

Zinc-threonine enriched yeast improved the growth and mineral composition of marine rotifer, *Brachionus plicatilis*

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Abstract

Growth and survival rate in the larval stages of marine fish are influenced by the live feeds. Different methods were used for enrichment of the live feeds with vitamins and fatty acids, however, those methods cannot be used for soluble materials such as zinc. So, in this research, the effects of zinc-threonine enriched *Saccharomyces cerevisiae*, on growth factors and mineral composition of marine rotifer, *Brachionus plicatilis* was investigated. For this purpose, rotifers in four groups including: 1) yeast without enrichment (the control), 2) yeast containing 18.22 mg g⁻¹ of zinc 3) yeast containing 23.76 mg g⁻¹ of zinc and 4) yeast containing 46.15 mg g⁻¹ of zinc were cultured for 10 days. Based on results, in group with 46.15 mg g⁻¹ of zinc-threonine the specific growth rate (SGR) and mineral composition of rotifers significantly improved ($p < 0.05$). Maximum number of rotifers and eggs were 219.3±2.0 and 30.3±11.0 number ml⁻¹, respectively. The eggs ratio (the number of eggs/total number of female rotifers) and SGR is related to the group of 23.76 mg g⁻¹ and 46.15 mg g⁻¹ of zinc-threonine, respectively. Highest amount of zinc in treatment 4 was 977± 4.99 mg kg⁻¹ of rotifers. Also the amount of Cu in treatment 4 was significantly higher than other groups. Conversely, by increasing zinc content, other ions levels like Fe and Mn were significantly decreased ($p < 0.05$). In conclusion, zinc-threonine enriched yeast could improve the growth, reproduction and mineral composition of marine rotifers, *Brachionus plicatilis*.

Keywords: Yeast, *Saccharomyces cerevisiae*, Zinc-threonine, Mineral composition, Rotifer, *Brachionus plicatilis*

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Introduction

Live foods such as the *Brachionus* genus of rotifers are currently used for the marine fish larvae in hatcheries (Zink *et al.*, 2013). The survival rate and larval growth of marine fish in aquaculture industry are influenced by the live feeds. Different methods were used for enrichment the live feeds with vitamins and fatty acids, however, those methods cannot be used for soluble materials such as zinc (Watanabe *et al.*, 1983; Takeuchi, 2001; Haga *et al.*, 2002; Park *et al.*, 2006; Matsumoto *et al.*, 2009). Primitive study shown that the feed used in the hatcheries such as *Saccharomyces cerevisiae* and algae affected the mineral composition of rotifers (Watanabe *et al.*, 1978a). The minerals are responsible for development of the nervous system, growth and survival rates of aquatic larvae, bone formation, maintenance and adjustment of the colloidal system and acid-base balance of aquatic organisms (Satoh *et al.*, 1983; Watanabe *et al.*, 1997). Although the minerals in the body of the fishes are low but zinc play important role in fish diet (Satoh *et al.*, 1983; Watanabe *et al.*, 1997; Apines-Amar *et al.*, 2004) and it is as accelerator performance for many enzymes (Vallee and Auld, 1990). It is reported that adding zinc to diet of fish is essential, because zinc has important role on healthy bone (Ma and Yamaguchi, 2001). The rotifers cultured in artificial environments have about 80 $\mu\text{g g}^{-1}$ of zinc (Watanabe *et al.*, 1978b), but in the natural zooplankton species such as *Acartia clausi* and *Calanus cristatus* the zinc

value are 9 times (about 700 $\mu\text{g g}^{-1}$) and 2 times (about 161 $\mu\text{g g}^{-1}$) higher than rotifer, respectively (Fujita, 1972). However, the comparison between these species is not directly possible because they are larger than rotifers. Albeit, it can be said that cultured rotifers contains less zinc than natural zooplanktons. The zinc requirement of marine fish is about 15-40 mg kg^{-1} (dry mater) (Watanabe *et al.*, 1997) and the zinc content in rotifers cultured in artificial conditions does not meet the needs of marine fish larvae. It is reported that direct addition of soluble zinc to the rotifer culture medium did not have a positive result (Matsumoto *et al.*, 2009) but with zinc enriched *Chlorella*, it resulted in a significant amount of zinc in the rotifer body. Also, it was reported (Stehlik-Tomas *et al.*, 2004) that yeast has been able to enrich significantly with minerals such as Zn, Cu and Mn. Therefore, in this research zinc-threonine enriched yeast as an organic source compound was used to study the growth rate and mineral composition of marine rotifer, *B. plicatilis*.

Material and methods

Preparation the materials

Rotifer *B. plicatilis* prepared from the Institute of Bushehr shrimp and using algae *Nannochloropsis* was scaled up to the mass density in live food laboratory of Artemia and Aquaculture institute, Urmia. Zinc-threonine was obtained by mixing the threonine amino acid with zinc sulfate as follows: A solution of ZnSO_4 (50.6 $\text{mg ZnSO}_4 \cdot 7\text{H}_2\text{O}$ in 6 ml of 50% ethanol) was added drop wise to

a threonine solution (5 ml of ethanol 50%, final concentration of 0.016 mol L⁻¹). Then 100 µl of water was added to it, after continuous stirring for an hour at room temperature, centrifuged at 8000 rpm for 15 minutes (Sigma, 8k, Cat≈10855) (Wang *et al.*, 2010).

Yeast enrichment in the non-growth phase was performed as follows: One gram of *S. cerevisiae* was cultured in 200 mL of YEPD (Yeast extract-peptone-dextrose) medium at 27°C, pH=5.8 and 160 rpm for 24 h on a shaker incubator (Biotech, South Korea). Then Zinc-threonine at concentration of 2.5, 5 and 7.5 mg mL⁻¹ was added into the medium and incubation was continued for 24 h at the same condition. After centrifugation at 3000 rpm for 1 min the yeast cells were harvested and washed with normal saline to remove the additional Zinc-threonine. Zinc-threonine enriched yeast with 18.22, 23.76 and 46.15 mg g⁻¹ of zinc were used for feeding to rotifer (Wang *et al.*, 2010).

Experimental design

Rotifers using zinc-threonine enriched yeast in four groups including: 1) yeast without enrichment (control) 2) yeast containing 18.22 mg g⁻¹ of zinc 3) yeast containing 23.76 mg g⁻¹ of zinc and 4) yeast containing 46.15 mg g⁻¹ of zinc were cultured.

Initial density of rotifers was 30 ind ml⁻¹ and rotifers were fed with a 1×10⁶ cell ml⁻¹ of algae and a 0.5g of zinc-threonine enriched yeast per million of rotifer on a daily. Water quality parameters including, salinity (30±1ppt), pH (7.6-8.3) and

temperature (24±1 °C) were checked daily. Gentle and continuous aeration oxygen to meet the needs of rotifers was performed. The total number of rotifers, the number of eggs and egg ratio, on a daily, were evaluated. Through the following equation, Specific growth rate (SGR) was calculated (Krebs, 1995). $SGR = (\ln N_t - \ln N_0) / t$, Where N_0 and N_t is respectively for initial and final population of rotifer, and t is for experiment period (days). R value was calculated in the exponential phase of population. At the end of the feeding period, the biometric factors in each of the treatment groups were measured using a microscope equipped with micrometer lenses.

Analysis of minerals

After centrifuging the yeast cells and rotifers (2000 rpm for 20 min) and discarding the supernatant materials, wet sample as a paste like were used for the analysis of minerals. Through the use of MLS-1200 MEGA Microwave, the samples were digested with nitric acid (30%) under cold water for 30 minutes. Using an atomic absorption (Nov AA 400, Analytic Jena, Germany) the mineral concentrations (Zn, Mn, Cu and Fe) of digested sample were obtained (Lowry and Lopez, 1946).

Statistics

SPSS Statistical software, version 21 was used to run ANOVA. Using the Levene's and Kolmogorov-Smirnov test, the data respectively were analyzed for homogeneity of variances and normality ($p < 0.05$). ANOVA were used

for analysis of groups and was followed with Duncan's test. The level of 0.05 was considered for differences among means. The data are displayed with Mean \pm SD.

Results

Specific growth rate

The SGR in rotifers fed zinc-threonine enriched yeast was higher than control group but no significant difference was found between them. Highest SGR of the rotifer fed 46.15 mg g⁻¹ of zinc threonine was 0.1822 (per day) and the

lowest SGR were observed in un-enriched yeast group (Fig. 1a).

After 10 days the total number of rotifers fed different concentration of zinc threonine was given in Table 1. The maximum number of rotifers significantly was associated with zinc-containing groups ($p < 0.05$). At the end of experiment, the highest number of rotifers (219.3 \pm 2.0 ind ml¹) was obtained in groups fed yeast contain 46.15 mg g⁻¹ of zinc. The group fed un-enriched yeast showed the lowest number of rotifers during the 10-day period.

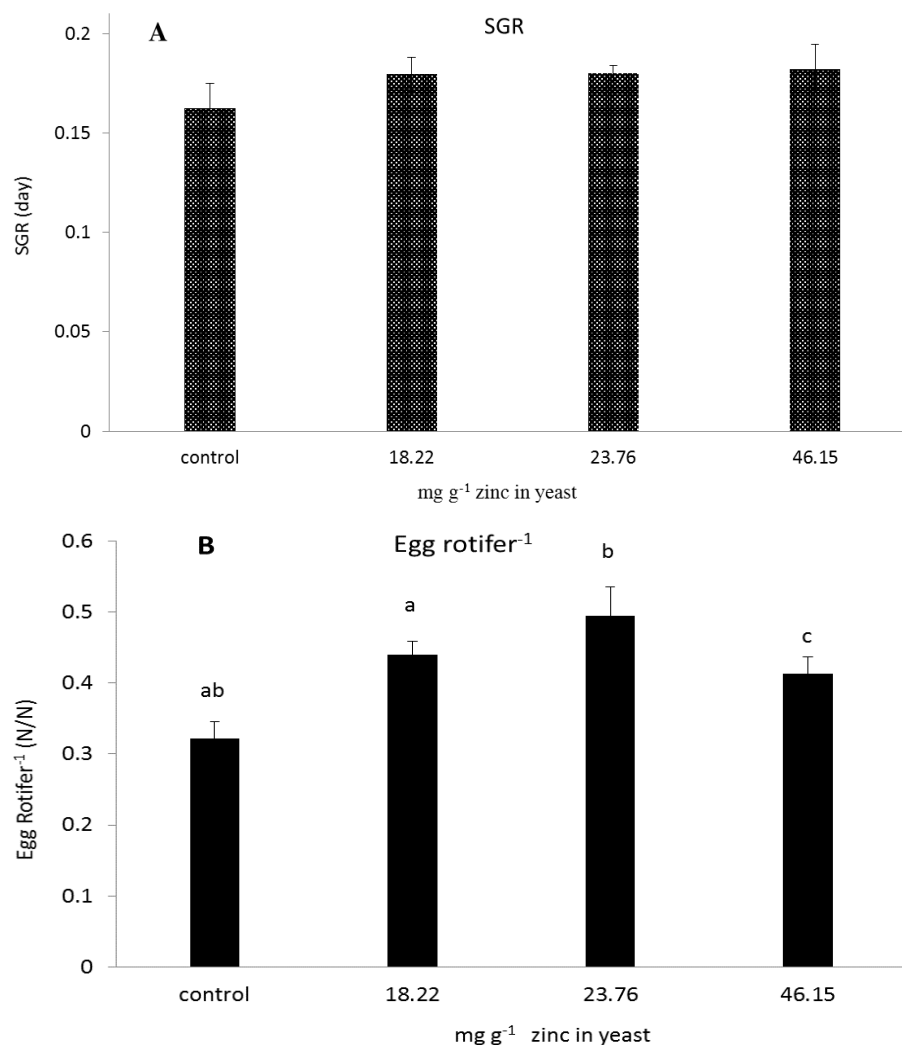


Figure 1: Specific Growth Rate (SGR) (A) and Egg ratio (the number of eggs/total number of female rotifers) (B) of rotifers fed zinc-threonine enriched yeast after 10 days. The data is Mean \pm SD.

Table 1: Mean number of rotifers fed zinc-threonine-enriched yeast with different content (18.22, 23.76 and 46.15 mg g⁻¹ of zinc in yeast) for 10 days (Mean ± SD).

Days*	Yeast zinc content (mg g ⁻¹)			
	18.22	23.76	46.15	un-enriched yeast
1	43.3±1.5	41.7±0.9	42.7±2.3	43.0±1.5
2	51.0±1.2	49.3±0.9	52.7±2.6	48.7±3.2
3	73.0±0.6 ^a	68.7±0.9 ^{ab}	72.7±1.5 ^a	67.3±3.4 ^{ab}
4	94.0±2.1 ^a	87.7±3.5 ^{ab}	92.3±1.5 ^{ab}	86.0±1.7 ^{bc}
5	115.3±1.9 ^a	108.7±0.9 ^{bc}	112.7±1.5 ^{ab}	107.3±3.4 ^{bc}
6	136.0±1.7 ^a	129.7±0.9 ^a	134.7±1.5 ^a	122.3±3.4 ^b
7	157.0±2.1 ^a	153.0±1.7 ^a	160.3±3.8 ^a	138.3±3.9 ^b
8	181.7±3.0 ^a	172.3±2.0 ^b	180.3±4.1 ^{ab}	153.0±2.3 ^c
9	167.7±32.5	193.3±2.6	196.3±2.4	173.7±2.4
10	218.0±2.5 ^a	210.7±2.7 ^a	219.3±2.0 ^a	185.3±5.8 ^b

* Data were analyzed in each days separately.

Different letters in each row have a significant difference ($p < 0.05$).

Daily total eggs and egg ratio

Total eggs in treatments for growing days are given in Table 2. The maximum number of eggs (30.3±11 number ml⁻¹) is related to the groups fed yeast contain 46.15 mg g⁻¹ of zinc on the day eight. In most days of experiment, the lowest total number of eggs was recorded in untreated yeast groups.

The mean egg ratio in treatments enriched with zinc-threonine for 10 days is shown in Fig. 1b. The maximum egg ratio is related to the treatment of yeast contain 23.76 mg g⁻¹ of zinc. As shown, mean egg ratios, ranging from 0.25 to 0.49, had significant differences between groups ($p < 0.05$).

Table 2: Total number of eggs in rotifers fed zinc-threonine-enriched yeast with different content (18.22, 23.76 and 46.15 mg g⁻¹ of zinc in yeast) for 10 days (Mean ± SD).

Days*	Yeast zinc content (mg g ⁻¹)			
	18.22	23.76	46.15	un-enriched yeast
1	7± 3.4 ^a	6.6±2.0 ^a	4.3±4.0 ^a	7.3±1.5 ^a
2	18.3±3.0 ^a	18.6±5.5 ^a	14.6±3.5 ^a	14.6±4.9 ^a
3	20.6±2.0 ^a	22.6±2.8 ^a	19.3±3.5 ^a	16±2.6 ^a
4	18.6±4.5 ^a	21.6±6.8 ^a	19.3±0.5	17.6±1.5 ^a
5	22.6±2.5 ^a	19± 6.08 ^{ab}	16± 4.3 ^{ab}	14.3± 2.3 ^{ab}
6	20.6± 4.1 ^a	21.6±3.0 ^a	20±1 ^a	16±4.3 ^a
7	24.6±6.6 ^a	25.3±5.1 ^a	23.6±3.0 ^a	11.3±5.5 ^b
8	23.3± 2.0 ^a	19±4.3 ^a	30.3±11.0 ^a	14.6±2.5 ^b
9	20±2.6 ^a	21.3±3.7 ^a	23.3±4.9 ^a	22±9.6 ^a
10	27.6±4.1 ^a	26.3±2.3 ^a	28.6±4.5 ^a	22.7±2.5 ^a

* Data were analyzed in each days separately.

Different letters in each row have a significant difference ($p < 0.05$).

Mineral compositions of rotifer

Using atomic absorption the contents of Fe, Cu, Mn and Zn in rotifers were obtained that the results are shown in

the Fig. 2. As seen, the maximum value of zinc (977±4.99 mg kg⁻¹) was in rotifer fed yeast containing 46.15 mg g⁻¹ of zinc. Conversely the amount of

Fe decreased with using the zinc-threonine enriched yeast and maximum value of that ($17.86 \pm 12.02 \text{ mg kg}^{-1}$) was shown in the rotifers fed un-enriched yeast. The amount of Cu and Mn in

rotifers was obtained slightly and there was little difference between treatments, although they were statistically significant.

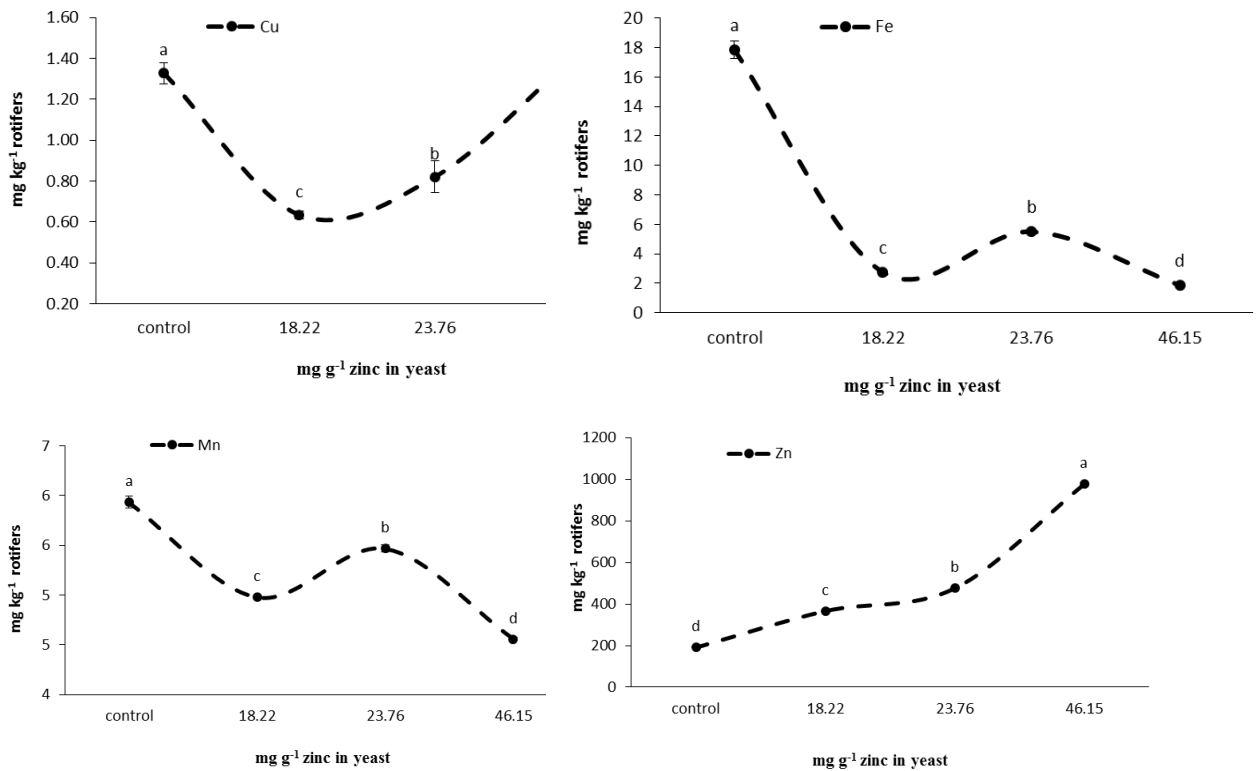


Figure 2: The amount of Zn, Fe, Mn and Cu of the rotifers fed zinc-threonine enriched yeast, Data are Mean \pm SD, n=3.

Discussion

The impact of zinc was evaluated in live feed (Matsumoto *et al.*, 2009; Penglase *et al.*, 2011; Nordgreen *et al.*, 2013). In this study, the organic form of zinc (zinc-threonine enriched yeast) showed a significant effect on growth and the zinc content of rotifer *B. plicatilis*. It has been suggested that to enriched rotifers with minerals, using enriched microorganism is better than direct addition the minerals in rotifer medium and it will be more effective (Penglase *et al.*, 2011). The findings of

the present study corroborate this conclusion.

Maximum density of rotifers was obtained in day ten on treatment 4 (yeast containing 46.15 mg g^{-1} of zinc). Also SGR and egg ratio that investigated in this study improved in rotifer fed zinc-threonine enriched yeast than rotifer fed un-enriched yeast. Specific growth rate of rotifer *B. plicatilis* was in the range of 0.1-2 but the most species show a SGR of less than 0.5 of the days (Sarma *et al.*, 2001) that correspond with the results of our study. In this study mean egg ratio (Fig.

1b) suggested a medium reproductive rate in compared to funding of Yúfera and Navarro (1995) that reported 0.6 under unlimited feed availability. Also it was reported an egg ratio between 0.025 and 0.37 by Zink *et al.* (2013). The medium egg ratio are probably due to the sampling time at the end of the feeding period, as the egg ratio is reduced rapidly in response to the exhaustion of feed intake (Zink *et al.*, 2013). Improving growth in rotifers is due to activity of the enzymes, improved metabolism and the immune system resistance (Nordgreen *et al.*, 2013). In particular the rotifer growth rates are depends on the amount of mineral used (Lubzens *et al.*, 1985).

This study showed that rotifer's ability to absorb of zinc was noteworthy. Fe and Mn ions content showed a decreasing trend with increasing zinc in the diet but the amount of measured copper in rotifers was low. Formerly, it was demonstrated that Mn ion levels has reduced with Zn ion enrichment of rotifer, and a reduced trend of Cu levels in rotifer was also observed by Matsumoto *et al.* (2009). Also in enriched *Artemia* with Zn and Mn ions at the same time, between Zn and Mn ions an antagonistic effect have seen and in compared to single enrichment, *Artemia* showed a lower concentration of Mn ion (Nguyen *et al.*, 2008). In addition, it was showed that the amount of Fe in zinc-enriched *Artemia* was lower.

Zinc is available in aquatic environment and can be used by organisms. Growing rotifers under artificial conditions such as in the

hatcheries of marine fish due to the limitations of nutrients, may result in inappropriate concentrations of zinc and other trace elements. Albeit, the amounts of minerals in the body are low, Zinc as trace element play important role in fish diet (Sato *et al.*, 1983; Watanabe *et al.*, 1997; Apines-Amar *et al.*, 2004; Sato *et al.*, 2008) and it acts as a cofactor for many enzymes (Vallee and Auld, 1990). Adding the zinc to diet of fish is essential because zinc has important role such as reduce deformities of bone and enhance marketing, growth and improve immune system (Ma and Yamaguchi, 2001). Changes in environmental conditions affect the growth of rotifers and the minerals including zinc can drive these changes in favor of aquatic organisms such as rotifer (Penglase *et al.*, 2011). Requirements the larvae of fish as the first consumer to mineral are impressive. In this study, the yeast was used as an intermediary for transfer zinc to rotifer. Further, biochemical composition of yeast is very important for the growth and reproduction of rotifer (Matsumoto *et al.*, 2009).

In this research zinc content of rotifers fed zinc-threonine enriched yeast was corresponded to those of zinc in their diets. The enrichment of about 5 times of rotifers indicates that zinc content in the body of rotifers can respond to larvae requirement of marine fish that at least those requirements is 15 to 40 mg kg⁻¹ of the diets. It is stated that zinc content of rotifers cultured in different conditions were 80 µg g⁻¹ (Watanabe *et al.*, 1978a; Watanabe *et*

al., 1978b) that this amount did not meet the requirement of fish. Also they have shown that culture media such as fresh or marine water *Chlorella* and baker's yeast greatly affected the body composition of rotifers, but it has been ineffective on their minerals. In contrast, other researchers (Fujita, 1972; Takahashi *et al.*, 2005; Matsumoto *et al.*, 2009) have been shown addition of zinc through the enrichment of yeast or algae have the positive impact on rotifer.

In this study, the effect of yeast contains three different content of zinc (18.22, 23.76 and 46.15 mg g⁻¹) on the growth indices and minerals of rotifers were evaluated that it was found that yeast with 46.15 mg g⁻¹ zinc-threonine had a better effect on the growth, reproductive factors and zinc content of rotifer's body. By feeding zinc threonine enriched yeast, the zinc content in rotifers was significantly increased about 5 times. Therefore in conclusion, by using zinc-threonine enriched yeast we can improve the zinc content of marine rotifer, *B. plicatilis* as a first feeding zooplankton.

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