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Effect of nitrilotriacetic acid on bioavailability of nickel during methane fermentation

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Abstract

In the case of anaerobic reactors for the treatment of wastewaters, the ubiquitous presence of sulfide and carbonate leads to extensive precipitation of metals like nickel, which are essential for the metabolism of the anaerobic microorganisms that carry out the mineralization of the pollutants present in the wastewater. This study is concerned with the complexation of nickel with nitrilotriacetic acid (NTA) and its effect on the biogas production by methanogenic archaea in the presence of sulfide. Methane production was enhanced 10, 30 and 48%, at sulfide concentration of 0.25, 0.5 and 1 mM, respectively, by the addition of 10 μM NTA. Accompanying with that, substrate degradation and methane production were accelerated and the carbonate and sulfide fraction of nickel in the biomass decreased 28.8 and 58.0%. NTA boosted the internalization of Ni, consequently enhancing the bioavailability of Ni for methanogenic archaea and promoting the methane production.

Keywords: Nitrilotriacetic acid (NTA); Anaerobic digestion; Methane; Metals; Uptake

1. Introduction

The trace element requirement of anaerobic microorganisms is specific because of the many cobalt-, nickel- and iron-containing enzymes involved in the biochemistry of fermentation and methane (CH_4) production [1,2]. Nickel is an important element in anaerobic microorganisms and plays a key role in methane formation. Methanogenic archaea use several pathways to reduce the various carbon substrates (e.g., methanol, acetate, and H_2/CO_2), but all pathways converge on the common intermediate methyl-S-CoM [3]. Methyl-S-CoM contains a nickel-harbouring tetrapyrolic structure, coenzyme F_{430}, present in all methanogens and exclusively found in methanogens [4]. In addition, many hydrogenase enzymes used to form or consume hydrogen gas possess nickel [5]. Carbon monoxide dehydrogenase (CODH), which possesses two nickel-containing metallocenters, is present in both acetoclastic methanogens and acetogenic microorganisms [6]. Nickel may also play a role in the stability of some methanogens, for instance in maintaining wall stability [7]. Therefore, lack of a single metal can severely limit the overall anaerobic conversion process. Previous studies showed that methane fermentation could be enhanced by addition of Ni^{2+}. Ni^{2+} added in concentrations as low as 10 μM significantly increased biogas production in a laboratory poultry waste digester utilizing excreta from laying hens as the organic energy source [8]. Canovas-Dlaz also found that butyric acid conversion by a mixed methanogenic population in a pilot scale anaerobic downflow fixed-film reactor (DFFR), was increased by the addition of nickel (30 mg/L $NiCl_2{\cdot}6H_2O$) [9].

However, in the case of anaerobic reactors for the treatment of wastewaters, the ubiquitous presence of sulfide resulting from sulfate reduction and organic matter mineralization will lead to extensive metal precipitation in the form of sulfides, from which the metals are not (directly) available for uptake by microorganisms. The speciation of metals may play a decisive role in their bioavailability [10]. A number of researchers have examined how the addition of yeast extract to the media affects methanogenesis. Baresi et al. [11] conducted studies on enriched cultures of *Methanosarcina* sp. by varying the concentration of yeast extract from 0.01 to 0.5%. Their results showed a dramatic increase in the rate of methane production in medium containing 0.1–0.5% yeast extract. They concluded that yeast extract may satisfy certain nutrient restrictions of the enrichment and stimu-

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Nomenclature

CH_4	methane
CO_2	carbon dioxide
COD	chemical oxygen demand (mg/L)
CODH	carbon monoxide dehydrogenase
CSTR	continuous stirred tank reactor
DFFR	downflow fixed-film reactor
H_2	hydrogen gas
MPA	methane production archaea
NTA	nitrilotriacetic acid
TSS	total suspended solids (g/L)
VSS	volatile suspended solids (g/L)

late the rate of methanogenesis. But the finding of Gonzalez-Gil et al. [10] suggested that this is due to the formation of dissolved bioavailable complexes, which favors the dissolution of metals from their sulfides. Trace doses of yeast extract may be effective in keeping additions of essential metals to anaerobic reactors at a minimum. Direct addition of yeast extracts to full-scale anaerobic reactors treating chemical wastewaters may not be economically feasible. So in this study we select NTA as a chelating agent to investigate the effect of NTA and Ni on the methane fermentation, according to the changes of methane production and speciation of Ni in the biomass. It is expected that the results obtained from this study could provide valuable information for the application of chelators on the enhancement of methane fermentation.

2. Materials and methods

2.1. Biomass

Biomass was collected from a full-scale anaerobic reactor treating beer wastewater (Wuxi, China). It had a pH value of 7.2. The total suspended solids (TSS) and volatile suspended solids (VSS) concentration of the biomass were 91 ± 1.8 and 65.5 ± 1.5 g/L, respectively. The synthetic wastewater was supplemented for acclimation of the biomass in a 2 L continuous stirred tank reactor (CSTR) digester, which had been operated at $35 \pm 2\,^{\circ}C$. Components of the synthetic wastewater were described in Section 2.2 (except that Ni^{2+} concentration was 20 μM). The hydraulic retention time was 5 days and the acclimation period was 2 months. Prior to an experimental run, several (3–4) successive subcultures in nickel deficient synthetic wastewater were generated for the acclimated biomass.

2.2. Synthetic wastewater

Synthetic wastewater was used in the study. It contained the following components in ultrapure water: 85 mM sodium acetate, 0.50 mM KH_2PO_4, 0.50 mM Na_2HPO_4, 3.7 mM NH_4Cl, 0.50 mM $MgCl_2$, 0.89 mM $CaCl_2$, and 1.0 mM Na_2S. Acid and alkaline solutions of trace elements (1 mL of each per liter) was used. The acid trace element solution contained the following components: 7.5 mM $FeCl_2$, 1.0 mM H_3BO_3, 0.50 mM $ZnCl_2$, 0.10 mM $CuCl_2$, 0.50 mM $MnCl_2$, 8.5 mM $CoCl_2{\cdot}6H_2O$ and 50 mM HCl. The alkaline trace element solution contained the following components: 0.10 mM Na_2SeO_3, 0.10 mM Na_2WO_4, 0.10 mM $Na_2MoO_4{\cdot}2H_2O$, and 10 mM NaOH. Nickel was added as required in the form of $NiCl_2{\cdot}6H_2O$.

2.3. Operating conditions

All experiments were carried out in 150 mL glass serum bottles. The bottles were properly capped and connected to liquid displacement system for recording methane production with time. The liquid in the system was 15% NaOH solutions. Schematic view of experimental set-up was presented in Fig. 1.

Required amount of biomass was added to serum bottles which contained 100 mL synthetic wastewater to get VSS concentration of 5 g/L. After seeding and adjusting pH to 7.0 ± 0.2, the bottles were flushed with N_2 gas for 5 min to provide anaerobic conditions, then sealed with natural rubber stoppers and plastic screw-caps. They were incubated in a temperature-controlled room at $35 \pm 2\,^{\circ}C$ and gas production in each bottle was measured daily with the liquid displacement device. After gas measurement the bottles were manually shaken. In serum bottles with biomass and 100 mL ultrapure water (purged of oxygen with nitrogen gas), control experiments were conducted in parallel to determine the background gas production.

Additional reaction bottles were also arranged to test COD and pH changes in the anaerobic digestion. Each experiment was performed in triplicate, and averaged results were presented here.

2.4. Analysis

TSS, VSS and COD were measured according to standard methods [12]. Before the total dissolved Ni^{2+} concentrations were determined by atomic absorption spectrometry (SpectraAA220, Varian, USA), the samples were acidified with 0.1 M HNO_3.

The extraction procedure used in the present study is the scheme originally described by Stover et al. [13], in which the

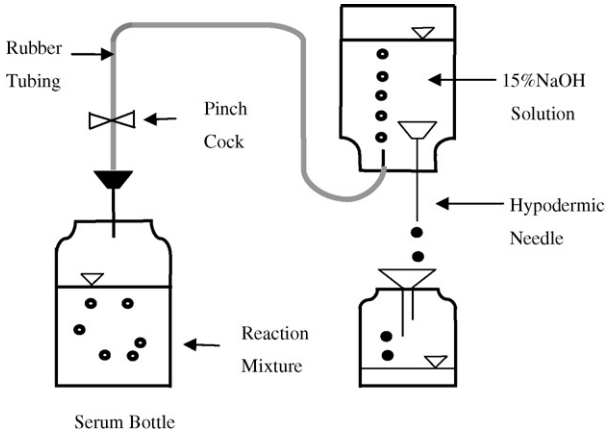

Fig. 1. Schematic of batch experiment set-up.

Table 1
Sequential chemical procedure used to fractionate metals in anaerobic samples

Fraction	Extracting agent	Extraction conditions	
		Shaking time[a]	Temperature
F1. Exchangeable	30 mL KNO_3 (1 M, pH 7)	16 h	20 °C
F2. Sorbed	48 mL KF (0.5 M, pH 6.5)	16 h	20 °C
F3. Organically bound	48 mL $Na_4P_2O_4$ (0.1 M)	16 h	20 °C
F4. Carbonates	48 mL EDTA (0.1 M, pH 6.5)	2 × 8 h	20 °C
F5. Sulfides	30 mL HNO_3 (1 M)	16 h	20 °C
F6. Residual	10 mL demineralized water and 10 mL aqua regia (HCl/HNO_3, 3:1)	26 min	Microwave-oven[b]

[a] Shaking was applied at 200 rpm.
[b] Extraction of the residual fraction in the microwave was equal to the pseudo-total extraction method.

reagents were used for the selective solubilisation of the metals. Details of the extraction procedure are shown in Table 1.

3. Results and discussion

3.1. Effects of total sulfide on methane production during anaerobic digestion

Because the biomass was acclimated for deficient of Ni^{2+}, we first studied the uptake of nickel as reflected by the biogas production by the enriched methanogenic archaea under different sulfide concentrations. The methane production due to the conversion of the substrate was measured over time. Ni concentration was 20 μM. As total sulfide increased from 0.25 to 1 mM, the methane production decreased from 188 to 132 mL (Fig. 2). This is unlikely to be due to sulfide toxicity effects, since 1 mM was previously found to be below toxic concentrations [14]. Because the solubility product constant (K_{sp}) of NiS is as low as 2×10^{-21}, S^{2-} can easily precipitate with Ni^{2+}. Therefore, as sulfide concentration increases, more Ni should exist as the precipitate. Then the availability of Ni for methane production archaea should be decreased. The software Visual MINTEQ was used to solve simultaneous chemical equilibrium and to calculate the theoretical concentration of metallic forms in anaerobic environments. Calculations were done based on the feed composition to demonstrate what the distribution of the metal looks like in an anaerobic environment without biomass. It can be seen from Table 2 that when sulfide concentration increased from 0.25 to 1 mM, the free Ni^{2+} concentration decreased from 5.8 to 1.4% of total Ni^{2+} concentration. So it seemed that the decrease in free Ni^{2+} concentration caused the reduced methane production.

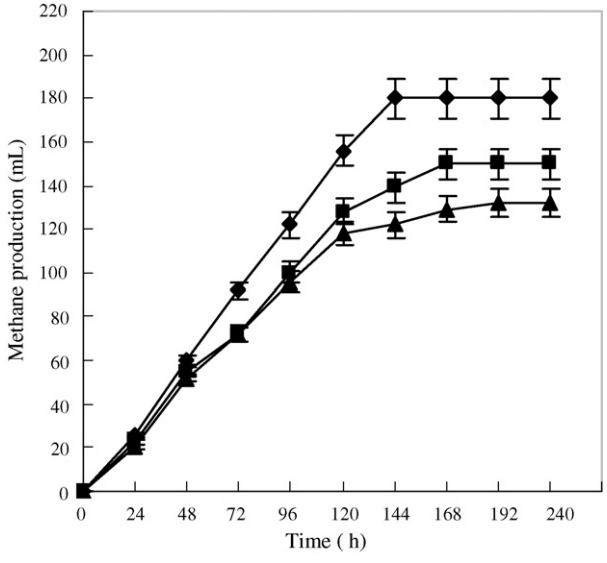

Fig. 2. Methane production due to the anaerobic conversion of acetate at various S^{2-} concentrations without NTA. Ni was added at 20 μM. Sodium sulfide was added at 0.25 (♦), 0.5 (■), and 1 mM (▲).

3.2. Effect of Ni concentration on methane production in the presence of sulfides

As sulfide concentration went up, the free Ni^{2+} concentration decreased (Table 2) and methane production was also cut down significantly (Fig. 2). So it showed that 20 μM Ni^{2+} could not meet the demand of methane production archaea (MPA) under sulfide concentration of 1 mM (Fig. 2). Previous study showed nickel at trace dosage could stimulate methane fermentation [15]. But the effect of higher nickel concentration on methane fermentation was not investigated. So tests were performed for nickel additions in the range of 0–20 mM, and sulfide was also added at 1 mM. The availability of a chemical element could be either too high causing toxicity or too low causing limitation of a microbial process. Limitation by a chemical element is shown if the rate of a process increases by addition of that element [16].

In the presence of sulfide, even at total nickel concentrations as high as 200 μM, metals seem to limit the rate of methanogenesis from acetate because the methane production was still higher at nickel concentration of 2000 μM than that at nickel concentration of 200 μM. The biogas production rate increases with increasing metal concentration (Fig. 3). When the Ni^{2+} concen-

Table 2
The fraction of Ni speciation in bottles estimated by use of software Visual MINTEQ

S^{2-} concentration (mM)	Ni^{2+} (%)	NiS (%)	$NiNH_3^{2+}$ (%)	$NiHPO_4$ (%)
0.25	5.8	86.2	0.1	0.35
0.50	2.9	93.1	0.03	0.2
1.00	1.4	96.6	0.02	0.08

The total Ni concentration was 20 μM. NTA was absent.

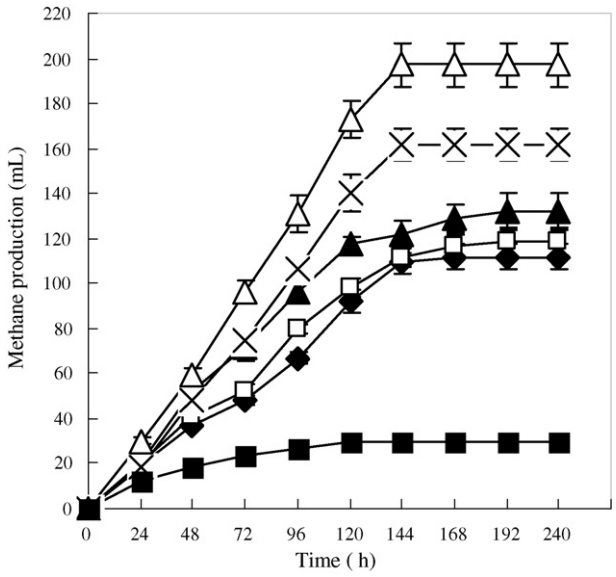

Fig. 3. Methane production due to the anaerobic conversion of acetate at various Ni^{2+}concentrations without NTA. Sodium sulfide was added at 1 mM. Ni was added at 0 (♦), 1 (□), 20 (▲), 200 (×), 2000 μM (△) and 20 mM (■).

tration was 1, 20, 200 and 2000 μM, methane production was 119, 132, 162 and 197 mL, which increased 6.30, 17.9, 44.6 and 65.5%, respectively, compared with the control. Gonzalez-Gil et al. [17] found that methane production rate increased sharply in anaerobic conversion of methanol with increasing Ni concentration from 0 to 400 μM. He also calculated equilibrium dissolved metal concentrations with different initial doses of Ni and Co. The calculated concentrations of free Ni and Co should increase sharply only when the initial dose of Ni and Co are more than 100 μM, corresponding to the moment that the amount of sulfide added becomes limiting for precipitation reactions. Our results (Fig. 3) here also confirmed that the metabolic activity of the anaerobic biomass is metal-limited. The chemical form of the metals (speciation) will determine their availability for the microorganisms. Metal sulfide precipitates are not directly available for uptake by biomass. Therefore, in practice, the metal is usually added in a large amount and this will increase costs.

On the other hand, too high concentration of nickel caused inhibition in methane production process. As can be seen from Fig. 3, when the nickel concentration was 20 mM, the methane production was inhibited severely and methane production was only 30 mL, which decreased 73.2%, compared with the control. Ni^{2+} was also a potent inhibitor of macromolecular synthesis such as RNA and proteins if its concentration is too high [18]. So the dosage should be paid more attention according to the condition of the reactors such as the concentrations of sulfides, carbonates and phosphate, which should precipitate with the metals.

3.3. Methane production under different sulfide concentrations by addition of NTA

When sulfide concentration was 1 mM, Ni^{2+} seemed to limit the rate of methanogenesis because of the formed precipitate with S^{2-}. A large amount should be added to the reactors so that methane production can be increased significantly. If the precipitate can be avoided or minimized, can the methane production be affected greatly by different sulfide concentrations (below 1 mM)? In order to promote the dissolution of metal ions from precipitates, such as metal sulfide, which was unavailable for methanogens, NTA is selected, which can form stable complexes with metals, to be added to the reaction bottles. Then the changes in methane production were investigated. NTA concentration was 10 μM.

Table 3
The fraction of Ni speciation in bottles estimated by use of software Visual MINTEQ

S^{2-} concentration (mM)	Ni^{2+} (%)	Ni-NTA (%)	NiS (%)
0.25	0.25	95.59	3.80
0.50	0.19	93.51	6.03
1.00	0.14	90.23	9.42

The total Ni concentration was 20 μM. Ten micromole NTA was added.

The software Visual MINTEQ was used to solve simultaneous chemical equilibrium and to calculate the theoretical concentration of metallic forms under different sulfide concentrations. It can be seen from Table 3 that when sulfide concentration increased from 0.25 to 1 mM, there was no great difference in the dissolved Ni^{2+} concentration. Most of nickel was complexed with NTA. Comparing Table 2 with Table 3, it can be seen that NiS was mostly dissolved. As the formation of precipitate between Ni and sulfides was avoided, there was also little difference in the methane production (Fig. 4). Nickel bioavailability in anaerobic treatment reactors is of especially importance because it is a component element of coenzyme F_{430}. Although there was no uniform theory about the bioavailability of metal complexes, it was observed from our results that Ni-NTA can be taken up by MPA, which was speculated from the

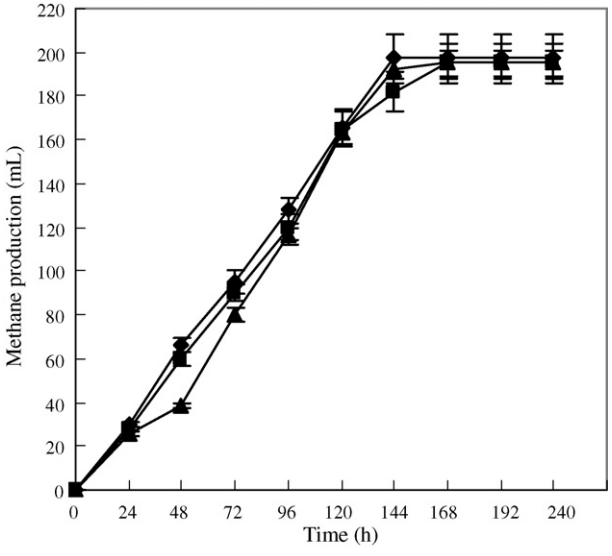

Fig. 4. Methane production due to the anaerobic conversion of acetate at various S^{2-}concentrations with 10 μM NTA. Ni was added at 20 μM. Sodium sulfide was added at 0.25 (♦), 0.5 (■), and 1 mM (▲).

changes in methane production. Compared Fig. 2 with Fig. 4, it was observed that at sulfide concentration of 0.25, 0.5 and 1.0 mM, methane production was enhanced 10, 30 and 48%, respectively, by addition of 10 μM NTA. Kida et al. [19] investigated the requirement of nickel and cobalt by the biomass. The methanogenic activity of the culture broth increased from 10 to 135 mL/(g-VSS h) with nickel addition. The minimum concentration of Ni^{2+} to meet the amounts in the biomass was estimated to be 27 μg/L of the synthetic wastewater. In our experiments the dissolved nickel concentration was only 16.8 μg/L under 1 mM of sulfide when NTA was absent (Table 2). So it cannot fulfill the demand of the biomass and the rate of methanogenesis was limited. When NTA was presence, dissolved nickel concentration was 1083 μg/L, it could meet the requirement of the biomass and the methane production was enhanced.

In addition, compared Fig. 3 with Fig. 4, it was observed that when 10 μM NTA was added, the methane production was 195 mL under 20 μM of Ni^{2+}, 1 mM of sulfides (Fig. 4). But if NTA was absent, only under 2000 μM of Ni^{2+} could the methane production be 197 mL (Fig. 3). Thus, the Ni^{2+} dosage could be cut down greatly by addition of NTA. It provides an attractive approach to keep the additions of essential metals to anaerobic reactors at a minimum.

3.4. Kinetics of anaerobic digestion with and without NTA

The substrate consumption and product formation kinetics were analyzed using Eqs. (1) and (2), a modified Gompertz equation [20], for a typical run at temperature of 35 °C, sodium acetate of 7 g/L, nickel of 20 μM and sulfide of 1 mM with or without NTA addition:

$$S_0 - S = S_{\max} \exp\left\{-\exp\left[\frac{R_{\max,s} \times e}{S_{\max}}(\lambda_s - t) + 1\right]\right\} \quad (1)$$

$$P = P_{\max} \exp\left\{-\exp\left[\frac{R_{\max} \times e}{P_{\max}}(\lambda - t) + 1\right]\right\} \quad (2)$$

where S_0 is the initial substrate concentration; S is the substrate concentration at fermentation time t; $S_{\max}$ is the potential maximum substrate degradation; $R_{\max,s}$ is the maximum rate of substrate degradation; λ_s is the lag time to exponential substrate degradation; P is the product formed at fermentation time t; $P_{\max}$ is the potential maximum product formed; $R_{\max}$ is the maximum rate of product formed; λ is the lag time to exponential product formed. It has also been used by Mu et al. [21] to describe the product formation in the hydrogen production process.

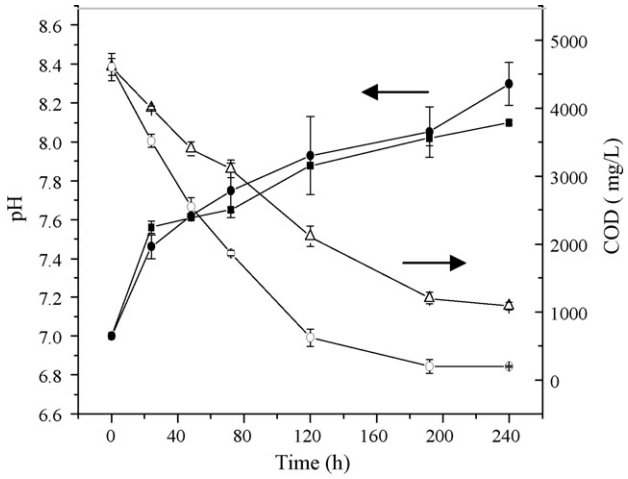

Fig. 5. COD and pH changes during anaerobic conversion of acetate. Sodium acetate concentration was 7 g/L, sulfide was 1 mM, Ni was added at 20 μM, and NTA was 10 μM. (●, pH value in NTA addition bottle; ■, pH value in control bottle; △, COD concentration in control bottle; ◯, COD concentration in NTA addition bottle).

As shown in Figs. 3–5, the acetate degradation, pH value and methane production increased with fermentation time. Its changing patterns were well described by Eqs. (1) and (2). The fitted curves equations and the parameters estimated for the acetate degradation and formation of methane were summarized in Table 4. Correlation coefficients of non-linear analysis by Eqs. (1) and (2) were over 98.6%, suggesting that the modified Gompertz equation was able to describe the process for anaerobic digestion of acetate. It also can be seen from Table 4 that $S_{\max}$ and $R_{\max,s}$ were 3724 mg/L and 23.3 mg/(L h) in control bottles, which were 4442 mg/L and 43.6 mg/(L h) in NTA amended bottles. Likewise, the potential maximum methane production $P_{\max}$ and the maximum methane production rate were indeed higher in 10 μM NTA added bottle than in no NTA added one. This also suggested that methane fermentation could be enhanced by addition of NTA.

3.5. Effect of NTA on speciation of Ni

The distribution of those metals into different fractions, that is, the speciation (organic, inorganic, free ion or chelated), deter-

Table 4
Calculated results using the modified Gompertz equation for substrate degradation and methane formation

NTA	The modified Gompertz equation	$S_{\max}$ (mg/L)	$R_{\max,s}$ (mg/(L h))	λ_s (h)	R^2
Substrate degradation					
None	$S_0 - S = 3724 \times \exp\{-\exp[23.3 \times e \times (3.7 - t)/3724 + 1]\}$	3724 ± 141	23.3 ± 2.3	3.7 ± 1.3	0.994
10 μM	$S_0 - S = 4442 \times \exp\{-\exp[43.6 \times e \times (2.8 - t)/4442 + 1]\}$	4442 ± 177	43.6 ± 1.7	2.8 ± 1.2	0.996
NTA	The modified Gompertz equation	$P_{\max}$ (mL)	$R_{\max}$ (mL/h)	λ (h)	R^2
Methane formation					
None	$P = 136.27 \times \exp\{-\exp[1.25 \times e \times (9.92 - t)/136.27 + 1]\}$	136.27 ± 3.15	1.25 ± 0.06	9.92 ± 0.83	0.992
10 μM	$P = 208.95 \times \exp\{-\exp[1.88 \times e \times (27.29 - t)/208.95 + 1]\}$	208.95 ± 5.67	1.88 ± 0.05	27.29 ± 0.43	0.986

Ni^{2+} addition concentration was 20 μM and sulfides concentration was 1 mM.

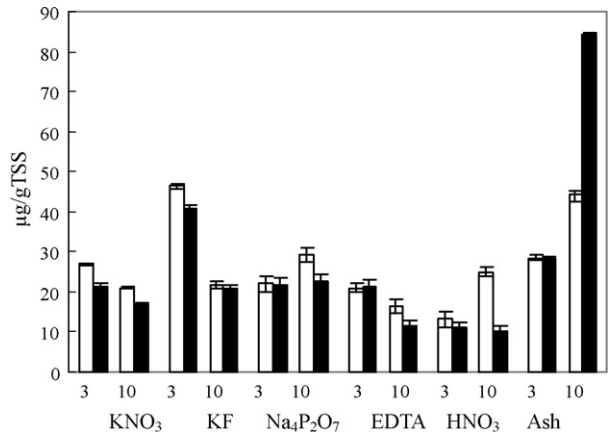

Fig. 6. Ni distribution in samples collected on days 3 and 10 from reaction bottles with and without 10 μM NTA (□, without NTA; ■, with NTA). Ni^{2+} concentration was 20 μM and sulfide concentration was 1 mM.

mines their availability for the metabolic activity [22]. Behaviour of metals in methanogenic biomass is further complicated by their precipitation as sulfides, phosphates, and carbonates [23].

The nickel speciation with the addition of 10 μM NTA were revealed in Fig. 6. Most of the Ni was more strongly adsorbed onto biomass ($Na_4P_2O_7$ extract) or internalized. In the control bottle, on days 10 most of nickel was precipitated as a carbonate or sulfide salt (EDTA and HNO_3 extract), which were 16.19 and 24.72 μg/g TSS, respectively. While in the NTA amended bottle, the corresponding values were 11.52 and 10.39 μg/g TSS, respectively. The carbonate and sulfide fraction of nickel decreased 28.8 and 58.0% with NTA addition. These results seem to indicate that the presence of NTA improved the methane production because it boosted the internalization of Ni, and as the amount of carbonate and sulfide fraction of nickel decreased a lot in the presence of NTA, the results may also suggest that NTA favored the dissolution of Ni^{2+} from their carbonate and sulfides and a direct uptake of the complex NTA–Ni might have occurred. These results tend to agree with the findings of Gonzalez-Gil et al. [10] and Aquino Sérgio and David [24], which showed that the addition of yeast or SMP increased the bioavailability of metals because of the complexation of metals with them.

4. Conclusions

The results presented in this paper showed that the presence of 10 μM NTA enhanced the methane production, and this seemed to be related to the increased bioavailability of metals caused by the complexation of nickel with NTA. By addition of NTA, substrate degradation and methane production were accelerated and the kinetic constant $R_{max,s}$ (the maximum rate of substrate degradation) and R_{max} (the maximum rate of product formed) were increased from 23.3 mg/(L h) and 1.25 mL/h to 43.6 mg/(L h) and 1.88 mL/h. The nickel distribution in anaerobic treatment systems amended with NTA suggested that NTA favored the dissolution of Ni^{2+} from their carbonates and sulfides. A direct uptake of the complex of NTA–Ni by the biomass might have occurred, hence the deficient of metal nutrients for methane production archaea was overcome and methane production was significantly improved.

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