Increased Thermal Tolerance of *T. fusca* β-Glucosidase *via* Directed Evolution

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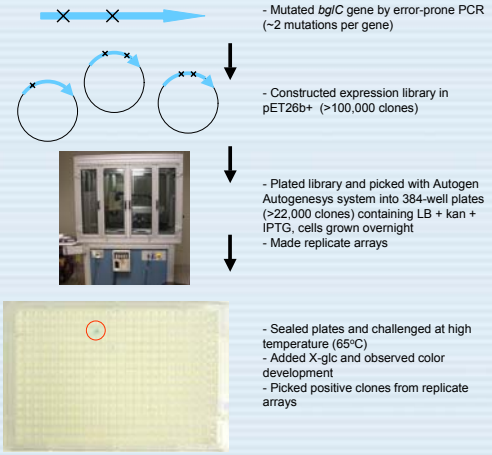
Summary

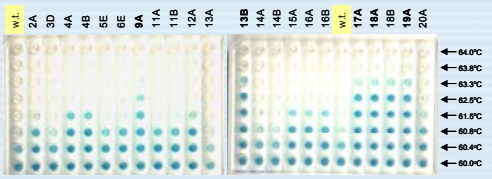
The use of heat-tolerant enzymes can improve turnover rates and tolerance to the stresses of large-scale processes. Directed evolution with high-throughput screening was used to increase the thermal tolerance of a Family 1 β-D-glucosidase encoded by the *bglC* gene of the cellulolytic actinomycete *Thermobifida fusca*. The *bglC* coding sequence (provided by David Wilson, Cornell University) was mutagenized by error-prone PCR. More than 22,000 clones were picked using an Autogen Autogenesys colony picker and screened *via* direct temperature challenge in 384-well plates. The resulting candidates were further screened using a novel temperature gradient plate assay. At least nine candidates showed enhanced thermal tolerance and were further characterized. Pair-wise combinations were then made of some of the most promising mutations. Recombinant mutant and wild type BglC proteins were purified from *E. coli*, and differential scanning microcalorimetry (DSC) and temperature challenge experiments were performed. One of the combinations generated a BglC protein with an increased thermal stability of at least 5°C over wild type. This protein was demonstrated to have a nine-fold increased half-life at temperatures of 62 and 64°C. Through successive rounds of mutation and screening, and additional combinations of mutations, it should be possible to further increase the thermal tolerance of this β-glucosidase.

High-Throughput Screen



- Mutated *bglC* gene by error-prone PCR (~2 mutations per gene)
- Constructed expression library in pET26b+ (>100,000 clones)
- Plated library and picked with Autogen Autogenesys system into 384-well plates (>22,000 clones) containing LB + kan + IPTG, cells grown overnight
- Made replicate arrays
- Sealed plates and challenged at high temperature (65ºC)
- Added X-glc and observed color development
- Picked positive clones from replicate arrays

Secondary Screen

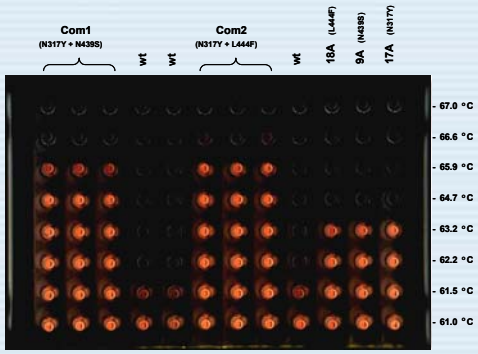


A secondary screen of thermal tolerance was performed on cell cultures using a gradient thermocycler. Saturated, IPTG induced *E. coli* cultures were dispensed into each column of a 96-well plate and challenged for 10 minutes over the temperature range indicated at the right of the figure. Remaining activity was assayed using X-glc as a substrate. Clones showing thermal tolerance significantly above that of wild type in this assay (e.g., 9A, 13B, 17A, 18A, and 19A) were examined further.

Results of Screening

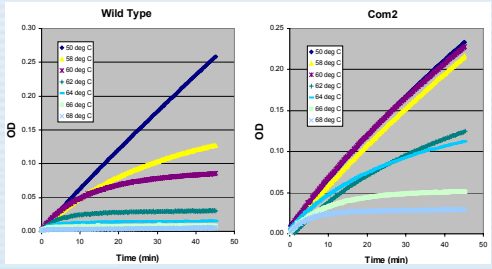
Mutant	Total # Changes	# Silent Changes	CodonChange	Amino Acid Change	Protein Position	Shorthand
9A	3	2	AAC->AGC	Asn->Ser	439	**N439S**
13B	1	0	CTC->TTC	Leu->Phe	444	**L444F**
17A	2	1	AAC->TAC	Asn->Tyr	317	**N317Y**
18A	1	0	CTC->TTC	Leu->Phe	444	**L44F (same as 13B)**
19A	2	1	AAC->TAC	Asn->Tyr	317	**N317Y (same as 17A)**
15A	2	1	AGC->TGC	Ser->Cys	319	**S319C**
16A	1	0	AAC->ATC	Asn->Ile	178	**N178I**
20F9	1	0	GCC->GTC	Ala->Val	433	**A433V**
21D	2	0	GGC->CGC TCG->ACG	Gly->Arg Ser->Thr	337 341	**G337R S341T**
Com1	Combination of mutants17A and 9A					**N317Y + N439S**
Com2	Combination of mutants17A and 18A					**N317Y + L444F**
Com3	Combination of mutant 16A and Com2					**N317Y + L444F+ N178I**
Com4	Combination of mutants 17A and 20F9					**N317Y + A433V**

Mutant Combinations



Mutant combinations analyzed using the thermal gradient technique described above. Combined mutations Com1 and Com2 show additive effects of single mutations (at right of figure). wt = wild type. Color is reversed for enhanced visualization.

Kinetic Assays



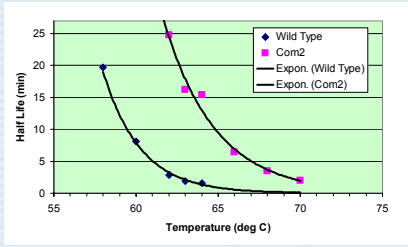
Kinetic assays were performed on purified proteins to assess protein activity over time at various temperatures. Comparison of Com2 to wild type is shown. PNPG was used as a substrate using protein loadings having equal activity at 50ºC. Half-lives of proteins were derived from these curves.

Half-Life Determinations

BglC Mutant	Amino Acid Changes	58°C	60°C	62°C	63°C	64°C	66°C	68°C	70°C
Wild Type	-	19.8	8.1 10.5	2.9 4.4	1.9	1.6 <3			
9A	N439S				6.9				
13B	L444F		28.1	11.7	7.4	6.0			
17A	N317Y		34.1 >45	21.2 25.7		7.7 8.2 9.7	3.7		
15A	S319C		17.1	8.6		3.5			
16A	N178I		20.8	10.2		3.9			
20F9	A433V		15.0 28.2	10.9 11.7		4.4 4.0 4.2	1.6		
21D	G337R S341T		22.4	9.5	2.8	3.7			
Com1	N317Y + N439S	Poor activity							
Com2	N317Y + L444F		>30 >45	24.8 26.0 >45	16.2	15.3 19.7 15.6 25.0	6.4 12.2	3.5 4.5	2.0
Com4	N317Y + A433V		28.3	30.9		14.1	7.7	3.0	

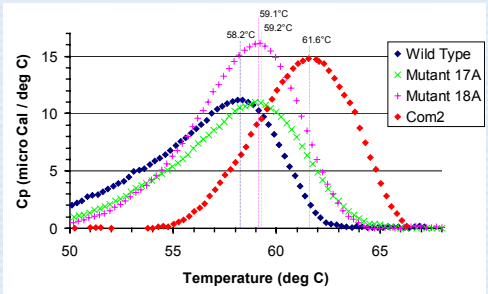
Half-lives given in minutes; colors represent different experiments

Half-Life vs. Temperature



Half-lives of Com2 and wild type proteins, as determined from kinetic assays, plotted as a function of temperature. Horizontal displacement of curves demonstrates a >5ºC improvement in thermal stability in Com2 over wild type. Vertical displacement of curves shows about a nine-fold increase in half-life at temperatures of 62 and 64ºC.

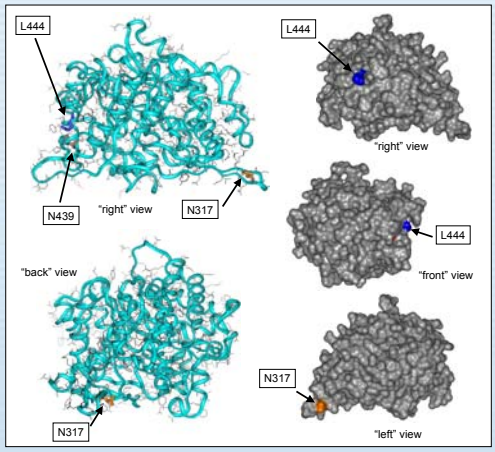
Differential Scanning Calorimetry



DSC assays on recombinant wild type, Com2, and the two single mutants comprising Com2. T_{max} values for each are indicated. A Microcal model VP-DSC was used with 50 ug/mL protein at pH 6.5, scanning at 60ºC/h.

3D Structure Predictions

Based on META PredictProtein Threadings



Conclusions

- Directed evolution and high-throughput screening were used successfully to identify enhanced thermal tolerance mutations in the BglC β-glucosidase of *T. fusca.*
- In a screen of >22,000 clones, more than seven different mutations were identified that affected thermal tolerance.
- Combining mutations gave additive or synergistic effects.
- One mutant combination, Com2, showed an increase in thermal tolerance of more than 5ºC based on activity. The half-life at temperatures of 62 and 64ºC increased about nine-fold.
- Differential Scanning Calorimetry supported an increase in thermal stability of the Com2 protein.
- Emerging structural information on this β-glucosidase will shed light on the mechanism by which these mutations increase thermal stability.

Acknowledgements

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