Directed Evolution Toward Improved Production of L-Ribose from Ribitol

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Abstract: Improvement of the one-step production of L-ribose from ribitol using a recombinant *Escherichia coli* is described. The gene encoding the enzyme mannitol-1-dehydrogenase (MDH) from *Apium graveolens* has previously been codon-optimized, cloned into the constitutive pZuc10 vector, and expressed in *E. coli*. This MDH catalyzes the NAD-dependent conversion of mannitol to D-mannose and has the ability to convert several polyols to their L-sugar counterparts, including ribitol to L-ribose. Here, three rounds of directed evolution using libraries generated through error-prone PCR and screened using a dinitrosalicylate reagent. Mutants were selected for improved conversion of L-ribose, and the best mutant was isolated by combining two round 2 mutations. Libraries were also selected for thermal stability and screened at increasingly higher temperatures with each round of mutagenesis. An overall 19.2-fold improvement was observed with a final conversion of 46.6 ± 1.7% and a productivity of 3.88 ± 0.14 $gL^{-1}d^{-1}$ in 50 mL shaken flasks at 34°C. Further characterization of the mutants suggests that increased enzyme thermal stability and expression are responsible for the increase in L-ribose production. The mutant *E. coli* production strain isolated represents an improved system for large-scale production of L-ribose.

Keywords: L-ribose, ribitol, directed evolution, mannitol-1-dehydrogenase MDH.

INTRODUCTION

Chiral chemicals are of great importance in the areas of drug discovery and natural product synthesis [1]. Of these chemicals, enantiomerically pure carbohydrates are essential building blocks for the synthesis of many chemical products important to pharmaceuticals [2-4]. However, traditional chemical synthesis of stereospecific carbohydrates is limited by multistep synthesis, low enantiomeric purity, and low yields [5, 6]. Enzyme catalyzed reactions via whole-cell bioconversion differ in the fact that they are simple, inexpensive, and time efficient [7], while maintaining high purity products [8]. Since carbohydrates play such a large role in cellular recognition, signaling, extra and intracellular targeting, and even the development of disease states, it is not surprising that an increasing number of drugs utilizing sugars of the L-conformation are currently on the market or undergoing clinical trails [2, 4, 9, 10]. Furthermore, interest in L-sugars in the biochemical and pharmacological research community is growing steadily [11]. While many methods of production of L-ribose have been described [12-15], high costs ($500/kg est.) remain inhibitory for intermediate stage development [6]. It is evident that a scaleable and economical process for the production of L-sugar intermediates is of critical importance to both industry and research.

Previous work demonstrated successful cloning, expression, and characterization of the NAD-dependent mannitol-1-dehydrogenase (MDH) from *Apium graveolens* (garden celery). The MDH is of particular interest because of both its regioselectivity at the 1-carbon position as well as its stringent stereoselectivity at the 2-carbon position [16-22]. Thus, the synthesis of various sugars in the L-conformation is permitted including L-ribose from ribitol (Fig. **1**), L-gulose from D-sorbitol, and L-galactose from galactitol [21]. The recombinant *Escherichia coli* adapted to express MDH was employed in a 1 L bioconversion using L-ribose as a model system. Conversion conditions were optimized to produce a 55% conversion at a concentration of 100 g/L in 72 hours, giving a volumetric productivity of 17.4 $gL^{-1}d^{-1}$ [11]. However, this system had some important drawbacks including the need for a temperature shift at induction (cooling is a significant manufacturing cost) and the requirement for a moderately expensive exogenous induction reagent, isopropyl β-D-1-thiogalactopyranoside (IPTG). Furthermore, the temperature of optimum conversion was significantly below the temperature of peak metabolic activity for *E. coli*, suggesting that improvements could be achieved in the time required for conversion.

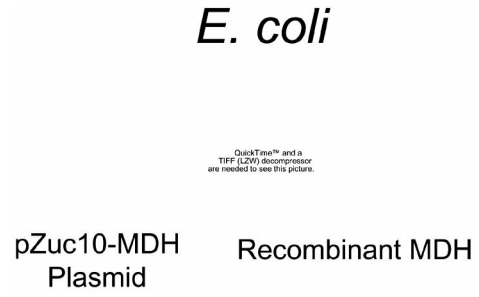

Fig. (1).

Directed evolution has been demonstrated as a successful means to improve enzyme function in the areas of activity and overall performance, enantioselectivity, substrate specificity, and enzyme stability [23]. The use of error-prone PCR as a method of mutagenesis and a unique dinitrosalicylate reagent for identifying reducing sugars allowed the production and screening of thousands of library members in a quick and simple manner. This method was applied to improve the activity of MDH towards an unnatural substrate, ribitol, opposed to the natural substrate, mannitol. Improved enzyme thermal stability allowed the

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conversion to proceed at an increased temperature and the need for an induction reagent and temperature shift were eliminated through use of a constitutive vector.

MATERIALS

Ribitol and L-ribose were purchased from CMS Chemicals Ltd. (Oxsfordshire, UK), while oligonucleotide primers were obtained from Integrated DNA Technologies (Coralville, IA). Kanamycin, ampicillin, NAD, IPTG, glycerol, $ZnCl_2$, 3,5-dinitrosalycylic acid (DNS) and lysozyme were obtained from Sigma-Aldrich (St. Louis, MO); other cell culture components were obtained from Difco (Sparks, MD). *E. coli* EC100 *(F⁻ mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80dlacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ⁻ rpsL nupG)* were obtained from Epicentre (Madison, WI) and *E. coli* BL21 (DE3) was purchased from Novagen (Madison, WI). Restriction enzymes EcoRI, BamHI, HindIII, T4 DNA ligase, pMal-C2x, and Taq Polymerase were purchased from New England Biolabs (Ipswich, MA). Aminex HPX 87P column (300 × 7.8 mm), de-ashing cartridge (30 × 4.6 mm), and Carbo-P micro-guard cartridge (30 × 4.6 mm) and SDS-PAGE gel and marker were obtained from Bio-Rad Laboratories (Hercules, CA). Commercial plasmid purification, PCR product purification and gel extraction kits were purchased from Qiagen (Valencia, CA).

METHODS

Library Preparation. An error-prone PCR was carried out as previously described [24] on the MDH gene template from the previously constructed pZuc10 (pTRP-MDH [11]) vector (containing tryptophan promoter, pMB1, ROP, and MDH gene). This was performed using the forward primer 5'-CGA ACT AGT TAA CTT TTA CGC AAG TTC-3' and reverse primer 5'-CCA TGG GTA AGT ATT TCC TTA AGG ATC C-3' (underlined bases designate BamHI restriction site) under the following conditions: 2 min 95°C, 23 cycles of 35 sec 95°C, 35 sec 55°C, 1 min 72°C, and finally 5 min 72°C. The resulting PCR product was digested with EcoRI and BamHI and ligated into a similarly prepared pZuc10 vector using T4 DNA ligase at 16°C overnight. *E. coli* EC100 competent cells were transformed with the purified plasmid, plated onto kanamycin (50 μg/mL) containing LB-agar plates and incubated overnight at 37°C. Plasmids were purified from the resulting colonies. *E. coli* Zuc174 (*Δ(araD-araB)567, lacZ4787(Δ)(::rrnB-3), lacIp-4000(lacIQ), l-, rph-1, Δ(rhaD-rhaB)568, hsdR514, Δ*(*ptsH-crr*)) was transformed with the resulting purified plasmids and the transformants were selected on kanamycin containing LB-agar plates and incubated at 37°C.

Library Screening. Ribitol Medium (RM) was prepared with final concentrations of 0.3 mM $ZnCl_2$, 50 g/L ribitol, 50 μg/mL kanamycin, and 0.1% glycerol in LB. RM (200 μL) was added to wells of 96-well plates containing sterile 3 mm glass beads. Colonies of the MDH gene library were picked into the wells (three wells per plate contained Zuc174/pZuc10 positive control) and shaken at 30°C. After 72 hours, a DNS reducing sugar assay was performed [25] on a 20-fold dilution of the culture broth. Cell cultures from wells that appeared darker than the positive control in the DNS assay were streaked onto kanamycin (50 μg/mL) containing LB-agar plates and incubated at 37°C overnight. Two individual colonies from each plate were grown in 5 mL cultures containing LB-kanamycin (50 μg/mL) and were used to inoculate culture tubes containing 3 mL of RM, which were shaken at 30°C for 72 hours. A second DNS reducing sugar assay was performed on a 20-fold dilution of the resulting culture broth to verify the hits. The mutants from tubes that produced the three darkest wells were selected for scale up to a 50 mL conversion in RM and grown at 30°C. Samples for HPLC analysis were prepared at 24-hour time points. Confirmed positives served as a template for subsequent rounds of mutagenesis and screening. Temperature was 30°C in the first round of screening, 34°C in the second round, and 37°C in the final round. Approximately 1,200 colonies were screened in the first round, 3,000 colonies in the second round, and 5,000 colonies in the final round. Verified positive mutations were sequenced (University of Illinois-Urbana-Champaign Biotechnology Center). To ensure improved conversion results were not strain based, plasmids were purified, *E. coli* were retransformed and strains were compared to original hits on a 50 mL flask conversion.

Combining Second Round Mutations. The mutation from mutant 2-H5 was incorporated into the parent template 2-H6 via the PCR based "megaprimer" site-directed mutagenesis method [26]. Briefly, a primer containing the 2-H5 mutation was designed, 5'-CGA AAA CAT CAT CGA AAC CAT CGG CAG C-3' (underlined base designates the mutagenic base), and a PCR was carried out using this and the pZuc10 reverse primer on the 2-H6-pZuc10 plasmid template. The resulting PCR product was used as a primer in a second PCR reaction with the pZuc10 forward primer and 2-H6-pZuc10 plasmid template. The resulting PCR product was subcloned into pZuc10. The combined mutation was successful as confirmed by sequencing (n=2).

Cloning of Maltose Binding Protein (MBP)-MDH Fusion. The MDH gene from successful mutants was amplified by PCR from pZuc10 plasmids using the designed pMal forward primer 5'- GCT ACG GGA TCC ATG GCG AAA AGC AGC G -3' (underlined bases designate BamHI restriction site) and pMal reverse primer 5'– GCC TGC AAG CTT TTA CGC GCC GAG GC –3' (underlined bases designate HindIII restriction site). Note that the wild-type MDH was amplified with the reverse primer previously described [11] since it did not contain a mutation found in the first round of directed evolution in the 3' primer region. Inserts were digested with restriction enzymes BamHI and HindIII and ligated into a similarly prepared pMal-C2x vector using T4 DNA ligase at 16°C overnight. *E. coli* EC100 competent cells were transformed with the resulting ligation and plated on kanamycin containing LB-agar plates. *E. coli* BL21 (DE3) competent cells were transformed with purified plasmids from colonies containing MDH insert (as confirmed by colony PCR).

Purification of MBP-MDH. An overnight culture of BL21 (DE3) pMAL-MBP-MDH (5 mL) was used to inoculate 350 mL Terrific Broth containing 20 mM glucose and 100 μg/mL ampicillin. After 2.5 h at 37°C, the temperature was reduced to 25°C and 0.5 mM IPTG was added. Cells were harvested 16 h post-induction by

centrifugation and resuspended in 50 mM HEPES (pH 7.2), 300 mM NaCl, 1 mg/mL lysozyme, and 10% glycerol (3 mL/g cells) and stored at -80°C overnight. MBP-MDH enzymes were purified as previously described [11] except a 2 mL column of amylose resin was used, and enzymes were concentrated by centrifugation using a Millipore Amicon Ultra-15 with an Ultracel membrane.

Characterization of MDH. The initial rates of reaction were determined by monitoring the increase in absorbance of NADH (ε = 6.22 mM $^{-1}$ cm $^{-1}$) at 340 nm for 0.6 min at 25°C using a Varian Cary 1E spectrophotometer. Reactions were initiated by addition of ~25 µg MBP-MDH to 600 µL of 100 mM tris buffer pH 8.5 containing substrates D-mannitol or ribitol and NAD. One unit (U) of activity was defined as 1 µmol NADH produced/min and specific activity was defined as 1 U/mg of total protein added. All assays were performed in duplicate per experiment and the average values from 2 or more separate experiments were utilized to calculate kinetic constants k_{cat} and K_M using least squares regression analysis for 5 or more concentrations of each substrate. Half-life values were found by adding enzyme that had been incubating at 43°C to 2.5 mM NAD and 250 mM D-mannitol at three-minute intervals. The values for $T_{1/2}$ were calculated using the first order decay $T_{1/2}$= -ln(2)/k, where k is the rate of decay as calculated by Microsoft Excel. Values were collected in at least two or more separate experiments with the average and standard deviation reported. Values are expressed as the average of all data collected (unless statistically irrelevant by Q test) with the associated standard deviation.

Modeling. Swiss-Model Workspace [27] was used to generate a model of the wild type MDH and the mutant MDH incorporating the sites of beneficial mutations. Rasmol [28] was used to view and edit the models.

MDH Expression and SDS-PAGE. Culture tubes containing 5 mL LB-kanamycin (50 µg/mL) were inoculated with pZuc10-MDH mutants and grown to log-phase at 37°C. Enzymes were expressed at room temperature for 4 hours and 1 mL of culture medium was pelleted and lysed in 1 mL 100 mM tris buffer containing 1 mg/mL lysozyme. Resuspended cells were stored at -20°C overnight, thawed at room temperature, and pelleted to clear cell debris. Protein expression was visualized by SDS-PAGE by the method of Laemmli [29] using lysate samples normalized to 10 µg of protein and Coomassie staining.

Analysis. The HPLC separation system consisted of a solvent delivery system (P2000 pump, Spectra-Physics, San Jose, CA) equipped with an autosampler (717, Waters Chromatography Division, Millipore Corp., Milford, MA), a refractive index detector (410 differential refractometer, Waters), and a computer software based integration system (Chromquest 4.0, Spectra-Physics). An ion moderated partition chromatography column (Aminex HPX-87P with De-ashing and Carbo-P micro-guard cartridges) was utilized at 85°C with a mobile phase of Milli-Q filtered water at a flow rate of 0.6 mL/min. Peaks were detected by refractive index and were identified and quantified by comparison to retention times and areas of authentic standards (ribose and ribitol).

RESULTS AND DISCUSSION

Directed Evolution. After the screening of about 1,200 library members at 30°C, one mutant was successfully isolated, 1-C1, that had a darker DNS results suggesting more reducing sugar (L-ribose) was being produced. The first round mutant served as a template for a second round library and approximately 3,000 members were screened at 34°C using the DNS reducing sugar assay. Two mutants, 2-H6 and 2-H5, were isolated. After the second round, two approaches were taken, since there were two different mutants of similar ability. In the first approach, the mutants were utilized as templates for two separate mutagenesis and screening experiments. After screening 5,000 library members from each template at 37°C, one improved mutant was isolated from the 2-H5 template, 3-G7. In the second approach, the two mutants from round 2 were combined as described in the Materials and Methods section. After combining the second round mutations 2-H6 and 2-H5, a final library was constructed from this template; however, after screening at 37°C, no improved mutants were identified.

Shaken Flasks. Shaken flask experiments were carried out as previously described using medium with glycerol and zinc for improved conversion [11] to quantitate the improvements in production of L-ribose from ribitol. The various mutants created by directed evolution were tested in 50 mL flask conversions at 34°C and compared to the wild-type MDH with samples analyzed by HPLC. As displayed in Table **1** and Fig. (**2**), a 19.2-fold overall improvement was demonstrated at 34°C, which achieved a 46.6 ± 1.7% conversion in the combined round 2 mutant. Additionally, improvements in space-time yield increased from 0.203 ± 0.004 to 3.88 ± 0.14 $gL^{-1}d^{-1}$. While the combined mutant performed the best, the third round mutant 3-G7, performed nearly as well. This evidence, along with the difficulty in finding hits in later round libraries suggests that the MDH is no longer the rate-limiting step towards L-ribose production and that further rounds of mutagenesis would not improve overall conversion.

Table 1. Conversion and Productivity of Mutants

Mutant	Conversion	STY ($gL^{-1}d^{-1}$)	Ratio to WT
WT	2.43 ± 0.05%	0.203 ± 0.004	1.0
1-C1	28.5 ± 0.3%	2.37 ± 0.03	11.7
2-H6	38.1 ± 0.2%	3.1 ± 0.2	15.7
2-H5	40.6 ± 1.1%	3.6 ± 0.2	17.8
3-G7	45.4 ± 1.1%	3.78 ± 0.09	18.7
Comb. Rnd 2	46.6 ± 1.7%	3.88 ± 0.14	19.2

Sequence Characterization. Upon sequencing, two amino acid substitutions were observed in the round 1 mutant (1-C1), F14I and S47C. Based on homology modeling using the previously solved crystal structure of sinapyl alcohol dehydrogenase [30] as a template (77% sequence identity [31]), mutation S47C is found in the active site of the enzyme. In sinapyl alcohol dehydrogenase, the

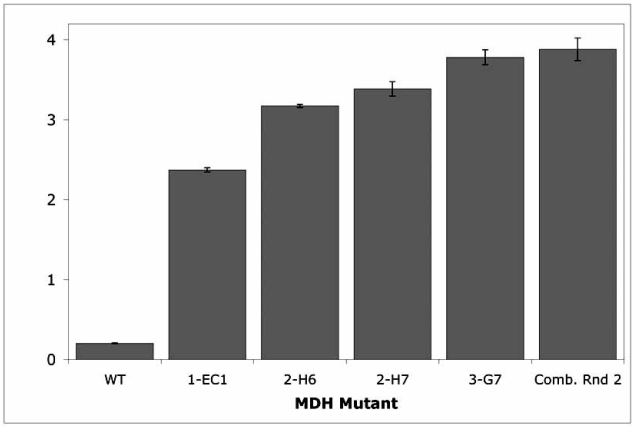

Fig. (2).

corresponding amino acid residue is responsible for coordinating the catalytic Zn^{2+} [30]. While the oxygen of serine has been reported as a zinc ligand [32], Cys-47 is more commonly found in alcohol dehydrogenases as a NADH binding ligand [33]. Mutant 2-H6 contained the amino acid substitution H54Y, and mutant 2-H5 contained the amino acid substitution D122E. The exchange of phenylalanine (aromatic) to isoleucine (hydrophobic) in the 1-C1 mutation and the exchange of histidine (positive charge) to tyrosine (aromatic) are significant substitutions. Models of the wild type MDH (**A**) with mutation sites in black and the mutant MDH with the substituted amino acids from the combined round 2 mutant in black (**B**) are shown in Fig. (**3**). The third round mutant, 3-G7, contained the amino acid substitutions E8V and I149V. It is curious that both mutations resulted in the substitution of a valine group. The exchange from glutamic acid (negative charge) to valine residue (hydrophobic) is a considerable change, however, no obvious loss or gain of function is expected from these mutations.

Purified Protein Data. The Michaelis constant (K_M) and the turnover number (k_{cat}) for each purified enzyme were determined for either ribitol or mannitol and NAD. Catalytic efficiency for mannitol was reduced in the mutants as a function of both lower k_{cat} and higher K_M values, which is summarized in Table **2**. The mannitol k_{cat} decreased 8-fold from 4.85 ± 0.24 s^{-1} in the WT to 0.59 ± 0.08 s^{-1} in the combined mutant. The decrease in activity was paralleled for the K_M values where a near 7-fold increase was observed from 46 ± 1 mM in the WT to 315 ± 7 mM in the combined mutant with mannitol as the substrate. There was no clear trend for the K_M values of the NAD substrate with mannitol. The values ranged from 61 ± 18 μM for the round 1 mutant to 193 ± 154 μM for round 2-H6. Overall, the catalytic efficiency decreased 55-fold from 0.11 to 0.002. The kinetic data suggests that the directed evolution rounds swayed the MDH away from mannitol as a substrate.

Interestingly, kinetic activity remained fairly constant with ribitol as the substrate between the WT and the combined round 2 mutant MDH. Regarding the k_{cat} values, a decrease in activity was observed in round 1 and round 2 mutants, but was maintained slightly above the level of the WT in the combined mutant. Conversely, the K_M values saw a modest decrease from the WT to the round 1 and round 2 MDH. However, the combined mutant resulted in a higher K_M of 173 ± 39 mM compared to the WT value of 112 ± 52 mM. The k_{cat} /K_M ratio showed no clear trend and varied only slightly from the highest value of 0.012 in the round 1 mutant to the lowest value of 0.006 in the combined mutant. Due to the lack of drastic changes with the substrate ribitol, kinetics values of the MDH are not likely a significant factor in the increase of the L-ribose production rate. The $K_{M,\ NAD}$ values with ribitol as a substrate decreased with each round, resulting in a near 4-fold decrease from the WT to the combined mutant. However, k_{cat} was not significantly affected in the combined mutant, although some intermediate mutants had reduced k_{cat} values.

Overall, the MDH with the best conversion, the combined round 2 mutant, had decreased activity on mannitol and similar activity on ribitol as compared to the WT. The ratio of catalytic efficiencies of mannitol to ribitol decreased from 13.8 in the WT to 0.3 in the combined mutant; a 44-fold change.

Thermal Stability. Since *Apium graveolens*, the parent organism from which the MDH gene originated, has an optimal vegetation temperature between 16°C and 21°C [34]

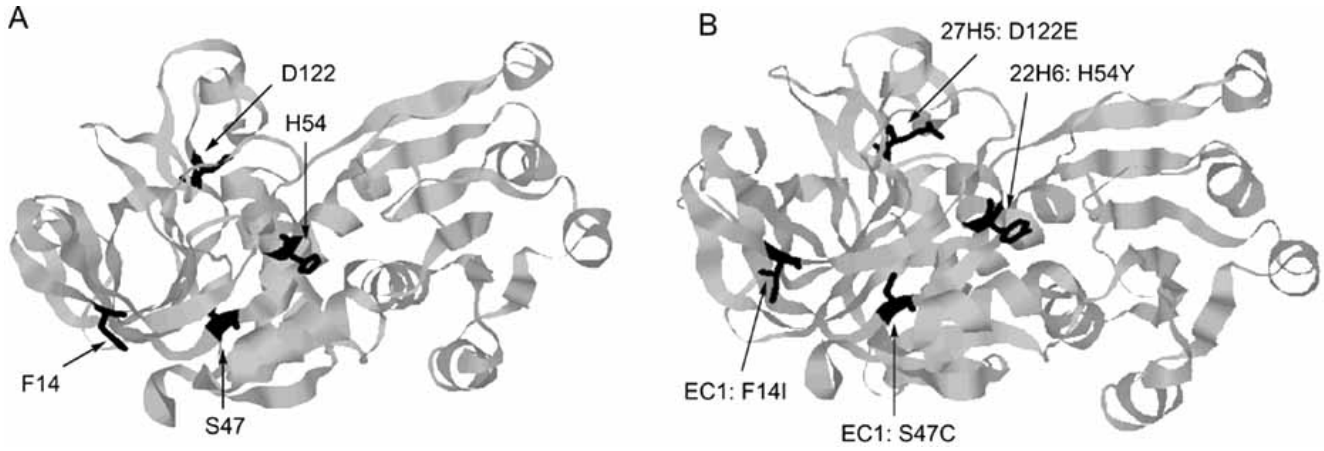

Fig. (3).

Table 2. Comparison of MDH Kinetic Values for Mannitol and Ribitol

Enzyme	Mannitol				Ribitol				Ratio k_{cat}/K_M
	k_{cat} (s^{-1})	k_M (mM)	k_{cat}/K_M	$K_{M, NAD}$ (μM)	k_{cat} (s^{-1})	k_M (mM)	k_{cat}/K_M	NAD $K_{M, NAD}$ (μM)	Mannitol:Ribitol
WT	4.85 ± 0.24	46 ± 1	0.11	88 ± 20	0.94 ± 0.09	112 ± 52	0.008	167 ± 80	13.8
1-C1	1.80 ± 0.15	24 ± 4	0.075	61 ± 18	0.34 ± 0.01	29 ± 10	0.012	69 ± 47	6.3
2-H6	0.97 ± 0.41	135 ± 4	0.007	193 ± 154	0.66 ± 0.06	88 ± 3	0.008	82 ± 72	0.9
2-H5	1.30 ± 0.17	110 ± 9	0.012	80 ± 35	0.27 ± 0.03	39 ± 5	0.007	55 ± 37	1.7
Comb. Rnd 2	0.59 ± 0.08	315 ± 7	0.002	104 ± 86	1.00 ± 0.09	173 ± 39	0.006	43 ± 34	0.3

it was not expected that proteins from the organism would achieve optimal activity at higher temperatures suitable for *E. coli* optimal growth (37°C). It was hypothesized that the later round MDH mutants would be selected for increased thermal stability as these rounds were screened at increasingly higher temperatures. To demonstrate improvements in the thermal stability of the enzymes, each MDH was incubated at 43°C and added to a reaction mixture of mannitol and NAD at incremental time points. Half-life values were found for each enzyme and are summarized in Table **3**. An overall 12-fold improvement from the WT MDH to the combined mutant was observed from half-lives of 3.5 ± 0.7 min to 42 ± 18 min. Although 37°C was the final screening temperature, L-ribose production is considerably better at 34°C for all the MDH strains, including those selected at 37°C. Also, the combined round 2 mutant, which consisted of mutations selected at 34°C, was the most thermal stable enzyme tested. The positive correlation between improved enzyme thermal stability and L-ribose production is a reasonable explanation for the improvements observed in the shaken flask conversions. The increased thermal stability observed may also be a desirable characteristic for future scale-up processing. Since cooling expenses are significant for industrial fermentations [35], strains that permit higher temperatures may be more economically feasible.

Table 3. Comparision of MDH Thermal Stability

Enzyme	$T_{1/2}$ @ 43°C (min)	Ratio $T_{1/2}$
WT	3.5 ± 0.7	1.0
1-C1	5.8 ± 1.6	1.7
2-H6	16.0 ± 2.1	4.6
2-H5	14.7 ± 1.8	4.2
Comb. Rnd 2	42 ± 18	12.0

SDS-PAGE Comparison of Expression. The expression of MDH mutants and the WT subcloned in the constitutive pZuc10 vector was analyzed by SDS-PAGE, which can be found in Fig. (**4**). The MDH expressed well with the expected monomer size of ~39 KDa (mutations did not change MW significantly). Compared to the WT (Lane 1), expression improved dramatically in the round 1 MDH (Lane 2) and remained consistent with round 2, 2-H6 and 2-H5 (Lanes 3 and 4, respectively), and combined mutation (Lane 5). It is expected that the vast increase in MDH as a percentage of total soluble protein in the cell contributes to the increased conversion rates seen in the shaken flask experiments.

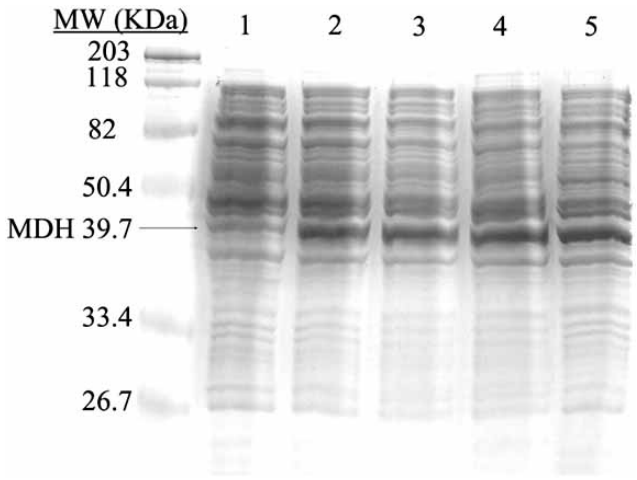

Fig. (4).

Although many chemical syntheses for L-ribose and other L-sugars have been demonstrated, such methods are not practical for industrial production due to use of expensive reagents and organic solvents, multi-step synthesis, and low yields [36-39]. A biochemical method has also been described that oxidizes ribitol to L-ribulose with *Acetobacter aceti* IFO 3281 washed cells and isomerizes the L-ribulose to L-ribose using L-ribose isomerase from *Acinetobacter* sp. strain DL-28 [40]. While overall yield is around 70% with a productivity of 11.7 $gL^{-1}d^{-1}$, the process requires two separate biochemical steps that may prove inefficient in an industrial setting [40].

Further applications of this work include greatly reducing the cost of many antiviral pharmaceuticals used for treating hepatitis B such as Tyzeka®, Clevudine®, Valtorcitabine® and Maribavir® [41, 42]. The number of people infected with hepatitis B worldwide is 350 million [43], with highly endemic areas including sub-Saharan Africa, the Pacific, and Asia [44]. The development of the recombinant *E. coli* L-ribose production strain could greatly reduce the cost of L-nucleoside drugs. Decreased cost may also help to make L-ribose available and affordable to the pharmaceutical and biochemical research community, thus further spurring the invention of novel and effective treatments.

It is expected that scale-up will prove to be simple and efficient. Previously, the WT MDH as a MBP fusion protein in a similar *E. coli* host achieved 55% conversion in 72 hours in a 1 L bioconversion [11]. Considering the vast improvements made via improvements made by directed evolution higher conversion rates and quicker bioconversion times are anticipated. Additionally, the bioconversion can be carried out at a higher temperature and does not require an induction step or expensive induction reagent, further reducing costs.

In summary, three rounds of directed evolution and a combining mutations step were performed on the mannitol-1-dehydrogenase gene from *Apium graveolens* subcloned into *E. coli*. The best mutant, which was a combination of two round 2 mutations, produced L-ribose at a 46.6 ± 1.7% conversion and 3.88 ± 0.14 $gL^{-1}d^{-1}$ productivity in shaken flasks; a 19.2-fold improvement compared to the WT. While enzyme activity for mannitol decreased substantially, activity for ribitol did not change significantly. It is expected that the large increase in thermal stability and expression are responsible for the improved conversion of L-ribose from ribitol.

ABBREVIATIONS

MDH = Mannitol-1-Dehydrogenase

DNS = 3,5-dinitrosalycylic acid

IPTG = Isopropyl β-D-1-thiogalactopyranoside

RM = Ribitol medium

MBP = Maltose Binding Protein

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