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ISOLATION AND CHARACTERIZATION OF A NEW COPROTHERMOBACTER PROTEOLYTICUS STRAIN FROM A THERMOPHILIC ANAEROBIC DIGESTOR

IZOLACJA I CHARAKTERYSTYKA NOWEGO SZCZEPU BAKTERII (COPROTHERMOBACTER PROTEOLYTICUS) Z BEZTLENOWEJ, TERMICZNEJ KOMORY FERMENTACYJNEJ

The genus Coprothermobacter represents a very deep-branching phylum within the domain Bacteria with only two species described so far. Sequences of 16S rDNA closely related to C. proteolyticus have been repeatedly reported in thermophilic anaerobic digestion processes, either in manmade or natural environments. Unfortunately, little is known about the diversity and the ecology of the Coprothermobacter genus in thermophilic anaerobic digestion. It has been suggested that thiosulfate reduction plays an important physiological role in non-sulfate reducing members of the Thermotogales and Coprothermobacter orders, and that a thiosulfate shunt is responsible for the depletion in the hydrogen production in cultures of C. platensis and C. proteolyticus. Nevertheless, further studies are still needed to understand the pathways of thiosulfate reduction in these and other related bacteria. We isolated and identified a new thermophilic Coprothermobacter strain designated IT3. The new isolate shared 99 % 16S rDNA similarity and 90 ± 4 % DNA-DNA homology with Coprothermobacter proteolyticus BT^T. Thiocyanate production for strains IT3 and BTT were almost identical at 55°C, but superior to the activity for the thermophilic Coprothermobacter platensis strain 3RT. For every examined temperature, maximum specific growth rates on 20 mM thiosulfate were determined in liquid culture using anaerobic techniques. The temperature profile of hydrogen production was similar to the temperature profile of growth on thiosulfate implying that the hydrogen production was growth-associated. Strain IT3 showed the highest growth-curve potentials between 45 and 65°C at 20 mM thiosulfate. Similarly, strain IT3 exhibited the highest cumulative hydrogen productions for 24 hours in the absence of thiosulfate. For strain IT3 at thermophilic range of 55-65°C, the hydrogen production in the absence of thiosulfate increased with the maximum specific growth rate on thiosulfate. At 55°C, strain IT3 maintained a lower specific growth rate but a higher hydrogen production compared to strain BT^T. However, correlation between hydrogen production and thiosulfate reduction (determined as specific growth rate on 20 mM thiosulfate) revealed a constant surplus of 4 mmol hydrogen per litre of medium with strain IT3. Analysis of the Arrhenius plot in the temperature range of 55-65°C showed that the apparent activation energy for growth of strain IT3 was 67 kJ/mole against 134 kJ/mole for Coprothermobacter proteolyticus BT^T. These results suggest that strain IT3 might be better suited for hydrogen production.

1. Introduction

Anaerobic digestion can be very useful for the treatment of organic waste and the generation of energy. In the thermophilic range, at temperatures between 55 °C and 65 °C, the residence times can be shorter but the thermophilic process operation requires heating and its stability might depend on the temperature sensitivity of the microorganisms involved (Jördening and Winter 2005).

Coprothermobacter proteolyticus is a moderately thermophilic proteolytic bacterium that can grow at temperatures up to 75 °C with an optimum at 63 °C (Klingeberg et al. 1991; Kersters et al. 1993; Ollivier et al. 1985). Although *C. proteolyticus* was initially described as belonging to a deep-branching phylum within the domain *Bacteria* (Rainey and Stackebrandt 1993), analysis of its 16S rRNA gene revealed that it is related to *Thermoanaerobacter* sp. It is currently assigned to the family *Thermodesulfobiaceae* (Mori et al. 2003) within the order *Thermoanaerobacterales* and is the first sequenced genome from that family (Galperin 2008). Since the description of *Coprothermobacter platensis* (Etchebehere et al. 1998), there are only two species described so far in the genus *Coprothermobacter*. Sequences of 16S RNA gene closely related to *Coprothermobacter* sp. have been repeatedly found in manmade or in natural mainly thermophilic environments (Kawagoshi et al. 2005; Kersters et al.1994; Nazina et al. 2006; Tatara et al. 2008; Wrighton et al. 2008). Unfortunately, little is known about the diversity and the ecology of the *Coprothermobacter* genus in thermophilic anaerobic digestion. It has been suggested that thiosulfate reduction plays an important physiological role in non-sulfate reducing members of the *Thermotogales* and *Coprothermobacter* orders (Ravot et al. 1995), and that a thiosulfate shunt is responsible for the depletion in the hydrogen production in cultures of *C. platensis* and *C. proteolyticus* (Etchebehere and Muxí 2000). Studies on the microbial ecology of these organisms could beneficiate from the characterization of new isolates capable of using thiosulfate as an electron acceptor. Moreover, further studies are still needed to understand the pathways of thiosulfate reduction in these and other related bacteria.

In this paper, we report the isolation of a novel strain belonging to the genus *Coprothermobacter* from a thermophilic (55 °C) biogas plant treating dairy cow manure. This isolate designated strain IT3 was identified as a member of the *Coprothermobacter proteolyticus* species and was compared to the type strain, showing improved hydrogen production at corresponding growth rates on thiosulfate.

2. Materials and methods

2.1. Bacterial strains

C. proteolyticus BT^T (DSM 5265), *C. platensis* $3R^T$ (DSM 11748) were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany).

2.2. Media

Prereduced media were prepared using Hungate technique and autoclaved for 15 min at 121 °C (Miller and Wolin 1974). The following media from DSMZ were used during this study: medium 481 (*C. proteolyticus*) and medium 834 (*C. platensis*). Strain IT3 was maintained under N_2 atmosphere at 55 °C in the basal medium (BM) from the *Dictyoglomus* medium 388 with the following modifications (per liter): 1g polypeptone, 1g yeast extract, and without soluble starch and sodium bicarbonate, and the initial pH was adjusted to 7.2 using 1M NaOH. The modified PY medium was prepared without vitamin K_1 (Gerhardt et al. 1994). The BCYT medium, a basal medium with carbonate, yeast extract (0.5 g/l) and trypticase (0.5 g/l), was prepared under N_2:CO_2 with a ratio 80:20 (Etchebehere et al. 1998). Sodium sulfate and sodium thiosulfate were added from anaerobic filter sterilized solutions to a final concentration of 20 mM.

2.3. Enrichment and isolation

A digestion sample from a thermophilic biogas plant treating dairy cow manure in Obihiro University of Agriculture and Veterinary Medecine (Hokkaido, Japan) was anaerobically incubated at 55 °C while methanogenesis was being inhibited with a final concentration of 10 mM of sodium 2-bromoethanesulfonate. The subsequent enrichment was monthly transferred at 55 °C into fresh BM by using 10% (v/v) inocula and 2.5g/l of lignin organosolv (Aldrich) which was removed before the isolation and purification steps in BM. Isolation was conducted in roll tubes in solidified BM with agar (1.5%, w/v) and incubated at 55 °C. After one week incubation, colonies were picked and reisolated three times in agar-shake tubes. A representative purified strain IT3 was grown under N_2 atmosphere, in BM at 55 °C for further characterization.

2.4. Microscopic examination

Cell morphology and arrangements were examined under microscope (Olympus BH-2). Microphotographs were taken after gram-staining was applied (Gerhardt et al. 1994).

2.5. Thiocyanate assay

The formation of thiocyanate from cyanide and thiosulfate was tested by colorimetric measurement after incubation at 55 °C of the reaction mixture in cell suspensions (Ravot et al. 2005). All the strains were precultured for two days in their respective medium. Measurements were performed with a spectrophotometer uv-visible mini-1240 (Shimadzu). One U corresponded to 1 μmol thiocyanate produced per minute. Single determinations were performed.

2.6. Growth experiments

The growths of strains IT3 and BT^T on thiosulfate were followed in unstirred BM (10 ml) supplemented with 20 mM thiosulfate in 20 ml vials, using 5 % (v/v) inocula from one-week old cultures in BM (OD at 660 nm about $\leq$0.3 and $\geq$0.4 for strain BT^T and strain IT3, respectively). The results were means of triplicate vials and the growth curves were obtained from parallel experiments. Optical density measurements were performed at 660 nm with direct insertion of the vials in a uv-visible 1200 spectrophotometer (Shimadzu). Negative controls without inoculum were prepared in duplicate. Sulfate reduction was also tested in BM at 20 mM.

The modified Gompertz equation was used to describe changes of microbial numbers with time; it is one of the most used empirical models providing interpretable growth parameters, and it is well suited for batch culture since exponential growth cannot occur indefinitely (Grijspeerdt and Vanrolleghen, 1999). The modified Gompertz equation was fitted to the growth curves by nonlinear regression with CurveExpert 1.4 and the following parameters were determined: the maximum specific growth rate μ_{max} (h^{-1}), the asymptotic growth potential P or maximum value reached, and the lag time λ (h) (Zwietering et al. 1990):

$$Y = P \exp\{-\exp[\mu_{max} e(\lambda - t)/P + 1]\} \quad (1)$$

where $Y = LN[(OD-OD')/OD_O]$ (2) represents the logarithm of the bacterial cell concentration (assuming the ODs are proportional to the bacterial cell number), with OD and OD_O respectively the actual and initial optical densities for the strain growing in the 20 mM thiosulfate medium, and OD′ the optical density of the medium (negative control) ; t is the incubation time (h); e is the base of natural logarithm (LN).

Hydrogen sulfide production from thiosulfate was also tested in BM (Gerhardt et al. 1994). Hydrogen produced after one day was measured in 30-ml Hungate tubes (Sanshin Kogyo Co. Ltd., Yokohama, Japan) containing 9 ml of modified PY medium and inoculated with 1 ml of a 24-hours preculture in modified PY medium, in water baths at different temperatures.

2.7. Analytical procedures

The headspace hydrogen and carbon dioxide contents were measured by gas chromatography using a gas chromatograph GC-8A with a thermal conductivity detector (Shimadzu) equipped with a Unibeads C 60/80 column (GL Sciences) and argon as the gas carrier.

2.8. Amplification and sequence analysis of 16S rRNA gene

DNA from the strain IT3 was obtained by repeated freeze-thaw and purification by ethanol-sodium acetate precipitation. The ribosomal 16S rRNA gene was amplified by PCR using the following universal primers: 27F (5'-AGAGTTTGATCMTGGCTCAG-3'), corresponding to positions 8-27 in forward *Escherichia coli* numbering, and 1492R (5'-TACGGYTACCTGTTACGACTT-3'), corresponding to positions 1513-1492 in reverse *E. coli* numbering. The following temperature cycles were performed: 95 °C for 15 min, 35 cycles of 94 °C for 60 s, 53 °C for 60 s, 72 °C for 2 min, followed by final incubation for 20 min at 72 °C. The PCR products were purified as above and sequenced

on an Applied Biosystems 3100 DNA sequencer. After the base calling was examined (Sequence Scanner v1.0, Applied Biosystems, Inc.), a contiguous consensus sequence was obtained for strain IT3 (TIGR Assembler 2.0). The region of the consensus sequence from the beginning up to the 27F primer region was then manually removed (about 300 bp). Finally, the sequence was 1386 bp long, corresponding to a segment between positions 25 and 1411 of the 16S rRNA gene of *E. coli*.

2.9. DNA-DNA hybridization

Genomic DNA was isolated by cetyltrimethylammonium bromide (CTAB) lysis (Ausubel et al. 2002). DNA-DNA hybridization was evaluated by slot blot using the DIG labeling system (Roche Diagnostics GmbH) according to the method of Hänninen et al. (1991). The intensity of hybridization in each application area was determined at different dilution ratios using the public software ImageJ 1.38x. DNA homology was calculated as a weighted average of the serial dilution intensities.

3. Results

3.1. Identification

3.1.1. DNA-DNA hybridization

DNA similarity between strains IT3 and BT^T was 90 ± 4 % (n=3) using genomic DNA of BT^T as a template for probe. The reciprocal evaluation with IT3 as a probe showed averages ranging from 85 % to 92 %.

3.1.2. Phylogenetic analysis

BLAST comparison with rDNA sequences available in databases revealed that strain IT3 (GenBank accession No. GU363592) was closely related to *C. proteolyticus* strain BT^T (genome GenBank accession No. CP00145.1) and to *C. platensis* $3R^T$ with 99 % and 94 % similarities respectively.

3.2. Microscopic examination

Cells of strain IT3 grown in BM medium were straight rods, occurring singly or in pairs at 55 °C and 60 °C. Young cultures of strain IT3 stained gram-negative, as well as of strain BT^T. During the growth of strains IT3 and BT^T in modified PY medium, long filaments appeared at the temperature range between 60 °C and 65 °C (Fig. 1). In our conditions, we were not able to observe neither growth activity nor viable cells when *C. platensis* was inoculated in modified PY medium.

3.3. Thiocyanate assay

All the strains were previously grown with 20 mM thiosulfate, and the enzymatic activity, reported by volume and normalized using optical density (cell density) , corresponded to 0.770 ± 0.013, 0.881 ± 0.015, 0.858 ± 0.015 U/ml per OD_{660} respectively for *C. platensis*, strain IT3, *C. proteolyticus*. These results show that thiocyanate productions of strain IT3 and BT^T, while similar to each other, were significantly higher to the value of *C. platensis*.

3.4. Growth experiments

Growth of strains IT3 and BT^T was followed as an increase in optical density in BM medium with 20 mM thiosulfate. The temperature profile of hydrogen production was similar to the temperature profile of growth between 55 °C and 65 °C, implying that the hydrogen production was growth-associated.

Strains IT3 and BT^T were able to reduce thiosulfate into hydrogen sulfide. No significant differences in growth were observed in BM supplemented with sulfate and in the control cultures lacking sulfate, indicating that both strains did not utilize sulfate as an electron acceptor. At 55 °C in BCYT, the hydrogen concentration in the headspace decreased from ca. 50% to approximately 8% after 14 days when 20 mM of thiosulfate were included for each strain, that is, approximately 84% diminution of the hydrogen concentration in the headspace (data not shown).

Growth curves were fitted by the modified Gompertz equation at different temperatures. The values of μ_{max}, P and λ determined by best fitting the optical densities are reported for both strains in Tables 1 and 2 respectively. The coefficients of correlation R^2 indicated a strong correlation between the experimental data and the fit and random normal distribution of residuals of less than 5 % was demonstrated by the calculation of values D of Kolmogorov-Smirnov test (NIST Dataplot^{TM}). Strain IT3 usually grew faster and to a higher optical density than strain BT^T except at 55 °C and 70 °C as respectively suggested by the values of μ_{max} and P.

When grown on 20 mM thiosulfate in BM at 45 °C, strain BT^T exhibited a very long lag phase of about 5 days, but only a few hours for IT3 (Tables 1 and 2). The effect of the temperature on the growth on thiosulfate revealed two different profiles. In strain IT3, the maximum specific growth rate was increasing monotonically with the temperature, while strain BT^T showed a peak at 55 °C followed by a decrease at 60 °C and finally increased with the temperature.

In modified PY medium lacking thiosulfate, the effect of temperature on the hydrogen production was also studied for both strains. Hydrogen production for strain IT3 was higher and increased from 55 °C up to 65 °C, which is consistent with the reported optimal temperature of 63 °C for *C. proteolyticus* (Ollivier et al. 1985). Hydrogen production for strain BT^T significantly decreased at 60 °C but increased afterwards until it reached a plateau at 70 °C while the hydrogen content for strain IT3 continued to decrease from 65 °C to 75 °C. In our hands, the thermophilic strain *C. platensis* $3R^T$ did not actively produced hydrogen presumably because of cell lysis at this temperature range. Morphological observations in modified PY medium showed that filaments of strain BT^T already appeared at 60 °C but filaments of strain IT3 were rather absent at this temperature. Filamentation occurred in both strains IT3 (Fig. 1) and BT^T at 60-65 °C

accordingly to the reported optimal growth temperature for *C. proteolyticus* (Brock and Freeze 1969; Ferroni and Inniss 1974; Ollivier et al. 1985).

Correlation of the metabolic rate during thiosulfate reduction with the hydrogen production without thiosulfate revealed an additional production of ca. 4 mmol/L of molecular hydrogen in modified PY medium inoculated with strain IT3 in the temperature range of 55 °C-65 °C (Fig. 2).

4. Discussion

Strain IT3 shared 98 % identity based on an early 16S rRNA gene sequence of *C. proteolyticus* strain BT^T (Rainey and Stackebrandt 1993). This finding prompted us to check for DNA-DNA relatedness between these two strains (Stackebrandt and Goebel 1994). The values were in good agreement with the analysis of Goris and collaborators (2007) on the correlation between DNA-DNA hybridization values and 16S rRNA gene sequence identities of a pairs of strains belonging to the same species. These authors showed that within very close similarity based on 16S rDNA (>97 %), one could except to unravel the diversity within a particular microbial species. Based on their conclusions and a careful analysis of the phylogenetic data, strain IT3 could represent an important element of diversity within the *Coprothermobacter* genus in general, and within the *C. proteolyticus* species in particular.

In order to further reveal the particularity of strain IT3, growth analysis on thiosulfate was conducted at different temperatures. These data (Tables 1 and 2) allowed us to deduce the activation energies using the Arrhenius plot. Hence, between 45 °C and 65 °C, strain IT3 required an activation energy of 67 kJ/mol, which is in the range of the activation energies measured for the psychrophilic sulfate-reducing bacteria (Isaksen and Jørgensen 1996; Knoblauch and Jørgensen 1999). Although not apparent, further analysis retrieved a constant activation energy of 134 kJ/mol for strain BT^T if one ignored that the Arrhenius plot shifted down at 60 °C coinciding with early filamentation of strain BT^T at this temperature, which could indicate that growing cells of strain BT^T needed more energy in order to double their population. Although twice the activation energy needed for strain IT3, the activation energy for strain BT^T was still in the range of the "effective" activation energy for growth $U_g \approx 110$ kJ/mol described by Price and Sowers (2004). At the currently high nutrient level (Odum 1971), the difference in activation energies is consistent with the idea that individual cells preferentially grow bigger before they divide if the proper nutrient leading to filamentation (Janssen 1991) is available and/or a sensor responsible for the regulation of growth is activated (Weart 2007). Thus, strain BT^T seemed to show an energetic load on both hydrogen production and metabolic rates whereas strain IT3 seemed to favor catabolic activities over biomass yield (Fig. 1) (Von Stockar et al. 2006).

Overall, the hydrogen production was proportional to the thiosulfate reduction. In our study, thiosulfate reduction was associated with the thiocyanate production and the depletion in hydrogen production. Substrate specificity towards thiosulfate during thiocyanate production is related to a rhodanese-like activity in the absence of sulfate reduction (Ravot et al. 2005). Transcriptional analysis of biofilm formation processes in *Thermotoga maritima* revealed that a cysteine desulfurase (SufS) gene was up-regulated in iron-sulfur cluster assembly (Pysz et al. 2004). SufS that belongs to the aspartate aminotransferase superfamilly of pyridoxal 5′-phosphate-dependent enzymes catalyzes

the decomposition of L-cysteine to L-alanine and sulfane sulfur via the formation of an enzyme-bound persulfide intermediate (Mihara and Esaki 2002). Tatara and co-workers (2008) showed that the population of *Coprothermobacter* sp., along with the population of methanogenic archea, was principally detected in the biofilm fraction of a thermophilic anaerobic packed-bed reactor. Evidence of direct transfer of the sulfane sulfur on SufS to a rhodanese has been also provided and it seems that the transfer occurs by covalent bond with rhodanese (Forlani et al. 2005). At least, one putative SufS (GenBank accession No. ACI17047.1) has been identified in the genome of strain BT^T. A rhodanese catalytic motif (residues 202-207, CVPGHK) is present in the binding pocket of this SufS enzyme and shares the same SufS catalytic lysine 207 residue. The primary cellular substrate of SufS is cysteine, but we could consider a putative rhodanese activity involving the binding of thiosulfate at the binding pocket. No rhodanese gene is currently found in the *C. proteolyticus* genome.

The sulfur from thiosulfate could be mobilized via a persulfide at the first cysteine residue and the subsequent sulfurated form could transfer *in vitro* the sulfur to cyanide. Indeed, the thiocyanate production was comparable to the activity of the rhodanese-like enzyme of *Halanaerobium congolense* determined by Ravot and co-workers (2005). It has also been demonstrated that positive charge at the last amino acid greatly increased the thiosulfate sulfurtransferase activity (Krepinsky and Leimukühler 2007). Therefore, we also speculate that the positive lysine residue activated during cysteine desulfuration plays a similar role in the *Coprothermobacter* genus. Whether the above proposed mechanism in SufS is operational in members of the *Coprothermobacter* genus has yet to be investigated, but SufS offers an important target to elucidate both thiocyanate production and hydrogen depletion since it probably participates to sulfur mobilization required for proper cellular function. Hence, one could assume that these two phenotypes are formally described by equations (3) and (4), respectively.

$$S_2O_3^{2-} + CN^- \leftrightarrow SCN^- + SO_3^{2-} \quad (3)$$

$$S_2O_3^{2-} + H_2 \leftrightarrow HS^- + HSO_3^- \quad (4)$$

But the reduction potential of the thiosulfate/hydrogen sulfide-sulfite redox couple ($E_o' = -0.40$ V) (Thauer et al. 1977) does not solely lead to enough free energy-change allowing rapid growth of these strains on thiosulfate, unless an alternative pathway is made available for disposing of reducing equivalents. The introduction of thiosulfate triggers such a pathway involving the formation of alanine concomitantly with the underproduction of molecular hydrogen (Etchebehere and Muxí 2000) but the exact mechanism of thiosulfate reduction remains unclear in these organisms. The present comparison between the two strains of *C. proteolyticus* was made possible because of their close relationship, yet two distinct metabolic phenotypes were observed. These results call for further physiological investigations, especially to determine how fermentation balance is defined in strain IT3 and other *Coprothermobacter* strains.

In our study, strain IT3 was identified and characterized as closely resembling the type strain, *Coprothermobacter proteolyticus* strain BT^T. Nevertheless, we found some useful differences between these two strains mainly based on the analysis of growth on thiosulfate at thermophilic range. Because of the discrimination it revealed between strain IT3 and the type strain BT^T, thiosulfate might be helpful in isolating and describing other novel members of the *Coprothermobacter* genus and in further establishing the bacterial diversity of this group, especially at high DNA-DNA relatedness. The questions of what kind of *Coprothermobacter* strains are efficient in thermophilic anaerobic digestion and what is their ecological role in these systems need to be addressed.

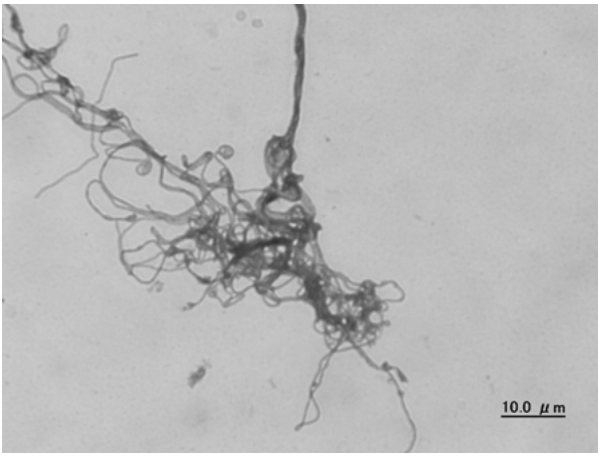

Fig.1. Microphotographs of gram-stained cells of strain IT3 grown at 65 °C. Bars, 10 µm.

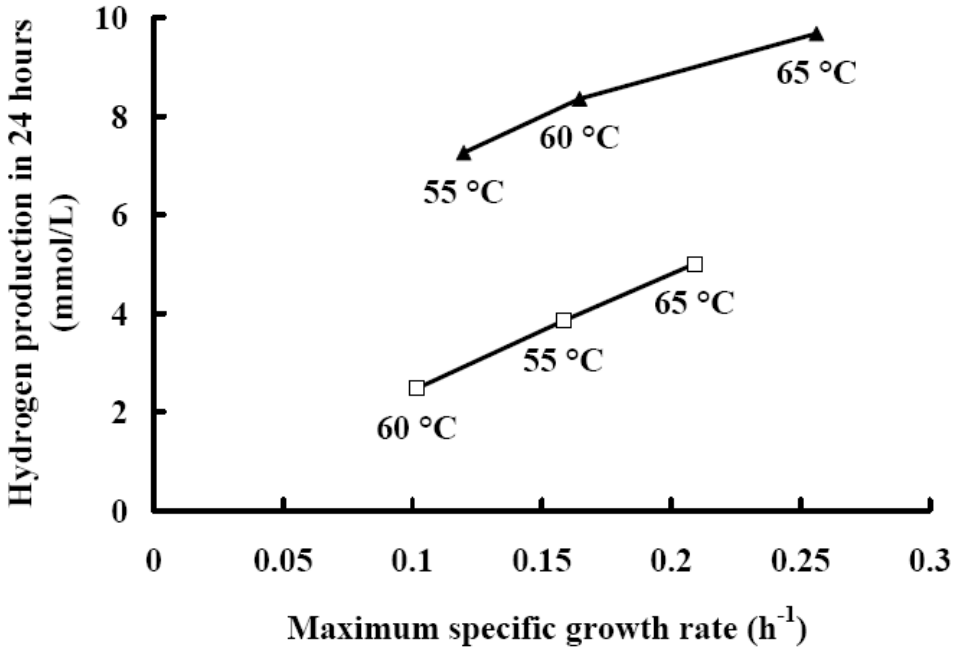

Fig. 2. Variation of the hydrogen production as a function of the maximum specific growth rate at corresponding temperatures. Symbols: strain IT3 (▲); C. proteolyticus strain BT^T (□). Hydrogen production in modified PY medium was measured in headspace after 24 hours and is expressed as mmoles per litre of culture medium. Maximum specific growth rates were determined in BM amended with 20 mM thiosulfate.

Tab. 1. Parameters of the modified Gompertz equation for strain IT3

Temperature	μmax (in h^{-1})	P	λ (in h)	n	R2	D
45 °C	0.0558	3.38	7.52	40	0.997	0.0787
55 °C	0.120	3.76	0.64	17	0.994	0.1634
60 °C	0.165	3.60	0.13	17	0.991	0.1170
65 °C	0.256	2.41	2.53	6	0.996	0.2472
70 °C	0.273	2.09	2.50	6	0.996	0.2410

Tab. 2. Parameters of the modified Gompertz equation for C. proteolyticus strain BT^T

Temperature	μmax (in $h^{-1)}$	P	λ (in h)	n	R2	D
45 °C	0.0345	3.09	121.51	18	0.993	0.1075
55 °C	0.158	2.39	3.49	8	1.000	0.1794
60 °C	0.102	2.81	1.96	17	0.997	0.1292
65 °C	0.209	1.87	2.55	6	0.994	0.2819
70 °C	0.217	4.03	1.48	11	0.982	0.1998

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