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Enzyme Engineering: An Innovative Way to Manage the Economics of Biosimilars

Nov 2014

Naveen Kulkarni, CEO

About Polyclone

Capability Snapshot

Solutions for Bio-Pharma

Solutions for Healthcare

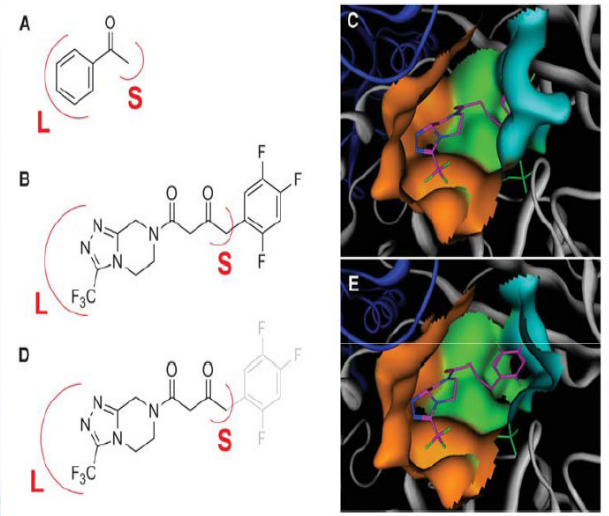
	Nucleic Acid Studies	Peptides, Proteins & Enzymes	Small Molecules	Cell & Molecular Biology
In Silico	Computational Oligo Design ePrime	Peptide analysis & design ePep Enzyme & Protein modifications eEF	Custom screening services for lead based research challenges	Cell culture services Isolation, enrichment & expansion of stem cells Molecular diagnostics
In Vitro	Custom projects in molecular biology & cell/ stem cell biology for discovery & validation			

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Need to engineer the enzyme

- The most common issue with the transamination reaction is the unfavourable reaction conditions
- The enzymes also suffer from
  - low turnover numbers
  - post reaction processing issues
  - poor thermostability
  - low solvent tolerance
  - limited substrate range (substrates with a substituent no larger than a methyl group at the position adjacent to the ketone), hence low activity towards the desired substrate

Transaminase
Engineered to produce Sitagliptin



- **27 mutations has given 10 to 13% increase in overall activity**

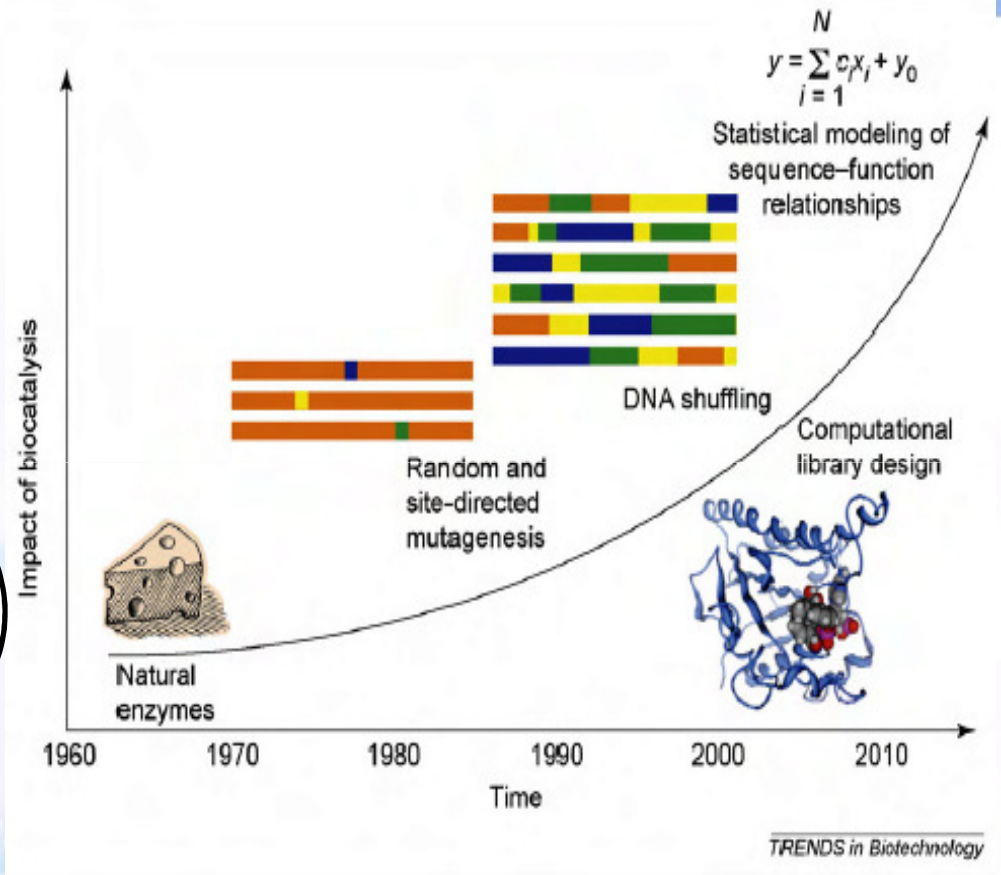
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Different approaches for engineering biocatalysis/ Enzymes

- Rational design
- Directed evolution

The number of success stories in enzyme engineering using these approaches is far lesser than the failures encountered

- Silent substitutions
- Even, Major modifications lead to useless changes



Reports say that computational tools hasten the process of making better enzymes and provide molecular details for further development

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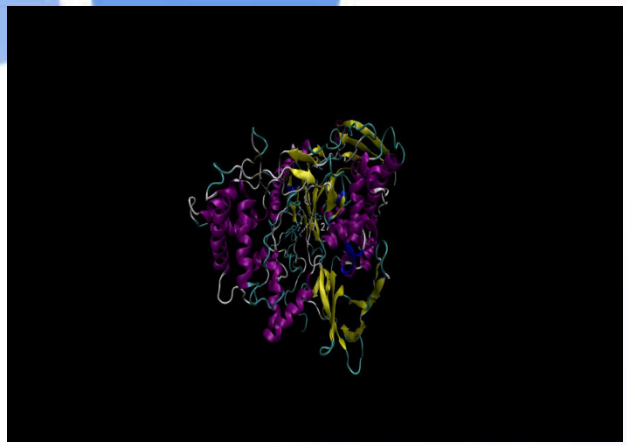
Screening and selection tools- in demand

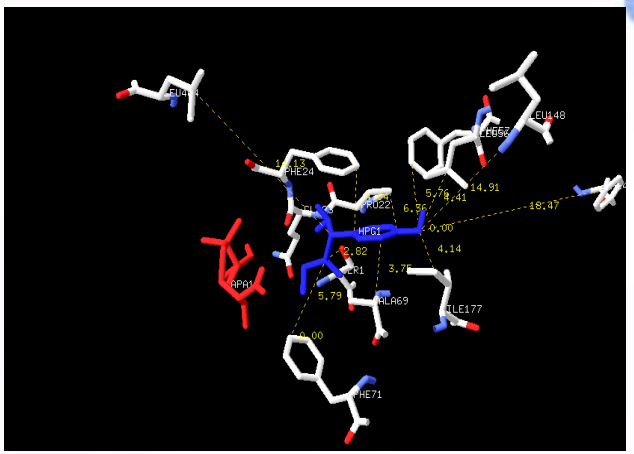
- The crucial step in enzyme engineering- Generating focused libraries
- Computational techniques – to generate focused libraries
  - Understanding of protein structure and function
  - Mimicking the experimental process of library screening

- But is there a technique that can accurately predict the kinetic properties of an enzyme considering factors like temperature, pH and the thermodynamics of the E-S reaction?
  - Sequence based methods predict protein stability- generalized prediction and does not consider catalytic binding mode
  - Docking considers catalytic binding on static structures, intermolecular energies shows no change if mutations are beyond the active site, no linear relationship
  - Molecular dynamics can reveal different transition state of the E-S reaction- many post simulation analysis to interpret the difference, time consuming

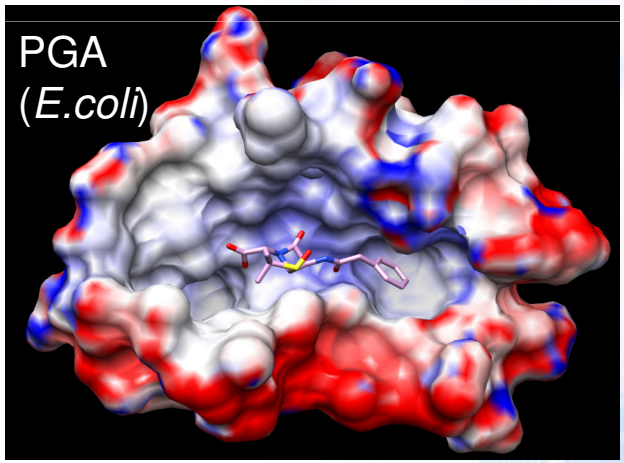
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“Computational techniques”- fastest way to understand proteins/enzymes



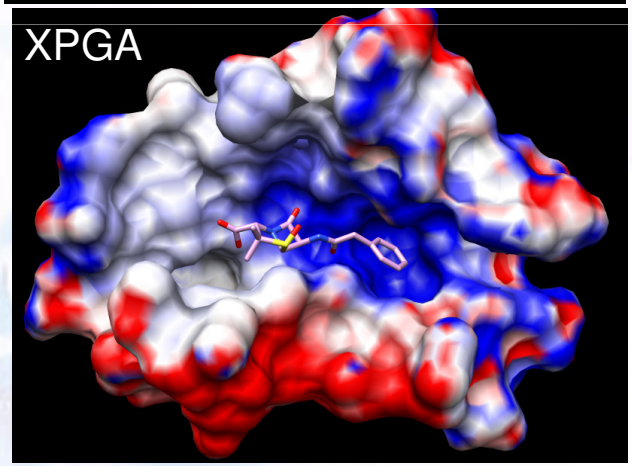


Hydrolysis
100%
Synthesis
25%



Sequence
similarity
90%

Active site
is 100 %
identical

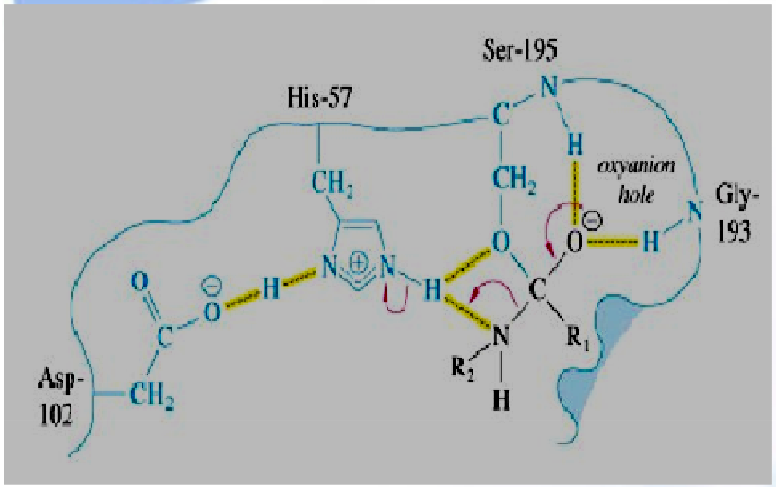


Hydrolysis
100%
Synthesis
50%

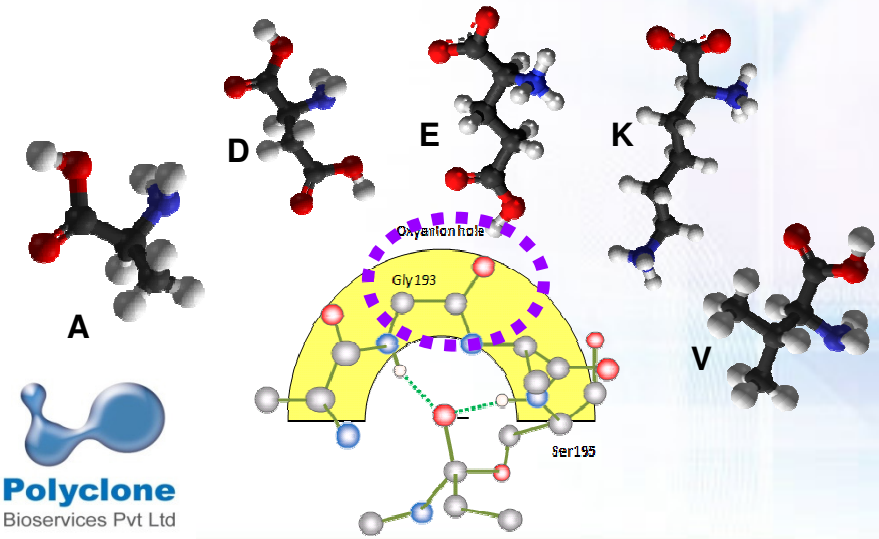
- *Simulation studies show clear change in the electrostatic potential of XPGA compared to PGA*
- *The XPGA having a stronger positively charged active site.*

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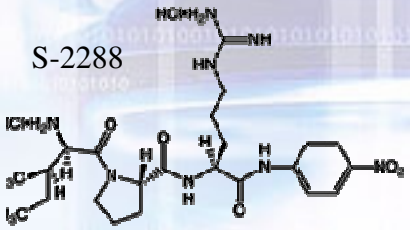
Enzyme of study-serine protease (Chymotrypsin)



Reported activity data of serine protease (Chymotrypsin)			
position 193	Substrates	Kcat values	Classification
A	S-2288	64.8	moderate
D	S-2288	4.2	low
E	S-2288	71.2	moderate
K	S-2288	30	low
V	S-2288	10	low
G	S-2288	110.2	high
A	S-2366	117	high
D	S-2366	40.8	low
E	S-2366	98	high
K	S-2366	114.2	high
V	S-2366	26.7	low
G	S-2366	145	very high

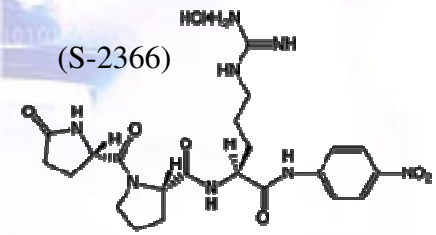


S-2288



H-D-Ile-Pro-Arg-p-nitroanilide

(S-2366)

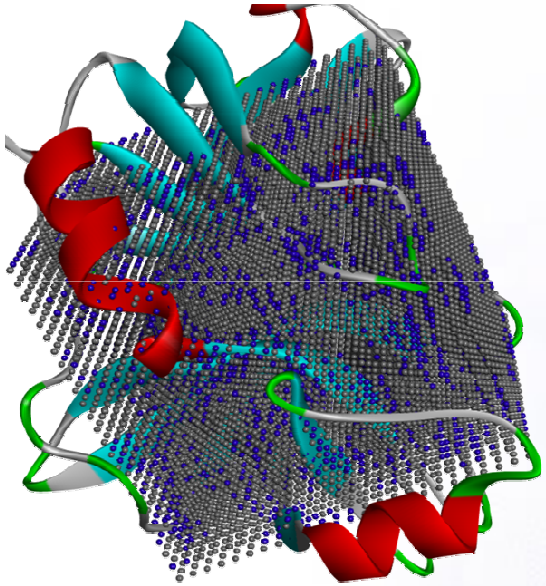


pyroGlu-Pro-Arg-p-nitroanilide

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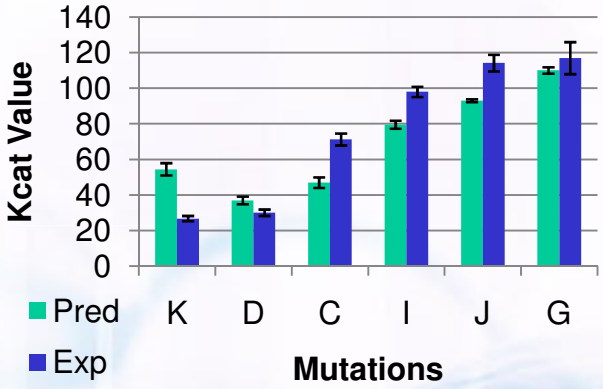
1.8 million data points were generated to select the relevant GCODs

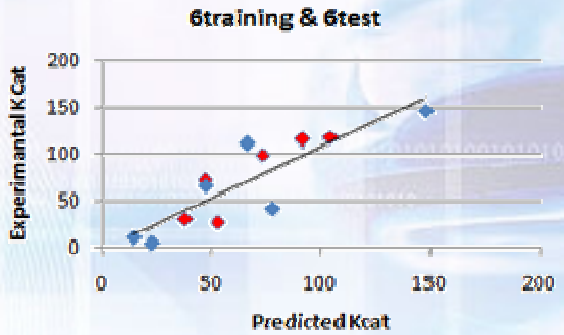
- Small spheres are GCODs
- 156,250 descriptors for each mutation
- 1,875,000 data points -overall for 12 E-S simulations



Blue spheres represent selected GCODs for generating QSAR model

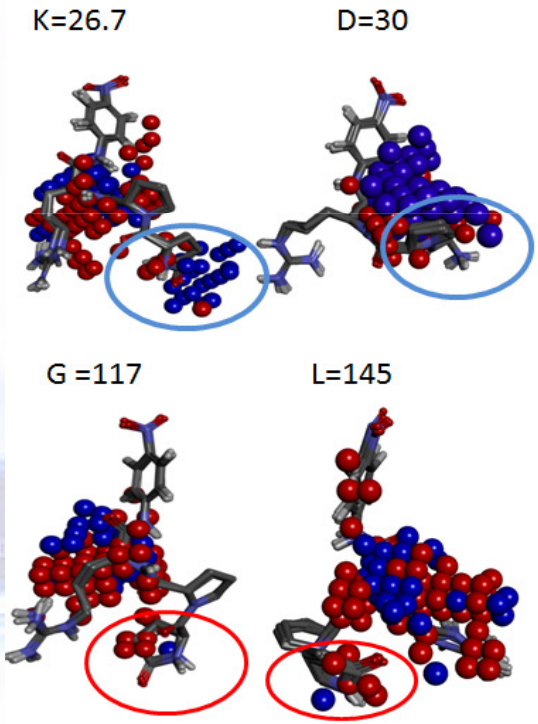
Predicted activity values correlated with the experimental values





Blue = training set
Red = test set

Showed a specific interaction with GLY 218 and GLU 217

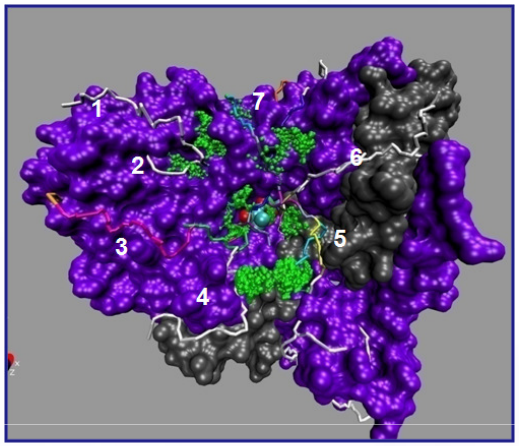


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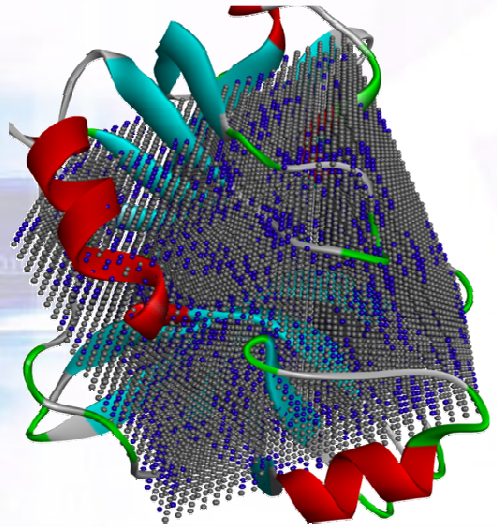
Enzyme engineering Framework - eEF

- eEF is used to engineer enzymes
  - against thermal & pH denaturation
  - for increased activity on novel substrates
  - to attain activity against a synthetic substrate
- Predicts Hotspots and enzyme modifications for better activity
  - The Hotspots are predicted in the catalytic site as well sites aiding catalysis which can be away from the active site
  - Grid based energy landscape
  - Impact on inter and intra domain
  - Per residue energy profiling
- Experiments conducted to predict Hotspots are carried out in different assay conditions like solvent, pH, temperature etc.

Path based Hotspot prediction



Grid Based Hotspot prediction

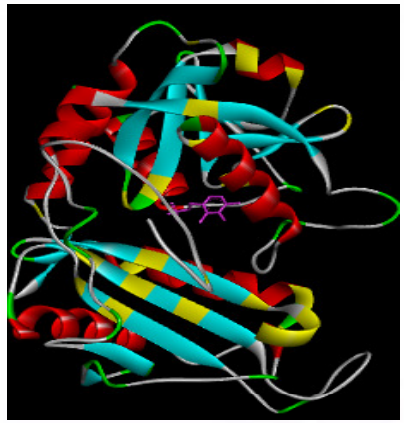


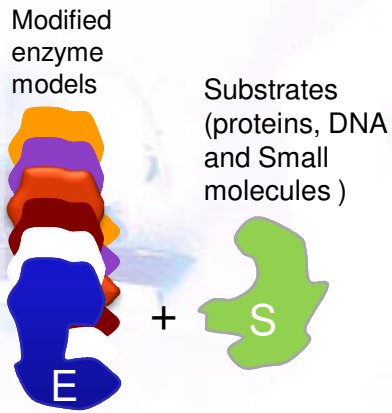
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Screening mutations

Never misses a potential modification !

Number of substitutions at each site	Number of modification sites																		Total number of modified enzymes
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
	12	12	12	12	12														248832
	6	6	6	8	12	12													248832
	4	4	5	5	6	9	12												259200
	3	4	4	5	5	6	6	6											259200
	3	3	3	3	3	5	6	6	6										262440
	3	3	3	3	3	3	3	4	5	6									262440
	2	2	2	2	2	4	4	4	4	4	8								262144
	2	2	2	2	2	2	2	3	3	4	5	12							276480
	2	2	2	2	2	2	2	2	3	3	3	3	12						248832
	2	2	2	2	2	2	2	2	2	2	2	4	4	8					262144
	2	2	2	2	2	2	2	2	2	2	3	3	3	3	3				248832
	2	2	2	2	2	2	2	2	2	2	3	3	3	3	3				248832
	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	4		262144
	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	262144





Intra-intermolecular energy based filter-transformed scoring functions

- Energies calculated based on the domain movement analysis
- Empirical and knowledge based screening
- The structure of the Enzyme & substrate is flexible

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Engagement: Sponsor can monitor the output at any given point of the process

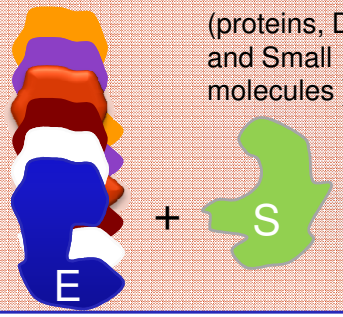
Input

Specify the requirement - Catalytic and physical properties for the enzyme of interest

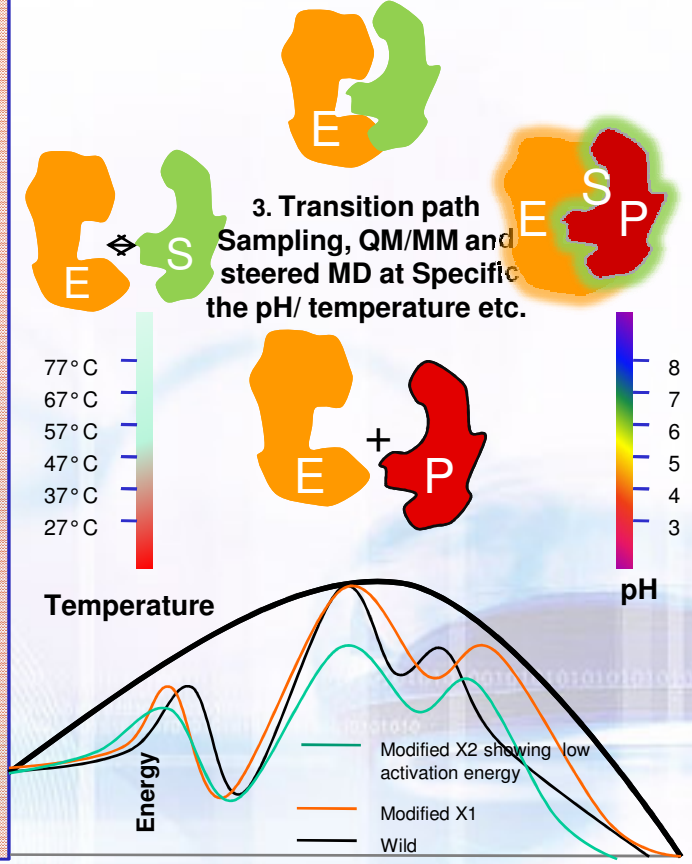
1. **Hotspot Identifier**
   - Molecular dynamics and 4D-QSAR studies
   - Model 200,000 modifications
2. **Intra-intermolecular energy based filter- transformed scoring functions**

Modified enzyme models

Substrates (proteins, DNA and Small molecules)



In silico Enzyme-Substrate reaction



Output

4. Predict catalytic and physical properties

- Activation energy, Rate limiting steps, Kcat, Km etc. of the modifications

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Protein engineering and biopharmaceutical market

- Is Biobetter better than Biosimilar development?
- Biobetters are proteins that are superior when compared to the innovative biologic, based on a molecular or chemical modification that may translate into an improvement over the reference protein product
- In order to improve their clinical potential, second generation recombinants are being developed, each exhibiting improved efficacy and potency depending on their applications
- With growing incidences of cancer, autoimmune disorders and infectious diseases, spurt of new opportunities for growth of the market is expected by the end of this decade
- Factors that restrain new biologics development
  - development costs
  - stringent regulatory issues
  - risk of failure

Examples

- ***Sequence modification ,Glycosylation, Pegylation***
- ***Interferon alpha(increased efficacy) , interleukin-2 (stable in solution)***

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Challenges that can be addressed

- Increase half life
- Decrease immunogenicity
- Increase protease resistance
- Increase solubility & stability
- Increase catalytic activity of therapeutic enzymes

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Requirements from sponsor

- ✓ Clearly define the objectives of the study
- ✓ 3D structure of the enzyme of interest
- ✓ Mode of binding and kinetic properties of the enzyme-substrate reaction
- ✓ Structure of enzyme-substrate complex
- ✓ Reported structure of enzyme complexed with other active substrates OR inhibitor/s
- ✓ Specify process conditions of the reaction
- ✓ Available literature study

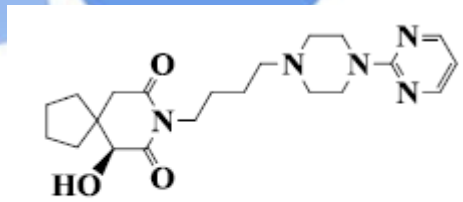
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Thank You !

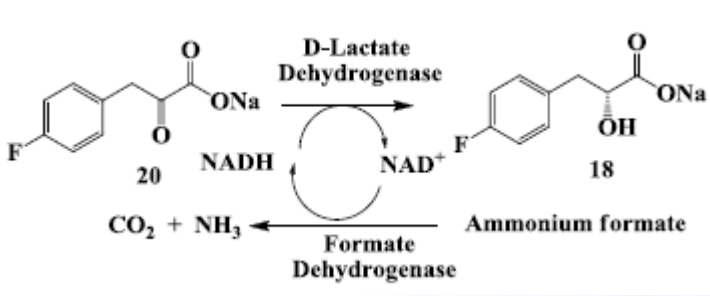
info@polyclonebio.com

Enzymatic preparation of Drugs

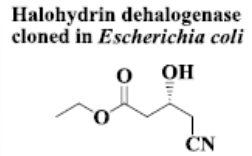


(*S*)-2
(*S*)-6-Hydroxybuspirone

- Antianxiety drug :Reductase

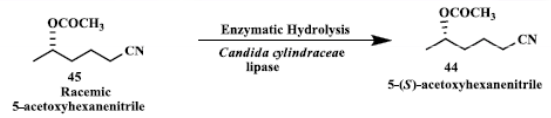


Rhinovirus
protease inhibitor



12
Ethyl (*R*)-4-Cyano-3-hydroxybutyrate

- Cholesterol lowering agent



45
Racemic
5-acetoxyhexanenitrile

44
5-(*S*)-acetoxyhexanenitrile

Anti-Alzheimer's drugs

Anticancer Drug: Enzymatic Preparation of (S)-2-Chloro-1-(3-Chlorophenyl)Ethanol

Anticancer drug: Enzymatic preparation of C-13 paclitaxel side-chain synthon.

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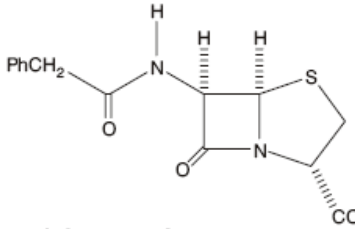
Advantage of using Transaminases for producing drugs

Antimigraine Drugs
Anti – diabetic drug

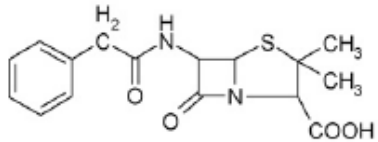
- Requires low-cost substrates
- Does not need an external cofactor to recycle the enzyme
- High enantioselectivity and reaction rate
- Ability to accept inexpensive amines, such as isopropylamine,as amino donors, in combination with their role in organic synthesis for the biocatalytic production of a wide variety of chiral amines and a-amino acid compounds of high enantiomeric excess

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Engineering penicillin G acylase for producing semi -synthetic antibiotics

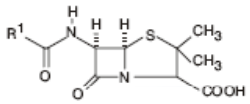


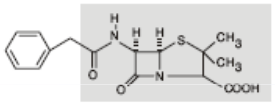
ß-lactam ring



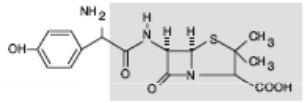
Penicillin G

Penicillin

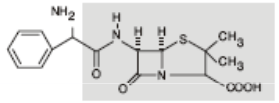




Benzylpenicillin

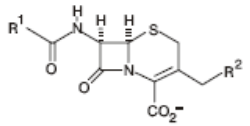


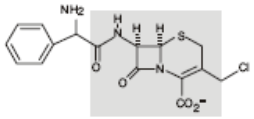
Amoxicillin



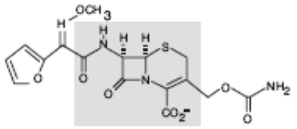
Ampicillin

Cephalosporin

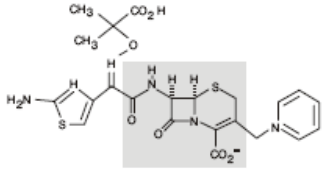




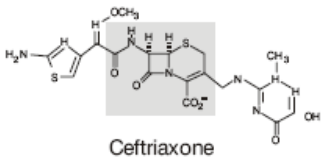
Cefaclor



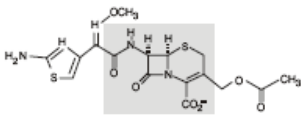
Cefuroxime



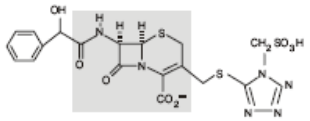
Ceftazidime



Ceftriaxone



Cefotaxime



Cefonicid

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Computational tools in enzyme engineering

- Multiple tools are used to address different parts of the problem. Ex: Tools like
  1. CC\PBSA,EGAD,FoldX,Hunter,I-Mutant2.0,Rsetta,Prethermut, pKD Server, PROPKA are used for thermal and pH stability
  2. Autodock, Gold, DOCK, Schrodinger for enzyme-substrate binding
  3. Gromacs, NAMD, Charmm for E-S simulation
- Knowledge based tools use sequence level information to predict hotspots
- Molecular modeling and dynamics are by far the most revealing techniques when it comes to studying the enzymatic reaction

- Need for specific expertise to shift from one tool to other and move on to the next to make sense
- Sequence based methods gives no information about the change in the catalytic binding modes and the thermodynamics of the reaction
- Molecular modeling and dynamics are computationally too expensive and very often the results are not comprehensive

There is no such integrated tool in the market that can produce 1000's of modifications and predict activation energy and rate limiting steps at different process conditions for the required biocatalysis

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