Introduction

Chemical production is progressively turning towards more sustainable feedstocks and processes. As an alternative to petroleum, biomass can serve as a source for important chemicals such as organic solvents, e.g. n-butanol, isobutanol or ethanol. Biotechnological approaches for conversion of biomass into some of these molecules are well established by fermentation [1,2]. However, these microbial processes show several limitations such as reduced product yield due to by-product formation, maintenance of the cells metabolism and, more importantly, low productivity and product titer due to the toxicity of the products formed [3]. One solution to these problems, which has recently found strong interest, is the elimination of the cell as production vehicle and the application of synthetic enzymatic cascades instead [4,5,6,7,8].

In Synthetic Cascade Biomanufacturing the process limits are given only by the limits of enzymes, which can be much more robust than cells recently we showed the prospect of this approach for the production of isobutanol and ethanol in a cell-free system [9]. This artificial, solely enzyme based cascade system shows remarkable advantages compared to fermentative processes. The enzymes used in the artificial cascade tolerate organic solvents to higher levels than cell based systems, and remain active up to 4%v/v isobutanol. At this concentration, microbial hosts are unable to survive or maintain active metabolism [1,2,3,10]. In contrast to fermentative approaches, the artificial cell-free process is based on thermostable enzymes and hence is optimized to be functional at higher temperatures, leading to faster conversion and a higher product yield.

The process is based on conversion of the substrate glucose, which represents the prevalent component of biomass. Glucose can easily be generated from different kinds of biomass. Starch is used for many biotechnological based processes by digestion of the polymer to its glucose monomers [5,11]. Following the biorefinery concepts, glucose will be supplied by pretreatment of lignocellulosic biomass in the near future [12,13,14]. In the artificial biocatalytic process, glucose is enzymatically converted to the organic solvents isobutanol and ethanol (Figure 1). One key reaction in the synthetic pathway is the oxidation of D-glyceraldehyde to D-glycerate. The corresponding enzyme, glyceraldehyde dehydrogenase (AlDH), ideally should combine the following properties: high thermostability and tolerance towards solvent, high activity, acceptance of NAD\(^+\) as cofactor and high substrate specificity.

Screening of scientific databases revealed a number of enzymes potentially suitable for the cell-free process. Table 1 shows a selection of these AlDHs whose catalytic functions have already been shown. All enzymes have a high temperature optimum suitable for the desired temperature of the process to be in the range of 50 to 60°C [9]. The desired AlDH has to accept NAD\(^+\) as cofactor and high substrate specificity.

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Figure 1. Function of glyceraldehyde dehydrogenase (AIDH) in our synthetic reaction cascade. Glucose is degraded enzymatically to pyruvate and glyceraldehyde. AIDH catalyzes the oxidation of \( \delta \)-glyceraldehyde to \( \delta \)-glycerate, which is then dehydrated to pyruvate. Ethanol and isobutanol are synthesized by further reaction steps. AIDH must not catalyze isobutyraldehyde or acetaldehyde oxidation since the irreversibly formed carboxylates are unwanted side products, thus lowering the overall yield. Important reactions are shown in detail (continuous arrows), while some reaction steps are summarized (dashed arrows).

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NAD<sup>+</sup> dependent. NAD<sup>+</sup> represents a cheaper and more stable alternative to NADP<sup>+</sup> [15]. As biocatalyst for the reaction of D-glyceraldehyde to D-glycerate, the desired AlDH needs to be highly substrate specific for glyceraldehyde because other aldehydes in the substrate chain must not be targeted for oxidation (Figure 1). In a final reaction step, acetaldehyde and isobutyraldehyde are reduced to ethanol and isobutanol respectively [17]. Once formed, the carboxylates cannot reenter the pathway without activation and the production yield for organic solvents would drop dramatically.

From the known AlDHs none appears to fulfill all requirements (Table 1). Enzymes with high substrate specificity are typically described to accept NADP<sup>+</sup>, whereas NAD<sup>+</sup>-dependent AlDHs appear to have broader substrate specificities. Since many NAD(P)<sup>+</sup> dependent aldehyde dehydrogenases have a strong preference for either NAD<sup>+</sup> or NADP<sup>+</sup> but some are generally accepting both cofactors [18,19,20], we chose AlDH from Thermoplasma acidophilum (TaAlDH) due its very high substrate specificity as most promising candidate for application in the cell-free production of ethanol and isobutanol. Here we thoroughly characterize TaAlDH in light of its application and provide protocols for its production in an active state.

**Materials and Methods**

**Chemicals**

All chemicals were purchased in analytical grade from Sigma-Aldrich (Munich, Germany), Carl Roth (Karlsruhe, Germany), Serva and Merck (Darmstadt, Germany).

**Cloning**

Codon-optimized *taaldh* gene was provided by Gencart (Regensburg, Germany); *taaldh* gene sequence was translated from protein sequence (NCBI accession number CAC11938.1). Construction of the expression vector was performed according to the protocol of Guterl et al. [9].

**TaAlDH Production**

For recombinant expression *E. coli* BL21 (DE3) (F<sup>−</sup> ompT hsdSB (rB<sup>−</sup> mB<sup>−</sup>) gal dcm), purchased from Novagen (Nottingham, UK), was transformed with pCBRHiC-*taaldh*, which codes for the C-terminal His-tag fusion of the protein.

Small scale protein expression was performed in shake flasks. Positive transformants of *E. coli* BL21 (DE3) were grown in auto-induction medium [21] at 37°C overnight. Cell lysis was performed with B-PER protein extraction reagent (Thermo Scientific, Ulm, Germany).

Large amounts of *TaAlDH* -expressing cells were produced by fed-batch fermentation according to the protocol of Neubauer et al. [22] in a 40 L Biostat Cplus bioreactor (Sartorius, Göttingen, Germany). Defined medium was supplemented with 30 mg/L kanamycin. After inoculation, cells were grown for 24 h at 37°C and then induced with 70 mg/L IPTG. Enzyme expression was performed for 3 h, yielding 10 g/L cells (wet weight).

Cells were harvested by centrifugation at 5,000 x g for 10 min at 25°C (Sorvall RC6+, Thermo Scientific), suspended in 10-fold the amount of loading buffer (200 mM NaCl, 20 mM imidazole, 2.5 mM MgCl<sub>2</sub>, 50 mM TRIS pH 8) containing 10 U/mL DNase I (Applichem, Darmstadt, Germany) and lysed with Basic-Z Cell Disruptor (Constant Systems, Northants, UK).

**TaAlDH Purification**

If not otherwise stated, purification steps were carried out at room temperature. Cell debris and protein aggregates were separated from soluble fraction by centrifugation at 30,000 x g for 45 min.

After heat treatment at 50°C for 30 min, soluble *TaAlDH* was further purified by nickel affinity chromatography using an AKTA UPC-900 FPLC-system (GE Healthcare, Freiburg, Germany). Supernatant was loaded on a HiTrap FF-column previously equilibrated with loading buffer. After a subsequent washing step, *TaAlDH* was eluted with imidazole buffer (200 mM NaCl; 500 mM imidazole; 50 mM TRIS pH 8) at a flow rate of 5 mL/min and fractions containing *TaAlDH* were combined. The buffer was switched to 20 mM [NH<sub>4</sub>]HCO<sub>3</sub> with HiPrep 26/10 desalting column and *TaAlDH* was lyophilized with an Alpha 2-4 LD Plus freeze dryer (Martin Christ, Osterode am Harz, Germany).

**Table 1. Characteristics of thermostable AlDHs for the reaction cascade shown in Figure 1.**

| AIDH origin | Expression system | A<sub>s</sub> (U/mg) | T<sub>opt</sub> (°C) | Cofactor | Substrate spectrum | Reference |
|-------------|-------------------|---------------------|-------------------|----------|--------------------|----------|
| Thermoplasma acidophilum | Native | 0.3 | 50–55 | NAD<sup>+</sup> | Glyceraldehyde, 3-phosphoglyceraldehyde, glycolaldehyde | [36] |
| Thermoplasma acidophilum | *E. coli* | 28.4 | 63 | NAD<sup>+</sup> | Glyceraldehyde, 3-phosphoglyceraldehyde, glycolaldehyde | [35] |
| Picrophilus torridus | *E. coli* | 10.9 | 63 | NAD<sup>+</sup> | Glyceraldehyde, 3-phosphoglyceraldehyde, glycolaldehyde | [35] |
| Flavobacterium frigidimarli | Native | 2.3 | 55–60 | NAD<sup>+</sup> | Glyceraldehyde, various aldehydes | [52] |
| Geobacillus thermoleovorans | *E. coli* | 0.1 | 50–55 | NAD<sup>+</sup> | Glyceraldehyde, long chain aldehydes | [53] |
| Bacillus stearotherophilus | *E. coli* | 36.3<sup>1</sup> | 50–60 | NAD<sup>+</sup> | Acetaldehyde, propionaldehyde, isobutyraldehyde, hexanaldehyde | [54] |

A<sub>s</sub>: Specific enzyme activity, T<sub>opt</sub>: Optimum temperature of enzyme activity.

<sup>1</sup>No activity with glyceraldehyde tested, data refers to acetaldehyde.

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Prior to refolding, insoluble TaAlDH inclusion bodies were purified to remove other insoluble materials from the pellet. First, inclusion bodies in the pellet were suspended in cleaning buffer (0.5% Triton X, 1 mM EDTA, 20 mM TRIS pH 8); the suspension was stirred for 20 min and centrifuged (30,000 x g, 4°C, 45 min). After that, the inclusion bodies were washed twice with washing buffer (1 mM EDTA, 20 mM TRIS pH 8). The purified inclusion body pellets were stored at −20°C.

Refolding TaAlDH

Purified TaAlDH inclusion bodies were dissolved in denaturation buffer (6 M guanidinium chloride (GdmCl), 2 mM dithiothreitol, 20 mM TRIS pH 8) at a protein concentration of 6.0 mg/mL and incubated for 1 h at 25°C. TaAlDH refolding was achieved by 30-fold dilution of the denaturation buffer and refolding yield was analyzed after dilution in different refolding buffers (20 mM sodium phosphate pH 6, 20 mM HEPES pH 7 or 20 mM TRIS pH 8) with additives (0–20% glycerol and 0–0.5 M NaCl). After incubation for 4 h at 25°C or 4°C, activities of these samples were measured under standard assay conditions (see below) and compared to the specific activity of soluble TaAlDH.

Matrix-assisted refolding was performed according to the protocol of Holzinger et al. [23] using 0.5 M NaCl, 20% glycerol, 20 mM TRIS pH 8 as refolding buffer. 6.0 mg/mL unfolded TaAlDH inclusion bodies were loaded on HiTrap FF-column and washed with denaturation buffer with a flow of 5 mL/min. In vitro refolding was performed by gradually increasing the refolding

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**Table 2. Purification of soluble TaAlDH from 10 g of E. coli cells produced in 1 L of fed-batch fermentation.**

|                | Total volume (mL) | Protein concentration¹ (mg/mL) | Total protein¹ (mg) | Volumetric activity² (U/mL) | Total activity² (U) | Specific activity¹,² (U/mg) |
|----------------|-------------------|-------------------------------|--------------------|-----------------------------|---------------------|-----------------------------|
| Soluble fraction | 120               | 2.62                          | 314.6              | n. d.                       | n. d.               | n. d.                       |
| Heat treatment  | 120               | 1.22                          | 146.7              | 0.002                       | 0.23                | <0.1                        |
| Filtration      | 120               | 1.18                          | 141.7              | 0.002                       | 0.26                | <0.1                        |
| Nickel affinity chromatography | 16           | 0.09                          | 1.4                | 0.019                       | 0.30                | 0.21                        |
| Desalting       | 20                | 0.07                          | 1.3                | 0.015                       | 0.30                | 0.22                        |
| Lyophilization  | 20                | 0.06/0.04                     | 1.2/0.86           | 0.013                       | 0.26                | 0.21/0.31                   |

n. d.: Not determinable.

¹Protein concentration was determined with Bradford Assay/additionally after lyophilization by UV absorption spectroscopy.

²Enzyme activity was analyzed using NAD⁺ as electron acceptor during purification.

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buffer concentration from 0–100% in 90 min. The treated enzyme was purified as described above for soluble purification.

Gel Filtration

Soluble and refolded TaAlDH were analyzed via gel filtration on a Superdex 200 column (GE Healthcare). Calibration of the column was performed according to the manufacturer’s instructions using thyroglobulin (M = 670 kDa), ferritin (M = 440 kDa), catalase (M = 232 kDa), \( \gamma \)-globulin (M = 158 kDa), aldolase (M = 158 kDa), ovalbumin (M = 44 kDa) and myoglobin (M = 17 kDa) as molecular mass standards; blue dextran was used as void volume marker. The elution of proteins was detected by UV absorption. The elution volume \( V_e \) was correlated with the molecular mass \( M \) by

\[
\log M = \frac{V_e - V_0}{V_t - V_0}
\]

where \( V_t \) and \( V_0 \) are the total volume and the void volume of the column, respectively. \( V_e \) of TaAlDH was determined by injection of 50 mg lyophilized TaAlDH, dissolved in 10 mL sample buffer (20 mM TRIS pH 8). M of TaAlDH oligomers were determined from elution profile \( V_e \) using the calibration described above.

Protein Determination

TaAlDH solubility and inclusion body purification steps were monitored by SDS-PAGE [24]. Insoluble fractions were solubilized in 8 M urea or homogenized in cleaning or washing buffer before SDS-PAGE sample preparation. Bradford assay [25] was used for protein quantification during purification, with BSA as standard. TaAlDH inclusion bodies were quantified between purification steps from homogenized samples. The molecular absorption coefficient of TaAlDH at 280 nm was calculated by ProtParam tool to be \( \varepsilon = 82.28 \text{mM}^{-1}\text{cm}^{-1} \) (www.expasy.org) [26] and applied to determine the exact protein concentration in further characterization experiments [27].

Activity Assays

Unless otherwise stated, measurements were performed at 50°C. TaAlDH activity was determined spectrophotometrically by measuring the rate of NAD\(^+\)/NADP\(^+\) reduction at 340 nm (\( \varepsilon_{\text{NADH/NADPH}} = 6.22 \text{mM}^{-1}\text{cm}^{-1} \)) in flat bottom microtiter plates (Greiner Bio-One, Solingen, Germany) with a Fluostar Omega Photometer (BMG Labtech, Ortenberg, Germany). One unit of activity was defined as reduction of 1 \( \mu \text{mol} \) of cofactor per minute. Standard assay mixtures (total volume 0.2 mL) contained 1 mM \( \alpha \)-glyceraldehyde, 2 mM NAD\(^+\) and appropriate amounts of enzyme in 100 mM HEPES pH 7.

The cofactor acceptance of TaAlDH for NAD\(^+\) and NADP\(^+\) was determined in 100 mM HEPES pH 6.2. The assay contained 0.02 mg/mL refolded TaAlDH, 5 mM \( \alpha \)-glyceraldehyde and either NAD\(^+\) (0–75 mM) or NADP\(^+\) (0–0.24 mM). NAD\(^+\) was soluble in all concentrations in 100 mM HEPES pH 6.2 at 25°C. Apparent \( v_{\text{max}} \) and \( K_m \) values of the cofactors were calculated by fitting initial rate data to the Michaelis-Menten equation using Sigma Plot.

| Buffer and pH | NaCl (M) | Glycerol (%) | Relative activity (%) refolded at 4°C | Relative activity (%) refolded at 25°C |
|---------------|----------|--------------|-------------------------------------|-------------------------------------|
| Phosphate pH 6| 0.5      | -            | <1                                  | <1                                  |
|               | 0.5      | 20           | <1                                  | <1                                  |
|               | -        | -            | <1                                  | <1                                  |
|               | -        | 20           | 11                                  |                                     |
| HEPES pH 7    | 0.5      | -            | <1                                  | 19                                  |
|               | 0.5      | 20           | 18                                  | 37                                  |
|               | -        | -            | <1                                  | <1                                  |
|               | -        | 20           | 8                                   | 25                                  |
| TRIS pH 8     | 0.5      | -            | <1                                  | 35                                  |
|               | 0.5      | 20           | 20                                  | 51                                  |
|               | -        | -            | <1                                  | 19                                  |
|               | -        | 20           | 2                                   | 44                                  |

Table 3. TaAlDH enzyme activity after refolding purified TaAlDH inclusion bodies by dilution under different renaturing conditions.
The substrate specificity of TaAlDH for different aldehydes was determined using 4 mM NAD$^+$ and 10 mM of the respective aldehyde (D-glyceraldehyde, acetaldehyde, propionaldehyde, n-butyraldehyde, pyruvaldehyde, isobutyraldehyde or n-glucose). Activity was determined at 50°C in 100 mM HEPES pH 7. The assay for D-glyceraldehyde was performed using 0.02 mg/mL refolded TaAlDH and the assays with other aldehydes were performed with up to 0.45 mg/mL refolded TaAlDH.

The activity of refolded TaAlDH was tested in presence of organic solvents. 0.20 mg/mL TaAlDH was incubated for 30 min in 100 mM HEPES pH 7 containing different organic solvents and organic solvent concentrations (0–8% v/v n-butanol, 0–9% v/v isobutanol or 0–20% v/v ethanol). After 10-fold dilution of supernatant, remaining activity was tested in respective incubation mixture.

The solvent dependent deactivation of TaAlDH was tested at 50°C. The enzyme (0.20 mg/mL) was incubated in 100 mM HEPES pH 7 containing 0% v/v, 0.3% v/v or 3% v/v isobutanol, respectively. For activity measurement, samples were diluted 10-fold to reduce the amount of enzyme in the measurement. Dilution was into identical buffer and solvent conditions (0% v/v, 0.3% v/v or 3% v/v isobutanol, respectively).

To analyze enzyme reactivation, TaAlDH was diluted from 3% v/v isobutanol to 0.3% v/v isobutanol. For this, 0.2 mg/mL TaAlDH was incubated in 100 mM HEPES pH 7 containing 3% v/v isobutanol for 30 min before 10-fold dilution with 100 mM HEPES pH 7 (giving a final concentration of 0.3% v/v isobutanol during activity measurement). To analyze the dependence of enzyme reactivation on time of inactivation, reactivation was also tested accordingly after different times (2 min, 10 min, 30 min, 1 h, 2 h and 24 h) of inactivation at 3% v/v isobutanol. To analyze the dependence of enzyme reactivation on time of reactivation, activity was also measured after different times (2 min, 10 min and 30 min) of incubation after dilution to 0.3% v/v isobutanol.

Table 4. Refolding of TaAlDH from 10 g of E. coli cells produced in 1 L of fed-batch fermentation.

| Step                        | Total volume (mL) | Protein concentration$^1$ (mg/mL) | Total protein$^1$ (mg) | Volumetric activity$^2$ (U/mL) | Total activity$^2$ (U) | Specific activity$^{1,2}$ (U/mg) |
|-----------------------------|------------------|----------------------------------|------------------------|-------------------------------|------------------------|---------------------------------|
| Insoluble fraction          | 120              | 3.28                             | 393.5                  | <0.1                          | <0.1                   | <0.1                           |
| 1st inclusion body washing  | 120              | 0.70                             | 83.6                   | <0.1                          | <0.1                   | <0.1                           |
| 2nd inclusion body washing  | 120              | 0.59                             | 70.8/49.86             | <0.1                          | <0.1                   | <0.1                           |
| Matrix-assisted refolding   | 42               | 1.40                             | 58.7                   | 0.260                         | 10.91                  | 0.19                           |
| Desalting                   | 60               | 0.99                             | 59.2                   | 0.211                         | 12.66                  | 0.21                           |
| Lyophilization              | 60               | 0.97                             | 58.3/41.02             | 0.190                         | 11.38                  | 0.20/0.28                      |

$^1$Protein concentration was determined with Bradford Assay/additionally after inclusion body washing and lyophilization by UV absorption spectroscopy.

$^2$Enzyme activity was analyzed using NAD$^+$ as electron acceptor during purification.

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Circular Dichroism

Circular dichroism (CD) spectra were recorded at room temperature (25 ± 0.1°C) on a Jasco J600 spectropolarimeter (Jasco, Milan, Italy). Far-UV CD of soluble TaAlDH (0.36 mg/mL) and refolded TaAlDH (0.27 mg/mL) in sample buffer were measured in cuvettes with layer thickness of 0.2 cm. Near-UV CD of soluble TaAlDH (0.34 mg/mL) and refolded TaAlDH (0.37 mg/mL) in sample buffer and unfolded TaAlDH (4.51 mg/mL) in denaturation buffer were measured in cuvettes with layer thickness of 1 cm. The observed ellipticity at wavelength \( \lambda \) (\( \theta_\lambda \)) was recorded with Jasco J600 spectropolarimeter using Spec-Man II software. The mean amino acid residue ellipticity \([\theta]_{MRW,\lambda}\) of TaAlDH was calculated with

\[
[\theta]_{MRW,\lambda} = \frac{\theta_\lambda \cdot M}{10^2 \cdot c \cdot d \cdot (N_A - 1)}
\]

where \( M \) is the molecular mass of the protein with 56.4 kDa for glyceraldehyde dehydrogenase, \( c \) is the enzyme concentration in mg/mL, \( d \) is the path length of the cell in cm and \( N_A \) is the number of amino acids in the protein [28]. The secondary structure contents of the proteins were predicted from \([\theta]_{MRW,\lambda}\) of far-UV CD spectra by the neural-network-based algorithm K2d from Dichroweb using reference protein dataset RDB3 [29].

Fluorescence Spectroscopy

Unfolding of TaAlDH was monitored by fluorescence spectroscopy, using a Varioskan Flash Multimode Reader (Thermo Scientific), in Nunc-Immuno™ MicroWell™ 96 well polystyrene plates (Sigma-Aldrich). Protein unfolding by chemical denaturants was tested by dissolving TaAlDH at a concentration of 0.04 mg/mL in sample buffer containing varying concentrations of GdmCl (0–6 M) and dithiothreitol (0–2 mM). After incubation for 1 h at room temperature (25 ± 0.1°C), fluorescence emission was measured at 330 nm upon excitation at 280 nm at incubation temperature (25 ± 0.1°C) and normalized (divided by the observed fluorescence emission maximum). For organic solvent induced protein unfolding, 0.04 mg/mL TaAlDH was dissolved in sample buffer containing 0–9% v/v isobutanol and 0–2 mM dithiothreitol. After incubation for 30 min at 40 ± 0.1°C, fluorescence emission was measured at 330 nm upon excitation at 280 nm at incubation temperature (40 ± 0.1°C) and normalized (divided by the observed fluorescence emission maximum).

Gibbs free energy of denaturation in absence of denaturant and solvent \( \Delta G^\circ_0 \) for TaAlDH was calculated according to Santoro and Bolen [30], assuming a 2-state model and that protein unfolding is reversible. With pre- and postdenaturational baselines

### Table 5. Kinetic parameters of refolded TaAlDH with different cofactors NAD\(^+\) and NADP\(^+\) at 50°C, pH 6.2.

| Cofactor | \( K_m \) (mM) | \( v_{max} \) (U/mg) |
|----------|----------------|---------------------|
| NADP\(^+\) | 0.022±0.001 | 0.849±0.007 |
| NAD\(^+\) | 22.13±1.43 | 1.859±0.047 |

\( ^1 \)0.02 mg/mL TaAlDH.

\[ \text{doi:10.1371/journal.pone.0070592.t005} \]

### Table 6. Substrate specificity of refolded TaAlDH at 50°C, pH 7.0.

| Substrate | Relative activity (%) |
|-----------|----------------------|
| D-Glyceraldehyde | 100.0 |
| Acetaldehyde | <0.1 |
| Propionaldehyde | <0.1 |
| n-Butyraldehyde | <0.1 |
| Pyruvaldehyde | <0.1 |
| Isobutyraldehyde | <0.1 |
| D-Glucose | <0.1 |

\[ \text{doi:10.1371/journal.pone.0070592.t006} \]

Figure 5. Michaelis-Menten kinetics of refolded TaAlDH with cofactors A) NAD\(^+\) and B) NADP\(^+\). Reaction rates were determined in 100 mM HEPES pH 6.2 at 50°C with 5 mM D-glyceraldehyde and various concentrations of NAD\(^+\) or NADP\(^+\), respectively.

\[ \text{doi:10.1371/journal.pone.0070592.g005} \]
where $Y_u$ and $Y_n$ are the normalized fluorescence of protein in complex with NADH aldehyde dehydrogenase (betB) from Staphylococcus aureus unfolding. was monitored by fluorescence spectroscopy as described for v/v isobutanol and 0–2 mM dithiothreitol. Refolding of isobutanol and diluted 30-fold in sample buffer containing 0–9% Ta solvent and its reactivation. Deactivation of refolded TaAlDH was measured after incubation for indicated time at 50°C in 3% v/v isobutanol (red) or 0.3% v/v isobutanol (blue). Furthermore, samples incubated in 3% v/v isobutanol were also measured after 10-fold dilution to 0.3% v/v isobutanol (green) to test reactivation within 2 min. doi:10.1371/journal.pone.0070592.g007

deactivation cooperativity. Denaturation curves were fitted for fluorescence at denaturant concentration $X$, and $m$ is related with unfolded and folded state, respectively, $Y_{obs}$ is the normalized parameter $X$ to $Y_{obs}$ by nonlinear regression using Sigma Plot.

To test the reversibility of enzyme unfolding, 1.2 mg/mL TaAlDH was dissolved in denaturation buffer and diluted 30-fold in sample buffer containing varying concentrations of GdmCl (0–100 mM HEPES pH 7 containing various concentrations of ethanol. To improve the overall yield of active protein, TaAlDH inclusion bodies were used for refolding experiments. Prior to refolding, inclusion bodies were purified by two washing steps. No loss of TaAlDH was observed during the washing steps and the final purity was estimated to be over 90% (Figure 2B). After dissolving purified inclusion bodies in 6 M GdmCl, different refolding conditions were tried in order to maximize the yield (Table 3). Activity after dilution in phosphate buffer pH 6 could only be detected when glycerol was supplemented and refolding took place at room temperature. Refolding of TaAlDH worked best at room temperature in TRIS pH 8 with glycerol and NaCl. Here, 51% of activity could be restored compared to the specific activity of soluble TaAlDH and total protein in inclusion bodies. The best refolding conditions (TRIS pH 8, 0.5 M NaCl, 20% glycerol) were applied to matrix-assisted refolding of large amounts of insoluble TaAlDH (Table 4).

After washing TaAlDH inclusion bodies, they were again dissolved in 6 M GdmCl and bound via His-tag to nickel-agarose. Elution with imidazole gave a final yield of 41.0 mg from 49.9 mg dissolved purified inclusion bodies in 6 M GdmCl, different refolding conditions were tried in order to maximize the yield (Table 3). Activity after dilution in phosphate buffer pH 6 could only be detected when glycerol was supplemented and refolding took place at room temperature. Refolding of TaAlDH worked best at room temperature in TRIS pH 8 with glycerol and NaCl. Here, 51% of activity could be restored compared to the specific activity of soluble TaAlDH and total protein in inclusion bodies. The best refolding conditions (TRIS pH 8, 0.5 M NaCl, 20% glycerol) were applied to matrix-assisted refolding of large amounts of insoluble TaAlDH (Table 4).

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Molecular Modeling

A homology model from TaAlDH amino acid sequence was created with phyre² based on the crystal structure of betaine aldehyde dehydrogenase (betB) from Staphylococcus aureus in complex with NAD⁺ as template (RCSB PDB ID 3FG0), which had 1.85 Å resolution. The model covered 98% of TaAlDH sequence and was identical to 34% [31].

Results

Production of Functional TaAlDH

The gene of TaAlDH was synthesized and cloned into a T7 based vector system for recombinant expression in E. coli. This host was chosen for its advantage that target thermostable enzymes can be separated from mesophilic host proteins with a simple heat incubation step [32] and because of future protein engineering for improvement of enzyme properties [33,34]. For its first partial characterization TaAlDH had been previously recombinantly produced in E. coli [35]. However, no information was given on the yield of its expression. We found E. coli to recombinantly produce TaAlDH mostly in its insoluble form. Despite efforts to improve solubility by varying E. coli host strains, growth medium and temperature, still over 95% of TaAlDH were inactive in the form of inclusion bodies (Figure 2A). After TaAlDH production via fed-batch fermentation, the soluble enzyme fraction was purified and finally lyophilized for easier storage and dosage (Table 2). Due to its low solubility, we obtained only 0.9 mg pure TaAlDH from 1 L fermentation broth.

To improve the overall yield of active protein, TaAlDH inclusion bodies were used for refolding experiments. Prior to refolding, inclusion bodies were purified by two washing steps. No loss of TaAlDH was observed during the washing steps and the final purity was estimated to be over 90% (Figure 2B). After dissolving purified inclusion bodies in 6 M GdmCl, different refolding conditions were tried in order to maximize the yield (Table 3). Activity after dilution in phosphate buffer pH 6 could only be detected when glycerol was supplemented and refolding took place at room temperature. Refolding of TaAlDH worked best at room temperature in TRIS pH 8 with glycerol and NaCl. Here, 51% of activity could be restored compared to the specific activity of soluble TaAlDH and total protein in inclusion bodies. The best refolding conditions (TRIS pH 8, 0.5 M NaCl, 20% glycerol) were applied to matrix-assisted refolding of large amounts of insoluble TaAlDH (Table 4).

After washing TaAlDH inclusion bodies, they were again dissolved in 6 M GdmCl and bound via His-tag to nickel-agarose. Elution with imidazole gave a final yield of 41.0 mg from 49.9 mg protein within the inclusion bodies. Imidazole had no influence on activity. Specific activity of refolded and soluble TaAlDH was comparable (0.28 U/mg and 0.31 U/mg, respectively). Total activity of lyophilized product from refolded TaAlDH was 11.38 U/L, while soluble purification resulted in only 0.26 U/L. Hence, with matrix-assisted TaAlDH refolding, the overall yield for total enzyme units could be increased 43-fold.
Figure 8. Fluorescence analysis of unfolding and refolding of TaAlDH in the presence of A) GdmCl (25°C) and B) isobutanol (40°C).

The fluorescence emissions of TaAlDH at λ = 330 nm were monitored upon excitation at λ_{max} = 280 nm. Data were collected for protein unfolding (red symbols) and refolding (green symbols) at indicated concentrations of GdmCl or isobutanol. The transition curve for protein unfolding is presented as the best fit using nonlinear regression (black curve).

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Structural Analysis of Functional TaAlDH

Previously, native and recombinant TaAlDH were reported to appear as tetramer and dimer, respectively [35,36]. In this study, we compared oligomeric states of soluble and refolded TaAlDH by size exclusion chromatography (Figure 3). Both preparations show a maximum around M = ~120 kDa and small amounts around M = ~260 kDa. According to the size estimation of TaAlDH monomer from SDS-PAGE (Figure 2) and the molecular mass calculated from the amino acid sequence (M = 56 kDa), soluble TaAlDH as well as refolded TaAlDH elution contain mainly dimers with small amounts of maybe tetramers. Refolded TaAlDH in addition showed the presence of very small amounts of impurities or possibly monomeric protein around M = ~50 kDa, which after elution from the column did not show any TaAlDH activity under standard assay conditions.

Refolded and soluble TaAlDH were also compared by CD spectroscopy. The far-UV CD spectra of both types of TaAlDH had one maximum at 192 nm and two minima at 209 nm and 220 nm. Evaluation of molar ellipticity in far-UV CD spectra indicated characteristics of ca. 37% α-helices and ca. 17% β-sheets for both soluble and refolded TaAlDH. These findings are in reasonable agreement with a TaAlDH homology model, which showed 40% α-helices and 20% β-sheets (Figure S1). Differences in the far-UV CD spectra between both enzymes were marginal and within standard deviation, indicating complete refolding of secondary structure with identical conformations (Figure 4A). In addition, the near-UV CD spectra of soluble protein and refolded protein were very similar (Figure 4B). All further characterization was therefore performed with refolded TaAlDH.

TaAlDH Activity

Previously, TaAlDH was described as not accepting NAD⁺ as cofactor [35,36]. Having larger amounts of protein available, we more thoroughly characterized TaAlDH in the oxidation of D-glyceraldehyde to D-glycerate with both cofactors and found it to be active with NAD⁺ as well (Table 5). Rate dependencies on the cofactors followed Michaelis-Menten kinetics (Figure 5). Compared to NAD⁺, K_{m} and v_{max} values for NADP⁺ were 1000-fold lower and 2-fold lower, respectively. v_{max} determined for refolded TaAlDH was lower in comparison to the earlier described recombinant enzyme, but in the range of the native enzyme [35,36]. While still being majorly an NADP⁺ dependent AlDH, the activity was high enough at 5 mM NAD⁺, the concentration which is used in the synthetic cascade.

Specificity of TaAlDH had already been tested in previous publications using various substrates [35,36]. However, activities with isobutyaldehyde or n-butyraldehyde, which are important regarding the solvent production cascade shown in Figure 1, have not yet been examined. Thus, these two aldehydes were tested along with other aldehydes appearing as intermediates in the cascade (Table 6). The lack of activity towards oxidation of acetaldehyde from previous publications could be confirmed. D-Glucose was tested to see whether TaAlDH would accomplish the first reaction step of the synthetic cascade, but its substrate specificity was too high, resulting in activity with D-glyceraldehyde only.

The Effect of Organic Solvent on TaAlDH Enzyme Activity

Within the cell-free process, participating enzymes are supposed to tolerate high product concentrations. The proper function of the enzymes must not be inhibited by the organic solvents ethanol or isobutanol. Accordingly, TaAlDH activity assays were performed in presence of these molecules. Since we are expanding our tool box for cell-free production continuously, we also tested TaAlDH in n-butanol.

The activity of TaAlDH decreased with higher organic solvent concentration (Figure 6). In 20% v/v ethanol, the activity was still at 40% of initial activity, while in the presence of isobutanol or n-butanol the activity dropped much quicker with increasing solvent concentrations. Here, less than 6% of the initial enzyme activity was detected at a final concentration of 9% v/v isobutanol or n-butanol after 30 min.

To further study the reversibility of inactivation by organic solvents, the time course of activity decrease in the presence of isobutanol was recorded (Figure 7). TaAlDH deactivation by 3% v/v isobutanol at 30°C occurred immediately within 2 min down to 80% of initial activity. After 30 min of TaAlDH deactivation, lower and 2-fold lower, respectively. v_{max} determined for refolded TaAlDH was lower in comparison to the earlier described recombinant enzyme, but in the range of the native enzyme [35,36]. While still being majorly an NADP⁺ dependent AlDH, the activity was high enough at 5 mM NAD⁺, the concentration which is used in the synthetic cascade.

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activity dropped to 55%, but even after 24 h at 3% v/v isobutanol a residual activity of 10% was retained. Slight inactivation was also observed in 0.3% v/v isobutanol leading to loss of ca. 50% activity after 24 h.

Inactivation of enzyme in the absence of isobutanol was similar to that in 0.3% v/v isobutanol (data not shown). Activity could be recovered partially within 2 min when isobutanol was diluted from 3% v/v to 0.3% v/v. After 24 h of inactivation this effect declined substantially indicating an increasing irreversibility of inactivation with enduring incubation. Reactivation times longer than 2 min (10 and 30 min) after dilution from 3% v/v to 0.3% v/v isobutanol did not lead to a further activity increase (data not shown), indicating that reactivation was very fast.

Characterization of TaAlDH Structural Stability

Ellipticity from soluble and refolded TaAlDH was positive between 260 nm and 280 nm indicating the presence of tertiary structure in the protein (Figure 4B). The signal decreased steeply below 260 nm, which is characteristic for $\alpha$-helical secondary structures of the folded variants. In contrast, ellipticity from TaAlDH in 6 M GdmCl was close to zero between 260 nm and 280 nm and also below 260 nm indicating complete loss of tertiary and $\alpha$-helical secondary structures, respectively.

To determine thermodynamic stability, GdmCl and isobutanol induced unfolding of TaAlDH was examined by fluorescent measurements (Figure 8). To test the full reversibility of enzyme unfolding samples of folded as well as fully unfolded TaAlDH were diluted into different concentrations of isobutanol or GdmCl. Identical fluorescence values at respective isobutanol or GdmCl concentrations were obtained indicating complete reversibility under assay conditions (Figure 8). Due to poor solubility of isobutanol in water (formation of streaks and separate organic phase above 9% v/v isobutanol), no well resolved baseline for unfolded TaAlDH in isobutanol could be obtained. Constant fluorescence emissions were assumed for folded TaAlDH in 0.0–0.4% v/v isobutanol and 0.0–0.2 M GdmCl as well as for unfolded TaAlDH in 4.0–5.8 M GdmCl and 8.0–8.8% v/v isobutanol. From the fitted GdmCl plot, the parameter of cooperativeness $n_{\text{GdmCl}}=−4.8±0.2$ kJ/(mol·M) and the free Gibbs energy change $\Delta G^{0}_D=7.9±0.4$ kJ/mol was calculated for TaAlDH. Fitting the plot for unfolding in isobutanol, the calculations indicated $n_{\text{Isobutanol}}=−2.1±0.1$ kJ/(mol·M) and $\Delta G^{0}_D=8.0±0.5$ kJ/mol for TaAlDH. Accordingly, more than 95% TaAlDH was unfolded in concentrations exceeding 3.2 M GdmCl or 7.6% v/v isobutanol.

Discussion

Synthetic Cascade Biomannufacturing can become a powerful technology, when the right enzymes are available, combining correct specificity, activity and stability. TaAlDH is such an enzyme for a key reaction step in the synthetic 4-enzyme glycolysis for the conversion of glucose to pyruvate. Being reported to be active with NAD$^+$, we found the enzyme to also accept NAD$^+$ under technically relevant conditions (cofactor >1 mM). Most importantly, TaAlDH has very high subrate specificity. Due to its thermophilic origin TaAlDH has an acceptable thermostability. The optimum temperature was reported to be 50–55°C [36]. Thermostability is often correlated with stability in organic solvents [37,38], however in this respect, TaAlDH does not show a remarkable tolerance. At 5% ethanol and 2% isobutanol or n-butanol a significant decrease in activity could be observed. An isobutanol induced unfolding transition accordingly revealed a thermodynamic stability ($\Delta G^{0}$ of folding) of $−8.0±0.5$ kJ/mol at 40°C, based on a 2-state model [30] and reversible unfolding, an assumption which was shown to be valid in this case (Figure 8). Stability measurement by GdmCl induced unfolding transition gave a similar value of $−7.9±0.1$ kJ/mol. Generally, this value is rather low for an enzyme of thermophilic origin [37,39], showing that there is a large potential for optimization [40,41]. To stabilize proteins in organic solvents, free energies of intramolecular interactions have to be increased relative to those for protein-solvent interactions. In this regard, Arnold suggested rules for protein stabilization. Exchange of amino acids should satisfy most of protein hydrogen bonds and increase the number of cross-linked salt bridges to stabilize protein substructures and raise the unfolded state energy, respectively. Furthermore, the increase of free energy of protein unfolding could be accomplished by a more compact packing of hydrophobic side chains, e.g. replacing threonine by smaller hydrophobic amino acids like valine or isoleucine [40,42]. These considerations could help in stabilizing the enzyme by protein engineering. First experiments are underway here.

Recombinant enzyme production using E. coli as host is common standard and was done previously for TaAlDH, however, no data on yields were given [35]. In our hands only very small amounts of enzyme could be obtained in soluble form, the major part of enzyme production was directed into inclusion bodies. This is not uncommon when working with enzymes of archaeal origin [43,44]. All attempts failed to increase the yield of soluble protein by altering culture conditions. Soluble, active enzyme in large amounts could, however, successfully be obtained in in vitro refolding from inclusion bodies. The catalytic properties of soluble and refolded TaAlDH were similar (0.3 U/mg) and spectroscopic analysis showed them to be identical (Figure 4). Protein refolding from inclusion bodies is working for small-scale enzyme production [45] and so TaAlDH was obtained in high yields as a functional enzyme. However, the method is very time consuming in comparison to soluble purification. Furthermore, the use of large amounts of buffers and the decrease in wear and tear of machines by chaotropic agents boost enzyme production costs. Thus, for large-scale industrial biocatalysis, TaAlDH needs to be more efficiently and recombinantly produced in soluble form, another target for enzyme engineering [46].

An objective for further studies on TaAlDH would be the examination of stereo selectivity. Although enantiomerically pure D-glycerate is already applied today for synthesis of non-chiral sugar acid derivatives [47,48,49], the enzyme may be useful for future applications in biocatalytic processes for chiral chemicals [50]. For L-glyceraldehyde production by a glycerol dehydrogenase from glycerol, TaAlDH could serve as cofactor recycling enzyme and would simultaneously oxidize D-glyceraldehyde out of a racemic mixture for L-glyceraldehyde enrichment [51].

This study emphasizes TaAlDH as a valuable option when enzyme specialists are needed for new biocatalytic production of chemicals.

Supporting Information

Figure S1 TaAlDH homology model, based on the crystal structure of betaine aldehyde dehydrogenase (betB) from Staphylococcus aureus (PDB ID 3FG0). $\alpha$-Helices and $\beta$-sheets are colored red and yellow, respectively. (TIF)
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Author Contributions

Conceived and designed the experiments: FS. Performed the experiments: FS. Analyzed the data: FS. Wrote the paper: FS VS.

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