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Extreme thermophilic biohydrogen producers

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Abstract

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

Methods and Results: A strict anaerobic extreme thermophilic hydrogen producing bacterial culture was enriched from a lab-scale digester treating household wastes at 70^{o}C. The enriched mixed culture consisted of two rod-shaped bacterial members growing at optimal temperature of 80^{o}C and optimal pH 8.1. The culture was able to utilize glucose, galactose, mannose, xylose, arabinose, maltose, sucrose, pyruvate and glycerol as carbon sources. Growth on glucose produced acetate, hydrogen and carbon dioxide. Maximal hydrogen production rate on glucose was 1.1 mmol..$L^{-1}.h^{-1}$ with a maximum hydrogen yield of 1.9 mole H_2 per mole glucose. 16S ribosomal DNA clone library analyses showed that the culture members were phylogenetically affiliated to the genera *Bacillus* and *Clostridium*. Relative abundance of the culture members, assessed by Fluorescence *In Situ* Hybridization (FISH), were 87 ±5 % and 13 ±5 % for *Bacillus* and *Clostridium*, respectively.

Conclusions: An extreme thermophilic, strict anaerobic, mixed microbial culture with H_2-producing potential was enriched from digested household wastes.

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

Keywords: hydrogen, extreme thermophilic, household wastes

INTRODUCTION

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

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

risk of contamination by non-H_2 producing mesophilic microbes (Van Niel *et al.* 2002; Kanai *et al.* 2005). The present knowledge about biological hydrogen production at high temperatures is limited to studies on pure cultures utilizing defined carbon sources for H_2 synthesis (van Ooteghem *et al.* 2004; Kanai *et al.* 2005). In these studies, the H_2-producing microorganisms were isolated from natural hot environments. Very little information is available about H_2 producing potential of extreme thermophiles (growing between 55^{o}C and 80^{o}C) in hot artificially created habitats, such as laboratory reactors digesting organic wastes. Kotsopoulos *et al.*, 2005 previously reported that dairy manure predigested at 55^{o}C was used as inoculum in a granular upflow anaerobic sludge blanket reactor (UASB) for biological H_2 production at 70^{o}C. No extreme thermophilic H_2 producing culture derived from artificial anaerobic environments has been isolated, characterized and identified hitherto. The identification of such cultures and subsequent characterization of growth properties may result in the development of potentially important biotechnological processes, such as direct fermentation of hot effluents (hydrolysates) derived from thermal pretreatment of energy crops (plants grown specifically for production of bioenergy) and crop residues (Klinke *et al.*2002). The utilization of such hot hydrolysates for biohydrogen production not only circumvents the need for prior cooling, but also saves considerable amounts of energy. This will lead to more sustainable biological H_2 production.

The aim of this work was to enrich, identify and characterize a strict anaerobic extreme thermophilic microbial culture with H_2-producing ability, using digested household solid waste as inoculum.

MATERIALS AND METHODS

Enrichment

Basal anaerobic (BA) medium was used for enrichment and routine cultivation as described previously (Angelidaki et al. 1990), except that no L-cysteine hydrochloride was added, and the concentration of $Na_2S.9H_2O$ was increased to 0.25 $g.L^{-1}$. The medium was supplemented with sterile solutions of glucose and yeast extract to final concentrations of 0.25 % (w/v) and 0.05 % (w/v), respectively. 40 mL medium was then dispensed in 100 mL serum bottles inoculated with 4 ml effluent from H_2 producing laboratory-scale continuously stirred tank reactor treating household solid wastes (HSW) at 70°C. HSW corresponded to a heterogeneous mixture of food residues, papers, garden wastes, plastics, stones, and metals as described in Liu *et al.* (2006). The reactor had been operated at 2 days solid retention time and organic loading rate of 11 g volatile $solids.L^{-1}.d^{-1}$ at 70°C for approx. one year, and was originally inoculated with the effluent of a mesophilic (38°C) full-scale biogas plant treating a mixture of wastewater sludge and municipal solid wastes (Grindsted, Denmark). After inoculation, the serum bottles were incubated in dark at 70°C until H_2 production and glucose consumption ceased. Six successive transfers of the enriched mixed culture were performed until steady hydrogen production rates were achieved.

Isolation

Isolation experiments were carried out anaerobically at 70°C using the roll tube technique (Hungate 1969). Dilutions of the enriched mixed cultures were transferred into fresh BA medium containing 2.5 g glucose L^{-1} and solidified with 11 g Gelrite L^{-1} and 1 g $MgCl_2$ L^{-}

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

Preparation of cell extracts.

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

Preparation of cultural supernatant.

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

Analytical methods.

Gas chromatography (GC) was used for identification of the fermentation products. Flame-ionization detection was used for volatile fatty acids and ethanol according to Sorensen *et al.* (1991). H_2 concentration in the gas phase was measured using a gas chromatograph (MicroLab, Arhus, Denmark) equipped with a s-m stainless column packed with Porapak Q (50/80 mesh) and coupled to a thermal conductivity detector (TCD). Lactate was quantified by high-pressure liquid chromatography as previously described (Hörber et al., 1998). Growth rates were determined by measuring culture

turbidity (OD_{578}) with glucose as sole substrate. Glucose was measured by spectrophotometry using the phenol-sulphuric acid method (Dubois *et al.* 1956).

Physiological tests

Prior to the determination of pH and temperature optima, the enriched mixed culture was adapted to each temperature and pH by transferring the enriched mixed culture three times into fresh BA medium supplemented with glucose. The effect of different incubation temperatures (55, 60, 65, 70, 75, 80, 85 and 90 ^{o}C) on H_2 production was 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 H_2 production was investigated at 70^{o}C. The final pH of the media was measured at the experimental temperature (70^{o}C). To determine the spectrum of substrate utilization, several carbohydrates (soluble starch, cellulose, maltose, sucrose, seduheptulose, mannose, galactose, xylose, arabinose, erythrose), organic acids (formate, acetate, propionate, butyrate, pyruvate) and alcohols (methanol, ethanol, glycerol) were investigated in BA medium. All substrates were added from sterile anaerobic stock solutions to reach a final concentration of 0.25 % (w/v).

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

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

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

PCR-DGGE analysis.

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

and Bacteria specific primer 7-f were used (Lane 1991). For *Archaea,* 1492-r and Archae-specific primer A109-f were used (Grosskopf et al, 1998). PCR was performed in a 50µl (total volume) reaction mixture containing 50mM KCL, 20mM Tris-HCl (pH 8.4), 5 mM $MgCl_2$, each deoxinucleotide triphosphate at a concentration of 200µM, 1 µl of *Taq* polymerase (2 U/µl; Sigma), 10 pmol of each primer, and 1µl of DNA extract. The thermal cycling program used for amplification was as follows: pre-denaturation at 95 ^oC for 5 min; 25 cycles of denaturation at 95^oC for 30 s, annealing at 52^oC for 40 s, elongation at 72^oC for 90 s, and post-elongation at 72^oC for 5 min. The reactions were subsequently cooled to 4 ^oC. The size of the amplicon was estimated on 1.5 % agarose gel. The amplicon was then used as DNA template for the subsequent PCR (PCR-2) to incorporate a GC clamp in the DNA fragment prior to DGGE (Muyzer and Smalla, 1998). For *Bacteria*, primer 518-r and 357-f (with 40 bp GC clamp at the 5' end) (Muyzer et al., 1993) were used to amplify the ~200 bp fragment of the V3 region. The PCR program corresponded to 20 cycles of three steps: 94°C for 1 min, 65°C for 0.75 min, and 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 1 min followed by a final step at 72°C for 10 min. PCR products were stored at 4°C and analysed on 1.5 % agarose before DGGE. DGGE analysis of the amplicons obtained from PCR-2 was performed as previously described by Zoetendal et al. (2001), using the Dcode Universal Mutation Detection system (Bio-Rad, Hercules, CA) with 8% (v/v) polyacrylamide gels and a denaturant gradient of 30–60%. A 100 % denaturing solution was defined as 7M urea and 40 % formamide. Electrophoresis was performed for 16 h at 70 V in a 0.5x TAE buffer at 60^oC. DGGE gels were stained with SYBR Green for 15 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

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

Growth characteristics and physiology

The final enriched mixed culture could grow at temperatures ranging from 55 to 90 ^{o}C (pH 7.2) ((Fig 1a) and at pH between 5.5 and 9.0 (70°C) (Fig. 1b). Specific growth rate reached its maximum of 0.14 h^{-1} at temperatures between 70-80 ^{o}C (pH 7.2) and pH between 7-8 (70°C). H_2 production rate on glucose was 1.1 mmol H_2 $.L^{-1}.h^{-1}$ and the H_2 yield was 1.9 mol H_2 per mole glucose. The presence of yeast extract was not found to be essential but stimulated the growth.

The enriched mixed culture grew and produced H_2 on a wide variety of carbon sources including glucose, galactose, mannose, maltose, sucrose, arabinose, xylose, glycerol and pyruvate (Table1) but not on sedoheptulose, erythrose, cellulose, soluble starch, acetate, formate, propionate, methanol and ethanol. Autotrophic growth on H_2/CO_2 (80:20) was also not observed. Yeast extract was also successfully utilized as a sole carbon and energy source for H_2 production.

At a temperature of 70^{o}C and pH 8.1, the fermentation of 0.55 mmol glucose generated 1.05 mmol H_2, 1.2 mmol acetic acid, 0.1 mmol propionic acid, 0.04 mmol ethanol and 0.02 mmol isovaleric acid. No lactic acid or butyric acid was detected.

PCR-DGGE

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

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

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

16S ribosomal DNA gene sequence analyses

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

FISH image analysis

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

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

DISCUSSION

In the present study an extreme thermophilic, strict anaerobic, chemo-organotrophic, bacterial mixed culture with H_2-producing ability was enriched for the first time from digested household waste. All attempts of microbial isolation failed considering cell extracts and cultural supernatants as sources of growth stimulating compounds as reported in other studies for isolation of thermophiles (Ohno *et al.* 2000; Rhee *et al*. 2000). Most probably colony formation was not possible under the isolating conditions, viz. heating of the medium up to 90^{o}C to melt before inoculations, or the solidifying agent itself (Koser 1948).

Similar extreme thermophilic and hyperthermophilic (growing above 80^{o}C) microorganisms were previously enriched and isolated from high-temperature natural environments like hot springs or habitats associated with volcanic activity (Daniel 1992) and from ambient–temperature habitats as cold seawaters (Stetter et al. 1993). Several studies reported extreme thermophiles and hyperthermophiles isolated from man-made biotopes such as Uranium mine (Fuchs *et al.* 1995), hot tap water environments (Brock 1997), smoldering coal refuse piles and geothermal power plants (Stetter 1996). Only one hyperthermophilic bacterium, *Dictyoglomus* (Mathrani and Ahring 1992) isolated from paperpulp cooling tank, was reported to produce low amounts of H_2. This microorganism was used only to demonstrate possibility for thermophilic production of ethanol from

xylan. Until now, no extreme thermophilic H_2 producers have been enriched from anaerobic digesters treating organic wastes. As already adapted to degrade organic household waste, these microorganisms can find variety of practical applications for hydrogen production combined with environmental depollution under extreme thermophilic conditions.

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

Clostridium sp. (Freier *et al.* 1988) has already been reported. However, to our knowledge, H_2 production from extreme thermophilic strains belonging to either of these genera coexisting in a mixed culture, has never been recorded. Only hydrogen production by mesophilic *Clostridium* sp in mixed culture with *Enterobacter* sp was reported till now (Harihuko et all., 1998). Furthermore, cellulose degradation and lactate production by the enriched culture were not observed unlike previous reports. Also a high 16S ribosomal DNA sequence similarity (99-93 %) of the enriched mixed culture members was observed with several uncultured extreme thermophilic bacteria, accentuating the fact that the those culture members are novel.

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

$$C_6H_{12}O_6 + 4H_2O \rightarrow 2CH_3COO^- + 4H_2 + 2HCO_3^- + 4H^+ \quad (1)$$

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

The isolation and identification of fermentative H_2 producers with a high yield and high production rate of H_2 are important for development of commercial sustainable fermentative biohydrogen production process. Continuous biohydrogen production system using a hyperthermophilic microorganism grown on define medium has already been reported (Kanai *et al.*, 2005). However, extreme thermophilic biohydrogen production from organic wastes has not been developed yet. Although hydrogen production rates and yields reported in this study are not high compared with previous studies of extreme thermophiles (Van Niel *et al*., 2002; van Ooteghem *et al*., 2004), the enriched mixed culture described here can be applied to assist start-up of biohydrogen reactors treating organic waste at similar conditions as the optimum conditions for growth of the enriched mixed culture.. Another possible application would be immobilization of the enriched mixed culture in reactor systems for H_2 production at high temperatures from a variety of complex preheated substrates such as hot hydrolysates from energy crops pretreatment.

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Legend to figures

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

Figure 2. a) PCR-1: arrow indicates ~1.5kb16S ribosomal DNA amplicons b) PCR-2: arrow indicates ~200bp amplicons corresponding to the V3 region tagged with 30bp GC clamp, Lane M corresponds to the 1kb DNA ladder c) DGGE: Lane 1- Control DNA from pure culture of *Bacillus coagulans* IIT-BT S1, Lane 2- PCR-2 product obtained from enriched mixed culture, arrows indicate two distinct bands.

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

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

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

[a]Standard deviations were based on triplicate analyses

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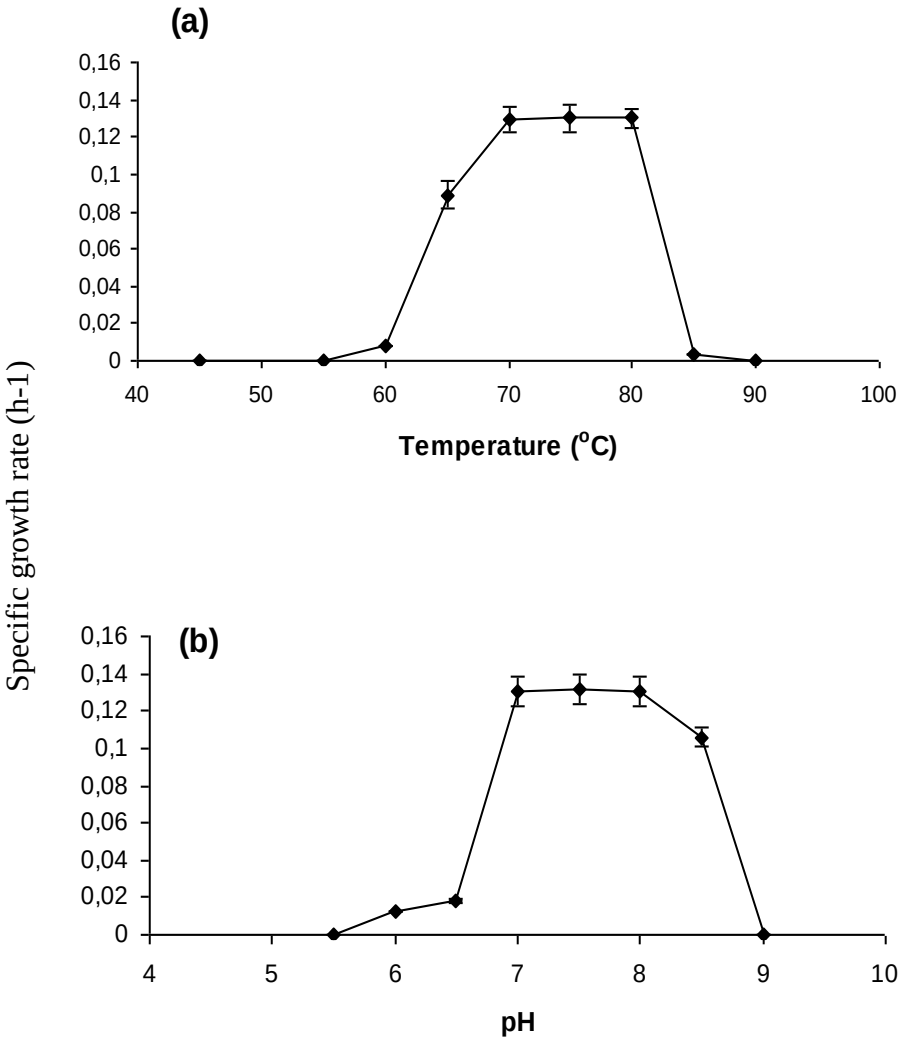
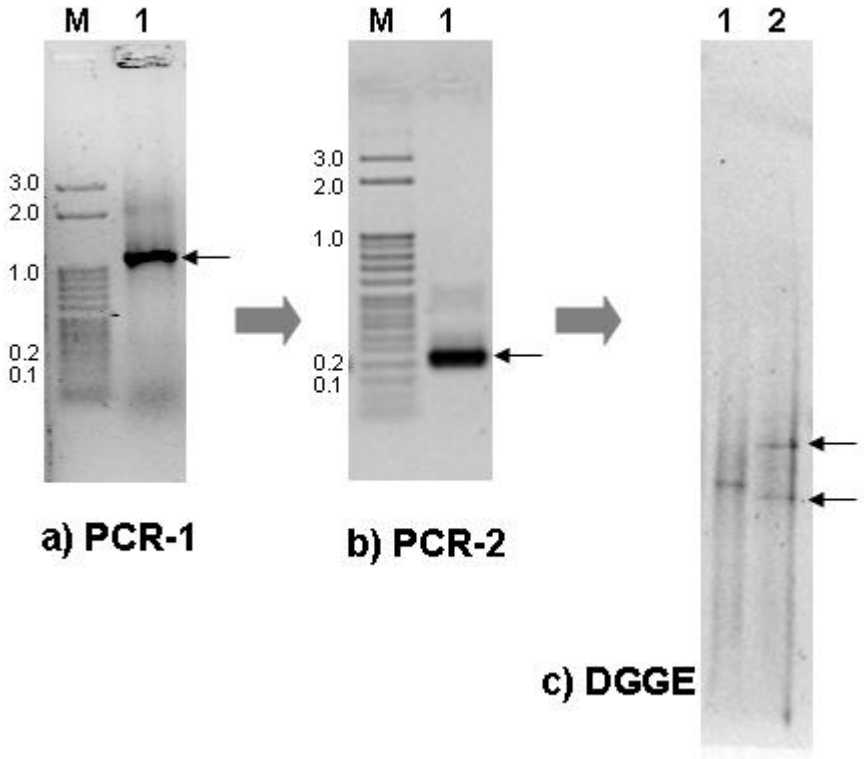


529

530 Figure 1.



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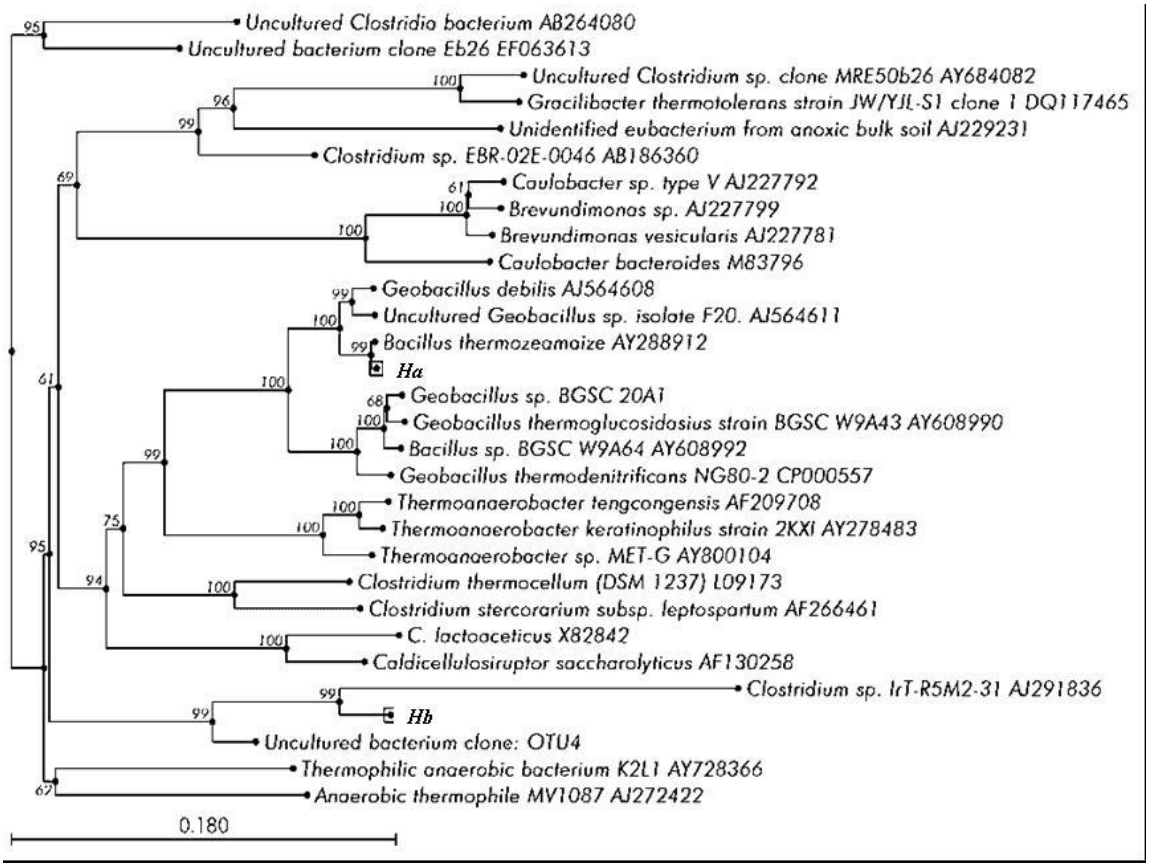
532 Figure 2.

533



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535 Figure 3.

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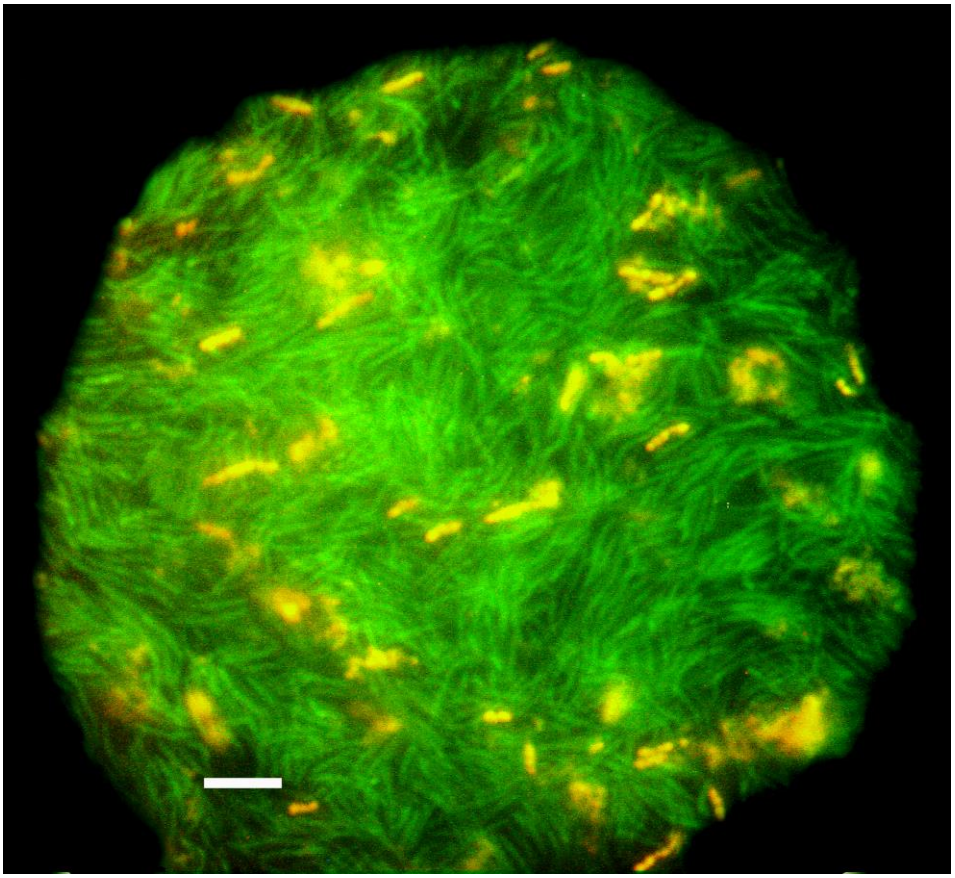

Figure 4.