

Applying a New Feeding Protocol for Enhancing Mass Culture and Nutritional Value of the Rotifer *Brachionus plicatilis* Müller, 1786.

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Abstract

The study tested a new feeding protocol to find out the best program using cheap and available products to enhancement the mass culture and the nutritional value of *B. plicatilis*. The feeding regime was six artificial treatments formulated from dried yeast (*Saccharomyces cerevisiae*) with sucrose sugar and yeast with molasses in different concentrations. Additionally, a live *Cyclotella* sp. had been applied as supplementary food to each treatment and as independent treatment (control). *B. plicatilis* was cultured in small scale to find the best food regime and technique to apply it in the large one. *B. plicatilis* attained its highest density in the small scale culture (370 Ind.ml⁻¹) with T1 (30 % yeast, 70 % sugar and *Cyclotella* sp.) at the 12th day, while the highest population growth rate (PGR) (0.65) was calculated at the 9th day. According to the analysis of variance (ANOVA) and PGR, T1 had been applied in the large scale culture. *B. plicatilis* samples were composed of 51.6% total protein with 16 amino acids and 33.01% total lipid with 19 identified fatty acids. The study concluded that the treatment (T1) is a suitable diet for enrichment the mass culture and the nutritional value of *B. plicatilis*.

Introduction

With the increase of the population around the world, the biggest challenge is food security, Particularly the animal protein. Thus, aquaculture is becoming very important as a potential resource for the sustainable production of animal protein throughout the world, especially fish production declines from their natural fisheries. Nevertheless, one of the most important challenges plagues the development and growth of the aquaculture industry is the provision of a cheap feed, where fish feeds consume at least 60 % of the total farm income (FAO, 2016). Another important challenge is formulation suitable feeds for small larval fishes, whose functional development of their alimentary canal is not yet complete (Pedersen & Hjelme land, 1988). Thus, the researchers were paying attention to solve these challenges by using live food (phytoplankton and

zooplankton) and reduce the dependence on artificial feeds (Yousef & Hegab, 2017).

Zooplankton is considered a strategically hidden treasure of the animal protein around the world. It plays an important role in any aquatic ecosystem, by occupying a transitional position in the food chain (Xie, Xiao, Tang, Lu & Cai, 2008). Moreover, it's very important as food for many fishes (Grubišić et al., 2012). Rotifers are considered an important live food for fish larvae in the aquaculture industry because they characterized by slow motility, rapid reproduction and adequate size (Lubzens, Tandler, & Minkoff, 1989), besides having the noticeable amount of enzymes which helps fish larvae to develop their digestive system (Demir & Diken 2011). Among all rotifer species, *B. plicatilis* is the most common one used in aquaculture to feed fishes, especially in their early life stages (Lubzens, Zmora, & Barr, 2001), it is essential for intensive culture

of marine larval fish in the hatcheries of aquaculture systems (Yoshimura, Hagiwara, Yoshimatsu, & Kitajima, 1996; Cavalin & Weirich, 2009). Moreover, it constitutes a major and in some cases the only food source for larval stages of several marine aquaculture fish and invertebrates (Lubzens et al. 1990), due to its high growth rate, adequate size, high nutritional value and its ability to feed on a variety of feed types and it has a complex life cycle (Jeeja, Joseph, & Raj, 2011; Kostopoulou, Carmona, & Divanach, 2012).

The culturing of *B. plicatilis* has been studied by many researchers from different aspects. However, its success in mass culture has still potential for improvement (Kostopoulou, Miliou, & Verriopoulos, 2015). So, this work aimed to find out the best enrichment program using cheap and available products (yeast, sucrose sugar and molasses) with a live *Cyclotella* sp. for enhancement the mass culture of *B. plicatilis* and its nutritional value to use as valuable direct food for the earlier larval stages of fish or as supplementary food for the adults.

Material and Methods

Collection and Isolation of the Rotifer *B. plicatilis*

Samples were collected from the northern part of Manzala Lake near the connection point between the Lake and Mediterranean Sea (Boughaz El-Gamil Region, Port Said, Egypt) by filtration of 100 litres of water using zooplankton net of 55 μ m mesh size. Then the rotifer *B. plicatilis* was isolated by fine pipette to start the culture in small and large scale depending on the training manual (Abidi, 1998). *Brachionus plicatilis* was cultured in two steps; Small scale culture to find the best food regime and technique to apply it in large scale culture as the following:

The Small Scale Culture

Culture Technique and Experimental Design

The feeding regime was six artificial treatments formulated from dried yeast (*Saccharomyces cerevisiae*) with sucrose sugar (commercial sugar) and yeast with molasses in different concentrations as shown in Table 1, based on preliminary trials preceded this study.

Cyclotella sp. was added day other day between 11.00:12.00 pm with 2.5×10^3 live cells as supplementary food to each treatment. Additionally, *B. plicatilis* was fed on only live *Cyclotella* sp. (2.5×10^3 live cells) as a control, which added day other day. *Cyclotella* sp. was derived from pond culturing at the National Institute of Oceanography and Fisheries, Inland water branch, Egypt. All tested cultures were conducted at water temperature $28 \pm 1^\circ\text{C}$ and salinity (20‰) with daily photoperiod of 12 hours, and aeration was slightly and continuously provided using an air pump. The culture water using in this study was sterilized by using solar energy. Each treatment was started by culturing 1000 individual of the species in glass beakers (1 litre; i.e. one Individual ml^{-1}). 1/2 gram of the treatments (yeast + sugar) and (yeast + molasses) with different levels (six treatments) were added at the beginning of the experiment. The density of *B. plicatilis* was investigated every 3 days during the experiment. The experiment was conducted for 18 days.

Population Growth Rate was calculated by the following equation according to Schlosser and Anger (1982) and Hotos (2003) to find the best treatment for enhancing *B. plicatilis*.

$$\mu (\text{PGR}) = (\ln N_t - \ln N_0) / t$$

where

μ = population Growth Rate (PGR)

N_0 = Initial density

N_t = Final density at day-t of the culture period

t = Culture period (day)

Data analysis

To find the best treatment, one-way analyses of variance (ANOVA with subsequent Tukey's honestly significant difference (HSD) tests) based on abundance and PGR of *B. plicatilis* between different tested treatments were done by XLstat 2016 software.

The Large Scale Culture (Mass Culture)

At the end of the small culture, the best treatment of the previous experiment was chosen to feed *B. plicatilis* in large scale. Ceramic ponds (3.00×4.00×0.5 m 6000 litres' capacity) was used in this experiment under

Table 1. The different concentrations of the artificial treatments (T1-T6).

Treatment	Artificial Diet Percentages		
	Yeast	Commercial sugar	Molasses
T 1	30 %	70%	0
T 2	50%	50%	0
T 3	70 %	30%	0
T 4	30 %	0	70%
T 5	50%	0	50%
T 6	70 %	0	30%

soba at El-Kanater El-Khayria Station, National Institute of Oceanography and Fisheries, NIOF Cairo. The culture was performed at $28 \pm 1^\circ\text{C}$ (controlled by air-conditioning), salinity (20‰) with daily photoperiod of 12 hours. Central aeration with 3/4 inch in diameter made of PVC pipe (30 mm) perforated with 0.50-mm holes was provided to maintain the dissolved oxygen levels by air blower (2 horses). The mass culture was started by 10 Individual l^{-1} of *B. plicatilis* and counted every 3 days to calculate the abundance of *B. plicatilis* and its population Growth Rate (PGR). Half of the pond (2500 litres) was filtrated through plankton net (55 μm mesh size) when *B. plicatilis* attained its peak based on the results of small scale culturing. The collection mas of *B. plicatilis* was washed many times by distilled water to get a pure mass of *B. plicatilis* without any suspended matter. The wet mass was weighted then dried at 45°C for 48 hours using a digital oven to estimate the dry weight and assess its biochemical composition.

Analyzing of the Biochemical Composition of *B. plicatilis* Samples

Proximate analysis was conducted on *B. plicatilis* samples. Crude protein, lipid content, Amino and Fatty Acids of the cultured *B. plicatilis* samples were

measured depending on dry weight basis according to the standard methods of AOAC (2012) by Regional Center for Food and Feed (RCFF), Giza, Egypt (with International certificates of accreditation ISO/IEC 17025; the serial number of samples: 138 and 139).

Results

The Small Scale Culture

The Population Growth of *B. plicatilis*

The density and the population growth rate of *B. plicatilis* showed diverse patterns with different treatments (Figure 1 and 2). The densities of *B. plicatilis* increased gradually during the experiment period till they reached their maximum values at the 12th day with the treatments T1, T4 and T5 with values of 370, 178 and 110 Ind. ml^{-1} respectively then densities decreased again after the 12th day. However, the population growth rate of *B. plicatilis* was high from the 3rd to the 6th day with all treatments, but it reached its maximum values of 0.65 / day after 9 days with T1. On the other hand, T2 stimulated *B. plicatilis* to reach high value (118 Ind. ml^{-1}) in the 15th day, while the other treatments (T3, T6 and *Cyclotella* sp.) did not show an

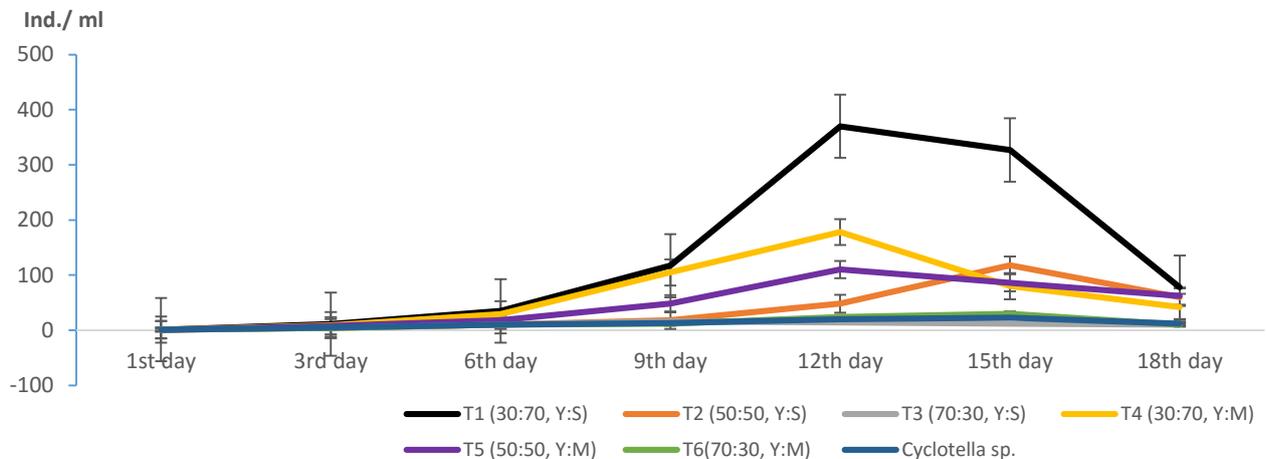


Figure 1. The densities of *B. plicatilis* (Ind. ml^{-1}) with different treatments during the experiment period of the small scale culture.

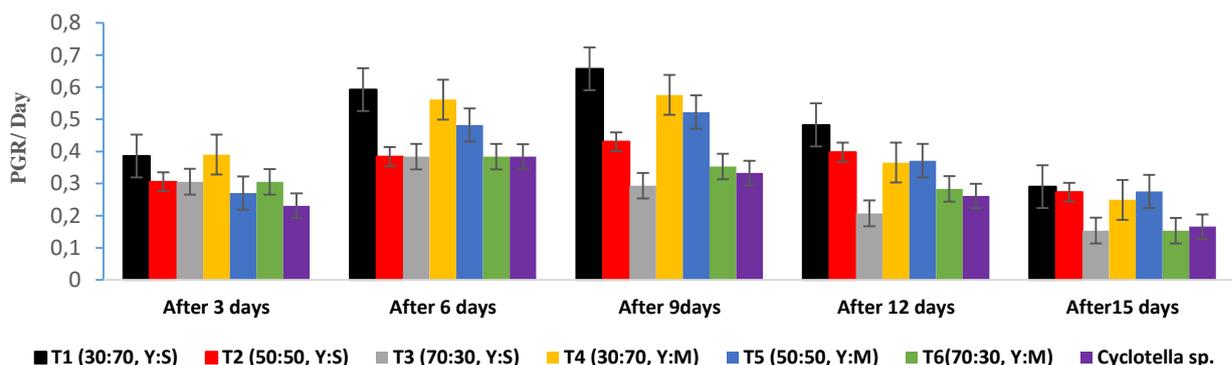


Figure 2. Population growth rate of *B. plicatilis* with different treatments during the experiment period of the small scale culturin

important rise in the densities of *B. plicatilis* during the experiment period. According to the analysis of variance (ANOVA), T1 showed the highest variation (based on the abundance and PGR of *B. plicatilis*) with the other treatments (Table 2). It was the best treatment for growing *B. plicatilis*.

The Large Scale Culture (Mass Culture)

According to the results of the small scale experiment, we selected the best treatment (T1: 30 % yeast: 70 % sugar) with *Cyclotella* sp. which gave the

highest growth rate to apply it as feed in the mass culture. The environmental conditions of mass culture were adjusted as the same conditions of the small scale experiment. The results showed that, the highest population growth rate (0.99 / day) was recorded after 6 days, however *B. plicatilis* attained its highest density (56000 Ind. l⁻¹) on the 12th day (Figure 3 and 4). At this time, *B. plicatilis* was harvested by filtration the half of the culturing pond before starting in the decline phase; it gave 600 and 32.2 gram for wet and dry weight, respectively.

Table 2. The variance analysis (ANOVA and HSD; a confidence interval of 95%) based on the abundance and PGR of *B. plicatilis* between the tested treatments.

Treatment	Abundance	PGR
LS means and pairwise comparison for treatments		
T1	172.000 a a	0.482 a a
T4	80.200 ab b	0.428 ab ab
T5	54.000 ab b	0.384 abc bc
T2	39.600 ab b	0.358 bcd bcd
T6	16.000 b b	0.296 cd de
<i>Cyclotella</i>	13.667 b b	0.243 d e
T3	11.200 b b	0.269 d e
Pr > F	0.003	0.000
Significant	Yes	Yes

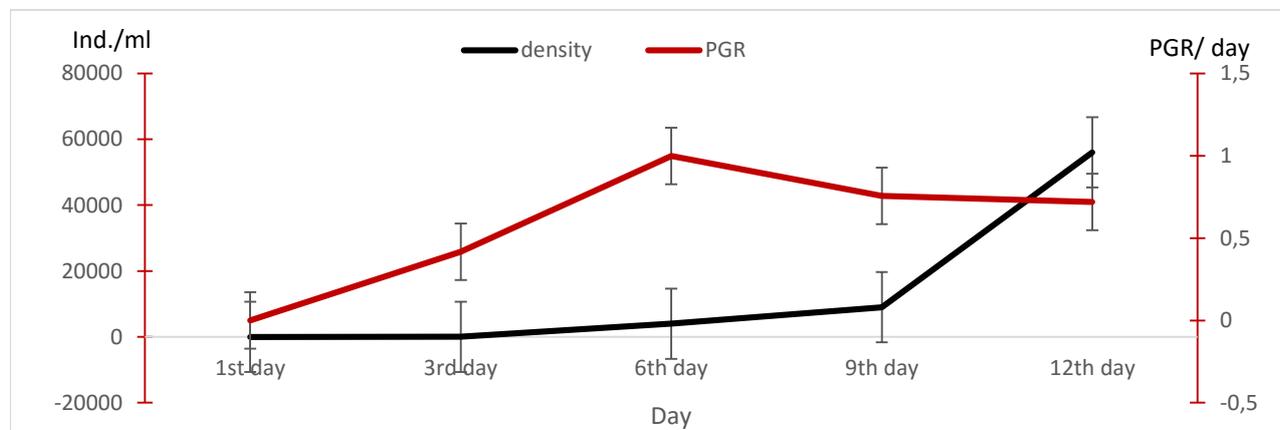


Figure 3. Density and population growth rate of *B. plicatilis* (Ind. l⁻¹) in the large scale (mass culture) under feeding with artificial diet of treatment T1 (30% yeast to 70% sugar) with *Cyclotella* sp..



Figure 4. The collection mass of rotifer *B. plicatilis* (brown colour) after filtration by plankton net (100µm mesh size).

Table 3. Standard Amino Acids content of the dry rotifer *B. Plicatilis* samples

Name	% of Total amino acids
Glutamic acid	4.63
Aspartic acid	3.24
Leucine*	2.84
Proline	2.5
Lysine*	2.45
Valine*	2.15
Phenylalanine*	2.05
Alanine	1.92
arginine*	1.88
Isoleucine*	1.81
Tyrosine	1.62
Glycine	1.57
Cysteine	1.38
Serine	1.35
Threonine*	1.35
Methionine*	0.74

*= essential amino acids

Table 4. Fatty Acids content of the dry rotifer *B. Plicatilis* samples

Fatty acid	Name	%
C 16:0	Palmitic acid	24.16
C 16:1w7	Palmitioleic acid	23.86
C14:0	Myristic acid	11.54
C 18:1 n7	Vaccinic acid	11.05
C 18:0	Stearic acid	5.33
C 15:0	Pentadecanoic acid	4.4
C 17:1	Heptadecanoic acid	2.63
C 18:2 w6	Linoleic acid	2.19
C20:1 w9	Gadolic acid	1.79
C 20:1 w7	Eicosaenoic acid	1.31
C 20:4 w6	Arachidic acid	1.2
C20:3 w3	Eicosatrienoic acid	0.99
C16:3 w4	Hexagonic acid	0.56
C18:3 w3	Linolenic acid	0.52
C16:2 w4		0.64
C18:1 n 13		2.76
C18:2 n 5		2.8
C18:2 n 7		0.88
C20:3 w6		0.62
unidentified fatty acids		0.77

Biochemical Composition of *B. Plicatilis*

Protein and Amino Acids Contents

Table 3 lists the amino acids composition of *B. plicatilis* samples. Protein content was determined in a high percent of 51.66%. The samples were composed of 16 amino acids, 8 of them are essential. The essential amino acids were formed about 15.27% of total amino acids. Leucine, Lysine, Valine and Phenylalanine were formed the high percent (2.84, 2.45, 2.15 and 2.05%, respectively) of the total essential amino acids. On the other hand, the non-essential amino acids were formed about 18.2% of total amino acids. Glutamic acid and Aspartic acid were the highest amino acids estimated of total protein contents.

Fatty Acids Contents

The data on the fatty acid contents of *B. plicatilis* samples is shown in Table 4. Lipid was determined with a high value of 33.01% with 19 identified fatty acids, in addition to other unidentified fatty acids, that formed about 8.47% of total fatty acids. Palmitic and Palmitioleic acids were formed the major components of total fatty acids with percentages of 24.16 and 23.86% respectively, while the remainder fatty acids were minor and occurred in small or trace quantities.

Discussion

Brachionus plicatilis is an essential food for aquaculture, where it is widely applied as a primary live

food for the initial larval stage of fish and crustacea (Ando, Kobayashi, Sugimoto, & Takamaru, 2004; Cheng, Aoki, Maeda, & Hino, 2004). *B. plicatilis* can feed on a variety of diets such as algae, protozoa, bacteria and organic materials in addition to artificial feeds (Jeeja et al. 2011).

The environmental conditions of *B. plicatilis* are very important for its growth and reproduction. Thus, in the current study, *B. plicatilis* was cultured at water temperature $28 \pm 1^\circ\text{C}$ with all treatments. This temperature degree is suitable to *B. plicatilis* culturing, where McVey and Moore (1983) reported that the optimum temperature to *B. plicatilis* is that between $20\text{--}30^\circ\text{C}$. Also, Altaff and Janakiraman (2015) adjusted the mass culture of *B. plicatilis* by maintaining water temperature between 26 and 31°C . We kept salinity at 20 ppt during the period of small and large scale culturing because the ideal reproduction of *B. plicatilis* can only happen when salinities below 35 ppt, although it can survive in a wide range of salinity from 1 to 97 ppt (Lubzens, 1987).

The yeast has been selected as food to *B. plicatilis*, because it characterizes by its solubility in water to very small particles and it has high nutritional value with high protein content (Andersen, 1991), therefore it is an acceptable food for *B. plicatilis*. Also, several studies (e.g. James, Bou-Abbas, Al-Khars, Al-Hinty, & Salman, 1983; James, Dias, & Salman, 1987; Demir & Diken, 2011; Sahandi & Jafaryan 2011) were enriched *B. plicatilis* by the yeast. While many studies (e.g. Arnold & Holt 1991; Dhert, Rombaut, Suantika, & Sorgeloos, 2001) applied oil as fatty acid and energy source to enrich rotifers, we replaced the oil by commercial sugar and molasses beside *Cyclotella* sp. as an energy source and fatty acids source to avoid the changes in water quality may happen by the oil as well as the importance of sugar in the activation of digestive enzymes of rotifers (Christiansen & Nielsen, 2002). Also, in the current experiment, *Cyclotella* sp. was chosen as supplementary food because of it rich with fatty acids, it is non-toxic, its size is suitable to *B. plicatilis* where, it ranges in size from 9 to $25\mu\text{m}$ (Neümuller, Cunningham, & McKee, 2002; Pahl, Lewis, King, & Chen, 2012).

In the current study, the highest density (370 Ind. ml^{-1}) and highest population growth rate ($0.65/\text{day}$) of *B. plicatilis* were observed in small scale with T1 (30% yeast:70% sugar with *Cyclotella* sp.). These results are considered higher than that recorded in several previous studies, where the highest density of *B. plicatilis* fed on only algae was less than 200 Ind. ml^{-1} (e.g.; Villegas, 1990; Konglkeo, 1991; Pi, 1991; Park, 1991; Alam & Shah, 2004; Abdel Rahman, Abdel Razek, Abou Zeid, & Mohamed 2008; Ferreira, Seixas, Coutinho, Fábregas, & Otero, 2011). Moreover, these results also considered higher than that recorded by some previous work that used artificial diet either supplemented with algae or not to enrich *B. plicatilis*, where their highest density was less than 200 Ind. ml^{-1}

(e.g. Arnold & Holt, 1991; Mostary et al., 2010; Sahandi & Jafaryan, 2011). The higher results of the current study compared to the previously mentioned studies may be attributed to the presence of sugar in our diet. Where, it plays an important role as an energy source as well as its importance in activation of digestive enzymes of rotifers, which may enhance the utilization of yeast (Christiansen & Nielsen, 2002). Nevertheless, some studies such as Hirayama and Funamot (1983); James et al. (1983); Mol (2016) achieved higher densities of *B. plicatilis* with artificial diet either supplemented with algae or not (572, 450 and 570 Org./ ml). These better results may be due to that the initial cultured densities ($50, 80$ and 100 Ind. ml^{-1}) used in these studies were higher than that used in our study (1 Ind. ml^{-1}). On the other hand, the culture of the current study was reached the highest density peaks on the 12th day. However, the highest population growth rate was calculated at the 6th and 9th day for small and mass culture, respectively. These results coincide with the fact that rotifers have the shortest life span (12 days) and can more reproductive after 3 days. Also, the egg-to-egg span is 2-3 days and 15-25 young are produced by an adult of *B. plicatilis* throughout its life span (Allan, 1976).

In the current study, the protein content (51.66 %) of *B. plicatilis* fed on yeast with sucrose sugar (30: 70%; Y: S) in addition to *Cyclotella* sp. was high compared with previous studies, that fed *B. plicatilis* on artificial diets supplemented by live algae. Where the protein content of *B. plicatilis* fed on baker's yeast with oil & *Chlorella* sp. and baker's yeast with Algamac 2000™ and yeast with *Chlorella* sp. and yeast with cod liver oil & vitamins & live algae *Isochrysis* sp. was nearly similar to our results, it ranged between 44.28% and 52.32% (Srivastava, 2006). Also, the protein content of *B. plicatilis* fed on *Chlorella* sp., with yeast and rotifer diet (oil blend, phospholipid, Croda oil; vitamin C, A, E, B1, Astaxanthin and Protein blend) were ranged between 37 and 45 % (Hamre, 2016). However, our recommended artificial diet (yeast with sugar) with *Cyclotella* sp. are cheaper and available. Furthermore, the protein contents of *B. plicatilis* fed on yeast with sucrose sugar (30:70%, Y: S) with *Cyclotella* sp. was higher than that ($46.02 \pm 0.226\%$) determined by other studies, that fed *B. plicatilis* on a mix of live algae, including *Nannochloropsis* sp. and *Isochrysis galbana* (Jeeja, et al., 2011). The high content determined by our study could be attributed to the presence of sugar, which activate the digestive enzymes of *B. plicatilis* and enhance the utilization of yeast (Christiansen & Nielsen, 2002). Generally, the biochemical composition of *B. plicatilis* depends on its dietary components, growth and feeding rates (Caric, Sanko-Njire, & Skaramuca, 1993; Nhu, 2004; Jeeja et al., 2011). The results of amino acids analysis showed that Glutamic acid was determined with the highest percentage of total amino acid contents of *B. plicatilis* samples during the current study. This amino acid is very important for fish and human, where it is important for energy production, the immune

system protection, supporting the muscle growth and removing toxic ammonia from the body (Hetteema, 2006). On the other hand, Glutamic acid synthesized in *B. plicatilis* maybe had been derived from yeast, where it forms about 9 % of total amino acids of yeast (Podpora, 2016).

In the current investigation, the lipid content (33.01%) of *B. plicatilis* was high, in contrast to that (4.5 to 28.5 %) determined by several studies, that fed *B. plicatilis* on only live algae (Dendrinis & Thorpe, 1987; James & Rezeq, 1988; Millmena, Aujero, & Borlcmgan, 1990; Frolov, Pankov, Geradze, Pankova, & Spektorova, 1991; King, Liang, & Rusch, 2002; Nhu, 2004). Also, it was higher than fed on only yeast. James et al. (1987) reported that rotifer feed on only yeast has a low lipid content. Nevertheless, Jeeja et al. (2011) determined high lipid content (36.1±0.07%) of *B. plicatilis*, which fed on a mix of live *Nannochloropsis* sp. And *Isochrysis galbana*. Thus, lipid and its fatty acid contents may be synthesized in *B. plicatilis* from yeast or/ and *Cyclotella* sp. Where, *Cyclotella* is rich with lipid contents; Polyunsaturated and free fatty acids, phospholipid, sterol and triglyceride classes (Pahl, Lewis, Chen & King, 2010). James and Rezeq (1988); Frolov et al. (1991); Caric et al. (1993) reported that the synthesis of the fat content in rotifers is depending on the quality of the diets applied in the culture system.

Conclusion

Brachionus plicatilis is the most common rotifer species used in aquaculture, However, its mass culture has still potential for improvement. So, this work aimed to find out the best enrichment program using cheap and available products for the enhancement of *B. plicatilis* culture in large scale and its nutritional value. From the present results, the study concluded that the treatment (T1: 30% yeast: 70% sugar) with *Cyclotella* sp. was a suitable diet for enrichment the rotifer *B. plicatilis* in a large scale culture. Also, the best time for *B. plicatilis* harvesting was 12th day before its density decline. the treatment 1 (T1) enhanced the nutritional value of *B. plicatilis*, where the protein content was 51.6% and lipid was 33.01%. Thus, *B. plicatilis* is acceptable food to use as valuable direct food for the earlier larval stages of fish or as supplementary food for the adults, due to its high nutritional value

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