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Alcohol dehydrogenase stabilization by additives under industrially relevant reaction conditions

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ABSTRACT

Alcohol dehydrogenases form one of the most important enzyme classes for the synthesis of chiral hydroxyl-compounds. High solvent concentrations may improve the efficiency of biocatalytic ADH reactions but, however, turned out to damage most enzymes.

In order to overcome the damage caused to these enzymes, this work describes the stabilization of five different alcohol dehydrogenases under very high solvent concentrations (up to 90% of the reaction volume). The reductive conversion of the ketone substrate into the corresponding alcohol was increased up to sevenfold by pre-incubating the enzymes with specific stabilizers. This technique is highly efficient and additionally facile as no prior immobilization, polymerization or deposition treatment is necessary. It was revealed that each ADH gained an optimal stabilization effect by one specific stabilizer and appropriate concentration. Furthermore, the results obtained on laboratory scale were transferred successfully to 4000 mL scale to verify the applicability of this technique for industrial use.

1. Introduction

Enzyme stabilization is a prerequisite to developing economically viable biocatalytic processes. The most frequently targeted aims of stabilization processes are extended storage stability, an increased resistance towards high temperature, extreme pH-value and high solvent concentrations.

Stabilization against high amounts of organic solvents is an interesting topic, as there are various advantages to working in aqueous media. One drawback of aqueous systems lies in the limited solubility of many substrates. Increasing the concentration of the organic solvent allows the use of higher substrate loadings, leading to enhanced conversion rates. Referring to thermodynamics, higher co-substrate loadings can shift the equilibrium towards the product site [1]. Both factors may enhance the economy of the process significantly and make it more competitive to chemical processes. Another interesting aspect is the facilitated product recovery when using solvents with low boiling points. Relating to the reaction itself, a low amount of water in the reaction medium avoids water dependent site-reactions [2]. Additionally, there are particular conversions, like esterifications or transesterifications, that require low water activity in order to allow them to take place [1,3].

In general, there are numerous techniques to stabilize proteins. One approach is to undertake protein engineering achieved through the use of random mutagenesis or rational protein design. Examples of the latter are through the insertion of proline amino acids, disulfide bridges, through the exchange of movable amino acids (high B-factor) or through polypeptide chain extensions [4–6].

A second stabilization method is through the use of physico-chemical modification of proteins. Examples are the mono-functionalization via amino acid derivatives, acylation and alkylation [7] or the bi-functionalization by cross-linking agents like glutaraldehyde or diimidates.

A third, and widely applied technique is the immobilization of enzymes. This is one of the most preferred stabilization methods as it offers other positive properties besides the stabilizing effect [8]. It facilitates an easy re-usage of the catalyst, simplified purification and it enables a more flexible reactor design.

In this work, the stabilization of different alcohol dehydrogenases against very high solvent concentrations by addition of stabilizing additives (Table 1) was examined. In the past, this

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Table 1
Additives that were tested for their stabilizing effects on the alcohol dehydrogenases. In most cases the substances can be assigned to groups of polyol, salt or polyethylene glycol.

Polyol 1	Polyol 2	Salt	PEG	Additional
Sucrose	Mannitol	KCl	400	Lysine
Erythritol	Glycerol	$(NH_4)_2SO_4$	4000	Glycine
Fructose	Sorbitol	NH_4Cl	6000	PEI
Glucose				
Maltose				

method was mainly applied to extend the storage stability of enzymes in aqueous media [9,10] or to protect bioactive peptides during the lyophilization [11,12]. Nevertheless, it was proven that protection against organic-aqueous media by additives is also possible [13–15].

The new aspect of this work was the stabilization of dissolved enzyme lyophilisate directly in the biocatalytic reaction without prior polymerization, immobilization or deposition to a supporting material. Additionally, very harsh reaction conditions were applied, the ratio of the organic compounds (isopropyl alcohol and substrate) was raised up to 90% of the reaction batch. The experiments were conducted with five different alcohol dehydrogenases (ADH030, ADH040, ADH270 and ADH380 from evocatal GmbH and LB-ADH (ADH from *Lactobacillus brevis*)). Beyond this, the focus of this work lay on the examination of the potential of this stabilization method for higher scale reactions.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals used were of analytical grade and purchased from Sigma Aldrich (Steinheim, Germany) or Carl Roth GmbH (Karlsruhe, Germany). All enzymes are commercially available from evocatal GmbH (Monheim am Rhein, Germany).

2.2. Alcohol dehydrogenase reactions

The reactions on laboratory scale (5 mL) were performed at room temperature.

2.2.1. ADH030, ADH270 and LB-ADH (ADH from Lactobacillus brevis)

The amount of the organic solvent in the batch was 90% and was composed of 3975 μL isopropyl alcohol as co-substrate for cofactor regeneration and 525 μL of the substrate acetophenone (final conc. 900 mM). Enzyme loading was 0.5 mg/mL, concentration of the cofactor NAD^+ was 0.15 mM (ADH030 and LB-ADH) and 0.5 mM $NADP^+$ (ADH270), respectively. Potassium phosphate was used as buffer (100 mM, pH 7.5) including 0.1 mM $ZnCl_2$ for ADH030 or 0.1 mM $MgCl_2$ for ADH270 and LB-ADH.

2.2.2. ADH040 and ADH380

The amount of isopropyl alcohol was 90% in the reaction batch of ADH040 and 50% in the reaction batch of ADH380 including 100 μL 2-Butanone (final concentration 200 mM). Enzyme loading was 5 mg/mL, concentration of the cofactor NAD^+ was 0.15 mM in 50 mM triethanolamine buffer (pH 7).

2.3. Stabilization protocol

Depending on the particular enzyme, the appropriate amount of lyophilisate was weighed out (see above) and dissolved for 15 min in 125 μL (2.5 vol.%) cofactor solution at 100 rpm in a 5 mL beaded rim glass. 375 μL (2375 μL in the case of ADH380) of a solution of additive (Table 2) in the appropriate enzyme buffer were added and the mixture was incubated for 60 min at 300 rpm.

Table 2
Concentrations in which the additives were supplemented in the first screening approach. The substances were inserted in two different quantities to reveal concentration-depending effects.

Additive	Concentration (mol/L)		Additive	Concentration (mol/L)	
	A	B		A	B
Sucrose	0.1	1	$(NH_4)_2SO_4$	0.1	1
Erythritol	0.1	1	NH_4Cl	0.1	1
Fructose	0.1	1	PEG400	0.1	1
Glucose	0.1	1	PEG4000	0.05	0.1
Maltose	0.1	0.5	PEG6000	0.025	0.05
Mannitol	0.05	0.1	Lysine	0.1	1
Glycerol	0.1	1	Glycine	0.1	1
Sorbitol	0.1	1			
KCl	1	3	PEI	1%	10%

To start the reaction, isopropyl alcohol and the particular substrate were added to the reaction medium. The conversion rate and the enantiomeric excess were measured by gas chromatography.

2.4. Enzyme preparation on larger scales

The recombinant *E. coli* strains, containing an expression plasmid (based on pET21a), were grown over night at 37 °C in LB-medium supplemented with 100 μg/mL ampicillin. Expression of the proteins was induced by adding 100 μM IPTG (final conc.) to a culture that was inoculated with the bacteria from the overnight culture at a density of OD_{580} = 0.05, when said culture had a density of OD_{580} = 0.5–0.7. Cultures were shaken at 200 rpm for about 18 h at 30 °C. The cells were harvested by centrifugation for 20 min at 8000 × *g* at 4 °C (J2-21M/E, Beckmann, High Wycombe, UK).

For subsequent cell disruption the cell pellet was re-suspended in a threefold amount (mL/mg cell wet weight) of buffer (100 mM Tris/HCl, pH 7.2; 1 mM $MgCl_2$) and subjected to ultrasonic treatment (Sonopuls, Bandelin electronic, Berlin, Germany). A crude protein extract was obtained by centrifugation of the lysed cells for 20 min at 14,000 × *g* at 4 °C. The supernatant was lyophilisated.

2.5. GC-analysis

The conversion of the substrates into the corresponding alcohols and the enantiomeric excess (*ee*) were measured by gas chromatography system Focus GC (Thermo Scientific, Milano, Italy) with a flame ionization detector. A FS-Innopeg 2000 (30 m × 0.25 mm × 0.25 μm) column for conversion and a Restek Rt-bDEXcst (30 m × 0.25 mm × 0.25 μm) column (CS Chromatographie, Langerwehe, Germany) for enantiomeric excess analysis were used.

100 μL of the reaction batch were merged with 500 μL tert-butylmethylether and dried with 50 mg Na_2SO_4. After mixing and centrifugating, 100 μL of the supernatant were analyzed by gas chromatography. The retention times for 2-butanone conversion analysis were 3.5 min for the ketone and 5.5 min for the alcohol compound at an oven temperature of 38 °C (carrier gas N_2, gas pressure constant 100 kPa, injector temperature 200 °C, detector temperature 250 °C). The retention times for enantiomeric excess analysis were 21.6 min for the (R)-alcohol and 22.8 min for the (S)-alcohol at an oven temperature of 38 °C (carrier gas N_2, gas flow-through 5 mL/min, injector temperature 200 °C, detector temperature 250 °C).

The retention times for acetophenone conversion analysis were 3.9 min for the ketone and 5.8 min for the alcohol compound at an