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Respirometric Activities of Heterotrophic and Nitrifying Populations in Aerobic Granules Developed at Different Substrate N/COD Ratios

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Abstract. Aerobic granules were successfully developed at substrate N/COD ratios ranging from 5/100 to 30/100 by weight. By measuring respective respirometric activities of heterotrophic, ammonia-oxidizing, and nitrite-oxidizing bacteria, it was found that the relative abundance of nitrifying bacteria over heterotrophs in aerobic granules was closely related to the substrate N/COD ratios. Results further showed that the populations of both ammonia and nitrite oxidizers were significantly enriched with the increase of the substrate N/COD ratio, while a decreasing trend of heterotrophic population was observed in the aerobic granules. These seem to indicate that high substrate N/COD ratio favors the selection of nitrifying bacteria in the aerobic granules, while the relative activity of nitrifying population against heterotrophic population evolved until a balance between two populations was reached in the aerobic granular sludge community. Moreover, cell elemental composition was correlated with the shift in microbial populations, e.g., the enriched nitrifying population in the aerobic granules resulted in a high cell nitrogen content normalized to cell carbon content. This study provides a good insight into microbial interaction in aerobic granules.

Industrial and municipal wastewaters often contain both organics and nitrogen. To date, many wastewater treatment plants need to be upgraded for nutrient removal owing to more and more stringent environmental regulations. A biofilm process has been commonly employed in plant upgrading for the removal of nitrogen from wastewater [3, 5]. Owing to the sensitivity of nitrifying bacteria to environmental factors as well as their extremely low growth rates, it is still difficult to obtain and maintain a sufficient nitrifying biomass in conventional suspended or fixed culture-based wastewater treatment systems [13]. Recently, intensive research effort was dedicated to aerobic granulation [4, 9, 14]. Aerobic granules are microbial aggregates that result from cell-to-cell immobilization; consequently, high biomass retention can be achieved [14]. It is obvious that to remove organics and ammonium from wastewater, both nitrifying and heterotrophic populations should co-exist in a biological treatment system, while the presence of organic carbon can affect the nitrification efficiency in both suspended and attached cultures [2, 8, 12]. Little is currently known about the development of hybrid aerobic granules for simultaneous organic oxidation and nitrification. Therefore, the main objectives of this study are to investigate the activity distribution of heterotrophic and nitrifying bacteria in aerobic granules developed at different substrate N/COD ratios, and further to show change in microbial elemental composition of aerobic granules with the substrate N/COD ratio. It is expected that these results would be useful for the development of aerobic granule-based bioreactors for simultaneous organic oxidation and nitrification.

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Materials and Methods

Experimental set-up. Four columns (80 cm height and 6 cm in diameter) with a working volume of 2.4 L were used as sequencing batch reactors, and each reactor has the same geometrical configuration. Four reactors (R1 to R4) were operated sequentially with a cycle time of 4 h. Effluent was discharged from the middle port of the reactor. R1 to R4 were inoculated with 650 mL of fresh activated sludge taken from the nitrification unit of a local municipal wastewater treatment plant. Synthetic substrate consists of ethanol as sole carbon source, ammonium chloride, sodium bicarbonate, and other necessary nutrients. The ethanol chemical oxygen demand (COD) concentration was fixed at 500 mg L^{-1}, while the ammonium–nitrogen concentration varied from 25 to 150 mg L^{-1} in R1 to R4, which gave a respective substrate N/COD weight ratio of 5/100 to 30/100. To

Table 1. Elemental formulas of aerobic granules

Sampling on	Substrate N/COD ratio			
	5/100	10/100	20/100	30/100
Day 60	$C_{5.8}H_{12.2}O_{4.0}NS_{0.05}P_{0.04}$	$C_{5.4}H_{10.8}O_{3.7}NS_{0.04}P_{0.04}$	$C_{5.3}H_{10.9}O_{3.4}NS_{0.04}P_{0.04}$	$C_{5.3}H_{10.8}O_{3.3}NS_{0.05}P_{0.04}$
Day 67	$C_{5.6}H_{11.4}O_{3.9}NS_{0.05}P_{0.04}$	$C_{5.5}H_{10.9}O_{3.8}NS_{0.05}P_{0.04}$	$C_{5.1}H_{10.3}O_{3.4}NS_{0.05}P_{0.04}$	$C_{5.1}H_{9.9}O_{3.1}NS_{0.05}P_{0.04}$
Day 86	$C_{5.4}H_{8.3}O_{3.3}NS_{0.05}P_{0.04}$	$C_{5.2}H_{7.7}O_{3.1}NS_{0.04}P_{0.05}$	$C_{5.1}H_{7.3}O_{3.2}NS_{0.04}P_{0.04}$	$C_{4.8}H_{7.1}O_{2.9}NS_{0.04}P_{0.04}$

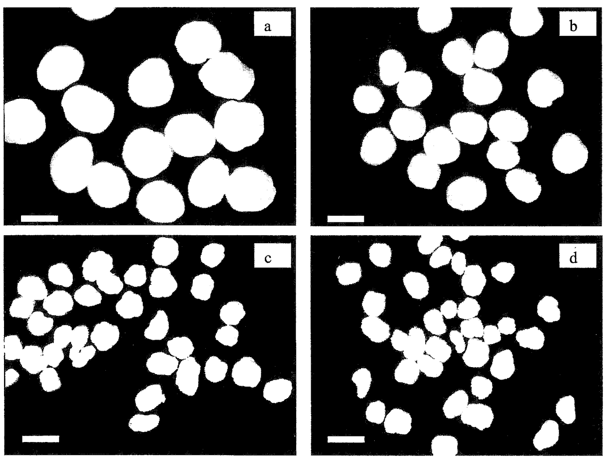

Fig. 1. Images of aerobic granules taken on day 60. (a) substrate N/COD of 5/100; (b) 10/100; (c) 20/100; (d) 30/100. Bar: 1 mm.

satisfy the growth requirement of nitrifying bacteria, the ratio of bicarbonate to ammonium-nitrogen was kept constant at a value of 8.0 mg mg^{-1} for all reactors. The micronutrients in the synthetic wastewater can be found elsewhere [6]. The minimum dissolved oxygen concentrations detected in the reactors were above 2.0 mg L^{-1}, while the reactor pH fell into a range of 8.2 and 7.5. The experiments were conducted in a temperature control room of 25°C.

Analytical methods. Ammonium, nitrite, and nitrate concentrations were measured by using a flow injection analyzer (QuikChem Method 10-107-06-1-I and QuickChem Method 10–107–04–1-F, Lachat Instruments, Inc.), while COD concentration was determined by a standard method [1]. The specific oxygen utilization rate $(SOUR)_H$ by heterotrophic bacteria and specific nitritation and nitratation oxygen utilization rates by ammonium oxidizers and nitrite oxidizers, namely $(SOUR)_{NH4}$ and $(SOUR)_{NO2}$, were determined by the methods developed by [8, 11]. The sum of $(SOUR)_{NH4}$ and $(SOUR)_{NO2}$ was used to describe the overall activity of nitrifying populations. The following are the details of the SOUR tests. A certain amount of granule sample was carefully washed with tap water and was put in a pre-cleaned BOD bottle. Then, the BOD bottle was fully filled with the pre-aerated nutrient and substrate solution, and the oxygen-sensing probe with stirring mechanism was immediately inserted into the BOD bottle. The DO concentration was recorded at intervals of 15 s. The DO concentration versus time gives a straight line, the slope of which equals the oxygen utilization rate (OUR) in terms of milligrams O_2 utilized per liter per minute. Hence, the specific oxygen utilization rate (SOUR) can be calculated as 60×OUR/the concentration of aerobic granules in BOD bottle (g L^{-1}). The respective substrate used for determination of $(SOUR)_H$, $(SOUR)_{NH4}$, and $(SOUR)_{NO2}$ was ethanol as sole carbon, NH_4Cl, and $NaNO_2$, while the biomass, COD, NH_4-N, and NO_2-N concentration was kept constant at 500, 400, 20, and 20 mg L^{-1}, respectively. All SOUR tests were conducted at 25°C.

Elemental composition of granules. In order to analyze the elemental compositions of aerobic granules, granule samples were dried until constant weight at 105°C, and then were pulverized [1]. Of the prepared sample, 1.0 mg was used to determine the cell C, H, N, S, and O contents by using CHNS/O analyzer (CHNS/O 2400 II, PerkinElmer), while another 0.1 g of the above sample was digested with nitric acid [1], and multi-elemental analysis was performed using inductively coupled plasma (ICP) emission spectrometer (PerkinElmer Optima 2000).

Physical characteristics of granules. The size of the granular sludge was obtained by using laser particle size analysis system (Malvern Mastersizer Series 2600) or image analyzer (Quantimnet 500 Image Analyzer, Leica Cambridge Instruments).

Results

R1 to R4 were operated at a constant influent ethanol concentration of 500 mg COD L^{-1}, while the initial ammonium concentration was in the range of 25 to 150 mg N L^{-1}, equivalent to a substrate N/COD weight ratio of 5/100 to 30/100 in R1 to R4, respectively. After 20 days' operation, tiny aerobic granules formed in four reactors. Microscopic observation shows that the aerobic granules developed in four reactors have a similar physical structure with a clear outer round shape (Fig. 1). Compared with the seed sludge with a mean floc size of 0.09 mm, the sizes of aerobic granules gradually stabilized at a mean diameter of 1.9 mm in R1, 1.5 mm in R2, 0.5 mm in R3, and 0.4 mm in R4 on day 60.

In this study, the respective respirometric activity of ammonia oxidizer and nitrite oxidizer was described by the specific nitritation oxygen utilization rate $(SOUR)_{NH4}$, and the specific nitratation oxygen utilization rate $(SOUR)_{NO2}$, while the activity of heterotrophic bacteria was quantified by its specific heterotrophic oxygen utilization rate $(SOUR)_H$. The activity evolution of heterotrophs, ammonia oxidizer, and nitrite oxidizer in the aerobic granules developed at different substrate N/COD ratios are shown in Fig. 2. It can be seen that both $(SOUR)_{NH4}$ and $(SOUR)_{NO2}$ increase with the increase of the substrate N/COD ratio, while the $(SOUR)_H$ tends to decrease with the substrate N/COD ratio. These results imply that high substrate N/COD ratio seems to favor the selection of a nitrifying

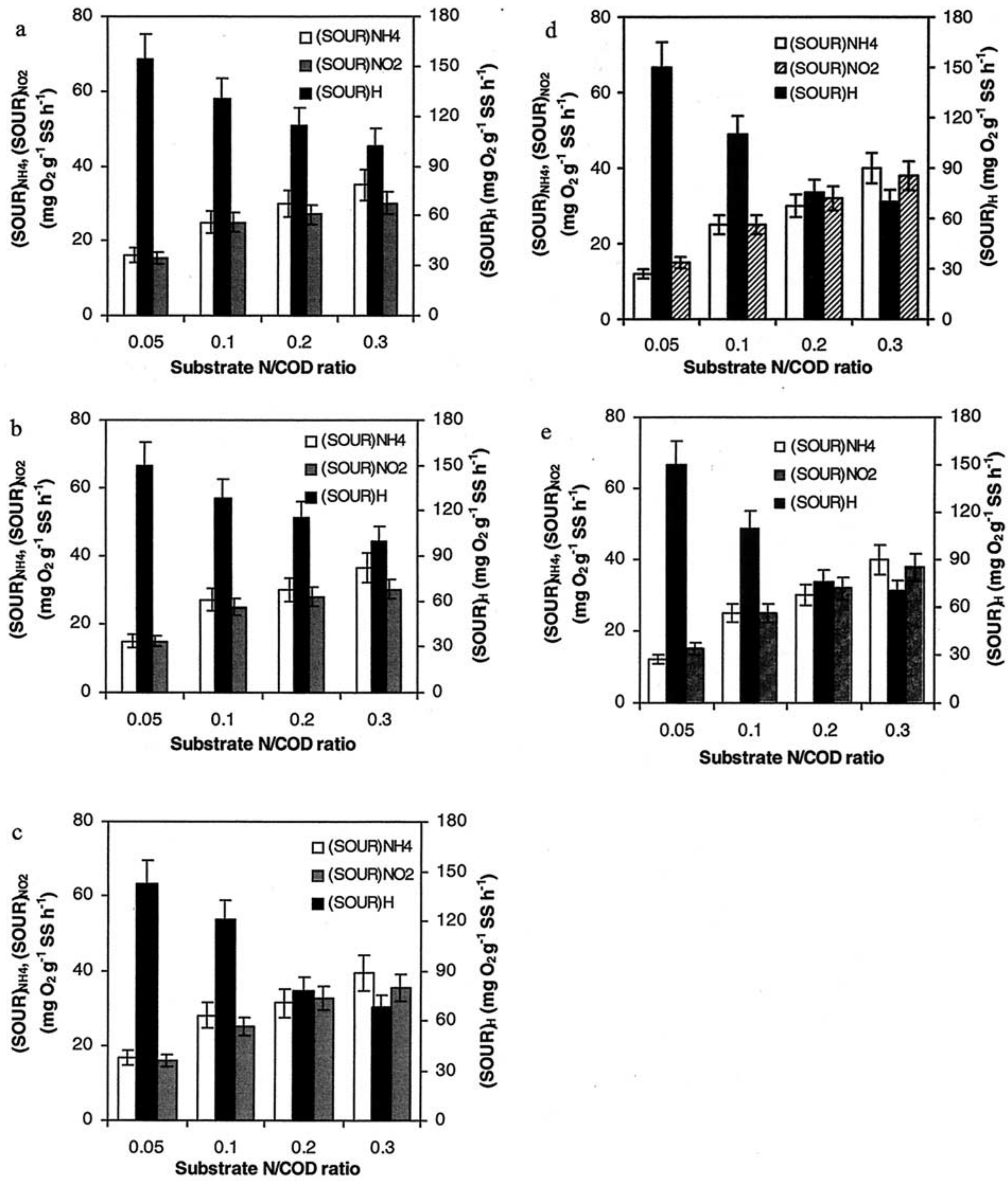

Fig. 2. Respirometric activities of heterotrophs, ammonia oxidizer, and nitrite oxidizer in aerobic granules developed at different substrate N/COD ratios. (a) day 60; (b) day 67, (c) day 86, (d) day 162 and (e) day 334.

population in the aerobic granules. It is reasonable to consider that the sum of $(SOUR)_{NH4}$ and $(SOUR)_{NO2}$, namely $(SOUR)_N$, represents the overall activity of nitrifying bacteria. Figure 3 further shows the evolution of relative abundance of a nitrifying population over a heterotrophic population in aerobic granules. From day 86 onwards, a balance between two populations was achieved.

Table 1 summarizes the elemental formulas of the

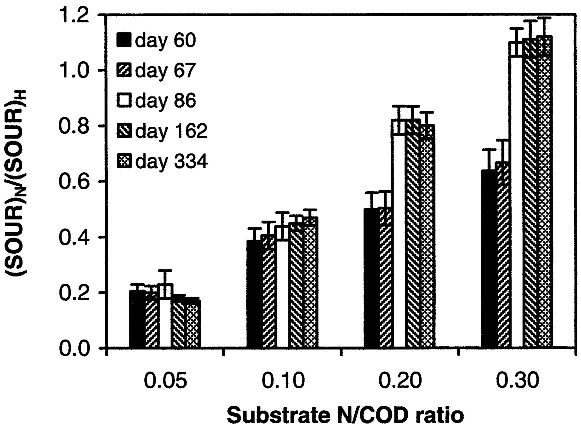

Fig. 3. Evolution of relative activity of nitrifying population over heterotrophic population in aerobic granules.

aerobic granules cultivated at different substrate N/COD ratios and sampled on operation day 60, 67, and 86. The results indicate that aerobic granules mainly comprise six major elements, i.e., C, H, O, N, P, and S. It was found that an increased $(SOUR)_N/(SOUR)_H$ ratio results in an increased cell N/C ratio. It seems that the elemental compositions of aerobic granules are highly correlated with the population distribution in the aerobic granules, which is determined by the substrate N/COD ratio, as shown in Table 1 and Figs. 2 and 3.

Discussion

Figure 1 suggests that aerobic granules can form in a wide range of substrate N/COD ratios from 5/100 to 30/100 after 20 days' operation. This implies that the aerobic granular sludge reactors can be started up in 2–3 weeks, as opposed to upflow anaerobic sludge blanket (UASB) reactor, which requires 2–8 months of careful incubation for the formation of anaerobic granules [7]. Figure 2 shows a profound effect of the substrate N/COD ratio on the activity distribution of heterotrophic, ammonium-oxidizing and nitrite-oxidizing bacteria in the aerobic granules sampled on day 60, day 67, day 86, day 162, and day 334. It can be seen that both ammonium-oxidizing and nitrite-oxidizing activities significantly increased with the increase of the substrate N/COD ratio, whereas the heterotrophic activity in the aerobic granules decreased markedly. It is a reasonable consideration that the fraction of active biomass in a culture would be proportionally related to the respirometric activity [11]. Figure 2 seems to imply that the relative abundance of ammonium oxidizers and nitrite oxidizers in aerobic granules would increase with increasing substrate N/COD ratio, whereas the heterotrophs became less and less dominant, i.e., at a high substrate N/COD ratio, the nitrifying bacteria would be competitive to heterotrophs and become an important component of the aerobic granules. Similarity was also reported in study on biofilm [8, 11, 12]. It can be seen in Fig. 3 that a nitrifying population continued to build up over heterotrophic population in the aerobic granules until a balance between heterotrophic and nitrifying populations was reached on day 86 onwards. It may be expected that an aerobic granule-based compact and efficient bioreactor for simultaneous organic removal and nitrification would be developed in near future.

The results in Table 1 and Fig. 3 indicate that the cell N/C ratio increases with the $(SOUR)_N/(SOUR)_H$ ratio. This can be attributed to possible carbon storage under transient conditions or population shift in aerobic granules developed at different substrate N/COD ratios. However, a study on carbon fluxes in the growth of aerobic granules cultivated at different substrate N/COD ratios clearly showed that the carbon storage in the granules were negligible (data not shown). Thus, it seems certain that alteration of elemental composition of aerobic granules developed at different substrate N/COD ratios implies the change of microbial diversity distribution in the aerobic granules (Fig. 2). In fact, the elemental composition could be used to analyze the species distribution and physiological state of microorganisms [10]. It should be realized that variation in cell elemental composition may show how species interactions develop in ecosystems under different conditions of energy input and nutrient supply. Consequently, a change in microbial composition would result from the competitive growth kinetics of heterotrophic and nitrifying populations under given culture conditions.

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