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RESEARCH ARTICLE

Innovative method of culturing bdelloid rotifers for the application of wastewater biological treatment

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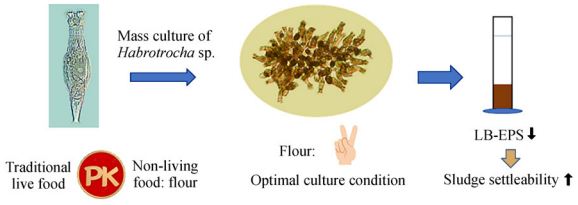
HIGHLIGHTS

- An innovative method of culturing bdelloid rotifer fed on flour was proposed.
- Rotifer fed on flour grew faster than that fed on bacteria or *Chlorella vulgaris*.
- The optimum mass culture conditions for rotifer fed on flour were investigated.
- The cultured rotifer could improve sludge settleability in the SBR.

GRAPHIC ABSTRACT



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ABSTRACT

This study aims to establish a simple and efficient method for the mass culture of bdelloid rotifers, which is the basis for the application of bdelloid rotifers as biological manipulators to improve wastewater biological treatment performance. A common bdelloid rotifer, *Habrotrocha* sp., in a wastewater biological treatment system was selected as the culture target. Rotifers fed on flour could reproduce faster than those fed traditional food such as *Chlorella vulgaris* or mixed bacteria. As a rotifer food, flour has the advantages of simple preparation, effortless preservation, and low cost compared to live *Chlorella vulgaris* or mixed bacteria, so it is more suitable for the mass culture of rotifers. The optimal rotifer culture conditions using flour as food were also studied. According to the experimental results, the recommended rotifer culture conditions are a flour particle size of 1 μm, a flour concentration of 6×10^6 cell/mL, a temperature of 28°C, a pH level of 6.5 and salinity of 100–500 mg/L. In addition, the sludge volume index in the sequencing batch reactor (SBR) with the addition of cultured rotifers was 59.9 mL/g at the end of operation and decreased by 18.2% compared to SBR without rotifer, which indicates that the cultured rotifers still retained the function of helping to improve sludge settling. This function may be related to the rotifer's role in inhibiting bacteria from producing loosely bound extracellular polymeric substances in the SBR.

1 Introduction

Bdelloid rotifers possess an elongated soft body, stretchable head, retractable feet, and paired gonads composed of germarium (Melone and Ricci, 1995b). They are a common microfauna observed in various wastewater biological treatment systems (Hu et al., 2013). For example, *Habrotrocha* sp. and *Philodina* sp. are abundant in pulp and paper industry wastewater (Lee and Welander, 1996) and the genus *Rotaria* is dominant in domestic wastewater (Sankai et al., 1997). Some researchers have hypothesized a connection between microfauna and wastewater biological treatment performance. This is especially true given that bdelloid rotifers were proposed as indicators of good treatment performance (Salvadó et al., 2004; Amaral et al., 2018). This indicator function of bdelloid rotifers may be associated with their beneficial role (Ding et al., 2017). Lapinski & Tunnacliffe (2003)

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found that the bdelloid rotifer *Philodina roseola* significantly improved the municipal wastewater clarification rate and degree of suspended particle removal in an experiment in which cultured *P. roseola* was added to a laboratory-scale mini activated sludge reactor. Consequently, the reduction in suspended particles may be due to rotifers' direct predation or enhanced aggregation. Our results showed that the bdelloid rotifer *Philodina erythrophthalma* could promote the growth and flocculability of two bioflocculation-producing bacteria *Brevundimonas vesicularis* and *Bacillus cereus* isolated from activated sludge, which directly confirmed the possibility of enhanced aggregation by bdelloid rotifers (Ding et al., 2017).

The ecosystem can be artificially changed or modified by adding or removing certain species through biomanipulation methods (Qu and Fan, 2010). An attractive strategy is to develop biomanipulation techniques involving the addition of cultured microfauna into a bioreactor so that wastewater biological treatment efficiency can be ecologically and economically improved. Protozoa *Paramecium caudatum* and Metazoa *Daphnia magna* were added to a biological system for municipal wastewater treatment, revealing the potential of microfauna to improve water quality (Shiny et al., 2005). Pous et al. (2021) found that the metazoan *Daphnia magna* not only effectively removes solids and pathogens, but also successfully promotes the removal of biological organic matter and nitrogen in pilot-scale reactor. Liang et al. (2006) used microfauna to reduce excess sludge and established a new method to scale the sludge reduction rate caused by microfauna. Among microfauna, bdelloid rotifers may be the most suitable for biomanipulation in wastewater biological treatment systems because they are usually the dominant species due to their large body size and adherent life style, allowing them to persist for a long time in a biological treatment system. The research undertaken by Lapinski & Tunnacliffe (2003) demonstrated the effectiveness of using bdelloid rotifers to manipulate the biological treatment of wastewater. Our study also confirmed this possibility (Ding et al., 2019).

To establish an engineerable and efficient mass culture method for bdelloid rotifers, a large quantity of rotifers needs to be inoculated to improve biological treatment efficiency. For the cultivation of rotifers, the current focus is almost entirely on free-swimming rotifers such as the genus *Brachionus*, which are usually used as food for rearing fish larvae (Kim et al., 2018). Although bdelloid rotifers live in benthos or attach to a substrate, unlike free-swimming rotifers, both rotifers feed on bacteria, yeasts, and microalgae (Ricci, 1984; Nandini and Sarma, 2001). Theoretically, living foods such as bacteria and microalgae can also be used as food to cultivate bdelloid rotifers. However, the traditional culture method has disadvantages. For example, preparation process for living food is complex with high production costs. In addition, differences in the size of food intake between attached rotifers and free-swimming rotifers should not be ignored. A suitable range of food particle sizes for the bdelloid rotifer *P. roseola* is 0.2–3 μm (Lapinski and Tunnacliffe, 2003). However, the free-swimming rotifer *Brachionus calyciflorus* showed active particle ingestion of silica beads 3–6 μm in size (Miquelis et al., 1998). Another study showed that *Brachionus plicatilis* could ingest particles ranging between 1.6 and 10 μm and had an optimum particle size of 4.5 μm (Baer et al., 2008). Therefore, suitable particle size for food is a factor to focus on while investigating methods for the mass cultivation of bdelloid rotifers.

The beneficial effects of microfauna on biological processing may be numerous. Ratsak (2001) counted the oligochaete worm *Nais elinguis* in four aeration tanks in a full-scale, thoroughly mixed, municipal activated sludge plant for a year and half and observed that the sludge volume index was considerably lower when worms were present in aeration tanks, and the worms had no effect on the effluent quality. In addition, extracellular polymeric substances (EPS) produced by microbial secretion or macromolecular cell cleavage are speculated to influence sludge's physicochemical properties (Hu et al., 2019; Zhang et al., 2019). Generally, EPS can be divided into loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS) (Pellicer-Nàcher et al., 2013). Excessive LB-EPS results in the deterioration of cell attachment and weakening of the floc structure, resulting in poor sedimentation. In contrast, the TB-EPS did not correlate with sludge settleability (Li and Yang, 2007). However, the relationship between microfaunas and EPS of activated sludge is unclear.

This study aimed to investigate the possibility of using wheat flour as an alternative to replace traditional foods for culturing the bdelloid rotifer *Habrotrocha* sp., which is commonly present in wastewater biological treatment systems. Important cultural factors, such as food size, food concentration and growing environmental conditions, were also studied to establish optimal culture conditions. To determine whether the rotifers artificially subcultured on flour can improve the biological treatment of wastewater, the sludge settling performance in a sequencing batch reactor was selected as an index to evaluate the activity of the cultured rotifer.

2 Materials and methods

2.1 Rotifer

The bdelloid rotifer *Habrotrocha* sp. was isolated from activated sludge of one municipal sewage treatment plant in Shanghai, China, under an inverted microscope (37XB, Shanghai CSOIF Co., Ltd, China). The main contents of

the rotifer subculture medium included 0.06 g of $CaCO_3$, 7 mL of 1 mg/L pH 6.5 phosphate buffer and 993 mL of distilled water. Adult rotifers cultured in subculture medium were used for the experiment. All chemicals (analytic grade) used in this study were purchased from Sinopharm Group Co. (Sinopharm, China).

2.2 Evaluation index

The specific growth rate (SGR) was used to evaluate the population growth characteristics of *Habrotrocha* sp. and calculated from the exponential phase with Eq. (1).

$$\mathrm{SGR} = (\ln N_t - \ln N_0)/t, \quad (1)$$

where SGR (d^{-1}) refers to specific growth rate, N_0 (ind/mL) represents the initial population density, N_t (ind/mL) represents the population density after time t, and t (days) represents the interval time of culture.

2.3 Experimental design

2.3.1 Growth characteristics of rotifer on different foods

The population growth rate and the highest density of *Habrotrocha* sp. fed three different kinds of foods, microalgae, mixed bacteria, and wheat flour, were investigated. Microalgae *Chlorella vulgaris* was obtained from the Wuhan Institute of Aquatic Biology, Chinese Academy of Sciences, China. Mixed bacteria were isolated from activated sludge bacteria that were sampled from the aeration tank of a municipal wastewater treatment plant and amplified by meat peptone medium for 24 h. The wheat flour was purchased from the market. The amount of food added to the culture medium was approximately 0.8 according to the OD_{600} value.

2.3.2 Effects of different flour particle sizes on the population reproduction of rotifers

Five experimental groups with 5 different sizes of flour particles as food were designed to evaluate the effects of particle size on the population growth rate of *Habrotrocha* sp. Five different sizes of flour particle sediments were obtained from centrifugation at 250, 650, 1000, 2000 and 5000 r/min and named the first- to fifth-grade particles, respectively (Table 1). The graded flour particle size was determined by a particle size analyzer (JL-1177, Shanghai Shuangxu Electronics Co., Ltd, China). The percentage of natural sedimentation particles of flour was determined by the wheat sedimentation index sodium dodecyl sulfate method (Carter et al., 1999). The experimental group using the first-grade particle was called the S1 group, and then the S2 group, S3 group, S4 group, and S5 group adopted second-, third-, fourth- and fifth-grade particles, respectively. In each experimental group, a 60 mm petri dish was utilized as a culture vessel in which the culture medium was composed of 6 mL of sterile water, 300 mg/L NaCl and an equal weight of the particle and inoculated with six adult rotifers. The main culture conditions included pH 6.5±0.8, dark conditions, a constant temperature of 25°C, and the addition of 20 μL of sterile water every 24 h to replenish the moisture lost by evaporation. The experimental period was designed to be 13 days because the growth of *Habrotrocha* sp. on day 12 usually declined based on our preliminary experiment. The numbers of *Habrotrocha* sp. were counted under an inverted microscope every 24 h. All experiments were repeated three times.

2.3.3 Effects of flour particle concentration on the population reproduction of rotifers

Five experimental groups with 5 particle concentrations of 1.0×10^6, 3.0×10^6, 5.0×10^6, 7.0×10^6 and 9.0×10^6 cell/mL were selected and named the C1 group, C2 group, C3 group, C4 group and C5 group, respectively. Meanwhile, they were designed to evaluate the effects of particle concentration on the population growth rate of *Habrotrocha* sp. In each experimental group, the main experimental conditions, including culture vessels, culture medium, inoculation number of *Habrotrocha* sp., culture conditions, experimental period and rotifer count method, were the same as Section 2.3.2 with an average food particle size of 0.94 μm. All experiments were repeated three times.

2.3.4 Effects of the growth environment on the population reproduction of rotifers

Based on the above results, the effects of the three growth environmental factors, temperature, pH, and salinity on the population growth of *Habrotrocha* sp. were explored. In all 3 experiments assessing the impact of growth environment, the main experimental conditions were the

Table 1 Characteristics of particle size distribution and natural sedimentation of five grades of particles

Item	First-grade particle	Second-grade particle	Third-grade particle	Fourth-grade particle	Fifth-grade particle
Average particle size (μm)	16.8	5.89	2.38	0.94	0.58
80% particle size range (μm)	11.4–27.8	0.87–12.5	0.48–5.61	0.26–1.65	0.18–1.05
Percentage of natural sedimentation particle (%)	91.5	18.4	0.5	0	0

same as Section 2.3.2, except for using fourth-grade particles with a concentration of 5.0×10^6 cells/mL and specific growth environment conditions. In the case of the temperature experiment, the culture temperatures were 20°C, 25°C, 30°C and 35°C. In the case of the pH experiment, the pH values were 5, 6, 7, 8 and 9, and the pH of the culture medium was adjusted by NaOH or HCl. In the case of the salinity experiment, the five salinity levels were 100, 300, 500, 700, 900 and 1100 mg/L. The configured NaCl concentration determines the salinity level. All experiments were repeated three times.

2.3.5 Effects of cultured rotifers on the sludge settleability

Two SBR reactors with a 1 L working volume were used for this experiment. Reactor I was only inoculated with sludge as a control, Reactor II was inoculated with sludge and approximately 100000 cultured *Habrotrocha* sp. fed on flour. The inoculated sludge was collected from a secondary clarifier of a municipal wastewater treatment plant in Shanghai, China. The raw water used in the experiment was synthetic wastewater. The synthetic wastewater contained the following components: NH_4Cl 125 mg/L, KH_2PO_4 10 mg/L, $CaCl_2$ 10 mg/L, $MgSO_4$ 10 mg/L, KCl 10 mg/L, $NaHCO_3$ 24 mg/L, $C_6H_{12}O_6$ 50 mg/L, $FeSO_4$ 1.5 mg/L, $CuSO_4$ 1.5 mg/L, $ZnSO_4$ 1.5 mg/L and $MnSO_4$ 0.6 mg/L. The pH of synthetic wastewater was adjusted to 7.2 using hydrochloric acid and sodium hydroxide. The operation duration of the bioreactor was generally divided into several phases, which consisted of 5 min of water influent, 60 min of anaerobic/anoxic reaction without agitation, 250 min of aerobic reaction, 40 min of settling, and 5 min of effluent discharge. An air bubble was delivered from the bottom of the bioreactor to maintain the dissolved oxygen (DO) concentration at 2.5–5.5 mg/L in the aerobic phase. The water temperature of the bioreactor was maintained at 25°C–27°C. The sludge volume index (SVI) was measured according to the standard analysis method (APHA, 1998). The extraction method for loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS) was performed according to a sonication/thermal method (Ma et al., 2017).

3 Results and discussion

3.1 Growth characteristics of rotifer on different foods

To explore the feasibility of replacing the traditional live food consisting of *C. vulgaris* and mixed bacteria with the nonliving food wheat flour for the culture of *Habrotrocha* sp., the growth characteristics of rotifers were studied using the above 3 foods (Fig. 1). SGR of *Habrotrocha* sp. fed on microalgae, and bacteria, flour were 0.39 d^{-1}, 0.5 d^{-1}, and 0.55 d^{-1}, respestively and their highest densities were 45.38 ind/mL, 65.16 ind/mL and 110.67 ind/mL, respectively. The SGR and the highest density of *Habrotrocha* sp. fed on flour were higher than those fed on *C. vulgaris* or mixed bacteria, demonstrating that flour was a better food for culturing *Habrotrocha* sp. than *C. vulgaris* or mixed bacteria. Compared to *C. vulgaris* and mixed bacteria as food for *Habrotrocha* sp., flour has more advantages, such as easy preparation and storage because it is a nonliving food and easily available from the market.

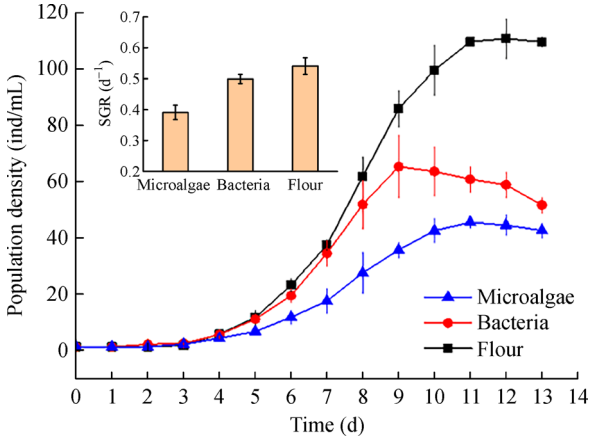

Fig. 1 Population growth characteristics of *Habrotrocha* sp. cultured with three different foods.

Chlorella, as a microalgae, is often used as a food in fish cultures and even as a human health supplement (Panahi et al., 2016), apparently due to its nutritionally rich properties (Makareviciene et al., 2021). However, in this study, the SGR and the highest density of rotifers using *C. vulgaris* as food were significantly lower than those using mixed bacteria or wheat flour, which may be related to the larger food particle size of 5–10 μm (Scragg et al., 2003) because we observed that the rotifer was not able to ingest *C. vulgaris* under the microscope.

The growth rate of rotifers fed mixed bacteria was close to that of rotifers fed flour, but the rotifers almost stopped growing after the ninth day of the experiment as shown in Fig. 1. At this time, a large bacterial floc could be observed by eye; that is, the previously scattered bacteria together formed zooglea, making it difficult for the rotifers to feed. Floc formation by bacteria is beneficial in the activated sludge process, but it is not suitable for the mass culture of rotifers.

It has been reported that the growth rate of *P. roseola* is 0.25 d^{-1} when fed on *C. vulgaris* (Nandini and Sarma, 2001) and only 0.135 d^{-1} when fed on *Escherichia coli* (Ricci, 1984). In this study, the SGR of *Habrotrocha* sp. fed on *C. vulgaris*, mixed bacteria and wheat flour with the culture conditions of pH at 6.2, a temperature of 25°C, a salinity of 100 mg/L, and an average particle size of 5.89 μm were 0.39 d^{-1}, 0.5 d^{-1}, and 0.55 d^{-1}, respectively.

The growth rates of rotifers fed on bacteria or *C. vulgaris* in this study were all higher than the results reported in the literature, which may be due to the different species of cultivated rotifers, although they were all bdelloid rotifers.

Moreover, the culture of *Chlorella vulgaris* or bacteria requires aseptic procedures, so that special aseptic equipment is required during culture and storage. However, flour can be directly used as rotifer food with convenient operation and simple equipment. To calculate the cost of the culture medium, if the culture density was 100 ind/mL and the culture medium volume was 1 m^3, the costs of bacteria, *Chlorella vulgaris*, and flour were 1350 Chinese Yuan, 450 Chinese Yuan and 150 Chinese Yuan, respectively, so the cost of using flour as rotifer food was significantly reduced.

3.2 Effects of flour particle size on the population growth of rotifers

During the 13-day culture period, the population growth curves of *Habrotrocha* sp. fed 5 different sizes of flour particles ranging from 0.58 μm to 16.8 μm are displayed in Fig. 2. In the S1 group to S5 group, the SGRs of rotifers were 0 d^{-1}, 0.51 d^{-1}, 0.55 d^{-1}, 0.645 d^{-1} and 0.616 d^{-1}, and their maximum densities were 0 ind/mL, 61.6 ind/mL, 84.7 ind/mL, 106 ind/mL and 104 ind/mL, respectively. This revealed that SGR and the maximum population density of the rotifer became larger with decreasing food particle size. When the average particle size was 16.8 μm, the population of the rotifer almost stopped reproducing. When the average particle size was 5.89 μm, the rotifer population reproduced significantly, suggesting that the upper limit of the particle size that can be ingested and utilized by *Habrotrocha* sp. is between 5.89 μm and 16.8 μm.

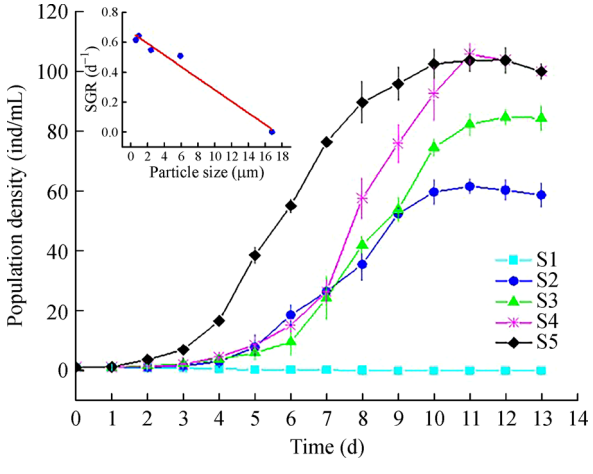

Fig. 2 Population growth characteristics of *Habrotrocha* sp. cultured with five different flour particle sizes. Abbreviations: S1–S5, S1, S2, S3, S4, and S5 group. Points are the mean±standard error based on three replicates. The inset shows the relationship between particle size and SGR of *Habrotrocha* sp.

The bdelloid rotifer *P. roseola* can efficiently ingest particles in a size range from 0.2 to 3 μm, which correlates with more than 95% of the suspended particles in wastewater, while particle sizes up to 10 μm are limited (Lapinski and Tunnacliffe, 2003). This study not only clarified that the suitable range of particle size was 0.58–0.94 μm as food for another bdelloid rotifer, *Habrotrocha* sp. but also revealed a significant linear relationship between the reproduction rate of rotifers and food particle size (Fig. 2).

Furthermore, *Habrotrocha* sp. like *P. roseola* can ingest small-sized particles, which may contribute to the removal of microscopic suspended particles that cannot settle naturally in a secondary sedimentation tank. Therefore, it is necessary to develop mass culture bdelloid rotifer techniques to use cultured bdelloid rotifers in biological treatment systems to improve biological treatment efficiency. Incidentally, the wheat flour culture method developed in this study will also help with the study of the feeding habits of other microfauna.

3.3 Effects of flour particle concentration on the population growth of rotifers

To investigate the effect of food concentration on the population growth rate of *Habrotrocha* sp., five experimental groups with flour particle concentrations of 1.0×10^6, 3.0×10^6, 5.0×10^6, 7.0×10^6 and 9.0×10^6 cell/mL were used and named the C1 group, C2 group, C3 group, C4 group, and C5 group, respectively. The population density of *Habrotrocha* sp. cultured with different concentrations of flour particle for the 13-day culture period is illustrated in Fig. 3. In the C1 to C5 culture groups, the corresponding SGRs of *Habrotrocha* sp. were 0.768 d^{-1}, 0.683 d^{-1}, 0.645 d^{-1}, 0.599 d^{-1}, and 0.515 d^{-1}, and their maximum densities were 54.3 ind/mL, 66.4 ind/mL, 106 ind/mL, 103 ind/mL and 88.7 ind/mL, respectively. There was a significant negative linear correlation ($P < 0.01$) between the concentration of food particles and SGR of the rotifer (Fig. 3), indicating that the higher the food concentration, the slower the rotifer grows. The maximum population density of rotifers in the C1 to C4 groups showed an increasing trend with increasing food particle concentration. In the C5 group, the maximum population density may be higher than that in the other groups, as the growth of the rotifer did not show a downward trend on the last day of the experiment. Moreover, the time for the rotifer population to enter the logarithmic growth period was delayed by increasing the food concentration. Based on the above results, the selection of food concentration for practical applications may require considering both reproductive speed and maximum density.

When the food concentration was higher than a certain range, the growth rate of *Habrotrocha* sp. decreased, which may be related to its feeding characteristics.

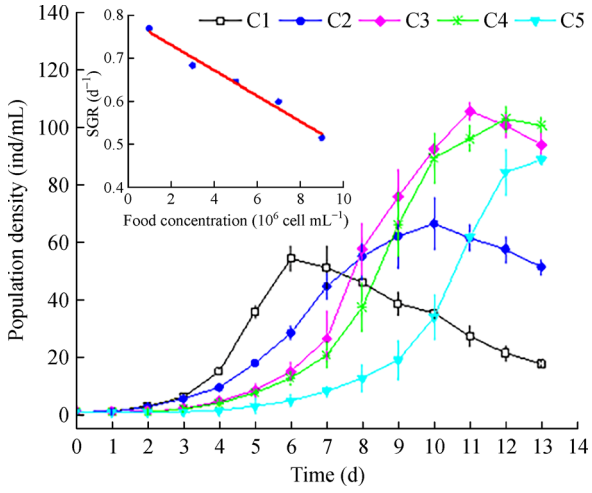

Fig. 3 Population growth characteristics of *Habrotrocha* sp. fed on five different food concentrations. Points are the mean±standard error based on three replicates. Abbreviations: C1–C5, C1, C2, C3, C4, and C5 group. The inset shows the relationship between flour concentration and SGR of *Habrotrocha* sp.

Habrotrocha sp. has two coronas with ciliary rings in the upper part of the mouth (Melone and Ricci, 1995a). There is a small gap between the two ciliary rings. The cilia can beat sequentially at a high speed of 1200 complete strokes per minute and produce a vortex of water (Lansing and Lamy, 1961). The vortex draws food particles in the water into the rotifer's mouth through the gap. However, according to our observations under the microscope, if the particle size of food was larger than the gap between the two ciliary rings, the cilia stopped beating and allowed the particle to be flicked away due to the reaction force. After that, the cilia would continue to beat for prey. The reason that SGR of *Habrotrocha* sp. may have decreased with increasing particle concentration because the increase in particle concentration will increase the quantity of larger particles, which increases the chance of cilia stopping working and thus reduces the feeding efficiency of rotifers.

3.4 Effects of the growth environment on the population growth of rotifers

3.4.1 Temperature

Under constant temperature culture conditions of 20°C, 25°C, 30°C and 35°C, SGRs of *Habrotrocha* sp. were 0.420 d^{-1}, 0.645 d^{-1}, 0.661 d^{-1} and 0.427 d^{-1}, and their maximum densities were 28.7 ind/mL, 106 ind/mL, 119 ind/mL and 46.7 ind/mL (Fig. 4), respectively. At a culture temperature of 30°C, *Habrotrocha* sp. had the fastest population growth rate, the highest density, and the shortest time to enter the logarithmic phase. The SGR of rotifers was slightly lower at a culture temperature of 25°C than at a temperature of 30°C. Therefore, temperatures in the range from 25°C to 30°C are suitable temperature conditions for the rotifer growth. When the culture temperature was 20°C or 35°C, both SGRs and the maximum rotifer density were significantly reduced relative to culture temperatures of 25°C and 30°C, suggesting that temperature conditions lower than 20°C or higher than 35°C were not suitable for mass culture of bdelloid rotifers. It was reported that *P. roseola* grows fastest at 35°C in the tested temperature range of 5°C–35°C (Schaefer and Pipes, 1973), which is different from our research result. This may be because different species of bdelloid rotifers adapt to different growth environments.

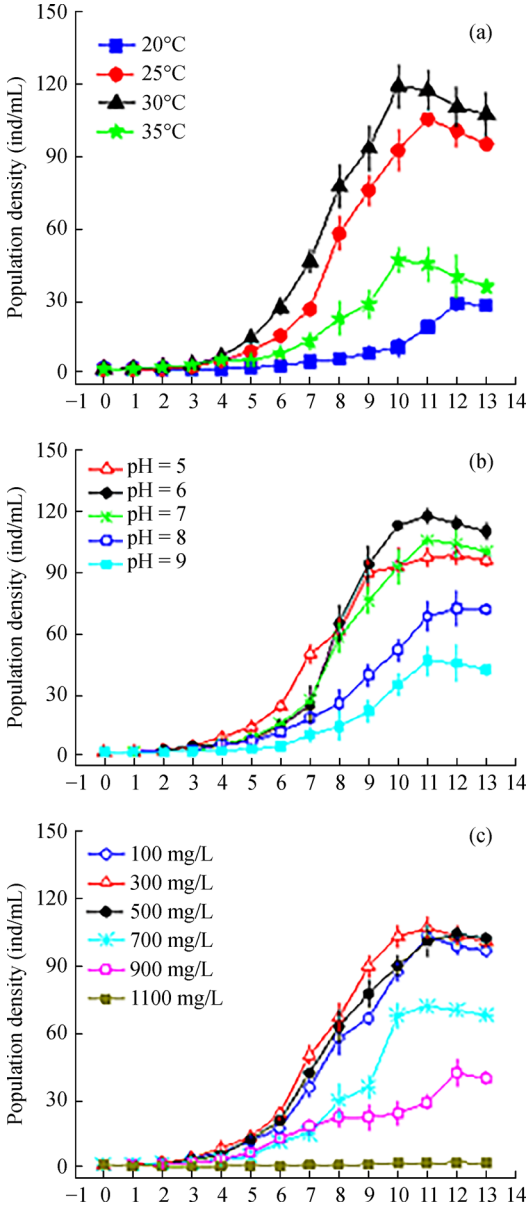

Fig. 4 Population growth characteristics of *Habrotrocha* sp. under different growth environmental conditions: (a) temperature; (b) pH and (c) salinity. Points are the mean±standard error based on three replicates

Habrotrocha sp. were detected in a natural environment with a water temperature of 12°C–30°C, while the genus *Philodina* sp. was detected in a natural environment with a water temperature of 36°C (Zeng et al., 2020).

3.4.2 pH

The population growth of *Habrotrocha* sp. at pH 5–9 during 13 days of culture is shown in Fig. 4. When the pH values were 5, 6, and 7, the corresponding SGRs of the rotifer were 0.651 d^{-1}, 0.749 d^{-1}, and 0.645 d^{-1}, and their maximum densities were 98.1 ind/mL, 118 ind/mL, and 106 ind/mL, respectively. The SGR and the maximum density of the rotifer were relatively close to the range of pH 5–7, and pH 6 was the most suitable for the reproduction of the rotifer.

When the pH values were 4 and 9, *Habrotrocha* sp. displayed a significant slowdown in growth rate and a decrease in maximum density. However, it was reported that the population growth and reproduction rate of *Habrotrocha rosa* Donner were higher at pH 4 than at pH 3, 5 or 6 (Błedzki and Ellison, 1998). The bdelloid rotifers *Cephalodella hoodi* and *Rotaria rotatoria* were found in lakes with a pH of 2.3 (Deneke, 2000), revealing that different species of bdelloid rotifers can live in widely different pH environments and can survive in extreme environments. *Habrotrocha* sp. were isolated from a sewage biological treatment system in this study. Therefore, the examined bdelloid rotifer is able to live in a near-neutral water environment. In addition, the pH value of the food suspension used in this study without adjustment was approximately 6.5±0.8, which is in the optimum pH range for the growth of *Habrotrocha* sp. Therefore, there is no need to adjust the pH of the culture medium when flour is applied as food in practice.

3.4.3 Salinity

When the salinities of the culture medium were in the range of 100–500 mg/L, there was a similar variation curve of the population density of *Habrotrocha* sp. (Fig. 4). The SGR and maximum density of rotifers in 300 mg/L salinity culture medium were 0.651 d^{-1} and 107 ind/mL, respectively, which were higher than those in other culture media with different salinities. SGR and the maximum density of rotifers decreased significantly when the salinity level was 700 mg/L compared to salinities in the range of 100–500 mg/L. The growth of the rotifer population slowed down at a salinity of 900 mg/L and stopped at a salinity of 1100 mg/L in the culture medium, indicating that the salinity threshold limiting rotifer population reproduction was between 900 mg/L and 1100 mg/L.

Salinity is considered to be one of the most important abiotic factors, thus affecting the survival and abundance of zooplankton. The population dynamics of five free-swimming freshwater rotifers, *Anuraeopsis fissa, Brachionus calyciflorus*, *B. havanaensis*, *B. patulus* and *B. rubens* were adversely affected by 1.5–3.0 g/L NaCl (Sarma et al., 2006). Bdelloid rotifers *Philodina citrina* and *Rotaria rotatoria* were found in brackish water where the salinity levels were 2–10 g/L (Leasi and De Smet, 2020). Moreover, it was reported that *Habrotrocha* sp. can thrive under a salinity of 10 g/L (Ponce-Palafox et al., 2019). The *Habrotrocha* sp. used in this study seems to be a more rigorous type of freshwater rotifer compared with the abovementioned bdelloid rotifers.

3.5 Effects of cultured rotifers on sludge settleability

To determine whether rotifers fed wheat flour are still helpful for the biological treatment of wastewater, the effect of cultured *Habrotrocha* sp. on sludge settleability and EPS in the reactor were investigated. Two SBR reactors, of which Reactor I lacked rotifers and Reactor II was supplemented with rotifers, were used for this study. As shown in Fig. 5, during the first to sixth days of operation in the two reactors, the SVI decreased from 94.1 and 95.2 mL/g to 73.2 and 59.9 mL/g in Reactor I and

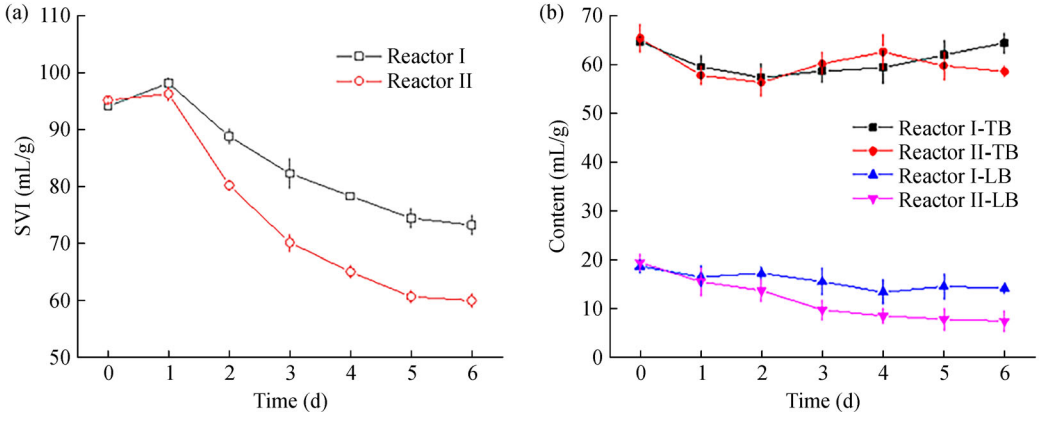

Fig. 5 Settleability of the sludge (a); LB-EPS and TB-EPS of sludge in SBR (b) with and without *Habrotrocha* sp. Points are the mean±standard error based on three replicates.

Reactor II, respectively. That is, the sludge settleability in both reactors showed a tendency toward improvement, but the performance of Reactor II was better than that of Reactor I.

To further understand the mechanism of action of the rotifer, the concentration of EPS was analyzed because EPS of sludge has an important influence on the sedimentation and flocculation performance of sludge (Ma et al., 2017; Wang et al., 2021) and LB-EPS in EPS has a negative effect on sedimentation and bioflocculation in particular (Li and Yang, 2007). The results of this study showed that the addition of *Habrotrocha* sp. did not affect TB-EPS. However, it significantly reduced the content of LB-EPS in sludge flocculation (Fig. 5), suggesting that the role of bdelloid rotifers may be related to the inhibition of bacteria to produce LB-EPS, thus improving the sludge settleability performance.

4 Conclusions

An innovative method for culturing the bdelloid rotifer *Habrotrocha* sp. using wheat flour in place of traditional live foods such as *C. vulgaris* or mixed bacteria was developed. The optimum rotifer culture conditions including food particle size, food concentration, and growth environment when using flour as food were also determined. The cultured *Habrotrocha* sp. fed on flour helped improve sludge settleability in the sequencing batch reactor, which may be related to the role of rotifer inhibiting bacteria from producing LB-EPS.

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