Microbial synthesis of high-value plant secondary products: Bioresource mining and engineering

Bo Yu, Ph.D

CAS Key Laboratory of Microbial Physiological and Metabolic Engineering, Institute of Microbiology, Chinese Academy of Sciences
Biotechnology: old
its underlying processes have been used by mankind for thousands of years. e.g. the production of wine and cheese.
Biotechnology: new

Modern biotechnology uses enhanced micro-organism like yeast, bacteria as ‘cell factories’, as well as enzymes, to produce a variety of goods.
Three Waves of Modern Biotechnology

<table>
<thead>
<tr>
<th>Red Biotechnology</th>
<th>Green Biotechnology</th>
<th>White Biotechnology</th>
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<tbody>
<tr>
<td>Medicines</td>
<td>Transgenic plants</td>
<td>Industrial products</td>
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</tbody>
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- **1980’s-**
- **1990’s-**
- **2000’s-**

Biotechnology has found its entry into medicine (red) and agriculture (green), and now a new wave of industry (white), also called industrial biotechnology.
Why Industrial Biotechnology?

Demand

People Health

Foods

?

Technology

Medical Biotechnol.

Red Biotechnology

Agricultural Biotechnol.

Green Biotechnology

Industrial Biotechnol.

White Biotechnology
Fossil: the basis of modern civilization

Refinery provides the essential chemicals and energy for modern society
The challenges for socio-economic

**People**

- Environment pollution

**Resource depletion**

**Climate change**

Environmental costs (white pollution, greenhouse, etc.)
The solutions: Biotechnol. process
-- from Petroleum to Biomass as materials

Oil

Coal

Petrochemical
Coal industries

RUBBER, FUEL, NYLON
PLASTICS, CHEMICALS

Biochemical
Engineering

GREEN & SUSTAINABLE

CHEMICALS, PESTICIDE,
FINE CHEMICALS

ANTIBIOTICS, VITAMINS
AMINO ACIDS, ENZYMES

Renewable biomass

Corn, Cassava

Industry exhaust

Modern Industry
Definition of Industrial Biotechnology

application of modern biotechnology for the industrial production of chemical substances and bio-energy, using living cells and their enzymes, resulting in inherently clean processes within minimum waste generation and energy use.

Nutraceuticals
Fine chemicals
Bulk chemicals
Can biotechnology be competent?
Bio-products fit with current industries

- Petroleum
  - Gasoline
  - Kerosine
  - Diesel
  - Hydrocarbons $C_nH_{2n+2}$

- Biomass
  - Bio-ethanol
  - Bio-diesel
  - Biogas, $H_2$
  - Carbohydrates $C_n(H_2O)_n$

- Chemical industries
  - Basic chemicals
  - Fine chemicals
  - Polymers
  - Oxygenate
  - Chiral chemicals

- Fuel & Energy
  - Bulk chemicals
  - Fine chemicals
  - Bio-polymers
Bio-products fit with current industries

- Carbonhydrates
  - starch
  - Hemicellulose
  - Cellulose
  - Lignin
  - Lipid
  - Protein

- Sugar
  - glucose
  - fructose
  - xylose
  - arabinose

- Polymers

- Thermochem
  - SG
  - C2
  - C3
  - C4
  - C5
  - C6

- Microorganism

- Modern Chemical Indust.

- Platform chem.

- H2 methane
  - Ethanol, ethylene
  - Lactic, glycerol acrylic acid 3-hydroxy-propionic acid
  - Fumaric acid Succinate Aspartate Malic acid
  - Itaconic acid Levulinic acid
  - Citric acid Gluconate Sorbitol

- Modern Chemical Indust.

- Platform chem.
I.B. reduces the processes and costs

Organic substrate

Traditional Chem.

Biotechnology Fermentation

- Materials < 37%
- Energy < 30%
- CO₂ em. < 63%
Case: 1,3-propanediol bio-production

- Modify >70 genes, 18 genes knockout or overexpressed
- Titer > 135 g/L, productivity > 3.5 g/L/h
- First industrialization example of bio-based chemical
Biotech makes industry green

Advanced Biocatalysts

- Efficient Genetic Manipulation
- Rapid Microbial Evolution
- Protein Engineering & Screening
- System Metabolic Engineering

Nutraceuticals

Fine chemicals

Bulk chemicals

Fuel
Plant secondary metabolites

More than 2,000 kinds of plant natural products such as isoprenoids, alkaloids and flavonoids, which are all plant secondary metabolites, have been used by human as flavors, fragrances and medicines.

(Chang et al., 2006)
Schematic overview of biosynthetic routes and precursors of the plant natural products

(Marienhagen, et al., 2013)
Isoprenoids

Isoprenoids comprise the largest class of the natural product products, encompassing more than 5,000 known compounds with an extremely diverse array of chemical structures, such as taxol, artemisinin, and carotenoids.
Alkaloids are nitrogen-containing, low-molecular-weight compounds and known for their medicinal use.
Glucosinolates constitute a natural class of organic compounds that contain sulfur and nitrogen and are derived from glucose and an amino acid.
Natural diversity of glucosinolates

They are synthesized from certain amino acids.

About 132 different glucosinolates are known to occur naturally in plants.

**Aliphatic glucosinolates** derived from mainly methionine, but also alanine, leucine, isoleucine, or valine.

**Aromatic glucosinolates** include indolic glucosinolates, derived from tryptophan, phenylalanine and tyrosine.
Enzymatic activation

The plants contain the endogenous thioglucosidases, called myrosinase, which, in the presence of water, cleaves off the glucose group from a glucosinolate. The remaining molecule then quickly converts to an isothiocyanate, a nitrile, or a thiocyanate; these are the active substances that serve as defense for the plant.

To prevent damage to the plant itself, the myrosinase and glucosinolates are stored in separate compartments of the cell and come together mainly under conditions of physical injury.
Isothiocyanates

Isothiocyanate was known for anti-carcinogenic activity, antimicrobial activity, and anti-inflammatory activity.

Studies have suggested inverse relations between the intake of cruciferous vegetables, such as broccoli, and cancer incidence.

The following compounds contribute to this function.

- Sulphoraphane (SFN)
- Benzyl Isothiocyanate (BITC)
- Phenylethyl Isothiocyanate (PEITC)
Natural diversity of isothiocyanates

Its variation in the side group that is responsible for the variation in the biological activities of these plant compounds.

Some glucosinolates:
• Sinigrin is the precursor to allyl isothiocyanate
• Glucotropaeolin is the precursor to benzyl isothiocyanate
• Gluconasturtiin is the precursor to phenethyl isothiocyanate
• Glucoraphanin is the precursor to sulforaphane
Shortcomings of natural extraction

- Natural diversity makes the extraction of separate pure compound high costly

- The enzymes of entire pathway in plants stored in separate compartments of the cell, make the synthesis inefficient

- Extraction process is complicated with high pollution
The comparison between plants and microorganisms as metabolic engineering platform

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<th>plant</th>
<th><em>E. coli</em></th>
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<tbody>
<tr>
<td>Growing rate</td>
<td>slow</td>
<td>rapid</td>
</tr>
<tr>
<td>Costs</td>
<td>high</td>
<td>low</td>
</tr>
<tr>
<td>Yield</td>
<td>low</td>
<td>high</td>
</tr>
<tr>
<td>Purity</td>
<td>low</td>
<td>high</td>
</tr>
<tr>
<td>Extraction process</td>
<td>difficult</td>
<td>simple</td>
</tr>
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on an industrial scale
Microbial production of ITCs

-- Benzyl Isothiocyanate as a case --

**Pathway design**

- **Gene mining**
- **Protein modification**

**E. coli**

**Functionally expressed enzymes**

- Phenylalanine
- Benzylisothiocyanate
P450 enzymes

Glucotropaeolin

The original biosynthesis pathway in plant
The difficulties and solution strategies for P450

1. Translational incompatibility of the membrane signal modules

   Modification of N-terminal membrane–binding domain

2. Absence of electron transfer machinery

   Fused with corresponding reductase via linker peptide

ATR1: NADPH--cytochrome P450 reductase 1

ATR2: NADPH--cytochrome P450 reductase 2

This enzyme is required for electron transfer from NADP to cytochrome P450 in microsomes.
Functional expression of CYP79A2

The first eight modified amino acid from bovine-derived CYP17α was confirmed to be advantageous for anchorage to the membrane for E.coli. The 25 to 74 amino acids from sorghum derived CYP79A1 were proven to be effective for higher expression level. The 73-711 amino acids were the functional domain without transmembrane sequence. 40-529 amino acids were the functional domain.
Functional expression of CYP83B1

a) MDLLLI IAGLVAAP AFFFL RSTKKSLRLPPGPKGLPIIG MALLLVAF

b) 1 8 15 494 73 711

17\alpha CYP83B1 (G_4S)_2 ATR2

ATR1: NADPH--cytochrome P450 reductase 1
ATR2: NADPH--cytochrome P450 reductase 2

Lane 1 and 2, fused with ATR1
Lane 3 and 4, fused with ATR2
The biosynthesis pathway of benzyl isothiocyanate
Design the pathway and reduce the step

Pathway in plants

Glutathione

GSTF

GGP1

Spontaneous reaction

Cysteine
Selection and expression of C-S lyase

C-S lyase was not redundant unlike the other enzymes in plant. SUR1 is the only C-S lyase for this pathway in plants.

We tried to optimize the induction conditions but still failed to get the functional enzyme for inclusion body.
Selection and expression of C-S lyase

The biosynthesis of methionine in \textit{E. coli} involves cystathionine $\beta$-lyase which catalyzes a reaction similar to SUR1.
MetC (β-lyase) was chosen for substituting SUR1.
Condon-optimized \textit{UGT74B1} from \textit{Broccoli rapa} was successfully expressed and purified which shows a high affinity for various types of thiohydroximate.

\textbf{UGT: UDP-glucose:thiohydroximate S-lucosyltransferase}
SOT16、SOT18 both show catalyze activity on benzyl-derived glucosinolate. We failed to obtain the soluble SOT16 and select *Arabidopsis thaliana* ecotype Col-0 derived SOT18 which was confirmed to have higher $V_{\text{max}}$ compared to the other variants.

**SOT18 Marker**

**SOT: desulfoglucosinolate:PAPS sulfotransferase**
Selection of suitable myrosinase

✓ Glycosylation modification was essential for plant-derived myrosinases to form activity configuration.

✓ Myrosinase from *Brevicoryne brassicae* which need no secondary modification was selected to express in *E.coli*.
The activity of Myrosinase from *Brevicoryne brassicae* was confirmed.
The modified biosynthesis pathway of benzyl isothiocyanate in *E. coli*

All enzymes have been functionally expressed in *E. coli*

- Black indicate the original element
- Red indicate the modified in *E. coli*
As a proof of concept, successful biosynthesis of BITC *in vitro* by functionally expressed enzymes from different sources was confirmed.
Conclusion

\[ \text{E. coli} \]

- **Gene mining**
  - MA2R2: NADPH, CO₂, NADP⁺ → N-OH
  - MB1R2: NADPH, CO₂, NAD⁺ → Cysteine

- **Protein modification**
  - Spontaneous reaction
  - METC, PLP

- **Pathway design**
  - BMVR, SOT18
  - UGT74B1

- **Functionally expressed enzymes**
  - Phenylalanine → Benzyl isothiocyanate
Functionalizing the synthesis *in vivo*

- Cofactor supplement: *cysteine* and PAPS
- Coenzyme balance and supplement: NADPH
- Remove substrate feedback inhibition and degradation: *cysteine*
Cofactor supplement

ATP regeneration

Regeneration
Remove feedback inhibition

SAT-m from Arabidopsis

Remove product inhibition

Knock-out degradation path.
NADPH balance (P450 enzymes needs NADPH)

Expression of zwf increases PPP pathway for NADPH supplement
Schematic map of BITC synthesis in vivo

MAP: MA2R2, MB1R2, zwf, BMYR, SOT18, UGT74B1

Reactions:
- MA2R2: O₂, NADPH, CO₂, H₂O → O-Acetylserine
- MB1R2: O₂, NADPH, CO₂, H₂O → Sulfide
- BMYR: Glucose-6-P → Glucose
- SOT18: PAPS → AMP
- UGT74B1: UDP → UDP-D-glucose

Pathway:
- Glucose → Glucose-6-P → O-Acetylserine → sulfide → O-Acetylserine
- Sulfide + Cysteine → SAT-m serine
- SAT-m serine → PAPS regeneration
- PAPS regeneration → AMP → APS
- AMP → ATP

Enzymes:
- CYSQ, APK2, METC, PLP

Metabolites:
- Glucose, Glucose-6-P, O-Acetylserine, sulfide, Cysteine, SAT-m serine, PAPS, AMP, APS, ATP
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Bo Yu
E-mail: yub@im.ac.cn