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Optimization of Indoor Production of Fresh Water Rotifer, *Brachionus calyciflorus*, b: Feeding Studies

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Abstract: *Brachionus calyciflorus* is commonly found in fresh water ponds. Its production depends on unrelenting supply of *Chlorella* in sufficient quantity. In the current studies a water sample was collected from fish culture ponds by Wisconsin plankton net (64 µm mesh). The freshly collected stock was concentrated and fractionated by passing through 600, 200, 125, 75 and 38 µm sieves arranged vertically with gradual decrease in pore size. The pure *Brachionus calyciflorus* were fed on *Chlorella* available in the laboratory. Algae was gradually replaced by yeast to reduce dependency on labour intensive live food. Maximum number of rotifers 413 ml^{-1}, was observed when they were fed on 160:32 yeast:algae ratio by weight combination. Ciliates and cyclops posed a major threat during culture and frequent crashes were observed due to this menace. Cyclops were selectively eradicated from the rotifer culture at 0.09 DDVP after 20 h of exposure but not ciliates. There was no selective mortality in ciliates at any stage. Nevertheless both ciliates and rotifers were dead at 0.2 ppm. Simple method of cyst preservation is mentioned.

Key words: *Brachionus calyciflorus*, *Chlorella* sp., yeast

INTRODUCTION

The larvae of culturable Indian and Chinese major carps hatch in relatively undeveloped state. They depend on the nutrients stored in the yolk sac for the first few days. On completion of yolk sac absorption, they demand immediate external food supply for nourishment. Microorganisms present in rearing environment, serve as the first food to fish larvae during the initial period of their life after they hatch from eggs and soon after mouth opening (Lubzens *et al*., 2001). In wild these organisms are available in the form of variety of zoo and phytoplankton but in captive fish culture environments, they need to be provided either from outside sources or from laboratory cultures. Hence culture of live food, rotifer for example, is an important component of a successful fish hatchery (Lee *et al*., 2002). Non-availability of appropriate food at this stage is a major cause of larval losses.

Rotifers have been widely used as essential food source in raising freshwater and marine fish larvae due to its unique characteristics (Lubzens, 1987; Dhert, 1996). It is easily digestible, has appropriate size, can survive in high stocking densities and swims slowly giving an ample opportunity to its predator for prey (Qie *et al*., 1997; Lubzens *et al*., 2001). It possesses apposite biochemical composition that suits the nutritional requirements of larval fish. In addition to the above it has the potential for enrichment with essential nutrients like fatty acids and vitamins if required and various therapeutants for production of healthy fish (Maragelman *et al*., 1985). Therefore, successful culture of fish and shrimp in various parts of the world can be attributed partly if not totally to successful mass cultivation of rotifers.

Rotifera (Rotatoria) belong to the smallest metazoan of which over 1000 species have been described, 90% of which inhabit freshwater habitats. *Brachionus* is one of the most common genera (Dhert, 1996). This genus is important zooplankton species as a primary live food source for early life of both marine and freshwater animal species. Its body is covered with a distinct cuticle, has bilateral symmetry and possesses sexual dimorphism. *Brachionus rotundiformis* and *Brachionus plicatilis* are euryhaline and common in marine environment while *Brachionus calyciflorus* and *Brachionus rubens* are common in freshwater environment.

With the remarkable developments in larval rearing technology of important food fishes, demand for rotifers has increased considerably. *Brachionus* is intensively used to cultivate marine fish larvae due to its essential role in first feeding of fish. Accordingly research on rotifers has enormously increased which is primarily devoted to the needs of aquaculture industry. Major focus is always on high stocking density culture, identification of appropriate food species and control of bio chemical factors which hinder their mass production. Not much attention has been given on its culture in

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freshwater in general and in our local environment in specific.
Our environment is not exception and neither our fish culture practices are. Similar terms and conditions apply to these fishes as applicable to those present in other parts of the world. We need this minuscule in abundance to make our fish hatcheries a successful venture. Therefore to address these concerns, we planned a study to introduce its culture and tried to replace algae, widely used rotifer feed, with cheaper feeds/ feed combinations without compromising its growth and production. Provision of good quality and sufficient micro algae as the sole food over an indefinite period is usually considered not only labor-intensive but expensive too.

MATERIALS AND METHODS

Experimental site: The studies were based on freshwater rotifer, *Brachionus calyciflorus* and were conducted simultaneously at two different places; Fish Hatchery Faisalabad and Fisheries Research and Training Institute, Lahore.

Preparation of stock culture of rotifers: Mixed population of zooplankton comprising of copepods, rotifers and cladocerans was collected from the wild. The water containing rotifers was sifted through 600, 200, 125, 75 and 38 µm mesh sieves arranged one above the other in a decreasing downward order. The material collected in the bottom most screen, was observed under microscope at 10x for confirmation of desired fauna which was saved for future rotifer culture.

Starter culture: The starter culture consisted of a static system and was limited to 500 ml Erlenmeyer's flasks. The inoculated flasks were placed at 2 cm from fluorescent light tubes (500 lux). The temperature was approximately constant at 28°C. Sufficient aeration was provided through the perforated stones based at the bottom of the flasks. The rotifers were inoculated @ 30 rotifers ml^{-1} and fed on fresh *Chlorella* containing 1.6 x 10^6 cells ml^{-1}. Rotifer culture was maximized to meet the requirement of subsequent trials.

Feeding trials: Density and production of rotifers are dependent on food availability and quality. The subsequent trials were on various food options. The *Chlorella vulgaris* was cultured in laboratory while the baker's yeast, *Saccharomyces cerevisiae* and vitamins were purchased locally. Based on algal density (cells ml^{-1}), quantities of algal supplies were determined by haemocytometer and algal volume was calculated following Nhu (2004):

$$V_1 = V\frac{N - N_2}{N_1 - N_2}$$

Where V_1 = Volume of algae supplied, V = Volume of rotifer culture, N = Target density of algae, N_1 and N_2 = Density of algae before and after inoculation of rotifers.

Supplementation of algae with vitamin C and B vitamins: Nine jars were arranged in order. They were randomly allotted to three treatment groups, three per treatment. Control group received only *Chlorella* while *Chlorella* in treatment 2 and 3 were supplemented with vitamin C and B respectively in a fixed proportions. All the three groups were fed thrice a day at 8.00, 12.00 and 16.00 h for 15 days. At the end of experimental duration each jar was randomly sampled for rotifer estimation (Table 1).

Algae-yeast trials: There were 4 treatment groups and a control. Control group was totally nourished on fresh *Chlorella*. In the subsequent treatment groups *Chlorella* was gradually replaced with baker's yeast. The sequence of treatments designed is given hereafter; (algae: *Chlorella*; 80:40, 64:80, 48:120, 32:160). Algae was fed in the morning while yeast in the afternoon. The yeast was well mixed with tap water and blended to form a uniform suspension before dispensing into respective jar for homogeneous particle dispersal. *Chlorella* was taken on wet weight while baker's yeast on dry weight basis (Table 2). Though various algae: yeast combinations were used but feeding rate was always 0.5 µg $rotifer^{-1}$.

Quantitative estimation of rotifers: All the culture water was poured and was filtered through a 45 µm Millipore membrane filter and rinsed well with several washings to remove the extraneous material. Rotifers were counted on Sedgewick-Rafter counting chamber (APHA, 2005) under Labomed light microscope model CX3. One ml of sample from each jar was transferred to Sedgewick-Rafter counter and cells were counted within 10 squares chosen randomly. The total cells were calculated using the following mathematical expression (Stirling, 1985; Rahman and Afzal Hussain, 2008 and modified by Ashraf, 2009 unpublished):

$$N = \frac{A \times 1000 \times C}{V \times F \times L \times 1000}$$

Where
N = Number of plankton cells ml^{-1} of original water
A = Total number of rotifer counted $field^{-1}$
C = Volume of final concentration of samples in ml
V = Volume of field cubic meter
F = Number of fields counted
L = Volume of original water in liters (optional)

Control of ciliates and cyclops: Ciliate contamination in algae or rotifers is common but their density depends on the hygiene and type of feed applied to the rotifers. Various dilutions of DDVP were prepared and applied to rotifer culture containers to control ciliates and cyclops (Table 3a, b and 4a, b, c).

Table 1: Effect of vitamin C and B on rotifer production in jars. Total water volume was 5 L and inocula size was 30 rotifers ml^{-1}. Number of algal cells provided $rotifer^{-1}$ were ~35000±4500 while Vitamin 'C' and 'B' were supplemented @ 8 g jar^{-1} in treatment 2 and 3 respectively

Rotifer data	Water quality data							
Treatment #	No. of rotifers ml^{-1}	Temp. °C	DO (ppm)	pH	Alkali-nity (ppm)	Hardness (ppm)	NH_3 (ppm)	CO_2 (ppm)
1	289±15.7^a	26±2	5.6±0.4	8.9±0.3	900±50	450±34	0.02	7.8±0.6
2	310±14.5^a	26±3	4.8±0.6	8.9±0.2	880±56	370±36	0.025	8.1±0.4
3	340±18.2^b	26±3	4.6±0.5	8.8±0.3	720±34	440±32	0.025	8.5±0.5

Table 2: Effect of various combinations of algae and yeast on the production of rotifers. Yeast was provided @ 0.5 µg $rotifer^{-1}$

Rotifer data	Water quality data								
Treatment #	Algae to yeast ratio	No. of rotifers ml^{-1}	Temp. °C	DO (ppm)	pH	Alkali-nity (ppm)	Hardness (ppm)	NH_3 (ppm)	CO_2 (ppm)
1	80:40	211±15.3^a	26±2	5.0±0.4	8.3±0.5	850±60	440±30	0.02	7.9±0.6
2	64:80	350±20.0^a	26±3	4.5±0.3	8.7±0.4	875±52	370±25	0.02	7.9±0.7
3	48:120	221±19.1^a	26±2	4.4±1.0	8.9±0.5	780±40	380±36	0.021	8.0±0.4
4	32:160	413±24.1^b	26±1	4.2±0.4	8.9±0.3	780±45	445±39	0.025	8.1±0.4
5	96:0	180±11.3^a	26±3	5.2±0.3	8.9±0.4	840±47	390±26	0.015	7.7±0.5

Daily protocol: Daily stirring of culture media, suspended the dregs at the bottom of the tank, which contained uneaten algae, unhealthy rotifers and accumulated fungal growth. All of this material was kept out of the harvest to maintain the quality of future cultures and preventing its accumulation in larval fish containers. Daily feeding was strictly monitored and excess was avoided. The water clarity (cloudiness) was gauged visually before each feeding. All the trials were conducted indoor and system was exposed to 16h:8h light: dark duration.

Water quality parameters: Temperature was recorded daily by mercury thermometer. Total ammonia and dissolved organic nitrogen were analyzed by water analysis kit (Hach, USA) after filtration of water sample through 0.45 µm filter. Un-ionized ammonia fraction was computed from the data of temperature and pH following Emerson *et al.* (1975) because analytical procedures do not differentiate between the two forms of ammonia in solution:

$$NH_3\text{-}N = TAN \times f$$

Where

$$f = 1/10^{pKa-pH} + 1$$

$$10^{pKa-pH} = (NH_3)(H^+)/(NH^+_4)$$

and

$$pKa = 0.09018 + 2729.92/ T + 273.15.$$

Oxygen was determined by YSI-D.O. meter (51B), pH by pH meter WTW model 735 and other parameters by Hach kit (Table 1 and 2). Any significant decrease in DO was immediately compensated with additional air stones to minimize DO fluctuations and to avoid accidental crashes.

Statistical analysis: Differences between groups were analyzed by one-way Analysis of Variance (ANOVA) and differences among treatment means were distinguished using Duncan's Multiple Comparison test at $\alpha = 0.05$ level. The results are presented as means ± SEM. The analysis was performed using SPSS statistical package (version 14).

RESULTS

Feeding studies were conducted on rotifers to maximize their production and make the culture cost effective. Algae was also supplemented with vitamin C and B in the first trial. In the second trial *Chlorella* and yeast were applied in various proportions. Rotifers produced were observed, counted and recorded.

Effect of vitamin C and B: Rotifers fed on *Chlorella* supplemented with vitamin B produced highest rotifer density (340 ml^{-1}) significantly($p<0.05$) higher than control and treatment 2. Vitamin C supplemented group was the second highest though not different from that of control group at $p<0.05$ (Table 1).

Effect of algae and yeast: All the feeding combinations performed equally except treatment group 4 (Algae:yeast; 32:160) which produced the highest number of rotifers(413 ml^{-1}) significantly ($p<0.05$)higher than its counterparts (Table 2).

Control of ciliates: Though various doses of DDVP (0.05-0.5 ppm) were applied to control ciliates but none of them was effective. Mortality was not selective and the death point was same both for ciliates and rotifers (Table 3a, b).

Control of cyclops: Similar to ciliate control various doses of DDVP (0.05-0.09 ppm) were devised and

Table 3a: Effect of various doses of DDVP on ciliates

	DDVP dose (ppm)				
Time	0.2	0.25	0.3	0.4	0.5
10 min	Rotifers + ciliates both alive	Rotifers + ciliates both alive	Rotifers + ciliates both alive	Rotifers + ciliates both dead	Rotifers + ciliates both dead
20 min	Both alive	Both alive	Rotifers dead ciliates present	Both dead	Both dead
30 min	Both alive	Rotifers dead, ciliates present	Both dead	Both dead	Both dead
1 h	Rotifers dead, ciliates present	Both dead	Both dead	Both dead	Both dead
2 h	Both dead	Both dead	Both dead	Both dead	Both dead

Table 3b: Effect of various doses of DDVP on ciliates

	DDVP dose (ppm)				
Exposure time	0.05	0.1	0.15	0.2	0.25
10 min	Rotifers + ciliates alive	Rotifers + ciliates alive	Rotifers + ciliates alive	Rotifers + ciliates both alive	Both alive but rotifers inactive
30 min	-do-	-do-	-do-	Rotifers were partially alive ciliates present	Rotifers dead ciliates alive
3 h	-do-	-do-	Rotifers dead, Ciliates alive	Both dead	Both dead
16 h	-do-	-do-	-do-	-do-	-do-

applied. DDVP dose of 0.09 showed the best results. It completely controlled cyclops permitting the normal growth and survival of the rotifers (Table 4a, b, c).

Water quality parameters: The range of physico-chemical parameters during culture of rotifers such as water temperature, pH, NH_3, Dissolved Oxygen (DO) and others did not vary much from treatment to treatment and remained within the suitable ranges during the course of experiment (Table 1 and 2).

DISCUSSION

Feeding studies were bifurcated in 2 trials. In trial 1 *Chlorella* was supplemented with vitamin C and B individually while in trial 2 with baker's yeast in different ratios.

Trial 1: Vitamin B supplementation gave the highest rotifer density significantly ($p<0.05$) higher than its counterparts (Table 1). Maruyama *et al.* (1990) indicated that B12 was essential for the population growth of rotifers. Rotifers cultivated with B12-enriched thraustochytrids (algae) contained 3.1% (w/w) of DHA and enhanced the population growth of rotifers. Lee and Park (2003) even got higher rotifer density when they supplemented condensed algae with vitamin B12. According to Shirasaka *et al.* (2005), propionyl CoA, a primer for odd numbered fatty acids was converted to succinyl CoA and it was consumed in TCA cycle in thraustochytrids, cultivated with vitamin B12. Vitamin B12-enriched thraustochytrids strain mh0186, enhanced the population growth of rotifers fed on the cells as sole feed (Hayashi *et al.*, 2007). Takao *et al.* (2005) investigated that the incorporation of Cobalt (Co) to green water produced higher number of rotifers and 3 times more vitamin B12. Yoshimatsu *et al.* (2006) further confirmed in their studies that addition of cobalt has increased the population of *Brachionus rotundiformis* by indirectly enhancing B12 production when added @ 0.01 mg ml^{-1}. Though our research work was not at cellular/molecular level which could explain all the working mechanisms of B12 nor we studied fatty acid levels in algae or rotifers, nevertheless like previous findings vitamin B12 did improve production of rotifers in current studies.

Rotifer density of vitamin C supplemented group was equal to control. Hapette and Poulet (1990) in their studies observed vitamin C in 26 species of the major zooplankton taxa. This confirms the ubiquity of this essential micronutrient in eukaryotes. Copepods and their fecal pellets were found substantial carriers of vitamin C constituting a potential pathway from phytoplankton to consumers. Treece and Davis (2000) in their findings further confirmed that vitamin A and B as dietary essentials for rotifer production but not vitamin C. Previous studies endorse ours with the affirmation that vitamin C is not a dietary essential for rotifers rather they meet their requirements from algae, the potential vitamin C producer for zooplanktons. Interesting results were however, found when all these groups were exposed to higher temperatures. All the rotifers in *Chlorella* and vitamin B12 group perished immediately at $34^{o}C$ but not vitamin C group which survived up to 40 ^{0}C temperature. Role of vitamin C in enhancing the resistance capability of higher organisms is well documented (Ashraf *et al.*, 2008) but was not clear in zooplanktons. Hien *et al.*

Table 4a: Effect of various doses of DDVP on control of cyclops

Exposure time (min)	DDVP dose (ppm) 0.05	0.1	0.15
10	Cyclops and rotifer both alive	Both alive	Both alive
20	-do-	-do-	-do-
30	-do	-do-	-do-
60	-do-	-do-	Both were dead

Table 4b: Effect of various doses of DDVP on control of cyclops

Exposure time (min)	DDVP dose (ppm) 0.11	0.12	0.13	0.14	0.15
10	Both alive	Both alive	Both alive	Both alive	Both alive
30	-do-	-do-	-do-	-do-	Cyclops dead + rotifer alive
60	Both dead	Both dead	Both dead	Both dead	Both dead

Table 4c: Effect of various doses of DDVP on control of cyclops

Exposure time	DDVP dose (ppm) 0.05	0.06	0.07	0.08	0.09
10 min	Rotifers+ cyclops both alive	Rotifers + cyclops both alive	Rotifers + cyclops Both alive	Rotifers + cyclops both alive	Rotifers + cyclops Both alive
30 min	Both alive	Both alive	Both alive	Both alive	Both alive
1 h	Both alive	Both alive	Both alive	Both alive	Both alive
2 h	Both alive	Both alive	Both alive	Both alive	Both alive
5 h	Both alive	Both alive	Both alive	Both alive	Both alive
11 h	Both alive	Both alive but cyclops inactive	Both alive but cyclops inactive	Rotifers alive cyclops half dead, half alive	Rotifer alive cyclops mostly dead. Some alive but inactive
20 h	Rotifers alive cyclops dead	Rotifers alive cyclops dead	Rotifers alive cyclops dead	Rotifers alive cyclops dead	Rotifer alive cyclops dead

(1999) determined the effects of various concentrations of vitamin C in diets, on the survival, metamorphosis and resistance capability of larval freshwater prawn (*Macrobrachium rosebergii*). Vitamin C enhanced survival up to 500 mg, with no effect on metamorphosis and improved resistance to bacterial infection up to 1000 mg incorporation kg^{-1} of diet. These studies substantiate our findings and further open new avenues for comprehensive investigative work on these lines.

Trial 2: Rotifers were cultured on *Chlorella vulgaris* alone and also with yeast supplementation. Purpose was to gradually replace or at least minimize the inclusion of expensive and labor intensive production of *Chlorella* ((Lubzens, 1987). Significantly ($p<0.05$) higher rotifer density (413 ml^{-1}) was observed at 32:160 *Chlorella:* yeast combination than its counterparts. There was no gradual trend in the increase of rotifer density with the increase in the ratio of yeast which demands further research. Oie *et al.* (1994) stated that quality and quantity of diet is the most important criteria that affects density and production of rotifers. Sarma *et al.* (2001) reported that *Brachionus calyciflorus* grew better on *Chlorella* alone while *Brachionus patulus* grew equally well when fed only on *Chlorella* or when mixed with *Saccharomyces cerevisiae* in equal proportions. *Chlorella* produced 100-150 individuals ml^{-1} while addition of baker's yeast enhanced it up to 562 individuals ml^{-1} meaning that species cultured also has lot of bearing on production. Previously Watanabe *et al.* (1983) found out that sudden rotifer crashes and larval losses can be prevented by culturing rotifers on both yeast and then feeding on marine *Chlorella*. Recently Mostary *et al.* (2007) obtained contradictory results to previous and our studies when he used dried, fresh *Chlorella* and baker's yeast for culture of rotifers. The mean population densities of *Brachionus angularis* recorded in treatment 1, treatment 2 and treatment 3 were 30.1±12, 37.4±14.6 and 21±6.1 ind. ml^{-1} respectively highest on fresh *Chlorella* while the lowest on yeast.

The rotifer density observed in current study was well close to the above observations which further validate our findings. Even copepods are not exception and show similar behaviour when cultured in captivity. Rhodes (2003) reported that copepod population fed formulated feed grew significantly faster and achieved significantly higher population densities ($p<0.01$) (the highest intrinsic growth rates than those fed on live algal food only). Use of baker's yeast is not something unusual but even ground shrimp meal, flour, rice bran, dried frozen algae and formulated diets have been extensively used

for culture of rotifers (Lubzens *et al.*, 1995) because rotifers can ingest food particles of up to 30 µm, including bacteria, baker's yeast, *Saccaromyces cerevisiae*. Baker's yeast has as such no nutritional value for rotifers but bacteria associated with the yeast is the source of nutrition for rotifers (Alessandro *et al.*, 1999). Still much higher rotifer densities can be achieved with better hand on bio-chemical factors and maintenance of proper hygienic conditions.

The range of physico-chemical parameters during culture of rotifers such as water temperature, dissolved oxygen, pH, NH_3 and others were within the acceptable limits and more or less similar in all the treatments. Though there were slight variations in some water quality parameters but that might not have any bearing on the integrity of findings (Table 1 and 2).

Ciliate contamination was a major problem during rotifer culture which badly affected stability and production of rotifers not only in our attempts but it was more prominent in the past studies (Hino, 1993) (Watanabe *et al.*, 1983). Halotricha and Hypotricha cilciates, such as *Uronema* sp. and *Euplotes* sp., are not desired in intensive cultures since they compete for feed with the rotifers. The appearance of these organisms is generally due to sub-optimal rearing conditions. They decrease the performance of rotifers and increase the chances of competition for food and air. Ciliates produce metabolic wastes which increase the NO_2-N level in the water and cause a decrease in pH. In current studies NH_3 remained in the normal range that might be due to low rotifer density hence its effects were not discernable. Dhert (1996) however, has different view point and states that presence of ciliates in the culture medium is not necessarily harmful except at high concentrations. He however, did not differentiate the high or low levels of ciliates in rotifer culture media. Nhu (2004) nullifies the Dhert (1996) and says that excessive presence of ciliates in mass cultures lead to considerable reduction in yield because of their activity which brings about an aggregation of food and thereby reduce the availability to rotifers. A lot were present in our cultures too which really has negative impact on rotifer proliferation. High concentrations of ciliates were related to low fertility of culture medium, low rotifer density and finally mass mortality in ours as well as in previous studies (Dhert, 1996). To achieve pure culture of the rotifer, *Brachionus calyciflorus*, Arimoro and Ofojekwu (2004) recommended the use of 'Basudine' an organophosphoric acid ester applied at the rate of 1.5 mg l^{-1}. At this concentration copepods and cladocerans and aquatic insects including mosquito larvae failed to flourish thereby permitting the rotifers to multiply. Ludwig (1993) found Trichlorfon, an organophosphate parasiticide that inhibits cholinesterase, very toxic to free swimming copepods and their nauplii but does not kill rotifers at dosages of 0.25 ppm (active ingredient). Arimoro (2006) renewed inoculum containing ciliates and other undesired insects with 10 ppm Oxyteteracycline 30 mg l^{-1}, Sarafloxacin or Lincospectin 30 mg l^{-1}. We used DDVP in our studies for control of ciliates and cyclops. Ciliates survived up to 0.3 ppm. They started dying at 0.2 ppm and totally died at 0.4 ppm but unfortunately at this concentration rotifers could not survive too (Table 3a and b). Cyclops however, proved much easier to control. They were selectively controlled at 0.09 ppm for 30 min exposure only without harming the rotifer stock (Table 4a, b and c).

Nevertheless we were able to attain 413 rotifers ml^{-1} on combination feed which has been achieved in the past in selected cases under different experimental and environmental conditions. We were able to control all the cyclops at 0.09 ppm DDVP which was far less than used by Arimoro (2006) which can increase the operational cost of the hatchery and may induce toxic effects to both rotifers and fish. Although with several accomplishments there were lot of failures too but it is really a major breakthrough in indoor culture of rotifers in this country. Presently rotifers are totally produced in wild and fish fry is left at the mercy of that unknown food sources whose both quality and quantity are uncertain. Further we have devised easy and cheaper methods for purification of rotifer culture. However, still lot needs to be done in this field especially in our local environment.

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