Characterization of Previously Mutated *Proteus mirabilis* Lipase in Methyl Acetate

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Abstract

Lipase catalyzed biodiesel production presents a potential methodology that exceeds the traditionally utilized acid/base protocols. Advantages include economic favorability and convenience due to more efficient catalysis, which leads to less time, starting material, and energy required for production. A lipase has been previously genetically engineered for methanol tolerance and was shown to be an effective catalysis for transesterification reactions utilizing methanol to produce fatty acid methyl esters, or biodiesel, and glycerol. An alternate acyl acceptor to methanol, methyl acetate, produces a fuel stabilizer, triacetin, as opposed to glycerol when used in the transesterification. This methodology has not been previously utilized with the characterization of these genetically engineered lipases, which are also described as dieselzymes. Dieselzyme mutations have been expressed in *E.coli* using a pET28 bacterial expression vector and purified utilizing immobilized metal ion affinity chromatography. Kinetic optimization was performed for three mutants using p-nitrophenyl palmitate as substrate at varying pHs and in methyl acetate versus methanol reactions. Further investigation of the dieselzymes includes use of synthetic triglycerides as substrate to continue kinetic optimization.

Introduction

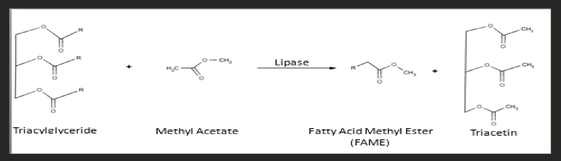


Figure 1: Lipase catalyzed triacylglycerol transesterification using methyl acetate

In traditional transesterification enzyme catalysis of triacylglycerol, methanol is utilized as the alkyl donor. In the transesterification process using a lipase, methyl acetate replaces methanol as the alkyl donor. This is due to methanol causing interruptions of lipase activity through the inference of key hydrogen bonds[4] . Due to many lipase's insolubility in the cytoplasm of the cell, many are expressed poorly in bacterial system. This results in the need for many lipases to be co-transformed with other chaperone proteins. *Proteus mirabilis* lipase (figure 2) was found to be soluble in *E. coli* expressions systems. It has been previously optimized in methanol conditions via directed evolution[2]. Other methods of transesterification utilizing methyl acetate as opposed to methanol have been developed[6]. These methods produce triacetin[3] instead of glycerol, making the method useful as a fuel additive.

Dieselzymes

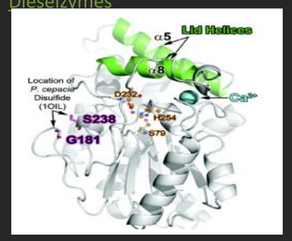


Figure 2[1]: Showing structure of wild-type *Proteus mirabilis* lipase (PML).
Lid helices shown in green are thought to open when exposed to non-polar interface
Catalytic triad (Asp-His-Ser) is shown in orange.
Homologous disulfide bridge is shown in purple to illustrate deviations in PML structure from other lipases in the same family (I.2 psychrophilic lipases).

Dieselzyme 1
• Mutated residues shown in purple to introduce disulfide bridge to increase stability of catalytic triad.

Dieselzyme 3
• Mutation of Dieselzyme 1
• Higher thermodynamic stability than Dieselzyme 1
• Disulfide bond mutant, Dieselzyme 1

Dieselzyme 4
• Disulfide bridge in dieselzyme 1
• Introduction of additional hydrogen bonds to prevent tertiary structure collapse in polar solvents
• Introduction of potential salt bridges near key helices for further tertiary structure stabilization.

Methods

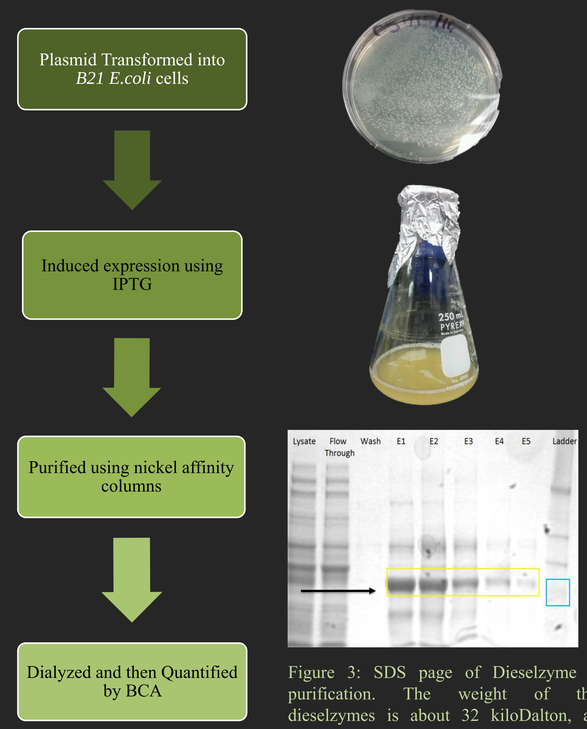
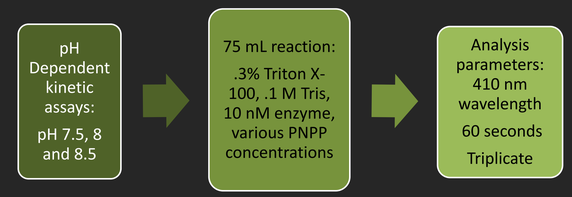


Figure 3: SDS page of Dieselzyme 3 purification. The weight of the dieselzymes is about 32 kiloDalton, as shown by the black arrow above.

Kinetic Analysis



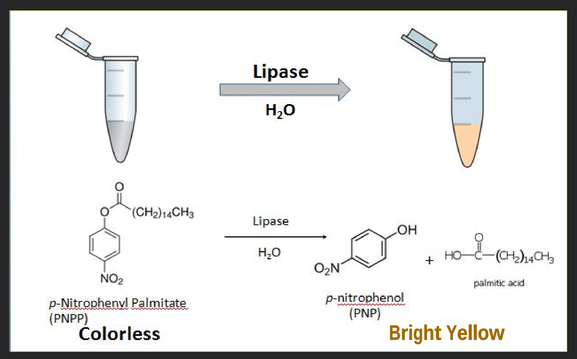


Figure 4: Mechanism of Colorimetric Analysis

Michaelis-Menton Kinetics

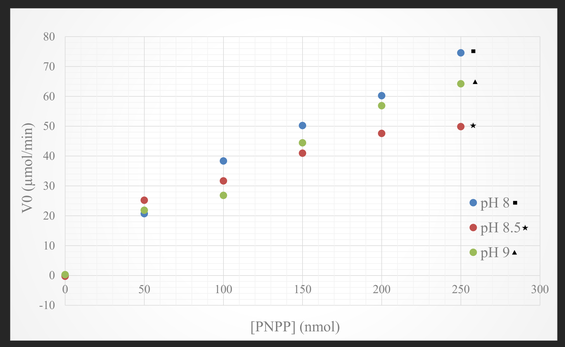


Figure 5: A plot of initial velocity of the enzyme against the concentration of the PNPP substrate was made to confirm Dieselzyme 3 fit the Michaelis-Menton model of kinetics when subjected to the 3 pH values, 8, 8.5 and 9. Using this data, Lineweaver-Burk plots were subsequently generated to determine the maximum velocity and turnover number and the amount of substrate needed for the enzyme to reach half its maximum velocity.

Results

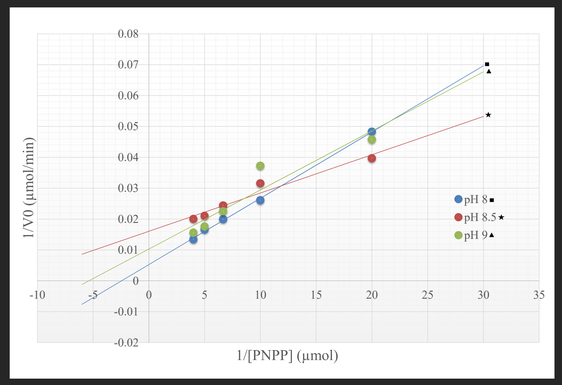


Figure 6: Lineweaver-Burk Plot of the inverse of initial velocity against inverse of PNPP concentration. Utilized to determine maximum velocity, turnover number and the amount of substrate needed for the enzyme to reach half its maximum velocity from the slope and y-intercept of the lines of best fit for pH 8, pH 8.5 and pH 9 analyses.

Table 1: Table 1 displays the kinetic parameters of Dieselzyme 3 conducted at pH 8, 8.5 and 9. Turnover numbers were calculated assuming one active site per molecule. Vmax and Km values were determined using Lineweaver-Burke plots after statistical analysis was done in triplicate.

pH	Slope (min^{-1})	Intercept (min/µmol)	Vmax (µmol/min)	Km (nmol)	Turnover Number (min^{-1})
8	0.00215	0.00527	189	407	$1.90*10^4$
8.5	0.00124	0.016	62.3	77.3	$6.23*10^3$
9	0.00191	0.0103	97.2	186	$9.71*10^3$

pH Dependence of Dieselzyme 3

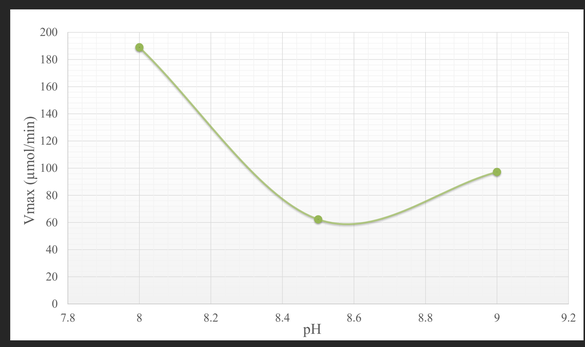


Figure 6: pH dependence of enzyme activity in Tris buffer. Vmax was determined at room temperature at 6 concentrations (0-250 nmol) of PNPP in reactions with 10nM enzyme, 0.3% Triton X-100 and 0.1M Sodium Phosphate at pH 8.0, 8.5 and 9.0 in triplicate.

Conclusions

- Observed higher activity at pH 8, optimal out of the three pH values tested.
- pH 9 displayed higher activity than 8.5
- Observed Dieselzyme 1 and 4 not perform well compared to 3
- Enzyme showed moderate activity at room temperature

Future Analysis

- Characterize Dieselzyme 3 in methanol versus methyl acetate
- Optimize mutations and reaction conditions for methyl acetate system
- Characterize transesterification of synthetic triacylglycerides utilizing gas chromatography for dieselzymes 1,3,and 4.
- Optimize purification of dieselzyme 1 and 4
- Improve purification and quatification methodology

Acknowledgments

Special thanks goes out to Tyler Korman and James Bowie (UCLA) for allowing us to make use of the Dieselzymes. Thank you Demetrius Carey for the allowance to utilize data previously collected to allow project continuation. Support for the research was provided by the University of Tampa Department of Biochemistry, Chemistry and Physics and through The University of Tampa David Delo Research Professor Grant (DSW) and The University of Tampa Dana Foundation Grant.

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