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Optimisation of growth conditions for continuous culture of the hyperthermophilic archaeon *Thermococcus hydrothermalis* and development of sulphur-free defined and minimal media

Anne Postec, Patricia Pignet, Valérie Cueff-Gauchard, Anne Schmitt, Joël Querellou, Anne Godfroy *

Laboratoire de Microbiologie des Environnements Extrêmes, UMR 6197 Ifremer, Centre de Brest, BP 70, 29280 Plouzané, France

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Abstract

The hyperthermophilic archaeon *Thermococcus hydrothermalis* was cultivated in continuous culture in a gas-lift bioreactor in the absence of elemental sulphur on both proteinaceous and maltose-containing media. Optimal conditions (pH, temperature and gas flow rate), determined on complex media that yielded maximal growth rate and maximal steady state cell density, were obtained at 80 °C, pH 6 and gas sparging at 0.2 $v\,v^{-1}\,min^{-1}$. Higher steady state cell densities were obtained on a medium containing maltose and yeast extract. In order to design a defined and minimal media, the nutritional requirements of *T. hydrothermalis* were then investigated using continuous culture in the absence of elemental sulphur in the gas-lift bioreactor. First, the complex nutriments were replaced and a defined medium containing maltose, 19 amino acids and the two nitrogenous bases adenine and thymine, was determined. Secondly, selective feedings and withdrawal of amino acids showed requirements for 14 amino acids.

Keywords: *Thermococcus hydrothermalis*; Gas-lift bioreactor; Culture optimisation; Defined and minimal media

1. Introduction

Within the hyperthermophilic archaea, species belonging to the order *Thermococcales* have been extensively studied in the fields of both physiology and genomics. In addition to their phylogenetic and ecological interest, hyperthermophilic archaea were early on considered to be potentially interesting organisms for the production of thermostable enzymes. *Thermococcales* species can grow in the absence of elemental sulphur, but for most of these species, the addition of sulphur greatly enhances growth in closed culture. Raven et al. [11] showed that a glass gas-lift bioreactor could be used to grow *Pyrococcus furiosus* at high cell densities in the absence of elemental sulphur. This experimental system was then used to develop a defined minimal medium that supported the growth of *P. furiosus* [12]. Using the same system, *Pyrococcus abyssi* ST549 was cultivated at high cell density on complex media containing yeast extract and peptone in the absence of elemental sulphur [7].

T. hydrothermalis was first isolated from a deep-sea hydrothermal vent of the East Pacific Rise. It has been shown to grow at temperatures ranging from 55 to 100 °C; it is strictly anaerobic and chemoorganotrophic. A descriptive study of this strain [6] revealed that *T. hydrothermalis* was able to grow on both proteinaceous substrates (or a mixture of amino acids) and on maltose, and a recent study confirmed that it could use maltose (in the presence of a small amount of yeast extract) and amino acids as carbon and energy sources in the presence of elemental sulphur.

Several enzymatic activities have been identified and characterised in *T. hydrothermalis*: α-amylase [9], pullulanase [5], α-glucosidase [8] and alcohol dehydrogenase [2].

* Corresponding author.
E-mail address: agodfroy@ifremer.fr (A. Godfroy).

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In this paper, we determined the optimal conditions for the cultivation of *T. hydrothermalis* in a gas-lift bioreactor in the absence of elemental sulphur and investigated its nutritional requirements in order to develop a defined minimal medium.

2. Materials and methods

2.1. Strain

T. hydrothermalis type strain AL662 (CNCM-I1319) was isolated in our laboratory [6].

2.2. Media

The growth medium was SME Y(1) P(2) medium [19] modified according to Sharp and Raven [11,18]. In further experiments, peptone was replaced by maltose at 2.5 or 5 $g\,l^{-1}$ [SME Y(1) mal(5) or SME Y(1) mal(2.5) media]. When yeast extract was replaced by the 20 amino acids, the latter were each first used at a concentration of 0.1 $g\,l^{-1}$ [SME 20AA(0.1) mal(2.5)]; then they were used at the same concentrations as in a 1 $g\,l^{-1}$ aqueous suspension of yeast extract [SME 19AA[Y] mal(2.5)]. Quantitative estimations of the concentration of each free amino acid in an aqueous suspension of yeast extract at 1 $g\,l^{-1}$ were determined by HPLC. The concentrations (per litre) were: Ala 42 mg, Arg 22 mg, Asn 14 mg, Asp 6.5 mg, Gln none, Glu 47 mg, Gly 16 mg, His 4 mg, Ile 25 mg, Leu 42 mg, Lys 23 mg, Met 4 mg, Phe 24 mg, Pro 14 mg, Ser 18 mg, Thr 12 mg, Trp 4 mg, Tyr 10 mg and Val 30 mg. Cys, used as reductant, was maintained at 0.5 $g\,l^{-1}$. The media were sterilised by filtration (Sartroban 0.22-µm filters, Sartorius) in 20 l Nalgene bottles previously sterilised by autoclaving.

2.3. Growth conditions

Closed cultures were performed in a 100 ml serum vial containing 50 ml of medium and sulphur as previously described [6]. Continuous culture experiments were performed using a gas-lift bioreactor, sparged with nitrogen as previously described by Raven [11,12,18]. The 2-litre volume glass vessel and Teflon top plate were fabricated by Radleys (UK). Temperature was controlled by a heated circulating bath filled with water, and temperature was monitored with a standard PT100 probe covered with Teflon. The pH was monitored using a combination gel pH electrode (Mettler Toledo), and acid and base were added with two peristaltic pumps (Masterflex). pH and temperature were controlled by a 4–20 mA controller and AFS Biocomand system from New Brunswick (Nijmegen, Netherlands). Fresh medium feeding and culture draw-off were performed using peristaltic pumps (Masterflex). Unless otherwise indicated, continuous cultures were carried out at 80 °C and pH 6, according to the descriptive study of the strain. Unless otherwise indicated, the dilution rate was 0.2 h^{-1} according to the previously determined growth rate [6] and under nitrogen sparging at 0.2 $v\,v^{-1}\,min^{-1}$.

2.4. Determination of cell density

Cell numbers were determined by direct cell counting using a Thoma cell (0.02 mm depth) under a phase contrast Olympus model BH-2 microscope. When necessary, samples were diluted in sterile water containing NaCl at 23 $g\,l^{-1}$. A steady state was considered to be obtained when the cell densities remained effectively constant for a period in excess of three culture volume changes, corresponding to 15 h at a dilution rate of 0.2 h^{-1} [11].

2.5. Growth rate determination

To determine the specific growth rate of *T. hydrothermalis* on the different media, batch culture experiments were carried out in the gas-lift bioreactor. A steady state continuous culture under the above culture conditions was washed out in order to significantly lower cell density. Medium feeding and drawing off were then stopped, and growth under batch conditions was followed by regular cell counting (every 15 min, 3 counts per sample). Growth rate were determined by performing a linear regression along the logarithmic part of the growth curve. When enough data were available, growth rate confidence limits are given ($\pm 2\times$ standard errors). Washout experiments were performed first to determine growth rates according to the formula $[1/x(\mathrm{d}x/\mathrm{d}t) = \mu_{\max} - D]$ [4], but problems with the reliability of media delivery rate measurement at high dilution rates ($D \geqslant 2$) were shown to significantly affect growth rate calculations.

2.6. Amino acids and maltose analysis by HPLC

The amino acids and maltose in the culture medium were analysed by means of HPLC (Alliance 2690; Waters), as described by Wery et al. [22].

3. Results

3.1. Determination of optimal conditions for growth of T. hydrothermalis in continuous culture on SME Y(1) P(2) medium

Optimal conditions of pH (pH 6) and temperature (80–85 °C) conditions for the growth of *T. hydrothermalis* were previously determined, in a flask, on a complex medium in the presence of elemental sulphur [6]. These conditions (pH 6 and 80 °C) were the first conditions used for the cultivation of *T. hydrothermalis* in the gas-lift bioreactor. Good growth was observed with a steady state cell densities ranging from 8×10^8 to 1×10^9 cell ml^{-1} at a dilution rate of 0.2 h^{-1}. The effect of gas sparging was studied by varying the nitrogen flow from 0.1 to 0.3 $v\,v^{-1}\,min^{-1}$. Maximal