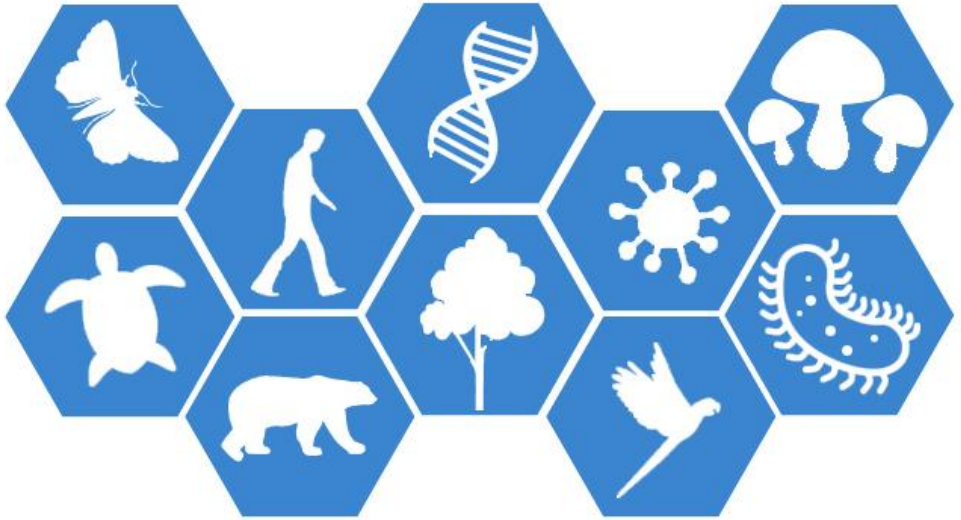


# Discovery and engineering of enzymes from termite gut metagenome for green industry

## Translating biodiversity to bioindustry



Verawat Champreda

Microbial Biotechnology and Biochemicals Unit

**BIOTEC**

# Bioeconomy



## Thailand 4.0

### Innovation & Sustainability

**Advanced Bio-industry and Biorefinery** will be one of the 5 new S-curve industries in the national agenda.

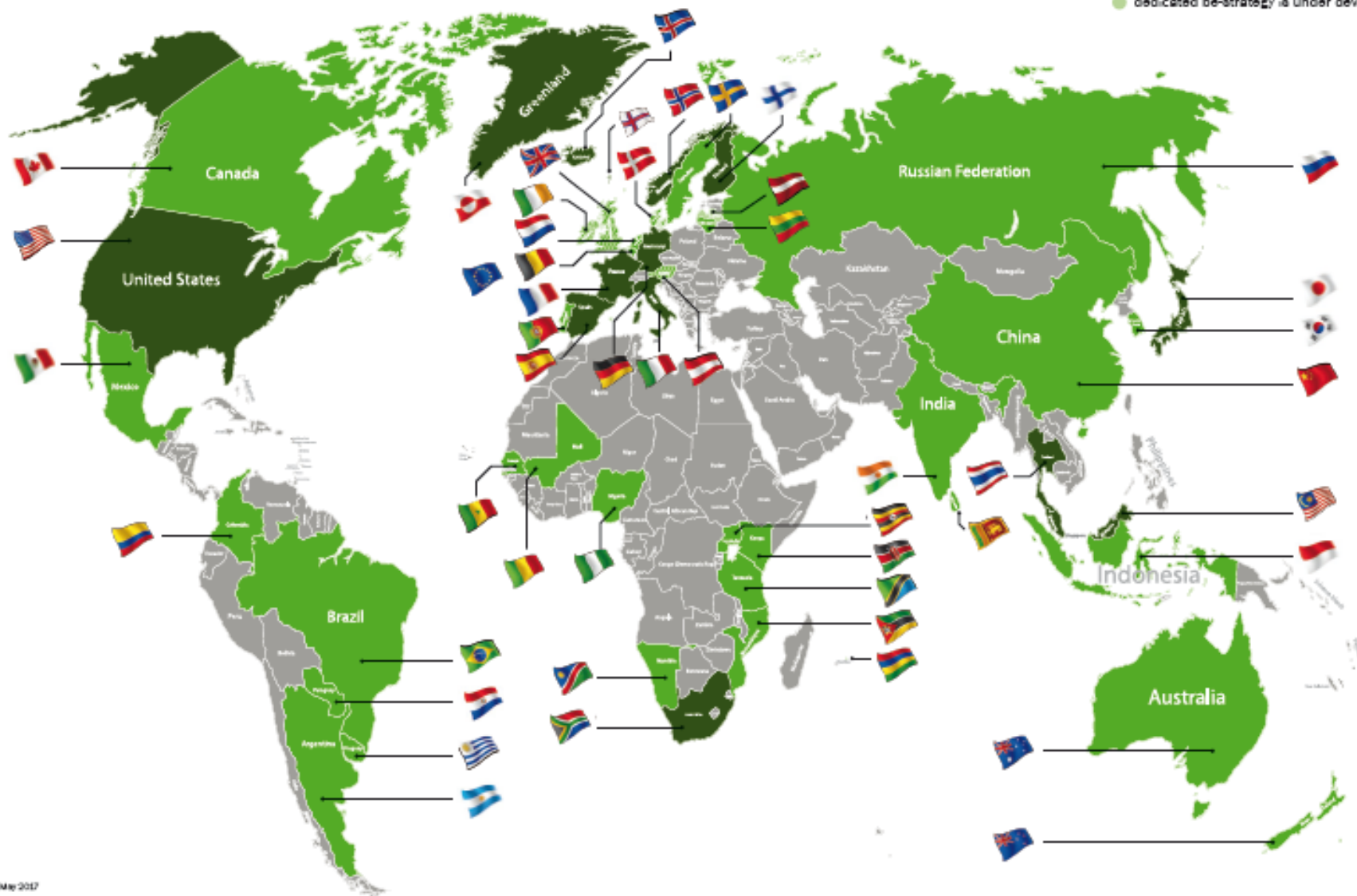
This is strongly linked to the country's established strength in agricultural sector, food industry and biofuel industry.

- Renewable resource
- Green & Clean processing
- Environmental & Economic sustainability



# Bioeconomy Policies around the World

- dedicated bioeconomy strategy
- bioeconomy-related strategy
- be-related strategy; dedicated be-strategy is under development
- dedicated be-strategy is under development



As of May 2017

© German Bioeconomy Council  
Hermannstraße 64 | 53173 Bonn | www.bio-step.eu

<http://www.bio-step.eu>

# Enzyme & Bio-based economy

Enzymes play an essential role in modern biotechnology related to production of commodity and specialty products in everyday life.

- Accelerating process reactions
- Additives in products for desirable properties



4620 M US\$  
6% growth/y



2500 M THB  
15% growth/y



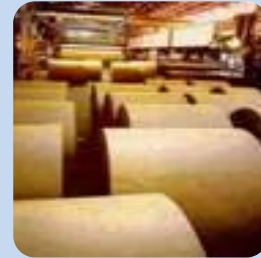
## Food & Feed

Pre-biotics/ supplements



## Biofuels & Chemicals

Saccharification/ processing



## Green processing

Pulp/ Textile/ Detergent



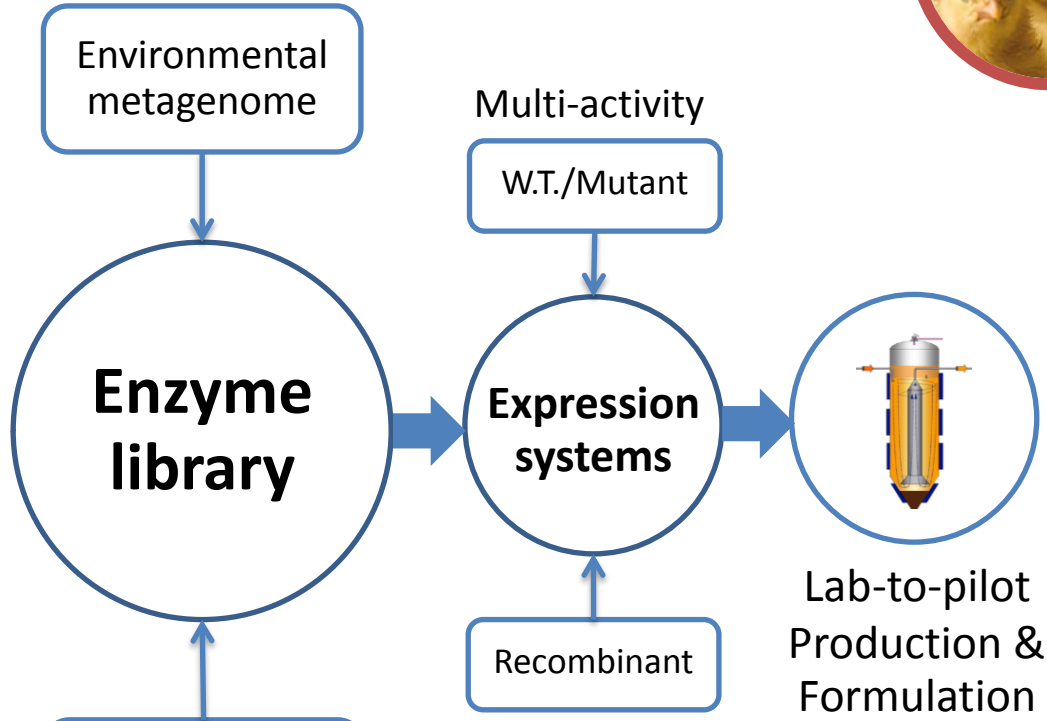
## Healthcares & Pharmaceuticals

Specialty high value enzymes



# Enzymes for green industry

From diversity to industry



Culture collection



BCC > 80,000 strains  
(1<sup>st</sup> in SEA)

Multi-activity  
W.T./Mutant

Expression systems

Recombinant

- Single activity
- *E. coli*
  - *P. pastoris*
  - *O. thermomethanolica\**
  - *Aspergillus*

Lab-to-pilot  
Production &  
Formulation



## Animal Feed

Enhancing nutrition/  
digestibility



## Biofuels & Biorefinery

Sugar platform  
conversion



## Green processing

Pulp/Textile  
Reducing chemicals  
and energy



## Specialty enzymes

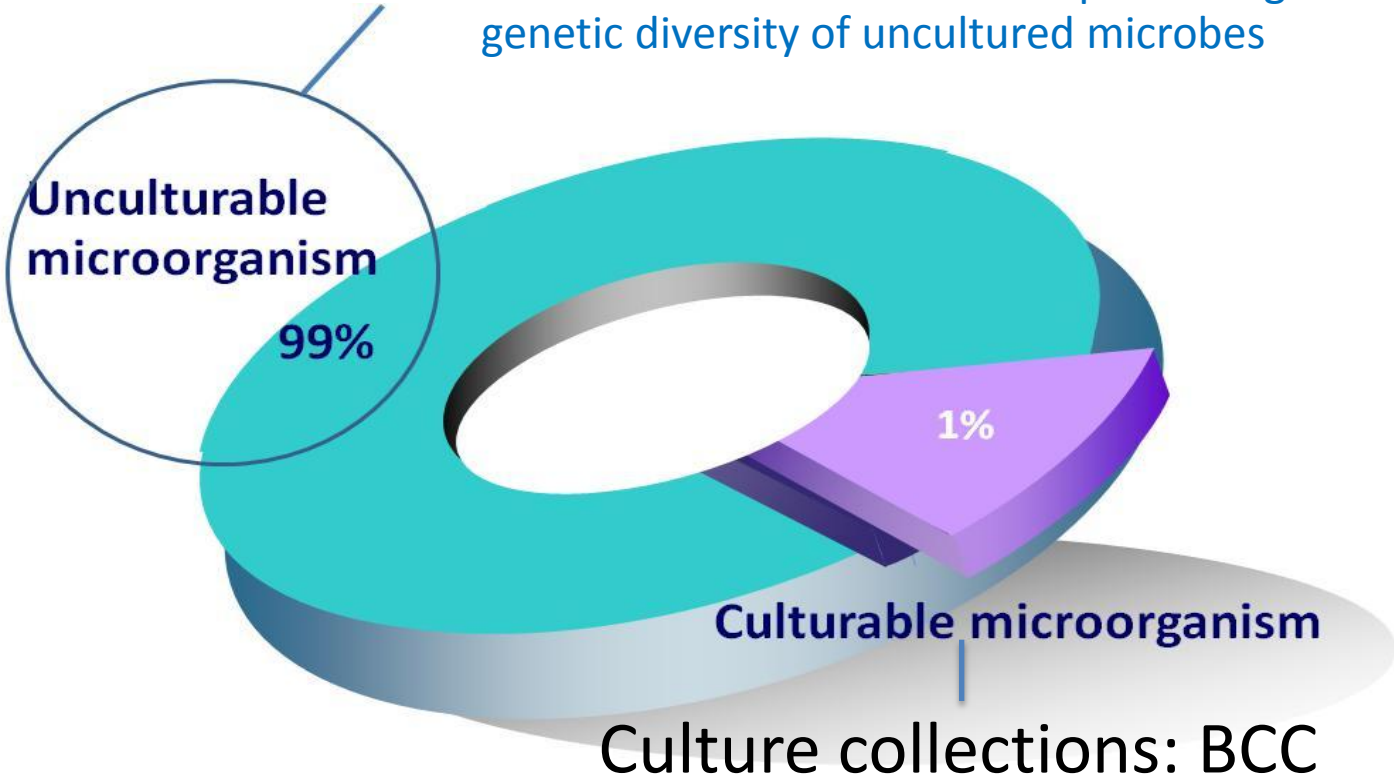
Healthcare products



A close-up photograph of a petri dish held by a gloved hand. The petri dish contains a clear agar medium with numerous bacterial colonies of various sizes and colors, including yellow, orange, and red. The background is dark and out of focus, showing a blurred yellow biohazard sign.

**1%** is cultivable.

**Metagenome:** The genomes of total microbiota found in nature representing the genetic diversity of uncultured microbes

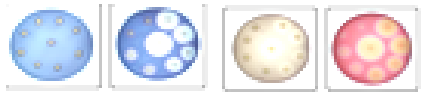


Culture collections: BCC



### Activity screening:

Enzyme function



### Sequence screening:

Conserved sequences



### Next-gen sequencing & bioinformatics annotation

- Tagged amplicon sequencing: 16S rRNA, ITS, metabolic marker



- Direct shotgun sequencing: metagenomic library

# Metagenomic exploration @BIOTEC



## Source

### Peat swamp forest

**Site:** Narathiwat, THA  
Organic carbon sink

## Method/ Target

### 16S rRNA on NGS

Biodiversity profiling  
Cellulose degradation

## References

Kanokratana et al. (2011)  
Microb. Ecol. 61, 518-528



### Bagasse collection site

**Site:** Chaiyapoom, THA  
Thermophilic lignocellulose decomposition

### Shortgun/16S rRNA Activity-based

Cellulose degradation  
and enzyme isolation

Mhauntong et al. (2015)  
Biotechnol. Biofuel 8:16



### Termite gut symbioint

**Source:** Trametes hindgut  
Alkaliphilic cellulose  
degradation

### Activity-based screening

Alkaliphilic  
cellulase/hemicellulase

Nimchua et al. (2012)  
J. Microbiol. Biotechnol.  
22, 462-469



### Carnivorous plant pitcher fluids

**Source:** 8 local Nepenthes sp.  
Acidic hydrolytic environment

### 16S rRNA on NGS

Biodiversity profiling  
Acidophilic microbes  
& enzymes

Kanokratana et al. (2016)  
Microb. Ecol. 72: 381-393



### Cow rumen

**Source:** Cow rumen fluid  
Anaerobic cellulose  
degradation

### 16S rRNA on NGS

Biodiversity profiling  
Cellulose degradation

Thoertkiattikul et al. (2012)  
Curr. Microbiol. 67, 130-137

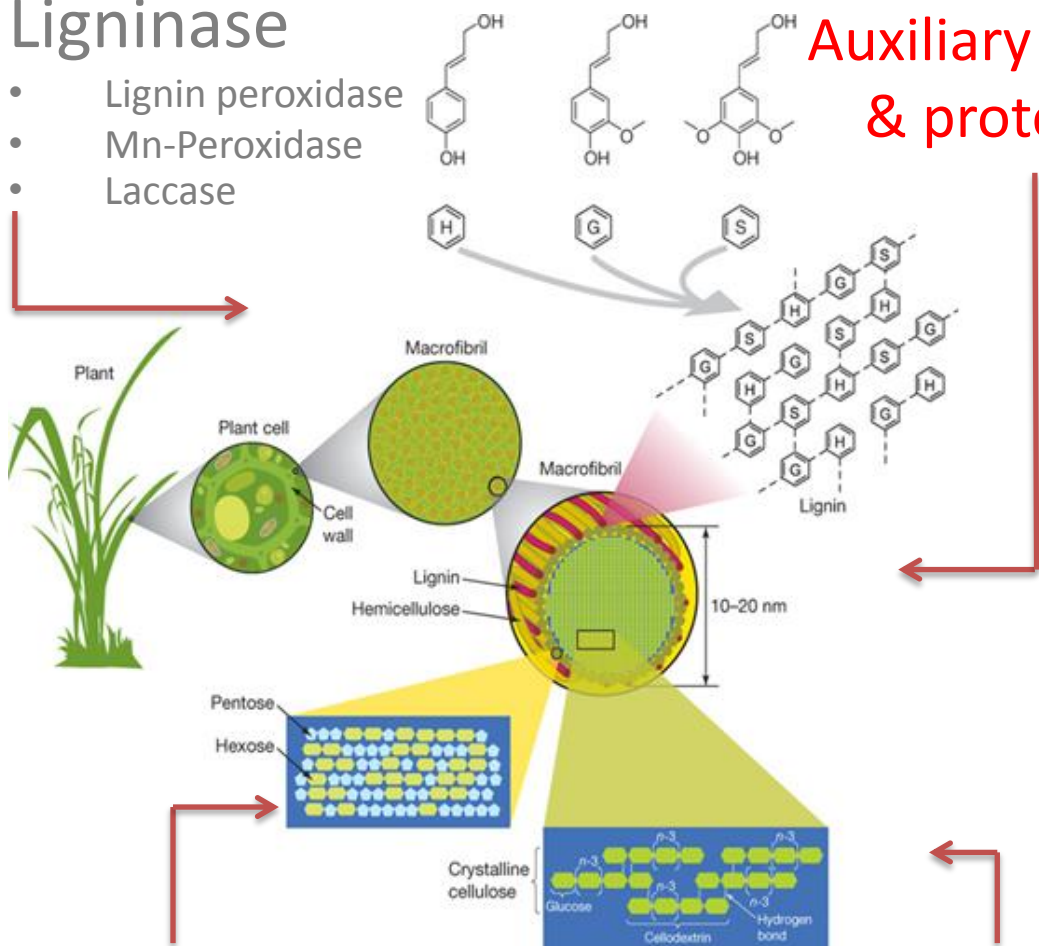


# Lignocellulose structure & decomposition

## Ligninase

- Lignin peroxidase
- Mn-Peroxidase
- Laccase

Auxiliary enz  
& proteins



## Hemicellulase

- Endo-acting:  
Xylanase/Mannanase
- Exo-acting/debranching

## Cellulase

- Endoglucanase
- Exoglucanase
- $\beta$ -Glucosidase

Bagasse



Rice straw



Corn stover



Agricultural waste



Animal feedstuff



Textile fiber




Paper pulp

Modified from Rubin (2008), Nature 454; 841-845

# Synergistic enzymes for biomass saccharification

## Gene pool

BIOTEC microbes



>80,000 (BCC)

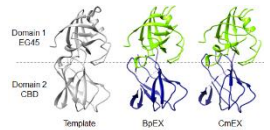
Metagenome



Public database

Bioinformatic tool

- Sequence
- Structure



## Enzyme library

**Core**

CBH1    CBH2    EG

**ACC**

$C_x$

BGL    XYN    XLD

PEC    MAN    AF

**AUX**

Low range

BpEX    CmE    SaEX

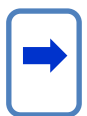
$C_1$

High range

AA9Tr    AA9Ct



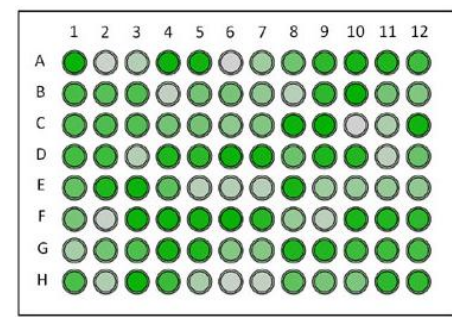
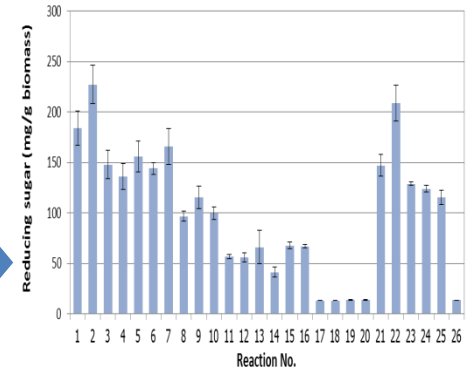
Crude multi-activity enzyme



Recombinant expression system  
*Ogataea thermomethanolica*/  
*P. pastoris*/*E. coli*

## Mixture formulation

### Synergy screening



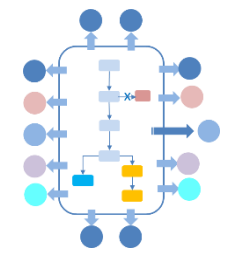
### Synergistic interaction

- DoE
- High-throughput

## Application



On-site enzyme production



Consolidated bioprocessing microbe



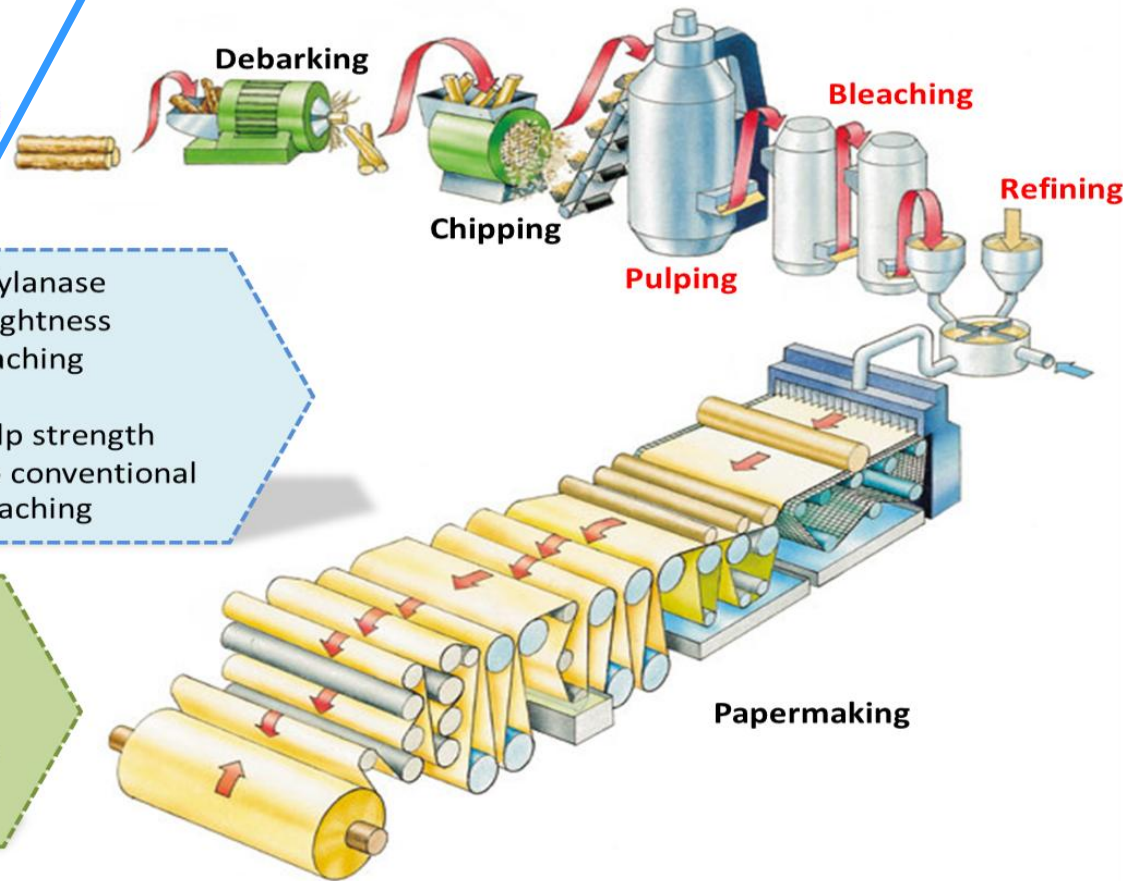
# Enzymes for green processing of pulp in paper industry

Alkaliphilic xylanase with no background cellulase activity is desirable

**Pulping:** Cellulase  
•Reduce energy and chemicals in pulping

**Bleaching:** Xylanase  
•Increase brightness  
•Reduce bleaching chemicals  
•Increase pulp strength compared to conventional chemical bleaching

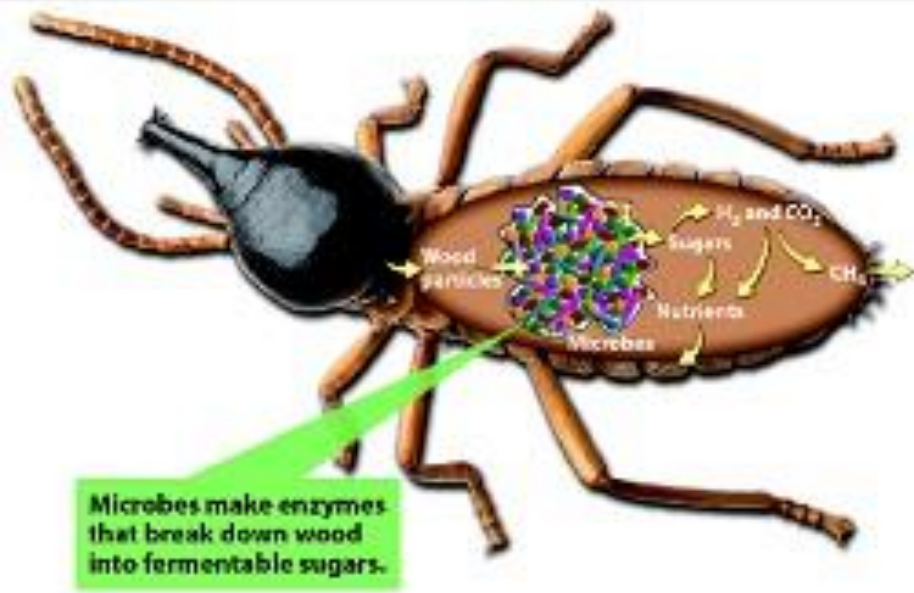
**Refining:** Cellulase  
•Reduce refining energy  
•Increase fibrillation and pulp strength



Partial xylan hydrolysis → Destruction of Lignin-Hemicellulose complex → Increase accessibility of bleaching chemicals → Chlorine usage ↓



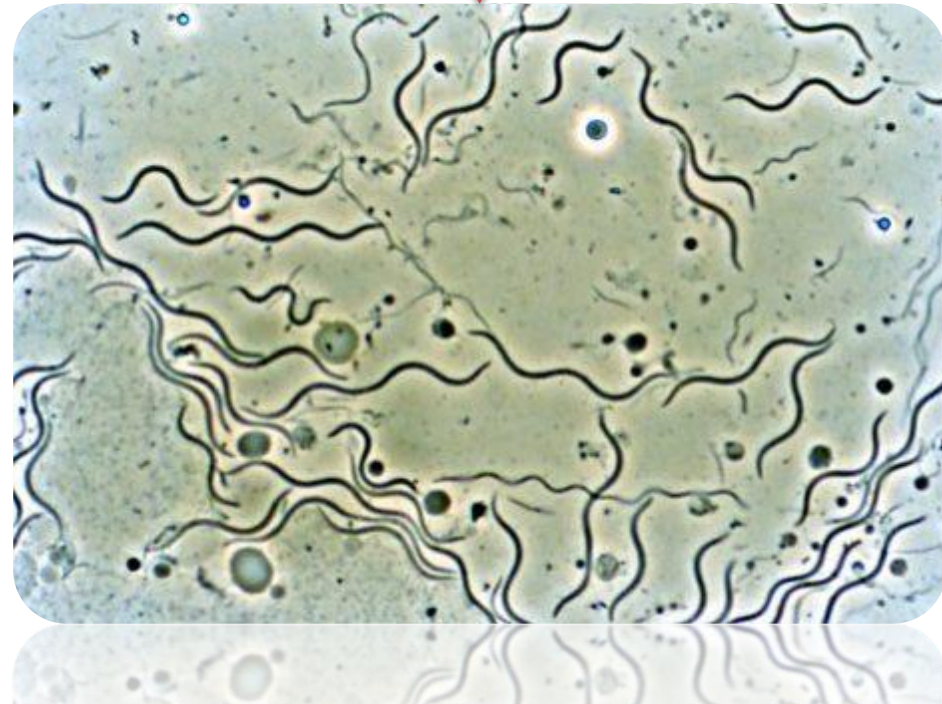
# Isolation of alkalophilic endo-xylanase from termite gut metagenome



Alkali pH with effective cellulolytic microbial community

Termite gut symbiont is one of the nature's most efficient model for the breakdown of wood. Genomic study revealed that almost 1,000 enzymes play a role in this process.

**Potential source for discovery of lignocellulose degrading enzymes for biotechnological application.**

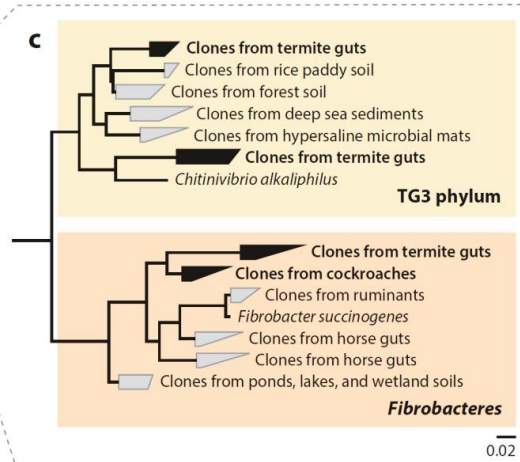
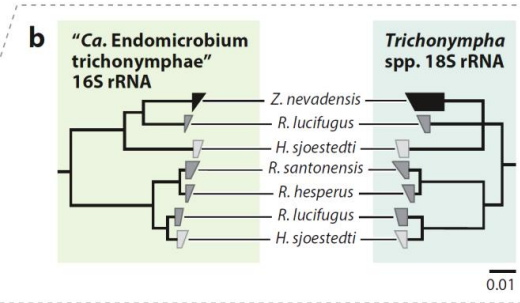
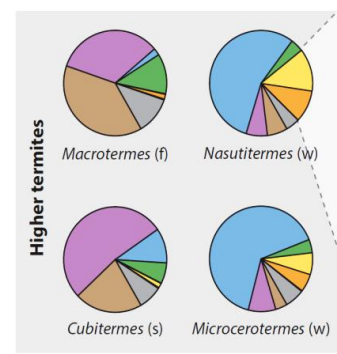
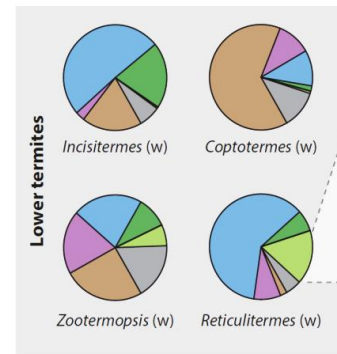
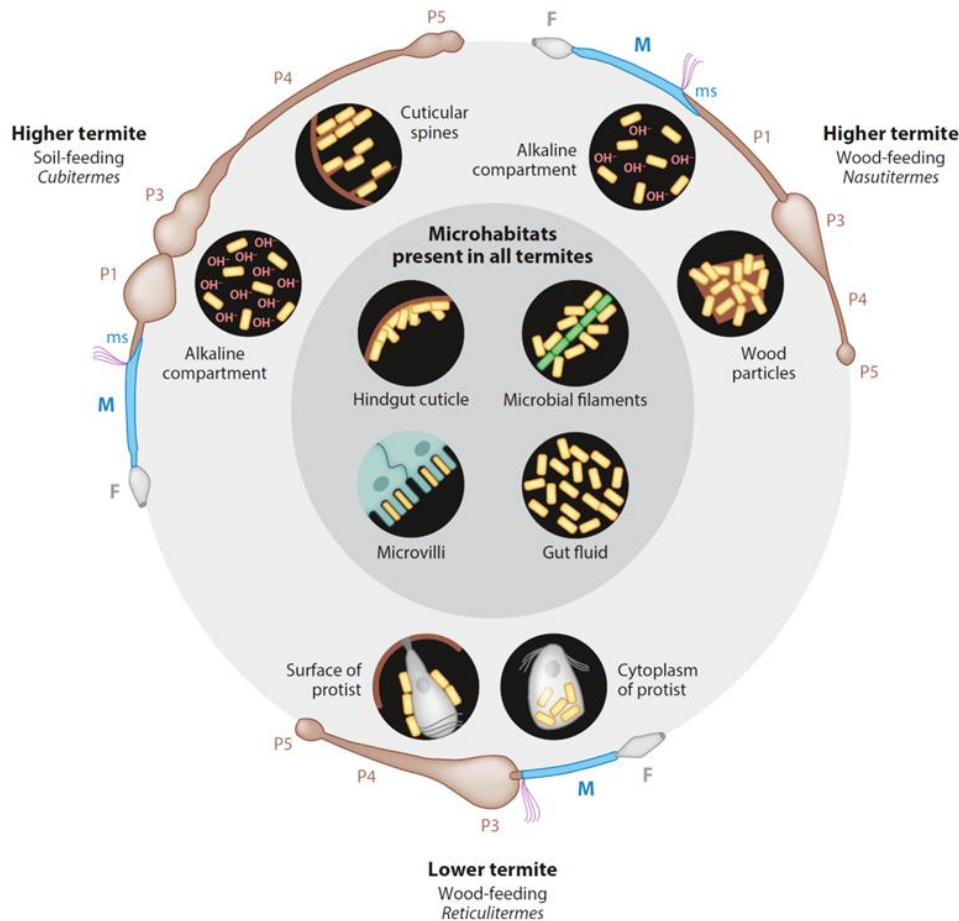
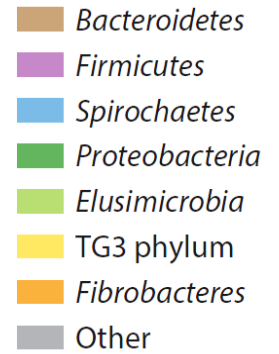




# Diversity of the gut microbiota

The termite gut microbiota comprises all three domains of life:

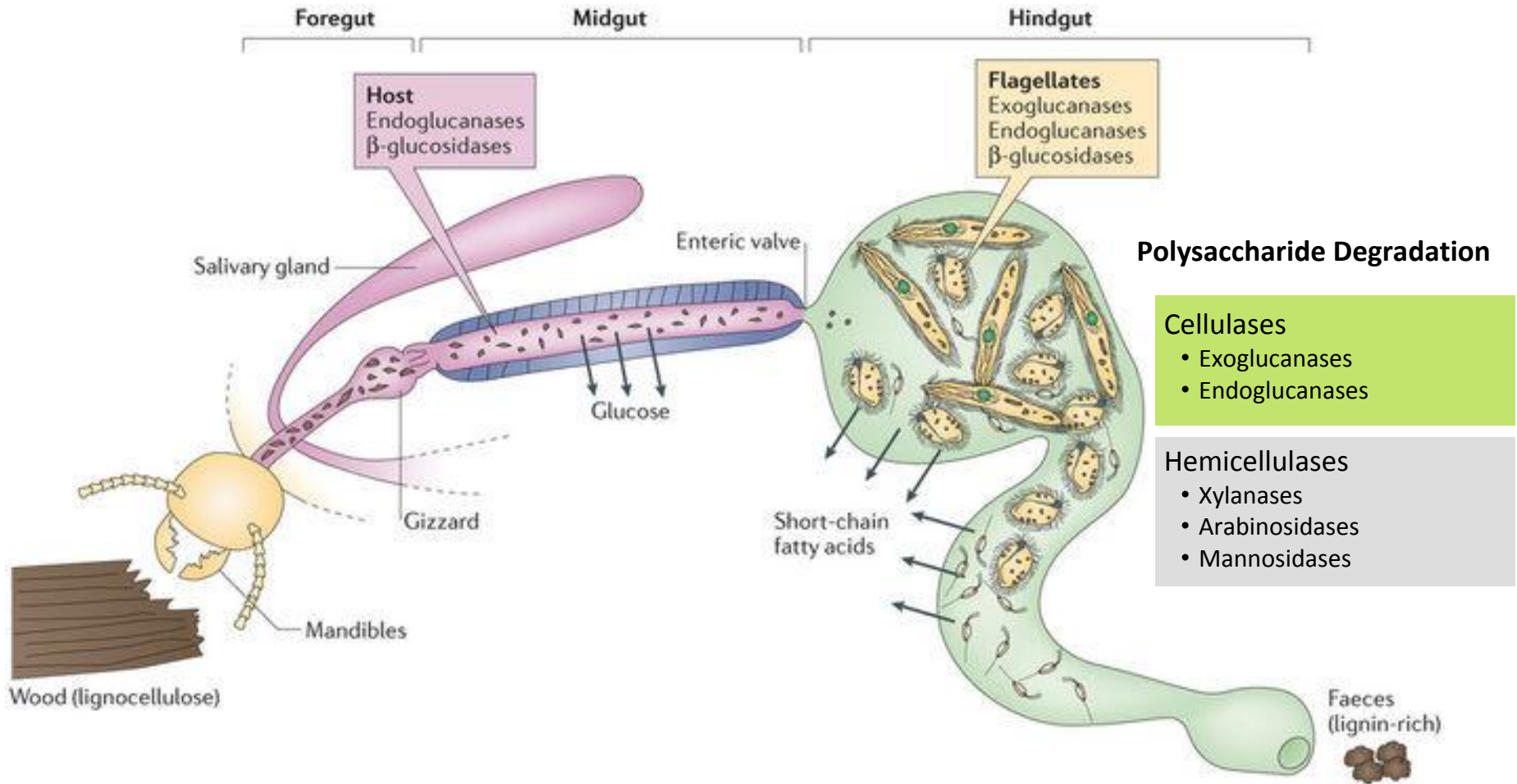
- Bacteria
- Archaea
- Eukarya (flagellate protists in lower termites)



Brune and Dietrich, 2015

# Major functional niches of the hindgut ecosystem

Alkaline pH



## Sequence analysis and annotation of the cellulase and xylanase genes retrieved from the metagenomic fosmid library of termite gut.

Fosmid clone	Predicted ORF	Protein length (aa)	Most homologous protein	Organism	Identity/similarity (%)	GH/conserved motif (range)/catalytic residues	GenBank Accession No.	References
CL1	<i>c0001</i>	404	GH5 (ABW39333) from metagenomes of termite gut	Uncultured bacterium	58/71	Cellulase/GH5 (82-372)/E205 and E338	GU721061	Warnecke <i>et al.</i> [36]
Xyn5	<i>x1088</i>	651	Endo-1,4- $\beta$ -xylanase (AAS85781) from metagenomes of adult lepidopteran moth	Uncultured bacterium	50/65	Xylanase/GH8 (8-405)/E44 and D276	GU721062	Brennan <i>et al.</i> [5]
Xyn12	<i>x0012</i>	273	Endo-1,4- $\beta$ -xylanase (AAS85784) from metagenomes of adult lepidopteran moth	Uncultured bacterium	56/73	Xylanase/GH11 (42-250)/E142 and E244	GU721063	Brennan <i>et al.</i> [5]
Xyn9	<i>x1098.1</i>	700	GH10 (ABN52146)	<i>C. thermocellum</i> ATCC27405	40/58	Xylanase/GH10 (38-378)/E184 and E306	GU721064	Unpublished
Xyn9	<i>x1098.2</i>	664	Xylanase (AAS85784) from metagenomes of adult lepidopteran moth	Uncultured bacterium	49/66	Xylanase/GH11 (55-260)/E147 and E253	GU721064	Brennan <i>et al.</i> [5]
Xyn9	<i>x1098.3</i>	529	Xylanase (AAS85783) from metagenomes of caterpillars	Uncultured bacterium	59/73	Xylanase/GH11 (32-244)/E145 and E242	GU721064	Brennan <i>et al.</i> [5]



## ENZ-Bleach: Alkaliphilic xylanase for pulp bio-bleaching



Metagenome of termite gut symbionts

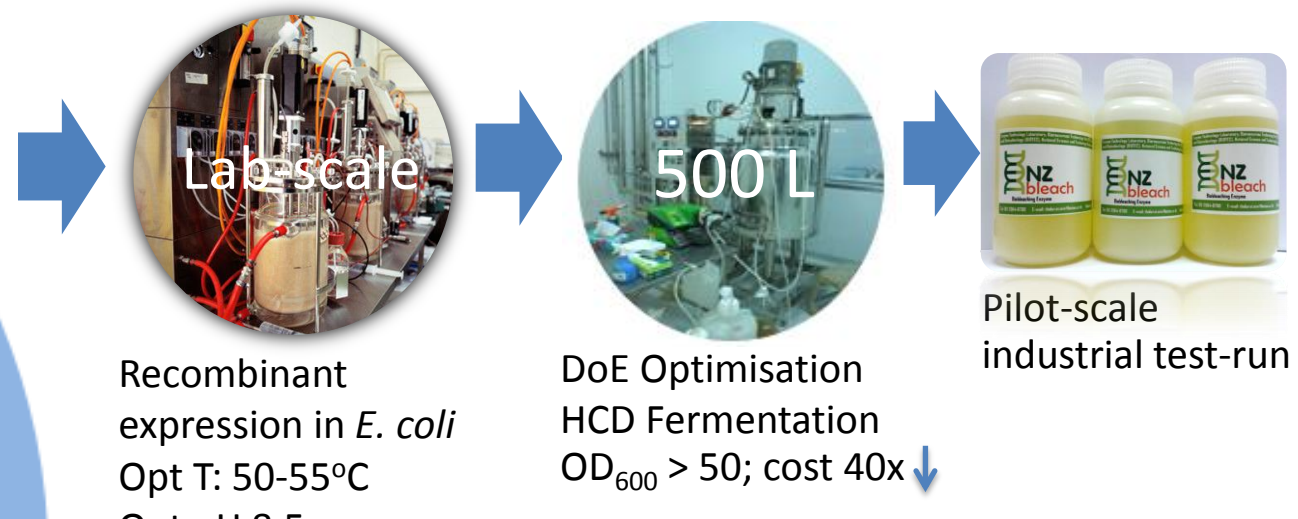
Alkaline hind gut

Fosmid library: 2 Gb

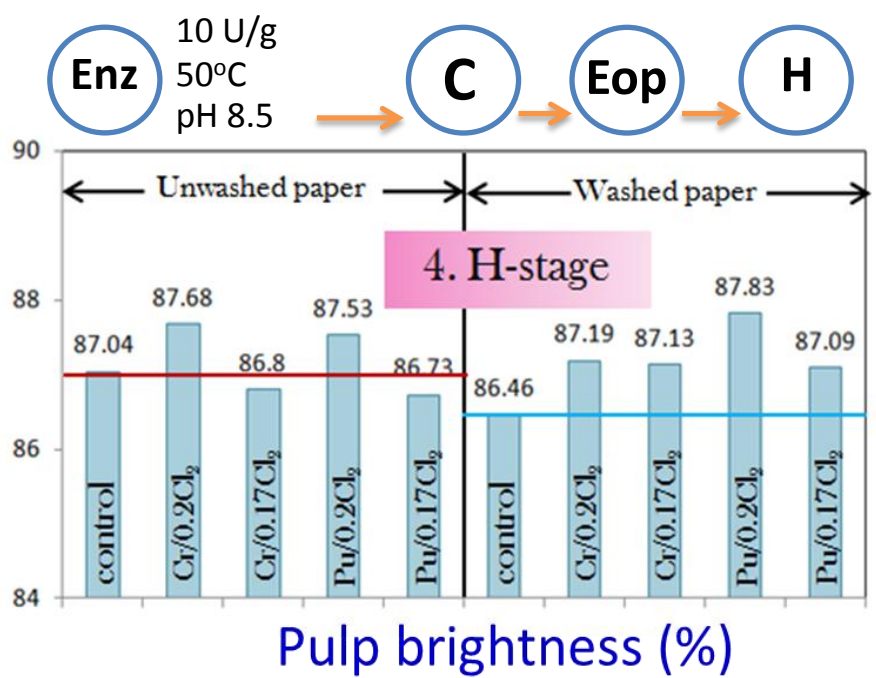
Activity-based screening: AZCL-xylan/ AZCL-HE-cel

12 Xyl genes  
2 Cel genes

Hit-rate:  $10^{-4}$



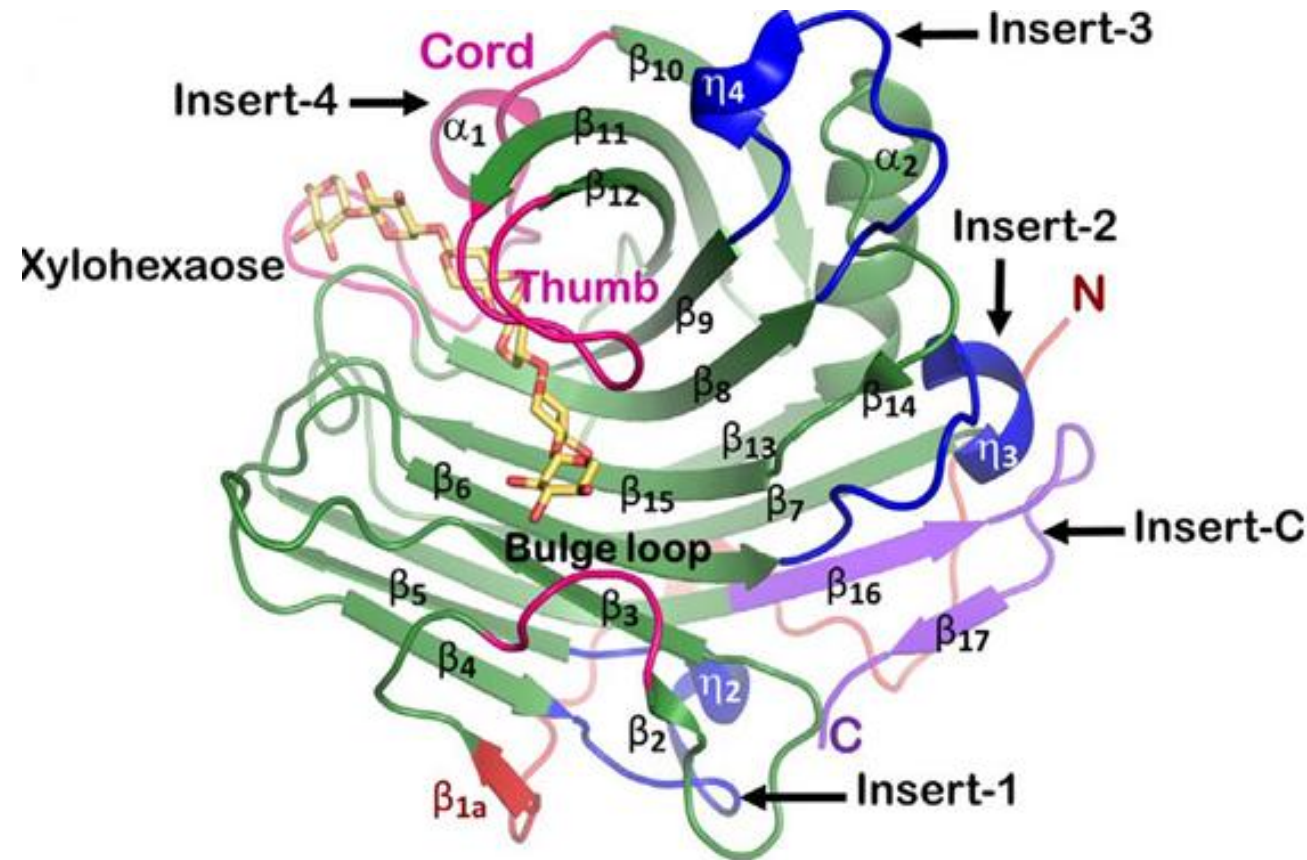
- ↑ Brightness
- ↓ Chlorine
- ↓ Chemical
- ↓ Energy
- ↓ Waste water



Nimchua et al. (2012) *J. Microbiol. Biotechnol.* 22: 462-469



# Structure-based protein engineering for thermostable and alkaliphilic enhancement of Xyn12.2 xylanase for applications in pulp bleaching



## Rational design

### Approach 1

Enhancing H-bonding

### Approach 2

Arginine replacement on polar surface

### Approach 3

S-S bond

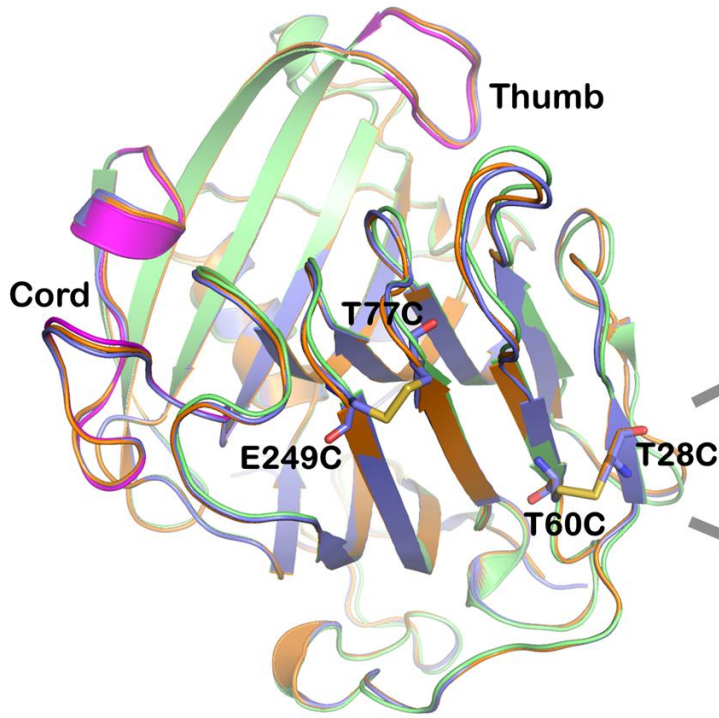
### Approach 4

Hydrophobic packing

## The overall structure of Xyn12.2.

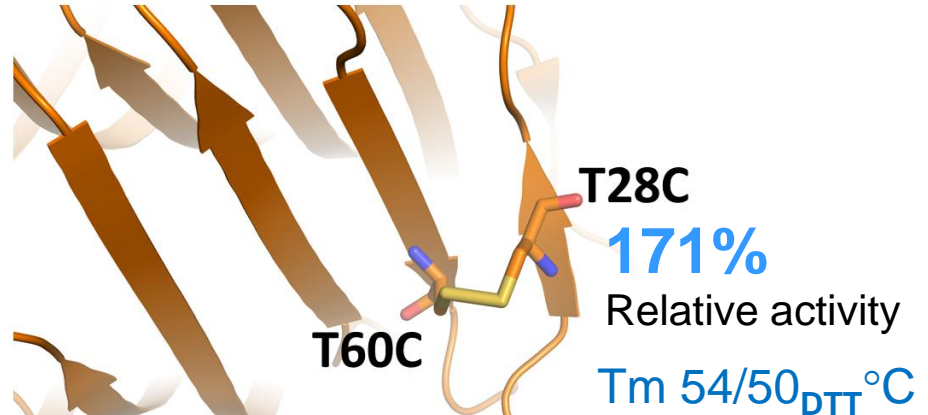
A cartoon representation of Xyn12.2 shows the right-handed  $\beta$ -jelly roll fold typical for GH11 xylanases. Positioning of the xylohexaose substrate was adopted from *T. reesei* xylanase crystal structure (PDB code 4HK8) and drawn as sticks to indicate the active site of Xyn12.2.

## Approach II: Addition of disulfide bonds (where is good for SS bond?)

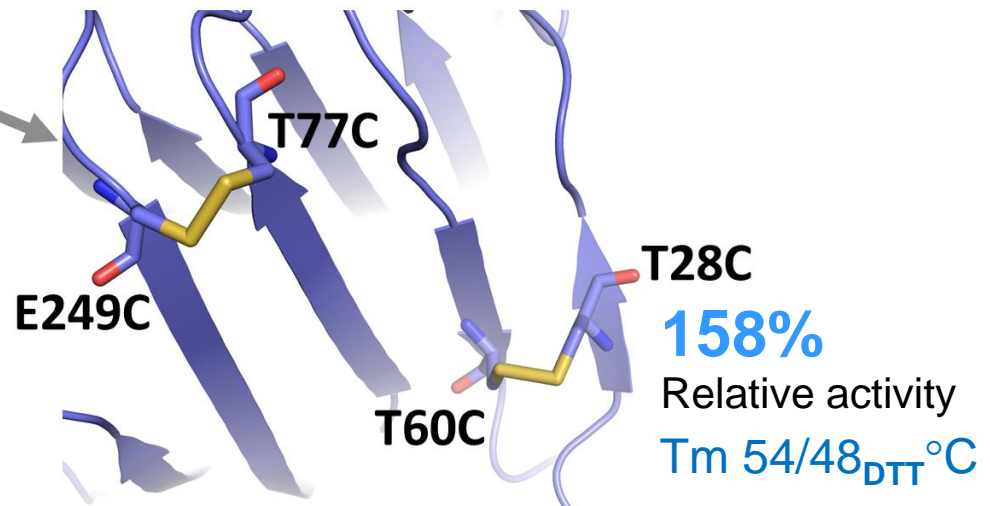


WT-Tm 50/50<sub>DTT</sub>°C

### One S-S bond in XynTT



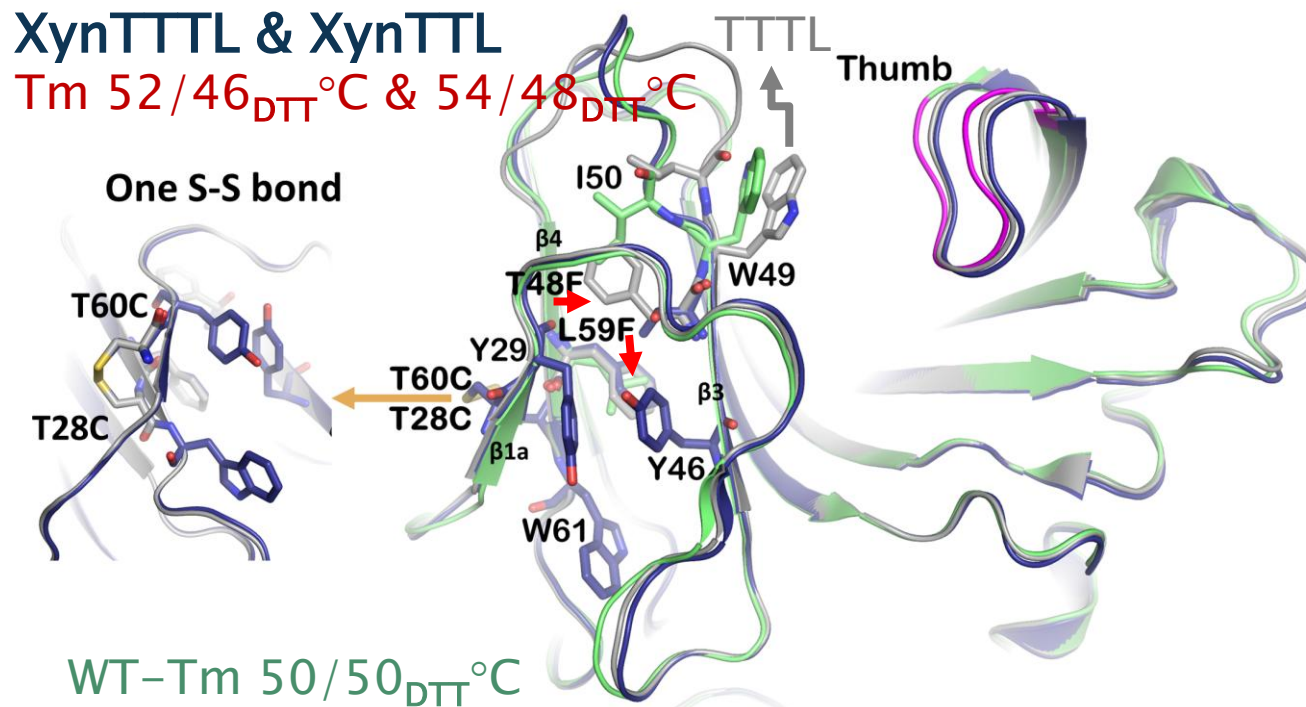
### Two S-S bonds in XynTTTE



XynTT and XynTTTE : exterior S-S bond

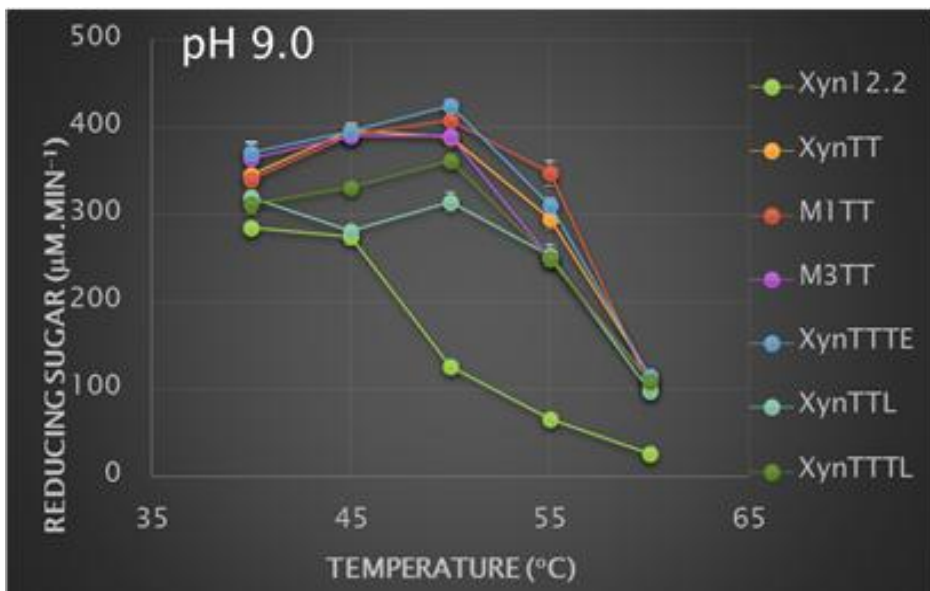
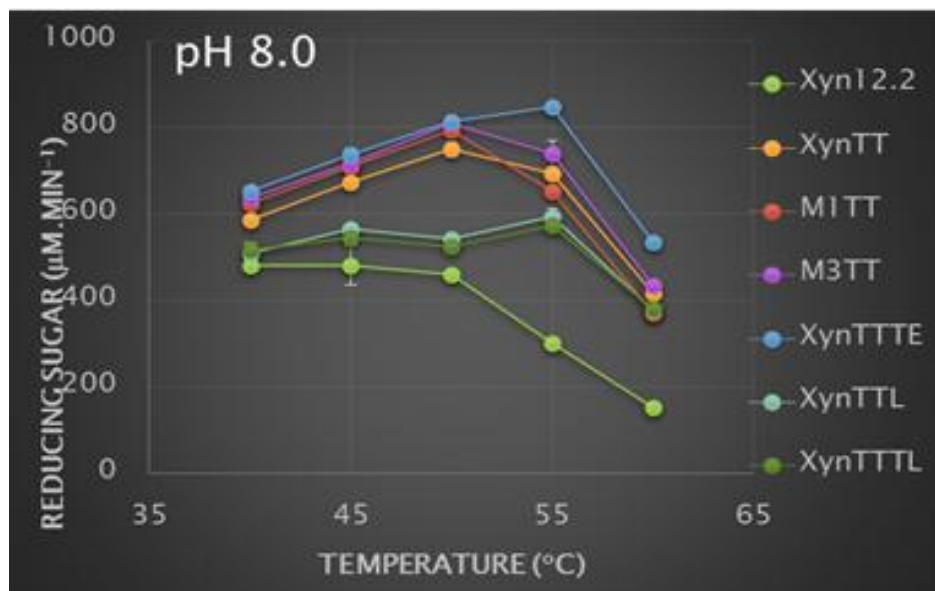
# Approach III: increasing enthalpy: Hydrophobic packing

Xylanase	Modifications	Mutations	$k_{cat}$ ( $s^{-1}$ )	%Relative activity
Xyn12.2 <sup>wt,c</sup>	wild type	-	1210±16	100
<b>Hydrophobic packing + disulfide bond formation</b>				
XynTTL <sup>2,c</sup>	hydrophobic core packing + S-S bond	T28C, T60C, L59F	1371±33	113
XynTTL <sup>1,c</sup>	hydrophobic core packing + S-S bond	T28C, T60C, T48F, L59F	1620±58	134
<b>Active site</b>				
E244A <sup>*</sup>	catalytic activity disruption	E244A	8±0	1
E244H <sup>*</sup>	catalytic activity disruption	E244H	1±0	0
E142H <sup>*</sup>	catalytic activity disruption	E142H	2±0	0



# Successful Xyn12.2 variants

Xylanase	Modifications	Mutations	$k_{cat}$ ( $s^{-1}$ )	%Relative activity
Xyn12.2 <sup>wt,c</sup>	wild type	-	1210±16	100
<b>Disulfide bond formation</b>				
XynTT <sup>1,c</sup>	exterior S-S bond	T28C, T60C	2070±47	171
XynTTTE <sup>1,c</sup>	two exterior S-S bonds	T28C, T60C, T77C, E249C	1912±22	158
<b>H-bond and engineered arginine + disulfide bond formation</b>				
M1TT <sup>1,c</sup>		V5N, V6N, K7R, K223R, K227R, T28C, T60C	1859±27	154
M3TT <sup>1,c</sup>		K73R, K185R, T28C, T60C	1807±26	149
<b>Hydrophobic packing + disulfide bond formation</b>				
XynTTL <sup>2,c</sup>	hydrophobic core packing + S-S bond	T28C, T60C, L59F	1371±33	113
XynTTTL <sup>1,c</sup>	hydrophobic core packing + S-S bond	T28C, T60C, T48F, L59F	1620±58	134





# Operational stability relationship to performance of enzyme variants

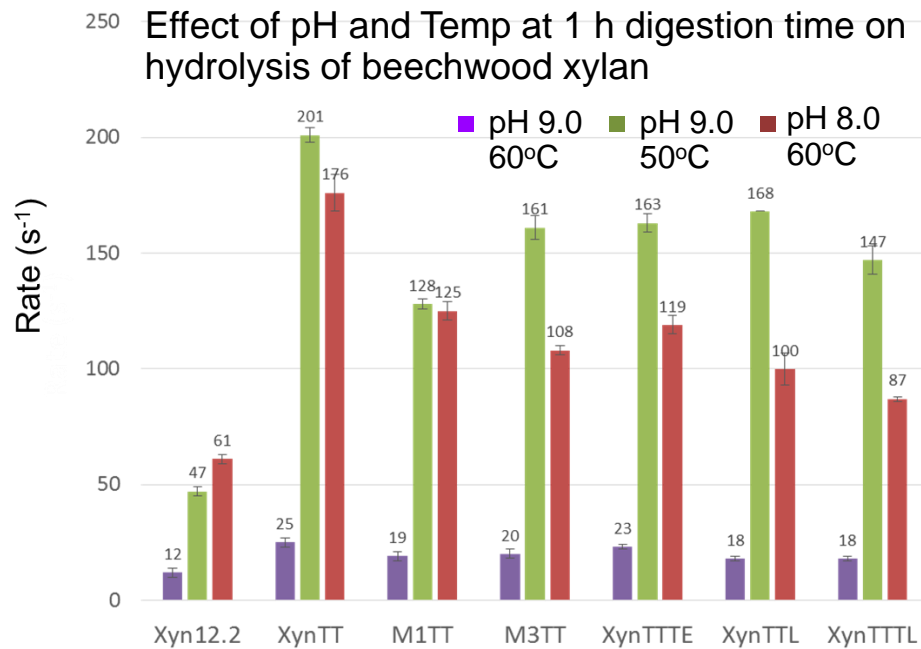
We can improve the performance of Xyn12.2 by addition of S-S bonds, high pKa Arg replacement, H-bonds, and hydrophobic interactions as shown in the variants XynTT, M1TT, M3TT, TTTE, XynTTL, and XynTTTL.

The performance of the variants are pH and temperature dependent, with higher threshold of tolerance than wild type.

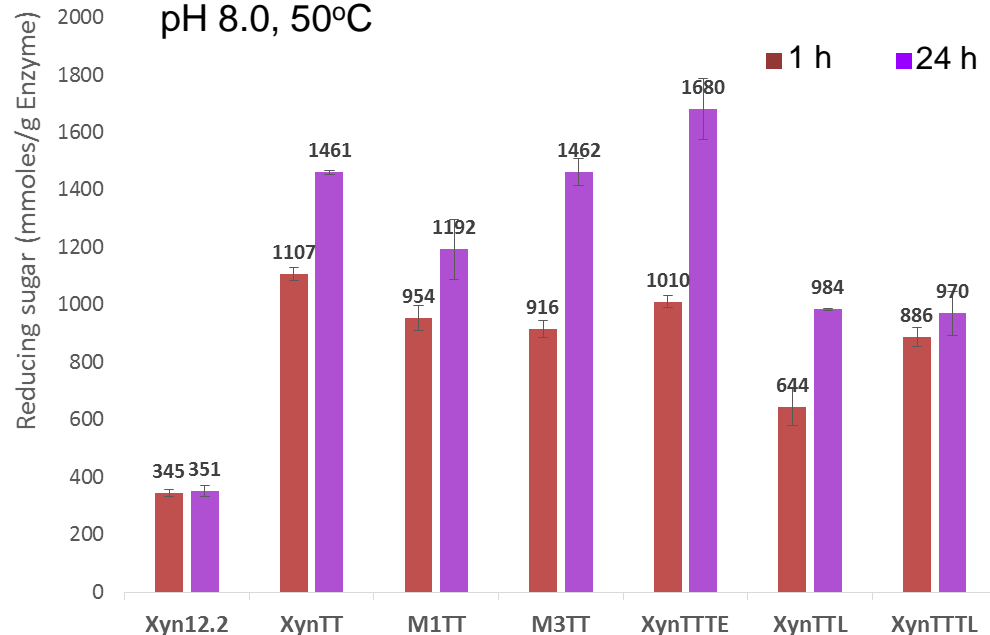
## Xyn TTTE quadruple mutants

- $k_{cat}$ : 270% at pH 8, 60°C
- $T_m$ : 3-6°C at pH 8-9
- Catalytic efficiency: 420% at pH 9, 50°C, 1 h

Effect of pH and Temp at 1 h digestion time on hydrolysis of beechwood xylan



Hydrolysis of alkaline-pretreated bagasse at pH 8.0, 50°C



# Enzyme R&D

## Enhancing capability of existing bio-industry for THAILAND 4.0



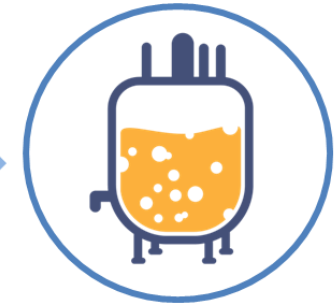
### Biobank & Bioservice

- National Enzyme library
- Quality control for product registration
- IP helpdesk



### Innovative R&D

- Enzyme engineering
- Strain development
- HTP bioprocess opt
- Formulation



### Demonstration plant (OEM/ODM)

- Non-GM/GM
- SmF/SSF
- Downstream processing
- Pre-processing unit

Platform technology build-up

Creating economic impact via value chain

producer → user → society



# Industrial & academic collaboration





# NSTDA: driving force for Thailand's S&T

## Acknowledgement

- National Center for Genetic Engineering and Biotechnology
- National Science and Technology Development Agency
- Enzyme Technology & Microbial Cell Factory Laboratory members
- JGSEE-BIOTEC Integrative Biorefinery Laboratory

Special thanks to Dr.Pemchit Chitnumsub