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Nitrate removal and extracellular polymeric substances of autohydrogenotrophic bacteria under various pH and hydrogen flow rates

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ABSTRACT

In recent years there has been an increasing interest in the use of autohydrogenotrophic bacteria to treat nitrate from wastewater. However, our knowledge about the characteristics of extracellular polymeric substances (EPS) releasing by these activities is not yet very advanced. This study aimed to investigate the change in EPS compositions under various pH values and hydrogen flow rates, taking into consideration nitrogen removal. Results showed that pH 7.5 and a hydrogen flow rate of 90 mL/min were the optimal operating conditions, resulting in 100% nitrogen removal after 6 hr of operation. Soluble and bound polysaccharides decreased, while bound proteins increased with increasing pH. Polysaccharides increased with increasing hydrogen flow rate. No significant change of bound proteins was observed at various hydrogen flow rates.

Introduction

Nitrate released by human activities, such as agriculture and industry enters groundwater through untreated discharge. Water containing high levels of nitrate adversely influences human health and other life forms. Specifically, methemoglobinemia in infants (Islam and Suidan, 1998) and gastric cancer in adults (Sunger and Bose, 2009) are considered to be caused by excessive doses of nitrate in drinking water. Furthermore, a residual amount of nitrate is capable of inducing eutrophication phenomenon which directly affects water quality and landscape of the natural water bodies. The removal of nitrate from water and wastewater is thus necessary to avoid its undesirable effects.

The reduction of nitrate into harmless nitrogen gas (*i.e.*, denitrification) by biological processes has become the most popular technology, compared to others such as coagulation, filtration and disinfection (Ghafari et al., 2008). Heterotrophic bacteria are mostly used in conventional biological systems in which these types of bacteria utilize organic matter to gain electrons for their metabolism. Nonetheless, heterotrophic bacteria do not show advantages when wastewater, mostly from industry, containing low concentration of organic matter is treated. Moreover, when using heterotrophic bacteria, the addition of an appropriate amount of organic carbon increases the operational cost of wastewater treatment plant (WWTP). To date then, a question of great interest in wastewater treatment is the presence of high concentrations of nitrate and low organic carbon in the influent of a WWTP. Autotrophic bacteria can alternatively be used in treatment of these types of wastewater. In some previous studies, autotrophic bacteria have been demonstrated to effectively remove nitrate from

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wastewater by using sulfur-limestone (Zhou et al., 2011) or hydrogen gas (Mousavi et al., 2014; Xia et al., 2010; Visvanathan et al., 2008) as an electron donor, and inorganic carbon such as CO_2 or HCO_3^- as carbon sources. Autohydrogenotrophic bacteria, a type of autotrophic bacteria which utilizes hydrogen gas as an electron donor, have been widely used to treat nitrate from ground water and wastewater in recent years. In mixed cultures, there are many types of bacteria which play different roles in sludge suspension. Ghafari et al. (2009a) showed that the denitrification process was carried out by "nitrate respiring" bacteria for the reduction of nitrate to nitrite and by "true denitrifying" bacteria for the reduction of nitrite to nitrogen gas. However, Chang et al. (1999) proposed that nitrate and nitrite reductase of *Alcaligenes eutrophus* were responsible for the reduction of nitrate to nitrite and nitrite to nitrogen gas. The denitrification reactions are given as below (Kurt et al., 1987):

$$NO_3^- + H_2 \rightarrow NO_2^- + H_2O \quad (1)$$

$$NO_2^- + 1.5H_2 + H^+ \rightarrow 0.5N_2 + 2H_2O \quad (2)$$

The overall reaction is:

$$NO_3^- + 2.5H_2 + H^+ \rightarrow 0.5N_2 + 3H_2O \quad (3)$$

In order to culture autohydrogenotrophic bacteria, various types of reactor has been proposed, such as a microporous membrane bioreactor (Mansell and Schroeder, 2002; Visvanathan et al., 2008), a sequencing batch reactor (Mousavi et al., 2014; Rezania et al., 2005), and a biofilm-electrode reactor (Chen et al., 2014). High nitrate removal rate (up to 100%) has been reported by many researchers (Ghafari et al., 2009b; Mansell and Schroeder, 2002; Vasiliadou et al., 2006). From another perspective, operational parameters, such as pH (Ghafari et al., 2010; Lee and Rittmann, 2003), temperature (Chen et al., 2014; Rezania et al., 2005), hydrogen flow rate (Khanitchaidecha and Kazama, 2012), or inorganic carbon source (Ghafari et al., 2009a) have gained much attention because of their direct effect on the performance of autohydrogenotrophic bacteria. Among them, pH and hydrogen flow rate are few of the important factors which directly affect nitrogen removal and nitrite accumulation. Lee and Rittmann (2003) showed that the optimal range of pH for autohydrogenotrophic bacteria was between 7.7 and 8.6, in which the maximum removal rate of nitrate was observed at pH 8.4. The accumulation of nitrite was dramatically increased at pH higher than 8.6. Rezania et al. (2005) reported that the optimum pH for nitrate and nitrite reduction was dependent on temperature (pH 9.5 at 25 ± 1°C and pH 8.5 at 12 ± 1°C). Another author suggested that appropriate pH values fell in range 6.0–7.0 (Chen et al., 2014). Glass and Silverstein (1998) found that no significant change of nitrate concentration was observed at pH values lower than 7.0 when carrying out experiments with heterotrophic denitrifying bacteria. Hydrogen gas is essential in controlling autohydrogenotrophic bacteria; its low solubility in aqueous solution (1.6 mg/L at 20°C), however, prevents the widespread use of this type of bacteria. Chang et al. (1999) found that nitrate reductase of *Alcaligenes eutrophus* was inhibited under low dissolve hydrogen concentration (<0.1 mg/L), while it was below 0.2 mg/L for nitrite reductase. Thus, an appropriate hydrogen flow rate and pH value must of necessity be considered in the system.

The bacteria in a sludge suspension are present not only in free form, but also as aggregates which are formed by many different types of microbes and materials such as extracellular polymeric substances (EPS). EPS are metabolic products excreted from microbial activities which play an important role in forming microbial aggregates. An EPS matrix has a significant influence on the morphology, structure, physicochemical and biological properties, as well as on the adhesion and cohesion phenomenon of microbial aggregates (Tsuneda et al., 2003). EPS may also protect cells from harsh living conditions, and even supply organic carbon as food for heterotrophic bacteria under starvation conditions (Liu and Fang, 2002; Zhang and Bishop, 2003). Furthermore, EPS is found to be an important factor in the flocculation, settling and dewatering in sludge suspensions (Liu and Fang, 2003). The main components of EPS are proteins and polysaccharides, which contribute up to 89% of total EPS compositions (Tsuneda et al., 2003). Hou et al. (2015) found that the concentration of polysaccharides in loosely bound extracellular polymeric substances increased to reduce the effect of CuO nanoparticles on bacteria. Although much research has been done on the nitrate treatment capability of autohydrogenotrophic bacteria, the effect of pH and hydrogen flow rates on the releasing of EPS from the activities of this type of bacteria has not been investigated to any extent. This study set out to investigate the change in compositions of EPS and to observe nitrate removal, nitrite accumulation and nitrogen removal under various pH values and hydrogen flow rates.

1. Materials and methods

1.1. Experimental setup

Activated sludge containing denitrification bacteria was collected from a sequencing batch reactor in Hsinchu, Taiwan. It was pre-treated to remove large particles and then inoculated in a specially designed 5-L reactor for culturing autohydrogenotrophic bacteria as can be seen from Appendix A Fig. S1. Herein, a continuous flow bioreactor equipped with a clarifier was set up. Synthetic wastewater was prepared with an initial nitrate concentration of 87.14 ± 6.07 mg N/L as sole electron acceptor, sodium bicarbonate as carbon source with C/N 0.5, $Na_2HPO_4{\cdot}12H_2O$ with N/P 5 and 1 mL/L trace solution with the following compositions: $MgSO_4{\cdot}7H_2O$ 10 g; $ZnSO_4{\cdot}7H_2O$ 2.2 g; $CaCl_2{\cdot}2H_2O$ 7.3 g; $MnCl_2{\cdot}4H_2O$ 2.5 g; $FeSO_4{\cdot}H_2O$ 5 g; $CuSO_4{\cdot}5H_2O$ 0.2 g; $CoCl_2{\cdot}6H_2O$ 0.5 g; KI 0.166 g; H_3BO_3 0.124 g; $Na_2MoO_4{\cdot}2H_2O$ 0.08 g, in 1 L deionized water. The reactor was continuously operated by a peristaltic pump (MasterFlex L/S, model 7518-10) with a flow rate of 5 mL/min and was mixed by a magnetic stirrer (Corning PC-310). The speed of the magnetic stirrer was adjusted so as to completely mix the sludge suspension. The concentration of mixed liquor volatile suspended solid (MLVSS) was 2372 ± 703 mg/L. Hydrogen gas was supplied as electron donor through a bubble stone with a flow rate of 50 mL/min. The pH was controlled by automatically adding phosphoric acid to keep at desired values. Throughout the experiment, dissolved oxygen (DO) was kept at ≤0.5 mg/L to ensure anoxic conditions. Hydraulic retention time was

kept at around 17 hr, while the temperature was controlled at 25°C.

1.2. Batch experiments and sample collection

Batch experiments were performed to investigate the effects of pH and hydrogen flow rate on the performance of autohydrogenotrophic bacteria and the composition of EPS from the reactor. Before each test, the peristaltic feed pump was temporarily stopped during the batch experiments. The feed solution was added to the reactor with an initial nitrate concentration of 87.14 ± 6.07 mg N/L. Bacteria were acclimated for at least three days before the experiments were carried out at each pH level. Each batch experiment was operated for 12 hr with two replicates. Samples were collected for each hour of operation. All the samples were then kept at 5°C for the following measurements.

1.3. Analytical methods

The concentrations of nitrate and nitrite were measured by using Ion Chromatography (Dionex, ICS-1000) with guard column. The eluent was prepared with a mixture of Na_2CO_3 (0.15 mmol/L) and $NaHCO_3$ (1.35 mmol/L). Samples were filtered through a 0.45 μm membrane filter (33 mm diameter, nylon, Millipore Millex-HN) before analyzed. Two separate peaks for nitrate and nitrite were found at the residence times of around 6.8 min and 3.8 min. The mixed liquor suspended solid (MLSS) and mixed liquor volatile suspended solid (MLVSS) was determined by drying the MLSS filter (47 mm diameter, Pall Corporation) at 105°C and 550°C. EPS was extracted using the formaldehyde-NaOH extraction method which has been demonstrated to be the most efficient procedure for extracting EPS from sludge suspension to obtain soluble and bound EPS (Liu and Fang, 2002). Soluble EPS was first collected by filtrating the suspension through 0.45 μm membrane filter (33 mm diameter, nylon, Millipore Millex-HN) after centrifuging (U-320R Boeco, Germany) at 4000 r/min and 4°C for 20 min. The solution was then re-suspended by adding up to 10 ml deionized water, and adding 0.06 ml 37% formaldehyde solution was added (at 4°C, 60 min) for cell fixing. Then 4 ml 1N NaOH was added (at 4°C, 180 min) to dissociate acidic groups. Bound EPS was obtained at the end of the EPS extraction procedure after centrifugation at 20,000 *g* (20 min, 4°C), filtration through 0.2 μm membrane filter (33 mm diameter, nylon, Millipore Millex-GN) and purification (at 4°C, 48 hr) by 3400 Da dialysis membrane (Snakeskin pleated dialysis membrane, Thermo). The phenol-sulfuric acid method (DuBois et al., 1956) was used to quantify the concentration of polysaccharides, while the Bradford method (Bradford, 1976) was used to measure the concentration of proteins.

Nitrogen removal performance of the reactor was calculated as follows:

$$\text{Nitrogen removal} = \left(1 - \frac{NO_2^- - N + NO_3^- - N}{NO_3^- - N_{inf}}\right) \times 100\% \quad (4)$$

where, $NO_2^- - N$ and $NO_3^- - N$ (mg/L) are the concentration of nitrite and nitrate at the sampling time and $NO_3^- - N_{inf}$ (mg/L) is the initial concentration of nitrate.

2. Results and discussion

2.1. Effect of pH on nitrate reduction, nitrite accumulation and nitrogen removal

The concentration of nitrate reduction, nitrite accumulation and nitrogen removal under different pH values (6.5, 7.5, 8.5 and 9.5) is shown in Fig. 1. Fig. 1a shows the effect of pH on the decrease of nitrate concentration in the presence of hydrogen gas (50 mL/min). Nitrate concentration decreased over the operational period for all pH values. At pH 6.5, the concentration of nitrate quickly decreased and was not detected after about 7 hr of operation, while it took 11 hr at pH 7.5 and 8.5. Nitrate was still present in reactor after 12 hr of operation at pH 9.5. The nitrate reduction rate at different pH values is given in Table 1. As can be seen, the highest reduction rate (16.56 ± 0.65 mg N/L/hr) occurred at pH 6.5 while the lowest reduction rate (7.97 ± 0.10 mg N/L/hr) occurred at pH 9.5. In other words, the nitrate reduction rate decreased with increasing pH. As mentioned above, "nitrate respiring" bacteria were responsible for the reduction of nitrate to nitrite (Ghafari et al., 2009a). These results of this study confirm that "nitrate respiring" bacteria are able to survive under a wide pH range 6.5–9.5 and are strongly active at low pH of 6.5. Fig. 1b shows the accumulation of nitrite which was converted from nitrate by "true denitrifying" bacteria (Ghafari et al., 2009a). The highest concentration of nitrite (approximately 70% of the total influent nitrogen) was observed at pH 6.5 after 7 hr of operation. At pH 7.5 the concentration of nitrite slightly increased and reached the highest value of around 16% of the total influent nitrogen and then decreased to zero after 12 hr of operation. In other words, all nitrogen in the form of nitrate and nitrite was removed. The nitrite accumulation rate at pH 8.5 (3.20 ± 0.54 mg N/L/hr) was seen higher than that at pH 7.5 (1.40 ± 0.62 mg N/L/hr) but lower than that at pH 6.5 (11.33 ± 0.35 mg N/L/hr) (Table 1). At pH 9.5 the increase of nitrite was insignificant with a very low nitrite accumulation rate of 0.57 ± 0.35 mg N/L/hr. The data yielded by this test showed that pH significantly affects the formation of nitrite when the reactor was operated under different pH values. The high accumulation of nitrite at pH 6.5 and 8.5 may be due to the repression of "true denitrifying" bacteria. In other words, it suggests that the ecological niche of "true denitrifying" bacteria is narrower than that of "nitrate respiring" bacteria. Interestingly, as can be seen in Fig. 1a and b, the concentration of nitrite peaked and went decreased only when the concentration of nitrate approached zero. This phenomenon could be explained as a result of the competition for electrons of "nitrate respiring" bacteria and "true denitrifying" bacteria, or nitrate reductase and nitrite reductase. The "nitrate respiring" bacteria seem to acquire electrons easier than the "true denitrifying" bacteria, and to repress the activities of "true denitrifying" bacteria until the nitrate is used up. These results are in agreement with those obtained by Glass and Silverstein (1998).

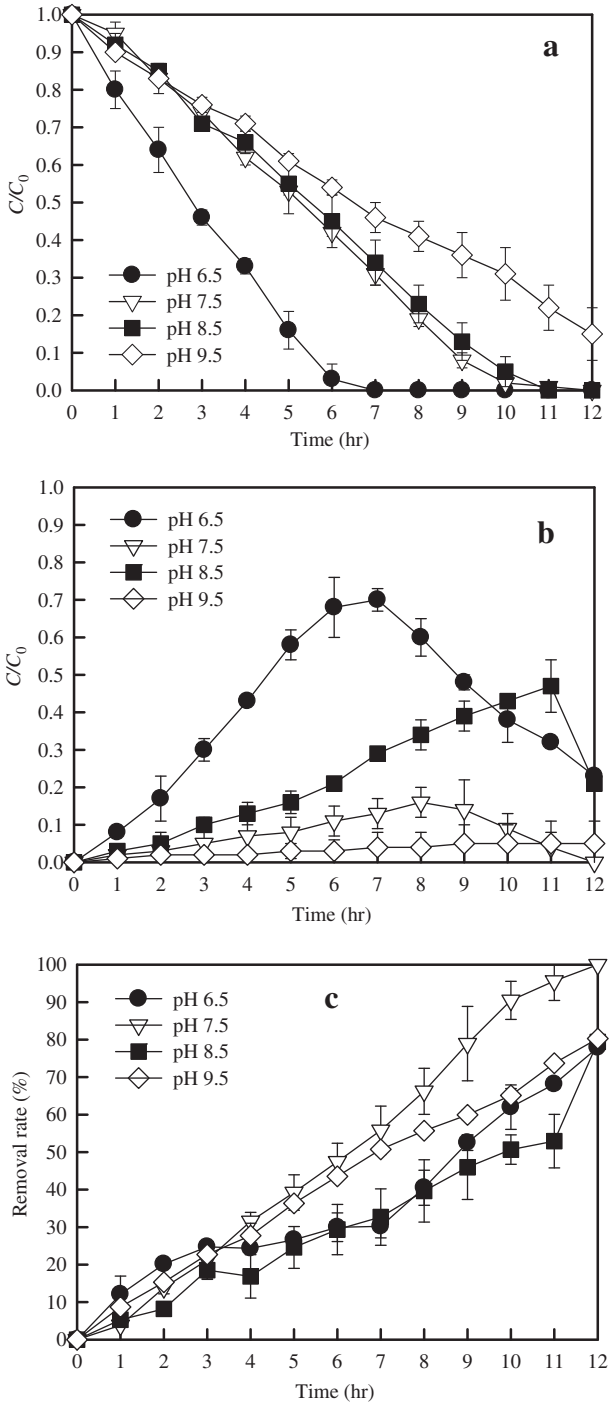

Fig. 1 – Nitrate reduction (a), nitrite accumulation (b) and nitrogen removal (c) under different pH values. C_0 (mg/L): initial concentration of nitrate; C (mg/L): concentration of nitrate or nitrite at a certain operation time.

Because of the presence of nitrite, the term "nitrogen removal" was used to describe the treatment efficiency of the reactor. Nitrogen removal was calculated as in Eq. (4). Fig. 1c shows the nitrogen removal of autohydrogenotrophic bacteria under different pH values. As can be seen, the best nitrogen removal of 100% was observed at pH 7.5, where nitrate and nitrite was totally removed from the reactor after 12 hr of operation, while nitrite was still present at pH 6.5 and 8.5, resulting in lower nitrogen removal which was 77.88% and 79.38%, respectively. Rezania et al. (2005) suggested that the performance of autohydrogenotrophic bacteria was inhibited at pH < 7 because carbon dioxide stripping led to a lack of carbon source. However, the results of this study were somewhat contradictory. If a deficiency of carbon source occurred, the reduction of nitrate might also be affected. Nevertheless, it is a fact that the nitrate reduction rate reached the highest values at pH 6.5. Thus, we conclude that low nitrogen removal at low pH values might not be a result of the carbon source deficiency, but possibly caused by the repression of "true denitrifying" bacteria. At pH 8.5, the lack of protons under a high alkalinity environment inhibited the occurrence of the reaction in Eq. (2) leading to a high nitrite accumulation, compared with pH 7.5. This is in line with other studies (Glass and Silverstein, 1998; Lee and Rittmann, 2003). By contrast, nitrate was not completely reduced to nitrite causing lower nitrogen removal (80.29%) at pH 9.5. It seems clear that a very high pH value slows down the activities of "nitrate respiring" bacteria resulting in the low reduction rate of nitrate during 12 hr of operation.

In summary, pH 7.5 was the optimal condition for the performance of autohydrogenotrophic bacteria. In addition, the pH adaptation of the "nitrate respiring" bacteria was greater than that of the "true denitrifying" bacteria.

2.2. Effect of hydrogen flow rates on nitrate reduction, nitrite accumulation and nitrogen removal

A series of hydrogen flow rates from 0 to 120 mL/min was also tested so as to determine the most effective hydrogen flow rate. Experiments were performed at pH 7.5 which was found to be the most suitable condition (Section 2.1) and temperature = 25°C. Fig. 2 illustrates nitrate reduction, nitrite accumulation and nitrogen removal under different hydrogen flow rates. As can be seen in Fig. 2a, the reduction of nitrate increased with the increase of hydrogen flow rate. Interestingly, the decrease of nitrate concentration was also observed when no hydrogen gas was supplied (hydrogen flow rate = 0 mL/min), when approximately 25% of nitrate was reduced after 12 hr of operation. This can be explained by the presence of heterotrophic denitrifying bacteria in the sludge suspension (Khanitchaidecha and Kazama, 2012). Even though no organic carbon was supplied in the influent, a rich source of organic carbon existed in the form of extracellular polymeric substances in the reactor, and becoming an alternative source of electrons for heterotrophic denitrifying bacteria (Liu and Fang, 2002). Another possible explanation is that hydrogen gas was produced during anaerobic biodegradation of organic substance and was then utilized by autohydrogenotrophic bacteria. As a result, nitrate concentration decreased after period when no hydrogen gas was supplied. These findings seem somewhat different from those of Chang et al. (1999), who recorded the inhibition of nitrate and nitrite reductase of *Alcaligenes eutrophus* under low dissolve hydrogen concentration, as noted in the Introduction section. Different nitrate reduction rates were observed at different hydrogen flow rates. Specifically, the higher the hydrogen flow rate applied, the faster the reduction trend of nitrate

Table 1 – Nitrate reduction rate and nitrite accumulation rate under different pH values.

pH	Nitrate reduction rate (mg N/L/hr)	Nitrite accumulation rate (mg N/L/hr)
6.5	16.56 ± 0.65	11.33 ± 0.35
7.5	8.03 ± 0.55	1.40 ± 0.62
8.5	8.00 ± 1.58	3.20 ± 0.54
9.5	7.97 ± 0.10	0.57 ± 0.35

Hydrogen flow-rate = 50 mL/min.

concentration was observed. Two similar decreasing trends of nitrate concentration were found at hydrogen flow rates of 90 and 120 mL/min, at which the highest reduction rate were also achieved, at 16.15 ± 0.07 and 17.00 ± 1.98 mg N/L/hr, respectively, as shown in Table 2. Obviously, a high hydrogen flow rate induced high concentration of dissolve hydrogen into the sludge suspension, thus leading to a higher nitrate reduction rate. Fig. 2b shows the nitrite accumulation in reactor under different hydrogen flow rate in the range of 0–120 mL/min. The concentration of nitrite quickly accumulated, reached a peak and then sharply decreased at a higher hydrogen flow rate. It is possible that the activities of both "nitrate respiring" and "true denitrifying" bacteria were promoted as high concentration of dissolved hydrogen was introduced. As can be seen, the peaks of nitrite accumulated were different at different hydrogen flow rates, and was found in the 3rd hour for a hydrogen flow rate of 120 mL/min, while it was in 4th, 6th, and 8th hour for a flow rate of 90, 70, and 50 mL/min, respectively. In the case of a hydrogen flow rate of 30 mL/min, no peak of nitrite accumulation was observed after 12 hr of operation. A very small increase in the nitrite accumulation rate at hydrogen flow rate of 0 mL/min was seen, compared to other hydrogen flow rates. Further, the nitrite accumulation rate increased from 0.33 ± 0.31 to 5.79 ± 1.34 mg N/L/hr with the increase of hydrogen flow rate from 0 to 120 mL/min (Table 2). These results indicate that high hydrogen flow rates lead to high nitrite accumulation rates which then reduced quickly after reaching peak values. As in Section 2.1, the nitrogen removal was calculated as illustrated in Fig. 2c. Nitrogen removal at hydrogen flow rate of 0 and 30 mL/min were 24.84% ± 6.4% and 33.4% ± 4.21%, respectively, after 12 hr of operation, while it was 100% for other flow rates. Although the nitrogen removal at hydrogen flow rates of 0 and 30 mL/min looked similar, the pathway was totally different. Specifically, the nitrate reduction at hydrogen flow rate of 0 mL/min was found two times lower than that at 30 mL/min (Fig. 2a). In another perspective, nitrite concentration at hydrogen flow rate of 30 mL/min (Fig. 2b) was observed to be higher than that at 0 mL/min (seems to not appear until the end of the test), resulting in similar trends of nitrogen removal in both situations. The reason for this phenomenon is that dissolved hydrogen concentration may not be high enough for autohydrogenotrophic bacteria, resulting in lower nitrogen removal at lower hydrogen flow rate. Mousavi et al. (2014) also found that an increase in H_2 sparging time did improve the denitrification rate. The complete removal of nitrate and nitrite was achieved at shorter operational time for higher hydrogen flow rate. In particular, no nitrate and nitrite was found after 6, 7 and 12 hr of operation for hydrogen flow rate of 120 and 90, 70, and 50 mL/min, respectively. Moreover, hydrogen flow rates higher than 90 mL/min showed similar result to that at 120 mL/min.

In summary, the hydrogen flow rate was found to significantly affect nitrogen removal of autohydrogenotrophic bacteria with an optimal hydrogen flow rate of 90 mL/min in this study. Further, a hydrogen flow rate of <50 mL/min resulted in a high accumulation of nitrite after 12 hr of operation.

2.3. Changes of EPS at different pH values and hydrogen flow rates

As noted above, EPS plays an important role in the aggregation of bacteria in a reactor, specifically autohydrogenotrophic bacteria in this study. In particular, the interaction between EPS and bacteria can gradually form microbial flocs or even granular sludge (Sheng et al., 2010). In this section, the change of EPS, mainly constituted of proteins and polysaccharides, was investigated under different pH values and hydrogen flow rates. Soluble and bound fractions of EPS were extracted to separately study the role of each to the behavior of autohydrogenotrophic bacteria when pH and hydrogen flow rates were changed.

2.3.1. Changes of EPS at different pH values

Since proteins and polysaccharides were considered as the main components of EPS, they were widely used to represent EPS in the wastewater treatment system. Fig. 3 describes the change of EPS in the form of soluble and bound fractions under different pH values. Fig. 3a shows the change of the concentration of soluble polysaccharides under the pH range of 6.5–9.5. As can be seen, the concentration of soluble polysaccharides significantly decreased with the increase of pH, with the concentration of soluble polysaccharides (97.87 ± 15.11 mg/L as glucose) at pH 6.5 three times higher than that at pH 9.5 (33.15 ± 14.32 mg/L as glucose). On the other hand, it was 78.06 ± 7.34 and 58.24 ± 4.52 mg/L as glucose at pH 7.5 and 8.5, respectively. Our results show that autohydrogenotrophic bacteria excrete more soluble polysaccharides into the sludge suspension under low pH conditions. Fig. 3b shows the concentration of bound polysaccharides and bound proteins and the ratio between bound proteins and bound polysaccharides which are given as bEPSp/bEPSc. The concentration of bound polysaccharides was similar at pH 6.5 (32.88 ± 4.68 mg/g MLVSS as glucose) and pH 7.5 (32.58 ± 3.75 mg/g MLVSS as glucose), while it decreased at higher pH of 8.5 and 9.5. The concentration of bound polysaccharides at pH 8.5 and 9.5 was 22.23 ± 9.74 and 17.99 ± 9.87 mg/g MLVSS as glucose, respectively. However, the concentration of bound proteins was opposite, increasing with an increase of pH: at pH 6.5, bound proteins reached a minimum concentration of 3.8 ± 1.03 mg/gMLVSS as Bovine Serum Albumin (BSA), compared to other pH values. Higher pH values resulted in higher concentration of bound proteins, and more than twice the concentration at pH 7.5 and 8.5 as that at pH 6.5 was observed, being at 2.38 and 2.73 times for pH 7.5 and 8.5 respectively. A slight decrease in bound proteins (9.01 ± 4.73 mg/g MLVSS as BSA) was seen at pH 9.5, compared to pH 8.5 (10.38 ± 1.1 mg/g MLVSS as BSA). This increase in bound proteins could be attributed to the high amount of exoenzymes in suspension (Yin et al., 2015). The ratio between bound proteins and bound polysaccharides rose from 0.12 to 0.5 with the increase of pH

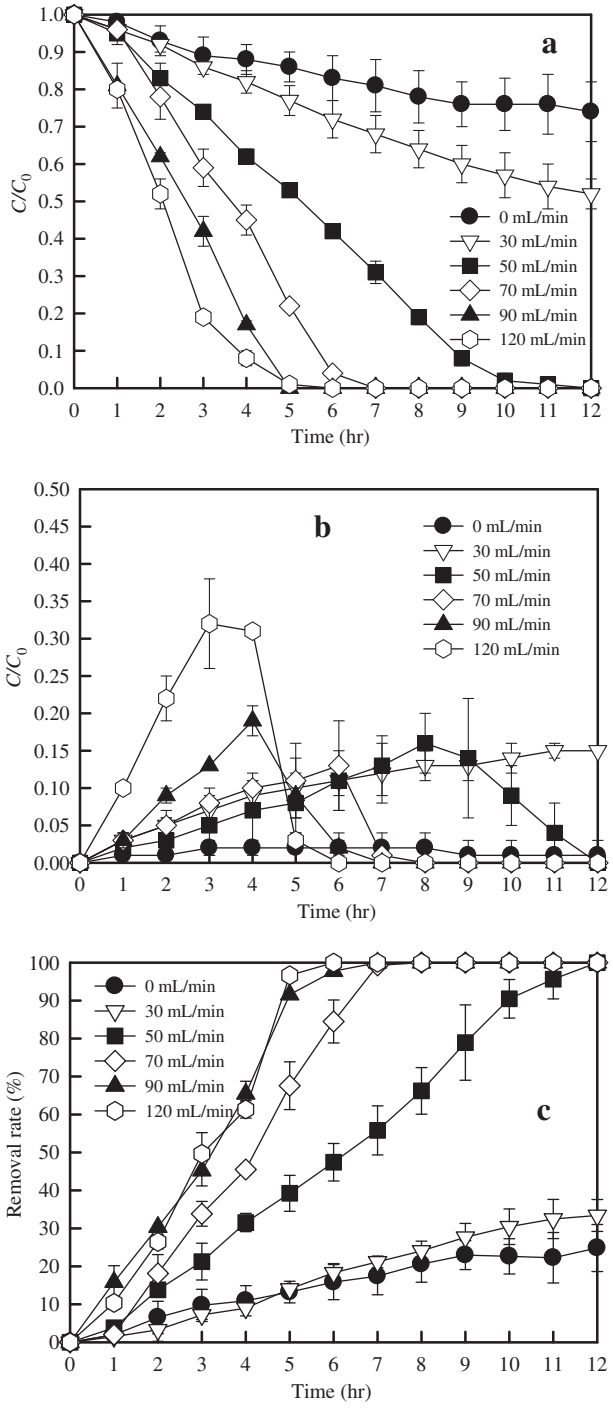

Fig. 2 – Nitrate reduction (a), nitrite accumulation (b) and nitrogen removal (c) under different hydrogen flow rates.

from 6.5 to 9.5, *i.e.*, the concentration of bound polysaccharides was approximately 2–8 times higher than that of bound proteins at different pH values. In other words, bound polysaccharides are the prominent substances in autohydrogenotrophic bacteria system. The results appear to be contradictory to previous studies (Liu and Fang, 2003; Lü et al., 2015). Lü et al. (2015) found that the amount of proteins was much higher than that of polysaccharides in anaerobic digestion of food waste, while Liu and Fang (2003) concluded that polysaccharides were dominant in pure cultures and proteins were dominant in mixed cultures.

In summary, the concentration of soluble and bound polysaccharides decreased with the increase of the pH values in the range of 6.5–9.5, while the concentration of bound proteins was reversed. Soluble proteins were undetectable at <1 mg/L in this study. Polysaccharides were dominant in the mixed culture of autohydrogenotrophic bacteria, compared with proteins.

2.3.2. Changes of EPS under different hydrogen flow rates

In order to understand the characteristics of autohydrogenotrophic bacteria under different hydrogen flow rates, EPS compositions were also investigated in this study. The concentration of soluble polysaccharides, bound proteins and polysaccharides are shown in Fig. 4. As the data shows, the concentration of soluble polysaccharides increased from 30.96 ± 9.8 mg/L to 76.83 ± 19.36 mg/L as glucose when the hydrogen flow rate was increased from 0 to 30 mL/min (Fig. 4a). As mentioned above, heterotrophic bacteria have a tendency to utilize organic carbon where there is no other electron donor (such as hydrogen gas). In other words, soluble polysaccharides might be used when no hydrogen gas is supplied, resulting in a lower concentration of soluble polysaccharides. This result seems to be consistent with that of Zhang and Bishop (2003), who suggested that EPS can be used as substrate in starvation conditions. The concentration of soluble polysaccharides remained stable at a hydrogen flow rate of 50 mL/min (78.06 ± 7.34 mg/L as glucose), and decreased slightly at flow rates of 70, 90 and 120 mL/min (65.16 ± 22.21, 51.18 ± 12.06 and 55.63 ± 18.77 mg/L as glucose, respectively). Clearly, when hydrogen gas was supplied (30, 50, 70, 90, and 120 mL/min), soluble polysaccharides were not utilized, leading to the higher concentration of soluble polysaccharides observed. Similar results were observed for the change in bound polysaccharides under different hydrogen flow rates. In particular, the lowest concentration of bound polysaccharides (19.86 ± 4.53 mg/g MLVSS as glucose) was found when the hydrogen flow rate was zero (Fig. 4b). The concentration then increased with the increase of hydrogen flow rate, and at 30, 50, and 70 mL/min, the concentration of bound polysaccharides was approximately 1.7 times higher than that at 0 mL/min. By contrast, the change of bound proteins seems insignificant with and without hydrogen flow. These results suggest that bound polysaccharides could also be used under starvation conditions, whereas bound proteins gave the opposite results. On the other hand, polysaccharides appeared the priority choice for bacteria under starvation conditions. Zhang and Bishop (2003) also found that bacteria used polysaccharides more readily than proteins. The ratio between bound proteins and bound polysaccharides was around 0.3 at all hydrogen flow rates. Results show that the effect of hydrogen flow rate on bEPSp/bEPSc ration was insignificant and that bound polysaccharides were dominant in EPS. Bound proteins were not affected by hydrogen flow rates.

In summary, soluble and bound polysaccharides seem to be used as electron donor source by heterotrophic bacteria when no hydrogen was available. Bound proteins concentration was independent of hydrogen flow rates. Polysaccharides were more dominant than proteins under all hydrogen flow rate conditions in the autohydrogenotrophic system.

Table 2 – Nitrate reduction rate and nitrite accumulation rate under different hydrogen flow rates.

Flow rate (mL/min)	Nitrate reduction rate (mg N/L/hr)	Nitrite accumulation rate (mg N/L/hr)
0	2.76 ± 0.93	0.33 ± 0.31
30	3.94 ± 0.25	1.58 ± 0.37
50	8.03 ± 0.55	1.40 ± 0.62
70	13.42 ± 0.54	1.93 ± 0.69
90	16.15 ± 0.07	2.92 ± 0.34
120	17.00 ± 1.98	5.79 ± 1.34

The reactor was operated at pH 7.5.

3. Conclusions

This study presents the effects of pH and hydrogen flow rates on the nitrate removal and EPS of autohydrogenotrophic bacteria in the treatment of nitrate-containing wastewater. The results show that pH 7.5 and 90 mL/min of hydrogen flow rate are the optimal operating conditions for autohydrogenotrophic bacteria in wastewater treatment system, in which the nitrate is completely removed after 6 hr of operation without intermediates. Change in EPS concentration is influenced by pH value and hydrogen flow rate. Polysaccharides, compared to proteins, are the dominant substances. Polysaccharides might be used as electron donor in the absence of hydrogen gas. Soluble proteins are not detected, while bound proteins increase with increasing pH and are independent of changes in hydrogen flow rate.

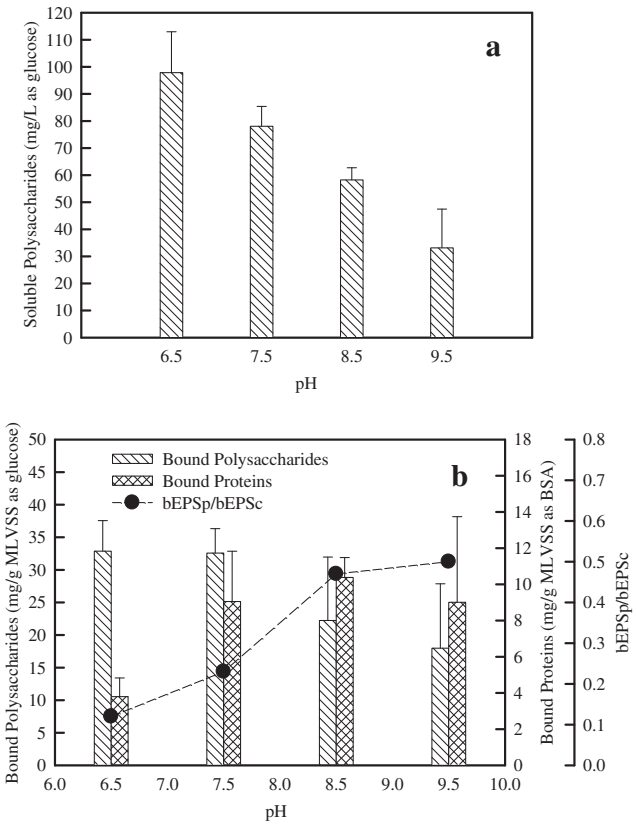

Fig. 3 – Soluble (a) and bound (b) fractions of extracellular polymeric substances (EPS) under different pH values.

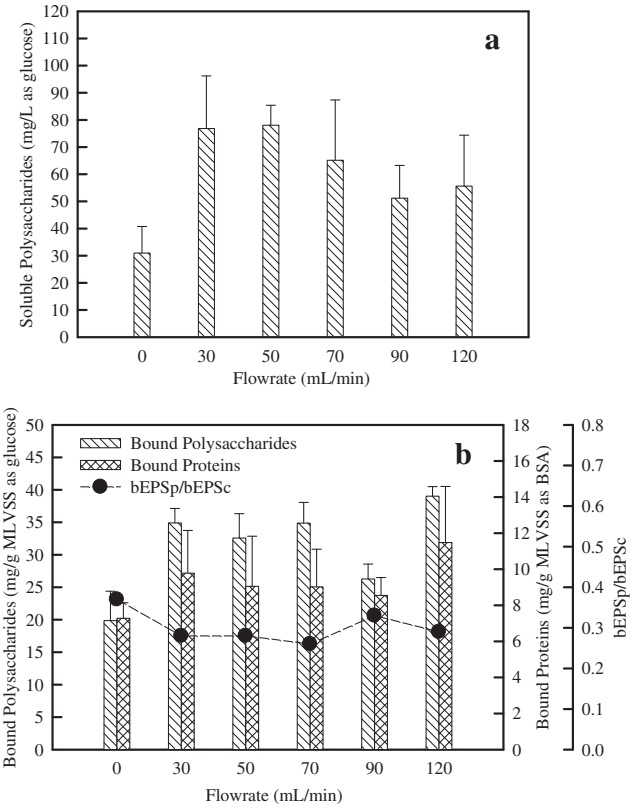

Fig. 4 – Soluble (a) and bound (b) fractions of EPS under different hydrogen flow rates.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jes.2017.01.005.

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