Adaptation of anaerobic biomass to saline conditions: role of compatible solutes and extracellular polysaccharides.

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Abstract

This study investigated the role of compatible solutes, extracellular polysaccharides (EPS), and nutrients on anaerobic biomass when stressed with salinity. When 1mM of osmoregulants glycine-betaine, α-glutamate and β-glutamate were added separately to serum bottles containing biomass not adapted to sodium, and fed with glucose and 35gNaCl/l, all the compatible solutes were found to alleviate sodium inhibition, although glycine-betaine was found to the most effective. The effect of glycine betaine on different anaerobic bacterial groups under salinity was monitored using VFAs, and showed that methanogens were more protected than propionate utilisers. Moreover, the addition of 1mM of glycine betaine to anaerobic biomass not adapted to salinity resulted in significantly higher methane production rates compared with anaerobic biomass that was exposed for 4 weeks to 35gNaCl/L. Interestingly, under saline batch conditions when the media was replaced totally the culture produced less methane than when only new substrate was added due to compatible solutes cycling between the media and the cell. The elimination of macronutrients from the medium was found to have a more pronounced negative effect on biomass under saline compared with non saline conditions, and because of the synthesis of N-compatible solutes sufficient nutrients should always be present. On the other hand, the absence from the medium of

micronutrients did not further reduce biomass activity under salinity. Finally, a higher production of EPS was obtained from biomass exposed to higher salt concentrations, and its composition was found to change under different saline conditions and time. As a result, biomass under saline conditions had a slightly higher mean flock size compared with the biomass that was not subjected to salinity.

Keywords: salinity, anaerobic biomass, acclimation potential, compatible solutes, extracellular polysaccharides, nutrients.

1. Introduction

Highly saline organic wastewaters are often poorly biodegraded in conventional wastewater treatment plants with non adapted biomass due to the toxic effect of sodium in the wastewater. The high concentrations of salt can cause cell plasmolysis and cell death due to the dramatic increase in osmotic pressure [1, 2]. There are two fundamental strategies for cells to survive under osmotic stress;

a) cells increase the intracellular ion concentration (mainly Potassium) in order to balance the external osmotic pressure, and all intracellular enzymes have to adapt to the new conditions. Anaerobic halophilic bacteria, whose entire physiology has been adapted to high saline environments, use this so called “salt in strategy” [3].

b) many microorganisms accumulate organic solutes called “compatible solutes”. The high external osmotic pressure is balanced within the cytoplasm by organic compatible solutes without the need for special adaptation of the intracellular enzymes, and compatible solutes also serve as protein stabilizers in the presence of high ionic strength inside the cell [2, 3]. These solutes can be synthesized by the cell, or provided by the medium, but for most species uptake from the medium is energetically more favorable than synthesis [4]. Methanogenic archae isolated from moderately saline environments

showed an accumulation of β-glutamine, α-glutamate, N-acetyl β-lysine and glycine betaine [3, 5, 6]. However, there are very few studies in the literature on the use of compatible solutes as osmoprotectants for anaerobic biomass treating saline wastewater. Yerkes et al [7] examined the addition of small concentrations of betaine (1-10mM) to *Methanosarcina* and *Methanosaeta* cultures in sucrose fed batch assays, CSTRs, fluidized bed reactors and UASB reactors; 1mM of betaine was found to be effective in reducing sodium toxicity. However, Vallero et al [8] using a mixture of compatible solutes found no significant reduction of sodium toxicity in thermophilic sulphate reducing biomass in a UASB reactor and batch assays. Nevertheless, to the best of our knowledge there are no studies in the literature on the effect of compatible solutes on a mixed anaerobic culture which is the biomass used in large scale wastewater treatment reactors.

In addition, the synthesis of compatible solutes not only depends on the form of salinity, but also on the available nutrients in the medium. In the absence of nutrients, halotolerant and halophilic bacteria can change the compatible solutes they are generating [9]; this study also investigated the role of macro and micro nutrients on the performance of anaerobic biomass under saline conditions. Apart from compatible solutes, anaerobic biomass also produce extracellular polysaccharides (EPS) to help them survive under sodium toxicity, but there has been very little work in this area. Liu et al [10] examined the accumulation of EPS under metal toxicity, and proposed that EPS exhibited chelating properties which mitigated toxicity. Moreover, under toxic conditions microorganisms generate EPS to act as a diffusion barrier between the cell

wall and extreme environments [11]. The generation of EPS can result in changes in the properties of anaerobic biomass or anaerobic granules. Lefebvre et al [12] noted degranulation occurring in a UASB reactor treating saline tannery wastewater, while Leerdam et al [13] observed small aggregates in a UASB reactor treating methanethiol at high salinity. However, Boardman et al [14] did not report any problems of degranulation using a UASB under high salinity, and Mendez et al [15] using a mesophilic anaerobic filter found that biomass had a strong tendency to form large floccules treating seafood processing wastewater.

Hence the aim of this work was to study a variety of different parameters that affect the acclimation potential of anaerobic biomass to salinity , such as : the effect of different compatible solutes in reducing sodium toxicity; the bacterial group which benefited the most from the presence of glycine betaine in the medium; and, the long term effect of glycine betaine even during the sudden removal of salinity. In addition, to compare the difference in acclimation potential between anaerobic biomass exposed for a long period to salinity, and for biomass that glycine betaine was added without previous pre-exposure to salinity. Finally, to study the performance of anaerobic biomass if macro and micro nutrients were absent from the medium, and the evolution of EPS under salinity over time.

2. Materials and methods

A batch reactor (5 litres) was inoculated with anaerobic sludge from a wastewater treatment plant (Mogden, UK), and operated under a stable organic loading rate (OLR)

of 2 gCOD/L.day for 6 months. The reactor was fed under batch mode every 7 days (6 days of mixing and 1 day for the biomass to settle and then the supernatant was removed and new feed added). Glucose was used as a substrate, and nutrients according to Owen et al [16] were also added. Another batch reactor (5 litres), after 5 months of operation as above, was subjected for 28 days to 30gNaCl/L and an OLR of 2 gCOD/L.day. Biomass was removed from the reactors for batch bioassays [16] and transferred into 165ml serum bottles (2gVSS/L) under anaerobic conditions by continuously flushing the bottles with a gas mixture of 70% N_2 and 30% CO_2 (0.5 l min^{-1}). To each serum bottle, 95 ml of media and 5 ml of a concentrated substrate (glucose) was added to give a final concentration of 2g COD/l before capping it with a leak proof Teflon seal. The serum bottles were placed in an Orbital Incubator Shaker at 37^0C and 200rpm.

To examine the absent of macronutrients the following compounds were excluded from the biomedia [16]: $(NH_4)_2PO_4$, NH_4Cl and $Na_2S.9H_2O$. To investigate the elimination of micronutrients the following compounds were omitted from the biomedia [16]: $MnCl_2.4H_2O$, $CoCl_2.6H_2O$, $CuCl_2.H_2O$, $ZnCl_2$, $FeCl_2.4H_2O$ and Na_2MO_4. Determination of VSS was performed according to Standard Methods [17]. The composition of biogas was determined using a Shimadzu GC-TCD fitted with a Porapak N column (1500 × 6.35 mm). The carrier gas was helium set at a flow rate of 50 ml/min, and the column, detector and injector temperatures were 28, 38 and 128°C, respectively. The peak areas were calculated and printed out on a Shimazdu Chromatopac C-R6A integrator. Samples of 1 ml were collected using 1 ml plastic

syringes (Terumo), and the coefficient of variance (COV) for 10 identical samples was ± 2%.

Volatile fatty acids (VFAs) were measured on a Shimadzu (model 10A) high performance liquid chromatography (HPLC) system with an auto-sampler using an Aminex HPX-87H ion exclusion column (300 mm x 7.8 mm). The sample volume was 50 μl, the column was maintained at 55^{o}C, and the eluent was 0.01 M H_2SO_4 at a flow rate of 0.7 ml/min. VFAs were detected with ultra-violet (UV) light at 210 nm The detection limit was 5 mg l^{-1} for VFAs, and the COV for 10 samples was within ± 8%. Particle size measurement was made using a Malvern Instruments Particle Size Analyser Model 2600C with a helium neon laser. The COV for 4 samples was ± 2%.

For EPS extraction the "steaming" method was employed [18]; 5mL of anaerobic sludge was centrifuged at 13,000rpm for 10 min at 4^{0}C, re-suspended in 10mL distilled water, and steamed in an autoclave at 80^{0}C and 1 bar for 10 minutes. After steaming the samples were re-centrifuged and the supernatant filtered (0. 2mm) to ensure that biomass was separated from the extracted EPS. For Size Exclusion Chromatography (SEC) an Aquagel OH-40 column (Polymer Labs) was used with DI water as the eluent at a flow rate of 0.75ml/min. The sample volume was 50μl, and the column was maintained at ambient temperature with both UV and refractive index (RI) detectors being used to detect the separated components. Standards of linear polyethylene oxide (PEO) and polyethylene glycol (PEG) were used; hence, the results obtained are quoted relative to these linear compounds.

3. Results and Discussion

3.1a. Addition of different compatible solutes to non adapted biomass adapting to sodium.

Anaerobic biomass not acclimated to sodium was exposed to 35 gNaCl/l, and three compatible solutes at a concentration of 1mM (glycine-betaine, α-glutamate and β-glutamate) were tested as antagonists to sodium toxicity. The concentration of glucose was adjusted in these bottles to allow for the COD of the compatible solutes such that the final concentration of substrate in each bottle was 2 gCOD/l, and a series of bottles were run without salinity to control for an excess of substrate. Under no salinity, compatible solutes did not result in any excess production of methane. The total methane for all bottles, under no salinity, was around 44ml CH_4 at 115 hours. From Figure 1 it can be seen that biomass exposed to 35 gNaCl/l without any addition of compatible solutes showed the slowest cumulative methane production. At this level of salinity, 1mM of glycine-betaine added to the biomass resulted in no increase in cumulative production of methane after 120 hours, however, after 310 hours there was a significant increase, and after 520 hours the methane production had increased by nearly 100% over the control. In contrast, the addition of 1mM α-glutamate only slightly enhanced methane production. From the above results glycine betaine was the most effective compatible solute, followed by β-glutamate and then α-glutamate. The glycine betaine, which is one of the most common compatible solutes in bacteria and archaea [19], seems to be the most effective antagonist for the anaerobic biomass to counteract sodium toxicity. In a media without compatible solutes, cells synthesise *de*

novo the compatible solutes so less substrate is available to be converted to methane. However, in the presence of compatible solutes in the medium, uptake from the medium is bioenergetically favored over biosynthesis [4, 6]. This explains the higher methane production rate of biomass when compatible solutes were added.

3.1b. Performance of different bacterial groups with and without glycine betaine at 35gNaCl/L.

Figure 2a shows the accumulation of VFAs for anaerobic biomass exposed to 35gNaCl/L, and the VFAs increase over time indicating inhibition of the system. Methanogens are affected more by sodium toxicity and as a result the acetic acid concentration was 885mg/L after 520 hours. The propionic acid utilisers were also inhibited and after 520 hours reached 220mg/L. In the case where 1mM of glycine-betaine was added (Figure 2b) the total VFAs after 520 hours were 110mg/L compared with 885mg/L in the control. The glycine –betaine was found to be more beneficial for the methanogens compared to the propionic acid utilisers. As can be seen in Figure 2b, after 450 hours there was no acetic acid while the propionic acid was 126mg/L. No conclusion can be drawn for the other bacterial groups as there was little inhibition of them under salinity.

3.1c. Comparison between anaerobic biomass pre-exposed to salinity and glycine betaine addition to a medium with 35gNaCl/L

Figure 3 shows that anaerobic biomass previously expose to salinity (30gNaCl/L in a batch reactor) for 28 days produced 15 ml of CH_4 in 380 hours compared with the 9 ml

of CH_4 in 380 hours for the biomass that was not subjected previously to salinity. However, addition of glycine betaine to the biomass not previously exposed to salinity resulted in 33ml of CH_4 in 380 hours, which is significantly higher than the anaerobic mixed culture exposed to salinity for 28 days. The addition of 1mM of glycine betaine to the biomass previously exposed to salinity resulted in 36ml of CH_4 in 380 hours which is slightly higher compared with the anaerobic biomass that was not exposed to salinity and 1mM of added glycine betaine. These results highlight the positive effect of glycine betaine in dramatically enhancing the acclimation of anaerobic biomass to salinity.

From the literature Rinzema et al [20] found that 12 weeks continuous exposure to salinity did not result in any acclimation of *Methanothrix*. To attain high COD removals of saline wastewater a strategy has to be followed that requires a gradual and long exposure to salinity [21, 22, 23]. However, from our data it appears that this acclimation time can be reduced significantly by supplying low concentrations of glycine betaine to the wastewater. Many industrial wastewaters can have widely varying concentrations of salinity [24], and this can have a negative effect on biological systems [25]. Hence a potential strategy for coping with a sudden increase in the salinity of a wastewater is by the addition of glycine betaine, a relatively cheap compatible solute. This strategy can be less time consuming and more practical than extensive dilution of the wastewater [26], or the gradual exposure of biomass to salinity over a long time period.

3.2 Different feeding strategies.

The effect of different ways of feeding can also have an impact on the tolerance of biomass to sodium. Initially anaerobic biomass was placed in serum bottles and fed with 2gCOD/L under 40gNaCl/L. The CH_4 production after 400 hours was negligible (3 ml CH_4), and different ways of re-feeding were attempted. In the first example (Figure 4), the media was removed by centrifugation and new media added with the same concentration of substrate and salinity. In the second assay, the original medium was not replaced and only concentrated substrate was added in order not to dilute the initial level of salinity (40 gNaCl/L). In the second assay, the biomass showed significantly higher methane production compared with the first. To test if this may have been caused by more available substrate in the second method of feeding, the media in the first feeding was replaced by a new medium with 4gCOD/l instead of 2gCOD/L. This excess of substrate did not result in any significant higher production of CH_4.

The higher methane production using the second method of feeding (not replacing the biomedia) can be explain by the work of Kunte [8]. According to this theory, the extraction and recovery of compatible solutes from the medium is a strategy used by cells to regulate the cytoplasmic solute concentration. Thus, compatible solutes will be cycled between the medium and the cytoplasm to achieve a certain equilibrium. In our study when the medium was removed (first method), all the compatible solutes that were excreted by the cell into the medium were removed, so the cycling of compatible solutes between the media and the cell was disrupted. Thus, after the next feeding, part

234 of the substrate was consumed in order to produce new compatible solutes which
235 reached equilibrium with the bulk media again. When the medium was not removed
236 (second method) and new substrate was added, there was a higher rate of CH_4
237 production since new compatible solutes did not need to be produced. Using this insight
238 the difference in performance of batch and continuous reactors under high salinity can
239 be explained. In batch reactors the media is removed before each feeding so the
240 compatible solutes are removed from the system, and the biomass has to generate new
241 compatible solutes to re-achieve equilibrium. In a continuous reactor the equilibrium
242 between cells and compatible solutes in the media is not changing as abruptly as in the
243 case of batch reactors, consequently higher performance is achieved. Slightly higher
244 performance in a continuous reactor (UASB – 10 hours HRT) compared with a batch
245 reactor was found by Rinzema et al. [20]. Also, Feijo et al. [27] found less inhibition to
246 sodium with continuous exposure than with batch exposure. However, more research
247 should be done to compare the performance of a continuous reactor and a batch reactor
248 exposed to salinity over a long period of time, and the effect of compatible solutes
249 cycling between the biomass and the medium.
250
251 *3.3. Elimination of Macro and Micro nutrients in the presence and absence of*
252 *35gNaCl/L.*
253 Under non-saline conditions the absence of macronutrients and micronutrients resulted
254 in 7 ml and 10.5 ml of CH_4 in 85 hours, respectively (Figure 5a), when a control
255 biomass produced 11.5ml of CH_4. In the presence of 30gNaCl/L (Figure 5b), the
256 anaerobic biomass without micronutrients produced 6.6 ml of CH_4 in 302 hours slightly

257 less with the control anaerobic biomass (7.4 ml of CH_4 in 302 hours). However, the
258 elimination of macronutrients resulted in 1.5 ml of CH_4 in 302 hours. Hence, the
259 performance of anaerobic mixed cultures without macronutrients is more inhibited in
260 the presence of salinity (activity is reduced 2.5 times) compared with normal
261 conditions. With micronutrients, activity was slightly reduced compared to the control
262 for normal and saline conditions. The reduced performance of biomass under salinity
263 without macronutrients can probably be attributed to their limiting ability to synthesize
264 N-containing compatible solutes. Halotolerant and halophilic bacteria under nutrient
265 limitations synthesize sugar and polyol solutes instead of N-containing compatible
266 solutes [8]. However, for non halotolerant organisms the shifting of the N-containing
267 solutes to polyol and sugars could be an energetically costly process. On the other hand,
268 to the best of our knowledge micronutrients are not involved in the synthesis of
269 compatible solutes by the cell. As a result the elimination of micronutrients do not
270 contribute to further inhibition under salinity. These results are in line with Boardman
271 et al. [13] who used palm processing wastewater in batch assays and found the same
272 activity, under salinity, for anaerobic biomass with extra micronutrients and without
273 extra micronutrients. The basic macronutrients (Nitrogen and Phosphorous) were
274 included in the wastewater so the supply of extra micronutrients did not contribute to
275 higher methane production.
276
277 *3.4.a. Production of EPS by anaerobic biomass under high salinity.*
278 Samples at different concentrations of salinity were analyzed by size exclusion
279 chromatography (SEC) using RI and UV detectors at 254nm and 210nm. Since there

was not much difference in the trend of the peaks at 254nm and 210nm, only the UV chromatographs at 254nm are presented. In SEC the organics with low molecular weight (MW) go deeper into the gel pores so take a longer time to elute, whereas the high MW compounds are preferentially excluded from the column. Calibration was taken place using PEG and PEO standards.

As can be seen from Figure 6a, the presence of salt (40 gNaCl/L) resulted in much higher output signals in the chromatogram compared to biomass exposed to no salt. The control biomass had two low peaks after 13 minutes that show that the EPS consisted of material with a MW greater than 58.4 kDa. With higher salinities, the peaks are at higher retention times showing that most of the EPS after 72 hours consisted of material around 10 kDa and 6.45 kDa for biomass exposed to 20 and 40gNaCl/l respectively. The peaks at around 20 minutes that appeared in all the samples were probably substrate intermediates. The composition of EPS from biomass not only changed under higher salinities, but also over time. For the control in Figures 6a at 72 hours and 6b at 24 hours the peaks appeared at the same retention time, but with a lower signal at longer times. The biomass exposed to a salinity of more than 20gNaCl/L produced EPS with a lower MW than at lower salinities. However, the signal at 20gNaCl/l was decreasing over time (from Figures 6 a and b), while with 40gNaCl/L the signal was increasing. The biomass at 20gNaCl/L appeared to acclimatize to the initial stress conditions (Figure 6c) so after 72 hours less EPS was produced than in 24 hours (Figures 6 a and b). During this period part of the EPS could have been released into the media, while part of it was biodegraded by the cell. In the biomass exposed to

40gNaCl/L, the EPS increased over time as a response of the biomass to counteract the high sodium toxicity. As a result, less substrate was available for methane production (Figure 6c). Biomass exposed to high salt concentrations increase their production of EPS as a natural response to osmotic stress outside the cell, and synthesize compatible solutes inside the cell. When biomass starts to acclimatize (eg. biomass at 20gNaCl/l after 24 hours), the EPS is reduced and the compatible solutes inside the cell increase. It is also clear that the composition of EPS during sodium toxicity changes over time due to biodegradation by the cell or release to the medium.

3.4.b. Particle size distribution at 48 and 144 hours for biomass exposed to normal conditions and 40gNaCl/L.

Figure 7 shows the particle size distribution for biomass exposed to low salinity (less than 1g/L), and to 40gNaCl/L for 48 and 144 hours. The distribution ranges from 1.25μm to 275 μm with a mean floc size of 23 μm for the first condition, while the biomass exposed to 40gNaCl/L had the same range of distribution but the mean floc size was 30 μm This slightly higher mean flock size with salinity can probably be attributed to the higher production of EPS that forms on the outer surface of the cell; as a result cells attached more easily to each other. However, Sowers and Gunsalus [28] found that the adaptation of thermophilic Methanosarcina to high salinity resulted in the loss of capacity to produce EPS, and the growth of bacteria in smaller aggregates.

4. Conclusions

- The addition of compatible solutes to the medium increases the performance of anaerobic biomass under saline conditions. Glycine-betaine was the most effective in counteracting sodium toxicity compared to β-glutamate and then α-glutamate.
- Methanogens are the most positively affected microbial group when glycine betaine is present in the saline medium, while propionate utilisers are less positively affected.
- Anaerobic biomass exposed for 28 days to 35gNaCl/L showed low acclimatization to salinity. On the other hand, anaerobic biomass that had not been exposed to salinity before but had glycine betaine added to the saline medium increased its acclimation potential significantly.
- Non-replacement of the medium with the addition of a substrate enhanced methane production, while the replacement of old media with the addition of new media and substrate resulted in significant inhibition due to disruption of the compatible solute balance.
- The elimination of macronutrients from the culture media resulted in more severe inhibition for anaerobic biomass with salinity than under non saline conditions. The removal of micronutrients from the culture media resulted in almost the same activity for anaerobic biomass under both conditions.
- Anaerobic biomass produces higher amounts of EPS under high salinity compared with non saline environments. The MW composition of EPS under sodium toxicity changes over time.
- Anaerobic biomass exposed to salinity resulted in a slightly higher mean floc size compared with anaerobic biomass exposed to non saline conditions.

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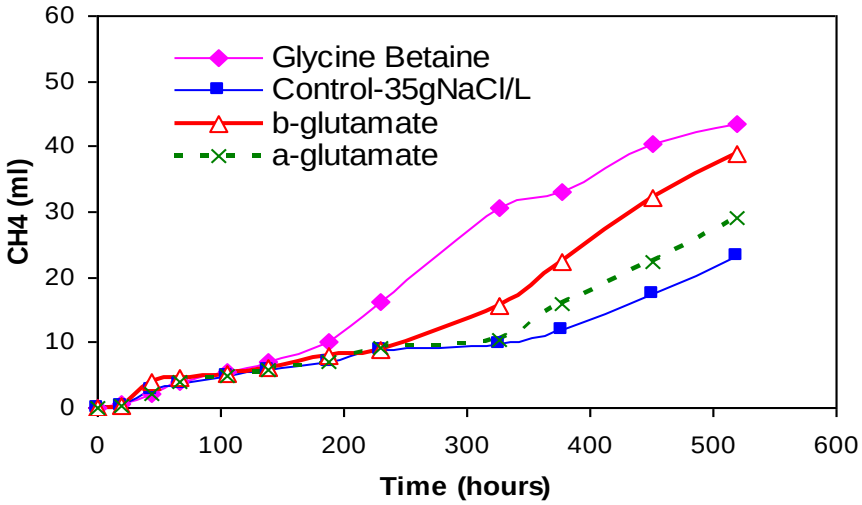

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434 Figure 1. Response of biomass to 35gNaCl/L in a control, and with 1mM of glycine betaine, α-

435 glutamate, β-glutamate added to the culture.

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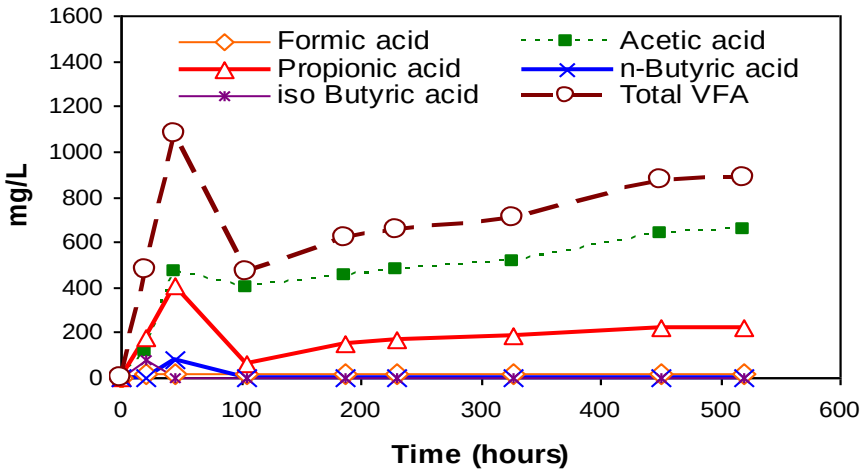

Figure 2a Production of VFAs over time for anaerobic biomass exposed to media containing 35gNaCl/L.

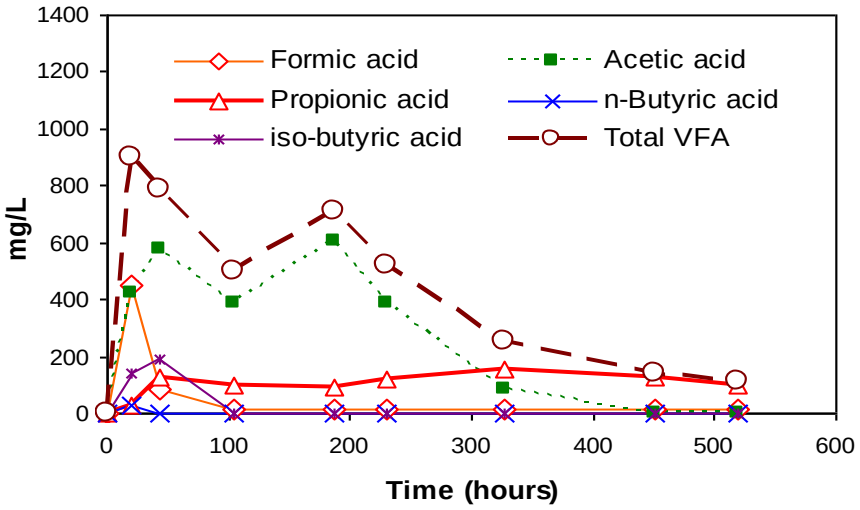

Figure 2b Production of VFAs over time for anaerobic biomass exposed to media with 35gNaCl/L and 1mM of glycine-betaine.

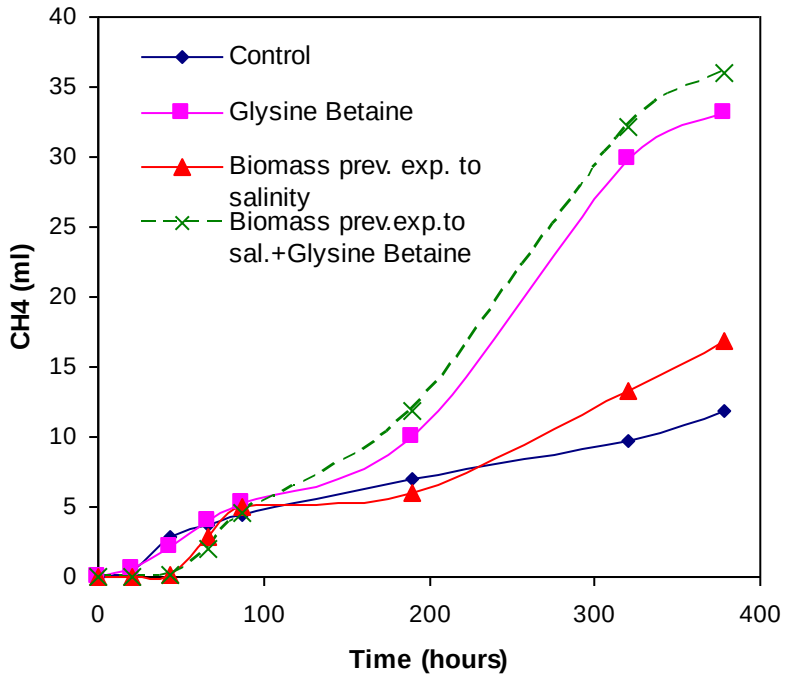

Figure 3. Methane production of anaerobic biomass with 35gNaCl/L medium (control), and when 1mM of glycine betaine is added, and when it was previously exposed to salinity for 28 days with and without glycine betaine added.

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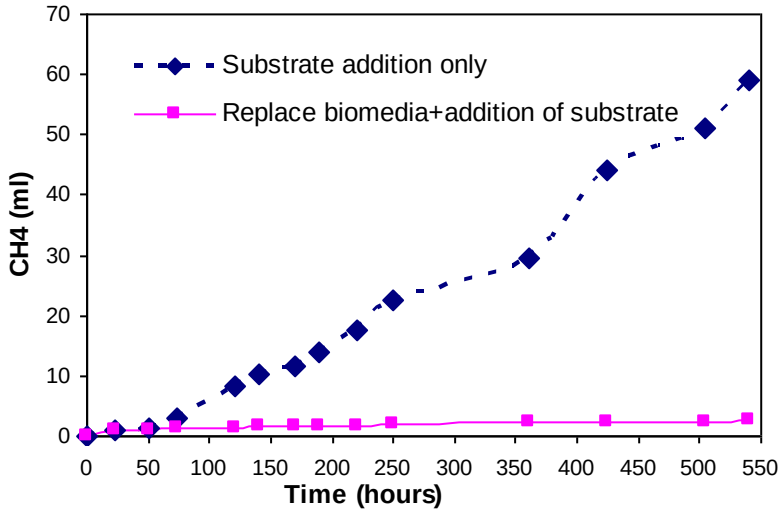

460

461 Figure 4. Batch feeding at 40gNaCl/l where all the media was replaced, and feeding at

462 40gNaCl/l where only substrate was added.

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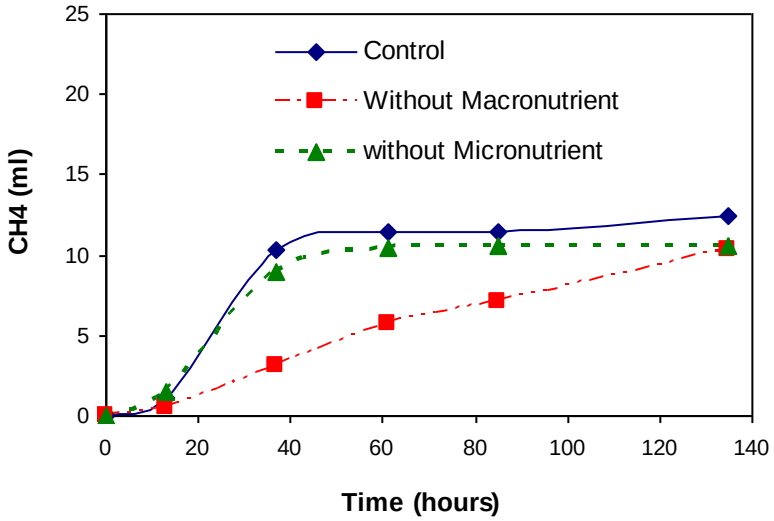


Figure 5a. Batch feeding under non saline conditions (less than 1gNaCl/L) for anaerobic biomass with Owen et al [16] media, and without macro and micronutrient from this media.

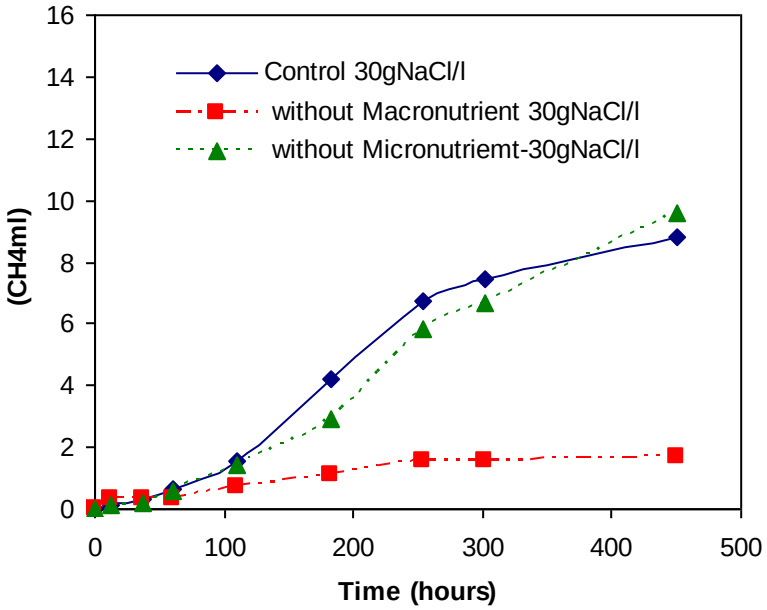


Figure 5b. Batch feeding under saline conditions (35 gNaCl/L) for anaerobic biomass with Owen et al [16] media, and without macro and micronutrients from this media.

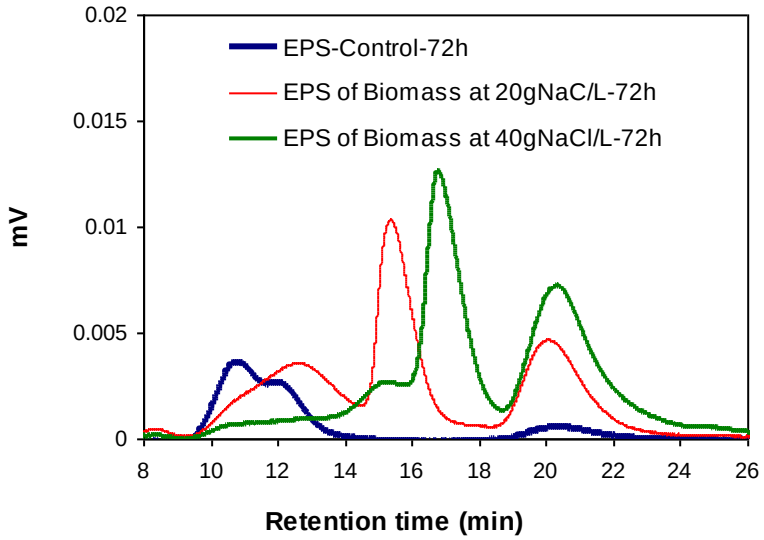


Figure 6a. EPS at 72 hours from biomass exposed to no salt, 20gNaCl/l and 40gNaCl/l

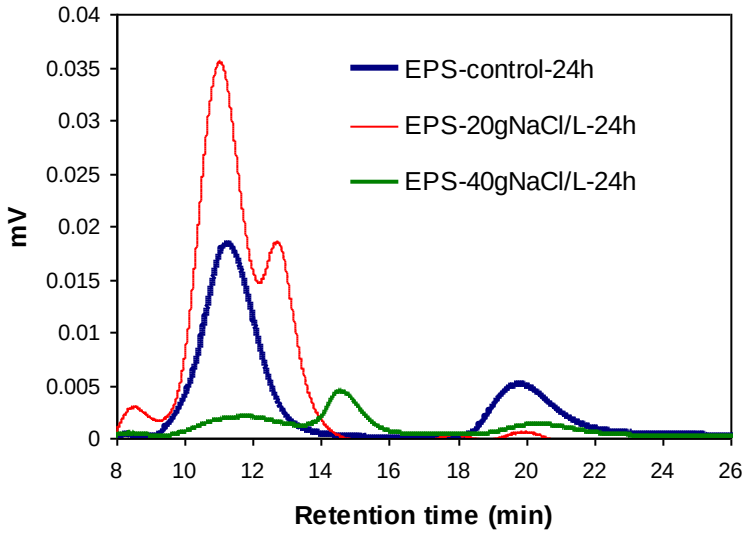
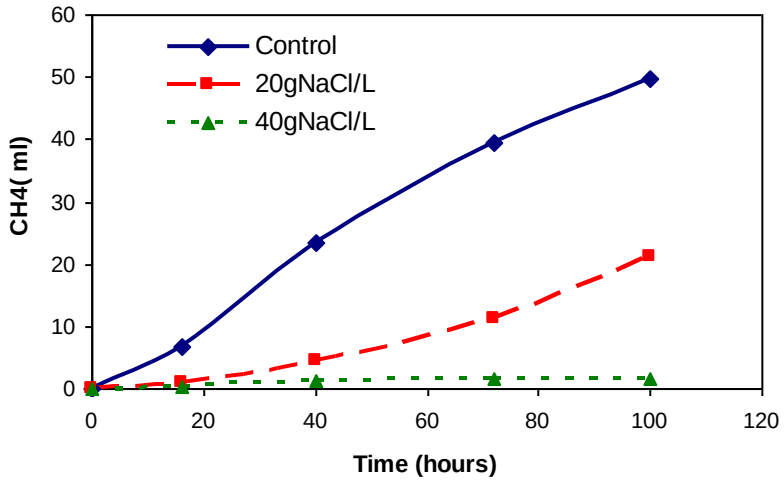


Figure 6b. EPS after 24 hours from biomass exposed to no salt , 20gNaCl/l and 40gNaCl/l



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483 **Figure 6c.** Cumulative CH_4 from biomass exposed to no salt, 20gNaCl/L and 40gNaCl/L.

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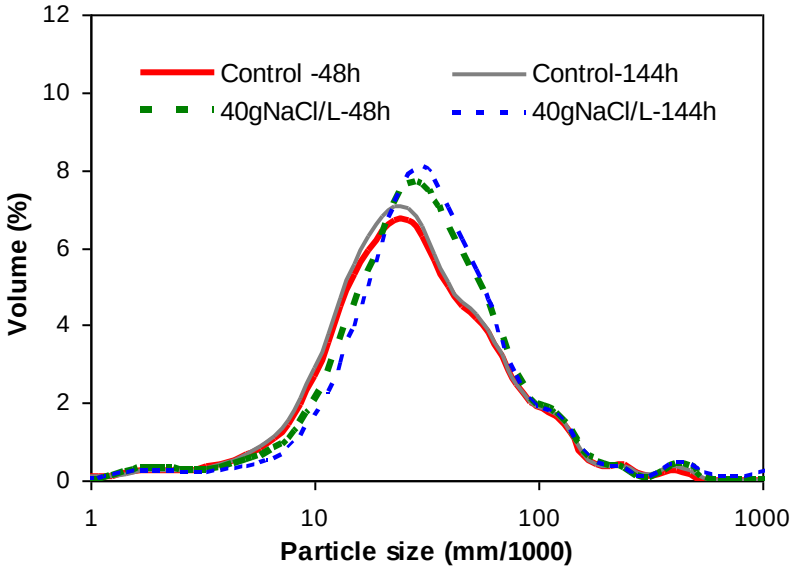

488

489 **Figure 7.** Particle size distribution for biomass exposed to 0 and 40gNaCl/L after 48 and 144

490 hours.