Industrial Biotechnology

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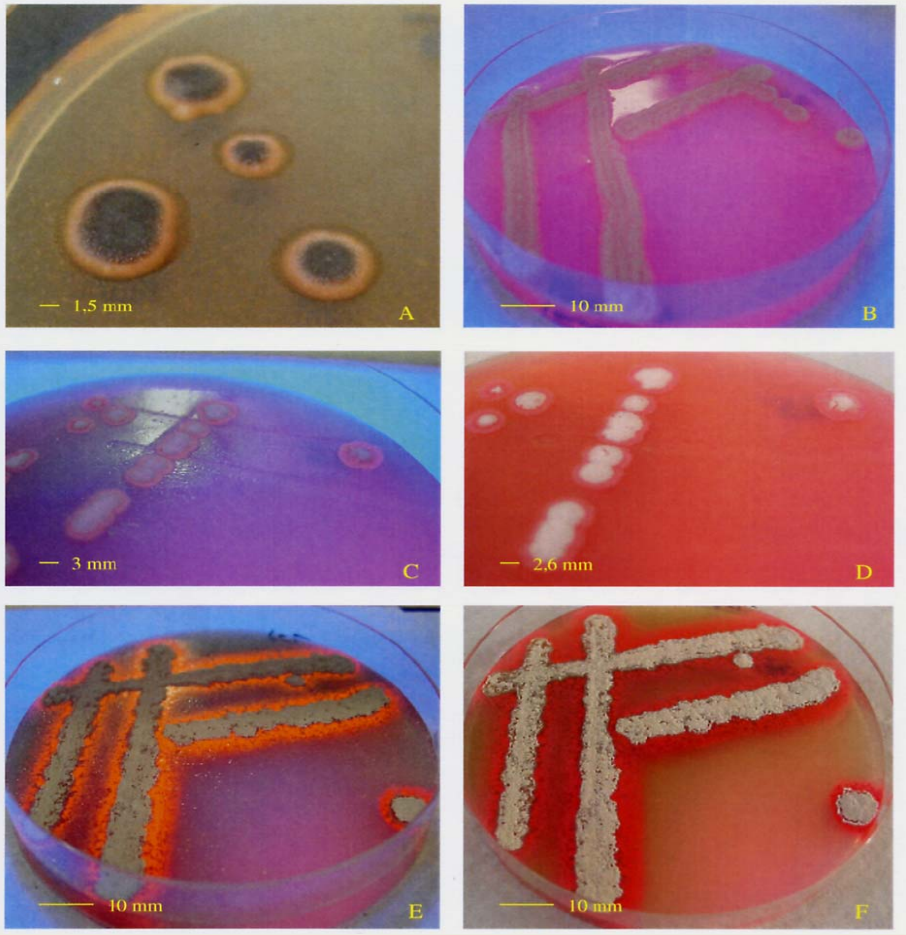
What is Industrial Biotechnology and why is is white?

Industrial Biotechnology is the application of nature's toolset to industrial production

EuropaBio (European Association for Bio-industries) 2003

Nature's toolset

- Microorganisms
  - Fungi
  - Yeasts
  - Bacteria
- Applications can be designed with
  - Whole microbial cells
  - Biocatalysts derived from microorganisms



Industrial production

- Implementation of biotechnology in industrial production processes
- Conversion of existing industrial processes to biobased ones
- Development of novel bioprocesses for industrial applications
- Use of renewable biomass to replace petrochemical feedstock

Bioprocess development

The colors of Biotech

- **Medicine, therapeutics, diagnostica, vaccines**
- **Agriculture, transgenic plants, nutrition**
- **Environment protection, waste treatment**
- **Therapeutics, new materials from marine organisms**

Industrial Biotechnology
fine and bulk chemicals
use of biomass

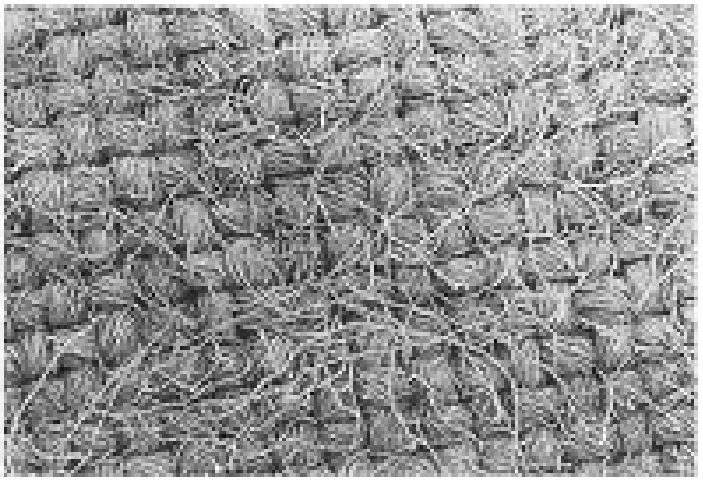
Products made by Industrial Biotechnology

- Fine chemicals
  - Highly functionalized
  - Often chiral building blocks
  - Typically produced in small quantities (<1.000 t/a)
- Bulk chemicals
  - Produced in large quantities >10.000 t/a
  - Opportunity for increased production by Industrial Biotech
- Industrial Enzymes
  - World enzyme demand will rise 6.8 percent annually to $8 billion in 2015
  - Targets: diagnostic, industrial biotechnology, animal feed, food markets
- Biofuels
  - Biogas, biodiesel, ethanol

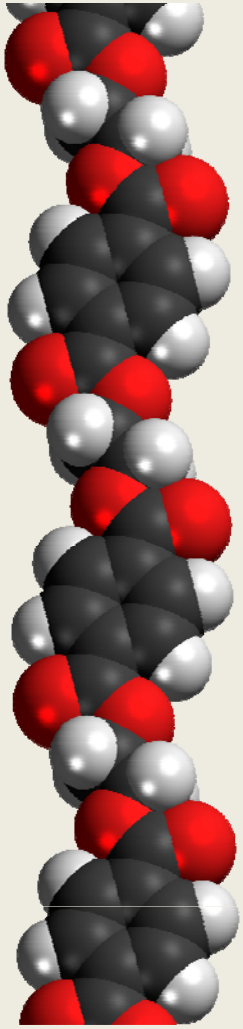
Enzymes for the detergent industry

- Enzymes already used in detergents
  - Proteases
  - Amylases
  - Cellulases
  - Lipases
- Novel enzymes: polyester hydrolases
  - Enabling laundry wash at lower temperatures and with less surfactant
  - Reduce pilling and improve color brightness of synthetic textiles

“Bio-finishing” of cotton textiles with cellulases

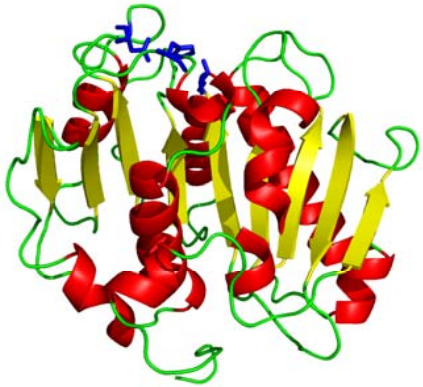


Most synthetic fibres are made from polyethylene terephthlate



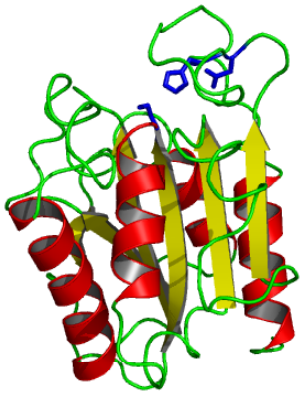
Effects of biocatalysts in PET hydrolysis and functionalization reactions

- Introduction of functional groups specifically on the surface of the polyester allows for the specific modification of polymer properties
- Increased hydrophilicity resulting in improved wetting and absorbancy properties in textile finishing
- Textile detergent additive: pilling reduction, oily stain removal
- Mild reaction conditions compared to chemical methods
- Reduced consumption of water, chemicals and energy when used in a bioprocess

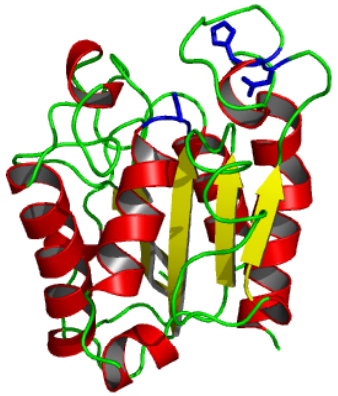


Microbial polyester hydrolases for the modification of synthetic polymers

Carboxylesterase TfCa *T. fusca*



Cutinase TfU_0882 *T. fusca*



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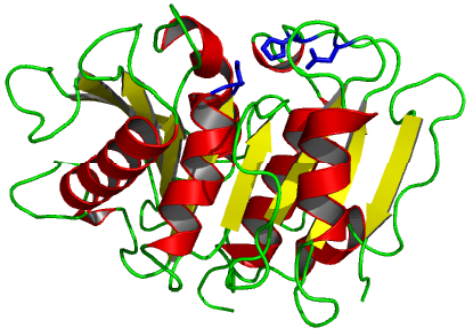
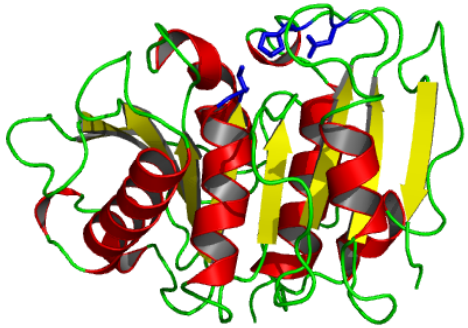
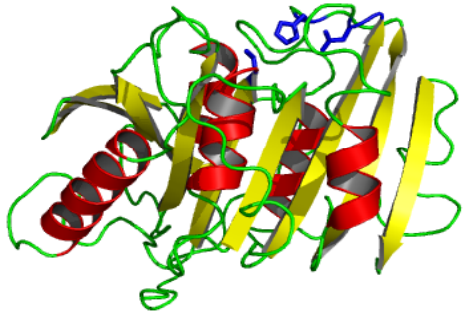


Cutinase *Fusarium solani*



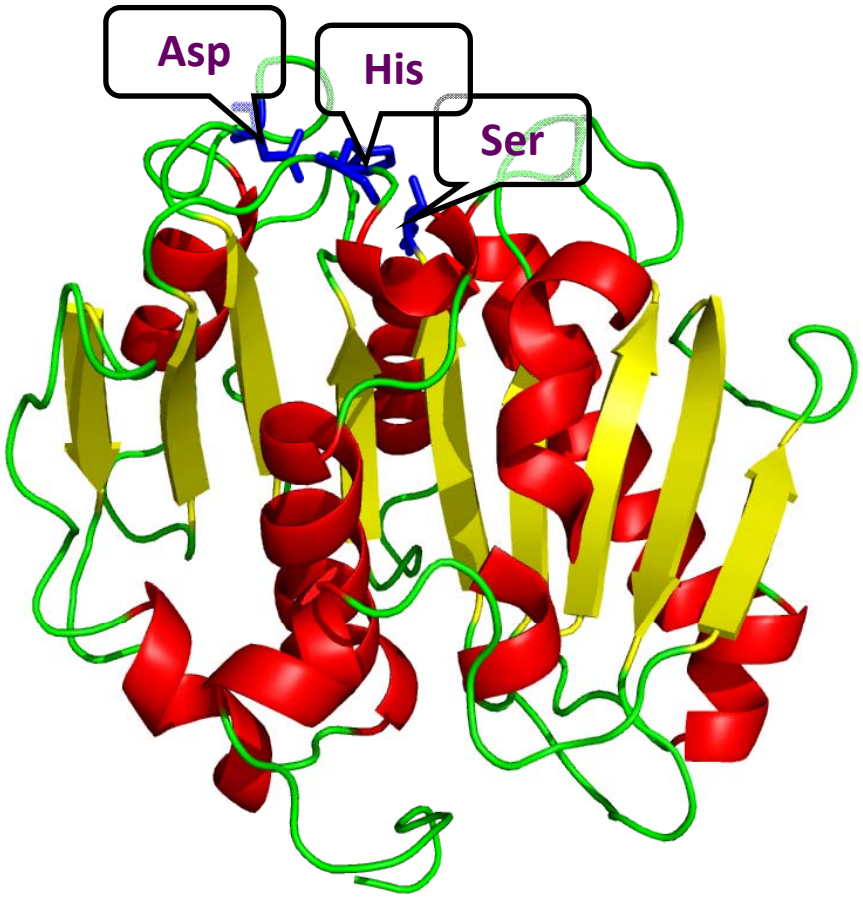
Cutinase Humicola insolens



Lipase *Pseudomonas mendocina*

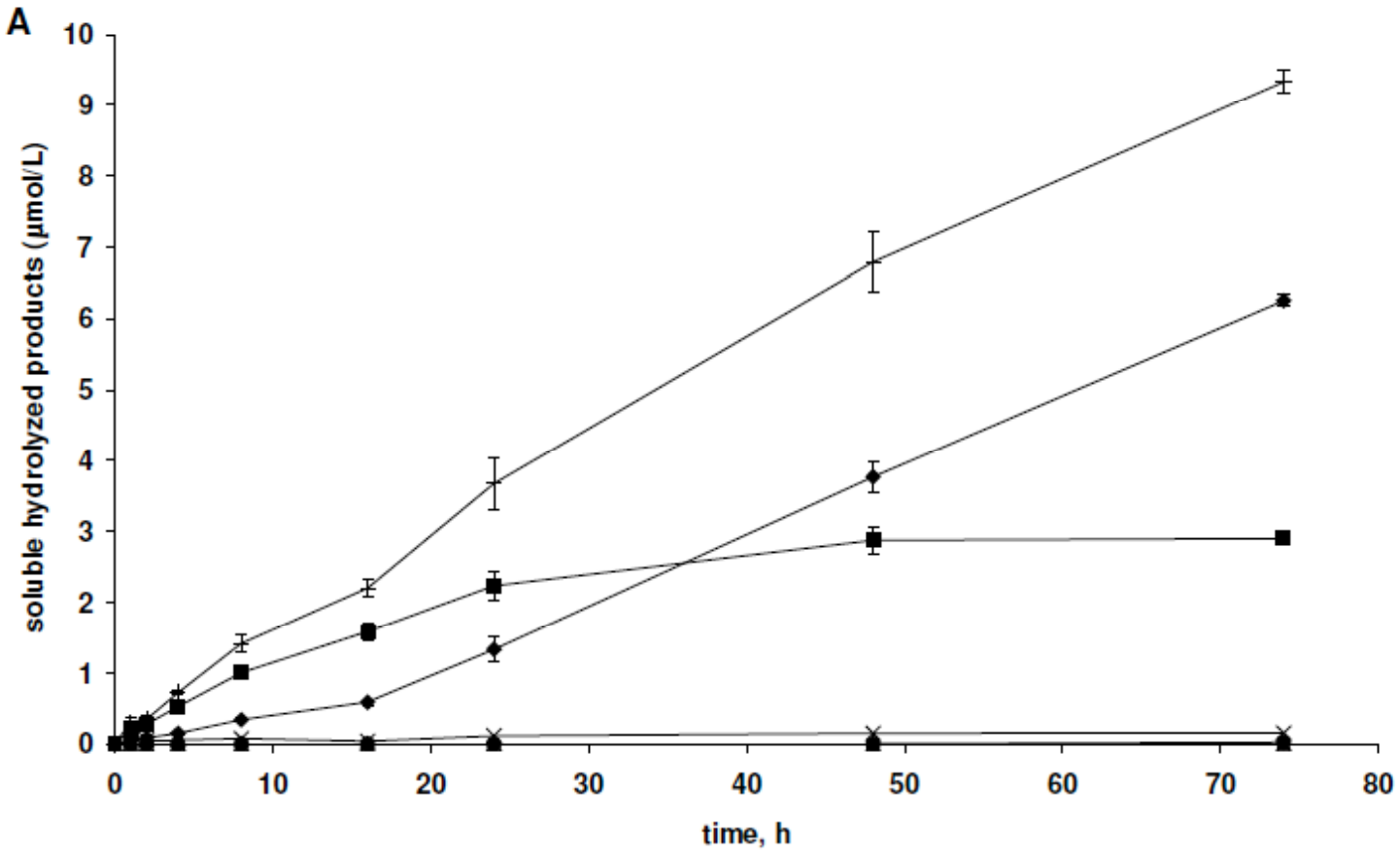
S Billig, T Oeser, C Birkemeyer, W Zimmermann. Appl Microbiol Biotechnol (2010) 87:1753–1764

Polyester hydrolase TfCut2 from *T. fusca*



- TfCut2 has high activity with PET and PCL
- Active site is located in a groove at the surface of the enzyme
- Stability: $T_{½}$= 50 h at 60°C
- Recombinant expression in *E. coli* and *Bacillus megaterium*

Hydrolysis of semi-crystalline PET fibers by the carboxylesterase TfCa from *T. fusca*



(♦) TPA

(■) MHET

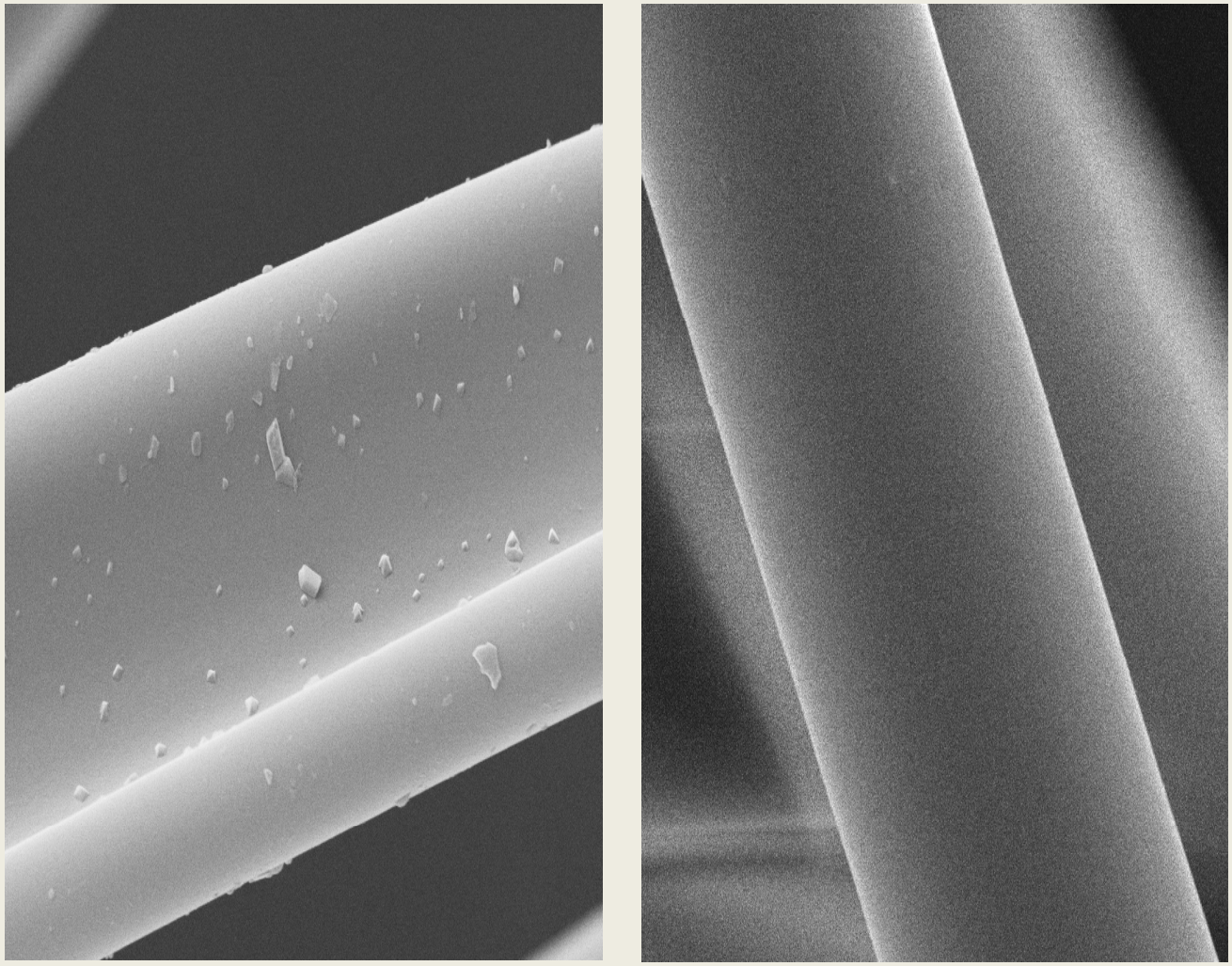
(▲) BHET

(●) EMT

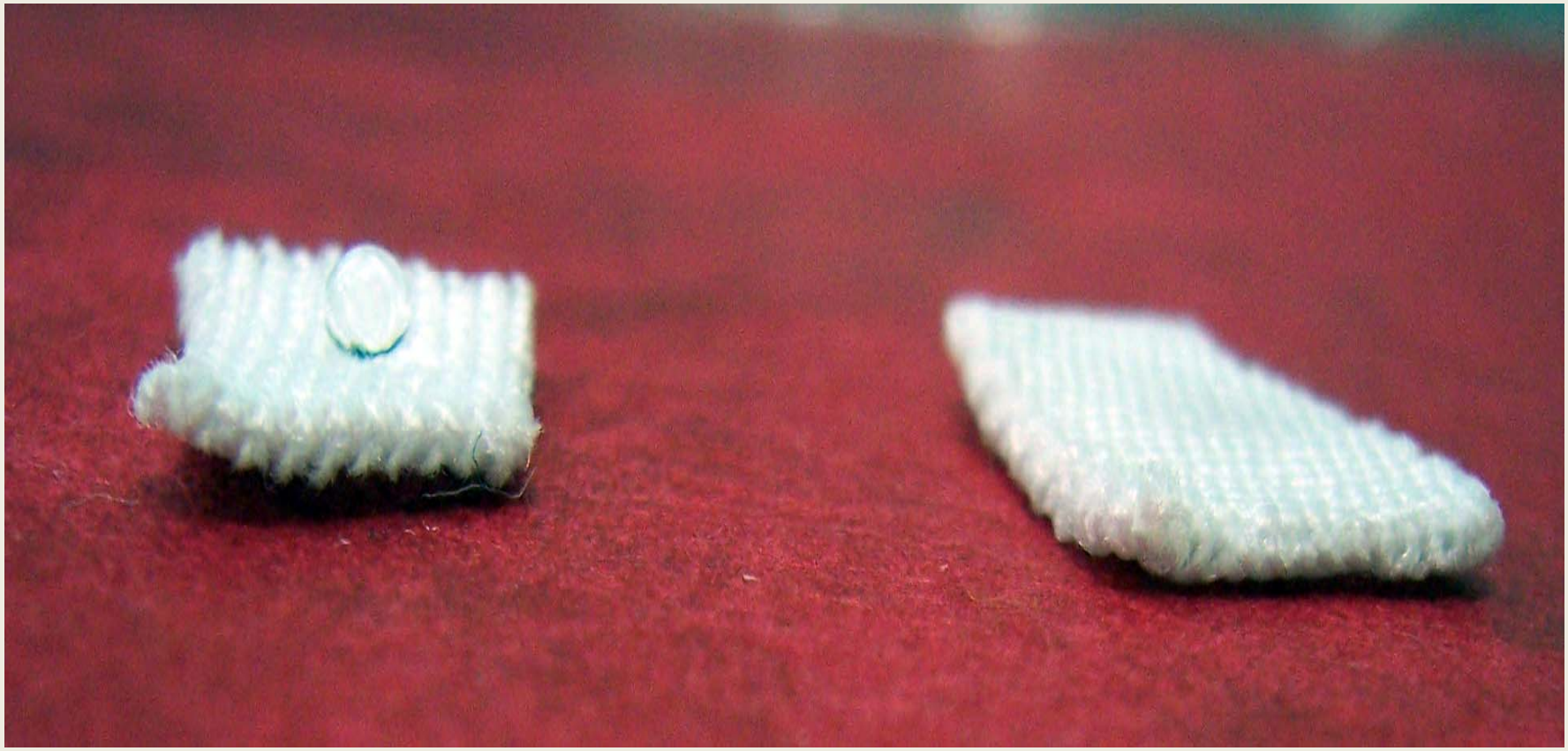
(x) EBT

(+) total released products

SEM of PET fiber surfaces (untreated, left) and PET hydrolase-treated (right)



Increased water absorption of PET textiles treated with a polyester hydrolase



Feuerhack A, Alisch-Mark M, Kisner A, Pezzin S, Zimmermann W, Andreaus J. Biocatalysis and Biotransformation, 26:357-364 (2008)

Challenges in biocatalyst development for synthetic polymer degradation

- The degradability of the synthetic polymer by an enzyme will depend on:
  - Aromatic components of the polymer
  - Accessibility of the polymer chain: degree of crystallinity
  - Efficient adsorption to the polymer surface
  - The reaction temperature > polymer chain mobility

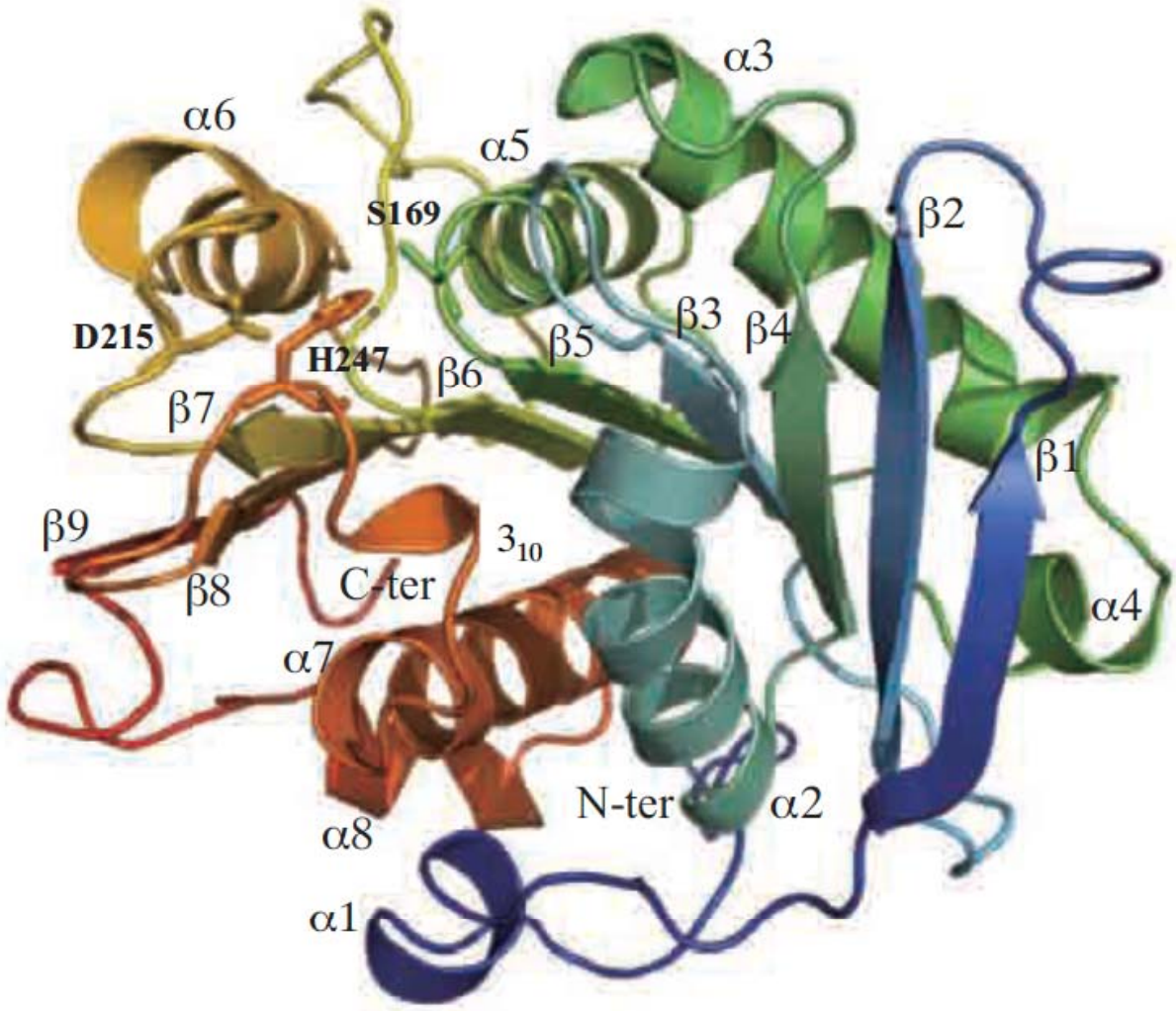
Strategies for tuning biocatalytic polyester hydrolysis activity

- Modification of the substrate binding pocket of the PET hydrolase → Identification of structural elements close to the active site responsible for polymer depolymerization

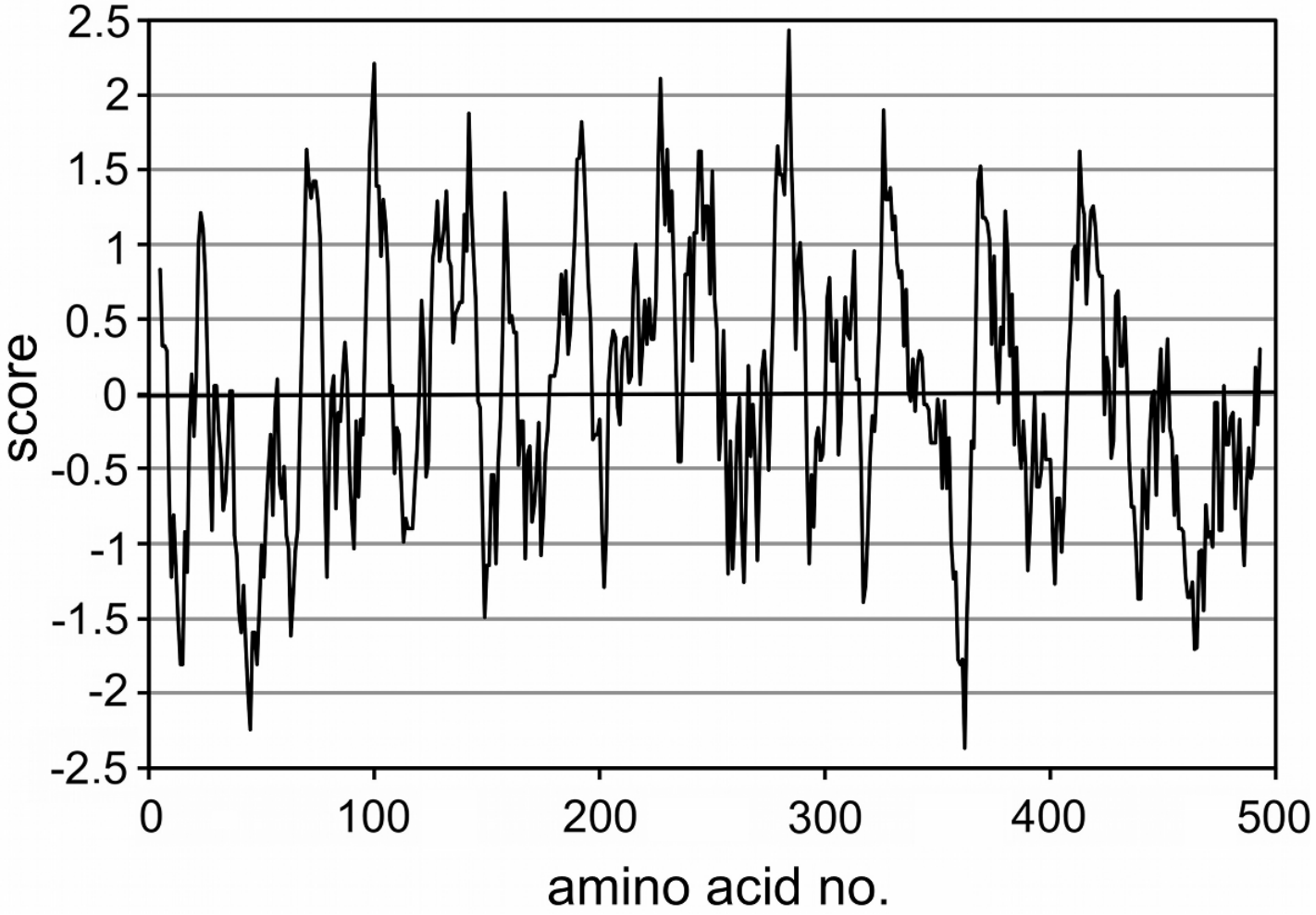
- Modification of substrate adsorption properties of the PET hydrolase → Improved interaction with the polymer chain

- Achieving PET hydrolase activity at higher temperatures → Increased polymer chain mobility and accessibility close to the glass transition temperature of PET at 67-81°C

Crystal structure of the polyester hydrolase from *Thermobifida alba*

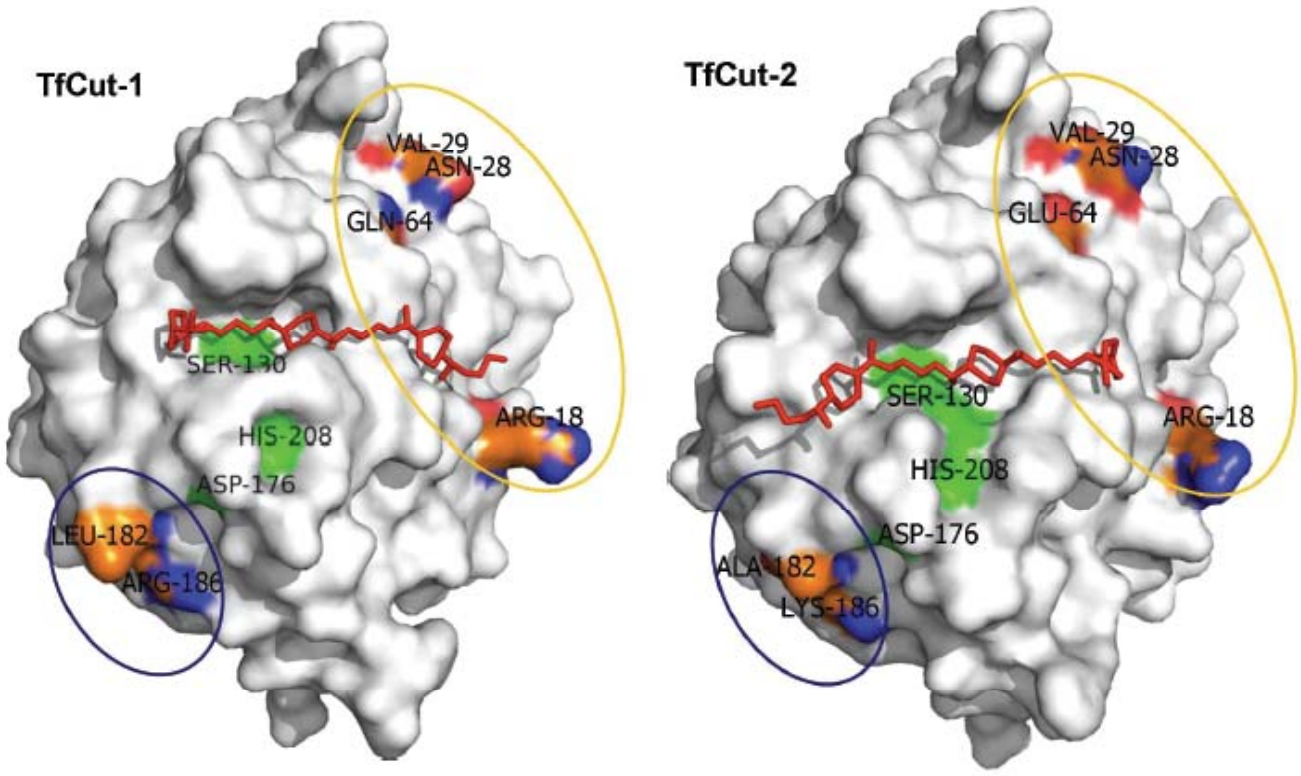


Hydrophobicity of the PET hydrolase TfCa from *T. fusca* KW3



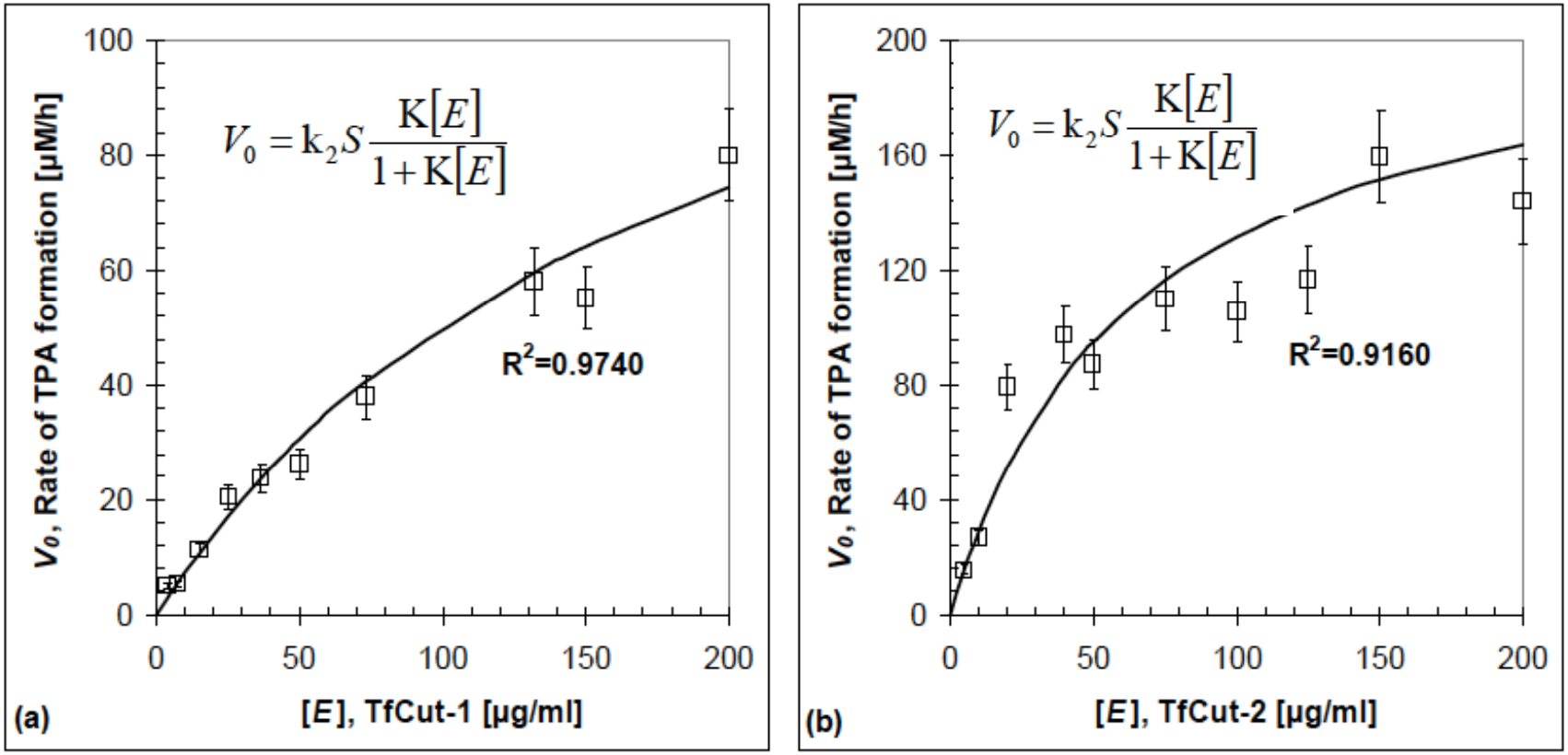
Kyte-Doolittle hydrophobicity plot of the amino acids of TfCa. Values for the amino acids above 0 indicate the hydrophobic character of the protein. The plot was prepared using ProtScale (Gasteiger et al., 2005).

Surface comparison of polyester hydrolase isoenzymes from *T. fusca*



Comparison of the surface of TfCut-1 and TfCut-2 shows two regions away from the active site with distinct hydrophobic and electrostatic properties

Initial rate of terephthalate formation from PET films by (a) TfCut-1 and (b) TfCut-2



TfCut-2 (b) shows a higher efficiency for PET hydrolysis compared to TfCut-1 (a)

Kinetic analysis of the release of terephthalate from PET films by TfCut1 and TfCut2

Variable residues on the surface of enzyme						K [ml/µg]	k_2 [nmol/cm^2/h]
No. AA	28	29	64	182	186		
TfCut1	Asn	Val	Gln	Leu	Arg	0.0054±0.0007	11.01±1.12
TfCut2	Asn	Val	Glu	Ala	Lys	0.0158±0.0038	16.56±3.39

TfCut2 shows a 3-fold higher adsorption equilibrium constant K and a 1.5-fold higher hydrolysis rate constant k_2 than TfCut-1, suggesting a higher affinity to PET films and also a higher hydrolysis rate compared to TfCut1

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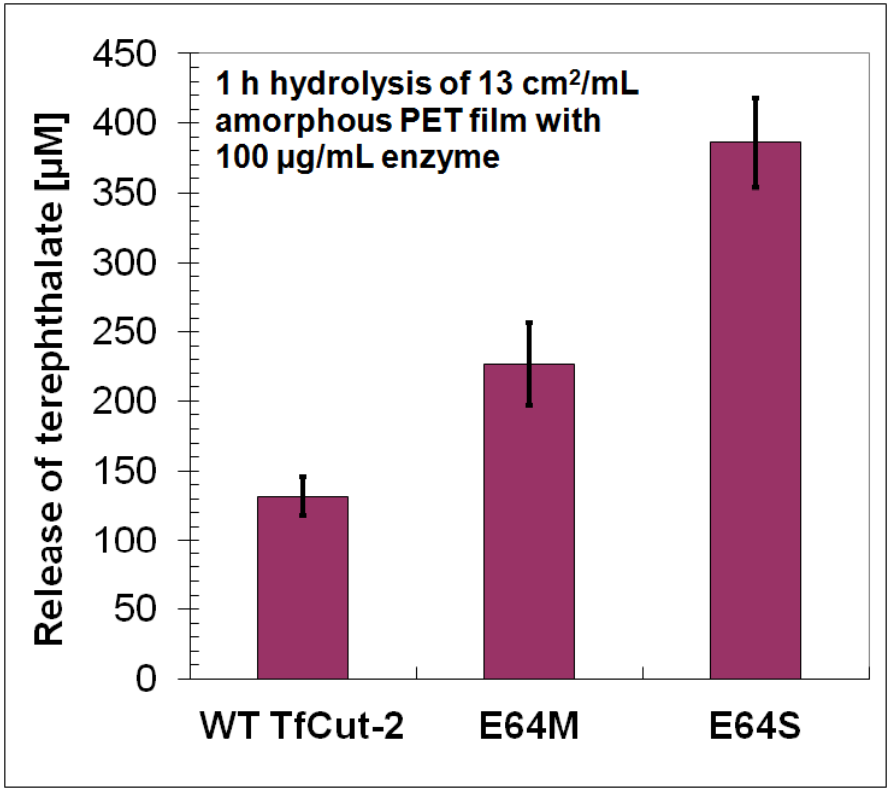
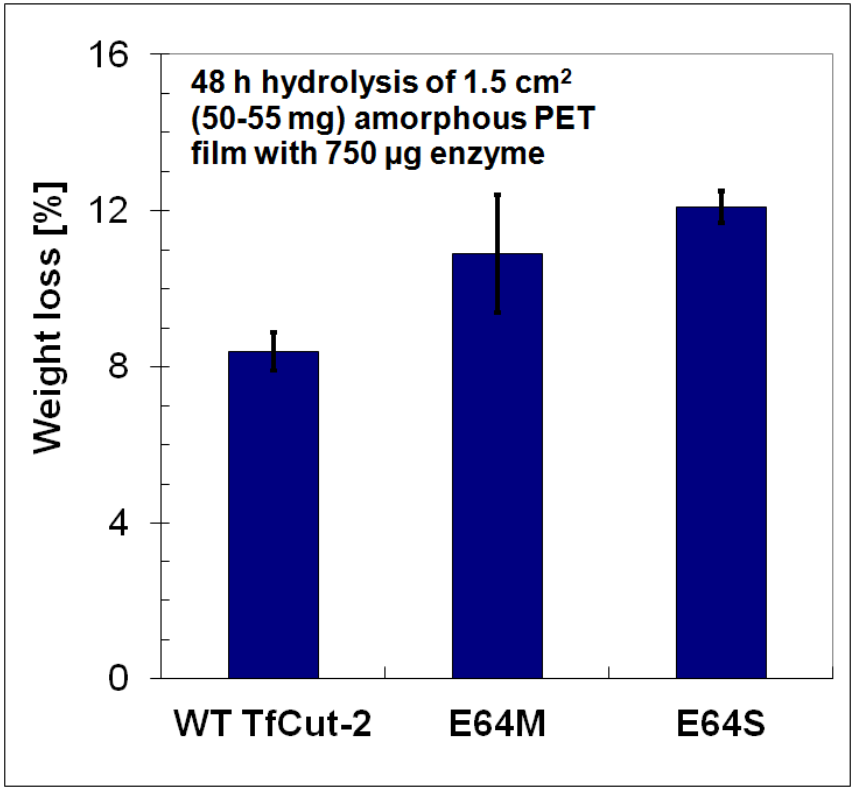
Site saturation mutagenesis of TfCut2 at Glu64, Ala182 and Lys186

Improved PET hydrolysis activity by TfCut2 variants

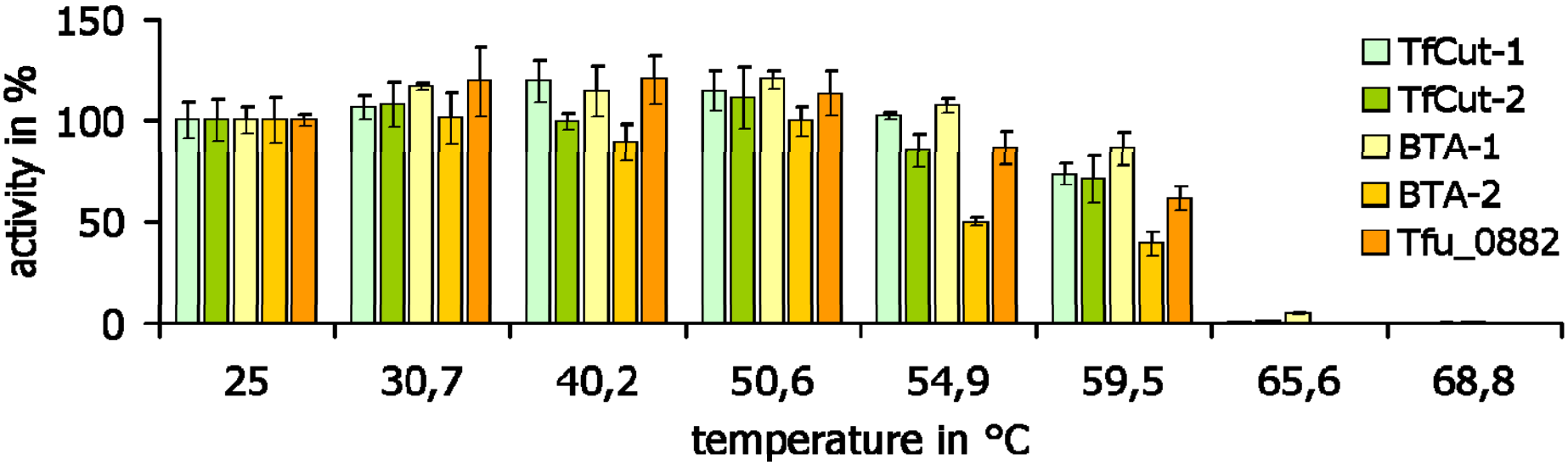




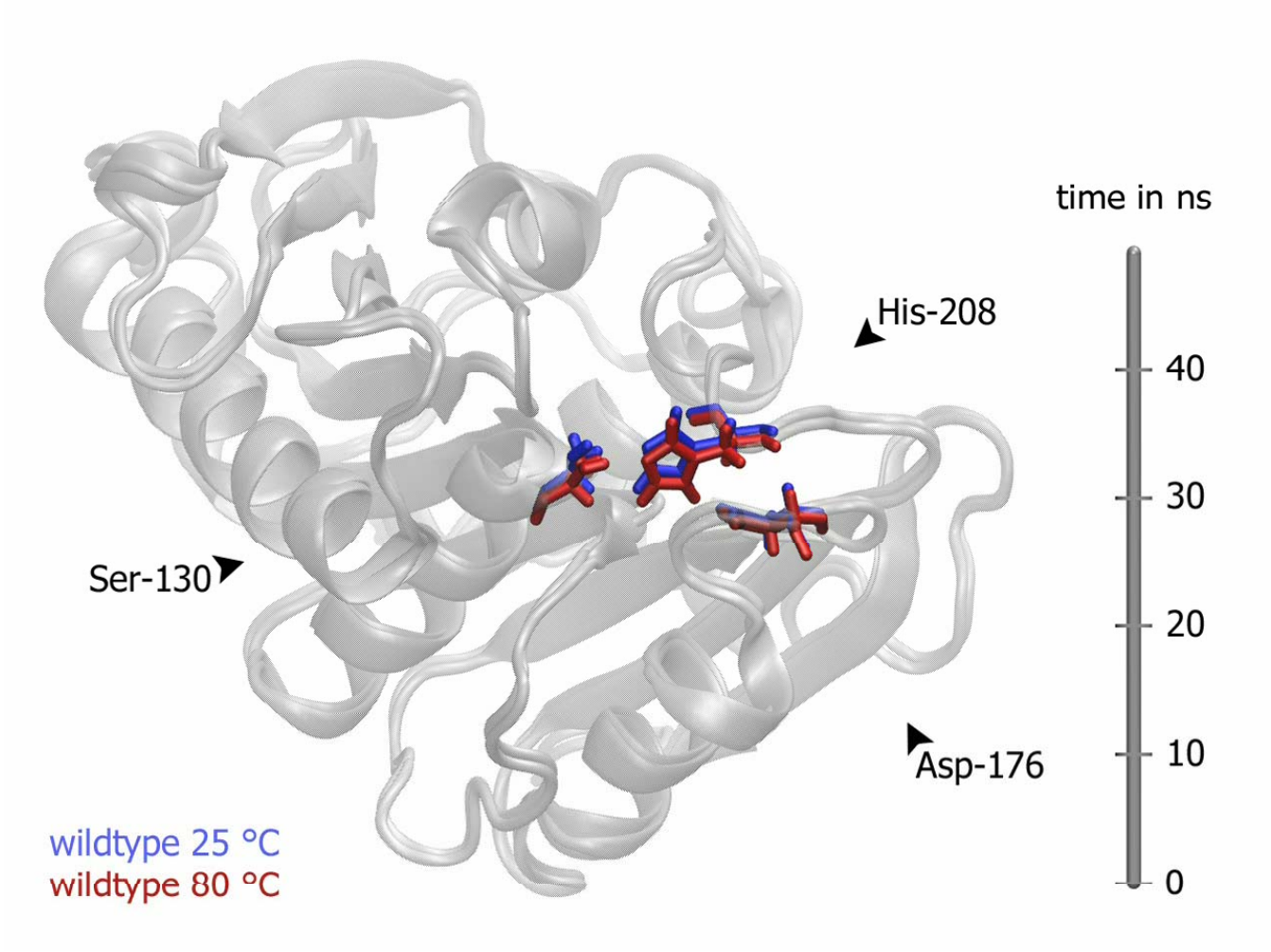
Compared to the wild-type TfCut2 the variants Glu64Met and Glu64Ser showed

- 1.7- and 2.9-fold higher amounts of terephthalate released
- 1.3- and 1.4-fold higher weight loss from PET films

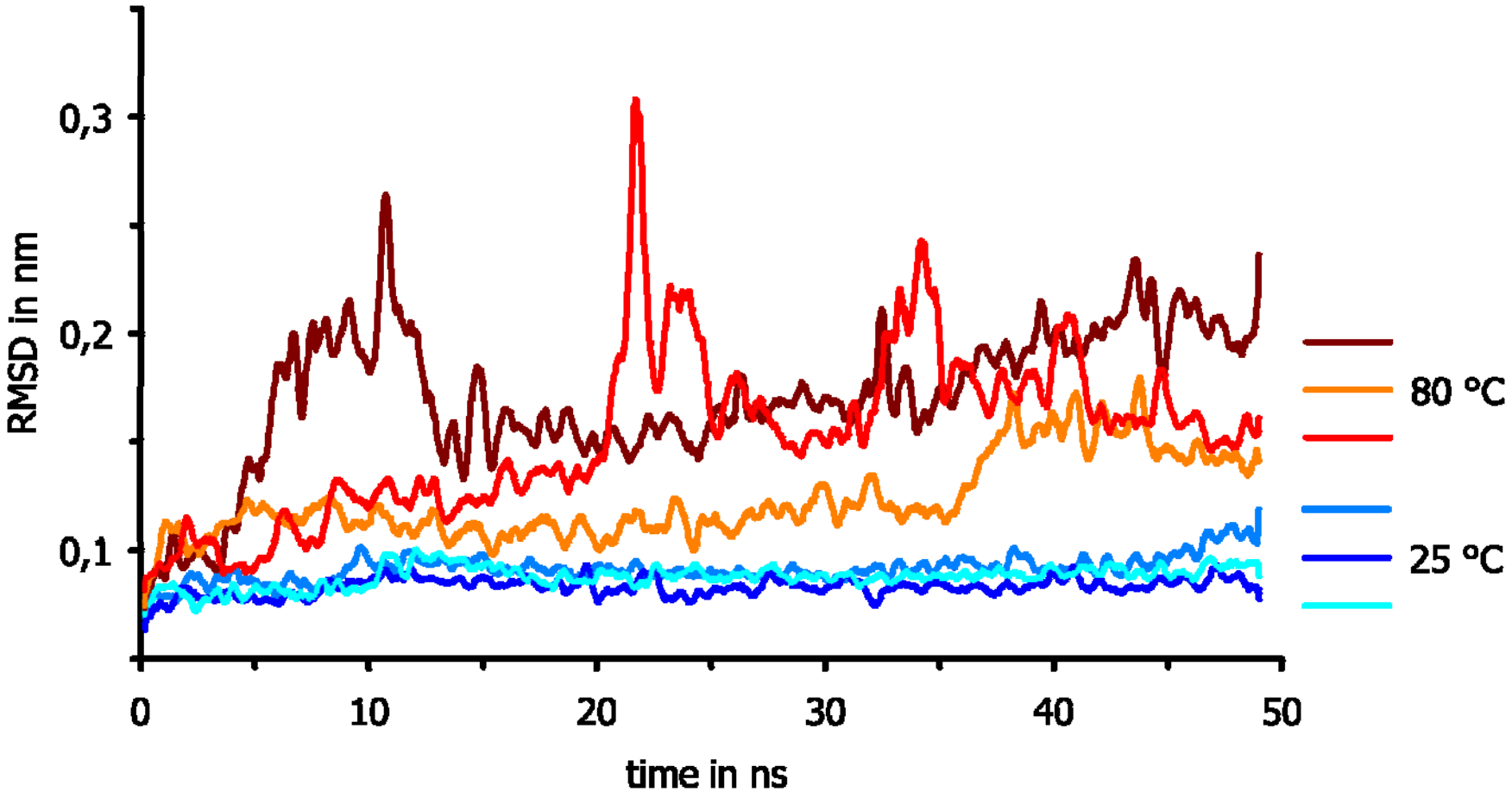
Thermostability of wild-type polyester hydrolases from thermophilic actinomycetes



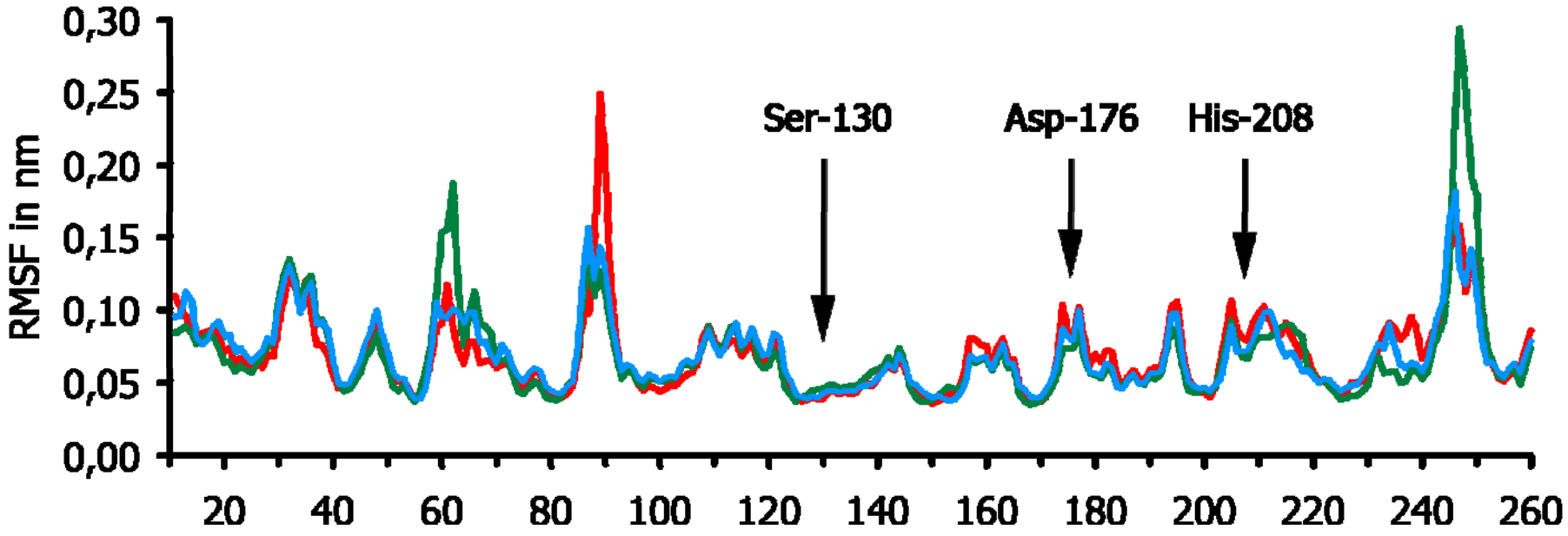
In silico prediction of polyester hydrolase thermostability



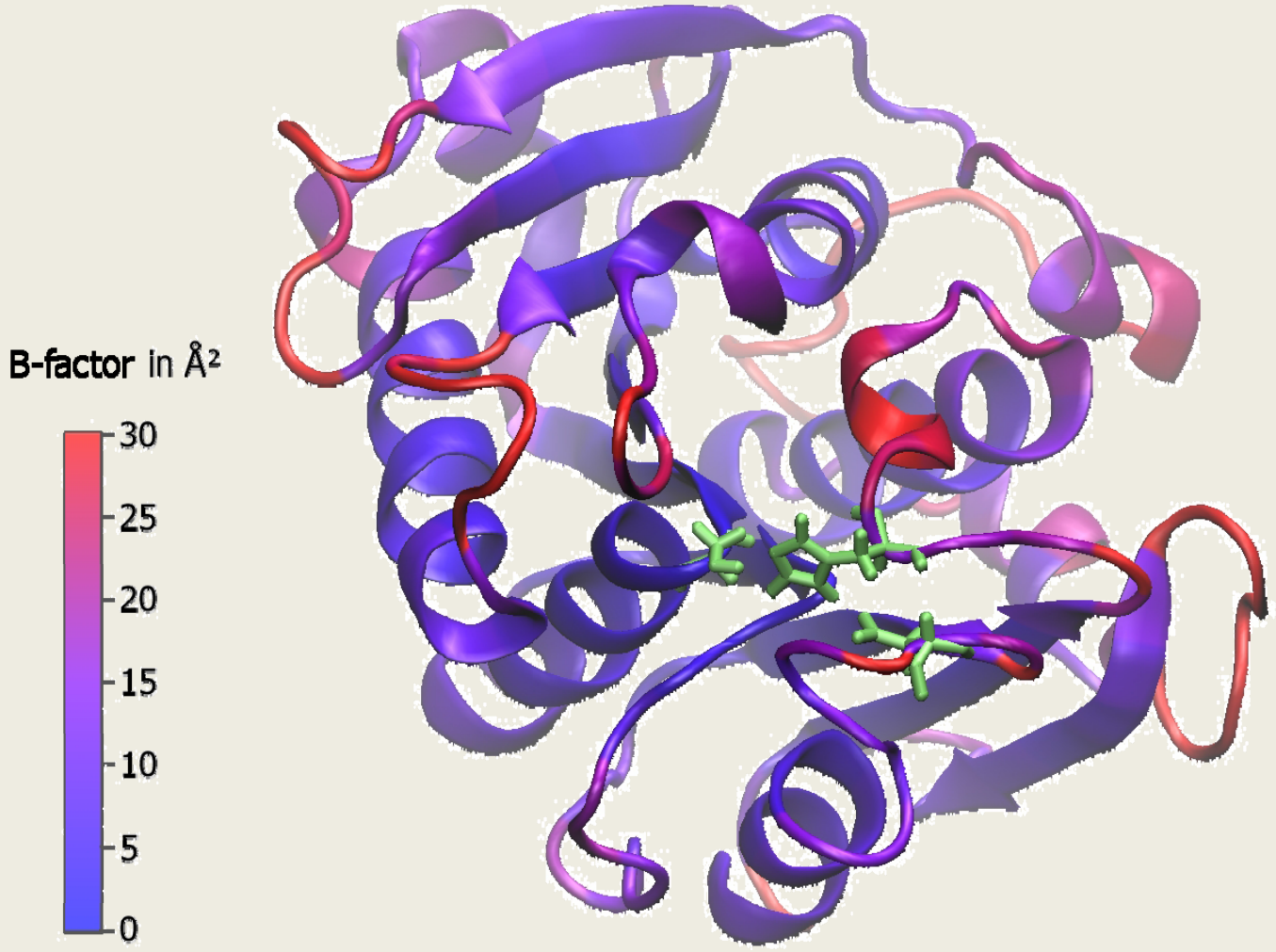
Identification of flexible areas in the polyester hydrolase structure



Flexibility profile of single amino acids of the polyester hydrolase



Selection of mutation sites for the polyester hydrolase



B-factor = influence of temperature, calculated from RMSF data

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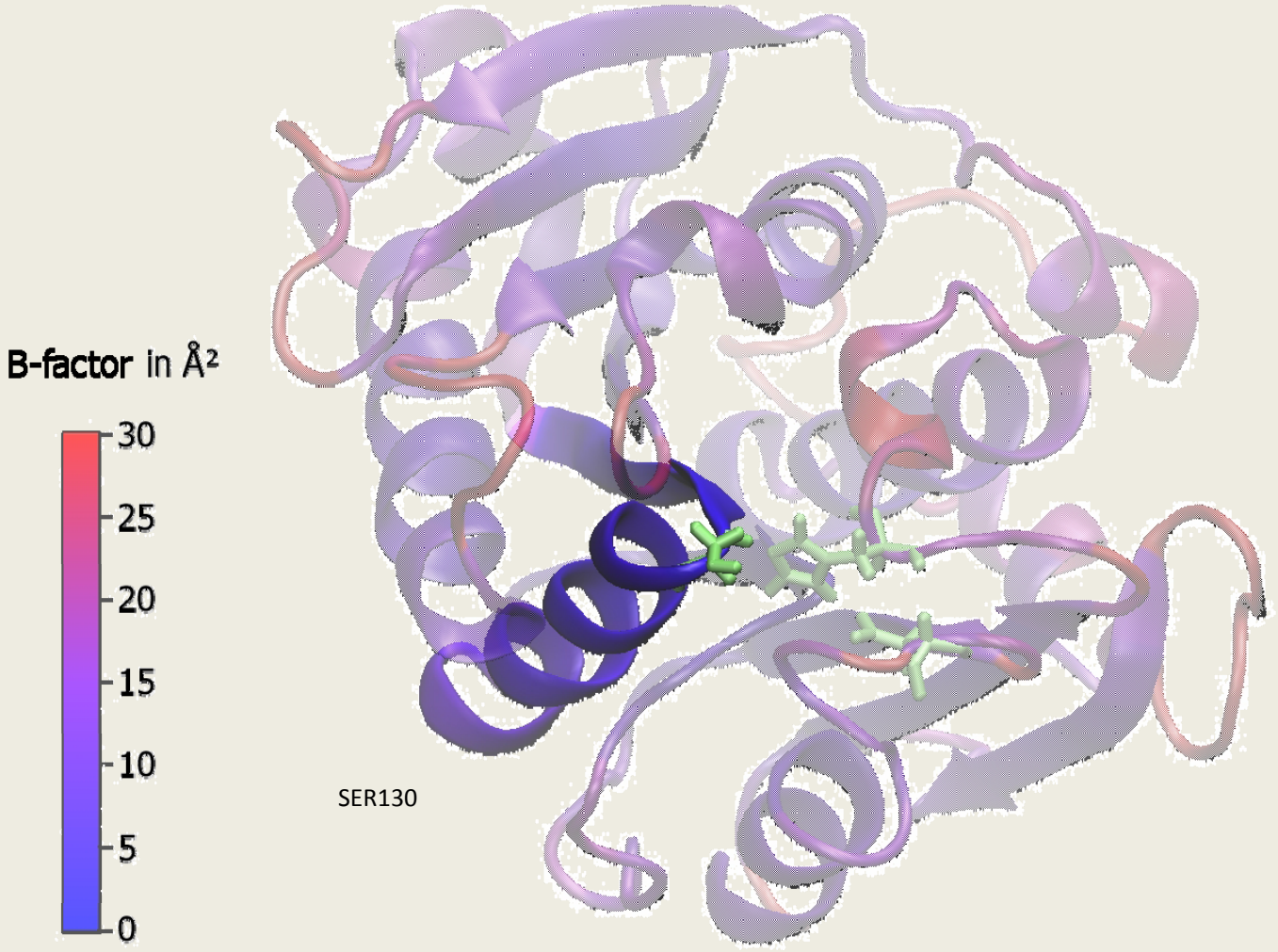

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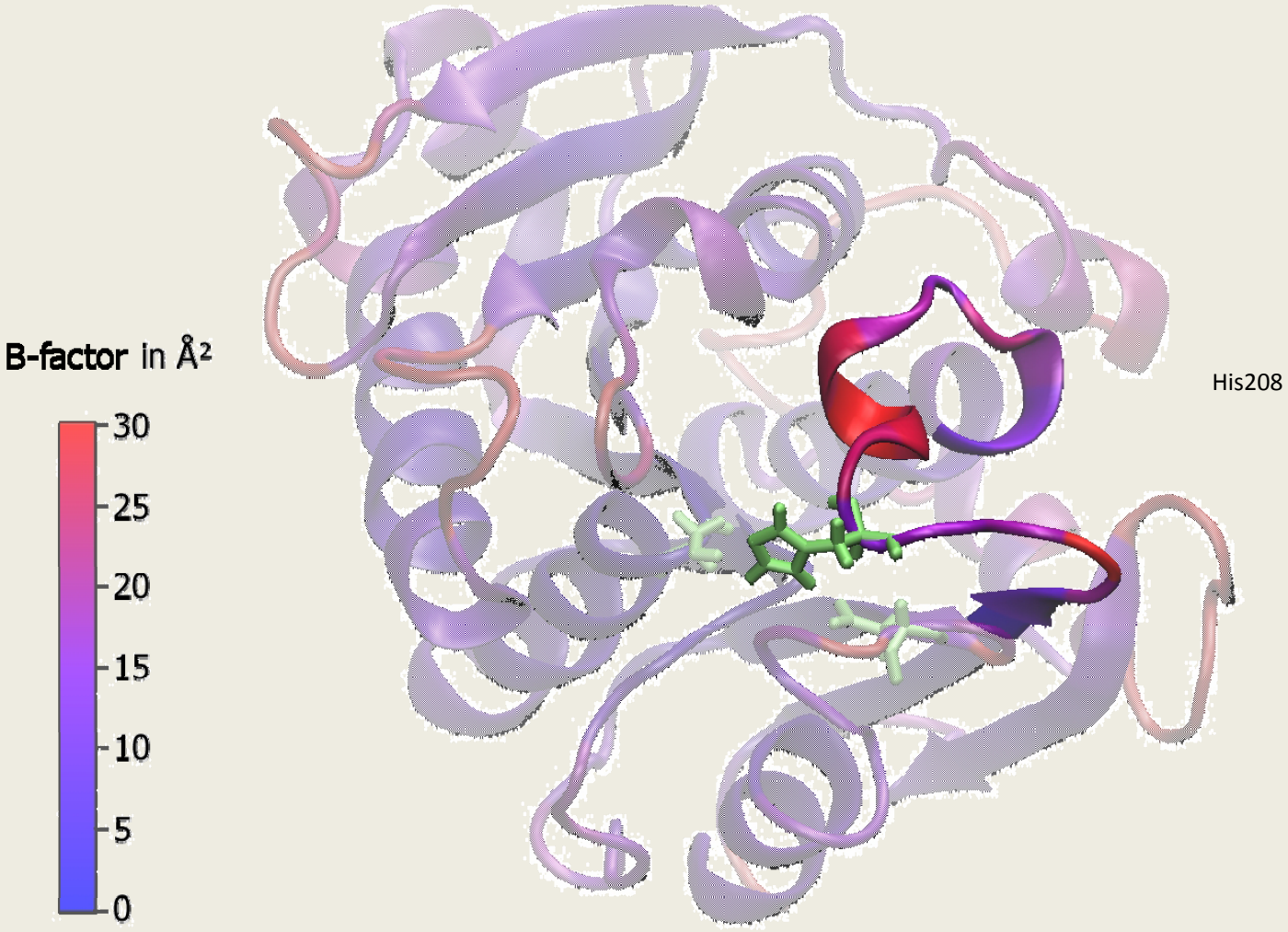

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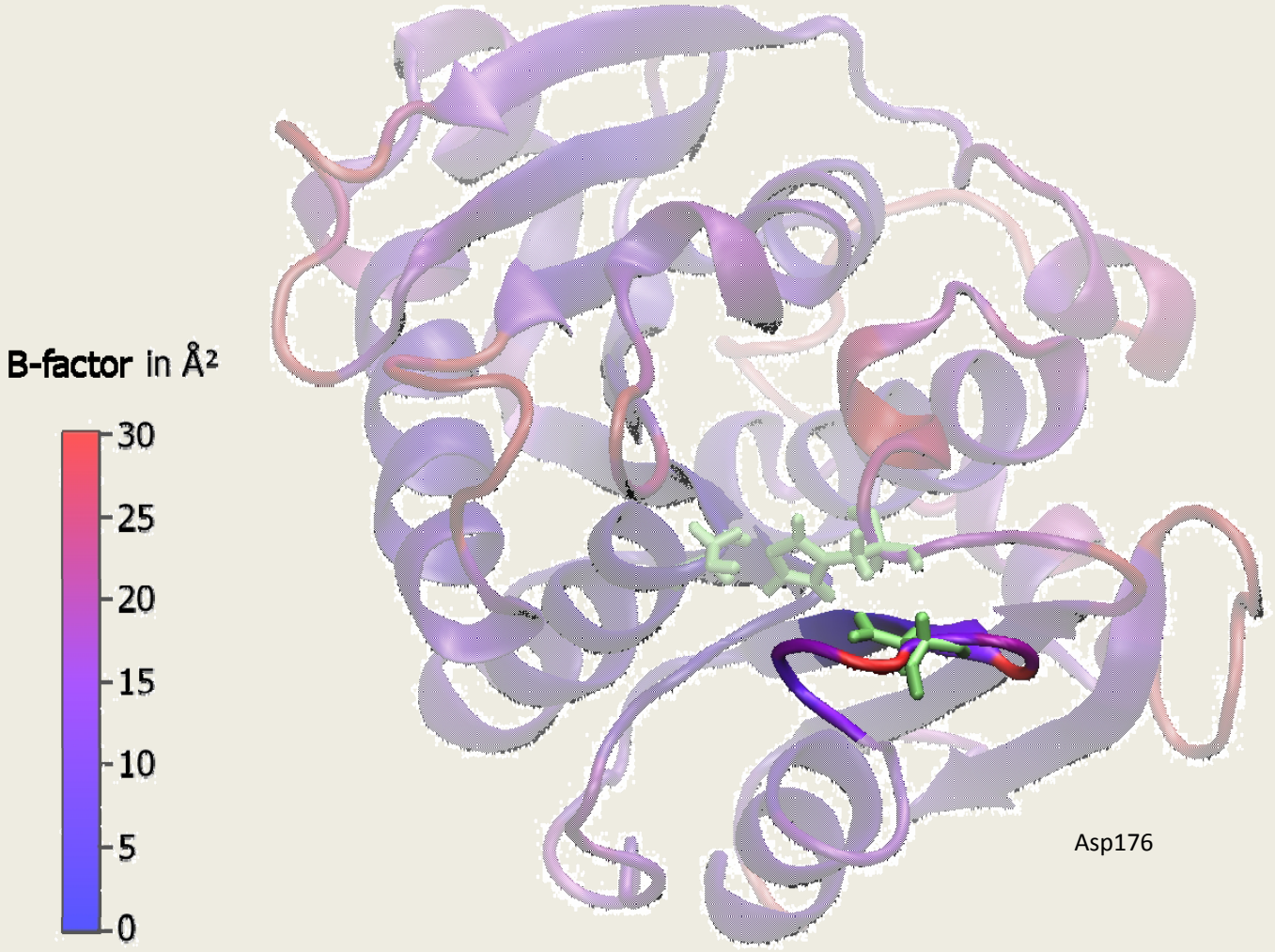

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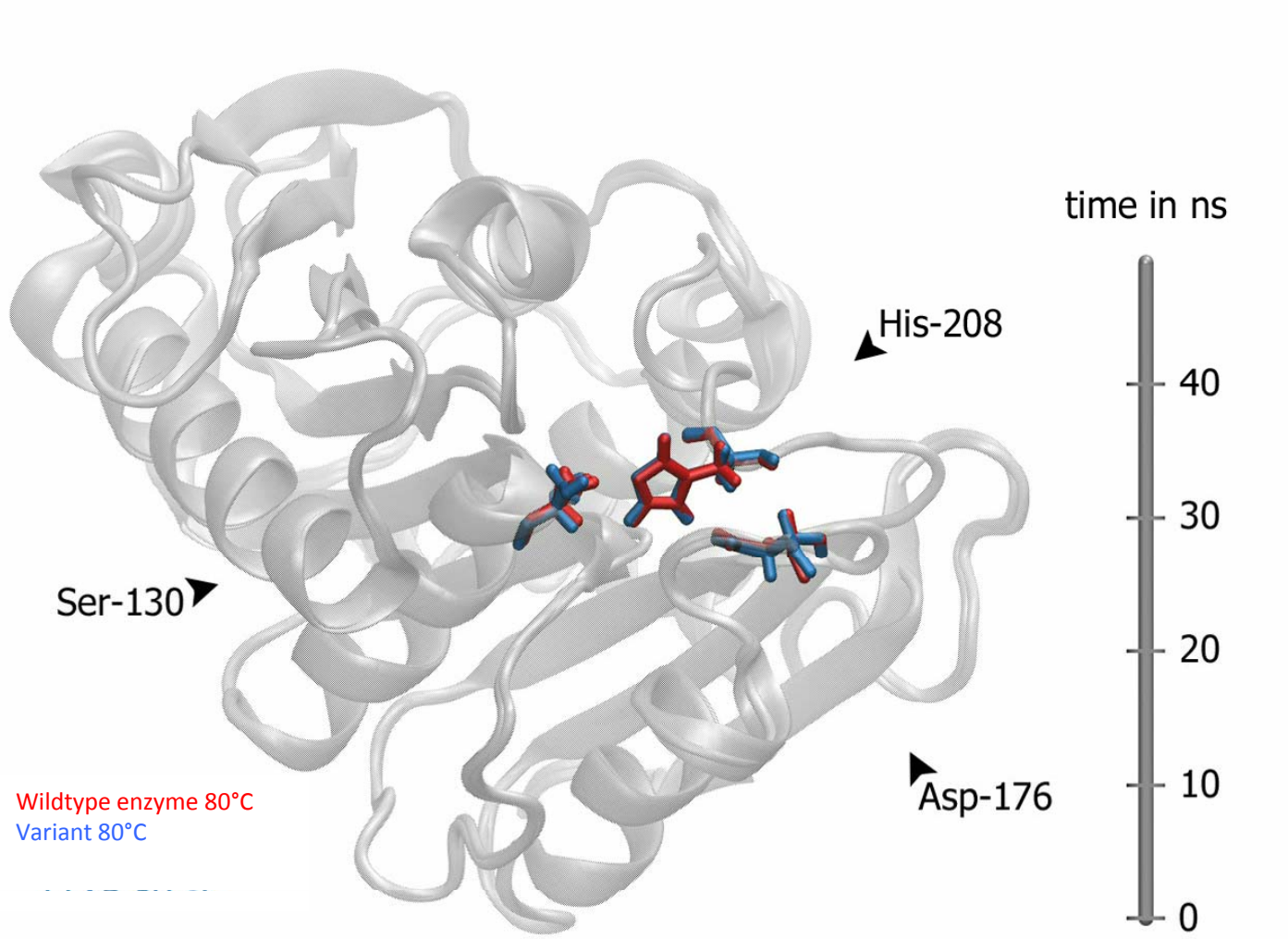
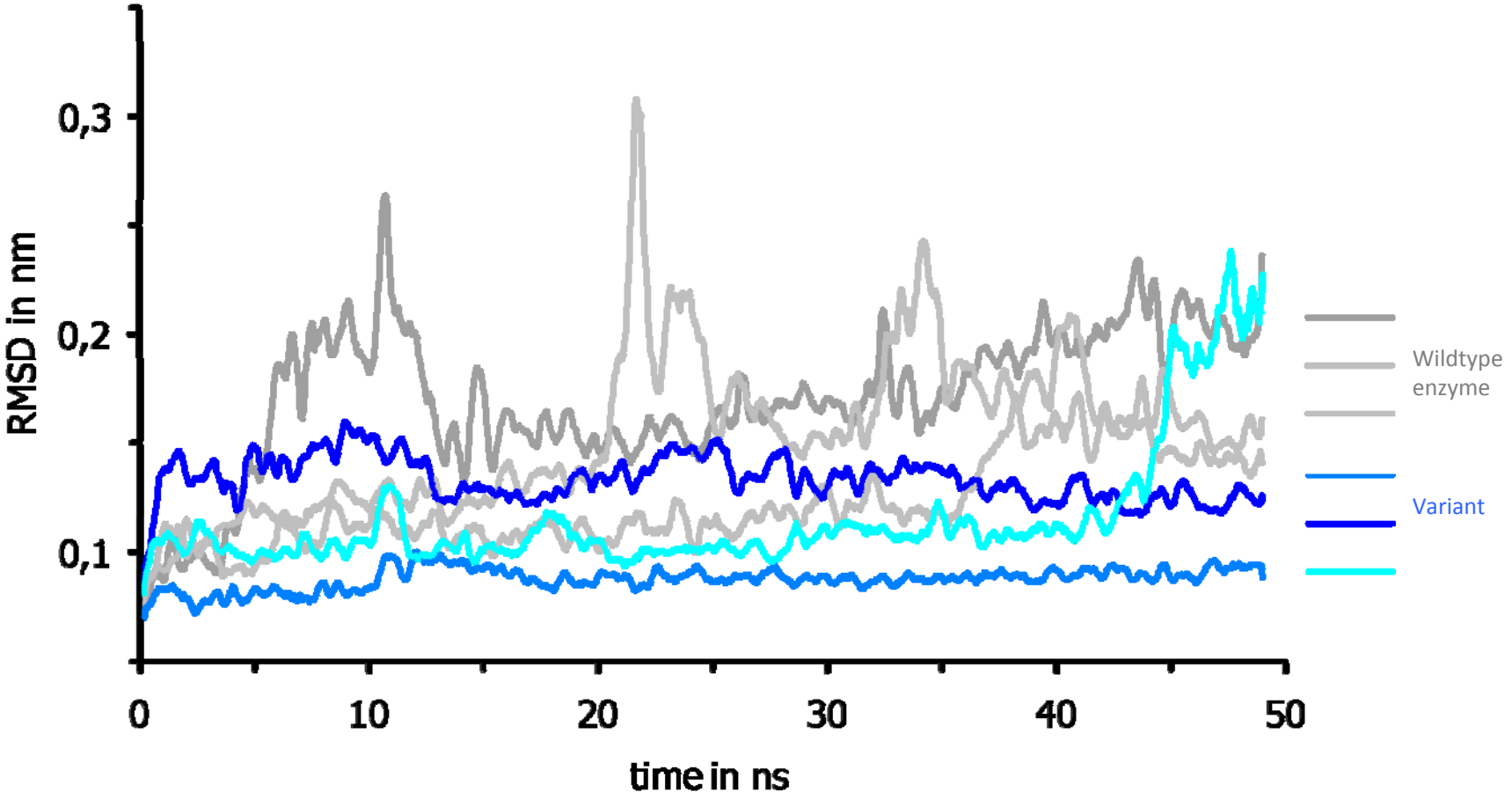
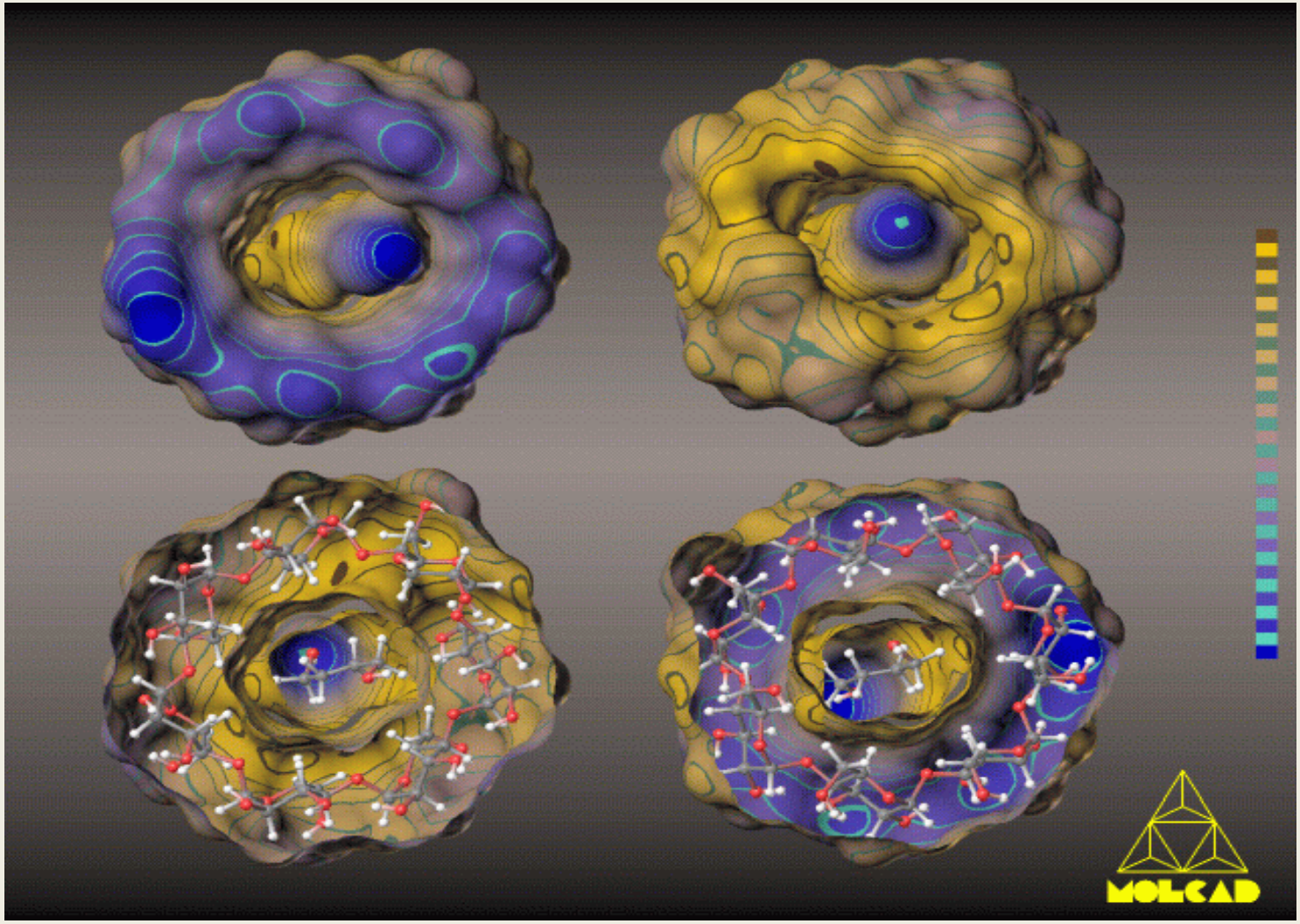
In silico prediction of thermostability of polyester hydrolase variants



Flexibility profile of the wildtype polyester hydrolase and the variant at 80°C



High value fine chemicals from renewable biomass: cyclodextrins



Renewable biomass: starch

Manihot esculenta Crantz

Biocatalytic starch modification: carbohydrate bioengineering

- Established industrial glucose and fructose production processes are using enzymes for the hydrolysis of starch
- By the utilization of specific enzymatic modification and synthesis reactions, carbohydrates with defined functional properties for new or improved products and processes can be obtained

Cyclodextrins

- Naturally occurring cyclic α-1,4-glucans
- Produced by biocatalytic synthesis from starch substrates
- The molecular structure of cyclodextrins resemble a truncated cone with a hydrophobic cavity
- Cyclodextrins can form inclusion complexes with guest molecules

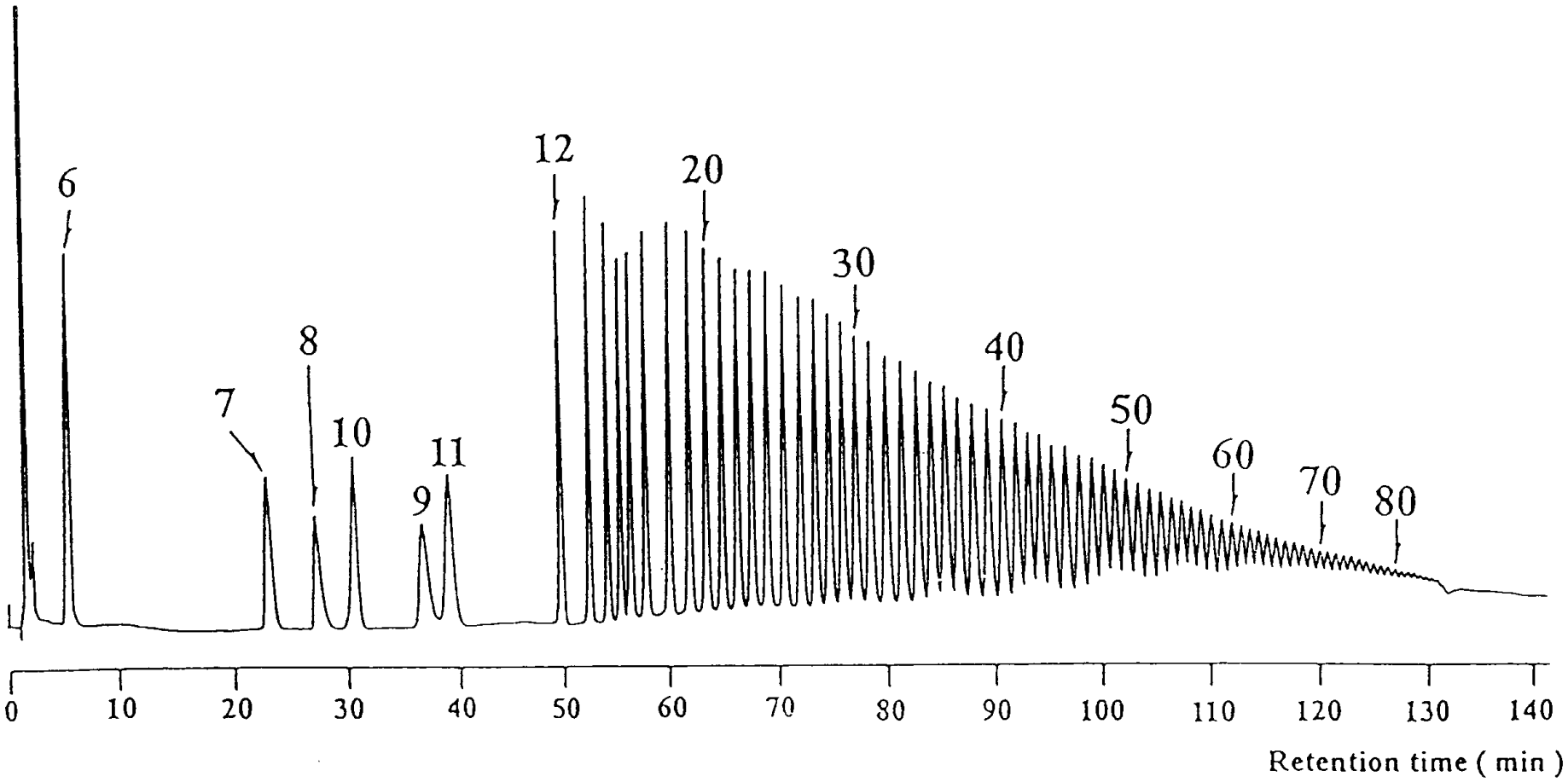
Enzymes synthesizing cyclodextrins

- Cyclodextrin glucanotransferase (CGTase, E.C. 2.4.1.19)
- Amylomaltase and D-enzyme (E.C. 2.4.1.25)
- 1,4-α-Glucan branching enzyme (E.C. 2.4.1.18)
- Glycogen debranching enzyme (GDE, E.C. 3.2.1.33)

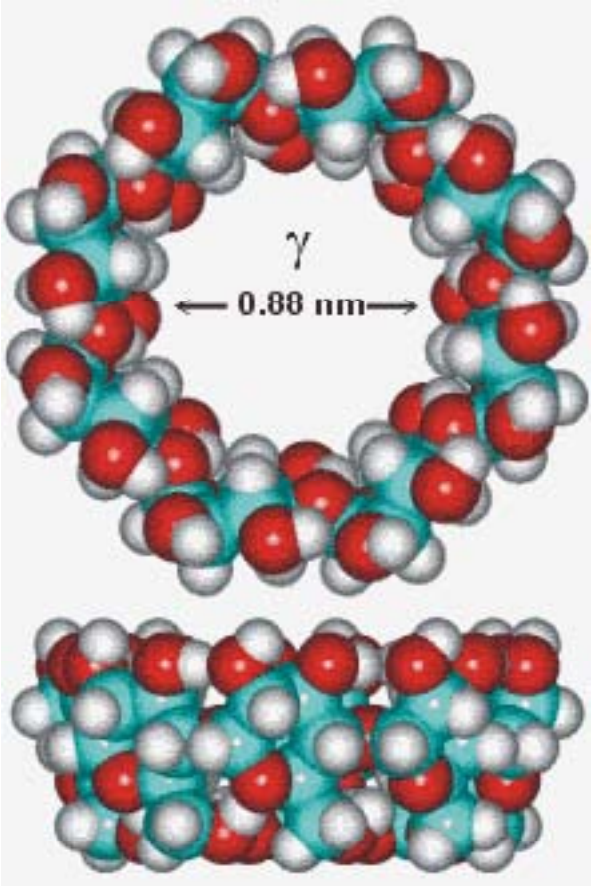
Specificity of biocatalytic cyclodextrin synthesis

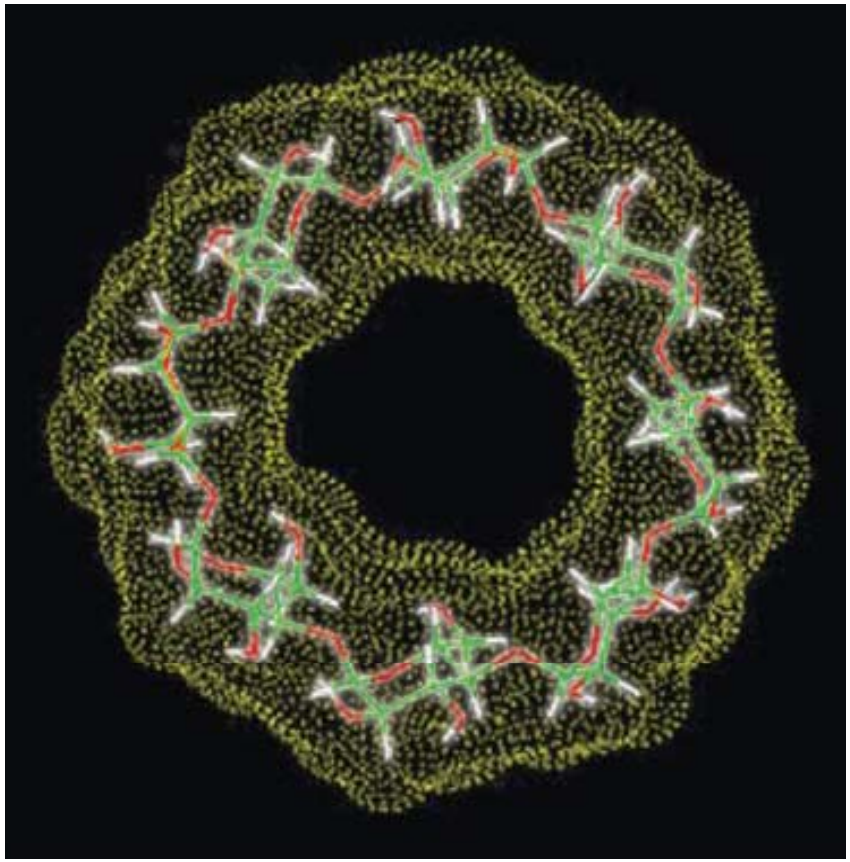
- The size and yield of the cyclodextrins obtained is depending on:
  - The selected starch substrate (branched/unbranched)
  - The balance of the different transferase and hydrolytic reactions catalyzed by the CGTase
  - Product specificity of the CGTase

HPAEC elution profile of cyclic α-1,4-glucans produced by CGTase from *Bacillus macerans*



Gamma-cyclodextrin (CD_8)





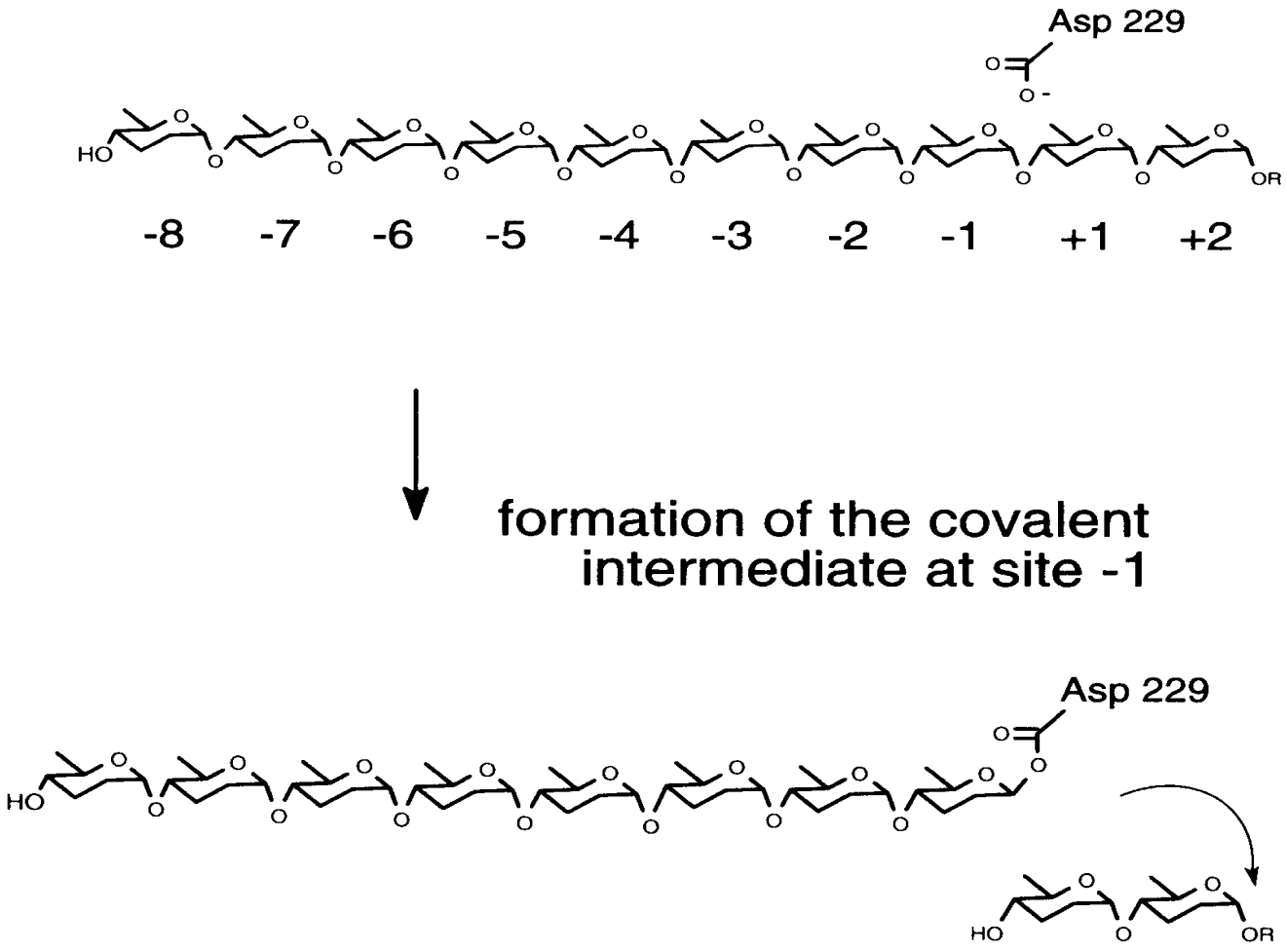
Gamma-cyclodextrin compared to the smaller alpha- and beta-cyclodextrins

- Larger internal cavity
- Accommodation of a wider range of large organic compounds such as macrocycles and steroids
- more flexible structure and much higher solubility in water (232 g/l, 25° C)
- Rapidly digested by human salivary amylase and pancreatic amylase
- Novel applications in the food and pharmaceutical industries

Screening for a suitable biocatalyst: a Gamma-CGTase from *Bacillus* sp.

- While most natural CGTases produce cyclodextrin mixtures, a few have shown a preference for CD_8 formation
- The alkalophilic *Bacillus* sp. G-825-6 produces a CGTase forming mainly CD_8
- Target: further improvement of the product specificity of the CGTase by site-directed mutagenesis

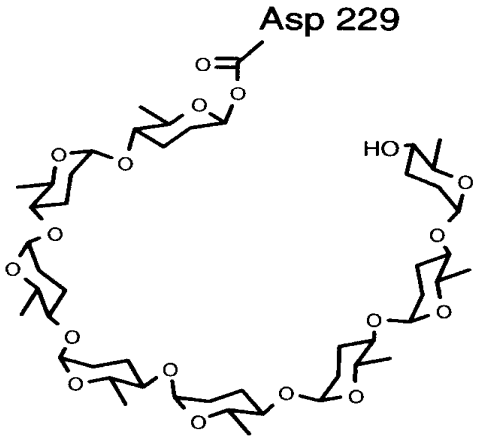
CGTase cyclization reaction I



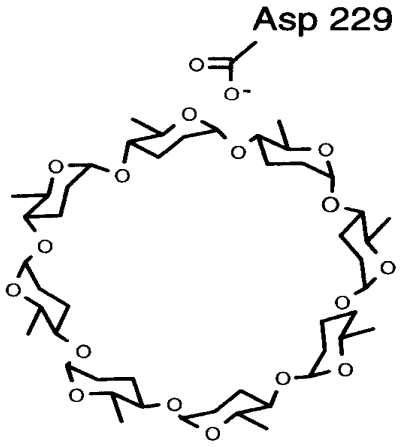
Uitehaag et al. 2000

CGTase cyclization reaction II

circularization

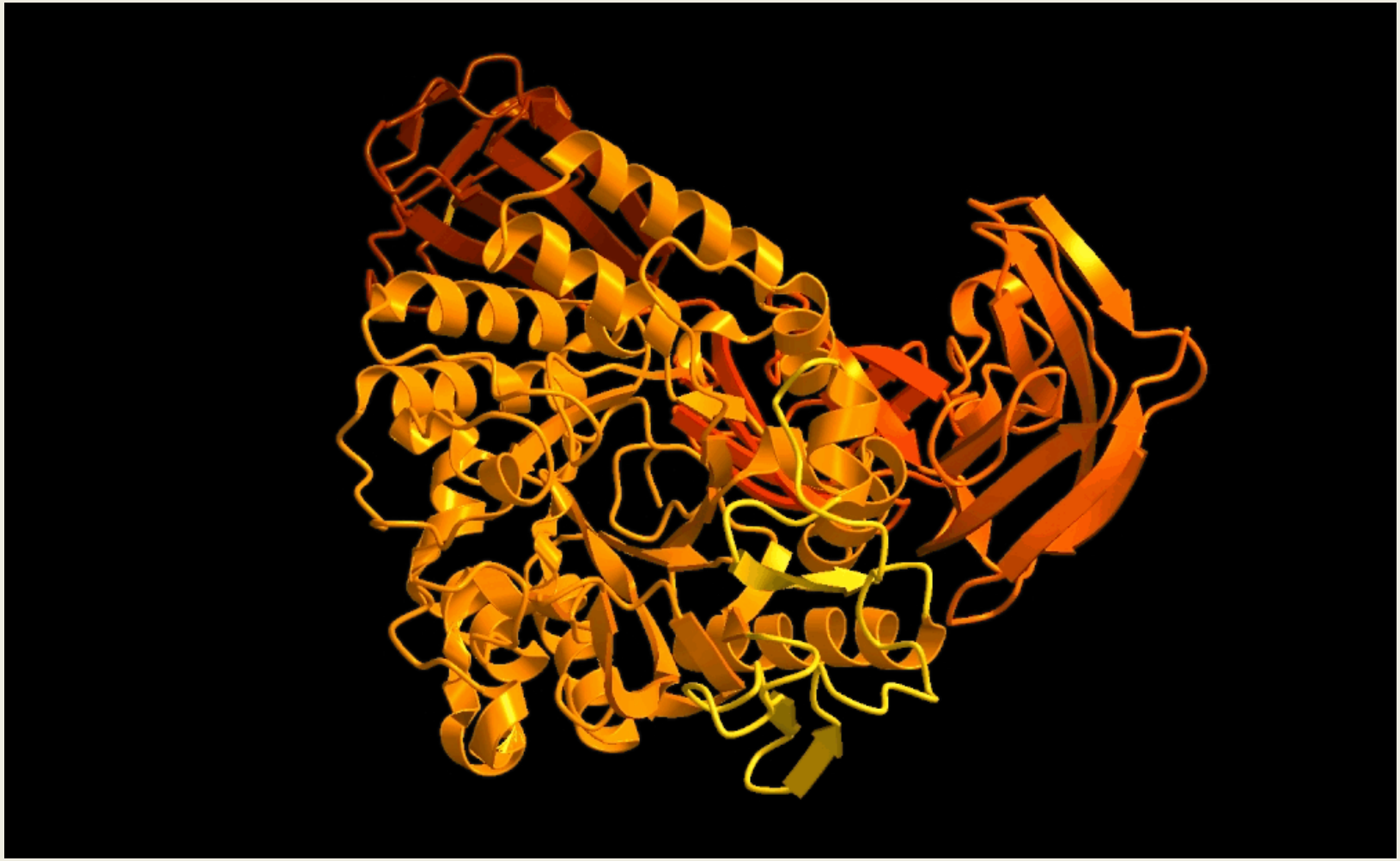


formation of a γ-cyclodextrin product



Uitehaag et al. 2000

Structure of the CGTase from *Bacillus circulans*

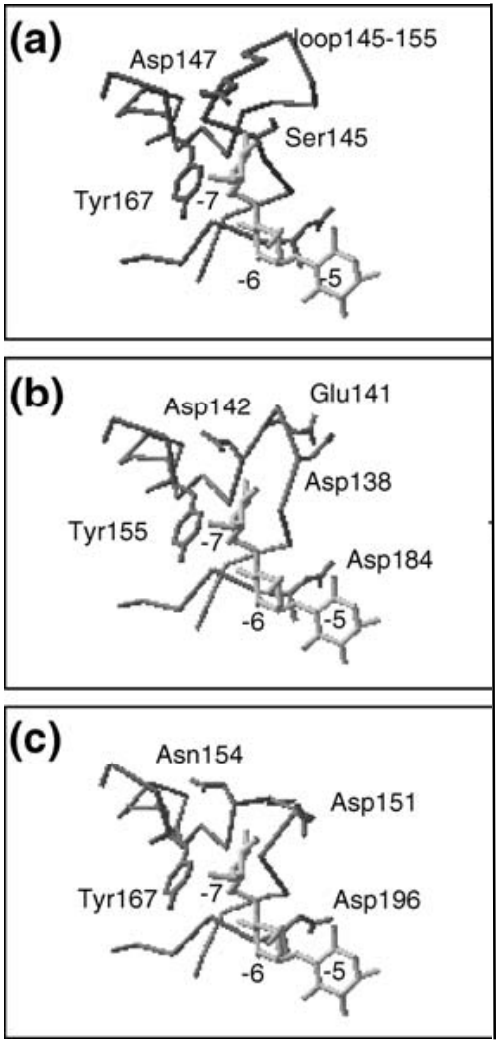


Uitehaag et al. 2000

Structural features involved in product specificity for CD_8 formation

- The active center of CGTases contains at least nine glycosyl binding subsites designated as −7 through +2
  - Loop AS 145–152 (numbering in *B. circulans* 251 CGTase) at subsite −7 > only two AS in ɣ-CGTases
  - Subsite −3, AS 47 and 87–94 (numbering in *B. circulans* 251 CGTase) > replaced by Threonin in ɣ-CGTases
  - Loop AS 87–93 and 94 > shorter in ɣ-CGTases
  - The changes create more space to accommodate CD_8 synthesis

Gluco-oligosaccharide interaction with subsite -7 in three CGTases



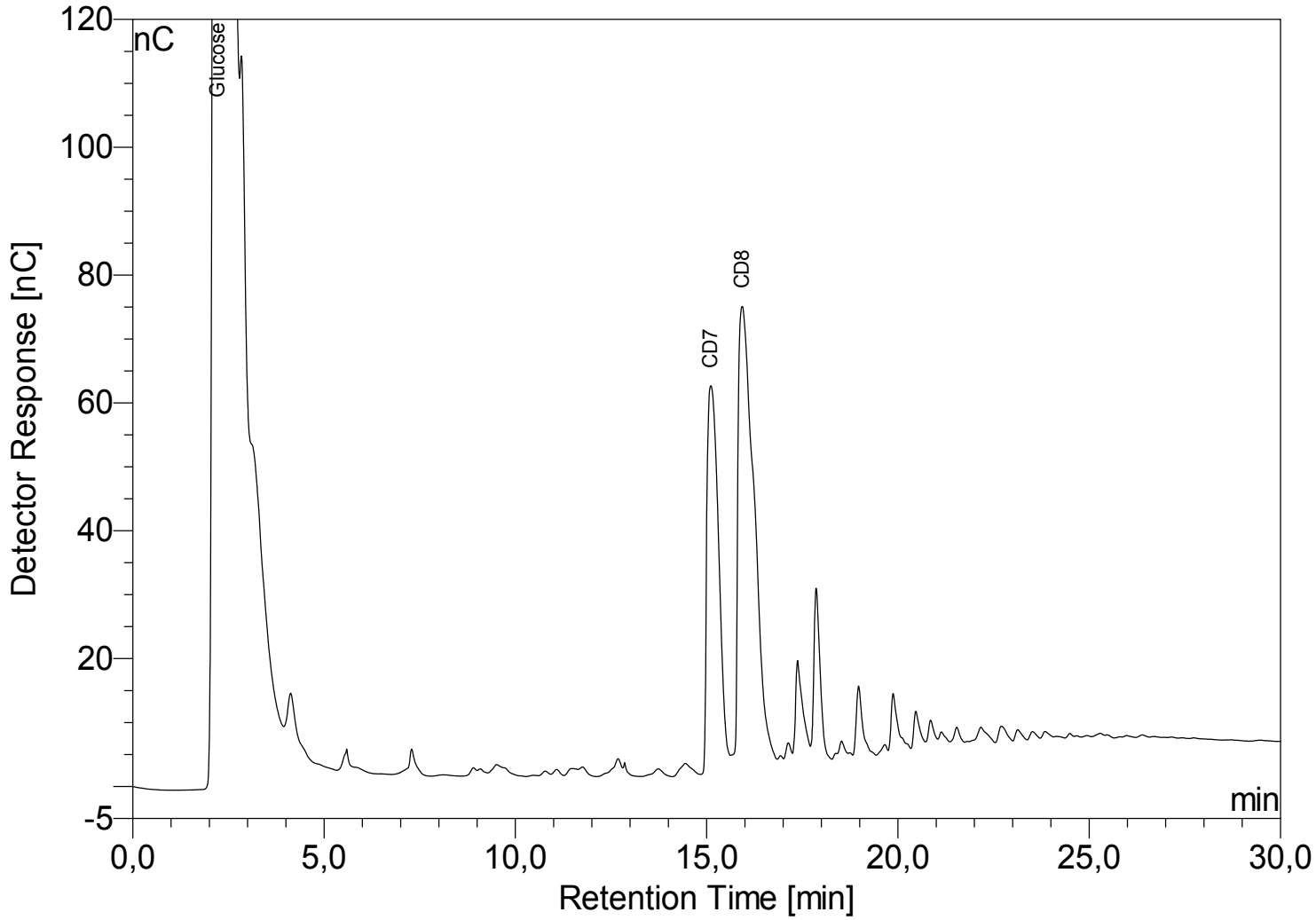
B. circulans 251 beta-CGTase
(Strokopytov et al. 1996)

Bacillus G-825-6 gamma-CGTase
(Hirano et al. 2009)

B. circulans No. 8 Δ(145–151)D-CGTase variant
(Parsiegla et al. 1998)

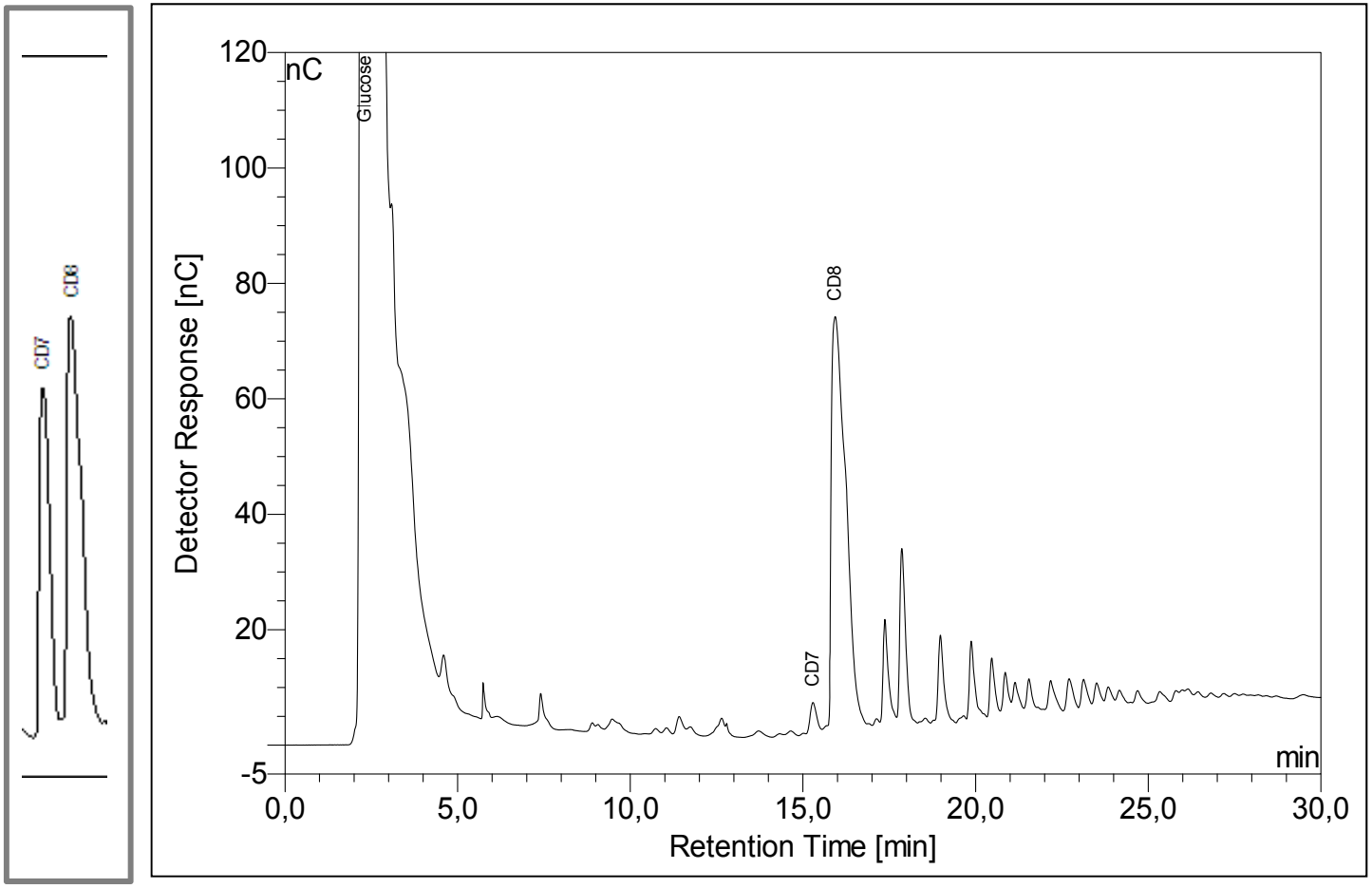
Hirano et al. Appl Microbiol Biotechnol (2006) 70: 193–201

Cyclodextrin products obtained with the wild-type gamma-CGTase

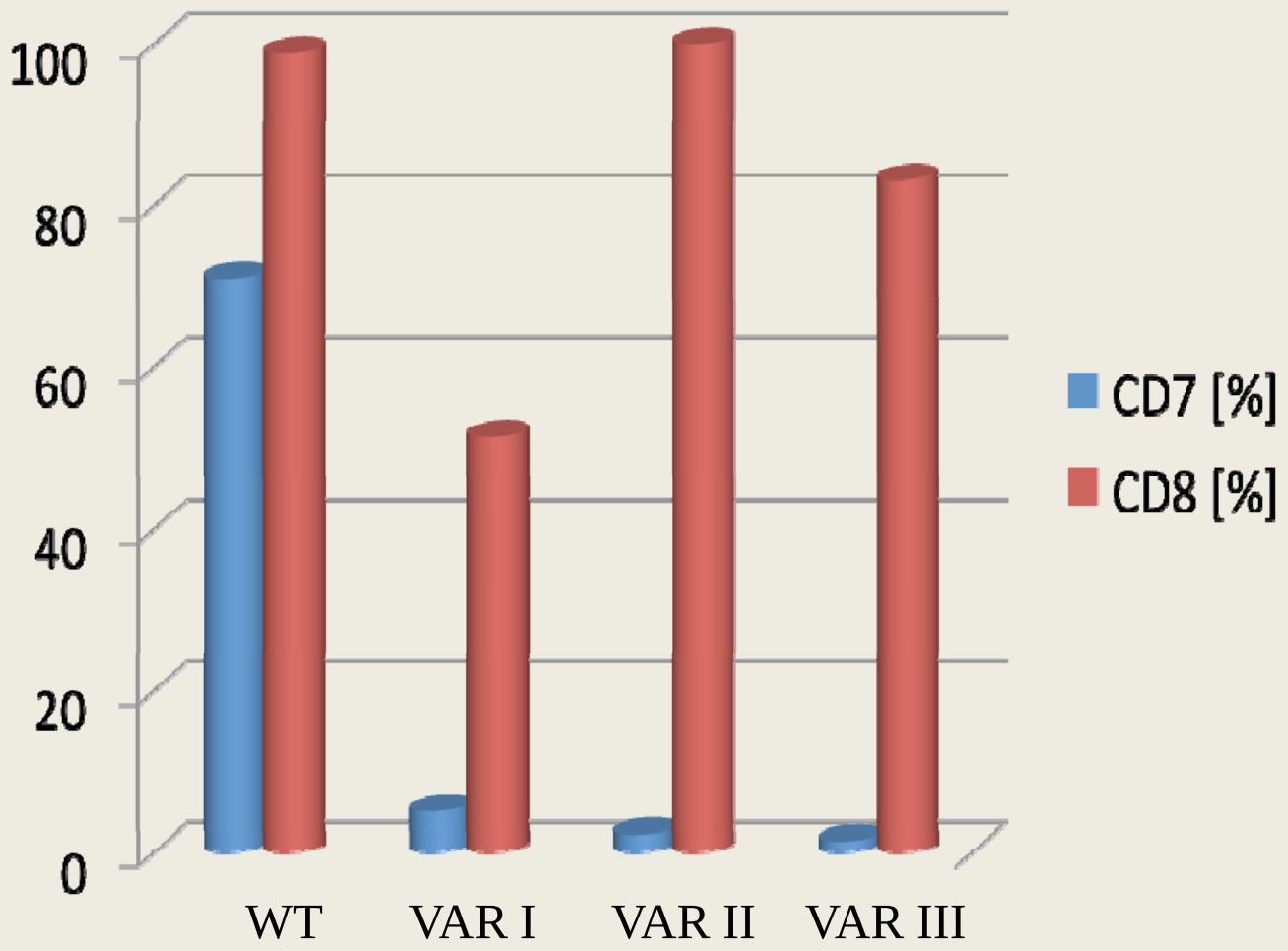


Cyclodextrin products obtained with the gamma-CGTase variant II (single mutation)

WT



Change of product specificity of the gamma-CGTase by rational design



Cyclodextrins: application examples

- Food industry
  - Stabilization and modification of food components, i.e. aroma development and release, gel formation
- Pharmaceutical industry
  - improve solubility of hydrophobic compounds
  - Drug delivery systems
- Nanobiotechnology
  - Molecular recognition for nanoscale applications and sensor techniques

Marketed pharmaceutical products containing cyclodextrins

Drug/cyclodextrin	Trade name	Formulation	Company (country)
α-Cyclodextrin (αCD)			
Alprostadil	Caverject Dual	Intravenous solution	Pfizer (Europe)
Cefotiam-hexetil HCl	Pansporin T	Tablet	Takeda (Japan)
Limaprost	Opalmon	Tablet	Ono (Japan)
PGE1	Prostavastin	Parenteral solution	Ono (Japan); Schwarz (Europe)
β-Cyclodextrin (βCD)			
Benexate HCl	Ulgut, Lonmiel	Capsule	Teikoku (Japan); Shionogi (Japan)
Cephalosporin	Meiact	Tablet	Meiji Seika (Japan)
Cetirzine	Cetrizin	Chewable tablet	Losan Pharma (Germany)
Chlordiazepoxide	Transillium	Tablet	Gador (Argentina)
Dexamethasone	Glymesason	Ointment, tablet	Fujinaga (Japan)
Dextromethorphan	Rynathisol	Synthelabo (Europe)	
Diphenhydramine and chlortheophylline	Stada-Travel	Chewable tablet	Stada (Europe)
Ethinylestradiol and drospirenone	Yaz	Tablet	Bayer (Europe, USA)
Iodine	Mena-Gargle	Solution	Kyushin (Japan)
Meloxicam	Mobitil	Tablet and suppository	Medical Union (Egypt)
Nicotine	Nicorette	Sublingual tablet	Pfizer (Europe)
Nimesulide	Nimedex	Tablets	Novartis (Europe)
Nitroglycerin	Nitropen	Sublingual tablet	Nihon Kayaku (Japan)
Omeprazole	Omebeta	Tablet	Betafarm (Europe)
PGE2	Prostarmon E	Sublingual tablet	Ono (Japan)
Piroxicam	Brexin, Flogene, Cicladon	Tablet, suppository	Chiesi (Europe); Aché (Brazil)
Tiaprofenic acid	Surgamyl	Tablet	Roussel-Maestrelli (Europe)
2-HHydroxypropyl-β-cyclodextrin (HPβCD)			
Cisapride	Propulsid	Suppository	Janssen (Europe)
Indometacin	Indocid	Eye drop solution	Chauvin (Europe)
Itraconazole	Sporanox	Oral and intravenous solution	Janssen (Europe, USA)
Mitomycin	MitoExtra, Mitozytrex	Intravenous infusion	Novartis (Europe)
Sulfobutylether β-cyclodextrin sodium salt (SBEβCD)			
Aripiprazole	Abilify	Intramuscular solution	Bristol-Myers Squibb (USA); Otsuka Pharm. (USA)
Maropitant	Cerenia	Parenteral solution	Pfizer Animal Health (USA)
Voriconazole	Vfend	Intravenous solution	Pfizer (USA, Europe, Japan)
Ziprasidone mesylate	Geodon, Zeldox	Intramuscular solution	Pfizer (USA, Europe)
Randomly methylated β-cyclodextrin (RMβCD)			
17β-Estradiol	Aerodiol	Nasal spray	Servier (Europe)
Chloramphenicol	Clorocil	Eye drop solution	Oftalder (Europe)
γ-Cyclodextrin (γCD)			
Tc-99 Teboroxime[a]	CardioTec	Intravenous solution	Squibb Diagnostics (USA)
2-Hydroxypropyl-γ-cyclodextrin (HPγCD)			
Diclofenac sodium salt	Voltaren Ophtha	Eye drop solution	Novartis (Europe)
Tc-99 Teboroxime[a]	CardioTec	Intravenous solution	Bracco (USA)

[a]An older product contained γCD but has been replaced by HPγCD in the current product.

Added value by Industrial Biotechnology

- Improvement of existing production routes
- Reduced consumption of chemicals, energy and water
- Lower environmental impact
- Increased sustainability of the production process
- Access to novel products
- Creation of new value chains with innovative products and processes

Contributors & Funding

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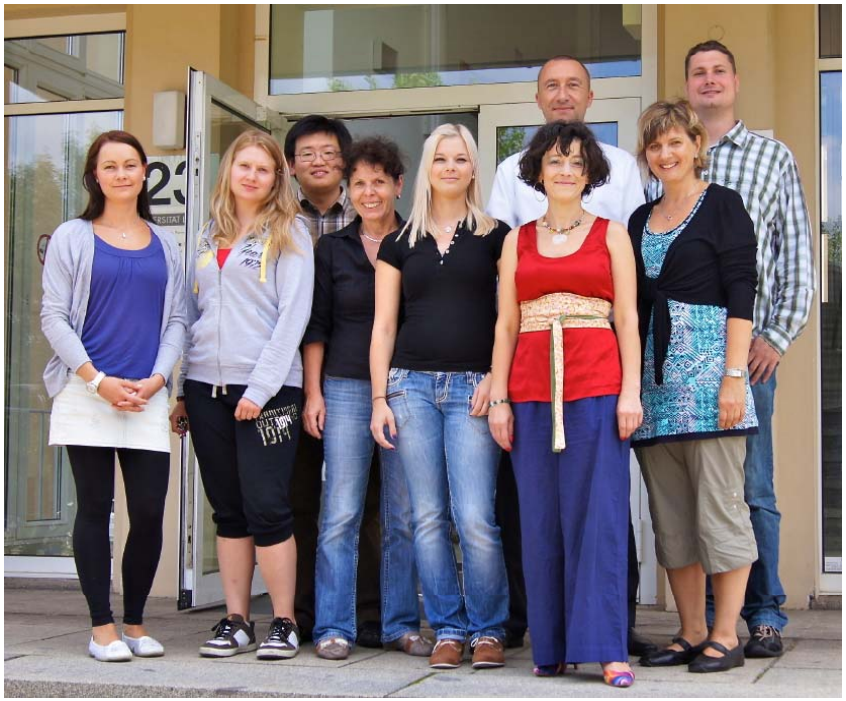

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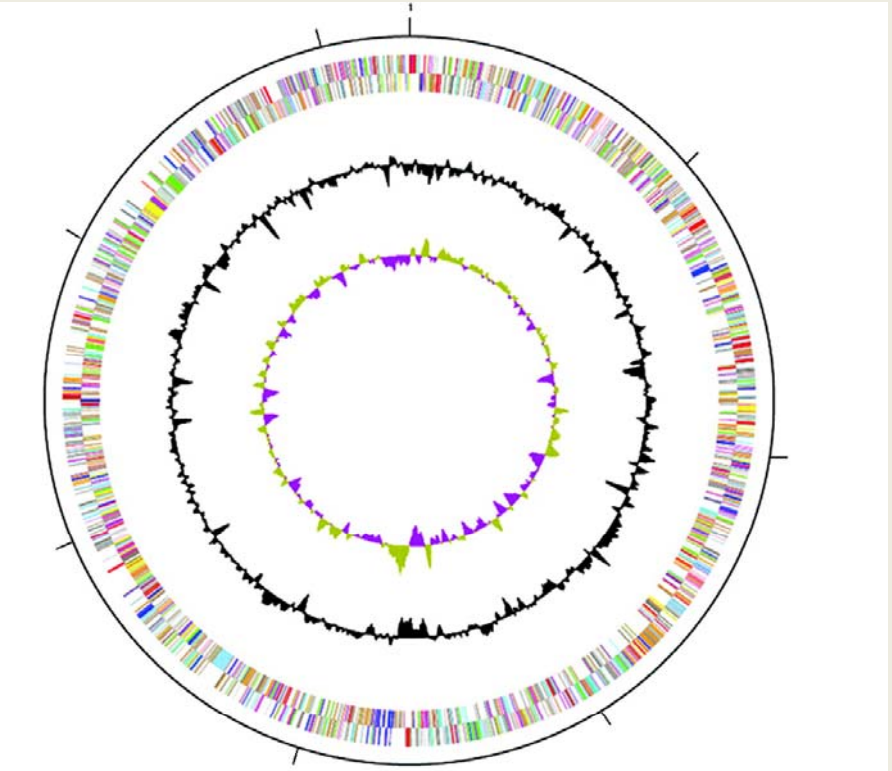
Site-directed mutagenesis of amino acids at the surface of a cutinase from *T. cellulosilytica* (Thc_Cut2)

	Wild-types		Mutants of Thc_Cut2					
	Thc_Cut1	Thc_Cut2	Arg19Ser	Arg29Asn	Ala30Val	Gln65Glu	Leu183Ala	Arg187Lys
Region 1	Ser19	Arg19	**Ser19**	Arg19	Arg19	Arg19	Arg19	Arg19
	Asn29	Arg29	Arg29	**Asn29**	Arg29	Arg29	Arg29	Arg29
	Val30	Ala30	Ala30	Ala30	**Val30**	Ala30	Ala30	Ala30
	Glu65	Gln65	Gln65	Gln65	Gln65	**Glu65**	Gln65	Gln65
Region 2	Ala183	Leu183	Leu183	Leu183	Leu183	Leu183	**Ala183**	Leu183
	Lys187	Arg187	Arg187	Arg187	Arg187	Arg187	Arg187	**Lys187**

Mutants Arg29Asn and/or Ala30Val showed a higher specific activity and higher k^{cat}/K^{M} *values on soluble substrates. Exchange of the positively charged arginine (Arg19 and Arg29) located on the enzyme surface to the non-charged serine and asparagine strongly increased the hydrolysis activity for PET.*

Herrero Acero et al. Biotechnology and Bioengineering 2013

Complete genome of *Thermobifida fusca YX*



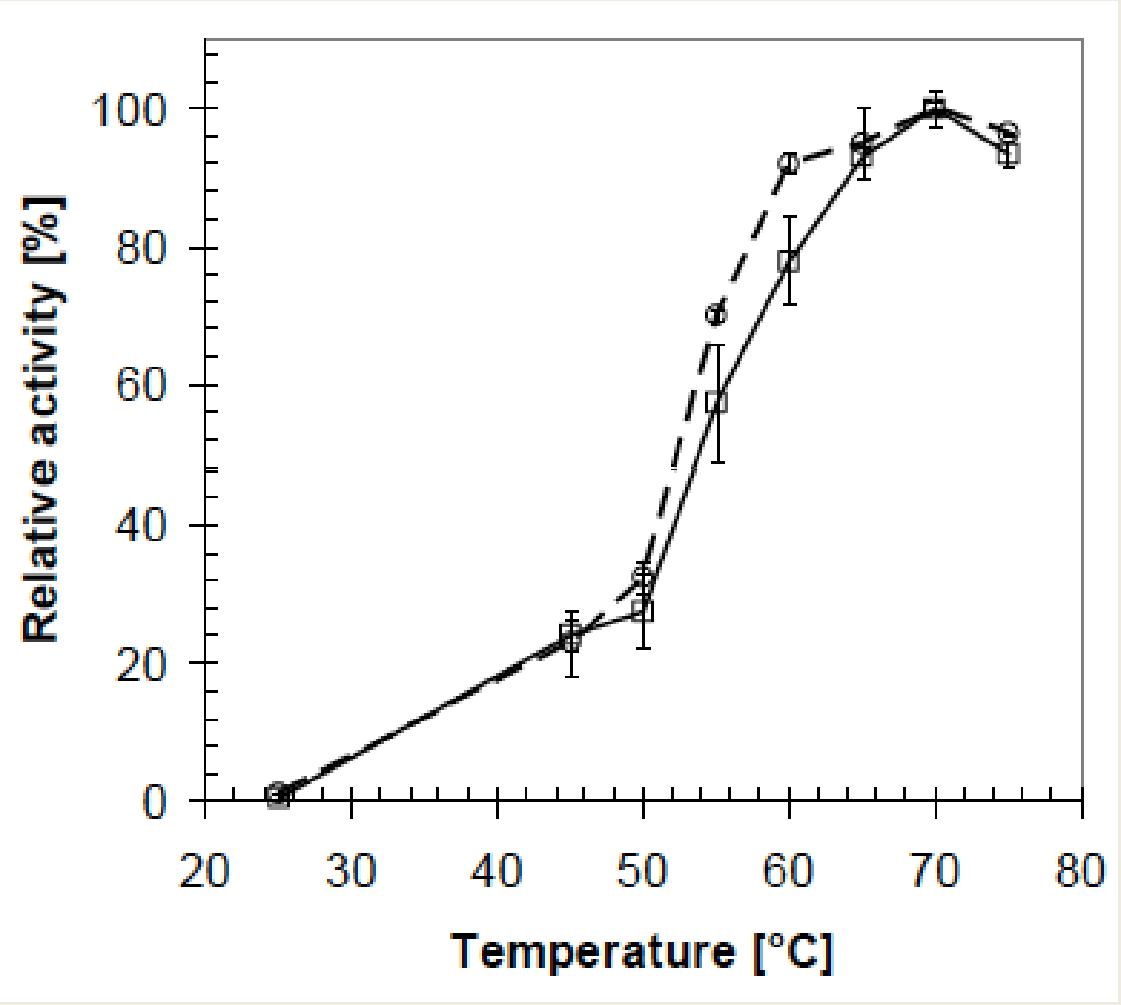
Total bases	3,642,249	Protein coding genes	3117
DNA coding bases	3,122,831 (85.74%)	With predicted function	2181 (68.54%)
GC%	67.5%	Without predicted function	936 (29.40%)
tRNA	53	Pseudogenes	7
5S/16S/23S	4/4/4		

From outside to inside, the first two circles represent the assignment for predicted coding sequences on the plus and minus strands, respectively. The two innermost circles represent the percent G+C content and G+C skew values, respectively.

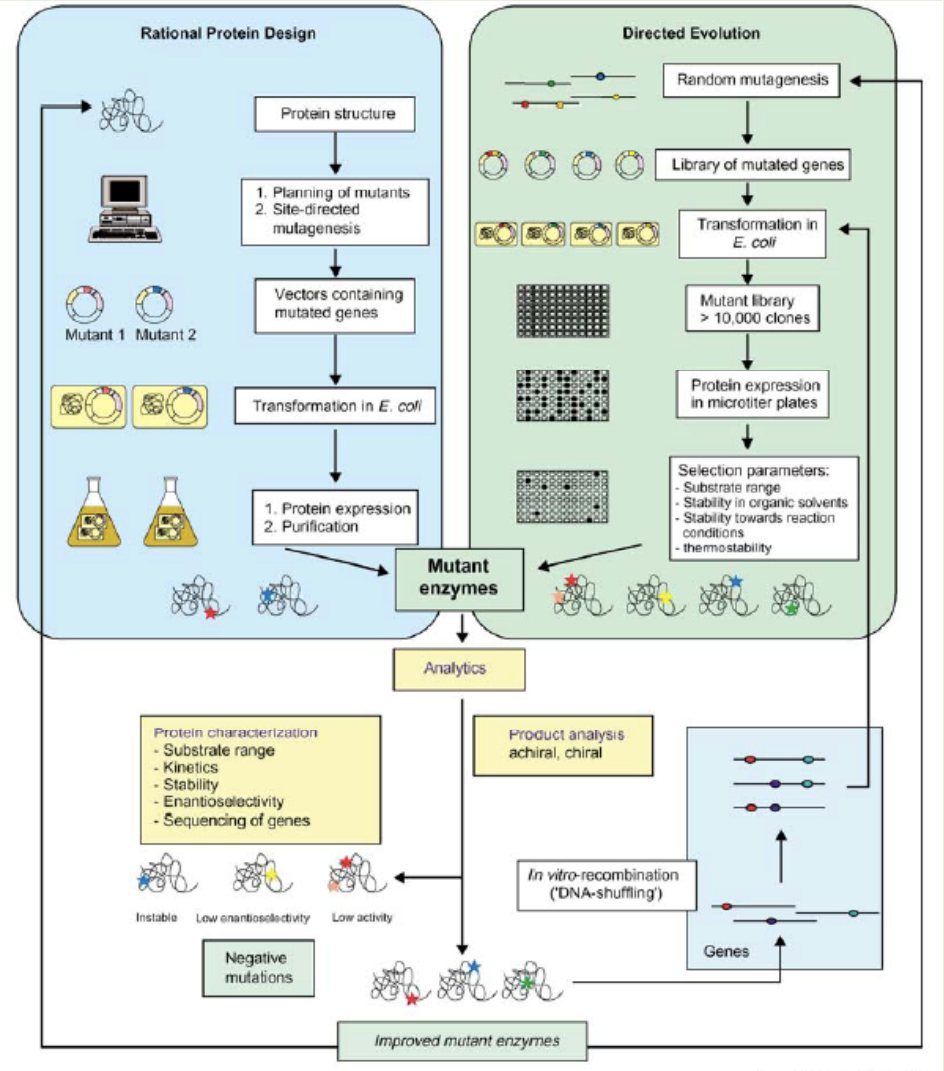
Lykidis A et al. J. Bacteriol. 2007;189:2477-2486

Digital inkjet-textile printing

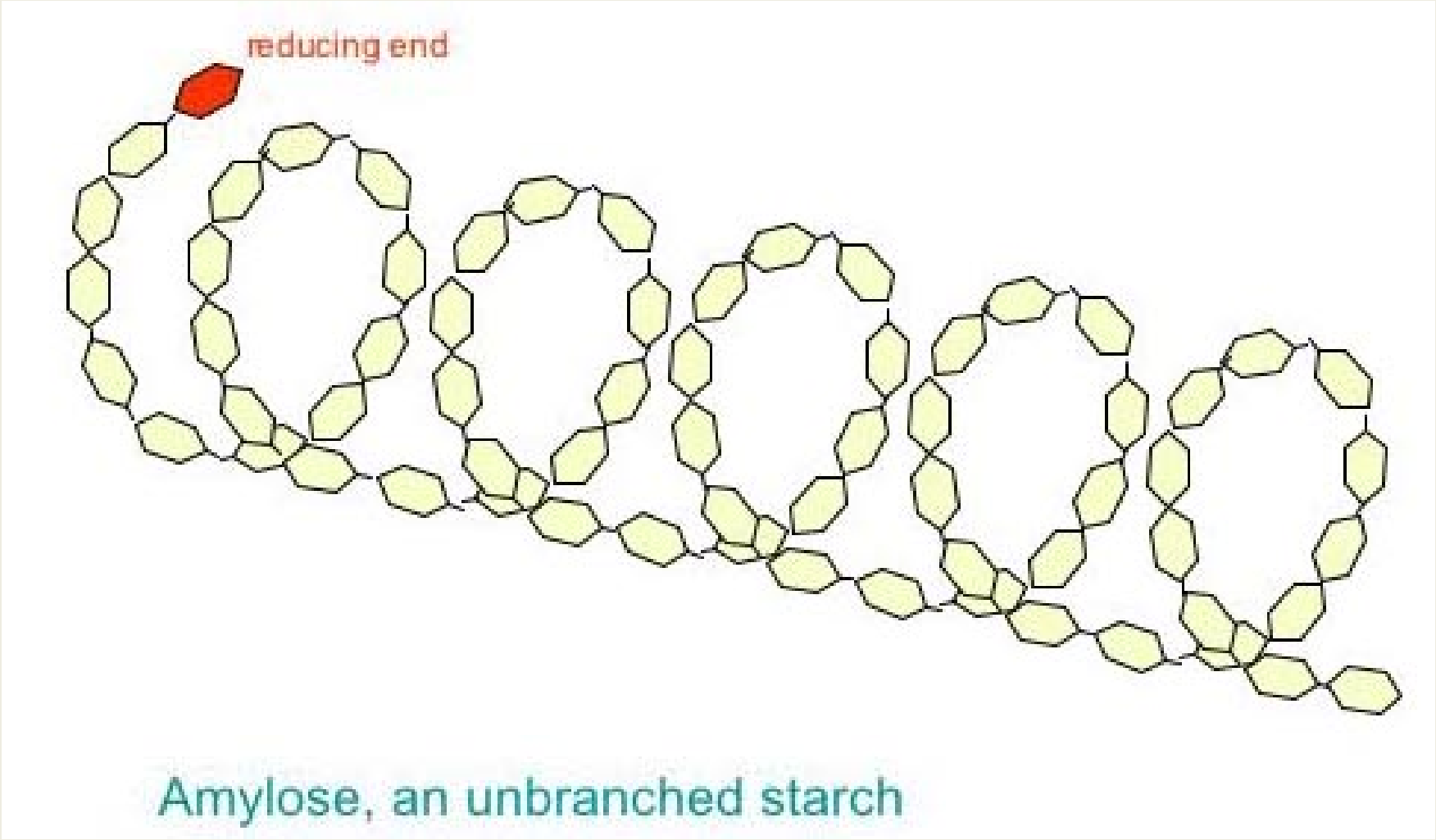
Effect of temperature on PET nanoparticle hydrolysis catalyzed by TfCut-1 (solid line) and by TfCut-2 (dashed line) from *T. fusca*



Change of the product specifity of the CGTase by rational protein design



Amylose structure



Amylose, an unbranched starch

Cyclodextrins as gene delivery vectors

- Synthetic vectors for delivery of DNA, RNA or nucleotides to cells
- Amphiphilic polycationic CD derivatives self-assemble into nanoparticles
- Formation of complexes with DNA which efficiently transfect cells

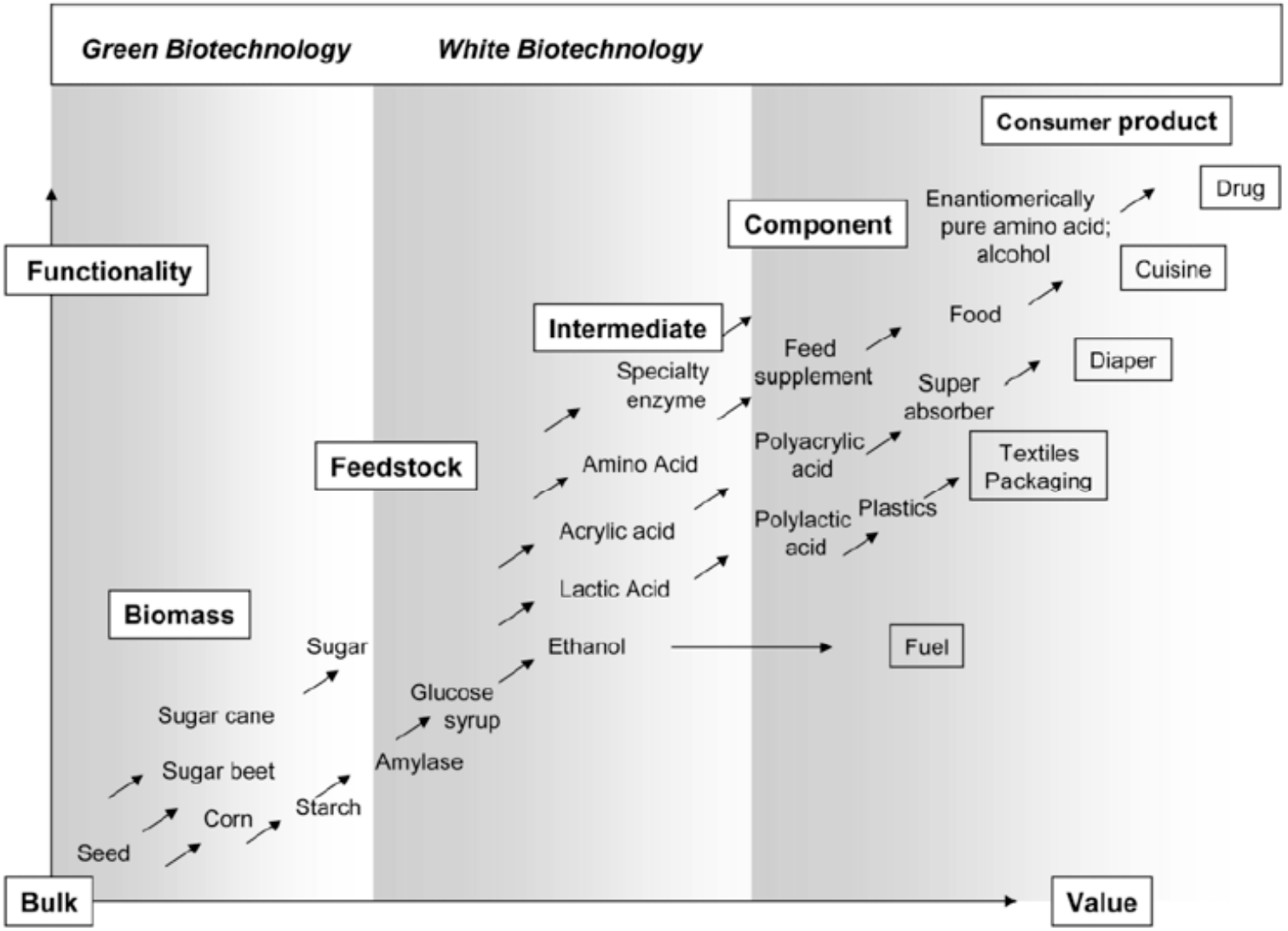
Cyclodextrin receptors in transducers for chemical sensing devices

- Gas sensing electrodes modified with cyclodextrins
- Microcantilevers with cyclodextrinsreceptors
- Quartz crystal microbalance (QCM) devices coated with cyclodextrins

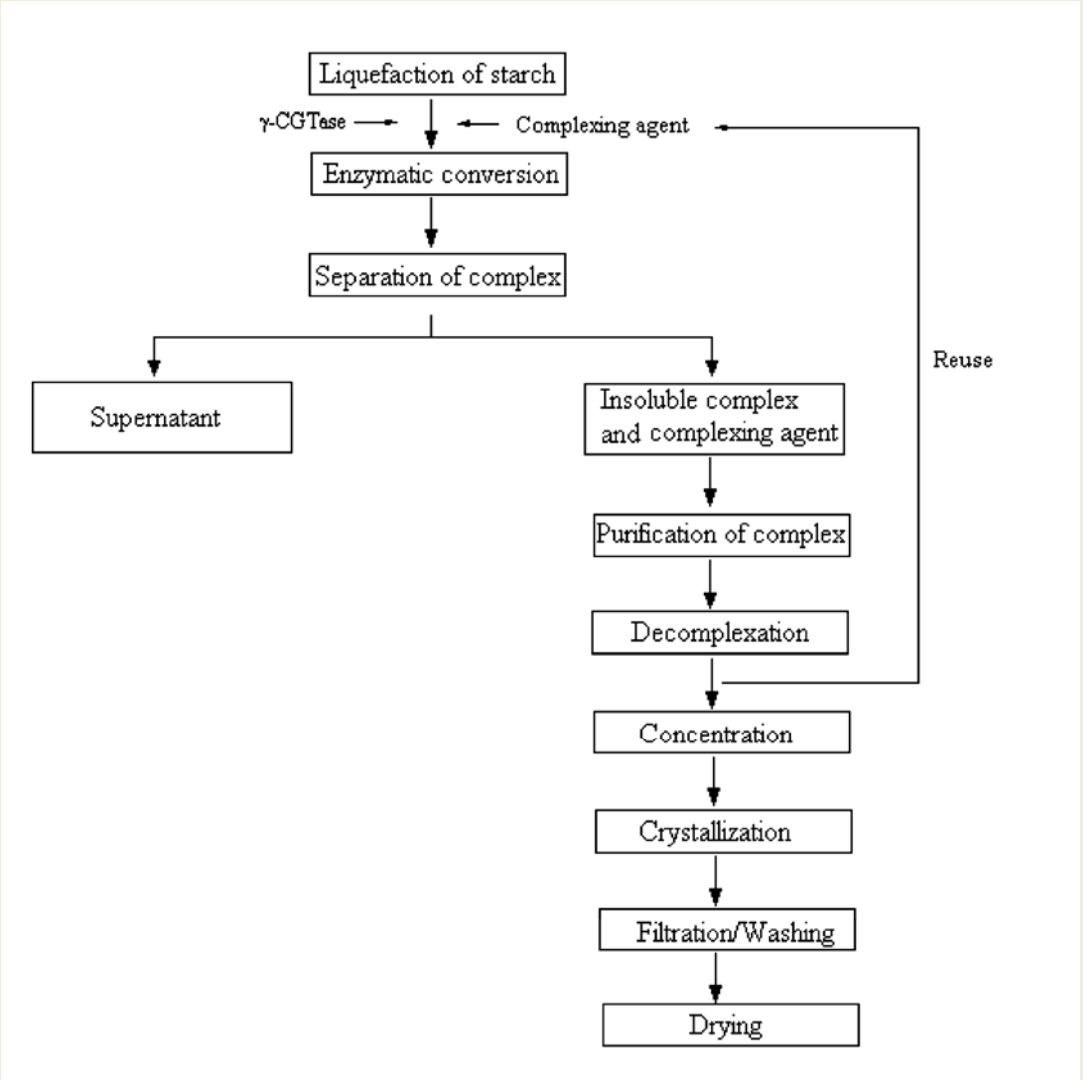
Molecular imprinted polymers: use of cyclodextrins in molecular recognition reactions

- Cyclodextrins display controllable receptor structures for molecular recognition processes
- The design of selective receptors for guest molecules of different sizes is feasible
- Molecular imprinted polymers with cyclodextrins as receptors for steroids and peptides have already been realized
- Applications
  - Sensors and assay systems
  - Polymers with controlled release properties
  - Separation processes: capillary electrophoresis, gas chromotography

Product value and functionality

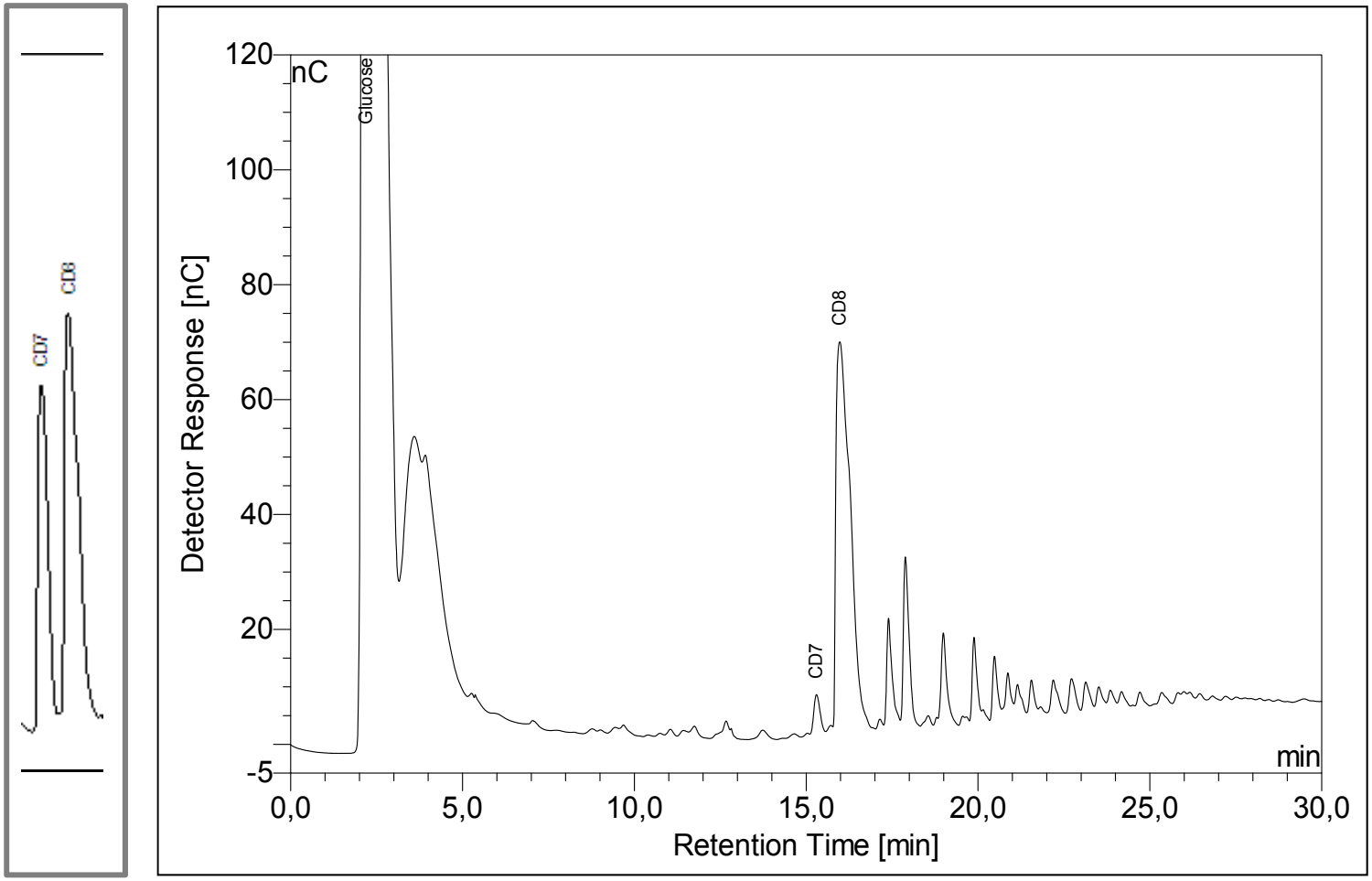


Solvent process for gamma-cyclodextrin production



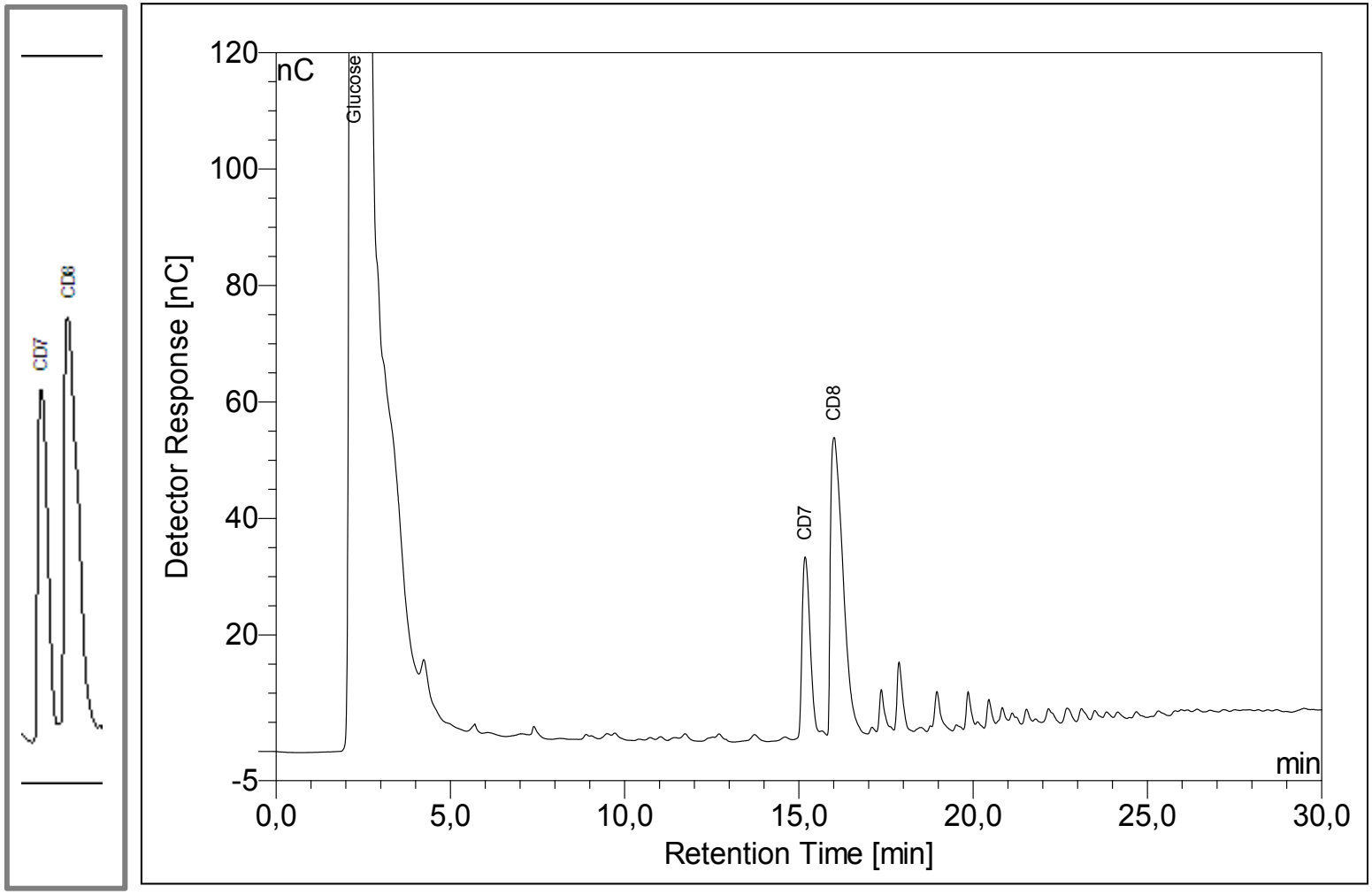
Cyclodextrin products obtained with the gamma-CGTase variant III (double mutation at sites 1 and 2)

WT



Cyclodextrin products obtained with the gamma-CGTase variant I (single mutation)

WT



Comparison of amino acid residues important for the cyclization reaction

Subsite	*B. circulans* 251 CGTase	Bacillus sp. G-825-6 CGTase	Identity
-7	Loop 145-151	deleted	
	S146	deleted	
-6	N193	N209	
	G180	G196	
	G179	G195	
	Y167	Y184	
-3	R47	T72	γ-CGTase *B. clarkii* 7364
	Y89	H112	CGTase *B. ohbensis*
	N94	F116	γ-CGTase *B. clarkii* 7364
	D196	D212	
	D371	**D386**	
+1	L194	L210	
	A230	A246	
	H233	H249	
+2	F183	F199	
	K232	A248	
	F259	F276	
Center	D229	**D246**	
	E257	**E274**	

AS in the catalytic triad in bold