Growth of hyperthermophilic microorganisms for physiological and nutritional studies

Anne Godfroy[1, *], Anne Postec[1] and Neil Raven[2]

[1] Laboratoire de Microbiologie des Environnements Extrêmes, UMR 6197, Ifremer, BP 7029280, Plouzané, France
[2] Health Protection Agency, Porton Down, Salisbury SP4 0JG, United Kingdom

*: Corresponding author : Anne Godfroy, email address : Anne.Godfroy@ifremer.fr

Introduction

Hyperthermophilic microorganisms grow at temperature of 80°C and above. Numerous species belonging to both the *Archaea* and *Bacteria* have now been isolated from both continental and volcanic areas. Initially following major isolation efforts, heterotrophic species were recovered, but this has now extended to species involved in all geo-biochemical cycles and employing a wide range of electron donors and acceptors.

In addition to their ecological and evolutionary interest, these organisms were rapidly identified as potential sources of stable biomolecules having potential application in industrial processes. However, an understanding of their growth characteristics and metabolism is necessary to realize their full biotechnological potential. A major limitation to their study has frequently been the small amount of biomass that can be generated.

Within the archaeal hyperthermophiles, some species have been more extensively studied: 1) thermoacidophilic species belonging to the order *Sulfolobales* 2) methanogens and 3) a number of species belonging to the order *Thermococcales.*

A key factor in the study of hyperthermophilic organisms has been the development of cultivation strategies allowing exploration of their metabolic behaviour and the optimisation of their growth parameters. At the same time this has allowed the production of biomass in sufficient quantities for further studies.

Initially, concerns about the damaging effect of highs level of reduced sulphur species by anaerobic hyperthermophiles, or sulphuric acid by oxidizing species, led to the development of multi-hundred litre capacity fermentors with ceramic liners (Kelly and Deming, 1988) Large scale cultivation of hyperthermophilic species was reported by Karl Stetter's group for biomass production of various hyperthermophilic species using an enamelled 300 litre fermentor (Stetter *et al.*, 1983; Blöchl *et al.*, 1997; Huber *et al.*, 2000). A range of cultivation systems have also been reported for the culture of

acidophilic hyperthermophiles (Schiaraldi *et al.*, 1999; Worthington *et al.*, 2003) and methanogens (Mukhopadhyay *et al.*, 1999).

CULTIVATION OF SULPHUR-REDUCING HYPERTHERMOPHILES IN BIOREACTORS

The cultivation of microorganisms at temperatures up to 110°C, in media that may contain high salt concentrations, and that actively produce hydrogen sulphide represents a major challenge to conventional laboratory fermentation equipment.

Thermococcales have been isolated from both deep and shallow marine ecosystems [with the exception of *Thermococcus sibiricus* (Miroshnichenko *et al.*, 2001) isolated from a high temperature oil reservoir and *Thermococcus ziligiii* (Ronimus *et al.*, 1997) and *Thermococcus waiotapuensis* (Gonzalez *et al.*, 1999) isolated from a continental hot spring]. The order *Thermococcales* includes 3 genera: *Thermococcus*, *Pyrococcus* and *Paleococcus*. These species are all anaerobic; they utilize peptides as carbon and energy sources and most of them exhibit better growth in the presence of elemental sulphur producing large amounts of hydrogen sulphide. Some are able to grow in the absence of elemental sulphur, but in serum vial batch culture the production of molecular hydrogen as a metabolic end product leads to early inhibition of growth.

Within the *Thermococcales*, the shallow marine species *Pyrococcus furiosus* has been the most extensively studied in terms of its physiology, genetics and enzymology, and is generally considered as the model species for heterotrophic hyperthermophiles. The pathways of both peptide and carbohydrate metabolism of this species have been well studied (Adams *et al.*, 2001).

Pressurized bioreactors have been developed in consideration that high pressure may be important for the growth of deep sea sulphur-reducing hyperthermophiles (Kelly and Deming, 1988; Miller

et al., 1988; Nelson *et al.*, 1992). However, most hyperthermophiles have been isolated at atmospheric pressure, although pressure effects have been reported for some of them (Erauso *et al.*, 1993; Marteinsson *et al.*, 1999a; Marteinsson *et al.*, 1999b; Canganella *et al.*, 2000). Simpler and less expensive approaches for the cultivation of hyperthermophiles have also been reported. These have been used successfully both to generate large quantities of biomass and to explore their physiology.

As they are relatively easy to grow, many systems have been developed for the cultivation of *Thermococcales* species, with particular emphasis on *P. furiosus*. Batch fermentation of numerous sulphur-reducing hyperthermophiles has been reported using conventional stainless-steel bioreactors, with proper attention to cleaning and maintenance operations, and numerous enzymes have been purified from the biomass produced (Adams, 1993; Gantelet and Duchiron, 1998; Raffin *et al.*, 2000). In the presence of sulphur, hydrogen sulphide is produced instead leading to moderate cells densities in closed culture (Fiala and Stetter, 1986; Godfroy *et al.*, 2000). Since hydrogen sulphide is reasonably soluble in water, this can lead to growth inhibition, the absence of a real stationary phase and cell lysis (personal observation). Moreover, reaction with many metals occurs to form extremely insoluble sulphide precipitates. However, high cell densities of *Thermococcales* can be obtained by sparging sulphur-containing cultures with anaerobic gases such as nitrogen, in both closed vials or in conventional glass fermentors (Brown and Kelly, 1989) or a modified one as described for the cultivation of *P. abyssi* (Godfroy *et al.*, 2000),

The reactor described by Brown and Kelly (Brown and Kelly, 1989) comprised a 2 litre glass round bottomed flask maintained at high temperature by a heating mantle controlled by a proportional controller. The reactor was operated at a volume of 1 litre. The vessel was fitted with a gas inlet sparger and the exit gas was passed through

sodium hydroxide to remove hydrogen sulphide. The medium was supplied from a polycarbonate reservoir using a peristaltic pump and anaerobicity maintained by sparging with nitrogen. After each change of dilution rate, sulphur was added to ensure it remained in excess. A constant volume was maintained via a dip tube connected in parallel to the medium feed pump.

When grown in the absence of elemental sulphur, species belonging to the order *Thermococcales* were shown to produce hydrogen as an end product of their metabolism and this appeared to limit growth severely unless removed. The glass continuous culture vessel described by Brown and Kelly (1989) has been used in many studies (Rinker and Kelly, 1996; Rinker and Kelly, 2000) for cultivation of *P. furiosus*, *T. littoralis* and the bacterium *Marinitoga maritima.*

Fermentation of *P. furiosus* in a conventional stirred bioreactor was described by Krahe *et al.* (1996) generating a high cell density, but it was shown that stirrer speeds above 1800 rpm had a negative effect on growth. Krahe also reported the utilisation of dialysis membrane bioreactor system for the cultivation of *P. furiosus* (Krahe *et al.*, 1996). Cultivation at high temperature was carried out using a Bioengeenering (Wald, Switzerland) membrane bioreactor consisting of two compartments (Holst *et al.*, 1997). The outer compartment comprises a cylindrical high strength polyamide foil fixed to a top and bottom stainless steel plate. The inner compartment is separated from the outer one by a tubular dialysis membrane. Gas sparging can be applied to either the inner or outer compartment, or both if necessary. The reactor is also equipped with two stirring units. Application of a high medium dilution rate in the outer compartment was shown to permit high cell densities to be reached compared to cultivation in closed glass bottle, primarily by the removal of a range of metabolic end products. This system was also used for cultivation of the thermoacidophilic species *Sulfolobus shibatae* (Krahe *et al.*, 1996).

A 2-litre bioreactor has also been developed for the cultivation of *P. furiosus* in continuous culture to high cell concentrations and biomass yields (Raven *et al.*, 1992). The intense sparging of sulphur-free, hydrogen evolving cultures has significant advantages, both in the quality of biomass produced and in the safety of the procedure. Since the risk of contamination of thermophilic and hyperthermophilic continuous cultures is relatively small, long-term steady state experiments both for biomass production and for the detailed investigation of physiological responses to culture perturbation are made possible.

CONTINUOUS CULTURE OF *THERMOCOCCALES* SPECIES IN THE GLASS GAS-LIFT BIOREACTOR

This experimental protocol, initially developed for optimisation of biomass production of *P. furiosus*, was then used for the development of a sulphur-free minimal medium for *P. furiosus* (Raven and Sharp, 1997), physiological study of *Pyrococcus abyssi* strain ST549 (Godfroy *et al.*, 2000), optimisation of culture conditions for the growth of *Thermococcus hydrothermalis* and the development of defined and minimal media (Postec *et al.*, 2005b). This protocol is now routinely used for biomass production of the *Pyrococcus abyssi* type strain GE5.

Description of the gas lift bioreactor and equipment

Glass vessel:

The glass vessel is a concentric tube reactor (Figure 1B) constructed by Radleys (UK) and comprising a central column (Figure 1A a) with a rounded base and an external glass jacket (Figure 1A b). Two glass

ports with Duran-type threads are fitted on opposite side (Figure 1A c), one at the bottom and one at the top. The base has a 45 mm diameter port with a glass Duran–type screw thread (Figure 1A d) for sparger insertion.

A glass draft tube (Figure 1A e) is placed within the central column section. It has two sets of 3 glass lugs near the top and bottom respectively. Silicone tubing is stretched over each lug and used to position the draft tube within the vessel.

Top plate:

A PTFE top plate (Figure 1C and 1A f) is clamped on top of the glass vessel using a stainless steel clamping ring (Figure 1A g) with a silicone gasket between.

The top plate is perforated by 13 ports:

- a single central port of 12 mm in diameter (Figure 1A h) used to fit a Mettler Toledo gel pH electrode.
- 2 x 8 mm ports (Figure 1A i) used to fit a PTFE-covered PT100 temperature probe and a glass condenser (Figure 1A i), through which cold water can be passed,
- 8 x 5 mm ports (Figure 1A k) used for acid and alkali addition; medium feed and culture draw-off; inoculation and sampling system using glass needles or 1ml glass pipettes. Water and gas tightness is ensured by compression of 5 ring gaskets. When not used, ports are sealed with small glass rods.

Medium/product and alkali/acids bottles:

Twenty litre Nalgene propylene bottles and 750 ml bottles were used for medium and product or acid and alkali respectively. Screw caps have 3 ports: for delivery, drawing-off and a gas vent fitted with a 0.2 µm PTFE filter. Empty bottles are sterilised by autoclaving and filled

with either medium or 1 M HCl or 1 M NaOH solutions, sterilised beforehand by filtration through 0.2 µm Sartobran autoclavable filters (Sartorius). All sterile connections are made using autoclavable quick coupling systems (Cole-Parmer).

Medium and product bottles can be kept under anaerobic conditions if necessary by sparging at a low rate with oxygen-free nitrogen.

Pumps and tubing:

Masterflex Tygon silicone and Pharmed tubing are used for liquids and gases respectively.

Peristaltic pumps are used for both medium delivery feeding and product draw-off and for alkali or acid feeding. For acid and alkali additions, peristaltic pumps are connected to the pH regulation system. The feeding pump is a programmable pump, while the product draw-off pump is a standard one.

Controls:

pH and temperature are controlled by a 4-20 mA Controller and AFS Biocommand system from New Brunswick (Nijmegen, the Netherlands) associated with a pH transmitter (Broadley James) and a temperature controlled heated circulated bath (Huber). For temperatures up to 95°C, water can be used for circulation, while mineral oil should be used for temperatures above this. Additionally, the gas lift vessel can be connected to any other standard fermentation control system.

Fermentation protocol

Medium:

The basal SME medium (Stetter *et al.*, 1983) was modified according to Sharp and Raven (Raven *et al.*, 1992; Sharp and Raven, 1997) (Table 1) and can be used as it is, or can be modified further for nutritional studies (Raven and Sharp, 1997; Postec *et al.*, 2005b). In this medium, L-cysteine is used as reductant.

Bioreactor set-up:

Temperature probes, pH probes (calibrated prior to use) and condensers are positioned in their appropriate ports. All other ports are closed with glass rods. The vessel can be sterilised (with ports closed and covered as appropriate) by placing in an autoclave, but given the fragility of the system, an *in situ* tyndallisation procedure can also be used: The vessel is filled with distilled water and heated at 100°C for 3 hours under aerobic conditions (by sparging the reactor with air) then cooled to room temperature; this procedure is repeated at least 4 or 5 times.

The condenser is then connected to a cold water supply (HS40, Huber refrigerated circulating bath filled with water). Sparging with nitrogen is initiated using a gas flow meter (Aalborg, Monsey, New York) with a 0.2 vol. vol^{-1}. min^{-1} flow rate. Media feed and product recovery bottles are connected aseptically to the bioreactor and water (used for the sterilisation step of the bioreactor) replaced by medium (at least 5 volume changes are needed). At the same time alkali and acid bottles can be connected aseptically to the bioreactor. A sampling device is also aseptically introduced into the corresponding port.

Temperature and pH regulation systems are switched on and the pH value of the medium standardized by measuring the pH of a small sample by comparison with a standard bench pH meter at room temperature. The system for continuous culture using the gas lift bioreactor is presented in Figure 2.

Starting culture:

When the temperature and pH have reached their set values, media feed and product draw off pumps are stopped prior to inoculation with a fresh grown vial culture (20 or 40 ml for a 2 litre culture) *via* the inoculation port.

Culture monitoring:

The cell density of the culture can be monitored by both direct cell counting using a 0.02 mm depth Thoma chamber (Weber Scientific International, UK) under a phase contrast microscope or by optical density at 600 nm.

When growth has started, the continuous process can be established by starting both feeding and draw-off pumps. For biomass production, a dilution rate of 0.2 h^{-1} or 0.4 h^{-1} is convenient (since from 9.6 to 19.2 l of culture per day are generated). For physiological and nutritional studies, the dilution rate can be lowered in order to obtain at least 3 volume changes in the bioreactor per day. This allows parameter such as pH, temperature and media composition to be altered sequentially each day. with results approximating to equilibrium values being obtained (Raven and Sharp, 1997; Postec *et al.*, 2005b).

Determination of growth parameters

In addition to steady state cell concentrations, the influence of various parameters (e.g. pH, temperature, gas flow rate or media composition) can also be measured by determination of maximal growth rate under sequentially modified conditions. As previously described (Rinker and

Kelly, 1996; Rinker *et al.*, 1999) wash out experiments can be performed to determine growth rates according to the formula

$$[1/x(dx/dt)= \mu_{max}-D]$$

where x =the cell density of the culture; t = time; μ_{max} = the maximal growth rate, and D is the dilution rate (Duarte *et al.*, 1994), but problems with the reliability of medium delivery rate measurements at high dilution rates ($D \geq 2h^{-1}$) have been shown to significantly affect growth rate calculations (personal observation). An alternative method to determine the specific growth rate can be achieved by performing batch culture experiments. A steady state continuous culture under the above culture conditions can be grown at supra-maximal dilution rates in order to lower the cell density significantly. Medium feeding and product draw off are then stopped, and growth under batch conditions followed by regular cell counting (e.g. every 15 min., 3 counts per sample). Growth rate can then be determined by performing a linear regression along the exponential part of the growth curve (Postec *et al.*, 2005b) .

Substrates and metabolic product analysis

The gas outflow from the bioreactor can be directly analysed using a MTI M200D micro gas chromatograph equipped with a thermal conductivity detector. A Molecular Sieve column with argon as the carrier gas and a temperature of 30°C was used to detect H_2 and CO_2, while H_2S was detected using a Poraplot U column at 100°C, with helium as the carrier gas.

Substrates (carbohydrates and amino acids) and metabolic end products (organic acids) can be analysed by means of HPLC (Alliance 2690; Waters) as described by Wery *et al.* (2001). Proteins from the supernatant of the culture are precipitated by a solution of 5-

sulfosalicylic acid at 2% (w/v). Amino acids may be analysed after derivatisation (with an ethanol / water / triethylamine / phenylisothiocyanate (7:1:1:1 by vol.) solution) according to the conditions given for the Waters Pico Tag method (WAT007360; Waters). Carbohydrates and linear organic acids are separated on an H^+ exclusion column (Polyspher OAKC 1.51270; Merck) at 60°C with an 18 mM H_2SO_4 elution solution (0.35 ml min^{-1}) and detected with a differential refractometer (Refractometer 410, Waters).

FUTURE PROSPECTS: CULTIVATION OF THERMOPHILIC AND HYPERTHERMOPHILIC MICROBIAL COMMUNITIES

While the gas lift bioreactor was originally developed for culture in the absence of sulphur, it has also been shown to be usable for culture in its presence. Particular care regarding gas exhaust elimination must be taken for safety reasons: hydrogen sulphide is trapped in 10 M NaOH and the off gas vented to the atmosphere. Cultures in the presence of sulphur should always be performed in well ventilated areas and in the presence of a hydrogen sulphide detector. The utilisation of the gas lift bioreactor has also been shown to be a useful tool for the cultivation of microbial communities in addition to pure cultures from deep sea hydrothermal chimneys.

Continuous enrichment cultures have been performed in the gas lift bioreactor using SME mineral and vitamin solutions added with various organic substrates at 60° and 90°C using a hydrothermal chimney sample as the inoculum. The microbial diversity present was then analysed using molecular tools. Cloning and sequencing of 16S rRNA genes of two independent samples from two independent cultures was performed. A denaturing gradient gel electrophoresis (DGGE) analysis of the 16S rRNA gene diversity was also performed over the course of the two cultures in order to monitor the dynamics of the microbial population. The results evidenced quite large diversity

compared to the diversity previously obtained in the same conditions by classical batch cultures in closed vessels. At 90°C, while archaeal diversity was limited to species belonging to the *Thermococcales*, unexpected bacterial diversity was obtained, including still uncultivated species and already described species growing at temperatures above their previously known optimal growth conditions (Postec *et al.*, in press). At 60°C, considerable bacterial diversity was achieved and both heterotrophic and autotrophic species shown to be cultivated, with some successfully purified and formally described (Postec *et al.*, 2005a). These preliminary experiments indicate that such continuous enrichment cultures in bioreactors may allow (i) access to as yet uncultivated microorganisms and (ii) the co-cultivation of thermophilic microbial deep sea hydrothermal vent species (involved in various biogeochemical cycles) permitting the study of the interactions between species under various experimental conditions with emphasis on better simulating environmental conditions.

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41762 Christy St.

Fremont, CA 94538, USA

Tel: 1-510-490-0900

Micro Gas Chromatograph

Waters

34 Maple Street

Milford, Massachusetts 01757, USA

Tel: 1-508-478-2000

http://www.waters.com

HPLC

Table 1. Composition of SME medium (Stetter *et al.*, 1983) modified according to Sharp and Raven (Raven *et al.*, 1992; Sharp and Raven, 1997).

Medium	per litre
NaCl	28 g
Yeast extract	1 g
Peptone	2 g
L-cysteine	0.5 g
Resazurine	0.5 mg
Magnesium salt solution	10 ml
Solution A	1 ml
Solution B	1 ml
Solution C	1 ml
Solution D	1 ml

Stock Solutions

Magnesium salt solution	
$MgSO_4.7H_2O$	180 g
$MgCl_2.6H_2O$	140 g

Solution A	
$MnSO_4.4H_2O$	9 g
$ZnSO_4.7H_2O$	2.5 g
$NiCl_2.6H_2O$	2.5 g
$AlK(SO_4)_2.12H_2O$	0.3 g
$CoCl_2.6H_2O$	0.3 g
$CuSO_4.5H_2O$	0.15 g

Solution B

$CaCl_2.2H_2O$	56 g
NaBr	25 g
KCl	16 g
KI	10 g

Solution C

K_2HPO_4	50 g
H_3BO_3	7.5 g
$Na_2WO_4.2H_2O$	3.3 g
$Na_2MoO_4.2H_2O$	0.15 g
Na_2SeO_3	0.005 g

Solution D in 1 M HCl

$FeCl_2.4H_2O$	10 g

Vitamins solution in ethanol/water (50 :50)

Pyridoxine.HCl	200 mg
Thiamine.HCl	100 mg
Riboflavin	100 mg
Nicotinic acid	100 mg
DL-calcium pantothenate	100 mg
Lipoic acid	100 mg
Biotin	40 mg
Folic acid	40 mg
Cyanocobalamin	2 mg

Legends to the figures

Figure 1. The gas lift bioreactor (A) drawing, (B) picture and (C) top plate.

Figure 2. Schematic diagram of the experimental system for continuous culture with the gas lift bioreactor. (a) pH probe; (b) pH controller; (c) acid and alkali bottles; (d) temperature probe; (e) heated circulating bath; (f) sparging assembly; (g) gas flow meter; (h) condenser; (i) condensate trap; (j) gas exhaust; (k) medium bottle; (l) medium pump; (m) product bottle; (n) drawing-off pump; (o) sampling system; and (p) inoculation septum.

sterile connection

filter

regulated valve

valve

peristaltic pump