
Physiology and continuous culture of the hyperthermophilic deep-sea vent archaeon *Pyrococcus abyssi* ST549

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Abstract: The deep-sea vent archaeon *Pyrococcus abyssi* strain ST549 was grown in batch cultures in closed bottles and by continuous culture in a gas-lift bioreactor, 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 *P. abyssi* ST549 is unable to use them as carbon sources. By contrast, proteinaceous substrates such as peptone and brain heart infusion were shown to be very good substrates for the growth of *P. abyssi* ST549 and allowed growth at high steady-state cell densities in continuous culture. 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 *P. abyssi* ST549.

Keywords: *Pyrococcus abyssi*; Hyperthermophile; Nutrition; Continuous culture; Sulfur requirement

1. Introduction

In addition to their phylogenetic and ecological interest, hyperthermophilic archaea have been identified from their initial isolation as potentially interesting organisms for the production of thermostable enzymes. *Pyrococcus furiosus* has become the most extensively studied of these hyperthermophiles both in the field of its physiology, metabolism [1-6]; and in the isolation and study of its thermostable enzymes [7-9].

Since the isolation of *Pyrococcus furiosus*, a range of organisms have been ascribed to the genus *Pyrococcus* primarily on the basis of a limited range of physiological properties. Among these were five strains isolated from a deep-sea hydrothermal vent in the North Fiji basin [10,11]. These were assigned to the species *Pyrococcus abyssi* [12], on the basis of their 16S rRNA sequences and DNA/DNA hybridisation [13]. One of these *Pyrococcus abyssi* isolates, ST549, was selected for further study since it exhibited a β -glucosidase activity [14]. This study examines some of the physiological properties of *Pyrococcus abyssi* strain ST549, including the determination of optimal growth parameters in closed bottle cultures and bioreactor continuous culture, both in the presence and absence of elemental sulphur.

2. Materials and methods

2-1 Organism

Pyrococcus abyssi strain ST549 was isolated by one of the authors (A. Godfroy) and deposited in the "Collection Nationale de Culture de Microorganismes", Institut Pasteur, Paris under the number : CNCM I.1318. GeneBank accession number for the 16S rRNA sequence is : AJ225071

2-2 Media

Unless otherwise indicated, 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 [15]. 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 were 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 sulphur was used, it was added at a concentration of 10 g l⁻¹. The media, 2216S, BHIS and 20AAS, were prepared as previously described [16]. For closed bottle cultures, the medium was sterilised by heating at 100°C for 30 min on two successive days. For continuous culture, the medium was sterilised by filtration as previously described [15].

2-3 Growth conditions

Closed culture experiments for the determination of growth parameters and sulphur requirement were performed as previously described [16].

Continuous culture experiments in the absence of sulphur were performed using a nitrogen sparged gas-lift bioreactor, at a dilution rate of 0.2 h⁻¹ as previously described [5,15]. Continuous culture experiments in the presence of sulphur were performed at a dilution rate of 0.2 h⁻¹ using a conventional glass 1.5 litre fermenter with a modified top plate and agitation system to minimise the corrosive effect of sea water, sulphur and hydrogen sulphide. The stainless steel top plate was replaced by Incolloy 625 and the agitation system replaced by a Vibromixer system (Chemap, Science Tec, Les Ullis, France) which consisted of a hollow shaft ended by a perforated disc and allowing the injection of nitrogen through it. Temperature was controlled by a heated-circulating bath filled with water and the temperature monitored by a PT100 class A probe. The pH was monitored using a combination gel pH electrode and controlled using a pH controller. Fresh medium was added and product removed using peristaltic pumps. The 20 l medium bottles containing elemental sulphur were sterilised by heating twice at 100°C for 30 min on two successive days. The medium was agitated by a magnetic stirrer. Hydrogen sulphide was trapped in 10M NaOH and the off gas vented to the atmosphere for safety. Cultures in the presence of sulphur were always performed in a well ventilated area and in the presence of an hydrogen sulphide detector.

2-4 Cell count

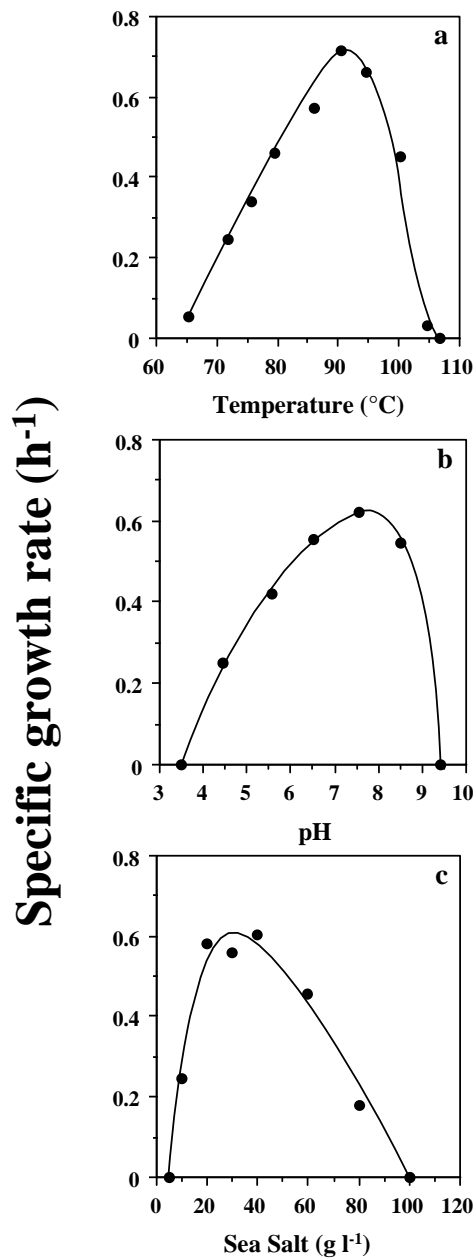
Cells densities were determined by direct cell counting [16].

3. Results

3-1 Determination of growth parameters

Pyrococcus abyssi ST549 grew over the range of 65°C to 103°C with an optimal temperature of 92°C (at 30 g l⁻¹ Sea Salt and pH 7.5) (fig 1A). Growth was observed over a pH range of 4.5 to 8.5 with optimal growth around 7.5 (at 30 g l⁻¹ Sea Salt and 90°C) . No growth was observed at pH 3.5 and 9.5 (fig 1B). Growth was observed over a total salt concentration from 10 to 80 g l⁻¹ with optimal growth at 30-40 g l⁻¹, corresponding to an NaCl concentration of 19.5 to 26 g l⁻¹ (at 90°C and pH 7.5) (fig 1C).

Figure 1. Temperature, pH and "Sea Salt" optima for growth of *Pyrococcus abyssi* ST549 grown on 2216 S medium : specific growth rate as function of temperature (30g l⁻¹ Sea Salt, pH 7.5) (A), pH (92°C, 30 g l⁻¹ Sea Salt)(B) and salinity (92°C, pH 7.5)(C).Growth rate were calculated using linear regression analysis along the logarithmic part of the resulting growth curves.



3-2 Sulphur requirement

In closed bottles, in the presence of elemental sulphur under a nitrogen atmosphere *Pyrococcus abyssi* ST549 exhibited rapid growth giving a final cell density of approximately $2-3 \times 10^9$ cell ml^{-1} . Sulphur could be replaced by cystine or polysulphide and in both cases large amounts of hydrogen sulphide were produced (data not shown). In the absence of elemental sulphur, 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 (fig 2).

3-3 Continuous culture in the absence of sulphur in a gas lift bioreactor on SME-Yeast extract/peptone medium

Growth of *Pyrococcus abyssi* ST549 was examined in a nitrogen sparged gas-lift bioreactor in an analogous manner to *Pyrococcus furiosus* [5]. At moderate gas flow rates (0.1 and $0.2 \text{ v v}^{-1}\text{min}^{-1}$) *Pyrococcus abyssi* ST549 reached significantly higher equilibrium cell densities than in closed cultures in the absence of elemental sulphur, indicating a positive effect of the stripping of metabolically produced hydrogen (Table 1). In contrast to *Pyrococcus furiosus*, this effect could not be extended to higher gas flow rates due to the build up of a surface foam which prevented efficient gas stripping. Additionally *Pyrococcus abyssi* ST549 also produced a black precipitate in the medium, presumed to be due to insoluble metallic sulphides.

Figure 2. Influence of sulphur and gas phase on the growth of *Pyrococcus abyssi* strain ST549 in closed vessels.

- (●) growth in the absence of sulphur under N_2 ;
- (○) growth in the absence of sulphur under H_2/CO_2 (80: 20);
- (▲) growth in presence of sulphur under N_2 .

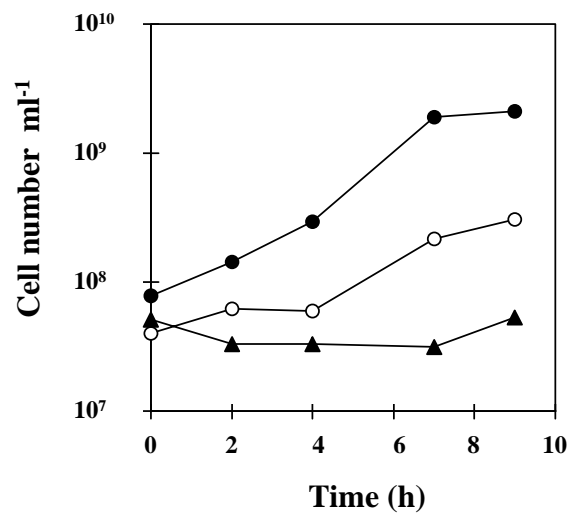


Table 1. Effect of nitrogen flow rate and of medium upon steady state cell density of *Pyrococcus abyssi* ST549 in continuous culture in the absence of elemental sulphur (90°C, pH 7.0, dilution rate 0.2 h⁻¹).

Medium	Nitrogen flow (ml min ⁻¹)	Steady State Cell density (cell ml ⁻¹)
SME peptone 2 g l ⁻¹ yeast extract 1 g l ⁻¹	200 (0.1vvm)	1.37 10 ⁹
	400 (0.2vvm)	1.96 10 ⁹
	600 (0.3vvm)	ND
SME peptone 1 g l ⁻¹ yeast extract 1g l ⁻¹ cellobiose 5 g l ⁻¹	200 (0.1vvm)	1.2 10 ⁹
	400 (0.2vvm)	1.58 10 ⁹
	600 (0.3vvm)	1.48 10 ⁹
SME peptone 1 g l ⁻¹ yeast extract 1 g l ⁻¹ maltose 5 g l ⁻¹	200 (0.1vvm)	2.3 10 ⁸
	400 (0.2vvm)	4.7 10 ⁸
	600 (0.3vvm)	1.25 10 ⁹
BHI medium NaCl 23 g l ⁻¹ Brain Heart Infusion 9 g l ⁻¹	200 (0.1vvm)	no growth
SME Brain Heart Infusion 9 g l ⁻¹	200 (0.1vvm)	1.62 10 ⁸
	400 (0.2vvm)	3.8 10 ⁸
	600 (0.3vvm)	8.1 10 ⁸
SME 20 amino acids 0.1 g l ⁻¹	200 (0.1vvm)	2 10 ⁶ *

* dilution rate 0.1 h⁻¹

3-4 Growth on cellobiose

Strain ST549 has been shown to contain high levels of β -glucosidase activity which was enhanced by the addition of cellobiose to the medium [14]. *Pyrococcus abyssi* was, therefore, tested for its ability to grow in continuous culture with cellobiose as the principal carbon source. To reduce its proteolytic and potentially autolytic activities, the peptone concentration in the medium was reduced to 1 g l⁻¹ and 5 g l⁻¹ cellobiose added. Under this condition, the steady state cell density peaked at 0.2 v v⁻¹min⁻¹ (1.58 x 10⁹ cells ml⁻¹), with a lower steady state cell density being achieved at 0.3 v v⁻¹min⁻¹ (1.48 x 10⁹ cells ml⁻¹). So no improvement in growth was observed with the provision of cellobiose, even if strain ST549 possess a β -glucosidase activity.

3-5 Growth on maltose

To investigate further carbohydrate utilisation by *Pyrococcus abyssi* ST549, the organism was tested for growth in the presence of maltose, since maltose is one of the few carbohydrates shown to be used by some members of the *Thermococcales* and it has been shown to be an excellent substrate for the growth of *Pyrococcus furiosus* [6]. Maltose was added to the medium at 5 g l⁻¹ and again the peptone concentration was reduced to 1 g l⁻¹. Under these conditions equilibrium cell densities were lower at each gas flow rate tested in comparison to

cellobiose, suggesting that maltose not only cannot enhanced the growth of *Pyrococcus abyssi* ST549, but that its presence has an inhibitory effect.

3-6 Proteinaceous and amino acids utilisation

Pyrococcus abyssi ST549 has been routinely grown on BHIS medium which consists of only brain heart infusion broth, sodium chloride and sulphur [16]. In closed bottle cultures, maximal cell densities of around 1×10^9 cells ml^{-1} were obtained (data not shown). *Pyrococcus abyssi* ST549 was tested for growth on this medium in absence of sulphur in the gas-lift bioreactor. Under these conditions no growth was observed. An aliquot of this culture was sampled and incubated overnight in a closed bottle in the presence of elemental sulphur and a cell density of 1.3×10^9 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, but lower equilibrium cell densities were obtained at each gas flow rate in comparison to growth on the SME yeast extract/ peptone medium. A maximal equilibrium cell density of 8.1×10^8 ml^{-1} was obtained at $0.3 \text{ v v}^{-1} \text{ min}^{-1}$.

In common with the type strain of *Pyrococcus abyssi* GE5 [12,17] and other deep-sea *Thermococcales* species [16,18,19], strain ST549 is able to grow on a medium containing 20 amino acids as the sole carbon source. In the presence of elemental sulphur or cystine in closed bottle cultures, maximal cell density of approximately 2×10^8 cells ml^{-1} were obtained. Growth was, therefore, tested in the gas-lift bioreactor with yeast extract and peptone being replaced by the mixture of 20 amino acids. Under these conditions extremely poor growth was observed, with an equilibrium cell density of 2×10^6 ml^{-1} being reached at a dilution rate of 0.1 h^{-1} . As described previously, incubation of an aliquot of this culture overnight on the same medium, but in the presence of sulphur, gave an increase in cell density to 1.2×10^8 cells ml^{-1} .

3-7 Continuous culture in the presence of sulphur

To confirm that the use by *Pyrococcus abyssi* ST549 of BHI and amino acids is influenced by the presence of sulphur in the culture medium, continuous culture experiments were performed in its presence. Each culture was started on medium containing yeast extract and peptone and SME mineral salts. The yeast extract and peptone were then removed and replaced by the 20 amino acids. This medium was in turn replaced by BHIS medium and a continuous culture maintained for 250h. A range of gas flow rates were tested and maximal cell densities were obtained for each medium at $0.4 \text{ v v}^{-1} \text{ min}^{-1}$, showing the positive effect of elevated gas sparging on hydrogen sulphide removal. On the yeast extract/peptone medium, a maximum equilibrium cell density of 3.31×10^9 ml^{-1} was reached at $0.4 \text{ v v}^{-1} \text{ min}^{-1}$ and a steady-state could be maintained indefinitely. On the medium containing the 20 amino acids a maximum equilibrium cell density of 9.26×10^8 cells ml^{-1} was obtained, again at a nitrogen flow rate of $0.4 \text{ v v}^{-1} \text{ min}^{-1}$. On BHIS medium, a maximum equilibrium cell density of 2.17×10^9 cells ml^{-1} was also obtained at $0.4 \text{ v v}^{-1} \text{ min}^{-1}$. These results are summarised in Table 2. Gas flow rates used in the continuous culture experiment in the presence of sulphur could not be compared to those used in the gas-lift bioreactor experiment, as gas stripping is probably less efficient in a conventional culture vessel than in a gas-lift vessel.

Table 2. Effect of nitrogen flow rate and of medium upon steady state cell density of *Pyrococcus abyssi* ST549 in continuous culture in the presence of elemental sulphur (90 °C, pH 7.0, dilution rate 0.2 h⁻¹)

Medium	Nitrogen flow (ml min ⁻¹)	Steady State Cell density (cell ml ⁻¹)
SME peptone 2 g l ⁻¹ yeast extract 1 g l ⁻¹	150 (0.1vvm)	5.28 10 ⁸
	300 (0.2vvm)	8.73 10 ⁸
	450 (0.3vvm)	1.75 10 ⁹
	600 (0.4 vvm)	3.31 10 ⁹
BHI medium NaCl 23 g l ⁻¹ Brain Heart Infusion 9 g l ⁻¹	150 (0.1vvm)	ND
	300 (0.2vvm)	7.15 10 ⁸
	450 (0.3vvm)	1.63 10 ⁹
	600 (0.4 vvm)	2.17 10 ⁹
SME 20 amino acids 0.1 g l ⁻¹	150 (0.1vvm)	ND
	300 (0.2vvm)	1.62 10 ⁸
	450 (0.3vvm)	4.37 10 ⁸
	600 (0.4 vvm)	9.26 10 ⁸

4. Discussion

The optimal temperature, pH and salinity conditions for growth of *Pyrococcus abyssi* strain ST549 were determined in closed culture in the presence of elemental sulphur and shown to be very similar to those of the type strain GE5 [12]. In closed culture bottles, *Pyrococcus abyssi* ST549 exhibited very slow growth in the absence of sulphur, due to inhibition by hydrogen evolved as an end product. This has been described previously for *Pyrococcus furiosus*, [20] and is confirmed by the inhibition of the growth of ST549 observed when hydrogen is added to the gas phase in the absence of sulphur. Continuous culture of *Pyrococcus abyssi* ST549 in a gas-lift bioreactor in the absence of elemental sulphur showed that this strain is cultivable at high cell densities at a nitrogen flow rate of 0.2 v⁻¹ min⁻¹.

Despite possessing high levels of β-glucosidase activity [14], growth of strain ST549 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. It also answer the question of the role of the enzyme *in vivo.*, Ladrat et al [14] suggested that the physiological role of the β-glucosidase in *P. abyssi* might be related to the deep-sea hydrothermal environment : in particular, the presence of animal with chitin shell (b-N-Acetyl-glucosaminidase activity) or the likely presence of marine polysaccharides like fucans (β-fucosidase activity). On the other hand, the probable transferase activity suggest the rôle of this enzyme is not limited to degradative processes but may also play a crucial role in the biosynthesis of glycocomponents of the microorganism.

Similarly, strain ST 549 appeared to be unable to reach high cells density by using of 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 90°C at a dilution rate of 0.2 h⁻¹. Strain ST549 has been screened for total amylolytic activities (i.e. α-amylase, α-glucosidase and pullulanase), however, none of these activities were detected in this strain [21]. *Pyrococcus abyssi* ST549 appeared to be unable to use disaccharides and this is consistent with previous growth studies which showed that *Pyrococcus abyssi* ST549 grew preferentially on proteinaceous substrates (Godfroy, data not shown). This is confirmed by the high levels of proteinase activity which have been identified in *Pyrococcus abyssi* ST549 and are presently under study [22]. Lack of growth on disaccharides may, however, be due to the absence of essential amino acids. This point is presently under investigation using both closed cultures in the presence of sulphur.

In the absence of elemental sulphur in continuous culture, *Pyrococcus abyssi* ST549 was unable to grow on a medium containing only brain heart infusion and NaCl, while very good growth had been previously observed on this medium in the presence of elemental sulphur in closed bottle cultures. This result was confirmed for continuous culture in the presence of sulphur. Brain heart infusion broth is frequently used to grow bacteria with stringent nutritional requirements and is considered to be a very rich medium. Growth on BHI as sole carbon source in the absence of elemental sulphur 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 sulphur is present in the medium. Traces element as Tungsten were shown to be involved in both *Pyrococcus furiosus* [23] and *Thermococcus litoralis* [24] enzymes which are involved in the metabolism of peptides.

The dramatic decrease in growth on amino acids in the absence of sulphur in comparison to the growth observed in its presence in both closed culture and continuous culture, indicates a significant role for sulphur under these conditions. This role might, therefore, not be restricted to the removal of metabolically-produced hydrogen by the formation of hydrogen sulphide, as was previously proposed for the type strain of *Pyrococcus abyssi* GE5 [12] and for *Pyrococcus furiosus* [20]. Even if sulphur reduction to hydrogen sulphide appears not to be coupled to energy conservation via sulphur respiration [25], higher molar growth yields for growth on maltose or cellobiose have been reported in the presence of sulphur [1,3]. Schicho and collaborators [3] have demonstrated that in a carbon substrate limited culture, the reduction of sulphur plays a dramatic role in the energy conservation of *Pyrococcus furiosus*. They suggest that sulphur does not conserve energy per se but may facilitate a process that in the absence of sulphur requires energy. An obvious possibility is that sulphur reduction is coupled to a transport process that in its absence requires energy. In *Pyrococcus abyssi*, therefore, sulphur reduction might be coupled to an energy requiring process such as amino acid transport.

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