UNIVERSITI PUTRA MALAYSIA

ASSESSMENT OF PUTATIVE BACTERIA AS PROBIOTICS IN JUVENILE FRESHWATER PRAWN, MACROBRACHIUM ROSENBERGII (DE MAN)

MEHRAN AVAKH KEYSAMI.

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By

MEHRAN AVAKH KEYSAMI

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

June 2006

DEDICATION

To my most beloved wife, Roya,
for all her understanding, patience and support during all difficulties and for her technical help during my study

To my lovely daughter, Romina,
for making every thing worthwhile

To my dearest parents,
for their true love, constant trust, principle guide and encouragement since my childhood

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

ASSESSMENT OF PUTATIVE BACTERIA AS PROBIOTICS IN JUVENILE FRESHWATER PRAWN, *Macrobrachium rosenbergii* (de Man)

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Chairman: Associate Professor Che Roos Saad, PhD

Faculty : Agriculture

A study focused on the use of putative bacteria as probiotics to reduce nutritional and disease problems in aquaculture industry was carried out. This study was conducted in 8 experiments to investigate juvenile *M. rosenbergii* putative bacterial flora as probiotics, for enhancement of growth and survival of juvenile *M. rosenbergii* during November 2002 to August 2005 at University Putra Malaysia.

Bacterial counts and the putative bacterial flora in juvenile *M. rosenbergii* cultivation (0.1-9.0 g), were investigated and the isolated strains were classified to genera. Total viable cell counts were $96\pm2.6\times10^4$ CFU/ml for culture water, $3100\pm169.0\times10^4$ CFU/ml for culture tank sediment, $2100\pm143.0\times10^4$ CFU/ml for prawn body surface, $14000\pm4.6\times10^4$ CFU/g for prawn digestive tracts and $5.6\pm2.8\times10^4$ CFU/g for prawn abdominal muscle. Altogether, 12 genera were identified from water, sediment and different organs of prawn. The most frequently isolated ones were *Bacillus*, *Aeromonas* and *Pseudomonas.*

The three groups of putative bacteria (*Bacillus subtilis, Micrococcus luteus and Corynebacterium ammoniagenes*), collectively showed antagonistic activity against the six pathogens in antibacterial activity test. *B. subtilis* showed greatest inhibition of pathogens in well and disc diffusion assays and cross-streak method. Addition of 10 ml of cell free supernatant of *B. subtilis* to pathogens resulted in complete suppression of it within 12 h. Maximum growth and maximum zone of inhibition against *A. hydrophila* was observed at 30 ^{o}C and at pH 8 with 1% NaCl, respectively.

A ranking index (RI) was calculated to screen potential aquaculture probiont. The RI is based on the doubling time (td) and lag period (λ) obtained from the growth profile of each bacterium. Three candidates (*B. subtilis, M. luteus and C. ammoniagenes*) and two pathogens (*A. hydrophila and Pseudomonas aeroginosa*) were grown in TSB broth and absorbance recorded at each 0.5 h during 24 h period. The *B. subtilis* grew equally as well as the pathogens and candidate *B. subtilis* had a faster specific growth rate (μ=0.034) than the other candidates, *M. luteus* (0.031) and *C. ammoniagenes* (0.032) respectively.

Sensitivity of juvenile prawns to two putative bacteria strains (*B. subtilis* and *A. hydrophila*) was examined using *in vitro* and *in vivo* pathogenicity challenge test. *In vitro* challenge test was done on juvenile prawn (0.1 g) using the immersion method. An *in vivo* challenge experiment was carried out using the feeding trial method. The result of *in vitro* and *in vivo*

pathogenicity tests indicated that the survival of juvenile (0.1 and 0.45 g) was not affected by the presence of *B. subtilis* in fresh water but, *A. hydrophila* infected juvenile *M. rosenbergii* and cause about 78.67% mortality and exhibited disease clinical signs.

A feeding trial was carried out to investigate the potential probiotic properties of *B. subtilis* and its suitable level in juvenile *M. rosenbergii*. Putative *B. subtilis* bacterium was added to commercial prawn feed as a probiotic. Six types of diets were prepared by mixing the prawn feed with *Bacillus* form in the ratio of 1:1, 2:1, 3:1, 4:1 and 5:1, with an unsupplemented control. After 60 days, the prawns fed diet at level 3:1, showed a higher mean weight gain (593.45g) or 170.29% increase in growth over control. Clearly, feed treated with *B. subtilis* appeared to enhance growth and survival of *M. rosenbergii* at a ratio of 3:1.

Another feeding trial was carried out to investigate the suitable methods of administration of *B. subtilis* to the commercial feed in juvenile *M. rosenbergii* culture. Four types of diets were prepared by mixing the prawn feed to *Bacillus* form in the ratio of 3:1 with an unsupplemented as a control. The different methods used were blending, soaking, spraying and bathing. After 60 days, the prawns fed diet under soaking method treated group, showed a higher mean weight gain (1.49 g) or 142.28% increase in growth over control.

To investigate the potential probiotic-ability of *B. subtilis* to combat the freshwater prawn diseases problem, *B. subtilis* bacterium was added to

prawn feed as a probiotic at a level of 10^{10} CFU/g feed by soaking method. Sixty days after the start of the *B. subtilis* feeding, the prawns were challenged by bath exposure to *A. hydrophila* (10^7 CFU/ml). After 28 days of post challenge, *B. subtilis* treated group had 88.33% survival, whereas the control group had only 20.83% survival, with an unhealthy external appearance.

After then a feeding experiment was conducted for juvenile *M. rosenbergii* (0.037 g) using three types of diets prepared with the incorporation of putative *B. subtilis*, non-putative *B. subtilis* and a commercial probiotic at level of 10^{10} (CFU/g) with an unsupplemented control. After 60 days the prawns fed putative *B. subtilis* added diets showed a higher suvival, mean weight gain (0.663± 0.017g) with 0.267 g increase in growth over control and best FCR value (2.33 ± 0.02 g).

Finally, the probiotic-ability of the *B. subtilis* was evident from properties consisting dominant isolated one, greatest antagonistic activity against pathogenic agents, highest RI, non-pathogenic to the host, growth enhancement, health improvement and more effectiveness than non-putative and commercial probiotics. This study demonstrated that among putative bacterial flora of juvenile *M. rosenbergii*, *B. subtilis* can be a promising probiotic for this prawn.

Abstrak tesis yang dikemukakan kepada senat Universiti Putra Malaysia sebagai memenuhi syarat untuk mendapatkan ijazah Doktor Falasafah.

PENILAIAN POTENSI BAKTERIA SEBAGAI PROBIOTIK DALAM UDANG GALAH JUVENIL, *Macrobrachium rosenbergii* (de Man)

Oleh

MEHRAN AVAKH KEYSAMI

Jun 2006

Pengerusi : Professor Madya Che Roos Saad, PhD

Fakulti : Pertanian

Satu kajian yang difokuskan kepada penggunaan probiotik untuk menyelesaikan masalah pemakanan dan penyakit dalam industri akuakultur telah dilakukan. Dalam kajian ini, sebanyak lapan eksperimen dilakukan untuk mengenalpasti flora bakteria daripada udang juvenil *M. rosenbergii* sebagai probiotik untuk meningkatkan pertumbuhan dan kemandirian udang untuk hidup, dari November 2002 hingga Ogos 2005 di Universiti Putra Malaysia.

Pengiraan bakteria dan flora bakteria putatif dalam ternakan udang juvenil *M. rosenbergii* (0.1 - 9.0 g) telah ditentukan dan jenis udang yang diasingkan kemudiannya dikelaskan kepada genera. Jumlah pengiraan sel hidup adalah sebanyak $96\pm2.6\times10^4$ CFU/ml bagi air kultur, $3100 \pm 169.0\times10^4$ CFU/ml bagi mendakan dalam tangki kultur, $2100\pm143.0\times10^4$ CFU/ml bagi permukaan badan udang, $14{,}000\pm4.6\times10^4$ CFU/g bagi salur pencernaan udang dan sebanyak $5.6\pm2.8\times10^4$ CFU/g bagi otot abdomen pada udang. Secara keseluruhannya, terdapat 12 genera telah dikesan daripada air, mendakan

dan organ-organ udang yang berbeza. Bakteria yang paling kerap diasingkan adalah daripada jenis *Bacillus*, *Aeromonas* dan *Pseudomonas.*

Sebanyak tiga kumpulan bakteria (*Bacillus subtilis*, *Micrococcus luteus* dan *Corynebacterium ammoniagenes*), telah menunjukkan aktiviti antagonis terhadap enam jenis patogen dalam ujian aktiviti antibakteria. *B. Subtiis* telah menunjukkan perencatan patogen yang tertinggi dalam kaedah telaga dan assei resapan cakera serta kaedah coretan lintang. Penambahan sebanyak 10 ml supernatan *B. subtilis* tanpa sel kepada patogen menyebabkan perencatan patogen dalam tempoh 12 jam. Pertumbuhan maksimum dan zon maksimum perencatan terhadap *A. hydrophila* diperhatikan pada 30 °C dan pada pH 8 dengan 1% NaCl, masing-masing.

Indeks kedudukan (RI) telah dikira sebagai saringan kepada probiont akuakultur yang berpotensi. RI dikira berdasarkan masa penggandaan (td) dan tempoh lanjut (λ) yang diperolehi daripada profil pertumbuhan bagi setiap bakterium. Tiga jenis bakteria (*B. subtilis, C. ammoniagenes dan M. luteus*) dan 2 jenis patogen (*A. hydrophila* dan *P. aeroginosa*) telah dikulturkan dalam agar TSB dan bacaan penyerapan (absorbans) direkodkan setiap setengah jam dalam tempoh masa 24 jam. Bakteria jenis *B. subtilis* membiak sama banyak dengan patogen-patogen dan mempunyai kadar pertumbuhan spesifik lebih tinggi (μ=0.034) berbanding spesis bakteria yang lain iaitu *M. luteus* (0.031) dan *C. ammoniagenes* (0.032) masing-masing.

Tahap sensitiviti udang juvenil kepada dua jenis bakteria putatif (*B. subtilis* dan *A. hydrophila*) telah ditentukan menggunakan ujian cabaran patogenisiti secara *in vitro* dan *in vivo*. Ujian *in vitro* telah dijalankan ke atas udang juvenil (0.1 g) menggunakan kaedah rendaman. Sementara ujian *in vivo* pula telah dijalankan menggunakan kaedah percubaan pemakanan. Hasil kedua-dua ujian menunjukkan bahawa kadar kebolehan udang juvenil untuk hidup (0.1 dan 0.45g) tidak dipengaruhi oleh kehadiran Bacillus dalam air tawar tetapi, *A. hydrophila* menyebabkan jangkitan kepada udang juvenil *M. rosenbergii* dan menyebabkan sebanyak 78.67% kematian dan menunjukkan ciri-ciri klinikal penyakit-penyakit tertentu.

Satu percubaan pemakanan telah dijalankan untuk menentukan potensi bakteria *B. subtilis* sebagai probiotik dan tahap yang sesuai dalam udang juvenil *M. rosenbergii*. Bakteria *B. subtilis* putatif telah ditambahkan ke dalam makanan udang komersil sebagai probiotik. Sebanyak enam jenis diet telah disediakan dengan mencampurkan makanan udang kepada *Bacillus* dengan nisbah 1:1, 2:1, 3:1, 4:1 dan 5:1, dan satu kumpulan kawalan. Selepas 60 hari, udang yang diberi makan diet dengan nisbah 3:1, telah menunjukkan peningkatan berat yang lebih tinggi (593.45 g) atau peningkatan pertumbuhan sebanyak 170.29% berbanding kumpulan kawalan. Dengan ini, didapati probiotik yang dirawat dengan *B. subtilis* dapat meningkatkan kadar pertumbuhan dan kebolehan udang juvenil *M. rosenbergii* untuk hidup pada nisbah 3:1.

Satu lagi percubaan pemakanan juga telah dijalankan untuk mengenalpasti kaedah yang paling sesuai untuk pemberian *B. subtilis* dalam makanan komersil untuk pengkulturan udang juvenil *M. rosenbergii*. Empat jenis diet telah disediakan dengan mencampurkan makanan udang dan bakteria *Bacillus* dengan nisbah 3:1 dengan satu kumpulan kawalan (tanpa campuran bacteria). Kaedah-kaedah yang dilakukan ialah penggaulan, rendaman dan penyemburan. Selepas 60 hari, kumpulan udang yang diberi makan diet yang disediakan dengan kaedah rendaman telah menunjukkan min berat tertinggi (1.49 g) atau peningkatan pertumbuhan sebanyak 142.28% berbanding kumpulan kawalan.

Untuk mengenalpasti potensi probiotik *B. subtilis* bagi mengatasi penyakit udang air tawar, bakteria *B. subtilis* telah ditambahkan kepada makanan udang sebagai probiotik pada tahap 10^{10} CFU/g makanan udang menggunakan kaedah rendaman. Enam puluh hari selepas pemberian makanan rawatan, kumpulan udang tersebut telah didedahkan kepada *A. hydrophila* (10^7 CFU/ml) dalam rendaman air. Selepas 28 hari, kumpulan udang yang dirawat dengan probiotik mempunyai 88.33% kemandirian untuk hidup, sementara kumpulan kawalan hanya mempunyai 20.83% kemandirian untuk hidup, dengan rupa luaran yang tidak sihat (sempurna).

Kajian selanjutnya telah dijalankan ke atas kumpulan udang juvenil *M. rosenbergii* (0.037g) dengan memberi makan tiga jenis diet yang disediakan menggunakan *B. subtilis* putatif, *B. subtilis* bukan-putatif dan probiotik komersil pada tahap 10^{10} (CFU/g) berbanding kumpulan kawalan. Selepas

60 hari, udang yang diberi makan diet bercampur *B. subtilis* putatif menunjukkan min pertambahan berat udang yang lebih tinggi (0.663± 0.017g) dan peningkatan pertumbuhan sebanyak 0.267 g berbanding kumpulan kawalan, di samping mempunyai nilai FCR yang terbaik (2.33± 0.02).

Sebagai kesimpulannya, potensi probiotik bagi bakteria *B. subtilis* telah dibuktikan berdasarkan bahan dominan yang diasingkan, kadar aktiviti antagonistik yang tinggi terhadap ejen patogenik, nilai RI yang tinggi, tidak mendatangkan penyakit kepada hos, peningkatan pertumbuhan dan kesihatan serta lebih efektif berbanding bakteria bukan putatif dan probiotik komersil. Kajian ini menunjukkan bahawa bakteria *B. subtilis* berpotensi sebagai probiotik dikalangan flora-flora bakteria juvenil *M. rosenbergii* yang lain.

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- Above all, to GOD almighty for making this study possible.

I certify that an Examination Committee has met on 12th June 2006 to conduct the final examination of Mehran Avakh Keysami on his Doctor of Philosophy thesis entitled "Assessment of Putative Bacteria as Probiotics in Juvenile Freshwater Prawn, *Macrobrachium rosenbergii* (de Man)" 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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Date : 11 JUL 2006

This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee are as follows:

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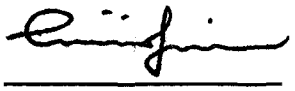

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Date 10 AUG 2006

DECLARATION

I hereby declare that this 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.

MEHRAN AVAKH KEYSAMI

Date: 10/7/06

Keysami

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