INVESTIGATION OF NOVEL CUTINASES FOR BIOTRANSFORMATIONS

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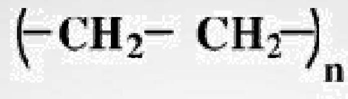
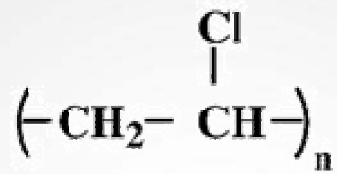
August 19th 2009

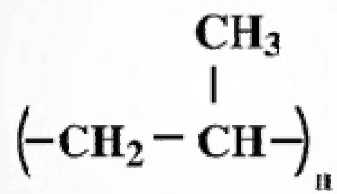
Conventional Petrochemical Thermo Plastics



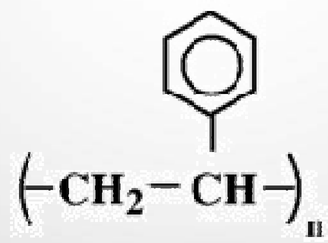
Polyethylene (PE)



Polyvinyl Chloride (PVC)



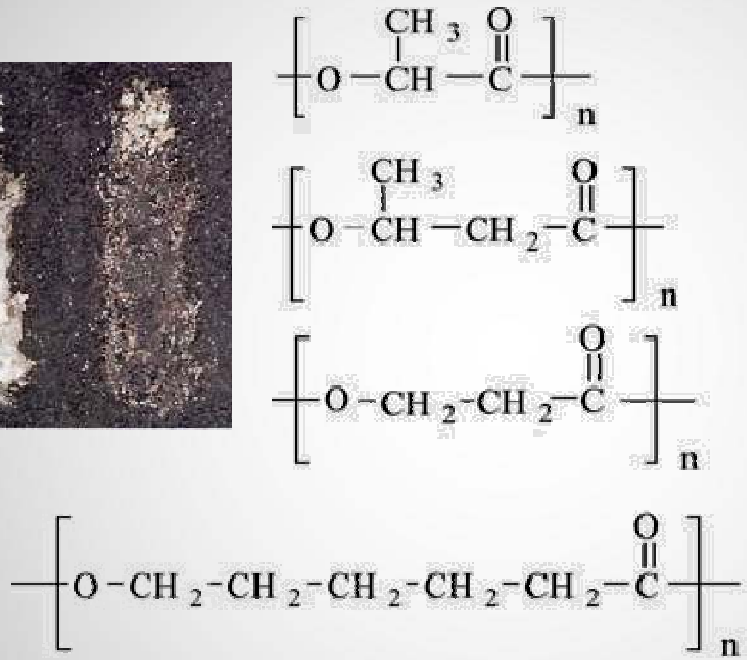
Polypropylene (PP)



Polystyrene (PS)

Pavia DL., *et.al.*, *Introduction to Organic Labrotory Techniques. 3rd Ed.* **1988**
Shah AA., *et. al. Biotechnology Advances*. **2008**, 246-65

Biodegradable Plastics



Poly(lactide) (PLA)

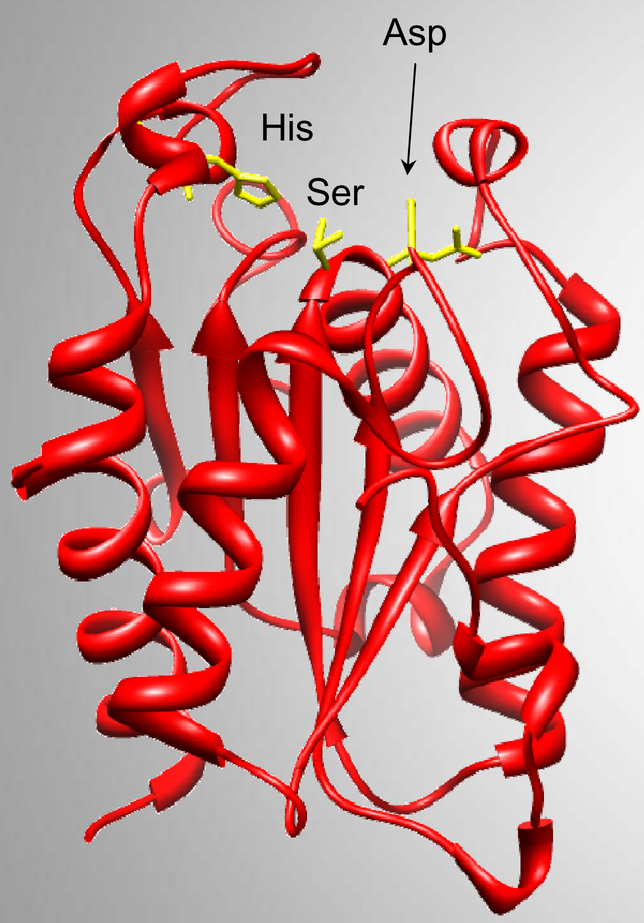
Poly(3-hydroxybutyrate) (PHB)

Poly(propiolactone) (PPL)

Poly(ε-caprolactone) (PCL)

Shah AA., *et. al. Biotechnology Advances*. **2008**, 246-65
Tokiwa Y., *et. al. Biotechnol. Lett*. **2004**, 1181-9
Azios, T., The Christian Science Monitor. **2007**.

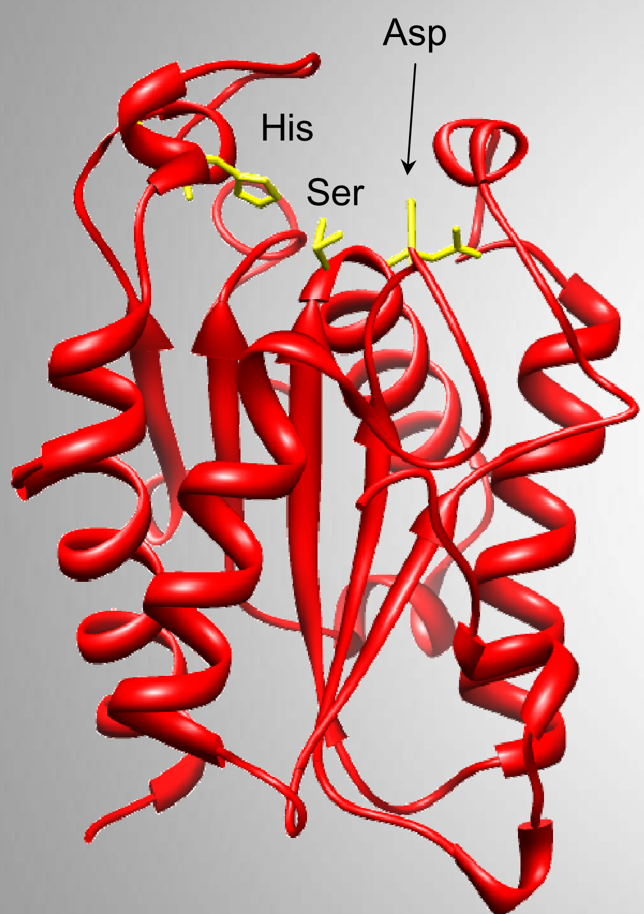
Hydrolytic Enzymes Employed in Organic Chemistry



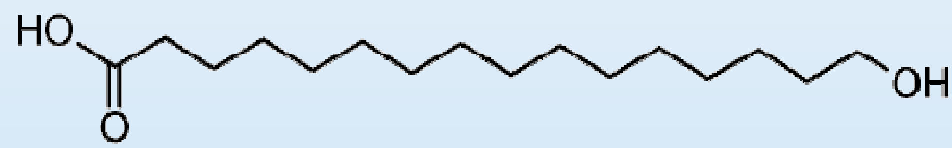
- Proteases, cellulases, lipases, amylases, and **cutinases**
- Cutinases member of serine hydrolase family
- Cutinases have a more exposed active site opening up different potential applications
- Catalytic triad composed of Ser, His and a carboxyl group
- Oxyanion hole Ser, Gln; which stabilize the transition state

Purdy RE., *et.al.*, *Biochemistry*, **1975,** 2832-40
Longhi, S., *et al.*, *J.Mol. Biol.*, **1997**, 779-99
Nicholas, A.,*et.al.*, *Biochemistry*, **1996**, 398-410
Pdb code: 1cex

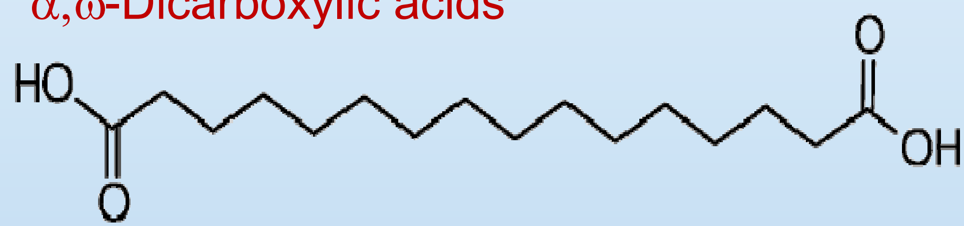
Fusarium solani Cutinase (FsC)



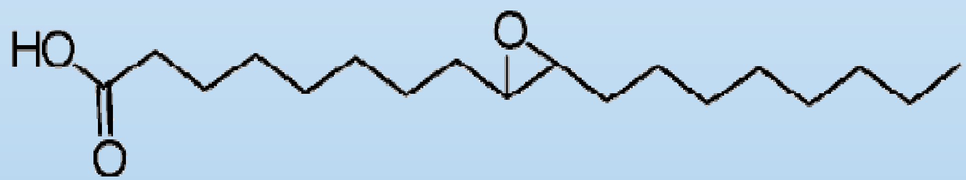
ω-Hydroxy fatty acids



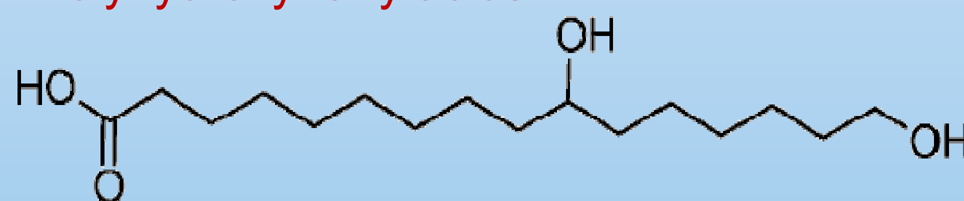
α,ω-Dicarboxylic acids



Epoxy-fatty acids



Polyhydroxy-fatty acids

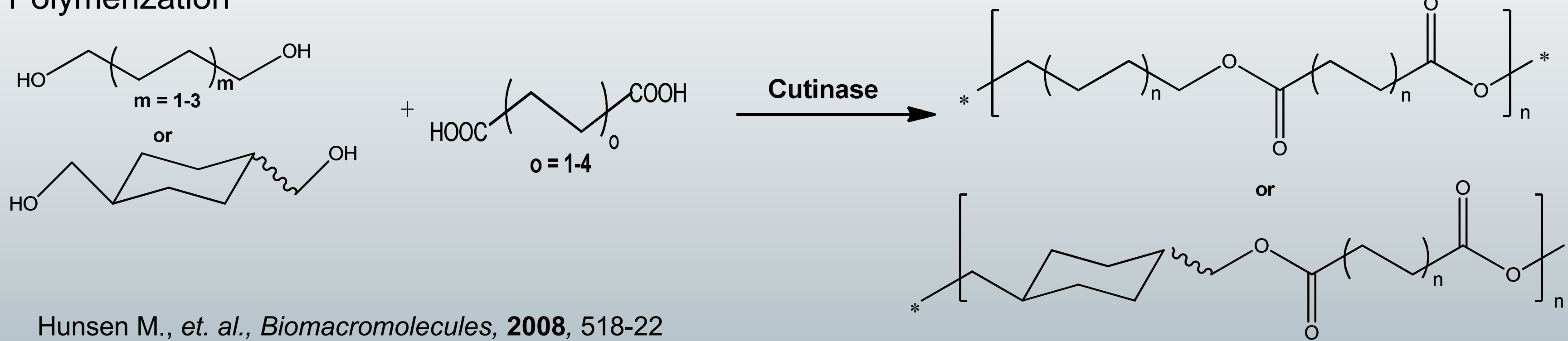


Purdy RE., *et.al.*, *Biochemistry,* **1975,** 2832-40
Longhi, S., *et al. J.Mol. Biol.,* **1997**, 779-99
Pollard M., *et.al. Trends in Plant Science*, **2008**, 1360-85
Pdb code: 1cex

Examples of Biotransformations using Cutinase

I.

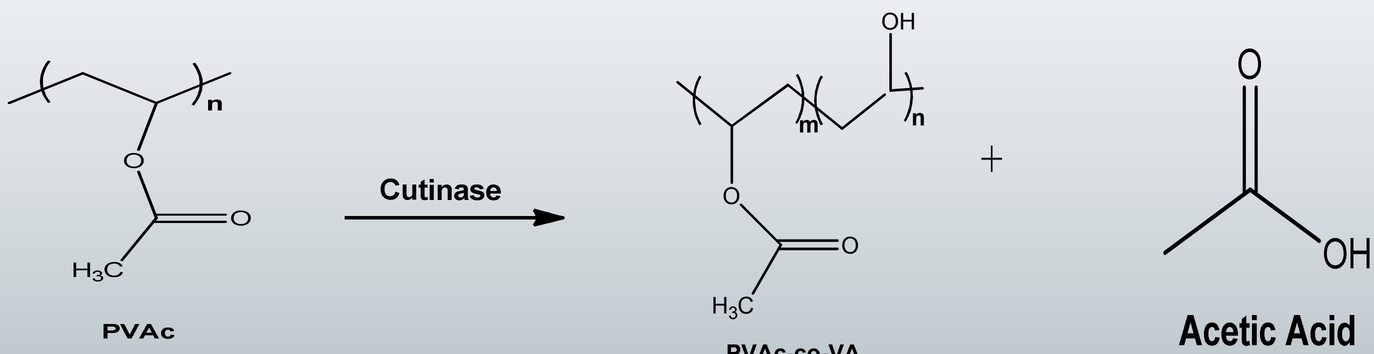
Polymerization



Hunsen M., *et. al., Biomacromolecules,* **2008***,* 518-22

II.

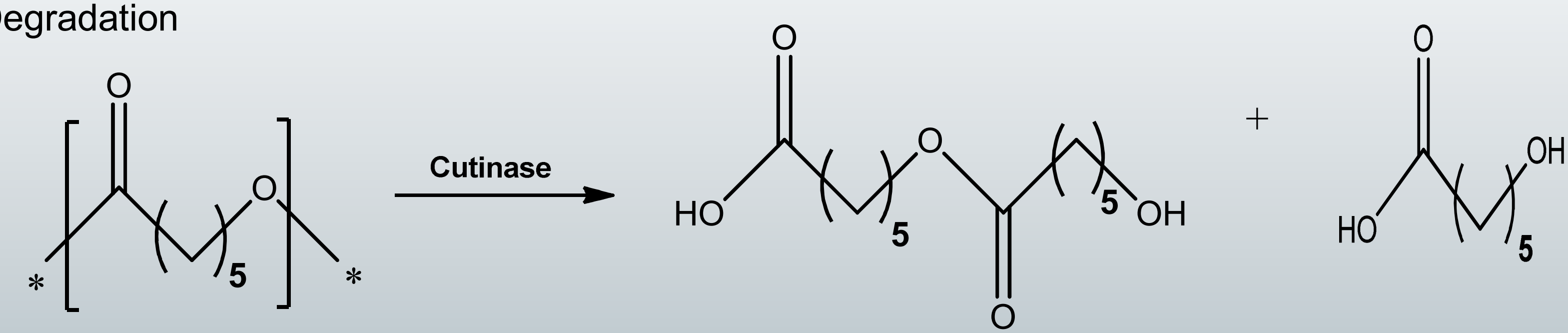
Modification



Ronkvist, A., PhD. Dissertation NYU:POLY

III.

Degradation



Liu Z., *et. al., JACS,* **2009**, under revision

Searching the Natural Diversity

```
AbC   --MMNLNLLLSKPC----------------QA-STTRNELETGSSDACPRTIFIFARGSTE
AfC   --MKFALLSLAAMAVASPV-----AIDVRQT-AITGDELRTG---PCEPITFIFARGSTE
AoC   MHLRNIVIALAATAVASPV-----DLQDRQL--TGGDELRDG---PCKPITFIFARASTE
HiC   --------------------------------GAIENGLESGSANACPDAILIFARGSTE
FsC   MKFFALTTLLAATASALPTSNPAQELEARQLGRTTRDDLINGNSASCRDVIFIYARGSTE
                                          : *  *   .*    :*:**.***

AbC   AGNMGALVGPFTANALESAYGASNVWVQGVGGPYTAGLVENALPAGTSQAAIREAQRLFN
AfC   PGLLGITTGPGVCNALKLS-RPGQVACQGVGPAYIADLASNFLPQGTSQVAIDEAAGLFK
AoC   PGLLGISTGPAVCNRLKLA-RSGDVACQGVGPRYTADLPSNALPEGTSQAAIAEAQGLFE
HiC   PGNMGITVGPALANGLES--HIRNIWIQGVGGPYDAALATNFLPRGTSQANIDEGKRLFA
FsC   TGNLG-TLGPSIASNLESAFGKDGVWIQGVGGAYRATLGDNALPRGTSSAAIREMLGLFQ
      .* :*   **  .. *:       :  ****  * * *  * ** ***.. * *   **

AbC   LAASKCPNTPITAGGYSQGAAVMSNAIPGLSAAVQDQIKGVVLFGYTKNLQNGGRIPNFP
AfC   LAASKCPDTKIVAGGYSQGAAVMHGAIRNLPSNVQNMIKGVVLFGDTRNKQDGGRIPNFP
AoC   QAVSKCPDTQIVAGGYSQGTAVMNGAIKRLSADVQDKIKGVVLFGYTRNAQERGQIANFP
HiC   LANQKCPNTPVVAGGYSQGAALIAAAVSELSGAVKEQVKGVALFGYTQNLQNRGGIPNYP
FsC   QANTKCPDATLIAGGYSQGAALAAASIEDLDSAIRDKIAGTVLFGYTKNLQNRGRIPNYP
       *  ***:: : *******:*:   ::  * . ::: : *..*** *:* *: * *.*:*

AbC   TSKTTIYCETGDLVCNGTLIITPAHLLYSDEAAVQAPTFLRAQID----SA
AfC   TDRTKIYCAFGDLVCDGTLIITPAHLSYGDDVP-SATSFLLSKV-------
AoC   KDKVKVYCAVGDLVCLGTLIVAPPHFSYLSDT-GDASDFLLSQLG------
HiC   RERTKVFCNVGDAVCTGTLIITPAHLSYTIEARGEAARFLRDRIR------
FsC   ADRTKVFCNTGDLVCTGSLIVAAPHLAYGPDARGPAPEFLIEKVRAVRGSA
       .:..::*  ** ** *:**::..*: *  :.   *. **  ::
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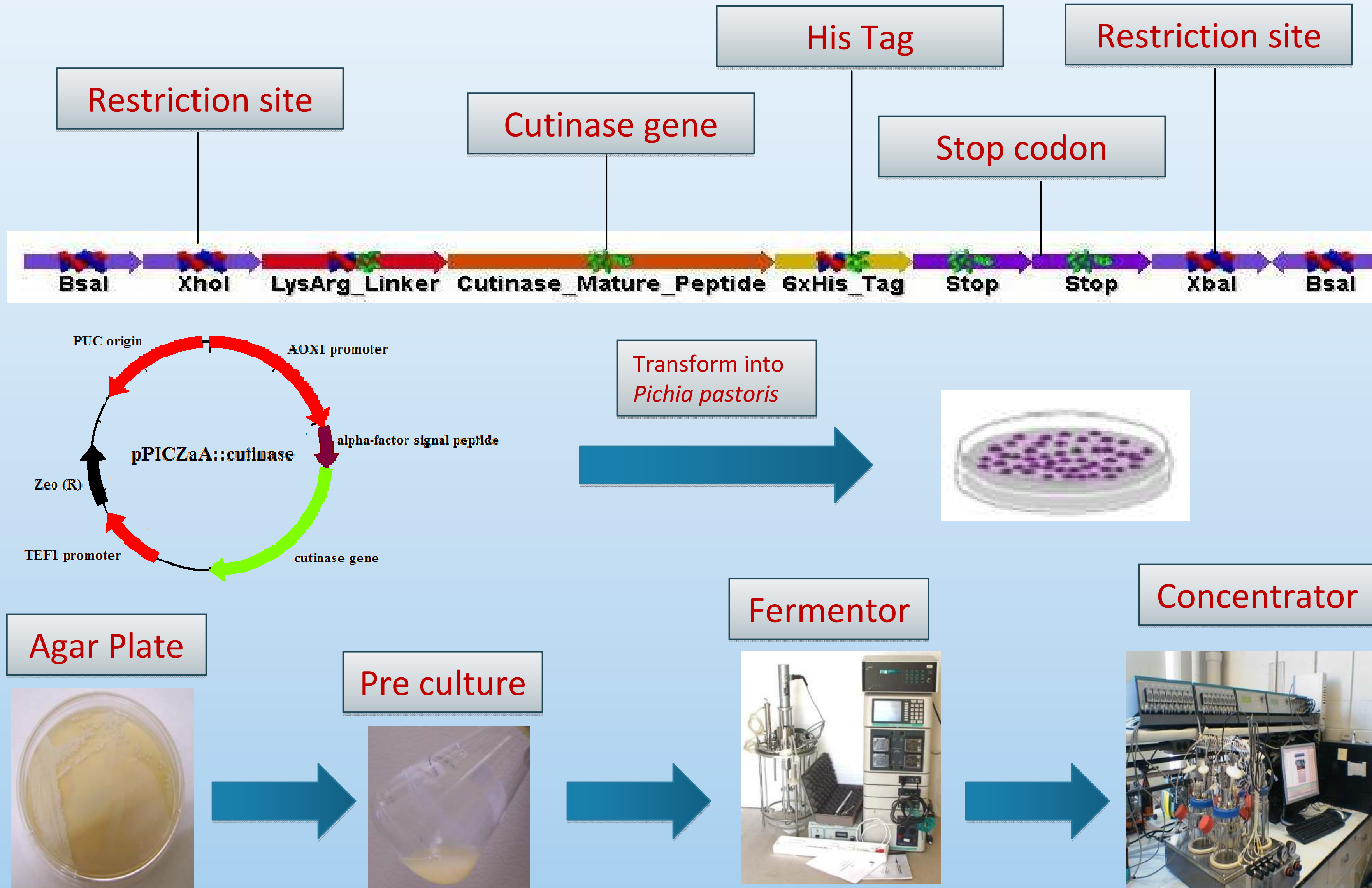
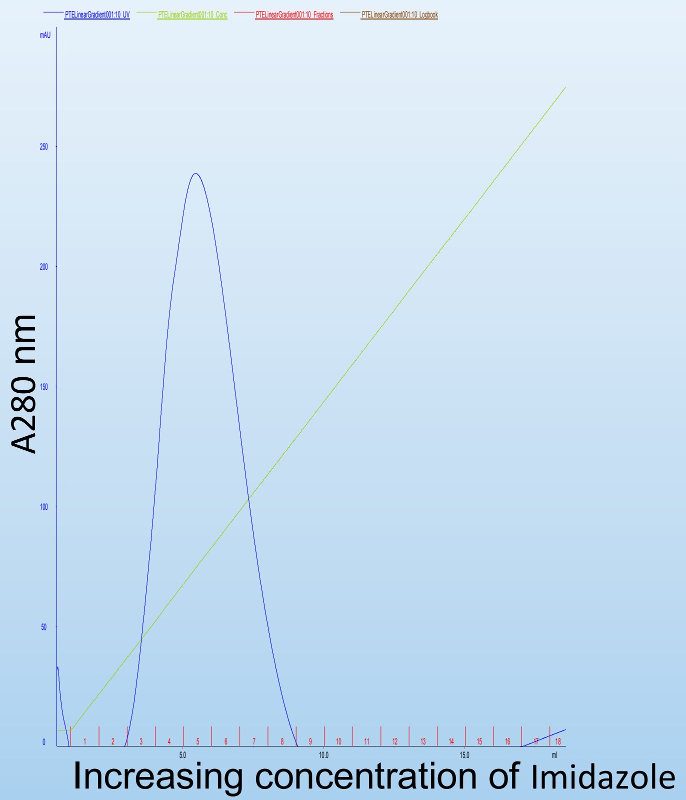
FsC as parental sequence

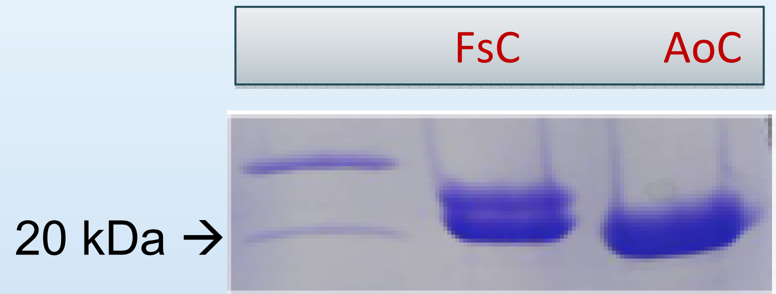
- 116 potential sequence were identified
- Sequences were eliminated if they were mutants or previously studies
- 4 sequences were selected which showed conservation of catalytic residues

Expression and Cloning of Cutinase



Purification of Cutinase

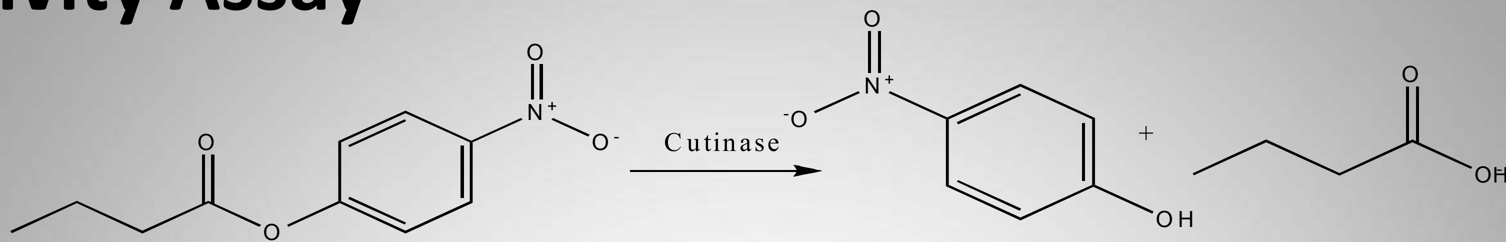




Estimated yield for a 3L expression ~600mg

Column: GE His Trap FF Column
Elution Buffer: 50 mM Na Phosphate
500 mM Imidazole, pH 8.0

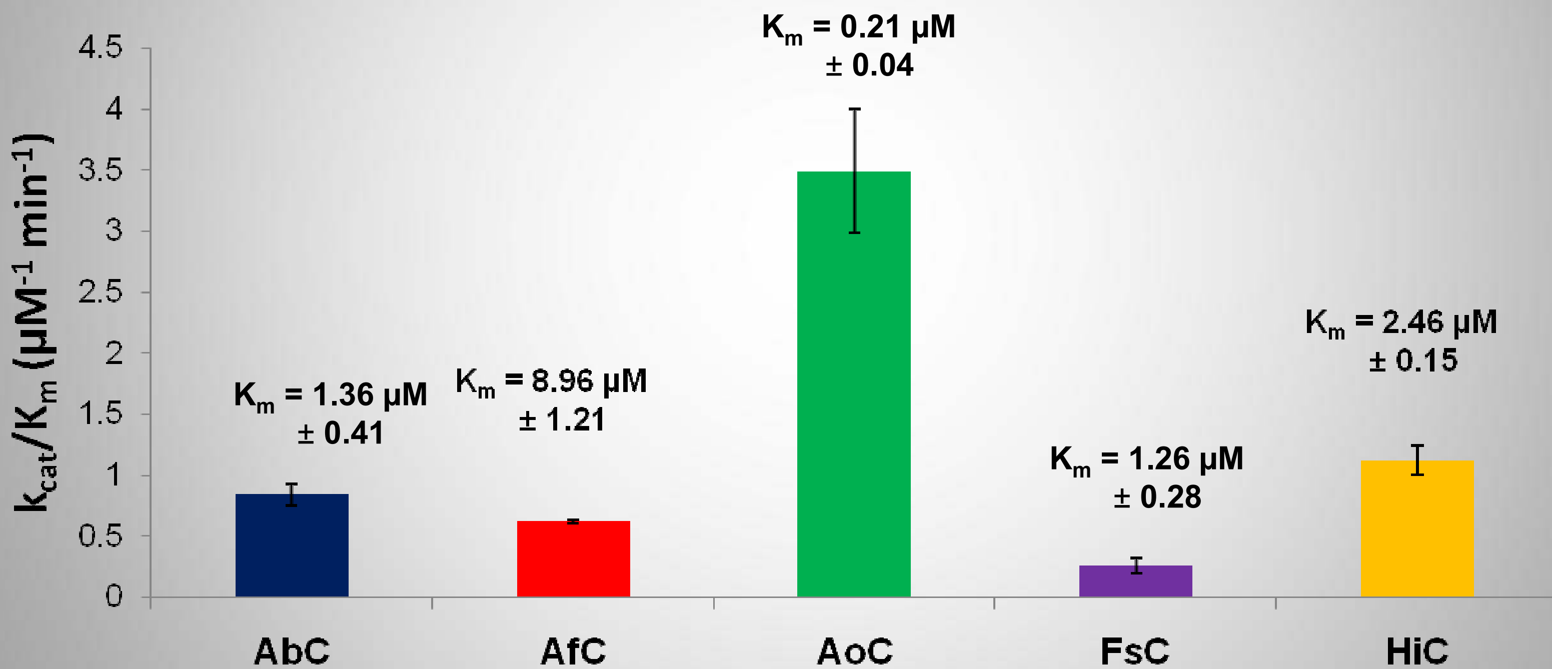
Activity Assay



p –nitrophenyl butyrate

p -nitrophenol
abs = 405 nm

butyric acid



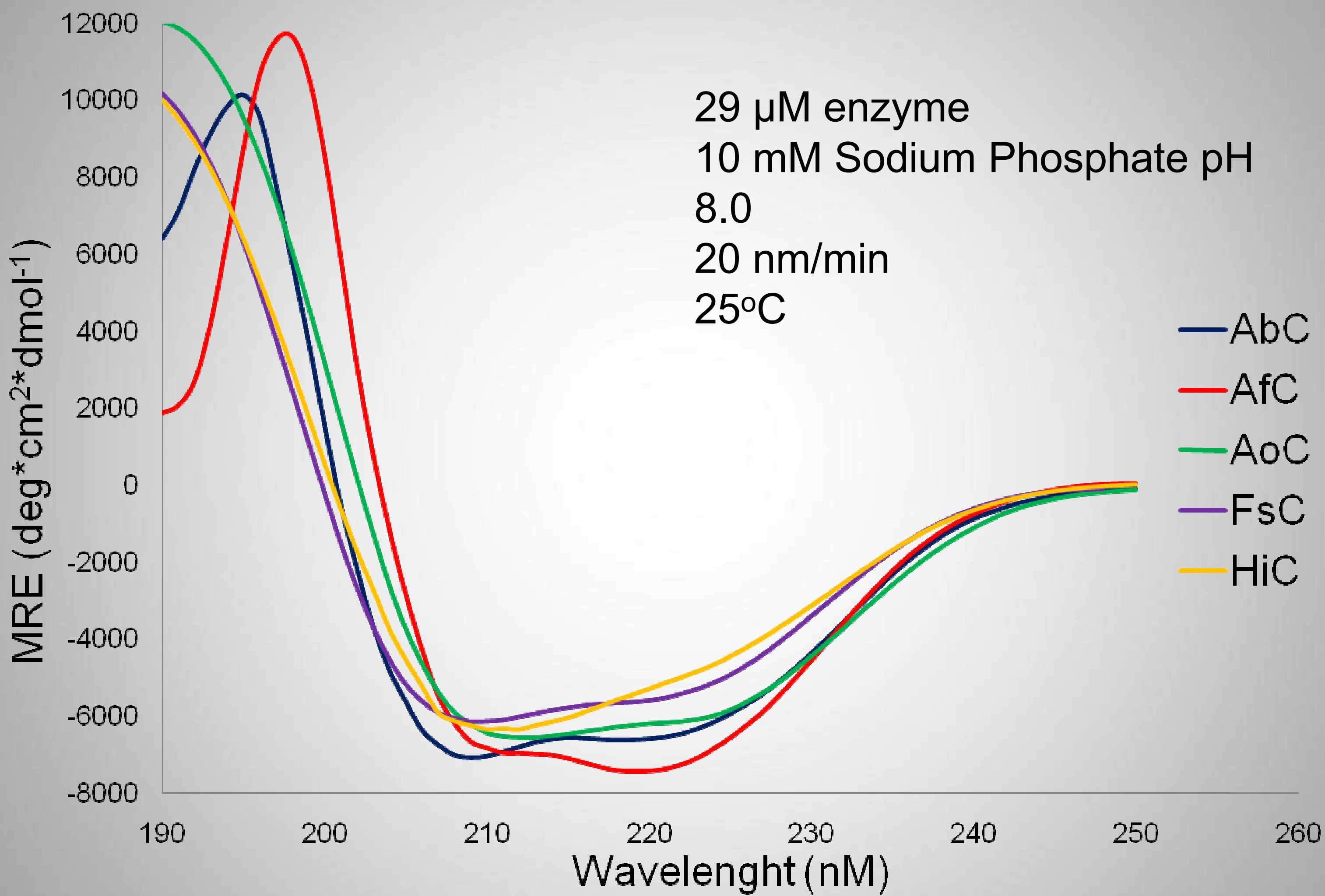
K_m : AoC > FsC > AbC > HiC > AfC

k_{cat}/K_m : AoC >HiC >AbC > AfC > FsC

<u>Reaction Condtions</u>
14.5 mM Tris, pH 7.5
0.75% Glycerol
25°C

Dantzing, A.H., *et.al.*, *J. Bacteriol.*, **1986,** 911-6

Secondary Structural Analysis



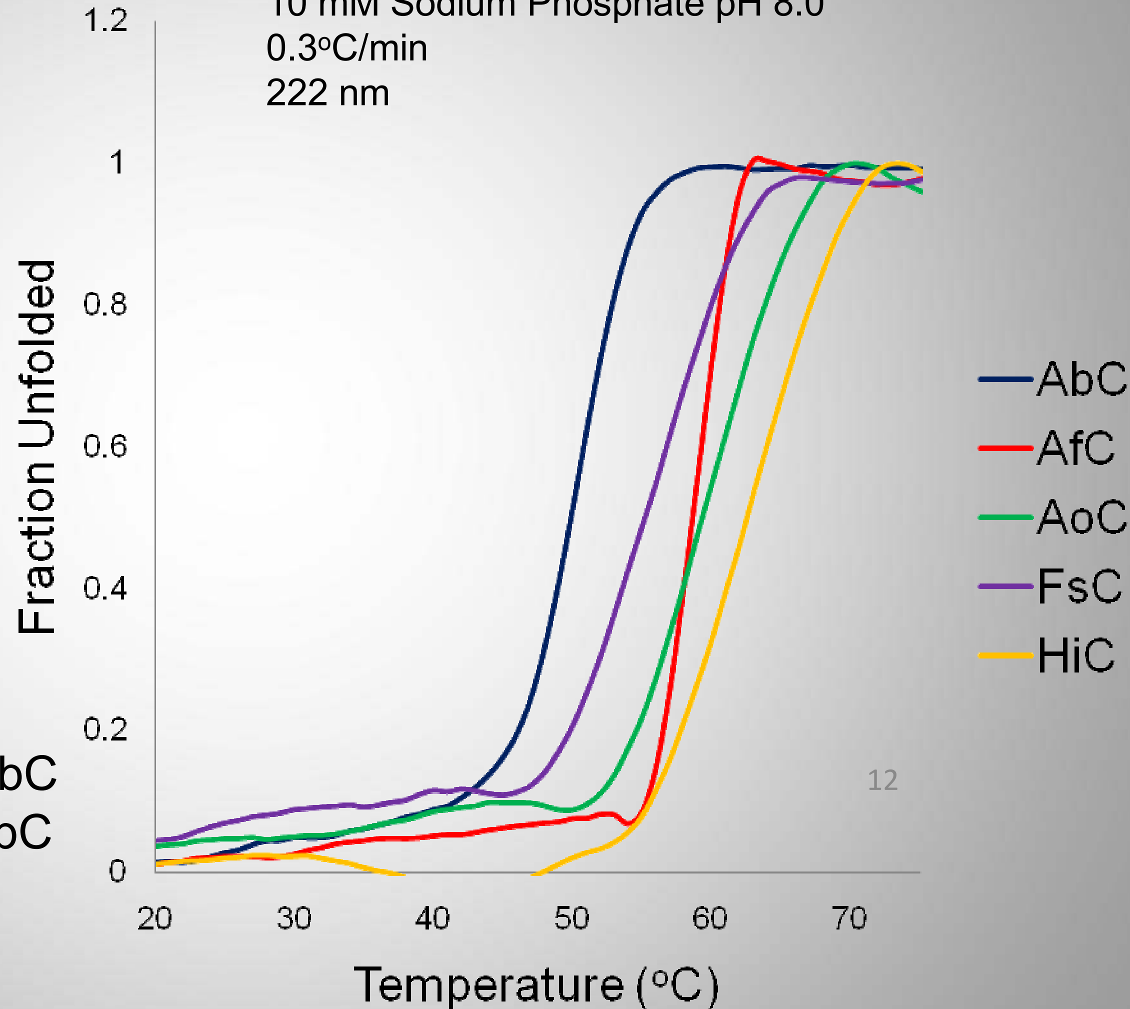
All proteins exhibit similar α/β secondary structure characteristics, hallmarked by a double minima at 208nm and 22nm

Biophysical Properties at pH 8.0

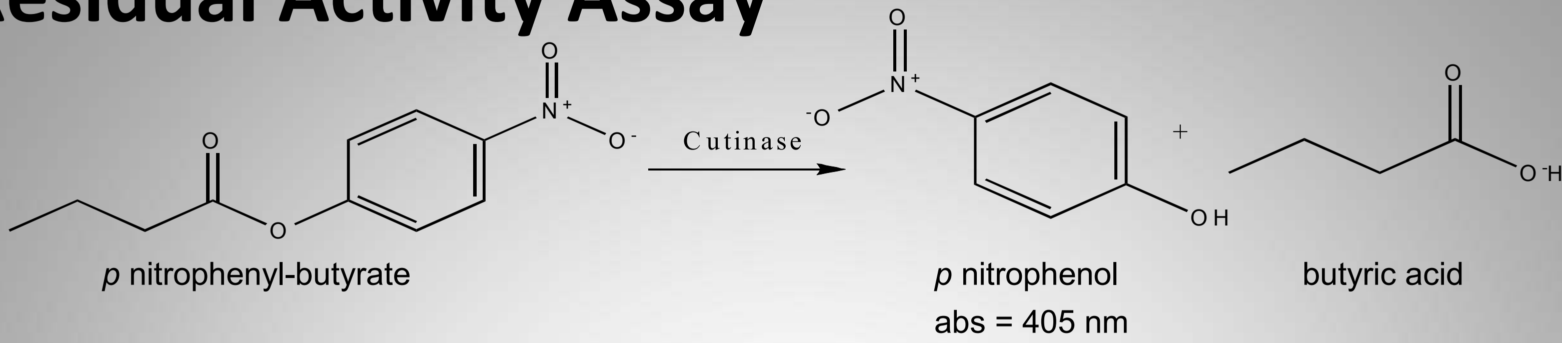
	T_m (°C)	ΔH (kJ/mol)
AbC	50	517.13
AfC	59	601.53
AoC	58	622.61
FsC	56	562.21
HiC	62	541.42

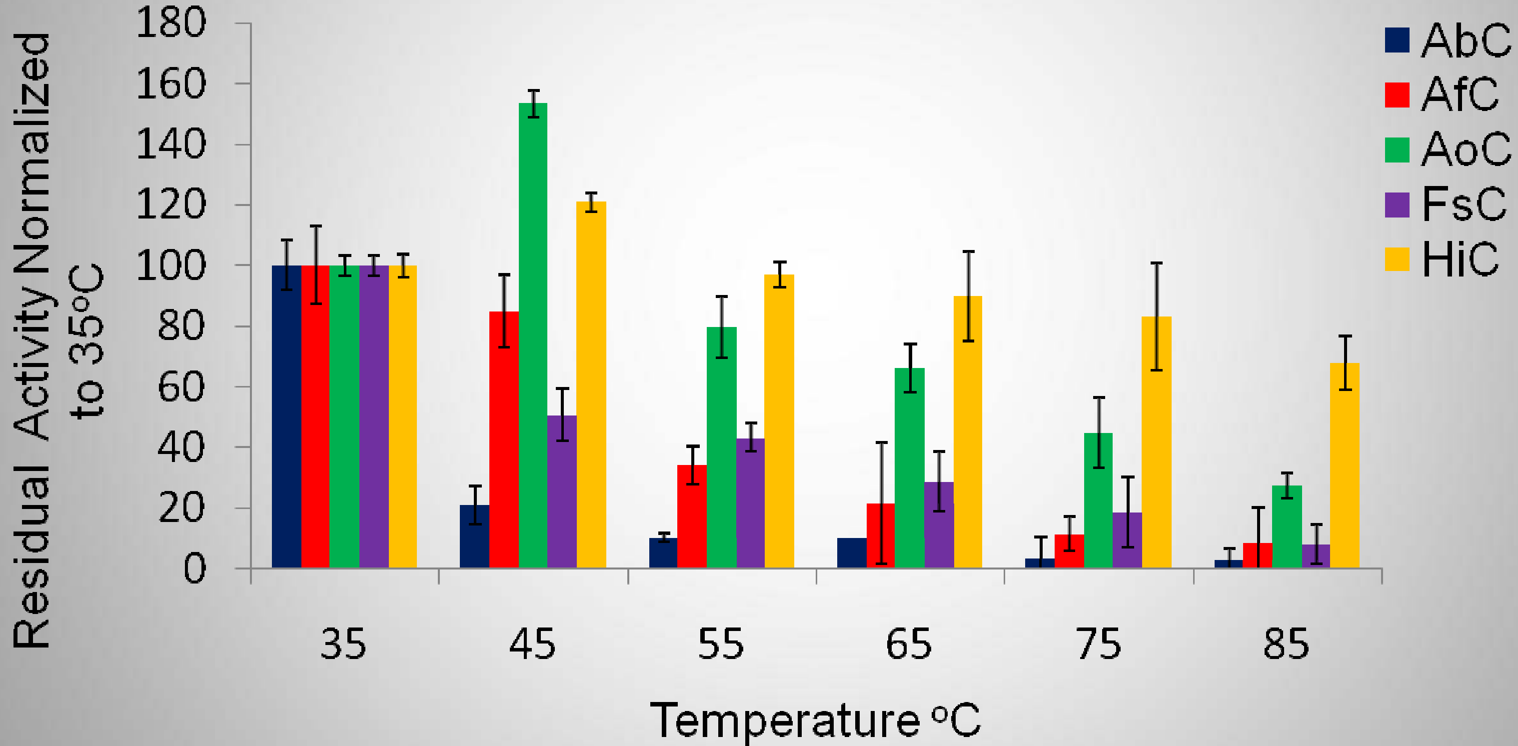
T_m: HiC > AfC > AoC > FsC > AbC
ΔH: AoC > AfC > HiC >FsC > AbC

29 µM enzyme
10 mM Sodium Phosphate pH 8.0
0.3°C/min
222 nm



Residual Activity Assay



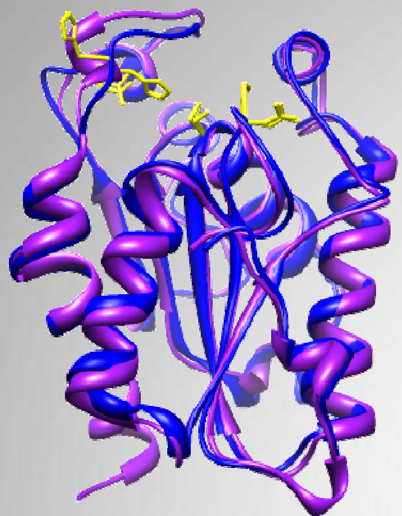


Reaction Condtions
14.5 mM Tris, pH 7.5
0.75% Glycerol

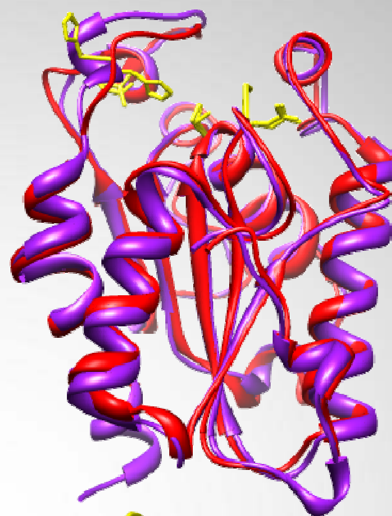
Dantzing, A.H., *et.al.*, *J. Bacteriol.*, **1986,** 911-6

Backbone Homology at pH 8.0

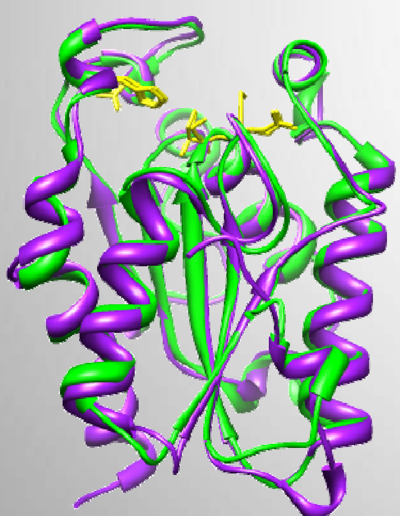
AbC
pI = 6.06



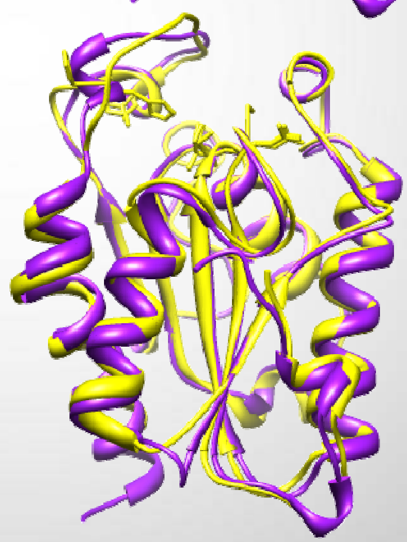
AfC
pI = 7.66



AoC
pI = 5.79



HiC
pI = 8.17

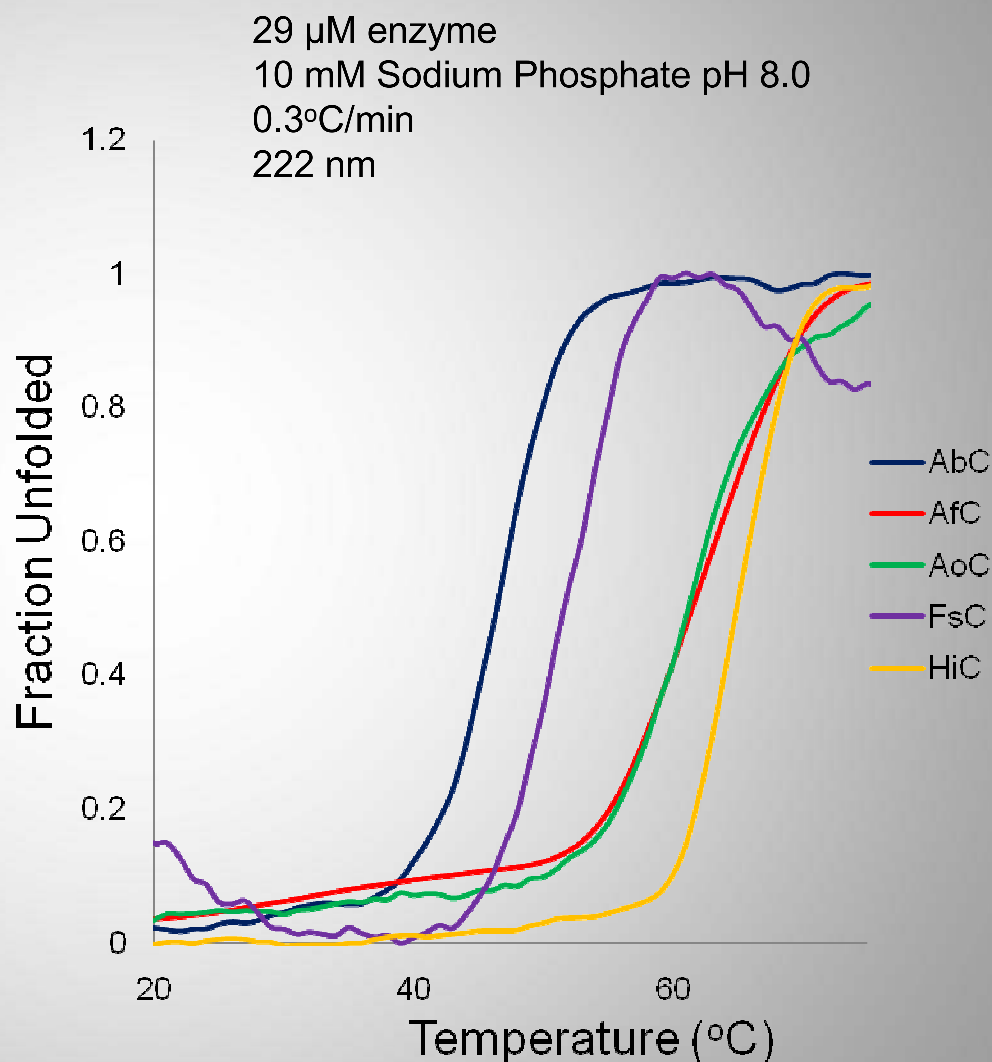


UCSF Chimera
Pettersen EF., *et.al.*, *J Comput. Chem.,* **2004**, 1605-12
Nyon MP., *et. al., J. Mol. Biol.,* **2009**, 226-35

Biophysical Properties at pH 5.0

	T_m (°C)	ΔH (kJ/mol)
AbC	50	445.69
AfC	64	675.00
AoC	61	612.34
FsC	53	536.77
HiC	63	563.06

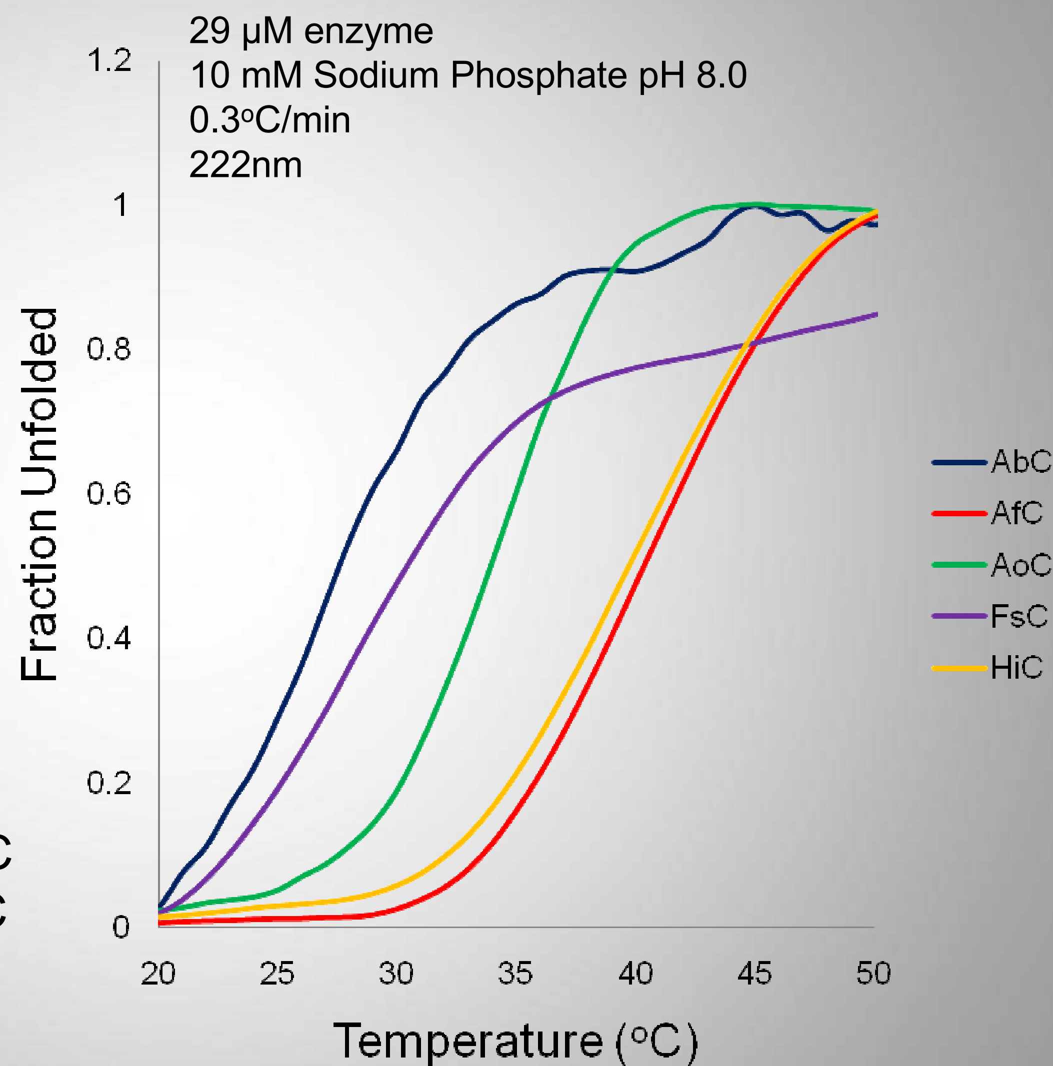
T_m: HiC > AfC > AoC > FsC > AbC
ΔH: AfC > AoC > HiC >FsC > AbC



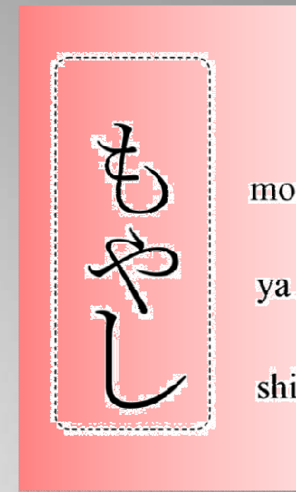
Biophysical Properties at pH 3.0

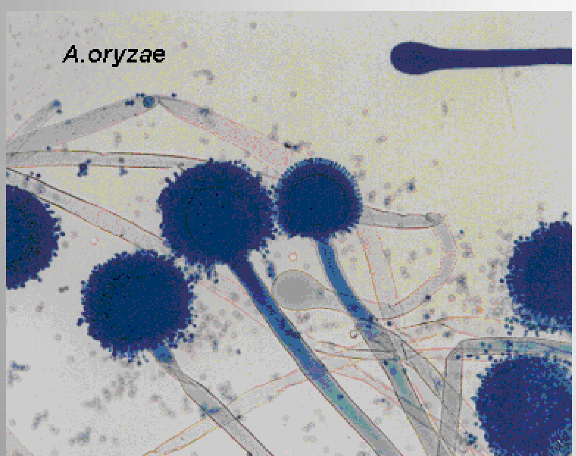
	T_m (°C)	ΔH (kJ/mol)
AbC	26	425.31
AfC	39	604.42
AoC	35	593.72
FsC	27	314.13
HiC	38	328.50

T_m: AfC > HiC > AoC > FsC > AbC
ΔH: AfC > AoC > AbC > HiC >FsC



Exploration of *Aspergillus oryzae*





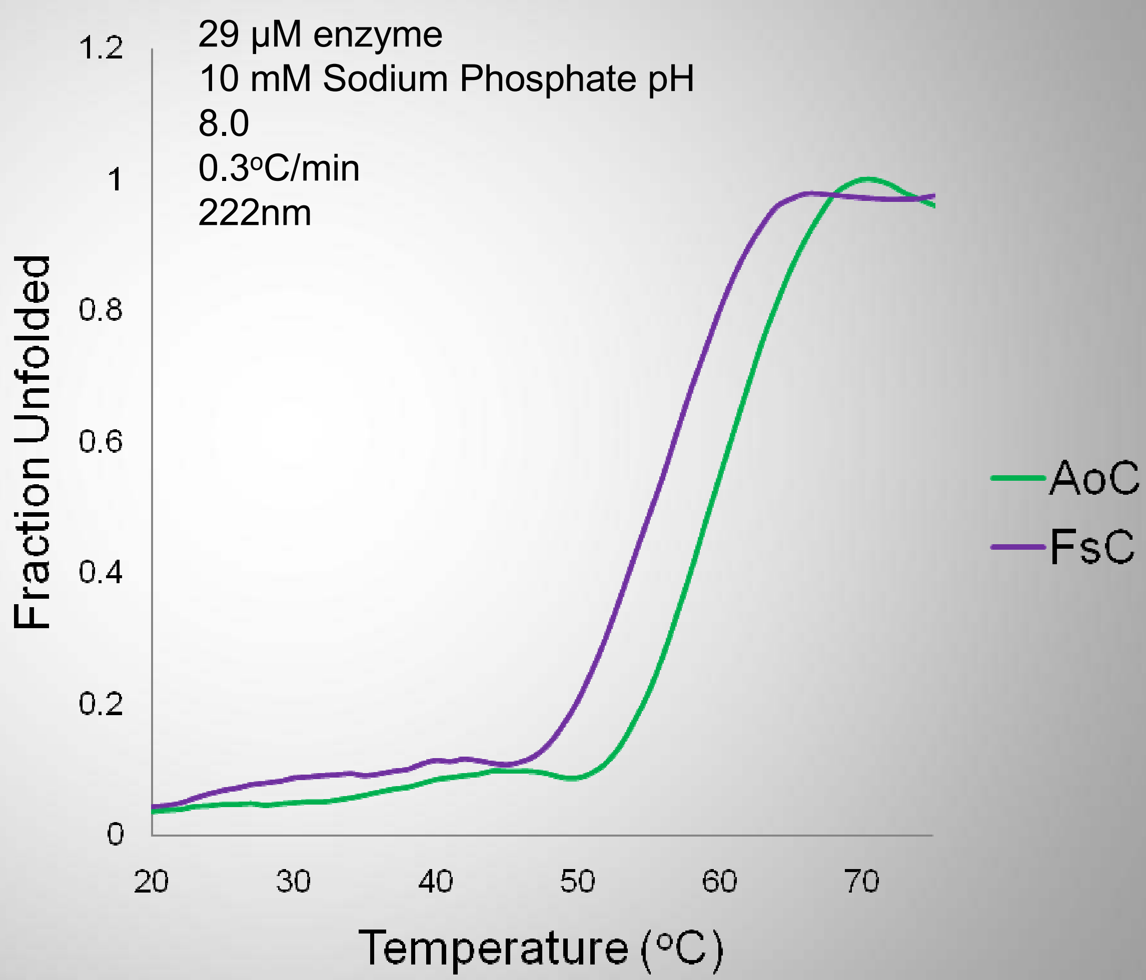
Aspergillus oryzae (koji mold)

- Filamentous fungus in the Japanese fermentation industry for the production sake, soy sauce, and miso
- *A. oryzae* is capable of living in broad range of pH and temperatures
- Fungi is capable of using biodegradable polymers as sole carbon source

Maeda H., *et. al., Appl. Microbiol Biotechnol.*, **2005**, 778-788
Machida M., *et. al.*, *Nature*, **2005**, 1157-61
Machida M., *et. al.*, *DNA Research*, **2008**, 173-83

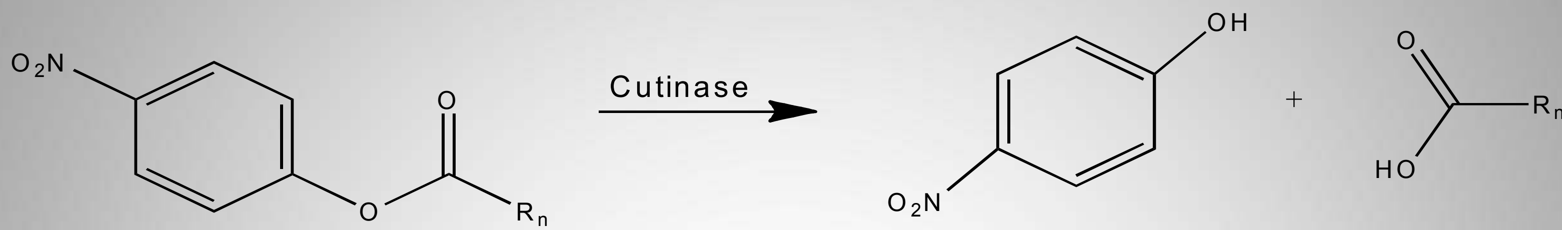
Comparison of FsC and AoC

	T_m (°C)
AoC	58
FsC	56



Liu Z., *et. al.*, *JACS*, **2009**, under revision

Kinetic Comparison of FsC & AoC



Rn = CH_3
Rn = C_2H_5
Rn = C_4H_9
Rn = C_5H_{11}

p nitrophenol
abs = 405 nm

<u>Reaction Condtions</u>
14.5 mM Tris, pH 7.5
0.75% Glycerol
25°C

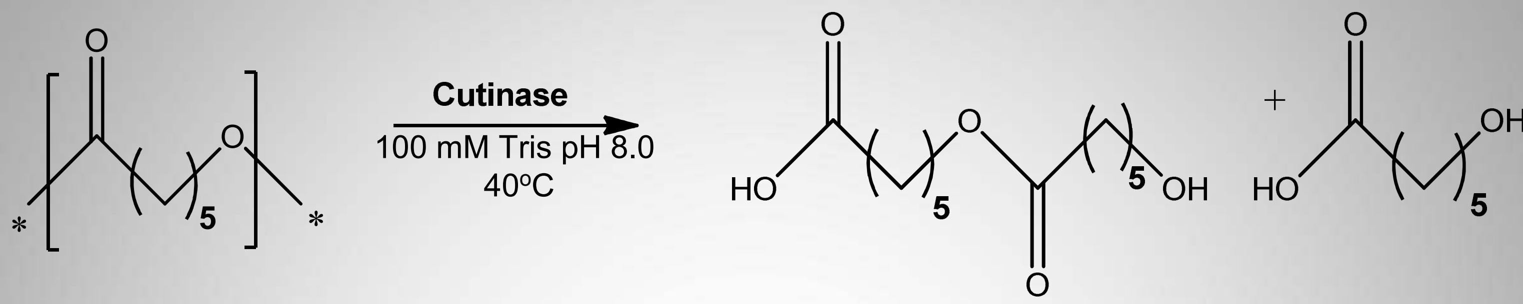
K_m (μM)	pNPA	pNPB	pNPV	pNPH
FsC	0.67 ± 0.23	1.26 ± 0.28	1.48 ± 0.56	1.50 ± 0.19
AoC	4.96 ± 0.11	0.21 ± 0.04	0.04 ± 0.01	0.29 ± 0.09

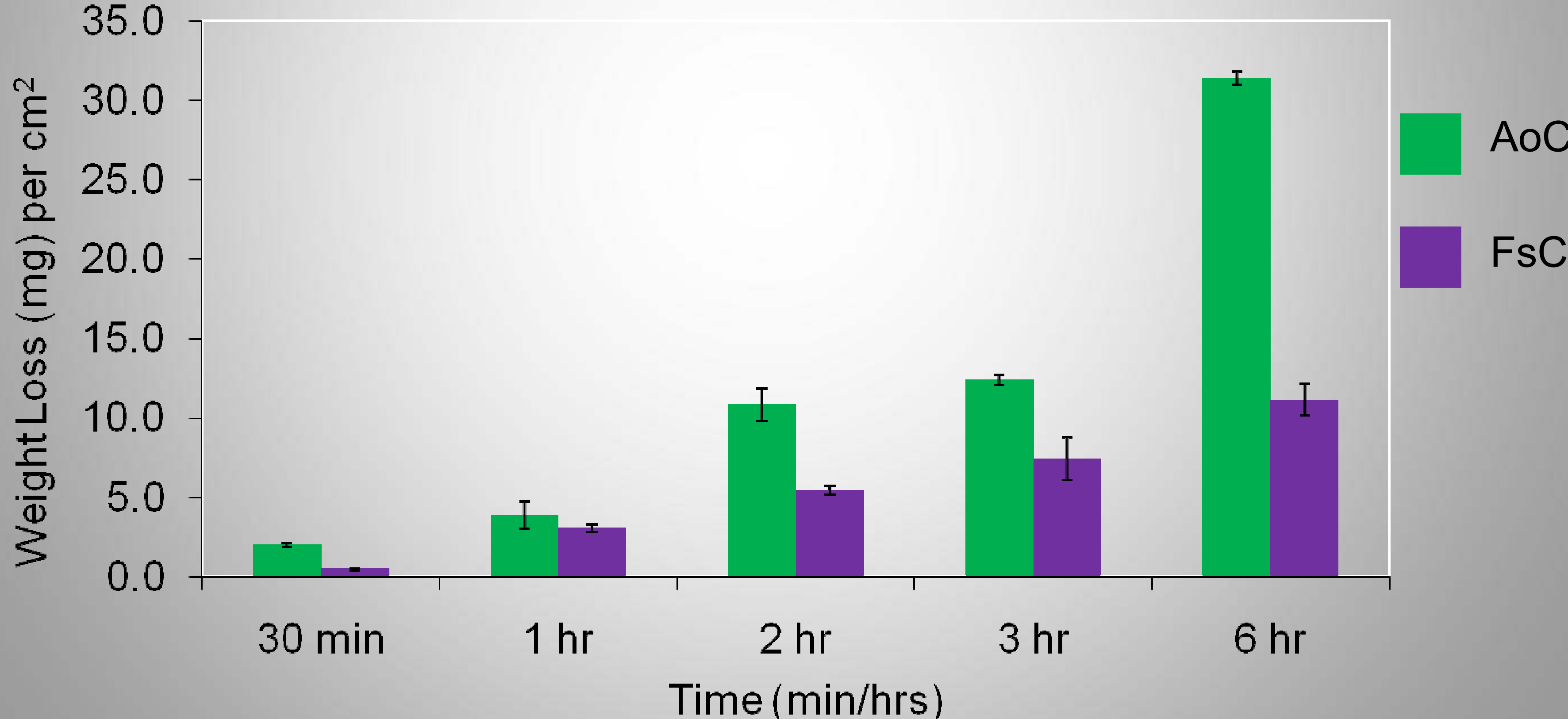
k_{cat}/K_m	pNPA	pNPB	pNPV	pNPH
FsC	2.53 ± 1.11	0.26 ± 0.06	0.61 ± 0.40	0.14 ± 0.02
AoC	0.07 ± 0.01	3.49 ± 0.51	3.32 ± -0.74	1.34 ± 0.48

AoC has a higher affinity towards the more hydrophobic substrates
AoC is more active towards the more hydrophobic substrates

Liu Z., *et. al.,* under revision, JACS, **2009**

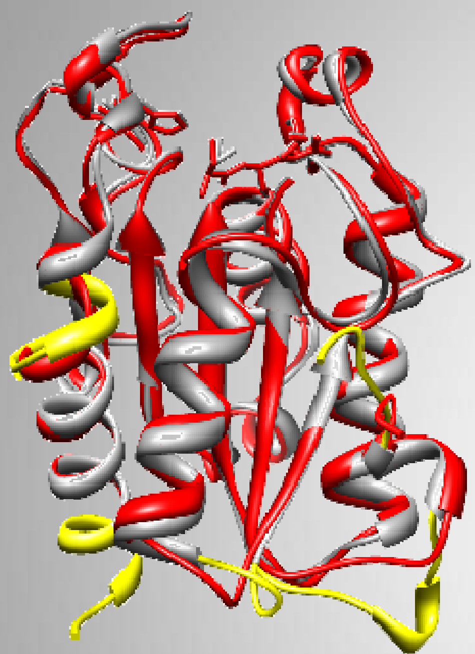
Poly(ε-caprolactone) degradation

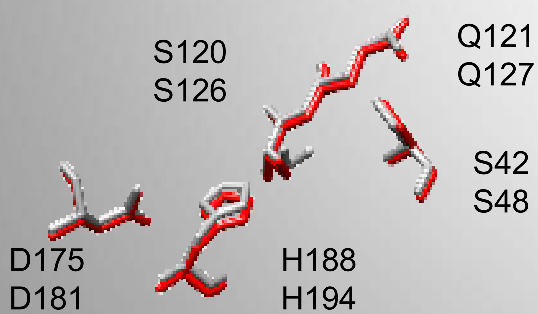




Liu Z., *et. al.*,*JACS*, **2009**, under revision

Backbone Comparison of FsC & AoC





Overlay of active site

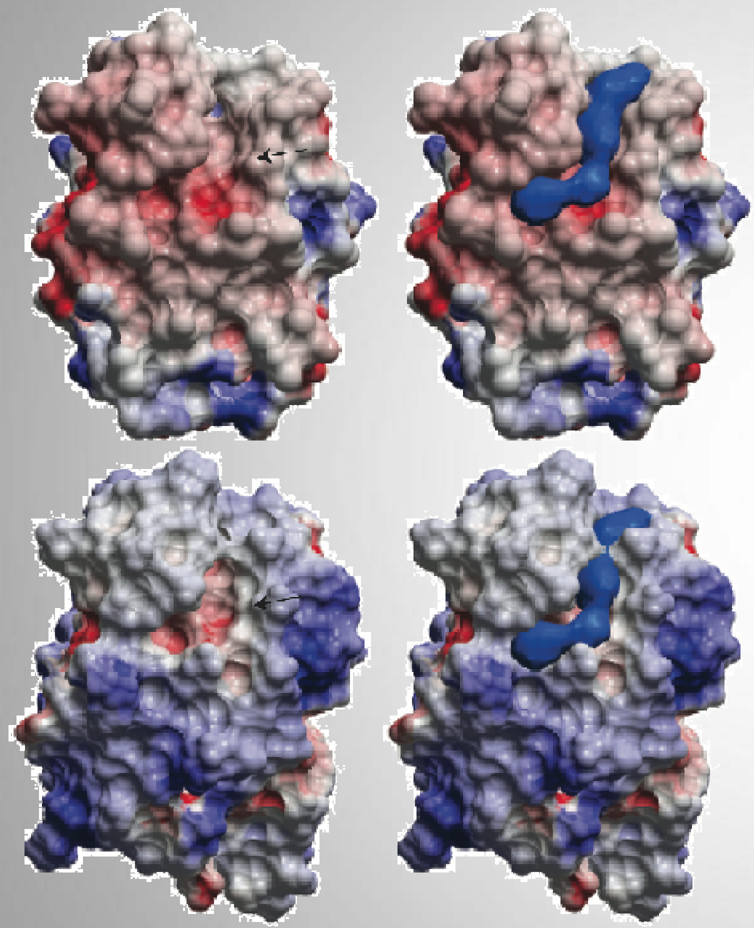
Superposition of AoC (red) and FsC (grey)

- Crystal structure was determined at 1.75 Å
- Overall rms deviation 1.02Å
- Main chain deviation 0.87Å
- AoC shorter in sequence than FsC
- AoC has one less β-strand than FsC
- An extra disulfide bond between Cys63-Cys76

- Backbone orientation and distances between the catalytic residues is similar

Liu Z., *et. al.*, *JACS*, **2009**, under revision

Comparison of Groove by Active site for FsC and AoC



- Distinguishable long and deep groove in AoC active site relative to FsC

- Although the sequence of catalytic site is conserved, the geometric arrangements of the hydrophobic regions are different

Liu Z., *et. al.*, *JACS*, **2009**, under revision

Conclusion

- Identified, expressed and characterized genetically distinct cutinase species
- Although these species exhibit similar structural similarities, they present different activities, thermostability and thermodynamics as a function of pH
- Cutinase from *Aspergillus oryzae* demonstates a higher affinity towards more hydrophobic substrates, this increased affinity is correlated to the protein surface of the enzyme
- Cutinase from *Aspergillus oryzae* is capable of degrading bioplastics

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- Wenhua Lv
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