UNIVERSITI PUTRA MALAYSIA

INVESTIGATION ON INDIGENOUS *BACILLUS* ISOLATES WITH BIOREMEDIATION PROPERTIES FOR IMPROVING WATER QUALITY AND SHRIMP HEALTH IN MALAYSIAN AQUACULTURE

DEVARAJA T.N.

FSAS 2002 19

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By

DEVARAJA T.N.

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia in Fulfilment of the Requirement for the Degree of Doctor of Philosophy

February 2002

Dedicated to my parents, who supported me to become what I want

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Chairperson: Professor Fatimah Md. Yusoff, Ph.D.

Faculty: Science and Environmental Studies

Indigenous marine bacteria of the genus *Bacillus* were selected to study their properties as potential use for bioremediation owing to their inherent versatility. Bacteria were isolated from water and sediment samples collected along the west coast of Peninsular Malaysia in brackishwater environment. Selected isolates were identified to species level using biochemical and API CH kit and three suitable isolates, *Bacillus pumilus* AB58, *B. subtilis* AB65 and *B. licheniformis* AB69 were selected for the study. Optimum growth requirements of temperature, NaCl and pH were 30°C, 1.5% and 7.5 respectively, determined for the isolates by measuring the optical density and corresponding cell number. The growth curves of the isolates were plotted and all of them reached maximum cell number during a 16-20 h incubation. The cell density in overnight cultures of *B. pumilus* AB58, *B. subtilis* AB65 and *B. licheniformis* AB69 were $5.7x10^9$ (± 0.8), $3.7x10^8$ (± 0.6), $5.0x10^9$ (± 0.6) cfu/ml respectively. They had the ability to tolerate ammonia levels of up to 20 mg/l without a considerable change in cell numbers for 48 h. However, the growth was suppressed completely at 25 mg/l of ammonia. At 40 ppt salinity, all the isolates survived for 4 days without significant change in initial cell numbers (10^8 cfu/ml). The selected isolates were found to secrete extracellular enzymes viz., protease, gelatinase, amylase and

lipase as detected by clear zone formation on substrate based agar plates. *Bacillus pumilus* AB58 and *B. subtilis* AB65 produced significantly ($P < 0.05$) bigger protease clear zones (19.0 ± 2.0 and 23.0 ± 4.0 diameter in mm respectively) than *B. licheniformis* AB69. However, *B. subtilis* AB65 secreted significantly ($P < 0.05$) more amylase (31.0 ± 5.0 diameter in mm) than the other two isolates. All the isolates were sensitive to most of the antibiotics tested on MHA plates. These isolates were compatible with each other in mixed culture conditions. They inhibited as well as excluded all the pathogenic vibrios (*Vibrio alginolyticus* M11, *V. alginolyticus* M12, *V. parahaemolyticus* M1, *V. parahaemolyticus* M3, *V. parahaemolyticus* M6, *V. alginolyticus* T, *V. parahaemolyticus* T, *V. harveyi* I and *V. parahaemolyticus* I) tested by diffusion disc, streak plate and common broth methods. Synergistic effect of isolates had significantly higher ($P < 0.05$) inhibition of all vibrios than the individual isolates. The isolates were confirmed for their non-pathogenicity to shrimp postlarvae (PL 29). All three isolates were tested for their effect on ammonia in simulated pond conditions. All non-aerated treatment tanks had significantly lower ammonia levels ($P < 0.05$) than the non-aerated control tanks, which were not treated with bacterial isolates both in case of single and combination treatments. Synergistic effect of isolates reduced the ammonia levels at a faster rate than the treatments with single isolate. Sediment properties were not significantly different between treated and control groups except for the total and available phosphorous levels, which were significantly higher in tanks treated with *B. licheniformis* AB69 ($P < 0.05$) compared to the others. The selected *Bacillus* isolates satisfied the criteria to qualify them for bioremediation in aquaculture.

Abstrak tesis dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi syarat untuk mendapat ijazah Doktor Falsafah

PENYIASATAN KE ATAS ISOLAT-ISOLAT *BACILLUS* TEMPATAN BERCIRIKAN BIOREMEDIASI BAGI MENINGKATKAN KUALITI AIR DAN KESIHATAN UDANG UNTUK AKUAKULTUR DI MALAYSIA

Oleh

DEVARAJA T.N.

Februari 2002

Pengerusi: Profesor Fatimah Md. Yusoff, Ph.D.

Fakulti: Sains dan Pengajian Alam Sekitar

Dalam kajian ini, spesies *Bacillus* marin tempatan telah dipilih untuk bioremediasi kerana sifat serba boleh yang semulajadi. Bakteria daripada sampel air dan sedimen daripada persekitaran air payau telah di kutip dari sepanjang pantai barat Semenanjung Malaysia. Isolat-isolat yang terpilih telah dikenalpasti ke peringkat spesies dengan menggunakan kaedah biokimia dan kit tersedia API CH dan tiga isolat terbaik, *Bacillus pumilus* AB58, *B. subtilis* AB65 dan *B. licheniformis* AB69 telah dipilih untuk kajian ini. Paras optimum keperluan-keperluan pertumbuhan asas; suhu, garam dan pH masing-masing adalah 30°C, 1.5% dan 7.5, yang mana telah ditentukan untuk isolat-isolat tersebut dengan mengukur densiti optik dan bilangan sel sejajar. Lengkuk pertumbuhan isolat-isolat telah diplot dan didapati bahawa semua isolat mencapai bilangan sel maksimum pada 16-20 jam (j) inkubasi. Densiti sel di dalam kultur semalaman *B. pumilus* AB58, *B. subtilis* AB65 dan *B. licheniformis* AB69 adalah $5.7x10^9$ (± 0.8), $3.7x10^8$ (± 0.6), $5.0x10^9$ (± 0.6) cfu/ml masing-masing. Mereka berupaya menahan paras ammonia sehingga ke 20 mg/l tanpa perubahan pada bilangan sel selama 48 j. Walau bagaimanapun, pertumbuhan terencat sepenuhnya apabila paras ammonia mencapai

tahap 25 mg/l. Pada saliniti 40 ppt, semua isolat berjaya hidup selama 4 hari tanpa perubahan yang signifikan pada bilangan sel permulaan (10^8cfu/ml). Isolat-isolat terpilih di dapati mengeluarkan enzim-enzim luar sel, iaitu protease, gelatinase, amilase dan lipase berdasarkan zon yang terang terhasil di atas piring agar. *Bacillus pumilus* AB58 dan *B. subtilis* AB65 lebih signifikan ($P < 0.05$) di dalam menghasilkan zon yang lebih terang (19.0 ± 2.0, dan 23.0 ± 4.0 diameter dalam mm masing-masing) berbanding dengan *B. licheniformis* AB69. Walau bagaimanapun, *B. subtilis* AB65 lebih signifikan di dalam merembes amilase ($P < 0.05$), iaitu (31.0 ± 5.0 diameter dalam mm) berbanding dengan kedua-dua isolat yang lain. Kesemua isolat adalah sensitif kepada kebanyakan antibiotik yang diuji di atas piring MHA. Isolat-isolat ini adalah serasi di antara satu sama lain dalam keadaan kultur campuran. Mereka merencat dan menyingkirkan semua vibrios patogenik (*Vibrio alginolyticus* M11, *V. alginolyticus* M12, *V. parahaemolyticus* M1, *V. parahaemolyticus* M3, *V. parahaemolyticus* M6, *V. alginolyticus* T, *V. parahaemolyticus* T, *V. harveyi* I and *V. parahaemolyticus* I) apabila diuji dengan cakera resapan, piring coretan dan medium biasa. Kesan sinergistik oleh isolat-isolat adalah secara signifikannya lebih tinggi ($P < 0.05$) ke atas perencatan semua vibrios berbanding dengan isolat tunggal. Isolat-isolat juga telah disahkan tidak patogenik terhadap pasca larval udang (PL 29). Ketiga-tiga isolat telah diuji kesannya terhadap ammonia dalam kolam simulasi. Semua tangki rawatan yang tidak mengandungi pengudaraan menunjukkan paras ammonia yang secara signifikannya lebih rendah ($P < 0.05$) berbanding dengan tangki kawalan yang tidak mengandungi pengudaraan, samada secara berasingan atau campuran. Kesan sinergistik isolat-isolat dapat mengurangkan paras ammonia pada kadar yang lebih cepat berbanding dengan

rawatan menggunakan isolat tunggal. Kandungan sedimen adalah tidak signifikan di antara kumpulan yang dirawat dan kawalan kecuali untuk jumlah dan tahap tersedia fosforus, yang mana secara signifikannya lebih tinggi di dalam tangki yang dirawat dengan *B. licheniformis* AB69 ($P < 0.05$) berbanding dengan yang lain. Isolat-isolat *Bacillus* yang terpilih memenuhi kriteria untuk melayakkannya sebagai agen bioremediasi di dalam akuakultur.

ACKNOWLEDGEMENTS

I would like to express my whole hearted indebtedness to my major advisor, Prof. Dr. Fatimah Md. Yusoff, Department of Biology, Faculty of Science and Environmental Studies, Universiti Putra Malaysia, Malaysia for her inestimable and propitious guidance throughout the course of research and in transcription of this thesis. Her counseling and constant encouragement has helped me a lot during the course of study. Her care throughout my stay in Malaysia is greatly acknowledged. Her meticulous nature of evaluating my manuscript has been instrumental in making this thesis a valuable one.

I am grateful to Prof. Dr. Shariff, M., Faculty of Veterinary Medicine, Universiti Putra Malaysia, Malaysia, member of my advisory committee for his invaluable guidance and encouragement throughout the course of study. His concern and timely exhortation shown towards me is deeply appreciated. I express my special thanks to him who gave me the opportunity to do Ph.D. in Malaysia.

I owe my heartfelt thanks to Dr. Arif, M.S., Associate Professor, Department of Biochemistry and Microbiology, Faculty of Science and Environmental Studies, Universiti Putra Malaysia, Malaysia, member of my advisory committee for his invaluable and kind suggestions and constant encouragement throughout the period of research.

I am thankful to Dr. Sanjoy Banerjee (dada), who stood next to me patting my back throughout this work and for his friendship filled with unfathomable patience. All my family members, my superb friends and their families and all those fantastic people whose blessings and support guarded me throughout this study cannot be thanked enough. Their immense love and affection has made me what I am today. I am grateful to each and every

one of them. Love and affection of my ayya, amma, anna, akka, bhava, atthige, manu, monika, and little queen leena have constantly kept me in good cheer. I am totally indebted to my love Gayi for entering and making a new beginning in my life when I was finishing Ph.D.

I thank heartily Universiti Putra Malaysia for giving me this opportunity to pursue my Ph.D. in Malaysia.

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LIST OF ABBREVIATIONS

AAHU	– aquatic animal health unit
ANOVA	– analysis of variance
Bl	– *Bacillus licheniformis* AB69
BOD	– biological oxygen demand
Bp	– *Bacillus pumilus* AB58
bp	– base pair
Bs	– *Bacillus subtilis* AB65
BWS	– bacterial white spot
cfu	– colony forming units
COD	– chemical oxygen demand
DDW	– double distilled water
DNA	– deoxyribo nucleic acid
dNTPs	– deoxyribonucleoside triphosphates
DO	– dissolved oxygen
DW	– distilled water
EDTA	– ethylene dinitro tetraacetic acid
ERMs	– environmentally relevant microorganisms
FAO	– Food and Agriculture Organisation
GEMs	– genetically engineered microorganisms
h	– hour
kDa	– kilo dalton
LD_{50}	– lethal dose 50

mmt	– million metric tonne
mt	– metric tonne
OD	– optical density
PCR	– polymerase chain reaction
ppt	- parts per thousand
RAPD	– random amplified polymorphic DNA
16S rRNA	- 16 subunit ribosomal ribose nucleic acid
S.E.	– standard error
TBE	– tris boric acid EDTA
TCBS	– thiosulphate citrate bile salt sucrose
TPC	– total plate count
TSA	– trypticase soy agar
TSB	– trypticase soy broth
UPM	– Universiti Putra Malaysia
USD	– Dollar of United States of America
VaM11	– *Vibrio alginolyticus* Malaysia 11
VaM12	– *Vibrio alginolyticus* Malaysia 12
VaT	– *Vibrio algninolyticus* Thailand
VhI	– *Vibrio harveyi* Indonesia
VpI	– *Vibrio paraheamolyticus* Indonesia
VpM1	– *Vibrio parahaemolyticus* Malaysia 1
VpM3	– *Vibrio parahaemolyticus* Malaysia 3
VpM6	– *Vibrio parahaemolyticus* Malaysia 6
VpT	– *Vibrio parahaemolyticus* Thailand

CHAPTER 1

GENERAL INTRODUCTION

1.1 Background and Scope of the Study

In recent decades aquaculture has become a major food production industry helping to meet the increasing demand for food. The world population has crossed six billion during the year 2000 (Census Bureau, 2000) causing an increase in demand for food. Therefore, food production by agriculture and capture fisheries has to be supplemented by other alternatives. Total world fish and shellfish production has reached 126.17 million metric tonnes (mmt) in 1999 (FAO, 2000) of which, 33.31 mmt come from aquaculture.

Dehadrai (1993) predicted that aquaculture has to fill the gap of 19.6 mmt by 2000, 37.5 mmt by 2010 and 62.54 mmt by 2020. Among various aquaculture practices, shrimp culture is gaining increasing importance world-wide due to the short period of culture and high profits. Even though shrimp farming has grown to become a booming export oriented industry, shrimp production and trade have undergone fluctuations during the past four years indicating uncertainty over its sustainability in days to come due to different social and environmental problems.

The shrimp industry has to develop strategies to tackle the viral disease problems, which have disrupted shrimp farming. Generally in shrimp farming, the ultimate goal is to maximise the production with high stocking density, increase feeding and increase water exchange often accompanied with heavy use of chemicals. This has

caused various other problems like disease outbreaks, environmental pollution and other socio-economic fall out (Primavera, 1994). The shrimp industry is now looking for ways to rebuild itself and aims at a long-term sustainability that will not pollute the environment or minimise pollution.

The incidence and severity of several infectious diseases largely depends upon the quality of environment in which the host lives. Outbreak of diseases might be avoided by maintaining a healthy environment through suitable water quality management practices. Healthy environment can also be achieved by stocking at optimum density, providing sufficient water exchange and aeration (Wang *et al.*, 1999a). In the past, water exchange was the only solution widely practised to get rid of the accumulated wastes but recent viral disease outbreak has limited this practice. The recent practice of using closed or semi-closed system with heavy chlorination to reduce introduction of viral infection is not popular due to the extra cost involved. Reducing stocking density and feeding rate to minimise the accumulation of organic matter has also indirectly decreased the rate of production thus reducing the profit margin (Phillips *et al.*, 1993). Frequent water exchange to flush out shrimp pond effluent eutrophicates the coastal environment.

The ultimate strategy is to manipulate the pond environment by enhancing the mineralisation process in culture ponds so as to maintain a healthy environment and minimise the nutrient load to reduce pollution before disposing the effluents (Phillips *et al.*, 1993). Several management practices have been adopted for maintaining optimal water and sediment quality like the use of chemicals, physical methods like bottom