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Evaluation of cation inhibition and adaptation based on microbial activity and community structure in anaerobic wastewater treatment under elevated saline concentration

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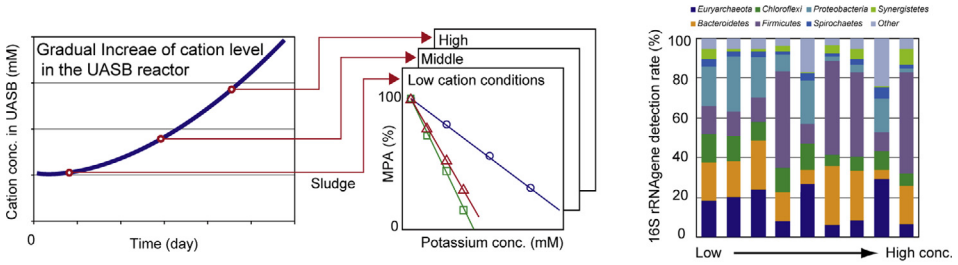
HIGHLIGHTS

- A UASB reactor was operated under elevated cation levels at 35 °C for 300 days.
- The 50% inhibitory K^+ concentration was 600 mM for H_2/CO_2 and 300 mM for acetate.
- Tolerance to cation inhibition and dominant archaea were not changed.
- Sludge adapted to moderate salinity had proper tolerance for high salinity water.

GRAPHICAL ABSTRACT



(A) UASB reactor experiment (35°C) (B) Cation inhibition assey (C) Microbial community analysis

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ABSTRACT

Continuous flow and batch experiments were carried out under mesophilic conditions (35 °C) to determine the effects of gradual increases in cation concentrations on specific methane producing activity (MPA) and microbial community structure in an upflow anaerobic sludge blanket (UASB) reactor fed with molasses wastewater for 300 days. The cation inhibition assay based on MPA as a function of potassium concentration was performed using the retained sludge. The 50% inhibitory concentration for potassium was 600 mM for H_2/CO_2 substrate, 300 mM for acetate, and 280 mM for propionate, and inhibition levels were clearly proportional to potassium concentration. The tolerance of retained sludge to potassium inhibition was not clearly changed from low to high saline conditions (day 59, 111, and 201). The 16S rRNA gene sequencing analysis revealed that *Methanobacterium* and *Methanosaeta* were the dominant archaea, and the archaeal community was not obviously changed at the genus level during operation.

1. Introduction

Bioethanol production and demand has grown rapidly in response to increased concerns regarding energy security and greenhouse gas (GHG) emissions [1,2]. However, bio-ethanol production processes generate a large amount of high strength wastewater, especially during distillation, which has the potential to cause severe water pollution, as well as considerable GHG production and release into the environment [3–5]. Therefore, use of appropriate wastewater treatments is necessary to reduce GHG emissions associated with bio-ethanol production. Stabilization ponds are commonly used as a simple and low-cost method of wastewater treatment in developing countries; however, these have been attributed to increasing GHG emissions [6]. Conversely, anaerobic digestion with biogas recovery has the potential to significantly reduce GHG emissions [6].

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Molasses is recognized as a promising feedstock for bio-ethanol production because it is a byproduct of sugar production that does not compromise food security. Proposed treatment systems for molasses wastewater include highly-efficient anaerobic reactors at a high organic loading rate (OLR) of 42 kg COD m^{-3} day^{-1} under both mesophilic [7] and thermophilic conditions [8]. However, molasses wastewater contains a significant amount of inorganic dissolved salt, especially potassium, as well as organic substances [3,4]. These compounds, which originate from the molasses itself, cause cation inhibition during anaerobic digestion [9]. Although cations of salts are associated with the anions, the inhibitory effects of salts on microorganisms are mainly related to the cations [10]. Contamination with high levels of salts causes dehydration of the bacterial cells under high-osmolarity growth conditions [11]. Even though they survive, high salt levels have deleterious effects on the enzyme catalytic rate [12], leading to decreased microbial activity of the retained sludge.

Dilution of high salinity wastewater with freshwater before anaerobic treatment effectively suppresses cation inhibition; however, this method consumes water, increases the volume of wastewater discharged, and increases operational costs. Accordingly, it is helpful to have reliable data describing cation inhibition levels based on microbial activity as a function of cation concentration. However, inhibition levels have varied in previous studies. For example, IC_{50} values for acetate-utilizing methanogens were shown to be K^+ 150 mM, Na^+ 320 mM, Mg^{2+} 80 mM, and Ca^{2+} 120 mM [13]. Conversely, the 50% inhibition level for acetate-utilizing microorganisms was reported to be K^+ 740 mM, with a threshold of 430 mM [14]. Rinzema et al. (1988) reported that this difference could be attributed to antagonistic and synergistic effects, differences in sensitivity between microorganisms, and differences in the test method [15].

Although the adaptation of biomass to high saline conditions is important for the design and operation of reactors, reports of adaptation have varied [9,16]. Jeison et al. (2008) reported a 50% inhibitory concentration of sodium based on an increase in the maximum specific methane production rate during continuous flow experiments [17]. In addition, Omil et al. (1995) showed significant adaptation of biomass in a pilot plant digester for the treatment of saline wastewater [18]. Conversely, Rinzema et al. (1998) found no adaptation of *Methanothrix* sp. (genera *Methanosaeta*) in the granular sludge of continuously fed UASB reactors to high sodium concentrations of 10 g L^{-1} within 12 weeks [15]. Similar to variations in cation inhibition levels, the conflicting results in adaptation might result from differences in the experimental methods and microbial community structure.

Despite adaptation to high saline conditions causing an abundance of tolerant microorganisms, the effects of cation inhibition on microbial community structure has not been thoroughly investigated. Therefore, this study was conducted to comprehensively evaluate the effects of cation inhibition on reactor performance, specific methane producing activity (MPA), and microbial community structure under elevated saline concentrations in an anaerobic reactor fed with molasses wastewater. A cation inhibition assay based on an MPA test as a function of potassium concentration was conducted to determine the inhibition levels. Simultaneously, temporal changes in the microbial community structure were determined by 16S rRNA gene sequencing analysis of samples collected under low to high salinity conditions.

2. Materials and methods

2.1. Reactor operating conditions

Two lab-scale upflow anaerobic sludge blanket (UASB) reactors with a total volume of 2.0 L (column 1.3 L and gas solid separator 0.7 L) were operated separately at 35 °C (Fig. S1). The UASB reactors were inoculated with granular sludge that had not been acclimated to high saline conditions from another UASB reactor treating molasses wastewater (max. 40,000 mg chemical oxygen demand: COD L^{-1}) at 35 °C that had been in operation for >300 days [7]. The inoculum in terms of the maximum cation concentration was approximately 50 mM K^+ and 190 mM Na^+ [19]. The inoculum volume was 6.1 g for total suspended solids (TSS) and 4.3 g for volatile suspended solids (VSS); VSS/TSS ratio was 0.71.

Two UASB reactors were operated in parallel. The operating conditions are shown in Table 1. The HRT of the UASB reactor was 1 day on Phase 1 and 2. The UASB reactor was operated under elevated cation concentrations, while the reference reactor operated under conditions of Phase 1 and 2. The concentration of influent applied to the UASB reactor was increased stepwise after being allowed to undergo pre-acidification for a few days. The synthetic wastewater was composed of raw molasses diluted with tap water for the desired COD levels. The raw molasses contained 915,000 mg/L COD, 1200 mM K^+, 87 mM Na^+, 160 mM Mg^{2+}, and 170 mM Ca^{2+} (Table S1). The OLR was maintained at 3 kg COD m^{-3} day^{-1} throughout the experiment, except for Phase 1 (day 0–9). A part of the effluent was recirculated to maintain the same upflow velocity. Sodium bicarbonate was used to provide alkalinity from day 54 to 97. The temperature of the reactor was kept at 35 °C throughout the experiment.

2.2. Cation inhibition assay based on methane producing activity

Cation inhibition levels were determined by MPA as a function of cation concentration. The cation inhibition assay was conducted using the retained sludge taken from the UASB reactor at the end of Phase 2, 3, and 4 (day 59, 111, and 201). The experimental procedure was shown in Fig. S2. For the assay, all retained sludge was taken from the UASB reactor temporarily, after which a part of the sludge sample was used for the test and the remaining sludge was returned to the UASB reactor. The sludge was washed with 25 mM phosphate buffer (pH 7.0) twice to eliminate the residual organic substances, then homogenized under anaerobic conditions. The homogenized sludge and medium were fed into 120 mL serum vials filled with nitrogen gas. The sludge concentration in the vials on day 59, 111, and 201 was 0.88, 0.91, and 0.63 g VSS L^{-1}, respectively. All assays were carried out in duplicate at 35 °C. The vials prepared the previous day without substrate were kept under experimental conditions overnight while shaking at 120 rpm. On the experimental day, the pH of the medium mixed with sludge

Table 1
Operating conditions of the UASB reactor. The reactor volume was 2 L. The reference reactor was operated under the same conditions as Phase 2 after day 10. The flow rate (influent flow rate + recirculation) was adjusted to approximately 4000 ml day^{-1} during the operational periods.

	Unit	Phase 1	Phase 2	Phase 3	Phase 4	Phase 5
Operating period	days	0–9	10–63	64–126	127–213	214–294
Influent COD	g L^{-1}	1.5	3.0	120	240	460
Influent flow rate	ml day^{-1}	2000	2000	50	25	13
Recirculation	ml day^{-1}	2000	2000	4000	4000	4000
COD loading	kg COD m^{-3} day^{-1}	1.5	3.0	3.0	3.0	3.0