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A marine bacterium, *Micrococcus* MCCB 104, antagonistic to vibrios in prawn larval rearing systems

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ABSTRACT: A marine bacterium, *Micrococcus* MCCB 104, isolated from hatchery water, demonstrated extracellular antagonistic properties against *Vibrio alginolyticus*, *V. parahaemolyticus*, *V. vulnificus*, *V. fluviallis*, *V. nereis*, *V. proteolyticus*, *V. mediterranei*, *V. cholerae* and *Aeromonas* sp., bacteria associated with *Macrobrachium rosenbergii* larval rearing systems. The isolate inhibited the growth of *V. alginolyticus* during co-culture. The antagonistic component of the extracellular product was heat-stable and insensitive to proteases, lipase, catalase and α-amylase. *Micrococcus* MCCB 104 was demonstrated to be non-pathogenic to *M. rosenbergii* larvae.

KEY WORDS: *Vibrio* · *Micrococcus* · Antagonism · *Macrobrachium* · Probiotic

INTRODUCTION

Among heterotrophic bacteria associated with larviculture and grow-out phases of crustaceans, *Vibrio* is a dominant genus responsible for much of the observed mortality (New 1995, Sung et al. 1999). Vibriosis has been a major hindrance to prawn culture in India and in other prawn-farming countries (Baticados et al. 1990, Singh 1990, Lightner 1996). To control vibrios, prophylactic and therapeutic use of antibiotics has been the choice in commercial hatcheries; however, this has led to resistance and the possible spread of vibrios in the environment (Weston 1996, Hameed et al. 2003).

As an alternate management measure, the introduction of selected bacterial cultures/products as probiotics with antagonistic properties has been proposed and applied (Gomez-Gil et al. 2000). Many bacterial isolates, which are common members of the non-pathogenic microflora of fish and shellfish culture systems, have been shown to inhibit fish and prawn pathogens *in vitro*. This has been demonstrated for lactic acid bacteria (Gatesoupe 1994), *Carnobacterium* (Robertson et al. 2000), *Bacillus* (Rengpipat et al. 2000), *Vibrio* (Austin et al. 1995), *Planococcus* (Austin & Billaud 1990) and *Pseudomonas* (Chythanya et al. 2002). Gomez-Gil (1995) and Verschuere et al. (2000) claimed that certain strains of bacteria associated with *Artemia* and prawn culture systems have the ability to control pathogens by means of competitive exclusion or by the production of inhibitory compounds. The antibacterial effect may be due to production of antibiotics (Williams & Vickers 1986), bacteriocins (Vandenbergh 1993), hydrogen peroxide, or alteration of pH by producing organic acids (Sugita et al. 1997). In aquaculture, *Micrococcus* has been documented by Austin & Allen (1982) and Prieto et al. (1987) in dehydrated *Artemia salina* cysts, cyst-hatching water and *A. salina* nauplii. Irianto & Austin (2002a) investigated the probiotic effect of a Gram-positive cocci (A1-6) to control furunculosis in rainbow trout, which apparently was *M. luteus* (Irianto & Austin, 2002b). Lalitha & Surendran

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(2004) reported *Micrococcus* in the environment of the farmed freshwater prawn *Macrobrachium rosenbergii*. However, there is a paucity of information on the antagonistic properties of the genus.

In the present work we have investigated the antagonistic properties of *Micrococcus* MCCB 104, an isolate obtained from hatchery seawater, to *Vibrio* spp. associated with prawn larval rearing systems. The *in vitro* antagonistic property of the isolate was evaluated using a disc diffusion assay, as well as co-culture methods. The pathogenicity of *Micrococcus* MCCB 104 towards *Macrobrachium rosenbergii* larvae in a bioassay system was also evaluated in order to explore its potential use as a 'probiotic with antagonism' for controlling vibrios in prawn larviculture.

MATERIALS AND METHODS

Bacterial isolates and culture conditions. Three groups of microorganisms served as targets for screening the antagonistic properties of *Micrococcus* sp. in this study (Table 1). They were 55 isolates of *Vibrio* (*V. cholerae* [12 isolates], *V. mediterranei* [7], *V. vulnificus* [8], *V. nereis* [11], *V. parahaemolyticus* [4], *V. fluvialis* [1], *V. proteolyticus* [2], *V. splendidus* II [2] and *V. alginolyticus* [8]) from larval rearing systems of *Macrobrachium rosenbergii* and isolates of *Bacillus* sp., *Pseudomonas* sp. and *Aeromonas* sp., associated with prawn culture systems (obtained from the culture collection of Centre for Fish Disease Diagnosis and Management, Cochin University of Science and Technology, Kerala, India), 7 isolates of marine yeasts and 6 marine actinomycetes (obtained from Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology). The antagonistic *Micrococcus* MCCB 104 was isolated from seawater sampled from a prawn hatchery in Kerala and identified following the scheme of Buchanan & Gibbons (1974). All isolates were grown either in ZoBell's 2216E agar or broth (HiMedia), prepared in 15 ppt seawater and incubated at 28°C unless otherwise indicated.

Antagonism assay. Antagonism of *Micrococcus* MCCB 104 towards the target microbial cultures was detected by the disc diffusion method; 6 mm diameter discs from Whatman No.1 filter papers (stack of 3 filter papers) were prepared, sterilised at 121°C for 15 min and dried. These discs were placed on ZoBell's marine agar plates, previously swabbed with the target bacte-

Table 1. Spectrum of inhibition of bacterial taxon by *Micrococcus* MCCB 104

Bacterial taxon	Code	Source
(A) Inhibited by *Micrococcus* MCCB 104		
Vibrio cholerae	MRCS 11, 12, 13, 16, 17, 19, 20, 21, 23, 35, 37, 39	*Macrobrachium rosenbergii* larvae
Vibrio mediterranei	MRCS 15, 18, 22, 32, 34, 36; MRQL 27	*Macrobrachium rosenbergii* larvae
Vibrio vulnificus	MRQL 5, 9, 10, 19, 20, 23, 33, 36	*Macrobrachium rosenbergii* larvae
Vibrio nereis	MRCS 24, 28, 29, 30, 31, 33; MRQL 13, 29, 32, 34, 35	*Macrobrachium rosenbergii* larvae
Vibrio parahaemolyticus	MRCS 1, 6, 8, 9	*Macrobrachium rosenbergii* larvae
Vibrio fluviallis	MRCS 26	*Macrobrachium rosenbergii* larvae
Vibrio proteolyticus	MRCS 4, 10	*Macrobrachium rosenbergii* larvae
Vibrio alginolyticus	MCCB 112 MRNL 1, 2, 4, 5, 6, 7, 8	Diseased *Macrobrachium rosenbergii* larvae
Aeromonas sp.	MRCS 2, 7 MRNL 9, 10, 16, 17	*Macrobrachium rosenbergii* larvae
(B) Not inhibited by *Micrococcus* MCCB 104		
Vibrio splendidus II	MRCS 3, 5	*Macrobrachium rosenbergii* larvae
Bacillus sp.	MCCB 101	Prawn grow-out
Pseudomonas sp.	PS-1, MCCB 103	Brackish water lagoon, prawn grow-out
Debaryomyces hansenii	S8, S87, S100, S169	Seawater, southwest coast of India
Candida sake	S165	Seawater, southwest coast of India
Candida tropicalis	S186	Seawater, southwest coast of India
Torulospora delbruecki	S303	Seawater, southwest coast of India
Streptomyces sp.	B272, B377	Seawater, southwest coast of India
Streptomyces fradiae	B451	Seawater, southwest coast of India
Streptomyces griseoflavus	B301	Seawater, southwest coast of India
Streptomyces pulveraceus	B361	Seawater, southwest coast of India
Streptomyces californicus	B30	Seawater, southwest coast of India

rial isolates. Aliquots (20 µl) of the *Micrococcus* MCCB 104 culture in ZoBell's marine broth (incubated for 5 d at 28°C) were pipetted onto the discs. The plates were incubated for 24 h at 28°C, and the formation of a zone of clearing around the discs was considered a positive indication of inhibitory activity.

Antagonism of cell-free culture supernatant. *Micrococcus* MCCB 104 was grown in ZoBell's broth for 5 d on a shaker (90 rpm) at room temperature (approx. 28°C). The cells were pelleted by centrifugation (10 000 × *g*, 10 min), the pH of the supernatant adjusted to 7.0 and then passed through a 0.2 µm pore-size cellulose-acetate membrane filter (Sartorius). Inhibitory activity on the target microbial cultures was detected by the disc diffusion method, as described above, and the zone of inhibition around the discs was recorded after 24 h using a HiAntibiotic ZoneScale (HiMedia).

Time course of growth and production of the antagonistic substance. Commencement of the antagonistic substance production, its peak activity and duration of sustained activity were determined during the growth cycle of *Micrococcus* MCCB 104. The culture was inoculated to an absorbance (*A*) of 0.01 at 600 nm (approx. 10^3 CFU ml^{-1}) in 500 ml of ZoBell's broth (pH 7.0) and incubated on a magnetic stirrer at room temperature (approx. 28°C). Starting at hour zero and then at periodic intervals, aliquots of 2 ml culture were drawn aseptically; 1 ml was used for monitoring growth ($A_{600\,nm}$) and 1 ml centrifuged at 10 000 × *g* at 4°C for 10 min to pellet the cells. The supernatant was filter-sterilised, as mentioned above, and antagonistic activity was tested against *Vibrio alginolyticus* MCCB 112, in triplicate, as mentioned earlier. The plates were incubated at 28°C for 24 h and observed for zones of clearing around the discs. This was continued until the culture entered the decline phase. Antagonistic activity was quantitatively expressed in terms of the diameter (mm) of the zone of inhibition around the discs.

Co-culture experiments. *Micrococcus* MCCB 104 and *Vibrio alginolyticus* MCCB 112 were precultured separately in ZoBell's broth at 28°C on a shaker (100 rpm) overnight. *V. alginolyticus* was then inoculated into 100 ml of ZoBell's broth in conical flasks (250 ml volume) to provide an initial cell density of about 10^3 CFU ml^{-1}, whereas the initial levels of *Micrococcus* MCCB 104 were 10^5, 10^6, 10^7 and 10^8 CFU ml^{-1}, respectively. All combinations were in duplicate. The flasks were incubated at 28°C on a shaker (100 rpm), and samples (1 ml) were withdrawn daily for *V. alginolyticus* counts. The cell count was taken by spread plating 0.2 ml aliquots of serially diluted broth suspensions on plates of thiosulphate citrate bile salts sucrose (TCBS) agar (HiMedia). The plates were incubated at 28°C for 2 d, and colonies were counted and expressed as CFU of *V. alginolyticus* in the coculture. This procedure was specifically chosen because *Micrococcus* MCCB 104 failed to grow on TCBS agar plates.

Preliminary characterisation of the antagonistic substance. The extracellular product having the antagonistic property was evaluated for the presence of bacteriocin, lipid, carbohydrate, hydrogen peroxide, acid and alkali by the disc diffusion method, as described earlier. To examine the presence of bacteriocin, the culture supernatant of *Micrococcus* MCCB 104, filtered through a 0.2 µm pore-size cellulose-acetate membrane filter (Sartorius) and neutralised to pH 7.0, was incubated at 37°C for 1 h with the proteolytic enzymes Proteinase K (1 mg ml^{-1}), pronase E (2 mg ml^{-1}), αchymotrypsin (5 mg ml^{-1}), trypsin (50 mg ml^{-1}) and lysozyme (1 mg ml^{-1}). For lipids and carbohydrates, the cell-free supernatant was treated with lipase (1 mg ml^{-1}) and α-amylase (1 mg ml^{-1}); for hydrogen peroxide, the cell-free supernatant was treated with catalase (2 mg ml^{-1}). All the enzymes were procured from Sigma-Aldrich. To examine alkali and acid production, the supernatant was neutralised to pH 7.0 by the addition of 1 N HCl or 1 N NaOH (Qualigens). Heat sensitivity of the inhibitory substance was tested by heating the cell-free supernatant in a water bath for 30 min at 60 and 80°C, and autoclaving for 15 min at 121°C. Each treated and untreated supernatant was tested for activity against *Vibrio alginolyticus* MCCB 112, and the presence of an inhibitory zone around the discs was determined after incubation for 24 h at 28°C.

Influence of growth conditions on the production of antagonistic substance. The influence of pH, temperature and NaCl concentration on production of the antagonistic substance by the *Micrococcus* MCCB 104 was studied. Sterilised nutrient broth supplemented with 1.5% NaCl (pH adjusted to 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, or 11.0) was inoculated with 0.1 ml of an overnight culture of *Micrococcus* MCCB 104 and incubated at 28°C for 24 h. Similarly, nutrient broth (pH 7.0, NaCl 1.5%) was inoculated, as described above, and incubated at 4, 15, 20, 25, 30, 35, 40, or 45°C for 24 h. The influence of different concentrations of sodium chloride was assessed by observing growth at 28°C in 1% tryptone broth (pH 7.0; HiMedia, India) containing 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, or 3.5% NaCl. A cell-free supernatant was obtained by centrifugation followed by filtration, as described earlier, to assess the inhibitory activity by the disc diffusion method on *Vibrio alginolyticus* MCCB 112.

Pathogenicity of *Micrococcus* MCCB 104 to *Macrobrachium rosenbergii* larvae. Pathogenicity of *Micrococcus* MCCB 104 was assessed on larvae of *Macrobrachium rosenbergii* (Stage PL1) brought from a commercial hatchery in Kerala. They were maintained for 3 d in 10 ppt seawater and were fed with sterile lab-

oratory-made egg custard. Apparently healthy larvae were then distributed, 50 each, to 15 l fibreglass tanks containing 10 l of 10 ppt, autoclaved seawater. *Micrococcus* MCCB 104 culture grown for 24 h was scraped from the surface of ZoBell's agar plates into sterile saline and centrifuged at 3000 × *g* for 10 min. The pellet was resuspended in fresh saline and adjusted to an A_{600nm} value of 1.0 (corresponding to 10^9 CFU ml^{-1}). The above suspension was added to the larval rearing water to give a final cell count of 10^8 CFU ml^{-1}. The control tank did not have any bacterial inoculum supplemented. The above experiment was done in triplicate. Larvae were monitored for 4 d and observed for mortality.

RESULTS

The bacterium MCCB 104 is Gram-positive and forms yellow pigmented colonies of non-motile cocci on ZoBell's agar with the ability to grow at pH 6.0, 37°C and in 7.5% NaCl. It is glucose non-fermentative, produces glucose acid, mannose acid, oxidase and catalase, but not arginine dihydrolase. MCCB 104 reacts positive to the nitrate reduction test and citrate utilisation test and negative to the indole test; it is able to utilise D-mannose, D-xylose, D-mannitol, sucrose, arabinose, raffinose, but not meso-inositol, D-trehalose, adonitol, D-cellobiose and galactose as sole carbon sources. It was resistant to antibiotic furazolidone and sensitive to novobiocin, bacitracin, ampicillin, streptomycin, rifampicin, neomycin, erythromycin, kanamycin, chloramphenicol, ciprofloxacin, oxytetracycline, nitrofurantoin and the vibriostatic compound O/129. It can hydrolyse gelatine, starch and cellulose, but not tributyrin and chitin. The above characteristics equate Culture No. MCCB 104 to the genus *Micrococcus* (Buchanan & Gibbons 1974). Accurate taxonomical positioning awaits results of 16S ribosomal DNA sequencing.

Table 2. *Micrococcus* MCCB 104. Effect of pH, temperature and salt (NaCl) concentration on growth (+: visible growth; ++: moderate growth; +++: excellent growth; –: no growth) and size of inhibition zone

Parameter	Growth	Zone of inhibition (mm) at 24 h
pH		
4, 5	–	0
6	+	13
7, 8, 9	+++	15
10, 11	++	10
Temperature of incubation (°C)		
4	–	0
15	+	0
20	+	12
25	++	14
30	++	14
35	+++	16
40	+++	16
45	–	0
Salt concentration (%)		
0	+	12
0.5, 1.0, 1.5	+++	16
2.0	++	14
2.5, 3.0	++	14
3.5	+	0

An antagonism assay by disc diffusion showed that *Micrococcus* MCCB 104 inhibited *Vibrio* and *Aeromonas* isolates (Table 1), with clearing zones of 15 to 20 mm diameter. However, yeasts, actinomycetes, *Bacillus* and *Pseudomonas* isolates, along with 2 isolates of *V. splendidus* II were resistant. An antagonism assay conducted with cell-free culture supernatant of *Micrococcus* MCCB 104 revealed similar inhibitory activity.

The antagonistic substance produced by *Micrococcus* MCCB 104 was detectable by disc diffusion assay in batch culture from 12 h of growth onwards. Growth was in stationary phase by 42 h and declined at about 48 h of post-inoculation. Production of the antagonistic substance peaked at the stationary phase, and maximum antagonistic activity was observed at 42 h of growth (Fig. 1).

In co-culture, growth of *Vibrio alginolyticus* MCCB 112 was inhibited by *Micrococcus* MCCB 104 when inoculated at an initial level of 10^5 to 10^7 CFU ml^{-1} (Fig. 2). All counts of the strain MCCB 104 in the co-culture

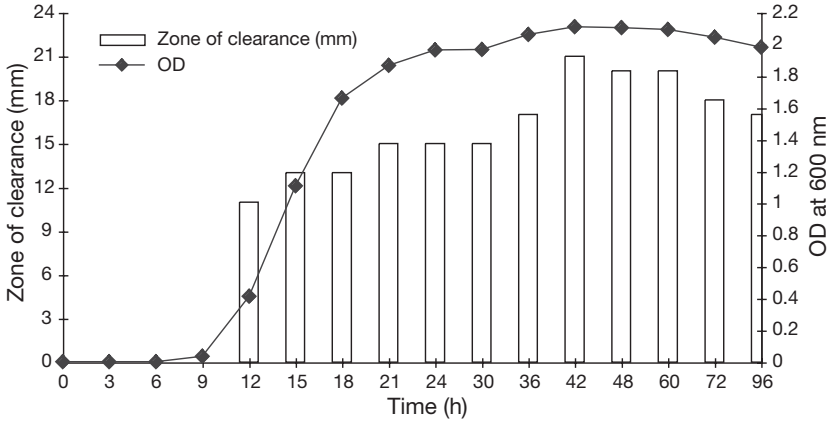

Fig. 1. *Micrococcus* MCCB 104. Growth and antagonistic activity against *Vibrio alginolyticus* MCCB 112. OD: optical density

Table 3. *Macrobrachium rosenbergii.* Pathogenicity test of *Micrococcus* MCCB 104 on larvae (PL1) (n = 3)

Treatment groups	Number of larvae that survived (of 50)			
	24 h	48 h	72 h	96 h
Micrococcus MCCB 104 (1.6×10^8 CFU ml^{-1})	48 ± 1	45 ± 1	44 ± 1	41 ± 2
Control	46 ± 2	44 ± 3	41 ± 2	36 ± 6

allowed initial growth of *V. alginolyticus*, but it never attained the cell count of the control. Flasks inoculated with the highest count (10^7 CFU ml^{-1}) of *Micrococcus* MCCB 104 showed the lowest count of *V. alginolyticus* (approx. 10^4 CFU ml^{-1}).

The activity of the cell-free culture supernatant remained relatively stable after the treatments with α-chymotrypsin, trypsin, Proteinase K, pronase E, lysozyme, lipase, catalase and α-amylase, as the zones of inhibition (16 mm) obtained were similar to that of control (untreated cell-free culture supernatant). Similar antagonistic activity was observed when the cell-free culture supernatant was neutralised to pH 7.0. There was no loss of antagonistic activity even when supernatants were autoclaved at 121°C for 15 min, indicating extreme heat stability of the substance.

pH 7.0, 8.0 and 9.0 were found to be the optima with a corresponding zone of inhibition of 15 mm diameter (Table 2). There was no growth at ≤pH 5.0. Growth was maximum at 35 and 40°C, with a 16 mm diameter zone of inhibition. There was no growth at 4 or 45°C. Maximum growth was observed in the medium containing 0.5, 1 and 1.5% NaCl, with a 16 mm diameter zone of inhibition, and minimal at 3 and 3.5% NaCl, with no antagonistic activity.

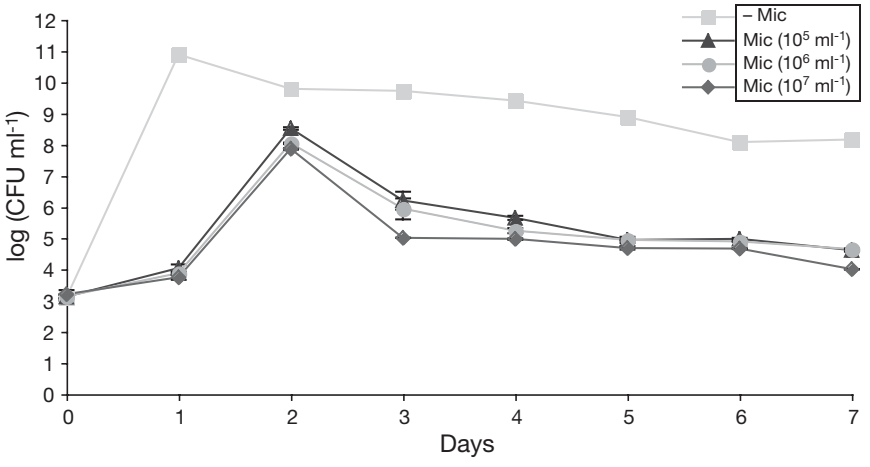

Fig. 2. *Vibrio alginolyticus* MCCB 112. Growth at 28°C in ZoBell's broth (15 ppt) with and without *Micrococcus* MCCB 104 (Mic) at different initial cell densities. Error bars are mean ±SE

Survival of *Macrobrachium rosenbergii* post-larvae was not affected by the challenge of *Micrococcus* MCCB 104 in larval rearing water (Table 3).

DISCUSSION

Micrococcus spp. are typical components of the heterotrophic bacterial microflora of various environments and have been recovered from soil (Rahman et al. 2002), seawater (Tanaka et al. 2001), marine sediments (Zhong et al. 2002) and prawn-rearing water (Phatarpekar et al. 2002). The *Micrococcus* MCCB 104 isolated in the present study from hatchery seawater inhibited several of the pathogenic isolates of *Vibrio* and *Aeromonas* spp. tested here. Results of the antagonism assay using the disc diffusion method with cell-free culture supernatant of *Micrococcus* MCCB 104 suggest that an extracellular product is likely to be responsible for the observed antagonism. Strains of *Micrococcus* spp. have been generally used as starter cultures in fermented beef and pork sausages (Bacus 1984) as these can prevent the growth of pathogenic-microorganisms by organic acid production (Schillinger & Lucke 1990). Irianto & Austin (2002a) reported that an isolate of unidentified Gram-positive cocci had potential in combating *A. salmonicida* infections in rainbow trout *Oncorhynchus mykiss* (Walbaum). It is noteworthy that many of the *Vibrio* species tested here are reported pathogens in aquaculture systems (Sindermann 1990, Austin & Austin 1993).

Co-culture experiments showed that the isolate *Micrococcus* MCCB 104 inhibited the growth of *Vibrio alginolyticus* when the initial count of the antagonist was 100 to 1000 times greater than that of the prawn pathogen. This indicates that the antagonist must be present at significantly higher levels than the pathogen, and the degree of inhibition increases with increased levels of the antagonist. Therefore, as a potential antagonistic probiotic, *Micrococcus* MCCB 104 has to be supplied in sufficient quantity and on a regular basis into the system.

Pridmore et al. (1996) reported that variacin, a bacteriocin produced by *Micrococcus varians*, inhibited other Gram-positive bacteria, but not Gram-negative bacteria. El-Shafei (1997) observed that the introduction of *Micrococcus* to fungal cultures resulted in lysis and inhibition of fungal growth, and attributed this to the pro-

duction of mycolytic enzymes. Preliminary characterisation of the antagonistic substance in the cell-free culture supernatant of *Micrococcus* MCCB 104 suggested that it was a non-proteinaceous compound. Moreover, the compound was found to be highly heat-stable and survived autoclaving. This thermostability should prove to be of greater use if the compound is incorporated into feed, where exposure to high temperature is unavoidable. At present, work is in progress to purify and further characterise the antagonistic substance from the culture supernatant.

The maximum production of antagonistic substance was observed at pH 7.0 to 9.0 and at temperatures of 35 to 40°C, which was also the optimum pH and temperature for the growth of the organism. This suggests a close relation between growth of *Micrococcus* MCCB 104 and the production of the antagonistic substance. This was further confirmed by the results in Table 2, which show that production of the antagonistic substance was maximum at NaCl concentrations between 0.5 and 1.5%, which was also optimal for growth of the organism.

One of the most important criteria for considering a bacterium as a candidate to be used in biocontrol programs in any culture system is its non-pathogenicity (Verschuere et al. 2000). This study has clearly shown that *Micrococcus* MCCB 104 is a non-pathogen to prawn larvae, even at 10^8 CFU ml^{-1}. Moreover, *Micrococcus* species have not been recognised and reported elsewhere as prawn pathogens. The present work strengthens the candidature of *Micrococcus* MCCB 104 as an 'antagonistic probiotic' in prawn larval rearing systems.

To our knowledge, this is the first report of an isolate of *Micrococcus* with properties antagonistic to vibrios associated with prawn larval rearing systems, although its identity is tentative and awaits DNA sequencing results. In this research it has been demonstrated that *Micrococcus* MCCB 104 can suppress vibrios *in vitro*. The nature of the antagonistic substance remains to be established; further work is underway in this regard. In conclusion, the study indicates the possibility of developing a biological method of suppressing or excluding vibrios associated with prawn larval rearing systems using *Micrococcus* MCCB 104.

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