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Effects of Vermiwash as Nutrient Supplement for Mass Production of Marine Microalga, *Chloroidium saccharophilum* for High Density Production of Marine Copepod, *Dioithona rigida* (Cyclopoida, Copepoda)

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Abstract: Microalgae are often used as nutritional supplements for aquatic animals and are widely used in the aquaculture industry, providing direct or indirect nutrients for many aquatic animals. The production of microalgae is a high economical investment due to the high costs of the culture medium to obtain microalgae biomass. Strategies such as alternative media should be undertaken to decrease costs without affecting the nutritional values of microalgae. Hence, in the present study we investigated the effects of different vermiwash extract as a supplementary for the mass production of marine microalgal biomass in larviculture. In this study, the three different combinations [VJSCS (Vermiwash was prepared from decomposed materials of *Jasminum sambac*), VCACS (Vermiwash was prepared from decomposed materials of *Celosia argentea*) and VPCCS (Vermiwash was prepared from decomposed materials Prawn shell compost)] of vermiwash were prepared with Conway media solution A (10 ml/l) and investigated the effects of growth factor, biomass production and high density production of marine copepod using vermiwash cultured microalga, *Chloroidium saccharophilum.* The growth rate, cell count and biomass production was increased in the *Chloroidium saccharophilum* using VPCCS when compared to other vermiwash materials. With reference to biomass production, the vermiwash was prepared from decomposed material prawn shell (VPCCS) has resulted in highest biomass concentration. With reference to the proximate composition analysis, the *C. saccharophilum* was grown in different VJSCS, VCACS and VPCCS media and showed maximum CHO (226±3.4 mg/g), protein (314.3±16.16 mg/g) and lipid (10.6±1.4 mg/g) composition in VPCCS. This proximate compositions were found significantly ($P<0.05$) higher in VPCCS. In VPCCS- *C. saccharophilum* diet, the *Dioithona rigida* reached a maximum density (20000±1000 individuals/l) on 21st day constituting the nauplii, copepodites and adults. With reference to fatty acid profile the VPCCS- *C. saccharophilum* diet has shown significant impacts ($p < 0.05$) on the profiles of saturated, monosaturated and PUFAs of *Dioithona rigida*. The contents of PUFAs including EPA, DHA and ARA in *D. rigida* thus depended on the *C. saccharophilum* diet when compared to other microalgal diet. Hence, the VPCCS has promoted the *C. saccharophilum* biomass and nutritive content and also VPCCS - *C. saccharophilum* diet promotes highest productivity of *D. rigida*.

Keywords: Microalgae, *Chloroidium saccharophilum, Dioithona rigida,* Vermiwash

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Introduction

Microalgae are good source of bioactive compounds, nutritional properties, long chain polyunsaturated essential fatty acids, and biofuels (Altaff and Vijayaraj, 2021). Microalgae cultivation requires large amount of fertilizers causing high cost during production of value added products (Kothari *et al.,* 2017; Siddiki *et al.,* 2022). The use of fertilizers/specific media for microalgae cultivation has negative impact on the environment (Herrera *et al.,* 2021). To achieve economically feasible products from microalgae, it is necessary to choose a cheap source such as waste water and agro-industrial wastes (León-Vaz *et al.,* 2019; Dragone, 2022). Alternatively, vermicompost can be used to cultivate microalgae. In natural system, earthworms along with soil microorganisms degrade organic waste material of soil and thus maintain nutrient flux and act as excellent biological agents for recovery of vermiwash and vermiprotein for wide use in agro-ecosystem, aquaculture and poultry (Cardoso *et al.,* 2013; Nath and Singh, 2016; Singh *et al.,* 2017). The coelomic fluid of earthworm is called as vermiwash. Recently vermiwash production has drawn the attention of commercial vermiculturists, because of its rich organic composition (Cardoso *et al.,* 2013; Nath and Singh, 2016; Singh *et al.,* 2017). Long tubular body of earthworm contains coelomic fluid secreted by the body itself that always keeps the body wet and facilitates the body functions (Cardoso *et al.,* 2013; Nath and Singh, 2016; Singh *et al.,* 2017). Concentration of nutrients like auxins, cytokinins, bacteria, fungi, calcium, phosphorus, potassium etc. present in vermiwash, depends on the raw material which is used in the production of vermicompost. Vermiwash from vermicomposting has rich source of nutrients, enzymes, plant growth hormones and vitamins (Das *et al.,* 2014; Bhagat *et al.,* 2022). Both macro and micronutrients are present in the vermiwash as a result of composting of organic matter by earthworms (Das *et al.,* 2014; Bhagat *et al.,* 2022). The nutrients present in vermiwash are easily available to plants. Since, vermiwash contains high concentrations of nutrients especially ammonia, urea, nitrates and phosphates, it can be a good source to promote the growth of microalgae (Musyoka and Nairuti, 2021). While many studies have shown that microalgae can be cultivated in various wastewaters, the cultivation of microalgae in nutrient rich vermiwash has received less attention. In this study, the viability of vermiwash was evaluated as a substrate under different dilutions to grow fresh water microalgae. The positive effect of vermiwash on microalgae was investigated in terms of promoting growth rate and biomass. Hence, the present study aimed to investigate high density biomass production of marine microalga, *Chloroidium saccharophilum* using vermiwash from different bioresources and act as supplementary regulator for microalgal growth.

Materials and Methods

Indoor cultivation of C. saccharophilum using different vermicompost:

The marine microalga, *Chloroidium saccharophilum* (OM337702.1) was obtained from Aquaculture Research Laboratory, Department of Marine Biotechnology, AMET Deemed to be University, Chennai, India. The *C. saccharophilum* was sub-cultured using Conway media and maintained at 29°C with vigorous aeration and photoperiod was set to a light/dark cycle of 12/12 h with a light intensity of 8000 Lux.

For indoor culture system, the different vermiwash media incorporated with Conway media solution A were used for culturing microalgae and the experiments were divided into 4 groups. Group 1- the vermiwash was prepared from commercially available vermicompost (10 ml/l) with Conway media solution A. Group 2- the vermiwash was prepared from decomposed materials of *Jasminum sambac* (VJSCS) using *Eudrilus eugeniae* worms (10 ml/l) with Conway media solution A. Group 3 - the vermiwash was prepared from decomposed materials of *Celosia*

argentea (VCACS) using *E. eugeniae* worms (10 ml/l) with Conway media solution A, and group 4 - the vermiwash was prepared from decomposed materials prawn shell compost (VPCCS) using *E. eugeniae* worms (10 ml/l) with Conway media solution A. For vermiwash preparation, the compost was immersed in water (1 kg/l), and stirred using a magnetic stirrer. The mixture was filtered with filter paper to remove non-soluble particulate solids. The liquid was stored at 4°C for further microalgal growth studies. The growth study was performed in triplicate using 250 ml of Erlenmeyer flasks. Subsequently, a volume of 10 ml of *C. saccharophilum* suspension with an optical density (OD_{660}) was introduced into each flask. The culture conditions were maintained at 29°C with vigorous aeration and photoperiod was set to a light/dark cycle of 12/12 h with a light intensity of 8000 lux. For this experiment, the microalgae were cultivated for 10 days. The growth of *C. saccharophilum* was determined by measuring the optical density (OD) using UV-Vis spectrophotometer (UV Shimadzu 1800) at 660 nm (Kwon *et al.,* 2005). The cell count (cells/ml) was determined using Sedgwick-Rafter counting chambers through a light microscope (APHA, 2005). Dry weight of microalgae was measured by harvesting the cells by centrifugation and washing with deionized water. The pellet was dried at 105°C for dry weight measurement. The *C. saccharophilum* cells from each flask were collected after 10 day growth, and centrifuged at 5000 rpm for 15 min. Supernatants were decanted and cell pellets were dried at 80 °C overnight to a constant level. The dried microalgal biomass was collected for further biochemical analysis. The proximate composition of carbohydrate (Dubois *et al.*, 1956), protein (Lowry *et al.*, 1951) and lipid (Folch *et al.*, 1957) contents were estimated using standard methods. All the experiments were carried out in triplicate and data are expressed as mean ± SD.

Indoor cultivation of Dioithona rigida using different combination of C. saccharophilum cells:

Dioithona rigida was obtained from ICAR-Central Institute of Brackishwater Aquaculture, Chennai, India and the experiments were carried out at Copepod Culture Unit, Aquaculture Research Laboratory, Department of Marine Biotechnology, AMET Deemed to be University, Chennai, India. For mass production experiment, the *D. rigida* was fed with three different microalgae *C. saccharophilum* cultured (VJSCS, VCACS and VPCCS) with 30,000 cells/ml diets and the optimized environmental parameter (29ºC temperature 30 psu salinity, 7.8 pH) of the culture medium was maintained. The microalgae were added daily to the *D. rigida* culture tanks to maintain the diet concentration to the copepod. To estimate the population density of copepods, the total number of copepods (nauplii, copepodites and adults), random sample were collected from the culture tanks and counted using Sedwick Rafter chamber. At the end of the experiments the *D. rigida* were harvested and subjected for further biochemical profile analysis. The proximate composition of carbohydrate (Dubois *et al.*, 1956), protein (Lowry *et al.*, 1951) and lipid (Folch *et al.*, 1957) contents were estimated using standard methods. The fatty acid profile was quantified by Gas Chromatograph (Agilent 6890 - Agilent Technologies, Santa Clara, CA). Triplicate samples were analyzed and mean ± SD was recorded.

All the data were statistically analyzed using One-Way ANOVA test (analysis of variance) using GraphPad Prism 8 Software. A value of 0.05 was considered to indicate statistical significance and results are expressed as mean ± SD.

Results and Discussion

For high density production of marine microalgal biomasss, the Culture medium also plays an important role in the growth of biomass and neutral lipid production of microalgae (Rawat *et al.,* 2013; Rosli *et al.,* 2020). The culture medium formula is crucial in the cultivation of microalgae to obtain high final cell concentration (Rawat *et al.,* 2013; Rosli *et al.,* 2020). Additionally, the culture medium components must satisfy the basic requirements for marine microalgae cell build up and metabolite production, by providing an

optimum supply of energy for marine microalgae metabolism reaction (Rawat *et al.,* 2013; Rosli *et al.,* 2020). Some researcher stated that using microelement in the soil extract will be beneficial for cultivation of microalgae (Teo *et al.,* 2016; Vonshak, 2017; Yaacob *et al.,* 2021). In addition, some report proved that soil extracts initiate rapid reproductive and initiate rapid growth in certain microalgae. In many previous research, cultivation on the F/2 medium and Walne medium in sea water have been used but no report state the effect of vermiwash to Conway medium in cultivation of microalgae. Hence, our study indicated the effect of vermiwash extract as a supplement for the mass production of marine microalgal biomass in larviculture.

The findings of the present study revealed the positive growth promoting effects of vermiwash in terms of growth rate and biomass in *C. saccharophilum* alga which is cultivated from the vermiwash prepared from decomposed materials prawn shell compost (VPCCS). Comparison of growth Control, VJSCS, VCACS and VPCCS indicated that the vermiwash, VPCCS could be used as an alternate for growth promoter for cultivating microalgae. Since vermicompost contains high concentrations of nitrates and phosphates, it is an excellent way to promote the growth of microalgae. Utilizing the vermiwash to improve algal biomass productivity and the use of vermiwash to cultivate microalga, *C. saccharophilum* is reported for the first time. Results of this study indicated that the role of vermiwash as growth media for microalgae, *C. saccharophilum* can be used as alternative media for marine microalgal culture. In the present study, the growth rate, cell count and biomass production was increased in the *C. saccharophilum* which was cultured in the vermiwash prepared from decomposed materials prawn shell compost (VPCCS) when compared to other vermiwash materials (Figs. 1-3). With reference to biomass production, the vermiwash was prepared from decompose materials prawn shell compost (VPCCS) has resulted in highest biomass concentration. Similarly, the higher growth yield of *Chlorella* sp. and *Scenedesmus quadricauda* on refuse compost extract has been reported earlier (Wong, 1985). In addition, the biowaste leachate was used to cultivate *Euglene gracilis*, *Selenastrum* sp. and *Chlorella sorokiniana* for growth and lipid production (Tossavainen *et al.,* 2018). In a study by Tan et al. (2017), chicken, goat and plant compost water was used to cultivate *Chlorella vulgaris* and the highest specific growth rate was obtained. With reference to the proximate composition analysis, the *C. saccharophilum* was grown in different VJSCS, VCACS and VPCCS media and showed maximum carbohydrates (226±3.4 mg/g), protein (314.3±16.1 mg/g) and lipid (10.6±1.4 mg/g) composition in VPCCS media (Table 1). This proximate compositions were found significantly ($P<0.05$) higher in VPCCS (Fig. 4).

In hatchery, an adequate supply of live food for first-feeding fish larvae is essential and nutritional quality of live food organisms can be improved through nutrient enrichment (Vijayaraj *et al.,* 2022). The use of live food organisms, especially at first feeding, is a requisite for most marine fish larvae (Altaff and Vijayaraj, 2021a; Vijayaraj *et al.,* 2022). In ocean, the marine fish larvae primarily feed on copepods, but the production protocols of copepods as live food is under developed in hatchery (Altaff and Vijayaraj, 2021b). The cyclopoids *Oithona* spp. are used as live feed in marine aquaculture (Altaff, 2020). The optimization of copepod feeding protocol is utmost important to improve their culture productivity, maintain favorable water quality parameters as well as saving operational costs by preventing production of unnecessary quantities of microalgae (Altaff and Vijayaraj, 2021a). In the present study, VPCCS - *C. saccharophilum* diet, the *D. rigida* reached a maximum density (20000±1000 ind./l) on 21st day constituting the nauplii, copepodites and adults (Table 2). Similarly, Nandakumar *et al.* (2015) and Jothiraj and Santhanam, (2019) reported that the *C. saccharophilum* diet have higher growth compared to the other feeds like *Dunaliella* sp., *Isochrysis galbana*, *Nannochloropsis* sp., *Coscinodiscus*

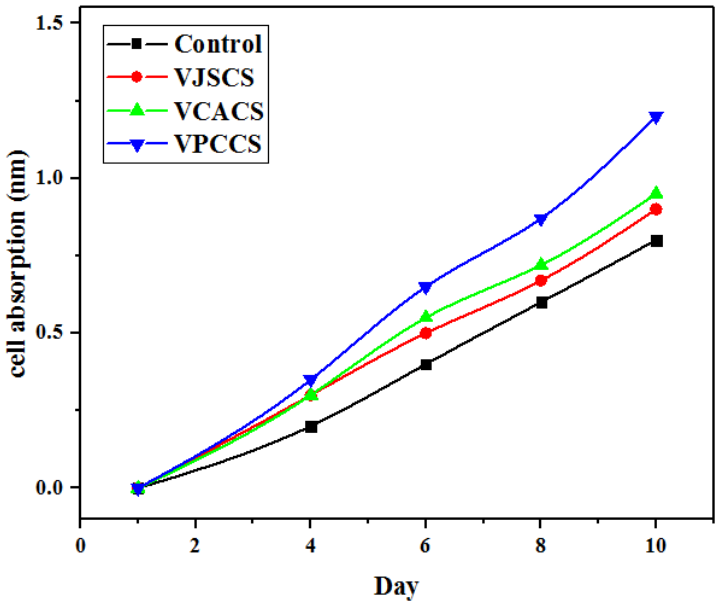

Fig. 1: Optical density of *C. saccharophilum* growth.

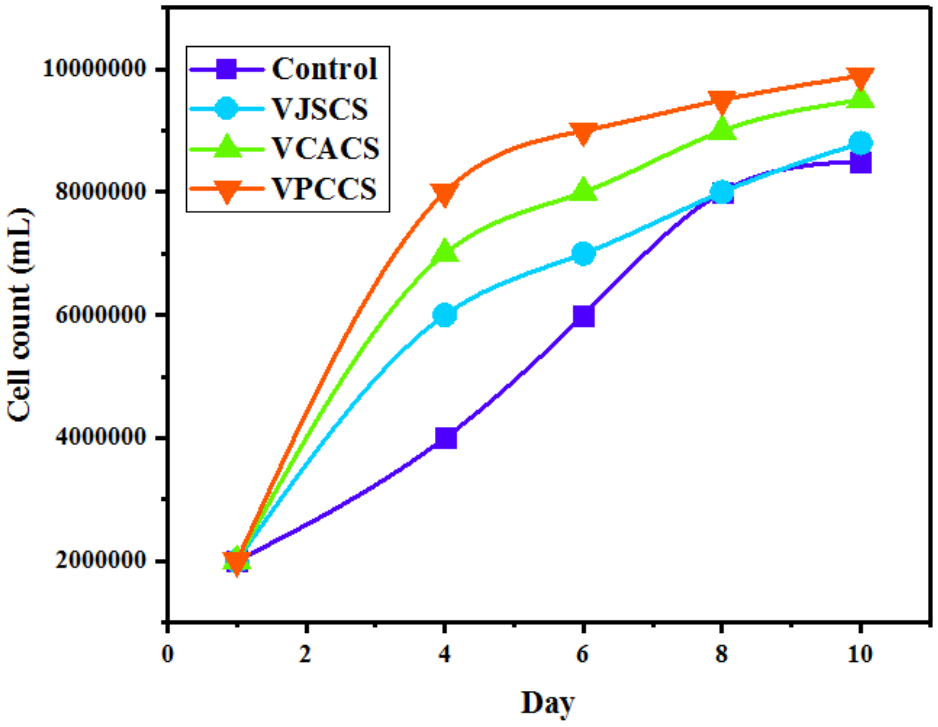

Fig. 2: Cell count of *C. saccharophilum* using different vermiwash promoters.

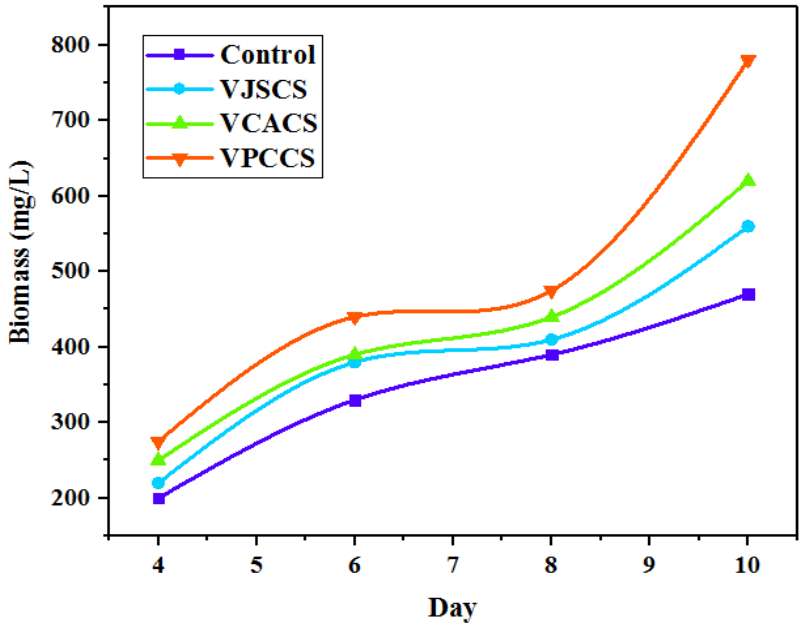

Fig. 3: Biomass of *C. saccharophilum* using different vermiwash promoters.

Table 1: Proximate composition of *C. saccharophilum* using different vermiwash promoters

Proximate composition	Control	VJSCS	VCACS	VPCCS
Carbohydrates (mg/g)	112±10.4	139±1.15	179±16.3	226±3.46
Protein (mg/g)	219±16.2	255±9.24	275±6.81	314.33±16.16
Lipid (mg/g)	6.8±1.04	7.8±0.35	8.17±0.75	10.66±1.44

The values are represented in Mean ± SD; The f-ratio value is 0.39784. The P value is 0.755998. The result is not significant (P < 0.05).

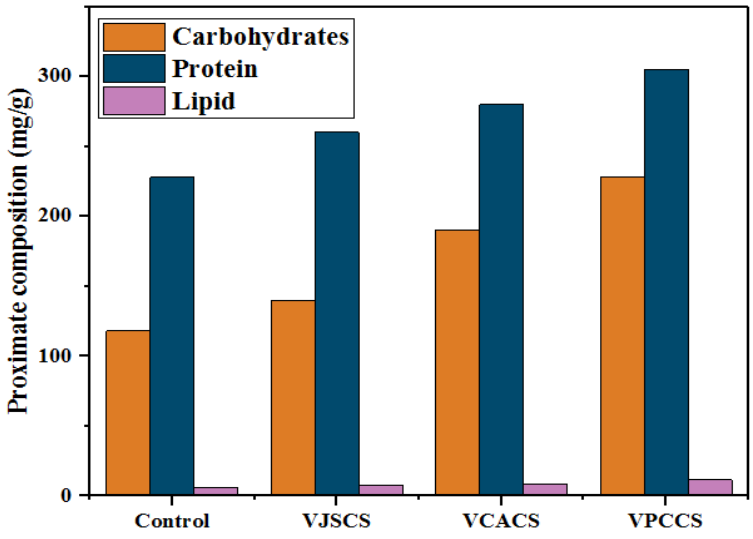

Fig. 4: Proximate composition of *C. saccharophilum* using different vermiwash promoters.

Table 2: High density production of *D. rigida* using different microalgal diets

Diet	Density (Ind./L)	Culture populations (ml)			
		Nauplii	Copepodites	Adults	Ovigerous
**Control	9333 ±1154	5±1	2±1	2±1	2±1
**VJSCS	12333±1527	7±1	3±1	2±1	2±1
**VCACS	14666±1154	9±1	3±1	2±1	2±1
*VPCCS	20000±1000	10±2	5±1	4±1	3±1

The values are represented in Mean ± SD. The f value is 15.17578. The P value is 0.000022. The result is significant (P < 0.05).

Table 3: Fatty acid profile of *D. rigida* using different microalgal diets

Fatty acid profile	Control	VJSCS	VCACS	VPCCS
C14:0	1.17±0.38	6.55±0.57	7.26±0.64	7.33±2.08
C 16:0	0	4.22±1.17	4.44±1.26	13.66±1.52
C 18:0	6.023±0.99	1.40±0.71	6.53±1.36	2.07±0.89
C 16:1 (n-7)	0.96±0.057	3.02±1.02	3.66±0.57	7.26±0.23
C 18:1 (n-9)	4.65±1	1.52±1.31	2.33±0.5774	4.33±1.52
C 18:1 (n-7)	21.66±2.88	11.77±3.46	14.25±1.55	2.49±1.32
C 18:2 (n-6)	6.34±0.57	10.66±2.30	12.09±1.81	3.5±0.5
C 18:3 (n-3)	48.73±5.7	45.46±2.88	41.29±3.74	5.33±1.15
C 18:4 (n-3)	4.41±2.3	0.79±0.254	0.65±0.56	5.81±1.28
C 20.4 (n-6)	2.7±0.43	0.42±0.15	0.22±0.19	5.83±0.76
C 20.5 (n-3)	0.26±0.23	3.77±2.88	1.40±1.21	16.33±1.52
C 22.6 (n-3)	3.06±0.11	7.22±0.57	6.63±0.54	26.93±4.35

The values are represented in Mean ± SD;. The f- value is 0.00236. The P value is 0.99984. The result is not significant (P < .05).

centralis, *Chaetoceros affinis* and *Skeletonema costatum*. The result of the present study indicated that the use of VPCCS - *C. saccharophilum* is a suitable diet for better growth of *D. rigida* leading to increase in the population density of the culture. Therefore, VPCCS - *C. saccharophilum* could be used as a preferred diet for culturing *D. rigida*. This might be possibly due to the significantly higher cellular density of this microalga. With reference to their nutritional properties, the fatty acids are energy sources and membrane constituents. They have biological activities that act to influence cell and tissue metabolism, function, and responsiveness to hormonal and other signals. The essential fatty acids in marine copepod ensured growth, survival and successful reproduction in marine fish seed production. Of these, especially eicosapentaenoic acid [EPA; 20:5 (n-3)] and docosahexaenoic acid [DHA; 22:6 (n-3)] are crucial, as copepods cannot

synthesize them and have to get them from food. Therefore, the abundant availability of good quality food is important for copepods. In the present study, VPCCS - *C. saccharophilum* diet has shown significant impacts ($p < 0.05$) on the profiles of saturated, monosaturated and PUFAs of *D. rigida*. The contents of PUFAs including EPA, DHA and ARA in *D. rigida* are depended on the *C. saccharophilum* diet when compared to other microalgal diet (Table 3). El-Sheekh *et al.* (2021) reported that the fatty acid profile of *C. saccharophilum* showed higher content of EPA, DHA and ARA than other microalgae. According to Nanton and Castell (1998) and Daase *et al.* (2011), the type of feed provided to copepods could influence their EPAs, in particular PUFAs, the long-chained omega-3 fatty acids EPA and DHA are produced exclusively by marine algae, and play a vital role in reproduction, growth, and metabolism of marine copepods.

Conclusion

Based on our experimental results it is concluded that the VPCCS - *C. saccharophilum* diet promotes highest productivity of *D. rigida* compared to other microalgal diet. The VPCCS - *C. saccharophilum* diet also enriches *D. rigida* with high total fatty acid and amino acid contents. Hence, the present study suggests VPCCS - *C. saccharophilum* as a suitable microalgal species for high density mass culture of this cyclopoid copepod species.

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