INDUSTRIAL FERMENTATION

RANGES OF FERMENTATION PROCESS

- Microbial cell (Biomass)
  - Yeast
- Microbial enzymes
  - Glucose isomerase
- Microbial metabolites
  - Penicillin
- Food products
  - Cheese, yoghurt, vinegar
- Vitamins
  - B12, riboflavin
- Transformation reactions
  - Steroid biotransformation

Fermentation

- Aerobic
- Bioreactors- adequate supply of sterile air
- In addition, these fermenters may have a mechanism for stirring and mixing of the medium and cells
- Example: Biomass, antibiotics, enzymes, vitamins.

- Anaerobic
- In anaerobic fermentation, a provision for aeration is usually not needed.
- Examples: Wine, lactic acid and ethanol

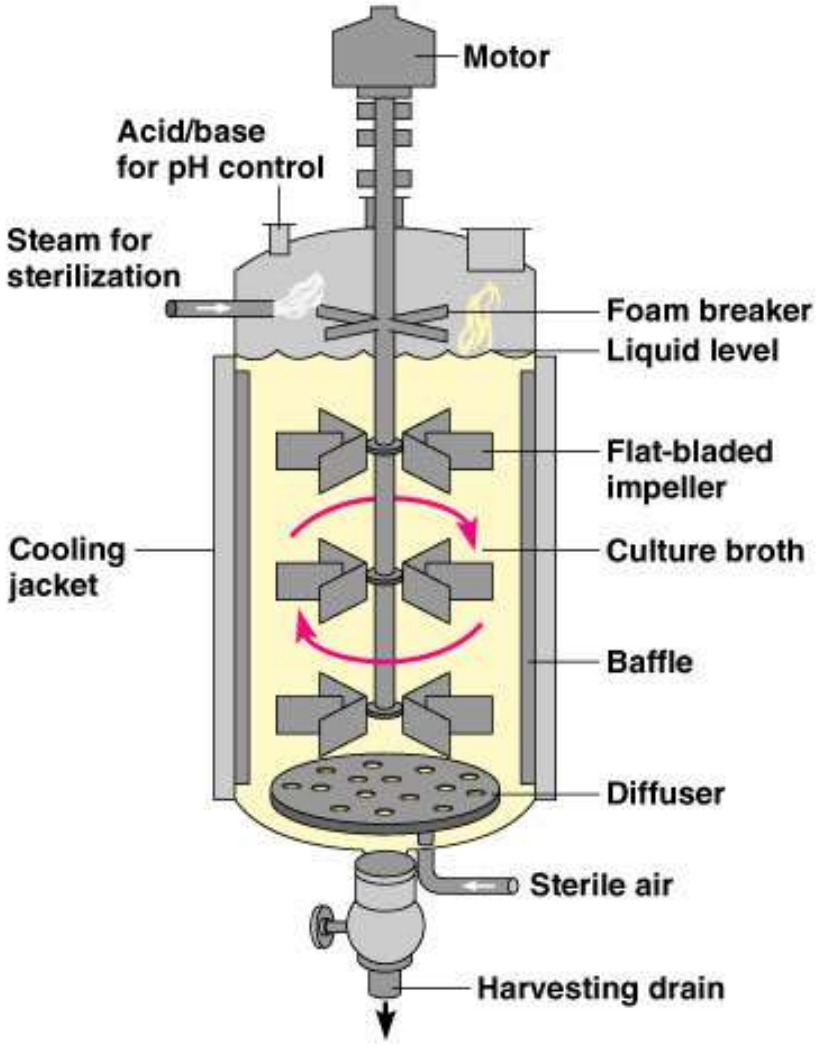
INDUSTRIAL FERMENTORS

- 125-250m^3
- Conditions in the fermenter are carefully monitored to regulate cell growth.
- Fermenter and all pipe work must be sterile before fermentation begins
- This is usually achieved by flushing the whole system with superheated steam before the production begins.
- Process if frequently aerobic so fermentor has to be well aerated.
- The aeration will be sufficient to mix many cultures
- If the culture is thick or sticky, additional stirring is required by a motor driven paddle called an **impeller.**

INDUSTRIAL FERMENTORS

- While initially the culture may need warming to start of the process – once it has started a **cooling system** is vital.
- Cooling is achieved by either a water jacket or cooling coils inside the fermenter.
- Are structures designed to optimize the growth conditions of the specific organisms that we want
  - *Control oxygen, pH, medium, temperature and nutrients antifoaming*
  - *Stirred tank reactor*
  - *Air lift reaction*

Fermentation Technology



(a) Section of a continuously stirred bioreactor

(b) A bioreactor tank is at the left.

Table 30.1 Fermentor sizes for various industrial processes

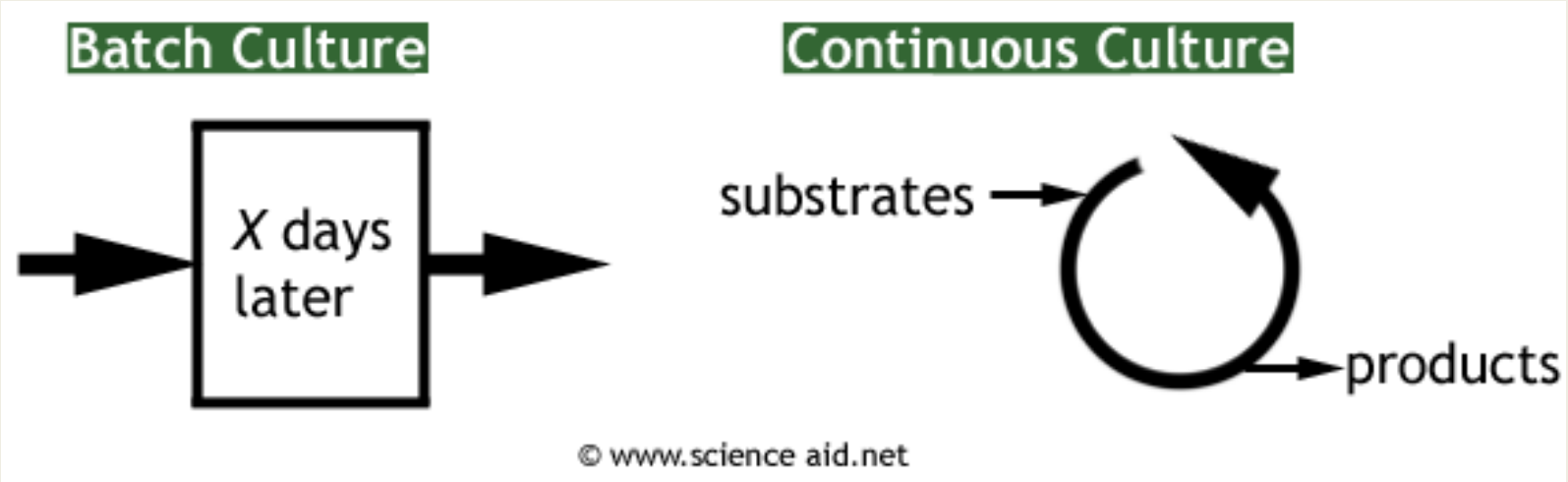
Size of fermentor (liters)	Product
1–20,000	Diagnostic enzymes, substances for molecular biology
40–80,000	Some enzymes, antibiotics
100–150,000	Penicillin, aminoglycoside antibiotics, proteases, amylases, steroid transformations, amino acids, wine, beer
200,000–500,000	Amino acids (glutamic acid), wine, beer

Table 30-1 Brock Biology of Microorganisms 11/e

Fermentation

- Fermentation could be:

- Batch mode
- Fed batch mode (continuous)



Batch fermentation

- Most fermentations are batch processes
- Nutrients and the inoculum are added to the sterile fermenter and left to get on with it!
- Anti-foaming agent may be added.
- Once the desired amount of product is present in the fermenter the contents are drained off and the product is extracted.
- After emptying, the tank is cleaned & prepared for a new batch.

Continuous fermentation

- Some products are made by a continuous culture system.
- Sterile medium is added to the fermentation with a balancing withdrawal of broth for product extraction.

Volume	10,000L to 100,000L+
Product value	Low (Low value added)
Product types	Biomass, Bulk chemicals, Antibiotics, Most enzymes
R & D development	Fermentation Technology/process engineering, strain and medium manipulation etc. to improve process economics
R & D Cost	Low

How can we improve process economics?

- Better Product Yields
- Higher Product Titres
- Improved Volumetric Productivity

Major Groups of Large Scale Processes

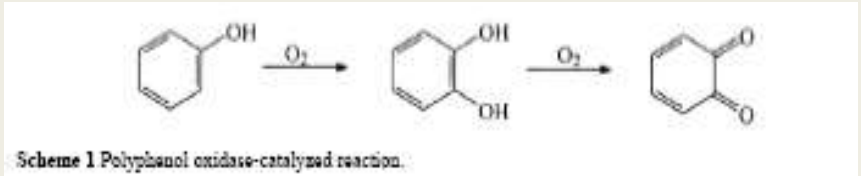
1. Biomass
2. Enzymes
3. Metabolites
   - *Primary Products of Catabolism e.g. Citric acid*
   - *Intermediates*

     e.g. glycine in Nitrogen metabolism
     - Production of MSG

       Corynebacterium used for production of MSG (Glutamate) and Lysine
   - *Secondary products e.g. penicillin*
4. **Biotransformations**

Growth = production

No Growth Needed



Scheme 1 Polyphenol oxidase-catalyzed reaction.

Commercial production of Microorganisms

- Fermentation projects (Beer and Wine)
- Biomass where the physical structure of the microbe is wanted
  - *Baking yeast (Saccharomyces cerevisiae)*
  - *Edible forms of bacteria (spirulina)*
  - *Bacterial Insecticides (Bacillus thuringensis)*
  - *Nitrogen Fixing Inoculants (bacteria: e.g. Rhizobium)*
  - *Single-cell protein (SCP)*
    - May concentrate toxic compounds
    - Nucleic acids in large numbers are toxic

Biomass

- Single cell protein:
  - ***For Animal feed***
    - Upgrading low value agricultural products:
      - *Cellulose*
      - *Starch*
    - Use yeasts or fungi
    - Profit margins very small – competitive market
  - ***For Human consumption***
    - Fungi (eg Quorn) *Fusarium venenatum* as fat substitute in milk and in cereals

Production of Yeast – Fermentation & Scale-up

- The basic stages of bakers yeast production includes:
  - Molasses is the substrate used in the production of bakers yeast.
  - Molasses, is a by-product of sugar refining (50% sugar), serves as the source of carbon and energy for the process.
  - It is supplemented with a number of nitrogenous compounds and vitamins such as biotin, which are required for the proper and efficient growth of the yeast cells.

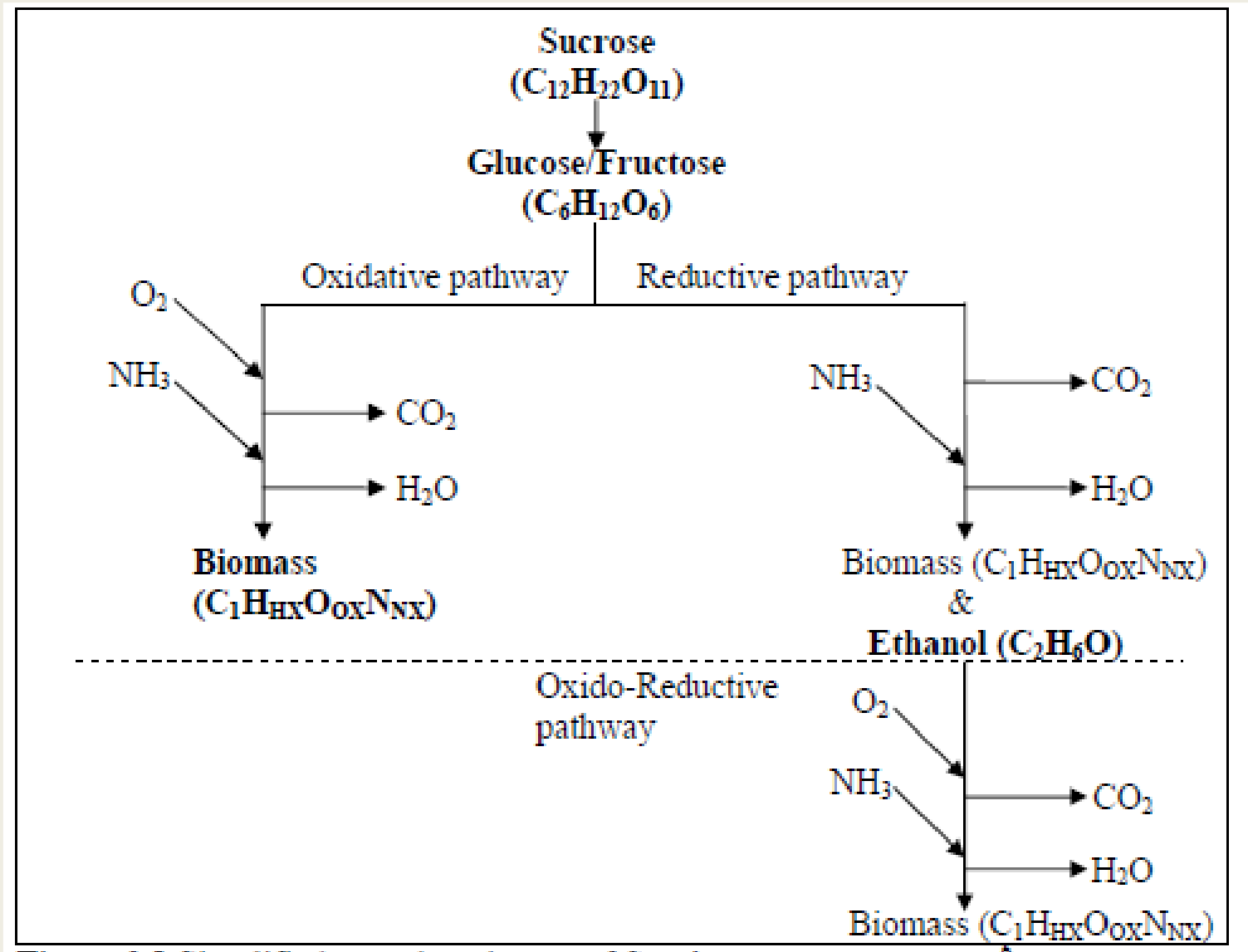


Figure 3.1 Simplified growth pathways of *Saccharomyces cerevisiae*.[6]

Production of yeast

- Commercial yeast production starts in the laboratory where a small quantity of a yeast culture is injected into a closed flask containing a sterile solution of molasses, ammonium salts to provide a source of nitrogen, and phosphate, necessary for yeast development and reproduction.
- The yeast culture is made up of a particular yeast strain, which is normally kept on an agar slant. An enormous number of strains of Saccharomyces cerevisiae exist, many of which have already been selected for baking.
- The closed flask that the culture is injected into is kept at a constant temperature and the yeast grows vigorously for 12 hours.

Production of yeast

- It is then transferred to a larger flask containing a further solution of molasses and nutrient material and more growth takes place.
- The transfer process is repeated again until a large enough culture of yeast is obtained to start the main yeast production process in the factory's large fermentation vessels.
- Fermentation vessels for yeast production range from 40,000 to 200,000L. The progressive increase of fermentor size used is known as **scale-up.**
- Until this stage the yeast cultures have been grown in the absence of air; this is known as anaerobic fermentation. Anaerobic fermentation is, however, inefficient in terms of yeast growth, and subsequent stages of yeast production take place with sterile air being blown/sparged through the growing yeast cultures; this is known as aerobic fermentation.

Production of yeast

- The reason why the early stages of yeast production take place in the absence of air is to favour the growth of yeast cells instead of other organisms, such as bacteria, which may gain access to the culture, since these would also grow rapidly and could decrease the efficiency of the process and affect the final yeast quality.
- A small amount of alcohol is produced during the early stages, which inhibits the growth of foreign organisms.
- The fermentation process continues with air being blown through the yeast cultures and molasses solution and nutrients being added continuously, at a constantly increasing rate that is directly proportional to the yeast cell population.
- By maintaining this supply at a level just sufficient for the amount of yeast present, together with an adequate supply of air, maximum yeast cell reproduction takes place with the minimum production of alcohol as indicated previously.

- At the end of the first stages of yeast growth, about 12 tonnes of yeast is produced and this is known as seed or mother yeast.
- The seed yeast is divided into portions and these are used to start other fermentations.
- These fermentations are carried on as before with increasing addition of air, molasses solution and nutrients, and each 3 tonnes of seed yeast produces about 11 tonnes of the final bakers yeast.
- Throughout the whole fermentation process stringent checks are carried out to ensure that yeast growth and quality are maintained, so that the final 40-50 tonnes of bakers yeast are of the same quality and have the same characteristics and properties as the original few milligrams of pure yeast culture that started the process.
- At the end of the fermentation stage the yeast is present as a suspension of cells in a dark brown liquid containing the residues of the molasses.

Production of yeast

- The yeast is removed from the fermentation liquid by a process of washing and separating in centrifugal separators, signaling the end of the fermentation and beginning of the downstream processing stage.

- Downstream Processing can be defined as the stages of processing that take place after the fermentation or bioconversion stage.
- The yeast broth which is produced by fermentation, containing approximately 5% solids, can be manipulated into two main types of bakers yeast product and an additional intermediate saleable product.
- These are cake yeast, granular yeast and cream yeast, each of which requires a downstream process to arrive to the desired product.

Enzymes

- Often depolymerases (eg. Amylases, Proteases)
- Large range of uses (and purities):
  - *Food*
  - *Pharmaceuticals*
  - *Detergents*
  - *Industrial Microbiology (Medium Preparation)*
  - *Leather Preparation*

Enzymes

- Organisms used for production:
  - *Bacteria (especially Bacillus)*
  - *Yeasts (eg Saccharomyces)*
  - *Fungi (eg Mucor)*
- Problems caused the cell's control systems (induction, repression) may need to be overcome:
  - *Mutate/engineer organism*
  - *Medium formulation*
  - *Process manipulation (substrate supply)*

Enzyme products

Enzyme	Use
Lipase	Enhances flavor in cheese making
Lactase	Lactose free milk products
Protease	Detergent additive, clear beer
α-Amylase	High fructose corn syrup
Pectinase	Reduces cloudiness in wine/juice

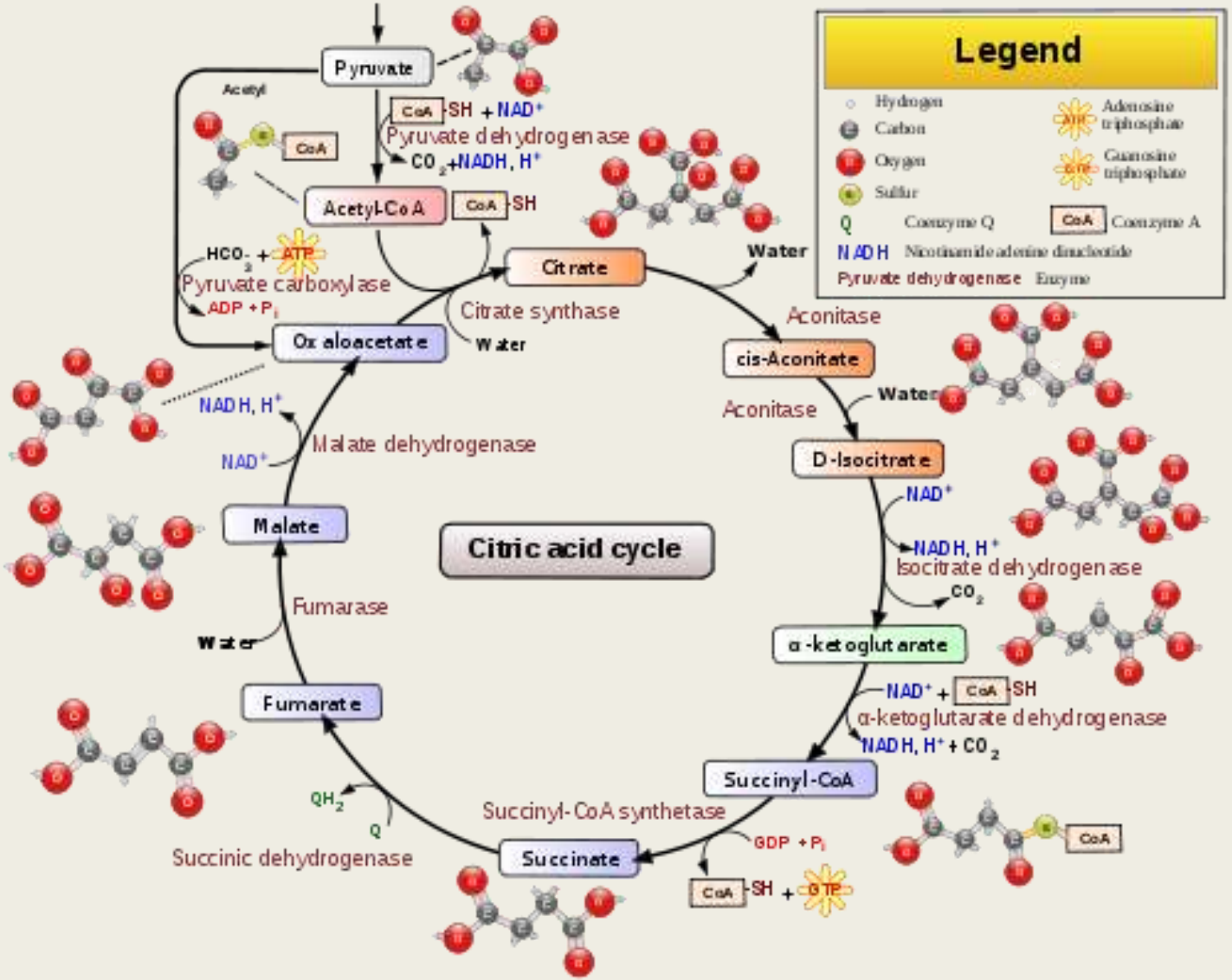
Table 1. Enzymes used in pretanning operations

Process	Enzyme	Microorganism
Soaking	Protease	*Aspergillus parasiticus*, *A. flavus*, *A. oryzae*, and *Bacillus subtilis*[19], *Rhizopus rhizopodiformis*[24]
	Carbohydrases	*Aspergillus awamori*[20]
Dehairing	Protease	*Aspergillus flavus*[33,35], *Aspergillus* sp.[34], *Bacillus subtilis*[41], *Lactobacillus* sp.[42], *Conidiobolus* sp.[36], *B. amyloliquefaciens*[64], *Streptomyces griseus*, *S. fradiae*[65], *S. moderatus*[66]
Bating	Protease	*A. parasiticus*[67,68], *S. rimosus* and *B. licheniformis*[48], *B. subtilis*[69], *Penicillium janthinellum*[70]
Degreasing	Lipase	*Rhizopus nodosus*[46], *A. oryzae* and *A. flavus*[71]

Primary Metabolites – Products of Catabolism

- Are produced during an organism's growth phase
- By-products of the cell's energy yielding processes
- "Normal" cells produce significant quantities (but we can improve it)
- Examples:
  - *Ethanol*
    - Alcoholic Beverages (€0.07/l)
    - Fuel (and industrial) Alcohol (€0.9/l)

Citric Acid Cycle



Primary Fermentation

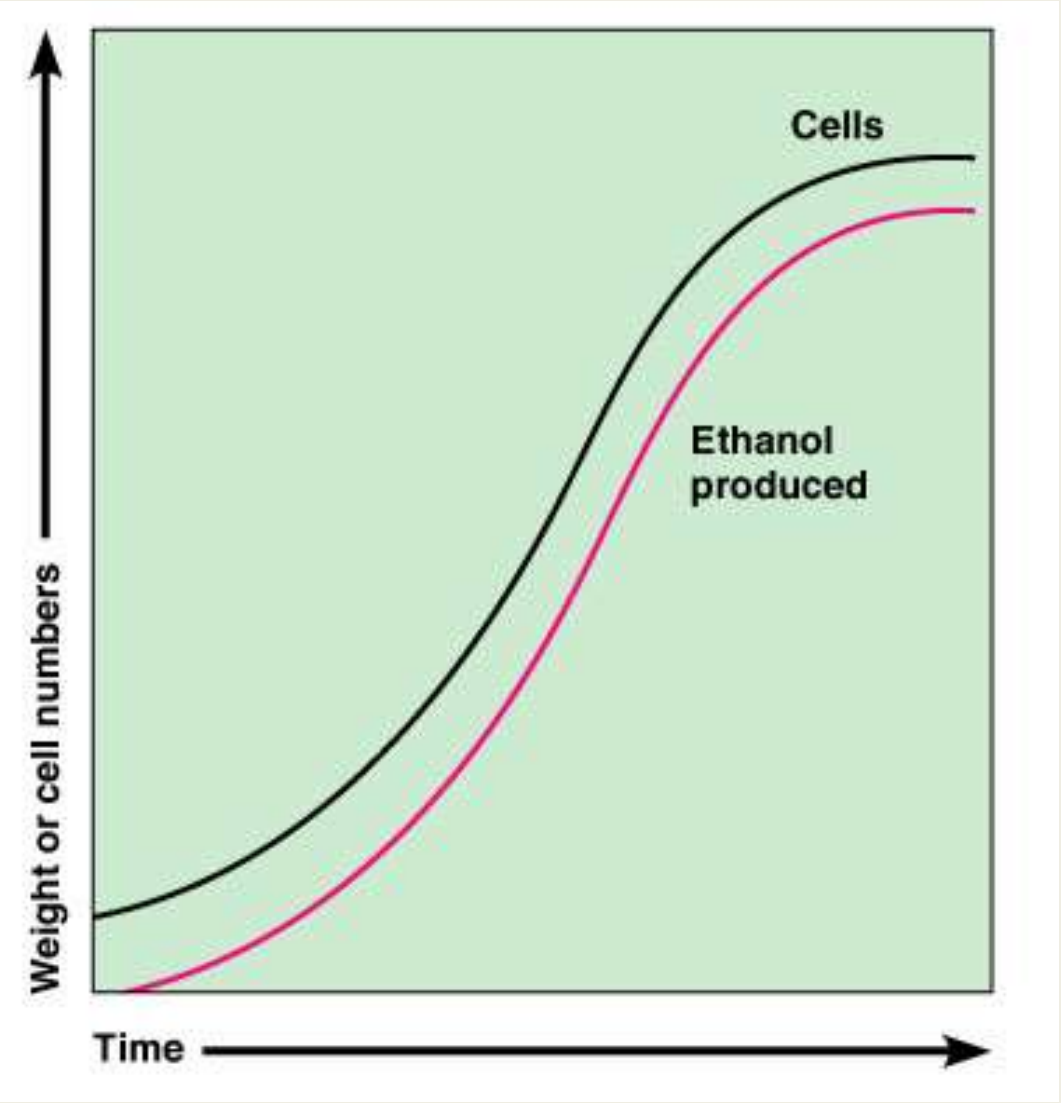


Figure 28.11a

Ethanol:

- *$C_3H_6O_3$ Converts to C_2H_5OH+ CO_2*
- *Beverages*
  - Organism: Yeast (*Saccharomyces cervisiae* or *uvarum*)
  - Some substrates immediately available:
    - *Grape juice (Wine, Brandy)*
    - *Sugar Cane (Rum)*
  - Some substrates need pre-treatment to depolymerise starch and protein:
    - *Malt (Beer, Whisky)*
    - *Cereals, potatoes etc. plus malt, enzymes etc (vodka, other spirits, some beers etc.)*
- *Post-fermentation treatment may include distillation (spirits) and/or maturation.*

Ethanol

- Fuel/Industrial Alcohol
  - *Organisms:*
    - Yeasts
    - Bacteria (*Zymomonas*): fast but sensitive to product.
  - *Substrates: Cheap Agricultural products:*
    - Sucrose (Sugar Cane)
    - Starch type products (Depolymerise with enzymes etc. or obtain organism with amylase activity)
  - *Very low value added/Competitive market (but Government support?).*
  - *Conventional distillation step can make the process uneconomical:*
    - Use vacuum (low temperature) distillation during fermentation.

Primary Metabolites –Metabolic Intermediates

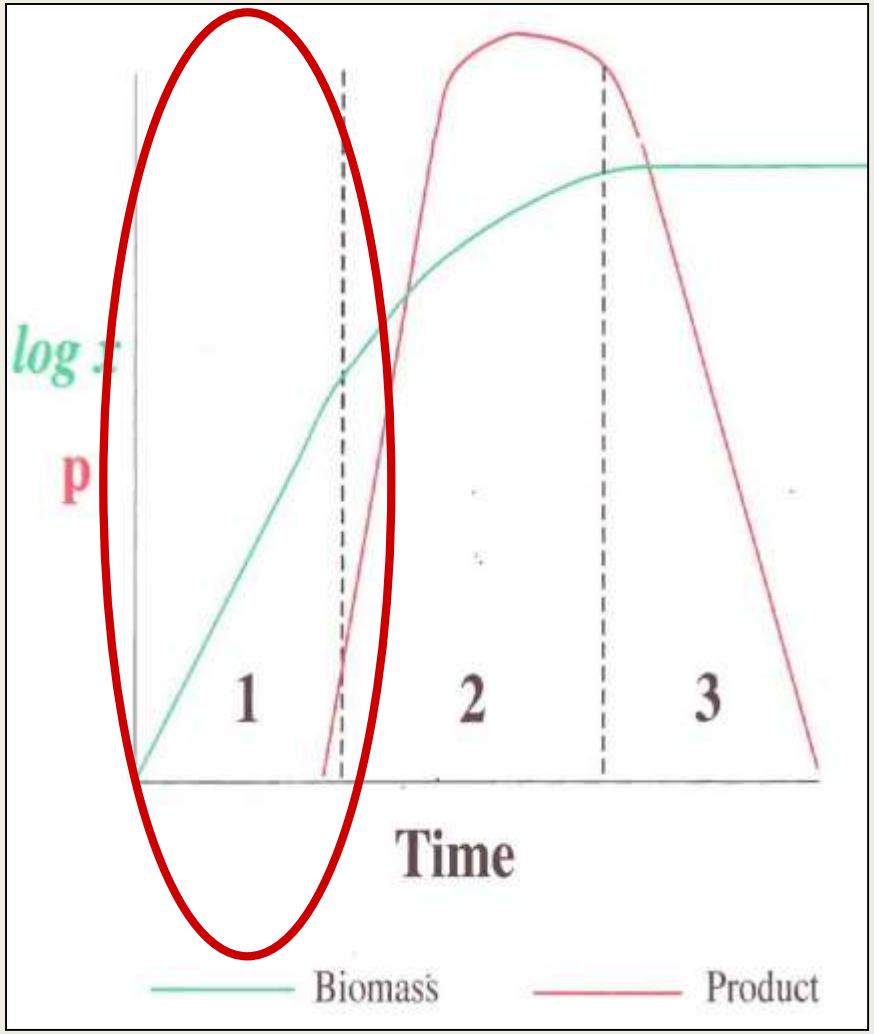
- Intermediates in metabolic pathways (TCA cycle, pathways leading to protein and nucleic acid production etc.).
- Levels of intermediate pools generally low in healthy “wild type” organisms
  - *Need to develop industrial strains:*
    - Overcome feedback inhibition/repression.
- Examples:
  - *Citric Acid (Soft Drinks, Foods etc.)*
  - *Lysine (Essential AA, Calcium absorption, Building blocks for protein)*
  - *Glutamic acid (Monosodium Glutamate precursor)*
  - *Phenylalanine (Aspartame precursor)*
- Organisms Yeasts. Fungi, Bacteria:
  - *Corynebacterium for amino acid production*

Secondary Metabolites

- Are not essential to cell growth or function.
- Not part of the “central” metabolic pathways
- Producers:
  - *Actinomycetes (eg Streptomyces)*
  - *Fungi (eg Penicillium)*
  - *Sporeforming bacteria (Bacillus)*
- Produced as growth slows/stops in batch cultures
- Antibiotics are of major industrial importance

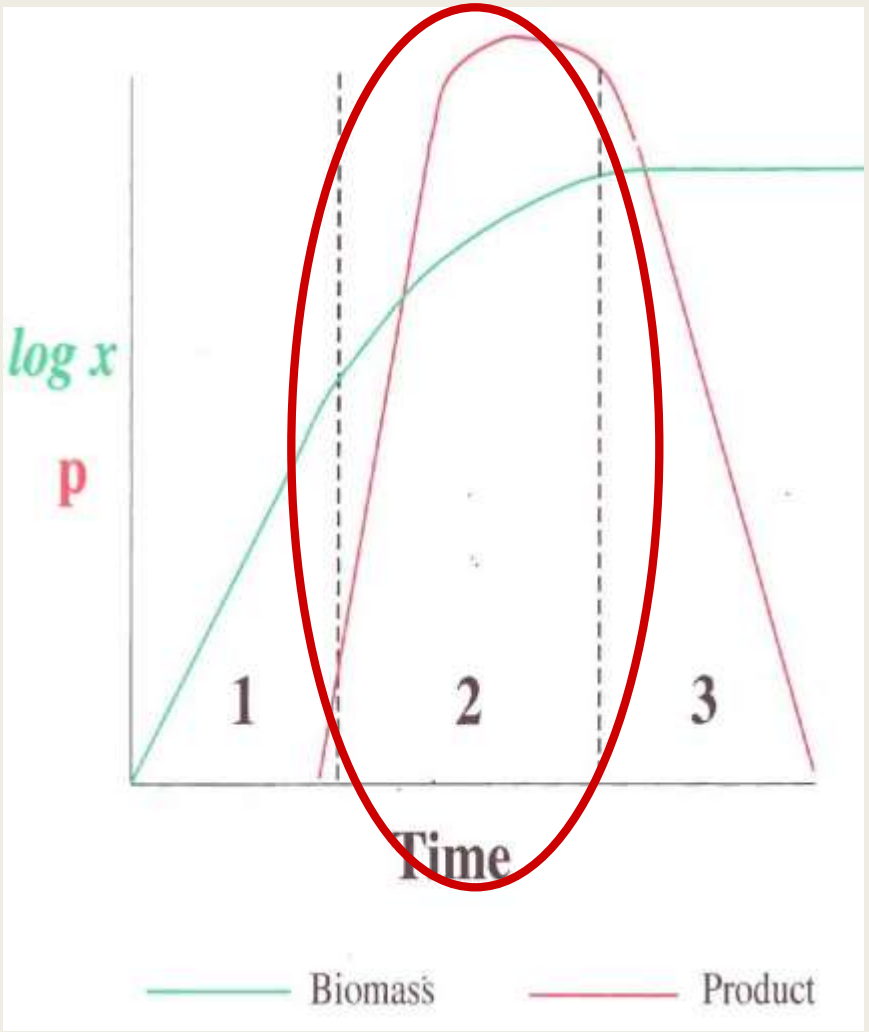
Secondary Metabolite production in Batch Culture

- 1. Trophophase
  - *Culture is nutrient sufficient*
  - *Exponential Growth*
  - *No Product Formation*



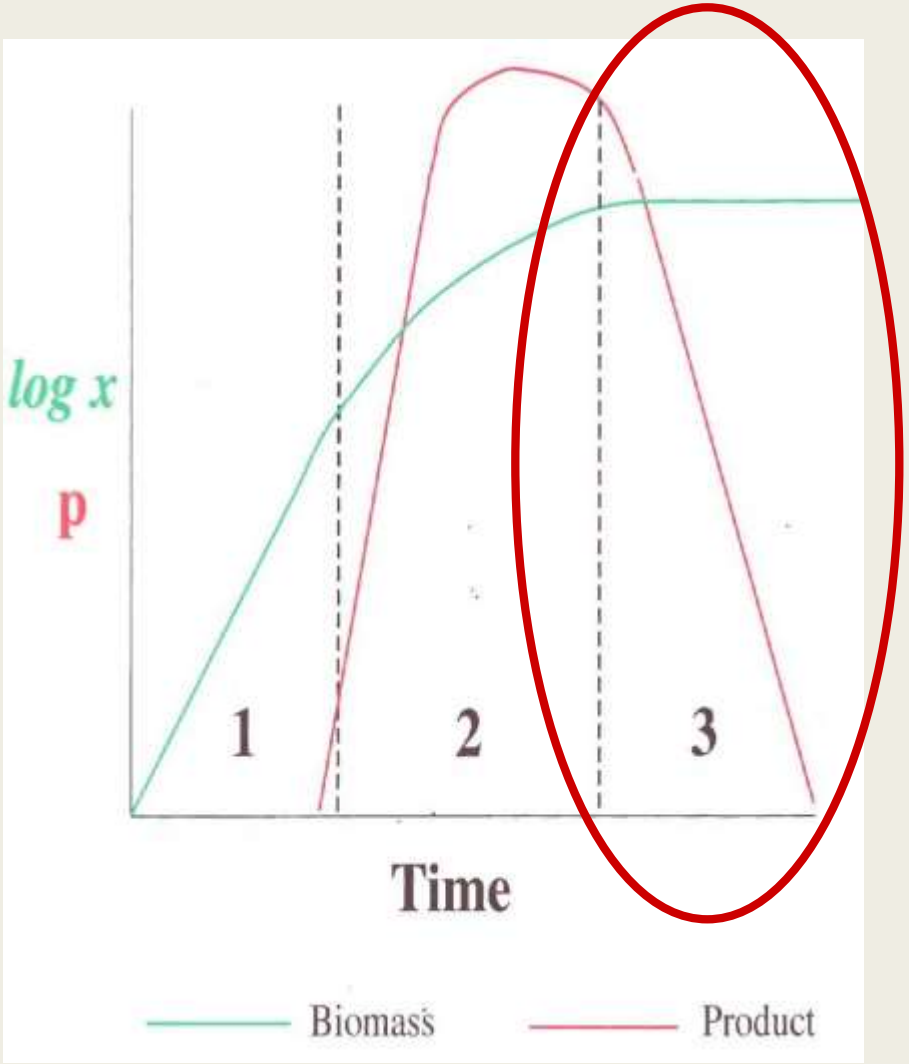
Secondary Metabolite production in Batch Culture

- 2 Idiophase
  - *Carbon limitation*
  - *Growth slowing or stopped*
  - *Product formation*
  - *HARVEST AT THE END OF THIS PHASE*



Secondary Metabolite production in Batch Culture

- 3 Senescence
  - *Product formation ceases.*
  - *Degeneration/lysis of mycelium (Fungi, Actinomycetes)*
  - *Product degraded/used by culture.*



Biotransformation (Bioconversion)

- Transformation of a chemical added to the medium into a commercially valuable compound
- Use cells as “catalysts” to perform one or two step transformation of substrate.
- Use cells several times:
  - *Fungal/Actinomycete mycelium*
  - *Immobilised bacteria or yeast cells packed into a column*
- Examples:
  - *Transformations* ***of plant sterols by Mycobacterium fortuitum”.***
  - *Ethanol to Acetic acid (immobilised Acetobacter)*

Bioconversions

- Example, bioconversion of steroids
  - *Chemical synthesis requires 37 steps*
  - *Bioconversion requires 11 steps, reduces the cost and shortens the time of manufacturing.*
- How these processes work
- Use of immobilized cells (cells localized in a matrix and the chemical is converted as it flows pas the column

Microorganisms represent an almost limitless supply of enzymatic reactions

- May reduce the risks and complexities of industrial syntheses
- Is less expensive
- By-products are usually less toxic
- Used in environmental cleanup (In situ)

Products from Microorganisms

Primary Metabolites	**Secondary Metabolites**
Amino Acids	Antibiotics
Vitamins	Pigments
Polysaccharides	Toxins
Ethanol	Alkaloids
Acetone and Butanol	Many pharmacological compounds

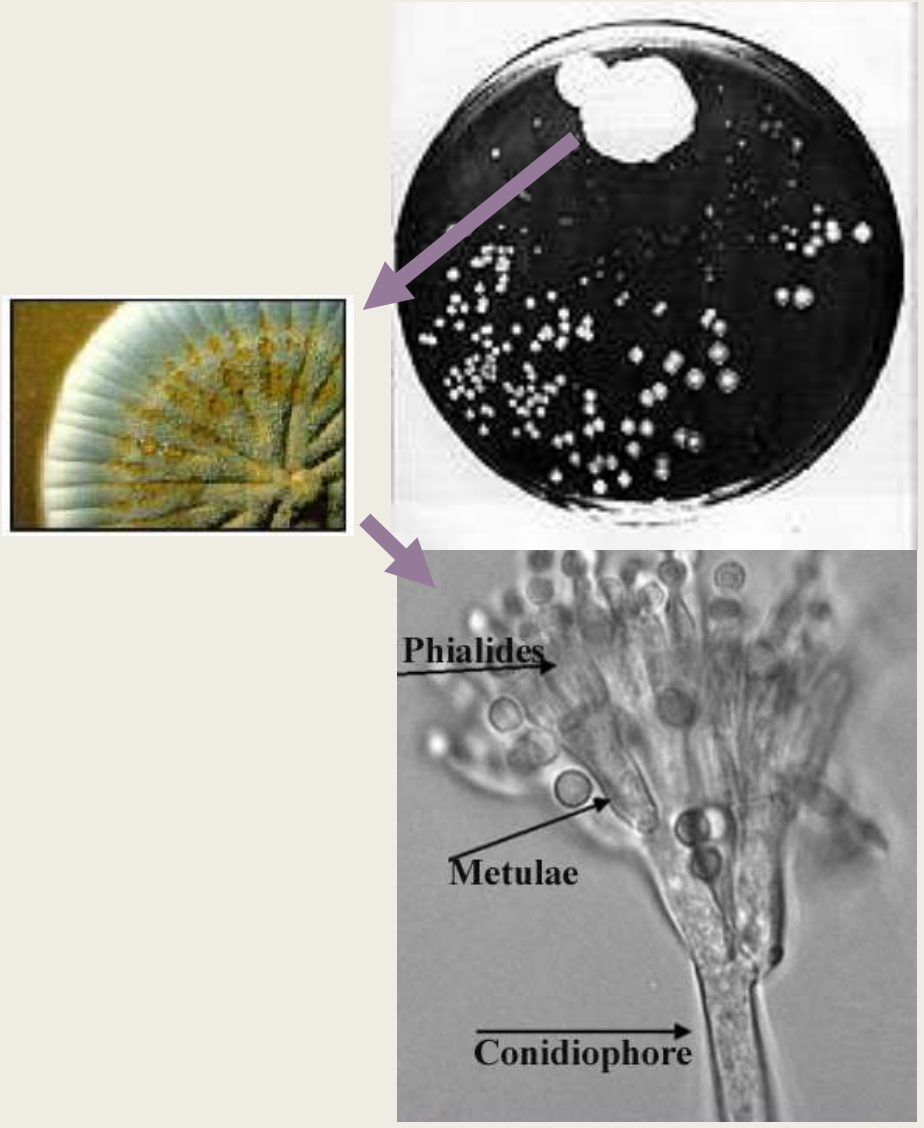
PRODUCTION OF ANTIBIOTICS

Some Antibiotics produced by Microorganisms

Antibiotic	*Producing microorganism*
Cephalosporin	*Cephalosporium acrimonium*
Chloramphenicol	*Streptomyces venezuelae*
Erythromycin	*Streptomyces erythreus*
Griseofulvin	*Penicillium griseofulvin*
Penicillin	*Penicillium chrysogenum*
Streptomycin	*Streptomyces griseus*
Tetracycline	*Streptomyces aureofaciens*
Gentamicin	*Micromonospora purpurea*

PRODUCTION OF PENICILLIN

- Fleming took a sample of the mold from the contaminated plate. He found that it was from the *Penicillium* family, later specified as *Penicillium notatum*.
- Penicillin was first produced on a large scale for human use in 1943. At this time, the development of a pill that could reliably kill bacteria was a remarkable development and many lives were saved during World War II because this medication was available.



MOA OF PENICILLIN and Spectrum

- All penicillin like antibiotics inhibit synthesis of peptidoglycan, an essential part of the cell wall.
- They do not interfere with the synthesis of other intracellular components.
- These antibiotics do not affect human cells because human cells do not have cell walls.

- Penicillins are active against Gram positive bacteria
- Some members (e.g. amoxicillin) are also effective against Gram negative bacteria but not *Pseudomonas aeruginosa*

PRODUCTION OF PENICILLIN

- Penicillin was the first important commercial product produced by an aerobic, *submerged* fermentation
- First antibiotic to have been manufacture in bulk.
- Used as input material for some semi synthetic antibiotics.
- It is fermented in a **batch culture**
- When penicillin was first made at the end of the second world war using the fungus *Penicillium notatum*, the process made 1 mg dm^{-3}.
- Today, using a different species (*P. chrysogenum*) and a better extraction procedures the yield is 50 g dm^{-3}.
- There is a constant search to improve the yield.

Production of Penicillin

- The yield of penicillin can be increased by:
- Improvement in composition of the medium
- Isolation of better penicillin producing mold sp. *Penicillium chrysogenum* which grow better in huge deep fermentation tank
- Development of submerged culture technique for cultivation of mold in large volume of liquid medium through which sterile air is forced.
- Like all antibiotics, penicillin is a secondary metabolite, so is only produced in the stationary phase.

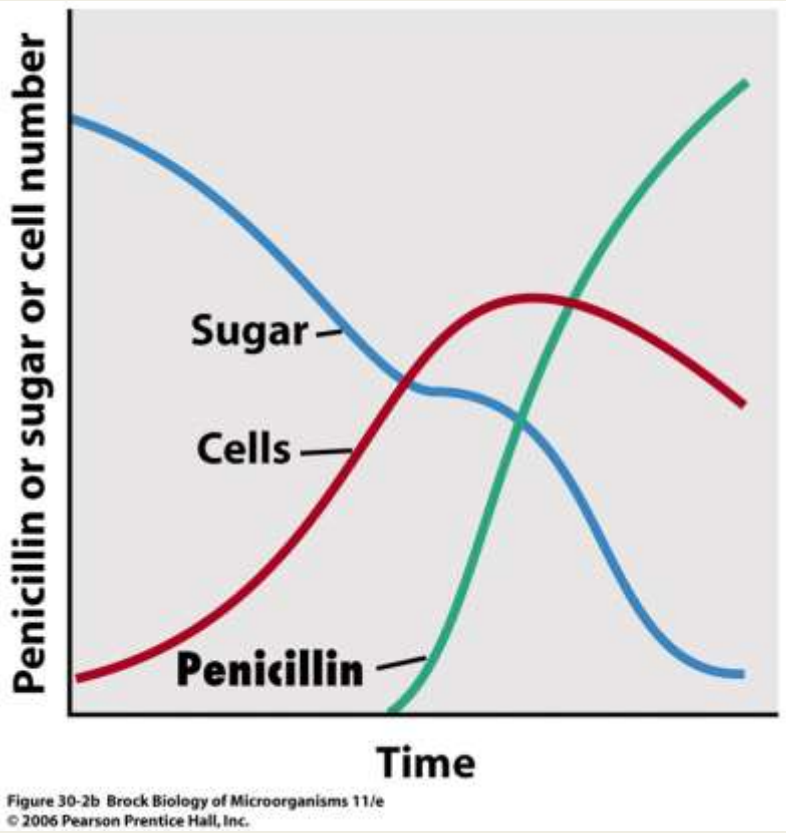


Figure 30-2b Brock Biology of Microorganisms 11/e

INDUSTRIAL PRODUCTION OF ANTIBIOTIC-PENICILLIN

- The industrial production of penicillin was broadly classified in to two processes namely,
- **Upstream processing**
  - *Upstream processing encompasses any technology that leads to the synthesis of a product. Upstream includes the exploration, development and production*
- **Downstream processing**
  - *The extraction and purification of a biotechnological product from fermentation is referred to as downstream processing.*

UPSTREAM PROCESSING

- **INOCULUM PREPARATION**
- The medium is designed to provide the organism with all the nutrients that it requires.
- Inoculation method- submerged technique
- Spores -major source of inoculum

- **RAW MATERIALS**
- **CARBON SOURCES:**

Lactose acts as a very satisfactory carbon compound, provided that is used in a concentration of 6%. Others such as glucose & sucrose may be used.

NITROGEN SOURCES:

- Corn steep liquor (CSL)
- Ammonium sulphate and ammonium acetate can be used as nitrogenous sources.

MINERAL SOURCES:

Elements namely potassium, phosphorus, magnesium, sulphur, zinc and copper are essential for penicillin production. Some of these are applied by corn steep liquor.

Calcium can be added in the form of chalk to counter the natural acidity of CSL

PAA (phenylacetic acid)- precursor

FERMENTATION PROCESS

- The medium is inoculated with a suspension of conidia of Penicillium chrysogenum.
- The medium is constantly aerated and agitated, and the mould grows throughout as pellets.
- After about seven days, growth is complete, the pH rises to 8.0 or above, and penicillin production ceases

STAGES IN DOWNSTREAM PROCESSING

Removal of cells

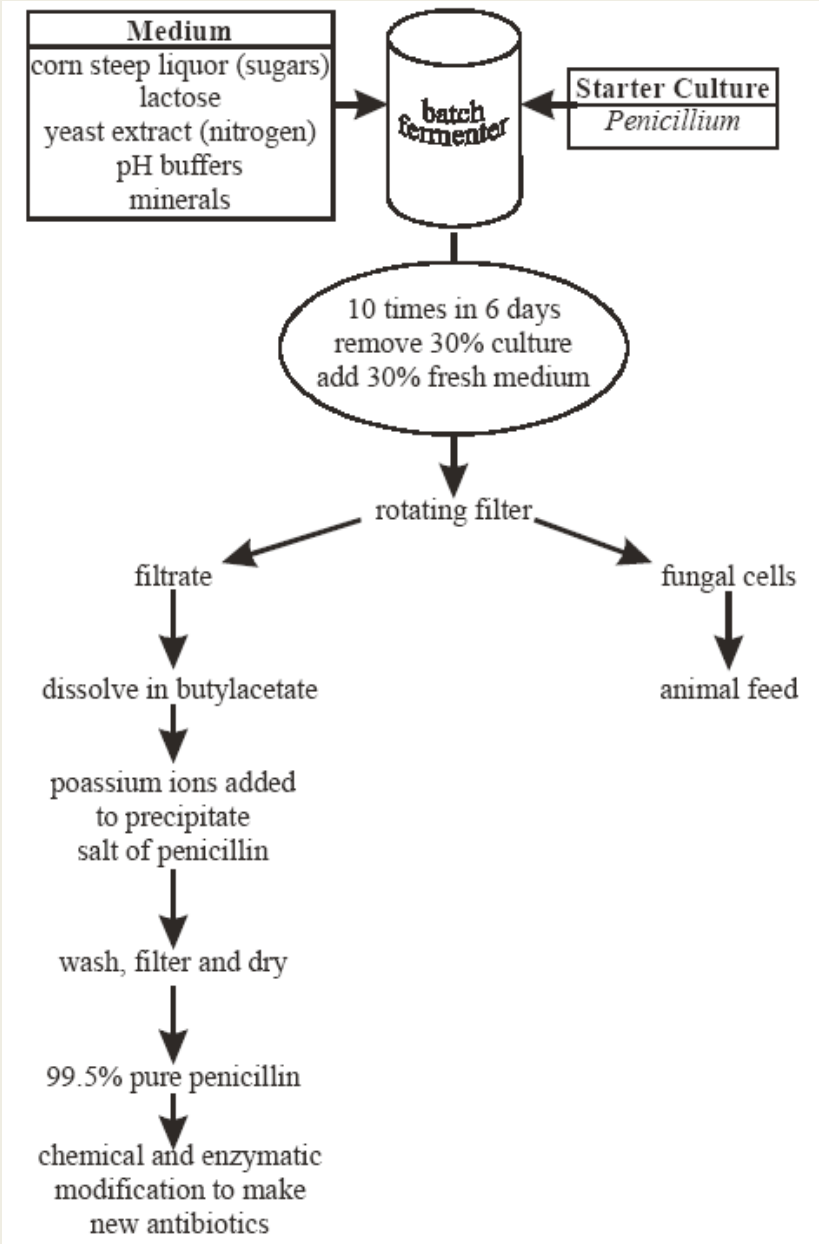
- The first step in product recovery is the separation of whole cells and other insoluble ingredients from the culture broth by technique such as filtration and centrifugation.

Isolation of benzyl Penicillin

- The PH is adjusted to 2-2.5 with the help of phosphoric or sulphuric acids.
- In aqueous solution at low PH values there is a partition coefficient in favor of certain organic solvents such as butyl acetate.
- This step has to be carried out quickly for penicillin is very unstable at low PH values.
- Antibiotic is then extracted back into an aqueous buffer at a PH of 7.5, the partition coefficient now being strongly in favor of the aqueous phase. The resulting aqueous solution is again acidified & re-extracted with an organic solvent.
- These shifts between the water and solvent help in the purification of penicillin.

- The treatment of the crude penicillin extract varies according to the objective, but involves the formation of an appropriate penicillin salt.
- The solvent extract recovered in the previous stage is carefully extracted back with aqueous sodium hydroxide.
- This is followed by charcoal treatment to eliminate pyrogens and by sterilization.
- Pure metal salts of penicillin can be safely sterilized by dry heat, if desired. Thereafter, the aqueous solution of penicillin is subjected to crystallization.
- For parental use, the antibiotic is packed in sterile vials as a powder or suspension.
- For oral use, it is tabletted usually now with a film coating.
- Searching tests (ex: for purity, potency) are performed on the appreciable number of random samples of the finished product.
- It must satisfy fully all the strict government standards before being marketed

1. A medium of corn steep liquor (a by product of starch manufacture), yeast extract and others substrates added to the fermenter.
2. After 40 hours, Penicillin begins to be secreted by the fungus
3. The mould mycellium (cell matter) is filtered from the harvested product.
4. Penicillin is extracted in the organic solvent: butylacetate, in which it dissolves.
5. Potassium salts are added and a penicillin precipitate is formed, this is washed and dried.



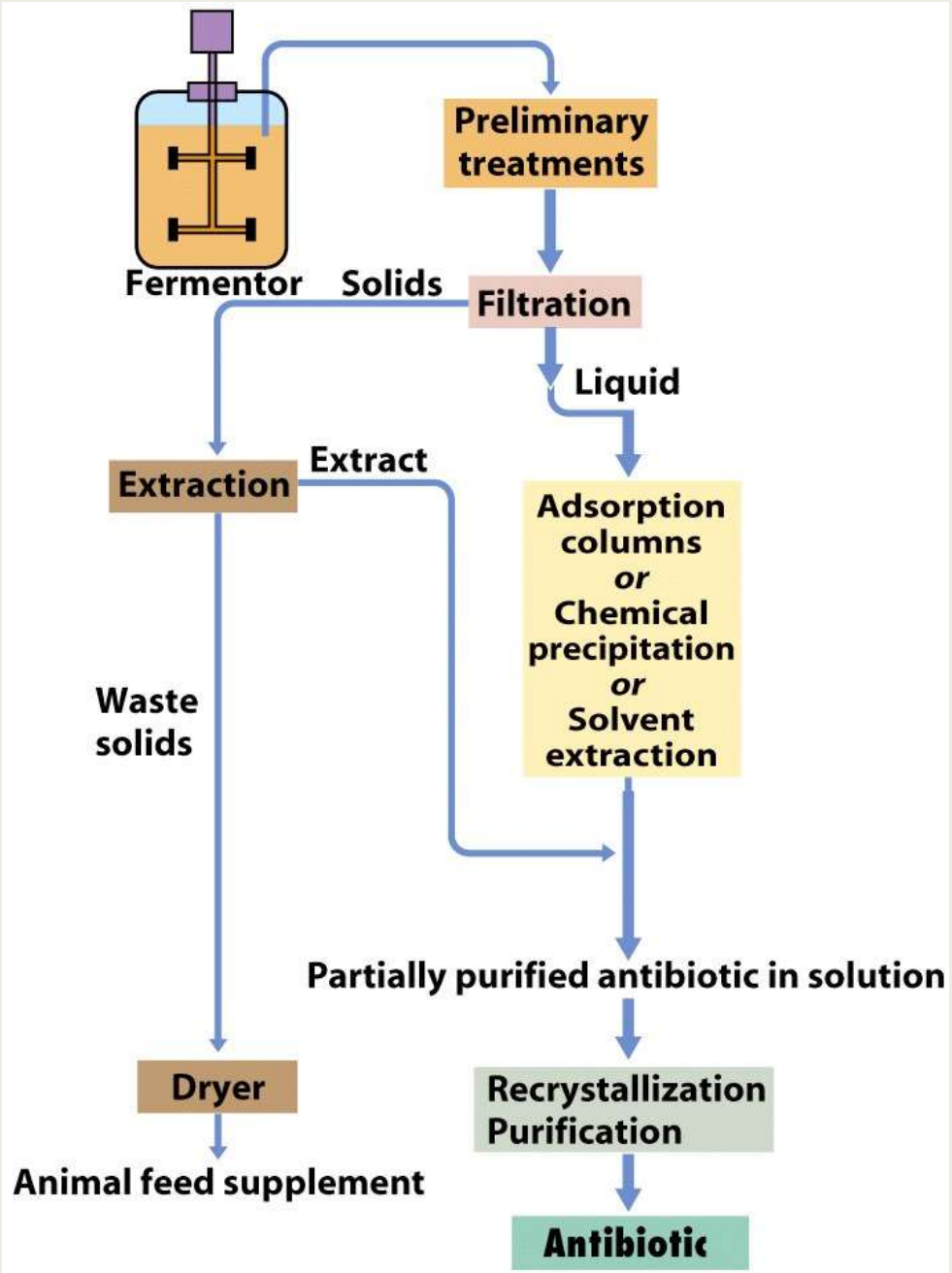
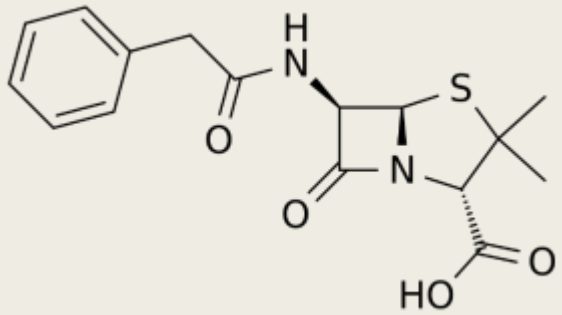


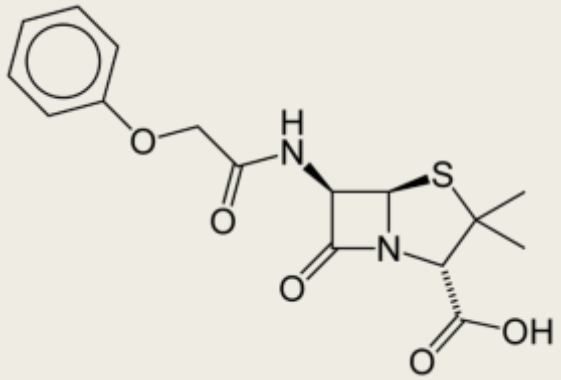
Figure 30-8a Brock Biology of Microorganisms 11/e

PRODUCTION OF PENICILLIN V

- Phenoxy methyl penicillin
- Addition of different Acyl groups to the medium.
- Phenoxyacetic acid as precursor instead of phenyl acetic acid.



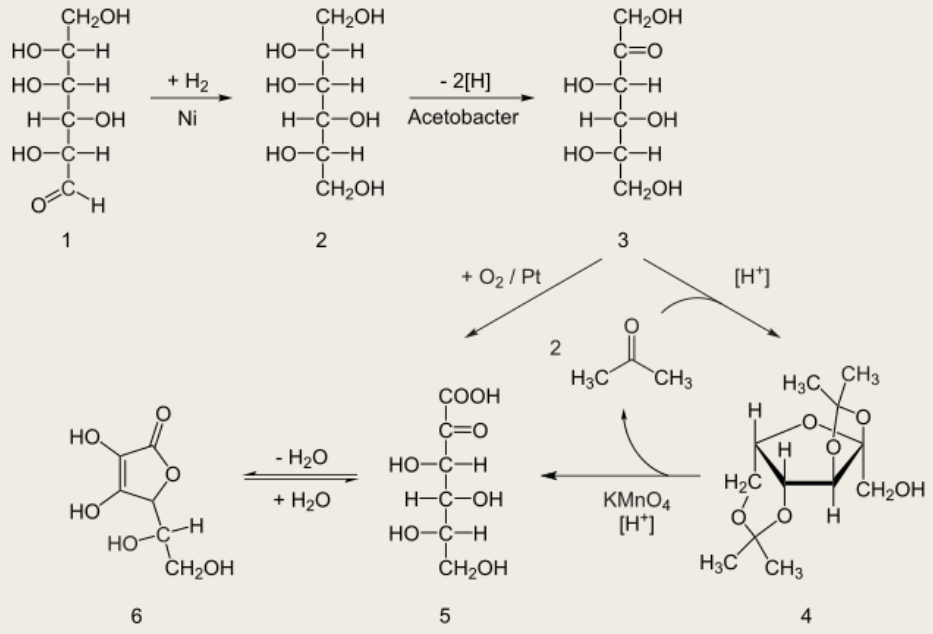
benzylpenicillin



Phenoxymethylpenicillin

Synthesis of l-Ascorbic Acid

- l-Ascorbic acid (vitamin C) is currently synthesized commercially by an expensive process starting with d-glucose that includes one microbial fermentation step and a number of chemical steps.
- The last step in this process is the acid-catalyzed conversion of 2-keto-l-gulonic acid (2-KLG) to l-ascorbic acid.



Trials to improve synthesis

- The current procedure for synthesizing ascorbic acid could be improved by producing 2-KLG from glucose by cofermentation with suitable organisms.
- Some bacteria (Acetobacter, Gluconobacter, and Erwinia) can convert glucose to 2,5-diketo-d-gluconic acid (2,5-DKG), and others (Corynebacterium, Brevibacterium, and Arthrobacter) have the enzyme 2,5-DKG reductase, which converts 2,5-DKG to 2-KLG.
- Limitations of cocultivation:
  - *the two fermenting organisms might have different temperature and pH optima. The medium requirements and growth rates also might differ in such a way that the fermentation conditions are optimal for one organism and suboptimal for the other. This situation leads to the eventual "washout" (depletion) of one of the organisms.*
  - *a tandem fermentation process where it requires two separate fermentations rather than one, and if the organisms have different growth requirements, it is difficult to run the process on a continuous basis. Therefore, the best way to convert glucose into 2-KLG would be to engineer a single microorganism that carried all of the required enzymes.*