Chapter 03 :

3. Microbial Metabolites of Economic Importance

3.1. Enzymes

- Enzymes are biological catalysts of complex protein nature. They are purified from various biological raw materials. Only microbial enzymes produced by fermentation have experienced significant expansion and are prepared industrially, as microorganisms offer numerous advantages as a source of enzymes: exponential growth and availability.
- For the production of an industrial enzyme, the selection of the appropriate strain is crucial, especially since, in most cases, applications for food purposes have seen significant development.

3.1.1. Isolation and Selection of Microbial Enzyme-Producing Strains

Microorganisms capable of producing enzymes that degrade certain compounds are generally found in environments where these substances are abundant. For example, microorganisms that secrete cellulases are numerous in forest soils. The isolation methods are classical methods using selective media. In the ideal selective medium, the enzyme substrate serves as the sole source of one of the essential elements. For instance, starch can be used as the sole carbon source to isolate microorganisms possessing amylase activity.

To be definitively selected, the isolated strains must meet several criteria:

- The ability to grow on a simple medium.
- Minimal production of secondary metabolites, such as antibiotics.
- Excretion of the enzyme in a way that allows easy separation and purification, without leading to various pollutants.
- Non-pathogenic nature and absence of toxic compound production.
- If the enzymatic preparation is intended for food contact, the strain must have food-grade status, meaning it belongs to the G.R.A.S. (Generally Recognized As Safe) category.

The strain's production capacity must be preserved, and any risk of contamination must be eliminated. To achieve this, an excessive number of successive cultures should be avoided.

3.1.2. Composition of Media for the Production of Microbial Enzymes

- Raw materials account for **60 to 80%** of the production cost in enzyme fermentation. The composition of the medium must be carefully defined, and extensive research focuses on replacing expensive compounds with cheaper, more abundant alternatives. These compounds should be utilized as efficiently as possible to minimize waste.

- The culture medium composition must consider the production and extraction stages. For the production of **pectinase, cellulase, catalase, invertase, and β-galactosidase**, the media used consist of:
  - **Carbon sources**: Cereal flour (soy, starch, potato, rice, bran, molasses).
  - **Nitrogen sources**: Fish meal, gelatin, soybean flour, bran, and peptone.
  - **Growth factors**: Yeast extract.

3.1.3. Enzymes in Various Industrial Sectors

- In nature, microorganisms use enzymes to break down **proteins, starch, pectin, lipids, and other large insoluble molecules**. These are reduced into monomers, which serve as sources of **carbon and energy** for the microorganisms themselves or other organisms in the environment.
- These **hydrolytic enzymes** are typically **extracellular** or located on the surface of bacteria or fungi. As extracellular enzymes, they can be easily recovered from the culture medium.
- Enzymes are used across all **fermentation industries**, including:
  - **Food processing** industries.
  - **Pharmaceutical industry** (antibiotics, organic acids, vitamins).
  - **Biomedical industry** (reagents).
  - **Cleaning and decontamination industries** (detergents, water, and surface treatment).
- The production of **bacterial proteases** is a major industrial process in both volume and value, with over **500 tons** of these enzymes produced annually.
  - They are primarily used in **detergent manufacturing and cheese production**.
  - Proteases used in detergents are derived from selected **Bacillus amyloliquefaciens** strains and are added to **enhance stain removal** from fabrics.
  - One major issue with using **proteases in detergents** is the **allergenic response** to bacterial proteins.

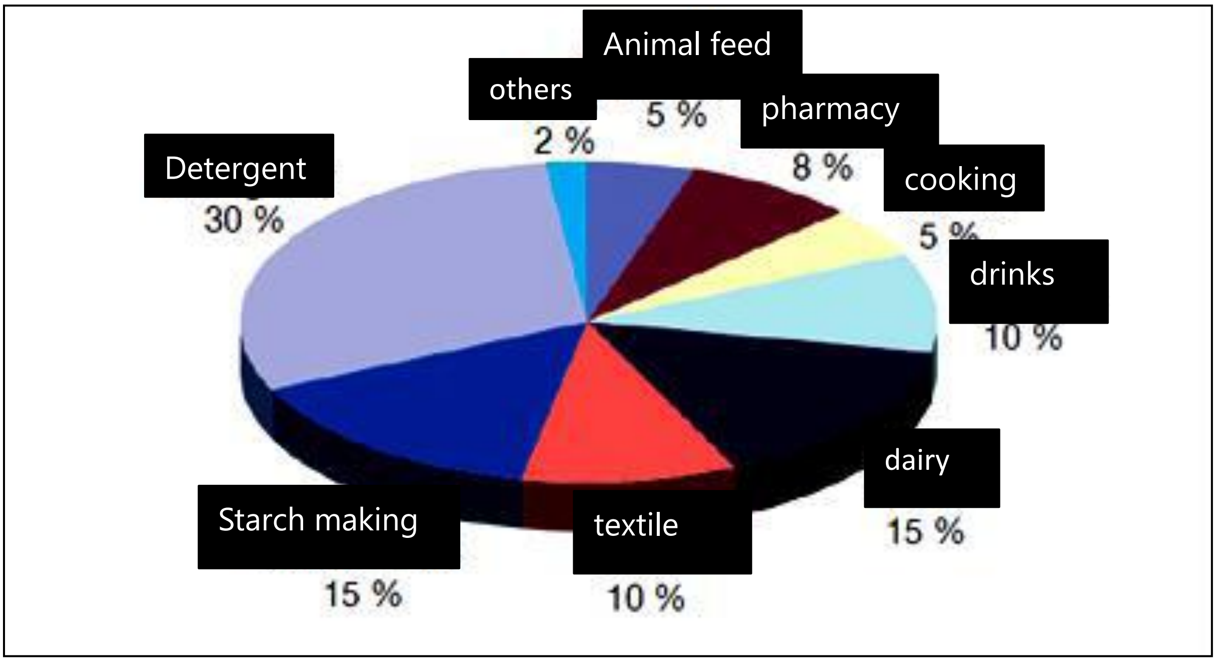

Figure: market shares occupied by enzymes in different sectors.

The industrial production of enzymes is obtained from industrial cultures of bacteria and fungi through fermentation. Once fermentation is complete, the enzymes are purified and then marketed in various forms: liquid, concentrated, or powder. The most widely used enzymes produced on an industrial scale are proteases and amylases.

3.1.4. Some Examples of Microbial Enzymes:

- **Amylases**: These are enzymes that hydrolyze starch. Several microbial amylases break down starch through different pathways to generate short-chain polymers (dextrins) and maltose. Other enzymes further hydrolyze dextrins and maltose into glucose. **α-Amylases** cleave internal **α-1,4 glycosidic bonds** and are produced by thermophilic bacteria of the ***Bacillus*** genus, which form endospores. Fungi such as ***Aspergillus*** species also produce these enzymes.
- **Glucoamylases** cleave glucose from the non-reducing end of starch. These enzymes are commercially used for the production of **fructose syrups**. Fructose is sweeter than glucose and is the preferred sugar in syrups and sweetened beverages. ***Aspergillus niger*** is a producer of glucoamylase.
- **Proteases**: Proteases hydrolyze proteins and peptides into their constituent units: **amino acids**. **Alkaline proteases** are used in **the bleaching industry, the meat industry, and cheese production**. The microorganisms used in the industrial production of proteolytic enzymes include bacteria from the ***Bacillus, Pseudomonas, Streptomyces*, and *Streptococcus*** genera, as well as fungi from the ***Penicillium* and *Aspergillus*** genera.

- **Penicillin Acylases**: These enzymes are produced by various bacteria and fungi, but industrial production is carried out using **selected mutant strains of E. coli**. These enzymes cleave penicillin into **6-aminopenicillanic acid** and **phenylacetic acid**. The free amine group of 6-aminopenicillanic acid can then be chemically modified to produce various **semi-synthetic penicillins**.

- **Taq Polymerase**: This enzyme is used for **polymerase chain reactions (PCR)** and is produced by ***Thermus aquaticus***, a thermophilic bacterium with an optimal growth temperature of **70°C**. As a result, this enzyme is **heat-stable**. It is widely used in **research, diagnostics, and forensic medicine**.

3.2. Ethanol

- The technology of fermentation originated with the earliest civilizations, which exploited the ability of microorganisms to produce alcoholic beverages, bread, and cheese. During the first half of the twentieth century, the production of wine, beer, and vinegar transitioned from ancient artisanal methods to established scientific techniques. As a result, large-scale microbial processes were developed for the production of citric and lactic acids in industry. Acetone, butanol, and ethanol were also produced through fermentation.

  The production of ethanol via fermenting yeasts plays a crucial role in the economy.
- Any material that can be converted into sugar, such as cellulose or starch, can be used to produce ethanol. Currently, commercially available ethanol is produced from sugar or starch. The main sugar-based crops are sugarcane and sugar beet. The most common starchy crops include corn, wheat, and cassava.

To obtain bioethanol (ETBE = Ethyl tert-butyl ether), sweet plant materials such as sugarcane, sugar beets, cereals, or potatoes are processed. A treatment extracts the sugar, which, under the action of yeast, undergoes alcoholic fermentation, producing ethanol along with some by-products. Through a synthesis process involving isobutene, ethanol is transformed into ETBE or bioethanol. Bioethanol can be blended with gasoline or used in its pure form in slightly modified internal combustion engines. One liter of ethanol provides 66% of the energy supplied by one liter of gasoline. It improves hydrocarbon combustion in vehicles, thereby reducing carbon monoxide emissions.

- **80% of global ethanol production comes from biotechnological sources.** It is a promising candidate as a biofuel (or additive) and, most importantly, as a starting component for a new chemical industry that replaces petroleum-based products.

3.2.1. Bioethanol Production Processes

3.2.1.1. Raw Materials Used

Ethanol is an ethyl alcohol that can be synthesized from hydrocarbons and/or biomass. However, microbial processing of biomass can produce what is known as "bioethanol." Ethanol is obtained through the fermentation of sugar-rich plants using microorganisms (yeasts, bacteria, etc.).

3.2.1.2. Materials Rich in Polysaccharides

Currently, bioethanol production is slightly dominated (53% of total production) by starch-based materials such as corn, wheat, and other cereals and grains. In these cases, the conversion process generally begins with the separation, cleaning, and milling of the grain. The starch is then typically converted into sugars through a high-temperature enzymatic process. The released sugars are then fermented by yeasts, and the resulting liquid is distilled to separate the bioethanol.

It is worth noting that raw materials rich in inulin (a polymer of fructose) are an important source of fructose, which can be obtained through the action of inulinase produced by various microorganisms.

3.2.1.3. Materials Rich in Simple Sugars

Sugarcane and sugar beets are plants that contain a high amount of simple sugars. These sugars are extracted through milling or diffusion and can be directly fermented. After fermentation, the liquid is distilled. It is important to note that so-called "first-generation" bioethanol is derived from the fermentation of raw materials that can also be used in human or animal food chains.

In contrast, ethanol produced from the fermentation of cellulosic materials—such as wood, leaves, and plant stems or waste products—is classified as second-generation bioethanol.

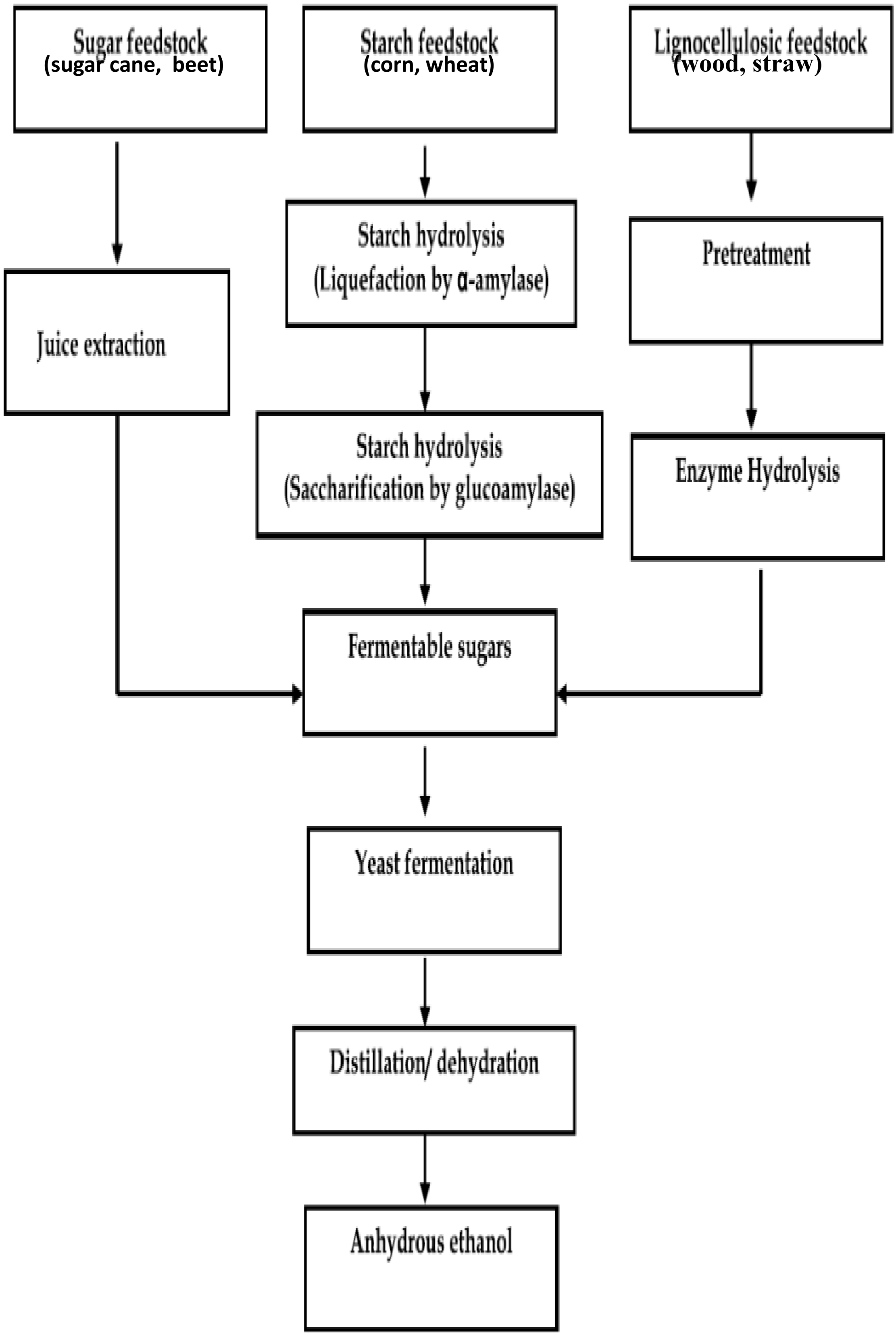

Fig. Schematic diagram of bio-ethanol production by fermentation process of sugar, starch and lignocelluloses feedstock

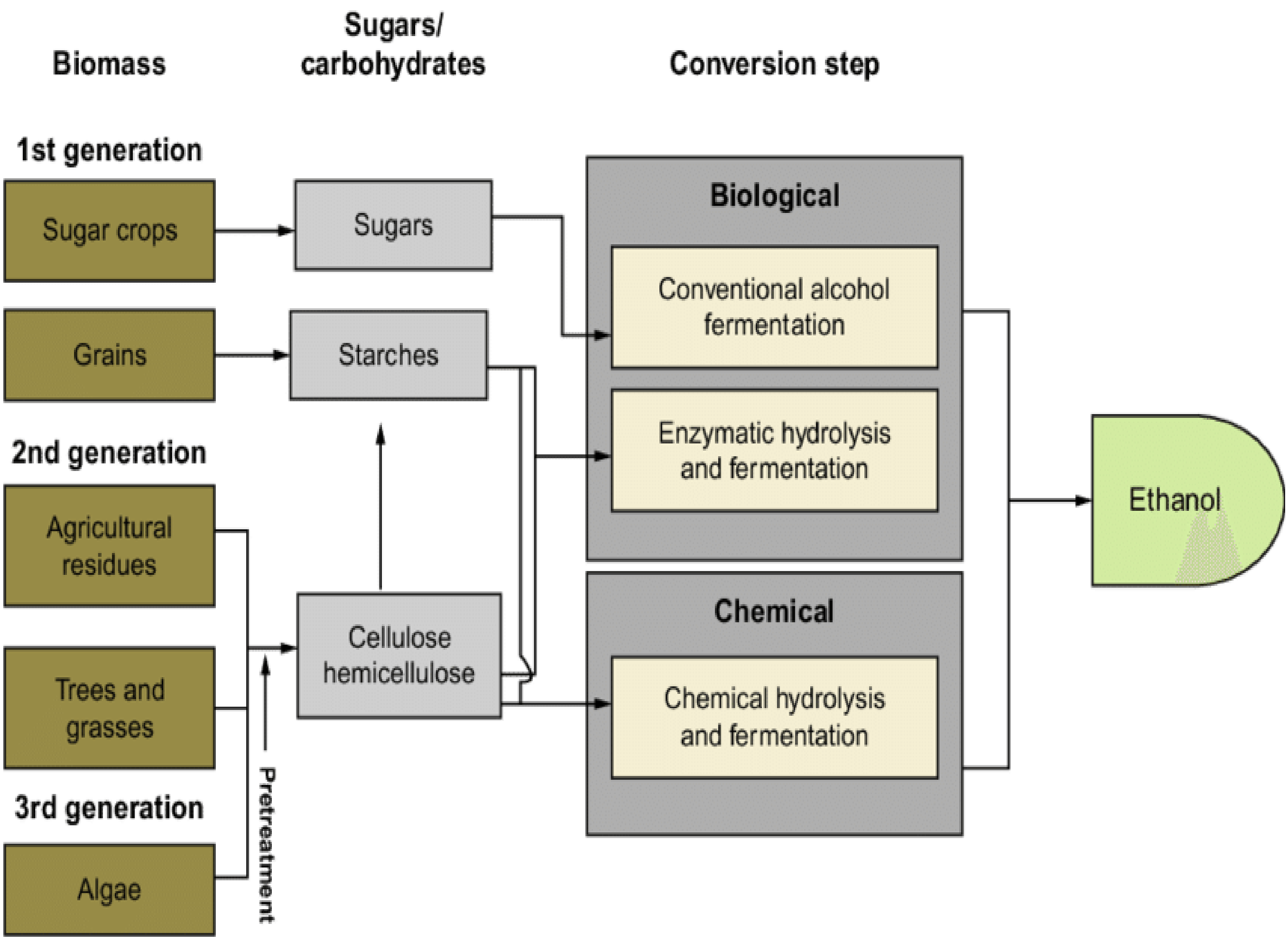

Fig. Schematic diagram of ethanol production processes from three different types of feedstocks.

3.2.2. Microorganisms Used in Microbial Ethanol Production

A wide variety of microorganisms can produce ethanol from sugars, but yeasts have the best advantages and the highest potential for this production. The most commonly used yeasts are ***Saccharomyces cerevisiae, Saccharomyces uvarum, Schizosaccharomyces pombe, and Kluyveromyces sp.***, each with its own advantages and drawbacks, depending on the substrate composition and the process used.

However, ***Saccharomyces cerevisiae*** remains the preferred choice because it offers **high production efficiency and growth at low pH (down to 4)**, an important factor that helps prevent contamination. Additionally, this yeast serves as an **experimental model** with several advantages, including **small size, a fully sequenced genome, ease of genetic manipulation, and extensive availability of scientific literature.**

3.3. Citric Acid

The main organic acids derived from microbial industries are acetic acid, glutamic acid, lactic acid, and especially citric acid. Their total annual production exceeds one million tons.

Citric acid (2-hydroxy-1,2,3-propane-tricarboxylic acid) is widely distributed in nature. It is a solid, white, colorless, and odorless substance. It plays a role in the metabolism of many animals and plants. It was isolated in crystalline form from lemon juice.

It is an important organic acid synthesized through microbial fermentation. More than 130,000 tons are produced annually worldwide. Citric acid was originally extracted from lemons (a lemon contains 7% to 9% citric acid). In 1923, a microbial fermentation process capable of producing high levels of citric acid was developed, and its price decreased with increased production. About two-thirds of the produced and marketed citric acid is intended for food and beverages, such as soft drinks, desserts, confectionery, frozen fruits, compotes, jellies, and ice creams.

In the pharmaceutical industry, iron citrate serves as a dietary source of iron. Citric acid is also used as a preservative for stored blood, medicines, and ointments. Citrate has also replaced polyphosphates in detergents due to the elimination of phosphates, which are environmental pollutants.

Today, 99% of the global citric acid consumption is produced biotechnologically. Citric acid is generally produced using molasses as the preferred carbon source for the species *Aspergillus niger*.

The majority of citric acid comes from fermentations in large stainless steel fermenters using strains of the fungus ***Aspergillus niger*.** Sucrose is also a substrate for citric acid production as a secondary metabolite during the idiophase. During the trophophase, mycelium is produced, and CO_2 is released. During the idiophase, glucose and fructose are directly metabolized into citric acid. Little CO_2 is produced. Under optimal conditions, about 70% of the sugar is converted into citric acid. The levels of iron and other inhibitory minerals are critical factors during fermentation. Therefore, the fermenter must be made of stainless steel or glass-lined, and the use of copper is prohibited.

3.3.1. Microorganisms used in CA Production

Aspergillus niger is the highly recommended industrial producer of citric acid. They flourish in sugar medium containing salts at pH 2.5-3.5 and excrete large volumes of CA. An estimated practical yield of CA is 70 % of the theoretical estimate which is 112g per 100g of sucrose.

Many organisms like ***Penicillin sp***., ***Eupenicillin sp***., ***Botrytis sp***., ***Absidia sp***., ***Ustulina vulgaris,*** and more can also accumulate citric acid in a sugar and inorganic salt medium. Yeast species belonging to the genera of ***Hansenula, Candida, Torula, Saccharomyces, Pichia,*** etc, can produce CA from carbohydrates and n-alkanes. However, the production of CA via these organisms may not seem economical due to the accumulation of unwanted by-products like isocitric acid. A remedial approach could be the development of a mutant strain with lower aconitase activity.

3.3.2. Industrial Production:

- Produced via microbial fermentation using *Aspergillus niger, Escherichia coli,* and other fungi or bacteria.
- Involves multiple regulated steps: sterilization, inoculation, media preparation, fermentation, recovery, and downstream processing.

3.3.3. Fermentation Process :

- A sterilized carbon source (e.g., molasses, sucrose) is inoculated with CA-producing microorganisms in a culture medium.
- The inoculated substrate is transferred to a bioreactor under optimal conditions (temperature, aeration, pH) for fermentation.
- Microorganisms metabolize the substrate, releasing citric acid as a by-product.

3.3.4. Recovery and Purification :

- After fermentation, the broth undergoes filtration, crystallization, and drying.
- The final product is anhydrous white citric acid crystals for commercial use.

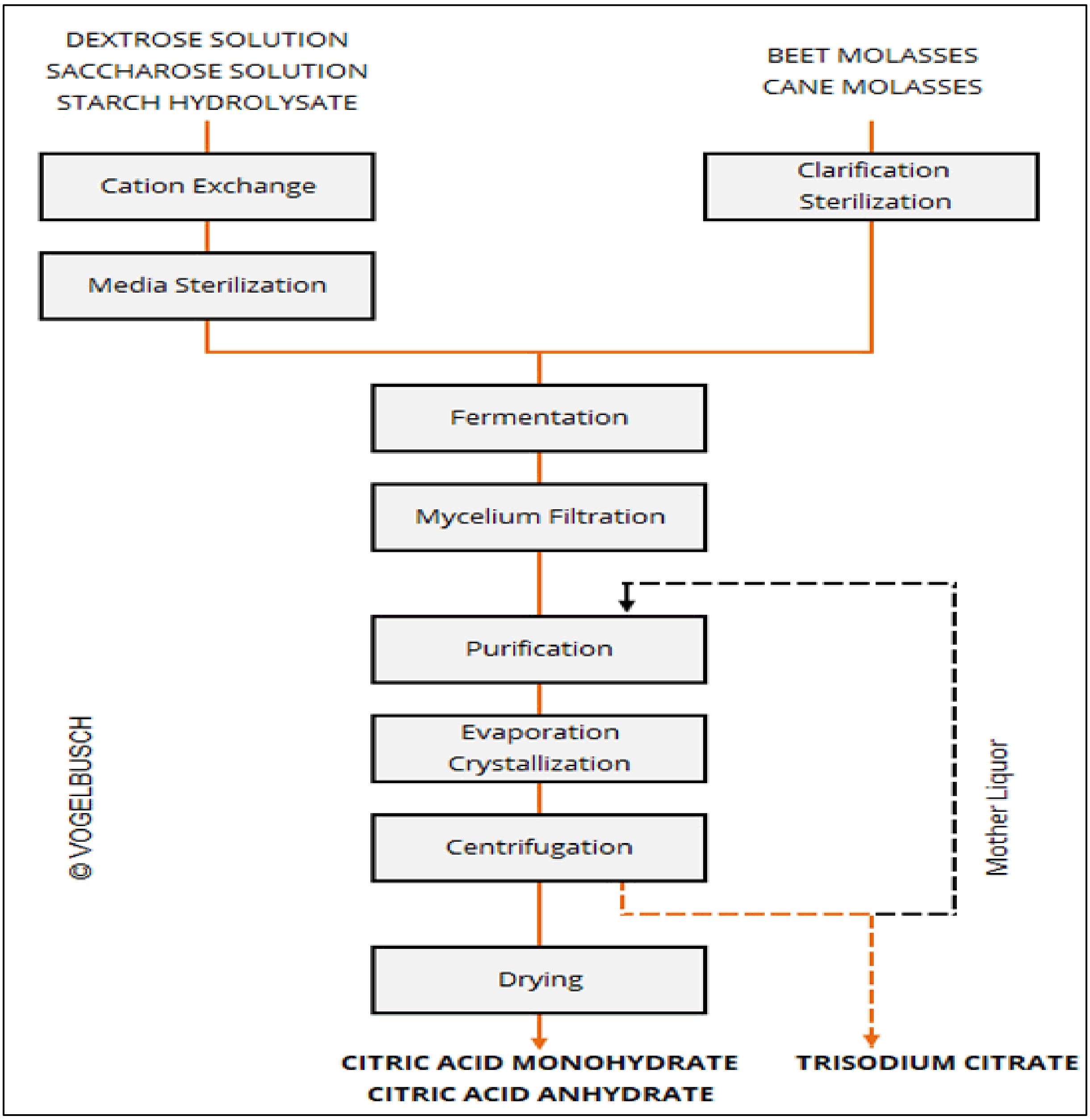

Fig. Schematic diagram of citric acid production by fermentation process

3.3.5. Applications of Citric Acid

3.3.5.1. Food Industry

- It is used as an additive (in beverages, jams, etc.). In beverages, it is generally used as a refreshing or effervescent agent and as an acidulant.
- In the manufacture of candies, the preservation of fruits, fish, ice creams, confectionery in general, sauces, fruit juices, and syrups.
- During harvests, as an acidifier for must.
- In white, rosé, and red wines, to adjust acidity during the production processes.
- It can be used as a cleaning agent for stainless steel due to its sequestering power.

- In plastics manufacturing in the form of esters.
- In metal purification due to its chelating power.

3.3.5.2. Pharmaceutical Industry

- Citric acid indirectly promotes bone growth by facilitating calcium absorption and regulating the size of calcium crystals in bones.
- Citric acid and its salts prevent blood coagulation in stored blood.
- It is used as a rinsing solution during root canal treatments in dentistry.
- In effervescent powders and tablets, the effervescent effect is achieved through citric acid and sodium bicarbonate.

3.4. Antibiotics

Approximately 10,000 different antibiotics have been characterized, and around 160 are industrially produced and marketed. Antibiotics are organic substances secreted as secondary metabolites by certain microorganisms or produced through chemical synthesis. The microorganisms capable of producing antibiotics are relatively few in number. The main groups used in the industrial production of antibiotics are filamentous bacteria of the genus *Streptomyces* and molds of the genera *Penicillium* and *Cephalosporium*.

Experience has shown, however, that bacteria with a life cycle involving spore formation (endospores or other types of spores) are the most effective in producing useful antibiotics.

Many antibiotics are currently available to the medical profession, but the search for new antibiotics continues, particularly against bacteria, fungi, viruses, and tumors. One of the major issues in the use of antibiotics and chemical therapeutics is the development of resistance in pathogenic agents. Antibiotic resistance also arises through mutation and selection processes.

Thus, the search for new antibiotics continues to combat the resistance developing against those currently in use. It is clear that new agents effective against bacteria, fungi, viruses, or tumors are now necessary.

3.4.1. Isolation of Antibiotic-Producing Microorganisms

Soil has become the main reservoir of antibiotic-producing microorganisms. The vast majority of antibiotic-producing strains have been isolated from soil. However, other ecological niches also contain microorganisms with the potential to produce secondary metabolites: decaying materials, sediments, waste, marine or freshwater environments, lichens, mosses, and plants.

One of the objectives of isolation is to search for new strains in the hope that they produce new secondary metabolites. Actinomycetes, and especially *Streptomyces*, are the best producers of antibiotics. However, *Streptomyces* have been heavily exploited. It is therefore necessary to continue isolating species from this genus while trying to find rare species.

3.4.2. Antibiotic Production by Actinomycetes

Actinomycetes play a very important role in the field of antibiotic biotechnology, despite the progress made in chemical synthesis. In fact, 45% of known antibiotics are naturally derived from actinomycetes, and more specifically from the *Streptomyces* genus, which is responsible for producing 75% of these antibiotics.

3.4.3. Examples of Some Antibiotics Produced by Microbial Processes

- *Penicillin*: This antibiotic is produced by *Penicillium chrysogenum* and contains a β-lactam ring, which is unusual in biological systems. This antibiotic has been used for over 50 years, and the development of resistant microorganisms has become a real problem. Semi-synthetic penicillins are more effective against microorganisms resistant to the natural product and are preferred antibiotics for clinical applications.
- *Cephalosporins*: This antibiotic, also part of the β-lactam family, is produced by the fungus *Cephalosporium acremonium*. Cephalosporins are less toxic than penicillin and have a broader spectrum of antimicrobial activity. They are also resistant to penicillinases produced by microorganisms resistant to penicillin. Cephalosporins are produced through fermentation, and some semi-synthetic derivatives are generated by chemical modification.
- *Streptomycin*: Many antibiotics are sugar derivatives, and streptomycin belongs to this group. It is produced by *Streptomyces griseus*. The fermentation medium used contains soy and glucose as a carbon source. Streptomycin is composed of amino sugars linked to other sugars by a glycosidic bond. Its discovery was of great medical importance because it was the first effective drug against the plague of tuberculosis. However, resistance to streptomycin has caused serious problems in tuberculosis therapy, and combinations of drugs are now administered over a specific period.
- *Erythromycin*: This antibiotic belongs to the macrolide family. It has large lactone rings linked to sugars. It is produced by *Streptomyces erythraeus*.
- *Rifamycin*: This is a macrocyclic lactone-type antibiotic produced by *Nocardia mediterranei*.
- *Rifampicin*: It is a specific inhibitor of bacterial DNA-dependent RNA polymerase. It is used in the treatment of tuberculosis in combination with isoniazid and pyrazinamide.

- *Tetracyclines*: Tetracyclines constitute a major group of antibiotics effective against both Gram-positive and Gram-negative bacteria. They are also effective against *Rickettsia*, *Mycoplasma*, *Leptospira*, *Spirochetes*, and *Chlamydia*. Some semi-synthetic derivatives have also been developed to counteract the problem of emerging bacterial resistance.
- *Spiramycin*: This is an antibiotic belonging to the macrolide family. Macrolides are basic lipophilic molecules composed of a lactone macrocycle or aglycone. The producing microorganism is *Streptomyces ambofaciens*.

3.5. Others

Other fermentation products and their applications are presented in the following table:

Production	**Microorganisms**	**Use**
Production of Pharmacologically Active Products		
Ergotamine	Claviceps purpurea	Migraine
Valiomaline	Streptomyces, hygroscopicus	Diabetes
Compactin	Penicillium citrinum	Cholesterol
Cyclosporin	Tolypocladium inflatum	Immunosuppressants
Prednisone	Curvularia lunata, Corynebacterium simplex	Asthma and other allergies
Aromas		
Anisaldehyde	Trametes suavolens	Anise aroma
Geraniol	Ceratiocystis variospora	Rose aroma
Methyl-phenylacetate	Trametes odorata	Honey aroma
Plant hormones		
Gibberellin	Phaeosphaeria sp.	Breaking dormancy, flowering stimulation
Lipids	Aspergillus fumigatus, Mucor miehei, Penicillium spinulosum	Food industry
Pigments		
Beta-carotene	Blakeslea trispora, Rhodotorula gracilis	Colorants