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Physiology and growth of Pyrococcus sp. HT3 in bach culture with cellobiose and maltose as the principal carbon sources

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ABSTRACT

The hydrothermal hot spring archaeonPyrococcussp. T3 was grown in batch cultures in closed bottles, both in the presence and in the absence of elemental sulfur. Growth on carbohydrates, proteinaceous substrates and amino acids was investigated. The disaccharides maltose and cellobiose were shown not to be able to enhance growth suggesting that Pyrococcussp. T3 is unable to use them as sole carbon sources. By contrast, proteinaceous substrates such as peptone and brain heart infusion were shown to be very good substrates for the growth of Pyrococcussp. T3. Growth on brain heart infusion was shown to require additional nutrients when sulfur was not present in the culture medium. Growth on amino acids only took place in the presence of sulfur. These results indicate that sulfur plays an important role in the metabolism and energetics of Pyrococcussp.HT3.

KEYWORDS

Pyrococcussp.HT3;
Hyperthermophile;
Nutrition;
Batch culture;
Sulfur requirement

INTRODUCTION

The advances in the discovery of stable enzymes from extremophiles have resulted in their increased use for applications such as organic synthesis and the production of specialty chemicals, pharmaceutical intermediates, and agrochemicals. The acceleration of enzyme discovery from this diverse class of organisms has helped facilitate the development of new industrial processes. Our understanding of the biochemical properties of these unique enzymes is finally starting to enable more creative applications.

Hyperthermophilic archaea have been identified from their initial isolation as potentially interesting organisms for the production of thermostable enzymes[29]. Pyrococcus furiosus has become the most extensively studied of these hyperther-

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mophiles both in the field of its physiology and metabolism[22,26,29] and in the isolation and study of its thermostable enzymes[15,20].

The main advantages of performing processes at higher temperatures are the reduced risk of microbial contamination, lower viscosity, improved transfer rates and improved solubility of substrates. However, co-factors, substrates or products might be unstable or other side reactions may occur.

Since the isolation of P. furiosus, a range of organisms have been ascribed to the genus Pyrococcus primarily on the basis of a limited range of physiological properties then on the basis of their 16S rRNA sequences and DNA/DNA hybridisation[6]. In our study, three isolates were obtained from hydrothermal hot spring "Hammam el biban" in Northern East of Algeria and, Pyrococcussp.HT3 was selected for further study since it exhibited á-glucosidase activity. This study examines some of the physiological properties of Pyrococcussp.HT3, including the determination of optimal growth parameters in closed bottle cultures, both in the presence and in the absence of elemental sulfur.

MATERIALS AND METHODS

Organism

Pyrococcussp.HT3 was isolated by one of the authors (M.K.) and deposited in the, DSMZ collection culture. The EMBL accession number for the 16S rRNA sequence is AM183944.

Media

Cultures were grown under anaerobic conditions at 90°C and pH 7.5. The growth medium was modified from SME medium according to Sharp and Raven[24,25]. For carbohydrate utilisation experiments, a peptone (Difco) concentration of initially 2 g l-1 was lowered to 1 g l-1 and maltose or cellobiose was added at a concentration of 5 g l-1. Yeast extract and peptone were replaced in further experiments by 9 g l-1 brain heart infusion (Difco) or by a mixture of the 20 classical amino acids, each at a concentration of 0.1 g l-1. When sulfur was used, it was added at a concentration of 10 g l-1. The media, 2216S and BHIS, were prepared as described by[8] and tested. For closed bottle cultures, the medium was sterilised by heating at 100°C for 40 min on three successive days.

Growth conditions

Closed culture experiments for the determination of growth parameters and sulfur requirement were performed as previously described[24,25].

The 2-l medium bottles containing elemental sulfur were sterilised by heating twice at 100°C for 30 min on two successive days. The medium was agitated with a magnetic stirrer. Hydrogen sulfide was trapped in 10 M NaOH and the off gas vented to the atmosphere for safety. Cultures in the presence of sulfur were always performed in a well-ventilated area and in the presence of a hydrogen sulfide detector.

Determination of growth kinetics was trained according to Gonzalez et al. method[9]. Growth rates (μ; h-1) were obtained from a regression lineof lnN plotted againstt, whereN is the number cell and t is the incubation time.

Cell count

Cells densities were determined by direct cell counting by direct cell counting using a Thoma chamber (depth 0.02 mm) hemocytometer and phase contrast microscopy at a magnification of 400 x (Olympus model BH-2 microscope).

RESULTS

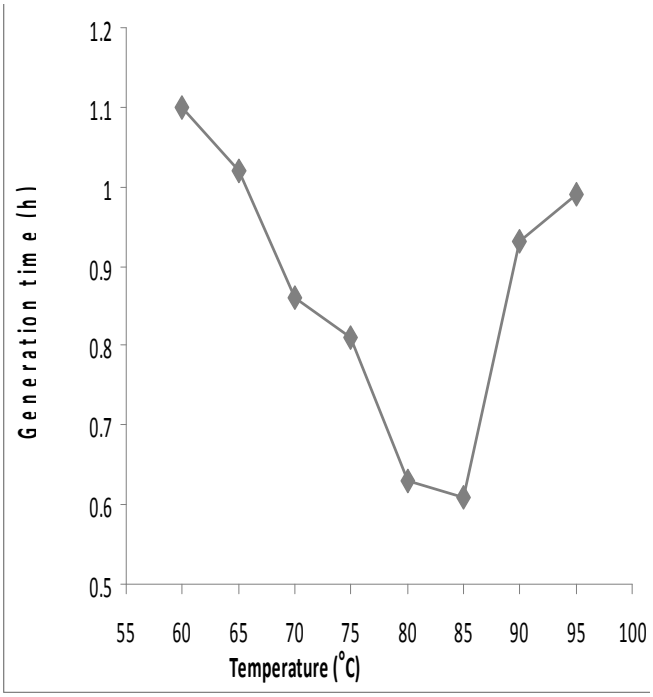
Determination of growth parameters

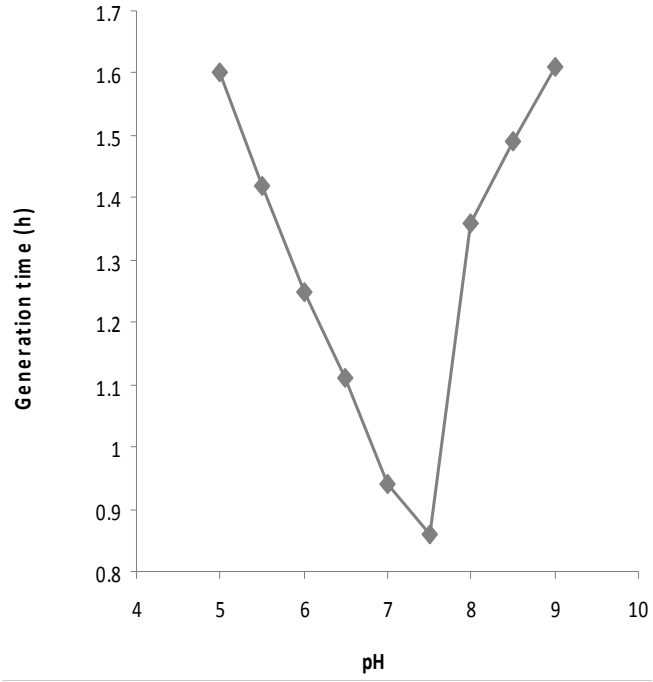
Pyrococcussp. HT3 was grown in penicillin-like bottles sealed with black butyl rubber stoppers containing a2216S medium. This isolate grew over the range of 65–95°C with an optimal temperature of 85°C (at 20 g l-1 sea salt and pH 7.5) (Figure 1a). Growth was observed over a pH range of 6.0–8.5 with optimal growth around 7.5 (at 20g l-1 sea salt and 85°C). No growth was observed at pH 5.5 and 9.5 (Figure 1b). Growth was observed over a total salt concentration from 10 to 40 g l-1 with optimal growth at 15–20 g l-1, of NaCl concentration (at 85°C and pH 7.5) (Figure 1c).

Sulfur requirement

In closed bottles, in the presence of elemental sulfur under a nitrogen atmosphere Pyrococcussp.HT3 exhibited rapid growth giving a final cell density of approximately 2.4×108_ cells ml-1. The fermentation product H2 is inhibitory to growth and the sulfur reduction seems serving to remove hydrogen[29], The solubility of elemental sulphur in water at elevated temperatures is not known[27].

It is not excluded that the soluble sulphur reaches substrate concentrations under these conditions. Sulfur might penetrate through the bacterial membrane, due to its lipophilic nature, to form polythionates in the presence of O2 or polysulfides in its absence at the more neutral pH that may preval in the cytoplasm.





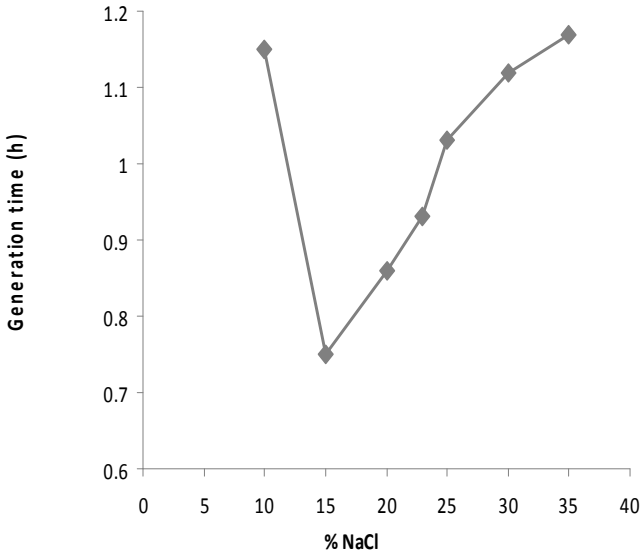


Figure 1 : Influence of temperature (1a), pH (1b) and NaCl concentration (1c) on the growth rate of the isolate Pyrococcussp.HT3. The generation times were alculated from the slopes of exponential portions of complete growth curves.

Sulfur could be replaced by cystine or polysulfide and in both cases large amounts of hydrogen sulfide were produced (data not shown). In the absence of elemental sulfur, growth terminated at a significantly lower cell density. When hydrogen was added to the gas phase, no growth was observed in the absence of sulphur[28,31].

Growth on cellobiose

This α-glucosidaseactivity was enhanced by the addition of cellobiose to the medium[19]. Pyrococcussp.HT3 was, therefore, tested for its ability to grow in bach culture with cellobiose as the principal carbon source according to[23]. To reduce its proteolytic and potential autolytic activities, the peptone concentration in the medium was reduced to 1 g l-1 and 5 g l-1cellobiose added. No important improvement in growth was observed with the provision of cellobiose. Strain Pyrococcussp.HT3 possesses α-glucosidase activity regarding to the glucose obtained.

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archaebacterium Pyrococcusabyssi has been studied[19]. A hyperthermophophilicα-1,4endoglucanase (family 5, cellulase) was identified in a hyperthermophilic archaeonPyrococcus horikoshii and found to be capable of hydrolyzing crystalline cellulose at high temperatures[17].

Growth on maltose

To investigate further carbohydrate utilisation by Pyrococcussp.HT3, the organism was tested as considering to[23] for growth in the presence of maltose, since maltose is one of the few carbohydrates shown to be used by some members of the

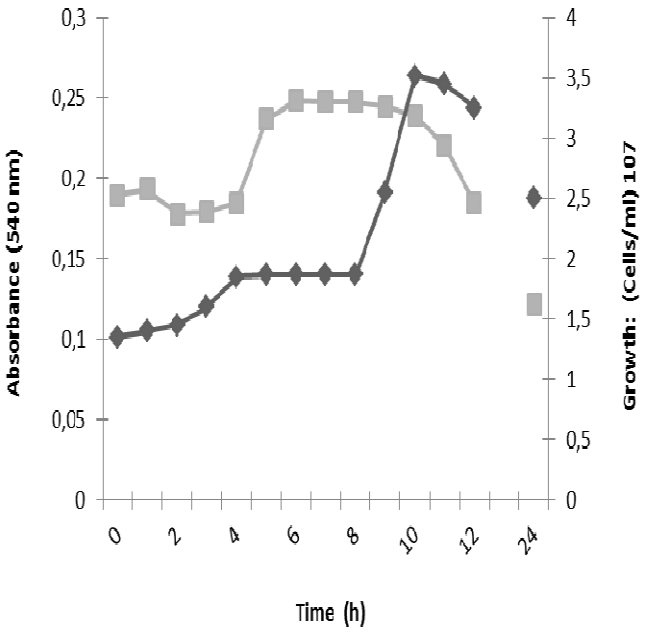

Figure 2 : Ability of Pyrococcus sp.HT3 to grow in culture with cellobiose as the principal carbon source on SME medium according to Sharp and Raven (Raven and al., 1992; Raven and Sharp, 1997)

Thermococcales and it has been shown to be an excellent substrate for the growth of P. furiosus. Maltose was added to the medium at 5 g l-1 and again the peptone concentration was reduced to 1 g l-1. The lower steady-state density obtained at the end of culture suggests that maltose not only cannot enhance the growth of Pyrococcussp.HT3, but that its presence has an inhibitory effect. Strain Pyrococcussp.HT3 possesses α-glucosidase activity regarding to the glucose obtained.

Proteinaceous and amino acid utilisation

Pyrococcussp.HT3 has been routinely grown on BHIS medium which consists of only brain heart infusion broth, sodium chloride and sulfur (Godfroy and al. 1996). In closed bottle cultures, maximal cell densities of around 1.2×108 cells ml

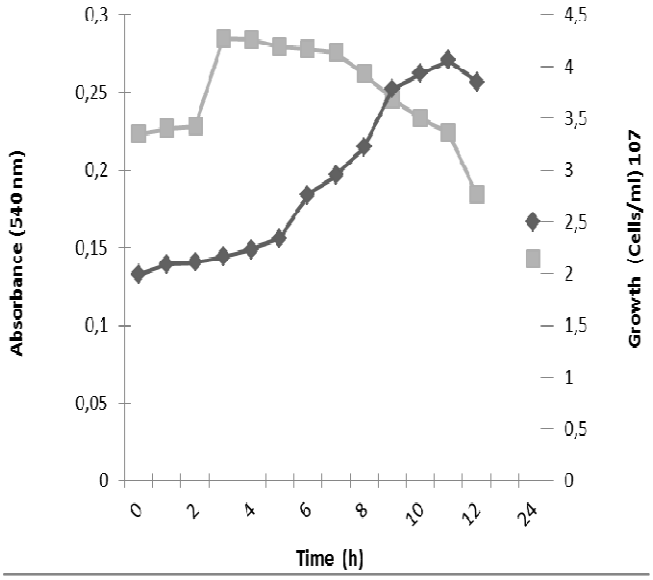

Figure 3 : Ability of Pyrococcus sp.HT3 to grow in culture with maltose as the principal carbon source on SME medium according to Sharp and Raven (N. Raven and al., 1992; N.D.H. Raven and R.J. Sharp 1997).

-1 were obtained[13]. Pyrococcussp.HT3 was tested for growth on this medium in the absence of sulfur. No growth was observed. An aliquot of this culture was sampled and incubated overnight in a closed bottle in the presence of elemental sulfur and a cell density of 1.1×108 ml-1 was obtained, showing that viable cells were clearly present. Addition of the minerals, trace elements and vitamins present in SME medium restored growth.

In common with other Thermococcales species strain Pyrococcussp.HT3 is able to grow on a medium containing 20 amino acids as the sole carbon source. In the presence of elemental sulfur or cystine in closed bottle cultures, a maximal cell density of approximately 3,4_×108 cells ml-1 was obtained.

DISCUSSION

Hyperthermophiles are characterized by a temperature optimum for growth between 80 and

110°C. They are considered to represent the most ancient phenotype of living organisms and thus their metabolic design might reflect the situation at an early stage of evolution. Their modes of metabolism are diverse and include chemolithoautotrophic and chemoorganoheterotrophic. No extant phototrophic hyperthermophiles are known. Lithotrophic energy metabolism is mostly anaerobic or microaerophilic and based on the oxidation of H2 or S coupled to the reduction of S, $SO4^{2-}$,

CO2 but rarely to O2. The substrates are derived from volcanic activities in hyperthermophilic habitats;

Our main interest was in a production process at elevated temperatures. This can have many advantages, amongst which is the possibility to increase the substrate concentration. Enzymes from thermophilic microorganisms have unique characteristics such as high temperature-, chemical- and pH stability

The optimal temperature, pH and salinity conditions for growth of Pyrococcussp.HT3 were determined in closed culture in the presence of elemental sulfur; In closed culture bottles, Pyrococcussp.HT3 exhibited very slow growth in the absence of sulfur, due to inhibition by hydrogen evolved as an end product. This has been described previously for P. furiosus[15] and is confirmed by the inhibition of the growth of Pyrococcussp.HT3 observed when hydrogen is added to the gas phase in the absence of sulfur. Both archaea are able to grow on a variety of α- and β-linked glucose saccharides and glucose[3,19]. Polysaccharides are degraded by specific extracellular glycosyl hydrolases to oligosaccharides[6] which are subsequently transported into the cell by either ABC (ATP-binding cassette)-type or secondary transporters (23,30-35). Sugar transport via the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) is apparently absent from archaea and eukaryotes. Transported oligosaccharides are hydrolysed further to glucose by specific intracellular glycoside hydrolases [1,2,10,11,12,14,15,18]. Glucose is metabolized to pyruvate via variants of two main sugar catabolic routes: the EM pathway, that is operating in Pyrococcus furiosus, or the ED pathway, which is found in S. solfataricus[19,20].

Growth of strain Pyrococcussp.HT3 was not enhanced by the addition of cellobiose as principal carbon source. As this enzyme has been shown to be intracellular, this could be explained by a deficiency in the transport of the disaccharide into the cell. The probable transferase activity suggests the role of this enzyme is not limited to degradative processes but may also be crucial in the biosynthesis of glycocomponents of the microorganism. Similarly, strain Pyrococcussp.HT3 appeared to be unable to reach high cell densities by using maltose as carbon source, in addition, its presence in the medium caused a degree of growth inhibition. This is observed despite the low mean residence time of the maltose at 85°C at a dilution rate of 0.I h-1. Strain Pyrococcussp.HT3 has been screened for two amylolytic activities (á-amylase, á-glucosidase), Pyrococcus sp.HT3 appeared to be able to use disaccharides and this is consistent with others growth studies which showed that Pyrococcusspecies grew preferentially on proteinaceous substrates[5,32].

Elemental sulfur is essential for growth and greatly stimulategrowth itis required for growth at High hydrogen concentrations, in the presence of S°, H2S is found.

In the absence of elemental sulfur and hydrogen,Pyrococcussp.HT3 was able to grow on a medium containing only brain heart infusion and NaCl, while good growth had been previously observed on this medium in the presence of elemental sulfur in closed bottle cultures. Brain heart infusion broth is considered to be a very rich medium. Growth on BHI as sole carbon source in the absence of elemental sulfur could only be restored by the addition of the minerals, trace elements and vitamins present in the SME medium. This suggests that one or more required elements are present in the mineral, trace element or vitamin solutions, and that these elements are not essential when sulfur is present in the medium[7].

The decrease in growth on amino acids in the absence of sulfur in comparison to the growth observed in its presence in closed culture indicates a significant role for sulfur under these conditions [27,28]. This role might, therefore, not be restricted to the removal of metabolically produced hydrogen by the formation of hydrogen sulfide. Sulfur may facilitate a process that in the absence of sulfur requires energy[31,4].

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The model for cellobiose gave a good description of the experiments. The enzyme was found to be uncompetitively inhibited by cellobiose and competitively inhibited by glucose. The use of a hyperthermostable enzyme was found to be positive. More substrate could be dissolved at higher temperatures, which benefited all reactions.

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