Water Research 147 (2018) 142–151

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

The potential of osmolytes and their precursors to alleviate osmotic stress of anaerobic granular sludge.

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ARTICLE INFO

Article history:
Received 3 August 2018
Received in revised form
27 September 2018
Accepted 29 September 2018
Available online 2 October 2018

Keywords:
Osmolytes
Anaerobic granular sludge
Methanogenic activity
Saline wastewater

ABSTRACT

Increasing amounts of saline (waste)water with high concentrations of organic pollutants are generated globally. In the anaerobic (waste)water treatment domain, high salt concentrations are repeatedly reported to inhibit methanogenic activity and strategies to overcome this toxicity are needed. Current research focuses on the use of potential osmolyte precursor compounds for osmotic stress alleviation in granular anaerobic sludges upon exposure to hypersalinity shocks. Glutamic acid, aspartic acid, lysine, potassium, gelatine, and tryptone were tested for their potential to alleviate osmotic stress in laboratory grown and full – scale granular sludge. The laboratory grown granular sludge was adapted to 5 (R5) and 20 (R20) g Na^+/L. Full-scale granular sludge was obtained from internal circulation reactors treating tannery (waste)water with influent conductivity of 29.2 (Do) and 14.1 (Li) mS/cm. In batch experiments which focused on specific methanogenic activity (SMA), R5 granular sludge was exposed to a hypersalinity shock of 20 g Na^+/L. The granular sludge of Do and Li was exposed to a hypersalinity shock of 10 g Na^+/L with sodium acetate as the sole carbon source. The effects on R20 granular sludge were studied at the salinity level to which the sludge was already adapted, namely 20 g Na^+/L. Dosing of glutamic acid, aspartic acid, gelatine, and tryptone resulted in increased SMA compared to only acetate fed batches. In batches with added glutamic acid, the SMA increased by 115% (Li), 35% (Do) and 9% (R20). With added aspartic acid, SMA increased by 72% (Li), 26% (Do), 12% (R5) and 7% (R20). The addition of tryptone resulted in SMA increases of 36% (R5), 17% (R20), 179% (Li), and 48% (Do), whereas added gelatine increased the SMA by 30% (R5), 14% (R20), 23% (Li), and 13% (Do). The addition of lysine, meanwhile, gave negative effects on SMA of all tested granular sludges. Potassium at sea water Na/K ratio (27.8 w/w) had a slight positive effect on SMA of Do (7.3%) and Li (10.1%), whereas at double the sea water ratio (13.9% w/w) had no pronounced positive effect. R20 granular sludge was also exposed to hyposalinity shock from 20 down to 5 g Na^+/L. Glutamate and N-acetyl-β-lysine were excreted by microbial consortium in anaerobic granular sludge adapted to 20 g Na^+/L upon this exposure to hyposalinity. A potential consequence when applying these results is that saline streams containing specific and hydrolysable proteins can be anaerobically treated without additional dosing of osmolytes.

1. Introduction

Economic sectors such as leather tanning, chemical and agro-food industries generate large amounts of saline (waste)water that is both rich in salt (NaCl) and organic matter (Lefebvre and Moletta, 2006). Le Borgne et al. (2008) reported that around 5% of the globally produced industrial (waste)water is either saline or hypersaline. This amount of saline industrial (waste)water is increasing due to growing industrial activities and recycling of (waste)water (Rozzi et al., 1999; Giustinianovich et al., 2018). A viable option for removing bulk organic pollutants from this water is anaerobic biological treatment. This approach also generates energy and reduces the amount of waste sludge. If sludge aggregation into microbial granules can be achieved, high rate anaerobic

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https://doi.org/10.1016/j.watres.2018.09.059
0043-1354/

reactors such as upflow anaerobic sludge blanket (UASB) should be considered. The excellent settling properties of granular sludge allows for a reduction in the bioreactor footprint (Vieira et al., 2005; Xiao and Roberts, 2010) and the high rate anaerobic reactors are already widely applied for industrial (waste)water treatment (Van Lier et al., 2015).

There are two factors of particular importance when treating (waste)water with high concentrations of soluble chemical oxygen demand (COD) with high rate anaerobic treatment: i) microbial activity; and ii) microbial sludge granulation and stability (O'flaherty et al., 1997; McHugh et al., 2003; Liu et al., 2009; Lu et al., 2013). However, high concentrations of monovalent salts have been repeatedly reported to inhibit the methanogenic activity (Rinzema et al., 1988; Liu and Boone, 1991; De Vrieze et al., 2016b) and cause granular sludge disintegration with subsequent biomass washout from the bioreactors (Rinzema et al., 1988; Ismail et al., 2008; Jeison et al., 2008; De Vrieze et al., 2016a).

Microbial cells have two different strategies for adapting to high osmotic pressure. The first is by increasing their intracellular ion concentration (mainly K^+ and Cl^-). The second is by up taking and/or synthesizing small organic molecules called compatible solutes (Sowers et al., 1990; Oren, 1999; Roberts, 2005). The first strategy is used by *Halobacteria* (Archaea) and *Haloanaerobiales* (Bacteria) and requires energy for adaptation of the cells enzyme machinery (Oren, 1999; Roeβler and Müller, 2001; Müller et al., 2005).

The second strategy, which is used by the majority of the living cells (Pflüger et al., 2005), does not require such adaptation. Additionally, microorganisms applying this mechanism can survive in a broader range of salinity levels (Roeβler and Müller, 2001; Müller et al., 2005; Pflüger et al., 2005). In compatible solute accumulating bacteria, the response to an osmotic shock can be divided into two phases. During the first phase, a rapid influx of potassium ion into the microbial cell is triggered. During the second phase, cellular levels of potassium are decreased and compatible solutes are accumulated instead (Pflüger et al., 2005). Similar responses to osmotic stress have been demonstrated in pure cultures of the methanogenic archaea *Methanococcus thermolitotrophicus* (Martin et al., 2000). Since salinity of (waste)water often fluctuates in time (Vyrides, 2015), this mechanism is likely to be utilized by the microbial consortium of bioreactors treating several types of saline (waste)water.

Several types of molecules can act as compatible solutes: sugars (trehalose, betaine), amino acids (glutamic acid, proline), derivatives of amino acids (N-ε-acetyl-β-lysine, ectoine) (Roberts, 2005), and polyols (Roeβler and Müller, 2001). Uptake from the bulk liquid of such molecules is bio-energetically more favourable than synthesis *de novo* (Oren, 1999). This explains why several studies focused on the potential of dosing osmolytes in anaerobic (waste)water treatment systems. Molecules like glycine betaine, α-glutamate, β-glutamate, trehalose and, N-acetyl-β-lysine (Yerkes et al., 1997; Vyrides and Stuckey, 2009; Vyrides et al., 2010; He et al., 2012) have all been demonstrated to have the potential of increasing methanogenic activity when flocculent biomass was exposed to osmotic stress. In a recent review Vyrides and Stuckey (2016) reported that glycine betaine is by far the most studied osmolyte to date. The effects of osmolytes on methanogenic activity of granular sludge are not researched so far. Moreover, the bottle neck for application of osmolytes in practice is the relatively high cost of such molecules (Vyrides and Stuckey, 2016).

Recently, robust anaerobic sludge granulation at high salinity levels (up to 20 g Na^+/L) was reported. The microbial activity was sufficient to achieve a high soluble COD removal efficiency (94%) at a loading rate of 16 g COD/L·d. The source of carbon in the synthetic (waste)water was a mixture of glucose, acetate, and tryptone (Sudmalis et al., 2018). Proteins contain a broad range of amino acids as their natural building blocks (Smith and Friedman, 1984; Di Gioia and Guilbert, 1999). These amino acids are known to act as osmo-protectant precursors in anaerobic microorganisms (Roberts, 2005; Zaprasis et al., 2014). It was hypothesised that, after hydrolysis, tryptone (a mixture of peptides formed by digesting casein with trypsin) supplied the microorganisms with the "building-blocks" for the osmo-protectants uptake/synthesis. Thus hydrolysable proteins are potentially a cheaper alternative to osmolytes if indeed they can alleviate osmotic stress.

In this work, the osmolytes present in granular sludge adapted to 20 g Na^+/L were first identified. Next it was investigated if these osmolytes, their precursors, and proteins were able to alleviate osmotic shock stress in four types of granular sludge. Furthermore, the potential of potassium to alleviate osmotic shock stress was studied, since its rapid uptake is reported as the first response to increase in salinity. The granular sludge was obtained from two full-scale anaerobic reactors treating saline tannery (waste)water and two from laboratory-scale reactors treating synthetic (waste) water at salinity levels of 5 and 20 g Na^+/L.

The results show that not only osmolytes, but also their precursors and proteins can alleviate osmotic stress in all four types of the anaerobic granular sludges.

2. Materials and methods

2.1. Inoculum

Full scale granular sludge was obtained from two internal circulation (IC) anaerobic reactors (Waterstromen, The Netherlands) treating tannery (waste)water in Dongen (Do) and Lichtenvoorde (Li) (The Netherlands). The average influent (waste)water conductivity was 14.1 and 29.2 mS/cm for the IC reactors in Lichtenvoorde and Dongen, respectively. The average influent total COD concentration was 6.8 g/L and 5 g/L in Lichtenvoorde and Dongen, respectively. The average influent chloride concentration was 4 g/L in Lichtenvoorde and 6.7 g/L in Dongen. The granular sludges from the full-scale reactors were pre-washed with 5 g Na^+/L nutrient medium (composition as described in Table 1) and were stored in the same medium at 4 °C before use in batch experiments. This was done in order to have a defined liquid phase ionic composition during the batch experiments.

The laboratory-scale granular sludge adapted to 5 and 20 g Na^+/L was formed from dispersed biomass, as described in detail in Sudmalis et al. (2018) and was kept at 4 °C before use in batch experiments. From this point forward, the granular sludge formed at 5 and 20 g Na^+/L is referred to as R5 and R20, respectively.

2.2. Batch experiments

Three types of batch experiments were performed in this study. The first involved use of tryptone at high COD proportion to study its potential for alleviating salt stress in R5 granular sludge. The second involved an abrupt decrease in salinity and identification of osmolytes in R20 granular sludge. The third type of experiments was performed to study the potential of potassium and potential precursors of osmolytes to alleviate osmotic stress in R5, R20, Do and Li granular sludge. A detailed description of each type of experiment is given in sections 2.2.1., 2.2.2. and 2.2.3.

The batch experiments with laboratory grown granules (R5 and R20) were performed in 118 mL serum bottles with a working volume of 50 mL. The experiments were performed in an incubator at 35 °C and with a 120 RPM mixing speed. The VSS concentration was set to 1 g/L and the COD:VSS ratio was 4:1 (w/w).

The experiments with full scale granules (Do and Li) were performed in Automatic Methane Potential Test System. (AMPTS) II