Linking nitrification characteristic and microbial community structures in integrated fixed film activated sludge reactor by high-throughput sequencing

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ABSTRACT

The primary goal of this study is to investigate ammonia removal, abundance of nitrifying bacteria and microbial community structures in a laboratory-scale integrated fixed film activated sludge (IFAS) reactor. The results of Illumina MiSeq sequencing based on 16S rRNA genes showed Proteobacteria and Bacteroidetes were the dominant phyla in both biofilm and suspended sludge samples in the IFAS reactor. The dominant ammonia-oxidizing bacteria (AOB) species was Nitrosomonas and the dominant nitrite-oxidizing bacteria species was Nitrospira. The contribution of biofilm to ammonia removal increased from 4.0 ± 0.9% to 37.0 ± 2% when the temperature decreased from 25°C to 10°C. The real-time polymerase chain reaction (PCR) result showed the abundance of AOB in suspended sludge was higher than that in biofilm at the same time. However, nitrification is more dependent on attached growth than on suspended growth in the IFAS reactor at 15°C and 10°C and the abundance of AOB in biofilm was also higher than that in suspended sludge. The more robust ammonia removal rate at low temperatures by biofilm contributed to the relatively stable ammonia removal, and biofilm attached on carriers in the IFAS reactor is advantageous for nitrification in low-temperature environment.

Key words | IFAS, Illumina MiSeq sequencing, microbial community structures, nitrification characteristic, real-time PCR

INTRODUCTION

The integrated fixed film activated sludge (IFAS) process is an upgrade option for the conventional activated sludge (CAS) process for pollutant removal in wastewater treatment. Compared to the CAS process, the IFAS process has several distinct advantages, including a decreased sludge yield coefficient, better sludge settling characteristics, higher organic loadings and stable nitrogen removal at low temperature (Onnis-Hayden et al. 2011; Regmi et al. 2011). These advantages have resulted in widespread acceptance of the IFAS process in the engineering community as an upgrade for obsolete wastewater treatment plants (Li et al. 2012). IFAS system has been evaluated by treatment performances and operating parameters. Studies showed that IFAS system provided enhanced and stable nitrogen and phosphorous removals (Randall & Sen 1996; Sriwiriyaarat & Randall 2005a; Stricker et al. 2009; Onnis-Hayden et al. 2011; Jabari et al. 2016). The contribution of biofilm to nitrification was determined as 40–70% of total NOx-N production (Moretti et al. 2015). A recent study suggested that IFAS process could be used for enhanced biological phosphorus removal (EBPR) with total nitrogen and total phosphorus removal efficiencies maintained at 70% and 81% (Bai et al. 2016). Aeration studies of IFAS and CAS processes indicated that the two processes had comparable standard oxygen transfer rate. However, the energy footprint per unit load oxidized of the IFAS may exceed 2.0 times that of the activated sludge process (Rosso et al. 2011). IFAS system could sustain a wider range of C/N ratio from 3:1 to 10:1 with sufficient carbon and nitrogen removals (Xia et al. 2008), while excessively high C/N ratio could lead to viscous bulking (van den Akker et al. 2010).

Temperature is an important environmental parameter for biological nitrification in wastewater treatment. Specific growth rate of nitrifying bacteria in activated sludge may decrease by 86% when temperature decreased from normal temperature (25°C–30°C) to temperatures lower
than 15 °C (Knowles et al. 1965; Antoniou et al. 1990). Several studies evaluated the influence of temperature on nitrification in IFAS systems. An early work comparing conventional biological nutrient removal (BNR) plant with two types of modified IFAS system under 10 ± 1 °C showed that, while complete nitrification was achieved by both IFAS and three-state BNR system, IFAS system provided EBPR at high solids retention time (SRT) and greater denitrification at moderate temperature (Sriwiriyarat & Randall 2005b). Another pilot-scale study showed that nitrification rate by biofilm was double the nitrification rate by activated sludge with operating temperature near 11.5 °C in an IFAS reactor (Di Trapani et al. 2011). Studies of a moving bed biofilm reactor system, similar to IFAS but without sludge recycle, demonstrated that nitrifying biofilm could quickly adapt to low temperature and recover ammonia removal rate (Zhang et al. 2015; Gilbert et al. 2014; Hoang et al. 2014). Previous studies on IFAS system provided multiple evidences for enhanced nitrification at low temperatures (Di Trapani et al. 2013). However, a detailed analysis of the nitrification rate and its correlation with nitrifying microorganisms is needed to distinguish the contributions of nitrification by suspended sludge and attached biofilm in IFAS system.

The traditional molecular biology methods such as fluorescence in situ hybridization, denaturing gradient gel electrophoresis and terminal restriction fragment length polymorphism have been used to investigate the functional bacteria in the IFAS system (Chae et al. 2008; Onnis-Hayden et al. 2011; Mahendran et al. 2012). These conventional methods enable the detection of several significant bacterial community members in function but lack sufficient sequences to capture comprehensive and systematic information for the analysis of complete community structures. Previous studies offered valuable insights of the dynamics of the microbial community in IFAS. Nonetheless, an investigation of the microbial community dynamics with deeper sequencing depth is needed to reveal more information of temperature adaptation of microbial communities in IFAS system. High-throughput sequencing platforms allow comparative analysis of whole microbial community diversity at greater sequencing depths (Loman et al. 2012). As one of the platforms, the Illumina MiSeq technology has been applied to screen pathogens in environmental samples (Cao et al. 2013; Kumaraswamy et al. 2014), and to study microbial community responses in a drinking water distribution system (Wang et al. 2014), biological wastewater and sludge treatment (Luo et al. 2015; Gilbert et al. 2014; Liang et al. 2014; Tan et al. 2015).

The objective of this study is to investigate ammonia removal, abundance of nitrifying bacteria and microbial community structures in a laboratory-scale IFAS reactor. Contribution of biofilm to nitrification was quantified. Abundances of ammonia oxidizing bacteria (AOB) in both suspended sludge and biofilm samples were quantified by real-time PCR (polymerase chain reaction). Using Illumina MiSeq sequencing this study provided new information regarding the microbial community of suspended sludge and attached biofilm at various temperatures. This study suggested that the more robust ammonia removal rate at low temperatures by biofilm contributed to the relatively stable removal of ammonia in the IFAS reactor.

**MATERIALS AND METHODS**

**Configuration and operating condition of IFAS reactor**

The experiment was conducted in a plexiglass laboratory-scale IFAS reactor for 260 days in a temperature controlled room. The rectangular reactor was 50 cm high, 25 cm long and 20 cm wide with a working volume of 20 L. The reactor was inoculated with activated sludge from a conventional municipal wastewater treatment plant. The mixed liquor suspended solids (MLSS) of inoculated activated sludge was 3,100 mg/L. Polyethylene carriers with a density of 0.95–0.98 kg/m³ and a specific surface area of 500 m²/m³ were added into the reactor. The carrier is a cylindrical shape with 8 mm height and 12 mm diameter. The reactor was filled with 4 L of carriers to achieve a filling fraction of 20% with total surface area of 2 m² for biofilm attachment. Influent was automatically pumped into the reactor at the flow rate of 2 L/h with a theoretical hydraulic retention time of 10 h. A settler was placed after the reactor for sludge separation. A peristaltic pump provided 0.6 L/h of returned activated sludge to the inlet of the rectangular reactor tank. SRT was controlled at 18 days by wasting an appropriate amount of activated sludge from the settler. Synthetic influent was prepared using the following recipe: 350 mg/L glucose, 150 mg/L NH₄Cl, 55 mg/L K₂HPO₄, 20 mg/L CaCl₂·2H₂O, 20 mg/L MgSO₄·7H₂O, 10 mg/L FeSO₄·7H₂O, 20 mg/L MnSO₄·H₂O, and 20 mg/L KCl. Information about trace elements is available elsewhere (Chae et al. 2008). Temperature, pH and dissolved oxygen (DO) were recorded using oxygen and pH meters. Concentrations of chemical oxygen demand (COD), ammonium, nitrite and nitrate were measured according to the State Environmental Protection Administration of China (SEPA)
standard methods after sample filtration through a 0.45 μm membrane (SEPA 2002). The pH of the mixed liquor was maintained approximately at 7.6 by adding NaHCO₃ solution. The reactor was aerated using filtered ambient air. The DO concentration in mixed liquor was maintained at 3.5 ± 0.5 mg/L. Reactor operation was planned in five phases with operating temperatures of 30, 25, 20, 15, and 10 °C. Biomass was collected at the end of each phase. Seed- ing activated sludge sample was named as J2. Suspended sludge and biofilm samples collected from the IFAS system at 30, 25, 20, 15, and 10 °C were named as S30, S25, S20, S15, S10 and B30, B25, B20, B15, B10, respectively.

Nitrification batch tests

Nitrification experiment was modified from a previous study (Kristensen et al. 1992). Nitrification experiments were conducted on days 101, 144, 185, 220, and 257. Each nitrification experiment of suspended sludge consisted of 400 mL mixed liquor. And each nitrification experiment of biofilm biomass consisted 13 carriers. A predetermined amount of synthetic wastewater, the same as reactor influent, was then added to six cylinders. For nitrification test of suspended sludge, the same MLSS concentration as for the IFAS reactor was used. For nitrification test of biofilm, the same carrier filling ratio (20% in this study) was adopted (Di Trapani et al. 2011). Aeration through a bottom diffuser provided mixing and maintained the same DO concentration of 3.5 ± 0.5 mg/L. Liquid samples were taken at 0, 45, 90, 135, 180, 225, 270, 315 and 360 min and were rapidly filtered through 0.45 μm membranes. NH₄⁺-N and MLSS concentrations were analyzed according to the standard method. As the study was limited by the relatively small reactor volume and carrier numbers, carriers were not sacrificed for measurement of biomass content of biofilm attached on the carriers. Carriers were returned to the IFAS reactor after the nitrification batch test.

Nitrification contribution tests of suspended sludge and biofilm in the IFAS reactor

To evaluate the contributions of biofilm and suspended sludge to NH₄⁺-N removal in the IFAS reactor, all the carriers in the IFAS reactor were temporarily removed out of the reactors at the end of each operation phase. The removed carriers were temporarily placed in a similar tank supplied with the same synthetic wastewater and aeration intensity. Reactor influent rate and location of effluent tubing were adjusted so that the same MLSS and ammonia loading rate were maintained. The effluent samples were taken every 2 hours until the effluent NH₄⁺-N concentration stabilized. Effluent samples were rapidly filtered through 0.45 μm membranes and then the NH₄⁺-N was analyzed. The contribution of biofilm to NH₄⁺-N removal was estimated using the following equation.

\[
R = \frac{Ne' - Ne}{N_0} \times 100\%
\]

where R is contributions of biofilm to NH₄⁺-N removal (%), \(N_0\) is the influent NH₄⁺-N concentration (mg/L), \(Ne\) is the effluent NH₄⁺-N concentration before removing carriers out of the reactors (mg/L), and \(Ne'\) is the stabilized effluent NH₄⁺-N concentration after removing carriers out of the reactors (mg/L).

DNA extraction and real-time PCR

The suspended sludge and biofilm samples of the reactor collected at 10, 15, 20, 25 and 30 °C were gathered in triplicate and preserved at −80 °C before use. DNA of suspended sludge and biofilm was extracted using a PowerSoil® DNA isolation kit (MO BIO Laboratories, CA, USA). The primer set of amoA-1F/amoA-2R was used to amplify the amoA gene. Primer characteristics and conditions for real-time PCR were adopted from a previous publication (Rotthauwe et al. 1997) and are included in Table S1 (available with the online version of this paper). Quantification was based on the fluorescence intensity of the SYBR Green dye in a total volume of 20 μL with ABSolute qPCR SYBR Green Rox, 1 μmol/L of each primer and 10 ng of biomass DNA. Reactions were performed in two independent PCR reactions in an ABI7500HT thermal cycler (Applied Biosystems, CA, USA). Briefly, all reactions were finished with a melting curve starting at 80 °C with an increase of 0.5 °C up to 95 °C. The PCR efficiency ranged between 90% and 100%. Standard curves were obtained using serial dilutions of linearized plasmids (pGEM®-T Easy Vector Systems, Promega, WI, USA) containing cloned amoA genes.

High-throughput 16S rRNA sequencing and data analysis

Sequencing library preparations and Illumina MiSeq sequencing were conducted at GENEWIZ, Inc. (Beijing, China). DNA samples were quantified using a Qubit® 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA) and DNA quality was checked.
RESULTS AND DISCUSSION

Reactor performance

Figure 1 and Table 1 show the COD removal and nitrification performance in 260 days of the reactor operation. It took the system nearly 40 days for start-up at 25°C after inoculation. The IFAS reactor was operated at 30, 25, 20, 15, and 10°C from Phase 1 to Phase 5. As shown in Figure 1(b), COD removal was stable under different temperatures with removal rate higher than 85%. COD removal by IFAS system at low temperature has been the subject of several studies. A study reported a total COD removal efficiency of higher than 76% by a pilot-scale IFAS reactor feeding with municipal wastewater at near 11.5°C (Di Trapani et al. 2011). Another study of full-scale IFAS system demonstrated higher than 94% COD removal at 10 ± 1°C (Sriwiriyarat & Randall 2005b). These findings suggest that IFAS system achieves sufficient COD removal in a wide range of operating temperatures.

Influent and effluent NH₄-N concentrations are reported in Figure 1(c). NH₄-N removal efficiency varied from 61.9% to 96.0% with an average of 85.1%. One study evaluated the ammonia concentration profile in an IFAS system across the treatment process and showed that ammonia removal efficiency may decrease after prolonged operation at low temperature (Zhang et al. 2013). It was observed in this study that when temperature decreased from 15°C to 10°C the ammonia removal rate of the IFAS system decreased from 81% to 61%. This phenomenon might be attributed to the decrease of nitrification kinetics and abundance of nitrifying microorganisms in the system at low temperature, which were further investigated in detail in this study.

Effluent nitrate and nitrite concentration ranged from 7.1 mg/L to 27.8 mg/L and from 0.03 mg/L to 1.10 mg/L, respectively Figure 1(d). The unexpected substantial amount of nitrogen removal seemed to be at variance with the design of IFAS reactor without anoxic zone.

However, a similar phenomenon was also observed in related study (Regmi et al. 2011). This could be attributed to the denitrifying bacteria living in oxygen starvation in the inner layer of biofilm in IFAS. Calculated nitrogen removal is reported in Figure S4 based on influent NH₄-N, effluent NH₄-N, and effluent NO₃-N concentrations (Figure S4 is available with the online version of this paper). The specific roles and denitrification mechanism of denitrifying bacteria in IFAS system required further study. A thorough understanding and good control of these functional denitrifying species would be of great significance for process management.

Ammonia oxidation rates of activated sludge and biofilm

Ammonia oxidation rates of suspended sludge and biofilm at different temperatures are shown in Figure 2. The ammonia oxidation rate was positively correlated with the temperature in the range of 10–25°C for biofilm (P = 0.002) and for activated sludge samples (P = 0.005), respectively. The nitrification rate of suspended biomass at 10°C is 12% of that at 25°C, whereas the nitrification rate of biofilm biomass at 10°C is 52% of that at 25°C. Sharp decrease of nitrification rate in wastewater treatment at low temperatures was observed due to lowered growth rate of nitrifying microorganisms (Painter & Loveless 1983; Antoniou et al. 1990) and decreased reaction kinetics (Sharma & Ahlert 1977). Interestingly, when temperature decreased from 15°C to 10°C the
ammonia oxidation rate of suspended sludge decreased by 66\% and that of biofilm was 25\%. As shown in Figure 1, NH$_4^+$-N removal efficiency of reactor was stable at different temperatures. The more robust ammonia removal rate at low temperatures by biofilm may contribute to the relatively stable removal of ammonia in this study.

In Figure 3, the contribution of biofilm to ammonia removal increased from 4.0 $\pm$ 0.9\% to 37.0 $\pm$ 2\% when the temperature decreased from 25°C to 10°C. This phenomenon could be mostly attributed to the special growth pattern of biofilm in IFAS process, which allows for decoupling of the growth rates of nitrifying organisms in biofilm from the SRT of the suspended mixed-liquor phase. This situation is particularly attractive for nitrogen removal process at low temperature since slow-growing nitrifying organisms can be retained on the carriers (Regmi et al. 2011), prolonging the retention time of nitrifying organisms to improve nitrification. However, the advantage of nitrification of biofilm could only be observed when activated sludge did not have sufficient nitrification capacity. At high temperature (30, 25 and 20°C), the contribution of sludge to ammonia removal was on average 90\%. The contribution

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**Figure 1** COD removal and nitrification performance of the IFAS reactor. Dashed lines indicate the days of temperature changes.
of sludge and biofilm to ammonia removal indicated where the nitrification took place at different temperature.

Diversity and abundance of nitrifying bacteria of suspended sludge and biofilm

The Illumina MiSeq sequencing data were used to assess the diversity of AOB and nitrite-oxidizing bacteria (NOB) based on 99% similarity of sequence alignments of 16S rRNA genes. The dominant AOB and NOB species were *Nitrosomonas* and *Nitrospira*, respectively. AOB and NOB genera observed in this study were consistent with the previous publications (Logemann et al. 1998; Layton et al. 2005).

Although nitrifying bacteria are classified in at least six different phylogenetic groups, only AOB within *Betaproteobacteria* and NOB of the genera *Nitrospira* and *Nitrobacter* are commonly found in biological wastewater treatment processes. In our study both Bacteria and Archaea 16S rRNA genes were targeted in Illumina MiSeq data. However, no ammonia-oxidizing archaea (AOA) were detected based on 16S rRNA gene in all samples. It was suggested that an extremely low DO zone might facilitate the growth of AOA in activated sludge process (Park et al. 2006; Zhang et al. 2009). In this study the IFAS reactor was operated at an average DO of 3.5 ± 0.5 mg/L in mixed liquor. While AOA have been detected in IFAS system (Short et al. 2013;
Veuillet et al. (2014); Malovanyy et al. (2015), the reactor operating condition in this study may not promote preferential niche specialization of AOA.

The abundance of AOB was quantified by real-time PCR (Figure 4). The AOB abundance was comparable to previous study at normal temperature (van den Akker et al. 2010). As shown in Figure 4, the abundance of AOB in suspended sludge was higher than that in biofilm when the IFAS reactor was operated at 25°C and 20°C while the relative abundances of AOB in suspended sludge was lower than that in biofilm when operated at 15°C and 10°C. With decreasing temperature from 25°C to 10°C, the abundance of AOB decreased by two orders of magnitude and one order of magnitude in suspended sludge and biofilm samples, respectively. Thus, biofilm biomass could better retain AOB compared with suspended sludge when temperature decreased to 15°C and 10°C. And this finding might explain the discrepancy of previous studies where Li et al. (2012) observed higher relative abundance of AOB in suspended sludge than in biofilm while other researchers observed the opposite (Kim et al. 2011; Onnis-Hayden et al. 2011; Mahendran et al. 2012). Further analysis by plotting the abundance of AOB against the nitrification rates of suspended sludge and biofilm samples indicated a diminishing effect of increased AOB abundance on nitrification rate (Figure S1, available with the online version of this paper). This phenomenon may be attributed to mass transfer limitation for both suspended sludge and biofilm. The high correlation may suggest that the AOB abundance obtained by real-time PCR targeting amoA gene may be a candidate parameter in IFAS model development. However, more studies will be needed to validate this hypothesis.

**Microbial community structure of suspended sludge and biofilm**

The different nitrification characteristics of sludge and biofilm may relate closely to the microbial community structure in IFAS system. Illumina MiSeq high-throughput sequencing was employed to investigate the microbial community of sludge and biofilm at different temperatures. After filtering, 1,896,991 effective sequences (J2: 175,915 sequences, S30: 115,583 sequences, S25: 129,871 sequences, S20: 200,944 sequences, S15: 230,087 sequences and S10: 127,875 sequences; B30: 204,698 sequences, B25: 135,721 sequences, B20: 223,825 sequences, B15: 129,127 sequences and B10: 302,874) were generated in total. Chao1, Shannon index, and abundance-based coverage estimator (ACE) of seeding sludge, suspended sludge, and biofilm are reported in Table 2. Rarefaction curves are shown in Figure S2 (available with the online version of this paper). Both microbial community diversity and richness decreased along reactor operation from Phase 1 to Phase 5.

One notable finding was that Chao1, the Shannon index, and ACE values of biofilm samples were significantly higher than those values of suspended sludge samples at all tested temperatures, indicating richer and more diverse microbial community structures in biofilm. Microbial community structures at phylum level in different samples are shown in Figure 5. In the IFAS system the predominant phyla were *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* for both suspended sludge and biofilm samples. These phyla were commonly observed in biological wastewater.

<table>
<thead>
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<th>Sample ID</th>
<th>Chao1</th>
<th>Shannon</th>
<th>ACE</th>
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<td>10.45</td>
<td>72,215</td>
</tr>
<tr>
<td>S30</td>
<td>44,294</td>
<td>7.79</td>
<td>39,615</td>
</tr>
<tr>
<td>S25</td>
<td>47,817</td>
<td>7.13</td>
<td>40,214</td>
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<td>S20</td>
<td>61,847</td>
<td>6.80</td>
<td>55,132</td>
</tr>
<tr>
<td>S15</td>
<td>26,061</td>
<td>5.51</td>
<td>25,837</td>
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<tr>
<td>S10</td>
<td>14,627</td>
<td>5.80</td>
<td>25,041</td>
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<td>6.68</td>
<td>25,041</td>
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</table>
treatment process and are ubiquitous in soil (Fierer et al. 2007; Xia et al. 2010; Zhang et al. 2012). The quantity of species in activated sludge was less than that in biofilm when operation temperature decreased to 10°C, indicating more diverse microbial community structures in biofilm at low temperature.

Principal coordinates analysis (Figure 6) was used to identify the different composition of sludge and biofilm community structures. The result clearly showed that the microbial communities in the IFAS reactor were distinct from seeding activated sludge (J2). Suspended sludge and biofilm samples collected during low-temperature period
(15 °C and 10 °C) were distantly related to the samples collected during high temperature period (30, 25 and 20 °C). This agreed with the nitrification characteristics of sludge and biofilm and suggested that two different growth stages divided by temperature existed in the IFAS reactor. Furthermore, the close cluster of suspended sludge and biofilm samples collected at the same temperature suggested a high likelihood of biomass exchange between the two forms of biomass. This phenomenon may be particularly important for nitrification in the IFAS system since bioaugmentation through biofilm growth and detachment may contribute to nitrification in suspended sludge (Boltz et al. 2009a, 2009b; Onnis-Hayden et al. 2011).

**CONCLUSIONS**

Temperature played a key role in shaping nitrification characteristic and microbial community structures in the IFAS reactor. The nitrification rate of suspended biomass at 10 °C is 12% of that at 25 °C, whereas the nitrification rate of biofilm biomass at 10 °C is 52% of that at 25 °C. The contribution of biofilm to ammonia removal increased from 4.0 ± 0.9% to 37.0 ± 2% when the temperature decreased from 25 °C to 10 °C. High-throughput sequencing showed that Proteobacteria and Bacteroidetes were the dominant phyla in both biofilm and suspended sludge samples in the IFAS reactor. The dominant AOB species was *Nitrosomonas* and the dominant NOB species was *Nitrospira*. Real-time PCR revealed that the abundance of AOB in biofilm was also higher than that in suspended sludge at 15 °C and 10 °C. The more robust ammonia removal rate at low temperatures by biofilm may contribute to the relatively stable removal of ammonia in this study. The understanding of the nitrification characteristic and microbial community structures in IFAS process in this study will be helpful in developing efficient strategies for IFAS wastewater treatment plant operation.

**ACKNOWLEDGEMENTS**

This research is supported by the National Science and Technology Major Project of the Ministry of Science and Technology of China (Grant No. 2012ZX07313-001 and Grant No. 2013ZX07314-003) and Natural Science Foundation of Jiangsu Province of China (Grant No. BK2011324).

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