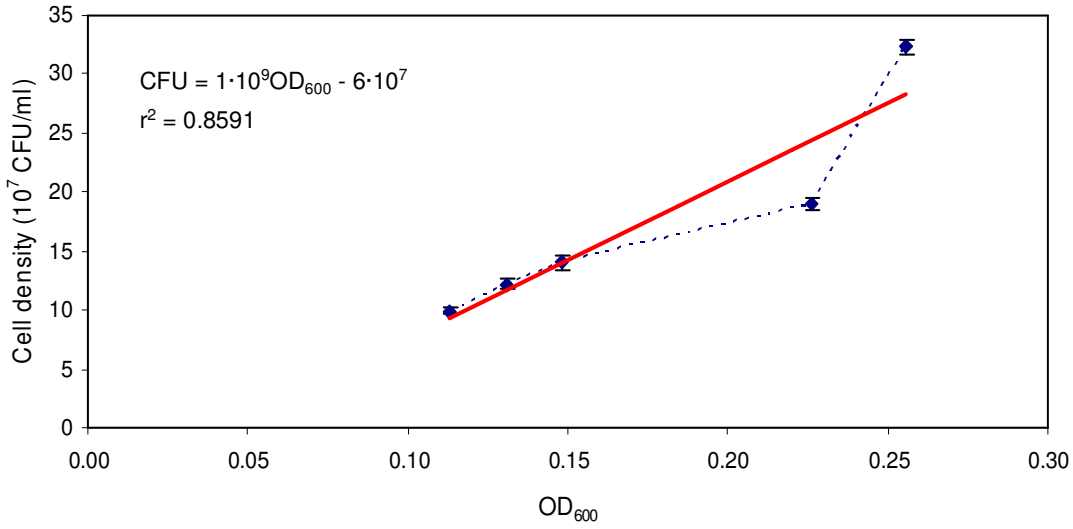

Figure A2.11. The relationship between cell density and optical density at 600 nm of *Y. ruckeri* VI 3629. Values are represented as means±standard error (vertical bars); n=3. The linear regression equation was used to calculate the bacterium cell density at particular optical density at 600 nm.

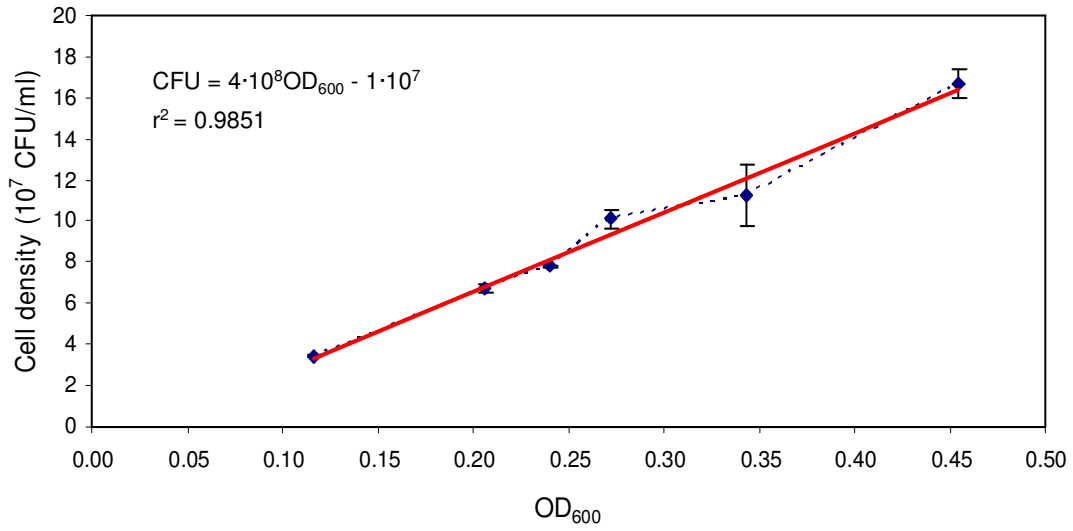

Figure A2.12. The relationship between cell density and optical density at 600 nm of *P. immobilis*. Values are represented as means±standard error (vertical bars); n=3. The linear regression equation was used to calculate the bacterium cell density at particular optical density at 600 nm.

Appendix 3

Minimal inhibitory concentration of synthetic cod piscidin

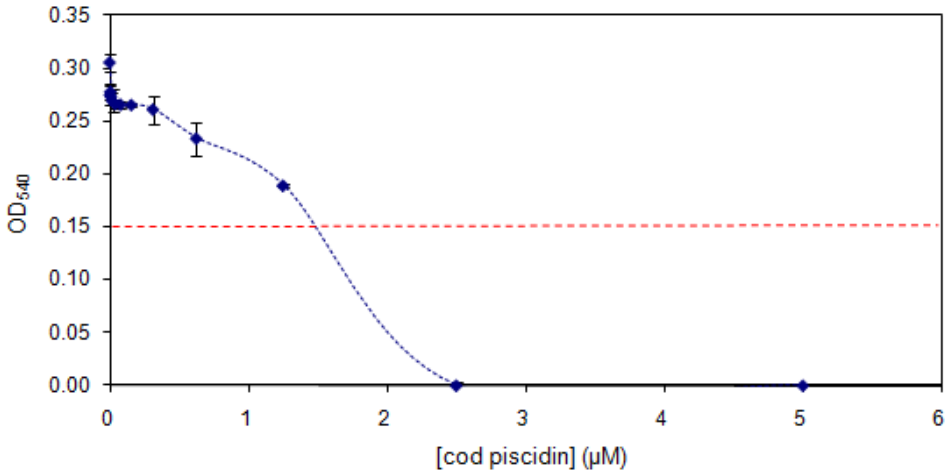

Figure A3.1. The different concentrations of synthetic cod piscidin were incubated with approximate 10^5 CFU/ml of *M. lysodeikticus*. The optical density at 540 nm was determined when the positive control, bacteria without peptide reached to 0.2 after incubated at appropriate temperature. The MIC values are represented in rage by the red line indicates the inhibition of bacterial growth by 50% compared to the positive control. Values are represented as means±standard error (vertical bars); n=3.

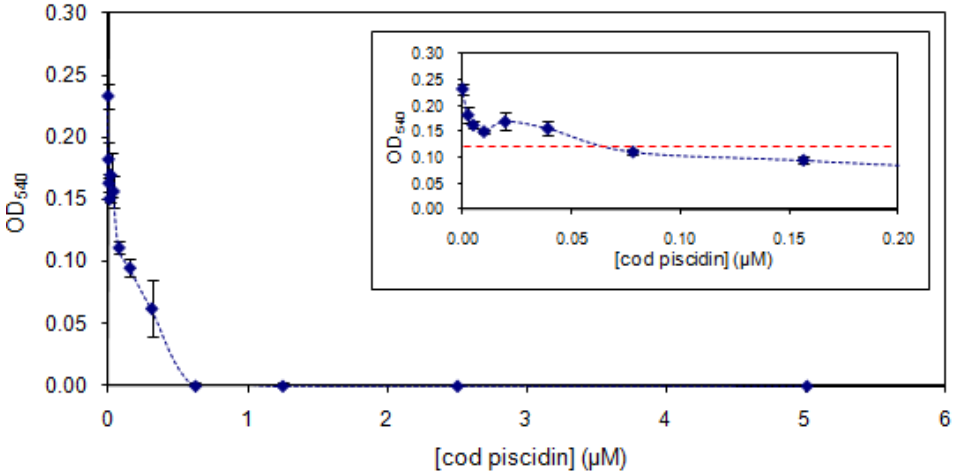

Figure A3.2. The different concentrations of synthetic cod piscidin were incubated with approximate 10^5 CFU/ml of *P. citreus*. The optical density at 540 nm was determined when the positive control, bacteria without peptide reached to 0.2 after incubated at appropriate temperature. The MIC values are represented in rage by the red line indicates the inhibition of bacterial growth by 50% compared to the positive control. Values are represented as means±standard error (vertical bars); n=3.

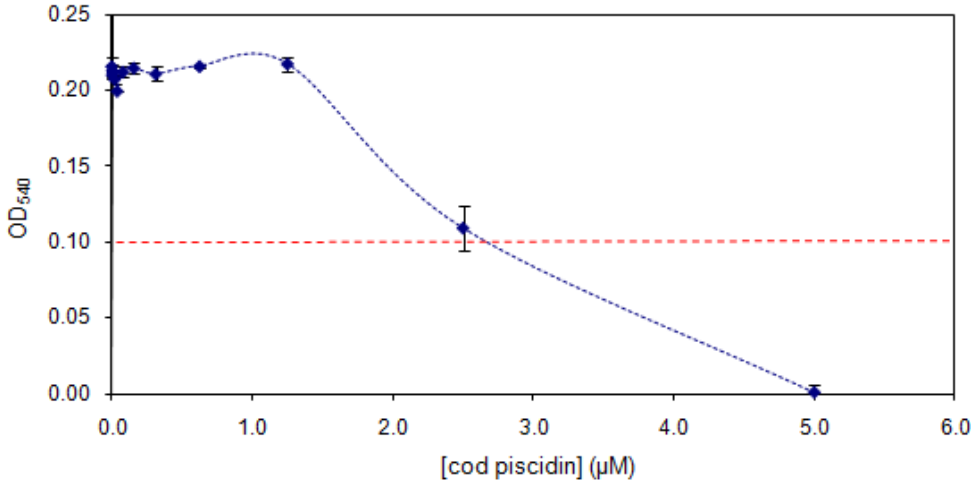

Figure A3.3. The different concentrations of synthetic cod piscidin were incubated with approximate 10^5 CFU/ml of *S. aureus* ATCC 9144. The optical density at 540 nm was determined when the positive control, bacteria without peptide reached to 0.2 after incubated at appropriate temperature. The MIC values are represented in rage by the red line indicates the inhibition of bacterial growth by 50% compared to the positive control. Values are represented as means±standard error (vertical bars); n=3.

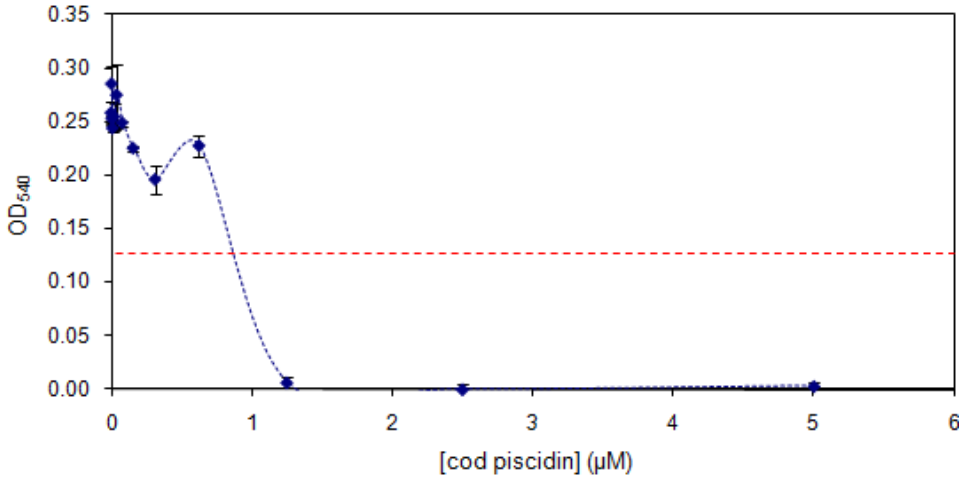

Figure A3.4. The different concentrations of synthetic cod piscidin were incubated with approximate 10^5 CFU/ml of *C. glutamicum* ATCC 13032. The optical density at 540 nm was determined when the positive control, bacteria without peptide reached to 0.2 after incubated at appropriate temperature. The MIC values are represented in rage by the red line indicates the inhibition of bacterial growth by 50% compared to the positive control. Values are represented as means±standard error (vertical bars); n=3.

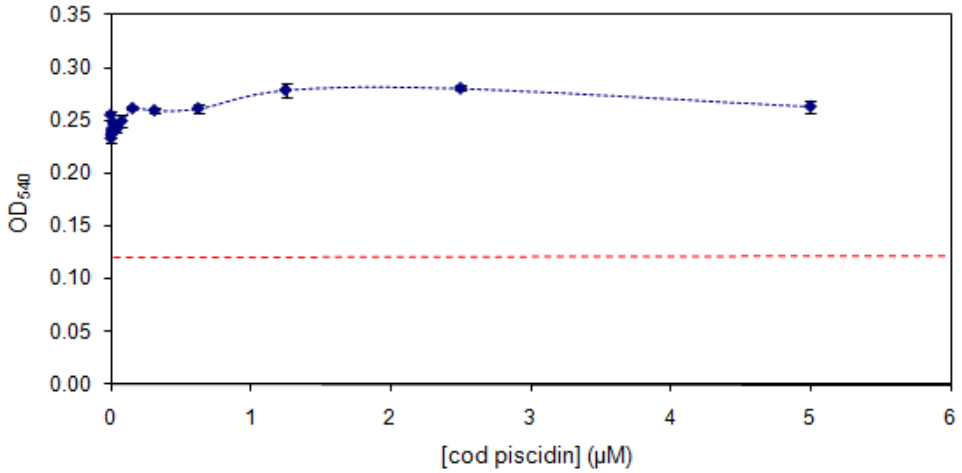

Figure A3.5. The different concentrations of synthetic cod piscidin were incubated with approximate 10^5 CFU/ml of *E. coli* ATCC 25922. The optical density at 540 nm was determined when the positive control, bacteria without peptide reached to 0.2 after incubated at appropriate temperature. The MIC values are represented in rage by the red line indicates the inhibition of bacterial growth by 50% compared to the positive control. Values are represented as means±standard error (vertical bars); n=3.

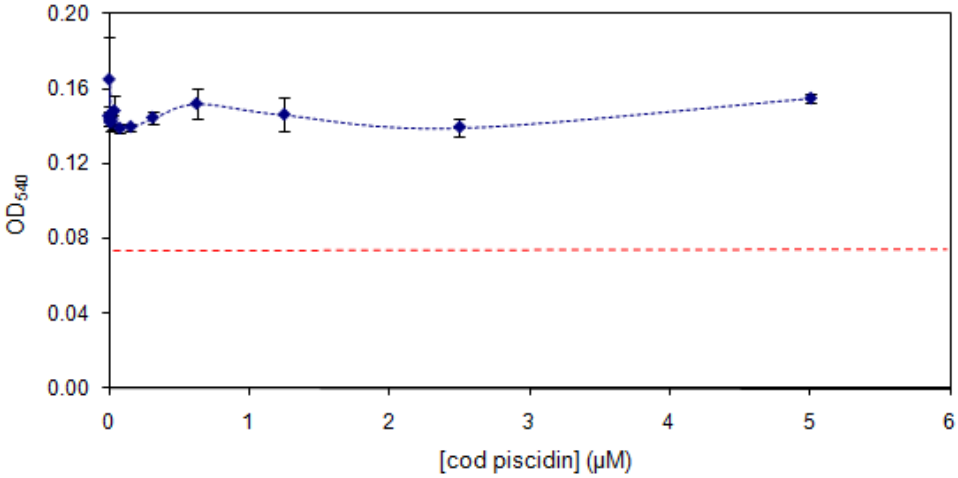

Figure A3.6. The different concentrations of synthetic cod piscidin were incubated with approximate 10^5 CFU/ml of *V. anguillarum* NCIMB 2133. The optical density at 540 nm was determined when the positive control, bacteria without peptide reached to 0.2 after incubated at appropriate temperature. The MIC values are represented in rage by the red line indicates the inhibition of bacterial growth by 50% compared to the positive control. Values are represented as means±standard error (vertical bars); n=3.

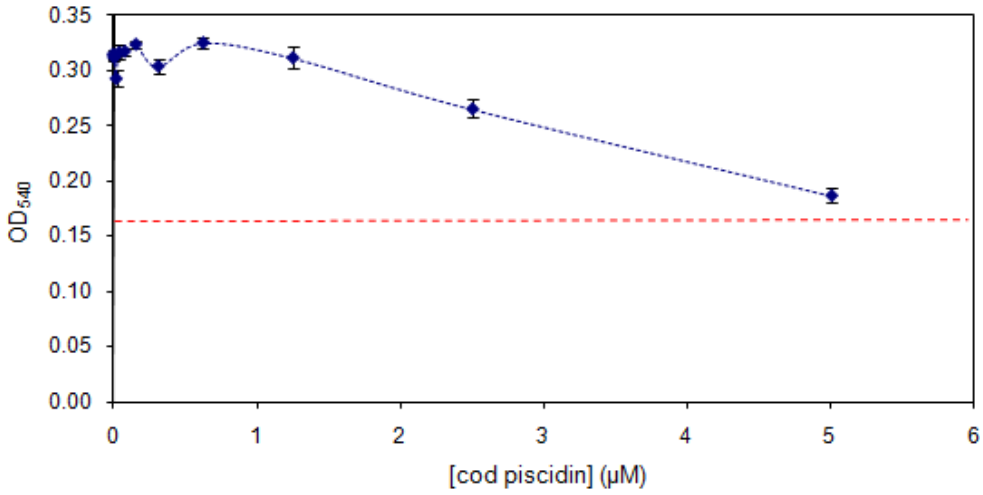

Figure A3.7. The different concentrations of synthetic cod piscidin were incubated with approximate 10^5 CFU/ml of *V. anguillarum* VI-F-258-3. The optical density at 540 nm was determined when the positive control, bacteria without peptide reached to 0.2 after incubated at appropriate temperature. The MIC values are represented in rage by the red line indicates the inhibition of bacterial growth by 50% compared to the positive control. Values are represented as means±standard error (vertical bars); n=3.

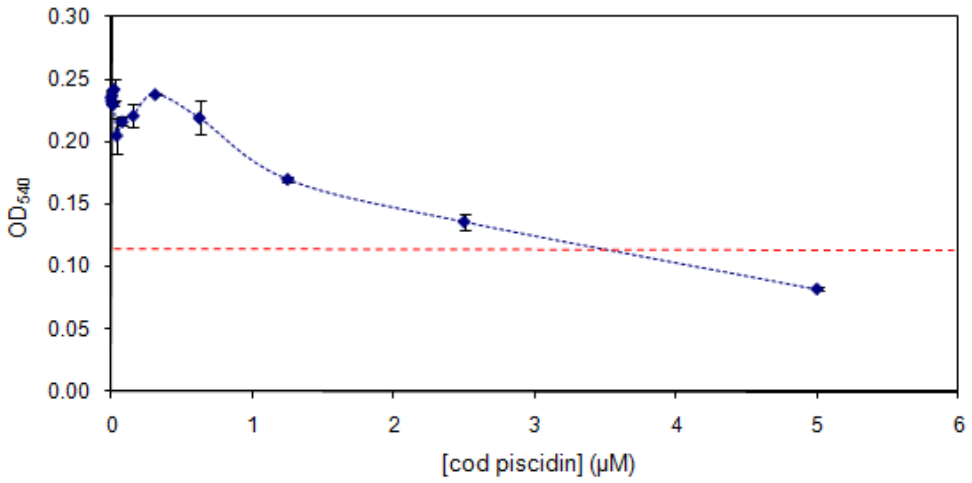

Figure A3.8. The different concentrations of synthetic cod piscidin were incubated with approximate 10^5 CFU/ml of *V. anguillarum*. The optical density at 540 nm was determined when the positive control, bacteria without peptide reached to 0.2 after incubated at appropriate temperature. The MIC values are represented in rage by the red line indicates the inhibition of bacterial growth by 50% compared to the positive control. Values are represented as means±standard error (vertical bars); n=3.

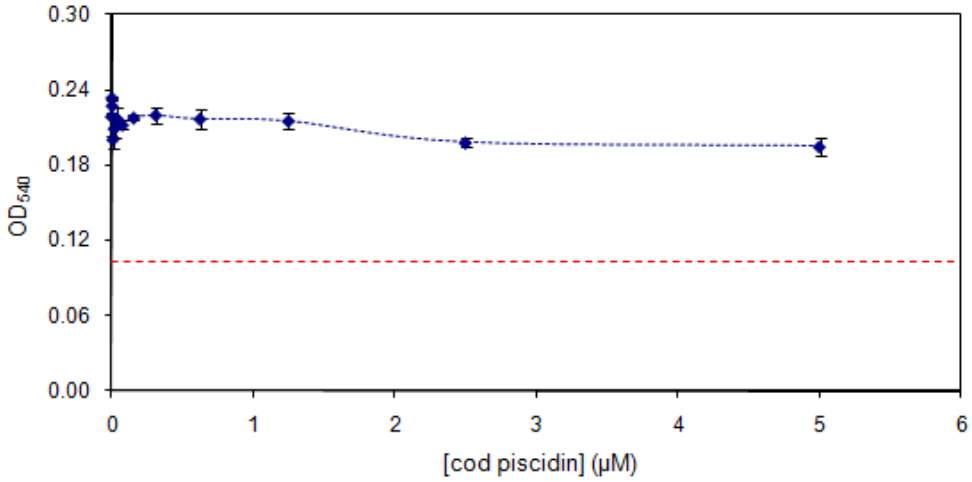

Figure A3.9. The different concentrations of synthetic cod piscidin were incubated with approximate 10^5 CFU/ml of *A. salmonicida* NCIMB 1102. The optical density at 540 nm was determined when the positive control, bacteria without peptide reached to 0.2 after incubated at appropriate temperature. The MIC values are represented in rage by the red line indicates the inhibition of bacterial growth by 50% compared to the positive control. Values are represented as means±standard error (vertical bars); n=3.

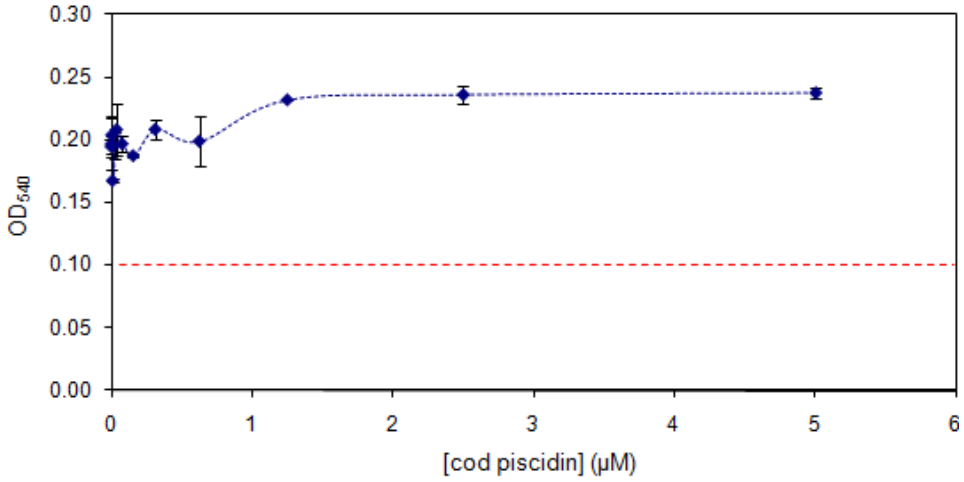

Figure A3.10. The different concentrations of synthetic cod piscidin were incubated with approximate 10^5 CFU/ml of *Y. ruckeri* NCIMB 2196. The optical density at 540 nm was determined when the positive control, bacteria without peptide reached to 0.2 after incubated at appropriate temperature. The MIC values are represented in rage by the red line indicates the inhibition of bacterial growth by 50% compared to the positive control. Values are represented as means±standard error (vertical bars); n=3.

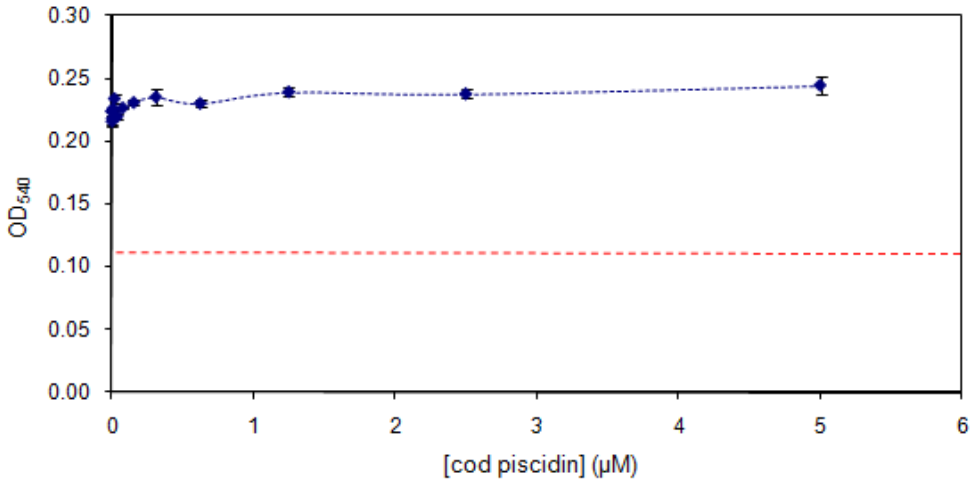

Figure A3.11. The different concentrations of synthetic cod piscidin were incubated with approximate 10^5 CFU/ml of *Y. ruckeri* VI 3629. The optical density at 540 nm was determined when the positive control, bacteria without peptide reached to 0.2 after incubated at appropriate temperature. The MIC values are represented in rage by the red line indicates the inhibition of bacterial growth by 50% compared to the positive control. Values are represented as means±standard error (vertical bars); n=3.

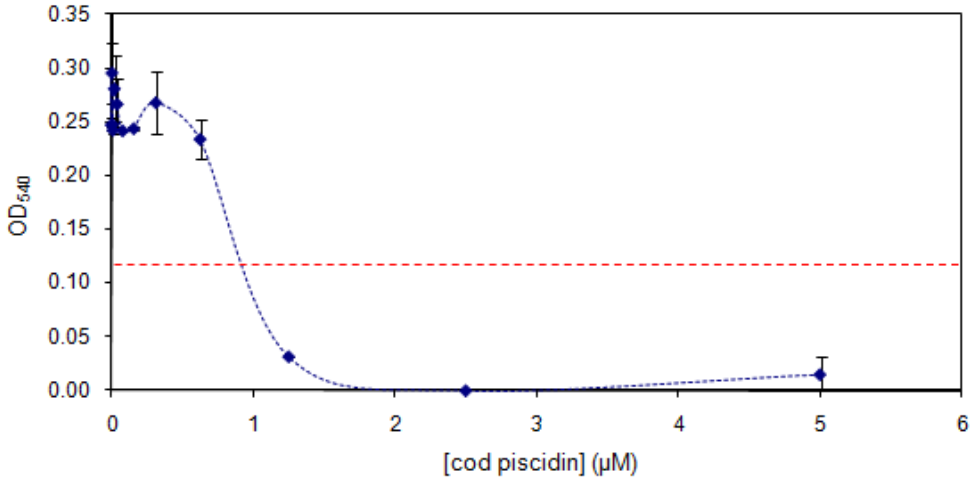

Figure A3.12. The different concentrations of synthetic cod piscidin were incubated with approximate 10^5 CFU/ml of *P. immobilis*. The optical density at 540 nm was determined when the positive control, bacteria without peptide reached to 0.2 after incubated at appropriate temperature. The MIC values are represented in rage by the red line indicates the inhibition of bacterial growth by 50% compared to the positive control. Values are represented as means±standard error (vertical bars); n=3.

Appendix 4

Minimal inhibitory concentration of antibiotics

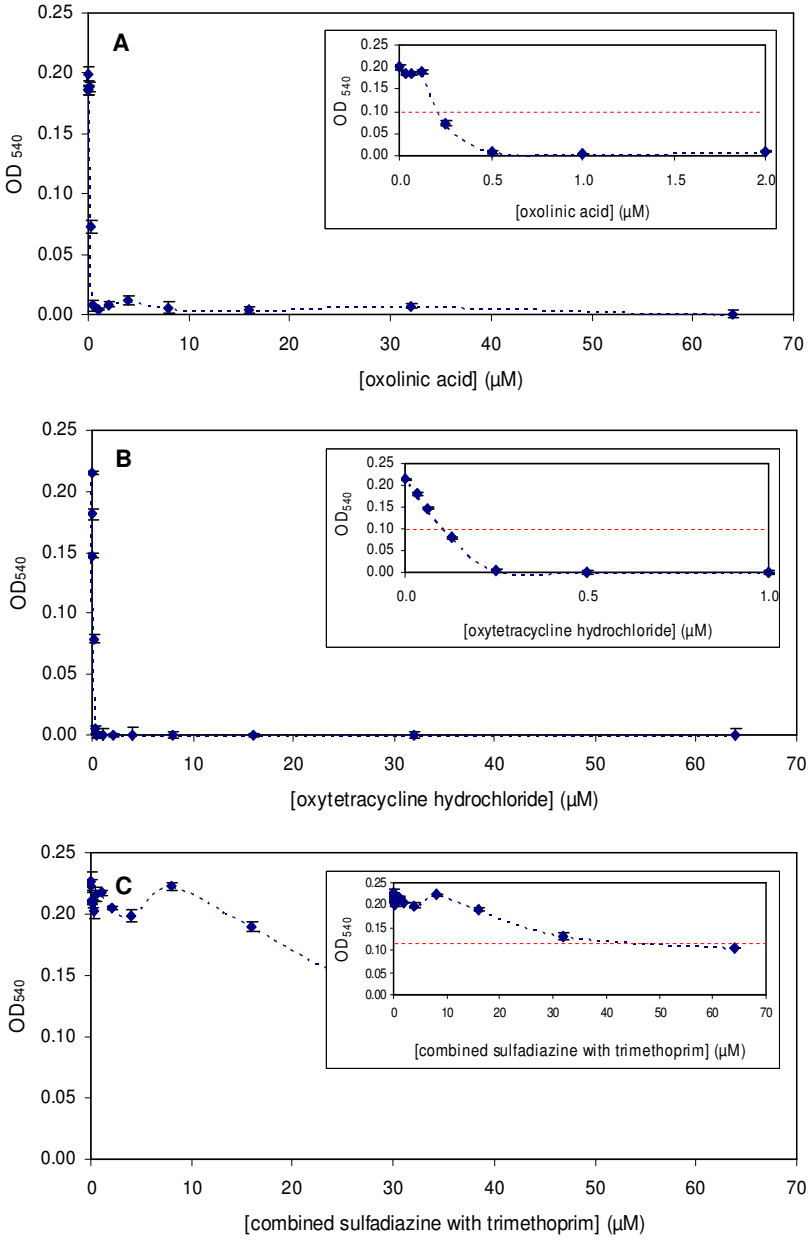

Figure A4.1. The different concentrations of antibiotics; oxolinic acid (A), oxytetracycline hydrochloride (B), and sulfadiazine/trimethoprim combination (C) were incubated with approximate 10^5 CFU/ml of *M. lysodeikticus*. The optical density at 540 nm was determined when the positive control reached to 0.2 after incubated at appropriate temperature. The MIC values are represented in rage by the red line indicates the inhibition of bacterial growth by 50% compared to the positive control. Values are represented as means±standard error (vertical bars); n=3.

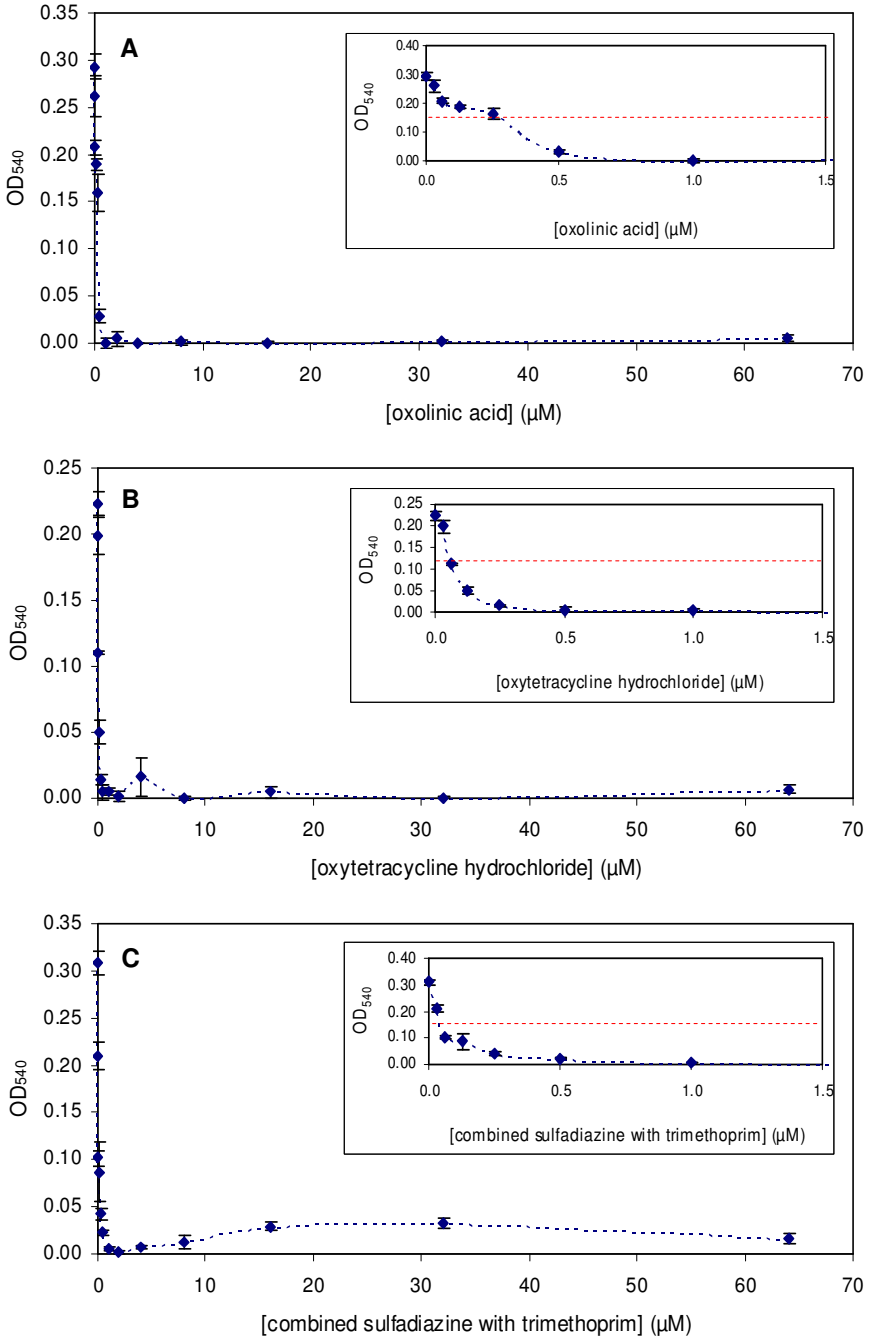

Figure A4.2. The different concentrations of antibiotics; oxolinic acid (A), oxytetracycline hydrochloride (B), and sulfadiazine/trimethoprim combination (C) were incubated with approximate 10^5 CFU/ml of *P. citreus*. The optical density at 540 nm was determined when the positive control reached to 0.2 after incubated at appropriate temperature. The MIC values are represented in rage by the red line indicates the inhibition of bacterial growth by 50% compared to the positive control. Values are represented as means±standard error (vertical bars); n=3.

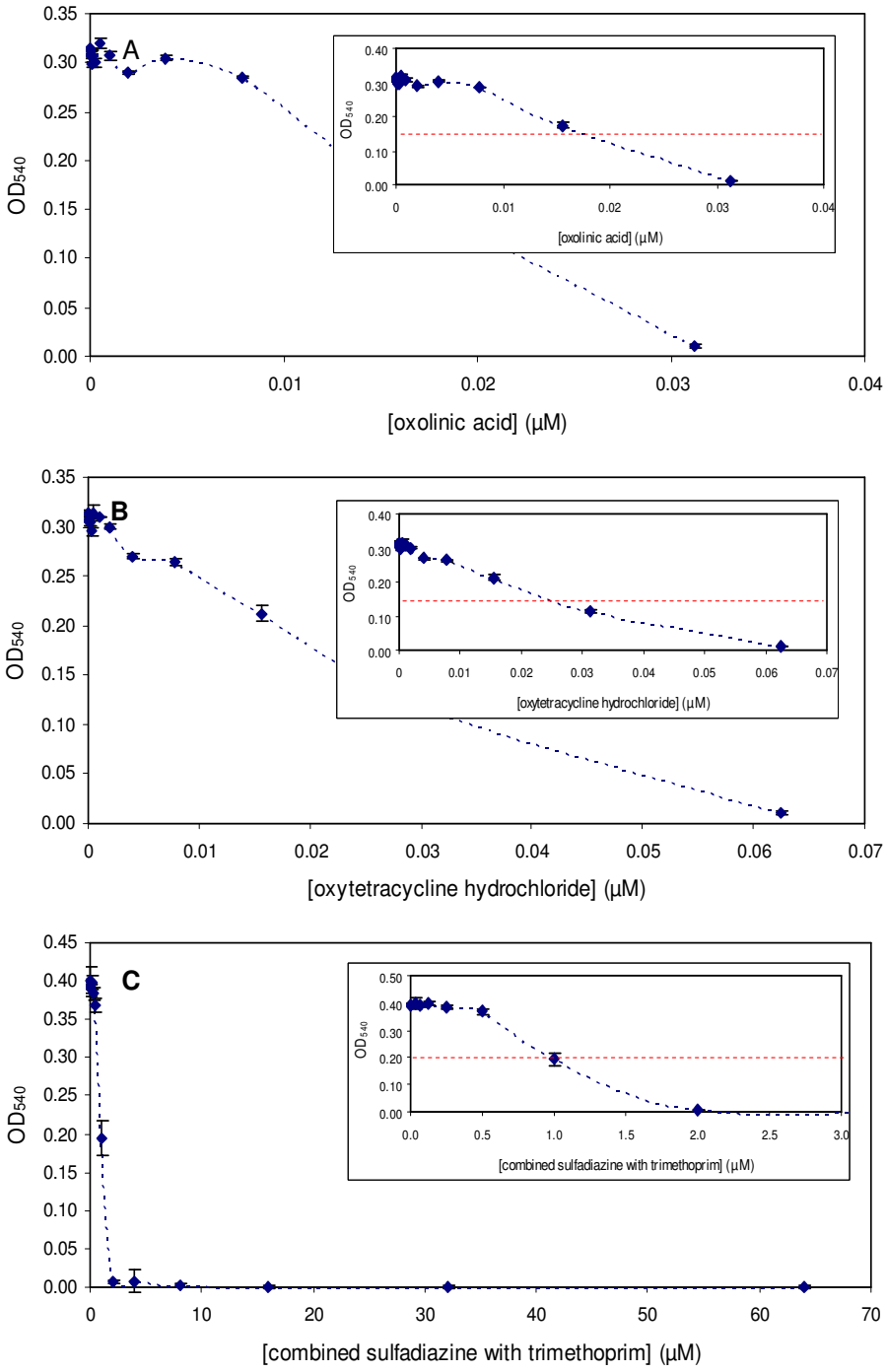

Figure A4.3. The different concentrations of antibiotics; oxolinic acid (A), oxytetracycline hydrochloride (B), and sulfadiazine/trimethoprim combination (C) were incubated with approximate 10^5 CFU/ml of *V. anguillarum* VI-F-258-3. The optical density at 540 nm was determined when the positive control reached to 0.2 after incubated at appropriate temperature. The MIC values are represented in rage by the red line indicates the inhibition of bacterial growth by 50% compared to the positive control. Values are represented as means±standard error (vertical bars); n=3.

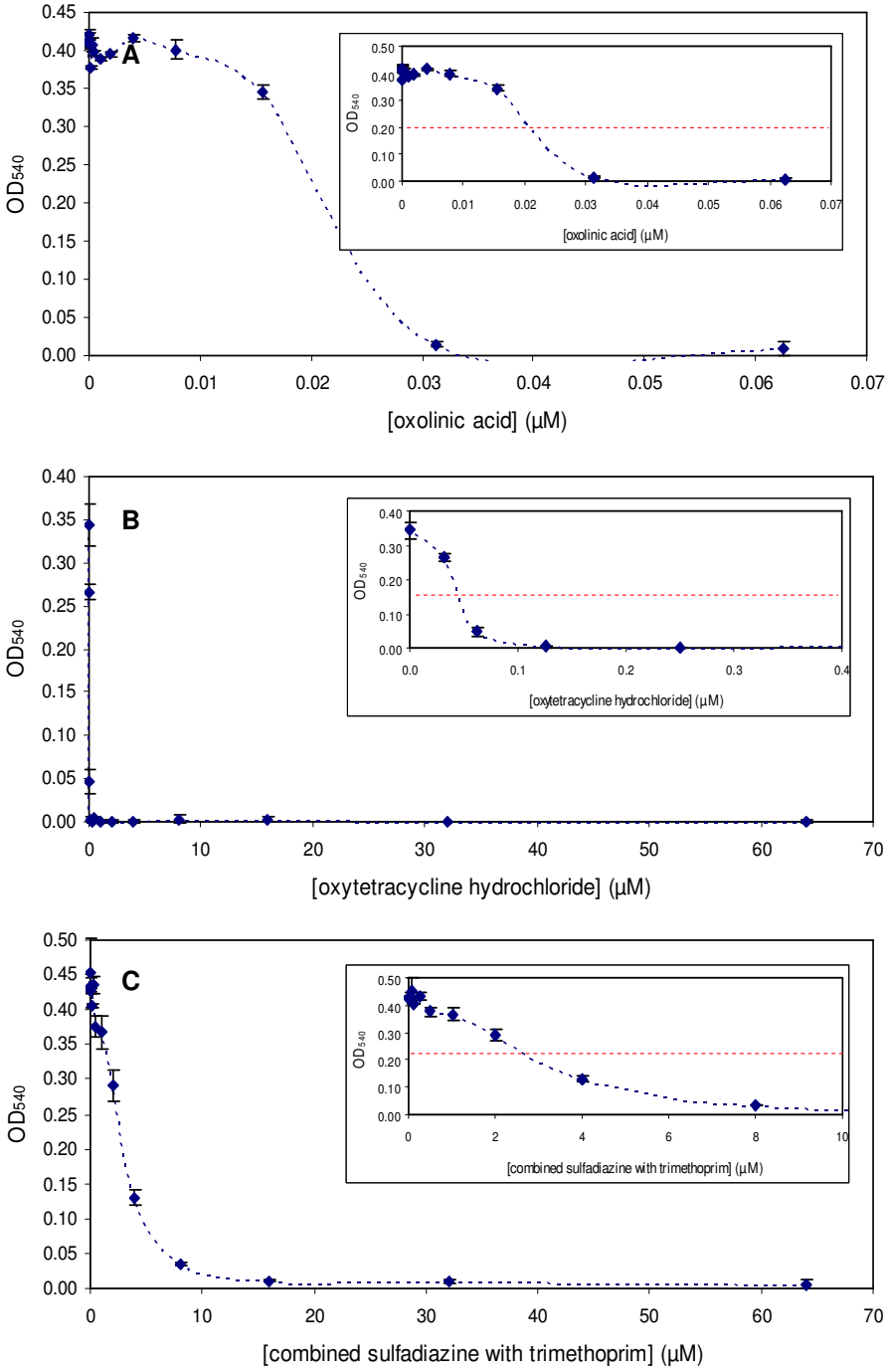

Figure A4.4. The different concentrations of antibiotics; oxolinic acid (A), oxytetracycline hydrochloride (B), and sulfadiazine/trimethoprim combination (C) were incubated with approximate 10^5 CFU/ml of *A. salmonicida* NCIMB 1102. The optical density at 540 nm was determined when the positive control reached to 0.2 after incubated at appropriate temperature. The MIC values are represented in rage by the red line indicates the inhibition of bacterial growth by 50% compared to the positive control. Values are represented as means±standard error (vertical bars); n=3.

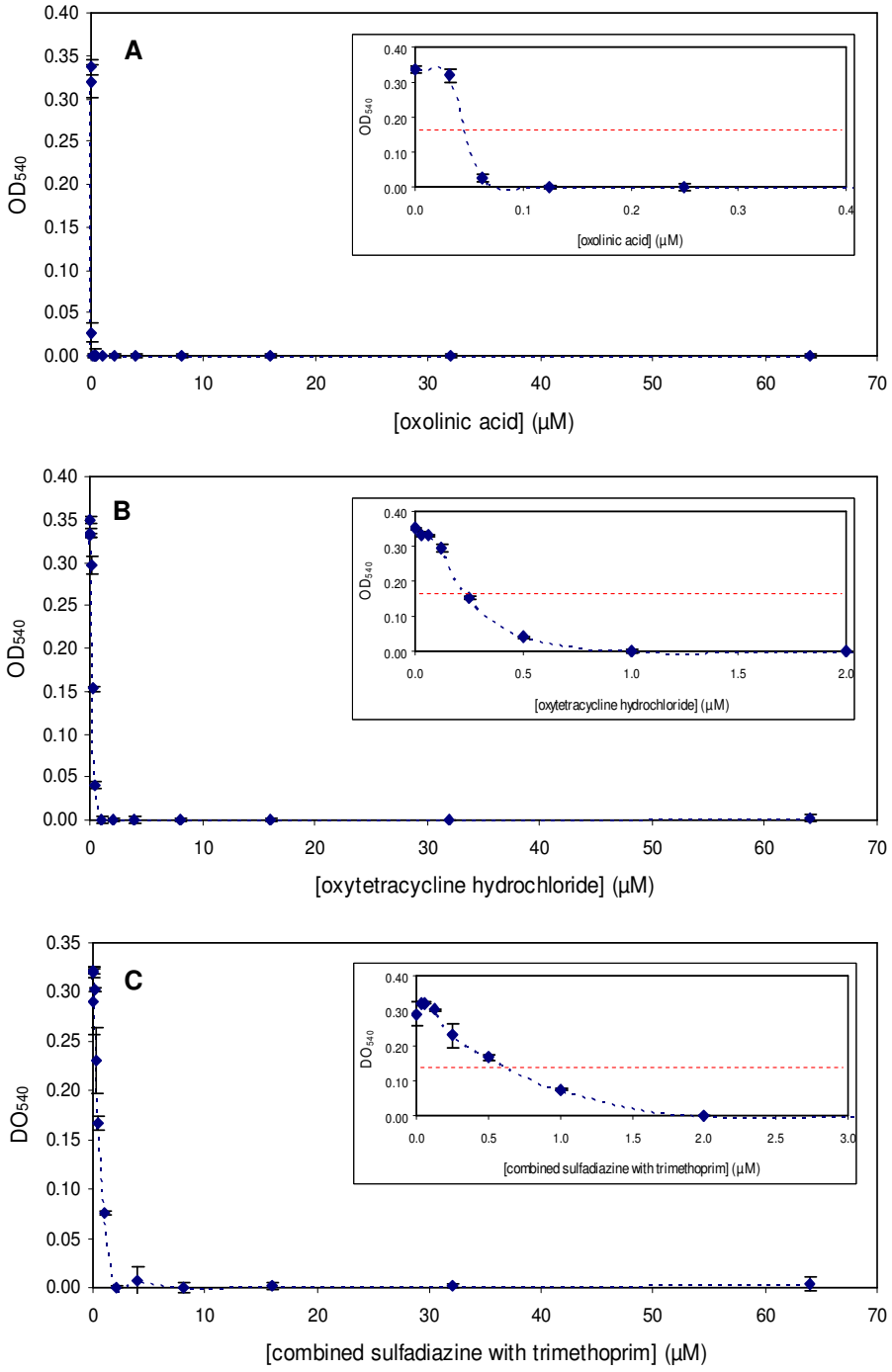

Figure A4.5. The different concentrations of antibiotics; oxolinic acid (A), oxytetracycline hydrochloride (B), and sulfadiazine/trimethoprim combination (C) were incubated with approximate 10^5 CFU/ml of *Y. ruckeri* VI 3629. The optical density at 540 nm was determined when the positive control reached to 0.2 after incubated at appropriate temperature. The MIC values are represented in rage by the red line indicates the inhibition of bacterial growth by 50% compared to the positive control. Values are represented as means±standard error (vertical bars); n=3.

Appendix 5

Minimal inhibitory concentration of combined antibiotic and synthetic cod piscidin

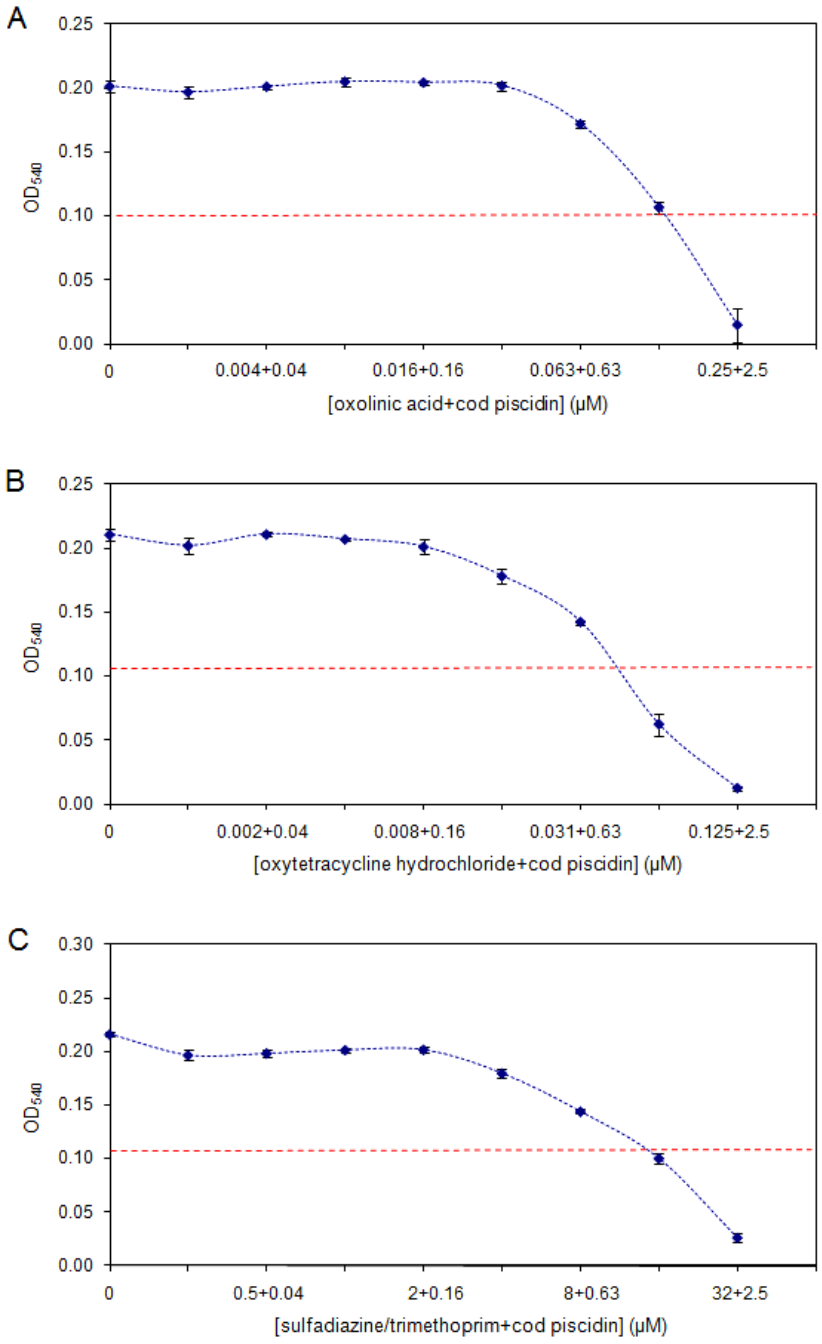

Figure A5.1. The MIC of combined synthetic cod piscidin with oxolinic acid (A), oxytetracycline hydrochloride (B), and sulfadiazine/trimethoprim (C) against *M. lysodeikticus*. The red line indicates the inhibition of bacterial growth by 50% compared to the positive control. Values are represented as means±standard error (vertical bars); n=3.

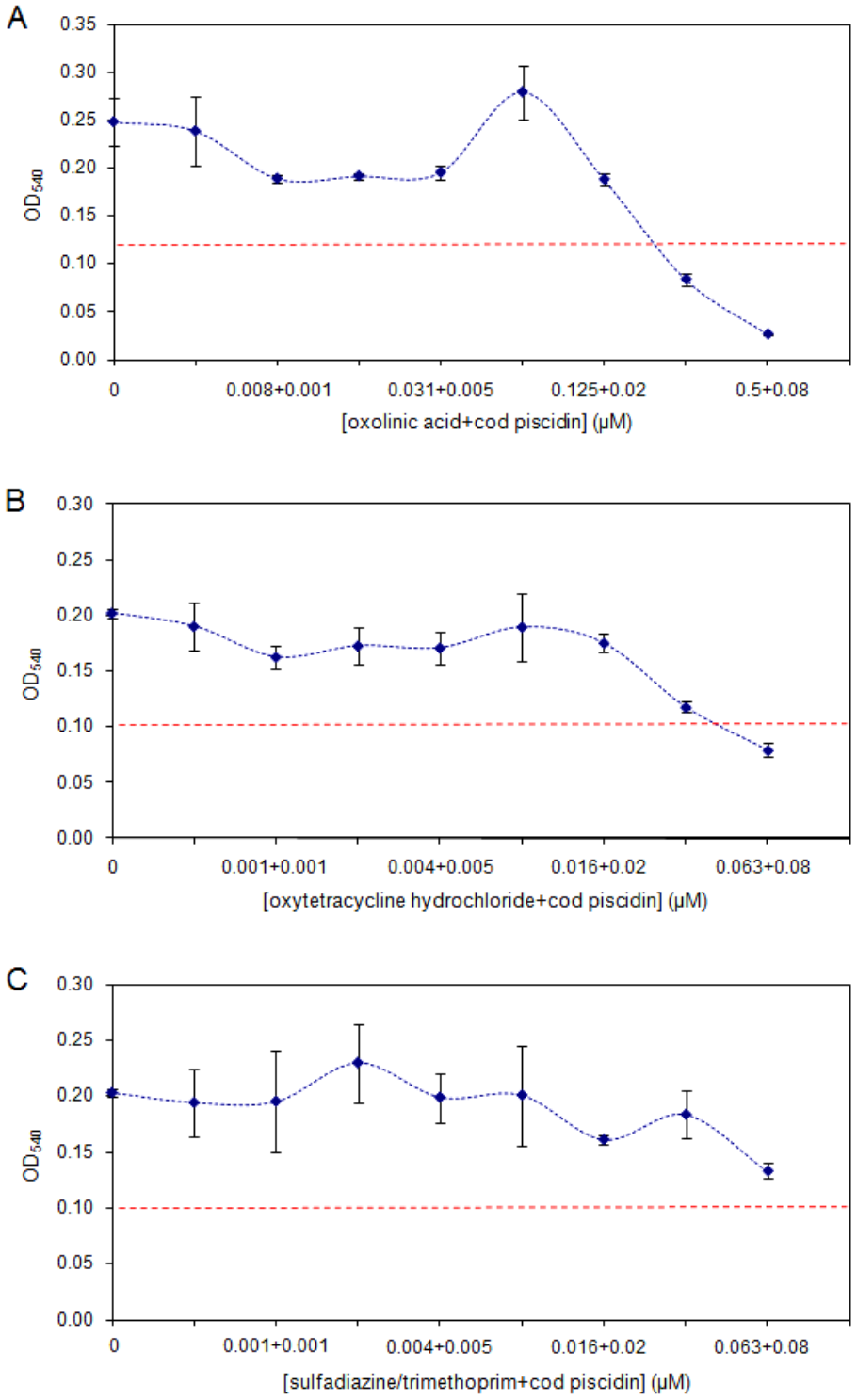

Figure A5.2. The MIC of combined synthetic cod piscidin with oxolinic acid (A), oxytetracycline hydrochloride (B), and sulfadiazine/trimethoprim (C) against *P. citreus*. The red line indicates the inhibition of bacterial growth by 50% compared to the positive control. Values are represented as means±standard error (vertical bars); n=3.

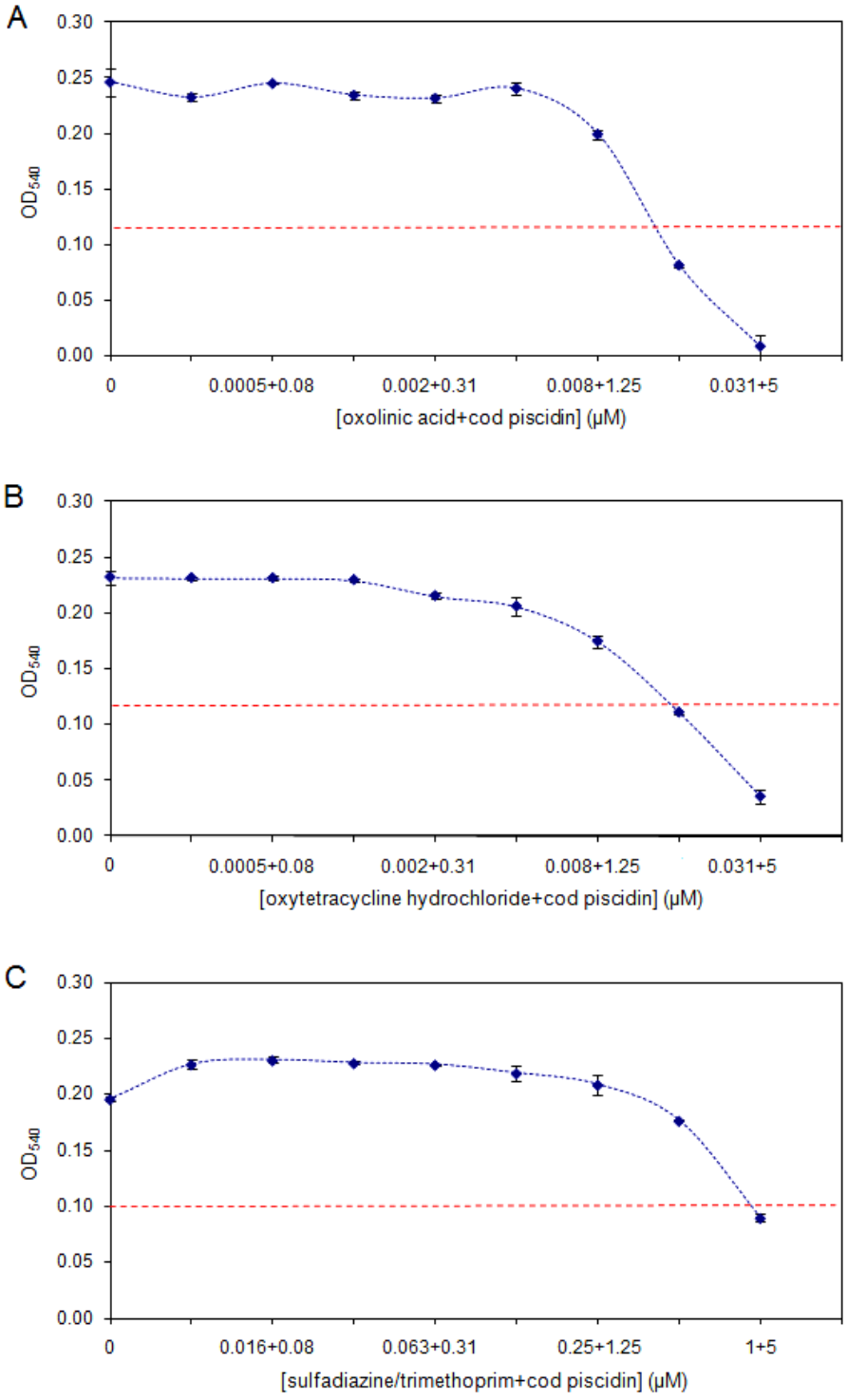

Figure A5.3. The MIC of combined synthetic cod piscidin with oxolinic acid (A), oxytetracycline hydrochloride (B), and sulfadiazine/trimethoprim (C) against *V. anguillarum* VI-F-258-3. The red line indicates the inhibition of bacterial growth by 50% compared to the positive control. Values are represented as means±standard error (vertical bars); n=3.

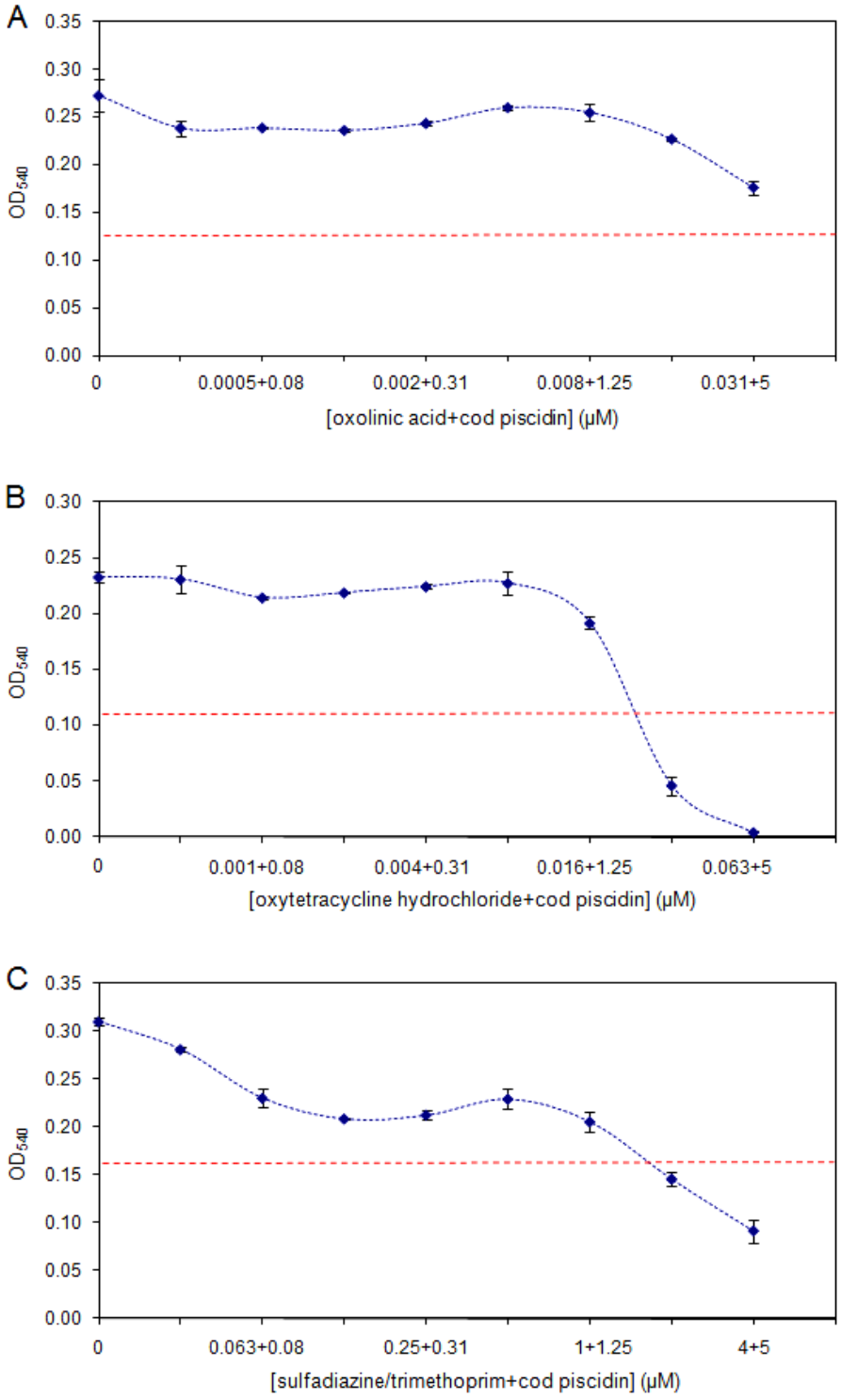

Figure A5.4. The MIC of combined synthetic cod piscidin with oxolinic acid (A), oxytetracycline hydrochloride (B), and sulfadiazine/trimethoprim (C) against *A. salmonicida* NCIMB 1102. The red line indicates the inhibition of bacterial growth by 50% compared to the positive control. Values are represented as means±standard error (vertical bars); n=3.

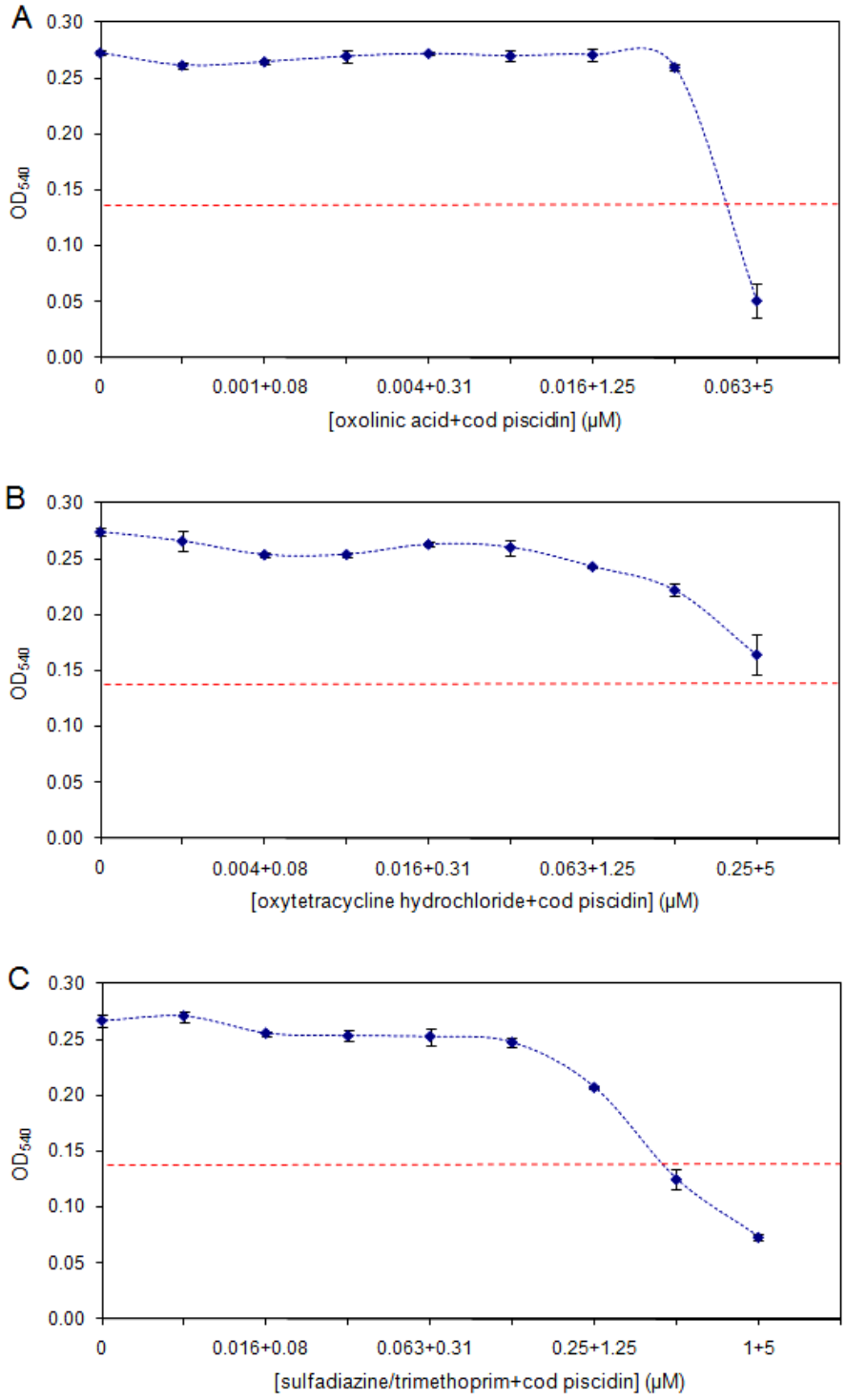

Figure A5.5. The MIC of combined synthetic cod piscidin with oxolinic acid (A), oxytetracycline hydrochloride (B), and sulfadiazine/trimethoprim (C) against *Y. ruckeri* VI 3629. The red line indicates the inhibition of bacterial growth by 50% compared to the positive control. Values are represented as means±standard error (vertical bars); n=3.

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