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Microbial probiotics for better fish survival and growth of Asian seabass (*Lates calcarifer*)

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Abstract
Survival and biomass production in seabass (*Lates calcarifer*) was assessed under different combinations of microbial mixtures of *Bacillus fluxus*, yeast and *Spirulina* by using the statistical approach of response surface methodology (RSM)**.** The fish biomass was 16% higher in probitoics mixture of bacillus (1.89 g/kg), yeast (2.77 g/kg) and Spirulina (5.27g/kg) than that fed with commercial feed alone. This work reiterated the potential of probiotics in better fish survival and growth of *Lates calcarifer* and the use of RSM in determining optimal probiotic mixtures.

Keywords: Probiotics, Bacillus, yeast, Spirulina, seabass, *Lates calcarifer*, fish biomass, survival.

1. Introduction
Lates calcarifer is called giant sea perch, Asian seabass or barramundi. This is euryhaline in nature and it can be reared at a wide range of salinities, and hence the seabass is considered to be a good candidate species for aquaculture, especially in South East Asian and Pacific countries [1]. The technology of seed production and larval rearing of the Seabass has been improved due to the application of the larval diets of *Brachionus*, *Atremia* and dry formulated diet. However, the cause of antimicrobial drugs, pesticides, chemicals and disinfectants in aquaculture usage for disease prevention and growth promotion has led to the questions of safety [2, 3]. In the recent years, the use of antibiotics such as oxytetracycline has been prohibited by several countries due to serious environmental hazards and carcinogenic effects in many teleost fish [4-6]. Hence, studies on safer diet supplements for cultivable fishes are of current importance.
Probiotics are defined as beneficial microbial diet supplements for the benefit of the cultivable organisms [7] such as fish and shrimps [8-11]. Several authors reported that lactic acid bacteria are commonly used as probiotics in animal nutrition which are *Lactobacillus bulgaricus*, *L. acidophilus*, *L. sporogenes*, *L. casei*, *L. plantarum* and *Streptococcus thermophillus* [5, 12, 13]. Cost of production is high due to intensive larval culture of carnivorous fishes like *Lates calcarifer* on live food. Hence, live feed along with the formulated diets can minimize the cost of production.
Response surface methodology (RSM) is a statistical technique, used for the optimization of particular response. It varied based on the factors used and optimized for the particular response. The present work was carried out for the optimization of the probiotic combination of different microbial biomass of *Bacillus flexus* along with yeast (*Pichia salcaria*) and cyanobacterium (*Spirulina*) for the enhanced survival and biomass production of seabass-*Lates calcarifer* by using RSM.

2. Materials and Methods
Microbial cultures
Bacillus flexus IML1 isolated from mangrove sediment was used for this experiment. In addition, yeast species (*Pichia salcaria*) and cyanobacterial species (*Spirulina* sp.) preserved in the Microbial Culture collections of CAS in Marine Biology, Annamalai University were also used. The cyanobaterial culture was developed in Marine SN medium, the bacillus culture in de Man Gogosa Sharpe medium and yeast culture in Yeast Malt broth medium. The biomass was shade-dried and incorporated with the commercial diet feed at different concentrations.

Optimization of the microbial probiotic combination for survival and biomass production of *Lates calcarifer* by Central Composite Desing (CCD)

The fish fries were procured from Central Institute of Brackish water Aquaculture (CIBA), Chennai, Tamil Nadu, India. For the transportation process the fish fries were packed with oxygen at the rate of 500 numbers per poly pack and without fed.

The present work was carried out for the optimization of the probiotic combination of different microbial biomass for the enhanced survival and biomass production of seabass-*Lates calcarifer* by using a statistical technique viz., Response Surface Methodology (RSM). In this regard, a randomized experiment was performed by 20 experimental runs of central composite design.

In this experimental study, fries of *Lates calcarifer* with size of 2 cm length and 0.1 g weight were stocked at the rate 5 individuals l^{-1}, fed in 20 aquaculture experimental tank with 100 l capacity, 110 cm length, 56 cm breath and 30 cm height, maintained with the water level at 20 cm height in the tank. The procured fries were acclimatized to the rearing conditions by placing the poly bags in experimental tanks for about 15 min followed by sprinkling of water from the tank to the poly bags before releasing the fry in to the tanks.

Different concentrations of microbial biomass such as *Bacillus flexus* IML1, yeast – (*Pichia salcaria*) and cyanobacterium (*Spirulina*) were used as feed to the fries of *Lates calcarifer* incorporated with the weaning feed (INVE) in the size of 500-800 micron and CIBA crumble feed 300-400 micron used daily, according to the 20 runs of batch experimental setup of response surface methodology (Table 1& Fig. 1). During larval rearing period, dissolved oxygen, salinity and pH were maintained at 4.5 mg.l^{-1}, 25 ppt and 7.8 respectively. The wastes from the water, faecal matter and uneaten feed were removed through siphoning method and made up with new water. This was done daily after a feed consumption was over. In order to monitor the growth, groups of larvae in triplicate were sampled from each tank at 7 days interval. Experimental and predicted responses of survival and biomass production are shown in Table 1. The coded values and actual factor value were calculated by using the following equations.

Yi (Survival of seabass) = $\beta_0 + \Sigma_i \beta_i X_i + \Sigma_i \beta_{ii} X_i^2 + \Sigma_{ij} \beta_{ij} X_i X_j$--- (1)

Y2 (Biomass production of seabass) = $\beta_0 + \Sigma_i \beta_i X_i + \Sigma_i \beta_{ii} X_i^2 + \Sigma_{ij} \beta_{ij} X_i X_j$ --- (2)

Where Yi is the predicted response, X_iX_j are independent variables, β_0 is the offset term, β_i is the i^{th} linear coefficient, βii is the ii^{th} quadratic coefficient, and β_{ij} is the ij^{th} interaction coefficient. The experiment design is presented in Table 1 along with experimental and predicted responses. However, in this study, the independent variables were coded as X_1, X_2, and X_3. Thus, the second order polynomial equation can be presented as follows equation 3:

$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1X_2 + \beta_{13}X_1X_3 + \beta_{23} X_2X_3$---------------------------------- (3)

The statistical model fitness was analyzed by variables of individual and interactions effects of the microbial biomass on the survival and biomass production of seabass by using ANOVA. It gives our model fitness and lack of the fit, if the case of our model was not significantly fit, it means that it is not an acceptable model for optimization of microbial probiotics. If the model probability was significant, it means that further analysis can be made on the interaction and individual effects on the case of survival and biomass production by drawing the surface plot and perturbation plots.

3. Results

Optimization of microbial probiotics for survival of *Lates calcarifer*

In order to optimize the combination of microbial probiotics on the fish survival, a randomized statistical model was used as a novel approach. Analysis of variance (ANOVA) of the regression model, interaction and combined effects of factors on the survival and biomass production were tested. The F value of 10.27 revealed that model was significant for fish survival (Table 2) as well as the model was also found to be significant, as was evident from the Fisher's F-test with a very low probability value ($P > 0.0006$). The quality of the model was tested by the determination coefficient R^2 and multiple correlation coefficients R^2. The value of adjusted R^2 (0.81) suggested that only 19% of the total variations in the fish survival response could not be explained by the model. The value of obtained R^2 (0.90) in the present experiment revealed the goodness of correlations between the experimental and predicted values of the fish survival. Lack-of-fit test was also analyzed. The F-value (54.32) obtained for the lack-of-fit test was not significant at 95% confidence limits, which further verified that the quadratic model was statistically valid. Validation of the model was also tested by plotting standard error in response (fish survival) as a function of a pair of factors. A plot of the standard errors in biomass production of responsible factors such as spirulina biomass and yeast biomass is shown in Fig. 2a. The shape of the low level of standard error plot and flat errors exhibiting circular contours and symmetrical shape around the centroid, representing ideal condition, the standard error value was fit with model and determined the standard error value of 0.52. It was the best value for the acceptable RSM statistical model. Generally the low level of the standard error is acceptable for a good experimental model. The regression analysis of the optimization study indicated that the model terms of bacillus and yeast biomass were significant on fish survival ($P < 0.05$). The response, fish survival (Y) was assessed by following regression equation (4):

$Y = 87.5 - 1.59 X_1 - 0.81 X_2 - 1.09 X_3 - 1.95 X_1^2 - 3.93 X_2^2 - 3.12 X_3^2 + 1.27 X_1X_2 + 1.52 X_1X_3 - 0.47 X_2X_3$ --------------------- (4)

Whereas: X_1 is yeast (%) (0–5 g/kg of feed), X_2 is spirulina (0–10 g/kg of feed), and X_3 is bacillus IML1 (0–5 g/kg of feed).

Further confirmation of the model fitness was plotted with experimental values and predicted value of model. This model was found fit with the experimental value (Fig. 2b,c). The final detection of the optimized value for the maximum fish survival is detected from perturbation plot (Fig. 2d).

Effect of combined of microbial biomass on survival of *Lates calcarifer*

In order to determine the optimal levels of each variable of microbial biomass for fish survival, three-dimensional response surface plots were constructed by plotting the response on the Z-axis against any two independent variables, while maintaining other variables at their central levels (Table 2, Fig. 3a-c). The higher survival was observed under the microbial probiotic combination of bacillus biomass of 1.96 g/kg, yeast biomass of 2.57 g/kg and spirulina biomass of 5.54 g/kg.

Optimization of the factors for the biomass of *Lates calcarifer*

In order to optimize the combination of microbial probiotics on the fish biomass, a randomized statistical model was attempted. Analysis of variance (ANOVA) of the regression model, interaction and combined effect of factors on the fish biomass production was tested. The probability value of 0.005 revealed that model was significant for fish biomass (Table 3). The quality of the model was tested by the determination coefficient R^2 and multiple correlation coefficients R^2. The value of adjusted R^2 (0.69) suggested that only 31% of the total variations in the fish biomass response could not be explained by the model. The value of obtained R^2 (0.83) in the present experiment revealed the goodness of correlations between the experimental and predicted values of the fish biomass. Lack-of-fit test was also not significant for the model response of fish biomass. The response, fish biomass (Y) was assessed by following regression equation (5):

$$Y\ (\text{Biomass}) = 1900.15 - 16.82X_1 - 161.07X_2 - 144.06\ X_3 - 108.51X_1^2 - 289.00_2^2 - 202.03X_3^2 + 16.25X_1X_2 + 10.5X_1X_3 + 27.25X_2X_3 \text{--} \quad (5)$$

Whereas: X_1 is yeast (%) (0–5 g/kg of feed), X_2 is spirulina (0–10 g/kg of feed), and X_3 is bacillus (0–5 g/kg of feed).

The final detection of the optimized value for the maximum biomass production was detected from perturbation plot (Fig. 4c).

Effect of combined of microbial biomass on fish biomass

In order to determine the optimal levels of each variable of microbial biomass for fish survival, three-dimensional response surface plots were constructed by plotting the response on the Z-axis against any two independent variables, while maintaining other variables at their central levels (Fig. 5a-c). The higher biomass production was observed in the fish fed with probiotic combination: bacillus (1.89 g/kg), yeast (2.77 g/kg) and spirulina (5.27 g/kg).

The biomass production (1905 g) was higher in the fish fed with optimal microbial mixed diet, than that (1641 g) in the control fish fed with commercial feed; Thus, there was an increment of about 16 % of biomass due to the microbial mixed feed as compared to commercial feed.

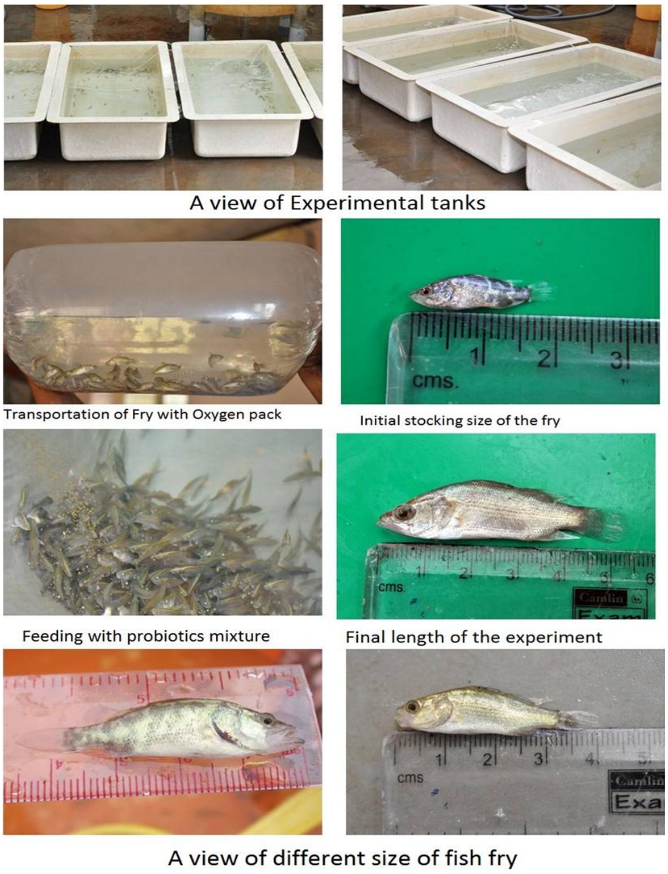

Fig 1: A view of experimental set up and formulation of mangrove-derived microbial probiotics for fish

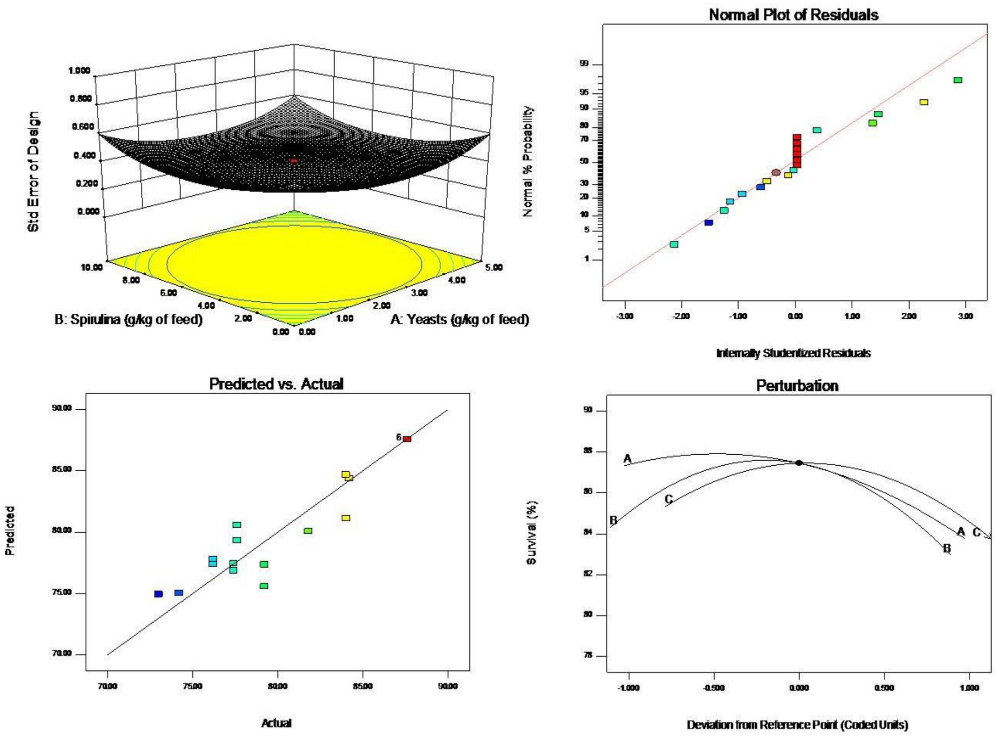

Fig 2: (a) Three-dimensional standard error plot for survival and biomass of *Lates calcarifer* (b) Normal plot for the residuals and normal percentage of probability for the response of predicted and experimental values (c) Predicted and actual experimental response for fish survival (d) perturbation plot for fish survival.

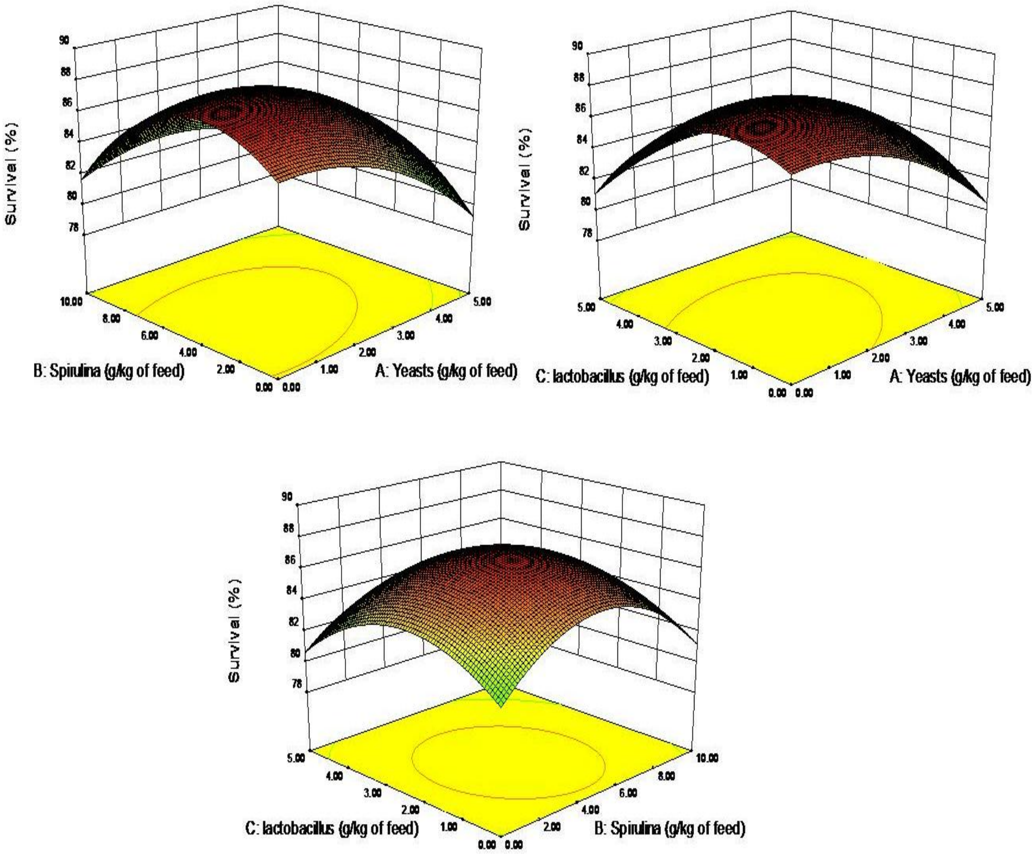

Fig 3: Three-dimensional response surface plot for the (a) Effect of yeast biomass and spirulina, (b) Effect of yeast and bacillus, (c) Effect of spirulina and bacillus, on response of fish survival.

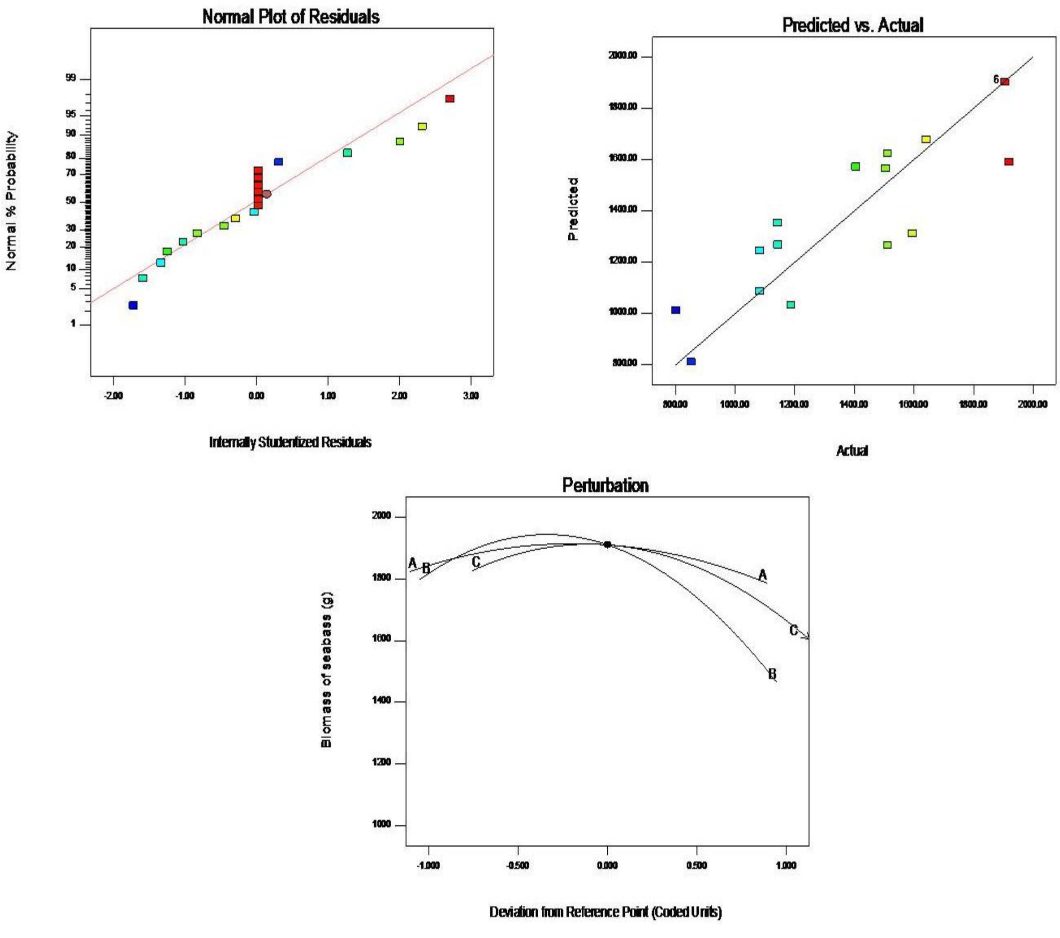

Fig 4: (a) Normal plot for the residuals and normal percentage of probability for the response of predicted and experimental values (b) Predicted and actual experimental response for fish biomass (c) Perturbation plot for fish biomass.

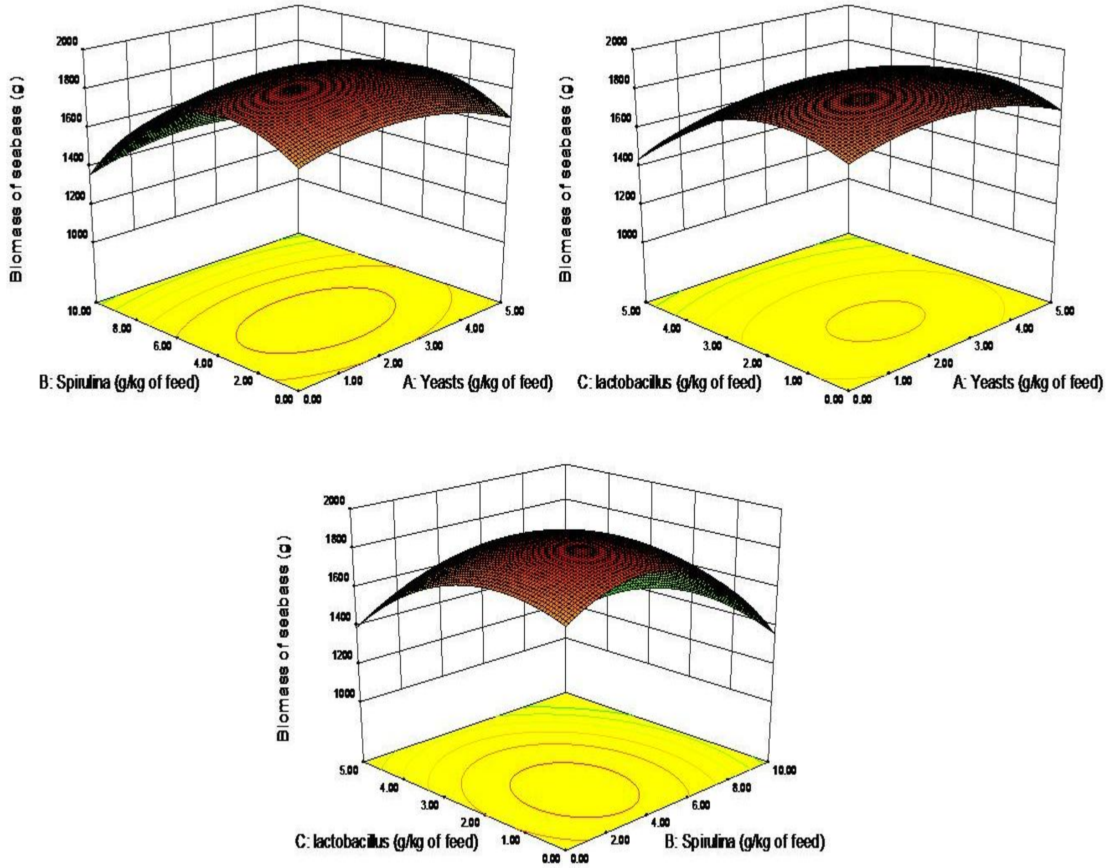

Fig 5: Three-dimensional response surface plot for the (a) Effect of yeast biomass and spirulina, (b) Effect of yeast and bacillus, (c) Effect of spirulina and bacillus, on response of fish biomass.

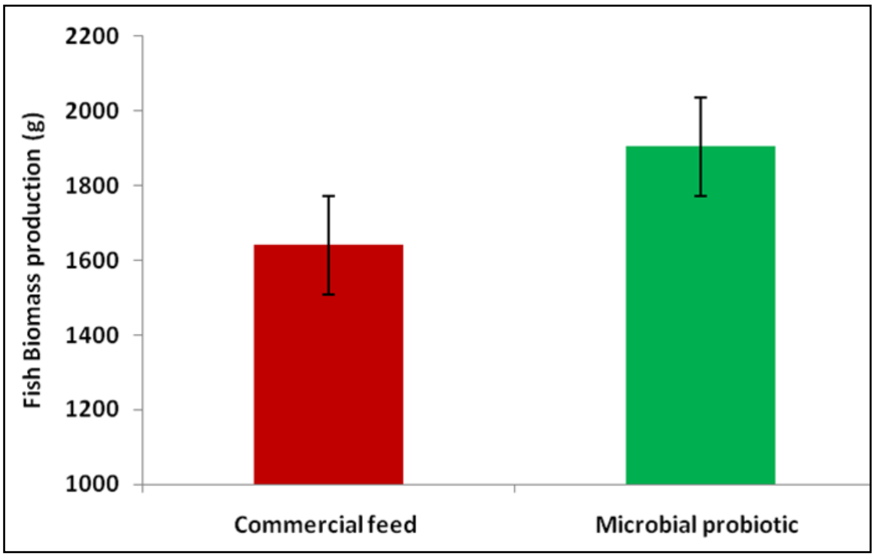

Fig 6: Comparison between microbial probiotics incorporated with commercial feed and commercial feed alone on fish biomass production in 30 days of culture

Table 1: Central composite design matrix for the experimental design and predicted responses for survival and fish biomass of *Lates calcarifer* (g/kg of feed)

Std	Run	Yeast (g/kg of feed)	Spirulina (g/kg of feed)	Bacillus (g/kg of feed)	Survival (%)		Fish Biomass (g)	
					Experi-mental	Predicted	Experi-mental	Predicted
1	20	0	0	0	84.2	84.3	1641	1676.5
2	15	5	0	0	79.2	75.5	1919	1589.4
3	8	0	10	0	84	81.1	1512	1267.4
4	12	5	10	0	77.4	77.4	1083	1245.2
5	6	0	0	5	81.8	80.0	1595	1312.9
6	2	5	0	5	76.2	77.3	1143	1267.7
7	16	0	10	5	73	74.9	803	1012.7

8	4	5	10	5	79.2	77.3	1188	1032.6
9	19	1.7	5	2.5	84	84.6	1512	1621.5
10	18	6.7	5	2.5	77.6	79.3	1505	1564.9
11	13	2.5	3.4	2.5	76.2	77.7	1143	1353.6
12	11	2.5	13.4	2.5	74.2	75.0	853	811.8
13	7	2.5	5	1.7	77.6	80.5	1405	1571.0
14	1	2.5	5	6.7	77.4	76.8	1083	1086.4
15	9	2.5	5	2.5	87.6	87.5	1905	1900.1
16	10	2.5	5	2.5	87.6	87.5	1905	1900.1
17	5	2.5	5	2.5	87.6	87.5	1905	1900.1
18	14	2.5	5	2.5	87.6	87.5	1905	1900.1
19	17	2.5	5	2.5	87.6	87.5	1905	1900.1
20	3	2.5	5	2.5	87.6	87.5	1905	1900.1

Table 2. Analysis of variance (ANOVA) for response surface methodology of main effects and interacting effects of parameters in quadratic model for fish survival

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	450.4697	9	50.05219	10.27295	0.0006***
A-Yeasts (%)	34.68213	1	34.68213	7.118327	0.0236*
B-Spirulina	9.125502	1	9.125502	1.872962	0.2011NS
C – Bacillus	16.33574	1	16.33574	3.352826	0.027*
AB	13.005	1	13.005	2.669209	0.1334NS
AC	18.605	1	18.605	3.81858	0.0792NS
BC	1.805	1	1.805	0.370467	0.5563NS
A^2	55.01348	1	55.01348	11.29123	0.0072**
B^2	223.0017	1	223.0017	45.76994	< 0.0001***
C^2	140.3338	1	140.3338	28.80279	0.0003***
Residual	48.7223	10	4.87223		
Lack of Fit	48.7223	5	9.744461	54.32	0.432NS
Pure Error	0	5	0		
Core Total	499.192	19			

Table 3: Analysis of variance (ANOVA) for response surface methodology of main effects and interacting effects of parameters in quadratic model for fish biomass

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	2352007	9	261334.1	5.824573	0.0055**
A-Yeasts (%)	3865.855	1	3865.855	0.086162	0.7751NS
B-Spirulina	354310.6	1	354310.6	7.896818	0.0185*
C-Bacillus	283462.3	1	283462.3	6.317763	0.0307*
AB	2112.5	1	2112.5	0.047083	0.8326NS
AC	882	1	882	0.019658	0.8913NS
BC	5940.5	1	5940.5	0.132401	0.7235NS
A^2	169711.4	1	169711.4	3.782501	0.0804NS
B^2	1203709	1	1203709	26.82807	0.0004***
C^2	588233	1	588233	13.11045	0.0047**
Residual	448675.1	10	44867.51		
Lack of Fit	448675.1	5	89735.02	54.34	0.876NS
Pure Error	0	5	0		
Cor Total	2800682	19			

Statistically significant *** (P <0.0001), *(P< 0.05), NS Non-significant

4. Discussion

The first application of probiotics in aquaculture used was *Bacillus toyoid* as food additive for increased growth rate of yellow tail *Seriola quinqueradiata* [14]. The use of probiotic *Streptococcus* strain as growth promoters of edible fishes has resulted in better growth of Nile tilapia (*Oreochromis niloticus*) [15]. Due to the commercial importance of this species, the effect of supplementing diet with probiotics (*Bacillus licheniformis* and *B. subtilis)* has produced an increase of 115.3% when commercial formulation is used at a concentration of 2% [16]. A commercial probiotic has reportedly increased survival and net fish production in catfish (*Ictalurus punctatus*) [17]. Taoka et al. [18] have studied effects of commercial probiotics formulated from mixed cultures of bacteria and yeast on survival of Japanese flounder *Paralichthys olivaceus*. The probiotics-treated fish have displayed significantly greater survival rate than the control fish [19]. Further studies have stressed probiotics ability to stimulate appetite, improve absorption of nutrients, and strengthen the host immune system [11,19].

Intensive culture practices for increased fish production often results in poor water quality, which in turn influences the growth of pathogenic microorganisms and disease problem. Use of probiotics is a promising way of bioremediation to overcome the issues of poor water quality and disease problem. This is proved with probiotics having nitrosomonas and nitrobacter species in fish ponds stocked with *Pangasius sutchi*, *Catla catla* and *Labeo rohita* [20]. The probiotics treatment reduces the concentrations of ammonia, nitrite and orthophosphates and also increases beneficial bacteria and

zooplankton as well as decreases pathogenic pseudomonas loads. This results in higher fish yields under the influence of probiotics [20].
The present work assessed the potential of microbial mixed probitics of bacillus, yeast and cyanobacterium for the enhanced growth and fish survival by using a randomized experimental model of response surface methodology, which was not used in the earlier experimental studies. The present work suggested that the probiotic combination of bacillus biomass of 1.89 g/kg, yeast biomass of 2.77 g/kg and spirulina biomass of 5.27 g/kg of feed to commercial diets could improve the fish biomass production (Tables 2, 3). Further research is required for understanding the specific role of probiotics of bacillus, yeast and spirulina in the fish nutrition. The role of the lactobacillus and yeasts is reportedly known as the probiotics for the growth of aquatic animals [3, 21]. The probiotics are able to modulate fish intestinal microbiota for both Gram positive of lactic acid bacteria and Gram negative bacteria [22]. This work reiterated the potential of probiotics in better fish survival and growth of *Lates calcarifer* and the use of RSM in determining optimal probiotic mixtures.

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