Heterotrophic Marine Bacteria as Supplementary Feed for Larval *Penaeus monodon*

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Introduction

Conventionally, penaeid shrimp larvae are reared by feeding microalgae (*Chaetoceros, Tetraselmis*, etc.) at the protozoea stage and *Artemia nauplii* during the mysis stage. Microalgal culture is technically demanding and expensive, representing 30 to 50% of hatchery operating costs (Jeffrey and Garland 1987) and several attempts have been made to find replacement feeds, with varying degrees of success (Jones et al. 1987). No effort has yet been directed to use bacterial biomass as a feed for larval shrimp.

Bacteria account for about 80% of the total 'bio-surface' in seawater and bacteriovory is widespread among a variety of the larvae of marine organisms (Azam et al. 1984). Intriago and Jones (1993) successfully used bacteria (*Flexibacter* sp.) as an exclusive diet for Artemia. The rotifer, *Brachionus plicatilis*, has also been grown with bacteria (Yasuda and Taga 1980; Gatesoupe et al. 1989).

The purpose of this study was to use autochthonously obtained, nonpathogenic heterotrophic marine bacteria as a substitute feed for microalgae in rearing larval *Penaeus monodon*.

Isolation and Identification of Bacteria

Water samples were collected aseptically from larval rearing tanks at two private shrimp hatcheries near Madras. After suitable dilution with sterile seawater, inocula were spread on Zobell 2216E agar plates. The colony and cell morphology of well isolated colonies were studied and identified by applying standard biochemical tests (Austin 1988).

All strains were checked for hemolytic properties using 5% sheep blood. Cultures were maintained by periodic subculturing and also as glycerol stocks (1 ml culture + 1 ml sterile glycerol). Eleven strains were isolated: *Micrococcus* (MCC), *Staphylococcus, Streptococcus, Bacillus* (two strains; BAC-1, BAC-2), *Pseudomonas* (two strains; PSM-1, PSM-2), *Vibrio parahemolyticus, V. fluviatilis, Moraxella* (MOR) and *Flavobacterium*. Six nonhemolytic strains were chosen for larval feed trials: BAC-1, BAC-2, PSM-1, PSM-2, MCC and MOR.

A single colony of each chosen strain was inoculated into 10-ml Zobell broth and kept in static condition. A 0.1% inoculum from this was used to grow the cells in 100-ml broth, kept in a orbital shaker at 200 rpm. Growth was monitored every two hours by measuring the optical density at 600 nm in a Hitachi U2000 spectrophotometer. The slope of the curve in mid-log phase was used to calculate the specific growth rate and the doubling time. Because vibrios are the major pathogens to shrimp larvae, the culture filtrate and lysed cells of all tested isolates were screened for

Table 1. Characteristics of seawater bacteria strains isolated for larval shrimp (*Penaeus monodon*) feeding trials. For explanation of abbreviations, see text.

Strain	Gram strain (±)	Shape	Size (µm)	Motility	Pigment	Doubling time (min)
BAC-1	+	Rods	0.5x3.5	-	-	58
PSM-1	-	Rods	0.5x2.0	+	-	63
PSM-2	-	Rods	0.5x3.0	+	-	32
BAC-2	+	Rods	0.5x2.5	-	-	30
MOR	-	Rods	0.75x1.0	-	Orange	144
MCC	+	Cocci	0.5-0.75	-	Yellow	74

vibriostatic properties by using the disc diffusion technique. *Vibrio alginolyticus* and *V. anguillarum* cultures were used for the assay.

The results of these culture experiments are summarized in Table 1. BAC-2 and PSM-2 were the fastest growing strains and MOR the slowest growing. None of the tested strains showed vibriostatic properties.

Proximate Analysis

For proximate analysis of strains, 1.5 ml of log-phase culture was centralized at 6 000 rpm and the pellets were washed with pH 7.4 phosphate-buffered saline (PBS). The pellets were dissolved in 200-µl lysis solution (10% sodium dodecyl sulphate, 1% mercaptoethanol), vortexed vigorously and kept in boiling water bath for 10 minutes. The cells were then sonicated four times at maximum wattage for 30 seconds each. From this solution, 10 µl aliquots were used for protein (Lowry et al. 1951), sugar (Miller 1959) and lipid (Folch et al. 1957) estimations. Moisture content was estimated by drying the cells to constant weight at 70°C.

Table 2 summarizes the results. PSM-2 had the highest protein content and MOR the lowest. Sugar content was broadly similar for all strains. MOR, PSM-1 and BAC-2 had relatively high lipid levels, compared to the others.

Larval Feed Trials

Unfed *Penaeus monodon* nauplius larvae (stage N_{5-6}), hatched at the Muttukadu hatchery of the Central Institute of Brackishwater Aquaculture near Madras were used for the study. Larvae from the same family were stocked at 100/l density in 1.5-l capacity clear

PVC beakers. Stored and settled seawater, filtered through a 10-µm filter bag, was used at 33-35 ppt salinity at a temperature of 28-31°C; and pH of 7.8. Gentle aeration was provided to each beaker through a plastic micropipette tip positioned in such a manner as to ensure uniform mixing.

For bacterial feed preparation, all six strains to be tested were grown in 250- or 1 000-ml Zobell broths in 1- or 5-l flasks, kept in a shaker at 200 rpm. Cells were harvested during their log-phase by centrifuging at 6 000

rpm. They were then washed twice with PBS and suspended in sterile seawater to make a final concentration of 10^8-10^9 colony forming units (CFU)/ml. The prepared biomass was stored at 4°C until use.

All experimental treatments were duplicated. Positive control larvae were fed exclusively with the non-chain forming diatom, *Chaetoceros calcitrans*, at a concentration of 10^5 cells/ml. Diatoms were cultured separately with nutrient enrichment and cultures in active growth phase were used. Diatom cell density was monitored by making replicate cell counts with a hemocytometer. All experimental treatments were fed daily and the total cell concentration was maintained at 10^5 cells/ml, except for the starved controls. About 90% of the seawater was exchanged daily to remove metabolic wastes. The larvae were counted and metamorphic stages noted every day. The experiment was terminated when larvae metamorphosed into postlarvae (P-1 stage). This was repeated for all six strains of bacteria. Details of the experimental treatments, with the concentrations of microalgae and bacteria, are given in Table 3.

Table 2. Proximate composition of marine bacteria; strains isolated for larval shrimp (*Penaeus monodon*), feeding trials. For explanation of abbreviations, see text.

Strain	Moisture (%)	Protein (mg/ml)	Reducing sugar (mg/ml)	Lipid (mg/ml)
BAC-1	80	4.08	0.33	0.95
PSM-1	77	5.93	0.35	1.19
PSM-2	79	8.70	0.39	0.96
BAC-2	82	2.33	0.33	1.34
MOR	83	2.19	0.31	1.56
MCC	83	7.50	0.31	0.97

Table 3. Details of experimental treatments in which bacterial biomass from different seawater strains was fed to *Penaeus monodon* larvae, as a possible substitute for microalgae. This set of treatments was duplicated for six bacterial strains.

Treatments	Microalgae (cells/ml)	Bacterial biomass (CFU/ml)
0% substitution (positive control)	100 000	-
10% substitution	90 000	10 000
30% substitution	70 000	30 000
50% substitution	50 000	50 000
70% substitution	30 000	70 000
100% substitution	-	100 000
Starved control	-	-

Results and Discussion

The percentage survivals of *P. monodon* larvae in different treatments are shown in Fig. 1. Starved controls neither metamorphosed from Z-1 stage nor survived beyond three days. Larvae fed with 100% bacterial biomass did not metamorphose beyond the Z-3 stage or survive beyond five days for any of the tested strains. This may be due to certain inherent limiting dietary factors. Bacteria may lack polyunsaturated fatty acids, sterols and certain amino acids (Philips 1984). Artemia have been grown to preadult stage solely on a *Flexibacter* strain, but best growth and biomass produced were observed with a mixture of *Flexibacter* and algae (Intriago and Jones 1993). They concluded that bacteria not only acted as food, but also assisted digestion of algae through the presence of exoenzymes.

Noticeably better survival than control was seen only with biomass of MCC and PSM-2, particularly at the 50% substitution level. In all other treatments, positive control larvae showed better survival. However, all larvae fed MCC and PSM-2 metamorphosed faster (nine days to P-1 stage) than controls larvae.

A comparison of the average survival among various treatments (Fig. 2) shows that PSM-2 strain gave the best survival, whereas all other treatments had lower survivals than the positive control. Larvae fed with MCC took the least time to metamorphose (Fig. 3) to the P-1 stage (two days less than controls). PSM-1, PSM-2, and MOR-fed larvae also took a day less to metamorphose than controls, where BAC-1 and BAC-2 fed larvae did not differ from controls in time to metamorphose.

The use of microorganisms in mariculture is recent (Maeda 1988). The concept of using nonpathogenic vibrios as probiotics in shrimp larviculture is now gaining ground, for example, in Ecuador (Garriques and Arevalo 1995). Probiotics may provide growth factors, produce metabolites that inhibit the proliferation of pathogens and stimulate the nonspecific immune response (Vanbelle et al. 1989).

Here, 50% replacement of *Chaetoceros* with MCC and PSM-2 biomass gave better survival and faster metamorphosis than with *Chaetoceros* alone. Not surprisingly, these two bacteria had the highest protein contents. Therefore, it is probable that, apart from other probiotic effects, MCC and PSM-2 also functioned as supplementary feed for the larvae.
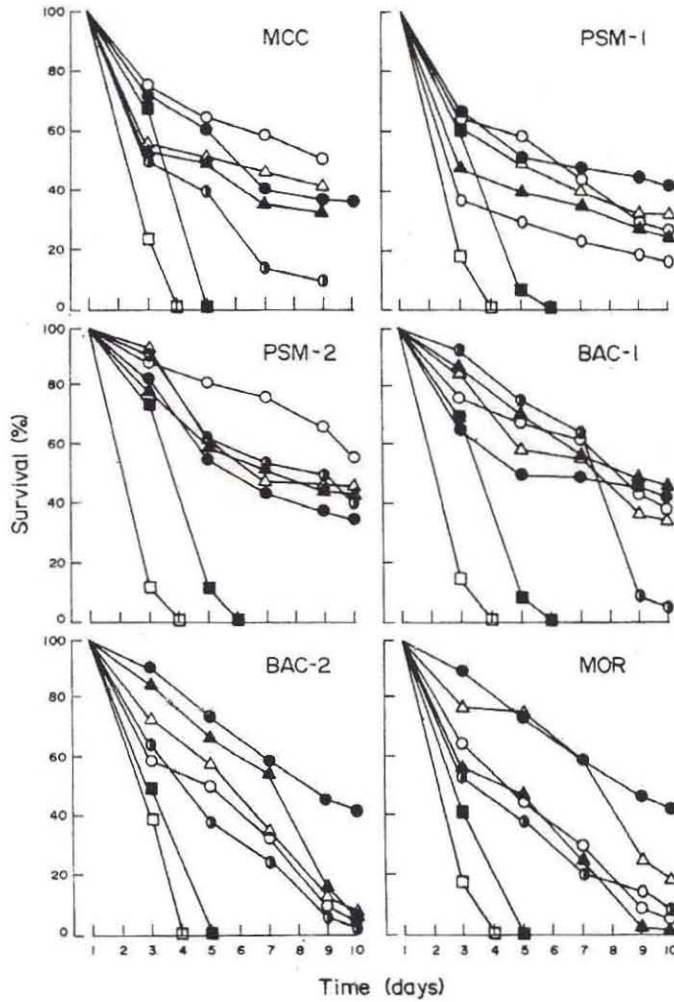

Fig. 1. Survival of Penaeus monodon *larvae fed with different percentages of bacterial biomass from the following strains:* ● *= 0% substitution;* △ *= 10% substitution;* ▲ *=30% substitution;* ◑ *= 50% substitution;* ○ *= 70% substitution;* ■ *= 100% substitution;* ❑ *= starved control.*
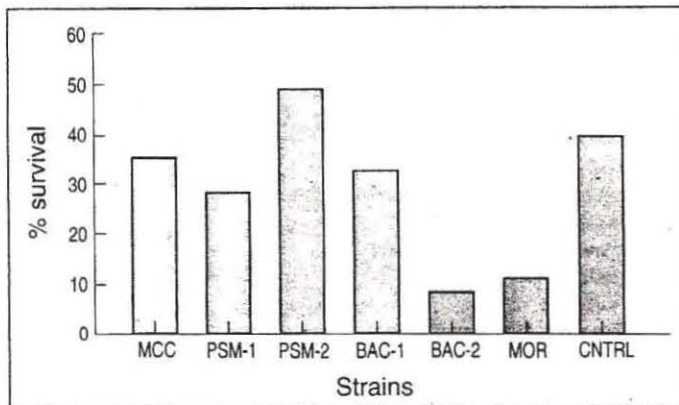

Fig. 2. *Average percentage survival (± standard errors) of* Penaeus monodon *larvae among various treatments and controls: mixed feeding on microalgae and bacterial biomass from the following strains:* Micrococcus *(MCC);* Pseudomonas *(PSM-1, PSM-2);* Bacillus *(BAC-1, BAC-2) and* Moraxella *(MOR). For further explanations of treatments, see Table 3.*
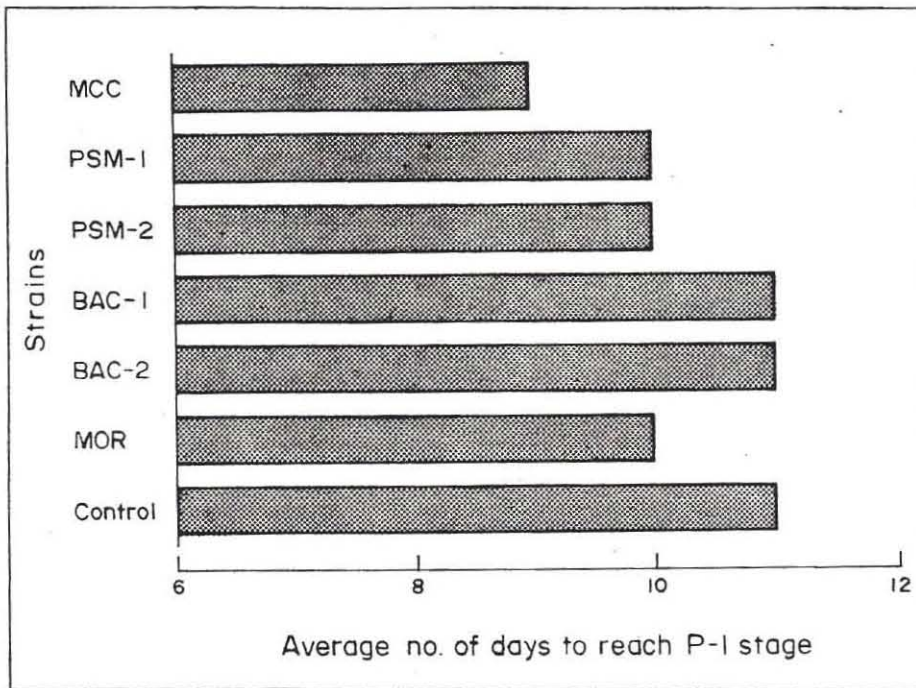
Fig. 3. Average time taken for Penaeus monodon to complete metamorphosis to the postlarval (P-1) stage for various treatments and controls based on mixed feeding on microalgae and bacterial biomass from the following strains: Micrococcus (MCC); Pseudomonas (PSM-1, PSM-2); Bacillus (BAC-1, BAC-2) and Moraxella (MOR). For further explanations of treatments, see Table 3.

There is no direct evidence as to the capacity of shrimp larvae to ingest bacterial particles of the size used in this study. Jones et al. (1987) reported that shrimp larvae could utilize 5-20 µm particles. However, in a study with fluorescent particles, Factor and Dexter (1993) demonstrated that decapod (crab) larvae could ingest particles in the size range of bacteria (1-2 µm).

Bacterial biomass might offer advantages over conventional plant and animal feed proteins for fish and shrimp nutrition (Tacon 1990). It can be mass-cultured rapidly in limited space on cheap substrates. For most bacteria, it is highly proteinaceous. Its nutritional composition could be enhanced by genetic manipulation. This present study demonstrates that bacterial biomass could be further investigated as a partial substitute for microalgae in penaeid shrimp larval rearing. The results are preliminary and wider screening could probably yield better bacterial strains for this purpose.

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