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Bioaugmentation treatment of nitrogen-rich wastewater with a denitrifier with biofilm-formation and nitrogen-removal capacities in a sequencing batch biofilm reactor

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Abstract

A strain with efficient biofilm-formation and aerobic denitrification capabilities was isolated and identified as *Pseudomonas mendocina* IHB602. In pure culture, strain IHB602 removed almost all NO$_3^-$-N, NO$_2^-$-N, and NH$_4^+$-N (initial concentrations 50 mg/L) within 24 h. The strain produced large amounts of extracellular polymeric substances (maximum 430.33 mg/g cell dry weight) rich in protein but containing almost no humic acid. This, and strong autoaggregation (maximum 47.09%) and hydrophobicity (maximum 85.07%), imparted strain IHB602 with biofilm forming traits. A sequencing batch biofilm reactor bioaugmented with strain IHB602 (SBBR1) had more rapid biofilm-formation than the control without strain IHB602 inoculation (SBBR2). During the stabilization period, the effluent removal ratios for NH$_4^+$-N (95%), NO$_3^-$-N (91%) and TN (88%) in SBBR1 were significantly higher than those in SBBR2 (NH$_4^+$-N: 91%, NO$_3^-$-N: 88%, TN: 82%). Microbial community structure analysis revealed that strain IHB602 successfully proliferated and contributed to nitrogen removal as well as biofilm formation.

Key words: Aerobic denitrification; Biofilm formation; Inorganic nitrogen removal; Bioaugmentation

1. Introduction

Ammonia (NH$_4^+$), nitrate (NO$_3^-$) and nitrite (NO$_2^-$) are the most common forms of inorganic ions in water ecosystems. Bacterial activity, characteristics of soil organic matter and mineral composition, intensive agricultural activity with fertiliser application and flooding of fields, increased urbanisation rate, irrigation and drainage all affect the form and emissions of inorganic nitrogen (Soldatova et al., 2017). The concentration of inorganic nitrogen in groundwater and surface water worldwide is increasing and has a significant impact on the water ecosystem.
Inorganic nitrogen pollution of aquatic ecosystems results in many environmental problems, including water acidification, eutrophication, toxic algal blooms, and direct toxicity to aquatic animals. Moreover, such pollution can adversely affect human health and the economy (Camargo and Alvaro, 2006). Therefore, it is urgently necessary to take effective measures to eliminate inorganic nitrogen pollution. Biological nitrogen removal technologies, especially applications of microorganisms, are key procedures for removing nitrogen from wastewater (Ma et al., 2016). Among efficient nitrogen removal methods, the aerobic denitrification pathway has become an attractive approach for wastewater treatment plants because it requires only a single reactor for nitrification/denitrification, uses fewer chemicals to adjust the system pH, and aerobic denitrifiers can be easily controlled during the treatment operation (Huang and Tseng, 2001).

A large number of aerobic denitrification bacteria have been reported, including *Pannonibacter phragmitetus* B1, *Acinetobacter sp.* YY1, *Sphingomonas sp.* YY2, *Methylobacterium gregans* DC-1 and *Pseudomonas sp.* YY3 (Bai et al., 2019; Lang et al., 2019; Hong et al., 2019). These denitrifiers have potential applications in nitrogen removal in a single reactor in aerobic conditions. However, investigations of these denitrifiers have mainly been performed in shake flasks using pure strains.

Biofilms, in which bacteria are immobilized, are a convenient way to keep functional bacteria in water treatment systems. Biofilm reactors have the advantages of strong adaptability, high organic matter and nitrogen removal rate, convenient operation and management, and low excess sludge production, resulting in an increasing demand for application in the upgrading of wastewater treatment plants all over the world (Escudie et al. 2011). Bacteria with strong biofilm formation abilities coaggregate with other microorganisms to develop biofilms (Rickard et al., 2004). Inoculating specific contaminant-degrading and biofilm-forming bacteria into biofilm reactors is a
highly efficient bioaugmentation method for pollutant removal in wastewater treatment (Li et al., 2013). However, there are some practical problems for treating wastewater. For example, the biofilm-forming bacteria may not effectively achieve pollutant removal, and the contaminant-degrading bacteria may not be effectively immobilized in biofilms, and may thus be easily washed out of the system. Moreover, simply mixing biofilm-forming bacteria with degrading bacteria may cause mutual growth inhibition, slow biofilm development, complex operation, and other issues (Li et al., 2008; Li et al., 2016). The presence of a single bacterial species with the ability to efficiently form biofilms and degrade pollutants simultaneously can achieve both fixation of functional bacteria in the wastewater treatment system and high-efficiency pollutant removal. Therefore, identifying such bacteria and testing their practical application in wastewater treatment is required.

A sequencing batch biofilm reactor (SBBR) is a sequencing batch reactor coupled with biofilms grown on packing media. SBBRs are effective in protecting growing microorganisms from being rinsed off and in reducing the concentration of toxic substances in wastewater (Wen et al., 2016). Compared with traditional activated sludge processes, SBBRs have stronger resistance to adverse effects and more stable ecosystems (Xiao et al., 2015). SBBR systems have been successfully operated to treat high concentrations of nitrogen and the good nitrogen removal performance benefited from protection of active bacteria by the biofilm (Mao et al., 2017).

In this study, an aerobic denitrifying bacterium with dual capacity for nitrogen removal and biofilm formation was isolated from the surface sediment of a high-altitude eutrophic lake. The biofilm-forming characteristics of the strain were analyzed, and treatment of nitrogen-rich wastewater in an SBBR augmented with this strain was evaluated. This work provided a promising
prospect for the bioaugmentation treatment to nitrogen-rich wastewater treatment with this type of
denitrifier in SBBR.

2. Materials and methods

2.1 Isolation and identification of aerobic denitrifiers

Strain IHB602 was isolated from the surface sediments of Dianchi Lake (Kunming, China). One
hundred grams of fresh sediment and 150 mL of denitrification broth medium (DBM) were placed
in 250 mL conical flasks and enriched in an incubator at 30 °C and 120 rpm for 7 days. DBM
comprised 16.67 g/L C₄H₄Na₂O₄·6H₂O, 10.55 g/L Na₂HPO₄·12H₂O, 5.0 g/L KNO₃, 1.5 g/L
KH₂PO₄, 0.3 g/L NH₄Cl, 0.3 g/L MgSO₄, and 2 mL/L of trace element solution and had a final pH
of 7.2. The trace element solution included (per liter): 1.8 g FeCl₂·4H₂O, 0.70 g MnCl₂·4H₂O, 0.5
g ZnCl₂, 0.5 g H₃BO₃, 0.25 g CoCl₂·6H₂O, 0.03 g Na₂MoO₄·2H₂O, 0.01 g NiCl₂·6H₂O, 0.01 g
Na₂SeO₃·5H₂O and 0.01 g CuCl₂·2H₂O. After incubation, the supernatant was decanted and diluted
10-fold. Two milliliters of supernatant were spread on BTB-agar plates with nitrate as the sole
nitrogen source and bromothymol blue as a chromogenic indicator of denitrification. After
incubation at 30 °C for 48 h, single bacterial colonies surrounded by blue circles were selected, and
purification was repeated at 30 °C and 120 rpm with 150 mL denitrification test medium (DTM),
which consisted of 50 mg/L NO₃⁻-N as the sole N source. A pure isolate with high nitrogen removal
efficiency was obtained and named strain IHB602. All media and containers were sterilized at
121 °C for 30 min. Cells were observed by scanning electron microscopy (SEM, S-3400 N, Hitachi,
Japan), where they were fixed in aqueous 2.5% glutaraldehyde for 12 h according to the method of
Sousa et al. (2015). Biochemical characterization of strain IHB602 was performed according to the
taxonomic outline of *Bergey’s Manual of Systematic Bacteriology* (second edition). 16S rRNA
genes were amplified by PCR using bacterial universal primers 8F (AGAGTTTGATCCTGGCTCAG)/1492R (GGTTACCTTGTTACGACTT). Genes were amplified at 94 °C for 7 min, 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min followed by extension at 72 °C for 4 min. 16S rRNA gene sequences were compared with available sequences in GenBank databases using BLAST on NCBI (http://www.ncbi.nlm.nih.gov). *napA* genes were amplified by primers NAP1 (TCTGGACCATGGGCTTCAG CCA) and NAP2 (ACGACGACCGCCAGCCAGCAG) following these procedures: 95 °C for 7 min, 37 cycles at 95 °C for 30 s, 59 °C for 30 s, 72 °C for 1 min and 72 °C for 4 min. A neighbor-joining phylogenetic tree based on 16S rRNA gene sequences was constructed with MEGA 6 software.

2.2 Nitrogen removal and biofilm formation properties of strain IHB602 in pure culture

2.2.1 Nitrogen removal test

For testing inorganic nitrogen removal, 50 mg/L nitrate-N, nitrite-N or ammonium-N was used as the sole N source. Other ingredients were the same: 16.67 g/L C₄H₄Na₂O₄·6H₂O, 1.5 g/L KH₂PO₄, 5.27 g/L Na₂HPO₄·12H₂O, 0.3 g/L MgSO₄ and 2 ml/L trace element solution. Four ml pre-cultured bacterial suspension was added to 500-ml flasks containing 200 ml N test medium (2% inoculum) and incubated at 30 °C and 120 rpm. Bacterial growth was analyzed by optical density at 600 nm (OD 600) in N removal medium. The concentrations of nitrate-N, nitrite-N and ammonium-N were measured periodically for all media. All experiments were performed in triplicate. Inoculation was done in a sterile environment and all used containers and media were sterilized at 121 °C for 30 min.

2.2.2 Extracellular polymeric substances (EPS) extraction and determination

Four milliliters bacterial suspension were inoculated in 200 ml nitrate test medium (120 rpm and
30 °C). Samples were taken periodically to extract EPS using the cation exchange resin (CER) method. Thirty milliliters of whole culture was centrifuged (8000 rpm, 15 min, 4 °C). The cell pellets were washed twice with phosphate-buffered saline (PBS; pH ~7.0) and resuspended to a volume of 30 ml with the addition of CER (70 g g$^{-1}$ dry cells). Centrifuge tubes were shaken at 250 rpm for 2 h at 4 °C and then kept static for 5 min to settle the CER. Then, the suspensions were centrifuged (10,000× g, 15 min, 4°C) and filtered using a 0.45-μm acetate filter membrane. Finally, the EPS was obtained and stored at −20 °C until analysis. The combined amount of protein and polysaccharide represented the total amount of EPS. The protein and polysaccharide contents were measured by the Lowry method and anthrone colorimetry, respectively. EPS, filtered with a 0.45 μm acetate membrane filter, was used to discriminate 3D excitation-emission matrix (EEM) fluorescence spectra using an Agilent Cary Eclipse fluorescence spectrophotometer (California, USA).

### 2.2.3 Auto-aggregation ability

A high auto-aggregation index indicates a strong tendency for cells for cells to agglomerate. Harvested cells were washed twice with PBS (pH 7.0), and then resuspended in PBS to an OD$_{600}$ of approximately to 0.6 ($A_0$). Thereafter, the suspension was stood for 5 h to allow aggregation, and the OD$_{600}$ was measured again ($A_t$). The auto-aggregation index was calculated by Eq. (1) to evaluate the auto-aggregation capacity of strain IHB602.

$$\text{Auto-aggregation index %} = \left(1 - \frac{A_t}{A_0}\right) \times 100 \quad (1)$$

### 2.2.4 Hydrophobicity

Hydrophobicity of cells was determined by measuring bacterial adhesion. Harvested cells were washed twice with PBS (PH 7.0) by centrifugation (5000× g for 10 min) and resuspended to an
OD\textsubscript{546} of 0.3. The cell suspensions (4 mL) alone or with 1 ml of hexadecane added and transferred to 10-mL sterile centrifuge tubes. The tubes were vortexed for 2 min, and the two phases were allowed to separate for 15 min. The aqueous PBS-cell phase was carefully removed, and its OD\textsubscript{546} was determined. The cell surface hydrophobicity was calculated with Eq. (2).

\[
\text{Hydrophobicity \%} = (1 - \frac{\text{OD}_{546}}{0.3}) \times 100 \tag{2}
\]

### 2.3 SBBR

To further investigate the biofilm formation and bioaugmentation of strain IHB602 in an SBBR, two parallel laboratory-scale SBBR systems (SBBR1 augmented with strain IHB602 and SBBR2 not augmented with strain IHB602, each with a total effective volume of 4 L) were fitted with polypropylene multifaceted hollow balls (12 valves and 25-mm diameter for each ball; 40 \% filling ratio per reactor) (Fig.1). A micro-porous aerator using an electromagnetic air pump (ACO-001, Zhejiang, China) was fixed at the bottom of the reactor, and the aeration rate was controlled by a glass rotameter (LZB-6WB, Fujian, China). The dissolved oxygen concentration in the SBBR systems was maintained at 4.5-5 mg/L, detecting by YSI (ProODO, Ohio, USA). A low speed automatic stirring rod was fixed above the reactor (M206-402, Hong Kong, China). The influent was controlled by a micro-peristaltic pump (LHZW001, Beijing, China) at a rate of 0.2 L/min, and the effluent was regulated by a microcomputer space-time switch (KG316T, Zhejiang, China). One running cycle was 12 hours. Both reactors were initially inoculated with 5 g/L MLSS activated sludge taken from the aeration tank of the Yunquan wastewater treatment plant (Anlu, China). In SBBR1, 500 mL of OD\textsubscript{600} \sim 1.0 pure-culture of strain IHB602 (dry cell weight about 4 g) were inoculated every 5 days during the biofilm-forming period. The dry weight ratio of strain IHB602 to activated sludge was about 1:5. SBBR2 was the control reactor with no added strain IHB602. A
55-day measurement analysis of the thickness and content of the biofilm was performed. A stable value, reached after ~50 days, was regarded as a sign of biofilm maturation. The influent components were approximately 50 mg/L nitrate-N and 50 mg/L ammonium-N. Sodium succinate hexahydrate was used to adjust the ratio of carbon to nitrogen (C/N) in four cycles, and C/N was maintained at approximately 15. Other ingredients were as follows: 7.5 g/L Na$_2$HPO$_4$·12H$_2$O, 1.5 g/L KH$_2$PO$_4$, 0.1 g/L MgSO$_4$, 0.1 g/L CaCl$_2$, 2 g/L NaCl and 2 mL/L of microelement solution. The ingredients of the microelement solution were the same as in DBM. Effluent samples were taken daily every two cycles to determine the concentrations of NH$_4^+$-N, NO$_3^-$-N, NO$_2^-$-N, and TN. The two SBBR units were placed in two clean laboratories, respectively. The devices were sterilized. The SBBR units were covered with large cartons twice a day and an ultraviolet lamp was used to sterilize the surrounding environment.

2.4 Analysis of microbial community structure in the SBBRs

The microbial community structure changes for long-term (100 days) stable operation were compared. Randomly selected carries were obtained from different sites in each SBBR and mixed to obtain biofilm as one sample for subsequent sequencing analysis. DNA was extracted from samples using an E.Z.N.A. Soil DNA Kit (Omega, USA) following the manufacturer instructions. The 16S rRNA primers 341F (CCTACGGGNGGCWGCAG )/805R (GACTACHVGGGTATCTAATCC) were used for sequencing amplification on an Illumina MiSeq platform (Illumina, USA) by LC-Bio Technology Co. (Hang Zhou, China). The raw Illumina reads obtained were deposited in the NCBI short-read archive under SRA accession PRJNA589909 (https://www.ncbi.nlm.nih.gov/sra/PRJNA589909). Sequences with 97% similarity were clustered as operational taxonomic units (OTUs) by Vsearch (v2.3.4). Taxonomic data were then assigned to
each representative sequence using the RDP Classifier (http://rdp.cme.msu.edu/). Alpha diversity was applied in analyzing the complexity of species diversity of the samples using the Good coverage and Simpson indices with QIIME (Version 1.8.0).

2.5 Analytical methods

The concentrations of TN and NH$_4^+$-N were measured by hydrochloric acid photometry and Nessler’s reagent photometry, respectively. NO$_3^-$-N and NO$_2^-$-N were determined using ion chromatography (ICS5000i, Thermo Fisher Scientific, MA, USA). The effluent samples were washed with deionized water three times after centrifugation (9000× g for 20 min), and freeze-dried for the determination of organic nitrogen, which was calculated from the dry weight and N content determined using an elemental analyzer (FLASH 2000, Thermo Fisher Scientific, USA). OD was detected using a UV-VIS spectrophotometer at 546 nm for hydrophobicity and 600 nm for cell growth and auto-aggregation (UV759, Shanghai, China). The weight of biofilm was measured according to Zhao et al. (2018) and was obtained after drying overnight at 105 °C and subtracting the weight of empty carrier balls from the weight of carrier balls with biofilm. Biofilm thickness was determined using microelectrodes following the methods of Ai et al. (2016). Nine measurements were made and averaged for each biofilm sample. After measuring the weight and thickness, the carrier balls were replaced to the reactors. The independent samples t-test was used to analyze data from the two SBBRs (p < 0.05 was considered a significant difference).

3. Results and discussion

3.1 Identification of strain IHB602

After the strain was cultured on BTB-agar plate for 2 days, the colonies showed off-white, convex, opaque and the surface was smooth, moist and thick. The cells were rod-shaped and about 0.4-
0.55×1.1-2.75 µm (width ×length) by SEM observation. Phylogenetic analysis based on the 16S rRNA gene sequence showed that strain IHB602 has 99.7% similarity with *Pseudomonas mendocina* strain W35 (KT380580.1). A neighbour-joining phylogenetic tree (Fig. 2a) further indicated close relationships of strain IHB602 with the members of *P. mendocina*. *napA* gene encodes the nitrate reductase used under aerobic conditions and is often used as a functional marker to identify aerobic denitrifying bacteria (Wen et al., 2019). A 848 bp fragment of the *napA* gene (MN641000) was amplified from strain IHB602 and had its highest homology (approximately 99%) with *napA* gene of *P. mendocina* strain MAE1-K (CP023641.1) (Fig. 2b). Biochemical tests of strain IHB602 showed that citrate utilization, hydrogen sulfide production, glucose utilization and aescine sodium presented positive properties and oxidase, urease, Voges-Proskauer reaction, starch hydrolysis, indole degradation, lactose utilization, gelatin degradation, inositol utilization, mannitol utilization, rhamnose utilization, arabinose utilization, growth at 4 and ONPG appeared negative properties. These biochemical features were also very similar to those of *P. mendocina*. Therefore, the strain was tentatively named as *Pseudomonas mendocina* IHB602.

### 3.2 Nitrogen removal performance of pure strain IHB602

As shown in Fig. 3a, the cell growth curve of strain IHB602 based on OD\textsubscript{600} values was fitted with a logistic equation, $y = y_0 - \frac{a}{1+(x/x_0)b}$. The values of parameters $a$, $b$, $x_0$ and $y_0$ were 2.1928, -3.2698, 18.3632 and 0.2086, respectively. The correlation coefficient ($R^2$) of 0.94 indicated that the logistic equation adequately described the growth characteristics of strain IHB602. Ammonium, nitrate, and nitrite were used as sole nitrogen sources to analyze the nitrogen removal performance of strain IHB602. As shown in Fig. 3a, the ammonium concentration decreased from 50.26 mg/L to 3.7 mg/L over 24 h with a removal ratio of 1.94 mg/L/h. Furthermore, nearly no nitrate and nitrite
were detected. This result is consistent with *Zobella taiwanensis* DN-7 (Lei et al., 2016) and *Pseudomonas stutzeri* T13 (Sun et al., 2017). As shown in Fig. 3b, strain IHB602 possessed aerobic denitrification ability, and could use NO$_3^-$-N as the nitrogen source for growth. Over 24 hours, the concentration of NO$_3^-$-N decreased from 50.36 mg/L to below the ion chromatography detectable limit (0.006 mg/L). The calculated average removal ratio of NO$_3^-$-N was 2.1 mg/L/h, which was more effective than some other previously reported aerobic denitrifiers such as *Pannonibacter phragmitetus* B1 (0.81 mg/L/h; Bai et al., 2019) and *Enterobacter cloacae* HW-15 (0.28 mg/l/h; Wan et al., 2017). Almost no accumulation of nitrite was observed during the process of nitrate degradation, but a small amount of NH$_4^+$-N was accumulated, probably due to the release of ammonium from dead bacterial cells (Yang et al., 2019). These results indicated that strain IHB602 exhibited an excellent capacity for aerobic denitrification. NO$_2^-$-N with an initial concentration of 49.64 mg/L was nearly depleted with no accumulation of NO$_3^-$-N or NH$_4^+$-N over the 24-h period (Fig. 2b). Therefore, strain IHB602 showed high efficiency in removing inorganic nitrogen.

### 3.2 Biofilm-formation properties of strain IHB602

In the process of strain IHB602 culture, it was observed that the bacteria obviously aggregated together and had biofilm formation characteristics. Auto-aggregation ability promotes biofilm development. Hydrophobicity affected bacterial adhesion to a biofilm. Characteristics of the EPS produced are strongly related to biofilm formation. Cell surface properties and the characteristics of EPS can be used as indicators for biofilm formation (Del Re et al., 2000; Malik et al., 2003; Karatan and Watnick, 2009). The biofilm-forming properties of strain IHB602 were further analyzed as follows:

#### 3.2.1 EPS characteristics of strain IHB602
Bacteria produce EPS, allowing cells of the microcolonies to adhere to each other to form mature biofilms (Terra et al., 2012). Quantitative analysis showed that the total amount of EPS produced by strain IHB602 gradually increased, from 147.13 mg/g cell dry weight at 12 h to 430.33 mg/g cell dry weight at 30 h, then began to decrease after 48 h. The protein content in the EPS of strain IHB602 was greater than that of polysaccharides (Fig. 4a). The EPS contained large amounts of protein, consistent with results of highly auto-aggregating Enterobacter sp. strain FL and biofilm-formanting Pseudomonas stutzeri strain XL-2 (Wang et al., 2018; Ding et al., 2019). Previous studies have shown that extracellular proteins could promote the formation of sludge aggregation flocs and biofilm and maintain the stability of the polymer structures (Higgins et al., 1997; Flemming and Wingender, 2010). Proteins contain negatively charged amino acids, which are more pronounced than the electrostatic bridging of the polysaccharides with divalent and multivalent cations (Laspidou et al., 2002). Three obvious fluorescent peaks from the EPS sample of strain IHB602 were observed by 3D EEM fluorescence spectra (Fig. 4b). Two peaks, observed at the excitation/emission wavelengths (Ex/Em) 205/300 and 230/300, were assigned tyrosine protein-like identification. Another peak at Ex/Em 235/350 was assigned tryptophan protein-like identification. No fluorescent peak was assigned for the presence of humic acid, suggesting that humic acid may not exist in the EPS of strain IHB602. No humus content was beneficial for maintaining a high sludge flocculation performance (Sheng et al., 2010). Therefore, EPS rich in protein and lacking humus may be important factors for biofilm formation.

3.2.2 Auto-aggregation and hydrophobicity performance of strain IHB602

The auto-aggregation ability of bacteria refers to the phenomenon of spontaneous aggregate formation during the process of biofilm formation. The hydrophobicity of bacteria is usually closely...
related to the aggregation and adhesion between bacteria or between bacteria and object surfaces. Higher hydrophobicity leads to better auto-aggregation ability. Understanding the mechanism of biofilm formation with good hydrophobic properties and auto-aggregation ability is important (Flemming and Wingender, 2010; Wang et al., 2018; Hong et al., 2019). As shown in Fig. 4c, the auto-aggregation capacity and hydrophobicity of this strain followed a similar trend as during the process of strain culturing. It increased gradually from 12 hours to 36 hours and reached the maximum values of 47.09% for auto-aggregation capacity and 85.07% for hydrophobicity. The auto-aggregation index of this strain was similar to that of Enterobacter sp. strain FL (~45% at 36h; Wang et al., 2018) and Streptococcus salivarius JIM8777 (~43%; Couvigny et al., 2018) but significantly higher than that of Methylobacterium gregans DC-1 (38.7%; Hong et al., 2019) and Lactobacillus paracasei subsp. paracasei BGNJ1-641 (~26%; Miljkovic et al., 2016). The hydrophobicity of strain IHB602 was higher than that of strains Pseudomonas sp.1-4 (61%; Zhu et al., 2012), Acinetobacter junii YB (68.5% in basal medium; Ren et al., 2014), and Lactobacillus sp. 2.17 (41.07%; Grajek et al., 2016). Strain IHB602 has high auto-aggregation capacity and hydrophobicity and thus might be more conducive to the formation of biofilms.

3.3 Bioaugmentation performance of strain IHB602 in an SBBR

Auto-aggregation of bacteria is a primary step in biofilm development (Malik et al., 2003). Bacteria with strong biofilm formation abilities coaggregate with other microorganisms to develop biofilms (Rickardet al., 2004). Adding strains with capacity for both biofilm formation and high-efficiency pollutant removal to augment wastewater treatment system would promote the coaggregation of other microorganism and further improve the development of biofilms. This would reduce the loss of bacteria from the bioreactors and enable more efficient removal of pollutants. In
this study, the bio-strengthening effect of strain IHB602 was investigated in an SBBR. The biofilm-forming process on the carriers and the effluent N concentrations in SBBR1 and SBBR2 were evaluated. As shown in Fig. 5a, on day 5, the biofilm weight and thickness on one carrier in SBBR1 averaged approximately 0.33 mg and 113 μm, respectively, but these values were only 0.12 mg and 47 μm, respectively, in SBBR2 (P< 0.05). On day 40, the biofilm weight and thickness on a single carrier in SBBR1 averaged approximately 7.4 mg and 2840 μm, respectively, and then remained unchanged. This indicated the maturation of the biofilm in SBBR1. However, the biofilm in SBBR2 continued to increase after day 40, and SBBR2 biofilm weight and thickness were close to those in SBBR1 by day 45. The high-efficiency of auto-aggregation capacity and hydrophobicity and large amount of EPS production imparted strain IHB602 with biofilm formation ability (Fig. 3). All these results suggest that strain IHB602 may promote the development of biofilm and thereby shorten the time for biofilm formation on carriers in SBBRs; the biofilm-forming time was slightly greater than the performance of P. stutzeri strain XL-2 (Zhao et al., 2018).

The effluent NH$_4^+$-N concentrations are shown in Fig 7b. In the initial 5 days, the effluent NH$_4^+$-N concentrations was 14.4-26 mg/L with removal ratios of 47-72% in both SBBR1 and SBBR2. From 5 to 15 days, the NH$_4^+$-N removal ratios gradually increased in both reactors. After 15 days, the effluent NH$_4^+$-N concentration in both reactors was less than 6 mg/L. The NH$_4^+$-N removal ratio stabilized at 95% in SBBR1 and 91% in SBBR2 for the long test (P< 0.05). In the first 6 days, the NO$_3^-$-N removal efficiency of both systems was not high, only 5.4 mg/ L in SBBR1 and 13.3 mg/ L in SBBR2, and the removal ratios were 89% and 73%, respectively (Fig. 7c). After 6 days, the average concentration of the stable NO$_3^-$-N was reduced to 2.5 mg/ L and 5.8 mg/ L (P< 0.05), and the removal ratios reached 95% and 88% for SBBR1 and SBBR2 (P< 0.05), respectively. The
minimum NO$_3^-$-N concentration reduced to 1 mg/ L in SBBR1 and 4 mg/ L in SBBR2, and the removal ratios were 98% and 92%, respectively. The average effluent NO$_3^-$-N concentration of the SBBR1 system was approximately 2 times than that of SBBR2. The reaction system also showed efficient removal of total nitrogen (Fig. 5b). During the first 5 days, the removal efficiency of both reactors fluctuated greatly: SBBR1 varied between 65% and 80%, and SBBR2 varied between 60% and 70%. The total nitrogen removal of SBBR1 was 79%-89%, and the total nitrogen removal of SBBR2 was 68%-86%, and the average removal ratios were 88% and 82% (P< 0.05), respectively. Overall, the total nitrogen removal efficiency of SBBR1 was approximately 6% higher than that of SBBR2. No accumulation of NO$_2^-$-N was detectable during this process. In addition, the nitrogen balance of the two reactors during the stable period was determined, further proving that bioaugmentation has a stronger effect on the removal of ammonia nitrogen, nitrate nitrogen and organic nitrogen (Table 1). The bioaugmentated SBBR1 showed faster development of biofilm and inorganic nitrogen removal capacity than non-bioaugmentated SBBR2.

3.4 Microbial community structure analysis

The microbial communities in SBBR1 and SBBR2 for the long-term (100 days) operation were analyzed (Fig. 6). Good coverage indices were 0.96 and 0.91 in SBBR1 and SBBR2, respectively. Similarly, the Simpson index was 0.98 in SBBR1 and 0.96 in SBBR2. This may indicate that adding strain IHB602 for bioaugmentation into a bioreactor does not result in a decrease of microbial diversity. The dominant phyla were **Proteobacteria** (76.8%), **Bacteroidetes** (14.2%) and **Firmicutes** (5.9%) (Fig. 6a) for both reactors. These results were consistent with other membrane reactors, such as bed reactor-membrane bioreactor (MBR) (Guadie et al., 2014) and particularly moving bed biofilm reactor (MBBR) (Su et al., 2016). Previous studies have shown that **Proteobacteria** and
Bacteroidetes could contribute to nitrification or denitrification, and Firmicutes could degrade organic pollutants and withstand extreme conditions (Chen et al., 2018; Fang et al., 2018). Additionally, the phylum Proteobacteria was reported as a key bacterial group in conventional wastewater treatment (Shi et al., 2015). For SBBR1 with the addition of strain IHB602, there was a higher abundance of Proteobacteria (86.14%) than in the control SBBR2 (67.47%), and its increase could promote pollutant removal (Maharjan et al., 2016). Further comparison of the microbial communities at the genus level showed that Pseudomonas mendocia in SBBR1 was significantly higher than in SBBR 2 (P< 0.05) (Fig. 6b), which implied that strain IHB602 successfully proliferated in the SBBR. Interestingly, comparing the top genera in the two systems, several genus related to denitrification were found to have significantly increased in SBBR1, such as Azoarcus (15.4% in SBBR1/0.03% in SBBR2; P=0), Paracoccus (10.08% in SBBR1/1.33% in SBBR2; P=0.01), Thauera (7.06% in SBBR1/0.09% in SBBR2; P=0.01), Rhodohacter (7.62% in SBBR1/1.41% in SBBR2; P=0.01). Azoarcus is an important genus for nitrogen removal in different biofilm systems, such as airlift inner-loop sequencing biofilm batch reactor and biological folded non-aerated filter (BFNAF) (Ruan et al., 2016; Chang et al., 2019). Paracoccus is a prevalent genus of nitrogen metabolism in biofilm reactors for wastewater treatment (Wei et al., 2019; Wang et al., 2019). Most Thauera exhibit capacities for aerobic or anaerobic (anoxic) denitrification and they might play a role in biofilm formation (Kondaveeti et al., 2014; Ju and Zhang, 2015; Zhao et al., 2018). Besides, Rhodohacteri showed the ability of denitrification in wastewater-treating biofilms (Satoh et al., 2006; Zhou et al., 2019). The abundance of Azonexus and Flavobacterium decreased in SBBR1 compared with SBBR2 and it is possible that these genera did not contribute much to the bioaugmented system. Although the abundance of strains IHB602 was not dominant in the system,
its bioaugmentation promoted nitrogen removal and biofilm formation from other denitrifying strains. This phenomenon was also observed in bioaugmentation with strain XL-2 in the treatment of ammonium-rich wastewater in a biofilm reaction system and strain NR in the treatment of nitrogen and organic carbon in a biofilm system (Zhao et al., 2018; Yang et al., 2018). Therefore, this type of strains with dual-capacity for biofilm formation and nitrogen-removal may enhance co-aggregation with other strains and increase the abundance of some nitrogen-removing strains to promote biofilm formation and nitrogen removal in SBBRs.

4. Conclusion

In this study, an aerobic denitrifier strain IHB602 was isolated from lake sediment. Strain IHB602 could efficiently remove NO$_3^-$-N, NO$_2^-$-N, and NH$_4^+$-N, and form biofilms. The strain produced large amounts of EPS rich in protein but containing almost no humic acid; this, and strong autoaggregation and hydrophobicity, imparted this strain with biofilm formation traits. Bioaugmentation of an SBBR with strain IHB602 successfully enhanced the inorganic nitrogen removal efficiency as well as biofilm formation in a single aerobic bioreactor.

Acknowledgments

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References


Figure Captions

Fig. 1 Schematic diagram of sequencing batch biofilm reactor. (1, Stirrer; 2, Carrier ball; 3, Heat layer; 4, Electromagnetic valve; 5, Aerator; 6, Peristaltic pump; 7, Air pump).

Fig. 2 The phylogenetic tree derived from neighbor-joining analysis of partial 16S rRNA gene sequences of strain DC-1 (a). Amplification results of napA (M: DL 2000 DNA marker) (b).

Fig. 3 Concentration variation of nitrogen compounds with the growth of strain IHB602 when NH$_4^+$-N, NO$_3^-$-N and NO$_2^-$-N served as nitrogen sources under aerobic conditions.

Fig. 4 Contents of proteins and polysaccharides in the EPS of strain IHB602 during aerobic cultivation (a). Fluorescence excitation-emission matrix (EEM) spectra of the EPS by strain IHB602 (b). Auto-aggregation and hydrophobicity during strain IHB602 cultivation (c).

Fig. 5 Average biofilm weight and average biofilm thickness during the biofilm-forming period (a). Changes in NH$_4^+$-N concentration (b), NO$_3^-$-N concentration (c), and TN concentration (d) for long period in SBBR 1 and SBBR 2. Values are given as mean ± SD.

Fig. 6 The relative abundances of bacterial at phylum level in SBBR1 and SBBR2 (a). Significant difference for Pseudomonas mendocina between SBBR1 and SBBR 2 at genus level (b).
Fig. 1
Fig. 2

Fig. 3

NO$_3^-$N as the sole nitrogen source

NO$_2^-$N as the sole nitrogen source
Fig. 4
Fig. 5
Table Captions

Table 1 Variation of N in the nitrogen balance.

<table>
<thead>
<tr>
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<th>Initial N (mg/L)</th>
<th>Final N (mg/L)</th>
<th>N lost (%)&lt;sup&gt;a&lt;/sup&gt;</th>
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Values are means ± SD for triplicates
<sup>a</sup> N lost (%) = 100 × (Initial N-Final N)/Initial N

Credit author statement

Pei Hong and Bangding Xiao designed the study. Pei Hong, Xingqiang Wu, Yilin Shu, Chunbo Wang, Cuicui Tian and Hailong Wu performed the experiments. Pei Hong, Xingqiang Wu and Yilin Shu analyzed the data. Pei Hong and Bangding Xiao drafted the manuscript. All authors read and approved the final manuscript.

Declaration of Interest Statement

The authors declare that they have no competing interests.

Highlights:

- Strain IHB602 was identified and removed almost all inorganic nitrogen within 24 hours.
- Strain IHB602 presented biofilm formation properties.
- Strain IHB602 showed better bioaugmentation performance on biofilm-formation and nitrogen-removal in SBBR.
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