Production of enzymes in microbes: Hydrolases

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Hydrolases

Learning outcomes. Be able to…

- **Describe the production and use of microbial cellulase, amylase, and lipase, with reference to:**

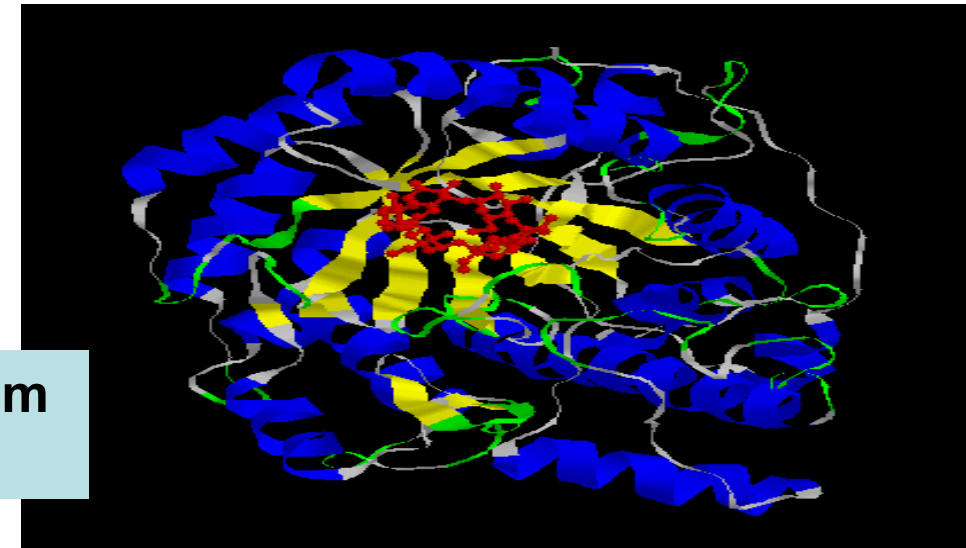
  - chemistry of substrate
  - origin of the enzyme
  - natural role in microbe
  - source (natural/recombinant)
  - specific biotech applications
  - methods of improvement

Proteins for household, industry + agriculture

- Enzymes are a valuable commodity
- Global market approx $4 billion & growing rapidly

Global value of bulk enzymes	
Food	45%
Detergents	34%
Textiles	11%
Leather	3%
Pulp+paper	1%
Others	6%

- Enyzme reactions have :
  - high specificity
  - mild conditions → No harsh solvents, acids, or heat i.e. environ. friendly
  - novel possibilites



Amylase structure from X-ray crystallography

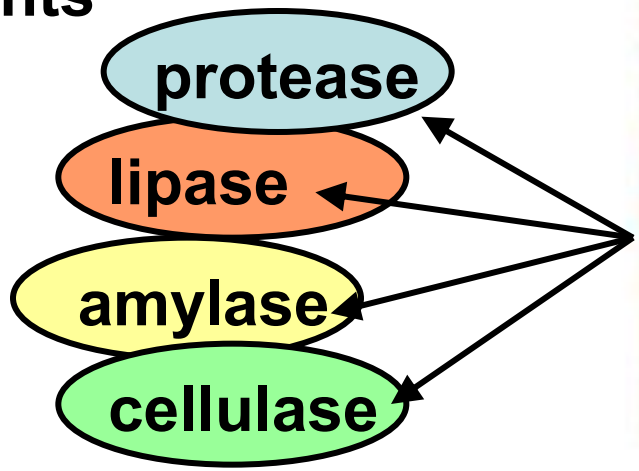
Proteins for household, industry + agriculture

- Hydrolytic enzymes (hydrolases) from bacteria and fungi are the most widely-used type of microbial enzymes

- Hydrolases are attractive due to:
  - high stability: easy to purify and handle
  - no cofactor requirements: cheap

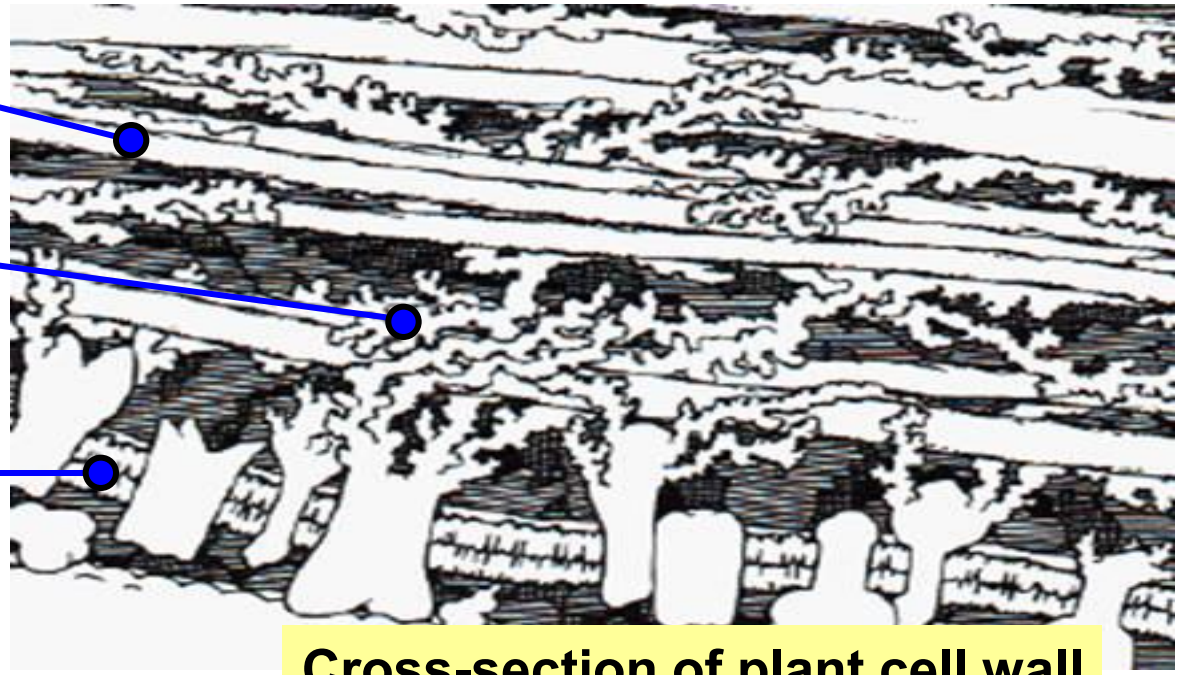
- Hydrolases are catalysts for breakdown of biopolymers,
  - to make higher-value products
  - as cleaning agents

A cocktail of microbial hydrolases is found in modern washing powder



Cellulase: cellulose as a substrate

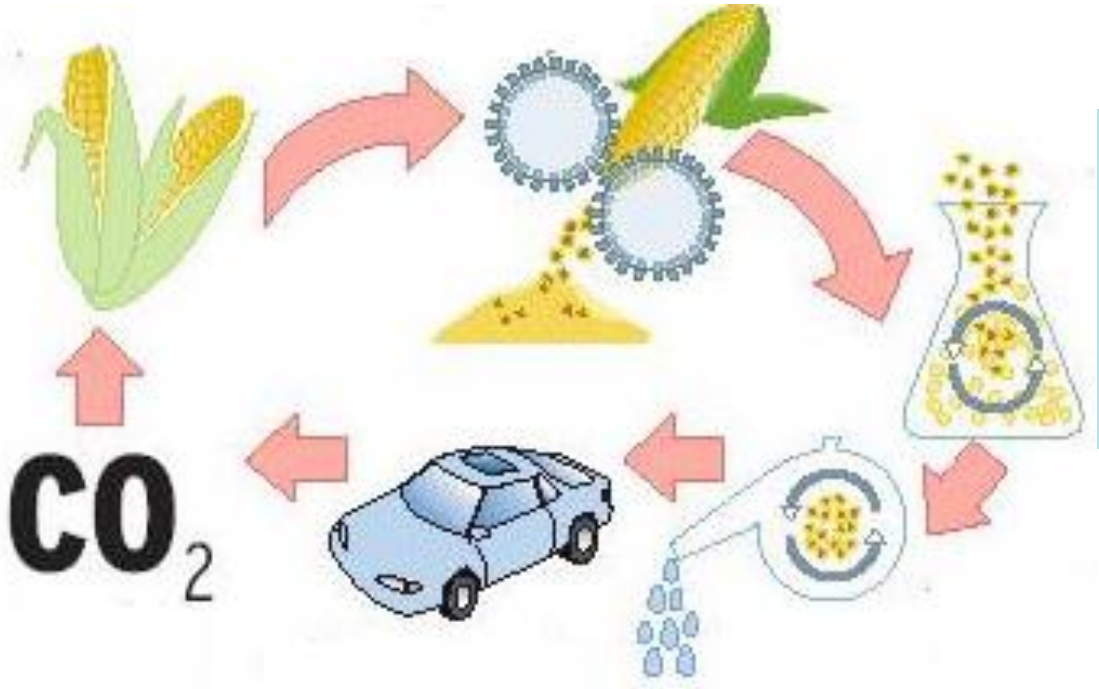
- **Cellulose:** most abundant organic molecule (~ 10^{11} tonnes); high turnover (~ 10^{10} tonnes per year)
- A huge potential reservoir of stored energy – synthesis rate equivalent to 50,000 barrels of oil per second
- Synthesized by plants + microbes as a structural material; in the form of rigid, crystalline, insoluble fibres



Cross-section of plant cell wall

Cellulase: industrial and domestic applications

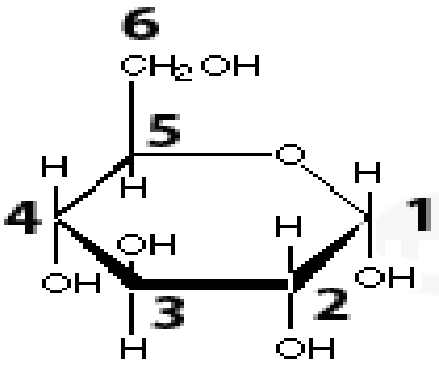
- **Textiles:** used to soften cotton fabrics and to produce 'stone-wash' effect in denim jeans
- **Detergents:** digestion of plant-based stains, brightening of fabric colours (removal of microfibrils)
- **Biofuels:** sugars from cellulose digestion used as feedstock for ethanol fermentation



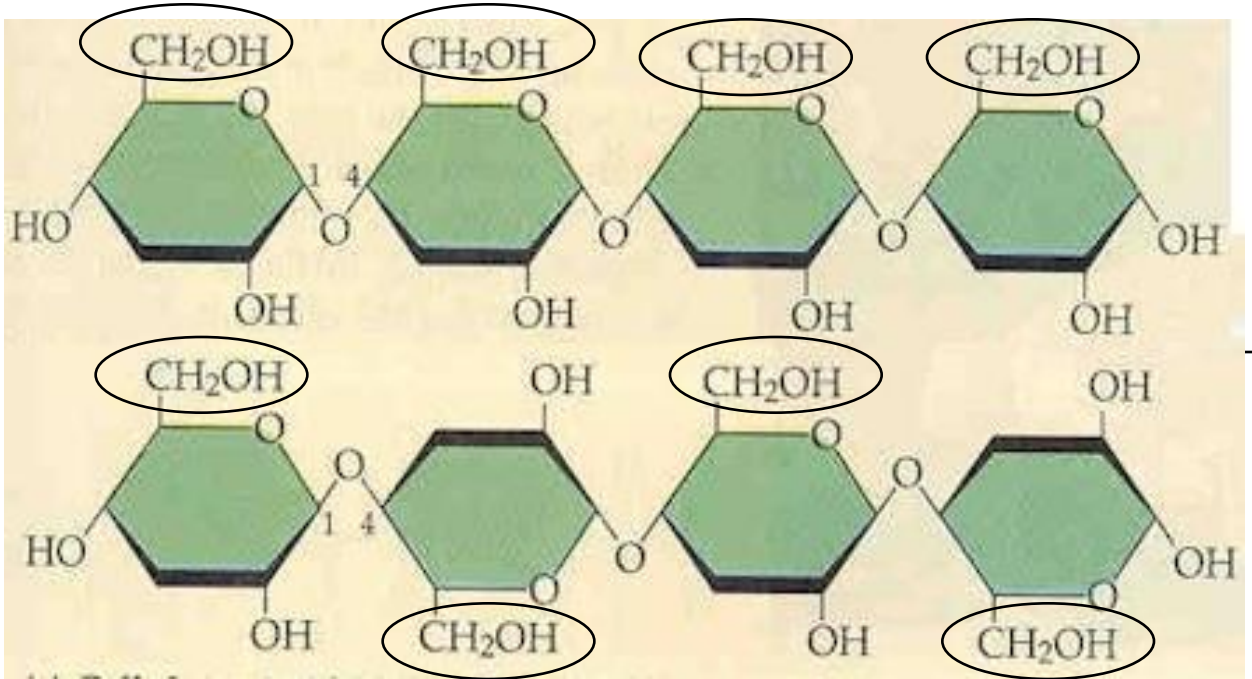
Fuel ethanol from biomass provides a 'closed loop' for carbon compounds
→ zero net greenhouse gas emissions

Cellulase: chemistry of cellulose

- **Cellulose is a linear homopolymer of glucose; ~1000-10,000 glucose molecules joined by β-1,4-linkages**



- **Essential features of glucose molecule:**
  - **six carbon atoms**
  - **multiple alcohol groups –OH**
  - **different linkages possible:**



Starch (amylose)

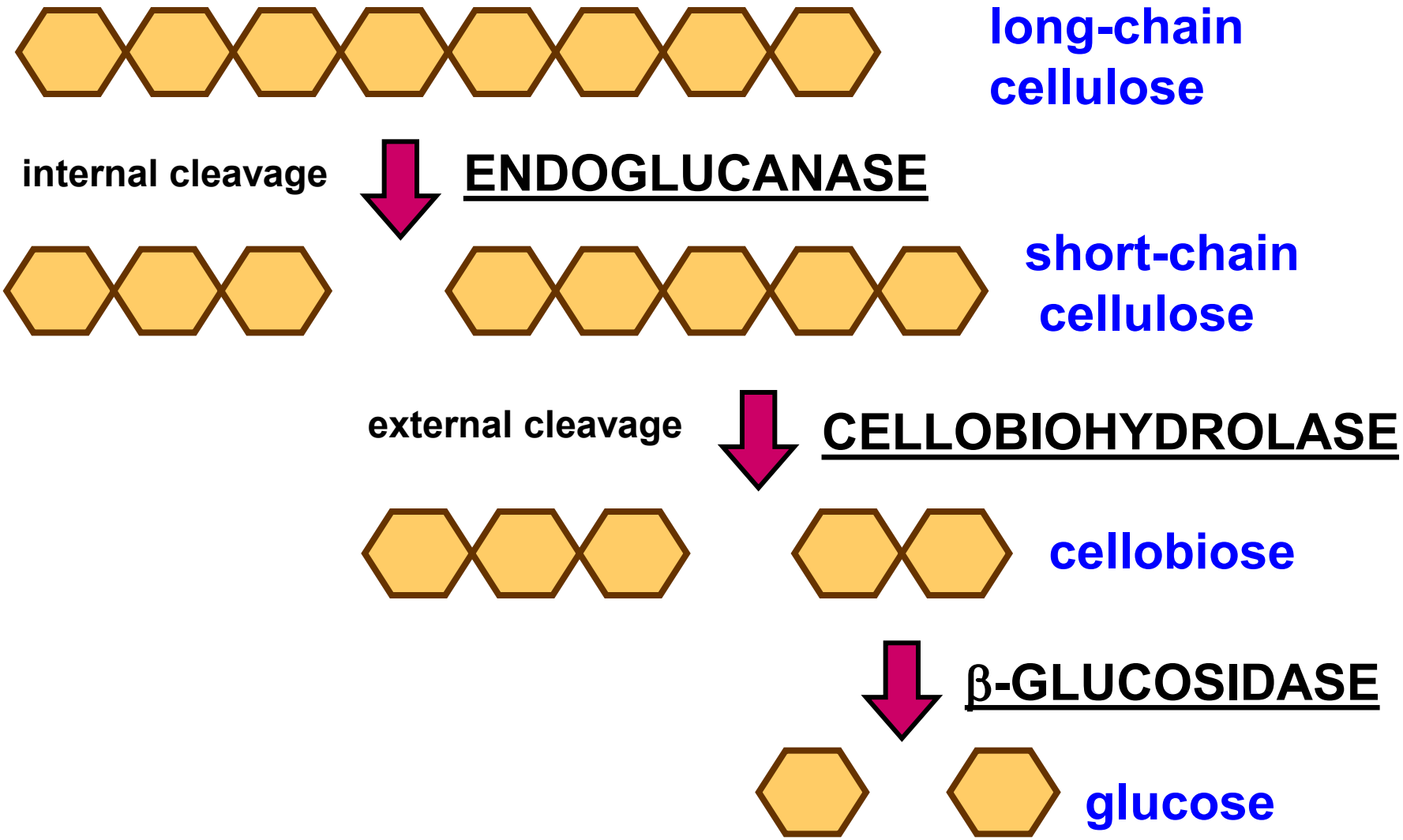
α-1,4- linkages

Cellulose

β-1,4- linkages

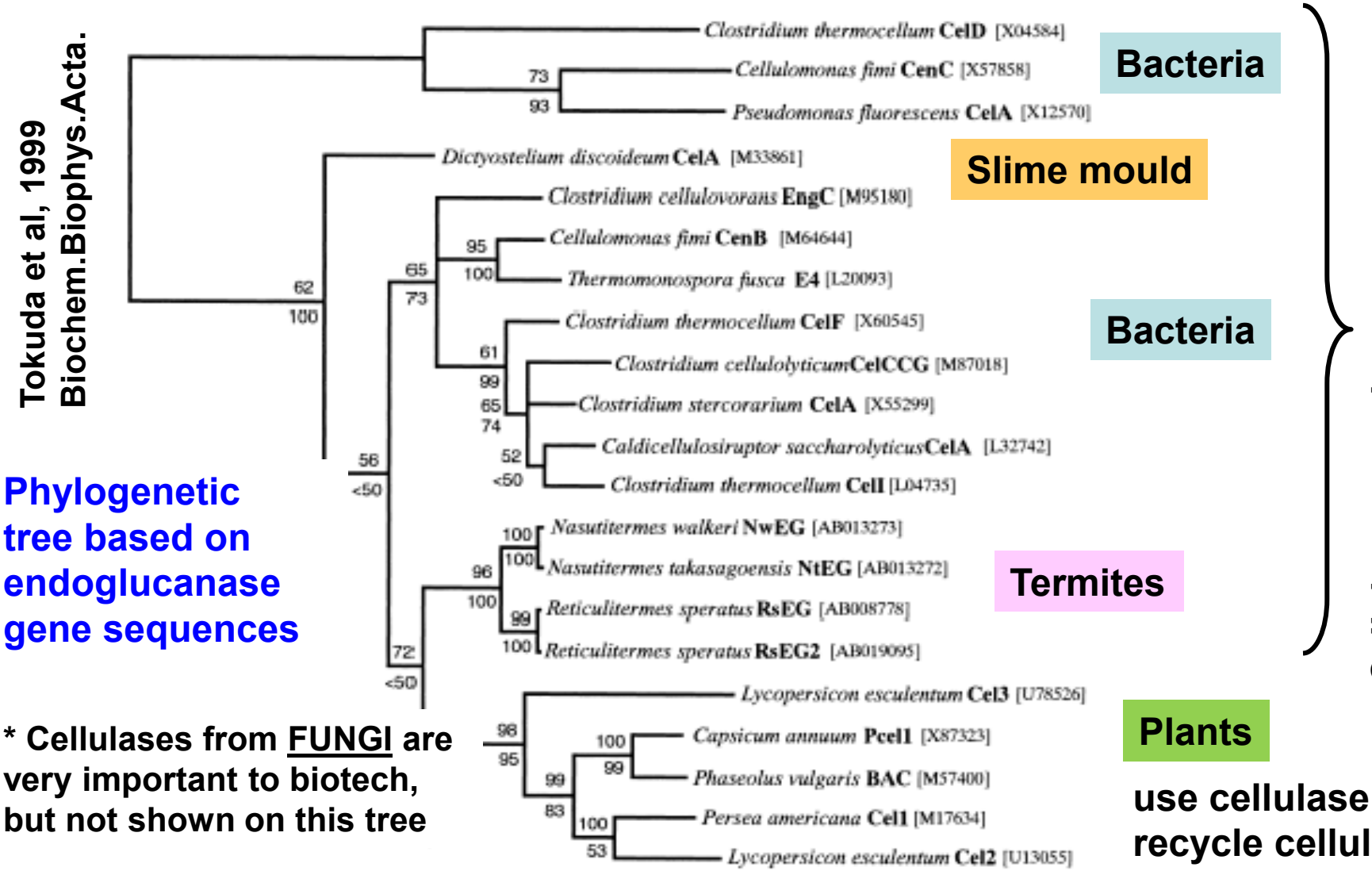
Cellulase: catalytic enzymes

- Different hydrolases act together synergistically to depolymerize cellulose → cellobiose → glucose



Cellulase: distribution and diversity

- **Cellulose acts as a carbon and energy source for diverse organisms; microbial cellulases most useful for biotech.**



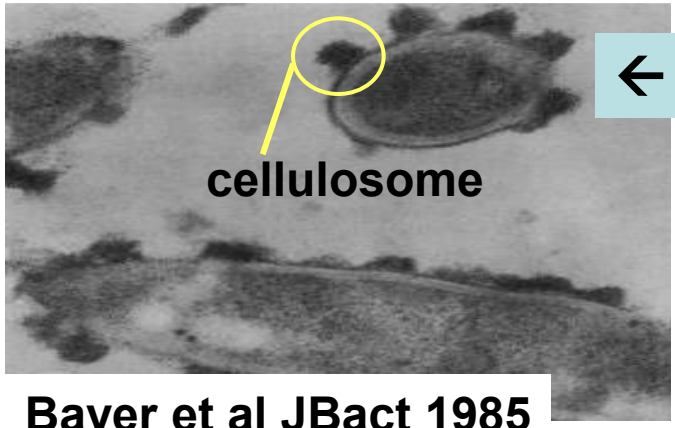
Phylogenetic tree based on endoglucanase gene sequences

*** Cellulases from FUNGI are very important to biotech, but not shown on this tree**

use cellulase to recycle cellulose

Cellulase: *Clostridium* as a source

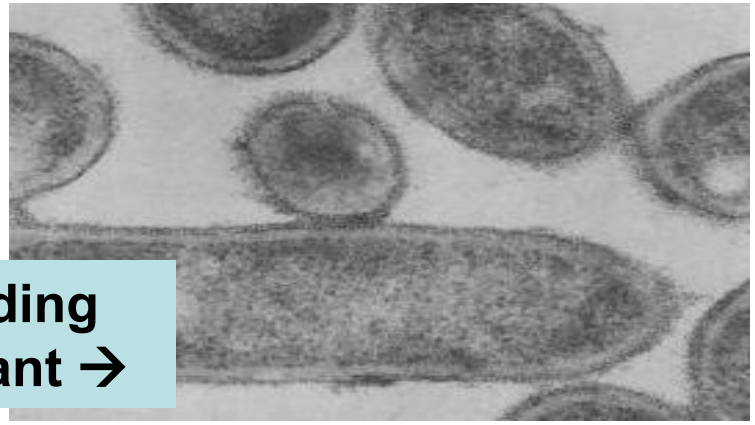
- Several species of *Clostridium* produce cellulase; eg. the thermophile *C. thermocellum*
- Clostridia useful when thermophilic enzyme (optimum at 70°C !) or growth under anaerobic conditions required
- Improvement of *Clostridium* cellulase production:
  - overexpression in *Bacillus* hosts
  - co-culture with ethanol-producing clostridia
  - site-directed mutagenesis of active site



Bayer et al JBact 1985

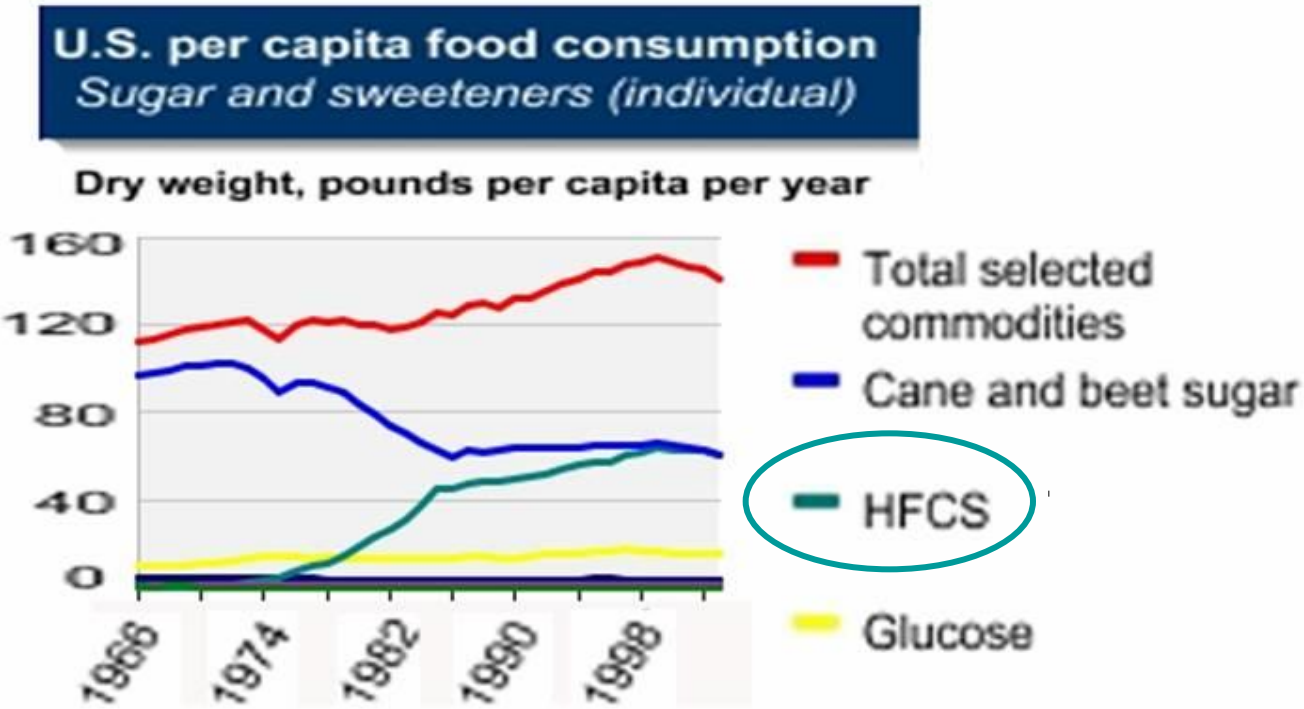
← Wild type

Cellulose-binding deficient mutant →



Amylase: Amylose (starch) as a substrate

- Amylose is main form of carbohydrate storage in plants
- Crops such as rice, wheat, corn, potato have been bred for high amylose content → high energy value
- Amylose is an important food source, and a useful feedstock for synthesis of diverse glucose derivatives



Amylase: applications in food processing

- Production of maltodextrins: used as nutrients, thickeners, stabilisers, ice-crystal inhibitors
- Production of high-fructose corn syrup sweeteners
- Production of sugar feedstocks for further fermentation to ethanol: used as beverage, solvent, biofuel

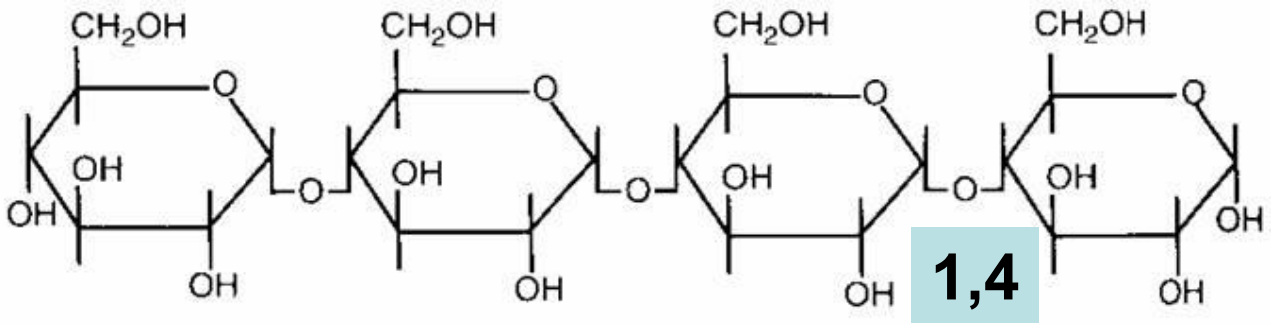
Maltodextrins: slow release nutrient

Raw amylose from plants

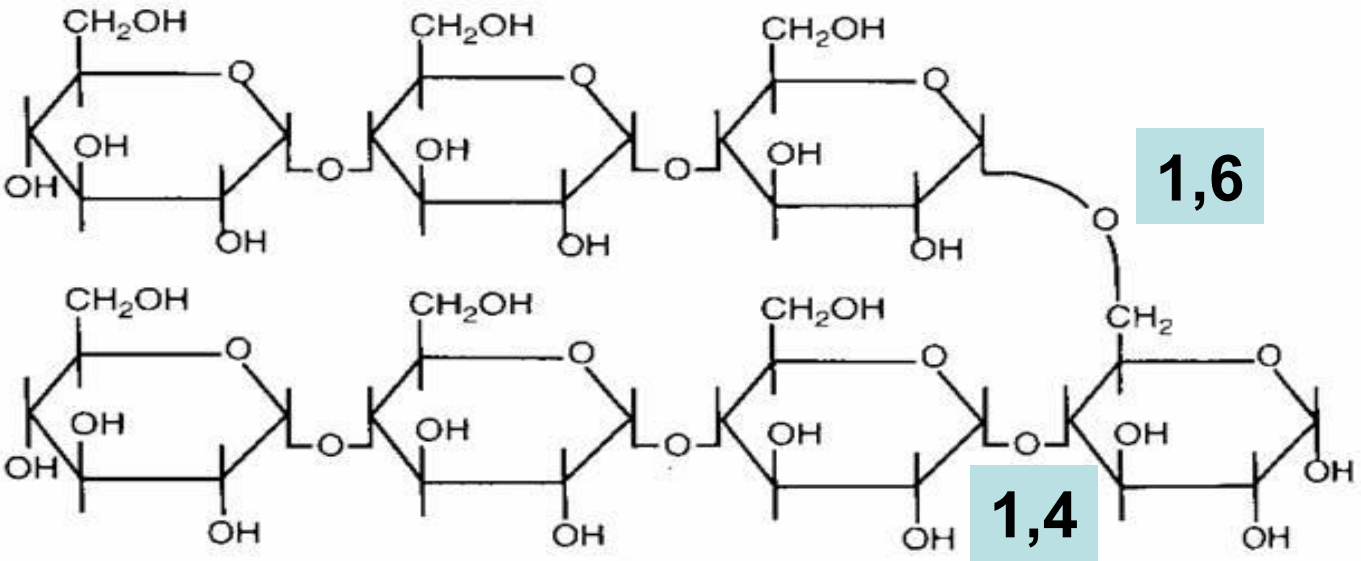
HFCS: sweetener, instant nutrient

Amylase: two types of amylose

- **Amylose:** **linear** **polymer of glucose;** **α-1,4-** **linkages only**

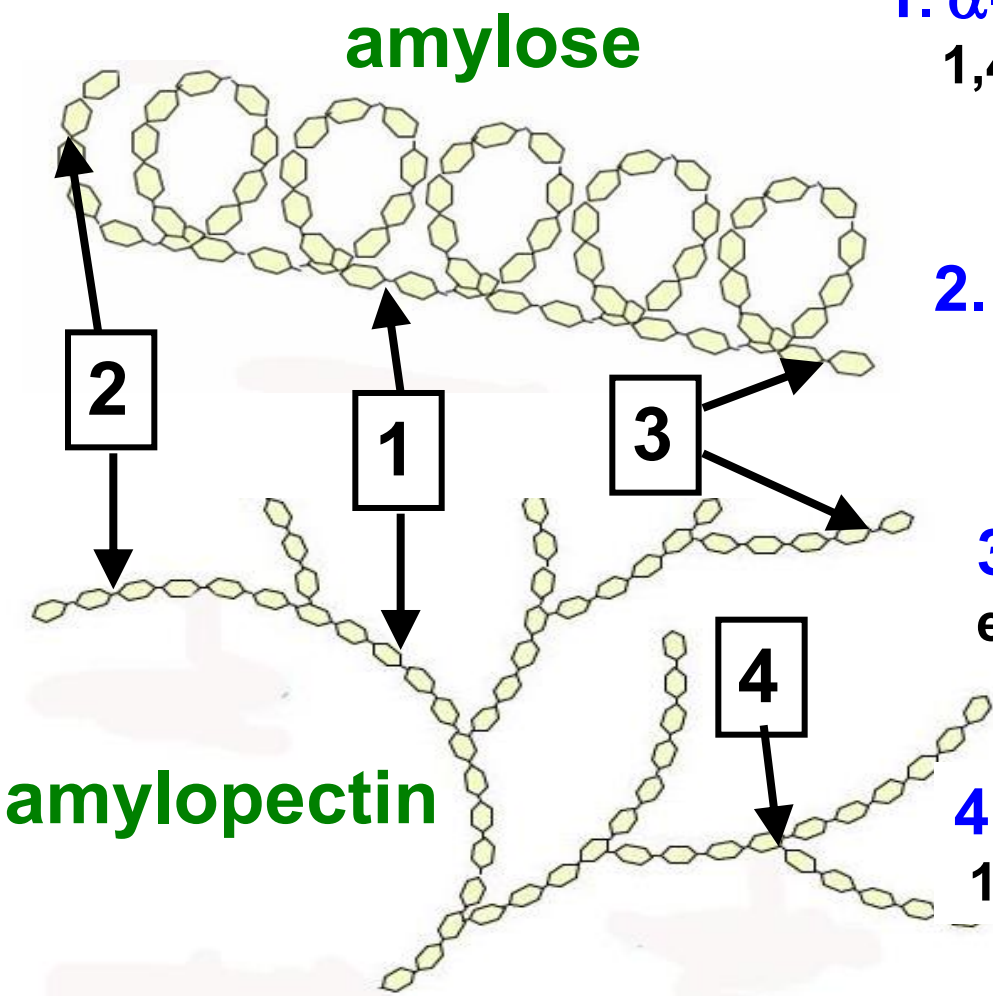


- **Amylopectin:** **branched** **polymer of glucose;**
  both α-1,4- and α-1,6- linkages



Amylase: Different enzymes, different targets

- **Amylose / amylopectin are large, insoluble compounds, enyzmes that attack starches must be extracellular**



1. α-amylase: attacks internal 1,4- bonds → maltodextrins

2. β-amylase: attacks external 1,4- bonds → maltose

3. glucoamylase: attacks external 1,4- bonds → glucose

4. pullulanase: attacks internal 1,6- bonds → debranching

Amylase: making high-fructose corn syrup (HFCS)

- **HFCS production requires multiple microbial enzymes**

STARCH (AMYLOSE, AMYLOPECTIN)

Liquefaction ↓ **95°C, α-amylase (*Bacillus*)**

MALTODEXTRINS

Saccharification ↓ **60°C, glucoamylase (*Aspergillus*)**
pullulanase (*Klebsiella*)
β-amylase (*Bacillus*)

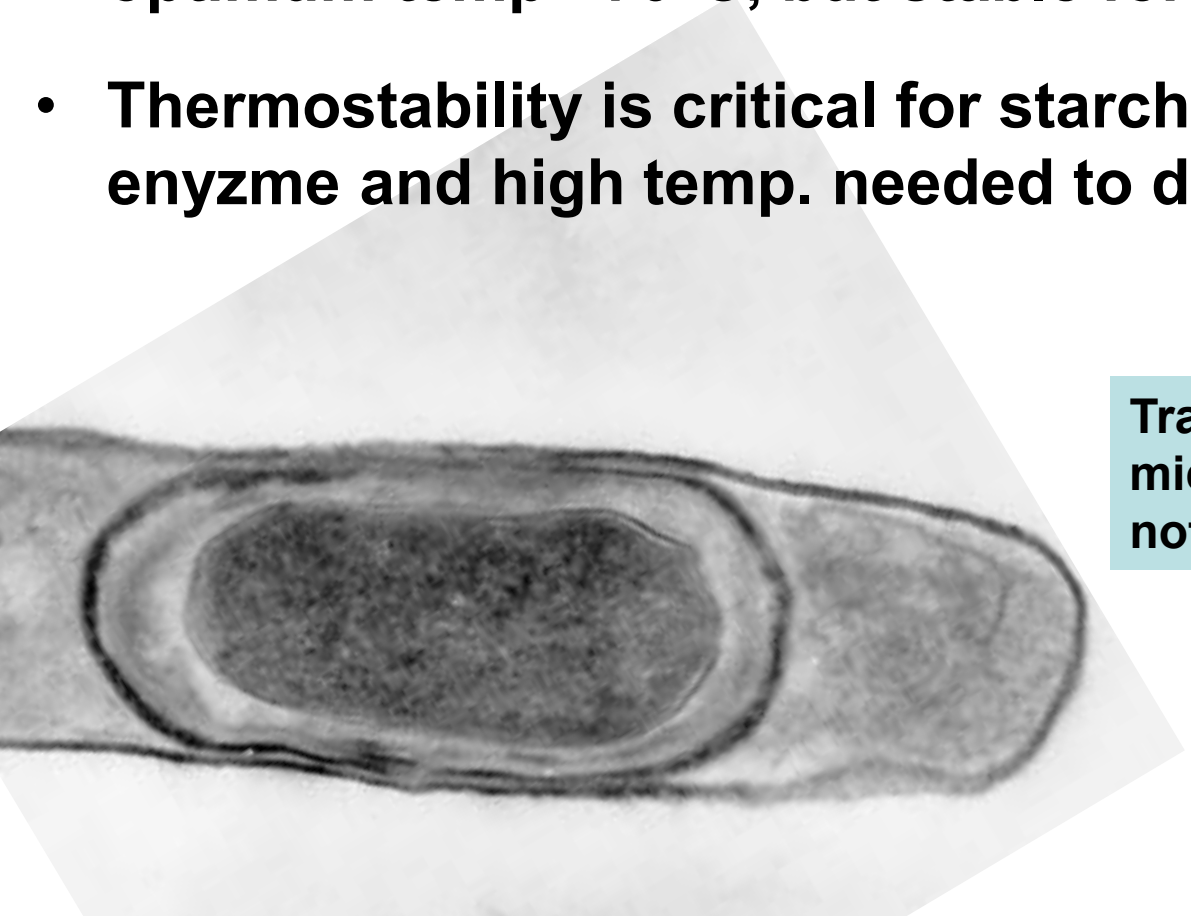
MALTOSE, GLUCOSE

Isomerization ↓ **Glucose isomerase (*Streptomyces*)**

FRUCTOSE

α-Amylase from *Bacillus licheniformis*

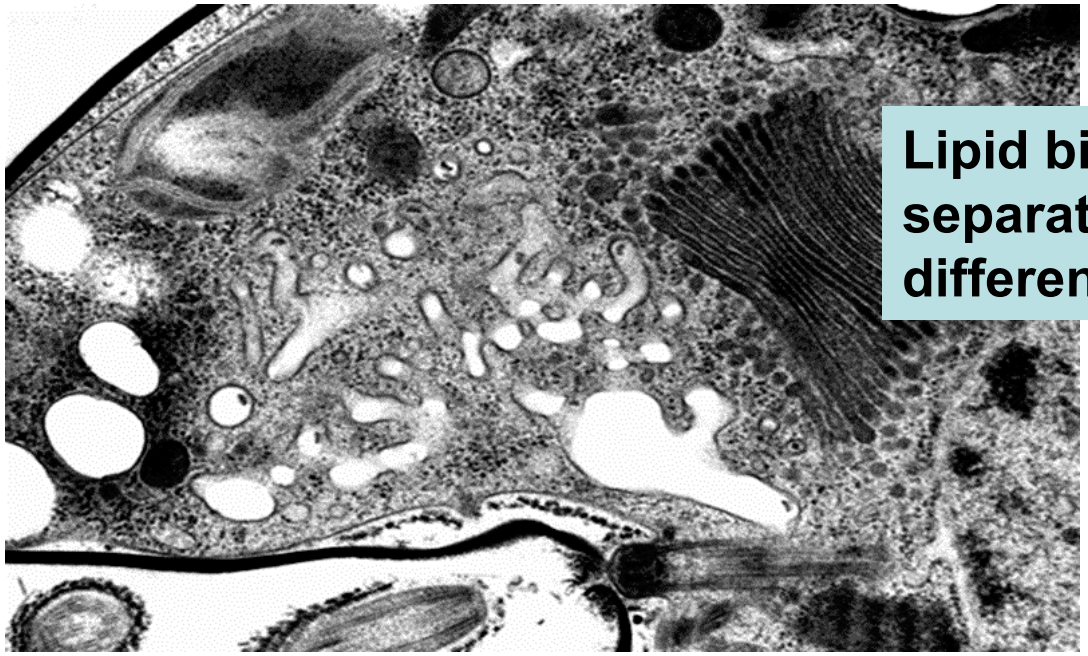
- ***Bacillus licheniformis*: thermophilic, facultative, Gram-positive, spore-former– grows on amylose as C source**
- ***B.lich.* expresses highly thermostable α-amylase: optimum temp ~70°C, but stable for >4 hours at 90°C**
- **Thermostability is critical for starch liquefaction; both enyzme and high temp. needed to dissolve substrate**



Transmission electron micrograph of *B.licheniformis*: note production of endospore

Lipase: What are lipids ?

- **Lipids:** hydrophobic biological molecules
  - **Structural** role in membrane: eg. phospholipids
  - **Storage** of carbon and energy: eg. triglycerides
  - **Hormones**: eg. steroids
  - **Vitamins**: eg. retinol (vitamin A)

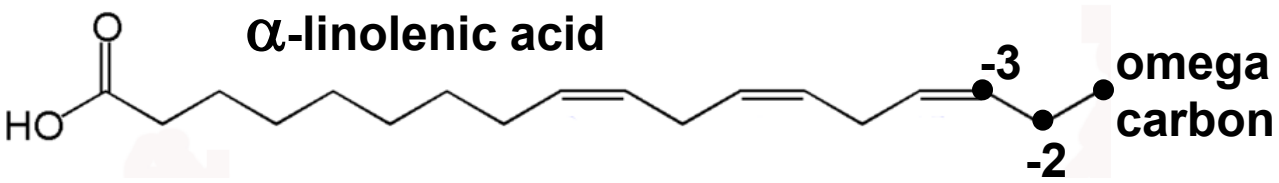


Lipid bilayer membranes allow separation of functions in different cellular compartments

Lipase: Domestic and industrial applications

- **Cheesemaking:**
  - Accelerated ripening & flavouring
- **Detergents:**
  - Hydrolysis of lipid stains
- **Fat and oil processing**:
  - Hydrolysis or trans-esterification

Oily fish and flax plants provide omega-3 fatty acids, can be enriched by lipase activity

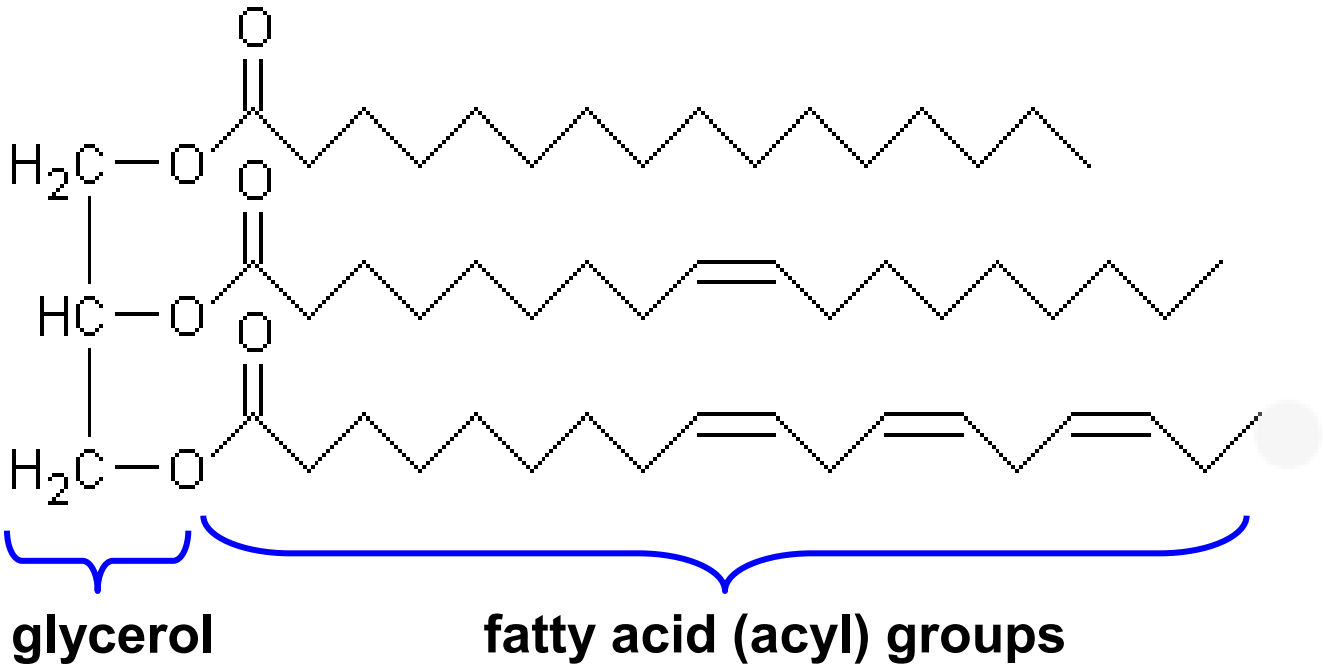


WHAT DOES "OMEGA-3" MEAN?

Lipases: chemistry of glycerides

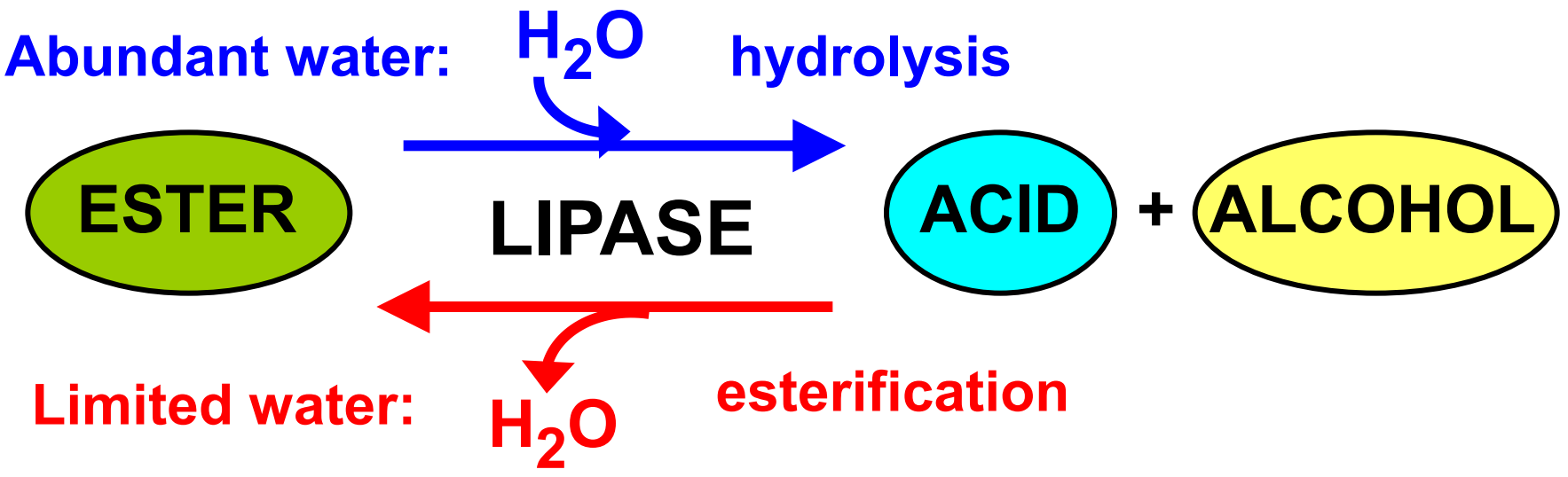
- Natural substrate of lipases is glycerides (a.k.a. fats)
- Glycerides: fatty acids joined to a glycerol backbone
- Mono, di, triglycerides: different number of acyl groups

Structure of a typical triglyceride:



Lipases: hydrolysis AND esterification

- Catalysis by lipases is reversible, with the direction of reaction largely determined by water availability



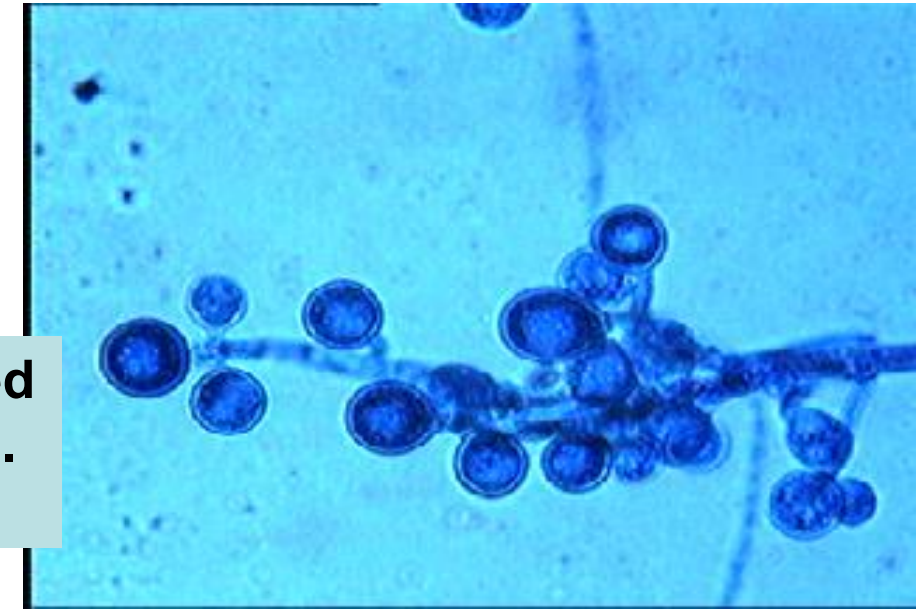
OR…

- Transesterification: lipase-mediated replacement of alcohol group of an ester with a different alcohol

Lipase from *Thermomyces*

- The fungus *Thermomyces* produces a thermophilic lipase when grown on vegetable oils as carbon sources

- *Thermomyces* lipase suitable for use in detergents:
  - alkaline pH optimum
  - good activity at 60°C
  - stable to surfactants



Thermomyces culture stained with lactophenol cotton blue. Note mycelia and sporangia

- *Thermomyces* lipase is only expressed at low levels; how can it be developed into a commercial product ?

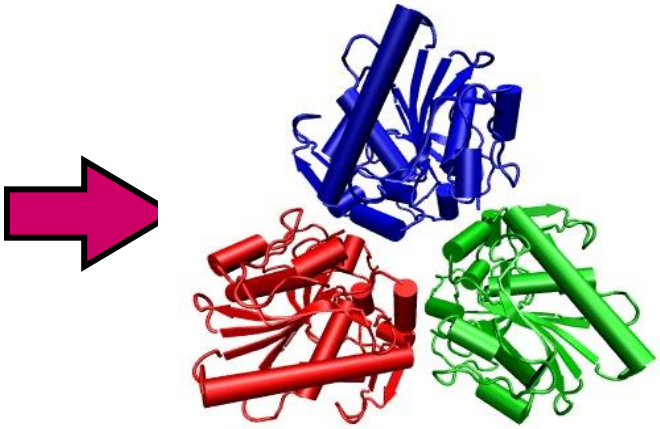
Lipase from *Thermomyces*

- **Reverse transcription of RNA from oil-grown *Thermomyces* culture yields cDNA of lipase gene**

Remember: eukaryotes like fungi have INTRONS in their genes

- **cDNA cloned into expression plasmid, then plasmid is transformed into *Aspergillus oryzae* host**
- **Enzyme is then purified for use in detergents**

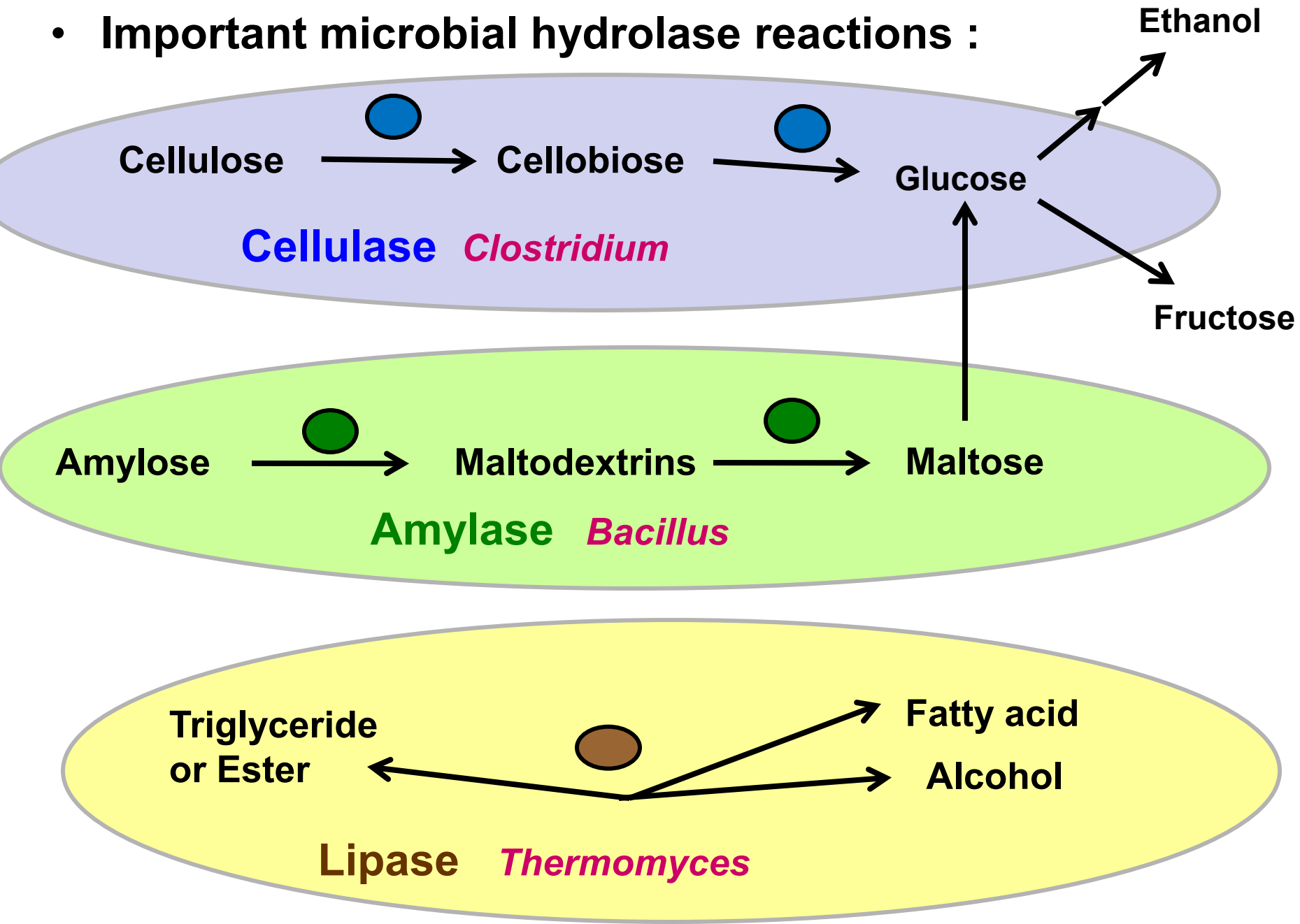
A.oryzae



Lipase

Detergent

Boel et al (1996) US Patent 55 36 661

Summary: Microbial hydrolases

- **Important microbial hydrolase reactions :**