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Nitrogen removal by mix-cultured aerobic denitrifying bacteria isolated by ultrasound: Performance, co-occurrence pattern and wastewater treatment

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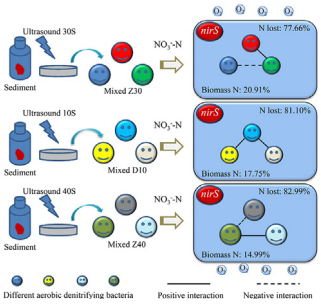
HIGHLIGHTS:

- Three high active Mix-CADB consortia were obtained.
- TN and TOC removal efficiencies of Mix-CADB were higher than 95% and 97%.
- Co-existence and interactions of strains drive the TN and TOC removal process.
- Mix-CADB was an efficient strategy for real nitrogenous wastewater treatment.

GRAPHIC ABSTRACT



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ABSTRACT

Single aerobic denitrifying bacterial strains have been studied extensively; considerably less attention has been paid to mix-cultured aerobic denitrifying bacteria (Mix-CADB). Herein, three novel Mix-CADB consortia were isolated from sediment using ultrasonic processor pretreatment. The nitrate and total organic carbon (TOC) removal efficiencies of the Mix-CADB were greater than 99% and 97% under aerobic conditions, respectively. Moreover, the optimal conditions for aerobic denitrification of the Mix-CADB were evaluated by a response surface methodology model. The *nirS* gene sequences indicated that the dominant phyla were Firmicutes and Proteobacteria. The relative abundance of mixed taxa changed over the culture time. Network analysis demonstrated that the total nitrogen (TN) and TOC removal performance were driven by the co-occurrence and interaction of *Bacillus subtilis*, *Pseudomonas stutzeri*, *Rhodococcus* sp., etc. Mix-CADB inoculation can remove 86% TN and 93% COD of real wastewater. The identified Mix-CADB isolated by ultrasonic pretreatment can be used for nitrogenous wastewater treatment.

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1. Introduction

Nitrogen (N) pollution is a challenging global environmental problem due to the fact that large amounts of chemical fertilizers have been used for agricultural plantation through human activities [1]. In recent decades, with the fast development of modern agriculture, there has been a massive increase in the quantity of N that enters water bodies like rivers, lakes, and reservoirs via agricultural runoff, livestock and poultry industry wastes [2]. In aquatic ecosystems, the escaped N from the effluents of industrial wastewater can contaminate freshwater and sediment, affecting human health and amphibian survival [3]. Furthermore, sediment enrichment with N can cause serious harmful algal blooms, and such events have increased dramatically with global warming [4]. Groundwater nitrate pollution and surface water eutrophication have occurred frequently in the past few decades [3]. It is therefore urgent to establish the best management strategies and develop novel N pollution control technologies (especially N removal) for the protection of aquatic ecosystems [4].

Previous studies have shown that biological N removal technology has been widely employed to reduce nitrate loading in wastewater [5], source water pretreatment [6], micro-polluted reservoirs [7], and wetlands [8] ecosystems. Compared to the traditional anaerobic denitrifying technique, aerobic denitrification attracts more attention from wastewater treatment engineers and environmental microbiologists because total nitrogen (TN) can be simultaneously removed with total organic carbon (TOC) under aerobic conditions [9]. For aerobic denitrifying bacteria, oxygen is an electron acceptor, and organic carbon acts as an electron donor [10]. During the past few years, aerobic denitrifying bacterial strains have been frequently isolated from ponds, reservoir sediments, domestic wastewater, and activated sludge, including *Bacillus* sp. YX-6 [10], *Rhodococcus* sp. CPZ24 [11], *Acinetobacter* sp. HA2 [12], *Paracoccus versutus* KS293 [13], and *Pseudomonas stutzeri* KTB [14]. As an example, Zhou et al. [14] isolated *Pseudomonas stutzeri* KTB and suggested that strain KTB can be used for biological removal of N compounds from nitrogenous wastewater. It is worth noting that although massive aerobic denitrifying bacteria have been isolated and many researchers have extensively studied the N removal performance of single and pure bacterial strains, less attention has been paid to mix-cultured aerobic denitrifying bacteria (Mix-CADB) and their practical application in real wastewater treatment.

Mix-cultured bacterial consortia have several advantages compared with single and pure bacterial strains, especially for the removal of combined pollutants [15–18]. For instance, the separation time of mixed bacteria from environmental samples is much less than that of single strains, because four to five rounds of purification are conducted to capture a pure strain [7]. From a microbial ecology point of view, in a mix-cultured bacterial consortium system, the co-existence and interaction of mixed bacterial consortia has novel biological functions, such as quorum sensing [19], the “cheating effect” [20], and mutualism [21–22]. Interestingly, Kaeberlein et al. [21] observed that a studied bacterial strain could not form a colony on the tested media alone but could grow well with the co-occurrence of other isolates. Bacterial species-species communication can enhance the biomass yield and intracellular enzyme activities and stabilize bacterial cooperative behaviors [23]. Furthermore, mix-cultured bacterial consortia are more effective for pollution removal because the combined diverse metabolic pathways and capabilities of mixed bacterial consortia are more powerful than those of a single pure isolate [18,24]). Mujtaba and Lee [22] studied a co-culture system in municipal wastewater treatment and found that the removal of ammonium and total organic carbon (TOC) was significantly increased in the co-culture system. Unfortunately, the nutrient removal performance of Mix-CADB has been not comprehensively understood.

Numerous techniques using different artificial media, optimizing the medium chemical composition, or pre-treating samples have been employed to improve the bacterial culturability from the natural environments to obtain diverse strains with high pollution removal efficiency [25–27]. Among these techniques, ultrasound technology is used as a pretreatment step in the microbe isolation process [28–31]. Matsumoto et al. [29] first isolated different bacterial strains from soil samples using an ultrasonic processor, and demonstrated that bacterial species harbored inside the soil aggregates can be isolated efficiently using ultrasonic processor pretreatment instead of other conventional methods. Likewise, Yang et al. [30] studied the effects of microwaves on the isolation of actinomycetes, and found more novel actinomycete strains after fracturing soil particles by physical microwave irradiation pretreatment. Jiang et al. [31] also explored the ultrasonic treatment of soil for actinomycete isolation and revealed that the abundance of bacterial colonies increases significantly with sonication treatment, while the appropriate treatment time varies among different soil types. However, to our knowledge, there has been far less attention focused on improving the isolation of aerobic denitrifying bacteria living in the sediment with sonication treatment, although the sediment has a similar texture as soil, as was previously reported [32].

The hypothesis of the present study is that Mix-CADB consortia with high TOC and N removal efficiency can be isolated using the ultrasonic pretreatment technique, while the co-existence and interaction among these genera may be vital for improving the aerobic denitrification process. The specific objectives of the present study were to (1) isolate mix-cultured aerobic denitrifying bacteria harbored in the sediment using sonication pretreatment; (2) determine the cell growth, TOC and N removal characteristics of three Mix-CADB consortia; (3) diagnose the dynamics of Mix-CADB compositions based on the *nirS* gene sequence; (4) optimize the influence of process parameters, including C/N ratio, pH, temperature, and shaking speed on the denitrification efficiency of the Mix-CADB, and finally (5) assess the practical application in real domestic sewage treatment.

2. Material and methods

2.1. Media

Based on previous studies [7,13], a denitrification medium (DM) was used in the present work [13]. The solid plate contained the DM liquid reagents plus 10 g of agar (Biowest, Spain) per litter. All media were autoclaved at 121 °C for 30 min before use.

2.2. Mix cultured denitrifying bacteria isolation

Polluted urban lake surface sediments (0–5 cm) were collected from three different sites in the urban lake, stored in a cooler (8 °C) and then transported immediately to the laboratory within 6 h. 200 mL fresh sediment was suspended in 500 mL DM liquid medium. Ten sterilized glass beads were put into the culture flask system, and shocked at 130 rpm with 30 min. The dissolved oxygen (DO) concentration was maintained with 4–6 mg/L, and enrichment at 30 °C in the dark. After 21 days of continuous culture, ultrasonic-assisted separation technology was used to isolate mix-cultured denitrifying bacteria. According to previous reports [29–31], the ultrasonic generator (KQ-500DE, Kunshan, China) with 500 W of power rating was selected, and the power rating was adjusted to 40%. The sediment suspension was ultrasonically treated with 0, 10, 20, 30, 40, and 50 s, respectively. The sediment suspension was diluted to 10^{-3} using the 10-fold serial dilutions method and then one mL of suspension with 10^{-3} was spread on the DM agar plate. The inoculated DM agar plates were then put into the chamber at 30 °C until colonies formed. The colonies grown on five plates of each treatment ($n = 5$) were washed with sterilized water and the mixed suspension was collected as seed culture. Three higher active Mix-CADB consortia cultures, termed A30, D10, and Z40, had the higher NO_3^- removal efficiencies, which were cultured in DM for further investigation. Mix-CADB consortia were maintained with 50% glycerol solution and stored at −80 °C.

2.3. Cell growth and TOC removal determination

To determine the cell growth characteristics of the Mix-CADB, 7.5 mL of seed cultures of A30, D10, and Z40 were inoculated into fresh liquid DM medium (150 mL) and cultured in the incubator in the dark at 30 °C at 125 rpm. The average value of OD_{600} was 6.5 mg/L. Under aerobic conditions, cells were grown aerobically. Cell optical density (OD_{600}) (1.0 OD_{600} = 0.23 g DW/L) values of A30, D10, and Z40 were measured by using a UV-spectrophotometer (UV-1240, Shimadzu, Japan). Two milliliters of each culture was sampled periodically for the determination of cell growth (OD_{600}). The total organic carbon (TOC) concentration was measured using a TOC analyzer (TOC-L, Shimadzu, Japan).

2.4. Denitrifying capacity determination

During incubation, cultures of A30, D10, and Z40 were sampled periodically using a sterilized pipette (Eppendorf, Germany), then centrifuged at 8000*g* for 10 min. The supernatant was selected to analyze the nitrate (NO_3^--N), ammonium (NH_4^+-N), nitrite (NO_2^--N), and TN concentrations through the standard method using a UV-spectrophotometer (DR 6000, HACH, USA).

2.5. Nitrogen balance analysis

To determine the nitrogen balance, the cultures of Mix-CADB were harvested after 72 h of cultivation and centrifuged at 8000*g* for 10 min. According to our previous study [7,13], the intracellular N was released from the cells using an ultrasonic cell disruptor (Ningbo Scientz Biotechnology, Ningbo, China). The calculations of biomass N and lost N as nitrogen gas were performed as described previously [7,13]. The assay was performed in triplicate (n = 3).

2.6. Mix cultured aerobic denitrifying bacterial composition

To identify ecologically associated species of Mix-CADB, the genomic whole DNA of the A30, D10, and Z40 cultures at 18 and 72 h was extracted using a DNA extraction kit (Omega, USA), and purified (Thermal Fisher Scientific, USA) according to the standard protocols. *nirS* gene specific primer sets (CD3A: 5′-GTSAACGTSAAGGARACSGG-3′ and R3CD: 5′-GASTTCGGRTGSGTCTTGA-3′) were selected [33]. Each PCR tube with 20 μL reaction liquid contained: 10 μL of PCR mix (Applied Biosystems, CA, USA), 10 ng of DNA, 0.8 μL of each primer, and a balance of ddH_2O. PCR was carried out on a thermal cycler (C-1000, Bio-red, USA) with the following program: 10 min at 96 °C, followed by 40 cycles of 15 s at 96 °C, 30 s at 60 °C, and 30 s at 72 °C, with a final extension for 10 min at 72 °C. PCR products were purified and checked as described before [33] The Illumina Miseq sequence was performed by Shanghai BIOZERON Biotechnology Co., Ltd. (Shanghai, China). Sequence data processing was conducted using the QIIME software [32]. Based on the Spearman's correlation coefficients, network analysis was performed to reveal the co-occurrence patterns and interactions of Mix-CADB and nutrient removal performance [33].

2.7. Optimization of the denitrification parameters

To optimize the denitrification parameters of three Mix-CADB, a Box-Behnken design with response surface methodology (RSM) was used for modeling and predicting the TN removal efficiency (response) regulated by the culture conditions [34]. According to our previous study [13], four variables including pH, temperature, C/N ratio, and shaking speed were selected, and twenty nine independent experimental measurements (Table S2) were carried out with three levels (−1, 0, and 1), as previously described [7]. The relationship between DO and shaking speed was measured as $DO_0 \approx 3.2$ mg/L, $DO_{65rpm} \approx 4.5$ mg/L, and $DO_{130rpm} \approx 6.9$ mg/L, respectively. Response surface graphics were constructed from the experimental models. Finally, the optimal conditions of the culture variables were generated from the RSM, by using the Design Expert software (version 7.1.5, Minneapolis, USA).

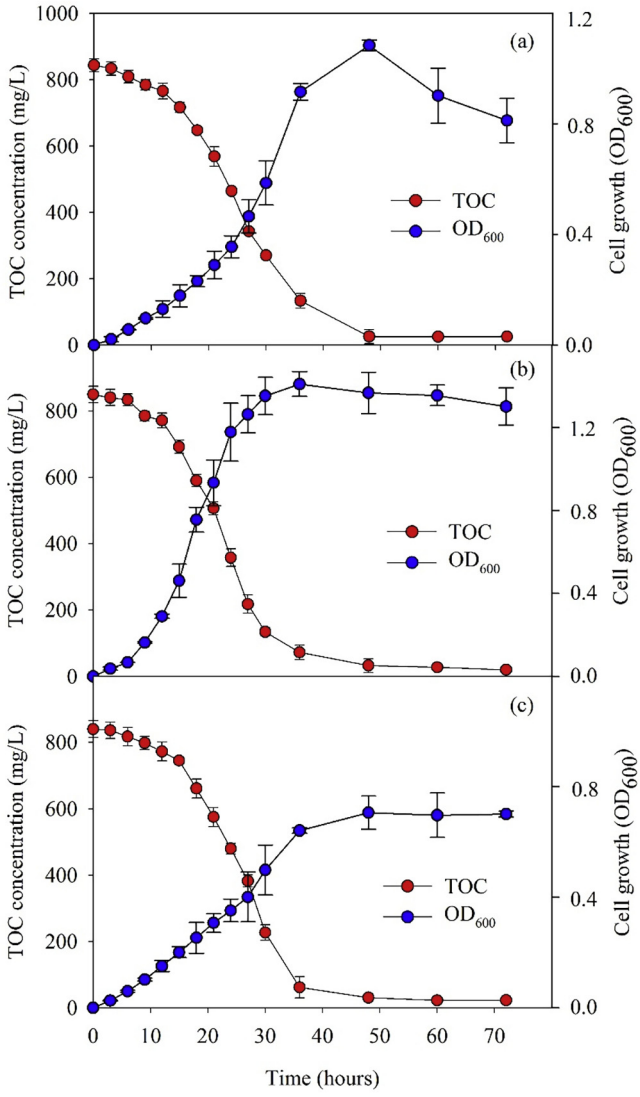

Fig. 1. Cell growth curve and TOC concentration of mix-cultured aerobic denitrifying bacteria (Mix-CADB) of A30 (a), D10 (b), and Z40 (c) inoculated in DM medium. Values are means ± standard deviation (SD) (n = 3).

2.8. Assessment of nitrate and COD removal in wastewater

To assess the application ability of A30, D10, and Z40, the TN and COD removal performances of A30, D10, and Z40 inoculated into wastewater were measured in a flask experiment. Domestic wastewater was sampled from domestic wastewater treatment plants (e.g., WWTP-1, WWTP-2, and WWTP-3, and treatment type were activated sludge process. Domestic wastewater was transported into WWTPs) in the city of Xi'an, transported to the laboratory within 6 h, and then inoculated with each of three Mix-CADB seed cultures (OD_{600} = 0.4) (10%, v/v). The TN and COD removal efficiencies (%) were measured based on the methods [35] after 24 and 72 h of cultivation.

2.9. Data analysis

To compare the cell growth, TOC and nitrogen removal efficiencies of Mix-CADB, one way-ANOVA followed by Tukey HSD post-hoc test

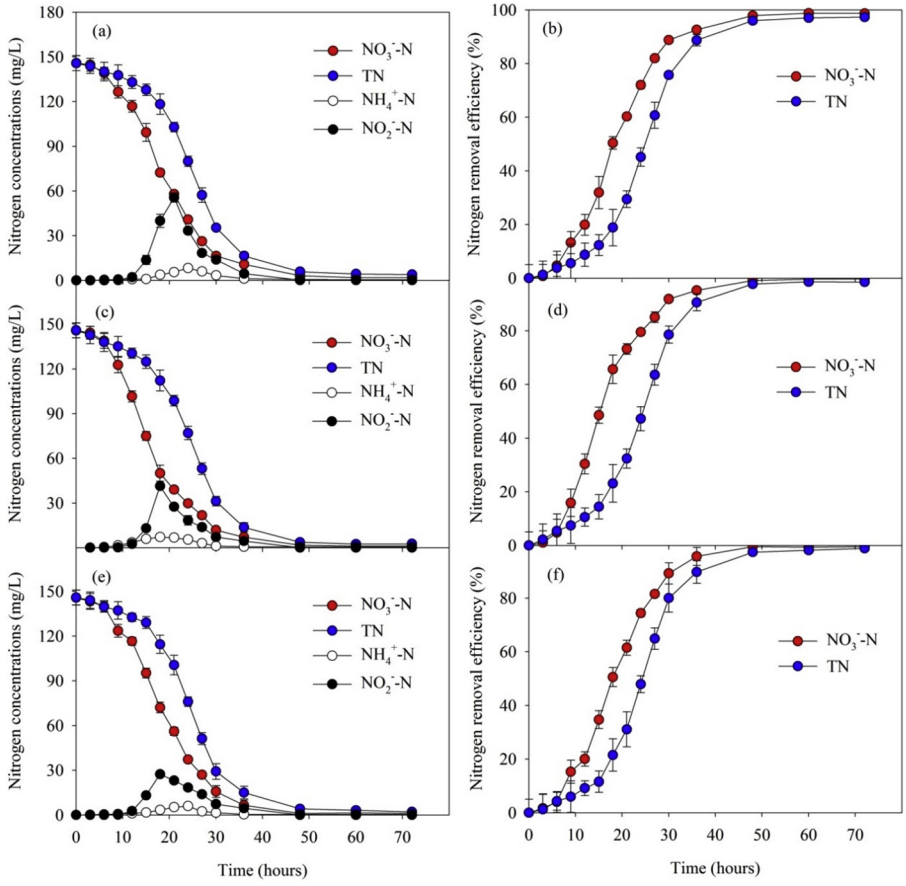

Fig. 2. Nitrogen (NO_3^--N, TN, NH_4^+-N, and NO_2^--N) concentrations, the NO_3^--N and TN removal efficiency of mix-cultured aerobic denitrifying bacteria (Mix-CADB) of A30 (a, b), D10 (c, d), and Z40 (e, f). Values are means ± standard deviation (SD) (n = 3).

Table 1
Nitrogen balance of the mix-cultured aerobic denitrifying bacteria (Mix-CADB) of A30, D10, and Z40 under aerobic conditions (unit: mg).

Mix-CADB	Initial TN	Final nitrogen				Intracellular nitrogen	N lost as nitrogen gas
		NO_3^--N	NO_2^--N	NH_4^+-N	Organic-N		
A30	21.75 ± 0.75	0.28 ± 0.05	0.02 ± 0.02	0.06 ± 0.00	1.12 ± 0.08	4.55 ± 0.06	16.89 ± 0.18
D10	21.75 ± 0.75	0.16 ± 0.02	0.08 ± 0.00	0.01 ± 0.00	0.55 ± 0.07	3.86 ± 0.14	17.64 ± 0.61
Z40	21.75 ± 0.75	0.17 ± 0.01	0.01 ± 0.00	0.06 ± 0.00	0.39 ± 0.04	3.26 ± 0.15	18.05 ± 1.15

TN: total nitrogen. Values are means ± standard deviation (SD) (n = 3).

was conducted by using SPSS software with significance considered at $P < 0.05$ (SPSS Statistics version 20.0). Co-occurrence and interaction of network analysis was performed using R software for Windows (version 2.15.3) and displayed with Gephi (https://gephi.org/) package.

3. Results and discussion

3.1. Cell growth and TOC removal

The cell growth characteristics and TOC removal performance of three Mix-CADB consortia (A30, D10, and Z40) are shown in Fig. 1. The cells of the Mix-CADB were grown aerobically in DM. After a 6 h lag phase, the concentrations of mixed cells increased steadily, reaching 1.084 (OD_{600}) at 48 h for A30, 1.41 at 36 h for D10, and 0.706 at 48 h for Z40. D10 had a significantly higher growth rate than A30 and Z40 (F = 16.56, $P < 0.01$). The equations of the growth curves were estimated as follows:

$$Y_{(A30)} = -0.0002X^2 + 0.0315X - 0.1487 (r^2 = 0.884) \quad (1)$$

$$Y_{(D10)} = -0.059X^2 + 0.2144X - 0.4345 (r^2 = 0.939) \quad (2)$$

$$Y_{(Z40)} = 0.0012X^2 + 0.0367X - 0.057 (r^2 = 0.979) \quad (3)$$

Successively, TOC concentrations decreased significantly during the culture periods (Fig. 1), with removal efficiencies of 96.98% for A30, 97.68% for D10, and 97.27% for Z40 under aerobic conditions, which were higher than that of *Pseudomonas stutzeri* strain ZF31 with 74% [7], *Bacillus subtilis* A1 with 71% [36], and *Acinetobacter calcoaceticus* HNR with 81% [37]. This pattern could be explained by the fact that the mix-CADB consortium has a different bacterial population, which increases its ability to degrade TOC based on a commensalism relationship. For instance, *Pseudomonas* spp. or *Rhodococcus* spp. can use toxic carbon (e.g., phenol) [38] and convert it into less toxic compounds, which can be consumed by *Paracoccus* spp. The highest TOC removal rate was observed in D10 with 23.41 mg/L/h, which was 26.4% and 38.1% higher than that of A30 and Z40, respectively (F = 5.28, $P < 0.05$). Meanwhile, TOC concentrations had a significant negative relationship

Table 2
Nitrogen removal efficiency of aerobic denitrifying bacteria in previous studies.

Aerobic denitrifying bacteria	Nitrate removal efficiency (%)	TN removal efficiency (%)	References
Mix-CADB A30	98.71	97.31	This study
Mix-CADB D10	99.26	98.17	This study
Mix-CADB Z40	99.25	98.57	This study
Mixed *Bacillus* sp.	NM	57.6	[24]
Paracoccus versutus KS293	99.8	84.3	[33]
Pseudomonas stutzeri X31	93.7	NM	[53]
Enterobacter cloacae HNR	92.0	91.5	[54]
Pseudomonas tolaasii Y-11	93.5	46.9	[45]
Acinetobacter sp. HA2	80	58.88	[12]
Pseudomonas stutzeri ZF31	97	73.3	[7]
Acinetobacter junii YB	99.05	51.5	[57]
Pseudomonas sp. ADN-42	NM	55.93	[58]
Paracoccus versutus LYM	95	67.17	[56]
Pseudomonas stutzeri XL-2	97.9	74.8	[41]
Marinobacter NNA5	100	42	[52]
Rhodococcus sp. CPZ24	67	NM	[11]
Marinobacter sp. F6	100	50.08	[60]
Psychrobacter sp. S1-1	100	46.48	[51]
Bacillus methylotrophicus L7	NM	53	[61]
Pseudomonas sp. Y2-1-1	52.9	NM	
Acinetobacter junii YB	86.55	89.98	
Pseudomonas mendocina 3-7	83.0	40.4	[50]
Bacillus subtilis A1	NM	50	[36]

NM: not mentioned in the literature. Mix-CADB: Mix-cultured aerobic denitrifying bacteria.

Table 3
Analysis of variance (ANOVA) for response surface quadratic model (*Y*).[a]

Source	Sum of squares	*df*	Mean square	*F*-value	*p*-value (Prob > *F*)	Statistics
Model[b]	18935.02	14	1352.50	17.88	< 0.0001	Significant
X_1	32.67	1	32.67	0.43	0.5217	NS
X_2	184.08	1	184.08	2.43	0.1411	NS
X_3	7120.92	1	7120.92	94.14	< 0.0001	Significant
X_4	5367.02	1	5367.02	70.96	< 0.0001	Significant
X_1X_2	25.05	1	25.05	0.33	0.5741	NS
X_1X_3	50.13	1	50.13	0.66	0.4292	NS
X_1X_4	12.57	1	12.57	0.17	0.6897	NS
X_2X_3	37.39	1	37.39	0.49	0.4935	NS
X_2X_4	158.51	1	158.51	2.10	0.1697	NS
X_3X_4	644.91	1	644.91	8.53	0.0112	Significant
X_1^2	318.37	1	318.37	4.21	0.0594	NS
X_2^2	223.19	1	223.19	2.95	0.1079	NS
X_3^2	1515.12	1	1515.12	20.03	0.0005	Significant
X_4^2	4589.00	1	4589.00	60.67	< 0.0001	Significant
Residual	1058.95	14	75.64			
Lack of Fit	1018.51	10	101.85	10.07	0.0197	Significant
Pure Error	40.44	4	10.11			
Cor Total	19993.98	28				

NS: not significant.
[a] The results were generated from the Design Expert software (version 8.0).
[b] X_1 is C/N ratio. X_2 is shaking speed. X_3 is temperature. X_4 is pH.

with cell growth ($r_{(A30)} = -0.980$, $r_{(D10)} = -0.969$, $r_{(Z40)} = -0.990$, $P < 0.001$ in all cases, Pearson's correlation, $n = 15$). This result is consistent with Sun et al. [39], who found that the reduction of the carbon source was strongly related to the cell growth. Under aerobic conditions, bacterial cell growth needs a sufficient carbon source to synthesize protein for growth and to produce electrons for aerobic denitrification [9,40]. Furthermore, the TOC reduction also demonstrates simultaneous removal of nitrogen and organic carbon [41]. Ultrasound treatment might effectively promote the aerobic denitrifying bacteria hydrolysis to decrease TOC. Low-frequency ultrasound has been demonstrated to enhance cell growth, enzyme activity (e.g., dehydrogenase activity), and COD removal [42]. Collectively, our results suggest that Mix-CADB can grow aerobically and remove TOC efficiently.

3.2. Nitrogen removal and balance

As shown in Fig. 2, three Mix-CADB consortia had excellent aerobic denitrifying ability. The mixed cells were cultivated for 72 h with the initial NO_3^--N concentration of 145 mg/L. For A30, the NO_3^--N concentration decreased from 145 mg/L to 1.88 mg/L, with 98.71% of NO_3^--N removed (Fig. 2a, b), which was higher than the removal by *Bacillus litoralis* N31 with 89.4% [43] and *Arthrobacter arilaitensis* Y-10 with 73.3% [44]. Simultaneously, for D10, after 36 h of cultivation, the NO_3^--N concentration decreased to 7.14 mg/L and approximately 95% of the NO_3^--N was removed aerobically (Fig. 2c, d), exceeding the removal by *Rhodococcus* sp. CPZ24 with 67% [11]. For Z40, the NO_3^--N concentration significantly decreased to 1.1 mg/L over 72 h in aerobic conditions. The TN removal rate of Z40 was 2.92 mg/L/h, which was higher than those of *Paracoccus versutus* LYM (0.89 mg TN/L/h) [45] and *Vibrio* sp. Y1-5 (1.38 mg TN/L/h) [46]. After 15 h of cultivation, the NO_3^--N removal rates reached as high as 3.10 mg/L/h, 4.72 mg/L/h, and 3.37 mg/L/h for A30, D10, and Z40, respectively, which were higher than those of *Pseudomonas tolaasii* strain Y-11 with 1.99 mg/L/h [45], *Rhodococcus* sp. CPZ24 with 0.93 mg/L/h [11], *Bacillus litoralis* N31 with 0.59 mg/L/h [43], *Diaphorobacter polyhydroxybutyrativorans* strain SL-205 with 2.13 mg N/L [32], and *Klebsiella pneumoniae* CF-S9 with 2.2 mg N/L [47]. It is clear that these three Mix-CADB consortia had greater N removal efficiency compared with previous reports, especially for TN removal. The most important reason is that the combination and mix of aerobic denitrifying bacterial species approximates natural conditions where different bacteria co-exist and combine their nitrogen metabolic enzyme activities (nitrate reductase) and denitrification genes (*nirS*, *nirK* and *narG*), which co-drive the removal of TN [21,22].

For these three Mix-CADB, although NO_2^--N concentrations increased during the 15–27 h cultivation periods, a smaller amount of NO_2^--N was detected at the end of the experiment. This pattern is consistent with the previous report by Zhao et al. [41], who observed that 47.7 mg/L NO_2^--N was accumulated at 12 h cultivation by *Pseudomonas stutzeri* XL-2 (initial NO_3^--N concentration = 100 mg/L). Under aerobic conditions, the NO_2^--N accumulated to 147 mg/L at 15 h by *Pseudomonas stutzeri* T13 [48]. A similar trend was also detected for NH_4^+-N, which may be released by the dead cells produced at higher cell growth rates [49]. It was previously reported that accumulated NH_4^+-N can stimulate NO_3^--N reduction during the aerobic denitrification process [44]. This phenomenon was probably due to the existence of the *narG* gene. In contrast, the accumulation of NH_4^+-N was not found in another aerobic denitrifying bacterial strain *Pseudomonas stutzeri* XL-2 [41].

To date, nearly all isolated aerobic denitrifying bacteria have been studied as single species. A great challenge is that TN removal efficiency is lower with single strain inoculation. For example, the TN removal efficiencies of *Pseudomonas mendocina* 3-7 [50], *Psychrobacter* sp. S1-1 [51], and *Marinobacter* strain NNA5 [52] were less than 50%. Indeed, compared with the reported literature [40,45,52,53], the three Mix-CADB studied here have the highest TN removal efficiency (Fig. 2, Table 2). Table 2 summarizes the NO_3^--N and TN removal efficiencies of aerobic denitrifying bacteria strains used in previous studies. For example, the TN removal efficiency was 60.7% by *Arthrobacter arilaitensis* Y-10 [45]. This finding is not surprising, given that we have known for some time that different aerobic denitrifying bacteria exhibit different metabolic pathways for driving TN removal. In mix-cultured systems, aerobic denitrifying bacteria species work together and drive aerobic denitrification effectively.

Next, we set out to establish the nitrogen balance of Mix-CADB. As shown in Table 1, the nitrogen balance revealed that 4.55 mg, 3.86 mg, and 3.26 mg N were metabolized for biomass synthesis in A30, D10, and Z40, respectively, while 16.89 mg, 17.64 mg, and 18.05 mg N,

Table 4
Analysis of variance (ANOVA) for response surface quadratic model (Y).[a]

Source	Sum of squares	*df*	Mean square	*F*-value	*p*-value (Prob > *F*)	Statistics
Model[b]	23347.90	14	1667.71	26.53	< 0.0001	Significant
X_1	101.50	1	101.50	1.61	0.2246	NS
X_2	216.16	1	216.16	3.44	0.0849	NS
X_3	8660.74	1	8660.74	137.76	< 0.0001	Significant
X_4	6667.01	1	6667.01	106.05	< 0.0001	Significant
X_1X_2	135.49	1	135.49	2.16	0.1642	NS
X_1X_3	137.71	1	137.71	2.19	0.1610	NS
X_1X_4	5.625E−003	1	5.625E−003	8.947E−005	0.9926	NS
X_2X_3	5.625E−003	1	5.625E−003	8.947E−005	0.9926	NS
X_2X_4	83.54	1	83.54	1.33	0.2683	NS
X_3X_4	913.85	1	913.85	14.54	0.0019	Significant
X_1^2	671.85	1	671.85	10.69	0.0056	Significant
X_2^2	454.80	1	454.80	7.23	0.0176	Significant
X_3^2	2162.71	1	2162.71	34.40	< 0.0001	Significant
X_4^2	5263.47	1	5263.47	83.72	< 0.0001	Significant
Residual	880.15	14	62.87			
Lack of Fit	842.27	10	84.23	8.90	0.0247	Significant
Pure Error	37.87	4	9.47			
Cor Total	24228.05	28				

NS: not significant.
[a] The results were generated from the Design Expert software (version 8.0).
[b] $X1$ is C/N ratio. X_2 is shaking speed. X_3 is temperature. X_4 is pH.

Table 5
Analysis of variance (ANOVA) for response surface quadratic model (Y).[a]

Source	Sum of squares	*df*	Mean square	*F*-value	*p*-value (Prob > *F*)	Statistics
Model[b]	21212.62	14	1515.19	16.22	< 0.0001	Significant
X_1	0.059	1	0.059	6.295E−004	0.9803	NS
X_2	0.11	1	0.11	1.221E−003	0.9726	NS
X_3	8271.90	1	8271.90	88.56	< 0.0001	Significant
X_4	5078.73	1	5078.73	54.37	< 0.0001	Significant
X_1X_2	29.16	1	29.16	0.31	0.5852	NS
X_1X_3	53.22	1	53.22	0.57	0.4629	NS
X_1X_4	26.99	1	26.99	0.29	0.5993	NS
X_2X_3	0.11	1	0.11	1.166E−003	0.9732	NS
X_2X_4	11.87	1	11.87	0.13	0.7268	NS
X_3X_4	464.19	1	464.19	4.97	0.0427	Significant
X_1^2	62.38	1	62.38	0.67	0.4275	NS
X_2^2	284.04	1	284.04	3.04	0.1031	NS
X_3^2	2239.52	1	2239.52	23.98	0.0002	Significant
X_4^2	6016.23	1	6016.23	64.41	< 0.0001	Significant
Residual	1307.69	14	93.41			
Lack of Fit	1213.27	10	121.33	5.14	0.0643	NS
Pure Error	94.42	4	23.61			
Cor Total	22520.31	28				

NS: not significant.
[a] The results were generated from the Design Expert software (version 8.0).
[b] X_1 is C/N ratio. X_2 is shaking speed. X_3 is temperature. X_4 is pH.

respectively, were converted to nitrogen gas. For Z40, approximately 83% of the initial nitrate was removed as gaseous products; there was no significant difference among the three Mix-CADB consortia ($P > 0.05$). Previously reported single aerobic denitrifying bacterial strains like *Pseudomonas stutzeri* XL-2 [41], *Enterobacter cloacae* HNR [54], *Agrobacterium* sp. LAD9 [11], and *Pseudomonas stutzeri* ZF31 [7], converted 62.4%, 70.8%, 50.1%, and 75% of nitrogen into gaseous products, respectively. Additionally, under anaerobic conditions, *Pseudomonas stutzeri* T13 assimilated approximately 50% of NO_3^--N into biomass, and the other half was reduced to NO_2^--N [55]. Similarly, *Acinetobacter* sp. HA2 [12], *Paracoccus versutus* LYM [56], and *Agrobacterium* sp. LAD9 [11] transformed 49%, 50%, and 41% of nitrogen into the biomass, respectively. Unfortunately, our nitrogen balance results did not provide the specific proportion of N_2, NO, and NO_2, which were presumed to convert to N_2 gas [24]. As described above, obviously, the Mix-CADB can convert much more NO_3^- into nitrogen gas and assimilate less NO_3^- into biomass, which is beneficial to wastewater treatment and sludge reduction. The most important reason for a higher amount of N being lost in Mix-CADB cultures is that different aerobic denitrifying bacterial species with diverse nitrogen metabolic pathways and the combined pathways and intracellular biochemical reactions can convert a higher metabolic flux into nitrogen gas.

3.3. Box-Behnken design for optimizing the parameters

To provide additional support for the aerobic denitrification of Mix-CADB, we established models. Based on the relationships among pH, shaking speed, temperature and the C/N ratio, the models of Mix-CADB were calculated as follows:

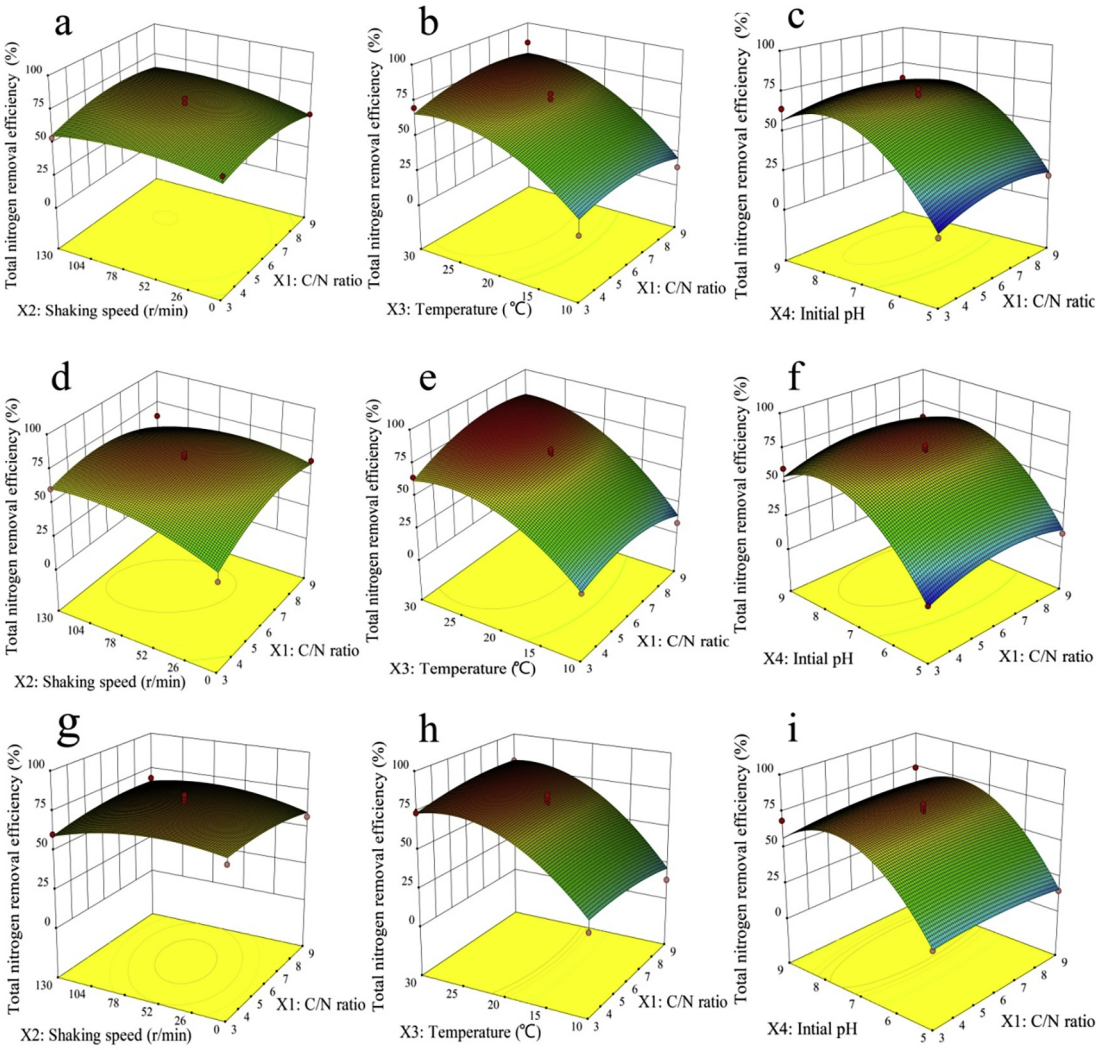

Fig. 3. Response surface (3D) plots for total nitrogen (TN) removal efficiency (%) of the mix-cultured aerobic denitrifying bacteria (Mix-CADB): A30 (a, b, c), D10 (d, e, f), and Z40 (g, h, i). Effects of pH and temperature, temperature and C/N ratio, shaking speed and C/N on total nitrogen (TN) removal efficiency.

$$Y_{(A30)}(\%) = -369.98 + 8.76X_1 - 0.08X_2 + 3.70X_3 + 89.60X_4 + 0.01X_1X_2 + 0.12X_1X_3 - 2.30X_1X_4 - 0.005X_2X_3 + 0.05X_2X_4 + 0.63X_3X_4 - 0.78X_1^2 - 0.001X_2^2 - 0.15X_3^2 - 6.65X_4^2 \quad (4)$$

$$Y_{(D10)}(\%) = -410.69 + 12.52X_1 - 0.26X_2 + 3.53X_3 + 94.05X_4 - 0.03X_1X_2 + 0.20X_1X_3 + 0.006X_1X_4 - 0.006X_2X_3 + 0.04X_2X_4 + 0.76X_3X_4 - 1.13X_1^2 - 0.002X_2^2 - 0.18X_3^2 - 7.12X_4^2. \quad (5)$$

$$Y_{(Z40)}(\%) = -411.24 - 2.18X_1 + 0.21X_2 + 5.50X_3 + 104.60X_4 + 0.02X_1X_2 + 0.12X_1X_3 + 0.43X_1X_4 + 0.003X_2X_3 - 0.02X_2X_4 + 0.54X_3X_4 - 0.35X_1^2 - 0.002X_2^2 - 0.18X_3^2 - 7.62X_4^2 \quad (6)$$

As shown in Tables 3–5, the models were checked using analysis of variance (ANOVA). For all combinations, the optimal conditions for aerobic denitrification of Mix-CADB based on a response surface methodology (RSM) model are listed in the Supplementary materials. Under optimal parameters, the TN removal efficiencies of A30, D10, and Z40 were 91%, 97%, and 91%, respectively (Table S5). Temperature can influence the aerobic denitrifying process (Fig. 3). As an example, *Acinetobacter* sp. HA2 possesses cold tolerance, and removed nitrates at 10 °C [12]. The aerobic denitrification efficiency was regulated by the DO concentration, which was related to the relative abundance of denitrifying genes (e.g., *nirS*, *nirK*, and *napA*) [13]. Moreover, the DO concentration in the medium was related to the shaking speed [53]. The shaking speed in this study was different from that used with *Pseudomonas stutzeri* strain XL-2 [41] and *Cupriavidus* sp. S1 [39], which suggested that 120 rpm was the optimal condition for denitrification. Nonetheless, this speed in agreement with a recent work conducted by Huang et al. [7], who suggested that a shaking speed of 54.2 rpm, C/N ratio of 6.7, 28 °C, and pH of 8 were the optimal parameters for *Pseudomonas stutzeri* ZF31, but the TN removal efficiency was 73%.

3.4. Mix-cultured aerobic denitrifying bacterial compositions and dynamics

Further research that profiles the denitrifying bacterial communities present during aerobic denitrification might be useful for identifying the key bacteria involved in this process. To systematically explore the taxa based on the *nirS* gene, the taxonomic compositions and dynamics of Mix-CADB were determined and displayed in Fig. 4. At the phylum level, A30, D10, and Z40 had different aerobic denitrifying bacterial taxa. Firmicutes, Proteobacteria, and bacteria-no rank were observed. Remarkably, the relative abundance of aerobic denitrifying bacteria

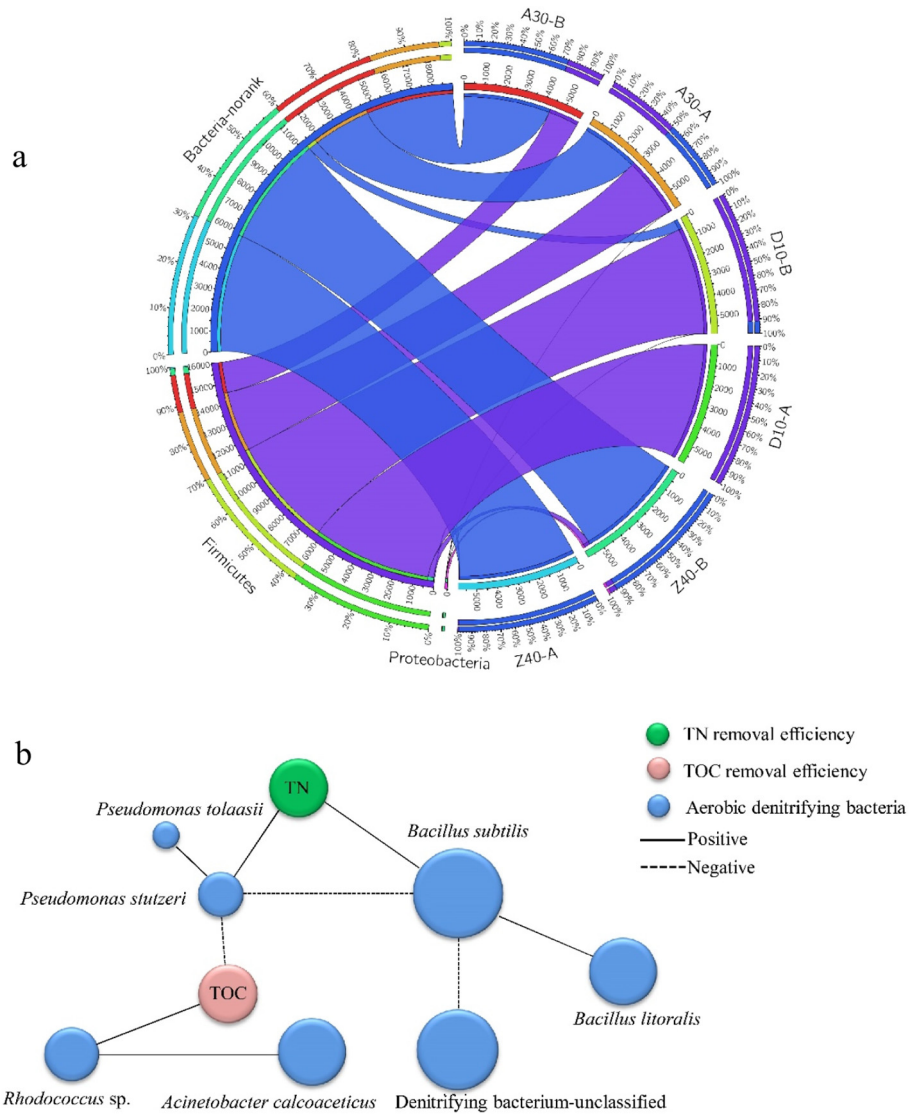

Fig. 4. (a) Compositions of mix-cultured aerobic denitrifying bacteria (Mix-CADB) of A30, D10, and Z40 based on *nirS* gene sequence at phylum level. B and A represent 18 and 72 h culture time, respectively. (b) Co-occurrence and interaction of mix-cultured aerobic denitrifying bacteria (Mix-CADB) during TN and TOC removal processes based on Spearman correlation analysis. TN: total nitrogen, TOC: total organic carbon.

was altered significantly with the cultivation times (Fig. 4a). According to the relative abundance, A30 consisted of 26% and 51% Firmicutes at 18 h and 72 h cultivation, respectively. The relative abundance of Bacteria-no rank decreased from 74% to 49%. In the D10 system, the dominate phylum was Firmicutes (96%) (Fig. 4a). Taxa dynamics in a mixed culture system are likely to be beneficial, forming cooperative cycles for nitrogen removal. Similarly, Kim et al. [24] demonstrated that the dominant *Bacillus* species changed with different C and N components in a mixed culture system (*B. cereus*, *B. subtilis*, and *B. licheniformis*). However, interactions between mixed *Bacillus* isolates in the removal of nitrogen have not been fully understood. This is likely to stem from the fact that metabolites of dominate isolate may be toxic to other species when they compete for nutrition and living space, or named as quorum sensing [19]. A network analysis can be used to explore the interactions of bacterial composition during aerobic denitrification processes. Meanwhile, changes in the relative abundance of populations can generate stable pollution removal in the face of environmental and nutrient concentration fluctuations, which is beneficial to wastewater treatment.

3.5. Co-occurrence and interaction for nitrate and TOC removal

Published works have shown lower TN removal efficiencies than those observed here [45,55–58]. There may be an incomplete conversion of the N and carbon sources for aerobic denitrification by single species, especially for TN removal. This limitation can be solved using a mixed culture; although mixed cultures have fewer applications, the use of mixed cultures is known to have great potential and, thus, should be explored in detail. Cooperative and competitive metabolic interactions in bacterial communities are important for nutrient reduction [59]. Thus, exploring co-occurrence patterns between aerobic denitrifying bacteria species can help to identify potential biotic interactions for nutrient removal [24]. Typically, bacterial strain forms close cooperative relationships, resulting in an indirect benefit to all species involved [59]. For this purpose, co-occurrence and interaction patterns among aerobic denitrifying bacteria taxa and nutrient removal variables were determined through network analysis. As shown in Fig. 4b, *Bacillus subtilis* was the “hub” taxa, and *Bacillus subtilis*, and *Pseudomonas stutzeri* were positive influence on the TN removal efficiency. These same genera have known cooperative relationships (e.g., *Pseudomonas tolaasii* and *Pseudomonas stutzeri*, *Bacillus litoralis* and *Bacillus*

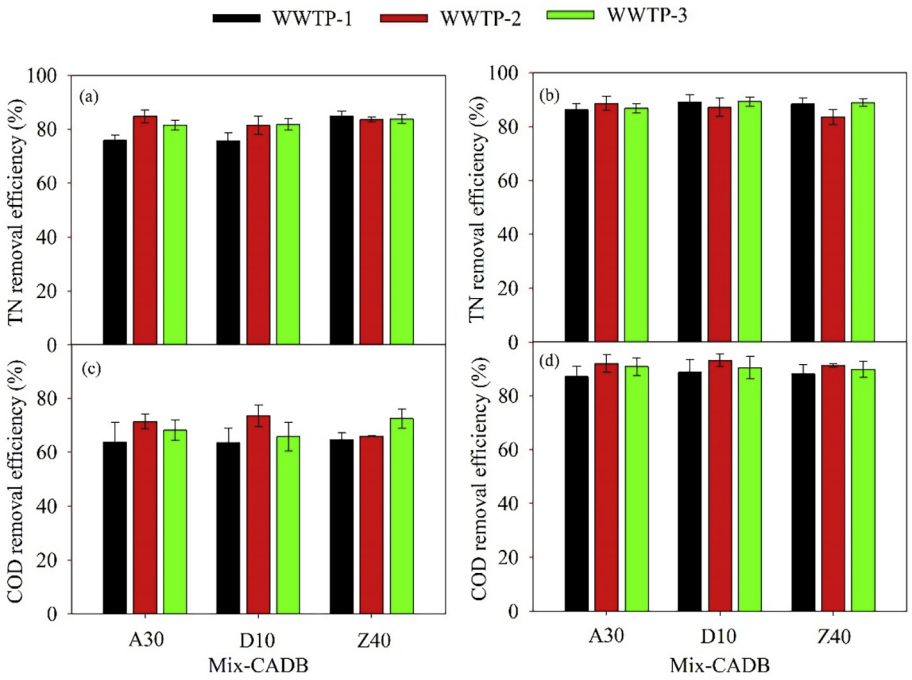

Fig. 5. TN (a, b) and COD (c, d) removal performance of real wastewater by mix-cultured aerobic denitrifying bacteria (Mix-CADB) inoculated of A30, D10, and Z40. (a) and (c) represent 24 h. (b) and (d) represent 72 h.

subtilis). *Rhodococcus* sp. combined with *Acinetobacter calcoaceticus* utilized more TOC in the co-culture systems. It could be possible that these microbes use similar pathways and have a similar nutrient requirement. As suggested here, a similar mixed culture study was conducted by Kim et al. [24], who found interactions among *Bacillus* strains during aerobic denitrification. In a mixed cell system, one bacterium produces metabolites (e.g., extracellular polysaccharides) that can be consumed by another species [59–61]. This linkage may suggest an explanation for the greater TN and TOC removal by mixed cultures than by single strains isolated using the conventional method (Table 2). Furthermore, positive interactions among the bacterial species of the consortium use species communication through horizontal gene transfer, signal compound transportation, cell densities, and the culture environment (e.g., pH and secondary metabolites) to regulate the growth of neighbors. Another explanation could be that species-level interactions can influence the stability of biological processes in bioreactors [15]. More work should be done to reveal the cell interactions and pathways by proteomic and RNA transcription methods. Overall, this finding is vital for the future design of Mix-CADB optimized for the bioremediation of wastewater. Accordingly, scaling from genera to co-occurrence and interaction may open the way to the development of a new isolating framework and technique to better understand the ecological mechanisms of aerobic denitrifying bacteria function in wastewater treatment.

3.6. Treatment of wastewater

The real wastewater sampled from three different wastewater treatment plants (WWTPs) was inoculated with a Mix-CADB consortium. As shown in Fig. 5, the Mix-CADB inoculation decreased the TN and COD concentrations in domestic sewage treatment. The highest COD removal efficiency was observed in the D10 treatment with 93% ($F = 6.89$, $P < 0.05$). The average TN removal efficiencies of A30, D10, and Z40 were 88%, 86%, and 88%, respectively ($F = 1.31$, $P > 0.05$), which were significantly higher than that of the WWTPs. In the practical systems of the WWTPs, the average TN removal efficiency was approximately 66% ($n = 18$, individual WWTP located in nine provinces of China) [33]. Similarly, Mujtaba and Lee [22] highlighted the use of a co-culture system (immobilized *Chlorella* sp. and activated sludge) in real wastewater treatment and found that the removal of nutrients was significantly increased in properly inoculated co-culture systems, exceeding TN removal in wastewater treatment plants. Owing to the diversity in nitrogen and carbon compounds, a single bacterium cannot degrade all the compounds. The use of a Mix-CADB consortium improves the variety of degradable nitrogen and carbon sources and develops an ecosystem of co-metabolism and commensalism. For example, *Pseudomonas stutzeri* can degrade toxic materials, and turn them into more soluble and less toxic compounds using extracellular enzymes, which can be used by *Bacillus subtilis*. In addition, mixed cultures have a greater resistance to other indigenous microorganisms harbored in real wastewater, and tolerate water quality fluctuation. In field conditions, mixed cultures are more resistant and flexible than single pure cultures [59] because the growth and activity of a bacterial inoculum may be inhibited when introduced at the field scale due to nutrient competition with other neighboring microbes present. It is therefore true that the Mix-CADB consortium can be used as a potential denitrifying bacterial agent in a practical wastewater treatment process and improve the treatment efficiency of WWTPs, especially for TN removal. This work has provided several new insights into Mix-CADB in engineering applications, and we plan to focus on elucidating the practical application of Mix-CADB consortia combined with nano-materials immobilization technology in wastewater treatment. In the theoretical aspect, the network analysis of C and N metabolic pathways should be further determined mechanistically using stable isotope (^{13}C and ^{15}N) and genetic synthetic biology.

4. Conclusions

In this investigation, three high efficiency Mix-CADB consortia named as A30, D10, and Z40 were isolated successfully from urban lake sediments using ultrasound pretreatment technology. The mixed cells can grow aerobically, and have excellent aerobic denitrifying ability. Mix-CADB consortia converted much more nitrates into nitrogen gas, and assimilate fewer nitrates into biomass. The RSM model generated

the following optimal conditions: C/N ratio of 7.18–8.55, temperature of 30–31 °C, pH of 8.1–8.5, and shaking speed of 72–98 rpm. The TN and TOC removal efficiencies of the Mix-CADB exceeded 95% and 97%, respectively. The co-existence and interactions of strains can drive nitrogen and carbon removal. For real wastewater treatment, the average TN removal efficiencies of A30, D10, and Z40 were 88%, 86%, and 88%, respectively.

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Conflict of interest

The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cej.2019.04.114.

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