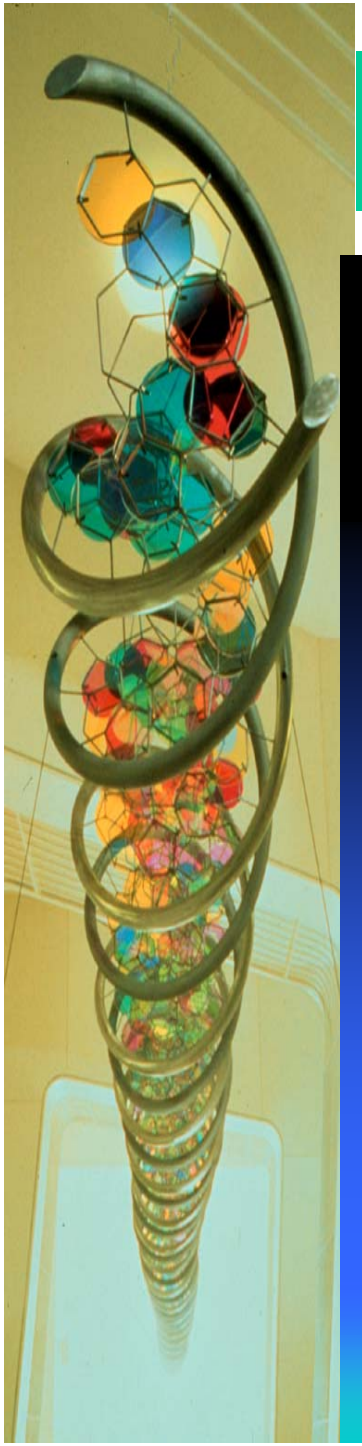
Biotechnology in Today's Commerce

CONNECTIONS

Fungal transformation
Fungal Biotechnology
Industrial Enzymes
Cherry Blossoms
Baby Calves
Designer Jeans
Biofuels
Lite beer



The Big Picture

- In agriculture, over 50 biotech crop products have been approved: vegetables with extended shelf life, plants with modified oils, and squash, corn, cotton, potatoes and soybeans with built-in disease, herbicide and pest-resistance.
- Over 60 animal biotech therapeutics have been developed
- 90% of industrial enzymes, widely used in the food and textile industry are biotechnology-produced.
- In the environment, biotechnology is used to treat hazardous waste in soil, water and the air, as well as in the prevention of pollution.

SOME KEY EVENTS IN THE HISTORY OF INDUSTRIAL MYCOLOGY

- Proving bread with leaven **prehistoric period**
- Fermentation of alcoholic beverages **prehistoric period**
- Knowledge of vinegar from fermented juices **prehistoric period**
- Cultivation of vines for wine **before 2000 BC**
- Manufacture of beer in Babylonia and Egypt **3rd century BC**
- Wine growing promoted by Roman Emperor Marcus Aurelius Probus **3rd century AD**
- Production of spirits of wine (ethanol) **1150**
- Vinegar manufacturing industry **14th century**
- Discovery of the fermentation properties of yeast by Erxleben **1818**
- Description of lactic acid fermentation by Pasteur **1857**
- Patenting of the first microbial enzyme **1894**
- Detection of fermentation enzymes in yeast by Buchner **1897**
- Discovery of penicillin by Fleming **1928/29**

In 2000, the global demand for industrial enzymes was approximately $2 billion with an annual growth rate of 5-10%.

The market for industrial enzymes is divided into the following sectors:

Technical Enzymes(detergent enzymes, enzymes for textile and leather manufacturing, enzymes for pulp and paper processing, enzymes for gas and oil production etc.)

Food Enzymes(enzymes for starch processing, sweetener production, baking, brewing, dairy products, distilling, juice and wine making etc.)

•The global market for industrial enzymes is estimated at $2 billion in 2004 and is expected to rise at an average annual growth rate (AAGR) of 3.3% to $2.4 billion in 2009.

•Volume growth of industrial enzymes is between a 4% and 5% AAGR, and is accompanied by decreasing prices.

•Growth of the animal feed enzyme sector is somewhat higher, at nearly a 4% AAGR, helped by increased use of phytase enzyme to fight phosphate pollution.

•Technical enzymes for detergent and pulp and paper manufacturing, among others, are the largest segment with a 52% share.

Fig. 16. Enzyme sales by industry.

Global market for industrial enzymes, 1999
Total market value: approx. DKK 11 billion

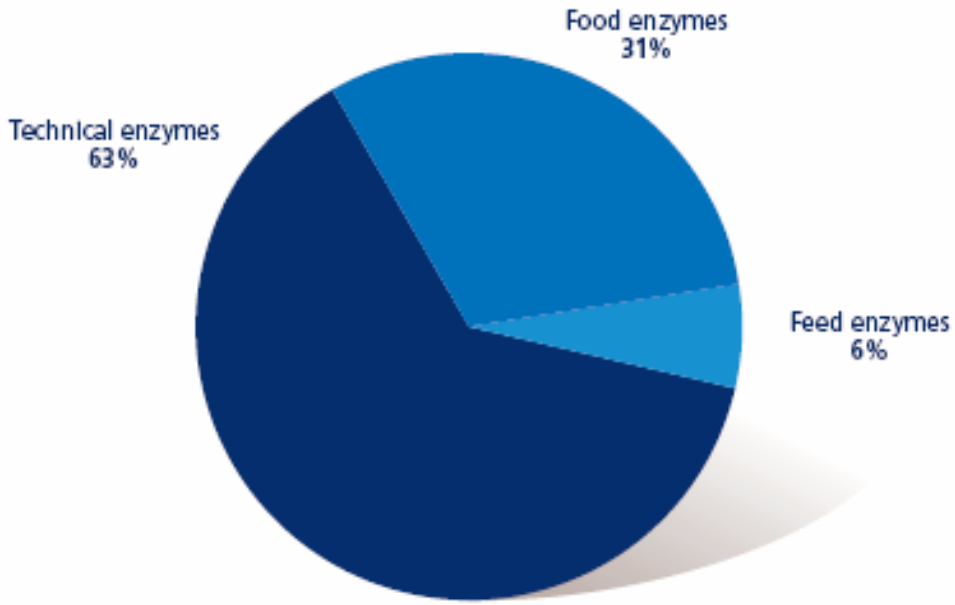
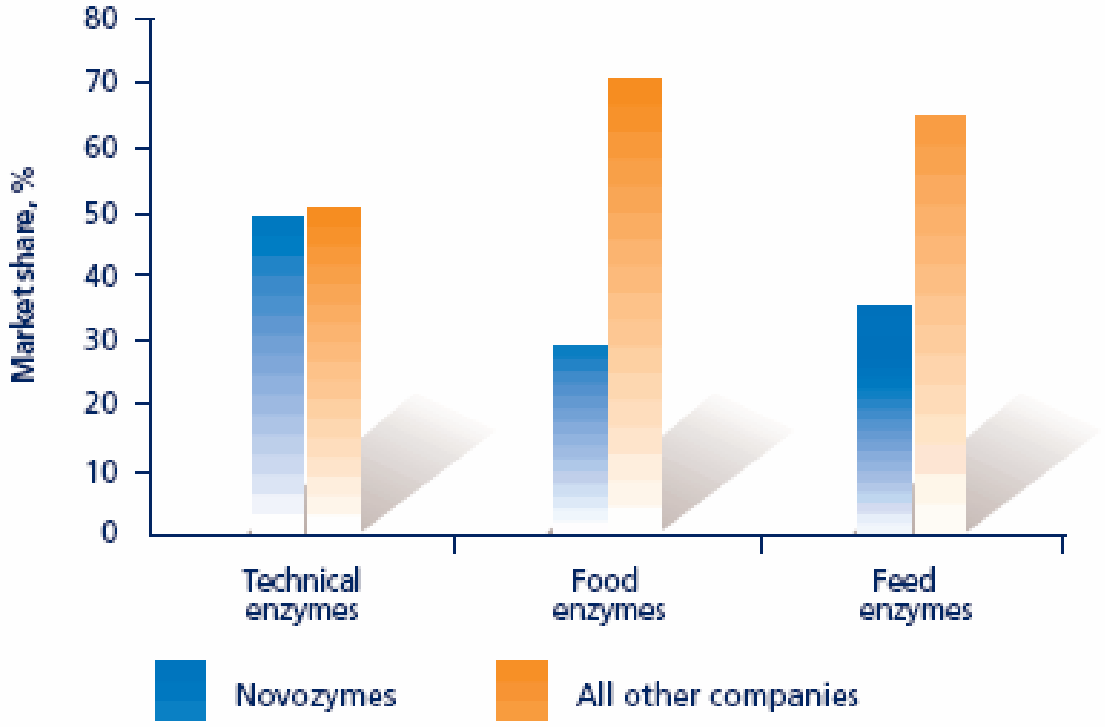


Fig. 17. Market shares 1999 in different segments.



Fungal Transformation and Secretion

- 90% of all industrial enzymes are produced by biotechnology
- About 40% of commercially available enzymes are derived from filamentous fungi.
- These enzymes are usually produced using species of the genera *Aspergillus* and *Trichoderma* as microbial factories via transformation.
- Because they secrete large amounts of protein into the medium, they can be grown in large-scale fermentation, and they are generally accepted as safe for the food industry.
- **Both of these fungi can be transformed by *Agrobacterium tumefaciens* -mediated transformation**.

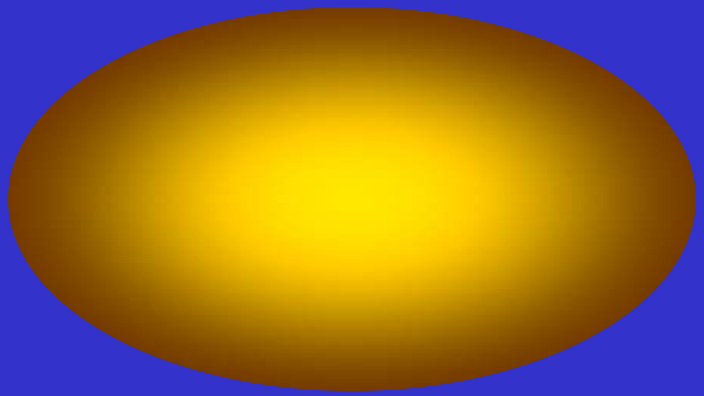
Nature Biotechnology 16, 839 - 842 (1998)

***Agrobacterium tumefaciens*-mediated transformation of filamentous fungi**

Marcel J.A. de Groot1, 2, Paul Bundock3, Paul J.J Hooykaas3 & Alice G.M. Beijersbergen1 1Unilever Research Laboratory Vlaardingen, Olivier van Noortlaan 120, 3133 AT Vlaardingen, The Netherlands

- *Agrobacterium tumefaciens* transfers part of its Ti plasmid, the T-DNA, to plant cells during tumorigenesis where it is routinely used for the genetic modification of a wide range of plant species.
- *A. tumefaciens* can also transfer its T-DNA efficiently to the filamentous fungus *Aspergillus awamori*, demonstrating DNA transfer between a prokaryote and a filamentous fungus.
- The T-DNA integrated into the *A awamori* genome in a manner similar to that described for plants.
- Other filamentous fungi, including *Aspergillus niger*, *Fusarium venenatum, Trichoderma reesei, Colletotrichum gloeosporioides, Neurospora crassa,* and the mushroom *Agaricus bisporus*, demonstrating the general applicability to filamentous fungi.

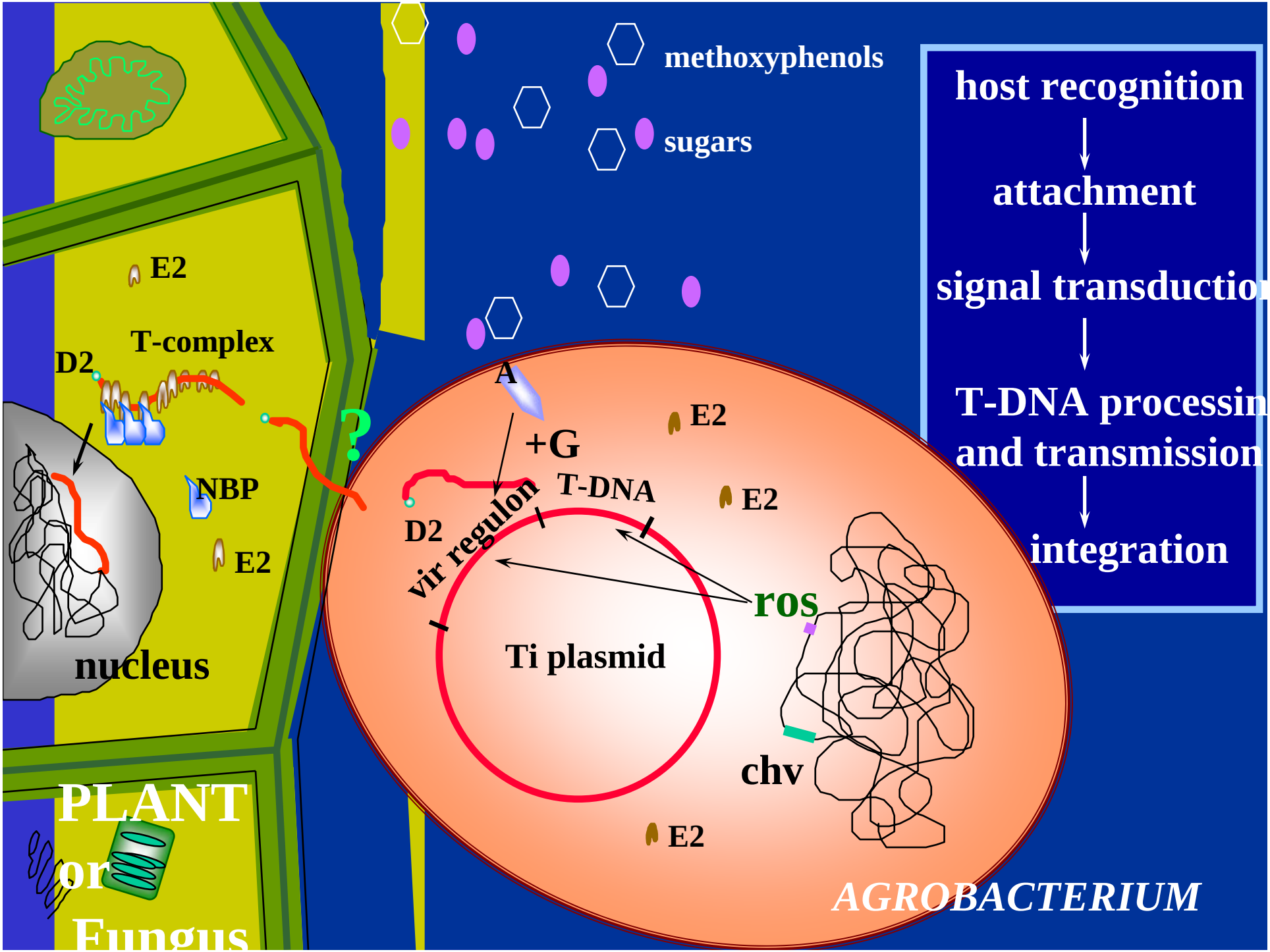
Promiscuous transmission of genes: example of the selfishness of DNA



A. tumefaciens

Angiosperms
Gymnosperms
Ascomycetes
Basidiomycetes
Actinomycetes
Human cells

[**Genetic Enginerring** 20:1-24 (1998)**;**
Antonie v. Leeuwenhoek 73:117-126 (1998)
***In* Horizontal Gene Transfer 1998]**

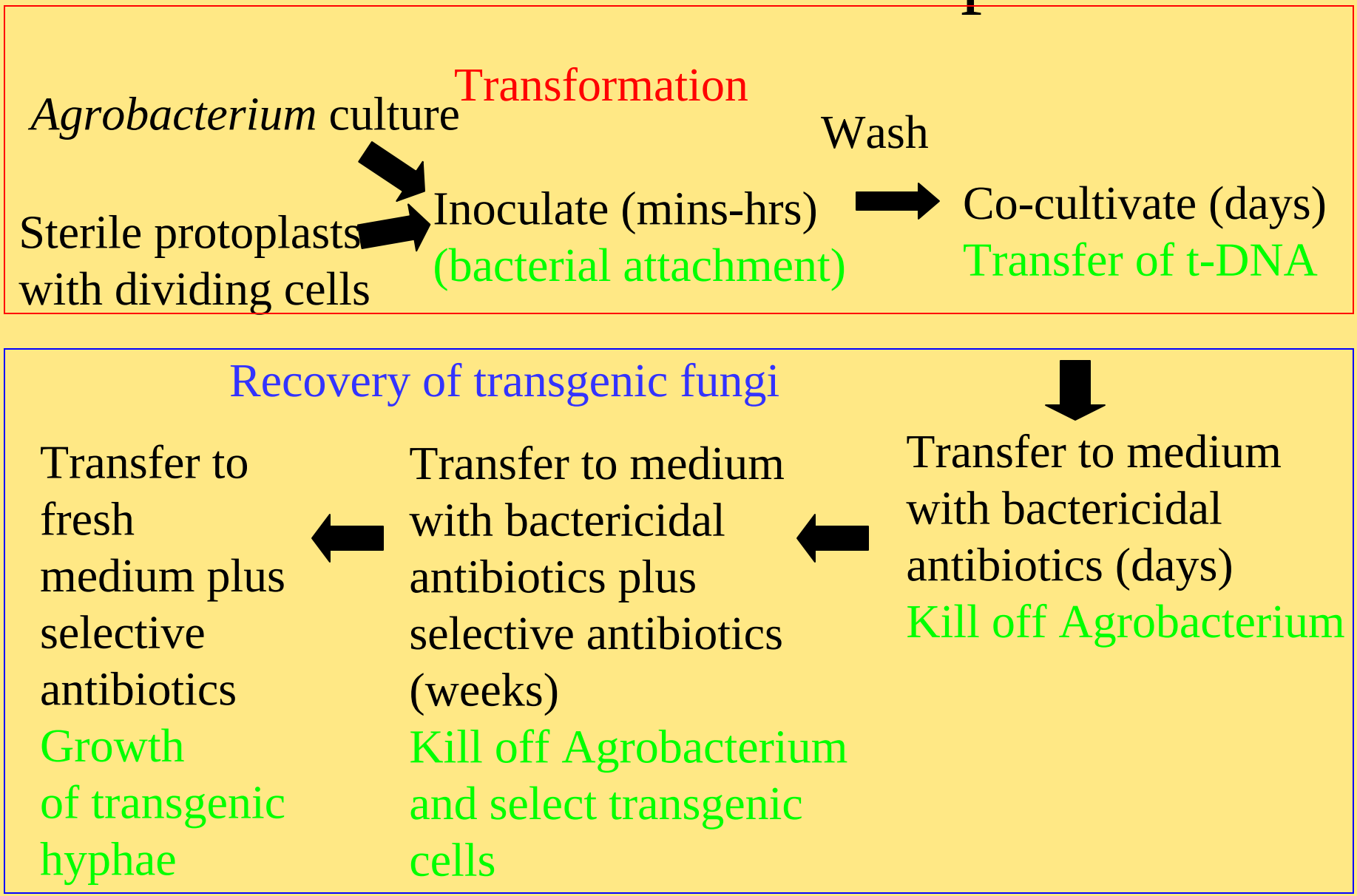

methoxyphenols
sugars
host recognition
attachment
signal transduction
T-DNA processing
and transmission
integration
E2
T-complex
D2
NBP
E2
nucleus
?
A
+G
E2
T-DNA
E2
D2
vir regulon
ros
Ti plasmid
chv
E2
PLANT
or
Fungus
AGROBACTERIUM

Agrobacterium-mediated transformation

- A natural genetic engineer
- 2 species
  - *A.tumefaciens* (produces a gall)
  - *A. rhizogenes* (produces roots)
- Oncogenes (for auxin and cytokinin synthesis) + Opines

- In the presence of exudates (e.g. acetosyringone) from wounded plants, Virulence (VIR) genes are activated and cause the t-DNA to be transferred to plants. Everything between the left and right border is transferred.

General transformation protocol



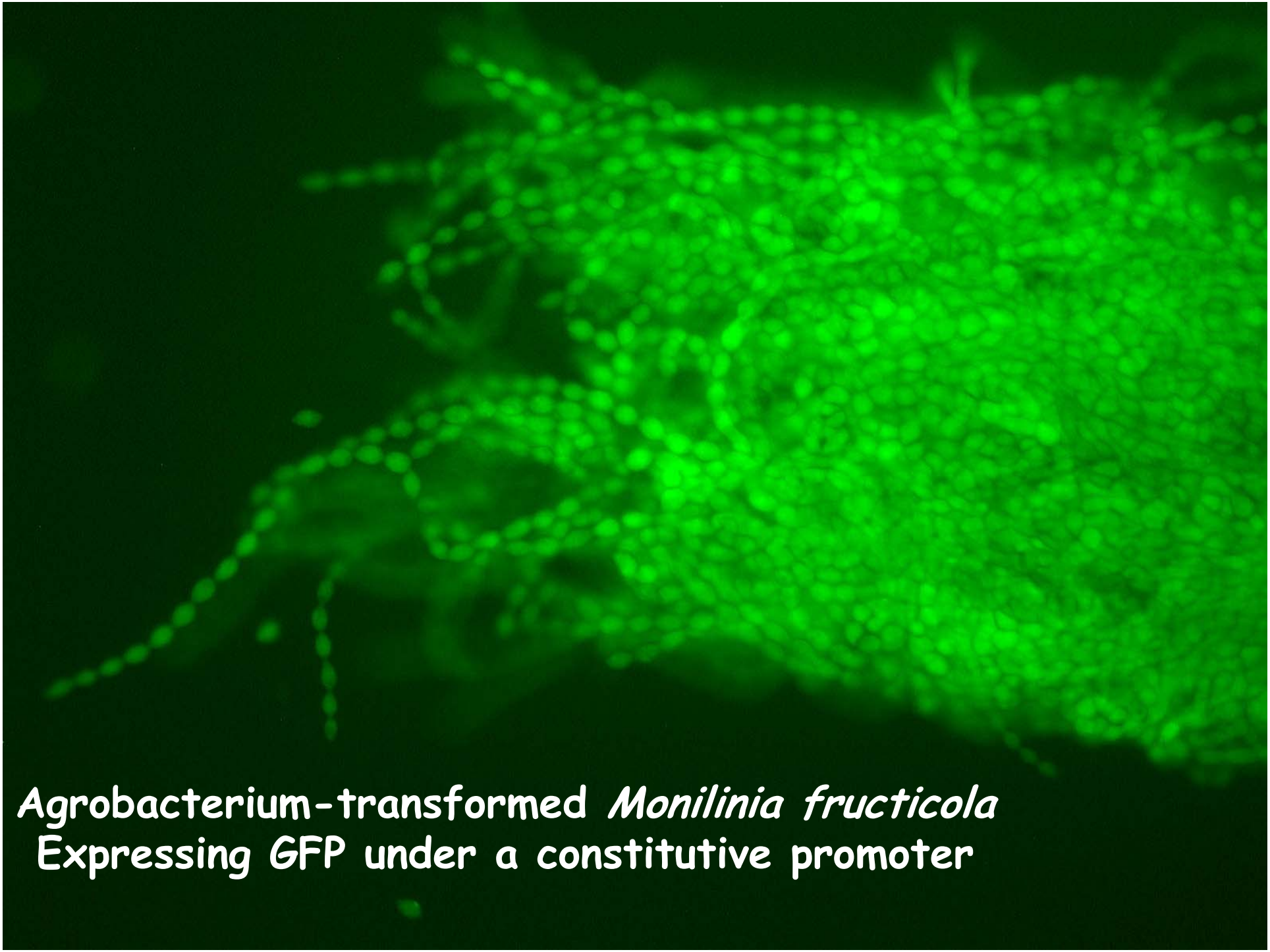

Agrobacterium-transformed *Monilinia fructicola*
Expressing GFP under a constitutive promoter

Table 2 Advantages of the *Aspergilli* as Expression/Secretion Hosts

- Eukaryotic host
- Good protein secretor
- F.D.A. approved (*A. niger*)
- Accepted by industry (organic acid and industrial enzyme production)
- Rapid growth on simple, inexpensive media
- Well characterized genetically (*A. nidulans*)
  - — available promoters with well-defined regulation
  - — many mutants
- Mitotically stable integrated transformants

Table 3 Biological Advantages of Extracellular Secretion as a Method of Prqtein Production

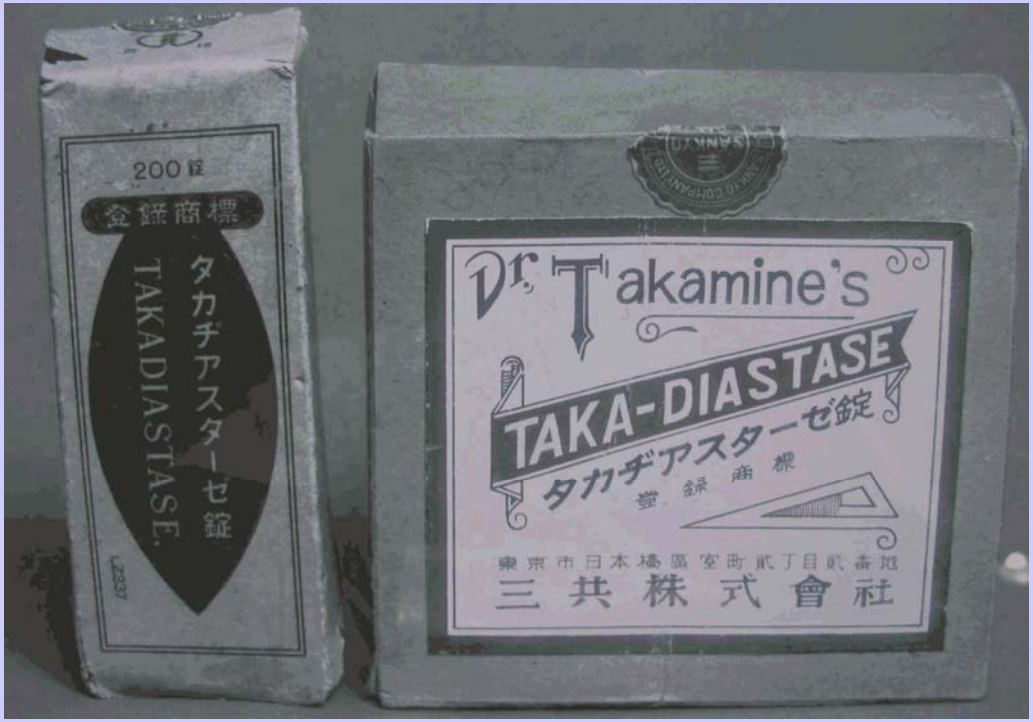
- Avoids intracellular accumulation of toxic levels of product
- Potentially avoids intracellular hydrolysis or undesirable modification of product
- Takes advantage of posttranslational events
  - — glycosylation
  - — protein folding
  - — other modifications
- Potential use of directed endoproteolysis

Examples of engineering fungi for industrial or pharmaceutical applications

Japanese father of American Biotechnology

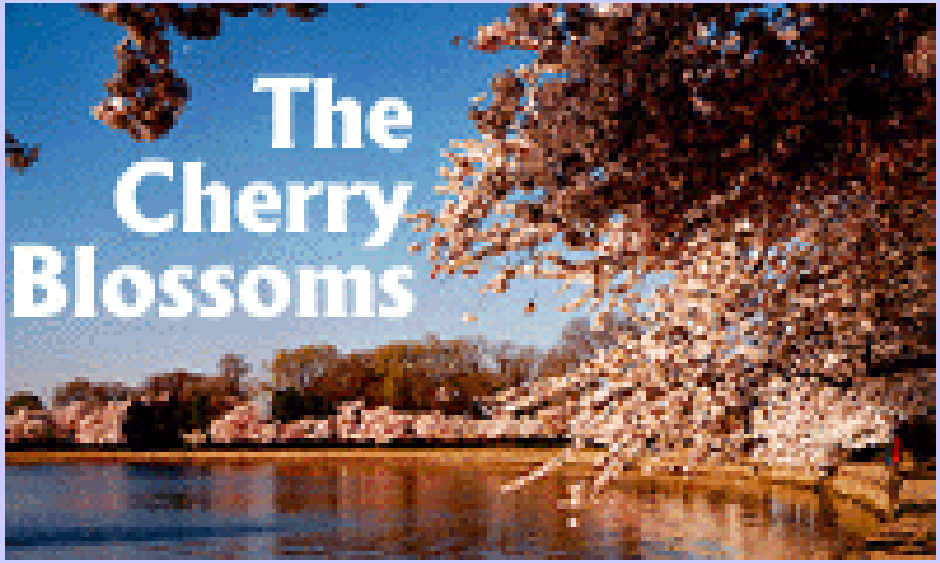
Invention of "TAKA-DIASTASE", an enzyme preparation, from the fungus Aspergillus oryzae, by Dr. Jokichi Takamine comprised of amylases used as a aid to digestion.

Patented the first microbial enzyme in 1894, Taka-Diastase



In 1901 he isolated and purified the hormone adrenaline, becoming the first to accomplish this for a glandular hormone.

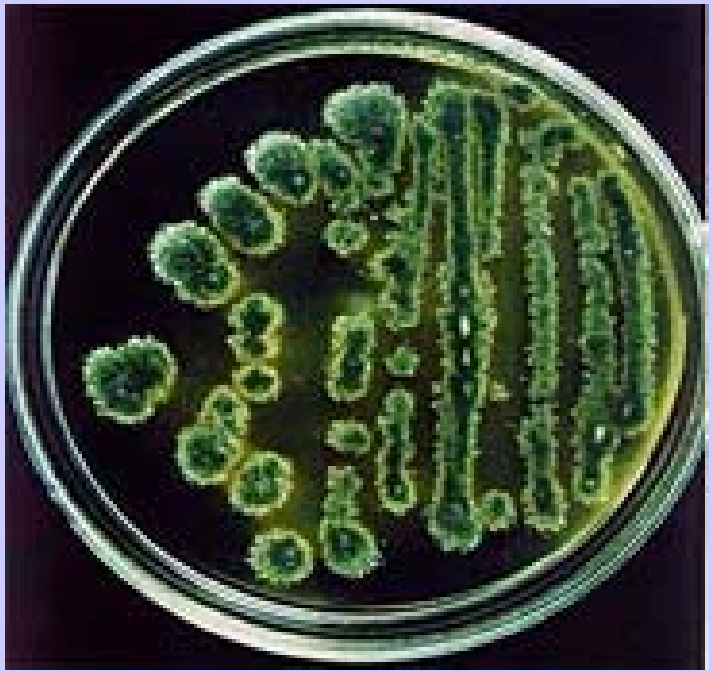
The cherry trees that ring the tidal basin in Washington DC were donated to the United States by Dr. Takamine in 1912. Cuttings from the original trees have been used to replant old and dying trees, preserving the genetic lineage to the original gift of 3,000 trees of which only about 125 trees remain.



Improved chymosin production in filamentous fungi

Chymosin

- Originally obtained from stomachs of 3-4 wk old calves
- Fungi produce chymosin but the fungal form leads to off flavors in the cheese
- Chymosin from *Endothia parasitica*, causal agent of chestnut blight works fine but is too aggressive
- Cloned the bovine chymosin, transformed into E. parasitica did fine but not enough yield
- Transformed T. reesi with the bovine gene and obtained > 30 g/L of chymosin
- Later Genencor transformed Aspergillus awamori to make a super producer.



Trichoderma reesei, as hosts for expression of genes originating from other organisms with a view of producing high yields of valuable gene products. *T. reesei* can secrete up to 40 g/L of extracellular protein therefore providing an effective host system for industrially relevant proteins such as chymosin. Enzyme-encoding genes from thermophilic microorganisms are of specific interest because of their industrial applications.

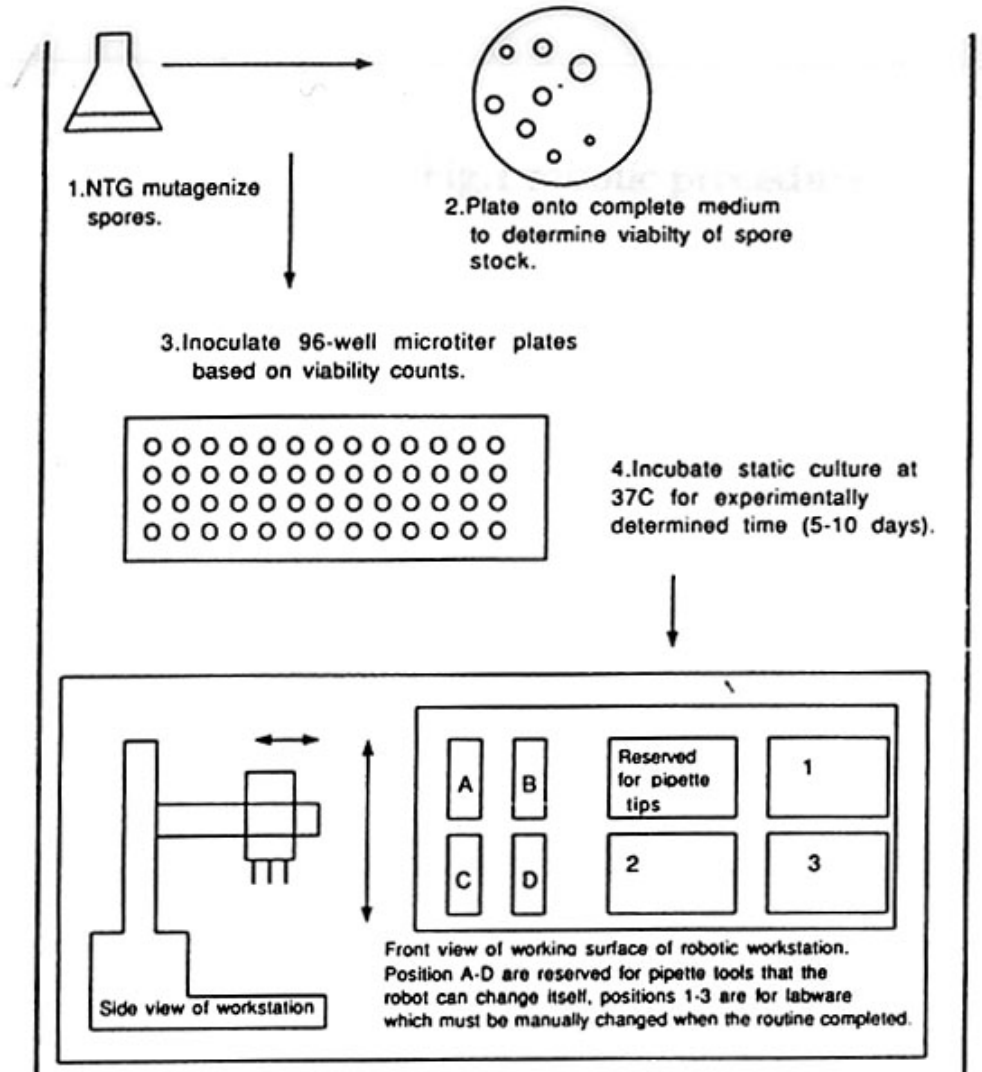


FIGURE 1 Robotic screening for the isolation of chymosin producing mutants.

Chymosin production (4-5 years)

2mg/L *A. nidulans*

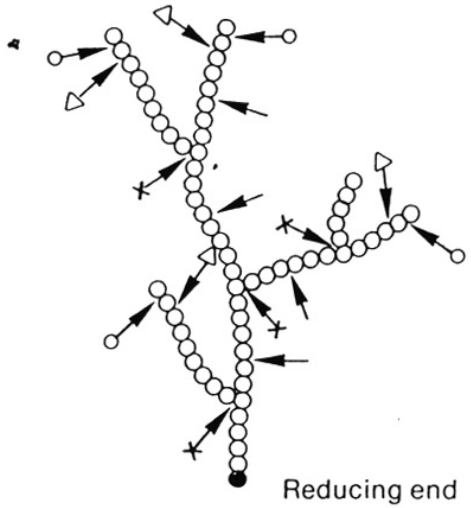
↓

40 mg/L *T. reesie*

↓

40 g/L *A. awamori*

STARCH HYDROLYSIS by AMYLASES



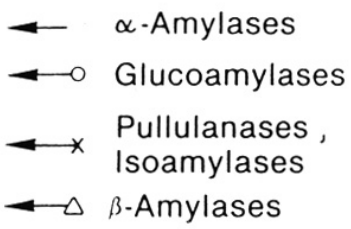
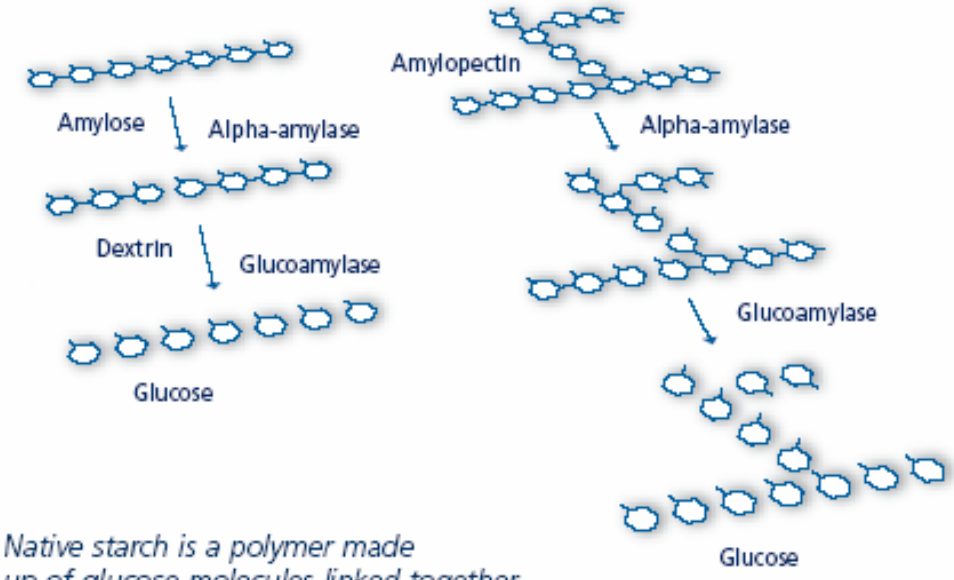


Figure 11.3 Mechanism of starch hydrolysis by amylases



Native starch is a polymer made up of glucose molecules linked together to form either a linear polymer called amylose or a branched polymer called amylopectin.

Fig. 6. Major steps in enzymatic starch conversion.

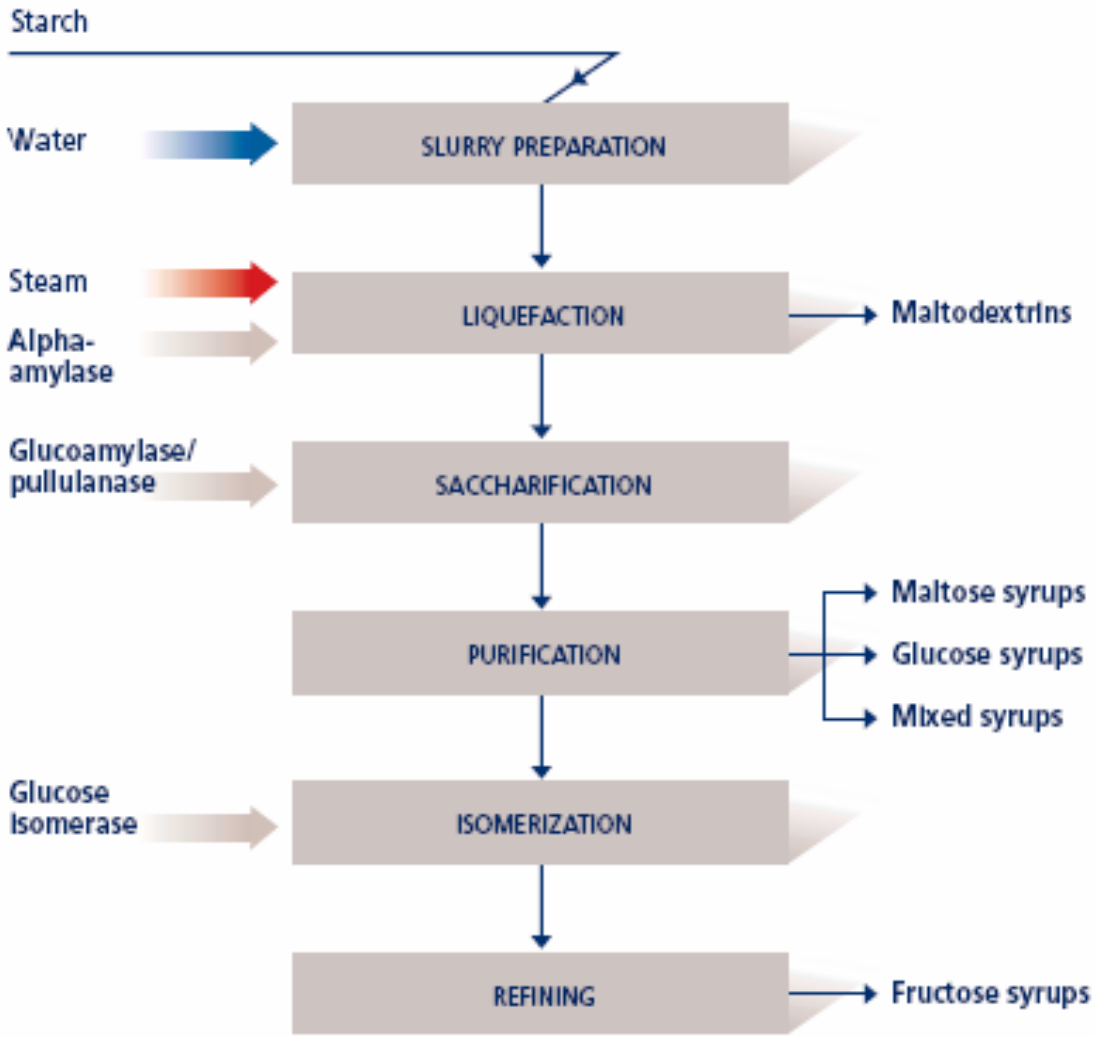


Table 1. Typical enzymes used in industrial processes.

CLASS	INDUSTRIAL ENZYMES
1: Oxidoreductases	Peroxidases Catalases Glucose oxidases Laccases
2: Transferases	Fructosyl-transferases Glucosyl-transferases
3: Hydrolases	Amylases Cellulases Lipases Pectinases Proteases Pullulanases
4: Lyases	Pectate lyases Alpha-acetolactate decarboxylases
5: Isomerases	Glucose isomerases
6: Ligases	*Not used at present*

Advantages of enzymes versus harsh chemicals or mechanical abrasion

1. Chemical reactions take place under mild conditions
2. Highly specific action
3. Very fast reaction rates
4. Numerous enzymes for different tasks

Enzymes for detergents and personal care

Enzymes have contributed greatly to the development and improvement of modern household and industrial detergents, the largest application area by far for enzymes today. They are effective at the moderate temperature and pH values that characterize modern laundering conditions. In laundering, dishwashing, and industrial and institutional (I&I) cleaning they contribute to:

- **in general, a better cleaning performance**
- **shorter washing times by quickly degrading dirt**
- **reduced energy consumption by lowering wash temperatures**
- **reduced water consumption through higher cleaning efficiency**
- **minimal environmental impact because they are readily biodegradable substances and make it possible to reduce pH levels in wash liquors**
- **rejuvenating the condition of cotton fabric through the action of cellulases on fibres.**

Laundry Detergents and Automatic Dishwashing Detergents

Their use in detergents began in the early 1930s with the use of pancreatic enzymes in pre-soak solutions. It was the German scientist Otto Röhm who first patented the use of pancreatic enzymes in 1913. The enzymes were extracted from the pancreases of slaughtered animals and they included proteases (trypsin and chymotrypsin), carboxypeptidases, alpha-amylases, lactases, sucrases, maltases and lipases. Thus, with the exception of cellulases, the foundation was already laid in 1913 for the commercial use of enzymes in detergents. Today, enzymes are continuously growing in importance for detergent formulators.

Each of the major classes of detergent enzymes – proteases, lipases, amylases and cellulases – provides specific benefits for laundering and automatic dishwashing. Historically, proteases were the first to be used extensively in laundering. Today, proteases have been joined by lipases and amylases in increasing the effectiveness of detergents, especially for household laundering at lower temperatures and, in industrial cleaning operations, at lower pH levels. Cellulases contribute to cleaning and to overall fabric care by rejuvenating or maintaining the appearance of washed garments.

Stone Washed Jeans Have Never Seen a Stone

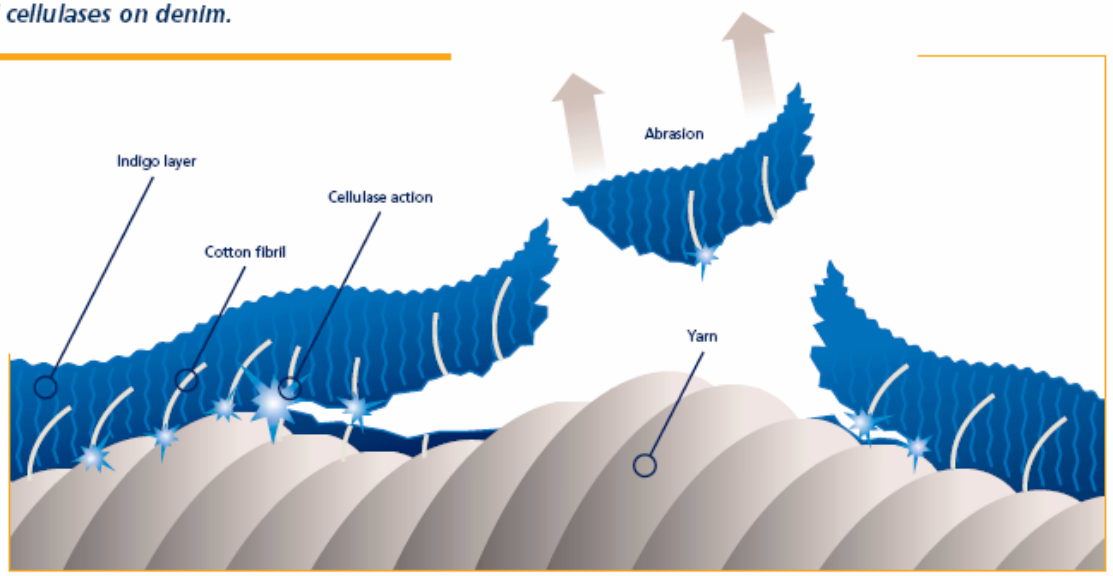
The denim industry is driven by fashion trends. The various cellulases available for modifying the surface of denim give fashion designers a pallet of possibilities to create new shades and finishes. The combination of new looks, lower costs, shorter treatment times and less solid waste has made abrasion with enzymes the most widely used fading process today. Incidentally, as the denim fabric is always sized, the complete process also includes desizing of the denim garment.

Sizing is the application of starch or other gelatinous material to fabric to protect it against mechanical damage during weaving or to make it more resistant to staining. Paper is treated in a similar manner.

Enzymes for denim finishing

The mode of action of cellulases is shown in Figure 3. Denim garments are dyed with indigo, which adheres to the surface of the yarn. The cellulase molecule binds to an exposed fibril (bundles of fibrils make up a fibre) on the surface of the yarn and hydrolyzes it, but leaves the interior part of the cotton fibre in the yarn intact. When the cellulases partly hydrolyze the surface of the fibre, the indigo is partly removed and light areas are created.

Fig. 3. The mode of action of cellulases on denim.



Removing hair from hides before tanning leather

The conventional and most widespread method of removing hair from bovine hides is to use lime and sodium sulphide in a hair-burning process. These chemicals dissolve the hair and open up the fibre structure of the hide.

Using a protease to help remove hair speeds up unhairing by improving the opening up of grain leather. Furthermore, sulphide requirements can be reduced by up to 40%, the strength of grain leather is increased, a cleaner grain surface is obtained and the area yield may also increase. The protease used must be active in the presence of lime at pH 12-13.

United States Patent 3986926
Method for preparing tannable pelts from animal skins and hides
Document: Abstract: A method for preparing tannable pelts from animal skins or hides, said method effecting concurrent softening, dehairing, opening of the hide structure, and bating in a single procedural step, which method comprises treating said skins or hides, free of preserving salt, with an aqueous bath having a pH between about 9 and about 12 and having dissolved therein: A. an effective amount of at least one member selected from the group consisting of a **fungus protease** whose optimum efficacy towards casein is at a pH above 7.0,

The starch industry began using industrial enzymes at an early date. Special types of syrup that could not be produced using conventional chemical hydrolysis were the first compounds made entirely by enzymatic processes.

Many valuable products are derived from starch. There has been heavy investment into enzyme research in this field and intensive development work on application processes. Reaction efficiency, specific action, the ability to work under mild conditions and a high degree of purification and standardization all make enzymes ideal catalysts for the starch industry. The moderate temperatures and pH values used for the reactions mean that few by-products affecting flavour and colour are formed. Furthermore, enzyme reactions are easily controlled and can be stopped when the desired degree of starch conversion is reached.

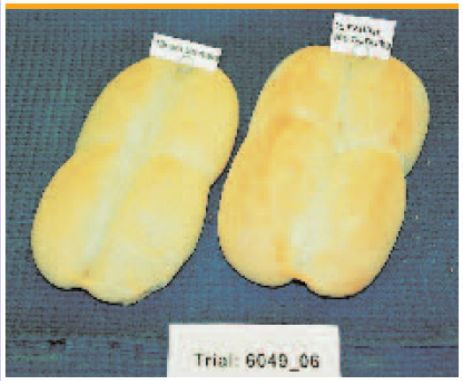
The first enzyme preparation for the food industry was glucoamylase in the 1960s and was a real turning point. This resulted in a rapid movement of the industry from processing starch by acid hydrolysis to enzymatic hydrolysis providing greater yields, a clearer product and easier crystallization. Even bigger was the introduction of glucose isomerase which made the industrial production of high fructose sugar possible and a multi-billion dollar industry in the US..

IMPROVEMENT IN BAKING THROUGH ENZYMES

For decades, enzymes such as malt and fungal alpha-amylases have been used in breadmaking. Rapid advances in biotechnology have made a number of exciting new enzymes available for the baking industry. The importance of enzymes is likely to increase as consumers demand more natural products free of chemical additives. For example, enzymes can be used to replace potassium bromate, a chemical additive that has been prohibited in a number of countries.

ENZYME	EFFECT
Amylase	Maximizes the fermentation process to obtain an even crumb structure and a high loaf volume.
Maltogenic alpha-amylase	Improves shelf life.
Glucose oxidase	Oxidizes free sulphydryl groups in gluten to make weak doughs stronger and more elastic.
Lipase	Dough conditioning by producing more uniform, smaller crumb cells and a silkier texture and whiter crumb colour.
Lipoxygenase	Bleaching and strengthening dough.
Xylanase	Dough conditioning. Easier dough handling and improved crumb structure.
Protease	Weakens the gluten to provide the plastic properties required in doughs for biscuits.

Fig. 11. Glucose oxidase and fungal amylase (right-hand loaf) were used to replace bromate in Marraquetta (South American bread).



Ingredients --
Potassium bromate
Chemical Formula: KBrO3
Synonyms
Bromated flour,
Bromic acid, potassium salt
Description
White crystals or powder.
Uses
Potassium bromate is used as a flour improver, where it strengthens the dough, allowing higher rising. It is an oxidizing agent, and under the right conditions, will be completely used up in the baking bread. However, if too much is used, or the bread is not cooked long enough or at a high enough temperature, then a residual amount will remain.
Potassium bromate has been banned in several countries as a carcinogen.

ELSEVIER Biotechnology Advances 18 (2000) 207–217

Research review paper

Production, purification, and characterization of the debittering enzyme naringinase

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Abstract

This review discusses the debittering enzyme naringinase and its essential role in the commercial processing of citrus fruit juice. Applications of this enzyme in other areas are identified. Characterization of the enzyme is detailed and its immobilized preparations are discussed. Production of microbial naringinase by fermentation is described.

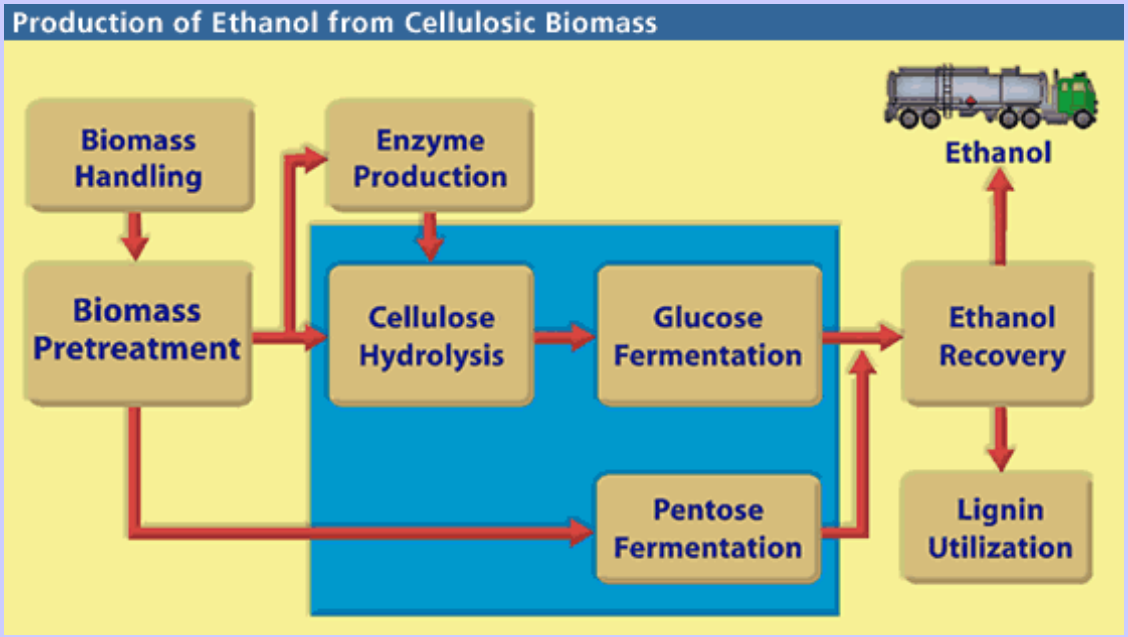
Keywords: Naringinase; Naringin; Debittering of fruit juice

Production of Food Enzymes

Genetic Engineering of Food Enzymes

- Make large quantities of a given enzyme
- Create a cheaper and more reliable source
- Improve the purity of the enzyme
- Improve the enzyme’s function in industrial use

Glucoamylase



- Saccharomyces used commercially for ethanol production from starch containing raw materials naturally lacks a glucoamylase gene necessary for full utilization of the raw material
- Cetus corporation engineered the glucoamylase gene from *Aspergillus awamori* into yeast leading to the ability of the transformed yeast to use up to 25% soluble starch as a sole carbon source and can produce up to 20 g/L in culture

THE DARK SIDE OF LIGHT BEER

Addition of fungal alpha amylases converts the unfermentable limit dextrins to fermentable simple sugars, resulting in a further conversion of sugar to alcohol: less carbohydrates and less body to the product and more alcohol.