

DETERMINATION OF PHYSICOCHEMICAL PROPERTIES OF FORMULATED FISH FEED SUPPLEMENTED WITH MICROALGAE FROM BIOREMEDIATION PROCESS

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Article history

Received

10 June 2018

Received in revised form

24 February 2019

Accepted

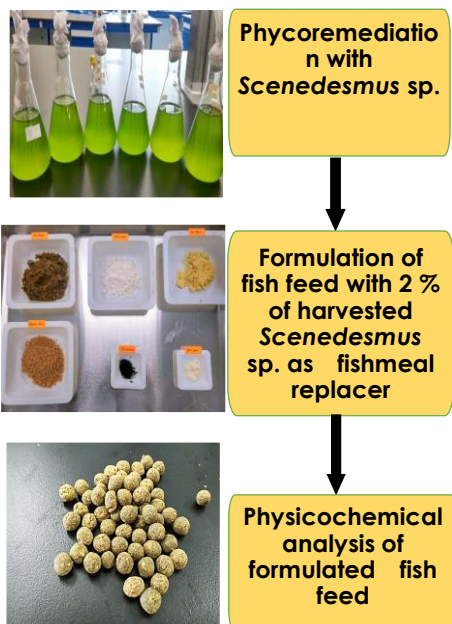
26 February 2019

Published online

18 April 2019

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Graphical abstract



Abstract

This study was conducted to determine the potential of *Scenedesmus* sp. from phycoremediation as fishmeal replacer in formulated fish feed targeted for *Oreochromis niloticus*. Formulated fish feeds were designated with Formulation 1 being the control; in Formulation 2 and 3, 2 % of fishmeal was substituted with *Scenedesmus* sp. cultivated in Bold's Basal Medium, and in artificial wastewater, respectively. It was found that supplementation with *Scenedesmus* sp. did not alter the hardness of the feed pellets ($p>0.5$), but the water stability of Formulation 2 and 3 was significantly lower than Formulation 1 ($p<0.05$). Zero correlation was established based on the fishmeal content with water stability ($p>0.05$). Negative correlation was determined between moisture content and water stability of Formulation 2 and 3 ($p<0.05$). This study showed the phycoremediation ability of *Scenedesmus* sp. in artificial wastewater with removal efficiency of 12.42 % and 49.25 % for Zn and Fe, respectively. It also found that biomass productivity of microalgae was lower in artificial wastewater compared to that in Bold's Basal Medium. Overall, the results of the study suggested the sustainability of wastewater-treated microalgae as fishmeal replacer had little effect on physicochemical properties of fish feed.

Keywords: *Scenedesmus* sp., formulated fish feed, physicochemical properties, water stability, phycoremediation

Abstrak

Penyelidikan ini bertujuan untuk mengkaji potensi *Scenedesmus* sp. dari fikoremediasi untuk dijadikan pengganti kepada tepung ikan dalam formulasi makanan ikan untuk *Oreochromis niloticus*. Tiga formulasi makanan ikan telah dirumuskan; dalam Formulasi 2 dan 3, tepung ikan telah diganti dengan *Scenedesmus* sp. yang dikulturkan dalam *Bold's Basal Medium* dan air kumbahan buatan pada 2 % sebagai protein tambahan. Hasil kajian menunjukkan bahawa penambahan makanan ikan dengan *Scenedesmus* sp. tidak mengubah kekerasan pelet makanan ikan tersebut ($p > 0.05$). Formulasi 2 and 3 mempunyai kestabilan dalam air yang lebih rendah ($p < 0.05$). Meskipun begitu, hubungan kait antara komposisi tepung ikan dalam makanan ikan dengan kestabilannya dalam air tidak dapat dibuktikan ($p > 0.05$). Korelasi negatif antara kandungan air dan kestabilan makanan ikan dalam air dibuktikan oleh Formulasi 2 dan 3 ($p < 0.05$). Kajian ini telah menunjukkan kapasiti *Scenedesmus* sp. untuk menjalankan fikoremediasi dengan kecekapan penyingkiran 12.42 % Zn and 49.25 % Fe. Walaubagaimanapun, produktiviti biojisim dalam air kumbahan buatan adalah lebih rendah apabila perbandingan dibuat dengan pertumbuhan dalam *Bold's Basal Medium*. Penemuan hasil kajian ini menunjukkan kesesuaian dan kelestarian untuk menggantikan tepung ikan dengan *Scenedesmus* sp. yang digunakan dalam rawatan air kumbahan kerana ianya mempunyai kesan yang kecil kepada sifat fisikokimia makanan ikan.

Kata kunci: *Scenedesmus* sp., formulasi makanan ikan, sifat fisikokimia, kestabilan makanan ikan, fikoremediasi

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1.0 INTRODUCTION

Malaysia is highly dependent on the import of animal feedstock especially for non-ruminant and aquaculture sectors [1]. Local production of fishmeal can sustain about 20 % of total requirements. However, due to the inferior nature of the raw materials (thrash fish or fishery wastes); the protein level is considerably lower with fluctuation between batches of products. The Malaysian government forecasted the annual consumption of fish to increase to 2.9 million tons per year in 2020 due to the increasing population and demand for fish protein [2]. The steep increase is bound to cause an increase in fish feed demand, and implicitly magnify the problem of inferiority and insufficiency of fishmeal supply in Malaysia.

Scenedesmus sp. is a species of green microalga under the Chlorophyceae family. It contains a high level of crude protein making it a perfect ingredient to be used in fish feed [3]. It has the potential to be a substitute for fishmeal due to the availability of essential amino acids required for fish growth. The flexibility and adaptability exhibited by the survival performance of *Scenedesmus* sp. allows it to be grown in different types of wastewater to perform phycoremediation, and give rise to low cost algal biomass that is ideal as fish feed supplement [4, 5]. Therefore, it is promising to use the sewage-cultivated *Scenedesmus* sp. as fishmeal substitutes.

Oreochromis niloticus, or commonly known as Nile tilapia, is one of the most cultured freshwater fish worldwide. In Malaysia alone, the production of Nile tilapia has reached 20.8 metric tonnes [6]. The five characteristics that make Nile tilapia one of the ideal candidates for aquafarming are high growth rate, versatility in environmental tolerance, stress and

disease resistance, high reproductive rate, and high acceptance for different diets [7]. The large-scale production of Nile tilapia in Malaysia is highly favoured if it is sufficient for both local supply and export. The farming of this hardy fish species can generate economic growth in the aquaculture industry of Malaysia. It is of utmost importance for local researchers to develop and lead the fish feed industry at a faster pace to make up for lost time, and alleviate reliance on imported formula feeds.

The use of *Scenedesmus* sp as human supplemental food has been explored, but it has yet to be commercialised. Some of the common microalgae that have been processed for human consumption are *Arthrospira*, *Dynaliella salina*, *Chlorella* spp. and *Aphanizomenon flos-aqua* [8]. It can be assured that the use of *Scenedesmus* sp. as animal feedstock will not incur an issue in food security for now. The potential of *Chlorella* spp. and *Scenedesmus* spp. as protein alternatives had also been studied [3]. The research provided an insight on the potential of *Scenedesmus* spp as a replacement for the comparatively more expensive fishmeal. The feasibility of sewage treated microalgae as animal feed had already been tested. According to Grau and Klein (1957) as cited by Lum *et al.* [9], feeding of broiler chickens with 20% substitution of sewage-grown *Scenedesmus* sp. in usual diet did not cause a negative effect in growth performance.

The objective of this study was to investigate the potential of *Scenedesmus* sp. from phycoremediation as fishmeal replacer in the formulation of a highly available, and economic fish feed for *Oreochromis niloticus*.

2.0 METHODOLOGY

2.1 Cultivation and Harvesting of *Scenedesmus* sp.

The freshwater microalgae used in this study were native from Endau-Rompin National Park, Upeh Guling, Johor. Fresh subculture was prepared by cultivating the microalgae in Bold's Basal Medium for 14 days. The intermediate-scale cultivation, in order to obtain the microalgae biomass, was performed by inoculating 1×10^6 cell/ml of *Scenedesmus* sp. into 5 gallon water bottles containing 10 litres of sterile BBM, and artificial wastewater, respectively. Before cell count and inoculation, the *Scenedesmus* culture was washed three times with sterile distilled water to remove residual chemicals. Cell count by observation of haemocytometer was performed under light microscope (Olympus LED BX 43, Japan).

The BBM used consisted of 10 ml of NaNO_3 25.0 g/l, $\text{CaCl}_2 \cdot 7\text{H}_2\text{O}$ 2.5 g/l, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 7.5 g/l, K_2HPO_4 7.5 g/l, KH_2PO_4 17.5 g/l, and NaCl 2.5 g/l each; EDTA 50 g/l, KOH 31.0 g/l, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 4.89g/l, H_2SO_4 1.0 ml/l, H_3BO_3 11.43 g/l, the micronutrients ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 8.82 g/l, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 1.44 g/l, MoO_3 0.71 g/l, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 1.57 g/l and $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$) were added at 1 ml each. On the other hand, artificial wastewater was composed of 4 ml of peptone 32 mg/l, meat extract 22 mg/l, and urea 6 mg/l each and 4 ml of NaCl 7 mg/l, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 4 mg/l, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 2 mg/l, NaNO_3 18.21 mg/l, NH_4Cl 76.43 mg/l and K_2HPO_4 16.84 mg/l each.

2.2 Growth Comparison and Phycoremediation Ability of *Scenedesmus* sp.

Cell concentration of 1×10^3 cell/ml was inoculated into 500 ml BBM and artificial wastewater each. Cell count was performed on day 15 of cultivation. The biomass productivity was computed according to Equation 1. Iron and zinc concentrations before and after 15-day phycoremediation by *Scenedesmus* sp. were analysed with Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) (Perkin Elmer AAnalyst 800, United States). The removal efficiency was computed using Equation 2.

$$\text{Biomass productivity} = \frac{\text{Nf-Ni}}{\text{Tf-Ti}} \quad (1)$$

$$\text{Removal efficiency (\%)} = \frac{\text{C1} - \text{C2}}{\text{C1}} \times 100 \quad (2)$$

Where C1 = input concentration; and C2 = output concentration

2.3 Formulation and Physicochemical Analysis of Fish Feeds

Three formulated fish feeds composed of fishmeal, soybean meal, wheat flour, and corn grain as basic ingredients were prepared. Each of the fish feed contained a different percentage of fishmeal, which were purely fishmeal, 98.0% fishmeal with 2.0% substitution of *Scenedesmus* sp., and 98.0 % fishmeal with 2.0 % substitution of *Scenedesmus* sp cultivated

in artificial wastewater. The weight of each ingredient is shown in Table 1.

Table 1 The formulation of fish feed with specific protein sources

Ingredients	Composition (%)		
	Formulation 1 Control	Formulation 2 98.0 % fishmeal + 2.0 % <i>Scenedesmu</i> <i>s</i> sp. cultivated in Bold's Basal Medium	Formulation 3 98.0 % fishmeal and 2.0 % <i>Scenedesmu</i> <i>s</i> sp. cultivated in artificial wastewater
Protein Supplement			
Soybean meal	19.60	19.60	19.60
Fishmeal	49.00	48.02	48.02
<i>Scenedesmu</i> <i>s</i> sp.	-	0.98	0.98
Basal Feed			
Corn grain	14.70	14.70	14.70
Wheat flour	14.70	14.70	14.70
Subtotal	98.00	98.00	98.00
Controlled ingredients			
Guar gum	2.00	2.00	2.00
Total	100.00	100.00	100.00

2.4 Physicochemical Analysis

AOAC Official Method 934.01 and 968.08 were employed for the determination of moisture content and ash, and mineral of the feeds. The water activity of the sample was determined using AquaLab Water Activity Meter (AL 1823, AquaLab LITE, US). Water stability of formulated fish feed was tested according to the method performed in previous research [10]. On the other hand, hardness of feed pellet was analysed with the application of texture analyser (Stable Micro Systems, UK). One-tailed t-test and one-way Analysis of Variance (ANOVA) were used to verify the data of the study (IBM-SPSS Statistics 22, US).

3.0 RESULTS AND DISCUSSION

3.1 Comparison of Microalgae Growth Rate in both Medium and Economic Feasibility of Cultivation Method

From the study, it was known that biomass productivity of microalgae was 1.11×10^4 g/l.d in BBM and 0.22×10^4 g/l.d in artificial wastewater. It was observed that the biomass productivity of *Scenedesmus* sp. in artificial wastewater was substantially lower than that in BBM. The lower biomass productivity observed from the growth of microalgae in artificial wastewater, suggested that there is an adaptation period for *Scenedesmus* sp. in the medium. In this experiment, the use of economic growth medium to cultivate microalgae strived as

the selling point. It is known that there has been an increase in the cost of inorganic fertilisers; the processing of microalgae fertilisers also led to undesirable environmental effect [11]. Fertiliser and fresh water makes up 50% of algal farming cost. Rather than relying on artificial media or freshwater, wastewater was used to mass-cultivate *Scenedesmus* sp. to improve the quality of wastewater quality, while giving rise to mass production of algae at the same time. Wastewater from food industry serves as a low-cost cultivating medium for algae by closing the loop of nitrogen and phosphorus cycle, and recovering nutrients that would otherwise be released as waste, and implicitly leading to harmful algal bloom [12].

3.2 Comparison of Mineral Biosorption of Microalgae in both Medium

The pH of artificial wastewater after phycoremediation was significantly different from the untreated one ($p < 0.05$). The pH range of sewage and industrial effluents limited by the government is from pH 6.0 to 9.0 for Standard A and pH 5.5 to 9.0 for Standard B according to Environmental Quality (Sewage and Industrial Effluents) Regulations 1979 [13]. The pH of sample was close to the limit according to EQA, but it was still acceptable. The pH of medium had become more alkaline after the treatment. The increase in pH could be explained by the photosynthesis of microalgae. The increase in pH signified the productivity of the *Scenedesmus* sp. in the culture medium, and the remediating ability of the microalgae by which it metabolized the inorganic carbon in the wastewater [14].

The effluent limit for iron concentration according to Standard A and Standard B is 1.0 and 5.0 mg/l, respectively, while for zinc concentration, it is 1.0 mg/l for both Standard A and Standard B. This study showed that the iron and zinc removal efficiency at 12.42 % and 49.25 % was desirable as it removed trace metals from wastewater effluent to diminish its adverse environmental effect, and the assimilation process supports the growth of *Scenedesmus* sp. Mineral recovery was achieved and when the microalgae was added into fish feed in order to substitute fishmeal, mineral fortification could be achieved. The microalgae could act as the mineral vehicles to supply trace metals for freshwater fish like Nile tilapia.

3.3 Physical Properties of Fish Feed

The following section discussed the physical properties of feed pellets and their interrelationships. All data on physical properties of feed is shown in Table 2.

Table 2 Physical properties of formulated fish feeds

Parameter	Fish feeds		
	Formulation 1 Control	Formulation 2 98.0 % fishmeal + 2.0 % Scenedesmus sp. cultivated in Bold's Basal Medium	Formulation 3 98.0 % fishmeal and 2.0 % Scenedesmus sp. cultivated in artificial wastewater
Feed characteristic	Sinking pellet	Sinking pellet	Sinking pellet
Diameter, mm	5.08 ± 0.054	5.05 ± 0.038	5.10 ± 0.046
Weight, g	0.158 ± 0.007	0.163 ± 0.015	0.167 ± 0.014
Colour			
L*	49.69 ± 0.074	37.39 ± 0.099	38.64 ± 0.085
a*	0.270	0.795	0.042
b*	6.02 ± 0.161	4.06 ± 0.479	5.82 ± 0.148
Water activity	0.65 ± 0.011	0.77 ± 0.008	0.64 ± 0.007
Hardness, N	14.05 ± 2.214	15.07 ± 1.202	15.38 ± 0.069

3.4 Physical Characteristics of Feed Pellets

Floatability of fish feeds is capable of manipulating the feeding motivation of Nile tilapia. In this study, the formulated feeds were classified as sinking pellets. Even though floating pellets showed higher uniformity and palatability, research suggested that floatability of feeds did not affect the survival rate of Nile tilapia [15, 16]. Nile tilapia could feed on pellets floating on the water surface and flowing in water column promptly after feed dispensation [17]. In this study, the average pellet diameter for the three feed formulations was 5.08 ± 0.025, which was larger than the suggested pellet size of 2.0 to 4.5 mm recommended by Liu *et al.* [18]. However, previous research had shown that pellet size did not affect the survival of Nile tilapia [19].

3.5 Colour of Feed Pellets

When data in the form of L-a-b system was analysed, it was found that the colour fish feeds of the three formulation were significantly different ($p < 0.05$). A post-hoc analysis using Tukey's HSD test found that the lightness of Formulations 2 and 3 was the same ($p > 0.05$); the redness of Formulations 1 and 3 was the same; and the yellowness of all three formulations were different ($p < 0.05$). Theoretically, it has been known that feeding motivation increased with

increasing darkness in the colour of the feeds [20]. It was deduced that Formulation 3, being intermediately dark while having close complexion to the red spectrum, would be a desirable fish feed formulation for Nile tilapia in order to improve growth performance of this commercial livestock.

3.6 Relationship of Moisture Content, Hardness and Water Stability of Feed Pellets

Moisture content of feed is usually formulated at a level lower than 11% in order to lengthen shelf life of the product; however, low moisture content could contribute to hardness of fish feed thus reducing feed intake. The hardness of fish feeds was also related to its water stability. Hardness of pellets correlated with higher water stability, but lower preference to fishes [17]. Results of one-way ANOVA test found the three formulations of feeds had the same hardness ($p > 0.05$). On the other hand, the moisture content of three formulated fish feeds was significantly different ($p < 0.05$), and Tukey's HSD test found that only the moisture content of Formulation 2 was significantly different from that of Formulations 1 and 3 ($p < 0.05$). The difference in moisture content between the samples might have been caused by failure in controlling the weight of pellets before and after the pelletizing and drying process and/or the small sample size. Correlation study suggested that there was no relationship between moisture content and hardness of the samples ($p > 0.05$).

Water stability of the three formulations was significantly different ($p < 0.05$). From Tukey's HSD analysis, it was found that when water stability test was conducted for 30 minutes, Formulations 2 and 3 had similar integrity ($p > 0.05$). Water stability test conducted for 1 hour and 2 hours, all formulations showed significantly different pellet integrity ($p < 0.05$). From the data obtained, it was observed that inclusion of *Scenedesmus* sp. had reduced the water stability of feed pellets for the three time frames (see Table 3). The observed condition could be explained by reviewing the formulation. As the protein content increased, the composition of starch reduced simultaneously; it was presumed that microalgae had higher protein content than fishmeal. It was noted that polysaccharide is considered as a common natural binder [21]. The designated feed formulations in this study involved the substitution of fishmeal with *Scenedesmus* sp.

It was postulated that the lower water stability observed in Formulations 2 and 3 was caused by the reduced level of fish protein, specifically gelatin found in fish skin and fish bones. There was zero correlation between moisture content and water stability ($p > 0.05$). When Pearson's correlation test was performed between moisture content and water stability of Formulations 2 and 3, a negative correlation was established ($r = -0.916$ at 30 minutes, $r = -0.997$ at 1 hour, and $r = -0.999$ at 2 hours; $p < 0.05$). This suggested that when moisture content increased, water stability of feed decreased. This

means that the reduction in water stability of Formulation 2 due to reduction of fishmeal content was masked by the high moisture content.

Table 3 Water stability test for formulated fish feeds

Time span for fish feed immersion (hour)	Water stability of feed pellet, %		
	Formulation 1 Control (pH 8.80 ± 0.071)	Formulation 2 n 2 98.0 % fishmeal + 2.0 % <i>Scenedesmus</i> sp. cultivated in Bold's Basal Medium (pH 8.41 ± 0.211)	Formulation 3 n 3 98.0 % fishmeal and 2.0 % <i>Scenedesmus</i> sp. cultivated in artificial wastewater (pH 8.49 ± 0.221)
0.5	94.95 ± 0.095	90.39 ± 0.407	91.41 ± 0.213
1	92.78 ± 0.220	60.76 ± 0.093	89.36 ± 0.663
2	88.27 ± 0.425	57.43 ± 0.161	84.79 ± 1.211

3.7 Mineral Composition of the Feeds

The mineral composition of the three formulated fish feeds is presented in Table 4. The results of one-way ANOVA for all six minerals showed that the composition of each mineral was different for the three fish feeds ($p < 0.05$). More information was obtained from post-hoc analysis using Tukey's HSD test. From the results of Tukey's HSD test, it was found that Formulations 2 and 3 had the same magnesium, iron and zinc content ($p > 0.05$). *Scenedesmus* sp. had increased the content of magnesium in fish feed by approximately 1000 times. Substitution of fishmeal with *Scenedesmus* sp. had substantially improved mineral profile of the formulated feeds. This study had proved the ability of *Scenedesmus* sp. to take up iron and zinc from the artificial wastewater, and it had increased the content of the aforementioned minerals in fish feeds, which was observable from iron and zinc content of Formulations 2 and 3. From the data, it could be deduced that Formulation 3 had a better mineral profile compared to Formulation 2.

Table 4 Mineral composition of formulated fish feeds

Mineral composition (mg/g)	Fish feeds		
	Formulation 1 Control	Formulation 2 98.0 % fishmeal + 2.0 % Scenedesmus sp. cultivated in Bold's Basal Medium	Formulation 3 98.0 % fishmeal and 2.0 % Scenedesmus sp. cultivated in artificial wastewater
Na	0.21 ± 0.002	1.22 ± 0.017	1.43 ± 0.021
K	0.08 ± 0.001	2.70 ± 0.034	3.67 ± 0.020
Mg	0.04 ± 0.208 × 10 ⁻³	0.30 ± 0.016	0.30 ± 0.038
Ca	1.45 ± 0.011	2.11 ± 0.006	2.24 ± 0.069
Fe	2.92 × 10 ⁻³ ± 0.776 × 10 ⁻³	10.9 × 10 ⁻³ ± 0.1 × 10 ⁻³	11.77 × 10 ⁻³ ± 0.577 × 10 ⁻⁶
Zn	35.64 × 10 ⁻³ ± 0.001	8.84 × 10 ⁻³ ± 0.1 × 10 ⁻³	9.65 × 10 ⁻³ ± 0.1 × 10 ⁻³

4.0 CONCLUSION

Scenedesmus sp. could perform phycoremediation to remove Fe and Zn in wastewater. From the physicochemical analysis of the formulated feeds, it could be deduced that *Scenedesmus* sp. could potentially substitute fishmeal at specific portion. Partial substitution of fishmeal with *Scenedesmus* sp. would not affect the hardness of the pellet but improve its colour to allow better visibility. The microalgae could act as mineral vehicle to improve mineral composition of the feeds. The feed pellets substituted with microalgae from phycoremediation would have higher availability, and it would be a more economic choice.

Acknowledgement

The authors wish to thank The Ministry of Education Malaysia under Fundamental Research Grant Scheme (FRGS) Vot No. K101, the Ministry of Energy, Science, Technology, Environment and Climate Change (MESTECC) under E-Science Fund (Vot No. 02-01-13-SF0135), and Universiti Tun Hussien Onn Malaysia under Research Fund and Geran Penyelidikan Pasca Siswazah (GPPS Vot No. H054) for supporting this research.

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