Pediococcus pentaceous-mediated fermentation of *Gracilaria corticate*: A sustainable reutilisation of renewable resource to enhance its nutritional profile, optimised through response surface methodology for improved growth and pathogenic resistance in *Oreochromis niloticus*

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Abstract
Seaweed, as a functional food and a sustainable alternative to synthetic additives, is gaining attention. It can enhance the nutritive value, improve antioxidant properties and mitigate oxidative stress induced by pathogens. This study investigates the utilisation of fermented seaweeds in feed formulations to reduce oxidative stress, improve fish health and enhance disease resistance. Seaweeds Gracilaria corticate, rich in bioactive compounds such as polyphenols and antioxidants, were fermented using probiotic Pediococcus pentosaceus MK459540.

Nile tilapia (Oreochromis niloticus) was fed a diet supplemented with fermented seaweed, which indicates lower levels of Superoxide Dismutase (SOD), Glutathione (GSH) and Glutathione-S-Transferase (GST) activities compared to control and non-fermented seaweeds when challenged with Vibrio harveyi, Aeromonas hydrophila and a mixture of both pathogens. These findings highlight the potential of seaweed, a sustainable and renewable marine resource in advancing aquaculture practices by promoting fish health and immunity.

Keywords: Solid state fermentation, Box-Behnken model, Functional foods.

Introduction

Inland aquaculture is the fastest-growing food sector, particularly in Asia, driven by the global demand for high-quality animal protein and essential nutrients to meet the needs of a growing population[5]. Aquaculture is essential for achieving Sustainable Development Goals (SDGs), especially in reducing poverty (SDG 1), combating hunger and enhancing food security (SDG 2) and fostering sustainable economic growth (SDG 8).

In India, it provides a reliable source of nutrition, creates employment opportunities and supports livelihoods. Thus, aquaculture contributes to both socio-economic development and global food sustainability[27]. Asia is the leading producer of farmed fish, with Nile tilapia (*Oreochromis niloticus*) being one of the most widely cultured species due to increasing consumption.

It is a freshwater fish capable of tolerating a wide range of osmotic and alkalinity stress and is now farmed in more than 135 countries and territories. Its popularity is due to its large size, fast growth, efficient breeding, affordability and mild, palatable taste[3]. However, the rapid spread of diseases caused by bacterial, viral and fungal pathogens poses a significant economic challenge due to high mortality rates and reduced production. Several pathogens, such as *Edwardsiella ictaluri*, a multidrug-resistant bacterium that is highly virulent, have caused 30 to 65% mortality in farmed tilapia in Vietnam[19]. Tilapia lake virus disease induces severe anaemia after seven days of intracoelomic exposure, with pathological findings including pale friable liver, pale intestine, dark shrunken spleen, histologically reduced RBCs and accumulation of melano-macrophage centres in the spleen[28].

Fungal pathogens such as *Ichthyophonus* spp. cause invasion of vascular organs, disrupting their function and *Branchiomycosis* spp. disrupts blood flow, affecting the gills[16]. The continuous use of antibiotics allows bacterial strains to develop resistance and survive, leading to the emergence of multidrug-resistant strains[8]. Given the increasing demand for tilapia as a source of high-quality fish protein, there is an urgent need to address the challenges related to its growth and disease resistance. This has led to the exploration of novel approaches, including natural feed additives, to improve tilapia farms, overall health and productivity. Among these additives, seaweeds, a macro alga, are rich in nutritional content and bioactive compounds, gaining attention as they are known to function as growth modulators, enhance the immune system and promote better antioxidant response[23].

Seaweeds, abundant in aquatic marine ecosystems, are bioactive compounds like polysaccharides, oligosaccharides, proteins, fatty acids, sterols, polyphenols, vitamins and minerals, most of which are antioxidants[10]. The bioactive compounds have been reported to strengthen the antioxidant defence system by reducing the oxidative stress of pathogenic infections[4]. Fermented seaweeds improve

immunomodulatory effects[2], modulate immune response, exhibit anti-inflammatory effects[25], prevent chronic diseases by increasing the bioavailability of polysaccharides, peptides, polyphenols and vitamins and improve gut health through the addition of probiotics and prebiotics, which promote the entire immune system[17].

Feed encapsulated with seaweed extract of *Gracilaria foliifera* and *Sargassum longifolium* with high phenolic content showed better resistance and increased survival percentage in *Oreochromis mossambicus* against *Aeromonas salmonicida* infection[26]. However, seaweed's complex cell wall structure, polysaccharides and antinutrients limit nutrient bioavailability[15]. Hence, developing effective and sustainable techniques to enhance seaweed protein digestibility helps to produce sustainable feed in aquaculture. Recent research has also focused on the fermentation of seaweeds, particularly through solid-state fermentation (SSF). SSF of *Padina gymnospora* seaweed fermented with *Lactobacillus* spp. showed better growth and increased biochemical composition of *Catla catla*[15].

Sargassum and *Gracilaria* species fermented with *Bacillus subtilis* as a formulated feed showed better growth, feed efficacy, survival rate and antioxidant profile when tested against the pathogen *Vibrio harveyi* and *Aeromonas hydrophila*[13]. Furthermore, investigating oxidative stress responses in *Oreochromis niloticus* is crucial for understanding the impact of pathogen-induced damage and evaluating the potential of fermented seaweed-based feeds in mitigating such damage. Oxidative stress is a key factor in fish health, influencing their ability to withstand infections and maintain cellular integrity under pathogen challenges. Nile tilapia exposed to *Aeromonas hydrophila*, leads to a disease, Aeromonas septicemia, which shows darkened skin, opercular hyperemia, gill congestion with elevated superoxide dismutase, catalase and glutathione-s-transferase[1].

This study explored the potential of *Gracilaria corticate* as a fermentation substrate, optimising the process through Response surface methodology to enhance its nutritional value. The goal was to improve the growth and weight gain of *Oreochromis niloticus* (Nile tilapia) while examining its Response to oxidative stress and pathogens.

The findings highlight the benefits of fermented seaweed feed in boosting growth performance and immune function in Nile tilapia. Beyond improving the economic sustainability of tilapia farming, these insights contribute to the broader development of functional feeds, offering sustainable nutrition solutions for various aquaculture species and supporting the long-term resilience of the industry.

Material and Methods

Seaweed collection: *Gracilaria corticate* was collected from the south-east coast of Tamil Nadu in Ramanathapuram district (9.285388°N latitude and 79.126979°E longitude), where a stable coverage of seaweed up to 1.0m depth was found. The samples were washed three times to remove the sand, salt, shells, debris and epiphytes. After rinsing with distilled water, the samples were dried in the shade for five days. Afterwards, they were ground into fine powder and stored for further use.

Optimisation of solid-state fermentation by Response surface methodology: Response surface methodology was used to optimise the process for maximising the production of simple sugars, proteins and lipids. Solid-state fermentation was conducted using *Pediococcus pentosaceus* MK459540. "Four key variables were considered: (a) fermentation time (hours), (b) Gracilaria quantity (g), (c) moisture content (%) and (d) prebiotic kidney bean content (%) (Table 1). Reducing sugars, proteins and lipid content were three responses considered following fermentation. The process was designed using Design Expert 13 software. A randomised Box Behnken model was employed with one block of 5 centre points, providing 29 runs. Reducing sugars were analysed using the DNS method, protein was analysed using the Lowry method and lipids were analysed using the organic solvent method.

Preparation of experimental feeds using fermented and non-fermented seaweed: The fermented and non-fermented *Gracilaria* were mixed with commercially available feed in a 3:7 ratio. Fermented and non-fermented *Gracilaria* served as experimental diets and commercial feed served as a control diet.
Control: Commercial feed (100%).

Feed 1: Non-fermented *Gracilaria* (30%) + commercial feed (70%).
Feed 2: Fermented *Gracilaria* (30%) + commercial feed (70%).

Experimental design for fish growth: The experiment was conducted in the Aqua lab, Department of Life Sciences, Christ University R and D facility.

Table 1
Box Behnken design employed ranges of 4 independent variables

S.N.	Name of Variables	Units	Low	High
1	Fermentation time	Hours	1	56
2	Seaweed quantity	Grams	1	5
3	Moisture content	%	15	45
4	Prebiotic quantity	%	10	40

The hatchlings of Nile tilapia were collected from the Fisheries Research and Information Centre (Inland), Hebbal, Bengaluru and equally distributed in food-grade high-quality plastic containers (50L) equipped with filters and aerators.

The filtration system was cleaned every day. Each tank had 15 fish; the initial fish weight was 1.56±0.10g. Fish were acclimatised for three days and the experiment was conducted over 60 days. Fish were fed once daily, at about 5% of their body weight. Weight measurements were taken every 10-day interval and feed was adjusted.

Growth assessment of the fish: Growth assessment was carried out using formulated feed diets. The following growth parameters were determined utilising mathematical formulas[20,21]. Weight gain percentage measures the increase in body weight over a specific period. The weight gain percentage can be calculated using the following formula:

$$\text{Weight Gain (\%)} = \frac{\text{Final weight(g)} - \text{Initial weight(g)}}{\text{Initial weight(g)}} \times 100$$

Specific growth rate percentage measures the relative growth of an organism per unit of time. It can be calculated using the following formula:

$$\text{Specific Growth Rate (\%)} = \frac{\ln(\text{Final weight}) - \ln(\text{Initial weight})}{\text{Time(days)}} \times 100$$

Feed conversion ratio measures how much feed an animal requires to gain a certain body weight. It can be calculated using the following formula:

$$\text{Feed Conversion Ratio} = \frac{\text{Amount of feed consumed (g)}}{\text{weight gain (g)}}$$

Feed efficiency ratio indicates the effectiveness of an animal's or an organism's ability to utilise feed for growth. It can be calculated using the following formula:

$$\text{Feed Conversion Ratio} = \frac{\text{weight gain (g)}}{\text{Amount of feed consumed (g)}}$$

Challenge studies on fish against the pathogen *Vibrio harveyi* and *Aeromonas hydrophila*

After 60 days of feed trial, fish were exposed to a 5-day acute exposure to pathogens like *Vibrio harveyi*, *Aeromonas hydrophila* and a combination of both. 30ml of broth with 10^9CFU/ml was added to 30L of tank to make it to 10^6CFU/ml. Antioxidant enzymes were estimated in the liver and muscle of the fish. The fish was dissected after the treatment. Liver and muscle samples were homogenised in PBS buffer (pH 7). Homogenate was centrifuged at 2000rpm and the supernatant was used as a sample for the assay. All these protocols were carried out in ice-cold conditions and samples were stored at -20ºC until further use.

Enzymatic glutathione-S-transferase (GST) assay: Glutathione-S-transferase (transferase type enzyme) catalyses the reaction of nucleophilic attack of the thiol group of reduced glutathione on the electrophilic carbon of 1-chloro-2,4-dinitrobenzene (CDNB), leading to the formation of thioether conjugate S-(2,4-dinitrophenyl) and byproduct HCl. A 100 µL homogenate sample was added to a reaction mixture containing 1 mL of 0.2 M phosphate buffer (pH 6.5), 0.1 mL of CDNB and 0.8 mL of distilled water. After thorough mixing, the mixture was incubated at 37°C for 5 minutes. Just before measuring, 0.1 mL of 20 mM GSH was added and absorbance at 340 nm was recorded every 30 seconds over 5 minutes[9].

$$\text{Reaction: GSH+CDNB} \xrightarrow{\text{GST}} \text{GS-DNB +HCl}$$

Enzymatic superoxide dismutase (SOD) assay: Superoxide dismutase (oxidoreductase type) is an antioxidant enzyme that catalyses superoxide conversion into less harmful chemicals. Homogenate sample (250ul) was added to the reaction mixture consisting of 650ul of sodium carbonate buffer (50mM, pH 10), 250ul of nitro blue tetrazolium dye (96uM), 250ul of Triton X-100 and 250ul of hydroxylamine hydrochloride. The reduction of NBT by producing superoxide radicals through autooxidation of hydroxylamine was measured as an increase in absorbance at 560 nm[11].

$$\text{Reaction: } 2O^{2-} + 2H^{+} \xrightarrow{\text{SOD}} O_2 + H_2O_2$$

Non-enzymatic reduced glutathione (GSH) assay: GSH is found in animal tissues, which is a potent antioxidant that reacts with Ellman's reagent (5,5'-dithiobis-(2 2-nitrobenzoic acid (DTNB)) to produce Glutathione disulfide (GSSG) and 2-nitro-5-thiobenzoic acid (TNB). Homogenate sample (100uL) was added to the reaction mixture consisting of 1000uL of DTNB(1mM) and 1900uL of phosphate buffer (0.2M). The rate of formation of TNB is measured at 412nm[6]. Reaction: DTNB+GSH⟶TNB+GSSG.

Statistical and Graph Analysis: Statistical analyses including analysis of variance (ANOVA), Fit statistics, standard deviation (SD) and Regression analysis(R^2), were conducted using Design Expert version 11. Response surface plots were generated using Design Expert version 11. All the X vs Y graphs were generated using Microsoft Excel.

Results and Discussion

Optimisation of solid-state fermentation using response surface methodology (Box-Behnken design): The solid-state fermentation of *Gracilaria corticate* using *Pediococcus pentosaceus* MK459540 was optimised using the Design Expert version 13 software. For fermentation involving seaweed, *Gracilaria* was studied under four parameters: fermentation time (hours), *Gracilaria* quantity (g), moisture content (%) and prebiotic kidney bean content (%). The first factor, fermentation time, was varied between 1 and 56

hours; the second factor, *Gracilaria* quantity, ranged from 1 to 5 grams; the third factor, moisture content, was set between 15% and 45% and the fourth factor, prebiotic kidney bean content, varied from 10% to 40%. The response variables for the fermentation process were yield in protein, reducing sugars and lipids. A Box-Behnken second-order model was employed to develop a quadratic design involving four factors, each at two levels, to identify the optimal conditions for maximum yield (Table 2). A regression analysis was conducted to assess the reducing sugar content, protein content and lipid content.

Reducing sugar analysis: Regression analysis indicated that the model F-value was 19.54 and the p-value was < 0.0001. It shows that the model is significant, explaining a variation in the data. In case of individual factors and their interactions, fermentation time (A) with F=199.81, p<0.0001; seaweed quantity (B) with F=43.40, p<0.0001; moisture(C) with F=11.88, p=0.0040 have significant effects, suggesting that they strongly influence the response. In contrast, Prebiotic kidney bean (D) with F=1.07 and p=0.3180 is insignificant, meaning that it has little to no significant impact. The analysis of interaction terms such as AB, AC, BC, BD and CD showed p-values>0.05 with no significant interaction.

Quadratic terms (A^2, B^2, C^2, D^2) and AD (p=0.1193) may have some borderline significance but are not substantial contributors. The residual sum of squares of 42.04 with 14 degrees of freedom indicates that this portion of variation is unexplained by the model. Lack of fit analysis showed F=5.82, p=0.0521, which indicates non-significance, which means the model fits the data well (Table 3). The predicted R^2 value of 0.7327 demonstrated reasonable agreement with the Adjusted R^2 value of 0.9026, with a difference of less than 0.2. Adequate precision, which measures the signal-to-noise ratio, had a value of 16.636, well above the desirable threshold of 4, indicating a proper signal. These results suggest that the model is reliable for navigating the design space. Additionally, the coefficient of determination (R^2) was 0.9513, reflecting a strong model fit (Table 4). A graphic representation in 3D (Fig. 1) was analysed for optimal regions.

Table 2
Design (actual) and results of yield response for *Gracilaria corticate*

RUN	Factor 1 Fermentation time (Hours)	Factor 2 *Gracilaria* quantity (Grams)	Factor 3 Moisture content (%)	Factor 4 Prebiotic quantity (%)	Reducing Sugars (mg/g)	Protein (mg/g)	Lipid (mg/g)
1	28.5	5	30	10	3.41	3.14	10
2	28.5	3	15	40	3.14	3.84	14
3	1	3	30	40	0.9	4.3	12
4	1	3	45	25	1.8	4.6	19
5	56	1	30	25	3.184	4.6	13
6	28.5	3	45	10	8.5	5.1	31
7	28.5	1	30	40	4.04	5.67	15
8	28.5	3	30	25	10.94	5.83	34
9	28.5	5	15	25	8.68	6.1	33.52
10	56	3	45	25	8.16	6.93	26
11	28.5	1	15	25	6.87	6.99	21
12	28.5	3	15	10	8.94	7.18	32.14
13	56	3	15	25	16	7.84	42
14	28.5	1	30	10	15	7.96	36
15	28.5	3	30	25	18.68	7.98	45
16	28.5	3	30	25	14.79	8.03	42.3
17	56	5	30	25	10.03	8.9	36.14
18	56	3	30	40	10.93	9.01	34.15
19	1	5	30	25	18.93	9.03	44
20	28.5	5	30	40	11	9.056	36
21	28.5	3	30	25	9.8	9.1	22
22	28.5	1	45	25	10	9.44	29.13
23	1	1	30	25	14.78	9.71	42
24	28.5	3	45	40	17.93	10.8	48
25	1	3	15	25	16.74	10.82	33
26	28.5	3	30	25	12.59	10.89	25
27	28.5	5	45	25	10.13	10.93	32.45
28	56	3	30	10	17.72	12.01	45.51
29	1	3	30	10	20.14	12.24	44.5

Table 3
ANOVA for the Quadratic model in response to reducing sugar

Source	Sum of squares	df	Mean Square	F-value	p-value	
Model	821.44	14	58.67	19.54	<0.0001	Significant
A-Fermentation time	600.05	1	600.05	199.81	<0.0001	
B-*Gracilaria* quantity	130.35	1	130.35	43.40	<0.0001	
C-Moisture content	35.67	1	35.67	11.88	0.0039	
D- Prebiotic Kidney bean content	3.22	1	3.22	1.07	0.3180	
AB	0.0110	1	0.0110	0.0037	0.9525	
AC	8.27	1	8.27	2.75	0.1193	
AD	1.86	1	1.86	0.6177	0.4450	
BC	4.88	1	4.88	1.63	0.2230	
BD	0.0529	1	0.0529	0.0176	0.8963	
CD	1.54	1	1.54	0.5120	0.4860	
A^2	11.32	1	11.32	3.77	0.0726	
B^2	10.96	1	10.96	3.65	0.0768	
C^2	1.82	1	1.82	0.6065	0.4491	
D^2	6.51	1	6.51	2.17	0.1631	
Residual	42.04	14	3.00			
Lack of Fit	39.34	10	3.93	5.82	0.0521	Not significant
Pure Error	2.70	4				
Cor Total	863.48	28				

Table 4
Fit statistics for SD and R^2 for response reducing sugar

Standard deviation	1.73	R^2	0.9513
Mean	10.86	Adjusted R^2	0.9026
Coefficient of variation	16.02	Predicted R^2	0.7327
		Adeq Precision	16.6362

Table 5
ANOVA for the Quadratic model in response to protein

Source	Sum of squares	df	Mean Square	F-value	p-value	
Model	154.04	14	11.00	6.12	0.0008	Significant
A-Fermentation time	54.15	1	54.15	30.11	< 0.0001	
B-*Gracilaria* quantity	35.74	1	35.74	19.88	0.0005	
C-Moisture content	9.77	1	9.77	5.43	0.0352	
D- Prebiotic Kidney bean content	1.12	1	1.12	0.6256	0.4422	
AB	0.5402	1	0.5402	0.3005	0.5922	
AC	0.0529	1	0.0529	0.0294	0.8663	
AD	0.2025	1	0.2025	0.1126	0.7421	
BC	0.3025	1	0.3025	0.1682	0.6879	
BD	1.02	1	1.02	0.5674	0.4638	
CD	2.80	1	2.80	1.56	0.2326	
A^2	31.93	1	31.93	17.76	0.0009	
B^2	7.52	1	7.52	4.18	0.0602	
C^2	0.3238	1	0.3238	0.1801	0.6778	
D^2	0.6097	1	0.6097	0.3391	0.5696	
Residual	25.17	14	1.80			
Lack of Fit	10.06	10	1.01	0.2663	0.9592	Not significant
Pure Error	15.11	4	3.78			
Cor Total	179.22	28				

The final equation for reducing sugar yield was: Reducing sugars= 10.19+7.07A+3.30B+1.72C+0.5180D-0.0525AB+ 1.44AC+0.6810AD+1.11BC0.1150BD+0.6200CD-1.32A^2 +1.30B^2+0.5299C^2+1.00D^2.

Protein analysis: Regression analysis indicated that the model F-value was 6.12 and the p-value was =0.0008. It shows that the model is significant, explaining a variation in the data. In case of individual factors and their interactions, A with F=30.11, p<0.0001; B with F=19.88, p=0.0005; C with F=5.43, p=0.00352; A^2 with F=17.16, p=0.0009 have significant effects, suggesting that they strongly influence the response. In contrast, prebiotic kidney bean (D) with F=1.12 and p=0.4422 is insignificant, meaning it has little to no impact. The analysis of interaction terms such as AB, AC, AD, BC, BD, CD, C^2 and D^2 showed p-values>0.100, with no significant interaction. Quadratic term B^2 may have some borderline significance, but they are not a strong contributor. The residual sum of squares with 25.17 and 14 degrees of freedom indicates that this portion of variation is unexplained by the model.

Lack of fit analysis showed F=0.2663, p=0.9592, which indicates non-significance, which means the model fits the data well (Table 5). The predicted R^2 value of 0.5449 demonstrated reasonable agreement with the adjusted R^2 value of 0.7191, with a difference of less than 0.2. Adequate precision, which measures the signal-to-noise ratio, had a value of 9.114, well above the desirable threshold of 4, indicating a proper signal. These results suggest that the model is reliable for navigating the design space. Additionally, the coefficient of determination (R^2) was 0.8595, reflecting a strong model fit (Table 6). A graphic representation in 3D (Fig. 2) was analysed for optimal regions.

The final equation for Protein yield was: Protein = 8.37+ 2.12A+1.73B+0.9022C+0.3062D+0.3675AB-0.1150AC+ 0.2250AD-0.2750BC-0.5050BD-0.8365CD-2.22A^2+1.08 B^2+0.2234C^2-0.3066D^2.

Lipid analysis: Regression analysis indicated that the model F-value was 21.87 and the p-value was < 0.0001. It shows that the model is significant, explaining a variation in the data. In case of individual factors and their interactions, A with F=226.01, p<0.0001; B with F=21.18, p=0.0004; C with F=29.84, p<0.0001 have significant effects, suggesting that they strongly influence the response. In contrast, D with F=2.69, p=0.1230 is insignificant, meaning it has little to no impact. The analysis of interaction terms such as AB, AC, AD, BC, BD, B^2, C^2, D^2 showed p values>0.05, with no significant interaction with each other. Quadratic terms A^2 and CD(p=0.0.0418) may have some borderline significance but are not substantial contributors.

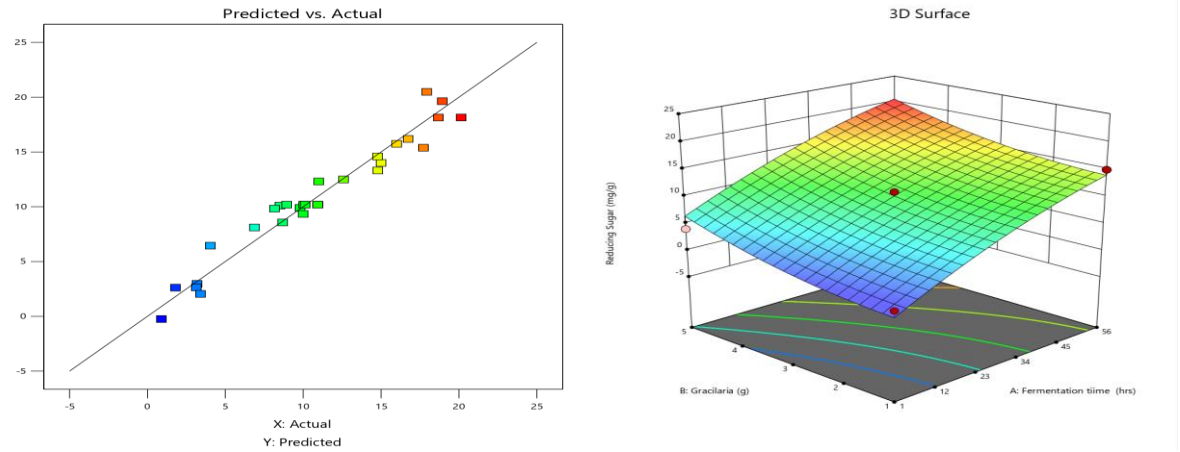

Fig. 1: Response surface plots for reducing sugar yield: a) Plot for predicted vs actual, b) 3D surface heat map

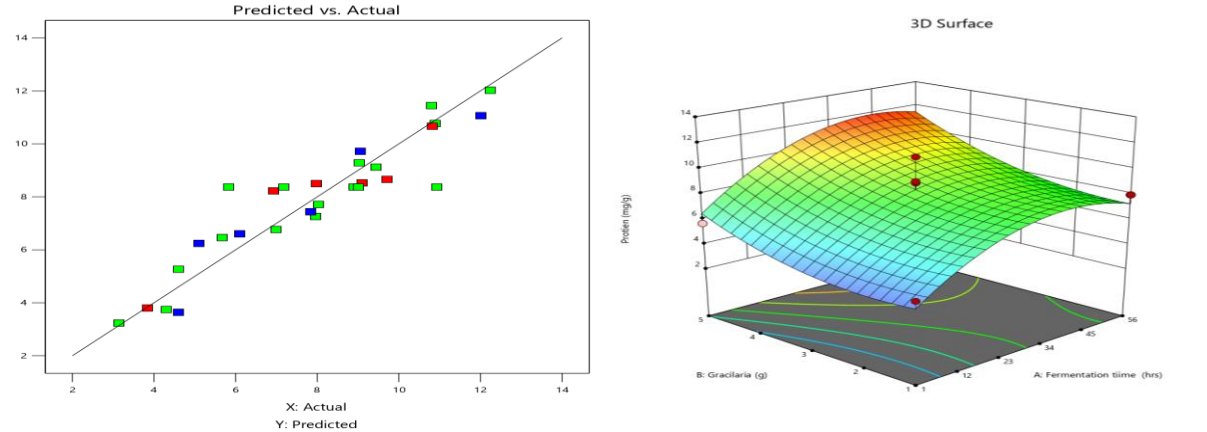

Fig. 2: Response surface plots for protein yield: a) Plot for Predicted vs Actual, b) 3D surface heat map

The residual sum of squares is 156.82 with 14 degrees of freedom, indicating that this portion of variation is unexplained by the model. Lack of fit analysis showed F=5.74, p=0.0534, which indicates non-significance; it means the model fits the data well (Table 7). The predicted R^2 value of 0.7601demonstrated reasonable agreement with the Adjusted R^2 value of 0.9125, with a difference of less than 0.2. Adequate Precision, which measures the signal-to-noise ratio, had a value of 17.9433, well above the desirable threshold of 4, indicating a proper signal. These results suggest that the model is reliable for navigating the design space. Additionally, the coefficient of determination (R^2) was 0.9563, reflecting a strong model fit (Table 8). A graphic representation in 3D (Fig. 3) was analysed for optimal regions.

The final equation for lipid yield was: Lipid=33.78+14.53A +4.45B+5.28C-1.59D+2.25AB-1.82AC+0.500AD+2.84BC -1.25BD+3.75CD-4.72A^2-1.18B^2-1.52C^2+0.6174D^2.

Table 6
Fit statistics for SD and R^2 for the response Protein

Standard deviation	1.34	R^2	0.8595
Mean	7.86	Adjusted R^2	0.7191
Coefficient of variation	17.05	Predicted R^2	0.5449
		Adeq Precision	9.1142

Table 7
ANOVA for the Quadratic model in response to lipid

Source	Sum of squares	df	Mean Square	F-value	p-value	
Model	3429.20	14	244.94	21.87	< 0.0001	Significant
A-Fermentation time	2531.71	1	2531.71	226.01	< 0.0001	
B-*Gracilaria* quantity	237.27	1	237.27	21.18	0.0004	
C-Moisture content	334.22	1	334.22	29.84	< 0.0001	
D- Prebiotic Kidney bean content	30.18	1	30.18	2.69	0.1230	
AB	20.25	1	20.25	1.81	0.2002	
AC	13.32	1	13.32	1.19	0.2939	
AD	1.0000	1	1.0000	0.0893	0.7695	
BC	32.32	1	32.32	2.89	0.1115	
BD	6.23	1	6.23	0.5557	0.4683	
CD	56.25	1	56.25	5.02	0.0418	
A^2	144.74	1	144.74	12.92	0.0029	
B^2	9.01	1	9.01	0.8047	0.3849	
C^2	14.99	1	14.99	1.34	0.2667	
D^2	2.47	1	2.47	0.2207	0.6457	
Residual	156.82	14	11.20			
Lack of Fit	146.61	10	14.66	5.74	0.0534	Not significant
Pure Error	10.21	4	2.55			
Cor Total	3586.03	28				

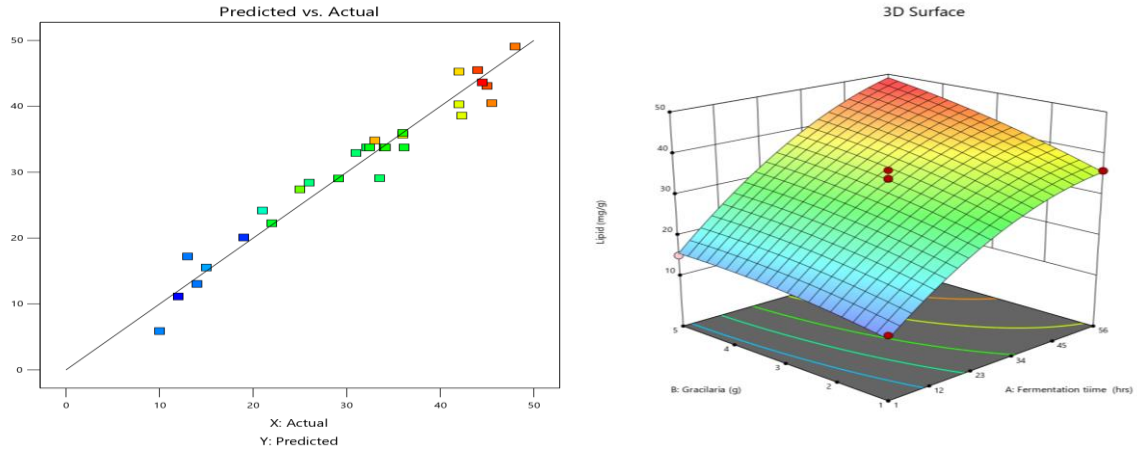

Fig. 3: Response surface plots for lipid yield: a) Plot for predicted vs actual, b) 3D surface heat map

Optimum final solution for the targeted response for each factor: The optimal conditions for maximising the yield of proteins, lipids and reducing sugars from Gracilaria fermented with *P. pentaceous* were determined to be 23.0691 hours of fermentation, 4.99g of seaweed, 44.99% moisture and 10% prebiotics, with a desirability of 0.831, indicating that it is a good model. These conditions yielded 16.53mg/g reducing sugars, 12.23mg/g protein and 40.30mg/g lipids (Fig. 4).

Growth performance and feed utilisation: In the present study, fingerlings of Nile tilapia showed significantly higher weight gain (%) upon completion of a 60-day feeding trial when fed with fermented feed (Feed 2) of 711.0±70.94 % in comparison to the control group, which showed 568.8±49.84% whereas non-fermented feed (Feed 1) showed less growth of 416.2±42.07% (Fig. 5). The specific growth rate percentage in a commercial feed showed moderate growth rate of 3.1±0.12% indicating a balanced nutrient meal, whereas in fermented feed, it showed significantly high SGR of 3.4±0.14% compared to non-fermented feed which showed 2.7±0.13% indicating that fermented feed improved the nutrient availability through fermentation by reducing antinutritional factors, leading to better absorption (Fig 6).

In commercial feed-fed fish, the feed efficiency ratio was 0.49±0.007 whereas compared to fermented and non-fermented feed, it showed 0.60±0.011 and 0.36±0.009, indicating that fermentation enhanced the nutrient availability and digestibility (Fig. 7). In case of the feed conversion ratio, commercial feed showed 2.03±0.03, fermented feed showed 1.64±0.03 and non-fermented feed showed 2.77±0.07, indicating fermented feed can significantly improve growth rate, reduce feed cost, minimise feed waste, be more sustainable and require less feed replacement (Fig. 8).

Effect of pathogen on stress levels in fish with different feed utilisation: This study evaluated the oxidative damage during pathogen infestation and how different feeds can avoid the damage. In this study, fish were exposed to 106CFU/ml of *Vibrio harveyi* (Vh) and *Aeromonas hydrophila* (Ah) and a mixture in a 1:1 ratio for 5 days.

Table 8
Fit statistics for SD and R^2 for the response lipid

Standard deviation	3.35	R^2	0.9563
Mean	30.96	Adjusted R^2	0.9125
Coefficient of variation	10.81	Predicted R^2	0.7601
		Adeq Precision	17.9433

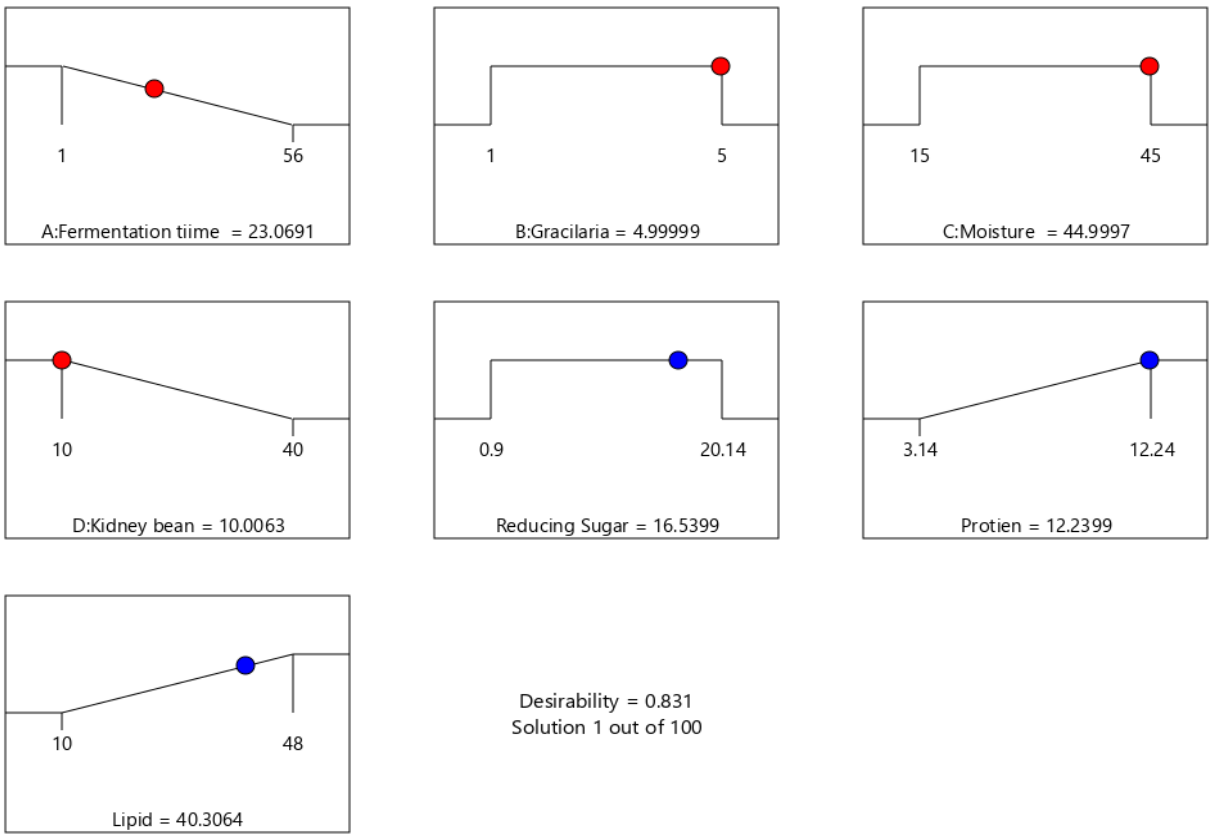

Fig. 4: Optimum Final solution for the targeted response for each factor

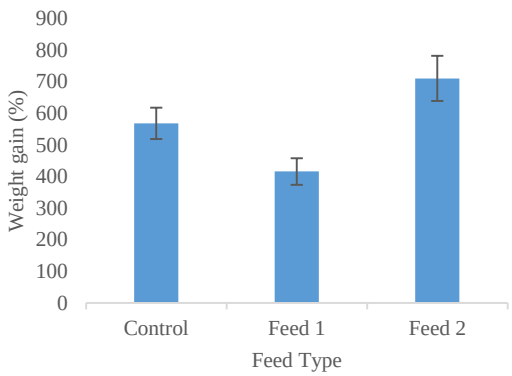

Figure 5: The weight gain percentage

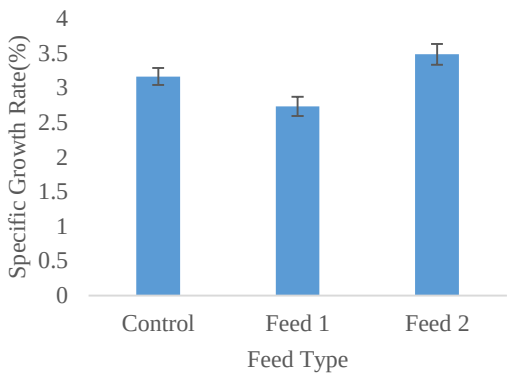

Figure 6: The specific growth rate percentage

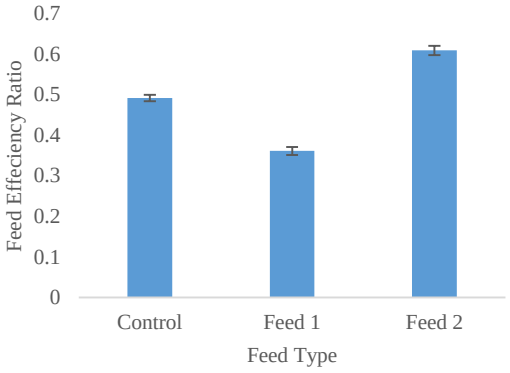

Figure 7: The feed efficiency ratio

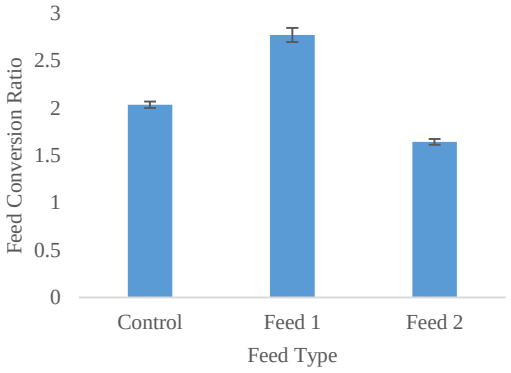

Figure 8: The feed conversion ratio

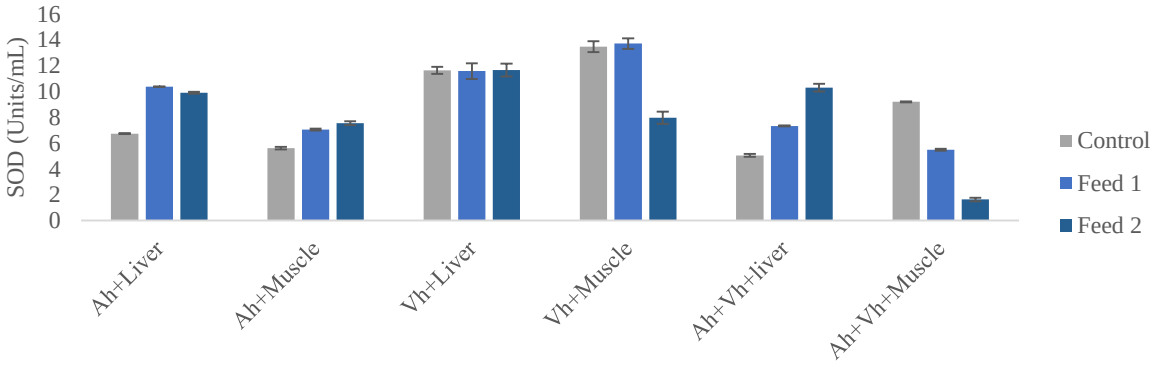

Figure 9: Superoxide dismutase activity in the liver and muscle of Nile tilapia exposed to the pathogen.

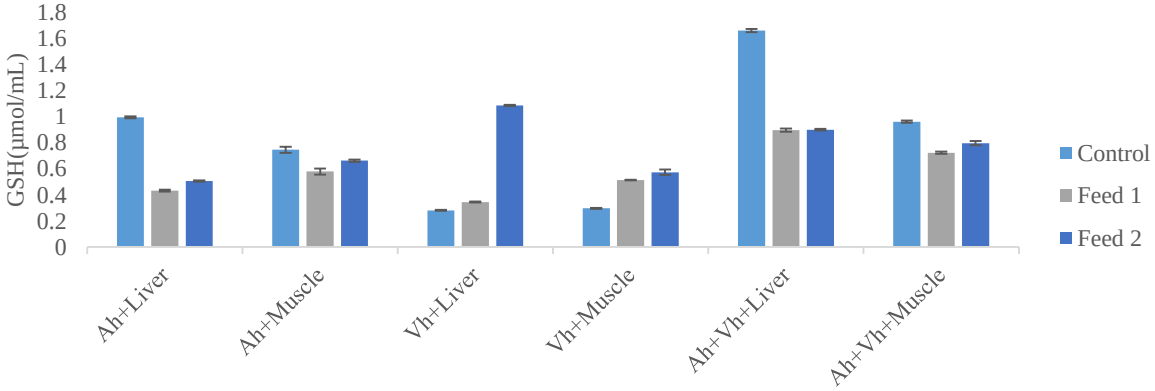

Figure 10: Glutathione activity in the liver and muscle of Nile tilapia exposed to the pathogen.

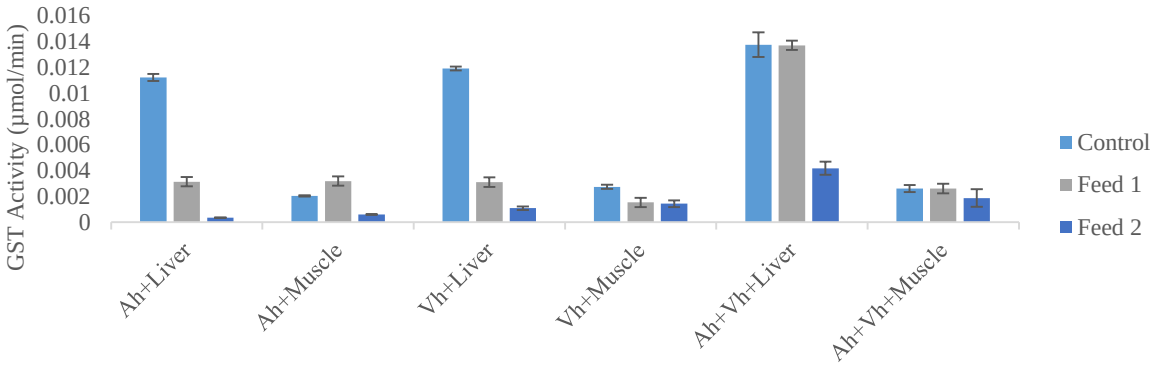

Figure 11: Glutathione S-transferase activity in the liver and muscle of Nile tilapia exposed to the pathogen.

Superoxide dismutase (SOD) activity: SOD enzymes regulate the levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS), thereby minimising their potential toxic effects [24]. In the Ah exposed groups, liver SOD levels, with feed 1 and feed 2, are slightly higher (10.36 and 9.89 Units/mL) and the control was marginally lower (6.7 Units/mL). In muscle, SOD activity was lower overall compared to liver tissue. Feed 2 showed the highest activity (7.53 Units/mL) followed by feed 1 (7.03 Units/mL). The control group had the lowest (5.6 Units/mL) indicating that fermented feed enhanced the muscular antioxidant response under Ah exposure. In Vh-exposed groups, liver SOD levels of all diets resulted in elevated SOD activity (11.56 to 11.64 Units/mL), with feed 2 showing the highest values (11.64 Units/mL whereas in muscles, feed 2 led to the moderate SOD levels (7.9 Units/mL), but showed very high values in control and feed 1(13.46 and 13.69 Units/mL).

In this combined stress group in a liver, feed 2 induced the highest SOD activity (10.28 Units/mL), outperforming feed 1 (7.32 Units/mL) and control (5.03 Units/mL) whereas, the control and feed 1 maintained relatively higher SOD activity (9.18 and 5.4 Units/mL, respectively). In comparison, feed 2 showed a sharp decline (1.62 Units/mL), indicating a possible muscle-specific antioxidant suppression or overconsumption of SOD in response to stress with fed fermented feed (Fig. 9). Similarly, infected with *Providencia rettgeri*, Nile tilapia led to increased oxidative stress in liver and kidney tissues. ROS and lipid peroxidation levels were high on day 14 post-infection, while antioxidant enzyme activity decreased, indicating a compromised antioxidant defence system in infected fish, potentially contributing to disease pathophysiology[7].

Nile tilapia fed with 0.5% and 1.0% ginger showed significant increases in haematological parameters and oxidative stress indices, including SOD and CAT enzyme activities in liver, gill and gut tissues compared to the control group[22].

GSH Activity: The GSH (glutathione) levels varied among different feeds when exposed to pathogens. In the Ah-exposed groups, liver GSH was highest in the control (0.99 µmol/mL), followed by feed 2 (0.505 µmol/mL) and lowest in feed 1 (0.432 µmol/mL). In muscle tissues, feed 1 and feed 2 showed slightly lower GSH (0.57 and 0.66 µmol/mL) than the control (0.74 µmol/mL). In Vh exposed groups, liver GSH was highest with feed 2 (1.084 µmol/mL) while the control and feed 1 were much lower (0.28 and 0.344 µmol/mL). Muscle GSH followed a similar trend, with feed 2 (0.57 µmol/mL) outperforming control and feed 1 (0.29–0.51 µmol/mL).

In the combined Ah and Vh group, the liver GSH peaked in the control (1.66 µmol/mL), with feed 1 and 2 showing similar and slightly reduced levels (0.89 µmol/mL). For muscle, GSH levels were comparable across treatments, with control marginally higher (0.96 µmol/mL) than feed 1 and feed 2 (0.72 and 0.79 µmol/mL) (Fig. 10). Similar results showed the potential of TrueAlgaeMax (TAM), a liquid seaweed extract, as a feed additive for Nile tilapia challenged with *Aeromonas hydrophila*. Different TAM concentrations (0%, 0.5%, 1%, 1.5% and 2%) were added to the diets, with 2% TAM exhibiting better glutathione activity, indicating betterment of non-specific immunity and growth performance[4].

GSH plays a key role in the conjugation of secondary metabolic toxins. A study revealed that when Nile tilapia, which are fed with Brown Alga (*Sargassum crassifolia*), were exposed to a sublethal dose of Nimbecidin, they showed a significant reduction in GSH activity compared to control groups. It indicates the combat of oxidative stress by removing toxins[18]. These studies emphasise the importance of GSH in detoxifying various pathogens and pollutants.

Glutathione S-transferase activity: In a detoxification process, GST plays a key role by catalysing the conjugation of glutathione with electrophilic toxins through localisation in the cytosol. The activity of this GST enzyme directly plays a role in the rate of toxin elimination[12]. When exposed to Ah in liver samples, the control group exhibited the highest GST activity at 0.0112 ± 0.0003 µmol/min. In contrast, feed 1 and feed 2 groups showed reduced activities of 0.0031 ± 0.0004 µmol/min and 0.00036 µmol/min respectively. Similarly, in muscle, GST activity declined from 0.00204 µmol/min (Control) to 0.0031 µmol/min (Feed 1) and 0.0006 µmol/min (Feed 2). In the Vh-exposed Liver, GST activity in the

control was 0.0119 µmol/min, while feed 1 and feed 2 groups showed values of 0.0031 µmol/min and 0.00109 µmol/min respectively.

The Vh Muscle group exhibited GST activities of 0.0027 µmol/min (Control), 0.0015 µmol/min (Feed 1) and 0.0014 µmol/min (Feed 2). The Ah+Vh Liver group had the highest overall GST activity in the control at 0.0137 µmol/min while feed 1 and feed 2 groups showed slightly reduced activities of 0.0137 µmol/min and 0.0041 µmol/min respectively. In Ah+Vh muscle, the control value was 0.0026 µmol/min, compared to 0.0026 µmol/min (Feed 1) and 0.00188 µmol/min (Feed 2) (Fig. 11). In a similar study, sugarcane bagasse powder (SB) used as dietary supplement on Nile tilapia at 20 and 40 g/kg showed improved growth performance compared to the control group, mucosal immunity. The notably enhanced immune response observed in Nile tilapia in this study may be linked to the presence of bioactive compounds and oligosaccharides found in the seaweeds[14].

Conclusion

In conclusion, using fermented seaweeds in fish feed formulations holds significant promise for mitigating oxidative stress induced by pathogen infections in fish. Fermented seaweeds, rich in bioactive compounds such as polysaccharides, peptides and polyphenols, exhibit enhanced antioxidant activity, modulate immune responses and improve growth performance in fish. The observed reductions in oxidative stress markers like superoxide dismutase (SOD), glutathione (GSH) and glutathione-S-transferase (GST) activity in fish fed with fermented seaweeds indicate their potential to combat oxidative stress effectively.

These findings highlight the importance of incorporating fermented seaweeds into fish diets to enhance fish health, disease resistance and overall performance in aquaculture systems. Further research is warranted to explore the practical applications and to optimise the use of fermented seaweeds in aquafeed formulations.

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