UNIVERSITI PUTRA MALAYSIA

EFFECTIVENESS OF *BACILLUS* SPP. ON AMMONIA REDUCTION AND IMPROVEMENT OF WATER QUALITY IN SHRIMP HATCHERY

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia in Fulfilment of the Requirement for the Degree of Master Veterinary Science

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Dedicated to my parents, my family and Kae Shin for their love and support

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Veterinary Science

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Chairman: **Professor Dato' Mohamed Shariff Mohamed Din, Ph.D.**

Faculty: **Veterinary Medicine**

Three *Bacillus* isolates, *B. subtilis* AB65, *B. pumilus* AB58 and *B. liheniformis* AB69 from brackish water environment were studied for their potential use in aquaculture bioremediation. In laboratory, all three *Bacillus* species grew well in Glucose-Ammonium-Peptone and reduced total ammonia nitrogen (TAN) effectively, however, combination of *B. subtilis* and *B. licheniformis* was the best in reducing TAN to 2.098 mg/l compared to control (without *Bacillus*) at 5.368 mg/l. All three *Bacillus* isolates significantly reduced TAN in water from shrimp culture tank as compared to the control (without *Bacillus*), and *B. pumilus* was the best in reducing TAN to 0.578 mg/l compared to control (without *Bacillus*) at 3.284 mg/l. Among the three agriculture products used, i.e. tapioca starch, sago starch and molasses, to enhance bioremediation, only *Bacillus* isolates supplemented with molasses were able to reduce TAN concentration whereby *B. pumilus* was the best isolate in reducing TAN to 0.92 mg/l.

Experiment conducted in prototype shrimp hatchery showed that addition of carbonaceous source (sugar) reduces TAN in tanks treated with *Bacillus* as well as in control tanks (without *Bacillus*). Combination of *B. pumilus* and *B. licheniformis* with sugar was the best treatment reducing TAN to 0.05 mg/l. Overall, studies conducted in laboratory showed that *B. pumilus* and *B. licheniformis* when used as individual species were the best ammonium bioremediator whereas study conducted in shrimp hatchery showed that combination of *B. pumilus* and *B. licheniformis* was the best bioremediator. The *Bacillus* isolates produced extracellular enzymes protease, lipase and amylase which were of alkalophilic characteristics. The optimum condition for enzyme activity was similar to the optimum culture condition of *Bacillus* isolates which ranged from 30°C to 35°C and pH 7.5 to pH 9. Feed 1 (F1), Feed 2 (F2) and casein were used to elicit the production of protease and amongst the three isolates, *B. licheniformis* (F2) produced the highest amount of protease activity at 40.93 U/ml followed by *B. pumilus* (F2) at 32.82 U/ml and *B. subtilis* (F2) at 29.7 U/ml when assayed at pH 9. Feed 1, F2 and olive oil were used to elicit the production of lipase and lipase activity was the highest in *B. licheniformis* (F2) followed by *B. pumilus* (F1) and *B. subtilis* (F1) at 1.5568, 1.5338, 1.4856 U/ml respectively. Starch was used to elicit the production of amylase and only *B. subtilis* produced amylase at 13.5255 U/ml. The *Bacillus* isolates proved to be able to bioremediate ammonium and hold promising future in aquaculture bioremediation.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi syarat untuk mendapat ijazah Master Veterinar Sains

KEBERKESANAN SPESIS *BACILLUS* DALAM PENURUNAN AMMONIA DAN PENINGKATAN KUALITI AIR DI DALAM TAPAK SEMAIAN UDANG

Oleh

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Pengerusi: **Profesor Dato' Mohamed Shariff Mohamed Din, Ph.D.**

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Tiga isolat *Bacillus*, *B. subtilis* AB65, *B. pumilus* AB58 dan *B. licheniformis* AB69 daripada persekitaran air payau telah dikaji potensinya untuk penggunaan di dalam bioremediasi akuakultur. Ketiga-tiga spesis *Bacillus* tersebut tumbuh dengan baik di dalam Glucose-Ammonium-Peptone dan menurunkan 'total ammonia nitrogen' (TAN) dengan berkesan. Walaubagaimanapun, gabungan *B. subtilis* dan *B. licheniformis* TAN adalah yang terbaik dalam menurunkan TAN ke 2.098 mg/l dibandingkan dengan kawalan (tanpa *Bacillus*) pada 5.368 mg/l. Ketiga-tiga isolat *Bacillus* menurunkan TAN dengan signifikan dalam air daripada tangki kultur udang dibandingkan dengan kawalan dan *B. pumilus* adalah yang terbaik (menurunkan TAN ke 0.578 mg/l) dibandingkan dengan kawalan (tanpa *Bacillus*) pada 3.284 mg/l. Antara tiga produk pertanian yang digunakan ie., tepung ubi kayu, tepung sagu dan molasses, untuk mengembangkan bioremediasi, hanya isolat *Bacillus* yang diberikan dengan molasses berjaya menurunkan TAN dimana *B. pumilus* adalah isolat terbaik dengan penurunan TAN ke 0.92 mg/l.

Eksperimen yang dijalankan di semaian udang prototaip telah menunjukkan bahawa penggunaan sumber karbon (gula) menurunkan TAN di dalam kolam yang dirawat dengan *Bacilli* dan kolam kawalan dimana gabungan *B. pumilus* dan *B. licheniformis* dengan gula merupakan rawatan terbaik yang menurunkan TAN ke 0.05 mg/l. Secara keseluruhannya, pengkajian dalam makmal mendapati bahawa *B. pumilus* dan *B. licheniformis* bila digunakan sebagai spesis individu adalah agen bioremediasi ammonium terbaik manakala pengkajian dalam tapak semaian udang mendapati bahawa gabungan *B. pumilus* dan *B. licheniformis* adalah yang terbaik. Isolat *Bacilli* juga menghasilkan enzim ekstrasel protease, lipase dan amylase yang bersifat alkalofilik. Persekitaran optima aktiviti enzim adalah menyerupai persekitaran optima pertumbuhan isolat *Bacillus* yang berjulat dari 30°C ke 35°C serta dari pH 7.5 hingga ke pH 9. 'Feed 1' (F1), 'Feed 2' (F2) and casein telah digunakan untuk penghasilan enzim protease daripada isolate *Bacillus*. Antara tiga isolat tersebut, *B. licheniformis* (F2) telah menghasilkan aktiviti protease yang tertinggi pada 40.93 U/ml diikuti dengan *B. pumilus* (F2) pada 32.82 U/ml dan *B. subtilis* (F2) pada 29.7 U/ml dimana protease tersebut dijalankan pada pH 9. 'Feed 1' (F1), 'Feed 2' (F2) and minyak zaitun telah digunakan untuk penghasilan enzim lipase daripada isolate *Bacillus* Aktiviti lipase adalah tertinggi dalam *B. licheniformis* (F2) diikuti *B. pumilus* (F1) dan *B. subtilis* (F1) pada 1.5568, 1.5338, 1.4856 U/ml masing-masing. Kanji telah digunakan untuk penghasilan enzim amylase daripada isolate *Bacillus* dan hanya *B. subtilis* sahaja yang menghasilkan amylase pada 13.5255 U/ml. Isolat *Bacillus* terbukti berupaya untuk bioremediasi ammonium dan mempunyai masa depan yang cerah dalam bioremediasi akuakultur.

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I certify that an Examination Committee met on 29th July 2004 to conduct the final examination of Ng Chi Foon on his Master of Veterinary Science thesis entitled "Effectiveness of *Bacillus* spp. On Ammonia Reduction and Improvement of Water Quality in Shrimp Hatchery" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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NG CHI FOON

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
B.L	*Bacillus licheniformis* AB69
B.P	*Bacillus pumilus* AB58
B.S	*Bacillus subtilis* AB65
BOD	biological oxygen demand
c	casein
cfu	colony forming unit
CMC	carboxymethylcellulose
Da	dalton
DDW	double distilled water
DOC	dissolved organic carbon
DOM	dissolved organic matter
FAO	Food and Agriculture Organisation
FDA	US Food and Drug Administration
F1	Feed 1
F2	Feed 2
GRAS	generally regarded as safe
h	hour
kDa	kilo dalton
LC_{50}	lethal concentration 50
mt	metric tonne
N	nitrogen

NH_3^+	ammonia
NH^4_+	ammonium
NH_3-N	ammonia-nitrogen
$(NH_4)_2SO_4$	anhydrous ammonium sulphate
NO_2^-	nitrite
NSW	non-sterile seawater
OD	optical density
o.o	olive oil
PCR	polymerase chain reaction
p.i	post inoculation
PL	postlarvae
PNP	p-nitrophenol
PNPB	p-nitrophenyl butyrate
POC	particulate organic carbon
PON	particulate organic nitrogen
POM	particulate organic matter
rpm	revolutions per minute
S	sucrose
SW	sterile seawater
TAN	total ammonia nitrogen
TSA	tryptic soy agar
TSB	tryptic soy broth
UN	United Nations

CHAPTER 1

INTRODUCTION

1.1 Background and Scope of the Study

Shrimp Industry

There has been a rapid expansion in the culture of shrimps over the last 20 years in Southern and Eastern Asia, Latin America and other tropical regions. In Southeast Asia, the production of cultured crustaceans has increased by over 500% between 1984 and 1995 (FAO, 1997). The UN FAO estimates that half of the worlds' seafood demand will be met by aquaculture in 2000, as wild capture fisheries are over exploited and are in decline. Shrimp (or prawn) culture is widespread throughout the tropical world. It is an industry set for a period of strongly growing demand, and is currently worth around USD 10 billion (Moriarty, 1999).

At present, aquaculture is the worlds' fastest growing food-production sector, providing an acceptable, protein rich supplement to, and substitute for, wild aquatic animals and plants. Over the last decade, aquatic production from captured fisheries and aquaculture has increased steadily, reaching 120.7 million mt in 1995, an increase of around 5.6 million mt since 1989. Much of this increase is attributable to aquaculture. The cultured shrimp and prawn subsector grew at an annual percent rate of 16.8 between 1984 and 1995. This increase was principally due to culture of penaeid shrimp species, which in 1995 accounted for 93.6% of all cultured shrimp and prawns. Penaeid production, notably of giant tiger prawn (*Penaeus monodon*) and other *Penaeus* species, increased from 31% or 54,000 mt and 2% or 2,100 mt

respectively, in 1984, to 54% or 503,000 mt and 18% or 165,000 mt in 1995 (Subasinghe *et al*., 1998).

The countries dominating the production of cultured marine shrimp in Southeast Asia are Thailand, Vietnam and the Philippines (Gräslund and Bengtsson, 2001, Table 1). In Southeast Asia, the production of cultured crustaceans increased by over 500% between 1984 and 1995. By 1998, the total shrimp aquaculture production in this region was 580,000 t, i.e. slightly more than the quantity of shrimps caught in the wild (FAO, 1997). The species dominating the marine shrimp culture in Southeast Asia are penaeid shrimp, especially *P. monodon*, commonly known as the black tiger shrimp (Gräslund and Bengtsson, 2001).

Table 1:Production of cultured shrimp in the countries dominating the Southeast Asia shrimp farming industry

Year	Thailand	Indonesia	Vietnam	Philippines	Malaysia
1995	220 000	80 000	50 000	30 000-50 000	5 260
1997	150 000	80 000	30 000	10 000	7 000
1999	200 000-210 000	100 000	40 000	40 000	15 539

Figures in metric tons. After Kongkeo (1997), Rosenberry (1995, 1997, 1999) and Malaysian Agricultural Directory and Index (1999).

However shrimp farming can generate severe environmental impacts which include destruction of wetland and mangroves forest in the construction of shrimp ponds,

seepage of brackish water from the cultured ponds into the groundwater and the most important impact being the effect of shrimp ponds effluent to the shrimp culture (Paez-Osuna *et al*., 2001).

1.2 Statement of the Issue

The effect of effluent from intensive marine shrimp farming has been a subject of increasing attention in the recent year. While extensive shrimp ponds produce few wastes, semi-intensive and intensive ponds produce high waste loads. Many shrimp farms have intensive earth ponds stocked with high densities of animals (25 to 100 animals/m^2) fed with high-protein formulated feeds. About 90% of the nitrogen (N) input to a pond comes from the formulated feed; however, much of the N (70 to 80%) is not retained by shrimp (Briggs and Funge-Smith, 1994). Instead, it enters the pond ecosystem and acts as an expensive fertilizer, stimulating the growth of bacteria and plankton (Burford *et al*., 1998).

Effluents from shrimp ponds can contain elevated concentrations of dissolved nutrients, phytoplankton, bacteria, and other suspended organic and inorganic solids (Ziemann *et al*., 1992). In prolong culture; the water quality of the shrimp pond will deteriorate causing oxygen depletion, light reduction, and eutrophication of the pond water. However, detailed studies have clearly shown that biological oxygen demand (BOD), ammonium and total suspended solid increase with stocking density (Tunyilai *et al*., 1993). In shrimp culture ammonia is toxic and can cause mortality of shrimp reared in a closed culture system (Chin and Chen, 1987; Spotte, 1979).

The microbial and plankton biomass will also increase and exerts considerable oxygen demand. Unless the ponds are flushed with cleaner water, hypoxic conditions that are detrimental to shrimp growth and health can readily develop. The discharge of eutrophic water with high loads of nitrogenous waste and suspended solids can damage the coastal environment, particularly in areas with high farm densities, by promoting harmful or nuisance algal blooms, increasing turbidity and creating anoxic conditions (Naylor *et al*., 1998).

The discharge has also been quoted as a source of deterioration of coastal natural resources and environment. The accumulated sediment or sludge, which is left on the ponds' bottom, also contain a lot of organic matter from uneaten feed and shrimp faeces or excreted matter. In a study conducted by Tunyilai *et al*. (1993), it was found that ammonia, chlorophyll *a*, and total suspended solids increase with stocking density. Although intensive shrimp culture systems produce substantially higher shrimp yields than do semi-intensive systems (Rosenberry, 1995), intensification will result in stressful environmental changes that can cause problems for shrimp (Lightner and Redman, 1998). The major problem due to intensification of the system is the occurrence of diseases (Primavera, 1994). Evidence suggests that serious shrimp farm production losses resulting from the outbreak of disease in Asia and Latin America are due to the environmental impacts of shrimp culture (Philips *et al*., 1993). The unregulated expansion of the industry, and a lack of understanding of the environmental consequences have also resulted in mass mortalities of shrimp, significantly affecting production in, for example, China and Taiwan (Chen, 1995; Qingyin *et al*., 1995).

Therefore there is a need to reduce the amount of ammonium in the pond so as to increase productivity as well as the survivality of the shrimp cultured. Several methods or management practices have been suggested to reduce ammonium concentration in ponds such as regular water exchange, regulating feed and feeding practices, water circulation and aeration, decreasing pond depth and bioremediation (Hargreaves, 1998). Bioremediation or bioaugmentation is a concept of reducing organic wastes to environmentally safe levels through use of micro or macroorganisms. Bacterial species belonging to genera *Bacillus, Pseudomonas, Acinetobacter, Cellulomonas, Rhodoseudomonas, Nitrosomonas* and *Nitrobacter* are known to help in mineralization of organic wastes (Thomas *et al.,* 1992).

Total organic load in shrimp ponds increases with time due to several factors, which includes shrimp feed and shrimp excreta deposition. The organic load increment is inevitable unless remedial intervention such as water exchange or the use of chemicals. This however is both time consuming and exhaust one's resources. The other alternative is to use microorganism seeds isolated from the aquatic environment itself.

Hypothesis: In the present work three *Bacillus* isolates were evaluated as a bioremediator. This microorganisms seeds must possess abilities to degrade organic compound in the receiving water such as protein, carbohydrate and lipid with the action of extracellular enzymes secreted by these microorganisms. The microorganism seeds must also be able to improve water quality of shrimp ponds in particular ammonium. The total number of microorganism to be applied, frequency of application and the mode of application have to be determined.

Objectives: The aim of this research is to evaluate the *Bacillus* spp. for bioremediation properties. The followings are the specific objectives:

1) Study the effect of selected *Bacillus* isolates on reduction of ammonium.
2) Characterization of extracellular enzymes (protease, cellulase, lipase, and amylase) produced by the *Bacillus* isolates.