Appl Microbiol Biotechnol
DOI 10.1007/s00253-015-7007-1

Production of halophilic proteins using *Haloferax volcanii* H1895 in a stirred-tank bioreactor

Eva Strillinger[1] · **Stefan Wolfgang Grötzinger**[2,3] · **Thorsten Allers**[4] · **Jörg Eppinger**[2] · **Dirk Weuster-Botz**[1]

Received: 15 July 2015 / Revised: 2 September 2015 / Accepted: 12 September 2015

Abstract The success of biotechnological processes is based on the availability of efficient and highly specific biocatalysts, which can satisfy industrial demands. Extreme and remote environments like the deep brine pools of the Red Sea represent highly interesting habitats for the discovery of novel halophilic and thermophilic enzymes. *Haloferax volcanii* constitutes a suitable expression system for halophilic enzymes obtained from such brine pools. We developed a batch process for the cultivation of *H. volcanii* H1895 in controlled stirred-tank bioreactors utilising knockouts of components of the flagella assembly system. The standard medium Hv-YPC was supplemented to reach a higher cell density. Without protein expression, cell dry weight reaches 10 g L^{-1}. Two halophilic alcohol dehydrogenases were expressed under the control of the tryptophanase promoter *p.tna* with 16.8 and 3.2 mg g_{CDW}^{-1}, respectively, at a maximum cell dry weight of 6.5 g L^{-1}. Protein expression was induced by the addition of L-tryptophan. Investigation of various expression strategies leads to an optimised two-step induction protocol introducing 6 mM L-tryptophan at an OD_{650} of 0.4 followed by incubation for 16 h and a second induction step with 3 mM L-tryptophan followed by a final incubation time of 4 h. Compared with the uncontrolled shaker-flask cultivations used until date, dry cell mass concentrations were improved by a factor of more than 5 and cell-specific enzyme activities showed an up to 28-fold increased yield of the heterologous proteins.

Keywords Gene expression · Halophile · *Haloferax volcanii* · Stirred-tank bioreactor · Biocatalysis · Biotechnology

Introduction

Biocatalysts commonly outperform chemical catalysts with respect to regio- and substrate selectivity and are active under

Eva Strillinger and Stefan Wolfgang Grötzinger contributed equally to this work.

✉ Jörg Eppinger
jorg.eppinger@kaust.edu.sa

✉ Dirk Weuster-Botz
d.weuster-botz@lrz.tum.de

Eva Strillinger
e.strillinger@lrz.tum.de

Stefan Wolfgang Grötzinger
stefan.grotzinger@kaust.edu.sa

Thorsten Allers
thorsten.allers@nottingham.ac.uk

[1] Institute of Biochemical Engineering, Department of Mechanical Engineering, Technische Universität München, Munich, Germany

[2] Division of Physical Sciences and Engineering, Biological & Organometallic Catalysis Laboratory, KAUST Catalysis Center (KCC), King Abdullah University of Science and Technology, Thuwal, Kingdom of Saudi Arabia

[3] Computer, Electrical and Mathematical Sciences and Engineering division, Computational Bioscience Research Center (CBRC), King Abdullah University of Science and Technology, Thuwal, Kingdom of Saudi Arabia

[4] Institute of Genetics, School of Biology, University of Nottingham, Queen's Medical Centre, Nottingham, UK

Published online: 01 October 2015

mild, sustainable and aqueous reaction conditions (Arends et al. 2007; Hatti-Kaul et al. 2007; Hollmann et al. 2011; Rozzel 1999). Today, enzymatic and whole-cell catalysis has found a broad range of applications, particularly in the production of fine chemicals, pharmaceuticals or agricultural intermediates (Meyer 2011). However, many enzymes, which are utilised currently, do not satisfy all industrial demands. High temperatures, high salt concentrations or high pressures (van den Burg 2003) pose major challenges for enzymatic production processes, given that most of the enzymes used originate from mesophilic organisms (Hough and Danson 1999) and show sufficient stability only at moderate reaction conditions. Correspondingly, interest in enzymes and microbes from extreme habitats such as brine pools, the Great Salt Lake, Antarctica, geysers or the Atacama Desert has increased sharply in recent years (Cao et al. 2008; Eichler 2001; Rothschild and Mancinelli 2001; Wang et al. 2013). Moreover, microorganisms thriving in these extreme habitats have developed various mechanisms to cope with high salinity and may be a source of novel metabolic pathways, which can be a valuable source of interesting transformations (Hough and Danson 1999).

The deep brine pools of the Red Sea, located along tectonic rift systems on its seabed, are considered an unexplored and remote source with high potential for the discovery of novel extremophilic enzymes (Grötzinger et al. 2014; Antunes et al. 2011; Eder et al. 2001). Temperatures in these brine pools vary from 23.3 °C (Kebrit) to 68.2 °C (Atlantis II) with salinities of 25 to 28 % (*w*/*v*) (Antunes et al. 2011; Wang et al. 2013). Archaea accumulate potassium chloride up to 5 M in the cytoplasm to balance the osmotic pressure (Kennedy et al. 2001). Hence, proteins of halophilic archaea evolutionarily adjusted their amino acid composition towards these conditions. Main differences to mesophilic enzymes are a higher amount of polar and charged residues on the protein surface to reduce the hydrophobicity, which result in the formation of a surface-bound hydration layer and the binding of hydrated salts (Danson and Hough 1998; Madern et al. 2000; Mevarech et al. 2000; Oren 2008). The use of *Escherichia coli* as a heterologous host for the expression of genes encoding halophilic proteins has been reported (Cao et al. 2008; Connaris et al. 1998a, b). However, the success of this approach depends strongly on the characteristics of the protein. The natural adaption of proteins to halophilic habitats poses significant challenges, and proteins may express inactive or form inclusion bodies. Refolding procedures may restore enzyme activity (Connaris et al. 1998a; Mullakhanbhai and Larsen 1975), but this method is not general, and many enzymes remain inactive (Timpson et al. 2012).

The exploitation of extremophilic microorganisms represents a reliable alternative to mesophilic systems. A typical example is the application of the archaea *Haloferax volcanii,* which was isolated from the Dead Sea by Benjamin Volcani in the 1940s (Mullakhanbhai and Larsen 1975). For optimal growth, it requires 2.6 M NaCl and a temperature between 42 and 45 °C (Mullakhanbhai and Larsen 1975).

To date, several strategies have been applied to engineer *H. volcanii for* improving heterologous gene expression resulting in the H1424 strain (Allers 2010; Allers et al. 2010). The corresponding plasmid to this strain is pTA963 with the inducible tryptophanase promoter *p.tna*, allowing the induction of intracellular protein overexpression by L-tryptophan (Allers 2010; Allers et al. 2004, 2010; Bitan-Banin et al. 2003; Large et al. 2007). The expression vector is based on the pHV2 origin which maintains the plasmid at a copy number of approximately 6 per genome equivalent (Allers 2010). To date, the *H. volcanii* expression system has been successfully applied for heterologous protein expressions on a shaker-flask scale (Karan et al. 2013; Timpson et al. 2012, 2013).

However, the high tendency of *H. volcanii* (Albers and Pohlschroder 2009; Frols et al. 2012; Ghosh and Albers 2011) to form biofilms prevents the use of this halophilic expression system in stirred-tank bioreactors because these biofilms interfere with sensors during fermentation. The genes Hvo_1033 and Hvo_1034, which encode an ATPase and an integral membrane protein of the pilus assembly system, are necessary for biofilm formation (Tripepi et al. 2013). Knockout of these genes successfully eliminated attachment of *H. volcanii* H1895 to surfaces of any kind while leaving cells motile (Tripepi et al. 2012, 2013).

This article describes the development of a simple batch process for efficient growth of *H. volcanii* without biofilm formation in a stirred-tank bioreactor followed by heterologous gene expression under control of the tryptophanase promoter. Genes encoding halophilic alcohol dehydrogenases ADH/D1 and ADH/A1 represent typical targets of halophilic gene expression. Both genes originate from the deep Red Sea brine pools (ADH/D1: Discovery brine pool and ADH/A1: Atlantis II brine pool) and were successfully expressed in preliminary studies (Grötzinger et al. 2014). Expression conditions were optimised using *bgaH* as a reporter gene. The protein codes for a halophilic β-galactosidase from *Haloferax alicantei* and was previously established as a model and reporting system for the expression of halophile proteins (Holmes et al. 1996; Holmes and Dyall-Smith 2000). Comparison with a mesophilic expression of ADH/D1 in *E. coli* illustrated the high efficiency and advantages of *H. volcanii* H1895 expression system.

Material and methods

Materials

Unless stated otherwise chemical reagents were purchased as analytical grade from Sigma-Aldrich (St. Louis, USA).

Experiments were performed with two mutants derived from the wildtype *H. volcanii* DS2 (DSM-3757, ATCC 29605), *H. volcanii* H1424 (Allers 2010) and the biofilm knockout strain *H. volcanii* H1895 which was cloned for this study. The vectors pTA963, pTA941-*bgaH* as well as pTA1228 were provided by the Institute of Genetics, School of Biology, University of Nottingham, Queen's Medical Centre Nottingham (UK). The vector pRE5 was provided by the University of Pennsylvania, Department of Biology (USA). The genes encoding the halophilic alcohol dehydrogenases ADH/D1 and ADH/A1 were integrated separately into the plasmid pTA963 and were provided by KAUST Catalysis Centre (King Abdullah University of Science and Technology, Saudi Arabia). *E. coli* BL21-CodonPlus(DE3)-RIPL cells were purchased from Stratagene (La Jolla, USA). The *E. coli* expression vector pET-3a was purchased from Novagen (Darmstadt, Germany). Restriction enzymes were purchased from New England Biolabs (Ipswich, Massachusetts).

Cloning of *H. volcanii* H1895

The mutant strain H1895 was derived by standard knockout techniques as described in Allers et al. from strain *H. volcanii* H1424 (Stroud et al. 2012). Therefore, the plasmid pRE5 (Esquivel and Pohlschroder 2014; Tripepi et al. 2013) was used to delete the genes Hvo_1034 and Hvo_1033. The knockout was confirmed by PCR using two primers adsorbing 700 bp (5′-AAGCTAGGATCCCGTCCTGCTGCCCCA-3′) in front and 700 bp (5′-GAACCTTCTAGACGAACTCGTCGGGCGCG-3′) (MWG Eurofins, Germany) behind the deleted genes.

Cloning of bgaH

The *bgaH* gene was cleaved from the plasmid pTA941 using the restriction sites *Nde* I and *Not* I. The gene was then integrated in pTA1228 using the same restriction sites. *E. coli* SDS110 (dam^-/dcm^-) was transformed with the plasmids. The accuracy of the sequence was confirmed by sequence analysis (forward primer 5′-GTTCGAACCGCCCTTTCCC-3′, reverse primer 5′-ATGACCATGATTACGCCAAG-3′ and internal primer 5′-GCCAGCAGTACGACGATT-3′) (MWG Eurofins, Germany). *H. volcanii* H1895 was then transformed with the non-methylated plasmids for protein expression. The transformation was performed as described in Holmes et al. (2008).

Growth in shaker-flasks

Cryo-stocks of *E. coli*-CodonPlus(DE3)-RIPL transformed with pET-3a-ADH/D1 were used to inoculate 100 mL of sterile LB Miller medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl). The cells were incubated overnight at 37 °C at 170 rpm. Afterwards, the culture was transferred to 1.6 L sterile LB Miller medium supplemented with 100 μg mL^{-1} ampicillin and 15 μg mL^{-1} tetramycin. The inoculated LB medium was distributed into four 1000-mL shaker-flasks with baffles (Bertani 1951). The culture was incubated at 37 °C at 170 rpm. To the culture, 1 mM isopropyl-d-thiogalactopyranosid (IPTG) was added to induce recombinant gene expression after OD_{600}=0.6 was reached. Nine hours after induction of gene expression at 30 °C, the cells were harvested by centrifugation (4500x*g*, 20 min, 4 °C). The cell pellets were stored at −20 °C.

Cryo-stocks of *H. volcanii* H1424 transformed with either pTA963-ADH/D1 or pTA963-ADH/A1 vectors were used to inoculate 50 mL sterile Hv-YPC medium (144 g L^{-1} NaCl, 21 g L^{-1} $MgSO_4 \cdot 7H_2O$, 18 g L^{-1} $MgCl_2 \cdot 6H_2O$, 4.2 g L^{-1} KCl, 5 g L^{-1} yeast extract, 1 g L^{-1} peptone, 1 g L^{-1} casamino acids, 12 mL of 121 g L^{-1} Tris, pH 7.5, 6 mL of 55.5 g L^{-1} $CaCl_2$) in a 250-mL shaker-flask (Allers et al. 2004). This pre-culture was used to inoculate 1 L of sterile Hv-YPC medium and incubated at 45 °C at 170 rpm. Recombinant gene expression was induced by addition of 222 mL (6 mM) of 27 mM L-tryptophan dissolved in 18 % salt water at OD_{650}=0.4 (Holmes et al. 2008). Gene expression was induced for a second time by adding further 3 mM (136 mL) of a 27 mM L-tryptophan solution to reach a final amount of 9 mM L-tryptophan, added after a process time of 16 h. Four hours later, the cells were harvested by centrifugation at 4500x*g* at 4 °C for 45 min. The cell pellet was stored at −20 °C.

Cryo-stocks of *H. volcanii* H1895 transformed with pTA1228-*bgaH* were used to inoculate 50 mL of sterile Hv-YPC medium in a 250-mL shaker-flask. The cells were incubated for 48 h at 45 °C and at 170 rpm. Fifty millilitres of sterile Hv-YPC medium in a 250-mL shaker-flask was inoculated with this 5-mL pre-culture. The shaker-flasks were incubated at 45 °C and 170 rpm. *bgaH* expression was induced as described in Table 1. The listed expression conditions were taken from literature. After the incubation period shown in Table 1, cells could be used directly for activity assays.

Growth in stirred-tank bioreactors

Simple batch cultures of *H. volcanii* H1895 transformed with pTA963-ADH/D1 or pTA963-ADH/A1 were performed on a 1-L scale with a temperature-, pressure-, pH- and DO-controlled stirred-tank bioreactor (1.8 L, Labfors, Infors, Bottmingen, Switzerland). The stirrer was equipped with two Rushton turbines. Sterile air with a flow rate of 2 vessel volumes per minute (vvm) was used to aerate the medium. The stirrer speed was varied between 200 and 1000 rpm (0.005 to 0.44 W L^{-1}) to provide a DO of >40 % air saturation. The Hv-YPC culture medium was used with 5 g L^{-1} lactate, 2 mM KH_2PO_4/K_2HPO_4 buffer, pH 7.5, 5 mM NH_4Cl, 2 g L^{-1} casamino acids and 10 g L^{-1} yeast extract. The

Table 1 *bgaH* expression in H1895 in 50 mL Hv-YPC performed in 250-mL shaker-flasks at different reaction conditions chosen from literature

Approach	Temp, °C	1. Induction			2. Induction			Source
		OD	Trp, mM	Incubation time, h	OD	Trp, mM	Incubation time, h	
1	45	n.d. (0.3)	14.7×10^{-3}	1	–	–	–	Tripepi et al. 2012 (Tripepi et al. 2012)
2	45	n.d. (0.3)	5	5	–	–	–	Lestini et al. 2013 (Lestini et al. 2013)
3	45	After 24 h	5	7	–	–	–	Timpson et al. 2012 (Timpson et al. 2012)
4	45	n.d. (0.3)	5	48	–	–	–	Liliensiek et al. 2013 (Liliensiek et al. 2013)
5	42	n.d. (0.3)	1	5–6	0.5	3	1	Allers et al. 2010 (Allers 2010)
6	45	0.3	3	16	n.d. (2.5)	1	4	Allers et al. (unpublished)
7	45	0.3	6	16	2.5	3	4	This report

n.d. refers to conditions that were not specified in the publication. Numbers in brackets indicate values for conditions, which were measured during this study

cultivation was performed at 45 °C. A pH of 7.5 was maintained by addition of 1 M NaOH to the stirred-tank bioreactor. When recombinant *H. volcanii* cells reached the exponential growth phase, recombinant gene expression was induced by addition of 6 mM of a 27 mM L-tryptophan solution dissolved in 18 % salt water. After an incubation period of 16 h at 45 °C, recombinant gene expression was induced for a second time by adding 3 mM of a 27 mM L-tryptophan solution to reach a final amount of 9 mM L-tryptophan added. After a process time of 4 h, cells were harvested by centrifugation at 4500x*g* at 4 °C for 45 min. The cell pellet was stored at −20 °C.

Automated batch cultivation in a parallel bioreactor

Studies aimed at the optimisation of gene expression in *H. volcanii* H1895 were performed in parallel-operated stirred-tank bioreactors operated in a magnetic inductive drive (bioREACTOR 48, 2 mag AG, Munich, Germany) with gas-inducing stirrers (Puskeiler et al. 2005). Forty-eight baffled single-use bioreactors (2 mag AG, Munich, Germany) with a nominal volume of 8 to 15 mL can be arranged in the magnetic inductive drive (Weuster-Botz et al. 2005).

For inoculum preparation, 10 mL Hv-YPC inoculated with frozen H1895_pTA1228-*bgaH* cells was incubated at 45 °C and 170 rpm for 48 h in a shaker-flask. The final cell solution was used to inoculate 50 mL sterile Hv-YPC in an unbaffled 250-mL shaker-flask. After an incubation time of 24 h at 45 °C and 170 rpm, the pre-culture was transferred into 500 mL sterile Hv-YPC. Of this culture, 10 mL was put into each millilitre-scale stirred-tank bioreactor. The headspace of each bioreactor was flooded with sterile gas at a flow rate of 0.1 L min^{-1}. Continuous gas dispersion into the liquid phase of the small-scale stirred-tank bioreactors was provided by the gas-inducing stirrers, which sucked the gas into the bioreactors. The stirrer speed was set to 2500 rpm (0.42 W L^{-1}) throughout the whole cultivation. The temperature in the liquid phase was maintained at 45 °C. The headspace of the gas phase was set to 6 °C to reduce evaporation.

The parallel stirred-tank bioreactor system was used to investigate appropriate reaction conditions for the recombinant expression of *bgaH* in *H. volcanii* H1895 in the batch process. Gene expression was induced by a single addition of L-tryptophan (27 mM in 18 % salt water) in a concentration varying from 1 to 9 mM. After a process time of 20 h, the optical density at 650 nm was determined, and the cells were used for the enzymatic assay. In comparison, gene expression was induced by a twofold addition of L-tryptophan solution. L-tryptophan amounts of 2, 4, 6, 9 or 10 mM, split into two doses for the first and the second induction step, were added. The incubation period between the first and the second steps was 16 h. After a process time of 4 h after the second induction step, OD_{650} was determined, and the cells were subjected to the enzyme assay. Moreover, the influence of the incubation time was investigated. The first incubation period was varied between 12 and 20 h, and the second between 1 and 6 h. After the incubation period, the OD_{650} was determined, and cells were used for the activity assay. All experiments were performed in triplicate.

Purification of proteins by immobilised metal ion affinity chromatography

For purification, the cell pellets of *H. volcanii* H1895 and *H. volcanii* H1424 were resuspended in buffer A (2 M NaCl, 10 mM HEPES, pH 7.5 buffer, with 10 mM imidazole) with 1 mM Pefabloc. For each 1 g of wet cells, 5 mL buffer A was added. Cell disruption was performed by sonication (600 W, 94 % amplitude, 50 % pulse, 1 h). Cell debris was removed by centrifugation at 25,000x*g* at 4 °C for 50 min. The supernatant was used for immobilised metal ion affinity

chromatography (IMAC) with Ni^{2+} as immobilised ion. The supernatant was loaded at a flow rate of 1 mL min^{-1} onto a HisTrap™ HP 5 mL (GE Healthcare, Little Chalfont, UK) which was pre-equilibrated with buffer A. Four column volumes (CV) of buffer A were used to remove non-specifically bound protein from the column. Elution of ADH/D1 was performed in two steps. First, two CVs of 11 % (*v*/*v*) buffer B (2 M NaCl, 10 mM HEPES; pH 7.5 and 300 mM imidazole) were used to elute impurities from the column. Following a linear gradient from 11 to 50 % (*v*/*v*) buffer B (slope: 1 % min^{-1}) the protein could be eluted.

For ADH/A1, the process was similar. The pellet was resuspended and sonicated as described above. After centrifugation (25,000x*g*, 4 °C, 50 min), the supernatant was loaded onto a HisTrap™ HP 5 mL column pre-equilibrated with buffer A. The flow rate was 1 mL min^{-1}. Impurities were removed from the column with 6 % (*v*/*v*) buffer B. Finally, the protein could be eluted following a linear gradient from 6 to 50 % (*v*/*v*) buffer B (slope 1 % min^{-1}). The whole purification process was monitored by UV at 280 nm. Fractions of 5 mL were collected and analysed by a 12.5 % (*w*/*v*) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using the protein marker BlueAQUA Prestained Protein Marker (GeneDireX) (Laemmli 1970). The SDS-PAGE was stained according to Fairbanks et al. (Fairbanks et al. 1971). Fractions of interest were combined and dialysed with Visking dialysis tubing (MWCO 10,000 kDa, Carl Roth GmbH, Karlsruhe, Germany). The process was performed overnight in a buffer consisting of 2 M NaCl, 10 mM HEPES, pH 7.5 in a dilution >1:100. The dialysed protein solution was concentrated using Amicon Ultracel® (MWCO 10.000 kDa, Millipore, Billerica, USA) by centrifugation at 4500x*g* at 4 °C. The extinction coefficients and the masses for both proteins were calculated with Protparam (Gasteiger et al. 2005). Proteins were stored at −20 °C.

Analytical procedures

Determination of acetate and lactate concentrations in the Hv-YPC complex medium was performed with enzyme assays (Roche Yellow-Line r-biopharm, Darmstadt, Germany). The technique for obtaining the cell dry weight followed Mironescu et al. (2003). A 1 mL aliquot of supernatant was removed following centrifugation for 10 min at 13,000x*g*. The cell pellet was dried at 80 °C for 2 days, and then the pellet was washed with 1 % NaCl (*w*/*v*) solution to remove the dried salt, which confounds measurement of cell dry weight. Protein concentration was determined by using a BCA Protein Assay kit (Thermo Fisher Scientific, Rockford, USA).

Determination of the L-tryptophan concentration was performed using a HPLC (Agilent, Santa Clara, CA, USA) with a C18 analytical reversed phase column (Phenomenex C18 column Synergi 4u Hydro-RP 80A, 150×2.0 mm). A 10 μL aliquot of the sample was loaded onto the column at ambient temperature and a flow rate of 0.4 mL min^{-1}. Bound substances were eluted by a gradient from 0 % B (20 mM TFA in dd-H_2O) to 100 % (*v*/*v*) B (95 % acetonitrile, 20 mM TFA in dd-H_2O) within 50 min. The L-tryptophan concentration was monitored at the specific wavelength of 254 nm. Absorption of the medium without L-tryptophan at the beginning and after incubation for 20 h was determined at 254 nm and subtracted from the samples.

Activity assays

Determination of the enzyme activity of the alcohol dehydrogenases ADH/D1 and ADH/A1 was performed following Timpson et al. (2012). All reactions were performed on a 1-mL scale in cuvettes (Semimicro, PMMA, Sigma-Aldrich, St. Louis, USA) at 50 °C in 3 M KCl, 50 mM glycine pH 10.0 with 0.1 mg mL^{-1} alcohol dehydrogenase, 43.7 mM 1-butanol as substrate and 12.5 mM NAD^+ at 60 °C. The reduction of NAD^+ to NADH was monitored at 340 nm using a photometer (Ultrospec 7000, GE healthcare, Fairfeld, USA) equipped with a water Peltier system (PCB 1500, DBS Vigonza, Italy). The enzyme activity was calculated using a molar extinction coefficient of 6.22 mM^{-1} cm^{-1}. All experiments were performed in triplicates. One unit of enzyme activity was as defined as the reduction of 1 μmol NAD^+ to NADH per minute.

Measurement of BGAH activity was performed at room temperature in cuvettes with a total volume of 1 mL. 700 μL bgaH-buffer (2.5 M NaCl, 50 mM Tris, pH 7.2, 10 μM $MnCl_2$, 0.1 % 2-mercaptoethanol) was mixed with 100 μL cell suspension and 100 μL 2 % (*v*/*v*) Triton X-100 and incubated for 5 min at room temperature. Then 100 μL ortho-nitrophenyl-para-galactoside (ONPG) solution was added (8 mg mL^{-1} ONPG in bgaH-buffer), the change of absorption was measured photometrically at 405 nm (Biomate 3, Thermo Spectronic, Germany) within 30 min (Holmes et al. 2008).

Background activity for BGAH was determined and subtracted from the specific activity. All experiments were performed in triplicate. Appropriate controls were included.

Results

Expression tests of ADH/D1 and ADH/A1 in *E. coli*-CodonPlus(DE3)-RIPL

Initially, expression of the two thermophilic alcohol dehydrogenases (ADH) ADH/D1 and ADH/A1 was tested in *E. coli*. The significant differences in archaeal and bacterial codon

usage mandates the codon usage enhanced strain *E. coli*-CodonPlus(DE3)-RIPL. Heterologous genes were introduced for expression through the standard pET3a vector. However, despite even for the *E. coli*-CodonPlus(DE3)-RIPL expression system, no recombinant expression of ADH/D1 or ADH/A1 was observed.

Expression of ADH/D1 and ADH/A1 in *H. volcanii* H1424 and purification

Cloning of the two genes encoding the thermohalophilic alcohol dehydrogenases ADH/D1 and ADH/A1 into pTA963 and subsequent transformation into *H. volcanii* H1424 led to two expression systems, which we designate as *H. volcanii* H1424-pTA963_ADH/D1 and *H. volcanii* H1424-pTA963_ADH/A1, respectively. Without protein expression, growth studies of *H. volcanii* H1424 revealed a final biomass concentration of 1.5 g L^{-1}.

After optimisation of the induction pattern (Table 1) and purification by Ni^{2+}-IMAC, 1 L of a shaker-flask culture yielded 3.53 mg protein per gram cell dry weight (g_{CDW}^{-1}) purified ADH/D1 and 0.44 mg g_{CDW}^{-1} purified ADH/A1. Successive expression tests using a stirred-tank bioreactor with various medium compositions as well as setups confirmed that *H. volcanii* H1424 cultures were prone to severe biofilm formation. None of the conditions tested were found to be suitable for further bioreactor cultivation studies of *H. volcanii* H1424.

Batch growth of *H. volcanii* H1895_pTA963-ADH/D1 in a stirred-tank bioreactor

Growth studies of *H. volcanii* H1895_pTA963-ADH/D1 (with knockouts of two flagella associated genes to prevent biofilm formation) in a temperature-, pH- and DO-controlled 1 L stirred-tank bioreactor achieved a cell density of 3.5 g L^{-1} for the Hv-YPC complex medium (Holmes et al. 2008), if no protein expression was induced. Supplementing the media with additional nutrition sources further improved cell densities. Addition of 10 g L^{-1} yeast extract and 2 g L^{-1} casamino acids increase amino acid and carbon availability. Augmenting the reaction media with 5 mM NH_4Cl and 2 mM of KH_2PO_4/K_2HPO_4 buffer (pH 7.5) provides ammonia and phosphate, which are readily used by *H. volcanii* for DNA and protein production. Lactate, glucose, acetate and succinic acid were tested as alternative carbon sources with lactate yielding a 20 % enhanced cell density compared with other carbon sources (data not shown). The combination of the supplements described above yields a final cell density of 10.5 g L^{-1} after 35 h in the batch-process. Figure 1 illustrates the growth of *H. volcanii* H1895_pTA963-ADH/D1 in Hv-YPC medium with and without supplements.

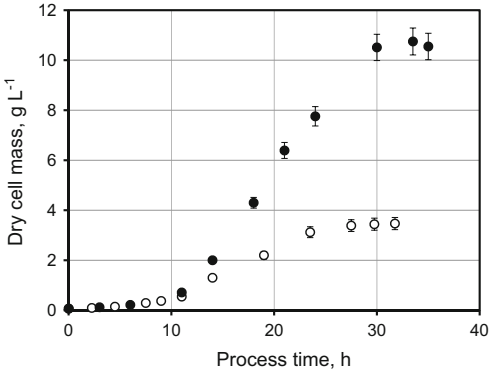

Fig. 1 Growth of *Haloferax volcanii* H1895_pTA963-ADH/D1 in a stirred-tank bioreactor batch culture (V=1 L, DO>40 %, pH 7.3, T= 45 °C). The comparison details the growth curves for Hv-YPC medium without (*white circles*) and with supplements (*black circles*; 10 g L^{-1} yeast extract, 2 g L^{-1} casamino acids, 2 mM K_2HPO_4/KH_2PO_4 pH 7.5, 5 mM NH_4Cl and 0.5 % (*v*/*v*) lactate)

Variation of expression conditions

After the establishment of optimal growth conditions for *H. volcanii* H1895, the next experiments focused on maximising the protein expression yields. Literature data on conditions for halophilic gene expression under control of the promoter p.tnA served as a starting point for the test system *H. volcanii* H1895_pTA1228-*bgaH*. The *bgaH* gene encodes a halophilic β-galactosidase, which was introduced into the vector pTA1228, an improved version of pTA963 vector containing an additional unique *NspI* site before the His-tag. Initial screens for BGAH expression yields were performed in shaker-flasks (Table 1, approach 5). Induction protocols 1 to 4 differ by the amount of added L-tryptophan (14.7×10^{-3} to 5 mM) and incubation time (7 to 48 h). Induction protocol 5 (with an initial L-tryptophan concentration of 1 mM) includes a second L-tryptophan induction after an interval of 6 h (L-tryptophan concentration 3 mM). For induction protocol 6 (initial L-tryptophan concentration of 3 mM), the incubation time between both inductions was extended to 16 h (second L-tryptophan addition 1 mM). An incubation period of 4 h followed the second induction step. Induction of protein expression using L-tryptophan also induces the expression of the native tryptophanase gene tnaA; hence, *H. volcanii* is able to metabolise the added L-tryptophan (Large et al. 2007) and correspondingly approach 6 gave the highest expression yields (Fig. 2). Further studies focused on the improvement of the two-step induction protocol. The final protocol 7 used an increased L-tryptophan concentration of 6 mM for the first induction and 3 mM for the second induction.

Figure 2 illustrates the measured specific β-galactosidase activities expressed in shaker-flasks under the conditions listed in Table 1. All cultures started from the same OD_{650}

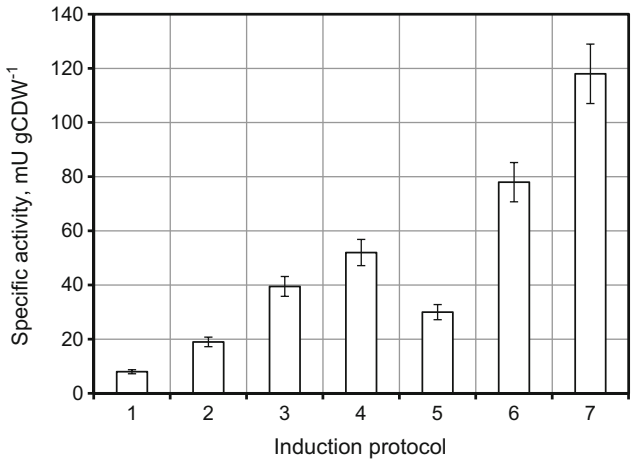

Fig. 2 Illustration of a specific β-galactosidase activity of *Haloferax volcanii* H1895_pTA1228-*bgaH* at different expression conditions in shaker-flasks. Protocol *1*: induction with 14.7×10^{-3} mM L-tryptophan, incubation 1 h, 45 °C (Tripepi et al. 2012). Protocol *2*: induction with 5 mM L-tryptophan, incubation 5 h, 45 °C (Lestini et al. 2013). Protocol *3*: induction with 5 mM L-tryptophan, incubation for 7 h, 45 °C (Timpson et al. 2012). Protocol *4*: induction with 5 mM L-tryptophan, incubation 48 h, 45 °C (Liliensiek et al. 2013). Protocol *5*: two-step induction with 1 mM L-tryptophan, incubation 5–6 h, 42 °C. Afterwards, second induction with 1 mM L-tryptophan, incubation 1 h, 42 °C (Allers 2010). Protocol *6*: two-step induction with 3 mM L-tryptophan, incubation 16 h, 45 °C. Afterwards, second induction with 1 mM L-tryptophan, incubation 4 h, 45 °C. Protocol *7* is a modification of protocol *6*: two-step induction with 6 mM L-tryptophan, incubation 16 h, 45 °C followed by a second induction with 3 mM L-tryptophan, incubation 4 h, 45 °C

of 0.3. Protocols 1 to 4 reveal a positive linear correlation of enzyme activity with L-tryptophan concentration (from 14.7×10^{-3} to 5 mM) and incubation time (1 to 48 h). A specific activity of 50 mU g_{CDW}^{-1} could be reached after an incubation period of 48 h with a 5 mM L-tryptophan concentration. The same correlation is observed for conditions 5 to 7. The short incubation period of 5 to 6 h between the first and second induction steps and 4 h after the second induction step in protocol 5 in combination with the low L-tryptophan concentration of 1 mM and 3 mM results in a low specific activity of 32 mU g_{CDW}^{-1}. Extending the incubation period to 16 h after the first and 4 h after the second induction step led to a specific activity of 80 mU g_{CDW}^{-1} (protocol 6). A further increase in tryptophan concentration resulted in the highest observed specific activity of 120 mU g_{CDW}^{-1} (protocol 7).

In shaker-flasks, both protein expression as well as the growth suffer from oxygen limitations and pH variations, which may influence the expression of β-galactosidase. Consequently, further studies were performed in parallel-operated stirred-tank bioreactors with DO and pH control on a 10-mL scale.

One-step protein expression protocols were performed first with *H. volcanii* H1895_pTA1228-*bgaH* using L-tryptophan concentrations between 1 and 9 mM and an extended incubation time of 20 h. Moreover, the L-tryptophan concentrations remaining after an incubation period of 20 h were measured (Fig. 3). In agreement with the data obtained from shaker-flask cultures, increased L-tryptophan concentrations typically correlated with increased protein expression levels. The measured specific β-galactosidase activity reached a maximum of 23 U g_{CDW}^{-1} for an L-tryptophan concentration of at least 7 mM. Interestingly, higher amounts of L-tryptophan decreased protein expression indicated by a drop of specific activity to 15 U g_{CDW}^{-1} (9 mM L-tryptophan). Analysis of the remaining L-tryptophan concentration after 10 h expression time showed that an up to 5 mM addition of L-tryptophan led to negligible consumption, whereas higher amounts of L-tryptophan are consumed resulting in a plateau concentration of approximately 5 mM in the medium. However, if the concentration of L-tryptophan exceeded 7 mM, L-tryptophan consumption is accompanied by an inhibition of the protein expression. Thus, well-controlled L-tryptophan levels are key to good expression yields, which argue for the development of a multi-induction protocol.

Correspondingly, inducer concentrations of 2, 4, 6, 9 or 10 mM L-tryptophan in the stirred-tank batch studies with *H. volcanii* H1895_pTA1228-*bgaH* were split into two portions, with a 16 h incubation time between both inductions and an additional 4 h cultivation time before cell harvest and β-galactosidase activity measurement. Again, increased L-tryptophan concentration directly translated into higher protein expression levels with the highest specific activity of 73 U g_{CDW}^{-1} for an addition of up to 9 mM total L-tryptophan split into two portions (6 and 3 mM L-tryptophan for the first and second inductions, respectively). L-tryptophan additions, exceeding in total a concentration of 9 mM, resulted in a decreased expression level and specific β-galactosidase activity. This result is

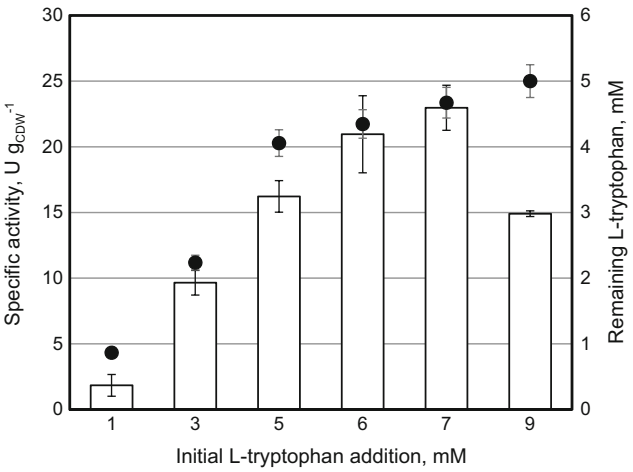

Fig. 3 Specific β-galactosidase activity of *Haloferax volcanii* H1895_pTA1228-*bgaH* with different initial L-tryptophan additions and remaining L-tryptophan concentration (*black circles*) after an incubation time of 20 h in batch-operated stirred-tank bioreactors (V=10 mL, T=45 °C, pH 7.3, air flow rate (head space)=0.1 L min^{-1}, n=2500 rpm with gas-inducing stirrers, DO>40 % air saturation)

illustrated in Fig. 4, where the total of 10 mM L-tryptophan (split in 7 and 3 mM) resulted in a specific activity of only 45 U g_{CDW}^{-1}. Quantification of the L-tryptophan, which remained after expression revealed that a significant consumption of L-tryptophan occurred only for additions exceeding a total of 6 mM, which was also the L-tryptophan level remaining in solution for higher inductor concentrations.

Apart from inductor concentration, induction time has a major influence on protein expression levels. Using the two-step induction protocol described above, the incubation time was varied after the first 6 mM addition of L-tryptophan between 12 and 20 h, while the second induction time following the 3 mM addition remained at 4 h. Analysis of the β-galactosidase activities revealed that an interval of 14 h is sufficient to reach the maximum activity of 70 U g_{CDW}^{-1} (Fig. 5a).

Analysis of the time interval between the second induction step and the cell harvest revealed that a second incubation time of 2 h is sufficient to reach the maximum β-galactosidase activity of 71 U g_{CDW}^{-1}, whereas shortening this period to 1 h led to decreased protein expression (35 U g_{CDW}^{-1}). In summary, it can be concluded that a total induction time of 18 h split in two intervals of 14 and 4 h (Fig. 5a) or 16 and 2 h (Fig. 5b) is sufficient to reach the maximum specific β-galactosidase activity.

Expression of thermohalophilic alcohol dehydrogenases in *H. volcanii* H1895 and purification

Two recently identified thermohalophilic alcohol dehydrogenases ADH/A1 and ADH/D1 (Grötzinger et al. 2014) were expressed in *H. volcanii* H1895 in a batch-operated stirred-tank bioreactor applying growth and induction protocols (two-step protocol with 16 and 4 h) as described above. Both genes originate from thermophilic hypersaline environments (63 °C and 17 % (*w*/*v*) salinity for ADH/A1 and 45 °C and 26 % (*w*/*v*) salinity for ADH/D1) and therefore can be considered typical test cases for a bioreactor expression protocol.

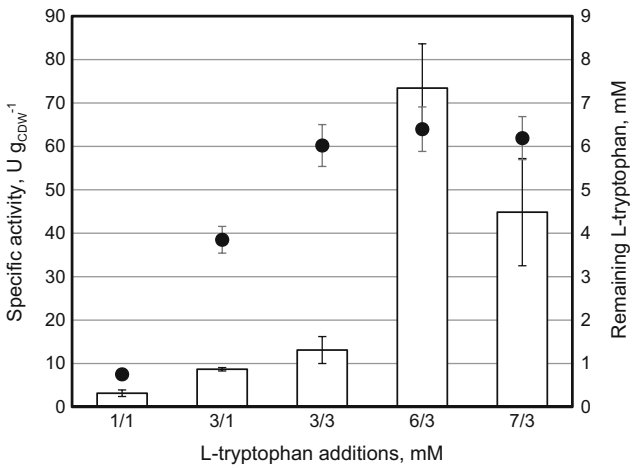

Fig. 4 Specific β-galactosidase activity of *Haloferax volcanii* H1895_pTA1228-*bgaH* with different L-tryptophan additions (initial addition/second addition after 16 h) and remaining L-tryptophan concentration (*black circles*) after an incubation time of 20 h in batch-operated stirred-tank bioreactors (V=10 mL, T=45 °C, pH 7.3, air flow rate (head space)=0.1 L min^{-1}, n=2500 rpm with gas-inducing stirrers, DO>40 % air saturation)

The initial addition of 6 mM L-tryptophan was timed to coincide with the onset of the exponential growth phase after a process time of 12 h. As illustrated in Fig. 6, the L-tryptophan concentration remained constant for 12 h with increasing dry cell mass concentrations up to 3.5 g L^{-1}. Subsequently, the L-tryptophan concentration decreased to 3.5 mM within 4 h. The second inducer addition (3 mML-tryptophan) resulted in an increase of L-tryptophan concentration to 7.8 mM, which was then depleted to 5.8 mM and then remained constant until cell harvest. Biomass concentration linearly increased to 6.5 g L^{-1} with process time from the onset of the logarithmic growth phase (cultivation time of 6 h) until 2 h before cell harvest.

Figure 7 compares the biomass, lactate, ammonia and acetate concentrations in the batch process for ADH/D1 expression with *H. volcanii* H1895_pTA963-ADH/D1 and the batch process for ADH/A1 expression with *H. volcanii* H1895_pTA963-ADH/A1. Biomass concentrations reached 6.5 g L^{-1} for ADH/D1 expression and 5.3 g L^{-1} for ADH/A1 expression (a). In both cultures, *H. volcanii* metabolised the supplemented carbon source (lactate) to a residual lactate concentration of 1 g L^{-1} (b). Acetate was formed as a by-product in the initial exponential growth phase of *H. volcanii* H1895_pTA963-ADH/D1. After reaching a peak of 0.15 g L^{-1}, continuous consumption decreased the acetate level to a final concentration of 0.05 g L^{-1}. During the expression of ADH/A1, the maximal acetate concentration was twice as high, lacking the subsequent metabolisation observed for ADH/D1 (c). Hence, the acetate accumulation in the aqueous phase reached a final concentration of 0.26 g L^{-1}. During the cultivation of *H. volcanii* H1895_pTA963-ADH/D1, the ammonia concentration did not change markedly within the first 24 h and then increased slightly before cell harvest. However, for ADH/A1 expression, the ammonia concentration decreased already within the first 10 h to a concentration of 0.25 g L^{-1} followed by an increase to 0.7 g L^{-1} (d).

Both alcohol dehydrogenases were isolated and purified using an immobilised metal ion affinity chromatography (IMAC) with Ni^{2+} as immobilised ion. Figure 8 shows the SDS-PAGE gel for the purified protein.

IMAC delivers largely pure proteins. In comparison to the expression in shaker-flasks, a markedly higher amount of protein was obtained in the bioreactor for both, ADH/D1 as well as ADH/A1 (Fig. 9). As observed in shaker-flasks, yields in

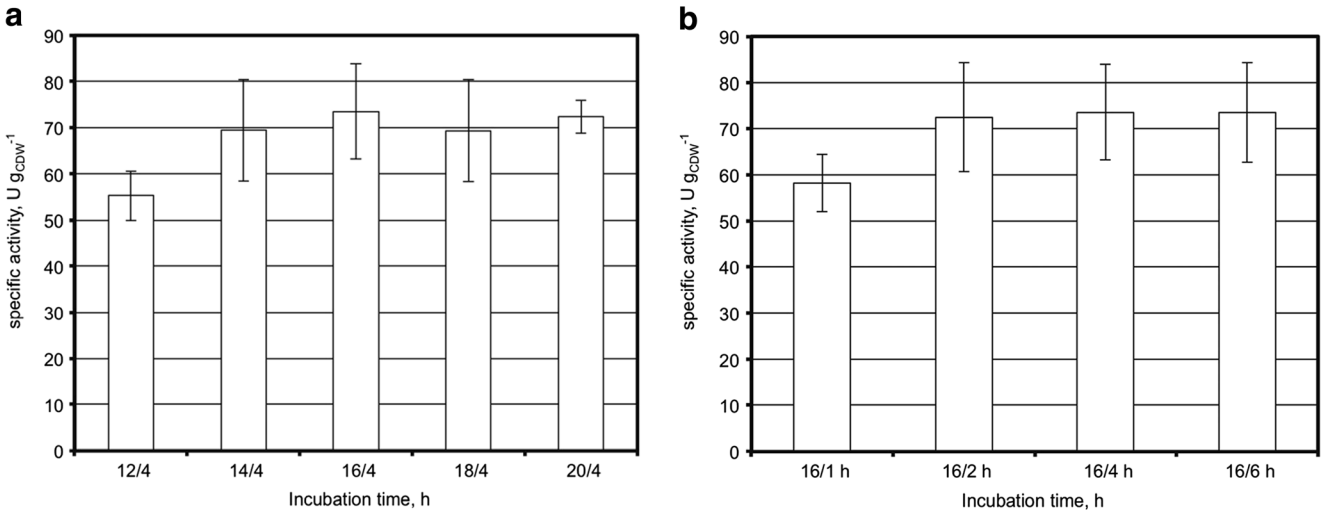

Fig. 5 Specific β-galactosidase activity of *Haloferax volcanii* H1895-pTA1228-*bgaH* induced with 6 mM initial L-tryptophan and 3 mM L-tryptophan after **a** a first incubation time of 12 to 20 h, followed by an incubation time of 4 h after the second L-tryptophan addition and **b** a first incubation time of 16 h followed by an incubation time of 1 to 6 h after the second L-tryptophan addition in batch-operated stirred-tank bioreactors (V=10 mL, T=45 °C, pH 7.3, air flow rate (head space)=0.1 L min^{-1}, n= 2500 rpm with gas-inducing stirrers, DO>50 % air saturation)

the bioreactor ADH/D1 were higher than those obtained for ADH/A1. After purification, 16.8 mg g_{CDW}^{-1} was obtained for ADH/D1 and 3.9 mg g_{CDW}^{-1} for ADH/A1.

Enzyme activities of ADH/A1 and ADH/D1

Both purified alcohol dehydrogenases were functional as indicated by the relatively rapid oxidation of 1-butanol to 1-butanal in the presence of 12.5 mM NAD^+. At 50 °C and pH 10 for 0.1 mg mL^{-1} enzyme and 43.7 mM substrate, specific activities of 1.97 mU mg^{-1} (ADH/D1) and 4.17 mU mg^{-1} (ADH/A1) were measured. A detailed study of the properties of both novel thermohalophilic ADHs is currently in progress and will be published elsewhere in due course.

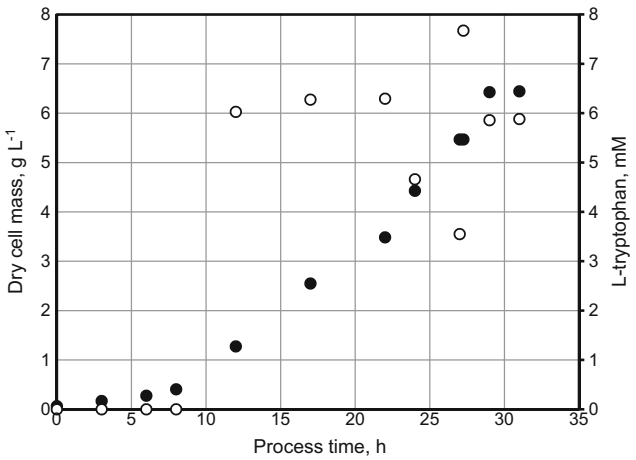

Fig. 6 Batch growth of *Haloferax volcanii* H1895_pTA963-ADH/D1 in a stirred-tank bioreactor (V=1 L, DO>40 % air saturation, pH=7.3, T= 45 °C). Initial induction of ADH/D1 expression at a process time of 12 h by adding 6 mM L-tryptophan followed by an incubation of 16 h and a second induction step with an addition of 3 mM L-tryptophan after 28 h (*black circles*, dry cell mass concentration; *white circles*, L-tryptophan concentration)

Discussion

A recent review on enzymes from extremophilic organisms stated that a fundamental challenge for the production of extremozymes is the availability of efficient large-scale expression systems (Elleuche et al. 2014). High-yielding protein expression favours the use of *E. coli* because of established molecular biology tools, a short generation time and comparatively straightforward handling. Nevertheless, there are limitations, particularly for the expression of genes originating from halophilic or thermohalophilic environments.

Accumulation of KCl in the cytoplasm of *H. volcanii* leads to a halophilic environment whereas *E. coli* reveals a mesophilic cytoplasmic environment. These large differences presumably explain why no recombinant expression of the alcohol dehydrogenase ADH/D1 was observed in *E. coli*. The engineered *H. volcanii* expression system (Allers 2010; Allers et al. 2004, 2010) was capable of producing moderate amounts of protein in shaker-flask cultures, the essential step for the biotechnological use of halophilic enzymes remains a large-scale production in bioreactors. The method established in this study facilitates straightforward batch cultivation of *H. volcanii* H1895 in a stirred-tank bioreactor using optimised settings and media. The halophilic reporter enzyme β-galactosidase (BGAH) enabled the development of a reliable and efficient protocol for protein expression in bioreactors. Using this method, two different, novel halophilic genes

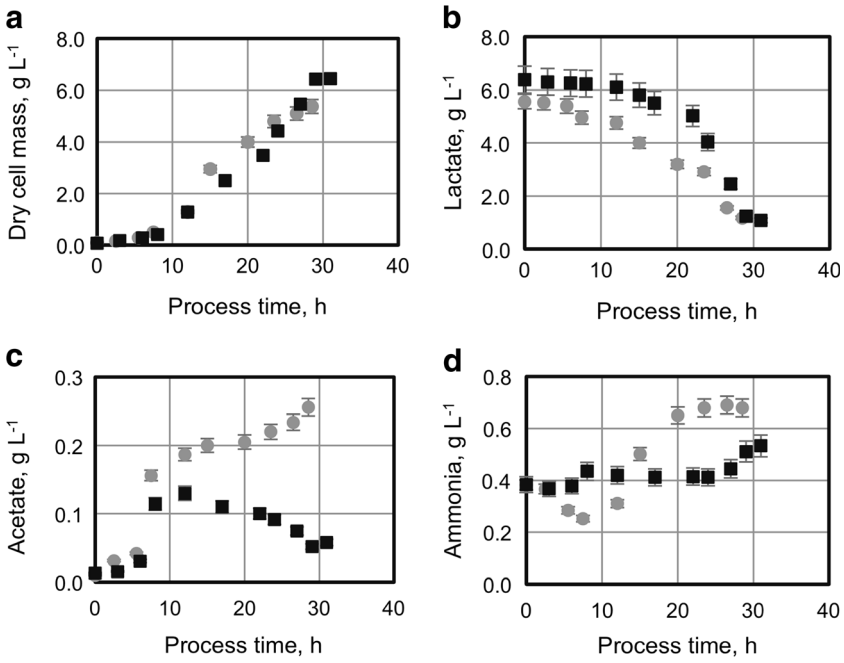

Fig. 7 Batch growth of *Haloferax volcanii* H1895_pTA963-ADH/D1 (*black squares*) and *Haloferax volcanii* H1895_pTA963-ADH/A1 (*grey circles*) in a stirred-tank bioreactor (V=1 L, DO>40 % air saturation, pH=7.3, T=45 °C). Initial induction of ADH/D1 and ADH/A1 expression at a process time of 12 h by adding 6 mM L-tryptophan followed by an incubation of 16 h and a second induction step with an addition of 3 mM L-tryptophan after 28 h. Comparison of dry cell mass concentration (**a**), lactate concentration (**b**), acetate concentration (**c**) and ammonia concentration (**d**)

encoding alcohol dehydrogenases were successfully expressed, isolated and purified. Activity tests proofed that both alcohol dehydrogenases were expressed as functional enzymes, which indicates that the protocol described in this article is broadly applicable.

Cultivation of microorganisms in a stirred-tank bioreactor has several advantages compared with cultivations in shaker-flasks. The supply of oxygen and nutrients, as well as, the pH can be controlled. Thus, microorganisms suffer neither from oxygen or nutrient limitations nor from changing pH, which strongly interferes with microbial growth and gene expression. Initial experiments showed that because of the strong tendency of *H. volcanii* H1424 to form biofilms, no reliable results could be achieved for this strain in stirred-tank bioreactors. *H. volcanii* has flagella on its cell surface that are responsible for surface adherence. The responsible genes Hvo_1033 and Hvo_1034 were knocked out, resulting in the new strain *H. volcanii* H1895 (Tripepi et al. 2013), which is motile but does not form biofilms. Accordingly, batch cultivations of this strain in a stirred-tank bioreactor were

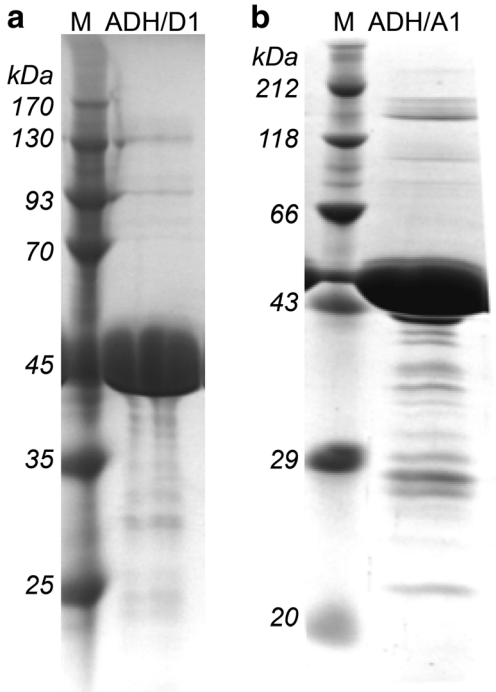

Fig. 8 SDS-PAGE analysis of purified ADH/D1 (44.3 kDa) (**a**) and ADH/A1 (40.2 kDa) (**b**). Lane *M*: molecular marker

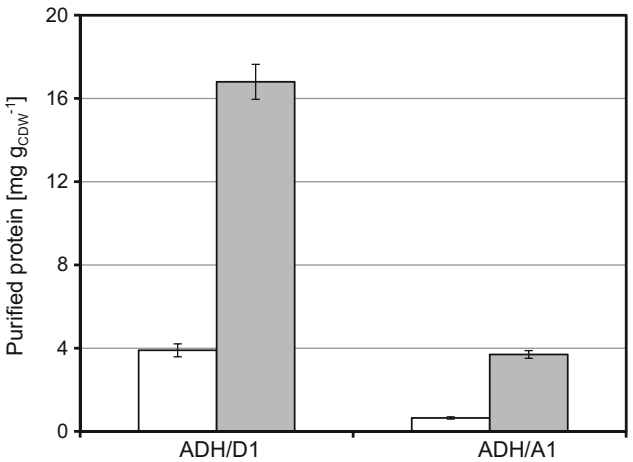

Fig. 9 Purified ADH/D1 and ADH/A1 expressed in shaker-flasks (*white bars*) (T=45 °C, 170 rpm, in total 4 L Hv-YPC medium) and the stirred-tank bioreactor (*grey bars*) (DO>40 %, T=45 °C and pH=7.3, in total 1 L Hv-YPC medium)

successful with the Hv-YPC complex medium at 45 °C, DO> 40 % and at pH 7.3. Several investigations were made to optimise the medium composition to gain a higher cell density. The complex medium was supplemented with 5 mM NH_4Cl, 2 mM K_2HPO_4/KH_2PO_4 (pH 7.5) and 0.5 % (*v*/*v*) lactate as additional carbon source. Moreover, the concentrations of yeast extract and casamino acids were tripled to 15 and 3 g L^{-1}, respectively. Cell densities of *H. volcanii* H1895 in the stirred-tank bioreactor cultures reached 10 g L^{-1} for batch experiments without induction of heterologous gene expression. This represents a sixfold increase over shaker-flask cultures.

Previously, Holmes et al. (2008) showed that the *bgah* gene, which encodes a halophilic β-galactosidase (Holmes et al. 1996) is well suited as a marker for heterologous gene expression levels in *H. volcanii*. BGAH expresses well in this archaeal host and the amount of active BGAH shows a linear correlation with the measured rates of the catalytic conversion of ONPG to ONP monitored at 405 nm. As expected, moving the expression from shaker-flasks into a bioreactor resulted in increased protein production, presumably owing to higher oxygen availability.

Analysis of different induction patterns, e.g. one-step or two-step induction, as well as the different concentrations of inductor, its consumption over cultivation time and different incubation periods revealed the following trends:

i. Given that protein expression is a time-consuming process, therefore, longer incubation periods were beneficial for expression, up to a certain threshold of 18 to 20 h.
ii. Higher concentrations of the inductor led to higher protein yields, with a maximum yield for L-tryptophan levels of about 6 mM. Higher amounts of L-tryptophan led to a decrease in protein expression. Presumably because at slightly increased concentrations, the intrinsic tryptophanase activity gets stimulated and at significantly higher concentrations apparently because of its dose-dependent toxicity (Gross et al. 1999).
iii. The highest expression was observed if L-tryptophan concentrations remained constant at a level of about 6 mM.
iv. Consumption of the inductor occurred towards the end of the exponential growth phase if the concentration of the inductor is >6 mM.

Taking these findings into account, heterologous gene expression was improved by introduction of a double induction pattern, with the first induction at the beginning of the exponential growth phase (6 mM L-tryptophan) followed by an incubation period of 16 h and a subsequent second induction (3 mM L-tryptophan) 2 to 4 h before cell harvest. This amounts to a total expression time of 18 to 20 h for maximal protein yields.

Screening experiments were performed in parallel-operated single-use bioreactors in a 10-mL scale. The results from this system can be scaled up directly to 1-L scale (Weuster-Botz et al. 2005).

Applying these optimised conditions for the expression of two halophilic alcohol dehydrogenases from the Red Sea brine pools gave a yield of 16.8 mg g_{CDW}^{-1} for ADH/D1 and 3.9 mg g_{CDW}^{-1} for ADH/A1 at final cell densities of 6.5 and 5.3 g L^{-1}, respectively. In contrast, from shaker-flasks, only 3.53 and 0.44 mg g_{CDW}^{-1} of purified ADH/D1 and ADH/A1 were obtained from final cell densities of 2.1 and 1.7 g L^{-1}. This comparison is illustrated in Fig. 9. The batch cultivation in the stirred-tank bioreactor led to a threefold increase in cell density and an increase in purified protein per cell dry weight by factors of 4.7 (ADH/D1) and 8.8 (ADH/A1). These values translate into total purified protein amounts of 109 mg (ADH/D1) and 20.6 mg (ADH/A1), which could be isolated from a 1-L scale stirred-tank bioreactor. This is an improvement in the yield of purified protein by the factor 15 (ADH/D1) and 28 (ADH/A1) over shaker-flask cultures. Apparently, for the weaker expressing ADH/A1, the benefit of bioreactor cultivation is higher.

Detailed analysis of the *H. volcanii* batch processes in stirred-tank bioreactors suggested reasons for the differences observed during the expression of the two alcohol dehydrogenases. In general, neither culture suffered from carbon limitation, as indicated by the observation that the initially added lactate was not metabolised completely (final concentration 1 g L^{-1} in both cases, Fig. 7b). During batch cultivation and expression of ADH/D1, negligible metabolism of complex components of the media to ammonia was evident, e.g. the ammonia concentration remained constant at 0.41 g L^{-1} for 25 h followed by an increase to 0.55 g L^{-1}. Both *H. volcanii* cultures formed acetate, which has an expression- and growth-reducing effect (Braesen and Schoenheit 2001; Wolfe 2005), yet in the case of ADH/D1, the maximum concentration of 0.12 g L^{-1} dropped to 0.08 g L^{-1} at harvest time. In contrast, the formation of high acetate levels (with a constant increase to 0.28 g L^{-1}) accompanied ADH/A1 expression and ammonia accumulated to a high concentration of up to 0.70 g L^{-1} (Fig. 7c, d) indicates a higher rate of metabolism of amino acids in the medium into ammonia. Apparently, in the case of ADH/A1 expression, the nutrients and amino acids available in the complex medium were not readily used for protein production and rather metabolised to growth-limiting side products. Correspondingly, the cell dry weight for ADH/A1-expressing cultures only reached approximately 80 % of that achieved for ADH/D1 cultures. Limited protein production translated into a yield of only 3.9 mg g_{CDW}^{-1} which is less than one fourth that of the stronger expressing alcohol dehydrogenase ADH/D1. These findings indicate that ADH/A1 is active inside the expression host and interacts with the metabolism of *H. volcanii* H1895. The enzyme ADH/A1

presumably catalyses the formation of a product, or consumes an educt, that is directly or indirectly correlated to the protein production machinery.

H. volcanii H1895 can now be used as an adequate and alternative expression system for halophilic proteins in the stirred bioreactor. Based on our studies, cultivation with cell densities of up to 10 g L^{-1} and protein expression can now be performed successfully and reliably in a stirred-tank bioreactor using a supplemented Hv-YPC medium and a two-step L-tryptophan induction. Using this protocol, two novel alcohol dehydrogenase genes isolated from the Red Sea brine pools were expressed and purified in good yields of up to 16.8 mg g_{CDW}^{-1}. Correspondingly, this protocol seems to be broadly applicable and opens the door to a large-scale production of proteins from a halophilic microorganism with *H. volcanii* in a batch-operated stirred-tank bioreactor set-up.

Acknowledgments Research reported in this publication was supported by the King Abdullah University of Science and Technology (KAUST). The authors gratefully acknowledge the support of Eva Strillinger and Stefan Grötzinger by the International Graduate School of Science and Engineering (IGSSE), Technische Universität München (TUM), Germany. We thank Anastassja Akal, Ram Karan and Lars Janoscheck for their assistance.

Compliance with Ethical Standards

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

This article does not contain any studies with human participants or animals performed by any of the authors.

References

Albers SV, Pohlschroder M (2009) Diversity of archaeal type IV pilin-like structures. Extremophiles 13(3):403–10. doi:10.1007/s00792-009-0241-7

Allers T (2010) Overexpression and purification of halophilic proteins in *Haloferax volcanii*. Bioengineered Bugs 1(4):288–290. doi:10.1128/AEM.02670-09

Allers T, Ngo HP, Mevarech M, Lloyd RG (2004) Development of additional selectable markers for the halophilic Archaeon *Haloferax volcanii* based on the leuB and trpA genes. Appl Environ Microbiol 70(2):943–953. doi:10.1128/aem.70.2.943-953.2004

Allers T, Barak S, Liddell S, Wardell K, Mevarech M (2010) Improved strains and plasmid vectors for conditional overexpression of His-tagged proteins in *Haloferax volcanii*. Appl Environ Microbiol 76(6):1759–69. doi:10.1128/AEM.02670-09

Antunes A, Ngugi DK, Stingl U (2011) Microbiology of the Red Sea (and other) deep-sea anoxic brine lakes. Environ Microbiol Rep 3(4):416–33. doi:10.1111/j.1758-2229.2011.00264.x

Arends I, Sheldon RA, Hanefeld U (2007) Introduction: green chemistry and catalysis green chemistry and catalysis I. WILEY-VCH Verlag GmbH & Co, KGaA

Bertani G (1951) Studies on lysogenesis. J Bacteriol 62(3):293–300

Bitan-Banin G, Ortenberg R, Mevarech M (2003) Development of a gene knockout system for the halophilic archaeon *Haloferax volcanii* by use of the pyrE gene. J Bacteriol 185(3):772–778. doi:10.1128/jb.185.3.772-778.2003

Braesen C, Schoenheit P (2001) Mechanisms of acetate formation and acetate activation in halophilic archaea. Arch Microbiol 175(5):360–368. doi:10.1007/s002030100273

Cao Y, Liao L, Xu XW, Oren A, Wang C, Zhu XF, Wu M (2008) Characterization of alcohol dehydrogenase from the haloalkaliphilic archaeon *Natronomonas pharaonis*. Extremophiles 12(3):471–6. doi:10.1007/s00792-007-0133-7

Connaris H, Chaushuri JB, Danson MJ, Hough DW (1998a) Expression, reactivation and purification of enzymes from *Haloferax volcanii* in *Escherichia coli*. Biotechnol Bioeng 64(1):38–45

Connaris H, West MW, Hough DW, Danson MJ (1998b) Cloning and overexpression in *Escherichia coli* of the gene encoding citrate synthase from the hyperthermophilic Archaeon *Sulfolobis solfataricus*. Extremophiles 2:61–66

Danson MJ, Hough DW (1998) Structure, function and stability of enzymes from the Archaea. Trends Microbiol 6(8):307–314

Eder W, Jahnke LL, Schmidt M, Huber R (2001) Microbial diversity of the brine-seawater interface of the Kebrit Deep, Red Sea, studied via 16S rRNA gene sequences and cultivation methods. Appl Environ Microbiol 67(7):3077–85. doi:10.1128/AEM.67.7.3077-3085.2001

Eichler J (2001) Biotechnological uses of archaeal extremozymes. Biotechnol Adv 19

Elleuche S, Schroder C, Sahm K, Antranikian G (2014) Extremozymes—biocatalysts with unique properties from extremophilic microorganisms. Curr Opin Biotechnol 29:116–23. doi:10.1016/j.copbio.2014.04.003

Esquivel RN, Pohlschroder M (2014) A conserved type IV pilin signal peptide H-domain is critical for the post-translational regulation of flagella-dependent motility. Mol Microbiol 93(3):494–504. doi:10.1111/mmi.12673

Fairbanks G, Steck TS, Wallach DFH (1971) Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry 10(13):2606–2617

Frols S, Dyall-Smith M, Pfeifer F (2012) Biofilm formation by haloarchaea. Environ Microbiol 14(12):3159–74. doi:10.1111/j.1462-2920.2012.02895.x

Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD, Bairoch A (2005) Protein identification and analysis tools on the ExPASy server. In: Walker JM (ed) The proteomics protocols handbook, vol 10, Springer. University of Hertfordshire, Hatfield, pp 571–607

Ghosh A, Albers SV (2011) Assembly and function of the archaeal flagellum. Biochem Soc Trans 39(1):64–9. doi:10.1042/BST0390064

Gross B, Ronen N, Honigman S, Livne E (1999) Tryptophan toxicity—time and dose response in rats. Tryptophan, serotonin and melatonin. Adv Exp Med Biol 467:507–516

Grötzinger SW, Alam I, Ba Alawi W, Bajic VB, Stingl U, Eppinger J (2014) Mining a database of single amplified genomes from Red Sea brine pool extremophiles—improving reliability of gene function prediction using a profile and pattern matching algorithm (PPMA). Front Microbiol 5:134. doi:10.3389/fmicb.2014.00134

Hatti-Kaul R, Tornvall U, Gustafsson L, Borjesson P (2007) Industrial biotechnology for the production of bio-based chemicals—a cradle-to-grave perspective. Trends Biotechnol 25(3):119–24. doi:10.1016/j.tibtech.2007.01.001

Hollmann F, Arends IWCE, Holtmann D (2011) Enzymatic reductions for the chemist. Green Chem 13(9):2285. doi:10.1039/c1gc15424a

Holmes ML, Dyall-Smith M (2000) Sequence and expression of a halobacterial beta-galactosidase. Mol Microbiol 36(1):114–122

Holmes M, Scopes RK, Moritz R, Simpson RJ, Englert C, Pfeifer F, Dyall-Smith M (1996) Purification and analysis of an extremely halophilic beta-galactosidase from *Haloferax alicantei*. Biochem Biophys Acta 1337:276–286

Holmes M, Kamekura M, Lam W, Nuttall S, Woods WG, Jablonski P, Serrano J, Ngui K, Antón J, Allers T (2008) The Halohandbook, vol 7

Hough DW, Danson MJ (1999) Extremozymes. Curr Opin Chem Biol 3: 39–46

Karan R, Capes M, DasSarma P, DasSarma S (2013) Cloning, overexpression, purification and characterization of a polyextremophilic beta-galactosidase from the Antarctic haloarchaeon *Halorubrum lacusprofundi*. BMC Biotechnol 13:3

Kennedy SP, Ng WV, Salzberg SL, Hood L, DasSarma S (2001) Understanding the adaptation of *Halobacterium* sp. NRC-1 to its extreme environment through computational analysis of its genome sequence. Genome Res 11(10):1641–50. doi:10.1101/gr.190201

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Large A, Stamme C, Lange C, Duan Z, Allers T, Soppa J, Lund PA (2007) Characterization of a tightly controlled promoter of the halophilic archaeon *Haloferax volcanii* and its use in the analysis of the essential cct1 gene. Mol Microbiol 66(5):1092–106. doi:10.1111/j.1365-2958.2007.05980.x

Lestini R, Laptenok SP, Kuhn J, Hink MA, Schanne-Klein MC, Liebl U, Myllykallio H (2013) Intracellular dynamics of archaeal FANCM homologue Hef in response to halted DNA replication. Nucleic Acids Res 41(22):10358–70. doi:10.1093/nar/gkt816

Liliensiek AK, Cassidy J, Gucciardo G, Whitely C, Paradisi F (2013) Heterologous overexpression, purification and characterisation of an alcohol dehydrogenase (ADH2) from *Halobacterium* sp. NRC-1. Mol Biotechnol 55(2):143–149 doi:10.1007/s12033-013-9666-4

Madern D, Ebel C, Zaccai G (2000) Halophilic adaption of enzymes. Extremophiles 4:91–98

Mevarech M, Frolow F, Gloss LM (2000) Halophilic enzymes: proteins with a grain of salt. Biophys Chem 86:155–164

Meyer H-P (2011) Sustainability and biotechnology. Org Process Res Dev 15:180–188

Mironescu M, Mironescu ID, Jascanu V, Posten C (2003) Influence of cultivation media on halobacteria I growth and biomass formation. ACTA Univ Cibiniensis 1(7):17–24

Mullakhanbhai M, Larsen H (1975) *Halobacterium volcanii* spec. nov., a Dead-Sea halobacterium with a moderate salt requirement. Arch Microbiol 104:207–214

Oren A (2008) Microbial life at high salt concentrations: phylogenetic and metabolic diversity. Saline Syst 4:2. doi:10.1186/1746-1448-4-2

Puskeiler R, Kaufmann K, Weuster-Botz D (2005) Development, parallelization, and automation of a gas-inducing milliliter-scale bioreactor for high-throughput bioprocess design (HTBD). Biotechnol Bioeng 89(5):512–23. doi:10.1002/bit.20352

Rothschild LJ, Mancinelli RL (2001) Life in extreme environment. Nature 409:1092–1101

Rozzel JD (1999) Commercial scale biocatalysts: myths and reality. Bioorg Med Chem 7:2253–2261

Stroud A, Liddell S, Allers T (2012) Genetic and biochemical identification of a novel single-stranded DNA-binding complex in *Haloferax volcanii*. Front Microbiol 3:224. doi:10.3389/fmicb.2012.00224

Timpson LM, Alsafadi D, Mac Donnchadha C, Liddell S, Sharkey MA, Paradisi F (2012) Characterization of alcohol dehydrogenase (ADH12) from *Haloarcula marismortui*, an extreme halophile from the Dead Sea. Extremophiles 16(1):57–66. doi:10.1007/s00792-011-0405-0

Timpson LM, Liliensiek AK, Alsafadi D, Cassidy J, Sharkey MA, Liddell S, Allers T, Paradisi F (2013) A comparison of two novel alcohol dehydrogenase enzymes (ADH1 and ADH2) from the extreme halophile *Haloferax volcanii*. Appl Microbiol Biotechnol 97(1):195–203. doi:10.1007/s00253-012-4074-4

Tripepi M, You J, Temel S, Onder O, Brisson D, Pohlschroder M (2012) N-glycosylation of *Haloferax volcanii* flagellins requires known Agl proteins and is essential for biosynthesis of stable flagella. J Bacteriol 194(18):4876–87. doi:10.1128/JB.00731-12

Tripepi M, Esquivel RN, Wirth R, Pohlschroder M (2013) *Haloferax volcanii* cells lacking the flagellin FlgA2 are hypermotile. Microbiology 159(Pt 11):2249–58. doi:10.1099/mic.0.069617-0

van den Burg B (2003) Extremophiles as a source for novel enzymes. Curr Opin Microbiol 6(3):213–218. doi:10.1016/s1369-5274(03)00060-2

Wang Y, Cao H, Zhang G, Bougouffa S, Lee OO, Al-Suwailem A, Qian PY (2013) Autotrophic microbe metagenomes and metabolic pathways differentiate adjacent Red Sea brine pools. Sci Rep 3:1748. doi:10.1038/srep01748

Weuster-Botz D, Puskeiler R, Kusterer A, Kaufmann K, John GT, Arnold M (2005) Methods and milliliter scale devices for high-throughput bioprocess design. Bioprocess Biosyst Eng 28(2):109–19. doi:10.1007/s00449-005-0011-6

Wolfe AJ (2005) The acetate switch. Microbiol Mol Biol Rev 69(1):12–50. doi:10.1128/MMBR.69.1.12-50.2005