

Extremophiles

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Cultivating the uncultured: limits, advances and future challenges

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Abstract:

Since the invention of the Petri dish, there have been continuous efforts to improve efficiency in microbial cultivation. These efforts were devoted to the attainment for diverse growth conditions, simulation of in situ conditions and achievement of high-throughput rates. As a result, prokaryotes catalysing novel redox reactions as well as representatives of abundant, but not-yet cultured taxa, were isolated. Significant insights into microbial physiology have been made by studying the small number of prokaryotes already cultured. However, despite these numerous breakthroughs, microbial cultivation is still a low-throughput process. The main hindrance to cultivation is likely due to the prevailing lack of knowledge on targeted species. In this review, we focus on the limiting factors surrounding cultivation. We discuss several cultivation obstacles, including the loss of microbial cell-cell communication following species isolation. Future research directions, including the refinement of culture media, strategies based on cell-cell communication and high-throughput innovations, are reviewed. We further propose that a combination of these approaches is urgently required to promote cultivation of uncultured species, thereby dawning a new era in the field.

Keywords: Culture - Isolation - Metabolism - Microbial community - Cell-cell communication - High-throughput cultivation

37 **Introduction**

38 Today, 1.8 million eukaryotic species are recognized, while estimates indicate that our planet
39 hosts 5 to 10-fold more species.. Until now, only 7031 prokaryotic species have been
40 described (and validated by the International Committee on Systematics of Prokaryotes) in
41 the one hundred and twenty years, since the invention of the Petri dish (Achtman & Wagner,
42 2008), even though we now realize that prokaryotes represent “the unseen majority”
43 (Whitman et al., 1998). The lack of an extensive and accurate picture of the microbial
44 diversity is partly due to a lack in technical advances in the microbiology cultivation field. .
45 Over the past three decades, molecular biology was an enormous driving force in
46 microbiology in uncovering the microbial diversity . Many new candidate divisions are now
47 recognized due to 16S rRNA sequence-based approaches and environmental metagenomics
48 (Curtis *et al.*, 2002). These findings exposed a gap between the known phyla and those
49 possessing cultured representatives (Fig. 1). In 1987, when much of our knowledge derived
50 from pure culture techniques, all the phyla known possessed cultured representatives. Twenty
51 years later, of the 100 bacterial phyla identified only 30 possess a cultivated representative
52 (Achtman & Wagner, 2008). The number of phylum-level divisions possessing cultured
53 representatives has increased at a linear and constant rate over the last two decades.
54 However, this increase was greatly augmented with the emergence of molecular-based
55 approaches. This augmentation is seen when candidate divisions are included in these
56 calculations (Fig. 1)..

57 From a quantitative point of view, the advances made by culture-dependent approaches may
58 appear trivial, especially since only 0.1% of the existing prokaryotes have been cultured so
59 far. Yet, this quantitative approach is reductive since the past 20 years of microbial cultivation
60 have led to unprecedented advances in our knowledge of the microbial world.

61

62 Molecular ecology and metagenomics have increased significantly our knowledge of the
63 genetic diversity and have led to interesting hypotheses (Hugenholtz & Tyson, 2008). The
64 advanced techniques have also revealed how far we are from measuring the full extent of
65 genetic diversity encoded by microbial life (Hugenholtz & Tyson, 2008; Pignatelli *et al.*,
66 2008). Considering that many of the genes stored in the databases have unknown functions or
67 are incorrectly annotated, it is probable that metagenomes alone will not offer sufficient
68 knowledge to cultivate all organisms. There is evidence that many of the candidate divisions
69 revealed by molecular approaches, and known only from molecular signatures, represent a
70 significant fraction of the microbial diversity. Some members of these ‘not-yet-cultured’ taxa
71 are probably key ecological players. Today, one of the main challenges for microbiologists is
72 to develop strategies to cultivate this uncultured majority. A comprehensive understanding of
73 biology and ecology of prokaryotes will require cultivation. Therefore, it is not surprising to
74 observe an increasing interest for the field of microbial cultivation (Leadbetter, 2003).

75 The aim of this review is to provide an overview of the new cultivation-based approaches
76 while documenting their limitations and outcomes. Further, we highlight how cultivation has
77 led to valuable advances in our understanding of microbial physiology and identify the future
78 challenges for microbiologists in the microbial cultivation field. Lastly, a discussion is
79 provided on the technical developments that may drive innovative research in the near future.

80

81 **I- Review on some cultivation successes**

82 Prokaryotic growth necessitates an energy source (light or chemical compounds), nutrients
83 and proper physicochemical conditions. Challenges for the microbiologist are to identify
84 required nutrients, to provide them in the growth medium in the appropriate concentrations to
85 sustain the microbial growth, and to avoid the co-precipitation of the introduced chemicals.
86 Since different organisms require a different set of nutrients in varying concentrations and

87 forms, the design of growth media remains a difficult task. Conversely, the intrinsic
88 selectivity of any growth medium imposes limitations on the type, diversity and number of
89 prokaryotes recovered from the natural environment. This phenomenon is known as the “great
90 plate count anomaly” (Staley & Konopka, 1985). Indeed, there is a difference of several
91 orders of magnitude between colony counts on laboratory medium and total numbers of
92 prokaryotic cells present in natural environments. Only a minor fraction of the naturally
93 occurring microbial community is recovered by conventional selective media (Skinner *et al.*,
94 1952; Amann *et al.*, 1995). Depending on the nature of the samples, the cultivation efficiency
95 of active cells by standard plating technique is estimated between 0.001% and 1% (Kogure *et*
96 *al.*, 1979; Staley & Konopka, 1985; Amman *et al.*, 1995). Thus, cultured microorganisms do
97 not reflect the functional and phylogenetic diversity present within any natural habitat.

98 Two main strategies are used for the isolation of pure cultures in microbiology. In both
99 strategies, enrichment culture is performed as a first step. The first strategy aims to isolate
100 colonies by repetitive streaking on solid medium (or alternatively by performing pour plates
101 or agar shake tubes), while the second strategy aims to isolate cells following repeated series
102 of dilutions in liquid medium. These classical approaches have led to the isolation of a large
103 number of strains belonging to taxa with few or no representatives in pure culture (Janssen *et*
104 *al.*, 1997; Joseph *et al.*, 2003). Nevertheless, the enrichment and pure culture isolation
105 strategies often select for opportunistic fast-growing organisms also called lab weeds. In
106 nutrient-rich artificial media, the community members with ‘r’-strategy, or fast-growers, often
107 overgrow and outcompete the naturally abundant ‘K’-strategists (Watve *et al.*, 2000).
108 Consequently, these conventional culture-dependent approaches do not reflect the actual
109 microbial communities (Amann *et al.*, 1995). Certain taxa are still severely under-represented
110 in pure cultures (Hugenholtz *et al.*, 1998). As direct consequence, most of our current

111 knowledge of the nutrition, physiology and biochemistry of prokaryotes is based on easily
112 cultivable organisms.

113 In recent years, novel cultivation strategies were developed to overcome these limitations.

114 Schematically, they are classified into four categories (Fig. 2).

115

116 **1- Refinement of standard cultivation strategies**

117 Different studies have demonstrated that a fraction of the “not-yet-cultured” groups of
118 prokaryotes can be grown by the refinement of classical approaches. Changes in the media

119 formulations, including the use of non-traditional electron donors, electron acceptors and

120 carbon sources have proven efficient in recovery of uncultured taxa (Köpke *et al.*, 2005).

121 Diversification of the media and multiplication of culture conditions are simple methods to

122 by-pass approaches that are selective by nature. For instance, the cultivable fraction from

123 coastal subsurface sediments was shown to yield a higher number and diversity of isolates

124 when culture collections were performed with diverse electron acceptors and carbon sources

125 (Köpke *et al.*, 2005). The qualitative composition of carbon sources is also a determinant

126 factor for cultivation efficiency . . As shown with seawater samples from the North Sea,

127 media prepared using several different carbon sources and complex compounds yield higher

128 number and more diverse isolates than similar media with only one carbon source. These

129 isolates obtained with a single substrate belonged almost exclusively to the

130 *Gammaproteobacteria* while representatives of four other classes grew on complex media

131 (Uphoff *et al.*, 2001). In recent years, it was shown that novel redox reactions, catalysed by

132 specific ecological communities of prokaryotes, can be identified within enrichment cultures

133 onto non-conventional media. Many novel physiotypes can be isolated using this method.

134 Significant advances were made in the field of the anaerobic degradation of hydrocarbons.

135 For example, different microbial consortia of Archaea and Bacteria, which couple the

136 anaerobic oxidation of methane to sulphate or nitrate reduction, were enriched from anoxic
137 marine or freshwater sediments. These findings enhanced our understanding of the global
138 biological cycles (Nauhaus *et al.*, 2002; Raghoebarsing *et al.*, 2006). Moreover, hydrocarbon-
139 degrading bacteria were identified using refined media containing only hydrocarbon/carbon
140 energy sources and nitrate, iron or sulphate as electron acceptors. These concurrent studies
141 led to a better understanding of the biochemistry and energetics of anaerobes. Several
142 biochemical mechanisms involved in the activation of some of these chemically non-reactive
143 compounds have been identified (Lovley and Lonergan, 1990; Rabus *et al.*, 1993; Galushko
144 *et al.*, 1999; Coates *et al.*, 2001; Widdel *et al.*, 2007). However, hydrocarbon-degrading
145 capacities, activation mechanisms and species or ecological guilds, which are involved in
146 hydrocarbon degradation, remain to be discovered. For example, a microbiological anaerobic
147 oxidation of methane with iron (III) or manganese (IV) as a terminal electron acceptor is
148 unknown, yet this reaction is thermodynamically possible (Raghoebarsing *et al.*, 2006). The
149 enrichment culture with anoxic ditch sediment, discovered in 1999 by Zengler and co-
150 workers, was another growth-supporting reaction of relevance. This team demonstrated that
151 the conversion of long-chain alkanes to methane, under strictly anoxic conditions, is
152 biologically performed by an ecological guild assumed to be acetogenic syntrophic bacteria
153 associated to acetoclastic and hydrogenotrophic methanogenic archaea (Zengler *et al.*, 1999).
154 The discovery of this process in nature might help to understand the terminal degradation of
155 organic matter in areas of deep and old marine sediments where sulphate is depleted. Another
156 novel physiotype recently identified due to advances in cultivation and isolation was an
157 autotrophic anaerobe, which couples the oxidation of phosphite (III) to sulphate reduction
158 (Schink and Friedrich, 2000). These novel physiotypes along with the newly recognized
159 biological redox reactions are only a few of the several examples of the significant advances

160 made in uncovering the microbial diversity through the enhancement of cultivating
161 techniques.

162 Some recent successes in improving traditional cultivation methods include the following.

163 The use of relatively low concentration of nutrients to increase the cultivability and to
164 improve the recovery of prokaryotes from different types of natural samples (Button *et al.*,

165 1993; Janssen *et al.*, 1997; Watve *et al.*, 2000; Cannon and Giovannoni, 2002; Rappé *et al.*,

166 2002; Sangwan *et al.*, 2005). The use of increased incubation periods to allow for the

167 development of strains from rarely isolated taxa (Sait *et al.*, 2002; Stevenson *et al.*, 2004;

168 Davis *et al.*, 2005; Sangwan *et al.*, 2005; Stott *et al.*, 2008) and, the addition of signalling

169 compounds known to mediate communication between bacteria (Bruns *et al.*, 2002; Bruns *et*

170 *al.*, 2003). Moreover, other less-documented approaches also yielded new isolates. These

171 included: the use of gellan gum (phytagel) as a gelling reagent instead of agar (Tamaki *et al.*,

172 2005; Stott *et al.*, 2008); the decrease in inoculum size (Davis *et al.*, 2005); the addition of

173 electron transporters to the culture media (Stevenson *et al.*, 2004); the addition of enzymes to

174 cope with reactive oxygen species (Stevenson *et al.*, 2004); the addition of inhibitors of

175 undesired organisms (Leadbetter *et al.*, 1999); and, the combination of an unusual energy

176 source with antibiotics to exclude Bacteria (Könneke *et al.*, 2005). Finally, sophisticated

177 single-cell isolation tools allowing for the manipulation of a targeted cell from a mixed

178 community (with a micro-capillary tube or with 'optical tweezers') have been developed

179 (Huber *et al.*, 1995; Fröhlich and König, 2000; Huber *et al.*, 2000). The principal limitations

180 of these single-cell isolation strategies include their labour intensive requirements and the

181 determination of the suitable growth conditions to cultivate prokaryotic cells of unknown

182 metabolism and systematic affiliation. The identification of a cell of interest among a complex

183 community in absence of clear distinctive morphological features is also challenging.

184 Altogether, these limitations account for the lack in isolating numerous undiscovered strains.

185

186 **2- Cultures *in situ* or cultures in simulated natural conditions**

187 Often, the laboratory conditions poorly mimic the natural environmental conditions.

188 Therefore, strategies aimed at simulating natural conditions or culturing *in situ* have been

189 proven efficient. Schematically, two types of “*in situ* colonization devices” have been

190 developed: the diffusion chambers and the carriers (of organic or inorganic nature)

191 (Kaeberlein *et al.*, 2002; Ferrari *et al.*, 2005; Yasumoto-Hirose *et al.*, 2006; Bollmann *et al.*,

192 2007). Diffusion chambers are apparatuses equipped with filter membranes which restrict the

193 movement of cells in the chamber. They allow for the removal of low-molecular weight

194 inhibitory end-products, as well as the exchange of chemicals between the chamber and the

195 environment, thereby making high density cultivation possible (Pörtner and Märkl, 1998;

196 Kaeberlein *et al.*, 2002). Different types of membrane-based systems have been developed to

197 grow microbial communities directly in the natural habitats (Kaeberlein *et al.*, 2002; Plugge

198 and Stams, 2002; Ferrari *et al.*, 2005; Bollmann *et al.*, 2007; Ferrari *et al.*, 2008). Uncultured

199 bacteria from soil, marine or activated sludge were grown in diffusion chambers. This led to

200 the hypothesis that *in situ* cultivation of environmental prokaryotes in diffusion chambers

201 either enriches sufficiently the strains for their subsequent isolation onto classical solid media

202 or conditions them for growth under otherwise prohibitive *in vitro* conditions (Bollmann *et*

203 *al.*, 2007). Interestingly, slow-growing organisms were cultivated using this method.

204 In natural ecosystems, many prokaryotes live attached to surfaces. This is well known for

205 microbes living in sediments, soils, or in association with eukaryotes. It is less recognized for

206 microbes living in aquatic habitats, where free-living forms were supposed to be dominant,

207 but other associations, with various interfaces, exist. The attached existence provides several

208 advantages for the prokaryotes. Attached cells escape grazing better than their free-living

209 neighbours. Attachment also allows cells to develop metabolic inter-relations, resistance to

210 different stresses and better access to adsorbed substrates (Schink, 1999). *In situ* colonization
211 carriers are useful tools to overcome cultivation limits induced by attachment of prokaryotes
212 to solid surfaces. Several publications describe the deposition in natural ecosystems of
213 different carriers such as glass, ceramic, titanium devices, porous inorganic substrates or
214 polyurethane foams (Araki *et al.*, 1999; Alain *et al.*, 2004; Yasumoto-Hirose *et al.*, 2006). *In*
215 *situ* collectors coated with selective substrates, are effective for the selective enrichment of
216 targeted prokaryotes (Yasumoto-Hirose *et al.*, 2006). In addition, specialized techniques
217 simulating one or several important spatial or physical parameters allow the cultivation of
218 novel phenotypes. For instance, gradient systems (Nelson and Jannasch, 1983; Emerson and
219 Moyer, 1997), high pressure reactors (Marteinsson *et al.*, 1999; Alain *et al.*, 2002), flow-
220 through devices (Houghton *et al.*, 2007) and gas-lift reactors (Postec *et al.*, 2005; Postec *et*
221 *al.*, 2007) were successfully used. . Pressure is also an essential parameter given that high-
222 pressure environments occupy the largest fraction of the known biosphere. This parameter was
223 often neglected in microbial cultivation. However, its effects on microbial life are as
224 important as those of temperature or salinity. Pressure acts upon physiology and upon
225 biochemical reactions. Thus, these types of reactors are effective to grow microbial strains
226 from high pressure habitats. However, only few piezophilic prokaryotes have been enriched
227 or isolated under elevated pressures (i.e. Yayanos *et al.*, 1979, 1981; Marteinson *et al.*, 1999;
228 Alain *et al.*, 2002; Houghton *et al.*, 2007). Recently, the first obligate piezophilic and
229 hyperthermophilic archaeon (*Pyrococcus* sp. strain CH1) was isolated from the deepest
230 hydrothermal vent field explored so far, using a high pressure reactor (Zeng *et al.*, in press).
231 Finally, different types of bioreactors are used to reproduce diverse environmental
232 conditions. The physical and chemical conditions of seafloor hydrothermal systems are
233 among the most difficult to reproduce in laboratories, considering the multiplicity of *in situ*
234 physical and chemical gradients. Gas-lift reactors and flow-through devices are powerful

235 tools to simulate hydrothermal vent *in situ* conditions (Postec *et al.*, 2005; Postec *et al.*, 2007;
236 Houghton *et al.*, 2007). Flow-through devices allow for continuous cultures under *in situ*
237 temperature, pressure and fluid flow. They make possible the simulation of the deep-sea vent
238 changes of fluid chemistry (Houghton *et al.*, 2007). Both systems allow for continuous
239 enrichment culturing under controlled conditions. Significant fractions of microbial
240 communities were grown using these systems (Postec *et al.*, 2007).

241

242 **3- Cultures of microbial communities**

243 The cultivation methods allowing for the growth of mixed populations offer great potential to
244 cultivate not-yet cultivated organisms. Indeed, in natural environments, most organisms live as
245 a part of a community in which distinct cells work in concert and communicate either by
246 trading metabolites, by exchanging dedicated signalling molecules, or by competition for
247 limited resources (West *et al.*, 2007; Nadell *et al.*, 2009). These relationships, in addition to
248 complex cell-cell communications, are hardly reproducible in monocultures. This “in group”
249 lifestyle, in biofilm or multi-cellular assemblage, is thought to generate robustness to
250 nutritional, biotic and abiotic changes by providing a capacity of adaptation to environmental
251 fluctuations. It is critical for microbial ecology and evolution. A striking example is biofilm,
252 in which cell-cell communication determines biofilm structure, maturation and microbial
253 niche construction thereby optimizing microbial survival and reproduction. Mixed microbial
254 assemblages have also the capacity to perform multiple-step functions that are often not
255 possible for individual strains or species. Examples are the degradation of cellulose or the
256 methanogenic conversion of complex organic matter (Brenner *et al.*, 2008). In some cases, the
257 cooperation within the microbial community is based on sharing metabolic intermediates,
258 micronutrients (e.g. vitamins) or chelating agents that either assist or compromise the growth
259 of other community members. When identified, such facultative dependencies can be

260 reproduced experimentally by supplying the medium with these micronutrients or co-
261 substrates. Besides facultative associations, syntrophic associations are often compulsory and
262 this interdependence cannot easily be by-passed or suppressed by the addition of factors to the
263 media (Schink, 1999). Many syntrophic associations are explained by unfavourable energetic
264 conditions. . Both facultative and syntrophic associations are widespread in natural habitats.
265 Consequently, approaches based on community cultures are effective methods to grow
266 facultative associations and syntrophic organisms. Using community culture approaches in
267 addition to dialysis membrane reactors, thermophilic syntrophic anaerobic glutamate-
268 degrading consortia from anaerobic sludge have been successfully enriched (Plugge and
269 Stams, 2002). Similarly, batch reactors operating under anaerobic-aerobic cycling conditions
270 have allowed the enrichment of mixed microbial sludge communities (Crocetti *et al.*, 2002).

271

272 **4- High-throughput automatable microbial culture formats**

273 The past decade was marked by the emergence of high-throughput cultivation methods
274 (Connon and Giovannoni, 2002; Zengler *et al.*, 2002; Zengler *et al.*, 2005; Ingham *et al.*,
275 2007). Several advances in high-throughput culture formats have originated from industrial
276 endeavours. Indeed, the myriad of organisms inhabiting our planet represents a tremendous
277 reservoir of bio-molecules for pharmaceutical, agricultural, industrial and chemical
278 applications. While culture-independent recombinant approaches are used to screen novel
279 molecules and enzymes from natural samples, cultivation of organisms greatly simplifies such
280 studies and allows the use of a strategy biased for the desired phenotype.

281 A very efficient high-throughput cultivation method resulted from modifying the pioneering
282 work, called extinction cultures, of Button and colleagues (Button *et al.*, 1993). Briefly,
283 extinction culturing requires dilutions of natural communities to a low number of cells (1 to 5
284 cells per ml) in natural environmental water followed by their incubation in defined

285 conditions. Following incubation, growth is measured. The initial procedure . was refined by
286 Giovannoni and colleagues to increase high throughput rates by using microtiter plates and by
287 fluorescence microscopy screening (Connon and Giovannoni, 2002; Stingl *et al.*, 2007). This
288 improved technique resulted in better sensitivity and cultivation efficiency. This technique
289 allows notably for the growth of slow-growers. It also allowed for the isolation of the first
290 representatives of two bacterial clades: the SAR11 clade, a ubiquitous alpha-proteobacterial
291 lineage found abundantly in marine pelagic environment (Rappé *et al.*, 2002) and the OM43
292 clade (Connon and Giovannoni, 2002).

293 Another technology for massive cultivation of prokaryotes was developed recently. This high-
294 throughput approach consists in the encapsulation of cells in gel microdroplets (GMDs)
295 incubated in a single column for long intervals of time under low nutrient flux conditions.
296 Micro-capsules are subsequently sorted by flow cytometry.. This technique is applied to
297 samples from different habitats and provides more than 10000 bacterial and fungal isolates
298 per natural sample (Zengler *et al.*, 2005). To our knowledge, this technology, which allows
299 the development and detection of micro-colonies (20 to 100 cells), has never been used for
300 anaerobes. It is advantageous as cell-containing micro-droplets are grown together in capsules
301 allowing for the exchange of signal compounds and metabolites between cells from different
302 micro-colonies but originating from the same natural community. Using the micro-
303 encapsulation approach, members of numerically abundant clades were isolated. Although
304 very promising, this technique is not yet easily implemented in microbiology laboratories
305 (Zengler *et al.*, 2002; Zengler *et al.*, 2005).

306 Finally, a multiwell microbial culture-chip was recently developed by a team of
307 microbiologists, nanotechnologists and micro-engineering experts (Ingham *et al.*, 2007). This
308 micro-Petri dish, is composed of a unique porous ceramic subdivided into millions of
309 compartments in which cultures can be separately grown. The growth of microorganisms is

310 sustained by the nutrients diffused through a porous membrane. The innovation lies in the
311 conception of the chip that is composed of micron-scale wells, is readily manufactured,
312 affordable and easy-to-use in microbiology laboratories not equipped with micromanipulator.
313 This system combines automation and miniaturisation, prerequisites for modern
314 microbiology.

315

316 All approaches described in this chapter aimed to optimize prokaryote cultivation efficiency
317 (fig. 2). Overall, these studies allow for the isolation of numerous novel species and permit
318 major breakthroughs in cultivation. Despite continuous efforts, culture dependent approaches
319 undergo changes characterized by gradual improvements rather than by a radical paradigm
320 shift. It is noteworthy that the so-called “Moore Law” in microbiology (Gefen and Balaban,
321 2008) applies to the volume used in culture and not to the output in number of novel species
322 described.

323

324 **II-Why do so many microbes resist cultivation?**

325 Despite extensive studies on ecosystems where sampling is conducted, the conditions used in
326 classical cultivating conditions are often far from endogenous abiotic and biotic conditions
327 required for microbial growth. Thus, it is not surprising that only a small fraction of the whole
328 microbial repertoire has been cultured so far.

329 The lack of efficient cultivation techniques stems from many factors that largely remain
330 unknown. These factors include limited knowledge of (i) the diverse organisms, (ii) the
331 chemistry of the natural habitats, (iii) the natural biotic and abiotic interactions and (iv) the
332 global functioning of the ecosystems at microbial level. Cultivation aims to create an artificial
333 system mimicking the *in situ* conditions. However, we do not have sufficient knowledge to
334 reproduce the natural conditions in the laboratory or to create viable synthetic conditions for

335 all organisms. This lack of knowledge has led to regrettable mistakes.. For example, traces of
336 tungsten element, which inactivates nitrogenase, have been included for a long time in the
337 culture media for *Spirochaetes* (Leadbetter, 2003). It was recently discovered that
338 *Spirochaetes* are able to grow *via* nitrogen fixation (Lilburn *et al.*, 2001), a property that can
339 be expressed only in tungsten-depleted media. Another remarkable example is the unexpected
340 discovery of nanoarchaea in enrichment cultures (Huber *et al.*, 2002). The discovery was
341 unattainable by current PCR-based approaches since the universal primers commonly used for
342 diversity studies could not target this group.

343 Another reason that can explain the failure to cultivate many prokaryotes is our lack of
344 patience and sensitive detection methods for low cell yields. As previously discussed, in some
345 cases, cultivation efficiency was significantly increased by long periods of incubation. This is
346 true for organisms originating from oligotrophic habitats and potentially in a non-growing or
347 dormancy state. It is important to note that transition from a non-growing to a growing state in
348 a synthetic laboratory medium is a critical and stressful event. Thus, adaptations of cells to
349 laboratory growth conditions may require increased cultivation times. For instance, the
350 duration of growth log phase can depend on the cell status (i.e. healthy, stressed or sub-
351 lethally damaged cells) and the gap between *in situ* and *in vitro* conditions (*de novo* synthesis
352 of an enzymatic set to grow in the synthetic medium, etc). Without permitting adequate time
353 intervals for growth, many organisms and many novel redox reactions would never have been
354 discovered. For example, without lengthy incubation periods, the observation that methane is
355 formed from long-chain alkanes under anaerobic conditions would not have been
356 documented. Under tested conditions, gas formation in the presence of hexadecane started
357 only after four-month incubation of the culture (Zengler *et al.*, 1999). While bioenergetics
358 calculations can predict thermodynamical feasibility of a reaction, our current knowledge can
359 not predict kinetics for ‘resuscitation’ and for the growth of *de novo* enrichment cultures *via* a

360 given reaction under a defined experimental conditions. Indeed, the characteristics of natural
361 uncultured organism(s) (physico-chemical optima for growth, transport systems, etc.) and of
362 their enzymes (catalytic rates, substrate affinities, regulation mechanisms) are unknown. At
363 best, we can suppose that enrichment cultures performed with highly stable substrates, weak
364 oxidants and calculated to have low net free energy gain, will require long-term incubations
365 and patience. Otherwise, extremely long growth kinetics or low cell densities are sometimes
366 observed in enrichment or pure culture. Inappropriate or non-optimal growth conditions might
367 be responsible for these low yields. Thus, once parameters that inhibit growth are identified,
368 “normal” growth kinetics and high yields can be restored (i.e. Flagan *et al.*, 2003). For the
369 organisms characterized by meagre yields, highly sensitive detection technologies such as
370 tangential flow filtration and concentration are critical (Giovannoni and Stingl, 2007).

371 Another rational put forward for prokaryotic resistance to cultivation is inherent to the *in vitro*
372 cultivation techniques widely used. More specifically, the *in vitro* cultivation techniques used
373 paradoxically aim to isolate strains in pure culture, while most organisms in nature live in
374 community and establish complex relationships (see previous chapter). The main
375 consequence of this general practice is a disruption of inter- and intra-species communication
376 during the very first stage of isolation. The impact of this perturbation on cultivability is
377 unknown for most prokaryotic species and might vary depending of the species. Cell-cell
378 communication (CCC) has gained considerable attention in recent years, in particular density-
379 dependent cell-signalling mechanisms known as quorum sensing (QS). Cell-cell interactions
380 have been investigated mainly in Bacteria due to their tremendous importance in health,
381 environmental and industrial applications. Bacteria respond to a wide range of signalling
382 molecules at intra-species level (species-specific compounds) and/or inter-species level
383 (Camilli and Bassler, 2006). Inter-species interactions appear to be ubiquitous among
384 prokaryotes and are not limited to signalling molecules such as autoinducer-2 (AI-2) and *N*-

385 acyl-homoserine lactones (*N*-AHLs) but extend to antibiotics at sub-inhibitory concentrations
386 (Ryan and Dow, 2008). Globally, prokaryotes and eukaryotes have co-existed for millions of
387 years and frequently have co-evolved in the same environments. Therefore it is not surprising
388 that interactions between them range from mutually beneficial to virulent. QS compounds like
389 AHLs found initially in Bacteria are involved in virulence, biofilm formation, motility,
390 antibiotic production and are recognized by eukaryotes with effects on immunomodulation,
391 intracellular calcium signalling and apoptosis (Hughes and Speriandio, 2008). QS
392 mechanisms have been extensively investigated during the past decade. Many Bacteria have
393 one QS circuit while some display two or three circuits to coordinate their population density.
394 Yet, these circuits are themselves under the control of a master QS regulator (Hooshangi and
395 Bentley 2008). Recently Kolodkin-Gal *et al.* (2007, 2008) showed that the mode of action of
396 antibiotics in *E. coli* is determined by the ability to communicate through the Extracellular
397 Death Factor (EDF) as a function of cell density. CCC was initially considered as an
398 exception limited to a few specialized bacteria, but has recently emerged as the norm in the
399 bacterial world. Several indications strengthen this trend: (i) the high frequency of QS among
400 genome-sequenced bacteria (40% of 800 sequenced bacterial genomes contain the *luxS* gene
401 suggesting that the AI-2 precursor functions as a universal signal (Pereira *et al.*, 2008)) and,
402 (ii) the discovery of signalling molecules in microbial metagenomic data. In addition, there is
403 growing evidence that QS signals provide to bacteria more information than cell density.
404 Surprisingly, the abundance of results on QS, biofilms and on cell-cell communication, at
405 intra-species or inter-species levels have been analysed in the perspective of pathology
406 (virulence, biofilm formation and control), biotechnology, synthetic biology (Hooshangi and
407 Bentley, 2008), evolution (Keller and Surette, 2006; West *et al.*, 2007), ecology (Nadell *et al.*,
408 2008) but seldom for cultivation purposes.

409 To account for CCC mechanisms in cultivation procedures remains a challenge due to our
410 lack of knowledge on the cell-cell interaction requirements of targeted species. Considering
411 the small fraction of prokaryotes cultivated, it seems likely that most cell-cell communication
412 mechanisms are unknown. CCC mechanisms described up to now, like those classically
413 observed with *Vibrio harveyi*, *Pseudomonas aeruginosa* and *Escherichia coli*, operate at high
414 cell densities. We cannot exclude that some CCC mechanisms could also act at low cell
415 densities (10^2 to 10^4 cells/ml) to regulate cell growth of some species. In that case, it may be
416 necessary to reconsider isolation procedures accordingly.

417 Finally, one last reason for which prokaryotes remain uncultured is due to enrichment-
418 isolation process whereby the abiotic interactions are broken down. This disruption of the bio-
419 geochemical factors, that collectively represent the environment, is a source of stress for the
420 organisms from natural habitats. The depreciation of these bio-geochemical factors from the
421 native extra-cellular habitats leads the experimentalists to design synthetic conditions which
422 might introduce a stressful parameter or a main change in resource type or concentration. For
423 example, a phenomenon similar to substrate-accelerated death might occur. Growth inhibition
424 is observed when cells are suddenly grown in the presence of concentrations greater than 1-10
425 μM of a given substrate (i.e. ammonia, phosphate or glucose) whereas they were previously
426 starved of this substrate (Overmann, 2006).

427

428 **III- Cultivating the uncultured: future directions**

429 There is current growing consensus among microbiologists that improvements are needed in
430 microbial cultivation. A comprehensive understanding of the microbial world will
431 undoubtedly require cultivation. It is clear that the keys of cultivation will not come from a
432 single technological breakthrough but will depend on our knowledge of the natural microbial
433 systems. The numerous examples of successes in cultivation that we have mentioned in this

434 review demonstrate that through patience and advances in technology, cultivation is a
435 surmountable obstacle for many organisms. We still have a lot to learn from pure culture
436 microbiology, even if these isolates might display unnatural behaviours in the synthetic
437 laboratory.. Pure culture microbiology still represents the best method to study microbial
438 physiology including detailed investigations on the role of genes, proteins, and metabolic
439 pathways.. This is true since several unexpected physiological discoveries were made after
440 isolation of novel species belonging to taxa presumed to be already well documented. In turn,
441 these data provide important guidance for the optimization of cultivation media.

442 While several difficulties remain in cultivating microorganisms, future directions can be
443 summarized as follows: refining culture medium, mimicking nature through *in situ* cultivation
444 systems or designing devices supporting CCC, and developing automated procedures through
445 robotics. Undoubtedly, combinations of these diverse approaches will yield successes in
446 cultivation.

447

448 ***1- Refinement of culture media***

449 Culture medium optimization will require the use of various complementary tools including:
450 (i) the ability to define the range of electron donors, acceptors and key elements sources based
451 on a better knowledge of the environment, (ii) molecular probes for screening novel species
452 and, (iii) high-sensitive methods with low detection thresholds to uncover rare and slow-
453 growing species in culture. In the future, results from single cell genomics and metagenomics
454 analyses will contribute to better isolation strategies for prokaryotes.

455

456 ***2- Design of isolation strategies based on cell-cell interactions***

457 As seen previously, microbial landscapes are dominated by biofilms or aggregates. The
458 isolation is not the preferred state of most Bacteria and Archaea. Despite the growing

459 knowledge on cell-cell signalling molecules, the use of antibiotics and auto-inducers in
460 culture is limited. Indeed, the vast majority of these compounds were characterized from
461 microorganisms in pure culture, some of which were subsequently retrieved in metagenomic
462 analyses. Metagenomics will yield numerous genes coding for novel auto-inducers or their
463 precursors, but they will be classified as unknown genes until discovered in culture. Therefore
464 it is not surprising that our previous analytical knowledge is hardly applicable to isolation
465 procedures of novel species. For isolation of a strain, one way to preserve the endogenous
466 CCC mechanisms is to grow it in microbial community conditions, using bioreactors, either
467 under planktonic stage, or incorporated in gel microdroplets (GMDs) (Zengler *et al.*, 2002),
468 or attached to a surface. So far, the community culture step is more often followed by an
469 isolation step through various means: streaking, dilution-to-extinction, sorting (mechanically
470 or by FACS). For the last method, single cells are distributed in wells or micro-wells filled
471 with appropriate medium and incubated for further culture. The major drawback is that the
472 appropriate medium used to grow the cells after the isolation step is different from the
473 medium that supported growth in the community culture, and that all chemical CCC are
474 suddenly disrupted. What is the impact on the cultivability of some strains? In order to
475 address this question it seems necessary to design novel culture equipments. The easiest
476 method would be to substitute culture microplates by micro-bioreactors in the Zengler's
477 method (Figure 3). In such a system, the flow from the community culture would nourish
478 each well of the micro-bioreactor with all the metabolites produced by the community culture.
479 This system would combine community culture and isolation. Further, it would allow for the
480 circulation of chemical compounds in the micro-bioreactor, partly preserving cell-cell
481 communication, even though physical cell-cell contacts would be disrupted. Since the
482 community culture would operate as a black box for signalling compounds, we cannot
483 exclude that some metabolites could have inhibitory effects. The benefits of such a

484 combination of enrichment culture and isolation remain to be assessed. By dissociating and,
485 in a following step, combining community culture and isolation, this system could address the
486 culture of consortia components and symbionts which are almost impossible to solve with
487 current practices.

488

489 ***3- High throughput issues***

490 A quiet revolution occurred during the past decade in liquid handling applied to life sciences
491 with the development of pipetting workstations. The main line of action was the reproduction
492 at high throughput of manual procedures by pipetting robots. Culture medium distribution in
493 micro-plate wells and inoculation of a single cell or GMD per well is now performed
494 routinely by robots. The combination of GMD encapsulation and flow cytometry sorting is
495 effective for aerobes (Akselband *et al.*, 2006). Further, these studies show that some cells
496 remain viable after staining and sorting, however these findings await confirmation. This
497 remains a challenge for obligatory anaerobes especially due to the sorting step with flow
498 cytometers. FACS equipment could be adapted to operate in anaerobic chambers, but might
499 prove inconvenient and costly. The development of direct sorting of GMDs on liquid
500 handling workstations would be an excellent alternative to avoid FACS analysis, to limit the
501 cost of equipments and, to allow dissemination of these approaches in microbiology
502 laboratories. What seems also to be certain is that the widespread use of high-throughput
503 cultivation procedures implies the simultaneous development of affordable high-throughput
504 identification procedures.

505

506 ***4-Combination principle***

507 Microbiologists have long recognized that our understanding of the microbial world critically
508 depends on the technological advances that broaden the knowledge-base for chemical,

509 biological and physical processes. Today, it is established that an integration of the
510 knowledge from multiple hierarchical levels of organization and from the molecule to the
511 ecosystem are also required. Despite these facts, most efforts in cultivation focused only in
512 the optimization of a limited set of parameters. We are convinced that this situation will
513 change in the near future for several reasons. First, a consensus is gradually emerging: culture
514 is not only needed to describe randomly novel species as a function of researcher main field
515 of interest. Culture appears more and more as the *sine qua non* condition for understanding
516 how the microbial world functions and is a prerequisite to predict changes in the frame of
517 global change. Secondly, it seems that the complexity of the microbial world emerging from
518 the growing knowledge of genomic and proteomic advances can not be analyzed only by
519 combining these approaches and relying on systems biology. Finally, the advent of
520 automation in culture and the combination of innovative methods lead to a new era in
521 cultivation, assuming that some specific equipments are developed to handle the cell-cell
522 interaction mechanisms during the clonal culture phase. Combinations theoretically possible
523 are detailed in Fig. 3.

524 Ecosystems encompass abiotic conditions, living species and all their interactions
525 (resource limitations, competition, predation, parasitism, among others). It is obvious that any
526 cultivation attempt is by nature highly reductive and cannot reproduce the conditions
527 observed in nature. The combination of existing methods and the development of novel
528 approaches will help to come close to conditions where a significant fraction of microbes is
529 amenable to culture (Fig. 2).

530

531 **Concluding remarks**

532 .

533 The extent of microbial diversity is still unknown and prokaryotes have undoubtedly many
534 secrets to reveal. The success of microbial life on Earth stems from its great physiological
535 diversity that collectively allows prokaryotes to derive energy from a wide range of redox
536 reactions and to colonize several types of habitats; including the extreme ones. It is
537 remarkable how much we have learned about microbial life by studying only a small fraction
538 of its diversity. New examples of microbial ingenuity were recently discovered due to
539 cultivation, either independently or in combination with metagenomics. There are still
540 important essentials hidden in the 99.9% of the microbial world that are not yet cultured.
541 They might change our understanding of biochemical processes, redox reactions,
542 physiological adaptations and microbial behaviours. We postulate that in the future major
543 advances in the understanding of microbial life will be achieved by innovative approaches in
544 cultivation, but not cultivation alone. Coordinated efforts of researchers studying microbial
545 systems at different levels should guide us to rethink culture strategies and to design growth
546 conditions as close as possible to the natural interactions and conditions. In conclusion, the
547 different cultivation successes discussed in this review demonstrate that contrary to widely
548 held beliefs, many prokaryotes can be cultivated. In view of the gap between the number of
549 phylotypes with or without representative cultured species, an additional question arises: is
550 there a need to define relative priorities between phylotypes? The division amongst
551 microbiologists at the international realm suggests that a consensus is not possible. The only
552 consensus that could emerge, and successfully funded, is the need to investigate novel
553 cultivation approaches, to develop new equipments and bring microbial cultivation in the 21st
554 century as a technologically advanced and a data rich discipline.

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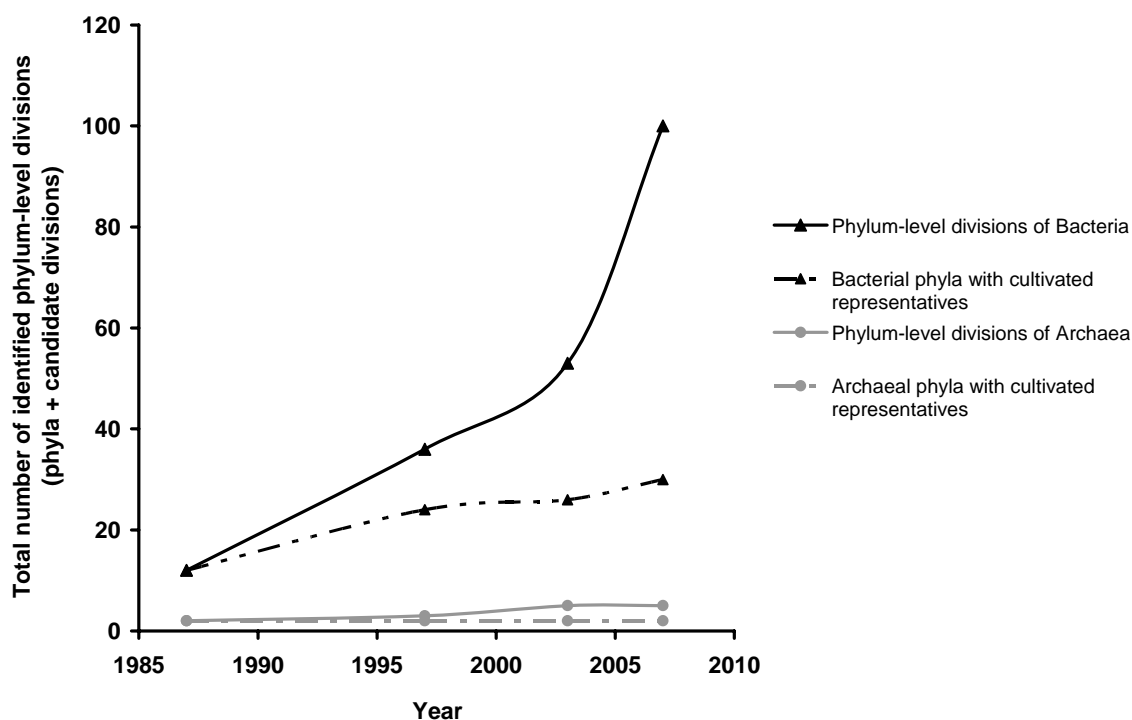
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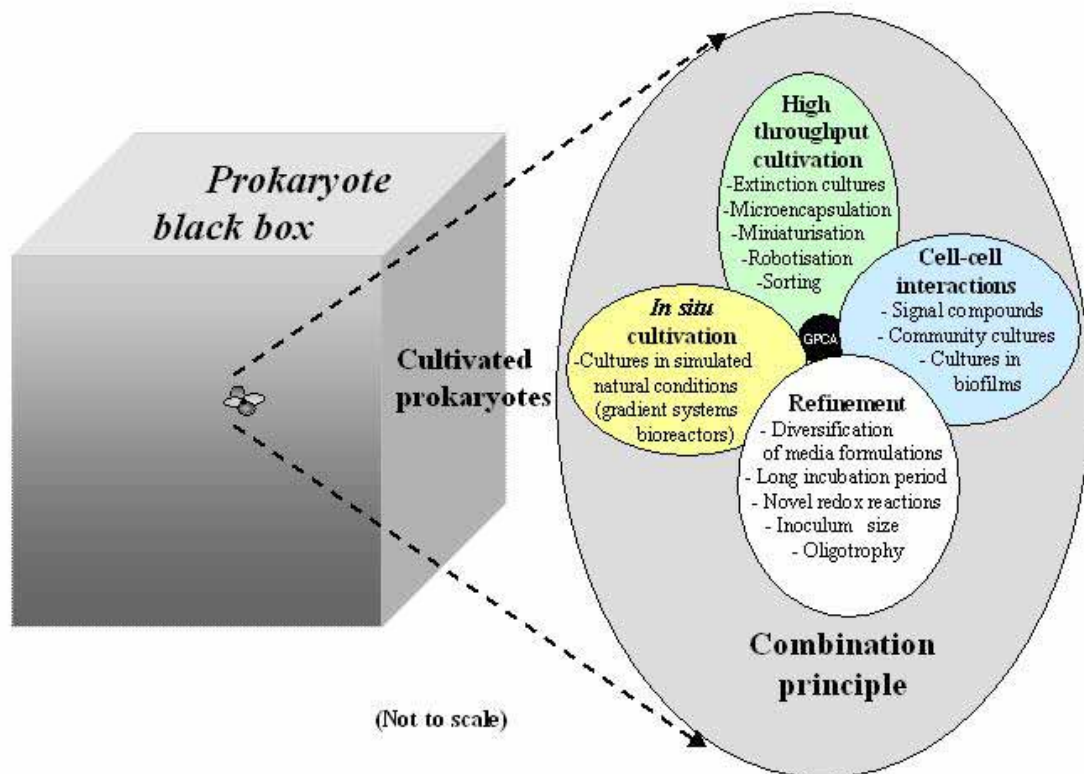
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 775 Fig. 1. Numbers of phylum-level divisions (phyla with cultivated members + candidate
 776 divisions with no cultivated representative) identified since 1987 among Bacteria (black line)
 777 and Archaea (grey line), and numbers of phyla with cultivated representatives (dotted lines).
 778 Adapted from Achtman & Wagner (2008).

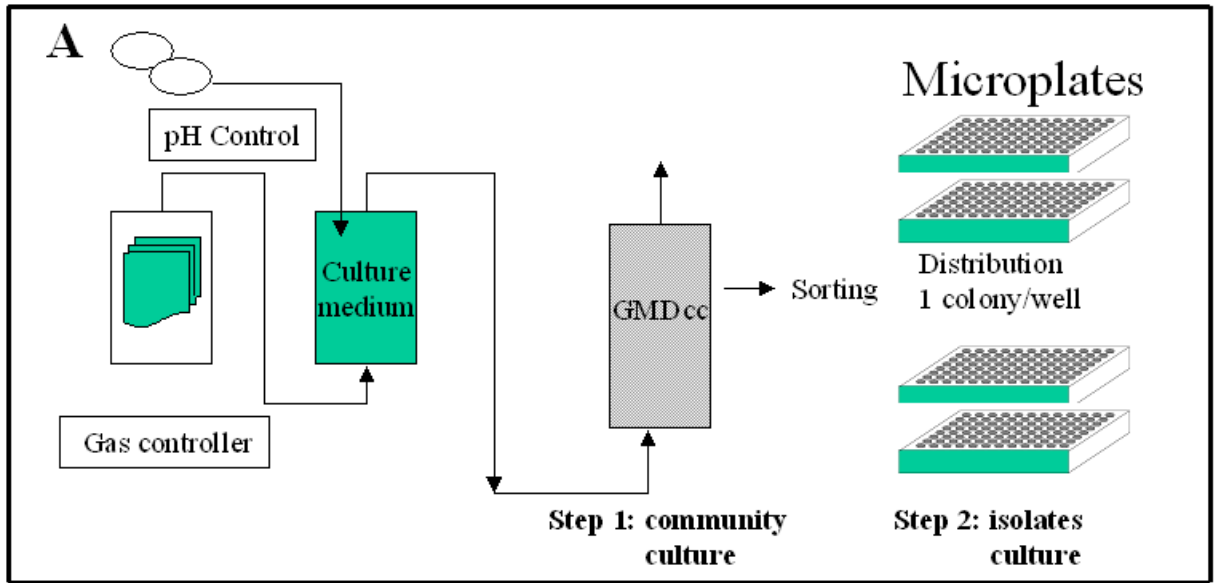
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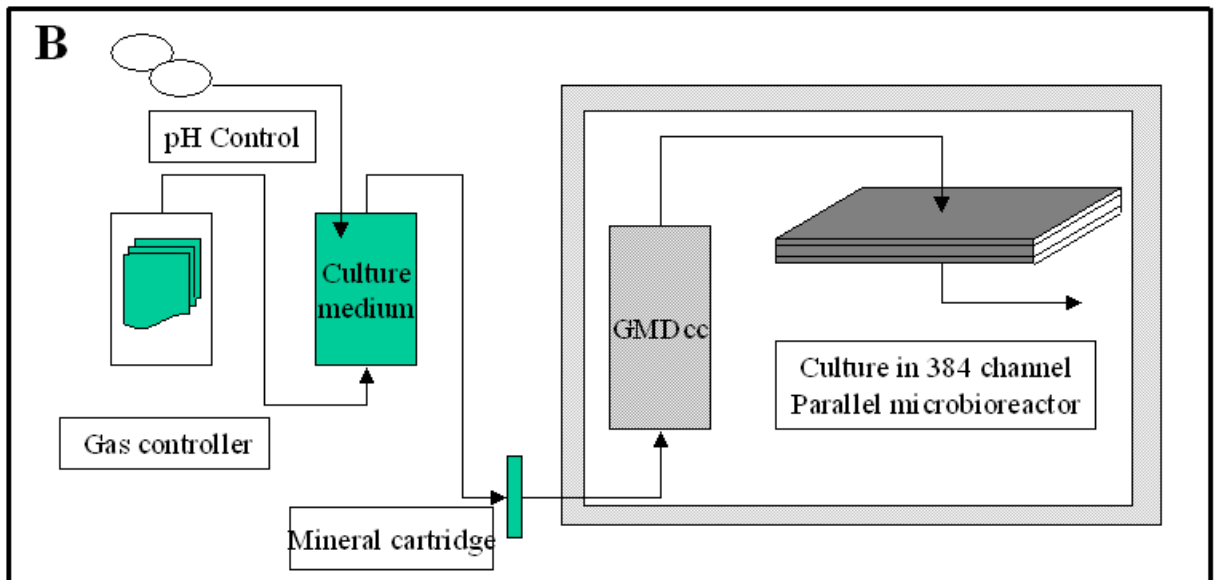
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780 Fig. 2. Schematic representation of the four main categories of cultivation strategies. The
 781 small black circle schematizes the minor fraction of a naturally occurring microbial
 782 community that are recovered onto conventional selective media, the so-called “Great Plate
 783 Count Anomaly”. Improved cultivation strategies developed so far aim to enhance one or few
 784 aspects of growth conditions among four main categories (represented by four bullets) and
 785 each allowed individually recovering more numerous and/or more diverse isolates than
 786 traditional approaches. None of these improved approaches is universal. We postulate that the
 787 combination (“the combination principle”) of already existing methods belonging to these
 788 four categories will help to come close to conditions where a significant fraction of microbes
 789 is amenable to culture.

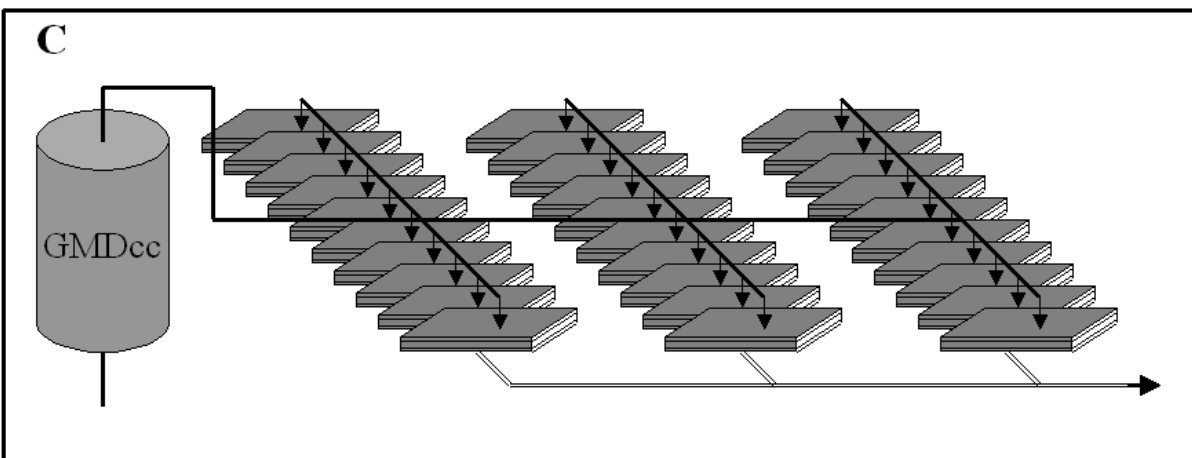
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793 Fig. 3. Schematic diagram of cultures and isolation procedures based on the combination
794 principle.

795 Abbreviation: GMDcc: community culture of microbial cells incorporated in gel micro-
796 droplets

797 A: GMD community culture followed by sorting and micro-plate cultivation (From Zengler *et*
798 *al.*, 2002, modified).

799 B: flowthrough culture in parallel micro-bioreactors nourished by community culture medium
800 and metabolite products. Micro-plates are replaced by micro-bioreactors directly connected to
801 the GMD community culture. This system can be operated in aerobic or anaerobic conditions.
802 Micro-bioreactors (either 96 or 384 parallel channels SBS format) and flowthrough
803 equipments awaiting development.

804 C: GMD and micro-bioreactors (384 channels) coupled in a nearly theoretical 10000 parallel
805 channels configuration.