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Short Communication

Aerobic granulation of single culture protist

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ABSTRACT

Thraustochytrium striatum was selected as a model protist to test the possibility of protist granulation for its good potential of organic waste valorization. Initially, only a selective settling velocity of 9 m h^{-1} was applied in a sequential batch reactor (SBR) with minimal granulation progress after three months. After the famine condition was introduced into the SBR with a feast-to-famine duration ratio of 0.2 by extending the cycle time and lowering the initial substrate concentration while keeping the selective settling velocity unchanged, protist granules with spherical shape and smooth surface formed and were dominated in the reactor after another month. The average particle size of protist granules was 728 μm, 90 times greater than its planktonic counterpart. This study evidenced the culture-independent phenomenon of aerobic granulation. Also, the combination of a low feast-to-famine duration ratio with a selective settling velocity was found to be required for inducing successful protist granulation.

1. Introduction

In the field of biological wastewater treatment, aerobic granulation has been researched for nearly two decades to induce co-aggregation of bacterial cells for the formation of aerobic granules with high biomass retention, good settleability, and tolerance of toxic and shock loading at lower energy and cost demand [1,2]. To date, almost all aerobic granulation has been achieved with mixed culture, and very little is available about its applicability to pure culture [2]. Based on the broadly reported aerobic granulation in wastewater, it was inferred that aerobic granulation phenomena should be culture-independent [2]. However, to our best knowledge, there was only one single culture aerobic granulation study in the literature, and the culture used was limited to the prokaryotic domain [3]. It is our interest to test whether this aerobic granulation technology is applicable to microbes from the eukaryotic domain, as well.

Thraustochytrium striatum is a marine protist that has received research attention in recent years for its special features suitable for advanced wastewater treatment [4,5]. This eukaryote is good at utilizing a broad range of carbon and nitrogen sources, including non-readily biodegradable organics such as aromatic and lignocellulosic compounds, for syntheses of cellular lipids and pigments, which can be further processed into value-added bioproducts [5–7]. Moreover, *T. striatum* is also good at tolerating severe conditions such as low pH, high salinity, and inhibition in wastewater [4,7]. Therefore, we selected *T. striatum* as a model eukaryote in this study for its good potential to become a robust catalyst to be applied in future biorefinery and bioremediation applications [4,6].

It is our hypothesis that *T. striatum* cells are able to form protist granules under appropriate cultivation conditions that can be extrapolated from the bacterial granulation studies, and then confer aforementioned advantages of aerobic granules on *T. striatum* cells in their application for advanced wastewater treatment. The selection pressure on bioparticle settling velocity together with an alternating feast/famine environment have been recognized to be important for inducing bacterial granulation [8–10]. By shortening the settling time of sequential batch reactor (SBR), a selective settling velocity can be created by the ratio of SBR discharge height to settling time, i.e., only bioparticles with settling velocity greater than this selective settling velocity can be retained in the reactor, while the rest slow settling cells will be washed out and eliminated [11,12]. Meanwhile, the cycle time of SBRs also can be easily manipulated to fine-tune the desired feast/famine conditions [13]. Therefore, *T. striatum* was inoculated into an SBR to test the aforementioned hypothesis. The goal of this study is to cultivate the very first protist granules in the area of pure culture granulation. The methods and data derived from this study will contribute to the development of a generalized aerobic granulation theory.

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2. Materials and methods

2.1. Reactor setup and operation

An aerobic pure culture granulation reactor with 200 mL working volume as shown in Fig. 1A and B comprised the main reactor, a feed pump, a waste pump, and an air pump for aeration. The reactor's inner diameter was 3 cm with a height of 30 cm. A baffle was added in the middle to turn the column reactor into an airlift reactor with internal hydraulic circulation (Fig. 1B). A 0.24-μm pore size air filter was installed in the air inlet for the sterilization of incoming air to ensure a sterile environment in the reactor. Before inoculation, the reactor and tubing were connected and sterilized in an autoclave at 121 °C for 30 min. The system was fed with a synthetic glucose medium stored in a 500-mL glass bottle. The synthetic glucose medium consisted of (per liter) 60 g of glucose, 12 g of yeast extract, and 12 g of peptone. The medium was prepared in artificial seawater comprised (per liter): 30 g of NaCl, 0.7 g of KCl, 10.8 g of $MgCl_2{\cdot}6H_2O$, 5.4 g of $MgSO_4{\cdot}7H_2O$, and 1.0 g of $CaCl_2{\cdot}2H_2O$. After three months of cultivation, this medium was diluted six times to introduce the famine condition. The medium was autoclaved at 121 °C for 15 min. After cooling it to room temperature, the medium stock bottle was connected to the reactor aseptically. The reactor was operated in the sequential batch mode and controlled by the timers (Ouzhrn, Shenzhen, China) in Fig. 1B with one min feed, 1440 min aeration, one min settling, and one min discharge. The discharge height (H in Fig. 1B) of the SBR was 15 cm. Thereby, a selective settling velocity equal to discharge height/settling time = 9 m h^{-1} was created to selectively retain bioparticles, i.e., only bioparticles settling faster than 9 m h^{-1} can be retained and prosper in the SBR while all the other slower settling bioparticles were washed out. The inlet gas flow was controlled to maintain dissolved oxygen (DO) concentration in the reactor > 2.0 mg L^{-1}. The temperature was maintained at 25 °C. The SBR cycle time was increased from 24 h to 72 h to facilitate the creation of famine phase after three months of cultivation with only selective setting velocity in place.

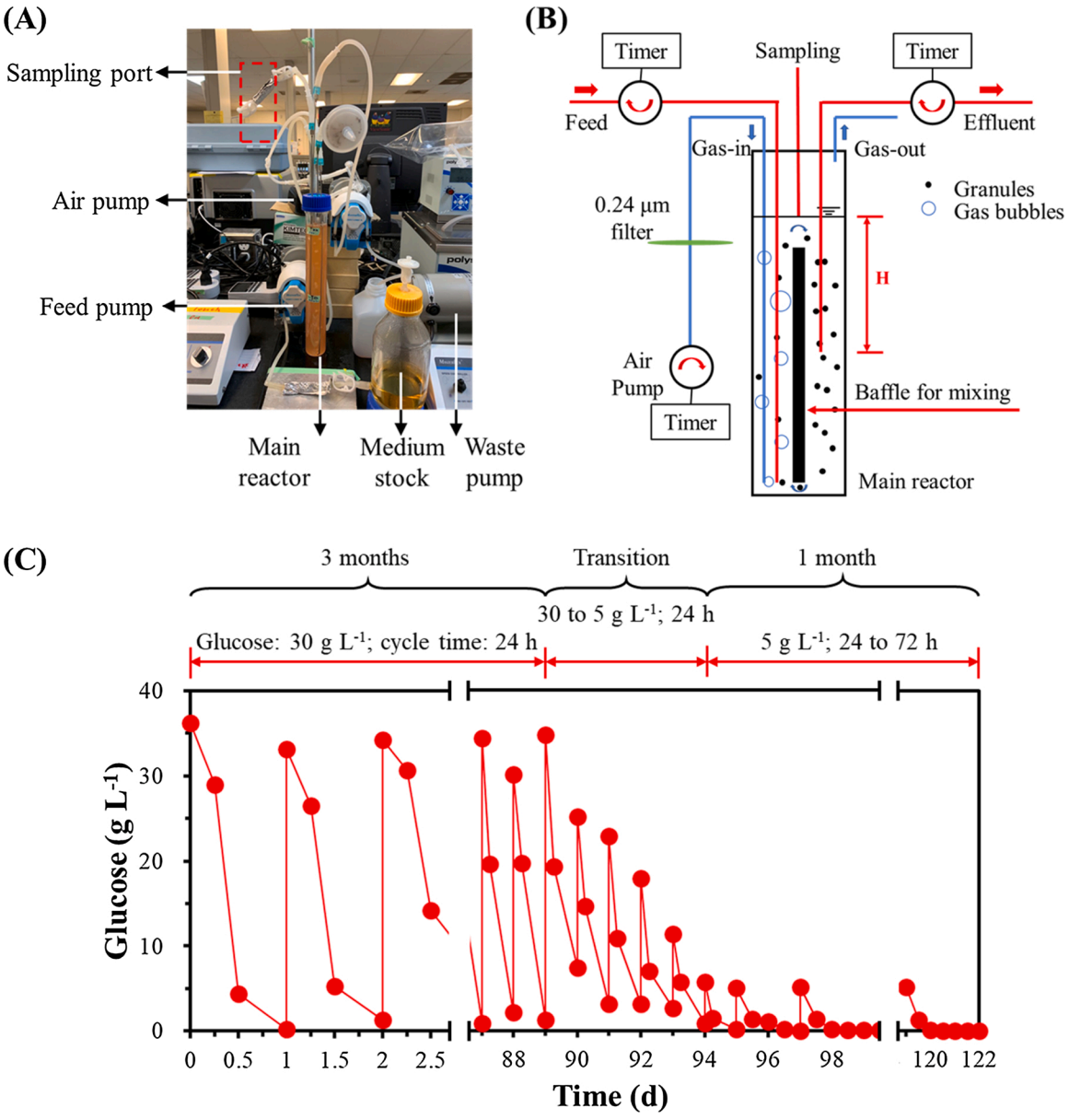

Fig. 1. Protist granulation reactor design and operation: (A) photographic and (B) schematic illustrations of the reactor setup; (C) the cyclic substrate profiles (–●–) and cycle time adjustment over four-month cultivation.

2.2. Inoculum preparation

The marine protist *T. striatum* ATCC 24473 was purchased from ATCC, and the seed culture was prepared in 250-mL flasks with 50 mL glucose medium according to the methods in a previous study [6]. The seed culture was initially inoculated into a glucose medium with only 10 g L^{-1} glucose to avoid the possible inhibition of seed culture after being directly transferred into a rich medium. The protist was cultivated in the dark under 25 °C with a shaking speed of 150 rpm for five days. Then, 10 mL culture was transferred to a glucose medium with 20 g L^{-1} glucose as a sub-culture. After five days, 10 mL sub-culture was then inoculated into the reactor, and all timers were started for each reaction cycle.

2.3. Analytical methods

DO was measured using a submersed, luminescent DO probe (Hach, Intellical™ LDO101, Loveland, CO, USA). The particle size distribution was analyzed using a laser scattering particle size analyzer (Horiba, LA-950, Kyoto, Japan). A Nikon Eclipse E200 microscope (Melville, NY, USA) was utilized to take microscopic images of aerobic granule morphology. The mixed liquor sample was separated with a 100-μm sieve to differentiate planktonic cells from granules. The biomass retained on the sieve was regarded as granules, while those passed through the sieve were defined as planktonic cells. The total suspended solids (TSS) of two streams of biomass were measured according to the standard method [14]. The glucose concentration was measured with a blood glucose meter (Ermaine, AimStrip, TX, USA) after filtration through 0.45 μm syringe filters.

3. Results and discussion

3.1. Cultivation of protist granules

Under cycle time of 24 h and with an initial glucose concentration of 30 g L^{-1}, the SBR in Fig. 1A and B has been operated for three months. However, protist granulation was not observed. Only planktonic cells with very similar characteristics of the seed *T. striatum* cells were cultivated (Fig. 2A). The substrate concentration profile in Fig. 1C shows that the glucose concentration can gradually drop to zero at the end of each cycle, however there was no famine condition within the first three months of cultivation. Given the importance of famine condition in inducing the aerobic granulation [8,10], the initial glucose concentration was gradually lowered to 5 g L^{-1} starting from day 90, and then the cycle time was gradually extended to 72 h in the course of a transitional period in order to create the feast/famine conditions in the SBR (Fig. 1C). With this adjustment, it only took another month to cultivate protist granules in the SBR. The morphology of the protist granules can be found in Fig. 2B and C. As can be seen, many spherical and compact aggregates with smooth surface showed up in the SBR (Fig. 2B and C). The red color was resulted from the carotenoid produced by *T. striatum,* which indicates good viability of the protist cells in these granules [6]. The particle size analysis in Fig. 3 revealed that 89 % of bioparticles cultivated in the SBR was with a mean size as large as 728 μm. The rest 11 % of bioparticles only had a mean size around eight μm which is the typical size of single cells of *T. striatum* as shown in Fig. 2A. The distinctive morphology and size difference of the cultivated bio-aggregates from the planktonic *T. striatum* cells evidenced that the *T. striatum* cells have successfully co-aggregated into protist granules in the SBR (Figs. 2 and 3). In addition, the significantly ($p < 0.05$) higher TSS concentration of *T. striatum* granules over that of its planktonic counterparts further revealed that protist granules have dominated the SBR (Fig. 4A). The settling velocity of protist granules was measured to

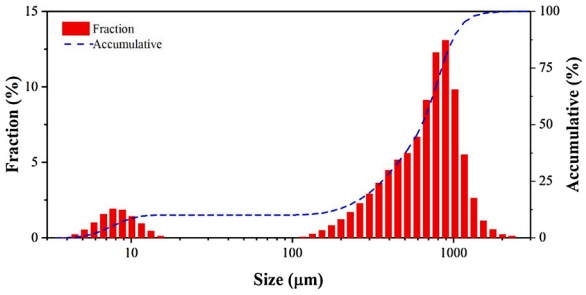

Fig. 3. The particle size distribution of *T. striatum* cells after 4-month cultivation.

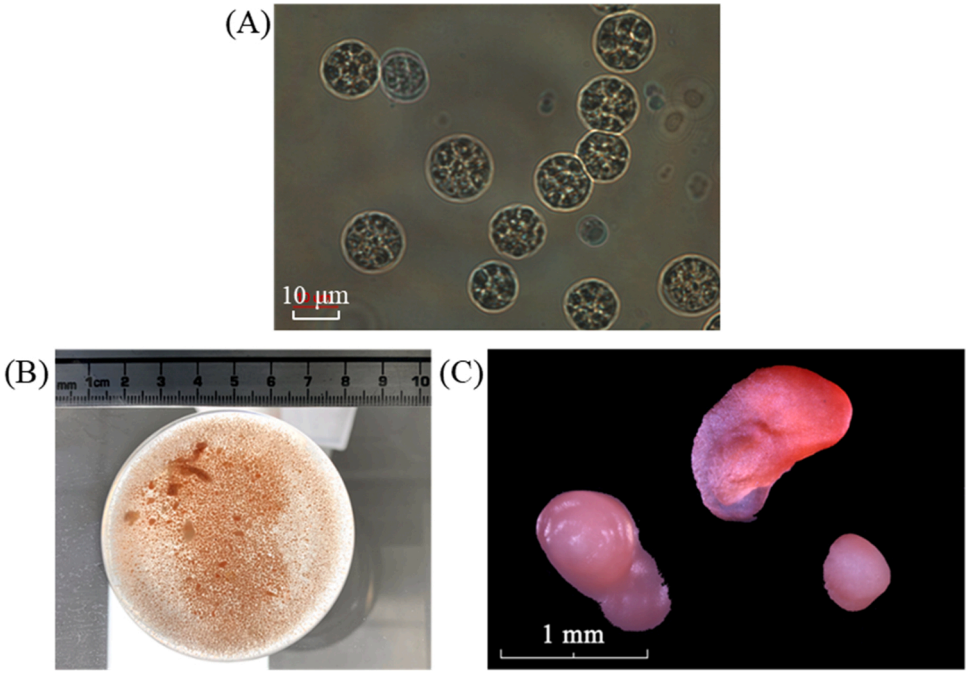

Fig. 2. Morphology of (A) planktonic *T. striatum* cells before granulation, and granules (B) in petri dish and (C) under microscope.

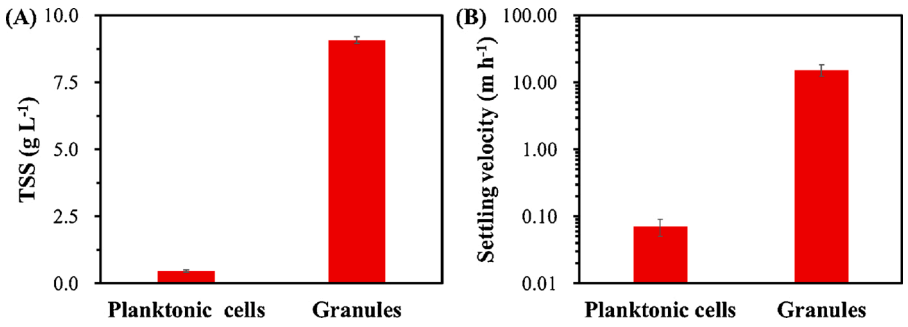

Fig. 4. (A) TSS and (B) settling velocity of planktonic *T. striatum* cells and granules in the SBR.

be as fast as 15.2 m h^{-1}, which was 300 times greater than that of their planktonic counterpart which was only 0.05 m h^{-1} (Fig. 4B). Such a significant improvement ($p < 0.05$) on the *T. striatum* cell settleability after the protist granulation is expected according to Stokes' law and in line with the typical characteristics of aerobic granules formed in mixed culture [2].

3.2. Importance of feast-to-famine ratios

Substrate concentration profiles in Fig. 5A revealed that only feast condition existed in the SBR operated with initial substrate concentration as high as 30 g L^{-1} and cycle time as short as 24 h within the first 90 days of the experiment in Fig. 1C. After three months of cultivation with only 9 m h^{-1} selective settling velocity as selection pressure and without a famine condition, the TSS concentration of planktonic cells was stabilized around 17 g L^{-1} at the end of each cycle (Fig. 5A). Based on the latest understanding from a recent publication [10], a low feast-to-famine ratio is favorable for aerobic granulation. To create an appropriate famine condition, the SBR cycle time was gradually increased from 24 to 72 h along with a decrease of initial substrate concentration from 30 to 5 g L^{-1} during the transitional period in Fig. 1C. As a result of this adjustment, a prolonged famine condition was created after 12 h of each cycle, giving a feast-to-famine duration ratio of 0.2 in Fig. 5B. It is noteworthy that a feast-to-famine ratio of 0.2 has been projected to be within the optimum range promoting aerobic granulation [10]. This is the range where the influence of feast-to-famine ratio can substantially compensate that of the selective settling velocity [10].

Over the seven days of this transitional period (Fig. 1C), the TSS concentrations of planktonic cells quickly decreased from 17 g L^{-1} in Fig. 5A to 10 g L^{-1} in Fig. 5B as a result of the starvation during the famine phase. However, one month later, this planktonic TSS further dropped to 0.5 g L^{-1} (Fig. 5B). This is because the majority of the planktonic *T. striatum* cells have co-aggregated into the form of biogranules (Fig. 4A). It should be realized that the selective settling velocity (9 m h^{-1}) was kept at the same level throughout the experiment without changes (Fig. 1C). Therefore, the success of protist granulation should be attributed to the introduction of a low feast-to-famine ratio in the SBR (Fig. 5B). It has been recognized from recent mixed culture studies that a ratio of feast-to-famine below 0.3 is ideal for inducing aerobic granulation [8,10]. The fact that controlling the feast-to-famine ratio within the similar range has also promoted successful single culture protist granulation implies that the aerobic granulation phenomenon is indeed culture-independent, and different domains of microorganism might share very similar environmental triggers required for promoting cell-to-cell aggregation.

3.3. Insight into the mechanism of single culture protist granulation

For a single culture of *T. striatum*, the shift of its growth mode from plankton to granules can be viewed as a phenotype change in the traits such as morphology and structure for adaptation to changing environments. The presence of phenotypic heterogeneity from the same genetic culture is a bet hedging strategy employed by organisms to ensure their DNA lineage survival in the history of evolution [15]. As an insurance policy in the face of environmental uncertainty, microbes not only reproduce the fast-growing planktonic cells to achieve fecundity but also invest in the persistent phenotype that offers longevity [16]. Aerobic granules are such a kind of persistent phenotype in that it allows microbes to survive the settling velocity selection (Fig. 4B). According to Stokes' law, the particle settling velocity squarely increases with the particle size but only linearly increase with the particle density. That being said, investing in producing the cells capable of co-aggregation offers a persistent phenotype that can survive the settling velocity selection (Fig. 4B). Interestingly, the rate of phenotype switch from normal cells to persistent cells has been found to depend strongly on the frequency of environmental changes but only weakly on the selective pressures of any given environment [15]. This might explain why the measure to create the alternating feast/famine conditions was effective in driving protist granule formation even though the settling velocity selection pressure was kept unchanged (Figs. 2–4). Similarly, in a recent *Escherichia coli* pure culture study, Merritt and Kuehn [16] were able to

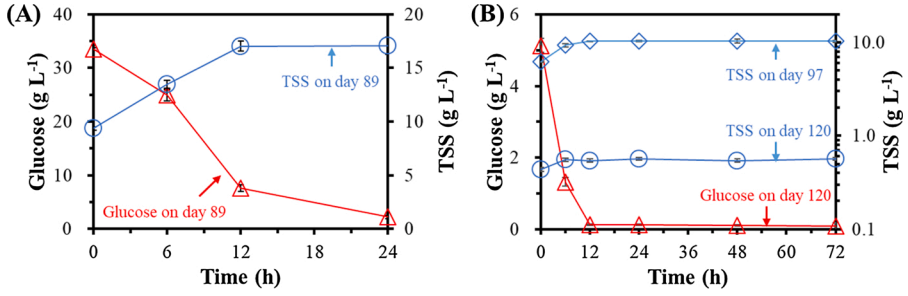

Fig. 5. Cyclic profiles of glucose concentrations (–△–) and TSS of planktonic cells on (A) day 89 (–○–); and (B) day 97 (–◇–) and day 120 (–○–).

induce pure culture *E. coli* to express co-aggregation phenotype by aggravating the famine condition under washout selection. It has been broadly recognized that environmental stress such as starvation is conducive to phenotype switch from plankton to biofilm, and vice versa when the nutrient availability was increased [17,18]. Aerobic granules can be regarded as spherical biofilms without abiotic carriers because microbial cells attach to each other's cell surfaces to form aggregate [2]. In response to feast/famine conditions, the pure culture bacteria were found to transition between free-floating aggregates [17], or surface-attached biofilms [19] and dispersed planktonic populations [20]. Hence, it is very likely that the reduced feast-to-famine ratio in the SBR in Fig. 1A and B have triggered the protist phenotype switch from planktonic to granulated growth mode, which allowed the protist cells to survive the settling velocity selection at the end of each SBR cycle (Figs. 2–5). In this sense, future work is warranted to focus on understanding the phenotype switch of protists from plankton to granules triggered by the feast/famine conditions in the SBR.

4. Conclusions

The following concluding remarks can be drawn from this study:

1 It was probably for the first time to show that single culture protist such as *T. striatum* can form aerobic granules.
2 It is verified in this study that the aerobic granulation theory developed from the mixed culture in prokaryotic domain actually can be applied to microbes in the eukaryotic domain.
3 In comparison with planktonic protists, the granulated protist possesses large particle size, compact structure, high biomass retention, and faster settling velocity.
4 A feast-to-famine ratio of 0.2 is required for inducing protist granulation despite the strong settling velocity selection.
5 Future studies are required to elucidate the mechanism of single culture aerobic granulation which might be relevant to phenotype switch.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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