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

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21 **Headline** : (Hyper)thermophiles entrapment in a polymers matrix

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25

26 **Abstract**

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

31 **Methods and Results:** The best conditions for immobilization were obtained at 80°C with
32 gellan and xanthan gums. After 5-week incubation, beads showed a good resistance to all
33 tested conditions except those simultaneously including high temperature (100 °C), low NaCl
34 (< 0.5 mol l⁻¹) and extreme pH (4/8). To confirm the method efficiency, batch cultures with
35 immobilized *Thermosipho* sp. strain AT1272 and *Thermococcus kodakarensis* strain KOD1
36 showed an absence of detrimental effect on cell viability and a good growth within and
37 outside the beads. **Conclusion:** This suggests that entrapment in a gellan/xanthan matrix
38 could be employed for the culture of anaerobic (hyper)thermophilic marine microorganisms.

39 **Significance and Impact of the Study:** (Hyper)thermophilic marine microorganisms possess a
40 high biotechnological potential. Generally microbial cells are grown as free-cell cultures. The
41 use of immobilized cells may offer several advantages such as protection against phage
42 attack, high cell biomass and better production rate of desired metabolites.

43 **Keywords**

44 Immobilization, Entrapment, Gellan, Xanthan, (Hyper)thermophilic Marine Microorganisms,
45 Anaerobiosis

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47

48 **Introduction**

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

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

93 **Materials and Methods**

94 Microbial strains and growth conditions

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

97 *Thermococcus kodakarensis* strain KOD1 (JCM 12380^T) a hyperthermophilic strain, were
98 used as models for the immobilization trials. *Thermosipho* sp. AT1272 and *Thermococcus*
99 *kodakarensis* KOD1 were routinely grown under nitrogen atmosphere respectively at 60 °C
100 and 80 °C in Ravot Modified Medium (RMM, pH 6.0) (Gorlas *et al.* 2013) reduced by the
101 addition (1 %, v/v) of Na₂S (0.2 mol l⁻¹). Growth experiments were performed under nitrogen
102 gaz in penicillin vials. Prior to immobilization, strains were subcultured twice for 16 h in
103 routine conditions. Their concentration was adapted in order to obtain *ca* 3×10⁸ cells ml⁻¹, and
104 4 ml of this suspension were mixed with the different polymer solutions under anaerobic
105 conditions in order to obtain a final concentration of *ca.* 6×10⁶ cells ml⁻¹ of polymer as
106 explained above, with the exception of beads used during the mechanical stability
107 experiments that were sterile. In the case of subculture in liquid medium for growth
108 comparison with cell immobilization, cells were inoculated around 6×10⁶ cells ml⁻¹.

109 Polymers preparation and immobilization procedure

110 Beads were prepared with two types of polymers, gellan and xanthan gums (Sigma-Aldrich,
111 France), either alone (gellan gum at 2.5 %, w/v) or as a mixture (gellan at 2.5 % and xanthan
112 at 0.25 %, w/v). Polymer powders were suspended in 150 ml of preheated (90 °C) distilled
113 water and mixed for 25 s in a blender. The polymer solution was then autoclaved for 15 min
114 at 121 °C just prior the immobilization. The immobilization procedure was adapted from
115 Cinquin *et al.* (2004), who developed an entrapment protocol for mesophilic bacteria. This
116 process is based on a two phase system composed of a polymer solution and oil under
117 agitation, this technique allows the production of beads recovered by sieving. In order to
118 apply this technique to the entrapment of strict anaerobic marine hyperthermophiles and
119 thermopiles, two different polymer compositions were tested with different NaCl
120 concentrations (0.15, 0.20, 0.27 and 0.38 mol l⁻¹ final concentrations). These concentrations

121 were tested in order to obtain conditions allowing the formation of a maximum of beads with
122 an average size of 1-2 mm while minimizing the osmotic stress. All solutions were
123 deoxygenated under N₂ flow, autoclaved and reduced with Na₂S (0.001 mol l⁻¹ final
124 concentration). The polymer solution, hardening solution (Ravot modified medium, RMM),
125 salt solution composed of NaCl (0.61 to 1.64 mol l⁻¹) and sodium citrate (0.06 mol l⁻¹), canola
126 oil (400 ml) and cell suspensions were transferred under an anaerobic hood. Fifty ml of NaCl
127 solution were added to the polymer solution (80 °C) that was inoculated, if necessary, with 4
128 ml of cell suspension, to reach a final concentration of *ca* 2 × 10⁷ cells ml⁻¹. After inoculation,
129 the polymer solution was stirred at 250 rpm min⁻¹ into oil at 80 °C to obtain a suspension of
130 aqueous droplets in oil. This suspension was cooled for 10 min at room temperature followed
131 by 10 min incubation on ice before being soaked 30 min under agitation in RMM for beads
132 hardening. After washing, beads of the appropriate size (1-2 mm diameter) were selected by
133 wet sieving.

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

139 Rheological tests

140 The viscoelastic behavior of the gellan and gellan plus xanthan gels was characterized in
141 absence and in presence of NaCl (0.20 mol l⁻¹ final concentration). Polymer solutions were
142 prepared as described above, and poured in Petri dishes. After cooling, 2-mm thick 25-mm
143 diameter cylindrical gel samples were cut and their rheological behavior was characterized
144 using oscillatory simple shear tests performed in the linear regime, at room temperature, using

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

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

150 • Experimental design methodology

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

163 • Design of experiments

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

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

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

170 In order to estimate the coefficients at best, a suitable experimental design was performed and
 171 more precisely, an asymmetrical optimal design $3^14^25^1$ in 16 experiments (Table 2). The
 172 experiments (Addelman 1962; Fedorov and Malyutov 1972) were replicated three times to
 173 evaluate the variance of experimental error. From the experimental results for each studied
 174 responses (Y_1, Y_2, Y_3), the estimation of the model coefficients β_{ij} were calculated by least
 175 squares regression. These coefficients could be graphically represented in order to show the
 176 behavior of the different levels for each variable.

177 • Experimental

178 Beads were produced as described before with a mixture of gellan (2.5 %, w/v) and xanthan
 179 (0.25 %, w/v) and a NaCl concentration of 0.2 mol l⁻¹. They were incubated in different
 180 conditions of pH (4.0 to 8.0), temperature (50 to 100 °C), NaCl (0.08 to 1.36 mol l⁻¹) and
 181 sulfur concentrations (0.03 to 0.15 mol l⁻¹) in modified SME medium. This medium is
 182 commonly used for the continuous culture of hydrothermal vent microbial communities
 183 (Postec *et al.* 2005). Basal modified SME medium without NaCl and sulfur was realized.
 184 NaCl was added, pH was adjusted according to the different conditions tested, and 50 ml were
 185 distributed in penicillin vials. Vials were autoclaved at 121 °C for 20 min followed by the
 186 addition of sterile colloidal sulfur. Twenty five grams of freshly produced sterile beads were
 187 added in each vial. Incubations were realized in triplicate for each condition tested (Table 2).

188 After incubation, beads size distribution was measured using a laser granulometer Beckman
189 Coulter LS™ 200 (Beckman Coulter Inc., Brea, USA), polymer release in the modified SME
190 medium was quantified using a colorimetric method adapted from Dubois *et al.* (1956). Two
191 ml of pure sulfuric acid and 0.5 ml of phenol 5 % (w/v) were added to 0.5 ml of sample,
192 incubated 15 min at 95 °C and 15 min at room temperature in dark condition before
193 measurement of absorbance at 492 nm. The general appearance of the beads was observed
194 with a binocular microscope.

195 Growth of immobilized microorganisms in batch experiments

196 Batch cultures were performed with 3 grams of beads containing freshly immobilized cells,
197 *Thermococcus kodakarensis* KOD1 or *Thermosipho* sp. AT1272, in 10 ml of RMM placed in
198 sealed penicillin vials under nitrogen atmosphere. The vials were incubated at different
199 temperatures (60, 65, 70 or 80 °C). Cell growth in beads and liquid medium was measured in
200 triplicates. Cell counting by regular methods being impossible in polymer beads, a protocol
201 based on the measure of cellular ATP was applied in beads and culture medium. The
202 correlations, $r^2 = 0.982$ for *T. kodakarensis* KOD1 and $r^2 = 0.998$ for *Thermosipho* sp.
203 AT1272, between cell counting using a Thoma cell counting chamber and ATP values, were
204 determined at different dilutions (10^0 , 10^{-1} , 10^{-2} , 10^{-3}) according to Gaboyer *et al.* (2014). The
205 appropriate correlation factor was applied to each sample tested, in order to evaluate the
206 number of cell ml^{-1} for each strain. The ATP content of bacterial suspensions in the liquid
207 culture mediums was determined with a Kikkoman Lumitester C-110 (Isogen Life Science)
208 using the BacTiterGlo Microbial Cell Viability assay (Promega) according to the
209 manufacturer's instructions: 100 μl of culture and 100 μl of BacTiter-Glo buffer were used.
210 Internal calibration was performed with 10 μl of a 100 nmol l^{-1} ATP solution and maximal
211 fluorescence emissions values were considered. In the case of beads, these ones (*ca* 100 mg)

212 were placed in a pre-weighted sterile hemolysis tube (Gosselin), they were washed thrice with
213 100 μl of sterile degazed saline solution. For ATP measurement, 100 μl of sterile distilled
214 water were added to the beads, which were vortexed for 10 s before adding 100 μl of
215 BacTiter-Glo buffer. As for liquid medium, internal calibration was performed with 10 μl of a
216 100 nmol l^{-1} ATP solution and maximal fluorescence emissions values were considered. All
217 manipulations were done in triplicates under sterile conditions.

218 **Results**

219 Influence of NaCl concentration and polymer matrix on beads formation

220 The immobilization of thermophilic and hyperthermophilic marine microorganisms
221 implicated the use of heat-stable polymers together with NaCl in order to preserve marine
222 cells from osmotic stress. The influence of NaCl concentration and the use of gellan with or
223 without xanthan gum were tested for the production of the largest volume of beads with the
224 right size (1-2 mm) and morphology (round), compatible with their use in batch or continuous
225 cultures. The increase in NaCl concentration within the gel (0.15, 0.20, 0.27 and 0.38 mol l^{-1})
226 during the emulsion step, dramatically perturbed beads formation, with or without addition of
227 xanthan. Addition of NaCl to the gellan solution induced a decrease in the total volume of
228 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
229 profoundly modified their surface. This one appeared much rougher at 0.38 mol l^{-1} NaCl
230 compare to 0.15 mol l^{-1} . Addition of xanthan gum induced a higher volume of produced beads
231 than gellan alone (113 ± 13 ml vs 74 ± 12 ml at 0.2 mol l^{-1} NaCl), and improve their
232 morphology (Fig. 1). Nor the addition of NaCl, nor the presence of xanthan significantly
233 modified beads diameter (average diameter of 1095 ± 365 μm).

234 Influence of NaCl concentration on gels viscoelastic behavior

235 Rheological tests showed an increase in the storage modulus (G') in parallel with the increase
236 in NaCl concentration (Fig. 2). Indeed, the storage modulus value stepped from 4×10^3 Pa
237 without NaCl, up to 2×10^5 Pa with 0.2 mol l^{-1} NaCl, with or without xanthan. As expected,
238 an increase in NaCl concentration induced a stiffening of the gel, which suggests a decrease
239 of the average mesh size of the gel network induced by NaCl. At least if the gel can be
240 considered as homogeneous. At last, it should be pointed out that the influence of xanthan on
241 the viscoelastic properties of the gellan/xanthan gels studied in this work is quite weak, even
242 if it is slightly more marked for the elastic properties (G') in the presence of NaCl.

243 Mechanical stability of beads

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

248 • Response Y_1 : granulometry

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

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

250 Before incubation, the average diameter of beads was of $1095 \pm 365 \mu\text{m}$. After 5-week
251 incubation in different conditions, their average diameters varied from $796 \pm 365 \mu\text{m}$ to 1156
252 $\pm 501 \mu\text{m}$, with the exception of the condition n°14 ($T^\circ 100 \text{ }^\circ\text{C}$, $\text{pH } 4.0$, 0.50 mol l^{-1} NaCl and
253 0.03 mol l^{-1} sulfur), which dramatically reduced beads diameter down to $297 \pm 273 \mu\text{m}$. From

254 the effect plot (Fig. 3), it can be observed that temperature very strongly influenced beads
255 diameter. When the temperature increased from 50 °C to 100 °C, the average diameter of
256 beads decreased down to 496 μm (minus 45 %). In a lesser extent, NaCl concentrations and
257 pH also significantly influenced bead diameters with a decrease of diameters at extreme pH
258 (especially pH 4.0) and low NaCl concentrations (0.50 mol l⁻¹). Sulfur concentrations induced
259 a slight but significant impact by reducing beads diameters at 0.03 and 0.15 mol l⁻¹.

260 • Response Y₂: polymer released

261 The regression coefficients of the model for polymer released are:

$$Y_2 = 0.71 - 0.14X_{1A} - 0.25X_{1B} + 0.09X_{2A} + 0.12X_{2B} + 0.05X_{2C} - 0.13X_{3A} \\ - 0.19X_{3B} - 0.03X_{3C} - 0.49X_{4A} - 0.48X_{4B} - 0.45X_{4C} - 0.40X_{4D}$$

262 From the effect plot (Fig. 4), it can be observed that temperature strongly influenced polymers
263 release after 5-week incubation. When temperature increased from 90 °C to 100 °C, the
264 average polymer release increased from 0.044 ± 0.01 g to 0.104 ± 0.04 g per vial, which is
265 equivalent to 0.18 % and 0.52 % of beads initial masses. We can also note a slight but
266 significant effect of pH and sulfur, with an increase in polymer release at extreme pH and at
267 0.15 mol l⁻¹ of sulfur. NaCl concentrations had no significant impact on polymer release. It
268 has to be noted that the maximal amount of polymer release reached 0.11 g, which
269 represented less than 1 % of polymer release after 5-week incubation.

270 • Response Y₃: visual aspect

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

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

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

276 After 5-week incubation, once again, temperature significantly affected beads morphology.

277 This was especially true when a high temperature was associated with a low NaCl
278 concentration, with a highly significant effect with 0.08 and 0.50 mol l⁻¹ of NaCl. An
279 association of high temperature (100 °C), low NaCl concentration (≤ 0.50 mol l⁻¹) and
280 extremes pH (4.0 and 8.0) induced a pronounced deterioration of the beads.

281 Immobilized cell growth in batch experiments

282 In order to validate this new entrapment protocol, a thermophilic bacteria *Thermosipho* sp.
283 AT1272 and a hyperthermophilic archaea *T. kodakarensis* KOD1 were used as models. The
284 two strains were immobilized in anaerobiosis. Their growth was monitored, both in the liquid
285 medium and within the beads after 24 h and 48 h of incubation at 60, 65, 70 or 80 °C in RMM
286 medium. Preliminary experimentations allowed associating ATP concentrations to cell
287 concentrations, for each strain. Consequently, it was possible to estimate cell concentrations
288 in beads and liquid medium along the incubation period. Just after immobilization, the amount
289 of cells within the beads was estimated at $2.4 \times 10^5 \pm 8.6 \times 10^4$ cells g⁻¹ for *Thermosipho* sp.
290 AT1272 and $2.3 \times 10^6 \pm 1.3 \times 10^5$ for *T. kodakarensis* KOD1 (Fig. 6). This represents a
291 percentage of viability of 2.7 % for *Thermosipho* sp. AT1272 inoculated at $9.1 \times 10^6 \pm 1.0 \times$
292 10^6 cells ml⁻¹ in the polymer solution, and of 54 % for *T. kodakenrensis* KOD1 inoculated at
293 $4.3 \times 10^6 \pm 9.7 \times 10^4$ cells ml⁻¹. After 24 h of incubation at 65°C, *Thermosipho* sp. AT1272
294 concentrations reached their maximum with $2.9 \times 10^7 \pm 1.9 \times 10^7$ cells g⁻¹ of beads, and $6.1 \times$

295 $10^8 \pm 5.7 \times 10^7$ cells ml⁻¹ in the liquid medium (Fig. 6). In the case of *T. kodakarensis* KOD1,
296 the highest cell concentrations were observed at 70°C after 24 h incubation, with $4.8 \times 10^7 \pm$
297 1.3×10^7 cells g⁻¹ of beads and $3.3 \times 10^8 \pm 2.4 \times 10^7$ cells ml⁻¹ in liquid medium. In comparison,
298 *Thermosipho* sp. AT1272 reached $2.4 \times 10^8 \pm 1.1 \times 10^7$ cells ml⁻¹ in free-cell culture, whereas *T.*
299 *kodakarensis* KOD1 reached $1.4 \times 10^8 \pm 9.8 \times 10^6$ cells ml⁻¹ in the same incubation conditions.

300

301 **Discussion**

302 The objective of this work was to develop a new protocol for the entrapment of thermophilic
303 and hyperthermophilic marine microorganisms in a polymer matrix. The judicious selection
304 of methods and polymers was critical to ensure the highest viability of entrapped cells,
305 together with the highest mechanical stability of beads at high temperatures (Rathore *et al.*
306 2013). The double-phase dispersion process previously described for the entrapment of
307 mesophilic microorganisms with a mixture of gellan and xanthan (Cinquin *et al.* 2004), was
308 adapted to high temperature and saline conditions. Gellan is an anionic exopolysaccharide
309 produced by *Sphingomonas elodea*, its commercial form is a low acyl, linear homopolymer.
310 It forms heat-stable gels (up to 90 °C) whose conformation and structure, depend on gellan
311 concentration, temperature, ionic strength, and type (monovalent or divalent) of stabilizing
312 cations in the aqueous solution. The commercial gellan powder is usually dissolved in
313 preheated distilled water at 90°C. The polymer solution is then autoclaved and maintained at
314 high temperature. When the temperature decreases, the gelation of the polymer solution
315 occurs by aggregation of the single-stranded helices in presence of monovalent (Na⁺ and K⁺)
316 and divalent (Ca²⁺ and Mg²⁺) cations. Aggregation stabilizes at higher temperature than their
317 melting point, which induces thermal hysteresis between gelation and melting (Giavasis *et al.*
318 2000; Morris *et al.* 2012). These properties, together with its low toxicity and resistance to

319 enzyme hydrolysis, explain that gellan is used in many applications, including cell
320 immobilization (Giavasis *et al.* 2000). Xanthan is a polysaccharide produced by *Xanthomonas*
321 *campestris*. It is a non-linear anionic and non-gelling polymer, that resists to high
322 temperatures and acidic pH (Giavasis *et al.* 2000). Its interactions with gellan, result in
323 different type of textures with different mechanical properties (hardness, brittleness,
324 elasticity) (Rodriguez-Hernandez and Tecante 1999). Xanthan is known to considerably
325 reduce the syneresis properties of gellan gel, and to increase its viscoelasticity, which is
326 particularly relevant for microorganism immobilization (Rodriguez-Hernandez and Tecante
327 1999; Giavasis *et al.* 2000).

328 In a first step, the effects of NaCl and xanthan, in gellan beads formation were tested.
329 Addition of increasing concentrations of NaCl during the emulsion step at high temperature
330 (80 °C) strongly decreased the total volume of produced beads, which became highly
331 deformed with rough surfaces for NaCl concentrations above 0.27 mol l⁻¹. This is not
332 surprising knowing the high reactivity of gellan to cations (monovalent Na⁺ in that case), that
333 increase its melting temperature and induce stiffer gel formation (Morris *et al.* 2012).
334 Addition of xanthan allowed the production of greater volumes of beads, with round shapes
335 and regular surfaces, up to a concentration of 0.27 mol l⁻¹ NaCl. Beads diameters were not
336 impacted by xanthan, whatever the concentration of NaCl. This is not surprising given that
337 beads size is rather linked to the emulsion speed. The rheological study demonstrated that
338 NaCl increased the elastic modulus and, therefore the stiffness properties of the gellan gel.
339 Contrary to the observations of Rodriguez-Hernandez and Tecante (1999), addition of
340 xanthan did not increase G', suggesting a lower impact on the gel viscose behavior. This is
341 probably due to the lower proportion of xanthan (10 %, w/v vs 20 %, w/v), together with the
342 higher concentration of gellan (2.5 %, w/v vs 0.5 %, w/v), used in the present study.
343 Rodriguez-Hernandez and Tecante (1999) showed that a mixture of gellan and xanthan

344 resulted in a heterogenic phase-separated gel with no interaction between gellan and xanthan
345 polymers. They also showed that addition of xanthan reduced gel syneresis. This effect is
346 particularly interesting for the viability of entrapped cells. A polymer matrix composed of
347 gellan and xanthan, with a concentration of 0.20 mol l^{-1} NaCl, was then chosen for further
348 experimentations. Indeed, it allowed the formation of a high volume of beads with NaCl
349 concentration compatible with marine microorganism viability.

350 In a second step, the mechanical stability of the beads was tested in incubation conditions
351 compatible with marine thermophiles /hyperthermophiles culture. Beads mechanical stability
352 is one of the major parameter allowing the successful use of beads in different types of culture
353 and fermentation procedures. In that case, the use of a marine culture medium was an
354 advantage due to its high ionic strength, allowing a good stability of the gel, even at high
355 temperatures. Indeed, Norton and Lacroix (2000) showed that bacterial immobilization in
356 gellan gum for incubation at 80°C was not possible, because of the gel weakness and its low
357 resistance to stress in dairy fluids. This was not the case in the present study, because of the
358 high ionic strength of the marine culture medium. According to our experimental design,
359 increasing temperature was the major factor affecting beads stability in terms of
360 granulometry, polymer release and general aspect of the beads. Extreme pH slightly increased
361 polymer release and decreased beads diameter, whereas low NaCl concentrations decreased
362 beads diameter. These results are in accordance with the known properties of gellan/xanthan
363 gels. Globally, the beads resisted very well to 5-week incubation in the sixteen tested
364 conditions. The modified SME medium allowed a good mechanical stability of the beads up
365 to 90°C , especially with NaCl concentrations above 0.50 mol l^{-1} , whatever the pH and the
366 sulfur concentrations. However, conditions simultaneously implicating a temperature of 100
367 $^{\circ}\text{C}$, NaCl concentration below 0.50 mol l^{-1} and extreme pH should be avoided. This once
368 again, is in accordance with literature data showing that the strength of gellan gels increases

369 in a pH range of 4.0 to 7.0, gellan being stable between pH 2.0 to 10.0, whereas xanthan
370 possesses a smaller range of pH stability of 4.0 to 8.0 (Giavasis *et al.* 2000).

371 In a third step, cell survival rate after immobilization, and cell growth capacity within and
372 outside the beads were tested in batch experiments. Cell viability of entrapped cells is usually
373 assessed after beads disruption and cell counting on agar plates (Sun and Griffiths 2000;
374 Cinquin *et al.* 2004). However, in that case, the stiffness of the beads did not allow their
375 disruption with physical and mechanical methods, at least in conditions compatible with cell
376 survival. Moreover, marine microorganisms do not always grow on agar plates. Consequently,
377 we estimated cell survival rate, cell concentration, and growth by analyzing their ATP
378 content. Immediately after immobilization, cell survival rate was of 2.7 % for *Thermosipho*
379 sp. AT1272 and 54 % for *T. kodakarensis* KOD1. These results are in accordance with
380 literature data showing survival rates between 1 and 40% for mesophilic bacteria (Cinquin *et*
381 *al.*, 2006). *Thermosipho* sp. AT1272 reached concentrations of $6.1 \times 10^8 \pm 5.7 \times 10^7$ cells ml⁻¹
382 in the liquid medium, and $2.9 \times 10^7 \pm 1.9 \times 10^7$ cells g⁻¹ in beads after 24 h incubation at
383 65°C, compared to $2.5 \times 10^8 \pm 1.1 \times 10^7$ cells ml⁻¹ for the free-cell cultures in the same
384 conditions. *T. kodakarensis* KOD1 reached $3.3 \times 10^8 \pm 2.4 \times 10^7$ cells ml⁻¹ and $4.8 \times 10^7 \pm$
385 1.3×10^7 cells g⁻¹, respectively in the liquid medium and in beads after 24 h incubation at
386 70°C, compared to $1.4 \times 10^8 \pm 9.8 \times 10^6$ cells ml⁻¹ in free-cell cultures. The high percentage of
387 the survival rates, together with the high concentrations of both strains in beads and liquid
388 medium, showed that entrapment and culture of immobilized anaerobic (hyper)thermophilic
389 marine strains is possible at high temperature.

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

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

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411 **Conflict of Interest**

412 Authors declare no conflict of interest.

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495 **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

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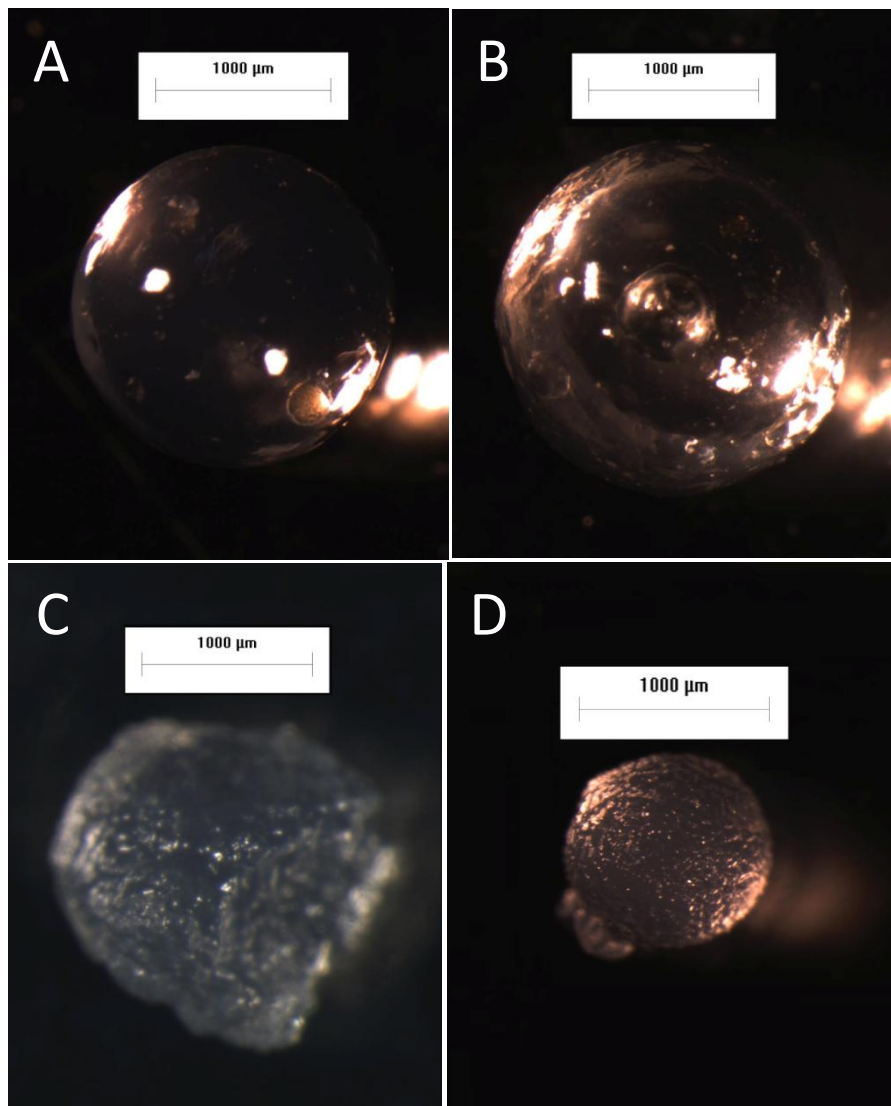
501 **Table 2** Description of tested factors in each performed conditions

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

502 *Each condition was performed in triplicate

503 **Figures**

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509 **Figure 1** Morphology of beads composed of gellan added of 0.15 mol l⁻¹ NaCl (A) or of 0.38
510 mol l⁻¹ NaCl final concentration (C); or beads composed of gellan and xanthan gums added
511 of 0.15 mol l⁻¹ NaCl (B) or of 0.38 mol l⁻¹ NaCl final concentration (D).

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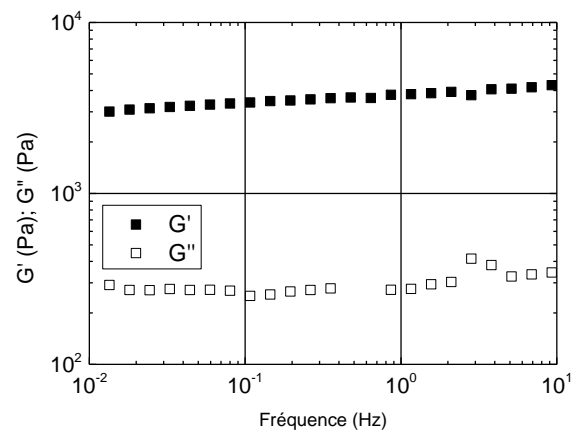
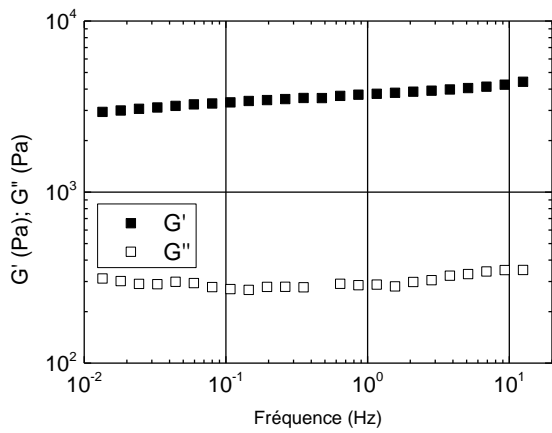
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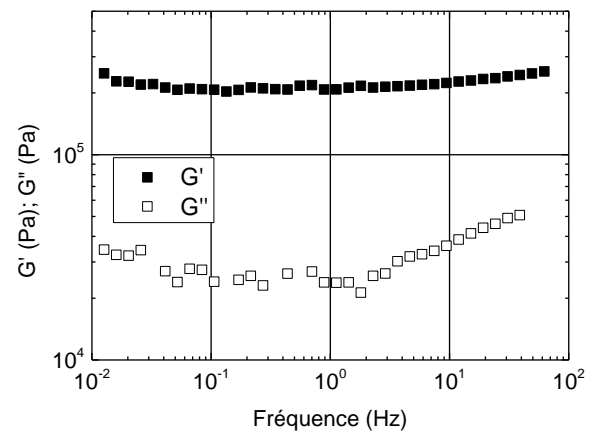
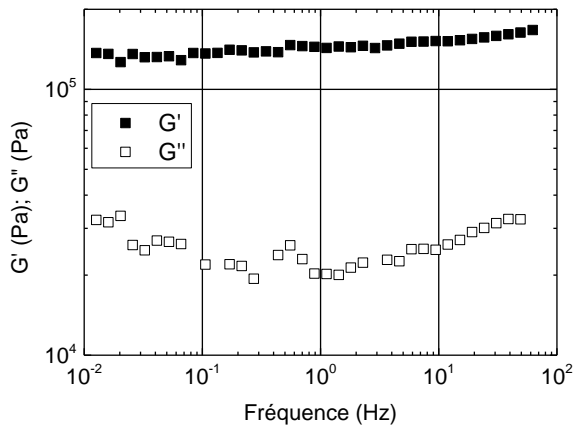
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529 **Figure 2** Elastic modulus (G') and viscous modulus (G'') for a gel composed of gellan gum

530 (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

531 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.

532 Results are from one experiment of at least three performed. The margin of error was of 5%.

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548 **Figure 3** Response Y1 granulometry, (■) effect plot of the coefficients. The experiments

549 were replicated three times to evaluate the variance of experimental error.

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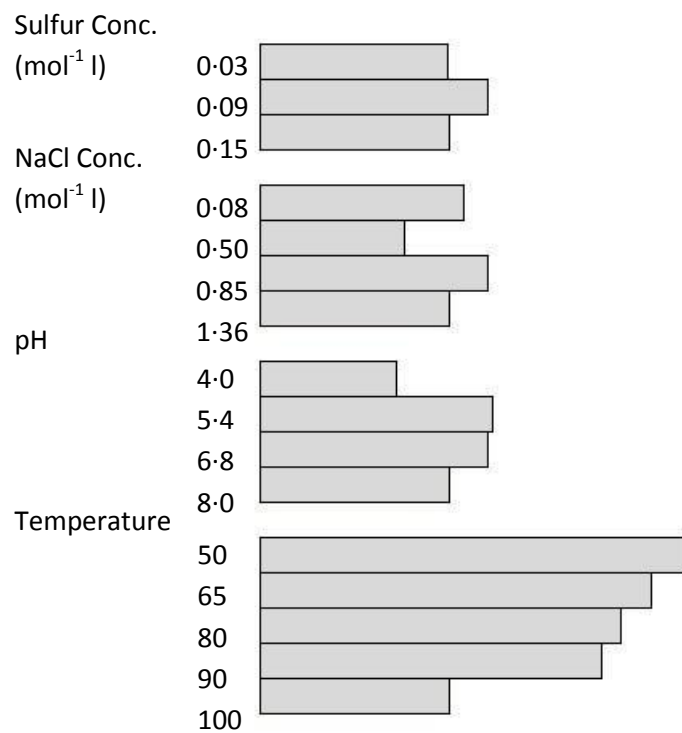
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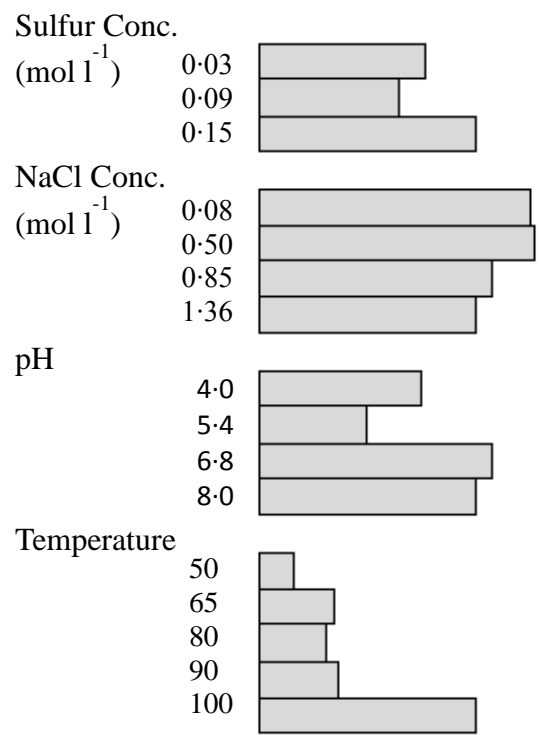
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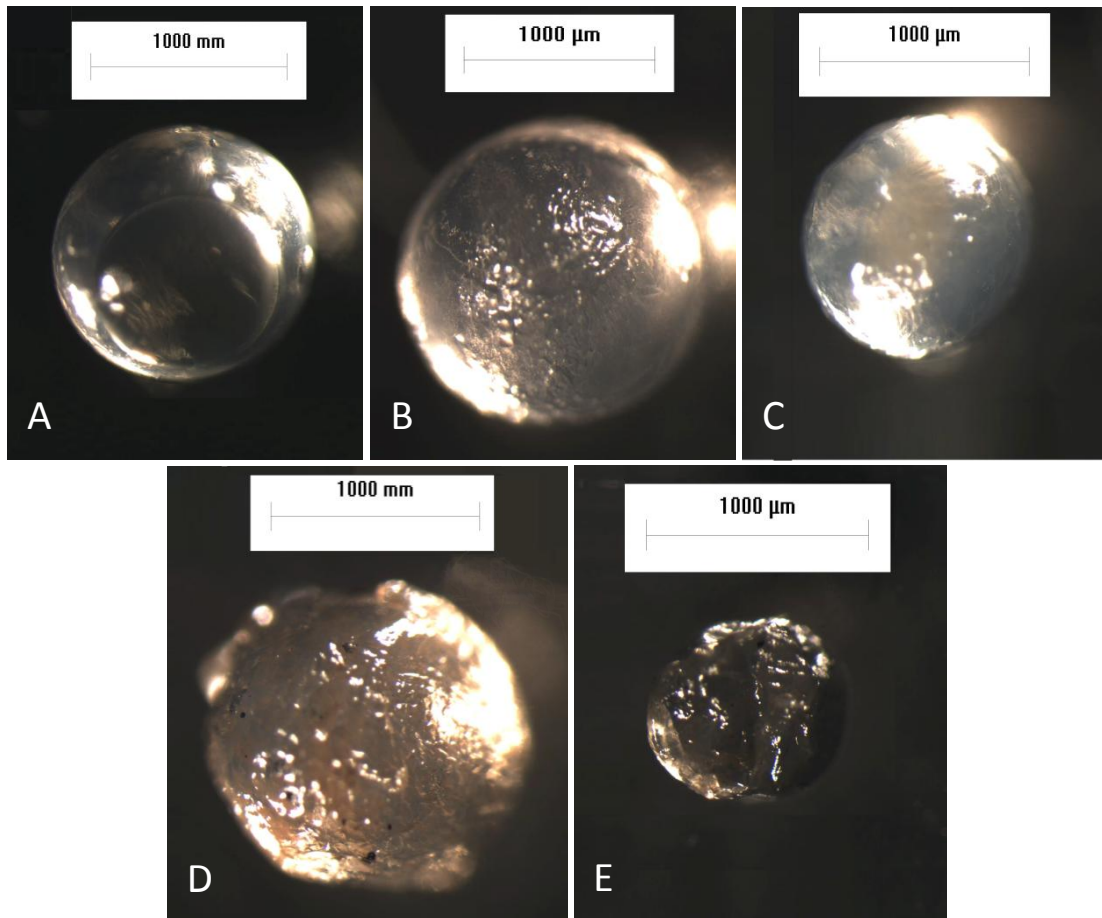
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571 **Figure 4** Response Y2 Polymer release, (■) effect plot of the coefficients. The experiments
572 were replicated three times to evaluate the variance of experimental error.

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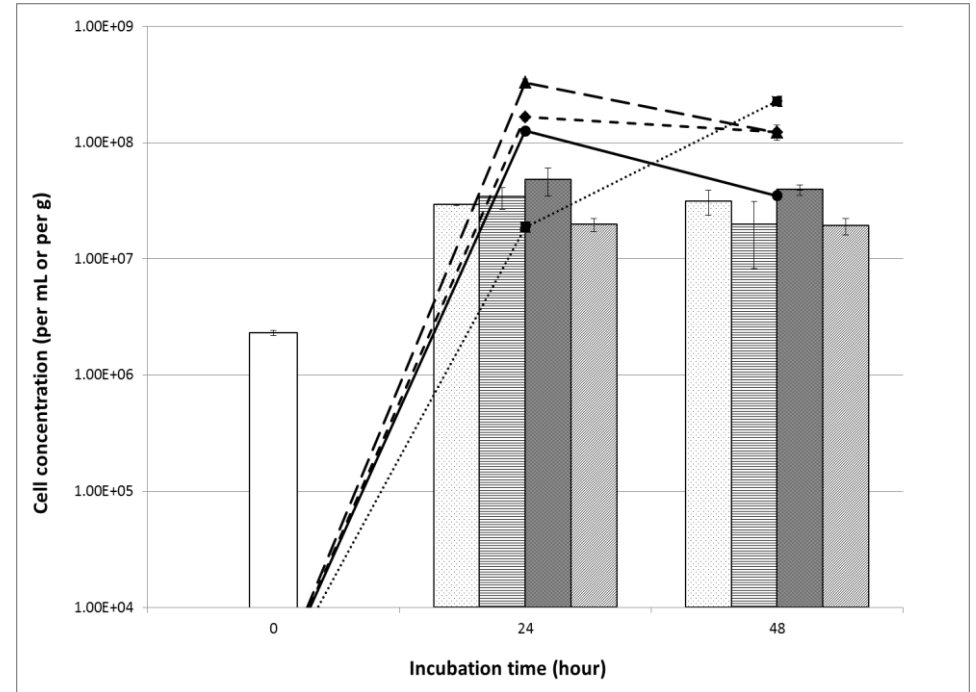
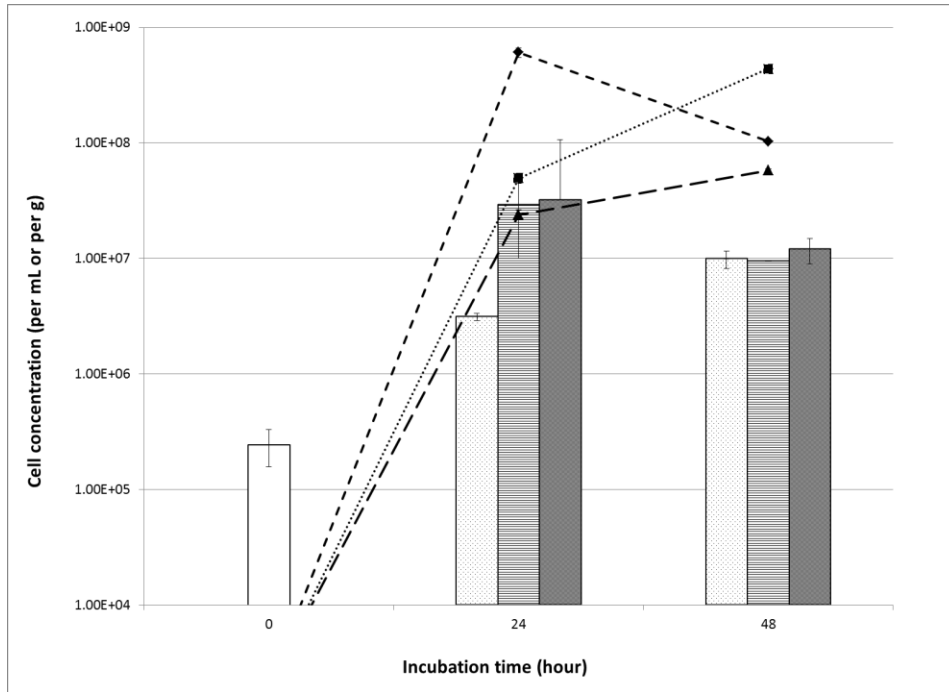
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584 **Figure 5** Response Y3: visual aspect of beads ranging from 1 (A) to 5 (E).



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587 **Figure 6** Cell growth at 60, 65, 70 or 80 °C in Ravot medium measured by ATPmetry for *Thermosipho* sp. AT1272 (A), and *Thermococcus*
 588 *kodakarensis* KOD1 (B). In beads (□) just after immobilization; (▣) at 60°C; (▤) at 65°C; (▥) at 70°C; and (▦) at 80°C. In supernatants
 589 (•••■•••) at 60°C; (—◆—) at 65°C; (—▲—) at 70°C; and (—●—) at 80°C.

