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Optimizing Functional Feed for Growth and Pathogen Resistance in *Oreochromis niloticus* using Fermented Seaweeds: A Comprehensive Approach Through Solid State Fermentation and Oxidative Stress Response

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ABSTRACT

The study aimed to explore the potential of seaweeds *Sargassum wightii* and *Gracilaria corticate* fermented using *Bacillus subtilis* MN960600 (CK4). Fermented seaweeds showed enhanced antioxidant activity in DPPH assays. A second-order model known as Box-Behnken was used to create an optimized quadratic design for fermentation parameters enhancing protein, reducing sugars, and lipid yields. This optimized feed demonstrated significant growth improvement of 18 to 20 % in *Oreochromis niloticus* when compared to commercial feed and a 35 to 40% higher growth in fermented and non-fermented feed groups. Additionally, fish fed formulated seaweeds exhibited resilience to *Vibrio harveyi* and *Aeromonas hydrophila* pathogen stress. Additionally, the study highlighted the ability of the formulated seaweed in reduction of oxidative stress caused by pathogens *Vibrio harveyi* and *Aeromonas hydrophila* in *Oreochromis niloticus*. The study emphasized the potential use of seaweeds and probiotic bacteria as a sustainable aquafeed.

INTRODUCTION

Aquaculture has emerged as a critical sector in meeting the escalating demand for high-quality fishery products as protein sources worldwide **(Ababouch *et al.*, 2023)**. In the quest to enhance aquaculture sustainability and productivity, there is a growing interest in optimizing functional feed formulations to promote growth and bolster disease resistance in farmed fish species **(Smith *et al.*, 2021)**. High stocking densities in aquaculture facilities can facilitate the rapid spread of diseases among fish populations. Pathogens, parasites, and bacteria thrive in such environments, exacerbated by stress factors like poor water quality or inadequate nutrition **(Lafferty *et al.*, 2015)**. Effective disease prevention and treatment strategies are therefore essential to maintain the health and productivity of aquaculture stocks. *Oreochromis niloticus*, commonly known as the Nile tilapia, have evolved as a pivotal species in global aquaculture due to its adaptability and economic significance **(Zhou *et al.*, 2019)**. As the demand for high-quality fish protein intensifies, there is a pressing need to develop innovative approaches that address the challenges of growth optimization and pathogen resistance in *Oreochromis niloticus*

farming. The quest for sustainable aquafeed solutions has led to the exploration of natural additives, particularly seaweeds, renowned for their rich bioactive compounds and nutritional benefits **(Lafarga *et al.*, 2020)**. In aquaculture, probiotics are live microbial supplements administered through feed. These additives play a crucial role in enhancing the gut microflora, thereby improving feed absorption, nutritional uptake, and bolstering immunity against harmful bacteria in the gut. Commonly employed probiotics in aquaculture encompass lactic acid bacteria like *Lactobacillus* sp., *Bacillus* sp., *Enterococcus* sp., and yeast strains such as *Saccharomyces cerevisiae* **(Bhandary *et al.*, 2023; Kurian & Paari, 2023)**. Recent studies have increasingly focused on leveraging the potential of fermented seaweeds to enhance fish health and performance. Fermentation processes, especially solid-state fermentation (SSF), have demonstrated promise in augmenting the bioavailability of nutrients and bioactive compounds, thereby amplifying their efficacy as feed supplements. Furthermore, the fermentation of seaweeds through solid-state fermentation (SSF) has emerged as a promising technique to enhance the nutritional quality of feeds by promoting the synthesis of bioactive compounds and by improving digestibility **(Gora *et al.*, 2018)**. Solid-state fermentation represents a biotechnological approach that utilizes the metabolic activities of microorganisms, such as bacteria and fungi, to transform the composition of substrates **(Xue *et al.*, 2022)**. The use of solid-state fermentation allows for the enrichment of functional feed with bioactive compounds, including prebiotics and probiotics, that can positively influence the digestive efficiency and immune response of *Oreochromis niloticus* **(Chakraborty *et al.*, 2019)**. This study aimed to comprehensively explore the potential of fermented seaweeds in optimizing functional feed for *Oreochromis niloticus*, with a focus on growth promotion and bolstering resistance against pathogenic challenges. This study employed a comprehensive solid-state fermentation process to enhance the nutritional content of seaweeds, aiming to maximize the growth potential and disease resistance of the Nile tilapia.

In addition to growth promotion, the study investigated the oxidative stress response of *Oreochromis niloticus* when fed with fermented seaweed-based diets challenged with fish pathogens *Vibrio harveyi* and *Aeromonas hydrophila*. Oxidative stress analysis is crucial in fish pathogen disease studies because it helps assess the impact of pathogens on antioxidant defenses, lipid peroxidation, and overall cellular damage, thereby elucidating disease mechanisms and potential therapeutic strategies. Oxidative stress is also crucial factor influencing the overall health and disease resistance of fish **(Peng *et al.*, 2022)**, and understanding the oxidative stress response will provide valuable insights into the effectiveness of the optimized functional feed in promoting resilience against pathogenic challenges. Through a multidisciplinary approach that integrates solid-state fermentation techniques and investigates the oxidative stress response, this research aimed to contribute to the development of sustainable aquaculture practices for *Oreochromis niloticus*. The findings of this study hold the potential to not

only enhance the economic viability of the Nile tilapia farming but also advance our understanding of functional feed optimization strategies that can be applied to other aquaculture species, thereby promoting the overall sustainability of global aquaculture.

MATERIALS AND METHODS

1. Seaweed collection and processing

The study involved collecting seaweeds from the intertidal and subtidal regions (up to 1.0m depth) along the southeast coast of Tamil Nadu, spanning from the Gulf of Mannar to the southern side of Mandapam Camp. Two stations (L1: N9.267268, E79.032702; L2: N9.289560, E79.136006) were chosen during spring tide periods, and their coordinates were recorded using a GPS device (GARMIN-eTrex30x). Seaweeds were rinsed with fresh water to remove sand, salt crystals, and epiphytes, then air-dried under controlled conditions. Morphological and anatomical analyses were conducted as demonstrated by **Sahoo (2010)** using taxonomic literature and keys. Samples were subsequently shade-dried for three days before solid-state fermentation processing.

2. Optimizing seaweed fermentation parameters in feed formulation through response surface methodology (RSM)

Solid-state fermentation was conducted using *Bacillus subtilis* MN960600 (CK4). The research employed response surface methodology (RSM) software design expert version 11.1.2 to optimize the procedure for achieving maximum yields in protein, reducing sugars, and lipid content during solid-state fermentation. A second-order Box-Behnken model was employed, creating a quadratic design with four factors and two levels per factor. The experimental factors, including fermentation time, seaweed quantity, moisture, and prebiotic quantity, were systematically varied. The response variables for seaweeds *Sargassum wightii* and *Gracilaria corticata* were assessed to determine the optimal conditions for maximizing protein, reducing sugar, and lipid yields. The software defined various experimental factors and their combinations for the conducted experimental trials.

3. Antioxidant studies

3.1. Total antioxidant capacity (TAC)

The determination of total antioxidant capacity was conducted following the phosphomolybdenum method **(Nazarudin *et al.*, 2022)**, with slight modifications. The reagent solution was prepared by combining equal volumes of 0.6M sulphuric acid, 28mM sodium phosphate, and 4mM ammonium molybdate and 0.3mL of the extract, with a concentration of 1.0mg/ mL. To reduce light interference, the test was performed under dark conditions due to its light-sensitive nature. The mixture was thoroughly mixed

and subsequently incubated at 95°C for 90 minutes. Once samples were brought to room temperature, the absorbance of each mixture was measured at a wavelength of 695nm. Blank samples were prepared using solvent without extract, and a standard curve was generated using ascorbic acid. The total antioxidant capacity was expressed as milligrams of ascorbic acid equivalent (mg AAE) per gram of extract.

3.2. Ferrous reducing antioxidant capacity assay (FRAC / FRAP)

The FRAP assay was conducted following the protocol of **Gomaa *et al.* (2022)** with minor modifications. Specifically, 1mL of 0.2 M potassium phosphate buffer (pH 6.6) and 1mL of 1% potassium ferricyanide was mixed with 1mL of extract. The reaction mixture was incubated at 50°C for 20 minutes. Subsequently, 1mL of 10% trichloroacetic acid (TCA) was added, followed by centrifugation at 3000rpm for 10 minutes. The upper layer of the resulting solution (1mL) was then mixed with 1mL of distilled water and 0.5mL of 0.1 % $FeCl_3.6H_2O$. The absorbance of the mixture was measured at 700nm. The FRAP value was reported in milligrams of ascorbic acid equivalent (mg AAE) per gram of extract.

3.3. DPPH assay

The antioxidant activity of fermented and nonfermented seaweed extract was evaluated using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method following the protocol of **Tong *et al.* (2019)**. Stock solution was prepared by dissolving 20mg of DPPH in 100mL of methanol. In a test tube, 3mL of workable DPPH solution was mixed with 100µL of seaweed extract and a standard solution of 3mL of DPPH in 100µL of methanol was used as positive control. The tubes were then placed in complete darkness for 30 minutes, after which the absorbance was measured at 517nm. The percentage of antioxidant activity was calculated using the formula:

$$\%\ of\ antioxidant\ activity = [(Ac - As) \div Ac] \times 100$$

Where, Ac is the absorbance of control sample and As is the test specimen absorbance.

4. Growth assessment of *Oreochromis niloticus* fed with fermented (SWSG and SWGR) and non-fermented seaweeds (NFSG and NFGR)

4.1. Preparation of experimental diets using non-fermented and fermented seaweeds

Growth analysis of *Oreochromis niloticus* was conducted using formulated diets containing fermented seaweeds, specifically *Sargassum wightii* (SWSG) and *Gracilaria corticate* (SWGR). The fermented seaweeds were combined with commercially available feed at a 1:1 ratio. Similarly, to the non-fermented seaweeds, *Sargassum wightii* (NFSG)

and *Gracilaria corticate* (NFGR), commercial feed was mixed in a 1:1 ratio, serving as the negative control, while commercial feed was considered as the positive control.

4.2. Experimental design for fish rearing

The experimentation was conducted at a dedicated aquaculture facility. The hatchlings were procured from farm and equally distributed into five food-grade plastic containers (60L), equipped with filtration systems and aerators. Tanks were cleaned morning and evening before feeding the fishes and fishes were fed 5% of their body weight twice a day. Treatment groups comprise of control group, NFSG, NFGR, SWSG, and SWGR, corresponding to their respective feeding regimes. Initial weight of the fish was recorded as 0.14 ± 0.002g. Following a week-long acclimation, the hatchlings were fed with standard commercial feed. Subsequently, they were fed with the formulated feed twice daily, at an approximate rate of 5% of their body weight per day, for a period of 56 days. Weekly weight measurements were recorded to monitor weight gain.

Feed efficiency (FE) **(Eya *et al.*, 2011)**, feed conversion ratio (FCR) **(Fry *et al.*, 2018)**, specific growth rate (SGR%), and weight gain (WG%) **(Rahman *et al.*, 2021)** were analyzed for fish samples from each respective tank, providing comprehensive insights into the effects of different diets on the growth performance of *Oreochromis niloticus*.

Growth parameter calculations:

$$FRC\ (g) = \frac{Dry\ feed\ intake\ (g)}{Wet\ body\ weight\ gain\ (g)}$$

$$FE\ (g) = \frac{Wet\ weight\ gain\ (g)}{Dry\ feed\ consumed\ (g)}$$

$$SGR\ (g.\%/day) = 100 \times (ln\ Final\ weight - ln\ Initial\ weight)/feeding\ days$$

$$WG\ \% = \frac{final\ weight - Initial\ weight}{Initial\ weight} \times 100$$

5. Oxidative stress analysis after pathogen challenge studies

5.1. Experimental design

To assess the oxidative stress effects of pathogen infestation in fish, individuals were fed a formulated diet and exposed to *Vibrio harveyi*, *Aeromonas hydrophila*, or a combination of both at a concentration of 10^6 CFU/mL for seven days. Oxidative stress responses in fermented and non-fermented seaweed-treated groups were evaluated using Superoxide Dismutase (SOD), Catalase (CAT), Glutathione (GSH), and Glutathione-S-Transferase (GST) assays, following the protocol of **Kaur *et al.* (2017)**.

Tissues, including organ systems (liver, kidney, gonads, and viscera) and muscle (10% w/v), were analyzed for oxidative stress markers. To measure oxidative stress-related parameters, soft tissues (liver, kidney, and viscera) and muscle samples were homogenized (1:10 w/v) in a glass Potter tube with Tris-HCl buffer (10 mM, pH 7.4). The homogenates were then centrifuged at 2000×g for 10 minutes, and the supernatant was used for SOD, CAT, GSH, and GST activity assays.

5.2. Superoxide dismutase (SOD) assay

The reaction mixture comprised 1.2ml of solution A (50mM sodium carbonate in 0.1nM EDTA buffer, pH 10.8), 0.5ml of solution B (96µM NBT), and 0.1ml of solution C (0.6% Triton X-100), incubated at 37℃ for 10min. The reaction was initiated by adding 0.1ml of 20mM hydroxylamine HCl (pH 6). The rate of NBT dye reduction by O-2 anion, generated due to the photoactivation of hydroxylamine HCl, was recorded at 560nm for 3min for the blank. PMS was immediately added after the addition of hydroxylamine HCl to record the SOD activity present in the source at 560nm for 3min.

5.3. Catalase (CAT) assay

The assay mixture consisted of 2.9mL of 12.5mM H_2O_2 and 0.067M phosphate buffer (pH 7.0) and 0.01mL sample (DDW for blank). The decrease in absorbance/30 sec at 240nm was measured for 3min.

5.4. Glutathione (GSH) assay

Homogenate sample (0.1mL) and 2.0mL of 0.2M phosphate buffer were mixed thoroughly, followed by the addition of 1mL of 1.0mM dithiobisnitrobenzoic acid (DTNB) prepared in 1% (w/v) potassium citrate. After centrifugation at 3000 rpm for 15min, the absorbance of the supernatant was measured against reference blank at 412nm.

5.5. Glutathione-S-Transferase (GST) Assay

Samples (0.1mL) were incubated in 1mL of 0.2 M phosphate buffer (pH-6.5), 0.1mL of 20mM 1-chloro-2, 4-dinitrobenzene (CDNB) prepared in 95% ethanol, and 0.8mL of DDW. After thorough mixing, incubation was carried out at 37°C for 5min. 0.1mL of 20mM GSH (dissolved in DDW) was added just before measuring the increase in absorbance/30 sec at 340nm for 5 minutes. In the case of the blank, 2.9mL phosphate buffer and 0.1mL CDNB were mixed.

6. Statistical analysis

The data attained in this study were analyzed using one-way ANOVA at a 5% significance level. All experiments were conducted in triplicate and were expressed as

mean ± SD. Further fermentation studies using RSM were performed using Design Expert software version 11.

RESULTS AND DISCUSSION

1. Seaweed collection and processing

The seaweeds were collected from two distinct locations along the southeast coast of Tamil Nadu, specifically from the intertidal and subtidal regions up to a depth of 1.0m. The collection sites extended from the Gulf of Mannar in the north to the southern side of Mandapam camp. Seaweeds were gathered at low tide from the depth around 1.0 meter and collection took place at two designated locations during spring tide periods. After harvesting, the seaweeds underwent a freshwater rinse to remove any external contaminants such as epiphytes, sand, and salts. The collected seaweeds were identified as *Sargassum wightii* and *Gracilaria corticata* species using reliable taxonomic keys of **Dawes and McIntosh (1981)** and **Sahoo (2010)**.

2. Experimental design and optimization of the four selected fermentation parameters using Box-Behnken design

The solid-state fermentation of seaweeds *Sargassum wightii* (SG) and *Gracilaria corticata* (GR) using *Bacillus subtilis* MN960600 (CK4) was studied considering four parameters: fermentation time (hours), seaweed quantity (g), moisture (%), and prebiotic quantity (%). The response variables for the fermentation process were yield in protein, reducing sugars, and lipids. A Box-Behnken second-order model was used to create a quadratic design with four factors and two levels per factor, aiming to determine the optimal levels for maximum yield.

Fermentation time ranged from 1 to 60 hours, seaweed quantity from 1 to 5 grams, moisture from 10 to 40%, and prebiotic (kidney bean powder) from 10 to 40%. A total of 29 experimental runs with various combinations were performed, and the responses are detailed in Tables (1, 2). Regression analysis was conducted to evaluate the influence of the selected variables on the yield of proteins, reducing sugars, and lipids.

Table 1. SWSG_CK4 Experimental combinations and results

Runs	Fermentation time	Seaweed quantity	Moisture	Prebiotic supplements	Protein	Reducing sugars	lipid
	Hours	G	%	%	mg/g	mg/g	mg/g
1	1	1	25	25	5.2	2.13	12
2	30.5	3	10	40	21.6	4.36	19
3	60	1	25	25	24.7	3.16	35
4	30.5	5	10	25	25	4.16	20

5	30.5	1	10	25	19	3.17	19
6	30.5	5	25	10	23.36	5.93	22
7	1	3	40	25	5.74	2.13	16
8	1	5	25	25	8.6	1.93	11
9	30.5	1	40	25	17.9	3.81	20.01
10	30.5	3	25	25	21.02	3.98	23.45
11	60	3	25	10	26.7	3.48	40
12	30.5	3	40	10	22.87	4.34	23.04
13	30.5	5	40	25	24.84	4.93	25
14	30.5	5	25	40	22.34	3.9	22
15	30.5	3	25	25	23.38	3.56	21.67
16	1	3	25	40	3.01	1.2	11
17	30.5	1	25	40	18.84	3.05	22.114
18	30.5	3	25	25	21.9	3.35	24.61
19	60	5	25	25	34.09	3.94	45.63
20	60	3	25	40	29.24	3.24	45.034
21	30.5	3	40	40	24.39	4.15	20
22	30.5	3	25	25	22.33	4.01	23
23	30.5	3	10	10	19.95	3.13	22
24	30.5	3	25	25	22.3	3.24	24
25	30.5	1	25	10	17.01	3	21
26	1	3	10	25	2.94	2	10
27	60	3	10	25	29.31	2.8	40
28	1	3	25	10	4.77	1.98	10
29	60	3	40	25	31.89	3.8	42

Table 2. SWGR_CK4 Experimental combinations and results

Runs	**Fermentation time**	**Seaweed quantity**	**Moisture**	**Prebiotic supplements**	**Protein**	**Reducing sugars**	**lipid**
	Hours	**G**	**%**	**%**	**mg/g**	**mg/g**	**mg/g**
1	1	1	25	25	1.6	4.3	14
2	30.5	3	10	40	19.21	9.1	21
3	60	1	25	25	24.25	7.96	34
4	30.5	5	10	25	22.74	10.89	24
5	30.5	1	10	25	14.7	6.99	21
6	30.5	5	25	10	24.91	12.01	35.52
7	1	3	40	25	2.4	4.6	18
8	1	5	25	25	4.12	5.67	13
9	30.5	1	40	25	16.15	9.44	29.13
10	30.5	3	25	25	20.16	10.93	31.45
11	60	3	25	10	30.1	7.84	40
12	30.5	3	40	10	25.4	9.056	36

13	30.5	5	40	25	32.06	12.24	37
14	30.5	5	25	40	22.58	10.82	33
15	30.5	3	25	25	23.6	7.18	32.14
16	1	3	25	40	2.36	3.84	13
17	30.5	1	25	40	14.13	6.93	26.21
18	30.5	3	25	25	23.64	8.9	36.14
19	60	5	25	25	39.32	10.8	47
20	60	3	25	40	34.17	7.98	42
21	30.5	3	40	40	27.9	9.71	38
22	30.5	3	25	25	24.14	9.01	34.15
23	30.5	3	10	10	17.73	5.1	31
24	30.5	3	25	25	24.7	5.83	34
25	30.5	1	25	10	14.9	6.1	33.52
26	1	3	10	25	2.82	3.14	12
27	60	3	10	25	26	8.03	40
28	1	3	25	10	3.05	4.6	13
29	60	3	40	25	36.15	9.03	44

For protein yield, the regression analysis showed significant results with an F-value of 69.72 for SWSG_CK4 and 64.24 for SWGR_CK4, indicating the model's significance. The Lack of Fit F-value was 3.65 for SWSG_CK4 and 1.16 for SWGR_CK4, suggesting that the lack of fit is not significant and the model error is minimal. The Predicted R^2 values were 0.9244 for SWSG_CK4 and 0.9282 for SWGR_CK4, in reasonable agreement with the Adjusted R^2 values of 0.9717 and 0.9693, respectively. These values indicate an adequate precision measure with a model ratio of 29.0408 for SWSG_CK4 and 28.7735 for SWGR_CK4, confirming an adequate signal. The determination coefficients (R^2) were 0.9859 for SWSG_CK4 and 0.9847 for SWGR_CK4, verifying the model's fitness.

The final equations for protein yield were:

SWSG_CK4 Protein = 22.19 + 12.14*A + 2.96*B + 0.8192*C + 0.3967*D + 1.5*AB - 0.055*AC + 1.07*AD + 0.235*BC - 0.7125*BD - 0.0325*CD - 4.62*A^2 - 0.2867*B^2 + 0.282*C^2 - 1.14*D^2 **[Equation 1]**

SWGR_CK4 Protein = 23.25 + 14.47*A + 5*B + 3.07*C + 0.355*D + 3.14*AB + 2.64*AC + 1.19*AD + 1.97*BC - 0.39* BD + 0.255*CD - 4.95*A^2 - 1.81*B^2 - 0.3311*C^2 - 1.18*D^2 **[Equation 2]**

Significant model terms for protein yield included factors A, B, and A^2 for SWSG_CK4, and A, B, C, and A^2 for SWGR_CK4. Three-dimensional response surface graphs depicted the optimal conditions for protein yield.

Table 3. ANOVA for quadratic model in response to SWSG_CK4 protein production

Source	Sum of Squares	df	Mean Square	F-value	*P* -value	
Model	2050.13	14	146.44	69.72	< 0.0001	significant
A-Fermentation Time	1768.31	1	1768.31	841.92	< 0.0001	significant term
B-Seaweed Quant	105.49	1	105.49	50.23	< 0.0001	significant term
C-Moisture	8.05	1	8.05	3.83	0.0705	-
D-Prebiotics	1.89	1	1.89	0.899	0.3591	-
AB	8.97	1	8.97	4.27	0.0578	-
AC	0.0121	1	0.0121	0.0058	0.9406	-
AD	4.62	1	4.62	2.2	0.1601	-
BC	0.2209	1	0.2209	0.1052	0.7505	-
BD	2.03	1	2.03	0.9668	0.3422	-
CD	0.0042	1	0.0042	0.002	0.9649	-
A^2	138.63	1	138.63	66	< 0.0001	significant term
B^2	0.5334	1	0.5334	0.2539	0.6222	-
C^2	0.5158	1	0.5158	0.2456	0.6279	-
D^2	8.38	1	8.38	3.99	0.0656	-
Residual	29.4	14	2.1	-	-	-
Lack of Fit	26.5	10	2.65	3.65	0.1116	not significant
Pure Error	2.9	4	0.7252	-	-	-
Cor Total	2079.54	28	-	-	-	-

Fit Statistics			
Std. Dev.	1.45	**R^2**	0.9859
Mean	19.8	**Adjusted R^2**	0.9717
C.V. %	7.32	**Predicted R^2**	0.9244
		Adeq Precision	29.0408

Table 4. ANOVA for quadratic model in response to SWGR_CK4 in protein production

Source	Sum of Squares	df	Mean Square	F-value	*P* -value	
Model	3184.53	14	227.47	64.24	< 0.0001	significant
A-Fermentation Time	2512.57	1	2512.57	709.59	< 0.0001	significant term
B-Seaweed Quant	300	1	300	84.72	< 0.0001	significant term
C-Moisture	113.22	1	113.22	31.98	< 0.0001	significant term
D-Prebiotics	1.51	1	1.51	0.4271	0.524	-
AB	39.38	1	39.38	11.12	0.0049	-
AC	27.93	1	27.93	7.89	0.0139	-
AD	5.66	1	5.66	1.6	0.2266	-

BC	15.48	1	15.48	4.37	0.0552	-
BD	0.6084	1	0.6084	0.1718	0.6848	-
CD	0.2601	1	0.2601	0.0735	0.7903	-
A^2	158.68	1	158.68	44.81	< 0.0001	significant term
B^2	21.16	1	21.16	5.98	0.0283	-
C^2	0.711	1	0.711	0.2008	0.6609	-
D^2	9.09	1	9.09	2.57	0.1315	-
Residual	49.57	14	3.54	-	-	-
Lack of Fit	36.86	10	3.69	1.16	0.4815	not significant
Pure Error	12.72	4	3.18	-	-	-
Cor Total	3234.11	28	-	-	-	-

Fit Statistics				
Std. Dev.	1.88		**R^2**	0.9847
Mean	19.83		**Adjusted R^2**	0.9693
C.V. %	9.49		**Predicted R^2**	0.9282
			Adeq Precision	28.7735

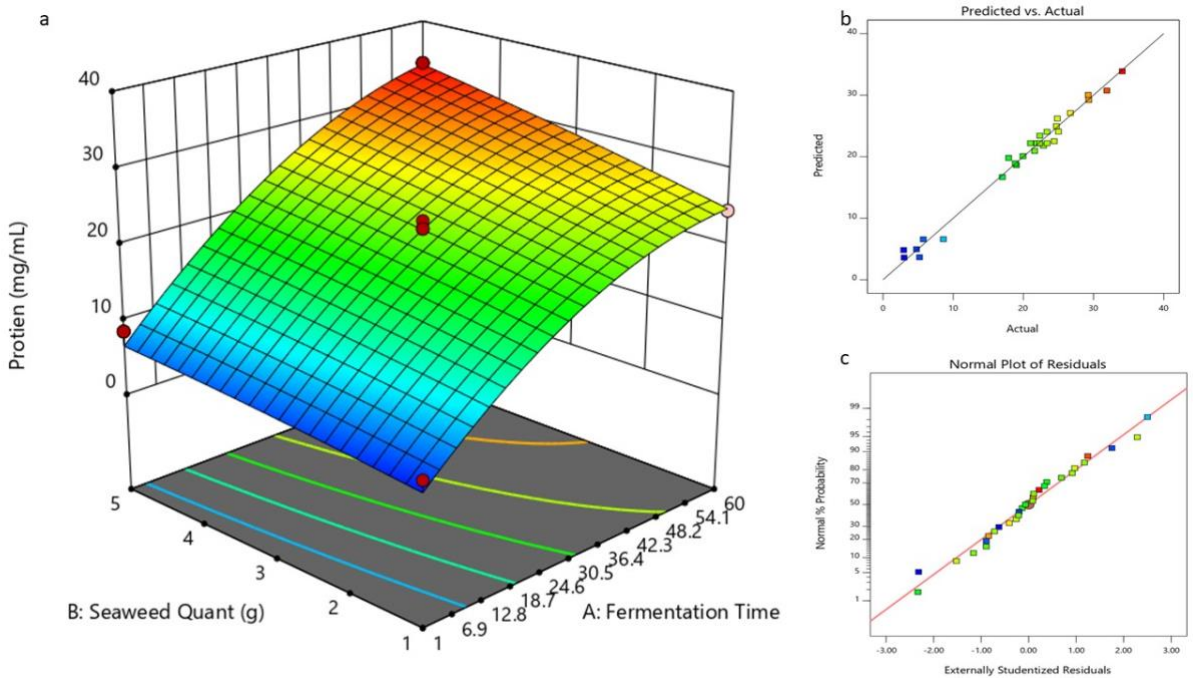

Fig. 1. SWSG_CK4 response surface plots for production of protein: **a)** Factor interaction heat map, **b)** Plot for predicted vs actual values, **c)** Plot for data distribution

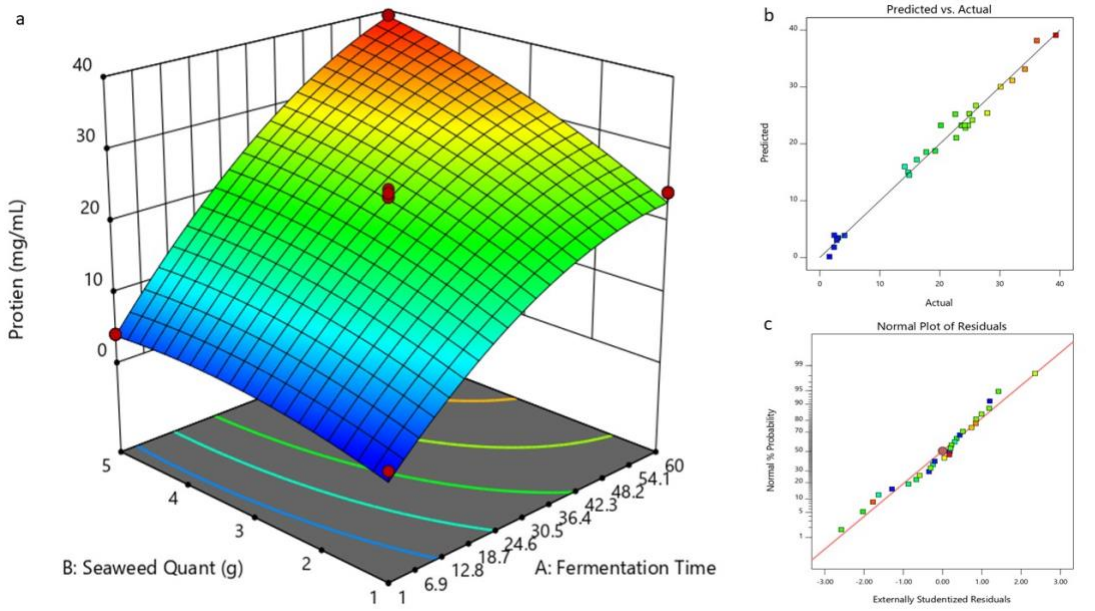

Fig. 2. SWGR_CK4 response surface plots for production of protein: **a)** Factor interaction heat map, **b)** Plot for predicted vs actual values, **c)** Plot for data distribution

For reducing sugar yield, the regression analysis showed significant results with an F-value of 7.8 for SWSG_CK4 and 6.12 for SWGR_CK4. The Lack of Fit F-value was 2.17 for SWSG_CK4 and 0.2663 for SWGR_CK4, indicating minimal model error. The Predicted R^2 values were 0.4194 for SWSG_CK4 and 0.5449 for SWGR_CK4, in reasonable agreement with the Adjusted R^2 values of 0.7726 and 0.7191, respectively. The model ratios were 10.6906 for SWSG_CK4 and 9.1142 for SWGR_CK4, indicating adequate signal. The determination coefficients (R^2) were 0.8863 for SWSG_CK4 and 0.8595 for SWGR_CK4.

The final equations for reducing sugar yield were:

SWSG_CK4 Reducing sugars = 3.63 + 0.7542*A + 0.5392*B + 0.295*C - 0.1633 D + 0.245* AB + 0.2175* AC + 0.135* AD + 0.0325* BC - 0.52* BD - 0.355 CD - 1.16*A^2 + 0.2531*B^2 + 0.2118*C^2 + 0.0843* D^2 **[Equation 3]**

SWGR_CK4 Reducing sugars = 8.37 + 2.12*A + 1.73*B + 0.9022*C + 0.3062*D + 0.3675*AB - 0.115*AC + 0.225* AD - 0.275*BC - 0.505*BD - 0.8365*CD - 2.22*A^2 + 1.08*B^2 + 0.2234*C^2 - 0.3066*D^2 **[Equation 4]**

Significant model terms for reducing sugar yield included factor A for SWSG_CK4, and A and A^2 for SWGR_CK4. Three-dimensional response surface graphs depicted the optimal conditions for reducing sugar yield.

Table 5. ANOVA for quadratic model in response to SWSG_CK4 in reducing sugars production

Source	Sum of Squares	df	Mean Square	F-value	*P*-value	
Model	25.14	14	1.8	7.8	0.0002	significant
A-Fermentation Time	6.83	1	6.83	29.63	< 0.0001	significant term
B-Seaweed Quant	3.49	1	3.49	15.15	0.0016	-
C-Moisture	1.04	1	1.04	4.53	0.0515	-
D-Prebiotics	0.3201	1	0.3201	1.39	0.2581	-
AB	0.2401	1	0.2401	1.04	0.3246	-
AC	0.1892	1	0.1892	0.8215	0.3801	-
AD	0.0729	1	0.0729	0.3165	0.5826	-
BC	0.0042	1	0.0042	0.0183	0.8942	-
BD	1.08	1	1.08	4.7	0.048	-
CD	0.5041	1	0.5041	2.19	0.1612	-
A^2	8.76	1	8.76	38.02	< 0.0001	significant term
B^2	0.4155	1	0.4155	1.8	0.2006	-
C^2	0.2911	1	0.2911	1.26	0.2799	-
D^2	0.0461	1	0.0461	0.2003	0.6613	-
Residual	3.22	14	0.2303	-	-	-
Lack of Fit	2.72	10	0.2722	2.17	0.2371	not significant
Pure Error	0.5023	4	0.1256	-	-	-
Cor Total	28.36	28	-	-	-	-

Fit Statistics				
Std. Dev.	0.4799		**R^2**	0.8863
Mean	3.37		**Adjusted R^2**	0.7726
C.V. %	14.22		**Predicted R^2**	0.4194
			Adeq Precision	10.6906

Table 6. ANOVA for Quadratic model in response to SWGR_CK4 in Reducing sugars production

Source	Sum of Squares	df	Mean Square	F-value	*P*-value	
Model	154.04	14	11	6.12	0.0008	significant
A-Fermentation Time	54.15	1	54.15	30.11	< 0.0001	significant term
B-Seaweed Quant	35.74	1	35.74	19.88	0.0005	-
C-Moisture	9.77	1	9.77	5.43	0.0352	-
D-Prebiotics	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	2.8	1	2.8	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	1.8	-	-	-
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	-	-	-	-

Fit Statistics			
Std. Dev.	1.34	**R^2**	0.8595
Mean	7.86	**Adjusted R^2**	0.7191
C.V. %	17.05	**Predicted R^2**	0.5449
		Adeq Precision	9.1142

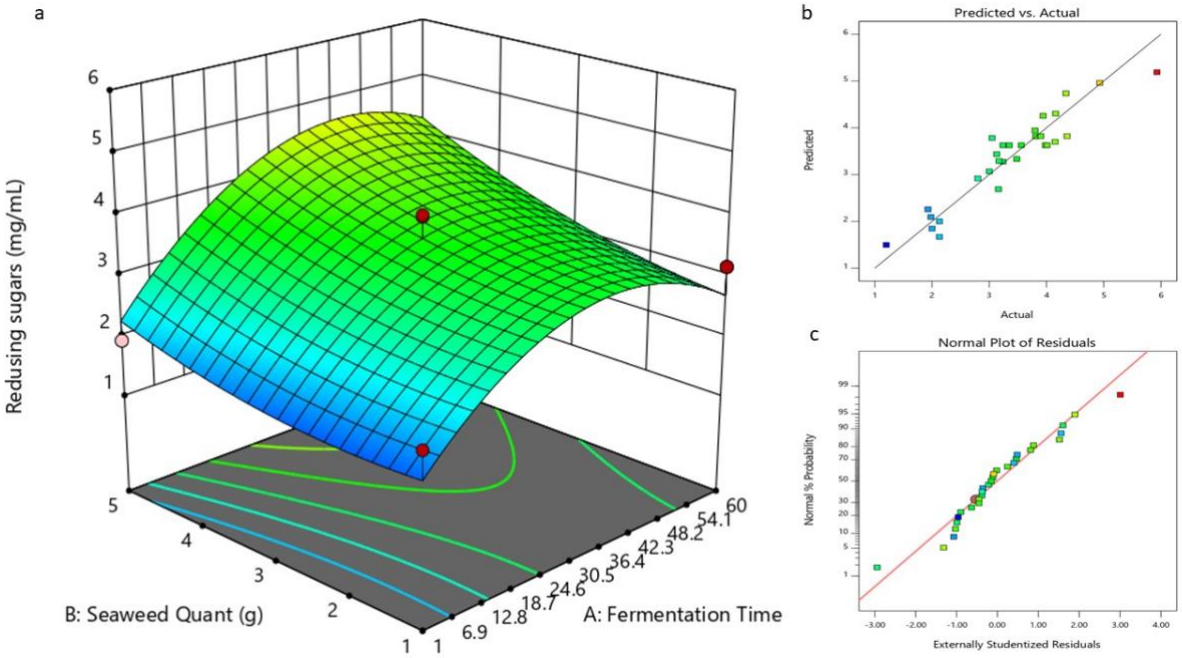

Fig. 3. SWSG_CK4 response surface plots for production of reducing sugars: **a)** Factor interaction heat map, **b)** Plot for predicted vs actual values, **c)** Plot for data distribution

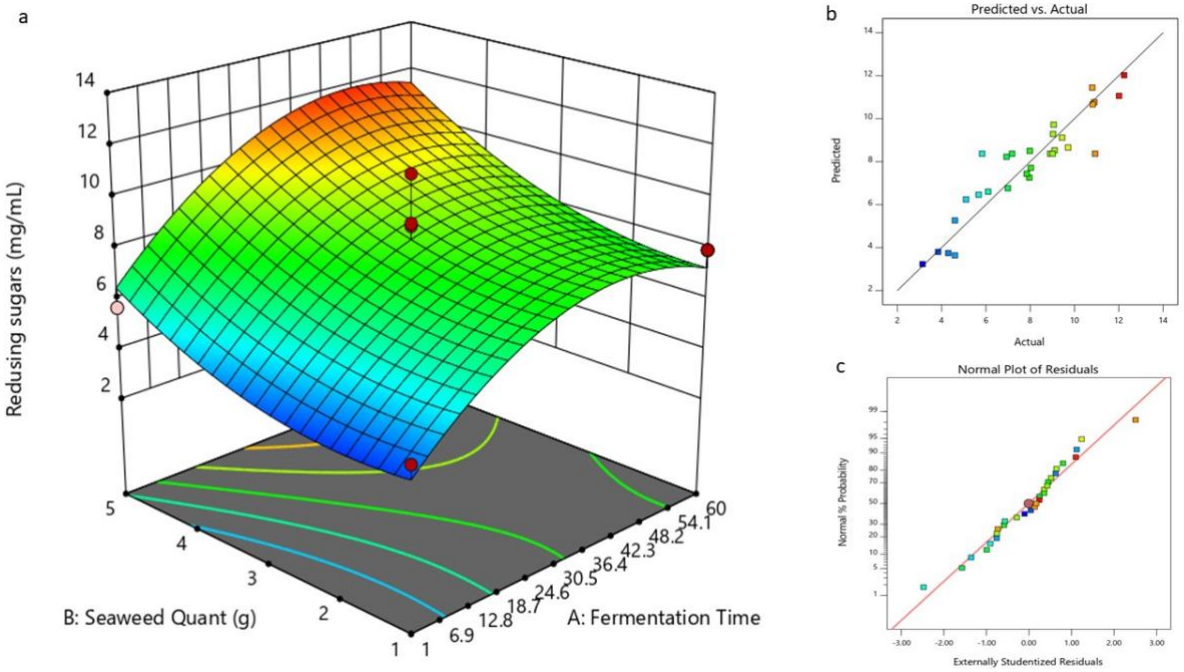

Fig. 4. SWGR_CK4 response surface plots for production of reducing sugars: **a)** Factor interaction heat map, **b)** Plot for predicted vs actual values, **c)** Plot for data distribution.

For lipid yield, the regression analysis showed significant results with an F-value of 57.38 for SWSG_CK4 and 30.6 for SWGR_CK4. The Lack of Fit F-value was 3.65 for SWSG_CK4 and 2.31 for SWGR_CK4, indicating minimal model error. The Predicted R^2 values were 0.9084 for SWSG_CK4 and 0.8373 for SWGR_CK4, in reasonable agreement with the Adjusted R^2 values of 0.9657 and 0.9367, respectively. The model ratios were 26.4645 for SWSG_CK4 and 20.5025 for SWGR_CK4, indicating adequate signal. The determination coefficients (R^2) were 0.9829 for SWSG_CK4 and 0.9684 for SWGR_CK4.

The final equations for lipid yield were:

SWSG_CK4 Lipid = 23.35 + 14.81*A + 1.38*B + 1.34*C + 0.0923*D + 2.91*AB – 1*AC + 1.01*AD + 0.9975*BC - 0.2785*BD - 0.01*CD + 4.17*A^2 - 1.2*B^2 - 1.03*C^2 - 0.8923*D^2 **[Equation 5]**

SWGR_CK4 Lipid = 33.58 + 13.67*A + 2.64*B + 4.43*C - 1.32*D + 3.5*AB - 0.5*AC + 0.5*AD + 1.22*BC + 1.2*BD + 3*CD - 4.51*A^2 - 2.34*B^2 - 1.87*C^2 - 0.4809*D^2

[Equation 6]

Significant model terms for lipid yield included factors A and A^2 for SWSG_CK4, and A and C for SWGR_CK4. Three-dimensional response surface graphs depicted the optimal conditions for lipid yield.

Table 7. ANOVA for quadratic model in response to SWSG_CK4 in lipid production

Source	Sum of Squares	df	Mean Square	F-value	*P* -value	
Model	2885.08	14	206.08	57.38	< 0.0001	significant
A-Fermentation Time	2630.37	1	2630.37	732.43	< 0.0001	significant term
B-Seaweed Quant	22.7	1	22.7	6.32	0.0248	-
C-Moisture	21.47	1	21.47	5.98	0.0283	-
D-Prebiotics	0.1023	1	0.1023	0.0285	0.8684	-
AB	33.81	1	33.81	9.42	0.0083	-
AC	4	1	4	1.11	0.3091	-
AD	4.07	1	4.07	1.13	0.3052	-
BC	3.98	1	3.98	1.11	0.3103	-
BD	0.3102	1	0.3102	0.0864	0.7731	-
CD	0.0004	1	0.0004	0.0001	0.9917	-
A^2	112.64	1	112.64	31.36	< 0.0001	significant term
B^2	9.29	1	9.29	2.59	0.1301	-
C^2	6.94	1	6.94	1.93	0.1861	-
D^2	5.16	1	5.16	1.44	0.2503	-
Residual	50.28	14	3.59	-	-	-
Lack of Fit	45.31	10	4.53	3.65	0.1118	not significant
Pure Error	4.96	4	1.24	-	-	-
Cor Total	2935.36	28	-	-	-	-

Fit Statistics				
Std. Dev.	1.9		**R^2**	0.9829
Mean	23.78		**Adjusted R^2**	0.9657
C.V. %	7.97		**Predicted R^2**	0.9084
			Adeq Precision	26.4645

Table 8. ANOVA for quadratic model in response to SWGR_CK4 in lipid production

Source	Sum of squares	df	Mean square	F-value	*P* -value	
Model	2834.38	14	202.46	30.6	< 0.0001	significant
A-Fermentation Time	2241.33	1	2241.33	338.72	< 0.0001	significant term
B-Seaweed Quant	83.53	1	83.53	12.62	0.0032	-
C-Moisture	235.23	1	235.23	35.55	< 0.0001	significant term
D-Prebiotics	20.88	1	20.88	3.16	0.0974	-
AB	49	1	49	7.41	0.0166	-
AC	1	1	1	0.1511	0.7033	-
AD	1	1	1	0.1511	0.7033	-
BC	5.93	1	5.93	0.896	0.3599	-
BD	5.74	1	5.74	0.8668	0.3676	-

CD	36	1	36	5.44	0.0351	-
A^2	132.06	1	132.06	19.96	0.0005	-
B^2	35.51	1	35.51	5.37	0.0362	-
C^2	22.7	1	22.7	3.43	0.0852	-
D^2	1.5	1	1.5	0.2267	0.6413	-
Residual	92.64	14	6.62	-	-	-
Lack of Fit	78.97	10	7.9	2.31	0.2177	not significant
Pure Error	13.67	4	3.42	-	-	-
Cor Total	2927.02	28	-	-	-	-

Fit Statistics				
Std. Dev.	2.57		**R^2**	0.9684
Mean	29.77		**Adjusted R^2**	0.9367
C.V. %	8.64		**Predicted R^2**	0.8373
			Adeq Precision	20.5025

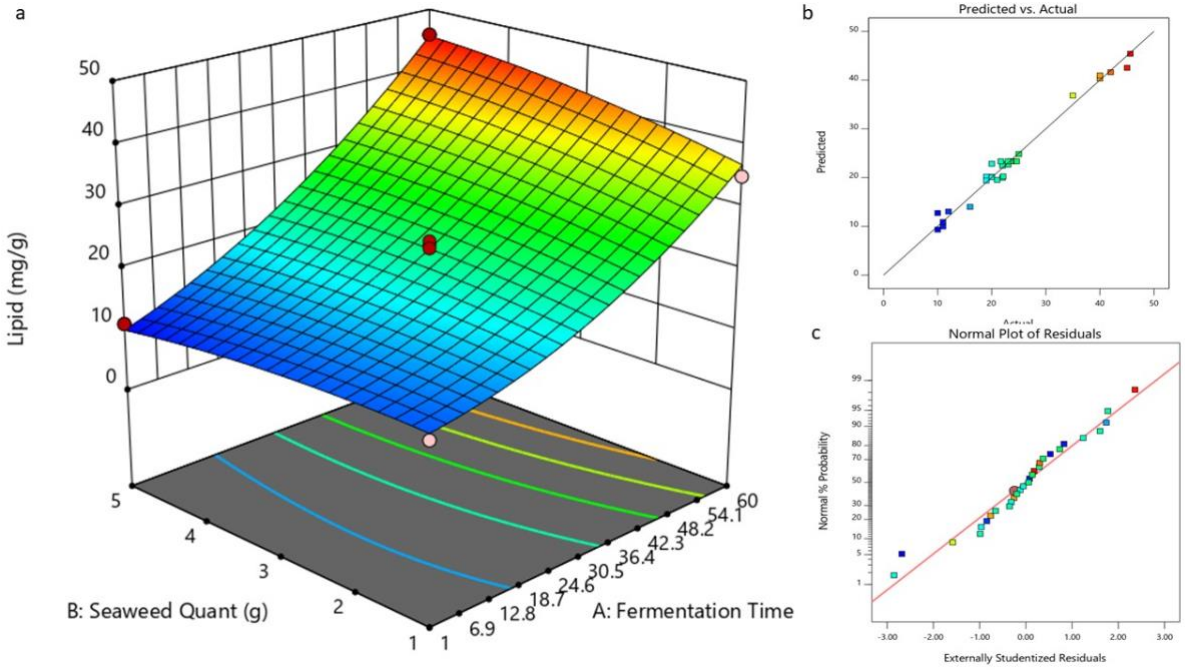

Fig. 5. SWSG_CK4 Response surface plots for production of lipids: **a)** Factor interaction heat map, **b)** Plot for predicted vs actual values, **c)** Plot for data distribution

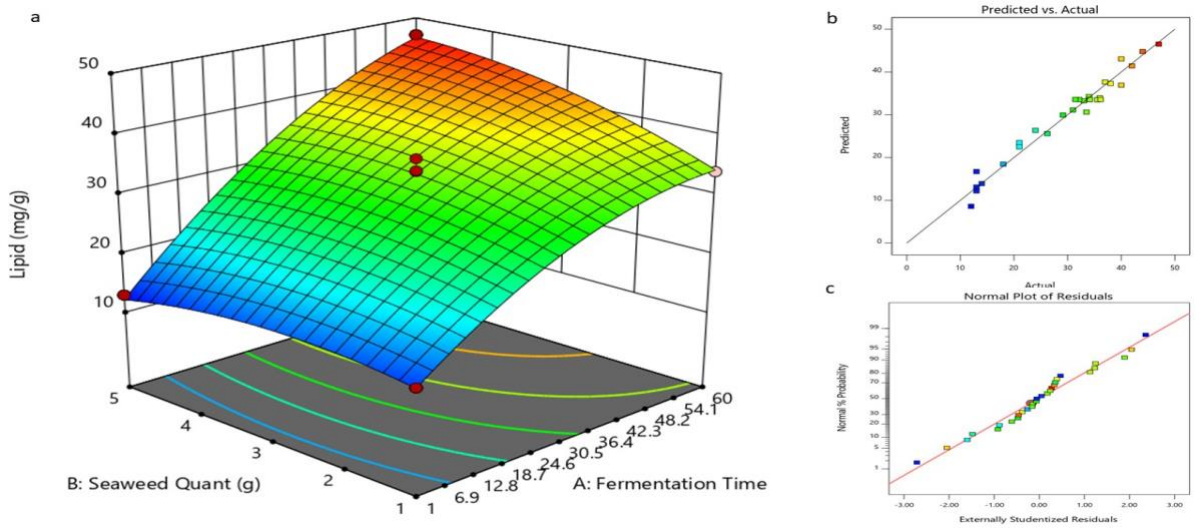

Fig. 6. SWGR_CK4 Response surface plots for production of lipids: **a)** Factor interaction heat map, **b)** Plot for predicted vs actual values, **c)** Plot for data distribution

The optimal conditions for maximum yield in proteins, reducing sugars, and lipids from *Sargassum wightii* (SWSG) fermented using *Bacillus subtilis* MN960600 (CK4) were found to be 59.9 hours of fermentation, 1g seaweed, 40% moisture, and 39.99% prebiotics, yielding 26.77mg/ g protein, 3.6mg/ g reducing sugars, and 35.6mg/ g lipids. For *Gracilaria corticata* (SWGR_CK4), the optimal conditions were 57.58 hours of fermentation, 2.421g seaweed, 40% moisture, and 30.88% prebiotics, yielding 35.057mg/ g protein, 8.926mg/ g reducing sugars, and 42.80mg/ g lipids.

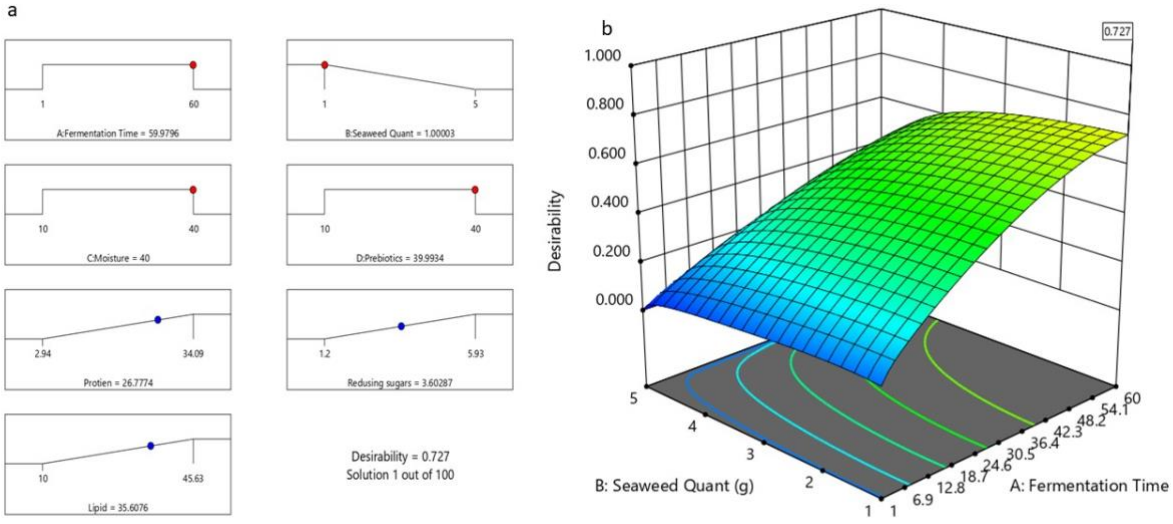

Fig. 7. SWSG_CK4 Optimal response of desired factor limits and desirability of the response model: **a)** Optimal response for the targeted response for each factor. **b)** Desirability of the computed model for optimal yield

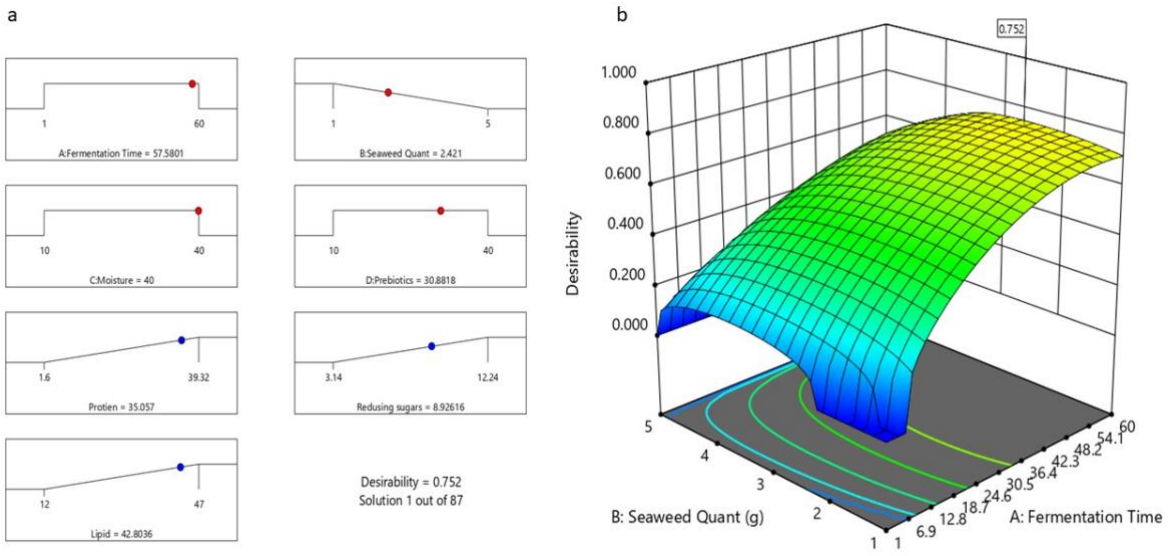

Fig. 8. SWGR_CK4 Optimal response of desired factor limits and desirability of the response model: **a)** Optimal response for the targeted response for each factor. **b)** Desirability of the computed model for optimal yield

Final feed formulation for both the seaweeds are as follows:

- Control feed composition- Fish meal (35.2%), ground nut oil cake (31.85%), rice bran (7.19%), corn flour (0.61%).
- NFSG - Fish meal (35.2%), ground nut oil cake (31.85%), rice bran (7.19%), corn flour (0.61%) and non-fermented seaweed S. wightii (25.15 %).
- NFGR - Fish meal (35.2%), ground nut oil cake (31.85%), rice bran (7.19%), corn flour (0.61%) and non-fermented seaweed G. corticate (25.15 %).
- SWSG_CK4 - Fish meal (35.2%), ground nut oil cake (31.85%), rice bran (7.19%), corn flour (0.61%) and fermented seaweed S. wightii (25.15 %).
- SWGR_CK4 - Fish meal (35.2%), ground nut oil cake (31.85%), rice bran (7.19%), corn flour (0.61%) and fermented seaweed G. corticate (25.15 %).

3. Total antioxidant capacity (TAC), ferrous reducing antioxidant capacity assay (FRAC / FRAP) and DPPH assay for both fermented and nonfermented seaweeds

In this study, the DPPH assay was employed to evaluate the antioxidative properties of various fermented and nonfermented seaweeds, specifically for their ability to function as proton radical scavengers or hydrogen donors. In the present study, seaweeds *Sargassum wightii* and *Gracilaria corticate* were fermented using a novel potent probiotic strain isolated from the dried anchovies (*Bacillus subtilis* MN960600). The total antioxidant activity was quantified as the amount of ascorbic acid equivalents in milligrams per gram of extract. Fig. (9a) clearly illustrates that the seaweed *Sargassum*

wightii (25.09 mg/g) and *Gracilaria corticata* (76.66 mg/g) exhibited contrasting total antioxidant capacity (TAC). *Sargassum* demonstrated a lower TAC ability, whereas the *Gracilaria* displayed the highest TAC ability. However, FRAP analysis reported no significant difference in both seaweeds. Our results align with the report of **Karthikeyan *et al.* (2015)**, where the extract of *Sargassum swartzii* (19.8mg of ascorbic acid/g) exhibited higher TAC activity compared to other seaweed species. Similarly, **Pirian *et al.* (2018)** reported comparable results, with *Sargassum vulgaris* exhibiting the greatest reduction activity and *Gracilaria corticata* displaying the highest total antioxidant capacity. Bioactive compounds prevalent in *Sargassum wightii* and *Gracilaria corticata* contribute for the antioxidant properties of the formulation. Presence of phenolic compounds, such as flavonoids and phenolic acids neutralize harmful free radicals and reduce oxidative stress. Carotenoids pigments such as beta-carotene and lutein in seaweed have strong antioxidant properties that can protect cells from oxidative damage and other environmental stressors **(Imchen & Singh 2022)**. Sulfated polysaccharides in *Sargassum wightii* and *Gracilaria corticata* have been found to exhibit antioxidant, anti-inflammatory, and immunomodulatory properties, contributing to the overall health benefits **(Olegovna *et al.*, 2022)**. Increased free radical scavenging activity was observed to be higher in seaweed *Sargassum wightii* fermented with *Bacillus subtilis* MN960600 (SWSG_CK4: 68.66%) among the rest of the combination followed by seaweed *Gracilaria corticata* fermented with *Bacillus subtilis* MN960600 (SWGR-CK4: 63.23%) (Fig. 9b). It was observed that fermented seaweeds have better antioxidant activity compared to non-fermented seaweeds *Sargassum wightii* (SGNF: 47.43%) and *Gracilaria corticate* (GRFN: 54.44%) which showed lower antioxidant activity.

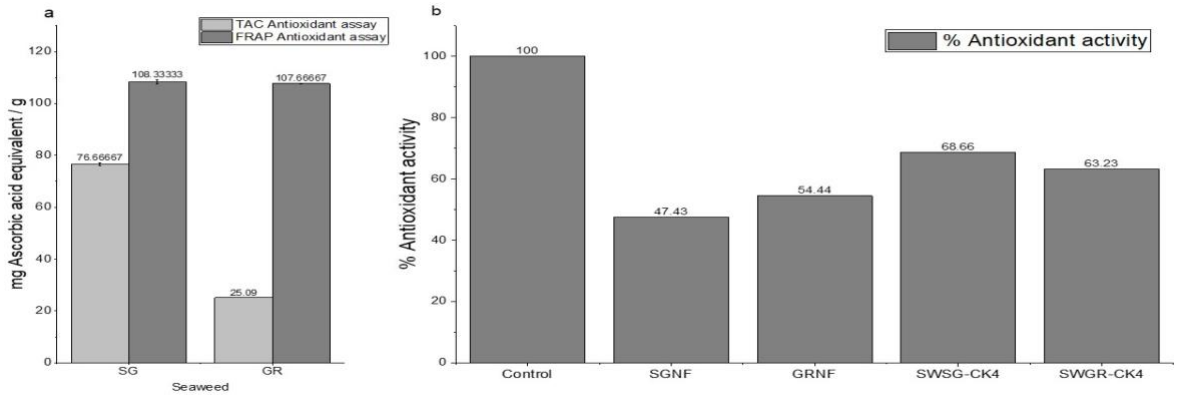

Fig. 9. Antioxidant studies namely TAC, FRAP and DPPH analysis for samples: SG: *Sargassum wightii*, GR: *Gracilaria corticata*, SGNF: *Sargassum wightii* non fermented, GRNF: *Gracilaria corticata* non fermented, SWSG-CK4: *Sargassum wightii* fermented with CK4, SWGR-CK4: *Gracilaria corticata* fermented with CK4. **a)** TAC and FRAP studies of nonfermented seaweeds. **b)** DPPH assay for fermented and nonfermented seaweeds

4. *Oreochromis niloticus* growth performance and feed utilization studies

The growth performance of *Oreochromis niloticus* in different treatment groups is depicted in Fig. (10a), representing the efficiency of the experimental diets. Following supplementation of the feeding, the initial body weight exhibited uniformity across all treatment groups. Upon the completion of 56 days of trials, a significant difference in growth rate was observed between the control group and the group treated with fermented seaweeds ($P< .05$). During the initial six weeks of the feed trials, there was no significant variations in growth rate between the two groups subjected to fermented seaweed treatment. However, this pattern changed after eight weeks, where a noticeable 14.29% surge in growth rate was determined in the SWGR_CK4 group compared to the SWSG_CK4 treated group. The difference in growth rate (42.86%) was well evident in fermented feeds compared to non-fermented seaweeds and the group supplemented with difference of 21.43% growth rate was observed between the control group and fermented seaweeds. Eventually, the data indicated that the feed conversion ratio (FCR) within the fermented seaweed-treated groups, namely SWGR_CK4 and SWSG_CK4, exhibited a significant lower value in comparison with the groups administered non-fermented seaweed *Sargassum wightii* (NFSG) and non-fermented seaweed *Gracilaria corticate* (NFGR) (Fig. 10b). Similarly, an increase in the value of feed efficiency (FE) was determined in feed trials in slots supplemented with SWGR_CK4 and SWSG_CK4, (Fig. 11a). Likewise, specific growth rate (SGR%) and weight gain (WG%) were higher in SWGR_CK4 and SWSG_CK4 slots supplemented with fermented seaweed compared to feed trials administered with non-fermented seaweed in NFSG and NFGR (Fig. 11b, c) respectively. These findings collectively shed light on the favourable effects of fermented seaweed supplementation, particularly in terms of growth performance metrics, suggesting its potential significance in enhancing the feed formulation strategies in *Oreochromis niloticus*.

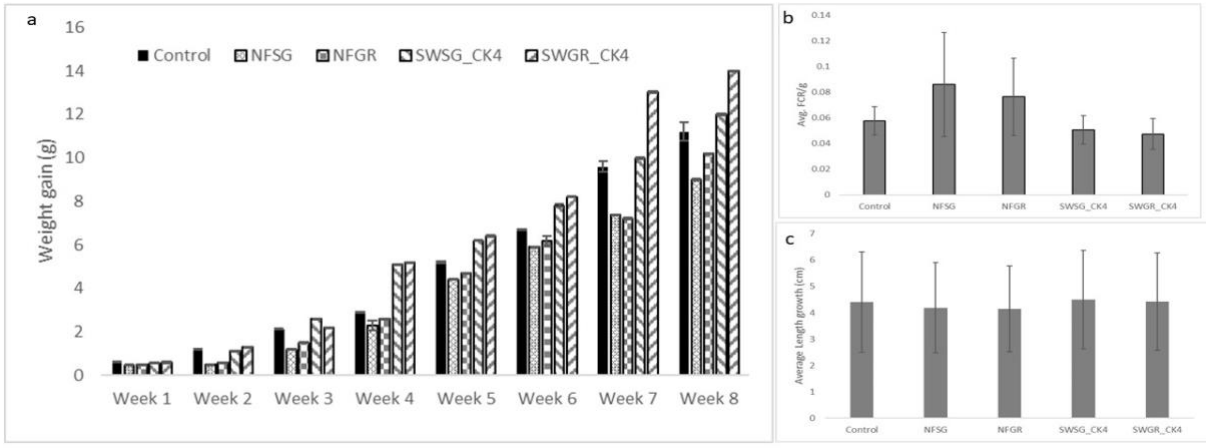

Fig. 10. Growth performance parameters of *Oreochromis niloticus* using both fermented and non-fermented seaweeds as a feed

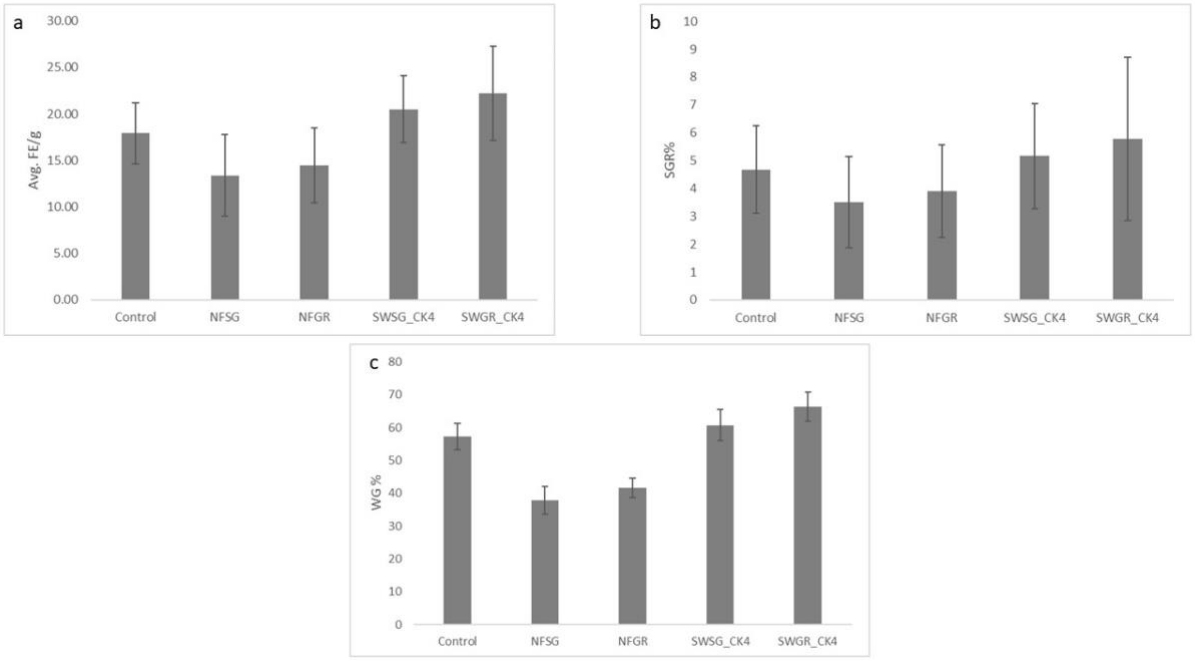

Fig. 11. Growth performance parameters feed efficiency (FE), specific growth rate (SGR), and weight gain (WG) of *Oreochromis niloticus* using both fermented and non-fermented seaweeds as a feed

5. Survival and oxidative stress analysis after pathogen challenge studies in each treatment groups

Oxidative stress as well as oxidative damage have been implicated in the initiation and progression of bacterial-induced diseases, contributing directly to disease pathogenesis of several bacterial fish diseases. The excessive formation of reactive oxygen species (ROS) inhibition to enzymatic antioxidant system, and the incapacity of the antioxidant defence system to scavenge and detoxify the excessive ROS production are linked to liver, kidney, and other tissue oxidative damage during bacterial infections, which contribute directly to disease pathogenesis and mortality of infected animals **(Souza *et al.*, 2019)**. Antioxidant enzymes have been used as a biomarker for detection of stress parameter in the Nile tilapia and its possibility as a potential candidate for tissue toxicity biomarkers **(Abdelazim *et al.*, 2018)**. Thus, the aim of the study was to evaluate whether tissue oxidative damage can be a reason linked to liver, kidney, and visceral damage during *Vibrio harveyi* and *Aeromonas hydrophila* infection using the Nile tilapia as an experimental model. In this study, when fish were exposed to pathogen *Vibrio harveyi* for one week, the mortality rate was observed to be 62.5% in control group, 12.5% in groups supplemented with non-fermented *Sargassum wightii* (NFSG), 12.5% in non-fermented *Gracilaria corticate* (NFGR), 0% in *Sargassum wightii* fermented using CK4 (SWSG_CK4) and 12.5% in group fed with *Gracilaria corticate* fermented using

CK4 (SWGR_CK4) (Table 9). Likewise, when exposed to *Aeromonas hydrophila* the mortality was observed to be 75% in control group, 37.5% in non-fermented *Sargassum wightii* (NFSG), 25% in non-fermented *Gracilaria corticate* (NFGR), 12.5% in *Sargassum wightii* fermented using CK4 (SWSG_CK4) and 25% in *Gracilaria corticate* fermented using CK4 (SWGR_CK4) (Table 10). However, when the fish were challenged with both the pathogenic bacteria together, the mortality was 100% in in control group, 75% in non-fermented *Sargassum wightii* (NFSG), 87.5% in non-fermented *Gracilaria corticate* (NFGR), 12.5% in *Sargassum wightii* fermented using CK4 (SWSG_CK4) and 37.5% in *Gracilaria corticate* fermented using CK4 (SWGR_CK4) (Table 11).

SOD enzymes control the levels of a variety of reactive oxygen species (ROS) and reactive nitrogen species, thus limiting both the potential toxicity of these molecules and controlling broad aspects of cellular life that are regulated by their signalling functions **(Sies *et al.*, 2020)**. The SODs convert superoxide radical into hydrogen peroxide and molecular oxygen, while the catalase and peroxidases convert hydrogen peroxide into water. In this way, two toxic species, superoxide radical and hydrogen peroxide, are converted to the harmless product water **(Weydert *et al.*, 2010)**. SOD activity in fish when challenged with pathogen *Vibrio harveyi,* both organ (liver, kidney, gonads, and viscera) and muscles were observed to be similarly stressed (Fig. 12 a). When challenged with *Aeromonas hydrophila* individually and in a mixed culture of *Vibrio harveyi* and *Aeromonas hydrophila* (1:1 ratio), the control group fishes were observed to have less SOD activity in organ level (liver, kidney, gonads, and viscera) than compared to muscles (Fig. 12b, c). In a related research, *Providencia rettgeri* infection in the Nile tilapia led to increased oxidative stress in the liver and kidney tissues **(Souza *et al.*, 2019)**. On day 14 post-infection, ROS and lipid peroxidation levels rose, and antioxidant enzyme activity decreased. These findings point to a compromised antioxidant defense mechanism in the infected fish, which may be relevant to the pathogenesis of the illness. **Sahan *et al.* (2016)** looked at the Nile tilapia that were given different amounts of ginger over a period of 90 days in another study. Comparing the 0.5 and 1.0% ginger groups to the control group, the former showed notable improvements in hematological parameters and oxidative stress indicators, including elevated SOD and CAT enzyme activity in the liver, gill, and gut tissues.

The % H_2O_2 inhibition levels were found to be 30 – 40 % lesser in SWSG_CK4 and SWGR_CK4 fed fish compared to commercial diet and nonfermented diet fed fish when challenged against *Vibrio harveyi* (Fig. 12d). Challenged with *Aeromonas hydrophila* individually and in a mixed culture of *Vibrio harveyi* and *Aeromonas hydrophila* (1:1 ratio), the H_2O_2 activity were not elevated as much of in organs (liver, kidney, gonads, and viscera) but was recorded higher in muscles. However, in fermented seaweed feeds SWSG_CK4 and SWGR_CK4 the H_2O_2 activity were found to be 8 to 9%

less in muscles and 4 to 6% less in organs (liver, kidney, gonads, and viscera) (Fig. 12e and f).

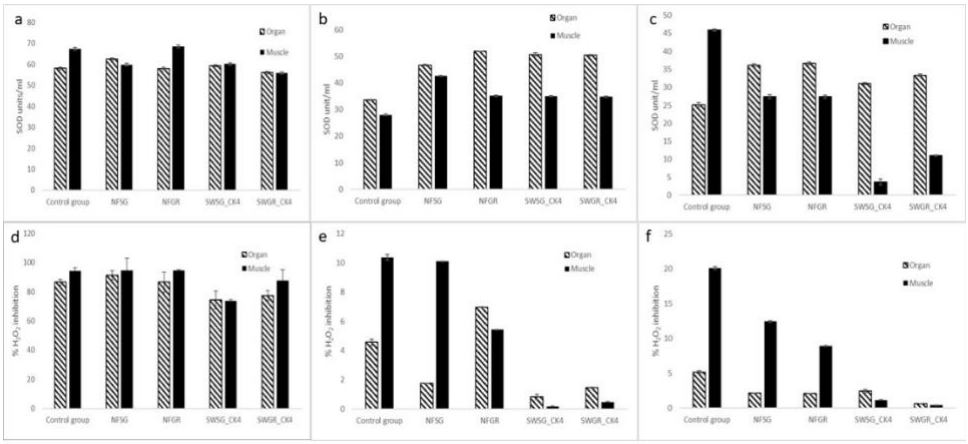

Fig. 12. Oxidative stress parameter (SOD and Catalase) in organs and muscle system

GSH is involved in protein glutathionylation and several other processes, such as the biosynthesis of leukotrienes and prostaglandins, and reduction of ribonucleotides **(Giacomo *et al.*, 2023)**. Glutathione (GSH), one of the most abundant low molecular weight non-protein thiols, modulates physiological levels of ROS and is involved in the cell's oxidative stress response **(Baba & Bhatnagar, 2018)**. GSH activity in organ system (liver, kidney, gonads, and viscera) were found to be 10 to 50 % less in all the three combination of challenge studies where the fishes were fed with seaweed formulated diets SWSG_CK4 and SWGR_CK4 (Fig. 13).

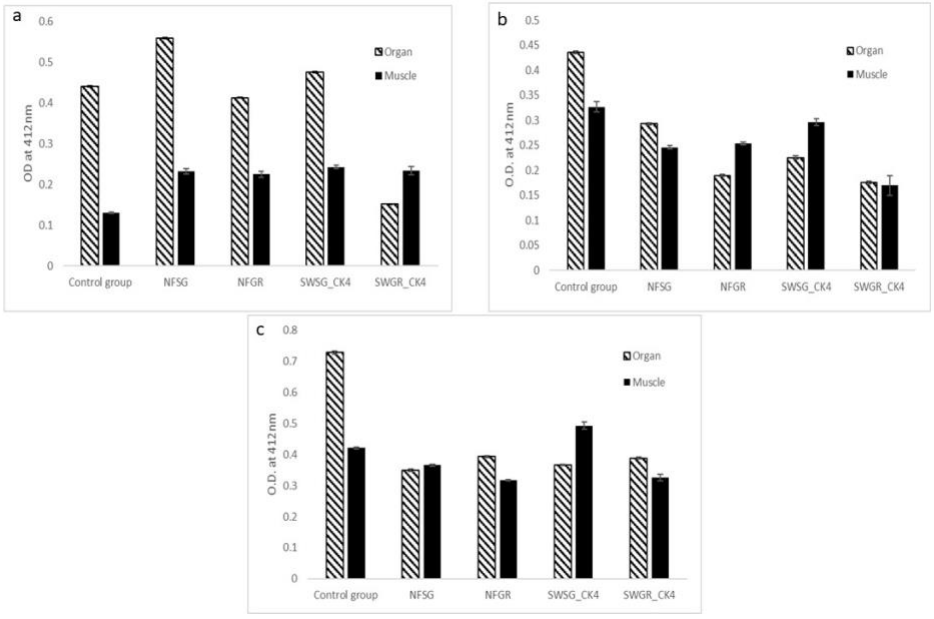

Fig. 13. Oxidative stress parameter (GSHs activity) in organs and muscle system

Glutathione S-transferases (GSTs) is a multifunctional enzyme, involved in detoxification processes. This role is achieved by catalytic conjugation of glutathione with a large number of electrophilic toxins **(Kumar & Trivedi, 2018)**. The glutathione transferases (GSTs; also known as glutathione S-transferases) are major phase II detoxification enzymes found mainly in the cytosol. The rate of increase in the absorption is directly proportional to the GST activity. In Fig. (14a), the % activity of GSTs in *Vibrio harveyi* infested fish were 80 to 90% lower in organs system (liver, kidney, gonads, and viscera) of fishes fed with SWSG_CK4 and SWGR_CK4 fermented feeds. Similarly, GSTs activity in muscles were found to be lower in fermented fed feed diets compared to non-fermented fed feed diets (Fig. 14b). GSTs activity in fish challenged with *Aeromonas hydrophila* exhibited similar results of that of fish challenged with *Vibrio harveyi* where the GST activity was found to be lower up to 80% in Organs and muscles (Fig. 14c and d). However, in mixed pathogen challenges the GSTs activity was higher in organs as well as muscles when compared to individual pathogen challenge studies. Although, still a significant lower GSTs activity was still observed in fermented feed diets in both organ system (liver, kidney, gonads, and viscera) and muscles (Fig. 14 e and f).

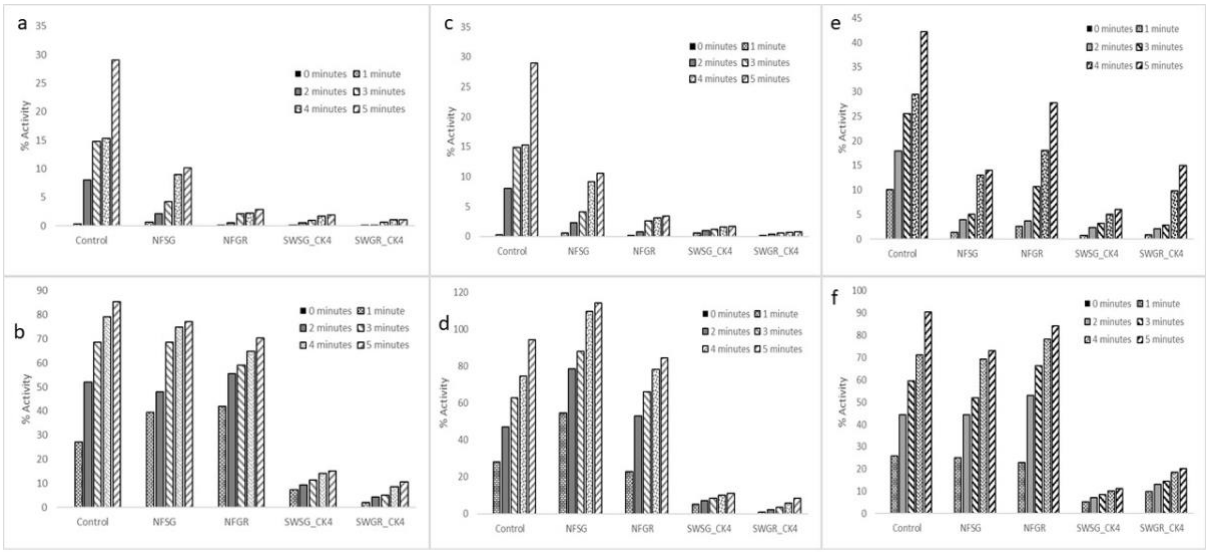

Fig. 14. Oxidative stress parameter (GSTs) in organs and muscle system

CONCLUSION

In the current study seaweeds *Sargassum wightii* and *Gracilaria corticata* were fermented using *Bacillus subtilis* MN960600 (CK4). The study explores the optimization of parameters via Box-Behnken design, among fermentation time, seaweed quantity, moisture, and prebiotic content for achieving maximal yields in proteins, reducing sugars, and lipids. Furthermore, the assessment of antioxidant capacities through total antioxidant capacity (TAC), ferrous reducing antioxidant capacity assay (FRAP), and DPPH assays

serves as a testament to reveal the enhanced antioxidative potential of fermented seaweeds. The evaluation of growth performance and feed utilization studies in *Oreochromis niloticus* reveals the benefits of fermented seaweed in the enhanced growth rates, improved feed efficiency, specific growth rate, and weight gain in *Oreochromis niloticus* fed with fermented seaweed formulations. The most compelling aspect of this study is the resistance conferred by fermented seaweeds against pathogenic bacteria *Vibrio harveyi* and *Aeromonas hydrophila*. The reduction in mortality rates, coupled with alterations in antioxidant enzyme activities and oxidative stress parameters reveal the potential of fermented seaweeds in mitigating the detrimental effects of pathogen infections in fishes. This research expands the scientific understanding of the fermentation process applied to seaweeds but also emphasizes their utility as functional feeds toward formulation as sustainable aqua feed.

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