
Cultivation of an immobilized (hyper)thermophilic marine microbial community in a bioreactor

Landreau M.^{1,2,3}, Duthoit Frederique^{1,2,3}, Roussel Erwan^{1,2,3}, Schönherr S.^{1,2,3}, Georges Myriam^{1,2,3}, Godfroy Anne^{1,2,3,*}, Le Blay Gwenaelle^{1,2,3,*}

¹ Université de Bretagne Occidentale (UBO, UEB), Institut Universitaire Européen de la Mer (IUEM)–UMR 6197, Laboratoire de Microbiologie des Environnements Extrêmes (LMEE), Place Nicolas Copernic, F-29280 Plouzané, France

² CNRS, IUEM – UMR 6197, Laboratoire de Microbiologie des Environnements Extrêmes (LMEE), Place Nicolas Copernic, F-29280 Plouzané, France

³ Ifremer, UMR 6197, Laboratoire de Microbiologie des Environnements Extrêmes (LMEE), Technopôle Pointe du diable, F-29280 Plouzané, France

* Corresponding author : Gwenaelle Le Blay, email address : gwenaelle.leblay@univ-brest.fr

Abstract :

Cultivation in a bioreactor of immobilized deep-sea hydrothermal microbial community was tested in order to assess the stability and reactivity of this new system. A community composed of 8 hydrothermal strains was entrapped in a polymer matrix that was used to inoculate a continuous culture in a gas-lift bioreactor. The continuous culture was performed for 41 days at successively 60°C, 55°C, 60°C, 85°C and 60°C, at pH 6.5, in anaerobic condition and constant dilution rate. Oxidic stress and pH variations were tested at the beginning of the incubation. Despite these detrimental conditions, 3 strains including 2 strict anaerobes were maintained in the bioreactor. High cell concentrations (3 × 10⁸ cells mL⁻¹) and high ATP contents were measured in both liquid fractions and beads. Cloning-sequencing and qPCR revealed that *Bacillus* sp. dominated at the early stage, and was later replaced by *Thermotoga maritima* and *Thermococcus* sp.. Acetate, formate and propionate concentrations varied simultaneously in the liquid fractions. These results demonstrate that these immobilized cells were reactive to culture conditions. They were protected inside the beads during the stress period and released in the liquid fraction when conditions were more favorable. This confirms the advantage of immobilization that highlight the resilience capacity of certain hydrothermal microorganisms after a stress period.

Keywords : immobilization, anaerobiosis, marine (hyper)thermophiles, continuous culture

INTRODUCTION

Culture of microbial consortia is a recently developed and useful approach with many applications in biotechnology and microbial ecology, notably because it can improve the comprehension of complex cell-cell interactions and metabolites exchanges or genes expression in natural consortia (Brenner *et al.*, 2008; Brune and Bayer 2012). Microbial consortia are widely spread in nature where they heavily influence the dynamic of many ecosystems, such as deep-sea (Vigneron *et al.* 2014), terrestrial (Gerbl *et al.* 2014) or intestinal ecosystems (Kau *et al.* 2011). They are often structured in biofilms or cell aggregates, sometimes embedded in viscoelastic extracellular matrices made of exopolysaccharides, DNA and proteins. These matrices protect the cells from external stress allowing them to persist in inhospitable environments (Stoodley *et al.* 2002; Keller and Surette 2006). Regarding these interesting properties, immobilization of microorganisms by entrapment in polymer matrices has been developed to improve microbial consortia stability and productivity in continuous cultures. Cell entrapment offers numerous advantages over free-cell cultures including high microbial concentration associated with a better protection of cells against harsh environmental conditions (pH, temperature, abiotic stresses and potential inhibitors) or bacteriophage attacks (Kanasawud *et al.* 1989). It also stabilizes the continuous culture systems by preventing the wash-out of less competitive or slow growing cells (Kumar and Schiigerl 1990; Rathore *et al.* 2013). Cell entrapment has been used for the continuous culture of thermophilic strains (Kanasawud *et al.* 1989; Klingeberg *et al.* 1990) or anaerobic mesophilic populations (Cinquin *et al.* 2004; Cinquin *et al.* 2006), but never for the study of anaerobic marine (hyper)thermophilic consortia from deep-sea hydrothermal vents. These ecosystems are characterized by sharp chemical and physicochemical gradients that rapidly change in time and space; they are at the origin of a large diversity of microorganisms possessing different metabolic pathways (Flores *et al.*, 2011). In this study, we applied the immobilization protocol recently developed (Landreau *et al.*, 2016) to test the behavior and resilience capacity of a (hyper)thermophilic community of microorganisms previously isolated from deep-sea hydrothermal vents during a continuous culture.

MATERIALS AND METHODS

Microbial community inoculum

The inoculum contained a mixture of (hyper)thermophilic microorganisms obtained from the DSMZ culture collection, *Thermosiphon* sp. DSM101094 and *Deferribacter abyssi* DSM14873; from the UBO culture collection, *Thermotoga maritima* UBOCC2576; and from the laboratory, *Marinitoga camini* AT1253, *Caminicella* sp. AT1249, *Bacillus* sp. ML01, *Thermococcus* sp. AT1260 and *Thermodesulfatator atlanticus* AT1325. These later strains were isolated from previous continuous enrichment cultures, performed in the same conditions and inoculated with chimney samples from an active black smoker of Rainbow field at Mid Atlantic Ridge (36°13'N 33°54'W, 2275 m) (Postec *et al.* 2005, Postec *et al.* 2007). The two chemolithoautotrophic strains are capable of using hydrogen as an electron donor; and sulfate as electron acceptor in the case of *T. atlanticus* (Alain *et al.*, 2010), or elemental sulfur and nitrate in the case of *D. abyssi* (Miroshnichenko *et al.*, 2003). The other strains are heterotrophs. The strains were reactivated and mixed in equivalent proportion in order to obtain a final concentration of 10^8 cells mL⁻¹.

Culture medium

The culture medium was the modified SME rich-medium containing a total amount of 3.1 g L⁻¹ of organic matter (composed of yeast extract, casaminoacids, glucose, dextrin, galactose, dextran, glycogen, pyruvate and acetate) and reduced by the addition of HCl-L-cystein (0.5 g L⁻¹) as described by Postec *et al.* (2007). The medium was autoclaved (121°C for 20 min) and transferred in a 1 L Nalgene bottle containing colloidal sulfur (3 g L⁻¹). The Nalgene bottle was previously decontaminated with peracetic acid (2%) and carefully washed, the colloidal sulfur was added and the Nalgene bottle was tyndallized by three successive sterilization steps (100°C for 20 min) at 24 h intervals.

Microbial entrapment

Cell entrapment was performed in an anaerobic chamber as previously described (Landreau *et al.* 2016). Beads were anaerobically transferred to the gas-lift bioreactor just after immobilization.

Culture conditions

The culture was performed during 41 days in a gas-lift bioreactor (Godfroy *et al.* 2006) (working volume: 250 mL) containing 30% (v/v) of freshly inoculated beads. Batch (6 hours) and fed-batch (days 0 to 4) were first carried out allowing the cells to grow inside the beads.

During the fed-batch period, 50 mL of SME medium were aseptically replaced every 24 h. After the batch/fed-batch period, the continuous culture was performed for 36 days (from days 5 to 41) by renewing the SME medium (maintained at 4°C) at a dilution rate of 0.04 h⁻¹ corresponding to a flow rate of 10 mL h⁻¹. The pH was set at 6.5 and regulated by addition of NaOH and HCl (1 mol L⁻¹). Anoxic culture conditions were maintained by sparging the medium with N₂. In an attempt to mimic the steep physicochemical variations undergone by deep-sea hydrothermal vent communities due to fluid fluctuations (Tivey, 2004; Prieur *et al.*, 2012), and to assess their impact on the stability and reactivity of the immobilized microorganisms, different culture parameters were applied. The temperature was set at 60°C during the batch/fed-batch period (days 0 to 4, first period), decreased at 55°C for 4 days (days 5 to 8, second period), increased again at 60°C for 12 days (days 9 to 20, third period) then at 85°C for 9 days (days 21 to 29, fourth period) and finally decreased at 60°C for the last 12 days (days 30 to 41, fifth period). From day 8, pH variations were performed for 48 h (24 h at 5.5 followed by 24 h at 8). During the first 8 days, the redox potential was maintained above -110 mV with a N₂ flow rate at 0.25 v v⁻¹ min⁻¹ (pinkish medium). On day 10, the redox potential increased above -51 mV during 6 hours due to the accidental stop of the gas station (Hungate 1969). After day 10, the redox potential was maintained below -110 mV by adjusting N₂ flow rate to 0.1 v v⁻¹ min⁻¹.

Samples (15 mL) from the liquid culture fraction were regularly collected directly from the bioreactor for cell counting, using a Thoma chamber (0.02 mm deep) with an Olympus CX41 microscope (Olympus, Rungis, France) and for ATP analysis. In parallel, larger volumes of effluents (*ca* 230 mL) were harvested over a 24 h period and maintained at 4°C for metabolite analysis and DNA extractions. Around 700 mg of beads were collected per sampling day, all along the continuous culture, but more especially at the end of each period, for ATP analyses. Sub-samples were directly stored at -80°C for DNA extraction, or previously placed in a glycerol/PFA 20% solution (9:1) for microscopic observations. Accurate counting of cells in polymeric beads has proven to be difficult; therefore, cell growth was estimated by measuring the amount of ATP in beads and in the liquid culture fraction as previously described (Landreau *et al.* 2016).

Total DNA extraction and 16S rRNA gene amplification, cloning sequencing and sequence analysis

Genomic DNA was extracted from beads using the PowerSoil® DNA isolation kit (MO BIO laboratories, Carlsbad, USA). For the liquid culture fraction samples, cell pellets were resuspended in 1 mL of TE 1X (100 mM Tris, 50 mM EDTA, pH 8.0) prior to a phenol/chloroform/isoamyl alcohol (PCI) extraction as described by Alain *et al.* (2002). In the present study, no extra lysis step was performed and no eukaryote DNA was added during the extraction. The 16S rRNA genes were amplified by PCR as previously described by Durand *et al.* (2010) using universal primers for Bacteria (E8F/U1492R) and Archaea (A8F/U1492R) and cloned in *E. coli* DH5 α cells using pGEM®-T Easy vector cloning kit (Promega) according to manufacturer's instructions. Archaeal and bacterial clone libraries were constructed for 5 liquid culture fraction samples collected after 4, 12, 20, 29 and 41 days of culture, and for beads after immobilization and after 20, 29 and 41 days of culture. Sequences were checked using the BLAST function on the NCBI website (<http://www.ncbi.nlm.nih.gov/BLAST>) (Altschul *et al.* 1990).

Quantitative real-time PCR

Real-time PCR quantifications were performed in triplicate using Perfecta™ SYBR® Green SuperMix ROX (Quanta Bioscience) according to manufacturer's recommendations. Three different pairs of primers were used for *Bacillus* sp. (BAC-391F 5'-TCGGGTCGTAAAGCTCTGTT-3' and BAC-628R 5'-CTCAAGTTCCCCAGTTTCCA-3'), for *T. maritima* (TTM-443F 5'-GCGGGGGAAGAATAAGGTAG-3' and TTM-612R 5'-ATTTACATCCGACACACCA-3'), and for *Thermococcus* sp. (ARC-787F 5'-ATTAGATACCCSBGTAGTCC-3' and ARC-1059R 5'-GCCATGCACCWCCTCT-3') at a final concentration of 0.6 μ M. Amplifications were performed with a first step of 2 min at 95°C followed by 40 cycles of 15 s at 95°C, 1 min at 60°C and 30 s at 72°C. For each strain, standard curves were obtained with 10-fold dilutions of their 16S rRNA gene ranging from 10² to 10⁸ copies μ L⁻¹ with R² above 0.9849 and efficiency above 93%. qPCR were performed in a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, Villebon sur Yvette, France).

Microscopic observation of beads

Microbial distribution within gel beads was analyzed just after immobilization and on days 5, 20, 29 and 41. Gel beads were fixed with 3% formaldehyde and stored at -20°C. After thawing, beads were washed with 80 μ L of Tris-EDTA buffer (1mM EDTA, 10 mM Tris,

pH8), placed on a glass slide and incubated 1 min in 30 μL of SYBR[®] Gold 1X in the dark. SYBR[®] Gold was then removed and beads were washed twice with 80 μL of TE buffer. Beads were covered with Citifluor[™] (Citifluor Ltd, Leicester, UK) and observed on an Axio Zoom V1.6 microscope (Zeiss, Marly le Roi, France).

Metabolite analyses

Acetate, propionate, lactate and formate concentrations were determined using a Dionex ICS-2000 Reagent-Free Ion Chromatography System equipped with an AS50 autosampler (Dionex Camberley UK). Chromatographic separation was conducted using two Ionpac AS15 columns in series at 30°C and the determination of species was carried out using an Anion Self-Regenerating Suppressor (ASRS 300 4-mm) unit in combination with a DS6 heated conductivity cell (35°C). The gradient program was as followed: 6 mM KOH (43 min), increase 27 mM KOH min^{-1} to 60 mM (39 min), decrease 54 mM KOH min^{-1} to 6 mM (5 min).

RESULTS AND DISCUSSION

Cell and ATP concentrations

The growth capacity and reactivity of the freshly immobilized (hyper)thermophilic microorganisms were tested in a continuous culture performed for 41 days in a gas-lift bioreactor in the same growth conditions as previously described by Postec *et al.* (2007). Despite a redox-potential above -110 mV observed during the batch/fed-batch period (days 0-4), cell growth was detected both in beads and in the liquid fractions with an increase in ATP concentrations reaching respectively 2.3 ± 0.24 pmoles g^{-1} and 369 ± 107.5 pmoles mL^{-1} on day 4 (Figure 1). The start of the continuous culture induced a strong decrease in the ATP concentration of the liquid fractions because of cell wash-out (days 5 to 7), but this was not the case in beads whose entrapped cells were maintained in the bioreactor. The pH stress on day 8 and the increase in the redox potential on day 10 (redox potential above -51 mV, the medium turned dark pink for a few hours), maintained the ATP concentrations very low (0.54 ± 0.04 to 0.94 ± 0.03 pmoles mL^{-1}) in the liquid fraction. However, three days after the end of the stress period, the system was highly colonized again, with ATP concentrations reaching 382 ± 30 pmoles mL^{-1} and 151 ± 42 pmol g^{-1} respectively in the liquid fraction and in beads

(day 13). ATP concentrations remained fairly stable until the end of the continuous culture with the exception of a transient decrease both in beads and in the liquid fractions at the beginning of the 85°C period. As expected, cell concentration detected in the liquid fraction globally followed the same behavior as ATP, and reached up to 3.2×10^8 cells mL⁻¹ (Figure 1) after the stress period, during which it decreased down to 10^6 cells mL⁻¹. These results showed that, despite a prolonged stress period that deeply affected cell activity and concentration, cells were maintained in the bioreactor and managed to recolonize the system when conditions became more favorable. Total cell concentration was in the same order of magnitude that the one obtained in Postec *et al.* (2007) after the same time of continuous culture at 60°C (5×10^8 cell mL⁻¹ at day 16).

Population dynamic

The analysis of the microbial diversity with cloning-sequencing showed that among the 8 species present in the inoculum, only *Bacillus* sp. and two strict anaerobes (*T. maritima* and *Thermococcus* sp.) were recovered in beads and in the liquid fraction at the end of the continuous culture. This suggests, as already described (Champagne *et al.* 1994; De Boever *et al.* 2000), that immobilization helped to preserve cell viability during the stress period, and prevented the strains to be washed-out of the system, at least for the least oxygen sensitive ones. Indeed, *T. maritima* can stand short oxygen exposures (Le Fourn *et al.* 2008, 2011; Khal *et al.* 2011).

Quantitative PCR analyses in the liquid fraction showed that *Bacillus* sp. was dominant (10^8 16S rRNA gene copy mL⁻¹) at the end of the batch/fed-batch period. Suggesting that its growth was promoted under microaerophilic conditions, while *T. maritima* and *Thermococcus* sp., more sensitive to oxygen than *Bacillus* sp., were detected in much lower amount (respectively 4.3×10^4 and 6.8×10^3 copy mL⁻¹) (Figure 2). The concentration of the three strains decreased on day 5 due to the start-up of the continuous culture, which partially washed-out the cells. Their concentrations remained low during the stress period, with *T. maritima* showing the lowest detected 16S rRNA gene copy number (5.2×10^2 copy mL⁻¹) on day 8. Two days after the end of this stress period, *Bacillus* sp. and *T. maritima* showed very high 16S rRNA gene copy numbers with respectively 1.4×10^8 and 2.5×10^7 copy mL⁻¹ (day 12). However, *Bacillus* sp. proliferation was transient since it strongly decreased between days 12 and 15, just before the increase of *Thermococcus* sp., whose growth was stimulated with the anoxic conditions, and that reached 1.4×10^7 copy mL⁻¹ (day 20). At the end of the

third period, both *T. maritima* and *Thermococcus* sp. showed high 16S rRNA gene copy number per mL. When the temperature increased to 85°C, the number of 16S rRNA gene copies of *T. maritima* and *Thermococcus* sp. transiently decreased, before stabilizing at high concentrations with *ca* 1.6×10^8 and 1.1×10^8 copies mL⁻¹ respectively. Their 16S rRNA gene copy numbers then decreased during the 60°C period to reach concentrations similar to the ones obtained at the end of the third period (60°C). In the same time, the 16S rRNA gene copy number for *Bacillus* sp. remained low with an average concentration of 2.6×10^5 copies mL⁻¹ between days 15 and 41.

Unfortunately, the other immobilized strains were not detected by PCR, nor by strain-specific qPCR in liquid culture fraction samples, although they were detected by cloning-sequencing in the beads just after immobilization. It is then possible that the period of higher redox potential and pH variation inhibited their growth.

Cell distribution within the beads

Just after immobilization, individual cells were homogeneously distributed into the gel matrix at a very low concentration (Figure 3). After 5 days of culture, only few cells were still present inside the beads. This decrease may be explained by the high redox potential observed during the first period that probably inactivated the most oxygen sensitive strains. At the end of the third and fifth periods (60°C), cells forming micro-colonies were very abundant, and were mainly located on the first external hundred micrometers of the bead. This cell distribution was already reported by Cinquin *et al.* (2006) and was explained by the diffusion limitations of substrates and inhibitory products (Doleyres *et al.* 2002). In contrast, at 85°C only few micro-colonies were visible on the peripheral layer. This decrease in the number of colonies within the beads was confirmed with the ATP concentrations that decreased on day 27 at 85°C. A similar decrease was observed in the liquid culture fraction a few days earlier (days 21 to 24). This phenomenon is probably due to the physiological adaptation of the cells to a higher temperature.

Organic acids production

Organic acids analyses revealed a high production of acetate that greatly increased during the third and fourth periods, a low production of propionate that transiently increased during the fourth period (85°C) and a production of formate that remained low all along the continuous culture (Figure 4). The variation of acetate all along the culture, that was parallel to

Thermococcus sp. and *T. maritima* concentrations, was probably due to carbohydrate fermentation (Huber *et al.* 1986; Pikuta *et al.* 2007). In the early days, acetate concentration slightly increased to reach 2300 μM on day 4. After a transient decrease associated with the start-up of the continuous culture (day 5), the acetate concentration strongly increased from day 12 to 14 (plus 4279 μM) in parallel to the increase of *T. maritima*, and between day 15 and 20 (plus 1392 μM) in parallel to the delayed growth of *Thermococcus* sp. at the end of the third period (60°C). During the fourth (85°C) and fifth periods (60°C), acetate concentration increased again in parallel to the 2 strains. Propionate concentrations remained very low (<110 μM) during the first 21 days, until a transient increase (up to 810 μM) during the fourth period (85°C), potentially linked to the growth of *Thermococcus* sp., a propionate producing strain (Legin *et al.* 1997). The formate concentration remained very low (below 570 μM) all along the culture. Overall, microbial community showed a great reactivity to culture condition changes in parallel in the beads and liquid culture fraction as observed by Cinquin *et al.* (2006).

Conclusion

In this report, we showed that it was effectively possible to immobilize and maintain (hyper)thermophilic marine microorganisms for several weeks in continuous culture. Total cell, organic acid concentrations and ATP monitoring showed that the polymer beads were highly colonized and that the microorganisms were released in the liquid fraction and reactive to culture conditions. Moreover, the use of an immobilized inoculum highlighted a certain resilience capacity of the system by allowing the microbial recolonization after a prolonged stress period. In the end, this new immobilization protocol provides a new tool for the study of hydrothermal vent chimney microbial communities during continuous cultures.

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CONFLICT OF INTEREST

No conflict of interest declared.

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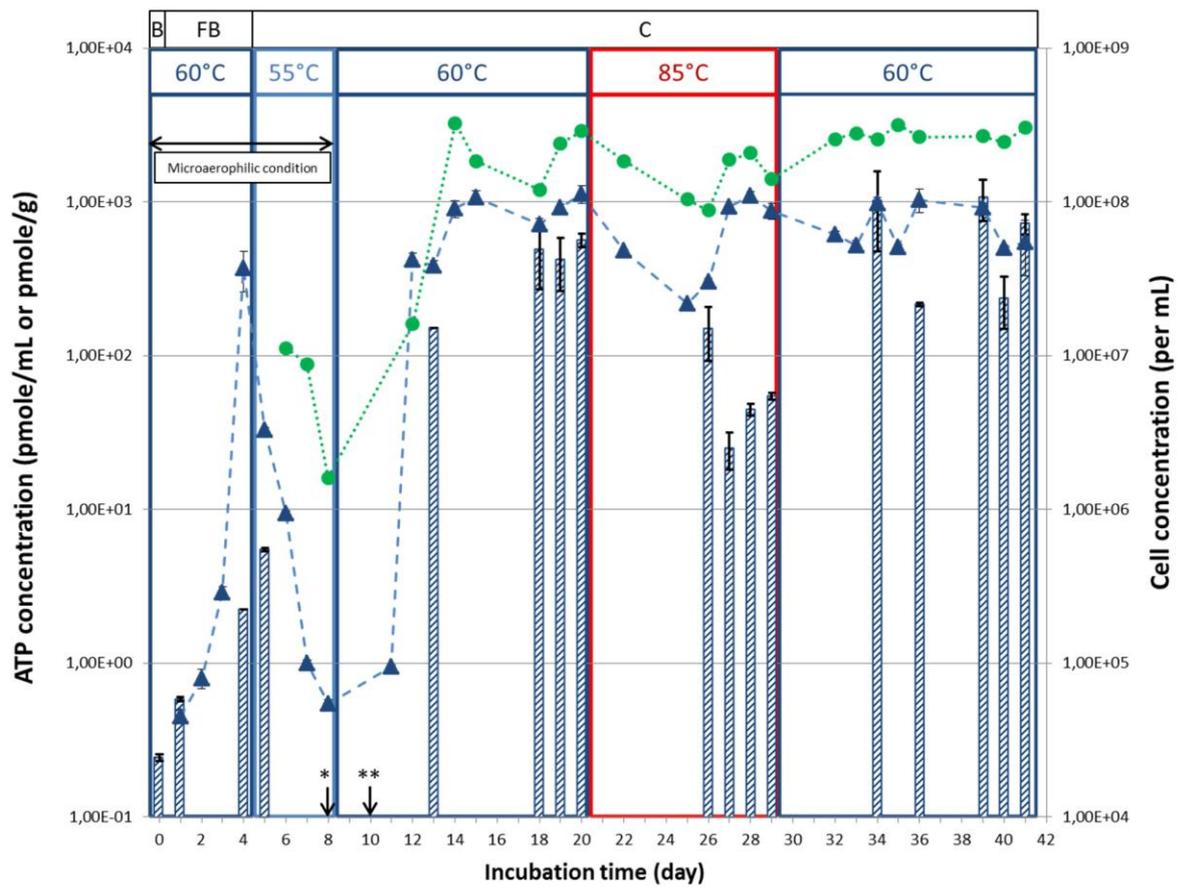


Figure 1. Continuous culture of immobilized (hyper)thermophilic microorganisms. B: Batch, FB: Fed batch, C: continuous culture; (▨) ATP concentration in beads, (▲) ATP concentration in liquid fractions, (●) cell concentration in liquid fractions. * pH stress (day 8), ** oxalic stress (day 10).

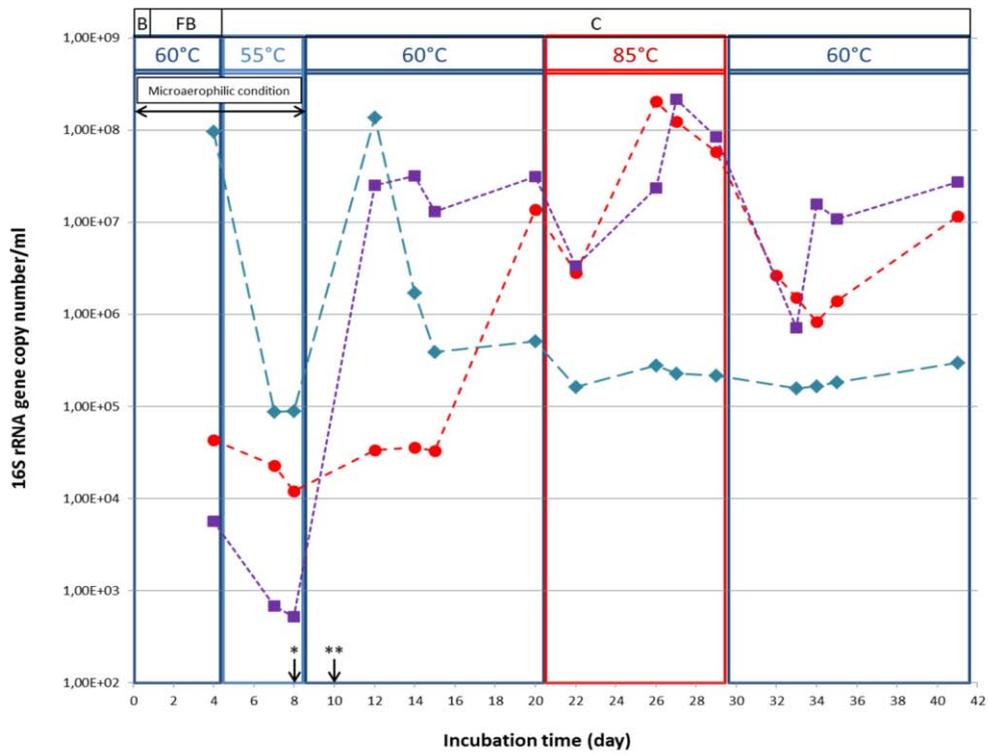


Figure 2. 16S rRNA gene copy number/ml in liquid fractions during the continuous culture of immobilized (hyper)thermophilic microorganisms. (■) *Thermotoga maritima*, (●) *Thermococcus sp.*, (◆) *Bacillus sp.* * pH stress (day 8), ** oxic stress (day 10).

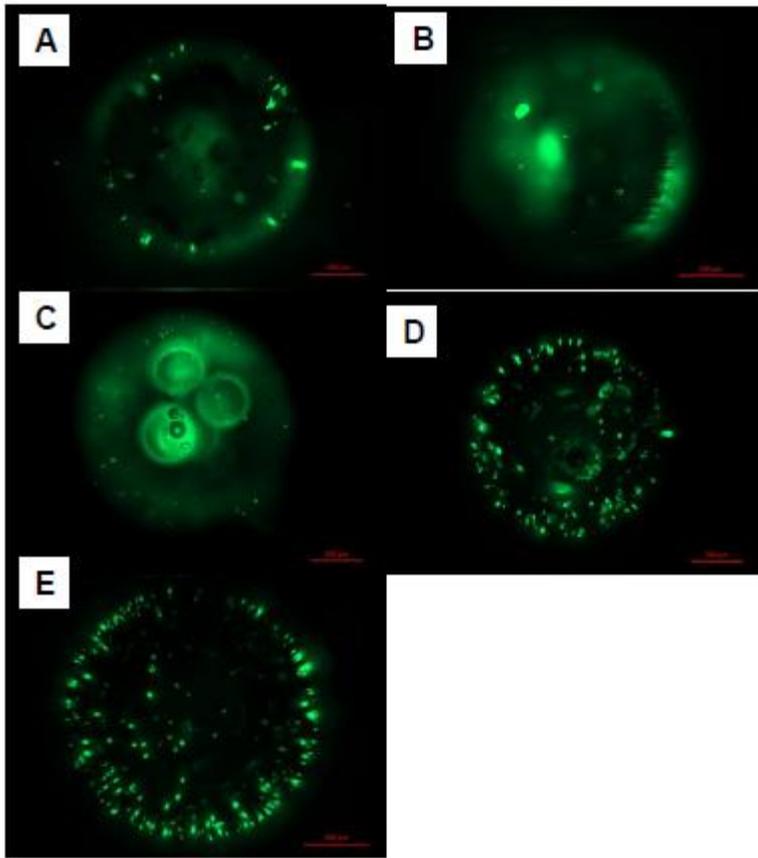


Fig. 3

Figure 3. Maximum intensity projection of beads stained with SYBR Gold after (A) 0, (B) 5, (C) 20, (D) 29 and (E) 41 days of culture.

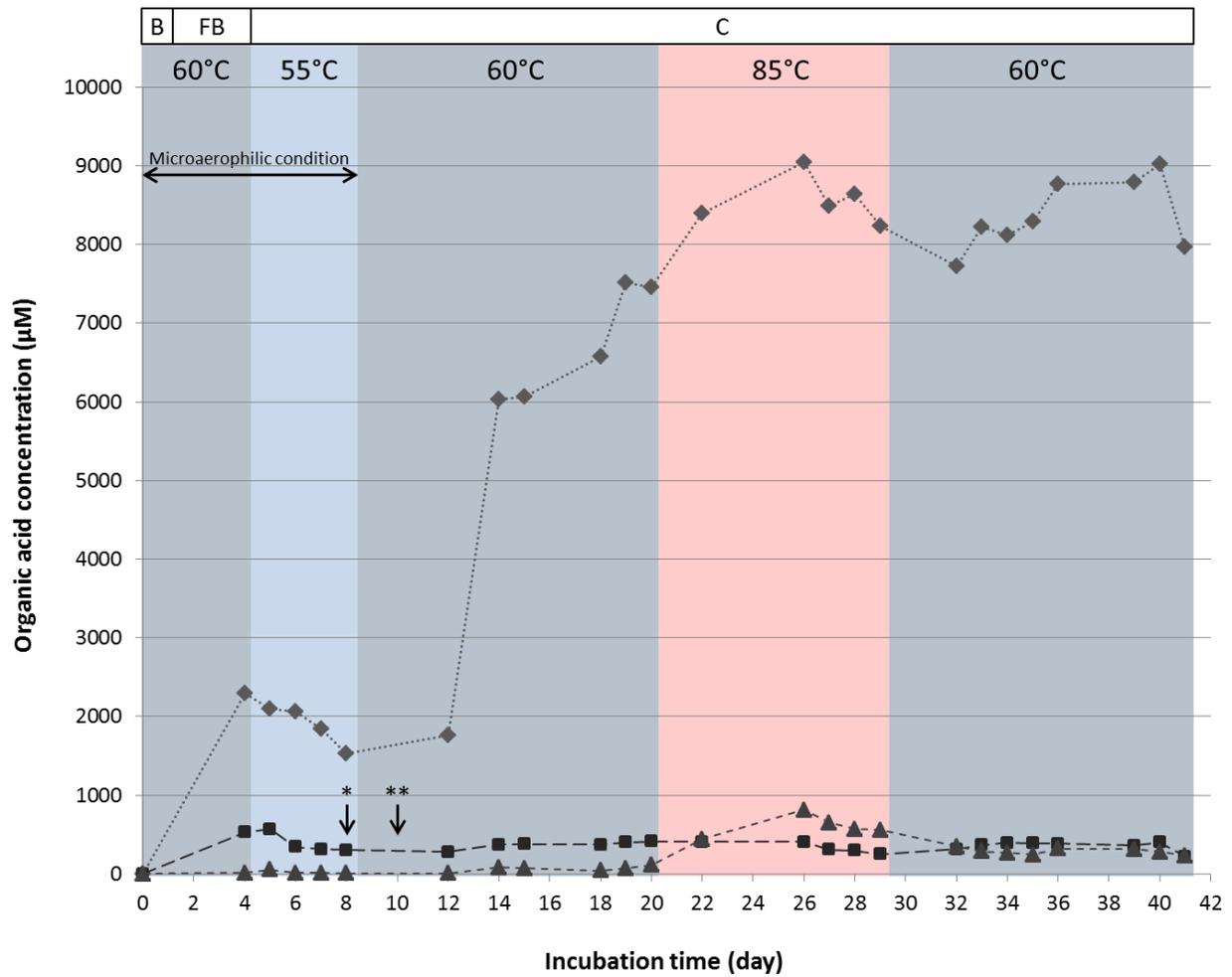


Figure 4. Organic acids concentration (μM) in liquid fractions during the continuous culture of immobilized (hyper)thermophilic microorganisms. (\blacklozenge) Acetate, (\blacksquare) formate, (\blacktriangle) propionate. * pH stress (day 8), ** oxalic stress (day 10).