Anaerobic Biofilm Enriched With an Ammonia Tolerant Methanogenic Consortium to Improve Wastewater Treatment in The Fishing Industry

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Abstract

The digestion efficiency of liquid industrial wastes increases when using bioreactors colonized by microbial biofilms. High concentrations of proteins derived from the fish processing industry lead to the production of ammonia, which inhibits methane production. Two bioreactors were constructed to compare methanogenic activity: one enriched with mMPA consortia (control bioreactor), and the second with NH₃ tolerant consortia (treatment bioreactor). Ammonia tolerant activity was assessed by applying an ammonia shock (755 mg NH₃/L). Methane production, consumption of total organic carbon (TOC) and the taxonomic composition of bacteria and archaea was evaluated using 16S rDNA in the acclimatization, ammonia shock, and recovery phases. The ammonia shock significantly affected both methane production and the consumption of TOC in the control reactor ($p < 0.05$) and taxonomical composition of the microbial consortia (OTU). These values remained constant in the treatment reactor. The analysis of biofilm composition showed a predominance of Methanosarcinaceae (*Methanomethylovorans* sp., and probably two different species of *Methanosarcina* sp.) in bioreactors. These results demonstrate that using acclimated biofilms enriched with ammonia tolerant methanogens control the inhibitory effect of ammonia on methanogenesis.

Introduction

A common waste management practice that controls odour and organic matter pollution is anaerobic digestion (Vidal et al. 2000). It is defined as the biological conversion of organic material into a variety of by-products, during which microorganisms perform cooperative and syntrophic processes to convert complex substrate mixtures into biogas that contains mainly methane (65–70%) and carbon dioxide (Kato & Watanabe 2010). This process allows energy to be recovered in the form of methane, which can replace fossil fuels for *in-situ* heating and electricity, reducing negative environmental effects, including global warming and acid rain (Gupta et al. 2012). Another advantage is that it is a relatively inexpensive process, making it a cost-effective way to produce energy (Angelidaki et al. 2011).

Treatment efficiency depends on the taxonomical composition of the microbial consortia. Methanogenesis is the limiting step in anaerobic processes, affected by physicochemical parameters such as high levels of H₂S and NH₃ in wastewater derived from the fishing industry (Sossa et al. 2004). The treatment of liquid industrial wastes (LIW) from the fishing industry faces periods of high organic and nutrient loads, as it is a seasonal activity. During this time, high concentrations of ammonia and sulphate cause the inhibition of methane production and poor bioreactor functionality. These bioreactors are containers with specific parameters for growing microbial communities that decompose organic material input. SRM (Sulphate Reduction Microorganisms) exhibit a higher affinity for common substrates when compared to MPA (Methane Production Archaea), therefore, reactions usually favour sulphate reduction (Mohan et al. 2005).

Moreover, protein hydrolysis produces α -acetoacids which are reabsorbed as nourishment and cause ammonia discharge, which increases alkalinity. It has been reported that free ammonia (NH₃) has higher

inhibitory effects on methanogenesis than NH_4^+ (Sossa et al. 2004). NH_3 concentration depends largely on the pH of the medium and, to a lesser degree, temperature. For example, the NH_3 concentration at pH 7 is close to 1% but increases to 10.2 % at pH 8 (Kayhanian 1999). High ammonia concentration and susceptibility of methanogenic archaea to ammonia have been demonstrated in several studies (Procházka *et al.* 2012). Decreases in methanogenic activity have been reported due to restricted availability of acetate as a carbon source for MPA (Sung & Liu 2003). However, microorganisms acclimated to high ammonia concentrations can sustain their growth and digestive activity in protein-rich effluents (Calli *et al.* 2005). Omil et al. (1995) recommend that substrates with high initial total ammonia nitrogen (TAN) and protein content (e.g., seafood processing industry) should be treated anaerobically using selected sludge containing up to 3000 mg/L of NH_3 (corresponding to 150 mg of free ammonia nitrogen (FAN)/L at pH 7.6). Strategies have been described in order to solve the problems associated with high H_2S production by enriching the microbial community with mMPA (methylaminotrophic Methane Production Archaea). Methylated amines are abundant in fishery waste and cannot be used by SRM as a substrate, but it has been reported that NH_3 effects can be minimized by using mMPA tolerant to high ammonia concentrations (Sossa et al. 2004).

Given this background, the main objective of this study was to evaluate the use of biofilms enriched with a consortium of ammonia tolerant mMPA in reactors used for treatment of industrial fishery waste, in order to improve ammonia shock tolerance during the purification process of liquid discharge effluents rich in proteins and sulphates, and to characterize the main taxonomic groups composing the biofilm before, during and after ammonia shock in a bioreactor, this improvement of the biofilm within the concept of bioaugmentation of microorganisms.

Materials And Methods

Isolation and growth kinetics of ammonia tolerant mMPA consortia biofilm

Ammonia tolerant mMPA consortia (hereinafter (NH_3) -mMPA) were isolated from a fishing industry methanogenic reactor exposed to increasing ammonia concentrations, following the physicochemical conditions and protocols described by Sossa et al. (2004). A final concentration of 1×10^6 cells/mL was inoculated into each vial to isolate consortia. Triplicate samples were taken every two days from the vials to determine methane concentrations in the biogas by using gas chromatography (Pham et al. 2013). Total mMPA concentration was assessed using the Most Probable Number technique, as recommended by the American Public Health Association (APHA 1992).

Scanning Electron Microscopy

To evaluate the attachment of (NH_3) -mMPA consortia on ceramic, the support was washed in a 35 kHz ultrasound bath and observed with a scanning electron microscope (SEM) (ETEC Autoscan U-1), as

described by Anderson *et al.* (1951).

Design of an anaerobic biofilm enriched with (NH₃)-mMPA under NH₃-N shock

Two anaerobic upflow fixed bed reactors were used: Control (CR) and Treatment (TR). CR was enriched with mMPA, while TR was enriched with (NH₃)-mMPA. Fixed bed reactors with retained biomass systems improve when the biofilms formed are fixed to ceramic rings, avoiding large dangerous pH fluctuations and liberating micronutrients to microbial communities (Urrutia *et al.* 1999). Total and bed volume for both CR and TR were 2 L. The bioreactors were semi-continuous fed with fishing industry model effluent medium (2.45 g/L Total Organic Carbon, 48.8mg NH₃-N/L, 82.3mg/L sulphate, 181mg/L sulphide and a salinity of 13.7 g/L, at pH 7.5, Sossa *et al.* 2004) at organic loading rate (OLR) of 0.98 g DOC_{added}/L-reactor/d and hydraulic retention time (HRT) of 5 d. Methylamine was used as the main carbon source. Ammonia concentration was 320–430 mg/L, in all cases, ammonium chloride was added to obtain the unionized ammonia concentration (mg NH₃-N/L). TR was inoculated with (NH₃)-mMPA at a final concentration of 10⁶ cells/mL.

In the enrichment phase (**phase A**), bacterial activity was inhibited with piperacillin (0.128 g/L), SRM activity with 2-mercaptoethane sodium sulfonate (mesna, 0.16 mg/L) and yeast activity with cycloheximide (0.4 g/L) in both reactors. The bioreactors were kept under these conditions for 3 months, allowing biofilm settlement (**phase A**) and acclimatization (**phase B**) before carrying out further analysis. Both bioreactors were supplemented with the fishing industry model effluent without inhibitors and maintained at a concentration of 37.7–128 mg NH₃-N/L in the medium at pH 7.5 for a hydraulic retention time of 5 days and incubated at 30°C for 3 months to allow anaerobic biofilm formation. After acclimatization (**phase B**), both bioreactors were exposed to an ammonia shock treatment (755 mg NH₃-N/L) (**phase C**). The methanogenic activity at different ammonia concentrations was monitored.

Chemical analysis of Bioreactor performed to evaluate performance

Total organic carbon (TOC), alkalinity, sulphate concentration, and volatile suspended solids were analysed according to standard methods (APHA 1992). Methane determination by gas chromatography consisted of the injection of 1 mL of biogas in a 1000 AGC Hach Carle Series chromatograph. Ammonia concentration was estimated (expressed as mg NH₃-N/L) according to the corrected formula (Clegg & Whitfield 1995).

Total microbial count

Biofilm analysis was carried out according to the protocol described by Hobbie *et al.* (1977). Samples were taken every 48 h from both CR and TR. Total bacterial count was performed using the direct epifluorescence technique with a LEICA Confocal Laser Microscope, mod. TCS NT.

amplification, cloning and sequencing of (NH₃)-mMPA consortia 16S rDNA: Genomic DNA of (NH₃)-mMPA consortia was extracted using a mini Beadbeater (Biospec Products, Bartlesville, Okla) and the FastDNA® SPIN Kit for Soil (MP Biomedicals LLC). Genomic DNA cloning was carried out by amplifying (NH₃)-mMPA 16S rDNA, using 21F (Abdallah et al. 2016) and 9R primers (Jurgens *et al.* 1997). The PCR amplification mix used was: DNA template, 1X PCR buffer (with 1.5 mM MgCl₂, Roche), 2 mM dNTPs, 2 pM of each primer and 0.04 U/μl pfu polymerase (Roche). The PCR program amplification cycling protocol for amplification was DNA melting at 95°C for 2 min, followed by 30 cycles of 30 s at 95° C, annealing at 40° C for 30 s, extension at 73° C for 3 min, and a final extension at 73° C for 5 min. PCR products were loaded in an agarose gel (1.5%), isolated and purified using the MiniElute gel purification kit (Qiagen), concentrated and quantified. Amplified fragments of purified rDNA were cloned using the Zero Blunt Cloning kit (Invitrogen). Amplified fragments obtained in the PCR reaction with M13F/M13R primers were cleaned using PCR clean-up kit plates and sequenced using ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing kit, using an AB3100 Genetic Analyser from Applied Biosystems (Hitachi).

PCR-DGGE Community comparison (gel Electrophoresis with denaturing gradient)

Biofilms obtained from the bioreactor were suspended and concentrated by centrifugation at 9.300g x 5 min for DNA isolation using the FastDNA® SPIN Kit for Soil (MP Biomedicals LLC). 16S rRNA gene was amplified using the primers described in the following section. A nested PCR was performed to obtain 16S rRNA gene fragments (Visser *et al.* 2009). The primers used for archaea were 21F/915R (PCR 1) and 344F-GC/915R (Abdallah et al. 2016) (PCR 2). A PCR program for archaeal DNA amplification was DNA melting at 95°C for 2 min, followed by 10 cycles of 30 s at 95°C, annealing at 71°C for 45 s (decreasing 1° C/cycle), extension at 72°C for 1 min, followed by 20 cycles of 30 s at 95°C, annealing at 61°C for 45 s, extension at 72°C for 1 min, and finally the extension at 72° C for 10 min. The primers used for bacteria were 341F/907R (Beck et al. 2013) (PCR 1) and 341F-GC/534R (PCR 2) (Dar et al. 2005). The PCR reaction mix contained DNA template 1X PCR buffer (with 1.5 mM MgCl₂, Promega), 0.2 mM dNTPs, 0.5 μM of each primer and 0.025 U/μL Gotaq polymerase. A PCR program for bacterial DNA amplification was DNA melting at 95°C for 2 min, followed by 20 cycles of 30 s at 95°C, annealing at 65°C for 45 s (decreasing 0.5°C/ cycle), extension at 72°C for 1 min, followed by 10 cycles of 30 s at 95°C, annealing at 55°C for 45 s, extension at 72°C for 1 min, and a final extension at 72°C for 2 min. PCR amplicon were kept at 4°C.

DGGE Primers used for archaea

Ar3F (5'-TTC CGG TTG ATC CTG CCG GA-3') (Zwiettering *et al.* 1994); Ar4F (5'-TCY GGT TGA TCC TGC CRG-3') (Hershberger et al., 1996); 21F (5'-TTC CGG TTG ATC CTG CCG GA) (Abdallah et al. 2016); 958R (5'-YCC GGC GTT GAN TCC AAT T-3') (Abdallah et al. 2016); Ar9R (5'-CCC GCC AAT TCC TTT AAG TTT C-3'); 915R (5'-GTG CTC CCC CGC CAA TTC C-3') (Abdallah et al. 2016); 69F (5'-TAA GCC ATG CAA GTC GAC G-3') (Leadbetter & Breznak 1996); 344F *5'-ACG GGG YGC AGC AGG CGC GA TCG CGC CTG CTC CCC GT-3' (The GC-clamp used, 5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3', was attached to the 5'-end of the 344F primer)(Raskin et al. 1994).

DGGE Primers used for most Bacteria and Archaea: 1492R (5'-GGY TAC CTT GTT ACG ACT T-3') (Leadbetter & Breznak 1996).

DGGE was performed using a Dcode Universal Mutation Detection System (Bio Rad Laboratories, Hercules, CA, USA), following system recommendations (Muyzer et al. 1993). Electrophoresis was performed at a constant voltage for 4 h (archaea) and 8 h (bacteria). Gels were stained with silver nitrate (Sanguinetti et al. 1994), while ethidium bromide (0.5 µg/mL) was used to obtain bands for subsequent sequencing. Excised gel bands were re-amplified (Valdebenito-Rolack et al. 2011), quantified and sequenced (Macrogen, Korea). Images (densitograms) were analysed by Quantity One software, BioRad version 4.2.3 using WPGAMA for cluster analysis (Chen et al. 2017, Putra et al. 2020).

Estimation of (NH₃)-mMPA consortia growth kinetics in biofilm

The growth kinetics of (NH₃)-mMPA in biofilms were estimated using the Gompertz model as described by Zwietering et al. (1994). Data were adjusted to the model by nonlinear regression and the most useful model was selected (Sokal & Rohlf 1968). To obtain the maximum specific growth rate (µ), lag phase duration (λ) and maximum count (A), results free R software for Windows was used. To compare the control reactor with the experimental reactor, t-tests were performed for independent groups, with a statistical significance of 0.005 using the SYSTAT Software package. Normal distribution was assumed, given that the measurements were performed in triplicate.

NUCLEOTIDE ACCESSION NUMBERS: The archaeal sequences obtained and described in this study of mMPA rRNA 16S gene sequence data were submitted to the NCBI GenBank database under accession numbers: EU544305, EU544306 and EU544307.

PHYLOGENETIC ANALYSIS

Sequences for selected clones in this study have been submitted to NCBI/GenBank. These were aligned with the NCBI nucleotide database using BLAST (Altschul et al. 1997), as those sequences were at least 90% identical and above. To compare the diversity of OTU in CR and TR in different phases of the bioreactors, a multiple alignment was performed using ClustalW (Mount 2004). Phylogenetic trees were calculated with MEGA4 software (Tamura et al. 2007), using the Kimura two-parameter correction and the Neighbour-Joining method.

Results And Discussion

Isolation and growth kinetics of ammonia tolerant consortia (NH₃)-mMPA biofilm

Ammonia tolerant consortia, (NH₃)-mMPA, were isolated from the reactor enriched with mMPA communities. Consortia showed a *Methanosarcinaceae* MPA family prevalence. Consortia were able to grow in a planktonic phase at 848.8 mg NH₃/L with methane representing a mean of approximately 70% of the produced biogas. (NH₃)-mMPA consortia were inoculated into vials with ceramic spheres as

support for 20 days, allowing biofilm formation. Growth kinetics determined by the Gompertz model showed a λ of 3.7 d (days) and μ_m of 0.67 d^{-1} , with $r^2 = 0.95$ (Fig. 1A). In addition, methane production was monitored during biofilm formation. Methane peaked at 80% of total biogas after 20 days of incubation. A time lag of 5–6 days was observed in methane production. By performing SEM analysis, (NH_3) -mMPA consortia were detected on the support surfaces after 4 days of incubation, consistent with the kinetic growth data that predicted micro-colony formation after 20 days of incubation (Fig. 1B).

Design of an anaerobic biofilm enriched with (NH_3) -mMPA under NH_3 shock

After a full year of operation, the control bioreactor (CR) showed a decrease of approximately 80% in TOC (Fig. 2A), with 85% methane content in the produced biogas. The methane production curve obtained for the treatment bioreactor (TR) gradually increased, approaching 70% of the biogas produced in the first 50 days (Fig. 2B). The alkalinity alpha factor remained below 0.3 in both reactors, TOC consumption varied between $80 \pm 20\%$ in CR and $62 \pm 17\%$ in TR. Methane content in the biogas ranged from $86 \pm 8\%$ in CR, and $81 \pm 18\%$ in TR (Fig. 2A and 2B).

Morphological and genotyping characterization of (NH_3) -mMPA consortia and ammonia Tolerant biofilm

To determine the microbial morphologies present in (NH_3) -mMPA consortia, concentrated planktonic cultures and ceramic support fragments were observed by SEM. Sarcina morphotypes characteristic of *Methanosarcinaceae* were found (Fig. 1B). Analysis of the 16S rDNA from (NH_3) -mMPA consortia revealed that gene sequences were grouped with *Methanosarcinaceae* members (*Methanosarcinaceae*, *Methanosarcinales*). Phylogenetic relationships of archaeal 16S rDNA partial sequences (EU544306; EU544307), archaeal mMPA ammonium tolerant strain 16S rDNA partial sequence (EU544305) and methanogenic species sequences from the Gene bank show that mMPA sequence EU544305 (868 bp), was grouped in the *Methanomethylovorans* genus. This is because it showed higher similarities with uncultured archaeon (99%) and *Methanomethylovorans hollandica* (Lomans 1999) (98%), and two different species of *Methanosarcina* genera which were predominant components of the consortia, and similar to cloned sequences. EU544306 (547 bp), uncultured *Methanosarcina* sp. clone HUB A5, and EU544307 (521 bp), were grouped into the *Methanosarcina* genus, showing high similarity with *Methanosarcina* sp. (95%) and *Methanosarcina baltica* (von Klein 2002) (95%). When a BLAST analysis compared the sequences with stored species Types of uncultured *Methanosarcina* sp. clone HUB A5, this is located near *Methanosarcina baltica* in a phylogenetic tree at a Neighbor Joining distance of 0.05 (not shown). *M. baltica* is an archaeon isolated from anoxic layers of sediment in the Baltic Sea that grows fast in marine culture, in methylamines, methanol, and acetate (vonKlein et al. 2002). Uncultured *Methanosarcina* sp. clone HUB A6 sequence EU544307 was also grouped in the *Methanosarcina* genus, showing high similarity to *Methanosarcina acetivorans* (Sowers 1984) (93 %) and uncultured *Methanosarcina* sp. (92%). Uncultured *Methanosarcina* sp. clone HUB A6 is located near *Methanosarcina acetivorans* in a phylogenetic tree at a Neighbor Joining distance of 0.2 (not shown), also described in anoxic marine environments and found as a single cell in marine cultures (Maeder et al. 2006).

Microbiological and molecular description of biofilm in bioreactors: Ammonia concentration in both bioreactor influents ranged between 37.7 and 128 mg NH₃-N/L until day 152, when both bioreactors were subjected to ammonia shock, increasing ammonia concentrations to 755 mg NH₃-N/L. Between day 180 and 208, ammonia concentrations returned to average levels (37.2–41.1 mg NH₃-N/L). Significant differences in ammonia concentration were observed in phases C and D in both TR and CR effluent outputs (Fig. 2A and 2B). The experimental bioreactor showed a lower concentration of ammonia residual (137–184 mg NH₃ L⁻¹) when compared with the control bioreactor (327 – 128 mg NH₃ L⁻¹). Shock NH₃-N effects in CR and TR are shown in Fig. 2A and 2B, respectively. Methanation in CR began at 90%, dropping to 70% during ammonia shock (Fig. 2A). A different response was observed in TR, where no significant variations were observed during the ammonia shock, reaching methanation levels above 90% throughout the experiment (t-test, p 0.0005-0.01) (Fig. 2B). In both cases (CR and TR), correlations between the percentage of TOC consumed and the concentration of methane produced were significant (r^2 : 0.72 $p \leq 0.05$ for CR, and $r^2 = 0.77$ $p \leq 0.05$ for TR) (Fig. 2). Production of methane specific was determined based on biomass as volatile suspended solids (VSS) were registered in both reactors. In CR, a production of 0.32 ± 0.04 mL CH₄/g (VSS * d) was registered, while TR showed a production of 0.65 ± 0.08 mL CH₄/g (VSS * d), this activity was re-controlled post ammonia shock where it was found a production of 0.30 ± 0.05 mL CH₄/g (VSS * d) in CR and 0.38 ± 0.04 mL CH₄/g (VSS * d) in TR. Increased methanogenic activity per biomass unit from day 152 to 166 was 20.5-fold higher in TR when compared to CR, which indicates that methanogenic archaea maintained their activity under ammonia shock conditions.

FIGURE 2. Total Organic Carbon consumption, methanization and ammonia concentration in an anaerobic filter type reactor enriched with mMPA. **2A.** CR. **2B.** TR. **Phase A:** CR anaerobic biofilm development with ammonia operating range of 27.45–75 mg NH₃-N/L; **Phase B.** Reactor acclimatization- Initial phase bioreactor functioning. Acclimatization at 37.7–128 mg NH₃-N/ L. **Phase C:** Ammonia shock at 755 mg NH₃-N/L, **Phase D:** Final phase bioreactor functioning, reactor recovery from 37.1 to 41.2 mg NH₃-N/L. ■- % Total Organic Carbon (TOC) consumption; □ -% methane; —●— NH₃ input concentration; —●— NH₃ output concentration.

When comparing the first 100 days of both reactors, the control bioreactor consumed significantly more TOC than the experimental bioreactor (t-test, $p < 0.0001$ –0.007). Methane production measure by gas chromatography was also significantly higher in the CR (89.48% – 95%) when compared to the TR (29% -75%) ($p < 0.0001$ –0.029). These differences diminished from day 54 to 82 (Fig. 2A and 2B). During and after the ammonia shock, (phase C - day 152 to 208), a higher consumption of TOC was observed in both bioreactors, although it was significantly higher in the TR until day 180, after which it decreased significantly to 65.78% on day 208. Methane production in the TR remained above 90%, significantly higher than in the CR ($p < 0.0005$ –0.0417), which showed a methane production that fluctuated between 69% – 86% (Fig. 2A and 2B). Significant differences (t-test, $\alpha \leq 0.05$) were observed for both methane production and TOC concentrations when comparing the CR and TR during the ammonia shock step. Figures 2A and 2B show how the ammonia shock caused a significant decline (t-test, $\alpha = 0.05$) in

methane production and TOC consumption in the CR. These results clearly indicate that the TR was resistant to the deleterious effect of ammonia, as the mMPA biofilm community was able to maintain its methanogen activity even under high ammonia concentrations.

Bands in the gel were conspicuous in phase B, the acclimatization phase (Fig. 3A, lane B), but tended to diminish in phase C, the ammonia shock, (Fig. 3A, lane C). However, the abundance of archaea increased between phase B, the recovery phase, and phase D, the final phase (Fig. 3A, lane D). Besides, lower archaea biodiversity was observed in the CR throughout the profile when compared with TR biofilm (Fig. 3C). Moreover, it was observed that (NH₃)-mMPA enriched biofilm produced fewer bands in the bacteria domain in the CR than biofilm enriched with mMPA tolerant to ammonia (Fig. 3B, lanes B, C and D). However, 16S rDNA bacterial profile similarities were higher in the CR than in the TR. Similarities were lower for archaea than bacteria, as shown in Fig. 3C and 3D.

Figure. 3. **A.** DGGE profiles of archaea. Gel bands recovered from 16S rDNA gels were sequenced and identified as EU544305 *Methanomethylovorans* sp., EU544306 and EU544307; two different species belonging to *Methanosarcina* sp. **B.** DGGE profiles of bacteria. **C.** Cluster analysis (WPGMA) using data from DGGE profiles of archaea in anaerobic biofilm to assess the similarity of taxonomic composition of consortia assigned to treatment of industrial waste rich in proteins, obtained from the control reactor (CR). **D.** Treatment Reactor (TR) lanes. B lanes- **phase B**, initial acclimatization at 37.7–128 mg NH₃-N/L; C lanes- **phase C**, ammonia shock at 755 mg NH₃-N/L; D lanes- **phase D**, final reactor recovery from 37.1 to 41.2 mg NH₃-N/L.

This study demonstrates that efficient wastewater treatment and stable methane production can be achieved under high ammonia concentrations by using anaerobic biofilms formed in fixed bed reactors containing (NH₃)-mMPA (ammonia tolerant archaea biofilms).

Zheng et al. (2015) demonstrate that the selection of a suitable support for fixed-bed reactors is crucial for the anaerobic digestion of ammonium-rich livestock wastes, since they found that using zeolites as support they achieve a methane production over 80%, compared to other types of support, which is comparable to that shown in this research. NH₃-tolerant microbial consortium, defined as a microbial community composed of archaea and bacteria, give rise to long-term stable communities whose tolerance to ammonia stress can be increased with an acclimated consortium. The main components (archaea) found in this study, of selected tolerant consortia shared general characteristics with the *Methanosarcinaceae* family, with the mMPA EU544305 sequence related to the *Methanomethylovorans* genus, and the other two sequences (EU544306 and EU544307) related to the *Methanosarcina* genus, the first one of which is considered an obligate methylotrophic methanogen (Supplementary Material SI 1. to SI 3.). Members of *Methanosarcinaceae* have been found in a wide variety of anaerobic environments where methane is produced, can grow on a diversity of substrates, and represent the most versatile group of Archaea (Oren 2014). The predominant archaea throughout the experiment were *Methanosarcina* and *Methanomethylovorans* (He et al. 2017; Cadavid-Rodríguez *et al.* 2019). Some researchers consider these species to be keystone species. For example, *Methanosarcina acetivorans* (Galagan et al. 2002), is a

unique species which may have a wide range of metabolisms that support the reactor's operation under ammonia shock.

In a study by Wu and Song (2021), adding inoculum through anaerobic co-digestion of waste activated sludge (WAS) and fish waste (FW), high methane production rates were obtained with mixtures of 1.5% and 3% of FW, but methanogenesis was inhibited with an addition of 6% of FW or higher, finding a high accumulation of ammonia and fatty acid. In a similar trial, Cadavid-Rodríguez *et al.* (2019) found that when using from 1.5% of FW in anaerobic digestion, methanogenesis is inhibited. Thus, the alternative of enriching the inoculum with ammonia-tolerant mMPA is attractive and feasible to avoid the inhibition of methanogenesis in the treatment of protein-rich fishery waste.

Furthermore, the analyses of the microbial community carried out by Wu & Song (2021) showed that methane production was dominated by hydrogenotrophic and methylotrophic methanogenic archaea, which is to be expected since fishery residues are rich in methylated amines, which would favour the growth and maintenance of the Ammonia tolerant mMPA consortia.

Physiological studies of this strain have reported that it tolerates NaCl concentrations of 100 mM. In this study, the NH₃ tolerant consortia were able to grow and maintain constant methanogenic activity at 400 mM NaCl, a characteristic that favours their use for fishery waste treatment at high salt concentrations (Aloui et al. 2009, Chen et al. 2018, He et al. 2017). Because salinity has been reported to deteriorate removal performance, activated sludge characteristics, and change microbial community in anaerobic reactors (Chen et al., 2017; He et al., 2017).

We identified a strong relationship between reactor performance and abundance of *Methanosarcina* cells. Until day 398 (750 FAN mg/L; 6000 TAN mg/L), highly active *Methanosarcina* cells were identified in large multicellular structures (clusters). Calli *et al.* (2005) found an abundance of *Methanosarcina* species at high ammonia concentrations (160–747 mg).

The experimental bioreactor showed constant low values of ammonia in the output effluent after an ammonia shock when compared to the control bioreactor. Similar results have been previously reported (Dai et al. 2016). The 16S rDNA profile of archaea and bacteria obtained by PCR-DGGE shifted at OTU (Operational Taxonomic Units) composition, confirming that an acetoclastic methanogenesis dominated acetate utilization in the bioreactor. Galagan et al. (2002) described the metabolic and physiological diversity of *Methanosarcina acetivorans*, suggesting that the formation of multicellular structures (cell envelope and extracellular matrix) is an adaptation to stress and likely plays an important role in the ability of *Methanosarcina* to colonise diverse environments. This species metabolises a broad spectrum of carbon compounds into methane, a process controlled by different methanogenesis genes. Individual copies of the duplicated genes may display differential regulation and kinetic properties, allowing the species to change between substrates, consistent with the reported plasticity that *Methanosarcina* species show in nature (Angelidaki et al. 2011), an important characteristic that allows the community to tolerate stress. High bacterial biodiversity was observed in the communities, but we did not find

significant differences between these communities. This idea has been expressed before in studies on stability in an anaerobic bioreactor (Fernández *et al.* 2000), where bacteria were found in the biofilm, even under stress. Nevertheless, according to our results, when the communities were exposed to changing conditions, the bacterial activity was modified, and archaea did not exhibit significant differences in abundance.

Our coastal zone is characterized by high primary productivity favoured by wind predominance and marine oceanographic current system in the South Eastern Pacific Ocean, that produce constant upwelling and for decades or centuries had been registered major fish strandings (Hernandez-Miranda *et al.* 2010, 2012, 2017). It is probable that an unknown endemic rich microbial ammonia methanogenic (bacteria and archaea) community potentially had evolved associated both sea water column and sediments because high protein content product of strandings. This potential microbial community sampled from effluent industrial fishery was used in the bioreactor and bioaugmented in the treatment bioreactor and our objective was characterized archaea components.

Declarations

To be used for life science journals + articles with biological applications

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- **Availability of data and material:** The data used for this article is available to be requested by the reviewers if they deem it appropriate.
- **Code availability:** Not applicable

Author Contribution

Manuel Alarcón-Vivero: Data curation, Formal analysis, Investigation, Methodology, Writing - original draft. Writing - review & editing

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Figures

Figure 1

A. Ammonia-tolerant isolated Methanosarcinaceae biofilm growth kinetics on ceramic supports at λ 3.7 days and $\mu = 0.67 \text{ d}^{-1}$ (r^2 0.95). Experimental values; - Values predicted by Gompertz model; $\blacktriangle$ methane in biogas (%). Bars represent standard deviation of average methane %. B. Image inside the graphic is a Microphotograph of isolated methanogenic ammonia tolerant Archaea (mMPA), Methanomethylovorans sp. (Methanosarcinaceae, Methanosarcinales) (5000X).

Figure 2

Total Organic Carbon consumption, methanization and ammonia concentration in an anaerobic filter type reactor enriched with mMPA. 2A. CR. 2B. TR. Phase A: CR anaerobic biofilm development with ammonia operating range of 27.45 - 75 mg NH₃-N/L; Phase B. Reactor acclimatization- Initial phase bioreactor functioning. Acclimatization at 37.7 -128 mg NH₃-N/ L. Phase C: Ammonia shock at 755 mg NH₃-N/L, Phase D: Final phase bioreactor functioning, reactor recovery from 37.1 to 41.2 mg NH₃-N/L. $\blacksquare$ - % Total Organic Carbon (TOC) consumption; $\square$ -% methane; $\bullet$ — NH₃ input concentration; $\circ$ — NH₃ output concentration.

Figure 3

A. DGGE profiles of archaea. Gel bands recovered from 16S rDNA gels were sequenced and identified as EU544305 *Methanomethylovorans* sp., EU544306 and EU544307; two different species belonging to *Methanosarcina* sp. B. DGGE profiles of bacteria. C. Cluster analysis (WPGMA) using data from DGGE profiles of archaea in anaerobic biofilm to assess the similarity of taxonomic composition of consortia assigned to treatment of industrial waste rich in proteins, obtained from the control reactor (CR). D. Treatment Reactor (TR) lanes. B lanes- phase B, initial acclimatization at 37.7 -128 mg $\text{NH}_3\text{-N/L}$; C lanes- phase C, ammonia shock at 755 mg $\text{NH}_3\text{-N/L}$; D lanes- phase D, final reactor recovery from 37.1 to 41.2 mg $\text{NH}_3\text{-N/L}$.