Full Length Research Paper

Evaluation of *in vitro Vibrio* static activity of *Shewanella algae* isolated from healthy *Penaeus monodon*

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To conquer disease problem in shrimp industries, probiotic biocontrol is a well known remedy now. The antagonistic ability of separated isolates from different parts of juvenile *Penaeus monodon* were screened against shrimp *Vibrio* pathogens; *Vibrio parahaemolyticus* and *Vibrio* alginolyticus. The most antagonistic effect was observed for an isolate that was primarily identified as *Shewanella algae* using conventional methods followed by Biolog microlog software. Since production of antagonistic agents rely on cultural conditions, antagonistic ability of candidate probioic against the mentioned *Vibrios* was assessed using Response Surface Methodology, with central composite design in which four independents variables were assumed: temperature (10 - 50°C), pH (6 -10), NaCl concentration (0 - 50%) and time (12 – 60 h). The coefficients of multiple determinations (R^2), for the responses of antagonistic effect of *S. algae* against *V. parahaemolyticus* and *V. alginolyticus* values were 0.807 and 0.805, respectively. Concentration of the NaCl exhibited least influence on the antibacterial effect of candidate probiotic while the other independent variables exhibited different degree of affect. The candidate probiotic revealed a reasonable antibacterial response in quite a wide range of temperature and pH in which the maximum levels were in the same range of optimum shrimp culture.

Key words: Antagonism, *Penaeus monodon*, probiotic, *Shewanella algae*, *Vibrio parahaemolyticus*, *Vibrio alginolyticus*

INTRODUCTION

Aquaculture industrial development has been accompanied by some practices potentially damaging to human and animal health (Goldburg and Naylor, 2005; Naylor and Burke, 2005) that include passing large amounts of veterinary drugs into the environment (Haya et al., 2000; Boxall et al., 2004) intended for disease prevention owing to intensification (Shariff et al., 2001). The search for technological solutions to problems related to high density aquaculture practices has resulted in a proliferation of often unproven and potentially dangerous solutions. Products and procedures such as chlorination, antibiotics, and even toxic insecticides are introduced as solutions for problems in aquaculture Balcázar and Cunningham, 2007). On account of the increasing concern over the potential harm of aquaculture effluents to

receiving water bodies, worries over the contamination of aquatic food products with bioaccumulative and potentially harmful chemicals and antibiotics, and human risks associated with contaminative aquatic products, research and application of probiotics is progressively increasing in aquaculture throughout the world (Wang et al., 2005). The use of microbial probiotics in aquaculture is now widely accepted for the control of pathogens as a better remedy than administering conventional antibiotics and disinfecting agents (Mohanty et al., 1993, 1996; Gatesoupe, 1999; Gomez-Gil et al., 2000; Sharma and Bhukhar, 2000; Irianto and Austin, 2002; Vine et al., 2006; Wang and Xu, 2006; Wang, 2007). The most recent studies concerned with the effects of probiotics on cultured aquatic species have emphasized on decreasing of mortality or, conversely, increasing of survival rate (Moriarty, 1998; Skjermo and Vadstein, 1999; Chang and Liu, 2002; Irianto, Austin, 2002). The potential of a candidate probiotic is due to its ability to antagonize the common putative pathogen bacteria (Jöborn et al., 1997), which may take place owing to competitive exclusion by

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which the probiotic antagonizes the potential pathogen by the production of inhibitory compounds or by competition for nutrients, adhesion sites or oxygen in the digestive tract (Fuller, 1987). *In vitro* antagonistic test is used as first step for a probiotic screening (Chythanya et al., 2002; Vaseeharan and Ramasamy, 2003; Ravi et al., 2007). The candidate probiotic need to be effective over a range of temperature and pH extremes and variations in salinity (Fuller, 1987).

Therefore the on going study was carried out after primary antagonistic study on 118 isolates separate from cultured *Penaeus monodon*. The *in vitro* antagonistic potential of the best probiotic candidate in extreme salinity, pH and temperature over a period of time was examined to evaluate conditions for best antagonistic response against shrimp *Vibirio* pathogens.

MATERIALS AND METHODS

Isolates and identification of bacteria flora of *P. monodon*

To prevent bias in sampling from commercial shrimp farms and hatcheries which usually utilize commercial probiotic additives, healthy post larvae of *P. mondon* was purchased from local hatchery and reared in the Hatchery complex, Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia. The seeds were kept in 4 circular fiber glass tanks (2 m, diameter and 1 m height) with conical bottoms equipped with air stones in a static water regime. They were feed with probiotic free commercial pellet for 2 months before being used for bacteria flora sampling. During rearing period, pH, salinity and temperature was monitored daily using a pH meter (YSI, USA) and a hand refractometer (Atago 8808). A total number of 25 juvenile shrimp from each tank were scooped randomly. The collected shrimps were weighted before being dissected. Then different parts of juvenile *P. monodon* shrimp, hepatopancreas and gut, muscles, and body surface together with tanks water and tanks sediments were subjected for isolating of bacteria flora in sterile condition. The pure cultures of isolate bacteria flora were kept in LB broth (Defco, USA) containing 15% glycerol at -80°C. The isolates were identified up to genus by conventional biochemical methods and confirmed using Biolog Microlog software (Olsson et al., 2004).

Pathogens and candidate probiotic

Two shrimp pathogens, *Vibrio parahaemolyticus* and *Vibrio alginolyticus* were previously isolated from a moribund *P. monodon*. These isolates were cultured on thiosulphate citrate bile salts sucrose agar medium, TCBS agar (Difco, USA) and were used as target organisms, against which the probiotic bacteria were screened for antagonistic activity from 118 isolate. A candidate probiotic which was isolated from digestive truck of a healthy *P. monodon* shrimp was identified primarily as *Shewanella algae*. Conventional biochemical methods (Finegold and Baron, 1986) followed by Biolog Microlog software (Olsson et al., 2004) were applied for all identifications.

Primary screening by well diffusion method

The pathogens, *V. parahaemolyticus* and *V. alginolyticus*, were cultured overnight in Mueller Hinton broth supplemented with 2% NaCl at 30°C, turbidity of the cultured broths were adjusted to 0.5 MacFarland standard, before being spread over Mueller Hiton agar

plates containing 2% NaCl, in triplicate. Wells with 4 mm diameter were punched on agar and 60 µl of over night cultured of candidate probiotic were dispensed into the wells after measuring their turbidities at 625 nm. Normal saline and 0.2 µg/µl of tetracycline served as negative and positive controls, respectively. Inhibitory zone around each well was measured and the bacteria which produced inhibitory zone were selected for further studies.

Assessment of antagonistic effect in different temperature, pH, NaCl concentration and time using cross streak method

Modified cross streak method was used for further assessment of the bacterium which previously showed the highest inhibitory zone against *V. parahaemolyticus* and *V. alginolyticus* (Chythanya et al. 2002). 18 h culture of candidate probiotic in Mueller Hinton broth containing 2% NaCl (Difco, USA) were streaked in 2 cm thick bands in diameter of Mueller Hinton agar plates (Difco, USA) which were supplemented with different levels of NaCl. Their pH was subsequently adjusted to different levels after the media has been prepared and autoclaved by titrating a sample with filtered sterile 1 N NaOH and 1 N HCl using a pH meter (Delta 320, China). Then the titration volume was applied to the whole media (Table 1). Plates were incubated for different period of times in variable temperatures as were documented in Table 1. After incubation in the desired temperature has been completed, the bacteria were scraped by a sterile slide, and the remained bacteria then were killed by exposing the plates to chloroform gas for 15 min. 18 h culture of two pathogen bacteria were streaked perpendicular to candidate probiotics band after their turbidities were adjusted to 0.5 MacFarland standard using a spectrophotometer (Thermospectronic, Genesys 20, USA) (Chythanya et al., 2002). Sterile normal saline and a 18 h cultured of candidate probiotic in Mueller Hinton agar after adjusting to 0.5 MacFarland standard were streaked as negative and positive control, respectively. The linear zones of inhibitory were recorded.

Experimental design

Response surface methodology (RSM) was selected design for study the *in vitro* linear antagonistic zone of *S. algae* cultured in different levels of four independent variables: temperature (x_1), pH (x_2), salinity (x_3) and time (x_4) against *V. parahaemolyticus* and *V. alginolyticus*. The coded and uncoded independent variables used in RSM designed are documented in Table 1. Central composite design with total 31 experimental runs containing 7 central points was used as listed in Table 2; the experiments were carried out in random order.

A second-order polynomial equation was applied to explain the *in vitro* antagonistic ability of *S. algae* against *V. parahaemolyticus* (Y_1) and *V. alginolyticus* (Y_2) as a function of the independent variables as follows:

$$Y = \alpha_0 + \sum_i \alpha_i x_i + \sum_{ii} \alpha_{ii} x_i^2 + \sum_{ij} \alpha_{ij} x_i x_j$$

Where Y_i represents the response variables, α_0 is a constant, α_i, α_{ii} and α_{ij} are the linear, quadratic and interactive coefficients, respectively. The coefficients of the response surface equation were determined using statistical software (Minitab 15, USA).

Statistical analysis

The possible differences of the temperature, pH and NaCl concentration within the larval rearing tanks were analyzed using Analysis of Variance (ANOVA). Experimental data was analyzed by

Table 1. Coding and ranges of different variables studied.

Ariable	Symbol	Coded levels				
		-α	-1	0	+1	+α
Temperature (°C)	x_1	10	20	30	40	50
pH	x_2	6	7	8	9	10
NaCl (‰)	x_3	0	12.5	25	37.5	50
Time (h)	x_4	12	24	36	48	60

Table 2. Central composite design for *Vibrio* static responses.

Run order	Temperature (°C)	pH	NaCl (%)	Time (h)	*Vibrio* static responses (mm)			
					V. parahaemolyticus		*V. alginolyticus*	
					Exp.	Pre.	Exp.	Pre.
1	40	9	37.5	24	24	16.8	22	18.3
2 (CP)	30	8	25.0	36	30	30.1	27	29.9
3	30	6	25.0	36	21	13.0	18	11.5
4	40	9	12.5	24	0	10.6	0	10.5
5	40	7	12.5	48	0	9.4	0	8.2
6	10	8	25.0	36	0	-4.3	0	-3.5
7	20	9	12.5	48	13	9.1	12	8.2
8	20	7	37.5	48	21	18.9	26	23.8
9	30	8	25.0	12	0	2.2	0	0.8
10	20	9	37.5	48	0	13.9	0	15.0
11	20	7	12.5	24	22	21.1	18	17.2
12	40	7	37.5	24	0	12.4	0	12.2
13	30	8	25.0	60	18	4.0	18	5.5
14 (CP)	30	8	25.0	36	31	30.1	30	29.9
15	40	9	37.5	48	0	9.4	0	9.2
16	30	8	50.0	36	52	40.5	60	44.5
17 (CP)	30	8	25.0	36	30	30.1	27	29.9
18 (CP)	30	8	25.0	36	30	30.1	30	29.9
19	50	8	25.0	36	0	-7.5	0	-8.2
20	40	7	37.5	48	0	4.2	0	6.0
21	40	7	12.5	24	33	22.4	30	18.3
22 (CP)	30	8	25.0	36	33	30.1	33	29.9
23	20	9	12.5	24	0	-1.0	0	-2.7
24	40	9	12.5	48	0	-1.6	0	-2.7
25	30	8	0.0	36	46	45.7	40	43.8
26	20	7	12.5	48	20	30.4	24	31.0
27	30	10	25.0	36	0	-3.8	0	-5.2
28 (CP)	30	8	25.0	36	27	30.1	29	29.9
29	20	7	37.5	24	0	4.9	0	6.0
30 (CP)	30	8	25.0	36	30	30.1	33	29.9
31	20	9	37.5	24	0	-0.9	0	0.2

CP: central point, Exp: experimental, Pre: predicted.

multiple regressions to fit the second order polynomial equation to all independent variables. Analysis of variance (ANOVA) was performed to evaluate significant differences between independent variables. To visualize the relationships between the responses and the independent variables, surface response and contour plots of the fitted polynomial regression equations were generated using statistical software (Minitab 15, USA).

RESULTS

The possible different among water quality (physicoche-

Table 3. ANOVA table for the regression model of Vibrio static responses of *S. algae* against *V. parahaemolyticus* and *V. alginolyticus*.

Source	V. parahaemolyticus		V. alginolyticus	
	Regression coefficient	p-Value	Regression coefficient	p-Value
x_0	-372.304	0.028	-420.738	0.016
x_1	4.630	0.069	4.387	0.089
x_2	81.488	0.018	88.845	0.012
x_3	-4.402	0.029	-3.911	0.053
x_4	-4.402	0.038	5.267	0.019
x_1^2	-0.090	0.000	-0.089	0.000
x_2^2	-6.379	0.003	-6.673	0.002
x_3^2	0.021	0.090	0.023	0.069
x_4^2	-0.047	0.002	-0.046	0.002
$x_1 x_2$	0.256	0.302	0.300	0.238
$x_1 x_3$	0.013	0.524	0.010	0.617
$x_1 x_4$	-0.046	0.034	-0.050	0.026
$x_2 x_3$	0.325	0.110	0.280	0.172
$x_2 x_4$	0.016	0.939	-0.063	0.763
$x_3 x_4$	0.008	0.628	0.007	0.688
R^2	0.807		0.805	

mical conditions) of shrimp larval raring tanks and shrimp body weight were analyzed. There were no significant difference between temperature, pH and salinity of different rearing tanks. Those were within suitable range for shrimp larvae culture, 29 ± 0.7°C, 7.9 ± 0.1, 25 ± 1%, respectively. No mass mortality or disease was observed within rearing period. The resultant statistical analysis between juvenile shrimp body weight of different larval tanks did not exhibited any significant difference (P<0.05), as well.

Fitting the model

The linear inhibitory zone of *S. algae* against *V. parahaemolyticus* and *V. alginolyticus* obtained from all the experiments are recorded in Table 2. The experimental data was used to calculate the coefficients of the quadratic polynomial equations, which were used to predict the values of linear inhibitory zone of candidate probiotic against mentioned shrimp pathogen *Vibrios* (Table 2). Analysis of variance (ANOVA) showed that the resultant quadratic polynomial models adequately represented the experimental data with the coefficients of multiple determinations (R^2) for the responses of *in vitro* antagonistic effect of *S. algae* against *V. parahaemolyticus* and *V. alginolyticus* values being 0.807 and 0.805, respectively. This indicates that the obtained quadric polynomial models were adequate to describe the influence of the studied independent variables on the linear inhibitory zone produced by candidate probiotic against the mentioned *Vibrios*.

Analysis of variance (ANONA) was used to evaluate the significance of the coefficients of the quadric polynomial models (Table 3). A larger F-value and a smaller P-value would indicate a more significant effect on the respective response variables, for each term in the model (Quanhong and Caili, 2005). Therefore, The most significant term for linear inhibitory zone of *S. algae* against *V. parahaemolyticus* is quadric term of temperature (P < 0.001) followed by quadric term of time and pH (P < 0.01), linear term of pH, linear term of NaCl concentration, interaction of temperature and time and linear term of time (P < 0.05). The rest of the terms did not exhibited any significant effect on inhibition of *V. parahaemolyticus* (P > 0.05).

The variables having the largest effect on inhibitory zone produced by *S. algae* against *V. alginolyticus* was quadric term of temperature (P < 0.001) followed by quadric term of pH and time (P < 0.01), linear term of pH, linear term of time and interaction of temperature and time (P < 0.05). None of the remained terms showed any significant effect on the inhibition of the growth of *V. alginolyticus*.

Surface response analysis

To visualize the effect of the independent variables on the dependent ones, surface response of the quadric polynomial models were generated by varying two of the independent variables within the experimental range while holding the other two constant at the central point. The linear zone of antibacterial effect of *S. algae* against both pathogen vibrios, *V. parahaemolyticus* and *V. alginolyticus*, followed same pattern when pH and temperature
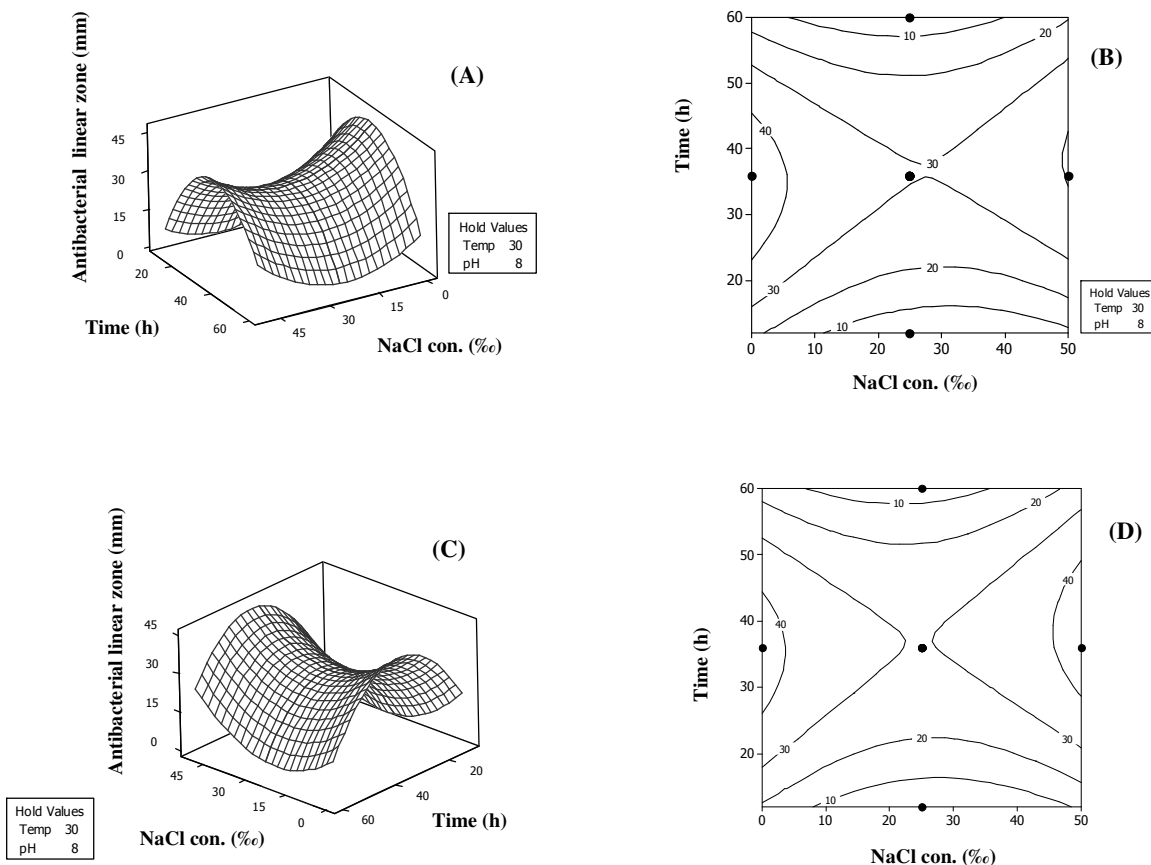
Figure 1. Surface and counter plots of antibacterial effect of *S. alga* against *V. parahaemolyticus*, (A and B) and *V. alginolyticus* (C and D) when temperature and pH were held constant at central point while time and NaCl concentration varied within experimental range.

were held constant at central point and varied time and NaCl concentration within experimental range. The highest antibacterial effect was observed in 25 - 45 h old cultured which have lowest and highest NaCl concentrations, zero and 50‰. The effect of time remained constant for both pathogens as the NaCl concentration changed. Antibacterial effect was highest in lowest NaCl concentration and decreased when its concentration increased up to 25‰, then antibacterial properties exhibited an incremental movement followed by increasing of NaCl concentration to the highest point, 50‰ (Figures 1A, B, C and D). Antibacterial property of *S. algae* against *Vibrio* pathogens was similar, when variables, pH and time, were varied within experimental range while temperature and NaCl concentration were held constant at central point. The highest antibacterial effect occurred at pH 7.5 - 8 in 35 - 40 h old culture followed by pH 7 - 9 and 25 - 50 h old culture (Figures 2A, B, C and D). When two critical shrimp growth factors, pH and NaCl concentration, were altered in experimental range, whilst the other two were held evenly at central point, the best antibacterial effect was exhibited in the lowest NaCl concentration in the pH range of 6 - 8.3. It thereafter reduced as NaCl concentration was raised to

25‰; incremental movement of antibacterial effect was raised after this to the highest NaCl concentration, 50%, even as pH range shifted to 7.5 – 9 (Figures 3A, B, C, and D). Antibacterial property of *S. algae* against mentioned *Vibrios* was evaluated when temperature and time differed within experimental ranges, at the same time as pH and NaCl concentration were held constant at central point. It has highest effect at around 20 - 40°C and in 25 – 55 h old cultured which is included the central points for temperature and time (Figures 4A, B, C and D). Antibacterial effect of candidate probiont against pathogen *Vibrios* were assessed when two crucial independent variables, temperature and NaCl concentration, were within the experimental range and other two were held at central point. The most effective range for temperature was 22 - 35°C which showed the highest activity at 30°C and remained relatively constant for different NaCl concentrations, but the highest antibacterial activity appeared in lowest zero (‰) NaCl concentration specially for *V. parahaemolyticus*. The antibacterial effect exhibited negative increment with increasing NaCl concentration to 25‰, then positive increment was observed continuously to the highest NaCl concentration, 50‰ (Figures 5A, B, C and D). The most
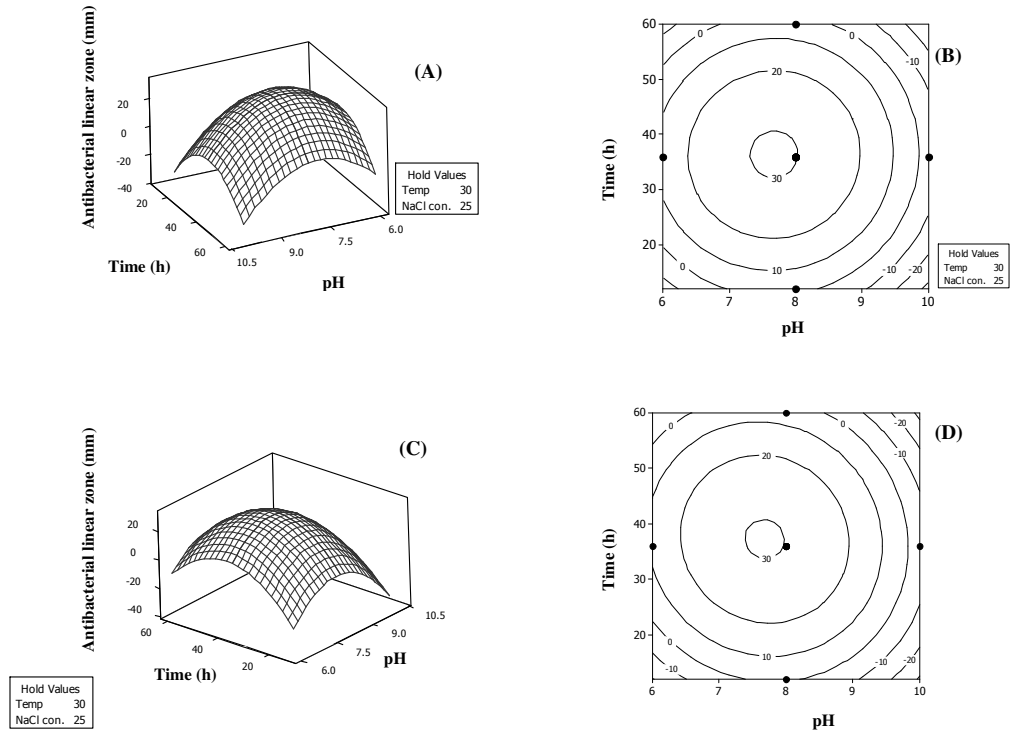
Figure 2. Surface and counter plots of linear antibacterial effect of *S. alga* against *V. arahaemolyticus*, (A and B) and *V. alginolyticus* (C and D) when temperature and NaCl concentration were held constant at central point while time and pH varied within experimental range.
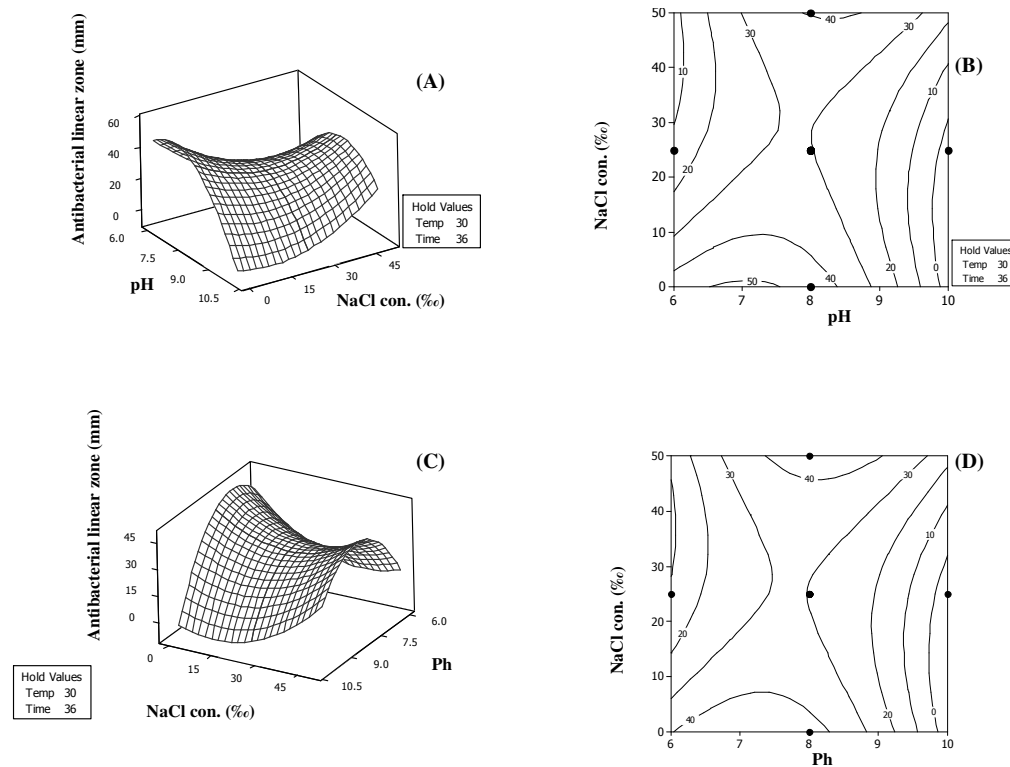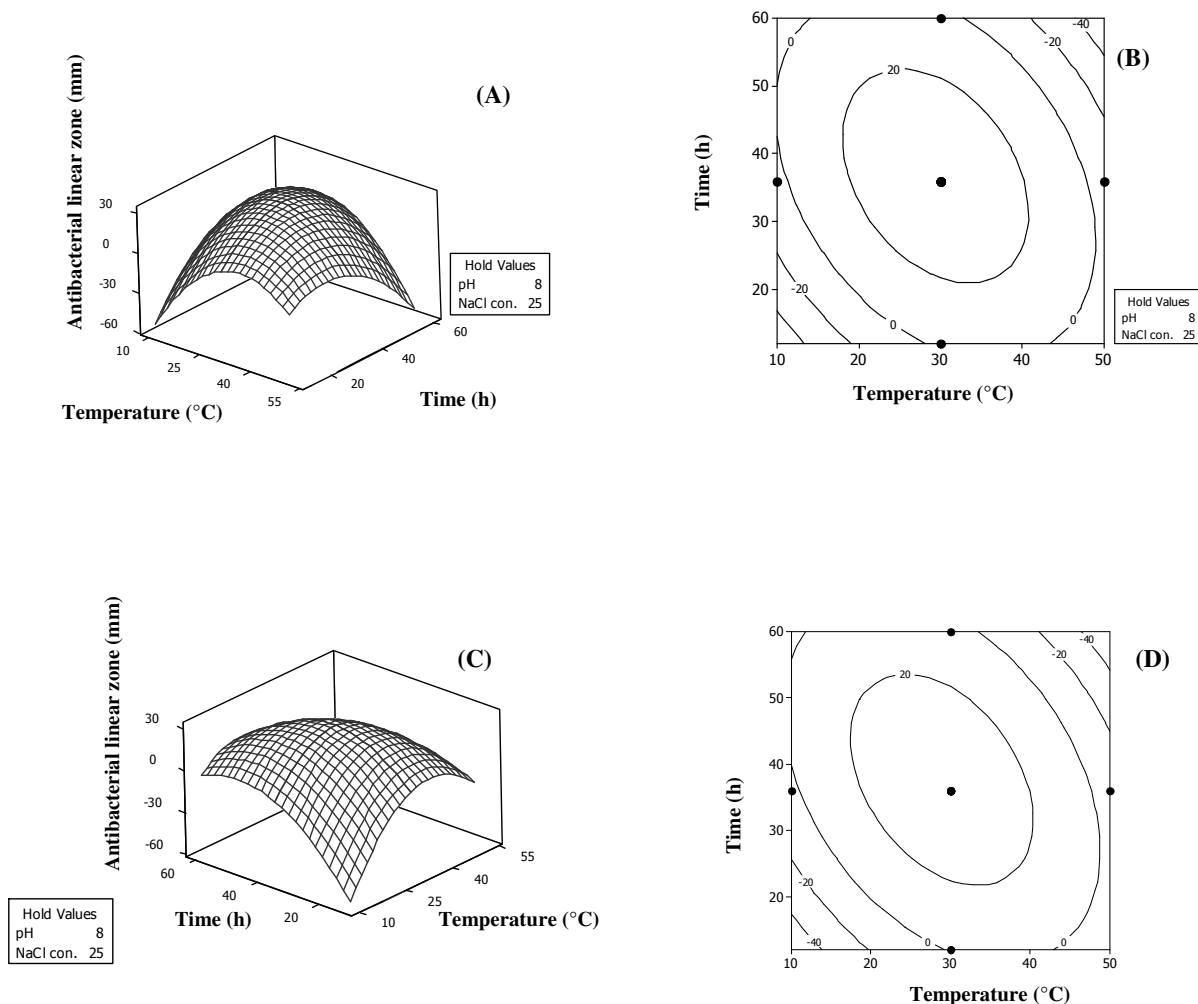

Figure 3. Surface and counter plots of antibacterial effect of *S. alga* against *V. parahaemolyticus* (A and B) and *V. alginolyticus* (C and D) when temperature and time were held constant at central point while pH and NaCl concentration varied within experimental range.

Figure 4. Surface and counter plots of antibacterial effect of *S. alga* against *V. parahaemolyticus* (A and B) and *V. alginolyticus* (C and D) when NaCl concentration and pH were held constant at central point while time and temperature varied within experimental range.

effective antibacterial propery of *S. algae* against experimental shrimp pathogen *Vibrios* was within 20 – 40°C and pH range of 6.5 - 9 (Figures 6A, B, C and D).

DISCUSSION

Attained results for antibacterial properties of *S. algae* against both experimental *Vibrios*, *V. parahaemolyticus* and *V. alginolyticus*, with different levels of four independent factors, temperature, pH, NaCl concentration and time were relatively similar. Antibacterial effect of *S. algae* against pathogen *Vibrios* is least affected by NaCl concentration which was the only alteration within results for the two *Vibrio* pathogens [NaCl concentration showed no significant ($P > 0.05$) effect on antibacterial properties of *S. alga* against *V. alginolyticus* while its linear coefficient was significant ($p < 0.05$) for *V. paraheamo-lyticus*].

It could be related to the unaltered antibacterial effect of candidate probiotic in different NaCl concentration against *V. alginolyticus*. Observation of relatively greater antibacterial effect against the two pathogen *Vibrios* in lowest NaCl concentration, zero ‰, minimum at central point, 25‰ NaCl concentration and again increasing to near maximum in high NaCl concentration, 50% (Figures 1 and 2) demonstrated surface and counter plots of NaCl concentration in opposition to time, pH and temperature, correspondingly. It might be due to synergistic effect of antibacterial production and lack of NaCl which is crucial for both *V. parahaemolyticus* and *V. alginolyticus* (Finegold and Baron, 1986). Although antibacterial effect in term of NaCl concentration at extreme experimental levels exhibited higher influence, it was indicated that appropriate NaCl concentration for *P. monodon* growth in ponds which is reported as 15 – 30‰ by Network of Aquaculture Centres in Asia, NACA,
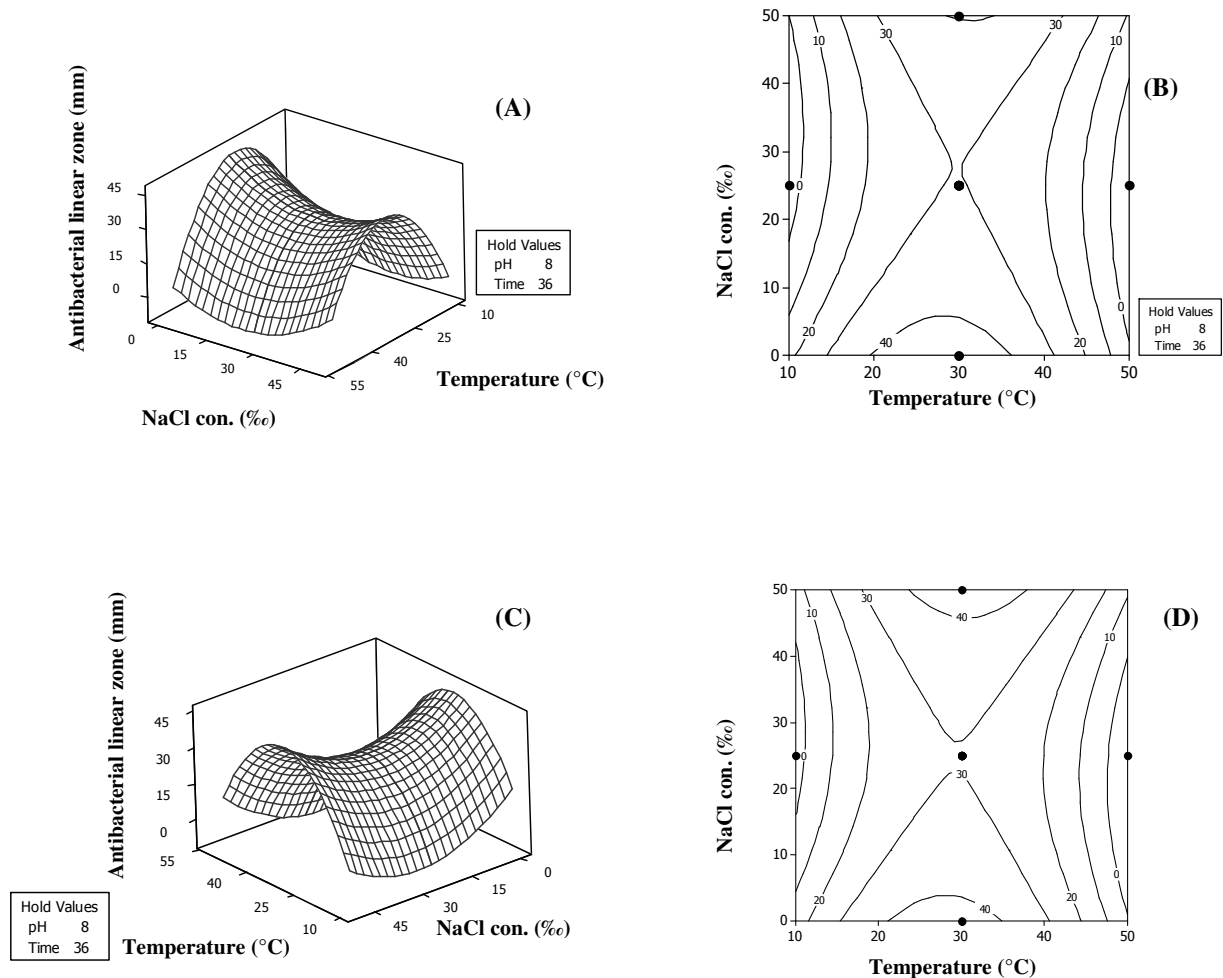
Figure 5. Surface and counter plots of antibacterial effect of *S. alga* against *V. parahaemolyticus* (A and B) and *V. alginolyticus* (C and D) when time and pH were held constant at central point while NaCl concentration and temperature varied within experimental range.

(Kungvankij and Chua, 1986) was not the most efficient concentration for inducing the highest antibacterial properties by candidate probiotic. Nevertheless, the existence of antibacterial potential at central point might be efficient to suggest the candidate probiotic for *in vivo* experiment. The ability of member of same genera, *Shewanella oneidensis* MR-1, to grow in broad range of temperature, 3 - 35°C (Abboud et al., 2005) and *Shewanella pealeana* which can grow in a range (6.5 – 7.5) of pH (Leonardo et al., 1999) has been previously acknowledged. However, many researchers have documented that production of bacteriocin compounds in extreme conditions are higher than the optimal growth conditions (Parente et al., 1994; Parente and Recciardi, 1994; Mortvedt-Abildgaard et al., 1995; Matsusaki et al., 1996; Krier et al., 1998). The highest antibacterial activity of *S. algae* against shrimp pathogen *Vibrios* was demonstrated in wide ranges of temperature (20 - 40°C)

and pH (6.5 – 8.8). In terms of temperature and pH, the highest antibacterial activity was observed in central point and lower than central point, 30°C and 7.5, respectively (Figures 1 and 2). These include the suitable temperature and pH ranges for shrimp culture (Kungvankij and Chua, 1986). Nevertheless, the range of pH and temperature in which *S. algae* exhibited highest antibacterial activity are completely broad and can compensate the *in vivo* fluctuations of pH and temperature. However, it quite agreed with the antibacterial linear inhibitory zone produced by *Pseudomonas* I-2 strain against *V. parahaemolyticus* at same temperature but 10‰ NaCl concentration during 24 h incubation period on Tripticase soy Agar (TSA) (Chythanya et al., 2002).

Though, the candidate probiotic exhibited relatively good *in vitro* antagonistic potential in wide range of temperature, pH and NaCl concentration, further *in vitro* and *in vivo* studies are required before introducing it as a
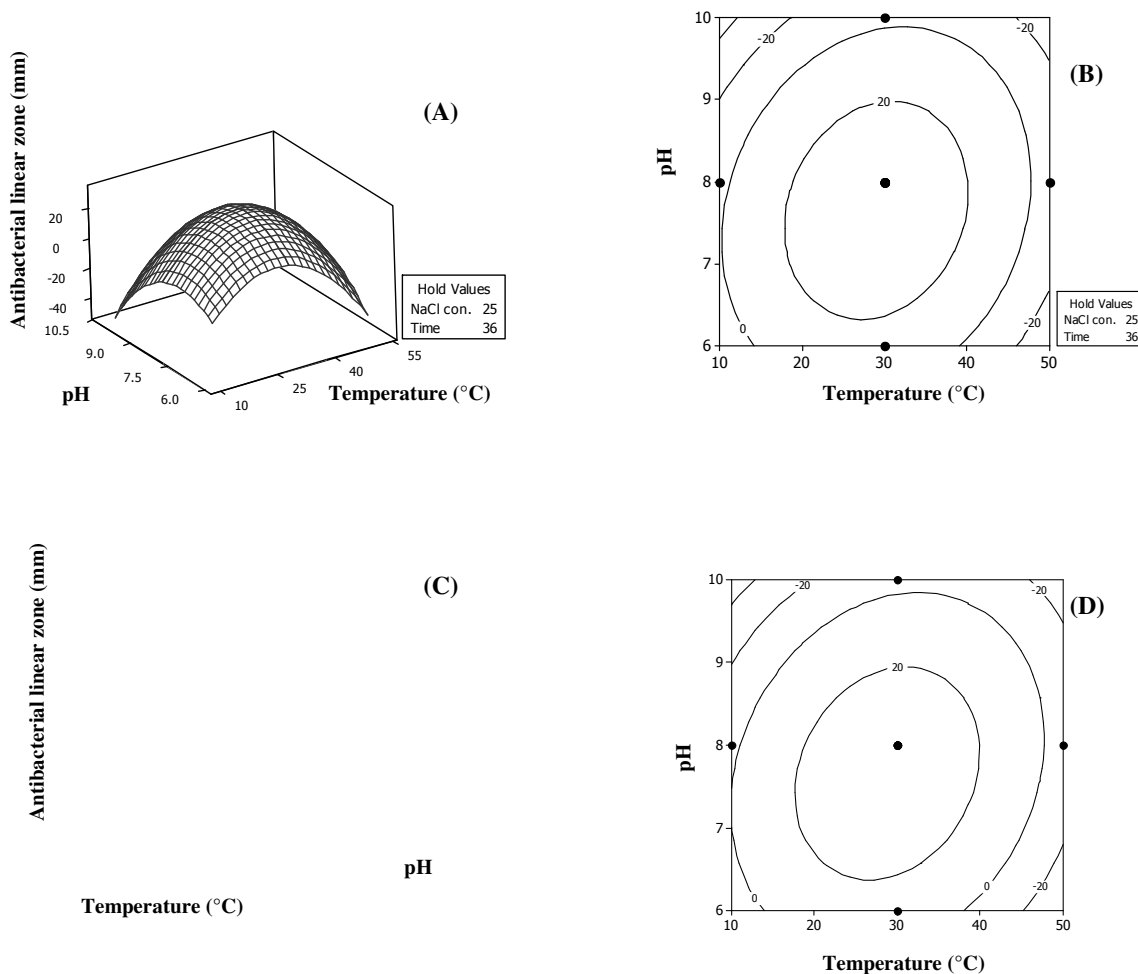
Figure 6. Surface and counter plots of antibacterial effect of *S. alga* against *V. parahaemolyticus* (A and B) and *V. alginolyticus* (C and D) when temperature and pH were held constant at central point while NaCl concentration and time varied within experimental range.

probiotic agent.

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