Experimental and statistical analysis of nutritional requirements for the growth of the extremophile *Deinococcus geothermalis* DSM 11300

Julie Bornot · César-Arturo Aceves-Lara · Carole Molina-Jouve · Jean-Louis Uribelarrea · Nathalie Gorret

Abstract Few studies concerning the nutritional requirements of *Deinococcus geothermalis* DSM 11300 have been conducted to date. Three defined media compositions have been published for the growth of this strain but they were found to be inadequate to achieve growth without limitation. Furthermore, growth curves, biomass concentration and growth rates were generally not available. Analysis in Principal Components was used in this work to compare and consequently to highlight the main compounds which differ between published chemically defined media. When available, biomass concentration, and/or growth rate were superimposed to the PCA analysis. The formulations of the media were collected from existing literature; media compositions designed for the growth of several strains of *Deinococcaceae* or *Micrococcaceae* were included. The results showed that a defined medium adapted from Holland et al. (Appl Microbiol Biotechnol 72:1074–1082, 2006) was the best basal medium and was chosen for further studies. A growth rate of 0.03 h^{-1} and a final OD_{600nm} of 0.55 were obtained, but the growth was linear. Then, the effects of several medium components on oxygen uptake and biomass production by *Deinococcus geothermalis* DSM 11300 were studied using a respirometry-based method, to search for the nutritional limitation. The results revealed that the whole yeast extract in the medium with glucose is necessary to obtain a non-limiting growth of *Deinococcus geothermalis* DSM 11300 at a maximum growth rate of 0.64 h^{-1} at 45 °C.

Keywords *Deinococcus geothermalis* · Nutritional requirements · Respirometry-based method · Principal Components Analysis

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Introduction

Deinococcus geothermalis belongs to the *Deinococcus–Thermus* group. It is a gram-positive and red-pigmented bacterium that forms spherical cells of 1.5–2 μm in diameter. It grows typically as diplococcus or tetracoccus. *Deinococcus geothermalis* was isolated for the first time in hot springs in Naples, Italy and in São Pedro do Sul, Portugal. It was identified at other locations including industrial paper machines where it can form biofilms (Väisänen et al. 1998; Kolari et al. 2002; Peltola 2011).

The genome of *D. geothermalis* is composed of a circular chromosome of 2,467,205 base pairs and two plasmids of 574,127 base pairs and 205,686 base pairs (Makarova et al. 2007). *D. geothermalis* can survive and repair its genome even after an exposition to high radiation levels (Ferreira et al. 1997; Brim et al. 2003). *Deinococcus geothermalis* is a moderate thermophile with an optimal growth temperature

between 45 and 50 °C. It can grow between pH 4.5–8.5 with an optimal at 6.5 (Ferreira et al. 1997).

D. geothermalis can tolerate high concentrations of solvents and reduce a variety of heavy metals which make it a good candidate for bioremediation of radioactive wastes and environments. For example, *D. geothermalis* is able to reduce Fe(III) and Cr(VI) and has been genetically modified to reduce Hg(II) (Brim et al. 2003). A strain of *D. geothermalis* isolated from soil samples (from hot springs areas, Krabi, Thailand) tolerates high concentrations of various solvents as decane, diethylphtalate, butyl acetate and ethyl acetate and can assimilate hydrocarbon solvents as carbon sources (Kongpol et al. 2008).

D. geothermalis is commonly cultivated in rich media containing at least one complex nutrient source such as yeast extract, peptone or tryptone (Ferreira et al. 1997; Brim et al. 2003). Just a few defined media compositions have been published for the growth of *D. geothermalis*. In these conditions, *D. geothermalis* is able to grow on ammonium sulfate as nitrogen source and glucose, fructose and toluene as carbon sources (Ferreira et al. 1997; Brim et al. 2003; Kongpol et al. 2008); but they were found to be inadequate for a good growth. The maximum growth rate at 45 °C was 0.12 h^{-1} (Kongpol et al. 2008). However, no quantitative studies on the nutritional needs are available in literature.

Furthermore, the nutritional requirements of the strain and physiological behavior were very difficult to evaluate from the published data because of the differences between the compositions of the media and the absence of biomass measurements and/or growth rates. Therefore, the main objective of this study was to determine the nutritional requirements of the reference strain *Deinococcus geothermalis* DSM 11300 to allow new insights into physiology of *D. geothermalis*. A synthetic defined medium is necessary to insure reproducible culture conditions to carry out physiological studies in well-controlled environmental conditions.

The multifactorial statistical method, Principal Component Analysis, was carried to compare and highlight the main differences in composition between media described in literature and media tested in flask experiments. Then, *Deinococcus geothermalis* DSM 11300 was cultivated in a bioreactor on a basal defined medium deducted from the PCA analyses. Finally, a respirometry-based strategy was chosen to study the effects of pulses of media components on oxygen uptake.

Materials and methods

Microorganism, media and growth conditions

Bacterial strain

The microorganism used in this study was *Deinococcus geothermalis* DSM 11300, provided by Deinol project partners (program DEINOL ISI of OSEO). The strain was inoculated into PGY medium broth (peptone 10 g L^{-1}, glucose 10 g L^{-1}, yeast extract 5 g L^{-1}) and incubated at 37 °C for 24 h. The bacterial cells were then stored at −80 °C with 20 % (v/v) glycerol.

Media and growth conditions

Pre-cultures of *D. geothermalis* DSM 11300 were carried out in a 5 mL tube containing 1.8 mL Complex Medium Glucose (CMG) incubated at 37 or 45 °C for 24 h on an orbital shaker (110 rpm). The Complex Medium Glucose was prepared by adding 10 g L^{-1} of glucose, 5 g L^{-1} of yeast extract, and 2 g L^{-1} of bacto-peptone to the mineral medium (see below). For each inoculum preparation, the strain was plated on PGY agar medium (PGY medium broth supplemented with agar 14 g L^{-1}) for 48 h at 37 °C. Only one colony was chosen for each inoculum. Two successive steps of pre-cultures, 15 and 150 mL for flask experiments or 18 and 180 mL for bioreactor cultures, were then carried out in CMG medium, in baffled Erlenmeyer flasks with an inoculum at 10 % (v/v). Each flask was incubated 12 h at 37 or 45 °C and 110 rpm. In Erlenmeyer flask experiments, the pH of the medium was between 6.5 and 6.8, the optimum pH for the growth of *Deinococcus geothermalis* being 6.5.

Due to technical constraints, the first experiments in flasks were conducted at 37 °C. Only four tests in baffled Erlenmeyer flasks and cultures in bioreactors were made at 45 °C. It is important to note that temperature positively affects growth rate, but has no effect on the maximal biomass concentration (Online Resource l).

After centrifugation 5 min at 4000*g* (Centrifuge 5810R, Eppendorf), the pellet was rinsed with physiological saline solution (9 g L^{-1} NaCl) before inoculation.

Basal mineral media compositions designed for the growth of various bacteria were tested: the mineral medium DM used in this study was adapted from a medium developed for the growth of *Deinococcus radiodurans* (Holland et al. 2006). The composition of the DM was designed and provided by Deinol project partners. Unlike the medium described by Holland et al. (2006), DM does not contain serine, glutamine and KH_2PO_4. In addition, glucose is used as carbon source instead of fructose and cobalamin (vitamin B12) is added at a concentration of 1 µg L^{-1} (0.01 µg L^{-1} in Holland et al. medium). The mineral medium DM was prepared as follows: 5.74 mM K_2HPO_4, 10 % v/v MOPS buffer mixture (400 mM MOPS acid, 200 mM NH_4Cl, 100 mM NaOH, 100 mM KOH, 2.76 mM Na_2SO_4, 5.28 mM $MgCl_2$ and 5 µM $CaCl_2$), 1 % v/v of a 2 mM $FeCl_3$ solution in 2 mM sodium citrate, 0.01 % v/v of a trace element 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 0.01 % v/v of each vitamin (niacin, thiamine hydrochloride, pyridoxine hydrochloride, cobalamin and biotin prepared separately at a concentration of 10 mg L^{-1}). The MOPS buffer mixture and 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 carbon source solutions were prepared and autoclaved separately and added to the mineral medium just before the inoculation.

The second medium was the chemically defined medium (MCD) usually used for the culture of lactic acid bacteria (Cocaign-Bousquet et al. 1995). The composition of the chemically defined medium designed for the growth of lactic acid bacteria was chosen because these bacteria are known to have numerous nutritional requirements to achieve an exponential growth without limitation. The MCD medium has been further implemented with the following bases adenine, guanine, uracile and xanthine to a concentration of 0.01 g L^{-1}.

The medium described by Little and Hanawalt for the culture of *Deinococcus radiodurans* (Little and Hanawalt 1973) was tested too and the last one was the MM medium developed for the culture of *Escherichia coli* (Sunya et al. 2012). The MM medium is a balanced mineral medium which provides all the essential nutrients required for the growth of *Escherichia coli.* It was used as described by Sunya et al. and twice concentrated or half diluted to evaluate the impact of several osmotic pressures on the growth of *D. geothermalis* DSM 11300.

A lot of media formulations were tested in baffled flasks; they are given in the electronic supplementary material (Online Resource 2).

Media supplementations

Yeast extract fractions were obtained by filtration of a 200 g L^{-1} yeast extract solution using Amicon Ultra-15 centrifugal filter units of 10, 30 and 50 kDa (Millipore, Germany) and Microsep centrifugal devices 3 kDa (Pall Life Sciences, USA). Each fraction was sterilized by filtration on Sartorius Minisart 0.2 µm.

Yeast extract mineral fraction was prepared by heating yeast extract powder at 550 °C during 12 h. The ashes were solubilised in concentrated acid solution (HCl or HNO_3) and diluted in ultra-pure water (18 MΩ MilliQ water). The solutions of ashes were autoclaved 20 min at 121 °C.

D. geothermalis DSM 11300 cellular extract was obtained by sonication of a 10-h cell culture in CMG. The sonication was carried out with a 13 mm diameter ultrasonic probe (vibracell 72412, Bioblock scientific, USA). 30 cycles of 20 s pulses on and off were applied. The cellular extract of *Deinococcus* was sterilized 20 min at 121 °C.

Chemicals products

Glucose, fructose, salts and oligo-elements were obtained from Prolabo (USA) and yeast extract and casamino acids vitamin-free from Difco Diagnostics (USA). Bacto-peptone was obtained from Becton–Dickinson (USA) and sucrose from Merck (Germany). $ZnSO_4$ and acetic acid were provided from Carlo Erba (France) and $MgCl_2$, vitamins, amino acids, lactic acid, egg yolk emulsion and the MOPS were provided from Sigma (USA). All products were of the highest analytical grade available.

Batch fermentations

Batch fermentations were performed in a bioreactor B Braun Biostat A (B Braun Biotech International, Germany) of 2 L working volume with a Micro DCU-300 measurement and control unit, a temperature probe (Biotech International, Germany) and dissolved oxygen and pH probes (Broadley-James Corporation, USA). Stirring and aeration were regulated to avoid oxygen limiting conditions but no pH regulation was applied. The mixing system consisted of four baffles and a three-blade marine impeller. The temperature was set at 45 °C. The antifoaming agent struktol J673 (Struktol, Germany) was used in case of foam formation during the fermentation. Online acquisition and regulation were done using MFCS/win 1.1 software package.

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

Analytical methods

Gaz analysis

The composition of exhaust gas of the bioreactor, dioxygen and carbon dioxide, was measured with a gas analyser (Innova 1313, LumaSense Technologies, USA), by photoacoustic spectroscopy for the carbon dioxide and by magneto-acoustic spectroscopy for the dioxygen. Compared with the composition of the air inlet, oxygen uptake and carbon dioxide, production rates were determined during the culture.

Biomass analysis

Biomass concentration was determined by optical density measurement at 600 nm (spectrophotometer Libra S4, Biochrom, UK) with a 2 mm absorption cell (Hellma, Germany) and cell dry weight measurements. Cell dry weight was estimated by filtration on polyamide membrane (Sartolon 0.2 µm—Sartorius, Germany) and drying to a constant weight for 48 h, at 60 °C under 200 mm Hg in a vacuum oven (Heraeus, France).

Supernatant analysis

During the fermentation, culture supernatant was obtained by centrifuging (MiniSpin Eppendorf, USA) fermentation broth samples in Eppendorf tubes at 12000*g* for 3 min. The glucose concentration in supernatant was measured with a YSI 2700 glucose analyzer (Yellow Springs Instruments®, USA).

Statistical analysis

PCA is a useful multifactorial statistical method for analyzing data of high dimension. It is a mathematical treatment that allows the reduction of dimension for the statistical exploration of complex quantitative information. Furthermore, its graphical representation is a way of expressing data to highlight their similarities and differences.

The data analyzed included the different formulations of synthetic media reported in the literature. The first column of the table corresponded to the authors having published the medium and in the other columns there were the concentrations of constituting nutrients. The data matrices used for the PCA are given in the electronic supplementary material (Online Resources 3 and 5). Obviously, if a compound was not present in the medium, its concentration was equal to zero. Variables have very heterogeneous variances so they were normalized before applying the PCA method. The standardized variables Xi* were calculated with the following formula: Xi* = (Xi−mean)/(variance)^(1/2). Then, PCA was carried out with Matlab (Statistics Toolbox, Matlab 6.5, Mathworks).

First, the data were plotted in a two-dimensional plane given by the two principal components (the component coefficient matrices are given in the electronic supplementary material: Online Resources 4 and 6), to give a descriptive view of the nutrients of the media highlighted by the analysis and the differences between the formulations. Then, with this representation of the data, the results of growth were manually reported to each medium. The comparison criteria were also the maximum exponential growth rate and biomass concentration (measured by OD at 600 nm), when they were available in the literature.

Results and discussion

Principal component analysis of the composition of defined media for the growth of *Deinococcaceae*

Fermentation media must satisfy all the nutritional requirements of microorganisms. Generally, they must provide carbon and nitrogen sources, phosphorous and sulfur, salts, oligo-elements and sometimes growth factors like vitamin(s) etc. Few results concerning nutritional requirements of *Deinococcus geothermalis* have been published to date (Ferreira et al. 1997; Brim et al. 2003; Kongpol et al. 2008). A multifactorial statistical analysis was chosen to compare the composition of the different defined media described for the culture of several strains of *Deinococcus radiodurans*, *Deinococcus geothermalis* and *Micrococcus*. Initially, *D. radiodurans* was named *M. radiodurans* because of its similarity to strains of the genus *Micrococcus*. In 1981, research on *M. radiodurans* properties showed that this strain had to be renamed and classified in a new genus, *Deinococcus* (Brooks and Murray 1981). *D. radiodurans* is the main mesophile strain of *Deinococcus* and is the most studied strain of *Deinococcaceae*. Most of the chemically defined medium has been designed for the growth of *D. radiodurans.* As described previously, PCA was chosen for this statistical analysis. PCA helped providing a graphical representation of the data and evaluating the main factors (defined medium components) that had a significant influence on the growth of *Deinococcus* and *Micrococcus* strains.

Eleven formulations were then integrated into this analysis: 10 culture media from the literature (7 for *Deinococcus* and 3 for *Micrococcus*) and 1 medium developed by Deinol partners. The PCA matrix is given in the electronic supplementary material (Online Resource 3). The best two-dimensional representation of the data was given by the first two principal components F_1 and F_2 which explained 54 % of the variance (Fig. 1). The others principal components were not retained because they explained less than 13 % of the variance. The compounds revealed by the first component F_1 are L-alanine, and L-arginine. Those revealed by F_2 are maltose, DL-aspartic acid, L-methionine, L-cysteine, L-histidine, L-tryptophan, and TRIS.

PCA revealed many elements with various concentrations between the different media compositions. After the superimposition of the growth data (maximum exponential growth rate and/or biomass concentration), it is difficult to highlight a link between compounds in the medium and the results of the growth. The maximum growth rate, 0.27 h^{-1}, was obtained for *D. radiodurans* with the medium described by Holland et al. (2006).

However, *Deinococcus geothermalis* DSM 11300 did not grow on Holland et al. (2006) medium but growth was possible on a simplified formulation of this medium provided by Deinol project partners (data not shown). This simplified medium, named DM, was chosen as a reference medium for the culture of the strain. A growth rate of 0.03 h^{-1} and a final OD_{600nm} of 0.55 (Fig. 2) were

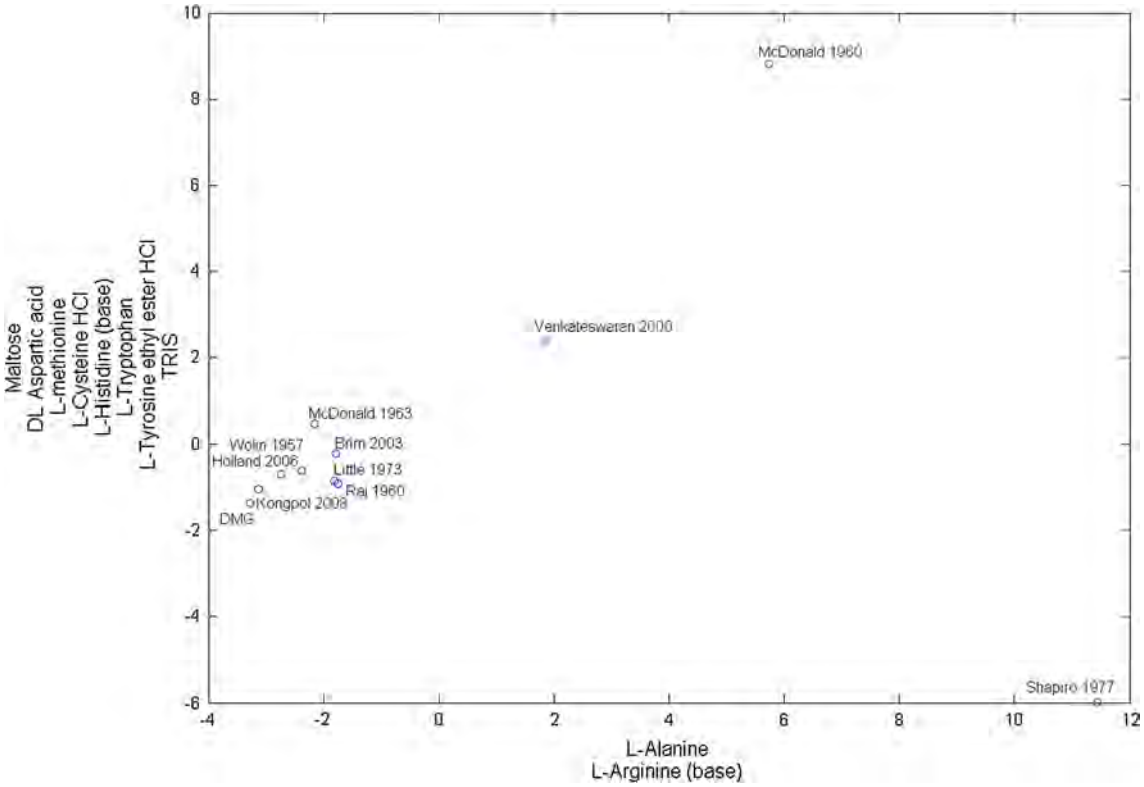

Fig. 1 Two-dimensional representation of the media formulations according to the principal components F_1 (34 %) and F_2 (20 %)

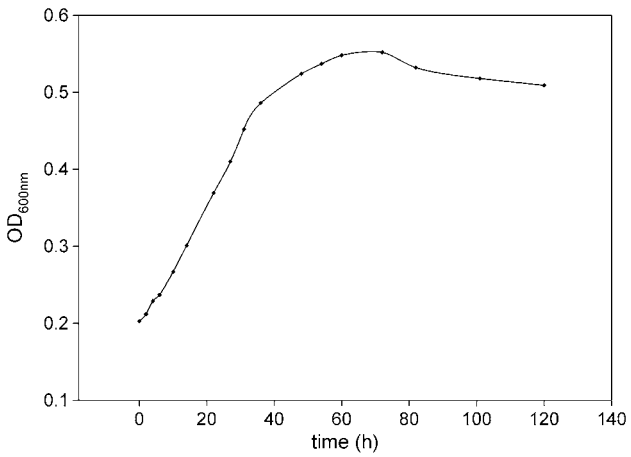

Fig. 2 Growth curve of *Deinococcus geothermalis* DSM 11300 on the mineral medium DM

obtained and the biomass yield on glucose was 0.23 g g^{-1}. However, when yeast extract was added to the medium, the growth rate and the OD_{600nm} reached 0.38 h^{-1} and 2.7, respectively. Further experiments were then necessary to test several media and component supplementations, to improve the growth of *D. geothermalis* DSM 11300 on a nutritionally controlled medium.

Growth of *Deinococcus geothermalis* DSM 11300 on several complex and defined media in Erlenmeyer flasks

The effects of the main components constituting a defined medium for the growth of microorganisms were investigated. All the media formulations tested are summarized in the electronic supplementary material (Online Resource 2). The experiments were done in baffled Erlenmeyer flasks to compare growth in terms of maximal cell concentration and kinetic (maximal OD_{600nm} and growth rate).

The results showed that fructose and sucrose could not be used as carbon source. On the other hand, glucose was assimilated by *D. geothermalis* DSM 11300 to produce biomass. At a temperature of 37 °C, the maximum OD_{600nm} and growth rate obtained with glucose 10 g L^{-1} as the sole carbon source were 1.5 and 0.08 h^{-1}, respectively, and the biomass yield reached 0.25 g g^{-1}. These results differed from results published by Ferreira who reported that *D. geothermalis* can utilize fructose and sucrose as carbon source (Ferreira et al. 1997). Lactate and acetate were not consumed as it was reported for *D. radiodurans* (Venkateswaran et al. 2000).

D. geothermalis DSM 11300 was not able to utilize $(NH_4)_2HPO_4$ or NH_4Cl. Unlike previous results (Brim et al.

2003), ammonia could not be the sole nitrogen source in the medium.

Many salts and oligo-elements (NH4Cl, $(NH_4)_6Mo_7O_{24}$, H_3BO_3, $CoCl_2$, $CuSO_4$, $MnCl_2$ $FeCl_3$, and $MnSO_4$) and vitamins (biotin, thiamine, pyridoxal, niacin, pantothenate, mesoinositol, aminobenzoic acid, and cobalamin) were added as medium supplementations. None of the minerals and growth factors tested could provide a non-limiting growth of the strain. A linear growth was observed, and the growth rate did not excess 0.03 h^{-1} as observed with the reference medium.

No growth was observed when manganese or iron was supplemented to the basal medium, even if it has been reported that manganese and iron play an important role in *D. radiodurans* metabolism and radiation resistance (Chou and Tan 1990; Daly et al. 2004).

Egg yolk emulsion, that could serve as a precursor for the unusual phospholipids and fatty acids identified by several authors in *Deinococcaceae* cell wall (Work 1964; Knivett et al. 1965; Thornley et al. 1965; Work and Griffiths 1968), was added to the reference medium. No growth was observed within these lipid-enriched conditions.

To focus on particular metabolism pathways associated with radiation resistance, arginine and glutamate were tested as specific growth factors for the culture of *D. geothermalis* DSM 11300. Genes encoding for nitric oxide synthase (NOS), enzymes catalyzing the production of nitric oxide (NO) from L-arginine, have been identified in *D. radiodurans* (Adak et al. 2002; Reece et al. 2009). Patel and Crane have reported that these bacterial NOS appear to be involved in preventing damage caused by irradiation and oxidation (Crane 2008; Patel et al. 2009). Arginine and glutamate pathways are linked but neither arginine nor glutamate was used as substrates in the defined medium; both supplementations could not improve the growth when glucose was present. This result was surprising because glutamate has been described as a preferred carbon and/or nitrogen source for *D. radiodurans* growth (Holland et al. 2006; He 2009).

A relationship between gluconate-induced Entner–Doudoroff pathway and the carbohydrate catabolism was considered. D-gluconate was added to the mineral medium formulation. Although the Entner–Doudoroff and the pentose phosphate pathways appear to be functional for the sugar catabolism in *Deinococcus radiodurans* (Venkateswaran et al. 2000; Makarova et al. 2001; Zhang et al. 2003), no growth of *D. geothermalis* DSM 11300 was observed on gluconate.

Finally, complex carbon and nitrogen sources were supplemented to the media formulations: the casamino acids vitamin-free provides all the essential amino acids (except tryptophan); tryptone and bacto-peptone contain oligopeptides and provide nitrogen, amino acids and vitamins in the culture medium. In addition, tryptone is rich in tryptophan. The observed growth in the presence of casamino acids vitamin-free, tryptone or bacto-peptone, added alone or with glucose, was linear. It was possible to achieve a maximum growth rate of 0.1 h^{-1} with these substrates as carbon and/or nitrogen sources (Liedert et al. 2012) or as growth factors. When yeast extract was added to the medium, an exponential growth was observed with growth rate of 0.38 and 0.64 h^{-1} at 37 and 45 °C, respectively. These results were in accordance with previous works which showed that *D. radiodurans* prefers other carbon sources than glucose, like amino acids in yeast extract (Venkateswaran et al. 2000). Ferreira et al. (1997) reported a growth rate of 0.75 h^{-1} at pH 7 and 45 °C for the growth of *D. geothermalis* DSM 11300, cultivated in the presence of tryptone and yeast extract.

These results did not highlight the nature of the essential(s) compound(s) for the growth of *D. geothermalis* DSM 11300. At least one or several growth factor(s) were lacking in the DM medium. Unless, yeast extract was added to the medium, none of the conditions described above made it possible to obtain a growth without limitation. This result was surprising because Ferreira et al. (1997) reported that yeast extract was not required for the growth of *D. geothermalis* DSM 11300; but no quantitative data of biomass concentration, OD or growth rate in a minimal medium without yeast extract were available in this published work. Nevertheless, all these experimental results implement the knowledge of the nutritional requirements for the growth of *Deinococcus geothermalis* DSM 11300.

Principal component analysis of published defined media and formulations tested in flask experiments for the growth of *Deinococcaceae*

A second PCA was carried out integrating data from literature and flask experiments.

As the previous analysis, the basic knowledge available on the substrates and various nutrient formulations for the growth of *Deinococcaceae* and *Micrococcaceae* was collected from the existing literature. The composition of the media used for flask experiments was implemented, resulting in a total of 48 media formulations; the PCA matrix is given in the electronic supplementary material (Online Resource 5).

In this case, F_1 and F_2 explained only 39 % of the variance; the others principal components were not retained because they explained less than 13 % of the variance (Fig. 3). The compounds highlighted by the first component F_1 are L-lysine, L-proline and L-valine. Those revealed by F_2 are glycerol, L-malic acid, potassium

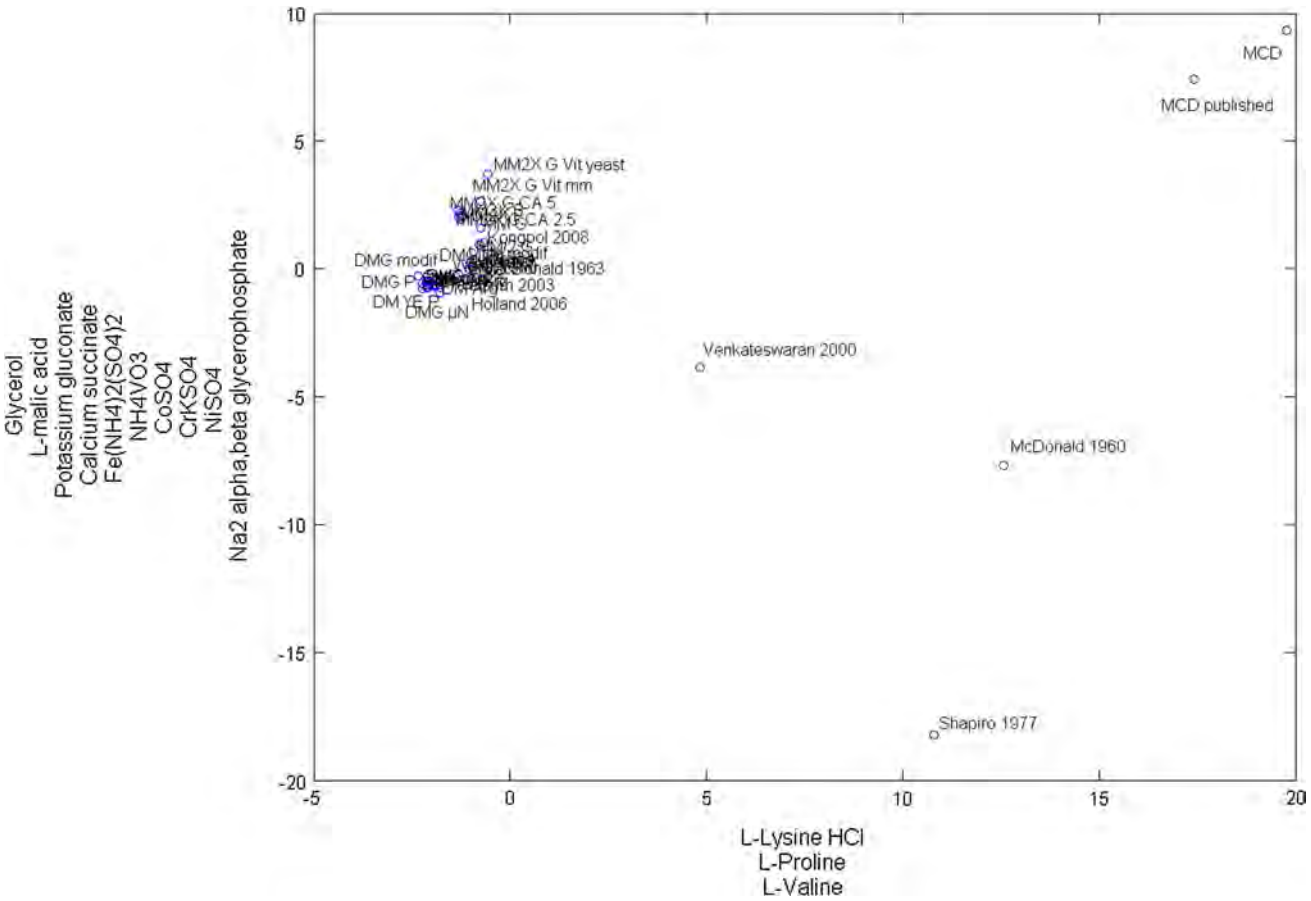

Fig. 3 Two-dimensional representation of the media formulations according to the principal components F_1 (25 %) and F_2 (14 %)

gluconate, calcium succinate, $Fe(NH_4)_2(SO_4)_2$, NH_4VO_3, $CoSO_4$, $CrKSO_4$, $NiSO_4$, and Na_2 α,β glycerophosphate.

This statistical analysis did not highlight significant differences in the composition of culture media which could explain the physiological behavior or the nutritional requirements of *D. geothermalis* DSM 11300. Too much variability between the media and the observations was observed to focus on just few compounds which can influence significantly the results.

Study of the influence of nutrient solution pulses on the growth of *Deinococcus geothermalis* DSM 11300 in fermentation conditions

Another strategy was then chosen to study the effect of specific nutrients on the growth of *D. geothermalis* in well-controlled environmental conditions. Culture in bioreactor were carried out to ensure no oxygen limitation and to monitor the pH value. A respiratory approach has been carried out to have access to respiration rate, a pertinent sensor for microbial activity. The oxygen uptake rate (OUR) is a physiological characteristic of a culture which could be directly related to the metabolic activity of the cells. OUR increases in the exponential growth phase because there is an increase of the metabolic activity of the microorganism due to a high substrate uptake rate (Garcia-Ochoa et al. 2010). Respirometry was previously used for the characterization of the microbial growth and the kinetic of biodegradation in organic wastes (Marsili-Libelli and Vaggi 1997; Tremier et al. 2005; Di Trapani et al. 2011). In addition, respirometric methods were applied to study the effect of nutrients pulses on the kinetic of growth of microorganisms (Oliveira et al. 2011). The OUR curve reflects the response of the microorganism, linked to its biological activity, when the nutritional environment is modified (Paca et al. 2010).

Pulses of carbon sources, nitrogen sources, salts, oligo-elements, vitamins, and casamino acids

1.8 L of defined medium DM was inoculated with *D. geothermalis* DSM 11300. After 30 min of decrease, the value of the pO_2 stabilized at 60 %. After 3 h, no increase in OD_{600nm} was observed traducing an absence of growth of the microorganism. After 3 h with constant dissolved oxygen pressure, pulses of nutritive element solutions were

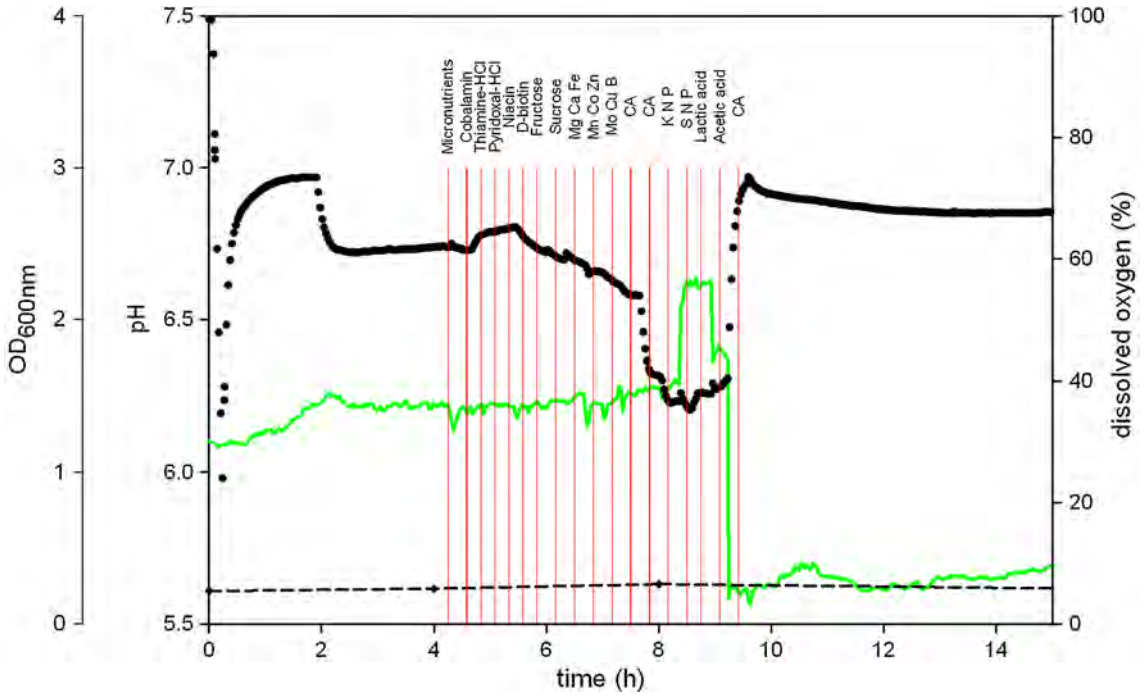

Fig. 4 Time course evolution of the dissolved oxygen (●), the OD_{600nm} (– ◆ –) and the pH (——) during the culture of *Deinococcus geothermalis* DSM 11300 (nutritional pulses are represented by the *vertical bars*—Mg = $MgSO_4$, Ca = $CaCl_2$, Fe = $FeSO_4$, Mn = $MnSO_4$, Co = $CoCl_2$, Zn = $ZnSO_4$, Mo = Na_2MoO_4, Cu = $CuCl_2$, B = H_3BO_3, CA = casamino acids vitamins-free, K N P = K_2HPO_4 + $(NH_4)_2HPO_4$, S N P = Na_2HPO_4 + $(NH_4)_2SO_4$ + NH_4Cl)

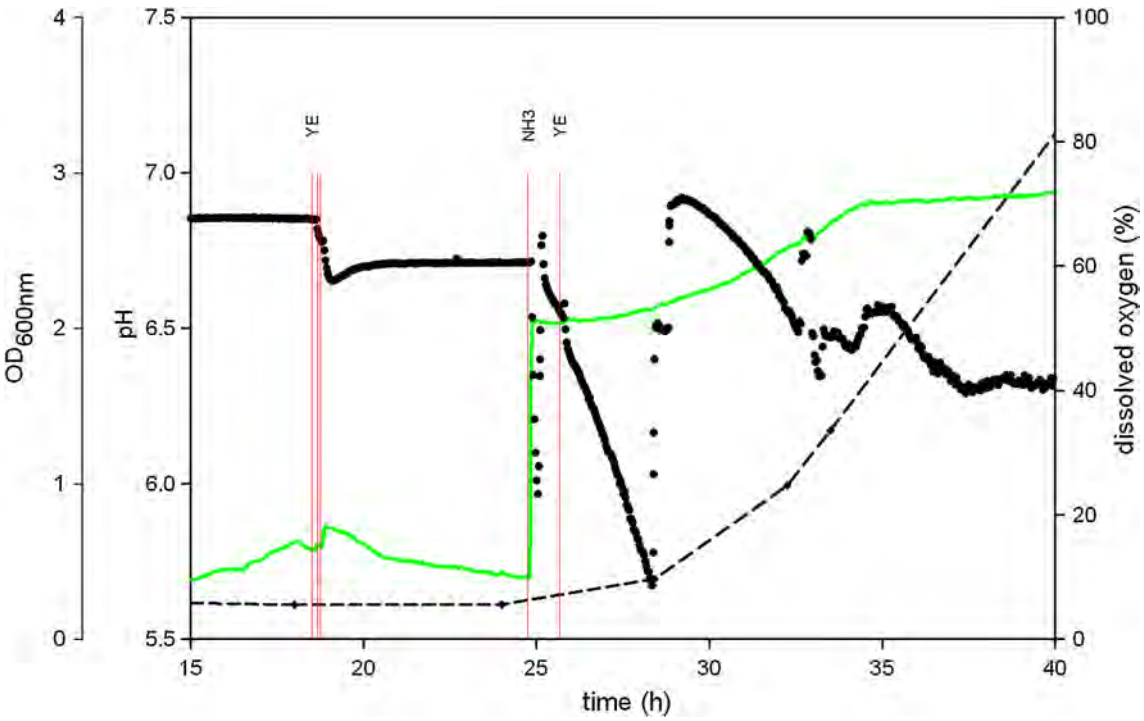

Fig. 5 Time course evolution of the dissolved oxygen (●), the OD_{600nm} (– ◆ –) and the pH (——) during the culture of *Deinococcus geothermalis* DSM 11300 (nutritional pulses are represented by the *vertical bars*—YE means yeast extract)

made. Their effects on the cellular activity of *D. geothermalis* DSM 11300 were quantified by measuring the dissolved oxygen pressure online. The experimental results are shown in Figs. 4 and 5.

The micronutrients were supplemented at a concentration tenfold higher than their initial concentration in the medium. Fructose and sucrose were added at 2 g L^{-1}, lactate and acetate at 1 g L^{-1} and the casamino acids at 25 and 250 mg L^{-1}. Yeast extract was added at a concentration varying from 0.5 to 2 g L^{-1}. Vitamins, salts and oligo-elements were added at a concentration equal to 10 % of their initial concentration. All these concentrations were calculated to induce detectable response on the oxygen uptake, in the case of a positive effect of the supplementations on the metabolic activity.

The pulses of solutions of micronutrients, vitamins, fructose, sucrose, oligo-elements (Mg, Ca, Fe, Mn, Co, Zn, Cu, H_3BO_3, Mo) had poor influence on the variation of the pO_2, there was no significant oxygen uptake (Fig. 4).

When the casamino acids vitamin-free was added for the first time, pO_2 decreased during 40 min. Nevertheless, no significant growth was observed regarding to the OD_{600nm}. The casamino acids may have been used as carbon source or provided one or several amino acids able to stimulate the metabolic activity but not enough to activate the growth. The pulses of solutions of salts (K, N, P and S) did not cause oxygen uptake. Those salts were not the factors limiting the growth (Fig. 4).

Lactic and acetic acids could not be used as source of carbon in these conditions. However, the addition of the acids caused an increase in the value of the pO_2 (Fig. 4). The pH variation had an important effect on the respiratory activity of *D. geothermalis*. In spite of the results of Ferreira et al. 1997, *Deinococcus geothermalis* seemed to be affected by the reduction of the pH value to 5.7. Indeed, even when casamino acids or yeast extract were added, it had a poor effect on the cellular activity at this pH value.

After 24 h of culture, there was no increase of the OD_{600nm} and only 0.9 g L^{-1} of glucose was consumed. After an addition of ammonia which increased the pH to 6.6 (Fig. 5), a pulse of yeast extract induced a significant oxygen uptake concomitant to glucose consumption. The value of the OD_{600nm} increased from 0.22 to 4.5 in 26 h, with a maximum growth rate of 0.25 h^{-1}. The growth factor(s) was (were) provided by yeast extract and was effective at pH close to 6.6.

Previous results with manganese and magnesium were checked and few nitrogen sources were tested (Fig. 6). During this experiment, a 4-h linear growth phase corresponding to less than one generation was obtained and the pO_2 value remained at 30 %. The oxygen uptake rate was constant, the OD_{600nm} increased from 0.69 to 0.94 at

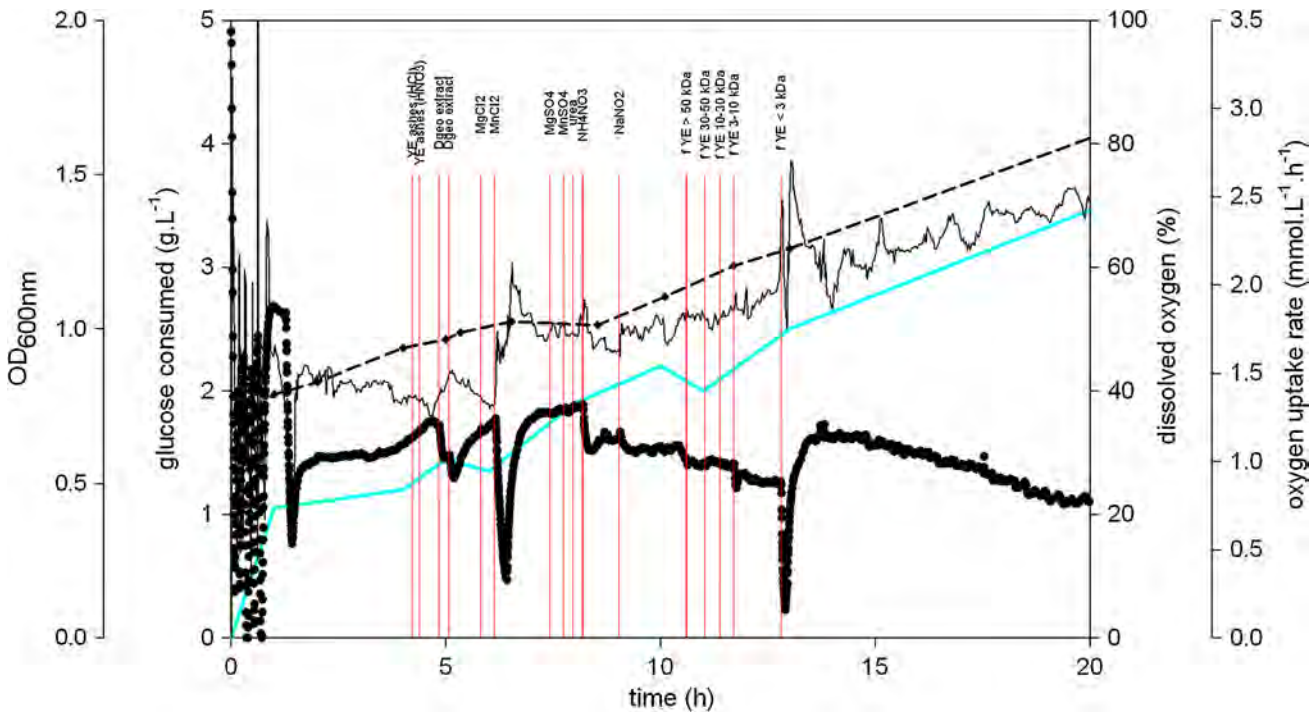

Fig. 6 Time course evolution of the dissolved oxygen (●), the glucose uptake (——), the OD_{600nm} (– ◆ –) and the oxygen uptake rate (——) during the culture of *Deinococcus geothermalis* DSM 11300 (nutritional pulses are represented by the *vertical bars*—YE means yeast extract, Dgeo extract means *Deinococcus geothermalis* DSM 11300 extract and f YE means fraction of yeast extract)

0.04 h^{-1} linear growth rate. To investigate the limitation in the medium, pulses of potential lacking compounds were made. The salts $MgCl_2$, $MgSO_4$ and $MnSO_4$ (1 mM each pulse) did not have any effect on the value of the pO_2. $MnCl_2$ increased the respiratory activity and the pO_2 fell to 9 % in 17 min. However, this pulse did not have a direct effect on the growth because no increase of the biomass was quantified, even if manganese was described as an important oligo-element for the biomass production of *Deinococcus geothermalis* (Zhang et al. 2000; Chou and Tan 1990). Then, nitrogen sources were added at a concentration equal to 1 mM. $NaNO_2$ and urea pulses had no influence. The addition of NH_4NO_3 resulted in a decrease of 8 % of pO_2 which was not considered as a significant response because no effect on the respiration of the microorganism was observed. Nevertheless, a linear growth was obtained after the pulse of NH_4NO_3. The OD_{600nm} increased at a growth rate of 0.05 h^{-1}. After 13 h of culture, an OD_{600nm} of 1.26 was reached (0.04 h^{-1} growth rate) and 2.5 g L^{-1} of glucose was consumed. During this culture, the respiratory quotient (RQ) was equal to 1.2. The effects of manganese and NH_4NO_3 were checked with experiments in Erlenmeyer flasks (data not shown). Neither manganese nor NH_4NO_3 improved the growth rate of *D. geothermalis* DSM 11300 cultivated on DM with glucose (10 g L^{-1}) as carbon source. A linear growth with a growth rate equal to 0.05 h^{-1} was obtained.

Pulses of several factions of yeast extract, whole yeast extract and Deinococcus geothermalis DSM 11300 extract

Since yeast extract was necessary to achieve good growth of *D. geothermalis*, its fractionation was done to observe which compound(s) may be responsible for the stimulating effect. According to Fig. 7, during the first 4 h which followed inoculation, there was no variation of the pO_2. The cells were viable and oxygen brought was sufficient to maintain the respiratory activity of the cells. However, no growth was observed, confirming the results of the previous fermentation. The growth being always limited after 4 h of culture, the pulses of nutritive elements in the DM started. The concentrations of the fractions of yeast extract added are listed in Table 1. Yeast extract ashes were added at a concentration equivalent to 0.1 g L^{-1} of yeast extract. Autoclaved and filtered yeast extract were added at a concentration of 0.1 g L^{-1} too. The results are shown in Figs. 7, 8 and in Table 1.

Five fractions of yeast extract were tested; the fraction containing compounds of molecular weight lower than 3 kDa induced a fast decrease of the pO_2, correlated with an increase of the oxygen uptake rate rO_2: it showed the presence of a growth factor within this solution. This growth factor could be a salt, a vitamin, an amino acid, a cofactor, a nitrogenous base, a peptide, etc. The addition of the fraction with compounds of molecular weight lower

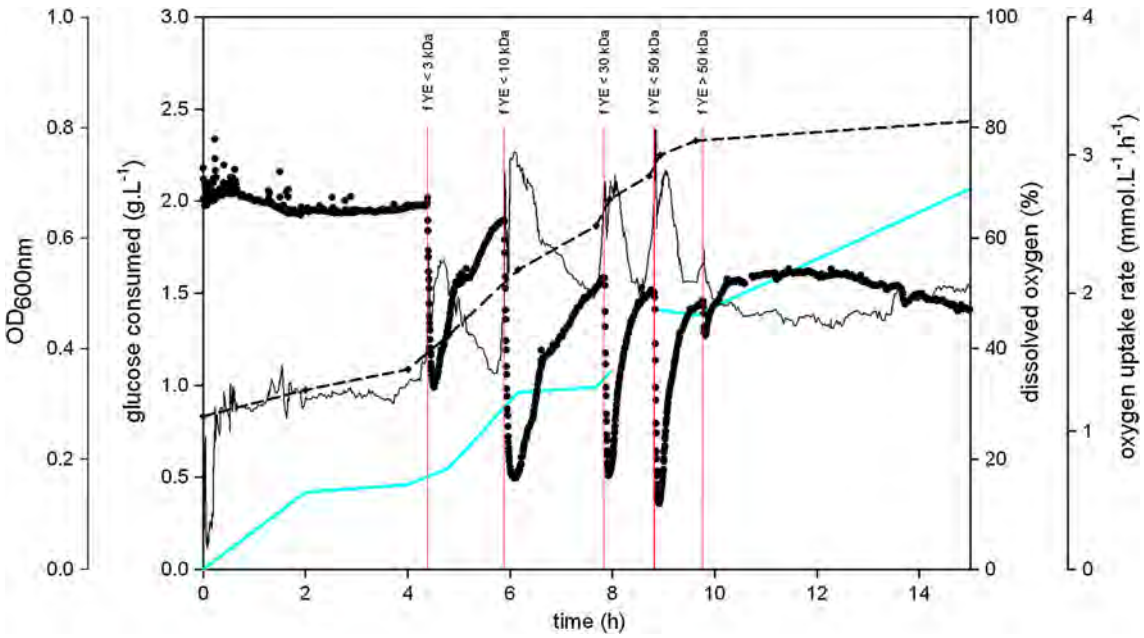

Fig. 7 Time course evolution of the dissolved oxygen (●), the glucose uptake (——), the OD_{600nm} (– ◆ –) and the oxygen uptake rate (——) during the culture of *Deinococcus geothermalis* DSM 11300 (nutritional pulses are represented by the *vertical bars*—f YE means fraction of yeast extract)

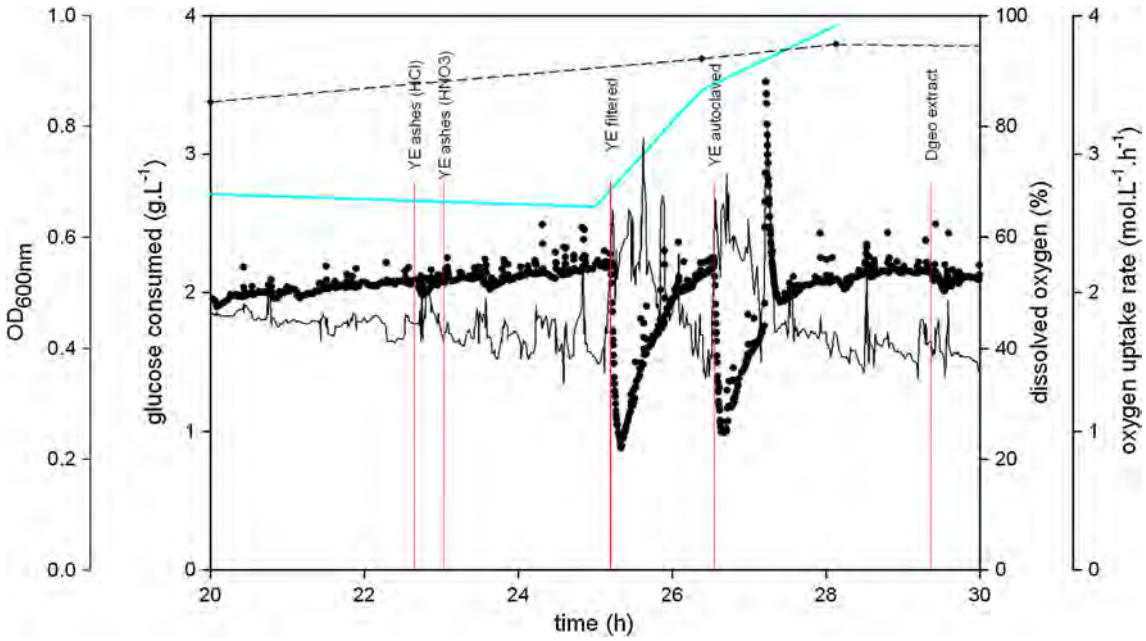

Fig. 8 Time course evolution of the dissolved oxygen (●), the glucose uptake (——), the OD_{600nm} (– ◆ –) and the oxygen uptake rate (——) during the culture of *Deinococcus geothermalis* DSM 11300 (nutritional pulses are represented by the *vertical bars*—YE means yeast extract and Dgeo extract means *Deinococcus geothermalis* DSM 11300 extract)

Table 1 Decrease of the dissolved oxygen according to the concentration of yeast extract fraction pulses

Fractions	Concentrations (g L^{-1})	Decrease of the dissolved oxygen (%)
$F < 3$ kDa	1	63 → 33
$F < 10$ kDa	0.3	63 → 17
$F < 30$ kDa	0.1	53 → 17
$F < 50$ kDa	0.1	50 → 12
$F > 50$ kDa	0.1	–

than 10 kDa resulted in an increase in the respiratory activity more important than the previous fraction. Moreover, the pO_2 did not return to its initial value. This fraction induced an increase in the oxygen uptake which was maintained after the pulse. The fractions with compounds of molecular weight lower than 30 kDa or lower than 50 kDa had the same effect on the pO_2 value but the pO_2 reached its initial value. The results observed in terms of respiratory activity were correlated with the growth of *D. geothermalis* DSM 11300. Indeed, the OD_{600nm} increased during the same time, from 0.36 to 0.78, with a growth rate of 0.14 h^{-1}. This growth rate is lower than the 0.64 h^{-1} obtained in flask experiments at 45 °C. It suggested that these fractions of yeast extract could increase the metabolic activity and the growth of *D. geothermalis*, but whole yeast extract was needed to achieve a non-limiting growth with the maximum growth rate. It resulted in a yield of 0.26 g g^{-1} of biomass produced per yeast extract added to the medium. During the same time, 0.9 g L^{-1} of glucose was consumed. Finally, the fraction with compounds of molecular weight higher than 50 kDa had no significant influence on the cellular activity of *Deinococcus geothermalis* DSM 11300. Despite the nutrients pulses, the RQ was constant at 1.2.

The mineral fraction of yeast extract (ashes) did not have any effect on the respiratory activity of *D. geothermalis* DSM 11300 (Fig. 8). It seemed that the growth factor was not a mineral one. The viability of the cells was checked by addition of 0.1 g L^{-1} of complete yeast extract. It caused a decrease of the pO_2 and an increase of the oxygen uptake rate. The OD_{600nm} increased after both pulses and 1.3 g L^{-1} of glucose were consumed during the same time. The increase of the OD_{600nm} from 0.80 to 0.95 is in accordance with a maximum growth rate of 0.63 h^{-1}. At 45 °C, when yeast extract was added in experiments in Erlenmeyer flasks, the growth rate was 0.64 h^{-1}. With the whole yeast extract, the growth was better than with the fractions. The yeast extract could provide a growth factor or been used as nitrogen and/or carbon source. In addition, two methods of yeast extract sterilization were tested: filtration and autoclave. Both pulses induced the same response. There was no difference between yeast extract filtered or autoclaved on the respiratory activity of *Deinococcus*. Then, the results revealed that a cellular extract of *Deinococcus geothermalis* cannot be used to induce the growth of *Deinococcus geothermalis*. This result may be

explained by the difference of composition between yeast extract and the bacterial extract. Brown et al. (1996) compared the biochemical composition of several marine strains of yeasts and bacteria. They found that yeasts and bacteria contained the same ratio of nucleic acid and lipids but yeasts contained less protein and ashes but more carbohydrates than bacteria. The results have shown that the composition in amino acids, fatty acids and sugars varied with the strain. In addition, the difference in composition may be related to the stage of harvest of the culture for the preparation of the extract.

Results shown in Fig. 6 confirmed that yeast extract ashes had no effect on the growth of *D. geothermalis* DSM 11300. With the pulses of 0.05 g L^{-1} of yeast extract fractions, it was confirmed that the substances having a significant effect on the pO_2 and on the cellular activity of *D. geothermalis* DSM 11300 were provided by the fraction of yeast extract with compounds of molecular weight lower than 3 kDa. There was an increase of the oxygen uptake rate, although the OD_{600nm} continued to increase and the glucose to be consumed by following the same dynamics.

Conclusions

The formulation of a synthetic culture medium well adapted to the culture of *D. geothermalis* DSM 11300 was assessed using a Principal Components Analysis (PCA) of eleven media compositions reported in the literature. Even if the PCA did not highlight a particular group of components associated with growth efficiency, the maximum growth rate of 0.27 h^{-1} was achieved on Holland et al. medium formulation with *D. radiodurans*. However, it provided a limited growth of the strain *D. geothermalis* DSM 11300, the simplified mineral medium DM adapted from Holland et al. was chosen as a reference medium. Thirty-seven complementary formulations were tested in Erlenmeyer flasks to implement the data used in PCA. Despite the number of formulations, the analysis could not discriminate compounds which would improve significantly the growth of *D. geothermalis* DSM 11300.

An original respirometry-based approach was conducted to study the effect of nutrients on the dynamics of growth and oxygen uptake of *D. geothermalis* DSM 11300 in a well-controlled bioreactor with the DM medium with glucose. Despite the large number of different solutions added to the medium, the growth factor(s) was (were) not revealed by this nutritional strategy of pulses. No growth or a maximum growth rate of 0.08 h^{-1} was obtained without yeast extract supplementation. Contrary to a previous work which showed that yeast extract was not necessary for the growth of *D. geothermalis* (Ferreira et al. 1997), this observation was in accordance with results published by Kongpol et al. (2008). He reported that the growth of *D. geothermalis* T27, a solvent-tolerant strain isolated in Thailand, was possible but poor in the absence of yeast extract.

Experimental results showed that only yeast extract or its fraction containing the compounds of molecular weight lower than 3 kDa had a positive effect on the growth. *Deinococcus geothermalis* DSM 11300 requires a single or several growth factor(s) with low-molecular weight (vitamin, amino acid, cofactor, nitrogenous base, and peptide etc.) to achieve a growth rate of 0.14 h^{-1}. However, the whole yeast extract is necessary to obtain a non-limiting growth with the maximum growth rate of 0.33 h^{-1} at 37 °C and 0.64 h^{-1} at 45 °C. Several explanations can be made to explain the positive effect of the growth factor(s). The growth factor is not a constitutive element of the microorganism, otherwise no growth at all would be obtained in its absence. So, it could be an element that (1) acts as a catalyst, (2) combines with a toxic compound present in the medium or (3) modifies physicochemical properties of the medium to permit the growth (Koser and Saunders 1938).

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Conflict of interest The authors declare that they have no competing interests.

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