



Disease resistance of Pacific white shrimp, *Litopenaeus vannamei*, following the dietary administration of a yeast culture food supplement

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Abstract

A yeast culture feed supplement (Diamond V XP Yeast Culture®, Diamond V Mills, Cedar Rapids, Iowa [IA]) was assessed for its impact on disease resistance in the Pacific white shrimp, *Litopenaeus vannamei*. Animals were fed a standard shrimp pellet diet supplemented with 0% (control with 1% grain carrier), 0.5% (with 0.5% carrier), or 1.0% XP daily for 4 weeks. To assess resistance to bacterial disease, at 1-week intervals 21 shrimp (0.5–2.5 g) from each test diet were injected intramuscularly with an LD₅₀ dose (2.0×10^5 /g body weight) of a gram-negative shrimp pathogen, *Vibrio* sp. 90-69B3. Survival was monitored every 4 h for 48-h post injection. Each week, three independent bacterial challenges were performed for each diet and the results expressed as the mean percent survival \pm standard error (S.E.). A two-way analysis of variance (ANOVA) showed a significant effect of diet ($p=0.003$, $df=31$), but not duration of feeding, on survival. A one-way ANOVA showed no differences among the treatment groups after 1 or 2 weeks. After 3 weeks, the mean survival of 1% XP-fed shrimp ($74.2 \pm 1.4\%$) was significantly higher than that of controls ($42.9 \pm 5.5\%$), while mean survival of shrimp fed 0.5% XP ($54.8 \pm 11.9\%$) was not significantly different from controls. After 4 weeks, mean survival of 1.0% XP-fed shrimp ($63.4 \pm 8.8\%$) remained higher than that of controls but the difference was not significant ($p=0.07$). An insufficient number of animals were available from the 0.5% XP-fed group to perform bacterial challenges at this timepoint. Mean survival of control shrimp declined significantly over the 4 weeks of study (slope of linear regression $\neq 0$, $p=0.005$, $df=11$), but no decline was observed in animals fed the 0.5% or 1.0% XP diets. After 4 weeks *L. vannamei* fed standard shrimp pellets, 0% XP control, or 1.0% XP diets showed no significant differences in weight, suggesting that the changes in disease resistance did not correlate with changes in growth rate among the treatment groups. These

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results indicate that dietary administration of Diamond V XP Yeast Culture® can protect shrimp against a decline in resistance to bacterial disease.

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1. Introduction

Productivity in aquaculture ponds is directly related to the growth rate and the survival of animals stocked into the culture system. Losses attributed to bacterial, viral and fungal diseases remain an important concern. Immunostimulants have been reported to increase resistance to these infectious diseases in teleost fish and shellfish (Raa, 1996; Sakai, 1999) by enhancing the nonspecific immune system, the set of defenses directed against all potentially invasive, disease-causing organisms. Two types of immunostimulants have received the most attention in shrimp aquaculture (1) fragments of bacterial cell walls, such as lipopolysaccharide (LPS), and (2) beta-glucans from one of several fungal or algal species (Raa, 1996). Dietary exposure to *Schizophyllum commune* glucan significantly increased resistance against white spot syndrome virus (WSSV) infection in *Penaeus monodon* post-larvae (15-day exposure) and juveniles (20-day exposure) (Chang et al., 1999) and against *Vibrio* sp. NU-1 in *Penaeus japonicus* (7-day exposure, Itami et al., 1994).

Despite these favorable reports, immunostimulants have not yet been incorporated into routine aquaculture of shrimp. Raa (1996) cited several concerns of large-scale producers, including conflicting reports regarding the dose, timing and frequency of administration. Matsuo and Miyazono (1993) reported that doses of peptidoglycan (PG) from the gram-positive bacterium *Bifidobacterium thermophilum* protected adult rainbow trout from vibriosis but reduced the resistance of juvenile fish to infection with *Vibrio* sp. Sung et al. (1994) found that the immersion of *P. monodon* in high concentrations of glucan (>1 mg/ml) had adverse effects on shrimp, causing tissue damage and decreasing resistance to *Vibrio* infection. In addition, Scholz et al. (1999) reported that a diet of 0.1% glucan over 7 weeks significantly reduced the ability of shrimp to clear live *Vibrio harveyi* from the hemolymph as compared to controls or to animals reared on diets containing 1% yeast, *Saccharomyces cerevisiae*.

With the exception of Itami et al. (1994) and Scholz et al. (1999), relatively little information is available regarding the performance of shrimp maintained on a diet supplemented with yeast. In the present study, we tested whether a dried yeast culture supplement (Diamond V XP Yeast Culture®, Diamond V Mills, Cedar Rapids, IA) might be used as an immunostimulant in the Pacific white shrimp, *Litopenaeus vannamei*.

2. Materials and methods

2.1. Diets

To produce XP Yeast Culture®, aerobically fermented *S. cerevisiae* and its molasses-based medium were mixed with cereal grains (ground yellow corn, hominy feed, corn

gluten feed, wheat middlings and rye middlings) to form a slurry and fermented a second time under anaerobic conditions. After drying, the mixture was ground to produce the desired final consistency of particle size. The grain carrier used for control diets consisted of the same cereal mixture in the same proportion as that used in the production of the XP.

Experimental diets were produced by combining commercial shrimp pellets (Rangen 35/2.5) with powdered XP and/or grain carrier and 50% gelatin (Knox) to produce three diets: (1) 1% XP, (2) 0.5% XP (containing 0.5% XP + 0.5% carrier), or (3) 0% Control XP (containing 1% carrier). Dry components were ground to a powder using a mortar and pestle. Gelatin (Knox), dissolved at 60 µg/ml in boiling water, was cooled to 25 °C and mixed at 50% v/w with the powdered diet. The resulting paste was spread evenly on aluminum foil and cooled at 4 °C until solid. Pellets were then cut to approximately 0.5 cm³ cubes and stored in sealed plastic bags at 4 °C for no longer than 7 days prior to use.

2.2. Disease resistance trials

Post-larvae of *L. vannamei* (Kona High Health Stock, High Health Aquaculture, Kona, HI) were grown to approximately 1 g juvenile stage animals at the Waddell Mariculture Center, Bluffton, South Carolina (SC). These juveniles were transported to the Grice Marine Laboratory, Charleston, SC, where they were placed in recirculating seawater at 30-ppt salinity and 23–26 °C throughout the experiments. Three days after transport, shrimp were placed on one of three experimental diets: 0% XP Control, 0.5% XP or 1% XP. Animals were fed ad lib one time each day. When shrimp had finished feeding (after 2 to 3 h), uneaten food was removed from the tanks. Ammonia, pH and temperature of the water were monitored daily and partial water exchanges were conducted as needed based on these measurements. After 1, 2, 3 and 4 weeks on the experimental feeds, animals from each group were tested for disease resistance using a bacterial challenge model.

Each challenge test employed 21 shrimp that were injected intramuscularly in the third abdominal segment with live *Vibrio* sp. 90-69B3 (kindly provided by D. Lightner and L. Mahone, University of Arizona). The 16S rRNA sequence of this strain places it in the *Vibrio parahaemolyticus/V. harveyi* family (unpublished, Eric Stabb, University of Georgia). After 1, 2 and 4 weeks on the experimental diets, shrimp weighing from 0.5 to 1.5 g (average approximately 1.0 g) were injected with 5 µl of 90-69B3 at a concentration of 4×10^7 cells/ml, based on an optical density (OD) value of 0.1 at 540 nm being equal to 1×10^8 bacteria/ml (Mikulski et al., 2000). Larger animals (1.5–3.0 g, average approximately 2.0 g) that were challenged after 3 weeks on the test diets were injected with 10 µl of 90-69B3 at 4×10^7 cells/ml, so that all animals in the study were challenged with approximately 2×10^5 bacteria per g body weight.

Following bacterial challenge, shrimp were held in a 19-l tank equipped with a recirculating biological filter. Survival was monitored every 4 h for 48 h. Ammonia levels were monitored and not allowed to exceed 1.0 mg/ml, although most readings were below 0.25 mg/ml. Groups of 21 animals from each of the three diet groups were challenged simultaneously using a single preparation of 90-69B3. Independent replicates with fresh bacterial preparations were performed on days 8, 9, 10 (designated week 1), 15, 16, 17 (designated week 2), 23, 24, 25 (designated week 3), 33 and twice on day 34 (designated week 4) with one exception. Animals from the 0.5% diet group were included in weeks 1,

2 and 3 but not in week 4 challenges because there were not enough remaining animals of appropriate size.

2.3. Growth trials

To compare the effects of diets on growth rate, 1 g juvenile stage animals with the same history as those used in the disease resistance trials were placed in recirculating seawater tanks at 30 ppt and 23–26 °C. Groups of 25–27 shrimp were placed in 76-l tanks equipped with recirculating biofilters and maintained on standard shrimp pellets (Rangen 35/2.5) or one of two experimental diets: 1% XP and 0% XP Control. Animals were fed ad lib daily and tanks maintained as described above. Individual shrimp were weighed each week for 4 weeks.

2.4. Statistical methods

A two-way Model I ANOVA was used to distinguish the effects of diet and duration of feeding on 48-h survival and on growth over the 4 weeks of study, while one-way ANOVAs were performed on 48-h survival data from each week. Post hoc pairwise multiple comparisons were conducted on significant effects using the Holm–Sidak method. Individual regressions were run on survival data within each diet group to assess changes over the feeding period. For all statistical tests, a p value of 0.05 was used to determine significance.

3. Results

For animals on all diets, mortalities following bacterial challenge began to occur at 8- to 12-h post injection and ceased by 48-h post injection (Fig. 1). Two-way ANOVA showed a significant effect of diet ($p=0.003$, $df=31$) but not duration of feeding ($p=0.193$, $df=31$) on 48-h survival. Post hoc pairwise multiple comparisons on the effect of diet showed significant differences in survival between animals on 1% XP and 0.5%XP ($p=0.048$) and between 1% XP and 0% XP Control diets ($p<0.001$).

After 1 or 2 weeks on the test diets, there were no significant differences in mean survival at 48 h following bacterial challenge. After 3 weeks, mean survival of *L. vannamei* fed 1.0% XP was significantly higher than that of control shrimp (Fig. 2, one-way ANOVA; $p=0.029$, $df=7$). Survivals after 48 h for 1% XP-fed shrimp in the three replicate trials were 71%, 75% and 76% (mean $74.2 \pm 1.4\%$ S.E.) as compared to 33%, 42% and 52% (mean $42.9 \pm 5.5\%$ S.E.) for controls. There was no significant difference in 48-h survival between shrimp fed 0.5% XP and 0% XP Control diets. After 4 weeks (Fig. 2), 48-h survivals of 1.0% XP-fed *L. vannamei* (52, 57, 81%) were higher than those of controls (29%, 35%, 48%), but the difference was not significant (one-way ANOVA; $p=0.065$, $df=5$). An insufficient number of animals from the 0.5% XP diet group were available for testing after 4 weeks. Within the 0.5% and 1.0% XP diet groups, 48-h survival did not change significantly over the 4 weeks of the feeding trial (Fig. 2; slope of linear regression $\neq 0$, $p=0.438$, $df=7$ and $p=0.922$, $df=11$, respectively). By

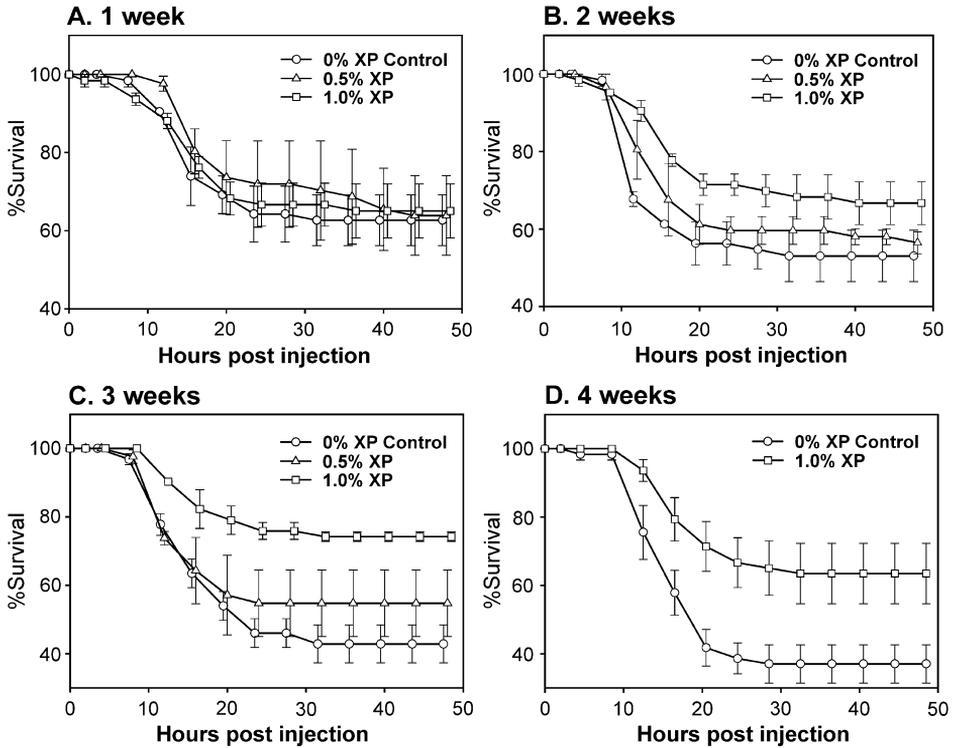


Fig. 1. Survival time course of *L. vannamei* fed one of three test diets and challenged with *Vibrio* sp. 90-69B3. Mean survivals \pm standard error (S.E.) ($n=3$, except C. 0.5% XP, $n=2$) are given at 4-h intervals over 48 h following bacterial challenge for each diet group on (A) week 1, (B) week 2, (C) week 3, (D) week 4.

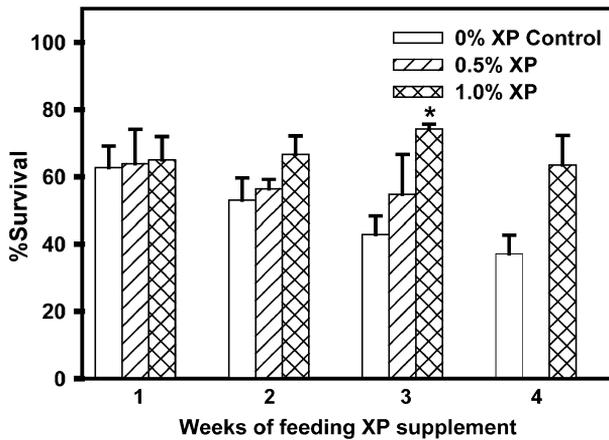


Fig. 2. Summary of 48-h survivals of *L. vannamei* after injection with *Vibrio* sp. 90-69B3. Values are mean survivals \pm S.E. ($n=3$, except 0.5% at 3 weeks $n=2$). (*) *L. vannamei* fed 1.0% XP had a significantly higher overall survival than control (0% XP) shrimp after 3 weeks of feeding.

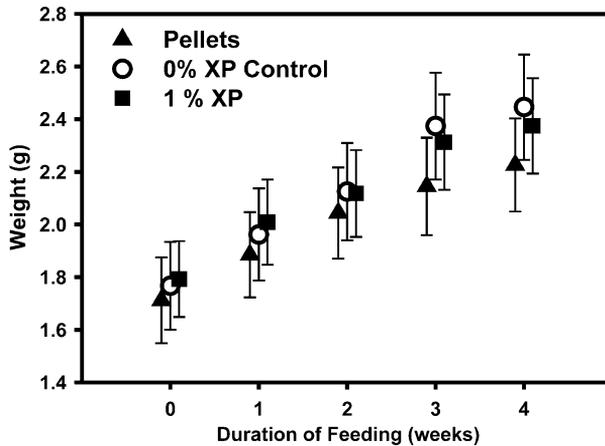


Fig. 3. Growth of *L. vannamei* ($n=25-27$) fed daily with standard shrimp pellets, 0% XP diet, or 1.0% XP diet for 4 weeks. Shrimp were weighed each week and mean weights \pm S.E. are presented. There were no significant differences among the weights of the three diet groups at any timepoint tested.

comparison, the survival of control shrimp decreased over the 4-week trial (Fig. 2; slope of linear regression $p=0.005$, $df=11$). There were no significant differences in the mean weights of animals among the three diet groups that were challenged in each week's trials, nor were there any significant differences among the average weights of animals used from week to week, with the exception of week 3 as described in Materials and methods (data not shown). Furthermore, there were no significant differences in the growth of animals maintained on 0% XP Control, 1% XP or the standard shrimp pellet diet (Fig. 3).

4. Discussion

The level of survival in response to bacterial challenge in *L. vannamei* remained constant in animals fed the 1% XP supplement, while survival declined in control shrimp fed only the grain-based carrier. These results are consistent with those of Su et al. (1995) and Chang et al. (2000) who observed greater viability in *P. monodon* fed a relatively low oral dose (0.2%) of beta-glucan from *S. commune* over 30 or 40 days. Similarly, combined immersion and dietary exposure to β -1,3-1,6-glucan from cell walls of the yeast *S. cerevisiae* for 51 days enhanced resistance of juvenile *P. monodon* to WSSV infection (Song et al., 1997). In contrast, the potentially negative effects associated with long-term use of immunostimulants (Matsuo and Miyazono, 1993; Sung et al., 1994) have led other investigators to recommend their periodic administration. Itami et al. (1998) fed PG to *P. monodon* at 0.2% of diet for seven consecutive days alternated with 7 days without the immunostimulant. These workers reported improved resistance to *Vibrio penaeicida* at 65 and 95 days in PG-fed animals as compared to controls.

The present study did not address the mechanism by which the dried yeast culture afforded protection. It is possible that the yeast-supplemented diet provided more optimal

nutrition than the control diet, but this was not reflected in the growth rates. The protective effect of the yeast supplement might also be attributed to its glucan content. Glucans are reported to enhance disease resistance by stimulating nonspecific components of the immune system or by improving processing and presentation of antigens during specific adaptive immune responses. For example, the protective effects of beta-glucan in crustaceans have been associated with activation of the prophenoloxidase system inducing antimicrobial activity in plasma and enhancing phagocytosis, cell adhesion and superoxide production in hemocytes (Song and Hsieh, 1994; Itami et al., 1994; Sung et al., 1996; Chang et al., 2000; Campa-Cordova et al., 2002).

Shrimp fed the control diet exhibited a decline in resistance to bacterial challenge. The reasons for this decline are not clear but may be attributed to one or more of the following interdependent factors. First, juvenile shrimp used in these studies came from closed recirculating production raceways that supported a dense microbial flora. These exposures might enhance the basal activity of the immune system in crustaceans. Once transferred to laboratory tanks with filtered seawater, test animals received much lower environmental and dietary exposures to bacteria, fungi or algae. With reduced exposure to activation signals from environmental or dietary microbes, the immune system of crustaceans may enter into a lower state of readiness. This phenomenon, called immunosuppression, has been documented in the purple sea urchin *Strongylocentrotus purpuratus* maintained for 18 months in UV-sterilized, artificial seawater on a diet of dried kelp (Gross et al., 1999, 2000). The yeast supplement might replace the absent microbial flora to maintain the activated state of immunological protection in the shrimp. Second, the laboratory conditions under which this study was conducted might have been stressful, leading to immunosuppression by any one of a number of mechanisms that have been suggested in the scientific literature. In this case, the yeast supplement may have protected against the negative effects of stress on the immune system. Finally, microbial flora in production raceways or ponds might normally serve as a nutritional supplement to the basal diet. Once transferred to filtered seawater, shrimp on the basal diet alone might have lost that component of dietary nutrition leading to a general decline in animal health. The yeast additive may have replaced the missing component of dietary nutrition. Regardless of the underlying reason(s) for its occurrence, this temporal decline in disease resistance among control shrimp maintained under laboratory conditions should be considered in evaluating the efficacy of immunostimulants.

Further laboratory studies as well as field trials are required to confirm the efficacy of Diamond V XP Yeast Culture®. Caution always must be taken in translating laboratory results to farm application. Nonetheless, it is clear that feed supplements that are readily available in large-scale quantities and have the capacity to adapt to high salt concentrations (Adler, 1994) deserve additional study as immunostimulants in aquaculture.

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References

- Adler, L., 1994. Response of the yeast *Saccharomyces cerevisiae* to salt stress. 3rd International Marine Biotechnology Conference, Program Abstracts. International Advisory Comm. of the Int. Marine Biotechnology Conference. Tromsø, Norway, p. 46.
- Campa-Cordova, A.I., Hernandez-Saaverdra, N.Y., DePhilippis, R., Ascencio, R., 2002. Generation of superoxide anion and SOD activity in haemocytes and muscle of American white shrimp (*Litopenaeus vannamei*) as a response to β -glucan and sulphated polysaccharide. *Fish Shellfish Immunol.* 353–366.
- Chang, C.F., Su, M.S., Chen, H.Y., Lo, C.F., Kou, G.H., Liao, I.C., 1999. Effect of dietary beta-1,3-glucan on resistance to white spot syndrome virus (WSSV) in postlarval and juvenile *Penaeus monodon*. *Dis. Aquat. Org.* 36, 163–168.
- Chang, C.-F., Chen, H.-Y., Su, M.-S., Liao, I.-C., 2000. Immunomodulation by dietary β -1,3 glucan in the brooders of the black tiger shrimp *Penaeus monodon*. *Fish Shellfish Immunol.* 10, 505–514.
- Gross, P.S., Al-Sharif, W.Z., Clow, L.A., Smith, L.C., 1999. Echinoderm immunity and the evolution of the complement system. *Dev. Comp. Immunol.* 23, 439–442.
- Gross, P.S., Clow, L.A., Smith, L.C., 2000. SpC3, the complement homologue from the purple sea urchin, *Strongylocentrotus purpuratus*, is expressed in two subpopulations of the phagocytic coelomocytes. *Immunogenetics* 51, 1034–1044.
- Itami, T., Takahashi, Y., Tsuchihira, E., Igusa, H., Konda, M., 1994. Enhancement of disease resistance of kuruma prawn *Penaeus japonicus* and increase in phagocytic activity of prawn hemocytes after oral administration of β -1,3-glucan (Schizophyllan). In: Chou, L.M., Munro, A.D., Lam, J.J., Chen, T.W., Cheong, L.K.K., Ding, J.K., Hooi, K.K., Khoo, H.W., Phang, V.P.E., Shim, K.F., Tan, C.H. (Eds.), *The Third Asian Fisheries Forum*. Asian Fisheries Society, Manila, Philippines, pp. 375–378.
- Itami, T., Asano, M., Tokushige, K., Kubono, K., Nakagawa, A., Takeno, N., Nishimura, H., Maeda, M., Kondo, M., Takahashi, Y., 1998. Enhancement of disease resistance of kuruma shrimp, *Penaeus japonicus*, after oral administration of peptidoglycan derived from *Bifidobacterium thermophilum*. *Aquaculture* 164, 277–288.
- Matsuo, K., Miyazono, I., 1993. The influence of long-term administration of peptidoglycan on disease resistance and growth of juvenile rainbow trout. *Nippon Suisan Gakkaishi* 59, 1377–1379.
- Mikulski, C.M., Burnett, L.E., Burnett, K.G., 2000. The effects of hypercapnic hypoxia on the survival of shrimp challenged with *Vibrio parahaemolyticus*. *J. Shellfish Res.* 19, 301–311.
- Raa, J., 1996. The use of immunostimulatory substances in fish and shellfish farming. *Reviews Fish. Sci.* 4, 288–299.
- Sakai, M., 1999. Current research status of fish immunostimulants. *Aquaculture* 172, 63–92.
- Scholze, U., Diaz, G.G., Ricque, D., Cruz Suarez, L.E.C., Albores, F.V., Latchford, J., 1999. Enhancement of vibriosis resistance in juvenile *Penaeus vannamei* by supplementation of diets with different yeast products. *Aquaculture* 176, 271–283.
- Song, Y.L., Hsieh, Y.T., 1994. Immunostimulation of tiger shrimp (*Penaeus monodon*) hemocytes for generation of microbicidal substances: analysis of reactive oxygen species. *Dev. Comp. Immunol.* 18, 102–209.
- Song, Y.L., Liu, J.J., Chan, L.C., Sung, H.H., 1997. Glucan-induced disease resistance in tiger shrimp (*Penaeus monodon*). *Fish Vaccinol.: Dev. Biol. Stand.* 90, 413–421.
- Su, M.S., Liu, K.F., Chang, C.F., Liao, I.C., 1995. Enhancement of grass prawn *Penaeus monodon* postlarvae viability by beta-1,3-glucan from *Schizophyllum commune*. *J. Taiwan Fish. Res.* 3, 125–132 (in Chinese with English abstract).
- Sung, H.H., Kou, G.H., Song, Y.L., 1994. Vibriosis resistance induced by glucan treatment in tiger shrimp (*Penaeus monodon*). *Fish Pathol.* 29, 11–17.
- Sung, H.H., Yang, Y.L., Song, Y.L., 1996. Enhancement of microbicidal activity in the tiger shrimp (*Penaeus monodon*) via immunostimulation. *J. Crustac. Biol.* 16, 278–284.