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Conversion of wastewater organics into biodiesel feedstock through the predator-prey interactions between phagotrophic microalgae and bacteria

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We herein report a new route of cultivating phagotrophic microalgae with wastewater for biodiesel feedstock production. A continuous-flow process has been developed, through which organic matter of wastewater is first converted into the biomass of bacteria and then the bacteria are consumed by phagotrophic microalgae for growth and lipid production.

Introduction

Responding to the declining reserve of crude oil, biodiesel has attracted much interest because it is renewable and environmentally friendly[1-3]. Biodiesel now relies on oil crops, tallow, and used oils for feedstock. However, tallow and used oils satisfy a rather small fraction of diesel demand[4] whereas the use of oil crops for biodiesel production causes sustainability concern as it competes with food crops for arable land[5]. Microalgae, requiring little arable land to cultivate, are logistically possible feedstock source for replacing a large fraction of fossil fuel[5]. The effort for microalgae-based biofuel so far has been focused on photosynthetic processes. Unfortunately, the limited light penetration and difficult maintenance of oleaginous strains as dominant population over indigenous algae have hampered the cell concentration achievable and made biodiesel production from photosynthetic microalgae not economically viable currently[6-7].

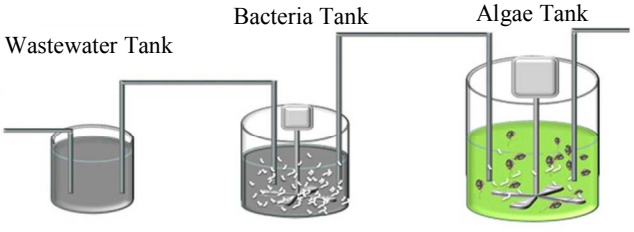

Fig. 1. Schematics of the continuous-flow process to convert wastewater organics into the biomass of phagotrophic microalgae.

Here, we report a new approach using phagotrophic microalgae for biodiesel feedstock production. This group of microalgae is capable of growing by ingesting bacteria[8-10]. By utilizing the bacteria-eating ability of these microalgae, we have designed a continuous-flow process, through which the organic matter of wastewater is converted to the biomass of these microalgae. The process consists of two stages (Figure 1): Bacterial Growth stage and Microalgal Growth stage. In the Bacterial Growth stage, wastewater continuously flows through the Bacteria Tank, where nutrients in wastewater are converted to bacterial biomass. Small bacteria have larger surface areas per unit volume to absorb nutrients and, thus, tend to grow faster than larger organisms. Under suitable operating conditions for the continuous-flow process to reach pseudo-steady state, the small fast-growing bacteria are expected to out-compete other organisms for essential nutrient(s)[11]. By controlling the retention time, all slower growers are washed out of the Bacteria Tank and at least one essential nutrient can be almost completely depleted. In the Microalgal Growth stage, the Bacteria Tank effluent enters the Algae Tank, where the phagotrophic microalgae ingest bacteria for growth and lipid accumulation. The microalgae can grow by obtaining all essential nutrients from the ingested bacteria while other organisms are growth-inhibited for lacking of the essential nutrient(s) depleted in the Bacteria Tank. The selection pressures designed in the process ensure the establishment of phagotrophic microalgae as the dominant population in the produced biomass.

In the studies, the species of phagotrophic microalgae, *Ochromonas danica,* was utilized. *O. danica* is capable of phagotrophic, phototrophic and heterotrophic metabolism[10]. The versatile abilities make it particularly suitable for the process. Firstly, the cultivation conditions for *O. danica* were investigated in batch cultures with dissolved nutrients as food sources. Then, the bacteria-ingesting ability of *O. danica* was researched in batch cultures with a variety of bacteria as food sources. Next, the continuous-flow process was tested with artificial wastewater, to determine the suitable retention times for the Bacteria and Algae Tanks. Lastly, studies with real wastewater were performed to further verify the feasibility of this new process.

Results and Discussion

Cultivation condition for *O. danica*

Table 1 summarizes the suitable pH, dissolved oxygen concentration (DO), and agitation for *O. danica*'s growth in ketchup-based media, with glucose, fructose and sucrose as primary carbon substrates[12]. The light effect on *O. danica*'s growth was also researched[13]. Results (Figure 2) demonstrated that heterotrophic *O. danica* growth is independent of light penetration/availability.

Table 1. Cultivation conditions for *O. danica*[12].

pH	*O. danica* can grow in a wide pH range from 3 to 7.5. Growth becomes much slower at pH lower than 3.5.
DO	*O. danica* can grow with DO ≥ 1% air saturation. However, the cell yield from consumed sugar was markedly lower at 1% DO than at DO ≥ 5%.
Agitation shear	Agitation with 6-blade turbine at impeller tip speed ≥ 0.68 m/s) was confirmed to cause shear damage to *O. danica* cells.

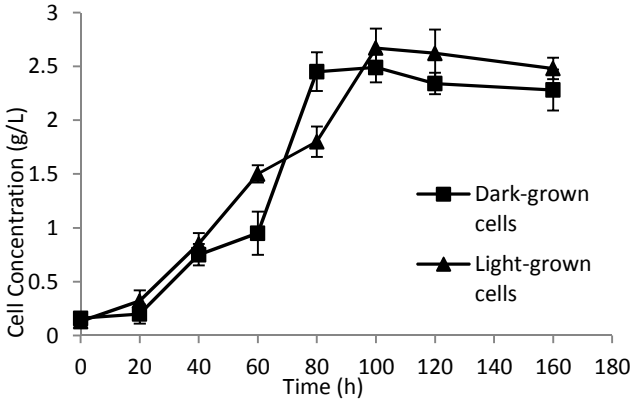

Fig.2 Light effect on heterotrophic growth of *O. danica* in a glucose-based medium[13].

Bacteria-ingesting ability of *O. danica*

The bacteria-ingesting ability of *O. danica* was tested by feeding a mixture of bacteria to *O. danica* in a batch system. The mixture of bacteria were cultured from waste activated sludge, which contains a variety of bacteria growing in wastewater. Figure 3A shows the dry weight concentration changes of *O. danica* and bacteria in the batch system: (96.4 ± 1.2) wt% of the fed bacteria were consumed within 24 h. The remaining bacteria were mainly in the form of aggregates that were too large for *O. danica* to ingest. The weight-based algal yield from consumed bacteria was (43 ± 1)%. The results demonstrate that *O. danica* can consume a variety of bacteria, which is in accord with the findings of previous studies[14-17]. The lipid productivity of *O. danica* was analyzed. The lipid content of *O. danica* grown on bacteria was around 30% of their dry weight. The algal lipid composition was examined by gas chromatography after lipid methylation[18]. The fatty acid methyl ester (major biodiesel components) yield from the algal lipids was (33±3)%. The methylated lipid profile (Figure 3B) shows that the fatty acid moiety of the algal lipids are mainly composed of myristic (14:0), palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), and linolenic (18:3) acids.

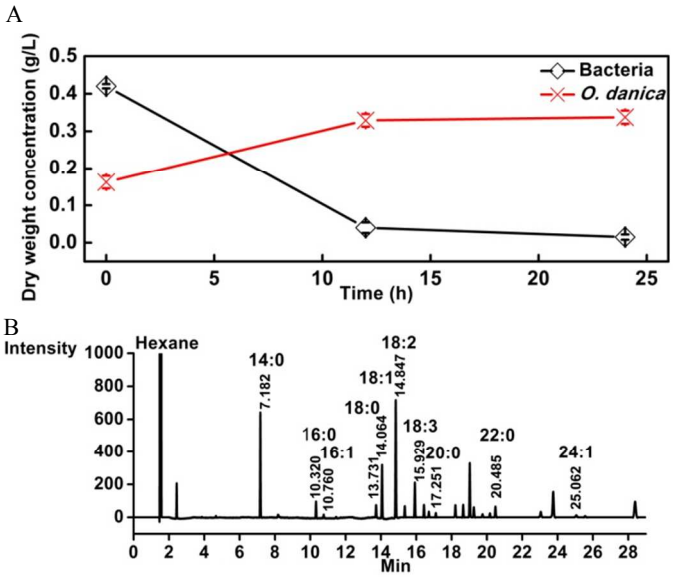

Fig.3 *O. danica*'s phagotrophic growth on wastewater bacteria. **(A)** Changes of algal and bacterial dry weight concentrations with time showing the growth of *O. danica* by consuming the bacteria provided at the beginning of a batch culture experiment. (**B)** The methylated lipid profile of *O. danica* cultured with bacteria.

Continuous-flow process tests with artificial wastewater

For continuous-flow culture systems, the retention time effect on the steady-state microorganism and limiting-substrate concentrations is well known[19], as shown in Figure 4A: at long retention times, the system approaches batch culture; the microorganism concentration is high around the maximum value and the limiting-substrate(s) is essentially consumed out. As the retention time is decreased, the limiting-substrate concentration increases and the microorganism concentration decreases until a critical retention time R_c is reached and the microorganism is totally washed out. Accordingly, a suitable range of Bacteria Tank retention time can be identified and used to wash out all but the fast-growing small bacteria and to almost exhaust the limiting-substrate, so that the effluent cannot support growth of microorganisms other than the bacteria-eating algae in the Algae Tank. For example, with the Algae Tank controlled at a retention time of 50 ± 2 h, the effect of Bacteria Tank retention time (R_b) was investigated. At R_b = 4 ± 0.3 h, bacteria stably dominated in the Bacteria Tank but could not deplete the limiting nutrient, leaving 55 ± 5 mg/L NH3-N in the effluent. (Nitrogen source was the limiting essential nutrient in the artificial wastewater.) In the Algae Tank fed with this effluent, significant yeast and fungal growth was observed (Figure 4B). *O. danica* could not phagocytize these large microorganisms, and the growth of yeast and fungi was not arrested because all necessary nutrients were present. On the other hand, at R_b = 5 ± 0.3 h, the dominant bacteria in the Bacteria Tank consumed all the nitrogen sources; and in the Algae Tank fed with the nitrogen-depleted effluent, *O. danica* populated without obvious amounts of yeast or fungi (Figure 4C).

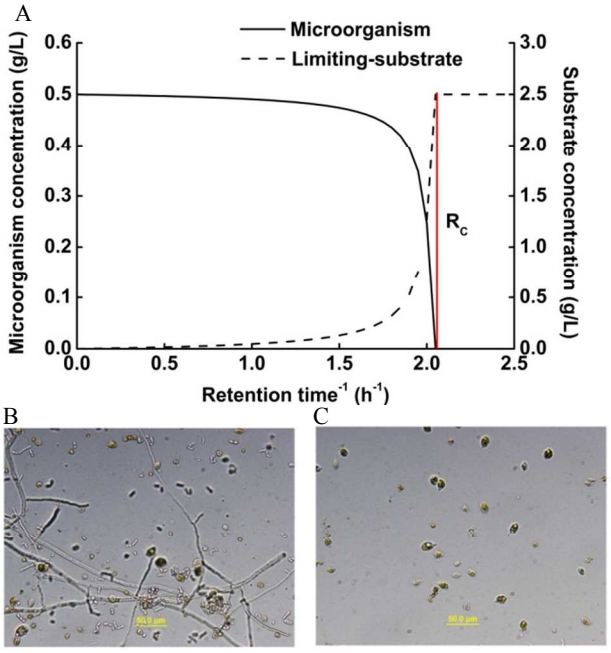

Fig.4 Retention time effects on the Bacteria Tank. **(A),** Theoretical profiles of steady-state cell and limiting-substrate concentrations in continuous culture systems at different retention times. The curved were calculated with the following growth constants: growth rate constant μ_m=2.079, saturation constant K_s=0.05, and the yield constant Y=0.2. **(B), (C),** Microscopic images showing the microbial populations established in the Algae Tank when the Bacteria Tank retention time was controlled at 4 and 5 h, respectively. The shorter Bacteria Tank retention time led to significant growth of yeast and fungi in the Algae Tank.

Next, with R_b controlled at 5 ± 0.3 h, the effect of Algae Tank retention time (R_a) was investigated. Results from six systems, R_a = 20, 30, 40, 50, 60, and 70 (± 2) h, are compared in Figure 5. At the shortest R_a of 20 h, *O. danica* was washed out. At higher R_a, *O. danica* predominated in the Algae Tank and, particularly at $R_a \geq 40$ h, almost all of the fed bacteria were consumed. The algal number concentration continued to increase with increasing Algae Tank retention time. The lipid content and lipid concentration in the Algae Tank at $R_a \geq 40$ h were much higher than those in the Bacteria Tank as shown in Figure 5B. In the Algae Tank at R_a = 30 h, the low lipid content and concentration was because of the lipid loss in the process of algae collection. In this system, significant amounts of extracellular polymers were produced by bacteria. Owing to the presentation of the extracellular polymers, algae in this system can only be collected at high centrifuge rate and the high pressure in this process lead to the broken of algal cells and the loss of lipid inside these cells. At $R_a \geq 40$ h, the lipid content and lipid concentration decreased with increasing Algae Tank retention time and the decline was probably because a higher portion of carbon source was spent on maintenance energy for a larger algal population. The protein originally in the artificial wastewater was completely consumed in the Bacteria Tank. About 88% of the glucose in the artificial wastewater was consumed in the Bacteria Tank, and the remaining 12% was consumed in the Algae Tank at all investigated retention times. The artificial wastewater study demonstrated the feasibility of cultivating phagotrophic microalgae through the continuous-flow process.

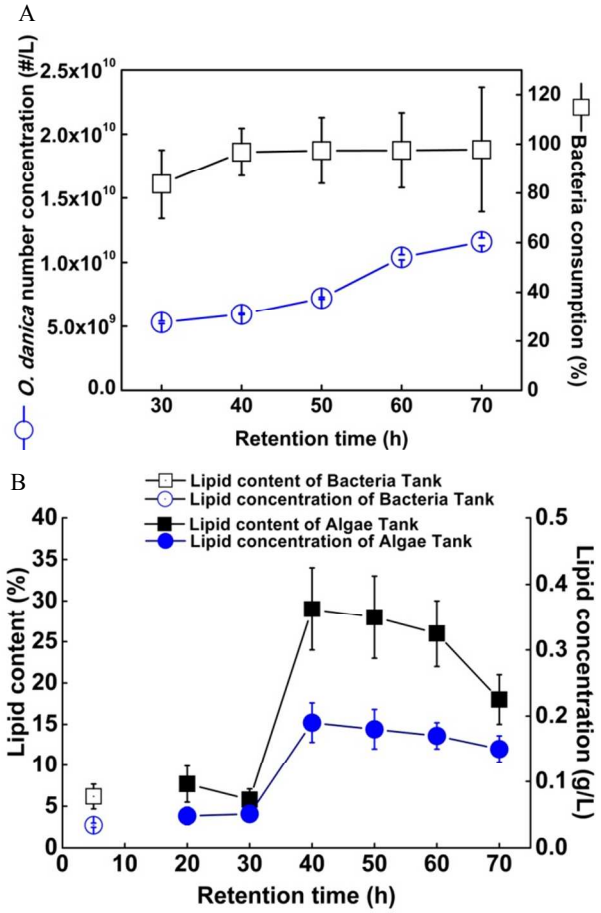

Fig. 5. Retention time effects on the Algae Tank. **(A),** The effects on algal number concentration and % bacteria consumed. **(B),** The effects on intracellular lipid content and overall lipid concentration.

Continuous-flow process tests with real wastewater

Lastly, we performed a study with real wastewater to further verify the feasibility of this new process. The retention times of Bacteria and Algae Tanks were controlled at 5 and 50 h, respectively. Compared to the study with artificial wastewater, the volumes of the Bacteria Tank and Algae Tank were scaled up from 0.15 L and 1.5 L to 1.2 L and 12 L respectively. The process was carefully maintained for 60 days to test the long-term stability. After 11 days pseudo-steady state was successfully reached with almost only small bacteria in the Bacteria Tank and *O. danica* in the Algae Tank. Small amounts of yeast and fungi were already present in the original wastewater. Concentrations of these microorganisms remained low in this process. The built-in selection pressure effectively prevented the interference from biotic components in the wastewater. The lipid yield from organics in the wastewater was (9.2 ± 1.4) % in the Bacteria Tank and it increased to (28 ± 5) % in the Algae Tank.

Conclusions

Here, for the first time, we investigated the possibility of cultivating phagotrophic microalgae for production of biodiesel feedstock. A continuous-flow process has been developed to convert the wastewater organics into the biomass of the bacteria-eating algae.

This process does not use arable land. Unlike photosynthetic microalgae, the phagotrophic microalgae cultivation is not limited by light availability and the process design has built-in selection pressure to establish the phagotrophic microalgae as the dominant population. The new process is particularly suitable to the wastewater having high organic concentrations, such as that from food, oil, aquaculture and animal farming industries. We anticipate the research can attract more effort to investigate the potential of using phagotrophic microalgae for production of biodiesel feedstock and to make biodiesel more sustainable and cost-competitive to petroleum fuel.

Experimental

O. danica was purchased from American Type Culture Collection (ATCC No. 30004). Waste sludge was obtained from Akron Water Pollution Control Station. The artificial wastewater consisted of 2.5 g/L glucose, 0.25 g/L peptone, 0.4 g/L $NaHCO_3$, 0.105 g/L NH_4Cl, 0.13 g/L KH_2PO_4, 0.06 g/L $MgSO_4{\cdot}7H_2O$, 0.1 g/L $CaCl_2{\cdot}2H_2O$, 0.006 g/L $MnCl_2{\cdot}4H_2O$, and 0.48 g/L NaCl. The real wastewater was collected from a grease trap on The University of Akron campus. The standard of fatty acid methyl ester mix was purchased from Sigma-Aldrich (Catalog No. 18918-1AMP).

Bacteria-ingesting ability of *O. danica*

Small volume of waste sludge were inoculated into artificial wastewater to grow a mixture of bacteria in a batch system. The pH of the batch system was maintained in the range from 6.5 to 7.5 by adding 0.2 M HCl or NaOH. The DO was controlled higher than 20%. After 24 hour, the cultured bacteria were collected by centrifuge and washed with deionized water once. Then, the collected bacteria were fed to *O. danica* in a batch system. During the batch culture, the the pH was controlled at 5 by adding 0.05 M HCl or NaOH. The DO was controlled higher than 10%.

Continuous-flow process tests with artificial wastewater

During the studies, the artificial wastewater was kept in refrigerator at 4 ^oC. In the Bacterial Growth stage, 7.5 ml of artificial wastewater was pumped into the Bacteria Tank every 15 minutes and 7.5 ml of bacteria solution were pumped out of the Bacteria Tank every 15 minutes to keep the volume constant. The pH of the Bacteria Tank was maintained at pH 6.5 to 7.5 by adding 0.2 M HCl or NaOH and the dissolved oxygen concentration (DO) was maintained at higher than 20% by stirring and pumping air (0.01 L/min). In the Microalgal Growth stage, 7.5 ml of bacteria solution from the Bacteria Tank was pumped into the Algae Tank and 7.5 ml of solution were pumped out of the Algae Tank to keep the volume constant. The pH of Algae Tank was maintained at pH 5 with 0.05 M HCl or NaOH and the DO was maintained at higher than 10% with mild agitation. Both the Bacteria Tank and Algae Tank were kept at room temperature.

Continuous-flow process tests with real wastewater

The retention times of Bacteria and Algae Tanks were controlled at 5 and 50 h, respectively. The pH of the Bacteria Tank was maintained at pH 6.5 to 7.5 by adding 0.2 M HCl or NaOH and the DO was maintained at higher than 20% by stirring and pumping air (0.01 L/min).The pH of Algae Tank was maintained at pH 5 with 0.05 M HCl or NaOH and the DO was maintained at higher than 10% with mixing. Both the Bacteria Tank and Algae Tank were kept at room temperature.

Notes and references

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