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CIBASTIM TECHNOLOGY FOR IMMUNE STIMULATION AND GROWTH PROMOTION IN SHRIMP

केन्द्रीय खारा जलजीव पालन अनुसंधान संस्थान
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Background

Mass mortalities associated with different species of *Vibrio* bacteria both in shrimp hatcheries and grow-out cultures have been extensively reported worldwide. Amongst the disease outbreaks *Vibrio* spp. are implicating number of syndromes *viz.*,

- Oral and enteric Vibriosis
- Appendage and cuticular Vibriosis
- Localized vibriosis of wounds
- Shell disease
- Systemic Vibriosis
- Septic hepatopancreatitis
- Tail rot disease
- Bacterial white tail disease
- Bright-red syndrome

Amongst the various inter related parameters, environmental stresses significantly suppress the immune system and increases the susceptibility to pathogenicity by *Vibrio* spp. Recurring disease outbreaks in shrimp aquaculture from 1993-94 onwards triggered to the development of safety measures like, crop holidays, implementation of better management practices (BMPs), use of probiotics, immunostimulants and many other commercial products. In a span of 4-5 years there was influx of commercial chemical and biological products in the market, the efficiency of many products is not known. There had been a rapid development of feed additives such as immunostimulants, which enhance shrimp resistance against potential pathogens and to counter the disease problems in shrimp aquaculture. The Central Institute of Brackishwater Aquaculture (CIBA), Chennai developed a microbial based immunostimulant product CIBASTIM to improve shrimp health and control diseases.

Development of CIBASTIM

Initially, *Vibrio anguillarum, V. alginolyticus, V. harveyi* and *Aeromonas hydrophila* bacteria were isolated from different internal organs of diseased shrimp and characterized based on biochemical characters and virulence. Inactivated whole cell bacterial product of individual or in combination of each species were prepared and administered to shrimps, *Penaeus monodon and Fenneropenaeus indicus* in different combinations and routes (immersion/oral through feed or bio-encapsulation). Parameters like, growth, survival, levels of pro-phenol oxidase and protection to challenge were evaluated. Based on these parameters, the best microbial product was selected for further study. The antimicrobial

defence in shrimp depends on a number of complex inter relationships between cellular and humoral activities. The mode of action of this vibrio bacterin is through modulation of the immune related molecules like, chemokinetic cell migration factors, bactericidins, and prophenoloxidase activating enzyme. The efficiency of the developed bacterin is attributed to the immune stimulatory activity and ability of the lipopolysaccharide (LPS) from gram negative bacteria cell wall. These LPS have the ability of binding to the pattern recognition proteins (PRPs) and stimulating the activation of proPO system of the semi-granulocytes and granulocytes (GC). Concurrently, the other immune elicitors such as total haemocyte count (THC), granulocytes count (GC), phagocyte index (PI) and bacterial clearance activity (BCA) are potential indicators of immune status. However, the success of immunostimulation and imparting disease resistance depends much on the dose, schedule and the route of application.

Standardisation of route of application

Effective administration of drugs and other biological products depends on the route and schedule adopted. Among the different routes of administration, enrichment of *Artemia nauplii* was found effective in stimulating the health and immunity in larval stages of shrimp. Oral administration as feed top dressing is the most convenient and practical method in shrimp aquaculture. Leaching of the immunostimulant can be countered through use of natural or synthetic binders. Yard and field trials, based on the method of top coating application for CIBASTIM on to feed demonstrated that there was enhancement of immune levels in the shrimp both in larval and grow-out stages. However, during larval stages in the shrimp hatchery both in combination or individual microbial base used through feed or immersion showed significant improvement in survival and growth (Table 1 and Fig. 1). Further, challenge test with pathogenic vibrio studies confirmed that shrimp fed with CIBASTIM developed protective responses as observed by the relative percentage survival (Fig. 2). Subsequent challenge studies showed that conferred protection was proportional to the duration of priming and boosting.

Table 1: Growth and survival (average values with S.E) of postlarvae of *P. monodon* immunostimulated using heat-killed *V. anguillarum* (primed and boosted)

Parameter	C	P (I)	P+B(I+I)	P+B(I+I+F)	P+ (I+F)
Survival (20 dpp)	$83.75^{a}\pm$ 1.13	$83.5^{a}\pm$ 0.75	80.75^{a} $\pm$ 1.0	89.75^{b} $\pm$ 2.0	79.0^{a} $\pm$ 1.75
Survival (40 dpp)	$71.5^{c}\pm$ 1.25	74.75^{c} $\pm$1.63	73.5^{c} $\pm$ 1.50	81.25^{d} $\pm$ 1.13	74.25^{c} $\pm$ 1.13
Survival (60 dpp)	$33.0^{e}\pm$ 4.0	$32.5^{e}\pm$ 1.25	32.25^{e} $\pm$ 0.75	34.0^{e} $\pm$ 1.25	38.5^{f} $\pm$ 1.50
Initial average weight (g)	$0.034^{g}\pm$ 0.004	$0.033^{g}\pm$ 0.005	$0.033^{g}\pm$ 0.005	$0.029^{g}\pm$ 0.002	$0.033^{g}\pm$ 0.004
Weight (g) at 30 dpp	$0.826^{h}\pm$ 0.074	$1.054^{i}\pm$ 0.037	$0.931^{i}\pm$ 0.074	$1.065^{i}\pm$ 0.073	$1.153^{j}\pm$ 0.068
Weight (g) at 60 dpp	$1.139^{k}\pm$ 0.054	$1.349^{l}\pm$ 0.066	$1.437^{l}\pm$ 0.088	$1.615^{m}\pm$ 0.050	$1.583^{m}\pm$ 0.052
Wg (g)	1.105	1.316	1.454	1.586	1.555
Wg (%) over C	0	19.15	31.64	43.61	40.74

C- control; P- priming; B- booster, I-immersion; F- in feed; Wg-weight gain; dpp – days post priming. Treatment means sharing common superscripts are not significantly (P>0.05) different from one another

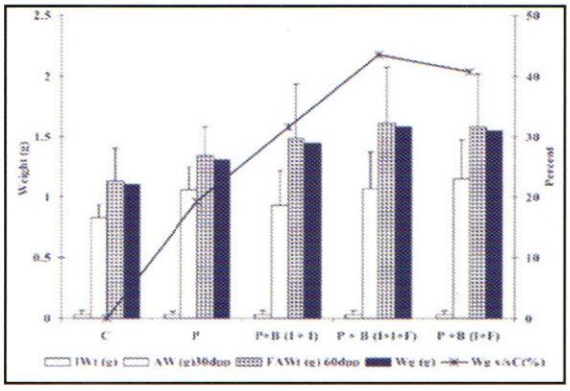


Fig. 1. Growth of immunostimulated (primed and boosted) and control postlarvae of *P. monodon* initially and at 30 and 60 days postpriming.

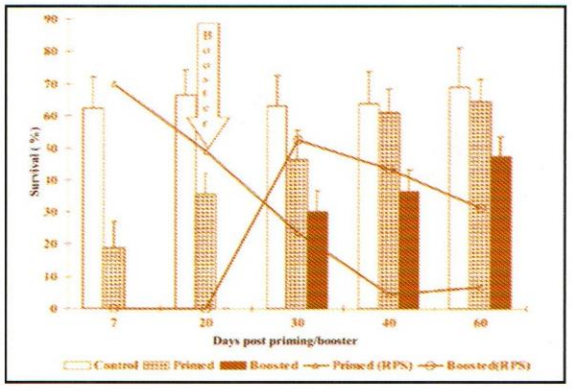


Fig. 2. Mortality upon challenge and protective response of the immunostimulated (primed and boosted) and control.

Standardisation of dose and schedule

Efficacy of dose is an important constituent in determining the desired immune stimulation and improvements in survival and growth. Study with different bacterial concentrations had showed that 10^8cfu kg^{-1} significantly improved the growth and survival of shrimp juveniles (Table 2). Higher concentration of the product dose not seem to significantly improve the growth and survival. Further the proximate analysis of the whole body revealed that the highest lipid and protein content was in group fed at 10^8cfu kg^{-1} concentration concurring that when the feed is top coated with optimal concentration ($2x10^8$cfu/kg feed) the product application helps in improvement of immunity levels (Table 3). The better performance in 10^8cfu kg^{-1}feed treatment was substantiated by improved values in proximate analysis of the whole body.

Table 2. Percentage survival and average body weight gain (g) of shrimp juveniles (n=20) administered with CIBASTIM at different concentrations (mean±SD).

Experimental Groups	Survival (%)	Av.weight at 0 days	Average body weight gain (g)		
			14th day	28th day	42nd day
Control	82.00±5.29	0.257±0.03	0.084±0.01^a	0.099±0.01^a	0.109±0.01^a
10^6 cfu kg^{-1}	85.66±3.05	0.240±0.01	0.103±0.00^a	0.119±0.01^{ab}	0.132±0.01^{ab}
10^8 cfu kg^{-1}	90.33±4.50	0.225±0.01	0.109±0.00^a	0.147±0.00^b	0.162±0.00^b
10^{10}cfu kg^{-1}	88.33±1.52	0.229±0.02	0.098±0.00^a	0.131±0.00^{ab}	0.156±0.01^b
10^{12}cfu kg^{-1}	86.33±3.21	0.258±0.02	0.100±0.00^a	0.143±0.01^b	0.154±0.01^b

Table 3. Proximate composition of lipid and protein in the carcass of PL (n=20) immunostimulated with different concentration of CIBASTIM (mean±SD).

Experimental Groups	Lipid composition	Protein composition
Control	15.34±3.40^d	54.97±0.68^b
10^6 cfu kg^{-1}	20.98±2.72^a	58.95±3.31^{bc}
10^8 cfu kg^{-1}	29.84±2.86^c	62.72±2.55^c
10^{10} cfu kg^{-1}	28.29±2.03^{bc}	58.50±1.65^{bc}
10^{12} cfu kg^{-1}	26.33±1.88^b	59.73±2.59^c

Evaluation in shrimp hatcheries and culture ponds

Yard experiments and field trials were conducted in shrimp hatcheries and shrimp culture ponds in several geographical locations like Tamil Nadu, Andhra Pradesh and Gujarat to establish the potential role and efficacy of whole cell microbial product 'CIBASTIM' in improving the shrimp health by enhancing immunity levels.

Evaluation of CIBASTIM in shrimp larval rearing system

In hatchery systems, inspite of the best management practices, the physico-chemical and microbial loads are significantly high in water, causing stress for the larvae leading to heavy mortality. Application of CIBASTIM during hatchery cycle trials improved the quality of water and the parameters were within the optimal range. The dynamics of mode of action of the product though not scientifically accredited, showed no deterioration of water quality with regard to pH changes, concentration of ammonia, nitrite, and turbidity in larval rearing tanks. However studies with CIBASTIM application suggest that there was decline of microbial population especially pathogenic forms in the larval rearing systems which influenced the improvement in survival rate. Trials in three commercial hatcheries showed significantly ($p<0.05$) higher survival (43.83±1.04 %) in treatment than in control groups (33.67±3.51 %).

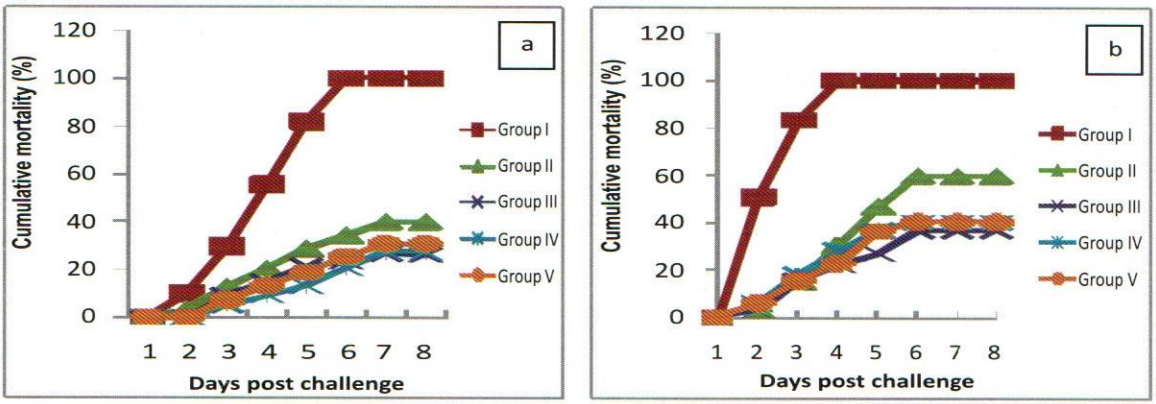


Fig 3. Percentage cumulative mortality of CIBASTIM administered hatchery raised juvenile shrimp challenged with (a) *V. anguillarum* and (b) *V. harveyi* (n=100). Group I (control), Group II (10^6cfu/ml), Group III (10^8cfu/ml), Group IV (10^{10}cfu/ml) and Group V (10^{12}cfu/ml)

Evaluation in juvenile shrimp – Testing in Yard experiments and shrimp culture ponds

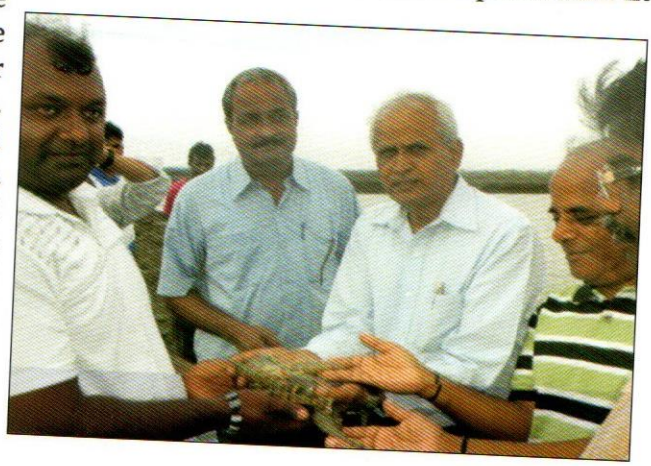
For commercialisation of a product it has to be tested first under controlled laboratory conditions and then to be evaluated under field conditions.

Yard experiments with shrimp postlarvae (PL 30) and feeding with top dressed product showed good survival and growth suggesting that the product had no secondary effects on the shrimp. The *V. anguillarum* challenge studies showed mortality post 48 h of challenge and by fifth day 100% of mortality in control groups. Comparatively treatment groups at the end of the seventh day observation showed cumulative mortality 40%, 26%, 27.33% and 30.67% in 10^6,10^8, 10^{10} and 10^{12}cfu kg^{-1} respectively (Fig. 3). Similarly in challenge test with

V. harveyi the mortality started after 24 h post challenge in all the groups. However 100% mortality was observed by 3rd day post challenge and in control group, 10^8cfu kg^{-1} showed significantly lowest mortality (36.67%) and highest at 10^6cfu kg^{-1}(60%) at the end of seventh day post challenge (Fig 3). The overall challenge studies indicated that with six week CIBASTIM administration @ 10^8cfu kg^{-1} significantly ($P \leq 0.05$) improved the resistance to pathogenic *Vibrio* spp. The improved antimicrobial defence may be attributed to non-specific stimulation of the antimicrobial factors in the shrimp immune system. Though the potential mechanism of resistance conferred is not clear, it is hypothesized that cell wall components of vibrio bacteria stimulates non-specifically the antimicrobial factors in the shrimp immune system.

Effect on immune parameters and resistance to challenge with *V.harveyi*

Haemocytes in crustaceans are potential indicators of immune status and their reduction in numbers makes the animal susceptible to infection. These cells are involved in a variety of defence responses in shrimp like, phagocytosis, encapsulation, melanisation and coagulation. Humoral defence molecules such as prophenoloxidase (proPO), antimicrobial peptides and clotting factors are released from haemocyte granules. Hence, the granular cell numbers are used as a more specific measure of the potential defence capacity related to these molecules. Since immunity and antimicrobial defence in shrimp depends on number of other complex interaction between cellular and humoral activities, the improvement in the efficiency of bacterial clearance rate indicates the mechanism behind the potential of immunostimulant to confer protection against invading pathogens. Thus, as a function of cellular and humoral immunity, the THC, GC, BCA and PA were studied in CIBASTIM administered shrimp. Significant improvement in THC, GC, BCA and PI were observed in shrimp from CIBASTIM treated compared to control ponds (Fig. 4). Challenge studies with virulent *V. harveyi* showed significantly higher cumulative mortality in controls (93.66 ±5.77% and 96.66±5.77%) compared to treatment (46±11.4%, 50±10 %) (Fig.4). The experimental trials with the bacterin showed enhanced immune parameters and the continuous administration of CIBASTIM has shown to induce the prolonged bacterial clearance capacity and cross-protection against virulent vibrio pathogen such as *V. harveyi*.



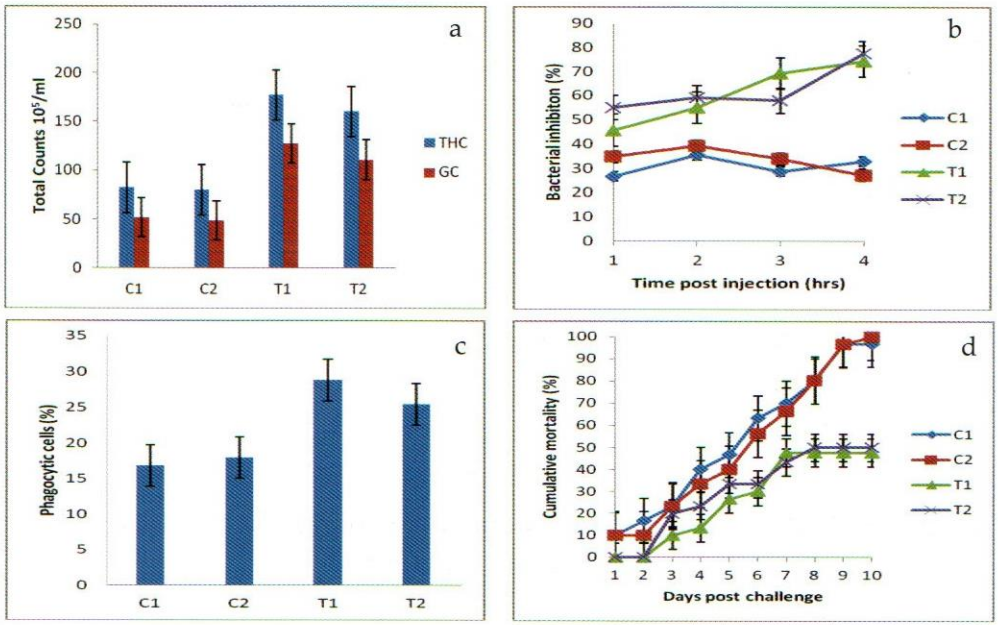


Fig.4. Haematological values and challenge protection (Mean ± SD in CIBASTIM administered pond reared shrimp. (a) Total haemocyte count (THC) and the granulocyte count (GC), (b) percentage bacterial clearance, (c) percentage phagocytosis and (d) percentage cumulative mortality. Control ponds (C1 and C2) and treatment ponds (T1 and T2).

Farm testing trails

Farm trails conducted in 26 commercial grow-out culture ponds (13 controls and 13 treatments) showed that average body weight (g), survival (%), production (kg/ha) and FCR (26.76 g ±7.28, 67.72 g ±17.39, 2518.16 g ± 1205.08, 1.66 g ± 0.52, respectively) were higher in treatment ponds compared to control ponds (23.41 g ± 7.29, 63.12 g ± 16.29, 2074.07 g ± 1154.49, 1.88 g ± 0.61, respectively).

Field demonstrations

Extensive multi-location testing was conducted in different eco-geographical areas across India. Extensive field (over 200 nos.) demonstrations of CIBASTIM have been conducted in the states of Tamil Nadu, Andhra Pradesh, Gujarat and West Bengal over a decade. In the demonstration trials, at the end of the culture period, treatment ponds showed more than 15% improvement in ABW, production and lower FCR over untreated control pond.

Apart from aiding as good growth promoter, CIBASTIM established the fact that it has a promising therapeutic effect on the shrimp as observed by the incidence of percentage deformities beyond 60 DOC. Poor growth and appendage deformities in the grow-out

shrimp culture is considered as indicator of low levels of bacterial resistance. Generally, the major deformities observed in shrimp during culture period beyond 60 DOC are biofouling, tail necrosis, rostral damage, black spots (melanisation on the body) and antenna cut. Field studies of CIBASTIM in grow-out shrimp cultures in Andhra Pradesh and Gujarat showed lesser percentage of these deformities and the shrimps were sturdy with fully opened uropod, flippery movements and good body colouration (Table 4 and 5). The low percentage of deformities in shrimp culture ponds administered with CIBASTIM suggest that the product acts as therapeutic agent to control the syndromes related to underlying vibrio bacterial infections. Other observations reported by farmers were pond bottom and water quality conditions in treatment ponds were better than the non- treated ponds. Ammonia in water was within the permissible range of 0.05 to 0.15 ppm with microbial loads significantly reduced in treatment ponds.

Table 4. Effect of CIBASTIM administration on appendage deformities (Mean±SD) in *P.monodon* culture ponds

Deformities	Treatment ponds (n=290)	Control ponds (n=228)
Antennae cut	5.06 [a] ±3.95	12.38 [b] ±1.92
Tail rot	4.57 [a] ±0.58	9.18 [b] ±0.88
Rostrum cut	4.80 [a] ±0.45	10.67 [b] ±1.03
Soft shell	7.13 [a] ±2.39	16.58 [b] ±0.87

Table 5. Levels of appendage deformities during culture period in CIBATIM treatment and control ponds (n=100)

DOC	Antennae cut		Tail rot	
	Treatment	Control	Treatment	Control
65	65	66	75	72
80	45	62	50	75
90	30	78	42	55
100	22	77	25	76
115	22	83	15	61
130	9	80	5	42

Effect on shrimp growth and production

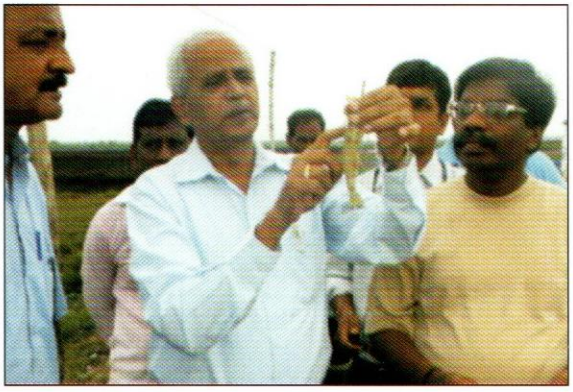
Ideally the immunostimulants improves ABW, production and lowers the percentage of deformities as a result the overall health which ultimately confers protection against invading pathogens was transformed into increase in production and productivity. Observation of improvement in growth and survival in treated ponds indicated the role of

CIBATIM in improving the shrimp health which was reflected in production (Table 6). The observations of the field study when replicated in yard experiments too confirmed the results. The effectiveness of oral administration of CIBASTIM as top dressing on feed in grow-out culture ponds was a simple, cost-effective method of delivery for enhancing high health, production and helps in generating higher income with better cost benefit ratio for the shrimp farmers.

Table 6. Effect of CIBASTIM administration on production and average body weight (Mean±SD) at varying stocking densities in *P.monodon* culture ponds

Stocking density (nos/m^2)	Production (kg/ha)		Average body weight (g)	
	Treatment	**Control**	**Treatment**	**Control**
<7 (n=15)	2103.68^{a}±720.62	1136.22^{b}±477.18	33.06^{a}±10.12	26.09^{a}±8.76
8-12 (n=50)	3129.31^{a} ±1229.07	2203.91^{b}±989.88	35.18^{a} ±7.11	33.63^{a} ±10.30
13-16 (n=11)	3892.38^{a}±449.25	1874.82^{b}±704.17	26.34^{a} ±4.59	28.40^{a}±7.79
>16 (n=9)	3376.12^{a} ±211.42	2550.53^{b}±461.21	25.80^{a} ±2.26	25.39^{a}±2.17

Note: Means in the same row with different superscript are significantly different (P<0.05)



5. CONCLUSION

The dose, schedule and route of application of CIBASTIM have been standardized successfully both under hatchery or pond culture conditions for *P. monodon* and subsequently for other commercially viable shrimp species (*F. merguiensis* / *L. vannamei*). Oral administration of formalin killed *V. anguillarum* as feed top dressing at the concentration of 10^{8}cfu/kg feed improves growth and survival of cultured shrimp and the cross protection from *V. anguillarum* and *V. harveyi*. The oral administration as feed-top dressing in grow out culture ponds is an easy, simple and cost-effective practical method

for controlling the vibriosis and improving health and production in commercial shrimp hatchery and grow-out systems. This is one of the first technologies using bacterial products with extensive field demonstrations and testing in different geo-ecological areas of shrimp aquaculture in India.

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