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THESIS

APPLICATION OF *BACILLUS* SPP.TO CONTROL THE PATHOGENIC BACTERIA OF AQUACULTURE

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The present study was aimed to select potential probionts from five different strains of *Bacillus* spp. The test pathogens included *Vibrio* spp. *of V. vulnificus, V. fluvialis, V. parahaemolyticus, V. alginolyticus, V. mimicus* and *V. cholerae* (non 01), which were isolated from the white feces disease infected pacific white shrimp (*Litopenaeus vannamei*), and *V. harveyi* which was isolated from the diseased post larvae of pacific white shrimp .*V. vulnificus* and *V. fluvialis* included two strains of yellow and green respectively. *Aeromonas hydrophila* was isolated from diseased Nile Tilapia (*Oreochromis niloticus*). The probiont strains were *Bacillus subtilis* B1, *B. pumilus* B2, *B. subtilis* B3, *B.subtilis* B4, and *B. subtilis* B5. The test of antagonism was carried out by cross streak and agar well diffusion plate assay (AWDA). Antagonistic activities were observed in B1, B2 and B5 against *A. hydrophila* by B1 and against all the *Vibrio* species by the other two strains. B1 and B5 were found to antagonize by inhibition to the pathogens while B2 colonized on the pathogens in cross streak method. In AWDA, these three probionts produced an antimicrobial substance evidenced by the presence of a clear zone after respective hours of incubation with the pathogens. B2 and B5 were found to retain antimicrobial activity up to seven days while B1 up to five days. There was no antagonism found in B3 and B4 against the selected pathogens. Based on the results of cross streak and AWDA, the probiontsB1, B2 and B5 were subjected to co-culture experiments where all the *Bacillus* species were cultured individually with the target pathogen for 120 hours and compared with the monoculture of each strain to determine the potentiality of competitive exclusion of the test probionts for the target pathogens. It was observed that B1 was able to reduce the growth of *A. hydrophila*by about 61.81%, while B2 and B5 inhibited the growth of *Vibrio* spp. by more than 90 and 85%, respectively, at the end of 120 hours of co-culture. For the characterization and partial purification of the antimicrobial substance the B2 was selected on the basis of the residual antimicrobial activity by the critical dilution method against the selected target pathogenic strain. The Cell free neutralized supernatant (CFNS) of B2 showed the moderate thermostability and could be stable up to 70°C for 60minutes and activity was greatly reduced while exposed to 80°Cfor 20minutes .The antimicrobials of B2 showed a stable activity within the pH range of 6 – 10 under room temperature (approximately 30°C) and 4°C . The residual antimicrobial activity of the crude CFNS showed a salinity tolerance up to 7 % of sodium chloride under 4 °C. The activity was absolutely loss while exposed to proteolytic enzyme like proteinase K and pepsin and there was no loss of activity while exposed to lipase. The partial purification of the CFNS was done by the amberlite XAD - 16 absorption after which it was subjected to SDS – PAGE for the determination of the molecular weight. The SDS –PAGE revealed a single protein band approximately 17 kDa. Initial characterization could categorize the antimicrobial substance in CFNS of B2 as bactericin like inhibitory substance. This study indicated that owing to the biochemical properties of B2 and antagonism produced by B2 and B5 against a wide range of pathogenic *Vibrio* spp. and *A . hydrophila* by B1 made these strains potential as a probiotic agent in aquaculture.

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Sanghamitra Nayak

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LIST OF ABBREVIATIONS

AM	*Aeromonus* spp**.** monoculture
A Co	*Aeromonus* spp**.** co-culture with *Bacillus* spp.
AU	Arbitrary activity unit.
AWDA	Agar well diffusion plate assay
CFNS	Cell free neutralized supernatant
CFU	Colony forming unit.
cm	Centimeter
°C	Degree celcius
EDTA	Ethylene diamine tetra acetic acid
gm	Gram
Hrs	Hour
kDa	Kilodalton
l	Liter
LAB	Lactic acid bacteria
M	Molar
MeOH	Methyl alcohol
min	Minute
ml	Milliliter
MW	Molecular weight.
N	Normal
NaCl	Sodium chloride
nm	Nanometer
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
rpm	Round per minute
SDS	Sodium dodecyl sulfate
TCBS	Thiosulfate –citrate bile salts sucrose agar.
TEMED	N, N, N', N'-tetramethylethylenediamine
TSB	Tryptic soy broth
TSA	Tryptic soy agar
µl	Microliter
VM	*Vibrio* spp. monoculture
V Co	*Vibrio* spp. co-culture with *Bacillus* spp.

APPLICATION OF *BACILLUS* SPP.TO CONTROL THE PATHOGENIC BACTERIA OF AQUACULTURE

INTRODUCTION

Aquaculture is the fast growing and rapidly expanding multibillion-dollar industry. It is contributing a major role in the economy of many Asian countries. And hence large scale production facilities are being introduced to meet the target of profitable and high economic turnover. This condition has led to the stressful condition for the aquatic animals that include the threat of diseases and of course the deterioration of environment, which in turn contributes in huge economic losses.

Prevention and control of diseases have led during recent decades to a substantial increase in the use of antibiotics. However, the utility of antibiotic as a preventive measure has been questioned, given extensive documentation of the evolution of antibiotic resistance among pathogenic bacteria. The massive use of antibiotics encourages natural emergence of antibiotic resistant bacteria, which can transfer their resistance genes to other bacteria that have never been exposed to the antibiotics (Karunasagar *et al*., 1994; Austin *et al*., 1995; Moriarty, 1997). At the same time the developed countries have raised the ban on the usage of antibiotics which is going to affect the export of the aquaculture products.

This situation led to the suggestions of suitable alternative of disease prevention methods, which could be the use of non-pathogenic bacteria as probiotic bio control agents (Austin *et al*., 1995; Moriarty, 1997; Gatesoupe, 1999; Mishra *et al*. 2001). The use of probiotics for disease prevention and improved nutrition in aquaculture is becoming increasingly popular due to an increasing demand for environment-friendly aquaculture. One of the commonly studied probiotics include the spore forming *Bacillus* spp. *Bacillus* spp. have been shown to possess adhesion abilities, produce bacteriocins (antimicrobial peptides) and provide immunostimulation (Cherif *et al*. 2001; Cladera-Olivera *et al*. 2004; Duc *et al*. 2004; Barbosa *et al*. 2005).

Probiotics protection can be due to different mechanisms such as nutritional competition or production of antibacterial substances. On the other hand, it has been reported that antibacterial substances are produced by aquatic bacteria isolated from various sources, and seem to play an important role in the antagonism of bacteria in aquatic ecosystems (Gauthier and Breittmayer, 1979; Nair and Simidu, 1987; Dopazo *et al*. 1988; Sugahara *et al*. 1988). Probiotic bacteria used in aquaculture are from a number of phylogenetic lineages, but are mainly derived from two bacterial divisions, the Gammaproteobacteria and the Firmicutes. The different menbers of the genus *Bacillus* are from Firmicutes lineage which includes *Bacillus subtilis* and *Bacillus pumilus*. Members of the *Bacillus* group are considered good producers of antimicrobial substances, including peptide and lipopeptide antibiotics, and bacteriocins. Antimicrobial substances produced by *Bacillus* species are the most important group of secondary metabolites that are widely exploited. This bacterium also competes for space on surfaces (e.g., gut wall) displacing other bacteria if they are present in high numbers. Also, they compete for nutrients and thus inhibit other bacteria from growing rapidly thereby exercising an inhibitory effect (Moriarty, 1998).

Proteases produced by *Bacillus* species are the most important group of secondary metabolites that are widely exploited (Ferrari *et al*. 1993). In addition, they also help in degrade organic accumulation in aquaculture ponds (Rengpipat *et al*. 1998; Verschuere *et al*. 2000).

Hence, the present study is going to analyze the potentiality of some strains of *Bacillus* species. The strains were obtained from Novozymes Biologicals, Inc., for experimental purposes only on the pathogenic bacteria isolated from white feces infected pacific white shrimps (*L.vannamei*) and diseased Nile Tilapia (*Oreochromis niloticus*) by focusing on the competitive and inhibitive potentiality against the selected pathogenic bacteria of aquaculture. An attempt was also made to partially purify and characterize the antimicrobial substance produced by *B. pumilus* (one of the selected strain) with special emphasis on the antagonistic activity of this antimicrobial substance on the selected pathogens.

OBJECTIVES

1. To study and preliminary selection of the potential *Bacillus* strains showed the better antagonism against the selected pathogen. The antagonism against the target pathogens was preliminary determined by the method of cross streak and Agar Well Diffusion Plate Assay.

2. To study the effect of competitive inhibition of the selected *Bacillus* strains against the target pathogens by the broth co-culture experiment.

3. To characterize and partially purify the antimicrobials produced by *B. pumilus* (one of the selected strain) with special emphasis on the antagonistic activity of this antimicrobial substance on the target pathogens.

LITERATURE REVIEW

Aquaculture – the global scenario - an overview

‘Aquaculture is the farming of aquatic organisms including fish, molluscs, crustaceans and aquatic plants. Farming implies some sort of intervention in the rearing process to enhance production, such as regular stocking, feeding, protection from predators, etc.’ (Code of Conduct for Responsible Fisheries of the Food and Agriculture Organization (FAO) of the United Nations; FAO, 1995). The intensification of aquaculture and globalization of the seafood trade have led to remarkable developments in the aquaculture industry.

Today, aquaculture is the fastest growing food producing sector in the world, with an average annual growth rate of 8.9% since 1970, compared to only 1.2% for capture fisheries and 2.8% for terrestrial farmed meat production systems over the same period. The most recent statistics indicate that the sector reached aquatic production of 9.4% annual percentage growth rate (APR) compared with meat production of farmed terrestrial animals such as pigs (APR of 3.1%), poultry (APR 5.1%), beef and meal (APR 1.2%), and mutton and lamb (APR 1.0%) (FAO, 2004). The per capita supply from aquaculture increased from 0.7 kg in 1970 to 6.4 kg in 2002, giving an annual growth rate of 7.2%. In 2002, the total world aquaculture production (including aquatic plants) was reported to be 51.4 million tonnes by volume and US$ 60.0billion by value. This represents an annual increase of 6.1% in volume and 2.9% in value, respectively, over reported figures for 2000. Asia produced 91.2% (by volume) and 82.0% (by value) of global aquaculture production. Of the world total, the China produced 71.2% of the total volume and 54.7% of the total value of aquaculture production. In 2002, the other nine top producers were India, Indonesia, Japan, Bangladesh, Thailand, Norway, Chile, Vietnam and the United States of America (Crespi, 2005). The majority of aquaculture production of fish, crustaceans and molluscs continues to come from the freshwater environment (57.7% by volume and 48.4% by value). Mariculture contributes 36.5% of production and 35.7% of the total value. Although brackishwater production represented only 5.8% of production volume in 2002, it contributed 15.9% of the total value, reflecting the prominence of high-value crustaceans and finfish (FAO, 2004).

Aquaculture is a significant socio-economic activity, especially for rural communities, contributing to livelihoods, food security and poverty alleviation through such mechanisms as income generation, employment, services, use of local resources, diversified farming practices, domestic and international trade and other economic investments serving the sector (NACA/FAO, 2001; Edwards *et al*. 2002).

Food fish has a nutritional profile superior to all terrestrial meat, being an excellent source of high quality animal protein and highly digestible energy, as well as an extremely rich source of omega-3 polyunsaturated fatty acids (PUFAs), fat-soluble vitamins (A, D and E) and water-soluble vitamins (B complex) and minerals (calcium, phosphorus, iron, iodine and selenium). At present, food fish represents the primary source of animal protein (contributing more than 25% of the total animal protein supply) for about one billion people within 58 countries worldwide, including many developing countries and low-income food-deficit countries (LIFDCs) (value excludes China). Consumption of omega-3 fatty acids from seafood products (including those from aquaculture) has been shown to prevent or ameliorate certain types of diseases (e.g., coronary heart disease and stroke; autoimmune disorders; cancers of the breast, colon and prostate; hypertension and rheumatoid arthritis) (Flick, 2004).

Diversification of Aquaculture

The aquaculture sector is highly diverse (NACA/FAO, 2001; Funge-Smith and Phillips, 2001.) in terms of:

(a) Species—an estimated 230 species of finfish, molluscs, crustaceans, aquatic plants, turtles, frogs, etc. are cultured.

(b) Culture systems—e.g., water-based systems, such as cages and pens, bottom/pole/rack/raft/long-line systems for molluscs, inshore and off-shore; land-based systems such as rain-fed ponds; irrigated or flow-through systems, tanks and raceways; land/ water-based systems, such as sea ranching; recycling systems such as high control enclosed systems, more open pond-based recirculation; monoculture and polyculture systems; integrated farming systems, such as livestock-fish, integrated agriculture-aquaculture, livestock-aquaculture.

(c) Culture environment—e.g., freshwater, brackish water, marine; inland, coastal and oceanic; temperate to tropical.

(d) Type of operation and scale—e.g., small-scale backyard ponds and hatcheries to commercial operations; hatchery holding of broodstock and production of seed, nursing systems, grow-out.

(e) Intensity of practice—e.g., extensive, semi-intensive, intensive.

(f) Type of management—from family to corporate ownership.

Constraints of Aquaculture

Taking into consideration the global population growth, it is clear that the future demand for aquatic products, even at the current level of per capita consumption, cannot be supplied by capture fisheries and therefore, the bulk will have to come from aquaculture. This goal will face considerable challenges, including management of aquatic animal health, which is already one of the major constraints to the development and expansion of the sector.

So to keep pace with the demanding situation, the current trend in aquaculture development is towards increased intensification and commercialization of aquatic production. Like other farming sectors, the likelihood of major disease problems occurring increases as aquaculture activities intensify and expand. Thus, the aquaculture industry has been overwhelmed with its share of diseases and problems caused by viruses, bacteria, fungi, parasites and other undiagnosed and emerging pathogens. Disease is now a primary constraint to the culture of many aquatic species, impeding both economic and social development in many countries. This situation can be attributed to a variety of multi-faceted and highly interconnected factors such as the increased globalization of trade in live aquatic animals and their products; the intensification of aquaculture through the translocation of broodstock, postlarvae, fry and fingerlings; the development and expansion of the ornamental fish trade (Subasinghe *et al*. 2001); the enhancement of marine and coastal areas through stocking aquatic animals raised in hatcheries; the misunderstanding and misuse of specific pathogen free (SPF) stocks (e.g. shrimps); unanticipated negative interactions between cultured and wild fish populations (Olivier, 2002); poor or lack of effective biosecurity measures; slow awareness on emerging diseases; climate change; all other human mediated movements of aquaculture commodities.

The control of endemic diseases imposes severe year-on-year costs on producers; for example, white-spot syndrome of shrimp virus (WSSV) has cost billions of dollars world-wide (Hill, 2002). The elimination of disease outbreaks, such as ISA (infectious salmon anaemia) in Scotland in 1998/1999, causes unexpected expenditure for both the industry and government. Emerging diseases also can have serious impacts on wild populations (Harvell *et al*. 1999). So the disease and disease control is one of the main focus to get a sustainable and profit making aquaculture industry.

The *Vibrio* infection in aquaculture

The disease by *Vibrio* infection is caused by gram negative bacteria in the family Vibrionaceae. This group of bacteria includes two important genera which can be significant fish pathogens. The genus *Aeromonas* includes several species which are important pathogens of freshwater fish, although they occasionally cause disease in marine species Bacteria in the genus *Vibrio* are other important pathogens of marine and brackish water fish, although they occasionally are reported in freshwater species.

Bacteria of the genus *Vibrio* are ubiquitous in marine and estuarine aquatic ecosystems in which shrimp occur naturally and are farmed. Several *Vibrio* spp. form part of the natural biota of fish and shellfish (Vanderzant *et al*. 1971; Colwell, 1984; Ruangpan and Kitao, 1991; Otta *et al*. 1999). The intensification of the penaeid shrimp culture industry and the transfer of aquatic organisms worldwide have been accompanied over the last two decades by an increased incidence of infectious pathogens. In this context, bacterial diseases due mainly to *Vibrio* species are often associated with low survival rates in hatchery or grow out conditions. Larval mortalities associated with the presence of *V. harveyi* have been reported in *Penaeus monodon* and *P. vannamei* in Indonesia (Sunaryanto and Mariam, 1986.), Thailand (Jiravanichpaisal *et al*. 1994.), India (Karunasagar *et al*. 1994.), Philippines (Baticados *et al*., 1990; Lavilla-Pitogo *et al*., 1990), Taiwan (Song and Lee, 1993; Liu *et al*. 1996) and Ecuador (Robertson *et al*. 1998). Disease outbreaks attributed to other *Vibrio* species such as *V. alginolyticus, V. damsela, V. parahaemolyticus, V. vulnificus* and *V. penaeicida* have been observed in nursery or growout ponds of *P. vannamei, P. monodon, P. japonicus* and *P. stylirostris* in Ecuador (Lightner, 1992), Malaysia (Anderson *et al*. 1988.), Taiwan (Song *et al*.1993; Lee *et al*., 1996.), Philippines (Alapide-Tendencia and Dureza, 1997.), Japan (Takahashi *et al*. 1985a; De La Pena *et al*. 1993.) and New Caledonia (Costa *et al*, 1998; ˜Mermoud *et al*. 1998..) Outbreaks of Luminous Vibriosis caused by *Vibrio harveyi* is a major constraint in shrimp production with potentially catastrophic impact (Lavilla-Pitogo *et al*. 1998; Ruangpan, 1998).

Disease signs range from localized cuticular lesions, oral and enteric infections to septicaemia (Lightner, 1996). The natural abundance of *Vibrio* spp., their ubiquity, multiplication rates and ability to adapt to environmental changes in shrimp culture ecosystems increase these preoccupations. Nevertheless, their precise etiological role remains uncertain and, as a consequence, they have been described in a wide range of shrimp disease either as opportunistic agents or true pathogens, and sometimes alternately.

Vibriosis occurs in cultured and wild marine fish in salt or brackish water, particularly in shallow waters during late summer. It was originally believed that scavenger fish feeding around the farms were the natural reservoir of *V. anguillarum*, and contact between fish seems to be an important factor for the spread of this pathogen. However, there is evidence that *V. anguillarum* is normally present in the intestinal microflora and food of cultured and wild healthy fish. The temperature and quality of the water, the virulence of the *V. anguillarum* strain and stress on the fish are important elements influencing the onset of disease outbreaks.

The occurrence of *Aeromonas hydrophila* in aquaculture

Aeromonas hydrophila has been recovered from a wide range of freshwater fish species worldwide (Austin and Adams 1996). Conflicting views have been expressed concerning whether *A. hydrophila* is a primary pathogen of freshwater fish or a secondary opportunistic pathogen of compromised or stressed hosts (Jeney and Jeney 1995). *A. hydrophila* has been associated with tail and fin rot, and epizootic ulcerative syndrome (EUS) (Austin and Adams 1996, Roberts 1997). *A. hydrophila* has also been described as the dominant infectious agent of 'fish-bacterial-septicaemia' in freshwater cultured cyprinid fishes, mainly crucian carp *Carassius carassius*, Wuchang bream *Megalobrama amblycephala* and silver carp *Hypophthalmichthys molitrix* in the Zhejiang province and other provinces in the South- east of China between 1989 to 1993 (Qian *et al*. 1997).

Aeromonas hydrophila causes disease in fish known as "Motile Aeromonas Septicemia" (MAS), "Hemorrhagic Septicemia," "Ulcer Disease," or "Red-Sore Disease." The many synonyms of this disease relate to the lesions caused by this bacterium which include septicemia where the bacteria or bacterial toxins are present within numerous organs of the fish, and ulcers of the fish's skin.

Aeromonas hydrophila is a ubiquitous gram-negative rod-shaped bacterium which is commonly isolated from fresh water ponds and which is a normal inhabitant of the gastrointestinal tract. The disease caused by this bacterium primarily affects freshwater fish such as cattfish, several species of bass, and many species of tropical or ornamental fish. Many have considered *Aeromonas hydrophila* to be an opportunistic pathogen. This seems like a contradiction in terms, since most bacteria which are termed"opportunistic" usually do not cause disease unless other factors are involved, and those bacteria which are considered a "pathogen" cart cause disease regardless of other factors. However, the term "opportunistic pathogen" conveys that

Aeromonas hydrophila always is capable of producing disease if given the chance.

Fish infected with *Aeromonas hydrophila* may have many different symptoms. These range from sudden death in otherwise healthy fish to lack of appetite, swimming abnormalities, pale gills, bloated appearance, and skin ulcerations. The skin ulcers may occur at any site on the fish and often are surrounded by a bright rim of red tissue. Other organs commonly affected with this disease include the gills, kidneys, liver, spleen, pancreas, and skeletal muscle. The symptoms vary since they are dependent upon a number of factors including the virulence of the organism, the resistance of the fish to infection, the presence or absence of bacteremia or septicemia, and stress factors associated with the fish. Because of the variability of these symptoms, the diagnosis of this disease based only upon symptoms is highly unreliable and may be economically disastrous to the fish producer.

Relevance of Probiotics

When faced with disease problems, the common response has been to turn to antimicrobial drugs (hereafter referred to as ADs). The livestock and aquaculture experienced widespread use of ADs in their practices. While the use of such products has an obvious benefit to treat animals infected by bacterial disease, the use of ADs has been either prophylactic (preventative), or for growth enhancement (Van den Bogaard and Stobberingh, 2000). Certain ADs have been shown to positively influence growth of livestock and used widely (Acar *et al*. 2000; Witte, 2000; Wierup, 2001; Phillips *et al*. 2004). Given this, and the desire to prevent establishment of pathogenic bacteria, it is argued that ADs have been widely overused (Aarestrup, 1999 and Schwarz *et al*. 2001). Schwarz *et al*. (2001) provided a good overview of AD use in animals and the potential hazards associated with this.

The use of ADs in agriculture and aquaculture has led to the emergence of antibiotic resistant bacteria (hereafter referred to as ARB) (Schwarz *et al*. 2001; Akinbowale *et al*. 2006). In aquaculture this was felt most dramatically in the shrimp industry where massive increases in production, overcrowding of animals and unchecked antibiotic usages led to the emergence of numerous ARB and production crashes in many Asian countries (Karunasagar *et al*. 1994; Moriarty, 1999). For example, production figures for shrimp in the Philippines dropped by 55% in 2 years; from 90,000 to 41,000 tonnes between 1995 and 1997. In fact, it has never recovered and, in 2002, a mere 37,000 tonnes was produced. An industry previously worth US $760 million is now worth only $240 million (FAO, 2007). Similarly, Thai shrimp production dropped by 40% between 1994 and 1997 due to disease problems (Moriarty, 1999); bacterial pathogens and shrimp viruses. Within aquaculture, there are numerous reports of ARB of farm origin (Karunasagar *et al*. 1994; Son *et al*. 1997; Molina-Aja *et al*. 2002; Chelossi *et al*. 2003; Sahul Hameed *et al*. 2003; Alcaide *et al*. 2005).

However, the risk is not just the potential loss to the farmer. The emergence of ARB on aquaculture farms could pose a risk to human health. There are many reports illustrating the transferral of resistant genes between bacteria (Son *et al*. 1997; Aarestrup, 1999; Van den Bogaard and Stobberingh, 2000; Witte, 2000; Schwarz *et al*. 2001). This process means ARB originating from a shrimp farm could potentially transfer plasmids to bacteria involved in human health problems. This is an area of current debate. Studies point to a farm animal origin in certain ARB genes that have made their way into human bacteria (Van den Bogaard and Stobberingh, 2000; Witte, 2000; Schwarz *et al*. 2001). However, recent reports argue this phenomenon (Acar *et al*. 2000; Phillips *et al*. 2004). The argument is based on the view that, although ARB have arisen in animal husbandry through use of antimicrobials, there is insufficient data to show a linkage to resistant gene transferral to humans. They argue in favor of the beneficial role antibiotics play in farming, and caution against premature, unscientific decisions in the restriction of antibiotic usage.

Regardless of which argument represents the true situation, governments and organizations have introduced much tighter restrictions for antibiotic usage in animal production. The European Union (EU) initially put a ban on the use of avoparcin in 1997, and in 1999, included virginiamycin, spiramcin, tylosin and bacitracin as banned growth promoters in animal feed (Turnidge, 2004; Delsol *et al*. 2005). In

2005, the EU implemented industries had a ban on the use of all non-therapeutic antimicrobials in animal production (Delsol *et al*. 2005). A leading example in the eradication of antibiotic use can be seen in the Norwegian salmon industry. After concern about the use of antibiotics in the late 1980s, there has been a 95% drop in usage from 50 tonnes to 1 tonne annually. During the same period, salmon production has increased 10-fold from about 5500 tonnes to 55,000 tonnes. Reasons for the turnaround have been attributed to the use of vaccines, better husbandry and selective breeding programs (Maroni, 2000).

There is a developing social attitude against unnecessary use of ADs and where possible, it is the move away from non- essential AD use that the responsible farmer now seeks. Given the threat that both ADs and bacterial pathogens pose to farmers, as well as in human health, alternatives are being sought. Probiotics is one field commanding considerable attention.

Probiotics the definition and principle

The term, probiotic, simply means “for life”, originating from the Greek words “pro” and “bios” (Gismondo *et al*. 1999). The most widely quoted definition was made by Fuller (1989). He defined a probiotic as “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance”. The concept of probiotic activity has its origins in the knowledge that active modulation of the gastrointestinal tract (GIT) could confer antagonism against pathogens, help development of the immune system, provide nutritional benefits and assist the intestinal mucosal barrier (Vaughan *et al*. 2002).

Typically, the lactic acid bacteria (LAB) have been widely used and researched for human and terrestrial animal purposes, and LAB are also known to be present in the intestine of healthy fish (Ringø and Gatesoupe, 1998; Hagi *et al*. 2004). Interest in LAB stems from the fact that they are natural residents of the human GIT with the ability to tolerate the acidic and bile environment of the intestinal tract. LAB also function to convert lactose into lactic acid, thereby reducing the pH in the GIT and naturally preventing the colonization by many bacteria (Mombelli and Gismondo, 2000; Klewicki and Klewicka, 2004). The most widely researched and used LABs are the lactobacilli and bifidobacteria (Corcoran *et al*., 2004; Ross *et al*. 2005; Senok *et al*. 2005).

Other commonly studied probiotics include the spore forming *Bacillus* spp. and yeasts. *Bacillus* spp. have been shown to possess adhesion abilities, produce bacteriocins (antimicrobial peptides) and provide immunostimulation (Cherif *et al*. 2001; Cladera-Olivera *et al*. 2004; Duc *et al*. 2004; Barbosa *et al*. 2005). The strains appear to be effective probiotics and commercial products containing such strains have been demonstrated to improve shrimp production to a level similar to that when antimicrobials are used (Decamp and Moriarty, 2006). *Bacillus* spp. hold added interest in probiotics as they can be kept in the spore form and therefore stored indefinitely on the shelf (Hong *et al*., 2005). The yeast, *Saccharomyces cerevisiae*, also has been commonly studied whereby immunostimulatory activity was demonstrated and production of inhibitory substances shown (Castagliuolo *et al*. 1999; Dahan *et al*. 2003; Van der Aa Kühle *et al*. 2005).

Multiple ways exist in which probiotics could be beneficial and these could act either singly or in combination for a single probiotic. These include inhibition of a pathogen via production of antagonistic compounds, competition for attachment sites, competition for nutrients, alteration of enzymatic activity of pathogens, immunostimulatory functions, and nutritional benefits such as improving feed digestibility and feed utilization (Fuller, 1989; Fooks *et al*. 1999; Bomba *et al*. 2002). It is often reported that a probiotic must be adherent and colonize within the GIT, it must replicate to high numbers, it must produce antimicrobial substances, and it must withstand the acidic environment of the GIT (Ziemer and Gibson, 1998; Dunne *et al*. 1999; Gismondo *et al*. 1999; Mombelli and Gismondo, 2000).

Several studies have demonstrated certain modes of probiotic action in effect in the aquatic environment. Bairagi *et al*. (2002) assessed aerobic bacteria associated with the GIT of nine freshwater fish. They determined that selected strains produced digestive enzymes, thus facilitating feed utilization and digestion. Ramirez and Dixon (2003), reported on the enzymatic properties of anaerobic intestinal bacteria isolated from three fish species, showing the potential role a probiotic could play. In a paper by Bairagi *et al*. (2004), the benefit of adding *B. subtilis* and *B. circulans* to the diet of rohu, *Labeo rohita*, was shown. In the search to replace fish meal with leaf meal in fish feed, they found that addition of the two fish intestinal *Bacillus* spp. increased performance as judged by a number of factors including growth, feed conversion ratio, and protein efficiency ratio. They attributed this to the extracellular cellulolytic and amylolytic enzyme production by the bacteria.

Although competition for adhesion sites has been widely suggested as a mode of action, there is little evidence in the literature to demonstrate this. There are studies reporting an adhesion of certain bacteria to intestinal mucus *in vitro*, but transferral of these to *in vivo* models has not produced supporting results (Hansen and Olafsen, 1999). Attachment ability of potential probiotics seen *in vitro* cannot be assumed to demonstrate the real effect *in vivo*. Vine *et al*. (2004.) demonstrated a competitive exclusion effect with five probiotics versus two pathogens on fish intestinal mucus. They found that the presence of one of the probiotics on the mucus inhibited the attachment of one of the pathogens tested. Interestingly, pre-colonization with the other probiotics encouraged attachment of the two pathogens. However, the general trend from their study showed that post treatment with the probiotics displaced the pathogen.

Although not directly concerning attachment competition, Yan *et al.* (2002) demonstrated that the production of antibiotic substances by two seaweed-associated *Bacillus* spp. was dependent on biofilm formation by the bacteria. This study highlighted a factor which might be important for some bacteria to be effective probiotics, i.e. surface attachment. This observation concurred with Fuller's (1989) definition of a probiotic, i.e. the requirement for GIT colonization. It has been proposed that the mechanism of competitive exclusion for attachment sites could be given a distinct advantage via addition of probiotic bacteria during the initial egg fertilization steps of larviculture, thereby “getting in there first” (Irianto and Austin, 2002a).

Application of probiotic in aquaculture

Aquatic animals have a much closer relationship with their external environment. Potential pathogens are able to maintain themselves in the external environment of the animal (water) and proliferate independently of the host animal (Hansen and Olafsen, 1999; Verschuere *et al*. 2000). These potential pathogens are taken up constantly by the animal through the processes of osmoregulation and feeding. A study with Atlantic halibut, *Hippoglossus hippoglossus*, showed the transition from a prevailing *Flavobacterium* spp. intestinal flora to an *Aeromonas* spp. / *Vibrio* spp. dominant flora occurred when first feeding commenced (Bergh *et al*. 1994). This study highlighted the impact that the external environment and feeding had on the microbial status of the fish.

Based on the intricate relationship an aquatic organism has with the external environment when compared with that of terrestrial animals, the definition of a probiotic for aquatic environments needs to be modified. Verschuere *et al.* (2000) suggested the definition “a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment”. In aquaculture, the range of probiotics evaluated for use is considerably wider than in terrestrial agriculture. Several probiotics either as monospecies or multispecies supplements are commercially available for aquaculture practices Apart from the nutritional and other health benefits (Smith *et al*,1993; Austin *et al*. 1995; Gildberg *et al*.1997; Gram *et al*. 1999; Carnevali *et al*. 2006.) certain probiotics as water additives can also play a significant role in decomposition of organic matter, reduction of nitrogen and phosphorus level as well as control of ammonia, nitrite, and hydrogen sulfide (Boyd *et al*. 1999). Numerous microbes have been identified as probiotics for aquaculture practices, many of which differ markedly in their mode of action. There are, however, some common mechanisms of action that have been reported for the majority of probiotic strains. Probiotics help in feed conversion efficiency and live weight gain (Al-Dohali *et al*. 2009; Saenz de Rdrignez *et al*. 2009.) and confer protection against pathogens by competitive exclusion for adhesion sites (Vine *et al*. 2004; Chabrillon *et al*. 2005.) production of organic acids (formic acid, acetic acid, lactic acid), hydrogen peroxide and several other compounds such as antibiotics, bacteriocins, siderophores, lysozyme (Silva *et al*. 1987; Sugita *et al*. 1998; Gibson *et al*. 1999; Yan *et al*. 2002; El- Dakar *et al*. 2007), and also modulate physiological and immunological responses in fish (Khattab *et al*. 2004; Balcazar *et al*. 2006.)

Effect of probiotics on systemic immunity

Among the numerous beneficial effects of probiotics, modulation of immune system is one of the most commonly purported benefits of the probiotics. The role of probiotics in modulating the immune system has been extensively investigated and reviewed in humans and animals (Fooks *et al*. 1999; Herich *et al*. 2002; Sartor *et al*. 2004; Galdeano *et al*. 2006) Most of the earlier studies in fish, dealt with growth promoting and disease protective ability of probiotics. However, in recent times much attention has been hitherto towards the immunmodulating effects of probiotics in piscine system. A lot of immunological studies have been performed in several fish using different probiotics and their potency to stimulate the teleost immunity both under in vivo and in vitro conditions is noteworthy (Aly *et al*. 2008; Zhou *et al*. 2009). Perusal of available literatures indicates that several probiotics either individually or in combination can enhance both systemic as well as local immunity in fish. The review is therefore, aiming to highlight the immunomodulatory activity of probiotics and also to evaluate the factors that regulate for the optimum induction of immune responses in piscine system.

Phagocytic activity

Phagocytic activity is responsible for early activation of the inflammatory response before antibody production and plays an important role in antibacterial defenses. Probiotics can effectively trigger the pahgocytic cells in host and enhancement of phagocytic activity by LAB group of probiotics such as *L. rhamnosus, L. lactis* and *Lactobacillus acidophilus* has already been observed in several animals .These probiotics are often used in aquaculture practices and supplementation of these probiotics either in viable or inactivated form is found to stimulate phagocytic activity in several fish species (Irianto *et al*. 2002, Panigrahi *et al*. 2004; Brunt *et al*. 2005; Brunt *et al*. 2007; Pieters *et al*. 2008).

Respiratory burst activity

Respiratory burst activity is an important innate defense mechanism of fish and shell fish. Several *in vitro* and *in vivo* studies showed significant increase in respiratory burst activity by various probiotics in many aquatic animals including fish. Probiotics like *Bacillus subtilis* and certain members of LAB group can stimulate respiratory burst activity in fish (Nikoskelainen *et al*. 2003; Salinas *et al*. 2005; Salinas *et al*. 2006; Zhou *et al*. 2009). Nevertheless 5×10^7 CFU/ml heat inactivated *Lactobacillus delbrueckii* subsp. *lactis* and *B. subtilis* under *in vitro* condition also found to enhance this activity of head kidney leucocytes of gilthead sea bream (*Sparus aurata*).

Lysozyme

Lysozyme, one of the important bactericidal enzymes of innate immunity is an indispensable tool of fish to fight against infectious agents Lindsay *et al*. (2004). Probiotics either single or in combination are found to trigger the lysozyme level in teleosts. The enhancement of lysozyme level by probiotics like *L. rhamnosus, Carnobacterium maltaromaticum*, *Carnobacterium divergens* in *O. mykiss* Panigrahi *et al*. (2004). *L. lactis* ssp. lactis, *L. mesenteroides* and *L. sakei* in brown trout (*Salmo trutta*) (Balcazar *et al*. 2007.) is reported.

Peroxidase and anti-protease activity

The peroxidase is an important enzyme that utilizes oxidative radicals to produce hypochlorous acid to kill pathogens. During oxidative respiratory burst, it is mostly released by the azurophilic granules of neutrophils. Dietary supplement of probiotic like *B. subtilis* alone or in combination with *L. delbrueck*ii ssp. *lactis* for 3 weeks lead to high serum protease activity. Probiotics like *E. faecium* also elevated the serum peroxidase level in *O. niloticus* when supplemented through water @1x10^7 CFU/ml in every 4 days for 40 days Wang *et al*. (2008).

Complement activity

Probiotics can enhance natural complement activity of fish (Panigrahi *et al*. 2005; Salinas *et al*. 2008.) and dietary as well as water treatment of many probiotics are often reported to stimulate the piscine complement components (Panigrahi *et al*., 2007; Salinas *et al*. 2008). It is also worth noting that non-viable probiotics can stimulate complement components in fish.

Cytokines

Cytokines are protein mediators produced by immune cells and contribute to cell growth, differentiation and defense mechanisms of the host (Peddie *et al*. 2002). Perusal of available literatures indicate that a number of probiotics can effectively modulate the production of pro-inflammatory cytokines such as interleukin-1 (IL-1), IL-6, IL- 12, tumor necrosis factor a (TNF-a), and gamma interferon (IFN-g) and anti-inflammatory cytokines such as IL-10 and transforming growth factor b (TGF-b) in many animals [87e89]. Probiotics like *Bifidobacterium longum, L. acidophilus, L. lactis, Lactobacillus paracascei* and *Lactobacillus plantarum* can up regulate the expression of various types of cytokines in various hosts. Probiotics like *L. rhamnosus, E. faecium* and *B. subtilis* are found to up regulate the pro-inflammatory cytokines like IL-1b1 and TGF- b in the spleen and head kidney of *O. mykiss*. (Panigrahi *et al*. 2007).

***Bacillus* spp and the antimicrobials**

Phylogenetically, bacteria belonging to the genus *Bacillus* belong to class I of the phylum Firmicutes i.e. the bacilli. Members of the genus *Bacillus* are Gram-positive, aerobic and endospore-forming bacteria that are characterized by their rod-shaped cell morphology, catalase production and their ubiquitous distribution. They are found in diverse environments such as soil and clays, rocks, dust, aquatic environments, vegetation, food and the gastrointestinal tracts of various insects and animals. This ability to survive and grow in such different ecosystems is based on the production of their robust endospores, their diversity in physiological properties and their growth requirements. *Bacillus* species are phenotypically and genotypically heterogenous and consequently, they exhibit quite diverse physiological properties such as the ability to degrade many different substrates derived from plant and animal sources, including cellulose, starch, proteins, agar, hydrocarbons and also biofuels (Lutz *et al*. 2006). Furthermore, some *Bacillus* species are heterotrophic nitrifiers, denitrifiers, nitrogen fixers, iron precipitators, selenium oxidizers, oxidizers and reducers of manganese, facultative chemolithotrophs, acidophiles, alkalophiles, psychrophiles, thermophiles and others (Priest, 1993; Slepecky and Hemphill, 2006). This diversity in physiological properties, which is reflected by the considerable diversity of *Bacillus* strains, thus allowed these bacteria to colonize a wide variety of ecological habitats. This ability of huge ecological diversification was potentiated by the production of spores, which are characterized by their remarkable ability of resistance and dormancy. The resistance of spores to heat, drying, disinfectants and other means of sterilization is of great relevance in food because of their economic concern in the food processing industry.

Members of the *Bacillus* group are considered good producers of antimicrobial substances, including peptide and lipopeptide antibiotics, and bacteriocins (Stein, 2005). The production of antimicrobial substances and sporulation capacity confer *Bacillus* strains with a double advantage in terms of their survival in different habitats. The presence of *Bacillus* species in food does not always imply spoilage or food poisoning, and some species or strains are even used in human and animal food production such as, for example, *Bacillus subtilis* strains that are used in Natto, an East Asian fermented food (Hosoi and Kiuchi 2003), production. Furthermore, specific *B. subtilis* strains are also used as a starter culture for fermenting soybeans into the traditional West African condiment dawadawa or for fermenting African mesquite seeds in the production of the Nigerian food condiment okpehe (Oguntoyinbo *et al*. 2007). A nontoxinogenic *Bacillus cereus* subspecies *toyoi* with probiotic properties is also used as an animal feed additive (Lodemann *et al*. 2008). On the other hand, other *Bacillus* species or strains have been implicated in food poisoning and food spoilage and these include *B. cereus*, *Bacillus coagulans*, *Bacillus mycoides*, *Bacillus weihenstephanensis*, *Bacillus licheniformis*, *B. subtilis*, *Bacillus pumilus*, *Bacillus thuringiensis* and *Bacillus sphaericus* (Jay *et al*. 2005; Granum, 2007; Stenfors Arnesen *et al*. 2008.) strains. Therefore, a rigorous selection process is required for the selection and development of *Bacillus* probiotic candidates (Senesi *et al*. 2001; Barbosa *et al*. 2005; Hong *et al*. 2005) or starter cultures, considering the intra species divergent virulence characteristics.

Antimicrobial peptides produced by *Bacillus* species

The capacity to produce antimicrobial peptides is widely spread among Gram-positive bacteria. These substances are directed against competitive microorganisms, and thereby generate a selective advantage for their producers. Bacterial antimicrobial peptides produced by ribosomal synthesis are commonly referred to as bacteriocins, and these are a heterologous group of proteinaceous antimicrobial substances that are produced by bacteria from every major lineage (Riley and Wertz 2002). They display a high degree of target specificity against related bacteria, although many have a wider spectrum of activity (Jack *et al*. 1995). Their proteinaceous nature implies a putative degradation in the gastrointestinal tract of humans and animals, suggesting their use as natural preservatives in foods (Cleveland *et al*. 2001). The first bacteriocin described was colicin produced by *Escherichia coli* (Cascales *et al*. 2007). However, currently, the bacteriocins most studied are those produced by lactic acid bacteria (LAB), because of their potential use as biopreservatives in the food industry, considering the 'generally recognized as safe' (GRAS) status of many strains and also those produced by industrially important *Bacillus* species, which have a history of safe use in food and industry . Many other antimicrobial substances that were not well characterized are known as bacteriocin-like inhibitory substances (BLIS). This term is often used when the peptide nature of the antimicrobial compound has not been confirmed. In the case of *Bacillus*, it is also important to corroborate the ribosomal synthesis of true bacteriocins, because this group of bacteria is prolific in the production of antimicrobial peptides (or lipopeptides) by nonribosomal synthesis (such as the iturins and others). Nonribosomally synthesized peptides are beyond the scope of this review, which focuses more specifically on bacteriocins or BLIS (whereby ribosomal synthesis is presumed, but may be unknown) produced by different *Bacillus* species.

Bacteriocins and BLIS produced by the *Bacillus* genus may be considered as the second most important after bacteriocins produced by the LAB. Strains from the *Bacillus* genus produce a diverse array of antimicrobial peptides, with several different basic chemical structures (Gebhardt *et al*. 2002; Stein, 2005). To date, no classification scheme has been devised for *Bacillus* bacteriocins despite all the classification efforts made with the bacteriocins of LAB. This is most probably a result of the lack of information on many of these bacteriocin amino acid sequences or the considerable diversity of the peptides/proteins produced by the bacilli. Some antimicrobial peptides have been included in the previously described groups or classes of the LAB bacteriocins. Examples are the lantibiotics, a unique class known for *Bacillus* antimicrobial peptides that are included in the Class I of the LAB bacteriocin classification scheme (Nes *et al*. 2007; Drider *et al*. 2006; Franz *et al*. 2007). Lantibiotics are ribosomally synthesized as precursor peptides. They are post-translationally modified by the dehydration of serine and threonine residues and subsequent intramolecular addition to cysteine, resulting in the formation of (β-methyl) lanthionine thioether bridges, the characteristic structural elements for lantibiotics (Chatterjee *et al*. 2005; Willey and van der Donk 2007; Bierbaum and Sahl 2009). Furthermore, several bacteriocins/BLIS produced by *Bacillus* species fall within Class II of LAB bacteriocins (as defined by Klaenhammer, 1993; Drider *et al*. 2006; Nes *et al*. 2007), which includes the pediocin-like bacteriocins (class IIa) and the two-peptide bacteriocins (class IIb). In this review, a simple classification scheme for the bacteriocins/BLIS produced by *Bacillus* species is being discussed.

Classification of *Bacillus* bacteriocins

The main classification scheme for antimicrobial peptides of ribosomal synthesis currently available is that of the LAB bacteriocins. The classification of LAB bacteriocins was previously well established by Klaenhammer (1993), and then adaptations or reclassifications were performed by van Belkum and Stiles (2000); Nes *et al*. (2007.) and then for the enterocin bacteriocins by (Franz *et al*., 2007). Some bacteriocins produced by *Bacillus* belong to the group of lantibiotics (Willey and van der Donk 2007; Asaduzzaman and Sonomoto 2009; Bierbaum and Sahl 2009). The lantibiotics have been divided previously into two groups or types: type A includes the elongated and positively charged lantibiotics, while type B includes the globular and noncharged lantibiotics (Jung, 1991). Type A lantibiotics can be further subgrouped (subgroups AI and AII) according to the enzymes that are involved in maturation of the lantibiotic molecules (Willey and van der Donk 2007). Class-AI lantibiotics are modified by the enzymes LanB and LanC, and processed by a dedicated serine protease LanP. Class-AII lantibiotics are modified by a single modification enzyme LanM, along with export, and are activated by an ABC-transporter LanT that has an N-terminal associated protease activity. In addition, the two-component lantibiotics, consisting of two post-translationally modified peptides that act synergistically, should be classified in a third group (Asaduzzaman and Sonomoto 2009). Nevertheless, two-component lantibiotics resemble subgroup AII lantibiotics in that each subunit is processed by a single modifying enzyme of the LanM type. Indeed, one of the subunits strongly resembles mersacidin, which is the prototype of AII subgroup. Other lantibiotics described recently (such as paenibacillin or sublancin 168) do not fit in any of the classes of lantibiotics described above. Given the increasing number of lantibiotics described within different bacterial groups, it is very difficult to establish a unifying classification scheme, and several other classifications have been proposed (Nes *et al*. 2007). Therefore, the *Bacillus* bacteriocin classification system should operate independent of that of LAB bacteriocin classifications, even though some compounds produced by both *Bacillus* and LAB has very similar characteristics.

Class I includes antimicrobial peptides that undergo different kinds of post-translational modifications. This class can be subdivided into four subclasses. Subclasses I.1–I.3 include peptides with modifications typical of lantibiotics (e.g. formation of lanthionine and β-methyl lanthionine residues), while subclass I.4 includes other unique modifications. Subclass I.1 includes type A lantibiotics with an enlongated structure, such as subtilin, ericin S and ericin A .Subclass I.2 includes the type B globular lantibiotic mersacidin, and other lantibiotics such as sublancin 168 and paenibacillin. Subclass I.3 includes the two-component lantibiotics, such as haloduracin and lichenicidin. Subclass I.4 includes the unique cyclic peptide subtilosin A that contains a head-to-tail peptide bond as well as particular sulfide bridges formed between cysteine groups and dehydrated amino acid residues. This classification is coherent with clustering obtained from the comparative analysis of published amino acid sequences. The subtilin and ericins form a coherent cluster, which is characterized by highly conserved regions in the mature peptide as well as in the leader peptide sequences. Interestingly, the A1 and A2 subunits of haloduracin and lichenicidin also form coherent clusters, suggesting that both two-peptide lantibiotics share a common origin. Mersacidin also shows homology with the A1 subunit of the two-peptide lantibiotics, and especially with the lichenicidin A1 subunit, with 25 conserved residues at identical positions, indicating its relatedness to the two-peptide lantibiotics.

Class II bacteriocins include small (0.77–10 kDa), ribosomally synthesized, nonmodified and linear peptides, which are heat and pH stable .This class can be subdivided into four subclasses. Subclass II.1 includes pediocin-like peptides with a conserved YGNGVXC motif near the N-terminus, and the coagulin produced by *B. coagulans* I_4, as well as the bacteriocins produced by *Bacillus circulans* and *Paenibacillus polymyxa* strains (SRCAM 37, SRCAM 602, SRCAM 1580) belong to this subclass. Subclass II.2 includes thuricin-like peptides with a conserved DWTXWSXL motif near the N-terminus, such as bacthuricin F4, thurincin H and thurincins S and 17 produced by *B. thuringiensis* strains, and cerein MRX1 produced by B. cereus strains. Comparative amino acid sequence analysis of thuricin-like peptides shows a coherent cluster. Subclass II.3 includes other linear peptides, such as lichenin produced by *B. licheniformis,* or *cereins* 7A and 7B.

Class III includes large proteins (>30 kDa) with phospholipase activity such as megacins A-216 and A-19213 produced by *Bacillus megaterium* strains.

Many other antimicrobial polypeptides of intermediate size (10–30 kDa) and other large antimicrobial proteins produced by bacilli are not included in this classification scheme due to the lack of data on their protein or gene sequences, although they will be described in the text under the category of BLIS.

Bacteriocin-like peptides

This section includes antimicrobial peptides and proteins that are not well characterized at either the genetic or the amino acid level, and that are active against bacteria, but not active against yeast or fungi The peptides produced by the different *Bacillus* species

Bacillus pumilus

Pumilicins include the plasmid-encoded peptide produced by B. pumilus PL10 (Lovett *et al*. 1976) and pumilicin 4 produced by *B. pumilus* WAPB4 (Aunpad and Na-Bangchang 2007). Pumilicin 4 with a molecular mass of 1994.62 Da, as determined by MS (mass spectrometry), is heat stable up to 121 °C for 15 min, active in the pH range of 3–9 and shows remarkable antibacterial activity against MRSA, vancomycin-resistant *E. faecalis* (VRE) and several Gram-positive test bacteria (Aunpad and Na-Bangchang 2007).

Bacillus subtilis

Bacillus subtilis strains are known to produce a wide variety of antibacterial and antifungal compounds (Stein, 2005). *Bacillus subtilis* ATCC 6633 produces several lantibiotics (Bierbaum *et al*. 1995; Paik *et al*. 1998; Stein *et al*. 2002, 2004, 2005), rhizocticin (Kugler *et al*. 1990) and two lipopeptides, surfactin and mycosubtilin, the latter being a member of the iturin family (Duitman *et al*. 2007). *Bacillus subtilis* strains may produce other antimicrobial substances, which have been characterized to a much lesser extent. Only the BLIS will be discussed in this section.

Betacin is a BLIS produced by *B. subtilis*, which is lysogenic for the temperate bacteriophage SPβ. It inhibits *B. subtilis* strains that do not harbor the SPβ prophage (Hemphill *et al*. 1980), *Bacillus subtilis* LFB112 from Chinese herbs produces a BLIS active against both Gram-positive and Gram-negative bacteria involved in domestic animal diseases, including *E. coli*, *Salmonella pullorum*, *P. aeruginosa*, *Pasteurella multocida*, *C. perfringens*, *M. luteus*, *Streptococcus bovis* and *S. aureus* (Xie *et al*. 2009). The active molecule of 6.3 kDa as estimated by SDS-PAGE, is heat and pH (3–10) stable and sensitive to proteinase K and proteinase E, but resistant to papain, trypsin and pepsin.

Bacillus subtilis 14B isolated from the rhizosphere of healthy plants (bitter almond) produces a BLIS (Bac. 14B) active against *Agrobacterium tumefaciens* (Hammami *et al*. 2009). Bac 14B has an estimated molecular weight of 21 kDa by SDS-PAGE and is stable to heat (up to 100 °C for 2 hrs) and sensitive to proteases. *Bacillus subtilis* 14B and Bac 14B are interesting as biocontrol agents, reducing the percentage of infections in plants caused by *A. tumefaciens* (Hammami *et al*. 2009).

Another uncharacterized antimicrobial substance was produced by *B. subtilis* LFE-1 isolated from an oil reservoir in Brazil (Korenblum *et al*. 2005). The antimicrobial substance produced by this strain was resistant to proteases, but sensitive to heat by autoclaving, and had a molecular weight between 3.5 and 12 kDa as estimated by dialysis.

Application and perspectives

Great efforts have been made in recent years to unravel the production of lantibiotic-type bacteriocins by *Bacillus* species, to decipher the enzymatic mechanisms involved in lantibiotic biosynthesis and to elucidate the genetic determinants and regulation of gene expression. Research in this field has been fuelled by massive genome sequencing, which allows the detection of putative new bacteriocins and to evaluate the distribution of key enzymes involved in bacteriocin synthesis between genomes of related and unrelated species. Hopefully, this streaming information will allow a better understanding of the significance of bacterial antagonism mediated by antimicrobial peptides in nature. The bacilli can also produce a wide array of nonmodified bacteriocins, some of which (such as the pediocin-like bacteriocins) seem to be widely disseminated among other bacterial species, while others (such as some thuricins) are commonly produced by many strains of the bacilli. This brings about many questions about the mechanisms of horizontal gene transfer involved in the dissemination of genes involved in bacteriocin production.

In spite of the knowledge already available on the production of true bacteriocins by bacilli, the scientific literature reports on many other not well-characterized antimicrobial substances of proteinaceous nature, the BLIS, most of which also fall in the range of low-molecular-weight peptides. Unfortunately, none of these have been sequenced at the protein or DNA level to provide insights into their nature or relatedness to already-known bacteriocins. Additional research efforts are clearly needed in this field, especially when considering the activity that these compounds display towards food-pathogenic bacteria or against fungi.

Bacteriocins from *Bacillus* species offer a much broader spectrum of potential applications compared with LAB bacteriocins. The use of *Bacillus* bacteriocins in food preservation is just starting to be investigated. This is a promising field of research, because many bacteriocins from bacilli may solve the limitations of LAB bacteriocins due to their sometimes broader inhibitory spectra against Gram-negative bacteria and even against fungi. Promising results have been reported already in the fields of human health, including the control of pathogenic bacteria such as MRSA, *G. vaginalis* and others, or inhibition of intestinal *C. difficile*. They could also be applied to improve animal health and the safety of animal products, such as for example in the control of animal mastitis, for inhibition of enteric pathogens (such as Campylobacter) in poultry, but also to improve rumen fermentation through the selective inhibition of undesired microbiota and selective proliferation of strains with improved hydrolytic activities on plant material.

Many *Bacillus* species are natural inhabitants of soil and elicit close growth-promoting relationships with plants. Some of the relevant mechanisms responsible for these symbiotic relationships are mediated by antimicrobial peptides that inhibit plant-pathogenic fungi or bacteria or even promote nodulations for nitrogen fixation (as in the case of thuricin 17). Bioprotectants based on bacteriocin-producing bacilli could be applied to improve plant health and also to avoid the postharvest decay of fruits and vegetables.

Last, but not the least, bacteriocin-producing *Bacillus* strains could be exploited for environmental applications such as, for example, in the control of SRB causing biocorrosion. Strains of *Bacillus* also produce other biomolecules of interest such as biosurfactancts, amylases or proteases, of interest for environmental applications. The bacteriocin production capacity could be exploited as a selective advantage for the proliferation of strains with technological interest in natural or even industrial environments.

Hopefully, the number of patents on applications of *Bacillus* bacteriocins will increase in the near future, and the antimicrobial arsenal of this group of bacteria, which are widely disseminated and adapted to survive under extreme conditions, will be exploited in a more rational way.

MATERIALS AND METHODS

Materials

Media and Chemicals

Tryptic Soy Agar and Tryptic Soy broth or TSA and TSB; Himedia
Thiosulfate-citrate-bile salts-sucrose agar or TCBS agar; Himedia
Sodium chloride; NaCl 1.5% and NaCl 0.85%
Sodium hydroxide; Merck
Hydrochloric acid; Merck
Proteinase K; Sigma Chemicals, St. Louis, Mo, USA.
Pepsin; Sigma Chemicals ,St. Louis, Mo, USA.
Lipase; Sigma Chemicals ,St. Louis, Mo, USA.
Trypsin; Sigma Chemicals ,St. Louis, Mo, USA.
Amberlite XAD-16; Sigma Chemicals. St. Louis, Mo, USA.
Ethanolin; Merck
Isopropanol; Merck
Acetic acid; Merck
Comassie Brilliant Blue G (Sigma)
Molecular weight markers (Gene Direx, USA)
N,N'-Methylene-bis-Acrylamide; Bio-Rad
SDS (Sodium dodecyl sulfate); Bio-Rad
Tris; Bio-Rad.
TEMED(N,N,N',N'-Tetramethylethylenediamine); Bio-Rad
Ammonium Persulfate; Bio-Rad
Bromphenol Blue; Sigma
Glycine; Bio-Rad.

Instruments

Laminar flow
Shaker
Hot air oven
Incubator
Centrifuge
Thermometer
pH meter
Analytical Balance (4digits)
Spectrophotometer
0.22-micron pore size membrane filter
Mini-Protean II Dual slab cell (Bio –Rad)
Glass wares and micropipette of different ranges.

Methods

1. Experimental Preparation for preliminary screening of antagonistic strains of *Bacillus* spp.

1.1 Bacterial strains and culture condition

1.1.1 Probionts

Five strains of *Bacillus* spp. including four different strains of *B. subtilis* and one strain of *B .pumilus* were taken.(Figure 2 and Figure 3)

B1. *B. subtilis*
B2. *B. pumilus*
B3. *B. subtilis*
B4. *B. subtilis*
B5. *B. subtilis*

All the strains of *Bacillus* spp. were provided by the Novozyme Biologicals Inc., for the experimental purpose only.

1.1.2 Pathogenic Bacteria

Seven species of *Vibrio* spp. including *V. vulnificus*, *V. fluvialis*, *V. parahaemolyticus*, *V. alginolyticus*, *V. mimicus*, *V. cholerae* (non 01), which were isolated from the white feces disease infected pacific white shrimps (*L.vannamei*) were selected for the experiment. *V. vulnificus* and *V. fluvialis* included two strains of yellow and green colony producer strains respectively. *V. harvey*i AQVH was isolated from the diseased post larvae of pacific white shrimp (*L.vannamei*). One strain of *Aeromonas hydrophila* AQAH was isolated from diseased Nile Tilapia (*Oreochromis.niloticus*). All these pathogenic strains (Figure 2) were obtained from Aquaculture Business Research Laboratory, Faculty of Fisheries, Kasetsart University.

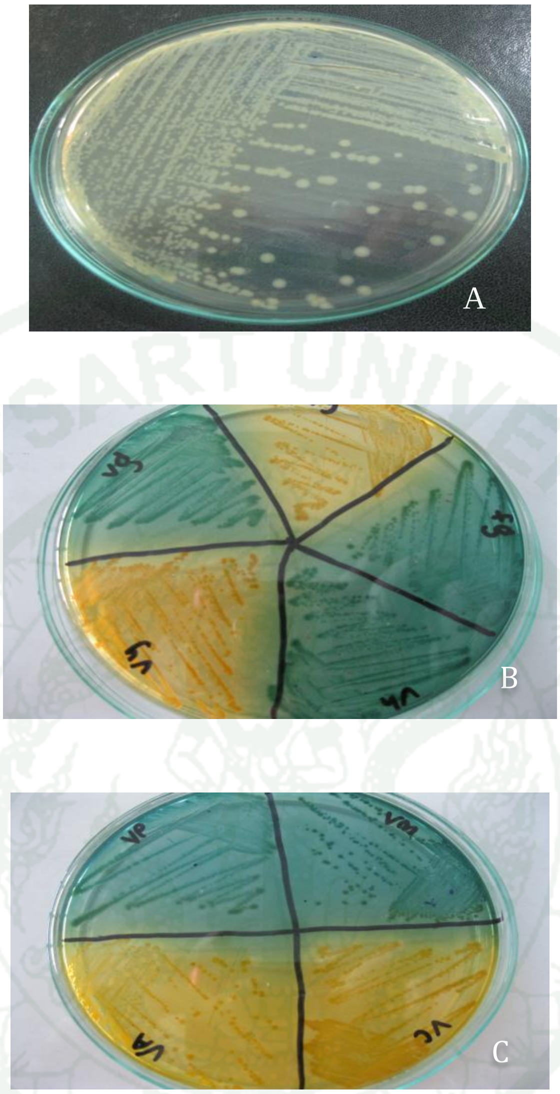


Figure 1 The selected pathogenic strains. (A) *A. hydrophila*; (B) (clock wise) Vg - *V. vulnificus* (Green), fy - *V. fluvialis* (yellow) , fg - *V. fluvialis* (Green), Vh - V. *harveyi*, vy - *V. vulnificus* (Yellow); (C) (clock wise) Vm - *V. mimicus* ,Vc - *V. cholerae* (non 01), Va - *V. alginolyticus*,Vp - *V. parahaemolyticus.*

Figure 2 Different *Bacillus* strains supplied by Novozymes Inc.

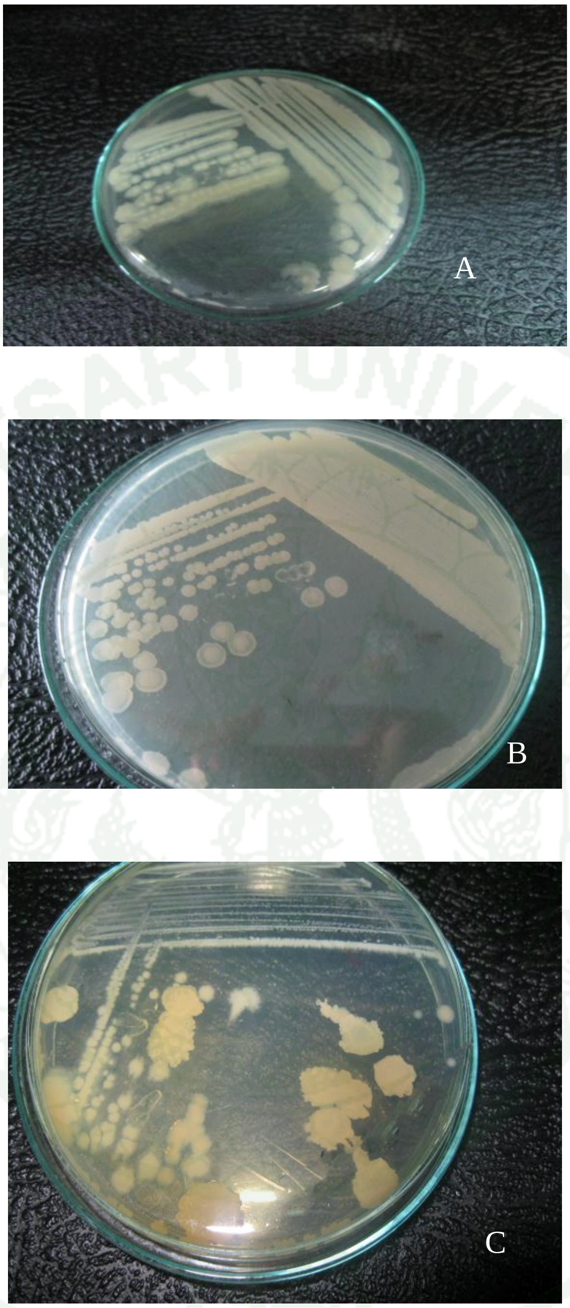


Figure 3 72 hours culture of B1, B2 and B5. (A) – *B1 Bacillus subtilis*, (B) – B2 *Bacillus pumilus* and (C) - B5 *Bacillus subtilis*.

Figure 4 Spore of B2, *Bacillus pumilus* (Gram staim)

1.2 Antimicrobial activity assay by Cross Streak method

Bacillus spp., *A. hydrophila* AQAH was cultured on Tryptic Soy Agar (TSA) and incubated at 30°C for 24 hours. *Vibrio* spp. was cultured on TSA supplemented with 1.5% NaCl (w/v). Antagonism test was carried out on TSA plate for *A. hydrophila* AQAH and TSA supplemented with 1.5% NaCl (w/v) for all the *Vibrio* spp. isolated from white feces diseases and and *V. harveyi* AQVH by cross streak method followed by the method of Lemos *et al*. (1985). Pathogenic bacteria were streaked in the first line and then *Bacillus* spp. was streaked perpendicular to it. Each type of bacterium streaking was done in triplicate and they were incubated at 30°C for 120 hours. Inhibition activities and colonization effect were observed at 24, 48, 72, 96 and 120 hours.

1.3 Antimicrobial activity assay by Agar Well Diffusion Plate Assay

1.3.1 Preparation of cell- free neutralized supernatant (CFNS)

To prepare the CFNS, the overnight culture of antimicrobial producer strains of all the *Bacillus* spp. was inoculated 1ml in 250 ml TSB and incubated at 30°C for 10 days at 150 rpm. After every 24 hours, aliquots of the culture was drawn from flask and subjected to centrifugation at 10000 x g for 10 min at 4°C to pellet the cells. (Figure 5) The supernatant was collected, pH was adjusted to 7.0 with either with 1 mol/L of NaOH or with1 mol/ litre of HCl and filtered through a 0.22µm (Millipore Ireland B.V.) membrane filter and used for the antagonistic activity assay by means of agar well diffusion plate assay. The prepared CFNS was used immediately.

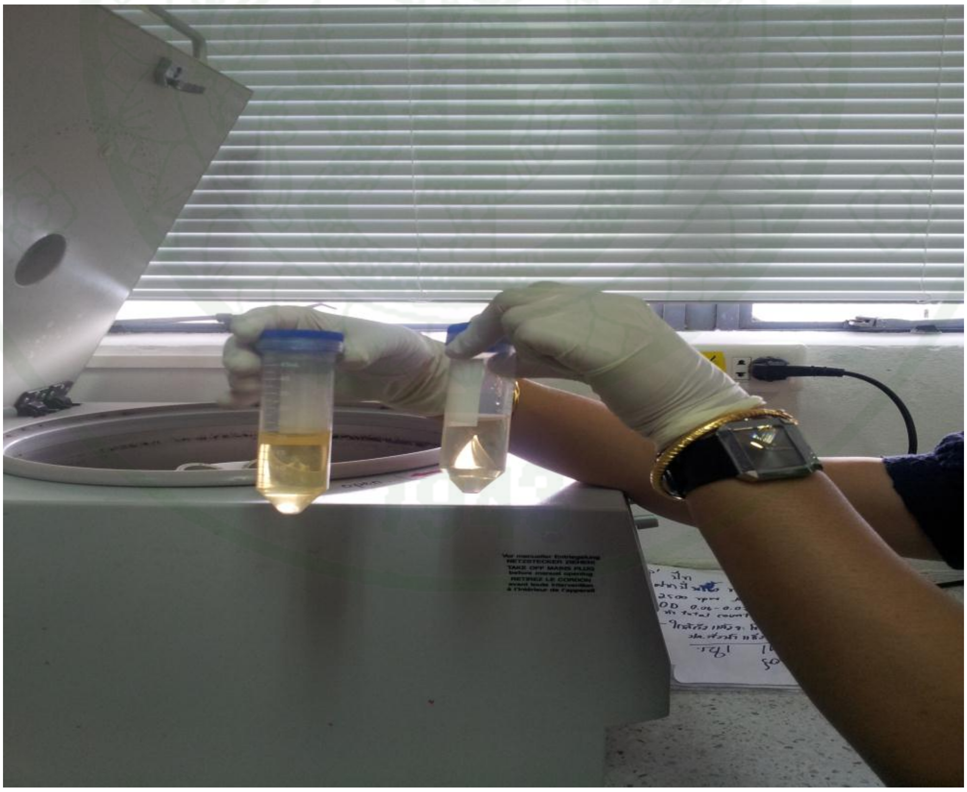


Figure 5 Extraction of CFNS of B2

1.3.2 The antimicrobial spectrum of the CFNS by Agar Well Diffusion Plate Assay

The agar well diffusion plate Assay method (Hence here after it is referred as AWDA) was carried out (Bauer *et al.*, 1966 with modification) to determine the antagonism. The indicator bacteria *Vibrio* spp. was cultured on TSB supplemented with 1.5% NaCl and *A. hydrophila* were cultured on TSB. Suspension from the over night culture of each indicator bacterial strain (100 µl) containing 10^7 CFU ml^{-1} was spread on the plates made up with Tryptic Soy Agar supplemented with 1.5% NaCl and TSA for *Vibrio* spp. and *A. hydrophila*, respectively and wells of 5 mm in diameter were punched in the agar with a sterile tip and filled with 50 µl of CFNS. Plates were pre-incubated for 2 hours at 4°C to allow the diffusion of any inhibitory metabolites into surrounding agar and then incubated at the optimum growth temperature (30°C) of the indicator microorganism. The plates were examined after 24 hours for the presence of a clear zone in the agar surrounding the well, which were considered as positive result. The measurements were recorded in triplicates. The diameter of the inhibition zone (clear zone) was measured in millimeters.

2. Experimental preparation to study the effect of competitive inhibition by the broth co-culture experiment.

2.1 Bacterial strains and culture condition

2.1.1 Selection of probionts

Based on the results of antagonism of the *Bacillus* spp. against the target pathogenic strains. The B1, B2 and B5 strains were selected for the study of broth co-culture experiment.

2.1.2 Pathogenic bacteria

Pathogenic bacteria are same as used in 1.1.2

2.2 Co-culture of *Bacillus* spp. with pathogenic bacteria in Tryptic Soy Broth

Based on the results of antagonism by the cross streak method and AWDA, *Bacillus* spp. was selected for the broth co-culture experiment with the pathogenic bacteria. B1 was co-cultured with *A. hydrophila*, B2 and B5 were co-cultured with all the *Vibrio* spp. but not with *A. hydrophila*. *Bacillus* and all the pathogenic bacteria were separately pre-cultured in 10 ml TSB for 24 hours with a shaking at 150 rpm. Tryptic Soy Broth of 10ml was inoculated with 10^5 CFU ml^{-1} pathogenic bacteria (from 24 hrs culture) which were inoculated together with 10^5 CFU ml^{-1}of *Bacillus* spp. of 24 hours grown culture. B1 was co-cultured with *A. hydrophila* AQVH in TSB. *Bacillus* spp. B2 and B5 were co-cultured with *V. harveyi* AQVH and all other strains of *Vibrio* spp.in TSB supplemented with 1.5% NaCl in the similar ratio of pathogen and probionts as 10^5 CFU ml^{-1} Each *Bacillus* and pathogenic bacteria had control group (mono-culture) where the TSB and TSB supplemented with 1.5% NaCl was inoculated only with *Bacillus* spp, *A. hydrophila*, and *Vibrio* spp. respectively at the same concentration that is 10^5 CFU ml^{-1} to compare the bacterial growth with the co-culture. Flasks were incubated at room temperature in a shaker with 150 rpm. All combinations were tested in triplicates. Samples were collected after 0, 24, 48, 72, 96 and 120 hours for enumeration of the number of bacteria. Counts of bacteria were taken in TSA plate for the total counts of bacteria and TCBS plate to count the number of *Vibrio* spp. (Only *Vibrio* spp. can grow on TCBS media). Competitive growth of the probionts and pathogens were analyzed by comparing the count of bacteria in co-culture with control or mono-culture.

3. Experimental procedure for the characterization and partial purification of the antimicrobials for one of the selected probiont strain

3.1 Determination of residual antimicrobial Activity of the probionts by the critical dilution method.

The antimicrobial activity of all the selected probionts (B1, B2 and B5) was further determined by the critical dilution method and the bacteriocin activity assay, described by Schillinger *et al*. (1993) and Cintas *et al*. (1995), respectively. To obtain the CFNS the *Bacillus* strain B2 and B5 were grown for 5 days at 30°C and B1 for 3 days and CFNS was prepared in the same method discussed before. The CFNS samples were sequentially diluted two times with 50µl distilled water. Each dilution was added to the well of 5mm diameter made up on the TSA (+1.5% NaCl) plate which was previously swabbed with the indicator strain of 10^7 CFU/ml. The arbitrary activity unit (AU) was defined as the reciprocal of the highest dilution that yielded a clear inhibition zone on the indicator strain and multiplied by dilution factor of 20 to obtain AU/ml of the original sample. So the titer of the antimicrobial solution, in AU/ml, was calculated as (1000/d) D, where D is the reciprocal of the dilution factor and d is the dose.

3.2 Selection of probiont for partial purification and characterization of antimicrobials

As per the result of the antimicrobial activity assay Arbitrary Activity Unit (AU/ml) and AWDA the antimicrobials of the *Bacillus pumilus* was selected for the partial purification and characterization.

3.2.1 Selection of pathogenic bacteria

Two strains of the pathogenic bacteria *V. parahamolyticus* (ABRC Laboratory) and *V. alginolyticus* (ABRC Laboratory) selected on the basis of the antagonism produced by B2 against these 2 strains as determined by Arbitrary Activity Unit (AU/ml) and AWDA.

3.3 Characterization of the antimicrobials by determination of the sensibility of heat treatment, proteolytic enzymes, range of pH, and salinity.

3.3.1 Sensibility to heat treatment

To evaluate the thermo stability of the neutralized supernatant of tested strain, *Bacillus pumilus*, was grown for 5 days in TSB at 30°C after which it was subjected to centrifugation to extract the CFNS in the same way as prepared for AWDA. The CFNS then heated at 40°C, 50°C, 60°C, 70°C, 80°C, 90°C, and 100°C for 60 mins and 121°C. for 15 min (in autoclave) and then cooled in ice water. The samples were withdrawn at each 20 and 60 minutes and the residual antimicrobial activity was determined by critical dilution method using *V. parahaemolyticus* (ABRC), and *V. alginolyticus* (ABRC) as indicator strains. And untreated sample was used as control.

3.3.2 Sensibility of antimicrobial substance to Enzymes

The CFNS of the tested strain of *Bacillus pumilus* was obtained after it was grown at 30°C for 5days and was treated with the proteolytic enzyme at a final concentration of 1mg /ml: proteinase k pH 7, pepsin pH 1.5 -2, and trypsin pH7 and also with lipase pH 8. All the chemicals were obtained from Sigma Fine Chemicals (St. Louis, Mo, USA.). After incubation at 37°C for 2 hours the samples were boiled 90°C for 5 minutes to inactivate the enzyme and cooled in ice water before analyzed for residual antimicrobial activity. Untreated CFNS (without enzyme) was used as control.

3.3.3 Sensibility of antimicrobial substance at different pH values

The tested strain of *Bacillus pumilus* B2 was grown for 5days at 30°C to obtain the CFNS by the process described above. The CFNS were adjusted to pH 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0 with 5mol/L NaOH and 5mol/L HCl. Now all the samples of the CFNS were divided in two groups, one was incubated at 30°C (room temperature) for 5 hours and the other was kept at 4°C for 24 hours. All the samples were adjusted to pH 6.5 - 7 before determining the antibacterial activity by critical dilution method.

3.3.4 Sensibility of antimicrobial substance to range of salinity

The effect of different concentration of NaCl on the antimicrobial activity was studied. Sodium chloride in a range of 0 – 10 % was added to the samples of the CFNS extracted in the same way as described before from the tested strain of *Bacillus pumilus* B2. .The samples were divided in to two groups. One group was kept at room temperature (approximately 30°C) for 24 hours, and another group was kept under 4°C for 24 hours, which were then subjected to determine the antimicrobial activity against the target pathogenic bacteria.

3.4 Partial purification of the antimicrobials from B2

3.4.1 Concentration of of antimicrobial substance using Amberlite XAD-16

The tested strain of B2 was grown at 1 L TSB broth for 5 days at 30°C. The culture was centrifuged at 10,000 g for 15 in at 4°C to obtain the CFNS. Purification was carried out as described by the Pilasombut *et al*., (2006). Antimicrobial substance adsorption was achieved by adding 20 gm of amberlite XAD-16 (Sigma, St.Lois, Mo) to 1L of CFNS and shaking at room temperature for 2hrs.(Figure 6) The hydrophobic amberlite adsorbing substances were collected (Figure 7), washed with 100ml of distilled water followed by 100 ml of 40%(v/v) ethanolin distilled water. Finally the antimicrobial substance was eluted with 100 ml of 70% isopropanol solution in distilled water. The eluate was evaporated to 5-10 ml by boiling in hot-plate.

3.5 Estimation of molecular weight of the antimicrobial substance by SDS-PAGE

The concentrated samples were analyzed for the antimicrobial assay against the target pathogen and loaded on SDS polyacrylamide gels to determine the molecular weight. Molecular weight markers for peptides ranging from 5 to 245 kDa (Gene Direx) were used as a Standard. After electrophoresis the gels were divided. One half containing the sample and molecular weight markers was stained.

The other half of the gel was fixed immediately for 2 hrs. in fixing solution (Appendix 10). It was then washed in several volumes of double distilled water. The gel was then cut according to the position of antimicrobial substance and overlaid on the agar plate (TSA +1.5% NaCl) seeded with the indicator strain (10^7 cfu/ml). The plate was incubated over night and examined for a zone of inhibition (Bhunia *et al*., 1987 with modification)

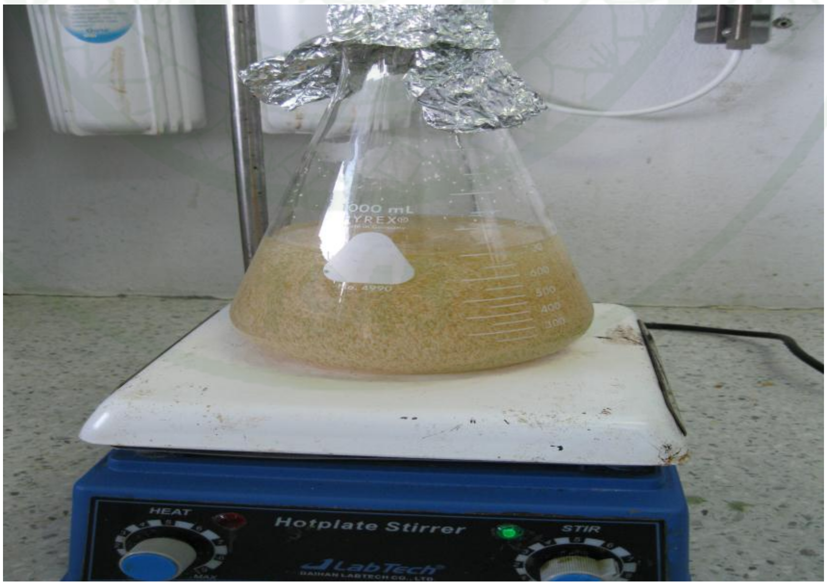


Figure 6 Concentrating the crude CFNS with amberlite - XAD 16 under gentle stirring.

Figure 7 Collection of the Amberlite XAD-16 bounded with CFNS in the column.

3.5.1 Materials for SDS- PAGE

Protein molecular weight standard marker, acrylamide (30%)/ bisacrylamide (0.8%); 4X Tris-CI/SDS (pH 8.8); 10% (w/v) ammonium persulfate; 10% (w/v) SDS; TEMED (N,N,N',N'-tetramethylethylenediamine); isobutyl alcohol (water-saturated); 4X Tris-CI/SDS (pH 6.8); 2X SDS/sample buffer; 1XSDS/electrophoresis buffer; Bromphenol Blue solution; 0.75-mm spacers; teflon combs.

3.5.2 Pouring the separating gel

1. The separating gel solution (16.5%) was mixed by adding 30% acrylamide 70.8% bisacrylamide, 4X Tris-CI/SDS, pH 8.8, and water to a 50 ml flask. 10% ammonium persulfate and TEMED was added to the solution and gently swirled to mix.

2. Using a Pasteur pipette, the separating gel solution was tranferred to the center of the sandwich along an edge of one of the spacers until the height of the solution in the sandwich was approximately 1.5 to 2 cm from the top of the shorter glass plate.

3. Using another Pasteur pipette, slowly the top of the gel was covered with a layer of isobutyl alcohol by gently squirting the isobutyl alcohol against the edge of one of the spacers.

4. The separating gel was allowed to polymerize fully.

3.5.3 Pouring the stacking gel

1. The layer of isobutyl alcohol was poured off completely and the separating gel top was rinsed three times with distilled water.

2. 4% stacking gel solution was prepared a by adding 0.45 ml of 30% acrylamide/0.8% bisacrylamide, 0.87 ml of 4X Tris-CI/SDS, pH 6.8, and 2.13 ml water. 20 µl of 10% ammonium persulfate and 3 µl TEMED was added to the solution and gently swirled to mix.

3. Using a Pasteur pipette, slowly allowed the stacking gel solution to trickle into the center of the sandwich along an edge of one of the spacers until the height of the solution in the sandwich was approximately 3 cm.

4. A Teflon comb was inserted into the layer of stacking gel solution and allowed the stacking gel solution to polymerize 30-45 min at room temperature.

3.5.4 Loading the sample

1. The concentrated antimicrobial substance was diluted to be analyzed at least 1:1 (v/v) with 2X SDS/sample buffer in a microcentrifuge tube.

2. The teflon comb was carefully removed without tearing the edges of the polyacrylamide wells.

3. Using a Pasteur pipette, the wells were filled with 1X SDS/electrophoresis buffer.

4. The upper buffer chamber was placed over the sandwich and locked the upper buffer chamber to the sandwich. 1X SDS/electrophoresis buffer was poured into the lower buffer chamber. Now the sandwich was placed attaching to the upper buffer chamber into the lower buffer chamber.

5. Partially the upper buffer chamber was filled with 1X SDS/electrophoresis buffer so that the sample wells of the stacking gel were filled with buffer.

6. Using a pipette, the antimicrobial samples were loaded into the wells by carefully applying the sample as a thin layer at the bottom of the wells.

7. The remainder of the upper buffer chamber was filled with additional 1X SDS/electrophoresis buffer.

3.5.4.1 Running the gel

7. The power supply was connected to the anode and cathode of the gel apparatus and run at 100 Volts.

8. After the Bromphenol Blue tracking dye had reached the bottom of the separating gel, the power supply was disconnected.

9. The upper buffer chamber and the attached sandwich was removed.

11. The gel orientation was done in a way so that the order of the sample wells were known, the sandwich was removed from the upper buffer chamber, and layed the sandwich on a sheet of absorbent paper or paper towels.

12. Carefully the spacers were slided out from the edge of the sandwich along its entire length and the gel was removed from the lower plate.

3.5.5 Staining of Protein Bands

1. Placed the gel in a small plastic box and cover with Coomassie Brilliant Blue R- 250 solution (Appendix 8) for staining with very slow agitation for 2 hours.

2. After around 2 hrs the staining solution was poured off and covered the gel with a solution of methanol/water/acetic acid (25%:7%:68% v/v/v) (Destaining Solution Appendix 9) and allowed to be agitated slowly for 30 min.

3. Destaining solution was discarded and replaced with fresh solution until the gel was clear except for protein bands.

3.5.6 Antimicrobial activity of the protein band

The other half of the gel containing the protein band was (not stained) studied for antibacterial activity by overlaying it on the plates seeded with the selected indicator strains (10^7cfu/ml) and examined for a zone of inhibition (Bhunia *et al*., 1987 with modification) after over night incubation at room temperature.

RESULTS AND DISCUSSION

Results

In this study the experiments were carried out in different steps to meet the criteria of the objective of the study .The results are discussed according to the experimental steps followed in the research.

1. Preliminary selection of probionts by the antagonistic assay against the selected pathogens

1.1 Bacterial strains and culture condition

The probiotic and the pathogenic bacteria, their culture condition and source as mentioned in the method has been illustrated in Table 1, Figure 1 and Figure 3.

Table 1 Indicator and test strains , source, culture medium and incubation condition

Bacterial Strain	Source	Medium	Incubation Conditions(°C/hr)
V. parahaemolyticus	ABRCVP 01	TSA + 1.5%NaCl	30/18-24
V. alginolyticus	ABRCVA01	TSA + 1.5%NaCl	30/18-25
V. harveyi	AQVH	TSA + 1.5%NaCl	30/18-26
V. vulnificus (G)	ABRCVV01	TSA + 1.5%NaCl	30/18-27
V.vulnificus(Y)	ABRC VV02	TSA + 1.5%NaCl	30/18-28
V.mimicus	ABRCVM 01	TSA + 1.5%NaCl	30/18-29
V.cholerae	ABRCVC01	TSA + 1.5%NaCl	30/18-30
V.fluvialis(Y)	ABRCVF 01	TSA + 1.5%NaCl	30/18-31
V. fluvialis (G)	ABRCVF02	TSA + 1.5%NaCl	30/18-32
A.hydrophila.	AQAH	TSA + 1.5%NaCl	30/18-33
Bacillus subtilis B1	Novozymes	TSA	30/18-34
Bacillus pumilus B2	Novozymes	TSA	30/18-35
Bacillus subtilis B3	Novozymes	TSA	30/18-36
Bacillus subtilis B4	Novozymes	TSA	30/18-37
Bacillus subtilis B5	Novozymes	TSA	30/18-38

1.2 Antagonism assay by Cross Streak method

After incubated for 48 hours with the pathogenic strains the *Bacillus* B2 and after 72 hours *Bacillus* B5 showed the antagonism against the selected pathogenic strains. B2 showed the colonization (where the *Bacillus* grew on the line of pathogenic bacteria) and B5 showed the inhibitory effects (Where the there was no growth of pathogenic bacteria near the *Bacillus* line) respectively against all the *Vibrio* spp. (Figure 8 and Table 2). However, these strains didn’t show any inhibitory effect against *A. hydrophila* AQAH. *Bacillus* B1 showed inhibitory effect against *A. hydrophila* AQAH after 72hours of incubation (Figure 8 and Table 2). The inhibitory effect was sustained upto 96 hrs for B1 and 120 hours for B2 and B5.. However the level of colonization was observed to increase for B2 up to 4th day after which there was no further increase observed. There was no inhibitory or colonization effect was found by *Bacillus* spp. B3 and B4 against any of the target pathogenic strain (Table 2).

1.3 Antagonism assay by Agar Well Diffusion Plate Assay (AWDA)

The AWDA assay was continued for 10 days for all the probionts and selected pathogens. The results showed that *Bacillus* B2 and B5 could produce antibacterial substance up to 7 days where the B1 could produce up to 5 days. (Table 3) B2 and B5 both the strains showed the antagonism against all the *Vibrio* spp (Table 4 and Table 5). And there was no antagonism by these strains (B2 and B5) against *A. hydrophila*. The B1 showed the antagonism against only *A. hydrophila* but could not exhibit antagonism against no other strains of *Vibrio* spp. It was found that among these bacilli B2 and B5 could produce antibacterial substances in highest level on 5th day (Figure 9 B, Figure 10 B and 10 C) while B1 could produce maximum on 72 hours (Figure 9 A Figure 10 A) as measured by the size of clear zone in AWDA. B3 and B4 showed no antagonism against none of the pathogenic strain (Table 2).

Table 2 The results of cross streak and AWDA by the *Bacillus* spp. against the pathogenic bacteria.

	Cross Strek	AWDA	Cross Strek	AWDA	Cross Strek	AWDA	Cross Strek	AWDA	Cross Streak	AWDA
V. parahaemolyticus	--	--	Col	CZ	--	--	--	--	Inh	CZ
V. alginolyticus	--	--	Col	CZ	--	--	--	--	Inh	CZ
V. harveyii	--	--	Col	CZ	--	--	--	--	Inh	CZ
V. vulnificus (G)	--	--	Col	CZ	--	--	--	--	Inh	CZ
V. vulnificus (Y)	--	--	Col	CZ	--	--	--	--	Inh	CZ
V. fluvialis (Y)	--	--	Col	CZ	--	--	--	--	Inh	CZ
V. fluvialis (G)	--	--	Col	CZ	--	--	--	--	Inh	CZ
V. cholarae(non 01)	--	--	Col	CZ	--	--	--	--	Inh	CZ
V.mimicus	--	--	Col	CZ	--	--	--	--	Inh	CZ
A.hydrophila .	Inh	CZ	--	--	--	--	--	--	--	--

CZ : Clear Zone Col: Colonization Inh: Inhibition

Table 3 The zone of inhibition produced by B1 against *A.hydrophilla* by AWDA

Bacillus Spp.	Indicator Strain	Day 0	Day1	Day 2	Day 3	Day 4	Day 5
B1	*Aeromonas sp.*	0	1±1.00^a	1±0.00 ^{a}	6±1.15 ^{a}	3±0.58 ^{a}	1±0.22 ^{a}

Means values within the same column sharing the same superscript are not significantly different at P=0.05.

Table 4 The zone of Inhibition produced by B2 against *Vibrio*. spp. by AWDA

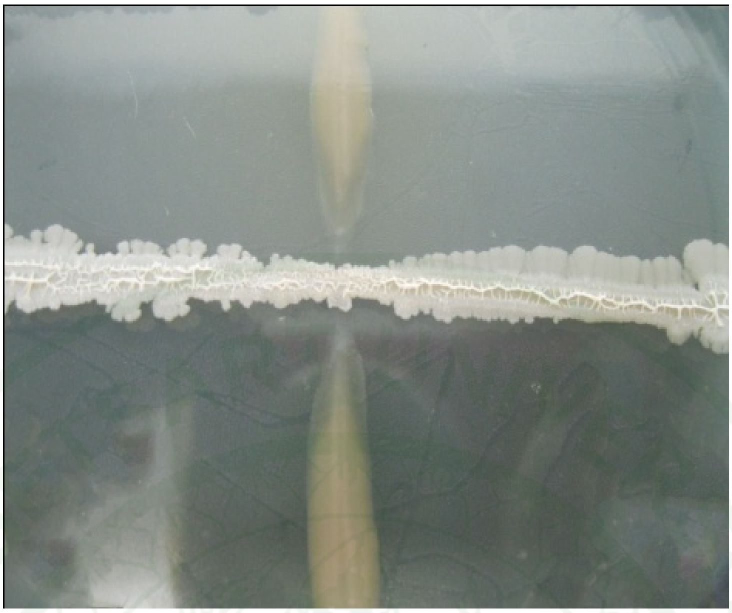
		Days										
Bacillus spp.	Indicator Strain	0	1	2	3	4	5	6	7	8	9	10
B 2	*V.parahaemolyticus*	0	5^{a} ±0.00	5^{a} ±1.00	6^{a} ±1.10	8^{a} ±2.10	22^{a} ±0.55	14^{a} ±1.00	5^{a} ±1.00	0	0	0
	V. alginolyticus	0	9^{a} ±1.00	10^{a} ±0.00	12^{a} ±0.00	14^{a} ±0.22	17^{a} ±0.11	11^{a} ±0.11	9^{a} ±0.00	0	0	0
	V. harveyi	0	5^{a} ±0.00	6^{a} ±1.11	9^{a} ±1.25	10^{a} ±0.22	12^{a} ±0.22	9^{a} ±0.15	4^{a} ±2.00	0	0	0
	V. vulnificus (G)	0	7^{a} ±1.15	8^{a} ±2.00	9^{a} ±0.88	10^{a} ±0.22	11^{a} ±0.00	8^{a} ±0.26	6^{a} ±1.11	0	0	0
	V.vulnificus(Y)	0	5^{a} ±2.00	5^{a} ±0.77	6^{a} ±1.10	6^{a} ±0.00	10^{a} ±1.00	7^{a} ±1.10	6^{a} ±2.00	0	0	0
	V.mimicus	0	5^{a} ±0.00	5^{a} ±1.00	6^{a} ±1.00	6^{a} ±2.00	10^{a} ±1.10	8^{a} ±0.30	5^{a} ±1.00	0	0	0
	V.choleriae	0	2^{a} ±0.00	4^{a} ±2.00	6^{a} ±1.00	6^{a} ±2.00	11^{a} ±0.22	8^{a} ±0.11	1^{a} ±0.15	0	0	0
	V.fluvialis(Y)	0	5^{a} ±1.00	7^{a} ±1.00	9^{a} ±1.00	10^{a} ±0.00	11^{a} ±0.55	7^{a} ±0.14	5^{a} ±0.00	0	0	0
	V. fluvialis (G)	0	6^{a} ±1.15	6^{a} ±0.00	7^{a} ±1.10	8^{a} ±0.00	10^{a} ±1.00	6^{a} ±0.00	5^{a} ±0.22	0	0	0
	A. hydrophila.	0	0	0	0	0	0	0	0	0	0	0

Means values within the same column sharing the same superscript are not significantly different at P=0.05

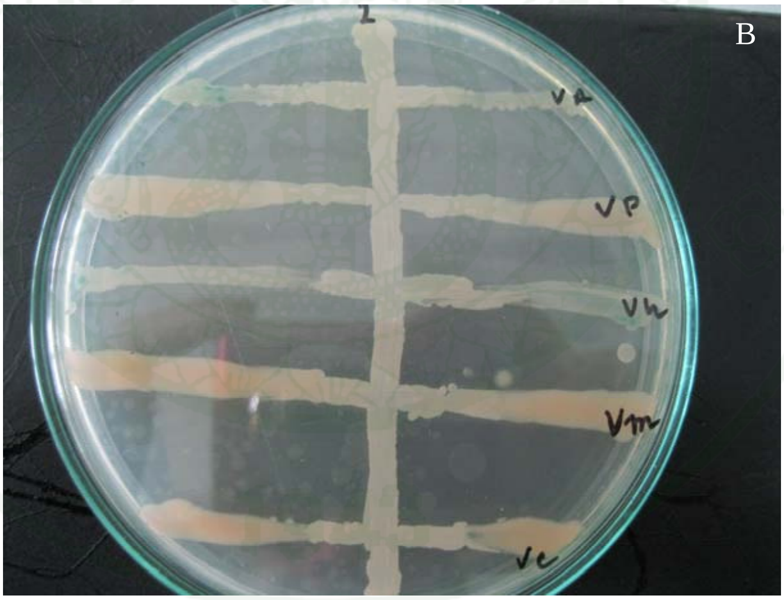
Table 5 The zone of inhibition showed by the B5 in AWDA against the *Vibrio* spp.

		Days										
Bacillus spp.	Indicator Strain	0	1	2	3	4	5	6	7	8	9	10
B5	*V. parahaemolyticus*	0	0	1±0.22^{a}	6±0.00^{a}	7±0.00^{a}	10±0.00^{a}	6±0.22^{a}	4±0.00^{a}	0	0	0
	V. alginolyticus	0	0	2±0.11^{a}	7±1.00^{a}	11±0.12^{a}	12±0.10^{a}	11±0.11^{a}	5±1.10^{a}	0	0	0
	V. harveyi	0	0	1±0.00^{a}	7±1.00^{a}	10±0.11^{a}	11±0.22^{a}	9±0.00^{a}	9±1.00^{a}	0	0	0
	V. vulnificus (G)	0	0	1±0.11^{a}	5±0.00^{a}	7±1.10^{a}	8±1.00^{a}	3±0.33^{a}	2±1.00^{a}	0	0	0
	V.vulnificus(Y)	0	0	1±0.22^{a}	4±1.00^{a}	5±0.10^{a}	6±0.00^{a}	3±0.00^{a}	16±0.00^{a}	0	0	0
	V.mimicus	0	0	1±0.25^{a}	4±0.22^{a}	7±1.00^{a}	8±0.00^{a}	5±1.00^{a}	4±0.00^{a}	0	0	0
	V.choleriae	0	0	2±0.00^{a}	7±0.11^{a}	8±1.15^{a}	10±1.00^{a}	9±0.00^{a}	7±0.22^{a}	0	0	0
	V.fluvialis(Y)	0	0	1±0.00^{a}	3±0.30^{a}	4±1.10^{a}	7±0.21^{a}	5±1.15^{a}	2±0.00^{a}	0	0	0
	V. fluvialis (G)	0	0	2±0.15^{a}	4±0.22^{a}	6±1.20^{a}	8±0.22^{a}	5±1.00^{a}	3±1.10^{a}	0	0	0
	A. hydrophila.	0	0	0	0	0	0	0	0	0	0	0

Means values within the same column sharing the same superscript are not significantly different at P=0.05

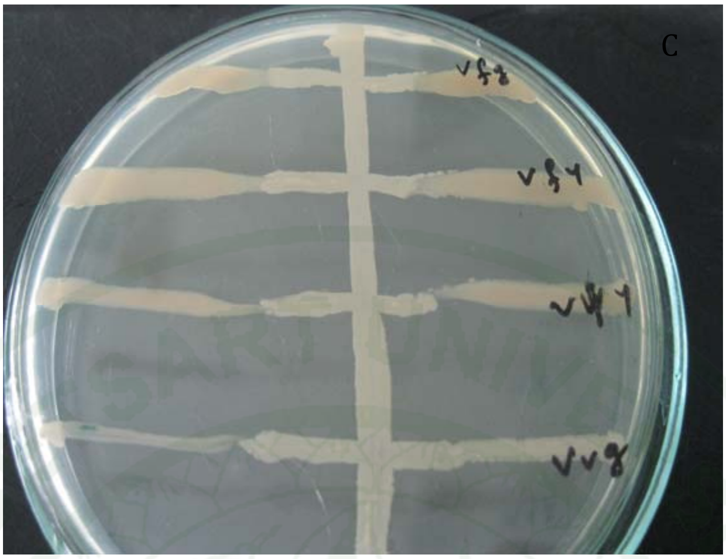


(A) Effect of inhibition (48 hrs) by B1 on *A. hydrophila* AQVH.

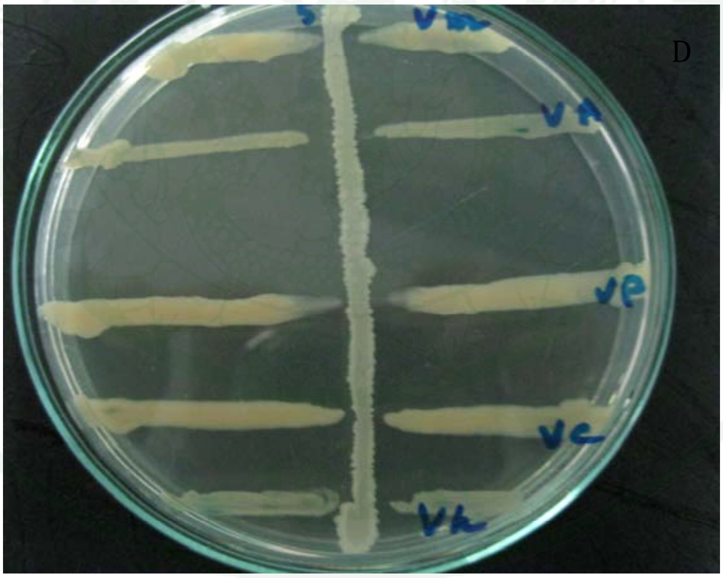


(B) Effect of inhibition (96 hrs) by B2 on (from top to bottom) *V. alginolyticus*, *V. parahaemolyticus*, *V.harveyi*, *V.mimicus*, *V. cholerae* (non01).

Figure 8 Results of Cross streak method in B1, B2 and B5

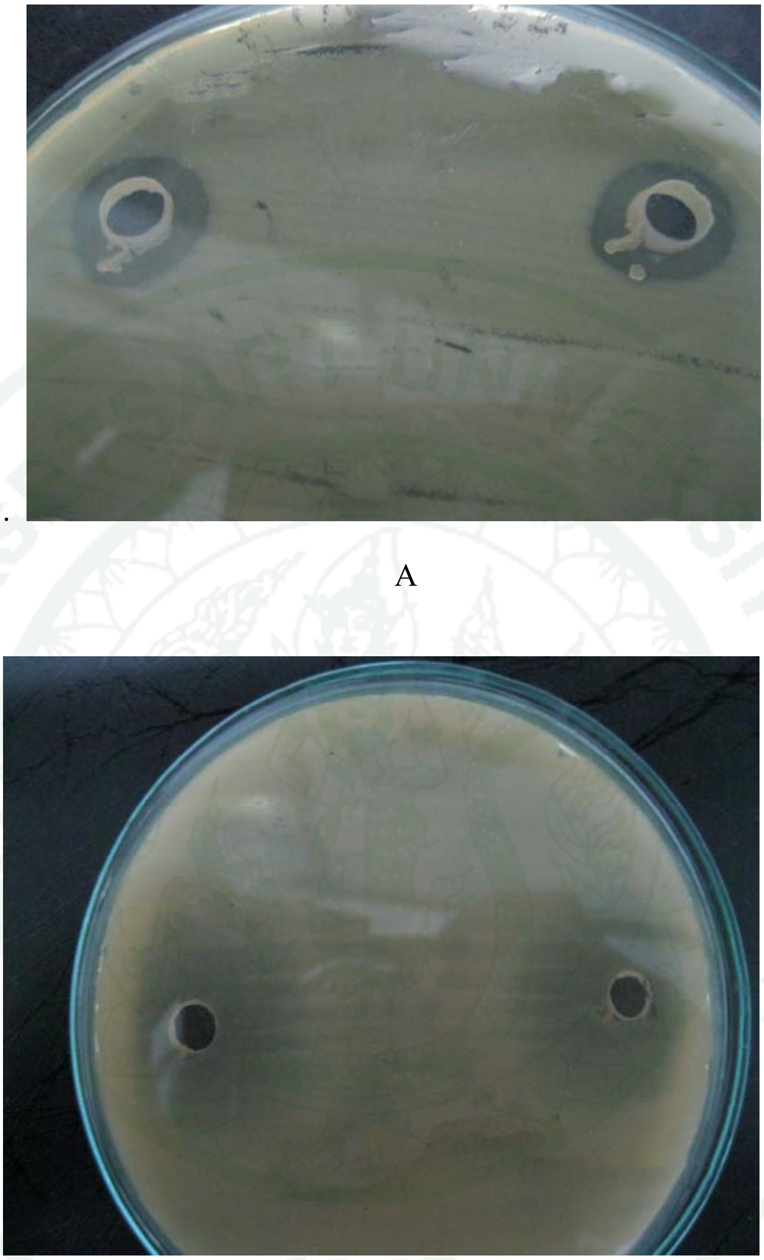


(C) Effect of colonization (96 hrs) by B 2 on (from top to bottom) *V.fluvialis* (Green), *V. fluvialis* (yellow), *V.vulnificus* (yellow), *V .vulnificus* (Green).



(D) Effect of inhibition by B5 (from top to bottom) on *V.mimicus*, *V. alginolyticus*, *V. parahaemolyticus*, *V. cholerae* (non01) and *V. harveyi*.

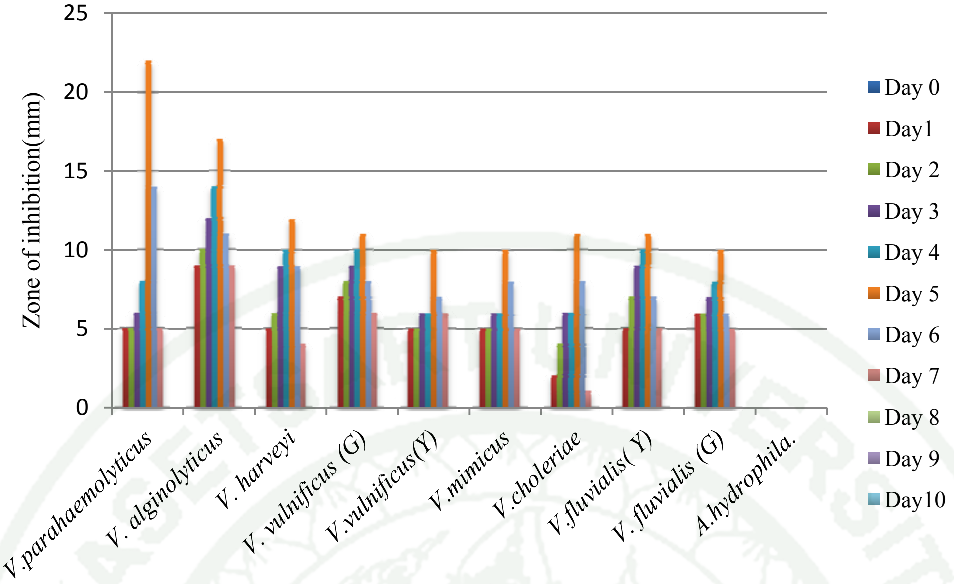
Figure 8 Continued



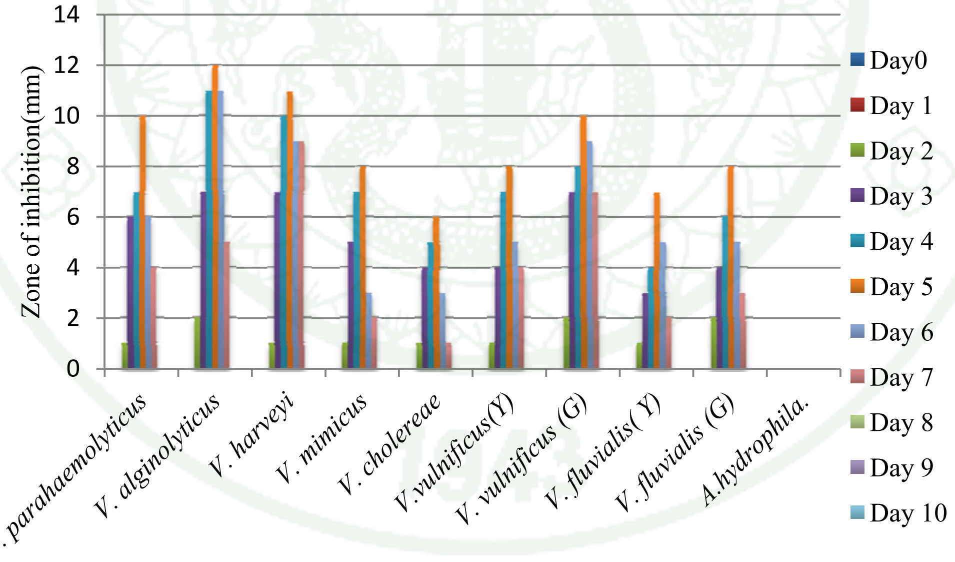
A

B

Figure 9 Zone of Inhibition (A) - by B1 at 72 hrs; (B) – by B5 at 120 hrs; agains *A. hydrophila* and *V harveyi*, respectively.

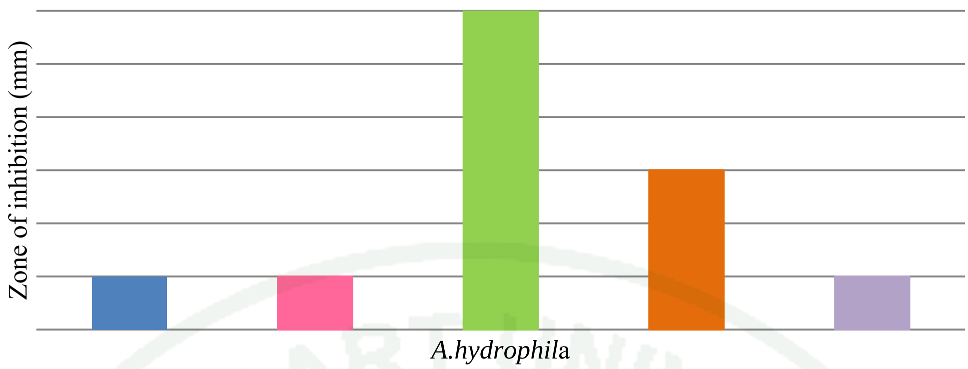


(A) B2 against *Vibrio* spp. (*V. parahaemolyticus, V. alginolyticus, V. harveyi, V. mimicus, V. cholerae* (non01) *V. vulnificus* (Y and G), *V. fluvialis* (Y and G).)



(A) B 5 against *Vibrio* spp. (*V. parahaemolyticus, V. alginolyticus, V. harveyi, V. mimicus, V. cholerae* (non01) *V. vulnificus* (Y and G), *V. fluvialis* (Y and G).)

Figure 10 Results of AWDA of B1, B2 and B5. as revealed by the measurement (mm) of clear zone by AWDA. The measurements are the mean value of three independent experiments.



(C) B1 against *A. hydrophila* AQAH.

Figure 10 Conmtinued

2.2 Co-culture of *Bacillus* spp. with pathogenic bacteria in Tryptic Soy Broth.

The result of preliminary selection of the probionts by cross streak and AWDA showed that only B1, B2 and B5 could inhibit the growth of selected pathogenic bacteria while B3 and B4 could not produce any antagonism. So the B1, B2 and B5 were selected for the broth co-culture experiment where the probionts were cultured with the pathogen in the same nutrient broth in the equal concentration to determine the inhibitiory effect of the probionts on the growth of pathogens in terms of comparing the number of pathogenic bacteria in co-culture and in control or mono-culture. At the same time the number of *Bacillus* also enumerated comparing between the co-culture and monoculture.

Based on the result of cross streak and AWDA antagonism assays the *Bacillus* B2 and B5 was co-cultured with all the *Vibrio* spp. and B1 was cultured with *A. hydrophila*. The enumeration was carried out in the following way

For the enumeration of *Vibrio* spp.

Total count of bacteria (TSA) = Number of total bacteria count from TSA plate.

The number of *Vibrio* spp. in co-culture (V Co) = The number of *Vibrio* spp. in TSB broth with *Bacillus* spp.

The number of *Vibrio* in co-culture (V Co) =TSA count – Number of bacteria in TCBS plate.

The number of *Vibrio* in monoculture (control) (V M) = Number of *Vibrio* cultured in TSB broth without *Bacillus.*

The percentage reduction of *Vibrio* = V Co - V M / V M * 100

For the enumeration of *A. hydrophila* :

Total count of bacteria (TSA) = Number of bacteria count from TSA plate.

The number of *A. hydrophila* in monoculture (control) (A M) = Number of *A. hydrophila* cultured in TSB broth without *Bacillus* .

The number of *A. hydrophila* in co-culture (A Co) = The number of *A. hydrophila* in TSB broth with *Bacillus* spp. (A Co) = TSA count –A M count

The percentage reduction of *A. hydrophila* = A M - A Co / A M * 100

For the enumeration of *Bacillus* spp. the same method was followed as described above.

The presence of *Bacillus* spp. (B2 and B5) led to a remarkable inhibition of growth of all the strains of different *Vibrio* spp. after 120 hours of incubation. Growth of *A. hydrophila* AQAH was found to get reduced 61.8% by B1 after 120 hours of co-culture (Table 6, Table 9). The growth reduction of the different strains of *Vibrio* spp. was more than 90% by B2 (Table 7, Table 9) and more than 85% (Table 8 , Table 9) at the end of 120 hours of co-culture experiment .While there were little difference observed in *Bacillus* spp. concentrations. The reduction of B1 was less than 5%, B2 was 4 to 7% and B5 was 4 to 8% respectively (Table 10) at the end of 120 hours of the co-culture experiment while compared to the control (mono-culture).The concentration of each pathogenic strain in the mono and co-culture with the the respective *Bacillus* spp. has been explained through (Table 6) and (Figure 29) for B1 and *A. hydrophila*. (Table 7) and (Figure 11,13,15,17,19,21,23, 25, 27) for B2 and (Table 8) and (Figure 12, 14, 16, 18, 20, 22, 24, 26, 28) for B5 with the pathogenic *Vibrio* spp. respectively.

Table 6 Concentration of B1 and *A.hydrophilla* in mono-culture and co-culture.

Bacteria	Time (hrs)					
	0	**24**	**48**	**72**	**96**	**120**
B1 M	2.9 ± 0.44 x 10^6	4.4 ± 0.52 x 10^9	2.6 ± 0.13 x 10^9	2.5 ± 0.12 x 10^9	5.5 ± 0.77 x 10^8	1.2 ± 0.80 x 10^8
Aero M	1.92 ± 0.35 x 10^6	1.3 ± 0.95 x 10^9	5.2 ± 1.0 x 10^8	2.2 ± 0.85 x 10^7	4.9 ± 0.37 x 10^7	1.1 ± 0.29 x 10^7
Aero Co	1.84 ± 0.69 x 10^6	1.0 ± 0.58 x 10^9	4.0 ± 0.88 x 10^8	9.9 ± 0.37 x 10^8	1.9 ± 0.45 x 10^7	4.2 ± 0.56 x 10^6
B1 Co	2.88 ± 0.39 x 10^6	4.2 ± 0.95 x 10^9	2.5 ± 0.56 x 10^9	2.4 ± 0.88 x 10^9	5.2 ± 0.88 x 10^8	1.15 ± 0.37 x 10^8

Mean value with same superscript are not significantly different at P = 0.05

Table 7 Concentration of B2 and *Vibrio* spp. in mono-culture and co-culture.

Bacteria	Time (hrs)					
	0	24	48	72	96	120
B2 M	8.9 ± 0.35 x 10^{6}	7.5 ± 0.43 x 10^{9}	5.5 ± 0.77 x 10^{9}	2.5 ± 0.33 x 10^{9}	6.5 ± 0.31 x 10^{8}	1.5 ± 0.44 x 10^{8}
Va M	3.9 ± 0.22 x 10^{6}	7.9 ± 0.31 x 10^{8}	2.3 ± 0.45 x 10^{8}	7.2 ± 0.34 x 10^{7}	1.2 ± 0.33 x 10^{7}	2.5 ± 1.1 ^{a}x 10^{6}
Vm M	7.9 ± 0.33 x 10^{6}	8.3 ± 0.57 x 10^{8}	2.1 ± 0.75 x 10^{8}	6.5 ± 0.82 x 10^{7}	2.5 ± 0.22 x 10^{7}	5.5 ± 0.12 x 10^{6}
Vvy M	8.4 ± 0.10 x 10^{6}	6.9 ± 0.80 x 10^{8}	4.2 ± 0.27 x 10^{8}	6.2 ± 0.36 x 10^{7}	2.1 ± 0.20 x 10^{7}	3.1 ± 1.0 x 10^{6}
Vvg M	8.1 ± 0.22 x 10^{6}	6.6 ± 0.27 x 10^{8}	3.9 ± 0.52 x 10^{8}	5.5 ± 0.32 x 10^{7}	1.5 ± 0.42 x 10^{7}	2.9 ± 0.37 x 10^{6}
Vh M	7.5 ± 0.18 x 10^{6}	6.1 ± 0.25 x 10^{8}	2.9 ± 0.37 x 10^{8}	5.9 ± 0.11 x 10^{7}	1.2 ± 0.21 x 10^{7}	4.2 ± 0.53 x10^{6}
Vfy M	3.3 ± 0.88 x 10^{6}	3.9 ± 0.71 x 10^{8}	2.5 ± 0.49 x 10^{8}	3.7 ± 0.22 x 10^{7}	1.1 ± 0.62 x 10^{7}	2.7 ± 0.13 x 10^{6}
Vfg M	2.7 ± 0.1 x 10^{6}	3.5 ± 0.19 x 10^{8}	2.2 ± 0.23 x 10^{8}	3.3 ± 0.42 x 10^{7}	1.2 ± 0.99 x 10^{7}	2.5 ± 0.12 x 10^{6}
Vp M	5.7 ± 0.75 x 10^{6}	5.9 ± 1.10 x 10^{8}	4.1 ± 0.51 ^{a} x 10^{8}	6.9 ± 0.73 x 10^{7}	1.8 ± 1.00 x 10^{7}	3.6 ± 0.52 x 10^{6}
Vc M	7.2 ± 0.12 x 10^{6}	6.4 ± 1.10 x 10^{8}	3.2 ± 0.51 ^{a} x 10^{8}	8.1 ± 0.45 x 10^{7}	3.1 ± 0.34 x 10^{7}	1.1 ± 1.00 x 10^{6}
B2 Co	8.85 ± 1.00 x 10^{6}	7.3 ± 0.56 x 10^{9}	5.13 ± 0.47 x 10^{9}	2.34 ± 0.32 x 10^{9}	6.14 ± 0.2 x 10^{8}	1.44 ± 0.15 x 10^{8}
Va Co	3.86 ± 0.42 x 10^{6}	5.0 ± 0.30 x 10^{8}	1.0 ± 0.25 x 10^{8}	1.8 ± 0.22 x 10^{7}	1.8 ± 0.77 x 10^{6}	1.30 ± 0.90 x 10^{5}
Vm Co	7.84 ± 1.25 x 10^{6}	5.4 ± 0.76 x 10^{8}	9.3 ± 0.79 x 10^{7}	1.69 ± 0.85 x 10^{7}	4.0 ± 0.36 x 10^{6}	4.2 ± 0.9 5 x 10^{5}
Vvy Co	8.35 ± 0.56 x 10^{6}	4.6 ± 0.32 x 10^{8}	1.9 ± 0.25 x 10^{8}	1.65 ± 0.67 x 10^{7}	3.2 ± 1.0 x 10^{6}	2.6 ± 0.37 ^{a}x 10^{5}
Vvg Co	8.0 ± 0.32 x 10^{6}	4.4 ± 0.45 x 10^{8}	1.9 ± 0.30 x 10^{8}	1.5 ± 0.36 x 10^{7}	2.7 ± 0.2 x 10^{6}	2.1 ± 0.56 x 10^{5}
Vh M	7.4 ± 0.1 x 10^{6}	4.0 ± 0.22 x 10^{8}	1.3 ± 0.62 x 10^{8}	1.5 ± 0.77 x 10^{7}	1.9 ± 0.90 x 10^{6}	3.5 ± 0.26 x 10^{5}
Vfy Co	3.2 ± 0.11 x 10^{6}	2.6 ± 1.75 x 10^{8}	1.1 ± 0.66 x 10^{8}	1.0 ± 0.52 x 10^{7}	1.6 ± 0.20 x 10^{6}	2.2 ± 0.42 x 10^{5}
Vfg Co	2.6 ± 0.11 x 10^{6}	2.4 ± 0.55 x 10^{8}	1.0 ± 0.0 x 10^{8}	1.0 ± 0.62 x 10^{7}	2.1 ± 0.57 x 10^{6}	2.2 ± 0.59 x 10^{5}
Vp Co	5.6 ± 0.39 x 10^{6}	3.9 ± 0.65 x 10^{8}	1.8 ± 0.76 x 10^{8}	1.8 ± 0.33 x 10^{7}	2.9 ± 0.22 x 10^{6}	2.1 ± 0.33 x 10^{5}
Vc Co	7.1 ± 0.57 x 10^{6}	4.4 ± 0.33 x 10^{8}	1.5 ± 1.0 x 10^{8}	2.3 ± 0.85 x 10^{7}	5.8 ± 0.76 x 10^{6}	1.18 ± 0.36 x 10^{6}

Mean value with same superscript are not significantly different at P = 0.05

Table 8 Concentration of B5 and *Vibrio* spp. in mono-culture and co-culture.

Bacteria	Time (hrs)					
	0	24	48	72	96	120
B5 M	7.8 ± 0.17 x 10^{6}	7.8 ± 1.5 x 10^{9}	6.2 ± 0.37 x 10^{9}	8.2 ± 0.76 x 10^{8}	2.2 ± 0.17 x 10^{8}	5.8 ± 0.33 x 10^{7}
Va M	3.9 ± 0.33 x 10^{6}	7.9 ± 0.31 x 10^{8}	2.3 ± 0.45 x 10^{8}	7.2 ± 0.34 x 10^{7}	1.2 ± 0.33 x 10^{7}	2.5 ± 1.1 x 10^{6}
Vm M	7.9 ± 0.33 x 10^{6}	8.3 ± 0.57 x 10^{8}	2.1 ± 0.75 x 10^{8}	6.5 ± 0.82 x 10^{7}	2.5 ± 0.22 x 10^{7}	5.5 ± 0.12 x 10^{6}
Vvy M	8.4 ± 0.10 x 10^{6}	6.9 ± 0.80 x 10^{8}	4.2 ± 0.27 x 10^{8}	6.2 ± 0.36 x 10^{7}	2.1 ± 0.20 x 10^{7}	3.1 ± 1.0 x 10^{6}
Vvg M	8.1 ± 0.22 x 10^{6}	6.6 ± 0.27 x 10^{8}	3.9 ± 0.52 x 10^{8}	5.5 ± 0.32 x 10^{7}	1.5 ± 0.42 x 10^{7}	2.9 ± 0.37 x 10^{6}
,Vh M	7.5 ± 0.18 x 10^{6}	6.1 ± 0.25 x 10^{8}	2.9 ± 0.37 x 10^{8}	5.9 ± 0.11 x 10^{7}	1.2 ± 0.21 x 10^{7}	4.2 ± 0.53 x 10^{6}
Vfy M	3.3 ± 0.88 x 10^{6}	3.9 ± 0.71 x 10^{8}	2.5 ± 0.49 x 10^{8}	3.7 ± 0.22 x 10^{7}	1.1 ± 0.62 x 10^{7}	2.7 ± 0.13 x 10^{6}
Vfg M	2.7 ± 0.11 x 10^{6}	3.5 ± 0.19 x 10^{8}	2.2 ± 0.23 x 10^{8}	3.3 ± 0.42 x 10^{7}	1.2 ± 0.99 x 10^{7}	2.5 ± 0.12 x 10^{6}
Vp M	5.7 ± 0.75 x 10^{6}	5.9 ± 1.10 x 10^{8}	4.1 ± 0.51 x 10^{8}	6.9 ± 0.73 x 10^{7}	1.8 ± 1.00 x 10^{7}	3.6 ± 0.52 x 10^{6}
Vc M	7.2 ± 0.12 x 10^{6}	6.4 ± 1.10 x 10^{8}	3.2 ± 0.51 x 10^{8}	8.1 ± 0.45 x 10^{7}	3.1 ± 0.34 x 10^{7}	1.1 ± 1.00 x 10^{6}
B5 Co	7.8 ± 0.23 x 10^{6}	7.2 ± 0.56 x 10^{9}	5.6 ± 0.87 x 10^{9}	7.4 ± 0.23 x 10^{8}	2.0 ± 0.35 x 10^{8}	5.3 ± 1.10 x 10^{7}
Va Co	3.9 ± 0.62 x 10^{6}	6.5 ± 0.25 x 10^{8}	1.3 ± 0.37 x 10^{8}	2.6 ± 0.33 x 10^{7}	3.1 ± 0.62 x 10^{6}	2.9 ± 0.52 x 10^{5}
Vm Co	7.9 ± 0.15 x 10^{6}	6.8 ± 0.39 x 10^{8}	1.2 ± 0.27 x 10^{8}	2.3 ± 0.66 x 10^{7}	6.1 ± 0.57 x 10^{6}	5.7 ± 0.42 x 10^{5}
Vvy Co	8.4 ± 0.22 x 10^{6}	5.7 ± 0.92 x 10^{8}	2.4 ± 0.35 x 10^{8}	2.3 ± 0.77 x 10^{7}	5.2 ± 0.47 x 10^{6}	3.2 ± 0.55 x 10^{5}
Vvg Co	8.0 ± 0.13 x 10^{6}	5.7 ± 0.87 x 10^{8}	2.3 ± 0.33 x 10^{8}	2.0 ± 0.75 x 10^{7}	2.9 ± 0.82 x 10^{6}	4.4 ± 0.23 x 10^{5}
Vh M	7.5 ± 0.39 x 10^{6}	5.0 ± 0.27 x 10^{8}	1.6 ± 0.37 x 10^{8}	2.2 ± 0.76 x 10^{7}	2.0 ± 0.55 x 10^{6}	4.2 ± 0.88 x 10^{5}
Vfy Co	3.3 ± 0.29 x 10^{6}	3.2 ± 0.89 x 10^{8}	1.4 ± 1.0 x 10^{8}	1.3 ± 1.0 x 10^{7}	1.9 ± 0.88 x 10^{6}	3.2 ± 0.37 x 10^{5}
Vfg Co	2.7 ± 0.37 x 10^{6}	2.9 ± 0.75 x 10^{8}	1.2 ± 0.76 x 10^{8}	1.2 ± 0.55 x 10^{7}	3.0 ± 1.9 x 10^{6}	4.2 ± 0.22 x 10^{5}
Vp Co	5.7 ± 0.77 x 10^{6}	4.8 ± 0.19 x 10^{8}	2.2 ± 0.73 x 10^{8}	2.5 ± 0.92 x 10^{7}	2.8 ± 0.33 x 10^{6}	4.0 ± 0.33 x 10^{5}
Vc Co	7.2 ± 0.33 x 10^{6}	5.2 ± 0.77 x 10^{8}	1.5 ± 0.27 x 10^{8}	3.0 ± 0.33 x 10^{7}	5.3 ± 0.23 x 10^{6}	1.5 ± 0.11 x 10^{6}

Mean value with same superscript are not significantly different at P = 0.05

Table 9 Reduction of growth of pathogenic bacteria (by percentage) in co-culture with *Bacillus* spp.

Bacteria species	Percentage decrease of *A. hydrophila* with B1					
	0hrs	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
A. hydrophila	0.51^{a}	23.^{a}	25.^{a}	54.99^{a}	61.22^{a}	61.81^{a}
	Percentage decrease of *Vibrio* spp. with B2					
		24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
V. alginolyticus	1.00^{a}	36.71^{a}	56.51^{a}	75.01^{a}	85.00^{a}	94.80^{a}
V. mimicus	0.76^{a}	34.94^{a}	55.72^{a}	73.70^{a}	84.00^{a}	92.43^{a}
V. vulnificus(Y)	0.60^{a}	33.33^{a}	54.39^{a}	73.39^{a}	84.76^{a}	91.61^{a}
V. vulnificus(G)	0.99^{a}	32.99^{a}	51.28^{a}	72.76^{a}	82.00^{a}	92.68^{a}
V. harveyi	0.67^{a}	34.23^{a}	54.82^{a}	74.57^{a}	84.11^{a}	91.65^{a}
V. fluvialis(Y)	0.61^{a}	31.43^{a}	56.00^{a}	72.97^{a}	85.48^{a}	91.85^{a}
V. fluvialis(G)	0.93^{a}	30.91^{a}	54.17^{a}	69.71^{a}	81.13^{a}	91.21^{a}
V. cholereae(non 01)	0.83^{a}	32.71^{a}	53.00^{a}	71.60^{a}	81.95^{a}	88.93^{a}
V. parahaemolyticuss	0.88^{a}	32.71^{a}	55.72^{a}	73.91^{a}	83.89^{a}	94.16^{a}
	Percentage decrease of *Vibrio* spp. with B5					
	0hrs	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
V.alginolyticus	0.49^{a}	17.47^{a}	43.48^{a}	63.89^{a}	74.16^{a}	88.4^{a}
V.mimicus	0.51^{a}	18.0^{a}	44.2^{a}	64.6^{a}	75.60^{a}	89.64^{a}
V.vulnificus(Y)	0.48^{a}	17.39^{a}	42.86^{a}	63.06^{a}	75.23^{a}	89.68^{a}
V. vulnificus(G)	0.49^{a}	13.6^{a}	41.79^{a}	62.00^{a}	80.67^{a}	84.83^{a}
V. harveyi	0.40^{a}	18.0^{a}	44.83^{a}	62.71^{a}	83.33^{a}	89.98^{a}
V. fluvialis(Y)	0.48^{a}	17.94^{a}	44.00^{a}	64.86^{a}	82.73^{a}	88.19^{a}
V. fluvialis(G)	0.44^{a}	17.14^{a}	45.4^{a}	63.64^{a}	75.00^{a}	83.23^{a}
V. cholerae(non 01)	0.56^{a}	17.97^{a}	53.1^{a}	62.96^{a}	82.90^{a}	86.36^{a}
V. parahaemolyticus	0.49^{a}	18.64^{a}	46.0^{a}	63.77^{a}	84.44^{a}	88.19^{a}

Mean value with the same superscript are not significantly different at P =0.05

Table 10 Reduction of growth of *Bacillus* spp. (in percentage) in co-culture with pathogenic bacteria.

	Percent decrease of B1with *A. hydrophila*					
	0hrs	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
A. hydrophila	0.68^{a}	4.50^{a}	3.84^{a}	4.00^{a}	5.45^{a}	4.16^{a}
	Percentage decrease of B2 with *Vibrio* spp.					
	0hrs	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
V.alginolyticus	0.21^{a}	4.00^{a}	7.27^{a}	8.41^{a}	8.18^{a}	7.33^{a}
V.mimicus	0.14^{a}	3.87^{a}	5.45^{a}	6.33^{a}	6.15^{a}	5.86^{a}
V.vulnificus(Y)	0.66^{a}	4.13^{a}	4.36^{a}	4.80^{a}	5.69^{a}	5.32^{a}
V.vulnificus(G)	0.79^{a}	1.33^{a}	3.64^{a}	6.09^{a}	6.00^{a}	6.00^{a}
V.harveyi	0.30^{a}	1.32^{a}	7.25^{a}	7.20^{a}	6.12^{a}	6.67^{a}
V.fluvialis(Y)	0.45^{a}	2.67^{a}	3.45^{a}	7.60^{a}	7.54^{a}	5.43^{a}
V.fluvialis(G)	0.67^{a}	2.00^{a}	3.64^{a}	7.96^{a}	7.69^{a}	6.65^{a}
V.cholereae (non 01)	0.56^{a}	2.56^{a}	6.73^{a}	6.40^{a}	5.54^{a}	4.00^{a}
V.parahaemolyticus	0.22^{a}	2.20^{a}	5.27^{a}	5.20^{a}	4.92^{a}	5.54^{a}
	Percentage decrease of B5 with *Vibrio* spp.					
	0hrs	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
V.alginolyticus	0.13^{a}	7.69^{a}	9.68^{a}	9.76^{a}	9.09^{a}	8.62^{a}
V.mimicus	0.28^{a}	6.41^{a}	6.13^{a}	6.10^{a}	5.87^{a}	5.34^{a}
V.vulnificus(Y)	0.77^{a}	6.15^{a}	6.61^{a}	6.22^{a}	6.36^{a}	6.55^{a}
V.vulnificus(G)	0.79^{a}	2.30^{a}	6.45^{a}	6.22^{a}	6.36^{a}	6.75^{a}
V.harveyi	0.52^{a}	5.77^{a}	8.39^{a}	8.41^{a}	7.27^{a}	7.2^{a}
V.fluvialis(Y)	0.65^{a}	7.95^{a}	7.26.^{a}	6.95^{a}	6.82^{a}	6.55^{a}
V.fluvialis(G)	0.90^{a}	1.96^{a}	3.23^{a}	4.90^{a}	5.00^{a}	5.17^{a}
V.cholereae(non 01)	1.03^{a}	2.57^{a}	4.83^{a}	4.88^{a}	4.55^{a}	4.82^{a}
V.parahaemolyticus	0.38^{a}	3.51^{a}	6.28^{a}	6.34^{a}	6.36^{a}	6.21^{a}

Mean value with the same superscript are not significantly different at P =0.05

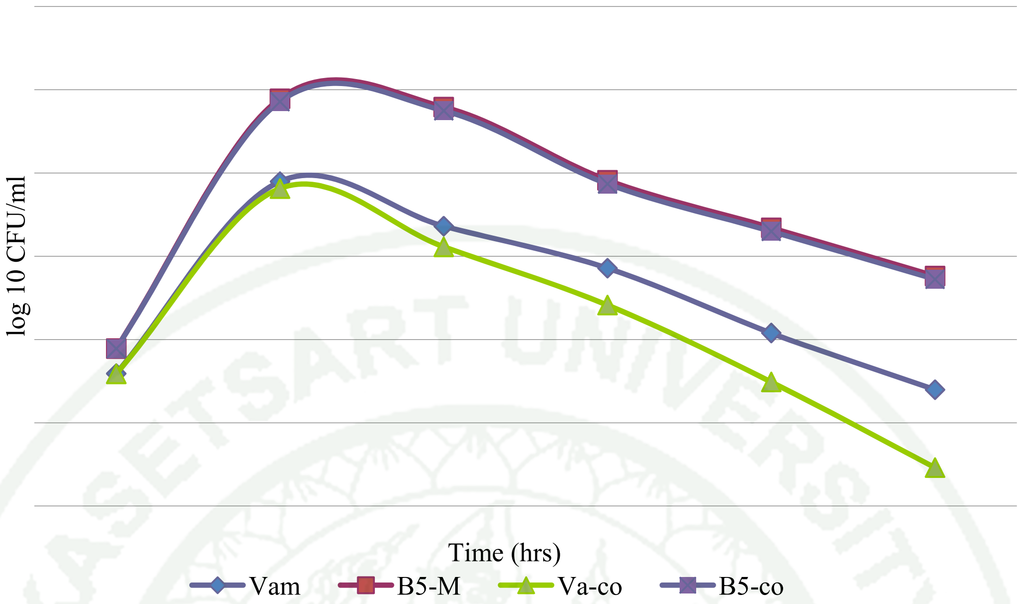


Figure 11 Growth of B2 and *V. alginolyticus* in co-culture and mono-culture.

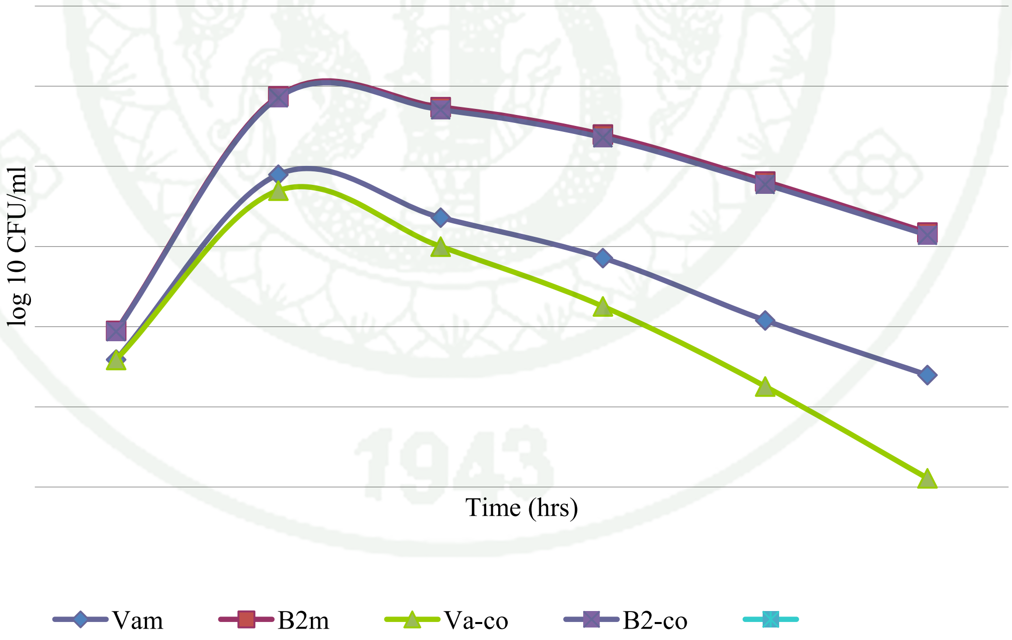


Figure 12 Growth of B5 and *V. alginolyticus* in co-culture and mono-culture.

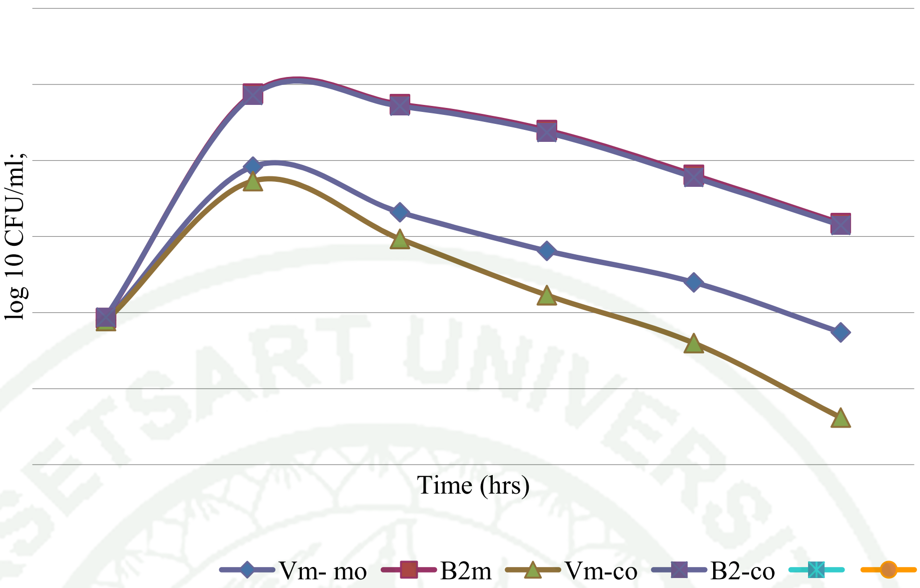


Figure 13 Growth of B2 and *V. mimicus* in co-culture and mono-culture.

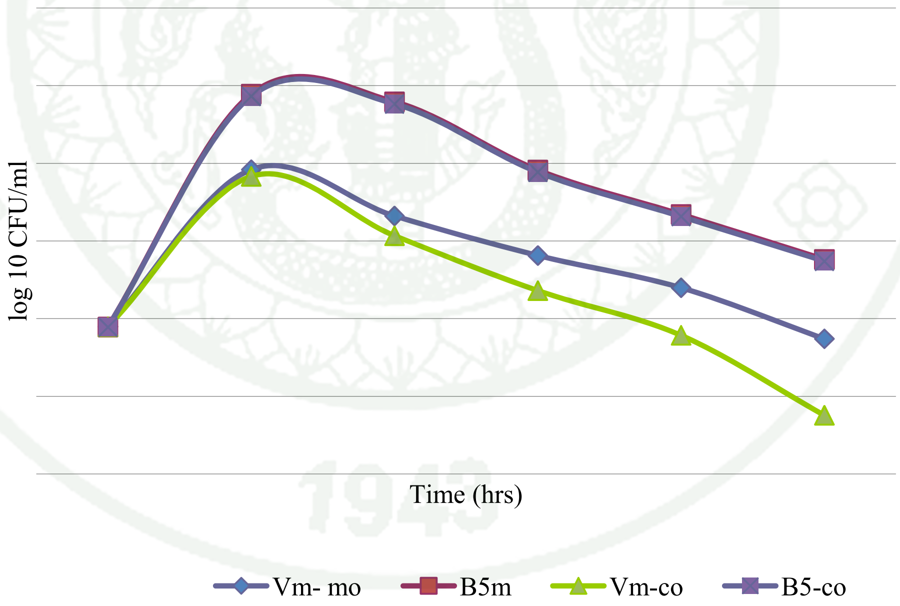


Figure 14 Growth of B5 and *V. mimicus* in co-culture and mono-culture.

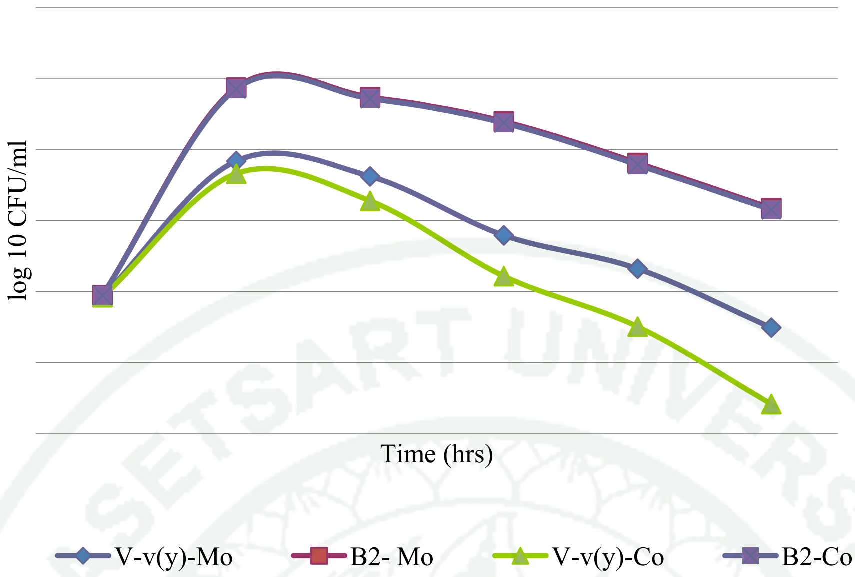


Figure 15 Growth of B2 and *V. vulnificus* (yellow) in co-culture and mono-culture.

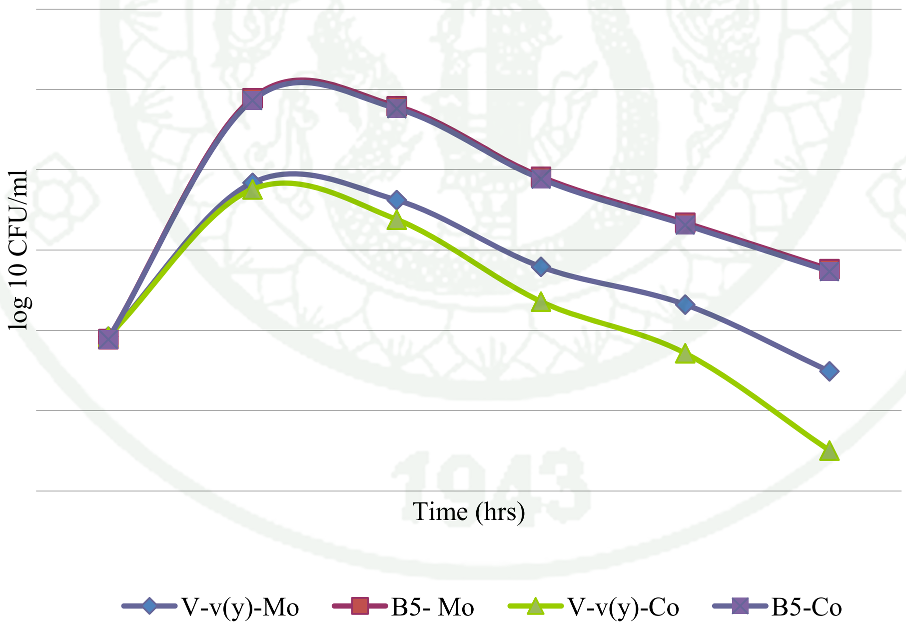


Figure 16 Growth of B5 and *V. vulnificus* (yellow) in co-culture and mono-culture.

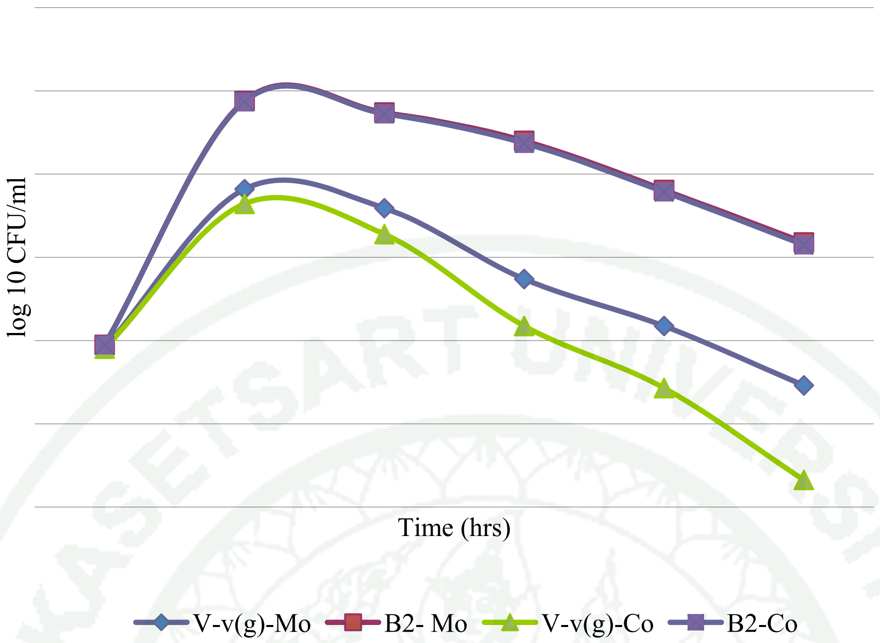


Figure 17 Growth of B2 and *V. vulnificus* (green) in co-culture and mono-culture.

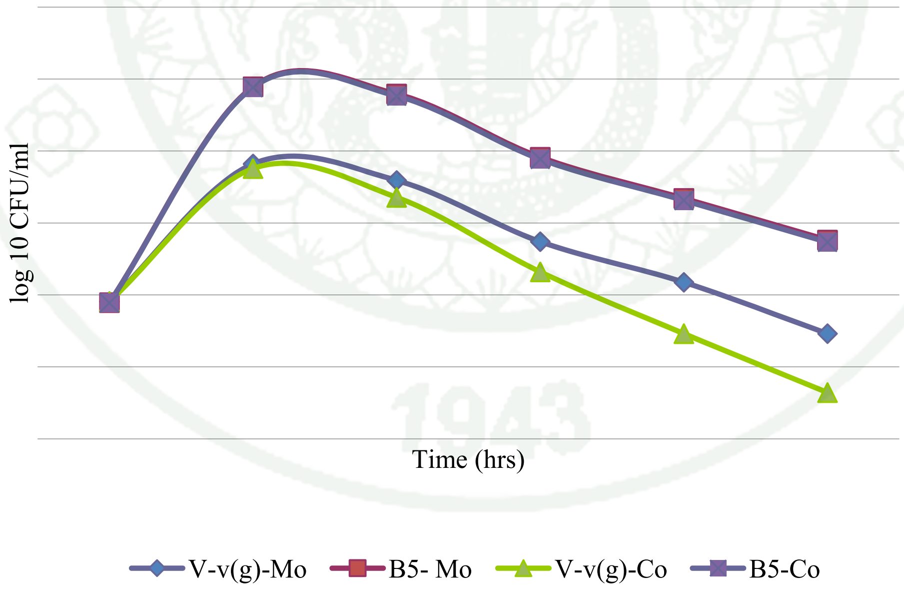


Figure 18 Growth of B5 and *V. vulnificus* (green) in co-culture and mono-culture.

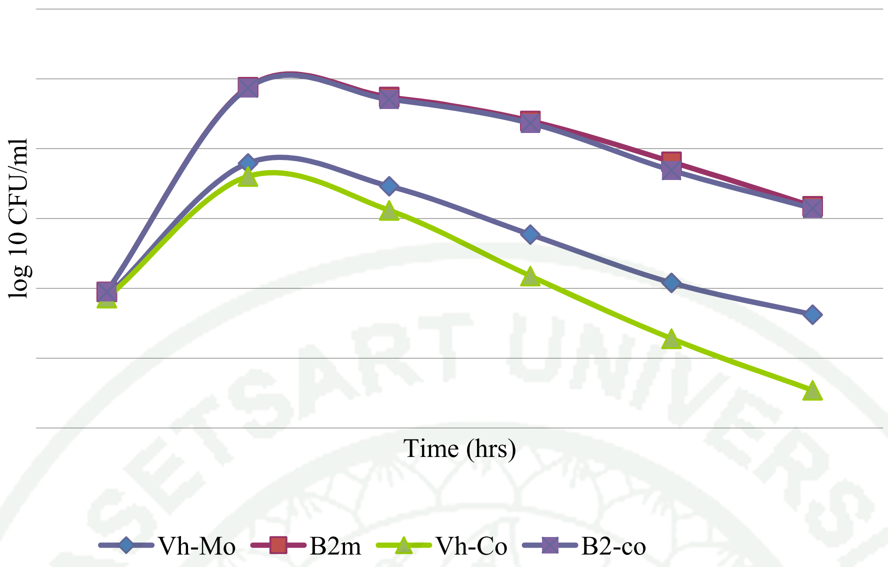


Figure 19 Growth of B2 and *V. harveyi* in co-culture and mono-culture.

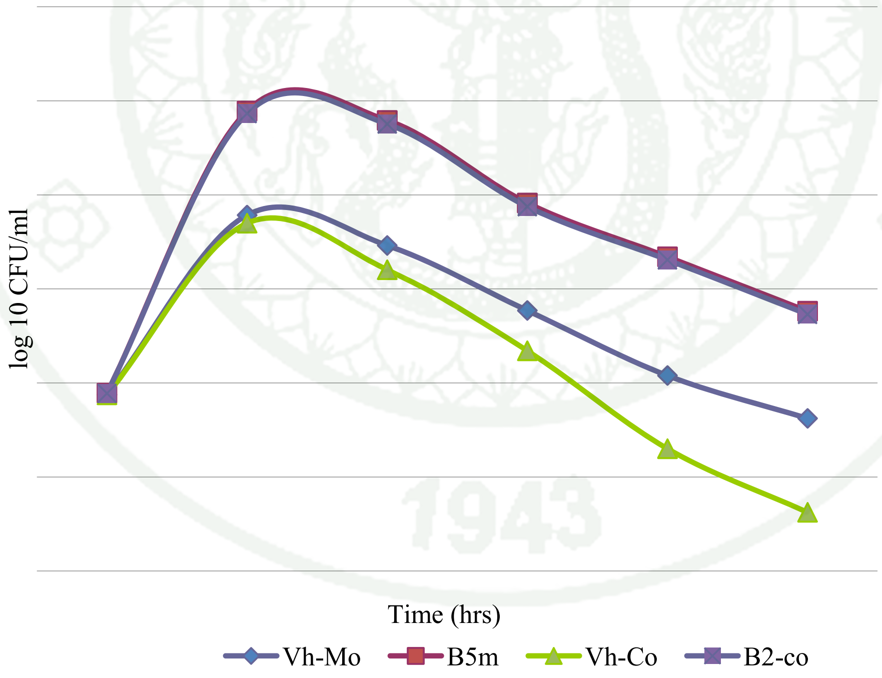


Figure 20 Growth of B5 and *V. harveyi* in co-culture and mono-culture.

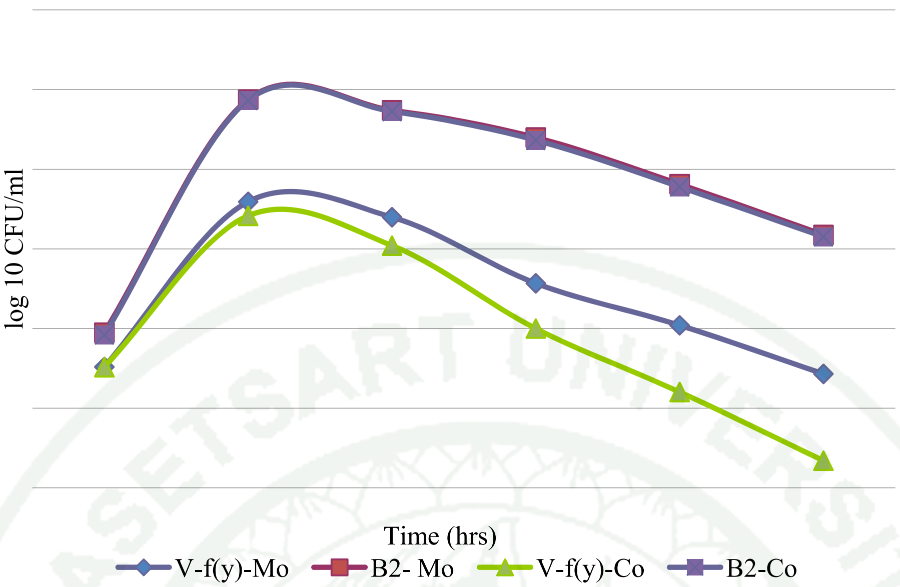


Figure 21 Growth of B2 and *V. fluvialis* (yellow) in co-culture and mono-culture.

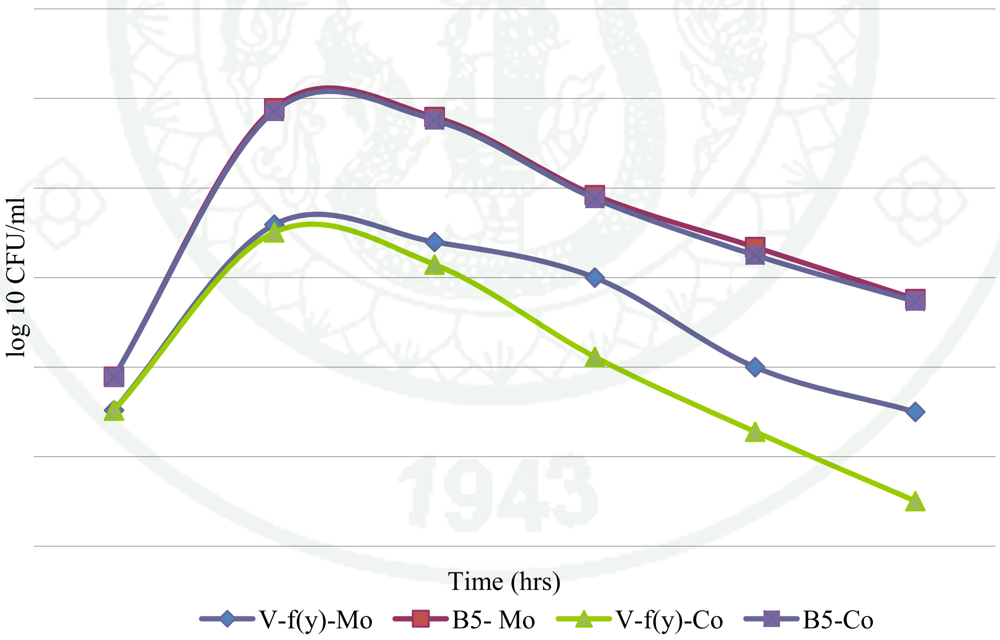


Figure 22 Growth of B5 and *V. fluvialis* (yellow) in co-culture and mono-culture.

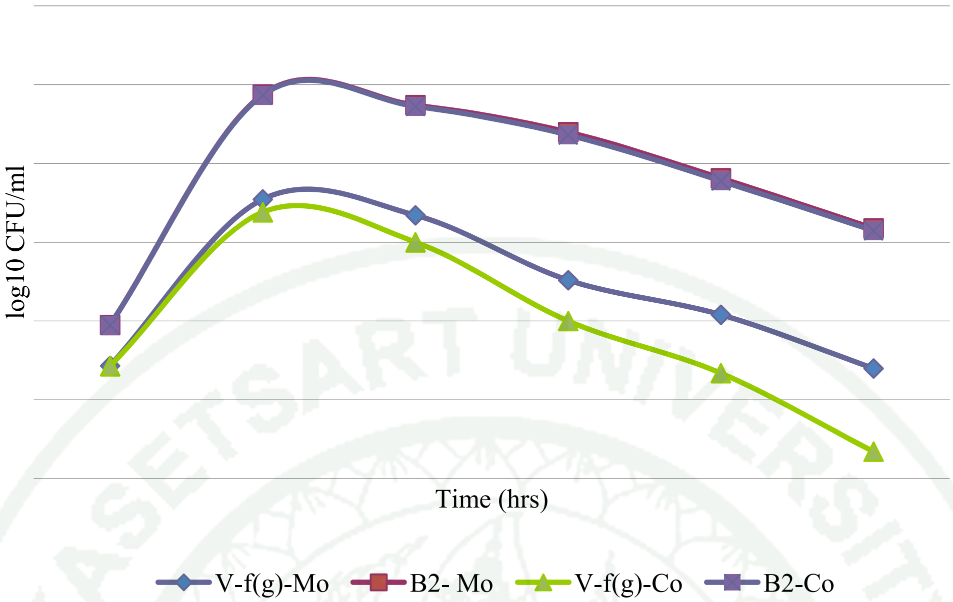


Figure 23 Growth of B2 and *V. fluvialis* (green) in co-culture and mono-culture.

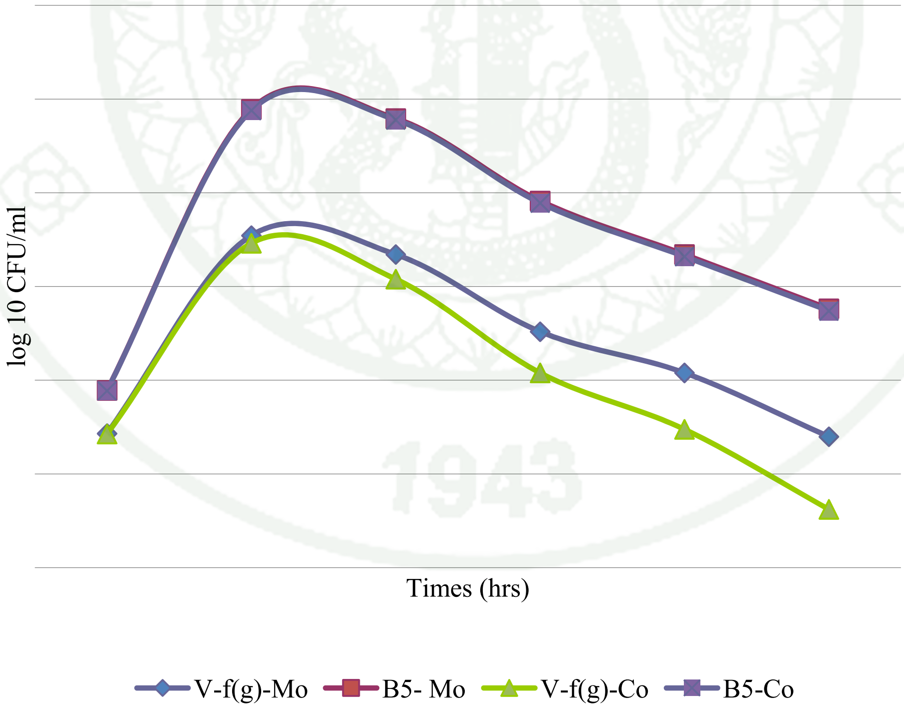


Figure 24 Growth of B5 and *V. fluvialis* (green) in co-culture and mono-culture

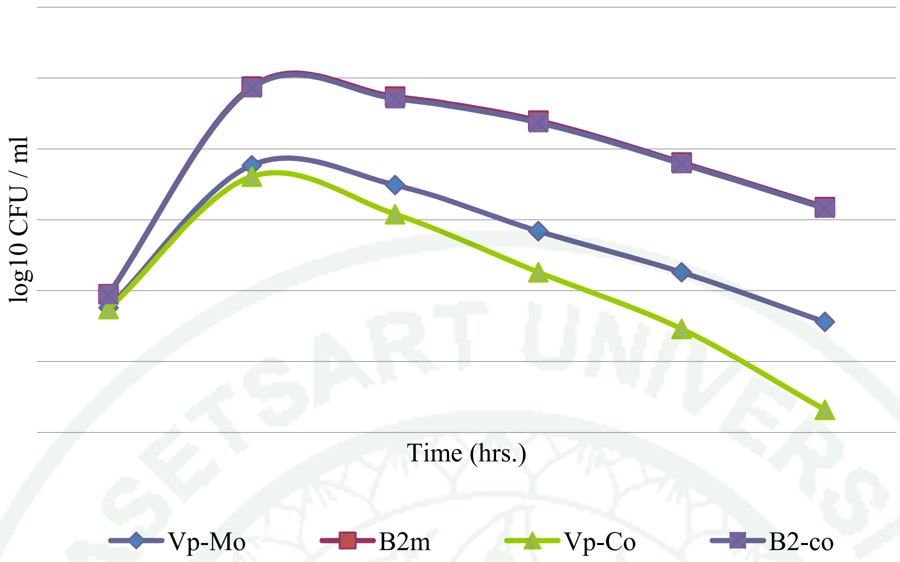


Figure 25 Growth of B2 and *V .parahemolyticus* in co-culture and mono-culture.

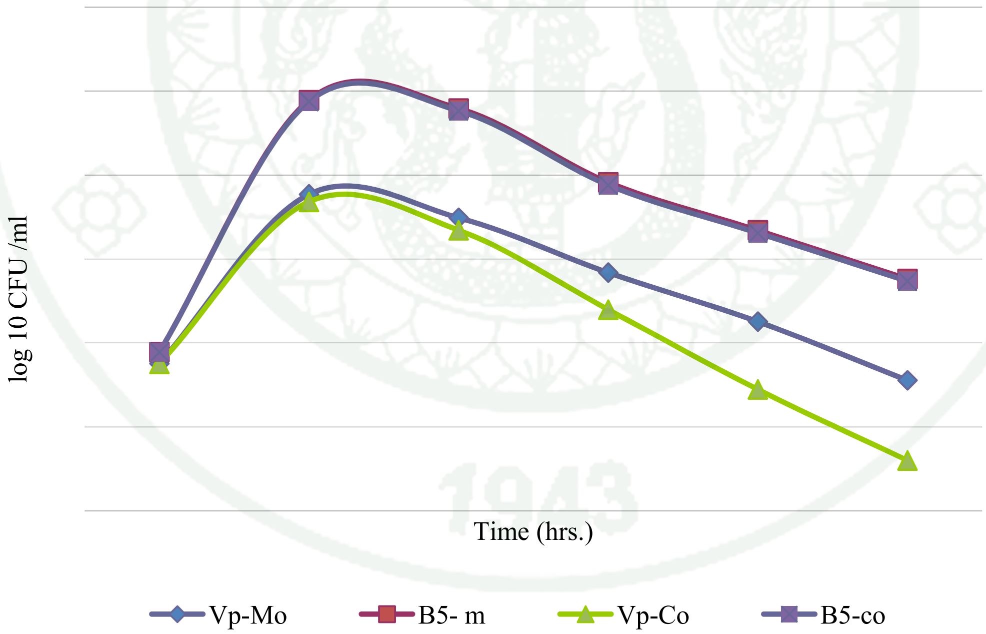


Figure 26 Growth of B5 and *V. parahemolyticus* in co-culture and mono-culture

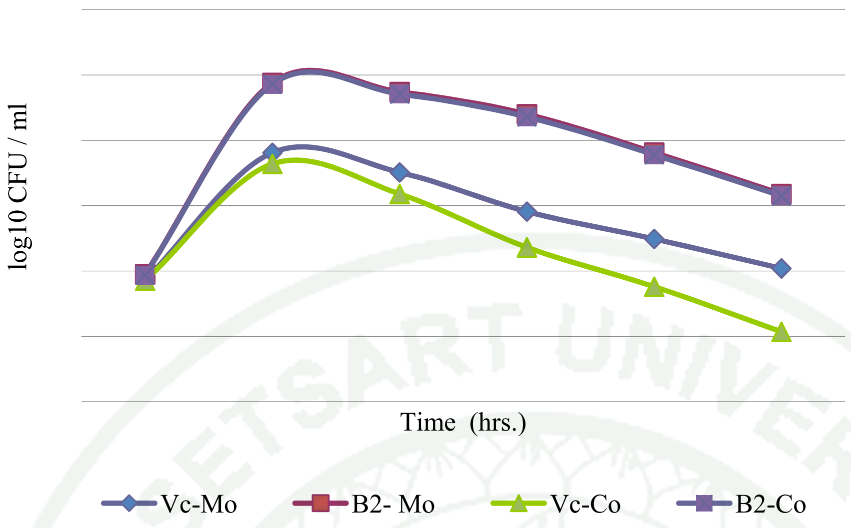


Figure 27 Growth of B2 and *V. cholarae* (non 01) in co-culture and mono-culture.

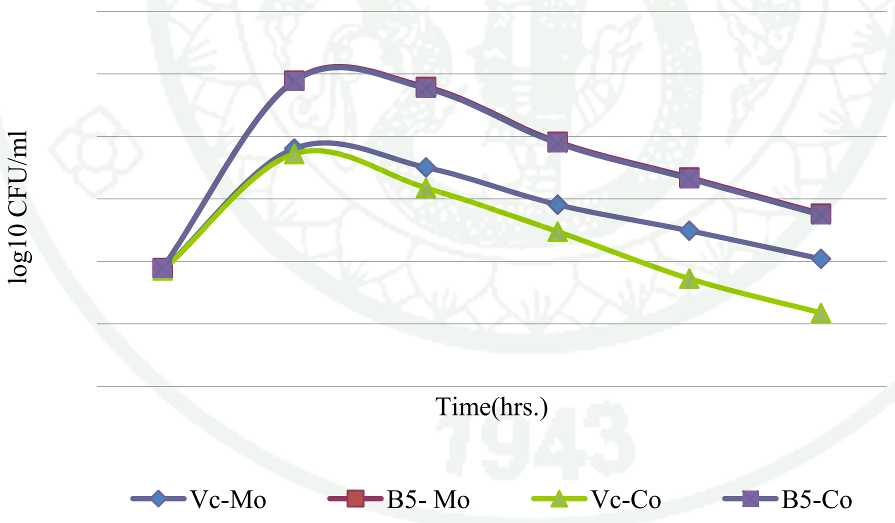


Figure 28 Growth of B5 and *V. cholarae* (non 01) in co-culture and mono-culture.

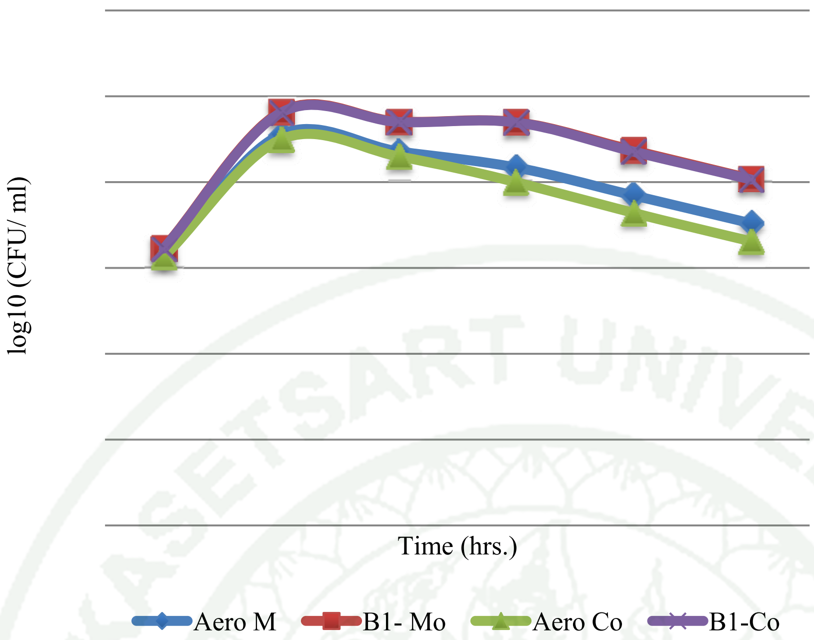


Figure 29 Growth of B1 and *A.hydrophila* in co-culture and mono-culture.

Data analysis of AWDA and broth co-culture experiment
All the data were analyzed by Analysis of Variance and Duncan's Multiple Range (SPSS 16).

3. Characterization and partial purification of the CFNS of the selected probionts.

3.1 Determination of residual antimicrobial activity of the probionts by the critical dilution method to select the probiont.

The CFNS of all the 3 *Bacillus* was tested by the critical dilution assay to determine the residual antimicrobial activity against the selected pathogenic strains against which these produce antagonism. Activity was measured as the reciprocal of the highest two-fold dilution showing antimicrobial activity. According to to the observations, 1/16 dilution of the crude supernatant is the highest dilution showing antimicrobial activity against indicator strain that are *V. parahaemolyticus* (ABRC Lab. Figure 31) and *V. alginolyticus* (ABRC Lab. Figure 30) which was used in all

measurements for bacteriocin or antimicrobial activity. Bacteriocin activity of cell-free supernatant of B2 (crude extract) was calculated as:16 x 20 = 320 AU/ml.
AU= Arbitrary Unit; 16= Reciprocal of the highest dilution (1/16); 20 = 1000 µl / 50µl (conversion factor)

Table 11 The residual antimicrobial activity of *Bacillus* spp. against the pathogenic bacteria showed by Critical Dilution method.

Indicator S rains	B1 AU/ml	B2 AU/ml	B5 AU/ml
V. parahaemolyticus	-	320	80
V. alginolyticus	-	320	80
V. harveyi AQVH	-	160	40
V. vulnificus (G)	-	160	40
V. vulnificus (Y)	-	160	40
V. mimicus	-	40	40
V. choleriae	-	80	40
V.fluvialis (Y)	-	80	20
V.fluvialis (G)	-	80	20
Aeromonas hydrophila	40	-	-

3.2 Selection of probiont for partial purification and characterization of antimicrobials

As per the result of the antimicrobial activity assay Arbitrary Activity Unit (AU/ml) and AWDA the B2 showed the most consistent antagonism against all the pathogenic strain except *A. hydrophila*. However B5 also could not produce the antagonism against *A. hydrophil*a. But in compare to B5. B2 showed the antagonism only from 24 hours of incubation which and also exhibited the highest antagonism on day 5 against all the pathogenic strain as indicated by AWDA and critical dilution method. B1 showed antagonism only against *A. hydrophila* but not against the other pathogenic *Vibrio* spp. So the antimicrobials of the *Bacillus pumilus* or B2 (Figure

32 and spore of selcted strain Figure 3) was selected for and characterization and the partial purification.

3.2.1 Selection of pathogenic bacteria

Two strains of the pathogenic bacteria *V. parahaemolyticus* (ABRC Laboratory, Figure 30) and *V. alginolyticus* (ABRC Laboratory, Figure 30) selected to determine and analyze the physic - chemical properties of the antimicrobials of B2 on the basis of the antagonism produced by B2 against these 2 strains as determined by Arbitrary Activity Unit (AU/ml) and AWDA .

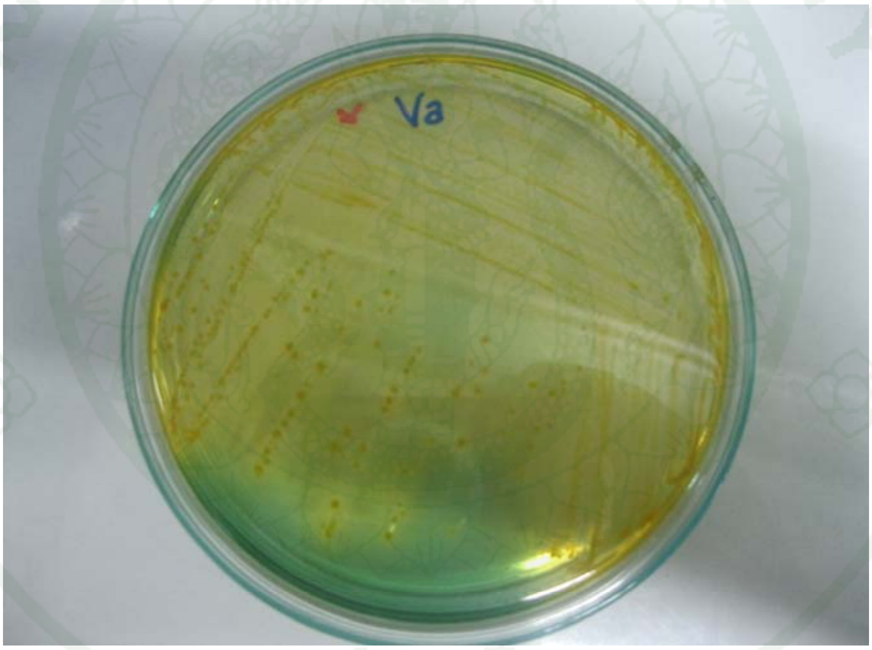


Figure 30 *Vibrio alginolyticus* of 24 hours culture.

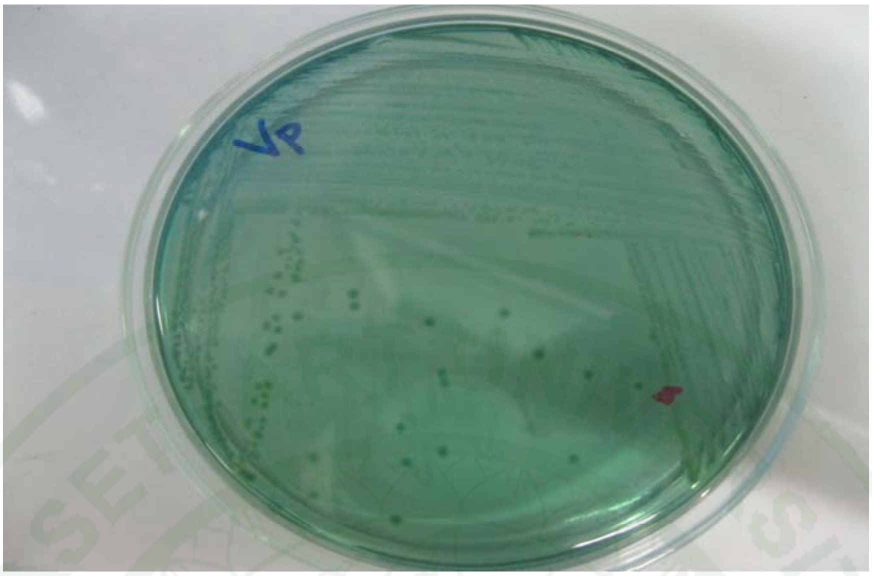


Figure 31 *Vibrio parahaemolyticus* of 24 hours culture.

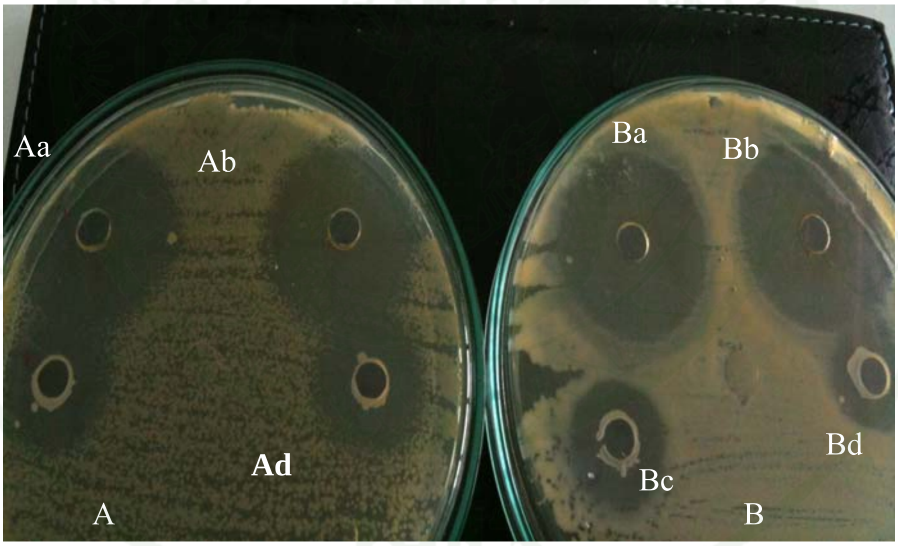


Figure 32 Zone of inhibition by the CFNS of B2 by critical dilution method. A- *V .alginolyticus*, B – *V. prahaemolyticus*, Aa and Ab – Crude CFNS(no dilution), Ac and Ad – 320AU/ml; Ba and Bb – Crude CFNS (no dilution), Bc and Bd – 320AU/ml

3.3.1 Sensibility to heat treatment

Effect of heat treatment on the antimicrobial activity was tested by keeping the crude extract at 45, 50, 60, 70, and 80° C for 20 and 60 minutes (Table 12, Figure 33 and Figure 34). As seen from Table, crude antimicrobial product was very stable to heat with respect to the all the temperatures and durations because the original activity of crude extract (320 AU/ml) was unchanged upon all the heat treatment regimes up to 70° C . The activity of the antimicrobials was found to reduce when kept at 80° C for 20 minutes where the residual activity dropped to 80AU/ ml. There was further reduction in activity found when heated at 90° C for 20 minutes which was 20 AU/ ml. No activity was found at any duration of 100 ° C and after autoclave at 121 ° C.

Table 12 Effect of different temperature on the antimicrobial activity of the CFNS of B2 against *Vibrio parahaemolyticus* (Vp) and *Vibrio alginolyticus* (Va).

Temperature	Vp/ 20min	Vp/60 min	Va 20min	Va 60min
40°C	320	320	320	320
50°C	320	320	320	320
60°C	320	320	320	320
70°C	320	320	320	320
80°C	80	40	80	40
90°C	20	0	20	0
100°C	0	0	0	0
121°C	0	0	0	0

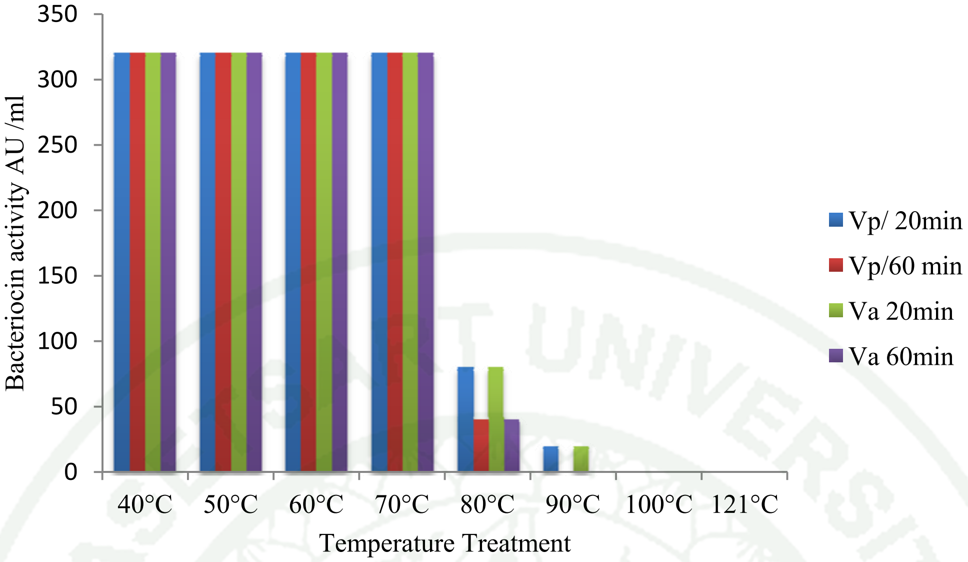


Figure 33 Antimicrobial activity of CFNS of B2 in different temperature range against *V. parahaemolyticus* (Vp) and *V. alginolyticus* (Va)

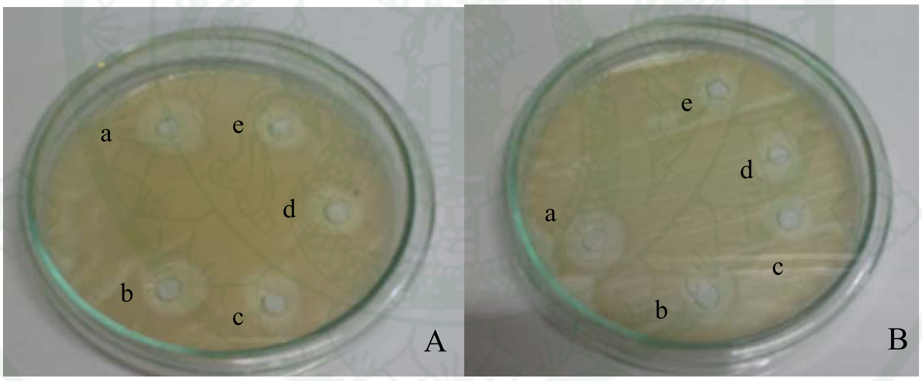


Figure 34 Zone of inhibition by the CFNS of B2 in different temperature range against *V. parahaemolyticus* (Vp) and *V. alginolyticus* (Va)
a – control (untreated), b - 40°C, C - 50°C, d - 60°C, e - 70°C.

3.3.2 Sensibility of antimicrobial substance to proteolytic enzyme

The cell-free culture fluid of B2 was treated by a variety of enzymes shown in Table to verify the protein nature of the inhibitor substance. The inhibitory activity of the supernatant was inhibited by all the proteases employed. Activity was not lost after treatment with lipasse. Complete inactivation of the antimicrobial activity was observed when the CFNS treated with pepsin and proteinase K. and the activity was reduced in great amount when treated with Trypsin 20 AU/ml. The activity remains unaltered 320AU/ml after the treatment with Lipase. The control where the CFNS was checked without the addition of enzymes remained unaltered. (Table 13, Figure 35, and Figure 36)

Table 13 Effect of proteolytic enzyme and lipase on the antimicrobial activity of the CFNS of B2 against *Vibrio parahaemolyticus* (Vp) and *Vibrio alginolyticus* (Va).

Enzyme	Vp(AU/ml)	Va (AU/ml)
Proteinase K	0	0
Pepsin	0	0
Trypsin	20	20
Lipase	320	320

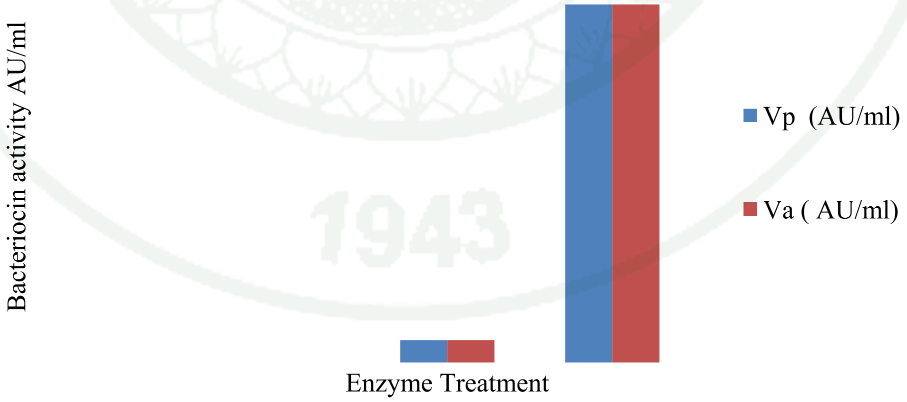


Figure 35 Antimicrobial activity of CFNS of B2 against different proteolytic enzymes and lipase against *V. parahaemolyticus* (Vp) and *V. alginolyticus* (Va).

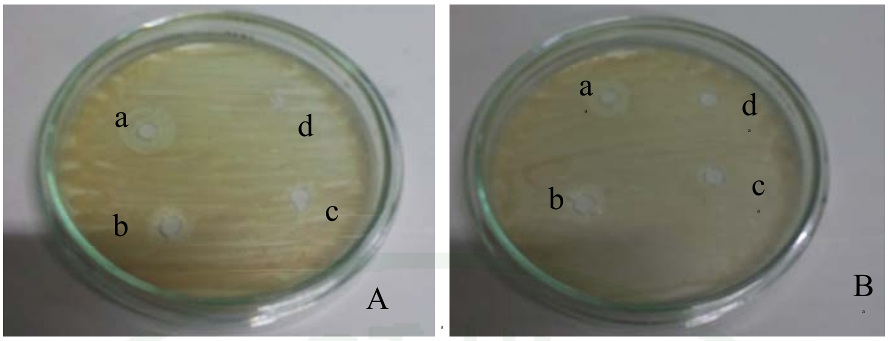


Figure 36 Zone of inhibition by the CFNS of B2 after the treatment with different proteolytic enzymes and lipase against *V. parahaemolyticus* (Vp) and *V. alginolyticus* (Va) ; a – Control (untreated), b – Lipase, c – Proteinase K, d - Pepsin

3.3.3 Sensibility of antimicrobial substance at different pH values

The CFNS of the tested strains were adjusted to various pH from 2.0 - 12.0 and incubated at room temperature and also at 4°C. The antimicrobials showed a stable residual antibacterial activity within a pH range 6 – 10, (Table 14, Figure 37, Figure 38), when incubated at 30°C (room temperature) and 4°C respectively. But the activity was reduced when exposed to pH below 6 and above 10. (Table 14) It showed the acitivity 80 AU/ml at pH 2, pH 3 and 40 AU/ml at pH 11. However it retained the activity 160 AU/ml at pH 4 when incubated at 30°C and 4°C respectively. No activity was observed at pH 12. The control CFNS in neutral pH showed the activity 320 AU /ml

Table 14 Effect of pH on the antimicrobial activity of the CFNS of B2 against *Vibrio parahaemolyticus* (Vp) and *Vibrio alginolyticus* (Va).

pH values	Vp 30°C AU/ml	Va 30°C AU/ml	Vp 4°C AU/ml	Va 4°C AU/ml
2	0	0	0	0
3	40	40	40	40
4	160	160	160	160
5	160	160	160	160
6	320	320	320	320
7	320	320	320	320
8	320	320	320	320
9	320	320	320	320
10	320	320	320	320
11	40	40	40	40
12	0	0	0	0

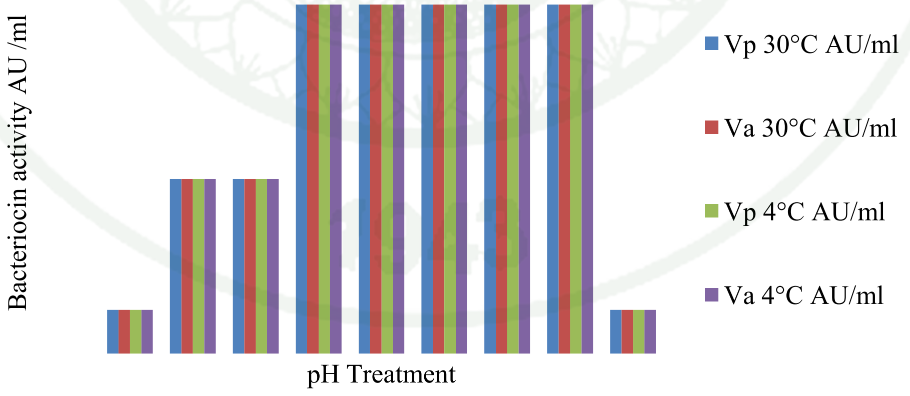


Figure 37 Antimicrobial activity of CFNS of B2 in different pH range at 30°C and 4°C against *V.parahaemolyticus* (Vp) and *V.alginolyticus* (Va).

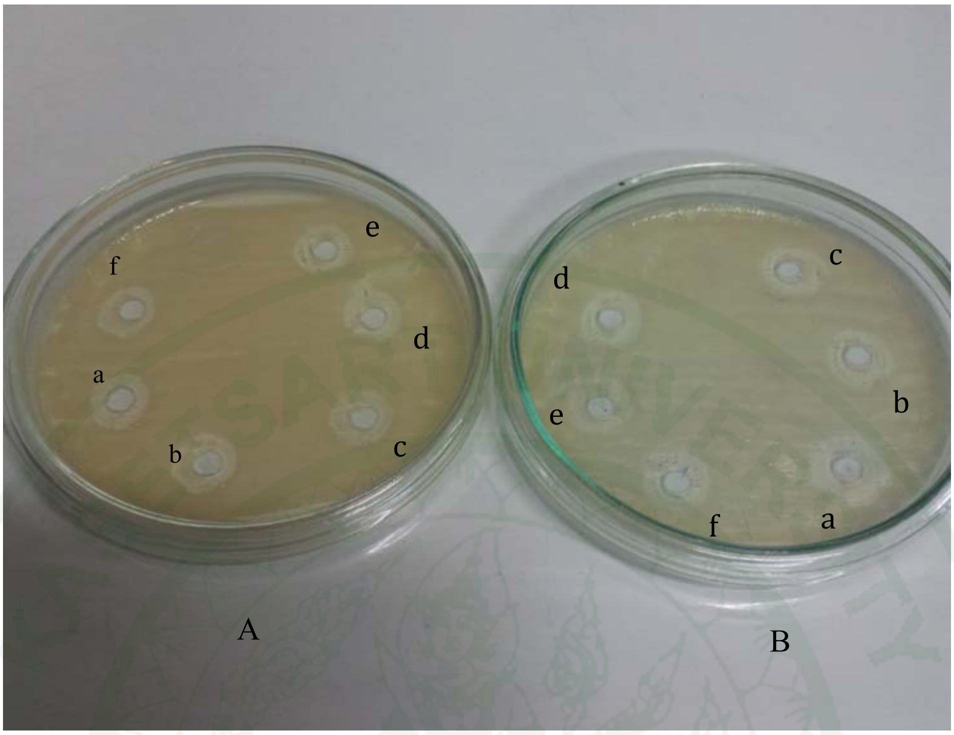


Figure 38 Zone of inhibition by CFNS of B2 in different pH range at 30°C against *V.parahaemolyticus* (Vp) and *V.alginolyticus* (Va); a – control (untreated), b – pH 6, c – pH 7, d – pH 8, e – pH 9, f – pH 10

3.3.4 Sensibility of antimicrobial substance to NaCl

The effect of different concentration of NaCl on the antimicrobial activity was studied. The CFNS when treated with NaCl in a range of 0 – 10%, in room temperature (approximately 30°C) and 4°C respectively for 24 hrs. The antimicrobials showed a stable residual antibacterial activity within a salinity range 0 to 7% under 4°C. The activity was reduced to 80 AU/ ml at salinity 8%. The Residual antimicrobial activity was found to be greatly reduced at salinity 8% and no activity was found at salinity 9% under 4°C. (Table 15 and Figure 39) The result of salinity tolerance under room temperature was different from that of 4°C. The activity was found to be reduced while exposed to above 1%. The activity was found 160 AU /ml at salinity 2. The activity was further declined above 2% range and it was found to show the inhibition of 80 AU/ ml in the range of 3 -7% of salinity..

While the activity retained only at 20 AU/ mi at salinity 8%. There was absolute loss of activity observed at salinity 9%. This was similar in both the condition of incubation.

Table 15 Effect of NaCl concentration (%) on the antibacterial activity of CFNS of B2 under 30°C and 4°C against *Vibrio parahaemolyticus* (Vp) and *Vibrio alginolyticus* (Va)

	30°C/24 hrs	4°C/hrs 24	30°C/24 hrs	4°C/24 hrs
NaCl %	Vp	Vp	Va	Va
0	320	320	320	320
1	320	320	320	320
2	160	320	160	320
3	80	320	80	320
4	80	320	80	320
5	80	320	80	320
6	80	320	80	320
7	80	320	80	320
8	20	80	20	80
9	0	0	0	0
10	0	0	0	0

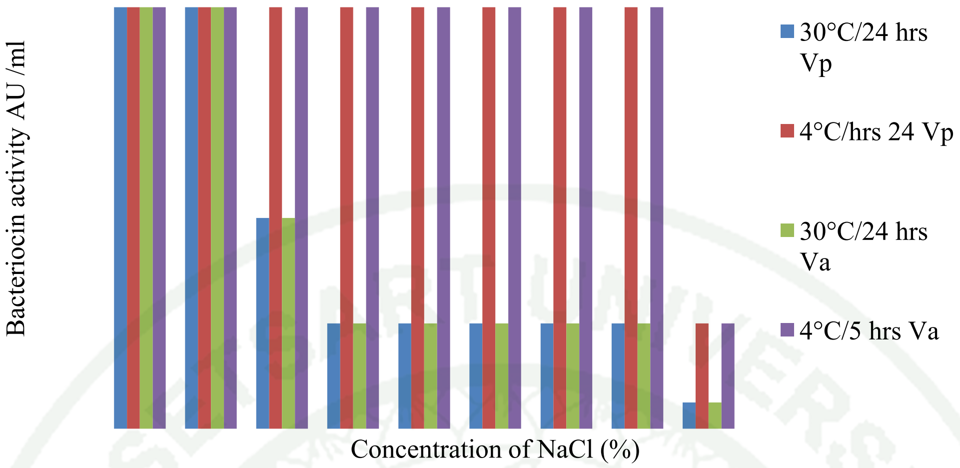


Figure 39 Antimicrobial activity of CFNS of B2 in different salinity range at 30°C and 4°C against *V.parahaemolyticus* (Vp) and *V.alginolyticus* (Va)

3.4 Concentrating the crude CFNS by amberlite XAD- 16 and partial purification

The concentrated CFNS was tested for the antimicrobial activity against the selected indicator strains by the critical dilution method where it showed the antimicrobial activity where the CFNS titer had reciprocal of the dilution factor 128 (D = 2560AU/ ml) (Figure 40)

3.5 Estimation of the molecular weight of the concentrated CFNS by SDS - PAGE

The preparation of the concentrated CFNS was used to determine the molecular weight of the sample. The part containing the sample and molecular marker was stained while the other part of the gel contained the sample only was fixed and then directly used for the antimicrobial activity. SDS –PAGE analysis revealed that there was only one protein band in the sample and hence can be explained that the partial purification could attain the homogeneity of the sample. The band corresponded to approximately 17 kDa.(Figure 41).

The sample containing part of the gel showed the zone of inhibition after 24 hours incubation in the TSA plate seeded with the target pathogens.

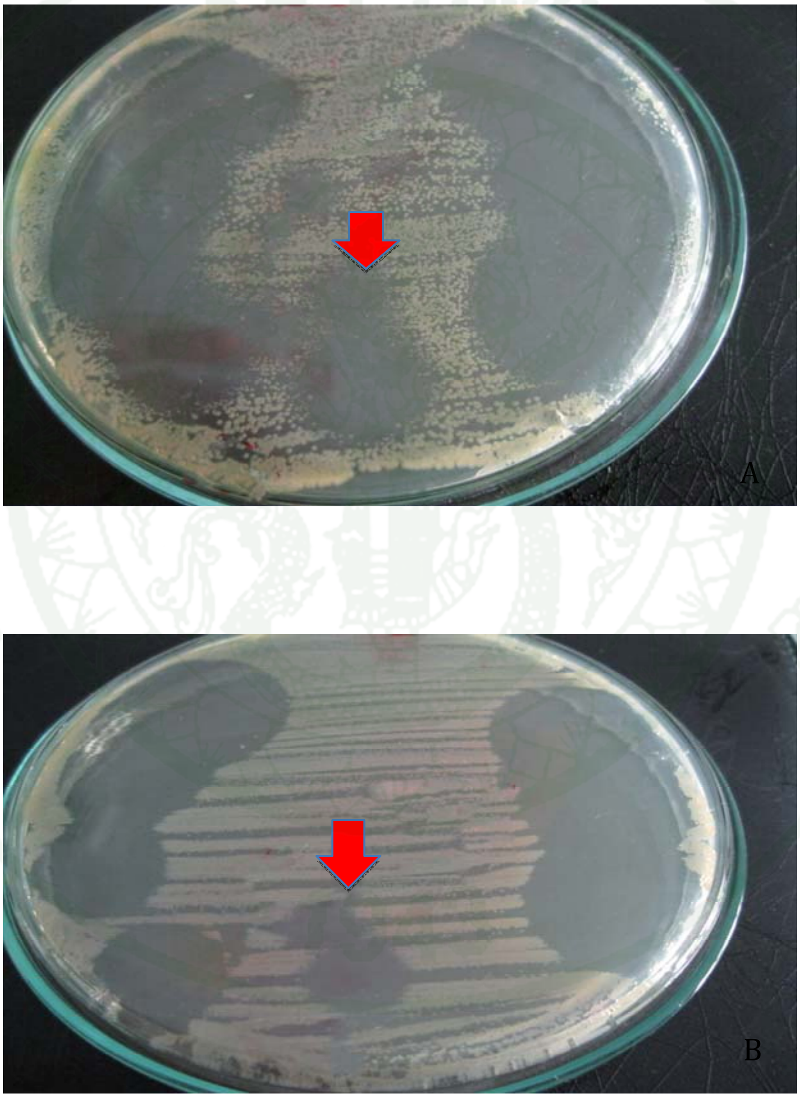


Figure 40 Antimicrobial activity of the partially purified CFNS of B2 against A - *V. parahaemolyticus* (Vp) and B - *V.alginolyticus* (Va). The arrow shows the clear zone obtained from the titer of the antimicrobials with the dilution factor 1/128.

Figure 41 SDS – PAGE of partially purified CFNS revealed the protein band (Lane 1-6 indicated by the arrow), corresponded to 17kDa as compared with the marker (M).

Discussion

Several studies on probiotics have been published during the last decade. The use of probiotics or beneficial bacteria, which control pathogens through a variety of mechanisms, is increasingly viewed as an alternative to antibiotic treatment. The use of probiotics in human and animal nutrition is well documented and recently, efforts to apply probiotics in aquaculture have started (Gatesoupe, 1999; Gomez-Gil *et al*. 2000; Verschuere *et al*. 2000). An expert with the Joint Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) stated that probiotics are live microorganisms, which when consumed in adequate amount; confer a health benefit for the host (FAO/WHO, 2001). Probiotics proposed as biological control agents in aquaculture belong to the following groups: lactic acid bacteria (*Lactobacillus* and *Carnobacterium*), genus *Vibrio*, genus *Bacillus*, and genus *Pseudomonas*. However other genera or species have also been mentioned such as *Aeromonas* and *Flavobacterium*.

Tests of antagonism, adhesion or challenge are essential in selecting a potential probiont. Antagonism may be due to competition for nutrients that favour the growth of probionts, or the expression of their inhibitory effects (Gatesoupe, 1999). Competitive exclusion has been mentioned as a possible mechanism for probiotic effects. In this study, we focused only on some strains of genus *Bacillus* spp., which, showed antagonistic activity to pathogenic bacteria in aquaculture in many studies. Preliminary antagonistic study has been done by cross streak and agar well plate diffusion method (AWDA). In this study we found that *Bacillus* B2 colonized all the strains of *Vibrio* spp. from 48 hours as seen in the cross streak method, with the level of colonization increasing up to 96 hours. The same strain started producing an antimicrobial substance after 24 hours of incubation (as revealed by the result of AWDA) against all target *Vibrio* pathogens. The secretion of this antimicrobial substance continued up to seven days. The highest production of antimicrobial substances was observed on the 5th day by this strain of *Bacillus* against almost all target pathogenic strains. This result could explain the reduction of

Vibrio strains in the co-culture experiment which strongly supports the evidence of 85-90% of reduction of *Vibrio* strains compared to the control (mono-culture) at the end of 120 hours while co-cultured with B2. *Bacillus* B5 started showing inhibition against all the target pathogenic *Vibrio* strains from 72 hours and the effect kept increasing up to 96 hours as revealed by the result of cross streak method. However production of antimicrobial substance for this strain was observed from 48 hours by AWDA. According to the result of AWDA the strain was able to produce the antimicrobial substance up to seven days against all the target pathogenic *Vibrio* strains and showed the highest level of activity on the 5th day of the experiment. This result also supported the growth reduction of all *Vibrio* strains at around 80-85% compared to control at the end of 120 hours while co-cultured with *Bacillus* B5. B1 showed the inhibitory effect only against *A. hydrophila* AQAH, with the zone of inhibition observed from 72 hours in cross streak method, and diameter of inhibition increasing until 96 hours. This result was different in the AWDA where we found that *Bacillus* B1 antagonized the target pathogen *A. hydrophila* AQAH from 24 hours of incubation. However, the production of antimicrobial substance continue until the 5th day while the highest level was observed at 72 hours, which was similar to the result of cross streak method. The use of the AWDA method in this experiment resulted in higher inhibition results than the use of the cross streak method. As a result, the inhibitory activity on indicator bacteria by the AWDA method was seen as better, but it could be because all metabolites and antimicrobial substance were present and being produced during the assay period. But the cross streak method, the inoculum of the *Bacillus* species were used, and conditions for the growing bacteria and/or the preparation of the bacteria as we had taken the inoculum from 24 hrs grown culture of the bacteial (probiont) strain which may result in a decrease of other metabolites inhibitory activity. So, the inhibition zone that was seen may be dependent solely on metabolic activity of the strain. Furthermore, the substances *Bacillus* species secreted might be in the log- or late log-phase of growth. However both the method proved to be effective in preliminary detection of antagonism. *Bacillus* B3 and B4 could not show the antagonism against none of the pathogenic strain in the whole assay.

Bacterial antagonism is a common phenomenon in nature; therefore, microbial interactions play a major role in the equilibrium between competing beneficial and potentially pathogenic microorganisms. However, the composition of microbial communities can be altered by husbandry practices and environmental conditions that stimulate the proliferation of selected bacterial species. It is well known that the microbiota in the gastrointestinal tract of aquatic animals can be modified, for example by ingestion of other micro-organisms; therefore, microbial manipulation constitutes a viable tool to reduce or eliminate the incidence of opportunist pathogens (Balcazar, 2002).

According to Bergey's Mannual of determinative Bacteriology (1994) *Vibrios* (Vibrionaceae strains) belongs to gamma proteobacteria, are gram negative, usually motile rods, are mesophilic and chemoorganotrophic, have a facultative fermentative metabolism, and are found in aquatic habitats in association with eukaryotes. They are able to grow on marine agar and also on salt supplemented selective medium thiosulfate – citrate bile salt sucrose agar (TCBS), and they are mostly oxidase positive. *Vibrios* are highly abundant in aquatic enviornments, including estuaries, marine coastal waters and sediments and aquaculture settings worldwide. Several cultivation dependents and independent studies showed that *Vibrios* appear particularly high densities in marine organisms, for example corals, fish, mollusks, seagrass, sponges, shrimps and zooplanktons. Isolation of *Vibrios* can be achieved in TCBS agar that had been additionally supplemented with 1.5% NaCl. This medium is selective for cultivation of shrimp pathogenic *Vibrios* and other enteropathogenic *Vibrio*s. The high concentrations of thiosulfate, citrate and the strong alkalinity in the medium inhibit the growth of enterobacteriociae. Ox bile and cholete suppress primarily the enterococci. Any coliform bacteria that may grow cannot metabolize sucrose. The mixed indicator thymol blue – bromothymol blue changes its color to yellow when acid is formed.

The first report of the existence in seawater of bacteria with an inhibitory effect against a *Vibrio* sp. has been attributed to Gaixa (1889). Since then the research has started to develop biological control agents. Competitive exclusion of potential pathogenic bacteria effectively reduces or eliminates the need for antibiotic prophylaxis in intensive larviculture systems (Garriques and Arevalo, 1995). In this study we did the broth co-culture experiment using the same concentration of pathogens and probionts for the periods of 120 hours to see the effect of competitive exclusion of the pathogens by the probionts. We have already discussed the amount of the reduction of pathogenic strains by the different *Bacillus* spp. The remarkable inhibition of *Vibrio* spp by B2 and B5 which correspond to 85 -90% by B2, 80 – 85% by B5 and 61.81% growth reduction was found in *A. hydrophila* by B1 at the end of 120 hrs of culture period . However, the reduction of *A. hydrophila* at the end of 96 hrs was 61.22% which was not much different from the percent decrease of *A. hydrophila* AQAH at the end of 120 hours. The reduction of *Bacillus* bacteria was 4.16% for B1, 4 to 8 % for B2, and 4 to 9% for B5, at the end of 120 hours of the co-culture experiment when compared to the mono-culture (control). These can be attributed to lack of nutrients in the culture media at the end of 120 hours of incubation. *Bacillus* B3 and B4 did not produce any antagonism by cross streak and AWDA against any of the pathogenic bacteria, therefore these strains were not selected for the co-culture experiment.

In this study (co-culture experiment) pathogenic bacteria were used at high concentrations. Previous studies have shown that the number of luminous bacteria in coastal areas ranged from $0.7\text{x}10^1$to$7\text{x}310^1$ CFUml^{-1}(Sudthongkong, 1996), while in freshwater areas, total bacteria varied from $3.1\text{x}10^1$to $1.0\text{x}10^3$ CFU ml^{-1} (de Sousa and Silva-Souza, 2001). In fish pond waters, total bacteria ranged from $1.8\pm 0.9 \text{ x}10^2$ to $6.0 \pm 1.2 \text{ x } 10^4$ CFU ml^{-1} (Al-Harbi, 2003). In this present study it was observed that in spite of applying a high amount of pathogenic bacteria (10^5CFU ml^{-1}) the experimental strains of *Bacillus* spp. (except B3 and B4) exhibited potential antagonism against the target pathogenic strains.

In this co-culture experiment, *Bacillus* spp. B2 and B5 could inhibit growth of *Vibrio*. spp. while B1 could inhibit growth of *A. hydrophila* AQAH, which was similar to reports by Purivirojkul and Areechon (2007). Many studies supported that *Bacillus* spp. could reduce pathogenic bacteria in aquaculture. Vaseeharan and Ramasamy (2003) reported *P. monodon* immersed in *Bacillus subtilis* BT23 at a density of 10^6-10^8 CFU/ml for 6 days showed 90% reduction in accumulated mortality when challenge with *V. harveyi* at 10^3 - 10^4 CFU/ml for 1 hour. Devaraja *et al*. (2002), used microbial products, *Bacillus* spp., *Saccharomyces* sp., *Nitrosomonas* sp. and *Nitrobacter* spp., in fish and shrimp pond by immersion for 110 days, the results showed that *Bacillus* spp. were dominant in all ponds and the bacterial populations were changed by use this probiotic. *Bacillus* spp. in monoculture and co-culture in this study, showed very little reduction in number that might be because of the lack of nutrient in the culture media during the experiment. The results from this study indicated the strong potential of these *Bacillus* spp. as another means for the control of pathogenic *Vibrio* spp. and a *A. hydrophila* as a probiotic for the application of aquaculture.

Moriarty (1998) and Rengpipat *et al*. (1998), reported that the possibility of increasing shrimp production in large bodies of water by adding the probiotic *Bacillus*. Similarly other studies have reported that probiotic could improve water quality (Homma and Shinohara, 2004; Manpal *et al*. 2003).

Table 11 showed sensitivity of the various strains to the antimicrobials produced by B1, B2 and B5 which was further measured with critical dilution method and the bacteriocin activity assay, described by Schillinger *et al*. (1993) and Cintas *et al*. (1995), respectively. Activity was measured as the reciprocal of the highest two-fold dilution showing antimicrobial activity. According to to the observations, 1/16 dilution of the crude supernatant is the highest dilution showing antimicrobial activity against indicator strain that are *V. parahaemolyticus* (ABRC Lab.) and *V. alginolyticus* (ABRC Lab.) which was used in all measurements for bacteriocin or antimicrobial activity. Bacteriocin activity of cell-free supernatant of B2 (crude extract) was calculated as:

16 x 20 = 320 AU/ml.

AU= Arbitrary Unit; 16= Reciprocal of the highest dilution (1/16); 20 = 1000 µl / 50µl (conversion factor)

The antibacterial spectra of the activity of the bacteriocin produced by B1, B2 and B5 was assayed against10 indicator strains) belonging to two different genera and 8 different species including *Vibrio* spp. and *A. hydrophila* (gram negetive). This assay was carried out to determine the residual antimicrobial activity of the antimicrobial product of these 3 strains of *Bacillus* spp. on the basis of which the selection of the antimicrobial product produced by the strain which showed the strongest inhibition to the pathogenic bacteria had been carried out.

The antimicrobial titer of B1 when tested against the selected pathogenic strain *A. hydrophila* it produced the moderate inhabitance that was 40 AU/ ml. The antimicrobial spectrum of the B5 against the *Vibrio* spp. had already been demonstrated through AWDA and cross streak. However the residual antimicrobial activity showed moderate to week inhibition of the pathogenic *Vibrio* spp. by B5 by the critical dilution method. The response of inhibition were 80 AU/ ml against *V. parahaemolyticus* and *V. alginolyticus* while 40 AU/ ml was found for the antimicrobial activity against *V. harveyi*, *V. vulnificus* (yellow and green producer strains) *V. cholerae* (non 01), *V. mimicus*, But *V. fluvialis* (Yellow and green colony producer strains respectively) was found to be weakly inhibited by the B5 where we found the residual antimicrobial activity was 20 AU /ml. The response of residual antimicrobial activity of the B5 against the pathogenic *Vibrio* could be attributed to the dilution of antimicrobial product which had reduced the strength of the antimicrobials.

For B2 the antimicrobial titer showed moderate to strong inhibition against the tested pathogenic strains. It showed the residual antimicrobial activity against *V. parahaemolyticus* and *V. alginolyticus* 320 AU /ml. The residual antimicrobial activity was exhibited against *V. harveyi*, *V. vulnificus* (yellow and green producer strains) 160 AU / ml. The residual antimicrobial activity was 80 AU/ ml against *V. cholerae* (non 01), *V. fluvialis* (Yellow and green colony producer strains respectively). However the *V. mimicus* was found to be moderately inhibited by B2 where the residual antimicrobial activity was 40 AU/ml.

The outer membrane of gram-negative bacteria prevents molecules like bacteriocins, antibiotics, dyes and detergents from reaching the cytoplasmic membrane and acts as a permeability barrier for the cell. The antimicrobial product of our tested strain *B. pumilus* showed the strong inhibition against the pathogenic *Vibrio* spp. which belong to gram negative strains.

Heat resistance is a major characteristic of many bacteriocins and bacteriocin-like compounds produced by lactic acid bacteria and can vary dramatically ranging from 60°C or 100°C for more than 30 min (e.g. lactocin 27, lactocin S, carnobacteriocins A and B) to autoclaving at 121°C for 15-20 min (e.g. lactacin B, lactacin F, nisin *etc.*) (De Vuyst and Vandamme, 1994).

Many of the bacteriocins particularly the ones of class I and class II, are described as small hydrophobic proteins containing little tertiary structure, which explains their heat stability. Other factors contributing to heat stability of the bacteriocin of LAB are stable cross-linkages, a high glycine content and occurrence of strongly hydrophobic regions. Such heat stability also excludes the possibility of the inhibitory action being due to bacteriophage (De Vuyst and Vandamme, 1994). In comparison with some of the bacteriocin produced by the lactic acid bacteria the bacteriocin of our tested strain of *B. pumilus* was not extremely heat stable. Effect of heat treatment on the bacteriocin activity was tested by keeping the crude extract 40, 50, 60, 70, 80, 90 and 100°C for various periods (Table 12). The crude bacteriocin was very stable to heat with respect to the temperatures and durations up to 70°C because the original activity of crude extract (320 AU/ml) was unchanged upon the heat treatment of 70°C. The activity started decreasing above this temperature. The activity found 80 AU/ml while heated for 20 minutes at 80°C and the further reduction was observed 40 AU/ml while heated for an hour at the same temperature.

The activity decrease at 20 AU/ ml while heated for 20 minutes at 90°C no activity was detected at 90°C while heated for 60 minutes. Complete loss of activity was found at 100°C and after autoclave at 121°C for 15 minutes. This study of temperature tolerance was similar as studied by the Nithya *et al*., 2010 where the supernatant of *B. pumilus* strain showed the activity up to 70°C for 10 minutes after which there was the decrease of activity was observed. However, the study of Anupad *et al*. (2007) had shown the heat tolerant strains of *B. pumilus* where there was no reduction of antimicrobial activity was found even after autoclave. Many of the *Bacillu*s spp. showed moderate to weak response to heat treatments. The study of Adtunji *et.al*. (2011), showed the antimicrobial activity up to 75° C and complete loss of activity at 100°C by some of the bacteriocin producing strains of *Bacillus* spp. The work of Karmen *et al*. (2003), supported that the four strains of *B. cereus* showed a thermal stability upon the the antimicrobial activity up to up to 45 ,60 and 75° C respectively and great reduction of activity observed while exposed to 90° C for 15 minutes and total loss of activity was observed after exposing to 100° C. The strain of *B. subtilis* studied by Lee *et al*. (2010), exhibited the temperature tolerance up to 80°C while the other strain of *B. subtilis* studied by Arai *et al*.(2011), showed a temperature tolerance of 100°C on exposure for 60 minutes upon the antimicrobial activity against the indicator strains.

The cell-free culture fluid of the *B. pumilus* was treated by different enzymes shown in Table to verify the protein nature of the inhibitor substance. The inhibitory activity of the supernatant was inhibited by all the proteases employed. Activity was not lost after treatment with lipase. These data clearly showed that the antimicrobial substance is of proteinaceous nature, containing cleavage-sites suitable for the mentioned proteases. Characterization of the proteinaceous inhibitor confirmed that the antimicrobial agent produced by the tested strain of *B. pumilus* was similar according to the criteria outlined by Tagg *et al*. (1976). The complete inactivation of the antimicrobial substance after the treatment with pepsin and proteinase k was observed and only 6% activity (320 AU/ ml has been considered the total activity as 100) was observed after the treatment with trypsin. This was one of the key criteria of bacteriocin characterization. There was no loss of activity was observed while treated with lipase enzyme (Table 13).These indicated that the bacterial peptide did not contain lipidic moiety. The pumilicin characterized by Anupad *et al*. (2007), from the *B.pumilus* strain also showed the inactivation against all the proteolytic enzymes and lipase which was similar to our study. However the antimicrobials of our tested strain had shown very reduced response against the trypsin.

The sensitivity of the antimicrobial substance against the proteolytic enzymes had also been reported from the other *Bacillus* spp., as the study by Gray *et al*.(2006), characterized the bacteriocin from *B. thuringiensis* and Hyronimus *et al*.(1998) , where the characterization of bactericin was carried out fron *B. coagulans*. The study also showed the antimicrobial compounds isolated from *B.amyloliquefaciens* (Lisboa *et al*., 2006, Sutyak *et al*, 2008), *B cereus* (Bizani *et al*. 2005), and *B. subtilis* (Arai *et al*. 2011) exhibited the sensitivity against the proteolytic enzymes.

Bacteriocins differ greatly with respect to their sensitivity to inactivation by changes in pH and temperature. Many of the bacteriocins and bacteriocin-like substances produced by lactic acid bacteria are only stable at acid and neutral pH (De Vuyst and Vandamme, 1994) and are inactivated even at a pH above 8.0 (e.g. nisin, lactostrepcins, pediocin AcH, leucocin A-UAL 187). This can be attributed to the solubility of the bacteriocins of LAB (lactic acid bacteria); the isoelectric points of the bacteriocins produced by LAB are around 8.0-9.0 and the solubility of the bacteriocins decreases with increasing pH.

The combined effect of temperature and pH was tested also by exposure of the crude bacteriocin, of which pH was pre-adjusted to 2-12, to room temperature and 4°C. The crude extract with a pH value between 2 and 12 was kept for 5 hrs at room temperature, for 24 h at 4°C and for 24 hrs at room temperature. It is seen from Table (14) that crude bacteriocin at 4°C within the pH range of 6 to 10 in both the condition was stable, activity losses occurred at pH 11 and below 6 only in spite of 24 hrs.

incubation at 4°C. At room temperature incubation for 5 hrs, the activity losses began to come out at pH 11, activity being dropped to 40 AU/ml from 320 AU/ml. In our study the optimum residual antimicrobial activity was observed between pH 6 to 10 in both the condition of room temperature (Approximately 30°C) and at 4°C. The bacteriocin activity was reduced to 40 AU/ml and 160 AU/ml at pH 3.0 and 4.0, and pH 5.0 respectively as explained in Table (14). The obvious stability of the bacteriocin at 4°C for 24 hrs incubation was due to the inhibition of the proteases at this temperature. No activity was detected at pH 2.0 and above 11.0. The activity was more stable in alkaline than in acidic condition. It was observed to have a wide range of activity under neutral to alkaline pH. The similar report was evidenced from the study of Anupad *et al*. (2007), where the Pumilicin 4 isolated from *B. pumilus* WAPB4 showed the activity under the pH range of 3.0 - 9.0 where the optimum activity was observed at alkaline pH range.The report of Lee *et al*. (2010) indicated about the antimicrobial activity under the the pH range from 4.0 to 10.0 of the strain of *B subtilis*., SC8. Karmen *et al*. (2003) reported of the pH tolerance of several strains of *B cereus* within 3.0 – 10.0. Teixeira *et al*. (2009) reported that the antimicrobial activity of *B. licheniformis* strain P40 showed under pH 3.0 – 10.0. Riazi *et al.* (2009) described the antimicrobial substance from *B. coagulans* ATCC 7050 was “Lactosporin” which retain it’s activity under pH value 3.0 - 7.0 with high range of thermostability remained active after the exposure to high temperature at 100°C. Similarly the *Bacillus* spp. MTCC 43 described by Sharma *et al*. (2009) also demonstrated the activity within a pH range of 4.0 – 10.0 with a temperature tolerance of 40 – 90°C. Kamoun *et al*. (2005), identified Bacthuricin F produced by *B. thuringiensis* which retained it’s antimicrobial activity when subjected to a range of temperature conditions 40 – 90°C for 30 min and also showed a stable activity under a pH range of 3.0 – 9.0.

Many studies had been conducted about the production of antimicrobial compounds under the influence of the range of salinity mostly in LAB. Paucities were there regarding the information of salinity tolerance of the bacteriocin of the *Bacillus* spp. The antimicrobial substance isolated from the tested strain of *B.*

pumilus was exposed to a range of salinity between 0 – 10% under room temperature and 4°C for 24 hrs. In both the condition of incubation there was no loss of activity was observed upto 0 -1 % of salinity. The activity strated declining above 1% of salinity at room temperature. The activity was found 160 AU /ml at salinity 2%. under room temperature of 24 hrs. The activity was further declined above 2% and it was found to show the inhibition of 80 AU/ ml in the range of 3 – 7%. And a great reduction of salinity was on 8% as 20 AU/ml. There was absolute loss of activity observed at salinity range of 9% which was similar in both the condition of incubation under room temperature and 4°C. However a stable activity was observed between 0 – 7% range of salinity when the crude supernatant of the *B. pumilus* was kept under 4°C. The activity was declined to 80 AU/ml when exposed to 8% salinity under the incubation of 4°C. At this temperature no activity was observed while exposed to the range of salinity 9%. As it was mentioned earlier that only little study had been done so far on the salinity tolerance of bacteriocin compounds from the *Bacillus* spp. However the report of Arai *et al*. (2011) differed from our finding where the antimicrobial product of *B. subtilis* B1 showed wide range of salinity 0 -15 % under 4°C. The activity was much reduced while exposed to room tenperature for 24 hrs which was similar to our findings. There was another report of a halotolerant strain of *B subtilis* BBK-1, (Roongsawang *et al*., 2002.) where the *Bacillus* spp. was found to produce biosurfactants up to the range of 8% salinity. The report of Wilaipun *et al*. (2002), showed that bacteriocin production by LAB strain *Enterococcus faecium* NKR-5-3 was inhibited when the broth contained more than 1% of NaCl. The similar findings was forwarded by N.S *et al*.(2012), where the activity of the bacteriocin produced by the LAB *Lactobacillus* L2 showed the optimum activity at 1% range of salinity under room temperature and the activity was subsequently declined at 2% ,3%, 4% and very little activity was recorded at 5% range of salinity. Study of karthikeyan *et al*. (2009), showed that the bactericin acitivity of the strain *L. acidophilus* can be optimum when it was exposed to 0.9% salinity.

The estimation of the apparent molecular weight of the semi-purified

Bacteriocin eluted from amberlite cextraction was made by SDS-PAGE. It revealed a major band at a molecular weight of 17 kDa. *Bacillus pumilus* B2 produced the highest molecular weight of antimicrobials for this particular species and to our knowledge; this study was the first to report on this *Bacillus* species (*B. pumilus*) against the pathogenic *Vibrio* spp. of acquaculture. The latter was 17 fold greater than pumilicin4 (1.994kDa) isolated from *B. pumilus* WAP4 by Anupad *et al*. (2007). Our antimicrobial is reported to be greater from *B. subtilis* bacillocin 22 and *B. subtilis* subtilosin A (3.4 kDa), *B. subtilis* CX1 bacteriocin (9.5 kDa) and also greater than *B. subtilis* KS03 iturin A2 (1.042 kDa). Those bacteriocins have not yet been reported for their antimicrobial activity against *Vibrio* spp. On the other hand the antimicrobial activity of the partially purified bacteriocin was detected on the portion of a unstained gel corresponded to the protein band over the indicator strain seeded plate following the SDS PAGE. However the report of lactosporin from *B. coagulans* ATCC7050 by Riazi *et al*. (2008) exhibited the molecular weight approximately 25 – 30 kDa . Hammami *et al*. (2008) described the bacteriocin bac 14 B from *B. subtilis* 14B had a molecular weight 21kDa which was unlike the other bacteriocin available from the same species of *Bacillus*.

Bacteriocins of Gram-positive bacteria are generally divided into four classes based on size, morphology, physical, and chemical properties (Lee and Kim, 2011). Class I bacteriocins are lantibiotics, which are small peptides (<5 kDa) (Field *et al*. 2007). They are post- translationally modified, incorporating non-traditional amino acids such as dehydroalanine, dehydrobutyrine, methyl-lanthione, and lantionine (Cleveland *et al*. 2001). This class is subdivided into Type A, B, and C with the distinction being that members of Type A are positively charged, linear peptides whereas those in Type B are either neutrally or negatively charged rigid globular peptides. Members of Type C require synergistic activity of two peptides to be active. This class includes the well-studied bacteriocins nisin and lacticin (McAuliffe *et al*. 2001).

Class II bacteriocins are small (<10 kDa), heat-stable peptides that are not post-translationally modified (Heng *et al*., 2007). Class II is also subdivided into two subgroups. Class IIa are pediocin-like or Listeria-active peptides, which contain a conserved N-terminal sequences (YGNGVxCxxxxCxV). Class IIb bacteriocins require the synergistic activity of two peptides to be fully active (Nissen-Meyer *et al*., 1992). Class III bacteriocins are generally large (>10 kDa), heat-labile peptides. They are subdivided into two subtypes. Type IIIa are bacteriolysins, which are bacteriolytic enzymes. Lysostaphin is the most studied bacteriocin in this subtype (Schindler and Schuhardt, 1964). Type IIIb are non-lytic bacteriocins. Helveticin J (37 kDa) produced by *Lactobacillus helveticus* belongs to this type (Joerger and Klaenhammer, 1986). Finally, Class IV bacteriocins have unique structural characteristics. The first and last amino acids of these bacteriocins are covalently bonded, thus they have cyclic structures. Enterocin AS-48 produced by *Enterococcus faecalis* subsp. *liquefaciens* S-48 was the first characterized bacteriocin belonging to this class (Maqueda *et al*. 2004).

The production of bacteriocin or bactericin like inhibitory substances (BLIS) had been reported by several research groups.For instance, polyfermenticin SCD is a heat labile proteinase k sensitive bacteriocin produced by *B.polyfermenticus* SCD (Lee *et al*. 2001) Subtilin is lantibiotic type produced by *B. subtilis*.. Hyronimus *et al. (*1998), reported a heat stable BLIS produced by *B. coagulans* similar with pediocin the bacteriocin produced by the *Pediococcus* spp. The molecular masses of alkaline proteases produced by several bacteria including *Bacillus* ranged from 15 kDa to 30 kDa, with few reports of higher molecular masses of 33 kDa (Samal *et al*. 1991), 36 kDa (Tsujibo *et al*. 1990) and 45 kDa (Kwon *et al*. 1994).

In this study we described a new antimicrobial compound produced by a strain of *B. pumilus* from the Novozyme Biologicals, which is heat labile, showed the activity under extreme pH and moderate salinity and activity was no longer detected while exposed to proteolytic enzymes. It didn’t contain any lipid moiety. The most recent classification of bacteriocin exclude class IV, of the remaining three classes, only class III gathers large size molecules which are heat labile .At the same time the class III bactericin showed the phospholipase activity which was not detected in our antimicrobial product. Many other antimicrobial polypeptides of intermediate size (10–30 kDa) and other large antimicrobial proteins produced by bacilli are not included in this classification scheme due to the lack of data on their protein or gene sequences, although they were described in the text under the category of BLIS (Bacteriocin Like Inhibitory Substance). Proteases produced by *Bacillus* species are the most important group of secondary metabolites that are widely exploited (Ferrari *et al*. 1993). The initial characterization of our protease showed the possibility to get included under this category of BLIS with the potential probiotic property.

CONCLUSION AND RECOMMENDATIONS

Conclusion

From the experimental works and discussion of this study the conclusion can be drawn as follow:

1. Among the five strains of *Bacillus* spp. including four different strains of *B.subtilis* and one strain of *B. pumilus* were taken (*B. subtilis* B1, *B. pumilus* B2, *B.subtilis* B , *B. subtilis* B4 and *B. subtilis* B5) for to test the antagonism against the selected target pathogens isolated from diseased Nile Tilapia and Pacific White shrimps. The pathogenic strains included Seven species of *Vibrio* spp. including *V. vulnificus, V. fluvialis, V. parahaemolyticus, V. alginolyticus, V. mimicus,* and *V. cholera (non 01)*, *V. harveyi* AQVH, *Aeromonas hydrophila* AQAH. *V. vulnificus* and *V. fluvialis* included two strains of yellow and green respectively.

2. Initial test of antagonism by cross streak and agar well diffusion assay revealed that the B2 and B5 was antagonistic to all the *Vibrio* spp. but not against the *A. hydrophila* while B1 was antagonistic against the *A.hydrophila* but not against the *Vibrio* spp.

3. The results showed that B2could produce a higher concentration of antibacterial substances than B5 and B1. Among these, *Bacilli* B2 and B5 could produce antibacterial substances at the highest level on day 5, while B1could produce the maximum level at 72 hours. However *Bacillus* B2 and *Bacillus* B5 showed the antagonism activity only against *Vibrio* spp. and this activity continued up to seven days, while B1 showed the antagonism only against *A. hydrophila* AQAH with the activity remaining up to five days. *Bacillus* spp. B3 and B4 showed no antagonism against any of the pathogenic strain

3. Broth- co-culture experiment of the *Bacillus* spp. with the pathogenic strains revealed that the presence of *Bacillus* spp (B2 and B5) led to a remarkable

inhibition of growth of all strains of *Vibrio* spp. after 120 hours of incubation. Growth of *A. hydrophila* AQAH was reduced by 61.8% by B1 after 120 hours of co-culture The growth reduction by B2 and B5 in the different strains of *Vibrio* spp. was more than 90%, and more than 85% respectively at the end of 120 hours of co-culture experiment whereas there was little difference observed in *Bacillus* spp. concentrations. The reduction of B1 was less than 5%, B2 was 4 to 7% and B5 was 4 to 8% at the end of 120 hours of the co-culture experiment compared to the control (mono-culture).

4. To select the strain of the *Bacillus* spp. for characterization and partial purification of the antimicrobial products, all the three strains were further analyzed for the antimicrobial activity against the selected pathogenic strains by critical dilution method where B2 was selected as it showed the highest antimicrobial activity as 320AU/ ml against *V. parahaemolyticus, V. alginolyticus* which were used as the indicator pathogenic strains for the characterization of the antimicrobials of B2.

5. The characterization of the antimicrobial substance of B2 revealed that it is moderately thermostable, had shown the tolerance of temperature up to 70°C for 60 minutes and activity was much reduced at 80°C , it showed a stable range of pH tolerance from pH 6 – 10 and more active in neutral to alkaline pH, there was completely loss of activity found while expose to proteolytic enzymes and no loss of activity was observed while treated with lipase which indicated that the antimicrobial product of our tested strain is a protease without any lipid moiety. The anitimicrobial is moderately halotolerant and showed the antimicrobial activity up to 7% of salinity when it was incubated under 4°C for 24 hours.

6. Estimation of the molecular weight by SDS –PAGE after the partial purifation of the antimicrobial substance by concentrating through Amberlite XAD - 16. revealed a single protein band of approximately 17kDa.

7. Initial characterization of the antimicrobial compound of our tested B2 strain placed it under the Bacteriocin Like Inhibitory Substance (BLIS).

Recommendations

The present study was an effort to establish the stains of *Bacillus* spp. as the effective probiotic to control the common pathogens of aquaculture. However, there are few areas are still left to be focused which are as follow:

1. Commercial scale *in vivo* experiments will be an area for future research where the individual *Bacillus* strain should be tested along with the pathogenic strains against the challenge of disease, growth parameter and water quality.

2. The elucidation of the mechanism of antagonistic action between these probionts and pathogens is needed to be done.

3. The characterization of the antimicrobial substance needs more research on their synthesis system, the response against the different inorganic substance, purification in more detailed way , amino acid sequencing. These are the certain areas which need to get focused to categorize the antimicrobial in more definitive way.

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APPENDIX

APPENDIX

PREPARATION OF REAGENTS OF SDS-PAGE

1.10% Ammonium persulfate

10 mg ammonium persulfate was dissolved in 100 ml water. It was prepared fresh daily.

2. N, N’-tetramethylene-ethylenediamine (TEMED**)**

TEMED was used from bottle, only pure, distilled TEMED was used. It was stored in cool, dry place and protected from light.

3. 30% Acrylamide/0.8% Bisacrylamide

30.0 g acrylamide and 0.8 g N, N’-methylene-bisacrylamide was taken in a total volume of 100 ml of distilled water. The solution was filtered through a 0.45-μm filter and stored at 4°C in the dark. It was discarded after 30 days, since acrylamide gradually hydrolyzed to acrylic acid and ammonia.

4. 4X Tris-CI/SDS, pH 6.8 (0.5 M Tris CI containing 0.4% SDS, also called 4X Upper Tris)

6.05 g Tris base and 4 ml of 10% SDS was dissolved in 40 ml of distilled water. The pH was adjusted to 6.8 with 1 N HCI. The distilled water was added to 100 ml of total volume. The solution was filtered through a 0.45 μm filter and store at 4°C.

5. Tris-CI/SDS, pH 8.8 (1.5 M Tris-CI containing 0.4% SDS, also called 4X lower Tris

18.17 g Tris base and 4 ml of 10% SDS was dissolved in 40 ml distilled water. The pH was adjusted to 8.8 with 1 N HCI. The distilled water was added to 100 ml to make the total volume. The solution was filtered through a 0.45-μm filter and stored at 4°C.

6. 5X SDS/Electrophoresis Buffer

15.1 g Tris base, 72.0 g glycine, and 5.0 g SDS was dissolved in about 800 ml of distilled water. After the solutes were dissolved, the volume was bring to 1.0 liter. The solution was filtered through a 0.45 μm filter and stored at 4°C. To prepare 1X SDS/electrophoresis buffer, one volume of the above solution was diluted with four volumes of distilled water.

7. 2X SDS/Sample Buffer

30 ml of 10% SDS, 10 ml glycerol, 5.0 ml 2-mercaptoethanol, 12.5 ml of 4X Tris-CI/SDS, pH 6.8, and 5-10 mg bromphenol blue was mixed and the volume to was adjusted to100 ml with distilled water.

8. Coomassie Brilliant Blue R-250

1) 40% Methanol

2) 7% Glacial Acetic Acid

3) 0.25% Coomassie Brilliant Blue R-250

To make 500 mL stain

8.1) Dissolve 1.25 g Coomassie Brilliant Blue R-250 in 200 mL MeOH with stirring in a glass beaker. Glass beakers were preferred here since cleaning following dye preparation is easier.

8.2) Slowly 35 mL glacial acetic acid was added with stirring

8.3) 265 mL deionized distilled water was added. The solution was allowed to stir for several minutes and it was checked periodically to see if particulates settle to the bottom of the beaker when stirring wais stopped.

8. 4) When dye appeared fully dissolved, filtered over Fisher filter paper to remove residual particulates.

5) Stored the dye at room temperature. Dye can be reused about 2-3 times if desired. Staining solution is stable for 2 - 3 weeks at 25°C.

9. Destaining Solution

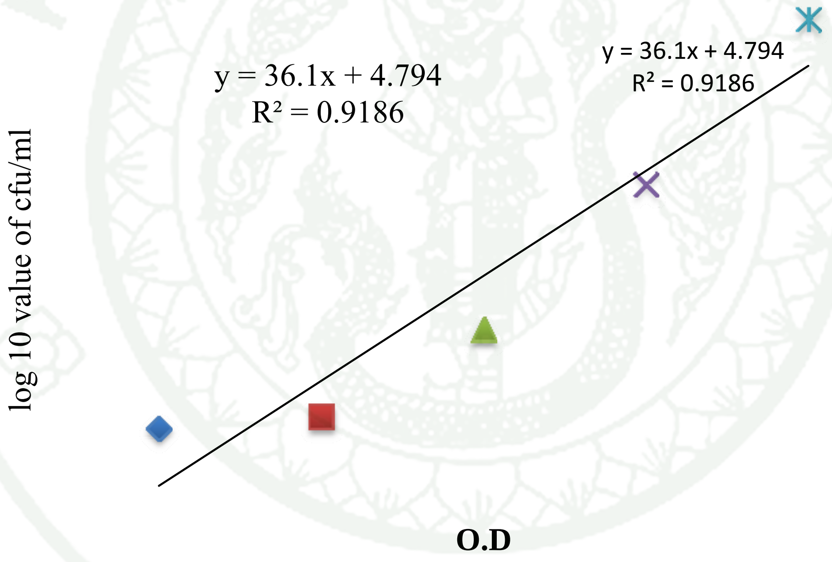
25% (v/v) methanol, 7% (v/v) acetic acid and 68% distilled water were mixed.

10. Fixing Solution

25% (v/v) methanol, 10% (v/v) acetic acid and 65% (v/v) distilled water were mixed.

Appendix Table 1 Number of bacteria (B2, CFU/ml) against the optical density (O.D).

OD	CFU/ml	log 10 cfu/ml
0.02	820000	5.91
0.04	990000	5.99
0.06	3900000	6.59
0.08	39000000	7.59
0.1	530000000	8.72

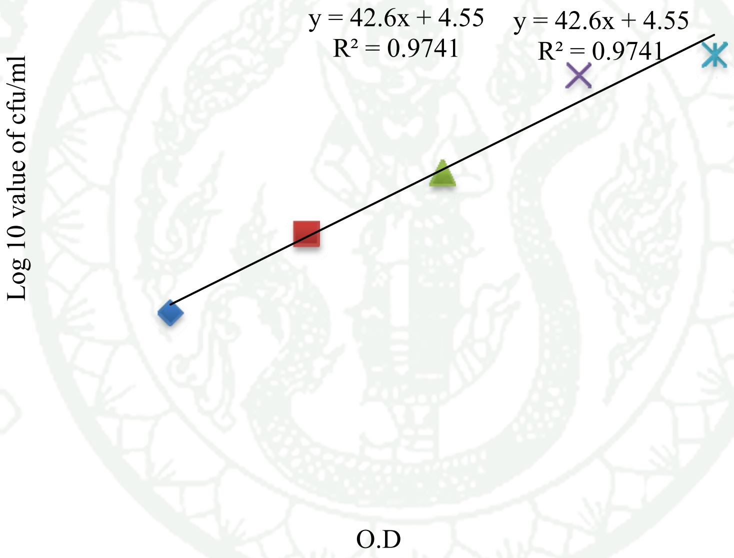


R = The correlation coefficient
Y = Number of colony (CFU / ml)
X = O.D

Appendix Figure 1 Standard curve of B2

Appendix Table 2 Number of bacteria (B5, CFU/ml) against the optical density(O.D).

OD	CFU/ml	log 10 of cfu/ml
0.02	200000	5.3
0.04	2000000	6.3
0.06	12000000	7.07
0.08	200000000	8.3
0.1	370000000	8.56

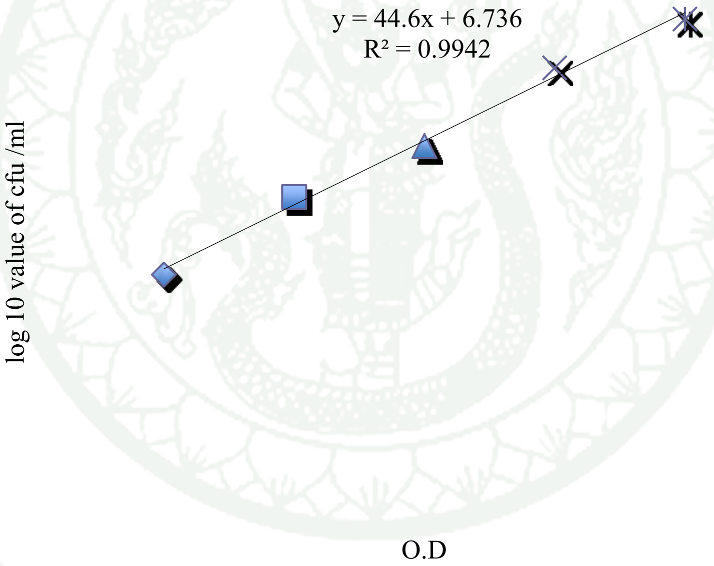


R = The correlation coefficient
Y = Number of colony (CFU / ml)
X = O.D

Appendix Figure 2 Standard Curve of B5

Appendix Table 3 Number of bacteria (B1, CFU/ml) against the optical density(O.D).

O.D	CFU /ml	log CFU/ml
0.02	36000000	7.55
0.04	434000000	8.63
0.06	2200000000	9.34
0.08	87000000000	10.43
0.1	1.3E+11	11.11

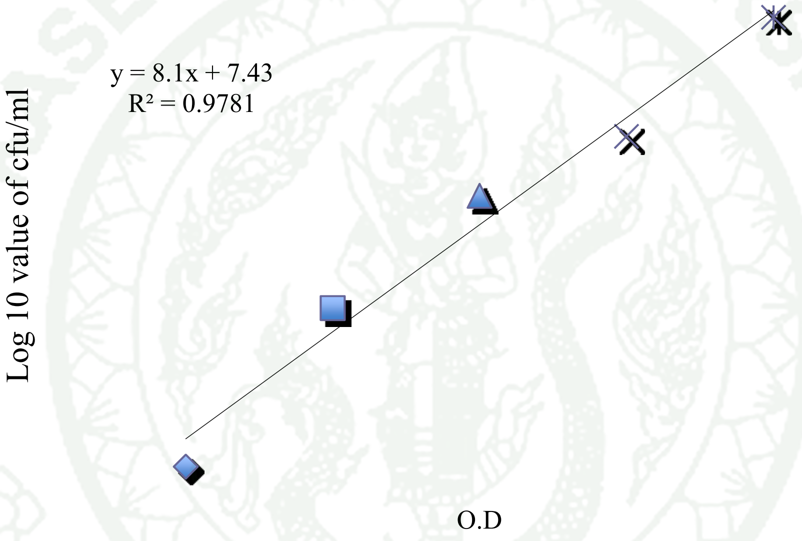


R = The correlation coefficient
Y = Number of colony (CFU / ml)
X = O.D

Appendix Figure 3 Standard Curve of B1

Appendix Table 4 Number of bacteria (*A.hydrophila*, CFU/ml) against the optical density(O.D)

O.D	CFU /ml	log 10 of CFU/ml
0.02	36000000	7.55
0.04	63000000	7.79
0.06	92000000	7.96
0.08	113000000	8.05
0.1	170000000	8.23

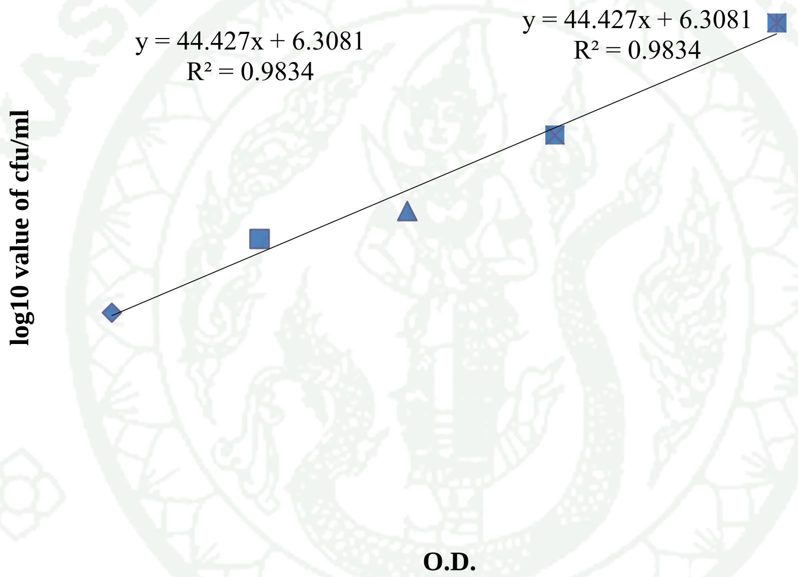


R = The correlation coefficient
Y = Number of colony (CFU / ml)
X = O.D

Appendix Figure 4 Standard Curve of *A.hydrophila.*

Appendix Table 5 Number of bacteria (*V.parahaemolyticus*, CFU/ml) against the optical density(O.D).

OD	CFU/ml	Log 10 of cfu/ml
0.01	6232000	6.79
0.03	69300000	7.84
0.05	1.72E+08	8.23
0.07	2.07E+09	9.31
0.1	8.05E+10	10.9

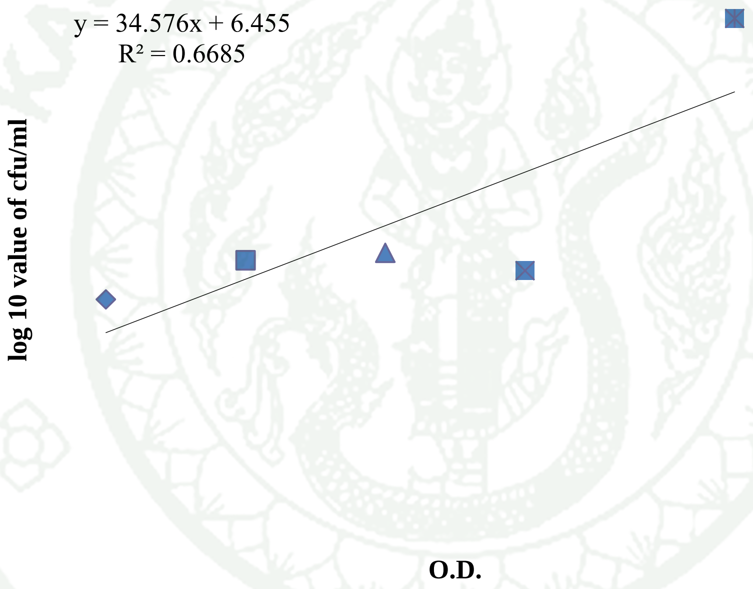


R = The correlation coefficient
Y = Number of colony (CFU / ml)
X = O.D

Appendix Figure 5 Standard Curve of *Vibrio parahaemolyticus.*

Appendix Table 6 Number of bacteria (*V.alginolyticus*, CFU/ml) against the optical density(O.D).

OD	cfu/ml	log10 of cfu/ml
0.01	17000000	7.23
0.03	54500000	7.73
0.05	68000000	7.83
0.07	40000000	7.6
0.1	7.3E+10	10.86

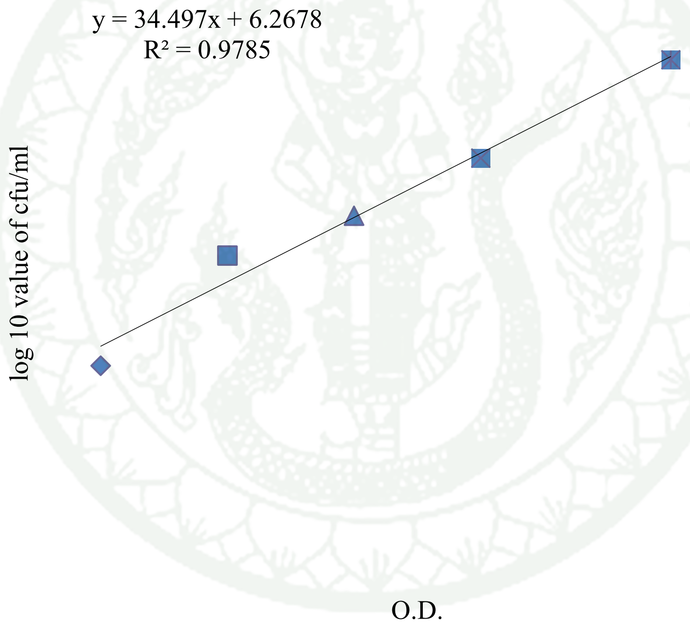


R = The correlation coefficient
Y = Number of colony (CFU / ml)
X = O.D

Appendix Figure 6 Standard Curve of *Vibrio alginolyticus.*

Appendix Table 7 Number of bacteria (*V.fluvialis* (Green), CFU/ml) against the optical density(O.D).

OD	CFU/ml	Log10 of cfu/ml
0.01	2524000	6.4
0.03	38350000	7.58
0.05	1.03E+08	8.01
0.07	4.25E+08	8.62
0.1	4.82E+09	9.68

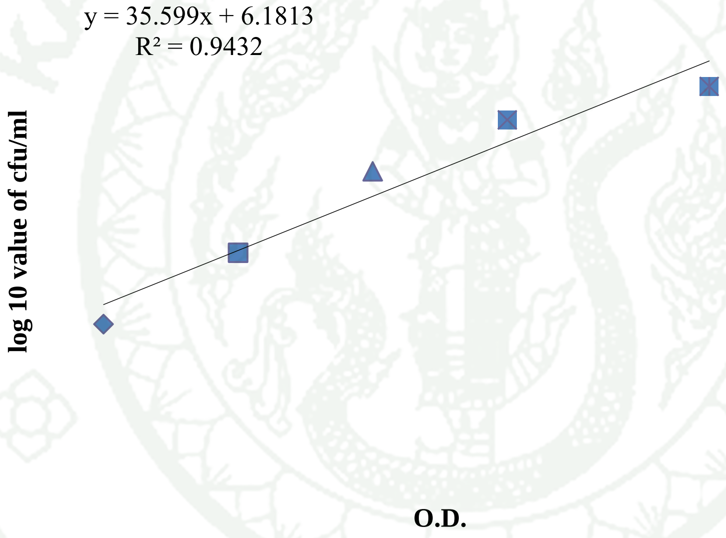


R = The correlation coefficient
Y = Number of colony (CFU / ml)
X = O.D

Appendix Figure 7 Standard Curve of *Vibrio fluvialis* (Yellow).

Appendix Table 8 Number of bacteria (*V.fluvialis* (Green), CFU/ml) against the optical density(O.D).

OD	CFU/ml	log10 of cfu/ml
0.01	1908000	6.21
0.03	16560000	7.21
0.05	195000000	8.29
0.07	925000000	8.96
0.1	2550000000	9.4



R = The correlation coefficient
Y = Number of colony (CFU / ml)
X = O.D

Appendix Figure 8 Standard Curve of *Vibrio fluvialis* (Green).

Appendix Table 9 Number of bacteria (*V.vulnificus* (Yellow), CFU/ml) against the optical density(O.D.)

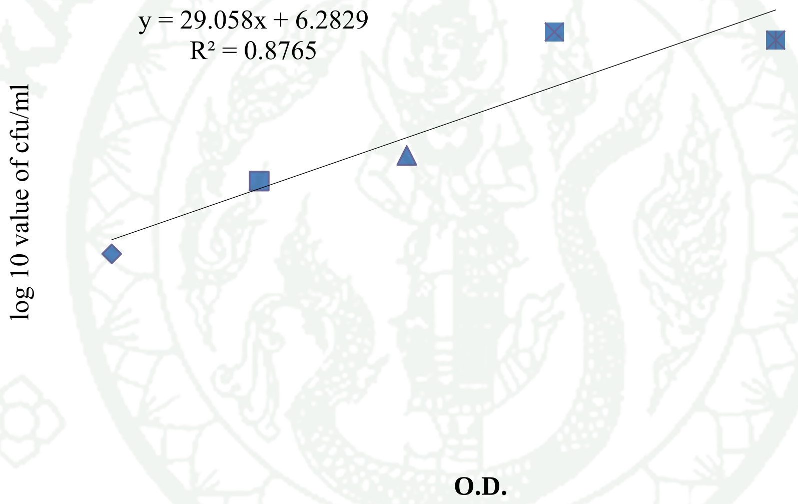
OD	CFU/ml	log10 of cfu/ml
0.011	47500000	7.67
0.029	3.45E+08	8.53
0.052	3.4E+08	8.53
0.07	7.45E+09	9.87
0.11	6.35E+10	10.8

R = The correlation coefficient
Y = Number of colony (CFU / ml)
X = O.D

Appendix Figure 9 Standard Curve of *Vibrio vulnificus* (Yellow).

Appendix Table 10 Number of bacteria (*V.vulnificus* (Green), CFU/ml) against the optical density(O.D)

OD	CFU/ml	log10 of cfu/ml
0.018	2584000	6.41
0.034	17400000	7.24
0.05	34050000	7.53
0.073	8.7E+08	8.93
0.185	7E+08	8.84

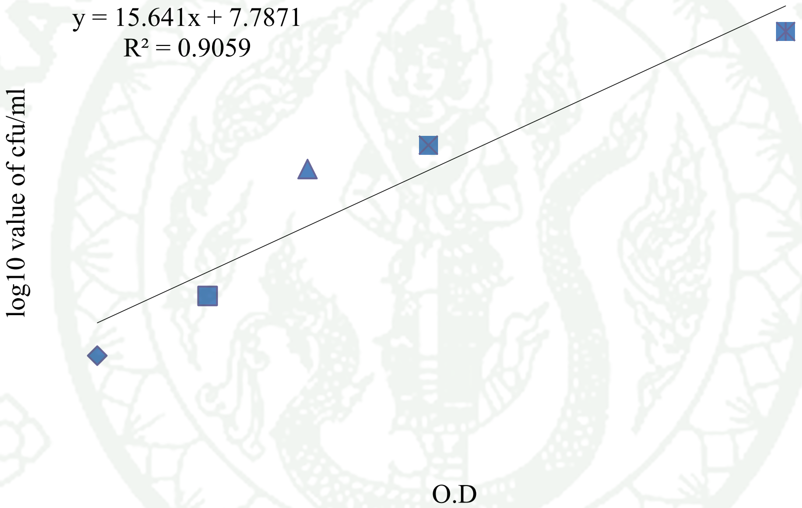


R = The correlation coefficient
Y = Number of colony (CFU / ml)
X = O.D

Appendix Figure 10 Standard Curve of *Vibrio vulnificus* (Green)

Appendix Table 11 Number of bacteria (*V.mumicus*, CFU/ml) against the optical density(O.D).

OD	CFU/ml	log10 of cfu/ml
0.012	58000000	7.76
0.033	1.41E+08	8.14
0.052	9.3E+08	8.96
0.075	1.33E+09	9.12
0.143	7.2E+09	9.85

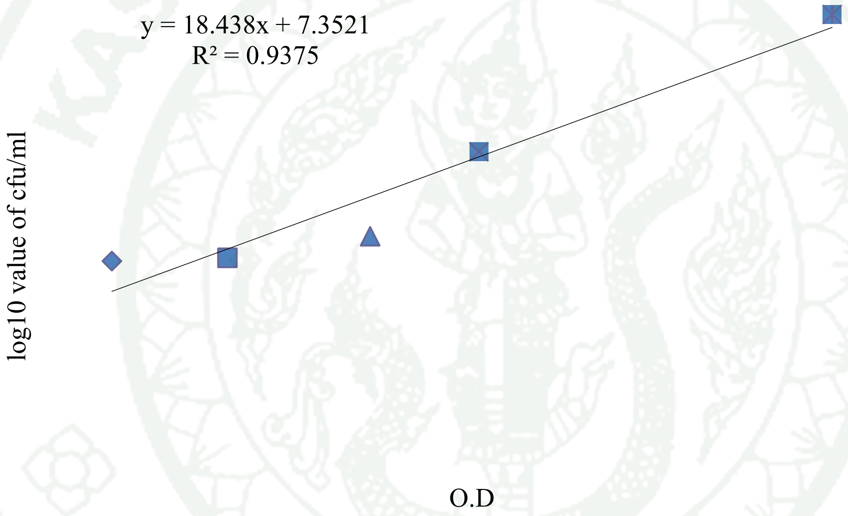


R = The correlation coefficient
Y = Number of colony (CFU / ml)
X = O.D

Appendix Figure 11 Standard Curve of *Vibrio mimicus.*

Appendix Table 12 Number of bacteria (*V.choleriae* (non 01), CFU/ml) against the optical density (O.D).

OD	CFU/ml	log10 of cfu/ml
0.017	77500000	7.88
0.034	82000000	7.88
0.055	1.19E+08	7.88
0.071	5E+08	7.88
0.123	5.2E+09	7.88

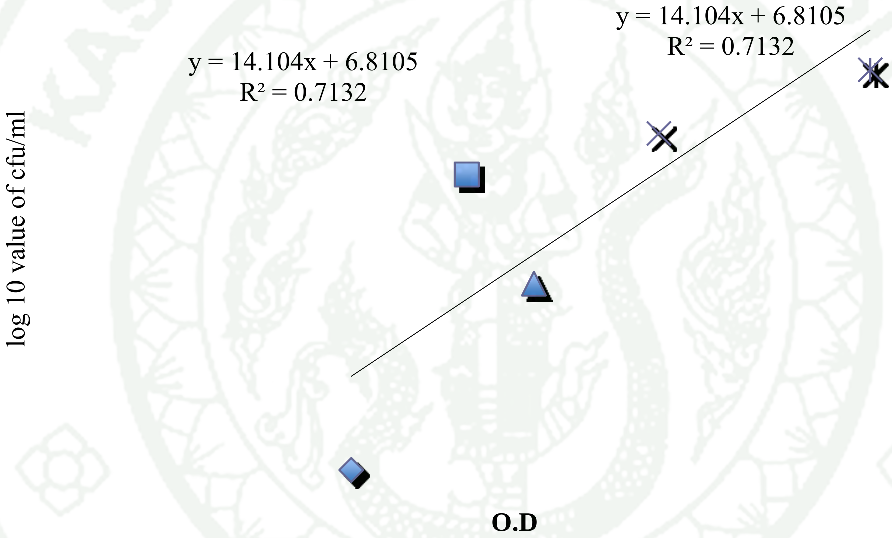


R = The correlation coefficient
Y = Number of colony (CFU / ml)
X = O.D

Appendix Figure 12 Standard Curve of *Vibrio choleirae* (non 01).

Appendix Table 13 Number of bacteria (*V.harveyii*, CFU/ml) against the optical density(O.D).

OD	CFU/ml	log 10 of cfu/ml
0.033	11850000	7.07
0.045	52500000	7.72
0.052	30900000	7.48
0.065	65800000	7.81
0.087	90300000	7.95



R = The correlation coefficient
Y = Number of colony (CFU / ml)
X = O.D

Appendix Figure 13 Standard Curve of *Vibrio harveyi.*

CURRICULUM VITAE

NAME : Mrs. Sanghamitra Nayak

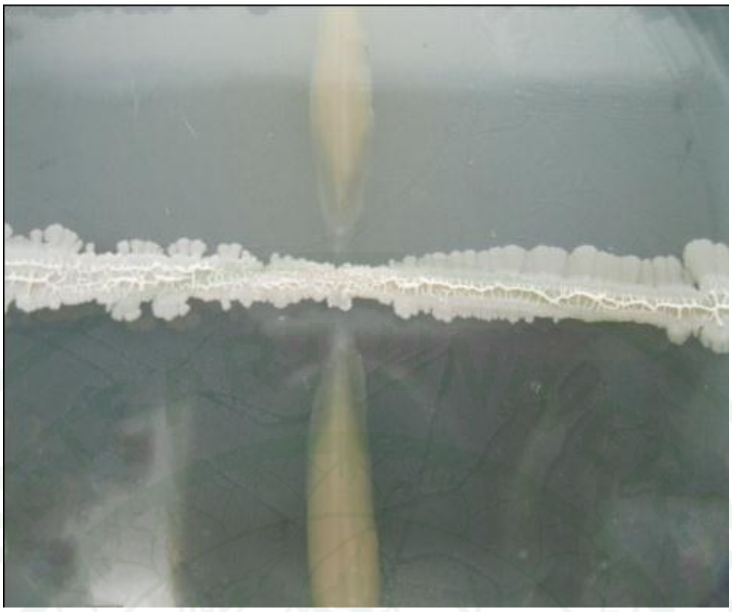
BIRTH DATE : June 28, 1973

BIRTH PLACE : Calcutta, India

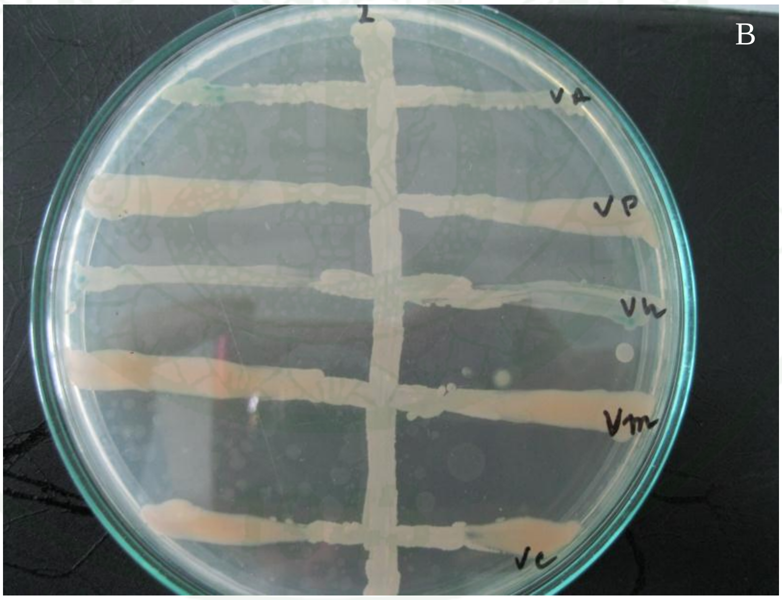
EDUCATION

YEAR	INSTITUTE	DEGREE/DIPLOMA
1996	Calcutta University	B.Sc (Zoology, Hons)
1998	Banaras Hindu University	M.Sc (Zoology)

POSITION/TITLE : Researcher
WORK PLACE : ABRC, Dept. of Fishery Biology, Kassetsart University

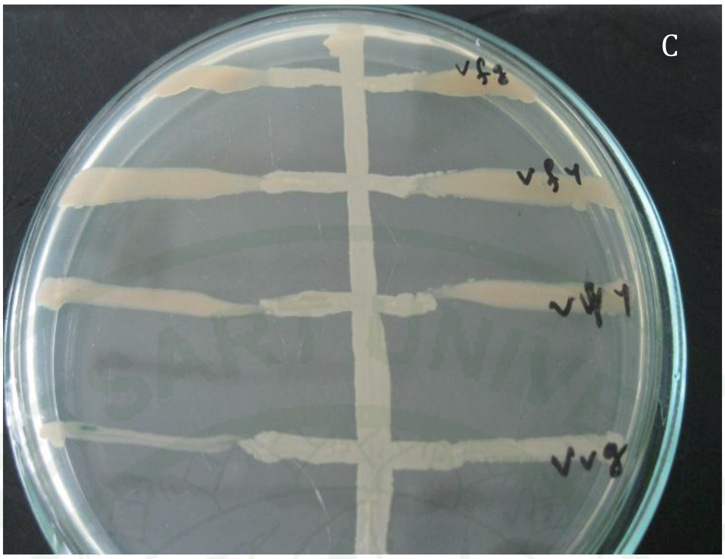


(A) Effect of inhibition (48 hrs) by B1 on *A. hydrophila* AQVH.

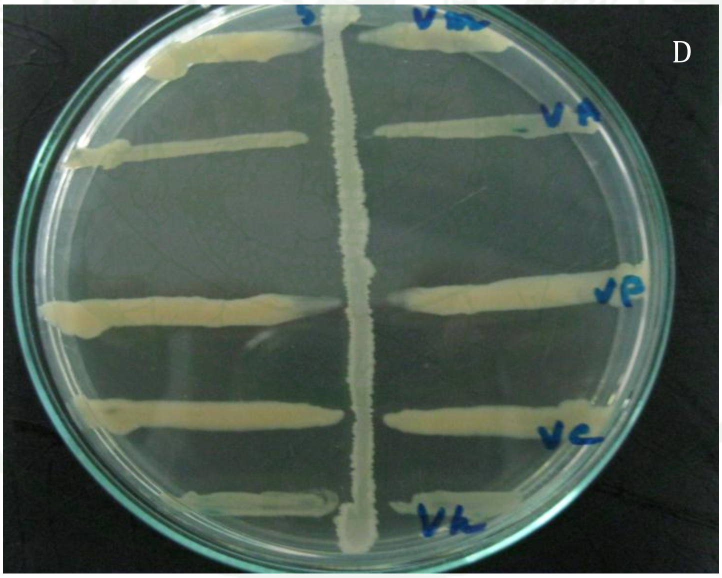


(B) Effect of inhibition (96 hrs) by B2 on (from top to bottom) *V. alginolyticus*, *V. parahaemolyticus*, *V.harveyi*, *V.mimicus*, *V. cholerae* (non01).

Figure 8 Results of Cross streak method in B1, B2 and B5

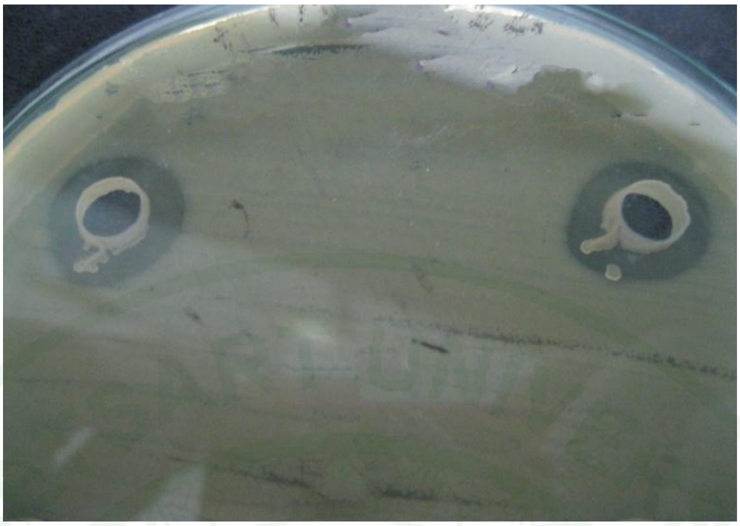


(C) Effect of colonization (96 hrs) by B 2 on (from top to bottom) *V.fluvialis* (Green), *V. fluvialis* (yellow), *V.vulnificus* (yellow), *V .vulnificus* (Green).

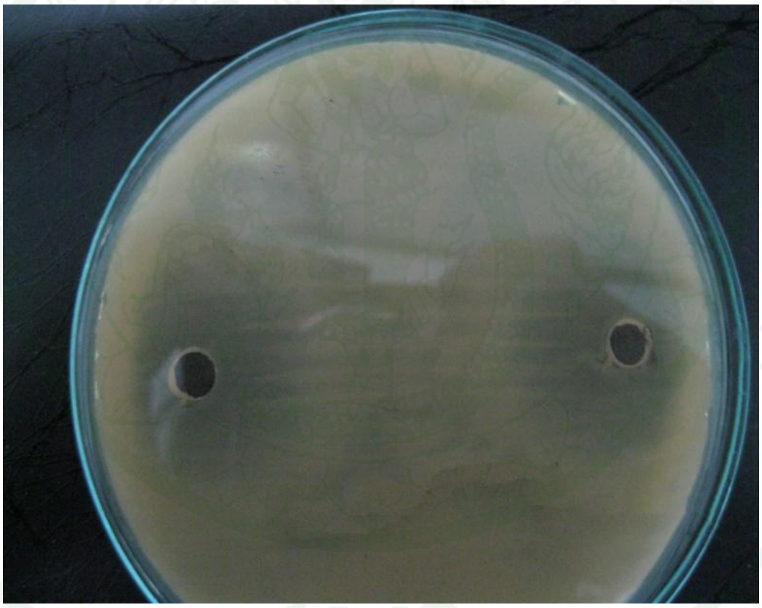


(D) Effect of inhibition by B5 (from top to bottom) on *V.mimicus,* *V. alginolyticus*, *V. parahaemolyticus*, *V. cholerae* (non01) and *V. harveyi*.

Figure 8 Continued

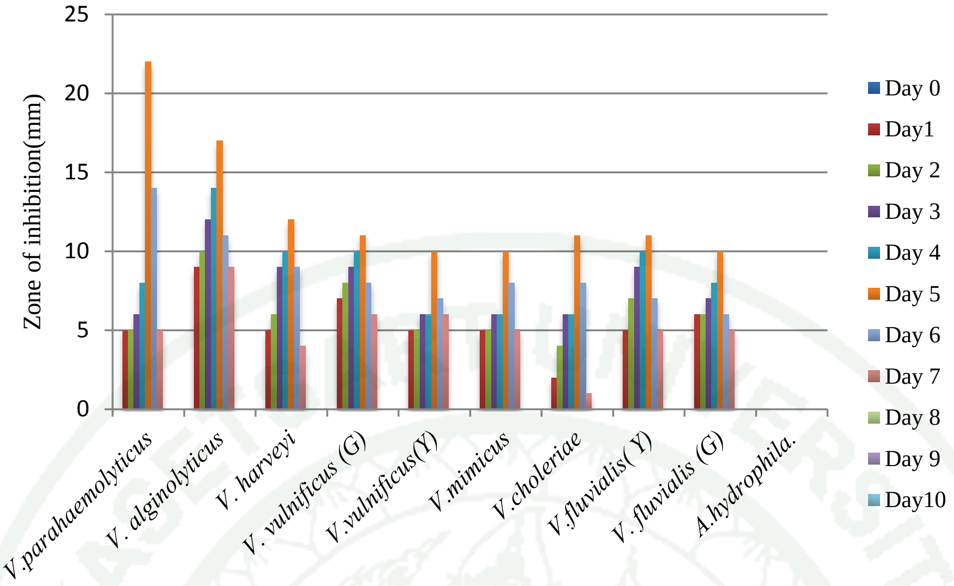


A

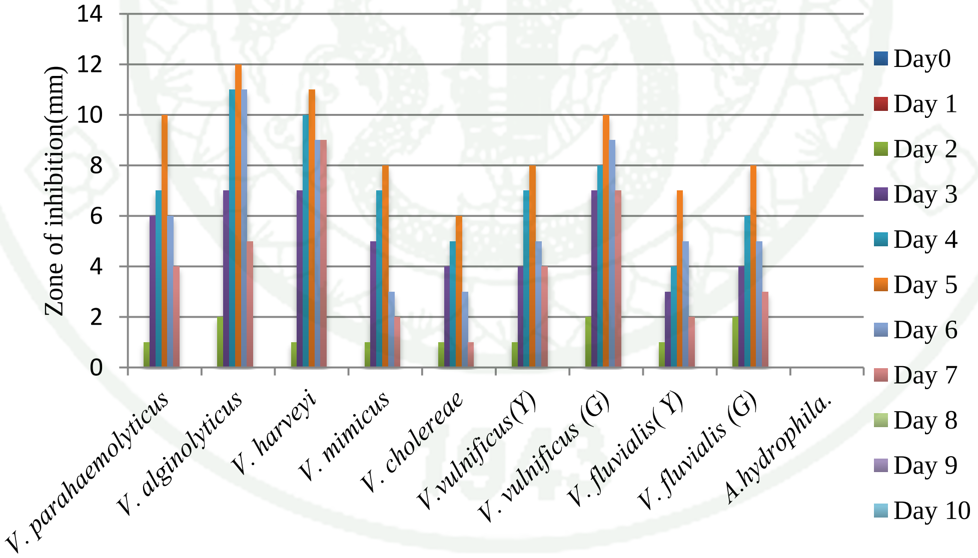


B

Figure 9 Zone of Inhibition (A) - by B1 at 72 hrs; (B) – by B5 at 120 hrs; agains *A. hydrophila* and *V harveyi*, respectively.

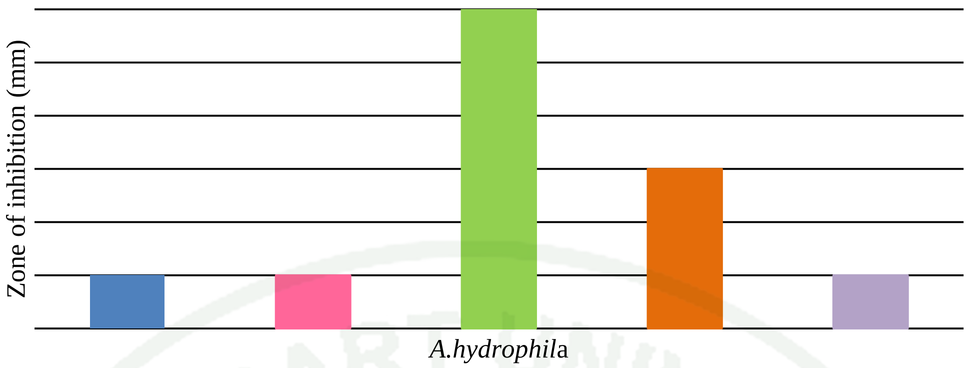


(A) B2 against *Vibrio* spp. (*V. parahaemolyticus*, *V. alginolyticus*, *V. harveyi*, *V. mimicus*, *V. cholerae* (non01) *V. vulnificus* (Y and G), *V. fluvialis* (Y and G).)



(A) B 5 against *Vibrio* spp. (*V. parahaemolyticus*, *V. alginolyticus*, *V. harveyi*, *V. mimicus*, *V. cholerae* (non01) *V. vulnificus* (Y and G), *V. fluvialis* (Y and G).)

Figure 10 Results of AWDA of B1, B2 and B5. as revealed by the measurement (mm) of clear zone by AWDA. The measurements are the mean value of three independent experiments.



(C) B1 against *A. hydrophila* AQAH.

Figure 10 Conmtinued

2.2 Co-culture of *Bacillus* spp. with pathogenic bacteria in Tryptic Soy Broth.

The result of preliminary selection of the probionts by cross streak and AWDA showed that only B1, B2 and B5 could inhibit the growth of selected pathogenic bacteria while B3 and B4 could not produce any antagonism. So the B1, B2 and B5 were selected for the broth co-culture experiment where the probionts were cultured with the pathogen in the same nutrient broth in the equal concentration to determine the inhibitiory effect of the probionts on the growth of pathogens in terms of comparing the number of pathogenic bacteria in co-culture and in control or mono-culture. At the same time the number of *Bacillus* also enumerated comparing between the co-culture and monoculture.

Based on the result of cross streak and AWDA antagonism assays the *Bacillus* B2 and B5 was co-cultured with all the *Vibrio* spp. and B1 was cultured with *A. hydrophila*. The enumeration was carried out in the following way

For the enumeration of *Vibrio* spp.

Total count of bacteria (TSA) = Number of total bacteria count from TSA plate.

The number of *Vibrio* spp. in co-culture (V Co) = The number of *Vibrio* spp. in TSB broth with *Bacillus* spp.

The number of *Vibrio* in co-culture (V Co) =TSA count – Number of bacteria in TCBS plate.

The number of *Vibrio* in monoculture (control) (V M) = Number of *Vibrio* cultured in TSB broth without *Bacillus.*

The percentage reduction of *Vibrio* = V Co - V M / V M * 100

For the enumeration of *A. hydrophila* :

Total count of bacteria (TSA) = Number of bacteria count from TSA plate.

The number of *A. hydrophila* in monoculture (control) (A M) = Number of *A. hydrophila* cultured in TSB broth without *Bacillus* .

The number of *A. hydrophila* in co-culture (A Co) = The number of *A. hydrophila* in TSB broth with *Bacillus* spp. (A Co) = TSA count –A M count

The percentage reduction of *A. hydrophila* = A M - A Co / A M * 100

For the enumeration of *Bacillus* spp. the same method was followed as described above.

The presence of *Bacillus* spp. (B2 and B5) led to a remarkable inhibition of growth of all the strains of different *Vibrio* spp. after 120 hours of incubation. Growth of *A. hydrophila* AQAH was found to get reduced 61.8% by B1 after 120 hours of co-culture (Table 6, Table 9). The growth reduction of the different strains of *Vibrio* spp. was more than 90% by B2 (Table 7, Table 9) and more than 85% (Table 8 , Table 9) at the end of 120 hours of co-culture experiment .While there were little difference observed in *Bacillus* spp. concentrations. The reduction of B1 was less than 5%, B2 was 4 to 7% and B5 was 4 to 8% respectively (Table 10) at the end of 120 hours of the co-culture experiment while compared to the control (mono-culture).The concentration of each pathogenic strain in the mono and co-culture with the the respective *Bacillus* spp. has been explained through (Table 6) and (Figure 29) for B1 and *A. hydrophila*. (Table 7) and (Figure 11,13,15,17,19,21,23, 25, 27) for B2 and (Table 8) and (Figure 12, 14, 16, 18, 20, 22, 24, 26, 28) for B5 with the pathogenic *Vibrio* spp. respectively.

Table 6 Concentration of B1 and *A.hydrophilla* in mono-culture and co-culture.

Bacteria	**Time (hrs)**					
	0	**24**	**48**	**72**	**96**	**120**
B1 M	$2.9 \pm 0.44 \times 10^6$	$4.4 \pm 0.52 \times 10^9$	$2.6 \pm 0.13 \times 10^9$	$2.5 \pm 0.12 \times 10^9$	$5.5 \pm 0.77 \times 10^8$	$1.2 \pm 0.80 \times 10^8$
Aero M	$1.92 \pm 0.35 \times 10^6$	$1.3 \pm 0.95 \times 10^9$	$5.2 \pm 1.0 \times 10^8$	$2.2 \pm 0.85 \times 10^7$	$4.9 \pm 0.37 \times 10^7$	$1.1 \pm 0.29 \times 10^7$
Aero Co	$1.84 \pm 0.69 \times 10^6$	$1.0 \pm 0.58 \times 10^9$	$4.0 \pm 0.88 \times 10^8$	$9.9 \pm 0.37 \times 10^8$	$1.9 \pm 0.45 \times 10^7$	$4.2 \pm 0.56 \times 10^6$
B1 Co	$2.88 \pm 0.39 \times 10^6$	$4.2 \pm 0.95 \times 10^9$	$2.5 \pm 0.56 \times 10^9$	$2.4 \pm 0.88 \times 10^9$	$5.2 \pm 0.88 \times 10^8$	$1.15 \pm 0.37 \times 10^8$

Mean value with same superscript are not significantly different at P = 0.05

Table 7 Concentration of B2 and *Vibrio* spp. in mono-culture and co-culture.

Bacteria	Time (hrs)					
	0	24	48	72	96	120
B2 M	8.9 ± 0.35 x 10^{6}	7.5 ± 0.43 x 10^{9}	5.5 ± 0.77 x 10^{9}	2.5 ± 0.33 x 10^{9}	6.5 ± 0.31 x 10^{8}	1.5 ± 0.44 x 10^{8}
Va M	3.9 ± 0.22 x 10^{6}	7.9 ± 0.31 x 10^{8}	2.3 ± 0.45 x 10^{8}	7.2 ± 0.34 x 10^{7}	1.2 ± 0.33 x 10^{7}	2.5 ± 1.1 ^{a}x 10^{6}
Vm M	7.9 ± 0.33 x 10^{6}	8.3 ± 0.57 x 10^{8}	2.1 ± 0.75 x 10^{8}	6.5 ± 0.82 x 10^{7}	2.5 ± 0.22 x 10^{7}	5.5 ± 0.12 x 10^{6}
Vvy M	8.4 ± 0.10 x 10^{6}	6.9 ± 0.80 x 10^{8}	4.2 ± 0.27 x 10^{8}	6.2 ± 0.36 x 10^{7}	2.1 ± 0.20 x 10^{7}	3.1 ± 1.0 x 10^{6}
Vvg M	8.1 ± 0.22 x 10^{6}	6.6 ± 0.27 x 10^{8}	3.9 ± 0.52 x 10^{8}	5.5 ± 0.32 x 10^{7}	1.5 ± 0.42 x 10^{7}	2.9 ± 0.37 x 10^{6}
Vh M	7.5 ± 0.18 x 10^{6}	6.1 ± 0.25 x 10^{8}	2.9 ± 0.37 x 10^{8}	5.9 ± 0.11 x 10^{7}	1.2 ± 0.21 x 10^{7}	4.2 ± 0.53 x10^{6}
Vfy M	3.3 ± 0.88 x 10^{6}	3.9 ± 0.71 x 10^{8}	2.5 ± 0.49 x 10^{8}	3.7 ± 0.22 x 10^{7}	1.1 ± 0.62 x 10^{7}	2.7 ± 0.13 x 10^{6}
Vfg M	2.7 ± 0.1 x 10^{6}	3.5 ± 0.19 x 10^{8}	2.2 ± 0.23 x 10^{8}	3.3 ± 0.42 x 10^{7}	1.2 ± 0.99 x 10^{7}	2.5 ± 0.12 x 10^{6}
Vp M	5.7 ± 0.75 x 10^{6}	5.9 ± 1.10 x 10^{8}	4.1 ± 0.51 ^{a} x 10^{8}	6.9 ± 0.73 x 10^{7}	1.8 ± 1.00 x 10^{7}	3.6 ± 0.52 x 10^{6}
Vc M	7.2 ± 0.12 x 10^{6}	6.4 ± 1.10 x 10^{8}	3.2 ± 0.51 ^{a} x 10^{8}	8.1 ± 0.45 x 10^{7}	3.1 ± 0.34 x 10^{7}	1.1 ± 1.00 x 10^{6}
B2 Co	8.85 ± 1.00 x 10^{6}	7.3 ± 0.56 x 10^{9}	5.13 ± 0.47 x 10^{9}	2.34 ± 0.32 x 10^{9}	6.14 ± 0.2 x 10^{8}	1.44 ± 0.15 x 10^{8}
Va Co	3.86 ± 0.42 x 10^{6}	5.0 ± 0.30 x 10^{8}	1.0 ± 0.25 x 10^{8}	1.8 ± 0.22 x 10^{7}	1.8 ± 0.77 x 10^{6}	1.30 ± 0.90 x 10^{5}
Vm Co	7.84 ± 1.25 x 10^{6}	5.4 ± 0.76 x 10^{8}	9.3 ± 0.79 x 10^{7}	1.69 ± 0.85 x 10^{7}	4.0 ± 0.36 x 10^{6}	4.2 ± 0.9 5 x 10^{5}
Vvy Co	8.35 ± 0.56 x 10^{6}	4.6 ± 0.32 x 10^{8}	1.9 ± 0.25 x 10^{8}	1.65 ± 0.67 x 10^{7}	3.2 ± 1.0 x 10^{6}	2.6 ± 0.37 ^{a}x 10^{5}
Vvg Co	8.0 ± 0.32 x 10^{6}	4.4 ± 0.45 x 10^{8}	1.9 ± 0.30 x 10^{8}	1.5 ± 0.36 x 10^{7}	2.7 ± 0.2 x 10^{6}	2.1 ± 0.56 x 10^{5}
Vh M	7.4 ± 0.1 x 10^{6}	4.0 ± 0.22 x 10^{8}	1.3 ± 0.62 x 10^{8}	1.5 ± 0.77 x 10^{7}	1.9 ± 0.90 x 10^{6}	3.5 ± 0.26 x 10^{5}
Vfy Co	3.2 ± 0.11 x 10^{6}	2.6 ± 1.75 x 10^{8}	1.1 ± 0.66 x 10^{8}	1.0 ± 0.52 x 10^{7}	1.6 ± 0.20 x 10^{6}	2.2 ± 0.42 x 10^{5}
Vfg Co	2.6 ± 0.11 x 10^{6}	2.4 ± 0.55 x 10^{8}	1.0 ± 0.0 x 10^{8}	1.0 ± 0.62 x 10^{7}	2.1 ± 0.57 x 10^{6}	2.2 ± 0.59 x 10^{5}
Vp Co	5.6 ± 0.39 x 10^{6}	3.9 ± 0.65 x 10^{8}	1.8 ± 0.76 x 10^{8}	1.8 ± 0.33 x 10^{7}	2.9 ± 0.22 x 10^{6}	2.1 ± 0.33 x 10^{5}
Vc Co	7.1 ± 0.57 x 10^{6}	4.4 ± 0.33 x 10^{8}	1.5 ± 1.0 x 10^{8}	2.3 ± 0.85 x 10^{7}	5.8 ± 0.76 x 10^{6}	1.18 ± 0.36 x 10^{6}

Mean value with same superscript are not significantly different at P = 0.05

Table 8 Concentration of B5 and *Vibrio* spp. in mono-culture and co-culture.

Bacteria	Time (hrs)					
	0	24	48	72	96	120
B5 M	7.8 ± 0.17 x 10^6	7.8 ± 1.5 x 10^9	6.2 ± 0.37 x 10^9	8.2 ± 0.76 x 10^8	2.2 ± 0.17 x 10^8	5.8 ± 0.33 x 10^7
Va M	3.9 ± 0.33 x 10^6	7.9 ± 0.31 x 10^8	2.3 ± 0.45 x 10^8	7.2 ± 0.34 x 10^7	1.2 ± 0.33 x 10^7	2.5 ± 1.1 x 10^6
Vm M	7.9 ± 0.33 x 10^6	8.3 ± 0.57 x 10^8	2.1 ± 0.75 x 10^8	6.5 ± 0.82 x 10^7	2.5 ± 0.22 x 10^7	5.5 ± 0.12 x 10^6
Vvy M	8.4 ± 0.10 x 10^6	6.9 ± 0.80 x 10^8	4.2 ± 0.27 x 10^8	6.2 ± 0.36 x 10^7	2.1 ± 0.20 x 10^7	3.1 ± 1.0 x 10^6
Vvg M	8.1 ± 0.22 x 10^6	6.6 ± 0.27 x 10^8	3.9 ± 0.52 x 10^8	5.5 ± 0.32 x 10^7	1.5 ± 0.42 x 10^7	2.9 ± 0.37 x 10^6
,Vh M	7.5 ± 0.18 x 10^6	6.1 ± 0.25 x 10^8	2.9 ± 0.37 x 10^8	5.9 ± 0.11 x 10^7	1.2 ± 0.21 x 10^7	4.2 ± 0.53 x 10^6
Vfy M	3.3 ± 0.88 x 10^6	3.9 ± 0.71 x 10^8	2.5 ± 0.49 x 10^8	3.7 ± 0.22 x 10^7	1.1 ± 0.62 x 10^7	2.7 ± 0.13 x 10^6
Vfg M	2.7 ± 0.11 x 10^6	3.5 ± 0.19 x 10^8	2.2 ± 0.23 x 10^8	3.3 ± 0.42 x 10^7	1.2 ± 0.99 x 10^7	2.5 ± 0.12 x 10^6
Vp M	5.7 ± 0.75 x 10^6	5.9 ± 1.10 x 10^8	4.1 ± 0.51 x 10^8	6.9 ± 0.73 x 10^7	1.8 ± 1.00 x 10^7	3.6 ± 0.52 x 10^6
Vc M	7.2 ± 0.12 x 10^6	6.4 ± 1.10 x 10^8	3.2 ± 0.51 x 10^8	8.1 ± 0.45 x 10^7	3.1 ± 0.34 x 10^7	1.1 ± 1.00 x 10^6
B5 Co	7.8 ± 0.23 x 10^6	7.2 ± 0.56 x 10^9	5.6 ± 0.87 x 10^9	7.4 ± 0.23 x 10^8	2.0 ± 0.35 x 10^8	5.3 ± 1.10 x 10^7
Va Co	3.9 ± 0.62 x 10^6	6.5 ± 0.25 x 10^8	1.3 ± 0.37 x 10^8	2.6 ± 0.33 x 10^7	3.1 ± 0.62 x 10^6	2.9 ± 0.52 x 10^5
Vm Co	7.9 ± 0.15 x 10^6	6.8 ± 0.39 x 10^8	1.2 ± 0.27 x 10^8	2.3 ± 0.66 x 10^7	6.1 ± 0.57 x 10^6	5.7 ± 0.42 x 10^5
Vvy Co	8.4 ± 0.22 x 10^6	5.7 ± 0.92 x 10^8	2.4 ± 0.35 x 10^8	2.3 ± 0.77 x 10^7	5.2 ± 0.47 x 10^6	3.2 ± 0.55 x 10^5
Vvg Co	8.0 ± 0.13 x 10^6	5.7 ± 0.87 x 10^8	2.3 ± 0.33 x 10^8	2.0 ± 0.75 x 10^7	2.9 ± 0.82 x 10^6	4.4 ± 0.23 x 10^5
Vh M	7.5 ± 0.39 x 10^6	5.0 ± 0.27 x 10^8	1.6 ± 0.37 x 10^8	2.2 ± 0.76 x 10^7	2.0 ± 0.55 x 10^6	4.2 ± 0.88 x 10^5
Vfy Co	3.3 ± 0.29 x 10^6	3.2 ± 0.89 x 10^8	1.4 ± 1.0 x 10^8	1.3 ± 1.0 x 10^7	1.9 ± 0.88 x 10^6	3.2 ± 0.37 x 10^5
Vfg Co	2.7 ± 0.37 x 10^6	2.9 ± 0.75 x 10^8	1.2 ± 0.76 x 10^8	1.2 ± 0.55 x 10^7	3.0 ± 1.9 x 10^6	4.2 ± 0.22 x 10^5
Vp Co	5.7 ± 0.77 x 10^6	4.8 ± 0.19 x 10^8	2.2 ± 0.73 x 10^8	2.5 ± 0.92 x 10^7	2.8 ± 0.33 x 10^6	4.0 ± 0.33 x 10^5
Vc Co	7.2 ± 0.33 x 10^6	5.2 ± 0.77 x 10^8	1.5 ± 0.27 x 10^8	3.0 ± 0.33 x 10^7	5.3 ± 0.23 x 10^6	1.5 ± 0.11 x 10^6

Mean value with same superscript are not significantly different at P = 0.05

Table 9 Reduction of growth of pathogenic bacteria (by percentage) in co-culture with *Bacillus* spp.

Bacteria species	Percentage decrease of *A. hydrophila* with B1					
	0hrs	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
A. hydrophila	0.51^{a}	23.^{a}	25.^{a}	54.99^{a}	61.22^{a}	61.81^{a}
	Percentage decrease of *Vibrio* spp. with B2					
		24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
V. alginolyticus	1.00^{a}	36.71^{a}	56.51^{a}	75.01^{a}	85.00^{a}	94.80^{a}
V. mimicus	0.76^{a}	34.94^{a}	55.72^{a}	73.70^{a}	84.00^{a}	92.43^{a}
V. vulnificus(Y)	0.60^{a}	33.33^{a}	54.39^{a}	73.39^{a}	84.76^{a}	91.61^{a}
V. vulnificus(G)	0.99^{a}	32.99^{a}	51.28^{a}	72.76^{a}	82.00^{a}	92.68^{a}
V. harveyi	0.67^{a}	34.23^{a}	54.82^{a}	74.57^{a}	84.11^{a}	91.65^{a}
V. fluvialis(Y)	0.61^{a}	31.43^{a}	56.00^{a}	72.97^{a}	85.48^{a}	91.85^{a}
V. fluvialis(G)	0.93^{a}	30.91^{a}	54.17^{a}	69.71^{a}	81.13^{a}	91.21^{a}
V. cholereae(non 01)	0.83^{a}	32.71^{a}	53.00^{a}	71.60^{a}	81.95^{a}	88.93^{a}
V. parahaemolyticuss	0.88^{a}	32.71^{a}	55.72^{a}	73.91^{a}	83.89^{a}	94.16^{a}
	Percentage decrease of *Vibrio* spp. with B5					
	0hrs	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
V.alginolyticus	0.49^{a}	17.47^{a}	43.48^{a}	63.89^{a}	74.16^{a}	88.4^{a}
V.mimicus	0.51^{a}	18.0^{a}	44.2^{a}	64.6^{a}	75.60^{a}	89.64^{a}
V.vulnificus(Y)	0.48^{a}	17.39^{a}	42.86^{a}	63.06^{a}	75.23^{a}	89.68^{a}
V. vulnificus(G)	0.49^{a}	13.6^{a}	41.79^{a}	62.00^{a}	80.67^{a}	84.83^{a}
V. harveyi	0.40^{a}	18.0^{a}	44.83^{a}	62.71^{a}	83.33^{a}	89.98^{a}
V. fluvialis(Y)	0.48^{a}	17.94^{a}	44.00^{a}	64.86^{a}	82.73^{a}	88.19^{a}
V. fluvialis(G)	0.44^{a}	17.14^{a}	45.4^{a}	63.64^{a}	75.00^{a}	83.23^{a}
V. cholerae(non 01)	0.56^{a}	17.97^{a}	53.1^{a}	62.96^{a}	82.90^{a}	86.36^{a}
V. parahaemolyticus	0.49^{a}	18.64^{a}	46.0^{a}	63.77^{a}	84.44^{a}	88.19^{a}

Mean value with the same superscript are not significantly different at P =0.05

Table 10 Reduction of growth of *Bacillus* spp. (in percentage) in co-culture with pathogenic bacteria.

	Percent decrease of B1with *A. hydrophila*					
	0hrs	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
A. hydrophila	0.68 ^{a}	4.50 ^{a}	3.84 ^{a}	4.00 ^{a}	5.45 ^{a}	4.16 ^{a}
	Percentage decrease of B2 with *Vibrio* spp.					
	0hrs	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
V.alginolyticus	0.21 ^{a}	4.00 ^{a}	7.27 ^{a}	8.41 ^{a}	8.18 ^{a}	7.33 ^{a}
V.mimicus	0.14 ^{a}	3.87 ^{a}	5.45 ^{a}	6.33 ^{a}	6.15 ^{a}	5.86 ^{a}
V.vulnificus(Y)	0.66 ^{a}	4.13 ^{a}	4.36 ^{a}	4.80 ^{a}	5.69 ^{a}	5.32 ^{a}
V.vulnificus(G)	0.79 ^{a}	1.33 ^{a}	3.64 ^{a}	6.09 ^{a}	6.00 ^{a}	6.00 ^{a}
V.harveyi	0.30 ^{a}	1.32 ^{a}	7.25 ^{a}	7.20 ^{a}	6.12 ^{a}	6.67 ^{a}
V.fluvialis(Y)	0.45 ^{a}	2.67 ^{a}	3.45 ^{a}	7.60 ^{a}	7.54 ^{a}	5.43 ^{a}
V.fluvialis(G)	0.67 ^{a}	2.00 ^{a}	3.64 ^{a}	7.96 ^{a}	7.69 ^{a}	6.65 ^{a}
V.cholereae (non 01)	0.56 ^{a}	2.56 ^{a}	6.73 ^{a}	6.40 ^{a}	5.54 ^{a}	4.00 ^{a}
V.parahaemolyticus	0.22 ^{a}	2.20 ^{a}	5.27 ^{a}	5.20 ^{a}	4.92 ^{a}	5.54 ^{a}
	Percentage decrease of B5 with *Vibrio* spp.					
	0hrs	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
V.alginolyticus	0.13 ^{a}	7.69 ^{a}	9.68 ^{a}	9.76 ^{a}	9.09 ^{a}	8.62 ^{a}
V.mimicus	0.28 ^{a}	6.41 ^{a}	6.13 ^{a}	6.10 ^{a}	5.87 ^{a}	5.34 ^{a}
V.vulnificus(Y)	0.77 ^{a}	6.15 ^{a}	6.61 ^{a}	6.22 ^{a}	6.36 ^{a}	6.55 ^{a}
V.vulnificus(G)	0.79 ^{a}	2.30 ^{a}	6.45 ^{a}	6.22 ^{a}	6.36 ^{a}	6.75 ^{a}
V.harveyi	0.52 ^{a}	5.77 ^{a}	8.39 ^{a}	8.41 ^{a}	7.27 ^{a}	7.2 ^{a}
V.fluvialis(Y)	0.65 ^{a}	7.95 ^{a}	7.26. ^{a}	6.95 ^{a}	6.82 ^{a}	6.55 ^{a}
V.fluvialis(G)	0.90 ^{a}	1.96 ^{a}	3.23 ^{a}	4.90 ^{a}	5.00 ^{a}	5.17 ^{a}
V.cholereae(non 01)	1.03 ^{a}	2.57 ^{a}	4.83 ^{a}	4.88 ^{a}	4.55 ^{a}	4.82 ^{a}
V.parahaemolyticus	0.38 ^{a}	3.51 ^{a}	6.28 ^{a}	6.34 ^{a}	6.36 ^{a}	6.21 ^{a}

Mean value with the same superscript are not significantly different at P =0.05

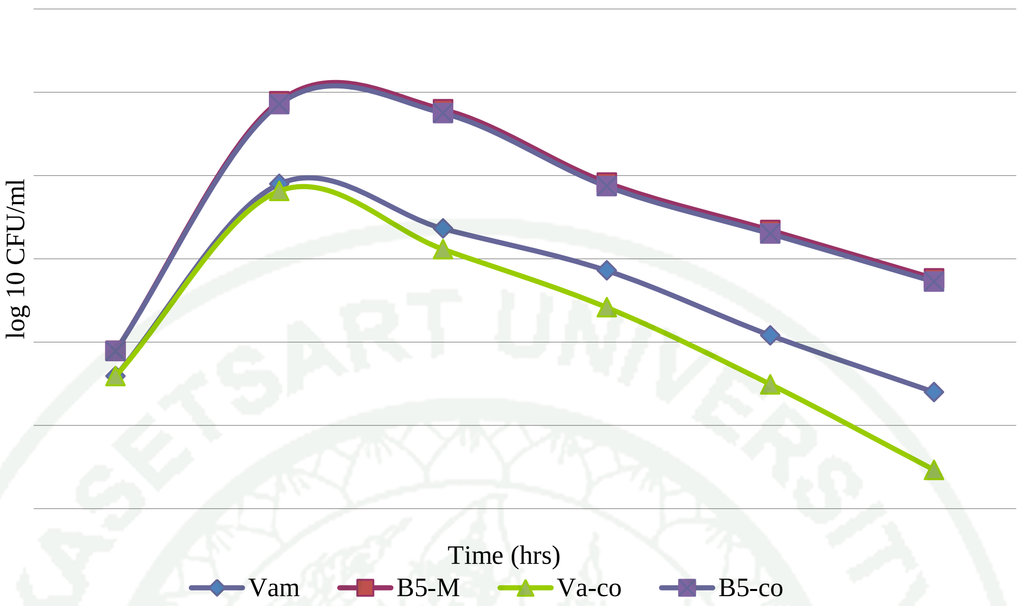


Figure 11 Growth of B2 and *V. alginolyticus* in co-culture and mono-culture.

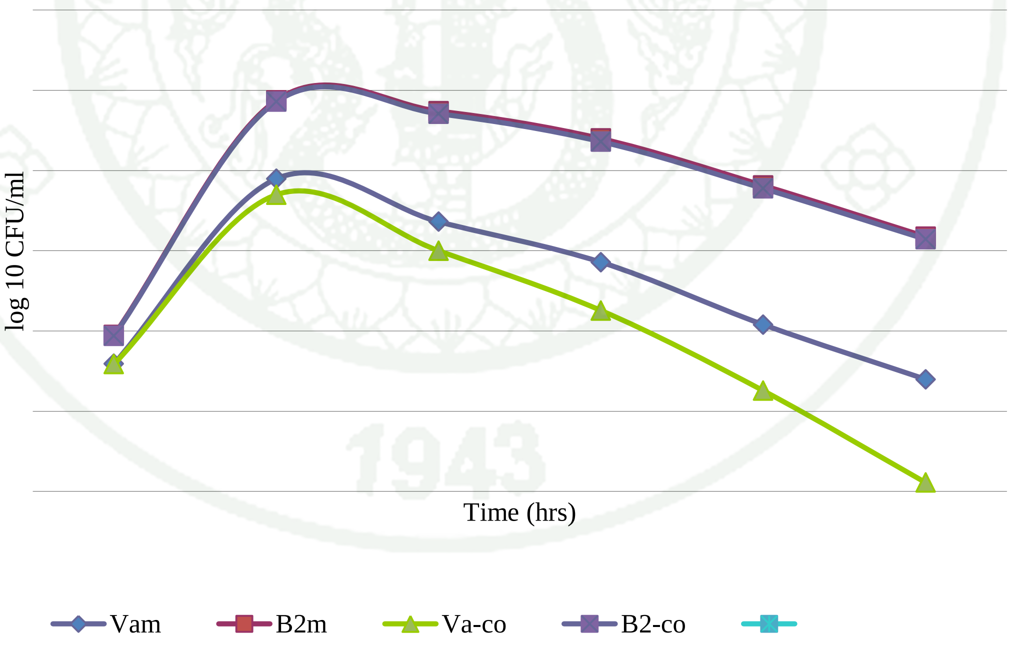


Figure 12 Growth of B5 and *V. alginolyticus* in co-culture and mono-culture.

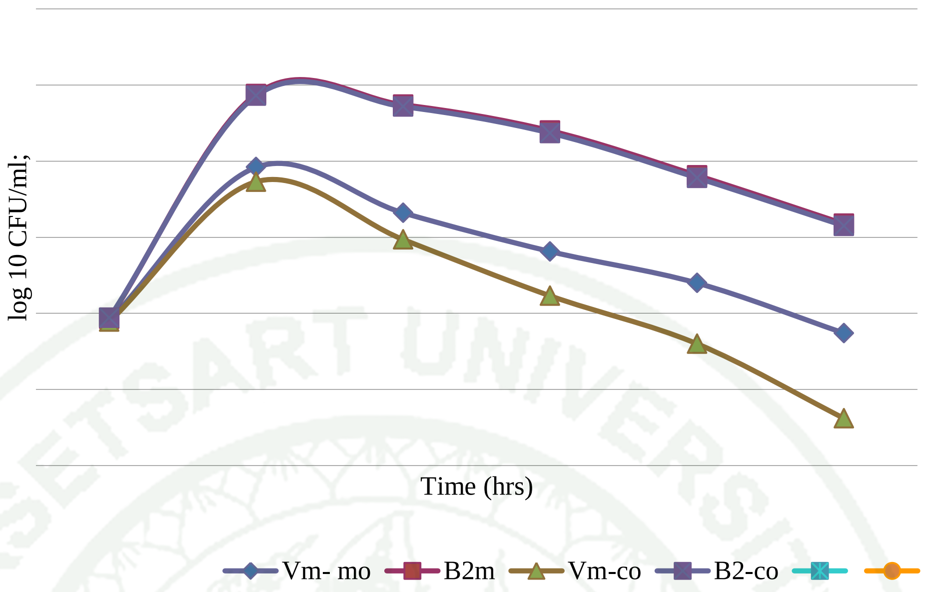


Figure 13 Growth of B2 and *V. mimicus* in co-culture and mono-culture.

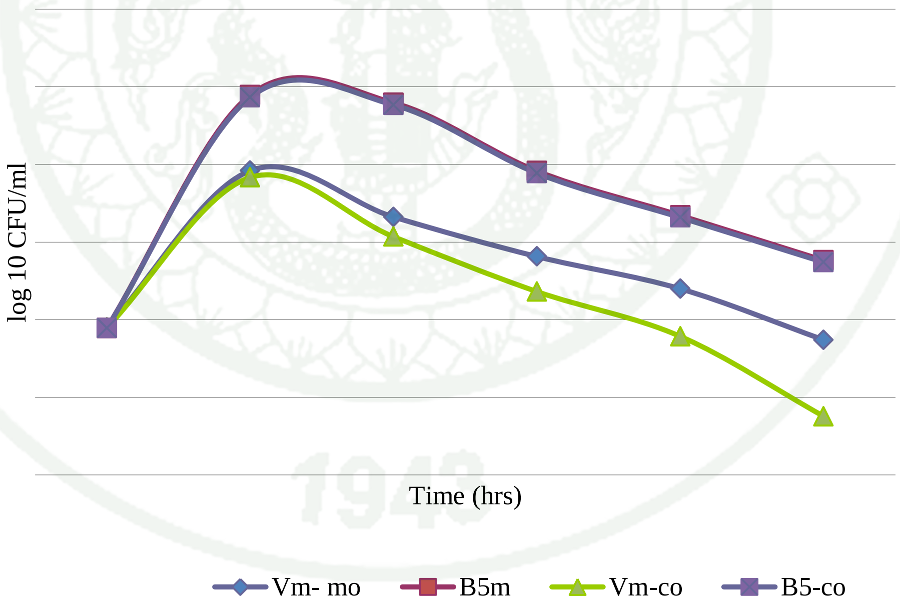


Figure 14 Growth of B5 and *V. mimicus* in co-culture and mono-culture.

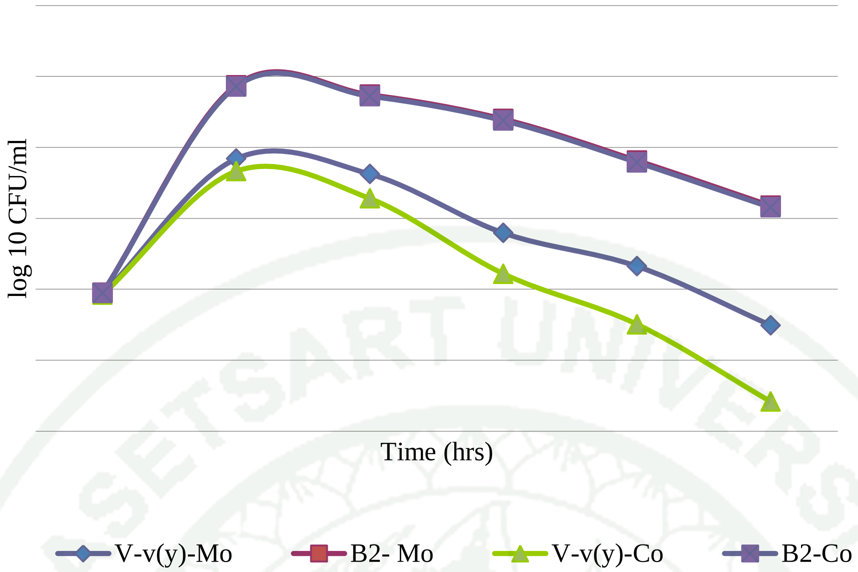


Figure 15 Growth of B2 and *V. vulnificus* (yellow) in co-culture and mono-culture.

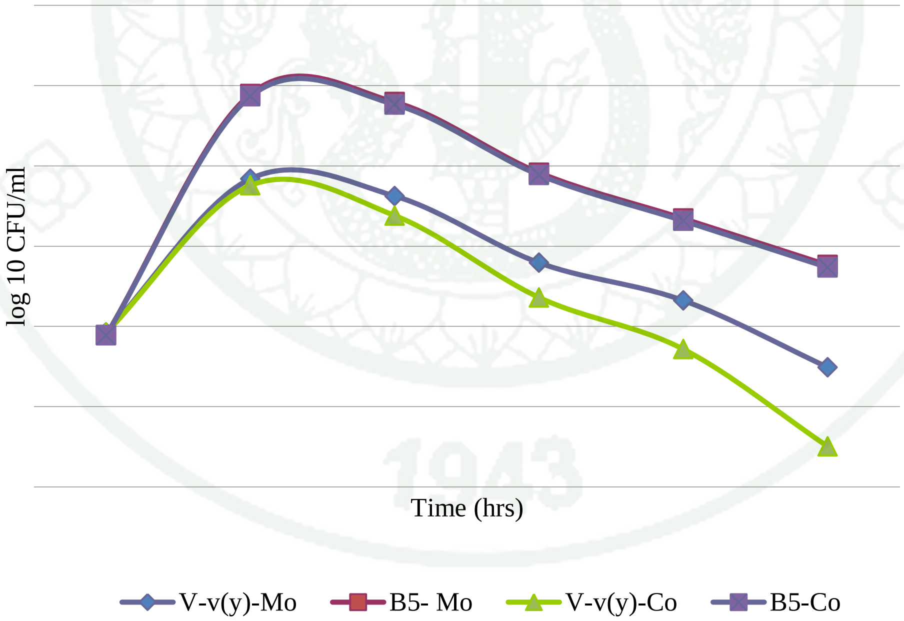


Figure 16 Growth of B5 and *V. vulnificus* (yellow) in co-culture and mono-culture.

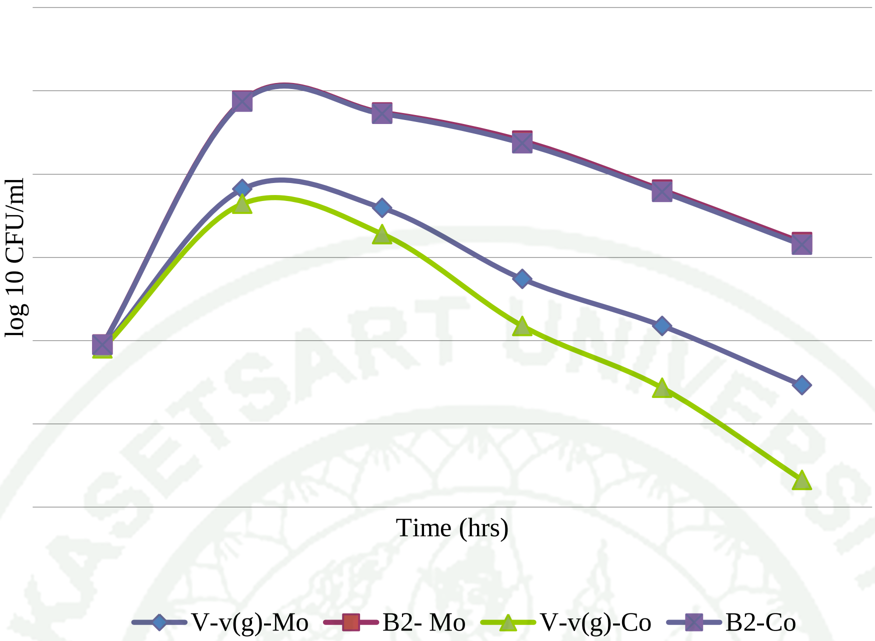


Figure 17 Growth of B2 and *V. vulnificus* (green) in co-culture and mono-culture.

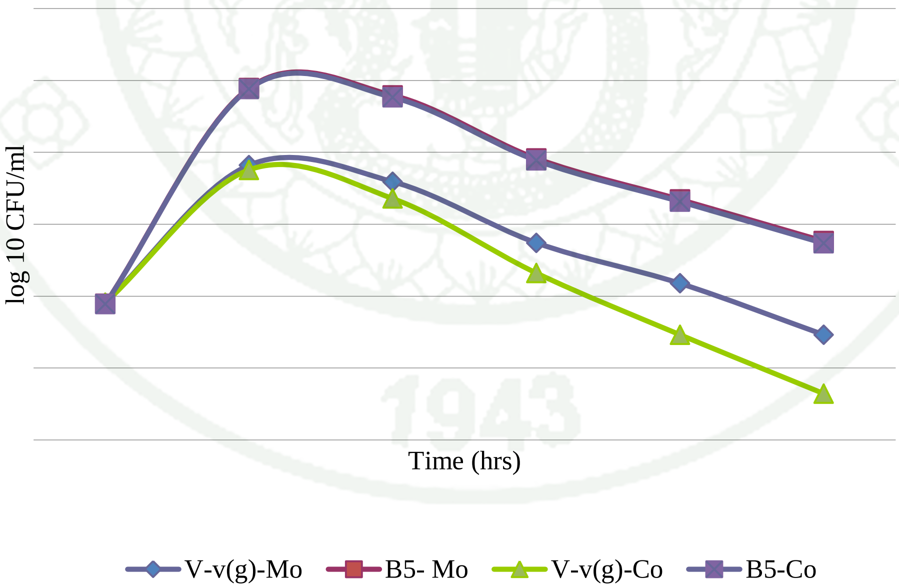


Figure 18 Growth of B5 and *V. vulnificus* (green) in co-culture and mono-culture.

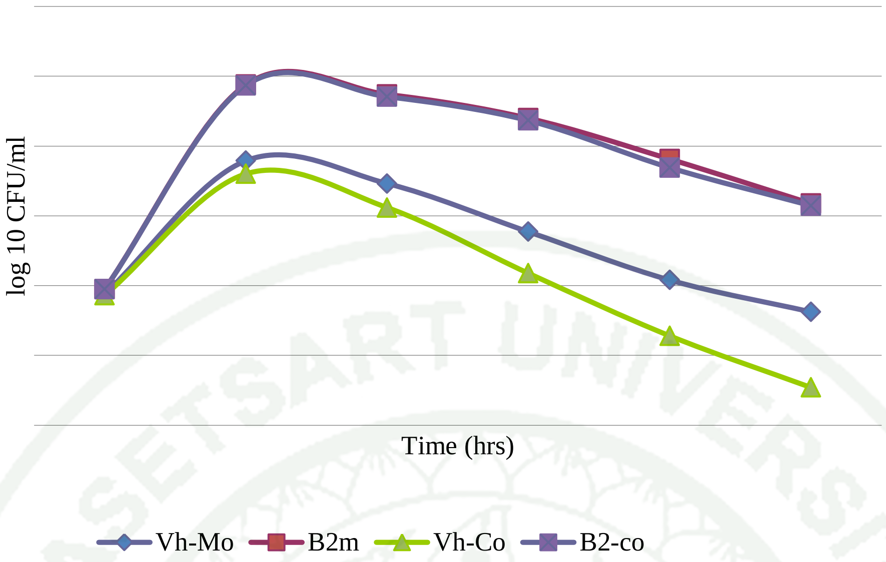


Figure 19 Growth of B2 and *V. harveyi* in co-culture and mono-culture.

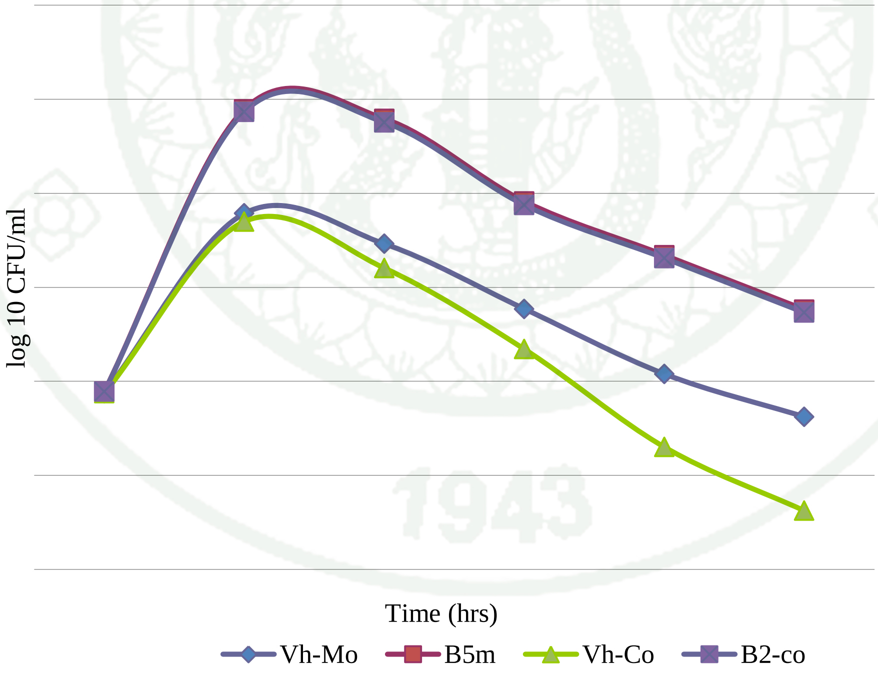


Figure 20 Growth of B5 and *V. harveyi* in co-culture and mono-culture.

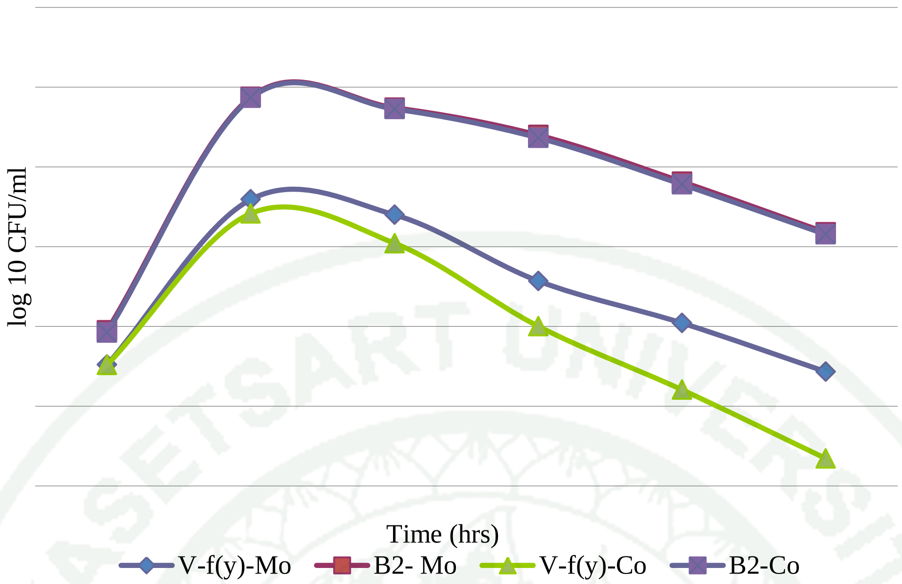


Figure 21 Growth of B2 and *V. fluvialis* (yellow) in co-culture and mono-culture.

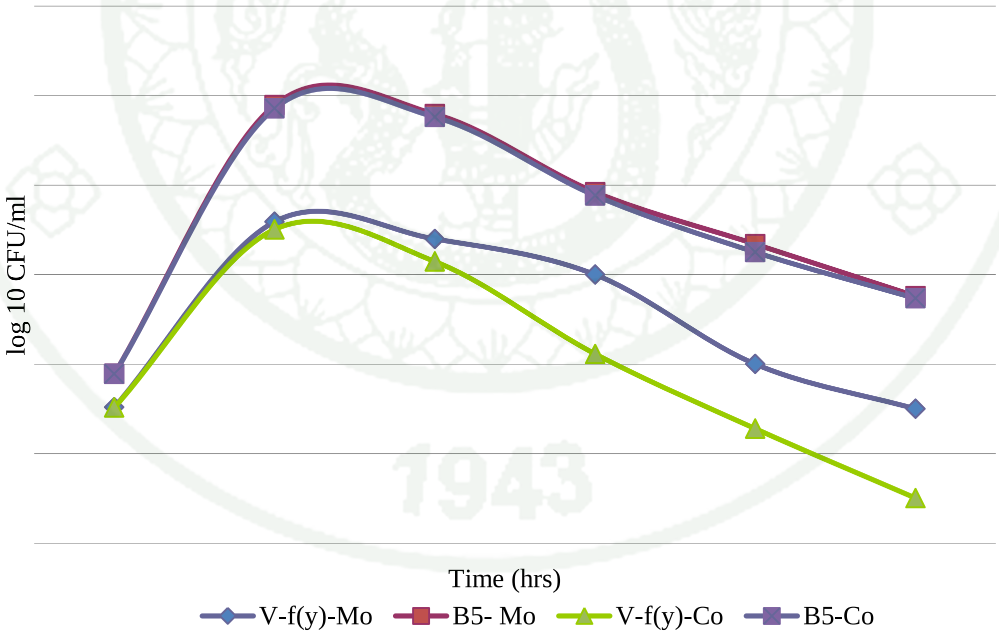


Figure 22 Growth of B5 and *V. fluvialis* (yellow) in co-culture and mono-culture.

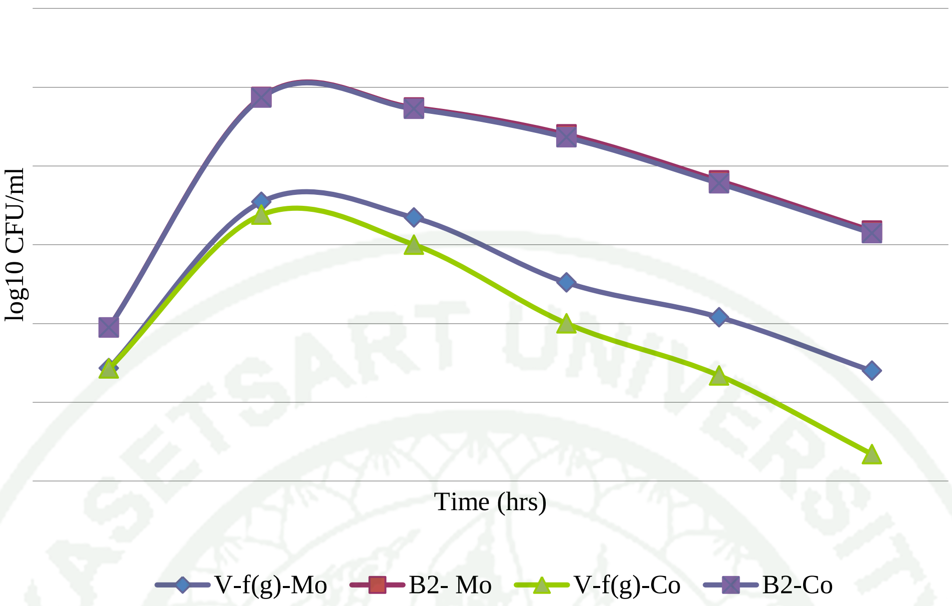


Figure 23 Growth of B2 and *V. fluvialis* (green) in co-culture and mono-culture.

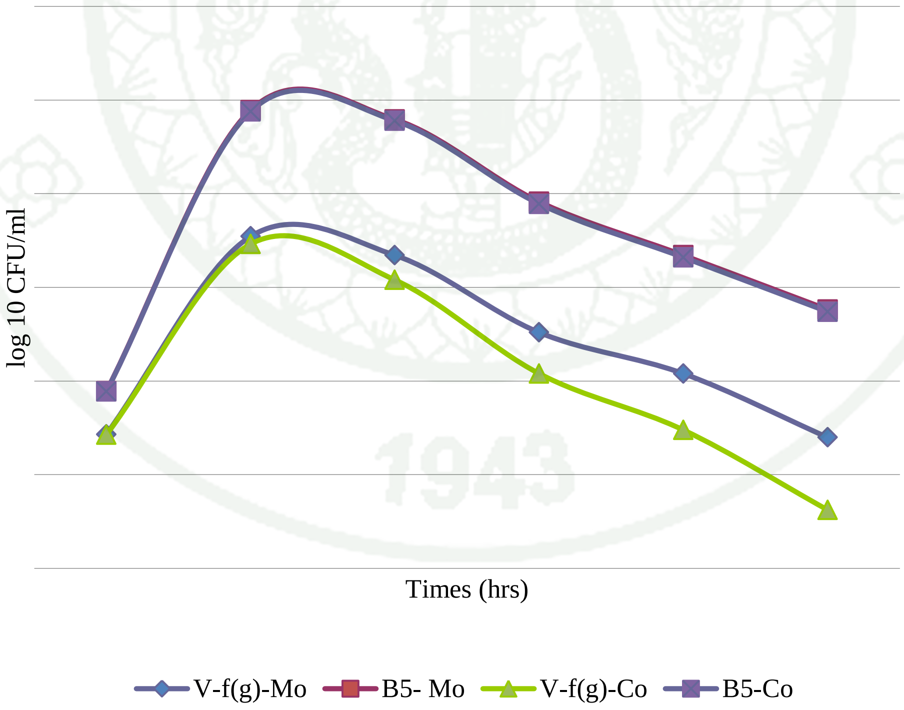


Figure 24 Growth of B5 and *V. fluvialis* (green) in co-culture and mono-culture

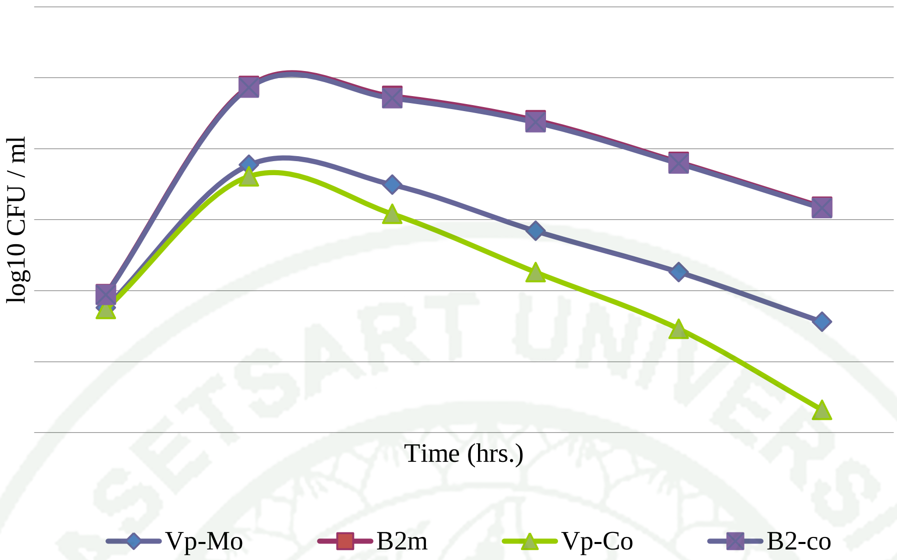


Figure 25 Growth of B2 and *V .parahemolyticus* in co-culture and mono-culture.

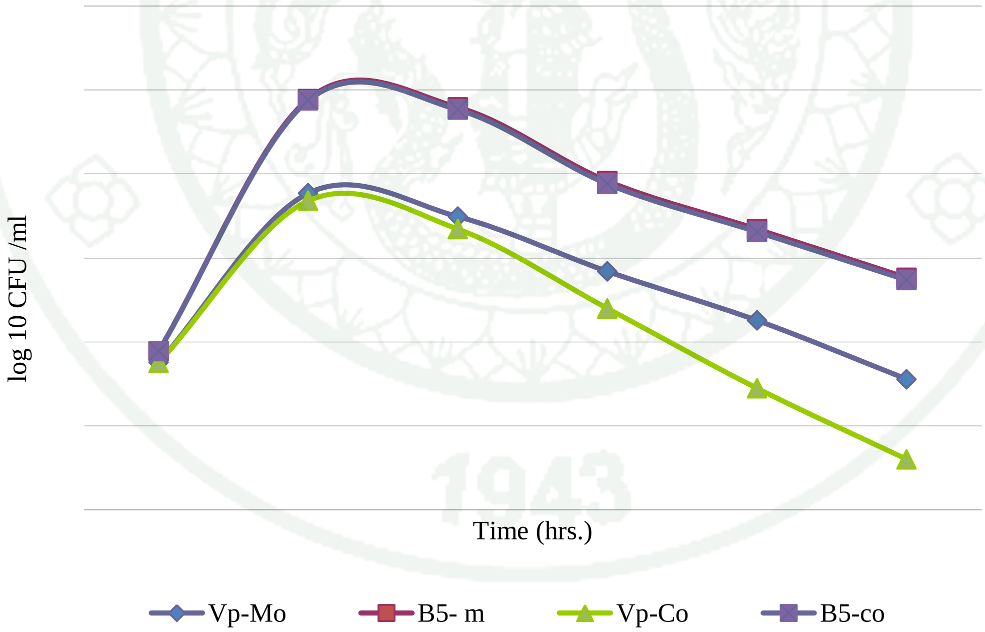


Figure 26 Growth of B5 and *V. parahemolyticus* in co-culture and mono-culture

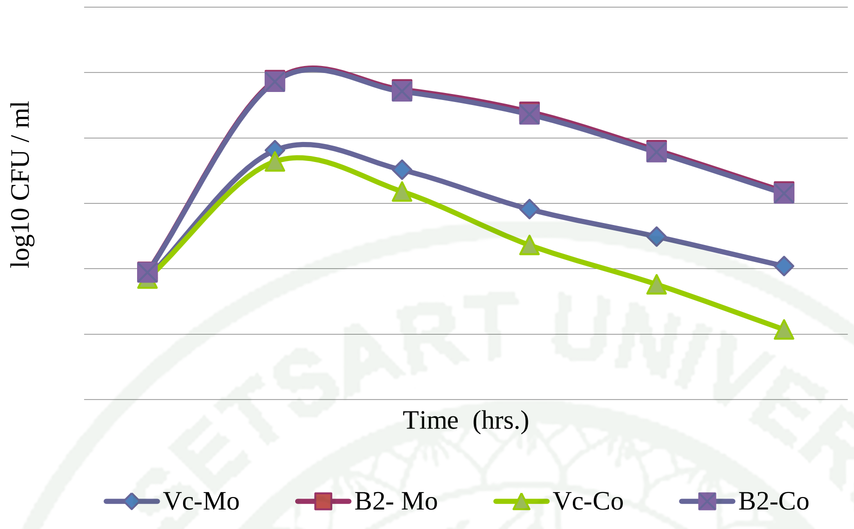


Figure 27 Growth of B2 and *V. cholarae* (non 01) in co-culture and mono-culture.

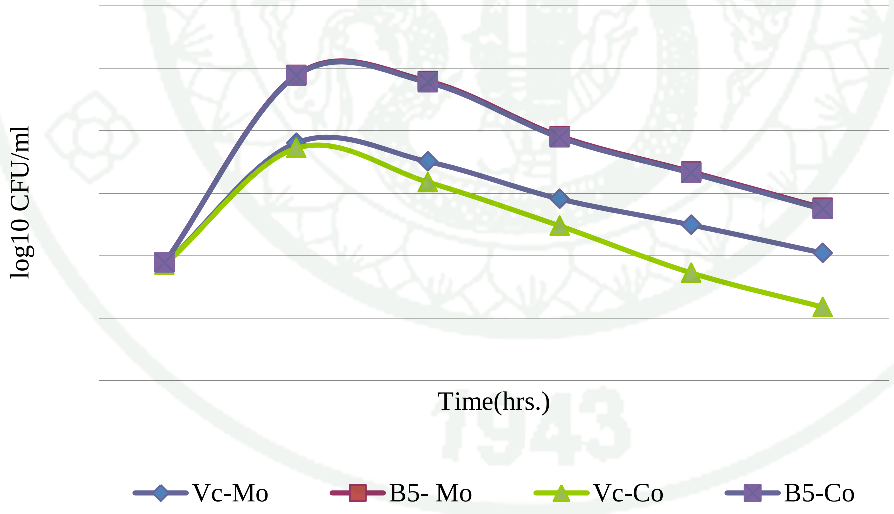


Figure 28 Growth of B5 and *V. cholarae* (non 01) in co-culture and mono-culture.

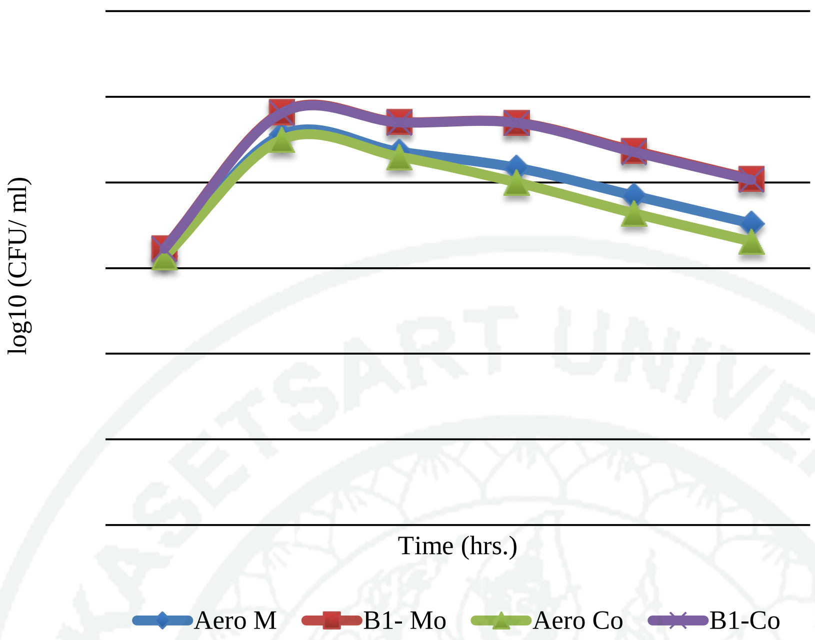


Figure 29 Growth of B1 and *A.hydrophila* in co-culture and mono-culture.

Data analysis of AWDA and broth co-culture experiment
All the data were analyzed by Analysis of Variance and Duncan's Multiple Range (SPSS 16).

3. Characterization and partial purification of the CFNS of the selected probionts.

3.1 Determination of residual antimicrobial activity of the probionts by the critical dilution method to select the probiont.

The CFNS of all the 3 *Bacillus* was tested by the critical dilution assay to determine the residual antimicrobial activity against the selected pathogenic strains against which these produce antagonism. Activity was measured as the reciprocal of the highest two-fold dilution showing antimicrobial activity. According to to the observations, 1/16 dilution of the crude supernatant is the highest dilution showing antimicrobial activity against indicator strain that are *V. parahaemolyticus* (ABRC Lab. Figure 31) and *V. alginolyticus* (ABRC Lab. Figure 30) which was used in all

measurements for bacteriocin or antimicrobial activity. Bacteriocin activity of cell-free supernatant of B2 (crude extract) was calculated as:16 x 20 = 320 AU/ml.
AU= Arbitrary Unit; 16= Reciprocal of the highest dilution (1/16); 20 = 1000 µl / 50µl (conversion factor)

Table 11 The residual antimicrobial activity of *Bacillus* spp. against the pathogenic bacteria showed by Critical Dilution method.

Indicator S rains	B1 AU/ml	B2 AU/ml	B5 AU/ml
V. parahaemolyticus	-	320	80
V. alginolyticus	-	320	80
V. harveyi AQVH	-	160	40
V. vulnificus (G)	-	160	40
V. vulnificus (Y)	-	160	40
V. mimicus	-	40	40
V. choleriae	-	80	40
V.fluvialis (Y)	-	80	20
V.fluvialis (G)	-	80	20
Aeromonas hydrophila	40	-	-

3.2 Selection of probiont for partial purification and characterization of antimicrobials

As per the result of the antimicrobial activity assay Arbitrary Activity Unit (AU/ml) and AWDA the B2 showed the most consistent antagonism against all the pathogenic strain except *A. hydrophila*. However B5 also could not produce the antagonism against *A. hydrophil*a. But in compare to B5. B2 showed the antagonism only from 24 hours of incubation which and also exhibited the highest antagonism on day 5 against all the pathogenic strain as indicated by AWDA and critical dilution method. B1 showed antagonism only against *A. hydrophila* but not against the other pathogenic *Vibrio* spp. So the antimicrobials of the *Bacillus pumilus* or B2 (Figure

32 and spore of selcted strain Figure 3) was selected for and characterization and the partial purification.

3.2.1 Selection of pathogenic bacteria

Two strains of the pathogenic bacteria *V. parahaemolyticus* (ABRC Laboratory, Figure 30) and *V. alginolyticus* (ABRC Laboratory, Figure 30) selected to determine and analyze the physic - chemical properties of the antimicrobials of B2 on the basis of the antagonism produced by B2 against these 2 strains as determined by Arbitrary Activity Unit (AU/ml) and AWDA .

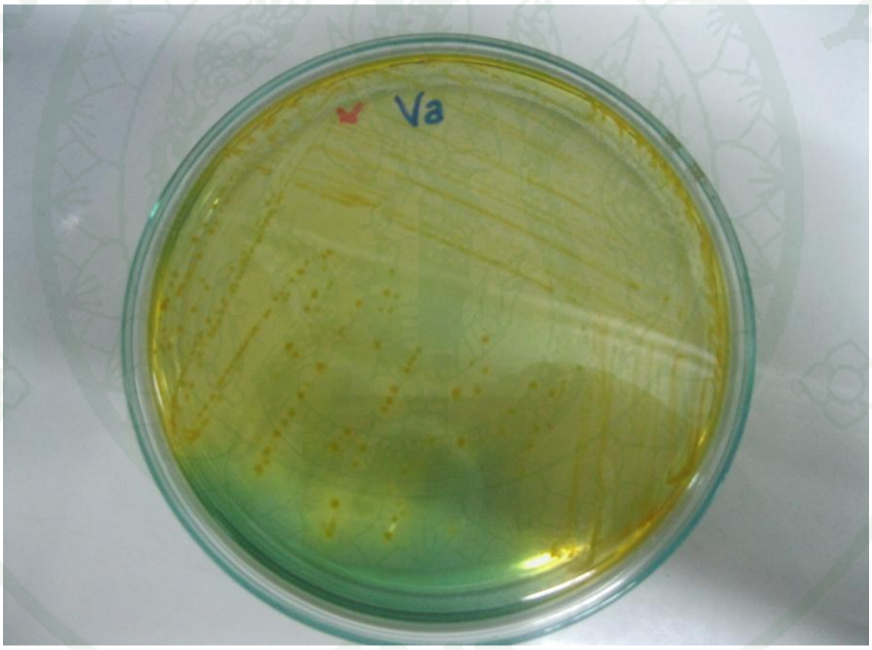


Figure 30 *Vibrio alginolyticus* of 24 hours culture.

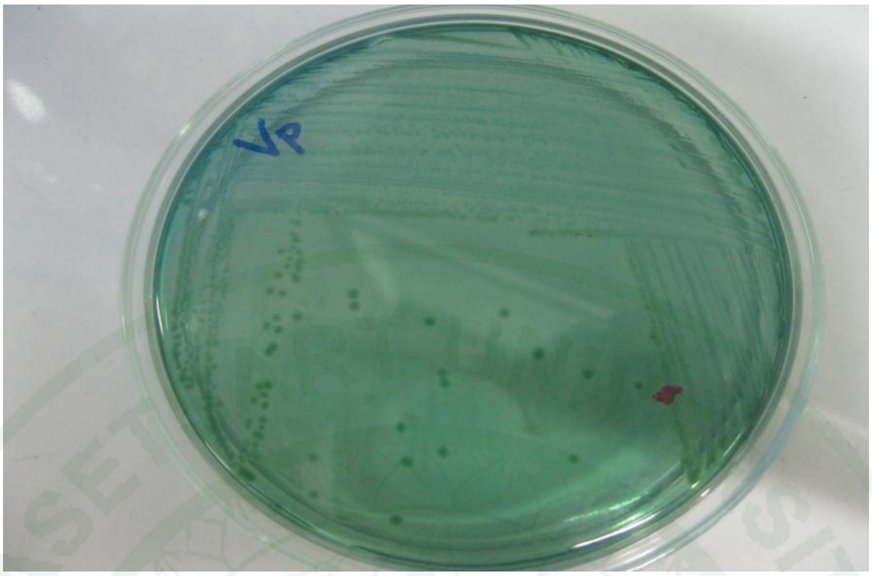


Figure 31 *Vibrio parahaemolyticus* of 24 hours culture.

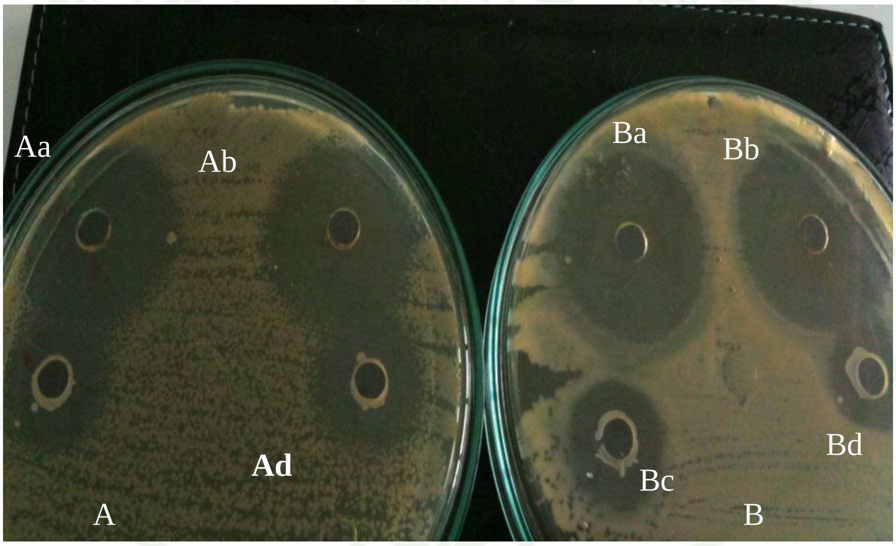


Figure 32 Zone of inhibition by the CFNS of B2 by critical dilution method. A- *V .alginolyticus,* B – *V. prahaemolyticus,* Aa and Ab – Crude CFNS(no dilution), Ac and Ad – 320AU/ml; Ba and Bb – Crude CFNS (no dilution), Bc and Bd – 320AU/ml

3.3.1 Sensibility to heat treatment

Effect of heat treatment on the antimicrobial activity was tested by keeping the crude extract at 45, 50, 60, 70, and 80° C for 20 and 60 minutes (Table 12, Figure 33 and Figure 34). As seen from Table, crude antimicrobial product was very stable to heat with respect to the all the temperatures and durations because the original activity of crude extract (320 AU/ml) was unchanged upon all the heat treatment regimes up to 70° C . The activity of the antimicrobials was found to reduce when kept at 80° C for 20 minutes where the residual activity dropped to 80AU/ ml. There was further reduction in activity found when heated at 90° C for 20 minutes which was 20 AU/ ml. No activity was found at any duration of 100 ° C and after autoclave at 121 ° C.

Table 12 Effect of different temperature on the antimicrobial activity of the CFNS of B2 against *Vibrio parahaemolyticus* (Vp) and *Vibrio alginolyticus* (Va).

Temperature	Vp/ 20min	Vp/60 min	Va 20min	Va 60min
40°C	320	320	320	320
50°C	320	320	320	320
60°C	320	320	320	320
70°C	320	320	320	320
80°C	80	40	80	40
90°C	20	0	20	0
100°C	0	0	0	0
121°C	0	0	0	0

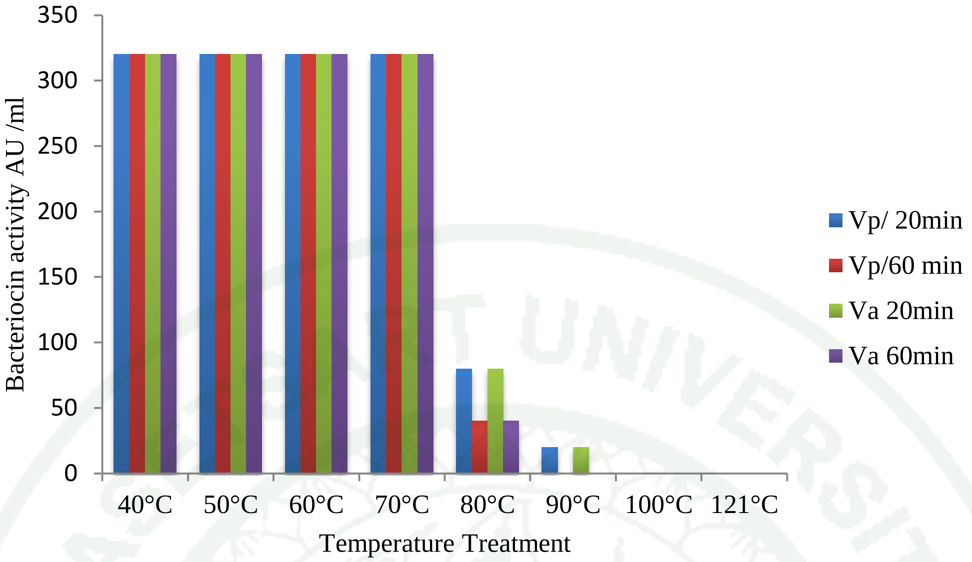


Figure 33 Antimicrobial activity of CFNS of B2 in different temperature range against *V. parahaemolyticus* (Vp) and *V. alginolyticus* (Va)

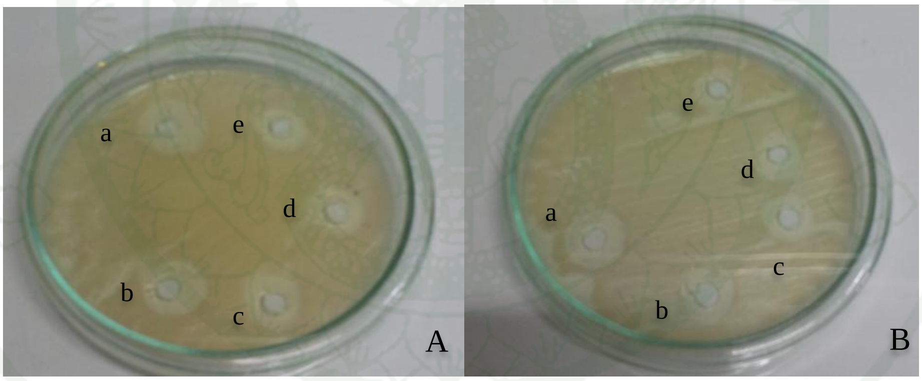


Figure 34 Zone of inhibition by the CFNS of B2 in different temperature range against *V. parahaemolyticus* (Vp) and *V. alginolyticus* (Va)
a – control (untreated), b - 40°C, C - 50°C, d - 60°C, e - 70°C.

3.3.2 Sensibility of antimicrobial substance to proteolytic enzyme

The cell-free culture fluid of B2 was treated by a variety of enzymes shown in Table to verify the protein nature of the inhibitor substance. The inhibitory activity of the supernatant was inhibited by all the proteases employed. Activity was not lost after treatment with lipasse. Complete inactivation of the antimicrobial activity was observed when the CFNS treated with pepsin and proteinase K. and the activity was reduced in great amount when treated with Trypsin 20 AU/ml. The activity remains unaltered 320AU/ml after the treatment with Lipase. The control where the CFNS was checked without the addition of enzymes remained unaltered. (Table 13, Figure 35, and Figure 36)

Table 13 Effect of proteolytic enzyme and lipase on the antimicrobial activity of the CFNS of B2 against *Vibrio parahaemolyticus* (Vp) and *Vibrio alginolyticus* (Va).

Enzyme	Vp(AU/ml)	Va (AU/ml)
Proteinase K	0	0
Pepsin	0	0
Trypsin	20	20
Lipase	320	320

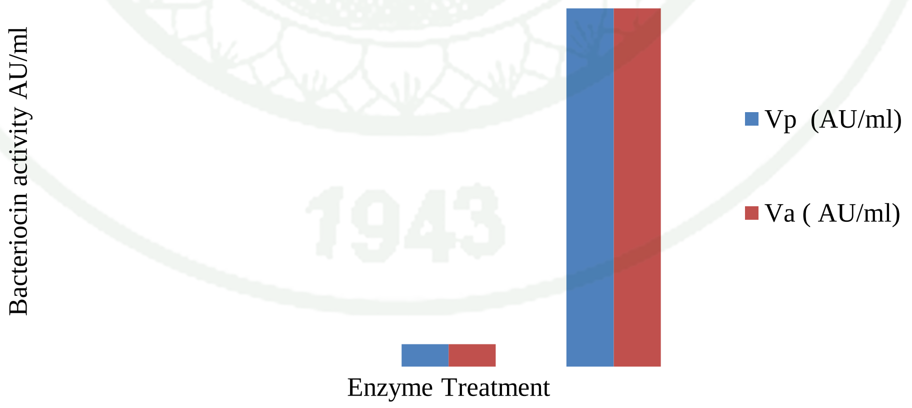


Figure 35 Antimicrobial activity of CFNS of B2 against different proteolytic enzymes and lipase against *V. parahaemolyticus* (Vp) and *V. alginolyticus* (Va).

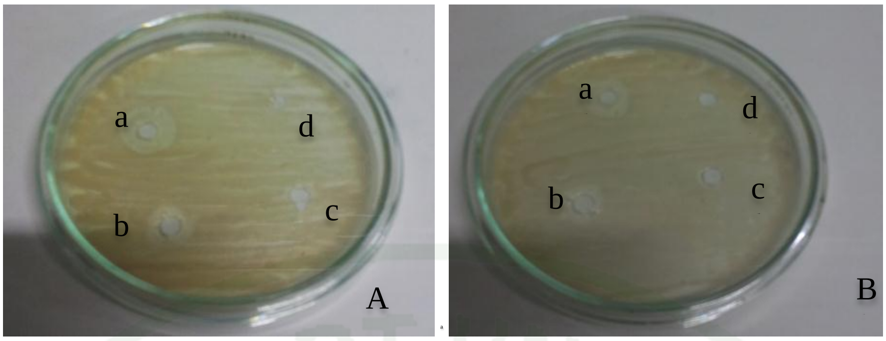


Figure 36 Zone of inhibition by the CFNS of B2 after the treatment with different proteolytic enzymes and lipase against *V. parahaemolyticus* (Vp) and *V. alginolyticus* (Va) ; a – Control (untreated), b – Lipase, c – Proteinase K, d - Pepsin

3.3.3 Sensibility of antimicrobial substance at different pH values

The CFNS of the tested strains were adjusted to various pH from 2.0 - 12.0 and incubated at room temperature and also at 4°C. The antimicrobials showed a stable residual antibacterial activity within a pH range 6 – 10, (Table 14, Figure 37, Figure 38), when incubated at 30°C (room temperature) and 4°C respectively. But the activity was reduced when exposed to pH below 6 and above 10. (Table 14) It showed the acitivity 80 AU/ml at pH 2, pH 3 and 40 AU/ml at pH 11. However it retained the activity 160 AU/ml at pH 4 when incubated at 30°C and 4°C respectively. No activity was observed at pH 12. The control CFNS in neutral pH showed the activity 320 AU /ml

Table 14 Effect of pH on the antimicrobial activity of the CFNS of B2 against *Vibrio parahaemolyticus* (Vp) and *Vibrio alginolyticus* (Va).

pH values	Vp 30°C AU/ml	Va 30°C AU/ml	Vp 4°C AU/ml	Va 4°C AU/ml
2	0	0	0	0
3	40	40	40	40
4	160	160	160	160
5	160	160	160	160
6	320	320	320	320
7	320	320	320	320
8	320	320	320	320
9	320	320	320	320
10	320	320	320	320
11	40	40	40	40
12	0	0	0	0

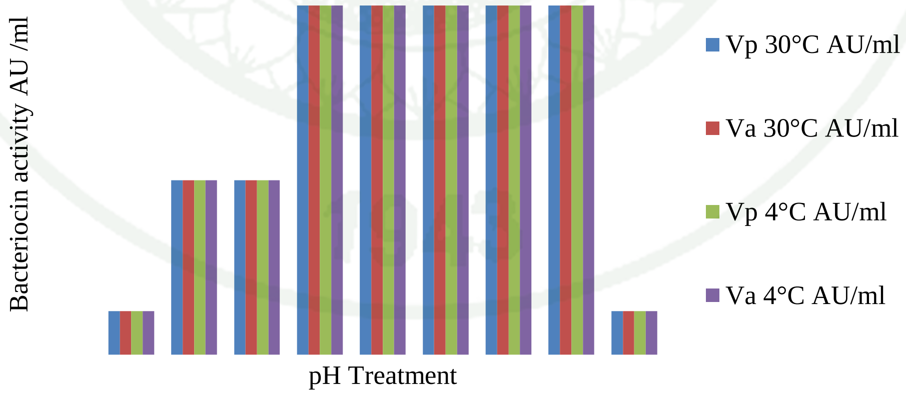


Figure 37 Antimicrobial activity of CFNS of B2 in different pH range at 30°C and 4°C against *V.parahaemolyticus* (Vp) and *V.alginolyticus* (Va).

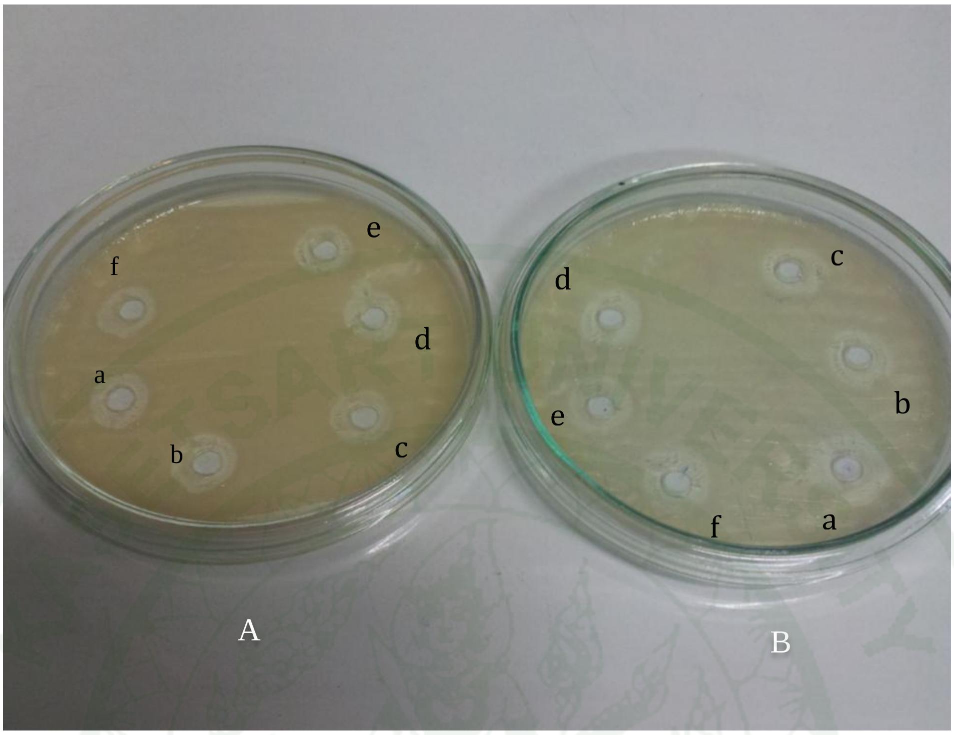


Figure 38 Zone of inhibition by CFNS of B2 in different pH range at 30°C against *V.parahaemolyticus* (Vp) and *V.alginolyticus* (Va); a – control (untreated), b – pH 6, c – pH 7, d – pH 8, e – pH 9, f – pH 10

3.3.4 Sensibility of antimicrobial substance to NaCl

The effect of different concentration of NaCl on the antimicrobial activity was studied. The CFNS when treated with NaCl in a range of 0 – 10%, in room temperature (approximately 30°C) and 4°C respectively for 24 hrs. The antimicrobials showed a stable residual antibacterial activity within a salinity range 0 to 7% under 4°C. The activity was reduced to 80 AU/ ml at salinity 8%. The Residual antimicrobial activity was found to be greatly reduced at salinity 8% and no activity was found at salinity 9% under 4°C. (Table 15 and Figure 39) The result of salinity tolerance under room temperature was different from that of 4°C. The activity was found to be reduced while exposed to above 1%. The activity was found 160 AU /ml at salinity 2. The activity was further declined above 2% range and it was found to show the inhibition of 80 AU/ ml in the range of 3 -7% of salinity..

While the activity retained only at 20 AU/ mi at salinity 8%. There was absolute loss of activity observed at salinity 9%. This was similar in both the condition of incubation.

Table 15 Effect of NaCl concentration (%) on the antibacterial activity of CFNS of B2 under 30°C and 4°C against *Vibrio parahaemolyticus* (Vp) and *Vibrio alginolyticus* (Va)

	30°C/24 hrs	4°C/hrs 24	30°C/24 hrs	4°C/24 hrs
NaCl %	Vp	Vp	Va	Va
0	320	320	320	320
1	320	320	320	320
2	160	320	160	320
3	80	320	80	320
4	80	320	80	320
5	80	320	80	320
6	80	320	80	320
7	80	320	80	320
8	20	80	20	80
9	0	0	0	0
10	0	0	0	0

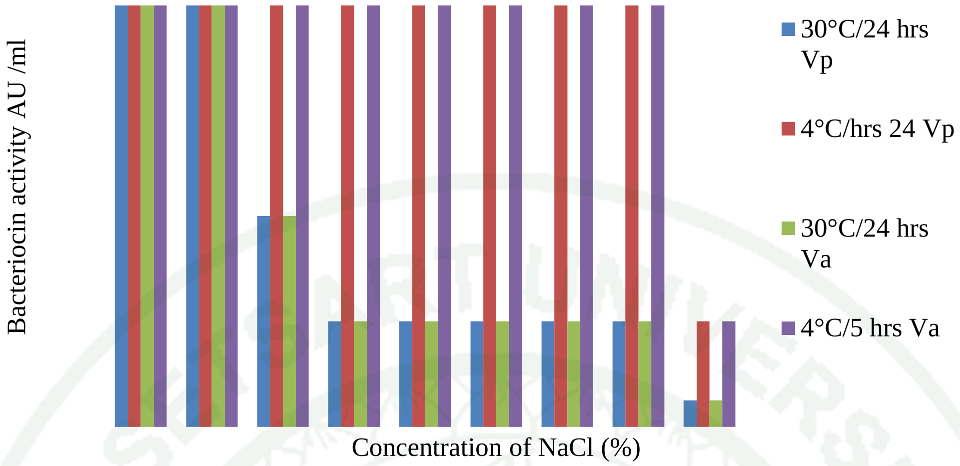


Figure 39 Antimicrobial activity of CFNS of B2 in different salinity range at 30°C and 4°C against *V.parahaemolyticus* (Vp) and *V.alginolyticus* (Va)

3.4 Concentrating the crude CFNS by amberlite XAD- 16 and partial purification

The concentrated CFNS was tested for the antimicrobial activity against the selected indicator strains by the critical dilution method where it showed the antimicrobial activity where the CFNS titer had reciprocal of the dilution factor 128 (D = 2560AU/ ml) (Figure 40)

3.5 Estimation of the molecular weight of the concentrated CFNS by SDS -PAGE

The preparation of the concentrated CFNS was used to determine the molecular weight of the sample. The part containing the sample and molecular marker was stained while the other part of the gel contained the sample only was fixed and then directly used for the antimicrobial activity. SDS –PAGE analysis revealed that there was only one protein band in the sample and hence can be explained that the partial purification could attain the homogeneity of the sample. The band corresponded to approximately 17 kDa.(Figure 41).

The sample containing part of the gel showed the zone of inhibition after 24 hours incubation in the TSA plate seeded with the target pathogens.

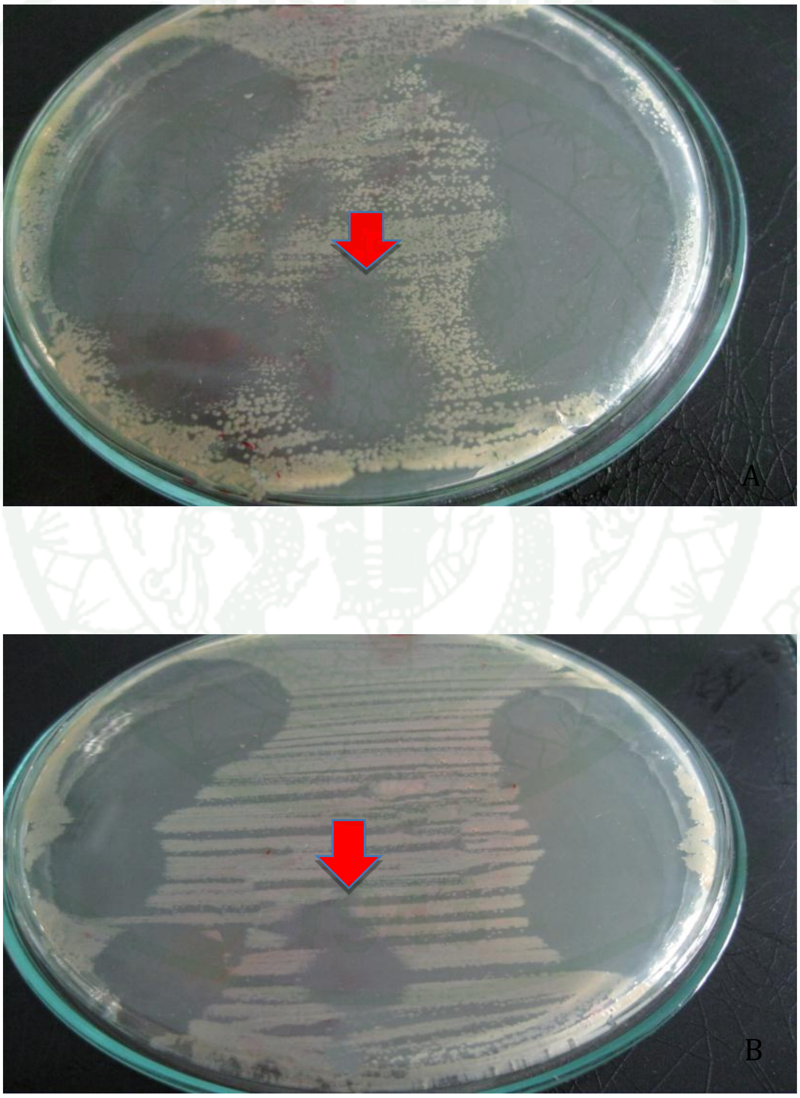


Figure 40 Antimicrobial activity of the partially purified CFNS of B2 against A - *V. parahaemolyticus* (Vp) and B - *V.alginolyticus* (Va). The arrow shows the clear zone obtained from the titer of the antimicrobials with the dilution factor 1/128.

Figure 41 SDS – PAGE of partially purified CFNS revealed the protein band (Lane 1-6 indicated by the arrow), corresponded to 17kDa as compared with the marker (M).

Discussion

Several studies on probiotics have been published during the last decade. The use of probiotics or beneficial bacteria, which control pathogens through a variety of mechanisms, is increasingly viewed as an alternative to antibiotic treatment. The use of probiotics in human and animal nutrition is well documented and recently, efforts to apply probiotics in aquaculture have started (Gatesoupe, 1999; Gomez-Gil *et al*. 2000; Verschuere *et al*. 2000). An expert with the Joint Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) stated that probiotics are live microorganisms, which when consumed in adequate amount; confer a health benefit for the host (FAO/WHO, 2001). Probiotics proposed as biological control agents in aquaculture belong to the following groups: lactic acid bacteria (*Lactobacillus* and *Carnobacterium*), genus *Vibrio*, genus *Bacillus*, and genus *Pseudomonas*. However other genera or species have also been mentioned such as *Aeromonas* and *Flavobacterium*.

Tests of antagonism, adhesion or challenge are essential in selecting a potential probiont. Antagonism may be due to competition for nutrients that favour the growth of probionts, or the expression of their inhibitory effects (Gatesoupe, 1999). Competitive exclusion has been mentioned as a possible mechanism for probiotic effects. In this study, we focused only on some strains of genus *Bacillus* spp., which, showed antagonistic activity to pathogenic bacteria in aquaculture in many studies. Preliminary antagonistic study has been done by cross streak and agar well plate diffusion method (AWDA). In this study we found that *Bacillus* B2 colonized all the strains of *Vibrio* spp. from 48 hours as seen in the cross streak method, with the level of colonization increasing up to 96 hours. The same strain started producing an antimicrobial substance after 24 hours of incubation (as revealed by the result of AWDA) against all target *Vibrio* pathogens. The secretion of this antimicrobial substance continued up to seven days. The highest production of antimicrobial substances was observed on the 5th day by this strain of *Bacillus* against almost all target pathogenic strains. This result could explain the reduction of

Vibrio strains in the co-culture experiment which strongly supports the evidence of 85-90% of reduction of *Vibrio* strains compared to the control (mono-culture) at the end of 120 hours while co-cultured with B2. *Bacillus* B5 started showing inhibition against all the target pathogenic *Vibrio* strains from 72 hours and the effect kept increasing up to 96 hours as revealed by the result of cross streak method. However production of antimicrobial substance for this strain was observed from 48 hours by AWDA. According to the result of AWDA the strain was able to produce the antimicrobial substance up to seven days against all the target pathogenic *Vibrio* strains and showed the highest level of activity on the 5th day of the experiment. This result also supported the growth reduction of all *Vibrio* strains at around 80-85% compared to control at the end of 120 hours while co-cultured with *Bacillus* B5. B1 showed the inhibitory effect only against *A. hydrophila* AQAH, with the zone of inhibition observed from 72 hours in cross streak method, and diameter of inhibition increasing until 96 hours. This result was different in the AWDA where we found that *Bacillus* B1 antagonized the target pathogen *A. hydrophila* AQAH from 24 hours of incubation. However, the production of antimicrobial substance continue until the 5th day while the highest level was observed at 72 hours, which was similar to the result of cross streak method. The use of the AWDA method in this experiment resulted in higher inhibition results than the use of the cross streak method. As a result, the inhibitory activity on indicator bacteria by the AWDA method was seen as better, but it could be because all metabolites and antimicrobial substance were present and being produced during the assay period. But the cross streak method, the inoculum of the *Bacillus* species were used, and conditions for the growing bacteria and/or the preparation of the bacteria as we had taken the inoculum from 24 hrs grown culture of the bacteial (probiont) strain which may result in a decrease of other metabolites inhibitory activity. So, the inhibition zone that was seen may be dependent solely on metabolic activity of the strain. Furthermore, the substances *Bacillus* species secreted might be in the log- or late log-phase of growth. However both the method proved to be effective in preliminary detection of antagonism. *Bacillus* B3 and B4 could not show the antagonism against none of the pathogenic strain in the whole assay.

Bacterial antagonism is a common phenomenon in nature; therefore, microbial interactions play a major role in the equilibrium between competing beneficial and potentially pathogenic microorganisms. However, the composition of microbial communities can be altered by husbandry practices and environmental conditions that stimulate the proliferation of selected bacterial species. It is well known that the microbiota in the gastrointestinal tract of aquatic animals can be modified, for example by ingestion of other micro-organisms; therefore, microbial manipulation constitutes a viable tool to reduce or eliminate the incidence of opportunist pathogens (Balcazar, 2002).

According to Bergey's Mannual of determinative Bacteriology (1994) *Vibrios* (Vibrionaceae strains) belongs to gamma proteobacteria, are gram negative, usually motile rods, are mesophilic and chemoorganotrophic, have a facultative fermentative metabolism, and are found in aquatic habitats in association with eukaryotes. They are able to grow on marine agar and also on salt supplemented selective medium thiosulfate – citrate bile salt sucrose agar (TCBS), and they are mostly oxidase positive. *Vibrios* are highly abundant in aquatic enviornments, including estuaries, marine coastal waters and sediments and aquaculture settings worldwide. Several cultivation dependents and independent studies showed that *Vibrios* appear particularly high densities in marine organisms, for example corals, fish, mollusks, seagrass, sponges, shrimps and zooplanktons. Isolation of *Vibrios* can be achieved in TCBS agar that had been additionally supplemented with 1.5% NaCl. This medium is selective for cultivation of shrimp pathogenic *Vibrios* and other enteropathogenic *Vibrio*s. The high concentrations of thiosulfate, citrate and the strong alkalinity in the medium inhibit the growth of enterobacteriociae. Ox bile and cholete suppress primarily the enterococci. Any coliform bacteria that may grow cannot metabolize sucrose. The mixed indicator thymol blue – bromothymol blue changes its color to yellow when acid is formed.

The first report of the existence in seawater of bacteria with an inhibitory effect against a *Vibrio* sp. has been attributed to Gaixa (1889). Since then the research has started to develop biological control agents. Competitive exclusion of potential pathogenic bacteria effectively reduces or eliminates the need for antibiotic prophylaxis in intensive larviculture systems (Garriques and Arevalo, 1995). In this study we did the broth co-culture experiment using the same concentration of pathogens and probionts for the periods of 120 hours to see the effect of competitive exclusion of the pathogens by the probionts. We have already discussed the amount of the reduction of pathogenic strains by the different *Bacillus* spp. The remarkable inhibition of *Vibrio* spp by B2 and B5 which correspond to 85 -90% by B2, 80 – 85% by B5 and 61.81% growth reduction was found in *A. hydrophila* by B1 at the end of 120 hrs of culture period . However, the reduction of *A. hydrophila* at the end of 96 hrs was 61.22% which was not much different from the percent decrease of *A. hydrophila* AQAH at the end of 120 hours. The reduction of *Bacillus* bacteria was 4.16% for B1, 4 to 8 % for B2, and 4 to 9% for B5, at the end of 120 hours of the co-culture experiment when compared to the mono-culture (control). These can be attributed to lack of nutrients in the culture media at the end of 120 hours of incubation. *Bacillus* B3 and B4 did not produce any antagonism by cross streak and AWDA against any of the pathogenic bacteria, therefore these strains were not selected for the co-culture experiment.

In this study (co-culture experiment) pathogenic bacteria were used at high concentrations. Previous studies have shown that the number of luminous bacteria in coastal areas ranged from 0.7x10^1to7x310^1 CFUml^{-1}(Sudthongkong, 1996), while in freshwater areas, total bacteria varied from 3.1x$10^1$to 1.0x10^3 CFU ml^{-1} (de Sousa and Silva-Souza, 2001). In fish pond waters, total bacteria ranged from 1.8± 0.9 x10^2 to 6.0 ±1.2 x 10^4 CFU ml^{-1} (Al-Harbi, 2003). In this present study it was observed that in spite of applying a high amount of pathogenic bacteria (10^5CFU ml^{-1}) the experimental strains of *Bacillus* spp. (except B3 and B4) exhibited potential antagonism against the target pathogenic strains.

In this co-culture experiment, *Bacillus* spp. B2 and B5 could inhibit growth of *Vibrio*. spp. while B1 could inhibit growth of *A. hydrophila* AQAH, which was similar to reports by Purivirojkul and Areechon (2007). Many studies supported that *Bacillus* spp. could reduce pathogenic bacteria in aquaculture. Vaseeharan and Ramasamy (2003) reported *P. monodon* immersed in *Bacillus subtilis* BT23 at a density of 10^6-10^8 CFU/ml for 6 days showed 90% reduction in accumulated mortality when challenge with *V. harveyi* at 10^3 - 10^4 CFU/ml for 1 hour. Devaraja *et al*. (2002), used microbial products, *Bacillus* spp., *Saccharomyces* sp., *Nitrosomonas* sp. and *Nitrobacter* spp., in fish and shrimp pond by immersion for 110 days, the results showed that *Bacillus* spp. were dominant in all ponds and the bacterial populations were changed by use this probiotic. *Bacillus* spp. in monoculture and co-culture in this study, showed very little reduction in number that might be because of the lack of nutrient in the culture media during the experiment. The results from this study indicated the strong potential of these *Bacillus* spp. as another means for the control of pathogenic *Vibrio* spp. and a *A. hydrophila* as a probiotic for the application of aquaculture.

Moriarty (1998) and Rengpipat *et al*. (1998), reported that the possibility of increasing shrimp production in large bodies of water by adding the probiotic *Bacillus*. Similarly other studies have reported that probiotic could improve water quality (Homma and Shinohara, 2004; Manpal *et al*. 2003).

Table 11 showed sensitivity of the various strains to the antimicrobials produced by B1, B2 and B5 which was further measured with critical dilution method and the bacteriocin activity assay, described by Schillinger *et al*. (1993) and Cintas *et al*. (1995), respectively. Activity was measured as the reciprocal of the highest two-fold dilution showing antimicrobial activity. According to to the observations, 1/16 dilution of the crude supernatant is the highest dilution showing antimicrobial activity against indicator strain that are *V. parahaemolyticus* (ABRC Lab.) and *V. alginolyticus* (ABRC Lab.) which was used in all measurements for bacteriocin or antimicrobial activity. Bacteriocin activity of cell-free supernatant of B2 (crude extract) was calculated as:

16 x 20 = 320 AU/ml.

AU= Arbitrary Unit; 16= Reciprocal of the highest dilution (1/16); 20 = 1000 µl / 50µl (conversion factor)

The antibacterial spectra of the activity of the bacteriocin produced by B1, B2 and B5 was assayed against10 indicator strains) belonging to two different genera and 8 different species including *Vibrio* spp. and *A. hydrophila* (gram negetive). This assay was carried out to determine the residual antimicrobial activity of the antimicrobial product of these 3 strains of *Bacillus* spp. on the basis of which the selection of the antimicrobial product produced by the strain which showed the strongest inhibition to the pathogenic bacteria had been carried out.

The antimicrobial titer of B1 when tested against the selected pathogenic strain *A. hydrophila* it produced the moderate inhabitance that was 40 AU/ ml. The antimicrobial spectrum of the B5 against the *Vibrio* spp. had already been demonstrated through AWDA and cross streak. However the residual antimicrobial activity showed moderate to week inhibition of the pathogenic *Vibrio* spp. by B5 by the critical dilution method. The response of inhibition were 80 AU/ ml against *V. parahaemolyticus* and *V. alginolyticus* while 40 AU/ ml was found for the antimicrobial activity against *V. harveyi*, *V. vulnificus* (yellow and green producer strains) *V. cholerae* (non 01), *V. mimicus*, But *V. fluvialis* (Yellow and green colony producer strains respectively) was found to be weakly inhibited by the B5 where we found the residual antimicrobial activity was 20 AU /ml. The response of residual antimicrobial activity of the B5 against the pathogenic *Vibrio* could be attributed to the dilution of antimicrobial product which had reduced the strength of the antimicrobials.

For B2 the antimicrobial titer showed moderate to strong inhibition against the tested pathogenic strains. It showed the residual antimicrobial activity against *V. parahaemolyticus* and *V. alginolyticus* 320 AU /ml. The residual antimicrobial activity was exhibited against *V. harveyi*, *V. vulnificus* (yellow and green producer strains) 160 AU / ml. The residual antimicrobial activity was 80 AU/ ml against *V. cholerae* (non 01), *V. fluvialis* (Yellow and green colony producer strains respectively). However the *V. mimicus* was found to be moderately inhibited by B2 where the residual antimicrobial activity was 40 AU/ml.

The outer membrane of gram-negative bacteria prevents molecules like bacteriocins, antibiotics, dyes and detergents from reaching the cytoplasmic membrane and acts as a permeability barrier for the cell. The antimicrobial product of our tested strain *B. pumilus* showed the strong inhibition against the pathogenic *Vibrio* spp. which belong to gram negative strains.

Heat resistance is a major characteristic of many bacteriocins and bacteriocin-like compounds produced by lactic acid bacteria and can vary dramatically ranging from 60°C or 100°C for more than 30 min (e.g. lactocin 27, lactocin S, carnobacteriocins A and B) to autoclaving at 121°C for 15-20 min (e.g. lactacin B, lactacin F, nisin *etc*.) (De Vuyst and Vandamme, 1994).

Many of the bacteriocins particularly the ones of class I and class II, are described as small hydrophobic proteins containing little tertiary structure, which explains their heat stability. Other factors contributing to heat stability of the bacteriocin of LAB are stable cross-linkages, a high glycine content and occurrence of strongly hydrophobic regions. Such heat stability also excludes the possibility of the inhibitory action being due to bacteriophage (De Vuyst and Vandamme, 1994). In comparison with some of the bacteriocin produced by the lactic acid bacteria the bacteriocin of our tested strain of *B. pumilus* was not extremely heat stable. Effect of heat treatment on the bacteriocin activity was tested by keeping the crude extract 40, 50, 60, 70, 80, 90 and 100°C for various periods (Table 12). The crude bacteriocin was very stable to heat with respect to the temperatures and durations up to 70°C because the original activity of crude extract (320 AU/ml) was unchanged upon the heat treatment of 70°C. The activity started decreasing above this temperature. The activity found 80 AU/ml while heated for 20 minutes at 80°C and the further reduction was observed 40 AU/ml while heated for an hour at the same temperature.

The activity decrease at 20 AU/ ml while heated for 20 minutes at 90°C no activity was detected at 90°C while heated for 60 minutes. Complete loss of activity was found at 100°C and after autoclave at 121°C for 15 minutes. This study of temperature tolerance was similar as studied by the Nithya *et al*., 2010 where the supernatant of *B. pumilus* strain showed the activity up to 70°C for 10 minutes after which there was the decrease of activity was observed. However, the study of Anupad *et al*. (2007) had shown the heat tolerant strains of *B. pumilus* where there was no reduction of antimicrobial activity was found even after autoclave. Many of the *Bacillu*s spp. showed moderate to weak response to heat treatments. The study of Adtunji *et.al*. (2011), showed the antimicrobial activity up to 75° C and complete loss of activity at 100°C by some of the bacteriocin producing strains of *Bacillus* spp. The work of Karmen *et al.* (2003), supported that the four strains of *B. cereus* showed a thermal stability upon the the antimicrobial activity up to up to 45 ,60 and 75° C respectively and great reduction of activity observed while exposed to 90° C for 15 minutes and total loss of activity was observed after exposing to 100° C. The strain of *B. subtilis* studied by Lee *et al*. (2010), exhibited the temperature tolerance up to 80°C while the other strain of *B. subtilis* studied by Arai *et al*.(2011), showed a temperature tolerance of 100°C on exposure for 60 minutes upon the antimicrobial activity against the indicator strains.

The cell-free culture fluid of the *B. pumilus* was treated by different enzymes shown in Table to verify the protein nature of the inhibitor substance. The inhibitory activity of the supernatant was inhibited by all the proteases employed. Activity was not lost after treatment with lipase. These data clearly showed that the antimicrobial substance is of proteinaceous nature, containing cleavage-sites suitable for the mentioned proteases. Characterization of the proteinaceous inhibitor confirmed that the antimicrobial agent produced by the tested strain of *B. pumilus* was similar according to the criteria outlined by Tagg *et al*. (1976). The complete inactivation of the antimicrobial substance after the treatment with pepsin and proteinase k was observed and only 6% activity (320 AU/ ml has been considered the total activity as 100) was observed after the treatment with trypsin. This was one of the key criteria of bacteriocin characterization. There was no loss of activity was observed while treated with lipase enzyme (Table 13).These indicated that the bacterial peptide did not contain lipidic moiety. The pumilicin characterized by Anupad *et al*. (2007), from the *B.pumilus* strain also showed the inactivation against all the proteolytic enzymes and lipase which was similar to our study. However the antimicrobials of our tested strain had shown very reduced response against the trypsin.

The sensitivity of the antimicrobial substance against the proteolytic enzymes had also been reported from the other *Bacillus* spp., as the study by Gray *et al*.(2006), characterized the bacteriocin from *B. thuringiensis* and Hyronimus *et al*.(1998) , where the characterization of bactericin was carried out fron *B. coagulans*. The study also showed the antimicrobial compounds isolated from *B.amyloliquefaciens* (Lisboa *et al*., 2006, Sutyak *et al*, 2008), *B cereus* (Bizani *et al*. 2005), and *B. subtilis* (Arai *et al*. 2011) exhibited the sensitivity against the proteolytic enzymes.

Bacteriocins differ greatly with respect to their sensitivity to inactivation by changes in pH and temperature. Many of the bacteriocins and bacteriocin-like substances produced by lactic acid bacteria are only stable at acid and neutral pH (De Vuyst and Vandamme, 1994) and are inactivated even at a pH above 8.0 (e.g. nisin, lactostrepcins, pediocin AcH, leucocin A-UAL 187). This can be attributed to the solubility of the bacteriocins of LAB (lactic acid bacteria); the isoelectric points of the bacteriocins produced by LAB are around 8.0-9.0 and the solubility of the bacteriocins decreases with increasing pH.

The combined effect of temperature and pH was tested also by exposure of the crude bacteriocin, of which pH was pre-adjusted to 2-12, to room temperature and 4°C. The crude extract with a pH value between 2 and 12 was kept for 5 hrs at room temperature, for 24 h at 4°C and for 24 hrs at room temperature. It is seen from Table (14) that crude bacteriocin at 4°C within the pH range of 6 to 10 in both the condition was stable, activity losses occurred at pH 11 and below 6 only in spite of 24 hrs.

incubation at 4°C. At room temperature incubation for 5 hrs, the activity losses began to come out at pH 11, activity being dropped to 40 AU/ml from 320 AU/ml. In our study the optimum residual antimicrobial activity was observed between pH 6 to 10 in both the condition of room temperature (Approximately 30^{c}C) and at 4°C. The bacteriocin activity was reduced to 40 AU/ml and 160 AU/ml at pH 3.0 and 4.0, and pH 5.0 respectively as explained in Table (14). The obvious stability of the bacteriocin at 4°C for 24 hrs incubation was due to the inhibition of the proteases at this temperature. No activity was detected at pH 2.0 and above 11.0. The activity was more stable in alkaline than in acidic condition. It was observed to have a wide range of activity under neutral to alkaline pH. The similar report was evidenced from the study of Anupad *et al*. (2007), where the Pumilicin 4 isolated from *B. pumilus* WAPB4 showed the activity under the pH range of 3.0 - 9.0 where the optimum activity was observed at alkaline pH range.The report of Lee *et al*. (2010) indicated about the antimicrobial activity under the the pH range from 4.0 to 10.0 of the strain of *B subtilis*., SC8. Karmen *et al*. (2003) reported of the pH tolerance of several strains of *B cereus* within 3.0 – 10.0. Teixeira *et al*. (2009) reported that the antimicrobial activity of *B. licheniformis* strain P40 showed under pH 3.0 – 10.0. Riazi *et al.* (2009) described the antimicrobial substance from *B. coagulans* ATCC 7050 was “Lactosporin” which retain it’s activity under pH value 3.0 - 7.0 with high range of thermostability remained active after the exposure to high temperature at 100°C. Similarly the *Bacillus* spp. MTCC 43 described by Sharma *et al*. (2009) also demonstrated the activity within a pH range of 4.0 – 10.0 with a temperature tolerance of 40 – 90°C. Kamoun *et al.* (2005), identified Bacthuricin F produced by *B. thuringiensis* which retained it’s antimicrobial activity when subjected to a range of temperature conditions 40 – 90°C for 30 min and also showed a stable activity under a pH range of 3.0 – 9.0.

Many studies had been conducted about the production of antimicrobial compounds under the influence of the range of salinity mostly in LAB. Paucities were there regarding the information of salinity tolerance of the bacteriocin of the *Bacillus* spp. The antimicrobial substance isolated from the tested strain of *B.*

pumilus was exposed to a range of salinity between 0 – 10% under room temperature and 4°C for 24 hrs. In both the condition of incubation there was no loss of activity was observed upto 0 -1 % of salinity. The activity strated declining above 1% of salinity at room temperature. The activity was found 160 AU /ml at salinity 2%. under room temperature of 24 hrs. The activity was further declined above 2% and it was found to show the inhibition of 80 AU/ ml in the range of 3 – 7%. And a great reduction of salinity was on 8% as 20 AU/ml. There was absolute loss of activity observed at salinity range of 9% which was similar in both the condition of incubation under room temperature and 4°C. However a stable activity was observed between 0 – 7% range of salinity when the crude supernatant of the *B. pumilus* was kept under 4°C. The activity was declined to 80 AU/ml when exposed to 8% salinity under the incubation of 4°C. At this temperature no activity was observed while exposed to the range of salinity 9%. As it was mentioned earlier that only little study had been done so far on the salinity tolerance of bacteriocin compounds from the *Bacillus* spp. However the report of Arai *et al*. (2011) differed from our finding where the antimicrobial product of *B. subtilis* B1 showed wide range of salinity 0 -15 % under 4°C. The activity was much reduced while exposed to room tenperature for 24 hrs which was similar to our findings. There was another report of a halotolerant strain of *B subtilis* BBK-1, (Roongsawang *et al*., 2002.) where the *Bacillus* spp. was found to produce biosurfactants up to the range of 8% salinity. The report of Wilaipun *et al*. (2002), showed that bacteriocin production by LAB strain *Enterococcus faecium* NKR-5-3 was inhibited when the broth contained more than 1% of NaCl. The similar findings was forwarded by N.S *et al*.(2012), where the activity of the bacteriocin produced by the LAB *Lactobacillus* L2 showed the optimum activity at 1% range of salinity under room temperature and the activity was subsequently declined at 2% ,3%, 4% and very little activity was recorded at 5% range of salinity. Study of karthikeyan *et al*. (2009), showed that the bactericin acitivity of the strain *L. acidophilus* can be optimum when it was exposed to 0.9% salinity.

The estimation of the apparent molecular weight of the semi-purified

Bacteriocin eluted from amberlite cextraction was made by SDS-PAGE. It revealed a major band at a molecular weight of 17 kDa. *Bacillus pumilus* B2 produced the highest molecular weight of antimicrobials for this particular species and to our knowledge; this study was the first to report on this *Bacillus* species (*B. pumilus*) against the pathogenic *Vibrio* spp. of acquaculture. The latter was 17 fold greater than pumilicin4 (1.994kDa) isolated from *B. pumilus* WAP4 by Anupad *et al*. (2007). Our antimicrobial is reported to be greater from *B. subtilis* bacillocin 22 and *B. subtilis* subtilosin A (3.4 kDa), *B. subtilis* CX1 bacteriocin (9.5 kDa) and also greater than *B. subtilis* KS03 iturin A2 (1.042 kDa). Those bacteriocins have not yet been reported for their antimicrobial activity against *Vibrio* spp. On the other hand the antimicrobial activity of the partially purified bacteriocin was detected on the portion of a unstained gel corresponded to the protein band over the indicator strain seeded plate following the SDS PAGE. However the report of lactosporin from *B. coagulans* ATCC7050 by Riazi *et al*. (2008) exhibited the molecular weight approximately 25 – 30 kDa . Hammami *et al*. (2008) described the bacteriocin bac 14 B from *B. subtilis* 14B had a molecular weight 21kDa which was unlike the other bacteriocin available from the same species of *Bacillus*.

Bacteriocins of Gram-positive bacteria are generally divided into four classes based on size, morphology, physical, and chemical properties (Lee and Kim, 2011). Class I bacteriocins are lantibiotics, which are small peptides (<5 kDa) (Field *et al*. 2007). They are post- translationally modified, incorporating non-traditional amino acids such as dehydroalanine, dehydrobutyrine, methyl-lanthione, and lantionine (Cleveland *et al*. 2001). This class is subdivided into Type A, B, and C with the distinction being that members of Type A are positively charged, linear peptides whereas those in Type B are either neutrally or negatively charged rigid globular peptides. Members of Type C require synergistic activity of two peptides to be active. This class includes the well-studied bacteriocins nisin and lacticin (McAuliffe *et al*. 2001).

Class II bacteriocins are small (<10 kDa), heat-stable peptides that are not post-translationally modified (Heng *et al*., 2007). Class II is also subdivided into two subgroups. Class IIa are pediocin-like or Listeria-active peptides, which contain a conserved N-terminal sequences (YGNGVxCxxxxCxV). Class IIb bacteriocins require the synergistic activity of two peptides to be fully active (Nissen-Meyer *et al*., 1992). Class III bacteriocins are generally large (>10 kDa), heat-labile peptides. They are subdivided into two subtypes. Type IIIa are bacteriolysins, which are bacteriolytic enzymes. Lysostaphin is the most studied bacteriocin in this subtype (Schindler and Schuhardt, 1964). Type IIIb are non-lytic bacteriocins. Helveticin J (37 kDa) produced by *Lactobacillus helveticus* belongs to this type (Joerger and Klaenhammer, 1986). Finally, Class IV bacteriocins have unique structural characteristics. The first and last amino acids of these bacteriocins are covalently bonded, thus they have cyclic structures. Enterocin AS-48 produced by *Enterococcus faecalis* subsp. *liquefaciens* S-48 was the first characterized bacteriocin belonging to this class (Maqueda *et al*. 2004).

The production of bacteriocin or bactericin like inhibitory substances (BLIS) had been reported by several research groups.For instance, polyfermenticin SCD is a heat labile proteinase k sensitive bacteriocin produced by *B.polyfermenticus* SCD (Lee *et al*. 2001) Subtilin is lantibiotic type produced by *B. subtilis*.. Hyronimus *et al. (*1998), reported a heat stable BLIS produced by *B. coagulans* similar with pediocin the bacteriocin produced by the *Pediococcus* spp. The molecular masses of alkaline proteases produced by several bacteria including *Bacillus* ranged from 15 kDa to 30 kDa, with few reports of higher molecular masses of 33 kDa (Samal *et al*. 1991), 36 kDa (Tsujibo *et al*. 1990) and 45 kDa (Kwon *et al*. 1994).

In this study we described a new antimicrobial compound produced by a strain of *B. pumilus* from the Novozyme Biologicals, which is heat labile, showed the activity under extreme pH and moderate salinity and activity was no longer detected while exposed to proteolytic enzymes. It didn’t contain any lipid moiety. The most recent classification of bacteriocin exclude class IV, of the remaining three classes, only class III gathers large size molecules which are heat labile .At the same time the class III bactericin showed the phospholipase activity which was not detected in our antimicrobial product. Many other antimicrobial polypeptides of intermediate size (10–30 kDa) and other large antimicrobial proteins produced by bacilli are not included in this classification scheme due to the lack of data on their protein or gene sequences, although they were described in the text under the category of BLIS (Bacteriocin Like Inhibitory Substance). Proteases produced by *Bacillus* species are the most important group of secondary metabolites that are widely exploited (Ferrari *et al*. 1993). The initial characterization of our protease showed the possibility to get included under this category of BLIS with the potential probiotic property.

CONCLUSION AND RECOMMENDATIONS

Conclusion

From the experimental works and discussion of this study the conclusion can be drawn as follow:

1. Among the five strains of *Bacillus* spp. including four different strains of *B.subtilis* and one strain of *B. pumilus* were taken (*B. subtilis* B1, *B. pumilus* B2, *B.subtilis* B , *B. subtilis* B4 and *B. subtilis* B5) for to test the antagonism against the selected target pathogens isolated from diseased Nile Tilapia and Pacific White shrimps. The pathogenic strains included Seven species of *Vibrio* spp. including *V. vulnificus, V. fluvialis, V. parahaemolyticus, V. alginolyticus, V. mimicus,* and *V. cholera (non 01), V. harveyi* AQVH, *Aeromonas hydrophila* AQAH. *V. vulnificus* and *V. fluvialis* included two strains of yellow and green respectively.

2. Initial test of antagonism by cross streak and agar well diffusion assay revealed that the B2 and B5 was antagonistic to all the *Vibrio* spp. but not against the *A. hydrophila* while B1 was antagonistic against the *A.hydrophila* but not against the *Vibrio* spp.

3. The results showed that B2could produce a higher concentration of antibacterial substances than B5 and B1. Among these, *Bacilli* B2 and B5 could produce antibacterial substances at the highest level on day 5, while B1could produce the maximum level at 72 hours. However *Bacillus* B2 and *Bacillus* B5 showed the antagonism activity only against *Vibrio* spp. and this activity continued up to seven days, while B1 showed the antagonism only against *A. hydrophila* AQAH with the activity remaining up to five days. *Bacillus* spp. B3 and B4 showed no antagonism against any of the pathogenic strain

3. Broth- co-culture experiment of the *Bacillus* spp. with the pathogenic strains revealed that the presence of *Bacillus* spp (B2 and B5) led to a remarkable

inhibition of growth of all strains of *Vibrio* spp. after 120 hours of incubation. Growth of *A. hydrophila* AQAH was reduced by 61.8% by B1 after 120 hours of co-culture The growth reduction by B2 and B5 in the different strains of *Vibrio* spp. was more than 90%, and more than 85% respectively at the end of 120 hours of co-culture experiment whereas there was little difference observed in *Bacillus* spp. concentrations. The reduction of B1 was less than 5%, B2 was 4 to 7% and B5 was 4 to 8% at the end of 120 hours of the co-culture experiment compared to the control (mono-culture).

4. To select the strain of the *Bacillus* spp. for characterization and partial purification of the antimicrobial products, all the three strains were further analyzed for the antimicrobial activity against the selected pathogenic strains by critical dilution method where B2 was selected as it showed the highest antimicrobial activity as 320AU/ ml against *V. parahaemolyticus*, *V. alginolyticus* which were used as the indicator pathogenic strains for the characterization of the antimicrobials of B2.

5. The characterization of the antimicrobial substance of B2 revealed that it is moderately thermostable, had shown the tolerance of temperature up to 70°C for 60 minutes and activity was much reduced at 80°C , it showed a stable range of pH tolerance from pH 6 – 10 and more active in neutral to alkaline pH, there was completely loss of activity found while expose to proteolytic enzymes and no loss of activity was observed while treated with lipase which indicated that the antimicrobial product of our tested strain is a protease without any lipid moiety. The anitimicrobial is moderately halotolerant and showed the antimicrobial activity up to 7% of salinity when it was incubated under 4°C for 24 hours.

6. Estimation of the molecular weight by SDS –PAGE after the partial purifation of the antimicrobial substance by concentrating through Amberlite XAD - 16. revealed a single protein band of approximately 17kDa.

7. Initial characterization of the antimicrobial compound of our tested B2 strain placed it under the Bacteriocin Like Inhibitory Substance (BLIS).

Recommendations

The present study was an effort to establish the stains of *Bacillus* spp. as the effective probiotic to control the common pathogens of aquaculture. However, there are few areas are still left to be focused which are as follow:

1. Commercial scale *in vivo* experiments will be an area for future research where the individual *Bacillus* strain should be tested along with the pathogenic strains against the challenge of disease, growth parameter and water quality.

2. The elucidation of the mechanism of antagonistic action between these probionts and pathogens is needed to be done.

3. The characterization of the antimicrobial substance needs more research on their synthesis system, the response against the different inorganic substance, purification in more detailed way , amino acid sequencing. These are the certain areas which need to get focused to categorize the antimicrobial in more definitive way.

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APPENDIX

APPENDIX

PREPARATION OF REAGENTS OF SDS-PAGE

1.10% Ammonium persulfate

10 mg ammonium persulfate was dissolved in 100 ml water. It was prepared fresh daily.

2. N, N’-tetramethylene-ethylenediamine (TEMED)

TEMED was used from bottle, only pure, distilled TEMED was used. It was stored in cool, dry place and protected from light.

3. 30% Acrylamide/0.8% Bisacrylamide

30.0 g acrylamide and 0.8 g N, N’-methylene-bisacrylamide was taken in a total volume of 100 ml of distilled water. The solution was filtered through a 0.45-µm filter and stored at 4°C in the dark. It was discarded after 30 days, since acrylamide gradually hydrolyzed to acrylic acid and ammonia.

4. 4X Tris-CI/SDS, pH 6.8 (0.5 M Tris CI containing 0.4% SDS, also called 4X Upper Tris)

6.05 g Tris base and 4 ml of 10% SDS was dissolved in 40 ml of distilled water. The pH was adjusted to 6.8 with 1 N HCI. The distilled water was added to 100 ml of total volume. The solution was filtered through a 0.45 µm filter and store at 4°C.

5. Tris-CI/SDS, pH 8.8 (1.5 M Tris-CI containing 0.4% SDS, also called 4X lower Tris

18.17 g Tris base and 4 ml of 10% SDS was dissolved in 40 ml distilled water. The pH was adjusted to 8.8 with 1 N HCI. The distilled water was added to 100 ml to make the total volume. The solution was filtered through a 0.45-μm filter and stored at 4°C.

6. 5X SDS/Electrophoresis Buffer

15.1 g Tris base, 72.0 g glycine, and 5.0 g SDS was dissolved in about 800 ml of distilled water. After the solutes were dissolved, the volume was bring to 1.0 liter. The solution was filtered through a 0.45 μm filter and stored at 4°C. To prepare 1X SDS/electrophoresis buffer, one volume of the above solution was diluted with four volumes of distilled water.

7. 2X SDS/Sample Buffer

30 ml of 10% SDS, 10 ml glycerol, 5.0 ml 2-mercaptoethanol, 12.5 ml of 4X Tris-CI/SDS, pH 6.8, and 5-10 mg bromphenol blue was mixed and the volume to was adjusted to100 ml with distilled water.

8. Coomassie Brilliant Blue R-250

1) 40% Methanol

2) 7% Glacial Acetic Acid

3) 0.25% Coomassie Brilliant Blue R-250

To make 500 mL stain

8.1) Dissolve 1.25 g Coomassie Brilliant Blue R-250 in 200 mL MeOH with stirring in a glass beaker. Glass beakers were preferred here since cleaning following dye preparation is easier.

8.2) Slowly 35 mL glacial acetic acid was added with stirring

8.3) 265 mL deionized distilled water was added. The solution was allowed to stir for several minutes and it was checked periodically to see if particulates settle to the bottom of the beaker when stirring wais stopped.

8. 4) When dye appeared fully dissolved, filtered over Fisher filter paper to remove residual particulates.

5) Stored the dye at room temperature. Dye can be reused about 2-3 times if desired. Staining solution is stable for 2 - 3 weeks at 25°C.

9. Destaining Solution

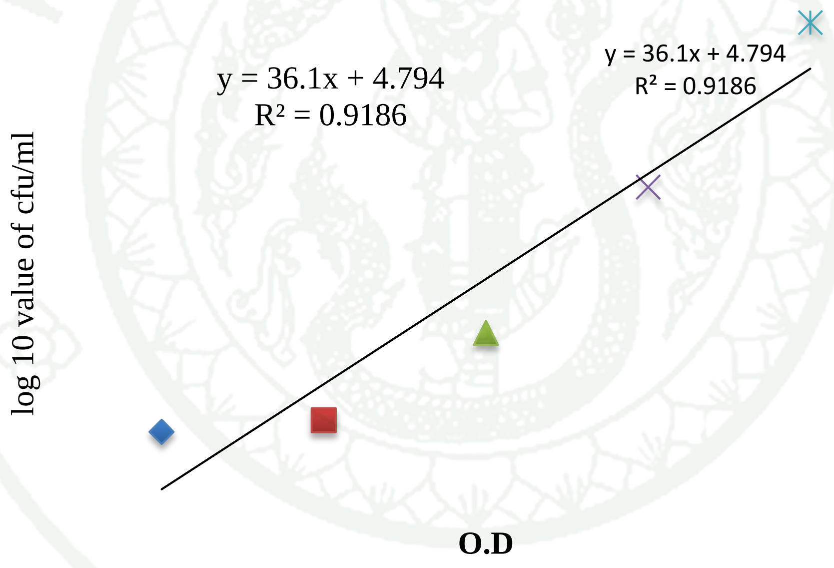
25% (v/v) methanol, 7% (v/v) acetic acid and 68% distilled water were mixed.

10. Fixing Solution

25% (v/v) methanol, 10% (v/v) acetic acid and 65% (v/v) distilled water were mixed.

Appendix Table 1 Number of bacteria (B2, CFU/ml) against the optical density (O.D).

OD	CFU/ml	log 10 cfu/ml
0.02	820000	5.91
0.04	990000	5.99
0.06	3900000	6.59
0.08	39000000	7.59
0.1	530000000	8.72

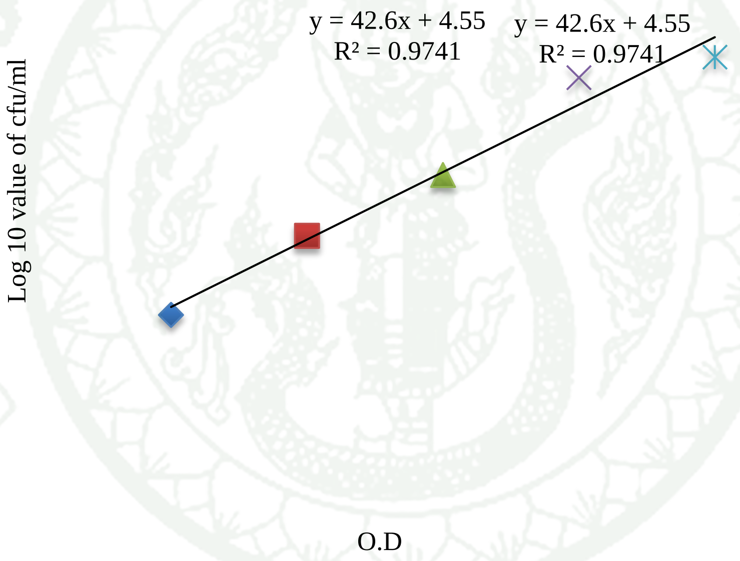


R = The correlation coefficient
Y = Number of colony (CFU / ml)
X = O.D

Appendix Figure 1 Standard curve of B2

Appendix Table 2 Number of bacteria (B5, CFU/ml) against the optical density(O.D).

OD	CFU/ml	log 10 of cfu/ml
0.02	200000	5.3
0.04	2000000	6.3
0.06	12000000	7.07
0.08	200000000	8.3
0.1	370000000	8.56

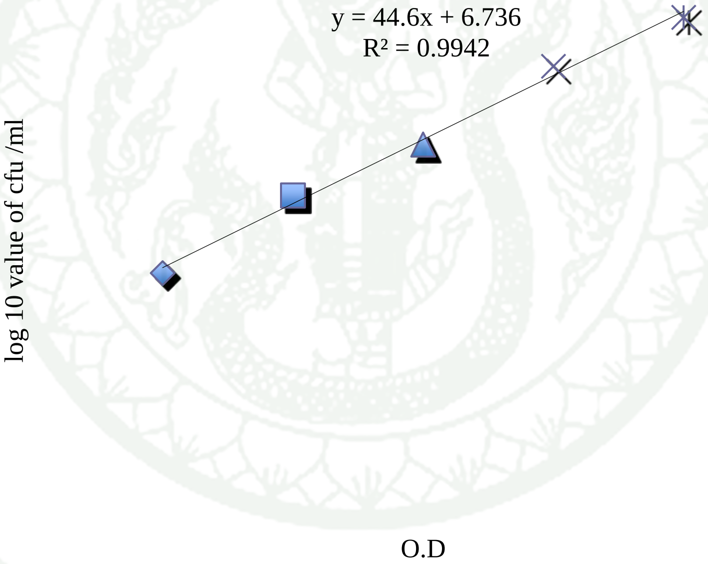


R = The correlation coefficient
Y = Number of colony (CFU / ml)
X = O.D

Appendix Figure 2 Standard Curve of B5

Appendix Table 3 Number of bacteria (B1, CFU/ml) against the optical density(O.D).

O.D	CFU /ml	log CFU/ml
0.02	36000000	7.55
0.04	434000000	8.63
0.06	2200000000	9.34
0.08	87000000000	10.43
0.1	1.3E+11	11.11

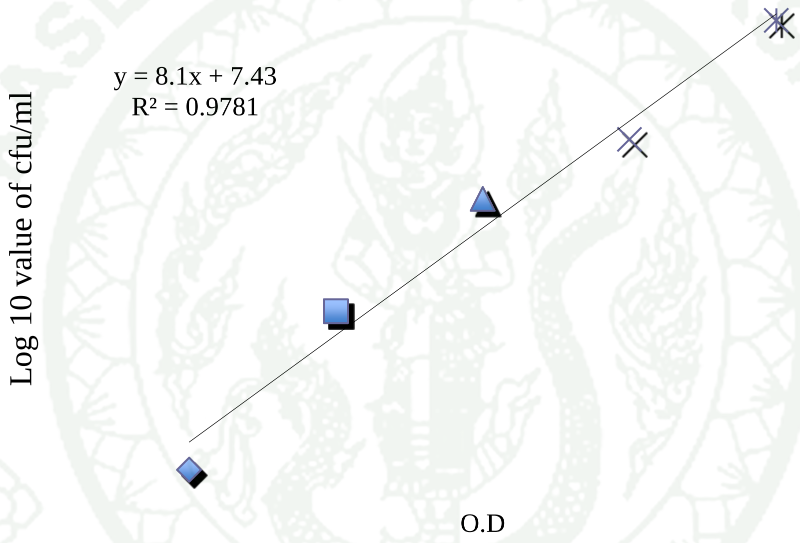


R = The correlation coefficient
Y = Number of colony (CFU / ml)
X = O.D

Appendix Figure 3 Standard Curve of B1

Appendix Table 4 Number of bacteria (*A.hydrophila*, CFU/ml) against the optical density(O.D)

O.D	CFU /ml	log 10 of CFU/ml
0.02	36000000	7.55
0.04	63000000	7.79
0.06	92000000	7.96
0.08	113000000	8.05
0.1	170000000	8.23

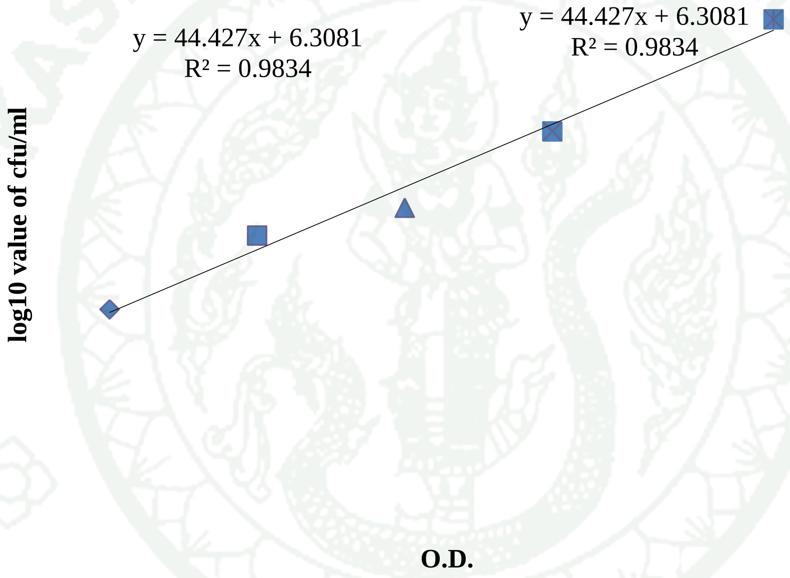


R = The correlation coefficient
Y = Number of colony (CFU / ml)
X = O.D

Appendix Figure 4 Standard Curve of *A.hydrophila*.

Appendix Table 5 Number of bacteria (*V.parahaemolyticus*, CFU/ml) against the optical density(O.D).

OD	CFU/ml	Log 10 of cfu/ml
0.01	6232000	6.79
0.03	69300000	7.84
0.05	1.72E+08	8.23
0.07	2.07E+09	9.31
0.1	8.05E+10	10.9

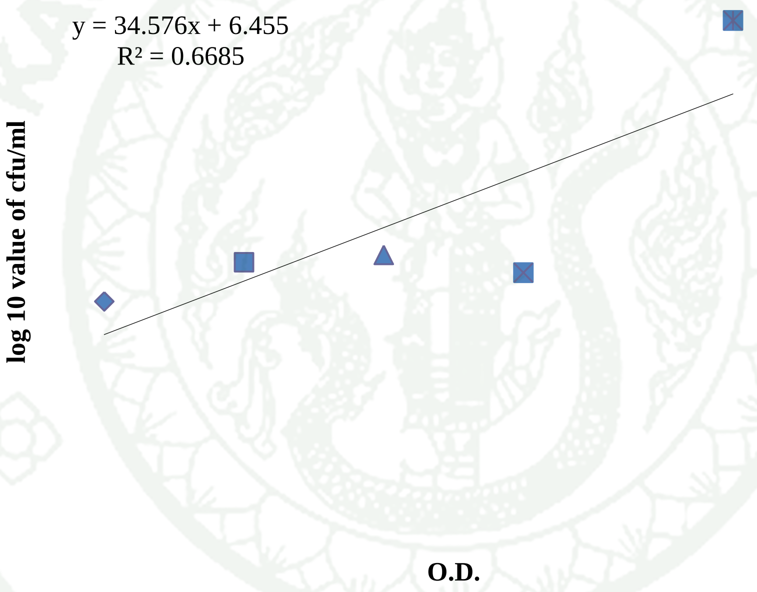


R = The correlation coefficient
Y = Number of colony (CFU / ml)
X = O.D

Appendix Figure 5 Standard Curve of *Vibrio parahaemolyticus.*

Appendix Table 6 Number of bacteria (*V.alginolyticus*, CFU/ml) against the optical density(O.D).

OD	cfu/ml	log10 of cfu/ml
0.01	17000000	7.23
0.03	54500000	7.73
0.05	68000000	7.83
0.07	40000000	7.6
0.1	7.3E+10	10.86

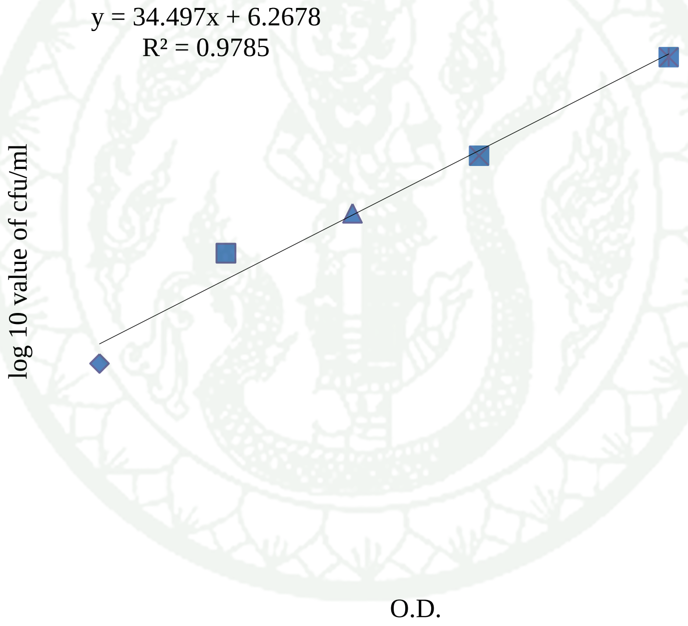


R = The correlation coefficient
Y = Number of colony (CFU / ml)
X = O.D

Appendix Figure 6 Standard Curve of *Vibrio alginolyticus.*

Appendix Table 7 Number of bacteria (*V.fluvialis* (Green), CFU/ml) against the optical density(O.D).

OD	CFU/ml	Log10 of cfu/ml
0.01	2524000	6.4
0.03	38350000	7.58
0.05	1.03E+08	8.01
0.07	4.25E+08	8.62
0.1	4.82E+09	9.68

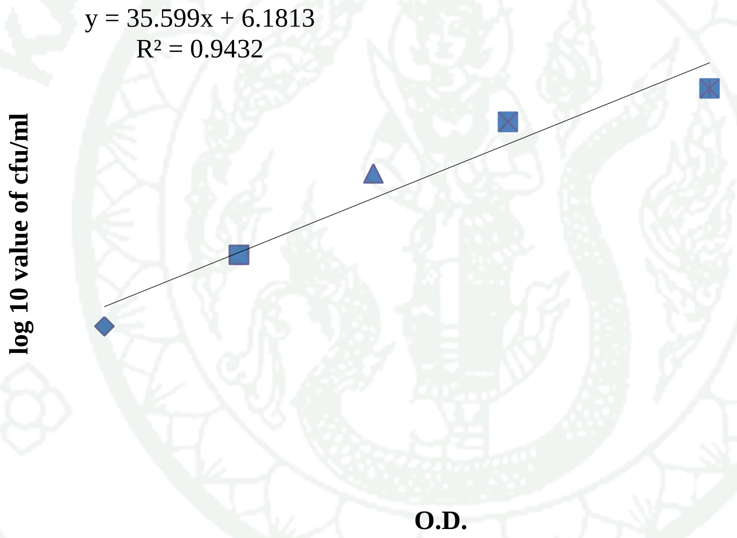


R = The correlation coefficient
Y = Number of colony (CFU / ml)
X = O.D

Appendix Figure 7 Standard Curve of *Vibrio fluvialis* (Yellow).

Appendix Table 8 Number of bacteria (*V.fluvialis* (Green), CFU/ml) against the optical density(O.D).

OD	CFU/ml	log10 of cfu/ml
0.01	1908000	6.21
0.03	16560000	7.21
0.05	195000000	8.29
0.07	925000000	8.96
0.1	2550000000	9.4

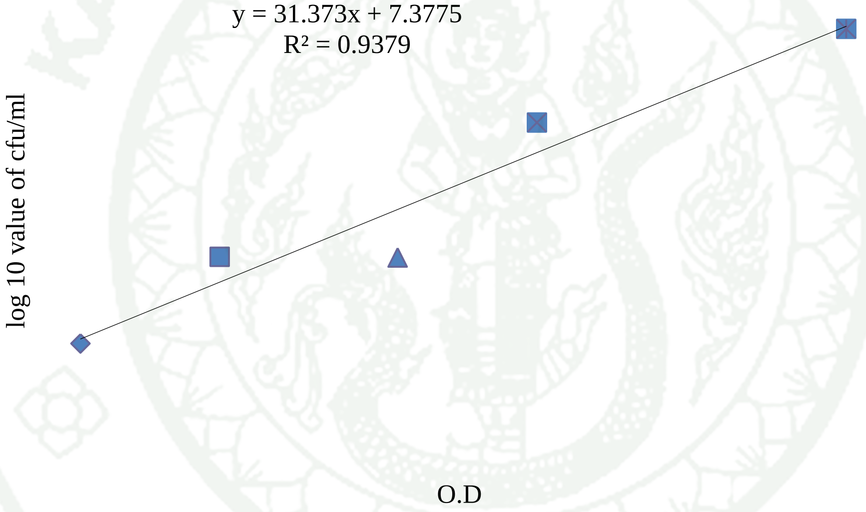


R = The correlation coefficient
Y = Number of colony (CFU / ml)
X = O.D

Appendix Figure 8 Standard Curve of *Vibrio fluvialis* (Green).

Appendix Table 9 Number of bacteria (*V.vulnificus* (Yellow), CFU/ml) against the optical density(O.D.)

OD	CFU/ml	log10 of cfu/ml
0.011	47500000	7.67
0.029	3.45E+08	8.53
0.052	3.4E+08	8.53
0.07	7.45E+09	9.87
0.11	6.35E+10	10.8

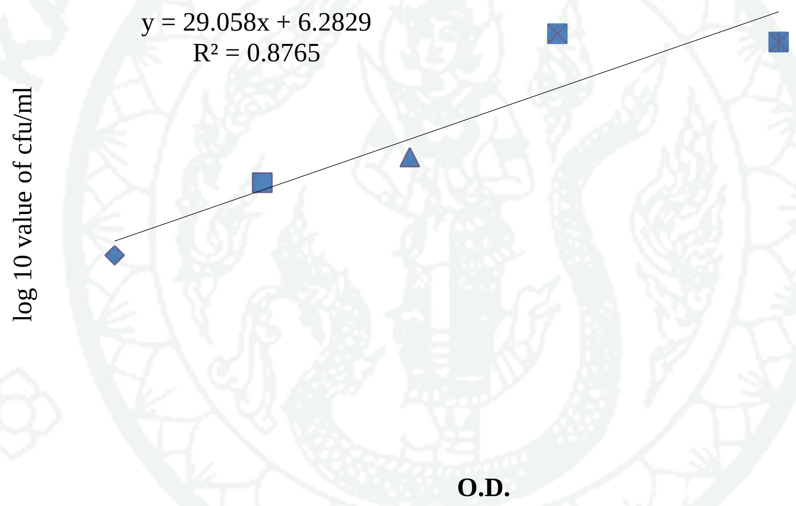


R = The correlation coefficient
Y = Number of colony (CFU / ml)
X = O.D

Appendix Figure 9 Standard Curve of *Vibrio vulnificus* (Yellow).

Appendix Table 10 Number of bacteria (*V.vulnificus* (Green), CFU/ml) against the optical density(O.D)

OD	CFU/ml	log10 of cfu/ml
0.018	2584000	6.41
0.034	17400000	7.24
0.05	34050000	7.53
0.073	8.7E+08	8.93
0.185	7E+08	8.84

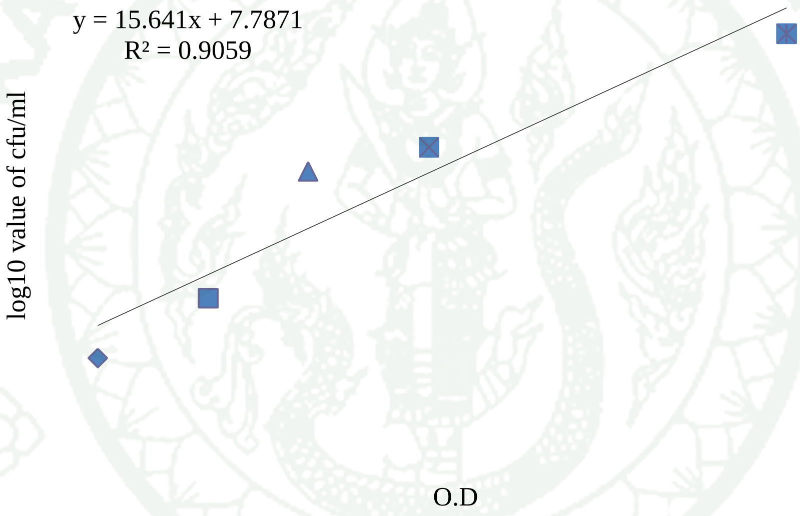


R = The correlation coefficient
Y = Number of colony (CFU / ml)
X = O.D

Appendix Figure 10 Standard Curve of *Vibrio vulnificus* (Green)

Appendix Table 11 Number of bacteria (*V.mumicus*, CFU/ml) against the optical density(O.D).

OD	CFU/ml	log10 of cfu/ml
0.012	58000000	7.76
0.033	1.41E+08	8.14
0.052	9.3E+08	8.96
0.075	1.33E+09	9.12
0.143	7.2E+09	9.85

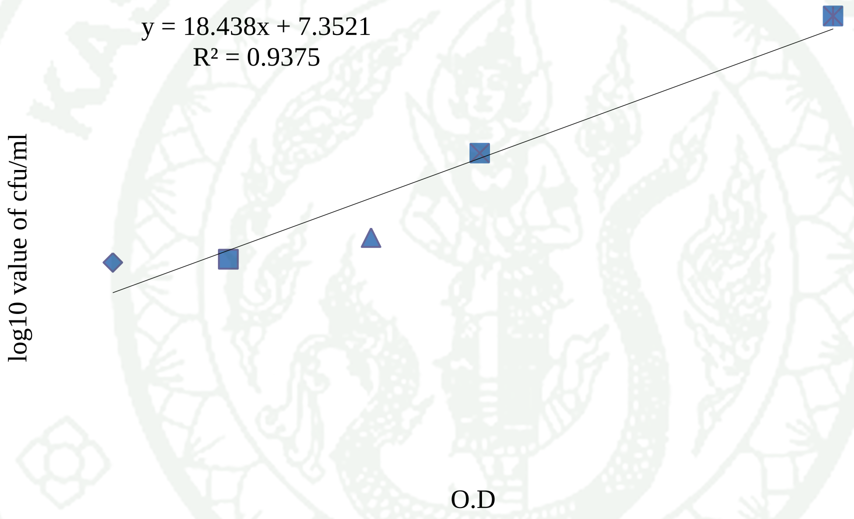


R = The correlation coefficient
Y = Number of colony (CFU / ml)
X = O.D

Appendix Figure 11 Standard Curve of *Vibrio mimicus.*

Appendix Table 12 Number of bacteria (*V.choleriae* (non 01), CFU/ml) against the optical density (O.D).

OD	CFU/ml	log10 of cfu/ml
0.017	77500000	7.88
0.034	82000000	7.88
0.055	1.19E+08	7.88
0.071	5E+08	7.88
0.123	5.2E+09	7.88

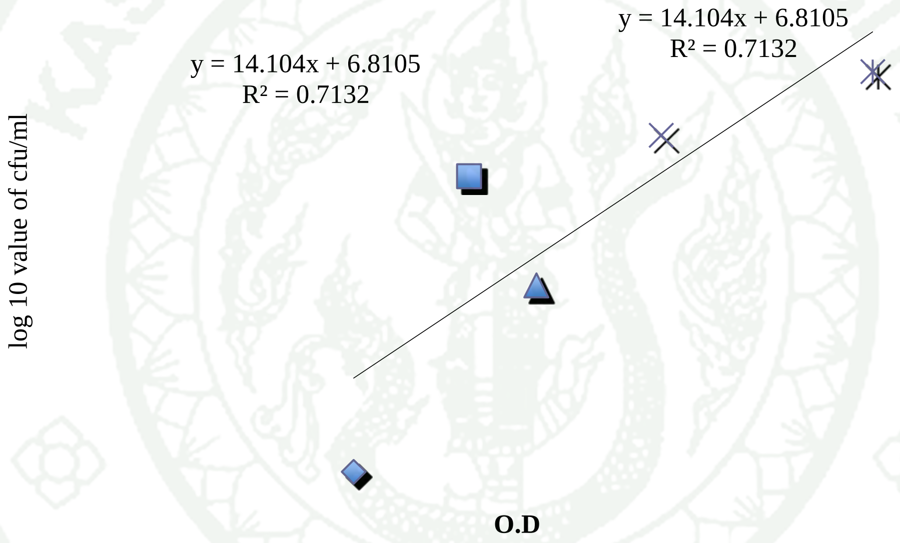


R = The correlation coefficient
Y = Number of colony (CFU / ml)
X = O.D

Appendix Figure 12 Standard Curve of *Vibrio choleirae* (non 01).

Appendix Table 13 Number of bacteria (*V.harveyii*, CFU/ml) against the optical density(O.D).

OD	CFU/ml	log 10 of cfu/ml
0.033	11850000	7.07
0.045	52500000	7.72
0.052	30900000	7.48
0.065	65800000	7.81
0.087	90300000	7.95



R = The correlation coefficient
Y = Number of colony (CFU / ml)
X = O.D

Appendix Figure 13 Standard Curve of *Vibrio harveyi*.

CURRICULUM VITAE

NAME : Mrs. Sanghamitra Nayak

BIRTH DATE : June 28, 1973

BIRTH PLACE : Calcutta, India

	YEAR	INSTITUTE	DEGREE/DIPLOMA
EDUCATION	1996	Calcutta University	B.Sc (Zoology, Hons)
	1998	Banaras Hindu University	M.Sc (Zoology)

POSITION/TITLE : Researcher
WORK PLACE : ABRC, Dept. of Fishery Biology, Kassetsart University