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ORIGINAL PAPER

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Influence of temperature on the production of an archaeal thermoactive alcohol dehydrogenase from *Pyrococcus furiosus* with recombinant *Escherichia coli*

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Abstract The heterologous production of a thermoactive alcohol dehydrogenase (AdhC) from *Pyrococcus furiosus* in *Escherichia coli* was investigated. *E. coli* was grown in a fed-batch bioreactor in minimal medium to high cell densities (cell dry weight 76 g/l, OD_{600} of 150). Different cultivation strategies were applied to optimize the production of active AdhC, such as lowering the cultivation temperature from 37 to 28°C, heat shock of the culture from 37 to 42°C and from 37 to 45°C, and variation of time of induction (induction at an OD_{600} of 40, 80 and 120). In addition to the production of active intracellular protein, inclusion bodies were always observed. The maximal activity of 30 U/l (corresponding to 6 mg/l active protein) was obtained after a heat shock from 37 to 42°C, and IPTG induction of the adhC expression at an OD_{600} of 120. Although no general rules can be provided, some of the here presented variations may be applicable for the optimization of the heterologous production of proteins in general, and of thermozymes in particular.

Keywords Protein production · Thermoactive ADH · *Pyrococcus furiosus* · *Escherichia coli* · Overexpression

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Introduction

Thermoactive enzymes from thermophilic microorganisms have become subject to intense research in recent years. There is a growing demand for thermoactive enzymes because of their increased stability at high temperatures and in organic solvents (Bruins et al. 2001; Zeikus et al. 1998). Of the thermoactive enzymes, thermoactive alcohol dehydrogenases (ADHs) are of special interest in the field of white biotechnology. Radianingtyas and Wright (2003) reported more than 100 known ADHs from thermophilic mircroorganisms. ADHs can be used for the synthesis of high value optically pure compounds (Ernst et al. 2005; Haberland et al. 2002). Due to the low water-solubility of some substrates and products, the conversions of the latter compounds should be performed in the presence of an organic solvent. Therefore, the stability of the ADH in organic solvents is of great importance (Kosjek et al. 2004).

The application of enzymes is often hampered by their low abundance in their natural host. Thermophilic microorganisms usually grow to low cell densities and in general require complex media (Bustard et al. 2000), so the isolation of the target protein is often laborious and inefficient. A better way to produce sufficient amounts of enzyme is the overexpression in mesophilic host organisms like *E. coli*, *Bacillus subtilis* or *Pichia pastoris*. The thermostable proteins can easily be purified by a heat treatment and the host organisms can grow on mineral media. The heterologous production of (thermo-) active protein at mesophilic conditions, however, can be limited by unfavorable equilibria at several stages of the protein folding process. In the present study the functional production of *Pyrococcus furiosus* ADH is optimized by variation of several parameters during the cultivation process. In its original host, this ADH is folded at extremely high temperatures (100°C optimal growth temperature of the wildtype). In *E. coli* the same protein has to be produced and folded correctly at 37°C.

To date, relatively few thermoactive ADH have been overexpressed heterologously, in mesophilic host organisms. The patent DE10218689 reports the overproduction of a thermoactive and thermostable ADH from *Rhodococcus erythropolis* in *E. coli*, but no activities or protein production rates are provided (Hummel et al. 2003). Also, the company Jülich Chiral Solutions offers a recombinantly produced thermoactive ADHs from *Thermoanaerobacter* sp. without giving any further references. Burdette et al. (1996) and Holt et al. (2000) cloned and overexpressed a thermostable ADH from the extreme thermophilic bacterium *Thermoanaerobacter ethanolicus* but presented little information about the growth of the recombinant host organism.

Some archaeal ADH have previously been cloned in *E. coli* (Antonie et al. 1999; Cannio et al. 1996; Van der Oost et al. 2001). In the latter paper, the authors reported the cloning of two ADHs from *P. furiosus* in *E. coli*. Recently, another 17 putative ADH-encoding genes from *P. furiosus* have been identified and overexpressed in *E. coli* (Machielsen et al. 2002). Four of them are able to convert secondary alcohols such as 2,3-butanediol and were therefore investigated in more detail. The thermoactive enzyme used in this article is one of those. It is a mid-chain, zinc-containing, NAD-dependent alcohol dehydrogenase (EC 1.1.1.1) from *P. furiosus* DSM 3638, referred to as AdhC.

Material and methods

Cloning and sequencing of the alcohol dehydrogenase encoding gene

The identification of the gene encoding an alcohol dehydrogenase was based on significant sequence similarity to several known alcohol dehydrogenases. The *P. furiosus adh*C gene (PF0991, GenBank accession number AE010211 region: 3490–4536, NCBI) was identified in the *P. furiosus* database (http://www.genome.utah.edu). The *adh*C gene (1,047 bp) was PCR amplified from chromosomal DNA of *P. furiosus* using the primers BG1279 (5′-GCGCGCCATGG**GCA**TCCGAGAAGATGGTTGCTATCA, sense) and BG1297 (5′-GCGCGGGATCCTCATTTAAGCATGAAAACAACTTTGCC, antisense), containing *Nco*I and *Bam*HI sites (underlined in the sequences). In order to introduce an *Nco*I restriction site, an extra alanine codon (GCA) was introduced in the *adh*C gene by the forward primer BG 1279 (bold in the sequence). The fragment generated was purified using Qiaquick PCR purification kit (Qiagen, Hilden, Germany). The purified gene was digested with *Nco*I/*Bam*HI and cloned in *E. coli* XL1-Blue using an *Nco*I/*Bam*HI-digested pET24d vector. Subsequently, the resulting plasmid pWUR78 was transformed into *E. coli* BL21(DE3) harboring the tRNA helper plasmid pSJS1244 (Kim et al. 1998). The sequence of the expression clone was confirmed by sequence analysis of both DNA strands.

The AdhC is an intracellularly produced enzyme. The ADH-producing recombinant *E. coli* is indicated as *E. coli* ADHC in this paper. Its name in the *P. furiosus* genome project is PF0991, (Robb et al. 2001); its NCBI gene identifier is 18893044. The sequence belongs to COG1063 (Threonine dehydrogenase and related Zn-dependent dehydrogenases).

Medium

For the growth of *E. coli* ADHC, a modified medium from Horn et al. (1996) was used. The medium contained (g/l): KH_2PO_4 16.6, $(NH_4)_2HPO_4$ 4, citric acid 2, $MgSO_4{\cdot}7H_2O$ 1.5. Trace elements (mg/l): H_3BO_3 3.8, $MnCl_2{\cdot}2H_2O$ 15.4, EDTA·$2H_2O$ 10.5, $CuCl_2{\cdot}2H_2O$ 1.9, $Na_2MoO_4{\cdot}2H_2O$ 3.1, $CoCl_2{\cdot}6H_2O$ 3.1, $Zn(CH_3COO)_2{\cdot}2H_2O$ 10, Fe(III)citrate hydrate 75. Antibiotics were used in the following concentrations: Kanamycin 0.1 mg/l, Spectinomycin 0.1 mg/l. Glucose 15 g/l was used as the carbon source in the batch phase.

One liter of feed consisted of 520 mL H_2O, 700 g glucose monohydrate, trace elements as denoted in the medium, and $MgSO_4{\cdot}7H_2O$ 19.8 g. The glucose concentration in the feed was 636 g/l; the density of the feed was 1,220 g/l. Pre-cultures were grown on LB medium with antibiotics in the same concentration as in the mineral medium. At the time of induction 2.5 M $ZnCl_2$ solution was added to the bioreactor, to a final concentration of 0.25 mM.

Growth of recombinant *E. coli*

E. coli cells were stored in 1 ml 80% glycerol aliquots at −80°C. One aliquot was transferred into 50 ml LB-medium in a 250 ml shaking flask with baffles and shaken at 175 rpm, 25 mm diameter for 4 h at 37°C. Depending on the OD of the pre-culture, 5–20 ml of the mid-exponential culture was inoculated into the bioreactor containing 1,200 ml mineral medium.

A 2 l Visual Safety Reactor (Bioengineering, Wald, CH) was used for all experiments. The bioreactor was equipped with a pH probe and a dissolved oxygen probe. pH was titrated to 6.8 by addition of 25% NH_3 (aq.) solution. Unless stated otherwise, the cultivation temperature was 37°C. The bioreactor was stirred constantly at 3,000 rpm and aerated with a mixture of air and oxygen at 0.8 vvm. The maximum oxygen concentration in the inlet gas was 40%. The gas composition was regulated manually, to keep the DO between 50 and 120% air saturation. Due to the manual control the dissolved oxygen concentration showed great fluctuations but always was well above the critical value of 30% (Castan and Enfors 2002).

The culture grew overnight to an OD_{600} of 15 at the end of the batch phase. After glucose was consumed completely, acetic acid (which was produced during unlimited growth) was used as the carbon source. This

could be verified by an increase in dissolved oxygen and pH. After this increase of DO and pH, exponential feeding (Korz et al. 1995) was started. Before induction the growth rate was set to $0.30/h^{-1}$, and was lowered if necessary when glucose was accumulating in the bioreactor. After induction, constant feeding of 20 g/h was applied if not stated otherwise. During growth, optical density and glucose concentration were measured at-line. Optical density was measured with a Thermo Spectronic Genesys 10 VIS spectrometer. Glucose was measured with a YSI 2700 Select (Yellowsprings Instruments, Yellow Springs, OH, USA) enzyme reactor. Dissolved oxygen was measured on-line; cell dry weight was measured off-line. The production of enzyme was induced with 0.1 mM IPTG at an OD_{600} of 40 (corresponding to approximately 20 g/l cdw, early exponential phase) unless otherwise stated. The cultivation was stopped when the increase in OD_{600} ceased or the constant feeding could no longer be maintained without the accumulation of glucose in the bioreactor.

Sample preparation and activity assay

Samples taken from the bioreactor were diluted with 20 mM Tris–HCl buffer pH 7.5 1:2 to 1:10 and sonicated for 10 min on ice. The sample was centrifuged for 10 min at 10,000 *g* at 4°C. The cell free extract (CFE) was incubated at 80°C for 30 min and centrifuged again for 10 min at 10,000 *g* and 4°C yielding the heat stable cell free extract (HSCFE).

ADH activity was detected by the oxidation of 2,3-butanediol to acetoin at 70°C and the parallel reduction of NAD to NADH. The test cuvette contained 880 µl 50 mM glycin buffer pH 10, 100 µl 100 mM 2,3-butanediol solution (racematic mixture), and 10 µl 28 mM NAD solution. The test was started by addition of 10 µl HSCFE. Adsorption at 340 nm was monitored for 4 min. A parabola was fitted through the measured values. The slope at $t=0$ multiplied by the molar extinction coefficient of NADH at 340 nm ($6.22\ mM^{-1}\ cm^{-1}$) resulted in the volumetric activity. One unit is defined as the conversion of 1 µmol NAD in 1 min at standard conditions (pH 10, $T=70$°C, 100 mM 2,3-Butanediol, 0.28 mM NAD).

To determine the specific activity of the pure AdhC the HSCFE was purified to homogeneity by a Q-Sepharose anion-exchange step as described by Van der Oost et al. (2001) for another ADH from *P. furiosus*. The pure AdhC has a specific activity of 5 U/mg at standard conditions. Protein content was measured according to Bradford (1976). Bovine serum albumin was used as the protein standard.

SDS and native PAGE

A Mini-Protean II (Biorad, Hercules, CA, USA) system was used for gel electrophoresis. Ten percent polyacryamide gels were used (Laemmli 1970). Samples were incubated in 1:4 dilution with sample buffer (10% SDS, 10 mM DTT, 10% (v/v) glycerol, 0.2 M Tris pH 6.8, and 0.05% bromophenole blue for SDS gel; 100 µl glycerol (80%), 100 µl bromophenole blue (1%), 300 µl Tris pH 6.8 for native gel) at 90°C for 5 min. The gel ran first 30 min at 50 V and then 2 h at 100 V; afterwards the gels were Coomassie-stained. Precision Plus Protein Unstained Standards (Biorad, USA) ranging from 10 to 250 kDa were used as markers.

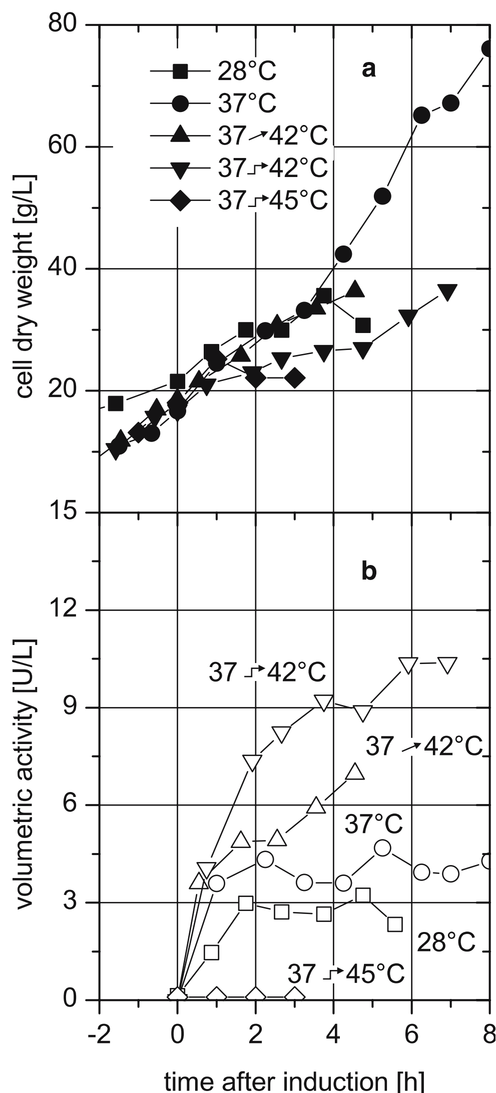

Fig. 1 Fermentation of *E. coli* ADHC at different temperatures and temperature profiles. Growth of microorganism (**a**) and enzyme production (**b**) are shown. Growth on minimal medium in a 2 l Bioreactor with an exponential feeding stategy. Induction was done with 0.1 mM IPTG at OD_{600} 40 which corresponds to ~20 g/L cdw. 64×202 mm (600×600 DPI)

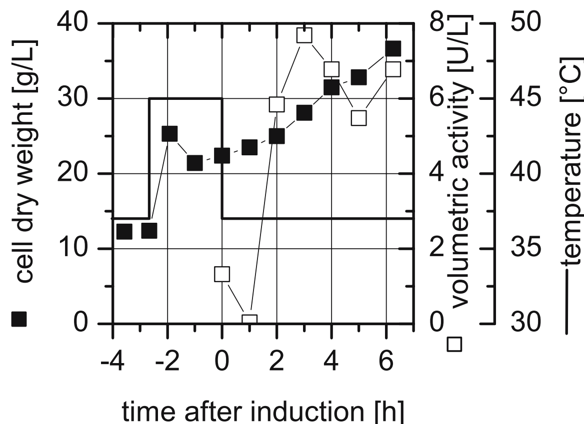

Fig. 2 Influence of heat shock proteins on the production of active enzyme. The heat shock proteins were produced by increasing the fermentation temperature from 37 to 45°C 2.5 h before induction. At induction, the temperature was set back to 37°C. Induction at OD_{600} of 45 with 0.1 mM IPTG. 92×70 mm (600×600 DPI)

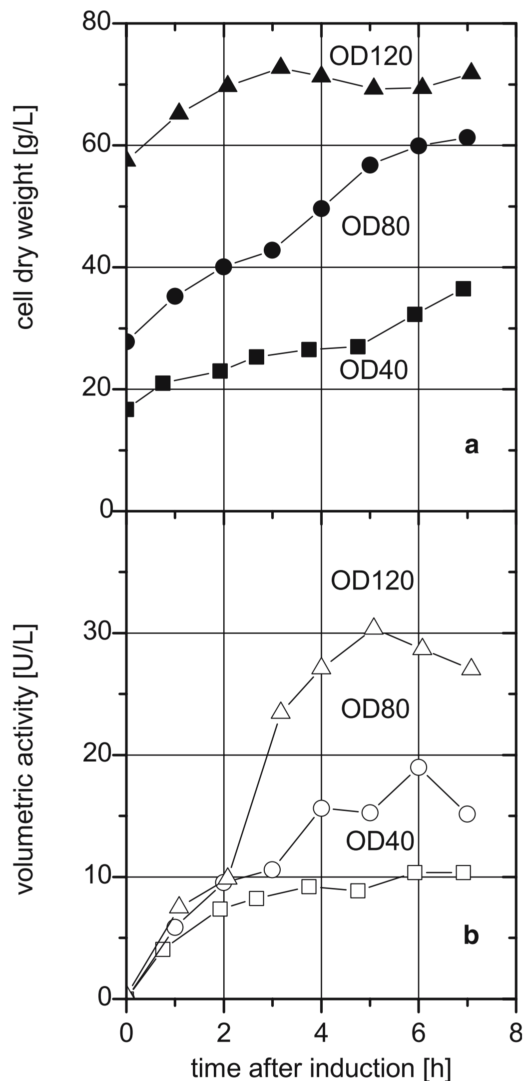

Fig. 3 Increase of ADH production depending on time of induction. Induction at early (OD_{600} 40), mid- (OD_{600} 80) and late exponential (OD_{600} 120) growth phase with 0.1 mM IPTG and temperature shock to 42°C. Growth of biomass (**a**) and enzyme production (**b**) is shown. 77×152 mm (600×600 DPI)

Results

Influence of *E. coli* ADHC growth temperature on active enzyme production

To examine the influence of growth temperature on the protein production, *E. coli* ADHC was grown at different temperatures. Two cultivations were performed at constant temperatures of 28 and 37°C. One cultivation was done with a stepwise increase of the temperature of 0.5°C/30 min from 37 to 42°C starting 5 h before induction. Two cultivations were done with a sudden increase of temperature at the time of induction, from 37 to 42°C and from 37 to 45°C. All experiments were induced with 0.1 mM IPTG at an OD_{600} of 40. At induction the feeding was changed from exponential feeding to constant feeding of 20 g/h feed solution.

The volumetric activities and cell dry weights are shown in Fig. 1. Throughout all cultivations, production of inclusion bodies was observed. Generally, a higher bioprocess temperature yielded higher enzyme activities. The maximal activities were 3 U/l at 28°C, 4.6 U/l at 37°C, 7 U/l with a slow increase of temperature from 37 to 42°C within 5 h, and 10.4 U/l with a sudden increase of temperature from 37 to 42°C. However, with a sudden increase of temperature from 37 to 45°C no activity was found. Also, the growth ceased at 45°C and strong foaming occurred.

The fast increase from 37 to 42°C led to a higher activity than the slow increase within 5 h. This may indicate that heat shock proteins help the active ADH fold correctly. To verify this assumption, the culture was heated at 45°C for 2.5 h and then cooled back to 37°C for induction. The enzyme production was compared to the cultivation at 37°C without heat shock before induction. The result is shown in Fig. 2. The highest activity of 7.7 U/l was reached 3 h after induction at a cell density of 28 g/l. Without heat shock and at a constant cultivation temperature, 3.6 U/l and 33 g/l cdw was reached 3 h after induction. The production of biomass was reduced after the heat shock, but with less biomass more active protein was produced.

Influence of induction point on enzyme production

Three experiments have been conducted to determine the optimal point of induction. Enzyme production was induced at OD_{600} of 40, 80 and 120. The temperature was set to 42°C after induction, the feed was set constant to 20, 40 and 60 g/h of feed solution, respectively, in these experiments. Figure 3 displays the experimental result. The highest activities reported after 5–6 h of

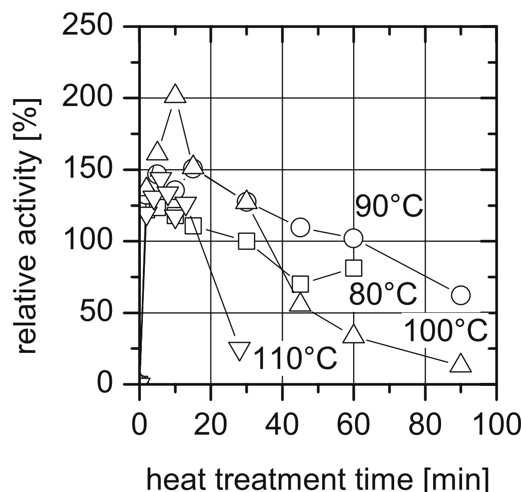

Fig. 4 Effect of heat treatment of the CFE at 80, 90, 100, and 110°C on the activity. All activities are related to standard conditions (30 min at 80°C = 100%). 77×74 mm (600×600 DPI)

induction were 10, 19 and 30 U/l. The highest enzyme activity was obtained by induction at an OD_{600} of 120, which is late exponential growth.

Dependence of heat treatment of cell free extract on ADH activity

The standard procedure for the heat treatment was varied. Cell free extract (CFE) was incubated for 90 min at 80, 90, 100 and 110°C, and samples were taken at regular intervals. The impact of the heat treatment in the purification step is shown in Fig. 4. Without heat treatment no activity is measured. The activity increases at the beginning of the heat treatment. The highest activity was measured after 10 min at 100°C. Here, the activity was doubled compared to normal heat treatment procedure. Longer incubation and higher temperature led to thermal inactivation of the enzyme.

Analysis of HSCFE

Figure 5 shows a SDS-PAGE and a native gel from the experiment with induction at $OD_{600} = 120$. The first lane is from the time of induction and the next lanes show samples taken every hour after induction. On the SDS gel two major bands are visible at 40 and 37 kDa. The upper band occurs after induction and is the AdhC monomer. The lower band is visible before induction but cannot be found on the native gel. Here, the homotetramer of AdhC appears at 150 kDa. The calculated size from the amino acid sequence of the enzyme is 38 kDa for the monomer and 152 kDa for the tetramer. On the gel, bigger proteins are also visible with sizes larger than 300 kDa. Bradford analyses of this sample indicate a protein content of 0.5 g/l in the HSCFE.

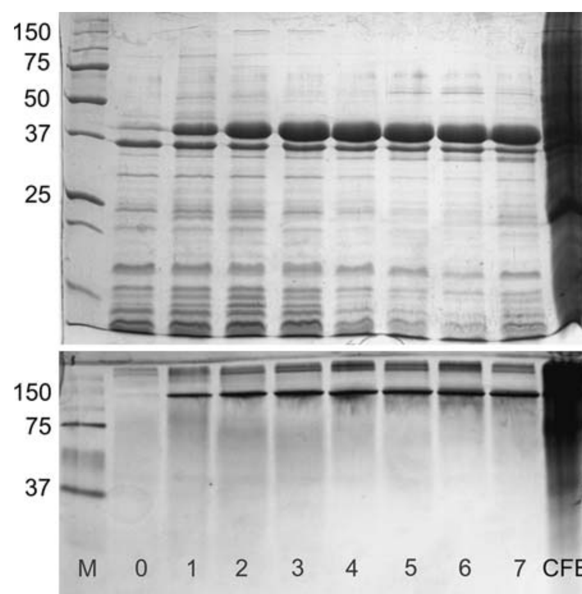

Fig. 5 SDS (*upper*) and native (*lower*) PAGE of the HSCFE of the experiment with induction at OD600 120. *M* standard protein marker, *0* time of induction, 1-7 mark the hours after induction. *CFE* without heat treatment. The 40 kDa band is the ADHC monomer. On the native gel the tetrameric structure of the ADH becomes evident with the band at 150 kDa

Discussion

The correct folding of the AdhC from hyperthermophilic *P. furiosus* in mesophilic recombinant *E. coli* was greatly influenced by the cultivation temperature. When grown at temperatures above the optimal growth temperature, *E. coli* produces heat shock proteins to prevent protein aggregation (Ehrnsperger et al. 1997). Heat shock proteins are known for their chaperonin activity, i.e., they help the protein folding and are responsible for an efficient protein quality control. When heated at 45°C for 2.5 h prior to induction, an increase of the AdhC activity was monitored compared to the standard cultivation at 37°C. The fast increase to 42°C yielded more active enzyme than the slow increase to 42°C. This suggests that heat shock proteins either assist in the correct folding of the AdhC, or maybe even allow for resolubilization of (partially) denatured molecules. The cultivation at 45°C was not successful, no activity could be found after induction and temperature increase. When grown in rich medium, *E. coli* has a temperature maximum of 49°C (Ingraham and Marr 1996). However, Ron and Bernard (1971) reported that production of methionine is inhibited at elevated temperatures. This is a possible explanation of the cessation of growth at 45°C on minimal medium which does not contain proteins or amino acids but just glucose and ammonia.

Also, the temperature of the heat treatment contributes to activity of AdhC. Without heat treatment the AdhC shows no detectable activity; but after short incubation at higher temperatures the activity is

revealed. The same effect was reported by Antoine et al. (1999) for recombinant ADH from *Thermococcus hydrothermalis*, which is also a tetramer.

Interestingly, a monomeric short chain recombinant ADH from *P. furiosus* does not show this effect; the enzyme activity can be measured without heat treatment, and no increase of activity can be monitored in the first minutes of heat treatment (data not shown).

Five hours after induction, in the experiment with abrupt temperature shift from 37 to 42°C and induction at OD_{600} of 120 the HSCFE contained 30 U/l and 0.5 g/l protein. The purified enzyme has a specific activity of 5 U/mg. From these values, we derive that only 1.2% of the total protein in the HSCFE corresponds to the active AdhC. The SDS- and native PAGE indicate minor impurities (Fig. 5), which cannot explain the 98.8% non-active thermostable protein. This indicates that most of the thermostable protein, still able to form tetramers and higher oligomers, is either inhibited or it requires a (probably minor) structural rearrangement; it is likely that the stimulating effect of a heat shock may bring about such an adjustment.

It is very difficult to extract general conclusions from the literature on the production of thermostable protein in general and of archaeal thermostable ADHs in particular. Different types of ADHs are hard to compare. Generally ADHs have a broad substrate spectrum, but there is no substrate which all ADHs can convert at comparable rates. The activity of the enzyme depends on substrate and co-substrate, and the concentrations thereof. High substrate concentrations often inhibit ADH. A possible way to circumvent the problem of different activities is to compare the amounts of functionally produced enzyme. In this paper the active enzyme yield was increased from 0.06 mg/l in shaking flask experiments (cultivation conditions according to pET system manual, Novagen 2003) to 5 mg/l in the optimized cultivation.

For the overexpression of genes from thermophilic organisms few protein yields are available. Riessen and Antranikian (2001) reported the expression of a keratinase from the extreme thermophilic bacterium *Thermoanaerobacter keratinophilus*; Brodersen (2005) optimized the production of this keratinase in recombinant *E. coli* and reached 290 mg/l active protein. Zappa et al. (2002) optimized overproduction of the thermoactive alkaline phosphatase from *Pyrococcus abyssi* and achieved protein yields of 8.3 mg/l with an *E. coli* host system. The same authors also tried a *Pichia pastoris* expression system with secretion of the target protein and reached approximately 4 mg/l in the supernatant of a fed batch culture.

An important question is whether or not *E. coli* is a good expression system for proteins from hyperthermophilic archaea. From our experimental results we can expect that application of a high-temperature expression system might result in more active enzyme. However, very few high-temperature expression systems are available. Lucas et al. (2002) constructed a shuttle vector which maintained a high copy number in *Pyrococcus abyssi*. Using anaerobic archaea as cell factories for thermostable, protein production is limited by the low cell density of these archaea in fermentation processes (Biller et al. 2002; Raven and Sharp 1997). Alternatively, *Sulfolobus* species can be used as thermophilic host organism since it grows to higher cell densities (Krahe et al. 1996; Schiraldi et al. 1999). An expression system is also available. Contursi et al. (2003) have developed a generic system for a *Sulfolobus sulfataricus* host and overexpressed ADH from the moderate thermophile *Bacillus stearothermophilus*. Aravalli and Garrett (1997) developed shuttle vectors which could be implemented in *Sulfolobus acidocaldarius*. The authors used an ADH of *S. solfataricus* and successfully overexpressed the enzyme. It is obvious that the thermophile expression systems also require optimization. Hence, for the time being, it may be worth the effort to enhance the functional production of a target protein in *E. coli* by optimizing the cultivation conditions as described in this study. However, it should be kept in mind that no general rules can be provided for the production of different proteins, sometimes a single amino acid change results in dramatic differences. Still, some of the here presented variations may be applicable for the optimization of the heterologous production of proteins in general, and of thermozymes in particular.

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