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Growth of the extremophilic *Deinococcus geothermalis* DSM 11302 using co-substrate fed-batch culture

Julie Bornot · Carole Molina-Jouve · Jean-Louis Uribelarrea · Nathalie Gorret

Abstract *Deinococcus geothermalis* metabolism has been scarcely studied to date, although new developments on its utilization for bioremediation have been carried out. So, large-scale production of this strain and a better understanding of its physiology are required. A fed-batch experiment was conducted to achieve a high cell density non-limiting culture of *D. geothermalis* DSM 11302. A co-substrate nutritional strategy using glucose and yeast extract was carried out in a 20-L bioreactor in order to maintain a non-limited growth at a maximal growth rate of 1 h^{-1} at 45 °C. Substrate supplies were adjusted by monitoring online culture parameters and physiological data (dissolved oxygen, gas analyses, respiratory quotient, biomass concentration). The results showed that yeast extract could serve as both carbon and nitrogen sources, although glucose and ammonia were consumed too. Yeast extract carbon-specific uptake rate reached a value 4.5 times higher than glucose carbon-specific uptake rate. Cell concentration of 9.6 g L^{-1} dry cell weight corresponding to 99 g of biomass was obtained using glucose and yeast extract as carbon and nitrogen sources.

Keywords *Deinococcus geothermalis* DSM 11302 · Fed-batch culture · Nutritional strategy · Growth conditions

Jean-Louis Uribelarrea and Nathalie Gorret contributed equally to the supervision of this work.

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Introduction

Deinococcus geothermalis belongs to the bacterial family *Deinococcaceae*. Members of the genus *Deinococcus* are known for their resistance to extreme environmental conditions like solvent and heavy metal presence, desiccation, oxidative stress, and ionizing radiations (Battista 1997; Blasius 2008; Cox and Battista 2005; Kongpol 2008; Mattimore and Battista 1996; Slade and Radman 2011). The main thermophilic strain of *Deinococcus*, *D. geothermalis*, has been isolated for the first time in hot springs at the Termi di Agnano near Naples in Italy (Ferreira 1997). The strain DSM 11302 is a type strain of *D. geothermalis* which has been discovered in water samples from São Pedro do Sul in Portugal. Its optimum growth temperature is between 45 and 50 °C and its optimum pH is 6.5 (Ferreira 1997).

Radiation and oxidation resistance properties of *Deinococcaceae* have been extensively investigated (Battista 1999, 1997; Blasius 2008; Daly 2009, 2006; Makarova 2007). Brim et al. have studied the possibility of using *D. geothermalis* properties for the bioremediation of radioactive wastes and irradiated environments (Brim 2003). Whereas advances in genetic engineering have been reported, metabolism of *D. geothermalis* and more generally of *Deinococcaceae* has been scarcely investigated (Ferreira 1997; Liedert 2012). *D. geothermalis* is currently cultivated in rich media containing at least one complex nutrient source as yeast extract, peptone or tryptone (Brim 2003; Ferreira 1997). Previous works have reported that growth in the absence of yeast extract was possible but low (Kongpol 2008). Nevertheless, the role of yeast extract is still unclear; it can be a source of constitutive nutrients or of growth factors. In 2009, growth of *D. radiodurans* was firstly quantified under controlled conditions in a 20 L bioreactor (He 2009): at pH 7 and 37 °C, the composition of tryptone glucose yeast extract medium was optimized to achieve a final $OD_{600\ nm}$ of 40

corresponding to 18 $g_{WCW}L^{-1}$ of biomass. A batch experiment with an addition of glucose 16 h after the inoculation was carried out. Moreover, kinetic of growth of *D. geothermalis* in a bioreactor under controlled parameters has never been investigated and reported yet.

Previous works have been done in order to define a synthetic medium for the growth of *D. geothermalis* (unpublished data). Experiments have been carried out in flasks and in batch-mode bioreactor cultures. Since yeast extract was necessary to achieve a non-limiting growth of *D. geothermalis*, the influence of a lot of vitamins, salts, amino acids, and fewer fractions of yeast extract on biomass production and oxygen uptake by *D. geothermalis* have been tested. The results have revealed that whole yeast extract was needed to achieve a non-limiting growth with the maximum growth rate. The main purpose of this study was then to contribute to a better understanding of the physiology of *D. geothermalis* DSM 11302, under controlled nutritional and growth conditions (temperature, pH, aeration). The strain was cultivated with glucose and yeast extract using an adaptive fed-batch culture mode. Fed-batch culture offers numerous advantages (Korz 1995; Lee 1999; Riesenberg and Guthke 1999) as production of high cell densities, control of growth rate, and study of microorganism physiology in response to nutritional environment changes. Moreover, fed-batch mode can reduce feedback inhibition or toxicity due to high substrate concentrations, and by-product production from overflow metabolism can be avoided by limiting substrate feed to the amount required solely for growth. The fed-batch mode with a feeding strategy using glucose and yeast extract as co-substrates was then carried out in this work: allowing flexibility in the nutrient supply, the possibility of growth control, and the role of yeast extract as a source of nitrogen, carbon, and/or growth factors were investigated.

Materials and methods

Bacterial strain

The microorganism used in this study was *D. geothermalis* DSM 11302, purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ; Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). The strain was inoculated into the peptone glucose yeast extract (PGY) medium broth (peptone 10 g L^{-1}, glucose 10 g L^{-1}, yeast extract 5 g L^{-1}) and incubated at 45 °C for 24 h. Bacterial cells were then stored at −80 °C with 20 % (*v*/*v*) glycerol.

Media and growth conditions

For inoculum preparation, one glycerol stock was streaked on PGY agar medium (PGY medium broth supplemented with agar 14 g L^{-1}) and incubated for 48 h at 45 °C. Preculture of *D. geothermalis* DSM 11302 was carried out in a 5 mL tube containing 1.5 mL yeast extract glucose medium (YEGM), at 45 °C for 24 h on an orbital shaker (110 rpm). Only one colony was used for the preculture. YEGM was prepared by adding 4 g L^{-1} of glucose, 5 g L^{-1} of yeast extract, and 0.4 M of 3-(N-morpholino)propanesulfonic acid (MOPS) (used as pH buffering) to 1 L of the mineral medium prepared for the bioreactor culture (see below). The successive steps of precultures were carried out in baffled Erlenmeyer flasks with increasing volumes (10, 100, and 450 mL YEGM). The inoculum size was 10 % (*v*/*v*) and the cultures were incubated 12 h at 45 °C and 110 rpm. The 450 mL preculture was used to inoculate 9 L of mineral medium in a 20 L bioreactor. The mineral medium (10 L) was prepared as follows: 5.74 mM K_2HPO_4, 20 mM NH_4Cl, 10 mM NaCl, 10 mM KCl, 278 μM Na_2SO_4, 528 μM MgCl, and 0.5 μM $CaCl_2$. After sterilization of this solution by autoclave, 100 mL of 2 mM $FeCl_3$ in 2 mM sodium citrate, 1 mL of a micronutrient solution (3×10^{-5} M $(NH_4)_6Mo_7O_{24}$, 4×10^{-3} M H_3BO_3, 3×10^{-4} M $CoCl_2$, 1×10^{-4} M $CuSO_4$, 25×10^{-4} M $MnCl_2$, and 10×10^{-5} M $ZnSO_4$), and 1 mL of each vitamin (niacin, thiamine hydrochloride, pyridoxine hydrochloride, cobalamin, and biotin prepared separately at a concentration of 10 mg L^{-1}) were added. The solutions of iron, micronutrients, and vitamins added were sterilized by filtration. The initial pH was adjusted to the required value 6.8 with NH_4OH. The mineral medium used in this study was adapted from a medium developed for the growth of *Deinococcus radiodurans* (Holland 2006).

Two substrate solutions were prepared for fed-batch experiments: glucose at 730 g L^{-1} and yeast extract at 385 g L^{-1}. A feeding salt solution containing 9.45×10^{-4} M $CaCl_2$, 5.58×10^{-5} M $CoCl_2$, 8.24×10^{-5} M $ZnSO_4$, 1.08×10^{-5} M H_3BO_3, 2.29×10^{-3} M K_2HPO_4, 9.49×10^{-3} M $MgCl_2$, 5.34×10^{-4} M $FeSO_4$, 1.48×10^{-5} M $CuSO_4$, 8.20×10^{-5} M $MnCl_2$, 3.53×10^{-6} M $AlCl_3$, 1.70×10^{-2} M H_3PO_4, 6.46×10^{-3} M H_2SO_4, and 7.93×10^{-6} M Na_2MoO_4 was also prepared. The composition was determined using calculation of excess factor for each element, as described in published works of Egli and Fiechter (1981) and Schneebeli and Egli (2013). The calculation was done under the assumption that the mineral elements presents in yeast extract solution are not consumed for the growth. Theoretical growth yields per element for *D. geothermalis* have been set based on average growth yields for microorganisms whose nutritional requirements are known. The pH of the concentrated saline solution was 1. These solutions were sterilized by autoclave for 20 min at 121 °C.

Chemicals

Glucose, orthophosphoric acid, ammonia, salts and oligo-elements were obtained from Prolabo (USA), yeast extract from Difco Diagnostics (USA), and sulfuric acid from Fisher

(USA). The salts $ZnSO_4$ and $MgCl_2$, vitamins, and the MOPS were provided from Sigma (USA) and $AlCl_3$ from Fluka (USA). All products were of the highest analytical grade available.

Cultures in bioreactor

Fed-batch experiments were performed in a 20 L bioreactor BBraun Biostat E (Braun, Melsungen, Germany) in situ sterilizable. Stirring and aeration were manually adjusted to avoid oxygen-limiting conditions. The mixing system consists of four baffles and three Rushton turbines. The reactor was instrumented with dissolved oxygen probe (Ingold, Urdorf, Switzerland), pH probe (Fermprobe Broadley-James Co., Santa Ana, USA), temperature, and pressure sensors. The temperature was maintained at 45 °C with a water-filled jacket connected to a thermostated vessel, the water temperature being regulated by a heat exchanger and a cooling water circulation system. The pH was kept at 6.8 with the addition of 14 % (*v*/*v*) ammonia solution or 8.5 % (*v*/*v*) orthophosphoric acid solution. The bioreactor was maintained to a relative pressure of 0.3 bar, and the antifoaming agent struktol J673 (Struktol, Germany) was used in case of foam formation during the fermentation. The solutions of ammonia, acid, and antifoaming agent were supplied with three peristaltic pumps (BBraun).

For fed-batch culture, medium in the bioreactor was supplied with three sterile feeds, yeast extract, glucose, and concentrated salts, using peristaltic pumps (BBraun, Masterflex and Gilson). The glucose feed concentration was 730 g L^{-1}, the second feed was a 385 g L^{-1} yeast extract solution, and the third feed was the concentrated salt solution. Yeast extract was added using two pumps: one with a flow range from 0 to 5 L h^{-1} (Masterflex 7521-35, Cole-Parmer Instrument Company, USA), the other one (BBraun FE411, Braun, Melsungen, Germany) with more precise flow, from 0 to 0.15 L h^{-1}. The glucose and salt solutions were added with two Minipuls 3 pumps (Gilson, Gilson Inc., USA) with a maximal flow of 0.15 L h^{-1}. The density of these feeding solutions was measured with a density meter (DE40, Mettler Toledo, USA), and masses of yeast extract and glucose added to the bioreactor were measured online by weighing (IB16000S and CPA16001S, Sartorius, Goettingen, Germany).

The bioreactor was connected to a computer with home-made software for online acquisition, monitoring, and regulation of the controlled parameters (stirring rate, pH, temperature, partial pressure of dissolved oxygen, pH-regulating solution and struktol additions). The inlet airflow was measured by a mass flowmeter (Brooks, USA). The composition of exhaust gas of the reactor was measured with an analyzer EGAS 8 (Braun, Germany), by infrared spectrometry for carbon dioxide and by paramagnetism for oxygen. These measurements, with mass balance equations, allowed calculating the oxygen uptake and carbon dioxide production rates and respiratory quotient (RQ). RQ is defined by the ratio between carbon dioxide production rate (r_{CO_2}) and oxygen uptake rate (r_{O_2}); its value depends on the degrees of reduction of the carbon source (γ_S), the final electron acceptor (γ_{O_2}), and the biomass (γ_X).

$$RQ = \left|\frac{r_{CO_2}}{r_{O_2}}\right| = \left|\gamma_{O_2} \times \frac{1 - Y_{S,X}}{\gamma_S - \gamma_X \times Y_{S,X}}\right|$$

Throughout the experiment, samples were harvested and stored at −20 °C.

Glucose, salts, and yeast extract feeding strategy

Glucose and yeast extract solution flows were adapted to microorganism needs to ensure a constant and maximal growth rate without nutrient limitation. Non-limiting conditions were ensured by dissolved oxygen monitoring. During 5 h, glucose was added by pulse to maintain a residual concentration higher than 0.4 g L^{-1} in order to be in glucose non-limiting conditions. Then, the glucose uptake rate of the strain was calculated using experimental data obtained since the beginning of the growth; the feeding rate was set at the glucose uptake rate value. The yeast extract feeding strategy was to adjust the yeast extract solution flow to the uptake rate of *D. geothermalis* DSM 11302, to avoid yeast extract limitation during the culture. It was made by online monitoring the dissolved oxygen. The salt supply was correlated to carbon substrate feeding. The flow of the salt pump was set at 1/10 of the total flows of glucose and yeast extract pumps. This value was chosen as a compromise between the nutritional requirements of the microorganism, the volume increase due to saline solution supply and technical constraints for feeding.

Biomass determination

Biomass concentration was determined by optical density ($OD_{600\ nm}$) measurements at 600 nm (spectrophotometer Libra S4, Biochrom, UK) and dry cell weight (DCW) measurements. Dry cell weight was estimated by filtration on polyamide membrane (Sartolon 0.2 μm, SARTORIUS®) and drying to a constant weight for 48 h at 60 °C under 200 mmHg in a vacuum oven (Heraeus, France).

Biomass analyses

Elemental composition of the biomass was analyzed. After centrifugation of the sample (4,000×*g* for 10 min), the pellet was washed three times with physiological water at 4 °C and then frozen at −80 °C. Fermentation samples were freeze dried and sent for analyses to the Central Service of Analysis (C.N.R.S., Solaize, France). Elements C, H, O, and N are

detected and analyzed after combustion of the sample by a katharometer or by a specific infrared detector. The protein concentration in the samples was determined by spectrophotometry, according to the colorimetric method of Biuret (Gornall 1949). To measure proteins of the biomass, the protocol was the following: a volume of 1 mL of fermentation medium was centrifuged at 12,000×*g* for 3 min and the pellet was suspended in ultra-pure water (qsp 1 mL). Cells had to be broken to release the protein contents: a step of heating at 100 °C in a water bath during 5 min in the presence of NaOH is necessary before the addition of the reactive $CuSO_4$ (Stickland 1951). For quantification, the standard used was a solution of bovine serum albumin.

Glucose, organic acids, proteins, and ammonia determination in supernatant

During fermentation, culture supernatant was obtained by centrifuging (MiniSpin Eppendorf, USA) fermentation broth samples in Eppendorf tubes at 12,000×*g* for 3 min. Glucose concentration was measured with an YSI 2700 glucose analyzer (Yellow Springs Instruments®, USA). Glucose and organic acid concentrations of filtered supernatants (Minisart filters 0.2 μm size pore, Sartorius, Germany) were determined by HPLC (Ultimate 3000, Dionex, USA) using an Aminex HPX-87H+ column (Bio-Rad, USA) under the following conditions: 50 °C, 5 mM H_2SO_4 as mobile phase at a flow rate of 0.5 mL min^{-1}, and detection with a refractometer and an UV detector at 210 nm. The samples were previously diluted in deionized water. External standards (0.2 to 5 g L^{-1}) were used for compound identification and quantification. Chromatograms were analyzed with Chromoleon software 6.80. The protein concentration in samples was determined by spectrophotometry, according to the colorimetric method of Biuret (Gornall 1949). The residual ammonium ion concentration in the supernatant was quantified using an ammonia electrode (Ammonia Gas-Sensing Electrode 95-12, Orion Research, Canada) and a pH/ISE meter (710 A). Total nitrogen and total organic carbon in the samples were measured with a total organic carbon (TOC)–total nitrogen (TN) analyzer (Shimadzu, USA). For TOC analysis, carbon dioxide was measured by an infrared detector after catalytic thermal oxidation at 680 °C. TN was determined after catalytic thermal decomposition at 720 °C and oxidation; NO* produced was quantified by chemiluminesce.

Results

Feeding strategy and microbial growth kinetic

D. geothermalis DSM 11302 fed-batch culture was performed applying a glucose yeast extract co-substrate nutritional strategy. Feed cycles, mass of added substrates, and growth results are shown in Fig. 1. Yeast extract solution was supplied to the medium using an adaptive command to closely base the feeding profile on the substrate uptake rate of the microorganism. A change in the dissolved oxygen dynamic was considered as a nutritional limitation. The feed cycles were controlled via pO_2 monitoring in order to avoid any limitation and accumulation in the course of the culture. The resulting feed ratios were calculated afterwards using mass balances: during the first 4 h, yeast extract was supplied with a ratio varying between 3.4 and 3.9 g (yeast extract (YE))/g(X), and after 4 h of culture, 2.5 g(YE)/g(X) were added.

The glucose flow was set based on the microorganism needs while maintaining a residual glucose concentration higher than 0.4 g L^{-1}. During 5 h, glucose was added by pulses, then glucose uptake rate was calculated and the flow rate was set up to adjust the glucose requirement. Glucose uptake rate increased from 0.7 to 0.9 g_{Glc} L^{-1} h^{-1} which corresponded to a specific uptake rate of 0.10 g_{Glc} $g_X{}^{-1}h^{-1}$. As shown in Fig. 1, no accumulation of this substrate in the culture medium was observed, and glucose was consumed throughout the growth.

The dynamic metabolic behavior of *D. geothermalis* DSM 11302 during growth in fed-batch experiments could then be analyzed in details in three phases:

Phase I (from 0 to 1.75 h)

After 30 min, the first pulse of yeast extract was made. This pulse induced a fast and immediate decrease of dissolved oxygen during few minutes. The growth rate varied from 0.85 to 1.05 h^{-1}. Then, oxygen continued to be uptaken but slower and the growth rate decreased. As a pulse of glucose was made without any change of the growth dynamic, the absence of glucose limitation was revealed.

Phase II (from 1.75 to 4 h)

Exponential yeast extract feed started. That caused a significant decrease of dissolved oxygen. The growth rate on yeast extract reached 0.97 h^{-1}. This result showed that before the addition of yeast extract, glucose was the sole substrate available in the medium and the growth was thus limited by yeast extract. The growth on yeast extract was possible until 4 h of culture, and 3.8 generations were obtained; then, in spite of yeast extract feed and pulses of glucose, the dynamic of growth and oxygen uptake slowed down.

Phase III (from 4 to 6 h)

The glucose was still consumed, but the growth rate decreased from 0.9 to 0.5 h^{-1}. The growth was linear and the biomass increased of 1.6 generations. Although both substrates were continuously added, dissolved oxygen remained constant from 4.7 to 5.7 h.

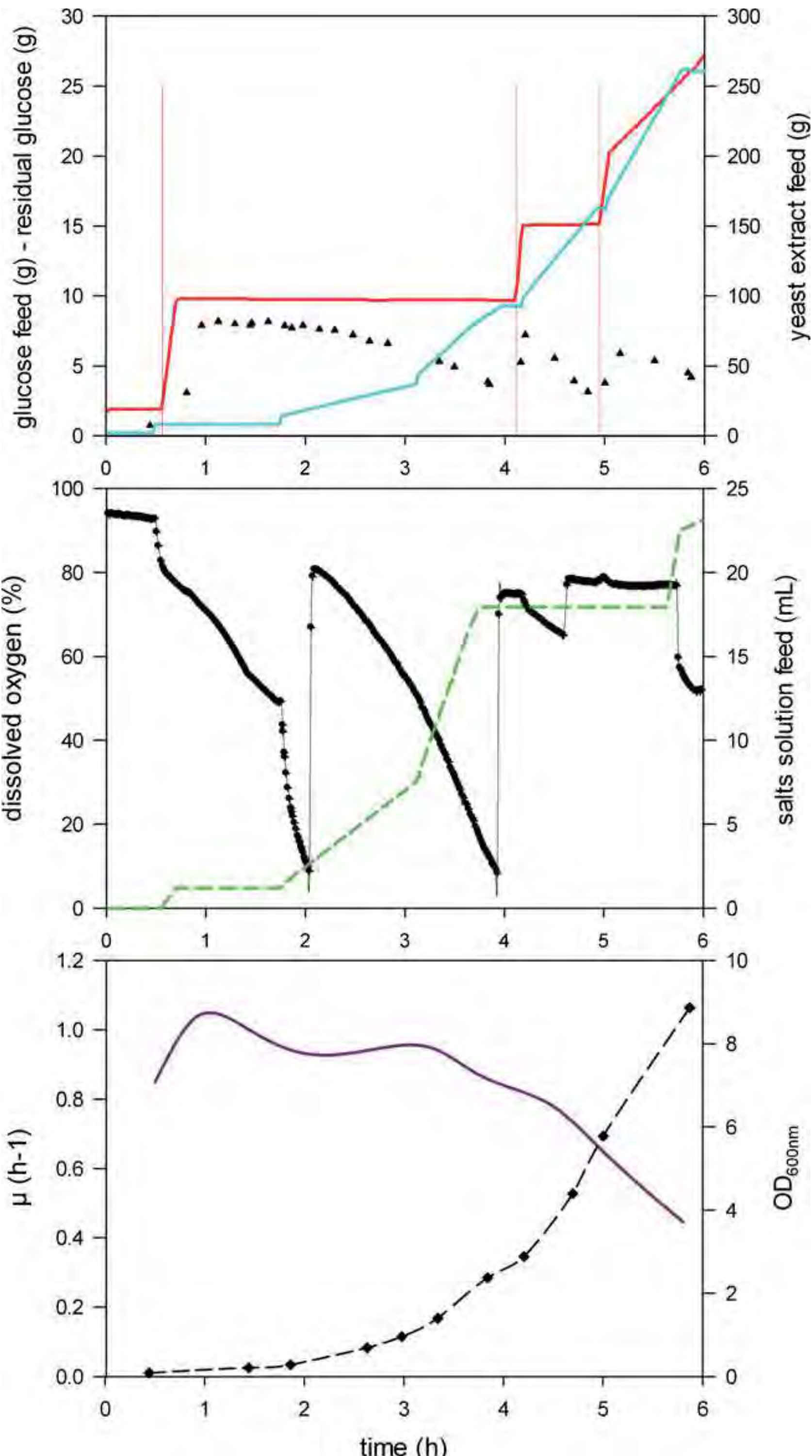

Fig. 1 Yeast extract (—), glucose supplies (—), residual glucose (▲), dissolved oxygen (●), salt solution feed (– –), growth rate (—), and $OD_{600\ nm}$ (◆) evolution during fed-batch culture of *D. geothermalis* DSM 11302 (*vertical bars* represent glucose pulses)

The saline solution supply was set at 1/10 of the total substrate flow. However, in order to investigate a potential salt toxicity, the saline solution feed was suspended during the phase III from 3.8 to 5.6 h of culture (Fig. 1). A dissolved oxygen value decrease would have revealed a salt inhibition, whereas an increase of dissolved oxygen value would have revealed that saline solution feed is required to ensure a salt non-limiting growth. The dissolved oxygen uptake and growth rate decreased, which could reveal a limitation by the salt solution. However, when the salt feed started again, it was not possible to obtain the dynamic of growth observed during phases I and II.

Fed-batch balances and yields obtained with the co-susbtrate nutritional strategy are summarized in Table 1. Residual substrate quantities in preculture medium broth have been quantified (residual glucose was analyzed and residual yeast extract was estimated with an average yield determined in previous experiments). Carbon and nitrogen sources carried by the preculture were considered in yield and carbon/nitrogen balance calculations. The final biomass concentration reached 9.6 $g_{DCW}L^{-1}$ corresponding to a total biomass of 99 g_{DCW}. This biomass was obtained by the addition of 253 g_{YE} and 26 g_{Glc}; yield on yeast extract added was 0.39 $g_X g_{YE}^{-1}$ and on glucose was 3.81 $g_X g_{Glc}^{-1}$.

The presence of the organic acids formate, malate, succinate, pyruvate, acetate, lactate, gluconate, and citrate in supernatants was analyzed; none of them was detected in samples collected until 3.5 h of culture. Then, due to the complex nutrient source yeast extract, a lot of peaks on chromatograms interfered with the analysis method. So, these organic acids were not quantified in samples harvested from 3.5 to 6 h of culture.

The elemental composition of biomass of *D. geothermalis* DSM 11302 grown on yeast extract and glucose was determined: $C_{1.00}H_{1.79}O_{0.51}N_{0.24}$. This corresponds to a molecular weight of 25.28 g $Cmol^{-1}$. This result was consistent with biomass composition of other bacteria; elemental compositions of *Escherichia coli*, *Aerobacter aerogenes*, and *Paracoccus denitrificans*, were, respectively, $C_{1.00}H_{1.77}O_{0.49}N_{0.24}$, $C_{1.00}H_{1.83}O_{0.55}N_{0.25}$, and $C_{1.00}H_{1.81}O_{0.51}N_{0.20}$ (Roels 1983).

Microscopic observations were made during the fed-batch culture of *D. geothermalis* DSM 11302. This bacterium grew as clusters of at least two cells (diplococci) in rich media under aerobic conditions (Fig. 2). *Deinococcaceae* are cells generally in pairs or tetrads (Holt and Bergey 1994) and *D. radiodurans* has been described as a bacterium typically growing as diplococci and tetracocci in rich media, due to its cell division (Daly 2004; Murray 1983).

Table 1 Mass balance, carbon balance, and yields for *D. geothermalis* DSM 11302 fed-batch culture

Substrate supplies and biomass production	Yeast extract added	Glucose added	Biomass (dry cell weight)
Substrate mass (g)	253	26	99
Substrate carbon (Cmol)	10.6	0.9	3.9
Biomass yield, $Y_{X/S}$ ($g_X g_S^{-1}$)	0.39	3.81	–
Biomass yield, $Y_{X/S}$ ($Cmol_X Cmol_S^{-1}$)	0.37	4.52	–

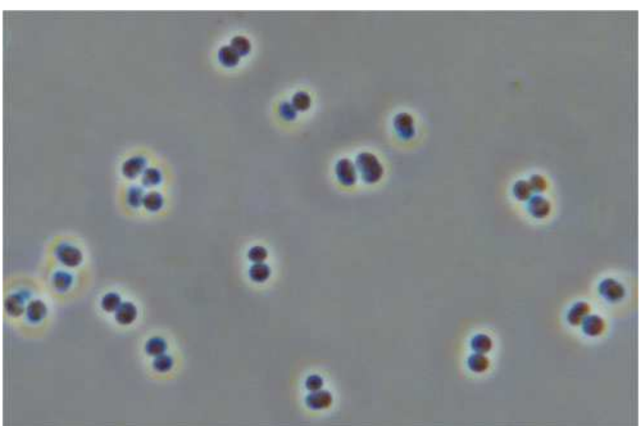

Fig. 2 *D. geothermalis* DSM 11302 grown on glucose and yeast extract in fed-batch conditions (microscopic observation ×1,000)

Carbon substrate catabolism

The results in Fig. 3 and in Table 2 confirmed that glucose was consumed throughout the culture, but it was not the preferential carbon source. At the beginning of the culture (phase I), glucose was used as the sole carbon source and carbon of yeast extract was not consumed yet. Then, between 1.4 and 2.6 h of culture, the yeast extract carbon-specific uptake rate increased up to an average of 0.68 Cmol $Cmol_X^{-1}h^{-1}$, while that of glucose-carbon decreased from 0.39 to 0.15 Cmol $Cmol_X^{-1}h^{-1}$. As soon as the exponential yeast extract feed began (phase II), the preferential carbon source was yeast extract even if glucose carbon uptake rate remained at 0.15 then 0.10 Cmol $Cmol_X^{-1}h^{-1}$. At the end of the culture (phase III), in spite of both substrate supplies, yeast extract carbon-specific uptake rate decreased from 0.68 to 0.17 Cmol $Cmol_X^{-1}h^{-1}$.

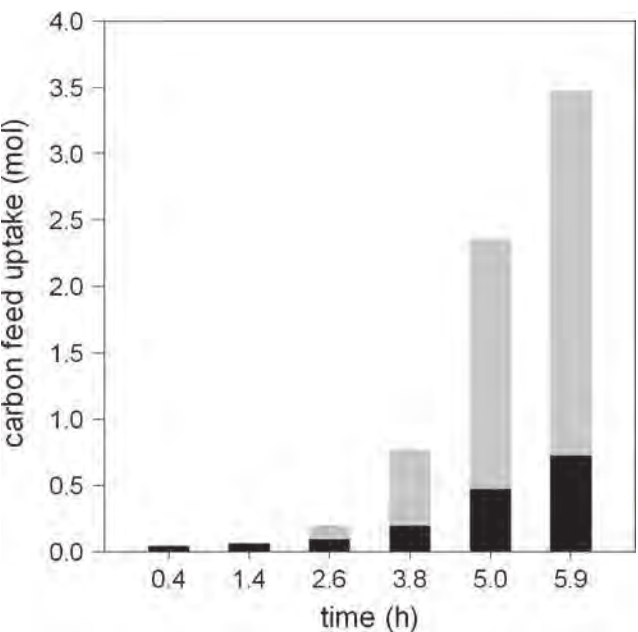

Fig. 3 Carbon substrate uptakes: yeast extract ( ) and glucose ( ) during fed-batch culture of *D. geothermalis* DSM 11302

Table 2 Carbon substrate-specific uptake rates during the three stages of *D. geothermalis* DSM 11302 culture

Culture stage	I	II	III
Glucose carbon-specific uptake rate ($Cmol\ Cmol_X^{-1} h^{-1}$)	0.39	0.15	0.10
Yeast extract carbon-specific uptake rate ($Cmol\ Cmol_X^{-1} h^{-1}$)	0 increased to 0.32	0.68	0.68 decreased to 0.17

Nitrogen source catabolism

Nitrogen accumulated in the culture medium was organic (proteins/peptides/amino acids) and mineral (ammonium), these two forms being found in similar quantities (Fig. 4). Ammoniacal nitrogen accumulation could be explained by yeast extract amino acid catabolism. Proteins/peptides/amino acids of yeast extract were partially metabolized and used as nitrogen source for biomass production.

Acidic and alkaline regulation

The correlation between acidic/alkaline regulation and yeast extract supply is presented in Fig. 5. During 4 h, low quantity of orthophosphoric acid was provided; it represented less than 2.5 mmol H^+, while 23 mmol OH^- was added with ammonia regulation. Then, alkaline regulation stopped and orthophosphoric acid supply started. At the end of the culture, 23 mmol OH^- and 166 mmol H^+ were added by the acidic/alkaline regulation.

Gas analyses

The composition of exhaust gas and air inlet of the reactor was analyzed to quantify r_{O_2} , r_{CO_2} , and RQ during the culture (Fig. 6). r_{O_2} absolute value and r_{CO_2} increased from 0.5 to 4.2 h of culture, carbon dioxide production rate being slightly lower than oxygen uptake rate. After 4.2 h, growth kinetic decreased, and after 4.5 h, the carbon dioxide production rate was higher than the oxygen uptake rate; these two rates remained constant until the end of the culture. Moreover, when yeast extract supply started at 1.75 h, RQ value increased from 0.6 to 0.9 in 2 h, and during the two following hours, it increased up to 1.1.

Discussion

First, to define a synthetic medium optimized for a non-limiting growth of *D. geothermalis*, previous experiments have been carried out in Erlenmeyer flasks and bioreactors in batch culture mode (unpublished data). The results have revealed that yeast extract and glucose are needed to ensure favorable nutritional conditions and reach *D. geothermalis* maximum growth rate. Fed-batch strategy when both glucose and yeast extract feedings can be perfectly controlled to prevent initial toxic effect of high glucose and yeast extract concentrations is the most adapted culture mode for the quantification of physiological behavior and the elucidation of the role of each substrate during the growth.

A fed-batch strategy was then developed to produce *D. geothermalis* DSM 11302 cells at a maximal growth rate, without inhibition or limitation of growth. Aerobic experiments were carried out in fed-batch culture mode with a co-substrate feeding approach. This required well-balanced substrate feed with online metabolism descriptor monitoring. The culture began with glucose as the sole carbon source, but the growth was limited. Exponential feed of yeast extract resulted in a change of catabolic behavior: when yeast extract was supplied to the medium, it was used as a preferred carbon

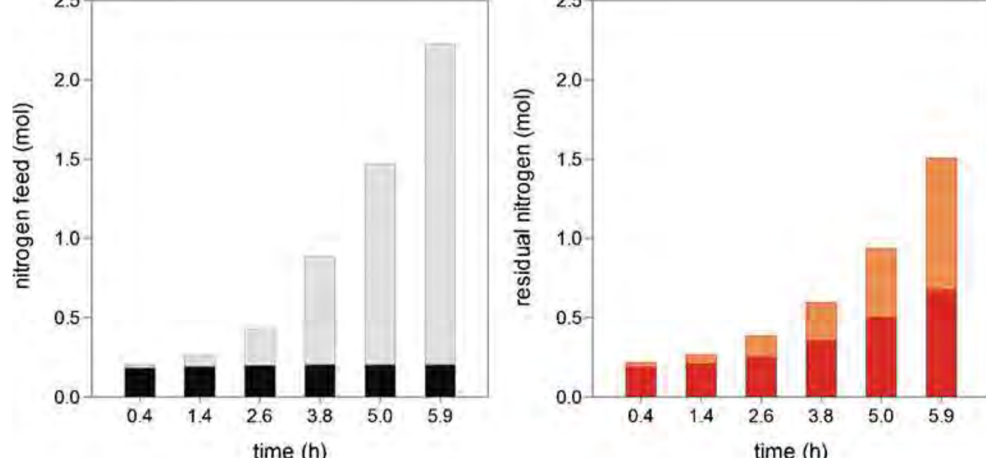

Fig. 4 Nitrogen substrate feed (*left*): yeast extract ( ) and ammonium ( ), and organic ( ) and mineral ( ) residual nitrogen (*right*) during fed-batch culture of *D. geothermalis* DSM 11302

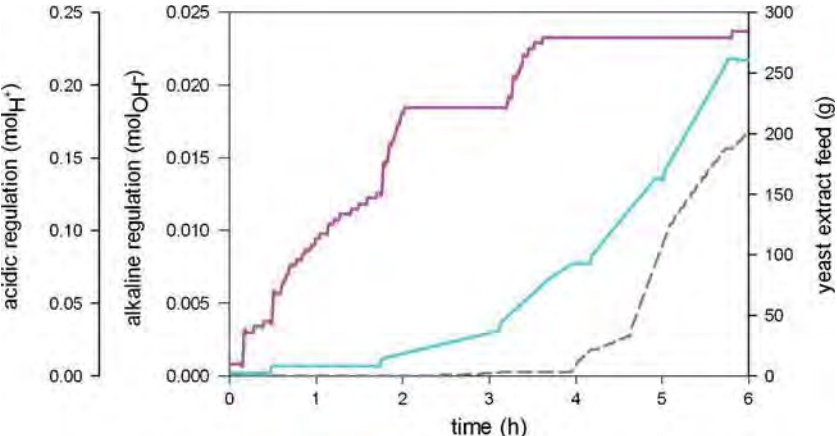

Fig. 5 Acidic (– –) and alkaline regulation (——) and yeast extract supply (——) evolution during fed-batch culture of *D. geothermalis* DSM 11302

source to produce biomass, even if there was residual glucose in the culture medium.

RQ value, which depends on the degree of reduction of substrates, increased from 0.5 to 1.1; values lower than 1 could be explained by a catabolism of compounds more reduced than glucose, probably from yeast extract (e.g., proteins, lipids). This study confirmed that nitrogen source could be mineral or organic (proteins). With the strain *D. geothermalis* DSM 11300, Brim (2003) reported that ammonia could be the sole nitrogen source in the medium, but published works have already reported that amino acids were the preferential carbon source for the growth of *D. radiodurans* grown in complex nutritive media (He 2009; Holland 2006; Venkateswaran 2000). The accumulation of mineral residual nitrogen in the medium could be explained by the catabolism of organic nitrogen sources like proteins, peptides, and amino acids. This catabolism required acidic regulation due to ammonium excretion; only 2.5 mmol H^+ was added by pH regulation. The medium buffering capacity and the carbon dioxide produced by the metabolism were sufficient to maintain the pH at 6.8. The results revealed that 30 to 34 % of the organic nitrogen sources were used as carbon source, but not as nitrogen source. The remaining fraction of organic nitrogenous compounds can be catabolized for both carbon and nitrogen utilization; amino acids could be integrated directly into the metabolic pathways too.

Nevertheless, after 4.5 h of culture, it was difficult to maintain the growth dynamic in spite of increasing yeast extract and glucose supplies. RQ stabilized at 1.1, the growth kinetic slowed down (phase III), and alkaline regulation stopped while acidic regulation started. Both substrates were consumed as carbon source. Moreover, as it is shown in Fig. 1, the saline solution supply was stopped between 3.8 and 5.7 h of culture. This strategy was chosen to check both the need of concentrated salts besides those found in yeast extract solution for the growth of *D. geothermalis* DSM 11302 and the absence of inhibition due to this saline solution. The growth decrease, in terms of oxygen uptake rate and growth rate, started just after the stop time of salt feed. Twenty minutes after the stop time of the concentrated salt feed, the specific substrate uptake rates were divided by 2 for glucose and 5 for yeast extract. So, two hypotheses could explain these

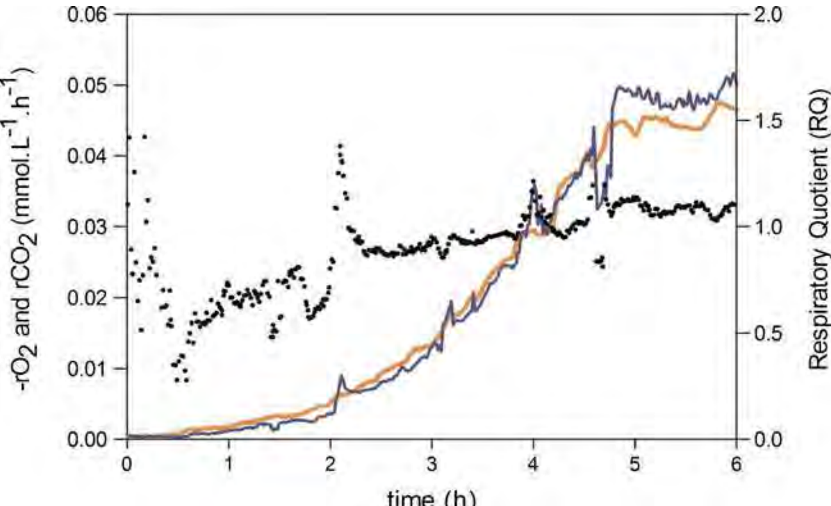

Fig. 6 Oxygen uptake rate (——), carbon dioxide production rate (——), and RQ (●) evolution during fed-batch culture of *D. geothermalis* DSM 11302

observations: it could result from a salt limitation because as soon as the saline solution started again, significant oxygen uptake was quantified. It revealed obviously that there was no inhibition due to the concentrated saline solution too. Nevertheless, this growth phase lasted only 30 min and oxygen uptake rate reached a plateau after 4.7 h of culture, corresponding to 5.7 generations. The second hypothesis was an inhibition due to the accumulation of yeast extract residual compounds or metabolic products which are toxics for the microorganism.

In fed-batch experiments, *D. geothermalis* was grown to 9.6 $g_{DCW}L^{-1}$ and reached 1.05 h^{-1} with a glucose and yeast extract adaptive feed command. This is the best growth performances reported up to date with a *Deinococcaceae* strain. In literature, He reported a final concentration of 18 $g_{WetCellWeight}L^{-1}$ of *D. radiodurans* (He 2009); the biomass has been harvested with a continuous centrifuge, and this result corresponds to cell paste and has not been quantified in dry cell weight. It has been obtained in 21 h, with a maximal growth rate corresponding to 0.3 h^{-1}, using a semi-defined medium; this medium provided 10 g L^{-1} of tryptone, 5 g L^{-1} of YE, and 10 g L^{-1} of glucose as potential carbon sources. Using the co-substrate feed strategy, the results showed that although glucose was consumed, it was not a preferential substrate for growth, and carbon and nitrogen brought by yeast extract were partially uptaken to produce biomass. By adding 253 g of yeast extract and 26 g of glucose in the culture medium, 99 g_{DCW} of biomass was obtained in this work. Yeast extract limiting factor being not identified yet, the whole yeast extract was supplied as substrate. Accumulation of yeast extract components in the culture broth could be toxic toward cells and enables reaching higher biomass concentration. Biomass production yields on yeast extract added and glucose added were, respectively, 0.4 $g_X g_{YE}{}^{-1}$ and 3.8 $g_X g_{Glc}{}^{-1}$, which represents on the whole carbon substrates of 0.4 $Cmol_X{}^{-1}Cmol_S{}^{-1}$ added.

This work reports the first quantitative description of the dynamic behavior of *Deinococcus* strain in well-controlled environmental conditions. Kinetic parameters (growth rate, substrate uptake rates, respiration rate, respiratory quotient) and conversion yields implement the knowledge on quantitative physiology of *D. geothermalis*. Such data are of interest to compare growth conditions and growth performances of several *D. geothermalis* strains grown in rich medium. This can help in defining further culture strategies.

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Conflict of interest The authors declare that they have no conflict of interest.

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