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Article

Activity of *Lactobacillus brevis* Alcohol Dehydrogenase on Primary and Secondary Alcohol Biofuel Precursors

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Abstract: The *R*-specific alcohol dehydrogenase (ADH) from *Lactobacillus brevis* LB19 (*Lb*ADH) was studied with respect to its ability to reduce a series of 3- through 5-carbon 2-alkanones and aldehydes of relevance as biofuel precursors. Although active on all substrates tested, *Lb*ADH displays a marked preference for longer chain substrates. Interestingly, however, 2-alkanones were found to impose substrate inhibition towards *Lb*ADH, whereas aldehyde substrates rendered no such effect. Inhibition caused by 2-alkanones was furthermore found to intensify with increasing chain length. Despite demonstrating both primary and secondary ADH activities, a preliminary sequence analysis suggests that *Lb*ADH remains distinct from other, previously characterized primary-secondary ADHs. In addition to further characterizing the substrate range of this industrially important enzyme, this study suggests that *Lb*ADH has the potential to serve as a useful enzyme for the engineering of various novel alcohol biofuel pathways.

Keywords: alcohol dehydrogenase; *Lactobacillus brevis*; butanol; biofuel

1. Introduction

With numerous applications in both *in vitro* and whole cell biotransformations, alcohol dehydrogenases (ADHs) catalyze a diversity of reduction reactions of importance to industrial biotechnology. Since its

discovery nearly 20 years ago, ADH from *Lactobacillus brevis* (*Lb*ADH) has been among the most comprehensively studied and employed enzymes to this end (see previous comprehensive reviews by Hummel [1], Nakamura, *et al.* [2], and Leuchs and Greiner [3]). An NADPH-dependent homotetramer and member of the short-chain dehydrogenases/reductases (SDR) extended enzyme family, *Lb*ADH boasts several attributes of importance as a versatile biocatalyst. For example, in addition to its high stability at elevated temperatures [4] as well as in non-conventional reaction media (e.g., organic solvents) [5], *Lb*ADH also displays high activity on a broad range of substrates. With known secondary alcohol dehydrogenase (SADH) activity, *Lb*ADH has most commonly been employed for the asymmetric reduction of prochiral ketones. More specifically, as it demonstrates *R*-specific functionality and excellent enantioselectivity, *Lb*ADH catalyzes stereoselective reductions with high enantiomeric excess (e.e. >99%). It has been reported that the preferred substrates of *Lb*ADH are generally ketones including a small alkyl group as one substituent and a "bulky", often aromatic moiety as the second [6]. Accordingly, substrates evaluated to date have predominantly included aromatic ketones and keto-esters.

Although less extensively investigated, a number of other studies have shown that *Lb*ADH also displays activity on select aliphatic 2-alkanones. For example, using a two-phase system (with an ionic liquid serving as a "substrate reservoir"), Eckstein *et al.* demonstrated that *Lb*ADH efficiently catalyzes the reduction of 2-octanone to (*R*)-2-octanol (88% conversion in 3 h with >99% e.e.) [7]. Several studies, meanwhile, have studied the ability of *Lb*ADH to catalyze the stereoselective reduction of 2-butanone [8–10]. Erdmann *et al.*, for example, recently demonstrated that whole cells of recombinant *Escherichia coli* expressing *Lb*ADH could be employed in a novel continuous reactor process to convert 2-butanone to (*R*)-2-butanol at >99% conversion and >96% e.e., while also achieving space time yields of ~2300 g/L-d [8].

In addition to their roles as building-block chemicals [8], short chain aliphatic alcohols are of particular interest as the potential gasoline alternatives. Among aliphatic alcohols, those with >2 carbons continue to emerge as attractive second-generation biofuel targets [11]. Compared to ethanol, for example, higher alcohols such as n-butanol and 2-butanol possess greater energy densities of (29.2 and 29.1 MJ/L, respectively, *versus* 19.6 MJ/L for ethanol) as well as physicochemical properties that improve their compatibility with conventional infrastructure and engines [12]. Through metabolic engineering and *de novo* pathway construction, novel microbes have recently been engineered for the production of various primary and secondary alcohols with potential biofuel applications, including *n*-propanol [13], 2-propanol [14], *n*-butanol [15,16], *iso*-butanol [17], and *n*-pentanol [18]. In all cases, ADHs play key roles as the terminal enzyme step in each of the respective biosynthetic pathways.

Inspired by its previously demonstrated and efficient role of *Lb*ADH in (*R*)-2-butanol production, the purpose of this study was to investigate its function and performance with respect to synthesizing other short-chain alcohol biofuels, thereby further evaluating its potential as a broadly useful and robust enzyme for future metabolic engineering studies. Meanwhile, in addition to assaying its well-known SADH activity, the function of *Lb*ADH towards the synthesis of primary short-chain alcohols was also explored. Taken together, the present study provides a comprehensive account of the function and relative activity of *Lb*ADH on a collection of 3- through 5-carbon 2-alkanones and aldehydes of potential interest as advanced biofuels (Figure 1).

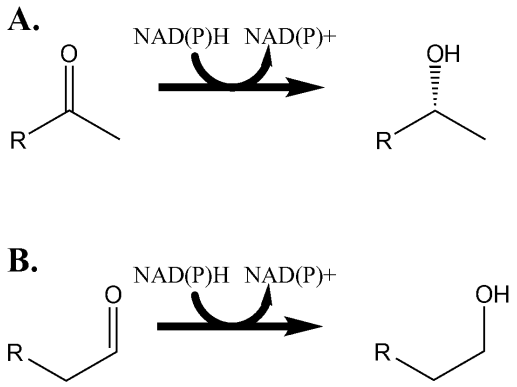

Figure 1. The *R*-specific alcohol dehydrogenase (ADH) from *Lactobacillus brevis* LB19 (LbADH) was evaluated for its ability to reduce short-chain (**A**) 2-alkanones and (**B**) aldehydes to their respective secondary and primary alcohols, here R = CH_3, CH_2CH_3, or $CH_2CH_2CH_3$.

2. Experimental Section

2.1. Chemicals

All media components and chemicals used in this study were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Microorganisms and Media

All bacterial strains used in this study are listed in Table 1. *Escherichia coli* NEB10-beta (New England Biolabs; Ipswich, MA, USA) was used for routine cloning as well as for the storage and propagation of plasmids. *E. coli* BW25113 was obtained from the Coli Genetic Stock Center (CGSC) at Yale University and was used as the wild-type parent. *E. coli* BW25113(DE3), which was generated by λDE3 prophage integration into BW25113 using the λDE3 Lysogenization Kit (EMD Biosciences; San Diego, CA, USA), was used for recombinant *Lb*ADH expression in support of all *in vitro* and *in vivo* transformation studies. *L. brevis* LB19 was purchased from the Centre International de Ressources Microbiennes Bacteries d'Interet Alimentaire, Institut National de la Recherche Agronomique (CIRM-BIA; Rennes, France). *E. coli* strains were routinely cultured in LB broth supplemented with appropriate antibiotics, as required, whereas *L. brevis* was cultured in MRS media.

2.3. Plasmid Construction

Table 1 lists all DNA plasmids constructed and used in this study. The expression vector pACYCDuet-1 was purchased from Novagen (Billerica, MA). Standard molecular biology techniques [15] and/or manufacturer protocols were used for all gene cloning. Plasmid and genomic DNA purification was performed according to manufacturer protocols using the Zyppy™ Plasmid Miniprep and Genomic DNA Clean & Concentrator kits, respectively, from Zymo Research (Orange, CA, USA). Phusion High-Fidelity DNA Polymerase, all restriction enzymes, and T4 DNA Ligase were purchased from New England Biolabs (Ipswich, MA, USA). *Lb*ADH was PCR amplified from *L. brevis* LB19 genomic DNA

using custom oligonucleotides primers (specifically, 5′-ATTCATATGTCAAACCGGTTA-3′ and 5′-ATTCTCGAGTTATTGAGCGGT-3′) synthesized by Integrated DNA Technologies (San Diego, CA, USA). The amplicon was purified using the DNA Clean & Concentrator kit (Zymo Research) before both it and pACYCDuet-1 were digested by treatment with NdeI and XhoI. Digested fragments were gel purified using the Zymoclean Gel DNA Recovery kit (Zymo Research) before then being ligated together at 4 °C overnight using T4 DNA ligase. Ligations were transformed into chemically competent NEB10-beta and plated on LB solid agar with 34 mg/L chloramphenicol overnight at 30 °C for selection. This resulted in the construction of pACYC-*Lb*ADH. To aid in its purification, *Lb*ADH was as additionally re-cloned between the BamHI and EcoRI sites of pACYCDuet-1 (using the primer pair 5′-ATTGGATCCTTCGACACTCTT-3′ and 5′-ATAGAATTCGGGGCTTACAAC-3′) to incorporate an N-terminal poly-His tag, resulting in the construction of pACYC-*Lb*ADH-His.

Table 1. Strains and plasmids constructed and/or used in this study.

Strain	***Genotype or Description***	***Source***
E. coli NEB10-beta	*araD139*Δ(*ara-leu*)7697 *fhuA lacX74 galK* (ϕ80 Δ(*lacZ*)*M15*) *mcrA galU recA1 endA1 nupG rpsL* (Str^R) Δ(*mrr-hsdRMS-mcrBC*)	*New England Biolabs*
E. coli BW25113	*F'* λ⁻ Δ*(araD-araB)567,* Δ*lacZ4787(::rrnB-3), lambda⁻, rph-1,* Δ*(rhaD-rhaB)568, hsdR514*	CGSC
E. coli BW25113(DE3)	λDE3 lysogen of BW25113λ	This study
L. brevis LB19	Genetic source of *Lb*ADH	CIRM-BIA
Plasmid	***Features***	***Source***
pACYCDuet-1	Expression vector, Cm^r, P_{T7}, pACYC184 Ori	Novagen
pACYC-*Lb*ADH	*Lb*ADH from *L. brevis* LB19 inserted between NdeI and XhoI sites of pACYCDuet-1	This study
pACYC-*Lb*ADH-His	*Lb*ADH from *L. brevis* LB19 inserted between BamHI and EcoRI sites of pACYCDuet-1	This study

2.4. Whole Cell Conversion of 2-Butanone to 2-Butanol by E. coli Growing Cells

E. coli BW25113(DE3) was transformed with pACYC-*Lb*ADH and plated on LB solid agar with 34 mg/L chloramphenicol overnight. Colonies were selected from the resultant pool of transformants and used to inoculate 5 mL LB media with chloramphenicol and cultured at 37 °C overnight. These seed cultures were next used to inoculate (1% vol.) 50 mL LB with 2 g/L glucose and chloramphenicol in 250 mL shake flasks fitted with foam plug stoppers to maintain aerobic conditions. A culture of *E. coli* BW25113(DE3) was also analogously prepared to serve as a control. Cultures were incubated aerobically at 37 °C and induced by addition of IPTG at a final concentration of 0.5 mM upon reaching optical density at 600 nm (OD_{600}) of ~0.7. At the same time, 2-butanone was also added to each flask at an initial concentration of 1 g/L. Culturing continued under the same conditions for up to 24 h with periodic sampling for analysis of 2-butanone and 2-butanol by high performance liquid chromatography (HPLC), as described below. Meanwhile, to assess the potential for substrate or product loss by volatilization, control flasks consisting of 1 g/L 2-butanone or 2-butanol in sterile water were also analogously prepared and incubated in parallel.

2.5. Whole Cell Conversion of 2-Butanone to 2-Butanol by E. coli Resting Cells

Seed cultures of *E. coli* BW25113(DE3) pACYC-*Lb*ADH and *E. coli* BW25113(DE3) (control) were prepared as above and used to inoculate (1% vol.) 50 mL LB with 2 g/L glucose and chloramphenicol in 250 mL shake flasks. Cultures were again incubated aerobically at 37 °C and induced by addition of 0.5 mM IPTG upon reaching OD_{600} of ~0.7. Following overnight incubation, cultures were centrifuged to collect cells by pelleting. Cell pellets were washed once with and then re-suspended in 25 mL pH 7.0 PBS buffer to a final OD_{600} of 0.4 (~0.14 g/L cell dry weight (CDW)). To reduce the potential for volatile losses, 5 mL of each cell suspension was transferred to a glass Hungate tube fitted with a butyl rubber lined septa cap. Prior to sealing, 2-butanone was added to each tube at an initial concentration of 0.5 g/L. Cultures were incubated at 37 °C while shaking for a period of 10 h, with periodic sampling to monitor substrate depletion and product formation by HPLC. All experiments were performed in triplicate to provide an assessment of error.

2.6. Metabolite Analyses

Aqueous concentrations of 2-butanone and 2-butanol in whole cell cultures were determined via HPLC analysis (1100 series, Agilent, Santa Clara, CA, USA). Separation was achieved on a ZORBAX Eclipse XDB-C18 column (Agilent), operated isothermally at 50 °C. The mobile phase consisted of a solution of 5 mM H_2SO_4, pumped at a constant flow rate of 1.0 mL/min. Analytes were detected using a refractive index detector. For each species, standard solutions of known concentration were prepared in water to provide external calibration and determine concentrations.

2.7. In Vitro Enzyme Assays

E. coli BW25113(DE3) was transformed with pACYCD-*Lb*ADH-His and plated on LB solid agar with 34 mg/L chloramphenicol overnight. Colonies were selected from the result transformant pool and used to inoculate 5 mL LB media with chloramphenicol which was then cultured at 37 °C. Cultures were incubated aerobically at 37 °C and induced upon reaching an OD_{600} of ~0.7 by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.5 mM. Following overnight incubation, cultures were centrifuged to collect cells by pelleting. Cells were re-suspended in 900 μL of His-binding buffer to which 100 μL of 10x FastBreak Cell Lysis Reagent (Promega, Madison, WI, USA) was added. The mixture was vortexed at room temperature for 15 min before centrifuging the lysate for 2 min to pellet. Following manufacturer protocols, the His-Spin Protein Miniprep kit (Zymo Research) was then used to purify *Lb*ADH from the lysate. Purification of expressed *Lb*ADH was confirmed by gel electrophoresis (not shown). Protein concentration in the sample was determined via Bradford assay using BSA as a standard. Purified *Lb*ADH was used in *in vitro* activity assays according to the following protocol. To a cuvette with a total aqueous volume of 0.5 mL was added 5 μL of purified *Lb*ADH solution (whose typically protein content in this study was about 0.21 ± 0.03 mg/mL), the requisite amount of substrate (as appropriate to vary initial substrate concentrations), and the balance of pH 7.0 potassium phosphate 50 mM buffer. To initiate the reaction, 1 μL 100 mM NADPH solution was then added. In all cases, reaction progress was monitored by following the depletion of NADPH (initially 0.1 mM), as measured at 340 nm every 10 s using a spectrophotometer (DU800, Beckman Coulter, Brea, CA, USA).

A molar extinction coefficient of 6.22 × 10^3 M^{-1} cm^{-1} was used for NADPH. Reaction rates were measured by the method of initial velocities, as determined via least squares regression of those data obtained in the first 1–2 min of each experiment. All assays were performed at room temperature for each of the following substrates: acetone, 2-butanone, 2-pentanone, propionaldehyde, butyraldehyde, and valeraldehyde. All experiments were performed in triplicate to provide an assessment of error.

2.8. Modeling Enzyme Kinetics

Enzyme activity data was modeled according to one of two established kinetic models. In cases where substrate inhibition was not observed, the standard form of the Michaelis-Menten model was chosen [16]:

$$v = \frac{k_{cat}[E_0][S]}{K_M + [S]} \quad (1)$$

where v is velocity, $[E_0]$ is the total enzyme concentration, $[S]$ is substrate concentration, and the constants k_{cat} and K_m represent the turnover number and Michaelis-Menten constant, respectively. Meanwhile, for those substrates that caused observable substrate inhibition, a modified form of the Michaelis-Menten model was used [17]:

$$v = \frac{k_{cat}[E_0][S]}{K_M + [S] + [S]^2/K_I} \quad (2)$$

where v, $[E_0]$, S, k_{cat}, and K_M are as above, and K_I is an inhibition constant. Nonlinear least-squares regression was performed to estimate all parameters, as achieved using the intrinsic MATLAB® function nlinfit.

3. Results and Discussion

3.1. Confirming the In Vivo Function of Recombinant LbADH for 2-Butanol Production

As previously discussed, LbADH possesses the known ability to reduce 2-butanone to 2-butanol. Accordingly, to first confirm its functional expression under the conditions of interest in this study, a series of whole assays were performed using *E. coli* BW25113(DE3) pACYC-LbADH and exogenous 2-butanone as substrate. Reduction of 2-butanone to 2-butanol was first tested using growing cells under aerobic conditions. When initially provided with 1 g/L 2-butanone, 2-butanol levels reached a maximum of 0.56 g/L by 18 h with no residual 2-butanone detected. In contrast, no 2-butanol production was detected using the control strain (*i.e.*, *E. coli* BW25113(DE3)), however, only 0.45 g/L 2-butanone remained. Volatile losses were presumed to be a source of significant 2-butanone depletion (note: 2-butanone is ~4.5-times more volatile than water under the culture conditions performed), as subsequently confirmed through control experiments (*i.e.*, only trace levels of 2-butanone remained in a water solution that initially contained 1 g/L 2-butanone following incubation for 18 h under analogous conditions). Accordingly, to restrict volatile losses, additional whole cell assays were subsequently performed in this case using sealed culture tubes. To reduce oxygen requirements, resting cells were accordingly used in this case. As seen in Figure 2, the initial biotransformation of 2-butanone to 2-butanol occurred at a volumetric rate of ~0.15 g/L-h and a specific rate of ~1.1 g/g_{CDW}-h. In contrast no conversion of 2-butanone

to 2-butanol was observed by the control strain (*i.e.*, *E. coli* BW25113(DE3)). Meanwhile, whereas a yield of only ~50% was achieved in this case, this was likely a consequence of limited availability of the required NADPH cofactor; a limitation that has been addressed in other LbADH studies via coupled enzymatic co-factor regeneration [10].

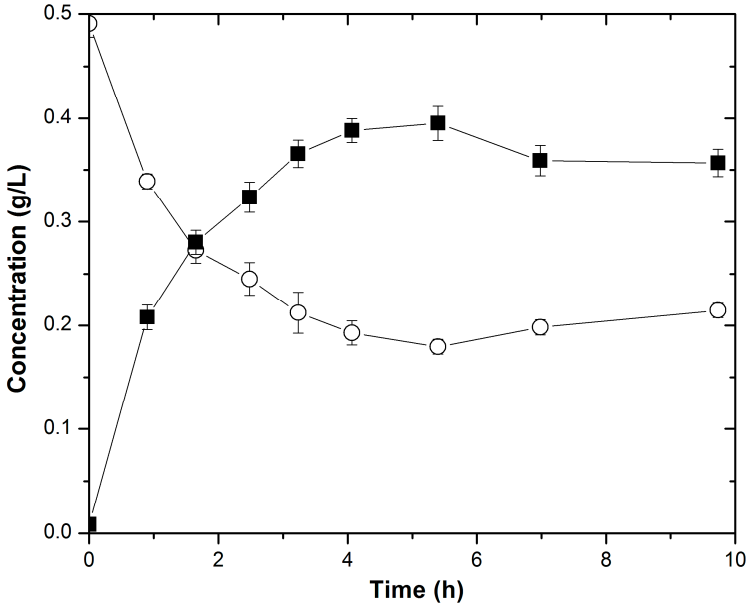

Figure 2. *In vivo* conversion of 2-butanone (open circles) to 2-butanol (solid squares) by resting cells of *E. coli* BW25113(DE3) pACYC-LbADH. Error bars reported at one standard deviation from triplicate experiments.

For comparison, Erdmann *et al.* also studied the LbADH catalyzed reduction of 2-butanone to 2-butanol using recombinant *E. coli* whole cells, with initial volumetric rates reported to reach as high as 24.9 g/L-h under batch conditions [8]. It should be noted, however, that the initial 2-butanone and CDW concentrations used were 83- and 179-times greater, respectively, than those employed here. Accordingly, when compared on the basis of CDW, the specific rate of 0.99 g/g_{CDW}-h demonstrated in said works compares well to this study. Most importantly, these results confirm that LbADH was indeed functionally expressed in *E. coli* according to the engineered plasmid construct prepared in this study.

3.2. Characterizing the In Vitro *Activity of Recombinant LbADH on Short-Chain 2-Alkanones and Aldehydes*

Next, following its recombinant expression by *E. coli* BW25113(DE3) pACYC-*Lb*ADH-His, the subsequent purification of *Lb*ADH (whose monomer mass is 26 kDa [3]) was confirmed by SDS-PAGE gel analysis (Figure 3).

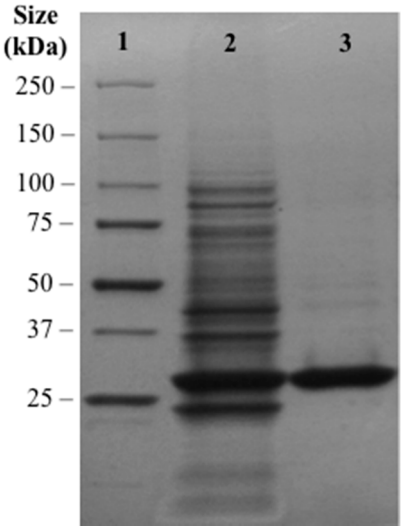

Figure 3. SDS-PAGE analysis of LbADH expression by *E. coli* BW25113(DE3) pACYC-LbADH-His and its subsequent column purification. Lane 1: Precision Plus Protein Standard (Bio-Rad); Lane 2: crude lysate; Lane 3: purified product.

The activity of *Lb*ADH towards the panel of 3- through 5-carbon 2-alkanones and aldehydes of interest was next investigated through *in vitro* enzyme assays. As seen in Figures 4 and 5, *Lb*ADH was indeed functional on all of the 2-alkanone and aldehyde substrates tested, demonstrating first and foremost that *Lb*ADH displayed both secondary and primary alcohol dehydrogenase activities. Clearly, however, characteristic differences were noted among the observed behaviors. At elevated concentrations, *Lb*ADH was subject to substrate inhibition by 2-alkanone substrates, however, not but the corresponding aldehydes. Accordingly, *Lb*ADH activity was fit to enzyme kinetic models given by Equations (1) and (2), respectively, with the resultant best-fit parameter estimates compared in Table 2. As seen from Figures 4 and 5 as well as comparison of the kinetic model parameters, *Lb*ADH in general displayed greater affinity yet lower activity (decreasing K_M and k_{cat} in Table 2) towards substrates with increasing carbon chain lengths. A decrease in K_M with increasing chain length is consistent with past reports of the preference of *Lb*ADH for substrates with "bulky" ligands [3]. Meanwhile, in the case of 2-alkanones, inhibition of SADH activity by *Lb*ADH increased (*i.e.*, lower K_I) alongside increases in the substrate chain length, with the greatest levels of substrate inhibition observed for 2-pentanone. The present findings demonstrate that the broad substrate specificity of *Lb*ADH also includes 3- through 5-carbon 2-alkanones and aldehydes. Moreover, the observation of both primary and secondary alcohol dehydrogenase activities suggests that *Lb*ADH is not strictly a SADH. Whereas, to the best of our knowledge, such a prospect has not before been thoroughly investigated, past studies have reported that *Lb*ADH can in fact catalyze the reduction of benzaldehyde to benzyl alcohol, albeit with low activity [1].

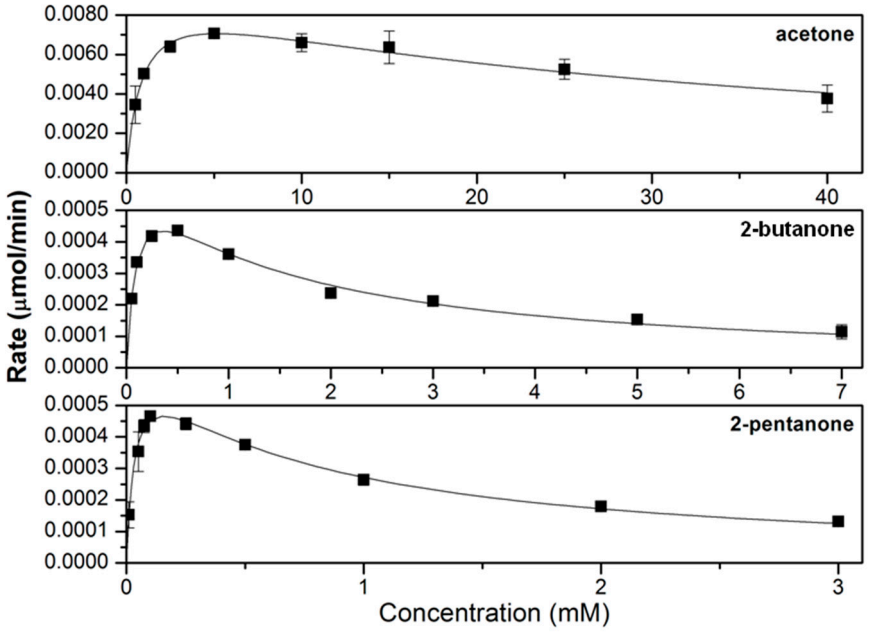

Figure 4. Comparing the activity of recombinant LbADH towards the reduction of short-chain 2-alkanones to their corresponding secondary alcohols. Substrates tested include acetone (upper), 2-butanone (middle), and 2-pentanone (lower). Error bars reported at one standard deviation from triplicate experiments.

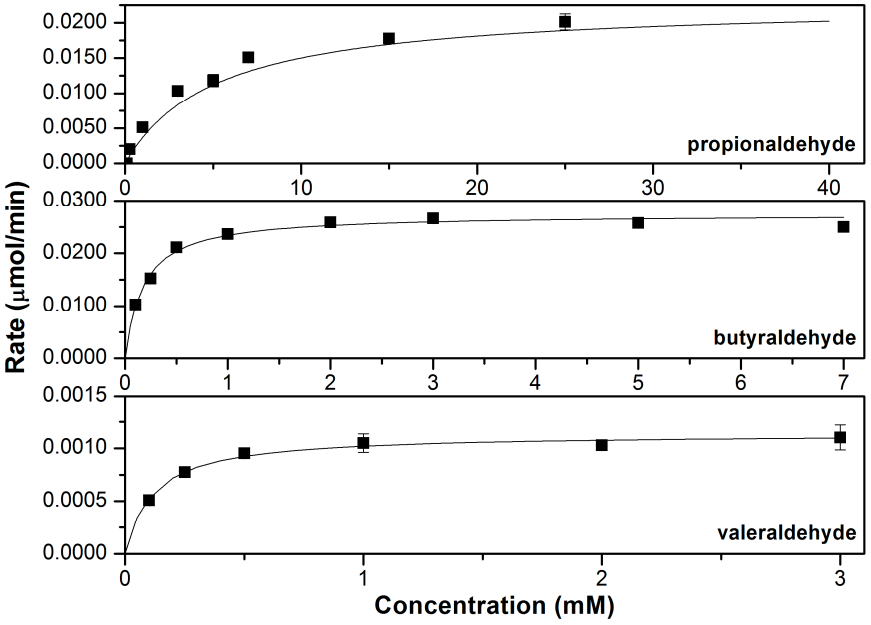

Figure 5. Comparing the activity of recombinant LbADH towards the reduction of short-chain aldehydes to their corresponding primary alcohols. Substrates tested include propionaldehyde (upper), butyraldehyde (middle), and valeraldehyde (lower). Error bars reported at one standard deviation from triplicate experiments.

Table 2. Best-fit enzyme kinetic model parameters for 3–5 carbon 2-alkanones and aldehydes. "N.D.", not determined. Error associated parameter estimates reported at one standard deviation.

Product Alcohol	Substrate	k_{cat} (s^{-1})	K_M (mM)	K_I (mM)	k_{cat}/K_M ($mM^{-1}s^{-1}$)
2°	Acetone	1.52 ± 0.01	0.88 ± 0.16	30.7 ± 6.8	1.73 ± 0.05
	2-Butanone	0.11 ± 0.01	0.10 ± 0.02	1.34 ± 0.26	1.12 ± 0.28
	2-Pentanone	0.11 ± 0.01	0.04 ± 0.01	0.68 ± 0.17	3.03 ± 1.05
1°	Propionaldehyde	3.36 ± 0.19	3.1 ± 0.7	N.D.	1.09 ± 0.25
	Butyraldehyde	4.42 ± 0.11	0.17 ± 0.02	N.D.	25.5 ± 3.5
	Valeraldehyde	0.18 ± 0.01	0.12 ± 0.03	N.D.	1.57 ± 0.41

3.3. Comparing LbADH with Other Bacterial ADHs

Though perhaps a novel finding for *Lb*ADH, enzymes displaying both primary and secondary ADH activities are not uncommon. Numerous microbes have been reported to possess similar abilities to reduce the same and/or related substrates to their corresponding alcohols. In the case of 2-butanone reduction to 2-butanol, for example, this includes ADHs from bacteria *Burkholderia* sp. [19], *Pseudomonas* sp. [20], and *Rhodococcus* sp. [21], along with yeasts including *Rhodotorula glutinis* [22], *Candida parapsilosis* [23], and *Geotrichum candidum* [24]. From the comparison provided in Table 3, it can be seen that, among specific bacterial ADHs whose activity on 2-butanone has been characterized, *Lb*ADH shows among the highest affinities. ADH from *C. beijerinckii* NRRL B593, however, possesses significantly greater k_{cat} and k_{cat}/K_M values; perhaps not surprising as 2–3 carbon 2-alkanones and aldehydes are intermediates native to this acetone-butanol-ethanol (ABE) fermenting microbe. Unlike *Lb*ADH, however, *C. beijerinckii* ADH has been reported to display decreasing affinity towards substrates with bulkier ligands, as seen by its higher K_M value for 2-butanone relative to acetone. In contrast, the two butanol dehydrogenases (BDHI and BDHII, respectively) from *C. acetobutylicum* ATCC 824, meanwhile, have been reported to display 2.2- and 3.6-fold higher activities towards butyraldehyde than with acetaldehyde [25], indicating that, much like *Lb*ADH, these ADHs too display a preference towards longer chain substrates.

It has previously been reported by Ismaiel *et al.* that ADHs with activity on both short-chain 2-alkanones and aldehydes (in said case each of acetone, acetaldehyde, and butyraldehyde) from *Clostridium beijerinckii* B593, *Thermoanaerobacter brockii*, and *Methanobacterium palustre* each shared significant homology (*i.e.*, 67% identity) in terms of their respective N-terminal sequences [26]. As shown in Figure 6, further alignment of the N-terminus of *Lb*ADH with these ADHs was performed as part of this study, however, a low alignment score was obtained. Furthermore, pairwise alignment of the entire coding sequences rendered similarly poor results with respect to overall homology (no more than ~12% identity in each case). A subsequent nucleotide BLAST search using *Lb*ADH did, however, reveal a noteworthy result. More specifically, 34% identity was observed between *Lb*ADH and a furfural transforming, short-chain dehydrogenase/reductase (SDR) from *C. beijerinckii* NCIMB 8052. With respect to enzymes of the greater SDR family, Kallberg *et al.* noted that sequence identities of only 15%–30% are in fact typical [27]. Thus, lying at the higher end of this range, it is concluded that said observed homology is significant. Furthermore, in contrast to the poor homology observed in Figure 6, as seen in Figure 7 very high N-terminus

homology (62%) was also observed between *Lb*ADH and the SDR from *C. beijerinckii* NCIMB 8052. In addition to furfural, SDR from *C. beijerinckii* NCIMB 8052 has been reported to display high activity on other bulky aldehydes (including benzaldehyde), as well as limited functionality on butyraldehyde [28].

Table 3. Comparing activities of other bacterial ADHs on 2-butanone and other substrates relevant to this study. "-" indicates not determined/reported.

Organism Enzyme(s)	Substrate(s)	K_M (mM)	k_{cat} (s-1)	k_{cat}/K_M ($mM^{-1}s^{-1}$)	Relative Activity	Reference
L. brevis LB19 *Lb*ADH	2-butanone	0.096	0.107	1.12	-	This Study
Clostridium beijerinckii NRRL B593 ADH	acetone 2-butanone	0.98 1.5	139 64.2	142 43.3	- -	[26]
Rhodococcus sp. GK1 SADH	acetone 2-octanone	65 2.1	- -	- -	- -	[21]
C. acetobutylicum ATCC 824 BDH I BDH II	Butyraldehyde butyraldehyde	3.6 14	- -	- -	- -	[25]
Burkholderia sp. AIU652 ADH	acetone 2-butanone 2-pentanone	0.065 0.040 -	- - -	- - -	100% 83% 44%	[19]
Pseudomonas sp. PED ADH	2-butanone 2-pentanone	- -	- -	- -	100% 6%	[20]

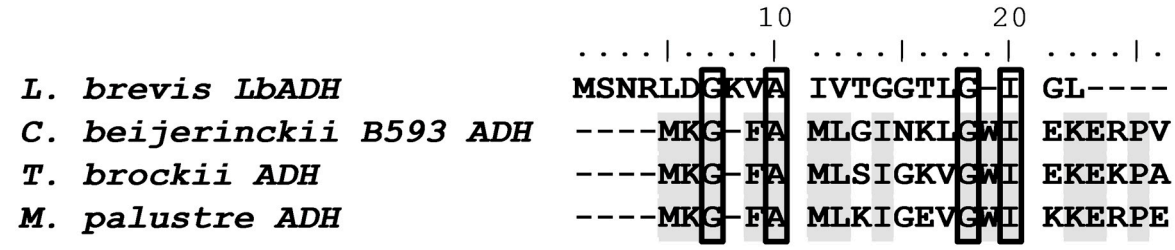

Figure 6. N-terminus alignment of LbADH with ADHs from Clostridium beijerinckii B593, Thermoanaerobacter brockii, and Methanobacterium palustre. Amino acids conserved in all four enzymes are shown enclosed in boxes whereas gray shading shows those residues conserved in only the latter three enzymes.

Figure 7. N-terminus alignment of LbADH with SDR from C. beijerinckii NCIMB 8052. Conserved amino acids are shown in enclosed in boxes.

4. Conclusions

Despite the traditional focus on *Lb*ADH as a biocatalyst for performing asymmetric reductions of 2-alkanone aromatic substrates, increasing evidence continues to point to its further ability to also reduce short-chain aliphatic substrates. Moreover, with its demonstrated activity on both 2-alkanone and aldehyde substrates, *Lb*ADH possesses significant potential as a versatile enzyme for engineering next generation biofuel production pathways.

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Author Contributions

David R. Nielsen planned the experiments. Ibrahim Halloum, Brian Thompson and Shawn Pugh performed the experiments. Ibrahim Halloum and David R. Nielsen prepared the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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