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Research Article

Catalytic Modules in Non-Natural Butanol Biosynthesis: Conversion of the Key Intermediate Crotylalcohol to N-Butanol via a Designed Enzyme Cascade

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Submitted: 06 August 2013

Accepted: 02 September 2013

Published: 04 September 2013

Keywords

- n-Butanol
- Cell-free production system
- Tailor made bio-manufacturing
- Computational biosynthesis route
- 2-Enoate reductase
- Alcohol dehydrogenase
- Substrate promiscuity
- Non-natural enzyme cascade

Abstract

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 predicated 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.

INTRODUCTION

Climate change and dwindling fossil resources drive industrial developments of renewable, biomass based processes for fuels, chemicals and commodity products [1,2]. Industrial alcohols such as bio-ethanol are key in the development of renewable processes, as they are versatile platform chemicals for fuel and commodity production. However, due to its low energy density and low hydrophobicity, bio-ethanol is no ideal replacement for fossil fuels [3]. An emerging bio-fuel alternative is n-butanol, which due to its unlimited miscibility with fossil fuels and its higher energy density is poised to be a key next generation renewable building block [3]. Conventionally, n-butanol is produced via the anaerobe *Clostridium acetobutylicum* based acetone (A), butanol (B) and ethanol (E) fermentation process. However, the conventional cell based ABE process suffers from marginal economic viability and low product titres due to end-product toxicity above 1 - 2 %(v/v) and accumulation of undesired metabolic products [4]. An emerging alternative is the development of non-natural, cell-free enzyme cascades that selectively convert sugars into platform chemicals such as butanol [5]. Cell free enzymatic processes can potentially result in higher product yields as there is no requirement to maintain viable cellular functions and the rapid adaptability of component enzymes to harsh industrial reaction conditions [2]. Therefore, cell-free production systems enable the targeted, mass efficient production of chemical products.

Starting from the universal glycolytic intermediate pyruvate, the native, coenzyme-A dependent n-butanol biosynthesis pathway of *C. acetobutylicum*, involves seven enzymatic steps that required different co-factor systems to yield n-butanol [6]. Recently, consolidated, computational models of non-natural n-butanol biosynthesis from pyruvate have been proposed, which could guide experimental reaction engineering towards a consolidated cell-free process [5,7-9]. In our quest to construct a

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-natural, 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 predicated biosynthetic modules involved in non-natural 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, deoxyribonucelotides 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). Other chemicals as well as nicotinamide adenine dinucleotide disodium salt (NADH and NAD^+) were purchased from Carl Roth (Karlsruhe, 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) (F^- *recA1 hsdR* (r_{K12}^- m_{K12}^+) (DE3) (Rif^R)) was purchased from Merck KGaA (Darmstadt, Germany), *E. coli* XL1-Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proAB lacI*q*Z*Δ*M15* Tn*10* (Tetr)] from Stratagene (Waldbronn, 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 *Nco*I and *Xho*I. The open reading frame of *B. subtilis yqjM* was amplified by PCR, applying the primers 5' YqjM_pET28 (GCG CCA TGG CCA GAA AAT TAT TTA CAC CTA TTA) and 3' YqjM_pET28a (ATA TCT CGA GCC AGC 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 OD_{600} = 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 lysate was prepared with Emulsiflex-B15 (Avestin, Mannheim, Germany) and cell debris removed by centrifugation (25.000 g, 4 °C, 20 min). A Ni^{2+}-NTA column (column volume (cv) ~ 4 ml; Thermo Scientific, Schwerte, Germany) was equilibrated with 50 mM Hepes pH 8.0, 20 mM imidazole. His_6 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

(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

Crotylalcohol and crotonaldeyhde 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 crotonaldeyde, 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 are 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 (ΔG_0 = -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-enoate reductases accepts a wide range of α-, ß-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-enoate 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-, ß-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 []. This feature is of particular importance in the construction of a consolidated reaction cascade, since NADH can be used as the sole redox 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 reduction 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_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-enoate reductases could be determined spectrometrically by measuring the decrease in NADH absorbance. However, for reactions of YqjM in aerobe 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.

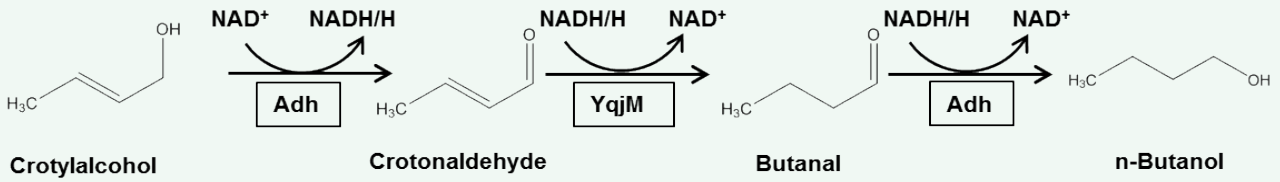

Figure 1 Non-natural enzyme cascade for conversion of crotylalcohol to n-butanol. Adh: commercial horse liver alcohol dehydrogenase, YqjM: recombinant 2-enoate reductase from *B. subtilis*.

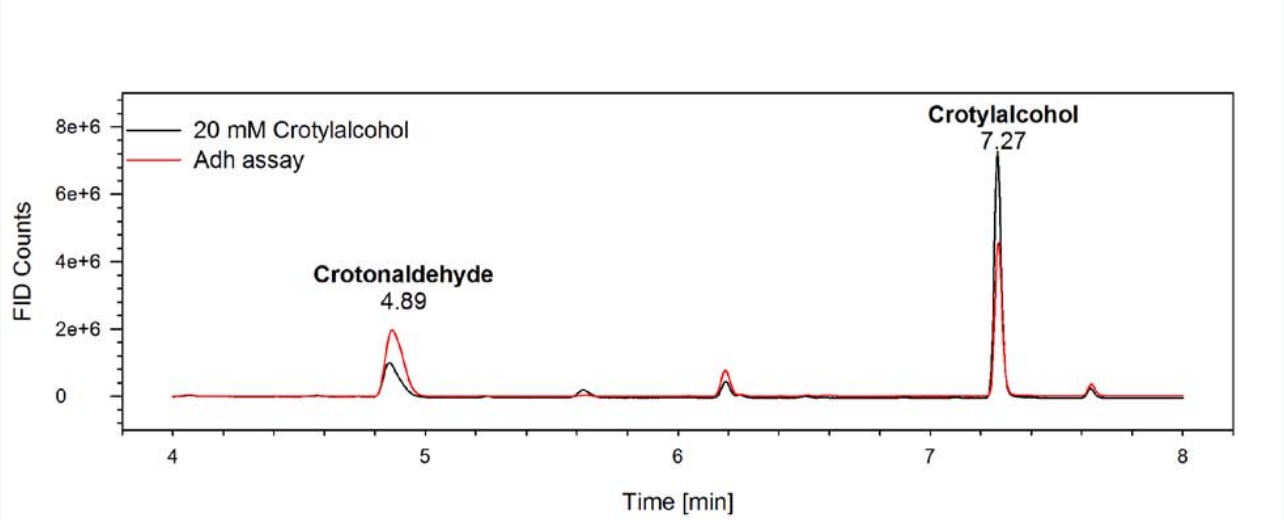

Figure 2 Reactivity of horse liver Adh towards crotylalcohol via GC-FID measurements: Adh assay with crotylalcohol as substrate (in red) and 20 mM crotylalcohol as control (black); The assay was conducted in 50 mM Hepes buffer (pH 7), 20 mM crotylalcohol and 20 mM NAD+ at 40 °C. Crotylalcohol has a retention time of 7.27 min. A butanol contamination from the crotylalcohol stock resulted in a peak at 6.2 min.

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-His_6 variant was indeed catalytically active and able to convert crotonylaldehyde to butanal (Figure 3).

In contrast, to our data, literature describes the YqjM-His_6 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-His_6 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 His_6-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_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^+/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.

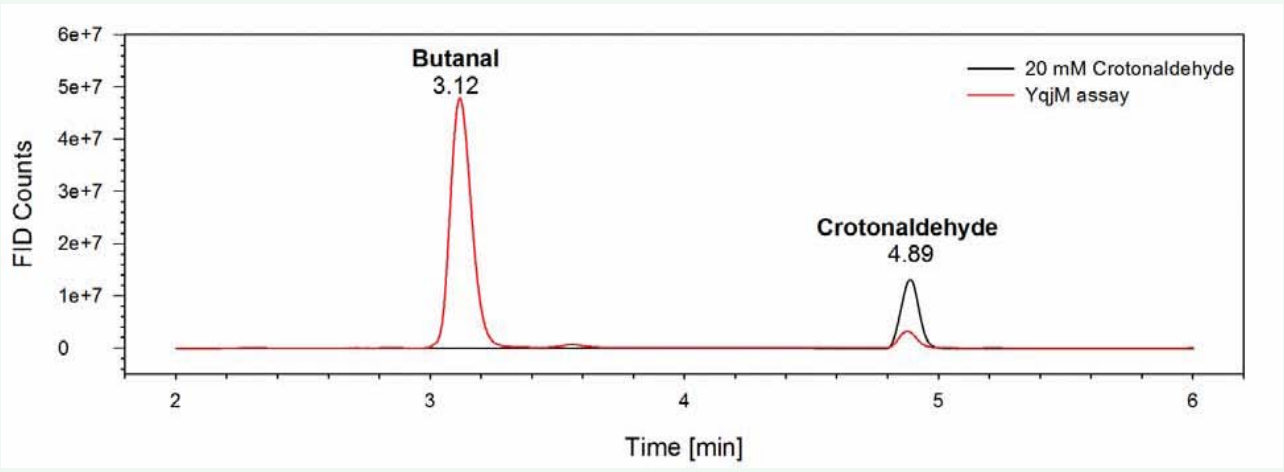

Figure 3 **Conversion of crotonaldehyde to butanal with recombinant YqjM: GC measurements: YqjM assay with the crotonaldehyde substrate (in red) and 20 mM butanal as control (in black); the assay was conducted in 50 mM Hepes buffer (pH 7) with 20 mM crotonaldehyde, 20 mM NADH and 0.05 mM FAD^+ at 40 °C.**

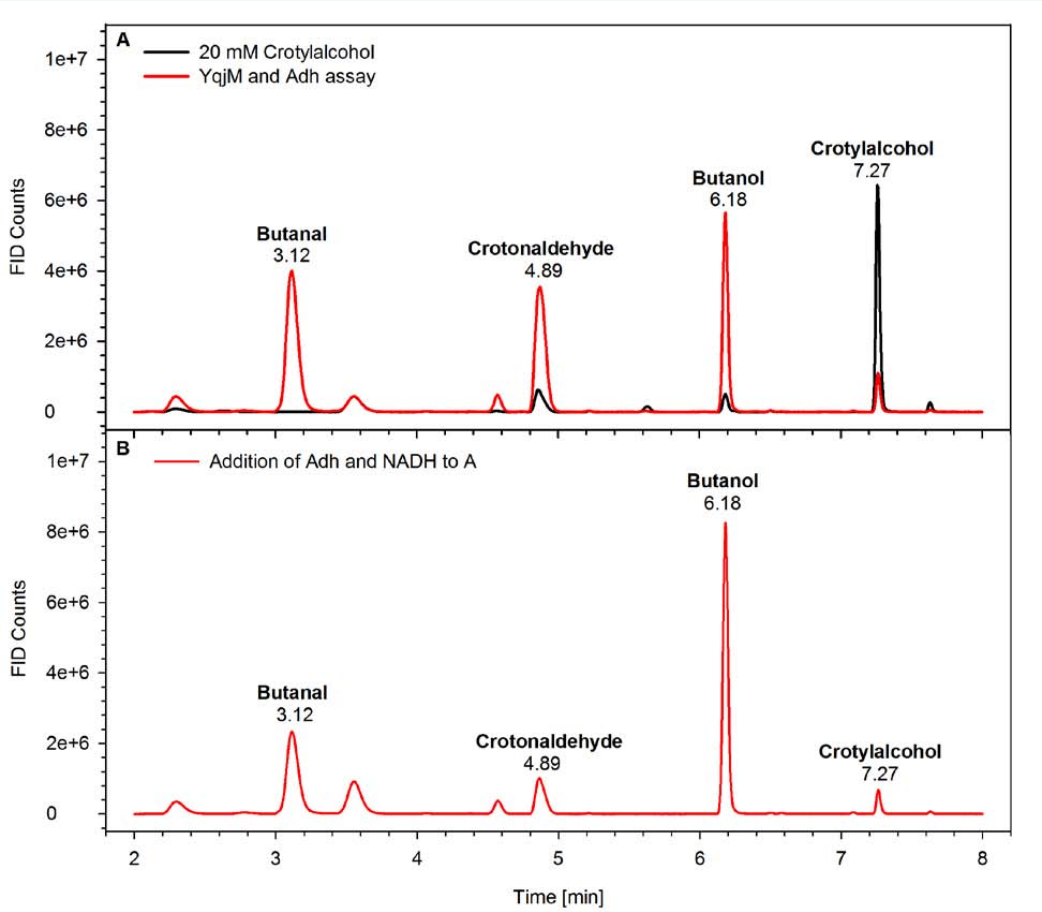

Figure 4 Chromatograms of GC measurements; (A) Result of YqjM and Adh assay with crotylalcohol as substrate (in red) and 20 mM crotylalcohol as control in (black); (B) To assay (A) Adh and NADH were again added and incubated at 40 °C for another hour. Crotylalcohol has a retention time of 7.27 min, n-butanol of 6.18 min, crotonylaldheyde of 4.89 min and butanal of 3.1 min.

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.

ACKNOWLEDGEMENTS

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.

Financial disclosure

This work was supported by the German Federal Ministry of Education and Research (BMBF) through grant No. 0315485B and Clariant AG, a for-profit company pursuing commercialization of bio-based processes.

REFERENCES

1. Himmel ME, Ding SY, Johnson DK, Adney WS, Nimlos MR, Brady JW, et al. Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science. 2007; 315: 804-807.
2. Guterl JK, Garbe D, Carsten J, Steffler F, Sommer B, Reiße S, et al. Cell-free metabolic engineering: production of chemicals by minimized reaction cascades. ChemSusChem. 2012; 5: 2165-2172.
3. Durre P. Biobutanol: an attractive biofuel. Biotechnol J. 2007; 2: 1525-1534.
4. Steen EJ, Chan R, Prasad N, Myers S, Petzold CJ, Redding A, et al. Metabolic engineering of Saccharomyces cerevisiae for the production of n-butanol. Microb Cell Fact. 2008; 7: 36.
5. Zhang YH. Production of biocommodities and bioelectricity by cell-free synthetic enzymatic pathway biotransformations: challenges and opportunities. Biotechnol Bioeng. 2010; 105: 663-677.
6. Yan Y, Liao JC. Engineering metabolic systems for production of advanced fuels. J Ind Microbiol Biotechnol. 2009; 36: 471-479.
7. Wu D, Wang Q, Assary RS, Broadbelt LJ, Krilov G. A computational approach to design and evaluate enzymatic reaction pathways: application to 1-butanol production from pyruvate. J Chem Inf Model. 2011; 51: 1634-1647.
8. Ranganathan S, Maranas CD. Microbial 1-butanol production: Identification of non-native production routes and in silico engineering interventions. Biotechnol J. 2010; 5: 716-725.

9. Nielsen DR, Leonard E, Yoon SH, Tseng HC, Yuan C, Prather KL. Engineering alternative butanol production platforms in heterologous bacteria. Metab Eng. 2009; 11: 262-273.

10. Pace CN, Vajdos F, Fee L, Grimsley G, Gray T. How to measure and predict the molar absorption coefficient of a protein. Protein Sci. 1995; 4: 2411-2423.

11. Elisabeth Gasteiger, Christine Hoogland, Alexandre Gattiker, S'everine Duvaud, Marc R. Wilkins, Ron D. Appel, et al. Protein identification and analysis tools on the ExPASy server, in The proteomics protocols handbook. Springer. 2005; 571-607.

12. Stoll VS, Blanchard JS. Buffers: principles and practice. Methods Enzymol. 2009; 463: 43-56.

13. Orbegozo T, de Vries JG, Kroutil W, Biooxidation of primary alcohols to aldehydes through hydrogen transfer employing janibacter terrae. European J Org Chem. 2010; 2010: 3445-3448.

14. Light DR, Dennis MS, Forsythe IJ, Liu CC, Green DW, Kratzer DA, et al. Alpha-isoenzyme of alcohol dehydrogenase from monkey liver. Cloning, expression, mechanism, coenzyme, and substrate specificity. J Biol Chem. 1992; 267: 12592-12599.

15. Petersen DJ, Welch RW, Rudolph FB, Bennett GN. Molecular cloning of an alcohol (butanol) dehydrogenase gene cluster from Clostridium acetobutylicum ATCC 824. J Bacteriol. 1991; 173: 1831-1834.

16. BRAND L, EVERSE J, KAPLAN NO. Structural characteristics of dehydrogenases. Biochemistry. 1962; 1: 423-434.

17. Jankowski MD, Henry CS, Broadbelt LJ, Hatzimanikatis V. Group contribution method for thermodynamic analysis of complex metabolic networks. Biophys J. 2008; 95: 1487-1499.

18. Fitzpatrick TB, Amrhein N, Macheroux P. Characterization of YqjM, an Old Yellow Enzyme homolog from Bacillus subtilis involved in the oxidative stress response. J Biol Chem. 2003; 278: 19891-19897.

19. Stueckler C, Hall M, Ehammer H, Pointner E, Kroutil W, Macheroux P, et al. Stereocomplementary bioreduction of alpha,beta-unsaturated dicarboxylic acids and dimethyl esters using enoate reductases: enzyme- and substrate-based stereocontrol. Org Lett. 2007; 9: 5409-11.

20. Kitzing K, Fitzpatrick TB, Wilken C, Sawa J, Bourenkov GP, Macheroux P, et al. The 1.3 A crystal structure of the flavoprotein YqjM reveals a novel class of Old Yellow Enzymes. J Biol Chem. 2005; 280: 27904-27913.

21. Fitzpatrick TB, Auweter S, Kitzing K, Clausen T, Amrhein N, Macheroux P. Structural and functional impairment of an Old Yellow Enzyme homologue upon affinity tag incorporation. Protein Expr Purif. 2004; 36: 280-291.

Cite this article

Sommer B, Haack M, Garbe D, Brück T (2013) Catalytic Modules in Non-Natural Butanol Biosynthesis: Conversion of the Key Intermediate Crotylalcohol to N-Butanol via a Designed Enzyme Cascade. JSM Biotechnol Bioeng 1(2): 1010.