BIOREMEDIATION AND BIOMASS UTILIZATION

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Bioremediation

• Bioremediation is defined as the process whereby organic wastes are biologically degraded under controlled conditions to an innocuous state, or to levels below concentration limits established by regulatory authorities.
• Bioremediation uses naturally occurring bacteria and fungi or plants to degrade or detoxify substances hazardous to human health and/or the environment.
• The microorganisms may be indigenous to a contaminated area and stimulated in activity (biostimulation) or they may be isolated from elsewhere and brought to the contaminated site (bioaugmentation).
Problems with bioremediation

- Work *in vitro*, may not work in large scale. Work well in the laboratory with simulation, may not work in the field. Engineering approach is needed.
- Alternatively, select adapted species on site (indigenous species) to remediate similar damage.
- Most sites are historically contaminated, as a results of the production, transport, storage or dumping of waste. They have different characteristics and requirements.
- Those chemicals are persistent or recalcitrant to microbial breakdown.
Use of bacteria in bioremediation

- Greatly affected by unstable climatic and environmental factors from moisture to temperature.
- For examples, pH in soil is slightly acidic; petroleum hydrocarbon degrading bacteria do not work well < 10 C.
- These microbes are usually **thermophilic anaerobic**.
- Fertilizers are needed. Seeding or bioaugmentation could be useful too.
- They contain monooxygenases and dehydrogenases to break down organic matters including most toxic substances.
Biomass Utilization

Renewable Bioresource Feedstock
- Plants
  - crops
  - trees
  - algae
- Animals, fish
- Microorganisms
- Organic residues
  - municipal
  - industrial
  - agricultural
  - forestry
  - aquaculture

Bioprocess Technology
- Biocatalysis (Enzymes)
- Fermentation (Microorganisms)

Physical – Chemical Process Technology
- Extraction
- Pyrolysis
- Gasification

Industrial Bioproducts
- Bioenergy and Biofuels
- Manufactured products:
  - biochemicals
  - biosolvents
  - bioplastics
  - ‘smart’ biomaterials
  - biolubricants
  - biosurfactants
  - bioadhesives
  - biocatalysts
  - biosensors
The diffusion of biotechnology and green chemistry across the economy

**Biotechnology**

**Green Chemistry**

- **Health and Medicine**
  - Biopharmaceuticals
  - Vaccines
  - Diagnostic kits
  - Artificial organs
  - Gene therapies

- **Agriculture and Food**
  - Disease and drought resistant crops
  - Functional foods
  - Nutraceuticals
  - Biopesticides

- **Industrial Bioproducts**
  - Biofuels and bioenergy
  - Manufactured products
    - Biochemicals
    - Bioplastics
    - Biolubricants
    - Biocatalysts
    - Biosensors

- **Additional ‘Sectors’**
  - Informatics
  - Genomics
  - Nanotechnology
  - Security
  - Services

**First Wave**
**Second Wave**
**Third Wave**
Advantages of Biomass Utilization
→ feedstock waste → Energy

Fossil Carbon Economy -
carbon is used and discarded

- Waste is a disposal problem
- Growth from increasing production volume

Bioproducts Economy -
carbon is recycled

- Waste is a feedstock opportunity
- Growth from increasing value added

Carbon Cycle in Industry
Carbon Cycle in Nature
Biomass Utilization

(1603~1867)
- Rice straw
- Sandals Shoes
- Mat
- Rope
- Rain coat

Future (2010~2020)
- Biomass Refinery
- Bioplastics

Future (2010~2020)
- Plastics, Fabrics, Synthetic rubber, etc.
- Rain coat
- Umbrella
- Sandals Shoes
- Mat
- Rope

Future (2010~2020)
- Fertilizer
- Chemicals

Biomass Utilization

Rice straw

Biomass Refinery

Bioplastics

Recycle

Waste

Compost

Composting

Biogas

Alcohol

Energy

Oven

Gas oven

Electric oven

Cars

Petroleum

(1989~2010)

Energy

Compost

CO₂
Why BIOMASS?

1. To substitute or complement drying up petroleum.
2. To reduce the amount of CO$_2$ emission.
3. To decrease (urban/agricultural/forestry) organic wastes.
4. To re-activate rural life and economy.
Special waste bioprocessing

-- Degradation of oil in spills

-- Degradation of xenobiotics (chlorinated insecticides, herbicides and fungicides)

-- Degradation of polymers (plastics)

-- Transformation of toxic metal species into less or non-toxic forms.
Pseudomonas

- Genetically engineered bacteria (*Pseudomonas*) with plasmid producing enzymes to degrade octane and many different organic compounds from crude oil.
- However, crude oil contains thousands of chemicals which could not have one microbe to degrade them all.
- Controversial as GE materials involved.
Degradable organic compounds in the environment

1) Biodegradable: undergoes a biological transformation. Complete degradation results in CO$_2$ and some inorganic compounds

2) Persistent: do not biodegrade in certain environments

3) Recalcitrant: resist biodegradation in most environments

Not always beneficial process:

1) non toxic compound $\rightarrow$ toxic metabolite,

2) toxic chemical $\rightarrow$ more toxic chemical (mercury waste transformed into very toxic methyl mercury compounds).
Microbes have the ability to consume natural or synthetic organic substances as nutrient and energy sources.

Pseudomonas group – digest more than 100 organic compounds.

Microbes have an extensive set of enzymes that can serve as a raw material for further mutagenesis.

Modified enzymes = Digesting new substrates, working in altered environment or having improved kinetics.
“Catabolic plasmids” of Pseudomonas

1) Encode enzymes for waste-degradation pathway

2) Could be transferred between species

3) Transfer across the community increases in response to novel nutrient use, or exposure to a potentially toxic compound.

Enzymes of catabolic plasmids able to degrade:
- octane
- naphthalene
- toluene
- benzene
- pesticides
- herbicides...
Examples of naturally occurring “catabolic plasmids” capable to interspecies transfer

<table>
<thead>
<tr>
<th>Compound</th>
<th>Plasmid</th>
<th>Size</th>
<th>Bacterium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biphenyl</td>
<td>pBS241</td>
<td>195 kb</td>
<td>P. putida</td>
</tr>
<tr>
<td>Camphor</td>
<td>pPG1(CAM)</td>
<td>Near 500 kb</td>
<td>Pseudomonas sp.</td>
</tr>
<tr>
<td>Octane</td>
<td>OCT</td>
<td>Near 500 kb</td>
<td>P. oleovorans</td>
</tr>
<tr>
<td>Chlorobiphenyl</td>
<td>pSS50</td>
<td>53.2 kb</td>
<td>Alcaligenes spp.</td>
</tr>
</tbody>
</table>

From Microbial Ecology, 19:1-20, 1990
Ananda Chakrabarty’s “Super-Pseudomonas”

1974 Chakrabarty created super-Pseudomononas degrading oil in spills

Plasmids CAM (camphor degradation), OCT (octane degradation), NAH (naphtaline degradation) and XYL (Xylol degradation) were combined in the same cell
Creation of Super-Pseudomonas

CAM (camphor degradation), OCT (octane degradation), NAH (naphtaline degradation) XYL (Xylol degradation)
*Pseudomonas putida* mt-2 (pWWO) is a very good bacteria capable of decontaminating organic substances including solvents, such as toluene, one of the components of gasoline. It is very common in soil and plant rhizosphere. It promotes plant growth, is a biocontrol agent for plant pathogens.
Degradation of benzene by pseudomonads (cited in Galzer and Nikaido, Microbial Biotechnology, Freeman and Company, NY)

Acetyl CoA
Succinate

Different benzene derivatives induce different degradative pathways; TOL cleavage supply P. putida with energy

Meta-cleavage pathway (TOL catabolic plasmid), activated when chromosomal pathway overloaded

Acetaldehyde
Pyruvate

Benzene

1,2-dihydro-1,2-dihydroxybenzene

TOL catabolic plasmid

Meta-cleavage

Ortho-cleavage

Ortho-cleavage pathway (chromosomal)
Burkholderia cepacia makes molecule difficult to degrade.
Most biodegradation-performing bacteria are mesophilic (20-40°C is their growth optimum).

Rivers or soils that need to be cleaned are at 0-20°C and are difficult to clean!!

Conjugation

Psychrophilic strain (cold-liking)

Result: Psychrophilic strain degrading both Salicilate and Toluene
Difficulties with releasing biodegrading “lab” microbes into the environment

“Oh dear! I didn’t realize ‘in the field’ would be like this! We should have stayed in the laboratory.”

Plant carbohydrates is a valuable material for industry

**STARCH**

Starch: 1-4 linkage of α glucose

**Cellulose**

Cellulose: 1-4 linkage of β glucose
Starch – edible for humans.
Cellulose – non-edible for humans
Crystalline Region
Amorphous Region
Cellulose
Lignin
Hemicellulose

Pretreatment gives enzyme accessible substrate
STARCH
Cellulose
Starch

Amylose

(100 – 400,000 glucose monomeres)

Amylopectin

(10,000 – 4,000,000 glucose monomer)

corn starch from waxy maize contains only 2% amylose

but that from amylomaize is about 80% amylose.
## Annual world production of commercial starches

### in millions of metric tons

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<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>Maize</td>
<td>11.6</td>
<td>20</td>
<td>26.3</td>
<td>35.2</td>
</tr>
<tr>
<td>Potato</td>
<td>1.4</td>
<td>2.4</td>
<td>2.5</td>
<td>2.1</td>
</tr>
<tr>
<td>Tapioca &amp; others</td>
<td>1.5</td>
<td>1.7</td>
<td>3.2</td>
<td>3.8</td>
</tr>
<tr>
<td>Wheat</td>
<td>0.6</td>
<td>1.5</td>
<td>1.7</td>
<td>2.8</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>15.1</strong></td>
<td><strong>25.6</strong></td>
<td><strong>33.7</strong></td>
<td><strong>43.9</strong></td>
</tr>
</tbody>
</table>
Components of plant cell walls

- Cellulose
- Hemicellulose
  - *need special yeast to convert to ethanol*
- Lignin
- Extractives
- Ash

Fermentable sugars obtained from cellulose in 1819

Chapple, 2006; Ladisch, 1979
Uses of non-usable banana starch
Straw degradation in flask

Control

Inoculated

day 0

day 4

day 8

day 10
Major Microorganisms in the consortium
GlucoAmylase is able to digest branching points

Alpha-Amylase genes from B. amyloliquietaeciems; B. Stearoothermophilus
(both expressed in B. subtilis)

GlucoAmylase is derived from Aspergillus niger; A. awamori

Starch granules
- 35% in cold water
- pH 6.5
- 40 ppm Ca^{2+}

Starch slurry
- 106°C, 5 min
- Bacterial α-amylase, 1500 U kg\(^{-1}\)

Gelatinised starch (< 1 DE)
- 95°C, 2 h

Liquefied starch (1 1 DE)
- 0.3% D-glucose
- 2.0% maltose
- 97.7% oligosaccharides

Saccharification
- pH 4.5
- Glucoamylase, 100 U kg\(^{-1}\)
- Pullulanase, 100 U kg\(^{-1}\)
- 60°C, 72 h

Glucoamylase

Maltozose syrup (44 DE)
- 97% D-glucose
- 1.5% maltose
- 0.5% isomaltose
- 1.0% other oligosaccharides

Glucose sirup
- 4% D-glucose
- 56% maltose
- 28% maltotriose
- 12% other oligosaccharides
Yeast Metabolism: pentose fermentation

**Glucose**
- Glucose → Glucose-6-P
- Glucose-6-P → Fructose-6-P
- Fructose-6-P → Glyceraldehyde-3-P
- Glyceraldehyde-3-P → 3-Phosphoglycerate
- 3-Phosphoglycerate → Phosphoenolpyruvate
- Phosphoenolpyruvate → Pyruvate → Acetaldehyde

**Xylose**
- Xylose → Xylitol
- Xylitol → Xylose
- Xylose → Xylose-5-P
- Xylose-5-P → PPP

**TCA Cycle**
- Pyruvate → Acetaldehyde

**NAD(P)H**
- NADH → NAD+
- NAD+ → NADH

**Ho et al**
Yields of Ethanol from Corn Stover (Cellulose Ethanol)

From Cellulose: 50 to 55 gal / ton
From Xylan: 30 to 35 gal / ton
Total: 80 to 85 gal / ton.

Corresponds to about 250,000 tons /yr for 20 million gal per year plant

Requires engineered yeast, pretreatment cellulase enzymes
Ethanol is renewable energy source (better than oil)

Every bushel of corn can produce 2.5 gallons of ethanol

U.S. Corn Used for Ethanol

- 35% less CO
- 42% less NOx
- 43% less NMHC (nonmethane hydrocarbon)
- 39% less PM (particulate matter)
- 79% less CO2 over life cycle

Source: USDA / Economic Research Service
Previous pictures were about starch; it will be nice to transform cellulose (woods) into ethanol too...
Cellulose waste

Cellulose itself
Cristalline; amorhous

Lignin
Complex aromatic (organic) polymer
-- no simple repeat unit
-- hydrophobic-repels water
-- acts as an adhesive in cell wall
-- stiffens wood

Hemi-Cellulose
Combinations of 5 or 6 sugars;
Side groups (branching)
Amorphous

-- acts as an adhesive in cell wall
-- stiffens wood
Hemicellulose
(a mix of many monomers)

Usually, all of the pentoses are present.

The pentoses are also present in rings that can be 5-membered or 6-membered.

Xylose is always the sugar present in the largest amount.
Problems with commercial glucose production from cellulose waste

1. typical waste cellulose material contains less than half cellulose (lignin and pentosans are non-degradable contaminants).

2. A combination of enzymes is needed to solubilize and degrade cellulose waste.

3. Natural enzymes are comparatively unstable or have low activity against complex of the lignin and cellulose. Enzymes are subject to both substrate and product inhibition.

   cost of cellulose $\rightarrow$ glucose conversion is excessive

   Strach is more expensive to produce than cellulose, but starch glucose is much cheaper
Most pure cellulose sources (most easily transformed to glucose) are most expensive ones

<table>
<thead>
<tr>
<th>Raw material</th>
<th>Lignin</th>
<th>Cellulose</th>
<th>Hemicellulose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pine wood</td>
<td>27.8</td>
<td>44.0</td>
<td>26.0</td>
</tr>
<tr>
<td>Birch wood</td>
<td>19.5</td>
<td>40.0</td>
<td>39.0</td>
</tr>
<tr>
<td>Sugar cane bagasse</td>
<td>18.9</td>
<td>33.4</td>
<td>30.0</td>
</tr>
<tr>
<td>Rice straw</td>
<td>12.5</td>
<td>32.1</td>
<td>24.0</td>
</tr>
<tr>
<td>Cotton</td>
<td>None</td>
<td>80–95</td>
<td>5–20</td>
</tr>
</tbody>
</table>

Cellulose is associated with lignin and pentosans, so as to resist biodegradation; dead trees take several years to decay even in tropical rainforests.
**Cellulases**

- **Cellulases**
  - **cellbiohydrolase (CBH)**: hydrolyse the cellulose chain from one end.
  - **endoglucanase (EG)**: Cut randomly inside the cellulose chain.

**Cellulose**

- **Cellulose (crystalline)**
  - **Cellulose (non-crystalline)**
    - **Exo-cellbiohydrolase**
      - **Celllobiose**
        - **Celllobiose**
          - **Cellulase**
            - **D-glucose**

Hydrolyse cellulose cooperatively, i.e. they act in synergy.
first, hemicellulases may digest hemicellulose (and open up wood for bleaching, cellulose to be extracted etc...)

Enzyme source: 
Fermentation of a nonpathogenic strain of *Aspergillus niger*.

Enzyme complex: 
arabinosidase, mannanase, mannosidase and xylanase.

Xylose is produced from hemicellulose in large amounts!!!
Xylose is major component of waste. Would be great to transform it to ethanol somehow!!!
Ideal biocatalyst microorganism:

-- Creates ethanol from glucose (from cellulose)
-- Creates ethanol from xylose (from hemicellulose)
-- Capable to high-yield ethanol production
-- Growth in industrial settings

No “ideal bug” exists yet

Promising Biocatalysts

- *Zymomonas*
- Recombinant *Saccharomyces*
- Recombinant *E. coli*
- *Lactobacillus*
- Xylose-Assimilating Yeasts
- *Clostridium*
Zymomonas mobilis

• Advantages:
  – Natural fermentative microorganism
  – Near theoretical ethanol yield from glucose
    (92%-94% versus 88%-90% for yeast)
  – Shorter fermentation time (300%-400% faster than yeast)
    – No oxygen requirement
    – Tolerant to inhibitors in hydrolysates
    – High ethanol tolerance
    – Fermentation at low pH
    – Grows at high sugar concentrations
    – High specific productivity

• Limitations:
  – Narrow substrate utilization range (only pure glucose)
a strain of *Zymomonas mobilis* that produces ethanol from both xylose and glucose was created

DOE is assisting Arkenol Inc. (California) in building a new biomass-to-ethanol plant that will provide 8 million gallons per year of ethanol from rice straw using the *Zymomonas mobilis* process.

This project provides an alternative to rice straw burning, which is being phased out in California for environmental reasons.
Fermentation Profile by *Z. mobilis* 206C/pZB301 grown on Glu:Xyl:Ara (30:30:20 g/l) at pH = 5.5, T = 31.5°C

Similar result achieved with genome-integrated copies of XYL-ARA genes (greater strain stability)
The problem of non-degradable lignin remains:
Lignin

Hardwoods
softwoods

Amorphous co-polymer of p-coumaril alcohols
Lignin derivatives used as fuel additives (to increase octan number)

(Lignin)

(Chemical process)

Hydrodeoxygenation

Hydrocracking

Hydrogenolysis

Hydrogenolysis

Etherification

BCD Product

R = H, CH₃

Hydrocarbon Fuel Additive

R = H, CH₃, OMe

Oxygenate Fuel Additive
System of Biomass Utilization

- Rice production
- Fallow rice pad
- Ethanol Station
- Fields
- Horticulture
- High School Public Education
- Town Office Volunteer Center
- Sightseeing Hotel Restaurants Brewery Others
- Other usage of Ethanol
- Local Economy
- Local Government
- Forest
- Wood waste
- Wastes
- Ethanol bus
- Ethanol car
- Ethanol truck
- Ranch
- Compost Center Waste Treatment Center
- Cooperate
- Local Education
- High School Public Education
- Agricultural Products Distribution
- Non-eatable parts
- Straw/Husk
- Industrial crops
Future: New Sources
Sunlight to Biodiesel?

CO₂ → Plant Cellulose → Sugars

CO₂ → Algae

E. coli → Bakers Yeast

Biodiesel
Ethanol Blend
Thank You

Microbes make the world go around

Marvellous microbes