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Entrapment of anaerobic thermophilic and hyperthermophilic marine microorganisms in a gellan/xanthan matrix

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Abstract :

Aims : The aims of this study were (i) to develop a protocol for the entrapment of anaerobic (hyper)thermophilic marine micro-organisms; (ii) to test the use of the chosen polymers in a range of physical and chemical conditions and (iii) to validate the method with batch cultures.

Methods and Results : The best conditions for immobilization were obtained at 80°C with gellan and xanthan gums. After 5-week incubation, beads showed a good resistance to all tested conditions except those simultaneously including high temperature (100°C), low NaCl (<0·5 mol l−1) and extreme pH (4/8). To confirm the method efficiency, batch cultures with immobilized Thermosipho sp. strain AT1272 and Thermococcus kodakarensis strain KOD1 showed an absence of detrimental effect on cell viability and a good growth within and outside the beads. Conclusion This suggests that entrapment in a gellan–xanthan matrix could be employed for the culture of anaerobic (hyper)thermophilic marine micro-organisms. Significance and Impact of the Study (Hyper)thermophilic marine micro-organisms possess a high biotechnological potential. Generally microbial cells are grown as free-cell cultures. The use of immobilized cells may offer several advantages such as protection against phage attack, high cell biomass and better production rate of desired metabolites.

Keywords : (hyper)thermophilic marine micro-organisms, anaerobiosis, entrapment, gellan, immobilization, xanthan.

Introduction

Microorganism immobilization is commonly used in many fields including food, pharmaceutical, agricultural, therapeutics, environmental and research applications (Cassidy *et al*. 1996). This technology is generally used for biomass production and/or for the production of various compounds such as amino acids, organic acids, antibiotics, steroids and enzymes, either in batch, fed-batch or continuous cultures. Extensive applications of immobilized cells have been proposed in the industry using different strategies of immobilization, such as adsorption or attachment to inert surfaces, self-aggregation of cells by flocculation, encapsulation in polymer gels or entrapment in different type of matrices (Rao and Satyanarayana 2009). Cell immobilization offers several advantages over free-cell cultures such as high cell biomass, enhance survival, and may increase production rate of desired metabolites (Rathore *et al*. 2013). Among the different types of immobilization, cell entrapment in polymer matrices is commonly used for a wide variety of microorganisms that do not flocculate or naturally attach to inert substrates, and because it induces a high cell viability (Kanasawud *et al*. 1989; Rathore *et al*., 2013). Cell entrapment allows the diffusion of small molecules that sustain the viability, activity and growth of the entrapped cells. In addition, they are protected against abiotic stress and potential inhibitors present in the culture medium, bacteriophages attacks and shear forces (D'Souza 2002; Nussinovitch 2010). Their biological stability is increased with small loss of plasmids and the physical retention of cells within the bioreactor prevents wash-out of slow growing cells in case of continuous cultures (Champagne *et al*. 1994; Lamboley *et al*. 1999). Beads containing the immobilized cells may be recovered, stored and reused. Entrapment protocols for (hyper)thermophilic microorganisms have been poorly described in literature data. Only few thermophilic bacterial species such as *Thermus* spp*., Bacillus* spp. and *Geobacillus* spp. have been entrapped in polymer matrices (gellan, sol-gel silica, κ-carrageenan, alginate, agarose and polyacrylamide)

(Klingeberg *et al*. 1990; Norton and Lacroix 2000; Kabaivanova *et al*. 2005; Rao and Satyanarayana 2009). To our best knowledge nobody has never developed an entrapment protocol for the culture of thermophilic and hyperthermophilic anaerobic marine microorganisms despite their high biotechnological potential as source of novel enzymes and active compounds (Huber and Stetter 1998; Bustard *et al*. 2000; Schiraldi and De Rosa 2002; Trincone 2011). We propose to develop a protocol for the entrapment of thermophilic and hyperthermophilic marine microorganisms in a polymers matrix. The judicious selection of polymers and conditions for cell entrapment was here critical for ensuring beads production and mechanical strength, together with the maintaining of cell viability in conditions compatible with microorganisms growth. Gellan and xanthan polysaccharides appeared to be good candidates because of their non-toxic, heat-resistant and pH resistant gelling properties. Beads size and mechanical resistance through long-term culture being of primordial importance in immobilized cell culture, general mechanical properties of the beads was studied in order to determine beads behavior in different incubation conditions (salinity, pH, temperature and sulfur concentration) mimicking different growth conditions. The objectives of this study were (i) to develop a protocol for the entrapment of thermophilic and hyperthermophilic anaerobic marine microorganisms, (ii) to test the mechanical stability of the beads in different physico-chemical conditions, and (iii) to validate the method with batch cultures of immobilized marine microorganisms with *Thermococuus kodakarensis* strain KOD1 and *Thermosipho* sp. strain AT1272 used as model organisms.

Materials and Methods

Microbial strains and growth conditions

Thermosipho sp. strain AT1272 (DSM 101094), a thermophilic strain previously isolated from a Rainbow hydrothermal chimney sample in our laboratory (Postec *et al*. 2005), and

Thermococcus kodakarensis strain KOD1 (JCM 12380^{T}) a hyperthermophilic strain, were used as models for the immobilization trials. *Thermosipho* sp. AT1272 and *Thermococcus kodakarensis* KOD1 were routinely grown under nitrogen atmosphere respectively at 60 °C and 80 °C in Ravot Modified Medium (RMM, pH 6·0) (Gorlas *et al*. 2013) reduced by the addition (1 %, v/v) of Na_2S (0·2 mol l^{-1}). Growth experiments were performed under nitrogen gaz in penicillin vials. Prior to immobilization, strains were subcultured twice for 16 h in routine conditions. Their concentration was adapted in order to obtain *ca* 3×10^{8} cells ml^{-1}, and 4 ml of this suspension were mixed with the different polymer solutions under anaerobic conditions in order to obtain a final concentration of *ca*. 6×10^{6} cells ml^{-1} of polymer as explained above, with the exception of beads used during the mechanical stability experiments that were sterile. In the case of subculture in liquid medium for growth comparison with cell immobilization, cells were inoculated around 6×10^{6} cells ml^{-1}.

Polymers preparation and immobilization procedure

Beads were prepared with two types of polymers, gellan and xanthan gums (Sigma-Aldrich, France), either alone (gellan gum at 2·5 %, w/v) or as a mixture (gellan at 2·5 % and xanthan at 0·25 %, w/v). Polymer powders were suspended in 150 ml of preheated (90 °C) distilled water and mixed for 25 s in a blender. The polymer solution was then autoclaved for 15 min at 121 °C just prior the immobilization. The immobilization procedure was adapted from Cinquin *et al*. (2004), who developed an entrapment protocol for mesophilic bacteria. This process is based on a two phase system composed of a polymer solution and oil under agitation, this technique allows the production of beads recovered by sieving. In order to apply this technique to the entrapment of strict anaerobic marine hyperthermophiles and thermopiles, two different polymer compositions were tested with different NaCl concentrations (0·15, 0·20, 0·27 and 0·38 mol l^{-1} final concentrations). These concentrations

were tested in order to obtain conditions allowing the formation of a maximum of beads with an average size of 1-2 mm while minimizing the osmotic stress. All solutions were deoxygenated under N_2 flow, autoclaved and reduced with Na_2S (0·001 mol l^{-1} final concentration). The polymer solution, hardening solution (Ravot modified medium, RMM), salt solution composed of NaCl (0·61 to 1·64 mol l^{-1}) and sodium citrate (0·06 mol l^{-1}), canola oil (400 ml) and cell suspensions were transferred under an anaerobic hood. Fifty ml of NaCl solution were added to the polymer solution (80 °C) that was inoculated, if necessary, with 4 ml of cell suspension, to reach a final concentration of *ca* 2×10^7 cells ml^{-1}. After inoculation, the polymer solution was stirred at 250 rpm min^{-1} into oil at 80 °C to obtain a suspension of aqueous droplets in oil. This suspension was cooled for 10 min at room temperature followed by 10 min incubation on ice before being soaked 30 min under agitation in RMM for beads hardening. After washing, beads of the appropriate size (1-2 mm diameter) were selected by wet sieving.

For each NaCl concentration tested during the immobilization step, beads size distribution was measured using a laser granulometer Beckman Coulter LS™ 200 (Beckman Coulter Inc., Brea, USA), total volume of formed beads (1-2 mm diameter) was measured by water-displacement, and the general appearance of the beads was observed with a binocular microscope SDF PLAPO 1XPF (Olympus, Tokyo, Japon).

Rheological tests

The viscoelastic behavior of the gellan and gellan plus xanthan gels was characterized in absence and in presence of NaCl (0·20 mol l^{-1} final concentration). Polymer solutions were prepared as described above, and poured in Petri dishes. After cooling, 2-mm thick 25-mm diameter cylindrical gel samples were cut and their rheological behavior was characterized using oscillatory simple shear tests performed in the linear regime, at room temperature, using

a Bohlin Gemini constant stress rheometer equipped with parallel plates (diameter = 25 mm; gap = 2 mm). Waterproof abrasive paper of equivalent roughness of about 10 μm was put on both plates in order to prevent sample slippage. These experiments were done in triplicates.

Mechanical stability of sterile beads in different incubation conditions using experimental design

- Experimental design methodology

Design of experiments consist of a group of mathematical and statistical techniques that can be used to organize experiments at best in order to quantify the relationship between the output variables (called responses) and the input variables (called factors). They allow real advantages in terms of reduced experimental effort and increased quality of information (Lewis *et al*. 1999; Cela *et al*. 2009). Because of cost and run-time of experiments, this methodology was chosen to limit the number of experiments, judiciously selected, to study the influence of four parameters (factors) on beads mechanical stability. These four factors (sulfur and NaCl concentrations, pH and temperature) (Table 1) were considered as potentially influential on beads mechanical stability over time (5-week incubation). The variation range for each factor was determined based on a preliminary study and their effects were evaluated by granulometry (beads size distribution) (Y_1), polymer release (Y_2) and beads general deterioration (Y_3).

- Design of experiments

As the aim of this study was a direct comparison of three or more values, a screening study was performed and an additive mathematical model was postulated. The reduced reference state model used can be written as follows:

$$\eta = \beta_0 + \beta_{1A}X_{1A} + \beta_{1B}X_{1B} + \beta_{2A}X_{2A} + \beta_{2B}X_{2B} + \beta_{2C}X_{2C} + \beta_{3A}X_{3A} + \beta_{3B}X_{3B} + \beta_{3C}X_{3C} + \beta_{4A}X_{4A} + \beta_{4B}X_{4B} + \beta_{4C}X_{4C} + \beta_{4D}X_{4D}$$

where X_{ij}=1 when the level j of the variable i is present and X_{ij} = 0 for the other cases. The coefficient β_{ij} represents the variation of the response, replacing one level of the variable i, considered as a reference stats (arbitrarily the last level), by the level j.

In order to estimate the coefficients at best, a suitable experimental design was performed and more precisely, an asymmetrical optimal design $3^1 4^2 5^1$ in 16 experiments (Table 2). The experiments (Addelman 1962; Fedorov and Malyutov 1972) were replicated three times to evaluate the variance of experimental error. From the experimental results for each studied responses (Y_1, Y_2, Y_3), the estimation of the model coefficients β_{ij} were calculated by least squares regression. These coefficients could be graphically represented in order to show the behavior of the different levels for each variable.

- Experimental

Beads were produced as described before with a mixture of gellan (2·5 %, w/v) and xanthan (0·25 %, w/v) and a NaCl concentration of 0·2 mol l^{-1}. They were incubated in different conditions of pH (4·0 to 8·0), temperature (50 to 100 °C), NaCl (0·08 to 1·36 mol l^{-1}) and sulfur concentrations (0·03 to 0·15 mol l^{-1}) in modified SME medium. This medium is commonly used for the continuous culture of hydrothermal vent microbial communities (Postec *et al*. 2005). Basal modified SME medium without NaCl and sulfur was realized. NaCl was added, pH was adjusted according to the different conditions tested, and 50 ml were distributed in penicillin vials. Vials were autoclaved at 121 °C for 20 min followed by the addition of sterile colloidal sulfur. Twenty five grams of freshly produced sterile beads were added in each vial. Incubations were realized in triplicate for each condition tested (Table 2).

After incubation, beads size distribution was measured using a laser granulometer Beckman Coulter LS™ 200 (Beckman Coulter Inc., Brea, USA), polymer release in the modified SME medium was quantified using a colorimetric method adapted from Dubois *et al*. (1956). Two ml of pure sulfuric acid and 0·5 ml of phenol 5 % (w/v) were added to 0·5 ml of sample, incubated 15 min at 95 °C and 15 min at room temperature in dark condition before measurement of absorbance at 492 nm. The general appearance of the beads was observed with a binocular microscope.

Growth of immobilized microorganisms in batch experiments

Batch cultures were performed with 3 grams of beads containing freshly immobilized cells, *Thermococcus kodakarensis* KOD1 or *Thermosipho* sp. AT1272, in 10 ml of RMM placed in sealed penicillin vials under nitrogen atmosphere. The vials were incubated at different temperatures (60, 65, 70 or 80 °C). Cell growth in beads and liquid medium was measured in triplicates. Cell counting by regular methods being impossible in polymer beads, a protocol based on the measure of cellular ATP was applied in beads and culture medium. The correlations, r^2 = 0·982 for *T. kodakarensis* KOD1 and r^2 = 0·998 for *Thermosipho* sp. AT1272, between cell counting using a Thoma cell counting chamber and ATP values, were determined at different dilutions (10^0, 10^{-1}, 10^{-2}, 10^{-3}) according to Gaboyer *et al*. (2014). The appropriate correlation factor was applied to each sample tested, in order to evaluate the number of cell ml^{-1} for each strain. The ATP content of bacterial suspensions in the liquid culture mediums was determined with a Kikkoman Lumitester C-110 (Isogen Life Science) using the BacTiterGlo Microbial Cell Viability assay (Promega) according to the manufacturer's instructions: 100 μl of culture and 100 μl of BacTiter-Glo buffer were used. Internal calibration was performed with 10 μl of a 100 nmol l^{-1} ATP solution and maximal fluorescence emissions values were considered. In the case of beads, these ones (*ca* 100 mg)

were placed in a pre-weighted sterile hemolysis tube (Gosselin), they were washed thrice with 100 μl of sterile degazed saline solution. For ATP measurement, 100 μl of sterile distilled water were added to the beads, which were vortexed for 10 s before adding 100 μl of BacTiter-Glo buffer. As for liquid medium, internal calibration was performed with 10 μl of a 100 nmol l^{-1} ATP solution and maximal fluorescence emissions values were considered. All manipulations were done in triplicates under sterile conditions.

Results

Influence of NaCl concentration and polymer matrix on beads formation

The immobilization of thermophilic and hyperthermophilic marine microorganisms implicated the use of heat-stable polymers together with NaCl in order to preserve marine cells from osmotic stress. The influence of NaCl concentration and the use of gellan with or without xanthan gum were tested for the production of the largest volume of beads with the right size (1-2 mm) and morphology (round), compatible with their use in batch or continuous cultures. The increase in NaCl concentration within the gel (0·15, 0·20, 0·27 and 0·38 mol l^{-1}) during the emulsion step, dramatically perturbed beads formation, with or without addition of xanthan. Addition of NaCl to the gellan solution induced a decrease in the total volume of beads of 1-2 mm (from 93 ± 1·4 ml at 0·15 mol l^{-1} NaCl to 11 ml at 0·38 mol l^{-1}), and profoundly modified their surface. This one appeared much rougher at 0·38 mol l^{-1} NaCl compare to 0·15 mol l^{-1}. Addition of xanthan gum induced a higher volume of produced beads than gellan alone (113 ± 13 ml vs 74 ± 12 ml at 0·2 mol l^{-1} NaCl), and improve their morphology (Fig. 1). Nor the addition of NaCl, nor the presence of xanthan significantly modified beads diameter (average diameter of 1095 ± 365 μm).

Influence of NaCl concentration on gels viscoelastic behavior

Rheological tests showed an increase in the storage modulus (G') in parallel with the increase in NaCl concentration (Fig. 2). Indeed, the storage modulus value stepped from 4×10^3 Pa without NaCl, up to 2×10^5 Pa with 0·2 mol l^{-1} NaCl, with or without xanthan. As expected, an increase in NaCl concentration induced a stiffening of the gel, which suggests a decrease of the average mesh size of the gel network induced by NaCl. At least if the gel can be considered as homogeneous. At last, it should be pointed out that the influence of xanthan on the viscoelastic properties of the gellan/xanthan gels studied in this work is quite weak, even if it is slightly more marked for the elastic properties (G') in the presence of NaCl.

Mechanical stability of beads

Analyses of the experimentation results were performed with the NEMRODW software (Mathieu *et al*., 2009), and coefficients were estimated for each response. To facilitate the interpretation, coefficient values were plotted in order to visualize the behavior of each level of the studied factors.

- Response Y_1: granulometry

For granulometry results, the model can be written as follows:

$$\mathbf{Y_1 = 639 \cdot 35 - 3 \cdot 97X_{1A} + 80 \cdot 14X_{1B} + 28 \cdot 63X_{2A} - 97 \cdot 28X_{2B} + 78 \cdot 45X_{2C} - 111 \cdot 44X_{3A} + 89 \cdot 84X_{3B} + 98 \cdot 23X_{3C} + 496 \cdot 13X_{4A} + 423 \cdot 02X_{4B} + 358 \cdot 82X_{4C} + 319 \cdot 93X_{4D}}$$

Before incubation, the average diameter of beads was of 1095 ± 365 μm. After 5-week incubation in different conditions, their average diameters varied from 796 ± 365 μm to 1156 ± 501 μm, with the exception of the condition n°14 (T° 100 °C, pH 4·0, 0·50 mol l^{-1} NaCl and 0·03 mol l^{-1} sulfur), which dramatically reduced beads diameter down to 297 ± 273 μm. From

the effect plot (Fig. 3), it can be observed that temperature very strongly influenced beads diameter. When the temperature increased from 50 °C to 100 °C, the average diameter of beads decreased down to 496 μm (minus 45 %). In a lesser extent, NaCl concentrations and pH also significantly influenced bead diameters with a decrease of diameters at extreme pH (especially pH 4·0) and low NaCl concentrations (0·50 mol l^{-1}). Sulfur concentrations induced a slight but significant impact by reducing beads diameters at 0·03 and 0·15 mol l^{-1}.

- Response Y_2: polymer released

The regression coefficients of the model for polymer released are:

$$Y_2 = 0 \cdot 71 - 0 \cdot 14X_{1A} - 0 \cdot 25X_{1B} + 0 \cdot 09X_{2A} + 0 \cdot 12X_{2B} + 0 \cdot 05X_{2C} - 0 \cdot 13X_{3A} - 0 \cdot 19X_{3B} - 0 \cdot 03X_{3C} - 0 \cdot 49X_{4A} - 0 \cdot 48X_{4B} - 0 \cdot 45X_{4C} - 0 \cdot 40X_{4D}$$

From the effect plot (Fig. 4), it can be observed that temperature strongly influenced polymers release after 5-week incubation. When temperature increased from 90 °C to 100 °C, the average polymer release increased from 0·044 ± 0·01 g to 0·104 ± 0·04 g per vial, which is equivalent to 0·18 % and 0·52 % of beads initial masses. We can also note a slight but significant effect of pH and sulfur, with an increase in polymer release at extreme pH and at 0·15 mol l^{-1} of sulfur. NaCl concentrations had no significant impact on polymer release. It has to be noted that the maximal amount of polymer release reached 0·11 g, which represented less than 1 % of polymer release after 5-week incubation.

- Response Y_3: visual aspect

Different numbers were assigned to beads, depending on their visual aspect. Fresh non degraded beads (round and smooth) were noted 1, whereas the most degraded beads were noted 5 (Fig. 5). Beads noted up to 4 harbored a shape compatible with immobilized cell

culture, which was not the case with beads noted 5 that were highly degraded. The regression coefficients of the model are:

$$Y_3 = 3 \cdot 61 - 0 \cdot 01X_{1A} + 0 \cdot 62X_{1B} + 1 \cdot 24X_{2A} + 0 \cdot 92X_{2B} - 0 \cdot 48X_{2C} + 0 \cdot 27X_{3A} - 0 \cdot 10X_{3B} + -0 \cdot 35X_{3C} - 1 \cdot 82X_{4A} - 2 \cdot 16X_{4B} - 1 \cdot 23X_{4C}1 \cdot 54X_{4D}$$

After 5-week incubation, once again, temperature significantly affected beads morphology. This was especially true when a high temperature was associated with a low NaCl concentration, with a highly significant effect with 0·08 and 0·50 mol l^{-1} of NaCl. An association of high temperature (100 °C), low NaCl concentration (≤ 0·50 mol l^{-1}) and extremes pH (4·0 and 8·0) induced a pronounced deterioration of the beads.

Immobilized cell growth in batch experiments

In order to validate this new entrapment protocol, a thermophilic bacteria *Thermosipho* sp. AT1272 and a hyperthermophilic archaea *T. kodakarensis* KOD1 were used as models. The two strains were immobilized in anaerobiosis. Their growth was monitored, both in the liquid medium and within the beads after 24 h and 48 h of incubation at 60, 65, 70 or 80 °C in RMM medium. Preliminary experimentations allowed associating ATP concentrations to cell concentrations, for each strain. Consequently, it was possible to estimate cell concentrations in beads and liquid medium along the incubation period. Just after immobilization, the amount of cells within the beads was estimated at $2{\cdot}4 \times 10^5 \pm 8{\cdot}6 \times 10^4$ cells g^{-1} for *Thermosipho* sp. AT1272 and $2{\cdot}3 \times 10^6 \pm 1{\cdot}3 \times 10^5$ for *T. kodakarensis* KOD1 (Fig. 6). This represents a percentage of viability of 2·7 % for *Thermosipho* sp. AT1272 inoculated at $9{\cdot}1 \times 10^6 \pm 1{\cdot}0 \times 10^6$ cells ml^{-1} in the polymer solution, and of 54 % for *T. kodakenrensis* KOD1 inoculated at $4{\cdot}3 \times 10^6 \pm 9{\cdot}7 \times 10^4$ cells ml^{-1}. After 24 h of incubation at 65°C, *Thermosipho* sp. AT1272 concentrations reached their maximum with $2{\cdot}9 \times 10^7 \pm 1{\cdot}9 \times 10^7$ cells g^{-1} of beads, and 6·1 ×

$10^8 \pm 5{\cdot}7 \times 10^7$ cells ml^{-1} in the liquid medium (Fig. 6). In the case of *T. kodakarensis* KOD1, the highest cell concentrations were observed at 70°C after 24 h incubation, with $4{\cdot}8\times10^7\pm 1{\cdot}3\times10^7$ cells g^{-1} of beads and $3{\cdot}3\times10^8 \pm 2{\cdot}4\times10^7$ cells ml^{-1} in liquid medium. In comparison, *Thermosipho* sp. AT1272 reached $2{\cdot}4\times10^8 \pm 1{\cdot}1\times10^7$ cells ml^{-1} in free-cell culture, whereas *T. kodakarensis* KOD1 reached $1{\cdot}4\times10^8 \pm 9{\cdot}8\times10^6$ cells ml^{-1} in the same incubation conditions.

Discussion

The objective of this work was to develop a new protocol for the entrapment of thermophilic and hyperthermophilic marine microorganisms in a polymer matrix. The judicious selection of methods and polymers was critical to ensure the highest viability of entrapped cells, together with the highest mechanical stability of beads at high temperatures (Rathore *et al*. 2013). The double-phase dispersion process previously described for the entrapment of mesophilic microorganisms with a mixture of gellan and xanthan (Cinquin *et al*. 2004), was adapted to high temperature and saline conditions. Gellan is an anionic exopolysaccharide produced by *Sphyngomonas elodea*, its commercial form is a low acyl, linear homopolymer. It forms heat-stable gels (up to 90 °C) whose conformation and structure, depend on gellan concentration, temperature, ionic strength, and type (monovalent or divalent) of stabilizing cations in the aqueous solution. The commercial gellan powder is usually dissolved in preheated distilled water at 90°C. The polymer solution is then autoclaved and maintained at high temperature. When the temperature decreases, the gelation of the polymer solution occurs by aggregation of the single-stranded helices in presence of monovalent (Na^+ and K^+) and divalent (Ca^{2+} and Mg^{2+}) cations. Aggregation stabilizes at higher temperature than their melting point, which induces thermal hysteresis between gelation and melting (Giavasis *et al*. 2000; Morris *et al*. 2012). These properties, together with its low toxicity and resistance to

enzyme hydrolysis, explain that gellan is used in many applications, including cell immobilization (Giavasis *et al*. 2000). Xanthan is a polysaccharide produced by *Xanthomonas campestris*. It is a non-linear anionic and non-gelling polymer, that resists to high temperatures and acidic pH (Giavasis *et al*. 2000). Its interactions with gellan, result in different type of textures with different mechanical properties (hardness, brittleness, elasticity) (Rodriguez-Hernandez and Tecante 1999). Xanthan is known to considerably reduce the syneresis properties of gellan gel, and to increase its viscoelasticity, which is particularly relevant for microorganism immobilization (Rodriguez-Hernandez and Tecante 1999; Giavasis *et al*. 2000).

In a first step, the effects of NaCl and xanthan, in gellan beads formation were tested. Addition of increasing concentrations of NaCl during the emulsion step at high temperature (80 °C) strongly decreased the total volume of produced beads, which became highly deformed with rough surfaces for NaCl concentrations above 0·27 mol l^{-1}. This is not surprising knowing the high reactivity of gellan to cations (monovalent Na^{+} in that case), that increase its melting temperature and induce stiffer gel formation (Morris *et al*. 2012). Addition of xanthan allowed the production of greater volumes of beads, with round shapes and regular surfaces, up to a concentration of 0·27 mol l^{-1} NaCl. Beads diameters were not impacted by xanthan, whatever the concentration of NaCl. This is not surprising given that beads size is rather linked to the emulsion speed. The rheological study demonstrated that NaCl increased the elastic modulus and, therefore the stiffness properties of the gellan gel. Contrary to the observations of Rodriguez-Hernandez and Tecante (1999), addition of xanthan did not increase G", suggesting a lower impact on the gel viscose behavior. This is probably due to the lower proportion of xanthan (10 %, w/v vs 20 %, w/v), together with the higher concentration of gellan (2·5 %, w/v vs 0·5 %, w/v), used in the present study. Rodriguez-Hernandez and Tecante (1999) showed that a mixture of gellan and xanthan

resulted in a heterogenic phase-separated gel with no interaction between gellan and xanthan polymers. They also showed that addition of xanthan reduced gel syneresis. This effect is particularly interesting for the viability of entrapped cells. A polymer matrix composed of gellan and xanthan, with a concentration of 0·20 mol l^{-1} NaCl, was then chosen for further experimentations. Indeed, it allowed the formation of a high volume of beads with NaCl concentration compatible with marine microorganism viability.

In a second step, the mechanical stability of the beads was tested in incubation conditions compatible with marine thermophiles /hyperthermophiles culture. Beads mechanical stability is one of the major parameter allowing the successful use of beads in different types of culture and fermentation procedures. In that case, the use of a marine culture medium was an advantage due to its high ionic strength, allowing a good stability of the gel, even at high temperatures. Indeed, Norton and Lacroix (2000) showed that bacterial immobilization in gellan gum for incubation at 80°C was not possible, because of the gel weakness and its low resistance to stress in dairy fluids. This was not the case in the present study, because of the high ionic strength of the marine culture medium. According to our experimental design, increasing temperature was the major factor affecting beads stability in terms of granulometry, polymer release and general aspect of the beads. Extreme pH slightly increased polymer release and decreased beads diameter, whereas low NaCl concentrations decreased beads diameter. These results are in accordance with the known properties of gellan/xanthan gels. Globally, the beads resisted very well to 5-week incubation in the sixteen tested conditions. The modified SME medium allowed a good mechanical stability of the beads up to 90 °C, especially with NaCl concentrations above 0·50 mol l^{-1}, whatever the pH and the sulfur concentrations. However, conditions simultaneously implicating a temperature of 100 °C, NaCl concentration below 0.50 mol l^{-1} and extreme pH should be avoided. This once again, is in accordance with literature data showing that the strength of gellan gels increases

in a pH range of 4·0 to 7·0, gellan being stable between pH 2·0 to 10·0, whereas xanthan possesses a smaller range of pH stability of 4·0 to 8·0 (Giavasis *et al*. 2000).

In a third step, cell survival rate after immobilization, and cell growth capacity within and outside the beads were tested in batch experiments. Cell viability of entrapped cells is usually assessed after beads disruption and cell counting on agar plates (Sun and Griffiths 2000; Cinquin *et al*. 2004). However, in that case, the stiffness of the beads did not allow their disruption with physical and mechanical methods, at least in conditions compatible with cell survival. Moreover, marine microorganisms do not always grow on agar plates. Consequently, we estimated cell survival rate, cell concentration, and growth by analyzing their ATP content. Immediately after immobilization, cell survival rate was of 2·7 % for *Thermosipho* sp. AT1272 and 54 % for *T. kodakarensis* KOD1. These results are in accordance with literature data showing survival rates between 1 and 40% for mesophilic bacteria (Cinquin *et al*., 2006). *Thermosipho* sp. AT1272 reached concentrations of $6{\cdot}1 \times 10^{8} \pm 5{\cdot}7 \times 10^{7}$ cells ml^{-1} in the liquid medium, and $2{\cdot}9 \times 10^{7} \pm 1{\cdot}9 \times 10^{7}$ cells g^{-1} in beads after 24 h incubation at 65°C, compared to $2{\cdot}5\times10^{8} \pm 1{\cdot}1 \times10^{7}$ cells ml^{-1} for the free-cell cultures in the same conditions. *T. kodakarensis* KOD1 reached $3{\cdot}3\times10^{8} \pm 2{\cdot}4\times10^{7}$ cells ml^{-1} and $4{\cdot}8\times10^{7} \pm 1{\cdot}3\times10^{7}$ cells g^{-1}, respectively in the liquid medium and in beads after 24 h incubation at 70°C, compared to $1{\cdot}4 \times 10^{8} \pm 9{\cdot}8\times10^{6}$ cells ml^{-1} in free-cell cultures. The high percentage of the survival rates, together with the high concentrations of both strains in beads and liquid medium, showed that entrapment and culture of immobilized anaerobic (hyper)thermophilic marine strains is possible at high temperature.

We successfully developed for the first time a protocol dedicated to the entrapment of anaerobic thermophilic and hyperthermophilic marine strains. We showed that despite difficulties associated with the work at high temperatures, in strict anaerobic and saline

conditions, it was possible to use a polymers matrix made of gellan and xanthan to immobilize and grow thermophilic and hyperthermophilic strains. Moreover, the beads showed a very good mechanical resistance in a large panel of conditions compatible with growth conditions of numerous marine thermophiles/hyperthermophiles microorganisms. Cell entrapment is a useful technology that can be applied in a multiplicity of ways and may help to solve certain problem associated with culture of marine thermophiles/hyperthermophiles such as low biomass and low productivity in a context of metabolite or biomass production, or to the culture of slow growing strains in a context of community cultures. Indeed, this technology together with new media design could be used for the continuous culture of pure strains and/or microbial consortia of (hyper)thermophilic marine strains with biotechnological applications such as heat-stable enzyme production (amylases, glycosidases, lipases, xylanases etc..) or heavy metals and pollutants detoxification.

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Conflict of Interest

Authors declare no conflict of interest.

References

Addelman, S. (1962) Orthogonal main effect plans for asymmetrical factorial experiments. *Technometrics* **4**, 489-495.

Bustard, M.T., Burgess, J.G., Meeyoo, V., and Wright, P.C. (2000) Novel opportunities for marine hyperthermophiles in emerging biotechnology and engineering industries. *J Chem Technol Biotechnol* **75**, 1095-1109.

Cassidy, M.B., Lee, H., and Trevors, J.T. (1996) Environmental applications of immobilized microbial cells. *J Ind Microbiol* **16**, 79-101.

Cela, R., Claeys-Bruno, M., and Phan-Tan-Luu, R. (2009) Screening strategies. *Compr Chem* **1**, 251-300.

Champagne, C., Lacroix, C., and Sodini-Gallot, I. (1994) Immobilized cell technology for the dairy industry. *Crit Rev Biotechnol* **1**, 109-134.

Cinquin, C., Le Blay, G., Fliss, I., and Lacroix, C. (2004) Immobilization of infant fecal microbiota and utilization in an *in vitro* colonic fermentation model. *Microb Ecol* **48**, 128-138.

Cinquin, C., Le Blay, G., Fliss, I., and Lacroix, C. (2006) New three-stage *in vitro* model for infant colonic fermentation with immobilized fecal microbiota. *FEMS Microbiol Ecol* **57**, 324-36.

D'Souza, S.F. (2002) Trends in immobilized enzyme and cell technology. *Indian J Biotechnol* **1**, 321-338.

Dubois, M., Gilles, K., Hamilton, J.K., Rebers, P.A., and Smith, F. (1956) A colorimetric method for the determination of sugars. *Anal Chem* **28**, 350-356.

Fedorov, V.V., and Malyutov, M.B. (1972) Optimal design in regression experiments. *Math Operationsforsch Stat* **14**, 237-324.

Gaboyer, F., Vandenabeele-Trambouze, O., Cao, J., Ciobanu, M.C., Jebbar, M., Le Romancer. M., and Alain, K. (2014) Physiological features of *Halomonas lionensis* sp. nov., a novel bacterium isolated from a Mediterranean Sea sediment. *Res Microbiol* **165**, 490-500.

Giavasis, I., Harvey, L.M., and McNeil, B. (2000) Gellan gum. *Crit Rev Biotechnol* **20**, 177-211.

Gorlas, A., Alain, K., Bienvenu, N., and Geslin, C. (2013) *Thermococcus prieurii* sp. nov., a hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent. *Int J Syst Evol Microbiol* **63**, 2920-2926.

Huber, H., and Stetter, K.O. (1998) Hyperthermophiles and their possible potential in biotechnology. *J Biotechnol* **64**, 39-52.

Kabaivanova, L., Dobreva, E., Dimitrov, P., and Emanuilova, E. (2005) Immobilization of cells with nitrilase activity from a thermophilic bacterial strain. *J Ind Microbiol Biotechnol* **32**, 7-11.

Kanasawud, P., Hjiirleifsdottir, S., Hoist, O., and Mattiasson, B. (1989) Studies on immobilization of the thermophilic bacterium *Thermus aquaticus* YT-1 by entrapment in various matrices. *Appl Microbiol Biotechnol* **31**, 228-233.

Klingeberg, M., Vorlop, K.D., and Antranikian, G. (1990) Immobilization of anaerobic thermophilic bacteria for the production of cell-free thermostable alpha-amylases and pullulanases. *Appl Microbiol Biotechnol* **33**, 494-500.

Lamboley, L., Lacroix, C., Artignan, J.M., Champagne, C.P., and Vuillemard, J.C. (1999) Long-term mechanical and biological stability of an immobilized cell reactor for continuous mixed strain mesophilic lactic starter production in whey permeate. *Biotechnol Prog* **15**, 646-654.

Lewis, G., Mathieu, D., and Phan-Tan-Luu, R. (1999) Pharmaceutical experimental design. *J Chemometr* **14**, 93-94.

Mathieu, D., Nony, J., and Phan-Tan-Luu, R. (2009) Logiciel NEMRODW. (LPRAI, ed.), Marseille.

Morris, E.R., Nishinari, K., and Rinaudo, M. (2012) Gelation of gellane- A review. *Food Hydrocolloids* **28**, 373-411.

Norton S, and Lacroix, C. (2000) Gellan gum gel as entrapment matrix for high temperature fermentation processes: a rheological study. *Biotechnol Tech* **4**, 351-356.

Nussinovitch, A. (2010) Bead formation, strengthening, and modification. *In Polymer Macro- and Micro-Gel Beads: Fundamentals and Applications*, ed. Nussinovitch, A. pp 27-52 New York, USA: Springer-Verlag.

Postec A, Urios L, Lesongeur F, Ollivier B, Querellou J, and Godfroy A (2005) Continuous enrichment culture and molecular monitoring to investigate the microbial diversity of thermophiles inhabiting deep-sea hydrothermal ecosystems. *Curr Microbiol* **50**, 138-144.

Rao JL, and Satyanarayana T (2009) Hyperthermostable, Ca(2+)-independent, and high maltose-forming alpha-amylase production by an extreme thermophile *Geobacillus thermoleovorans*: whole cell immobilization. *Appl Biochem Biotechnol* **159**, 464-477.

Rathore S, Desai PM, Liew CV, Chan LW, and Heng PWS (2013) Microencapsulation of microbial cells. *J Food Eng* **116**, 369-381.

Rodriguez-Hernandez AI, and Tecante A (1999) Dynamic viscoelastic behavior of gellan iotacarrageenan and gellan-xanthan gels. *Food Hydrocolloids* **13**, 59-64.

Schiraldi C, and De Rosa M (2002) The production of biocatalysts and biomolecules from extremophiles. *Trends Biotechnol* **20**, 515-521.

Sun W, and Griffiths MW (2000) Survival of bifidobacteria in yogurt and simulated gastric juice following immobilization in gellan-xanthan beads. *Int J Food Microbiol* **61**, 17-25.

Trincone A (2011) Marine biocatalysts: enzymatic features and applications. *Mar Drugs* **9**, 478-499.

Table 1 Description of factors and variable levels

Factors	Variables	Levels	
		Coded values	Real values
Sulfur concentration	X_1	A	0·03 mol l^{-1}
		B	0·09 mol l^{-1}
		C	0.15 mol l^{-1}
NaCl concentration	X_2	A	0·08 mol l^{-1}
		B	0·50 mol l^{-1}
		C	0·85 mol l^{-1}
		D	1·36 mol l^{-1}
pH	X_3	A	4·0
		B	5·4
		C	6·8
		D	8·0
Temperature	X_4	A	50°C
		B	65°C
		C	80°C
		D	90°C
		E	100°C

Table 2 Description of tested factors in each performed conditions

Experiment	Sulfur (mol l^{-1})	NaCl (mol l^{-1})	pH	Temperature (°C)
1	0·03	0·08	4·0	50
2	0·03	0·50	5·4	65
3	0·03	0·85	6·8	80
4	0·03	1.36	8·0	90
5	0·09	0·50	6·8	90
6	0·09	0·85	8·0	100
7	0·15	0·08	6·8	100
8	0·15	0·50	8·0	50
9	0·15	0·85	4·0	65
10	0·15	1·36	4·0	80
11	0·15	0·08	5·4	90
12	0·09	0·08	8·0	65
13	0·09	1·36	5·4	100
14	0·03	0·50	4·0	100
15	0.03	1·36	6·8	65
16	0.03	0·08	8·0	80

*Each condition was performed in triplicate

Figure legends

Figure 1 Morphology of beads composed of gellan added of 0·15 mol l^{-1} NaCl (A) or of 0·38 mol l^{-1} NaCl final concentration (C); or beads composed of gellan and xanthan gums added of 0·15 mol l^{-1} NaCl (B) or of 0·38 mol l^{-1} NaCl final concentration (D).

Figure 2 Elastic modulus (G') and viscous modulus (G'') for a gel composed of gellan gum (2·5%, w/v) at (A) 0 mol l^{-1} NaCl or (C) 0·20 mol l^{-1} NaCl and for a gel composed of gellan gum (2·5%, w/v) and xanthan gum (0·25%, w/v) at (B) 0 mol l^{-1} or (D) 0·20 mol l^{-1} NaCl. Results are from one experiment of at least three performed. The margin of error was of 5%.

Figure 3 Response Y1 granulometry, ( ) effect plot of the coefficients. The experiments were replicated three times to evaluate the variance of experimental error.

Figure 4 Response Y2 Polymer release, ( ) effect plot of the coefficients. The experiments were replicated three times to evaluate the variance of experimental error.

Figure 5 Response Y3: visual aspect of beads ranging from 1 (A) to 5 (E).

Figure 6 Cell growth at 60, 65, 70 or 80 °C in Ravot medium measured by ATPmetry for *Thermosipho* sp. AT1272 (A), and *Thermoccocus kodakarensis* KOD1 (B). In beads ( ) just after immobilization; ( ) at 60°C; ( ) at 65°C; ( ) at 70°C; and ( ) at 80°C. In supernantants ( ) at 60°C; ( ) at 65°C; ( ) at 70°C; and ( ) at 80°C.

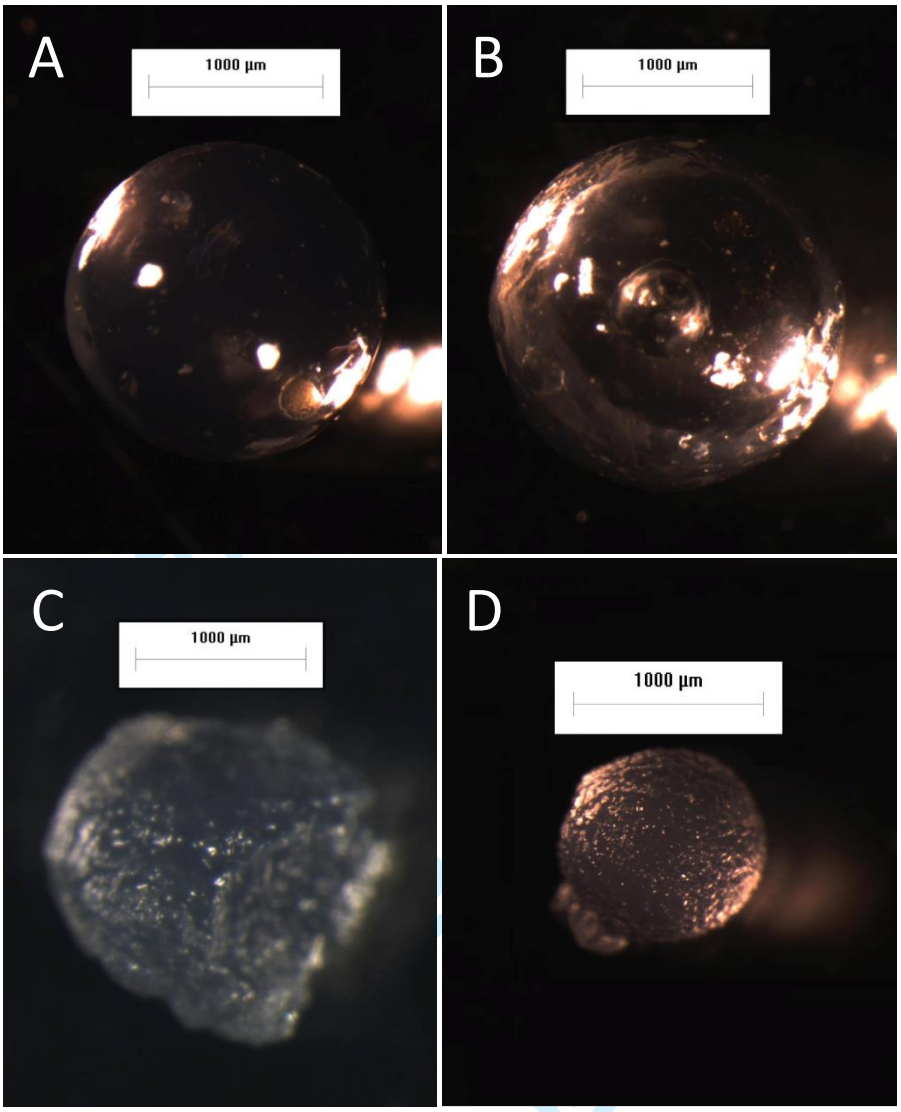

Figure 1

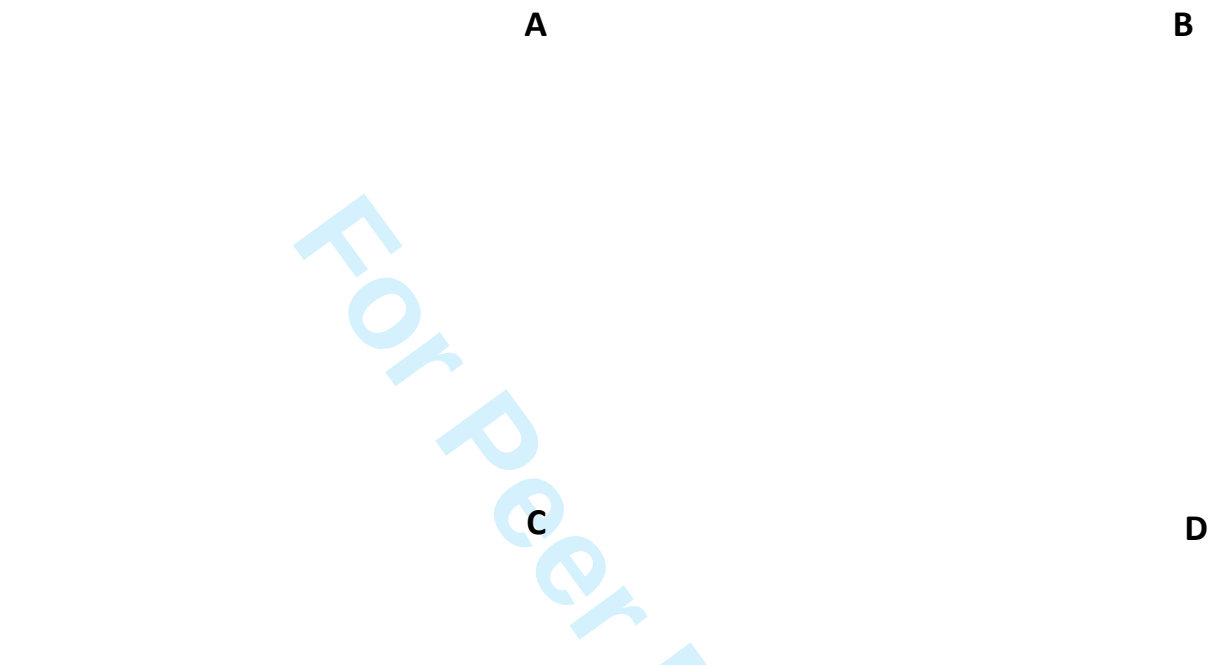

Figure 2

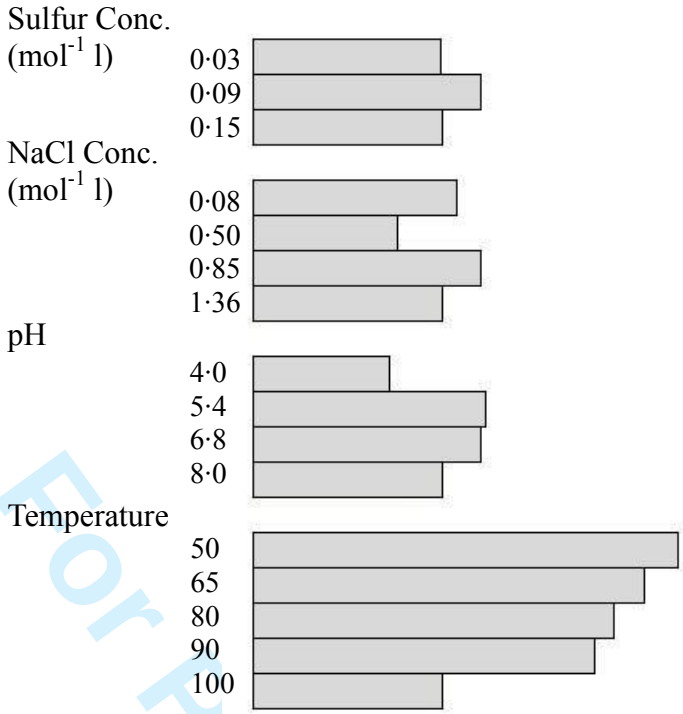

Figure 3

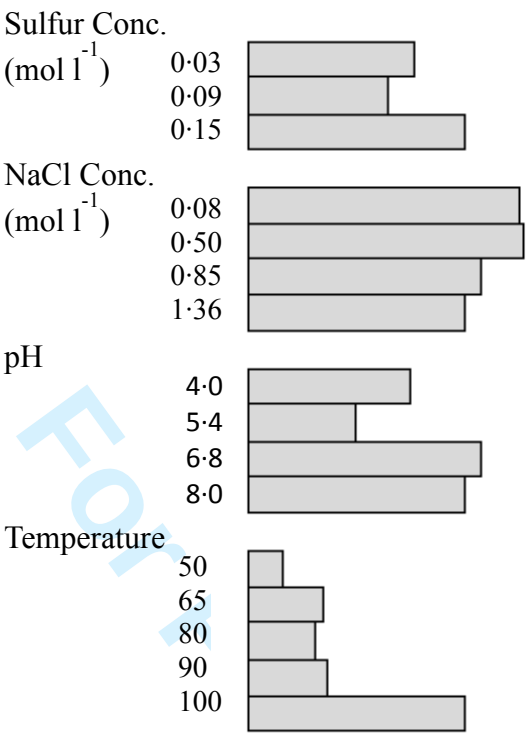

Figure 4

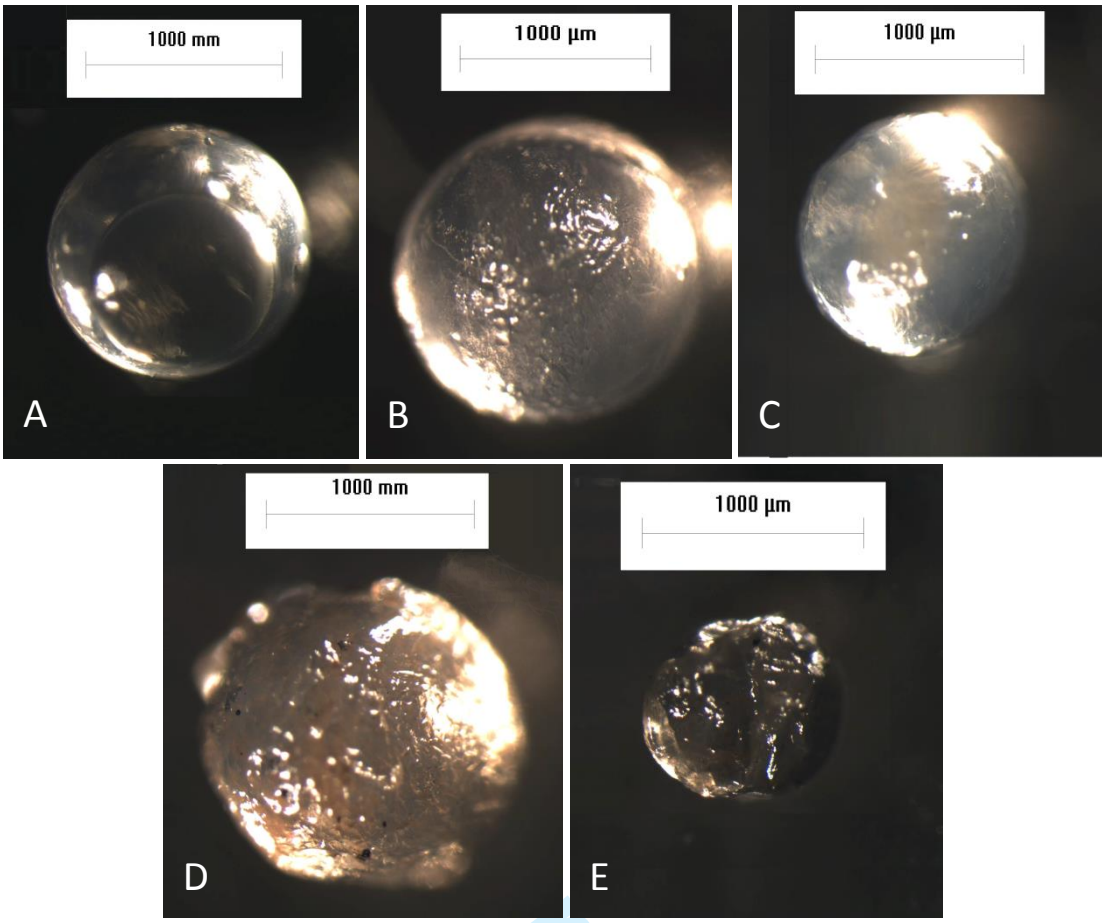

Figure 5

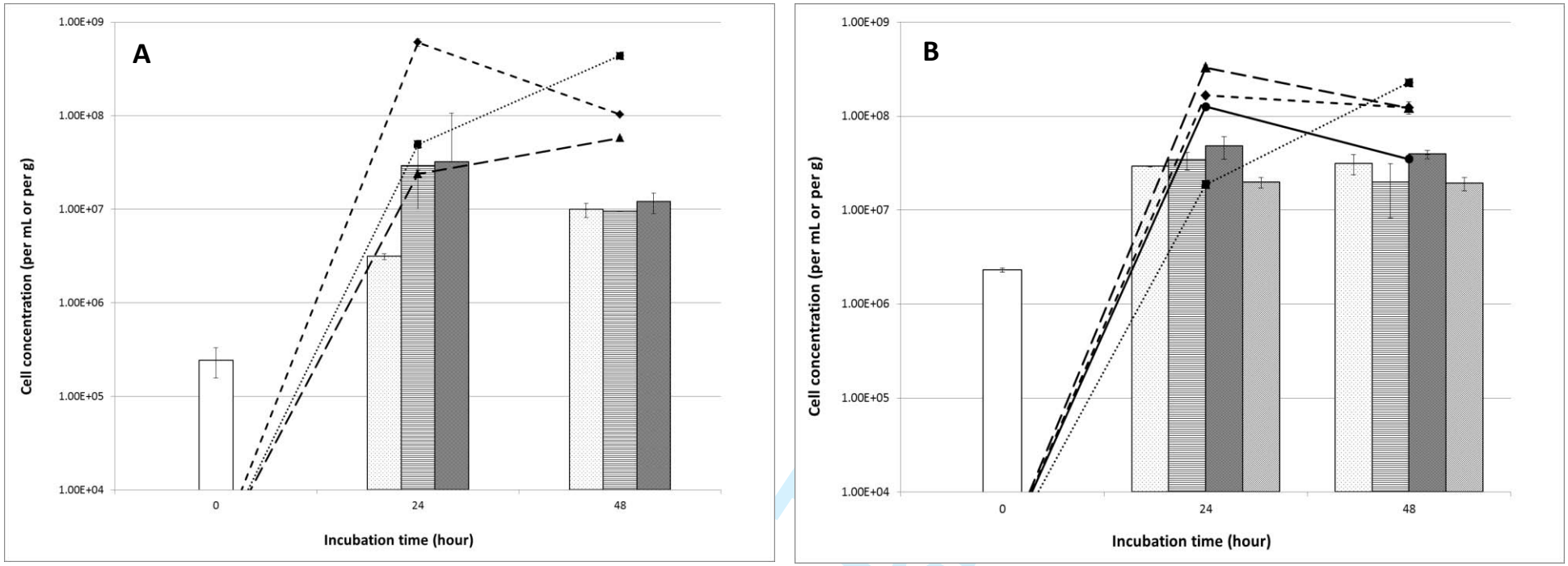
A
B
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
Cell concentration (per mL or per g)
0
24
48
Incubation time (hour)

Figure 6