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1 **A strict anaerobic extreme thermophilic hydrogen - producing culture enriched**
2 **from digested household waste.**

3

4

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6

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11

12 Extreme thermophilic biohydrogen producers

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15 **Abstract**

16 **Aims:** Enrichment, characterization and identification of strict anaerobic extreme
17 thermophilic hydrogen producers from digested household solid wastes.

18 **Methods and Results:** A strict anaerobic extreme thermophilic hydrogen producing
19 bacterial culture was enriched from a lab-scale digester treating household wastes at
20 70°C. The enriched mixed culture consisted of two rod-shaped bacterial members
21 growing at optimal temperature of 80°C and optimal pH 8.1. The culture was able to
22 utilize glucose, galactose, mannose, xylose, arabinose, maltose, sucrose, pyruvate and
23 glycerol as carbon sources. Growth on glucose produced acetate, hydrogen and carbon
24 dioxide. Maximal hydrogen production rate on glucose was 1.1 mmol.L⁻¹.h⁻¹ with a
25 maximum hydrogen yield of 1.9 mole H₂ per mole glucose. 16S ribosomal DNA clone
26 library analyses showed that the culture members were phylogenetically affiliated to the
27 genera *Bacillus* and *Clostridium*. Relative abundance of the culture members, assessed by
28 Fluorescence *In Situ* Hybridization (FISH), were 87 ±5 % and 13 ±5 % for *Bacillus* and
29 *Clostridium*, respectively.

30 **Conclusions:** An extreme thermophilic, strict anaerobic, mixed microbial culture with
31 H₂-producing potential was enriched from digested household wastes.

32 **Significance and Impact of the Study:** This study provided a culture with potential to
33 be applied in reactor systems for extreme thermophilic hydrogen production from
34 complex organic wastes.

35 **Keywords:** hydrogen, extreme thermophilic, household wastes

36

37 INTRODUCTION

38 Nowadays, hydrogen gas (H_2) is attracting attention as it is a sustainable, clean and
39 non-polluting alternative to fossil fuels. However, H_2 is not a primary energy resource
40 and must be produced from other sources. The main industrial process to produce H_2
41 consists of steam reforming from natural gas and petroleum, a process which depends on
42 fossil fuels and generates carbon dioxide as byproduct (Rostrup-Nielsen 2001). Another
43 source is electrolysis of sea water, which could be sustainable if electricity is generated
44 from renewable resources, but at present there is no significant surplus of sustainable
45 electricity capacity. One of the methods to circumvent the dependence of H_2 production
46 from fossil fuels is to utilize the potential of H_2 -producing microorganisms to derive
47 hydrogen from widely available organic sources.

48 Due to technical limitations, the biological production of hydrogen in full-scale
49 systems has not been fully developed yet (Angenent *et al.* 2004). The biological H_2
50 production at lab and pilot-scale is achieved through anaerobic “dark” fermentation or
51 photosynthesis (Nandi and Sengupta 1998). The dark fermentative H_2 production is a low
52 cost alternative of the photoprocesses which require larger space and power-consuming
53 light sources. This has already been widely investigated with several mesophilic pure and
54 mixed cultures (Hawkes *et al.* 2007, Li and Fang, 2007). However, only a little
55 information is available about the potential of extreme thermophilic H_2 producing
56 microorganisms (growing above 65°C). In comparison to mesophilic biohydrogen
57 production, the fermentation process under high temperatures offers several advantages
58 such as faster H_2 production rates, higher bioavailability of the substrate (higher solubility
59 of organic compounds), better H_2 extraction rates from liquid to gas phases, and lower

60 risk of contamination by non-H₂ producing mesophilic microbes (Van Niel *et al.* 2002;
61 Kanai *et al.* 2005). The present knowledge about biological hydrogen production at high
62 temperatures is limited to studies on pure cultures utilizing defined carbon sources for H₂
63 synthesis (van Ooteghem *et al.* 2004; Kanai *et al.* 2005). In these studies, the H₂-
64 producing microorganisms were isolated from natural hot environments. Very little
65 information is available about H₂ producing potential of extreme thermophiles (growing
66 between 55°C and 80°C) in hot artificially created habitats, such as laboratory reactors
67 digesting organic wastes. Kotsopoulos *et al.*, 2005 previously reported that dairy manure
68 predigested at 55°C was used as inoculum in a granular upflow anaerobic sludge blanket
69 reactor (UASB) for biological H₂ production at 70°C. No extreme thermophilic H₂
70 producing culture derived from artificial anaerobic environments has been isolated,
71 characterized and identified hitherto. The identification of such cultures and subsequent
72 characterization of growth properties may result in the development of potentially
73 important biotechnological processes, such as direct fermentation of hot effluents
74 (hydrolysates) derived from thermal pretreatment of energy crops (plants grown
75 specifically for production of bioenergy) and crop residues (Klinke *et al.* 2002). The
76 utilization of such hot hydrolysates for biohydrogen production not only circumvents the
77 need for prior cooling, but also saves considerable amounts of energy. This will lead to
78 more sustainable biological H₂ production.

79 The aim of this work was to enrich, identify and characterize a strict anaerobic
80 extreme thermophilic microbial culture with H₂-producing ability, using digested
81 household solid waste as inoculum.

82

83 MATERIALS AND METHODS

84 Enrichment

85 Basal anaerobic (BA) medium was used for enrichment and routine cultivation as
86 described previously (Angelidaki et al. 1990), except that no L-cysteine hydrochloride
87 was added, and the concentration of $\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$ was increased to $0.25 \text{ g}\cdot\text{L}^{-1}$. The medium
88 was supplemented with sterile solutions of glucose and yeast extract to final
89 concentrations of 0.25 % (w/v) and 0.05 % (w/v), respectively. 40 mL medium was then
90 dispensed in 100 mL serum bottles inoculated with 4 ml effluent from H_2 producing
91 laboratory-scale continuously stirred tank reactor treating household solid wastes (HSW)
92 at 70°C . HSW corresponded to a heterogeneous mixture of food residues, papers, garden
93 wastes, plastics, stones, and metals as described in Liu *et al.* (2006). The reactor had been
94 operated at 2 days solid retention time and organic loading rate of $11 \text{ g volatile solids}\cdot\text{L}^{-1}\cdot\text{d}^{-1}$
95 at 70°C for approx. one year, and was originally inoculated with the effluent of a
96 mesophilic (38°C) full-scale biogas plant treating a mixture of wastewater sludge and
97 municipal solid wastes (Grindsted, Denmark). After inoculation, the serum bottles were
98 incubated in dark at 70°C until H_2 production and glucose consumption ceased. Six
99 successive transfers of the enriched mixed culture were performed until steady hydrogen
100 production rates were achieved.

101

102 Isolation

103 Isolation experiments were carried out anaerobically at 70°C using the roll tube technique
104 (Hungate 1969). Dilutions of the enriched mixed cultures were transferred into fresh BA
105 medium containing $2.5 \text{ g glucose L}^{-1}$ and solidified with $11 \text{ g Gelrite L}^{-1}$ and $1 \text{ g MgCl}_2\cdot\text{L}^{-1}$

106 ¹. To stimulate colony formation, media was supplemented with sources of potential
107 microbial growth factors. Two types of media were chosen for isolation - supernatant
108 from the enriched mixed culture, and cell extract from the enriched mixed culture. After
109 sterilization by membrane filtration (0.45µm), supernatant and the cell extract were added
110 to media at final concentration of 20 % (w/v) and 1 % (w/v) respectively. Cell extracts
111 and the supernatant were prepared as described below.

112 **Preparation of cell extracts.**

113 Cells extract was prepared by sonication method described elsewhere (Bae *et al.* 2005). A
114 12 hrs old culture with stable hydrogen production rates was used for cell extracts
115 preparation.

116 **Preparation of cultural supernatant.**

117 Supernatant solution of the 12 hrs old enriched mixed culture was prepared by
118 centrifugation of the cultural broth for 5 min at 5000 g.

119

120 **Analytical methods.**

121 Gas chromatography (GC) was used for identification of the fermentation products.
122 Flame-ionization detection was used for volatile fatty acids and ethanol according to
123 Sorensen *et al.* (1991). H₂ concentration in the gas phase was measured using a gas
124 chromatograph (MicroLab, Arhus, Denmark) equipped with a s-m stainless column
125 packed with Porapak Q (50/80 mesh) and coupled to a thermal conductivity detector
126 (TCD). Lactate was quantified by high-pressure liquid chromatography as previously
127 described (Hörber *et al.*, 1998). Growth rates were determined by measuring culture

128 turbidity (OD₅₇₈) with glucose as sole substrate. Glucose was measured by
129 spectrophotometry using the phenol-sulphuric acid method (Dubois *et al.* 1956).

130 **Physiological tests**

131 Prior to the determination of pH and temperature optima, the enriched mixed culture was
132 adapted to each temperature and pH by transferring the enriched mixed culture three
133 times into fresh BA medium supplemented with glucose. The effect of different
134 incubation temperatures (55, 60, 65, 70, 75, 80, 85 and 90 °C) on H₂ production was
135 assessed at pH 7-7.1. The effect of the initial pH (5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9) on
136 H₂ production was investigated at 70°C. The final pH of the media was measured at the
137 experimental temperature (70°C). To determine the spectrum of substrate utilization,
138 several carbohydrates (soluble starch, cellulose, maltose, sucrose, seduheptulose,
139 mannose, galactose, xylose, arabinose, erythrose), organic acids (formate, acetate,
140 propionate, butyrate, pyruvate) and alcohols (methanol, ethanol, glycerol) were
141 investigated in BA medium. All substrates were added from sterile anaerobic stock
142 solutions to reach a final concentration of 0.25 % (w/v).

143

144 **Microscopic observations and Fluorescent *In Situ* Hybridization (FISH) procedure**

145 Gram staining of fresh 24 h enriched mixed cultures was performed according to
146 Standard Methods (Gerhardt 1981). Fresh 24 h cultures of *Bacillus* sp. and *Pseudomonas*
147 sp. were used as positive and negative controls, respectively. Staining procedure was also
148 performed in an anaerobic gloveless chamber according to Johnson *et al.*, 1995 to cross-
149 check the consistency of the Gram staining results obtained under aerobic conditions.

150 The enriched mixed cultures were observed using FISH. The fixation and hybridization
151 procedures were performed according to Hugenholtz *et al.* (2001). The hybridization
152 temperature was 46°C and the formamide concentration was 35 % (v/v). Specific 16S
153 rDNA probes were used to target the bacterial division of Firmicutes gathering *Bacillus*
154 *sp.* and *Clostridium sp.* (YSGAAGATTCCCTACTGC – probe LGC354deg adapted
155 from Meier *et al.*, 1999), and the *Clostridium* genus (CAAACGCAGTCCATGAGT –
156 probe CLOS621 from Liu *et al.*, 2002). The Firmicutes bacteria and *Clostridium sp.*
157 were marked in green using fluorescein (FITC) label and in red using indocyananine
158 (Cy3) label, respectively. The slides were examined using a Olympus BX60
159 epifluorescence microscope. Appropriate excitation lasers and emission filters were used
160 for FITC and Cy3 labels. Microscopic pictures were collected using the Nikon
161 DXM1200F and ACT-1 software v.2.63. Relative abundances of individual members
162 were then estimated from image analysis of 30 microscopic pictures using Image J v.1.4
163 software. Relative abundance of *Clostridium sp.* and *Bacillus sp.* corresponded to the
164 percentage of yellow colored pixels, i.e. CLOS621/LGC354deg stained cells, and green
165 colored pixels, i.e. LGC354deg stained cells, from total colored pixels, respectively.

166

167 **PCR-DGGE analysis.**

168 PCR-DGGE was used here to study microbial community structure in the enriched mixed
169 culture. Two ml of the 24h fresh enriched mixed culture was centrifuged at 5000 g for 5
170 min and pelleted cells were resuspended in 50µl sterile MilliQ water. Genomic DNA was
171 extracted and purified using QIAamp DNA Stool Mini kit (QIAGEN, 51504). DNA
172 extract was used as the template for PCR (PCR-1). For *Bacteria*, universal primer 1492-r

173 and Bacteria specific primer 7-f were used (Lane 1991). For *Archaea*, 1492-r and Archae-
174 specific primer A109-f were used (Grosskopf et al, 1998). PCR was performed in a 50µl
175 (total volume) reaction mixture containing 50mM KCL, 20mM Tris-HCl (pH 8.4), 5 mM
176 MgCl₂, each deoxinucleotide triphosphate at a concentration of 200µM, 1 µl of *Taq*
177 polymerase (2 U/µl; Sigma), 10 pmol of each primer, and 1µl of DNA extract. The
178 thermal cycling program used for amplification was as follows: pre-denaturation at 95 °C
179 for 5 min; 25 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 40 s,
180 elongation at 72°C for 90 s, and post-elongation at 72°C for 5 min. The reactions were
181 subsequently cooled to 4 °C. The size of the amplicon was estimated on 1.5 % agarose
182 gel. The amplicon was then used as DNA template for the subsequent PCR (PCR-2) to
183 incorporate a GC clamp in the DNA fragment prior to DGGE (Muyzer and Smalla,
184 1998). For *Bacteria*, primer 518-r and 357-f (with 40 bp GC clamp at the 5' end) (Muyzer
185 et al., 1993) were used to amplify the ~200 bp fragment of the V3 region. The PCR
186 program corresponded to 20 cycles of three steps: 94°C for 1 min, 65°C for 0.75 min, and
187 72°C for 1 min, 10 cycles of three steps: 94°C for 1 min, 55°C for 0,75 min, and 72°C for
188 1 min followed by a final step at 72°C for 10 min. PCR products were stored at 4°C and
189 analysed on 1.5 % agarose before DGGE. DGGE analysis of the amplicons obtained from
190 PCR-2 was performed as previously described by Zoetendal et al. (2001), using the
191 Dcode Universal Mutation Detection system (Bio-Rad, Hercules, CA) with 8% (v/v)
192 polyacrylamide gels and a denaturant gradient of 30–60%. A 100 % denaturing solution
193 was defined as 7M urea and 40 % formamide. Electrophoresis was performed for 16 h at
194 70 V in a 0.5x TAE buffer at 60°C. DGGE gels were stained with SYBR Green for 15
195 min and analyzed on GelDoc XR 1708170 system (Bio-Rad).

196

197 Construction of 16S ribosomal DNA clone library

198 16S ribosomal DNA clone library was used for identification of community members.
199 The amplicons obtained from PCR-1 were ligated into pGEM T-Easy vector and
200 transformed into JM109 competent cells using the pGEM T-Easy vector system
201 (Promega). Thus obtained clones were subcultured in LB+amp and the plasmid DNA was
202 extracted using GenElute Plasmid Miniprep Kit (SigmaAldrich). The inserts were
203 confirmed by restriction digestion with EcoRI (SigmaAldrich) and then analyzed using
204 PCR-DGGE as described above. Finally, 40 clones were picked and the DNA extracted
205 from those clones was sequenced at MWG, Germany.

206

207 Phylogenetic analysis

208 The obtained 16S ribosomal DNA sequences were analyzed using the BLAST program
209 of NCBI. The sequenced were aligned to their nearest neighbours and phylogenetic tree
210 was constructed using CLCBio Free software version 3.2.4. The final sequences have
211 been submitted to the Genbank database under the accession numbers **EF661579** (1550
212 bp) and **EF661580** (1437 bp).

213

214 RESULTS**215 Microscopic observations of the isolates**

216 A population of rod-shaped bacteria (Gram positive and Gram negative) was observed in
217 the enriched mixed culture (data not shown). All attempts to isolate the enriched mixed

218 culture members on roll tubes with and without sources of growth factors were
219 unsuccessful.

220

221 **Growth characteristics and physiology**

222 The final enriched mixed culture could grow at temperatures ranging from 55 to 90 °C
223 (pH 7.2) ((Fig 1a) and at pH between 5.5 and 9.0 (70°C) (Fig. 1b). Specific growth rate
224 reached its maximum of 0.14 h⁻¹ at temperatures between 70-80 °C (pH 7.2) and pH
225 between 7-8 (70°C). H₂ production rate on glucose was 1.1 mmol H₂ .L⁻¹.h⁻¹ and the H₂
226 yield was 1.9 mol H₂ per mole glucose. The presence of yeast extract was not found to be
227 essential but stimulated the growth.

228 The enriched mixed culture grew and produced H₂ on a wide variety of carbon
229 sources including glucose, galactose, mannose, maltose, sucrose, arabinose, xylose,
230 glycerol and pyruvate (Table1) but not on sedoheptulose, erythrose, cellulose, soluble
231 starch, acetate, formate, propionate, methanol and ethanol. Autotrophic growth on
232 H₂/CO₂ (80:20) was also not observed. Yeast extract was also successfully utilized as a
233 sole carbon and energy source for H₂ production.

234 At a temperature of 70°C and pH 8.1, the fermentation of 0.55 mmol glucose
235 generated 1.05 mmol H₂, 1.2 mmol acetic acid, 0.1 mmol propionic acid, 0.04 mmol
236 ethanol and 0.02 mmol isovaleric acid. No lactic acid or butyric acid was detected.

237 **PCR-DGGE**

238 PCR-1 with *Bacteria*-specific primer resulted in clear amplification of the bacterial
239 16S rDNA (Figure 2a). PCR-2 resulted in amplification of the V3 region from the

240 bacterial 16S rDNA (Figure 2b). Two distinct bands were obtained in preliminary DDGE
241 analysis of the enriched mixed culture (Figure 2c).

242 No *Archaea* were found in the enriched mixed culture. All attempts to amplify
243 *Archaea* 16S rDNA based on *Archaea*-specific primer with variations of PCR running
244 conditions, and Mg ion concentrations in the PCR mixture were not successful (data not
245 shown).

246

247 **16S ribosomal DNA gene sequence analyses**

248 Construction of 16S ribosomal DNA clone library and subsequent sequence analysis
249 revealed that the clones represented two community members (phylotypes), named as Ha
250 and Hb. A phylogenetic tree depicting relationship of community members with other
251 thermophilic and extreme thermophilic bacteria is presented in Figure 3. It shows that the
252 phylotypes Ha and Hb were related to species of the genera *Bacillus* and *Clostridium*,
253 respectively. The closest relatives to Ha and Hb were *Bacillus thermozeamaize* AY288912
254 and *Clostridium* sp. IrT-R5M2-31 respectively with 99 % sequence similarity in both
255 cases.

256

257 **FISH image analysis**

258 The FISH observations (Figure 4) showed the presence of *Bacillus* sp and *Clostridium* sp
259 in the mixed culture which was consistent with the taxonomic affiliation of the individual
260 members obtained by PCR-DGGE and 16S ribosomal DNA sequence analysis. All cells
261 were equally distributed and no cell aggregation structures were observed. No additional

262 minority microorganisms were detected. Relative abundances of *Bacillus* sp. and
263 *Clostridium* sp. were 87 ± 5 % and 13 ± 5 % respectively.

264

265

266 **DISCUSSION**

267 In the present study an extreme thermophilic, strict anaerobic, chemo-organotrophic,
268 bacterial mixed culture with H₂-producing ability was enriched for the first time from
269 digested household waste. All attempts of microbial isolation failed considering cell
270 extracts and cultural supernatants as sources of growth stimulating compounds as
271 reported in other studies for isolation of thermophiles (Ohno *et al.* 2000; Rhee *et al.*
272 2000). Most probably colony formation was not possible under the isolating conditions,
273 viz. heating of the medium up to 90°C to melt before inoculations, or the solidifying
274 agent itself (Koser 1948).

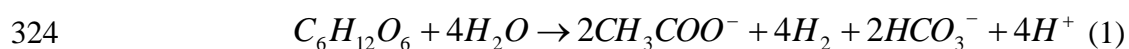
275 Similar extreme thermophilic and hyperthermophilic (growing above 80°C)
276 microorganisms were previously enriched and isolated from high-temperature natural
277 environments like hot springs or habitats associated with volcanic activity (Daniel 1992)
278 and from ambient-temperature habitats as cold seawaters (Stetter *et al.* 1993). Several
279 studies reported extreme thermophiles and hyperthermophiles isolated from man-made
280 biotopes such as Uranium mine (Fuchs *et al.* 1995), hot tap water environments (Brock
281 1997), smoldering coal refuse piles and geothermal power plants (Stetter 1996). Only one
282 hyperthermophilic bacterium, *Dictyoglomus* (Mathrani and Ahring 1992) isolated from
283 paperpulp cooling tank, was reported to produce low amounts of H₂. This microorganism
284 was used only to demonstrate possibility for thermophilic production of ethanol from

285 xylan. Until now, no extreme thermophilic H₂ producers have been enriched from
286 anaerobic digesters treating organic wastes. As already adapted to degrade organic
287 household waste, these microorganisms can find variety of practical applications for
288 hydrogen production combined with environmental depollution under extreme
289 thermophilic conditions.

290 Based on 16S ribosomal DNA sequence analysis, the enriched mixed culture
291 described here consisted of two members of the *Bacteria* phylum, Firmicutes- one
292 phylogenetically related to *Bacillus* and the second related to *Clostridium*. In general,
293 *Clostridium* sp. and *Bacillus* are known to be Gram positive however, certain strains like
294 *Clostridium hathewayi* (Allen *et al.* 2003; Elsayed and Zhang 2004) and *Bacillus*
295 *massiliensis* (Glazunova *et al.* 2006) have been reported to be Gram negative. It has been
296 observed that fixing and Gram staining of anaerobic bacteria in the absence of oxygen
297 (anaerobic chamber) provides improved staining results and more accurate presumptive
298 identification of obligate anaerobes (Johnson *et al.* 2005). Therefore, to confirm the same,
299 the staining was performed under strict anaerobic conditions in an anaerobic gloveless
300 chamber. However, there was no difference in Gram reaction obtained under aerobic and
301 anaerobic conditions. FISH results confirmed microbial community composition obtained
302 by PCR-DGGE and clone library. Relative quantitative analysis based on FISH image
303 (Figure 4) clearly revealed that *Bacillus* dominated over *Clostridium* in the mixed culture.
304 As dominant community member, *Bacillus* sp was likely the support of hydrogen
305 production by either direct involvement or more likely through interactions with
306 *Clostridium*,sp. under extreme thermophilic conditions. Hydrogen production from pure
307 cultures of mesophilic *Bacillus* sp. (Kotay and Das, 2007) and moderate thermophilic

308 *Clostridium sp.* (Freier *et al.* 1988) has already been reported. However, to our
309 knowledge, H₂ production from extreme thermophilic strains belonging to either of these
310 genera coexisting in a mixed culture, has never been recorded. Only hydrogen production
311 by mesophilic *Clostridium sp* in mixed culture with *Enterobacter sp* was reported till now
312 (Harihuko *et al.*, 1998). Furthermore, cellulose degradation and lactate production by the
313 enriched culture were not observed unlike previous reports. Also a high 16S ribosomal
314 DNA sequence similarity (99-93 %) of the enriched mixed culture members was
315 observed with several uncultured extreme thermophilic bacteria, accentuating the fact that
316 the those culture members are novel.

317 With regard to the physiology, the growth rates and the optimal pH and temperature
318 ranges of the enriched mixed culture were in accordance with data reported for other
319 extreme thermophiles (Mladenovska and Ahring 1995; Huang *et al.* 1998; Van Niel *et al.*
320 2002). Fermentation balance performed showed that acetate was the major fermentation
321 end product detected in the fermentation broth, approx. 2 mole of acetate per mole
322 utilized glucose was registered. Considering this following reaction for hydrogen
323 production from glucose was constructed, according to Claasen *et al.*, 1999:



325 Maximal theoretical hydrogen yield according to this reaction is 4 mole hydrogen per
326 mole glucose. However, a lower practical hydrogen yield (around 50 % from the
327 theoretical yield) and the lack of butyrate and lactate in the cultural broth could be
328 explained by the contingency of mixed fermentation pathways leading to synthesis of
329 more reduced metabolites, such as alanine (Kengen and Stams 1994).

330 The isolation and identification of fermentative H₂ producers with a high yield and
331 high production rate of H₂ are important for development of commercial sustainable
332 fermentative biohydrogen production process. Continuous biohydrogen production
333 system using a hyperthermophilic microorganism grown on define medium has already
334 been reported (Kanai *et al.*, 2005). However, extreme thermophilic biohydrogen
335 production from organic wastes has not been developed yet. Although hydrogen
336 production rates and yields reported in this study are not high compared with previous
337 studies of extreme thermophiles (Van Niel *et al.*, 2002; van Ooteghem *et al.*, 2004), the
338 enriched mixed culture described here can be applied to assist start-up of biohydrogen
339 reactors treating organic waste at similar conditions as the optimum conditions for growth
340 of the enriched mixed culture.. Another possible application would be immobilization of
341 the enriched mixed culture in reactor systems for H₂ production at high temperatures
342 from a variety of complex preheated substrates such as hot hydrolysates from energy
343 crops pretreatment.

344

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351

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494 **Legend to figures**

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496 Figure 1. (a) Effect of temperature on growth of the enriched mixed culture. (b) Effect of
497 pH on the growth of the enriched mixed culture. Error bars represent standard deviations
498 from triplicate analyses.

499 Figure 2. a) PCR-1: arrow indicates ~1.5kb 16S ribosomal DNA amplicons b) PCR-2:
500 arrow indicates ~200bp amplicons corresponding to the V3 region tagged with 30bp GC
501 clamp, Lane M corresponds to the 1kb DNA ladder c) DGGE: Lane 1- Control DNA
502 from pure culture of *Bacillus coagulans* IIT-BT S1, Lane 2- PCR-2 product obtained
503 from enriched mixed culture, arrows indicate two distinct bands.

504 Figure 3. Phylogenetic relationships of the enriched mixed culture members Ha and Hb
505 and their closely related sequences described in the literature. The tree was built using a
506 neighbor-joining method. Numbers at nodes represent the bootstrap values above 50%,
507 for 100 bootstrap calculations. The scale bar represents 0.180 substitutions per
508 nucleotide. Accession numbers of sequences are indicated.

509 **Fig. 4.** Fluorescent In Situ Hybridization microscopic observations of a 20 times
510 concentrated enriched mixed culture. The sample was hybridized with the FITC-labelled
511 Firmicutes probe LGC354deg, and the Cy3-labelled *Clostridium* probe CLOS621. All
512 Firmicutes bacteria appeared in green and more specifically red-coloured *Clostridium* sp.
513 appeared in yellow. Bar indicates 5µm.

514

515 Table 1. Maximal growth rates on different substrates.

Substrate	Maximal growth rate (h⁻¹) ± SD^a
Glucose	0.13 ± 0.06
Galactose	0.075 ± 0.003
Mannose	0.05 ± 0.002
Maltose	0.05 ± 0.002
Sucrose	0.075 ± 0.004
Arabinose	0.00625 ± 0.0003
Xylose	0.025 ± 0.001
Glycerol	0.05 ± 0.0025
Pyruvate	0.021 ± 0.001

516 ^aStandard deviations were based on triplicate analyses

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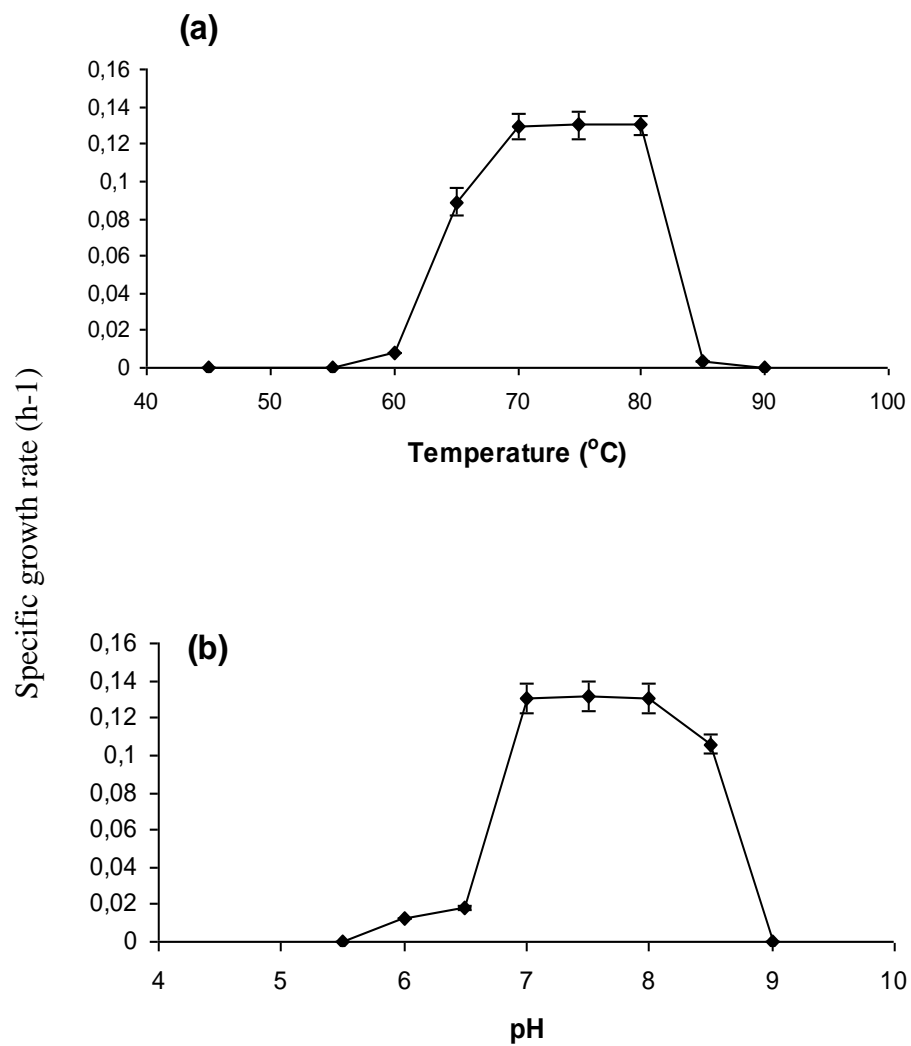
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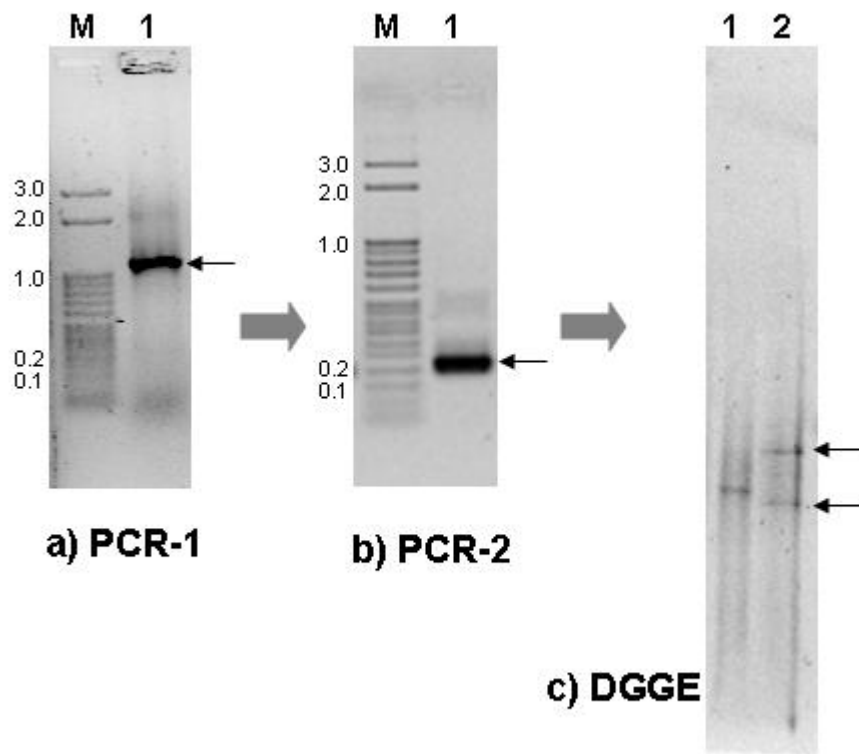
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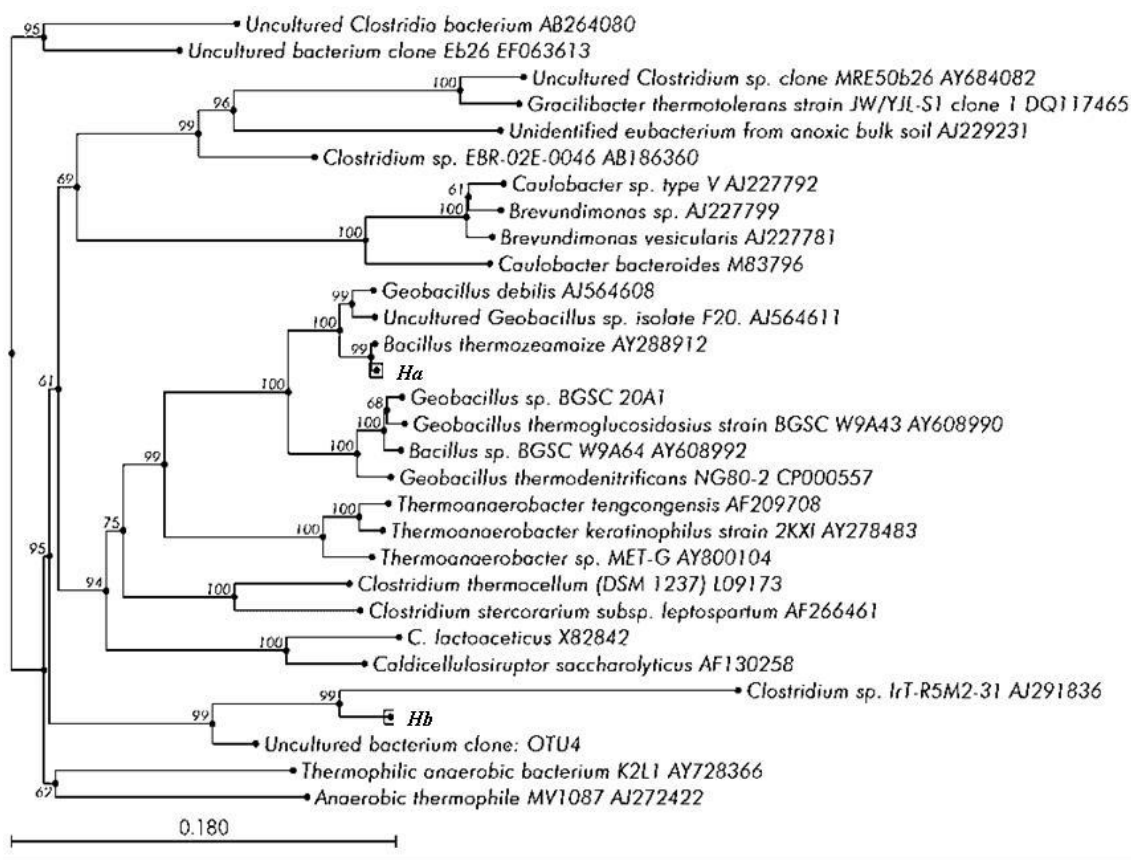
530 Figure 1.



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532 Figure 2.

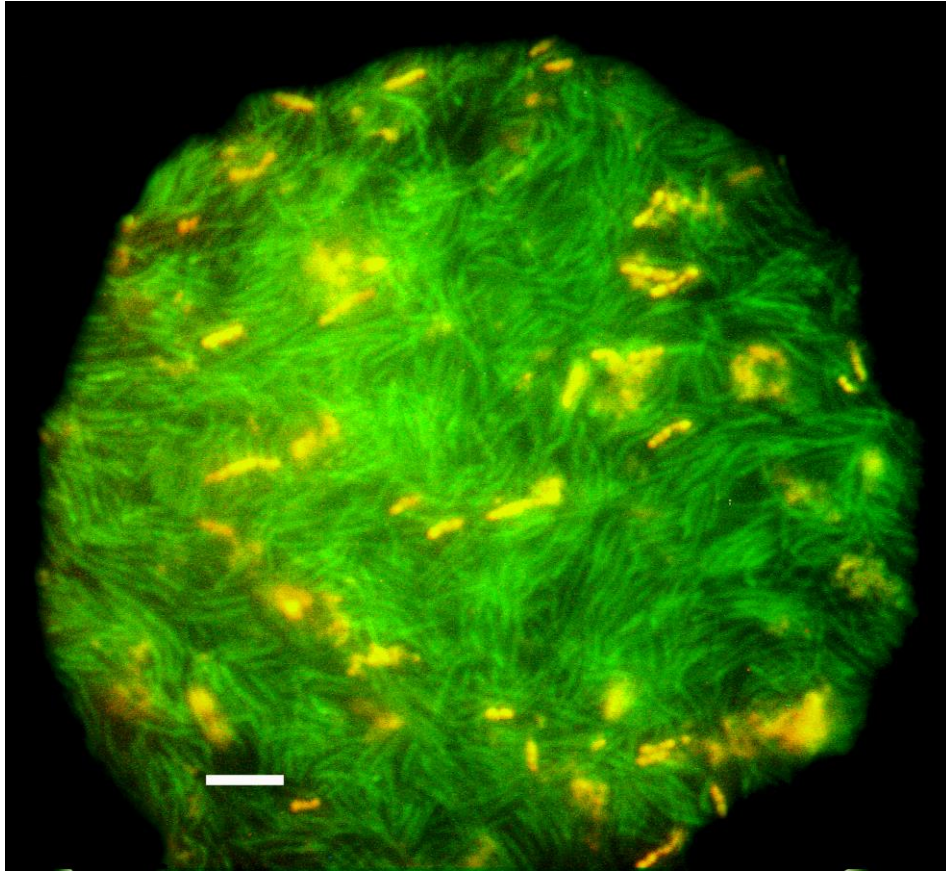
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535 Figure 3.

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538 Figure 4.

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