INTRODUCTION

Climate change and dwindling fossil resources drive industrial developments of biomass based processes for chemical and fuel applications. Butanol is poised to be a next generation renewable building block due to its improved energy content and hydrophobicity compared to bio-ethanol. Conventionally, bio-butanol is produced via the anaerobe ABE fermentation that employs Clostridia species as cellular production systems. However, economic viability of this cell based process is limited due to end product toxicities above 2% v/v and accumulation of alternative metabolic products. Alternatively, tailor-made, cell-free enzyme cascades are emerging as alternative production systems, which hold the promise of rapid adaptability to harsh process conditions and improved n-butanol yields. However, the molecular complexity of natural n-butanol biosynthesis currently prohibits realization of a robust cell-free n-butanol production process. Recently, simplified, non-natural n-butanol production pathways have been predicted by computational methods. However, enzyme systems that allow consecutive conversion of predicted intermediates to n-butanol have not been identified. A key biosynthetic module in computationally predicted n-butanol production is the conversion of crotylalcohol to n-butanol. We have designed a non-natural enzyme cascade that allows the three step conversion of crotylalcohol to n-butanol using just two enzymes. The involved enzyme systems, horse liver alcohol dehydrogenase and 2-enoate reductase from Bacillus subtilis, show pronounced substrate promiscuity, which allows the consolidated conversion of crotylalcohol to n-butanol. Further, the designed enzyme cascade allows production of n-butanol using only the NAD+/NADH redox couple as a unified electron shuttle, which significantly reduces the molecular complexity of the cell-free reaction cascade. This is the first study that could experimentally validate a reaction module of a computationally predicted n-butanol production pathway. The development of designed, cell-free reaction cascades will pave the way towards mass- and cost-efficient n-butanol production at an industrial scale.
consolidated, tailor-made cell-free n-butanol production system we have evaluated several biosynthetic modules that have been predicted in the conversion of pyruvate to n-butanol [6-8]. A key intermediate in the predicted n-butanol production cascade is crotonaldehyde [6-8]. However, at present no enzyme system capable of catalyzing the conversion of crotonaldehyde to butanal has been identified.

This study reports a non-national, three step enzyme cascade, which allows the conversion of crotylalcohol to crotonaldehyde, which is then further, elaborated via butanal to n-butanol. Interestingly, we could achieve this three step reaction with only two enzyme systems utilizing their respective substrate promiscuity. Each reaction module was established individually prior to combining enzymatic reactions to allow consolidated aerobic conversion of crotylalcohol to n-butanol. For the first time we could identify an enzyme system that catalyses the dedicated conversion of crotonaldehyde to butanal. Further, this is the first report on the experimental validation of computationally predicted biosynthetic modules involved in non-national n-butanol biosynthesis. The data will contribute to the development of mass efficient n-butanol production systems, which are prerequisite for an advanced bio-butanol process design.

MATERIAL AND METHODS

Chemicals and enzymes

Restriction enzymes, T4 ligase, Taq-polymerase, deoxyribonucleotides as well as DNA and protein-standards were purchased from Thermo Scientific (Schwerte, Germany). DNA-sequencing was provided and oligonucleotides were ordered by eurofins mwg operon (Ebersberg, Germany). Crotonaldehyde was purchased from Sigma Aldrich (Taufkirchen, Germany) and crotylalcohol (2-buten-1-ol, cis and trans) from VWR (Darmstadt, Germany). The pET28a-vector was provided by Novagen (Darmstadt, Germany). Alcohol dehydrogenase (Adh) from horse liver was purchased from evocatal GmbH (Düsseldorf, Germany). Adh was resuspended in the respective assay buffer and used as received.

Strains and plasmids

E. coli HMS174 (DE3) [P recA1 hsdR (rK+ mK12+) (DE3) (Rif®)] was purchased from Merck KGaA (Darmstadt, Germany). E. coli XL1-Blue [recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F proAB lacIqZAM15 Tn10 (TetR)] from Stratagene (Waldbrohn, Germany). The pET28a-vector was provided by Novagen (Darmstadt, Germany).

Isolation of genomic DNA

Isolation of the genomic DNA from B. subtilis was conducted using the genomic DNA isolation kit from Thermo Scientific (Schwerte, Germany), according to the manufacturer’s instructions.

Cloning of yqjM

B. subtilis yqjM gene was cloned into the vector pET28a pre-digested with Ncol and Xhol. The open reading frame of B. subtilis yqjM was amplified by PCR, applying the primers 5’ YqjM_pET28 (GGG GCA TGG GCA GAA AAT TAT TTA AACTCTTATA) and 3’ YqjM_pET28a (ATA TCT CGG AGG CTC TTT CGT ATT GAA CAG GG). The restriction sites contained in the primer’s sequences are underlined.

Heterologous expression and purification of YqjM

Enzyme expression was performed using E. coli HMS174 (DE3) as host strain. YqjM was expressed in TB medium supplemented with 50 µg/ml kanamycin. After inoculation, cells were grown to an OD600 = 0.5 - 0.7 at 37 °C and subsequently induced with 1 mM IPTG. For further cultivation temperature was kept at 25 °C for 20 h. Afterwards the cells were harvested (4.500 g, 4 °C, 30 min).

The cell pellet was resuspended in 50 mM Hepes pH 8.0, 20 mM imidazole. Cell lysis was prepared with EmulsiFlex-B15 (Avestin, Mannheim, Germany) and cell debris removed by centrifugation (25.000 g, 4 °C, 20 min). A Ni²⁺-NTA column (column volume (cv) ~ 4 ml; Thermo Scientific, Schwerte, Germany) was equilibrated with 50 mM Hepes pH 8.0, 20 mM imidazole. His, tagged enzyme was loaded on the column and washed with 3 cv of equilibration buffer. The tagged enzyme was eluted with 50 mM Hepes pH 8.0 and 500 mM imidazole. The protein containing fractions were well visible, because of the yellow protein colour. Desalting of the protein was performed with PD-10 columns (GE Healthcare). The protein was stored as liquid stock with 10 % glycerol at -80 °C.

Protein concentration of the unfolded protein dissolved in 8 M urea was measured at 280 nm [10]. The corresponding extinction coefficient was calculated by ExPASY’s ProtParam tool [11].

GC-FID analysis

Reaction mixtures for analysis of YqjM activity with gas chromatography (GC) contained 50 mM Hepes buffer pH 7, 20 mM crotonaldehyde, 20 mM NADH and 0.05 mM FMN. Reaction mixtures of the Adh assays contained 50 mM Hepes buffer pH 7, 20 mM crotylalcohol and 20 mM NAD⁺. Additionally, the pH-value of the Hepes buffer was adjusted to 40 °C according to Stoll [12]. For coupled YqjM and Adh assay, reaction mixtures consisted of 50 mM Hepes buffer pH 7, 20 mM crotylalcohol, 20 mM NADH and 20 mM NAD⁺. YqjM was added to a final mass of 0.34 mg and Adh to 0.28 mg, respectively.

All substrates and products could be quantified by a Trace GC Ultra (Thermo Scientific, Schwerte, Germany), equipped with a Headspace Tri Plus auto sampler, an agitator and a flame ionization detector (FID). The GC analysis was performed on a StabiWax column (length 30 m, internal diameter 0.25 mm, film thickness 0.25 µm (Macherey-Nagel, Düren, Germany), with helium (1.2 ml min⁻¹) as carrier gas. Injector and detector temperature were 200°C, whereas the oven temperature programme was 50 °C for 2 min, raised to 200 °C with a ramp of 10 °C min⁻¹, the end temperature was held for 1 min. The samples (500 µl in a 10 ml gas-tight headspace vial) were incubated at 40 °C for 15 min. For the analysis 700 µl of the headspace were injected (headspace syringe 100 °C) in the split mode with a flow of 10 ml min⁻¹. For quantification of the crotylalcohol and crotonaldehyde concentrations, the FID response in each sample was related to control measurements of known concentrations.
Conversion of crotylalcohol to crotonaldehyde

Crotylalcohol and crotonaldehyde are key building blocks in a computational reaction cascade that predicts conversion of pyruvate to n-butanol [7]. The in-silico predicted reaction cascade involves the initial oxidation of crotylalcohol to crotonaldehyde, which is then reduced to provide butanal. Finally, butanal is reduced to yield n-butanol as the end-product. Enzymatic conversion of crotylalcohol to crotonaldehyde and butanal to n-butanol has been reported utilizing microbial and mammalian alcohol dehydrogenases [13-15]. By contrast, no enzyme systems for the dedicated conversion of crotonaldehyde to butanal have been identified yet. To experimentally establish the desired reaction cascade, we first screened for enzyme systems that could individually catalyse each step involved in the conversion of crotylalcohol to n-butanol (Figure 1). As the reactivity of alcohol dehydrogenases towards crotylalcohol and butanal is established, we primarily screened for enzymes which could accept both substrates. The identification of a single Adh capable of catalysing two reactions would allow for construction of a consolidated reaction cascade. Further, we selected a 2-enoate reductase, which could accept the substrate crotonaldehyde and catalyse its conversion to butanal. Ultimately, the selected enzyme reactions were combined to allow a consolidated conversion of crotylalcohol to n-butanol (Figure 1).

Conversion of crotylalcohol to crotonaldehyde

Literature data indicated that horse liver alcohol dehydrogenase (Adh) is an ideal candidate for the oxidation/reduction of C4 compounds due to its relatively high activity towards butanal [14]. As this reaction is relevant for downstream catalytic steps, we have evaluated, if Adh also shows activity towards the primary substrate, crotylalcohol (Figure 2). Therefore, Adh was incubated in 50 mM Hepes buffer (pH 7, at 40 °C) with the co-factor NAD+ and crotylalcohol as the sole substrate for one hour. As Adh is a mesophilic enzyme a reaction temperature of 40 °C was chosen to prevent denaturation over the assay period [16].

Adh was able to effectively catalyse the conversion of crotylalcohol to crotonaldehyde (Figure 2). However, GC data indicated that the reaction was not quantitative, as only one third of the substrate was converted to crotonaldehyde. Since the conversion of crotylalcohol to crotonaldehyde is thermodynamically unfavourable ($\Delta G^\circ = -5.78$ kcal/mol; [17]), it is reasonable that the reaction reached an equilibrium. To allow efficient conversion of crotylalcohol to n-butanol, it is therefore imperative that one of the subsequent enzymatic steps is capable of quantitatively converting either crotonaldehyde or butanal. This is the first report of a characterized Adh accepting both crotylalcohol and butanal as substrates. The substrate promiscuity of Adh is the basis for construction of a consolidated reaction cascade towards n-butanol.

Conversion of crotonaldehyde to butanal by YqjM

At present no enzyme system for the dedicated conversion of crotonaldehyde to butanal has been identified. This is partially due to the transient reactivity of crotonaldehyde in aqueous reaction systems. Since the enzyme family of 2-enol reductases accepts a wide range of α-, β-unsaturated aldehydes and ketones [18-19], we have applied an in vitro screening effort to identify an enzyme capable of converting crotonaldehyde to butanal. In these screening efforts we identified the NADH dependent 2-enol reductase YqjM derived from B. subtilis (EC 1.3.1.31) as a key candidate.

YqjM belongs to the family of Old Yellow Enzymes (OYE) (EC 1.6.99.1) [18]. OYEs were the first enzyme family reported to feature a catalytically active flavin (FMN) moiety as part of the active site. In biological redox reactions enzyme-bound flavin serves as a temporary sink of electrons, which are further passed on to an electron-accepting protein or substrate species.

Interestingly, YqjM displays pronounced substrate promiscuity, accepting a range of chemically different substrates including quinones, α-, β-unsaturated aldehydes and ketones [18,19]. Additionally, it has been demonstrated that YqjM acts as a detoxification enzyme in the antioxidant defence system [18], suggesting that YqjM has multiple physiological substrates. Further, YqjM is promiscuous in its co-factor choice as it can utilize both NADH and NADPH in molar amounts [18]. This feature is of particular importance in the construction of a consolidated reaction cascade, since NADH can be used as the sole reductant mediator (Figure 1), which significantly reduces the molecular complexity of the entire system. The observed substrate promiscuity of YqjM (pdb-ID: 1Z48) is further corroborated through its open catalytic domain [20].

In this study, we have utilized YqjM’s substrate promiscuity for the targeted redox conversion of crotonaldehyde to butanal.

i. Characterisation of recombinant YqjM: The yqjM gene was isolated from B. subtilis genomic DNA by PCR and cloned into a standard expression vector pET28a. Subsequently, YqjM was expressed in E. coli HMS174 (DE3) with a C-terminal His$_\text{6}$Tag. After cell disruption YqjM was found to be equally distributed between the soluble and insoluble fractions. The molecular weight (Mr = 38 kDa) of recombinant YqjM was consistent with the literature [12]. Prior to in vitro application, soluble YqjM was purified to homogeneity by application of Ni$^{2+}$-NTA affinity chromatography.

ii. Reactivity of YqjM towards crotonaldehyde: The activity of 2-enol reductases could be determined spectrophotometrically by measuring the decrease in NADH absorbance. However, for reactions of YqjM in aerobic media, this spectrophotometric measurement is complicated by a side-reaction with molecular oxygen, which leads to the non-productive depletion of the NADH pool [18,21].

In this study, no reliable data on YqjM activities could be collected using spectrophotometric measurements. Therefore, we applied a validated GC based methodology to accomplish quantitative measurements of YqjM activity towards crotonaldehyde.

(20 mM). The concentrations of butanal and n-butanol in each sample were determined by correlating the GC-FID response to control samples (butanol: 10 mM; butanal: 10 mM) of a separate GC run.

RESULTS AND DISCUSSION

Central and crotylalcohol as the sole thermodynamically unfavourable (∆G

Since the conversion of crotylalcohol to crotonaldehyde is one third of the substrate was converted to crotonaldehyde.
In analogy to the Adh reaction, YqjM activity was determined at 40 °C (t = 1 h) in a Hepes buffer system (pH 7) containing FMN and NADH as co-factors and crotonaldehyde as the sole substrate.

The resulting GC measurements revealed that recombinant YqjM-His₆ variant was indeed catalytically active and able to convert crotonaldehyde to butanal (Figure 3).

In contrast, to our data, literature describes the YqjM-His₆ variant as catalytically inactive [21]. However, this literature evidence is based on spectrophotometric data, which are inconclusive due to the parallel reaction of YqjM with molecular oxygen present in the reaction [21]. That the YqjM-His₆ variant (catalytic activity = 1 U/mg) may be less active than the wild-type enzyme can be deduced by structural analysis of YqjM (pdb-ID: 1Z48). Structurally intact YqjM is a homo-tetramer. Apparently, a close interaction of the C-terminus of each YqjM monomer with its neighbouring subunit results in formation of basic AB dimers, which are required to maintain structural integrity and catalytic viability. Introduction of the C-terminal His₆-tag in YqjM impairs the formation of the AB dimer and interferes with substrate binding, ultimately constraining catalysis [20,21].

To our knowledge, this is the first report describing the targeted enzymatic conversion of crotonaldehyde to butanal.

Consolidated conversion of crotylalcohol to butanol in the presence of NADH/NAD⁺ as the sole redox couple

This study aims to create an enzyme cascade for the conversion of crotylalcohol to n-butanol. It was demonstrated that horse liver Adh is able to catalyse the reduction of crotylalcohol to crotonaldehyde and butanal to n-butanol. Further, we identified YqjM to catalyse the intermediate reaction involving the conversion of crotonaldehyde to butanal. Therefore, combining horse liver Adh and YqjM allows for consolidated conversion of crotylalcohol to n-butanol using the NADH/NAD⁺ as the sole redox couple, which significantly reduced the molecular complexity of the reaction system.

The consolidated conversion of crotylalcohol to n-butanol was initially carried out overnight (15 h) at 40 °C using a Hepes buffer system (pH 7) containing YqjM and Adh as well as the co-factors FMN (required by YqjM), NAD⁺ and NADH. All reaction intermediates and products were quantified using an established GC methodology (Figure 4).

The GC data indicated that in the reaction system horse liver Adh could only partially convert crotylalcohol to crotonaldehyde (c = 7.8 mM, corresponding to 39 %). YqjM was capable to convert crotonaldehyde to butanal (c = 15 mM, corresponding to 76 %), while horse liver Adh was able to convert a significant amount of butanal to the end product n-butanol (c = 10.3 mM).
Over the entire reaction cascade approximately 51.5\% of the initial crotylalcohol could be converted to n-butanol. This is the first proof of concept that the non-natural tailor-made enzyme cascade in this study could carry out the chemical conversions predicted by computational methods [7]. In an attempt to further optimize the reaction cascade, we added fresh Adh and NADH after the overnight incubation period. This was reasonable as Adh and NADH have limited stability at elevated temperatures [2,16]. Due to the reaction stoichiometry for NADH utilization (Figure 1) and the non-productive reaction of YqjM with O\textsubscript{2}, it was expected that the system’s NADH pool was rapidly depleted [21].

After addition of Adh and NADH, the reaction was allowed to continue for an additional hour. The addition of Adh and NADH resulted in a 25\% increase in n-butanol (c = 14.5 mM, corresponding to 72.5\%) yield. Therefore, titration of NADH during the reaction may significantly increase the n-butanol product yield, thereby counteracting the effects of molecular oxygen (Figure 4B). Similar observations have been made in the cell-free production of isobutanol [2].

This is the first evidence that the synergistic action of YqjM and Adh allow concerted conversion of crotylalcohol to n-butanol. There are significant efforts to establish consolidated biosynthetic pathways in cellular and cell-free production systems [5,7,8,14]. Computational models of alternative pathways can guide experimental approaches to validate alternative, non-natural reaction systems. For the first time we could realise an enzyme cascade that carries out an essential reaction module in non-natural aerobic n-butanol biosynthesis predicated by computational models [7].

CONCLUSIONS

Due to its enhanced energy content and hydrophobic properties, bio-based n-butanol is a desired next generation building block for renewable chemical and fuel processes [1,2]. While cellular n-butanol production systems suffer from limited productivity due to end-product toxicities and unproductive metabolic side reactions [3], cell-free enzyme cascades have emerged as alternative production systems that provide mass efficient production of platform chemicals and rapid adaptability to harsh process conditions [5].

Natural biosynthetic pathways involved in cellular n-butanol production are highly complex, involving multiple reaction steps and several cofactors [3]. More recently, computational methodologies have been employed to predict non-natural reaction steps for the conversion of pyruvate to n-butanol [7]. These computationally predicted reaction modules allow for a significant reduction in molecular complexity compared to their natural counterparts. Hence, computational pathway design may guide experimental efforts to design consolidated enzyme cascade for n-butanol production. A key reaction module in the predicted n-butanol production pathway is the conversion of crotylalcohol to n-butanol. In this study, we presented a new, non-natural enzyme cascade that allows for the consecutive conversion of crotylalcohol to n-butanol via crotonaldehyde and butanal. Horse liver Adh could be identified to catalyse both the conversion of crotylalcohol to crotonaldehyde and the final oxidation of butanal to n-butanol with good yields. Further, the 2-enoate reductase from B. subtilis YqjM could be identified to catalyse the intermediate conversion of crotonaldehyde to butanal.

Interestingly, both Adh and YqjM can utilize the same NAD\textsuperscript{+}/NADH redox system, which allowed the design of a cell-free enzyme cascade with significant reduction of molecular complexity. Application of both Adh and YqjM allowed the consolidated three step conversion of crotylalcohol to n-butanol utilizing the pronounced substrate promiscuity of the employed enzyme systems. Reaction analysis indicated that the non-productive side reaction of YqjM with molecular oxygen derived from the reaction medium, leads to rapid depletion of the available NADH pool, thereby limiting n-butanol yields. Initial optimisation of the reaction system indicates that NADH titration during the reaction can increase conversion efficiency of crotylalcohol to n-butanol. Alternatively, the reaction may be conducted under anaerobic conditions, thereby eliminating the reaction of oxygen with YqjM.

Figure 3 Conversion of crotonaldehyde to butanal with recombinant YqjM: GC measurements: YqjM assay with the crotonaldehyde substrate (in red) and 20 mM butanal as control (in black); the assay was conducted in 50 mM Hepes buffer (pH 7) with 20 mM crotonaldehyde, 20 mM NADH and 0.05 mM FAD\textsuperscript{+} at 40 °C.
This is the first report demonstrating that non-natural enzyme systems can be used to validate computational models of alternative n-butanol production pathways. In conclusion, computationally predicted reaction pathways can guide the experimental development of non-natural biosynthesis routes for molecular efficient, consolidated n-butanol production systems. These developments are essential for the design of molecular and cost efficient industrial processes.

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Author contribution

T.B. and D.G. conceived this study and finalized the manuscript. B.S. drafted the manuscript and conducted the experimental procedures. M.H. was involved in GC measurements.

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